# Molecular Bases of the Reduced Osteogenic Differentiation Potential in *Nf1* Deficient Osteoprogenitors

By

Seyedmohammad Ebrahim Tahaei

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

January 31, 2018

Nashville, Tennessee

Approved:

Joey Barnett, Ph.D.

Florent Elefteriou, Ph.D.

Alan Brash, Ph.D.

Jeffrey M. Davidson, Ph.D.

Ana Carneiro, Ph.D.

For my family

# Acknowledgement

This dissertation is the product of years of constant presence- both mentally and physically- in Dr. Florent Elefteriou's lab. The dedication that was required in getting this degree and finishing graduate school was beyond imagination. The will and perseverance that I showed, facing all the negative data and day to day hardships of being a graduate student, surpassed many expectations. I hope that as the result of this experience, I have become a more mature and better individual.

Graduate school could have not been possible without financial assistance from the Vanderbilt International Scholar Program Fellowship and Children's Tumor Foundation Young Investigator Award YIA-2015-01-015 to me, and development funds from Vanderbilt University Medical Center department of Medicine and Baylor College of Medicine department of Molecular Human Genetic to Dr. Florent Elefteriou.

I am thankful today for the many helping hands along my way, and would like to acknowledge here the role of my teachers, colleagues, friends, and family. First, thanks to Dr. Florent Elefteriou, my supervisor, who has taught me to look at science in a different light. Thanks, too, to all the members of my thesis committee who have spent so many hours in meetings, discussing data and asking thought-provoking questions. Without your guidance, I could not have finished my Ph.D. You have always pushed me towards becoming a better and more independent scientist.

Next, thanks to my friends, both inside and outside the University. Your love and support are like soft cushions during hard days, and helped me to not lose my mind. I hope I can return the favor. Finally, and most importantly, my deepest gratitude goes to my family. To my mom, who always encouraged my curious nature, nurtured my childhood interest in science, and cheered along my

every success since then. To my brother who has always had my back and given me good advice. To my sister who has brought so much joy and happiness into my life. To my father who had an undeniable role in my success. I could not have gotten where I am today without you, and I want to thank you for believing in me. This work is dedicated to you.

# Table of contents

Page

# Table of figures

Page

# List of tables

# Abstract

Neurofibromatosis type 1 results from mutations in *NF1,* a gene that encodes Neurofibromin. This common genetic condition is associated with tibial pseudarthrosis (PA), whose etiology is unknown but thought to involve defective bone-repairing osteoprogenitors. The main objective of my thesis was to delineate the causal determinants of the poor osteogenic potential of *Nf1*$^{-/-}$ osteoprogenitors. I showed that increased Epiregulin and TGFβ1 expression does not contribute to the reduced osteogenic differentiation of *Nf1*$^{-/-}$ osteoprogenitors, and contrary to all expectations, that this phenotype is likely independent from MAPK/ERK constitutive signaling. Using a RNA-Seq approach, I identified changes in pro-inflammatory and extracellular matrix gene signatures as putative determinants of the impaired differentiation of *Nf1*$^{-/-}$ osteoprogenitors. Finally, I obtained preliminary data pointing to inhibition of RUNX2 activity upon loss of *Nf1* function. These results suggest unexpected interactions between Neurofibromin and proximal cell signaling/adhesion components that impact not one but multiple downstream signaling pathways.

# Chapter
# I. Introduction and Background

## Overview

The skeleton is one of the major organs affected by Neurofibromatosis type 1 (NF1). Some of these NF1 associated bone manifestations are associated with high morbidity and have an unknown etiology, thus preventing the design of targeted pharmacological therapies. Major NF1 bone manifestations include dystrophic scoliosis and pseudarthrosis (PA) or non-union after fracture. PA in NF1 is usually unilateral and anterolateral [1]. The focus of my dissertation is on identifying the cause of the poor osteogenic potential associated with loss of *Nf1* function as a way to better understand the biology of *Nf1* in the osteoblast lineage and to identify new potential therapeutic targets to prevent or treat pseudarthrosis. In order to explain my findings, first I will introduce the formation of the skeleton and the process of bone repair, the NF1 skeletal maladies and what is known about the *NF1* gene product: Neurofibromin. In chapter 2, I will present my findings that demonstrated that Transforming growth factor β1 (TGFβ1) and Epidermal growth factor receptor (EGFR) signaling do not contribute to the reduced osteogenic phenotype observed in *Nf1*-deficient osteoprogenitors. The third chapter will present a set of independent approaches and results supporting the controversial hypothesis that this phenotype is Mitogen-activated protein kinase (MAPK/ERK)-independent. In the fourth chapter, I will present the results of an unbiased approach to identify genes and pathways underlying the poor osteogenic potential of *Nf1*-deficient osteoblasts, and further supporting the existence of a MAPK/ERK-independent mechanism. In the fifth chapter, I will present preliminary data showing that reduced RUNX2 activity may contribute

to the reduced osteogenic differentiation of *Nf1*-deficient osteoprogenitors. In the last chapter, I will discuss my findings and expand on the future directions for this project.

## Bone biology

### Bone structure

Bone is a dense connective tissue that composes around 15% of the human adult body weight. Bone has multiple physiological and anatomical roles. Anatomically, the skeletal musculature connects to the appendicular skeleton and uses the bone as a platform for locomotion; ribs facilitate respiration by providing the attachment site for the intercostal muscles; bone also protects vital internal organs such as heart and brain. Physiologically, long bones are composed of a red marrow made of hematopoietic and mesenchymal stem cells (HSC and MSC, respectively), pericytes and endothelial cells. HSCs sustain blood cells populations by the process of hematopoiesis. MSCs located in the marrow are a reservoir for osteoprogenitors that can differentiate towards bone-forming osteoblasts. Bones also provide a reservoir for calcium and phosphate, two major inorganic ions in the body [2]. Recently, it has been shown that bone is an endocrine organ that has an important role in the regulation of glycemia and insulin sensitivity [3–5] and can regulate male fertility and testosterone production [6,7].

Anatomically, there are multiple types of bones in the adult skeleton. The long bones are composed of three parts: a hollow cylinder, or diaphysis; a cone-shaped metaphysis below the growth plates; and spherical epiphyses above the growth plates [8]. Cortical (compact) bone is the outer layer of bones. This layer has high matrix mass and very small size pores. These features make this structure suited for the mechanical loading role of the skeleton. The other type of bone that is

located underneath cortical bone is trabecular bone. This type of bone has more porosity compared to the cortical bone and plays a role in energy absorbing and weight distribution in the body. The diaphysis is composed primarily of dense cortical bone whereas the metaphysis and epiphysis are rich in trabecular bone. The cortical to trabecular bone ratio in different skeletal elements differs from 25:75 in the vertebra to 95:5 in the radial diaphysis [9].

The outermost and innermost layer of the cortical bone contain cells that have osteogenic potential [10]. The outermost layer is a membranous layer called periosteum and the innermost layer adjacent to marrow is called endosteum. These two membranous layers act as reservoirs for mesenchymal stem cells. After fracture, MSCs located in these layers undergo differentiation towards osteoblasts and chondrocytes and produce the extracellular matrix necessary for regaining the mechanical integrity of the bone [11]. Additionally, Maes and colleagues showed that pericyte-like cells that reside in the wall of invading vasculature to the sites of new bone formation have an expression pattern and morphological similarities to osteoprogenitors and contribute to trabecular bone formation, whether it is in the bone development process during embryogenesis or after birth in the process of bone fracture healing [12].

Microscopically, bone is divided into two structural categories: woven and lamellar bone. The major difference between these two types of bones is in their collagen orientation. Woven bone has irregular collagenous fibrils, and in adults it is only observed post-fracture and in pathological conditions. On the other hand, the lamellar bone has organized collagen fibrils and the majority of the bone in adults is composed of this type of bone. Woven bone is suited in conditions where speed of bone laying is more important than stiffness. Woven bone formation might happen during normal physiological conditions e.g. fracture healing and pathological conditions e.g. Paget's disease.

## Extracellular bone matrix

Bone in general is composed of two phases, extracellular matrix and cells residing in bone. Bone matrix is composed of two components. An organic component comprised of different proteinaceous matrix proteins and a mineral component. The organic component of the matrix is made of different types of collagenous (the major type in bone is Collagen type I) and non-collagenous (e.g. Osteocalcin) matrix proteins. These proteins have different functions and mutations in their encoding genes can lead to severe skeletal conditions. For example, Osteogenesis Imperfecta (OI) is the result of disruption in the formation of Collagen type 1 fibrils, and is characterized by high risk of fracture [13].

The most prevalent protein in the human body is type 1 collagen, whose synthesis and location is not limited to osteoblasts. Fibroblasts also make this protein in skin [8]. Collagen synthesis is a complex process involving different enzymes and cofactors, e.g. Lysil Oxidase and Vitamin C that assist with collagen cross-linking. The final product is composed of triple helix chains that are wrapped around each other in a right-handed fashion [14]. The collagenous matrix gives bone an elasticity-similar to skin, which makes the skeleton capable of absorbing energy from environmental impacts [15]. Bone would be brittle without this collagenous matrix, which would lead to increased fracture risk similar to what is observed in OI patients.

An important non-collagenous protein in bone is Alkaline phosphatase (Alp). This enzyme can be found in two forms: bound to the outer plasma membrane of osteoblasts (through Phosphoinositol) or free form in the extracellular matrix. It converts Pyrophosphate (PPi)- an inhibitor of matrix mineralization- to inorganic Phosphate (Pi)-the raw material for matrix mineralization. *Alpl* expression is used as a marker of the osteoblast lineage and differentiation both *in vitro* and *in vivo* [16].

Another major protein in bone that composes 8-12% of non-collagenous proteins is Integrin binding sialoprotein, IBSP. This structural protein belongs to the family of Small Integrin-Binding Ligand N-linked Glycoproteins (SIBLING) [17]. *Ibsp*-deficient osteoprogenitors show impaired osteogenic differentiation. Mice lacking this gene also have reduced healing response to injuries such as bone drilling [17].

The mineral component of the skeleton is composed mostly of hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_2$] with small traces of Magnesium, Bicarbonate and Potassium. Hydroxyapatite crystals in bone are smaller than those found in rocks, which makes these crystals more accessible for metabolic regulations and release into the blood stream. The mineral component gives the bone its strength and acts as a form of reservoir that, alongside with the kidney, regulates calcium and phosphate homeostasis in the body. Without mineral components, the bone would be similar to rubber and could not support body weight. The mixture of organic (collagenous and non-collagenous proteins) and non-organic (hydroxyapatite) components makes this organ suitable for providing both elasticity and strength.

## Bone cells

Bone is composed of two major cell types, namely osteoblasts and osteoclasts. These cells originate from different lineages and have different roles in bone. Osteoblasts form bone, while osteoclasts destruct bone. Bone homeostasis requires that the activity of these two cell types be controlled rigorously to keep bone mass constant in adulthood [15]. Osteoblasts are cuboidal shaped mononuclear cells with a high amount of Golgi apparatus and mitochondriae that is suited for their role as the producer of copious amount of extracellular matrix in the skeleton. These cells

differentiate from mesenchymal stem cells (MSCs). It is generally accepted that osteoblast-forming MSCs in adults reside in the bone marrow, periosteum and endosteum layers of bone. However, there are some reports that pericyte cells that reside along the vessels and provide integrity to vessels might also have stem-like features and differentiate towards osteoblasts upon receiving osteogenic cues [18].

In general, osteoblasts are defined as cells that lay down a unique extracellular matrix enriched in collagen type I, osteopontin and osteocalcin (this combination of proteinaceous matrix is called osteoid) and have the ability to mineralize this proteinaceous matrix. Failure to mineralize osteoid leads to a pathological condition called osteoidosis, which reduces bone strength and favors fracture. This condition is commonly observed in Neurofibromatosis type 1 (NF1) Pseudarthrotic biopsies [19].

After performing their role in secreting ECM, osteoblasts have three fates: First, they can become quiescent and line the bone surfaces; second, they can undergo apoptosis; third, they can become embedded in their own matrix and become osteocytes.

Osteocytes are the largest population of cells in the bone. They are long-lived cells with stellar shape and long dendritic processes that support their different roles in skeletal biology. These cells are the mechanosensors of the skeleton. They detect mechanical loading and have a major instrumental role in the reaction of the skeleton to this type of stimuli, hence they play a paramount role in the stimulatory effect of exercise on bone formation, in weightlessness-induced bone loss, and in modeling and remodeling of the skeleton. Osteocytes can negatively control the formation of osteoblasts by secreting inhibitors of the Wnt pathway e.g. Sclerostin, SFRP1, and DKK1 and can activate osteoclastogenesis by secreting RANKL [20]. Anti-Sclerostin antibodies have shown

therapeutic potential in mouse models of Osteogenesis Imperfecta and in randomized clinical trials for the treatment of this disease [21,22]. Additionally, osteocytes control phosphate metabolism by the secretion of Fibroblast growth factor 23 (FGF23) [23]. This hormone regulates phosphate level of serum by controlling the excretion rate of phosphate from the kidney. Overexpression of FGF23 by osteocytes leads to hypophosphatemic rickets [24], a condition where low phosphate and calcium level in the body lead to a weak skeleton.

Osteoclasts are multinuclear cells that are formed from maturation and fusion of monocytes/macrophages, cells that derive from HSCs [25]. Osteoclasts are bone-destructing cells. Bone resorption is a multistep process. First, activated osteoclasts form a ruffled membrane that binds to the matrix, and then form a sealing zone that encircles the specific part of the bone that is going to be resorbed; in the next step, HCl is secreted in the ECM via a $H^+$ pump and a Cl- transporter to dissolve hydroxyapatite crystals. To digest matrix proteins, Cathepsin K secreted from osteoclasts degrades collagens and other proteins in the bone extracellular matrix [25].

## Bone formation during embryonic development

The skeleton is formed from three distinct lineages during embryonic development. The axial skeleton (trunk and skull) is formed from paraxial mesoderm (somites), the appendicular skeleton (limbs) is formed from the lateral plate mesoderm, and craniofacial bones and cartilage are formed from the cranial neural crest. Regardless of the source, skeleton formation starts with the migration of the embryonic mesenchymal cells into future location of bones to form condensation centers. At this step, depending on the type of bone to be formed, these condensation centers will have two fates. MSCs in one group will later generate flat bones, directly differentiating towards osteoblasts

in a process called intramembranous ossification. However, the majority of bones in the body are formed through a distinct process called endochondral ossification [15].

In the endochondral ossification process (**Figure 1**), MSCs in the condensation centers are divided into two groups; the inner group of MSCs differentiate towards chondrocytes and begin secreting a cartilaginous extracellular matrix that forms an anlagen or template for future bones. Cells around this template form the perichondrium that will eventually become periosteum. Inner chondrocytes will start to divide and secrete Collagen type II, and later on enlarge, become hypertrophic and secrete both Collagen type X and Matrix Metaloproteinase 13 (MMP13). These two enzymes resorb Collagen type II matrix to allow the invasion of vessels through the perichondrium which delivers osteoblast progenitors, hematopoietic stem cells and endothelial cells. It was thought for many years that these hypertrophic chondrocytes undergo apoptosis, however a recent study by Yang and colleagues showed that some of these cells might likely become osteoblasts after the hypertrophic stage [27]. Based on the accepted common dogma, osteoblasts delivered by vascular invasion begin forming trabecular bones at primary ossification center. This is the first part of the cartilaginous bone that becomes mineralized. HSCs and endothelial cells form a central medullary canal that later contains the bone marrow. As the embryo develops, the primary ossification center expands and bone elongates. Secondary ossification centers form in one or both ends of the developing bone as vasculature invades the bone ends. This results in the development of epiphyseal growth plate cartilage, which is responsible for the longitudinal growth of bones postnatally [15,28,29].

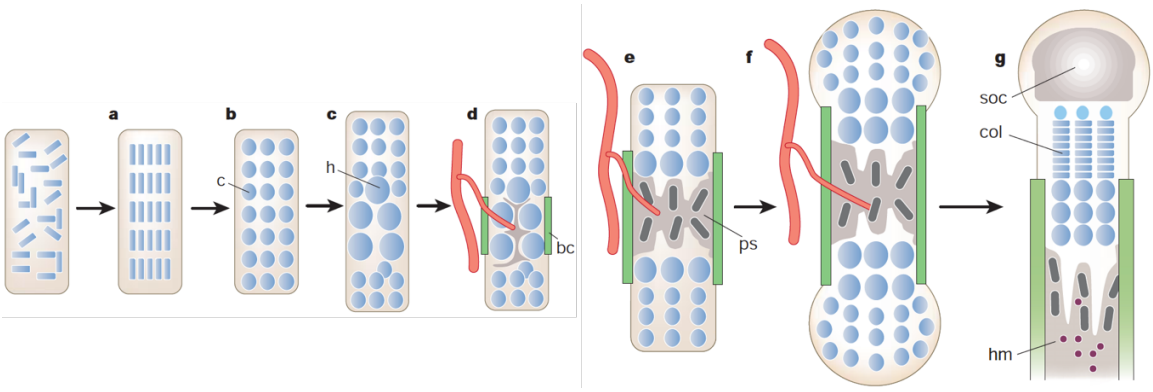



**Figure 1. Endochondral ossification**
Endochondral ossification begins when mesenchymal progenitor cells (**a**) condense and (**b**) differentiate into chondrocytes (c) to form a cartilaginous anlage. (**c**) Proliferative chondrocytes mature to hypertrophy (h) and (**d**) promote differentiation 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 (From Kronenberg HM, 2003 [26]).

## Bone formation during fracture healing

In the majority of healthy individuals, the bone healing process after fracture reestablishes biomechanical properties, tissue integrity and physiology of the affected bone within weeks [30]. It is of critical importance to understand the process of normal bone healing and to identify the main regulatory elements of this process to identify the culprits in cases of impaired bone healing, which occurs in 10% of individuals with fracture.

Multiple studies have shown that the process of bone healing to some extent recapitulates the embryonic development of limbs [31–34]. This includes the morphogenetic pathways and genes that are expressed during organogenesis [35]. The process of fracture healing has four phases (**Figure 2**). Inflammation; soft callus formation; hard callus formation, and remodeling. The disruption of any of these phases may lead to delayed healing [36,37].

In the first phase, as the result of vasculature network disruption, inflammatory cells from the peripheral circulatory system plus HSC-derived cells that are already present in the marrow form a hematoma that keeps the bone pieces together and acts as a mold for future callus formation. Callus is a temporary tissue that is formed around and between the fracture site and at early stages of fracture healing. It is composed of a fibrocartilaginous extracellular matrix and at later stages becomes ossified and in the last phase is resorbed by osteoclasts. Cells that are in and around hematoma release cytokines such as Macrophage colony stimulating factor (M-CSF), Interleukin-1 (IL-1) and Tumor Necrosis Factor-α (TNF-α) [38,39]. These cytokines play several roles: firstly, they stimulate the recruitment of more inflammatory cells; secondly, they increase angiogenesis; thirdly, they stimulate other cells in the vicinity of fracture such as periosteal cells to secrete growth factors that both force existing MSCs towards differentiation and also recruit more MSCs towards

the fracture site [40–42]. In the second phase, MSCs that are in the hematoma differentiate towards chondrocytes, start proliferating and secrete copious amount of extracellular matrix including Collagen type II and proteoglycans [39]. At the end of this phase, the cartilaginous matrix is calcified and chondrocytes undergo hypertrophy and apoptosis. Additionally, the mineralized matrix is resorbed by the action of osteoclasts that are recruited by chemo-attractants such as TNF-α, M-CSF and Receptor activator of nuclear factor κ B ligand (RANKL). In the third phase, newly recruited MSCs undergo osteoblastic differentiation [39]. These cells secrete type I Collagen and other structural extracellular matrix proteins found in calcified bone. Alkaline phosphatase (ALP), present at the cell surface of osteoblasts or secreted in the matrix, alongside calcium and phosphate granules secreted from osteoblasts form hydroxyapatite crystals on the deposited osteoid. At the end of this phase a mechanically weak woven bone is formed [44]. Although the cartilaginous transformation to woven bone is similar to endochondral ossification during embryonic bone development, a process similar to intramembranous ossification occurs around the edges of the fractured bones. MSCs residing in the periosteal area undergo direct differentiation towards osteoblasts and form the cortex of the bony callus, which is the first semi-rigid bony structure that is formed during long bone fracture healing [30].

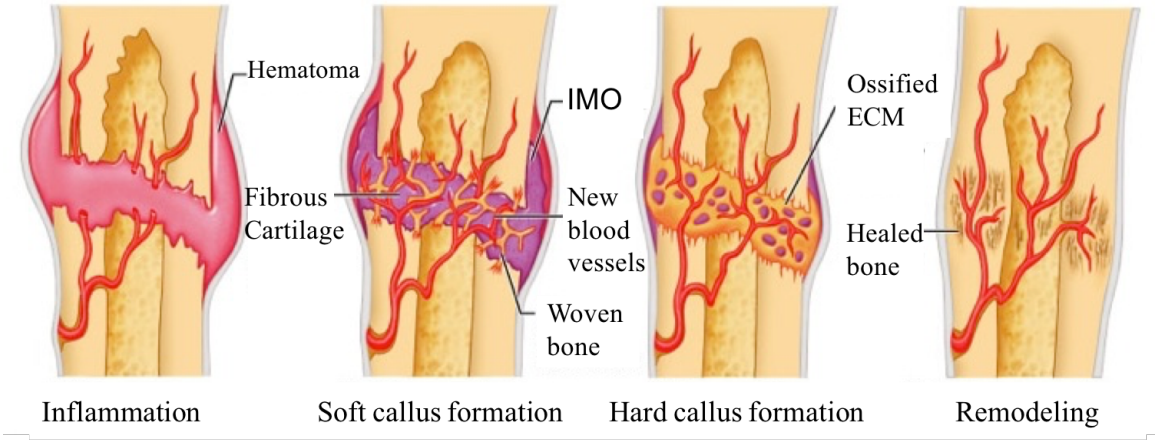



**Figure 2. Bone healing process**

Fracture healing begins with formation of hematoma, a mixture of diverse cell types such as inflammatory cells trapped in the fibrin clot. In the second stage, two process occurs; as the result of angiogenic signals, vasculature bed is restored and MSCs undergo endochondral ossification between fractured bones and intramembranous ossification (IMO) at the edges of fracture bone and thus early callus forms. At the third stage, chondrocytes undergo apoptosis and osteoblasts replace them (hard callus). At the last stage, the hard callus is remodeled by orchestrated activity of osteoclasts and osteoblasts.
( from [43]).

In the last phase of fracture healing, by the orchestrated actions of osteoclasts and osteoblasts, the woven bone will be resorbed and lamellar bone will be laid down [33]. Lamellar bone has structured and organized collagen fibrils and is stronger than woven bone mechanically. In humans the remodeling phase might take years to complete, while in mouse it only takes few weeks [45]. Most importantly bone healing process requires fracture stability; failure in providing this requirement leads to generation of fibrotic tissue and Pseudarthrosis (PA) [46,47].

## Post fracture non-union or pseudarthrosis

The majority of fractured bones in healthy individuals heal in a few months. However, a small percentage of individuals suffer from delayed union- a pathologic condition called pseudarthrosis or post fracture non-union. There is a debate about the exact definition of PA between clinicians. However, it is generally accepted that cases of fractured bones that do not heal after six months are considered cases of non-union. The incidence of non-union is affected by diverse factors such as age, smoking, nature of the fracture, stability of fracture, and severity of the fracture [47]. Open fractures, which are fractures where bone is exposed, are more likely to have delayed healing and non-unions. Additionally, the location of the open fractures could compound the rate of non-union. For example, lower limbs have higher rates of non-union [48]. In contrast, closed fractures of the tibia and mid-shaft fractures are generally less severe with minimal soft tissue damage. External fixation and intramedullary nailing can help to achieve fracture stabilization and minimize non-union in these cases. In the absence of any underlying problem such as infection, autogenous bone grafting and substitute bone graft materials have been used successfully in treatment of delayed or non-union fracture [49]. Bone healing in general and more specifically at anatomical locations of

poor bone repair, such as the tibia, can be worsened in the presence of an underlying genetic skeletal disorder such as Neurofibromatosis type 1.

One of the important cell type in the fracture healing process are osteoblasts that are formed from osteogenic differentiation of MSCs. In the next section, I will review the important stages and factors that affect osteogenic differentiation, a process critical to bone formation and regeneration.

## Osteoblast differentiation

Osteoblasts originate from osteogenic commitment and differentiation of mesenchymal stem cells (MSCs). "Stemness" of these cells give them the multipotency to become different cell types. The fate of MSCs' differentiation depends on the nature and amount of molecular and mechanical signals that these cells receive and their sensitivity to these signals. MSCs can differentiate towards four different lineages: Myoblasts that form muscles, adipocytes that form adipose tissue, chondrocytes that form cartilage, and osteoblasts that form calcified bone. In some cases, these cells at some point in their life can trans-differentiate towards another cell type. However, this notion is to some extent controversial [50,51]. In some physiologic or pathogenic cases, this multi-potency is shifted towards one type of cell lineage at the expense of the other. For example, in aging or obesity, a high proportion of MSCs differentiate towards adipocytes and hence the marrow becomes "yellow" [52]. This change has physiological consequences, e.g. increased fracture risk.

Osteoblast differentiation mechanisms and regulation have been studied extensively using murine models, human primary osteoprogenitor cells and skeletal cancer cell lines from osteosarcoma patients [28,53–59]. Osteogenic differentiation is a multi-step process that is regulated temporally and

spatially. Disruption of these steps lead to failure of MSC osteogenic differentiation, which based on our current knowledge, is a common observation in cells extracted from NF1 PA biopsies [60,61].

Osteogenic differentiation is commonly considered a three-step process (**Figure 3**). In the first step, upon receiving osteogenic cues, MSCs commit to the osteoblast lineage and express markers of this lineage including RUNX2, Osteopontin (*Spp1*) and Collagen type I (*Col1a1)* and downregulate expression of progenitor markers e.g. inhibitor of DNA binding 4 (*Id4*) [62,63]. These cells are pre-osteoblastic and are still able to proliferate. For *in vitro* studies, ascorbate treatment is used to initiate collagen maturation and osteogenic differentiation [14]. Additionally, recent studies have shown that ascorbate treatment can epigenetically change chromatin and hence cells fate [64]. *In vivo*, ascorbate play similar roles in the maturation of collagen, while Indian Hedgehog (Ihh) and Sonic hedgehog (shh) signaling are important in the stimulation of the osteogenic differentiation process during embryonic development [65]. Members of TGFβ superfamily are another class of growth factors that play an important role in bone formation and osteogenic differentiation *in vivo* [66]. RUNX2 is an important factor in bone formation in both endochondral and intramembranous bone formation and in the commitment of MSCs towards pre-osteoblasts (see below). In the absence of Runx2 expression/activity, osteoprogenitors do not commit to osteoblast lineage and a mineralized skeleton does not form[67,68].

In the second stage of osteogenic differentiation, RUNX2 activates the expression of *Osx* (aka *Sp7*) and as the result of synergistic transcriptional activity of RUNX2 and SP7, the production of Collagen type 1 increases. Cells begin to express Integrin binding sialoprotein (*Ibsp)* and Alkaline phosphatase (*Alpl)*. At that point, the bone matrix is mainly composed of non-mineralized collagen and cells stop proliferating and assume the characteristic cuboidal shape of osteoblasts [16].

In the final phase, cells express *Alpl* and *Bglap* and mineralization of the extracellular matrix begins. Two major functional activity at this stage are mediated by Osteopontin and Alkaline phosphatase. Alkaline phosphatase breaks down pyrophosphate and provide phosphate for formation of hydroxyapatite and Osteopontin creates a center that crystals of hydroxyapatite form around it [69].

Over expression of *Runx2* and *Sp7* in immature osteoblasts has negative effect on the skeleton, and is associated with increased numbers of immature osteoblasts (expressing Osteopontin) and reduced number of mature osteoblasts expressing Osteocalcin. Hence these mice develop osteopenia and reduced cortical thickness [70,71]. This example highlights the importance of the temporal regulation of transcription factors during osteogenesis. Ectopic expression of *Runx2* in chondrocytes during development can have pathological consequences including formation of bone in cartilage tissues that are never calcified in normal physiology. These examples illustrate the importance of spatial regulation of these transcription factors during osteogenesis [72].

### Transcriptional regulation of osteogenesis

There are multiple transcription factors that control osteogenic commitment and osteogenic differentiation. The master regulator of osteogenesis is RUNX2 (a protein discovered in Drosophila important for body patterning [73]), a Runt-containing DNA-binding transcription factor that controls the expression of many osteogenic markers including SP7, Alkaline phosphatase and Osteocalcin (*Bglap)*. Mouse models with global deletion of *Runx2* lack any mineralized bone and die postpartum as the result of respiratory failure [67]. Because one chapter of my thesis is dedicated to the biology of Runx2 and its role in the reduced osteogenic differentiation of *Nf1*-deficient

osteoprogenitors, readers are directed to that chapter of my thesis for further information on the biology of this master transcription factor.

Osterix (SP7) is a zinc finger transcription factor with homology to the SP transcription factor family. Global deletion of this gene leads to an unmineralized skeleton in embryos accompanied with the normal expression of Runx2 [74]. Similar to *Runx2* null mice, *Osx* null mice die shortly postpartum as a result of respiratory failure [74]. These data show the mineralization of the skeleton requires the presence and action of both Ruxn2 and Sp7 simultaneously and that RUNX2 is an upstream regulator of *Sp7*. Osteoblast-specific deletion of *Sp7* resulted in osteopenia due to the inhibition of osteoblast differentiation in adult mice [75]. This protein binds to promoter of several osteogenic genes including *Col1a1 and Ibsp* and activates their transcription [76,77]. However, overexpression of *Sp7* in mature osteoblasts was shown to reduce the bone formation and decrease the expression of osteogenic markers e.g. *Ibsp* [71].

ATF4 –contrary to RUNX2 and SP7- is a transcription factor that regulates the maturation of osteoblasts and act as an accessory transcription factor for RUNX2. This protein binds to the *Bglap* promoter (although to a separate recognition site in the *OG2* promoter than the RUNX2 recognition site[78]) and induces the expression of Osteocalcin. Lack of *Atf4* causes delayed skeletal formation and bone postnatal accrual [78,79]. An earlier study by Yang and colleagues showed that this transcription factor is important for amino acid transport into osteoblasts and hence matrix formation [78]. Elefteriou and colleagues proposed a causal relationship between the increased activity of ATF4 and the bone phenotype in the $Nf1_{Col1}^{f/f}$ mouse model [80]. Although this mouse model show some of the phenotypes observed in NF1 patients e.g. delayed bone healing and increased osteoidosis, this mouse model does not recapitulate other clinical findings such as

reduced bone mineral density (BMD). We currently do not know whether any of these transcription factors are affected by *Nf1* loss of function.

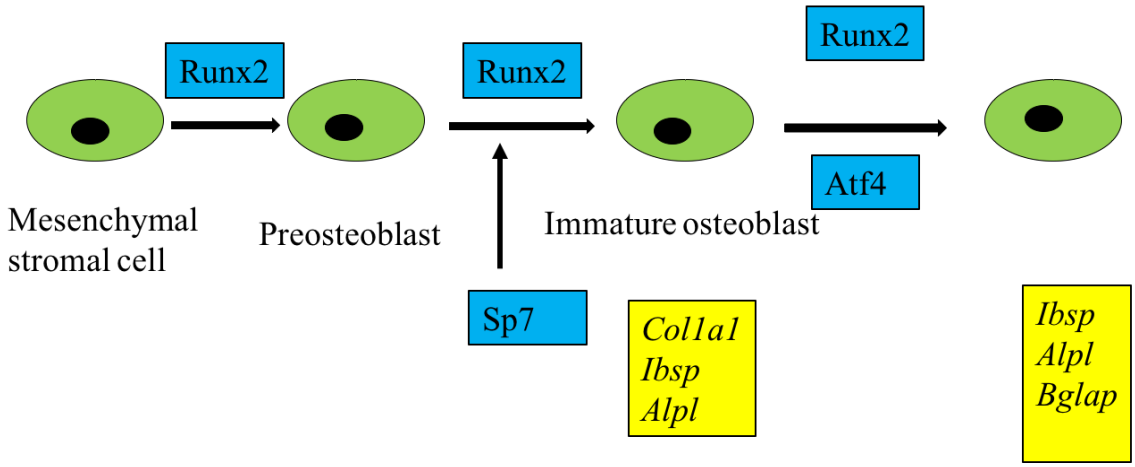



**Figure 3. Simplified schematic of the osteoblast differentiation process**
Osteogenic differentiation is a multi-step process that is precisely regulated. Mesenchymal stromal cells upon Runx2 activity can promote osteogenic differentiation. In later stages two other transcription factors Sp7 and Atf4 induce the maturation of committed osteoprogenitors. Mature osteoblasts express unique markers e.g. *Bglap.*

## Cytokines

### Bone morphogenetic protein (BMPs)

Bone morphogenetic proteins (BMPs) belong to the TGFβ superfamily. Members of this superfamily have diverse roles in bone formation (**Figure 4**) [81–86]. They bind to heterodimeric serine threonine kinase receptors (Activin receptor Like Kinase: ALKs) and activate the transcription of their target genes. The identity of the target genes depends on the ligand, the receptor dimer that ligand binds to and the intracellular downstream signaling that is activated. BMPs are the best-known example of factors that stimulate osteogenic differentiation and have been used extensively in the study of molecular mechanisms important for bone formation and osteoblast differentiation.

Upon binding of BMPs to one of several isoforms of BMPRI receptors (ALK1-3 & ALK6), a conformational change occurs that makes this receptor a target for BMPRII, a kinase that is constitutively active. Phosphorylation of BMPRI by BMPRII activates downstream canonical and non-canonical signal transduction pathways. The canonical downstream signaling pathway is initiated with phosphorylation of receptor regulated-SMADs (SMAD1,5,8). These SMADs upon phosphorylation co-translocate to the nucleus with common-SMAD (SMAD4) and regulate the transcription of their target genes [87]. This regulation occurs either with direct binding of SMADs to the SMAD Binding Elements (SBEs) in the promoter of these genes such as inhibitor of differentiation 1 (*Id1*) [88] or through interaction with other transcription factors (e.g. RUNX2) [89].

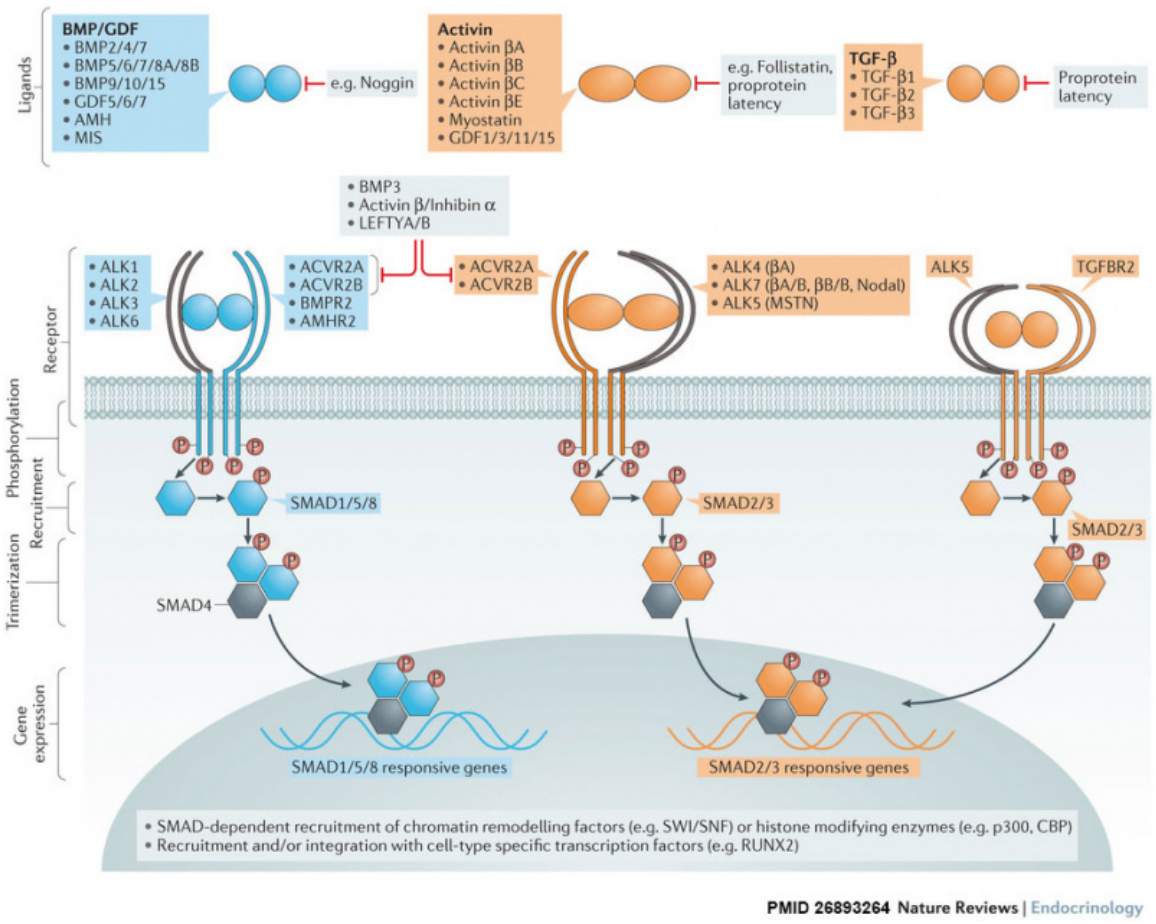



**Figure 4. TGFβ superfamily**

TGFβ superfamily is composed of three separate families that bind preferentially to their specific receptor and activate unique intracellular pathways. Members of BMP family after binding to their receptor, activate SMAD 1,5,8, while members of TGFβ and Activin family, activate SMAD2,3. All three families have antagonists in extracellular matrix such as Follistatin and Noggin which sequester the ligands and inhibit the binding of ligands to their specific receptors. (From Salazar VS, *et al*, 2016 [90]).

In non-canonical signaling pathways, after activation of BMPRI and BMPRII, the intermediate signaling molecules TAB1, TAK1, and XIAP become active and activate p38 and JNK [91]. ERK activation upon BMP2 treatment has been documented too [92].

### Transforming Growth Factor β (TGFβ)

TGFβ family members are the most abundant cytokines in the bone matrix. They are secreted in an inactive precursor form and are embedded in bone until they get activated by enzymatic activity of proteins such as matrix metaloproteinases (MMPs). Upon release from their precursor form, these mature cytokines bind to one of type 1 receptors (ALK4,5,7) which causes conformational change in the receptor. This conformational change forms phosphorylation sites for the TGFBR2 receptor. Constitutive kinase activity of this receptor leads to active TGFBR heterodimer that can phosphorylate and activate SMAD2,3. pSMAD2,3 translocate to the nucleus along with SMAD4 and there SMADs can activate the transcription of target genes. Similar to members of the BMP family, TGFβ family members can activate non-canonical (e.g. MAPK) signaling pathway (**Figure 4**).

There are three isoforms of TGFβs with distinct physiological roles in embryonic development [93–95]. Studies of the role of these factors on bone formation and osteogenic differentiation has led to contradictory results. For example, although Noda and colleagues reported that local injection of pig TGF-β1 in rat tibial bone led to increase bone formation [96], Edwards and colleagues reported that daily injection of anti-TGF-β1 antibody (1D11) has anabolic effect on bone formation in mice [97]. In general, TGFβs increase migration and the rate of proliferation of osteoprogenitors [98]. Studies in patients have shown that both lack and increased activity of TGFβ1 lead to skeletal maladies [99].

Similarly, *in vitro* studies on the role of this growth factor has shown pro-osteogenic and anti-osteogenic roles at different stages of differentiation. In a study to assess the effect of TGFβ1 on human osteoprogenitors and immature osteoblast populations, TGF-β1 treatment increased proliferation and Alkaline phosphatase activity, whereas it repressed the commitment of osteoprogenitors cells to the osteoblast lineage [100]. TGF-β1 treatment on early osteoprogenitors decreased the expression of *Runx2* and *Bglap* and hence reduced osteogenic differentiation [101]. These studies highlight the importance of temporal regulation of this cytokine during osteogenic differentiation. It has been shown that TGF-β1 could inhibit the formation of mature osteoblasts [101,102], through inhibition of ATF4 function [103].

### Insulin and insulin like growth factors

Insulin and insulin like growth factor (IGF-1) are two evolutionary conserved proteins whose effect on body growth has been known for decades. Mutations in these genes lead to poor bone quality and increase risk of fracture [104,105]. Targeted deletion of the IGF1 receptor in osteoblasts, using the *Igf1r*$^{f/f}$ mouse model, leads to decreased mineralization of newly formed bone and reduced trabecular bone volume, although the number of osteoblasts is not reduced, which suggests that osteoblast activity was affected in this condition [106]. Similarly, deletion of the Insulin receptor in *Insr* KO mouse model causes failure to reach peak bone mass, however, contrary to *Igf1r* KO mice, *Insr* KO mice have reduced osteoblast number [107]. The pro-osteogenic action of Insulin and IGF-1 relies on their modulation of the osteogenic master transcription factor, Runx2 [108,109].

### Fibroblast growth factors (FGFs)

Fibroblast growth factors (FGFs) are a family of cytokines composed of 22 members that bind to four different types of receptors (FGFRs) [110] and activate several downstream signaling pathways including PI3K, MAPK and PLCγ. Mutations in FGFs and FGFRs cause skeletal maladies from dwarfism to craniosynostosis [111]. This diversity of symptoms highlights the important role that this family of growth factors plays during both embryonic development and postnatal skeletal accrual maintenance. From these 22 members, FGF2 is the most pro-osteogenic factor. FGF2 increases the proliferation rate of osteoprogenitors, the activity of RUNX2 and osteogenic marker gene expression. FGF2 activates PI3K, ERK1,2 and PKC [112–116]. Another important member of FGF family, FGF4, has similar effects on bone formation and its deletion leads to reduced bone formation in mice [111].

### Epidermal Growth Factors (EGFs)

The Epidermal Growth Factor (EGF) family is composed of seven structurally similar growth factors. These include Amphiregulin (Areg), Betacellulin (Btc), Epidermal growth factor (EGF), Epigen (Epgn), Epiregulin (Ereg), heparin-binding EGF like growth factor (HB-EGF) and transforming growth factor-α (TGFA). These members share a common structure and are expressed as a transmembrane precursor that are cleaved and become active upon action of various peptidases e.g. Matrix Metalloproteinases (MMPs) [117]. EGF family members bind to a family of Receptor Tyrosine Kinases (RTKs) called epidermal growth factor receptors (EGFRs) or ErbBs. There are four known members in this family and each EGF family member has a different affinity for EGFR family members (**Figure 2**). Upon binding of the cognate ligand to its receptor, receptor dimerization (either homo-dimerization or hetero-dimerization) reaction occurs, the intracellular

domain of EGFRs auto-phosphorylate themselves and this conformational change creates a docking site for interaction with adapter molecules such as Src Homology domain-containing proteins Grb2. This in turn activates Son of Sevenless (SOS), a GTP Exchange Factor (GEF) that facilitates the exchange of GDP-RAS (inactive form of RAS) to GTP-RAS (active form of RAS) and RAS downstream signaling pathways including MAPKs become active.

*Egfr* KO mice die postnatally in the first week of their life as a result of respiratory problems and epithelial immaturity in various organs [118]. These mice develop skeletal defects both in the craniofacial and long bones during embryonic development [119,120]. The same phenotype is observed in hypomorphic mouse models of *Egfr*, however, this mouse line has longer lifespan and hence the phenotypes in adult mice can be investigated [121]. These mice have reduced weight and larger hypertrophic zone in their growth plate [121].

*Ex vivo* studies on *Egfr* KO and *Egfr* hypomorphic calvaria osteoprogenitors have shown reduced proliferation and premature osteogenic differentiation [121]. These findings suggest that EGFR signaling in osteoprogenitors stimulates their proliferation and inhibits their osteogenic differentiation, a behavior characterizing osteoprogenitors deficient for *Nf1*.

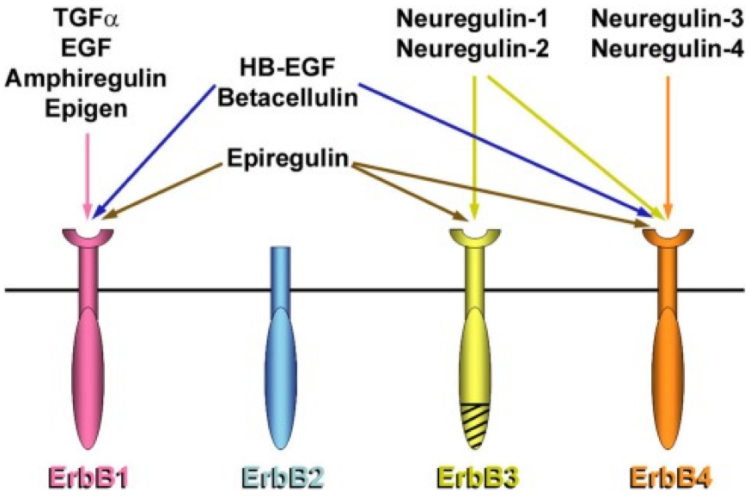



**Figure 5. EGFR family members and their ligands preference**
Four isoforms of the ErbB (EGFR) family are depicted here with their preferred ligand specificity. Epiregulin can bind preferably to ErbB1 and ErbB4 and activates EGFR downstream signaling (From Clasadonte J, *et al*, 2011[122]).

*In vitro* studies in rodent osteoprogenitors have provided further confirmation that EGF signaling in osteoprogenitors has an inhibitory effect on differentiation and stimulatory effect on proliferation. EGF treatment of murine osteoprogenitors increased the rate of DNA synthesis and reduced the rate of collagen synthesis [123,124].

Treatment of rat bone marrow stem cells with increasing doses of EGF decreased *Opn* expression, ALP activity, and mineralized nodule formation (the latter two are proxies for osteoblast function) [125]. Other studies have shown that treatment with EGF reduces the expression of the osteogenic gene markers *Opn*, *Alpl*, *Ibsp* and *Bglap* [126]. This effect is probably mediated through decreased expression of two master osteogenic transcription factors, Runx2 and Osterix [127]. Two other members of EGF family, HB-EGF and Betacellulin- also have similar inhibitory effect on osteogenic differentiation [128,129].

## Hormones

### Parathyroid hormone

Parathyroid hormone (PTH) belongs to the family of peptide hormones. PTH is secreted from the parathyroid glands and its major role is to control the level of calcium via bone resorption and calcium reabsorption from kidneys [130]. Administration of PTH can have two opposite outcomes on the skeleton: PTH intermittent injections increase bone formation (anabolic effect), while continuous administration leads to increased bone resorption (catabolic effect) [131]. Over expression of PTH as the result of hyperparathyroidism lead to bone loss [132]. Therapeutically, administration of the first 34 amino acid or the complete form of this peptide hormone has shown promising

results in osteoporotic patients [133,134]. PTH administration can trigger bone formation through three distinct mechanisms. PTH can directly act on osteoblasts to promote osteoblastogenesis [135]. *In vitro* and *in vivo* studies have shown that PTH treatment leads to increased expression of osteoblastic markers, *Runx2*, *Col1a1* and *Bglap* [136]. PTH can also decrease the apoptosis of the osteoblasts [137], via inactivation of the pro-apoptotic protein BAD, increased expression of survival proteins such as BCL-2, and increase of DNA repair [138,139]. Lastly, there are some evidences that suggest that PTH exposure can reactivate bone-lining cells, which are quiescent cells believed to be physiologically inactive on bone surfaces that could explain the increased numbers of observed osteoblasts in PTH-treated animals [140]. Continuous PTH treatment can trigger increased osteoclast activity and is mediated through increase in the expression of RANKL in osteocytes and osteoblasts [141].

### Estrogen

Estrogen is important hormone whose role in bone homeostasis is well known. Around 80 years ago, Albright characterized an idiopathic osteoporosis that was observed mostly in postmenopausal women [142]. Further studies showed that these cases were attributed to the loss of estrogen function in bone and later studies showed that estrogen therapy could reduce the risk of fracture in postmenopausal women [143]. Although, estrogen deficiency leads to increase osteoblastogenesis, this increase is nullified by increase apoptosis of these osteoblasts [144,145]. On the other hand, estrogen reduces the membrane expression of RANKL and hence decrease osteoclast formation, which is mediated through regulation of Runx2 [146,147]. Hence, the net effect of estrogen deficiency is increased osteoclastogenesis and bone resorption. Murine studies have shown that estrogen deficiency also leads to increase expression of pro-inflammatory cytokines

e.g. TNFα and IL-1. Human trials have shown that blocking these cytokines can abrogate the bone loss caused by estrogen deficiency [148].

## Mechanical signaling

It is a well-known concept that physical exercise increases bone density. A very good example is observed in tennis players, who have higher bone density in the dominant arm compared to the contralateral arm [149]. The opposite is also true; reduced loading as the result of metabolic diseases (e.g. body weight loss), injury (e.g. bedrest) and professional conditions (e.g. spaceflight) leads to reduced bone mass [150]. The effect of unloading on skeleton biology is especially apparent in microgravity conditions. Various groups have reported that astronauts on flight missions lose up to 1.5 % of their bone density for each month in outer space. This condition in turn, increases the risk of fracture and reduce the number of space missions that these astronauts can participate [151–153]. These observations support the presence of biomechanical loading sensors in cells responsible for bone homeostasis. Furthermore, it was shown that constant low magnitude mechanical signals can increase the volume fraction of trabecular bone, even in the presence of high fat diet [154].

Studies on the role of mechanical strain in bone suggest that every cell type in the osteoblast lineage from MSCs to osteocytes can act as mechanosensors. Mechanical strain in MSCs activates ERK1,2 and enhances *Runx2* expression and matrix mineralization [155–157]. Osteoblasts also can be stimulated by mechanical signals. It is shown that secretion of prostaglandins, that can activate osteoblast activity, is increased in osteoblast-like cells (MC3T3) after fluid shear stress [158]. Lastly, osteocytes, the major cell type in the bone, are specifically suited to detect and respond to the

mechanical signals because of the presence of canaliculi (a network of protrusions from osteocyte cell body similar to axons of a neuron).

Mechanical signals such as shear fluid stress can activate intracellular signaling via different pathways (**Figure 6**). It is proposed that sensing mechanical signals in bone cells cannot be attributed to only one receptor type, but rather to the synchronized action of several receptors that can sense and cause the orchestrated response to these stimuli. Lipid rafts in the plasma membrane can detect shear fluid stress and this causes change in various intracellular signaling molecules such as PKC. Cells tether to their extracellular matrix using different molecules, such as Integrin. Integrin are formed from hetero-dimerization of one alpha and one beta chain, where different combinations can have various expression pattern in different cell types and play diverse roles in different organs[159]. Mechanical stimulation leads to aggregation of Integrin molecules and this aggregates creates a docking site for other signaling molecules such as focal adhesion kinase (FAK), that leads to targeted increase in activation of intracellular signaling including MAPKs [160]. Another cell surface proteins that can act as mechanoreceptor are Cadherin molecules These are a class of integral membrane glycoproteins that associate with several cytoplasmic proteins such as α and β-Catenin [161]. Mechanical stimulation can cause dissociation of β-catenin from N-Cadherin [162]. Released β-Catenin can be translocated to the nucleus and activate the transcription of its target genes [163]. As a result of coordinated action of these receptors bone cells sense mechanical signals and bone formation and resorption is regulated.

Mechanical signals are important for MSCs linage determination. Adipocyte and osteoblast formation are reciprocal processes that can be impacted by mechanical stimuli. It has been shown that exercise increases the osteoblast population of bone marrow and reduce adipocytes formation

[165]. *In vitro* studies also showed that mechanical signaling can affect osteogenic differentiation through β-catenin and GSK-β [163]. Plating MSCs on stiff substrates -without any exogenous growth factors- increases the expression of *Runx2, Alpl* and *Bglap* in a β1 Integrin-dependent manner [166,167]. These findings suggest that osteoprogenitors differentiation can be impacted by changes in the biomechanical properties of their ECM.

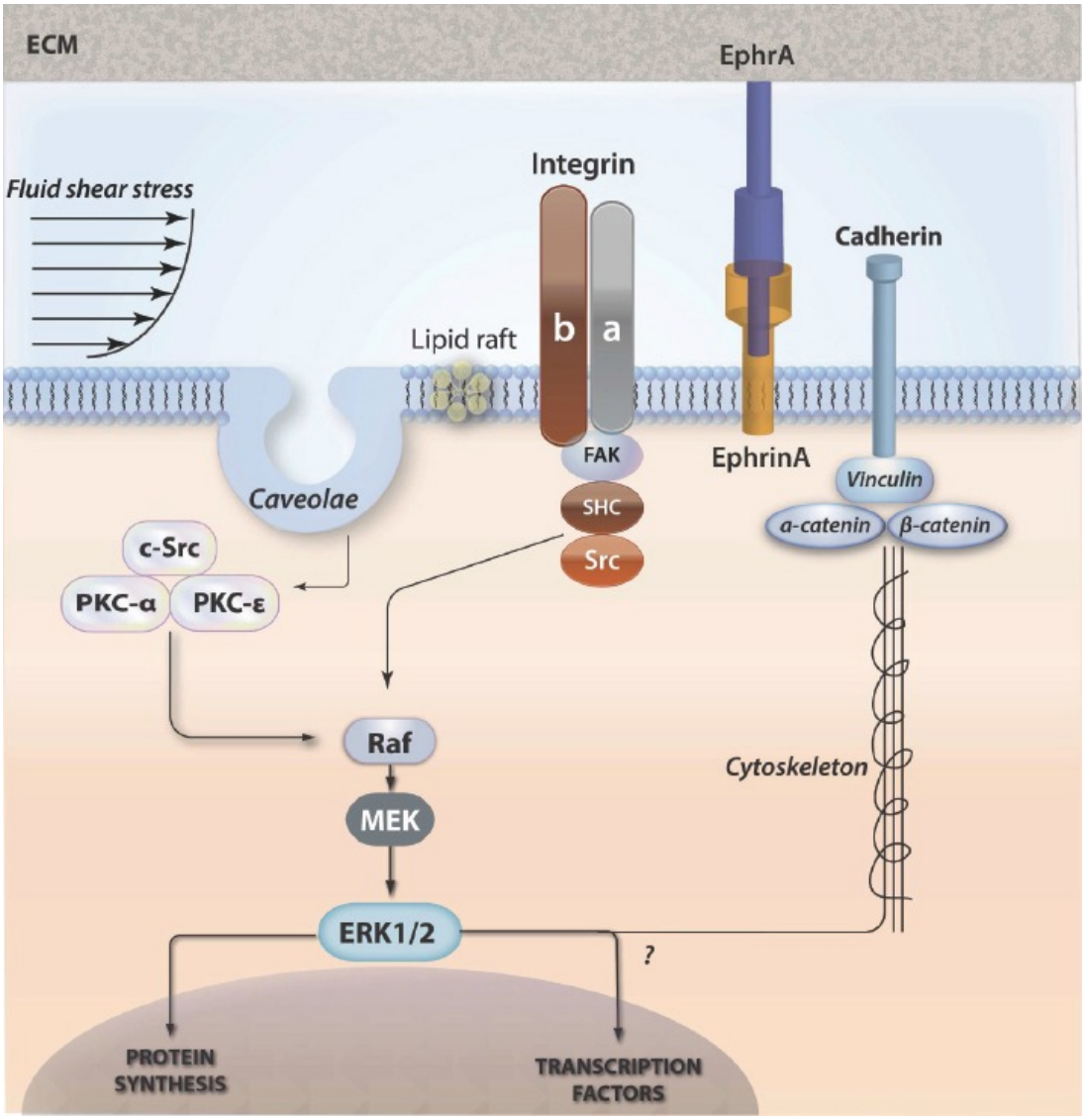



**Figure 6. Mechanoreceptors**

Mechanical signals are detected by various structures in bone cells. In this schematic representation, a few examples of mechanosensors are displayed. Lipid rafts, Integrin, cell to cell transmission of signals via Ephrins and Cadherins are among some of the candidate mechanoreceptors that bone cells use to detect mechanical signals such as fluid shear stress in their extracellular environment. Activation of mechanosensors changes intracellular signaling pathways such as ERK1,2, which affect protein synthesis e.g. Collagen type 1 and impact transcription factors such as *Runx2* (From Yavropoulou MP, Yovos JG, 2016 [164]).

## Role of MAPK in osteogenic differentiation

Mitogen activated protein kinases are a family of well-conserved signal transduction molecules that regulate multiple activities in the cells, from proliferation to differentiation and apoptosis. These molecules respond to extracellular signals such as growth factors, stress, radiation and phosphorylate their target proteins on serine threonine residues. There are three classical types of MAPKs, that are commonly known by their final effectors: ERKs (extracellular signal regulated kinase), JNKs (c-Jun Terminal kinase), and p38. Activation of these kinases is a multi-tiered process (including three kinases) that is finely controlled. These three tiers include MAP Kinase Kinase (MKKK), MAPKK (MKK) and MAPK. MAPKKKs is the upstream activator that receives the signal, MAPKK is the intermediate transducer and MAPK is the final effector. In the case of classical MAPK (ERK1,2), the upstream activators are RAFs (A, B, C) that receive the signal from RAS, the intermediates transducers are MEK1,2 and the final effectors are ERK1,2 (**Figure 7**).

The main focus of my dissertation is the classical MAPK ERK1,2 and hence in this section, I will focus mostly on the role of this kinase in skeletal homeostasis and osteogenic differentiation. The role of JNK and p38, other classical MAPKs, will be only briefly mentioned.

Deletion of members of p38 family of MAPK (*Mkk3*, *Mkk6* and *Mk14* and *Mk11*) in skeletal cells lead to decreased bone mass, which is secondary to an osteoblast differentiation defect [169]. In line with these genetic data, inhibition of p38 using the pharmacological inhibitor SB203580, has inhibitory effect on the differentiation of WT calvarial cells. It has been shown that RUNX2- the master transcription regulator of osteogenesis- is phosphorylated and activated by p38 and p38 kinase activity is necessary for proper function of RUNX2 [170].

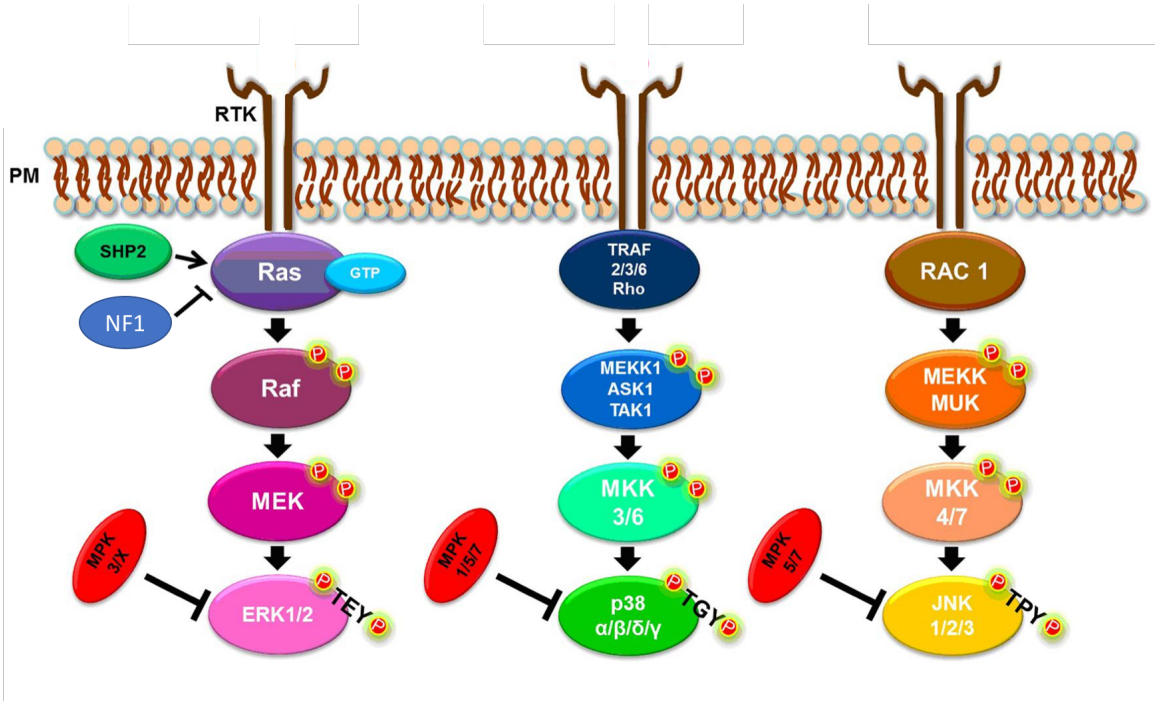



**Figure 7. Simplified schematic of MAPK pathways**

Activation of cell surface receptors such as receptor tyrosine kinases, activate a transducer that interact at the cytoplasmic side of the plasma membrane with these activated receptors. The signals from this transducer is transmitted to MKKKs, MKKs and the final effector MKs. For example, RAS activation signal is transferred to RAF, then MEK1,2 and at the end to ERK1,2. Proteins such Src Homology protein 2 (SHP2) act as guanine exchange factors (GEFs) for RAS and facilitate the exchange of GDP with GTP and hence activate them. Neurofibromin on the other hand negatively regulates RAS signaling by facilitation of RAS GTP to RAS GDP and hence inhibits the activation of ERK1,2 activity (modified Soares-Silva M, *et al*, 2016 [168]).

JNK contribution in proliferation, differentiation and response to extracellular stimuli is documented [171]. There are three isotypes of JNK: JNK1, JNK2, and JNK3. JNK major role is in response to extracellular stress signals such as ultraviolet radiation and reactive oxygen species. It has been shown that global deletion of *Mapk8* leads to decrease apoptosis in response to U.V radiation [172]. JUN, the target of JNK kinase activity, also becomes active in response to pro-inflammatory signals. These signals have negative effect on osteogenic differentiation and increase osteoclastogenesis [173,174].

ERK inhibition by a retrovirus encoding a dominant negative ERK1 form led to decrease Alkaline phosphatase activity (a proxy for osteoblast differentiation) and calcium nodules deposition (a proxy for ECM mineralization capacity of osteoblasts) in human mesenchymal osteoblasts [175]. Additionally, it has been shown that ERK1,2 inhibition, whether through introduction of a dominant negative ERK protein or a MEK1,2 pharmacological inhibition, leads to a switch from osteogenic differentiation and activation of RUNX2 to adipogenic differentiation and activation of PPARγ [176]. Murine calvarial cells expressing dominant negative form of MEK (DN MEK) under control of the mouse Osteocalcin promoter (*mOG*) showed decreased osteogenic markers gene expression, Alkaline phosphatase activity and mineral deposition *ex vivo*. On the other hand, expression of constitutive activated MEK (sp MEK) under control of the same promoter led to increased osteogenic differentiation [177]. The *in vivo* study of these mice showed increased mineralization and femur length that was growth plate-independent in sp MEK mice and the opposite phenotype was observed in a DN MEK mouse model. This mouse model shows that ERK1,2 activity in mature osteoblasts has anabolic effects on long bones.

Inactivation of ERK1,2 in osteoprogenitors by using the $Erk1^{-/-}$; $Erk2^{f/f}{}_{prx}$ mouse model caused severe skeletal malformations. Expression of *Runx2* and *Col1a1* was not affected in these mice,

which indicated that the expression of these early markers is not controlled by ERK activity. However, *Bglap* expression was downregulated and ECM mineralization was reduced in these mice, which is in accordance with previous reports showing that Osteocalcin expression is dependent on RUNX2 activity, which in turn depends on ERK activity. Another interesting observation from this study was that lack of ERK causes cartilage formation in the perichondrium where osteoblasts normally form, which suggests that absence of RUNX2 activity, which to some extent is dependent on ERK1,2 phosphorylation in addition to other factor [178], is necessary for MSCs lineage fate determination [179]. All in all, these studies indicate that ERK activity is required for osteoblast formation and activity and that this signaling pathway may have different roles at different differentiation stages.

At the molecular level, ERK1,2 activation in osteoprogenitors leads to the phosphorylation of RUNX2, especially at Serine 308, an activating phosphorylation site [54,178]. This phosphorylation increases the transcriptional activity of RUNX2 by increasing the binding of RUNX2 to the promoter of its target genes [180]. It also has been reported that p-ERK1,2 physically interacts with chromatin and the interaction of these two molecules is necessary for transcription of osteogenic marker genes [181]. This study showed that RUNX2 is necessary for p-ERK1,2 recruitment to chromatin and while the interaction of RUNX2 with chromatin remains constant, p-ERK1,2 interaction with chromatin increases over the course of osteogenic differentiation. Overall, previous studies have shown stimulatory effect of ERK1,2 on osteogenic differentiation. This effect is mediated through proliferation and increasing the number of osteoprogenitors, phosphorylation and hence activation of RUNX2 and induction of osteogenic marker genes. Therefore, interruption of this pathway, has deleterious effects on osteogenic differentiation.

Because of the importance of these signaling molecules, many pathological conditions are associated with the mutations in the genes that encode different members of this pathway. One of these conditions is called Neurofibromatosis type 1 (NF1).

## Neurofibromatosis type 1

Neurofibromatosis type 1 (NF1) is a common genetic disorder that occurs in 1 out of 3500 live births [182]. The NF1 condition has been known for many years as a neuro-cutaneous conditions. Accurate description of this condition was finalized by a physician named Von Recklinghausen, and to his honor it was named after him [183]. The term Neurofibroma, a benign peripheral nerve sheath tumors, was introduced in the end of 19$^{th}$ century [183].

NF1 is a pleiotropic disease with manifestations in multiple organs [184]. Most common manifestations include pigment abnormalities such as skin fold freckling and café au lait macules in skin; Neurofibroma in peripheral nerves, Lisch nodules (benign hamartoma of the eye) and skeletal maladies. In addition, NF1 can cause malignant peripheral nerve sheath tumor (MPNST) and optic nerve glioma, both associated with high mortality [185].

The NF1 skeletal manifestations are divided into two categories: general and focal. General manifestations include osteopenia (mild bone mineral density loss) and short stature. Focal manifestations include asymmetry of facial bones (sphenoid bone dysplasia), chest wall abnormalities, idiopathic or dystrophic scoliosis, and unilateral anterolateral bowing of tibia; the latter condition can progress towards fracture and non-union after fracture or pseudarthrosis (PA) (**Figure 8**) [186].

NF1 patients are born with only one functional copy of the *NF1* gene. Tibia bowing and pseudarthrosis in NF1 patients is usually unilateral. This observation motivated researchers to investigate the possibility of double hit mutations in the population of the cells that make-up the PA site. Sequencing the biopsies from the PA site of the patients has revealed loss of heterozygosity in the majority of NF1 PA cases [187,188]. Therefore, it is currently thought that the genetic set up of the bowed NF1 tibia consists of a mixture of *NF1* heterozygous cells and cells characterized by double hit *NF1* inactivating mutations, whose presence alters bone quality, strength and repair.

### NF1 gene and protein

NF1 is caused by mutations in the *NF1* gene that encodes Neurofibromin, a 320 kDa protein with diverse molecular function (**Figure 9**) [189,190]. The *NF1* gene was identified at the end of the 80's by linkage analysis [191–193]. It is located in the centromeric region of the long arm of chromosome 17, spans over 350 kb of DNA, and encompasses over 62 exons. Neurofibromin binds and regulates the activity of several signaling molecules including protein kinase A, Focal adhesion kinase and Ras-MAPK [189,190,194,195]. The latter interaction is the most studied function of Neurofibromin and is believed to be responsible for the formation of neurofibromas and MPNST. Therefore, ongoing therapies target the MAPK regulated function of Neurofibromin. Modulation of RAS/MAPK signaling by Neurofibromin is mediated through action of its GTPase activating related domain (GRD). Other characterized domains of Neurofibromin include a Cysteine/Serine rich domain (CSRD), Sec14-PH, and a FAK binding domain.

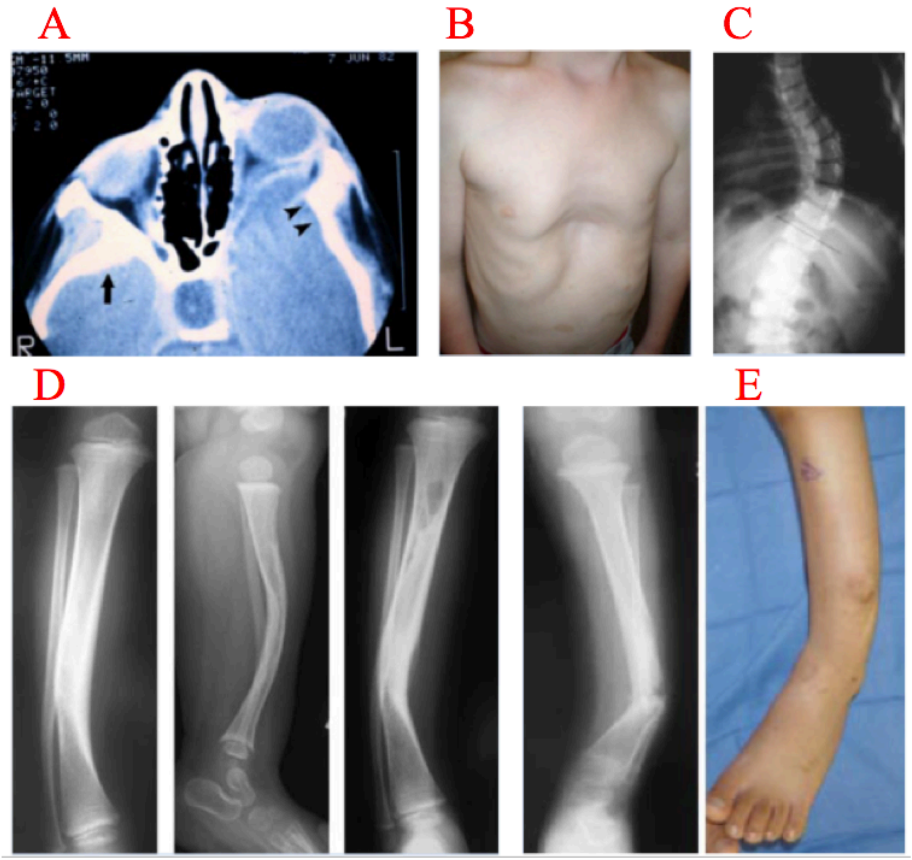



**Figure 8. Focal NF1 Bone Manifestations**
Focal NF1 bone manifestations include: (**A**) sphenoid wing dysplasia in the left side of the bone (arrowheads) showing abnormal structure compared to the contralateral side (arrow), (**B**) pectus excavatum, (**C**) scoliosis (both non-dystrophic, shown, 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) (From Elefteriou F, *et al*, 2009 [1]).

Neurofibromin is an orthologue of p120 GAP. The major known function of Neurofibromin is the negative regulation of RAS signaling. RAS proteins acts as mediator of signaling and can become active by the action of multiple mediators including receptor tyrosine kinases, e.g. EGFR or FGFR. Inactivation of these proteins occurs when the nucleotide in the central active domain of RAS is converted to GDP. Based on the spontaneity of this reaction, RAS superfamily is divided into two groups: In one group this reaction happens very fast and hence these proteins do not need further assistance with the GTP exchange. However, members of the second group –including RAS p21- need another modulator protein that facilitate the conversion of the GTP form to the GDP form.

Neurofibromin acts as the negative regulator of RAS by facilitating the conversion of RAS-GTP (active form of RAS) to RAS-GDP (inactive form of RAS). The GTPase-related domain (GRD) of Neurofibromin is located in the center of the linear sequence of the protein. However, the position of the domain in regard to the 3-dimensional structure of the protein remains to be determined. Mutations in this gene cause chronic activation of MAPK signaling. Hence the focus of the NF1 field is mostly concentrated on the effect of chronic active MAPK signaling on the pathology of this disorder, although *NF1* mutation associated with NF1 pathologies are known to occur throughout the entire gene and in other domains of the gene.

### Mouse models of NF1

Similar to many genetic disorders, mechanistic studies in NF1 patients have been hampered by many factors, including the rarity of patients' samples. This is an especially restricting factor in the context of the skeletal conditions associated with NF1, because the focal skeletal manifestations are rare among NF1 patients (3-4% of the patients). The heterogeneous genetic makeup of the human population also causes additional complexity in interpretation of the

mechanistic studies. Finally, human bone samples can be obtained only after fracture, thus the early events leading to bowing and fracture cannot be assessed. Despite these limitations, mechanistic studies have been performed *ex vivo* on biopsies from samples of the pseudoarthrotic site from NF1 patients, which have provided some invaluable clues about the etiology of NF1 PA [60,61] (see below). To overcome these limitations, several mouse models have been developed to study the NF1 disease and its pathogenesis.

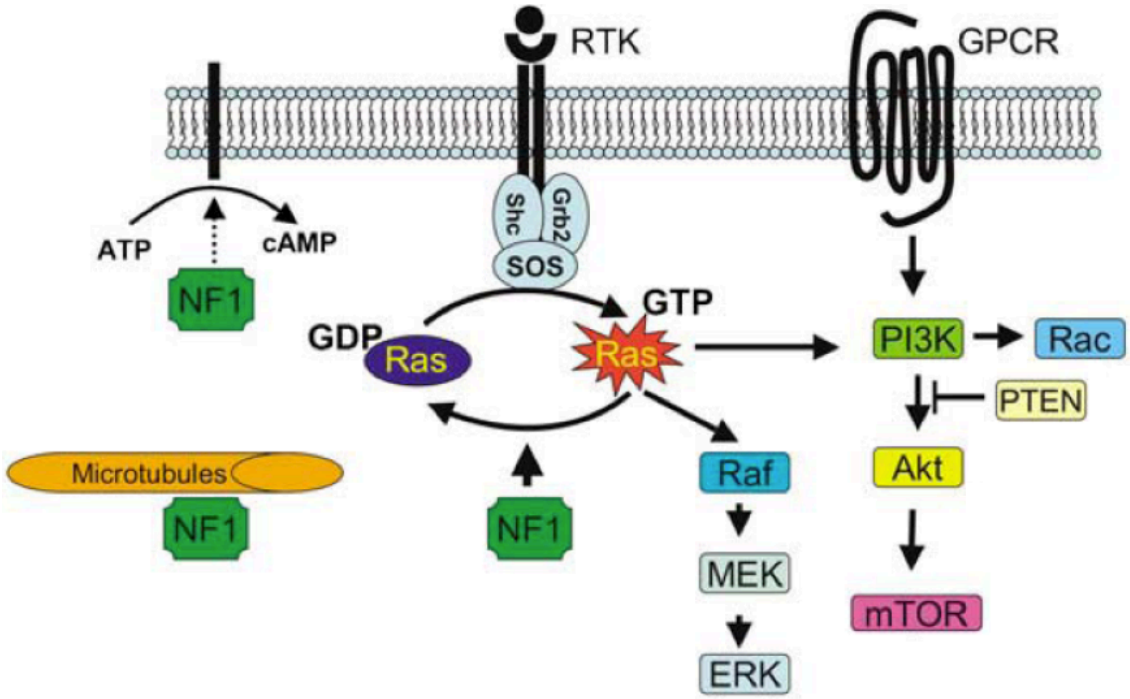



**Figure 9. The functions of Neurofibromin**
The major role of Neurofibromin is to facilitate the hydrolysis of Ras-GTP to Ras-GDP, thus inactivating main downstream signaling modulators of RAS that are MAPK and PI3K. Neurofibromin can also simulate Adenylyl cyclase activity and interact with microtubules. Modified and used with permission (From Le LQ, Parada LF, 2007 [196]).

The first approach to generate a global *Nf1* KO mouse model was done simultaneously by two groups through targeting exons 31 of the *Nf1* gene (this specific exon is important for the GTPase activity of Neurofibromin). These researchers discovered that the Mendelian ratio was disturbed and upon genotyping the offspring they discovered that *Nf1* global inactivation leads to embryonic lethality at day 13 post-coitum as the result of abnormal cardiac development [197,198]. Embryonic lethality explains the observation that there is no known NF1 patient with global *NF1* loss of function.

*Nf1* heterozygote mice show tumor disposition at old age, including pheochromocytoma, a benign form of adrenal gland tumor. No skeletal problems were detected in *Nf1* haploinsufficient mice [199–201]. However, at the cellular and molecular levels, changes in RAS activity and osteoclastogenesis were detected in *Nf1* haploinsufficient mice, especially upon challenges such as ovariectomy [202]. In regards to fracture healing, there is a report that delayed healing in *Nf1* haploinsufficient mice depends on the site of the fracture. Mid-shaft tibia fractures heal normally in these mice, while fracture in distal tibia showed delayed healing [201]. However, it is important to note that delayed fracture healing in this study was observed between mid-shaft fractures and distal tibia fractures in *Nf1* WT mouse which suggests that other factors at the site of fracture might influence the rate of delayed bone healing.

Because the *Nf1* global KO mouse model is not viable, the Flox/CRE system has been used for the study of NF1-related pathologic conditions since early 2000s [203] (**Figure 10**). This provides the necessary tools for studying the role of distinct cell types at various stages of differentiation on the skeletal phenotype of NF1.

*Nf1* deletion in mature osteoblasts using CRE Recombinase under control of *2.3 col1a1,* a promoter element that is active in immature committed osteoblasts, was first used by Elefteriou and colleagues [80], who showed that these mice have increased bone mass, associated with osteoidosis (accumulation of non-mineralized matrix) and increased osteoclastogenic activity. However, this increased bone density is in contradiction with the observed phenotype in patients [80]. Upon fracture, these mice show delayed bone healing, with an increased callus size (hypertrophic) [204]. Although NF1 patients can present with delayed bone healing, the callus size in these patients is on the contrary reduced (atrophic) [204]. This model thus does not recapitulate the skeletal conditions in NF1 patients, indicating that the cell of origin for NF1 PA is likely not a committed osteoblast.

Various groups then began to use other CRE recombinase under control of different promoters to target osteoprogenitors and osteochondroprogenitors. The first progenitor targeted promoter used was the *Prx-Cre* mice. The *Prx* promoter is active in the limb buds during embryonic development. $Nf1_{prx}^{-/-}$ mice were short because of severe growth plate abnormalities. More importantly, these mice displayed tibial bowing and fracture healing defect [205,206]. However, there are several drawbacks in using this mouse model. The fact that the *Prx-cre* transgene is active in all of the cells in limb buds (including chondrocytes, muscle and endothelial cells) make this transgene non-specific for osteoprogenitors. Hence because of its nature, this model does not allow one to dissociate the role of *Nf1* in chondrocytes versus osteoblasts. Additionally, *Prx* is only expressed in the appendicular skeleton, while NF1 patients have defects in their axial skeleton as well. All of these factors led researchers to use other systems.

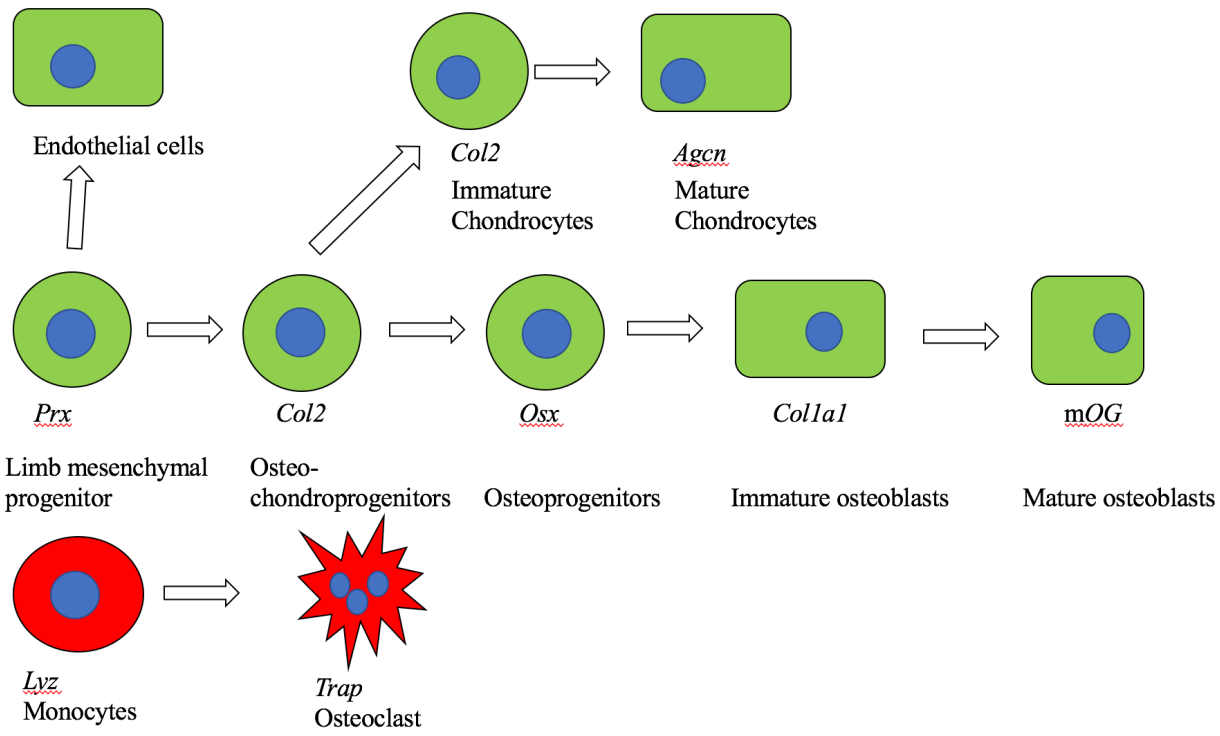



**Figure 10. CRE promoter systems commonly used to study role of *Nf1* in skeletal cells**
CRE systems targeting osteochondroprogenitors (green cells) and HSC progenitors (red cells) at different stages of differentiation are shown here. *Prx* CRE system targets other cell types e.g. endothelial cells. *Col2* CRE system during embryonic development targets both osteoblast and chondrocyte lineage.

Wang and colleagues used the CRE recombinase system under control of the *Col2a1* promoter. These mice exhibited progressive, dystrophic scoliosis, sternal, and craniofacial abnormalities with tibial bowing, osteoidosis, and cortical porosity, the latter phenotype being also observed in human bone biopsies from NF1 PA patients. Osteoprogenitors derived from the bone marrow of these mice showed that *Nf1* deficiency impaired osteogenic differentiation. However, these mice were severely dwarfed and often died shortly after weaning, making the study of fracture healing impossible in this model [207].

The next mouse model that was used to study the effect of *Nf1* deficiency in osteoprogenitors was the inducible $Nf1_{osx}^{-/-}$ mice. N'Dong and colleagues in my laboratory showed that deletion of *Nf1* in adult mice (upon tetracycline withdrawal from drinking water at weaning) have normal size, but their bones exhibited poor biomechanical properties and elevated cortical porosity. Similar to the *Col2* CRE mouse model, BMSCs from this model were also characterized by impaired osteogenic differentiation and mineralization *in vitro*. These mice also have delayed bone healing with an atrophic callus, similar to patients [208,209].

### *Localized inactivation of Nf1*

Each CRE system that has been introduced so far targets the osteoblast lineage in the entire targeted skeleton system, which makes the study of a focal condition, pseudarthrosis, difficult. Because of these shortcomings, some researchers have started to use a more local inactivation system to study NF1 PA.

The unilateral and localized incidence of pseudarthrosis cannot be accurately recapitulated using CRE systems that were mentioned in previous subsections. In order to overcome this obstacle, the

Schindeler group in Australia devised a local injection of a Cre-adenovirus into the site of fracture as an alternative tool for studying the role of *Nf1* in fracture healing [210]. Delayed fracture healing, increased osteoclast number at the fracture site and the presence of myofibroblasts showed the relevance of this mouse model in the recapitulation of these features found in patients. However, this model has a major flaw, as there is no specificity in virus infectivity and all the cells that come into contact with the virus will be *Nf1* null, which is not the case in patients. Therefore, the role of *Nf1* in osteoprogenitors cannot be studied separately from the microenvironment.

### Clinical management of NF1 pseudarthrosis

There are different approaches to manage patients with NF1 tibia bowing and NF1 pseudarthrosis. One approach for the management of NF1 tibia bowing and prevention of fracture is the use of bracing techniques in an attempt to avoid fracture until the age of puberty, after which incidence of tibial fracture decreases.

If fracture occurs, the first approach is the surgical removal of affected fibrotic tissues at the fracture site. In this technique surgeons remove the affected tissue by dissecting, and scraping the fibrotic tissue sub-periosteally at the PA site, until reaching "bleeding" (healthy) bones. This technique can improve union rate when is used in conjunction with intramedullary or external bone stabilization approaches to mechanically stabilize the lesion site, however the fibrotic tissues can regrow and might contribute to refracture of the affected limb. A possible explanation for this failure is that the *NF1* null cells -with double hit mutations- are scattered along the affected limb at both proximal and distal sites (Dr. Legius Laboratory, CTF annual meeting 2016, Austin, TX), and hence the surgical removal of all of the affected tissue/cells cannot be successful. After surgery, remaining *NF1* null cells can proliferate and repopulate the affected limb and affect bone

repair again. To increase the rate of surgery success, marrow tissue from a non-affected bone from the same patient (autologous grafting) is used to provide cells to boost fracture healing [211,212]. However, there are major limitations to this technique, namely rarity of graft tissue and morbidity at the donor site, which makes this approach challenging. Surgery success rate can also be impacted by the age of patient at surgery time, the degree of angulation and deformity of the affected limb, the involvement of fibula and severity of shortening [213–216].

### *Bisphosphonate*

Bisphosphonates are a class of anti-resorptive drugs that have been used extensively in the past decade for the treatment of osteoporosis. This drug, targeting the mevalonate pathway, inhibits osteoclast differentiation and stimulates osteoclast apoptosis [217]. Its use in patients with NF1 PA comes from the observation that *Nf1* haploinsufficiency leads to increased osteoclastogenesis, and thus could be anti-catabolic in the context of bone repair [218,219]. However, because this drug is not treating the underlying problem-osteoblast differentiation and bone mineralization impairment, this class of drug needs to be used in combination with other drugs. In 2011, *Schindeler* et al published a study where the effect of combination therapy (rhBMP-2 and zoledronic acid) was assessed. They showed that use of rhBMP-2 reduced non-union to 75% and the combination therapy –rhBMP-2 and Zoledronic acid- halved this number and hence they concluded that combination therapy is more effective than BMP-2 therapy alone [220]. However, there is a limited number of clinical trials that have assessed the additional efficiency of bisphosphonate on recalcitrant bone healing in PA patients [221]. *Ex vivo* studies on the biopsies from NF1 patients have shown that treatment with bisphosphonate does not improve osteogenic differentiation in the harvested tissue [222]. Additionally, use of bisphosphonate in NF1 children with high bone turnover

rate should be pursued with caution, since studies in rat models have shown that bisphosphonate can have deleterious effect on the growth plate and high turnover bones [223]. This effect was not observed in the mouse models [224]. This disparity highlights the difficulties in generalizing the results of animal studies across species.

### *BMP-2,7*

In some cases, surgeons during surgery wrap the fractured bone with BMP-2 or BMP-7 soaked sponges. This practice has shown some promising results [225–228]. In the clinical setting, these growth factors –especially BMP-2,7- have been used extensively for improving fracture healing rate [227,229–232]. However, one concern with the use of BMPs is their effect on ectopic calcification in soft tissues in proximity of the fracture. In addition there are reports of an enlargement of glioma in one child after receiving rhBMP-2 and sarcomas formation in an adult patient [233,234]. Studies of the tumorigenic effect of BMP-2 has generated contradictory results. In a meta-study, researchers found that although BMP family members might exert anti-proliferative effect, they increase the tumorigenic properties e.g. invasiveness in different cancer types [235]. Hence using this factor in children that are prone to development of cancer should be with caution.

## Proposed Therapeutic Strategies

Current therapies are performing below expectation for the management of the orthopedic manifestations in NF1 patients because they do not target the causal factor/mechanisms of the disease. Hence targeted pharmacological therapies to promote bone union in NF1 pseudarthrosis are highly desired. Our laboratory and others in the NF1 field have focused on the development of

new translationally relevant mouse models and on acquiring a better understanding of the molecular etiology of recalcitrant bone healing in NF1 to identify new targets and propose more effective treatments.

### *RAS-MAPK inhibitors*

Neurofibromin is a negative regulator of RAS activity. RAS inhibitors that are currently on the market are targeted against mutated variants of RAS and hence are not suitable for NF1. Current efforts related to designing a targeted therapy for native RAS is focused on either inhibition of RAS activation or inhibition of downstream signaling effectors of RAS. A potentially novel approach takes advantage of the action of the FDA-approved Statin drugs that inhibit HMG-CoA Reductase. These compounds also inhibit the prenylation of RAS that is a required step for RAS activation and therefore, represents an effective mechanism to inhibit RAS constitutive activity using a drug known to be safe in humans.

The first study that used this approach in the bone field was done by Kolanczyk and colleagues. These researchers used high doses of lovastatin in the $Nf1_{Prx}^{-/-}$ mice to overcome first pass metabolism of the drug in the liver (the target organ of lovastatin), and found that osteogenic gene expression and bone parameters were improved [206]. A problem with this strategy is the use of high dose of Lovastatin to reach therapeutic concentration in the affected limb of NF1 children with no hypercholesteremic indication may have potential toxic effects. Thus Wang and colleagues in my laboratory hypothesized that inhibition of RAS activation by local delivery of Lovastatin could be more therapeutically-relevant in post fracture non-union and they tested this hypothesis in the osteoblast specific *Nf1*-deficient mice [204]. It was observed that bone properties post-fracture were improved in $Nf1_{Col1}$-/- mice. However, as it was mentioned above, this mouse model is

translationally not relevant. These studies support the utility of statins for the treatment of NF1 PA, but validation awaits studies to be performed in more clinically relevant models.

A second approach to target RAS is through manipulating the downstream signaling including MAPK/ERK. The efficiency of MEK inhibitors in the preclinical mouse models of NF1 neurofibromas and MPNST clinical trials led researchers in the bone field to address the potential and efficacy of MEK inhibitors on osteogenic differentiation impairment and on the bone phenotype of different mouse models [236–238]. However, contrary to neurofibroma and MPNSTs, two separate *in vivo* studies have shown that MEK inhibitors could not rescue the delayed bone healing of mouse models of NF1 PA, nor could it improve their bone mechanical properties [209,239]. These studies also showed that administration of BMP-2, a potent bone anabolic agent, cannot correct the delayed bone healing of these models, nor could it rescue the *ex vivo* differentiation impairment observed in BMSCs extracted from long bones of $Nf1_{Osx}^{-/-}$ mice. However, the combination of both low dose BMP-2 and MEK inhibitor (Trametinib) did improve bone mechanical properties and bone cell differentiation impairment *ex vivo*. In follow up studies, we have shown in the *Elefteriou* laboratory that BMP-2 treatment can improve the *ex vivo* reduced differentiation potency of *Nf1*-deficient BMSCs and MEFs, although this response was blunted compared to WT cells.

### *TGFβ1 inhibitors*

Accelerated cell proliferation, lack of osteogenic cell differentiation and presence of fibrotic tissue in the PA site is similar to the bone phenotypes of other conditions characterized by excessive TGFβ signaling, including Camurati-Engelmann, Marfan and Loeys-Dietz syndromes [82,240,241]. This observation in addition to excessive osteoclastogenesis led Rhodes and colleagues to assess

the role of TGFβ1 signaling in the *Nf1*$_{Col2.3}$*-/-* mouse model. They found that *Tgfb1* expression was upregulated in this mouse model and that the use of SD-208 (a TGFBRI inhibitor) could rescue the delayed bone healing and improve bone properties in these mice [242].

Several caveats of this study are outlined here: Firstly, TGFβ1 in the early stages of oncogenesis has an inhibitory effect on cancer progression [243] and hence, the use of anti TGFβ1 therapy in NF1 patients that are cancer prone could be problematic. Secondly, these researchers used an osteoblast specific mouse model (2.3kb*Col1*-CRE) whose clinical relevance in NF1 patients is debatable. Thirdly and most importantly, data from our laboratory- presented in chapter two- revealed that *Tgfb1* expression was not increased in *Nf1*-deficient osteoprogenitors, and that the conditioned medium of these cells does not induce TGFβ1 signaling. However, the role of SD-208 and other TGFβ1 inhibitors showed promise in our *in vitro* model, which can be attributed to the effect of TGFβ1 signaling inhibition on *Nf1* competent cells (WT or +/-) [97].

### *C-type Natriuretic peptide (CNP)*

C-type natriuretic peptide (CNP) is a small peptide with inhibitory function on RAF activity. Mechanistically, CNP binds to and activates its receptor NPR-B, which in turn activates guanyl cyclase that converts GMP to cyclic GMP, which is activator of Protein Kinase G (PKG). PKG phosphorylates and inactivates RAF, thus preventing terminal activation of ERK [244]. In the past, our laboratory targeted the RAS-RAF-MEK-ERK axis with systemic administration of a recombinant form of CNP in the *Nf1*$_{Col2}^{-/-}$ mouse model and has shown promising results in correcting its stature and growth plate organization [245]. However, this approach has had limited success in *ex vivo* system using *Nf1*-deficient osteoprogenitors, although these cells like

chondrocytes do express NPR-B, the receptor for CNP (data shown in the third chapter) and chondrocytes (data not shown). Therefore, CNP might have limited effect in the context of fracture healing.

### *Asfotase alpha*

Asfotase alpha is a recombinant, bone-targeted, human TNSALP developed to treat hypophosphatasia [246]. Presence of hypo-mineralized tissues in the PA site of NF1 patients [19] and the presence of osteoidosis in preclinical mouse models of NF1 disease [80,207] led our laboratory to hypothesize that *Nf1* modulates matrix mineralization. Indeed, it was found that *Nf1* deficiency in the *Nf1 osx-cre* and *Nf1 Col2* mouse models leads to accumulation of pyrophosphate (PPi), a known inhibitor of matrix mineralization, and this accumulation was through a MAPK/ERK dependent pathway [208,247]. *Nf1* deficiency increases the expression of proteins responsible for production and transport of PPi into the extracellular matrix, namely ENPP1 and ANK. The administration of Asfotase alpha was able to improve the bone mechanical properties and bone mineralization in two mouse models of NF1 skeletal dysplasia [208]. However, despite its beneficial effect on bone mineralization, it is important to note that administration of this enzyme may not promote the osteogenic differentiation of *Nf1*-deficient osteoprogenitors.

### *EGFR Inhibitors*

RAS proteins act as signal transducers that usually transmits signals from the Receptor Tyrosine Kinases (RTKs) and activates their diverse downstream signaling molecules. The upstream activators of Neurofibromin-regulated RAS in osteoprogenitors remains unknown. In the second

chapter of my thesis, I will present data that point toward EGFR as a potential modulator of RAS in osteoprogenitors lacking *Nf1*. I followed this hypothesis based on the premise that by modulation of upstream regulator of Neurofibromin aka EGFR1, we could find a therapeutic approach for the management of pseudarthrosis.

## NF1 reduces osteogenic differentiation

One of the NF1 skeletal manifestations with the highest morbidity is pseudarthrosis. As it was mentioned in the previous sections, there are currently no satisfactory pharmacological management option for this condition. This stems from the fact that the exact mechanism of pseudarthrosis formation is not well understood. One important cell type in the process of fracture healing and bone formation is the mesenchymal stem cell. These cells undergo osteogenic differentiation to become osteoblasts that can produce a calcified extracellular matrix that restores the strength and biomechanical properties to the fractured bones. Osteoblasts are post-mitotic cells that express osteogenic markers e.g. *ALPL, IBSP and BGLAP.* It is assumed that a defect in these cells plays a central role in the delayed fracture healing in NF1 PA.

In support to this assumption, one study using biopsies from a NF1 pseudarthrotic site showed increased rate of proliferation in cells from the biopsy (presumably *NF1*-deficient) compared to cells from healthy individuals [222]. Other studies have shown reduced osteogenic gene expression, Alkaline phosphatase activity and calcified nodule formation in cells extracted from the pseudarthrotic site compared to healthy individuals (**Figure 11**) [60,222,248]. A similar pattern has been observed in *Nf1*-deficient osteoprogenitors in the $Nf1_{osx}^{-/-}$ and $Nf1_{Col2}^{-/-}$ mouse models [207,209,210,249].

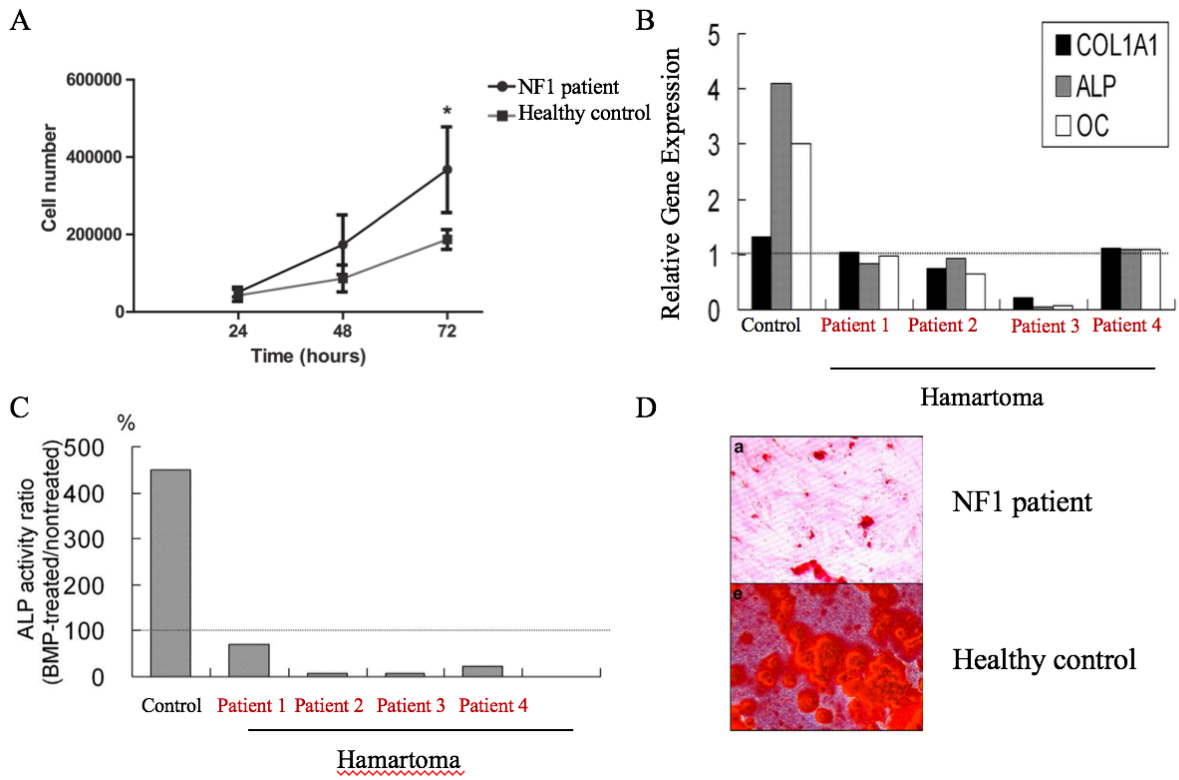



**Figure 11. Cells from the pesudarthrotic site have increaed proliferation and reduced ostegenic potential**

A: osteoprogenitor cells from the pseudarthrotic site of NF1 patients proliferate faster than osteoprogenitors from healthy individuals [222]. B&C: periosteal cells from patients show reduced osteogenic gene expression and ALP activity compared to healthy individual [60] D: osteoprogenitor cells from pseudarthrotic site of NF1 patients show reduced calcified nodule formation compared to healthy individual (From Madhuri V, et al, 2016 [222]).

All of these studies have compared samples from patients with healthy controls, which will introduce inter-individual differences. There are two ways to overcome this issue, using either the mouse models that have been mentioned before or using samples from a non-pseudarthrotic site from the same patients. Recently, our collaborator Dr. Rios at the Scottish Rite Hospital published a study that utilized high throughput sequencing and showed that the expression of the EGFR ligand, *EREG,* was highly upregulated in cells extracted from the NF1 pseudarthrotic site compared to the iliac crest of the same patient. In the next chapter I will explain why this report was exciting and present my findings on the role of EGFR signaling in the reduced osteogenic potential of *Nf1*-deficient osteoprogenitors.

# II. The reduced osteogenic potential of *Nf1* deficient osteoprogenitors is EGFR independent

This is modified version of an article published in the journal of Bone following peer review. The version of the record is: *S.E. Tahaei, G. Couasnay, Y. Ma, N. Paria, J. Gu, B. F. Lemoine, X. Wang, J.J. Rios and F. Elefteriou. The reduced osteogenic potential of Nf1-deficient osteoprogenitors is EGFR-independent. 106; 103-111.*

## Introduction

As discussed in Chapter I, in contrast to most cases of fractures in children, which usually progress towards bone union within weeks, 2-5% of children with NF1 present with recalcitrant bone healing despite multiple attempts with surgical stabilization and anabolic and anti-catabolic treatments. The phenotype starts in early childhood with an initial and unilateral bowing of the tibia that often progresses towards fracture and non-union (Pseudarthrosis). Pseudarthrosis (PA) has one of the highest morbidity among NF1 skeletal complications, with little clinical management options [186,215,250], and often leads to amputation of the affected limb [226]. Bone Morphogenic Proteins (BMPs) are currently used off label with variable success, and under clinical investigation for efficacy [230,232,251], although BMP2 did not show a beneficial effect when used alone in preclinical models [209,239]. Hence, finding new therapeutic options for the management of this condition is a significant clinical need.

In search for finding genes that are differentially changed in pseudarthrotic sites and might cause pseudarthrosis, Paria and colleagues used biopsies from NF1 PA patients and compared

them with biopsies from iliac crest of the same patients for RNA-Seq. They identified a significant upregulation of *EGFR* and *EREG* in cells from pseudarhtrotic sites [187]. Epiregulin, encoded by *EREG*, is one of the seven Epithelial Growth Factor (EGF) family members that preferentially binds to and activates EGFR1 and Erb-B4 forms among the four cloned EGFRs [252–254]. These findings sparked great interest because 1) increased EGFR signaling is known to inhibit osteoprogenitor cell differentiation [126–129,255–259]; 2) drugs are clinically available to block EGFR signaling, thus raising the possibility of rapidly repurposing EGFR inhibitors to promote the differentiation of *NF1*-deficient osteoprogenitors and potentially bone healing in cases of NF1 bone non-union. Based on these observations, we hypothesized that sustained EGFR signaling in *Nf1*-deficient osteoprogenitors contributes to their differentiation defect and EGFR inhibitors can be used for improving the recalcitrant fracture in NF1 PA patients.

## Materials and methods

### BMSC cultures

The institutional animal care and use committee Baylor College of Medicine approved all the mouse procedures. Mice were housed 2-5 per cage. Mouse BMSCs were extracted from long bones of 2-3 month-old $Nf1^{f/f}$ mice [203] by centrifugation at 3000 g for 3 minutes, as previously described [260]. Extracted marrow was plated in 10 cm dishes in α-MEM medium (without ascorbic acid) supplemented with 10% fetal bovine serum and 100 U/ml Penicillin/ Streptomycin (15140-122, ThermoFisher) for three days. At that time, non-adherent cells were discarded by changing the medium. Cells were trypsinized after reaching 80% confluence and were seeded in 6-well plates at 10,000 cells/$cm^2$ for adenovirus transduction. After reaching

60% confluence, cells were incubated with the adenovirus solutions (Ad-GFP or Ad-CRE recombinase, Baylor College of Medicine vector development lab) in the presence of Gene Jammer reagent (Agilent technologies; Cat# 204132), as described previously [261]. Briefly, Gene Jammer was added at a final concentration of 1% to FBS- and antibiotic-free α-MEM medium. The solution was vortexed briefly and incubated for 10 minutes at room temperature before adding the virus at a MOI of 400 and incubating for further 10 minutes. Final mixture was added to each well and cells were incubated with the virus solutions for 24 hours. The media was then changed to fresh complete α-MEM medium containing 10% FBS and Pen/Strep (Thermofisher Cat# 15140122). Mouse BMSCs were differentiated in osteogenic medium containing ascorbic acid (50 μg/ml) and β-glycerophosphate (5mM) in α-MEM medium for 7 days. Medium was changed every other day.

For conditioned medium (CM) collection, mBMSCs infected with either Ad-GFP or Ad-CRE were washed with PBS two times and were grown with FBS-free α-MEM medium for 1 day. The CMs were centrifuged at 1000 g for 5 minutes to remove debris, and the supernatant was collected and were kept at -80°C until use.

A431 cells were grown in DMEM supplemented with 10% FBS and 100 U/ml Penicillin/ Streptomycin (15140-122, ThermoFisher). After reaching 80% confluence, cells were starved in serum-free DMEM overnight. Cells were then treated with the conditioned media plus normal goat IgG control (AB-108-C, R&D Systems) or Epiregulin neutralizing antibody (AF1068-SP, R&D Systems) at the final concentration of 0.4 μg/ml. Cell lysates were extracted after ten minutes and the amount of p-EGFR, EGFR (Cat. # 3777S and 4267S from Cell signaling Technology, respectively) and β-actin (A5316 from Sigma) were measured by Western blotting.

To measure Smad2 activation, mBMSCs were grown in α-MEM until they reached 80% confluence and were then starved overnight in FBS free medium before treatment with either recombinant activated TGFβ-1 (R&D systems, Cat# 766-MB-005) or the conditioned medium from *Nf1* WT and KO BMSCs for 30 minutes. Cells were then scraped in RIPA buffer and after protein extraction, the amount of Smad2,3 (Cat. # 3102, CST) and p-Samd2 (3108, CST) levels were measured.

### Calvaria cultures

The calvariae from 4 days-old *Nf1*$^{f/f}$ pups were extracted and digested consecutively three times in digestion medium, prepared by dissolving collagenase P at a final concentration of 0.1 mg/ml (Sigma, Cat# 11213865001) in 0.25% Trypsin (ThemoSisher, Cat# 25200-056). After the last digestion, bone fragments were plated in 10 cm dish in α-MEM medium (without ascorbic acid) supplemented with 10% FBS and 100 U/ml Penicillin/Streptomycin (Cat# 15140-122, ThermoFisher). Medium was changed after 4-5 days. Cells were trypsinized after reaching 80% confluence and were replated in 6 well plates before infection with a GFP- or CRE adenovirus as indicated above.

### Human primary cell culture and sorting

Human BMSCs isolated from tibial PA of one NF1 patient with an inherited mutation c.1381C>T (p.R461X) and a somatic deletion (c.1642_6999del; p.Asn510_Lys2333del) in the *NF1* gene [187] were cultured in α-MEM (without ascorbic acid) supplemented with 10% FBS and 1% PS. Cells were trypsinized and resuspended in 500ul of PBS containing 10% FBS and 2.5mM EDTA. The

7AAD live cell marker dye was added to the cell suspension and live single cells were sorted using an Aria Cell Sorter (BD Biosciences) into 96-well plates containing 100ul of α-MEM media with 20% FBS. 100ul of conditioned media from the original "bulk" culture was added to help with the growth of single cell clones. After reaching confluence, cells were expanded into 6-well plates and cultured again with fresh medium complemented with 50% of bulk culture conditioned media. DNA from clonal lines was extracted using the QiaAmp DNA mini kit (Qiagen) and sequenced for the presence or absence of the deleted allele.

### Single-cell mRNA sequencing and analysis

Single cells were isolated and cDNAs were generated using the Fuidigm C1 instrument and SMARTer Ultra Low RNA Kit (Clontech Cat#634834). Sequencing libraries were generated using the Nextera XT Library Preparation Kit (Illumina ref#15032354) and sequenced using the Illumina HiSeq 2500 generating paired-end 100bp reads. A single bulk sample of 100-200 clonal *NF1*$^{-/-}$ cells were isolated and processed in the same manner, except that the BioRad Thermal Cycler was used in place of the C1.

FASTQ sequence reads were trimmed using Flexbar read trimmer and mapped to the human reference genome (GRCh38) using HISAT2 [262,263]. Mapped reads were compared to the GENCODE transcriptome (version 24) and counted using HTSeq [264]. Following filtering, 78 cells (N=50 iliac crest *NF1*$^{+/-}$ and N=28 clonal *NF1*$^{-/-}$) were used for differential gene expression analysis using DESeq2 [265]. One bulk sample of clonal *NF1*$^{-/-}$ cells were also included for comparison. Log counts per million (CPM) mapped reads was calculated and visualized using the R package *beeswarm*. Significance values (p-value) are adjusted for multiple comparisons.

## Luciferase assay for TGFβ1 activity measurements

The conditioned medium from mBMSCs was harvested as described above, and the TGFβ1 reporter cell line MDA-scp28 was used to quantify active TGFβ1 in this CM [46]. Briefly, 50,000 MDA-scp28 cells/well were plated in a 96-mutliwell culture plate in high glucose DMEM supplemented with 10% FBS and 100 U/ml Penicillin/ Streptomycin. The MDA-scp28 were then starved for 24hr in FBS-free DMEM high glucose medium and were treated with the CM of mBMSCs or recombinant TGFβ1 for 8hr. Luciferase activity was detected by the Dual Luciferase kit (Promega, E1960), following the manufacturer instructions. Firefly luciferase activity was normalized by the Renilla luciferase activity (ratio F-Luc/R-Luc).

## TGFβ1 ELISA

Total TGFβ1 in supernatants of WT and Nf1-deficient mBMSCs was quantified by ELISA (R&D, DY1679). Briefly 100 µl of supernatant were acidified with 20 µl of 1N HCl and incubated 10 min at room temperature. Acidity was then neutralized by the addition of 20 µl of 1.2N NaOH/0.5M HEPES. Total TGFβ1 concentrations of prepared samples were measured according to the manufacturer's instructions.

## Drugs

AG-1478 (Selleckchem Cat# S2728), Poziotinib (Selleckchem, Cat# S7358) and SD208 (Sigma, Cat# S7071) were reconstituted in DMSO (Vehicle).

## ALP activity

Cells were washed with PBS, harvested and lysed in 250 µl of 0.05% Triton plus two cycles of freezing/thawing at -80°C/37°C. Cell lysate were then centrifuged for 20 minutes at 16,000g at 4°C and supernatants were used for protein (BCA method; Life Technologies, Cat# 23225) and ALP activity

measurements. ALP activity was measured using a colorimetric assay. Briefly, a PNPP ((4-nitrophenyl phosphate disodium salt hexahydrate, Sigma Cat# P4744) solution was prepared in water and was mixed with AMP (2-amino-2-methyl-1-propanol, Sigma Cat # A65182) buffer. Cells lysate were added to the mix (1:5) and incubated at 37°C for 30 minutes. Absorbance was read at 405 nm and normalized to protein content.

## Gene expression assays

Total RNA was extracted using TRIzol (Thermofisher, Cat# 15596026), and contaminating genomic DNA was digested by treatment with DNAse I (Promega, Cat# M6101). cDNAs were synthesized from 1ug RNAs using the high capacity cDNA reverse transcription kit (Thermofisher, Cat# 4368814),). Quantitative qRT-PCR was performed using the following TaqMan primers/probes: *Ccnd1* (Mm00432359_m1), *Ibsp* (Mm00492555_m1), *Egfr1* (Mm01187858_m1), *Tgfb1* (Mm03024053_m1), *Alpl* (Mm00475834_m1), and the normalizer *Hprt* (Mm03024075_m1) from Thermofisher, or SYBR green primers: *Nf1* (forward: GTATTGAATTGAAGCACCTTTGTTTGG; reverse: CTGCCCAAGGCTCCCCCAG); *Ereg* (forward: TTGTGCTGATAACTGCCTGTAGAA; reverse: CACCGAGAAAGAAGGATGGAGAC). SYBR qPCR specificity of amplification was verified by the presence of a single peak on the dissociation curve.

## Western blot

Proteins were extracted from cell cultures using RIPA buffer. Protein concentration was measured using BCA assay (Thermo-Fisher). Ten µg of total protein was run on SDS gel before transfer to a nitrocellulose membrane. Membranes were blocked using 5% non-fat powder milk in TBST

buffer. Epiregulin antibody (AF1068-SP, R&D Systems), β-actin: (A5316, Sigma), Tgbfb1 (55052, BD) and Cell Signaling Technologies antibodies Smad2,3 (3102), p-Samd2 (3108), EGFR (4267S) and p-EGFR (3777S) were diluted in blocking buffer at 1:1000 to 1:2000 dilution and incubated with the membranes overnight at 4°C. Following washing, membranes were then incubated with an HRP-conjugated secondary antibody (goat anti mouse Santa Cruz Cat # sc-2005, goat anti-rabbit Santa Cruz Cat# sc-2030) diluted in blocking buffer at room temperature for one hour. Membranes were washed and incubated with ECL solution for 2 minutes and exposed to photographic film.

### Statistical analyses

For comparison between WT and KO cells, a student t-test was performed. For multiple treatments, a two-way analysis of variance (ANOVA) was used to determine whether there was a statistically significant difference in treated vs. non-treated cells between genotypes. P-value less than 0.05 was considered significant. Statistical analysis was performed using Graph Pad PRISM (v6.0a, La Jolla, CA, USA). Data are provided as mean +/- SD.

## Results

### *EREG* expression is increased, while there is no increase in *TGFB* ligands nor *TGFBR*s expression in human bone cells characterized by *NF1* double hit mutations

Consistent with previously published data [187], single-cell sequencing confirmed highly significant upregulation of *EREG* in *NF1*$^{-/-}$ clonal cells that harbor a germline p.R461X variant and a somatic p.Asn510_Lys2333del large deletion extracted from PA site (Cells positive for loss of

heterozygocity; LOH), compared to patient-matched *NF1*$^{+/-}$ cells extracted from iliac crest (**Figure 12A**). *EGFR* expression was slightly, though not significantly, higher in the *NF1*-deficient clonal cell line (**Figure 12B**).

Rhodes *et al* reported that excess *Tgfβ1* expression in *Nf1*-deficient mouse osteoblasts might be involved in reduced osteogenic potential of *Nf1* deficient osteoprogenitors [242]. However, no significant differences in gene expression were observed for genes encoding *TGFβ* ligands nor *TGFβ* receptors in loss of heterozygosity (LOH) positive human cells (**Figure 12C-H**). This finding is in contrast to a previous report using *Nf1*-deficient osteoblasts extracted from the *Col1*$_{2.3kb}$-cre;*Nf1*$^{f/f}$ mouse model [242].

### *Nf1* deficient murine osteoprogenitors show no difference in expression or activity of TGFβ1

These observed differences in *TGFB* expression led us to assess the expression of *Tgfb1* in WT and *Nf1*-deficient mouse bone marrow stem cells (mBMSCs). For this purpose, *Nf1*$^{f/f}$ mBMSCs were cultured and infected with a cre-expressing adenovirus (herein referred to as *Nf1*-deficient) or a GFP-expressing adenovirus (herein referred to as WT control).

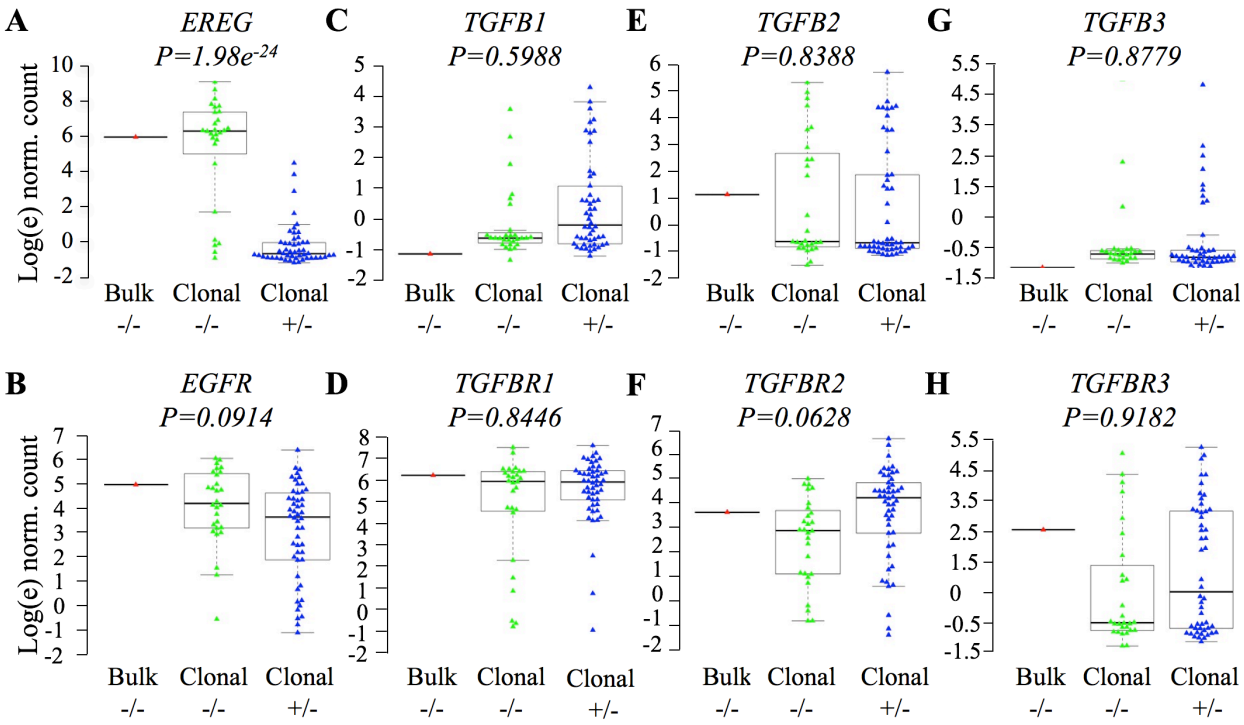



**Figure 12. Gene expression profile of cells extracted from NF1 PA site**

A-B: Expression of *EREG* and its main receptor *EGFR*. C-H: Gene expression of TGFβ family ligands and receptors. Bulk: mixed cells from the NF1 PA site; Clonal -/-: Single cells from the NF1 PA site; Clonal +/-: single cells from the iliac crest cultures from the same patient.

Loss of *Nf1* gene expression following cre-expressing adenovirus transduction was confirmed by a significant reduction (>90%) in *Nf1* gene expression by qRT-PCR compared to Ad-GFP control (**Figure 13**). Expression of *Tgfb1* level and TGFβR activity was measured (Figure 10). No difference in *Tgfb1* expression was found in *Nf1*-deficient mBMSCs (**Figure 14A**). *Tgfb1* was also expressed at similar levels in WT and *Nf1*-deficient mouse embryonic fibroblasts (MEFs) isolated from WT and $Nf1^{-/-}$ embryos, considered to be more immature mesenchymal progenitor cells than mBMSCs (**Figure 14B**) and in WT and *Nf1*-deficient calvaria-derived cells that are considered more committed to the osteoblast lineage (**Figure 14C**). No detectable difference in the amount of soluble total TGFβ1 (measured by ELISA, **Figure 14D**) nor secreted active TGFβ1 (measured by Western Blot, **Figure 14E**) was observed between the conditioned medium (CM) from WT and *Nf1*-deficient mBMSCs. Finally, the CM from WT and *Nf1*-deficient mBMSCs resulted in similar levels of activation of a sensitive SMAD-responsive luciferase reporter MDA231 cell line (**Figure 14F**) [266], and to similar level of p-SMAD2 activation in treated WT BMSCs (**Figure 14G**). Collectively, these data strongly suggest that increased TGFβ1 production by *Nf1*-deficient osteoprogenitors is not the main cause of the impaired osteogenic potential of these cells.

### TGFβ1 inhibition has beneficial effects on osteogenic differentiation

Rhodes and colleagues assessed the efficiency of anti- TGFβR (SD-208) in *Nf1* mouse model and discovered that peritoneal injection of TGFβR1 antagonist can rescue the bone phenotype and fracture healing delay in osteoblast specific *Nf1* mouse model [242]. However, these researchers failed to show the effect of SD208 on osteogenic differentiation impairment phenotype of *Nf1* deficient osteoprogenitors *in vitro*.

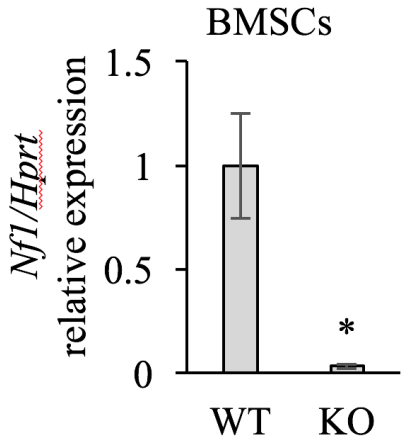



**Figure 13. *Nf1* knock down efficiency**
*Nf1* expression in Adenovirus CRE treated mBMSCs is reduced to 90% of *Nf1* expression in Adenovirus GFP treated cells

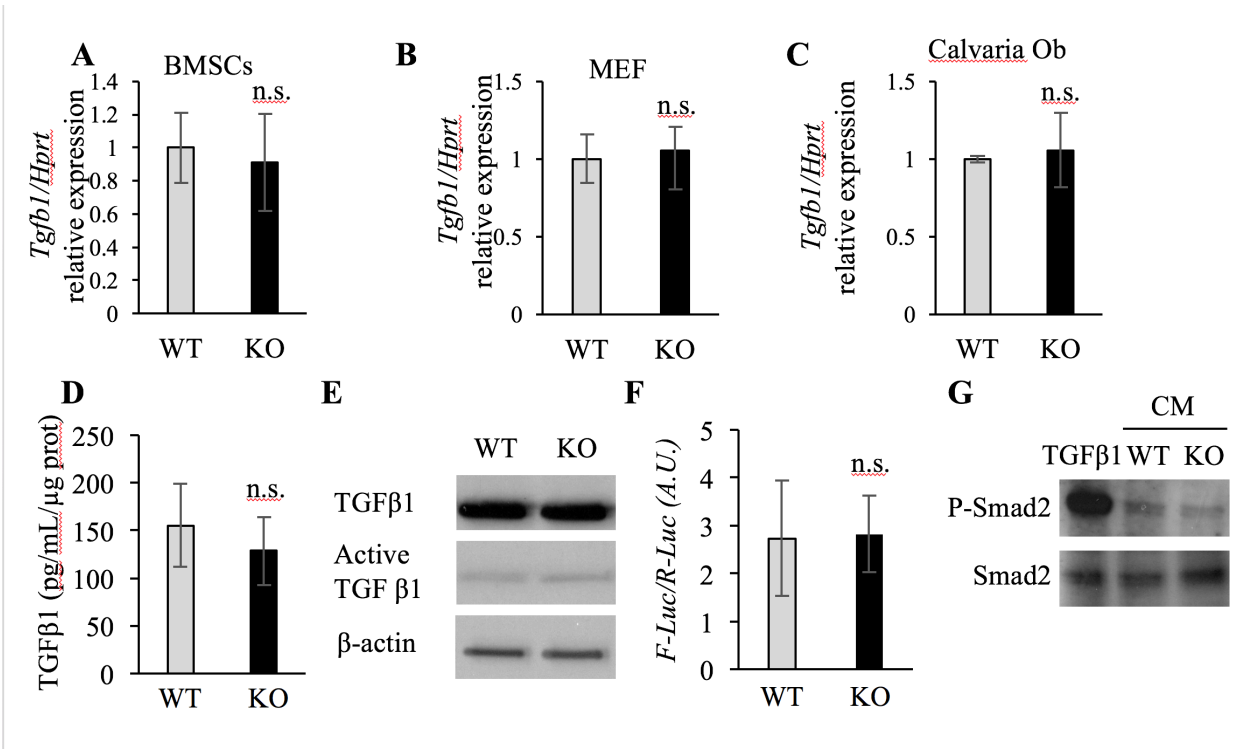



**Figure 14. *Nf1* deficiency does not change *Tgfb1* expression and TGFβ1 activity**
**A-C**: *Tgfb1* expression in murine WT and *Nf1*-deficient mBMSCs (**A**), MEF cells (**B**) and calvariae cells (**C**)(qPCR, n=3). **D-E**: TGFβ1 protein expression in WT and *Nf1* KO BMSCs using ELISA (**D**, n=3) and Western blotting (**E**, n=3). **F-G**: Measurement of TGFβ-1/SMAD signaling activity in the conditioned medium collected from cultures of WT and -deficient mBMSCs (n=3) using Luciferase assay (**F**, n=3) and p-SMAd2 level (**G**, n=3, TGFβ1 positive control: 5ng/ml). n.s: non-significant, *: $p < 0.05$ between genotypes, qPCR gene expression is normalized by *Hprt* expression.

In a previous section of this chapter, I showed that *Tgfb1* expression and TGFβ1 activity were not increased in both human and murine *Nf1* deficient osteoprogenitors cells (**Figure 12 & 14**). However, my finding did not rule out a possible beneficial effect of SD-208 on the differentiation of *Nf1*-deficient osteoprogenitors *in vitro*. In order to assess this question, I treated WT and *Nf1* deficient osteoprogenitors with the same dose of SD-208 as was used in the *Rhodes* study and measured the expression of the osteogenic marker gene *Alpl* (**Figure 15**). Treatment of *Nf1* WT and *Nf1* KO BMSCs increased the expression of *Alpl.* Although this increase in *NF1* KO cells was statistically significance compared to control that received DMSO, it was statistically lower than in the *Nf1* WT SD208 treated group.

This finding can be interpreted in 3 ways: First, because there is no increase in Tgfb1 expression/activity in *Nf1* deficient cells, this anabolic effect could be through this drug off-target activity. This possibility could be tested using other structurally non-related TGFβR1 inhibitors/anti-TGFβ1neutralizing antibody. The other possibility is that the positive effect of this drug on *Nf1* deficient population of cells is through its effect on non-recombined cells. Although using flox system has its advantages, it appears that it fails to generate a homogenous population of KO cells. In order to address the question of effect of this drug on non-recombined cells, I suggest that the effect of this drug be assessed on a homogenous population of *Nf1* KO cells (e.g. MEF cells). The last possibility is that TGFβ1 receptors are more sensitive in *Nf1* deficient cells compared to *Nf1* WT cells and as the result of higher intrinsic activity, the reduced osteogenic differentiation could be modulated by TGFβR1 antagonist –SD-208. This increase in sensitivity could be explained by increase plasma membrane presence of the receptors, increased/decreased association of accessory proteins with these receptors that could potentially modulate the response of cells to TGFβ-1. This third possibility will be revisited in chapter IV of this thesis.

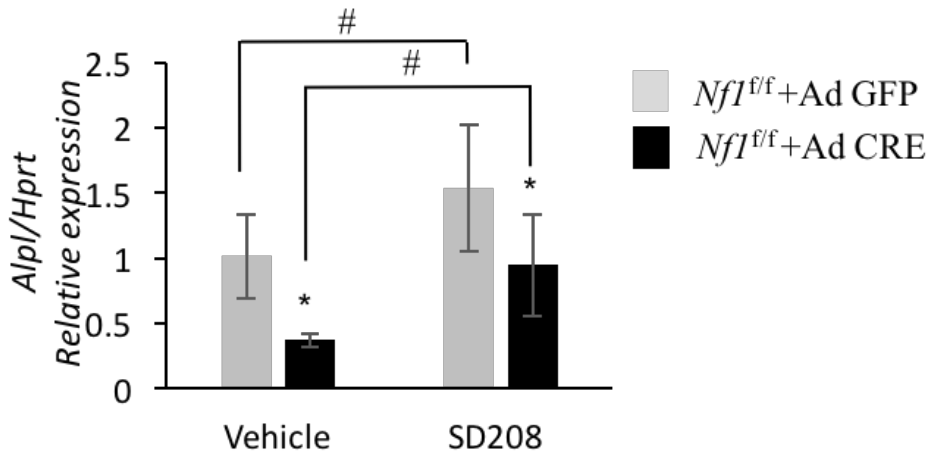



**Figure 15. TGFβR inhibitor SD208 stimulates osteoblast differentiation**
Expression of *Alpl* in response to TGFβR inhibition by SD-208 following 7 days of osteogenic differentiation (qPCR, n=3, * and #: $p<0.05$ between genotypes and treatments, respectively). qPCR gene expression is normalized by *Hprt* expression.

### Epiregulin is ectopically expressed and active in *Nf1*-deficient mBMSCs

Because single-cell sequencing confirmed that *NF1* deficiency in human bone cells was associated with *EREG* over-expression, we sought to determine whether this phenotype was conserved in mBMSCs. Using the same strategy of *ex vivo Nf1* ablation as indicated above, we found *Ereg* to be expressed in *Nf1*-deficient mBMSCs at three times the level of WT mBMSCs (**Figure 16A**). This increase was confirmed at the protein level (**Figure 16B**). In contrast, expression of *Egfr* was not altered in *Nf1*-deficient mBMSCs (**Figure 16C**), though EGFR protein abundance was higher in these cells (**Figure 16D**). The expression of other EGFR ligands, including *Betacellulin* (*Btc*), *Epidermal Growth Factor* (*Egf*), *Transforming Growth Factor a* (*Tgfa*) and *Amphiregulin* (*Areg*), was undetectable in both WT and *Nf1*-deficient mBMSCs (data not shown). These results suggest that Neurofibromin signaling represses *Ereg* expression in both human and mouse BMSCs and that EGFR protein synthesis or stability is regulated by mechanisms that are Neurofibromin-dependent and post-transcriptional.

Epiregulin is synthesized as a precursor membrane-bound protein that must be cleaved for biological activity and activation of EGFR [253]. To determine if *Nf1*-deficient mBMSCs generate higher amount of active epiregulin than WT mBMSCs, a cell line highly sensitive to EGFR ligands (A431 cells) [267] was treated with the CM from WT and *Nf1*-deficient mBMSCs.

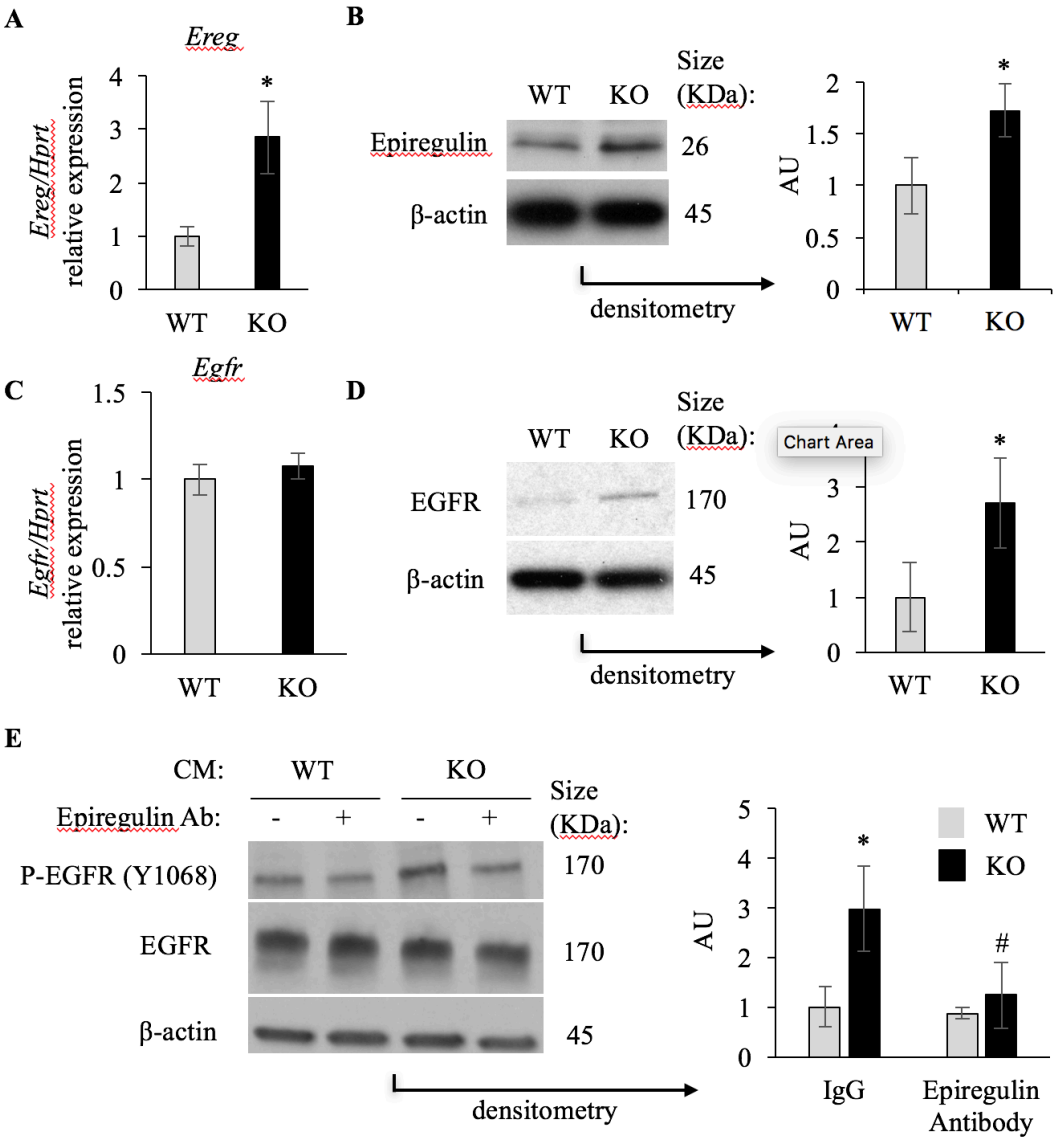



**Figure 16. *Nf1* deficiency increases Epiregulin expression and activity in mBMSCs**
**A**: *Ereg* expression in WT and *Nf1*-deficient mBMSCs (qPCR, n=3). **B**: Epiregulin protein expression in WT and *Nf1*-deficient mBMSCs (Western blot, n=3, Right graph: densitometric analysis). **C**: *Egfr* expression in WT and *Nf1*-deficient mBMSCs (qPCR, n=3). **D**: EGFR protein expression in WT and *Nf1* deficient mBMSCs (Western blot, n=3, Right graph: densitometric analysis). **E**: Level of phosphorylated EGFR (p-EGFR), EGFR and β-actin in A431 cells treated with the conditioned medium (CM) from WT (grey bar) and *Nf1*-deficient (KO, black bar) mBMSCs in the presence of IgG control or an epiregulin neutralizing antibody (Western blot, n=3, Right graph: densitometric analysis). * and #: $p<0.05$ between genotypes and treatments, respectively. qPCR gene expression is normalized by *Hprt* expression.

Both CMs led to EGFR activation (phosphorylation), but the CM from *Nf1*-deficient mBMSCs was three times more potent than the one from WT mBMSCs (**Figure 16E**). EGFR activity following treatment with the *Nf1*-deficient CM was also blocked following addition of an Epiregulin-neutralizing antibody (**Figure 16E**). These results suggest that the CM of *Nf1*-deficient mBMSCs contains higher amount of active epiregulin compared to WT mBMSCs.

## Inhibition of EGFR signaling fails to rescue the osteogenic differentiation of *Nf1*-deficient mBMSCs

Because chronic activation of EGFR leads to inhibition of osteogenic differentiation [126–129,255–258] and *Nf1*-deficient mBMSCs overexpress both EGFR and its ligand epiregulin, we sought to block EGFR signaling to determine if excessive EGFR signaling contributed to the reduced osteogenic potential of these cells. Firstly, I tested the anti-EGFR efficacy of the Ag-1478 and Poziotinib on *Nf1* WT BMSCs (**Figure 1**7). *Nf1* WT and *Nf1*-deficient mBMSCs were prepared as described above and treated from the start of differentiation (Day 0) with AG-1478 (0.5 and 1 μM), a potent and selective EGFR kinase inhibitor (IC50=3nM in a cell-free system [268]), for 7 days in osteogenic medium, and early osteogenic differentiation was assessed by measuring *Alkaline phosphatase* (*Alpl*) and *Integrin binding sialoprotein* (*Ibsp*) expression. As expected, the expression of *Alpl* and *Ibsp* in *Nf1*-deficient mBMSCs was reduced to 10-20% of WT controls (Vehicle in **Figure 18A-B**). However, inhibition of EGFR signaling with AG-1478 failed to rescue the reduced expression of these genes in *Nf1*-deficient mBMSCs (**Figure 18A, B**), which was confirmed by measuring ALP activity (**Figure 18C**). Poziotinib, an irreversible pan-EGFR inhibitor (IC50=3.2nM for HER1, 5.3nM for HER2 and 23.5nM for HER4 [269]) tested at two concentrations (100nM or

400nM) also failed to increase the expression of *Alpl* and *Ibsp* (**Figure 18D, E**) and ALP activity (**Figure 18F**) in *Nf1*-deficient mBMSCs following osteogenic induction.

It remained possible that epiregulin signals via receptors other than EGFR or ERB-B4. To address this hypothesis, WT and *Nf1*-deficient mBMSCs were grown in osteogenic conditions for 7 days in the presence of an epiregulin-neutralizing antibody (0.4 ug/ml). Although this neutralizing antibody was added to the medium in large excess and successfully blocked EGFR activation in human A341 cells (see **Figure 16E**), it failed to rescue the osteogenic differentiation of *Nf1*-deficient mBMSCs (**Figure 18G-I**). Together, these results suggest that the increase in Epiregulin and EGFR expression in *Nf1*-deficient osteoprogenitors does not contribute to their defective differentiation potential.

## Discussion

EGFRs are a family of receptor tyrosine kinases that play an important role during embryogenesis with roles in proliferation and differentiation in diverse cell types [270]. Previous studies have shown a proproliferative and antidifferentiation role in osteoprogenitors [124,125,127,271]. Hence, when transcriptome profiling of bone cells cultured from a case of NF1 tibial PA indicated that *NF1*-deficiency was associated with over-expression of *EREG*[187], we formulated the hypothesis that the *Nf1* associated reduced osteogenic differentiation might be EREG/EGFR related. I investigated the role of EREG/EGFR signaling in the reduced osteogenic potential of *Nf1* deficient osteoprogenitors with the prospect that clinically available EGFR inhibitors may promote bone union in challenging surgical cases of NF1 PA. I showed here that *Ereg* was overexpressed in *Nf1*-deficient mouse BMSCs, as observed in human cells with *NF1* biallelic mutations.

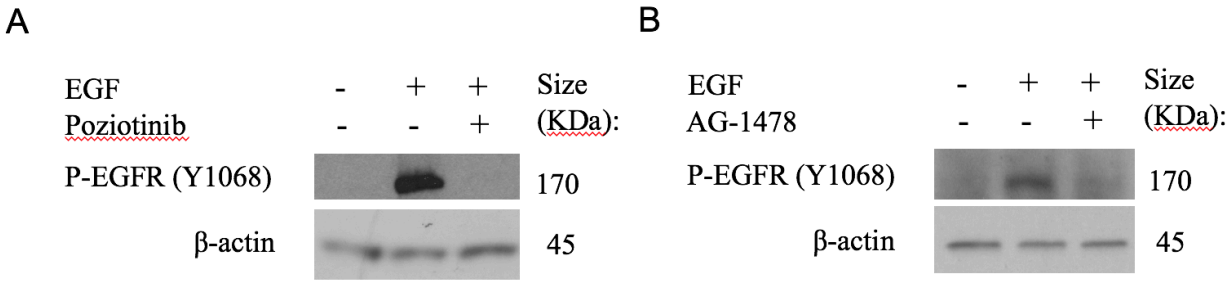



**Figure 17. Efficacy of EGFR inhibition**
A: Poziotinib (100nM) inhibit phosphorylation (activation) of EGFR in mBMSCs
B: Ag-1478 (500nM) inhibit phosphorylation (activation) of EGFR in mBMSCs

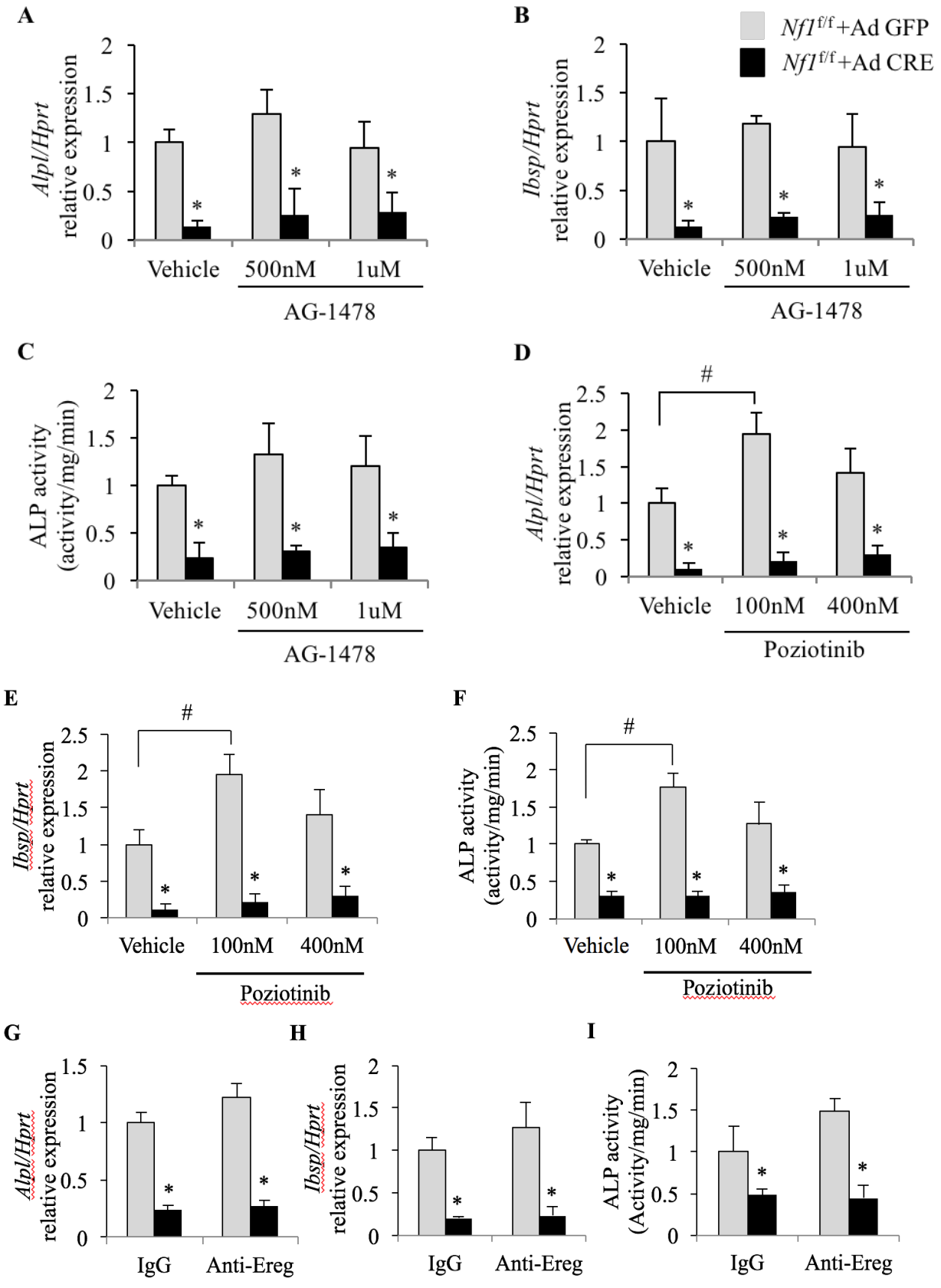

A
Alpl/Hprt
relative expression
2
1.5
1
0.5
0
*
*
*
Vehicle
500nM
1uM
AG-1478
B
Nf1f/f+Ad GFP
Nf1f/f+Ad CRE
Ibsp/Hprt
relative expression
2
1.5
1
0.5
0
*
*
*
Vehicle
500nM
1uM
AG-1478
C
ALP activity
(activity/mg/min)
2
1.5
1
0.5
0
*
*
*
Vehicle
500nM
1uM
AG-1478
D
#
Alpl/Hprt
relative expression
2.5
2
1.5
1
0.5
0
*
*
*
Vehicle
100nM
400nM
Poziotinib
E
#
Ibsp/Hprt
relative expression
2.5
2
1.5
1
0.5
0
*
*
*
Vehicle
100nM
400nM
Poziotinib
F
#
ALP activity
(activity/mg/min)
2.5
2
1.5
1
0.5
0
*
*
*
Vehicle
100nM
400nM
Poziotinib
G
Alpl/Hprt
relative expression
1.5
1
0.5
0
*
*
IgG
Anti-Ereg
H
Ibsp/Hprt
relative expression
2
1.5
1
0.5
0
*
*
IgG
Anti-Ereg
I
ALP activity
(Activity/mg/min)
2
1.5
1
0.5
0
*
*
IgG
Anti-Ereg

**Figure 18. anti-EGFR treatment does not resuce reduced osteogenic potential of *Nf1* deficient mBMSCs**

A-B, D-E and G, H: Expression of early osteoblast marker genes (Alpl, Ibsp) in response to EGFR or Epiregulin inhibition during osteogenic differentiation (A-B: AG-1478, D-E: Poziotinib and G, H: epiregulin-neutralizing antibody) in WT and Nf1-deficient (KO) mBMSCs (qPCR, n=3, * and #: $p<0.05$ between genotypes and treatments, respectively). C, F and I: ALP activity in response to AG-1478, Poziotinib and Anti-Ereg neutralizing antibodies, respectively (n=3, * and #: $p<0.05$ between genotypes and treatments, respectively). qPCR gene expression is normalized by *Hprt* expression.

Although evidence for increased epiregulin protein production by *Nf1*-deficient mBMSCs and EGFR signaling activity were observed, both pharmacological EGFR inhibition and epiregulin ligand blockade failed to correct the differentiation defect of these cells. These results led us to conclude that the upregulation of Epiregulin expression and EGFR activation induced by *Nf1* deficiency in osteoprogenitor cells does not cause the reduced osteogenic potential of *Nf1* deficient cells, and indicate that pathways other than EGFR signaling contribute to this phenotype.

The finding that the level of TGFβ1 was increased in the culture medium of BMSCs isolated from the $Nf1_{Col2.3kb}^{f/-}$ mice is consistent with the phenotypic overlap between the cellular abnormalities in NF1 PA and other conditions characterized by excessive TGFβ signaling, including Camurati-Engelmann, Marfan and Loeys-Dietz syndromes [82,240,241]. It is also consistent with the known pro-proliferative and anti-osteogenic differentiation activities of TGFβ [101,103,272–275], which mimic the *in vitro* behavior of *Nf1*-deficient osteoprogenitors. However, our analyses did not allow us to confirm increased levels of TGFβ1 expression in *Nf1*-deficient bone cells, including MEFs, BMSCs and calvaria primary cells, and the reason for this may stem in the differences between the cell types that were used in these two studies. *Rhodes et al.* prepared $Nf1^{-/-}$ MSCs from the bone marrow of $Nf1_{Postn}^{f/-}$ mice, and differentiated them after 5-10 passages in osteogenic medium [242]. This is in contrast with our cultures, that were prepared from undifferentiated mBMSCs extracted from $Nf1^{f/f}$ mice, infected *ex vivo* with a GFP- or CRE-adenovirus, and not passaged after infection. Although the adenovirus infection may impact to some extent the behavior of the cultures, this approach has the advantage of comparing clearly-defined genotypes and cultures, whose behavior starts to differ after *ex vivo* infection with Adenovirus and hence avoid the impact of cell-cell interactions *in vivo*, whereas the approach from Rhodes *et al.* relies on extensively passaged primary cells, whose differentiation and behavior may

be impacted *in vivo* before extraction and plating, and *ex vivo* because of multiple passages [242]. A consequence from these different experimental conditions is that the two studies may have compared osteoblasts at different differentiation stages, with the *Rhodes* study based on more differentiated osteoblast cultures used in our study [242], which used undifferentiated, plastic-adherent bone marrow osteoprogenitors and *Nf1* ablation induced shortly thereafter before induction of differentiation by confluency and addition of osteogenic medium. This is important to note because the progressive and long-term nature of tibia bowing and non-union in NF1 patients, and data from genetic mouse models related to this condition, all support the idea that the cell of origin for this condition is a proliferating, undifferentiated mesenchymal progenitor, prior to the expression of *Col2* and *Osx* [209,245]. Hence, the traits and behavior of *Nf1*-deficient undifferentiated osteoprogenitors are likely to be more clinically relevant than the characteristics of *Nf1*-deficient mature osteoblasts or osteocytes for instance [276], that are unlikely to be ever generated based on the defective differentiation of *Nf1*-deficient osteoprogenitors.

It is still important to recognize the beneficial effect of TGFβ1 blockade on bone mass and fracture healing reported by Rhodes *et al* [242], and the clinical relevance of these findings. A similar comment applies to the findings by Ghadakzadeh *et al* [277], showing improved bone healing upon use of Nefopam treatment to block the increase in β-catenin expression they detected in *Nf1*-deficient mBMSCs [277,278]. An important note related to these published studies is that they all use *Nf1* conditional floxed cells to achieve gene ablation following CRE activity. A caveat with this approach is that gene recombination is rarely complete. Therefore, a detectable increase in osteogenic differentiation in these cultures following treatment can reflect an osteogenic response of non-recombined cells to osteogenic treatments like BMP2, nefopam or blockade of TGFβR. This is supported by the observation that SD-208, a TGFβR inhibitor, increases *Alpl* expression in

both *Nf1*-deficient and WT mBMSCs (**Figure 15**) and bone mass in both WT and *Nf1* $_{Col2.3kb}^{f/-}$ mice [242]. Interpretation of results must account for this effect of treatment on non-KO cells, and the extent of this confounding factor should be assessed by the use of appropriate controls, which include treatment of the WT cells. Taking this comment into consideration, we conclude that TGFβ1 and β-catenin blockade has preclinical value as pharmacological approach to improve bone union in children with NF1 PA, but the stimulatory effect of SD-208 treatment on WT cells and our inability to detect an increase in TGFβ1 expression in *Nf1*-deficient bone cells question the contribution of increased TGFβ1 levels to the impaired osteogenic potential of *Nf1*-deficient BMSCs.

*NF1* deficiency led to increased expression of *EREG* in human and murine osteoprogenitors. However, our results do not support increased *Ereg* expression and signaling as a major component of the defective differentiation potential of *Nf1*-deficient osteoprogenitors, while sharing that there might some beneficial effect for use of TGβR1 inhibitor SD-208. In order to identify specific NF1 signaling-related molecular targets/nodes amenable to pharmacological treatment, we thus decided to further investigate the contribution of the most studied signaling pathway in NF1 patients, MAPK signaling.

# III. Chronic MAPK activity may not cause the osteogenic differentiation impairment in *Nf1*-deficient osteoprogenitors

## Background

Mitogen Activated Protein Kinases (MAPK) are an ancient kinase system in mammalian cells that regulate different aspects of organ and cell physiology and responses to different stimuli [279]. While *Erk1* KO mouse are viable with defective thymocyte development, *Erk2* KO mice die at an early embryonic developmental stage [280,281]. Disruption of MAPK specifically in the skeleton has deleterious effect on embryonic skeletal development and skeletal homeostasis. But what is very important to emphasize here is that the stimulatory effect of increased ERK signaling in WT osteoblasts sharply contrasts with the inhibitory effect of chronic ERK activation in Nf1 KO osteoblasts. This observation questions the very important assumption of causality between MAPK/ERK chronic activation and reduced osteogenic differentiation observed in *Nf1*-deficient osteoprogenitors. Secondly, the majority of the search on therapeutic options for PA are currently based upon this notion that constitutive MAPK/ERK signaling is responsible for the pathologic findings in NF1 PA patients. Thus, by assessing the putative irrelevance of this constitutive activation of RAS/ERK signaling on PA, the search could be redirected towards the correct path. Hence this chapter will focus on investigating the possible role of MAPK/ERK in the reduced osteogenic differentiation potential of *Nf1*-deficient osteoprogenitors.

## Materials and methods

### BMSC culture

The institutional animal care and use committee Baylor College of Medicine approved all the mouse procedures. Mice were housed 2-5 per cage. Mouse BMSCs were extracted from long bones of 2-3 month-old *Nf1*$^{f/f}$ and *Nf1*$^{f/-}$ mice [198,203] by centrifugation at 3000 g for 3 minutes, as previously described [260]. Extracted marrow was plated in 10 cm dishes in α-MEM medium (without ascorbic acid) supplemented with 10% fetal bovine serum and 100 U/ml Penicillin/ Streptomycin (15140-122, ThermoFisher) for three days. At that time, non-adherent cells were discarded by changing the medium. Cells were trypsinized after reaching 80% confluence and were seeded in 6-well plates at 10,000 cells/cm$^2$ for adenovirus transduction. After reaching 60% confluence, cells were incubated with the adenovirus solutions (Ad-GFP or Ad-CRE recombinase, Baylor College of Medicine vector development lab) in the presence of Gene Jammer reagent (Agilent technologies; Cat# 204132), as described previously [261]. Briefly, Gene Jammer was added at a final concentration of 1% to FBS- and antibiotic-free α-MEM medium. The solution was vortexed briefly and incubated for 10 minutes at room temperature before adding the virus at a MOI of 400 and incubating for further 10 minutes. Final mixture was added to each well and cells were incubated with the virus solutions for 24 hours. The media was then changed to fresh complete α-MEM medium containing 10% FBS and Pen/Strep (Thermofisher Cat# 15140122). Mouse BMSCs were differentiated in osteogenic medium containing ascorbic acid (50 μg/ml) and β-glycerophosphate (5mM) in α-MEM medium for 7 days. Medium was changed every other day.

## Drugs

U0126 (Sigma Cat# U120-1MG) was reconstituted in DMSO (Vehicle). Modified C-Natriuretic peptide (BMN 111) was from Biomarin Inc and was reconstituted in PBS.

## Gene expression assays

Total RNA was extracted using TRIzol (Thermofisher, Cat# 15596026), and contaminating genomic DNA was digested by treatment with DNAse I (Promega, Cat# M6101). cDNAs were synthesized from 1ug RNAs using the high capacity cDNA reverse transcription kit (Thermofisher, Cat# 4368814),). Quantitative qRT-PCR was performed using the following TaqMan primers/probes: *Ccnd1* (Mm00432359_m1), *Ibsp* (Mm00492555_m1), *Alpl* (Mm00475834_m1), and the normalizer *Hprt* (Mm03024075_m1) from Thermofisher, or SYBR green primers: *Nf1* (forward: GTATTGAATTGAAGCACCTTTGTTTGG; reverse: CTGCCCAAGGCTCCCCCAG); *Bglap* (forward ACCCTGGCTGCGCTCTGTCTCT; reverse TAGATGCGTTTGTAGGCGGTC). SYBR qPCR specificity of amplification was verified by the presence of a single peak on the dissociation curve.

## Western blot

Proteins were extracted from cell cultures using RIPA buffer supplemented with Protease cocktail inhibitor (Sigma # P8340-1ML) and Phosphatase cocktail inhibitor (Sigma #P0044-1ML). Protein concentration was measured using BCA assay (Thermo-Fisher). Ten µg of total protein was run on SDS gel before transfer to a nitrocellulose membrane. Membranes were blocked using 5% non-fat powder milk in TBST buffer. β-actin (A5316, Sigma), p44/42 MAPK; ERK1/2 (CST#4695) and Phospho-p44/42 MAPK; ERK1/2 (CST#4376) were diluted in blocking buffer at 1:1000 to

1:2000 dilution and incubated with the membranes overnight at 4°C. Following washing, membranes were then incubated with an HRP-conjugated secondary antibody (goat anti mouse Santa Cruz Cat # sc-2005, goat anti-rabbit Santa Cruz Cat# sc-2030) diluted in blocking buffer at room temperature for one hour. Membranes were washed and incubated with ECL solution for 2 minutes and exposed to photographic film.

### Statistical analyses

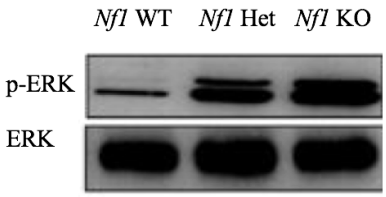
For comparison between *Nf1* WT and KO cells, a student t-test was performed. For multiple treatments, a two-way analysis of variance (ANOVA) was used to determine whether there was a statistically significant difference in treated vs. non-treated cells between genotypes. P-value less than 0.05 was considered significant. Statistical analysis was performed using Graph Pad PRISM (v6.0a, La Jolla, CA, USA). Data are provided as mean +/- SD.

## Results

### *Nf1*+/- BMSCs are characterized by increased ERK activation but, in contrast to *Nf1*-/- BMSCs, do not show reduced osteogenic differentiation potential *in vitro*

*Nf1* heterozygote osteoprogenitors such as Mouse Embryonic Fibroblasts (MEFs) show increased ERK activity compared to *Nf1* WT cells (**Figure 19A**) [200,218,282]. However, *Nf1* heterozygote mice do not show any apparent skeletal abnormalities compared to *Nf1* WT mice [197,198].

A




B

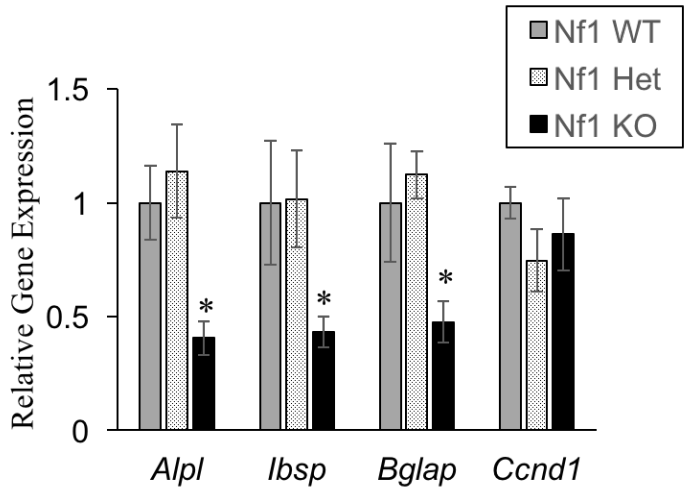



**Figure 19. *Nf1* heterozygocity does not cause reduced osteogenic diferentiation potential**
A: Western blot for p-ERK and ERK in *Nf1* WT, *Nf1* Heterozygote and *Nf1* null MEF cells (From Shapira S, Barkan B, Fridman E, Kloog Y, Stein R. The tumor suppressor neurofibromin confers sensitivity to apoptosis by Ras-dependent and Ras-independent pathways. *Cell Death Differ*. 2007;14(5):895-906. [283]). B: BMSCs were grown in osteogenic medium for 10 days and the expression of osteogenic gene markers was measured between different genotypes. Expression of osteogenic gene markers is similar between *Nf1* WT and *Nf1* heterozygote BMSCs. *Ccnd1* expression is included as the control for RNA and cDNA quality N=3 * p-value <0.05.

This observation led us to assess the osteogenic differentiation potential of *Nf1* heterozygote BMSCs. For this purpose, $Nf1^{f/-}$ (heterozygote) and $Nf1^{f/f}$ mice were sacrificed and their BMSCs were extracted. $Nf1^{f/f}$ BMSCs were either transduced with Ad-GFP (WT) or Ad-CRE (KO), while $Nf1^{f/-}$ BMSCs were transduced with Ad-GFP (Het).

BMSCs were grown in osteogenic medium for 10 days and gene expression was assessed (**Figure 19B**). Data in this graph show that there is no difference in the expression of osteogenic gene markers between *Nf1* WT and *Nf1* heterozygote BMSCs, while the expression of osteogenic markers in *Nf1*-deficient cells is decreased. Similar results were obtained using BMSCs from WT and Nf1+/- mice (data not shown). These data show that increased MAPK/ERK activity is not sufficient for causing the osteogenic differentiation phenotype of *Nf1*-deficient osteoprogenitors.

## RAF and MEK normalization do not rescue the reduced osteogenic differentiation potential of *Nf1* deficient BMSCs

In order to further investigate the role of chronic MAPK/ERK signaling on *Nf1*-associated reduced osteogenic differentiation, we decided to normalize the chronic activation of ERK in *Nf1*-deficient osteoprogenitors at different nodes of the MAPK signaling cascade, using pharmacological inhibitors (**Figure 20A**). For this purpose, *Nf1* WT and *Nf1*-deficient BMSCs were grown in regular medium in the presence of either vehicle (DMSO), the MEK1,2 specific inhibitor (U0126 1μM) or RAF inhibitor (C-Natriuretic peptide 10μM) for seven days (**Figure 20B**). Chronic activation of ERK1,2 was reduced in *Nf1* KO BMSCs in response to U0126 and CNP. In the next step, *Nf1* WT and *Nf1*-deficient osteoprogenitors were grown in osteogenic medium supplemented with the aforementioned concentrations of U0126 or CNP and the differentiation of BMSCs was assessed by measuring the expression of osteogenic gene markers (**Figure 20C**).

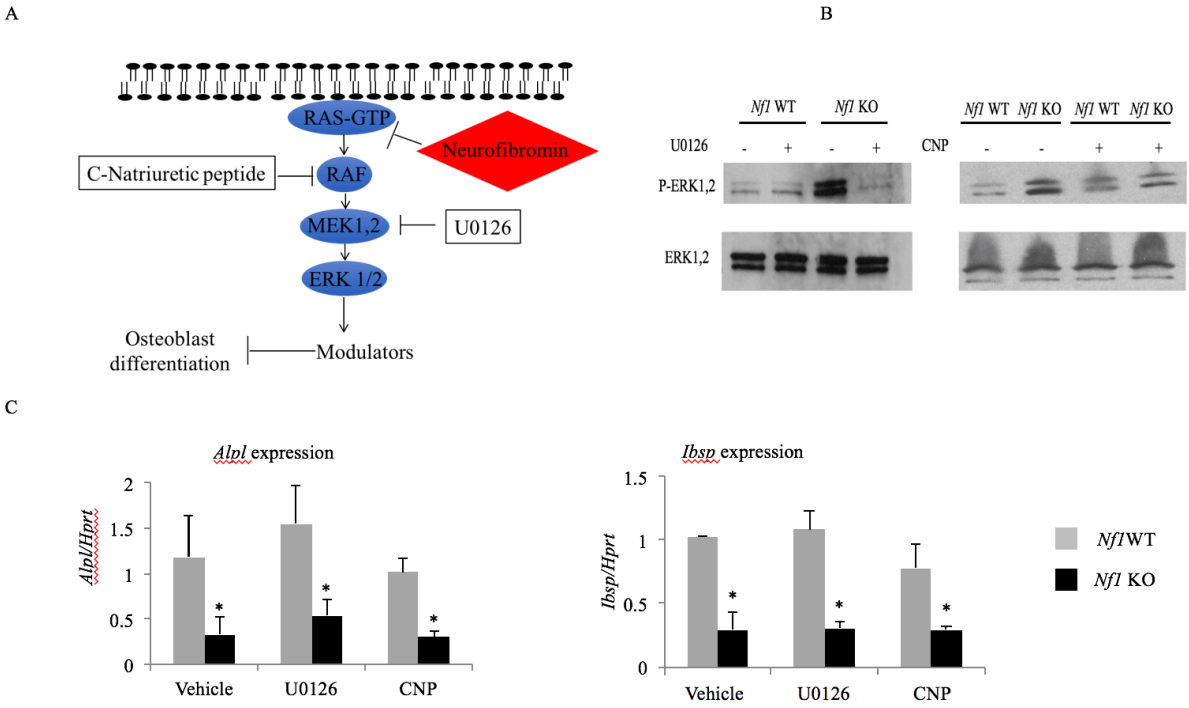



**Figure 20. Normalization of MAPK signaling does not rescue reduced osteogenic differentitiation in *Nf1* deficient osteoprogenitors**

A: Simplified schematic of the steps of MAPK normalization. B: Western blot depicting the efficacy of U0126 (1μM) and CNP (10μM) to normalize ERK activity. The CNP blot is courtesy of Dr. Matthew Karolak. C: BMSCs were grown in osteogenic medium supplemented with vehicle (left), 1μM U0126 (middle) and 10μM CNP. Gene expression show reduced osteogenic gene marker expression in *Nf1*-deficient cells N=3 * p-value <0.05.

The rationale for using C-Natriuretic peptide was that this molecule has reduced cytotoxicity compared to other RAF inhibitors due to its specificity for MAPK targeting, since CNP requires a receptor at cell surface to exert its effect. Previously our laboratory has shown that CNP has beneficial effect on the growth plate in the context of *Nf1* deficiency. I observed that there was no inhibitory effect of treatment on osteogenic marker gene expression of *Nf1* WT cells in the U0126 or CNP-treated groups (**Figure 20B**). However, these data also revealed that neither U0126 nor CNP treatment could rescue the reduced osteogenic differentiation observed in *Nf1*-deficient cells (**Figure 20C**). To further support this finding, we used MEFs, a different type of osteoprogenitors, and obtained similar results.

### Among all "Rasopathies", only NF1 is associated with pseudarthrosis

Rasopathies are a group of disorders that have in common chronic activation of RAS signaling. Chronic activation can be caused either as the result of activating mutation in RAS or other downstream signaling molecules or as a result of inactivating mutations in the negative regulators of RAS signaling e.g. *NF1*, *PTPN1* (**Table 1**). Similar to NF1, other Rasopathies also have musculoskeletal manifestations (**Figure 21**). Those include short stature, chest wall deformities and low BMD (osteopenia). Importantly, pseudarthrosis of the tibia is reported only in patients with NF1. This observation suggests that short stature, chest wall deformities and osteopenia are likely RAS/ERK-dependent, whereas NF1 pseudarthrosis might be caused by a RAS/ERK-independent mechanism negatively impacting the differentiation and function of osteoprogenitors to be recruited to the fracture site.

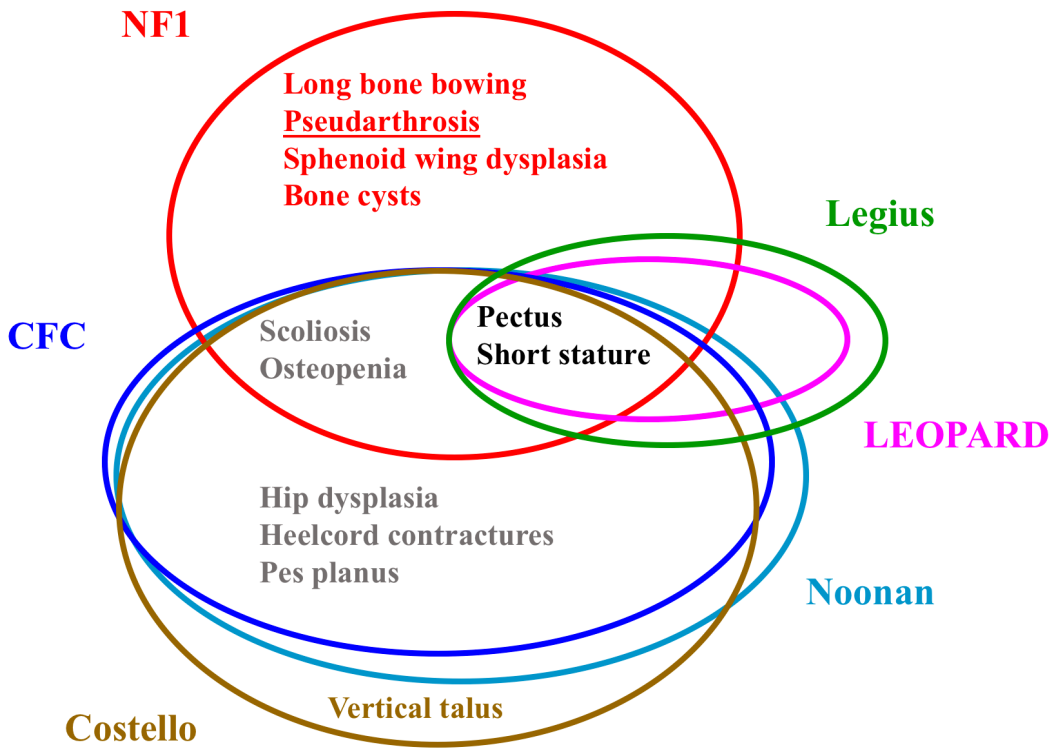
**Table 1**. List of common Rasopathies

| Name | Gene | Phenotypes |
|---|---|---|
| Noonan | *PTPN11*, *SOS1*, *KRAS*, *RAF1*, *NRAS*, *CBL*, *BRAF*, and *SHOC2* | kyphosis, lordosis, scoliosis, chest wall deformities, and short stature[284] |
| Costello | *HRAS*[285] | kyphosis, scoliosis, anterior chest wall anomalies, hand anomalies, short stature, and osteopenia/osteoporosis [284] |
| Cardiofaciocutaneous Syndrome (CFC syndrome) | *BRAF*, *MAP2K1*, *MAP2K2*, and *KRAS* [284] | non-dystrophic scoliosis, low BMD, and chest abnormalities |
| LEOPARD (multiple lentigines syndrome) | *PTPN11* [286] | short stature, chest wall deformities and scoliosis |
| Legius | *SPRED1* | short stature, macrocephaly and pectus excavatum and café au lait spots, Neurofibroma[287]. |

Of note is that features involving cartilage formation and physiology in human NF1 PA biopsies and in mouse models of skeletal *Nf1* deficiency could be attributed to the negative effect of MEK signaling on growth plate physiology and chondrocyte differentiation [288]. Our laboratory has shown that treatment of chondrocyte-specific *Nf1*-deficient mice with a CNP analogue (NC-2), which blocks chronic RAS/RAF-1 activation, could rescue the stature and growth plate abnormalities observed in these mice. [245]. The short stature of patients with RASopathies also suggests a growth plate defect caused by chronically active RAS/ERK signaling. Taken together, these observations suggest that RAS/RAF/ERK chronic activation in absence of *Nf1* negatively impacts the commitment or differentiation of chondrocytes, but is not the causal determinant of the poor osteogenic potential of osteoprogenitors committed to the osteoblast lineage.

## Discussion

*NF1* deficiency leads to chronic activation of the RAS-MAPK signaling pathway [190,289,290]. Many of the manifestations of NF1 have been attributed to this chronic activation of MAPK signaling and thus therapies have been designed to counteract ERK activation [204,237,238]. For example, it has recently been shown that transgenic expression of the GRD domain of Neurofibromin in committed osteoblasts could rescue some phenotypes associated with *Nf1* deficiency, including increased Tgfb1 production and its downstream signaling molecules Smad2,3 [242]. However, as it was discussed in chapter II of this thesis, these findings are not translationally relevant because these researchers used a CRE system that is active in committed osteoblasts, while in the absence of Neurofibromin in osteoprogenitors, committed osteoblasts cannot form.




*Modified from Dr. D. Stevenson (Stanford)*

**Figure 21. NF1 is a unique condition among Rasopathies**
When comparing the phenotypes of known human Rasopathies, we see an overlap of phenotypes such as short stature. This suggest a common path in the genetics of these phenotypic features. However, pseudarthrosis is a unique feature of *NF1* deficiency, therefore suggests that delayed bone healing may stem from Neurofibromin unique biology independent of its RAS modulatory role.

MAPK signaling is required at different stages of skeleton formation and osteoblast differentiation. It stimulates osteoblast differentiation from osteoprogenitors thus it could be expected that chronic activity of MAPK in *Nf1*-deficient osteoprogenitors should promote osteoblast differentiation. However, *Nf1*-deficient osteoprogenitors show reduced osteogenic differentiation. This suggests that there may be a signaling pathway downstream of MAPK signaling that *Nf1* deficiency negatively impacts to blunt the response of *Nf1*-deficient cells to osteogenic cues.

If chronic MAPK signaling was causing the reduced osteogenic differentiation in *Nf1*-deficient osteoprogenitors, one would expect a reduction in osteogenic differentiation in *Nf1* heterozygote cells, which are also characterized by increased MAPK activity. However, these cells did not show reduced osteogenic differentiation compared to *Nf1* competent cells. This represents a second evidence that the osteoblast differentiation potential of *Nf1*-deficient osteoprogenitors is MAPK-independent. There might be a possible inhibitory threshold for MAPK activity that *Nf1* heterozygote cells fail to reach, although we could not detect by Western blot a difference WT and KO cells. This possibility could be further investigated by transduction of increasing amount of constitutive active MEK in *Nf1* competent MSCs and measurement of osteogenic differentiation potential. Another more sensitive readout, such as an Elk1-reporter system, could also be used [291]. (ELK1 is a major nuclear substrate for ERK). In addition, normalization of MAPK signaling in two primary cell types, using different inhibitors could not rescue the reduced osteogenic differentiation potential in *Nf1*-deficient osteoprogenitors. These lines of evidence show that MAPK constitutive activity is not sufficient for causing the reduced osteogenic differentiation of *Nf1*-deficient osteoprogenitors, and therefore, there may be another signaling pathway that is impacted as the result of *Nf1* deficiency.

Another line of evidence that support this conclusion comes from clinical observations of Rasopathies. These syndromes share several features that probably stem from the same genetic path between NF1 and other members of this family. However, pseudarthrosis is a unique feature of NF1 only. This represents a fourth evidence suggesting that MAPK chronic signaling is not involved in the pathology of NF1 pseudarthrosis.

The existence of defects caused by RAS-MAPK independent changes in the behavior of *Nf1*$^{-/-}$ cell types other than bone cells has been explored previously. One of the other functions of Neurofibromin, in addition to the control of RAS-MAPK activity, was discovered in Drosophila. *nf1* null flies indeed have impaired memory and this phenotype is cAMP-dependent (it was rescued by introduction of PKA) [194]. The notion that learning disability associated with *Nf1* deficiency is PKA dependent has been further studied using mouse models. Investigators showed that *Nf1*-deficient neuroprogenitors show reduced neurogenic potential and increased proliferation rate. Restoring RAS activity to its normal level could normalize their proliferation rate, but it was ineffective in rescuing the differentiation phenotype [292].

Ismat and colleagues showed that embryonic lethality associated with *Nf1* deficiency could be partially rescued in *Nf1* null murine embryos which express the human HA-tagged GTPase-related domain of Neurofibromin (HA-GRD) ubiquitously. However, ubiquitous expression of this transgene could not rescue all of the phenotypes associated with *Nf1* deficiency in these mice and *Nf1* null pups died shortly postpartum. Although the cardiac phenotype was rescued in these pups, cells originated from the ectoderm still showed gross defects, which suggest that there are some phenotypes associated with *Nf1* deficiency that are NF1-GRD independent [293].

All in all, at least 4 lines of evidences point toward a MAPK-independent pathway being involved in the reduced osteogenic differentiation phenotype of *Nf1*$^{-/-}$ BMSCs: 1) different studies have shown that MAPK signaling has a stimulatory effect on osteogenic differentiation of WT osteoprogenitors and committed osteoblasts, whereas increased MAPK signaling in *Nf1*$^{-/-}$ BMSCs inhibited it; 2) MEK and RAF-1 pharmacological inhibition was unable to rescue the differentiation phenotype of *Nf1*$^{-/-}$ BMSCs; 3) both *Nf1*$^{-/-}$ and *Nf1*$^{+/-}$ BMSCs are characterized by increased RAS/ERK signaling, but only *Nf1*$^{-/-}$ display a defect in osteogenic differentiation potential and 4) RASopathies are conditions characterized by high RAS/MAPK signaling and share several skeletal abnormalities but pseudarthrosis is only observed in NF1 patients. Based on these findings, I chose to investigate further the possible existence of a RAS or MAPK-independent mechanism underlying the poor osteogenic potential of *Nf1*$^{-/-}$ BMSCs, by using a nonbiased approach.

# IV. A non-candidate, unbiased approach to identify the dysregulated signaling pathway(s) in *Nf1*-deficient osteoprogenitors

## Background

Until this stage of these studies, my main hypothesis was that the reduced osteogenic differentiation observed in *Nf1*-deficient osteoprogenitors was caused by MAPK dysregulated signaling and to correct this phenotype, MAPK signaling or its downstream modulators should be normalized. However, multiple lines of evidence presented in the previous chapter led us to pursue the hypothesis that a MAPK-independent pathway causes the reduced osteogenic differentiation phenotype in *Nf1* deficient osteoprogenitors. This hypothesis does not preclude that there is not a pathogenic role for the chronic activation of ERK1,2 in the skeleton of NF1 patients. Good evidence suggests that increased RAS/ERK signaling contributes to abnormal chondrocyte proliferation and maturation and to the matrix mineralization defect observed in *Nf1* deficient murine osteoprogenitors [208]. However, there is no direct evidence that suggest this mineralization phenotype contributes to the differentiation defect of *Nf1*-deficient BMSC. Therefore, the identification of the cause of this differentiation defect remains a priority.

Candidate approaches for the identification of risk or causative factors in different pathological conditions have been used extensively. However, in this case they have failed, therefore I decided to use RNA-Seq as an unbiased high-throughput approach as a first step to identify mediators of the *Nf1* differentiation defect. I compared the differential gene expression between *Nf1* WT and *Nf1* deficient BMSCs and also identified differences between genotypes that were MAPK-independent.

## Materials and methods

### BMSC cultures

The institutional animal care and use committee Baylor College of Medicine approved all the mouse procedures. Mice were housed 2-5 per cage. Mouse BMSCs were extracted from long bones of 2-3 month-old *Nf1*$^{f/f}$ mice [203] by centrifugation at 3000 g for 3 minutes, as previously described [260]. Extracted marrow was plated in 10 cm dishes in α-MEM medium (without ascorbic acid) supplemented with 10% fetal bovine serum and 100 U/ml Penicillin/ Streptomycin (15140-122, ThermoFisher) for three days. At that time, non-adherent cells were discarded by changing the medium. Cells were trypsinized after reaching 80% confluence and were seeded in 6-well plates at 10,000 cells/cm$^2$ for adenovirus transduction. After reaching 60% confluence, cells were incubated with the adenovirus solutions (Ad-GFP or Ad-CRE recombinase, Baylor College of Medicine vector development lab) in the presence of Gene Jammer reagent (Agilent technologies; Cat# 204132), as described previously [261]. Briefly, Gene Jammer was added at a final concentration of 1% to FBS- and antibiotic-free α-MEM medium. The solution was vortexed briefly and incubated for 10 minutes at room temperature before adding the virus at a MOI of 400 and incubating for further 10 minutes. Final mixture was added to each well and cells were incubated with the virus solutions for 24 hours. The media was then changed to fresh complete α-MEM medium containing 10% FBS and Pen/Strep (Thermofisher Cat# 15140122). After one day in cel culture, cells received either DMSO or U0126 (1μM) and were kept in cell culture for 8 hours before RNA/protein extraction.

## Chemicals and reagents

U0126 (Cell Signaling Technology, Cat. # 9903) and Bay-11-7085 (Sigma Cat. # B5681-10MG) were reconstituted in DMSO (Vehicle).

## Gene expression assays

Total RNA was extracted using TRIZOL (Thermofisher, Cat# 15596026), and contaminating genomic DNA was digested by treatment with DNAse I (Promega, Cat# M6101). cDNAs were synthesized from 1ug RNAs using the high capacity cDNA reverse transcription kit (Thermofisher, Cat# 4368814). Quantitative qRT-PCR was performed using the following TaqMan primers/probes for the normalizer *Hprt* (Mm03024075_m1), *Ibsp* (Mm00492555_m1), and *Alpl* (Mm00475834_m1 from Thermofisher, or SYBR green primers: *Nf1* (forward: GTATTGAATTGAAGCACCTTTGTTTGG; reverse: CTGCCCAAGGCTCCCCCAG); SYBR qPCR specificity of amplification was verified by the presence of a single peak on the dissociation curve.

## Western blot

Cell rinsed with PBS. Proteins were extracted from cell cultures using RIPA buffer supplemented with Protease cocktail inhibitor (Sigma # P8340-1ML) and Phosphatase cocktail inhibitor (Sigma #P0044-1ML). Protein concentration was measured using BCA assay (Thermo-Fisher). Ten µg of total protein was run on SDS gel before transfer to a nitrocellulose membrane. Membranes were blocked using 5% non-fat powder milk in TBST buffer. β-actin (A5316, Sigma), p44/42 MAPK (Erk1/2) (CST#4695) and Phospho-p44/42 MAPK (Erk1/2) (CST#4376) were diluted in blocking buffer at 1:1000 to 1:2000 dilution and incubated with the membranes overnight at 4°C. Following

washing, membranes were then incubated with an HRP-conjugated secondary antibody (goat anti mouse Santa Cruz Cat # sc-2005, goat anti-rabbit Santa Cruz Cat# sc-2030) diluted in blocking buffer at room temperature for one hour. Membranes were washed and incubated with ECL solution for 2 minutes and exposed to photographic film.

## RNA-Seq

All of RNA-Seq raw data file and library synthesis was performed in Scottish Rite hospital molecular biology center. Whole-transcriptome profiling (RNA-seq) was performed using RNA extracted from BMSCs treated with either DMSO or U0126. Samples are run on the Agilent Tapestation 4200 to determine level of degradation thus ensuring only high-quality RNA is used (RIN Score 8 or higher). The Qubit fluorometer is used to determine the concentration prior to staring library prep. One microgram of total DNAse treated RNA is then prepared with the TruSeq Stranded Total RNA LT Sample Prep Kit from Illumina. Total RNA is depleted of its rRNA and fragmented before strand specific cDNA synthesis. cDNA are then a-tailed and indexed adapters are ligated. After adapter ligation, samples are PCR amplified and purified with AmpureXP beads, then validated again on the Agilent Tapestation 4200. Before being normalized and pooled, samples are quantified by Qubit then run on the Illumina NextSeq 500 using V2 reagents. Fastq files were checked for quality using fastqc and fastq screen and were quality trimmed using fastq-mcf [294]. Trimmed fastq files were mapped to mm10 (UCSC version from igenomes) using TopHat [295]. Low-quality reads were filtered using Samtools and duplicates were marked using picard-tools, read counts were generated using featureCounts[296] and differential expression analysis was performed using edgeR implemented in the R statistical framework [297]. Quality measures of RNA-sequencing were investigated. Pairwise correlation of gene expression between control samples

calculated using counts per million (CPM) ranged from 0.60 to 0.85. To measure reproducibility between sequence runs, technical replicates were re-sequenced for a single WT/KO pair.

### Statistical analyses

For comparison of differentially expressed genes between *Nf1* WT and KO cells, a student t-test was performed. For comparison of the effect of different doses of BAY-11-7085 treatment between genotypes ANOVA test was used. Statistical analysis was performed using Graph Pad PRISM (v6.0a, La Jolla, CA, USA). Data are provided as mean +/- SD.

## Results

To compare the differential gene expression between *Nf1* WT and *Nf1* deficient BMSCs and the putative changes between genotypes that were MAPK-independent, I used *Nf1* WT and *Nf1* KO BMSCs treated with either vehicle or the MEK1,2 inhibitor U0126 (1 μM) for 8hrs, two days post adenovirus GFP or CRE transduction.

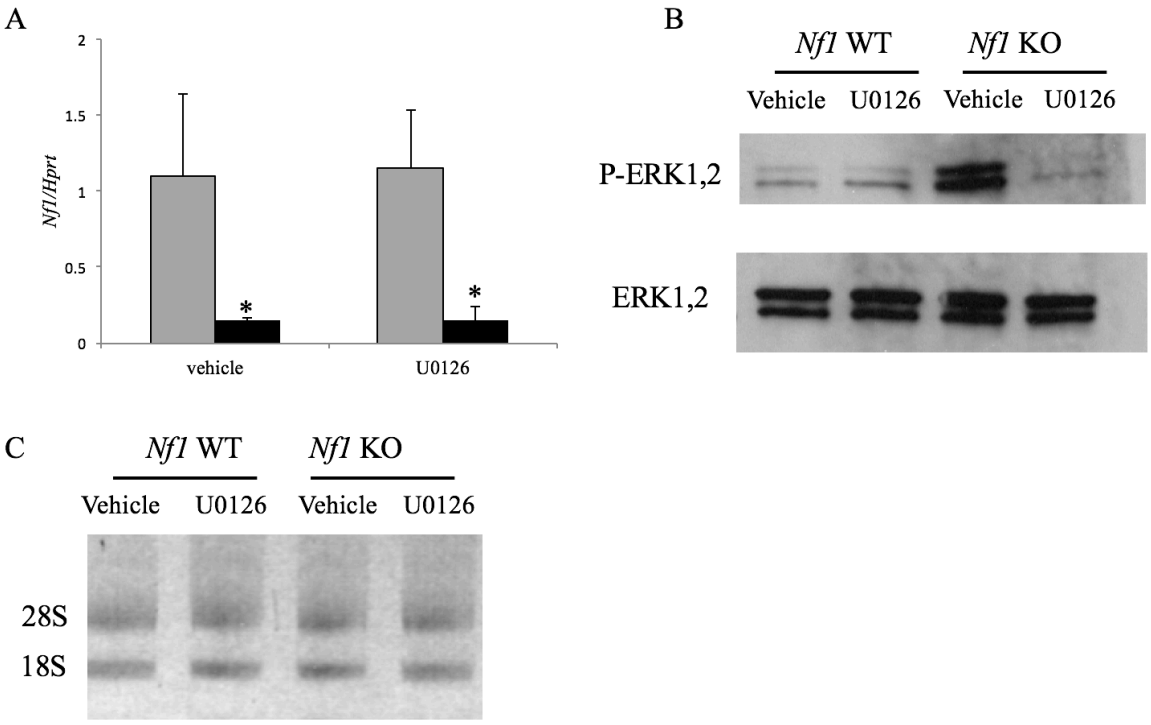



**Figure 22. Quality control for RNA seq submitted samples**
**A**: Expression of *Nf1* in adenovirus CRE-treated (*Nf1* KO) BMSCs compared to adenovirus GFP-treated (*Nf1* WT) BMSCs. qPCR, n=3, *: $p<0.05$ between genotypes, multiple t-tests. **B**: ERK phosphorylation is increased in *Nf1* KO BMSCs and is normalized after U0126 treatment in *Nf1* KO BMSCs. No effect of treatment was observed in WT BMSCs. **C**: RNA samples (1ug) had clear 28S and 18S bands and 28S>18S, confirming good RNA quality.

Several levels of quality controls were used prior to submission of the RNA samples for RNA-Seq assay. These include: 1) the efficacy of *Nf1* gene expression knock down was assessed using qRT-PCR for *Nf1* expression (**Figure 22A**); 2) Functionality of *Nf1* knock down and efficacy of U0126 treatment was validated by the level of p-ERK1,2 in *Nf1* WT and KO cells treated with U0126 (**Figure 22B**); 3) RNA integrity was confirmed (**Figure 22C**) by the presence and density of 28S and 18S bands. Importantly, the chosen concentration of U0126 did not reduce MEK activity in *Nf1*-competent cells, but normalized the p-ERK level in *Nf1* KO osteoprogenitors. This was done to avoid the deleterious effect of MEK inhibition on osteogenic differentiation [177].

A summary of the average number of reads in each group is depicted in **Figure 23A**. For this comparison, CPM cut off was implemented, rather counting more than 1 in all four groups of experimental condition was used for inclusion in this table. More than 13,000 genes could be detected in each experimental condition. Of these genes, normalized expression of BMSC markers (*Cd81*, *Cxcl12*, *Vim*, *Igfbp7*) [298,299] between all four conditions were high (**Figure 23B**), while markers of endothelial cells *Pecam1*[300] and *Cdh5* [301] and markers of HSC lineage (*Cd45* and *Cd150*) were either absent or their expression was very low (*Cd244* and *Cd34*) [302] (**Figure 23C**), thus confirming the osteoblastic nature of the cultures and validating the RNAseq data.

Four important comparisons (Analyses) were performed to answer several important biological questions, which are elaborated further in the next sections (**Figure 24A**). Cut off for the statistical significance was False Discovery Rate (FDR) smaller than 0.05, Fold change more than 2-fold, and normalized count per million (CPM) reads more than or equal to 10 in at least one experimental condition (*Nf1* WT BMSCs + Vehicle, *Nf1* WT BMSCs + U0126, *Nf1* KO + vehicle and *Nf1* KO + U0126). The summary of statistically significant Differentially Expressed Genes (DEGs) in the four starting comparisons is depicted in (**Figure 24B**).

A

| *Nf1* WT | Vehicle | U0126 |
|---|---|---|
| Reads | 32.1 x $10^6$ | 33.1 x $10^6$ |
| Genes | 13,509 | 13,575 |
| *Nf1* KO | Vehicle | U0126 |
| Reads | 38.3 x $10^6$ | 31.7 x $10^6$ |
| Genes | 13,604 | 13,585 |

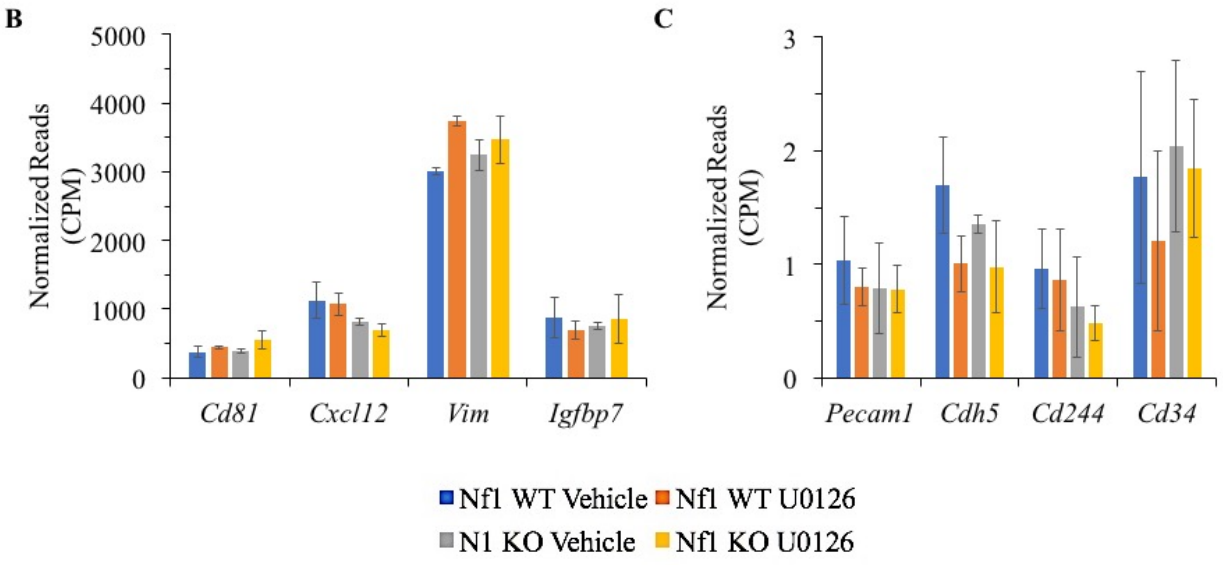



**Figure 23. Schematic of the RNA-Seq experimental groups and analyses of cell specific markers**

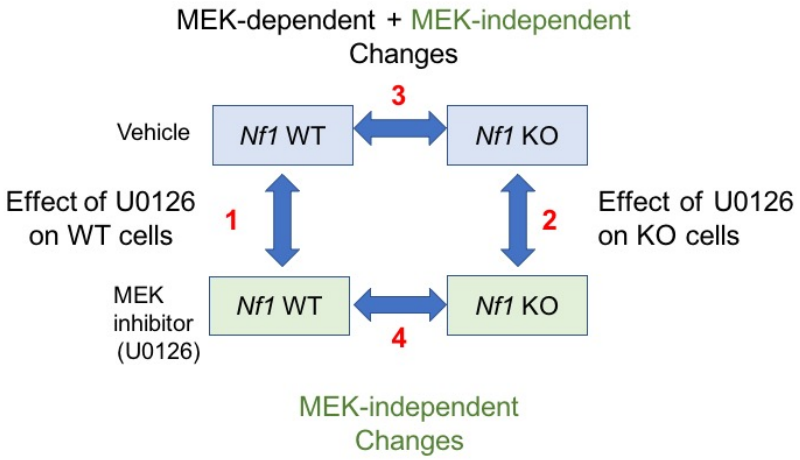
**A**: summary of the number of the reads in each experimental group B: Normalized expression of several BMSC markers between four groups. C: Normalized expression of several Endothelial and Hematopoietic Stem Cells (HSC) markers between four experimental groups.

After importing the list of differentially expressed genes from all four comparisons into the Ingenuity pathway analyses (IPA) software, the subcellular location of the predicted proteins encoded by these DEGs was given. In addition to four cellular location Cytoplasm, Extracellular matrix, Nucleus and plasma membrane, some of these genes were categorized as "other", which were not included in the final count. This list was imported into Excel and graphed (**Figure 24C**). Distribution of subcellular location of the DEGs between the first three comparisons was similar. However, a drastic decrease in DEG encoded proteins in nucleus and increase in DEG encoded proteins in extracellular space was observed in comparison No.4 (*Nf1* KO + U0126 vs. *Nf1* WT U0126). This finding suggests that DEGs in analysis No.4 (genes in *Nf1* KO cells that are non-responsive to MAPK normalization) are enriched in the extracellular matrix. This finding is important, as composition and signaling originated from extracellular matrix play key roles in the process of osteoblast differentiation and skeleton biology.

### Comparison No.1 (Effect of U0126 treatment on *Nf1* WT BMSCs)

MAPK is a critical signaling pathway and modulation of its activity can cause diverse changes in gene expression. Although, western blotting of *Nf1* WT BMSCs treated with U0126 for 8 hours did not show any apparent effect on the status of MAPK activity, there was still a possible non-detected subtle effect of U0126 treatment on the expression of direct target genes in *Nf1* WT cells. Hence, comparison No.1 was performed between *Nf1* WT cells treated with U0126 or vehicle (DMSO). The aim of this comparison was to investigate the effect of U0126 treatment on gene expression in WT BMSCs. In this comparison, we observed less than 100 genes changed significantly compared to comparison number 2 (effect of U0126 on KO cells). Very limited number of DEGs, in line with our objective not to negatively impact the behavior of these cells.

A




B

| Analysis | Comparison | Up-regulated genes | Down-regulated genes | Total |
|---|---|---|---|---|
| 1 | WT U0126 vs. WT Vehicle | 15 | 32 | 47 |
| 2 | KO U0126 vs. KO Vehicle | 300 | 904 | 1204 |
| 3 | KO Vehicle vs. WT Vehicle | 107 | 264 | 371 |
| 4 | KO U0126 vs. WT U0126 | 16 | 15 | 31 |

C

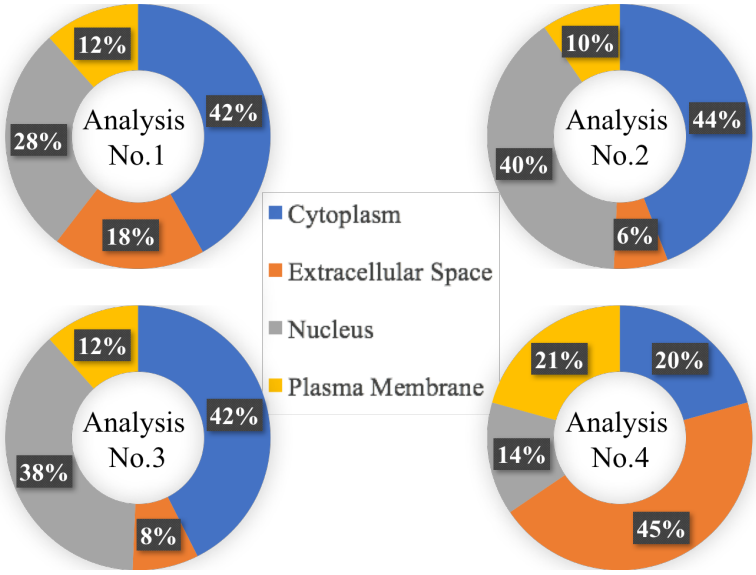



**Figure 24 Summary of analyses performed on RNA-Seq data**

A: Schematic representation of four analyses performed. Next to each arrow, a brief phrase is put that explain what each comparison provides. B: Summary of statistically significant differentially expressed genes between four analyses .C: Subcellular location of encoded proteins between four analyses.

This set of data shows that MEK inhibition does not overtly impact gene expression in *Nf1* WT BMSCs. However, we detected some inhibitory effect on the expression of genes that are known to be under control of ERK signaling such as *Ank and Mgp* [208,303], confirming biological activity of U0126.

In order to have a better understanding of the overall changes in the molecular biology of these cells, I used a Gene ontology online software (PANTHER Overrepresentation Test (release 20170413)/ GO Ontology Database Released 2017-10-24). This software uses the list of imported genes and compares them with the database of annotated genes in its dataset. The list of DEGs for this analysis was not enriched for any biological process or cellular component. The only enriched parameter was structural constituent of ribosomes as a molecular function (Enrichment score 15.71). This finding suggests that U0126 treatment impacted ribosomes and, by extension, translation process in these cells.

Another tool for studying high throughput datasets is the Ingenuity Pathway Analysis (IPA) software developed by QIAGEN. Based on the list of the DEGs in dataset, IPA can calculate a z-score that could be used for inference of activation state of any given upstream or causal transcription regulator and overall p-value that can be used for measuring statistical significance. IPA performs this prediction based on the comparison of prior acquired knowledge in the literature with the list of imported DEGs using Fisher's Exact test. These upstream transcription regulators could be drugs, proteins and siRNAs and hence the list of upstream regulators can be very broad. Z-score is calculated based on the relationships between any given transcription regulator (TR) and the set of observed genes that are under affected by that TR. Upstream signaling analysis considers a z-score of more than 0 as activated state of transcription regulator and less than 0 as inhibited transcription regulator.

For example, if based on the literature, activation of transcription regulator A upregulates the expression of gene B and downregulates the expression of gene C, and in observed dataset, the same trend is observed, the z-score will be positive and vice versa. It is important to keep in mind that there might be some discrepancies between the expected gene expression based on the calculated z-score and status of the observed gene expression. This can be explained by taking into account the fact that there are multiple transcription regulators that can interact with each other and gene expression in any given system is the result of this interaction. The other feature of this software is prediction of causal relationship between signature genes and known transcriptional modifiers, that similarly to upstream regulator analysis calculates a Z-score that can be used for inference.

For comparison No.1, the upstream regulator analysis contained a list of different kinase inhibitors such as MAPK antagonist, U0126, as transcriptionally activated. On the other side of the spectrum, growth factors such as EGF and Vascular endothelial growth factor (VEGF) were shown to be transcriptionally inhibited. This is expected and shows that the U0126 treatment was effective and targets of U0126 were changed (the direction of their gene expression change depending on U0126). The causal analysis showed that DEGs in U0126 treatment cells can cause similar gene signature to the effect of PDCD4, a known tumor suppressor protein [304]. Similarly, gene signature predicted that KAT5, a known acetyltransferase enzyme important for histone modification and activation of proliferation [305], is inhibited. Both of these predictions suggest that MAPK inhibition have negative effect on cell proliferation, which was expected based on the effect of MAPK signaling on proliferation. Comparison No.1 showed that although the number of genes affected in *Nf1* WT treated cells with U0126 was limited, some genes important for proliferation and protein translation were affected. This may have some implication regarding the use of any MAPK

inhibitor in the context of fracture healing, suggesting that such drugs should be used with caution and use be considered temporally, since one of the early important phases of fracture healing is the proliferation of diverse cell types at the site of injury.

### Comparison No.2 (Effect of MAPK normalization on *Nf1* KO BMSCs)

One of the most characterized and incriminated signaling pathways in NF1 is MAPK/ERK signaling. Therapies for NF1-associated malignancies have been developed and are in clinical trials with promising results [236]. However, although MEK blockade was shown to be inefficient to improve bone healing in two distinct mouse models of NF1 PA, there are no studies designed to assess the effect of MEK inhibition on gene expression of *Nf1* KO BMSCs. Comparison No.2 was performed between *Nf1* KO cells that were incubated with U0126 vs. vehicle to assess the effect of MAPK normalization on *Nf1* KO cells. Surprisingly, here we observed the highest number of DEGs between all four comparisons (Figure 3B). This indicated that gene expression in *Nf1* KO cells is far more sensitive to U0126 treatment compared to *Nf1* WT cells and also suggested that normalization of MAPK, in addition to normalizing genes that are dysregulated by MAPK constitutive activity, might also shift RAS constitutive activity towards other signaling pathways that are not affected in non-treated *Nf1* KO BMSCs. Additionally, this suggests that RAS-MAPK pathway is indeed one of the main pathways regulated by Neurofibromin in osteoprogenitors, although, this pathway does not seem to underlie the differentiation defect of $Nf1^{-/-}$ BMSCs.

Similar to Comparison No.1, I performed gene ontology for DEGs in this dataset. As the number of the DEGs in this comparison was high, an FDR less than 0.02 and fold enrichment score more than 2 was chosen for statistical significance. The summary of gene ontology is depicted in **Table 2.**

**Table 2:** Comparison No. 2 (Gene ontology analysis of genes impacted by U0126 treatment in *Nf1* KO BMSCs).

| GO biological process complete | **Fold Enrichment** | **FDR** |
|---|---|---|
| Histone H3-K27 trimethylation | 12.3 | 1.35E-02 |
| Nucleosome positioning | 12.3 | 1.34E-02 |
| Translational initiation | 5.09 | 1.65E-04 |
| GO molecular function complete | | |
| Translation initiation factor activity | 4.42 | 8.78E-03 |
| Histone binding | 3.34 | 2.47E-05 |
| Ribonucleoprotein complex binding | 3.08 | 1.69E-03 |
| mRNA binding | 2.98 | 5.12E-05 |
| Ubiquitin protein ligase activity | 2.46 | 2.48E-03 |
| GO cellular component complete | | |
| Astral microtubule | 13.12 | 1.70E-02 |
| Cytoplasmic ubiquitin ligase complex | 8.2 | 1.82E-02 |
| Nuclear nucleosome | 7.65 | 3.18E-03 |
| U1 snRNP | 7.25 | 4.02E-03 |
| Exon-exon junction complex | 6.89 | 5.08E-03 |
| Mitotic spindle pole | 5.62 | 5.75E-03 |
| Condensed nuclear chromosome, centromeric region | 5.51 | 1.41E-02 |

One of the top biological processes enriched in this comparison relate to genes that are important for the methylation of histones. Methylation on H3K27 (amino acid lysine residue No.27 of Histone 3) has negative effect on expression of modified genes [306]. As MAPK activation stimulate transcription of diverse genes that are involved in physiological processes such as proliferation [307], epigenetic modification of Histones may be one of the routes that U0126 could implement its inhibitory effect on MAPK signaling pathway. In addition to epigenetic modification of promoters and hence gene transcription, U0126 also impact translation and ribosomes. U0126 treatment affects molecular function such as Histone, mRNA and Ribonucleoprotein complex binding. It also enriches genes that are important for splicing such Small Nuclear Ribonuceloprotein Sm D1 (SNRP1). Enrichments of all of these pathways and molecular functions depict that U0126 treatment in *Nf1* KO cells impact the gene expression and translation of MAPK target genes at the level of transcription, mRNA maturation and protein synthesis. This enrichment might stem from the fact that loss of *Nf1* affect the translation and protein synthesis and U0126 treatment may normalize this dysregulation.

Upstream regulator IPA analysis provided more detailed insights into the possible signaling pathway changes in *Nf1* KO U0126-treated BMSCs. One of the top activated upstream regulators in this comparison was HRAS. This finding was expected, because Neurofibromin modulate RAS activity and inhibition of MEK using U0126 should not affect RAS constitutive activity and RAS constitutive activity should be rechanneled to new signaling pathways. In addition, PTEN a negative regulator of PI3K was inhibited, which suggests that in absence of MAPK signaling, PI3K might become the major activated signaling in *Nf1* KO BMSCs. Another observation to support the possible role of PI3K signaling pathway, in the presence of MAPK inhibitor, in *Nf1* KO osteoprogenitors comes from the detection of several growth hormones known to activate PI3K

signaling, such as PDGF and HGF as transcription regulators with positive (activated) z-score [308,309].

IPA causal network analysis showed TATA binding protein (TBP) as one of transcription regulators with negative z-score (inhibited). This finding shows that U0126 treatment impact the transcription of the genes that are under transcriptional control of TBP. Another inhibited transcription regulator was Nuclear Co-Activator 6 (NCOA6). As its name implies, this protein activates the transcription of diverse genes by its interaction mostly with other nuclear receptors such as vitamin D3. Its mutation and activation is associated with several cancers such as lung [310]. Inhibited state of transcription factors important factors in *Nf1* KO cells treated with U0126 is in line with the anti-proliferative and anti-tumor properties of MAPK antagonists in NF1 associated malignancies.

### Comparison No.3 (Identification of differentially expressed genes between WT and *Nf1*-deficient BMSCs)

*Nf1* loss of function causes reduced osteogenic differentiation. To identify DEGs as the result of *Nf1* deletion, Comparison No.3 was performed between *Nf1* WT and *Nf1* KO cells that were incubated with vehicle (DMSO) to identify genes and pathways differentially regulated between genotypes (in a MAPK-dependent and MAPK-independent manner). A brief overview of the genes that are dysregulated in this condition shows that they encompass proteins with diverse cellular functions that ranges from controlling arginine metabolism (*Ass1*) to regulate RNA splicing and binding (*lsm5*). This diversity of function show that Neurofibromin impacts different aspects of cell physiology.

The results of gene ontology analysis are shown in **Table 3**. The cut off for the inclusion was enrichment higher than 2 and FDR less than 0.05. DNA replication and cell division were among the enriched biological processes, in line with the fact that Neurofibromin loss of function in different cell types causes constitutive activity of RAS and increases proliferation rate [311–313]. Of interest, Neurofibromin deficiency affects rate of cell proliferation depending on the cell type. Ono and colleagues in our laboratory showed that *Nf1* deficiency reduced cell proliferation in the growth plate of *Nf1*-deficient osteochondroprogenitors [245] whereas Kolanczyk and colleagues showed that *Nf1*-deficient osteoblasts have increased proliferation rate [205]. It has been shown that *Nf1* deficiency increases the proliferation rate of neural cells [314]. Enrichment of genes controlling proliferation disappears in *Nf1* KO BMSCs upon MEK inhibition (Comparison 4), which suggests that this activated process is MEK-dependent, in accordance with known function of ERK1,2 in stimulating the proliferation.

Similar to previous comparisons (effect of U0126 on BMSCs), *Nf1* loss of function affects the protein synthesis process in *Nf1*-deficient BMSCs. These changes include enrichment in mRNA binding and export, and ribosome assembly. Elefteriou and colleagues have shown that protein synthesis was dysregulated in *Nf1*-deficient osteoblasts and low protein diet could rescue some of the phenotypes observed in the $Nf1_{col1}^{-/-}$ mouse model [80]. However, treatment with a MEK inhibitor cancelled this enrichment, thus indicating the MEK dependence of increased protein synthesis.

The next enriched set of genes detected in this analysis included regulators of cell substrate adhesion to the matrix and genes located in the extracellular matrix. *Nf1* deficiency increases the adhesion of MEF cells to different types of substrate, e.g. fibronectin and Collagen type I, through a FAK-mediated pathway [315]. This finding is observed in BMSCs as well [316].

**Table 3**: comparison No. 3 (Gene ontology analysis of genes impacted by *Nf1* deficiency in BMSCs).

| GO biological process complete | **Fold Enrichment** | **FDR** |
|---|---|---|
| DNA-dependent DNA replication maintenance of fidelity | 10.69 | 2.72E-02 |
| Ribonucleoprotein complex export from nucleus | 7.14 | 1.78E-02 |
| Ribosome assembly | 7.14 | 1.76E-02 |
| Positive regulation of DNA replication | 6.97 | 4.49E-02 |
| Nuclear export | 6.48 | 5.23E-03 |
| Response to organophosphorus | 6.02 | 1.74E-02 |
| Response to purine-containing compound | 5.3 | 3.01E-02 |
| mRNA transport | 5.15 | 3.50E-02 |
| Regulation of cell-substrate adhesion | 4.23 | 1.08E-02 |
| Positive regulation of cell migration | 2.83 | 1.08E-02 |
| Cell division | 2.83 | 1.08E-02 |
| Actin filament-based process | 2.68 | 2.34E-02 |
| Regulation of mitotic cell cycle | 2.67 | 1.79E-02 |
| Regulation of cell cycle | 2.16 | 2.46E-02 |
| Cytoskeleton organization | 2.22 | 1.41E-02 |
| Cellular response to stress | 2.15 | 1.59E-03 |
| Positive regulation of catalytic activity | 2.12 | 1.76E-02 |
| GO molecular function complete | | |
| Structural constituent of ribosome | 5.16 | 2.01E-03 |
| mRNA binding | 3.88 | 2.65E-02 |
| GO cellular component complete | | |
| THO complex part of transcription export complex | 37.86 | 5.72E-04 |
| Nuclear euchromatin | 9.47 | 3.29E-02 |
| Cytosolic small ribosomal subunit | 9.25 | 2.83E-03 |
| Nuclear matrix | 6.16 | 2.76E-03 |
| Cytosolic large ribosomal subunit | 6.12 | 1.96E-02 |
| Nuclear chromosome, telomeric region | 4.59 | 3.14E-02 |
| Focal adhesion | 4.08 | 4.94E-06 |
| Chromosome, centromeric region | 3.54 | 2.22E-02 |
| Spindle | 2.96 | 4.40E-02 |
| Nucleolus | 2.43 | 5.54E-04 |
| Extracellular matrix | 2.3 | 3.91E-02 |
| Catalytic complex | 2.02 | 9.74E-04 |

The increased adhesion phenotype is also seen in *Nf1*$^{+/-}$ monocytes and play an important role in increased activity of Nf1+/- osteoclasts [218]. This is a valuable indication that there might be a dysregulated change in communication between the extracellular matrix and intracellular machinery of *Nf1*$^{-/-}$ BMSCs that interestingly does not disappear upon MAPK inhibition.

Another feature of IPA software is its phenotype and disease association analysis. Similar to other aforementioned analyses (Upstream regulator and causal network), this feature also calculates a z-score that could represent the relationships between any given disease or phenotype and DEGs. For comparison No.3, two interesting phenotypes were observed. These included firstly, stimulation of protein synthesis and protein expression (discussed previously) and secondly, inhibition of differentiation of diverse cell types such as neurons, endothelial cells and bone marrow cells (**Figure 25**). This is in accordance with published studies that show loss of *Nf1* negatively impacts differentiation of cells in nervous system, endothelial cells and cells from connective tissue such as osteoprogenitors [209,293,314,317,318]. From this observation, it can be hypothesized that there may be one or some common transcriptional regulators in these diverse cell types whose activity is controlled by Neurofibromin and hence loss of *Nf1* reduces the differentiation of these cells through interruption of these interactions. One of the common nodes between differentiation of neurons and bone marrow cells is Jak2. Jak2 is a signaling molecule involved in transducing signals in osteoprogenitors from growth hormone receptor (GHR) and stimulating IGF-1 synthesis [319]. As it was discussed in chapter I, IGF-1 is one of the important growth factors that have stimulatory effect on osteogenic differentiation. This finding might suggest that the lack of some unknown paracrine or hormonal factor or insensitivity to these factors impacts the differentiation of *Nf1*-deficient osteo- and neuro-progenitors and inhibition of migration of endothelial cells during development.

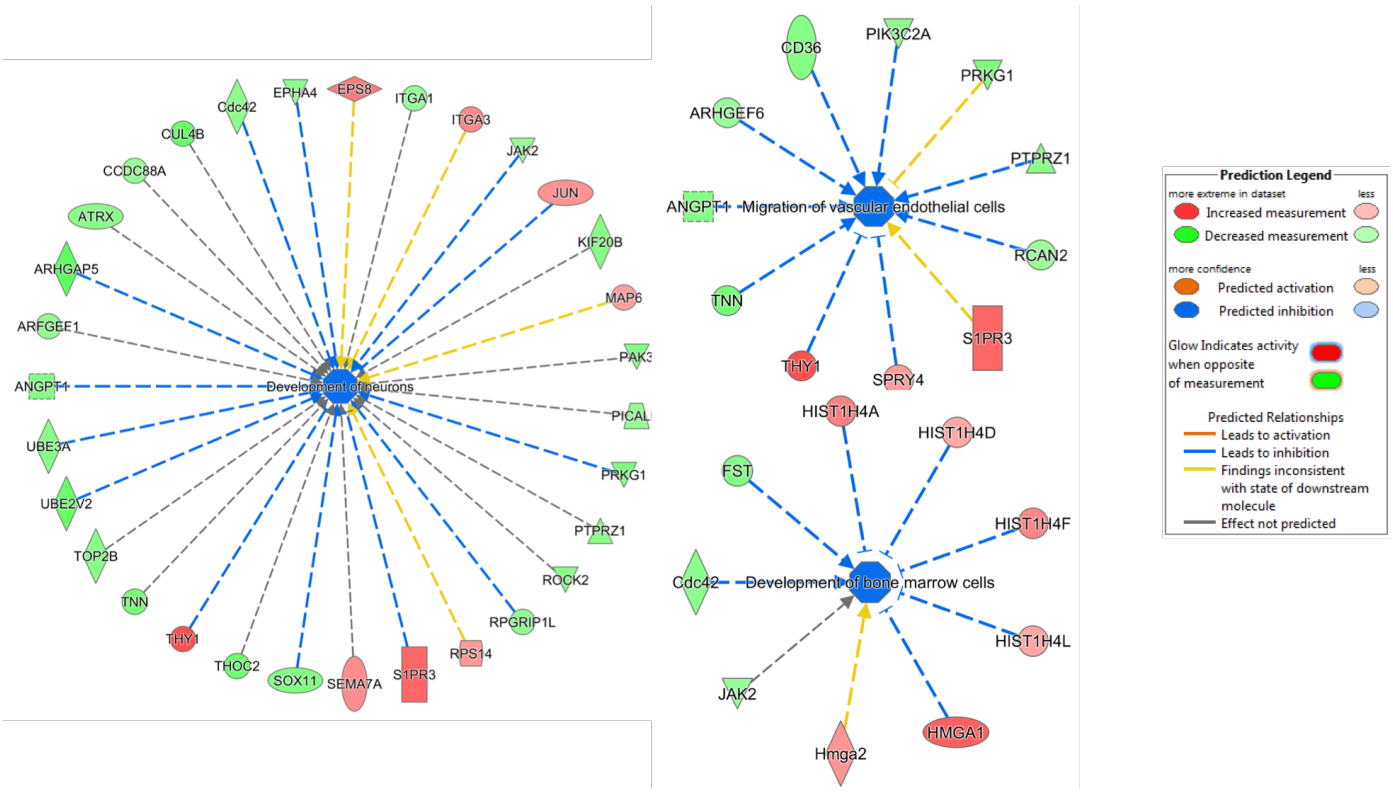



**Figure 25. Loss of *Nf1* is associated with reduced differentiation in diverse cell types .**
A: Graph on the left shows that *Nf1* deficiency causes dysregulation of genes important for development of neurons B: *Nf1* deficiency affects development of heart. In this graph, the list of the genes important for migration of endothelial cells such as *Angpt4* is depicted. C: *Nf1* loss of function impacts the development of connective tissue e.g. bone, by dysregulating the expression of genes such as *Hmga2*.

Upstream IPA analysis depicted several possible transcriptional regulators for this comparison. Because of the lower number of the genes compared to comparison No.2, only few upstream regulators had absolute Z-score value of more than 2. IPA software designates the z-score more than +2 as activated and less than -2 as inhibited transcriptional regulators. The list of the predicted regulators with absolute z-score more than 1 is shown in the **Table. 4**. Cells with pale blue filling are predicted upstream regulators whose transcriptional activity might have been inhibited. Conversely, marigold cells are the activated transcriptional regulators. Of these regulators, I will discuss some of them that have known impact on osteoblast differentiation and bone homeostasis. However, this does not exclude a possible role for other transcriptional regulators.

The most promising observation among upstream transcriptional regulators was the presence of molecules involved in pro-inflammatory signaling pathways. IPA predicts that loss of *Nf1* induces the inhibition of inhibitors of pro-inflammatory signaling such as Interleukin 13 (IL13), an anti-inflammatory cytokine, NFKBIA, an inhibitor of NFκB signaling and JNK antagonist, SP600125 and activators of pro-inflammatory signaling such as JUN. Pro-inflammatory signals such as TNFα can activate multiple signaling pathways including Jun N-terminal kinase (JNK) and NFκB. Individual with chronic inflammatory diseases lose bone as a result of disruption of two homeostatic processes; osteoclastogenesis (bone resorption) and osteoblastogenesis (bone formation) [320]. NF1 patients often present with a mild form of osteopenia which has been attributed to increase osteoclastogenesis [200], however, the role of the pro-inflammatory signals in osteoblastogenesis in these patients and different mouse models have not been studied.

The highest activated upstream regulator belonged to Brain Derived Neurotrophic Factor (BDNF). BDNF is a factor with an important role in neural differentiation. Interestingly, depletion of this

factor in the central nervous system caused increase in bone mass in mouse models [321]. Conversely, treatment of cementoblasts (cells important for generating cements that covers the tooth root) with BDNF increased osteogenic gene expression e.g. *Alpl* [322]. Thus, BDNF may have opposite effects on bone formation peripherally and centrally and its effect depends on the cell type. Whether it may affect the differentiation of BMSCs is currently unknown.

Based on IPA analysis, DTX1 is an inhibited transcription factor upstream of *Nf1*. DTX1 is an activator of the Notch family and its inhibition signature may suggest inhibition of NOTCH signaling pathway in *Nf1*-deficient cells. In mesenchymal cells, NOTCH signaling inhibits osteoblast differentiation and bone formation [323]. NOTCH signaling pathway has been implicated in the osteoarthritis through activation of non NFκB signaling [324]. This is in contradiction with the known phenotype of *Nf1* deficient BMSCs, where these cells do not differentiate towards osteoblast lineage. These 2 latter examples suggest that the stringency of our analysis could be increased in an attempt to "weed" the detection of such genes whose relevance to the impaired osteogenic potential of Nf1KO BMSCS is doubtful.

**Table 4**. List of upstream transcriptional regulators for Comparison No.3 (Loss of *Nf1* effect on gene expression)

| Upstream Regulator | Molecule Type | Activation z-score | p-value |
|---|---|---|---|
| IL13 | Cytokine | -2.257 | 0.0319 |
| DTX1 | Transcription regulator | -2 | 0.000533 |
| NFKBIA | Transcription regulator | -1.511 | 0.000000159 |
| SP600125 | JNK inhibitor | -1.474 | 0.00035 |
| Methotrexate | Anti-tumor | -1.463 | 0.00388 |
| Thapsigargin | Inhibitor of the sarcooplasmic reticulum $Ca^{2+}$ ATPase | -1.392 | 0.018 |
| ESR1 | Nuclear receptor | -1.366 | 0.049 |
| 12-(3-adamantan-1-yl-ureido) dodecanoic acid | Inhibitor of epoxide hydrolase | -1.364 | 0.00269 |
| HIC1 | Transcription regulator | -1.342 | 0.00203 |
| FBXO32 | enzyme | -1.254 | 0.000241 |
| Forskolin | Adenylate Cyclase activator | -1.245 | 0.00679 |
| IGF2 | Growth factor | -1.227 | 0.0175 |
| Mifepristone | Progesterone blocker | -1.175 | 0.0319 |
| HOXA10 | Transcription regulator | -1.134 | 0.017 |
| Fulvestrant | Anti-estrogen | -1.065 | 0.00696 |
| CEBPA | Transcription regulator | -1.05 | 0.0168 |
| Captopril | Angiotensin-converting enzyme (ACE) inhibitor | 1.067 | 0.0369 |
| Rapamycin | mTOR inhibitor | 1.114 | 0.00695 |
| Phorbol myristate acetate | PKC activator | 1.121 | 0.0386 |
| 17-alpha-ethinylestradiol | Estrogen derivative | 1.172 | 0.0225 |
| D-glucose | Sugar | 1.43 | 0.0026 |
| GnRH analog | Biologic drug | 1.667 | 0.0269 |
| JUN | Transcription regulator | 1.724 | 0.00747 |
| BDNF | Growth factor | 2.159 | 0.00911 |

IPA causal network analysis in this comparison predicted several possible molecules that may cause the observed changes in *Nf1*-deficient cells. Mitogen activated protein kinase 13 (MAPK13) was one of the activated causal regulators (**Figure 26**). MAPK13 also known as stress-induced protein kinase 4 or p38δ, is activated by pro-inflammatory cytokines and cellular stress and belong to a four members family. Contrary to p38α and p38β, whose role in osteoblast is stimulation of differentiation and in skeleton bone formation [325,326], the role of p38δ has not been investigated in the osteoblast lineage. However, based on the factors activating this MAPK, i.e. stress and pro-inflammatory signals, one can assume that inhibition of this MAPK could show a pro-osteoblastogenic phenotype. This is also another indication that gene expression in these cells might be affected by pro-inflammatory signals.

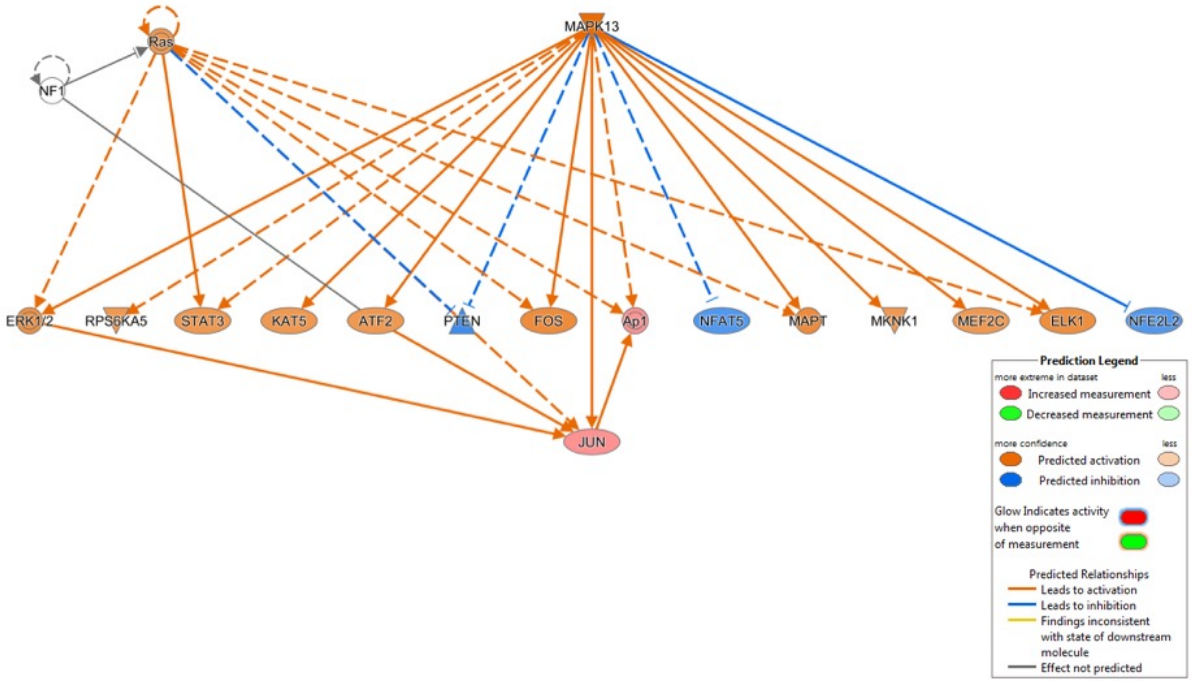



**Figure 26. Causal regulator predicts MAPK13 as the cause of DEGs**

### Comparison No.4 (Identification of DEGs in WT and *Nf1* KO BMSCs treated with U0126)

In the previous chapter, several lines of evidence suggested that the osteogenic differentiation phenotype of *Nf1* deficient cells was MAPK-independent. Hence, the next comparison was performed to assess the effect of MAPK treatment between *Nf1* KO and *Nf1* WT BMSCs to answer the question: what are the DEGs between genotypes that are MEK-independent (and are responsible for the reduced osteogenic potential in *Nf1* osteogenic BMSCs)? GO analysis (**Table 5**) showed that the only enriched set of genes in this analysis for biological processes was osteoblast differentiation (Enrichment score 28.75). This is an important finding, which supports our hypothesis that the poor osteogenic differentiation potential of *Nf1* KO cells is MAPK-independent. Analysis of cellular location of DEGs showed that similar to Comparison No.3 extracellular matrix was the top-enriched cellular component. This finding strengthens the results of the previous analysis and suggests that the differentiation phenotype of *Nf1* KO BMSCs stems from a MAPK-independent change, possibly in signals originating from the extracellular matrix.

ECM elasticity and stiffness could affect the differentiation fate of stem cells. Stem cells plated on soft surface preferentially express neurogenic markers, while plating stem cells on hard substrate promote osteogenic differentiation [327]. A recent study showed that plating MSCs on stiff matrix caused up-regulation of Integrin α2 and disruption of this upregulation using siRNA annulled the osteogenic differentiation stimulatory phenotype [167]. Integrin α2 is a known receptor for collagen type I, the major component of extracellular matrix in bone. It has been shown that culturing osteosarcoma cells on collagen type I stimulates osteogenic differentiation of these cells [328]. Disruption of collagen organization either through mutations in the Collagen encoding genes or the genes that are responsible for post-translational modification of Collagen e.g. Lysyl oxidase (*Lox)* leads to Osteogenesis imperfecta (OI).

**Table 5.** Comparison No. 4 (Gene ontology analysis of genes impacted by *Nf1* deficiency in BMSCs that are MEK independent).

| GO biological process complete | **Fold Enrichment** | **FDR** |
|---|---|---|
| Osteoblast differentiation | 28.75 | 1.46E-02 |
| **GO cellular component complete** | **Fold Enrichment** | **FDR** |
| proteinaceous extracellular matrix | 11.93 | 3.82E-03 |
| extracellular space | 3.35 | 4.60E-04 |

Patients suffer from a plethora of skeletal problems e.g. brittle bone. This highlights the importance of Collagen type I function in skeleton physiology. MSCs grown on the de-cellularized extracellular matrices (ECM) of mature osteoblasts promote osteogenic differentiation, while ECM from myoblasts promote myogenic differentiation [329]. This suggests that there are factors in the ECM of osteoblasts that promote the differentiation of MSCs towards osteogenic lineage. Although this observation might be the result of increased modulus (stiffness) of the extracellular matrix from mature osteoblasts (which is calcified), it could also point towards the presence of osteogenic growth factors that are embedded in the extracellular matrix of differentiating osteoblasts. BMP superfamily members such as TGFβ1 are embedded in the extracellular matrix in an inactive form, and upon release by physiological factors such as matrix metalloproteinases, become biologically active and can stimulate MSC differentiation toward the osteoblastic lineage. All in all, this finding may provide a possible explanation for the pathological findings in NF1 PA patients: *NF1* deficiency in MSCs may cause changes in the maturation process of their ECM, that in turn affect the differentiation of MSCs towards osteoblasts. This new hypothesis is currently tested in our laboratory.

Similar to comparison No.3, the most prominent phenotype associated with DEGs in this comparison was differentiation of connective tissue cells and mineralization of cells (**Figure 27**). The term mineralization of cells is misleading and needs clarification. Cell don't mineralize, rather they mineralize their extracellular matrix. Mineralization of ECM and differentiation of connective tissue cells are two phenotypes that are associated with DEGs in this comparison. This is in accordance with the known function of Neurofibromin in the osteoprogenitors and osteoblast lineages, where loss of Neurofibromin function causes reduced mineralization and differentiation in several mouse models of NF1 disease and in vitro studies [208,209,317].

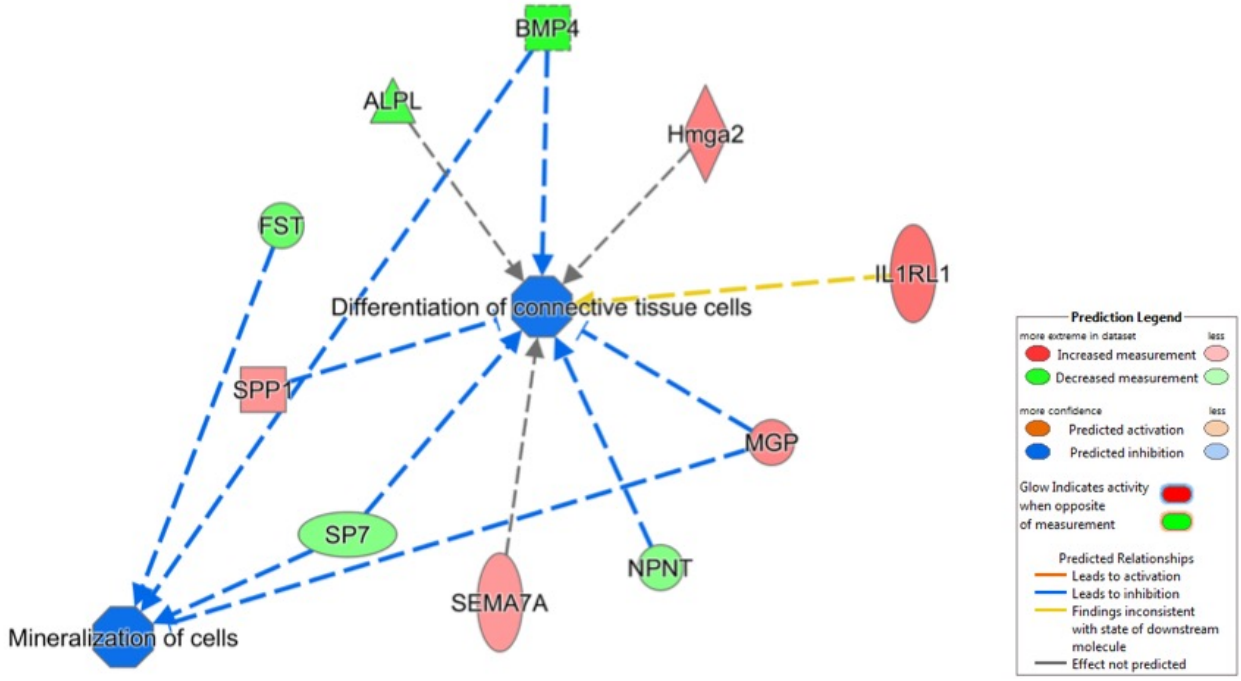



**Figure 27. Loss of *Nf1* is associated with reduced differentiation in cells from connective tissue origin**

The result of the IPA upstream regulator analysis is shown in **Table 6**. The most important common upstream regulator between Comparison No.3 and Comparison No.4 is the presence of pro-inflammatory signals. Three possible modulators of this pathway, namely ligands, cell surface receptors and transcriptional regulators were among upstream transcriptional regulator in this comparison. Pro-inflammatory cytokines such as TNF-α and Interferon γ, TREM1 (a receptor belonging to the Ig family that regulates the release of the pro-inflammatory cytokines in immune cells) and Jun (a transcription regulator) are indicators of possible activated pro-inflammatory signaling in *Nf1* KO BMSCs treated with MEK inhibitor. Jun activity has inhibitory effect on the activity of RUNX2, a master osteogenic transcription factor, exerted by phosphorylation of RUNX2 at Serine 104 moiety [330] (see next chapter). Inflammatory cytokines such as TNF-α, upon binding to its receptor, activate downstream signaling, including the canonical NF-κB pathways, whose activity is regulated at different levels [331] and suppress osteogenic differentiation [173,332].

HRAS is a predicted upstream regulator in *Nf1*-deficient cells. This is an expected observation, because none of the experimental conditions were targeted in normalizing RAS activity (activated by Nf1 loss of function) and because MEK is not the only pathway downstream of RAS signaling. Interestingly, HRAS is the only enriched upstream regulator between three RAS isoforms. Although an early study showed that all three isoforms of RAS were expressed in murine bone marrow [333], further studies showed that the expression is not the only factor controlling the function of RAS [334]. The current finding that HRAS is a predicted upstream regulator in *Nf1* KO cells might point towards a dominant regulatory effect of *Nf1* on H-RAS in osteoprogenitors. Alternatively, this finding might suggest the importance of HRAS as the dominant RAS isoform in bone marrow stem cells.

**Table 6**: Upstream regulators in comparison 4 (DEGs in MEK-treated *Nf1* KO and WT cells).

| Upstream Regulator | Molecule Type | Activation z-score | p-value of overlap |
|---|---|---|---|
| WNT3A | Cytokine | -1.937 | 0.000305 |
| PD98059 | MEK1,2 inhibitor | -1.767 | 0.0000721 |
| OSM | Cytokine | -1.131 | 0.00489 |
| PAX7 | Transcription regulator | -1.067 | 0.00000221 |
| FAS | Transmembrane receptor | -1.067 | 0.000125 |
| TGFB1 | Growth factor | 1.029 | 0.00000732 |
| Cg | Complex | 1.036 | 0.00000723 |
| ESR2 | Ligand-dependent nuclear receptor | 1.067 | 0.000232 |
| PPARG | Ligand-dependent nuclear receptor | 1.096 | 0.00333 |
| IFNG | Cytokine | 1.111 | 0.000000534 |
| CDKN2A | Transcription regulator | 1.213 | 0.0000343 |
| beta-estradiol | Natural Estrogen | 1.233 | 0.00768 |
| HRAS | Enzyme | 1.308 | 0.0000987 |
| Phorbol myristate acetate | PKC activator | 1.377 | 0.0159 |
| MYC | Transcription regulator | 1.457 | 0.000431 |
| TNF | Cytokine | 1.546 | 0.000000129 |
| SMARCA4 | Transcription regulator | 1.671 | 0.00000976 |
| tretinoin | Vitamin A derivative | 1.689 | 1.9E-09 |
| TREM1 | Transmembrane receptor | 1.71 | 0.00000531 |
| EGFR | Kinase | 1.711 | 0.00359 |
| HGF | Growth factor | 1.925 | 0.000434 |
| FGF2 | Growth factor | 1.937 | 0.000108 |
| PDGF BB | Complex | 1.938 | 0.000837 |
| TP53 | Transcription regulator | 1.945 | 0.000622 |
| Ca2+ | Chemical | 1.953 | 0.000656 |
| IL5 | Cytokine | 1.982 | 0.000472 |
| JUN | Transcription regulator | 2.174 | 0.000263 |
| PRL | Cytokine | 2.224 | 0.0000291 |

### Comparison No.5 (common genes between Analysis 3 and Analysis 4)

The comparison No.4 is based on the assumption that removing the genes that are responsive to MEK inhibition, the remainder of DEGs were MEK-independent genes. Comparison No.1 and 2 earlier in this chapter showed that U0126 treatment affect gene expression on the exposed cells in both genotypes. In order to remove the effect of U0126 treatment and study only the genes that change as the result of *Nf1* deficiency and are not responsive to MAPK inhibition, the list of genes between Comparison No. 3 and Comparison No.4 were compared and a list of common genes was created that was used for analyses in this section (**Table 7**). The log fold change and FDR for each gene was chosen from the Comparison No.3 (DEG between *Nf1* KO vs *Nf1* WT cells treated with vehicle) so the numbers would be closer to the normal physiological condition in *Nf1*-deficient BMSCs. Cells with genes that have known function in osteoblasts and skeleton biology are filled green. (this does not imply that other genes in this table might not be important for osteoblast differentiation).

One of the genes that are down-regulated in *Nf*-deficient osteoprogenitors is *Abi3bp*. *Abi3bp* KO MSCs *in vitro* show increased proliferation and reduced differentiation in towards different linages including the osteoblast lineage. Similar to *Nf1* deficiency, loss of *Abi3bp* causes constitutive activation of RAS and ERK1,2 in MSCs [335]. ABI3BP is secreted into extracellular matrix and interact with β1 integrin. Hence, it could be a possible modulator in the extracellular matrix that negatively regulate RAS. It was mentioned in the first chapter that plating MSCs on a stiff matrix can induce osteogenic differentiation in a β1 integrin-dependent manner [166]. Hodgekinson and colleagues showed that over expression of Akt1 could upregulate the expression of this gene [335]. However, in *Nf1* deficient cells, even with increased activity of RAS and possibly its downstream modulator AKT1, the expression of *Abi3bp* is reduced. This could be explained by the fact that in

the Hodgkineson paper, observed overexpression of *aAbi3bp* was in a non-physiologic condition in cells forced to express high levels of *Akt1* and the role of AKT1 in regulation of *Abi3bp* expression was not studied using AKT1 pharmacological inhibitors in normal conditions. Alternatively, this observed downregulation of *Abi3bp* in *Nf1*-deficient cells could be explained by the presence of other unknown factors that control the expression of *Abi3bp* and their effect is dominant over AKT1 activity.

The next two genes in the list, high mobility group AT-hook 1 and 2, *Hmga1* and *Hmga2,* are known transcription regulators involved in embryogenesis and tumorigenesis. As their name implies these transcription regulators bind to AT rich sequences of DNA and change gene expression. It has been proposed that HMGA2 is important in keeping MSCs in an un-differentiated state and hence for maintaining the MSC pool [336]. Wang and colleagues in a recent study showed that HMGA2 could inhibit osteogenic differentiation of a murine calvarial cell line, MC3T3, and mechanical stimulation could promote the differentiation by reducing HMGA2 level in these cells [337]. These two findings can be basis for a possible hypothesis, where as a result of *Nf1* deficiency and changes in the extracellular matrix, possibly as the result of ABI3BP deficiency, mechanical signaling required for osteoblast differentiation does not occur and hence *Nf1*-deficient BMSCs remain in the MSCs state or become "tumor like" with increased proliferation rate.

**Table 7.** The list of common DEGs between *Nf1* deficient cells treated with U0126 and vehicle

| Gene ID | Log FC | FDR | Gene name |
|---|---|---|---|
| Abi3bp | -1.777 | 0.000127 | ABI family member 3 binding protein |
| Alpl | -1.155 | 0.00207 | Alkaline Phosphatase |
| Arhgap22 | 2.256 | 0.00000312 | Rho GTPase activating protein 22 |
| Ass1 | 1.586 | 0.0000167 | Arginosuccinate 1 |
| Eps8 | 1.532 | 0.000113 | Epidermal growth factor receptor pathway substrate 8 |
| Fst | -1.246 | 0.000951 | Follistatin |
| Gm5424 | 1.586 | 0.0000167 | Unknown protein 5424 |
| Hmga1 | 1.844 | 0.0000232 | high mobility group AT-hook 1 |
| Hmga2 | 1.214 | 0.00499 | high mobility group AT-hook 2 |
| Krt9 | 1.529 | 0.000144 | Keratin 9 |
| Mest | -1.835 | 0.0000989 | mesoderm specific transcript |
| Myom1 | -1.319 | 0.000111 | Myomesin1 |
| Ptx3 | -2.236 | 1.06E-09 | Pentraxin 3 |
| S1pr3 | 1.782 | 0.000000271 | sphingosine-1-phosphate receptor 3 |
| Sema7a | 1.316 | 0.000311 | semaphorin 7A |
| Serpinb2 | 1.56 | 0.00706 | serpin family B member 2 |
| Spp1 | 1.483 | 0.0000616 | secreted phosphoprotein 1 |

Since approaches to address the functional relevance of every member of this gene set- over expression of downregulated gene or inhibition of upregulated ones- is time consuming and costly, I used data analysis software PANTHER and IPA. Because of the low number of the genes in this dataset, gene ontology software did not reveal any enriched molecular function or cellular component. The alternative explanation is that these genes affect diverse pathways and their low number prevents their enrichment in any given molecular function or cellular compartment. The only enriched biological process was enrichment of response to stimulus term, which is a broad term including all of the genes entered in this comparison, as these genes are all responsive to one stimulus.

IPA upstream analysis provided several possible signaling pathways (**Table 8**). TGFβ1 is one of the predicted upstream regulators in the set of genes that were present in *Nf1* KO BMSCs regardless of treatment with U0126. The anti-osteogenic effect of this factor has been described in detail in chapter I of this thesis. The predicted role of TGFβ1 as an upstream regulator may at first seem contradictory to chapter II findings, where no increase in expression of *Tgfb1* at mRNA and protein level was observed in murine *Nf1*-deficient cells (confirmed in human RNA-Seq data, where *TGFB1* expression was not changed). However, this prediction can be explained by the notion that TGFβ1 signaling in *Nf1* KO cells may be activated through action of the TGFβ1 precursor that is embedded in the extracellular matrix with TGFβR receptors. As it was mentioned in chapter II, capability of TGFβR antagonist SD208 in normalizing the reduced osteogenic differentiation might stem from a higher sensitivity of the TGFβR1 receptors in *Nf1*-deficient cells. However, this notion is weakened by the observation that *Nf1*-deficient cells have blunted response to TGFβR antagonist, which suggests that there might be another unknown factor that plays a role in the reduced differentiation observed in these cells.

The list of upstream regulators in this analysis, similar to two previous comparisons, also point towards pro-inflammatory signaling pathway. Pro-inflammatory ligands such as IL1β and IFN γ and transcription regulator JUN were among these transcriptional regulators. The fact that Upstream analysis using IPA showed several upstream regulators whose change was in the same direction between *Nf1* KO cells vs. *Nf1* WT cells in both treated and non-treated cells and common genes between these two analyses (Comparison No.5) strengthens this hypothesis that *Nf1* loss of function may cause an increased pro-inflammatory signaling gene signature. As it was mentioned in the previous sections, the stimulatory role of this signaling on bone destruction and inhibitory function on osteoblast differentiation has been studied extensively. Together, these data led us to study the putative role of disturbed NF-κB in the impaired osteogenic differentiation of *Nf1* osteoprogenitors.

**Table 8**: List of upstream regulators among genes common in *Nf1*-deficient cells treated with U0126 and vehicle. Pale blue represents inhibited upstream regulators and marigold shows activated upstream regulators

| Upstream Regulator | Molecule Type | Activation z-score | p-value of overlap |
|---|---|---|---|
| PD98059 | MEK inhibitor | -1.114 | 0.000569 |
| Forskolin | Adenylate Cyclase agonist | -0.928 | 0.0000897 |
| Dexamethasone | chemical drug | -0.453 | 0.00624 |
| Cycloheximide | chemical reagent | -0.391 | 0.00000531 |
| NFkB (complex) | complex | -0.391 | 0.0000582 |
| IL1B | cytokine | 0.258 | 7.99E-08 |
| TGFB1 | growth factor | 0.44 | 0.000123 |
| TGM2 | enzyme | 0.577 | 0.0000177 |
| Cg | complex | 0.581 | 0.00000479 |
| TNF | cytokine | 0.739 | 0.000138 |
| ESR1 | ligand-dependent nuclear receptor | 0.849 | 0.00174 |
| CEBPA | transcription regulator | 0.882 | 0.0000119 |
| CDKN2A | transcription regulator | 1 | 0.0000465 |
| phorbol myristate acetate | chemical drug | 1.026 | 0.00807 |
| SMARCA4 | transcription regulator | 1.091 | 0.000755 |
| IFNG | cytokine | 1.102 | 0.00000159 |
| lipopolysaccharide | chemical drug | 1.151 | 0.0000116 |
| D-glucose | chemical - endogenous mammalian | 1.238 | 0.00113 |
| tretinoin | chemical - endogenous mammalian | 1.394 | 0.00000447 |
| JUN | transcription regulator | 1.931 | 0.000248 |

In order to determine if pro-inflammatory signals contribute to the reduced osteogenic potential of *Nf1*-deficient osteoprogenitors, and because the cytokine or intracellular changes affected were unknowns, I first targeted the canonical inflammatory signaling pathway. A major component of this pathway is activation of NF-κB and its translocation to the nucleus, where it activates the transcription of inflammatory response genes. For this purpose, I used a known pharmacological inhibitor of the NF-κB pathway: Bay11-7085. This inhibitor specifically inhibits the phosphorylation of IκB kinase (IKK), an important step in the activation and release of NF-κB. Inhibition of IKK prevents IκB being targeted for degradation and hence stops the subsequent release of NF-κB and its translocation into the nucleus. I cultured *Nf1* WT and *Nf1* KO cells in osteogenic medium in the presence of this inhibitor for seven days at increasing concentrations with the maximum concentration of 10 μM (shown to be effective in reducing IκB phosphorylation) and assessed osteogenic differentiation by gene expression [338–340].

Incubation with Bay11-7085 at any of the doses tested was unable to correct the reduced osteogenic differentiation in *Nf1* KO osteoprogenitors (**Figure 28**). One possibility for the lack of drug effect could on improving reduced osteogenic differentiation could be a biologically inactive drug. Hence, use of another NF-κB pathway inhibitor such as SN-50 is recommended. Alternatively, higher doses of BAY11-7085 could be tested. The observation that there was no detectable increase in NF-κB protein (p65) could be detected (data not shown), might point towards the role of a non-canonical pro-inflammatory signaling pathway in the poor osteogenic differentiation potential of *Nf1*-deficient BMSCs. Jun activation for instance can be triggered by non-canonical inflammatory pathways and as it was mentioned earlier can negatively impact RUNX2 activity.

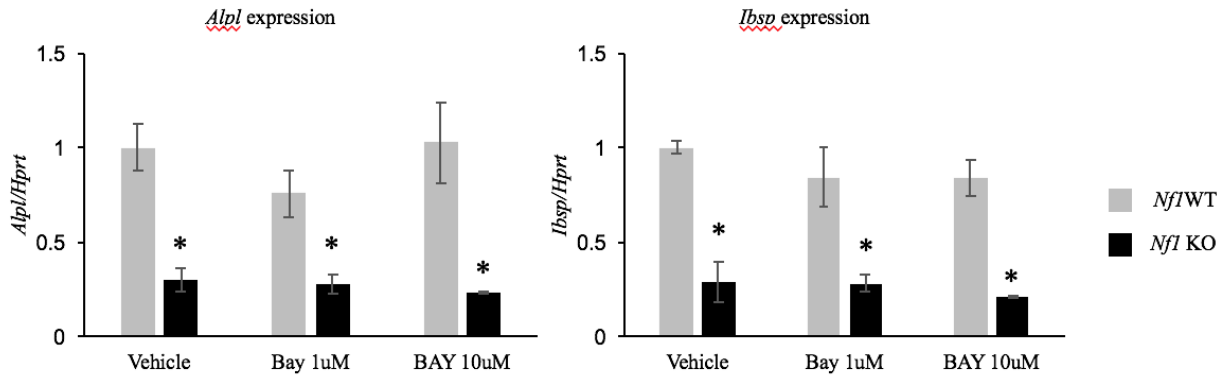



**Figure 28. Inhibition of NFκB signaling does not rescue osteogenic differentiation of *Nf1* deficient ostoprogenitors**

BMSCs were grown in osteogenic medium for 7 days supplemented with either vehicle (left bars), Bay11-7085 at 1 μM (middle bars) and 10 μM (right bars) and expression of *Alpl* (left panel) and *Ibsp* (right panel) was measured. N=3 q-RT-PCR * p-value < 0.0.

Upstream regulator analysis among different comparison suggest that JUN might be activated in *Nf1*-deficient cells, although a preliminary study could not find any increase in active JNK in these cells (data not shown). A recent paper proposed a role for increased JNK- Jun N-terminal kinase-activity in the reduced osteogenic differentiation of *Nf1*-deficient calvarial cells [341]. However, I was not able to reproduce this finding, which may be due to the fact that the dose that was used in the prior study with calvaria cells was cytotoxic to BMSCs. To study the possible role of non-canonical inflammatory signaling and involvement of JUN in *Nf1* reduced osteogenic differentiation phenotype, the next step will be to first assess the level of JNK and JUN activity in the *Nf1*-deficient cells and provided the level of activated JNK or JUN is increased, JNK inhibitors should be used to assess the functional relevance of this increased activity.

## Discussion

In this chapter, I described a nonbiased approach that I used to investigate the genes/pathways important for the reduced osteogenic differentiation potential observed in *Nf1*-deficient osteoprogenitors. This is the first study that we are aware of that investigates the changes in gene expression and MAPK dependence in murine *Nf1* deficient osteoprogenitor cells. Previously, Paria and colleagues reported changes in gene expression profiles using cells extracted from human biopsies [187]. Their study suggested a possible role for *EREG* in the pathogenesis of PA which was the basis of the hypothesis for the second chapter of this thesis.

We have acquired multiple evidence supporting the notion that the reduced osteogenic potential in *Nf1*-deficient osteoprogenitors stems from MAPK-independent changes. Because we did not have any indication of what those changes might be, we decided to investigate the gene expression

signature in *Nf1*-deficient osteoprogenitors using a high throughput unbiased analysis by RNA-Seq. We found that there were close to 400 genes whose expression were significantly changed (FC more than 2 and FDR less than 0.05) in *Nf1*-deficient when compared to *Nf1* WT osteoprogenitors. As we dissected the MAPK-dependent from MAPK-independent differential gene expression in these cells, we discovered that the majority of the genes that remained statistically significant after MAPK normalization between KO and WT cells were genes important for osteoblast differentiation. This is an additional indication that the osteogenic differentiation phenotype of *Nf1* KO BMSCs is MEK-independent.

Sun and colleagues performed a microarray to detect RAS/MEK independent differential gene expression in *Nf1*-deficient MPNST cell lines. Their study showed RAS/MAPK-independent increase in several members of the Bmp family such as *BMP4* [342]. However, this was not observed in my dataset where there was a decrease in the expression of *Bmp4* independent of MEK signaling in *Nf1*-deficient cells when compared to WT cells. This discrepancy may be explained by the different origins of the cells used in two studies.

**Table 7** provides a list of possible modulators of *Nf1* reduced deficiency. The functional importance of *Abi3bp, Hmga2* and other genes listed in the **Table 7** can be assessed in several stages. Firstly, using qRT-PCR the dysregulation status of these genes should be confirmed. However, before evaluating the functional relevance of these genes in reduced osteogenic differentiation, one could compare this list of genes with the data from NF1 PA human RNA-Seq to find genes that are common between human and murine cells. Next step would be the functional evaluation of these genes. For this purpose, one can use Adenovirus expression systems to express exogenously the downregulated genes or express shRNAs to downregulate the suppress the expression of up-regulated genes

Another interesting finding in this chapter was the inhibition of Jak2 in IPA analysis of *Nf1* deficient cells. As it was mentioned earlier, IGF-1 is an important factor in osteoblast differentiation and bone biology [343]. Deletion of receptor of this factor (*Igfr1*) in mouse models cause osteoblast differentiation problems [106]. JAK2 is important signaling molecule in insulin related growth factor 1 (IGF-1) signaling [319]. The predicted inhibition of JAK2 signaling may suggest the inhibition of IGF-2 signaling. This lead could be followed up by first measuring the expression of important factors in this pathway: the ligand (IGF-1), the receptor (IGF-1R) and the ligand antagonist (IGFBPs). Provided the IGF-1 signaling is dysregulated in the *Nf1* deficient cells, one can assess the functional relevance of this pathway in reduced osteogenic differentiation observed in *Nf1* deficient cells.

Another important finding in this study was the identification of MEK-independent changes in genes encoding extracellular matrix proteins in *Nf1* KO BMSCs versus WT BMSCs. This finding is very intriguing because the extracellular matrix is an important component of the bone sensing system that detects loading/unloading and reacts by the stimulation of bone formation. The majority of KO models of genes encoding ECM proteins in bone have disturbed skeletal parameters, similar to *Nf1* cKO mouse models. It is tempting to speculate at this point that changes in the ECM composition or structure generated by *Nf1* KO cells might provide abnormal/pathologic signals to bone MSCs that reduce their differentiation potential, possibly via outside-in signaling and integrins. This notion is further strengthened by the presence of TGFβ1as an upstream regulator in genes that are changed as the result of *Nf1* deficiency. Indeed, preliminary data showed that plating *Nf1*-deficient cells on the matrix laid by differentiated *Nf1* competent cells (WT) can to some extent improve osteogenic differentiation of *Nf1*-deficient cells, whereas plating of WT BMSCs on a matrix laid down by KO BMSCs reduces differentiation. This suggests

that either *Nf1*-competent cells secrete some stimulatory factor(s) that promote the differentiation of *Nf1* KO BMSCs, or that the KO matrix lack a crucial component required for osteogenic differentiation. The other possibility is that the stiffness of the matrix laid down by WT cells can promote the differentiation of KO cells. This possibility is weakened because plating these cells on plastic does not promote the differentiation. Further studies are now required to measure extracellular matrix stiffness and other biomechanical properties between genotypes. The presence or lack of pro-osteogenic factors in the matrix could also be assessed using a proteomic approach. As it was mentioned before, it is possible that secreted and embedded TGFβ1 in the extracellular matrix of KO cells can become activated by some modulators. For example, *Fst* that is down regulated in *Nf1* KO cells regardless of U0126 treatment can sequester TGFβ1 and Activin, another member of TGFβ1 superfamily, in the extracellular matrix and inhibits interaction with their receptor, TGFBRI. Hence, the downregulation of *Fst,* might lead to increase bio-availability of TGFβ1 and its interaction with its receptor without any effect on expression of TGFβ1. To assess increase in TGFBRI signaling, one can study the activation of SMAD2,3 in *Nf1* KO osteoprogenitors and also measure active TGFβ1 in the matrix.

Through IPA upstream analysis, we also discovered enrichment of pro-inflammatory mediators in *Nf1*-deficient osteoprogenitors, which was independent of MAPK signaling. Inhibition of the NF-κB canonical pathway, however, with different doses of BAY11-085 did not normalize the reduced osteogenic differentiation of *Nf1*-deficient cells. This could be explained by the fact that I could not detect any changes in NF-κB in *Nf1*-deficient cells. The importance of non-canonical pathways activated by TNF-α/other inflammatory cytokines such as JNK signaling pathway, which has inhibitory effect on osteogenic differentiation [174,344], cannot be ruled out and further investigations are needed. Firstly, the dysregulation of this pathway should be assessed measuring activated form

of JNK (p-JNK) and JUN. In the next step, using specific inhibitors of JNK, one should assess the functional relevance of this pathway.

One last important finding in this chapter, was the observing dysregulated genes important for proliferation in *Nf1* deficient cells, which was MEK dependent. Cells at late stages of differentiation do not divide, so there might be a possibility that excessive proliferation in *Nf1* deficient cells can cause reduced differentiation. As MEK normalization does not improve osteogenic differentiation in *NF1* deficient cells and MEK normalization in KO cells, does normalize genes important for proliferation, one can assume that dysregulated proliferation may not be the cause of reduced osteogenic differentiation in *Nf1* deficient cells. However, the proliferation rate and its dependency on MEK constitutive activation in *Nf1* deficient cells should be directly measured.

The IPA analysis suggests that other pathways could be up-regulated or enriched to cause the reduced osteogenic differentiation of *Nf1*-deficient osteoprogenitors. For example, IPA analysis found forskolin as an inhibited upstream regulator in *Nf1* KO BMSCs. Forskolin is a known activator of adenylate cyclase. This finding may be a proxy for reduction in cAMP regulated pathways through cAMP responsive binding (CREB) proteins. CREB activity leads to activation of BMP-2, an important factor in the osteogenic differentiation pathway. In support of this theory small molecule inducers of PKA have provided promising results in stimulating osteogenic differentiation *in vitro* [345,346]. Hence our finding that one of the activators of PKA is reduced might point towards reduced activity of PKA. PKA activity has been shown to be increased in *Nf1*-deficient cells from $Nf1_{col1}$-/- [80] and this might explain their increased normalized bone volume. However, in osteoprogenitors specific mouse models of *Nf1* such as $Nf1_{osx}$-/- bone volume is decreased [209], which might suggest that PKA activity might be modulated in a differentiation state

dependent manner in osteoprogenitors. PKA reduced activity is responsible for cognitive impairment in Drosophila [194]. Hence PKA may be a promising target in the search for factors responsible for the reduced osteogenic potential of *Nf1* deficient cells.

# V. Preliminary data suggesting the contribution of reduced Runx2 activity to the poor osteogenic potential of *Nf1*-deficient osteoprogenitors

## Background

RUNX2 is a transcription factor that was originally identified as a binding partner and activator of *Bglap*, which encodes osteocalcin. Ostecalcin is an extracellular protein that binds to hydroxyapatite and that is specifically synthesized by mature osteoblasts [53]. *Runx2* is a member of Runt family of transcription factors, that were originally identified in Drosophila as an important regulator of segmentation and body patterning [347,348]. There are three murine orthologues of the runt gene with distinct pattern of expression and activity in different tissues [349]. Runx1 is important in the process of hematopoiesis and mutations in this gene leads to acute myeloid leukemia (AML) and blocks hematopoiesis [350,351]. *Runx2* plays a pivotal role in bone formation and haploinsufficiency of this gene leads to a hereditary disease called Cleidocranial dysplasia [67,68,352,353]. RUNX3 is considered important in gastric cancer formation and resistance to 5-fluorouracil and cisplatin chemotherapy [354–356].

*Runx2* expression during embryogenesis increases just before the formation of mesenchymal condensation centers in anlagens of murine embryos, around day 12 postcoitum [59]. In adults, RUNX2 expression was reported to be limited to cells derived from osteoprogenitors [59], however, further studies using a LacZ reporter under control of the *Runx2* promoter showed the presence of a RUNX2 signal in testis and hippocampus [357]. *In vitro* studies have shown that in response to

osteogenic cues expression of this transcription factor increases in both myogenic and osteoprogenitor cells [59,85,167,358] . Transgenic expression of dominant negative (DN) form of RUNX2 under control of type II collagen promoter led to lack of mineralization in mouse skeleton. Over expression of WT RUNX2 using the same system lead to mineralization of every cartilage structure in developing embryo excluding digits [72]. Over expression of WT RUNX2 under control of type I collagen led to slightly milder phenotype.

Bone formation is highly dependent on RUNX2 activity. Animals with global *Runx2* deletion develop a cartilaginous skeleton with no mineralized bone and die postpartum as a result of respiratory failure [67]. In these mice during embryogenesis, vasculature does not invade the cartilaginous skeleton, and as a result of lack of osteoclasts and osteoprogenitor migration into the forming bone structures, no marrow is formed. Global *Runx2* deficiency affects both endochondral and intramembranous ossification processes, which shows that Runx2 is important in the early stages of osteoprogenitor commitment and differentiation. Disruption of RUNX2 activity by expression of *dn-Runx2* (dominant negative RUNX2 protein that has higher DNA binding affinity towards RUNX2 targeted promoters) under the control of the osteocalcin promoter (*mOG2*) causes adult bone defects in the form of osteopenia [57] . Targeted disruption of *Runx2* expression by using a $Runx2_{Col2a1}^{-/-}$ system that targets osteochondroprogenitors has provided further evidence that RUNX2 plays a key role in early stages of osteoblast differentiation [359]. These mice have an almost indistinguishable skeleton from newborns with global *Runx2* deletion, except for normal calcified flat bones, namely clavicles and facial bones. However, targeted deletion of *Runx2* in committed or premature osteoblasts using $Runx2_{Col1a1}^{-/-}$ system results in normal embryogenic bone formation [359]. However, it must be noted that in this study, adult mice were not examined and hence the role of RUNX2 as maintenance factor in bone accrual cannot be ruled out. The difference between

these two CRE system is in the stage of CRE recombinase expression and hence disruption of RUNX2 activity at different phases of the differentiation process of bone osteoprogenitors. The *Col2a1*-cre model is considered to target early osteochondroprogenitors, while the *Col1a1*-cre is considered to target committed osteoblasts [360]. This observation indicates that early disruption of RUNX2 activity is deleterious in bone formation and osteoblast differentiation.

*In vitro* studies have provided evidence that RUNX2 activity is necessary for the expression of osteoblastic genes. Ducy and colleagues have shown that there are *Runx2* consensus binding sequences in the promoter of *Bglap* (*mOG2*) that are crucial for expression of Osteocalcin [53]. The use of *Runx2* antisense oligos has shown that inhibition of Runx2 can stop the production of calcified nodules and alkaline phosphatase activity *ex vivo* [361]. Further studies have shown that the expression of Osteopontin [362], Collagen type 1 [55], and Alkaline phosphatase activity are also dependent on the activity of RUNX2 [363,364]. On the other hand, forced expression of *Runx2* under the control of *2.3 kb Col1* promoter induces osteopenia and multiple fractures in mice, accumulation of osteopontin expressing cells in their bone and lack of mature osteoblasts that express osteocalcin [70].

RUNX2 transcriptional activity is modulated by posttranslational modifications such as phosphorylation. RUNX2 is phosphorylated at different residues by MAPK, p38, JNK and possibly other kinases with different physiological consequences. The most studied phosphorylation site of RUNX2 is at serine 319, which leads to stimulation of RUNX2 activity [365]. Conversely, phosphorylation at another Serine residue of RUNX2, S104 causes reduced activity [330]. Custom antibodies or mass spectrometry are two methods that have been used to study different phosphorylation sites in this transcription factor. Acetylation is another posttranslational modification that can activate Runx2 transcriptional activity [366]. Conversely, histone deacetylases

(HDACs) can inhibit the transcriptional activity of RUNX2 though two distinct pathways: Firstly, these enzymes deacetylate RUNX2 on lysine residues and hence reduce RUNX2 transcriptional activity [367]. Secondly, they can physically interact with RUNX2 through different domains- Runx2 N-terminal and c-terminal- and repress RUNX2 transcriptional activity [368]. These extensive posttranslational modifications on RUNX2 display the importance of RUNX2 and regulation of its activity as one of the most critical transcription factors for the generation of a normal skeleton and for the differentiation potential of bone MSCs.

## Material and methods

### BMSC culture

The institutional animal care and use committee Baylor College of Medicine approved all the mouse procedures. Mice were housed 2-5 per cage. Mouse BMSCs were extracted from long bones of 2-3 month-old *Nf1*$^{f/f}$ [203] by centrifugation at 3000 g for 3 minutes, as previously described [260]. Extracted marrow was plated in 10 cm dishes in α-MEM medium (without ascorbic acid) supplemented with 10% fetal bovine serum and 100 U/ml Penicillin/ Streptomycin (15140-122, ThermoFisher) for three days. At that time, non-adherent cells were discarded by changing the medium. After reaching 60% confluence, cells were incubated with the adenovirus solutions (Ad-GFP or Ad-CRE recombinase, Baylor College of Medicine vector development lab) in the presence of Gene Jammer reagent (Agilent technologies; Cat# 204132), as described previously [261]. Briefly, Gene Jammer was added at a final concentration of 1% to FBS- and antibiotic-free α-MEM medium. The solution was vortexed briefly and incubated for

10 minutes at room temperature before adding the virus at a MOI of 400 and incubating for further 10 minutes. Final mixture was added to each well and cells were incubated with the virus solutions for 48 hours. The media was then changed to fresh complete α-MEM medium containing 10% FBS and Pen/Strep (Thermofisher Cat# 15140122). Mouse BMSCs were differentiated in osteogenic medium containing ascorbic acid (50 μg/ml) and β-glycerophosphate (5mM) in α-MEM medium for 4 days.

### Gene expression assays

Total RNA was extracted using TRIzol (Thermofisher, Cat# 15596026), and contaminating genomic DNA was digested by treatment with DNAse I (Promega, Cat# M6101). cDNAs were synthesized from 1ug RNAs using the high capacity cDNA reverse transcription kit (ThermoFisher, Cat# 4368814),). Quantitative qRT-PCR was performed using the following TaqMan primers/probes: *Runx2* (Mm00501584_m1) and the normalizer *Hprt* (Mm03024075_m1) from ThermoFisher.

### Western blot

Proteins were extracted from cell cultures using RIPA buffer. Protein concentration was measured using BCA assay (Thermo-Fisher). Ten μg of total protein was run on SDS gel before transfer to a nitrocellulose membrane. Membranes were blocked using 5% non-fat powder milk in TBST buffer. β-actin (A5316, Sigma), Runx2 (CST# 12556S) were diluted in blocking buffer at 1:1000 to 1:2000 dilution and incubated with the membranes overnight at 4°C. Following washing, membranes were then incubated with an HRP-conjugated secondary antibody (goat anti mouse

Santa Cruz Cat # sc-2005, goat anti-rabbit Santa Cruz Cat# sc-2030) diluted in blocking buffer at room temperature for one hour. Membranes were washed and incubated with ECL solution for 2 minutes and exposed to photographic film.

### ChIP assay

ChIP assay was done using MAGnify™ Chromatin Immunoprecipitation System (ThermoFisher Cat# 492024) and was performed following the manufacturer's instructions. Briefly, *Nf1* WT and *Nf1* KO BMSCs were kept in either regular medium or osteogenic medium for 4 days. In the next step, cells were washed with PBS and formaldehyde at the final concentration of 1% was added in the medium, cells were incubated for 15 minutes in 37 degrees incubator. Equivalent of Glycine was added to neutralize the formaldehyde in the medium and cells were incubated for 15 minutes. Next step, cells were washed with PBS once and were scraped into an Eppendorf tube. After cell lysis and sonication, cell extracts were centrifuged (20,000G for 15 minutes) to remove debris. An aliquot of each condition was kept for input control before incubation with antibodies. Cell extracts were incubated with RUNX2 antibody (CST# 12556S) or IgG control (supplemented in the kit) overnight in the presence of Dynabeads® Protein A for Immunoprecipitation (ThermoFisher Cat# 10001D). After extensive washing, DNA was extracted from the beads. qRT-PCR was performed using SYBR green primers for *Ibsp* promoter for each condition and was normalized to the input. ChIP primers were as follow: *Ibsp* Fwd: CCAGTTTTCAAACATCCAAATCCATAGG *Ibsp* Rev TTGGCACTGGGAGATGTCCTCCCTT [369]. Because the CT values in ChIP assay were high, qRT-PCR products were run on the agarose gel to visualize the presence of amplified bands.

### Statistical analyses

For comparison between *Nf1* WT and KO cells, a student t-test was performed. P-value less than 0.05 was considered significant. Statistical analysis was performed using Graph Pad PRISM (v6.0a, La Jolla, CA, USA). Data are provided as mean +/- SD.

## Results

Based on the notion that the majority of the genes that are dysregulated in *Nf1* deficient BMSCs are the genes that are under control of Runx2, I hypothesized that the dysregulation of the osteogenic differentiation might be Runx2 mediated. In order to investigate this possibility, I looked at the different levels of RUNX2 regulation, namely expression and transcriptional activity in *Nf1* deficient cells.

### Runx2 expression

Osteogenic differentiation starts by increasing transcriptional activity or expression of the master osteogenic regulator, Runx2 [181]. We thus examined the *Runx2* mRNA and RUNX2 protein expression in BMSC cultures from *Nf1* WT and *Nf1* KO BMSCs to address whether Neurofibromin controls the expression of Runx2 in osteoprogenitors and whether its loss of function might explain the reduced osteogenic differentiation observed in *Nf1*-deficient cells. We observed that the expression of Runx2, at the mRNA and protein levels, was similar between *Nf1* WT and *Nf1* KO osteoprogenitors grown in osteogenic medium (**Figure 29A and B**).

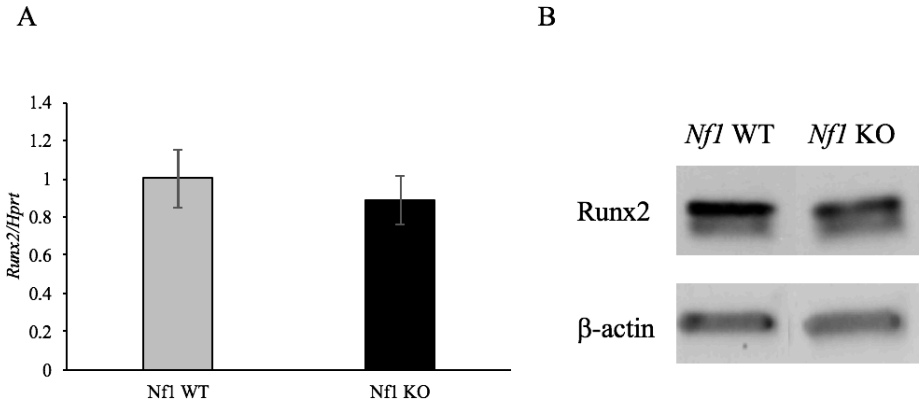



**Figure 29. Runx2 expression is not different between *Nf1* WT and *Nf1* KO BMSCs**
A) BMSCs were cultured in the presence of osteogenic medium for 4 days show no statistically significant difference in *Runx2* mRNA expression B) representative western blot showing similar Runx2 protein expression in *Nf1* WT and *Nf1* KO BMSCs. N=3

These data suggest that Neurofibromin does not regulate Runx2 expression and that reduced expression of Runx2 is not causal for the impaired osteogenic differentiation of *Nf1* KO BMSCs.

### RUNX2 transcriptional activity

Even though *Nf1* loss of function does not affect Runx2 expression in differentiating BMSCs, it could affect Runx2 activity, which is known to be modulated by multiple post-translational modifications, including phosphorylation, acetylation and ubiquitination [139,178,274,330,344,370]. These modifications can have stimulatory or inhibitory effects on the transcriptional activity of Runx2. In lieu of investigating each post translational modification, we hypothesized that the final effect of these putative changes could be observed by measuring the binding of Runx2 to its target promoters by ChIP. In this assay, an anti-rabbit nonimmune control antibody was included to account for nonspecific binding of antibodies to chromatin. The target gene of Runx2 transcriptional activity, *Ibsp* [369], was chosen to assess the binding activity of Runx2 in response to osteogenic medium.

In this assay four conditions were tested. *Nf1* WT and *Nf1* KO BMSCs were cultured for four days in the presence of either osteogenic medium and regular medium and the binding of Runx2 to the promoter of its target gene was tested. The gel is depicted in **Figure 30**.

ChIP assay showed increased Runx2 *Ibsp* promoter binding in *Nf1* WT osteoprogenitors in response to osteogenic medium, confirming the technical validity of the assay to assess the activity of Runx2. However, Runx2 binding to the promoter of *Ibsp* was comparatively much weaker in *Nf1* KO osteoprogenitors in response to osteogenic medium.

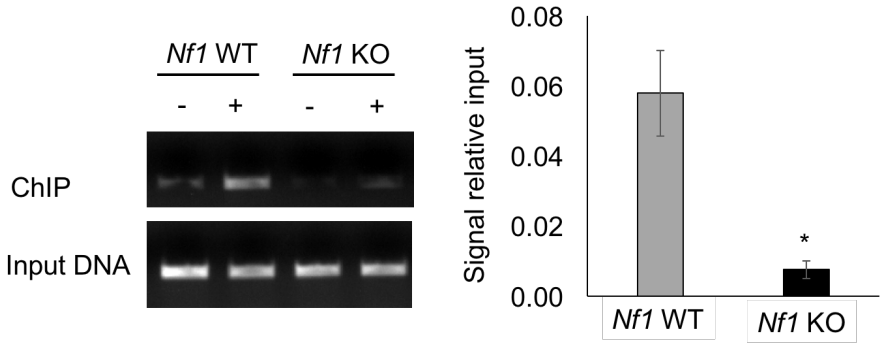



**Figure 30. ChIP assay shows reduced binding of RUNX2 to the promoter its target gene, *Ibsp***
ChIP assay was performed using anti-RUNX2 antibody on *Nf1* WT and *Nf1* KO BMSCs that were grown in the regular or osteogenic growth medium for four days. The harvested DNA was used for qRT-PCR. Primers were targeted for amplification of Runx2 binding promoter region of *Ibsp* A) Representative qRT-PCR amplification product of the *Ibsp* promoter post-ChIP (top) and input DNA normalizer (bottom). B) Normalized expression of PCR product by input, N=3. *: $p < 0.05$, t-test.

This finding must be strengthened by additional experiments but it suggests the existence of a factor or mechanism in *Nf1* KO cells that prevents the binding of Runx2 to its target gene promoter. The mechanism can involve a factor binding to or modifying Runx2 to inhibit its activity.

## Discussion

Previous data related to the reduced osteogenic potential of MSCs associated with NF1 PA and $Nf1_{osx}$-/- mouse model in addition to my experimental data shows that osteogenic marker genes whose expression is down regulated are targets of Runx2 transcriptional activity [60,222209]. This finding persuaded us to investigate the status of *Runx2* expression and RUNX2 activity in osteoprogenitors cells. In C2C12 myogenic cells, BMP-2 exposure promotes osteogenic differentiation and increases the expression of Runx2 [85,371]. A similar trend is observed in murine osteoblastic cell lines and primary cell cultures [167,358]. However, the increase in Runx2 mRNA and protein expression is not universal.

We observed that expression of Runx2 is not changed between *Nf1* WT and *Nf1* KO BMSCs. *Ex vivo* study of cells from PA biopsies of NF1 patients showed that *RUNX2* expression in the cells with reduced osteogenic differentiation was not different from healthy individuals as well [222]. This finding further supports that the blunted response to osteogenic differentiation signals in MSCs with loss of Neurofibromin function in both human and murine cells is independent of changes in RUNX2 expression. My results suggest that the transcriptional activity of RUNX2 in *Nf1* deficient osteoprogenitors could be compromised as demonstrated by reduced binding of RUNX2 to the promoter of its target gene *Ibsp* when compared to *Nf1* competent cells. This decreased binding of RUNX2 to its target promoters could be the result of several factors: 1) Neurofibromin direct

interaction could reduce RUNX2 transcriptional activity; 2) Neurofibromin through action of another protein can block RUNX2 from binding to its targets' promoters; this could include reduced/lack of a co-activator that facilitates RUNX2 binding to its target promoters or increase in an inhibitor that can spatially block the interaction; and 3) Neurofibromin could change the post translational modification of Runx2 protein which can modulate Runx2 transcriptional activity.

Regarding the first possibility, Neurofibomin is usually considered a cytosolic protein; however, there are some studies that have shown that Neurofibromin can translocate into the nucleus and there could bind to Runx2 and modulate its activity [372,373]. In order to study the physical interaction between Runx2 and Neurofibromin, nuclear extracts could be harvested and Runx2 could be immunoprecipitated and the presence of Neurofibromin could be checked by immunoblotting. In the next step, the functional importance of this interaction could be studied by using expression vectors for different domains of Neurofibromin.

There are several known factors that interact or compete with RUNX2 binding to its targeted promoters and hence affect its transcriptional activity. Msx2, YAP and TAZ, NRF2 and Myeloid Elf-1 like factor (MEF) act as antagonist for RUNX2 transcriptional activity, while Dlx5 helps RUNX2 binding to its target promoters. [374375–378] Expression of these genes were not changed in our RNA-Seq data, but their role has not been ruled out yet, mostly because the protein levels/activity of these proteins could still be changed without change in their gene expression.

The third possibility is the modification of Runx2 activity through post translational modifications, e.g. phosphorylation or acetylation. Study of *Nf1* deficiency effect on the phosphorylation status of Runx2 is hampered by the fact that there are no commercially available antibodies against all the Ser/Thr sites of Runx2. A similar difficulty exists for studying the acetylation status of Runx2.

Hence, mass spectrometry would be an appropriate approach to be used for this type of follow up study.

# VI. Discussion and Future Directions

## General discussion

To investigate the cause of the reduced osteogenic potential of osteoprogenitors characterized by *Nf1* loss-of-function, I took advantage of floxed *Nf1* mouse model to generate WT and *Nf1* KO BMSCs to determine the role of known modulated signaling pathways in these cells –namely EGFR and MAPK/ERK, *in vitro*. I also used a non-candidate approach as a first step to uncover differential signaling pathways between WT and *Nf1* KO BMSCs, and I addressed the effect of *Nf1* deficiency on the expression and function of Runx2, a master osteogenic transcription factor, whose target genes are downregulated upon *Nf1* deficiency in osteoprogenitors.

The first part of my studies was based on the results of a RNA-seq analysis generated from cells of a PA biopsy from a NF1 patient, which showed up-regulation of *EREG*, a member of the EGF family [187]. Based on the known inhibitory effects of EGFR signaling on osteogenic differentiation, I hypothesized that ectopic EGFR signaling might be a causal factor for the reduced osteogenic differentiation of *Nf1*-deficient cells. I showed evidence that although *Nf1*-deficient murine osteoprogenitors have increased *Ereg* expression at both mRNA and protein levels, as observed in human cells from a NF1 PA lesion, inhibition of EGFR signaling using two different EGFR inhibitors or Epiregulin neutralization by blocking antibodies could not normalize the reduced osteogenic differentiation potential of *Nf1*-deficient cells. However, the effect of these inhibitors on human MSCs from NF1 PA patients could not be assessed as the result of the rarity of these samples.

The NF1 pseudarthrotic site is composed of a genetically mixed population of *NF1* heterozygote and *Nf1* null cells. I showed here that EGFR blockade could not improve osteogenic differentiation of *Nf1*-deficient osteoprogenitors *in vitro*, however, in the context fracture healing *in vivo*, EGFR blockade could still have some benefits, as treatment of murine WT or *Nf1* heterozygote osteoprogenitors with the EGFR antagonist Poziotinib had stimulatory effect on osteogenic differentiation (data not shown). This could theoretically and to some extent compensate for the reduced osteogenic differentiation of *Nf1*-deficient osteoprogenitors in the fracture site. However, one has to keep in mind that the proliferative advantage and widespread distribution of *Nf1* deficient cells in the affected limb, which may limit this beneficial effect of treatment on Nf1+/- cells and might explain high failure rate of corrective surgery in NF1 pseudarthrosis patients. Hypothetically, EGFR blockade might prevent the proliferation advantage in *NF1* null cells, which might allow *NF1* heterozygote osteoprogenitors to participate in the healing process of fractured bone.

In the third chapter of my thesis, I addressed the question of the MAPK involvement in the reduced osteogenic differentiation phenotype of *Nf1*-deficient osteoprogenitors, which are characterized by increased MAPK/ERK activity *in vitro* and *in vivo* [245,318]. The notion that constitutive MAPK/ERK signaling could be the cause of the reduced osteogenic differentiation of these cells is well anchored in the NF1 bone field, but my data suggests otherwise. First, MAPK/ERK signaling is important for osteogenic differentiation in early osteoprogenitors and immature osteoblasts, as inhibition of this pathway leads to skeletal phenotypes that include severe bone deformity and reduced bone formation *in vivo*. On the other hand, *in vitro* expression of a constitutively active MEK increases the osteogenic differentiation of osteoprogenitors [177,179]. However, in *Nf1*-deficient osteoprogenitors that have increased MEK activity we do not observe increased osteogenic

differentiation, but rather the opposite phenotype which suggests a MAPK/ERK-independent pathway is causing the reduced osteogenic differentiation phenotype of these cells.

Second, although *Nf1* haploinsufficiency causes increased ERK activity, *Nf1* heterozygote mice, contrary to bone-targeted *Nf1*-deficient mouse models, do not show any skeletal phenotype. Additionally, I showed that *Nf1* heterozygote cells do not have reduced osteogenic differentiation compared to *Nf1* WT cells. If RAS/ERK chronic activity was the cause of the reduced osteogenic differentiation of *Nf1*-deficient BMSCs, *Nf1*+/- BMSCs should have shown reduced osteogenic differentiation as well, which was not the case.

Third, blocking experiments further support the notion of a MAPK/ERK-independent mechanism underlying the osteogenic differentiation phenotype of *Nf1*-deficient BMSCs. MAPK/ERK signaling normalization in two *Nf1*-deficient cell types (BMSCs and MEFs) at two different nodes (RAF and MEK) along the pathway did not normalize the osteogenic differentiation phenotype of these cells. It is important to notice that these inhibitors were used at a concentration that normalized ERK activity (they did not completely block this pathway). Similar results were independently obtained by the Schindeler group using different cells and pharmacological inhibitors [239].

Fourth, there are also clinical evidence leading us to question the contribution of the RAS/RAF/MEK/ERK pathway to the poor osteogenic potential of *Nf1*-deficient BMSCs and NF1 PA. NF1 belongs to a family of pathologic conditions that are generally called Rasopathies. Members of this group share similar chronic activation of the MAPK pathway downstream of RAS and skeletal problems, e.g. short stature, that could be attributed to disrupted growth plate physiology. However, pseudarthrosis is a unique feature of NF1 that is not observed in any of the

other RASopathies. This observation makes it questionable that NF1 PA stems from chronic activation of the MAPK/ERK pathway.

These 4 observations do not prove that the etiology of NF1 PA and the poor osteogenic potential of MSCs characterized by *Nf1* loss-of-function is RAS/MAPK-independent, but strongly suggest the existence of another dominant signaling pathway altered downstream of RAS or Neurofibromin.

MAPK is not the only signaling pathway that is regulated by the RAS/GRD molecular switch, which acts as a break on RAS downstream signaling pathways by facilitating the inactivation of RAS. RAS indeed regulates multiple downstream signaling pathways including PI3K, PKCζ, PLCε,.. [379]. Because there is a plethora of signaling molecules that could be regulated by RAS, it would be logical to first modulate RAS activity to investigate the role of RAS/GRD in the process of osteogenic differentiation. Farnesyl transferase inhibitors e.g. lovastatin could be used to reduce the membrane insertion step in the process of RAS activation and hence counteract a stimulatory effect of GRD absence caused by lack of Neurofibromin on RAS activity. However, because Lovastatin, similar to other members of statin family, have other known targets, one preferably needs to use genetic methods to further confirm/study the role of GRD. One approach could be to use *Nf1*-deficient cells that express exogenous HA-GRD to selectively rescue the absence of GRD in bone cells. If the RAS/GRD function of Neurofibromin contributes to the impaired osteogenic differentiation of *Nf1*-deficient BMSCs, introduction of GRD in these cells should rescue their reduced osteogenic differentiation. This method has been shown to be effective in dissecting the role of RAS/GRD in endothelial development in mouse models [293]. Another genetic approach to address the importance of RAS activity is the use of RAS heterozygote mouse models. Using the

flox system, one can remove one allele of RAS in osteoprogenitors of *Ras* $^{f/+}$; *Nf1*$^{f/f}$ mice using adenovirus encoding cre recombinase *ex vivo*, and thus address the contribution of increased RAS activity to the reduced osteogenic differentiation phenotype of *Nf1*-deficient osteoprogenitors. Because there are three different RAS isoforms and they have to some extent redundant, this approach will require the study and generation of each RAS isoforms in osteoprogenitors in the context of *Nf1* deficiency. However, based on IPA analysis KRAS might be the target of Neurofibromin regulation in these cells (see below).

Another possibility is that Neurofibromin controls the differentiation of osteoprogenitors through a RAS/GRD-independent pathway. *Nf1* encodes a 280 KDa protein, in which the GRD only comprises 10% of the protein. Other domains can interact with multiple structures and proteins. RAS/GRD-independent changes in NF1-associated phenotypes have been documented previously [195,293,380–382]. For addressing this possibility, different domains of this protein could be cloned into a lentivirus vector and *Nf1*-deficient osteoprogenitors could be infected with these lentiviruses to assess the potential of different domains in normalizing the reduce osteogenic differentiation of *Nf1*-deficient osteoprogenitors. This approach has been attempted in our laboratory but a high rate of random recombination within the cloned fragments hampered progress in this effort. The difficulty of transforming primary *Nf1*-/- BMSCs is also a challenge with this approach.

Studying any given phenotype using candidate gene approach has various limitations. Most importantly, it is based on *a priori* hypotheses. This approach cannot take into account the role of non-preditable genes that might play a role in the observed phenotype. In addition, there might be novel interactions between different genes in the context of each studied disease/system. Candidate gene approaches also fail to take into account the contribution of several genes in any studied

phenotype. This is most important in complex disorders such as pseudarthrosis, whose study is better suited through high throughput approaches.

Hence, a non-candidate RNA-Seq approach was another method that I used to investigate signaling pathways/modulators, under control of Neurofibromin, that regulate osteogenic differentiation in osteoprogenitors. Experimental conditions were designed to tease apart MAPK-independent changes in *Nf1*-deficient cells. Gene enrichment analysis provided a fifth evidence that *Nf1* controls osteogenic differentiation through a MAPK-independent pathway. Upstream regulator analysis using Ingenuity pathway analysis showed pro-inflammatory signals as possible mediators. Although the NFκB canonical pathway did not appear to be affected nor contributing to the differentiation phenotype of *Nf1*-deficient osteoprogenitors, an inflammatory non-canonical pathway could still be affected in these cells.

Because the majority of osteogenic genes that are down-regulated in *Nf1*-deficient osteoprogenitors appear to be under Runx2 transcriptional control, I assessed the expression and transcriptional activity of this transcription factor in WT and *Nf1*-deficient cells. In chapter V, I have shown preliminary evidence that although *Nf1* deficiency does not change Runx2 expression at both mRNA and protein levels, it reduced binding of Runx2 protein to the promoter of its target gene *Ibsp*. To confirm the reduced transcriptional activity of Runx2 in *Nf1*-deficient BMSCs, additional Runx2 target promoters will be assessed by Chip or EMSA. Another complementary approach will be to use a reporter assay based on the use of luciferase under control of Runx2 binding elements (6OSE2) [177,274,383,384]. Because of low DNA transfection efficiency into primary cells, I cloned a 6OSE2 luciferase construct into a lentivirus vector. However, the efficiency of BMSC lentivirus infection was not sufficient. To overcome this challenge, Adenovirus vectors which can transduce BMSCs more easily, may be used in the future.

Another open question is how Runx2 activity is modulated by Neurofibromin. Different possibilities are outlined in the discussion section of chapter V. Briefly, RUNX2 transcriptional activity modulation could be 1) the result of direct interaction of Neurofibromin with RUNX2; 2) overexpression of an inhibitor or absence of a co-activator and 3) the result of RUNX2 posttranslational modifications triggered by *Nf1* deficiency. Investigating these possibilities requires first the confirmation that RUNX2 transcriptional activity is decreased in *Nf1*-deficient cells. Assessment of changes in RUNX2 post-translational modifications will likely require mass spectrometry and immunoprecipitation approaches. These techniques should be able to dissect how *Nf1* deficiency could lead to a decrease Runx2 transcriptional activity and to the reduced osteogenic potential of *Nf1*-deficient osteoprogenitors.

## Conclusion

In closing, the research described in this dissertation has provided mechanistic evidence excluding the upregulation of *EREG* and *TGFB1* as main contributor of the osteogenic differentiation potential of cells characterized by *Nf1* loss of function. It also provided indirect evidence suggesting that the reduced osteogenic differentiation phenotype of *Nf1*-deficient osteoprogenitors does not stem from MAPK chronic activation. Finally, I obtained preliminary data supporting the hypothesis that Neurofibromin acts as a positive regulator of Runx2 transcriptional activity.

From a translational point of view, I have shown that targeting EGFR, TGFβ1 and MAPK signaling is unlikely to have a beneficial impact clinically because of absence of effect on bone cells characterized by *NF1* loss of function. However, my data does not exclude a possible positive effect on *NF1*+/- bone cells, whose differentiation and function might be promoted by these drugs.

An important question, if this holds true, is whether the presence of *NF1*-deficient cells, with their proliferative advantage and hypersecretion of the mineralization inhibitor PPi, will negatively impact the putative beneficial effect of drugs blocking EGFR or TGFβR. It is thus likely that the treatment of NF1 PA will require a combination drug strategy, and it is still an open field that needs further study. 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, et al. Skeletal abnormalities in neurofibromatosis type 1: approaches to therapeutic options. *Am J Med Genet A*. 2009;149A(10):2327-2338. doi:10.1002/ajmg.a.33045.
2. Weatherholt AM, Fuchs RK, Warden SJ. Specialized connective tissue: bone, the structural framework of the upper extremity. *J Hand Ther*. 2012;25(2):123-31; quiz 132. doi:10.1016/j.jht.2011.08.003.
3. Fukumoto S, Martin TJ. Bone as an endocrine organ. *Trends Endocrinol Metab*. 2009;20(5):230-236. doi:10.1016/j.tem.2009.02.001.
4. Lee NK, Sowa H, Hinoi E, et al. Endocrine regulation of energy metabolism by the skeleton. *Cell*. 2007;130(3):456-469. doi:10.1016/j.cell.2007.05.047.
5. Zee T, Settembre C, Levine RL, Karsenty G. T-Cell Protein Tyrosine Phosphatase Regulates Bone Resorption and Whole-Body Insulin Sensitivity through Its Expression in Osteoblasts. *Mol Cell Biol*. 2012;32(6):1080-1088. doi:10.1128/MCB.06279-11.
6. Oury F, Sumara G, Sumara O, et al. Endocrine Regulation of Male Fertility by the Skeleton. *Cell*. 2011;144(5):796-809. doi:10.1016/j.cell.2011.02.004.
7. Pi M, Chen L, Huang M-Z, et al. GPRC6A Null Mice Exhibit Osteopenia, Feminization and Metabolic Syndrome. Calbet JAL, ed. *PLoS One*. 2008;3(12):e3858. doi:10.1371/journal.pone.0003858.
8. Clarke B. Normal bone anatomy and physiology. *Clin J Am Soc Nephrol*. 2008;3 Suppl 3(Suppl 3):S131-9. doi:10.2215/CJN.04151206.
9. Eriksen EF, Axelrod DW MF. *Bone Histomorphometry*. New York: Raven Press; 1994.
10. Fell HB. The Osteogenic Capacity in vitro of Periosteum and Endosteum Isolated from the Limb Skeleton of Fowl Embryos and Young Chicks. *J Anat*. 1932;66(Pt 2):157-180.11. http://www.ncbi.nlm.nih.gov/pubmed/17104365. Accessed October 3, 2017.
11. Colnot C. Skeletal Cell Fate Decisions Within Periosteum and Bone Marrow During Bone Regeneration. *J Bone Miner Res*. 2009;24(2):274-282. doi:10.1359/jbmr.081003.
12. Maes C, Kobayashi T, Selig MK, et al. Osteoblast Precursors, but Not Mature Osteoblasts, Move into Developing and Fractured Bones along with Invading Blood Vessels. *Dev Cell*. 2010;19(2):329-344. doi:10.1016/j.devcel.2010.07.010.
13. Gajko-Galicka A. Mutations in type I collagen genes resulting in osteogenesis imperfecta in humans. *Acta Biochim Pol*. 2002;49(2):433-441. http://www.ncbi.nlm.nih.gov/pubmed/12362985. Accessed December 28, 2017.
14. Franceschi RT, Iyer BS, Cui Y. Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. *J Bone Miner Res*. 2009;9(6):843-854. doi:10.1002/jbmr.5650090610.
15. Berendsen AD, Olsen BR. Bone development. *Bone*. 2015;80:14-18. doi:10.1016/j.bone.2015.04.035.
16. Caetano-Lopes J, Canhão H, Fonseca JE. Osteoblasts and bone formation. *Acta Reum Port*. 32(2):103-110. http://www.ncbi.nlm.nih.gov/pubmed/17572649. Accessed December 8, 2014.
17. Bouleftour W, Juignet L, Bouet G, et al. The role of the SIBLING, Bone Sialoprotein in skeletal biology - Contribution of mouse experimental genetics. *Matrix Biol*. 2016;52-

54:60-77. doi:10.1016/j.matbio.2015.12.011.
18. Mills SJ, Cowin AJ, Kaur P. Pericytes, mesenchymal stem cells and the wound healing process. *Cells*. 2013;2(3):621-634. doi:10.3390/cells2030621.
19. Seitz S, Schnabel C, Busse B, et al. High bone turnover and accumulation of osteoid in patients with neurofibromatosis 1. *Osteoporos Int*. 2010;21(1):119-127. doi:10.1007/s00198-009-0933-y.
20. Nakashima T, Hayashi M, Fukunaga T, et al. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat Med*. 2011;17(10):1231-1234. doi:10.1038/nm.2452.
21. Grafe I, Alexander S, Yang T, et al. Sclerostin Antibody Treatment Improves the Bone Phenotype of *Crtap* $^{-/-}$ Mice, a Model of Recessive Osteogenesis Imperfecta. *J Bone Miner Res*. 2016;31(5):1030-1040. doi:10.1002/jbmr.2776.
22. Glorieux FH, Devogelaer J-P, Durigova M, et al. BPS804 Anti-Sclerostin Antibody in Adults With Moderate Osteogenesis Imperfecta: Results of a Randomized Phase 2a Trial. *J Bone Miner Res*. 2017;32(7):1496-1504. doi:10.1002/jbmr.3143.
23. Dallas SL, Prideaux M, Bonewald LF. The osteocyte: an endocrine cell ... and more. *Endocr Rev*. 2013;34(5):658-690. doi:10.1210/er.2012-1026.
24. Liu S, Rowe PSN, Vierthaler L, Zhou J, Quarles LD. Phosphorylated acidic serine-aspartate-rich MEPE-associated motif peptide from matrix extracellular phosphoglycoprotein inhibits phosphate regulating gene with homologies to endopeptidases on the X-chromosome enzyme activity. *J Endocrinol*. 2007;192(1):261-267. doi:10.1677/joe.1.07059.
25. Teitelbaum SL. Bone resorption by osteoclasts. *Science*. 2000;289(5484):1504-1508. http://www.ncbi.nlm.nih.gov/pubmed/10968780. Accessed October 3, 2017.
26. Kronenberg HM. Developmental regulation of the growth plate. *Nature*. 2003;423(6937):332-336. doi:10.1038/nature01657.
27. Yang L, Tsang KY, Tang HC, Chan D, Cheah KSE. Hypertrophic chondrocytes can become osteoblasts and osteocytes in endochondral bone formation. *Proc Natl Acad Sci*. 2014;111(33):12097-12102. doi:10.1073/pnas.1302703111.
28. Karsenty G, Wagner EF. Reaching a genetic and molecular understanding of skeletal development. *Dev Cell*. 2002;2(4):389-406. http://www.ncbi.nlm.nih.gov/pubmed/11970890. Accessed October 6, 2017.
29. Lefebvre V, Bhattaram P. Vertebrate skeletogenesis. *Curr Top Dev Biol*. 2010;90:291-317. doi:10.1016/S0070-2153(10)90008-2.
30. Einhorn TA, Gerstenfeld LC. Fracture healing: mechanisms and interventions. *Nat Rev Rheumatol*. 2014;11(1):45-54. doi:10.1038/nrrheum.2014.164.
31. Bolander ME. Regulation of fracture repair by growth factors. *Proc Soc Exp Biol Med*. 1992;200(2):165-170. http://www.ncbi.nlm.nih.gov/pubmed/1374563. Accessed October 2, 2017.
32. Ferguson C, Alpern E, Miclau T, Helms JA. Does adult fracture repair recapitulate embryonic skeletal formation? *Mech Dev*. 1999;87(1-2):57-66. http://www.ncbi.nlm.nih.gov/pubmed/10495271. Accessed October 2, 2017.
33. Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA. Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. *J Cell Biochem*. 2003;88(5):873-884. doi:10.1002/jcb.10435.
34. Vortkamp A, Pathi S, Peretti GM, Caruso EM, Zaleske DJ, Tabin CJ. Recapitulation of

signals regulating embryonic bone formation during postnatal growth and in fracture repair. *Mech Dev*. 1998;71(1-2):65-76. doi:10.1016/S0925-4773(97)00203-7.
35. Bais M, McLean J, Sebastiani P, et al. Transcriptional Analysis of Fracture Healing and the Induction of Embryonic Stem Cell–Related Genes. Sham MH, ed. *PLoS One*. 2009;4(5):e5393. doi:10.1371/journal.pone.0005393.
36. Gerstenfeld L, Cho T-J, Kon T, et al. Impaired Fracture Healing in the Absence of TNF-α Signaling: The Role of TNF-α in Endochondral Cartilage Resorption. *J Bone Miner Res*. 2003;18(9):1584-1592. doi:10.1359/jbmr.2003.18.9.1584.
37. Gerstenfeld LC, Cho TJ, Kon T, et al. Impaired intramembranous bone formation during bone repair in the absence of tumor necrosis factor-alpha signaling. *Cells Tissues Organs*. 2001;169(3):285-294. doi:47893.
38. Einhorn TA, Majeska RJ, Rush EB, Levine PM, Horowitz MC. The expression of cytokine activity by fracture callus. *J Bone Miner Res*. 2009;10(8):1272-1281. doi:10.1002/jbmr.5650100818.
39. Barnes GL, Kostenuik PJ, Gerstenfeld LC, Einhorn TA. Growth factor regulation of fracture repair. *J Bone Miner Res*. 1999;14(11):1805-1815. doi:10.1359/jbmr.1999.14.11.1805.
40. Kitaori T, Ito H, Schwarz EM, et al. Stromal cell-derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model. *Arthritis Rheum*. 2009;60(3):813-823. doi:10.1002/art.24330.
41. Wan C, Gilbert SR, Wang Y, et al. Activation of the hypoxia-inducible factor-1 pathway accelerates bone regeneration. *Proc Natl Acad Sci*. 2008;105(2):686-691. doi:10.1073/pnas.0708474105.
42. Yang X, Ricciardi BF, Hernandez-Soria A, Shi Y, Pleshko Camacho N, Bostrom MPG. Callus mineralization and maturation are delayed during fracture healing in interleukin-6 knockout mice. *Bone*. 2007;41(6):928-936. doi:10.1016/j.bone.2007.07.022.
43. Bone healing. https://www.orthobullets.com/basic-science/9009/fracture-healing. Accessed October 12, 2017.
44. Ketenjian AY, Arsenis C. Morphological and biochemical studies during differentiation and calcification of fracture callus cartilage. *Clin Orthop Relat Res*. 1975;(107):266-273. http://www.ncbi.nlm.nih.gov/pubmed/48443. Accessed October 2, 2017.
45. WENDEBERG B. Mineral metabolism of fractures of the tibia in man studied with external counting of Sr85. *Acta Orthop Scand Suppl*. 1961;52:1-79. http://www.ncbi.nlm.nih.gov/pubmed/13784324. Accessed October 2, 2017.
46. Green E, Lubahn JD, Evans J. Risk factors, treatment, and outcomes associated with nonunion of the midshaft humerus fracture. *J Surg Orthop Adv*. 2005;14(2):64-72. http://www.ncbi.nlm.nih.gov/pubmed/16115430. Accessed October 2, 2017.
47. Santolini E, West R, Giannoudis P V. Risk factors for long bone fracture non-union: a stratification approach based on the level of the existing scientific evidence. *Injury*. 2015;46:S8-S19. doi:10.1016/S0020-1383(15)30049-8.
48. Hayda RA, Brighton CT, Esterhai JL. Pathophysiology of delayed healing. *Clin Orthop Relat Res*. 1998;(355 Suppl):S31-S40. http://www.ncbi.nlm.nih.gov/pubmed/9917624. Accessed October 28, 2017.
49. Finkemeier CG. Bone-grafting and bone-graft substitutes. *J Bone Joint Surg Am*. 2002;84-A(3):454-464. http://www.ncbi.nlm.nih.gov/pubmed/11886919. Accessed October 28, 2017.

50. Petecchia L, Viti F, Sbrana F, Vassalli M, Gavazzo P. A biophysical approach to quantify skeletal stem cells trans-differentiation as a model for the study of osteoporosis. *Biophys Chem*. 2017;229:84-92. doi:10.1016/j.bpc.2017.05.011.
51. Clabaut A, Delplace S, Chauveau C, Hardouin P, Broux O. Human osteoblasts derived from mesenchymal stem cells express adipogenic markers upon coculture with bone marrow adipocytes. *Differentiation*. 2010;80(1):40-45. doi:10.1016/j.diff.2010.04.004.
52. Ambrosi TH, Scialdone A, Graja A, et al. Adipocyte Accumulation in the Bone Marrow during Obesity and Aging Impairs Stem Cell-Based Hematopoietic and Bone Regeneration. *Cell Stem Cell*. 2017;20(6):771-784.e6. doi:10.1016/j.stem.2017.02.009.
53. Ducy P, Karsenty G. Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. *Mol Cell Biol*. 1995;15(4):1858-1869. http://www.ncbi.nlm.nih.gov/pubmed/7891679. Accessed September 30, 2017.
54. Xiao G, Jiang D, Thomas P, et al. MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. *J Biol Chem*. 2000;275(6):4453-4459. http://www.ncbi.nlm.nih.gov/pubmed/10660618. Accessed July 22, 2015.
55. Kern B, Shen J, Starbuck M, Karsenty G. Cbfa1 Contributes to the Osteoblast-specific Expression of type I collagen Genes. *J Biol Chem*. 2001;276(10):7101-7107. doi:10.1074/jbc.M006215200.
56. Bialek P, Kern B, Yang X, et al. A twist code determines the onset of osteoblast differentiation. *Dev Cell*. 2004;6(3):423-435. doi:10.1016/S1534-5807(04)00058-9.
57. Ducy P, Starbuck M, Priemel M, et al. A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. *Genes Dev*. 1999;13(8):1025-1036. http://www.ncbi.nlm.nih.gov/pubmed/10215629. Accessed September 30, 2017.
58. Wagner EF, Karsenty G. Genetic control of skeletal development. *Curr Opin Genet Dev*. 2001;11(5):527-532. http://www.ncbi.nlm.nih.gov/pubmed/11532394. Accessed December 8, 2014.
59. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell*. 1997;89(5):747-754. http://www.ncbi.nlm.nih.gov/pubmed/9182762. Accessed September 30, 2017.
60. Lee DY, Cho T-J, Lee HR, et al. Disturbed osteoblastic differentiation of fibrous hamartoma cell from congenital pseudarthrosis of the tibia associated with neurofibromatosis type I. *Clin Orthop Surg*. 2011;3(3):230-237. doi:10.4055/cios.2011.3.3.230.
61. Mariaud-Schmidt RP, Rosales-Quintana S, Bitar E, et al. Hamartoma involving the pseudarthrosis site in patients with neurofibromatosis type 1. *Pediatr Dev Pathol*. 8(2):190-196. doi:10.1007/s10024-004-1004-1.
62. Zur Nieden NI, Kempka G, Ahr HJ. In vitro differentiation of embryonic stem cells into mineralized osteoblasts. *Differentiation*. 2003;71(1):18-27. doi:10.1046/j.1432-0436.2003.700602.x.
63. Kulterer B, Friedl G, Jandrositz A, et al. Gene expression profiling of human mesenchymal stem cells derived from bone marrow during expansion and osteoblast differentiation. *BMC Genomics*. 2007;8(1):70. doi:10.1186/1471-2164-8-70.
64. Aghajanian P, Hall S, Wongworawat MD, Mohan S. The Roles and Mechanisms of Actions of Vitamin C in Bone: New Developments. *J Bone Miner Res*. 2015;30(11):1945. doi:10.1002/JBMR.2709.
65. St-Jacques B, Hammerschmidt M, McMahon AP. Indian hedgehog signaling regulates

proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev*. 1999;13(16):2072-2086. http://www.ncbi.nlm.nih.gov/pubmed/10465785. Accessed November 14, 2017.
66. Chen G, Deng C, Li Y-P. TGF-β and BMP Signaling in Osteoblast Differentiation and Bone Formation. *Int J Biol Sci*. 2012;8(2):272-288. doi:10.7150/ijbs.2929.
67. Komori T, Yagi H, Nomura S, et al. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell*. 1997;89(5):755-764. doi:10.1016/S0092-8674(00)80258-5.
68. Otto F, Thornell AP, Crompton T, et al. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell*. 1997;89(5):765-771. http://www.ncbi.nlm.nih.gov/pubmed/9182764. Accessed September 30, 2017.
69. Neve A, Corrado A, Cantatore FP. Osteoblast physiology in normal and pathological conditions. *Cell Tissue Res*. 2011;343(2):289-302. doi:10.1007/s00441-010-1086-1.
70. Liu W, Toyosawa S, Furuichi T, et al. Overexpression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures. *J Cell Biol*. 2001;155(1):157-166. doi:10.1083/jcb.200105052.
71. Yoshida CA, Komori H, Maruyama Z, et al. SP7 Inhibits Osteoblast Differentiation at a Late Stage in Mice. Van Wijnen A, ed. *PLoS One*. 2012;7(3):e32364. doi:10.1371/journal.pone.0032364.
72. Ueta C, Iwamoto M, Kanatani N, et al. Skeletal malformations caused by overexpression of Cbfa1 or its dominant negative form in chondrocytes. *J Cell Biol*. 2001;153(1):87-99. doi:10.1083/jcb.153.1.87.
73. Canon J, Banerjee U. Runt and Lozenge function in Drosophila development. *Semin Cell Dev Biol*. 2000;11(5):327-336. doi:10.1006/scdb.2000.0185.
74. Nakashima K, Zhou X, Kunkel G, et al. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell*. 2002;108(1):17-29. http://www.ncbi.nlm.nih.gov/pubmed/11792318. Accessed November 8, 2017.
75. Baek W-Y, Lee M-A, Jung JW, et al. Positive Regulation of Adult Bone Formation by Osteoblast-Specific Transcription Factor Osterix. *J Bone Miner Res*. 2009;24(6):1055-1065. doi:10.1359/jbmr.081248.
76. Koga T, Matsui Y, Asagiri M, et al. NFAT and Osterix cooperatively regulate bone formation. *Nat Med*. 2005;11(8):880-885. doi:10.1038/nm1270.
77. Ortuño MJ, Ruiz-Gaspà S, Rodríguez-Carballo E, et al. p38 regulates expression of osteoblast-specific genes by phosphorylation of osterix. *J Biol Chem*. 2010;285(42):31985-31994. doi:10.1074/jbc.M110.123612.
78. Yang X, Matsuda K, Bialek P, et al. ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome. *Cell*. 2004;117(3):387-398. http://www.ncbi.nlm.nih.gov/pubmed/15109498. Accessed May 21, 2016.
79. Xiao G, Jiang D, Ge C, et al. Cooperative interactions between activating transcription factor 4 and Runx2/Cbfa1 stimulate osteoblast-specific osteocalcin gene expression. *J Biol Chem*. 2005;280(35):30689-30696. doi:10.1074/jbc.M500750200.
80. Elefteriou F, Benson MD, Sowa H, et al. ATF4 mediation of NF1 functions in osteoblast reveals a nutritional basis for congenital skeletal dysplasiae. *Cell Metab*. 2006;4(6):441-

451. doi:10.1016/j.cmet.2006.10.010.
81. Sanford LP, Ormsby I, Gittenberger-de Groot AC, et al. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development*. 1997;124(13):2659-2670.
82. Janssens K, Vanhoenacker F, Bonduelle M, et al. Camurati-Engelmann disease: review of the clinical, radiological, and molecular data of 24 families and implications for diagnosis and treatment. *J Med Genet*. 2005;43(1):1-11. doi:10.1136/jmg.2005.033522.
83. Kang JS, Alliston T, Delston R, Derynck R. Repression of Runx2 function by TGF-β through recruitment of class II histone deacetylases by Smad3. *EMBO J*. 2005;24(14):2543-2555. doi:10.1038/sj.emboj.7600729.
84. Alliston T, Choy L, Ducy P, Karsenty G, Derynck R. TGF-beta-induced repression of CBFA1 by Smad3 decreases cbfa1 and osteocalcin expression and inhibits osteoblast differentiation. *EMBO J*. 2001;20(9):2254-2272. doi:10.1093/emboj/20.9.2254.
85. Lee M-H, Kim Y-J, Kim H-J, et al. BMP-2-induced Runx2 Expression Is Mediated by Dlx5, and TGF- 1 Opposes the BMP-2-induced Osteoblast Differentiation by Suppression of Dlx5 Expression. *J Biol Chem*. 2003;278(36):34387-34394. doi:10.1074/jbc.M211386200.
86. Smaldone S, Clayton NP, Del Solar M, et al. Fibrillin-1 Regulates Skeletal Stem Cell Differentiation by Modulating TGFβ Activity Within the Marrow Niche. *J Bone Miner Res*. 2015. doi:10.1002/jbmr.2598.
87. Weiss A, Attisano L. The TGFbeta Superfamily Signaling Pathway. *Wiley Interdiscip Rev Dev Biol*. 2013;2(1):47-63. doi:10.1002/wdev.86.
88. López-Rovira T, Chalaux E, Massagué J, Rosa JL, Ventura F. Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene. *J Biol Chem*. 2002;277(5):3176-3185. doi:10.1074/jbc.M106826200.
89. Zhang YW, Yasui N, Ito K, et al. A RUNX2/PEBP2alpha A/CBFA1 mutation displaying impaired transactivation and Smad interaction in cleidocranial dysplasia. *Proc Natl Acad Sci U S A*. 2000;97(19):10549-10554. doi:10.1073/pnas.180309597.
90. Salazar VS, Gamer LW, Rosen V. BMP signalling in skeletal development, disease and repair. *Nat Rev Endocrinol*. 2016;12(4):203-221. doi:10.1038/nrendo.2016.12.
91. Sieber C, Kopf J, Hiepen C, Knaus P. Recent advances in BMP receptor signaling. *Cytokine Growth Factor Rev*. 2009;20(5-6):343-355. doi:10.1016/j.cytogfr.2009.10.007.
92. Lai C-F, Cheng S-L. Signal transductions induced by bone morphogenetic protein-2 and transforming growth factor-beta in normal human osteoblastic cells. *J Biol Chem*. 2002;277(18):15514-15522. doi:10.1074/jbc.M200794200.
93. Kulkarni AB, Huh CG, Becker D, et al. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci U S A*. 1993;90(2):770-774. http://www.ncbi.nlm.nih.gov/pubmed/8421714. Accessed October 8, 2017.
94. Sanford LP, Ormsby I, Gittenberger-de Groot AC, et al. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development*. 1997;124(13):2659-2670. http://www.ncbi.nlm.nih.gov/pubmed/9217007. Accessed October 8, 2017.
95. Kaartinen V, Voncken JW, Shuler C, et al. Abnormal lung development and cleft palate in mice lacking TGF–β3 indicates defects of epithelial–mesenchymal interaction. *Nat Genet*.

1995;11(4):415-421. doi:10.1038/ng1295-415.
96. Noda M, Camilliere JJ. In vivo stimulation of bone formation by transforming growth factor-beta. *Endocrinology*. 1989;124(6):2991-2994. doi:10.1210/endo-124-6-2991.
97. Edwards JR, Nyman JS, Lwin ST, et al. Inhibition of TGF-β signaling by 1D11 antibody treatment increases bone mass and quality in vivo. *J Bone Miner Res*. 2010;25(11):2419-2426. doi:10.1002/jbmr.139.
98. Makhijani NS, Bischoff DS, Yamaguchi DT. Regulation of proliferation and migration in retinoic acid treated C3H10T1/2 cells by TGF-? isoforms. *J Cell Physiol*. 2005;202(1):304-313. doi:10.1002/jcp.20128.
99. Grafe I, Yang T, Alexander S, et al. Excessive transforming growth factor-β signaling is a common mechanism in osteogenesis imperfecta. *Nat Med*. 2014;20(6):670-675. doi:10.1038/nm.3544.
100. Kassem M, Kveiborg M, Eriksen EF. Production and action of transforming growth factor-beta in human osteoblast cultures: dependence on cell differentiation and modulation by calcitriol. *Eur J Clin Invest*. 2000;30(5):429-437. http://www.ncbi.nlm.nih.gov/pubmed/10809903. Accessed November 17, 2017.
101. Alliston T, Choy L, Ducy P, Karsenty G, Derynck R. TGF-beta-induced repression of CBFA1 by Smad3 decreases cbfa1 and osteocalcin expression and inhibits osteoblast differentiation. *EMBO J*. 2001;20(9):2254-2272. doi:10.1093/emboj/20.9.2254.
102. Breen EC, Ignotz RA, McCabe L, Stein JL, Stein GS, Lian JB. TGF? alters growth and differentiation related gene expression in proliferating osteoblasts in vitro, preventing development of the mature bone phenotype. *J Cell Physiol*. 1994;160(2):323-335. doi:10.1002/jcp.1041600214.
103. Lian N, Lin T, Liu W, et al. Transforming growth factor β suppresses osteoblast differentiation via the vimentin activating transcription factor 4 (ATF4) axis. *J Biol Chem*. 2012;287(43):35975-35984. doi:10.1074/jbc.M112.372458.
104. Thrailkill KM, Lumpkin CK, Bunn RC, Kemp SF, Fowlkes JL. Is insulin an anabolic agent in bone? Dissecting the diabetic bone for clues. *AJP Endocrinol Metab*. 2005;289(5):E735-E745. doi:10.1152/ajpendo.00159.2005.
105. Laron Z, Klinger B, Silbergeld A. Patients with Laron Syndrome Have Osteopenia/Osteoporosis. *J Bone Miner Res*. 1999;14(1):156-156. doi:10.1359/jbmr.1999.14.1.156.
106. Zhang M, Xuan S, Bouxsein ML, et al. Osteoblast-specific Knockout of the Insulin-like Growth Factor (IGF) Receptor Gene Reveals an Essential Role of IGF Signaling in Bone Matrix Mineralization. *J Biol Chem*. 2002;277(46):44005-44012. doi:10.1074/jbc.M208265200.
107. Fulzele K, Riddle RC, DiGirolamo DJ, et al. Insulin receptor signaling in osteoblasts regulates postnatal bone acquisition and body composition. *Cell*. 2010;142(2):309-319. doi:10.1016/J.CELL.2010.06.002.
108. Qiao M, Shapiro P, Kumar R, Passaniti A. Insulin-like growth factor-1 regulates endogenous RUNX2 activity in endothelial cells through a phosphatidylinositol 3-kinase/ERK-dependent and Akt-independent signaling pathway. *J Biol Chem*. 2004;279(41):42709-42718. doi:10.1074/jbc.M404480200.
109. Fulzele K, Riddle RC, DiGirolamo DJ, et al. Insulin receptor signaling in osteoblasts regulates postnatal bone acquisition and body composition. *Cell*. 2010;142(2):309-319. doi:10.1016/J.CELL.2010.06.002.

110. Johnson DE, Williams LT. Structural and functional diversity in the FGF receptor multigene family. *Adv Cancer Res*. 1993;60:1-41. http://www.ncbi.nlm.nih.gov/pubmed/8417497. Accessed October 9, 2017.
111. Marie PJ. Fibroblast growth factor signaling controlling bone formation: An update. *Gene*. 2012;498(1):1-4. doi:10.1016/j.gene.2012.01.086.
112. Choi S-C, Kim S-J, Choi J-H, Park C-Y, Shim W-J, Lim D-S. Fibroblast Growth Factor-2 and -4 Promote the Proliferation of Bone Marrow Mesenchymal Stem Cells by the Activation of the PI3K-Akt and ERK1/2 Signaling Pathways. *Stem Cells Dev*. 2008;17(4):725-736. doi:10.1089/scd.2007.0230.
113. Park J, Park O-J, Yoon W-J, et al. Functional characterization of a novel FGFR2 mutation, E731K, in craniosynostosis. *J Cell Biochem*. 2012;113(2):457-464. doi:10.1002/jcb.23368.
114. Suzuki H, Suda N, Shiga M, et al. Apert syndrome mutant FGFR2 and its soluble form reciprocally alter osteogenesis of primary calvarial osteoblasts. *J Cell Physiol*. 2012;227(9):3267-3277. doi:10.1002/jcp.24021.
115. Nakanishi R, Akiyama H, Kimura H, et al. Osteoblast-targeted expression of Sfrp4 in mice results in low bone mass. *J Bone Miner Res*. 2008;23(2):271-277. doi:10.1359/jbmr.071007.
116. Kim HJ, Kim JH, Bae SC, Choi JY, Kim HJ, Ryoo HM. The protein kinase C pathway plays a central role in the fibroblast growth factor-stimulated expression and transactivation activity of Runx2. *J Biol Chem*. 2003;278(1):319-326. doi:10.1074/jbc.M203750200.
117. Schneider MR, Wolf E. The epidermal growth factor receptor ligands at a glance. *J Cell Physiol*. 2009;218(3):460-466. doi:10.1002/jcp.21635.
118. Miettinen PJ, Berger J, E., et al. Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature*. 1995;376(6538):337-341. doi:10.1038/376337a0.
119. Miettinen PJ, Chin JR, Shum L, et al. Epidermal growth factor receptor function is necessary for normal craniofacial development and palate closure. *Nat Genet*. 1999;22(1):69-73. doi:10.1038/8773.
120. Wang K, Yamamoto H, Chin JR, Werb Z, Vu TH. Epidermal growth factor receptor-deficient mice have delayed primary endochondral ossification because of defective osteoclast recruitment. *J Biol Chem*. 2004;279(51):53848-53856. doi:10.1074/jbc.M403114200.
121. Sibilia M, Wagner B, Hoebertz A, et al. Mice humanised for the EGF receptor display hypomorphic phenotypes in skin, bone and heart. *Development*. 2003;130(19):4515-4525. doi:10.1242/dev.00664.
122. Clasadonte J, Sharif A, Baroncini M, Prevot V. Gliotransmission by Prostaglandin E2: A Prerequisite for GnRH Neuronal Function? *Front Endocrinol (Lausanne)*. 2011;2. doi:10.3389/fendo.2011.00091.
123. Canalis E, Raisz LG. Effect of epidermal growth factor on bone formation in vitro. *Endocrinology*. 1979;104(4):862-869. doi:10.1210/endo-104-4-862.
124. Hata R, Hori H, Nagai Y, et al. Selective inhibition of type I collagen synthesis in osteoblastic cells by epidermal growth factor. *Endocrinology*. 1984;115(3):867-876. doi:10.1210/endo-115-3-867.
125. Matsuda N, Kimar NM, Ramakrishnan PR, Cho M-I. Role of epidermal growth factor

receptor in osteoblastic differentiation of rat bone marrow stromal cells. *J Bone Miner Metab*. 1996;14(3):137-145. doi:10.1007/BF02239481.
126. Chien HH, Lin WL, Cho MI. Down-regulation of osteoblastic cell differentiation by epidermal growth factor receptor. *Calcif Tissue Int*. 2000;67(2):141-150. http://www.ncbi.nlm.nih.gov/pubmed/10920219. Accessed February 14, 2015.
127. Zhu J, Shimizu E, Zhang X, Partridge NC, Qin L. EGFR signaling suppresses osteoblast differentiation and inhibits expression of master osteoblastic transcription factors Runx2 and Osterix. *J Cell Biochem*. 2011;112(7):1749-1760. doi:10.1002/jcb.23094.
128. Yu S, Geng Q, Ma J, et al. Heparin-binding EGF-like growth factor and miR-1192 exert opposite effect on Runx2-induced osteogenic differentiation. *Cell Death Dis*. 2013;4:e868. doi:10.1038/cddis.2013.363.
129. Genetos DC, Rao RR, Vidal MA. Betacellulin inhibits osteogenic differentiation and stimulates proliferation through HIF-1alpha. *Cell Tissue Res*. 2010;340(1):81-89. doi:10.1007/s00441-010-0929-0.
130. Civitelli R, Ziambaras K. Calcium and phosphate homeostasis: concerted interplay of new regulators. *J Endocrinol Invest*. 2011;34(7 Suppl):3-7. http://www.ncbi.nlm.nih.gov/pubmed/21985972. Accessed October 9, 2017.
131. Dobnig H, Turner RT. The Effects of Programmed Administration of Human Parathyroid Hormone Fragment (1–34) on Bone Histomorphometry and Serum Chemistry in Rats [1]. *Endocrinology*. 1997;138(11):4607-4612. doi:10.1210/endo.138.11.5505.
132. Stein EM, Silva BC, Boutroy S, et al. Primary hyperparathyroidism is associated with abnormal cortical and trabecular microstructure and reduced bone stiffness in postmenopausal women. *J Bone Miner Res*. 2013;28(5):1029-1040. doi:10.1002/jbmr.1841.
133. Neer RM, Arnaud CD, Zanchetta JR, et al. Effect of Parathyroid Hormone (1-34) on Fractures and Bone Mineral Density in Postmenopausal Women with Osteoporosis. *N Engl J Med*. 2001;344(19):1434-1441. doi:10.1056/NEJM200105103441904.
134. Greenspan SL, Bone HG, Ettinger MP, et al. Effect of recombinant human parathyroid hormone (1-84) on vertebral fracture and bone mineral density in postmenopausal women with osteoporosis: a randomized trial. *Ann Intern Med*. 2007;146(5):326-339. http://www.ncbi.nlm.nih.gov/pubmed/17339618. Accessed October 9, 2017.
135. Jilka RL. Molecular and cellular mechanisms of the anabolic effect of intermittent PTH. *Bone*. 2007;40(6):1434-1446. doi:10.1016/j.bone.2007.03.017.
136. Onyia JE, Helvering LM, Gelbert L, et al. Molecular profile of catabolic versus anabolic treatment regimens of parathyroid hormone (PTH) in rat bone: An analysis by DNA microarray. *J Cell Biochem*. 2005;95(2):403-418. doi:10.1002/jcb.20438.
137. Bellido T, Ali AA, Plotkin LI, et al. Proteasomal Degradation of Runx2 Shortens Parathyroid Hormone-induced Anti-apoptotic Signaling in Osteoblasts. *J Biol Chem*. 2003;278(50):50259-50272. doi:10.1074/jbc.M307444200.
138. Schnoke M, Midura SB, Midura RJ. Parathyroid hormone suppresses osteoblast apoptosis by augmenting DNA repair. *Bone*. 2009;45(3):590-602. doi:10.1016/j.bone.2009.05.006.
139. Bellido T, Ali AA, Plotkin LI, et al. Proteasomal Degradation of Runx2 Shortens Parathyroid Hormone-induced Anti-apoptotic Signaling in Osteoblasts. *J Biol Chem*. 2003;278(50):50259-50272. doi:10.1074/jbc.M307444200.
140. Leaffer D, Sweeney M, Kellerman LA, et al. Modulation of osteogenic cell ultrastructure by RS-23581, an analog of human parathyroid hormone (PTH)-related peptide-(1-34), and

bovine PTH-(1-34). *Endocrinology*. 1995;136(8):3624-3631. doi:10.1210/endo.136.8.7628402.
141. Silva BC, Bilezikian JP. Parathyroid hormone: anabolic and catabolic actions on the skeleton. *Curr Opin Pharmacol*. 2015;22:41-50. doi:10.1016/j.coph.2015.03.005.
142. ALBRIGHT F, SMITH PH, RICHARDSON AM. POSTMENOPAUSAL OSTEOPOROSIS. *J Am Med Assoc*. 1941;116(22):2465. doi:10.1001/jama.1941.02820220007002.
143. Cauley JA, Seeley DG, Ensrud K, Ettinger B, Black D, Cummings SR. Estrogen replacement therapy and fractures in older women. Study of Osteoporotic Fractures Research Group. *Ann Intern Med*. 1995;122(1):9-16. http://www.ncbi.nlm.nih.gov/pubmed/7985914. Accessed October 10, 2017.
144. Jilka RL, Takahashi K, Munshi M, Williams DC, Roberson PK, Manolagas SC. Loss of estrogen upregulates osteoblastogenesis in the murine bone marrow. Evidence for autonomy from factors released during bone resorption. *J Clin Invest*. 1998;101(9):1942-1950. doi:10.1172/JCI1039.
145. Kousteni S, Bellido T, Plotkin LI, et al. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell*. 2001;104(5):719-730. http://www.ncbi.nlm.nih.gov/pubmed/11257226. Accessed November 9, 2017.
146. Martin A, Xiong J, Koromila T, et al. Estrogens antagonize RUNX2-mediated osteoblast-driven osteoclastogenesis through regulating RANKL membrane association. *Bone*. 2015;75:96-104. doi:10.1016/j.bone.2015.02.007.
147. Martin A, Yu J, Xiong J, et al. Estrogens and androgens inhibit association of RANKL with the pre-osteoblast membrane through post-translational mechanisms. *J Cell Physiol*. 2017;232(12):3798-3807. doi:10.1002/jcp.25862.
148. Charatcharoenwitthaya N, Khosla S, Atkinson EJ, McCready LK, Riggs BL. Effect of Blockade of TNF-α and Interleukin-1 Action on Bone Resorption in Early Postmenopausal Women. *J Bone Miner Res*. 2007;22(5):724-729. doi:10.1359/jbmr.070207.
149. Jones HH, Priest JD, Hayes WC, Tichenor CC, Nagel DA. Humeral hypertrophy in response to exercise. *J Bone Joint Surg Am*. 1977;59(2):204-208. http://www.ncbi.nlm.nih.gov/pubmed/845205. Accessed October 28, 2017.
150. (US) O of the SG. Determinants of Bone Health. 2004. https://www.ncbi.nlm.nih.gov/books/NBK45503/. Accessed October 28, 2017.
151. Lang T, LeBlanc A, Evans H, Lu Y, Genant H, Yu A. Cortical and trabecular bone mineral loss from the spine and hip in long-duration spaceflight. *J Bone Miner Res*. 2004;19(6):1006-1012. doi:10.1359/JBMR.040307.
152. LeBlanc A, Lin C, Shackelford L, et al. Muscle volume, MRI relaxation times (T2), and body composition after spaceflight. *J Appl Physiol*. 2000;89(6):2158-2164. http://www.ncbi.nlm.nih.gov/pubmed/11090562. Accessed October 29, 2017.
153. LeBlanc A, Schneider V, Shackelford L, et al. Bone mineral and lean tissue loss after long duration space flight. *J Musculoskelet Neuronal Interact*. 2000;1(2):157-160. http://www.ncbi.nlm.nih.gov/pubmed/15758512. Accessed October 29, 2017.
154. Luu YK, Capilla E, Rosen CJ, et al. Mechanical Stimulation of Mesenchymal Stem Cell Proliferation and Differentiation Promotes Osteogenesis While Preventing Dietary-Induced Obesity. *J Bone Miner Res*. 2009;24(1):50-61. doi:10.1359/jbmr.080817.

155. Qi M-C, Hu J, Zou S-J, Chen H-Q, Zhou H-X, Han L-C. Mechanical strain induces osteogenic differentiation: Cbfa1 and Ets-1 expression in stretched rat mesenchymal stem cells. *Int J Oral Maxillofac Surg*. 2008;37(5):453-458. doi:10.1016/j.ijom.2007.12.008.
156. Sen B, Xie Z, Case N, Ma M, Rubin C, Rubin J. Mechanical strain inhibits adipogenesis in mesenchymal stem cells by stimulating a durable ??-catenin signal. *Endocrinology*. 2008;149(12):6065-6075. doi:10.1210/en.2008-0687.
157. Simmons CA, Matlis S, Thornton AJ, Chen S, Wang C-Y, Mooney DJ. Cyclic strain enhances matrix mineralization by adult human mesenchymal stem cells via the extracellular signal-regulated kinase (ERK1/2) signaling pathway. *J Biomech*. 2003;36(8):1087-1096. doi:10.1016/S0021-9290(03)00110-6.
158. Genetos DC, Geist DJ, Liu D, Donahue HJ, Duncan RL. Fluid shear-induced ATP secretion mediates prostaglandin release in MC3T3-E1 osteoblasts. *J Bone Miner Res*. 2005;20(1):41-49. doi:10.1359/JBMR.041009.
159. Campbell ID, Humphries MJ. Integrin structure, activation, and interactions. *Cold Spring Harb Perspect Biol*. 2011;3(3):a004994. doi:10.1101/cshperspect.a004994.
160. Thompson WR, Rubin CT, Rubin J. Mechanical regulation of signaling pathways in bone. *Gene*. 2012;503(2):179-193. doi:10.1016/j.gene.2012.04.076.
161. Nelson WJ, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science*. 2004;303(5663):1483-1487. doi:10.1126/science.1094291.
162. Norvell SM, Alvarez M, Bidwell JP, Pavalko FM. Fluid Shear Stress Induces ?-Catenin Signaling in Osteoblasts. *Calcif Tissue Int*. 2004;75(5):396-404. doi:10.1007/s00223-004-0213-y.
163. Case N, Ma M, Sen B, Xie Z, Gross TS, Rubin J. Beta-catenin levels influence rapid mechanical responses in osteoblasts. *J Biol Chem*. 2008;283(43):29196-29205. doi:10.1074/jbc.M801907200.
164. Yavropoulou MP, Yovos JG. The molecular basis of bone mechanotransduction. *J Musculoskelet Neuronal Interact*. 2016;16(3):221-236. http://www.ncbi.nlm.nih.gov/pubmed/27609037. Accessed January 8, 2018.
165. David V, Martin A, Lafage-Proust M-H, et al. Mechanical Loading Down-Regulates Peroxisome Proliferator-Activated Receptor γ in Bone Marrow Stromal Cells and Favors Osteoblastogenesis at the Expense of Adipogenesis. *Endocrinology*. 2007;148(5):2553-2562. doi:10.1210/en.2006-1704.
166. Olivares-Navarrete R, Lee EM, Smith K, et al. Substrate Stiffness Controls Osteoblastic and Chondrocytic Differentiation of Mesenchymal Stem Cells without Exogenous Stimuli. van Wijnen A, ed. *PLoS One*. 2017;12(1):e0170312. doi:10.1371/journal.pone.0170312.
167. Shih Y-R V, Tseng K-F, Lai H-Y, Lin C-H, Lee OK. Matrix stiffness regulation of integrin-mediated mechanotransduction during osteogenic differentiation of human mesenchymal stem cells. *J Bone Miner Res*. 2011;26(4):730-738. doi:10.1002/jbmr.278.
168. Soares-Silva M, Diniz FF, Gomes GN, Bahia D. The Mitogen-Activated Protein Kinase (MAPK) Pathway: Role in Immune Evasion by Trypanosomatids. *Front Microbiol*. 2016;7:183. doi:10.3389/fmicb.2016.00183.
169. Greenblatt MB, Shim J-H, Zou W, et al. The p38 MAPK pathway is essential for skeletogenesis and bone homeostasis in mice. *J Clin Invest*. 2010;120(7):2457-2473. doi:10.1172/JCI42285.
170. Ge C, Yang Q, Zhao G, Yu H, Kirkwood KL, Franceschi RT. Interactions between extracellular signal-regulated kinase 1/2 and p38 MAP kinase pathways in the control of

RUNX2 phosphorylation and transcriptional activity. *J Bone Miner Res*. 2012;27(3):538-551. doi:10.1002/jbmr.561.
171. Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell*. 2000;103(2):239-252. http://www.ncbi.nlm.nih.gov/pubmed/11057897. Accessed November 1, 2017.
172. Behrens A, Sibilia M, Wagner EF. Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. *Nat Genet*. 1999;21(3):326-329. doi:10.1038/6854.
173. Bandow K, Maeda A, Kakimoto K, et al. Molecular mechanisms of the inhibitory effect of lipopolysaccharide (LPS) on osteoblast differentiation. *Biochem Biophys Res Commun*. 2010;402(4):755-761. doi:10.1016/j.bbrc.2010.10.103.
174. Hah Y-S, Kang H-G, Cho H-Y, et al. JNK signaling plays an important role in the effects of TNF-α and IL-1β on in vitro osteoblastic differentiation of cultured human periosteal-derived cells. *Mol Biol Rep*. 2013;40(8):4869-4881. doi:10.1007/s11033-013-2586-3.
175. Lai C-F, Chaudhary L, Fausto A, et al. Erk Is Essential for Growth, Differentiation, Integrin Expression, and Cell Function in Human Osteoblastic Cells. *J Biol Chem*. 2001;276(17):14443-14450. doi:10.1074/jbc.M010021200.
176. Jaiswal RK, Jaiswal N, Bruder SP, Mbalaviele G, Marshak DR, Pittenger MF. Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase. *J Biol Chem*. 2000;275(13):9645-9652. http://www.ncbi.nlm.nih.gov/pubmed/10734116. Accessed November 1, 2017.
177. Ge C, Xiao G, Jiang D, Franceschi RT. Critical role of the extracellular signal–regulated kinase–MAPK pathway in osteoblast differentiation and skeletal development. *J Cell Biol*. 2007;176(5):709-718. doi:10.1083/jcb.200610046.
178. Ge C, Xiao G, Jiang D, et al. Identification and functional characterization of ERK/MAPK phosphorylation sites in the Runx2 transcription factor. *J Biol Chem*. 2009;284(47):32533-32543. doi:10.1074/jbc.M109.040980.
179. Matsushita T, Chan YY, Kawanami A, Balmes G, Landreth GE, Murakami S. Extracellular signal-regulated kinase 1 (ERK1) and ERK2 play essential roles in osteoblast differentiation and in supporting osteoclastogenesis. *Mol Cell Biol*. 2009;29(21):5843-5857. doi:10.1128/MCB.01549-08.
180. Vimalraj S, Arumugam B, Miranda PJ, Selvamurugan N. Runx2: Structure, function, and phosphorylation in osteoblast differentiation. *Int J Biol Macromol*. 2015;78:202-208. doi:10.1016/j.ijbiomac.2015.04.008.
181. Li Y, Ge C, Franceschi RT. Differentiation-dependent association of phosphorylated extracellular signal-regulated kinase with the chromatin of osteoblast-related genes. *J Bone Miner Res*. 2010;25(1):154-163. doi:10.1359/jbmr.090705.
182. Huson SM, Compston DA, Clark P, Harper PS. 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*. 1989;26(11):704-711. http://www.ncbi.nlm.nih.gov/pubmed/2511318. Accessed December 1, 2014.
183. Brosius S. A History of von Recklinghausen's NF1. *J Hist Neurosci*. 2010;19(4):333-348. doi:10.1080/09647041003642885.
184. Friedman JM, Birch PH. Type 1 neurofibromatosis: a descriptive analysis of the disorder in 1,728 patients. *Am J Med Genet*. 1997;70(2):138-143. http://www.ncbi.nlm.nih.gov/pubmed/9128932. Accessed December 8, 2014.
185. Hirbe AC, Gutmann DH. Neurofibromatosis type 1: a multidisciplinary approach to care.

*Lancet Neurol*. 2014;13(8):834-843. doi:10.1016/S1474-4422(14)70063-8.
186. Stevenson DA, Little D, Armstrong L, et al. Approaches to treating NF1 tibial pseudarthrosis: consensus from the Children's Tumor Foundation NF1 Bone Abnormalities Consortium. *J Pediatr Orthop*. 2013;33(3):269-275. doi:10.1097/BPO.0b013e31828121b8.
187. Paria N, Cho T-J, Choi IH, et al. Neurofibromin deficiency-associated transcriptional dysregulation suggests a novel therapy for tibial pseudoarthrosis in NF1. *J Bone Miner Res*. 2014;29(12):2636-2642. doi:10.1002/jbmr.2298.
188. Stevenson DA, Zhou H, Ashrafi S, et al. Double inactivation of NF1 in tibial pseudarthrosis. *Am J Hum Genet*. 2006;79(1):143-148. doi:10.1086/504441.
189. Ballester R, Marchuk D, Boguski M, et al. The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. *Cell*. 1990;63(4):851-859. http://www.ncbi.nlm.nih.gov/pubmed/2121371. Accessed December 1, 2014.
190. Xu GF, O'Connell P, Viskochil D, et al. The neurofibromatosis type 1 gene encodes a protein related to GAP. *Cell*. 1990;62(3):599-608. http://www.ncbi.nlm.nih.gov/pubmed/2116237. Accessed December 1, 2014.
191. Barker D, Wright E, Nguyen K, et al. Gene for von Recklinghausen neurofibromatosis is in the pericentromeric region of chromosome 17. *Science*. 1987;236(4805):1100-1102. http://www.ncbi.nlm.nih.gov/pubmed/3107130. Accessed October 4, 2017.
192. Goldgar DE, Green P, Parry DM, Mulvihill JJ. Multipoint linkage analysis in neurofibromatosis type I: an international collaboration. *Am J Hum Genet*. 1989;44(1):6-12. http://www.ncbi.nlm.nih.gov/pubmed/2491784. Accessed October 4, 2017.
193. O'Connell P, Leach RJ, Ledbetter DH, et al. Fine structure DNA mapping studies of the chromosomal region harboring the genetic defect in neurofibromatosis type I. *Am J Hum Genet*. 1989;44(1):51-57. http://www.ncbi.nlm.nih.gov/pubmed/2562822. Accessed October 4, 2017.
194. The I, Hannigan GE, Cowley GS, et al. Rescue of a Drosophila NF1 mutant phenotype by protein kinase A. *Science*. 1997;276(5313):791-794. http://www.ncbi.nlm.nih.gov/pubmed/9115203. Accessed July 22, 2015.
195. Tsai P-I, Wang M, Kao H-H, et al. Neurofibromin mediates FAK signaling in confining synapse growth at Drosophila neuromuscular junctions. *J Neurosci*. 2012;32(47):16971-16981. doi:10.1523/JNEUROSCI.1756-12.2012.
196. Le LQ, Parada LF. Tumor microenvironment and neurofibromatosis type I: connecting the GAPs. *Oncogene*. 2007;26(32):4609-4616. doi:10.1038/sj.onc.1210261.
197. Brannan CI, Perkins AS, Vogel KS, et al. Targeted disruption of the neurofibromatosis type-1 gene leads to developmental abnormalities in heart and various neural crest-derived tissues. *Genes Dev*. 1994;8(9):1019-1029. http://www.ncbi.nlm.nih.gov/pubmed/7926784. Accessed July 22, 2015.
198. Jacks T, Shih TS, Schmitt EM, Bronson RT, Bernards A, Weinberg RA. Tumour predisposition in mice heterozygous for a targeted mutation in Nf1. *Nat Genet*. 1994;7(3):353-361. doi:10.1038/ng0794-353.
199. Yu X, Chen S, Potter OL, et al. Neurofibromin and its inactivation of Ras are prerequisites for osteoblast functioning. *Bone*. 2005;36(5):793-802. doi:10.1016/j.bone.2005.01.022.
200. Yang F-C, Chen S, Robling AG, et al. Hyperactivation of p21ras and PI3K cooperate to alter murine and human neurofibromatosis type 1-haploinsufficient osteoclast functions. *J Clin Invest*. 2006;116(11):2880-2891. doi:10.1172/JCI29092.

201. Schindeler A, Morse A, Harry L, et al. Models of tibial fracture healing in normal and Nf1-deficient mice. *J Orthop Res*. 2008;26(8):1053-1060. doi:10.1002/jor.20628.
202. He Y, Rhodes SD, Chen S, et al. c-Fms Signaling Mediates Neurofibromatosis Type-1 Osteoclast Gain-In-Functions. Glogauer M, ed. *PLoS One*. 2012;7(11):e46900. doi:10.1371/journal.pone.0046900.
203. Zhu Y, Romero MI, Ghosh P, et al. Ablation of NF1 function in neurons induces abnormal development of cerebral cortex and reactive gliosis in the brain. *Genes Dev*. 2001;15(7):859-876. doi:10.1101/gad.862101.
204. Wang W, Nyman JS, Moss HE, et al. Local low-dose lovastatin delivery improves the bone-healing defect caused by Nf1 loss of function in osteoblasts. *J Bone Miner Res*. 2010;25(7):1658-1667. doi:10.1002/jbmr.42.
205. Kolanczyk M, Kossler N, Kühnisch J, et al. Multiple roles for neurofibromin in skeletal development and growth. *Hum Mol Genet*. 2007;16(8):874-886. doi:10.1093/hmg/ddm032.
206. Kolanczyk M, Kühnisch J, Kossler N, et al. Modelling neurofibromatosis type 1 tibial dysplasia and its treatment with lovastatin. *BMC Med*. 2008;6:21. doi:10.1186/1741-7015-6-21.
207. Wang W, Nyman JS, Ono K, Stevenson DA, Yang X, Elefteriou F. Mice lacking Nf1 in osteochondroprogenitor cells display skeletal dysplasia similar to patients with neurofibromatosis type I. *Hum Mol Genet*. 2011;20(20):3910-3924. doi:10.1093/hmg/ddr310.
208. de la Croix Ndong J, Makowski AJ, Uppuganti S, et al. Asfotase-α improves bone growth, mineralization and strength in mouse models of neurofibromatosis type-1. *Nat Med*. 2014;20(8):904-910. doi:10.1038/nm.3583.
209. Ndong J de la C, Stevens DM, Vignaux G, et al. Combined MEK inhibition and BMP2 treatment promotes osteoblast differentiation and bone healing in Nf1OSX (-/-) mice. *J Bone Miner Res*. 2014. doi:10.1002/jbmr.2316.
210. El-Hoss J, Sullivan K, Cheng T, et al. A murine model of neurofibromatosis type 1 tibial pseudarthrosis featuring proliferative fibrous tissue and osteoclast-like cells. *J Bone Miner Res*. 2012;27(1):68-78. doi:10.1002/jbmr.528.
211. Weiland AJ, Weiss AP, Moore JR, Tolo VT. Vascularized fibular grafts in the treatment of congenital pseudarthrosis of the tibia. *J Bone Joint Surg Am*. 1990;72(5):654-662. http://www.ncbi.nlm.nih.gov/pubmed/2355026. Accessed October 1, 2017.
212. Pho RW, Levack B, Satku K, Patradul A. Free vascularised fibular graft in the treatment of congenital pseudarthrosis of the tibia. *J Bone Joint Surg Br*. 1985;67(1):64-70. http://www.ncbi.nlm.nih.gov/pubmed/3968148. Accessed October 1, 2017.
213. Shah H, Rousset M, Canavese F. Congenital pseudarthrosis of the tibia: Management and complications. *Indian J Orthop*. 2012;46(6):616-626. doi:10.4103/0019-5413.104184.
214. Bobotas K, Lallos SN, Nikolaou VS, Korres DS, Efstathopoulos NE. Successful treatment of congenital pseudarthrosis of the tibia: still a challenge. *Eur J Orthop Surg Traumatol*. 2013;23 Suppl 2(S2):S303-10. doi:10.1007/s00590-012-1072-2.
215. Stevenson DA, Birch PH, Friedman JM, et al. Descriptive analysis of tibial pseudarthrosis in patients with neurofibromatosis 1. *Am J Med Genet*. 1999;84(5):413-419. http://www.ncbi.nlm.nih.gov/pubmed/10360395. Accessed December 1, 2014.
216. Johnston CE, Birch JG. A tale of two tibias: a review of treatment options for congenital pseudarthrosis of the tibia. *J Child Orthop*. 2008;2(2):133-149. doi:10.1007/s11832-008-

0084-2.
217. Drake MT, Clarke BL, Khosla S. Bisphosphonates: Mechanism of Action and Role in Clinical Practice. *Mayo Clin Proc*. 2008;83(9):1032-1045. doi:10.4065/83.9.1032.
218. Rhodes SD, Yang H, Dong R, et al. Nf1 Haploinsufficiency Alters Myeloid Lineage Commitment and Function, Leading to Deranged Skeletal Homeostasis. *J Bone Miner Res*. 2015;30(10):1840-1851. doi:10.1002/jbmr.2538.
219. Wu X, Chen S, He Y, et al. The haploinsufficient hematopoietic microenvironment is critical to the pathological fracture repair in murine models of neurofibromatosis type 1. *PLoS One*. 2011;6(9):e24917. doi:10.1371/journal.pone.0024917.
220. Schindeler A, Birke O, Yu NYC, et al. Distal tibial fracture repair in a neurofibromatosis type 1-deficient mouse treated with recombinant bone morphogenetic protein and a bisphosphonate. *Bone Joint J*. 2011;93-B(8):1134-1139. doi:10.1302/0301-620X.93B8.25940.
221. Birke O, Schindeler A, Ramachandran M, et al. 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*. 2010;4(6):507-517. doi:10.1007/s11832-010-0293-3.
222. Madhuri V, Mathew SE, Rajagopal K, Ramesh S, Antonisamy B. Does pamidronate enhance the osteogenesis in mesenchymal stem cells derived from fibrous hamartoma in congenital pseudarthrosis of the tibia? *Bone Reports*. 2016;5:292-298. doi:10.1016/j.bonr.2016.10.003.
223. Erdogan M, Bereket C, Ozkan N, et al. The effect of zoledronic acid on growth plates and high turnover bones. *Bratisl Lek Listy*. 2014;115(3):131-135. http://www.ncbi.nlm.nih.gov/pubmed/24579680. Accessed October 4, 2017.
224. Zhu ED, Louis L, Brooks DJ, Bouxsein ML, Demay MB. Effect of Bisphosphonates on the Rapidly Growing Male Murine Skeleton. *Endocrinology*. 2014;155(4):1188-1196. doi:10.1210/en.2013-1993.
225. Tressler MA, Richards JE, Sofianos D, Comrie FK, Kregor PJ, Obremskey WT. Bone morphogenetic protein-2 compared to autologous iliac crest bone graft in the treatment of long bone nonunion. *Orthopedics*. 2011;34(12):e877-84. doi:10.3928/01477447-20111021-09.
226. Richards BS, Anderson TD. rhBMP-2 and Intramedullary Fixation in Congenital Pseudarthrosis of the Tibia. *J Pediatr Orthop*. 2016. doi:10.1097/BPO.0000000000000789.
227. Richards BS, Oetgen ME, Johnston CE. The use of rhBMP-2 for the treatment of congenital pseudarthrosis of the tibia: a case series. *J Bone Joint Surg Am*. 2010;92(1):177-185. doi:10.2106/JBJS.H.01667.
228. Spiro AS, Babin K, Lipovac S, et al. Combined treatment of congenital pseudarthrosis of the tibia, including recombinant human bone morphogenetic protein-2: a case series. *J Bone Joint Surg Br*. 2011;93(5):695-699. doi:10.1302/0301-620X.93B5.25938.
229. Govender S, Csimma C, Genant HK, et al. Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures: a prospective, controlled, randomized study of four hundred and fifty patients. *J Bone Joint Surg Am*. 2002;84-A(12):2123-2134. http://www.ncbi.nlm.nih.gov/pubmed/12473698. Accessed October 1, 2017.
230. Lee FY-I, Sinicropi SM, Lee FS, Vitale MG, Roye DP, Choi IH. Treatment of Congenital Pseudarthrosis of the Tibia with Recombinant Human Bone Morphogenetic Protein-7

(rhBMP-7): A Report of Five Cases. *JBJS Case Connect*. 2006;os-88(3):627-633. doi:10.2106/JBJS.D.02201.
231. Fabeck L, Ghafil D, Gerroudj M, Baillon R, Delincé P. Bone morphogenetic protein 7 in the treatment of congenital pseudarthrosis of the tibia. *J Bone Jt Surg - Br Vol*. 2006;88-B(1):116-118. doi:10.1302/0301-620X.88B1.16619.
232. Anticevic D, Jelic M, Vukicevic S. Treatment of a congenital pseudarthrosis of the tibia by osteogenic protein-1 (bone morphogenetic protein-7): a case report. *J Pediatr Orthop B*. 2006;15(3):220-221. doi:10.1097/01.bpb.0000194439.75378.ac.
233. Oetgen ME, Richards BS. Complications Associated With the Use of Bone Morphogenetic Protein in Pediatric Patients. *J Pediatr Orthop*. 2010;30(2):192-198. doi:10.1097/BPO.0b013e3181d075ab.
234. Steib J-P, Jean-Paul S, Bouchaïb J, et al. Could an osteoinductor result in degeneration of a neurofibroma in NF1? *Eur Spine J*. 2010;19 Suppl 2(S2):S220-5. doi:10.1007/s00586-010-1416-8.
235. Tian H, Zhao J, Brochmann EJ, Wang JC, Murray SS. Bone morphogenetic protein-2 and tumor growth: Diverse effects and possibilities for therapy. *Cytokine Growth Factor Rev*. 2017;34:73-91. doi:10.1016/j.cytogfr.2017.01.002.
236. Jessen WJ, Miller SJ, Jousma E, et al. MEK inhibition exhibits efficacy in human and mouse neurofibromatosis tumors. *J Clin Invest*. 2013;123(1):340-347. doi:10.1172/JCI60578.
237. Jousma E, Rizvi TA, Wu J, et al. Preclinical assessments of the MEK inhibitor PD-0325901 in a mouse model of neurofibromatosis type 1. *Pediatr Blood Cancer*. 2015;62(10):1709-1716. doi:10.1002/pbc.25546.
238. Wu J, Dombi E, Jousma E, et al. Preclincial testing of Sorafenib and RAD001 in the Nfflox/flox;DhhCre mouse model of plexiform neurofibroma using magnetic resonance imaging. *Pediatr Blood Cancer*. 2012;58(2):173-180. doi:10.1002/pbc.23015.
239. El-Hoss J, Cheng T, Carpenter EC, et al. 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*. 2014;96(14):e117. doi:10.2106/JBJS.M.00862.
240. Kirmani S, Tebben PJ, Lteif AN, et al. Germline TGF-? receptor mutations and skeletal fragility: A report on two patients with Loeys-Dietz syndrome. *Am J Med Genet Part A*. 2010;152A(4):1016-1019. doi:10.1002/ajmg.a.33356.
241. WILNER HI, FINBY N. SKELETAL MANIFESTATIONS IN THE MARFAN SYNDROME. *JAMA*. 1964;187:490-495. http://www.ncbi.nlm.nih.gov/pubmed/14084820. Accessed June 18, 2017.
242. Rhodes SD, Wu X, He Y, et al. Hyperactive transforming growth factor-β1 signaling potentiates skeletal defects in a neurofibromatosis type 1 mouse model. *J Bone Miner Res*. 2013;28(12):2476-2489. doi:10.1002/jbmr.1992.
243. Jakowlew SB. Transforming growth factor-β in cancer and metastasis. *Cancer Metastasis Rev*. 2006;25(3):435-457. doi:10.1007/s10555-006-9006-2.
244. Ahluwalia A, Hobbs AJ. Endothelium-derived C-type natriuretic peptide: More than just a hyperpolarizing factor. *Trends Pharmacol Sci*. 2005;26(3):162-167. doi:10.1016/j.tips.2005.01.005.
245. Ono K, Karolak MR, Ndong J de la C, Wang W, Yang X, Elefteriou F. The ras-GTPase activity of neurofibromin restrains ERK-dependent FGFR signaling during endochondral

bone formation. *Hum Mol Genet*. 2013;22(15):3048-3062. doi:10.1093/hmg/ddt162.
246. Millán JL, Narisawa S, Lemire I, et al. Enzyme Replacement Therapy for Murine Hypophosphatasia. *J Bone Miner Res*. 2007;23(6):777-787. doi:10.1359/jbmr.071213.
247. Johnson K, Goding J, Van Etten D, et al. Linked deficiencies in extracellular PP(i) and osteopontin mediate pathologic calcification associated with defective PC-1 and ANK expression. *J Bone Miner Res*. 2003;18(6):994-1004. doi:10.1359/jbmr.2003.18.6.994.
248. Cho T-J, Seo J-B, Lee HR, Yoo WJ, Chung CY, Choi IH. Biologic characteristics of fibrous hamartoma from congenital pseudarthrosis of the tibia associated with neurofibromatosis type 1. *J Bone Joint Surg Am*. 2008;90(12):2735-2744. doi:10.2106/JBJS.H.00014.
249. Kawanami A, Matsushita T, Chan YY, Murakami S. Mice expressing GFP and CreER in osteochondro progenitor cells in the periosteum. *Biochem Biophys Res Commun*. 2009;386(3):477-482. doi:10.1016/j.bbrc.2009.06.059.
250. Ippolito E, Corsi A, Grill F, Wientroub S, Bianco P. Pathology of bone lesions associated with congenital pseudarthrosis of the leg. *J Pediatr Orthop B*. 2000;9(1):3-10. http://www.ncbi.nlm.nih.gov/pubmed/10647102. Accessed August 3, 2016.
251. Fabeck L, Ghafil D, Gerroudj M, Baillon R, Delincé P. Bone morphogenetic protein 7 in the treatment of congenital pseudarthrosis of the tibia. *J Bone Jt Surg - Br Vol*. 2006;88-B(1):116-118. doi:10.1302/0301-620X.88B1.16619.
252. Riese DJ, Cullum RL. Epiregulin: roles in normal physiology and cancer. *Semin Cell Dev Biol*. 2014;28:49-56. doi:10.1016/j.semcdb.2014.03.005.
253. Shirakata Y, Komurasaki T, Toyoda H, et al. Epiregulin, a novel member of the epidermal growth factor family, is an autocrine growth factor in normal human keratinocytes. *J Biol Chem*. 2000;275(8):5748-5753. http://www.ncbi.nlm.nih.gov/pubmed/10681561. Accessed March 2, 2015.
254. Toyoda H, Komurasaki T, Uchida D, et al. Epiregulin. A novel epidermal growth factor with mitogenic activity for rat primary hepatocytes. *J Biol Chem*. 1995;270(13):7495-7500. http://www.ncbi.nlm.nih.gov/pubmed/7706296. Accessed February 14, 2015.
255. Qin L, Tamasi J, Raggatt L, et al. Amphiregulin is a novel growth factor involved in normal bone development and in the cellular response to parathyroid hormone stimulation. *J Biol Chem*. 2005;280(5):3974-3981. doi:10.1074/jbc.M409807200.
256. Krampera M, Pasini A, Rigo A, et al. HB-EGF/HER-1 signaling in bone marrow mesenchymal stem cells: inducing cell expansion and reversibly preventing multilineage differentiation. *Blood*. 2005;106(1):59-66. doi:10.1182/blood-2004-09-3645.
257. Antosz ME, Bellows CG, Aubin JE. Biphasic effects of epidermal growth factor on bone nodule formation by isolated rat calvaria cells in vitro. *J Bone Miner Res*. 1987;2(5):385-393. doi:10.1002/jbmr.5650020505.
258. Nicolas V, Nefussi JR, Collin P, Forest N. Effects of acidic fibroblast growth factor and epidermal growth factor on subconfluent fetal rat calvaria cell cultures: DNA synthesis and alkaline phosphatase activity. *Bone Miner*. 1990;8(2):145-156. http://www.ncbi.nlm.nih.gov/pubmed/2306559. Accessed June 16, 2017.
259. Nakamura T, Toita H, Yoshimoto A, et al. Potential Involvement of Twist2 and Erk in the Regulation of Osteoblastogenesis by HB-EGF-EGFR Signaling. *Cell Struct Funct*. 2010;35(1):53-61. doi:10.1247/csf.10001.
260. Mulcrone PL, Campbell JP, Clément-Demange L, et al. Skeletal Colonization by Breast Cancer Cells Is Stimulated by an Osteoblast and β2AR-Dependent Neo-angiogenic

Switch. *J Bone Miner Res*. 2017. doi:10.1002/jbmr.3133.
261. Fouletier-Dilling CM, Bosch P, Davis AR, et al. Novel compound enables high-level adenovirus transduction in the absence of an adenovirus-specific receptor. *Hum Gene Ther*. 2005;16(11):1287-1297. doi:10.1089/hum.2005.16.1287.
262. Dodt M, Roehr J, Ahmed R, Dieterich C. FLEXBAR—Flexible Barcode and Adapter Processing for Next-Generation Sequencing Platforms. *Biology (Basel)*. 2012;1(3):895-905. doi:10.3390/biology1030895.
263. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods*. 2015;12(4):357-360. doi:10.1038/nmeth.3317.
264. Anders S, Pyl PT, Huber W. HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics*. 2015;31(2):166-169. doi:10.1093/bioinformatics/btu638.
265. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15(12):550. doi:10.1186/s13059-014-0550-8.
266. Korpal M, Yan J, Lu X, Xu S, Lerit DA, Kang Y. Imaging transforming growth factor-beta signaling dynamics and therapeutic response in breast cancer bone metastasis. *Nat Med*. 2009;15(8):960-966. doi:10.1038/nm.1943.
267. Hunter T, Cooper JA. Epidermal growth factor induces rapid tyrosine phosphorylation of proteins in A431 human tumor cells. *Cell*. 1981;24(3):741-752. http://www.ncbi.nlm.nih.gov/pubmed/6166387. Accessed August 19, 2016.
268. Fry DW, Kraker AJ, McMichael A, et al. A specific inhibitor of the epidermal growth factor receptor tyrosine kinase. *Science*. 1994;265(5175):1093-1095. http://www.ncbi.nlm.nih.gov/pubmed/8066447. Accessed March 22, 2017.
269. Nam H-J, Kim H-P, Yoon Y-K, et al. Antitumor activity of HM781-36B, an irreversible Pan-HER inhibitor, alone or in combination with cytotoxic chemotherapeutic agents in gastric cancer. *Cancer Lett*. 2011;302(2):155-165. doi:10.1016/j.canlet.2011.01.010.
270. Herbst RS. Review of epidermal growth factor receptor biology. *Int J Radiat Oncol Biol Phys*. 2004;59(2 SUPPL.):21-26. doi:10.1016/j.ijrobp.2003.11.041.
271. Kumegawa M, Hiramatsu M, Hatakeyama K, et al. Effects of epidermal growth factor on osteoblastic cellsin vitro. *Calcif Tissue Int*. 1983;35(1):542-548. doi:10.1007/BF02405091.
272. Ottmann OG, Pelus LM. Differential proliferative effects of transforming growth factor-beta on human hematopoietic progenitor cells. *J Immunol*. 1988;140(8). http://www.jimmunol.org/content/140/8/2661.short. Accessed June 18, 2017.
273. Fine A, Goldstein RH. The effect of transforming growth factor-beta on cell proliferation and collagen formation by lung fibroblasts. *J Biol Chem*. 1987;262(8):3897-3902. http://www.ncbi.nlm.nih.gov/pubmed/3493244. Accessed June 18, 2017.
274. Kang JS, Alliston T, Delston R, Derynck R. Repression of Runx2 function by TGF-β through recruitment of class II histone deacetylases by Smad3. *EMBO J*. 2005;24(14):2543-2555. doi:10.1038/sj.emboj.7600729.
275. Samarakoon R, Dobberfuhl AD, Cooley C, et al. Induction of renal fibrotic genes by TGF-β1 requires EGFR activation, p53 and reactive oxygen species. *Cell Signal*. 2013;25(11):2198-2209. doi:10.1016/j.cellsig.2013.07.007.
276. Kamiya N, Yamaguchi R, Aruwajoye O, et al. Targeted Disruption of NF1 in Osteocytes Increases FGF23 and Osteoid With Osteomalacia-like Bone Phenotype. *J Bone Miner Res*. 2017. doi:10.1002/jbmr.3155.

277. Ghadakzadeh S, Kannu P, Whetstone H, Howard A, Alman BA. β-Catenin modulation in neurofibromatosis type 1 bone repair: therapeutic implications. *FASEB J*. 2016. doi:10.1096/fj.201500190RR.
278. Baht GS, Nadesan P, Silkstone D, Alman BA. Pharmacologically targeting beta-catenin for NF1 associated deficiencies in fracture repair. *Bone*. 2017;98:31-36. doi:10.1016/j.bone.2017.02.012.
279. Cargnello M, Roux PP. Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases. *Microbiol Mol Biol Rev*. 2011;75(1):50-83. doi:10.1128/MMBR.00031-10.
280. Pagès G. Defective Thymocyte Maturation in p44 MAP Kinase (Erk 1) Knockout Mice. *Science (80- )*. 1999;286(5443):1374-1377. doi:10.1126/science.286.5443.1374.
281. Yao Y, Li W, Wu J, et al. Extracellular signal-regulated kinase 2 is necessary for mesoderm differentiation. *Proc Natl Acad Sci U S A*. 2003;100(22):12759-12764. doi:10.1073/pnas.2134254100.
282. Wu M, Wallace MR, Muir D. Nf1 haploinsufficiency augments angiogenesis. *Oncogene*. 2006;25(16):2297-2303. doi:10.1038/sj.onc.1209264.
283. Shapira S, Barkan B, Fridman E, Kloog Y, Stein R. The tumor suppressor neurofibromin confers sensitivity to apoptosis by Ras-dependent and Ras-independent pathways. *Cell Death Differ*. 2007;14(5):895-906. doi:10.1038/sj.cdd.4402057.
284. Stevenson DA, Yang FC. The musculoskeletal phenotype of the RASopathies. *Am J Med Genet Part C Semin Med Genet*. 2011;157(2):90-103. doi:10.1002/ajmg.c.30296.
285. Aoki Y, Niihori T, Kawame H, et al. Germline mutations in HRAS proto-oncogene cause Costello syndrome. *Nat Genet*. 2005;37(10):1038-1040. doi:10.1038/ng1641.
286. Digilio MC, Conti E, Sarkozy A, et al. Grouping of Multiple-Lentigines/LEOPARD and Noonan Syndromes on the PTPN11 Gene. *Am J Hum Genet*. 2002;71(2):389-394. doi:10.1086/341528.
287. Brems H, Chmara M, Sahbatou M, et al. Germline loss-of-function mutations in SPRED1 cause a neurofibromatosis 1-like phenotype. *Nat Genet*. 2007;39(9):1120-1126. doi:10.1038/ng2113.
288. Murakami S, Balmes G, McKinney S, Zhang Z, Givol D, de Crombrugghe B. Constitutive activation of MEK1 in chondrocytes causes Stat1-independent achondroplasia-like dwarfism and rescues the Fgfr3-deficient mouse phenotype. *Genes Dev*. 2004;18(3):290-305. doi:10.1101/gad.1179104.
289. Cichowski K, Jacks T. NF1 tumor suppressor gene function: narrowing the GAP. *Cell*. 2001;104(4):593-604. http://www.ncbi.nlm.nih.gov/pubmed/11239415. Accessed December 1, 2014.
290. Bollag G, Clapp DW, Shih S, et al. Loss of NF1 results in activation of the Ras signaling pathway and leads to aberrant growth in haematopoietic cells. *Nat Genet*. 1996;12(2):144-148. doi:10.1038/ng0296-144.
291. Hirata Y, Brems H, Suzuki M, et al. Interaction between a Domain of the Negative Regulator of the Ras-ERK Pathway, SPRED1 Protein, and the GTPase-activating Protein-related Domain of Neurofibromin Is Implicated in Legius Syndrome and Neurofibromatosis Type 1. *J Biol Chem*. 2016;291(7):3124-3134. doi:10.1074/jbc.M115.703710.
292. Hegedus B, Dasgupta B, Shin JE, et al. Neurofibromatosis-1 regulates neuronal and glial cell differentiation from neuroglial progenitors in vivo by both cAMP- and Ras-dependent

mechanisms. *Cell Stem Cell*. 2007;1(4):443-457. doi:10.1016/j.stem.2007.07.008.
293. Ismat FA, Xu J, Lu MM, Epstein JA. The neurofibromin GAP-related domain rescues endothelial but not neural crest development in Nf1 mice. *J Clin Invest*. 2006;116(9):2378-2384. doi:10.1172/JCI28341.
294. Aronesty E. Comparison of Sequencing Utility Programs. *Open Bioinforma J*. 2013;7(1):1-8. doi:10.2174/1875036201307010001.
295. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol*. 2013;14(4):R36. doi:10.1186/gb-2013-14-4-r36.
296. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*. 2014;30(7):923-930. doi:10.1093/bioinformatics/btt656.
297. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010;26(1):139-140. doi:10.1093/bioinformatics/btp616.
298. Igarashi A, Segoshi K, Sakai Y, et al. Selection of Common Markers for Bone Marrow Stromal Cells from Various Bones Using Real-Time RT-PCR: Effects of Passage Number and Donor Age. *Tissue Eng*. 2007;13(10):2405-2417. doi:10.1089/ten.2006.0340.
299. Wieczorek G, Steinhoff C, Schulz R, et al. Gene expression profile of mouse bone marrow stromal cells determined by cDNA microarray analysis. *Cell Tissue Res*. 311(2):227-237. doi:10.1007/s00441-002-0671-3.
300. Newman PJ, Berndt MC, Gorski J, et al. PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily. *Science*. 1990;247(4947):1219-1222. http://www.ncbi.nlm.nih.gov/pubmed/1690453. Accessed December 29, 2017.
301. Lampugnani MG, Resnati M, Raiteri M, et al. A novel endothelial-specific membrane protein is a marker of cell-cell contacts. *J Cell Biol*. 1992;118(6):1511-1522. http://www.ncbi.nlm.nih.gov/pubmed/1522121. Accessed December 29, 2017.
302. Challen GA, Boles N, Lin KK-Y, Goodell MA. Mouse hematopoietic stem cell identification and analysis. *Cytometry A*. 2009;75(1):14-24. doi:10.1002/cyto.a.20674.
303. Kyono A, Avishai N, Ouyang Z, Landreth GE, Murakami S. FGF and ERK signaling coordinately regulate mineralization-related genes and play essential roles in osteocyte differentiation. *J Bone Miner Metab*. 2012;30(1):19-30. doi:10.1007/s00774-011-0288-2.
304. Lankat-Buttgereit B, Göke R. The tumour suppressor Pdcd4: recent advances in the elucidation of function and regulation. *Biol Cell*. 2009;101(6):309-317. doi:10.1042/BC20080191.
305. He W, Zhang M-G, Wang X-J, et al. KAT5 and KAT6B are in positive regulation on cell proliferation of prostate cancer through PI3K-AKT signaling. *Int J Clin Exp Pathol*. 2013;6(12):2864-2871. http://www.ncbi.nlm.nih.gov/pubmed/24294372. Accessed January 6, 2018.
306. Dong X, Weng Z. The correlation between histone modifications and gene expression. *Epigenomics*. 2013;5(2):113-116. doi:10.2217/epi.13.13.
307. Whitmarsh AJ. Regulation of gene transcription by mitogen-activated protein kinase signaling pathways. *Biochim Biophys Acta - Mol Cell Res*. 2007;1773(8):1285-1298. doi:10.1016/J.BBAMCR.2006.11.011.
308. Zhang H, Bajraszewski N, Wu E, et al. PDGFRs are critical for PI3K/Akt activation and

negatively regulated by mTOR. *J Clin Invest*. 2007;117(3):730-738. doi:10.1172/JCI28984.
309. Ye M, Hu D, Tu L, et al. Involvement of PI3K/Akt Signaling Pathway in Hepatocyte Growth Factor–Induced Migration of Uveal Melanoma Cells. *Investig Opthalmology Vis Sci*. 2008;49(2):497. doi:10.1167/iovs.07-0975.
310. Mahajan MA, Samuels HH. Nuclear receptor coactivator/coregulator NCoA6(NRC) is a pleiotropic coregulator involved in transcription, cell survival, growth and development. *Nucl Recept Signal*. 2007;4:e002. doi:10.1621/nrs.06002.
311. Chen Y-H, Gianino SM, Gutmann DH. Neurofibromatosis-1 regulation of neural stem cell proliferation and multilineage differentiation operates through distinct RAS effector pathways. *Genes Dev*. 2015;29(16):1677-1682. doi:10.1101/gad.261677.115.
312. Gitler AD, Epstein JA. Regulating heart development: the role of Nf1. *Cell Cycle*. 2(2):96-98. http://www.ncbi.nlm.nih.gov/pubmed/12695655. Accessed July 22, 2015.
313. Bajenaru ML, Zhu Y, Hedrick NM, Donahoe J, Parada LF, Gutmann DH. Astrocyte-specific inactivation of the neurofibromatosis 1 gene (NF1) is insufficient for astrocytoma formation. *Mol Cell Biol*. 2002;22(14):5100-5113. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=139771&tool=pmcentrez&rendertype=abstract. Accessed December 13, 2015.
314. Zhu Y, Harada T, Liu L, et al. Inactivation of NF1 in CNS causes increased glial progenitor proliferation and optic glioma formation. *Development*. 2005;132(24):5577-5588. doi:10.1242/dev.02162.
315. Kweh F, Min Z, Kurenova E, Wallace M, Golubovskaya V, Cance WG. Neurofibromin physically interacts with the N-terminal domain of focal adhesion kinase. *Mol Carcinog*. 2009;48(11):1005-1017. doi:10.1002/mc.20552.
316. Zhou Y, He Y, Sharma R, et al. Hyperactive RAS/PI3-K/MAPK Signaling Cascade in Migration and Adhesion of Nf1 Haploinsufficient Mesenchymal Stem/Progenitor Cells. *Int J Mol Sci*. 2015;16(6):12345-12359. doi:10.3390/ijms160612345.
317. Tahaei SE, Couasnay G, Ma Y, et al. The reduced osteogenic potential of Nf1 -deficient osteoprogenitors is EGFR-independent. *Bone*. 2017;106:103-111. doi:10.1016/j.bone.2017.10.012.
318. Wang W, Nyman JS, Ono K, Stevenson DA, Yang X, Elefteriou F. Mice lacking Nf1 in osteochondroprogenitor cells display skeletal dysplasia similar to patients with neurofibromatosis type I. *Hum Mol Genet*. 2011;20(20):3910-3924. doi:10.1093/hmg/ddr310.
319. Darvin P, Joung YH, Yang YM. JAK2-STAT5B pathway and osteoblast differentiation. *JAK-STAT*. 2013;2(4):e24931. doi:10.4161/jkst.24931.
320. Hardy R, Cooper MS. Bone loss in inflammatory disorders. *J Endocrinol*. 2009;201(3):309-320. doi:10.1677/JOE-08-0568.
321. Camerino C, Zayzafoon M, Rymaszewski M, Heiny J, Rios M, Hauschka P V. Central depletion of brain-derived neurotrophic,factorin mice results in high bone mass and metabolic phenotype. *Endocrinology*. 2012;153(11):5394-5405. doi:10.1210/en.2012-1378.
322. Kajiya M, Shiba H, Fujita T, et al. Brain-derived neurotrophic factor stimulates bone/cementum-related protein gene expression in cementoblasts. *J Biol Chem*. 2008;283(23):16259-16267. doi:10.1074/jbc.M800668200.
323. Zanotti S, Smerdel-Ramoya A, Stadmeyer L, Durant D, Radtke F, Canalis E. Notch

Inhibits Osteoblast Differentiation and Causes Osteopenia. *Endocrinology*. 2008;149(8):3890-3899. doi:10.1210/en.2008-0140.
324. Zhang H, Hilton MJ, Anolik JH, et al. NOTCH inhibits osteoblast formation in inflammatory arthritis via noncanonical NF-κB. *J Clin Invest*. 2014;124(7):3200-3214. doi:10.1172/JCI68901.
325. Cong Q, Jia H, Biswas S, et al. p38α MAPK Regulates Lineage Commitment and OPG Synthesis of Bone Marrow Stromal Cells to Prevent Bone Loss under Physiological and Pathological Conditions. *Stem cell reports*. 2016;6(4):566-578. doi:10.1016/j.stemcr.2016.02.001.
326. Browne AJ, Göbel A, Thiele S, Hofbauer LC, Rauner M, Rachner TD. p38 MAPK regulates the Wnt inhibitor Dickkopf-1 in osteotropic prostate cancer cells. *Cell Death Dis*. 2016;7(2):e2119. doi:10.1038/cddis.2016.32.
327. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell*. 2006;126(4):677-689. doi:10.1016/j.cell.2006.06.044.
328. Celic S, Katayama Y, Chilco PJ, Martin TJ, Findlay DM. Type I collagen influence on gene expression in UMR106-06 osteoblast-like cells is inhibited by genistein. *J Endocrinol*. 1998;158(3):377-388. http://www.ncbi.nlm.nih.gov/pubmed/9846167. Accessed November 27, 2017.
329. Rao Pattabhi S, Martinez JS, Keller TCS, III. Decellularized ECM effects on human mesenchymal stem cell stemness and differentiation. *Differentiation*. 2014;88(4-5):131-143. doi:10.1016/j.diff.2014.12.005.
330. Wee H-J, Huang G, Shigesada K, Ito Y. Serine phosphorylation of RUNX2 with novel potential functions as negative regulatory mechanisms. *EMBO Rep*. 2002;3(10):967-974. doi:10.1093/embo-reports/kvf193.
331. Vallabhapurapu S, Karin M. Regulation and Function of NF-κB Transcription Factors in the Immune System. *Annu Rev Immunol*. 2009;27(1):693-733. doi:10.1146/annurev.immunol.021908.132641.
332. Gilbert L, He X, Farmer P, et al. Inhibition of osteoblast differentiation by tumor necrosis factor-alpha. *Endocrinology*. 2000;141(11):3956-3964. doi:10.1210/endo.141.11.7739.
333. Leon J, Guerrero I, Pellicer A. Differential expression of the ras gene family in mice. *Mol Cell Biol*. 1987;7(4):1535-1540. http://www.ncbi.nlm.nih.gov/pubmed/3600635. Accessed November 28, 2017.
334. Castellano E, Santos E. Functional specificity of ras isoforms: so similar but so different. *Genes Cancer*. 2011;2(3):216-231. doi:10.1177/1947601911408081.
335. Hodgkinson CP, Naidoo V, Patti KG, et al. Abi3bp is a multifunctional autocrine/paracrine factor that regulates mesenchymal stem cell biology. *Stem Cells*. 2013;31(8):1669-1682. doi:10.1002/stem.1416.
336. Pfannkuche K, Summer H, Li O, Hescheler J, Dröge P. The High Mobility Group Protein HMGA2: A Co-Regulator of Chromatin Structure and Pluripotency in Stem Cells? *Stem Cell Rev Reports*. 2009;5(3):224-230. doi:10.1007/s12015-009-9078-9.
337. Wang H, Sun Z, Wang Y, et al. miR-33-5p, a novel mechano-sensitive microRNA promotes osteoblast differentiation by targeting Hmga2. *Sci Rep*. 2016;6(1):23170. doi:10.1038/srep23170.
338. Passacquale G, Phinikaridou A, Warboys C, et al. Aspirin-induced histone acetylation in endothelial cells enhances synthesis of the secreted isoform of netrin-1 thus inhibiting monocyte vascular infiltration. *Br J Pharmacol*. 2015;172(14):3548-3564.

doi:10.1111/bph.13144.
339. Yang Y-H, Zhou H, Binmadi NO, Proia P, Basile JR. Plexin-B1 activates NF-κB and IL-8 to promote a pro-angiogenic response in endothelial cells. *PLoS One*. 2011;6(10):e25826. doi:10.1371/journal.pone.0025826.
340. Tang Q-L, Xie X-B, Wang J, et al. Glycogen Synthase Kinase-3 , NF- B Signaling, and Tumorigenesis of Human Osteosarcoma. *JNCI J Natl Cancer Inst*. 2012;104(10):749-763. doi:10.1093/jnci/djs210.
341. Sullivan K, El-Hoss J, Little DG, Schindeler A. JNK inhibitors increase osteogenesis in Nf1-deficient cells. *Bone*. 2011;49(6):1311-1316. doi:10.1016/j.bone.2011.09.043.
342. Sun D, Haddad R, Kraniak JM, Horne SD, Tainsky MA. RAS/MEK-independent gene expression reveals BMP2-related malignant phenotypes in the Nf1-deficient MPNST. *Mol Cancer Res*. 2013;11(6):616-627. doi:10.1158/1541-7786.MCR-12-0593.
343. Zhang W, Shen X, Wan C, et al. Effects of insulin and insulin-like growth factor 1 on osteoblast proliferation and differentiation: differential signalling via Akt and ERK. *Cell Biochem Funct*. 2012;30(4):297-302. doi:10.1002/cbf.2801.
344. Huang Y-F, Lin J-J, Lin C-H, Su Y, Hung S-C. c-Jun N-terminal kinase 1 negatively regulates osteoblastic differentiation induced by BMP2 via phosphorylation of Runx2 at Ser104. *J Bone Miner Res*. 2012;27(5):1093-1105. doi:10.1002/jbmr.1548.
345. Kim J-M, Choi JS, Kim Y-H, et al. An activator of the cAMP/PKA/CREB pathway promotes osteogenesis from human mesenchymal stem cells. *J Cell Physiol*. 2013;228(3):617-626. doi:10.1002/jcp.24171.
346. Lo KW-H, Kan HM, Ashe KM, Laurencin CT. The small molecule PKA-specific cyclic AMP analogue as an inducer of osteoblast-like cells differentiation and mineralization. *J Tissue Eng Regen Med*. 2012;6(1):40-48. doi:10.1002/term.395.
347. Nüsslein-Volhard C, Wieschaus E. Mutations affecting segment number and polarity in Drosophila. *Nature*. 1980;287(5785):795-801. http://www.ncbi.nlm.nih.gov/pubmed/6776413. Accessed September 30, 2017.
348. Gergen JP, Wieschaus EF. The localized requirements for a gene affecting segmentation in Drosophila: analysis of larvae mosaic for runt. *Dev Biol*. 1985;109(2):321-335. http://www.ncbi.nlm.nih.gov/pubmed/3996753. Accessed September 30, 2017.
349. Simeone A, Daga A, Calabi F. Expression of runt in the mouse embryo. *Dev Dyn*. 1995;203(1):61-70. doi:10.1002/aja.1002030107.
350. Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell*. 1996;84(2):321-330. http://www.ncbi.nlm.nih.gov/pubmed/8565077. Accessed September 30, 2017.
351. Wang Q, Stacy T, Binder M, Marin-Padilla M, Sharpe AH, Speck NA. Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc Natl Acad Sci U S A*. 1996;93(8):3444-3449. http://www.ncbi.nlm.nih.gov/pubmed/8622955. Accessed September 30, 2017.
352. Mundlos S, Otto F, Mundlos C, et al. Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. *Cell*. 1997;89(5):773-779. http://www.ncbi.nlm.nih.gov/pubmed/9182765. Accessed September 30, 2017.
353. Lee B, Thirunavukkarasu K, Zhou L, et al. Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia. *Nat Genet*. 1997;16(3):307-310. doi:10.1038/ng0797-307.

354. Li QL, Ito K, Sakakura C, et al. Causal relationship between the loss of RUNX3 expression and gastric cancer. *Cell*. 2002;109(1):113-124. doi:10.1016/S0092-8674(02)00690-6.
355. Wang N, Sui F, Ma J, et al. Site-specific Hypermethylation of RUNX3 Predicts Poor Prognosis in Gastric Cancer. *Arch Med Res*. 2016;47(4):285-292. doi:10.1016/j.arcmed.2016.07.011.
356. KATAOKA J, SHIRAHA H, HORIGUCHI S, et al. Loss of Runt-related transcription factor 3 induces resistance to 5-fluorouracil and cisplatin in hepatocellular carcinoma. *Oncol Rep*. 2016;35(5):2576-2582. doi:10.3892/or.2016.4681.
357. Jeong J-H, Jin J-S, Kim H-N, et al. Expression of Runx2 transcription factor in non-skeletal tissues, sperm and brain. *J Cell Physiol*. 2008;217(2):511-517. doi:10.1002/jcp.21524.
358. Schneider A, Cama G, Ghuman M, Hughes FJ, Gharibi B. Sprouty 2 , an Early Response Gene Regulator of FosB and Mesenchymal Stem Cell Proliferation During Mechanical Loading and Osteogenic Differentiation. *J Cell Biochem*. 2017;118(9):2606-2614. doi:10.1002/jcb.26035.
359. Takarada T, Hinoi E, Nakazato R, et al. An analysis of skeletal development in osteoblast-specific and chondrocyte-specific runt-related transcription factor-2 (Runx2) knockout mice. *J Bone Miner Res*. 2013;28(10):2064-2069. doi:10.1002/jbmr.1945.
360. Elefteriou F, Yang X. Genetic mouse models for bone studies—Strengths and limitations. *Bone*. 2011;49(6):1242-1254. doi:10.1016/j.bone.2011.08.021.
361. Banerjee C, McCabe LR, Choi JY, et al. Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone-specific complex. *J Cell Biochem*. 1997;66(1):1-8. http://www.ncbi.nlm.nih.gov/pubmed/9215522. Accessed September 30, 2017.
362. Sato M, Morii E, Komori T, et al. Transcriptional regulation of osteopontin gene in vivo by PEBP2αA/CBFA1 and ETS1 in the skeletal tissues. *Oncogene*. 1998;17(12):1517-1525. doi:10.1038/sj.onc.1202064.
363. Harada H, Tagashira S, Fujiwara M, et al. Cbfa1 isoforms exert functional differences in osteoblast differentiation. *J Biol Chem*. 1999;274(11):6972-6978. http://www.ncbi.nlm.nih.gov/pubmed/10066751. Accessed September 30, 2017.
364. Prince M, Banerjee C, Javed A, et al. Expression and regulation of Runx2/Cbfa1 and osteoblast phenotypic markers during the growth and differentiation of human osteoblasts. *J Cell Biochem*. 2001;80(3):424-440. http://www.ncbi.nlm.nih.gov/pubmed/11135373. Accessed September 30, 2017.
365. Li Y, Ge C, Long JP, et al. Biomechanical stimulation of osteoblast gene expression requires phosphorylation of the RUNX2 transcription factor. *J Bone Miner Res*. 2012;27(6):1263-1274. doi:10.1002/jbmr.1574.
366. Ling M, Huang P, Islam S, et al. Epigenetic regulation of Runx2 transcription and osteoblast differentiation by nicotinamide phosphoribosyltransferase. *Cell Biosci*. 2017;7(1):27. doi:10.1186/s13578-017-0154-6.
367. Schroeder TM, Kahler RA, Li X, Westendorf JJ. Histone deacetylase 3 interacts with Runx2 to repress the osteocalcin promoter and regulate osteoblast differentiation. *J Biol Chem*. 2004;279(40):41998-42007. doi:10.1074/jbc.M403702200.
368. Jensen ED, Schroeder TM, Bailey J, Gopalakrishnan R, Westendorf JJ. Histone Deacetylase 7 Associates With Runx2 and Represses Its Activity During Osteoblast

Maturation in a Deacetylation-Independent Manner. *J Bone Miner Res*. 2007;23(3):361-372. doi:10.1359/jbmr.071104.
369. Artigas N, Ureña C, Rodríguez-Carballo E, Rosa JL, Ventura F. Mitogen-activated protein kinase (MAPK)-regulated interactions between Osterix and Runx2 are critical for the transcriptional osteogenic program. *J Biol Chem*. 2014;289(39):27105-27117. doi:10.1074/jbc.M114.576793.
370. Jeon E-J, Lee K-Y, Choi N-S, et al. Bone morphogenetic protein-2 stimulates Runx2 acetylation. *J Biol Chem*. 2006;281(24):16502-16511. doi:10.1074/jbc.M512494200.
371. Lee MH, Javed A, Kim HJ, et al. Transient upregulation of CBFA1 in response to bone morphogenetic protein-2 and transforming growth factor beta1 in C2C12 myogenic cells coincides with suppression of the myogenic phenotype but is not sufficient for osteoblast differentiation. *J Cell Biochem*. 1999;73(1):114-125. http://www.ncbi.nlm.nih.gov/pubmed/10088730. Accessed October 12, 2017.
372. Li C, Cheng Y, Gutmann DA, Mangoura D. Differential localization of the neurofibromatosis 1 (NF1) gene product, neurofibromin, with the F-actin or microtubule cytoskeleton during differentiation of telencephalic neurons. *Dev Brain Res*. 2001;130(2):231-248. doi:10.1016/S0165-3806(01)00190-0.
373. Vandenbroucke I, Van Oostveldt P, Coene E, De Paepe A, Messiaen L. Neurofibromin is actively transported to the nucleus. *FEBS Lett*. 2004;560(1-3):98-102. doi:10.1016/S0014-5793(04)00078-X.
374. Shirakabe K, Terasawa K, Miyama K, Shibuya H, Nishida E. Regulation of the activity of the transcription factor Runx2 by two homeobox proteins, Msx2 and Dlx5. *Genes Cells*. 2001;6(10):851-856. http://www.ncbi.nlm.nih.gov/pubmed/11683913. Accessed January 5, 2017.
375. Zaidi SK, Sullivan AJ, Medina R, et al. Tyrosine phosphorylation controls Runx2-mediated subnuclear targeting of YAP to repress transcription. *EMBO J*. 2004;23(4):790-799. doi:10.1038/sj.emboj.7600073.
376. Hong J-H, Hwang ES, McManus MT, et al. TAZ, a Transcriptional Modulator of Mesenchymal Stem Cell Differentiation. *Science (80- )*. 2005;309(5737):1074-1078. doi:10.1126/science.1110955.
377. Hinoi E, Fujimori S, Wang L, Hojo H, Uno K, Yoneda Y. Nrf2 Negatively Regulates Osteoblast Differentiation via Interfering with Runx2-dependent Transcriptional Activation. *J Biol Chem*. 2006;281(26):18015-18024. doi:10.1074/jbc.M600603200.
378. Kim Y-J, Kim B-G, Lee S-J, et al. The suppressive effect of myeloid elf-1-like factor (MEF) in osteogenic differentiation. *J Cell Physiol*. 2007;211(1):253-260. doi:10.1002/jcp.20933.
379. Rajalingam K, Schreck R, Rapp UR, Albert ??tefan. Ras oncogenes and their downstream targets. *Biochim Biophys Acta - Mol Cell Res*. 2007;1773(8):1177-1195. doi:10.1016/j.bbamcr.2007.01.012.
380. Deraredj Nadim W, Chaumont-Dubel S, Madouri F, et al. Physical interaction between neurofibromin and serotonin 5-HT 6 receptor promotes receptor constitutive activity. *Proc Natl Acad Sci*. 2016;113(43):12310-12315. doi:10.1073/pnas.1600914113.
381. Arun V, Wiley JC, Kaur H, Kaplan DR, Guha A. A novel neurofibromin (NF1) interaction with the leucine-rich pentatricopeptide repeat motif-containing protein links neurofibromatosis type 1 and the french canadian variant of leigh's syndrome in a common molecular complex. *J Neurosci Res*. 2013;91(4):494-505. doi:10.1002/jnr.23189.

382. Shapira S, Barkan B, Friedman E, Fridman E, Kloog Y, Stein R. The tumor suppressor neurofibromin confers sensitivity to apoptosis by Ras-dependent and Ras-independent pathways. *Cell Death Differ*. 2007;14(5):895-906. doi:10.1038/sj.cdd.4402057.
383. Xiao G, Jiang D, Thomas P, et al. MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. *J Biol Chem*. 2000;275(6):4453-4459. http://www.ncbi.nlm.nih.gov/pubmed/10660618. Accessed November 2, 2017.
384. Zhang F, Xu L, Xu L, Xu Q, Karsenty G, Chen CD. Histone demethylase JMJD3 is required for osteoblast differentiation in mice. *Sci Rep*. 2015;5:13418. doi:10.1038/srep13418.