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Exploring ERK1/2 influence on BMP signaling in skeletogenesis of CFP1 knockout cells

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Thesis

EXPLORING ERK1/2 INFLUENCE ON BMP SIGNALING IN SKELETOGENESIS OF CFP1 KNOCKOUT CELLS

by

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ABSTRACT

Epigenetics provides a possible explanation for the often-observed difference between genotype and the final phenotype. Epigenetics is regulated through a variety of means such as histone methylation. The focus of this paper is on the transcription factor CFP1 and its affects on cell signaling within bone growth and differentiation. CFP1 has been shown to regulate DNA and histone methylation, which affects gene expression. Epigenetics within bone development has been linked to numerous disorders and thus any insights into the underlying mechanisms may help lead to therapeutic solutions. With two primary forms of skeletogenesis, endochondral and intramembranous ossification, three different cell lines were utilized in this paper; each of these representing different stages of differentiation within skeletogenesis. This thesis' focuses on studying the interaction of two of the primary cell signaling pathways that regulate skeletogenesis, FGF and BMP. The ERK MAPK pathway is one of the FGF signaling pathways that has been shown to previously interact with BMP. Results showed in CFP1 knockout cells that there was a decrease in BMP signaling while there was an increase in the phosphorylation of ERK, the final step in ERK MAPK pathway. In order to possibly restore BMP signaling, inhibitors of the phosphorylation of ERK were utilized. Through Western Blot analysis, BMP signaling could be raised with inhibition of ERK demonstrating the

antagonistic relationship between them. It appears that CFP1 has an overall inhibitory role in the ERK MAPK pathway, and that when it has been removed there is a decrease in BMP signaling, which can affect proper bone growth and differentiation.

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LIST OF ABBREVIATIONS

BMP ..... Bone Morphogenetic Protein

BSA .....Bovine Serum Albumin

CFP1 ..... CXXC Finger Protein 1

CGIs ..... CpG Islands

Col10a1 ..... Collagen Type X Alpha 1

Col2a1 ..... Collagen Type II Alpha 1

DNA ..... Deoxyribonucleic Acid

DNMTs ..... DNA Methyltransferases

ERK ..... Extracellular Signal-Regulated Kinase

FBS ..... Fetal Bovine Serum

FGF ..... Fibroblast Growth Factor

FGFR ..... Fibroblast Growth Factor Receptor

HAT ..... Histone Acetyltransferase

HDAC ..... Histone Deacetylation

I-Smads ..... Inhibitory Smads

MAPK ..... Mitogen-Activated Protein Kinase

MLB ..... Mouse Limb Buds

PBST ..... Phosphate Buffered Saline with Tween

pERK ..... Phosphorylated ERK

pSmad ..... Phosphorylated Smad

RNA .....Ribonucleic Acid

R-Smads ..... Receptor Smads

TGF-β ..... Transforming Growth Factor-β

INTRODUCTION

Epigenetics

Epigenetics is the unique inheritance of gene expression patterns that does not alter the inherent deoxyribonucleic acid (DNA) structure. It does this primarily by affecting the chromatin and controlling its accessibility for transcription (Allis, 2016). Epigenetics helped better explain for the observed difference between the genotype and the final phenotype (Goldberg, 2007).

The evolutionary demand for epigenetic control became warranted with the rise of eukaryotes. Eukaryotes had to have an efficient system for packing, storing, and accessing their genetic material due to the sheer volume compared to prokaryotes (Ordog, 2012). For this to occur eukaryotes utilize chromatin, a nucleoprotein complex that is made up of histones and nonhistone proteins, which properly stores and indexes DNA. Chromatin can condense or expand depending on what the cell requires. Chromatin must be “opened” or expanded for gene expression to occur, as the ribonucleic acid (RNA) polymerase complex II needs the space to transcribe the DNA (Ordog, 2012).

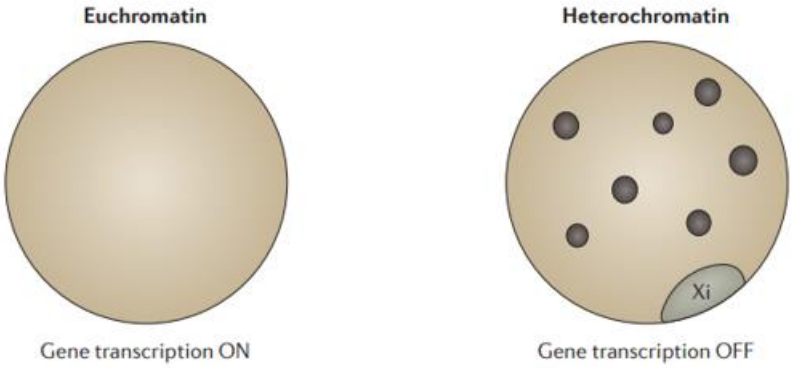

Figure 1: Visual schematic representation of euchromatin versus heterochromatin (Allis, 2016).

The condensation and expansion of chromatin have the official names of heterochromatin and euchromatin, respectively. The main difference between these two states is the accessibility of the DNA for transcription. As seen in Figure 1 in the heterochromatic state, the chromatin becomes more condensed and makes the DNA inaccessible and can be seen as dark-staining under a microscope. On the other hand, the euchromatic state is when chromatin is uncoiled and can be seen as a lighter staining as the DNA becomes more accessible for transcription (Allis, 2016). Although these mechanisms appear to be binary in nature, accessible or not, there is a great number of dynamics that allow DNA transcription to be tunable depending on the needs of the cell (Bowman, 2015).

Chromatin is made up of repeating units called nucleosomes, that are made up of octamers, which consists of two copies of four core histones: H2A, H2B, H3 and H4 (Figure 2). From here the double helix DNA is wrapped around the histones. The bending of the DNA inevitably brings the phosphate backbones of the double helix closer and is

energetically unfavorable, but is maintained through neutralization of charges from the multiple arginine and lysine side chains found in the histone (Bowman, 2015). The bending of the DNA around the histones is demonstrated in Figure 2, but the arginine and lysine cannot be seen. In the middle of Figure 2, the core histone proteins are organized so that there are two main groupings: (H3 and H4)$_2$ tetramer as well as two H2A, H2B dimers (Ordog, 2012).

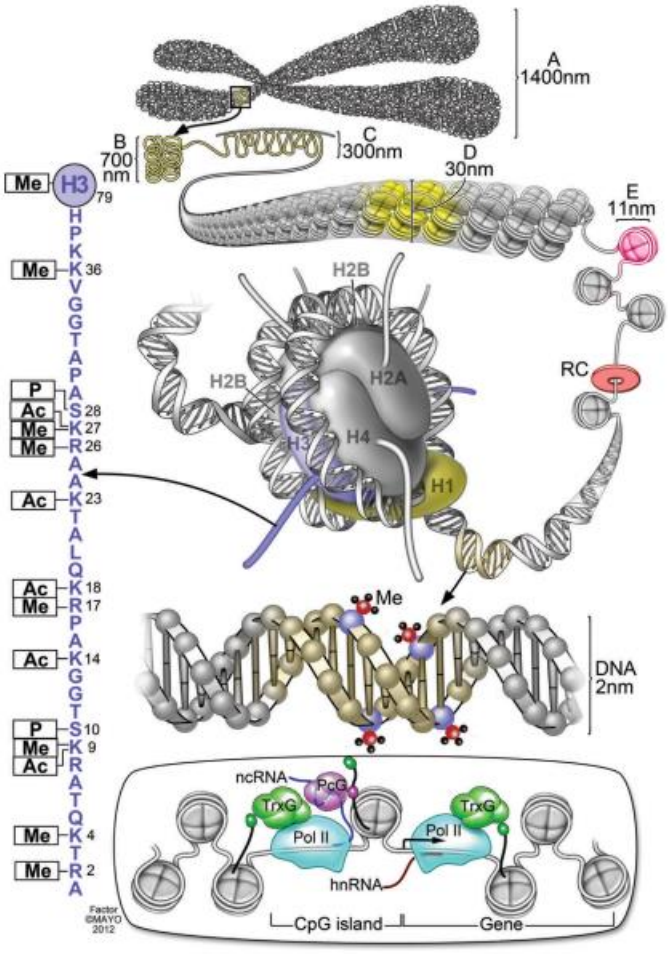

Figure 2: Chromatin organization and structure. At the top is chromatin which is then zoomed in to show how the nucleosomes pack and have DNA wrapped around them. The DNA is zoomed in on to show methyl groups attached and then even closer to

demonstrate how the RNA polymerase complex would bind and transcribe DNA (Ordog, 2012).

Acetylation & Methylation

Some of the first epigenetic mechanisms that were discovered were histone acetylation and DNA methylation. Acetylation occurs from a histone acetyltransferase (HAT) enzyme that places an acetyl group on the histone and opens the chromatin. Alternatively, recent studies have shown that histone deacetylation (HDAC) has been linked with gene repression. Although the mechanisms of HAT and HDAC have become more elucidated, the exact process by which acetylation brings about an open state of chromatin is still unknown (Allis, 2016).

DNA methylation is one of the most studied epigenetic means of control. The process involves adding a methyl group to the nucleic acids, most frequently the cytosine on its 5^{th} carbon, and is crucial of certain cellular process (Ordog, 2012). These processes including development, X-chromosome inactivation, as well as imprinting. The methylation of cytosine allows another layer of coding capabilities of the genome with methylation patterns being heritable and essential for maintaining cellular identity (Goldberg, 2007).

DNA methylation is carried out primarily by DNA methyltransferases (DNMTs). There are two different types of DNA methyltransferases with each of their own functions: maintenance methyltransferases and de novo methyltransferases. Maintenance DMNTs' function is to make sure during DNA replication that the newly synthesized strand brings with it the original strand's methylation pattern, ensuring heritability

through cell division. Whereas, de novo methyltransferases establish new methylation patterns critical for cellular development and differentiation (Jones, 2012; Baubec, 2015).

Acetylation and methylation appear to have the guidelines of activation and repression, respectively, yet these are in general terms – not as a rule. Certain methylation patterns have been shown to open centromeres and thus increase gene transcription (Stimpson, 2011). Therefore, acetylation and methylation demonstrate some of the intricate processes necessary for proper cell growth and differentiation.

CpG Islands

DNA methylation primarily affects cytosine residues in areas with repeating cytosine and guanines, termed CpG islands (CGIs). CGIs are filled with CpG dinucleotides that are predominantly non-methylated and service as important sites for initiation of transcription and also epigenetic regulation. DMNTs carry a methyl and attach it to the cytosines within these CGIs which is correlated with decreased transcription (Goldberg, 2007).

CGIs are important due to being located near the transcriptional start sites of genes. Notably, about 50% of human and mouse genes are found near unmethylated CGIs (Carlone, 2005). Thus, the methylation state of CGIs is critical for epigenetic regulation with most CpG dinucleotides being unmethylated within these regions compared to the rest of the DNA, where CpGs are often methylated (Thomson, 2010). Not only being methylated or not, but the specific timing for methylation is essential for

normal mammalian development with mutations in methyltransferase enzymes leading to a variety of syndromes (Carlone, 2005).

CXXC Finger Protein 1

CGIs are often the site for key epigenetic regulators such as CXXC Finger Protein 1 (CFP1) to aid in proper gene expression. CFP1 is a protein that primarily aids in DNA and histone methylation. CFP1 is encoded by the Cxxc1 gene and in the absence of this protein embryos fail to gastrulate (Carlone, 2005). In addition, selective removal of CFP1 from stem cells blocks their ability to differentiate into mature cell types (Carlone, 2005; Chun, 2014). Depletion of CFP1 in zebrafish has also resulted in a total decrease in cytosine methylation (Tate, 2009).

CFP1 has many important domains that facilitate and enable its interactions. These include not only a domain to bind directly to DNA, but also to histone modifiers. The core structure of CFP1 is the CXXC (two cysteines with two amino acids in between repeated) domain which directly binds to unmethylated CpG dinucleotides (Tate, 2009). This domain allows CFP1 to be able to recognize specific gene promoters and regulate epigenetic modifications that could critically define cellular identity.

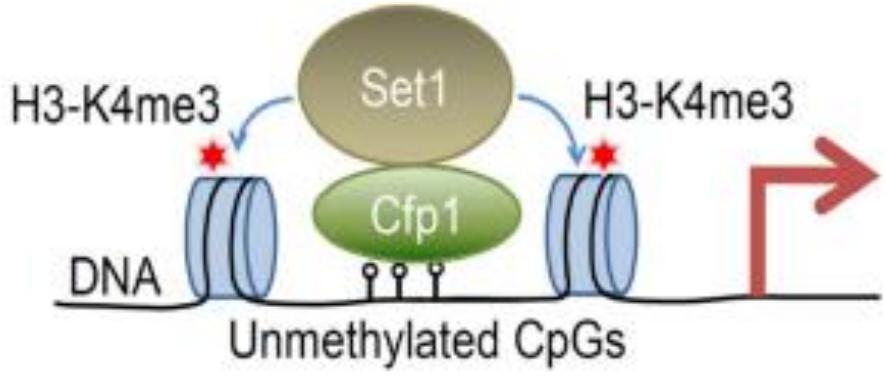

Figure 3: Proposed schematic courtesy of Dr. Diana L. Carlone on how CFP1 aids in guiding the methylation of Set1 protein complex. CFP1 binding to both the DNA as well as the Setd1A histone H3K4 methyltransferase complex.

Other domains within CFP1 interact with certain histone modification complexes such as a protein interaction domain. This domain binds onto chromatin and help regulate specific methylation of H3K4me3 (Tate, 2009). H3K4me3 means that a nucleosome's H3 protein is tri-methylated (three methyl groups attached) on its fourth lysine residue (K). CFP1 regulates this methylation by interacting with Setd1A histone H3K4 methyltransferase complex and helps localize the complex to where methylation is necessary (Figure 3). These specific marks are generally associated with the euchromatic state and thus allows for transcriptional activation (Tate, 2009). It is of note, that this is an example of methylation leading to increase gene expression. In stem cells lacking CFP1, they are shown to be viable but with increased levels of histone methylation. Not only increased histone methylation, but mislocalization of the Setd1A histone H3K4 methyltransferase complex was increased in these stem cells (Tate, 2009). Taken as a whole, CFP1's structure leads to its functionality, which has great impact on regulating embryogenesis.

Given CFP1's pivotal effect of regulating methylation, it is likely to also play an important role in tissue-specific differentiation particularly in bone. The Carlone lab, the focuses on investigating the role that CFP1 plays in bone formation and differentiation.

Epigenetics in Bones & Abnormalities

Within medicine, there are numerous malformations and congenital abnormalities that can occur that affect bone formation. These can significantly impact an individual's quality of life as one's bones are quintessential for general structural integrity and movement.

DNA methylation is an extremely important field of research for bone biology due to the number of musculoskeletal diseases that have been found associated with it. Osteoarthritis, musculoskeletal disease, has been linked to increased methylation at promoters of important transcription factors in bone homeostasis (Gordon, 2015).

Epigenetic control is integral for bones to properly form. Specific timing of regulating signaling pathways is important for the various stages of cell differentiation. If there are any defects in the receptors, enzymes, or coenzymes in any of these signaling pathways, there are specific musculoskeletal diseases that follow depending on what is defective. For example, any mutation in a single allele of *Sox9,* which encodes for SOX9, a transcription factor required for chondrogenesis, leads to a particular skeletal malformation syndrome: Campomelic Dysplasia (Bi, 2001). Figure 4 shows skeletal staining of the rib cage for wild type and Sox9 mutants, demonstrating the inability of proper bone growth.

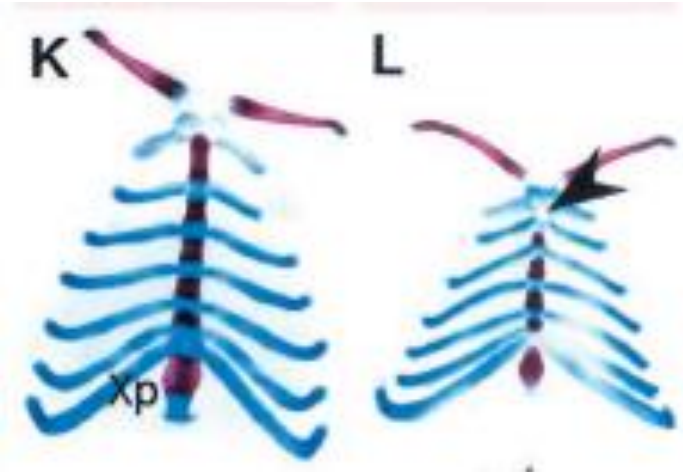

Figure 4: *Difference between wild type (left) and Sox9 mutant's (right) rib cages. The arrow pointing to the missing manubrium sterna (Bi, 2001).*

Bone Formation

Skeletogenesis begins with the mesenchymal stem cell that through a variety of transcription factors and signaling molecules can either directly commit to the osteoblast (bone forming cell) via an osteoprogenitor cell intermediate, a process called intramembranous ossification, or it can become a chondroprogenitor cell giving rise to the chondrocyte lineage, referred to as endochondral ossification. With intramembranous ossification the mesenchymal stem cells directly become osteoprogenitor cells. For endochondral ossification, first cartilage is laid down to serve as a mold, and then later bone matrix is laid on top of the cartilage. Intramembranous ossification is how many of the craniofacial bones are made, while endochondral ossification is primarily how all the other bones are formed such as the tibia, fibula, and femur (Long, 2013).

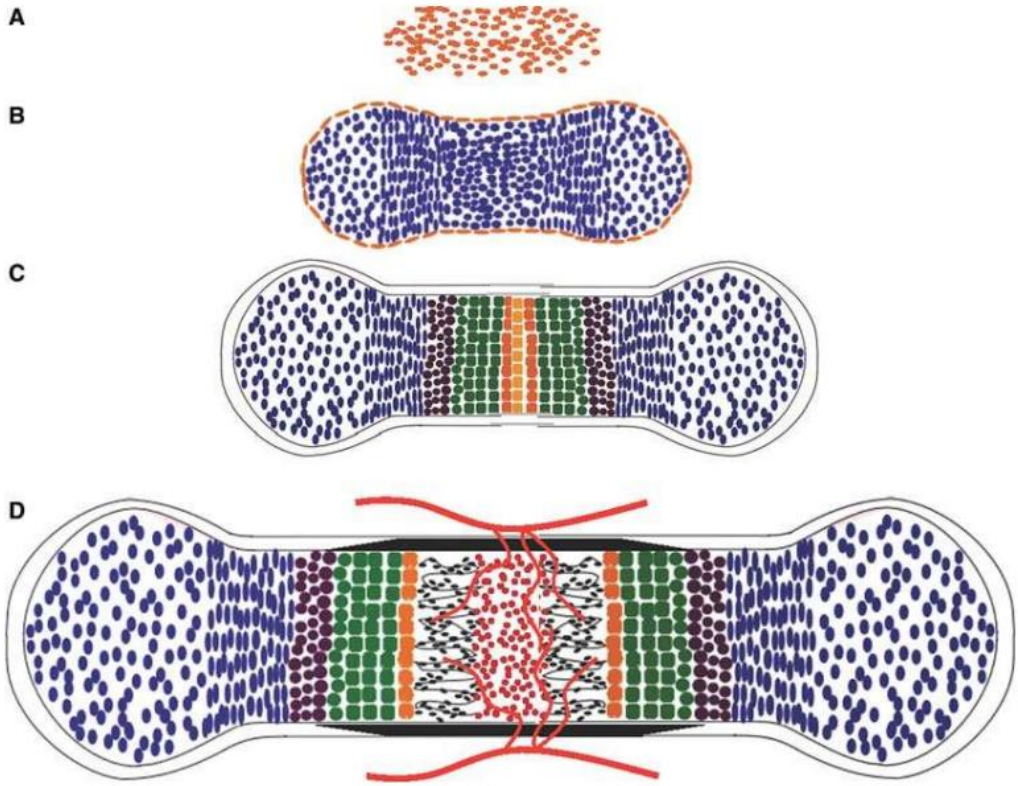

Figure 5: Endochondral ossification (Long, 2013).

Referring to Figure 5 endochondral ossification beings with mesenchymal condensation. Mesenchymal cells begin to group and condense, with the core beginning to differentiate into chondrocytes, while the peripheral cells form the perichondrium. The central chondrocytes then begin secreting collagen type II alpha 1 (Col2a1), IX, and XI to form a cartilage matrix. Along with collagen, a particular proteoglycan is created, aggrecan (Long, 2013). The production of aggrecan and collagens are markers of the beginning of chondrogenesis. The transcription factor, SOX9, regulates the expression of some of these matrix proteins and is considered essential for chondrogenesis throughout many different steps (Long, 2013).

The chondrocytes undergo proliferation which invariably drives the outward linear growth. Chondrocytes within the center begin to mature and eventually become hypertrophic. This stage of chondrogenesis, early hypertrophic, is marked by the secretion of collagen type X alpha 1 (Col10a1) which occurs in tandem with loss of Sox5, Sox6, Sox9, Col2a1 and aggrecan expression (Kozhemyakina, 2015). After more growth, the late hypertrophic chondrocyte region forms, which begins to express matrix metalloproteinase 13, loses Col10a1 expression, and allows for vascular invasion within the hypertrophic cartilage that brings with it osteoprogenitor cells. These osteoprogenitor cells differentiate into osteoblasts that begin to create the primary ossification site (Long, 2013). With this, a series of zones form in which the maturation of chondrocytes appears to linearly progresses through. From epiphysis (articular end) to diaphysis (midshaft), the zones are proliferation, hypertrophy and bone formation (Long, 2013). The epiphysis is the end of the bone and is found within what is called the growth plate, in which a secondary ossification center will arise and separate the growth plate from the ends of the bone (Kozhemyakina, 2015).

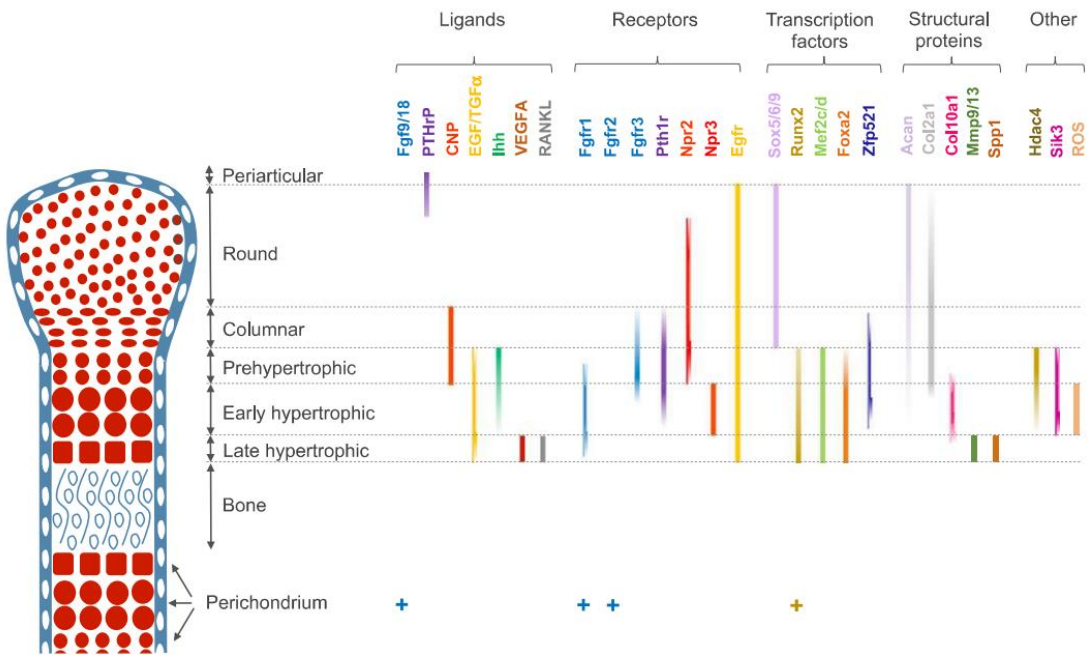

Figure 6: Overview of gene expression within endochondral ossification (Kozhemyakina, 2015).

This distinction of zones means that within each area there are specific proteins, ligands, and transcription factors being produced (Figure 6). For the timing and coordination of this growth to occur, there are a series of signaling pathways within the cells that allow for communication between each other and their environment. This communication, through signaling pathways, can go on to alter gene expression, which leads to differences in cellular make up between the different zones in endochondral ossification.

Signaling Pathways

Cell signaling pathways are complex networks of molecular interactions that govern communication between and within cells. These pathways are how cells interact

with their environment and allow them to execute a wide variety of functions necessary for life. Specific ligands (e.g. hormones, neurotransmitters, growth factors) bind to receptors and are necessary for the activation of certain pathways. Deviation from the usual control of signaling pathways has been linked to a variety of diseases such as cancer, Alzheimer's disease, and Parkinson's disease (Kim, 2009).

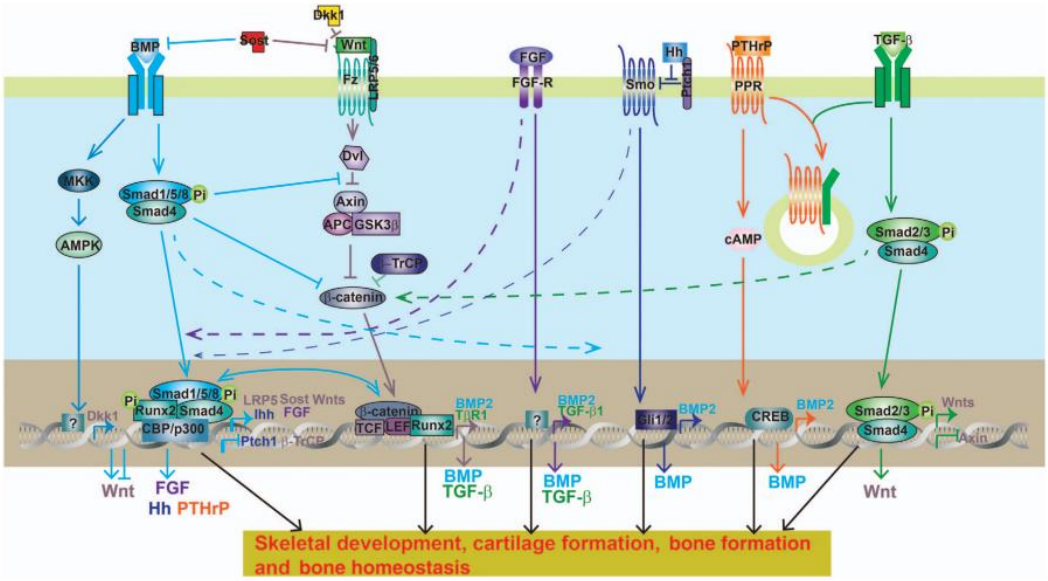

Figure 7: The amount of crosstalk between the various signaling pathways necessary for proper bone formation and growth (Wu, 2016).

There are multiple signaling pathways that are vital for the proper growth and maintenance of bones (Figure 7). These other pathways include but are not limited to: BMP, FGF, Notch, Hedgehog, and Wnt (Wu, 2016). The primary focus within the Carlone laboratory is are the BMP and FGF signaling pathways.

Bone Morphogenic Protein

One of the main signaling pathways for bone formation and growth is the Bone Morphogenetic Protein (BMP) signaling pathway. The BMP signaling pathway is a part of the transforming growth factor-β (TGF-β) superfamily. This superfamily act through a heteromeric receptor complex: made up of a type I and type II receptor at the surface of the cell. The signaling process begins with the BMP ligand binding to the receptor, which then initiates a cascade that goes onto influence gene expression and differentiation of mesenchymal stem cells into either osteoblast or chondrocytes (Wu, 2016). The BMP signaling pathway is essential for the early stages of mesenchymal stem cell condensation by helping maintain Sox9 expression. This pathway is also important for later stages with the induction of collagen type II and X (Retting, 2009).

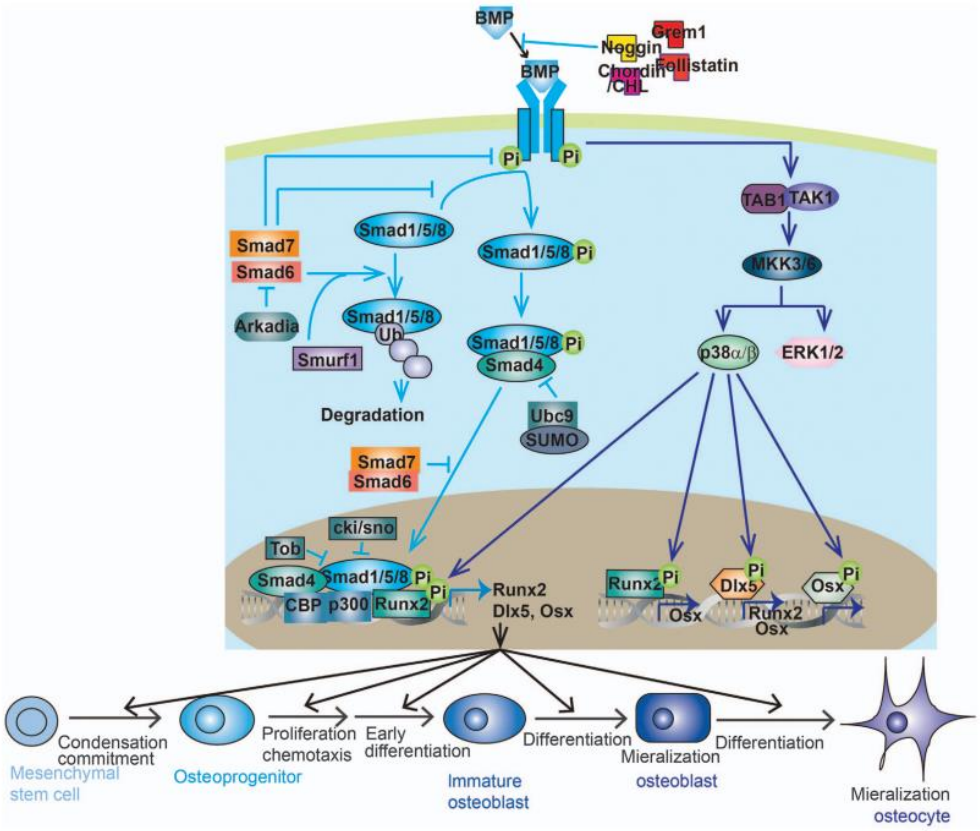

Figure 8: BMP signaling in bone development (Wu, 2016).

There are two primary paths that follow after BMP binds to the receptors: canonical Smad-dependent pathway and non-canonical Smad-independent pathway (Figure 8). These two pathways exert their effect specifically on transcription factors like Runx2, which is necessary for osteoblast differentiation (Wu, 2016). The Carlone laboratory focuses on the canonical Smad-dependent pathway and its effects on bone growth and formation.

Canonical Smad-dependent pathway begins with BMP binding to type II receptors which then form a heteromeric complex with type I receptors. Type II receptors go onto then phosphorylate the type 1 receptors (Wu, 2016). The activated type I receptor

then phosphorylates receptor Smads (R-Smads), specifically Smad 1, 5, and 8. These R-Smads are then activated and form a complex with common-mediator Smad: Smad 4. Together the complex translocate to the nucleus and goes on to regulate gene expression by interacting with other transcription factors, repressors, or activators, which invariably leads to various cellular responses (Long, 2013). R-Smads contain two main domains that are connected mainly by a linker region. The R-Smads are phosphorylated on the C-terminus by the type I BMP receptors, whereas, phosphorylation in the linker region marks R-Smads for degradation by Smurfs, an E3 ubiquitin ligase (Retting, 2009).

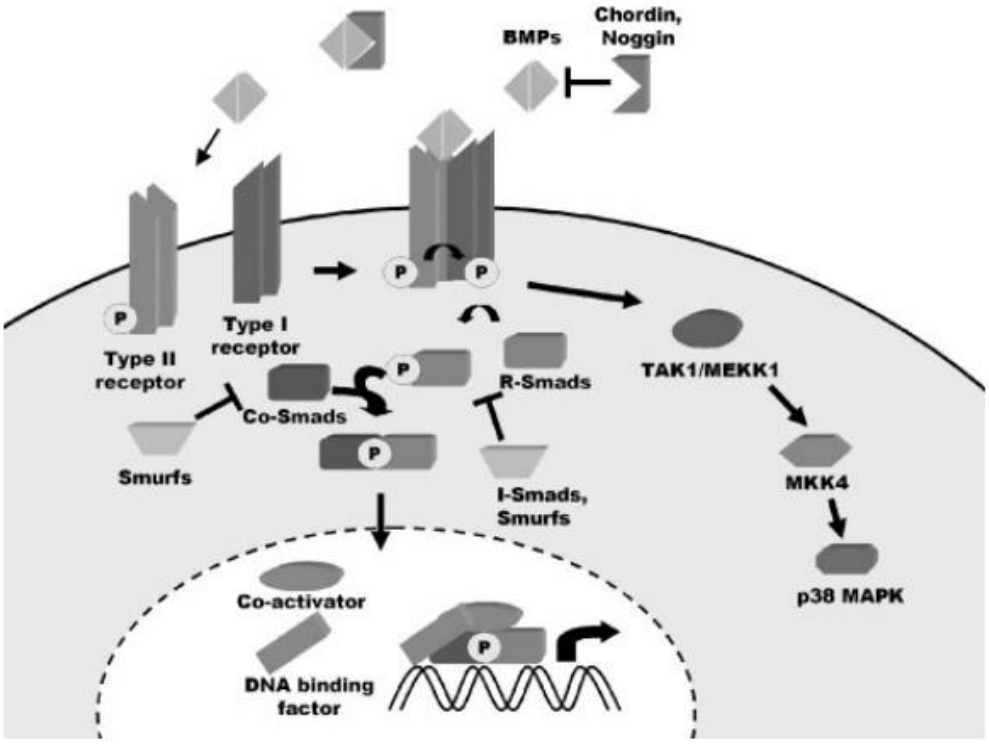

Figure 9: Simplified image of BMP canonical and non-canonical signaling pathway (Yoon, 2004).

There are various points of regulation within the canonical Smad-dependent pathway of BMP. The main form of regulation for this pathway is through inhibitory

Smads (I-Smads) with the help of Smurfs (Figure 9). I-Smads, specifically Smad 6 and 7, function as a negative feedback regulator making sure that this pathway is properly controlled with no excessive response. I-Smads inhibit signaling in multiple different ways: inhibiting R-Smad from translocating to the nucleus, competitively binding to type I receptor, or recruiting Smurf1/2 (Wu, 2016).

Fibroblast Growth Factor

Another important signaling pathway within bone development is that of the Fibroblast Growth Factor (FGF) family. FGF signaling starts with the binding of a particular ligand to a specific cell surface tyrosine kinase receptor, which then phosphorylates various other signaling modules: mitogen-activated protein kinase (MAPK), signal transducer and activation of transcription 1, phosphoinositide 3-kinase, and protein kinase C (Long, 2013). The Carlone lab primarily focuses on the MAPK signaling pathway, in particular that of the ERK MAPK pathway.

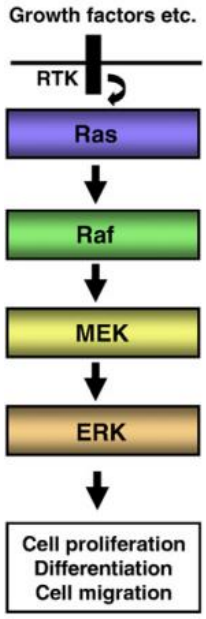

Figure 10: One of the MAPK signaling pathways, ERK MAPK, utilized for cellular differentiation and proliferation (Kim, 2015)

The ERK MAPK pathway begins with a wide-variety of growth factors or cytokines binding to the FGF receptor (FGFR) which activates the small G protein Ras (Figure 10). Following which, Ras phosphorylates Raf which then goes onto phosphorylate MEK1 and MEK2. Lastly, MEK1 and MEK2 phosphorylate extracellular signal-regulated kinase (ERK) 1 and 2. ERK1 and ERK2 then phosphorylate different nuclear and cytoplasmic proteins that affect gene expression within the cells (Matsushita, 2012).

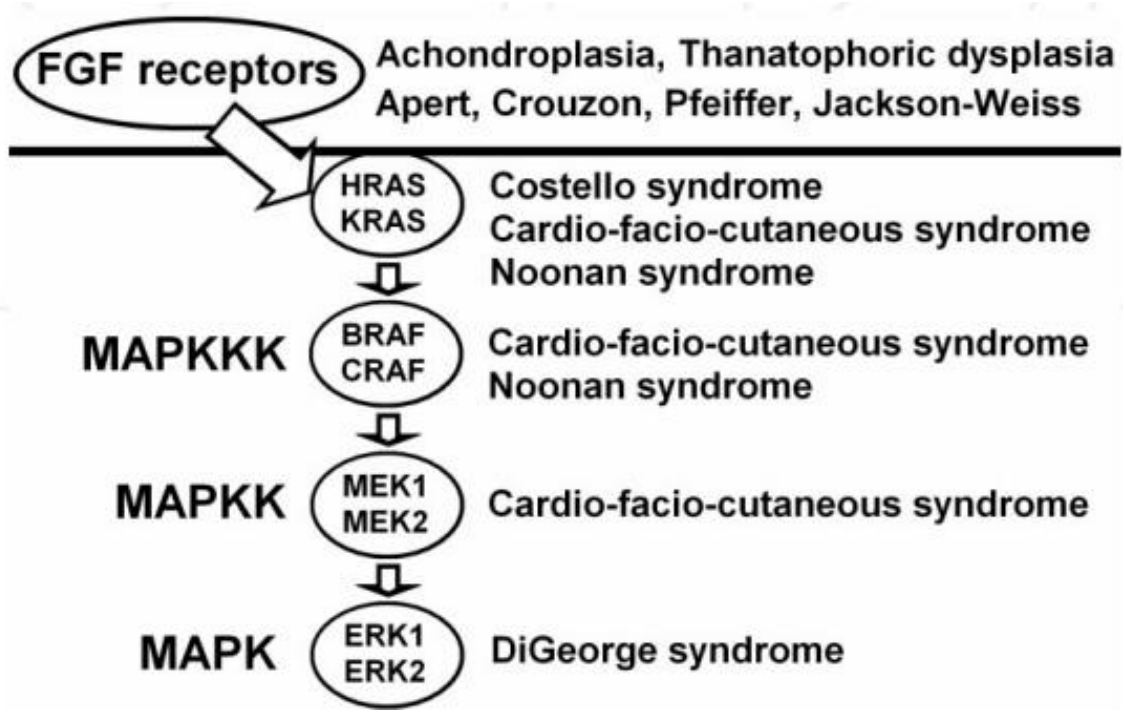

Figure 11: List of disorders for the ERK MAPK pathway and the enzyme or receptor the mutation happens in (Matsushita, 2012).

FGF signaling pathway is important for bone development and differentiation, and mutations in either the receptors or any of the enzymes can lead to various musculoskeletal disorders. Figure 11 lists the disorders that arise from mutations in the enzymes or receptors of the ERK/MAPK pathway (Matsushita, 2012). Not only do mutations lead to musculoskeletal disorders, but mutations of enzymes in the ERK MAPK pathways have also been found in numerous cancers (Roskoski, 2012).

ERK proteins 1 and 2 have a similar kinase structure to other kinases but have a specific N- and C- terminal extension that allows for signaling. These extensions are called the amino-terminal lobe and carboxyterminal lobe. Between these two is the catalytic site, such that when ERK1/2 is catalytically active these lobes are brought closer together, while their distance expands when inactive. To activate ERK1/2, two residues,

sequence Thr-Glu-Tyr, in the activation lip must be phosphorylated by MEK (Roskoski, 2012).

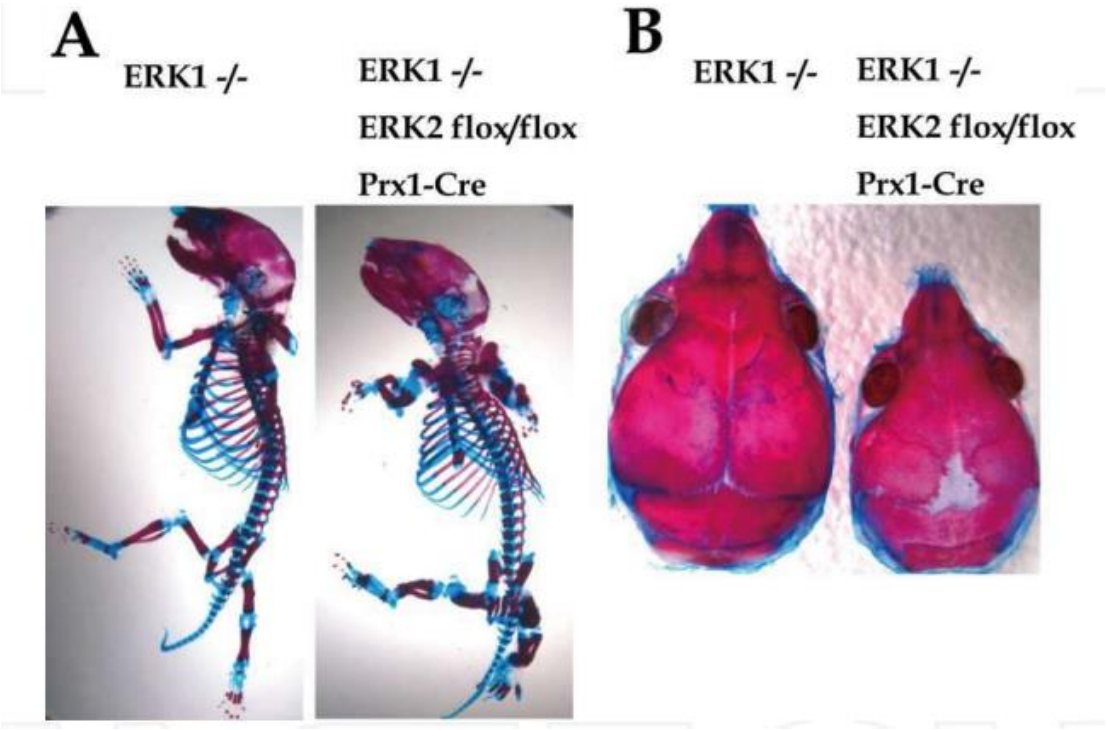

Figure 12: Skeletal preparation with alizarin red and alcian blue in ERK1/2 knockout mice. Deformities in long bones and delayed closure of cranial suture (Matsushita, 2012).

ERK1/2 have been found to play a vital role in bone development and differentiation. When ERK is inactivated in mice, there are severe limb deformities and defects such as decreased long bone growth (Figure 12). This may be because inactivation of ERK1/2 also causes an expansion of the terminally differentiated chondrocytes as well as a decrease in osteoclast formation (Matsushita, 2009). When MEK1 is constitutively active in mesenchymal cells, there is an increase in bone formation and ultimately an inhibition of cartilage formation (Matsushita, 2012).

For bone growth, FGF and BMP appear to have an antagonistic relationship. If BMP was the gas on the car, FGF would be the brakes. This antagonistic relationship is primarily mediated by ERK proteins. ERK proteins have been found to phosphorylate Smads on their linker region which ultimately leads to degradation (Retting, 2009). This degradation is done by Smurf1/2 which binds to R-Smads only when the linker region has been phosphorylated. Smurf binding leads to proteasome-mediated degradation (Sapkota, 2007).

Proposed Schematic

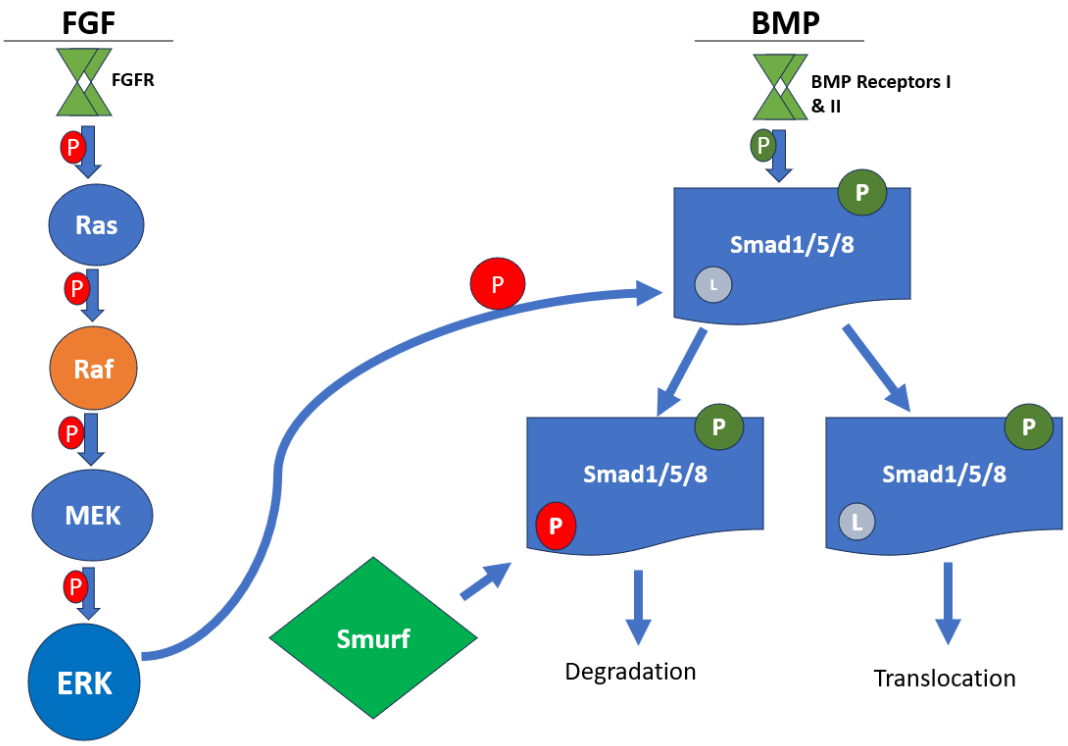

Figure 13: Proposed Schematic for interaction between FGF and BMP. Circles with the letter "P" represent phosphorylation. The circle "L" on the Smad 1/5/8 is to represent the linker region.

The proposed schematic (Figure 13) is an attempt at creating a visual representation of how FGF and BMP signaling pathways communicate with each other.

On the left is the FGF signaling pathway in which the FGF receptor begins the phosphorylation cascade, ending with phosphorylated ERK. On the right is the BMP signaling pathway, in which Smad proteins are phosphorylated by BMP receptors type I and phosphorylated ERK proteins. Following the color of the phosphorylation, the Smad if phosphorylated by ERK, will be targeted for degradation by Smurf1/2. If Smad is phosphorylated by the BMP receptor, then it will go onto translocate to the nucleus where it will affect gene expression.

AIMS

To gain greater insights into CFP1's regulation of BMP and FGF interactions, the aims of this thesis are:

Aim 1: To determine if inhibition of ERK MAPK signaling restores phosphorylated Smad levels in CFP1 knockout cells.

Aim 2: To explore ERK protein and phosphorylation levels within different cell lines representing different stages of skeletal cell differentiation.

METHODS AND MATERIALS

Cell Culture

In the Carlone lab there are three different cell lines that the experiments are conducted with that come from various stages in endochondral ossification or intramembranous ossification. For endochondral ossification, the cell line mouse limb bud (MLB) is a chondrocyte progenitor cell line that through CRISPR-Cas-9 have had CFP1 knocked out. For intramembranous ossification, the W20 cells are bone mesenchymal stem cells that will become osteoblasts, while MC3T3 cells are a more differentiated osteoprogenitor cell. Both have had CFP1 knocked out with CRISPR-Cas-9.

Cells were unfrozen after being stored in 90% fetal bovine serum (FBS) and 10% dimethyl sulfoxide in liquid nitrogen. The cells are plated in media (DMEM with 10% FBS, 1% penicillin/streptomycin) to grow. Cells are seeded into 6 or 12 well plates at a concentration of 5×10^5 cells/well. Cells were incubated until confluency reached up to 70-90%. After cells were treated with inhibitor or had reach set confluency, the media is aspirated and the plates are stored in -80 degrees Celsius until ready for protein extraction.

Inhibitor PD98059 and U0126 were resuspended into a 10mM stock solution using dimethyl sulfoxide. The stock solution was subsequently diluted (using dimethyl sulfoxide) to a concentration of 1mM. This concentration was used to generate the various doses of inhibitor used in the studies. To ensure that any changes observed with the inhibitor was not due to the vehicle (dimethyl sulfoxide), a similar volume of

dimethyl sulfoxide, corresponding to the highest dose of inhibitor, was added to cells and referred to as 0µM.

Protein Extraction

For protein extraction, first the cell culture plate was taken out of the freezer and 100µL of RIPA buffer was added to the plate. The RIPA buffer contained: anti-phosphatase, anti-protease and sodium dodecyl sulfate. After which, the plate was shaken and the cell lysis was collected into a microfuge tube. The samples were then sonicated and boiled. Following boiling, samples were centrifuged and the protein-containing supernatant collected into a final microfuge tube.

Protein concentrations were determined using a BCA standard curve. A 96 well plate was used with 2µL of the samples in duplicates. Pierce BCA protein assay reagent was made with A: B=50:1 and 100µL was added to each well. The plate was then incubated for 30 minutes in 37 degrees Celsius and then measured for absorbance (562 nm) on the BioTek Epoch plate reader. The readings were then used to calculate protein concentrations using a BCA template.

Western Blots

A sample buffer was made with 2-mercethanol and Laemmli buffer (1:5) and then 20uL was added to the samples. The samples were then boiled for 1 minute, centrifuged and into Bio-Rad gels. Running buffer made up of tris-base (0.025 M), glycine (0.092

M), and sodium dodecyl sulfate (0.1%) and H_2O was used. The samples undergo running at 80V for 20 minutes and then 100V for 50 minutes with Bio-Rad PowerPac.

After running, polyvinylidene fluoride membranes were soaked in methanol for 5 minutes before transferring. The gels are then moved and a sandwich is made with a cassette, fiber pad, filter paper, gel and membrane. Transfer buffer was made up of glycine (0.192 M), tris-base (0.025), and 20% of methanol with H_2O. The transfer is done in an ice bucket at 100V for an hour and a half with Bio-Rad PowerPac.

After transferring, the membrane was placed in with blocking buffer which contained 1% bovine serum albumin (BSA) in phosphate buffered saline with 0.1% Tween (PBST) with added 1:100 NaN_3 (final concentration of 2%). The membranes were blocked for 1-2 hours on a shaker at room temperature.

The membranes were then incubated with primary antibody overnight. For the Carlone lab, there are multiple primary antibodies used. Two different antibodies were used to determine phosphorylated and total levels of ERK. Both of these were used at a dilution of 1:1000 in BSA. Similarly, antibodies for total Smad and phosphorylated Smad (pSmad) at a dilution of 1:1000 in BSA. Lastly to have an internal control for protein loading, an antibody to Vinculin was used at a dilution of 1:20,000 in BSA.

After overnight incubation, the membranes were washed in PBST and then incubated in secondary antibodies for 1.5-3 hours. These antibodies were used at similar dilution as the primary antibodies but diluted in PBST. For Vinculin an anti-mouse antibody was used, while the others were anti-rabbit.

After secondary incubation, the membranes were washed three times with PBST for 5 minutes. Then to image the membranes, a mixture of signaling reagent was made 1:1 between A: B. The membranes were incubated in the signaling mixture for 1-3 minutes and then taken to the imager.

To reuse the membranes, the blots were washed with PBST 3 times for 5 minutes and then incubated in stripping buffer for 15 minutes to remove antibodies. The membranes were washed again with PBST 6 times for 5 minutes incubated in BSA for 30 minutes to block and then new primary antibody was added.

After Western blot images were collected, ImageJ was utilized to quantify the intensity of each protein band. Calculations involved were phosphorylated Smad divided by Smad, Smad divided by Vinculin, and phosphorylated ERK (pERK) divided by ERK. To determine statistical significance, two-tailed Student's *t*-test was used to compare groups of two and one-way ANOVA with Turkey's post-hoc analysis was used for multiple pairwise comparisons. Significance was set at $p < 0.05$.

RESULTS

To begin investigating Aim 1, an inhibitor (PD98059) for MEK1 phosphorylation of ERK was used in cell culture. The MLB cell line was seeded and grown in a 12 well plate as triplicates and then were incubated with 0 µM, 1 µM, or 10 µM of the inhibitor for 24 and 48 hours. The cells were then collected and a Western blot analysis was conducted for Vinculin, pSmad, Smad for both the 24 and 48 hours (Figure 14). ERK and phosphorylated ERK levels were also assessed but only at the 48 hours timepoint.

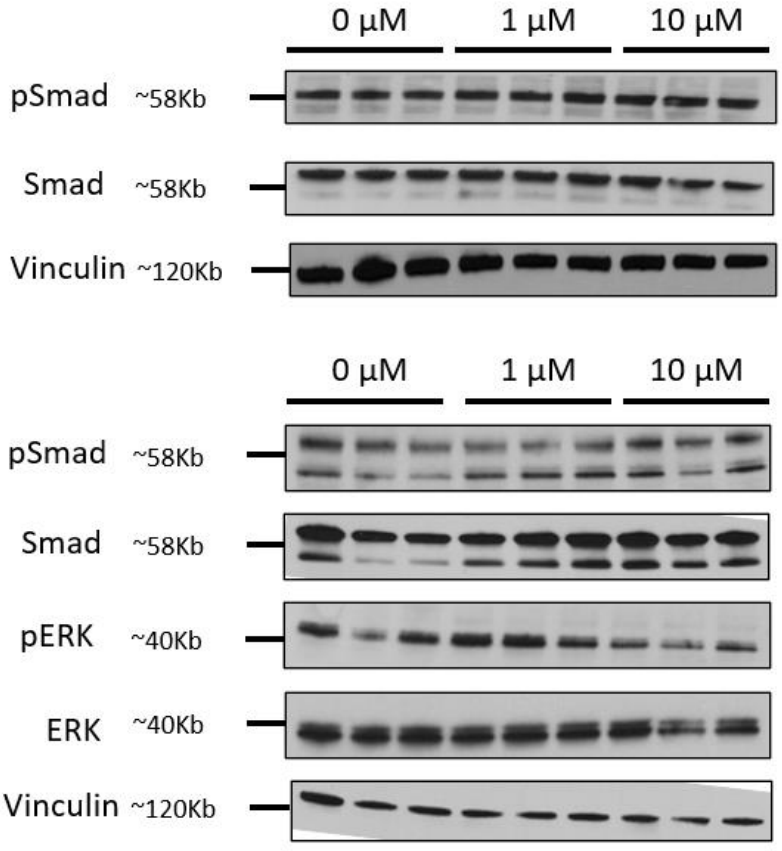

Figure 14: (Top): 24 hours exposure to inhibitor PD98059 at dosage of 0 µM, 1 µM, or 10 µM. Name of antibodies imaged on the left with estimated molecular weight of molecule. (Bottom): 48 hours exposure of the inhibitor.

Westerns were then quantified using ImageJ. Smad levels were controlled for with Vinculin and ratios were collected for phosphorylated/total protein levels for both Smad and ERK.

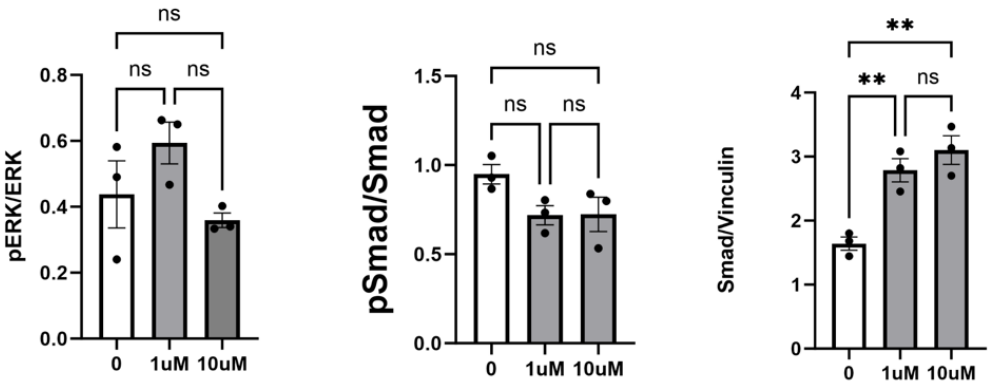

Figure 15: Ordinary one-way ANOVA presentation for 48 hours exposure of MLB cells to inhibitor PD98059

There was no statistical significance (ns) for pERK/ERK levels in an ordinary one-way ANOVA for either dosage to control or each other (Figure 15). There were similar results for pSmad/Smad levels. Statistical significance (** = $p < 0.01$) was found for Smad 1/Vinculin levels when compared to vehicle for both doses. No difference was found between the different dosages.

The experiment was repeated but with a higher dosage of inhibitor (0 µM, 10 µM, 25 µM). The experiment was only conducted at an exposure time of 48 hours and similar western images as last experiment were collected.

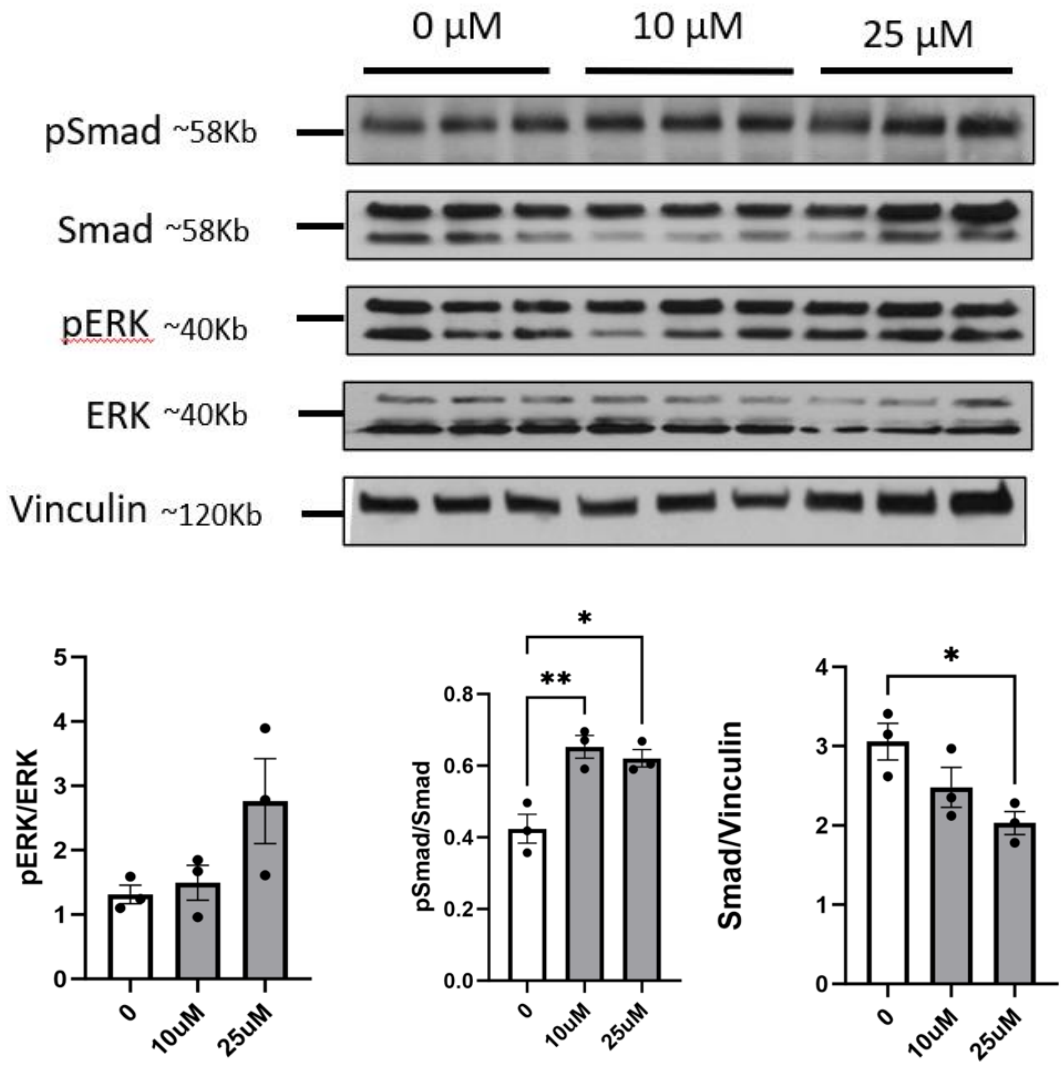

Figure 16: (Top): Western images for higher dosage of inhibitor with antibodies imaged on the left. (Bottom): Ordinary one-way ANOVA conducted between 0 µM and doses (10 µM and 25 µM).

No statistical significance was found for pERK/ERK levels between the 0 µM and 10 µM or 25 µM doses (Figure 16). Cells treated with 25 µM of inhibitor having large variation. Statistical significance (** = $p < 0.01$) was found in pSmad/Smad 1 levels between 0 µM and 10 µM. Statistical significance (* = $p < 0.05$) appeared between vehicle and 25 µM dosage for pSmad/Smad levels as well as Smad/Vinculin levels.

Due to lack of results showing decreased pERK/ERK levels using the PD98059 inhibitor, another MEK1 inhibitor, U0126, was utilized. Following similar preparations as previous experiments, cells were plated in triplicates for 24 or 48 hours of exposure to varying dosage (0 µM, 1 µM, and 10 µM) of inhibitor. Western blot images were collected and similar calculations were conducted as before. There was no statistical significance for either 24 hours or 48 hours for any of the previously calculated levels (Figure 17).

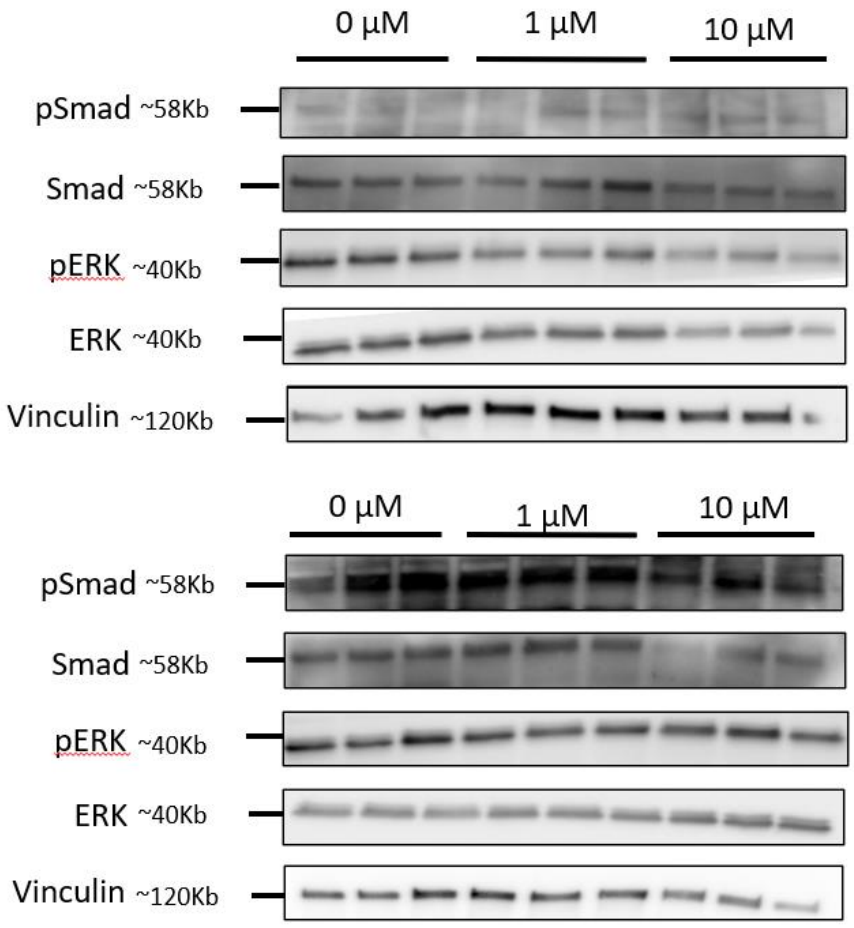

Figure 17: (Top): Western images of 24 hours of incubation with inhibitor U0126. (Bottom): Western images of 48 hours of incubation with inhibitor U0126.

The next experiment was to increase the number of samples at doses 0 and 10 µM at 24 hours of incubation with inhibitor U0126 and see if there is any change in pSmad/Smad levels. There were 6 samples at each dosage. Cells were grown and protein extracted for western imaging.

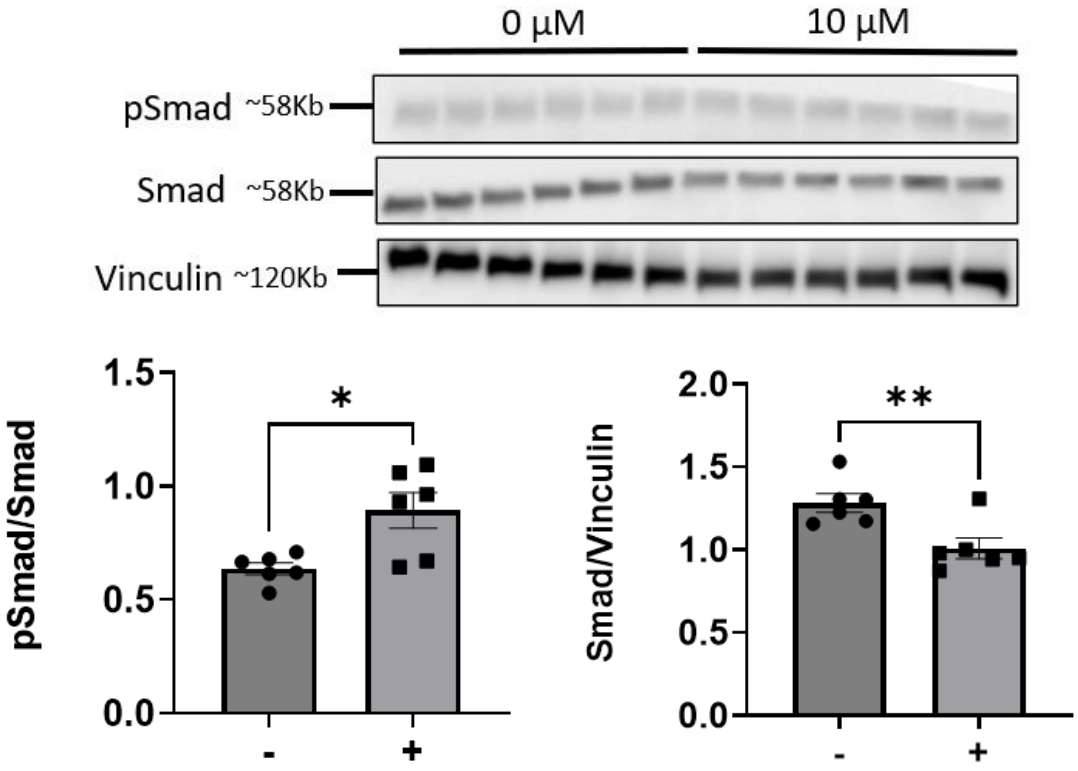

Figure 18: Increasing the number of samples (Top): Western images of pSmad/Smad levels. (Bottom): Student t-test comparing 0 µM (-) and 10 µM (+)

Statistical significance (* = $p < 0.05$) was found between the doses for pSmad/Smad levels and statistical significance (** = $p < 0.01$) was found for Smad/Vinculin levels with 0 µM showing a higher concentration of Smad protein levels (Figure 18).

On another plate, triplicates were seeded for doses with 0 and 10 µM of inhibitor U0126 at an exposure of 1 hour. This plate was to see if an hour exposure would decrease pERK levels. Cells were collected and protein extracted for Western blot analysis. Statistical significance (* = $p < 0.05$) was found between the two different doses showing that the inhibitor stopped the phosphorylation of ERK (Figure 19).

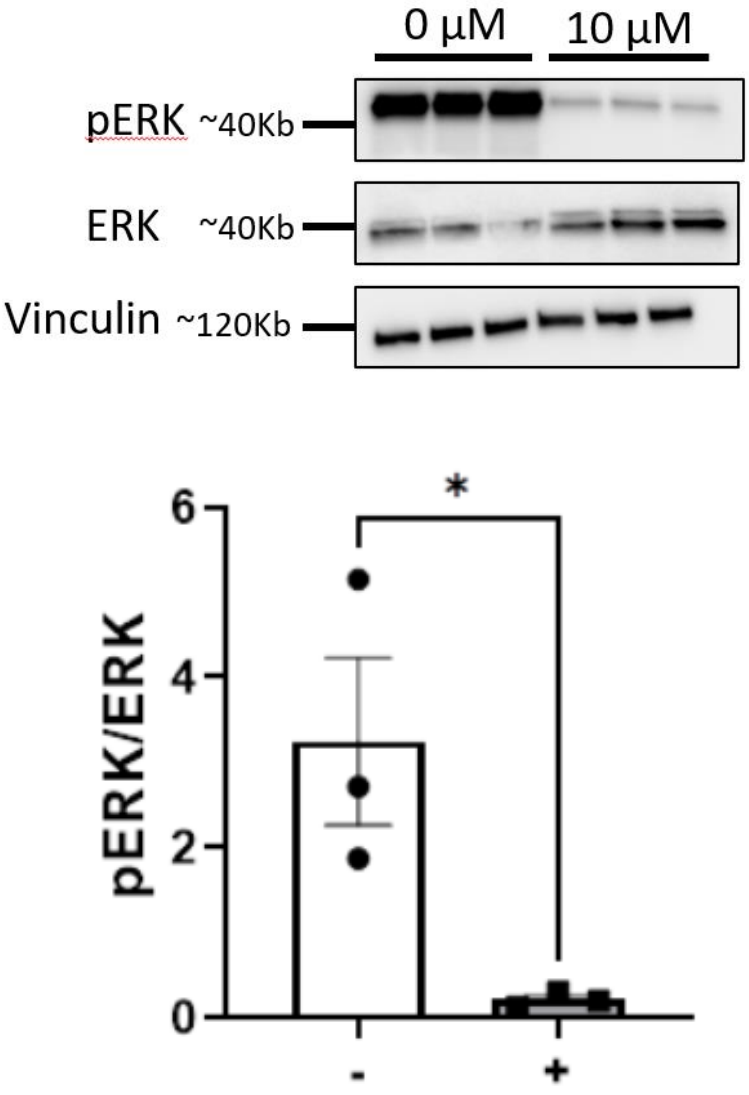

Figure 19: (Top): Western images of cells treated for an hour with inhibitor U0126. (Bottom): Student t-test comparing 0 µM (-) and 10 µM (+)

For Aim 2, similar experiments were conducted as Aim 1 but with cell lines MC3T3 and W20, which represent a different pathway to bone formation. Control (V1) and CFP1 knockout (G1-3) MC3T3 cells were plated and grown in triplicates in a 6 well plate and protein was then collected. Western blot analysis for Vinculin, pERK, and ERK was performed and statistical analysis was conducted using Student *t*-test comparing control and knockout. Statistical significance (* = $p < 0.05$) was found between knockout and control cells for pERK/ERK levels (Figure 20).

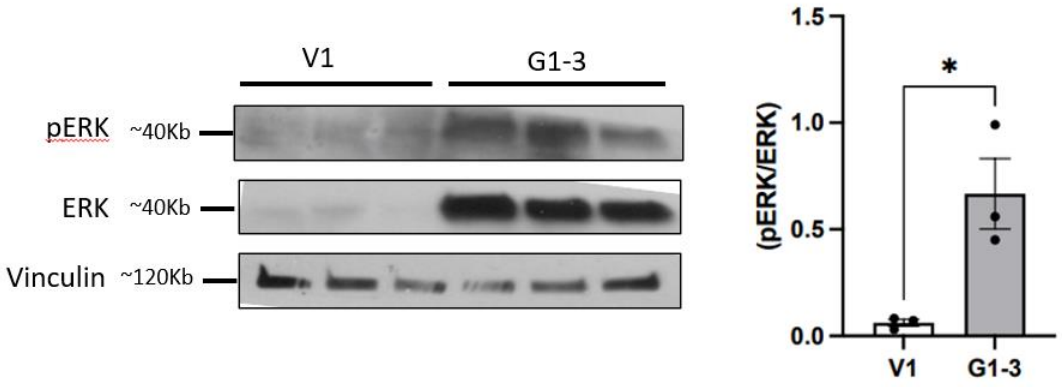

Figure 20: (Left): Western images showing triplicates for both control (V1) and CFP1 knockout (G1-3) MC3T3 cells for Vinculin, pERK, and ERK. (Right): Student t-test for pERK/ERK levels between vector and guide

This experiment was repeated with the MC3T3 cells plated in triplicates, as well as W20 cells: control (V1) and CFP1 knockout cells (G9). Both cell lines were grown to confluency and protein was collected for Western blot analysis.

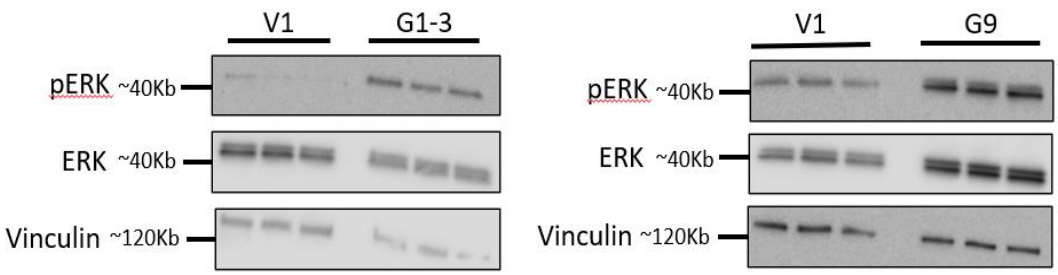

Figure 21: (Left): Western images of MC3T3 cells control (V1) and CFP1 knockout (G1-3) for pERK, ERK, and Vinculin (Right): Western images of W20 cells control (V1) and CFP1 knockout (G9) for pERK, ERK, and Vinculin.

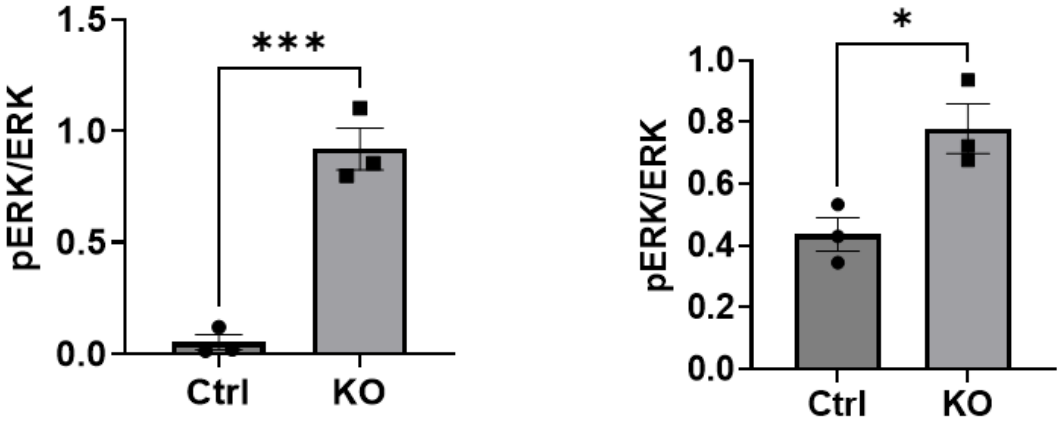

Figure 22: (Left): Student t-test for MC3T3 control (Ctrl) versus CFP1 knockout (KO). (Right): Student t-test for W20 control (Ctrl) versus CFP1 knockout (KO).

Similar results as previous experiment with control MC3T3 cells exhibiting low pERK levels compared to knockout cells with a statistical significance of $p < 0.001$ (***). A similar increase in pERK levels was detected in knockout W20 cells compared to controls (* = $p < 0.05$). Taken together, these data confirm CFP1 is a negative regulator of ERK MAPK signaling in skeletal cells.

DISCUSSION

Aim 1

In exploring the effects of ERK on BMP signaling, the first step was to inhibit the phosphorylation of ERK and see if pSmad levels were restored. To begin, the inhibitor PD98059 was used in order to stop the phosphorylation of ERK in the hope that with less pERK there would be less phosphorylated Smad in its linker region and thus less degradation. Figure 14 and 15 show that the inhibitor did not decrease the amount of total pERK, but at 48 hours of exposure at both doses the amount of Smad 1 proteins increased. So, although the total amount of pSmad hasn't been increased, the total amount of Smad 1 proteins that can be phosphorylated has increased.

Since there was no direct decrease in pERK levels, a high dosage of inhibitor PD98059 was used. Forty-eight hours of exposure was continued from last experiment. pSmad/Smad levels were higher for both the dosage with 10 μM being particularly significant. This is a more observed difference from the previous experiment. It is of interest that even though 10 μM was used for both experiments, there is an inconsistency of results with the pSmad levels. The variation in Smad levels can be possibly explained by an increased level of toxicity at 25 μM, in which that could put the cells in a stressful environment and thus alter their signaling pathways and how they communicate. The lack of an effect on pERK/ERK levels by the inhibitor could be indicative that the inhibitor may not be working.

When the number of samples were increased, there was shown to be an increase in pSmad levels. At the same time there appears to be a decrease in overall Smad protein

levels, which is the opposite of previous findings. The inhibitor U0126 lowered pERK levels when the cells are only exposed for one hour, but with the increase in exposure time this difference does not appear as large.

While the inhibitor appears to blunt ERK phosphorylation, with short exposure, with longer exposure it appears as though the cells shift under the new conditions and re-establish a new norm. Possibly with initial exposure to the inhibitor, pERK is decreased and then only after being exposed for long enough does the cell start to increase production of Smad 1 protein levels. This can then go on to increase the ratio of phosphorylated to not Smad proteins, but only after there has been an increase in production of Smad 1.

Aim 2

With the preliminary data analyzing pERK and ERK protein levels, the other cell lines demonstrate CFP1's possible regulatory effect on FGF and BMP signaling. Given that in both the W20 and MC3T3 cells there is a great increase in pERK/ERK levels when CFP1 is absent, it appears that CFP1 has some inhibitory role for the FGF signaling pathway. Whether BMP signaling is also altered in these cells, similar to MLB cells, remains to be assessed. But given BMP's vast role in skeletogenesis it is highly likely.

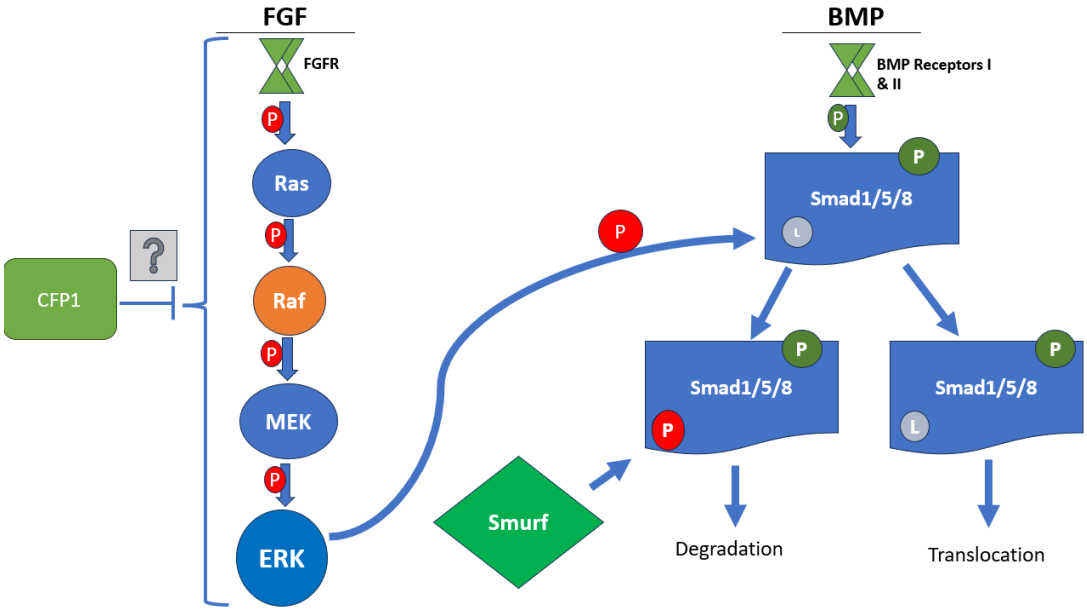

Figure 23: Proposed schematic with CFP1 involvement

Although it is unclear where in the ERK MAPK pathway CFP1 acts, there is still a clear contrast between control and knockout. CFP1 appears to inhibit some part of the phosphorylation cascade or above.

Limitations

There was great variation in the inhibitory results with the different inhibitors. This is not uncommon as studying the ERK MAPK pathway is known to be quite variable in its results (Kim, 2010). This variability is possibly due to cell confluency. The cells were collected in the range of 70-90% confluency, but there may be variation in cellular make up depending on the confluency of cells as with higher confluency there may be more emphasis on the FGF pathway rather than BMP. In contrast, at a lower

confluency there may be less signaling for the FGF pathway and rather more focus on the BMP pathway.

Future Research

In the future, there needs to be more clarity on exactly what protein CFP1 is inhibiting within the FGF phosphorylation cascade. These experiments would be looking at the phosphorylation and protein levels of the enzymes above ERK, such as MEK1, Raf, or Ras. There may be a protein above ERK that does not demonstrate difference between control and CFP1 knockout. Another route would be to study the gene expression levels by qPCR analysis for some of FGFs target genes and note lack of difference between control and knockout.

Another possible route is to study the non-canonical pathway of BMP signaling. This pathway is of particular note because it goes on to phosphorylate ERK1/2 but through TAK1 phosphorylation. The non-canonical pathway may be also activated and could possibly explain the variation in pERK levels as the BMP signaling pathway may self-regulate between phosphorylating Smad or TAK1, which could help mitigate Smad proteins effect on gene expression.

Lastly, confluency of the cells could be studied as a possible avenue to limit the variability in results as well as gain insights on the influence confluency has on results. To begin, the pERK/ERK levels at various confluency should be analyzed to confirm the variation of results. From there, it would be to study variation within BMP signaling with pSmad and Smad protein levels.

CONCLUSION

Through investigating the influence of ERK1/2 on BMP signaling, there is a confirmation of the antagonistic relationship between FGF and BMP signaling. It appears that when CFP1 is knocked out there is a removal of inhibition on the ERK MAPK signaling pathway, which then goes on to more robustly inhibit the BMP pathway. By inhibiting the ERK MAPK pathway, there shows a slight restoration of BMP signaling. These findings may help elucidate the interactions between these two pathways and ultimately how CFP1 regulates them during bone development and growth.

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