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Loss of Dot1L function in cartilage impairs skeletal growth

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Loss of Dot1L Function in Cartilage Impairs Skeletal Growth

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Physiology and Neurobiology Honors Thesis
University of Connecticut

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ABSTRACT

Cartilage is essential for the proper formation, growth, and function of long bones. In the process of endochondral ossification, cartilage serves a pre-cursor to long bone. Many studies have primarily focused on the transcription factors, growth factors, and signaling molecules that regulate this process. In recent years, epigenetic mechanisms have surfaced as key regulatory elements of gene expression. Some important epigenetic mechanisms include DNA methylation and post-translational modification of histone residues, such as histone acetylation and histone methylation. Our research focuses on a unique histone methyltransferases known as disruptor of telomeric silencing 1-like (Dot1L) and its role in embryonic and postnatal skeletal development. Genetic variants of Dot1L have been associated with skeletal growth, human height, and cartilage degeneration. Dot1L's enzymatic activity catalyzes the methylation of lysine residue 79 in histone H3 and is associated with gene activation. There remains a gap in our knowledge regarding epigenetic regulators of cartilage development and endochondral ossification. For this reason, our research aims at understanding the role of Dot1L in regulating endochondral ossification in growth plate cartilage and its implications in skeletal growth. We utilize a Cre recombinase system to genetically inactivate the Dot1L gene in embryonic and postnatal cartilage and perform phenotypic and histological analyses and comparisons of the skeletons of control mice and mice expressing loss of Dot1L in cartilage. Results show that loss of Dot1L in mice expressing the Cre enzyme impairs skeletal growth. In addition, preliminary histological analyses of growth plate cartilage from mice with postnatal loss of Dot1L in cartilage suggest that Dot1L is important for controlling chondrocyte differentiation.

INTRODUCTION

Cartilage plays an important role in the formation, growth, and function of embryonic development and post-natal growth, acting as a precursor to long bones. The majority of cartilage structure and function is regulated by chondrocytes, which are cells responsible for secreting the extracellular matrix (ECM). Chondrocytes of articular (joint) cartilage have distinct functions from chondrocytes of the growth plate. Growth plate chondrocytes regulate the growth of epiphyseal plates. Chondrocytes are metabolically active cells, since they are responsible for producing ECM components and are derived from mesenchymal stem cells (MSCs) (Akkiraju and Nohe, 2015). The replacement of cartilage by bone occurs in the growth plate, a transient structure, and is regulated by key regulatory and transcription factors, which will be discussed in greater depth later on.

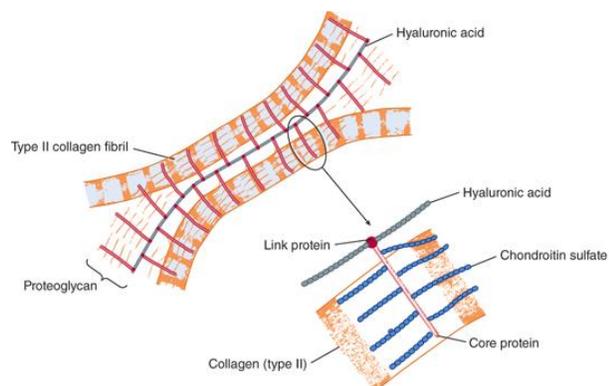
The process by which cartilage in the growth plate of longitudinal bones is gradually replaced by bone is known as endochondral ossification. Chondrocytes undergo proliferation, hypertrophy, and apoptosis. The cartilaginous scaffold provides a surface for attachment of osteoblasts, which replace cartilage with bone. Because of the importance of chondrocyte differentiation in the formation and longitudinal growth of the skeleton, disruption in the regulation of endochondral ossification will result in stunted growth or bone deformities. We therefore look at key regulatory factors to better understand the regulation of endochondral ossification in growth plate cartilage.

Components and origin of cartilage

Classified as a connective tissue found throughout the body, cartilage functions in connecting and supporting bones. It has unique properties such as tensile stretch and mechanical properties that can be attributed to the organization of the extracellular matrix (ECM) (Horkay,

2012). The ECM is made up 10-25% collagen, 5-15% proteoglycans, 70-80% water, and smaller concentrations of noncollagenous proteins and glycoproteins (Horkay, 2012). Collagens are macromolecules responsible for stabilizing the matrix by providing tensile and shear strength to the tissue (Akkiraju and Nohe, 2015). Specifically, type II collagen is the major fibrous component responsible for providing cartilage's shear strength. Imbedded within the fibrous network of collagen are charged proteoglycans. The major proteoglycan in cartilage is the aggrecan molecule, which interacts with hyaluronic acid and contributes to the proper functioning of articular cartilage by endowing it with load-bearing properties (Horkay, 2012). As previously mentioned, chondrocytes are the cells responsible for producing components of the ECM. Chondrocytes facilitate fluid exchange within the matrix to maintain proper cartilage functions. This fluid exchange is important for absorption of nutrients and removal of waste products (Horkay, 2012). Chondrocytes are the cells that undergo endochondral ossification during bone growth and repair. Despite the ability of cartilage to undergo repair, it is limited in its capacity to repair damage due to its avascular structure, which causes slow healing, and the restrictions placed by the fibrous structure of the collagenous network.

Figure 1- Components of Cartilage



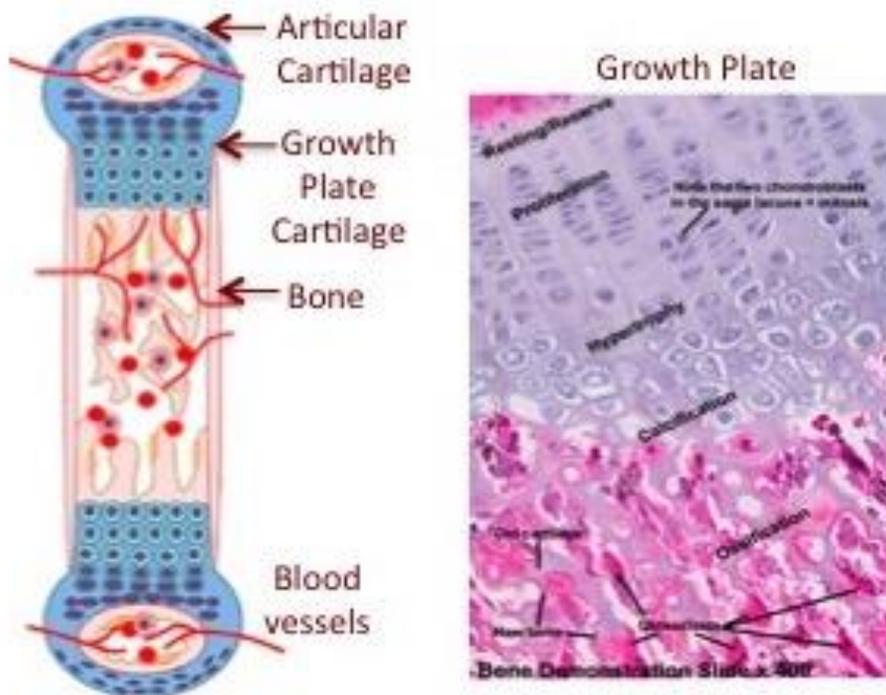
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The diagram above shows the components of cartilage, specifically the interactions between Type II collagen fibril, proteoglycans and hyaluronic acid (HA).

There are different types of cartilage specialized to perform different functions in the body. For example, articular cartilage functions to protect the ends of long bones and joints by providing a lubricated surface to reduce friction during movements. On the other hand, growth plate cartilage provides the template for long bone formation. This paper puts an emphasis on studying the regulatory factors that dictate endochondral ossification, which occurs in the growth plate. During this process, the ECM of chondrocytes transition from deposits of collagen 2 (Col2) to collagen X (ColX), which serves as the base for osteoblast invasion (Li et al., 2015). To better understand the regulation of growth plate cartilage, we first look at the organization of it.

Transient structure of the growth plate and regulatory transcriptional factors

In longitudinal bones, the growth plate is a transient structure where chondrocytes undergo endochondral ossification. Chondrocytes undergo successive stages of proliferation, hypertrophy (cell maturation), and apoptosis. Thus, cartilage is important since it provides a stable template for bone formation and is the source of longitudinal bone growth (Mackie et al., 2008). The transient nature of the growth plate consists of several histologically and functionally distinct zones within the structure, each of which is regulated by transcription factors that regulate chondrocyte gene expression under extracellular factors.

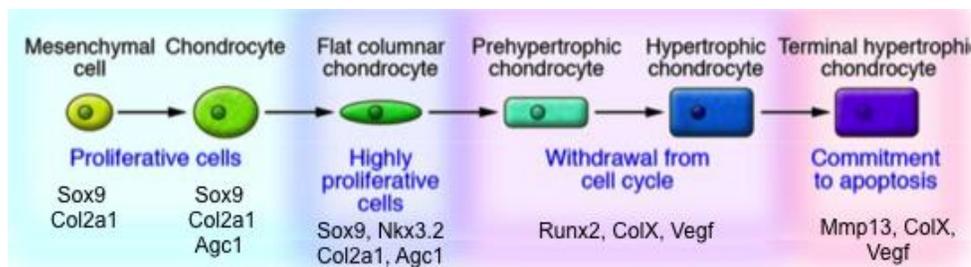
Figure 2- Organization of Growth Plate Cartilage

Growth plate organization depicts different zones of chondrocyte differentiation, starting from resting zone all the way to zone of ossification.

The resting zone is furthest away from the ossification front, where cartilage is replaced by bone. Stem-cell like precursors lie in the resting zone. These cells are capable of self-renewal and, more importantly, gives rise to proliferative chondrocytes (Luis et al., 2016). In the zone of proliferation, proliferating cells become flattened as they begin to arrange themselves into multicellular clusters (Mackie et al., 2008). The proliferative zone secretes factors that regulate a number of genes. Sex-determining region Y (SRY)-box 9, or Sox9, is the master transcriptional factor of cartilage. Sox9 is expressed by proliferative cells and is responsible for inducing the expression of one of the major components of cartilage matrix, type II collagen (Col2a) (Mackie, et al. 2008). Col2a and Agreccan (Agc) are also found in proliferating chondrocytes. These genes are specific to cartilage. Col2a encodes for type II collagen, a major component of the

chondrocyte's extracellular matrix (Mackie et al., 2008). As the cells undergo maturation, they begin secreting type X collagen (ColX), vascular endothelial growth factor (VegF), and matrix metalloproteinase (Mmp13). ColX codes for the matrix of hypertrophic chondrocytes and VegF is a growth factor that facilitates vascular invasion of growth cartilage in the hypertrophic zone. Since cartilage is an avascular structure, VegF is an important growth factor whose secretion is characteristic of hypertrophic chondrocytes. In addition, Runt-related transcription factor 2 (Runx2) is a transcription factor necessary for hypertrophy of chondrocytes (Nishimura et al., 2018). As the chondrocytes undergo apoptosis, their matrix remains and becomes a template for the formation of new bone. Mmp13 is one of the enzymes that degrade the cartilaginous matrix, making room for osteoblasts to replace it with bone and allow blood vessels to come in (Nishimura et al., 2018).

Figure 3- Model of endochondral bone formation



Process of chondrocyte differentiation involves important markers of each stage of differentiation. Markers of each stage are shown. Sox9 is a key regulatory transcription factor in the differentiation of cartilage. Col2a1 codes for type II collagen, which forms the matrix of prehypertrophic chondrocytes. As chondrocytes proliferate, their matrix is replaced by ColX.

As depicted by the schematic above, the process of endochondral ossification is tightly regulated by a series of transcription and growth factors. If disrupted, stunted growth or bone deformities may present, since bone will no longer be able to replace cartilage, resulting in no longitudinal growth of bone. Although there are many studies on the transcriptional regulation of

this complex process, there is little known about the epigenetic controls. Thus, this research further explores the epigenetic controls of endochondral ossification in the growth plate *in vivo*.

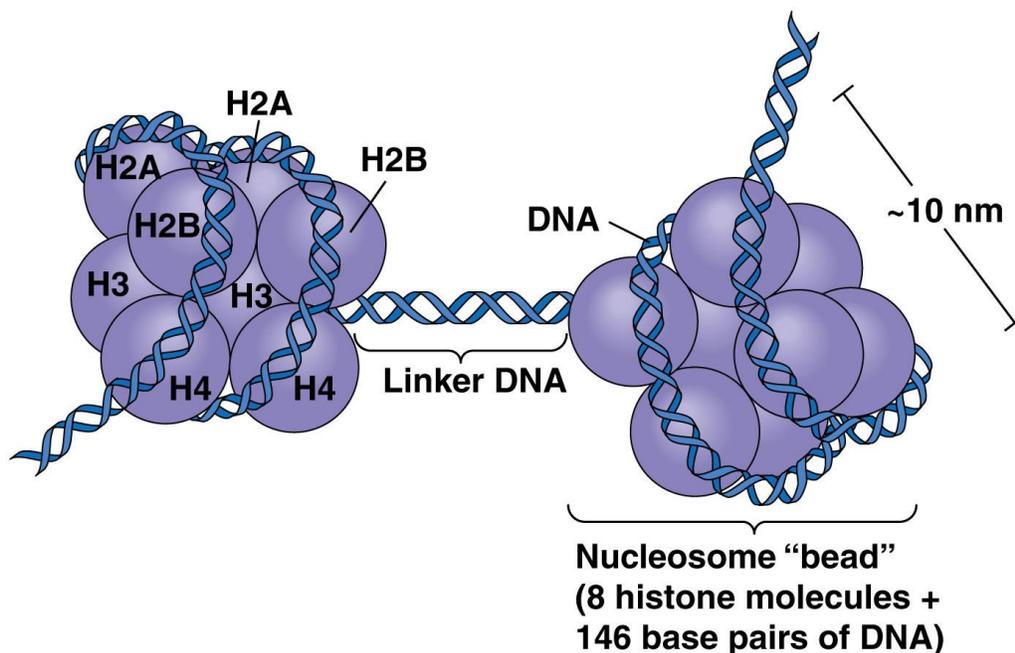
Epigenetic controls as a higher level of regulation for gene expression

Epigenetic controls refer to control of gene expression outside of altering the genetic sequence (Barter et al., 2012). Modifications involve the regulated additions of chemical groups to DNA (DNA methylation) and histones (histone modification). Together, these mechanisms allow the cell to adapt to environmental changes and are often inherited during cell division (Barter et al., 2012). Specific enzymes, called writers, dictate these modifications. Effector proteins, or readers, recognize the modifications. Finally, enzymes called erasers are responsible for removing the marks of modification, rendering epigenetic marks reversible (Nicholson et al., 2015). Writers, readers, and erasers work together to control post-translational modifications (PTMs).

One of the most well documented epigenetic regulatory systems is DNA methylation, which is conserved between species (Hata, 2015). DNA methylation involves the addition of a methyl group to DNA at CpG dinucleotides on the carbon 5 position of cytosine (Hata, 2015). This type of methylation converts cytosine to 5-methylcytosine and is regulated by DNA methyltransferases. Methylation at the promoter region of genes prevents certain transcription factors from binding to binding elements, which represses transcription (Hata, 2015). Several studies have been done to assess the relationship between DNA methylation and cartilage degeneration, including osteoarthritis (OA). In patients exhibiting OA, damaged chondrocytes show an increased level of methylation in the promoter region of the gene Sox9 (the master transcriptional regulator of cartilage) (Hata, 2015). Hypermethylation of this gene is associated with decreased Sox9 expression, and subsequently impaired chondrogenesis.

Histones modification, another illustration of PTM, is an example of a chromatin modification. Chromatin refers to the protein/DNA complex, in which the histone is the special protein that encompasses the DNA double helix (Annunziato, 2008). The basic repeating structural unit of chromatin is the nucleosome, which is comprised of eight histone proteins and about 146 base pairs of DNA (Van Holde, 1988; Wolffe; 1999). Nucleosomes are made up of a dimer of each of the following histones: H2A, H2B, H3, and H4 (Annunziato, 2008). The histones come together to form an octamer, which binds and wraps about 146 bp of DNA (Annunziato, 2008). Histone H1 wraps around another 20 base pairs, which forms two full turns around the octamer, forming the complete structure known as a chromatosome (Annunziato, 2008). Histones are positively charged proteins that bind tightly to the negatively charged phosphate groups of the phosphate-sugar backbone of DNA.

Figure 4- Overview of histone complex



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Histones H2A, H2B, H3, and H4 come together to form an octamer. Histone complex binds and wraps 146 bp of DNA (Pearson Education, 2012).

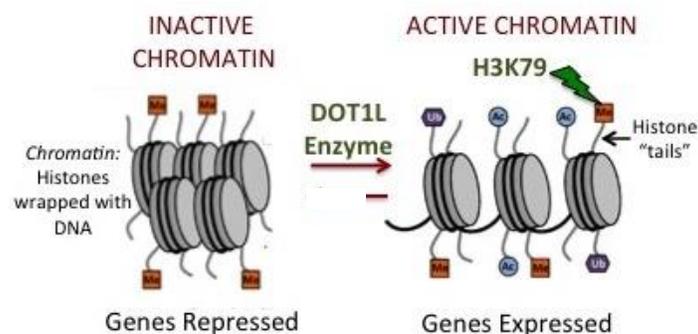
Histones undergo post-translational modifications, such as acetylation, phosphorylation, ubiquitination, and methylation. Acetylation of histones occurs when an acetyl group is added to positively charged lysine residues on the N-tail of histone H3 (Hata, 2015), which neutralizes the tail and reduces the binding affinity of histone H3 with negatively charged DNA. The decrease in the electrostatic bond between the histone and the DNA allows for easy access of transcriptional proteins to the transcription sites (Hata, 2015). Thus, histone acetylation is associated with increased transcriptional activity. In contrast, histone deacetylation inhibits transcription. The enzymes responsible for histone acetylation and histone deacetylation are known as histone acetylases (HATs) and histone deacetylases (HDACs), respectively. From a chondrogenic standpoint, HAT enzymes are known to positively regulate chondrocyte gene expression through interactions with Sox9 (Hata, 2015). Conversely, studies on HDACs reveal that inhibition of HDACs protects cartilage from destruction by increasing *Col2a* gene expression in articular chondrocytes (Hata, 2015). Histone acetylation plays an important role in maintaining chondrocyte differentiation, as the enzymes responsible for this PTM interacts with the Sox9 regulatory gene and *Col2a1* gene, which codes for matrix collagen.

Another common protein modification is methylation. This paper focuses primarily on regulation of histone methylation and its effects on growth plate chondrocytes. Histone methylation plays a key role in the formation of active and inactive genomic regions, and is associated with both transcriptional activation and silencing. Whereas acetylation adds acetyl groups to lysine residues, methylation adds methyl groups to these residues but does not affect their charge. Depending on the target site, histone methylation/demethylation is associated with transcriptional repression or activation. Moreover, there are three types of methyl-lysine molecular structures: mono methyl (me1), di-methyl (me2), and tri-methyl (me3). After

methylation/demethylation, the structure of chromatin can take two forms: a condensed transcriptionally “inactive” form and an open “active” form. Despite the fact that the significance of histone methylation has been studied in many cellular events including differentiation and proliferation, there remains a gap in our knowledge of the regulatory mechanisms of histone methylation during chondrocyte differentiation.

We discuss one enzyme in particular, disruptor of telomeric silencing like-1 or Dot1L, known for its methylation of lysine-79 on histone 3. Genetic variants of the epigenetic regulator Dot1L has been associated with skeletal growth and cartilage degeneration (Betancourt 2012 and Monteagudo 2017). Methylation of residue 79 in the globular domain of histone 3 lysine by Dot1L establishes a more relaxed state in chromatin that results in transcriptional activation. Dot1L is unique because it is the only enzyme that adds methyl groups to lysine-79 on histone 3. By studying the effects of Dot1L deletion in cartilage, we are able to better understand the cellular and molecular mechanisms involved in skeletal differentiation and growth.

Figure 5- Dot1L-mediated epigenetic regulation



Dimethylation of lysine 79 in histone 3 (H3K79me₂) by Dot1L is associated with open chromatin and gene activation.

Regulatory functions of the histone methyltransferase DOT1L

Dot1L plays a critical role in regulating gene transcription, development, cell cycle progression, somatic reprogramming, and DNA damage repair (Wong et al., 2015). This enzyme

is responsible for the methylation of H3K79. Mono- and di-methylation of H3K79 is associated with increased gene transcription, whereas tri-methylation results in gene repression (Wong et al., 2015). DOT1L is the homolog gene to DOT1 (disruptor of telomeric silencing), which was first identified in proteins whose over-expression lead to disruptions in telomeric silencing in yeast (Singer et al., 1998). DOT1L has been found in a range of species, including mammals such as humans and mice. It functions in adding methyl groups in a non-progressive manner, which means it is forced to dissociate and reassociate to H3K79 (Wong et al., 2015).

As mentioned before, DOT1L is involved in many cell processes throughout the body. Studies on H3K79 methylation on human histones through the use of mass spectrometry shows that mono-methylation of H3K79 (H3K79me) is the most abundant form and is associated with active gene transcription sites (Wong et al., 2015). In contrast, H3K79me₃ is predominantly present in silent gene regions in comparison to active regions, which is why tri-methylation of H3K79 is associated with gene repression in human cells. H3K79me₃ has been linked to cell cycle regulation: arrested cells in the G₀ phase of the cell cycle show higher levels of H3K79me₃ (Wong et al., 2015). Inhibition of Dot1L correlates with enhanced reprogramming of cell types via silencing of lineage-specific programs of gene expression (Wong et al., 2015). In this manner, Dot1L inhibition yields pluripotent stem cell colonies. The wide range of functions of Dot1L contributes to its complexity. To add to this complexity, single nucleotide polymorphisms (SNPs) of Dot1L in humans have been linked to increased human height (Lango Allen, 2010). While inhibition of Dot1L and tissue-specific knockouts have a variety of consequences across the human genome, we are focused on the role of Dot1L in skeletal development and joint cartilage.

The role of Dot1L in skeletal growth and human height

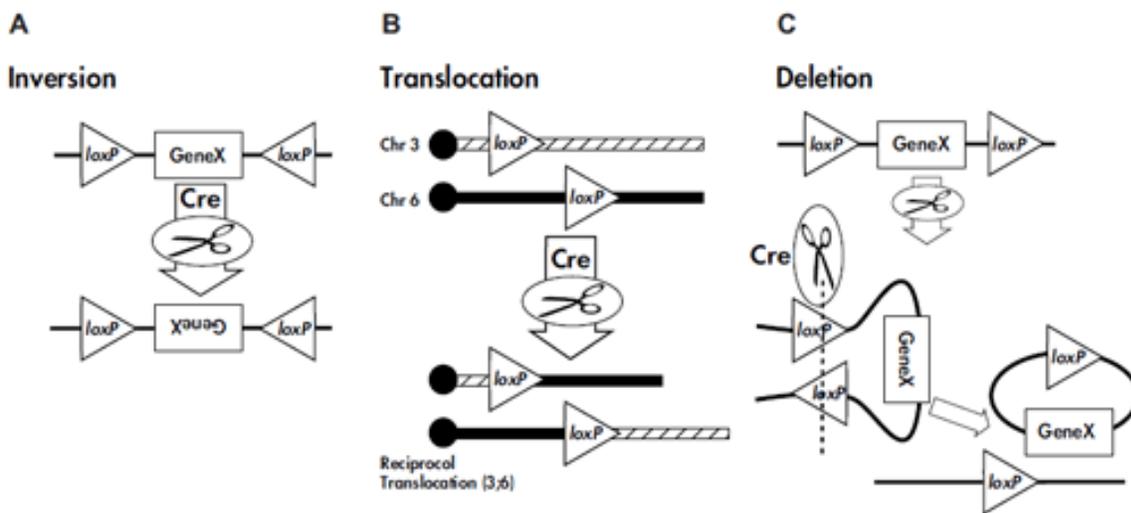
In a study done by Betancourt (2012), immunohistochemical staining of the DOT1L protein in mice highlights its role in chondrogenic differentiation and adult articular cartilage (Betancourt, 2012). Results show silencing of Dot1L inhibits chondrogenesis *in vitro*, because Dot1L knockdown reduces proteoglycan and collagen content, and subsequently mineralization during this process (Betancourt, 2012). Loss of Dot1L disrupts the molecular regulation of healthy chondrocytes and can lead to osteoarthritic in mice (Monteagudo et al., 2017). Furthermore, embryonic stem cells that are Dot1L-deficient present with a global loss of H3K79 methylation (Jones et al., 2008). Thus, we know that Dot1L and H3K79 methylation play important roles in heterochromatin formation and embryonic development.

H3K79 methylation is associated with transcriptional activation and is a critical transcriptional regulator (Kim et al., 2014). The loss of Dot1L results in reduced proliferation of cells (Kim et al., 2014), and in cartilage, loss of Dot1L disrupts cartilage homeostasis (Monteagudo et al., 2017). Studies link variations in the gene with increased human height (Jones et al. 2008 and Simeone and Alberti 2014). In a study by Simeone and Alberti (2014) on *Epigenetic heredity of human height*, genome-wide SNP analyses identified genomic variants associated with increased human heights. Among them are variants in Dot1L; polymorphisms in Dot1L are associated with taller pubertal stature and accelerated growth rate (Jones et al., 2008). Although Dot1L has been linked to skeletal growth and increased human height, the specific role of Dot1L in postnatal skeletal development remains obscure. Thus, we developed a mouse model where we specifically inactivate Dot1L in cartilage in order to examine its role *in vivo* in regulating endochondral ossification and skeletal growth.

Using cartilage specific Cre-recombinase to study the role of Dot1L in vivo

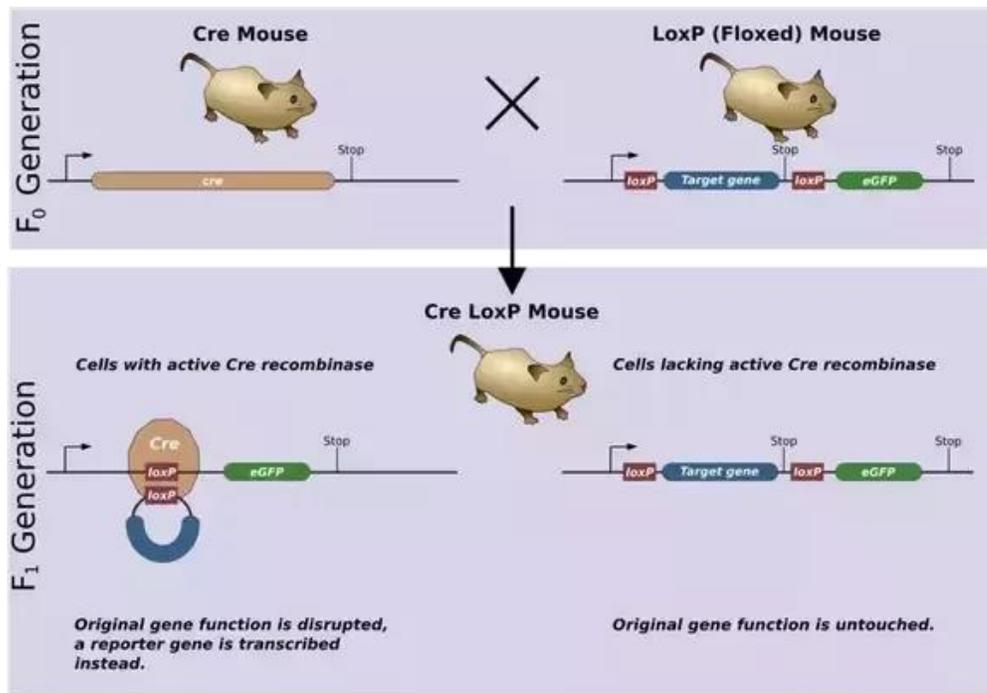
Our lab developed a mouse model of Dot1L loss of function to explore the physiological role of Dot1L in skeletal development. This model utilizes a Cre-recombinase system to specifically inactivate the expression of the Dot1L gene in cartilage in an inducible manner. Conditional inactivation of genes in the adult mouse has been plausible with the introduction of the Cre-loxP recombination system. Systematically deleting a gene would be lethal to an embryonic subject; the Cre-Lox system allows for temporal and tissue-specific control of gene deletion.

The Cre-loxP system involves a conditional allele and a Cre driver, in which the conditional allele is the targeted gene located between loxP sites near critical exons or regulatory elements of the gene of interest (Henry et al., 2009). The enzyme Cre can recognize specific sites, called loxP sites, on a gene sequence. LoxP sites are 34 base pairs long and include a spacer region 8 base pairs long as well as two flanking palindromic sequences that are 13 bp long each (de la Pena, 2015). Expression of the Cre protein leads to recognition of LoxP sites. The Cre protein binds to the 13 bp palindromic regions in the site to form a dimer. This dimer binds to another dimer in another loxP site, forming a tetramer. The target sequence is cleaved by the Cre protein. The orientation of the loxP sequence is important, since it determines whether the Cre will produce a deletion, inversion, or translocation of the target sequence. If the two loxP sequences go in the same direction, they produce a deletion. If the sites are oriented in opposite directions, they produce an inversion. Finally, if the loxP sites are located in different positions on the chromosome, Cre recombination produces a translocation.

Figure 6- Directionality of LoxP Sequences

Different orientations of LoxP sequences and cleaving produce different results. (A) Two LoxP sites oriented in opposite directions produce an inversion. (B) LoxP sites on different positions on the same chromosome produce a translocation. (C) LoxP sites go in opposite directions produce deletion (de la Pena, 2015).

We are able to engineer transgenic mice that have loss of Dot1L function in cartilage by using a cartilage specific Cre, called Aggrecan Cre, as the Cre driver. As mentioned before, Aggrecan is a major ECM protein in growth plate cartilage and thus specifically found in cartilage (Henry et al. 2009). To delete Dot1L in cartilage, we use an Aggrecan-Cre (Agc-Cre) mouse. These transgenic mice are generated such that the expression of Cre is controlled by tissue specific regulatory elements, in this case cartilage. Thus, when aggrecan is expressed, so is the Cre. To control for the timing of gene activation (i.e. embryonically or postnatally), we create a polypeptide that combines the Cre recombinase with a mutant ligand-binding domain of estrogen receptor CreERT2 (Henry et al., 2009). Administration of tamoxifen, an estrogen analog, activates the Cre recombinase by translocating the Cre from the cytosol into the nucleus. Excision of floxed sites and subsequently inactivation of Dot1L becomes dependent upon the administration of tamoxifen in Agc-Cre mice expressing the CreERT2 transgene.

Figure 7- Model for generation of Cre LoxP Mouse

The schematic above explains how to generate a Cre LoxP mouse. For this research, we use an aggrecan-Cre mouse to direct cartilage specific inactivation of *Dot1L*. Mice with *Dot1L* are floxed, as the LoxP sites are located on either side of exon 5 of the DNA sequence. These mice are then bred with aggrecan-Cre mice and produce two different genotypes of mice. Cells with active Cre recombinase will have a disruption in the target gene's function. Cells lacking active Cre recombinase will have the original gene function intact (de la Pena, 2015)

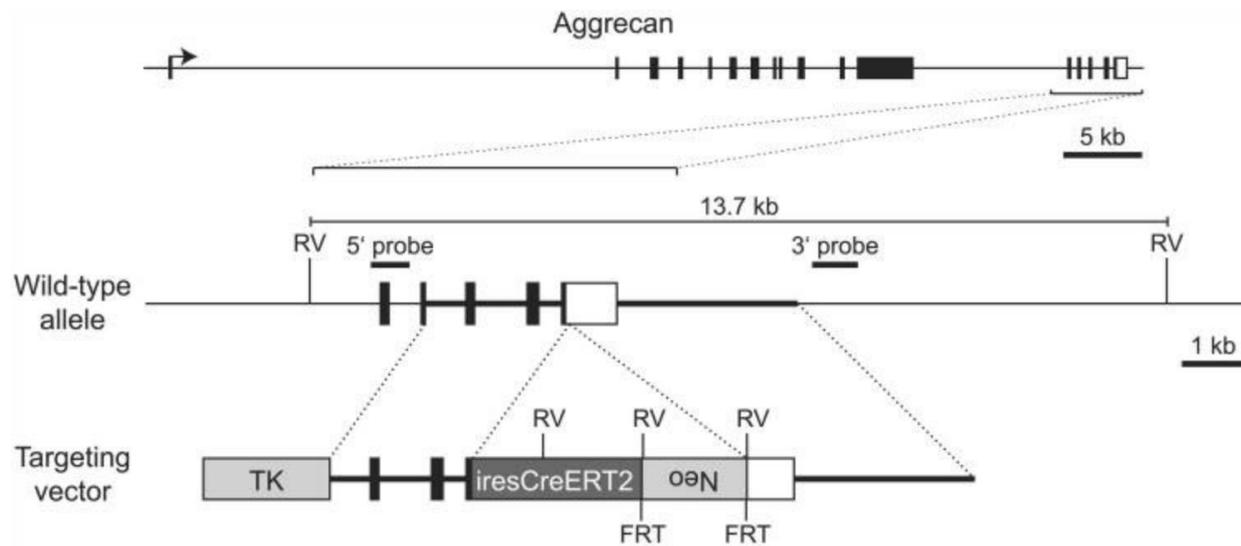
Objective

This research focuses on the Dot1L histone methyltransferase, and its physiological effects on postnatal skeletal development. Development of a mouse model of Dot1L loss of function enables us to explore the physiological role of Dot1L in skeletal development. This model system utilizes a Cre-recombinase system to specifically inactivate the expression of the Dot1L gene in cartilage in an inducible manner. The objective is to validate a mouse model that utilizes the Cre recombinase system to genetically inactivate the Dot1L gene in postnatal and embryonic cartilage. In addition, phenotypic and histological analyses will be performed on mice skeletons with postnatal and embryonic loss of Dot1L expression in cartilage. We will determine if the activity of Dot1L methyltransferase *in vivo* in cartilage is necessary for the regulation of endochondral ossification and skeletal growth. Studying the role of Dot1L in regulating endochondral ossification is essential to revealing the mechanism by which it epigenetically modifies cartilage-specific genes. Loss of Dot1L function in cartilage is hypothesized to impair postnatal skeletal growth.

MATERIALS & METHODS

Dot1L conditional loss of function model and DNA isolation from ear notches

Dot1L transgenic mice harboring flox (FL) sites flanking exon 5 were used (See Figure 8 below) (Henry et al. 2009). Aggrecan-CreERT2 mice express Cre recombinase in the nucleus of chondrocytes only when injected with tamoxifen, an estrogen analog. Tamoxifen was administered in two ways, either indirectly or directly. Embryonic mice were given tamoxifen through moms by intraperitoneal injections (IP) on day 15. Postnatally, tamoxifen was injected directly into the mice on P5. The amount of tamoxifen administered to each mouse was 1.5mg/10 grams of body weight. Mice were weighed and amount of tamoxifen delivered was determined using the aforementioned ratio. Dot1L floxed mice were mated with mice carrying the tamoxifen inducible cartilage specific Cre recombinase gene. Mice were genotyped using genomic DNA harvested from ear biopsies. Ear notches were stored at -20°C in 1mL test tubes. To isolate the DNA, 50uL of Alkaline Lysis Reagent (25mL water, 62.5uL of 10N sodium hydroxide, 10uL of 0.5M disodium EDTA) was added to the tube. Ear fragments were completely submerged in the reagent. Tubes were then incubated at 94°C for at least one hour, then centrifuged briefly to ensure accumulation of fluid at the bottom of the tube. Next, 50uL of Neutralization Reagent (24mL of water, 1mL of 1M Tris-HCl) was added to each tube using a pipette. Contents were mixed using the tip of the pipette to break up the tissue. Contents were then used for PCR reaction.

Figure 8- Gene-targeting strategy for the Aggrecan-CreERT2

(Top) *Wild-type allele*: panel depicts the genomic structure of the mouse *aggrecan* gene. (Bottom) *Targeting vector*: an inducible *Cre recombinase* structure (*CreERT2*) is integrated in the 3' untranslated region (UTR) of the endogenous mouse *aggrecan* gene. The *CreERT2* is preceded by a 5' internal ribosome entry sequence (*IRES*) and is inserted 63 nucleotides downstream of the stop codon of the *aggrecan* gene. (Henry et al., 2009)

Polymerase Chain Reaction (PCR)

DNA isolated from ear notches were stored at -20°C . DNA was amplified through polymerase chain reaction (PCR). Polymerase chain reaction is a method used to synthesize new strands of DNA complementary to the targeted strand of DNA (template strand). Primers (15-30 bp long) are used to target specific DNA sequences that can be further amplified. PCR was performed using primer pairs that specifically amplify (i) wild type *Dot1L* (200 bp); (ii) floxed *Dot1L* allele (300 bp); (iii) wild type *aggrecan* (299 bp); and (iv) *aggrecan-Cre* (200 bp) were used¹⁰. The following PCR conditions were used for *Dot1L*: incubation for 5 min at 94°C , followed by 35 amplification cycles of 30 s of denaturation at 94°C followed by 30 s of annealing-elongation at 56°C and 30 s at 72°C . 2% agarose gel migration of amplicons were

performed to determine the specificity of the PCR. Primers used for PCR analysis of Dot1L (Integrated DNA Technologies) are listed below:

| PRIMER | PRIMER SEQUENCE (5' → 3') |
|---------------------------------|----------------------------------|
| Dot1L-F_floxed (FORWARD PRIMER) | CAG AGG ATG ACC TGT TTG TCG |
| Dot1L-R_floxed (REVERSE PRIMER) | CAT CCA CTT CCT GAA CTC TCG |

The following PCR conditions were used for AgcCre: incubation for 2 min at 94 °C, followed by 10 amplification cycles of 30 s of denaturation at 94 °C followed by 15 s of annealing-elongation at 65°C (-0.5°C per cycle decrease) and 10 s at 68°C. For the next 28 cycles: 15 s of denaturation at 94 °C followed by 15 s of annealing-elongation at 60°C and 10 s at 72°C. Samples underwent 2 min in 72°C and were held at 4°C until ready for gel electrophoresis. 2% agarose gel migration of amplicons were performed to determine the specificity of the PCR. Primers used for PCR analysis of AgcCre (Integrated DNA Technologies) are listed below:

| PRIMER | PRIMER SEQUENCE (5' → 3') |
|-----------------------------|----------------------------------|
| Aggrecan-F (FORWARD PRIMER) | GTG GAG AGT CTT CTG GCA TTA C |
| Aggrecan-R (REVERSE PRIMER) | CAC TGA GTT CCA CAG ATC CTA AC |

Alcian blue staining of histological sections

To distinguish the cartilage matrix on histological sections, Alcian blue dye (purchased from Poly Scientific) was used to visualize the proteoglycan-rich matrix of the cartilage. Alcian blue stained the proteoglycan-rich cartilage matrix, while nuclear red stained the nuclei. The blue dye specifically forms electrostatic bonds with tissue polyanions, specifically the glycoproteins

in the matrix of cartilage (detects sulfated and carboxylated acid mucopolysaccharides). Histological sections (7µm) of paraffin-embedded hindlimbs were deparaffinized in xylene, then rehydrated to distilled water in the following sequence: 10 min in xylene, 5 min in xylene, 2 min x 2 in 100% ethanol, 2 min x 2 in 95% ethanol, 2 min in 70% ethanol, 2 min in 50% ethanol, and 2 min in water. Slides were then placed in 3% acetic acid solution for 3 minutes. Sections were then stained with Alcian blue solution pH 2.5 for 20 minutes at 37°C, then washed in water for 10 minutes and then rinsed in distilled water in a container (water in container was changed every minute for 5 minutes). Slides were then counterstained in 0.1% nuclear red fast solution for 4 minutes, followed by a wash with copious amounts of tap water for 5 minutes. Sections were then dehydrated (reverse of rehydration process stated above) and mounted with appropriate media (Cytoseal 60).

Immunohistochemistry of paraffin slides

Immunohistochemistry was performed on paraffin embedded slides of Dot1L floxed mice. Sections were deparaffinized in xylene and rehydrated in the following sequence: 10 min in xylene, 5 min in xylene, 2 x 2 min in 100% ethanol, 2 x 2min in 95% ethanol, 2 min in 70% ethanol, 2 min in 50% ethanol, and 2 min in water, then indefinitely in PBS. Sections were outlined with a hydrophobic pen. Heat-induced epitope retrieval was performed using 4N HCl solution (solution was prewarmed to 37°C prior to use) or pepsin (added dropwise) for 10 minutes in 37°C. Next, sections were treated with 3% hydrogen peroxide in water for 30 minutes at room temperature to inactivate endogenous peroxidase activity. Slides were rinsed in PBS for 3x5 minutes. Then, sections were made permeable to antibodies by placing slides in 0.1% Triton-X-100 (in PBS) for 10 minutes, followed by washing in PBS for 3x5 minutes. Next, sections were blocked in normal goat serum for 1 hour at room temperature

and incubated overnight at 4 °C with the primary antibodies against Col2a (Abcam, ab58632; dilution 1:250 in EPZ-5676-injected mice and 1:400 in cartilage-specific *Dot1l* knockout mice), *Dot1L* (Novusbio, NB10-40845; dilution 1:750), Sox9 (Abcam, ab185230; dilution 1:100) or H3K79me2 (Abcam, ab177183; dilution 1:100).

Slides were washed prior to secondary antibody incubation in PBS for 10 minutes (1X) followed by washing in PBS for 10 minutes (3-4X). Secondary antibody incubation occurred for 1.5-2 hours at room temperature in appropriate HRP-linked secondary antibody in 1% BSA. The following secondary antibodies were used: peroxidase anti-mouse IgG (Vector Laboratories PI-2000, 1:200), peroxidase anti-rabbit IgG (Vector Laboratories PI-1000, 1:200), and Peroxidase anti-goat IgG (Vector Laboratories PI-9500, 1:200). Slides were then washed with PBS for 10 minutes (3-4X). The revelation step was done using a Vector AEC (RED) Substrate Kit with a Buffer Stock Solution, and AEC Stock Solution, and a Hydrogen Peroxidase Solution. Solutions were mixed together in a test tube and 30-50uL of the solution was added per slide section. Sections were monitored every 2-3 minutes to watch for color changes (browning of sections). Slides were then rinsed in 2-3 changes of PBS and places in slide rack for remaining steps.

Nuclei was counterstained by dipping slides in hematoxylin for 2 seconds and then rinsed immediately in copious amounts of distilled water. Slides were drained and dipped in ammonia-water for 3-5 seconds and rinsed again in distilled water. Slides were mounted in appropriate media and cover slipped.

Immunofluorescent staining of cultured cells

Immunofluorescent staining was done on cultured cells. Isolated chondrocytes were fixed in formalin, blocked in 1% bovine serum albumin, then incubated with primary antibodies for

Col2a, Dot1L, or H3K79me2 for one hour at 37°C. After washes in PBS, cells were incubated with fluorescently labeled secondary antibodies (anti-mouse Alexafluor-488, green; or anti-rabbit Alexafluor-594, red) for one hour at 37°C. Cells were washed in PBS, then cover slipped prior to microscopy.

Embryonic whole skeletal staining

Alcian blue staining was used on eviscerated whole mice embryos. The skin and muscles were cleared off of these mice before the staining procedure was done. Alcian blue stains for cartilage and Alizarin red stains bone. Comparisons between stained control skeletons (Dot1L FL/FL) and stained transgenic skeletons (Dot1L FL/FL x AgcCre^{ERT2}) allowed us to determine the effects of embryonic deletion of Dot1L in cartilage. Solutions used included 100% ethanol (EtOH: Pharmco-AAPER Cat#111000200), 100% acetone, glycerol (Fisher Cat# BP229-4), Alcian Blue stain (200 mls; 0.03% (w/v) in 80% EtOH, 20% acetic acid), and Alizarin Red stain (200 mls; 0.05% in 1% w/v potassium hydroxide).

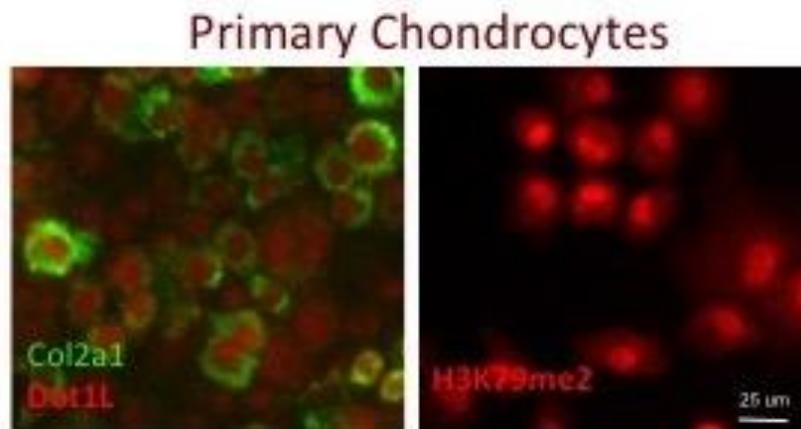
At autopsy, embryos were placed in tap water for 1-5 hours, then scalded in hot tap water (~65°C) for 20-30 seconds to allow for easier maceration of the tissue. Forceps were used to eviscerate the embryo. Embryos were fixed in 95% EtOH overnight; bubbles were removed from body cavity. Embryos were then transferred to 100% acetone and incubated overnight at room temperature. Next, cartilage staining was done by placing enough Alcian blue stain to cover the body. Embryos were stained overnight at room temperature. The next day, embryos were rinsed twice in 95% EtOH and destained in 95% EtOH overnight at room temperature. Samples were cleared by placing them in 1% KOH for 1 hour, then counterstaining of bone was performed by placing embryos in Alizarin Red stain for 3-4 hours. Samples were then cleared by placing them in 1% KOH of decreasing strengths: 1% KOH for 1-3 days at 4°C,

80:20 of 1% KOH to glycerol and clear overnight at room temperature, 50:50 of 1% KOH to glycerol and cleared overnight at room temperature, 20:80 of 1% KOH to glycerol and clear overnight at room temperature, then stored indefinitely in 20:80 of 1% KOH to glycerol.

RESULTS

Dot1L expression in primary chondrocytes

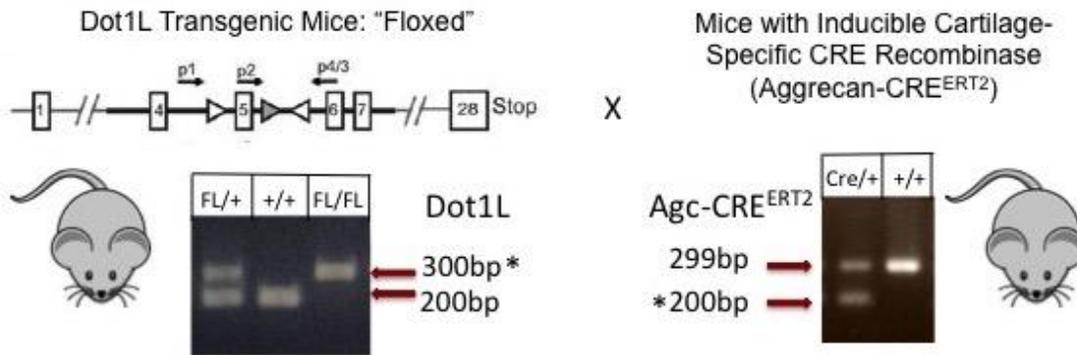
In order to visualize the expression of Dot1L in chondrocytes, we examined isolated chondrocytes from wild-type mice. Chondrocytes that expressed Dot1L in the nucleus would validate the efficiency of the Dot1L being used. Antibody staining was used to determine co-expression of Col2a (type II collagen), Dot1L, and H3K79me2 (dimethylation of lysine-79 on H3) in chondrocytes harvested from wild type postnatal day 6 mice. Chondrocytes were harvested from the long bones of wild type postnatal day 6 mice. Cells were co-stained using Dot1L and Col2a1-specific antibodies. Cultured cells were co-stained in order to distinguish between the matrix and the nucleus of the chondrocytes. Co-expression of the two proteins was necessary, because Col2a validates the existence of chondrocytes, so Dot1L should also be present in these chondrocytes. Col2a (green) is expressed in the matrix whereas Dot1L (red) is expressed in the nucleus. Antibodies were used to detect the proteins. Red fluorescence correlated with Dot1L expression and green fluorescence correlated with Col2a expression. Additionally, nuclear staining of H3K79me2 (dimethylation of lysine-79) was also observed in wild type primary mouse chondrocytes (red). The red staining on the right panel depicts activity of Dot1L, since it is the only enzyme that methylates lysine 79 residue on histone 3.

Figure 9- Dot1L expression in primary mouse chondrocytes

(Left) Chondrocytes were harvested from the long bones of wild type postnatal day 6 for cultured. Cells were co-stained using *Dot1L* and *Col2a1*-specific antibodies. IF microscopy showed nuclear expression of *Dot1L* (red) in mice co-express *Dot1L* and *Col2a*. *Col2a* (green) is expressed in the matrix, whereas *Dot1L* (red) is expressed in the nucleus. (Right) Nuclear staining of H3K79me2 (dimethylation of lysine-79) was also observed in wild type primary mouse chondrocytes.

Developing a mouse model for the conditional loss of *Dot1L* function in cartilage

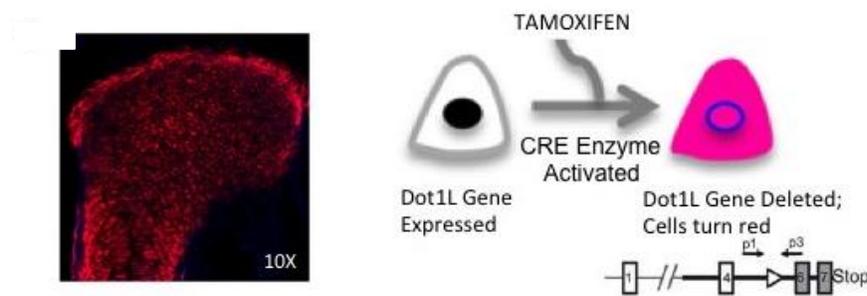
Dot1L transgenic mice harboring flox sites flanking exon 5 were generated as described in Henry et al. These mice were bred with mice carrying the tamoxifen inducible cartilage specific Cre recombinase gene (Aggrecan-CRE^{ERT2}). In order to uniquely identify each offspring from the mouse model, we performed PCR reactions and gel electrophoresis to determine the genotypes. DNA from ear notches of each mice were amplified through PCR. Primers specific to *Dot1L* and Agc-Cre were used to amplify specific regions of DNA. Based on the fragment size and number, the corresponding genotype could be determined. For example, a WT *Dot1L* transgenic mice presented with one band at 200bp. +/+ denotes wild type genotype. FL/FL represents mice homozygous for the floxed *Dot1L* alleles. Cre/+ is indicative of a mouse that is heterozygous for the Cre allele.

Figure 10- Breeding of Dot1L Transgenic Mice and AgcCreERT2 Mice

Dot1L transgenic mice harboring loxP sites at exon 5 were bred with mice carrying the tamoxifen inducible cartilage specific Cre recombinase gene. Genotypes were determined by PCR analyses. +/+ denotes WT; FL/FL denotes mice homozygous for the floxed *Dot1L* alleles. Cre/+ is indicative of a mouse that is heterozygous for the Cre allele.

Validation of cartilage-specific recombinase activity in tamoxifen-treated mice

To activate the cartilage-specific Cre recombinase, mice must be injected with tamoxifen, an estrogen analog that causes the Cre to translocate into the nucleus. In the nucleus, the Cre cleaves the floxed sites it recognizes on *Dot1L*, rendering the enzyme nonfunctional. Cre both deletes *Dot1L* from the sequence and produces a red fluorescence in mice that lose *Dot1L*. Administration of tamoxifen in mice lacking *Dot1L* results in aggrecan-expressing cartilage cells turning red. Results show red fluorescence around the surface of the femur, which represents the articular cartilage. The red fluorescence is seen due to the Ai9 reporter allele in the mice. Ai9 is a Cre reporter mouse that has a loxP STOP cassette that prevents the transcription of a CAG promoter driven red fluorescent protein variant (tdTomato). Following Cre-mediated recombination, mice with Ai9 Cre reporter alleles express red fluorescence (Madisen et al. 2010).

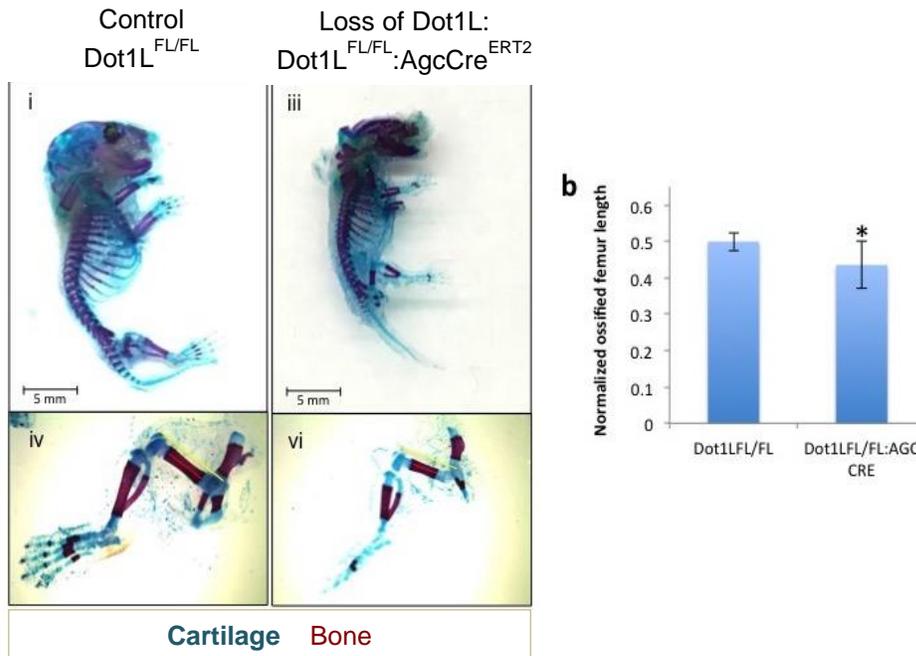
Figure 11- Tamoxifen activated CRE Enzyme

Injection of tamoxifen induces translocation of Cre into the nucleus. Cre is expressed and the gene for Dot1L is deleted, producing a red fluorescence to visualize the deletion. (Left) Aggrecan expression cells turn red in mice lacking Dot1L.

Embryonic deletion of Dot1L in cartilage impaired skeletal growth

Embryonic whole skeletal staining was done in order to differentiate between bone and cartilage in cleared skeletons. The physical consequences of deletion of Dot1L in cartilage on an embryonic level reveals that mice with Agc-Cre are physically shorter than the control mice. These mice were not directly given tamoxifen, but rather given tamoxifen indirectly through the mom, who received intraperitoneal injection (IP). Deletion of Dot1L was induced by tamoxifen on embryonic day 15 and pups were harvested at day 17.5. In addition to comparing the overall lengths of the skeletons, we measured the ratio of ossified femur to overall femur length to determine the effects that the loss of Dot1L has on the ossification of femur length. We observed a significant difference between the Agc-Cre mice and the control mice. There was a reduction in the length of the mineralization of the femur in Agc-Cre mice, which suggests that deletion of Dot1L in these mice lead to impaired skeletal growth. Thus, homozygous Dot1L^{FL/FL} mice are phenotypically normal. Cartilage-specific inactivation of Dot1L in embryonic and postnatal mice by tamoxifen-induced Cre recombinase activity (Dot1L^{FL/FL}:AgcCre^{ERT2}) resulted in impaired skeletal growth.

Figure 12- Embryonic comparison of Dot1L deletion in $Dot1L^{FL/FL}$ and $Dot1L^{FL/FL};AgcCre^{ERT2}$



*Deletion of Dot1L was induced by tamoxifen on embryonic day 15. Embryos were harvested on day 17.5. Intact skeletons were stained with Alcian blue (cartilage) and Alizarin red (bone). Staining and quantitative analyses of femur length showed reduced ossified femur length in mice with Dot1L loss of function. * $p < 0.05$.*

Postnatal deletion of Dot1L in cartilage impaired skeletal growth

Qualitative observations were made on the effects of postnatal deletion of Dot1L in cartilage. The images depict the effects of Dot1L deletion postnatally on both Control ($Dot1L^{FL/FL}$) and transgenic ($Dot1L^{FL/FL}, AgcCre$) mice. Pups were injected directly with tamoxifen on postnatal day 7-10. Images were taken at 3 weeks and showed impaired growth in Tam-treated $Dot1L^{FL/FL};AgcCre^{ERT2Ki/+}$ mice. X-rays showed shortening of tibias in Dot1L deleted mice vs. control mice.

Figure 13- Postnatal comparison of Dot1L deletion in $Dot1L^{FL/FL}$ and $Dot1L^{FL/FL};AgcCre^{ERT2}$

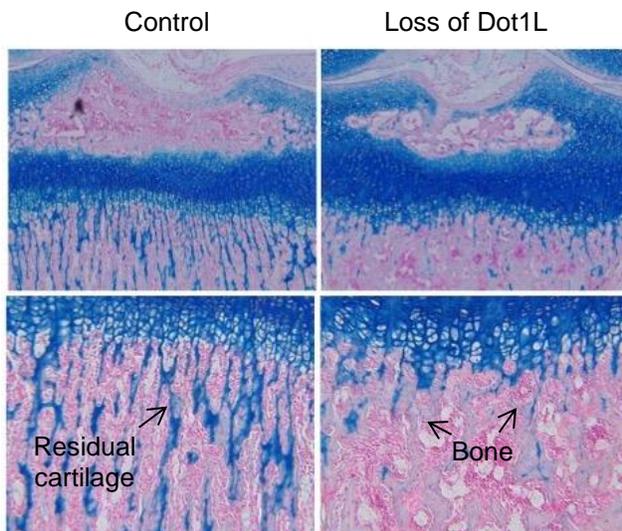


Comparisons between control ($Dot1L^{FL/FL}$) and loss of Dot1L mice depict shortening of hindlimbs in Dot1L deleted mice.

Postnatal deletion of Dot1L affected the growth plate structure

Histological sections of the mouse tibia growth plates from 3-week-old control and Dot1L postnatal loss of function mice were stained with Alcian blue to visualize cartilage. Staining depicts that control mice have more residual cartilage than the AgcCre mice, which poses the question of how exactly does Dot1L affect endochondral ossification.

Figure 14- Tibia growth plate comparisons of control mice and mice exhibiting loss of Dot1L postnatally



Alcian blue staining of control mice and loss of Dot1L mice depict differences in the amount of residual cartilage present in the growth plate. Mice exhibiting loss of Dot1L present with faster bone formation nearer to the primary ossification center.

DISCUSSION

Previous studies on the regulation of endochondral ossification and its effects on skeletal growth have focused on regulation by systemic and local factors, such as growth hormone, insulin-like growth factors (IGFs), thyroid hormone, parathyroid hormone related peptide, growth factors, and transcription factors such as Sox9 and Runx2. Recently, however, epigenetic control of gene expression and cell behavior has gained attention and the effects of these processes on chondrocyte hypertrophy are being studied. One of the key modifications that regulate chromatin structure and gene expression is histone acetylation. Histone deacetylase 4 (HDAC4) is the first histone-modifying enzyme to be associated in cartilage development. HDAC4 inhibits hypertrophy by suppressing the activity of Runx2 and MEF2C (myocyte-specific enhancer factor 2C), which are key transcription factors that promote hypertrophic differentiation in endochondral ossification (Vega et al., 2004; Arnold et al., 2007). Mutations in the human HDAC4 gene have been associated in a genetic condition with several skeletal defects, known as Brachydactyly mental retardation syndrome (Williams et al., 2010). More recently, studies have shown HDAC3 is also implicated in growth plate regulation (Bradley et al. 2013). HDAC3 knockout mice exhibit acceleration of chondrocyte hypertrophy but smaller cell size, which was attributed to an increase in the expression of phosphatase-rich repeat phosphatase 1 (Phlpp1) in HDAC3-deficient chondrocytes. Phlpp1 levels affect Akt signaling; an increase in Phlpp1 levels suppress Akt signaling, which is a positive regulator of chondrocyte hypertrophy (Peng et al., 2003; Ulici et al., 2008, 2009; Rokutanda et al., 2009). These studies show that there is a direct relationship between the epigenetic regulation of gene expression to cell size increase.

Many other posttranslational modifications control histone function, but very few have been studied in relation to chondrocyte differentiation. Recent studies have demonstrated that the histone methyltransferases ESET is involved in hypertrophy (Lawson et al., 2013; Yang et al., 2013). ESET has been associated with HDAC4 in suppressing Runx2 activity and subsequently hypertrophy. ESET deficient chondrocytes have been shown to undergo accelerated hypertrophy. Interestingly enough, our study of loss of Dot1L function in cartilage also shows increased hypertrophic differentiation in growth plate chondrocytes. Figure 14 staining shows that postnatal deletion of Dot1L affected the growth plate structure. Control mice have more residual cartilage than the AgcCre mice. From the histological sections, it appears that Dot1L impairs the differentiation of chondrocytes, because staining from mice with a loss of Dot1L show chondrocyte differentiation and maturation progressing more rapidly, which is why there is more bone formation. This rapid progression from cartilage to bone, however, is not indicative of more longitudinal growth, as these mice still appear to be shorter. Thus, the histological analyses of the growth plates from mice with postnatal loss of Dot1L suggest that Dot1L is involved in controlling chondrocyte differentiation. We hypothesize that Dot1L affects the later stages of endochondral ossification, as a smaller hypertrophic layer means there is less cartilage to be replaced. In comparison to HDAC KO mice, which are associated with acceleration of hypertrophy but smaller cell size, Dot1L KO mice show similar patterns of accelerated differentiation, but shorter bone lengths.

Linking loss of Dot1L to impaired skeletal growth

It is important to study the mechanisms that control chondrocyte differentiation in order to develop a thorough understanding of the formation of longitudinal bones. Defects in the process of forming long bones are associated with chondrodysplasias, which are linked to

dwarfism and stunted growth. Dot1L is a histone methyltransferase that has been identified as a regulator of cartilage health and disease (Monteagudo et al., 2017). Variations of Dot1L are also associated with human height (Lango Allen et al., 2010). Dot1L is specifically studied, because genome wide association studies (GWAS) have showed that common single variants in the gene protect against osteoarthritis, but how the gene affects OA is still unknown. These studies have prompted us to investigate the role of Dot1L in embryonic and postnatal skeletal development. We address the question “is the activity of Dot1L methyltransferase in cartilage necessary for the regulation of endochondral ossification and skeletal growth?” The objective of this research was to develop and validate a mouse model that utilizes the Cre recombinase system to genetically inactivate the Dot1L gene in embryonic and postnatal cartilage. In addition, we aimed to perform phenotypic and histological analyses of skeletons of mice with embryonic and postnatal loss of Dot1L expression in cartilage.

Examination of isolated chondrocytes from the long bones of wild-type postnatal day 6 mice depict co-staining of Dot1L and Col2a specific antibodies. The antibodies were linked to a fluorescent tag in order to help us visualize between the two proteins. Dot1L antibodies presented as red tags and Col2a presented as green tags under immunofluorescent microscopy. This observation was necessary, because Col2a validates the existence of chondrocytes, since it is the gene responsible for making type II collagen. Dot1L staining was important, because we needed to distinguish between the matrix and the nucleus. Dot1L is expressed in the nucleus of chondrocytes. Thus, Col2a (green) expression corresponds to the matrix of chondrocytes, whereas Dot1L (red) staining represents the nucleus. This can be seen in the left panel of Figure 9. Additional nuclear staining of H3K79me2 was observed in WT primary mouse chondrocytes.

Methylation of lysine-79 is indicative of Dot1L activity. The right panel of Figure 9 shows the red fluorescence, which corresponds to the activity of Dot1L.

Our mouse model utilizes the Cre recombinase system to genetically inactivate the Dot1L gene in embryonic and postnatal cartilage. As indicated by the results, the Cre used in our experiments was Aggrecan Cre, which is a cartilage specific Cre. Mice expressing Cre recombinase were crossed with tdTomato reporter mice to produce mice that expressed red fluorescence. Mice that were injected with tamoxifen and lacked Dot1L showed red fluorescence around the surface of the femur, which represents articular cartilage. This red fluorescence, characteristic of the tdTomato reporter, validates the Cre recombinase activity by using immunofluorescence. This means that the cells turn red if the Dot1L gene is deleted.

To address the effects of embryonic and postnatal deletion of Dot1L on skeletal growth, we looked at the physical consequences of the deletions on mice limbs and whole skeletons. Embryonic deletion of Dot1L in cartilage rendered mice with a $Dot1L^{FL/FL}; AgcCreERT2$ genotype, compared to the control mice ($Dot1L^{FL/FL}$). Control mice are phenotypically normal, and had no stunted growth, as depicted by whole skeletal staining. In contrast, mice that presented with loss of Dot1L embryonically had impaired skeletal growth. This observation was supported by comparing the ratios of ossified femur to overall femur length to determine how the loss of Dot1L affects ossification of the femur. Quantitative analysis of femur length showed reduced ossified femur length in mice with Dot1L loss of function. This indicates that Dot1L is necessary in order for ossification to occur.

Postnatal deletion of Dot1L in cartilage also depicted impaired skeletal growth. This type of deletion was represented in a more qualitative manner. Pups who were directly injected with tamoxifen had the genotype $Dot1L^{FL/FL}; AgcCreERT2$. Tamoxifen, as mentioned above, is

necessary for the activation of Cre. Once activated, the Cre enzyme is able to target the exon on the Dot1L gene responsible for its enzymatic activity, cleaving it and rendering it inactive. X-rays show that mice treated with tamoxifen have shorter hindlimbs in comparison to control mice, who were not treated with tamoxifen.

Limitations and Future Studies

In this study, we observed that loss of Dot1L postnatally and embryonically in mice resulted in retarded skeletal growth. However, we did not examine the factors that regulate Dot1L activity in growth plate cartilage. Thus, future studies are aimed at understanding the role of Dot1L in endochondral ossification and uncovering the genes whose expressions are regulated by Dot1L in cartilage. In addition, comprehensive phenotypic, histological, and immunohistochemical analyses are underway to determine the specific role of Dot1L in chondrocyte differentiation in the growth plate. Studies have suggested that transcriptional control may be a mechanism to regulate Dot1L activity (Monteagudo et al., 2017). We know that regulation of Dot1L activity directly controls H3K79 methylation and affects gene transcription. Dot1L is the only known histone methyltransferases for lysine 79 of histone H3, but there are no known demethylases for H3K79. Demethylation of H3K79 occurs through histone renewal and cell division. Further studies can identify a specific demethylase of H3K79 through chromatin immunoprecipitation (ChIP) procedures. ChIP is a technique used to analyze histone modifications (Milne et al. 2014). Through this technique, we may be able to identify a demethylase of H3K79 and observe if it affects Dot1L's control of H3K79, and subsequently, differentiation in growth plate cartilage.

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