A study of cis-regulatory sequences of *wnt1* in the disease Osteogenesis Imperfecta

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A Senior Honors Thesis

Presented to

the Faculty of the Department of Biology and Biochemistry

University of Houston

In Partial Fulfillment of the Requirements for the Degree Bachelor of Science

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By

Maria Saad Dalibalta

May 2021

Maria Dalibalta

Approved:

Dr. Arne Lekven, (Thesis Advisor)

Dr. Erin Kelleher

Dr. Marc Hanke

Dr. Dan Wells, Dean of the College of Natural Sciences and Mathematics

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Abstract

wnt1 is known to be mutated in the inherited cases of the disease Osteogenesis Imperfecta, a bone genetic disorder also known as the brittle bone disease. The canonical Wnt signaling pathway has a known role in bone homeostasis and development and studies have shown that *wnt1*, an evolutionarily conserved member of the Wnt signaling pathway, is particularly important for the Wnt canonical pathway in osteoblast differentiation. This suggests a potential role of *wnt1* in bone homeostasis. Understanding the regulation of *wnt1* expression by cis-regulatory elements during development is important to understand the role it plays in bone development and homeostasis. This project uses ATAC-seq (Assay for Transposase-Accessible Chromatin) datasets available to the public to study regions within and surrounding the *wnt1* gene to find open chromatin regions, as this is associated with enhancer activity. The enhancers that are shown to be active are studied closely using the ENCODE project to obtain information on the expression profiles of the enhancers and the tissue in which they may be active. The activity of the enhancers was compared in tissues where *wnt1*-signaling is known to be important: hindbrain tissue, adipose tissue, and bone marrow macrophage and two enhancers with the highest activity were identified. By knowing these cis-regulatory sequences of *wnt1*, now it could be easier to possibly use *wnt1* expression as a therapeutic target in the disease Osteogenesis Imperfecta.

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List of Abbreviations

OI Osteogenesis Imperfecta

UCSC University of California Santa Cruz

Introduction

Background on Osteogenesis Imperfecta (OI), the canonical Wnt pathway, and the wnt1 gene

Osteogenesis Imperfecta (OI) is a genetic bone disorder which is characterized by abnormal bone fragility, skeletal deformity and other extraskeletal symptoms (Etich et al., 2020). The Wnt signaling pathway is known to have importance in regulating bone homeostasis and development (Baron et al., 2013). Several studies have shown that canonical Wnt signaling regulates osteoclast function and controls bone resorption (Albers et al., 2013). The *wnt1* gene is known to be mutated in many cases of this disease; missense and nonsense mutations of wnt1 have been identified in people diagnosed with Osteogenesis Imperfecta (Laine et al., 2013). Therefore, the *wnt1* gene could play a possible role in the regulation of bone homeostasis, and a good understanding of its regulation would allow future perspectives in treating bone diseases like Osteogenesis Imperfecta.

wnt1 is part of a Wnt gene cluster which comprises *wnt1* and *wnt10b,* which function redundantly at least during zebrafish embryonic development, but whether these genes are required in other contexts it is still unknown (Lekven et al., 2003). Interestingly enough, *wnt10b* is known to have a role in regulation of bone mass in mouse, by inducing of the expression of osteoblastogenic transcription factors and ultimately stimulating osteoblastogenesis, This idea is supported by analyses of *wnt10b*-/- mice which show decreased trabecular bone mass (Bennett et al., 2005). The importance of *wnt10b* in bone mass regulation, and its presence in the same locus as *wnt1* have strengthened the possibility that *wnt1* transcriptional regulation is an interesting candidate in the understanding of the disease Osteogenesis Imperfecta.

The complex spatial and temporal expression of genes during the general development of an organism is regulated by cis-regulatory sequences called enhancers. Therefore, the close study

of enhancers that regulate *wnt1* would help understand its role in OI. Generally, enhancer sequences that are accessible to transcription machinery are known to be active, and those sequences are typically found in open chromatin regions. Therefore, experimental methods that identify open chromatin sequences, such as ATAC-seq, are important tools for identifying novel active enhancers of *wnt1*.

Another important point which strengthens the argument that the Wnt canonical pathway should be studied to understand OI, is mutations have been identified in key signaling Wnt mediators such as low-density lipoprotein receptor-related protein 5 (LRP5) in diseases with high or low bone-mass phenotypes, such as OI (Laine et. al, 2013). Furthermore, canonical Wnt signaling has been proven to be essential for normal skeletal development, as it induces bone formation and osteoclast differentiation in early osteoblast progenitors (Laine et. al, 2013). Mice which lack Fzd9, the Wnt receptor, have shown cell-autonomous defects in bone formation. The Wnt1 ligand, in particular, is important for the Wnt canonical pathway in osteoblast differentiation as experiments have shown that abnormal Wnt1 proteins stimulated less bone mineralization than wildtype Wnt1 did (Laine et. al, 2013). This was tested on osteoblast precursor cell lines derived from mouse, where mutant Wnt1 proteins were expressed to assess their effect on the endogenous targets of Wnt signaling. Wildtype Wnt1, but not the mutant Wnt1, induced the expression of the downstream β-catenin targets (Laine et al, 2013). These findings, along with other studies, have proven that *wnt1* is important for the regulation of bone mass and could therefore be an interesting therapeutic target in osteogenesis imperfecta (Laine et. al, 2013).

The availability of genome wide sequencing data online has made it possible for researchers to explore these massive datasets in different contexts and utilize knowledge gained from the analyses of these published data to advance research in different areas. The goal of this

thesis is to perform data mining experiments to identify enhancers responsible for controlling different aspects of *wnt1* expression, especially in bone.

- Chapter 1 – Analysis of publicly available ATAC-seq data to identify putative *wnt1* enhancers -

The regions of DNA that are exposed to transcription machinery are expressed in the genome. The open chromatin regions of the chromosome are accessible and in which genes are transcribed, and a widely used tool to identify these regions is a method called Assay for Transposase-Accessible Chromatin with high throughput sequencing, or ATAC-seq (Buenrostro et al., 2015). This molecular biology technique is used to assess genome-wide chromatin accessibility. The advantage of it in comparison with other genome sequencing techniques, such as FAIRE-Seq or DNase-Seq, is that it requires a significantly lower number of cells and a shorter time to process samples (Buenrostro et al., 2013).

How ATAC-Seq works

In the nuclei of cells, DNA is packaged with histone proteins and forms a complex called chromatin. Chromatin is active when it is open and accessible to transcription factors and ATAC-Seq can be used to map this in the genome. Open chromatin regions are typically nucleosome depleted regions and are often bound by different protein factors (Zhang et al., 2017). To search for genome-wide accessible chromatin regions, a Tn5 transposase can be used to insert sequencing adapters into the chromatin regions in the nucleus that are open and have accessibility (Figure 1A). The transposition leads to fragmented DNA, and the adapters are completed with a 72˚C extension step. Then, the DNA is amplified using PCR where additional sequence is added within the adapters, which consist of a sequencing barcode and common sequencing ends (Figure 1B). High throughput sequencing is used to create a genome-wide map of the Tn5 transposon insertions and thus a genome-wide measure of chromatin accessibility.

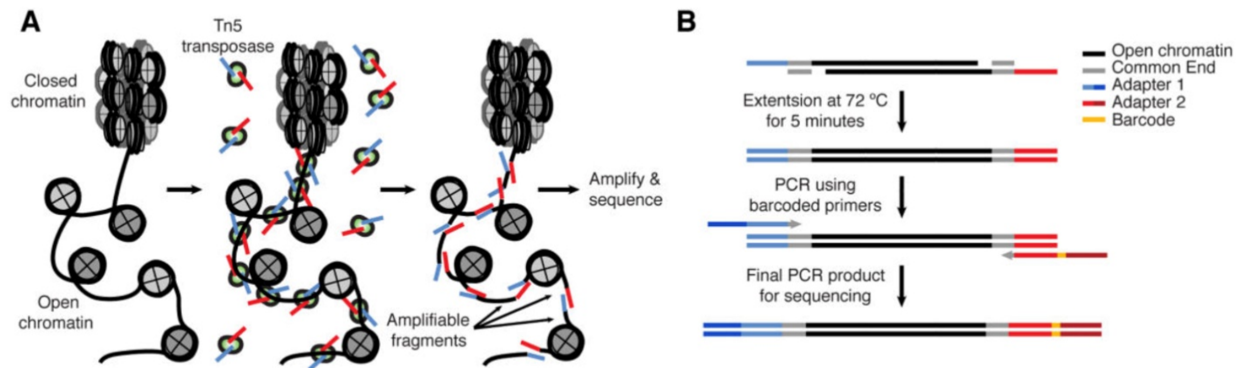

Figure 1 **ATAC-Seq Methods** (A) Function of transposase in ATAC-Seq. (B) Extension and amplification of resulting DNA fragments (Buenrostro et al., 2015).

After assembly of the genome, PCR duplicates and mitochondrial DNA are removed to avoid confusion when analyzing data. The fragments of DNA are used to find open chromatin regions, nucleosome free regions, as well as other components of genome transcription such as transcription factor binding sites that can be identified as over-represented sequences in accessible regions that have similar profiles. All this information can be used to compare sequences and find differences or similarities between transcription in the cells. In this project, ATAC-Seq data that is publicly available was used to look closely at the *wnt1* regulatory locus to find potential cis-regulatory sequences of the *wnt1* gene.

Methods of datamining using publicly available ATAC-seq data

In order to pinpoint potential cis-regulatory sequences of *wnt1* in relation to OI, the chromatin accessibility of the DNA surrounding *wnt1* is needed. Studying ATAC-Seq data is the ideal approach to profile this chromatin accessibility, many publications report an extensive number of ATAC-seq analyses of genomic accessibility from a large number of diverse tissues, as this could help in understanding several aspects of gene regulation and expression. Because these data are publicly available and have not been analyzed specifically for putative *wnt1* enhancers,

these are a valuable source of potential information to be mined for putative *wnt1* enhancers. Thus, an atlas of adult mouse ATAC-Seq profiles was used as a data source for this project.

Liu et al., 2019 performed an array of ATAC-seq experiments from diverse mouse tissues. This paper provided the chromatin accessibility data used to scan the *wnt1* locus to find previously unknown and potential enhancers that regulate *wnt1* expression in the bone. The model organism used in this paper were adult mice and the published atlas consisted of 66 ATAC-seq profiles from 20 different primary tissues from male and female mice (Figure 2) (Liu et al., 2019). 296,574 accessible elements were identified in their project, and a raw count of chromatin accessibility was displayed as a table in the supplementary materials section of the paper.

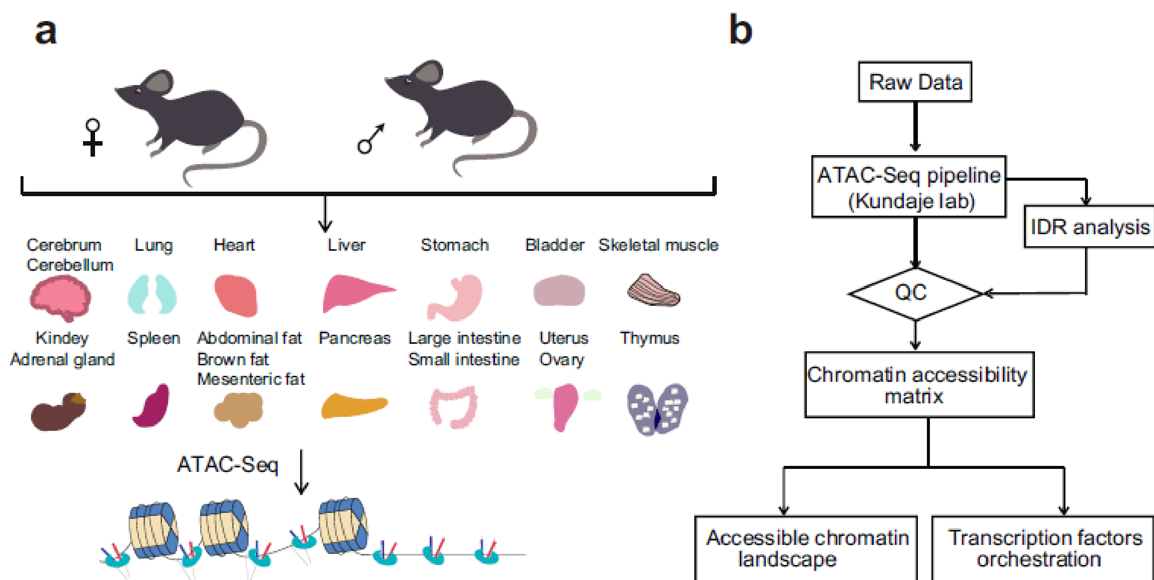

Figure 2 **Experimental and data analysis workflow of the Nature paper "An ATAC-seq atlas of chromatin accessibility in mouse tissues"** (a) The ATAC-seq profiling was done on 20 different tissue from adult mice. (b) The workflow used to produce the ATAC-seq profile.

Each open chromatin region was identified and labeled with the chromosome number and location, and the tissues in which these regions are found were indicated. The chromosome

location of the *wnt1* gene was mapped on the University of California Santa Cruz Genome browser, and potential cis-regulatory sequences of *wnt1* were given by the website. This interactive website provides genome sequence data from many model organisms, including several vertebrate and invertebrate organisms. The UCSC genome browser was an essential tool for the project, as it provided a map of the gene of interest (*wnt1*), in addition to other genes in close proximity and allowed the viewing of the cis-regulatory sequences alongside the genes (Figure 3).

To study the ATAC-Seq data provided by the paper (Liu et al., 2019), the chromosome locations identified as open chromatin regions were aligned with the chromosome locations of the candidate cis-regulatory sequences of *wnt1* given by the UCSC genome browser. The regulatory sequences of interest became clear when they were found to be within coordinates that are open chromatin in the chromosome, and the regions of DNA that were in closed chromatin were filtered out (Table 1).

Results of datamining and the putative cis-regulatory sequences of wnt1 in OI

Table 1: **The open chromatin region coordinates of *wnt1* aligned with enhancers which fall in this region.**

Wnt 1 ATAC-Seq open chromatin coordinates	Enhancer aligned with coordinates
chr15_98726254_98726740	none
chr15_98728516_98729049	none
chr15_98735152_98735587	none
chr15_98746468_98746827	E0611272/enhD: chr15:98746732-98746895
chr15_98747591_98749055	E0611274/enhD: chr15-98747591-98749055
chr15_98753046_98753681	none
chr15_98754954_98755507	none
chr15_98758041_98758503	none
chr15_98758913_98759515	none
chr15_98763618_98765397	E0611282/enhP: chr15:98763650-98763818
chr15_98765779_98766158	none
chr15_98771302_98771679	none
chr15_98782501_98782870	none
chr15_98783242_98784345	none
chr15_98784964_98785814	none
chr15_98799481_98799792	none
chr15_98800068_98800577	none
chr15_98801283_98802659	none
chr15_98803412_98804085	none
chr15_98807629_98808087	E0611329/enhP: chr15:98807421-98807729
chr15_98808133_98808789	E0611331/enhP: chr15:98808279-98808564, E0611332/enhP: chr15:98808567-98808785
chr15_98813882_98814278	none
chr15_98815638_98816568	none

Column 1 indicates which regions of chromosome 15 were found to be open chromatin. Column 2 indicates which enhancers of *wnt1* fall within the corresponding coordinates on chromosome 15. “None” in column 2 indicate that no enhancers were found within the corresponding coordinates in column 1.

The *wnt1* gene region studied was in location Chr15: 98,727,142 – 98,8143,839, which spans a region larger than just the *wnt1* transcription unit by around 84,000 base pairs. We chose this region because enhancer sequences can be proximal or distal to the gene sequence, and previous publications suggest this encompasses a Wnt1 topological association domain (TAD), a structure hypothesized to spatially constrain the interactions of enhancers. The data given in the ATAC-seq paper (Liu et al., 2019) allowed the identification of open chromatin regions within or around these coordinates, as shown in column 1 of Table 1. Following the collection of this information, these coordinates were analyzed in the UCSC genome browser for their position

relative to *wnt1* and candidate enhancers located within these coordinates were recorded in column 2 of Table 1. These enhancers were identified by the ENCODE Data Analysis Center based on biochemical signatures such as histone modifications, H3K4me3 and H3K27ac.

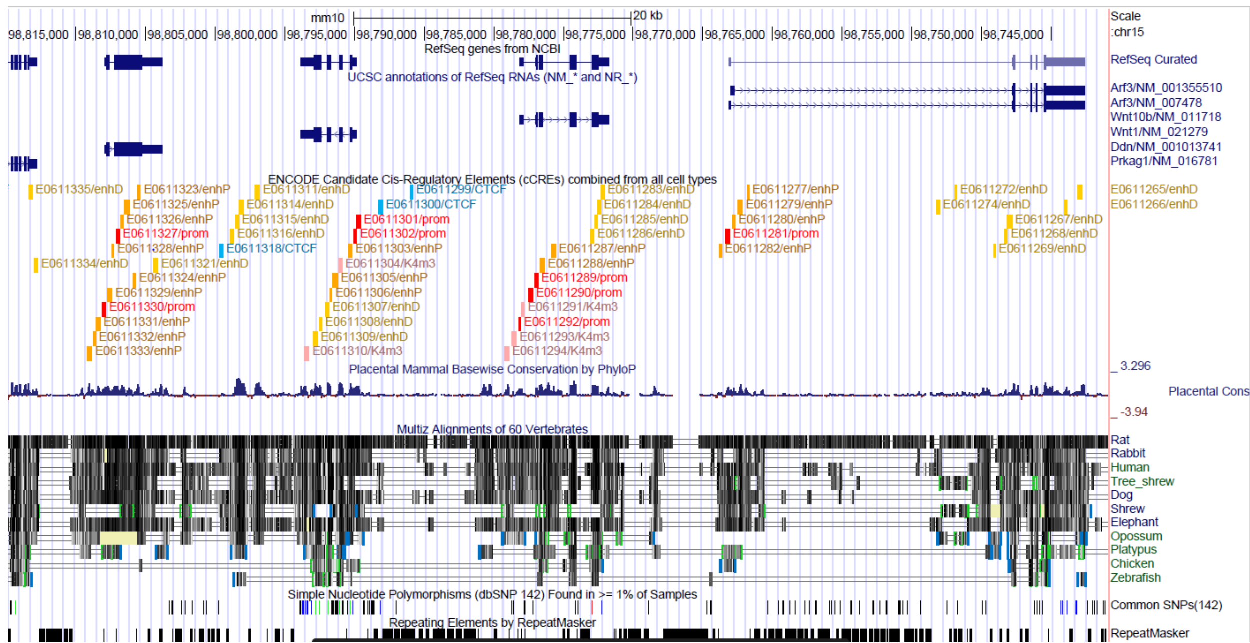

Figure 3: The location of the candidate enhancers of *wnt1* with respect to *wnt1* and neighboring genes *wnt10b, ddn* and *arf3* on chromosome 15, produced by the BLAT search tool on the UCSC genome browser.

The enhancers E0611272/enhD and E0611274/enhD are a few thousand base pairs away from each other and are located in the first intron of *arf3*, around 48,000 base pairs upstream of *wnt1*. Enhancer E0611282/enhP is located a few hundred base pairs upstream to *arf3,* and around 30,000 base pairs upstream of *wnt1*. Enhancer E0611329/enhP is in the first exon of *ddn* and is approximately 12,500 base pairs downstream of *wnt1*. As for enhancers E0611331/enhP and E0611332/enhP, they are located roughly 200 base pairs away from each other and they are a few hundred base pairs upstream of *ddn,* and around 13,500 base pairs downstream of *wnt1*. As enhancers are known to function independently of their location with respect to the gene they

regulate, all the above-mentioned enhancers can potentially be regulating wnt1 expression in different tissues.

- Chapter 2: Analysis of chromatin accessibility and histone modification information available through the ENCODE Project to identify putative *wnt1* enhancers -

The Encode Project

After identifying potential *wnt1* region cis-regulatory sequences, these sequences were further studied using the Encyclopedia of DNA Elements (ENCODE) project to identify any expression profiles which the enhancers may have in relation to certain tissue types. ENCODE was another important tool for this project as it dives deep into the regulation and organization of genes, and it provides valuable information on transcription, histone modification, and chromatin structure. More importantly, gene regulation is studied in the project by associating candidate regulatory elements with each other and with expressed genes, providing expression profiles of those elements (The ENCODE Project Consortium, 2012). The ENCODE portal has a search engine under “search for candidate cis-regulatory elements” in which the enhancer names were entered, and “Mouse mm10” was selected, also known as GRCm38 (Genome Reference Consortium Mouse Build 38). A profile was returned for each enhancer and provided different pieces of information on its expression in several tissue types and at varied ages of mouse.

Tissues selected for studying activity of putative enhancers of wnt1

The ENCODE project provided data on the epigenetic modifications to the histone H3, a DNA packaging protein, in the sequences of the enhancers. This was labeled as H3K27ac, and it means the acetylation of a lysine residue at position 27 of the N-terminal of the histone H3 protein. H3K27ac is defined as a mark of active enhancers, as it is associated with higher levels of activation of transcription. This mark was used as a point for comparison and the ENCODE project studied it in several tissue types of mouse such as bone marrow macrophage, hindbrain of embryo,

adipose tissue of adults, and embryonic fibroblasts in the pursuit of finding which enhancers show the highest activities in which tissues (Table 2). The different tissue types were chosen for a particular reason. Bone marrow macrophage was studied as *wnt1*'s importance in bone development is well known, and because macrophages have been demonstrated to interact with bone cells and influence bone formation (Gu et al., 2017). Embryonic fibroblast was chosen as a control, as *wnt1* and *wnt10b* are not expected to have activity. Adipose tissue was studied, as it plays a role in fat development and *wnt10b* is known to be important for this development. Hindbrain tissue was considered as it is part of the nervous system and wnt-1-mediated cell signaling is critical in the development of many regions of the central nervous system, including hindbrain. The identified candidate enhancers of *wnt1* were in open chromatin regions in these specific tissues, as shown by the data from the Liu et al., 2019 study, which provides support to continue the study of the activity of the enhancers in these tissues, for the regulation of *wnt1*.

Results

***Table 2*: The Z-score of H3K27ac of enhancers in different tissue types**

	Z score of H3K27ac			
Enhancer	Bone marrow macrophage (male adult 8 weeks)	Hindbrain embryo 13.5 days	Adipose tissue male adult 24weeks	Embryonic fibroblast male 13.5 weeks
E0611332/enhP	0.31	0.33	-0.21	-0.77
E0611331/enhP	0.67	0.5	0.2	2.03
E0611282/enhP	0.32	2.28	0.15	0.5
E0611329/enhP	0.22	0.68	-1.5	0.5
E0611272/enhD	0.67	0.4	0.2	2.03
E0611274/enhD	0.08	-0.49	-0.54	0.61

A Z-score for H3K27ac activity was given for each enhancer in different tissue types. The highlighted scores are those of enhancers with the highest activity in each tissue type.

Marks of activity were studied for enhancers shown to be open chromatin, such as the marks associated with higher activation of transcription (H3K27ac) which indicates active enhancers. The cis-regulatory elements are part of the representative DNase hypersensitivity sites

(rDHSs) and are supported by histone modifications (H3K27ac). For each rDHS, Z-scores were computed for the log10 of H3K27ac signals in every biosample. This is important because ENCODE uses DNase-seq and CHIP-seq data which give different types of signals for each biosample and Z-score computation is needed to be able to compare these signals. Z-scores describe the number of standard deviations which a particular measurement lies above or below the population mean, which allows comparison across measures (Curtis et al., 2016).

The Z-scores of H3K27ac were compared for each enhancer in the other tissues (Table 2). The embryonic fibroblast tissue of male mouse at 13.5 weeks was used as a control, as *wnt1* and *wnt10b* are not expected to have activity there. However, interestingly, the Z-score of H3K27ac was high under this tissue particularly for enhancers E0611272/enhD and E0611331/enhP which showed a Z-score of 2.03. This could be due to the importance of these enhancers in regulating expression of other genes, not *wnt1* in particular, as *wnt1* and *wnt10b* are known not to have activity in embryonic fibroblast tissue. Furthermore, E0611272/enhD and E0611331/enhP also showed the highest Z-score of 0.2 in adipose tissue of male adults at 24 weeks. For hindbrain of embryos of 13.5 days, the enhancer E0611282/enhP showed the highest Z-score of 2.28. As for bone marrow macrophage of male adults at 8 weeks, the enhancers E0611272/enhD and E0611331/enhP showed the highest Z-score of 0.67. There was a clear pattern of very similar activity in the enhancers E0611272/enhD and E0611331/enhP in tissues that are known to have dependence on the *wnt1* gene (bone marrow macrophage and adipose tissue). The enhancer E0611282/enhP showed high activity as well in hindbrain tissue, however it had fluctuating activity in the other tissues.

Conclusions

The results indicate the importance of the putative enhancers E0611272/enhD and E0611331/enhP in the regulation of *wnt1*, and in particular in the role of *wnt1* in bone formation. These findings will aid in efforts to use *wnt1* as a therapeutic target in the disease Osteogenesis Imperfecta. Future directions for this project would be to test the two enhancers E0611272/enhD and E0611331/enhP and their regulation of *wnt1* in bone cells. Zebrafish can be used as a model organism and these enhancers can be amplified by PCR, ligated to a plasmid with a reporter gene and transgenic fish can be recovered. If these enhancers show activity in zebrafish (e.g., they are able to drive EGFP reporter fluorescence in zebrafish embryos), this would prove the conservation of those sequences in zebrafish. The presence of orthologous enhancers in mouse and zebrafish would further confirm their importance in *wnt1* regulation, and this is a strong tool for future studies in understanding *wnt1* and OI and proposing therapeutics.

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