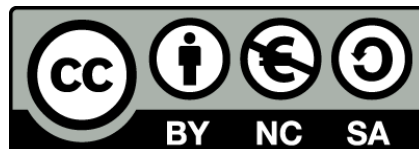




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Role of Wnt pathway genes in complex and monogenic phenotypes of low and high bone mass

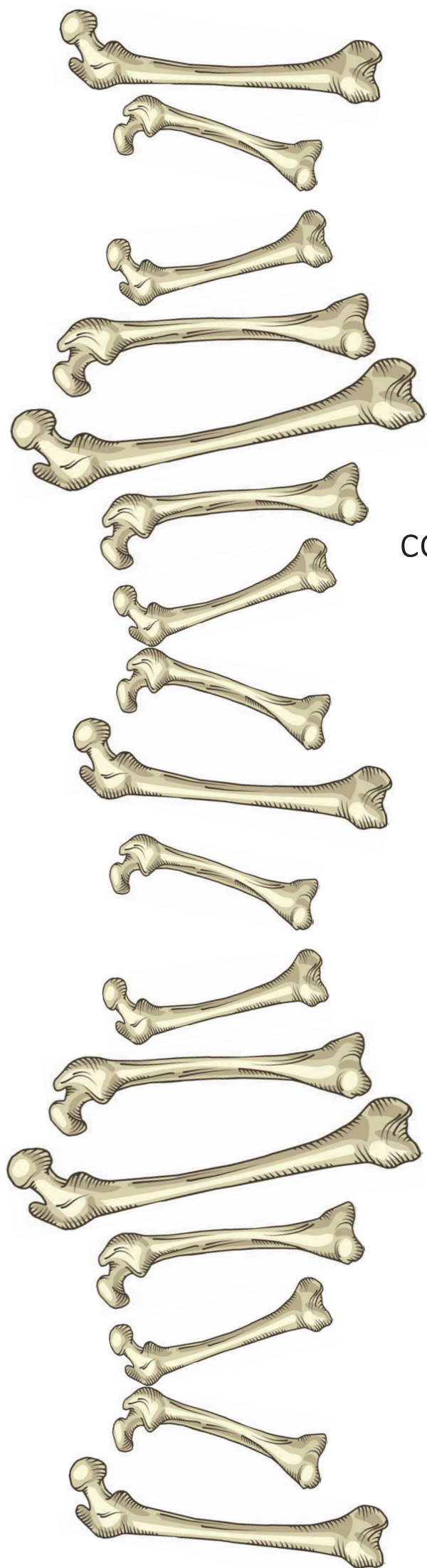
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Role of Wnt pathway genes in
complex and monogenic phenotypes
of low and high bone mass

Núria Martínez-Gil
2020

Tesi Doctoral

Universitat de Barcelona

**Role of Wnt pathway genes in complex and monogenic phenotypes
of low and high bone mass**

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Núria Martínez-Gil

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Departament de Genètica, Microbiologia i Estadística

Tesi dirigida pel Dr. Daniel Grinberg Vaisman (codirector) i la Dra. Susanna Balcells Comas (codirectora i tutora)



Dr. Daniel Grinberg Vaisman
(codirector)



Dra. Susanna Balcells Comas
(codirectora i tutora)



Núria Martínez Gil

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ABSTRACT

Osteoporosis is a complex disease characterized by low bone mass, microarchitectural deterioration, and increased fracture risk. It is the most common bone disease that affects more than 200 million people worldwide and is responsible for more than 8.9 million fractures every year, especially affecting the elderly. This makes it one of the major causes of morbidity and mortality and generates a high social-health cost, which is expected to grow as life expectancy has been increasing considerably over the last decades. Although nowadays there are different treatments available that reduce bone loss, few of them target bone formation enhancement. Therefore, it is essential to discover new anabolic therapeutic targets that restore bone mass and skeletal architecture. In this doctoral thesis, I have used two tools to identify new therapeutic targets by elucidating the genetic bases of the disease.

On the one hand, I have performed a search for variants in three Wnt pathway genes, that might explain the GWAS significant association with different bone parameters, and subsequently I characterized them functionally. Specifically, I have studied *DKK1*, *SOST*, and *WNT16*, identifying common and rare variants that may explain the susceptibility to the complex disease of osteoporosis or the extreme high bone mass (HBM) phenotype. Regarding *DKK1*, I have shown the loss of inhibitory function associated with missense mutations found in patients with HBM and/or in the general population. I have also detected for the first time a physical contact between the region enriched in BMD-GWAS signals and the *DKK1* promoter and also with, *LNCAROD*, a lncRNA known to regulate *DKK1* expression. For *WNT16*, I have identified a promoter region in intron 2 that interacts with different regulatory elements located in introns of the neighboring gene *CPED1*. Finally, in *SOST*, I have shown a decrease in gene expression associated with the minor allele of a variant that modifies an extended TATA box motif, a reduction in protein expression of a missense variant in the signal peptide found in an HBM women, and, for the first time, physical interactions between the *SOST* proximal promoter and *ECR5* and several enhancer elements upstream of it. These functional studies reinforce the importance of the Wnt pathway in bone homeostasis and demonstrate the need to study in-depth the GWAS signals that often do not correspond to a functional variant.

On the other hand, using whole-exome sequencing (WES), I have also investigated a family case of HBM in the search for a causal gene. Manual filtering of the low-frequency variants found in this case suggests that mutated *VAV3* - along with other variants in *SIK3*- could determine the HBM phenotype in the family due to an inhibitory effect on osteoclastogenesis. Importantly, the results have pointed at novel genes and regions which may constitute a new therapeutic target for osteoporosis.

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ABBREVIATIONS

4C: Circular chromosome conformation capture

AD: Autosomal dominant

AFF: Atypical femoral fracture

ALP: Alkaline phosphatase

APC: Adenomatous polyposis coli

AR: Autosomal recessive

AXIN1: Axis inhibition protein 1

BARCOS: Barcelona osteoporosis (name of the cohort of Spanish postmenopausal women)

BF: Bone formation

BM: Bone mass

BMD: Bone mineral density

BMI: Body mass index

BMP: Bone morphogenetic proteins

BMU: Bone multicellular units

BP: Bisphosphonates

BR: Bone resorption

BSAP: Bone specific alkaline phosphatase

BSP: Bone sialoprotein

BTM: Bone turnover markers

BUA: Broadband ultrasound attenuation

CADD: Combined annotation dependent depletion

CAMKII: Calmodulin-dependent protein kinase II

cCRES: candidate cis-regulatory elements

CDC42: Cell division control protein 42

CK1: Casein kinase 1

cKO: Conditional KO

CLC-7: Chloride channel 7 alpha subunit

CMI: Chiari malformation type I

CNV: Copy number variations

CRD: Cysteine rich domain

CRISPR: Clustered regularly interspaced short palindromic repeats

CTSK: Cathepsin K

CTX1: C-terminal cross-linking telopeptides of type I collagen

DAAM1: DVL-associated activator morphogenesis

dCAS9: deficient Cas9

DKK: Dickkopf

dKO: double KO

DMP1: Dentin matrix acidic phosphoprotein 1

DVL: disheveled

DXA: Dual-energy X-ray absorptiometry

eBMD: estimated BMD

ECM: Extracellular matrix

ECR5: Evolutionarily conserved region 5

ELISA: Enzyme-linked immunosorbent assay

ENCODE: Encyclopedia of DNA Elements

eQTL: expression quantitative trait locus

ER: Endoplasmic reticulum

ERES: Estrogen response elements

ERK: Extracellular signal-regulated kinases

ERT: Estrogen replacement therapy

ESE/ISE: Exonic/intronic splice enhancers

ESS/ISS: Exonic/intronic splice silencers

EXAC: Exome aggregation consortium

FDA: Food and drug administration

FGF: Fibroblast growth factors

FN-BMD: Femoral neck BMD

FNL: Femur neck length

FNSA: Femur neck axis angle

FNSM: Femoral neck section modulus

FPS: Farnesyl pyrophosphate synthase

FRAX: Fracture risk assessment

FZD: Frizzled receptor

GARFIELD: GWAS analysis of regulatory or functional information enrichment with LD correction

GEFOS: Genetic factor for osteoporosis

GENOMOS: Genetic markers for osteoporosis

gnomAD: genome aggregation database

GoF: Gain-of-function

GSK3 β : Glycogen synthase kinase 3 β

GTex: Genotype-tissue expression

GWAS: Genome wide association studies

H2: Heritability

HA: Hydroxyapatite

HBM: High bone mass

het KO: heterozygous KO

HGMD: Human gene mutation database

hOB: human osteoblast

HR-pQCT: High resolution peripheral QCT

HRT: Hormone replacement therapy

IFMRS: International federation of musculoskeletal societies

IMPC: International phenotyping consortium

IP3: Inositol 1,4,5 triphosphate

iPSC: induced pluripotent stem cells

iRNA: interference RNA

JNK: JUN kinase

KD: Knock-down

KO: Knock-out

KRM: Kremen

LBM: Low bone mass

LCL: Lymphoblastoid cell lines

LD: Linkage disequilibrium

LF: Low frequency

LGR: Leucine-rich repeat-containing G-protein-coupled receptor

LoF: Loss-of-function

LRP: Low-density lipoprotein receptor-related protein

LS-BMD: Lumbar spine BMD

MAF: Minor allele frequency

MAPK: Mitogen-activated protein kinase

MCS: Multiple cloning site

M-CSF: Macrophage colony stimulating factor

MEF2C: Myocyte-enhancer factor 2c

MHC: Major histocompatibility complex

miRNA: microRNA

MMP9: Matrix metalloproteinase 9

MPRA: Massively parallel reporter assay

MRI: Magnetic resonance imaging

MSC: Mesenchymal stem cells

MSK-KP: Musculoskeletal knowledge portal

NCBI: National center of biotechnology information

ncRNA: non-coding RNA

NFAT: Nuclear factor of activated T cells

NF- κ B: Nuclear factor kappa B

NGS: Next generation sequencing

NNW: Narrow neck width

NTX1: N-terminal cross-linking telopeptides of type I collagen

NV: No viable

OA: Osteoarthritis

OB: Osteoblast

OBCD: Origins of bone and cartilage disease

OC: Osteoclast

OCN: Osteocalcin

Ocy: Osteocyte

OI: Osteogenesis imperfecta

ONFH: Osteonecrosis of the femoral head

OP: Osteoporosis

OP: Osteoporosis

OPG: Osteoprotegerin

OPN: Osteopontin

OPPG: Osteoporosis-pseudoglioma syndrome

OSX: Osterix

P1CP: Type I serum procollagen C-terminal propeptide

P1NP: Type I serum procollagen N-terminal propeptide

PCP: Planar cell polarity

PDB: Protein data bank

PKC: Protein kinase C

PKD1/2: Polycystin-1/2

PLC: Phospholipase C

PMD: Plasma membrane disruption

PRCN: Porcupine

PROMPTS: Promoter upstream transcript

PTH: Parathyroid hormone

QCT: Quantitative computed tomography

QUS: Quantitative ultrasound

RA: Rheumatoid arthritis

RAC: Ras-related C3 botulinum substrate

RANK: Receptor activator of nuclear factor- κ B

RANKL: Receptor activator for nuclear factor κ B ligand

REF: References

REMS: Radiofrequency echographic multi spectrometry

RHOA: Ras homolog gene family member A

RNAPII: RNA polymerase II

RNF43/ZNRF3: Ring finger protein 43/ Zing and Ring finger 3

ROCK: Rho kinase

ROR1/2: Receptor tyrosine kinase-like orphan receptor 1 or 2

RSPO: R-spondin ligand family members

RUNX2: Runt related transcription factor 2

RYK: receptor-like tyrosine kinase

SD: Standard deviation

SERM: Selective estrogen receptors modulators

SFRP: Secreted frizzled-related proteins

SRA: Sequence read archive

TALEN: Transcription activator-like effector nuclease

TCF/LEF: T-cell factor/lymphoid enhancer factor

TF: Transcription factor

Tg: Overexpression transgenic mice.

TGF β : Transforming growth factor beta

TLE: Transducin-Like Enhancer of Split proteins

TRAP: Tartrate-resistant acid phosphatase

TR-BMD: Trochanteric BMD

TRPV4: TRP cation channel subfamily V member 4

TSS: Transcription start sites

UCSC: University of California Santa Cruz

UTR: Untranslated regions of mRNA

VANGL: Van Gogh-like

VGCC: L-type voltage-gated calcium channel

VOS: Speed of sound

VP: Viewpoint

WES: Whole exome sequencing

WGS: Whole genome sequencing

WHO: World Health Organization

WIF1: Wnt inhibitory factor 1

WLS: Wntless.

YAP/TAZ: Yes-associated protein/transcriptional co-activator with a PDZ-binding domain

β -TrCP: β -Transducin repeat-containing protein

INTRODUCTION

1. THE BONE TISSUE

1.1. Generalities

The skeletal system is the biological system comprising bone and cartilage tissues, and has several important functions, including: i) mechanical: providing structural support for the body and thus allows movement and locomotion, ii) protective: acting as a physical barrier against external threats for all the vital organs (such as the brain, spinal cord, heart, and lungs) and bone marrow that lie within, iii) metabolic: maintaining mineral homeostasis and pH balance, iv) environmental: generating optimal conditions for bone marrow hematopoiesis and v) reservoir: acting as a source of growth factors and cytokines (1,2).

1.2. Structure and types of bone

Mature long bones are structured by a central part, also called diaphysis, and two peripheral ends, also called epiphyses (Figure 1). Epiphyses and diaphysis join through metaphysis, that contains the growth plate and a layer of hyaline cartilage in a growing bone or an epiphyseal line of osseous tissue in the early adulthood. While the epiphysis is filled with trabecular bone tissue with red marrow, the walls of the diaphysis are composed of cortical bone and the central area contains the medullary cavity, which is filled with yellow marrow. Covering the medullary cavity, we find the endosteum, a delicate fibrous layer of connective tissue that contains bone cells. Covering the diaphysis and epiphysis we find the periosteum, a fibrous and resistant connective tissue membrane, which contains blood and lymphatic vessels and nerves. Both the endosteum and the periosteum are responsible for the bone growth, remodeling and repair of fractures. The periosteum covers the entire external surface of the bone except for the ends of the epiphysis where the articular cartilage is found to form the joints. El articular cartilage allows the sliding of the surfaces in close contact (1,3).

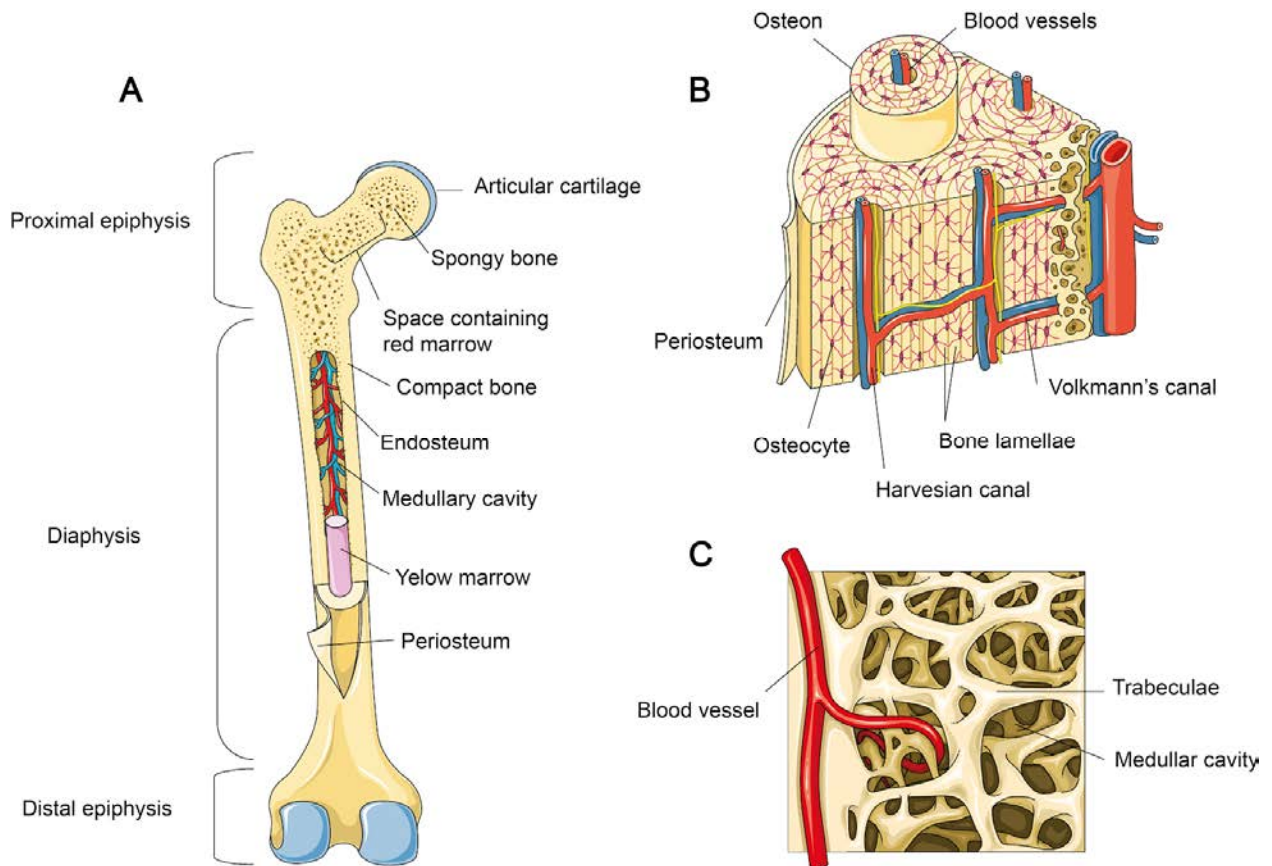


Figure 1. (A) Structure and histology of long bones. (B) Three-dimensional diagram of a block of cortical bone from the diaphysis of a long bone. (C) Diagram of a trabecular bone of the epiphysis of a long bone. Image created with Servier Medical Art.

According to histology, we can classify bone tissue into cortical bone or trabecular bone (Figure 1B and C). While cortical bone is responsible for mechanical activity, trabecular bone is responsible for the metabolic functions and harbors the red bone marrow. For these reasons, trabecular bone houses the largest area of bone remodeling, and it is in this region that most bone pathologies are concentrated. Adult bone tissues are composed of 80% cortical bone and 20% trabecular bone, although this proportion may vary depending on the location within the body. The cortical bone is dense, solid and mostly calcified. It can be found in the diaphysis of long bones, in the inner and outer region of flat bones and in different areas of the rest of the bones. Cortical bone is made up of concentric layers of bone lamellae (osteons), which are nourished by the Haversian ducts and Volkmannian ducts that contain the blood vessels. In contrast, the trabecular bone has a porous bone matrix, without osteons. It has interstitial sheets of irregular shape, forming the trabeculae and between these trabeculae is located the red bone marrow. The trabecular bone can be found in the

epiphysis of the long bones and in the interior of flat bones. Unlike cortical bone, blood vessels penetrate directly into the trabecular bone (1,3,4).

According to its shape, we find 4 categories of bone: long, short, flat and irregular bones. Long bones are those that have a length greater than the thickness and width. These include the clavicle, humerus, ulna radius, metacarpals, femur, tibia, fibula, metatarsals, and phalanges. The short bones have similar sizes in all 3 dimensions, and among these are the carpus, tarsus, patella and sesamoids. The flat bones are thin and wide and protect the organs. Among these we find the skull, jaw, shoulder blades, sternum and ribs. Finally, irregular bones have irregular shapes and perform different functions. In this group we find the vertebrae, sacrum, coccyx and hyoid (5).

1.3. Bone constituents

The bone tissue is formed by a mineralized extracellular matrix (ECM) and different types of cells.

1.3.1. Extracellular matrix

The bone ECM is composed of a mineralized fraction (~ 70%) and an organic fraction (~ 30%). The mineralized portion is mainly made up of calcium and phosphate in the form of hydroxyapatite crystals ($\text{Ca}_{10}(\text{PO}_4)(\text{OH})_2$) that are lodged between the collagen fibers, which endows the bone with its stiffness and resistance. The organic fraction is composed mainly of type I collagen (~ 90%) that provides elasticity, flexibility and the ability to absorb mechanical energy. The remaining 10% is made up of proteoglycans and non-collagen proteins that are essential for bone development, growth, remodeling and repair. Some of these are Alkaline Phosphatase (ALP), Osteocalcin (OCN), Osteonectin and Osteopontin (OPN). In addition, the extracellular matrix also houses numerous cytokines and growth factors, such as Transforming Growth Factor β (TGF β) or Bone Morphogenetic Proteins (BMP) (3,6).

1.3.2. Cells

We categorized the bone cells into two different lineages: the bone forming cells and the bone resorbing cells.

1.3.2.1 Bone forming cells

The osteoblasts are the cells responsible for bone formation and mineralization. They originate from mesenchymal stem cells (MSC), but may also derive from bone lining cells and potentially from chondrocytes (7,8). MSCs can also differentiate into chondrocytes, adipocytes, myocytes, and fibroblasts (9,10). The cell fate depends on the presence of specific stimuli. In the case of the differentiation of MSC to osteoblasts, a process termed osteoblastogenesis (Figure 2), the transcription factors (TF) Runt related transcription factor 2 (RUNX2) and Osterix (OSX), play an essential role (11,12). MSCs will give rise to osteoprogenitor cells that differentiate into osteoblast precursors and finally into mature osteoblasts (Figure 2). They have a cuboidal morphology, with a large and clear nucleus, abundant cytoplasm, enlarged endoplasmic reticulum and Golgi apparatus, and a large number of free ribosomes due to the abundance of proteins they synthesize (6,13). Osteoblasts are found in bone surface sites where bone formation is active, where they secrete type I collagen and non-mineralized ECM, OCN, OPN and ALPL, for subsequent mineralization (1).

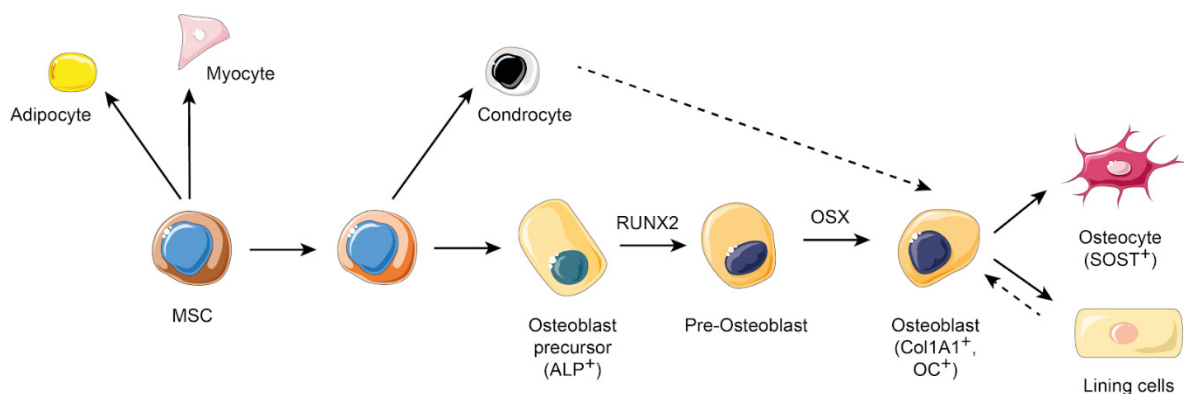


Figure 2. Osteoblastogenesis. MSC cells give rise to mature osteoblasts that differentiate into osteocytes or lining cells. Moreover, osteoblasts can also derive from chondrocytes or lining cells (dashed lines). Some of the biochemical markers for each cell type are highlighted in parentheses. RUNX2 and OSX are two key TFs important for the differentiation process. Image created with Servier Medical Art.

Once differentiated, osteoblasts can undergo apoptosis (50-70%) or mature, becoming osteocytes or lining cells. When the osteoblast is surrounded by the extracellular matrix itself, it becomes an osteocyte. Osteocytes are terminally differentiated bone cells enclosed in the mineralized bone matrix, and they are

responsible for mechanotransduction, control of bone remodeling and calcium homeostasis. They are the most abundant cell type in bone tissue representing 90% of all adult bone cells (14), and present a flattened dendritic morphology, with small rough endoplasmic reticulum and Golgi apparatus. They have filopodia, generating an extensive canalicular network that allows communication and the exchange of molecules with neighboring cells (13,15,16). Unlike osteoblasts, osteocytes do not express much type I collagen or ALP, but instead begin to express other proteins such as sclerostin or Dentin matrix acidic phosphoprotein 1 (DMP1). Osteocytes do not divide and have an average life of 25 years. In addition, inactive osteoblasts can mature into the lining cells that cover the bone surface when there is no active growth. The lining cells have a flattened and elongated structure and regulate the flow of mineral ions from bone extracellular fluid, thus serving as a blood–bone barrier. They retain the ability to redifferentiate to osteoblasts through stimulation by parathyroid hormone (PTH) or different mechanical forces (6,17).

1.3.2.2 Bone resorbing cells

The cells responsible for bone resorption are the osteoclasts, large phagocytic multinucleated cells. These cells are the product of the fusion of mononucleated osteoclast precursors derived from the myeloid lineage of hematopoietic stem cells. In the differentiation process, termed osteoclastogenesis (Figure 3), specific molecular signals are necessary for the mononuclear cell differentiation and fusion process. Among these we highlight Receptor Activator for Nuclear Factor κ B Ligand (RANKL), critical for the formation of osteoclasts, and macrophage colony stimulating factor (M-CSF), necessary for the proliferation, survival and differentiation of osteoclast precursors and for the cytoskeletal changes necessary for bone resorption. These two molecular signals are expressed by the osteoblasts and osteocytes, while the osteoclast progenitors express the respective receptors M-CSF and Receptor activator of nuclear factor-kappa B (RANK). In addition to RANKL and M-CSF, osteoblasts express osteoprotegerin (OPG) a decoy receptor of RANKL, which inhibits the RANKL–RANK interaction to suppress bone resorption (18). Mature osteoclasts promote structural changes in the cytoskeleton and cell adhesion and are found on the bone surface where the bone is injured, being removed or remodeled. The part of the osteoclast membrane that is in contact with the bone surface presents invaginations that constitute the ruffled border, with many mitochondria and lysosomes

that release protons and lysosomal hydrolases during resorption in order to degrade the ECM (19).

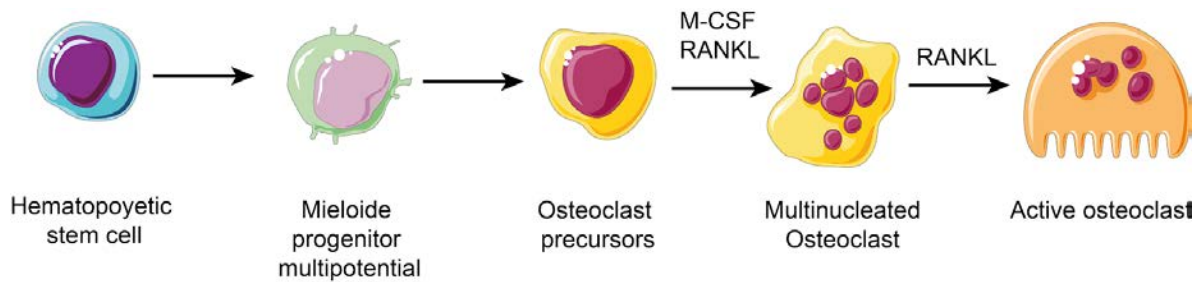


Figure 3. Osteoclastogenesis. Hematopoietic stem cells will give rise to myeloid progenitors that in the presence of M-CSF and RANKL differentiated into active osteoclast. Image created with Servier Medical Art.

1.4. Skeletal development

Bones are formed during embryonic development by two different processes: **intramembranous ossification** and **endochondral ossification**. In both cases, mesenchymal cells first aggregate to form mesenchymal condensations that prefigure the shape of future skeletal elements. In intramembranous ossification, this condensation will lead to the direct differentiation of the MSCs into osteoblasts, which secrete an osteoid that slowly becomes mineralized. The flat bones of the skull are formed through this process. In contrast, in endochondral ossification, condensation will lead to the differentiation of MSCs into chondrocytes that will produce a cartilage template of the future bone, and, upon hypertrophy and apoptosis of chondrocytes, will be populated by blood vessels and osteoblasts precursors and eventually replaced by bone tissue. A thin layer of cartilage remains between the diaphysis (central portion) and the epiphysis (rounded end) of the bone, forming the epiphyseal plate so that the bone can continue to grow until adulthood, when the epiphyseal plate is completely ossified. The long bones, vertebrae, pelvis, and skull base bones are formed by endochondral ossification. Both processes are regulated at the molecular level by a large number of growth factors and TFs, among them various components of the Wnt pathway (20).

1.5. Bone remodelling

The homeostasis of adult bone mass under healthy conditions is maintained through the exquisite balance of bone resorption by osteoclasts and bone formation by

osteoblasts. Bone remodelling is the process of renewing bone tissue with the aim of maintaining mineral homeostasis and bone resistance, avoiding the accumulation of microdamage in the bone. Bone remodeling takes place throughout life, but it only has a positive balance until the age of 30, when under normal conditions, between 5 and 10% of the bone is renewed each year (13). Bone remodeling occurs in special units called bone multicellular units (BMU) which are made up of different cell types and are spatially organized and temporally regulated. The process consists of five overlapping phases:

1. **Activation:** First, a detection of an initiating remodelling signal (direct mechanical strain or hormones) results in the recruitment and activation of circulating osteoclast precursors. Next, the lining cells on the bone surface are removed to allow osteoclast precursor to bind to the bone matrix. Multiple mononuclear cells fuse to form multinucleated pre-osteoclasts which bind to the bone matrix forming the resorption pit.
2. **Resorption:** A sealing zone is formed between the osteoclast and the bone surface. The plasma membrane of the osteoclast is enlarged, generating a ruffled border with proton pumps (H^+ -ATPase) and chloride channels (Chloride channel 7 α subunit; CLC-7) that generate an acidic extracellular environment (pH 4.5) to dissolve the bone mineral. Next, cytoplasmic lysosomes release phosphatases (e.g. tartrate-resistant alkaline phosphatase; TRAP), proteases (e.g. cathepsin K; CTSK) and matrix metalloproteinases (e.g. Matrix metalloproteinase 9; MMP9) that allows digestion of the organic matrix; the resorption phase is completed by mononucleated cells after multinucleated cells undergo apoptosis (21–24).
3. **Reversal:** Bone resorption changes to bone formation; the osteoblast lineage cells are recruited and remove bone matrix leftovers, subsequently deposit a first layer of proteins in the resorption pits and finally, form a cement line to enhance osteoblastic adherence (25,26).
4. **Formation:** The reabsorption of the bone mineral matrix results in the release of growth factors and TFs (fibroblast growth factors (FGF), $TGF\beta$, BMPs, interleukins) responsible for the recruitment and differentiation of osteoblasts. The osteoblasts synthesize a new organic matrix called osteoid (especially type

I collagen and proteoglycans) and gradually mineralize it by deposition of calcium phosphate crystals (27).

5. **Termination:** 50–70% of osteoblasts enter apoptosis, or become osteocytes or lining cells. The end result of the bone remodeling process is the production of a new osteon.

Bone remodeling takes 120 to 200 days in cortical and trabecular bone, respectively, and three-quarters of this time are intended for the bone formation process (13).

1.6. Bone mechanotransduction

Bone tissue has the capacity to adapt to mechanical forces such as compression, tension, shear forces and gravity. Mechanotransduction in bone comprises the intracellular signaling pathways that are activated in response to external mechanical forces and will ultimately result in bone formation or resorption. Osteocytes are the main responders to those forces (28).

Within bone, mechanical loading causes a fluid movement from the high-pressure to low-pressure regions, where the osteocytes undergo transient plasma membrane disruption (PMD) that allows for calcium influx into the cell, which in turn initiates mechanotransduction (28–30). Gap junction proteins like the connexin family and ion channels such as the TRP cation channel subfamily V member 4 (TRPV4), L-type voltage-gated calcium channel (VGCC) and polycystin-1/2 (PKD1/2) act as mechanosensors (30). Both calcium and ATP are important messengers of mechanotransduction (29). The opening of these channels is followed by the activation of mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases (ERK) and Wnt signaling (31,32). The mechanical loading through expression regulation promoted osteoblast differentiation and bone formation while osteoclastogenesis and bone resorption are reduced. Unloading results in the opposite effect (28,30,33). Interestingly, both the volume and quality of the bone are increased by mechanical loading (exercise), as the formation of new bone is stimulated on highly strained surfaces while surfaces that experience lower strain remain quiescent. Furthermore, unloading or immobilization has been documented to cause rapid bone loss (28,30,33).

2. GENETIC SKELETAL DISORDERS

Genetic disorders are a class of diseases caused by a change in the DNA sequence that produces an abnormal molecular function, and can be categorized in monogenic or polygenic. The monogenic disorders are caused by rare highly penetrant alleles affecting a single gene and generally follow mendelian inheritance, even though they can also be influenced by genetic or environmental modifiers. On the other hand, the polygenic disorders are caused by the additive effects of many susceptibility variants with small individual effects, and are highly influenced by environmental factors.

Genetic skeletal diseases are those that present significant skeletal involvement. In their last nosology and classification (34), 461 different genetic skeletal disorders are described for which the genetic cause of 425 (92%) has been identified. Among them, we can find both monogenic diseases, for example osteogenesis imperfecta (OI), and polygenic diseases such as, osteoporosis. OI type I is a rare pathology of bone fragility characterized by growth delay and frequent fractures and caused in 85% of the cases by rare mutations in *COL1A1* or *COL1A2* genes (35,36). In contrast, osteoporosis is a common bone fragility disease caused by small additive effects combined with environmental factors. Among the genetic skeletal diseases there is a wide range of affectations ranging from neonatal lethality to only mild growth retardation or deformity. The diagnosis of these pathologies includes the description of the growth pattern and other clinical characteristics, skeletal radiographs, biochemical diagnostics and genetic tests.

In this section, I will introduce the main characteristics of three genetic skeletal disorders. Specifically, we will review Osteoporosis (OP), the High Bone Mass (HBM) phenotype and Chiari type I malformation.

2.1. Bone mineral density (BMD)

Within genetic skeletal diseases we find a very large subgroup containing skeletal dysplasia that affects the bone mass. Therefore, the parameter used to measure the state of bone and therefore for the diagnosis of these bone pathologies is the complex quantitative trait of bone mineral density (BMD). BMD is normally distributed in the population, with two small groups at the extremes with very high (HBM) or very low (LBM: Low Bone Mass) BMD values (Figure 4).

The BMD of an individual increases with age until it reaches its maximum peak approximately between 25 and 30 years, where the synthesis and resorption of bone are equivalent. From this point onwards, the resorption overcomes the formation, leading to a gradual loss of BMD. The peak value of BMD is lower in women than in men and, in women during the first years of menopause, bone mass decreases sharply due to decreased production of estrogens (Reviewed in (37)).

The BMD value is expressed in grams of mineral per square centimeter (g/cm^2) and is measured by a non-invasive medical densitometry test, such as the dual-energy X-ray absorptiometry (DXA). BMD is generally measured in the neck of the femur (FN-BMD) and in the lumbar spine (LS-BMD). From the BMD values, the relationship with the population mean is calculated in terms of standard deviations. Two types of scoring can be used for this calculation: T-score and Z-score. The T-score compares the patient's BMD with the average peak value of bone mass in adults of the same sex and population. The T-score value is expressed as the number of standard deviations (SD) with respect to the value of said peak. Similarly, the Z-score is expressed as the number of SD from a reference value, but this time this value is the average BMD of adults of the same sex, population and age (38,39).

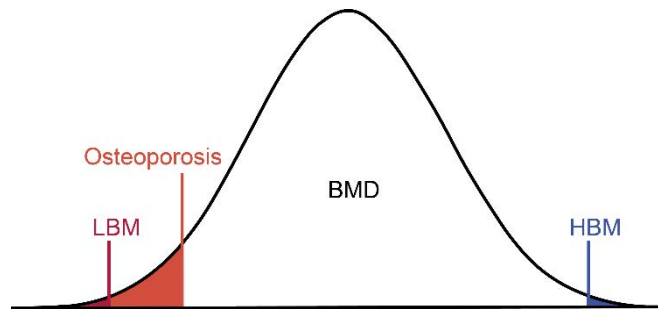


Figure 4. BMD distribution in the general population. Gaussian curve showing the BMD distribution in the population. In red the rare LBM phenotypes, such as Osteogenesis Imperfecta, in orange the complex disease osteoporosis and in blue the HBM phenotypes, such as Osteopetrosis.

In most cases, BMD is a good marker of bone status, which explains 50-70% of the variation in bone strength and is an excellent predictor of the occurrence of fractures. Despite this, the BMD only gives us information on the quantity of tissue and not on bone quality (40–42). To fully characterize the basic components of the bone strength (composition, microarchitecture, size and shape) various *in vivo* imaging techniques are being developed, including magnetic resonance imaging (MRI), qualitative computed tomography (QCT), high resolution peripheral QCT (HR-pQCT), quantitative ultrasound (QUS), radiofrequency echographic multi spectrometry (REMS) and microindentation (43).

In addition to these techniques, biochemical bone turnover markers (BTMs) and metabolic tests that measure the products of collagen metabolism can be quantified to determine bone structure and strength. These markers are very useful to determine the speed of bone loss, the risk of fracture and to check the functioning of the therapy. Despite this, clinical use is still limited, and more research is needed to determine its usefulness. Some examples of these BTMs are summarized in Table 1 (44–46).

Table 1. BTMs and metabolic tests to know the state of the bone.

Biochemical markers	Examples	Function
Bone Resorption Markers	C-terminal and N-terminal cross-linking telopeptides of type I collagen (CTX1 and NTX1)	Proteolysis of osteoclasts
	Collagen Pyridinium Cross Links (Deoxypyridinoline and Pyridinoline)	Collagen Cross Link Peptides
	Hydroxyproline	Released in urine during bone collagen breakdown
	TRAP	Dephosphorylation of bone matrix proteins, generates reactive oxygen species for bone matrix degradation
	Cathepsin K	Collagen Breakdown
Bone Formation Markers	Sialoprotein bone	Binds to integrin $\alpha v \beta 3$, nuclear hydroxyapatite and enhances bone resorption.
	Type I serum Procollagen , N-terminal Propeptide (P1NP)	Synthesis of Collagen in Bone
	Type I serum procollagen, C-terminal propeptide (P1CP)	Synthesis of collagen in bone
	Serum OCN	Osteoid mineralization
	Serum bone specific alkaline phosphatase (BSAP)	Bone matrix calcification

2.2. BMD heritability

The percentage of genetic contribution of a character is expressed through the calculation of heritability. Heritability (h^2) is the proportion of the total variance of the character that is determined by genetic factors. This is calculated through twin and family studies. BMD shows considerable heritability; between 50 and 85% of the variance in the peak BMD is genetically determined (47–51). This heritability varies

depending on the measurement site, the population used and the sex. Heritability is normally found to be higher in men than in women and in axial sites (spine or hip) than in the forearm (52,53), the head being the place with the highest heritability (54).

There is also a hereditary component to age-related bone loss, but the genetic contribution is less than in peak bone mass establishment (55). In women, about 40% of the variance in bone loss in the wrist and lumbar spine is genetically determined, while no significant genetic component was found for bone mass loss in the femoral neck (56–58). This is consistent with the fact that the age of menopause is genetically determined (59), with estrogen deficiency after menopause being the main determinant of bone loss in women. In man, there is no evidence of a genetic component in wrist bone loss, but far fewer studies have been conducted (60).

In addition, the genetic influence on bone metabolism has been determined for geometric parameters ($h^2 = 30\text{--}70\%$) (61), bone ultrasound ($h^2 = 40\text{--}50\%$) (53), bone microarchitecture, predicted by HR-pQCT ($h^2 = 20\text{--}80\%$), (62) and BTMs such as BSAP ($h^2 = 67\text{--}80\%$), OCN ($h^2 = 14\text{--}44\%$) and PTH ($h^2 = 54\text{--}65\%$) (63).

2.3. Osteoporosis

2.3.1. General description and symptoms

Osteoporosis is a generalized disease of the skeletal system characterized by loss of bone mass and microarchitecture deterioration of bone tissue, which compromises bone resistance and consequently causes increased fragility and susceptibility to fractures (64) (Figure 5). Osteoporosis can be considered as a pathological imbalance of bone remodelling processes, where bone resorption dominates over bone formation, resulting in a negative total bone balance (55). Osteoporosis does not present symptoms so the diagnosis is made through

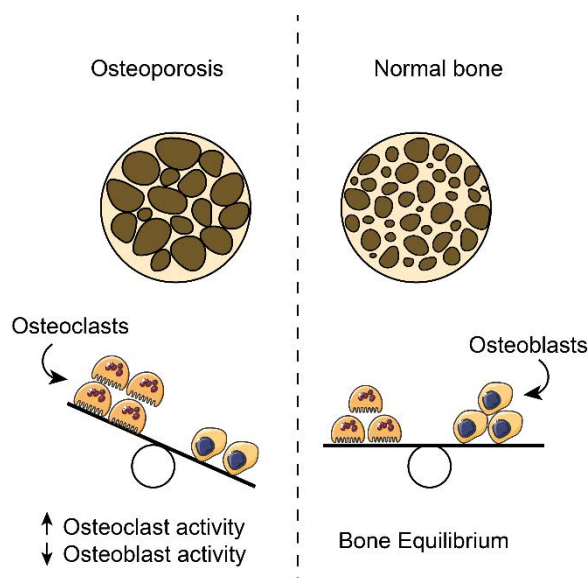


Figure 5. Graphical representation of trabecular bone BMD and osteoclast-osteoblast equilibrium osteoporosis and normal bone.

the quantitative measurement of BMD. The clinical importance of the disease lies in osteoporotic or fragility fractures. Osteoporotic fractures occur in situations where healthy people would not suffer a bone fracture. These fractures normally occur at major skeletal sites such as vertebrae (spine), proximal femur (hip), distal forearm (wrist), or shoulder (44). These fractures lead to high morbidity/mortality and a reduction in the quality of life, so that the social-health cost of this disease is high and increases with increasing life expectancy (65,66).

2.3.2. Heritability of osteoporosis features

In addition to BMD, osteoporotic fracture also has considerable heritability, estimated at 54-68% (67,68). Fracture is a very complex phenotype, mainly due to the fact that most fractures are due to a fall, and the risk of falling is a complex character with a very high environmental contribution. Therefore, the heritability of the fracture decreases with age, since the environmental component increases (68).

Other determinants of bone strength such as porosity, cortical thickness, morphology of the trabecular bone and intrinsic properties indicative of the quality of bone tissue, also contribute to the genetic predisposition of osteoporotic fractures but have been less studied and require larger samples due to its high polygenic component (Reviewed in (69)).

2.2.3. Diagnosis

Osteoporosis is a silent pathology with no symptoms or evidence until a fracture occurs. The diagnosis of osteoporosis is made through BMD values and through cases of fracture in adulthood in the absence of major trauma. In 1994, the World Health Organization (WHO 1994), by standardizing the bone mineral density values, defined the following diagnostic categories for osteoporosis (Figure 6):

- Normal: when the BMD T-score is greater than -1 SD.
- Osteopenia: when the BMD T-score is between -1 and -2.5 SD.
- Osteoporosis: When the BMD T-score is less than -2.5 SD, and BMD Z-score less than -1 SD.
- Severe or established osteoporosis: When the presence of fractures is added to the criterion of osteoporosis.

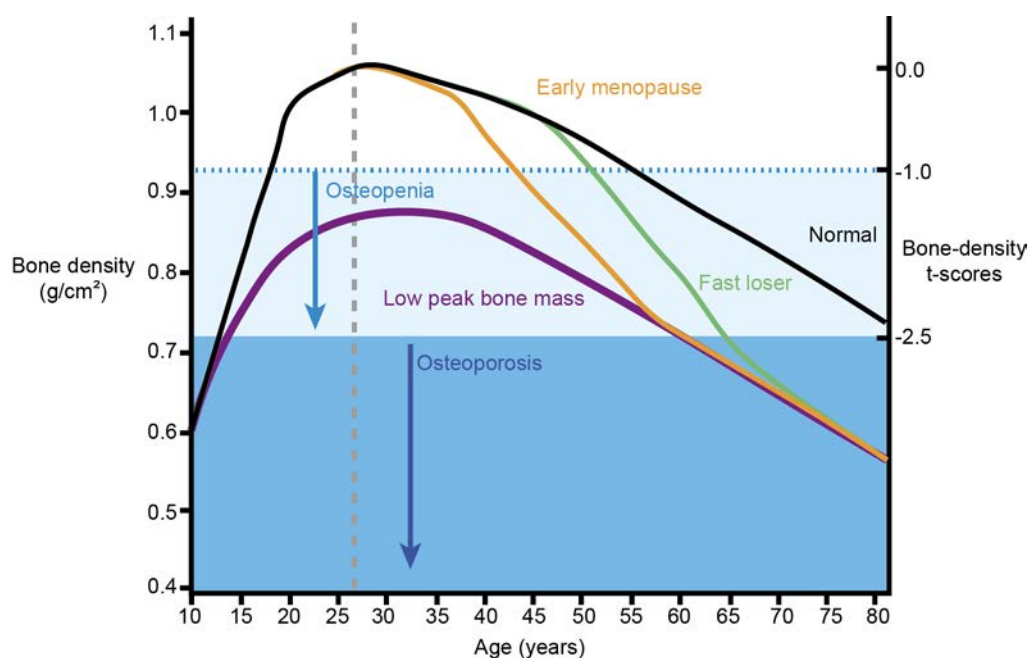


Figure 6. Variation in BMD of women at different ages. In black the normal situation, with the BMD peak around 27 years and gradual loss of BMD accentuated at menopause. We show 3 conditions for which osteoporosis can occur. In orange the early menopause where the age of menopause is earlier, so the steepest decline is advanced in time. In green the fast loser, those women who do not lose BMD gradually but do so at high speed. In purple the low peak bone mass where the maximum peak of BMD is lower so the gradual loss will lead to osteoporosis earlier. In the two shades of blue, the t-score and g/cm² values from which osteoporosis or osteopenia are considered. Adapted from (70).

In addition to these diagnostic techniques, to apply a pharmacological treatment, the diagnosis must be complemented with laboratory tests (blood or urine tests) that exclude secondary causes of osteoporosis (e.g. hyperparathyroidism, osteomalacia) (Office of the Surgeon General (US) (2004); National Osteoporosis Foundation (2003)).

Furthermore, fracture risk calculators, for example the FRAX tool (Fracture Risk Assessment; www.nof.org) have been developed to calculate the probability of fracture risk to be calculated at 10 years based on clinical risk factors, with or without the FN-BMD value. Some of the clinical features it takes into account are age, sex, ethnicity, previous fractures, body mass index (BMI), family history of fractures, use of oral glucocorticoids, rheumatoid arthritis, and secondary causes of osteoporosis including tobacco and alcohol consumption (71,72).

2.3.4. Classification

Osteoporosis is generally classified into two types: primary or secondary osteoporosis. Primary osteoporosis constitutes the largest group and includes osteoporosis cases that are not directly caused by another disease. Within primary osteoporosis, 2 subcategories are distinguished according to age range: younger than 70 years (primary osteoporosis type 1) and older than 70 years (primary osteoporosis type II). Postmenopausal type I or primary osteoporosis is the one that generally occurs in postmenopausal women and is dependent on hormonal level. It is characterized by an accelerated and disproportionate loss of bone. Primary type II or senile osteoporosis affects individuals of both sexes as a consequence of a hormonal deficit and metabolic changes associated with the normal aging process. It is characterized by fractures of the neck of the femur, and of the proximal portion of the humerus and pelvis, and both cortical and trabecular bone are affected. Two other types of primary osteoporosis are: disuse osteoporosis, caused by long periods of inactivity or immobilization that induces bone resorption and inhibits bone formation ; and juvenile osteoporosis, a very rare pathology normally caused by hypercalciuria or by low calcium absorption affecting children between 8 and 14 years (73, 46).

Secondary osteoporosis are all those cases of osteoporosis that are a consequence or an accompanying manifestation of other diseases or their treatment. Some secondary causes of osteoporosis are endocrinological diseases (e.g. hypogonadal states, endocrine pathologies, diabetes), gastrointestinal diseases, genetic disorders (e.g. hypophosphatasia), hematological disorders (e.g. leukemias), rheumatic diseases (e.g. rheumatoid arthritis), organ transplantation, drugs (e.g. anticoagulants) and nephritic diseases. These can cause poor development of bone mass or accelerate and enhance the loss of bone mass.

2.3.5. Risk factors

Osteoporosis is a complex disease determined both by genetic factors, by environmental factors and by the interaction of both. Thanks to different epidemiological studies, many markers and risk factors have been identified that carry a greater probability of developing osteoporosis. The main risk factors are detailed in Table 2. Some with higher specific weight are: more than 10 years of menopause,

history of previous fracture, family history of osteoporosis (genetic factors) and age (39,44,45,74).

Table 2. Osteoporosis Risk Factors

Advanced age
Female gender
Associated with the female risk factor:
- Early menopause
- Late menarche
Low BMI
Previous fragility fractures
Medication (e.g. glucocorticoids, anticoagulants, anticonvulsants)
Current smoking
Excessive alcohol intake
Depressed mood
Hormonal status (estrogens and testosterone)
Family history (genetic factors)
Vitamin D deficiency
Caucasian or Asian ethnicity
Malnutrition (low calcium, phosphorus, magnesium, protein insufficiency)
Low physical activity
Falls and risks that increase falls
Other diseases (e.g. diabetes, rheumatoid arthritis)
Bone geometry

2.3.6. Epidemiology

Osteoporosis is the most common bone disease that affects more than 200 million people worldwide and is responsible for approximately 8.9 million fractures annually (65). Because osteoporosis is the most common complex disease of bone, it constitutes a major public health problem. The incidence of osteoporosis varies between geographic areas. In Europe, 27.6 million people suffer osteoporosis with more than 3.5 million fractures per year (75,76). The incidence of osteoporosis is up to 3 times higher in women than in men, mainly due to two factors, the lower peak of bone mass in women and the rapid loss of BMD after menopause. In line with this, the

overall probability of osteoporotic fractures is much higher in women than in men, being especially high for trabecular bone fractures. Furthermore, the incidence of fractures increases exponentially with age as BMD falls. Fifty percent of women and 25% of men over 50 will suffer an osteoporotic fracture (77).

2.3.7. Management

The most effective and promising treatment to reduce the incidence of osteoporosis, and therefore the osteoporotic bill, is prevention. Decreasing the modifiable risk factors can increase the peak BMD and decrease the rate of bone loss. A balanced diet rich in calcium and vitamin D, physical exercise, minimizing the risk of falls and alcohol consumption, and not smoking can help prevent osteoporosis (78,79).

Existing pharmacological treatments are very effective in reducing the incidence of osteoporotic fractures, maintaining bone strength, and alleviating fracture symptoms, but they do have some side effects that need to be considered. We can divide the treatments into two categories: anti-resorptive and anabolic. Anti-resorptive treatments are those that block the resorption carried out by osteoclasts. In this group we find bisphosphonates (BPs), denosumab, strontium ranelate, estrogen replacement therapy (ERT) and selective estrogen receptor modulators (SERMs). On the other hand, anabolic treatments stimulate bone formation carried out by osteoblasts. Among these we find the derivatives of the PTH (teriparatide, abaloparatide) and more recently Romosozumab. In addition, these treatments are supplemented with vitamin D and calcium. It should be noted that not all treatments reduce the risk of fracture at all sites of the bone skeleton (80), therefore, the choice of treatment must take into account the site of the fracture, contraindications and side effects (44,45,81).

In addition to these treatments, new treatments that target bone metabolism proteins are in different preclinical stages. These have been elucidated thanks to in-depth molecular studies of the mechanisms of bone remodelling. One of the most promising is CTSK Inhibitors. This collagenase is osteoclast-specific and is responsible for the dissolution of the organic components of the resorption lacuna. Therefore, CTSK inhibitors inhibit proteolysis of bone matrix proteins and reduce resorption. Odanacatib is the only CTSK inhibitor drug that is in the clinical phase.

However, this inhibitor is not very specific and affects other members of the cathepsin family (82–84).

2.4. High bone mass disorders

In the last edition of the Nosology and Classification of Genetic Skeletal Disorders (34), 45 of the 461 genetic skeletal diseases are classified as High bone mass disorders. As their name suggests, high bone mass disorders are characterized by generalized increased BMD. Through the biological mechanism by which this high BMD is reached, we can categorize them into three types. First, reduction in bone resorption, such as osteopetrosis that presents skeletal sclerosis and abnormally dense but brittle bones (85). Second, increased bone formation, which includes different pathologies of bone overgrowth caused by an activation of the Wnt pathway. Two examples are the sclerosteosis and van Buchem disease, two rare diseases of excessive bone overgrowth caused by a reduction of Wnt pathway inhibition by sclerostin (86–89). Third, disturbed balance between both bone formation and bone resorption. In this thesis we will focus on a phenotype with an increased BMD caused by an increase in bone formation, specifically the High Bone Mass (HBM) phenotype.

2.4.1. The High Bone Mass (HBM) phenotype

The HBM phenotype (AKA Osteosclerosis, Endosteal hyperostosis, Osteopetrosis type 1 and Van Buchem disease type 2; MIM 144750; 607634; 607636) is a condition of bone overgrowth, characterized by unusually dense bones and a very strong skeleton that results in total absence of fractures (90) (Figure 7). Patients with this phenotype present high bone synthesis (evaluated through serum markers) and thickening of the trabecular and cortical bone but equal bone resorption, bone architecture, and serum levels of calcium, phosphate, PTH and vitamin D (91,92). Although the first cases described were asymptomatic, some

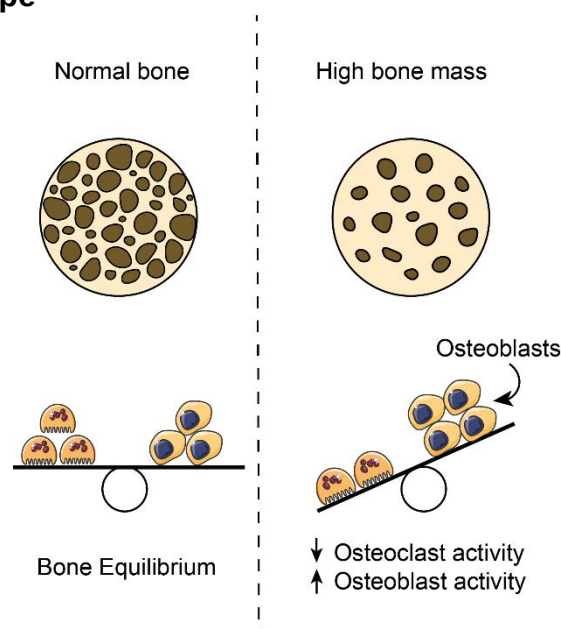


Figure 7. Graphical representation of trabecular bone BMD and osteoclast-osteoblast equilibrium normal bone and high bone mass phenotype.

secondary complications associated with excessive bone formation have now been described. Some of the most common are widening of the jaw, generalized sclerosis of the skull, compression of the cranial nerve, severe headache, and torus palatinus (93). Even with all this, most patients do not require treatment. The definition of HBM is based on the BMD value from DXA, but the thresholds used in the literature are variable (91,92,94). In this doctoral thesis we have used the definition of Little et al. 2002, where HBM is characterized by the sum of the Z-scores measured in the LS and the FN is greater than 4 (92,95). Although the calculation of the prevalence of the trait is challenging, it has been estimated at 0.2-1% (92,96–98). Regarding the genetic cause, the HBM phenotype can be monogenic or polygenic (85). Among the HBM-monogenic we highlight the gain-of-function (GoF) mutations in the coreceptors of the Wnt pathway LRP5 and LRP6 (92,93,95,99). These mutations produce a loss of affinity for extracellular inhibitors DKK1 and sclerostin, thus preventing internalization of LRP5/6 (92,93,95,99–103). Consistently, all the *LRP5/6*-HBM mutations described are located in the first β -propeller domain, which interacts with the NXI domain of extracellular inhibitors of the Wnt pathway (104–106). In addition to the mutations in canonical Wnt pathway genes, other mutations have been found in other genes that may cause the phenotype, as is the case of the rare dominant missense mutation in *SMAD9* (p.Leu22Pro) that was found co-segregating with this trait in a family and also in two unrelated patients (107). *SMAD9* acts inhibiting BMP-dependent target gene transcription, thus reducing osteoblasts activity (107). Regarding the HBM-polygenic, they arise from the carriage of the protective alleles of BMD-associated variants. In this sense, genome wide association studies (GWAS) have been carried out in cohorts with extreme BMD that have made it possible to identify common variants associated with the phenotype (discussed below).

2.5. Chiari malformation type I

Chiari malformation type I (CMI; OMIM 118420) is a neurological and spinal disorder characterized by downward herniation of the cerebellar tonsils below the foramen magnum by at least 5 mm without involving the brain stem (108). This results in compression of the neural tissue at the cranio-vertebral junction leading to neurological dysfunction, which may or may not be accompanied by syringomyelia, an abnormal increase in cerebrospinal fluid in a fluid-filled sac within the brainstem or spinal cord. CMI is only diagnosed in adulthood and it's primary, and often only,

symptom, is headache located in the occipital area and extending towards the cranial carota. CMI is not a genetic skeletal disease, but we included it in this thesis because one of the proposed mechanisms for the pathogenesis of primary CMI is a defect in the mesoderm involving sclerotomes during the development of the occipital bone. This results in a smaller and shallow posterior fossa to accommodate the normal size of the cerebellum (109,110). The diagnosis is made through MRI or head and neck X-rays. Although the prevalence of CMI is challenging (many carriers being asymptomatic), it is the most common type of Chiari malformation and has been found in approximately 1% of patients who undergo MRI (111).

CMI may have genetic or acquired origin. The genetic origin is based on the description of family history of the disease (family aggregation), twin studies, and overlap with known genetic conditions (112–118). For example, genome-wide linkage analysis has identified regions that co-segregate with the disease that are also associated with other pathologies, such as Klippel-Feil syndrome or Marfan syndrome (119,120). Recently, a population-based description of the familial clustering of 2871 patients with CMI provided evidence for a genetic contribution to disease predisposition (121). Genes involved in the early development of the paraxial mesoderm, important for the formation of the mammalian skull, including growth differentiation factors *GDF3* and *GDF7* (120) as well as *CDX1*, *FLT1* and *LDH1A1* (122), and have been associated with familial occurrence of CMI. Furthermore, mutations in *LRP4* and *DKK1* are found co-segregating with CMI in two different families (123). The most likely inheritance pattern is oligogenic with variable penetrance (124,125). In addition to the genetic origin, CMI can be acquired, for example after lumbar traumatic or repeated punctures or ventriculo-peritoneal bypass.

3. IDENTIFICATION OF GENES RELATED TO BONE PATHOLOGIES

Over the years, many genes causing or contributing to the phenotype of different bone pathologies have been identified. Monogenic diseases are generally caused by very rare mutations with a very large effect. On the other hand, complex diseases are normally caused by the additive effect of numerous variants with very small effects and which are frequent in the general population (Figure 8). However, rare variants

with larger effects can also contribute to the establishment of complex diseases. Examples are the variant in *LGR4* and *EN1* (126,127) (Figure 8). In this section we will review the main approaches for the identification of the genetic bases of skeletal genetic disorders.

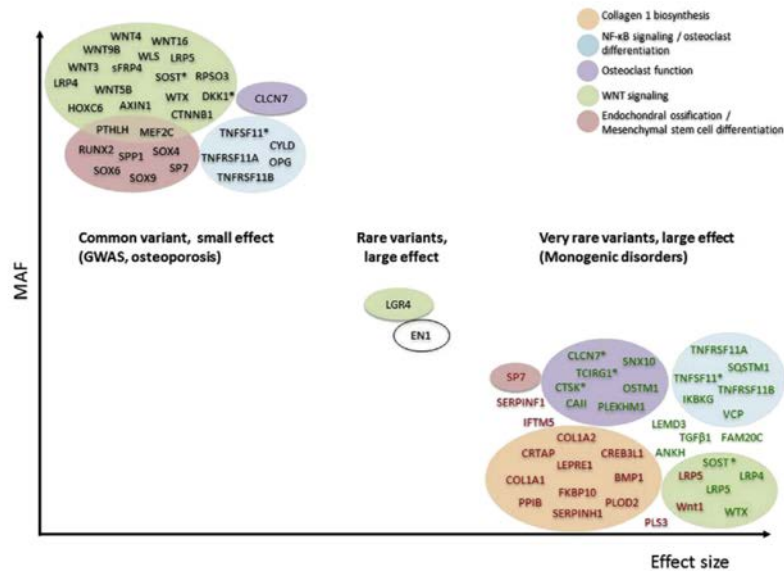


Figure 8. Minor allele frequency (MAF) and effect size of some of the genes identified in bone pathologies. Genes in green are those associated with pathologies with high BMD and genes in red with pathologies with low BMD. * Represents genes that have been approved or are being studied as a therapeutic target. Excerpted from Ref. (128).

The search for the genetic cause of monogenic diseases focuses on the search for rare variants in patients, isolates or members of a family. Sometimes a detailed clinical description can be informative in predicting a number of genes as the genetic cause of the disease. However, in many cases, candidate genes are not available, and it is therefore necessary to carry out hypothesis-free approaches. These approaches include parametric linkage analysis, array microchips covering a selection of putative candidate genes and high-throughput sequencing techniques such as whole exome sequencing (WES) and whole genome sequencing (WGS).

The search for the genetic cause of complex diseases focuses on statistical associations between regions or variants and the phenotype or trait. Some of the most common approaches include nonparametric linkage analysis, association studies of candidate genes and genome wide association studies (GWASs). In addition, in recent

years, WES and WGS are also used in the identification of rare variants contributing to the complex phenotype.

In this section we will review some of the techniques used to identify genetic variants in monogenic and complex diseases, with particular emphasis on the diseases of interest in this thesis.

3.1. Linkage analysis

Linkage studies are based on a search of genetic markers spread throughout the genome that co-segregate with the disease in one family. Classically, 400 to 800 microsatellites separated by 5-10 cm from each other were genotyped as genetic markers, but SNP panels have been used in recent years (129). Despite the fact that the SNPs are less polymorphic, therefore less informative, they are highly abundant, distributed throughout the genome and are very easy to genotype.

There are two types of linkage studies: parametric and nonparametric. In parametric linkage studies, it is necessary to specify the inheritance model of the disease in a family by observing the pattern of segregation. This type has been used very successfully for the identification of genes causing monogenic diseases. An example is the linkage found between markers at 11q12-q13 and two diametrically opposite bone phenotypes. In 1996 Gong *et al.* identified the linkage of this region to osteoporosis-pseudoglioma syndrome (OPPG)(130), an autosomal recessive pathology characterized by skeletal and eye abnormalities. A year later Johnson identified linkage between this same region and the HBM phenotype. Positional cloning in this region led to the identification of the *LRP5* gene. The difference between the two types of disease-causing mutations is that OPPG results from loss-of-function (LoF) mutations causing *LRP5* to be unable to activate the canonical Wnt pathway. In contrast, the mutations responsible for the HBM phenotype are GoF and cause a loss of affinity for the extracellular inhibitors DKK1 and sclerostin. In this case, *LRP5* can no longer be internalized and remains available in the membrane for Wnt pathway activation (100–103,105,131,132).

In non-parametric linkage studies, it is not needed to specify the inheritance pattern of the disease. These types of linkage studies have been used, although without much success, in the search for genes that influence complex characters. In these cases,

identifying a *locus* with a small effect requires many families with many individuals. In the case of age-related diseases, it is very difficult to find multigenerational families with the disease phenotype, as is the case of osteoporosis, where the phenotype appears at advanced ages. This is why only few *loci* associated with BMD have been obtained (55,133).

A 20p12.3 region shows genome-wide significant linkage with osteoporotic fracture and BMD, in a large number of families with osteoporosis in Iceland (134). This genomic region contains the *BMP2* gene, a member of the TGF β superfamily that plays an important role in bone and cartilage development. Another study involving 39 pedigrees with osteoporosis found linkage with BMD in the 1p36 region (135). A compelling candidate in this region is the *RERE* gene, which codes for a protein of the atropine family and, when overexpressed, triggers apoptosis. Nevertheless, these associations could not be replicated in other populations, and in the largest linkage study (n = 11842) using BMD as the phenotype, no region was found that obtained the level of genome-wide significance (133).

3.2. Whole exome sequencing (WES)

The exome is the part of the genome composed of all the exons, which include untranslated regions of mRNA (UTRs) and the coding sequence of the genes. The human exome consists of approximately 20,000 genes and constitutes only 1% of the whole genome. Nevertheless, 85% of the described monogenic diseases are caused by mutations in the exome. For this reason, WES, is an efficient tool to identify mutations in these regions that might result in defects in the final protein. Thanks to the latest advances in next generation sequencing techniques during the last decades, WES is now a fast, cost-effective and widely used approach for the diagnosis of monogenic diseases. Although as a disadvantage they still have a relatively short read length (35 bp- 700 bp) between the different sequencing methods available.

Using this approach, mutations in three candidate genes were found in two Italian families with a CMI. All three candidates could potentially explain the defects in the craniofacial development observed in the patients. In a family of 4 members with 3 affected individuals (father and two children) a heterozygous missense mutation in *DKK1* (p.Ala41Thr) was found to co-segregate with the disease. In the second family of 3 members with 2 affected individuals (mother and child), the researchers found a

missense mutation in *LRP4* (p.Thr581Glu) and a missense mutation in *BMP1* (p.Arg314His), in both cases in heterozygosity, that co-segregated with the disease (123). Although their important role in bone metabolism suggests that DKK1, LRP4 and BMP, could be involved in CMI development, a functional study to confirm the causality of the mutations is still missing.

3.3. Whole genome sequencing (WGS)

The complete genome sequencing (WGS) technique consists of sequencing the entire genome of an individual. As such, when applied to both monogenic and complex diseases alike, WGS permits the identification of every variant, even in non-coding regions of the genome. Moreover, using long-read massive WGS (PacBio or Oxford Nanopore), we can accurately identify variants located within repetitive sequences and retrotransposons, which was not possible with previous sequencing or genotyping approaches.

One of the first WGS studies on bone parameters was conducted in an Icelandic population and identified a rare new nonsense variant in the *LGR4* gene that was associated with low BMD and increased risk of fracture (126). Three years later, the same increased the size of the cohort and identified a rare variant in *COL1A2* associated with low BMD (136). The second major WGS was carried out in 2015 by the GEFOS consortium (127). This study combined data from WGS (n = 2882) and WES (n = 3459) to impute variants that were not covered in previous GWAS. Using this approach, 2 new low-frequency variants were identified in non-coding regions with a very large effect size. One of the variants maps 53 kb downstream of the *EN1* gene and showed an association with high LS-BMD and reduced risk of fracture. Interestingly, this gene had never been associated with any bone parameter. The second variant is a low-frequency variant in the Wnt ligand, *WNT16*.

3.4. Association studies

Association studies have been a powerful tool to identify genes involved in complex diseases. These studies consist of establishing an association between the genetic or allelic frequency of a variant and the studied phenotype. Association studies are distinguished depending on whether the studied phenotype is categorical (e.g. fracture) or quantitative (e.g. BMD). In the first type, a case-control association study

is carried out, where the genotypic or allelic frequencies of a variant are compared between the two groups. In the case of quantitative traits, a mean and standard deviation of the trait are calculated for each genetic group, and a statistical test is used to compare if there are differences between the groups. In the association studies, sample size, standardization of phenotyping and genotyping, and chosen population are very important factors.

Among the association studies we can distinguish two classes: the candidate gene association studies and the GWAS. In this section we will describe these two approaches and the role they have in the discovery of genes related to osteoporosis.

3.4.1. Candidate gene association studies

Candidate gene association studies analyze polymorphic variants of candidate genes that are chosen for their role in bone biology and are classified into 3 main types, according to the selection criteria used:

- Functional candidate: For genes with a role in the development of monogenic bone diseases or in the regulation of bone metabolism (e.g. *LRP5*, *SOST*, *VDR*, etc.)
- Positional candidate: For genes located in a region in linkage with the phenotype (e.g. 11q12-q13)
- Expression candidate: For a regulatory region that changes the expression levels of a gene (e.g. *ECR5*).

More than 200 candidate gene association studies with BMD and/or osteoporotic fracture have been performed, and many common SNPs have found associated (Reviewed in (137)). However, these results have not always been replicated. This lack of replication is mainly due to sample size (unpowered studies), the ethnic difference of the populations used, and the different pattern of linkage disequilibrium (LD) in these diverse populations (138).

To increase statistical power and find more robust associations, large consortia have been created that aim at determining the genetic contribution of complex diseases testing candidate gene polymorphisms (139). The GENOMOS consortium (Genetic Markers for Osteoporosis; www.genomos.eu) was funded in 2003 with the

participation of groups in seven EU countries, with the aim to perform studies using standardized methods of genotyping and phenotyping (139). Likewise, a consortia of 5 international, multicenter, population-based studies evaluated 150 candidate genes, testing 36,000 SNPs spread across or flanking them in a cohort of 19,000 individuals, and found that only 9 of these candidate genes (*ESR1*, *LRP4*, *ITGA1*, *LRP5*, *SOST*, *SPP1*, *TNFRSF11A*, *TNFRSF11B* and *TNFSF11*) were significantly associated with BMD, all with a small effect (140). Furthermore, 4 of them (*LRP5*, *SOST*, *SPP1*, *TNFRSF11A*) also showed a significant association with fracture risk (140).

3.4.2. GWAS

GWAS studies analyze the relationship between millions of SNP-like markers distributed throughout the genome and a specific pathology or phenotype. This approach currently represents the most comprehensive and unbiased tool for the study of complex diseases. Contrary to candidate gene association studies, this is a hypothesis-free approach, allowing new genes or signaling pathways associated with the studied phenotype to be discovered. The establishment of GWAS has been possible thanks to advances in genomic high-throughput technologies, study design, improved statistical analyses, and the availability of large biobanks. Due to the number of statistical tests and corrections performed, and the small effect of each variant on complex diseases, very large cohorts are necessary. This has been obtained with the GWAS meta-analyses, where different studies are brought together with the aim of increasing the sample size. The increased statistical power allows to estimate the effect of individual genetic variants with higher accuracy (141,142). However, large samples do not necessarily result in more associated variants, as when the sample size is increased, the heterogeneity of the sample is also increased, which results in loss of population-specific variants (128).

In the field of osteoporosis, more than 40 GWAS and meta-analyses have been performed (Table 3), identifying more than 500 genes associated with different bone phenotypes. Even so, all these *loci* only explain a small fraction of the genetic contribution on the FN-BMD (143). The first GWAS study using different bone parameters as phenotypes (namely, FN-BMD, LS-BMD, trochanteric BMD (TR-BMD), risk of fracture and QUS of the calcaneus) was published in 2007 (144). In this study, 71,000 autosomal SNPs were genotyped in 1,141 individuals, and although some

nominal significance was found, none exceeded the multiple testing corrections. The following year two simultaneous studies identified 5 genes (*TNFRSF11B*, *LRP5*, *ESR1*, *ZBTB40* and *MHC*) with variants associated with genome-wide significance ($p < 5 \times 10^{-8}$) to BMD (145,146). The Stykarsdottir group expanded their cohort to 15,375 individuals and identified four new associations: *TNFRSF11A*, *SOST*, *MARK3* and *SP7* (147). These results demonstrate that the success of the GWAS depends largely on the sample size, but also on the effect size of the causal variants, the frequency of these variants, and the LD of the genotyped variant and the causal variants (141). The first major meta-analysis was published in 2009 by the GEFOS (GEnetic Factors for Osteoporosis) consortium, and included 19,195 samples (148). In this work, 13 new loci associated with BMD were identified. Three years later, the second multi-ethnic meta-analysis was published by the GEFOS consortium with 32961 samples in the discovery cohort and 83894 in the total cohort from 17 different GWAS⁽¹⁴⁹⁾. In this study, 56 loci were identified associated with BMD (FN-BMD, LS-BMD, or both), 32 of which were previously unknown. Of these 56 loci, 14 were also associated with fracture risk and 6 of them belong to the Wnt pathway (*WNT16*, *SOST*, *DKK1*, *CTNNB1*, *LRP5*, *WNT4*).

In the recent years, the use of estimated BMD (eBMD) from the QUS heel values of individuals from UK Biobank as a phenotype has allowed the identification of many new genes associated with BMD. Three large studies have been carried out with this phenotype. The first in 2017 where 307 SNPs were identified in 203 loci associated with eBMD, of which 153 were previously unknown (150). In 2018, Kim *et al.* (151) identified 1,362 SNPs at 899 loci of which 613 were novel. Finally, Morris *et al.*, (143) identified 518 loci, of which 301 were new, 13 of which were also associated with osteoporotic fracture (Table 3).

Due to the difficulty of recruiting large numbers of participants with fractures, bone fragility studies are limited. These studies have shown that the genes that have an impact on BMD are not necessarily the same ones that influence the risk of fracture. Still, most studies on osteoporotic fracture have been done testing the BMD-GWA loci, but a few GWAS studies have been performed with fractures alone (152,153). In 2018, Trajanoska *et al.* (154) published the largest GWAS study with fractures at different sites, with 37,857 cases and 22,711 controls in the discovery cohort and 300,000 in

the replication cohort (with 147,200 cases). In this study, 15 *loci* with medium effect were identified, many of which were also associated with BMD, thus reinforcing the relationship between BMD and fracture risk. In any case, the effect of the variants associated with fracture is lower than in BMD.

Some age, sex, and skeletal site-specific *loci* have been found in different GWAS studies (149,155–160). Furthermore, although most studies have been performed using BMD or fracture risk as phenotype, some have also been performed with different bone parameters such as heel broadband ultrasound attenuation (BUA) and speed of sound (VOS), trabecular volumetric measurements of BMD measured by peripheral QCT, hip structure parameters such as femur neck axis angle (FNSA), femur neck length (FNL), neck section modulus of femur (FNSM) and narrow neck width (NNW) (161–169).

Most GWAS studies use SNP with frequencies greater than 5%, thus losing the association of rare variants that are likely to have a large effect size. It has been described that in bone pathologies (like in many other genetic diseases) the rare variants have a greater effect on the phenotype (170). To detect them, one strategy that we have already introduced is to employ WGS. However, another strategy in identifying variants with a larger effect size is to significantly increase the frequency of the variant in the cohort by including only individuals with extreme BMD values. In this sense, Gregson and collaborators published the most exhaustive study with extreme BMD cases (171). The cohort was made up of 240 individuals with extremely high BMD (Z-score > 3.2), 1055 individuals with high BMD and 900 individuals with low BMD. In this study, they identified two *loci* (*NPR3* and *SPON1*) associated with LS-BMD and hip-BMD, respectively.

Even with all these approaches, many genes that have been experimentally associated with bone have not shown genome-wide significance in human studies. This is an indicator that the technique still needs to be refined further, including an improvement in data acquisition methods, in the homogeneity of the cohorts and considering rare variants.

Table 3. Different GWAS studies where extracellular modulators from the Wnt pathway have been found associated with bone parameters.

Study	Trait	N	Hits	Genes
Richards 2008 (145)	LS and FN-BMD, OF	8557	2	<i>LRP5</i>
Styrkarsdottir 2009 (147)	LS and FN-BMD, OF	15375	7	<i>WNT4/ZBTB40, SOST</i>
Rivadeneira 2009 (148)	LS-BMD and FN-BMD	19195	20	<i>SRF4/STARD3NL, LRP5, LRP4, WNT4/ZBTB40</i>
Duncan 2011 (94)	Extreme high or low TH-BMD	21789	23	<i>RSPO3, SOST, WNT4/ZBTB40, LRP5, SFRP4/STARD3NL</i>
Estrada 2012 (149)	LS and FN-BMD	83894	56	<i>SOST, WNT16, WNT5B/ERC1, DKK1/MBL2, WNT4/ZBTB40, LRP5, SFRP4/STARD3NL, RSPO3</i>
Medina-Gomez 2012 (156)	TB-BMD, skull-BMD	13712 *	1	<i>WNT16</i>
Zheng 2012 (169)	CT, FA-BMD, FAF	5878	1	<i>WNT16</i>
Koller 2013 (172)	LS-BMD, FN-BMD	9658	2	<i>WNT16</i>
Styrkarsdottir 2013 (126)	LS, TB and hip-BMD, OF	73965	2	<i>LGR4</i>
Zhang 2014 (160)	LS, FN and TH-BMD	27061	15	<i>WNT4/ZBTB40, WNT16, LRP5</i>
Moayyeri 2014 (161)	BUA, VOS, heel eBMD	58878	7	<i>WNT16, DKK1/MBL2, RSPO3</i>
Kemp 2014 (155)	TB, skull and UL - BMD,	9395 *	13	<i>WNT4/ZBTB40, WNT16/CPED1</i>
Chesi 2015 (173)	DR-BMD, BMC	1885 *	2	<i>WNT16/CPED1</i>
Zheng 2015 (127)	LS, FN and FA-BMD, OF	508253	36	<i>WNT4, CTNNB1, SFRP4, WNT16</i>
Styrkarsdottir 2016a (136)	LS and Hip-BMD, OF	209379	5	<i>LGR4</i>
Styrkarsdottir 2016b (174)	LS and Hip-BMD, OF	274941	14	<i>RSPO3, SOST</i>
Nielson 2016 (165)	LS-vBMD, clinical VF, radiographic VF	38714	5	<i>WNT4/ZBTB40</i>
Chesi 2017 (159)	LS, FN, DR and TH-BMD	1419 *	5	<i>WNT16/CPED1</i>

Mullin 2017 (162)	BUA, VOS, OF	16627	8	<i>WNT16, RSPO3</i>
Kemp 2017 (150)	Heel eBMD, OF	142487	203	<i>SOST, WNT16/CPED1, WNT1, WNT5B, DKK1/MBL2, LRP4, SFRP4/STARD3NL, WNT2B, LRP5, KRM1</i>
Medina-Gomez 2018 (158)	TB-BMD (LC)	66628	80	<i>SOST, WNT16/CPED1, WNT1, WNT4/ZBTB40, WNT5B, DKK1/MBL2, LRP4, SFRP4/STARD3NL, FZD4, LRP5</i>
Pei 2018 (175) Joint study of meta-analysis	LS and FN-BMD,	40449	9	<i>SOST, WNT16/CPED1, DKK1/MBL2, SFRP4/STARD3NL, LRP5</i>
Kim, 2018 (151)	Heel eBMD, OF	394929	899	<i>SOST, WNT16/CPED1, WNT1, WNT5B, WNT7B, DKK1/MBL2, LRP4, SFRP4/STARD3NL, WNT2B, , LRP5, KRM1, LRP6, LGR4, KRM1, LGR6</i>
Trajanoska 2018 (154)	OF	562258	15	<i>SOST, WNT16/CPED1, DKK1/MBL2, SFRP4/STARD3NL, LRP5, RSPO3</i>
Gregson 2018 (171)	Extreme low or high TH and LS-BMD,	2195	4	<i>WNT4/ZBTB40</i>
Hsu 2019 (176)	FNL, FNSA, NNW, FNSM, BMD, OF	27053	4	<i>LRP5</i>
Morris 2019 (143)	Heel eBMD, OF	426824	518	<i>SOST, WNT16/CPED1, WNT1, WNT4/ZBTB40, WNT5B, WNT7B, DKK1/MBL2, LRP4, SFRP4/STARD3NL, WNT2B, LRP5, FZD4, KRM1, LRP6, LGR4, KRM1</i>
Styrkarsdottir 2019 (177)	Hip and LS-BA, BMD, OF	50336	13	<i>WNT4/ZBTB40</i>

The study is represented by the first author and the year. In bold meta-analysis. In italics the WGS studies *In children. LC: Life course. N: Sample size. Hit: Total number of GWAS loci. The genes shown are the extracellular modulators of the Wnt pathway. BA: Bone area; BMD: Bone mineral density; BUA: Broadband ultrasound attenuation; CT: cortical thickness; DR: distal radius; eBMD estimated BMD; FA: forearm BMD; FAF: Femoral atypical fracture; FN: femoral neck; FNL: femoral neck length; FNSA: femoral neck-shaft angle; FNSM: femoral neck section modulus; LS: Lumbar spine; NNW: narrow neck width; OF: osteoporotic fracture; TB: Total body; TH: total hip; UL: upper limb; vBMD: volumetric BMD; VF: vertebral fracture; VOS: velocity of sound.

3.4.3. Amount of genetic variance explained by the associated variants

The information from genetic studies could help us to improve the prediction of the risk of a specific pathology and use this information to improve the prevention, diagnosis,

prognosis and treatment of the pathology (178). The calculation of the risk of disease through the GWAS hits is called Polygenic risk score. This prediction consists of a summation of the standardized effect size multiplied by the genetic dosage of each variant of a subset of variants selected for their association (179).

The percentage of BMD variance explained by the GWAS has increased substantially in recent years thanks to recent GWAS with very large sample sizes. In 2012, with the *loci* identified in Estrada et al.(149), only 6% of the BMD variation was explained, which is lower than the percentage explained with traditional risk factors such as age or weight (8-9% of variation). In contrast, with the *loci* determined in the last large GWAS (143), 20% of the BMD variation is explained. Nowadays, the combination of genetic factors, weight, height, age and sex, allow us to explain 25% of the variation in eBMD (151).

Still, there is a difference between the variability explained by genetic factors and the heritability of the disease. This may be due to an overestimation of heritability or to not considering other genetic factors. To increase the percentage of variability explained by genetic factors several approaches can be used, including increased sample size, deepened study of rare variants through WGS or GWAS with extreme phenotypes, but also study other types of genetic variants or epigenetics.

3.4.3.1. Copy number variations (CNV)

Copy number variations (CNV) are DNA segments of >1 kb present in a variable number of copies in the population compared to a reference genome (180). To date, only 3 GWAS for osteoporosis using CNV markers have been performed, in which *UGT2B17*, *VPS13B131*, and 6p25.1 locus have been associated with osteoporotic fracture, BMD, and hip geometry (181–183).

3.4.3.2 Epigenetics

Epigenetics is the study of changes in gene expression that are not due to a modification of the nucleotide sequence of DNA. Epigenetic changes are i) heritable, ii) reversible, iii) cell type and tissue specific, iv) dependent on interaction with the environment. The epigenetic mechanisms are associated with aging, supporting the association with diseases linked to age, such as osteoporosis. There are three main

epigenetic mechanisms: modification of histones, non-coding RNAs, and DNA methylation (reviewed in (184,185)).

Modification of histones consists of post-translational modifications at the N-terminal tails of histones, and includes acetylation, methylation, phosphorylation, ubiquitination or sumoylation. Histone modifications can drive changes in the interaction of DNA with histones or with other DNA binding complexes. One example important in bone homeostasis is the deacetylation of the lysine 9 of the histone 3 (H3K9) in the nucleosomes within the *SOST* promoter performed by Sirtulin 1. This deacetylation reduces gene expression leading to an increase in the Wnt pathway and a subsequent increase in bone formation (186).

Although the majority of genomic DNA is transcribed, a large amount (98% of the transcribed RNA) is not translated into proteins and is known as non-coding RNA (ncRNA). Within the ncRNAs, the types that have been most studied and associated with human pathologies are the long non-coding RNA and the microRNA (miRNA)(187). MiRNAs are short sequences of non-coding RNA (~ 21nt), single-stranded and evolutionarily conserved that can regulate gene expression through binding to mRNA. In most cases, miRNAs bind to the 3'UTR sequence of the mRNA to inhibit its expression. However, cases in which they bind to other regions of the gene have also been described. Many miRNAs regulate bone remodeling including the differentiation, proliferation and function of osteoblasts and osteoclasts (184). An example of this is the regulation of the Wnt pathway through specific miRNAs. It has been described that the miR29a, miR218 and miR355-5p inhibit the expression of antagonists of the Wnt pathway such as *DKK1*, *DKK2*, *SFRP2* and *SOST*, which leads to pathway activation (188–190). In the same line, some SNPs, both in the miRNAs or in the miRNA binding sequences, can affect the miRNA regulation (191). One example is SNP rs11614913 in the precursor MIR196A2, found to be associated with FN-BMD, LS-BMD, LS-area and hip fractures (177,192). Furthermore, it was found that the risk allele is less effective in repressing the target genes of hsa-miR-196a-5p (177). Another example involves the SNPs in miR3679 and miR4274 associated with FN-BMD in a cohort of Spanish postmenopausal women (193). The aberrant expression of miRNAs is associated with a variety of human pathologies, including skeletal bone diseases. For example, several studies showed differences in circulating miRNAs

between osteoporotic and control subjects (194). Finally, a miRNAs important feature is that they are secreted in extracellular fluids, a property that makes miRNA a potential biomarker for different diseases (195).

Finally, DNA methylation consists of the binding of a methyl group [CH₃] to carbon 5 of the cytosine inside the dinucleotides CpG. DNA methylation is generally associated with an inhibition of gene expression. For example, the CpG-rich region in the *SOST* promoter, exhibits different methylation patterns between osteoblasts (hypermethylated) and osteocytes (hypomethylated). Therefore, in osteoblasts, the binding of certain TFs (such as OSX) is blocked, which leads to low gene expression (196,197). In addition, different studies have suggested that DNA methylation has a relevant role in osteoblast differentiation, for instance in the methylation of *OPG* and *RANKL*, two key genes in the differentiation of osteoblasts and osteoclasts (197,198).

4. FUNCTIONAL STUDIES

Once a putative mutation causing or contributing to the disease has been identified, it is necessary to corroborate the finding by performing functional studies. In monogenic diseases, functional studies are simpler since the effect of the variable is larger. However, in complex diseases such as osteoporosis, the effect of a single variant is less obvious.

4.1. *In silico* tools and online resources

In silico tools allow us to assess the putative effect of variants, in order to predict their potential impact and prioritize them for subsequent functional validation. The *in-silico* tools and the online resources now available allow us to predict the impact of genetic variants at DNA, RNA or protein level. The first step to understand the functional potential of a variant is the search in the literature (PubMed) and databases (Ensembl, National Center of Biotechnology Information (NCBI), University of California Santa Cruz (UCSC) genomics institute)(199). These databases provide information related to the known variants, the correlation between the genotype and the phenotype, the variants frequency, etc. In addition to these databases, we want to highlighted dbSNP , with information on the genetic variants with an rs# identifier, and ClinVar, that links the variants with the phenotypic data (199,200). Repositories of exonic (Exome Aggregation Consortium; Exac) or genomic (Genome Aggregation database;

gnomAD) data from many individuals exist (60,706 and 15,496, respectively) that allow us to know the frequency of rare variants and to calculate the degree of gene intolerance to variants (201). Once the available databases have been consulted, free prediction programs can be used to score the variant “deleteriousness”. Numerous prediction programs (e.g. SIFT (202) and Polyphen-2 (203)) rely on sequence or structure information to predict changes at the protein level, to distinguish whether the amino acid change is neutral or disease-causing. These programs use the information on the variant's position in the protein, the nature of the amino acid change, the conservation between species, and other parameters to score its pathogenicity. In addition, there are programs to predict if a variant affects the stability of the protein, whether we have the protein structure in the Protein Data Bank (PDB) (Site-Directed Mutater (204)) or through machine learning programs (MUpro (205)). To detect changes at the RNA level, we have programs such as i) Human Splicing Finder (206) and Splice Port (207) to evaluate the effect of a variant on RNA splicing (5' donor, 3' acceptor, intronic branch points, exonic/intronic splice enhancers [ESE/ISE] or silencers [ESS/ISS]); ii) miRBase to evaluate the binding of miRNAs depending on the SNP allele, or iii) Rtools to assess the secondary structure of the generated mRNA what will affect the RNA stability and production (199). Finally, to detect changes at the DNA level, it is possible to determine evolutionary conservation at the DNA position or consult the data on regulatory elements published by various projects including Blueprint, Encyclopedia of DNA Elements (ENCODE) and Roadmap epigenomics (208–210). These projects include information on RNA expression, DNA methylation and accessibility, methylation and acetylation of different histones, and the presence of tissue-specific enhancers in different cell types, including human osteoblasts. Furthermore, we can determine if a variant is acting as a cis-eQTL for a given gene in different tissues in the Genotype-Tissue Expression (GTEx) database (although, unfortunately, bone tissue is not available)(211). In addition to these tools, Combined Annotation Dependent Depletion (CADD) (212), uses a machine learning approach to score the deleteriousness of both variants in coding and non-coding regions. More recently, the International Federation of Musculoskeletal Societies (IFMRS) has created a Musculoskeletal knowledge portal (MSK-KP; (213)) with the aim to integrate all the information available for skeletal diseases, including GWAS and bone phenotypes in KO mice, to facilitate the prioritization of genes with important roles.

In this sense, in recent years, integrative *in silico* analyses have been carried out on osteoblasts to map the variants to functional regions. Morris *et al.*, (143) mapped the variants associated with BMD in an ATAC-seq (assay for transposase-accessible chromatin using sequencing) of Saos2 cells (human osteosarcoma cell line) and public data of DNase I hypersensitivity sites in primary human osteoblasts (hOBs) from the ENCODE database. The authors found that the associated SNPs were located in rich regulatory regions marked by ATAC-seq or by sites of DNase I hypersensitivity. This suggests that intergenic spaces SNPs are marking an important regulatory region for the phenotype.

4.2. Reporter genes: Luciferase assay

One of the most widely used approaches to study gene regulation and cell physiology are reporter gene assays. A reporter gene is the one that produces a protein that allows a rapid quantification of its expression. Notable examples are the GFP and the luciferase gene, both of which use light emission and allow for a direct quantification of gene expression. In particular, the luciferase gene codes for the enzyme luciferase (from the firefly *Photinus pyralis*) that generates oxyluciferin, PPi, CO₂ and a luminescent signal easy to quantify in the presence of the substrate Luciferin, O₂ and ATP (Figure 9A). Commercial Dual-Luciferase Reporter Assay plasmids contain the luciferase gene and a Multiple Cloning Site (MCS) that allows us to clone the region to be tested in front of the gene (Pgl3-Basic; to test if the region acts as a promoter), in front of a basal promoter and the luciferase gene (Pgl3-promoter; to test if the region acts as an enhancer), or on the 3'UTR of the luciferase gene (pmirglo; to test if the region has any effect on the 3'UTR). In addition to the luciferase signal, a vector with another luciferase gene (from *Renilla reniformis*) under the control of a basal promoter is included in the assay, which in the presence of Coelenterazine and O₂ generates Coelenteramide, CO₂ and a luminescent signal. This second luciferase activity is used as a control to minimize the experimental variability caused by differences in cell viability or by transfection efficiency. In a standard assay, these two constructs are co-transfected into the desired cell type and luminescence is quantified in the presence of Luciferin and in the presence of Coelenterazine, thereby determining the expression of the firefly luciferase gene under the effect of the regulatory region of study.

One of the most widely used applications of the luciferase assays is to determine if a region of interest acts as a promoter region or as an enhancer (Figure 9B). To this aim, the region is cloned in front of the luciferase gene or in front of a basal promoter, respectively. This approach was successfully used to describe the *SOST* ECR5 enhancer (214), where 7 conserved regions (~ 200 bp) of the selected region in Van Buchem patients were cloned in front of the luciferase gene and the *SOST* promoter and transfected in rat osteosarcoma cells. The authors found that under the ECR5 region, the expression of the luciferase gene was stimulated 4-fold. The role of this region as a *SOST* enhancer has been confirmed by many others with different approaches.

Another use of luciferase assay is the study of the 3'UTR region of a gene (Figure 9C). In this case the 3' region of the gene of interest is cloned, which allows us to determine if there are any miRNA binding sites or some other regulator elements in that region that are affecting expression. With such a reporter assay, Tang *et al.*, (215) demonstrated that microRNA-433-3p binds to the 3'UTR region of *DKK1* in osteoblasts, leading to a reduction in gene expression. In contrast, when the binding site was mutated, this reduction in expression was not observed. Those results were also verified with other techniques (215).

Finally, luciferase assays can be used to recreate signaling pathways and see the effect that inhibitors or ligands have on it (Figure 9D). In this sense, Balemans *et al.* (131), described a luciferase study specific for the canonical Wnt pathway, where the luciferase gene was downstream of 5 TCF/LEF binding sites. Therefore, this technique allows to determine the activity of the canonical Wnt pathway through the quantification of luciferase activity. To recreate the pathway, different members of the Wnt pathway are also co-transfected, including *LRP5*, Wnt1 and mesdc2 (a chaperone necessary for the folding of *LRP5*). With this model, the effect of inhibitors on the Wnt pathway, other ligands, or the effect of mutations on members of the Wnt pathway can be tested. In particular, with this system, the authors tested the effect of 6 GoF mutations of *LRP5*, and found that neither *DKK1* nor *SOST* could efficiently inhibit the canonical pathway of Wnt in the presence of these mutations (131).

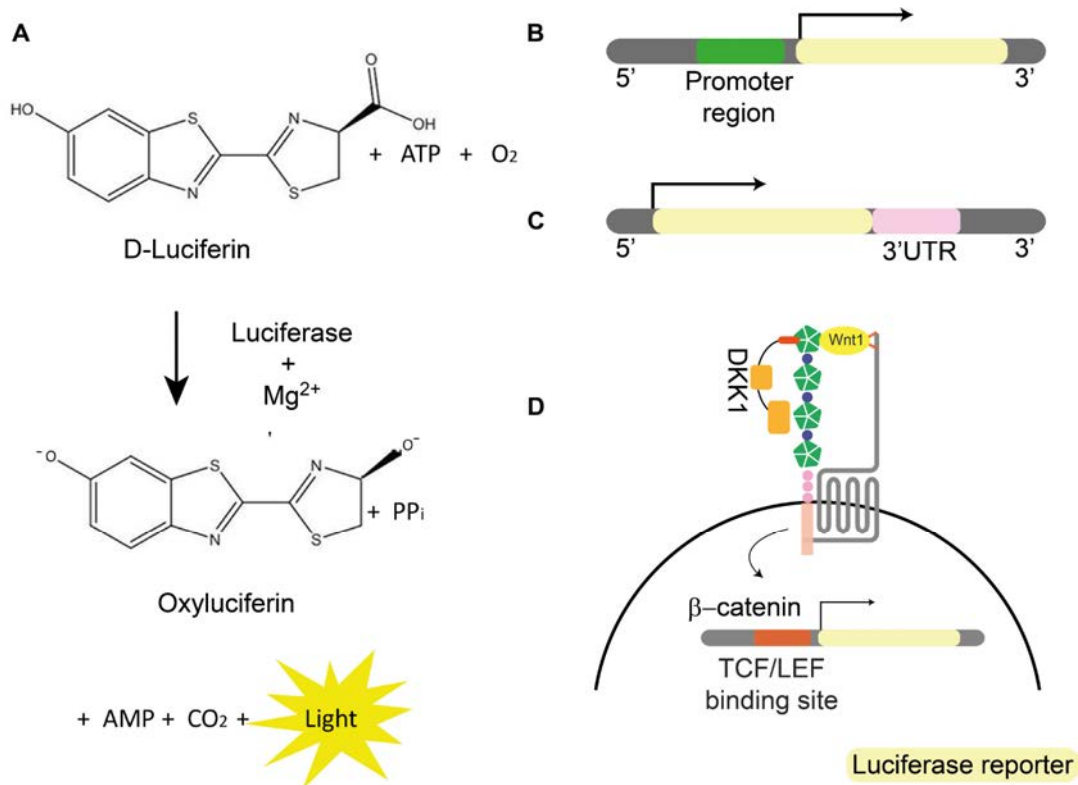


Figure 9. Luciferase reporter gene assay scheme. A) Chemical reaction carried out by the enzyme luciferase, where light is generated from the reagent D-luciferin. B) Luciferase promoter reporter assay: To check the promoter activity of the candidate region, it is cloned 5' of the luciferase gene. C) Luciferase 3'UTR Reporter Assay: To check the effect of a 3'UTR region on gene expression, the candidate region is cloned 3' of the luciferase gene. D) Wnt pathway specific luciferase reporter gene assay: To test the activity of the canonical Wnt pathway, five TCF/LEF sites are cloned at 5' of the luciferase gene, therefore the expression of the luciferase gene will correlate with the activity of the Wnt pathway.

4.3. Chromatin conformation capture

One of the most frequent assumptions in the annotations of the GWAS studies is to mark the gene closest to the GWAS hit as the cause of this association. An example of this is the characterization of the *FTO* locus with obesity. Different GWAS studies found SNPs in the *FTO* locus associated with the risk of obesity, so it was concluded that the *FTO* gene was the cause of this association. However, the intronic region where these associated SNPs are found is an enhancer that regulates the expression of the *IRX3* and *IRX5* genes, which are the true cause of this association (216–218). It is well established that there are important regulatory elements, such as enhancers or silencers, in the non-coding regions of the genome, and genetic variants in these regions can modify these elements and confer disease susceptibility through the

physical binding to their target gene. The identification of regulatory regions and their effectors is complicated by the observation that, due to the ternary conformation of DNA within chromatin in live cells, the distance in bp between a regulatory element and the regulated gene is not always related to the physical distance separating them within the nucleus. Thus, two genomically separated regions may be spatially contacting and interacting.

The chromosome conformation capture techniques developed by Job Dekker et al. in 2002 (219) allows to characterize the spatial chromatin organization and to quantify the physical distance between two genomic regions (Figure 10). These techniques involve the fixation of the nuclei with formaldehyde, which crosslinks and stabilizes the physical contacts between DNA segments that are associated through proteins. Depending on the interactions of interest, different approaches exist: 3C, 4C, 5C, Hi-C and Capture-C. For example, with the 4C approach (circular chromosome conformation capture) one can identify which regions of the entire genome are interacting with a specific region of interest (220). In recent years these technologies have been used to characterize the GWAS hits and to define topologically associating domains (TAD), in which interactions are enriched and variant effects are stronger.

In order to characterize different GWAS signals, Chesi et al., (221) carried out a high-resolution genome-wide Capture-C assay focused on promoters from osteoblasts differentiated from mesenchymal stem cells combined with ATAC-seq data. In this study, the authors analyzed 273 loci associated with BMD and they were able to map 95 variants (corresponding to 46 loci) to 81 active promoters. Among these promoters, the *ING3* and *EPDR1* genes were selected and functionally verified, which showed strong effects on osteoblastic and adipogenic differentiation. These two genes had not been previously associated with BMD, and the corresponding GWAS hits were at 260 Kb and 160 Kb, respectively. This study demonstrates how the combination of complementary approaches such as GWAS studies, chromosome conformation capture techniques and functional analysis holds the key us to gain biological insights that otherwise would not be elucidated by using one of these techniques alone. Moreover, two other studies from the same group used publicly-

available Hi-C data to identify the target genes for some associated GWA-SNPs and found that the SNPs were cis-eQTLs of the genes they interacted with (222,223).

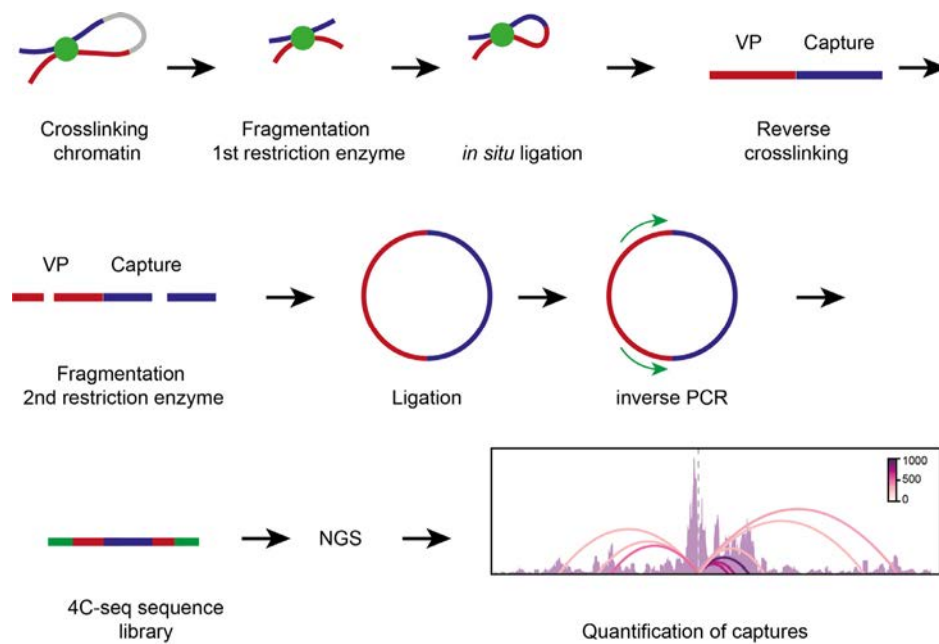


Figure 10. Outline of the 4C-seq procedure. (1) Genomic regions close in space (blue and red) are fixed by formaldehyde by protein-protein and protein-DNA cross-links (green). (2) DNA is fragmented with the primary restriction enzyme. (3) The fragments that have been fixed by proximity are ligated. (4) The crosslink is eliminated and the fragments that were close in space remain united. (5) A second round of fragmentation occurs using secondary enzymes. (6) A circularization occurs in the second round of ligation. (7) An inverse PCR is performed with primers located in the region of interest (viewpoint, VP) (8) A library is generated with the 4C-seq sequence (9) The amplicons are analyzed using next generation sequencing (10) and are located and quantify the sequence reads with which the viewpoint interacts (Adapted from (220)).

4.4. Expression quantitative trait locus (eQTL)

Variation in gene expression plays a very important role in the susceptibility to complex diseases. A given locus can affect the expression levels of a gene (expression Quantitative Trait Locus; eQTL) in a cell type, tissue and developmental-stage specific manner (Figure 11). These eQTLs can regulate the expression of a genes close (cis-eQTL) or distant (trans-eQTL) to their genomic locations. Using the GTEx database, it has been possible to identify a plethora of genome variants that are regulating the expression of different genes in more than 48 tissues. Unfortunately, among these tissues, we did not find any type relevant to bone. Importantly, studies of eQTLs in cell types relevant for a given disease or trait, are another approach to elucidate the

functional role of variants derived from GWAS. In this sense, different studies have integrated the information from GWAS and eQTLs to determine the functional basis of the associated variants (224–226). In the field of bone, some studies have been carried out with osteoblasts and osteoclasts.

Grunberg *et al.*, (2009) demonstrated the importance of choosing the correct cell type or tissue by comparing the results of a study of eQTLs in hOBs with public data from lymphoblastoid cell lines (LCL) and testing 2000 SNPs associated with BMD for cis-eQTL. The authors found that in hOB there was more than 3-fold convergent enrichment in cis-eQTLs compared to LCL. They also used the data from the cis-eQTL study in hOB to prioritize *loci* associated with BMD with p values $<10^{-3}$ from the Styrkarsdottir *et al.*, 2008 study (146). Finally, they identified a novel gene associated with BMD (the *SRR* gene) which, upon alterations in its promoter, affected its own gene expression.

In 2017, Mullin *et al.*, (162) conducted a study of eQTLs in osteoclasts using *in vitro* differentiated cells from 158 donors and found that 24 variants that were associated with BMD in GWAS studies were also associated with differences in the expression of 32 genes in osteoclasts. Some of these genes have a very important role in bone, such as *IQGAP1*, *CYP19A1*, *CTNNB1* and *COL6A3*.

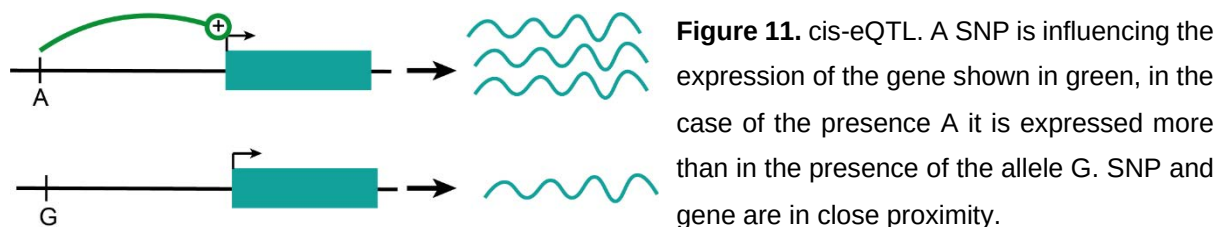


Figure 11. cis-eQTL. A SNP is influencing the expression of the gene shown in green, in the case of the presence A it is expressed more than in the presence of the allele G. SNP and gene are in close proximity.

5. THE WNT SIGNALLING PATHWAY IN BONE

The Wnt pathway (Wingless-type mouse mammary tumor virus integration site) is a ubiquitous pathway involved in a wide variety of cellular processes, and is highly conserved across species. It was first described for its role in carcinogenesis, and later demonstrated to be essential for embryonic patterning and for maintaining adult tissues (227–229). In the skeletal tissue, the Wnt pathway is involved in the differentiation of osteoblasts, osteoclasts and chondrocytes. In particular, the canonical Wnt pathway stimulates the process of differentiating MSCs into the

osteoblastic lineage, suppresses apoptosis in osteoblast precursors before determining the fate of cell differentiation and stimulates preosteoblast replication and matrix mineralization (230–235). Therefore, it has a very important role in both skeletal development and bone remodeling. Additionally, it has an essential function in mechanical communication (28).

Historically, the Wnt pathway is categorized into β -catenin-dependent (canonical pathway) and β -catenin independent (non-canonical pathways) (Figure 12). Although some studies postulate that different Wnts activate either the canonical or the non-canonical pathways, it has also been reported that some Wnt ligands (e.g., WNT5A) can act on both pathways (236,237). In all cases, the Wnt ligands bind to one of the 10 Frizzled family receptors (FZD) and optionally to a co-receptor that can be from the low-density lipoprotein receptor-related protein (LRP) family (LRP5/6) or a transmembrane tyrosine kinase (ROR1/2 or RYK)(238–240). The FZDs are seven-pass transmembrane G-protein receptors that can transmit signals through dishevelled proteins or through heterotrimeric G proteins (241,242). Although the canonical pathway has received much attention, there is increasing evidence on the role of the non-canonical pathways in bone and the crosstalk between the pathways (31,243,244). The canonical Wnt pathway begins with the formation of a heterotrimeric complex consisting of an FZD, a Wnt ligand and an LRP co-receptor (Figure 12). This binding results in the stabilization and translocation of β -catenin into the nucleus, where it activates the transcription of important target genes. When the pathway is not active, the 'destructive complex' phosphorylates and sequesters β -catenin in the cytoplasm for its subsequent degradation in the proteasome. The canonical Wnt pathway is regulated by a series of extracellular inhibitors common to all the Wnt pathways (e.g., Secreted frizzled-related proteins (SFRP) and Wnt Inhibitory factor 1 (WIF1)) but also by specific canonical Wnt pathway inhibitors that block the LRP5/6 binding (e.g., sclerostin and DKK1) (31,244). In addition to the canonical pathway, Wnt ligands can activate multiple signaling cascades independent of β -catenin. Within this category the most widely studied are the WNT-planar cell polarity (WNT-PCP) and the WNT-calcium (WNT- Ca^{2+}) pathways (Figure 12) (245–248). The WNT-PCP pathway begins with the binding of a Wnt ligand, such as WNT5A, to FZD and to ROR1/2 or RYK co-receptors. This binding activates small G proteins, such as RAC and RHO, which are implicated in the establishment of cell polarity and cell migration (249). In

addition, the activation of these pathways may inhibit the canonical Wnt pathway (250,251). The WNT- Ca^{2+} pathway regulates calcium release from the endoplasmic reticulum in order to control intracellular calcium levels. It begins with the binding of a Wnt ligand, such as WNT5A, to FZD, which in turn activates the phospholipase C (PLC). Cell adhesion, migration and embryonic development are regulated through this pathway (252).

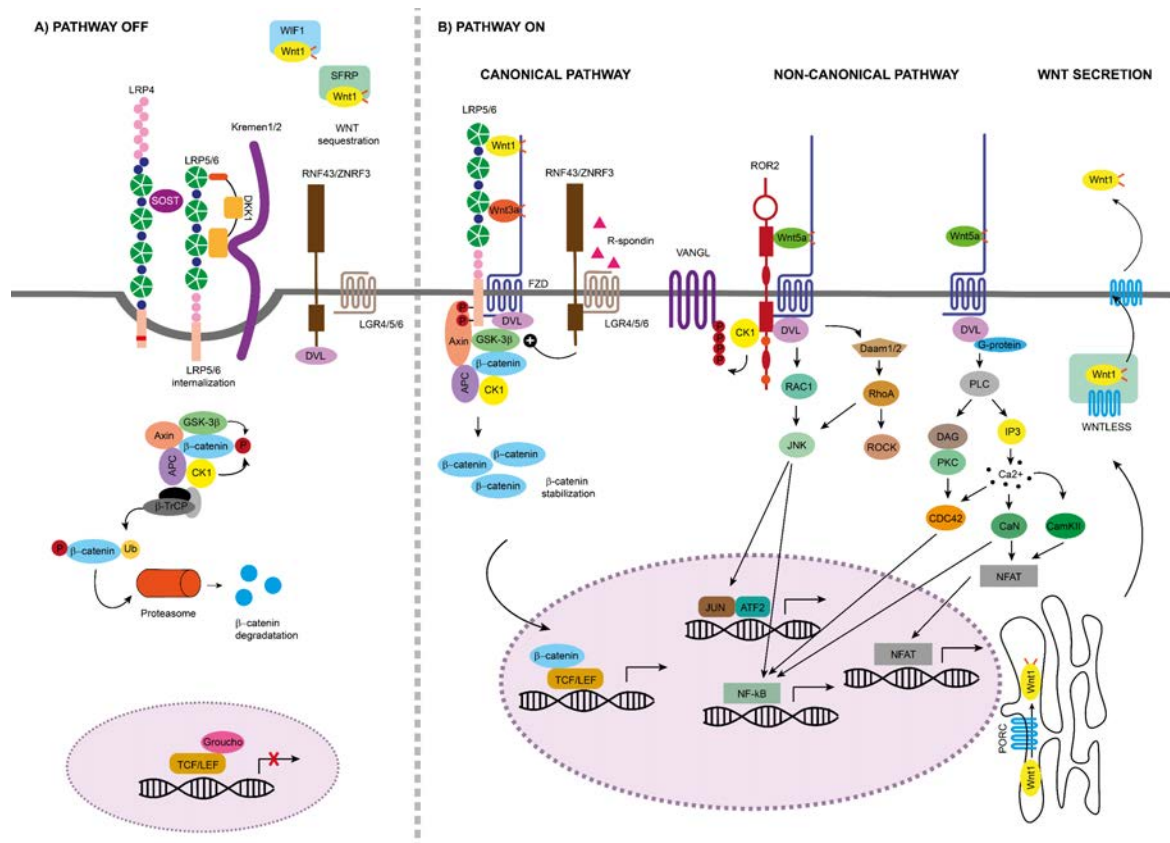


Figure 12. Wnt pathway. **Wnt pathway OFF:** The Wnt pathway is inactive in absence of Wnt ligands or by the effect of extracellular inhibitors that prevent the activation of the pathway. We can group the extracellular inhibitors into two categories depending on the action they perform: (i) binding to the Wnt ligands and preventing their binding to the membrane receptors, such as SFRP1–5 and WIF-1 and, (ii) interfering with the LRP binding to FZD, such as DKK1 and sclerostin. Due to their mechanism of action, the first type of inhibitors can act on both canonical and non-canonical pathways, while the second type is specific to the canonical Wnt pathway. The inhibition performed by DKK1 and sclerostin is mediated or enhanced by receptors such as Kremen proteins and LRP4 (253,254). When the canonical pathway is not activated, β -catenin is phosphorylated at three critical residues and sequestered in the cytoplasm by the destruction complex. The destruction complex is formed by scaffold proteins APC and AXIN1; Ser/Thr kinases, CK1 α , ϵ and δ and GSK3 β ; and transcriptional regulators, YAP/TAZ of the Hippo pathway (255). This phosphorylated β -catenin is polyubiquitinated for its degradation in the proteasome by the complex containing, among others, β -TrCP protein. Thus, it is not available in the nucleus and in its absence a complex is formed by TCF/LEF and TLE/Groucho, inhibiting the transcription of target

genes. **Wnt pathways ON:** The binding of a Wnt ligand to FZD/LRP or ROR1/2/RYK activates the Wnt pathway that will result in a different transcriptional regulation. **Canonical Wnt Pathway ON:** The pathway begins at the cell surface with the formation of a heterotrimeric complex consisting of a Wnt ligand (19 different Wnts), an LRP transmembrane co-receptor (LRP5/6), and an FZD receptor (10 different FZDs). The formation of the LRP-FZD-Wnt complex results in phosphorylation of the LRP5/6 co-receptor by CK1 α and GSK3 β . Then, the DLV (also called DSH) polymerizes and is activated, inhibiting the destruction complex. This produces stabilization and accumulation in the cytoplasm of β -catenin that will translocate to the nucleus. Once there, it displaces the TLE/Groucho repressors forming an active complex with TCF/LEF proteins, that results in the recruitment of coactivators and activation of transcription of genes important for the differentiation and formation of bone such as WISP1 and RUNX2 (31,255). **Non-canonical Wnt Pathway ON: i) WNT/PCP:** The WNT-PCP pathway begins with the binding of a Wnt ligand, such as WNT5A, to FZD and the co-receptors ROR1/2 or RYK. Then, DVL is recruited and activated, resulting in the activation of the scaffold protein VANGL. DVL forms a complex with DAMM1, which activates the small GTPase RHOA and RAC1 which in turn activate ROCK and JNK. This leads to rearrangements of the cytoskeleton and/or the induction of transcription through ATF2 and/or NFAT. **ii) WNT/Ca²⁺:** The binding of the ligand to an FZD receptor results in a recruitment and activation of DVL. Then, DVL binds to the small GTPases which activate PLC. This activation leads to the division of PIP2 into DAG and IP3. When IP3 binds to its receptor on the endoplasmic reticulum, calcium release occurs. This concentration of calcium and DAG through PKC can activate CDC42. Increased intracellular calcium can also activate calcineurin and CaMKII, which in turn can induce the activation of the NFAT TF or NF- κ B (252,256). **WNT signalling modulators:** The binding of RSPO to an LGR and RNF43/ZNRF3 maintain the Wnt signaling preventing the polyubiquitination and endocytosis of FZD performed by RNF43/ZNF3 (257). **WNT production:** Wnt precursors undergo posttranslational modifications such as porcupine-mediated palmitoylation, other lipid modifications and glycosylation in the ER. Then, the transmembrane protein WLS transports the functional Wnt ligands to the plasma membrane via the Golgi apparatus. Wnt ligands are secreted from the cell by solubilization, exosome formation, or on lipid-protein particles. Wnt is detached from the cell membrane as components of exosomes or lipid protein particles (258–260). APC: adenomatous polyposis coli; AXIN1: axis inhibition protein 1; β -TrCP: β -Transducin repeat-containing protein; CAMKII: calmodulin-dependent protein kinase II; CDC42: cell division control protein 42; CK1: casein kinase 1; DAMM1: DVL-associated activator morphogenesis; DVL: disheveled; ER: endoplasmic reticulum; FZD: Frizzled; GSK3 β : glycogen synthase kinase 3 β ; IP3, inositol 1,4,5 triphosphate, JNK: JUN kinase; LGR: Leucine-rich repeat-containing G-protein-coupled receptor; NFAT: nuclear factor of activated T cells; NF- κ B: nuclear factor kappa B; PKC: protein kinase C; PLC: Phospholipase C; PRCN: Porcupine; RAC: Ras-related C3 botulinum substrate; RHOA: Ras homolog gene family member A; RNF43/ZNRF3 : Ring finger protein 43/ Zing and Ring finger 3; ROCK: Rho kinase; ROR1/2: bind tyrosine kinase-like orphan receptor 1 or 2; RSPO; R-spondin ligand family members; RYK: receptor-like tyrosine kinase; SFRP: Secreted frizzled-related proteins; TCF/LEF: T-cell factor/lymphoid enhancer factor; TLE: Transducin-Like Enhancer of Split proteins; VANGL: Van Gogh-like; YAP/TAZ: Yes-associated

protein/transcriptional co-activator with a PDZ-binding domain; WIFI: Wnt Inhibitory factor 1; WLS: Wntless.

The importance of this pathway in bone was revealed by the description of mutations in *LRP5* that caused two diametrically opposed bone phenotypes: osteoporosis pseudo glioma syndrome (OPPG), and the high bone mass (HBM) phenotype (95,261,262) (Table 4). In addition, many rare and complex diseases are associated with gene members of the Wnt pathway (Table 4). Since many treatments for bone density diseases target genes that cause rare monogenic bone diseases, a comprehensive understanding of the causal variants and genes is fundamental.

Despite being the subject of extensive studies, the Wnt pathways display a great degree of complexity, which has so far made it challenging to gain a comprehensive understanding of all their biological functions. In particular, the expression of the target genes is tightly regulated by a myriad of extracellular components, including 19 ligands, 12 inhibitors and several receptors and co-receptors. As part of the present doctoral work, I have performed an extensive literature search with the aim of gathering all information available to date on the role of the extracellular modulators, with a special emphasis on human diseases and the animal models used to study them. This work has resulted in a publishable review which is adjoint to this doctoral thesis in Annex1.

For the purpose of the doctoral work and of this thesis, I focused on 3 members of the Wnt pathway previously associated with BMD and risk of fracture in many GWAS studies, namely *DKK1*, *SOST* and *WNT16*.

Table 4. Disease or traits affecting BMD caused by mutations in extracellular modulators of the Wnt pathway

Disease/Trait	OMIM	Phenotype	Gene	Inh	Comment	Ref.
OI XV	615220	- Low bone mass - Increased bone fragility	<i>WNT1</i>	AR AD	LoF	(263–265)
OPPG	259770	- Congenital blindness - Severe juvenile-onset OP - Spontaneous fractures	<i>LRP5</i>	AR	LoF	(105,261)
Osteoporosis	615221	- Low bone mass	<i>WNT1</i>	AD	LoF	(263)
	166710	- Risk of fractures	<i>LRP5</i>	AD	LoF	(266–268)
Cranio-diaphyseal dysplasia	122860	- Massive generalized hyperostosis - Sclerosis, especially involving the skull and facial bones	<i>SOST</i>	AD	LoF and dominant negative	(269)
Sclerosteosis	269500	- Progressive skeletal overgrowth	<i>SOST</i>	AR	LOF	(86–88)
	614305	- Syndactyly	<i>LRP4</i>	AD AR	LOF	(270,271)
van Buchem disease	239100	- Hyperostosis of the skull, mandible, clavicles, ribs and diaphyses of the long bones, as well as the tubular bones of the hands and feet.	<i>SOST</i> *	AR	* 52kb deletion downstream of <i>SOST</i>	(89)
HBM phenotype ¹	144750	- High bone mass, affecting especially the skull and tubular bones	<i>LRP5</i>	AD	GoF	(93,95,262)
	607634		<i>LRP6</i>	AD	GoF	(99)
	607636	- Low fracture risk	<i>SOST</i>	AR	LoF	(272)

OP: Osteoporosis; OI: Osteogenesis imperfecta; OPPG: Osteoporosis-pseudo glioma syndrome; AR: Autosomal recessive; AD: Autosomal dominant; LoF: Loss-of-function; GoF: Gain-of-function; REF: References¹: AKA: Osteosclerosis; Endosteal hyperostosis; Osteopetrosis type 1, Van Buchem disease type 2

5.1. DKK1

The *DKK1* gene codes for a secreted glycoprotein with the same name that belongs to the Dickkopf (Dkk) family. *DKK1* has two Cysteine rich domains (CRD1/2) and an NXI domain (Figure 13), and acts as an extracellular inhibitor of the canonical Wnt pathway (273,274). *DKK1* is extensively studied due to its crucial role in head morphogenesis (253,275), and specifically in bone tissue, since the GoF mutations HBM-LRP5 actually impair *DKK1* binding, thus producing a more active Wnt pathway (105,132). *DKK1* performs its function by inhibiting the canonical Wnt pathway through

binding to LRP5/6 coreceptors. The CRD2 domain binds, with high affinity, to the first and third β -propeller domains of the LRP5/6 and to kremen (KRM) coreceptors, and the NXI domain binds, with moderate affinity, the first β -propeller domains of the LRP5/6 coreceptors (101,104,106,276–279) (Figure 13). The binding between KRM and DKK1 facilitates the inhibition by forming a heterotrimeric LRP5/6-DKK1-KRM1/2 complex that induces rapid internalization of LRP5/6, decreasing the availability of these receptors in the cell membrane (280,281). However, it can also have an inhibitory effect in the absence of KRM1/2 (280). DKK1 is detected at low levels in most human tissues except the placenta (282,283), but in a body-wide screen of Dkk1 expression in young and adult mice, strong expression of Dkk1 was found to be mainly restricted to bone (284). This expression is most abundant in bone from young mice and decreases dramatically in adolescent and adult mice (285,286). Dkk1 expression has been demonstrated in osteoprogenitors, osteoblasts, osteocytes, and adipocytes (101,287–289), but there is controversy over whether the main source is early osteoblasts or mature osteocytes (290–292).

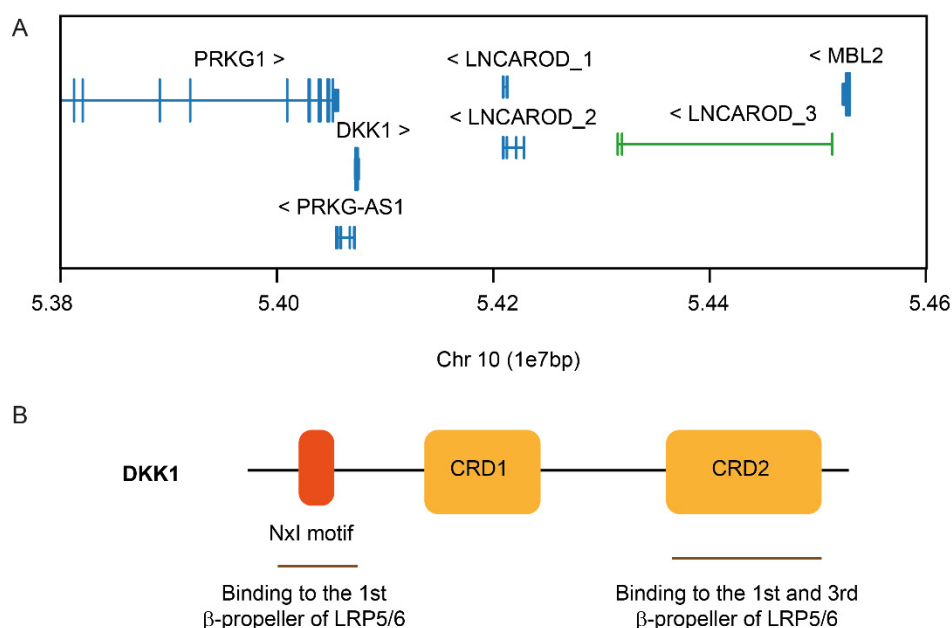


Figure 13. DKK1. A) Locus containing DKK1 and its neighbouring genes from GRCh37/hg19. In green the LNCAROD_3 transcript from GENCODE GRCh38/hg38. B) Schematic representation of DKK1 protein highlighting its functional domains. CRD: Cysteine rich domain.

DKK1 has been implicated in the pathogenesis of various diseases affecting bone and is a crucial modulator of glucocorticoid-induced (293–295) and estrogen-

deficiency-induced bone loss (294,296). Deregulation of DKK1 expression levels is associated with various bone-related disorders such as osteonecrosis of femoral head (ONFH) (297) and Paget disease; diabetes mellitus; autoimmune diseases such as rheumatoid arthritis (RA), osteoarthritis (OA), ankylosing spondylitis, and systemic lupus erythematosus; and various types of cancer including multiple myeloma, breast cancer, and prostate cancer (298,299). Very little data exists on the potential role of *DKK1* in bone mass in humans. To date, no disease-causing mutation has been reported in the HGMD. No SNPs have been found to be associated with BMD or with different bone parameters in GWASs. However, many SNPs located in a 350 kb-downstream region of *DKK1* have been found to be associated with BMD (143,149–151,158,161,162,175,300,301).

Dkk1 total knock-out (*Dkk1*-KO) mouse models die shortly after birth and exhibit developmental defects, including head defects and limb dysmorphogenesis (302) (Table 5). In contrast, the heterozygous *Dkk1*-KO mouse, the hypomorphic (doubleridge, *db*) mouse, the tamoxifen-inducible at 7 weeks mouse, and the osteolineage-specific mouse model show an increase in BMD and bone formation (287,290,303–305)(Table 5). By studying the allelic combination of null or *db* mice (*Dkk1*^{+/db}; *Dkk1*^{+/-}; *Dkk1*^{db/db}; *Dkk1*^{db/-}; *Dkk1*^{-/-}) it was seen that the increase in BMD correlates dose dependently with decreased expression of *Dkk1* (303,304). In contrast, transgenic *Dkk1* overexpression causes osteopenia with a decrease in the number of osteoblasts and bone formation (Table 5) (284,306). In double-KO (dKO) mouse models, it was shown that the developmental lethality in the *Dkk1*-KO and head morphogenesis were rescued by reduced *Lrp6* or *Wnt3* expression (303,307). While all models share an effect on bone formation, some of the generated mouse models show no difference in bone resorption, whereas osteoblast lineage cell-specific or tamoxifen-inducible conditional-KO (cKO) mice show a significantly reduced number of osteoclasts and a reduced RANKL/OPG ratio (287,290,303,304). In addition to these bone phenotypes, it was found that the response to dynamic tibial loading was augmented in mice lacking *Dkk1*, but that *Dkk1* does not seem to be essential for transducing the response to cyclic loading (33,308,309).

Thanks to *in vitro* and *in vivo* experiments, DKK1 has been confirmed to affect osteoblast function and has been linked to inhibition of the differentiation of osteoblasts and chondrocytes. Moreover, DKK1 also provokes the stimulation of

adipogenesis and the activation of osteoclasts, increasing the RANKL/OPG ratio (284,287,288,290,298).

Table 5. Mouse models used to test the role the DKK1.

Model	Tissue expression	Phenotype			Comments	Ref
		B.M.	B.F.	B.R.		
KO	Total KO	-	-	-	NV	(302)
Het KO	Heterozygous KO	↑	↑	=		(287,304)
doubleridge	Hypomorphic	↑	-	-	Deletion of a downstream regulatory region	(303,304)
Rosa26-CreERT2	Tamoxifen inducible at 7 weeks	↑	↑	↓ ¹	¹ Only the # OC	(290)
cKO-OSX-Cre	Pre-OB	↑	↑	↓ ¹	¹ Only the # OC	(290)
cKO-Dmp1-Cre	OCy	↑	↑	↓ ¹	¹ Only the # OC	(290,305)
Tg-Col1a1	OB	↓	↓	=		(284,306)

B.M.: Bone mass; B.F.: Bone formation; B.R.: Bone resorption; KO: Knock-out; cKO: conditional KO; het KO: heterozygous KO; NV: No viable; OB: osteoblast; OC: osteoclast; OCy: osteocyte; Tg.: Overexpression transgenic mice.

5.2. Sclerostin

SOST codes for the sclerostin cysteine-rich glycoprotein classified in the DAN family of BMP antagonists for his cysteine knot motifs (310,311)(Figure 14) and have been described as inhibitor of the BMP pathway and as WNT antagonist (312–316).

Sclerostin acts as a potent antagonist of the Wnt pathway, exerting its effect by blocking LRP5/6 co-receptors (312,313). To perform this function, sclerostin binds to LRP4, thereby enhancing its suppressive effect on the canonical Wnt pathway (270,317,318). The binding of sclerostin to LRP5/6 occurs through the first β -propeller domain (Figure 14), so sclerostin can inhibit the function of the Wnt ligands that bind to this domain (e.g., Wnt1) but not those that bind through the third β -propeller domain (e.g., Wnt3a) (106). This function is confirmed by mutations causing the LRP5-HBM

phenotype, which show a loss of sclerostin affinity (100,103). Due to its abundant expression in osteocytes, sclerostin was originally categorized as a protein exclusive to mature osteocytes (319). Accordingly, the highest levels of sclerostin were found in osteocytes surrounded by mineralized bone and distant from active bone surfaces (320). However, new studies have reported, through histological examination, that *SOST* transcripts are also found in bone marrow, cartilage, kidney, liver, lung, heart and pancreas (321). In relation to bone, in addition to osteocytes, sclerostin expression has been found in osteoblasts, osteoclasts, mineralized hypertrophic chondrocytes, odontoblasts, and cementocytes (322). *SOST* expression has also been found in the developing embryo, where it plays a role in limb patterning, which explains the cases of syndactyly in patients with sclerosteosis (323).

In humans, *SOST* mutations are associated with conditions characterized by excess bone formation: sclerosteosis, van Buchem disease, craniodiaphyseal dysplasia and the HBM phenotype (Table 4 and Figure 14). Indeed, the first evidence of the role of *SOST* in bone was the identification of LoF mutations responsible for sclerosteosis, a very severe autosomal recessive disease, caused by a lack of functional sclerostin (86–88,324). Furthermore, deletion of a regulatory region 52 Kb downstream of the gene, necessary for the correct expression of *SOST*, has also been found in patients with van Buchem disease (89). Van Buchem disease is less severe than sclerosteosis and the difference in severity lies in the fact that in van Buchem patients there is some expression of the *SOST* gene, whereas in patients with sclerosteosis there is none (325,326). Moreover, craniodiaphyseal dysplasia is an extremely rare sclerosing bone dysplasia associated with missense mutations in the signal peptide of sclerostin that cause a decrease in its extracellular secretion (269). Gregson et al. (272) also associated mutations in *SOST* with the HBM phenotype. In addition to these diseases, different GWAS studies have shown their association with BMD and the risk of fractures (143,149–151,158,174,175,301). Furthermore, altered expression of the gene has been found under special or pathological conditions. For example, although no sclerostin protein expression has been found in articular cartilage in healthy joint tissue, many studies have reported sclerostin protein expression in articular chondrocytes isolated from osteoarthritic joints or in rheumatic joint diseases. Nevertheless, it is unclear whether sclerostin plays a protective role or whether it mediates disease pathogenesis (327). Several groups have shown elevated

serum sclerostin levels in postmenopausal women (328,329), with aging (330–334), in patients with excess glucocorticoids (335), in multiple myeloma patients correlating with osteoblast function and low survival (336), in prostate cancer and breast cancer (337,338) and in vascular calcifications (339,340).

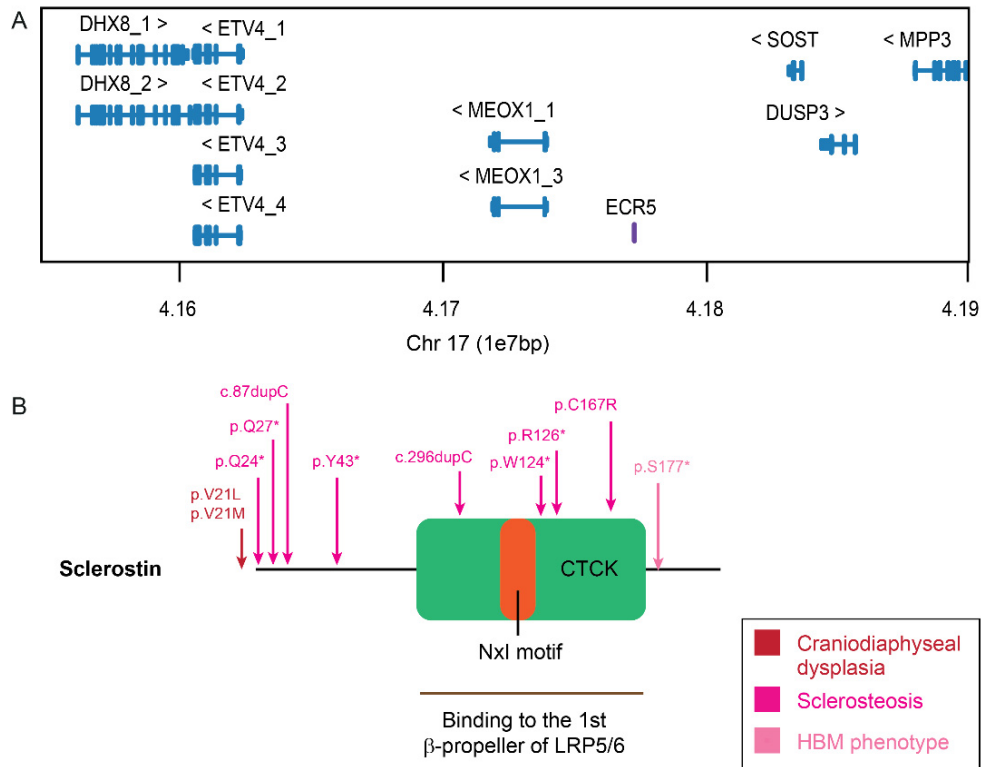


Figure 14. *SOST*. A) Locus containing *SOST* and its neighbouring genes from GRCh37/hg19. In purple the *ECR5* regulatory region. B) Schematic representation of Sclerostin protein highlighting its functional domains and the mutations described causing human skeletal diseases. CTCK: C-terminal cysteine knot-like.

The importance of the *SOST* gene in the determination of BMD has been demonstrated using animal models. *Sost*-KO mice show a phenotype of high BMD and bone strength with a high number of osteoblasts and increased bone formation that reproduce the phenotype observed in patients with sclerosteosis or van Buchem disease (341,342)(Table 6). In these mice, no changes were observed in the number of osteoclasts or in bone resorption markers, although an increase in OPG was observed (341). In order to identify the cell-type specific contributions to HBM of the *Sost*-KO, Yee *et al.* (343) generated different cKOs and observed that despite the fact that osteocytes are the main source of sclerostin, the cKO that best phenocopies the HBM of the *Sost*-KO is the *Sost*-cKO in osteoprogenitor cells of the limb mesenchyme

rather than the *Sost*-cKO in early stage osteocytes (Table 6). In contrast, mice that overexpress this gene present a low bone mass, lower bone resistance, a low number of osteoblasts and reduced bone formation (214,344–346). In addition, the mouse model with specific overexpression in osteocytes shows high levels of *Rankl* (344,346)(Table 6). All these data lead to the conclusion that sclerostin is a potent inhibitor of bone formation and that it may also influence the stimulation of bone resorption in some way.

Table 6. Mouse models used to test the role the Sclerostin.

Model	Tissue expression	Phenotype			Comments	Ref
		B.M.	B.F.	B.R.		
KO	Total KO	↑↑	↑	=		(341,342)
cKO-Prx2-Cre	Limb mesenchyme	↑↑	-	=		(343)
cKO-Col1a1-Cre	OB	↑	-	=		(343)
cKO-Dmp1-Cre	OCy	↑	-	=		(343)
cKO-Col10a1-Cre	CD	↑	-	=		(343)
Tg-Dmp1-SOST	OCy	↓	↓	=		(344,346)
Tg-Oc-SOST	OB	↓	↓	=		(345)
Tg-SOST	Total Tg.	↓	↓	=	Insertion of ~158-kb from 3'end of the <i>DUSP3</i> to <i>MEOX1</i> .	(214)

B.M.: Bone mass; B.F.: Bone formation; B.R.: Bone resorption; KO: Knock-out; cKO: conditional KO; OB: osteoblast; Ocy: osteocyte; CD: chondrocytes; Tg.: Overexpression transgenic mice.

The transcription of the *SOST* gene is finely regulated by a large number of signals. *SOST* expression is controlled by the proximal promoter region of the gene and by a 225-bp bone-specific distal enhancer called Evolutionary conserved region 5 (ECR5) 52 Kb downstream of the gene (214,347,348). The proximal promoter region (~ 1.4 kb upstream from the *SOST* gene) contains binding sites to RUNX2, SP7 and SMAD2/3, among others, which promote *SOST* transcription (196,349–353). In addition, the proximal promoter contains three estrogen response elements (ERES) that negatively control the expression of *SOST* through estrogen receptor B (354). Furthermore, it has been determined that this region is subject to epigenetic

modifications that regulate its expression, such as methylation (197,355) or acetylation (186). In addition to the proximal promoter, the ECR5 enhancer region regulates the correct expression of *SOST* in bone cells. The ECR5 region is deleted in patients with van Buchem disease, and its deletion in mice causes a drastic decrease in *SOST* levels in osteocytes (348). ECR5 stimulates the expression of *SOST*, in part through the binding of TF Myocyte-enhancer factor 2c (Mef2c) (214,348). The importance of the Mef2c TF in the enhancing activity of ECR5 was confirmed with the Mef2c-cKO mouse in osteocytes/osteoblasts, which presented HBM and low levels of sclerostin (356). Besides regulation through the proximal promoter, there is a large number of regulators of the expression of *SOST* in bone whose action is mediated via ECR5. The most relevant negative regulators of *SOST* are: PTH, cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), mechanical loading, and the IL-6 family of cytokines.

Sclerostin is the first molecular mediator described in osteocytes as responsible for the coupling of mechanical forces to the anabolic response of the skeleton (33,357). The mechanical loading of bone results in a dramatic reduction in *SOST* expression resulting in increased bone formation, and conversely unloading increases it (33). This is consistent with the fact that the *Sost*-KO mouse does not lose BMD after unloading (358), while the *SOST* transgenic overexpression mouse shows a reduction in load-induced bone formation (346). The mechanical loading negatively regulates *SOST* expression by mechanisms that involve prostaglandin E2 production, nitric oxide and periostin expression (359–362). While *SOST* is absolutely necessary for the anabolic actions of mechanical loading, it appears dispensable for PTH-induced bone gain (347,363). In addition to these effects, *SOST* has been shown to enhance adipocyte differentiation and promote adipogenesis and adipose hypertrophy via suppression of the Wnt pathway in *in vitro* and *in vivo* experiments (364–366).

Humanized monoclonal antibodies against sclerostin have been developed for the treatment of different bone pathologies such as osteoporosis and OI (367–372). Antibody treatment against sclerostin stimulates bone formation, decreases bone resorption and increases bone mass and strength while decreasing the risk of fragility fracture in both animals and humans (373–375). Thanks to positive results in clinical phase 2 and 3 trials for the treatment of postmenopausal osteoporosis (367–370), it is

currently approved for the market. The negative aspects associated with anti-sclerostin antibody treatment are that the increase in bone formation is not sustained in the long term and that it is contraindicated in patients with hypocalcemia or a high risk of ischemic heart disease or cerebrovascular disorder, therefore requiring caution before the implementation of treatment (376).

5.3. WNT16

Wnt ligands are highly conserved secreted glycoproteins formed by 350–400 amino acids, 23–24 of which are conserved cysteine residues. For secretion of an active Wnt ligand, Porc-mediated lipidation by palmitic acid is necessary in a conserved serine residue (Figure 15). Following this it is secreted into the extracellular matrix through Wntless (Wls) binding (258,259,377). However, some authors have questioned the essential nature of this step (259,378,379).

Although the degree of sequence identity between some Wnt family members is only 18%, it is thought that all Wnt proteins form a similar three-dimensional structure, which is shaped like a fist with a thumb and index finger protrusions (377,378,380). There are four main regions of concentrated amino acid conservation that map onto the Wnt structure: the tips of the thumb and index finger loops, the core of the N-terminal α -helical domain (NTD), and a large continuous patch in between, facing the exterior (Figure 15). The two main contact points between the Wnt ligand and the FZD receptor are the tips of the thumb and index loops (377,378). The CRD at the second point of contact with the receptor is similar to that of sFRP and ROR1/2, among others (381). On the NTD, the lipidation of the conserved serine creates hydrophobic forces with the receptor that, combined with shape complementarity, facilitate binding (378). Speer *et al.* argued that the lipid is not necessary for this interaction to occur, and thus could be kept outside the cleft (379). Nevertheless, the importance of the fatty acid binding seems worth noting, as mutations in the residues of the cleft lead to defects in receptor structure, and the presence of the lipid seems to activate the dimerization and activation of the receptor (382). The thumb loop residues contact with the CRD domain of the FZD receptors with poorer shape complementarity. The other conserved regions of the protein located opposite the FZD binding region bind to co-receptors such as LRP5/6/RYK (377,378,383). The amino acid differences between Wnt ligands enable their differential binding to receptors. For

example, WNT1 binds to the first β -propeller domain of LPR5/6 whereas WNT3a binds to the third β -propeller domain (106).

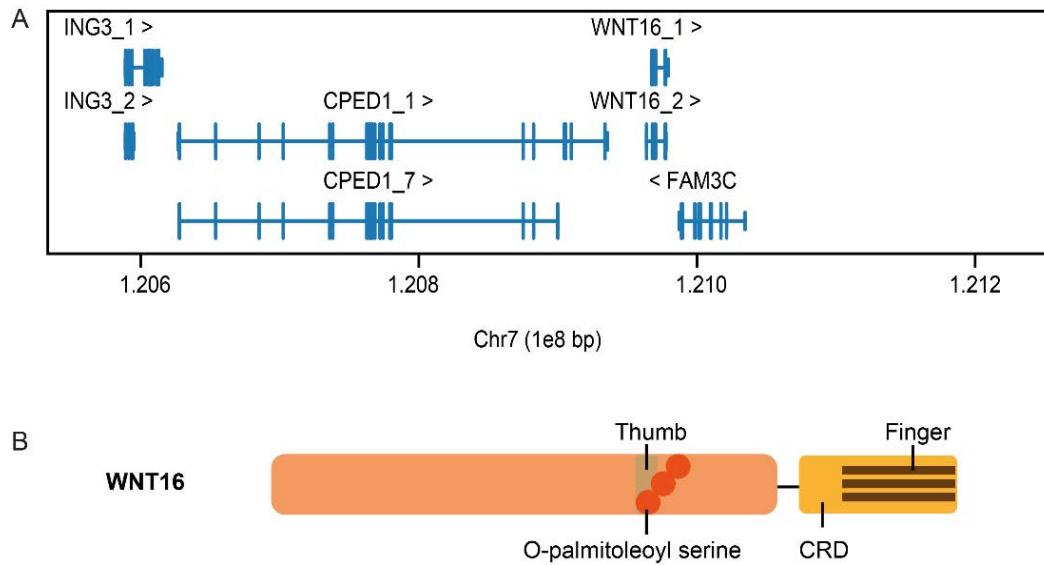


Figure 15. WNT16. A) Locus containing WNT16 and its neighbouring genes from GRCh37/hg19. B) Schematic representation of WNT16 protein highlighting its functional domains. CRD: Cysteine rich domain.

WNT16 is an important Wnt ligand involved in the regulation of postnatal bone homeostasis. The precise mechanism by which WNT16 acts on the different signaling pathways of WNT has not been fully identified. Different studies postulate that the effect of WNT16 on these pathways is cell-type specific. While there are studies showing that it activates the canonical pathway in MC3T3-E1 preosteoblasts and in Saos2 osteoblast-like cells, WNT16 does not activate this pathway in HEK293 cells (384–386). There is evidence that in chondrocytes, MC3T3-E1 and perivascular stem/stromal cells, WNT16 is also capable of acting through non-canonical pathways (PCP/JNK (387) and JNK (384,388)). Altogether, these results indicate that WNT16 regulates both canonical and non-canonical Wnt targets in osteoblasts (389).

In humans, the genomic region of *WNT16* is one of the most consistent GWAS signals, and has been associated with different skeletal phenotypes, including BMD, bone strength, geometric parameters, cortical bone thickness and fracture risk (127,143,149,150,156,158,160–162,169,172,173,175,300,301,390). In addition, the effect of WNT16 has been associated, through various pieces of evidence, with OA. First, the expression of *WNT16* is dramatically upregulated after cartilage injury or in OA onset (391). Wnt16-deficient mice develop severe OA because of reduced lubricin

and increased chondrocyte apoptosis (392). Using chondrocyte-cKO animal models and overexpression of Wnt16 by intra-articular injection of adenoviral Wnt16 vectors, Tong *et al.* determined that Wnt16 inhibited chondrocyte hypertrophy in both skeletal development and OA pathology, therefore producing positive effects on cartilage and ongoing OA (387). Contrary to these experiments, Törnqvist *et al.* (393) found that overexpression of Wnt16 in osteoblasts increased the subchondral bone mass but had no impact on OA in young adult female mice.

Wnt16-KO and the osteoblast-specific Wnt16-cKO mouse models show spontaneous fractures as a result of low BMD, low cortical thickness, reduced bone strength, and high cortical porosity, while the trabecular bone volume is not altered (156,169,384,394)(Table 7). Consistent with this, Wnt16 was found to be more highly expressed in cortical bone than trabecular bone (384). The phenotype of the osteocyte Wnt16-cKO mouse model is modest compared with the early osteoblast Wnt16-cKO (384). On the other hand, when Wnt16 is overexpressed in osteoblasts and osteocytes, the mouse models show higher BMD and better bone resistance in both trabecular and cortical bone (395–397) (Table 7). The difference in effect in the trabecular bone phenotype between the KO and KI mice might be due to the low expression of Wnt16 in the trabecular compartment. Despite the obvious bone phenotype that Wnt16 produces in mouse models, the molecular mechanisms by which Wnt16 regulates bone metabolism are not yet fully understood. Some authors suggest that Wnt16 acts by inhibiting osteoclastogenesis both indirectly, through the increase in OPG, and directly, acting on the osteoclast progenitors by osteoblast-derived Wnt16 (384,398). Other studies have shown that Wnt16 also exerts an effect on bone formation, increasing both the activity and the number of osteoblasts (394). Different effects have also been observed in transgenic mouse models overexpressing Wnt16 in osteoblast cells; some do not show differences in bone formation and resorption (397), while others show a large increase in bone formation and a moderate decrease in bone resorption (395,396). Moreover, Shen *et al.* demonstrated *in vitro* that Wnt16 promotes osteogenic differentiation of human perivascular stem/stromal cells (388). Taking all these studies into account, it would appear that Wnt16 has a dual anabolic and anti-resorptive effect. Different animal models show that the effect of Wnt16 and estrogen are independent (397), although it has been shown that the expression of Wnt16 in bone varies according to estrogen status (397,399). Moreover, Wnt16 is a crucial regulator of cortical bone in young adults and old mice (400). These

discoveries make WNT16 a good candidate as a therapeutic target for the treatment of osteoporosis in postmenopausal women. Another interesting potential application of Wnt16 treatment would be in bone loss due to glucocorticoid treatment. In reference to this, studies in animal models with Wnt16 over-expression and glucocorticoid treatment have been performed, with contradictory results. While some report partial protection against glucocorticoid-induced bone loss (401), others report that Wnt16 overexpression does not prevent it (402). Therefore, more studies will be needed to clarify whether WNT16 could be a good therapeutic solution.

Table 7. Mouse models used to test the role the Wnt16.

Model	Tissue expression	Phenotype			Comments	Ref
		B.M.	B.F.	B.R.		
KO	Total KO	↓	=/↓ ^I	↑	in cortical bone ^I ↓ (394); =(384)	(156,169, 384,394)
cKO-Runx2-Cre	OB	↓	-	-	In cortical bone	(384)
cKO-Dmp1-Cre	Ocy	=	-	-		(384)
CAG-Cre-ER	Tamoxifen inducible	↓	↓	↑	In cortical bone	(400)
Tg-Col1a1-Wnt16	OB	↑	=/↑ ^{II}	=/↓ ^{III}	^{II} = (397); ↑ (395) ^{III} = (397); ↓(395)	(395,397)
Tg-Dmp1-Wnt16	OCy	↑	↑	=		(396)

B.M.: Bone mass; B.F.: Bone formation; B.R.: Bone resorption; KO: Knock-out; cKO: conditional KO; OB: osteoblast; Ocy: osteocyte; Tg.: Overexpression transgenic mice.

OBJECTIVES

The two main objectives of this thesis are 1) to elucidate and functionally characterize the causal variants corresponding to bone mineral density GWAS signals within or close to the Wnt pathway genes, and 2) to determine genes that could explain the HBM phenotype.

To address them, the following specific aims were defined:

1. Identification of rare and common variants in three Wnt pathway genes associated with bone mineral density.
 - Resequence the coding and regulatory regions of *WNT16*, *DKK1* and *SOST* in a truncate selection of the BARCOS cohort in order to detect putative functional variants in LD with the sentinel variant from the BMD-GWAS meta-analysis of Estrada et al. (149).
 - Examine the detected variants through association analysis in the complete BARCOS cohort and through *in silico* prediction of functional effects, and define a list of the most interesting variants.
2. Functional characterization of the most interesting variants in *WNT16*, *SOST* and *DKK1* determined in objective 1.
 - Characterize rare missense variants in *DKK1* present in HBM women and in the general population.
 - Analyze the BMD-GWA-SNP-rich region located 350 kb downstream of *DKK1*.
 - Explore a putative relationship between *DKK1* variants, Chiari malformation type I and BMD.
 - Perform a detailed functional analysis of the region containing the variant rs142005327 in *WNT16*.
 - Perform a functional analysis of a low frequency variant *SOST* missense variant associated with HBM.
 - Characterize the regions interacting with the *SOST* proximal promoter.

- Conduct a functional analysis of a rare variant located in the extended TATA box of *SOST*.
3. Identification of genes that could explain Mendelian cases of the HBM phenotype.
- Resequence the candidate genes *SOST* in a small cohort of HBM cases from the Barcelona area.
 - Perform a whole exome sequencing analysis in a family segregating the HBM phenotype (family HBM9).

RESULTS

THESIS SUPERVISORS' REPORT ON THE CONTRIBUTION OF THE PHD CANDIDATE TO THE ARTICLES INCLUDED IN THIS DOCTORAL THESIS

Thesis title: “Role of Wnt pathway genes in complex and monogenic phenotypes of low and high bone mass”

Author: Núria Martínez-Gil

Supervisors: Dr. Daniel Grinberg Vaisman and Dr. Susanna Balcells Comas

The undersigned, Daniel Grinberg Vaisman and Susanna Balcells Comas, acting as PhD Supervisors of the Thesis by Núria Martínez Gil entitled “Role of Wnt pathway genes in complex and monogenic phenotypes of low and high bone mass” and presented as a compendium of 6 research articles, hereby inform about the precise contribution of the candidate in each publication.

CHAPTER 1: Identification of rare and common variants in three Wnt pathway genes associated with bone mineral density.

Article 1

Title: Common and rare variants of *WNT16*, *DKK1* and *SOST* and their relationship with bone mineral density.

Authors: Núria Martínez-Gil, Neus Roca-Ayats, Anna Monistrol-Mula, Natàlia García-Giralt, Adolfo Díez-Pérez, Xavier Nogués, Leonardo Mellibovsky, Daniel Grinberg & Susana Balcells

Journal: Scientific Reports

Number: 8 (2018)

Pages: 10951

Impact Factor (2018 JCR Science Edition): 4.011

Contribution of the PhD candidate: The candidate has undertaken most if not all of the work presented in the paper, which constitutes the starting point of her thesis, upon which

several other objectives have been built. She was involved in selecting the 50 women with highest BMD and 50 women with lowest BMD from the BARCOS cohort; she personally resequenced the coding regions of the 3 genes in these 100 samples and documented all the variants present in them; she then carried out *in silico* tests of pathogenicity, calculations of burden tests, and genotyping of selected variants in the complete BARCOS cohort. She performed the statistical analyses of association and drafted all the tables and figures of the MS. She was involved in the drafting of the MS and in the subsequent revisions after the feed-back from coauthors and the comments from reviewers.

CHAPTER 2: Functional characterization of the most interesting variants in *DKK1*, *SOST* and *WNT16* determined in objective 1.

Article 2

Title: Functional assessment of coding and regulatory variants from the DKK1 locus

Authors: Núria Martínez-Gil, Neus Roca-Ayats, Nurgül Atalay, Marta Pineda-Moncusí, Natàlia Garcia-Giralt, Wim Van Hul, Eveline Boudin, Diana Ovejero, Leonardo Mellibovsky, Xavier Nogués, Adolfo Díez-Pérez, Daniel Grinberg, Susanna Balcells

Journal: JBMR plus

Status: Accepted DOI: 10.1002/jbm4.10423

Impact Factor (2019 JCR Science Edition): NA

Contribution of the PhD candidate: Núria Martínez-Gil conceived and performed most of the experiments in this study. In particular, she built the plasmids, performed the mutagenesis, and performed the luciferase reporter assays (Wnt and 3'UTR pathway). She also genotyped all three SNPs, performed the RT-qPCR analysis in samples from primary human osteoblasts and performed the statistical analysis required for the eQTL assay. Next, she performed the alternative splicing test and the 4C-seq experiments, including their design of the cell culture and chromatin fixation of the cells. Finally, she completed the analysis of the significant interacting regions using the AfourC software.

She performed the global analysis and interpretation of data, the elaboration of figures and tables, and drafted the manuscript. She also handled the comments by co-authors and reviewers to reach a final accepted MS.

Article 3

Title: On the associations between Chiari malformation type I, bone mineral density and DKK1: a pilot study.

Authors: Núria Martínez-Gil, Leonardo Mellibovsky, Damian Manzano-Lopez Gonzalez, Daniel Grinberg and Susanna Balcells

Journal: Submitted to Journal of Applied Genetics

Contribution of the PhD candidate: Núria Martínez-Gil conceived the work and managed the coordination among clinicians specialized in CMI and those with expertise in bone mass phenotypes. She performed the wet lab experiments, prepared the tables and figures and drafted the initial MS. Upon rejection by a first journal, she has also handled the re-writing and resubmission to a second journal.

Article 4

Title: Genetics and genomics of *SOST*: functional analysis of variants and genomic regulation in bone cells

Authors: Núria Martínez-Gil, Neus Roca-Ayats, Mónica Cozar, Natàlia Garcia-Giralt, Diana Ovejero, Xavier Nogués, Daniel Grinberg, Susanna Balcells

Journal: Submitted to International Journal of Molecular Sciences

Contribution of the PhD candidate: Núria Martínez-Gil conceived and performed all the experiments in this study. She built the plasmids, performed the mutagenesis, and performed the luciferase reporter assay for the TATA-box variant; prepared the plasmids, performed cell transfections and the western blots to test the p.Val10Ile missense variant. Finally, she performed the 4-C experiment of the *SOST* promoter and completed the

analysis of the significant interacting regions using the AfourC software. She interpreted the data, created the figures and tables, and drafted the manuscript. She also handled the comments by co-authors prior to submission.

Article 5

Title: Regulation of WNT16 in bone involves upstream enhancers within CPED1

Authors: Núria Martínez-Gil, Neus Roca-Ayats, Carlos Herrera-Ubeda, Nicola Gritti, Nerea Ugartondo, Natàlia Garcia-Giralt, Diana Ovejero, Xavier Nogués, Jordi Garcia-Fernandez, Daniel Grinberg, Susanna Balcells

Journal: To be submitted to Genome Biology

Contribution of the PhD candidate: Núria Martínez-Gil conceived and performed most of the experiments in this study, including the design, actual cloning of selected regions and performance of the luciferase promoter/enhancer tests and also the design and performance of the chromatin conformation experiments (cell culture and chromatin fixation of the cells used). She has set up the collaboration with Dr. Gritti for the development of the AfourC software and with Dr. Herrera-Ubeda for the bioinformatic analysis of RNA-seq data from the WNT16 intron 2 region. She has carried out the overall data analysis and interpretation and has prepared the figures. She has drafted the first version of the MS and modified it according to the comments by co-authors and reviewers.

CHAPTER 3: Identification of genes that could explain Mendelian cases of the HBM phenotype.

Article 6

Title: A missense mutation in VAV3 in a familial case of high bone mass

Authors: Núria Martínez-Gil, Diana Ovejero, Natalia Garcia-Giralt, Leonardo Mellibovsky, Xavier Nogués, Raquel Rabionet, Daniel Grinberg, Susanna Balcells

Journal: To be submitted

Contribution of the PhD candidate: The candidate has participated in the conception of the WES study, has prepared the DNA samples for WES and performed the manual filtering of the variant list. She has also verified the selected variants by Sanger sequencing and performed the cosegregation analysis. She has coordinated with clinicians for the collection of the bone formation and bone resorption phenotypes in the family members and has drafted the MS.

Barcelona,



Daniel Grinberg



Susanna Balcells

CHAPTER 1: IDENTIFICATION OF RARE AND COMMON VARIANTS IN THREE WNT PATHWAY GENES ASSOCIATED WITH BONE MINERAL DENSITY.

Article 1

Common and rare variants of *WNT16*, *DKK1* and *SOST* and their relationship with bone mineral density.

Summary:

Numerous GWAS and candidate gene studies have highlighted the role of the Wnt pathway in bone biology. Our objective has been to study in detail the allelic architecture of three Wnt pathway genes: *WNT16*, *DKK1* and *SOST*, in the context of osteoporosis. We have resequenced the coding and some regulatory regions of these three genes in two groups with extreme bone mineral density (BMD) ($n = \sim 50$, each) from the BARCOS cohort. No interesting novel variants were identified. Thirteen predicted functional variants have been genotyped in the full cohort ($n = 1490$), and for ten of them (with $MAF > 0.01$), the association with BMD has been studied. We have found six variants nominally associated with BMD, of which 2 *WNT16* variants predicted to be eQTLs for *FAM3C* (rs55710688, in the Kozak sequence and rs142005327, within a putative enhancer) withstood multiple-testing correction. In addition, two rare variants in functional regions (rs190011371 in *WNT16b* 3'UTR and rs570754792 in the *SOST* TATA box) were found only present in three women each, all with BMD below the mean of the cohort. Our results reinforce the higher importance of regulatory versus coding variants in these Wnt pathway genes and open new ways for functional studies of the relevant variants.

Reference: Núria Martínez-Gil, Neus Roca-Ayats, Anna Monistrol-Mula, Natàlia García-Giralt, Adolfo Díez-Pérez, Xavier Nogués, Leonardo Mellibovsky, Daniel Grinberg & Susana Balcells. Common and rare variants of *WNT16*, *DKK1* and *SOST* and their relationship with bone mineral density. *Scientific Reports*. 2018; 8:10951 | DOI:10.1038/s41598-018-29242-8

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Common and rare variants of *WNT16*, *DKK1* and *SOST* and their relationship with bone mineral density

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Núria Martínez-Gil¹, Neus Roca-Ayats¹, Anna Monistrol-Mula¹, Natàlia García-Giralt², Adolfo Díez-Pérez², Xavier Nogués², Leonardo Mellibovsky², Daniel Grinberg¹ & Susana Balcells¹

Numerous GWAS and candidate gene studies have highlighted the role of the Wnt pathway in bone biology. Our objective has been to study in detail the allelic architecture of three Wnt pathway genes: *WNT16*, *DKK1* and *SOST*, in the context of osteoporosis. We have resequenced the coding and some regulatory regions of these three genes in two groups with extreme bone mineral density (BMD) ($n \sim 50$, each) from the BARCOS cohort. No interesting novel variants were identified. Thirteen predicted functional variants have been genotyped in the full cohort ($n = 1490$), and for ten of them (with $MAF > 0.01$), the association with BMD has been studied. We have found six variants nominally associated with BMD, of which 2 *WNT16* variants predicted to be eQTLs for *FAM3C* (rs55710688, in the Kozak sequence and rs142005327, within a putative enhancer) withstood multiple-testing correction. In addition, two rare variants in functional regions (rs190011371 in *WNT16b* 3'UTR and rs570754792 in the *SOST* TATA box) were found only present in three women each, all with BMD below the mean of the cohort. Our results reinforce the higher importance of regulatory versus coding variants in these Wnt pathway genes and open new ways for functional studies of the relevant variants.

In the last decade, numerous genome wide association studies (GWAS) have been conducted as a strategy to identify new genes and variants associated with complex diseases. In the field of osteoporosis, a number of GWAS have been performed to determine the association with bone mineral density (BMD) and osteoporotic fracture. BMD is a complex quantitative trait that is mostly genetically determined, therefore it is a good biological marker for the determination of bone status. Initial GWAS studies^{1–6} have identified more than 50 *loci* associated with BMD, among which the Wnt/ β -catenin signaling pathway was especially enriched. Specifically, in the study by Estrada *et al.*⁶, 14 BMD-associated *loci* were also found to be associated with fracture risk, and 6 of them belonged to the Wnt pathway: *LRP5*, *SOST*, *DKK1*, *WNT4*, *WNT16* and *CTNNA1*. More recently a low-frequency variant with large effects on BMD has been described in the *WNT16* gene in a whole genome sequencing study⁷. In fact, several monogenic bone phenotypes are caused by rare mutations in genes of the Wnt pathway. For example, sclerosteosis^{8–10} and van Buchem^{11,12} – two monogenic bone overgrowth diseases – are caused by rare loss of function mutations or the deletion of an enhancer element (ECR5) of *SOST*, and the osteoporosis-pseudoglioma syndrome is caused by rare loss of function mutations of *LRP5*¹³.

The Wnt pathway regulates a wide variety of cellular processes during embryogenesis and in adult regenerative tissues, as is the case for bone. Wnt proteins can activate up to 3 signaling pathways: 2 non-canonical and one canonical pathway or β -catenin-dependent pathway. When this signaling pathway is activated in osteoblasts, a series of important genes for the differentiation and formation of bone are induced¹⁴. The canonical pathway is finely regulated by extracellular inhibitors, including *DKK1* and sclerostin, encoded by *DKK1* and *SOST*,

¹Department of Genetics, Microbiology and Statistics, Faculty of Biology, University of Barcelona, IBUB, IRSJD, CIBERER, Barcelona, Spain. ²Musculoskeletal Research Group, IMIM (Hospital del Mar Medical Research Institute), Centro de Investigación Biomédica en Red de Fragilidad y Envejecimiento Saludable (CIBERFES), ISCIII, Barcelona, Spain. Daniel Grinberg and Susana Balcells jointly supervised this work. Correspondence and requests for materials should be addressed to S.B. (email: sbalcells@ub.edu)

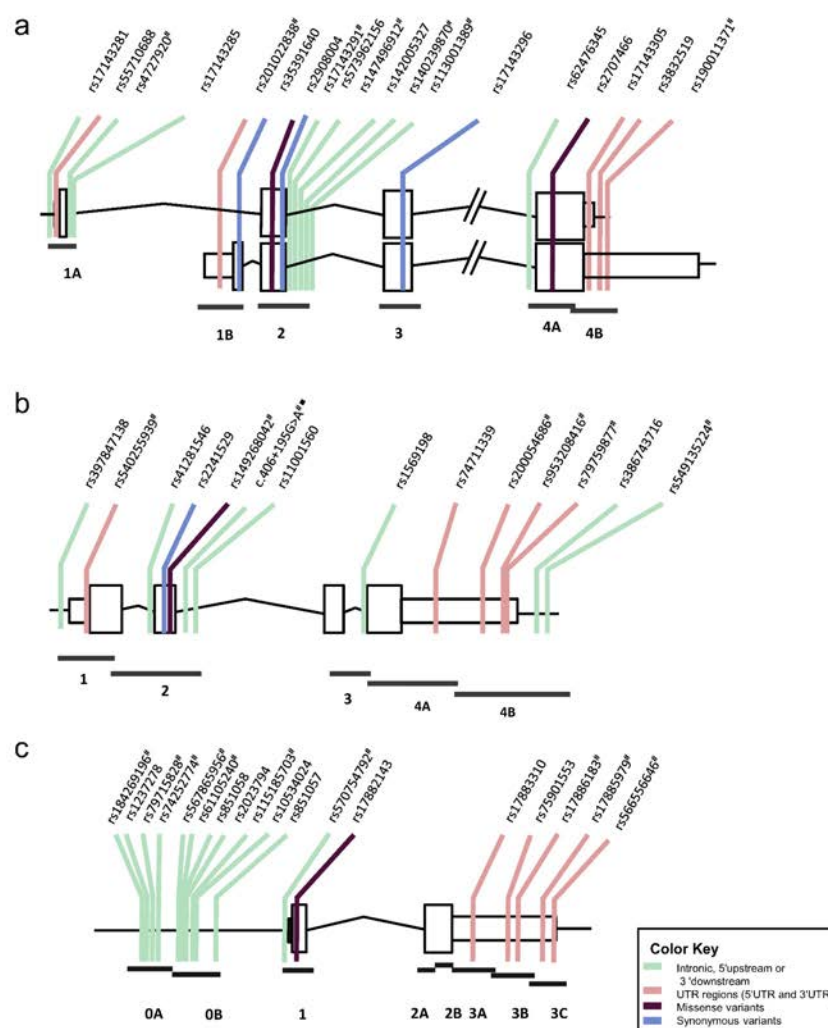


Figure 1. Variants found in *WNT16* (a), *DKK1* (b) and *SOST* (c). Horizontal lines below the genes indicate amplicons used to resequence the exons and flanking intronic regions (not to scale). *Rare variants (MAF < 0.01). ■ Novel variant.

respectively. Sclerostin is produced mainly in osteocytes, although it is also expressed in other cell types differentiated from the mineralized matrix, such as cementocytes or chondrocytes¹⁵.

Despite the large number of *loci* associated with BMD, they account for only a small part of the genetic impact on the femoral neck BMD (<6%)⁶. Thus, the variability of BMD cannot be explained solely by tag SNPs, which is why a search for new polymorphisms and rare variants is necessary. Our objective has been to identify variants within 3 genes of this pathway: *WNT16*, *DKK1* and *SOST*. We have approached the characterization following a two-step process. First, we have resequenced the coding regions of these genes in two extreme BMD groups of postmenopausal women of the BARCOS cohort to look for rare variants responsible for the phenotype, or common variants associated with BMD. Secondly, we analyzed the association of the common and low frequency variants with BMD, in the full cohort, and we explored the distribution of the rare variants.

Results

Search for Variants in a Truncated Selection of Samples from the BARCOS Cohort and Analysis of their Frequencies in the Extreme Groups. We resequenced *WNT16*, *DKK1* and *SOST* coding exons, intronic flanks, UTRs and regulatory regions, in 108 postmenopausal women in the extremes of the BMD distribution of the BARCOS cohort (55 with HBM and 53 with LBM, see Methods). A total of 19, 14 and 18 single nucleotide variants (SNVs) were identified in *WNT16*, *DKK1* and *SOST*, respectively (Tables 1–3 and Fig. 1), of

Variant definition			rs Number	Type		MAF (EUR)	Number of alleles		Functional prediction
<i>WNT16</i> (genomic)	<i>WNT16a</i>	<i>WNT16b</i>		<i>WNT16a</i>	<i>WNT16b</i>		HBM	LBM	
g.120965352 C > T	g.120965352 C > T	g.120965352 C > T	rs17143281	5'UP	5'UP	0.036 (T)	7	3	eQTL
g.120965467_120965470dup CCCC	p.Met1?	g.120965467_120965470dup CCCC	rs55710688	Fs	5'UP	0.233 (CCCC)	31	23	eQTL
g.120965562 A > G	c.65 + 28 A > G	g.120965562 A > G	rs4727920	I	5'UP	0.003 (G)	3	1	
g.120965585 T > A	c.65 + 51 T > A	g.120965585 T > A	rs17143285	I	5'UP	0.041 (A)	3	7	
g.120969332 C > A	c.66–289 C > A	c.–15 C > A	rs201022838	I	5'UTR	0 (A)	1	0	
g.120969412 G > C	c.66–209 G > C	p.Leu22Leu	rs35391640	I	S	0.038 (C)	4	2	
g.120969769 G > A	p.Gly72Arg	p.Gly82Arg	rs2908004	M	M	0.44 (A)	51	38	eQTL; T/B/P
g.120969825 C > A	p.Thr90Thr	p.Thr100Thr	rs17143291	S	S	0.009 (A)	0	2	
g.120969922_120969924delCTC	c.316 + 51_316 + 53delCTC	c.346 + 51_346 + 53delCTC	rs573962156	I	I	0.015 (-)	1	2	
g.120969929 G > T	c.316 + 58 G > T	c.346 + 58 G > T	rs147496912	I	I	0.009 (T)	1	1	
g.120969974_120969975dupCT	c.316 + 103_316 + 104dupCT	c.346 + 103_346 + 104dupCT	rs142005327	I	I	0.254 (CT)	30	23	eQTL; T/B/P
g.120970018 A > G	c.316 + 147 A > G	c.346 + 147 A > G	rs140239870	I	I	0.006 (G)	0	2	
g.120970045 C > A	c.316 + 174 C > A	c.346 + 174 C > A	rs113001389	I	I	0 (A)	1	0	
g.120972015 G > A	p.Arg200Arg	p.Arg210Arg	rs17143296	S	S	0.012 (A)	1	2	
g.1209778801 T > C	c.604_134T > C	c.634_134T > C	rs62476345	I	I	0.018 (C)	3	0	
g.120979528 C > T	p.Thr253Ile	p.Thr263Ile	rs2707466	M	M	0.44 (T)	51	38	eQTL
g.120979512 C > T	c.*113 C > T	c.*113 C > T	rs17143305	3'UTR	3'UTR	0.162 (T)	16	11	miRNA; eQTL
g.120979527_120979528insCTCT	g.120979527_120979528insCTCT	c.*128_*129insCTCT	rs3832519	3'D	3'UTR	0.177 (CTCT)	19	16	
g.120979568 G > C	c.*169 G > C	c.*169 G > C	rs190011371	3'D	3'UTR	0 (C)	0	1	miRNA

Table 1. Variants found in the resequencing of *WNT16*. Fs: Frameshift; I: Intronic; M: Missense; S: Synonymous; 5'UP: 5'upstream; 3'D: 3' downstream. In bold, SNPs genotyped in the complete BARCOS cohort. In italics, rare variants genotyped in the complete BARCOS cohort. MAF (EUR) from 1000 genomes. miRNA: Variant that affect the binding of miRNA (see Supplementary Table S1); eQTL: Variant described in GTEx as an eQTL in different tissues (see Supplementary Table S2). T: Tolerated by SIFT; B: Benign by PolyPhen-2; P: Polymorphism by Mutation Taster.

which only the *DKK1* variant c.406 + 195 G > A was novel. Fifteen of them have frequencies above 5% (common variants), 12 have frequencies between 1–5% (low frequency variants, LFV), and the 24 remaining have frequencies below 1% (rare variants), according to EUR population in 1000 genomes (sequencing chromatograms of the 24 rare variants are shown in Supplementary Figures S1–3). Only the *DKK1* LFV rs74711339 showed significant differences between the genotype frequencies of the two extreme groups (Fisher's exact test, $p = 0.0224$). This SNP is located in the 3'UTR and the minor allele (G) was found overrepresented in the LBM group (Table 2).

In silico Analysis of Functionality of the Identified Variants. To better assess the importance of the identified variants we have explored their possible functionality using *in silico* tools and databases.

Four of the variants were missense, of which 2 common SNPs in *WNT16* (rs2908004; p.Gly72Arg/p.Gly82Arg and rs2707466; p.Thr253Ile/p.Thr263Ile) and one LFV in *SOST* (rs17882143; p.Val10Ile) were predicted to be tolerated and benign by SIFT and PolyPhen-2, respectively. The other missense change, the *DKK1* rare variant rs149268042 (p.Arg120Leu), was predicted to be harmful (deleterious by SIFT, probably damaging by PolyPhen-2, and disease causing by Mutation Taster).

Fifteen other variants might play some regulatory role. One of them, the rs55710688 SNP, located in the 5'UTR of *WNT16a*, is an insertion of 4 nt (CCCC) that affects the Kozak consensus sequence, and might alter translation initiation rate. The *DKK1* intron 3 SNP rs1569198 was predicted to affect splicing, where the G allele might generate a new splice acceptor site 42 nt upstream of the original one, although with a lower score. One rare variant in *SOST*, rs570754792, located 23 bp upstream the transcription start site, lies within the extended TATA box motif. The change is a C (most probable in the consensus TATA box sequence) for a T (least probable). Five 3'UTR variants were predicted to have an effect on the binding of 13 miRNAs (Supplementary Table S1): 3 SNPs in *SOST* (rs17883310, rs17886183, rs75901553), 1 SNP in *WNT16* (rs17143305) and 1 rare variant in *WNT16b* (rs190011371), not present in the other mature transcript of this gene. Several variants were shown to be eQTLs of *WNT16*, *DKK1* or *SOST*, according to GTEx. The *WNT16* SNP rs55710688 that affects the Kozak sequence mentioned above, was also described as an eQTL of *WNT16* in adipose tissue. Regarding *DKK1*, two SNPs (rs74711339 and rs41281546) are eQTLs in transformed fibroblasts. For the *SOST* gene, we have found 3 SNPs (rs1237278, rs851058 and rs10534024) that act as eQTLs in more than one tissue. This information together with additional eQTL information relative to other genes, is shown in Supplementary Table S2. Finally, the *WNT16* SNP rs142005327 (a 2-bp intronic insertion) and the rare variant rs13001389 lie in a potential regulatory site in osteoblasts, according to ENCODE data (and rs142005327 is described as an eQTL of *CPED1* and *FAM33C*).

Variant definition		rs Number	Type	MAF (EUR)	Number of alleles		Functional prediction
DKK1					HBM	LBM	
g.54073904dupC	g.54073904dupC	rs3456455	5'UP	0.012 (C)	3	5	
g.54074079 C > A	c.-116C > A	rs540255939	5'UTR	0.001 (A)	0	1	
g.57074660 C > T	c.244-23 C > T	rs41281546	I	0.056 (T)	8	12	eQTL
g.54074757 A > G	p.Ala106Ala	rs2241529	S	0.458 (A)	36	39	
g.54074798 G > T	p.Arg120Leu	rs149268042	M	0.008(T)	1	0	D/PD/DC
g.54075040 G > A	c.406 + 195 G > A	—	I	—	1	0	
g.54075127 T > C	c.406 + 282 T > C	rs11001560	I	0.445 (T)	28	39	
g.54076271 A > G	c.548-43 A > G	rs1569198	I	0.51 (A)	45	52	eQTL; Splicing
g.54076649 A > G	c.*82 A > G	rs74711339	3'UTR	0.043 (G)	5	11	eQTL
g.54076944delT	c.*377delT	rs200054686	3'UTR	0 (-)	0	1	
g.54077319 C > T	c.*752 C > T	rs953208416	3'UTR	—	1	0	
g.54077322 G > A	c.*755 G > A	rs79759877	3'UTR	0 (A)	1	0	
g.54077566_54077571delCAGTATinsTAA	g.54077566_54077571delCAGTATinsTAA	rs386743716	3' D	—	8	13	
g.54077585 G > A	g.54077585 G > A	rs549135224	3' D	0.001 (A)	2	0	

Table 2. Variants found in the resequencing of *DKK1*. I: Intronic; M: Missense; S: Synonymous; 5'UP: 5'upstream; 3'D: 3' downstream. In bold, SNPs genotyped in the complete BARCOS cohort. MAF (EUR) from 1000 genomes. eQTL: Variant described in GTEx as an eQTL in different tissues (see Supplementary Table S2); Splicing: Predicted to affect splicing (see Text). D: Deleterious by SIFT; PD: Probable Damaging by PolyPhen-2; DC: Disease Causing by Mutation Taster.

Variant definition		rs Number	Type	MAF (EUR)	Number of alleles		Functional prediction
SOST			HBM		LBM		
g.41838340 G > A	g.41838340 G > A	rs184269196	5'UP	0.002 (A)	2	1	
g.41838229 C > T	g.41838229 C > T	rs1237278	5'UP	0.355 (C)	45	40	eQTL
g.41838130 G > A	g.41838130 G > A	rs79715828	5'UP	0.005 (A)	1	0	
g.41838012 G > T	g.41838012 G > T	rs74252774	5'UP	0.002 (T)	1	0	
g.41837786 C > T	g.41837786 C > T	rs567865956	5'UP	0.003 (T)	2	1	
g.41837720 C > T	g.41837720 C > T	rs611105240	5'UP	0 (T)	1	0	
g.41837719 G > A	g.41837719 G > A	rs851058	5'UP	0.402 (A)	47	42	eQTL
g.41837660 T > C	g.41837660 T > C	rs2023794	5'UP	0.044 (C)	6	3	eQTL
g.41837530 C > A	g.41837530 C > A	rs115185703	5'UP	0 (A)	1	0	
g.41837510_41837512delTCC	g.41837510_41837512delTCC	rs10534024	5'UP	0.354 (TCC)	45	43	
g.41837264 G > C	g.41837264 G > C	rs851057	5'UP	0.123 (G)	12	14	
g.41836179 G > A	g.41836179 G > A	rs570754792	5' UP	0.002 (A)	0	1	
g.41836082 C > T	p.Val10Ile	rs17882143	M	0.018 (T)	4	4	T/B/P
g.41832390 G > A	c.*320 C > T	rs17883310	3'UTR	0.013(T)	3	1	miRNA
g.41831844 G > A	c.*866 C > T	rs75901553	3'UTR	0.069 (T)	8	9	miRNA
g.41831706 C > T	c.*1004 G > A	rs17886183	3'UTR	0.004 (A)	4	1	miRNA
g.41831307delC	c.*1404delG	rs17885979	3'UTR	0.001 (-)	1	0	
g.41831153 C > G	c.*1557 G > C	rs566556646	3'UTR	0.001 (G)	1	0	

Table 3. Variants found in the resequencing of *SOST*. M: Missense; 5'UP: 5'upstream. In bold, SNPs genotyped in the complete BARCOS cohort. In italics, rare variants genotyped in the complete BARCOS cohort. MAF (EUR) from 1000 genomes. miRNA: Variant that affect the binding of miRNA (see Supplementary Table S1); eQTL: Variant described in GTEx as an eQTL in different tissues (see Supplementary Table S2). T: Tolerated by SIFT; B: Benign by PolyPhen-2; P: Polymorphism by Mutation Taster.

Association of Common and Low Frequency Variants with BMD in the Complete Cohort. In total, six common variants and four low frequency variants were genotyped in the complete BARCOS cohort (n = 1490) to test their association with BMD (Tables 1–3, in bold). For all of them, we obtained MAF values that are similar to those found in 1000 genomes for the European population (Supplementary Table S3). Nominal significant differences were obtained for lumbar spine BMD (LS-BMD) with the *WNT16* SNPs rs55710688, rs142005327, rs2908004 and rs2707466, under an additive model, and for the *SOST* SNP rs17882143, under a dominant model (Table 4, italics). In all cases, the minor allele had a protective effect on LS-BMD (Supplementary

SNP	Position	Type	P value LS-BMD			P value FN-BMD		
		A	R	D	A	R	D	
WNT16								
rs55710688	p.Met1? g.120965467_120965470dupCCCA	Fs 5'UP	0.0037	0.0283	0.0116	0.0479	0.1026	0.0991
rs2908004	p.G72R/p.G82R	M	0.0263	0.2152	0.0192	0.0510	0.0894	0.1254
rs142005327	c.346 + 103_104dupCT c.316 + 103_104dupCT	I	0.0037	0.008	0.0235	0.0191	0.1454	0.0285
rs2707466	p.T253I/p.T263I	M	0.0182	0.1252	0.0188	0.0167	0.0504	0.0439
rs3801387 ^a	c.603 + 2747 A > G c.633 + 2747 A > G	I	0.0081	0.0060	0.0609	0.0416	0.1032	0.0862
DKK1								
rs1569198	c.548-43 A > G	I	0.3626	0.726	0.2635	0.0095	0.0362	0.0307
rs74711339	c.*82 A > G	3'UTR	0.7960	0.0752	0.9498	0.0682	0.3901	0.0801
rs1373004 ^a	10:g.54427825 T > G	I	0.0543	0.1582	0.0867	0.585	0.4641	0.4006
SOST								
rs1237278	g.41838229 C > T	5'UP	0.9395	0.7643	0.9395	0.0955	0.4533	0.0718
rs2023794	g.41837660 T > C	5'UP	0.5142	0.4199	0.6018	0.6703	0.6838	0.5965
rs17882143	p.Val10Ile	M	0.0669	0.1204	0.0352	0.9888		
rs17883310	c.*320 C > T	3'UTR	0.2236	0.6254	0.3454	0.6152	0.7935	0.6319
rs4792909 ^a	g.41798824 G > T	5'UP	0.2158	0.1895	0.4355	0.1757	0.1537	0.3874

Table 4. Association between LS-BMD and FN-BMD and genotypes of common and LFV *WNT16*, *DKK1* and *SOST* variants. Fs: Frameshift; I: Intronic; M: Missense; 5'UP: 5'upstream; 3'D: 3' downstream. Values in italics indicate nominal significance. Values in bold indicate Bonferroni's significance. ^aSNP previously genotyped in BARCOS (Estrada *et al.*)⁶. A: Additive model; R: Recessive model; D: Dominant model. Reference allele for the dominant and recessive models was the minor allele.

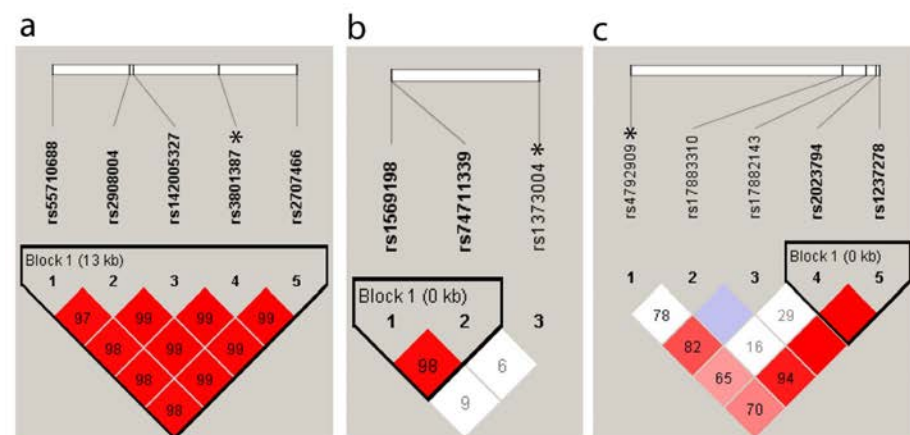


Figure 2. Haploview linkage disequilibrium plots of variants of *WNT16* (a), *DKK1* (b) and *SOST* (c) genotyped in the BARCOS cohort. ^aGWAS hits from Estrada *et al.*⁶. The numbers within the squares and the color scale both refer to D'/LOD values (with bright red: D' = 1 and LOD ≥ 2; white: D' < 1 and LOD < 2; blue: D' = 1 and LOD < 2 and shades of pink/red: D' < 1 and LOD ≥ 2).

Figure S4). Moreover, nominal significant differences were obtained in the comparison of the femoral neck BMD (FN-BMD) with the *WNT16* SNPs rs55710688, rs142005327 and rs2707466 and for the *DKK1* SNP rs1569198, under an additive model. Only the *WNT16* SNPs rs55710688 and rs142005327 remained significant after Bonferroni's correction ($p < 0.0038$) (Table 4, bold). No significant association was found between the genotyped variants and fractures. The *SOST* SNP rs17882143 presented the largest effect size ($\beta = 0.041$) of all associated SNPs described (Supplementary Table S4). In our cohort, the associated SNPs in *WNT16* and *SOST* were found to be in linkage disequilibrium with the respective GWA hits (rs3801387, *WNT16*; rs4792909, *SOST*) of Estrada *et al.*⁶ (Fig. 2a,c), while the *DKK1* SNP rs1569198 was not (Fig. 2b).

Haplotype analyses of *WNT16* associated SNPs. We next tested whether haplotypes might display a stronger association with BMD than any given *WNT16* SNP alone. We built haplotypes including the 4 associated

Haplotype	Freq	pValue	β
DGDAC	0.5479	<i>0.02743</i>	−0.01245
IAIGT	0.2586	<i>0.005418</i>	0.01815
DADAT	0.1522	0.9496	−0.00051
DAIGT	0.0212	0.5045	−0.01368

Table 5. Haplotype association with LS-BMD. Haplotype: rs55710688, rs2908004, rs142005327, rs3801387 and rs2707466. Nominal significant values are in italics.

Haplotype	Extreme BMD		Complete BARCOS cohort	
	HBM (n = 110)	LBM (n = 106)	Upper half (n = 1159)	Lower half (n = 1295)
DGDAC	60	67	653	748
IAIGT	30	21	345	310
DADAT	18	16	183	220
DAIGT	0	1	20	29
p-value ^a	0.351		<i>0.012</i>	
p-value ^b	0.1865		0.0022	

Table 6. Haplotype distribution in extreme BMD groups, and in the two halves of the complete BARCOS cohort according to LS-BMD mean (upper half > 0.8509; lower half < 0.8509). ^ap-value for the 2 × 4 contingency table. ^bp-value for the 2 × 2 contingency table including only the 2 associated haplotypes. Nominal significant values are in italic, Bonferroni's significant values are in bold.

SNPs and the *WNT16* tag-SNP from Estrada *et al.*⁶ in the complete BARCOS cohort and could detect 4 different haplotypes with frequencies above 2% (Table 5). The two most frequent ones consisted on the combination of all the major alleles or all the minor alleles, as expected, given the strong LD present in the region. We tested association between each of the 4 major haplotypes and LS-BMD and found that only the two most frequent (DGDAC and IAIGT) were nominally significantly associated (Table 5), although they did not reach significance after Bonferroni's correction. DGDAC had a BMD-lowering effect, opposite to IAIGT, which was protective. These effects are in agreement with those of the individual SNPs. We could not find an association of any haplotype with FN-BMD (data not shown).

We also tested for significant frequency differences of the 4 major haplotypes between the HBM and LBM groups of the truncate selection, and found none. We only observed a slight overrepresentation of DGDAC in LBM and a slight overrepresentation of IAIGT in the HBM group, as expected (Table 6). Finally, DGDAC was found to be significantly overrepresented in the lower half of the LS-BMD distribution in the complete BARCOS cohort (Table 6).

Analysis of Rare and Low Frequency Variants. In the resequencing of *WNT16*, *DKK1* and *SOST* in the extreme BMD groups, we found 24 rare and 12 low frequency variants. In *WNT16*, the minor alleles of these variants were distributed between 23 HBM and 22 LBM women. In *DKK1*, they were found in 8 HBM and 6 LBM women. Finally, in *SOST*, they were distributed in 16 HBM and 9 LBM women. However, this difference did not reach significance.

Three rare variants found in two LBM and one HBM women and located in putative regulatory elements (two in *WNT16* and one in *SOST*), were genotyped in the complete BARCOS cohort (in italics in Tables 1 and 3, respectively). For the LBM variants (rs190011371 and rs570754792), the rare allele was found in heterozygosis in 3 women whose BMD was below the mean of the BARCOS cohort (0.737 g/cm² and 0.7610 g/cm² vs. 0.8509 g/cm²). For the HBM variant rs113001389 (*WNT16*), no other instance of it was found in the complete BARCOS cohort.

Discussion

WNT16, *DKK1* and *SOST* have been previously associated with osteoporosis⁶. In this study, we have investigated variants within these genes as possible causes of the association. Specifically, we resequenced them in a truncate selection of women of the BARCOS cohort based on their Z-score. We found 19, 14 and 18 variants, respectively, 15 of which were common, 12 low frequency and 24 rare variants. Ten variants, with *in-silico* predicted functionality, were tested for association in the complete BARCOS cohort, and 6 of them (4 in *WNT16*, one in *SOST* and one in *DKK1*) gave nominal significant results with LS-BMD, FN-BMD or both. After correction for multiple testing, only two *WNT16* variants (one affecting the Kozak sequence and the other a putative intronic enhancer) remained significant. Finally, 2 rare variants present in putative regulatory sites (TATA box of *SOST* and 3'UTR of *WNT16b*) were each found in only 3 women of the BARCOS cohort, all 6 with BMD values below the cohort average.

Association with BMD. Of the 4 *WNT16* variants nominally associated with BMD, rs2707466 and rs2908004 were missense. These results agree with those published by others in different cohorts using several

bone parameters^{16–21}. Although these missense variants were predicted to be tolerated and benign by SIFT and PolyPhen-2, respectively, rs2707466 is a change of a threonine to an isoleucine that directly abolishes a phosphorylation site, which causes a deleterious effect on the WNT16 protein¹⁸, while rs2908004 is a change from a glycine to an arginine, two amino acids with very different biochemical properties. These functional changes might somehow contribute to the association.

The other two *WNT16* variants nominally associated with BMD remained so after multiple-testing correction and thus, deserve further attention. One, rs55710688, modifies the Kozak sequence of *WNT16a*, and has been shown to produce changes in the translation of this transcript²¹. The transcription of *WNT16* generates two RNA isoforms, *WNT16a* and *WNT16b*, which differ in the first exon and in the 3'UTR region and are independently controlled by two alternative promoters²². The expression of the *WNT16a* is restricted to the pancreas, whereas *WNT16b* is expressed in multiple organs²², although bone expression data are not available. It would therefore be interesting to know which isoform is being expressed in bone. In the work of Hendrickx *et al.*²¹, they carried out *in vitro* translation studies and showed a higher translation efficiency for the minor allele (insCCCA). Also, this allele was found over-represented in the HBM group, similar to our results. Interestingly, the rs55710688 variant is listed as an eQTL for *WNT16* (subcutaneous adipose tissue) and also for *FAM3C* (skin). The other variant, rs142005327, is an insertion of 2 nucleotides in intron 2, which lies in a potential regulatory site in osteoblasts, and is an eQTL of *FAM3C* (skin and tibial nerve) and of *CPED1* (tibial artery). Consistent with our results, Hendrickx *et al.*²¹ found differences between genotype frequencies of this variant in two extreme BMD groups, where the minor allele (insCT) was over-represented in the HBM group. Overall, our results for these two variants are in the same line of those of Hendrickx *et al.*²¹, and we describe, for the first time, a significant association in a full cohort, reinforcing their importance as determinants of bone mass.

The 4 variants of *WNT16* described above are in high linkage disequilibrium with the GWAS hit from Estrada *et al.*⁶, rs3801387, which lies in the last intron of the gene. We note that this variant is also an eQTL of *FAM3C* (skin). Given its location next to *WNT16*, there has been a debate on the role of *FAM3C* in BMD determination (for example see ref.¹⁶). This gene encodes a cytokine-like factor involved in epithelial to mesenchymal transition and recently has been shown to modulate osteogenic differentiation through Runx2 downregulation²³. It is tempting to speculate that the biological effect of these 3 significantly associated variants of *WNT16* happens in part through their effects on *FAM3C* expression.

The *DKK1* rs1569198, found nominally associated with FN-BMD, is predicted to affect splicing. This same variant was found associated with hip axis length (HAL) by PETERS *et al.*²⁴, in young Caucasian men. However, Koromila *et al.*²⁵ failed to find association with BMD or bone turnover markers in Greek postmenopausal women. This SNP is not in LD with the GWA hit (rs1373004, Estrada *et al.*⁶), which lies 3.5 kb downstream of the gene, and therefore may constitute an independent signal. On the other hand, the *DKK1* LFV rs74711339, that showed significant differences in the two extreme groups and is a *DKK1* eQTL, showed a trend for association with FN-BMD, but did not reach significance in the full cohort, probably due to lack of power.

The only *SOST* variant found nominally associated with BMD was rs17882143 (p.Val101Leu), a missense change affecting the signal peptide of the sclerostin protein. This change may alter the cellular localization of the protein as shown for another signal peptide missense variant (p.Val21Leu) by Kim *et al.*²⁶ To our knowledge, this is the first time that the rs17882143 SNP is found associated with BMD. It is in linkage disequilibrium with the *SOST* GWAS hit of Estrada *et al.*⁶, rs4792909, which, interestingly, did not show association in our cohort (see Table 4). We did not obtain association with BMD for the rest of the *SOST* SNPs tested, including rs2023794, which Zhang *et al.*²⁷ had found associated with LS-BMD in postmenopausal Chinese women.

Interesting rare variants. The *WNT16b* 3'UTR rs190011371, found in one LBM woman, was genotyped in the full cohort and two other cases presented with the minor allele, both with LS-BMD values below the mean of the cohort. According to *in silico* predictors, only the major allele (G) would bind the miRNA hsa-miR-383. Interestingly, this miRNA was found significantly under-expressed in osteoporotic bone (our unpublished results; see Supplementary Table S1). It is tempting to speculate that the inability of this miRNA to bind to the mutated 3'UTR from *WNT16b* would have a similar biological outcome, and this would be in agreement with the presence of the variant in 3 women with LS-BMD below the mean. Similarly, the *SOST* variant rs570754792, located within the extended TATA box motif, was found in one LBM woman, and was also present in two other women of the full cohort with a BMD below the mean. It should be noted that when rare and low frequency variants of each gene were taken into account, only those of *SOST* presented an unbalanced distribution, with an over-representation in the HBM extreme group. If we assume that most of them might be damaging, this would agree with the loss of the inhibitory activity of sclerostin in HBM women.

Additional variants. Beside the 13 variants chosen to be genotyped in the complete BARCOS cohort due to the evidence of functionality, there are other variants which deserve discussion. Among them, we highlight the SNPs rs17143305 and rs75901553 in the 3'UTR region of the *WNT16* and *SOST* genes, respectively. According to various functional predictors, rs17143305 may affect the binding of miRNAs hsa-miR-541 and hsa-miR-4263 and has been reported as an eQTL for genes *RP11-3L10.1* and *FAM3C* in different tissues. Interestingly, hsa-miR-541 has been described as a negative regulator of osteoblastic differentiation²⁸, in the only study in which it is studied in relation to bone tissues, to the best of our knowledge. On the other hand, as explained above, *FAM3C* was reported to have a negative effect on osteogenic differentiation²³. Regarding rs75901553, different functional predictors suggest that the binding of up to 8 miRNAs might be affected, two of them being hsa-miR-98-5p and hsa-miR-let-7f. The former is known to regulate the expression of BMP2, and consequently the osteogenic differentiation of hBMSC²⁹, while the latter was shown to be crucial for the regulation of β -catenin activity and osteogenic differentiation by TIMP-1³⁰. For these reasons, it may be interesting to further study the association of these variants with BMD, as well as their functionality.

Strengths and weaknesses. The resequencing approach in a truncate selection carried out in this study, together with deep *in silico* functional assessment, allowed us to describe potentially relevant common and rare variants present in extreme BMD groups in our cohort. We were able to identify variants that are influencing BMD in the general population, as well as rare variants that may explain extreme phenotypes. This has enabled us to find the rare variants rs190011371, rs113001389 and rs570754792, which would have gone unnoticed in association studies. We have performed a comprehensive analysis of the whole coding region, intronic flanking regions, 5' and 3'UTR and relevant regulatory regions of three important Wnt pathway genes. On the other hand, our approach has two main limitations: the sample size of the study and the lack of analysis of most of the introns and far regulatory regions.

Final remarks. This work provides variants with indications of functionality in 3 important genes of the Wnt pathway. Specifically, 4 missense variants have been found, three of them nominally associated with BMD. However, the only variants significantly associated were two putative regulatory variants in *WNT16*: rs55710688 (Kozak sequence) and rs142005327 (putative enhancer). In addition, two rare variants in functional regions (*WNT16* in 3'UTR and *SOST* in TATA box) may help determine a low BMD phenotype. Therefore, this work reinforces the importance of regulatory variants in these genes in bone function and opens new ways of research. To confirm our hypotheses, future research may include functional studies to validate the *in silico* predictions of the associated variants and replication of the association studies in different (and preferably larger) cohorts.

Methods

Study Cohort. The BARCOS cohort consisted of 1490 postmenopausal women from the Barcelona area, monitored at the Menopausal Unit of the Hospital del Mar (Barcelona, Spain), all of Spanish descent. BMD of all participants was measured at the lumbar spine (LS) and the femoral neck (FN) by dual energy X-ray absorptiometry (DXA). The following data were also recorded: age, age of menarche and menopause, number of fractures and anthropometric measures such as weight and height. DNA is available from all samples of the cohort. Details of the cohort and DNA extraction have been described previously^{31,32}.

For the resequencing of the candidate genes, two extreme BMD groups were selected from the BARCOS cohort. For the election of the extreme groups the statistical Z-score was used. The 55 women with the highest Z-score scores (from 2.98 to 0.695) were included in the HBM (High Bone Mass) group and the 53 women with the lowest Z-scores (−2.40 to −4.26) were included in the LBM (Low Bone Mass) group.

Written informed consent was obtained from all patients in accordance with the regulations of the Clinical Research Ethics Committee of Parc de Salut Mar, which approved the study. Samples from patients were obtained in accordance with the Helsinki Declaration, as revised in 2000. All experiments and protocols were approved by the Bioethics Committee of Universitat de Barcelona (IRB00003099).

Re-sequencing. Resequencing of the *WNT16*, *DKK1* and *SOST* genes was performed in two extreme subgroups of the BARCOS cohort according to the Z-score statistic score. The selection of fragments to be amplified from the *WNT16*, *DKK1* and *SOST* genes and the design of the primers were based on the consensus sequences ENSG00000002745, ENSG00000107984 and ENSG00000167941, respectively (GRCh37.p13, ENSEMBL). *WNT16* was amplified into 6 fragments; *DKK1* in 5 fragments and *SOST* in 7 fragments. The resulting amplicons included all of the coding exons, the UTR regions, the corresponding flanking intronic regions and the two different protein coding transcripts of *WNT16*. Finally, a pair of primers were used for the amplification of the 252bp of the ECR5 enhancer of *SOST*. Amplification of each of these fragments was done by PCR using Go Taq Flexi DNA polymerase (Promega). The temperature and magnesium conditions of each PCR, and the sequence of the primers are presented in Supplementary Table S5. The PCR fragments were analyzed by agarose gel electrophoresis and purification was done in MultiScreen TM Vacuum Manifold 96-well plates (Merck Millipore). The purified PCR products were sequenced by the Sanger method by the CCiTUB genomic service (Genómica, Parc Científic, Barcelona, Spain). The tagging kit used is BigDye™ Terminator v3.1 Cycle Sequencing Kit, detection and electrophoresis are performed on automated capillary sequencer models 3730 Genetic Analyzer and 3730xl Genetic Analyzer.

SNV Genotyping. Genotyping of 13 selected variants in the complete BARCOS cohort was carried out at LGC Genomics (Hoddesdon, UK). In addition, as BARCOS was included in the replication phase of the study by Estrada *et al.*⁶, the genotyping results of SNPs rs3801387 (*WNT16*), rs1373004 (*DKK1*) and rs4792909 (*SOST*) were also available. The genotyping of 12% of the samples was performed in duplicate, as a genotyping quality control, and showed a concordance above 99%.

Linkage Disequilibrium Calculation. The Haploview software from HapMap (<http://hapmap.ncbi.nlm.nih.gov/>) was used to calculate and represent the degree of linkage disequilibrium between genotyped polymorphic variants³³ using the default parameters.

Haplotype construction and association. Haplotypes of *WNT16* associated SNPs were built using PLINK (<http://pngu.mgh.harvard.edu/purcell/plink>) with default parameters³⁴. Association of haplotypes with LS- or FN-BMD was assessed with the same software. Correction for multiple testing was performed using the Bonferroni's method. Fisher's exact test was used to assess the *WNT16* haplotype frequency differences between the two extreme groups of BMD, and between the two halves of the LS-BMD distribution of the complete BARCOS cohort.

Statistical Analysis. Statistical analyses were performed using Rstudio version 3.3.2. Hardy-Weinberg equilibrium (HWE) was calculated using Chi-square test (χ^2). *p*-values less than 0.01 were considered significant.

Fisher's exact test was used to determine the differences between the two extreme groups of BMD on the genotype frequencies of all detected variants. Linear regression analysis adjusted by years since menopause (YSM), using SNPAssoc package, was used to determine the association between BMD and the genotype of each genotype variant. All analyses were performed by testing the additive, the recessive and the dominant model. *p*-values less than 0.05 were considered nominally significant. Correction for multiple testing was performed using the Bonferroni's method for the number of SNPs tested ($n = 13$).

Bioinformatic Analysis and in silico Predictions of the Effect of the Variants. To identify and characterize all variants, we used information from the Ensembl database GRCh37.p13 (<http://www.ensembl.org>). Information was sought on the MAF of each of the variants found in the European population (EUR) and in the Iberian Peninsula (IBS). We used SIFT (<http://sift.bii.a-star.edu.sg/>), PolyPhen (<http://genetics.bwh.harvard.edu/pph2/>) and MutationTaster (<http://www.mutationtaster.org/>) to test the effect of the missense variants. MiRNSNP (bioinfo.bjmu.edu.cn); miRNA-SNP (www.bioguo.org/miRNASNP); SNP Function Prediction (<https://snpinfo.niehs.nih.gov/snpinfo/snpfunc.html>); MicroSNiPer (<http://epicenter.ie-freiburg.mpg.de/services/microsniper/>); miRdSNP (<http://mirdsnp.ccr.buffalo.edu>); miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/>) were used to analyze the binding of miRNA to the variants in the 3'UTR region. The GTEx database (www.gtexportal.org/home/) was used to identify variants that act as eQTLs. Human Splicing Finder (umd.be) and SplicePort (<http://spliceport.cbc.umb.edu/>) were used to evaluate the predicted splicing sites. To characterize regions that include the variants found, the UCSC Genome Browser GRCh37/hg19 was enriched with ENCODE data from osteoblasts.

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Author Contributions

Study design: N.G.G., A.D.P., X.N., L.M., D.G., S.B. Performance of experiments: N.M.G., N.R.A., A.M. Data interpretation and analysis: N.M.G., N.R.A., N.G.G., D.G., S.B. Draft composition: N.M.G., D.G., S.B.

Additional Information

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Common and rare variants of *WNT16*, *DKK1* and *SOST* and their relationship with bone mineral density

Núria Martínez-Gil¹, Neus Roca-Ayats¹, Anna Monistrol-Mula¹, Natàlia García-Giralt², Adolfo Díez-Pérez², Xavier Nogués², Leonardo Mellibovsky², Daniel Grinberg^{1#}, Susana Balcells^{1#*}

¹Department of Genetics, Microbiology and Statistics, Faculty of Biology, University of Barcelona, IBUB, IRSJD, CIBERER, Barcelona, Spain

²Musculoskeletal Research Group, IMIM (Hospital del Mar Medical Research Institute), Centro de Investigación Biomédica en Red de Fragilidad y Envejecimiento Saludable (CIBERFES), ISCIII, Barcelona, Spain

Supplementary Table S1. Variants that affect the binding of miRNA at least in 3 different miRNA databases (MiRNSP; miRNA-SNP; SNP Function Prediction; MicroSNiPer; miRdSNP; miRTarBase).

Gene	Variant	miRNA	Gain/loss	Log FC #	Adj. p-val #
WNT16	rs17143305	hsa-miR-541	Gain	*	Ns
		hsa-miR-4263	Gain	*	Ns
	rs190011371	hsa-miR-383	Loss	-0.527	0.00408
	rs17883310	hsa-miR-1915-3p	Loss	1.58	0.00104
SOST	rs17886183	hsa-miR-5583	Gain	*	Ns
	rs75901553	hsa-miR-98-5p	Loss	*	Ns
		hsa-miR-3190	Gain	*	Ns
		hsa-miR-let-7a (and b.c.d.f.g)	Loss	-1.819	0.007

Gain: Minor allele creates a new binding site for the miRNA

Loss: Minor allele abolish the binding site for the miRNA

Data from De Ugarte et al. (2015)¹. Data not shown in the publication.

logFC: Logarithm (Osteoporotic/Non-osteoporotic)

* logFC < |0.5|; Ns < 0.05

Supplementary Table S2. GTEx eQTL data for all the SNVs found in the resequencing of *WNT16*, *DKK1* and *SOST*

SNP	Gene	p value	NES	Tissue
WNT16				
rs17143281	<i>FAM3C</i>	1.3e-11	0.65	Muscle - Skeletal
rs55710688	<i>FAM3C</i>	0.0000020	0.20	Skin - Not Sun Exposed (Suprapubic)
		0.000016	0.18	Skin - Sun Exposed (Lower leg)
	WNT16	0.000019	0.29	Adipose - Subcutaneous
rs2908004	<i>CPED1</i>	0.000029	0.097	Artery - Tibial
	<i>FAM3C</i>	0.000086	-0.15	Skin - Sun Exposed (Lower leg)
rs142005327	<i>FAM3C</i>	5.9e-8	0.22	Skin - Not Sun Exposed (Suprapubic)
		9.5e-7	0.20	Skin - Sun Exposed (Lower leg)
		0.000032	0.13	Nerve - Tibial
	<i>CPED1</i>	0.000029	0.11	Artery - Tibial
rs2707466	<i>FAM3C</i>	0.000050	-0.15	Skin - Sun Exposed (Lower leg)
	<i>CPED1</i>	0.000058	0.094	Artery - Tibial
rs17143305	<i>RP11-3L10.1</i>	7.5e-14	0.87	Testis
	<i>FAM3C</i>	2.9e-29	-0.52	Skin - Sun Exposed (Lower leg)
		5.9e-26	-0.51	Skin - Not Sun Exposed (Suprapubic)
		2.7e-8	-0.21	Nerve - Tibial
		4.6e-8	-0.22	Thyroid
		7.8e-8	-0.27	Stomach
		8.8e-8	-0.34	Esophagus - Mucosa
		1.0e-7	-0.42	Adrenal Gland
		1.0e-7	-0.29	Muscle - Skeletal
		1.1e-7	-0.77	Brain - Spinal cord (cervical c-1)
		4.8e-7	-0.20	Adipose - Subcutaneous
		9.5e-7	-0.31	Brain - Anterior cingulate cortex (BA24)
		0.0000028	-0.35	Pancreas
		0.0000031	-0.21	Adipose - Visceral (Omentum)
		0.0000032	-0.24	Breast - Mammary Tissue
		0.0000058	-0.17	Artery - Tibial
		0.0000087	-0.18	Esophagus - Muscularis
		0.000022	-0.28	Brain - Frontal Cortex (BA9)
		0.000027	-0.30	Brain - Cortex
DKK1				
rs41281546	DKK1	8.6e-7	-0.29	Cells - Transformed fibroblasts
rs1569198	<i>PRKG1-AS1</i>	0.000047	0.16	Cells - Transformed fibroblasts
rs74711339	DKK1	1.3e-7	-0.35	Cells - Transformed fibroblasts
SOST				
rs1237278	SOST	8.9e-18	-0.26	Artery - Tibial
		3.6e-9	-0.61	Brain - Cortex
		5.4e-9	-0.27	Artery - Aorta
		1.1e-7	-0.43	Artery - Coronary
		0.000024	-0.28	Heart - Atrial Appendage
		0.000013	0.22	Thyroid
rs851058	<i>MPP3</i>	0.000063	-0.17	Cells - Transformed fibroblasts
	<i>MPP2</i>			
	SOST	3.4e-20	-0.28	Artery - Tibial
		1.1e-10	-0.29	Artery - Aorta
		1.2e-8	-0.47	Artery - Coronary
		4.2e-7	-0.35	Heart - Atrial Appendage
rs2023794	<i>DUSP3</i>	0.000069	0.10	Cells - Transformed fibroblasts
	<i>DUSP3</i>	6.0e-7	0.29	Cells - Transformed fibroblasts
	<i>MPP2</i>	0.000022	0.46	Skin - Not Sun Exposed (Suprapubic)
		0.000048	0.50	Skin - Sun Exposed (Lower leg)

NES: Normalized Effect Size

Supplementary Table S3. Minor allele frequency (MAF) from 1000 genomes project: total population (ALL), european population (EUR) and Iberian population in Spain (IBS) and the complete BARCOS cohort frequency.

SNP	Minor allele	ALL	EUR	IBS	BARCOS cohort
WNT16					
rs55710688	CCCA	0.252	0.233	0.276	0.257
rs2908004	A	0.510	0.443	0.416	0.445
rs142005327	CT	0.257	0.254	0.29	0.28
rs113001389	A	0.004	0	0	0.0007
rs2707466	T	0.503	0.440	0.407	0.463
rs190011371	C	0.001	0	0	0.002
DKK1					
rs1569198	A	0.682	0.507	0.477	0.468
rs74711339	G	0.019	0.043	0.033	0.057
SOST					
rs1237278	C	0.436	0.355	0.425	0.392
rs2023794	C	0.074	0.044	0.037	0.056
rs570754792	A	0.0004	0.002	0.005	0.001
rs17882143	T	0.007	0.018	0.033	0.023
rs17883310	A	0.009	0.013	0.023	0.018

Supplementary Table S4. Effect size of the associated variants

SNP	Position	Type	Effect size (β and OR)	
			LS-BMD	FN-BMD
WNT16				
rs55710688	p.Met1?	Fs	0.018 (0.006, 0.031)	0.009 (0.0001, 0.019)
	g.120965467_120965470dupCCCA 5'UP		1.019 (1.006, 1.031)	1.01 (1.0001, 1.019)
rs2908004	p.G72R/p.G82R	M	0.012 (0.0015, 0.023) 1.012 (1.001, 1.023)	
rs142005327	c.346+103_104dupCT	I	0.018 (0.006, 0.03)	0.011 (0.002, 0.02)
	c.316+103_104dupCT		1.018 (1.006, 1.031)	1.011 (1.002,1.02)
rs2707466	p.T253I/p.T263I	M	0.014 (0.002, 0.026) 1.014 (1.002, 1.026)	0.01 (0.002, 0.019) 1.01 (1.002,1.019)
	c.603+2747A>G	I	0.017 (0.005, 0.03)	0.01 (0.0004, 0.019)
rs3801387 [#]	c.633+2747A>G		1.017 (1.005, 1.03)	1.01 (1.0004, 1.019)
DKK1				
rs1569198	c.548-43A>G	I		0.011 (0.003, 0.019) 1.011 (1.003,1.019)
SOST				
rs17882143	p.Val10Ile	M	<u>0.041 (0.003, 0.079)</u> <u>1.042 (1.003, 1.082)</u>	

Underline and Italics β and OR under dominant model

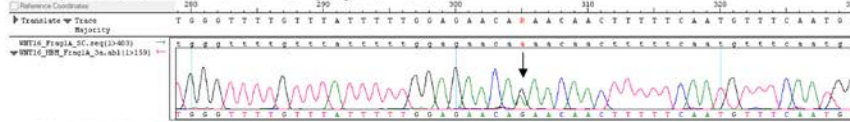
[#] SNP genotyping previously in BARCOS (Estrada *et al.* (2012)²).

Supplementary Table S5. PCR conditions. Sequence of the primers (5'→3'), annealing temperature (°C), amount of magnesium (mM) and elongation time at 72°C (seconds).

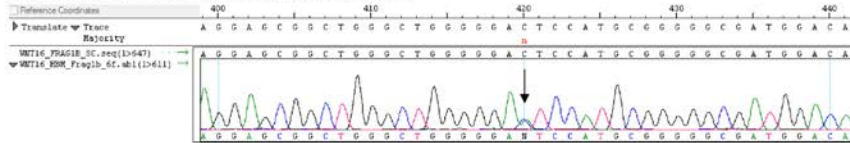
Primers	Sequence (5'→3')	T _m	Mg ²⁺ (mM)	Elongation time (s)
WNT16-Frag1a-F	GGTAGCTCCAGTAAGAGATTC	62	2	15
WNT16-Frag1a-R	CAGATTACCGTGTCTTTGGGT			
WNT16-Frag1b-F	CGGAGCCGCTCTCCACCA	62	2	15
WNT16-Frag1b-R	ATTAGGTCACCTCGTCTAAGGG			
WNT16-Frag2-F	ACTTTCAACTGAGGCTGGGG	62	2	15
WNT16-Frag2-R	CTGGAAGTGGGGAGTCAGG			
WNT16-Frag3-F	CTTCCTTTCTAAATATGTACTCG	58	2	15
WNT16-Frag3-R	AGGGCTGCCAGTGTTTGGTT			
WNT16-Frag4-F1	TGGGACAAAAACCAAAGGACG	62	2	15
WNT16-Frag4-R1	TGACCACATGGGTGTTGTAAC			
WNT16-Frag4-F2	AGGATGATCTGCTCTATGTTAAT	62	2	15
WNT16-Frag4-R2	CCCACCATTATTGAGTCCTGT			
DKK1-Frag1-F	GCGCTGATCACAGTCCTTATC	63	2	15
DKK1-Frag1-R	TGCTATAACGCTCGCTGGTA			
DKK1-Frag2-F	GCAGTGGGCAGTAACAGGT	62	2.5	35
DKK1-Frag2-R	TGTATTGAATCATTGAGGGACA			
DKK1-Frag3-F	GAGGAAGTTTGGCTTGTGTTT	62	2	15
DKK1-Frag3-R	CGAAGGAGAAGACAGTAGGAAA			
DKK1-Frag4a-F	GAACCACCTTGTCTTCAAAAATG	63	1.5	20
DKK1-Frag4a-R	TCCAAGAGATCCTTGCGTTC			
DKK1-Frag4b-F	AGGTGCTGCACTGCCTATTT	63	1.5	20
DKK1-Frag4b-R	CCGTATCCTCATTCCAATCAA			
SOST_Frag0a_F	CCGAGTTGGGCAGATCACC	62	1.5	15
SOST_Frag0a_R	TTAATGCAGACGGTCCAGCC			
SOST_Frag0b_F	ACGCGTTCCAGGGATGAATC	62	1.5	15
SOST_Frag0b_R	GGCCAAGGCAGCATTCTCTC			
SOST_Frag1_F	GCTAGAGGAGAAGTCTTGG	62	2	10
SOST_Frag1_R	CCATTCTTCCCCACCTCC			
SOST_Frag2a_F	AGATGTTTCAGGGGCAAAAGC	63	2	30
SOST_Frag2a_R	GGAAGTCGGGCCCCACTAG			
SOST_Frag2b_F	GTCACCGAGCTGGTGTGCT	63	2	30
SOST_Frag2b_R	CTCAGGGCCTGGAAGGTCT			
SOST_Frag3a_F	CTCAAGGACTTCGGGACCGA	62	2	15
SOST_Frag3a_R	AAATGAGGGTGGAGGTGG			
SOST_Frag3b_F	AGTCCTGGCTCTGCCACTAA	60	2	15
SOST_Frag3b_R	GGACACATTTCTGCCTAGAAAA			
SOST_Frag3c_F	GGGGGAAAACTACAAGTGC	62	2	15
SOST_Frag3c_R	TCCTTTCCAAACCCAGACC			
ECR5_F	TCCTTGCCACGGGCCACCAGCTTT	62	2	5
ECR5_R	CCCCCTCATGGCTGGTCTCATTG			

WNT16

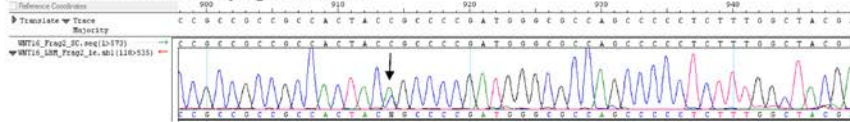
rs4727920; g.120965562A>G; c.65+28A>G (WNT16a) / g.120965562A>G (WNT16b); example of one HBM woman carrying the variant.



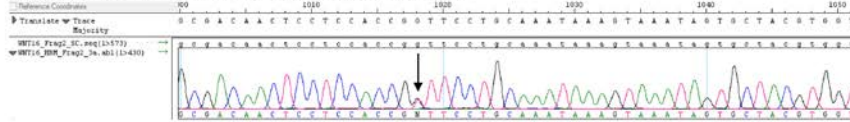
rs201022838; g.120969332C>A; c.66-289C>A (WNT16a) / c.-15C>A (WNT16b); example of the only HBM woman carrying the variant.



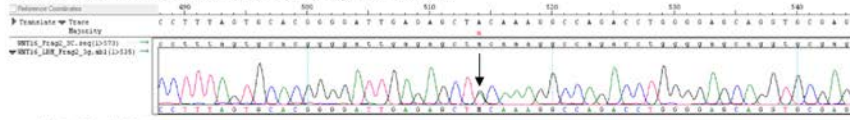
rs17143291; g.120969825C>A; p.Thr90Thr (WNT16a) / p.Thr100Thr (WNT16b); example of one LBM woman carrying the variant.



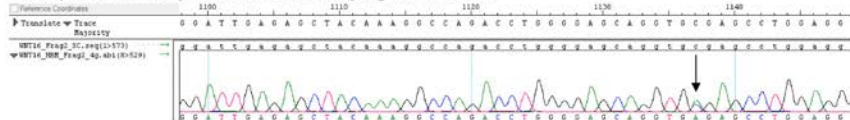
rs147496912; g.120969929G>T; c.316+58G>T (WNT16a) / c.346+58G>T (WNT16b); example of one HBM woman carrying the variant.



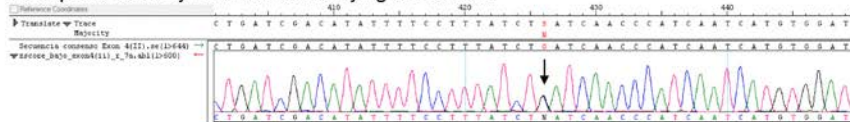
rs140239870; g.120970018A>G; c.316+147A>G (WNT16a) / c.346+147A>G (WNT16b); example of one LBM woman carrying the variant.



rs113001389; g.120970045C>A; c.316+174C>A (WNT16a) / c.346+174C>A (WNT16b); example of the only LBM woman carrying the variant.



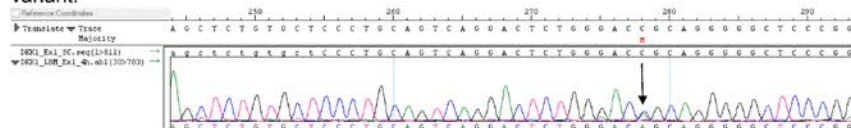
rs190011371; g.120979568G>C; g.120979568G>C (WNT16a) / c.*169G>C (WNT16b); example of the only LBM woman carrying the variant.



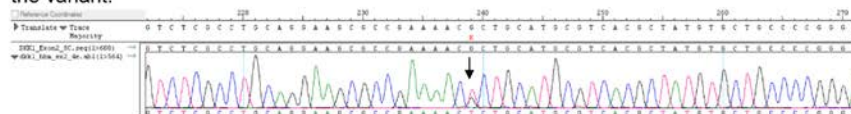
Supplementary Figure S1. Examples of DNA sequence chromatograms from various individuals, each containing one of the *WNT16* rare variants described in this paper. In all cases, the variants are in heterozygosis and are signaled by arrows.

DDK1

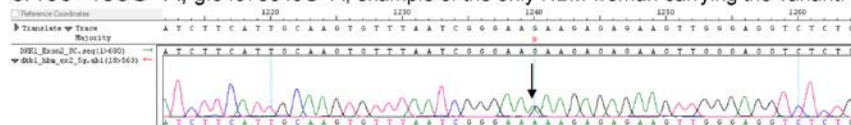
rs540255939; g.54074079C>A; c.-116C>A; example of the only LBM woman carrying the variant.



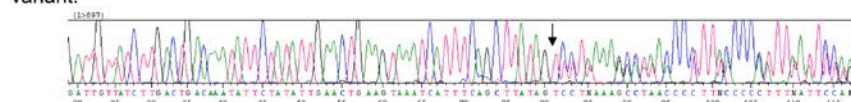
rs149268042; g.54074798G>T; p.Arg120Leu; example of the only HBM woman carrying the variant.



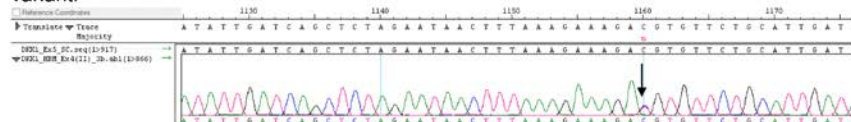
c.406+195G>A; g.54075040G>A; example of the only HBM woman carrying the variant.



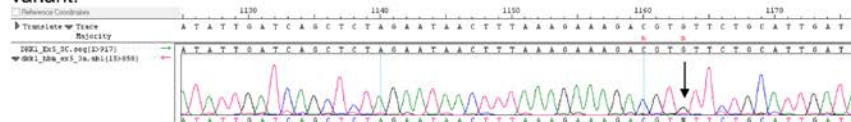
rs200054686; g.54076944delT; c.*377delT, example of the only LBM woman carrying the variant.



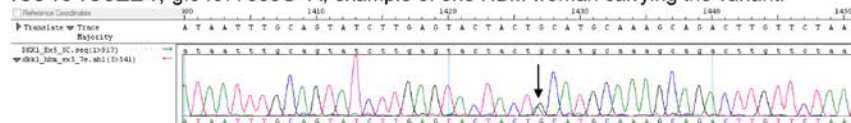
rs953208416; g.54077319C>T; c.*752C>T, example of the only HBM woman carrying the variant.



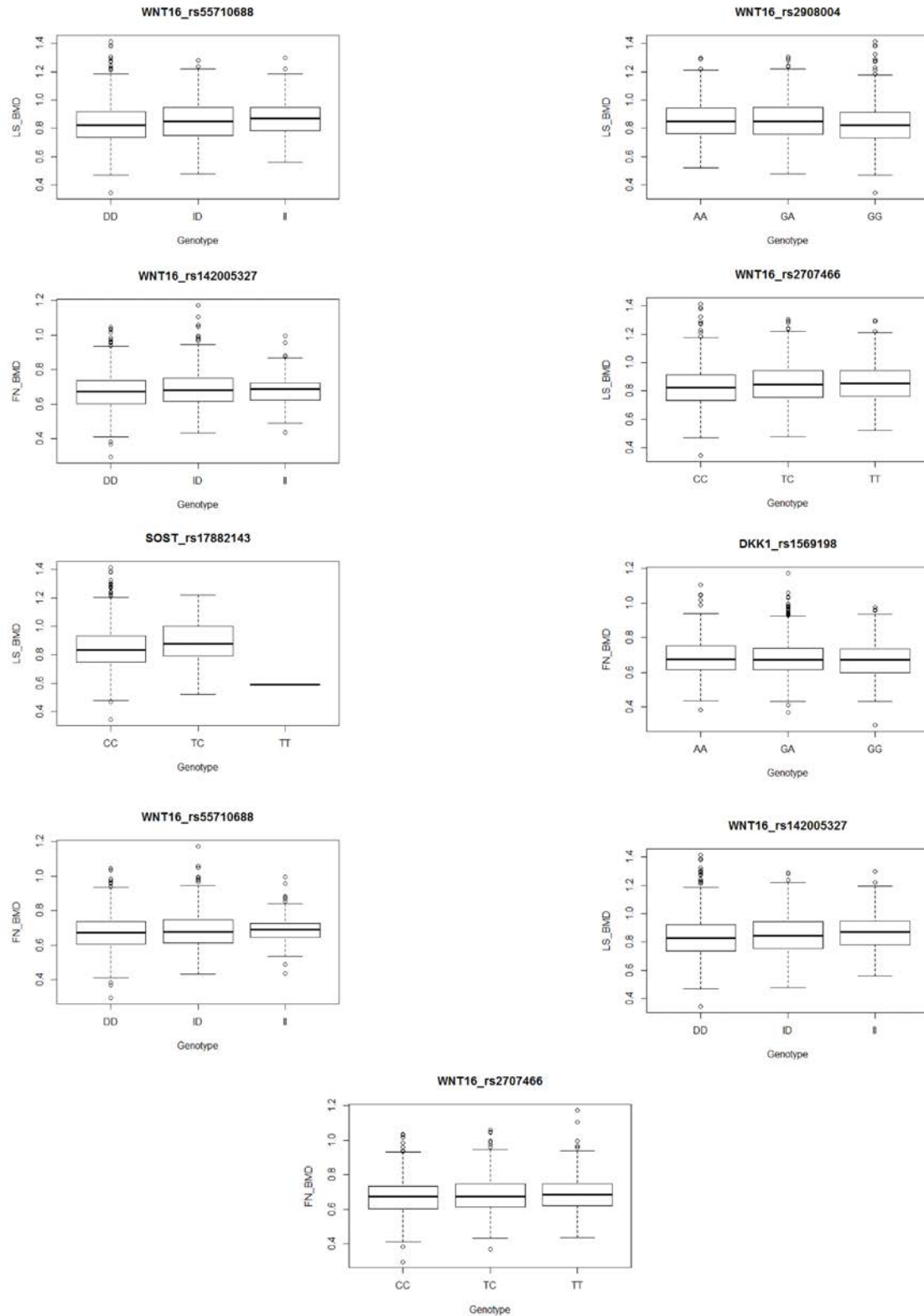
rs79759877; g.54077322G>A; c.*755G>A; example of the only HBM woman carrying the variant.



rs549135224; g.54077585G>A; example of one HBM woman carrying the variant.



Supplementary Figure S2. Examples of DNA sequence chromatograms from various individuals, each containing one of the *DDK1* rare variants described in this paper. In all cases, the variants are in heterozygosis and are signaled by arrows. In the case of rs20054686, which is a heterozygous deletion, the whole chromatogram to the right of it is affected.



Supplementary Figure S4. Boxplots for the nominally significant associated SNPs rs55710688, rs2908004, rs142005327, rs2707466, rs17882143, rs1569198 with LS-BMD or FN-BMD.

References

1. De-Ugarte, L. et al. MiRNA profiling of whole trabecular bone: identification of osteoporosis-related changes in MiRNAs in human hip bones. *BMC Med. Gen.* 8:75 (2015)
2. Estrada, K. *et al.* Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. *Nat. Genet.* **44**, 491–501 (2012).

CHAPTER 2: FUNCTIONAL CHARACTERIZATION OF THE MOST INTERESTING VARIANTS IN *DKK1*, *SOST*, AND *WNT16* DETERMINED IN OBJECTIVE 1.

Article 2

Functional assessment of coding and regulatory variants from the *DKK1* locus

Summary:

The *DKK1* gene encodes an extracellular inhibitor of the Wnt pathway with an important role in bone tissue development, bone homeostasis, and different critical aspects of bone biology. Several BMD genome-wide association studies (GWASs) have consistently found association with SNPs in the *DKK1* genomic region. For these reasons, it is important to assess the functionality of coding and regulatory variants in the gene. Here, we have studied the functionality of putative regulatory variants, previously found associated with BMD in different studies by others and ourselves, and also six missense variants present in the general population. Using a Wnt-pathway-specific luciferase reporter assay, we have determined that the variants p.Ala41Thr, p.Tyr74Phe, p.Arg120Leu, and p.Ser157Ile display a reduced *DKK1* inhibitory capacity as compared with WT. This result agrees with the high-bone-mass (HBM) phenotype of two women from our cohort who carried mutations p.Tyr74Phe or p.Arg120Leu. On the other hand, by means of a circularized chromosome conformation capture- (4C-) sequencing experiment, we have detected that the region containing 24 BMD-GWA variants, located 350-kb downstream of *DKK1*, interacts both with *DKK1* and the *LNCAROD* (LncRNA-activating regulator of *DKK1*, AKA *LINC0148*) in osteoblastic cells. In conclusion, we have shown that some rare coding variants are partial loss-of-function mutations that may lead to a HBM phenotype, whereas the common SNPs associated with BMD in GWASs belong to a putative long-range regulatory region, through a yet unknown mechanism involving *LNCAROD*.

Reference:

Núria Martínez-Gil, Neus Roca-Ayats, Nurgül Atalay, Marta Pineda-Moncusí, Natàlia García-Giralt, Win Van Hul, Eveline Boudin, Diana Ovejero, Leonardo Mellibovsky, Xavier Nogués, Adolfo Díez-Pérez, Daniel Grinberg & Susana Balcells. Functional Assessment of Coding and Regulatory Variants From the DKK1 Locus. *JBMR Plus*. 2020 | DOI: 10.1002/jbm4.0423

ORIGINAL ARTICLE



Functional Assessment of Coding and Regulatory Variants From the *DKK1* Locus

Núria Martínez-Gil,¹ Neus Roca-Ayats,¹ Nurgül Atalay,¹ Marta Pineda-Moncusí,² Natàlia Garcia-Giralt,² Wim Van Hul,³ Eveline Boudin,³ Diana Ovejero,² Leonardo Mellibovsky,² Xavier Nogués,² Adolfo Díez-Pérez,² Daniel Grinberg,¹ and Susanna Balcells¹

¹Department of Genetics, Microbiology and Statistics, Faculty of Biology, Universitat de Barcelona, Centro de Investigación Biomédica en Red en Enfermedades Raras (CIBERER), Institut de Biomedicina de la Universitat de Barcelona (IBUB), Institut de Recerca Sant Joan de Déu (IRSJD), Barcelona, Spain

²Musculoskeletal Research Group, Hospital del Mar Medical Research Institute, Centro de Investigación Biomédica en Red en Fragilidad y Envejecimiento Saludable, ISCIII, Barcelona, Spain

³Center of Medical Genetics, University of Antwerp & University Hospital Antwerp, Antwerp, Belgium

ABSTRACT

The *DKK1* gene encodes an extracellular inhibitor of the Wnt pathway with an important role in bone tissue development, bone homeostasis, and different critical aspects of bone biology. Several BMD genome-wide association studies (GWASs) have consistently found association with SNPs in the *DKK1* genomic region. For these reasons, it is important to assess the functionality of coding and regulatory variants in the gene. Here, we have studied the functionality of putative regulatory variants, previously found associated with BMD in different studies by others and ourselves, and also six missense variants present in the general population. Using a Wnt-pathway-specific luciferase reporter assay, we have determined that the variants p.Ala41Thr, p.Tyr74Phe, p.Arg120Leu, and p.Ser157Ile display a reduced *DKK1* inhibitory capacity as compared with WT. This result agrees with the high-bone-mass (HBM) phenotype of two women from our cohort who carried mutations p.Tyr74Phe or p.Arg120Leu. On the other hand, by means of a circularized chromosome conformation capture- (4C-) sequencing experiment, we have detected that the region containing 24 BMD-GWA variants, located 350-kb downstream of *DKK1*, interacts both with *DKK1* and the *LNCAROD* (LncRNA-activating regulator of *DKK1*, AKA *LINC0148*) in osteoblastic cells. In conclusion, we have shown that some rare coding variants are partial loss-of-function mutations that may lead to a HBM phenotype, whereas the common SNPs associated with BMD in GWASs belong to a putative long-range regulatory region, through a yet unknown mechanism involving *LNCAROD*. © 2020 The Authors. *JBMR Plus* published by Wiley Periodicals LLC on behalf of American Society for Bone and Mineral Research.

KEY WORDS: WNT/ β -CATENIN/LRPs; CELLS OF BONE OSTEOBLASTS; BONE DISEASES AND DISORDERS; OSTEOPOROSIS; GENETIC RESEARCH; MOLECULAR PATHWAYS REMODELING

Introduction

For the last two decades, genome-wide association studies (GWASs) have been a powerful tool to identify genes associated with complex diseases. Regarding osteoporosis, more than 500 loci associated with BMD have been determined. However, these loci only explain 20% of the BMD variability; few have been attributed a clear functional mechanism.⁽¹⁾ The challenge of current genetics is to establish the underlying functional basis of all these associations.

One of the most enriched pathways in genome-wide significant signals for BMD is the Wnt pathway. Polymorphisms in several of its genes have been associated with BMD and fracture

risk.^(1–10) Moreover, rare and penetrant mutations causing Mendelian bone phenotypes have been described in Wnt pathway genes, such as the loss-of-function and gain-of-function mutations in the LRP5 coreceptor, causing the two diametrically opposed phenotypes of osteoporosis-pseudoglioma syndrome⁽¹¹⁾ and high bone mass (HBM),^(12–14) respectively. The Wnt pathway is finely regulated by several extracellular inhibitors, among which sclerostin and *DKK1* stand out. These two proteins prevent the formation of the heterotrimeric complex LRP5/6-FZD-Wnt⁽¹⁵⁾ by forming other heterotrimeric complexes, together with LRP5 and LRP4 (in the case of sclerostin)^(16,17) or with LRP5 and Kremen1/2 (in the case of *DKK1*).⁽¹⁸⁾ *DKK1* binds both the first and third β -propeller domains of LRP5,^(19,20) and

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Address correspondence to: Susanna Balcells, Department of Genètica, Microbiologia i Estadística, Facultat Biologia, Av. Diagonal, 643, 08028 Barcelona, Spain. E-mail: sbalcells@ub.edu

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it is in the first β -propeller domain where mutations causing the HBM phenotype are clustered.^(21–23) Different studies have highlighted the important role of the DKK1 protein in the development of skeletal tissue, in bone homeostasis and in different critical aspects of bone biology.^(15,24,25) In mice, the homozygous *Dkk1* KO is lethal at birth and displays severe defects in head formation that result in anterior truncations.⁽²⁶⁾ In contrast, the heterozygous KO, the hypomorphic (doubleridge), the tamoxifen inducible at 7 weeks, or the osteolineage-specific mouse models show an increase in BMD and an increase in bone formation.^(27–31) By studying the allelic combination of null or doubleridge mice (*Dkk1*+/*db*; *Dkk1*+/-; *Dkk1db/db*; *Dkk1db*-/-; *Dkk1*-/-), a gene dose-dependent inverse correlation with BMD was observed.^(28,29) On the other hand, transgenic overexpression of the *Dkk1* gene in osteoblasts produces a relative decrease in the number of osteoblasts, thus producing a decrease in bone formation.^(32,33) In the last decade, thanks to the direct effect on the inhibition of osteoblastogenesis and the indirect activation of osteoclastogenesis, sclerostin and DKK1 have become interesting targets for the anabolic treatment of osteoporosis. In particular, monoclonal antibodies against DKK1 stimulate bone formation in younger animals and to a lesser extent in adult animals, and enhance fracture healing.⁽³⁴⁾

Given the importance of DKK1 in bone physiology and pathology, it is essential to reach a comprehensive understanding of the functional roles of the *DKK1* variants. In a previous study, we resequenced the transcribed regions and the intronic flanks of the gene in two patient groups with opposed and extreme BMD values from the Barcelona osteoporosis (BARCOS) cohort of postmenopausal women, and found three interesting variants in potentially functional regions: a rare missense variant (rs149268042; p.Arg120Leu), a rare 3'UTR (untranslated region) variant (rs74711339), and a common variant predicted to affect splicing (rs1569198), which was also found nominally associated with femoral neck BMD in the complete cohort.⁽³⁵⁾ In addition to these variants, a region between *DKK1* and *MBL2* is relevant because many GWASs on BMD found strong associations with SNPs clustered in it and not with SNPs inside the *DKK1* gene.^(1,3–5,36–41)

In this study, our objective was to test the underlying functionality of selected variants found in *DKK1*, as well as to determine if the region harboring the genome-wide significant SNPs is actually regulating the *DKK1* gene, the *MBL2* gene, or both. We carried out functional studies specific for each variant (Fig. 1), and a 4C-seq experiment to study a GWA-significant SNP-rich region. With this, we determined that the missense variants p.Ala41Thr, p.Tyr74Phe, p.Arg120Leu, and p.Ser157Ile reduce the DKK1 inhibitory capacity, and that the GWA-SNP region is indeed interacting with *DKK1* and also with the neighboring lncRNA *LNCAROD* (AKA *LINC0148*) gene.

Materials and Methods

Cell culture

The human osteosarcoma cell line Saos-2 was used for luciferase reporter assays and 4C-seq assays. It was obtained from the American Type Culture Collection (ATCC HTB-85) and grown in DMEM (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% FBS (Gibco–Life Technologies, Grand Island, NY, USA) and 1% penicillin/streptomycin (p/s; Gibco–Life Technologies), at 37°C and 5% of CO₂. The human fetal osteoblast 1.19 (hFOB) cell

line and human medulla-derived mesenchymal stem cells (MSCs) were used for 4C-seq assays. The hFOB 1.19 cell line was obtained from ATCC (ATCC CRL-11372) and grown in DMEM:F12 (1:1) medium without phenol red (Gibco–Life Technologies), supplemented with 10% FBS and 0.3 mg/mL geneticin (Gibco–Life Technologies) at 34°C and 5% of CO₂. MSCs were kindly provided by Dr. José Manuel Quesada Gómez from Instituto Maimónides de Investigación Biomédica, Hospital Universitario Reina Sofía, Córdoba, Spain.⁽⁴²⁾ These cells were grown in α -MEM medium (Gibco–Life Technologies), supplemented with 10% FBS, 1% p/s, and 1x glutamax (Gibco–Life Technologies) at 37°C and 5% of CO₂. Human primary osteoblasts (hOBs) used for expression quantitative trait loci (eQTLs) assays are described in Roca-Ayats and colleagues.⁽⁴³⁾

Luciferase reporter constructs and *DKK1* site-directed mutagenesis

The mouse *Wnt1*-V5 expression vector, *mesdc2* expression vector, human WT *LRP5* expression vector, pRL-TK, pGL3-OT reporter, and human *DKK1*-FLAG expression vector are described in Balemans and colleagues.⁽⁴⁴⁾ The mutations p.Ala41Tyr, p.Tyr74Phe, p.Pro84Leu, p.Ala106Thr, p.Arg120Leu, and p.Ser157Ile were introduced into the expression vector *DKK1*-FLAG using the QuickChange Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA), following the manufacturer's instructions. A 300-bp fragment of the *DKK1* 3'UTR containing the SNP rs74711339 in a central position was cloned (in each of the two allele versions) into the pmirGLO dual luciferase miRNA target expression vector (Promega, San Luis Obispo, CA, USA). Constructs were cloned using the XhoI and SdaI restriction sites. All primers used are detailed in Supplementary Information Table S1. In all cases, the presence of point mutations and absence of errors were verified through Sanger sequencing.

Luciferase gene reporter assay

Two luciferase gene reporter assays were performed: one to test the inhibitory activity of WT and mutant DKK1 proteins (Wnt pathway reporter assay) and the other to test the effect of the 3'UTR variant rs74711339 (3'UTR reporter assay). For both experiments, 3×10^5 Saos-2 cells per well were cultured in 6-well plates, 24 hours before the transfection. For the Wnt pathway reporter assay, we cotransfected: mouse *Wnt1*-V5 (64 ng), *mesdc2* (128 ng), human WT *LRP5* (128 ng), pRL-TK (160 ng), pGL3-OT reporter (1600 ng), and DKK1 (48 ng). When necessary, the empty pcDNA3 vector was used to equalize the total amount of DNA transfected in each experiment (total DNA = 2128 ng). For the miRNA reporter assay, we transfected at a 1:1 ratio, the pGFP vector and either the empty pmirGLO or with the *DKK1*-3'UTR fragment with one or the other allele (total DNA = 1.6 μ g). Eugene HD was used following the manufacturer's instructions. Forty-eight hours after transfection, cells were lysed and the luciferase activities of *Photinus pyralis* and *Renilla reniformis* were measured using a Glo-max Multi + luminometer (Promega), following the instructions of the dual-luciferase reporter assay system (Promega).

Western blot assay

For the Western blot assay, 3×10^5 Saos-2 cells per well were cultured in 6-well plates, 24 hours before the transfection. We transfected 2 μ g of the *DKK1* expression plasmid (WT or the missense variant: p.Ala41Thr, p.Tyr74Phe, p.Pro84Leu, p.Ala106Thr, p.Arg120Leu, p.Ser157Ile). For the negative control, we transfected

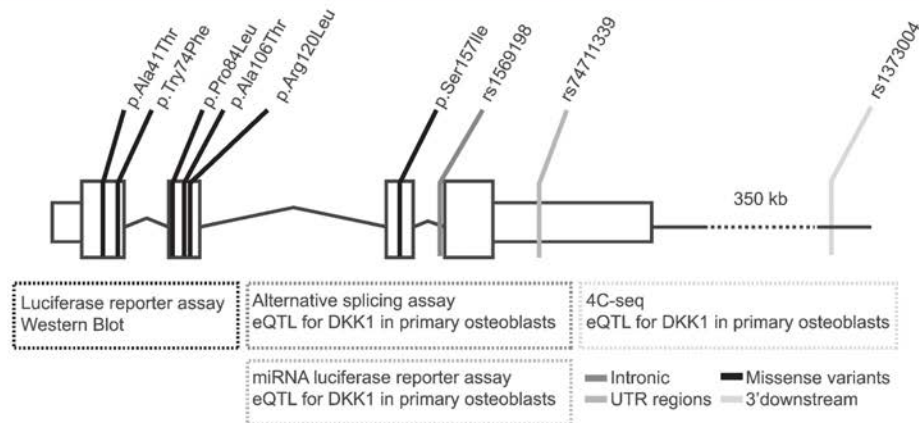


Fig 1. Scheme of the *DKK1* gene including coding exons (boxes), introns (lines), 5' and 3'UTR (small boxes) and the location of all the variants tested. Experiments performed to test the functionality of each type of variant are indicated below the scheme. eQTL = Expression quantitative trait loci; miRNA = microRNA; UTR = untranslated region.

2 µg of the empty pcDNA3 vector. Fugene HD was used following the manufacturer's instructions. After 24 hours, the medium was changed, reducing from 2 to 1 mL of DMEM, without FBS or antibiotics. Forty-eight hours after transfection, the supernatant (conditioned medium) of each condition was collected and were concentrated using 10 K Amicon Ultra filters (Millipore, Watford, UK). Extracellular proteins (concentrated conditioned medium) were quantified using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Proteins from concentrated conditioned medium (5 µg/lane) were separated by electrophoresis in an SDS-PAGE and transferred to a hydrophobic polyvinylidene fluoride transfer membrane (Millipore). The ab109416 antibody against DKK1 (Abcam, Cambridge, UK) was used. The images were developed using a peroxidase-conjugated secondary antibody (anti-rabbit IgG; A0545) for DKK1 antibody. All the experiments were performed in three independent biological replicates.

eQTL assay

DNA was extracted from cultured hOBs using the Wizard Genomic DNA Purification kit (Promega), according to the manufacturer's instructions. The concentration of the purified DNA was analyzed on a spectrophotometer (NanoDrop; Thermo Fisher Scientific). Genotypes for rs1569198, rs74711339, and rs1373004 were assessed by Sanger sequencing using the Big-Dye Terminator v3.1 (Applied Biosystems, Warrington, UK) in the genomics facilities of the Universitat de Barcelona. Primers (Invitrogen, Carlsbad, CA, USA; Thermo Fisher Scientific) were designed using the Primer3 Input 0.4.0 (Supplementary Information Table S1). RNA was extracted from cultured hOBs using the High Pure RNA Isolation kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. RNA was quantified using a NanoDrop spectrophotometer and retrotranscribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Thermo Fisher Scientific), according to the specifications of the manufacturer. qPCR was performed using UPL probes (Roche) on a LightCycler 480 Instrument II (Roche). *HMB3* gene expression was used as normalizing control, and fold changes were calculated by relative quantification, using the

second derivative method. Primers are summarized in Supplementary Information Table S1.

Alternative splicing analysis

hOB cDNA from homozygous women for the rs1569198 SNP was amplified using two primers (Fw: TCCGAGGAGAAATTGAGGAA and R: TCCATGAGAGCCTTTCTCC). The forward primer is located at the 3' end of exon 3 and the reverse primer is located at the 5' end of exon 4. This pair of primers would amplify a 254-bp amplicon in the case of WT splicing, and a 296-bp amplicon in the case of alternative splicing.

Circularized chromosome conformation capture sequencing

Circularized chromosome conformation capture-sequencing (4C-seq) experiment was carried out at the Functional Genomics Service of the Centro Andaluz de Biología del Desarrollo (Sevilla, Spain). 4C-seq libraries were generated from Saos-2, hFOB 1.19 and hMSC cell lines as described previously.^(45,46) 4-bp cutters were used as primary (DpnII) and secondary (Csp6I) restriction enzymes. For each cell line, a total of 1.6 µg of library was PCR amplified (primers used: AGTAAGCTGTGGAATCAATGAC and CTGAGCCTCTCTCTCGGATC, chr10:54427977–54428133, GRCh37). Samples were sequenced with Illumina Hi-Seq technology according to standard protocols at the Genomics Service of the Centro Nacional de Investigaciones Cardiovasculares (CNIC, Madrid, Spain). 4C-seq data were generated as described previously.⁽⁴⁷⁾ Briefly, raw sequencing data were demultiplexed and mapped to the corresponding reference genome (GRCh37). Reads located in fragments flanked by two restriction sites of the same enzyme, in fragments smaller than 40 bp or within a window of 10 kb around the viewpoint were filtered out. 4C-seq data were normalized by the total weight of reads within ±2 Mb around the viewpoint.

4C-seq data were analyzed with the AFourC software (publicly available at <https://github.com/Nikoula86/AFourC.git>), following and adapting previously described pipelines.^(48–50) Briefly, we

assumed that the 4C signal profile relative to the viewpoint v with coordinate x_v on chromosome N is formed by three independent contributions: a constant background level, a negative exponential representing the monotonic decay of the 4C signal with the genomic distance from the viewpoint,⁽⁵⁰⁾ and N gaussians representing the interaction peaks:

$$S_{4C}(x) = B + I_{x_v} \cdot e^{-(x-x_v)/\lambda} + \sum_{i=1}^N P(x_i) \cdot e^{-(x-x_i)^2/(2\sigma^2)}$$

To estimate the genomic distance-dependent monotonic decay, we assumed a symmetric trend around the viewpoint and performed the exponential fit on the left-right averaged profile. Statistically significant peaks were detected using a p value of 0.0005.

Statistical methods

Wnt pathway assay

The ratio of the Firefly and Renilla luciferase measurement was calculated (relative luciferase units; RLUs). For each mutant, the test factor had the following levels: Empty (refers to the luciferase activity resulting from the endogenous Wnt pathway), Active (luciferase activity produced by the Wnt pathway in the presence of exogenous Wnt1, medc2, and LRP5), Inhibitor (luciferase activity in the presence of exogenous Wnt1, medc2, LRP5, and WT DKK1 inhibitor), and Mutant (luciferase activity in the presence of exogenous Wnt1, medc2, LRP5, and each of the mutant DKK1 proteins). We analyzed the data distribution and heteroscedasticity via the Shapiro-Wilk test for normality and the Breusch-Pagan test, respectively. We normalized replicates per day using the DKK1-WT inhibition to reduce the nuisance factor. Then, we assessed the differences between luciferase activities through the Kruskal-Wallis test. A post hoc test for multiple comparisons of groups was performed according to pairwise comparisons using the Wilcoxon rank sum test, adjusting p values with Bonferroni. Statistical analyses were performed using R software v. 3.5.3 (R Foundation for Statistical Computing, Vienna, Austria; <https://www.r-project.org/>), and $p < 0.05$ was considered significant. GraphPad (GraphPad Prism 8; GraphPad Software, La Jolla, CA, USA) was used for graphic representation. Each experiment included a minimum of three replicates and was repeated independently in three separate experiments.

3' UTR assay

The ratio of the Firefly and Renilla luciferase measurement was calculated (RLUs). We analyzed the data distribution and homogeneity of variances via the Shapiro-Wilk test for normality and the Bartlett test, respectively. We normalized replicates per day using the empty vector activity to reduce the nuisance factor. We assessed the differences between luciferase activities through an ANOVA test. The Tukey HSD (honestly significant difference) test was used to perform the post hoc test for multiple group comparisons, testing the effect between all the conditions in the miRNA assay. Statistical analyses were performed using R software v. 3.5.3 (R Foundation for Statistical Computing), and $p < 0.05$ was considered significant. GraphPad (Graphpad Prism 8) was used for graphic representation. Each experiment included three replicates and was repeated independently in four separate experiments.

eQTL assay

For the study of the eQTLs, the WGA association function in R software v.3.5.3 was used.⁽⁵¹⁾ This function carries out an association analysis between SNPs and a dependent variable (DKK1 expression levels) under five different genetic inheritance models: codominant, dominant, recessive, overdominant, and log-additive.

Results

Functional evaluation of six DKK1 missense variants

To measure the inhibitory activity of six mutant DKK1 proteins (Fig. 1) on the canonical Wnt pathway, we performed a specific luciferase reporter gene assay. The transfection of *Wnt1*, *mesdc2*, and *LRP5*, together with a TCF/LEF-luciferase reporter, in Saos-2 cells resulted in an approximate fivefold stimulation of the endogenous Wnt pathway as previously described.⁽²²⁾ Likewise, addition of WT DKK1 resulted in a one-third inhibition of the pathway (35.2%; Fig. 2). In contrast, addition of either of the four DKK1 mutant proteins, p.Ala41Thr, p.Tyr74Phe, p.Arg120Leu, and p.Ser157Ile, resulted in a significantly reduced inhibitory capacity as follows: p.Ala41Thr displayed a 21.9% inhibition of the fully stimulated Wnt pathway (37.8% loss of the inhibition related to WT DKK1; $p < 0.001$), p.Tyr74Phe, a 23.2% inhibition of the Wnt pathway (34% of WT DKK1; $p < 0.001$), p.Arg120Leu, a 15.6% inhibition (55.2% of WT DKK1; $p < 0.001$), and p.Ser157Ile, a 22.2% inhibition (37% of WT DKK1; $p < 0.001$). No significant

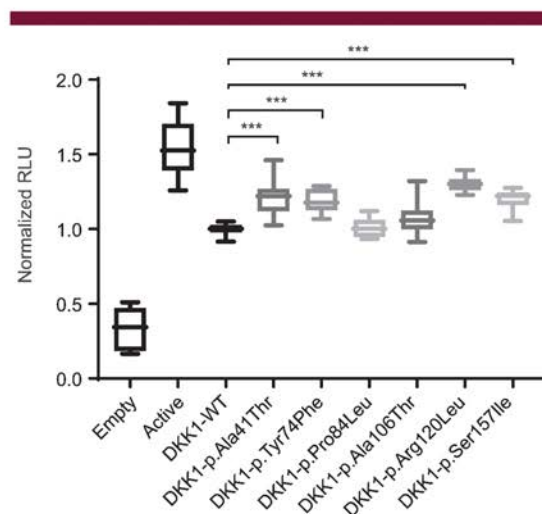


Fig 2. Luciferase reporter assays of six DKK1 missense variants. Boxplots of the normalized relative luciferase activity (RLU) of the following transfections: endogenous Wnt pathway (transfected only with the TCF/LEF reporter construct and the empty pcDNA3 vector; *Empty*), activated pathway (cotransfection: same as before plus Wnt1, LRP5 and mesdc2; *Active*) and the pathway inhibited by either the WT DKK1 or the six mutated DKK1 proteins (cotransfection as before plus the corresponding DKK1 constructs). Significant differences between the mutated DKK1 and the WT DKK1 (used as normalizer) are indicated as *** $p < 0.001$. Error bars represent the SD.

differences were observed for the remaining two missense variants (Fig. 2). By performing Western blot analyses of the different *DKK1* mutant proteins secreted to the extracellular space, we observed that only the p.Ser157Ile mutant was expressed at lower levels than those of WT *DKK1*. In contrast, all other mutants were expressed at similar or higher levels compared to the WT (Supplementary Information Fig. S1). Altogether, we have determined that four of the six missense variants do affect the *DKK1* inhibitory capacity.

Functional evaluation of variants rs74711339 (3'UTR) and rs1569198 (intronic)

To test the functional activity of the *DKK1*-3'UTR variant rs74711339, we performed a 3'UTR-luciferase reporter assay in Saos-2 cells. Significant differences were found between the empty vector and either allele, whereas no significant differences were detected between both alleles (Supplementary Information Fig. S2). This result indicates that 3'UTR of *DKK1* is involved in transcript expression or stabilization in Saos-2 cells, but the SNP tested is not affecting this regulation.

Variant rs1569198 is located in intron 3, 43 nucleotides upstream of the last exon of the gene. According to several in silico predictors, the G allele would generate a new splicing acceptor site (atgcaggtttagCA), producing a protein with 16 additional amino acids, encoded by the 3' end of intron 3. However, this alternative transcript has not been detected in primary osteoblast cDNA from 12 women homozygous for the G allele (Supplementary Information Fig. S3).

SNPs rs1569198 and rs74711339 are defined as eQTLs of *DKK1* in adrenal gland and transformed fibroblasts, respectively, according to GTEx (V8 release; Supplementary Information Table S2). Because bone tissue is not available in this database, we used an in-house collection of 45 primary osteoblasts to determine whether these two SNPs (plus rs1373004, found associated with BMD and risk of fracture in Estrada and colleagues⁽³⁾) are *cis*-eQTLs of *DKK1* in these cells. None of the tested SNPs resulted as eQTLs in our samples (Supplementary Information Fig. S4).

Chromatin interaction from the GWAS hit locus

We tested if the region containing 24 BMD-GWAS SNPs, located 350-kb downstream of *DKK1* (viewpoint, dashed gray line, in Fig. 3), is actually regulating *DKK1*, *MBL2*, or both. We performed a 4C-seq experiment in three types of bone-related cells (MSCs, hFOB 1.19, and the Saos-2 osteosarcoma cell line), and we applied an algorithm to discern the significant contacts (see Materials and Methods; arched lines in Fig. 3). We only observed interactions of this viewpoint with sequences included in a genomic region spanning from 628 kb centromeric to the viewpoint to 141 kb telomeric. No other interactions were detected elsewhere in the genome. Inside this interval, we detected two main significant interacting regions common to all three cell types. The first one coincides with the *DKK1* promoter; it is the strongest signal detected. The second corresponds to an extended region including several *LNCAROD* transcripts and enhancer signals according to the GeneHancer database⁽⁵²⁾ (Fig. 3). No significant interaction was detected with *MBL2*, except for an interaction with sequences located several kb upstream of it in Saos-2 cells. Altogether, we have concluded that the BMD-GWA-SNP-rich region is interacting with both *DKK1* and the lncRNA *LNCAROD* in three bone-related cell types.

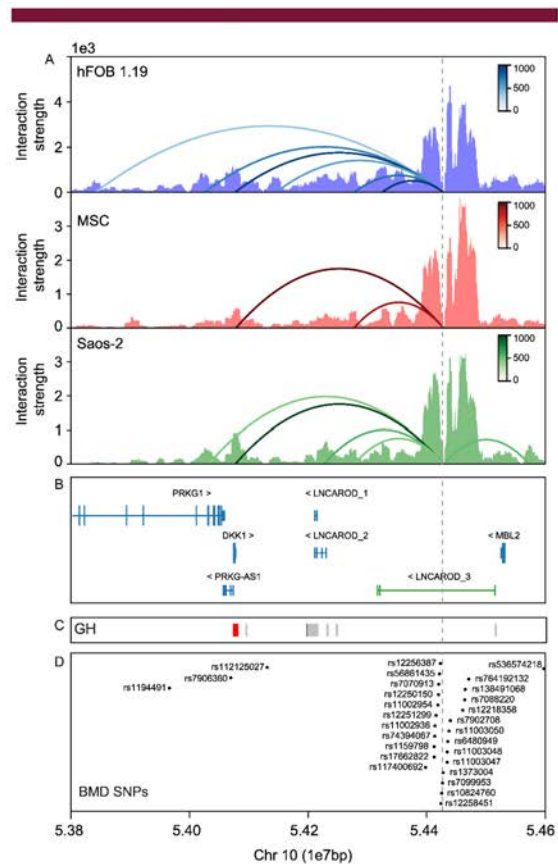


Fig 3. 4C-seq using the SNP rs1373004 region as viewpoint in human fetal osteoblast (hFOB 1.19), mesenchymal stem cells (MSCs), and Saos-2 cell line. (A) Graphical representations of the read depths (Interaction strength) across the *DKK1*-*MBL2* genomic region. Significant interactions are marked with arched lines and the color scale corresponds to the strength of these significant interactions. (B) Genes in this region: *PRKG1* (ENST00000373980.4), *PRKG1-AS1* (NR_038277.1), *DKK1* (ENST00000373970.3), *LNCAROD_1* (NR_120642.1), *LNCAROD_2* (NR_120641.1), and *MBL2* (ENST00000373968.3) from GRCh37/hg19, and in green the *LNCAROD_3* (ENST00000443523) from GENCODE V32.2 GRCh38/hg18. (C) Representation of the GeneHancer (GH) track from UCSC genome browser (enhancers -gray- and promoters -red-). (D) SNPs associated with bone parameters in different genome-wide association studies (GWASs), extracted from the GWAS catalog (<https://www.ebi.ac.uk/gwas/>).

Discussion

In this work, we have studied the functionality of intragenic *DKK1* variants and analyzed a region harboring a cluster of genome-wide significant SNPs from different GWASs. We have determined that four of six missense mutations affect the *DKK1* inhibitory capacity. Additionally, we have determined that the intergenic region containing the genome-wide significant signals interacts not only with *DKK1*, but also with the lncRNA *LNCAROD* gene in bone cells.

Missense variants

We have tested the functionality of six DKK1 missense variants. Two of them were found in two women with HBM in previous studies of our group, p.Try74Phe⁽⁵³⁾ and p.Arg120Leu⁽³⁵⁾ and five, including the latter, are among the most frequent missense variants in the general population according to gnomAD v3 (p. Arg120Leu, p.Ala106Thr, p.Ser157Ile, p.Pro84Leu and p. Ala41Thr). To test their possible functionality, we have performed a luciferase reporter assay specific for the canonical Wnt pathway and we have determined that mutations p. Ala41Thr, p.Tyr74Phe, p.Arg120Leu and p.Ser157Ile affect DKK1 inhibitory capacity whereas p.Pro84Leu and p.Ala106Thr did not. Interestingly, none of the variants tested are located in the CRD2 domain (amino acids 189–263) known to bind to the LRP5 first and third β -propeller to exert the inhibition (Fig. 4).

Variant p.Arg120Leu, which displays the strongest effect on DKK1 inhibitory capacity (55.2%), is the consistently predicted as deleterious, according the four most commonly used in silico tools (Table 1). In a previous work,⁽³⁵⁾ we detected it in a woman with HBM (sum Z-score = 6.20; menarche at 12 years; hysterectomy at 33 years; chronic mild renal insufficiency, arterial hypertension, and obesity). We propose that the loss-of-function of the protein with the p.Arg120Leu mutation may be contributing to the HBM phenotype of this woman, through insufficient inhibition of the Wnt pathway. In fact, this variant modifies the N-terminal cysteine-rich domain (CDR1; Fig. 4), described to exert independent signaling functions through other unknown receptors or pathways.⁽⁵⁵⁾ The loss-of-function that we have observed

for this variant does not agree with the study by Korvala and colleagues,⁽⁵⁶⁾ who did not find a lower inhibitory capacity for the same mutated protein. Technical differences may explain these negative results. In fact, these authors and others^(56,57) have found this mutation in patients with primary osteoporosis, a phenotype that does not fit with the loss of function of DKK1 and diametrically opposed to the HBM phenotype of our patient. Possible explanations for these discrepant findings may be a reduced penetrance for this variant and differences in patients' genetic background. Further investigation, preferably in vivo, may resolve this discrepancy.

The p.Arg120Leu and also p.Ala41Thr have been described as associated with type I Chiari disease (CMI).⁽⁵⁸⁾ This disease is defined as a downward herniation of the cerebellar tonsils across the foramen magnum caused by defects in the development of the occipital bone and the posterior fossa, which produces a neurologic dysfunction by direct compression of the neural tissue in the craniovertebral junction.^(59,60) Given the bone involvement in CMI, it will be interesting to study the possible relationship between mutations in *DKK1* and this disorder, which in many cases is asymptomatic and undiagnosed.

p.Ala41Thr is the variant with the second highest loss of inhibitory capacity (37.8%) and also predicted as deleterious by SIFT software (Sorting Intolerant From Tolerant; available from: <https://sift.bii.a-star.edu.sg/>; Table 1). This variant affects the NAIKN motif (amino acids 40–44; Fig. 4), crucial for binding to the first β -propeller of LRP5/6 proteins and present in all Wnt-pathway inhibitors.⁽²⁰⁾ Its frequency in the general population is 2.56 of every 10,000 individuals, but its effect in terms of bone

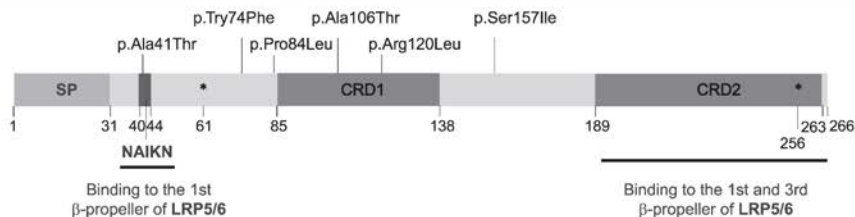


Fig 4. Domain structure of the DKK1 protein and position of the missense variants tested. The limits of the domains and the glycosylation sites (dots) are indicated below the scheme of the protein, together with the LRP5/6 binding domains (NAIKN motif and CRD2). CRD = Cysteine-rich domain; SP = signal peptide.

Table 1. Pathogenicity Predictions of the Six Missense Variants Tested

Mutations	In silico predictors				MAF (gnomAD v3)	Loss of inhibitory capacity (%)	Sign.
	SIFT	PolyPhen-2	CADD	REVEL			
p.Arg120Leu	D	PD	LD	LDC	0.00282	55.2	*
p.Ala41Thr	D	B	LB	LB	0.000128	37.8	*
p.Pro84Leu	T	PssD	LB	LB	0.000088	—	ns
p.Ser157Ile	T	B	LB	LB	0.000884	37.0	*
p.Try74Phe	T	B	LB	LB	0.0000201	34.0	*
p.Ala106Thr	T	B	LB	LB	0.00228	—	ns

Loss of inhibitory capacity and significance (Sign.) according to the results presented in this article. B = benign; CADD = combined annotation dependent depletion (available from: <https://cadd.gs.washington.edu/>; D = deleterious; gnomAD v3 = Genome Aggregation Database, version 3; LB = likely benign; LD = likely deleterious; LDC = likely disease causing; PD = probably damaging; MAF = minor allele frequency; ns = nonsignificant; PolyPhen-2 = polymorphism phenotyping version 2 (available from: <http://genetics.bwh.harvard.edu/pph2/>); PssD = possibly damaging; REVEL = rare exome variant ensemble learner⁽⁵⁴⁾; SIFT = sorting intolerant from tolerant (available from: <https://sift.bii.a-star.edu.sg/>); T = tolerated.

* $p < 0.001$.

mass is still unknown, as it happens for the p.Ser157Ile variant. The latter also displayed lower inhibitory activity, but in this case it may be a consequence of reduced secretion of the protein to the extracellular space (Supplementary Information Fig. S1). Finally, the variant p.Try74Phe was found in a previous study cosegregating in a family with HBM,⁽⁵³⁾ and the functional effect identified here is consistent with the cosegregation in the HBM family.

3'UTR and splicing variants

In contrast to missense variants, we were not able to find functional evidence for variants rs74711339 and rs1569198, located in the 3'UTR and in intron 3, respectively. The effect of these variants in other cell types or in different developmental stages cannot be ruled out.

GWAS hit locus

A group of 30 SNPs associated with bone parameters in 10 different GWASs exists in a 3.3-Mb window in 10q21.1,^(1,3–5,36–41) of which 24 are clustered within a 73-kb genomic region located 320 kb telomeric from *DKK1* and 54 kb centromeric from *MBL2* (Fig. 3D). However, no enhancer marks overlap with this region, only two of these 24 SNPs are described as eQTLs (rs7088220 and rs12218358 for *MBL2*; GTEx v8) and no interactions between this GWA-SNP-rich region and neighboring genes have been reported. We undertook a 4C-seq assay to determine its interactions across the genome in three bone cell types, and found that the GWA-SNP-rich region is interacting with both *DKK1* and regions that harbor several newly described transcripts of *LNCAROD*. Interestingly, this lncRNA has been recently shown to enhance *DKK1* transcription,⁽⁶¹⁾ requiring chromosomal proximity between the *A-ROD* and the *DKK1* loci. Our 4C-seq data are in agreement with these results because we found interactions with both *DKK1* and *LNCAROD*, which suggests a regulatory role for the GWA-SNP-rich region. It should be noted that in the study by Ntini and colleagues,⁽⁶¹⁾ they only considered short forms of *LNCAROD*, whereas the last build of the genome (hg38) includes new longer versions, overlapping with the GWA-SNP-rich region (see green lines in Fig. 3B). The regulatory role of the GWA-SNP-rich region is further supported by the double-ridge spontaneous mouse. This mouse model bears a 60-kb deletion (located 150 kb from *Dkk1* and 90 kb from *Mbl2*), probably corresponding to this human region, and displays a drastic drop in *Dkk1* expression levels (between 35% and <1% of WT), while keeping the levels of neighboring *Mbl2* and *Prkg1* genes intact.^(28,29) In this sense, it would be interesting to verify if *LNCAROD* is underexpressed in this mouse model, which would confirm the role of this lncRNA in *DKK1* regulation. Our results clearly show for the first time that this region is relevant for the regulation of *DKK1*, and it will be very interesting to assess the specific role of the different SNPs in this process.

Limitations

This work has two main limitations. One of them is the complex luciferase assay used for the study of missense mutations. This method involves the cotransfection of several vectors, which implies high variability. It is possible that some of the variants tested, expected to have a small effect, show differences in activity below the sensitivity of the reporter gene assay we have used. Regarding the eQTL study, the main limitation is the sample size. Because of difficulties in obtaining appropriate samples, we only

were able to use 45 primary osteoblasts, which precluded testing SNPs with low allele frequency. It would be useful to have a bank of primary osteoblasts with a much higher sample size to get information on different eQTLs important for bone; this information is lacking in current databases.

Conclusions

In conclusion, for the first time we show functional evidence for the relevance of *DKK1* in HBM phenotype and osteoporosis determination. Four missense variants in *DKK1* affect its inhibitory capacity, consistent with the HBM phenotype of a few individuals bearing these changes. Additionally, genomic interactions exist between a region rich in BMD-GWA-SNP and the *DKK1* gene, and also with a lncRNA known to enhance *DKK1* expression. These functional data open a way to study the mechanisms of *DKK1* regulation, which may define novel therapeutic targets for bone diseases.

Disclosures

Service on Advisory Board: DO: Kyowa Kirin; XN: Amgen, UCB; ADP: Amgen, UCB.

Honoraria or royalties for books or publications or for lectures (speaker fees), or participating in a speakers bureau: DO: Speaker fees in a couple of talks for Kyowa Kirin; XN: Speaker fees from Amgen, Lilly; ADP: Lilly, Amgen, UCB, Gilead, Theramex.

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Stock holdings and/or stock options in pharmaceutical, medical: ADP: Active Life Sci.

The other authors declare no conflicts of interest.

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Authors' roles: NMG, WVH, DG, SB were involved in the study conception and design. Material preparation and data collection were performed by NMG, NRA, NA, NGG, EB, DO, LM, XN, and ADP. The analyses were performed by NMG. The statistical analyses were performed by MPM. The first draft of the manuscript was written by NMG, DG, SB, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Author Contributions

Núria Martínez-Gil: Conceptualization; data curation; formal analysis; investigation; methodology; writing-original draft; writing-review and editing. **Neus Roca-Ayats:** Investigation; methodology; writing-review and editing. **Nurgül Atalay:** Investigation; methodology; writing-review and editing. **Marta Pineda-Moncusí:** Data curation; formal analysis; writing-review

and editing. **Natalia Garcia-Giralt:** Investigation; methodology; writing-review and editing. **Wim van hul:** Conceptualization; writing-review and editing. **Eveline Boudin:** Investigation; methodology; writing-review and editing. **Diana Ovejero:** Investigation; methodology; writing-review and editing. **Leonardo Mellibovsky:** Investigation; methodology; writing-review and editing. **Xavier Nogués:** Investigation; methodology; writing-review and editing. **Adolfo Diez-Perez:** Investigation; methodology; writing-review and editing. **Daniel Grinberg:** Conceptualization; funding acquisition; supervision; writing-original draft; writing-review and editing. **Susanna Balcells:** Conceptualization; funding acquisition; supervision; writing-original draft; writing-review and editing.

Data accessibility statement

The software package and the 4C data files used to make the statistical analysis, are publicly available at <https://github.com/Nikoula86/AFourC.git>. All data and material will be available upon request.

Peer Review

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Supplementary information:

Functional assessment of coding and regulatory variants from the *DKK1* locus

Núria Martínez-Gil¹, Neus Roca-Ayats¹, Nurgül Atalay¹, Marta Pineda-Moncusí², Natàlia Garcia-Giralt², Wim Van Hul³, Eveline Boudin³, Diana Ovejero², Leonardo Mellibovsky², Xavier Nogués², Adolfo Díez-Pérez², Daniel Grinberg¹, Susanna Balcells¹

1 Department of Genetics, Microbiology and Statistics, Faculty of Biology, Universitat de Barcelona, CIBERER, IBUB, IRSJD, Barcelona, Spain.

2 Musculoskeletal Research Group, IMIM (Hospital del Mar Medical Research Institute), Centro de Investigación Biomédica en Red en Fragilidad y Envejecimiento Saludable (CIBERFES), ISCIII, Barcelona, Spain.

3 Center of Medical Genetics, University of Antwerp & University Hospital Antwerp, Antwerp, Belgium.

Running Title: *DKK1* coding and regulatory variants

Corresponding author:

Susanna Balcells: sbalcells@ub.edu.

Dpt. Genètica, Microbiologia i Estadística

Facultat Biologia

Av. Diagonal, 643

08028 Barcelona

Tel +34 93 4035418

Table 1. Sequence of all the primers used

Experiment	Primer name	Sequence (5'-->3')
pmirGLO-rs74711339	rs74711339_F	ctcgagAAACCAGCTATCCAAATGCAG
	rs74711339_R	cctgcaggCAATCACAGGGGAGTTCCAT
seq-eQTLs	rs1569198 and rs74711339 F	AGGTGCTGCACTGCCTATTT
	rs1569198 and rs74711339 R	CCGTATCCTCATTCCAATCAA
	rs1373004_F	GAGCCACTTGATTAATGTCTCTGA
	rs1373004_R	TTGATTAAACAAAAGGTTACAAA
Site directed mutagenesis	p.Ala41Thr_F	GGGCAGGTTCTTGATAGTGTGGAATTGAGAACCG
	p.Ala41Thr_R	CGGTTCTCAATTCCAACACTATCAAGAACCTGCCC
	p.Try74Phe_F	CCCGGGCGGGAATAAGTTCCAGACCATTGACAAC
	p.Try74Phe_R	GTTGTCAATGGTCTGGAACCTATTCCCGCCCGGG
	p.Pro84Leu_F	GTCCTCTGCGCACAGGTACGGCTGGTA
	p.Pro84Leu_R	TACCAGCCGTACCTGTGCGCAGAGGAC
	p.Ala106Thr_F	TTTGACGCCTGTGTCCCCTCCGCG
	p.Ala106Thr_R	CGCGGAGGGGACACAGGCGTGCAAA
	p.Arg120Leu_F	CGTGACGCATGCAGAGTTTTCGGCGCTTC
	p.Arg120Leu_R	GAAGCGCCGAAAACCTCTGCATGCGTCACG
	p.Ser157Ile_F	GTGCTATGATCATTACCAAAGATTTCACTGATGGTTTCCTCAA
	p.Ser157Ile_R	TTGAGGAAACCATCACTGAAATCTTTGGTAATGATCATAGCAC
DKK1_qPCR	DKK1_qPCR_F	CTCCCGGACCCTGACTCT
	DKK1_qPCR_R	ACAAAGACCCGGGTAGCTC
HBMS_qPCR	HBMS_qPCR_F	TGCCCTGGAGAAGAATGAAG
	HBMS_qPCR_R	CAGCATCATGAGGGTTTTCC

Table 2. cis-eQTL information from GTEX database for the rs7471133 and rs1569198 SNPs.

SNP	Gene	P value	Tissue
rs74711339	<i>DKK1</i>	2.2e-10	Cells cultured fibroblasts
rs1569198	<i>PRKG1-AS1</i>	2.5e-10	Cells cultured fibroblasts
	<i>DKK1</i>	2.8e-5	Adrenal-Gland

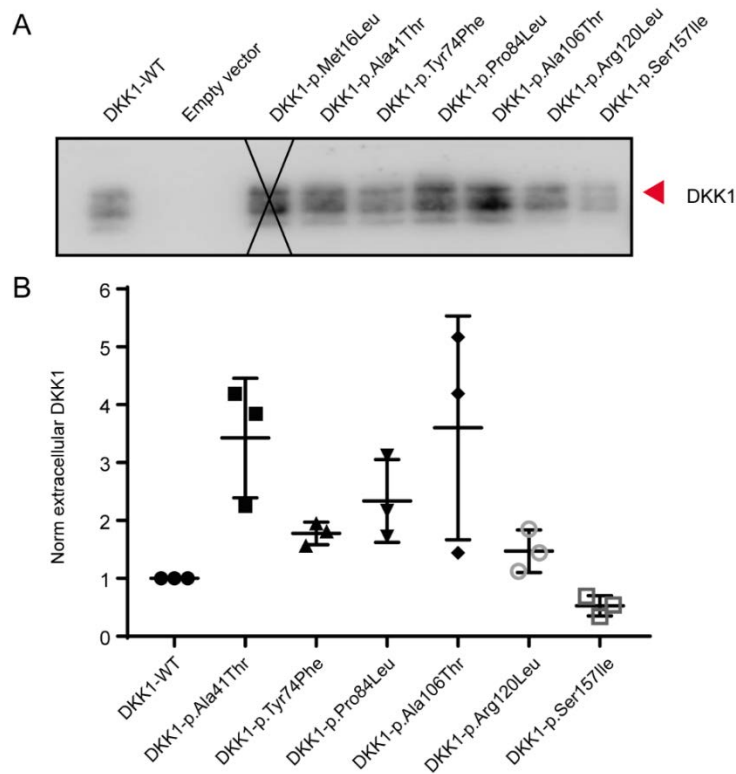


Fig. 1. Extracellular expression of six DKK1 missense variants. A) Western blot of heterologously expressed DKK1 proteins secreted to the extracellular space. Crossed-out lane: mutation unrelated to this work. B) Quantification of three western blot replicates normalized to the expression levels of DKK1-WT.

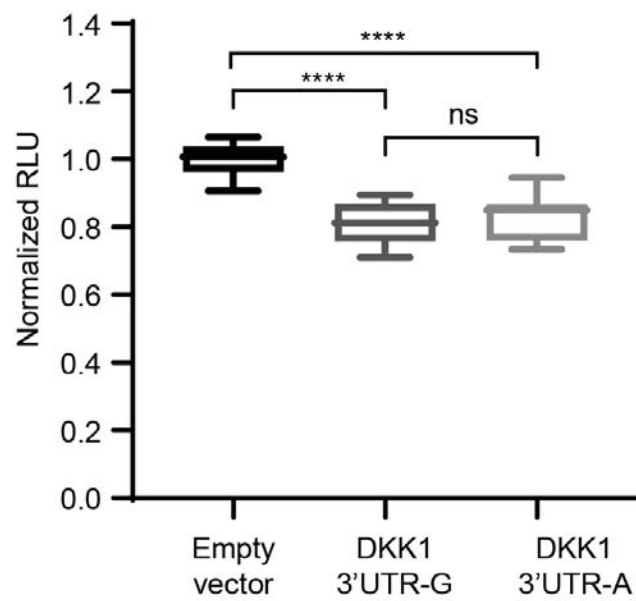


Fig. 2. Luciferase assays of the DKK1 3'UTR region. Boxplots of the normalized relative luciferase activity of the pmirGlo vector (*Empty vector*) and the constructs with the 3'UTR region of *DKK1*-containing the rs74711339 G and A alleles. Significant differences indicated as **** $p < 0.0001$. Error bars represent the SD.

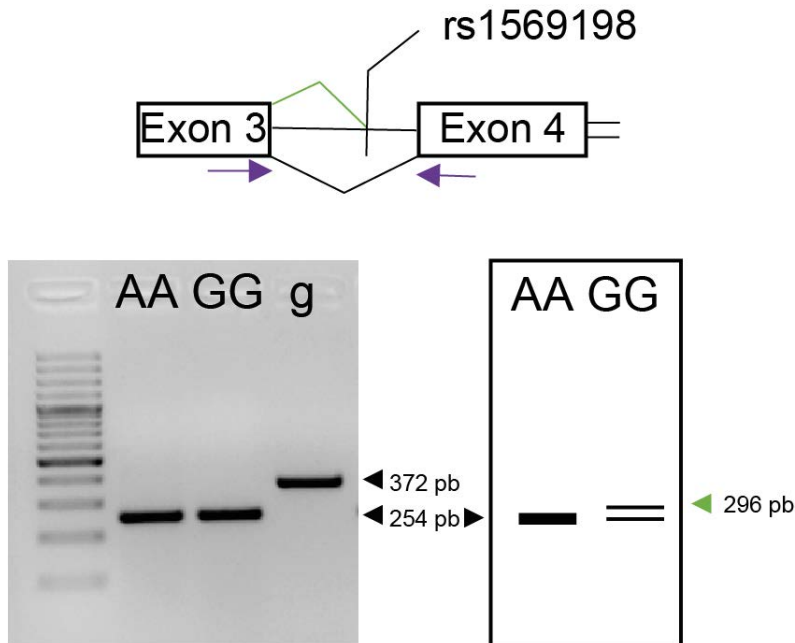


Fig. 3. RT-PCR amplification for hOB from homozygous women of each of the alleles of the SNP rs1569198. Top: Scheme intron 3 and flanking exons indicating the primers binding site (arrows) and the SNP position. Left: Representative electrophoresis of the RT-PCR products for the genotypes AA and GG all of 254 bp. Right: Result prediction under the hypothesis of a partial use of the alternative splice site (296 pb) generated by the G allele. g: PCR amplification of the genomic DNA to display an amplicon of 372 pb which includes the complete intron 3.

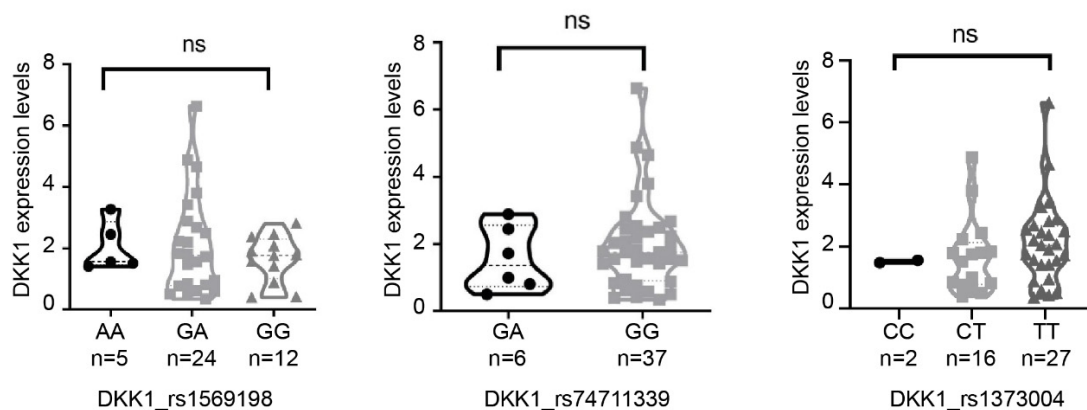


Fig. 4. Violinplot of cis-eQTL analysis of SNPs rs1569198, rs74711339 and rs1373004 on *DKK1* expression levels in hOB (n=45). ns: no significant

Article 3

On the associations between Chiari malformation type I, bone mineral density and *DKK1*: a pilot study.

Summary:

DKK1 is an inhibitor of the Wnt pathway with an important role in head formation and in bone mass determination. Recently, *DKK1* mutations were described in women with a high bone mass (HBM) phenotype and in patients with Chiari malformation type I (CMI), a cerebellum structural defect. Nevertheless, the association between HBM and Chiari malformation type I has never been established. Here, we systematically investigate the association between Chiari malformation type I and mutations in *DKK1* and between the Chiari malformation type I and bone mineral density (BMD). To this aim, we have recruited a small cohort of CMI patients (12 unrelated patients) to whom we have sequenced the coding regions of *DKK1* and have determined their BMD. In this pilot study, we did not identify any missense variant among the 12 patients, and only one patient had high BMD values, very close to the HBM phenotype values (Z-score Lumbar Spine-BMD + Femoral neck-BMD > 4). We encourage other researchers to analyse further CMI cases in order to gain sufficient power to precisely define the relationship between *DKK1* mutations CMI and HBM.

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On the associations between Chiari malformation type I, bone mineral density and *DKK1*: a pilot study.

Running title: Chiari malformation type I, bone mineral density and *DKK1*

NÚRIA MARTÍNEZ-GIL¹, LEONARDO MELLIBOVSKY², DEMIAN MANZANO-LOPEZ GONZALEZ³, DANIEL GRINBERG¹ and SUSANNA BALCELLS¹

¹ Department of Genetics, Microbiology and Statistics, Faculty of Biology, Universitat de Barcelona, CIBERER, IBUB, IRSJD, Barcelona, Spain.

² Musculoskeletal Research Group, IMIM (Hospital del Mar Medical Research Institute), Centro de Investigación Biomédica en Red en Fragilidad y Envejecimiento Saludable (CIBERFES), ISCIII, Barcelona, Spain.

³ Servicio de Neurocirugía, Hospital del Mar, Parc de Salut Mar, Barcelona, Spain.

Corresponding author:

Susanna Balcells: sbalcells@ub.edu

ABSTRACT

DKK1 is an inhibitor of the Wnt pathway with an important role in head formation and in bone mass determination. Recently, *DKK1* mutations were described in women with a high bone mass (HBM) phenotype and in patients with Chiari malformation type I (CMI), a cerebellum structural defect. Nevertheless, the association between HBM and Chiari malformation type I has never been established. Here, we systematically investigate the association between Chiari malformation type I and mutations in *DKK1* and between the Chiari malformation type I and bone mineral density (BMD). To this aim, we have recruited a small cohort of CMI patients (12 unrelated patients) to whom we have sequenced the coding regions of *DKK1* and have determined their BMD. In this pilot study, we did not identify any missense variant among the 12 patients, and only one patient had high BMD values, very close to the HBM phenotype values (Z-score Lumbar Spine-BMD + Femoral neck-BMD > 4). We encourage other researchers to analyse further CMI cases in order to gain sufficient power to precisely define the relationship between *DKK1* mutations CMI and HBM.

Key words: *DKK1*, Chiari malformation type I, HBM phenotype

1. INTRODUCTION

The Wnt pathway is highly conserved across species and is involved in a wide variety of cellular processes. In particular, it plays a very important role in both skeletal development and bone remodelling (Baron & Kneissel 2013). The activity of the canonical Wnt pathway is finely regulated by a number of extracellular components. Among them, DKK1 is an important inhibitor of the canonical pathway, through the binding to LRP5/6 co-receptors (Baron & Kneissel 2013). DKK1 has a crucial role in head formation and bone mineral density determination, as demonstrated by numerous studies performed in mouse models. For instance, the homozygous *Dkk1* knockout (KO) mouse is lethal at birth and displays severe defects in head formation that result in anterior truncations (Mukhopadhyay et al. 2001). Similarly, heterozygous *Dkk1* KO mice and those with conditional KO mutations restricted to the osteoblast lineage show an increased bone mass through increased bone formation (Morvan et al. 2006; Colditz et al. 2018; Witcher et al. 2018). Moreover, the specific overexpression of *Dkk1* in osteoprogenitors and in osteoblasts was shown to reduce bone formation in mice, causing osteopenia (Li et al. 2006; Yao et al. 2011). Despite the phenotypes described, very little data exist on the potential role of *DKK1* in bone mass in humans. To date, no clear disease-causing mutation has been reported in HGMD. Similarly, no SNPs in *DKK1* have been found associated with bone mineral density (BMD) or with different bone parameters in several GWAS. Nevertheless, the results obtained in mouse models highlight the need to perform further studies and investigate the possible role of *DKK1* in human bone development and homeostasis.

Recently, this hypothesis has been explored by studying *DKK1* variants present in different bone phenotypes. These studies identified two missense mutations in *DKK1* (p.Tyr74Phe and p.Arg120Leu) that are associated with a non-pathogenic high bone mass (HBM) phenotype in humans (Sarrión et al. 2014; Martínez-Gil et al. 2018). Subsequently, we tested the functionality of these two variants and of several missense variants present in the general population, including the missense variant p.Ala41Thr (Martínez-Gil et al. 2020). Moreover, it was determined that mutant *DKK1* proteins p.Ala41Thr, p.Tyr74Phe and p.Arg120Leu had lower Wnt pathway inhibitory activity compared to the wildtype, a result that fitted the associated HBM phenotype (Martínez-Gil et al. 2020). Therefore, we now have evidence of the association between *DKK1* variants and the HBM phenotype in humans.

Interestingly, two of the previous mutations, p.Ala41Thr and p.Arg120Leu, were identified in patients with Chiari malformation type I (CMI) (Merello et al. 2017), a neurological and spinal disorder characterized by downward herniation of the cerebellar tonsils through the foramen magnum without involving the brain stem (Barkovich et al. 1986). It has been proposed that CMI is generated via a mesodermal defect involving occipital bone sclerotomes, resulting in a cranial cavity which does not fully accommodate the cerebellum (Nishikawa et al. 1997; Marin-Padilla & Marin-Padilla 1981). The recent study of Merello et al. (Merello et al. 2017) shed some light on the association between *DKK1* variants and CMI.

Taken together, our results (Sarrión et al. 2014; Martínez-Gil et al. 2018; Martínez-Gil et al. 2020) and those of Merello et al., (Merello et al. 2017) suggest a possible mechanism by which loss-of-function of *DKK1* might contribute to CMI through increased bone formation at the skull. Supporting this hypothesis, Whyte et al. described one HBM woman with gain-of-function mutations in *LRP5*, that also present headache from Chiari malformation type I (Whyte et al. 2004). To confirm our hypothesis, 3 possible relationships may deserve further attention: (1) the association between the HBM phenotype and missense mutations in *DKK1*, suggested by our previous results (Sarrión et al. 2014; Martínez-Gil et al. 2018; Martínez-Gil et al. 2020); (2) the association between CMI and missense mutations in *DKK1*, suggested by Merello et al. (Merello et al. 2017) and (3) the association between CMI and the HBM phenotype (Figure 1). Here, we present a pilot study in which we further investigate the relationship between *DKK1* and CMI and, for the first time, we explore the association between HBM and CMI in a small cohort of CMI patients.

2. RESULTS& DISCUSSION

Here, we present preliminary data on the possible relationship among CMI, *DKK1* missense mutations and the HBM phenotype, which need further investigation.

In order to elucidate the relation between missense mutations in *DKK1* and Chiari malformation type I, we sequenced the coding regions and intron boundaries of *DKK1* in 12 patients with CMI, and in 8 relatives of these CMI patients (Table 1). None of the variants found were missense and they did not seem to be responsible for the phenotype (Table 2). If we consider the frequency of *DKK1* mutations found in Merello et al. (Merello et al. 2017) in 65 sporadic CMI patients (0.045), we expected to find

one case, as a maximum, in our cohort, which we did not. Therefore, it is very important to increase the cohort and sequence the *DKK1* gene in a larger number of CMI patients to properly replicate the results by Merello et al. (Merello et al. 2017). The fact that the two *DKK1* missense mutations so far associated with CMI have been proved to be functional (Martínez-Gil et al. 2020) is a compelling argument for an implication in CMI, which would appear to be a genetically heterogeneous condition. In this line, it would be interesting to also sequence other members of the Wnt pathway that may have an important role in skull development, such as *LRP4*, *LRP5* and *LRP6*. Additionally, the results of MRI performed in the two HBM patients bearing the *DKK1*-p.Tyr74Phe mutation showed that neither of them presented with CMI.

To study the relationship between CMI and BMD we have determined the BMD of a small group (n= 12) of CMI patients. In particular, for each patient, we have computed the sum of the BMD Z-scores for lumbar spine and femoral neck. According to this metric, HBM phenotype is defined by a value higher than 4 (Little et al. 2002). We found that the mean of the sum BMD Z-score of unrelated patients with available BMD (n = 9) was +0.5, while that of all available patients, related and unrelated, was +0.08 (n = 12). Taking into account the prevalence of the HBM phenotype (0.2-1%), under the hypothesis of no association, it would have been very unlikely to find a HBM case in this small cohort. However, patient CH5 presented a sum Z-score of 3.8, very close to the accepted threshold for HBM. For this reason, increasing the size of the cohort of patients with Chiari malformation type I with data on their BMD will provide a clearer view of this possible association (Table 1).

Finally, in order to elucidate the relationship between the *DKK1* missense variants and the HBM phenotype, it would be really interesting to measure the lumbar spine and femoral neck BMD of the patients from the paper by Merello and collaborators (Merello et al. 2017), who bear the p.Ala41Thr and p.Arg120Leu mutations, to determine if they have high BMD values and maybe even reach the HBM threshold.

In conclusion, although our study is clearly preliminary and underpowered, we consider the possible relationship among *DKK1* missense mutations, Chiari malformation type I and bone mineral density an interesting issue, with a few evidences all pointing in the same direction. We encourage the CMI research community to participate in this investigation to get to an adequate power.

3. MATERIAL AND METHODS

3.1 Ethics Statement

Both the Bioethics Committee of Universitat de Barcelona and the Clinical Research Ethics Committee of Parc de Salut MAR have emitted a favourable bioethical statement regarding the present research. Specifically, the protocol for obtention of peripheral blood from the CMI cohort was approved by both committees. Written informed consents were obtained from the participants in both instances.

3.2 Study Cohort

The study population included 12 unrelated CMI cases diagnosed at Hospital del Mar, Barcelona and invited to participate by personal interview and 8 relatives of 4 CMI patients (Table 1). Diagnosis of CMI was based on brain-MRI (Achieva 3.0 T, Philips, Amsterdam, Netherlands) demonstration of downward herniation >5 mm of the cerebellar tonsils on a mid-sagittal T1-weighted image in the presence of signs or symptoms indicating neural compression at the cranio-vertebral junction, syringo-hydromyelia, cerebellar dysfunction or intracranial hypertension. The brain MRI was performed to all CMI cases and to the two women with HBM who carried the p.Try74Phe variant (described in Sarrion et al. 2014). The cohort includes bone mineral density measurements obtained through dual-energy X-ray absorptiometry scans (DXA; QDR 4500 SL; Hologic, Waltham, MA, USA) and DNA samples. All DXA measurements were performed prior to any treatment that could increase bone mass. For individuals CH7, CH7a, CH10, CH11a, CH11b no DXA scan was available.

3.3 Sequencing *DKK1*

The genomic DNA of n = 20 CMI cases and their relatives was isolated from peripheral blood leukocytes using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. The four exons and flanking regions of *DKK1* were resequenced in all probands, as described in (Martínez-Gil et al. 2018).

Author's contributions: NMG, DG, SB were involved in the study conception and design. LM and DMLG recruited the Chiari malformation type I cohort and made bone mineral density measurements. NMG sequenced DKK1 gene. The first draft of the brief report was written by NMG, DG, SB and all authors commented on previous versions of the brief report and all authors read and approved the final version.

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Conflict of interest The authors declare that they have no conflict of interest.

Consent to participate and consent for publication: Both the Bioethics Committee of Universitat de Barcelona and the Clinical Research Ethics Committee of Parc de Salut MAR have emitted a favourable bioethical statement regarding the present research. **Availability of data and material:** All the data are available upon request.

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Table 1. Description of the Spanish CMI cohort

ID	Sex	Age	Family relationships	Disease status	BMD (Z-Score)		Sum Z-score
					LS	FN	
CH1	F	59		A	*	-1.3	
CH2	F	65		A	-0.9	-0.9	-1.8
CH2a	F	35	daughter of CH2	A	-0.4	-0.8	-1.2
CH2b	F	68	sister of CH2	A	0	-0.4	-0.4
CH2c	F	71	sister of CH2	N	+0.8	+0.8	+1.6
CH2d	F	72	sister of CH2	A	-1.8	-0.1	-1.9
CH3	F	46		A	+0.1	-0.1	0
CH4	M	24		A	+0.2	0	+0.2
CH5	F	45		A	+1.8	+2	+3.8
CH6	M	46		A	-0.4	+0.5	+0.1
CH7	M	61		A	NA	NA	NA
CH7a	F	63	sister of CH7	A	NA	NA	NA
CH8	M	47		A	-1.7	-0.6	-2.3
CH9	F	45		A	+1.3	+0.6	+1.9
CH10	M	74		A	NA	NA	NA
CH10a	F	NA	sister of CH10	N	+0.8	+1.4	+2.2
CH11	F	36		A	+0.8	+0.2	+1
CH11a	M	65	father of CH11	NA	NA	NA	NA
CH11b	F	67	mother of CH11	N	NA	NA	NA
CH12	F	83		A	+0.3	+1.3	+1.6

F: Female; M: Male; A: Affected N: Non-affected; FN: Femoral Neck; LS: Lumbar Spine; NA: Non available; * dysmorphic lumbar

Table 2. Variants found in the *DKK1* resequencing

Variant name (HGVS)	rs number	Type	MAF (EUR) ¹	MAF (CMI cohort)
c.548-43A>G	rs1569198	I	0.485 (G)	0.625 (G)
c.244-23C>T	rs41281546	I	0.075 (T)	0.083 (T) ²
p.Ala106=	rs2241529	S	0.447 (A)	0.292 (A)
p.His229=	rs148686867	S	0.0007(C)	0.042 (C) ³
c.*82A>G	rs74711339	3'UTR	0.063 (G)	0.042 (G) ⁴
g.54077566_54077571delinsTTA	rs386743716	3'D	-	0.042 (insTTA) ⁵

CMI cohort: n=12; ¹from GnomAD v2.1.1; ²CH7 and CH10; ³CH2; ⁴CH10; ⁵CH7

HGVS: Human Genome Variation Society nomenclature (den Dunnen et al., 2016); GRCh37.p13; I intronic variant; S: Synonymous variant; 3'UTR: 3'UTR variant; 3'D 3' downstream variant

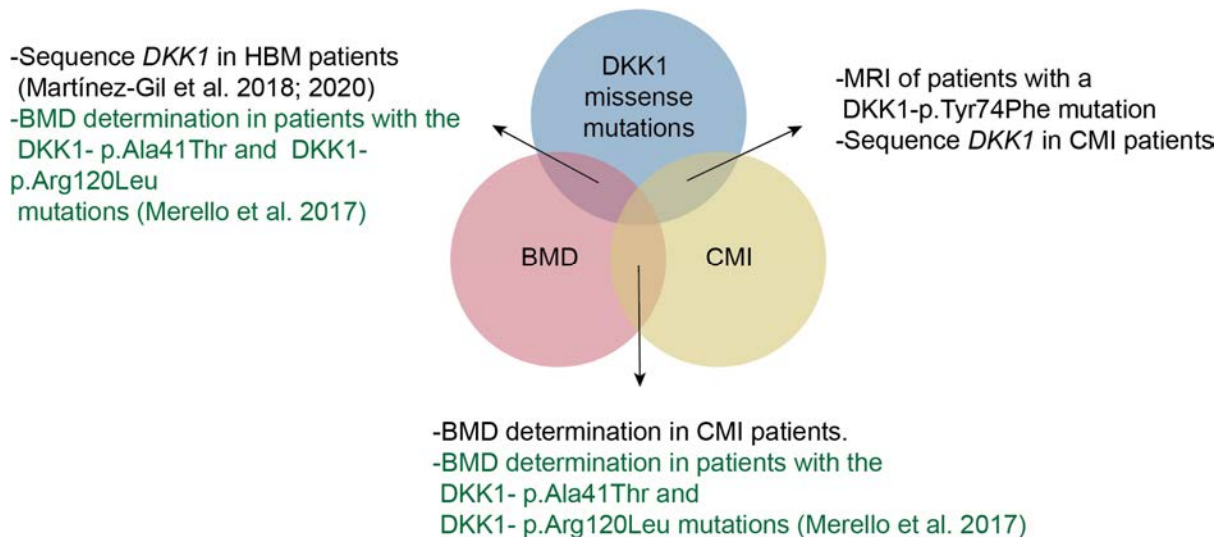


Figure 1. Overview of the 3 hypotheses to be tested to better understand the relationship between *DKK1* missense mutations-BMD-CMI. In italic, the determination that we suggest to Merello and collaborators (Merello et al. 2017).

Article 4

Genetics and genomics of *SOST*: functional analysis of variants and genomic regulation in bone cells

Summary:

SOST encodes the sclerostin protein, which acts as a key extracellular inhibitor of the canonical Wnt pathway in bone, playing a crucial role in skeletal development and bone homeostasis. The objective of this work was to assess the functionality of two variants previously identified (the SNP rs570754792 and the missense variant p.Val10Ile) and to investigate the physical interactors of the *SOST* proximal promoter region in bone cells. Through a promoter luciferase reporter assay we show that the minor allele of rs570754792, a variant located in the extended TATA box motif, displays a significant decrease in promoter activity. Likewise, through western blot studies of extracellular and intracellular sclerostin, we observe a reduced expression of the p.Val10Ile mutant protein. Finally, using a circular chromosome conformation capture assay (4C-seq) in 3 bone cell types (MSC, hFOB, Saos2), we have detected physical interactions between the *SOST* proximal promoter and the *ECR5* enhancer, several additional enhancers located between *EVT4* and *MEOX1* and a distant region containing exon 18 of *DHX8*. In conclusion, *SOST* presents functional regulatory and missense variants that affect its expression and displays physical contacts with far reaching genomic sequences, which may play a role in its regulation within bone cells.

Reference:

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Article

Genetics and genomics of *SOST*: functional analysis of variants and genomic regulation in bone cells

Núria Martínez-Gil ¹, Neus Roca-Ayats ¹, Mónica Cozar ¹, Natàlia Garcia-Giralt ², Diana Ovejero ², Xavier Nogues ², Daniel Grinberg ¹, Susanna Balcells ^{1*}

¹ Department of Genetics, Microbiology and Statistics, Faculty of Biology, Universitat de Barcelona, CIBERER, IBUB, IRSJD, Barcelona, Spain.²

² Musculoskeletal Research Group, IMIM (Hospital del Mar Medical Research Institute), Centro de Investigación Biomédica en Red en Fragilidad y Envejecimiento Saludable (CIBERFES), ISCIII, Barcelona, Spain.

* Correspondence: sbalcells@ub.edu

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Abstract: *SOST* encodes the sclerostin protein, which acts as a key extracellular inhibitor of the canonical Wnt pathway in bone, playing a crucial role in skeletal development and bone homeostasis. The objective of this work was to assess the functionality of two variants previously identified (the SNP rs570754792 and the missense variant p.Val10Ile) and to investigate the physical interactors of the *SOST* proximal promoter region in bone cells. Through a promoter luciferase reporter assay we show that the minor allele of rs570754792, a variant located in the extended TATA box motif, displays a significant decrease in promoter activity. Likewise, through western blot studies of extracellular and intracellular sclerostin, we observe a reduced expression of the p.Val10Ile mutant protein. Finally, using a circular chromosome conformation capture assay (4C-seq) in 3 bone cell types (MSC, hFOB, Saos2), we have detected physical interactions between the *SOST* proximal promoter and the *ECR5* enhancer, several additional enhancers located between *EVT4* and *MEOX1* and a distant region containing exon 18 of *DHX8*. In conclusion, *SOST* presents functional regulatory and missense variants that affect its expression and displays physical contacts with far reaching genomic sequences, which may play a role in its regulation within bone cells.

Keywords: Sclerostin, bone, 4c-seq, luciferase reporter assay, HBM

1. Introduction

Sclerostin (encoded by the *SOST* gene) is a potent antagonist of the canonical Wnt pathway and of bone formation, exerting its function by blocking LRP5/6 co-receptors [1,2]. To perform this function, Sclerostin binds to LRP4, which enhances its suppressive effect [3–5]. Sclerostin is a monomeric protein with a Nxl motif and an eight-membered cysteine knot motif, all of them implicated in LRP5/6 binding [6–8].

In humans, *SOST* mutations are associated with inherited high bone mass conditions characterized by excessive bone formation, with a sclerosis phenotype particularly prominent in the skull, jaw and long bones. The clinical spectrum of these conditions ranges from severe craniodiaphyseal dysplasia to non-pathological high bone mass, with the severity of the condition being inversely proportional to sclerostin abundance. Craniodiaphyseal dysplasia (CDD, MIM 122860) is the most severe sclerostin bone dysplasia, characterized by an absence of extracellular sclerostin and a dominant negative effect. It is an extremely rare autosomal dominant condition, with less than 20 cases reported [9,10]. Sclerosteosis (MIM 269500) is another severe sclerostin disease with autosomal recessive inheritance [11,12]. Nine loss-of-function mutations (either truncating or missense) have been described in sclerostin [12–19]. Additionally, three loss-of-function mutations in *LRP4*, have been described in

sclerosteosis-2 [4,20]. Van Buchem's disease (MIM 239100), a third *SOST* clinical entity, is caused by a 52-kb deletion of a regulatory region located 32 kb downstream of *SOST* and necessary for the correct expression of the gene [21,22]. The difference in severity lies in that in patients with Van Buchem, wild-type sclerostin is low, whereas in patients with sclerosteosis it is absent [23,24]. In line with these findings, a heterozygous truncating mutation in *SOST* has been associated with the non-pathological High Bone Mass (HBM) phenotype, which is characterized by unusually dense bones and a very strong skeleton practically eliminating fracture occurrence [25–28]. Furthermore, it was described that mutations in *LRP5* causing the HBM phenotype showed a loss of affinity for sclerostin [29,30].

Sclerostin is mainly expressed in mature osteocytes, although relatively high levels of mRNA have been found in different tissues [31]. In addition, *SOST*/sclerostin expression is altered in certain pathogenic conditions, including postmenopausal osteoporosis, osteoarthritis and rheumatic joint disease [32]. Genetically modified mice add another line of evidence to the role of *SOST* in bone mineral density (BMD) determination: Sclerostin-knock-out (KO) mice show high BMD and bone strength, with a high number of osteoblasts and increased bone formation that reproduce the phenotype observed in patients with sclerosteosis or Van Buchem's disease [33,34]. In contrast, mice that overexpress this gene present with low bone mass, lower bone resistance, low number of osteoblasts and reduced bone formation [35–38].

The transcription of *SOST* is finely regulated by a large number of signals. *SOST* expression is controlled by the proximal promoter region of the gene (~1.4 kb upstream *SOST*) and by a 225-bp bone-specific distal enhancer called *ECR5* (Evolutionary conserved region 5), located 32 kb downstream of the gene [31,39]. *ECR5* is deleted in patients with Van Buchem's disease, and its deletion in mice causes a drastic decrease in *Sost* levels in osteocytes [39]. *ECR5* stimulates the expression of *SOST*, in part through the binding of the transcription factor Myocyte-enhancer factor 2c (Mef2c) [35,39].

Humanized monoclonal antibodies against sclerostin have been developed for the treatment of different bone disorders such as osteoporosis or osteogenesis imperfecta, and they have been approved in Japan, US, EU and Canada [40–44]. Antibody treatment against sclerostin stimulates bone formation, decreases bone resorption and, thus, increases bone mass and strength, and decreases the risk of fragility fracture in both animals and humans [40–42,44–46].

Taking into account the crucial role of *SOST* in bone metabolism, we hypothesized that partial loss-of-function mutations in its gene might be associated with variations in BMD in the general population. In this line, we previously identified 2 variants in *SOST* that captured our interest. First, the rare variant rs570754792 located in the extended TATA box, which was only present in 3 women with low bone mass; and second, the low-frequency variant rs17882143 (p.Val10Ile), which modified the signal peptide of sclerostin, and whose minor allele was associated with high BMD [47].

Our objective in this work was to assess the functionality of these variants and to detect the main physical interactors of the *SOST* proximal promoter. We verified the loss of function of rs570754792 and rs17882143, which produced a decrease in gene expression and protein abundance, respectively. In addition, we were able to detect physical interactions of the *SOST* proximal promoter with several enhancers in the region, including the *ECR5* distal enhancer.

2. Results

2.1 The minor allele of a variant in the extended TATA box of *SOST* displays lower transcriptional capacity

The rare variant rs570754792 lies in the *SOST* proximal promoter, specifically in the extended TATA box. To test its effect, we performed a luciferase reporter assay in Saos-2 cells, testing a 520-bp fragment, which contains either of the variant alleles. Our results show that this region harbors promoter activity

(20-fold increased transcription vs. empty vector, for the wild-type allele) and show significant differences between the two alleles tested, where the minor allele displays lower transcription capacity (about 25% less than the major allele; Figure 1). The *COL1A1* promoter (SP construct in [48]), used as positive control, showed 31.6-fold increased promoter activity compared to the empty vector (Figure 1).

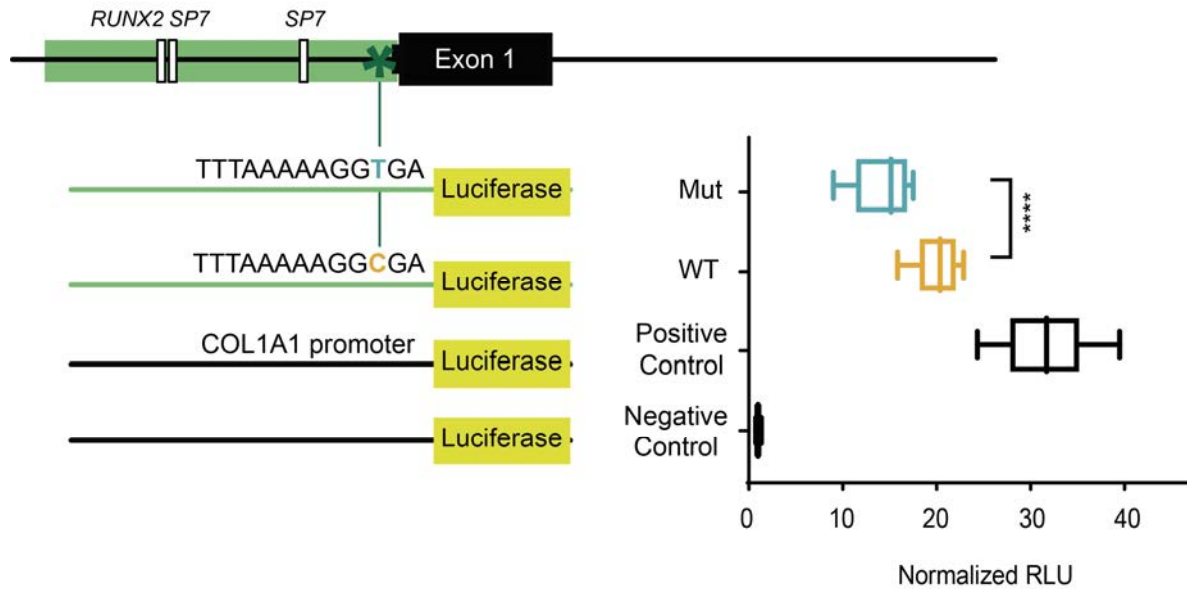


Figure 1. Functional testing of a rare variant in the extended TATA box of *SOST*. rs570754792 (g.41836179 G>A in GRCh37.p13) belongs to the extended TATA box of *SOST*, which is encoded in the negative strand of chromosome 17, thereby generating a C>T substitution. In black, a scheme of the location of the variant (asterisk) in the *SOST* upstream region and in green the constructs tested. We used the luciferase gene without promoter as a negative control and the *COL1A1* promoter as a positive control. Relative luciferase units (RLU; see material & methods) are normalized to the negative control. The experiment was performed three times in triplicate. Error bars indicate s.d. **** indicates $p < 0.0001$.

2.2. Lower protein abundance for the minor allele of rs17882143, both in the intracellular and extracellular spaces

The rs17882143 SNP is a missense change (p.Val10Ile) within the signal peptide of the sclerostin protein. To assess whether the change might affect the subcellular localization of the mutated protein or its abundance, we performed western blot studies with extracellular and intracellular protein extracts (Figure 2). In addition to this mutation, we tested 5 of the most frequent sclerostin mutations in the general population according to gnomAD (p.Arg19His, p.Gly150Arg, p.Asn53del, p.Ala28Val, p.Pro111Gln; Figure 2A), as well as two positive controls (p.Val21Leu and p.Cys167Arg; Figure 2A) whose absence in the extracellular space had been previously described [10,17]. All these changes are depicted along the sclerostin protein domains in Figure 2A. We verified that the positive control proteins SOST-p.Val21Leu and SOST-p.Cys167Arg showed a dramatic decrease in the extracellular space. However, while the SOST-p.Cys167Arg showed an increase in the amount of intracellular protein, the SOST-p.Val21Leu showed a decrease in intracellular levels (Figure 2B and C). Similarly to the results of these two positive control proteins (p.Val21Leu and p.Cys167Arg), we observed a lower abundance of SOST-p.Val10Ile and SOST-p.Gly150Arg proteins in the extracellular space and, for the SOST-p.Val10Ile, SOST-p.Val21Ile, SOST-p.Ala28Val, SOST-p.Asn53del and SOST-p.Pro111Gln, a lower

abundance in the intracellular compartment. The other mutant proteins behaved similarly to the WT protein (Figure 2B and C).

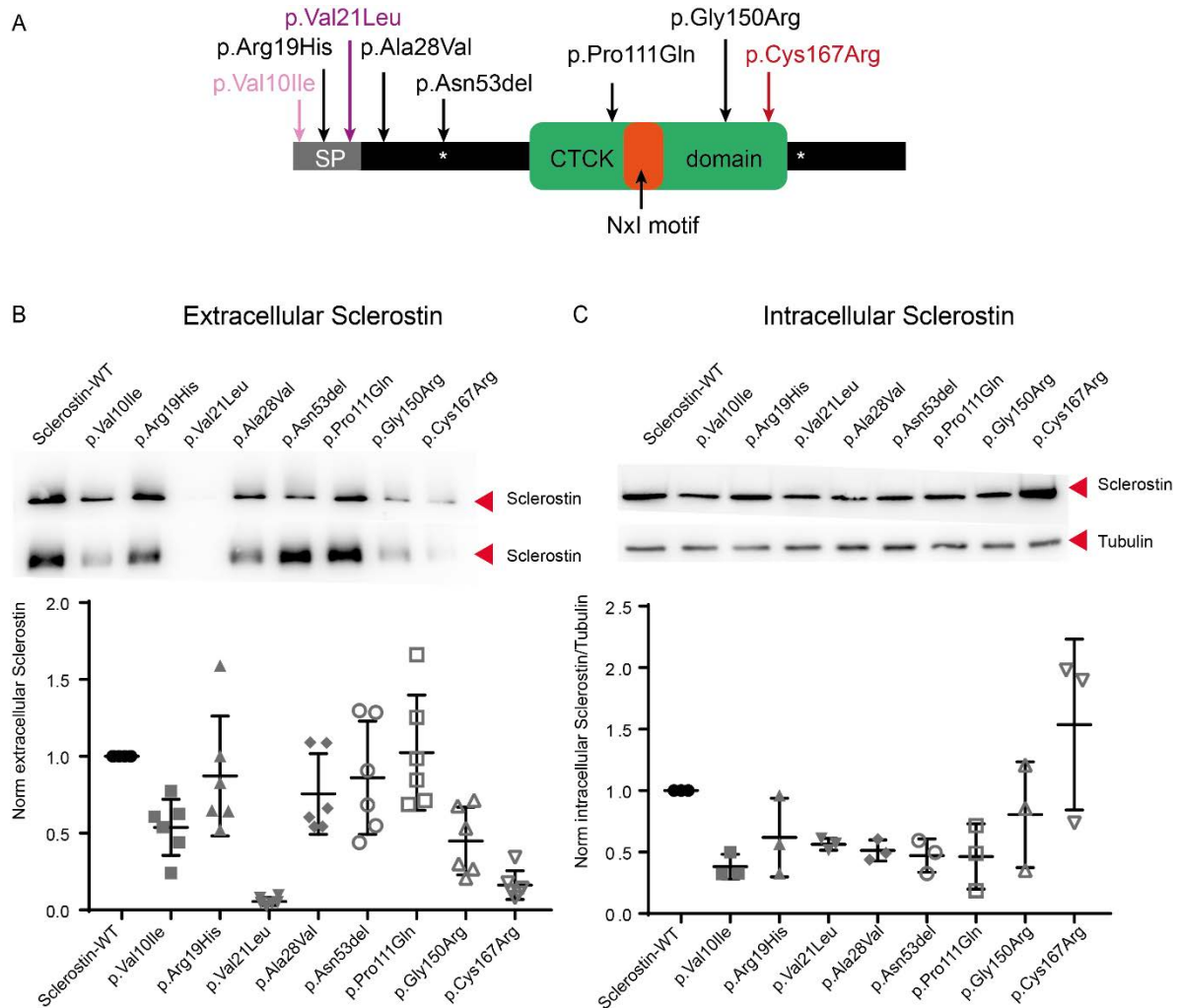


Figure 2. Western blot of variant sclerostin proteins transfected into Saos-2 cells. Variants were generated by *in vitro* mutagenesis into a *SOST* expression vector and subsequently transfected and heterologously expressed in Saos-2 cells. (A) Tested amino acid variants, displayed along a scheme of the sclerostin, with indication of specific domains (SP: signal peptide; CTCK: C-terminal cystine-knot; NXI: NXI(R/K) sequence motif; asterisks denote the glycosylation N-linked (GlcNAc) asparagine). In red, the mutation present in a patient with sclerosteosis [17]; in purple, the mutation found in a patient with craniofacial dysplasia [10] and in pale pink, the mutation associated with high BMD [47] (B) Detection of sclerostin in the extracellular media. Above, image of the first biological replicate; below, quantification of the sclerostin bands from images of 6 equivalent blots, normalizing to the extracellular levels of WT sclerostin. (C) Detection of intracellular sclerostin. Above, image of the first biological replica of sclerostin and their corresponding tubulin; below, the quantification of the ratios of sclerostin/tubulin protein from images of 3 equivalent blots, normalizing to the ratio of WT sclerostin/tubulin.

2.3. Physical interaction between the *SOST* proximal promoter and several distal regulatory elements in bone cells

To identify important regulators of *SOST* expression presence in three bone-related cells (Mesenchymal Stem Cells –MSC–; human fetal osteoblasts –hFOB– and an osteosarcoma cell line –Saos-2–), we performed a 4C-seq assay using the *SOST* proximal promoter (chr17:41838135-41838123, GRCh37/hg19) as a viewpoint (Figure 3). Using an algorithm to discern the significant contacts, we only observed interactions of this viewpoint with sequences included in a genomic region spanning 288 kb centromeric to the viewpoint. Within this region, we confirmed the strong physical interaction between the proximal promoter and the *ECR5* distal enhancer in the 3 cell types. Moreover, we found interactions in the three cell types between the viewpoint and several genomic regions located between *MEOX1* and *ETV4*, described as enhancers according to GeneHancer [49] and displaying strong H3K4me1, H3K4me2, H3K4me3, H3K27ac and H2AFz histone marks in osteoblasts (ENCODE; Supplementary Figure 1). In addition, we observed an interaction with a region containing exon 18 of the *DHX8* gene that displays strong H3K4me1 and H3K27Ac histone marks in osteoblasts (ENCODE), suggesting a potential regulatory role for this region, too. Finally we saw a significant interaction with the *DHX8* promoter in hFOB and Saos-2 cells, which displays H3K4me2, H3K4me3, H3K27ac, H3K79m3 and H2AFz histone marks in osteoblasts (ENCODE; Figure 3 and Supplementary Figure 1).

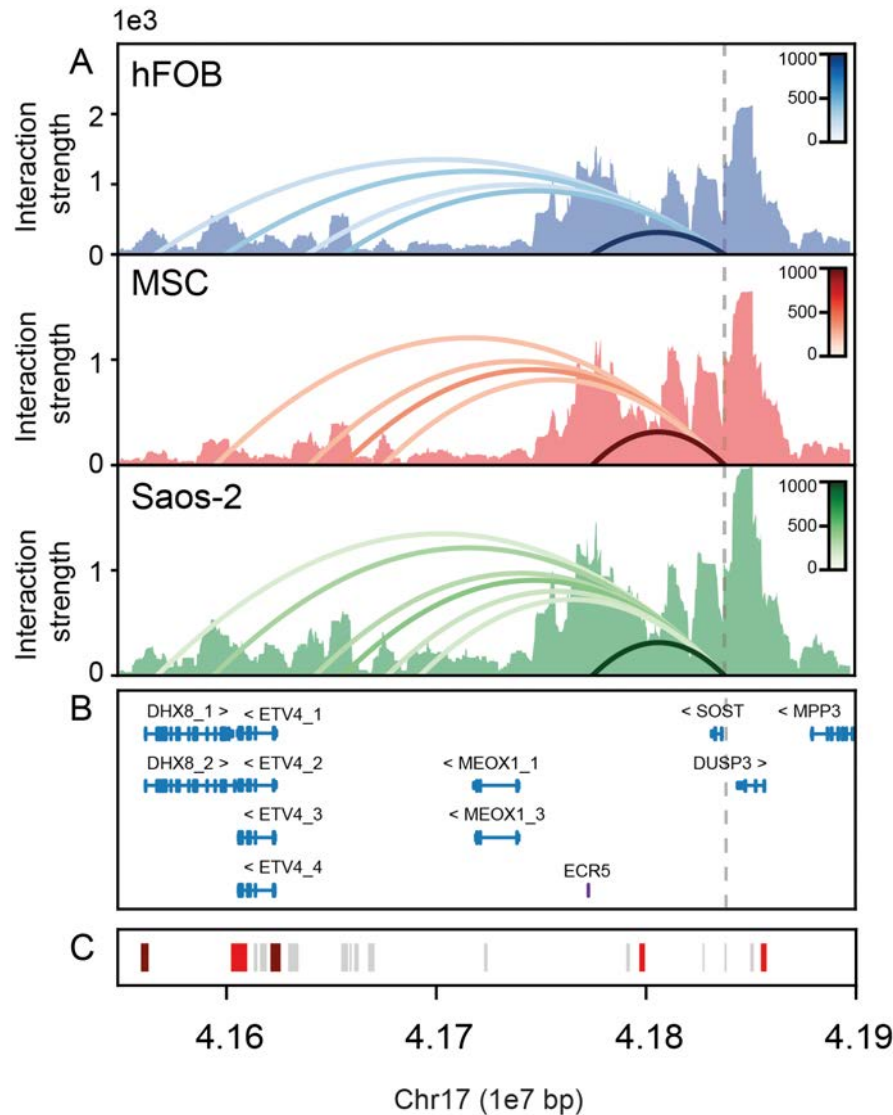


Figure 3. Chromatin interactions of the *SOST* proximal promoter region as detected by 4C-seq in human fetal osteoblasts (hFOB), mesenchymal stem cells (MSC) and the Saos-2 cell line. Panel A: Graphical representation of a 350-kb Chr.17 region containing *SOST* and all the genomic elements found to interact with it displaying both the read depths (Interaction strength) and their corresponding significance (arched lines). The dashed vertical line marks the *SOST* promoter, used as the viewpoint. Color scale corresponds to the strength of the significant interactions. Panel B: Genes in this region: *DHX8_1* (ENST00000262415.3), *DHX8_2* (ENST00000540306.1), *ETV4-1* (ENST00000319349.5), *ETV4_2* (ENST00000545954.1), *ETV4-3* (ENST00000591713.1), *ETV4_4* (ENST00000545089.1), *MEOX1_1* (ENST00000520305.1), *MEOX1_3* (ENST00000393661.2), *SOST* (ENST00000301691.2), *DUSP3* (ENST00000226004.3), *MPP3* (ENST00000398389.4) from GRCh37/hg19. In purple the *ECR5* position [35]. Panel C: Representation of the GeneHancer (GH) track (enhancers -grey- and promoters -red-).

2.4. The rs17882143 (p.Val10Ile) variant present in one HBM woman

To further explore the putative genetic association of *SOST* with bone density phenotypes, we resequenced the *SOST* gene and the *ECR5* enhancer, in 11 HBM women (described in [50]). We identified eight variants in *SOST* and one variant in *ECR5*, all of them previously described (Table 1). Interestingly, we found the variant p.Val10Ile in heterozygosity in one HBM woman (HBM13) with a high sum Z-score (lumbar spine + femoral neck BMD Z-score=5.2). In addition, we found the *ECR5* variant rs552004150 in heterozygosity in another HBM woman (HBM16) with a Sum Z-score=5.3. However, it did not co-segregate with the phenotype in the HBM16 family (Supplementary Figure 2a). This variant lies approximately 50 bp from the *MEF2C* binding site and does not modify the binding of any known transcription factor according to TRANSFAC (Supplementary Figure 2b). Variants rs1237278, rs851058, rs10534024, rs17882143, rs17886183 and rs17881550, found in our HBM cohort, are listed as eQTLs for different genes and tissues in the GTEx database (Supplementary Table 1). In addition, variants rs1237278, rs851058 and rs17885799 are described as affecting the binding site of various transcription factors, and the rare variant rs17885799 may generate a new exonic splicing enhancer (ESE) according to SNP function prediction (Supplementary Table 2). Furthermore, SNPs rs17883310 and rs17886183 show allele-specific differences in miRNA binding predictions (Supplementary Table 2).

Table 1. Variants in *SOST* and *ECR5* found in women with an HBM phenotype

	rs number	Type	MAF (EUR)	MAF (HBM cohort)	Predicted functionality
<i>SOST</i>					
g.41838229C>T	rs1237278	5'UP	0.355 (C)	0.45 (C)	eQTL; TFBS
g.41837719G>A	rs851058	5'UP	0.402 (A)	0.35 (A)	eQTL; TFBS
g.41837510_41837512del	rs10534024	5'UP	0.352 (TCCTCCT)	0.45 (TCCTCCT)	eQTL
p.Val10Ile	rs17882143	M	0.018 (T)	0.05 (T)	eQTL T;B;LB

c.-22G>A	rs17885799	5'UTR	-	0.05 (A)	TFBS, ESE
c.*320C>T	rs17883310	3'UTR	0.013 (T)	0.05 (T)	miRNA
c.*1004G>A	rs17886183	3'UTR	0.004 (A)	0.05 (A)	eQTL miRNA
c.*1266_*1267insG	rs17881550	3'UTR	0.412 (ins)	0.35 (ins)	eQTL
ECR5					
g.41774092G>A	rs552004150	3'D	0.003 (A)	0.05 (A)	

M: Missense variant; 5'UP: 5'Upstream; 5'UTR: 5'UTR variant; 3'UTR: 3'UTR variant; 3'D 3' downstream variant; MAF(EUR) from 1000 genomes. eQTL: described as eQTL in GTEx. TFBS: Transcription factor binding site; ESE: Exon Splicing enhancer; T: Tolerated by SIFT; B: Benign by Polyphen; LB: Likely Benign by CADD; miRNA: allelic differences in the miRNA binding prediction.

3. Discussion

Sclerostin is an important bone protein, strongly associated with different bone sclerosing dysplasias. In this work, we undertook a functional study to test two sclerostin variants (rs570754792 and rs17882143) and to define the physical interactions of the *SOST* proximal promoter region. We experimentally demonstrate the partial loss of function of these two variants that cause a loss of expression (rs570754792) and a loss of protein abundance (p.Val10Ile). Furthermore, we detect physical interactions of the *SOST* promoter with *ECR5*, with a region containing exon 18 of the *DHX8* gene and with several enhancers between *MEOX1* and *ETV4*, in 3 bone cell types.

Regarding the rare variant rs570754792, it was found in heterozygosity in only three women of the BARCOS cohort of postmenopausal women of the Barcelona area, all three with BMD values below the mean (LS-BMD: 0.767, 0.694, 0.822 vs 0.850 g/cm²)[47]. According to the established inhibitory role of *SOST* in the canonical Wnt pathway, we investigated whether *SOST* was expressed at levels higher than normal using a luciferase reporter assay. Surprisingly, we observed just the opposite: lower levels of expression in the presence of the minor allele (T). This result, however, is not totally unexpected, since the rare variant is part of the extended TATA box, and the major and minor alleles represent the canonical and non-canonical base at the position -6, respectively. At this point it is hard to reconcile the low BMD values observed in the three carriers and the lower *SOST* expression levels of the variant. The lack of genomic context of the luciferase experiments might provide an explanation for these contradictory results. On the other hand, it may just be that this variant has low penetrance, and in these women an alternative mechanism is responsible for their phenotype, (i.e. a different mutation or environmental component with a larger effect, masking that of the mutation in the extended TATA box of *SOST*). In fact, it could be a situation similar to that of van Buchem's disease, in which two copies of a low expressing allele are needed to show the phenotype.

Using western blot analysis, we studied the expression levels of sclerostin containing the SNP rs17882143 (*SOST*-p.Val10Ile). As this variant is located in the signal peptide of the protein, we expected a lower translocation to the extracellular space. However, we found lower amounts of it, both in the extracellular and the intracellular compartments, which seems to indicate a defect in a previous stage

to protein translocation and secretion. This result is supported by an analysis using the SignalIP signal peptide prediction program [51] that predicts no functional differences in the signal peptide. Our result could be explained by reduced transcription or translation levels or increased mRNA or protein degradation. In particular, it has been described that missense mutations can result in an altered secondary mRNA structure which compromises the accessibility of the protein translational machinery [52–54]. In our case, this hypothesis is supported by the Mfold web server [55], which predicts a secondary structure that has a different free energy depending on the allele of the *SOST*-p.Val10Ile. The p.Val10Ile mutation is the most frequent *SOST* missense variant according to gnomAD (MAF 0.0109). *In silico* functional predictors scored it as benign (SIFT; 0.43), tolerated (Polyphen; 0.001) and likely benign (CADD; 0 and REVEL; 0.123). In a previous study of our group [47], this variant showed nominal association with lumbar spine BMD, where the minor allele (Ile) was protective. Furthermore, we have now found that a woman with an HBM phenotype (with a sum Z-score of 5.2) is heterozygous for this variant (Table 1), and it was also detected in 8 additional HBM cases in the work of Gregson et al. [25]. All things considered, it could be speculated that people carrying this mutation show high non-pathogenic BMD due to a reduction of sclerostin expression.

The *SOST* p.Gln150Arg protein showed a decreased secretion to the extracellular space compared to the WT. This mutation, which is present in the general population with a frequency of 0.000333, is the third most frequent missense mutation in *SOST* and functional predictors consider it deleterious (SIFT; 0.02), possibly damaging (Polyphen; 0.651) or likely benign (CADD; 23 and REVEL; 0.295). Similarly to *SOST*-p.Val10Ile, it could be speculated that people carrying p.Gln150Arg may present a non-pathogenic HBM due to low levels of sclerostin in the extracellular space. In the future, BMD analyses of carriers will be helpful to test this prediction.

Regarding the two mutants previously described that we used as positive controls, we obtained similar results to those described by Piters et al. for p.Cys167Arg [17], while we obtained some discrepant results for p.Val21Leu, compared to data from Kim et al. [10]. In particular, we found that intracellular protein abundance of *SOST*-p.Val21Leu was approximately 50% of that of WT, while Kim et al. [10] showed values similar to wild-type sclerostin. Technical differences, including different cell types (HEK293 vs. Saos-2) and antibodies (anti-myc vs. anti-sclerostin), may explain this discrepancy.

Finally, through 4C-seq we showed for the first time the physical interaction between *ECR5* and *SOST* in 3 bone-relevant cell lines. Further, we identified the interaction of the proximal *SOST* promoter with several putative enhancer elements between *MEOX1* and *EVT4*, which display regulatory histone marks in osteoblasts (ENCODE). Even though they appear as non-tissue specific elements according to GeneHancer, in the future it will be interesting to investigate the role of these enhancers in bone-specific *SOST* expression. We also identified a significant interaction between the promoter and a distal regulatory element overlapping exon 18 of *DHX8*, a gene encoding a spliceosome component, which may be unrelated to bone mass. In the overall, emerging picture, several long-distance enhancers may participate in the regulation of *SOST* in bone cells. Whether they work in an additive way or as alternative redundant enhancers is an interesting question for future research.

Sclerostin is a key component of the canonical Wnt pathway in bone and, as such, it plays a crucial role in skeletal development and bone homeostasis. A detailed understanding of its developmental and tissue regulation together with the knowledge of the effect of variation at its locus is key to developing prediction algorithms or therapeutic strategies based on this gene/protein. Our results add functional evidence for the participation of the p.Val10Ile variant in non-pathogenic HBM phenotype and also provide regulatory evidence for certain enhancer elements in the *SOST* genomic region. In the future, more experiments are needed to gain a functional understanding of the regulation of sclerostin expression.

4. Materials and Methods

4.1. Cell Culture

The human osteosarcoma cell line Saos-2 was used for luciferase reporter assays, western blot experiments and 4C-seq assays. It was obtained from the American Type Culture Collection (ATCC® HTB-85™) and grown in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich), supplemented with 10% Fetal Bovine Serum (FBS; Gibco, Life Technologies) and 1% penicillin/streptomycin (p/s; Gibco, Life Technologies), at 37°C and 5% of CO₂. Human fetal osteoblast 1.19 (hFOB) cell line and human medulla-derived mesenchymal stem cells (MSCs) were used for 4C-seq assays. The hFOB 1.19 cell line was obtained from ATCC (ATCC® CRL-11372™) and grown in DMEM: F12 (1:1) medium without phenol red (Gibco, Life Technologies), supplemented with 10% FBS and 0.3 mg/ml Geneticin (Gibco, Life Technologies), at 34°C and 5% of CO₂. MSCs were kindly provided by Dr. José Manuel Quesada Gómez, from Instituto Maimónides de Investigación Biomédica, Hospital Universitario Reina Sofía, Córdoba, Spain [56]. These cells were grown in alpha-MEM medium (Gibco, Life Technologies), supplemented with 10% FBS, 1% p/s and 1x Glutamax (Gibco, Life Technologies), at 37°C and 5% of CO₂.

4.2. Luciferase reporter constructs, *SOST* expression vectors and site-directed mutagenesis

The 520-bp fragment of the *SOST* upstream region (chr17:41,836,117-41,836,636, GRCh37/hg19) was PCR-amplified and cloned into the pGL3 basic Luciferase Reporter Vector (Promega). Constructs harboring the two alleles of the rare variant rs570754792 were cloned using the KpnI and BglII restriction sites (Primers used: ATACTAGGTACCGTCAAACAGAAACGCCTT and TATCTTAAGATCTCTTCCAGTAGCACAGGC). The *SOST*/pcDNA3.1+ expression vector was a gift from Xi He (Addgene plasmid # 10842; <http://n2t.net/addgene:10842>; RRID: Addgene_10842). The missense mutations p.Val10Ile, p.Arg19His, p.Val21Leu (positive control), p.Ala28Val, p.Asn53del, p.Pro111Gln, p.Gly150Arg and p.Cys167Arg (positive control) were introduced into the expression vector using the QuickChange Site-Directed Mutagenesis kit (Agilent), following the manufacturer's instructions. In all cases, the presence of point mutations and absence of errors were verified through Sanger sequencing.

4.3. Luciferase gene reporter assay

For the luciferase gene reporter assay, 3x10⁵ Saos-2 cells per well were cultured in 6-well plates, 24h before the transfection. We transfected the cells either with the empty pGL3 basic vector (negative control), with the *SOST*-upstream region with each of the two alleles, or with the basal promoter of *COL1A1* (positive control from [48]). The total amount of DNA transfected per well was 2.2 µg. Fugene HD was used following the manufacturer's instructions. Forty-eight hours after transfection, cells were lysed and the luciferase activities of Photinus pyralis and Renilla reniformis were measured using a Glomax Multi+ luminometer (Promega), following the instructions of the Dual-luciferase reporter assay system (Promega). Each experiment included 3 replicates and was repeated independently in 3 separate experiments. The ratio of Firefly and Renilla luciferase measurements was calculated (relative luciferase units; RLU). All luciferase data were normalized using the empty vector activity (pGL3-basic), and expressed as mean values ± SD. The differences between the two alleles were evaluated using the Welch Two Sample t-test, and the homogeneity of variances was confirmed using Bartlett test and normality was confirmed using Shapiro test. Statistical significance was set at p-value<0.05. The statistical evaluation was carried out with Rstudio Version 3.6.1. The representations were performed using Graphpad (Graphpad Prism 8).

4.4. Western blot assay

For the western blot assay, 3×10^5 Saos-2 cells per well were cultured in 6-well plates, 24h before transfection. We transfected 2 μ g of the *SOST* expression plasmid (WT or any of the missense variants: p.Val10Ile, p.Arg19His, p.Val21Leu, p.Ala28Val, p.Asn53del, p.Pro111Gln, p.Gly150Arg and p.Cys167Arg). For the negative control, we transfected 2 μ g of the empty pcDNA3 vector (data not shown). Fugene HD was used following the manufacturer's instructions. After 24h, the medium was changed, reducing from 2 to 1 ml of DMEM, without FBS or antibiotics. Forty-eight hours after transfection, the supernatant (conditioned medium) of each condition was collected and the cells were lysed with RIPA buffer. Proteins in the conditioned medium were concentrated using 10K Amicon Ultra filters (Millipore). Extracellular proteins (concentrated conditioned medium) and intracellular proteins (RIPA extraction) were quantified using the PierceTM BCA Protein Assay Kit (ThermoFisher). Proteins from concentrated conditioned medium (10 μ g/lane) and intracellular proteins (5 μ g/lane) were separated by electrophoresis in an SDS-polyacrylamide gel (SDS-PAGE) and transferred to a hydrophobic PVDF transfer membrane (Millipore). We used the ab63097 antibody against sclerostin (Abcam) and antibody against Tubulin (Abcam) was used as a loading control. The images were developed using a peroxidase-conjugated secondary antibody (Anti-Rabbit IgG (A0545) for sclerostin antibody and Anti-Mouse IgG (A5906) for α -Tubulin antibody (Sigma-Aldrich). For the extracellular protein, samples were obtained from 3 biological replicates that were quantified twice by BCA. Each one of these quantifications was used to perform an SDS-PAGE experiment, producing a total of 2 experiments for each biological replica. For the intracellular protein, samples were obtained from 3 biological replicates and quantified once by BCA. We used the ImageJ 1.52a software to quantify the intensity of the bands and the GraphPad 8 software for the graphical representation.

4.5. PCR amplification and sequencing.

Resequencing of *SOST* and the *SOST* enhancer *ECR5* was performed in a small cohort of 11 HBM women described elsewhere [50]. Specifically, we resequenced the complete coding region, the 5' and 3' UTR regions and the intronic flanking regions of *SOST*, in addition to 1 Kb of the *SOST* proximal promoter and the *ECR5* enhancer region. PCR-amplification of each of these fragments was performed using the GoTaq Flexi DNA polymerase (Promega). The PCR fragments were analyzed by agarose gel electrophoresis and purification was done in MultiScreenTM Vacuum Manifold 96-well plates (Merck Millipore). The purified PCR products were sequenced by the Sanger method in the CCiTUB genomic service (Genómica, Parc Científic, Barcelona, Spain), using the BigDyeTM Terminator v3.1 Cycle Sequencing Kit. Detection and electrophoresis were performed on automated capillary sequencer models 3730 Genetic Analyzer and 3730xl Genetic Analyzer. The sequence of the primers and the conditions used were previously described [47].

4.6. Bioinformatic Analysis and in silico Predictions of the Effect of the Variants.

To identify and characterize all the variants, we used information from the Ensembl database GRCh37.p13 (<http://www.ensembl.org>). Information was sought on the MAF of each of the variants found in the European population (EUR) and in the Iberian Peninsula (IBS). We used SIFT (<http://sift.bii.a-star.edu.sg/>), PolyPhen (<http://genetics.bwh.harvard.edu/pph2/>), CADD (<https://cadd.gs.washington.edu/>) and REVEL [57] to test the effect of the missense variants. The SNP Function Prediction (snpinfo.niehs.nih.gov/snpinfo/snpfunc.html), MirSNP (<http://bioinfo.bjmu.edu.cn/mirsnp/>), PolymiRTS Database 3.0 (<http://compbio.uthsc.edu/miRSNP/search.php>) and MicroSNiPer (<http://vm24141.virt.gwdg.de/services/microsniper/>) were used to analyse the binding of miRNAs to the variants in the 3'UTR region. Only those results that coincided with at least two different programs have been considered. The GTEx database (www.gtexportal.org/home/) was used to identify variants that act as eQTLs. Human Splicing Finder (umd.be) and SplicePort (<http://spliceport.cbcb.umb.edu/>) were used to evaluate the predicted splicing sites. The SNP Function Prediction (snpinfo.niehs.nih.gov/snpinfo/snpfunc.html) was used to predict transcription factor binding sites

(TFBS) and new exonic splicing enhancers (ESE). To characterize regions that include the variants found, the UCSC Genome Browser GRCh37/hg19 was enriched with ENCODE data from osteoblasts.

4.7. 4C-seq

4C-seq was carried out at the Functional Genomics Service of the Centro Andaluz de Biología del Desarrollo (CABD, Sevilla, Spain). 4C-seq libraries were generated from Saos-2, hFOB 1.19 and hMSC cell lines as described previously [58,59]. Four-bp cutters were used as primary (DpnII) and secondary (Csp6I) restriction enzymes. For each cell line, a total of 1.6 µg of library was PCR amplified (primers used: CCTGCTGTGACATCTTCTGC and CCATTCAGAGGGGTGTGGATC). Samples were sequenced with Illumina Hi-Seq technology according to standard protocols at the Genomics Service of the Centro Nacional de Investigaciones Cardiovasculares (CNIC, Madrid, Spain). 4C-seq data were generated as described previously [60]. Briefly, raw sequencing data were demultiplexed and mapped to the corresponding reference genome (GRCh37). Reads located in fragments flanked by two restriction sites of the same enzyme, in fragments smaller than 40 bp or within a window of 10 kb around the viewpoint were filtered out. 4C-seq data were normalized by the total weight of reads within ±2 Mb around the viewpoint.

4C-seq data were analyzed with the AFourC software (Martínez-Gil N, Herrera C, Gritti N, Roca-Ayats N, Ugartondo N, Garcia-Giralt N, Ovejero D, Nogués X, Garcia-Fernandez J, Grinberg D & Balcells S, submitted) following and adapting previously described pipelines [61,62]. Briefly, we assumed that the 4C signal profile relative to the viewpoint v with coordinate xv on chromosome N is formed by three independent contributions: a constant background level, a negative exponential representing the monotonic decay of the 4C signal with the genomic distance from the viewpoint [62] and N gaussians representing the interaction peaks:

$$S_{4C}(x) = B + I_{x_v} \cdot e^{-(x-x_v)/\lambda} + \sum_{i=1}^N P(x_i) \cdot e^{-(x-x_i)^2/2 \cdot \sigma^2}$$

To estimate the genomic distance-dependent monotonic decay, we assumed a symmetric trend around the viewpoint and performed the exponential fit on the left-right averaged profile. Statistically significant peaks were detected using a p-value of 0.0005. The software package is publicly available at <https://github.com/Nikoula86/AFourC.git>.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/xxx/s1.

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Abbreviations

BMD Bone Mineral Density

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Supplementary information:

Article

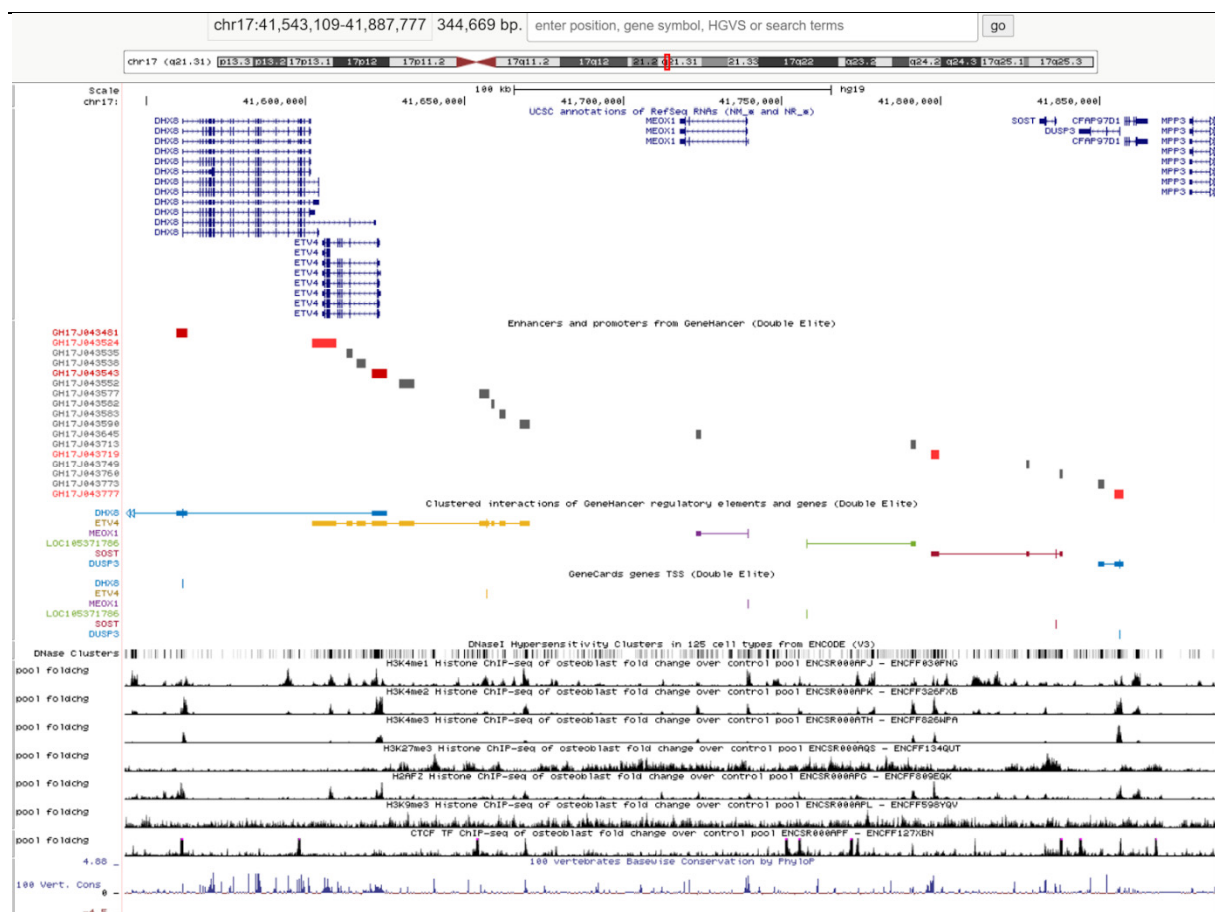
Genetics and genomics of SOST: functional analysis of variants and genomic regulation in bone cells

Núria Martínez-Gil ¹, Neus Roca-Ayats ¹, Mónica Cozar ¹, Natàlia Garcia-Giralt ², Diana Ovejero ², Xavier Nogues ², Daniel Grinberg ¹, Susanna Balcells ^{1*}

- 1 Department of Genetics, Microbiology and Statistics, Faculty of Biology, Universitat de Barcelona, CIBERER, IBUB, IRSJD, Barcelona, Spain.²
- 2 Musculoskeletal Research Group, IMIM (Hospital del Mar Medical Research Institute), Centro de Investigación Biomédica en Red en Fragilidad y Envejecimiento Saludable (CIBERFES), ISCIII, Barcelona, Spain.

* Correspondence: sbalcells@ub.edu

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Suppl. Figure 1. Schema of *SOST* genomic region from the UCSC genome browser with the following tracks (downwards): In red, the promoter regions and in grey the enhancer regions according to GeneHancer track. In different colors, the interaction regions according to GeneHancer track and the TSS for each gene. In black, osteoblast ChIP-seq profiles of H3K4me1, H3K4me3, H3K4me2, CTCF, H3K27ac, H3K9me3 and H2A.Z from ENCODE. In blue, vertebrate conservation.



Suppl. Figure 2. Study of the rs552004150 variant. (A) HBM19 family pedigree, in which 2 individuals (HBM16 and HBM16a) with the HBM phenotype are shown with filled symbols and with the SNP genotype; family members with no information on the bone density phenotype are displayed in white. (B) TRANSFAC result of the *ECR5* region. The position of the variant is indicated with a vertical arrow.

Suppl. Table 1. GTEx eQTL data for all variants found in the resequencing of *SOST*

SNP	Gene	Tissue
rs1237278	<i>SOST</i>	artery-tibial, aorta, coronary; lung; heart-atrial appendage; brain-cortex
	<i>MPP3</i>	thyroid
	<i>NAG3</i>	skin-sun exposed
rs851058	<i>SOST</i>	artery-tibial, aorta, coronary; lung; heart-atrial appendage; thyroid
	<i>C17orf105</i>	brain-cerebellum
	<i>CD300LG</i>	muscle-skeletal
	<i>DUSP3</i>	cells-cultured fibroblasts; whole blood; esophagus-mucosa
rs10534024	<i>MPP2</i>	skin-sun and not sun exposed
	<i>MPP3</i>	cells-cultured fibroblasts
	<i>NAG3</i>	lung; skin sun exposed
	<i>SOST</i>	artery-tibial, aorta, coronary; lung; heart-atrial appendage; brain-cortex, nerve-tibial
rs17882143	<i>MPP3</i>	thyroid; testis
	<i>DBF4B</i>	brain-caudate; thyroid
	<i>RPL27</i>	artery-aorta
rs17886183	<i>DUSP3</i>	whole blood

rs17881550	<i>SOST</i>	artery-tibial, aorta, coronary; heart-atrial appendage; lung; thyroid; nerve-tibial
	<i>AOC2</i>	lung
	<i>C17orf105</i>	brain-cerebellum
	<i>CD300LG</i>	muscle-skeletal
	<i>DUSP3</i>	esophagus-mucosa
	<i>MPP2</i>	skin-sun and not sun exposed
	<i>NAGS</i>	skin-sun exposed; lung

Suppl. Table 2. *In silico* predictions for the SNP in *SOST*

SNP	Prediction	Allele	
rs17885799	ESE	C	Two new Exonic Splicing Enhancer (ESE)
miRNA			
rs17883310	miRNA	C	hsa-miR-1915-3p hsa-miR-3141 hsa-miR-4685
rs17886183	miRNA	A	hsa-miR-5583-3p

ESE: New Exonic Splicing Enhancer predicted. miRNA: Differences in the miRNA binding site depending on the allele.

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Article 5

Regulation of *WNT16* in bone involves upstream enhancers within *CPED1*

Summary:

WNT16 stands up as an essential gene for bone homeostasis. Here, we present new evidence of the functional role of one *WNT16* region. Performing 4C chromatin conformation analysis in bone cells, we identify physical interactions between the proximal part of *WNT16* intron 2, shown here to be an active promoter in bone cells, and several putative regulatory regions within *CPED1*. Analysis of previously published RNA-seq data from hFOB cells disclosed low expression of a region located downstream of this promoter. Our results suggest a novel regulatory mechanism of *WNT16* in bone, mediated by physical interaction with various enhancer regions within *CPED1*.

Reference:

Núria Martínez-Gil, Carlos Herrera-Ubeda, Nicola Gritti, Neus Roca-Ayats, Nerea Ugartondo, Natàlia Garcia-Giralt, Diana Ovejero, Xavier Nogués, Jordi Garcia-Fernàndez, Daniel Grinberg, Susanna Balcells. Regulation of *WNT16* in bone involves upstream enhancers within *CPED1*. To be submitted to *Genome Biology*.

Regulation of *WNT16* in bone involves upstream enhancers within *CPED1*

Martínez-Gil N¹, Herrera-Ubeda C¹, Gritti N², Roca-Ayats N¹, Ugartondo N¹, Garcia-Giralt N³, Ovejero D³, Nogués X³, Garcia-Fernàndez J¹, Grinberg D¹, Balcells S^{1*}

¹ Department of Genetics, Microbiology and Statistics, Faculty of Biology, Universitat de Barcelona, CIBERER, IBUB, IRSJD, Barcelona, Spain.

² European Molecular Biology Laboratory (EMBL) Barcelona, 08003 Barcelona, Spain.

³ Musculoskeletal Research Group, IMIM (Hospital del Mar Medical Research Institute), Centro de Investigación Biomédica en Red en Fragilidad y Envejecimiento Saludable (CIBERFES), ISCIII, Barcelona, Spain.

Running title: WNT16 regulation through CPED1 enhancers

Corresponding author:

Susanna Balcells: sbalcells@ub.edu.

ABSTRACT

WNT16 stands up as an essential gene for bone homeostasis. Here, we present new evidence of the functional role of one *WNT16* region. Performing 4C chromatin conformation analysis in bone cells, we identify physical interactions between the proximal part of *WNT16* intron 2, shown here to be an active promoter in bone cells, and several putative regulatory regions within *CPED1*. Analysis of previously published RNA-seq data from hFOB cells disclosed low expression of a region located downstream of this promoter. Our results suggest a novel regulatory mechanism of *WNT16* in bone, mediated by physical interaction with various enhancer regions within *CPED1*.

Key words: Wnt pathway, Osteoblasts, Genetic research, Regulatory elements

INTRODUCTION

In recent years, genome-wide association studies (GWAS) have been widely used for the identification of genes involved in complex diseases. In particular, up to 40 GWAS have been published identifying more than 500 genetic *loci* associated with bone parameters ⁽¹⁻³⁾. However, these studies are limited in several crucial ways. First, it is possible that the hit, which is identified by the lowest p-value, is not the causal SNP. In fact, all the regions in linkage disequilibrium with the hit SNP are candidates to bear the causal variant/s. Second, the vast majority of associated SNPs are found in intergenic or non-coding regions, which makes functional tests difficult. Third, the gene responsible for the association is not always the closest gene. For instance, if the SNP is in a regulatory region it is possible that it regulates the expression of a gene that is many kb away. For these reasons, an in-depth functional study of associated variants is necessary for the identification of the effector gene, which can be of great help to reveal new therapeutic targets for treatment of common diseases, such as osteoporosis, the most common metabolic bone disorder. However, despite the large number of genetic *loci* identified, very few of them have been functionally characterized ⁽⁴⁻⁶⁾.

One of the most consistent “bone-GWAS” signals is the genomic region of *WNT16*, which has been associated with skeletal phenotypes ^(1-3,6-21). In humans, the *WNT16* protein has two alternative isoforms, *WNT16a* and *WNT16b*, and within bone cells is predominantly expressed in early osteoblasts ^(22, 23). A regulatory role of *WNT16* on bone has been confirmed by the analysis of Knock out (KO) and osteoblast-specific conditional KO mouse models, which showed spontaneous fractures as a result of low BMD and reduced bone strength ^(8,9,23). On the other hand, the transgenic overexpression of *Wnt16* in mouse osteoblasts results in higher BMD and bone resistance ⁽²⁴⁻²⁶⁾.

Given the contribution of *WNT16* to bone strength and its potential role as a therapeutic target for osteoporosis, it is essential to perform a comprehensive functional characterization and better understand its regulation. To this aim, we previously characterized the region corresponding to the *WNT16* rs3801387 GWAS sentinel signal ⁽⁷⁾, re-sequencing the *WNT16* gene in individuals with extreme BMD phenotypes. Next, we genotyped selected variants in a larger cohort ⁽²⁷⁾. As a result,

we found two regulatory variants associated with lumbar spine BMD and in high linkage disequilibrium with the GWAS sentinel SNP (rs3801387)⁽²⁷⁾. One of them affects the Kozak sequence and has already been functionally studied ⁽⁴⁾, while the other (rs142005327) is located in intron 2, overlapping a putative regulatory marks in primary osteoblasts (ENCODE).

RESULTS AND DISCUSSION

In the present work, we set out to perform a functional analysis of the *WNT16* intron 2 region containing the variant rs142005327 (Figure 1A, blue dot). To this aim, we focused on seven fragments in the intron 2 that show epigenetic marks typical of regulatory elements (Figure 1 B). In particular, this region shows hypersensitivity to DNase I, enrichment of transcription factor binding sites and a CpG island (Figure 1C). Moreover, despite at lower levels compared to the exons, analysis among vertebrates shows a conservation peak within intron 2 (Figure 1C). Interestingly, this region contains post-translational modifications of the histones in osteoblasts typical of promoters or enhancers, for example low H3K4me1/H3K4me3 ratio or H2AFz (Figure 1D). Accordingly, this region is recognized by GeneHancer as part of a larger promoter element containing the *WNT16b* canonical promoter. Similarly, it is classified as several enhancers regions, distinct from the *WNT16b* promoter, by ENCODE Registry of candidate cis-Regulatory Elements (cCREs; Figure 1E). To unambiguously determine the type of regulatory element of this region, we use a luciferase reporter assay and we tested the seven fragments for promoter or enhancer activities in Saos2 cells. None of the fragments tested displayed any relevant enhancer activity (Supplementary Figure 1). In contrast, five of them showed promoter activity (Figure 1B), with PC construct displaying the highest luciferase signal (6.3x fold compared to empty vector). On the other hand, PA, the smallest fragment containing rs142005327, failed to show promoter activity on its own. The *COL1A1*-promoter (SP construct in ²⁸), used as a positive control, showed 30.5x fold promoter activity compared to the empty vector (data not shown). Consistently, transcription factor ChIP-seq data from ENCODE and TRANSFAC data show that many transcription factors are predicted to bind to the C fragment (Supplementary Figure 2). The C region contains the variant rs73440215, which is in high linkage disequilibrium with the GWAS sentinel SNP and

has been shown to be associated with heel BMD ⁽³⁾. The promoter activity of the C fragment suggests that this variant could be the one responsible for the association with BMD and fracture risk.

Knowing that the C region is an active promoter in bone cells, we next decided to investigate whether the region immediately downstream from it is expressed in different cell types. To this aim, we consulted available RNA-seq data acquired from different tissues (Sequence Read Archive; SRA). Out of this large dataset, we found few reads that mapped to *WNT16* intron 2 restricted to hFOB cell types exclusively (Figure 1F and Supplementary Figure 3A). None of these reads is connected with the exon 3, which is consistent with the hypothesis that they come from enhancer/promoter RNA or other non-coding RNAs. Nevertheless, when translating the expressed region into protein and aligning it to all protein sequences with BLAST, we found a unique match with a Wnt16 protein in microbat. In contrast with the previous hypothesis, this result is consistent with the existence of an isoform of *WNT16* that uses the C region of intron 2 as promoter. After further analysis, we unexpectedly but excitingly found that the bat protein is formed by a first exon strikingly similar to the first exon of the putative isoform (Supplementary Figure 3B). The microbat protein is completed by 2 more exons that correspond to exons 3 and 4 of the human *WNT16*. These results confirm that, at least in one other species, a protein containing the amino acids coded by this previously undescribed human exon exists. In the future, experiments using different cell lines and developmental stages, will be crucial to elucidate whether such an isoform exists and, if appropriate, its role in humans.

To determine which regions might regulate the activity of this promoter, we performed a 4C assay on three relevant osteoblast-related cell types (Human fetal osteoblasts – hFOB-, an osteosarcoma cell line –Saos2-; and mesenchymal Stem Cells –MSC-) using the *WNT16* intron 2 region (*WNT16*-In2; chr7:120970581-120971655, GRCh37/hg19) as a viewpoint (Figure 2A and Supplementary Figure 4). To confirm the interactions, we performed four additional 4C-seq assays. Three of them were chosen using the regions with the strongest interaction with *WNT16*-intron 2 as viewpoints, namely the *CPED1* main promoter (*CPED1*-MP), the region between *CPED1* exons 11 and 12 (*CPED1*-R-11-12) and the region containing *CPED1* exon 17 (*CPED1*-R-17). Additionally, due to the important function of *FAM3C* in bone, we

chose a fourth viewpoint at the *FAM3C* main promoter (FAM3C-MP). The reciprocal 4C-seq assays confirmed the interactions with CPED1-R-11-12 and CPED1-R-17 in the three cell types used and with CPED1-MP for Saos2 and hFOB cells (black/grey arches in Figure 2A, see Methods). Among them, WNT16 and CPED1-R-11-12 showed the strongest interaction (Figure 2A, solid black arch) and was previously identified as an active promoter in the pre-osteoblast MC3T3-E1 cell line ⁽²⁹⁾. Unfortunately, we did not have sufficient MSC to test the CPED1-MP and FAM3C viewpoints, therefore we could not test these interactions for the MSC cell type. According to the Genenhancer database, both CPED1-R-11-12 and CPED1-R-17 are described as enhancers, while CPED1-MP and WNT16-In2 are described as promoters (Figure 2C). In addition, for every cell line and viewpoint, we have identified several non-reciprocal interactions (blue arrows in Figure 2A). Among them, we found interactions in the three cell types between the WNT16-In2 viewpoint and a genomic region within *CPED1* (exon 22-23; CPED1-R-22-23) that is described as a regulatory element (vertical dashed line in Figure 2A). Moreover, our data show that *FAM3C* does not strongly interact with either *CPED1* or *WNT16* (Figure 2A). Our results are in contrast with those of the GeneHancer database, which only includes either interactions between the main promoter of *CPED1* and some of the regulatory elements included in it, or between *WNT16* and a region located between the two genes (Supplementary Figure 5). The interaction between *CPED1* and *WNT16* that we report here is novel and may be bone cell type-specific. Despite these results, the resolution of the technique does not allow to discern whether the physical interaction with several regulatory elements of *CPED1* occurs with the alternative promoter described here or through the canonical promoter of *WNT16b*. In the future, studies at higher resolution or *in vivo* will be necessary to determine the interacting region precisely.

Taken together, our results demonstrate that the alternative promoter region of *WNT16* is physically interacting with *CPED1*. It is still an open question whether the *WNT16* region is regulating *CPED1* expression, or vice versa. However, previous studies have demonstrated that Wnt16-KO, including the alternative promoter region, does not affect *CPED1* expression in mouse models ⁽²³⁾. Therefore, the interaction observed here is likely due to a regulatory role of *CPED1* on *WNT16* expression. To

unambiguously demonstrate this regulation it will be necessary to establish a *CPED1*-KO model and quantify the expression of *WNT16* in bone cells.

The variants in the alternative promoter region of *WNT16* may be partially responsible for the association of this *locus* with BMD in GWAS. However, we cannot exclude the involvement of other genes in this association. For instance, Chesi *et al.* (2019) ⁽⁶⁾ performed a genome-wide promoter-focused Capture C and found that another gene in the region, *ING3*, plays an important role in the MSC cell fate decision to osteoblast lineage, which could synergize with *WNT16* functions. Interestingly, although these authors did not find looping between the SNP rs142005327 and putative enhancers in regions of open chromatin, the interacting pattern that they described for this *WNT16* promoter region is very much comparable with the one we present here, which essentially includes enhancers within *CPED1*. Chesi *et al.* ⁽⁶⁾ used only primary human MSC-derived osteoblasts, while we have used cell types at different stages along the differentiation path, from MSCs to terminally differentiated osteoblasts (Saos2). In the future it will be important to further dissect this regulatory region, for instance by studying different *in vivo* models.

CONCLUSION

In the present work we have performed a functional study of one region within *WNT16*, which harbours major consistent BMD/fracture GWAS signals. We have demonstrated the promoter activity of part of intron 2, and its physical interaction, in bone-related cells, with different putative regulatory regions within *CPED1*, including the main *CPED1* promoter. Furthermore, using previously published RNA-seq data from hFOB cells, we observed low levels of expression of a region at 3' of the intron 2 *WNT16* promoter. Our results suggest a novel regulatory mechanism of *WNT16* in bone cells that is mediated by physical interaction with various *CPED1* regions. Considering the important effect of *WNT16* in BMD determination, the characterization and evaluation of regulatory elements could be translated into therapeutic strategies to treat bone related disorders, such as osteoporosis.

MATERIAL AND METHODS

Cell Culture

The human osteosarcoma cell line Saos2 was used for luciferase reporter assay and 4C-seq assays. It was obtained from the American Type Culture Collection (ATCC® HTB-85™) and grown in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich), supplemented with 10% Fetal Bovine Serum (FBS; Gibco, Life Technologies) and 1% penicillin/streptomycin (p/s; Gibco, Life Technologies), at 37°C and 5% of CO₂. Human foetal osteoblasts 1.19 (hFOB) and human medulla-derived mesenchymal stem cells (MSCs) were used for 4C-seq assays. The hFOB 1.19 cell line was obtained from ATCC (ATCC® CRL-11372™) and grown in DMEM:F12 (1:1) medium without phenol red (Gibco, Life Technologies), supplemented with 10% FBS and 0.3 mg/ml Geneticin (Gibco, Life Technologies), at 34°C and 5% of CO₂. MSCs were kindly provided by Dr. José Manuel Quesada Gómez, from Instituto Maimónides de Investigación Biomédica, Hospital Universitario Reina Sofía, Córdoba, Spain ⁽³⁰⁾. These cells were grown in alpha-MEM medium (Gibco, Life Technologies), supplemented with 10% FBS, 1% p/s and 1x Glutamax (Gibco, Life Technologies), at 37°C and 5% of CO₂.

Luciferase reporter constructs

We cloned seven fragments (Full, Inv, 1, 2, A, B and C) of the WNT16-Intron 2 proximal region into the pGL3-promoter reporter vector (Promega) to create constructs EFull, EInv, E1, E2, EA, EB and EC, respectively, and into the pGL3-basic reporter vector (Promega) to create constructs PFull, PInv, P1, P2, PA, PB and PC, respectively (see Figure 1B). All fragments were cloned using the restriction enzymes XhoI and KpnI. The Full and Inv fragments correspond to the same region but in the opposite orientation. The size, genomic position, and primers used for cloning each region are specified in Supplementary Table 1. In all cases, the presence of the desired mutations and the absence of errors were verified by Sanger sequencing.

Luciferase gene reporter assays

We performed a luciferase gene reporter assays to test the regulatory function of the WNT16-Intron 2 region (regulatory reporter assay). For the regulatory reporter assay, 1.1 x10⁵ Saos2 cells per well were cultured in 12-well plates, 24h before the transfection. Each construct (empty, test fragment or positive control) was individually

co-transfected, at a 10:1 proportion, with pRL-TK (total DNA=1.1 µg). For enhancer assays, the empty vector was the pGL3-promoter backbone (Promega), test all the fragments and the SV40 enhancer was used as a positive control. For promoter assays, the empty vector was the pGL3-basic backbone (Promega), test all the fragments and the COL1A1-promoter (SP construct in ²⁸) was used as a positive control. Eugene HD was used following the manufacturer's instructions. Forty-eight hours after transfection, cells were lysed, and the luciferase activity of *Photinus pyralis* and *Renilla reniformis* was measured using a Glomax Multi+ luminometer (Promega), following the instructions of the Dual-luciferase reporter assay (Promega). Each experiment included three replicates and was repeated independently in three separate experiments.

4C-seq and AFourC software

4C-seq was carried out at the Functional Genomics Service of the Centro Andaluz de Biología del Desarrollo (Sevilla, Spain). 4C-seq libraries were generated from Saos2, hFOB 1.19, and MSC cell lines as described previously ^(31,32). Four-bp cutters were used as the primary (DpnII) and secondary (Csp6I) restriction enzymes. For each cell line, a total of 1.6 µg of the library was PCR amplified for 6 different viewpoints (Primers and coordinates in supplementary Material Table 2). Samples were sequenced with Illumina Hi-Seq technology according to standard protocols at the Genomics Service of the Centro Nacional de Investigaciones Cardiovasculares (CNIC, Madrid, Spain). 4C-seq data were analyzed as described previously ⁽³³⁾. Briefly, raw sequencing data were demultiplexed and mapped to the corresponding reference genome (GRCh37). Reads located in fragments flanked by two restriction sites of the same enzyme, in fragments smaller than 40 bp or within a window of 10 kb around the viewpoint, were filtered out. 4C-seq data were normalized by the total weight of reads within ± 2 Mb around the viewpoint.

4C-seq data were analyzed following and adapting previously described pipelines ⁽³⁴⁻³⁶⁾. Briefly, we assumed that the 4C signal profile relative to the viewpoint v with coordinate x_v on chromosome N is formed by three independent contributions. First, a constant background level common to all the chromosomes. Second, a negative exponential representing the monotonic decay of the 4C signal with the genomic distance from the viewpoint ⁽³⁴⁾. Third, N gaussians with maximum values P_i , widths σ_i

and centered around the coordinates x_i , representing the peaks obtained from the genomic regions that are in physical contact with the viewpoint v :

$$S_{4C}(x) = B + S_{4C}(x_v) \cdot e^{-(x-x_v)/\lambda} + \sum_{i=1}^N P(x_i) e^{-(x-x_i)^2/(2\sigma^2)}$$

To obtain the parameters of the 4C profile for a given chromosome N and viewpoint v , we first estimated the background level B by computing the mean of the signal above the 99th percentile excluding the chromosome of interest. We then estimated the minimum detectable peak prominence as 5% of the maximum value of the 4C profile on the chromosome of interest. Next, to estimate the genomic distance-dependent monotonic decay, we assumed a symmetric trend around the viewpoint and performed the exponential fit on the left-right averaged profile (Supplementary Figure 6). To find the profile peaks, we smoothened the curve with a moving average of window size 30 reads and found all local maxima. To find only statistically significant targets, we rejected peaks based on three criteria. First, using the exponential fit and its error, we estimated the residual of each peak from the mean and set a p-value threshold of 0.001. Next, to remove read errors and negligible peaks, we rejected peaks belonging to gaussians with a width smaller than 16 reads and a maximum value smaller than the estimated minimum detectable peak prominence. Finally, to avoid interactions due to close contact, we rejected peaks closer than 35 Kb from the viewpoint. With this method, we obtain statistically significant interaction for every viewpoint and cell line present in the paper in an automated fashion.

To obtain the mutual interactions in a given cell line, we filtered for mutual interactions that started at a viewpoint v_1 and landed in a target within 20 Kb of a second viewpoint v_2 . Finally, we normalize the interaction strength to the maximum interaction strength in the cell line. Other (non-mutual) interactions in the highest quartile (more than 75% of the strongest interaction within the same cell line) are also shown in the plot.

All software used for the analysis of the 4C data was implemented in Python and developed into a graphical user interface (GUI). The software package and the 4C data files used to make the statistical analysis, are publicly available at <https://github.com/Nikoula86/AFourC.git>.

Statistical methods

The ratio of the Firefly and Renilla luciferase measurement was calculated (Relative luciferase units). All luciferase data were normalized using the empty vector activity (Pgl3-promoter, pgl3-basic), and expressed as mean values $\pm$ SD. In the luciferase assay the differences between conditions were evaluated using the Welch Two Sample t-test, as homogeneity of variances was rejected using Bartlett test. The p-value was adjusted using Bonferroni's correction. Statistical significance was set at p-value<0.05. The statistical evaluation was carried out with Rstudio Version 3.6.1. The representations were performed using the Graphpad (Graphpad Prism 8).

Bioinformatic Analysis

SRA data selection

In order to obtain as many reads as possible mapping to the second intron of *WNT16*, we selected 13 Sequence Read Archive (SRA) files from control experiments involving human osteoblast cells that had at least a blast hit against the intronic sequence. After mapping them, we restricted our focus to just the human foetal osteoblast (hFOB) cells and selected all the available SRA files from hFOB control experiments. The selected experiment IDs are:

SRR7939699, SRR7939693, SRR7939687, SRR7141491, SRR7141490,
SRR7141489, ERR2603574, ERR2603576, SRR5087318, SRR4450223,
SRR4450224, SRR4450225, SRR4450226, SRR1186922, SRR1186921,
SRR1186916, SRR1186915, SRR1186910, SRR1186909, SRR3631725,
SRR3631724, SRR3631723, SRR3631722, SRR3631721, SRR3631720,
SRR3631719, SRR3631718, SRR3631717, SRR3631716, SRR3631714

Due to the mixture of single and paired-end reads, we ran two independent mappings for each class of reads.

SRA mapping and data visualization

The reads from the SRA files were extracted with fastq-dump (v2.8.1) and mapped against the human assembly GRCh38 ⁽³⁷⁾ using STAR (v2.7.0d) ⁽³⁸⁾ with the default options. Using SAMtools (v1.9) ⁽³⁹⁾ we extracted the reads mapping to *WNT16* (chr7:121319704-121346940) and used IGV (v2.8.2)⁽⁴⁰⁾ to visualize the resulting

output. The *WNT16* gene models and conservation data were obtained from the UCSC tracks ⁽⁴¹⁾ GENCODE v32 and Cons_30_Mammals, respectively.

Hypothetical isoform reconstruction

The intronic region where most of the reads mapped (chr7:121,330,337-121,330,889) was both translated and analysed for splicing donor sequences using Human Splicing Finder ⁽⁴²⁾ in order to recreate the most viable exon. This putative alternative reconstructed isoform was then aligned using blastp against the *Myotis davidii* Wnt16 (ELK35449.1)

Tissue Specificity Assessment

To assess the specificity of the expression we used available mapped reads from human tissues deposited in the EMBL-EBI ArrayExpress database (experiment E-MTAB-6814) ⁽⁴³⁾. The organs selected were: brain, cerebellum, heart, kidney, liver, ovary and testis, and the developmental stages were (when available) adult (29 years post birth) and 18-19 weeks post conception.

Author's contributions: NMG, DO, XN, DG and SB were involved in the Conceptualization. NMG was involved in the Data curation, Formal analysis. NMG, NRA, NU and NGG were involved in Investigation and Methodology. The bioinformatics study of the RNA-seq in hFOB cells was performed by CHU and JGF. The AFourC software was performed by NG. DG and SB were involved in the Funding acquisition. The first draft of the manuscript was written by NMG, DG, SB and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Disclosures: The authors declare no conflicts of interest.

Data availability: All data and material will be available upon request.

Code availability: The software package and the 4C data files used to make the statistical analysis, are publicly available at <https://github.com/Nikoula86/AFourC.git>.

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FIGURES:

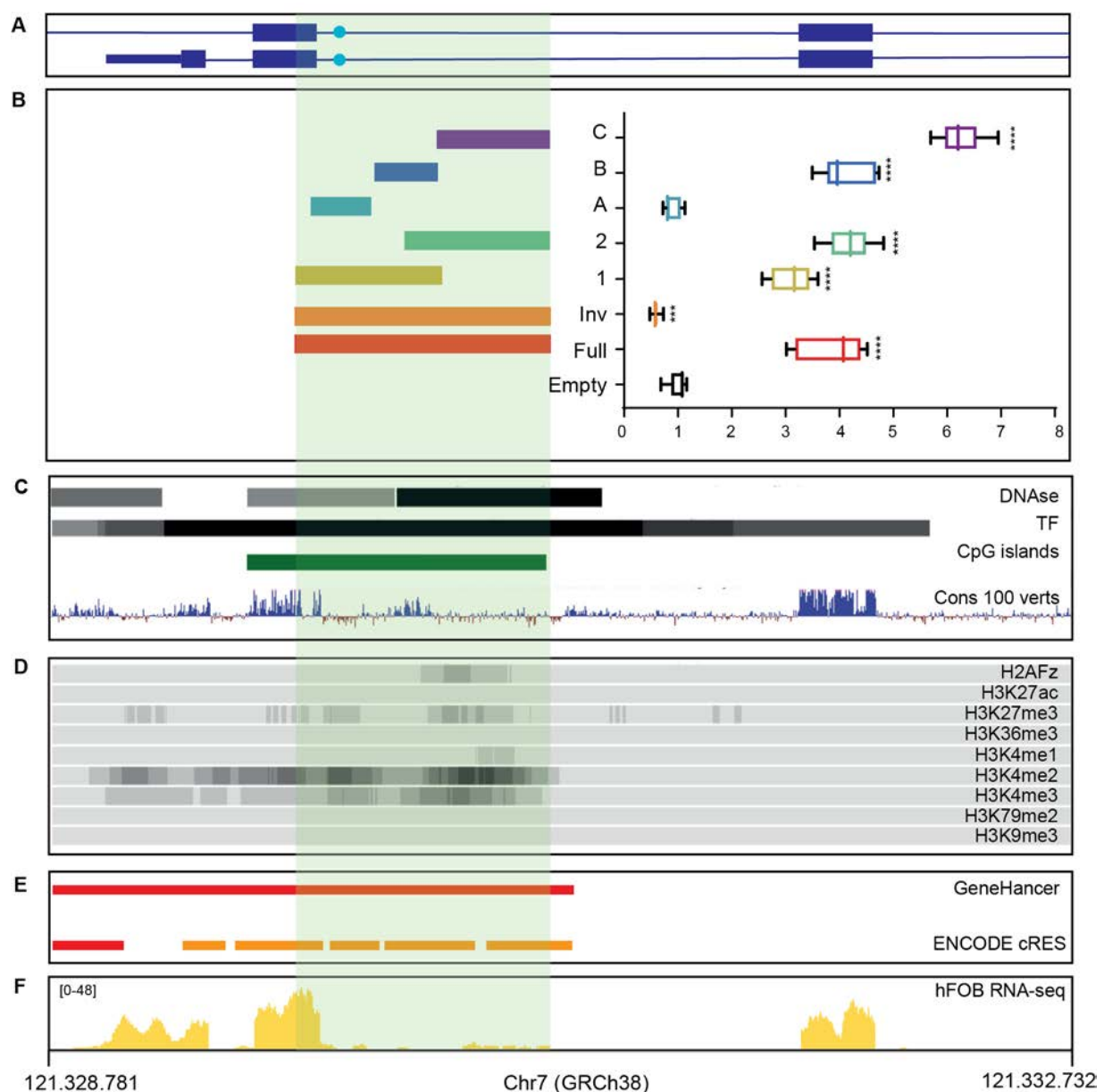


Figure 1. Characterization of a regulatory element in *WNT16* intron 2. **(A)** *WNT16* isoforms from UCSC annotations of RefSeq RNAs; light blue dots indicate the position of rs142005327 **(B)** Luciferase promoter assay of different *WNT16* intron 2 fragments. Normalized relative luciferase units (RLU; Ratio of *Photinus pyralis* and *Renilla reniformis* luciferase activities) of the different fragments cloned into the Pgl3-basic vector: Full, Ins, 1, 2, A, B, C (see details in methods). Values are normalized to the activity of the empty vector, arbitrarily set at 1. Error bars indicate s.d. ***p-value < 0.001; ****p-value < 0.0001. **(C)** DNase: Hypersensitivity to DNaseI (ENCODE project); TF: Transcription Factor ChIP-seq Clusters (ENCODE project); CpG islands from UCSC genome browser; Cons 100 Verts: Vertebrate Multiz Alignment & Conservation (100 Species). **(D)** Post-translational modifications of different histones in osteoblasts (ENCODE project). **(E)** GeneHancer database of human regulatory elements included in GeneCards and ENCODE Registry of candidate cis-Regulatory Elements (cCRES). **(F)** Expression levels of hFOB RNA-seq in SRA.

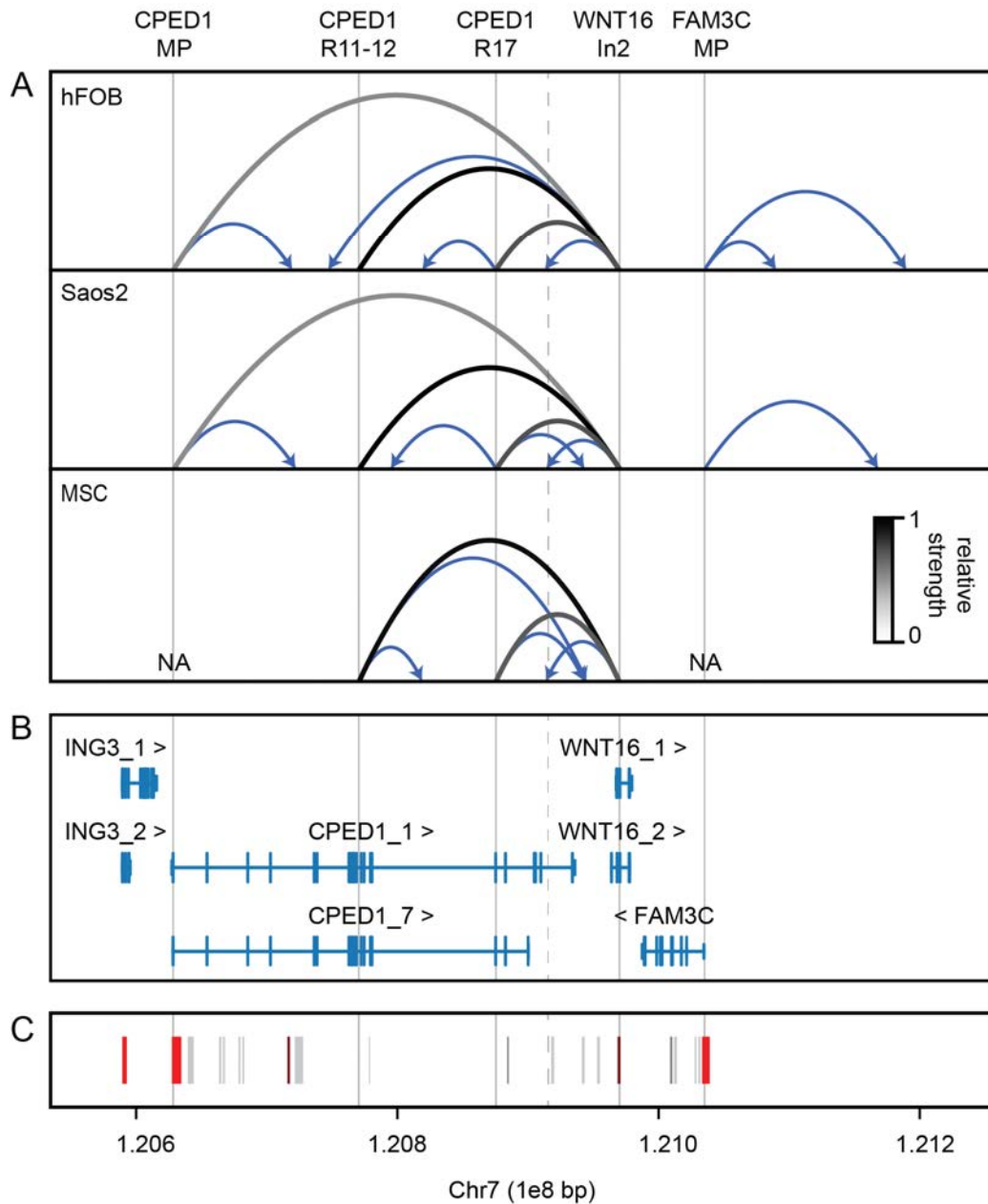


Figure 2. (A) Summary of results obtained in 4C-seq experiments using 5 viewpoints from the *CPED1-WNT16-FAM3C* region in human foetal osteoblasts (hFOB), Saos2 cells and mesenchymal stem cells (MSC). Observed contacts are depicted by arched lines. Grey scale arches represent reciprocal interactions and are color-coded according to the strength of the interaction (grey scale bar). Arrow-headed blue arches represent the non-reciprocal interactions with strength higher than 75% of the strongest interaction in the same cell line. Solid vertical lines represent the position of the viewpoints and the dashed line corresponds to the exon 22-23 *CPED1* region. (B) Displayed Ensembl/Havana genes in this region are *ING3_002* (ENST00000339121.5), *ING3_001* (ENST00000315870.5), *FAM3C*

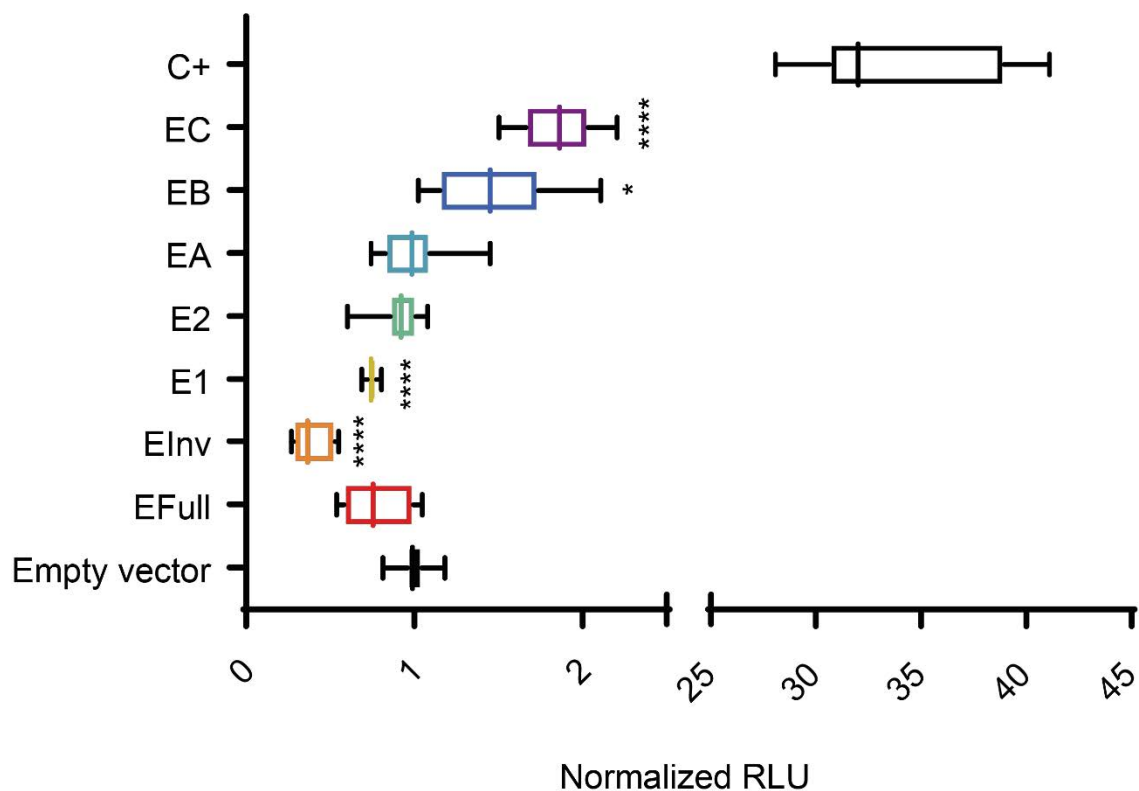
(ENST00000359943.3), WNT16_2 (ENST00000361301.2), WNT16_1 (ENST00000222462.2), CPED1_1 (ENST00000310396.5) and CPED1_7 (ENST00000450913.2) from GRCh37/hg19. (C) GeneHancer track from the UCSC genome browser, enhancers are shown in grey and promoters in red.

Supplementary information:**Table 1.** Coordinates (GRCh37/hg19) and primers used for cloning intron 2 fragments in luciferase reporter constructs

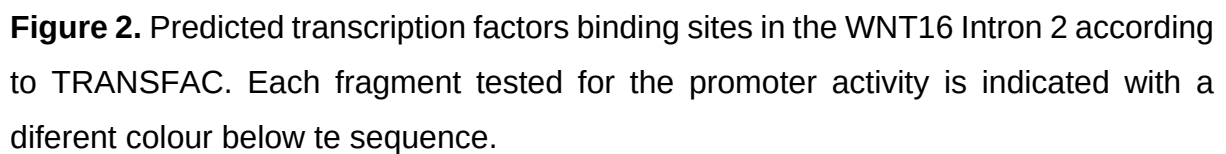
Fragments	Start	End	Forward	Reverse
Full	120969786	120970772	GGTACCCGAGAGAT GGAAGTGCATGA	CTCGAGTTTTCTCTCT ACGGGGACCA
Inv	120969786	120970772	CTCGAGCGAGAGAT GGAAGTGCATGA	GGTACCTTTTCTCTCT ACGGGGACCA
1	120969786	120970355	GGTACCCGAGAGAT GGAAGTGCATGA	CTCGAGTCTGTCAAC TCGGTGCTCCT
2	120970207	120970772	GGTACCGCTGGAGA AACATGATGTCG	CTCGAGTTTTCTCTCT ACGGGGACCA
VAR	120969846	120970079	GGTACCCCTCTTTG GCTACGAGCTGA	CTCGAGCTCTGGAAC TGGGGAGTCAC
CONS	120970092	120970339	GGTACCGGCTGGAG CTCTTCCTCTTT	CTCGAGTCCTAGGAG CCAATAAAGGTG
PIC	120970333	120970772	GGTACCCCTAGGAG CACCGAGTTGAC	CTCGAGTTTTCTCTCT ACGGGGACCA

Table 2. Coordinates (GRCh37/hg19) and primers for each viewpoint tested in the 4C-seq assay

Viewpoint	Start	End	Forward	Reverse
CPED1-MP	120628556	120630032	CCATGAGGTCT GAAAGGGATC	TAGGTCCCGAAAG TCTGAGG
CPED1-R-11-12	120771311	120771670	GGCAAATAAGG TGAATTCTGATC	GTAGTAGGCCAG CAGAAGG
CPED1-R-17	120875513	120877460	CCTAACCTCAGA TTCCCAGATC	GGTCAAATCCACT GTTTCTCTCC
WNT16-In2	120970581	120971655	CCAGTCCTAACA TTCTTTGGATC	GCCTTTAAGACTG TAAGAGGAGTAGC
FAM3C-MP	121035619	121036778	TGATGATTAGTG GCCCTAGATC	ACATCAAAGGGCT GGATAGC



Supplementary Figure 1. Luciferase enhancer assay of different WNT16 intron 2 fragments. Normalized relative luciferase units (RLU; Ratio of Photinus pyralis and Renilla reniformis luciferase activities) of the different fragments cloned into the Pgl3-promoter vector: Full, Ins, 1, 2, A, B, C (see details in methods). Values are normalized to the activity of the empty vector, arbitrarily set at 1. Error bars indicate s.d. ***p-value < 0.001; ****p-value < 0.0001.



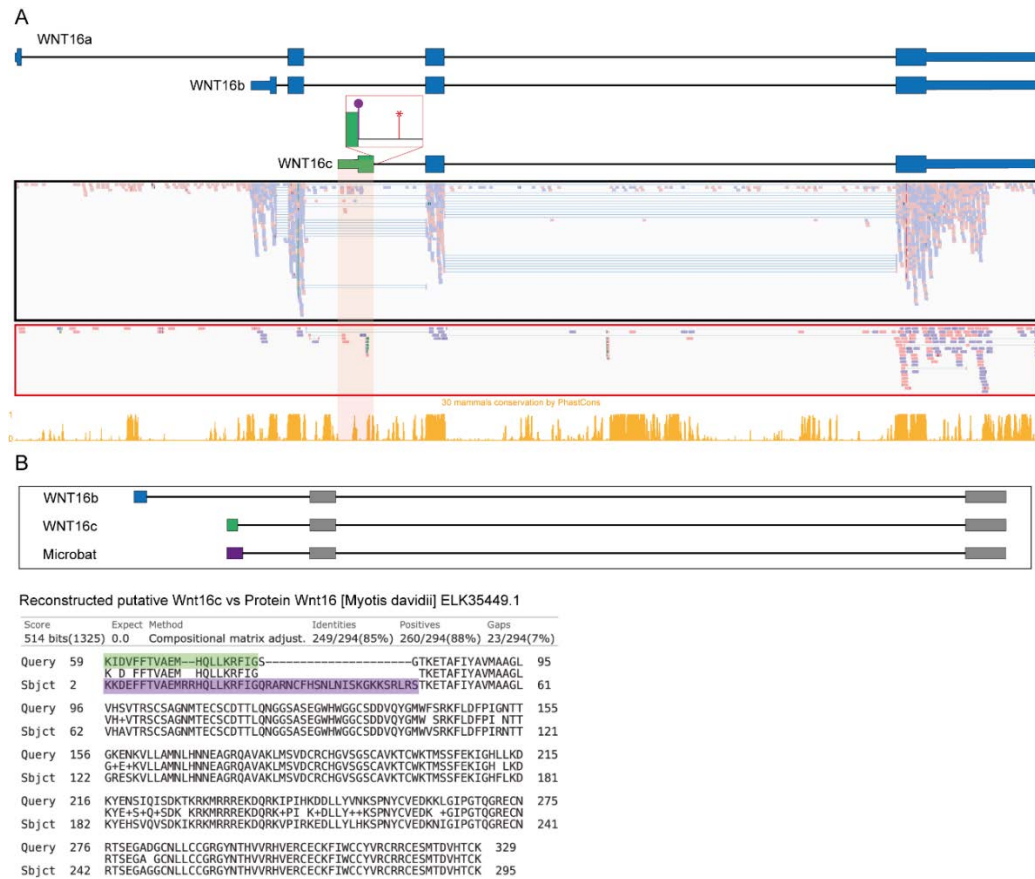


Figure 3. Reconstruction of the putative new WNT16c isoform. (A) Gene models for the canonical WNT16 isoforms (blue) and the new proposed isoform (new exon in green, rest of the exons in blue). In detail, position of a stop codon (red asterisk) relative to a donor splice site (purple dot). RNA-seq paired-end reads from hFOB cells are shown in the black frame while single-end reads from seven human tissues are shown in the red frame. Mismatches in the read sequence are represented as a stripe coloured according to the nucleotide (Green for A; Red for T; Gold for G; Blue for C). The track for the mammal conservation by PhastCons is present in yellow at the bottom. The region corresponding to the putative new exon is delimited in translucent orange across the image. (B) Comparison among the WNT6b, the putative WNT16c and microbat Wnt16 gene structures. The region shown for the latter corresponds to Myoluc.2.0 (GL430012:189-247-202,565). Wnt16 microbat gene is shown in purple, the canonical human WNT16 tBLASTn hit is shown in blue and the putative WNT16c alignment is shown in green. Below, sequence alignment between the microbat Wnt16 (Myotis davidii, ELK35449.1) and the putative reconstructed protein from the new isoform (WNT16c). The new proposed first exon is highlighted in green and the first exon of microbat is highlighted in purple.



Figure 4. Results obtained in the 4C-seq experiments using 5 viewpoints (top panels) from the *CPED1-WNT16-FAM3C* region in human fetal osteoblast (hFOB 1.19; panel A), Saos-2 cells (panel B) and mesenchymal stem cells (MSC; panel C). The strength of the interactions is shown on the Y axis and the interactions observed from each viewpoint are shown by arched lines. Each color represents a different viewpoint and the intensity of the color of the arches indicates the intensity of the interaction. Genes in these region (center panels): *ING3_002* (ENST00000339121.5), *ING3_001* (ENST00000315870.5), *FAM3C* (ENST00000359943.3), *WNT16_2* (ENST00000361301.2), *WNT16_1* (ENST00000222462.2), *CPED1_1* (ENST00000310396.5), *CPED1_7* (ENST00000450913.2), *PTPRZ1* (ENST00000393386.2) and *AASS-001* (ENST00000417368.2) from GRCh37/hg19. GeneHancer track (Bottom panels) in human regulatory elements (enhancers are shown in grey and promoters in red).



Figure 5. Schema of *ING3-CPED1-WNT16-FAM3C* locus from the UCSC genome browser with the following tracks (downwards). Genes in these locus from UCSC annotations of RefSeq RNAs. GeneHancer database of human regulatory elements included in GeneCards –in red promoters and in grey enhancers-. Interactions between the GeneHancer regulatory elements from Double Elite. In Black osteoblast ChIP-seq profiles of CTCF, H2A.Z, H3K27ac, H3K27me3, H3K4me1, H3K4me2, H3K4me3 and H3K9me3 (ENCODE project). ENCODE Registry of candidate cis-Regulatory Elements (cCREs). Vertebrate Multiz Alignment & Conservation (100 Species).

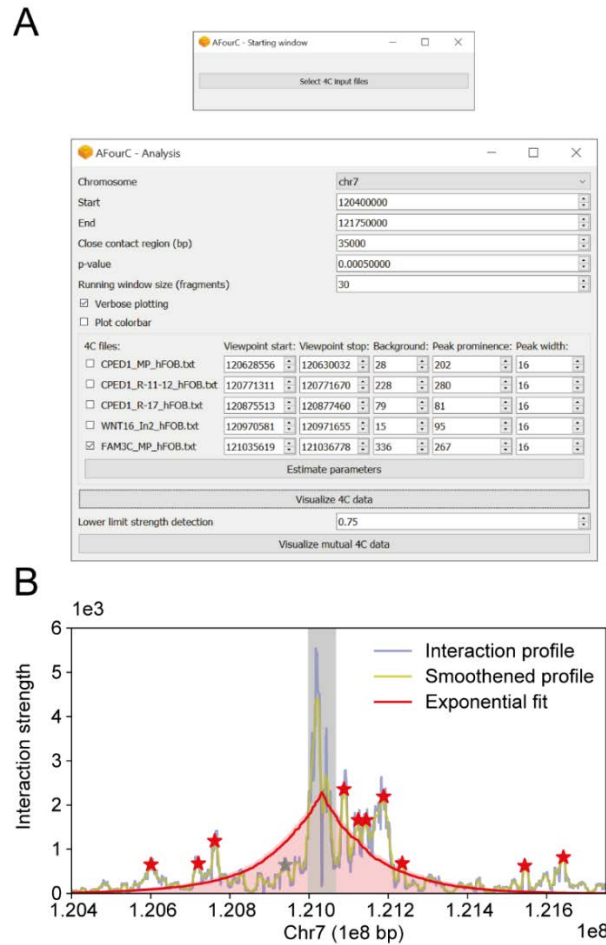


Figure 6. AFourC software. **(A)** User interface windows of the AFourC software. Any number of 4C input files can be read using the entry window (top) and are visualized in the second window (bottom). The following parameters are set to default values but can be manually adjusted: chromosome and region of interest (defined by start and end in units of base pair), region of close contact to the viewpoint, p-value for statistical filtering of peaks and window size for running mean. Within every 4C file, profile-specific parameters such as background level, peak prominence and peak width can be automatically estimated. For mutual 4C analysis, the minimum value of relative strength is set to 75%. **(B)** Visualization of the statistical analysis of interaction profile and peak detection for a given 4C dataset, as obtained by running the AFourC software in “*Verbose plotting*” mode (Panel A). Raw profile and profile smoothened with mean filter are shown in blue and yellow, respectively. Red curve: symmetric exponential fit. Transparent red area: exclusion region based on p-value. Transparent gray area: profile excluded from the analysis based on the close contact region. Black stars: non statistically significant peak. Red stars: statistically significant peaks used for further analysis.

CHAPTER 3. IDENTIFICATION OF GENES THAT COULD EXPLAIN MENDELIAN CASES OF THE HBM PHENOTYPE

Article 6

A missense mutation in *VAV3* in a familial case of high bone mass

Summary:

Osteoporosis is the most common bone disease worldwide, causing more than 8.9 million fractures annually, and representing a very high human and socioeconomic burden. Osteoporotic fracture is preceded by decreased bone mineral density (BMD), which greatly increases bone fragility. At the other end of the population BMD spectrum, some individuals present with very strong bones which fail to fracture. Both osteoporosis and high bone mass (HBM) are heritable and their genetic architecture encompasses both polygenic inheritance of common variants and some cases of monogenic highly penetrant variants in causal genes. These monogenic cases are especially attractive since they hold promise to the identification of novel therapeutic targets for osteoporosis. Here we have investigated the genetics of a family presenting with HBM segregating in an apparently mendelian dominant pattern. Since a polygenic cause had been previously discarded, as well as mutations in known HBM genes, we searched for rare causal variants in novel genes by whole exome sequencing (WES) in a trio comprising two affected cases and one non-affected family member. Rare coding variants fulfilling the inheritance pattern were further restricted to include only those genes that could be related to bone development, function or disease, leaving 28 variants in 27 genes. Of those, we highlight a missense variant in *VAV3*, a gene encoding a guanine nucleotide exchange factor with an important role in osteoclast activation and function. Although no previous cases of *VAV3* mutations have been reported associated with a bone phenotype in humans, *Vav3* KO mice display very dense bones, equivalent to the HBM phenotype present in our family. A second variant, which might play a secondary role, is a missense change in *SIK3*. However, the mouse and human bone phenotypes already described associated with this gene do not match so closely with HBM. Future functional assessment of the *VAV3* variant

will likely confirm VAV3 as a novel HBM gene and a potential therapeutic target for osteoporosis.

Reference:

Núria Martínez-Gil, Diana Ovejero, Natalia Garcia-Giralt, Leonardo Mellibovsky, Xavier Nogués, Raquel Rabionet, Daniel Grinberg, Susanna Balcells. A missense mutation in VAV3 in a familial case of high bone mass. To be submitted

A missense mutation in *VAV3* in a familial case of high bone mass

Núria Martínez-Gil¹, Diana Ovejero², Natalia Garcia-Giralt², Leonardo Mellibovsky², Xavier Nogués², Raquel Rabionet¹, Daniel Grinberg¹, Susanna Balcells¹

1 Department of Genetics, Microbiology and Statistics, Faculty of Biology, Universitat de Barcelona, CIBERER, IBUB, IRSJD, Barcelona, Spain.

2 Musculoskeletal Research Group, IMIM (Hospital del Mar Medical Research Institute), Centro de Investigación Biomédica en Red en Fragilidad y Envejecimiento Saludable (CIBERFES), ISCIII, Barcelona, Spain.

Abstract

Osteoporosis is the most common bone disease worldwide, causing more than 8.9 million fractures annually, and representing a very high human and socioeconomic burden. Osteoporotic fracture is preceded by decreased bone mineral density (BMD), which greatly increases bone fragility. At the other end of the population BMD spectrum, some individuals present with very strong bones which fail to fracture. Both osteoporosis and high bone mass (HBM) are heritable and their genetic architecture encompasses both polygenic inheritance of common variants and some cases of monogenic highly penetrant variants in causal genes. These monogenic cases are especially attractive since they hold promise to the identification of novel therapeutic targets for osteoporosis. Here we have investigated the genetics of a family presenting with HBM segregating in an apparently mendelian dominant pattern. Since a polygenic cause had been previously discarded, as well as mutations in known HBM genes, we searched for rare causal variants in novel genes by whole exome sequencing (WES) in a trio comprising two affected cases and one non-affected family member. Rare coding variants fulfilling the inheritance pattern were further restricted to include only those genes that could be related to bone development, function or disease, leaving 28 variants in 27 genes. Of those, we highlight a missense variant in *VAV3*, a gene encoding a guanine nucleotide exchange factor with an important role in osteoclast activation and function. Although no previous cases of *VAV3* mutations have been reported associated with a bone phenotype in humans, *Vav3* KO mice display very

dense bones, equivalent to the HBM phenotype present in our family. A second variant, which might play a secondary role, is a missense change in *SIK3*. However, the mouse and human bone phenotypes already described associated with this gene do not match so closely with HBM. Future functional assessment of the *VAV3* variant will likely confirm *VAV3* as a novel HBM gene and a potential therapeutic target for osteoporosis.

1. Introduction

High Bone Mass (HBM) is a condition of bone overgrowth characterized by unusually dense bones and a very strong skeleton that produces the total absence of fractures [1]. In some cases, secondary complications associated with excessive bone formation may develop, such as widening of the jaw, generalized sclerosis of the skull, compression of the cranial nerve, severe headache, and, in some families, torus palatinus [2], but in most cases, patients do not require treatment. The prevalence of HBM is 0.2-1% [3–6]. The HBM phenotype is normally diagnosed through bone mineral density (BMD) measured by bone densitometry (DXA). However, there is no consensus about the DXA cutoff value for an HBM diagnosis. While some authors use Z-score > or equal to +2.5 [7], others consider obtaining a value greater than four when performing the sum of Z-scores measured in the lumbar spine and the femoral neck [3].

Gain of function mutations in *LRP5* and *LRP6* have been described to cause HBM. Patients with these mutations present high bone synthesis (evaluated through serum markers) and thickening of the trabecular and cortical bone, but a normal rate of bone resorption, normal bone architecture, and unaltered serum levels of calcium, phosphate, parathyroid hormone (PTH) and vitamin D [3,8]. These variants produce a loss of affinity for the extracellular inhibitors DKK1 and sclerostin, thus preventing internalization of LRP5/6 [2,3,9–14]. All the *LRP5/6*-HBM mutations described are located in the first β -propeller domain, which interacts with the NXI domain of these extracellular Wnt pathway inhibitors [15–17]. In the same line, we have previously described two rare missense mutations in *DKK1* (p.Tyr74Phe and p.Arg120Leu) in two HBM women [4,18], and we recently have validated them functionally by demonstrating their partial loss of function [19]. In addition to the mutations in canonical Wnt pathway genes, mutations in other genes have been found as potential

candidates to cause the HBM phenotype. This is the case of a rare dominant missense mutation in *SMAD9* (p.Leu22Pro), which was found co-segregating with this trait in a family and in two unrelated patients [20]. *SMAD9* acts by inhibiting BMP-dependent target gene transcription in osteoblasts, thus limiting osteoblast activity [21]. In addition to these monogenic forms, the HBM phenotype can also be polygenic. A recent study of an HBM cohort has found an enrichment for common protective variants at BMD GWAS loci as compared with a reference cohort of normal BMD [22]. While a proportion of all HBM cases are expected to be monogenic, in most of these cases no causative mutations have been found, yet.

Given that several treatments for bone density diseases available in recent years stem from the identification of the causal genes of some rare monogenic bone diseases, the identification of novel genes causing the non-pathological HBM phenotype may provide additional novel therapeutic targets for bone density diseases. Here we have undertaken the genetic analysis of rare variants in a case of HBM previously described [4], in which family segregation is suggestive of a Mendelian inheritance and for which we have already collected evidence against polygenic inheritance.

2. Material and methods

Genomic DNA was extracted from peripheral blood leukocytes of all the family participants using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Trio whole-exome sequencing of I.1, II.1, and II.5 was performed at CNAG (Barcelona, Spain). Shortly, DNA was enzymatically fragmented and libraries were constructed and hybridized against the Nimblegene Human Clinical Exome Capture + Mitochondrial DNA probeset. Captured fragments were sequenced in an Illumina NovaSeq 6000 sequencer. The reads were then aligned to the hg38 reference genome with BWA-mem, duplicate-marked, recalibrated and sorted before calling variants with GATK's haplotype caller (V4). After quality-filtering following in-house hard filters, variants were annotated with Varaft [23], and prioritized under the hypothesis of an autosomal dominant segregation. We therefore filtered for heterozygous rare variants shared by I.1 and II.5 and absent in II.1. Variants located outside of the coding region (intergenic, 5' and 3' UTR, upstream, downstream, ncRNA or unknown variants), intronic variants not predicted to affect the splice site, synonymous variants, and those with a minor allele frequency > 0.01 were

filtered out, and 122 variants in 111 genes remained. Then, variants were prioritized based on gene functionality information (including known gene function, phenotype of animal models, human diseases and association in BMD GWAS), obtained from public databases (Musculoskeletal Knowledge Portal, Uniprot, OMIM, and Pubmed). Using this approach, we prioritized 28 variants in 27 different genes, which we sorted for pathogenicity score and gene function most closely related to the HBM phenotype. We tested these 28 variants for cosegregation in the rest of the family (III.1, III.2., III.3.) by Sanger sequencing at the CCiTUB genomic service (Genómica, Parc Científic, Barcelona, Spain) using BigDye™ Terminator v3.1 Cycle Sequencing Kit, followed by detection on automated capillary sequencer models 3730 Genetic Analyzer and 3730xl Genetic Analyzer.

For all the participating family members, a blood test, including a complete biochemical analysis of relevant parameters (kidney function, liver biology, thyroid hormones, and vitamin D) and BMD quantification, performed by dual-energy X-ray absorptiometry scans (DXA; QDR 4500 SL; Hologic, Waltham, MA, USA) were available. All DXA measurements were performed prior to any treatment that could increase bone mass. Also, pathologic phenotypes such as osteopetrosis or any other sclerosing bone disorders were ruled out based on the absence of radiologic alterations in the skull and long bones, the absence of any fragility fracture and the absence of any underlying disease.

In addition, a study of bone remodeling markers was carried out in the proband and her three daughters (II.5 and III.1, III.2 and III.3), including serum CTX (bone resorption marker), the levels of FaO and P1NP (bone formation markers) and those of Vitamin D.

Both the Bioethics Committee of Universitat de Barcelona and the Clinical Research Ethics Committee of Parc de Salut MAR have emitted favourable bioethical statements regarding the present research. Written informed consents were obtained from the participants in both instances. Blood samples and written informed consent were obtained in accordance with the regulations of the Clinical Research Ethics Committee of Parc de Salut MAR.

3. Case report

The proband (II.5 in Figure 1 and patient HBM9 in ref. [4]) had come to our osteoporosis unit for routine bone densitometry measurement due to postmenopause and she was invited to enroll in the BARCOS cohort of postmenopausal women from the Barcelona area [24,25]. Within BARCOS, a total of 1600 lumbar spine (LS) and femoral neck (FN) BMD measurements of the women were analysed in order to pinpoint those HBM cases in which the sum Z-score (LS+FN) was equal to or greater than 4 [3]. The proband was identified as a HBM case presenting a sum of Z-score of 7 ($Z_{LS} = 4.6$ and $Z_{Hip} = 2.4$; Table 1). Analysis of the number of BMD-risk alleles (as of [26]) carried by this HBM case yielded a surprisingly high number of them, which would predict that she would present with a low bone mass phenotype [4]. Because of this apparent contradiction, we hypothesized that she might be carrying an additional unknown rare and penetrant variant, which would be responsible for her HBM phenotype and which might segregate in a Mendelian fashion in her family. Subsequently, we analyzed her relatives, including her mother (I.1), brother (II.1) and her three daughters (III.1; III.2 and III.3). DXA analysis revealed that the mother presented a sum Z-score of 4.4 ($Z_{LS} = 2.4$ and $Z_{Hip} = 2.2$; Table 1), fulfilling the HBM definition according to [3] (Figure 1). In addition, one of the proband's daughters (III.1) presented a sum Z-score of 3.5 ($Z_{LS} = 1.3$ and $Z_{Hip} = 2.2$; Table 1), close but below the HBM threshold. The other two daughters, III.2 and III.3, presented sum Z-scores of 0.5 ($Z_{LS} = -0.2$ and $Z_{Hip} = 0.7$; Table 1) and -0.8 ($Z_{LS} = -0.4$ and $Z_{Hip} = -0.4$; Table 1) respectively, and the brother of the proband, II.1, presented a sum Z-score of 0.9 ($Z_{LS} = -0.1$ and $Z_{Hip} = 1$; Table 1), all rated as normal. Regarding the biochemical markers of bone turnover, all parameters fell within the normal range. Only the CTX marker was worthy of attention, as in the II.5 and III.1 samples it was found to be in the lower limit of normality (Table 1).

Trio WES analysis was undertaken using the two HBM samples (proband II.5 and her mother I.1) and the proband's brother (II.1) whose BMD was in the normal range. Prioritization of the rare and predicted pathogenic variants shared by the HBM cases (and absent in the non-HBM relative) yielded a set of 28 variants in 27 genes known to be involved in bone biology (Table 2). Of these, two rare variants (in *VAV3* and *S/K3*) stood out as the most likely candidates to be involved in the inherited HBM phenotype in this family. The proband and her mother shared the rare missense variants p.Thr124Ile in *VAV3* (ENST00000370056.9 ; rs20098001) and p.Gly19Glu

in *SIK3* (ENST00000375300.6; rs550430452), which were absent in the brother (II.1). Segregation analysis showed that they were also absent in the three proband's daughters (III.1, III.2, and III.3). Both variants are rare (MAF= 0.00009685 and MAF=0.006668 in GnomAD v2.1.1 in European non-finish population for *VAV3* and *SIK3*, respectively) and predicted to be deleterious by SIFT, although this prediction is not consistent across algorithms (Table 2). Given the known biological functions of these two genes (see below), these two variants might plausibly be involved in causing the HBM phenotype in this family. Table 2 lists other variants shared by the proband and her mother that could also be contributing to the phenotype or acting as modifiers.

4. Discussion

High bone mass is considered a phenotype that can be due to an accumulation of protective variants in genes involved in bone formation or bone maintenance, or to the presence of rare, highly penetrant mutations in genes that affect bone formation (*LRP5*, *LRP6*, *SMAD9*). Here we report a family with an unexplained HBM phenotype, apparently inherited in an autosomal dominant manner, and whose proband was shown to carry an excess number of osteoporosis risk alleles. Analysis of the exome sequence highlighted the presence of several rare variants in candidate genes related to bone phenotypes. Integration of genomic and phenotypic data, especially the hint of a mild bone resorption defect, suggests that a variant in *VAV3* is a likely candidate to cause the observed HBM.

The family includes a proband and her mother, both displaying a clear HBM phenotype, a brother and two daughters of the proband, displaying totally normal BMD values, and another daughter displaying notably high BMD, especially at the hip and not at the lumbar spine. Her sum Z score did not reach the threshold of a HBM definition and therefore, she could not be considered a case. For this reason, this sample was not subjected to WES but was later used to check presence/absence of selected variants. Our hypothesis assumed that there could be more than one variant at play to explain the HBM phenotype in the family, and that the daughter with notably high BMD might lack some of these.

VAV3 is a guanine nucleotide exchange factor (GEF) of the Rho family that has an important role in bone resorption. In particular, *VAV3* is essential for organizing the osteoclast's cytoskeleton and it has been shown to stimulate osteoclast activation *in*

vitro [27]. In addition, the Vav3-KO mouse presents high bone mass, reflecting an impaired osteoclast terminal differentiation and function, and protection against bone loss induced by PTH or RANKL [27]. The VAV3 protein presents a series of domains (Figure 2) through which it exerts its two main activities of guanine nucleotide exchange factor (GEF) and adaptor [28]. The catalytic activity of VAV3 is modulated by tyrosine phosphorylation at position Y141. When this tyrosine is not phosphorylated, the DH and Ac domains, together with the most N-terminal CH region, contact the catalytic domains, preventing interaction with the substrate and forming an autoinhibitory loop. Interestingly, the mutation identified in the proband maps to the N-terminal CH region and presumably, it could disrupt VAV3 activity by permanently forcing its autoinhibition. Considering this information, it is tempting to speculate that loss of function of VAV3, caused by the p.Thr124Ile mutation, could cause a defect in bone resorption and a HBM phenotype accompanied by the observed values at the limit of the population normal range of CTX resorption marker. While this explanation is plausible, this variant is shared by the proband and her mother, but not by the proband's daughter displaying nearly-HBM, suggesting that the VAV3 variant alone is not sufficient to fully explain the observed HBM in this family, and that other variants in other relevant genes may be contributing to it.

Next, we considered the involvement of a variant identified in *SIK3*, as the two HBM individuals also share a missense variant in this serine/threonine kinase. Several lines of evidence highlight *SIK3* as an essential regulator of skeletal biology, producing its effect on chondrocytes, osteoblasts/osteocytes, and osteoclasts. First, *Sik3* KO mice show growth plate abnormalities associated with delayed chondrocyte hypertrophy and primary bone formation [29]. Second, the combined ablation of *SIK2* and *SIK3* dramatically increased bone mass and bone turnover through the PTH/PTH1R signaling pathway [30]. In contrast, other works in primary pre-osteoblasts and osteocytes showed non effect on osteoblast differentiation or bone matrix mineralization and normal responsiveness to PTH [31,32]. Third, both the pharmacological inhibition (HG-9-91-01) and a *Sik3* KO model in RAW cells significantly inhibited osteoclastogenesis by dose-dependently reducing osteoclast differentiation markers and bone-resorbing activity [33]. Furthermore, in humans, a homozygous mutation in the catalytic domain of *SIK3* has been described as causing skeletal dysplasia [34]. All things considered, although *SIK3* indeed has a prominent role in bone biology, we acknowledge that the phenotype present in the proband and

her HBM mother does not resemble the patients' phenotype described in Csukasi et al. [34], nor that of the mouse deficient in *Sik3*. Therefore, we hypothesize that the *SIK3* variant might play a secondary role in defining the HBM phenotype in our family. Besides, the variant is absent in the proband's daughter presenting with elevated BMD.

In conclusion, we have identified a promising missense variant in *VAV3* which could play a major role in defining HBM, in combination with the variant in *SIK3*, which might enhance its effects. Further work in variant analysis will be necessary to assess the pathological role of these two variants and to find additional variants to explain the elevated BMD phenotype of the proband's daughter. If these findings are confirmed, *VAV3* might turn out to be a novel therapeutic target for the treatment of osteoporosis.

5. References

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TABLES:

Table 1. BMD Z-score values and BTM of all the family participants

	Z-score BMD		Bone remodelling markers			
	LS	Hip	P1NP (16-74)	BAP (4.3-20.1)	CTX (0.01-1.008)	Vit D (30-150)
I.1	2.4	2	NA	NA	NA	NA
II.1	-0.1	1	NA	8.65	NA	NA
II.5	4.6	2.4	27.5	11.8	0.085	NA
III.1	1.3	2.2	24.4	9.14	0.11	26
III.2	-0.2	0.7	32.7	10.4	0.269	35
III.3	-0.4	-0.4	55.9	8.5	0.248	44

LS: Lumbar spine, P1NP: Type I serum Procollagen , N-terminal Propeptide; BAP: Bone-specific alkaline phosphatase; CTX;C-terminal cross-linking telopeptides of type I collagen Vit D: Vitamin D.

Tabla 1. Priorized variants present in the HBM I.2 and II.5 and absent in II.1.

Gene	Variant	rs number	Function	BMD GWAS	Disease	Mouse model	Cosegreg.	Pathogenicity prediction				gnomAD
								CADD	PP	PV	SIFT	
ACOXL	p.G577X	rs189429375	Participate in fatty acid β -oxidation, fatty acid metabolic process, and oxidation reduction	Y	-	NA		37	.	.	.	0.0053
ARMC9	p.R536C	rs201558091	Involved in ciliogenesis	N	JBTS30	NA	Y	33	D	D	D	7.95E-06
PFAS	p.A466T	rs34939404	Phosphoribosylformylglycinamidinase synthase	N	-	NA	N	31	D	D	D	0.0051
RNF215	p.G241R	rs140327189	-	Y	-	NA	N	28.7	D	D	D	0.0018
ASB16	p.A218V	rs75036136	Involved in the pathway protein ubiquitination	Y		NA		27.7	D	D	D	0.0008
PARVG	p.V220F	rs139404110	Plays a role in the regulation of cell adhesion and cytoskeleton organization.	N	-	NA	N	27.7	D	D	D	0.0003
POMGNT1	p.D556N	rs74374973	Participates in O-mannosyl glycosylation	N	MDD G type A3, B3 and C3; RP76	NA		27	D	D	D	0.01
CCDC88B	p.Q106R	.	Positive regulator of T-cell maturation and inflammatory function.	Y	-	NA		26.6	D	D	D	.
EFL1	p.P830T,p.P881T	rs72749532	Involved in the biogenesis and translational activation of ribosomes	N	SDS2	NA	N	26.6	D	D	D	0.0072
KLHL17	p.V513E	rs372591096	Involved in the pathway protein ubiquitination.	Y	-	NA	N	26.5	P	D	D	1.27E-05

AMOTL1	p.Y605S	.	Inhibits the Wnt/beta-catenin signaling pathway.	N	-	NA	Y/ N	26.4	D	D	D	.
EFL1	p.V959I, p.V1010I	rs72749527	Involved in the biogenesis and translational activation of ribosomes	N	SDS2	NA		24.6	P	N	T	0.0072
TLR2	p.T491I	rs139078841	Cooperates with LY96 to mediate the innate immune response to bacterial lipoproteins.	N	-	Y	N	23.7	B	D	D	0.0002
UNC5B	p.G549S, p.G560S	rs180703790	Receptor for netrin required for axon guidance.	N	-	N		23.2	D	N	D	0.0004
VAV3	p.T124I	rs200980013	Exchange factor for GTP-binding proteins RhoA, RhoG and, Rac1.	N		Y	Y/ N	23.1	B	N	T	0.0002
XBP1	p.M139I, p.M144I, p.M89I,	rs143559198	Transcription factor during endoplasmic reticulum (ER) stress by regulating the unfolded protein response.	Y	-	Y	N	22.9	B	N	T	0.0003
PCDHGA10	p.V550L	.	Potential calcium-dependent cell-adhesion protein.	NA	-	NA		19.33	B	D	D	3.98E-06
PCDHGA12	p.V340L	rs151011884	Potential calcium-dependent cell-adhesion protein.	NA	-	NA		16.69	B	N	T	0.0021
KIAA1217	p.A597P, p.A914P	rs141672009	Required for normal development of intervertebral disks	Y	-	NA		16.57	D	N	T	0.0001
CDC14A	p.V428M	.	Dual-specificity phosphatase. Required for centrosome separation and productive cytokinesis during cell division.	N	-	N		15.64	B	N	T	.
PDE4D	p.T215M	rs145326116	Hydrolyzes the second messenger cAMP	N	ACRD YS2	N		13.37	D	D	D	0.001
HAS3	p.T267M	rs113161750	Catalyzes the addition of GlcNAc or GlcUA monosaccharides to the nascent hyaluronan polymer.	Y	-	N		12.99	B	N	D	4.82E-05
SIK3	p.G19E	rs550430452	Kinases involved in control skeletal actions.	N	SEMD K	Y	Y/ N	7.931	.	N	T	0.0044
SMC3	p.N435S	.	Central component of cohesin, a complex required for chromosome cohesion during the cell cycle	Y	CDLS 3	Y		7.918	B	N	T	.

CFAP97D1	p.K36R	.	-	Y	-	NA	6.657	B	N	T	.
MICALL2	p.V316M	rs371363067	Effector of small Rab GTPases	N		NA	0.063	B	N	T	0.0002
PCDHB8	p.V661L	rs2697541	Calcium-dependent cell-adhesion protein	N	-	NA	.	.	.	.	.
TENT5A	p.G39delinsGGDF GG	.	Nucleotidyltransferase	Y	Ol18	NA	N	.	.	.	.

List sort by CADD score. BMD GWAS: Genes associated with BMD in GWAS; Y: Yes; N: No; DISEASE: Gene associated with human diseases ACRDYS2: Acrodysostosis-2; CDLS3: Cornelia de Lange syndrome 3; JBT: Joubert syndrome 30; MDDG: Muscular dystrophy-dystroglycanopathy; SDS2: Shwachman-Diamond syndrome-2; SEMDK: Spondyloepimetaphyseal dysplasia, Krakow type; Ol: Osteogenesis imperfecta type 18; RT76: Retinitis pigmentosa type 76; MOUSE MODEL: KO mouse model with bone phenotype; Y: Yes; N: No; NA: Not available. Cosegreg. (absent in family members with normal BMD): Y (Yes) or N (No)/if Yes, present in member III.1 (with elevated BMD)?: Y (Yes) or N (No); PP: Polyphen-2; PV: PROVEAN; MAF: minor allele frequency from gnomAD.

FIGURES:

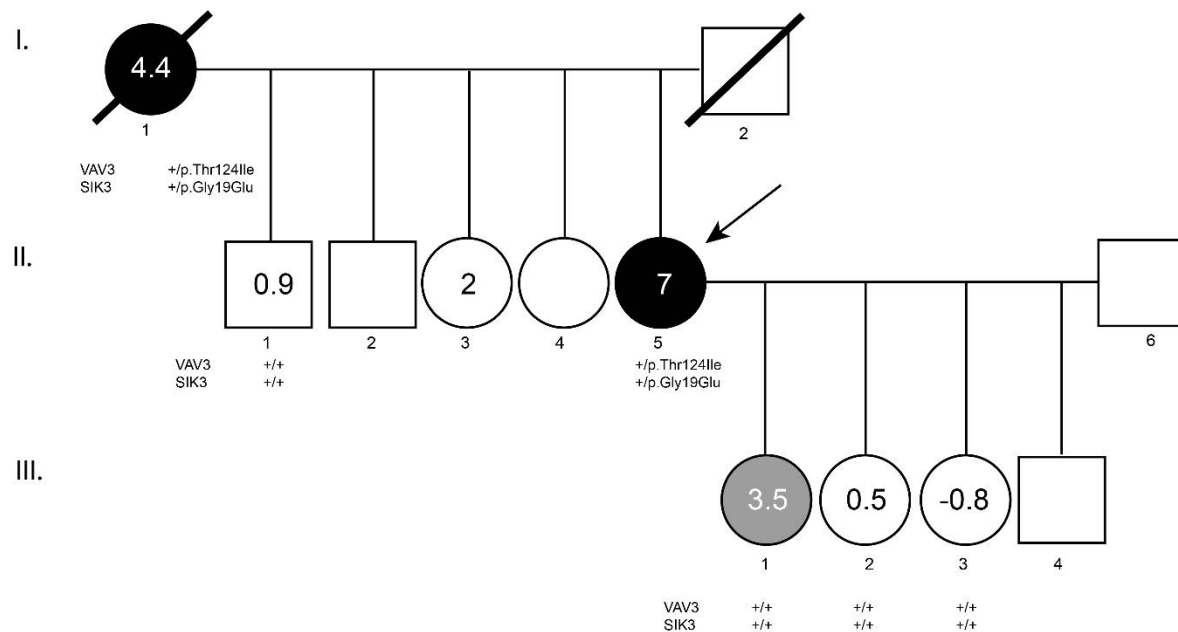


Figure 1. Pedigree of family HBM9 segregating HBM. Black filled symbols denote individuals presenting with the trait and the grey filled symbol denotes an individual with elevated BMD values not reaching the HBM threshold. The numbers inside the symbols correspond to the sum BMD Z-scores (LS+FN).

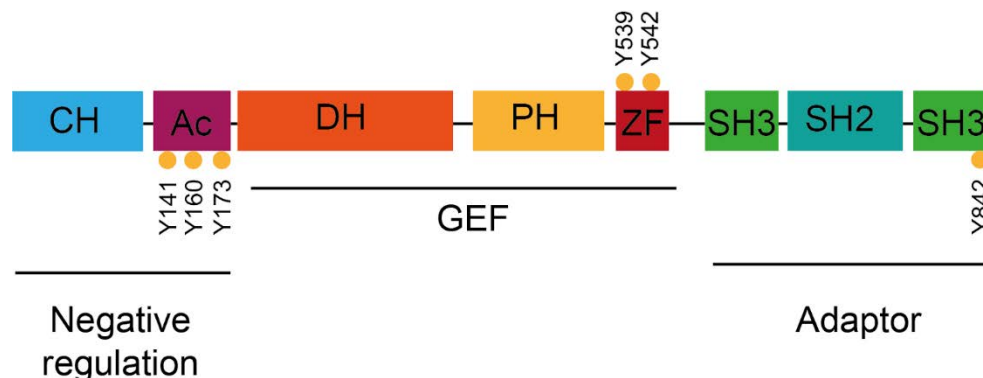


Figure 2. Domain structure of the VAV3 protein. From N-term to C-term: CH, calponin homology (blue); Ac, Acidic domain (burgundy); DH, DbI homology (orange); PH, Pleckstrin homology (yellow); ZF, Zinc finger (red); SH3, Src homology 3 (green); SH2, Src homology 2 (turquoise). Three main functions of VAV3 (negative regulation, Guanine exchange factor and adaptor) are ascribed to particular groups of domains, as indicated. Relevant tyrosine residues (Y) are depicted above and below the scheme with yellow dots.

DISCUSSION

In recent years, many efforts have been devoted to determining variants and genes that may be important for BMD and, in turn, various bone pathologies. On the other hand, an essential and much more time-consuming step is still challenging, namely the functional validation of these variants. My doctoral thesis has focused on i) the identification of variants in candidate genes highlighted through GWASs, ii) the identification of important new genes determining the genetic bases of monogenic diseases and iii) their functional characterization through *in vitro* experiments. Below I will discuss the main results obtained with emphasis on the pros and cons of the methods used and the biological effects obtained with the approaches performed.

1. THE GENETIC MEDICINE REVOLUTION

The improvement in sequencing techniques, Next Generation Sequencing (NGS), has allowed the identification of a plethora of genes associated with human inherited disorders. Consequently, this has helped to understand the molecular basis of genetic diseases. These techniques are based on massive high throughput analysis and allow to have a diagnosis in a short time and at low cost. NGS technology is widely and successfully used in many areas of genetic research including the diagnosis of heterogeneous diseases, in GWAS for common diseases and in functional genomic applications (4C-seq, ATAC-seq, ChIP-seq, RNA-seq, etc.) (403,404). Specifically, NGS techniques have revolutionized the diagnosis of skeletal disorders, going from knowing the cause of 58% (215 of 372) of skeletal diseases in 2006 to 92% (425 of 461) in 2019(34). This knowledge, together with careful clinical phenotyping, allows better patient care, both in terms of diagnosis and pharmacological therapy (405). Importantly, the identification of causative genes and defective proteins has proven essential to improve the diagnosis, prognosis and treatment of patients, thus paving the way to ever improving personalized therapy.

To identify the cause of a monogenic disease, different approaches can be taken. In the case of an obvious phenotype with a clear associated gene, a sequencing of the causal gene is sufficient to identify the mutations and, therefore, to perform the diagnosis. Similarly, if the number of associated genes is small, it is still possible to use commercial or custom panels to sequence the genes and identify the mutation. This, for instance, is the case of osteogenesis imperfecta. However, when the causal genes are unknown, it is necessary to adopt a hypothesis-free approach such as

whole exome or genome sequencing (WES or WGS), thus generating a list of candidate causal genes. While WES is relatively cost-effective and does not require large data storage nor analysis capacities, by design this approach is not capable of detecting mutations in introns or intragenic regions. Conversely, WGS is capable of detecting such mutations, however the costs are higher and the analysis is considerably more challenging. While the costs are rapidly decreasing, a possible work around to the analysis challenge is to first consider only exome data (thus performing a WES-like analysis), and keep the full WGS dataset for further investigation. In the present work, we have used NGS methods to identify the causative variants of the HBM phenotype (article 6). In this work, we have carried out a WES in a family where the HBM phenotype co-segregates. In this case, one rare missense variant in *VAV3* was prioritized as a possible cause of the HBM establishment together with another variant in *SIK3* that could act as a modifier enhancing the effect. On the other hand, in article 4 we use Sanger sequencing to resequence the candidate gene *SOST* and the *SOST* enhancer *ECR5* in a small HBM cohort. We found that the *SOST* variant rs17882143, present in an HBM woman, may be responsible for the phenotype. In the same way, in article 3, we resequenced the candidate gene *DKK1* in a small cohort of Chiari Malformation type I patients. None of the variants found seemed to be responsible for the phenotype.

Despite the successful application of NGS, the interpretation of its results is still challenging, as many of the genes discovered are novel. Therefore, a variant filtering or variant prioritization is needed to obtain a short list of putative causal variants, for subsequently perform a functional validation.

2. ASSOCIATION STUDIES

In the last decades, GWASs have been widely used, with outstanding success, to identify genes involved in complex diseases. In particular, in bone biology research, up to 40 GWAS have been published identifying more than 500 genetic loci associated with different bone parameters such as BMD, bone strength and risk of fracture (143,151,301). These studies have been very successful in i) confirming previously identified genes, such as *SOST* and *LRP5*, implicated in monogenic skeletal diseases and ii) identifying new candidate genes for which their role in bone biology is currently unknown. This has been achieved thanks to the hypothesis-free nature of GWASs.

Nowadays, GWASs are used as a tool not only to discover, prioritize and develop new compounds, but also to re-purpose existing drugs (406). GWAS results could also be used to predict the risk of disease development for individual patients, thus allowing us to design better preventive treatments (141). For all these reasons, GWASs have established themselves as an essential tool in both basic research and translational contexts.

Despite their evident success, GWASs present three crucial limitations. First, the markers used to test association with the phenotype are usually tagSNPs distributed homogeneously throughout the genome and that appear in a significant fraction of individuals ($MAF > 5\%$). Therefore, rare variants with strong phenotypic effects are inherently discarded in these studies. This challenge has been partially addressed by either analyzing the whole genome without any marker selection (WGS)(127) or using cohorts at the extremes of the phenotypic distribution (extreme truncated selection assays)(94). Second, the success of any GWAS heavily depends on the sample size. For this reason, recently the most common strategy has been to establish large consortiums involving different cohorts from all over the world. However, the resulting super-cohorts are genetically heterogeneous, thus hindering the potential of GWAS to discover population-specific variants. Third, GWAS reports the most statistically relevant SNPs, also called “sentinel” SNPs. However, even though this SNP could be the causal one, it is likely that it is simply marking the gene or genomic region and that other changes or variants in LD with the sentinel SNP are responsible for the disease. If the associated SNP is in a coding region and involves an amino acid change, there is a high probability that the SNP itself is causal, but in reality the vast majority of sentinel SNPs are found in non-coding regions (96%), both introns (41%) as well as intergenic (54%), and therefore cannot be easily linked to a candidate causal gene (407,408). Moreover, because of their non-coding nature, it is challenging to perform functional studies of these sentinel SNPs. Therefore, identifying the region that is marking the sentinel SNP and establishing the functional bases underlying a given association is the great goal of the post-GWAS era.

In this doctoral work, we have designed and implemented approaches to overcome the limitations of GWAS studies and showcased their applicability on three Wnt pathway bone-related genes. In particular, in article 1, we designed a two-step

approach aimed at finding the variants responsible for the association with BMD marked by a sentinel SNP. First, we discovered common as well as rare variants present in *DKK1*, *SOST* and *WNT16*, three Wnt pathway bone-related genes associated with BMD and risk of fracture in the GWAs performed by Estrada *et al.* (149). To this aim, we resequenced the coding and regulatory regions in a truncated selection cohort based on extreme high (HBM) and low (LBM) BMD Z-score values. Second, based on their frequency distribution within the extreme cohort or their putative functionality, we selected 13 interesting variants, including 3 rare, and genotyped them in the complete BARCOS cohort (n=1490). This approach allowed us to determine the association with BMD for the ten common or low frequency (LF) variants as well as the distribution in the full cohort of the three rare variants.

We found six common or LF variants associated with BMD (Figure 16). Two of the six associated variants withstood multiple statistical corrections: i) rs55710688, located in the Kozak sequence of *WNT16a* and ii) rs142005327, located in a putative regulatory site in intron2 of *WNT16*. While the former was previously functionally validated (409), the latter had not been studied before, and therefore we next performed a functional study in article 5. The other four associated variants (Figure 9) included two common missense variants of *WNT16* (rs2908004 and rs2707466), a *DKK1* variant predicted to affect splicing (rs1569198) and a low frequency missense variant in the signal peptide of *SOST* (rs17882143). For the last two, we have performed the functional validations in articles 2 and 5 respectively. The haplotype containing the four protecting alleles and risk alleles of the *WNT16* associated variants plus the *WNT16*-GWAS hit from Estrada *et al.* (149) show nominal association with the LS-BMD. Among the 4 genotyped variants that did not have association with BMD in the full cohort, one exhibited a significant difference in the genotypic frequency between the two extreme groups. This is a low frequency variant (rs74711339) found in the 3'UTR region of *DKK1*, and we functionally tested it in the work presented in article 2 (Figure 16).

The three rare variants selected are located in regulatory regions. In particular, we identified: i) rs57054792 (3 women), located in the extended TATA box motif of *SOST*, ii) rs190011371 (3 women), located in the 3'UTR region of *WNT16*, and iii) rs113001389 (1 HBM woman) located in the intron 2 putative regulatory region of

WNT16. In article 4, we further performed a functional characterization of the first variant located in the extended TATA box. Besides, we identified another rare missense variant, present in an HBM woman, rs149268042, for which a genotype analysis was not performed but that was functionally studied in article 2 (Figure 16).

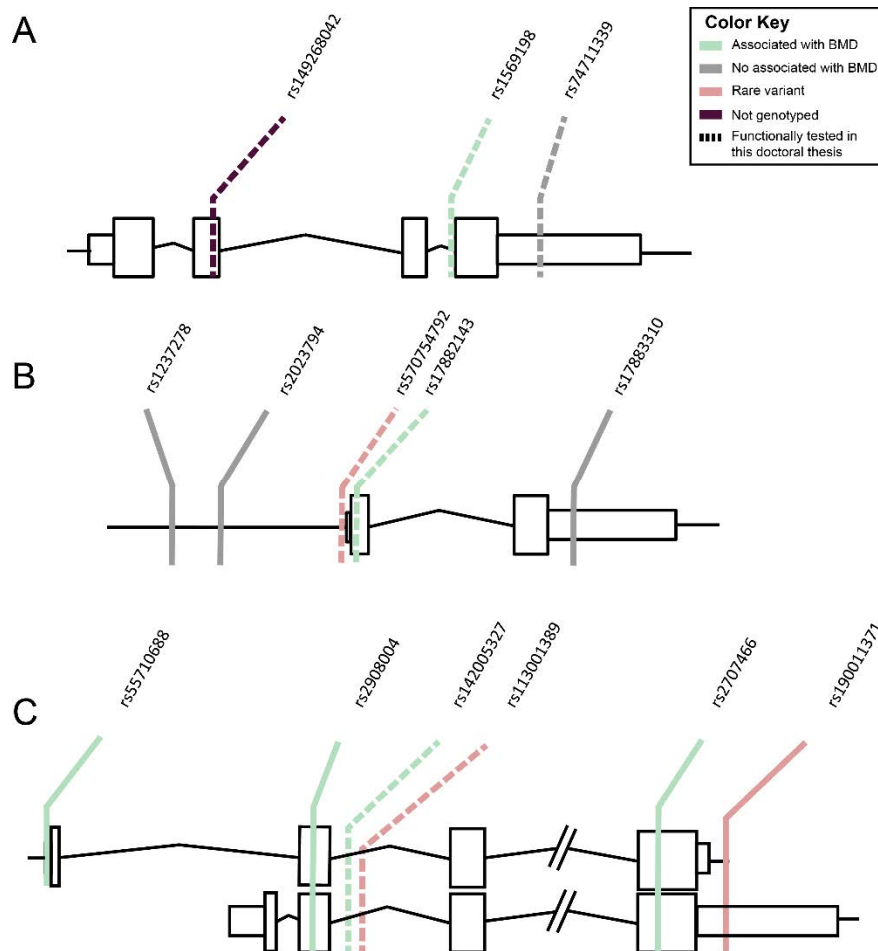


Figure 16. Thirteen variants identified in *DKK1*(a), *SOST* (b) and *WNT16* (c) in the resequencing of the extreme BMD groups and subsequently genotyped plus the rare variant rs149268042 not genotyped. Dashed lines shown the variants functionally tested in this doctoral thesis.

Therefore, we identified variants that could be functional and causal of the BMD association, including rare variants that might be responsible for the extreme phenotypes and were not identified by previous GWAS. In conclusion, our approach demonstrates the importance of performing a careful analysis of sentinel SNPs to identify the truly causal variants.

Still, we must consider some aspects that we will discuss below. i) Homogeneity of the cohort: The main means of diagnosing osteoporosis is through the quantitative measurement of BMD, which is also the main phenotype used in association studies.

BMD may vary depending on the measurement site and the technique used (410). For this reason, all individuals participating in the association test must have the BMD measured at the same site and using the same technique. For article 1, we have used the BARCOS (Barcelona Osteoporosis) cohort (411,412), which consists of 1490 postmenopausal women monitored at the Hospital del Mar (Barcelona). From this cohort, we discarded all women with any history of bone disease, metabolic or endocrine disorders, hormone-replacement therapy, or use of drugs that could affect bone mass. Moreover, it has been described that genetic determinants are highly specific and depend on several factors, including skeletal site, age and sex (149,155–160). Importantly, all the participants in the study had same sex, similar age and most of them had the LS-BMD and FN-BMD values measured by DXA, that is, at the same skeletal sites and using the same technique. Indeed, in article 1, we found specificity of variants to skeletal sites, as the rs1569198 variant associated with FN-BMD but not with LS-BMD, even though it had a larger sample size. Furthermore, all the women recruited have Spanish origin from the Barcelona area, and in a previous study, it was found that the cohort did not present population stratification (413). The population stratification may influence our results due to the allelic frequency of some polymorphisms, or the disease's prevalence variation between different ethnicities. In conclusion, the BARCOS cohort meets all requirements, and is therefore suitable for the association study we aim at.

ii) Statistical power of the cohort: The power of the cohort is the probability of detecting a true association. Several factors influence the statistical power of the cohort; the first and most important is the size of the cohort, but other factors include disease prevalence, genetic heterogeneity, LD between the marker and the causal variant and allelic frequencies and effect size of the variants (414). The BARCOS cohort's statistical power allows us to identify common variants with moderate effects in high LD with the causal variant but does not allow for the identification of variants with small effects or low frequency. This limitation is reflected by the lack of association in our cohort of variant rs4792909 in *SOST*, which showed an association in other large meta-analysis (149) or the lack of association of the low-frequency variant rs74711339 (MAF (EUR) = 0.043) in *DKK1* that only showed a trend towards significance.

iii) Covariable analysis: the BMD is a complex trait and many factors may influence the phenotype and are independent of the genetic component. That is why having specific measures in the cohort can allow us to control these variants, either by checking that they are homogeneously distributed in the cohort or

taken into consideration as a covariate. Some of the covariates that may influence BMD are gender, age, years since menopause, BMI, nutritional and hormonal status. Many of these covariates are recorded in the BARCOS cohort. In article 1, we have evaluated the influence that all available variables have on BMD, and we decided to use the years since menopause as a covariate in the association studies.

iv) Genotyping errors: It must be considered that, when many SNPs are genotyped in a large cohort, genotyping errors may occur that affect our results. This may be due to the low quantity or quality of the DNA samples or to the low reliability of the genotyping assay for a given variant. That is why quality controls are needed. In our case, we re-genotyped 12% of the samples, and found that the results showed a 99% concordance. Moreover, we discarded both DNA and SNPs that failed to give a result in 20% of the samples.

v) Multiple testing correction: When a high number of statistical tests are carried out, the probability that one of them is significant by chance increases, thus increasing the incidence of false positives. That is why it is necessary to apply a correction factor which depends on the number of tests performed. There is a wide variety of correction tests, from the most restrictive (e.g., Bonferroni) that prevents false positives and increases the occurrence of false negatives, to the least restrictive that give the opposite results. In article 1, we have used the Bonferroni correction, which consists of dividing the threshold of the value of the p statistic (0.05) by the number of independent tests performed. Using this test, the false-positive incidence is lower than 5%.

vi) Importance of rare variants: Rare or low frequency variants may have a more penetrant effect on the phenotype (415), but they require a larger sample size for association studies. Deep re-sequencing of previously associated regions provides an opportunity to detect and assess rare variants and structural variants comprehensively. In the case of the BARCOS cohort, the sample size is relatively small, thus we could not perform association study with rare variants. As an alternative approach, we grouped all rare variants found in a specific gene and compared them between the two extreme groups. However, we found that both rare variants in *WNT16* and *DKK1* were equally distributed among the two extreme BMD phenotypes, while the distribution of rare variants in *SOST* did not reach significance. Therefore, this approach did not prove useful for our cohort.

Taken together, it is important to note that the study of the BARCOS cohort is limited by its sample size. This is the case for both the full cohort as well as for the

truncated selection. Moreover, while our re-sequencing approach provides a useful means to overcome the limitation of standard GWAS studies, it is important to consider that we did not consider any intron and all the regulatory regions. Therefore, putative variants present in these regions could not be detected in our study.

While the discovery of putative causal variants is an essential aspect of this work, it is equally important to perform a functional analysis of the same variants in order to elucidate their effect and the causal relationship they have with the genetic disease. In the second chapter of this doctoral thesis, I have presented our functional characterization of the variants identified in the first article. In the following section, I will discuss our main results.

3. FUNCTIONAL STUDIES

As highlighted in the previous section, it is crucial to perform a functional study of the putative causal variants obtained with association studies or of the mutation revealed by WES. Starting from a list of putative variants identified through *in silico* studies, it is essential to characterize the effects that such variants have at the molecular and cellular levels. This is not only important to elucidate the mechanisms underlying the establishment of the phenotypic traits observed, but also to identify novel therapeutic targets and to provide a personalized treatment for the person bearing the variant. Importantly, these studies need a highly interdisciplinary approach, ranging from *in silico* analyses, such as pathogenicity predictor tools, to *in vitro* studies, including luciferase reporter or chromatin conformation assays, and *in vivo* studies using model organisms such as zebrafish or mouse. In this thesis, we have focused on *in vitro* studies to characterize the effects of the variants identified in article 1 in bone-related cells. In what follows, I will comment on the pros and cons of the main techniques used and discuss the results obtained.

3.1. *In silico* Functional Analysis of the Identified variants

The number of variants identified by both association tests and WES is massive, making the functional analysis an overwhelming task. For this reason, we need to prioritize the identified variants based on their potentially functional effect, which is determined using *in-silico* tools and online public resources. This approach not only allows us to select the most promising variants, but also provides an *a priori*

assumption of the molecular mechanisms by which a genetic variant may mediate its phenotypic impact. There is a wide variety of open source *in silico* tools available, and the appropriate one will depend on the type of variant.

In article 1 of the present thesis, among the 51 variants identified in the resequencing, 13 were selected based on different *in silico* tools for subsequent genotyping, including GTEx, Polyphen-2, SIFT and miRNA binding predictors (e.g. miRBase). In particular, 6 variants were chosen in *WNT16*. Two of them are the missense variants rs2908004 and rs2707466 and were chosen because, despite being categorized as tolerated and benign in SIFT and Polyphen-2, they are described as eQTLs of *FAM3C* and *CPED1* in GTEx and the amino acid changes involved affect biochemical properties. The third variant, rs55710688, was selected because it modifies the Kozak sequence of the *WNT16a* transcript and it might alter translation initiation rate. Furthermore, it is also described as eQTL of *FAM3C* and *WNT16*. The fourth and fifth variants (rs142005327 and rs113001389) were chosen because they map in intron 2, in a region enriched in histone marks typical of promoter/enhancer-type regulatory elements and the first of them is described as eQTL for *FAM3C* and *CPED1*. Finally, the sixth variant, rs190011371, was picked up as it is predicted to modify the 3'UTR of *WNT16b* protein and to affect the binding of certain miRNAs. In *DKK1*, we selected 2 variants. The first, rs1569198, is predicted to affect splicing, generating a larger exon, and is described as eQTL for *PRKG1-AS1*, while the second, rs74711339, was chosen because it is predicted to modify the 3'UTR, affecting the binding of certain miRNAs and is also described as eQTL for *DKK1*. In *SOST*, we selected 5 variants. One is the missense variant rs17882143, scored as tolerated and benign by SIFT and Polyphen-2, but predicted to modify the mRNA secondary structure. The second, rs570754792, was chosen because it lies within the extended TATA box motif. The third and fourth, rs1237278 and rs2023794, because they are described as eQTL of *SOST*, *MPP3* and *MPP2*, and *DUSP3* and *MPP2*, respectively. And the fifth, rs17883310, because it is predicted to modify the 3'UTR and to affect the binding of certain miRNAs. In addition to these variants, we conducted functional studies to test rs149268042, although it was not genotyped due to lack of budget. This is a *DKK1* rare missense variant present only in an HBM woman that is predicted as deleterious and probably damaging by SIFT and PolyPhen-2 and disease-causing by mutation taster.

In article 4, we used the SignallP prediction program to evaluate a defective cleavage site in mutations in the signal peptide and the Mfold web server to evaluate the most probable secondary mRNA structure for the missense variant rs17882143 located in the signal peptide of sclerostin. We obtained no functional differences in the cleavage site, however, in the presence of the minor allele, the secondary mRNA structure was predicted to be altered, which might compromise the accessibility of the protein translation machinery.

In article 5, we also made use of public databases to study regulatory elements of the genome. The clue that the region containing the rs142005327 and rs113001389 variants was a regulatory region was obtained by visualizing the post-translational modification data of histones in osteoblasts from the ENCODE project at UCSC. These marks caught our attention since, despite being an intronic region, it had typical activating regulatory elements. Classically, the regulatory elements are divided into two categories: i) promoters that recruit RNA polymerase II (RNAPII) and initiate transcription in transcription start sites (TSSs) and ii) enhancers, which amplify transcription by making contacts with the promoter region of the gene through TFs, and which can be found many bp away from the TSS but close to it in 3D conformation. In recent years, this classification has been disputed, as promoters can behave like enhancers and vice versa, and both have similar chromatin sequence and core promoter architecture (416). For example, when active, both elements initiate divergent transcription, thus generating two copies of RNA, termed sense and antisense. In the case of promoter regions, these are the messenger RNA and the promoter upstream transcript (PROMPTS), while in the case of enhancers, these are equally called eRNA. Both PROMPTS and eRNA are short RNAs that are typically degraded by the exosome. Importantly, both promoters and enhancers control gene expression in a cell-type and spatio-temporal specific manner (417) and are enriched in transcription factor binding sites (416). On the other hand, differences exist that allow us to distinguish the enhancer and promoter activities. First, 50% of the promoters fall in CpG islands, while this is not the case in enhancers, thus the overall content of CG is different between the two. Second, the amounts of associated transcriptional activity and RNAs produced is higher in the case of regions with promoter activity. Third, some histone marks such as H3K27ac (acetylation of lysine 27 in histone 3) is present in promoter regions but not in enhancer regions (416).

Similarly, the ratio of H3K4me1/H3K4me3 (mono-methylation and tri-methylation of lysine 4 in histone 3) is higher in regions with enhancer activity. Finally, while promoters present a specific orientation and position, it has been described that enhancers act independently of orientation, position, and distance.

Therefore, to characterize the *WNT16* intron 2 region containing the variants rs142005327 and rs113001389, we consulted all the characteristics provided by UCSC Genome Browser, ENCODE and GeneHancer such as histone marks, DNA methylation, binding of TFs, accessibility of chromatin, and conservation (detailed in figure 17). In addition, using the public RNA-seq data from the Sequence Read Archive (SRA) platform, we have consulted the expression levels of this region in bone cells, specifically hFOB.

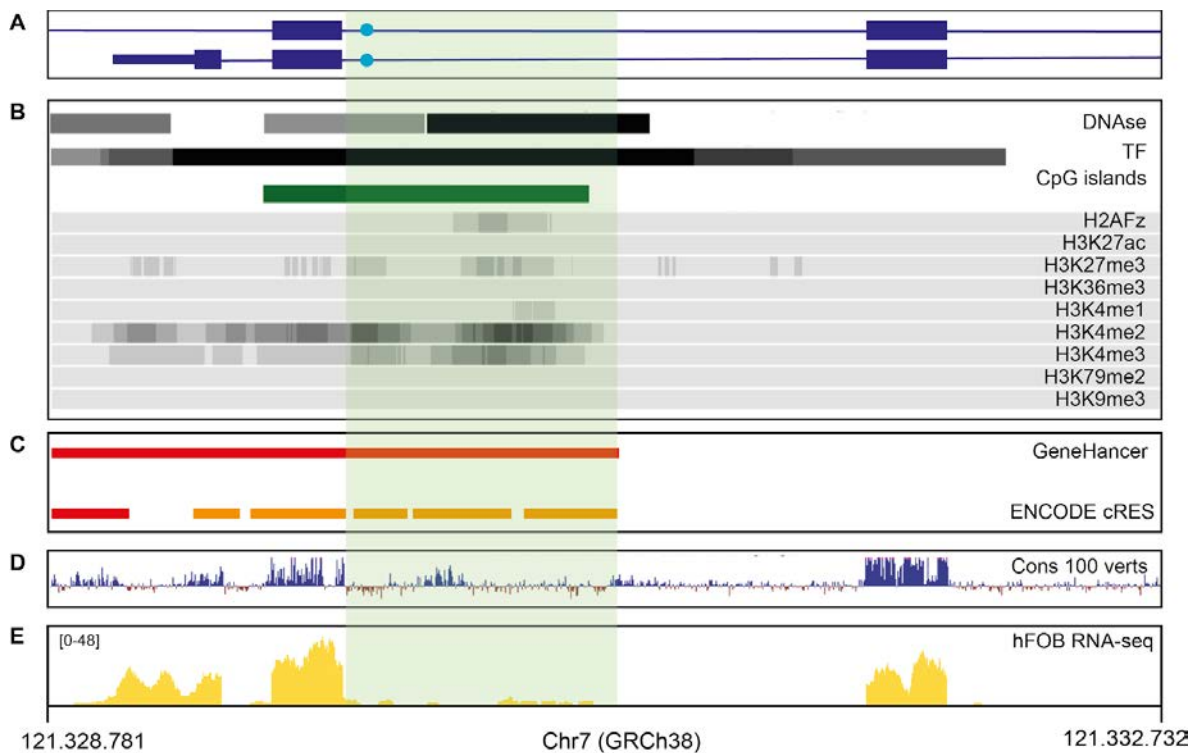


Figure 17. Clues for active regulatory region in *WNT16* intron 2. (A) *WNT16* isoforms from UCSC annotations of RefSeq RNAs; light blue dots indicate the position of rs142005327 and rs113001389 variants. (B) DNase: Hypersensitivity to DNaseI, information from 95 different cell types included in the ENCODE project. TF: Transcription Factor ChIP-seq Clusters, information from 340 factors and 129 cell types included in the ENCODE project. CpG islands from UCSC genome browser. Post-translational modifications of different histones in osteoblasts from the ENCODE project. (C) GeneHancer database of human regulatory elements included in GeneCards and ENCODE Registry of candidate cis-Regulatory Elements (cCREs) (D) Vertebrate Multiz Alignment & Conservation (100 Species) (E) Expression levels of hFOB RNA-seq in SRA.

Figure 17, panel A, shows the genes in chromosome 7 region 121328781-121332732 bp according to the *UCSC annotations of RefSeq RNAs*. Specifically, this region contains the two isoforms of *WNT16*: *WNT16a* (top) and *WNT16b* (bottom) that differ in their first exons and promoters (418). In addition, a light blue dot shows the location of the variants in intron 2, which are found close to the 3' end of exon 2. Panel B shows some of the epigenetic marks of the region of interest. Among them, we observe i) hypersensitivity to DNaseI in intron 2, which indicates accessible regions of chromatin functionally related to transcriptional activity, ii) enrichment of the TF binding sites, iii) a CpG island with 81 CpG sites, which are usually found at transcription initiation sites and are associated with promoter regions and iv) histone post-translational modifications in osteoblasts typical of promoters (low H3K4me1/H3K4me3 ratio), enhancers/promoters (H2AFz) or TF binding sites (H3K4me2). Taken together, these data suggest that the region contains a regulatory element. However, we also observe lack of H3K27ac and H3K36me3, which are typical of promoters or transcriptionally active regions, and presence of repression marks (H3K27me3) (416). Therefore, the interpretation of these results remains difficult. Panel C contains data from *GeneHancer* and *ENCODE Registry of candidate cis-Regulatory Elements (cCREs)*, which highlight the presence of regulatory elements in this region. In particular, while *GeneHancer* shows a single promoter activity mark (red) that contains the *WNT16b* promoter, *cCREs* detects several enhancer regions (orange) which are distinct from the *WNT16b* promoter region (red). Panel D shows the conservation among vertebrates: despite being lower than values observed for exons, there is a conservation peak within intron 2. Panel E represents RNAseq data publicly available from hFOB cells, showing low expression reads in the region of interest. Taken together, all these *in silico* data leads us to hypothesize that there is some active regulatory element in this region, and that its main activity and precise location are ambiguous and need further investigation. Therefore, we delimited the region of interest (light green) and carried out different functional studies to demonstrate its regulatory role (discussed below).

While in the previous paragraph *in silico* analysis was used to characterize a regulatory region, in article 6 we used it to prioritize the variants found through WES. Observing the inheritance pattern of the HBM phenotype in the HBM9 family, we hypothesized that the most likely pattern was an autosomal dominant inheritance.

Under the hypothesis that the causal variant is present in the proband and in her HBM mother in heterozygosity and absent in her brother who has a normal BMD, we found 8755 variants in 4027 genes. First, we discarded variants based on i) location outside of coding sequences (intergenic, 5' and 3' UTR, upstream, downstream, ncRNA or unknown variants), ii) frequency (greater than 0.01) and iii) prediction of no aberrant splicing for intronic variants (no effect or no intronic context). Of the original 8755, 122 variants in 111 genes passed these preliminary filters. Next, for every gene, we consulted public databases (MSK-KP, Uniprot, OMIM, and Pubmed) and obtained information on the functionality, phenotype of animal models, diseases described in humans and association in BMD GWAS. Using this approach, we prioritized 28 variants in 27 different genes. From this list, we tested their co-segregation with HBM in the family.

In this thesis, given the relatively low number of variants, it has been possible to perform a thorough manual characterization with the *in-silico* tools described so far. However, studies exist which generate a very large number of variants, thus making manual annotation extremely time consuming. In these cases, a more automated and high throughput approach is needed, such as -omic prediction tools, which allow to prioritize large collections of SNPs at the same time. For example, GARFIELD is a functional enrichment analysis approach (GARFIELD: GWAS analysis of regulatory or functional information enrichment with LD correction) that integrates association signals from GWAS with regulatory or functional annotations to find features relevant to a phenotype of interest (158).

Despite the usefulness of all these bioinformatics tools, functional prediction does not provide a definitive answer. For example, not all variants classified as "known pathogenic" have a clinical impact on their carriers. In a recent work, 17 variants were described and classified as pathogenic according to ClinVar and HGMD in 59 actionable genes. However, of the 24 confirmed carriers of these variants, only 3 cases developed a clinical event possibly caused by one of the variants (419). Therefore, direct functional analysis, for instance using *in vitro* models, is needed to fully characterize a variant.

3.2. Luciferase reporter assay

Thanks to its rapid and reliable quantification, reporter gene assays have been very useful for the study of gene expression and cell physiology. In the present doctoral thesis, I have used luciferase reporter gene assays to quantify the effect of different variants in three Wnt pathway genes (Figure 18). First, in article 4, I tested the impact of the rare variant rs570754792, located in the extended TATA box motif of *SOST*, using a promoter-luciferase approach (Figure 18A). Our results show promoter activity for the region containing the variant tested and significant differences between the two alleles, with the minor allele displaying lower transcription capacity. Second, in article 5, I use the same approach to characterize a *WNT16* intronic region containing SNP rs142005327. I tested several different intron 2 fragments and found that five of them showed promoter activity. In particular, the C-construct displayed the highest luciferase signal (Figure 18B). Third, in article 2, we used the luciferase reporter gene assays to test the effect of the DKK1-3'UTR variant rs74711339. Both alleles tested exhibited significant differences when compared with the empty vector, but they showed similar activity when compared to each other (Figure 18C). Fourth, in the same article, we studied the functionality of missense DKK1 variants with a luciferase reporter gene assay in which the transcription of the luciferase is controlled by 5 TCF/LEF sites. This allowed us to verify the activation level of the canonical Wnt pathway under different conditions. To have sufficient pathway activity, we co-transfected its three essential cDNAs, that is i) the Wnt1 ligand, ii) the LRP5 receptor and iii) mesdc2, the chaperone necessary for the correct LRP5 folding (Act. in Figure 18D). Together, we co-transfected either the WT or the mutated version of the DKK1 inhibitor. In particular, using this assay, we quantified the inhibiting capacity of DKK1 with missense mutations previously found in the general population and in women with HBM. Our results showed that the DKK1 proteins with mutations p.Ala41Thr, p.Try74Phe, p.Arg120Leu or p.Ser157Ile display decreased DKK1 inhibitory capacity (Figure 18D). In order to perform and interpret luciferase reporter assay experiments, one needs to consider several factors. In what follows, I will discuss these aspects and highlight the role they played into my work.

Luciferase reporter assays results are challenging to interpret as absolute values, but rather they need to be compared to known and quantifiable activity levels. Such levels of reference, called positive controls, provide a means to compare levels

of activity across experiments, thus providing value and credibility to the results obtained. In our studies, the positive control was chosen depending on the specific approach used. In articles 4 and 5, we have used the promoter of *COL1A1*, a gene we previously shown to be highly and reliably expressed in the cell type chosen⁽⁴²⁰⁾. We indeed found that it showed an increase in promoter activity of approximately 30 times compared to the empty vector, the maximum observed value among all the fragments and alleles tested. In article 2, in the assay of the missense variants in *DKK1*, we used the Wnt1-LRP5-mesdc2 co-transfection with or without WT *DKK1*. These two conditions exhibited the same reporter activity levels across replicates compared to the empty vector, thus representing the two extreme levels within which all mutant effects were found. In conclusion, using experiment-dependent controls, we could trust and interpret our luciferase results.

Luciferase reporter assay results heavily depend on the cell type used. All the tests have been carried out in Saos2 cells, a cell line derived from a primary osteosarcoma. This cell line has been determined to possess several osteoblastic features and could be useful as a permanent line of human osteoblast-like cells and as a source of bone-related molecules (421). The choice of cell type is particularly important for the experiments involving promoters and 3'UTR regions as it is necessary that the TF or miRNA that bind to them are expressed in it. That is why we cannot confirm that the SNP rs74711339, tested in article 2, does not affect other cell types, such as early-osteoblasts or chondrocytes, where some miRNA is expressed that might bind differently depending on the allele. In the same way, it could be that the promoter activity seen in articles 4 and 5 is higher or lower in different cell types. Therefore, it would be interesting to verify the effect in an *in vitro* system where all cell types that are important for bone determination are integrated. This and other approaches will be discussed in the next section.

It is important to consider that all the systems used the overexpression of genes in plasmid DNA, so they do not represent a physiological situation. For this reason, luciferase reporter assays do not consider the regulation in the native genomic context of the region, such as TF binding or DNA methylation. Moreover, it is challenging to control the molar amount of each transfected plasmid with sufficient accuracy and the transfection process itself is stochastic. These factors lead to cell-

to-cell variability in the amount of plasmid uptake and therefore to systematic variability in the expression levels. When the assay involves the co-transfection of several vectors, as is the case of the Wnt pathway luciferase, the variability increases further. Finally, it is possible that some of the variants tested, expected to have a small effect, show differences in the activity below the sensitivity of the reporter gene assay we have used. Therefore, several replicates of the same experiment are necessary to average out these sources of variability.

In conclusion, the luciferase reporter assay is an easy and fast approach that can be employed to test the effect of different variants, but, in the future, more physiologically-relevant methods will be necessary to confirm the results obtained.

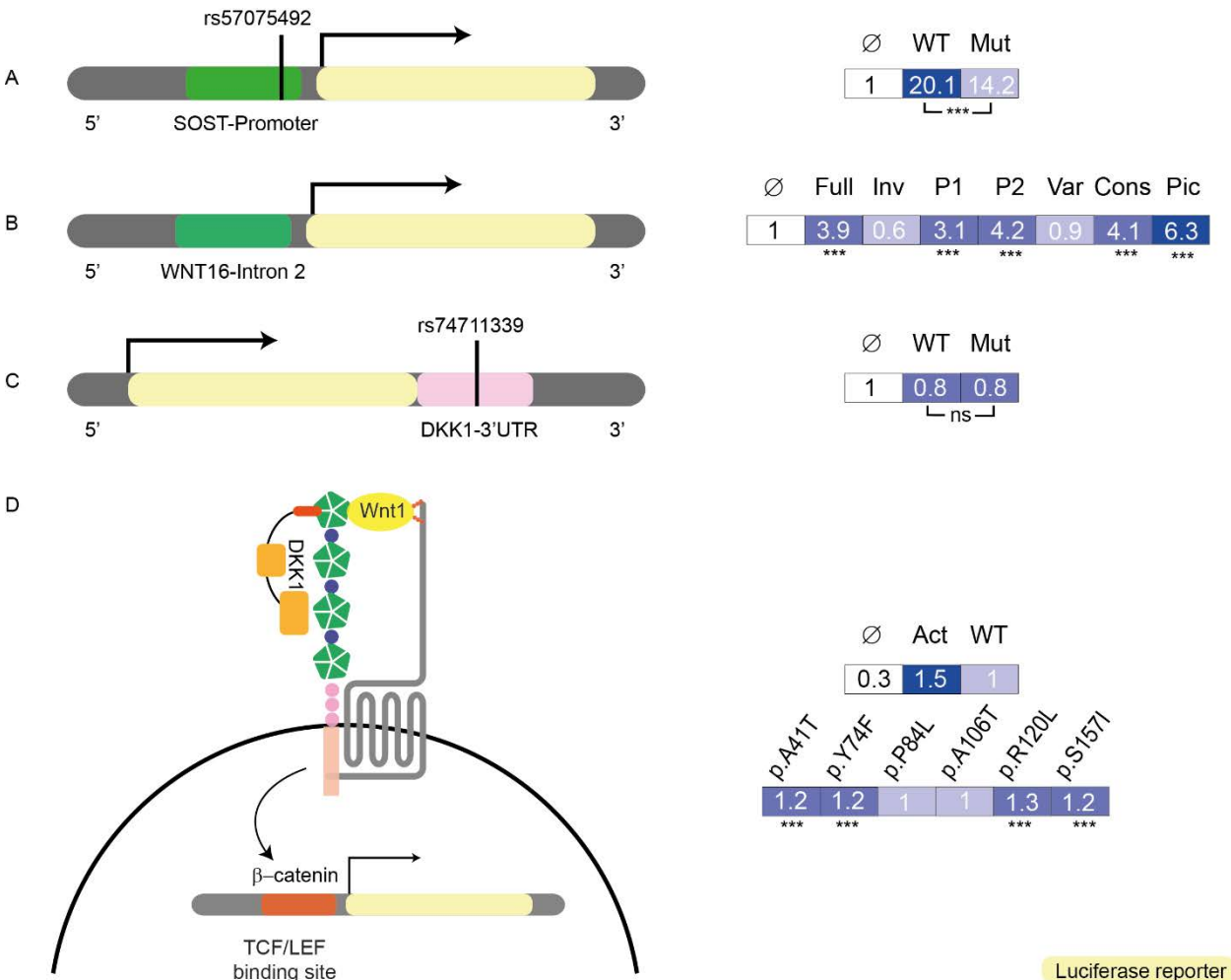


Figure 18. Design (right) and main **results** (left) of the luciferase reporter assays performed in this thesis. **Design:** A) Promoter reporter assay to test the variant rs57075492 in *SOST*. B) Promoter reporter assay to test several regions in intron 2 of *WNT16*. C) 3'UTR reporter assay to test the

variant rs74711339 in *DKK1*. D) Wnt pathway reporter assay to test the effect of missense mutations in *DKK1*. **Results;** the effects are shown with a number inside the box and a blue gradient to visualize the strength of said effect. Asterisks in panel B and D represent significance of each condition compared with the empty vector of each mutant compared with the *DKK1* WT activity, respectively. Mut. Mutant allele with respect to the ancestral one, Act : activated Wnt pathway.

3.3. Circularized Chromatin conformation capture (4C-seq)

Despite being a linear sequence of nucleotides, the genome is highly folded and organizes itself inside the nucleus in a complex 3D structure. Therefore, two regions widely separated in the number of bp can be found in close physical contact with each other. In recent years, it has been shown that this arrangement is dynamic and can vary among cell types and during cell differentiation (417,422). Importantly, it is well established that the 3D structure of the genome plays a major role in genomic regulation, as it allows promoters and enhancers to establish a TF-mediated interaction with each other, thus modifying the expression levels of the target genes. It is therefore clear that, if the target gene plays a role in disease establishment, variants in regulatory elements can still confer disease susceptibility (423). Evidence of the role of regulatory variant in disease susceptibility is found in GWAS studies, in which many of the associated SNPs are enriched in active regulatory regions (424). Nevertheless, because of the large genomic distance between the SNP region and the causal gene, establishing their association is still extremely challenging.

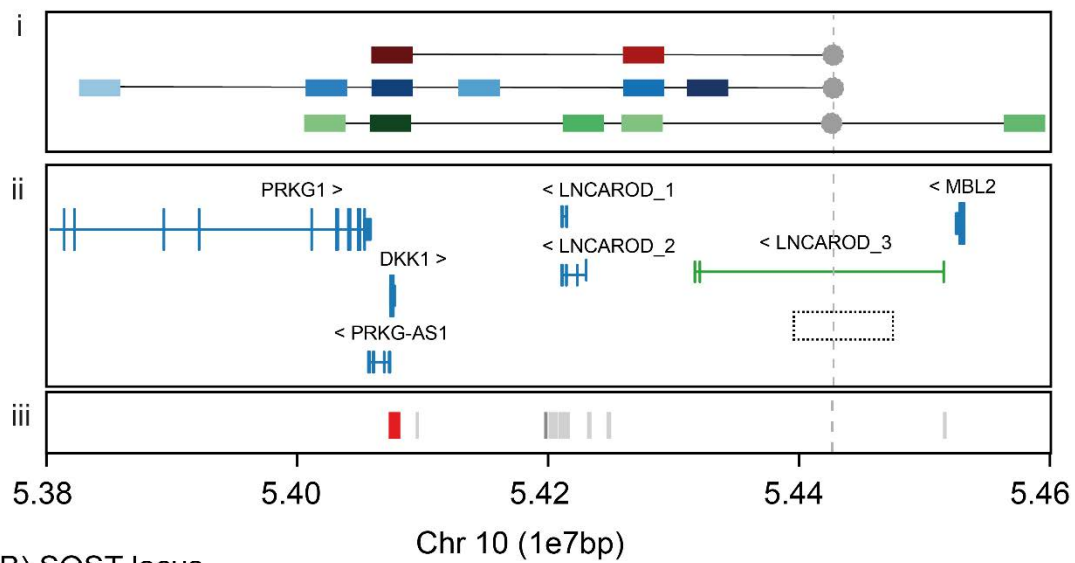
Chromosome conformation capture techniques allow us to characterize the spatial chromatin organization and quantify the physical interaction between two genomic regions. In this doctoral thesis, I have used 4C-seq techniques to identify the interactors of three genomic regions in three bone-related cell types (mesenchymal Stem Cells -MSC- red in Figure 19; human fetal osteoblasts –hFOB- blue in Figure 19; an osteosarcoma cell line –Saos2- green in Figure 19). First, in article 2 (Figure 19A), I have analyzed a region between the *DKK1* and *MBL2* genes, harboring a set of genome-wide significant SNPs associated with bone parameters in 10 different GWA studies (viewpoint, grey dot Figure 19). I performed a 4C-seq assay to determine its interactions across the genome and found that the GWA-SNP-rich region interacts with both *DKK1* and regions that harbor several newly described transcripts of

*LNCA*ROD. On the other hand, only a region several kb upstream of *MBL2* showed significant interaction in Saos2 cells. Second, in article 4, I used the *SOST* proximal promoter as a viewpoint (grey dot in Figure 19B) to elucidate important regulators of *SOST* expression. I confirmed, for the first time, the strong physical interaction between the proximal promoter and the *ECR5* distal enhancer. Moreover, I found interactions between the viewpoint and i) several genomic regions located between *MEOX1* and *ETV4*, described as enhancers according to GeneHancer, ii) a region containing exon 18 of the *DHX8* gene and iii) with the *DHX8* promoter in hFOB and Saos2 cells. Third, in article 5, I analyzed the physical interactors of the *WNT16* intron 2 region. I have first used this region as a viewpoint (grey dot in Figure 19C) and next confirmed the results using as viewpoints four of the interactors found in the first assay. In this way, I could identify reciprocal interactions (black squares) between the *WNT16* intron 2 region and different regions of *CPED1* that had been described as promoter or enhancer regions. Moreover, our data show that *FAM3C* does not strongly interact with either *CPED1* or *WNT16* (Figure 19C).

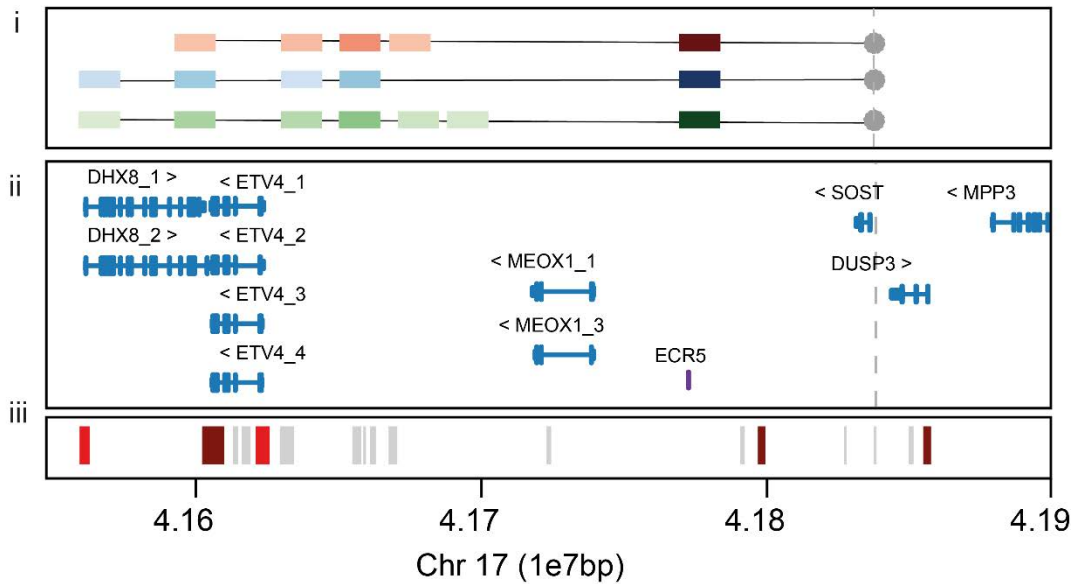
Although this technique has allowed me to successfully identify the physical interactions between the chosen viewpoints, it is limited in some crucial ways, which I will describe below. First, the resolution of the technique can be inadequate, as the capturing region contains hundreds of base pairs or even several kb, making it difficult to determine the minimum interacting region. This is particularly evident in article 5: due to the closeness between the canonical promoter of *WNT16b* and the new promoter described in the article, it is impossible to determine which of the two regions is the one that is interacting with the enhancers of *CPED1*. Second, similar to the luciferase reporter gene assays, cell type choice is crucial, since the interaction can be time- and tissue-specific. For all 4C-seq experiments performed in this thesis, I have used Saos2 cells as well as hFOB and MSCs. This strategy allowed me to identify time-specific interactions during osteoblastic differentiation. Despite using cell lines at different differentiation states, I have not found any relevant developmental stage-dependent interaction in the three experiments I have carried out. To identify tissue-specific interactions, I have analyzed the public data of the GeneHancer database, and compared my results with those for seven cell lines from different tissues. This allowed me to conclude that all the interactions observed in the *DKK1* and *SOST* regulatory regions, as well as the ones between *WNT16* and *CPED1*, are

bone-cell specific. However, interactions between *WNT16* and *FAM3C* were only found in other tissue types, but not in bone cells. Nevertheless, to confirm and extend these results, in the future it will be important to include other cell types that play a critical role in bone development and homeostasis, such as osteoclasts or chondrocytes. Lastly, it is important to identify statistically relevant interactions and to discard those that are close to the viewpoint or that do not show statistically significant strength. To this aim, we have developed a custom-written software based on previously published methods. This algorithm considers the probabilistic nature of the interactions and penalizes those that are very close to the viewpoint. In conclusion, while 4C-seq assays provide a powerful approach to help identify the complex interactions in the 3D genome, their limitations need to be considered carefully to design reliable experiments and obtain meaningful conclusions.

A) DKK1 locus



B) SOST locus



C) CPED1-WNT16-FAM3C locus

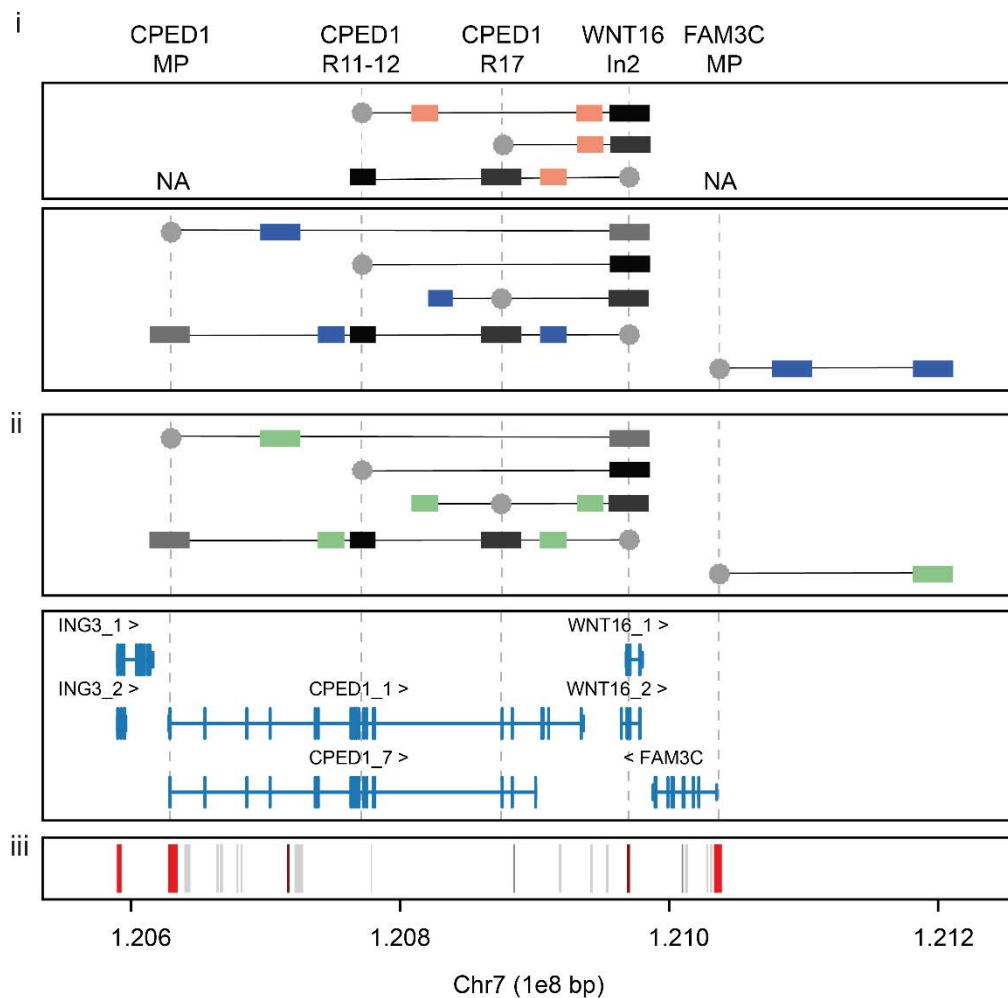


Figure 19. Main results of the 4C-seq assays. Panel A, B and C represent the interactions tested for DKK1, SOST and WNT16 locus. In Panel A,i) and B,i) the grey dot represents the viewpoint and the colored squares the interacting regions, with color intensity proportional to the interaction strength. In Panel C,i), the grey dots represent the viewpoint while the squares represent either reciprocal interactions (gray scaled according to interaction strength) or the strongest interactions in each cell line and for each viewpoint (colored). In all cases, red boxes represent interactions in MSC, blue boxes in hFOB and green boxes in Saos2 cells. Panels ii) contain the genes in the genomic region from GRCh37/hg19. Panels iii) contain the GeneHancer tracks. NA: Not available.

3.4. Western blot

Western-blot assays are among the most widely used techniques in molecular biology to detect the presence of a desired protein. In the present doctoral thesis, I have used this technique to detect the DKK1 and sclerostin proteins in articles 2 and 5. In article 2, I have verified the presence of DKK1 in the extracellular space, and I have seen that in almost all mutants it is expressed in equal or greater amounts than the WT

control. The exception was found in DKK1 protein with the p.Ser157Ile mutation which shows less protein in the extracellular space. In article 4, I have tested the presence of mutated sclerostin in the extracellular and intracellular space. This allowed us to demonstrate a reduction in protein expression for the protein with the p.Val10Ile mutation since it showed a decrease in expression both in the extracellular and intracellular space compared to the WT protein.

When performing western-blot assays, it is important to consider their limitations, and possibly complement the analysis using other techniques in parallel. The first limitation is that the method does not allow us to determine protein localization within the subcellular compartments. This could be solved with an immunocytochemical assay or using specific protein extraction protocols for every compartment. Furthermore, to perform a highly quantitative assay, other approaches exist, such as Enzyme-Linked ImmunoSorbent Assay (ELISA), which allows the quantification of protein expression levels and therefore represents an ideal tool to perform statistical analysis.

3.5. eQTLs

When assessing whether a variant influences the expression of a gene (eQTL), the cell type chosen is especially crucial since the effect can be tissue and time-specific. This was highlighted in a study that used single-cell RNA sequencing to generate expression profiles of ~ 25,000 peripheral blood mononuclear cells from 45 donors. This study identified eQTLs with opposite effects in different cell types (425). For this reason, in this doctoral thesis, I have used 45 primary osteoblast cells to test the putative eQTLs. These were obtained with an in-house collection pipeline since bone tissues are not available in the GTEx database. Using this collection, in article 2, we have studied the SNPs rs1569198, rs74711339, and rs1373004 and their relationship to the expression levels of *DKK1*. However, none of the tested SNPs resulted as eQTLs in our samples. The main limitation of our study is represented by the limited sample size. Due to the difficulties in obtaining appropriate samples, we only used osteoblasts from 45 donors, which precluded testing SNPs with low allele frequency. It would be useful to have a bank of primary osteoblasts with a much higher sample size to get information on different eQTLs important for bone, which are lacking in current databases.

4. OTHER *IN VITRO* APPROXIMATIONS AND CELL TYPES

To test the pathogenicity of a variant *in vitro* and recapitulate the phenotype observed, it is important to choose an appropriate cell type and mimic the genetic modification. First, cell type choice for functional analyses of variants involved in bone disease is challenging, as bone tissue requires a delicate interaction between different cell types for its correct function both in development and in bone homeostasis. Furthermore, the pathophysiology of a disease often involves the interaction of widely varied cell types, thus making it challenging to rely on a single cell type for *in vitro* studies. In this doctoral thesis, we have used cell lines derived from osteosarcoma (Saos2) for most of the experiments, and we used hFOB and bone marrow-derived MSC for chromatin conformation assays. For a complete characterization of bone tissue, it would be necessary to consider other cell types such as osteoclasts, osteocytes, lining cells, reversal cells, etc. (426,427). Alternative to the use of specific cell types, it is now possible to reprogram fibroblast cells from patients into induced pluripotent stem cells (iPSCs) and subsequently differentiate them into the bone cell of choice. Thanks to the fact that these cells have the genetic background of the patient, they resemble the real biological context to a higher degree (428,429). Despite the power of iPSCs, the most suitable option is the use of primary cells obtained from patient biopsies. However, it is extremely difficult to obtain such primary material, making these studies very complicated. In this thesis, we have had access to 45 human bone biopsies that we have used for the osteoblast-eQTL analyses in article 2. Despite the priceless value of the cohort, its statistical power is minimal, therefore it will be necessary to expand the number of primary osteoblasts to identify the association between genetic variants and expression levels.

Regarding the introduction of genetic modification, there is a wide variety of genetic engineering techniques that can be used. The simplest one, which was used in the present thesis, is the transfection of a plasmid containing the desired mutation. Furthermore, the expression of a specific gene can be eliminated by acting on the gene itself (knock-out; KO) or on the mRNA (knockdown; KD). In recent years, the simplest and fastest strategy to generate a KO is using the genome editing technique Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 (430,431). In addition to generating KO models, this technique offers the possibility of

introducing specific alterations in a given gene, such as insertions or point variants, and deleting regulatory elements. Interference RNA (iRNA) or a variant of CRISPR-based techniques with a deficient Cas9 (dCas9) can be used to generate a KD model. In our group, the iRNA knockdown of *GGPS1* was successfully used to generate a cellular model in osteoblasts and osteoclasts. Using this model, it was shown that osteoblasts expressing low levels of *GGPS1* have a strong mineralization reduction and a reduced expression of *OCN*, *OSX*, and *RANKL*, whereas osteoclasts with this same gene knocked down have lower resorption activity (432). Moreover, variants of the CRISPR-Cas9 system in which dCas9 is fused to repressors or activators can be used for the study of regulatory regions (416). These genome editing strategies can be applied in cell lines (for example, in Saos2) or in WT iPSCs that will later differentiate into a bone cell type, and are very useful when the primary material is not available. Once the cell model has been generated, it is necessary to characterize it. In the case of osteoblasts, their mineralization capacity can be tested through Alizarin Red S staining, while in the case of osteoclasts, their differentiation and function can be tested through TRAP staining. In addition, various markers for the correct function or differentiation can be measured both at the protein or RNA level. Another important *in vitro* technique is the massively parallel reporter assay (MPRA), which uses high throughput sequencing and barcode quantification to measure several putative enhancers and promoters simultaneously (416). In order to take into account the native chromatin context, lentiviral MPRA that test large numbers of candidate regulatory elements have been developed (433).

It is important to appreciate that, *in vivo*, the phenotypes emerge from the dynamic and complex interplay among several cell types. However, classical *in vitro* approaches usually use cultures of single cell types, thus severely simplifying the biological system. Co-culturing multiple cell types to reconstitute a complex structure would allow us to reach a better approximation of the *in vivo* physiology (434). Nevertheless, cultures of cells in 2D are fundamentally limited, as they lack essential elements of the *in vivo* biological context, such as the 3D environment, shear stresses and mechanotaxis. Alternatively, it is now possible to culture progenitor cells into a 3D biocompatible matrix or on complex microfluidic devices that allow to reproduce the native bone tissue environment (435,436). Moreover, using dynamic bioreactors that

introduce fluid movement, it is possible to facilitate a more homogenous environment, thus mimicking the *in vivo* physiology more closely (437).

A new tool that is being applied in the field in recent years is artificial intelligence (438). Both machine learning and deep learning can aid data analysis in several crucial ways, including but not limited to i) predict a fragility fracture by capturing underlying trends and patterns, which would be impossible with other modeling tools ii) segmentation of images from patients to detect bone features in an automated fashion and iii) prediction variant pathogenicity (e.g., CADD).

In conclusion, together with the establishment of patient-like cells, engineering and data analysis approaches will allow us to generate *in vitro* disease models that are experimentally tractable and to extract quantitative features and predictions. These techniques will thus advance our understanding of bone diseases, and have a great potential to aid the development of new therapeutic strategies.

5. IN VIVO APPROACHES

Animal models are essential tools to characterize the molecular and biological bases of a disease, to identify new genes involved in the disease, to explore the effect and resilience of drugs, and to develop new therapeutic approaches. Animal models can capture the complexity of the biological events in the organism and control other characteristics that may influence, such as environmental control and reproducibility (439). In particular, for the study of skeletal diseases, two vertebrate animal models stand up: mice and, more recently, zebrafish. Both meet a series of characteristics that make them optimal for the test of skeletal pathologies, including i) similarity to human genetic and skeletal system, ii) high fecundity, iii) short life cycle and gestation times and iv) availability of techniques for genome modification (440,441). Although, due to its close evolutionary relationship with humans, the most typical choice is the mouse, the use of zebrafish is increasing thanks to its cost-effective maintenance and the ease of doing microscopy imaging of the translucent embryos.

Within the genome modification techniques, a classical approach consists of random mutagenesis induced by chemicals such as N-ethyl-N-nitrosourea followed by skeletal phenotypic screening (303,304,442). An example is the hypomorphic doubleridge mouse that bears a 60-kb deletion (located 150 kb from *Dkk1* and 90 kb

from *Mbl2*) and displays a drastic drop in *Dkk1* expression levels (between 35% and <1% of WT) while keeping the levels of neighboring *Mbl2* and *Prkg1* genes intact (303,304). Interestingly, the deleted region could correspond to the BMD-GWAS-SNP-rich region studied in article 2, and that could contain some crucial regulatory elements for the expression of *DKK1*. Another approach involves the overexpression of exogenous genes in the animal model. Although of great value, the limitations of this type of animal model must be considered since overexpression occurs in all cell types, even in those where the gene is not usually expressed. This effect can be seen in the *Wnt16*-overexpressing mouse, which presents its phenotype in both cortical and trabecular bone, while the *Wnt16*-KO mouse only shows the phenotype in cortical bone. This difference lies in the fact that the expression of *Wnt16* is restricted to cortical bone (156,169,384,395,396). Additionally, it is possible to generate KO mice using gene-editing technologies such as meganucleases, zinc finger nucleases, transcription activator-like effector nuclease (TALENs) and CRISPR-Cas9 (443–445). This is a useful tool to understand the effect of a candidate gene on the bone phenotype. An example is the *Wnt16*-KO mouse, which presents spontaneous fractures due to low BMD, low cortical thickness, reduced bone strength, and high cortical porosity (156,169,384). In addition, in these mice, it was ruled out that the effect observed was due to the elimination of a regulatory element of neighboring genes since the quantification by RT-qPCR showed the expression levels of *Fam3c* and *Cped1* intact. Other approaches include conditional and inducible knockout models that can be generated using Cre/loxP recombination system to allow gene loss at specific cells and tissues and at the desired time (445). Finally, it is possible to generate KD models using antisense RNA morpholinos, iRNA, or dCas9 (446).

Regarding animal models used to study postmenopausal osteoporosis, the most popular one is the ovariectomized mouse, which displays drastically lowered estrogen levels and increased bone turnover (447). In zebrafish, chemically induced osteoporotic models using high iron stress (448) and glucocorticoids (e.g. prednisolone) have been shown to resemble an osteoporotic phenotype (449).

Once the animal model is generated, the phenotype can be characterized through biomechanical markers, BMD measurements, 3D microarchitecture, dynamic bone histomorphometry and serological analysis of bone formation and resorption

markers (450–452). Importantly, these models can also become a source of primary bone cells. Nevertheless, drawbacks exist that must be considered, such as the difficulty of *in vivo* cell imaging, the time required to generate the model (typically in the order of a year), statistical and ethical problems, and a lack of standardized analysis protocols. To address this last challenge, standardized high-throughput screens for KO have been generated by the International phenotyping consortium (IMPC; (453)) and by the origins of bone and cartilage disease (OBCD; (454)).

6. BIOLOGICAL SIGNIFICANCE AND EFFECT OF THE VARIANTS STUDIED

The approaches used in the present work allowed us to elucidate the functionality of the variants studied. In this section I will discuss the biological significance of our results.

6.1. DKK1

rs74711339: The 3'UTR LFV rs74711339 showed significant differences in the genotypic frequency between the two extreme BMD groups, but only a tendency in the association analysis in the full cohort. This apparent discrepancy could result from the statistical cohort power, which only allowed us to identify common variants with high or moderate effects. In addition, the functional validation of this variant indicated that a fragment of the 3'UTR of DKK1 containing the rs74711339 variant is involved in transcript expression or stabilization in Saos2 cells. However, this regulation was independent of the specific allele tested. A regulatory role of this variant in other cell types or different developmental stages cannot be ruled out.

Missense variants of DKK1: The DKK1 missense variants that showed a decrease in inhibitory capacity (p.Ala41Thr, p.Try74Phe, p.Arg120Leu and p.Ser157Ile; article 2) are some of the most frequent missense variants of this gene in the general population. Among them, the p.Try74Phe and p.Arg120Leu mutations have been found in HBM patients. Considering the results obtained, we propose that the loss-of-function of the protein with the minor allele of p.Arg120Leu and p.Try74Phe mutations may be contributing to the HBM phenotype of these women through insufficient DKK1-mediated inhibition of the Wnt pathway. The p.Arg120Leu mutation modifies the CRD1 domain, which exerts independent signaling function (other than the canonical

Wnt pathway), through unknown receptors or pathways (455). However, this functional result obtained for this mutation does not agree with the study by Korvala et al. (266), in which a lower inhibitory capacity was not found. These authors and others (266,456) have found this mutation in patients with primary osteoporosis, a phenotype that does not fit with the loss of function of DKK1 and is opposed to the HBM phenotype of our patient. These discrepancies may be due to a reduced penetrance, that depending on the genetic background in which it is found, it may not show its effect. Similarly, the p.Ala41Thr and p.Ser157Ile variants present in the general population could cause a non-pathological increase in BMD that has not been reported yet. The p.Ala41Thr is located in the NXI motif domain (amino acids 40-44), which is crucial for binding to the 1st β -propeller of LRP5/6 proteins and is present in all Wnt pathway inhibitors (106). For these reasons, the loss of inhibitory capacity can result from the loss of affinity of DKK1 to the LRP5/6 coreceptors, thus making the inhibition of the Wnt pathway impossible. Finally, the minor allele of the p.Ser157Ile mutation produces a reduced secretion of the protein to the extracellular space, which, by itself, could explain the loss of inhibitory capacity. This may be due, among other reasons, to the fact that the mutation reduces the stability of the mRNA or protein, thus causing their degradation before reaching their final destination. All the missense variants that showed significant differences also presented small effects. It is important to notice that the variants studied are often found in heterozygosity, both in the HBM women and in the general population, and therefore their effect is further reduced. In conclusion, in this thesis, we have been able to identify and functionally characterize several variants that might be responsible for the HBM phenotype of two women for which the origin of the trait had not been previously identified.

In this thesis, we have also worked on the relationship between missense mutations in DKK1, BMD, and Chiari malformation type I (CMI). In particular, we were motivated by the previous observation that the mutations p.Arg120Leu and p.Ala41Thr had been associated with CMI (123) and that bone development plays a major role in CMI establishment. Among the 12 CMI patients studied in our preliminary work, we did not identify any missense variant in DKK1. Moreover, only one of the patients had high BMD values, very close to the HBM phenotype values. Therefore, the putative relationship between DKK1 mutations, CMI and HBM is still unknown. In

the future, it will be important to extend these results using a larger cohort in order to test our hypothesis.

BMD GWAS-SNP-RICH region: We have identified significant physical interactions between a region rich in BMD-associated GWAS signals and 1) the *DKK1* gene and 2) several transcripts of the lncRNA *LNCAROD*. In contrast, we have found no relevant interaction with *MBL2*, its closest gene. Interestingly, the *LNCAROD* has been recently shown to enhance *DKK1* transcription, and requires chromosomal proximity between the A-ROD and the *DKK1* loci (457). Our 4C-seq data agree with these results since our viewpoint shows interactions with both *DKK1* and *LNCAROD* and suggests a regulatory role for the GWA-SNP-rich region. It should be noted that in the study by Ntini et al. (457), only short forms of *LNCAROD* were considered, while the last build of the genome (hg38) includes new longer versions that overlap with the GWA-SNP-rich region. The regulatory role of the GWA-SNP-rich region is further supported by the doubleridge spontaneous mouse that displays a drastic drop in *Dkk1* expression levels caused by a 60 kb deletion (located 150kb from *Dkk1* and 90kb from *Mbl2*)(303,304). This deletion probably overlaps with the GWAS-SNP-rich region, and for this reason, it would be interesting to verify if *LNCAROD* is under expressed in this mouse model, which would confirm the role of the lncRNA in *DKK1* regulation. Our results clearly show, for the first time, that this region is relevant for the regulation of *DKK1*, and, in the future, the assessment of the specific role of the different SNPs in this process will be investigated.

6.2. SOST

rs570754792: The rare variant rs570754792 was found in heterozygosity in only three women of the BARCOS cohort, all three with BMD values below the mean (LS-BMD: 0.767, 0.694, 0.822 vs. 0.850 g/cm²). According to the established inhibitory role of SOST in the canonical Wnt pathway, we investigated whether SOST was expressed at levels higher than average. However, we observed low levels of expression in the presence of the minor allele (T). This result is not unexpected since the rare variant is found in the extended TATA box, and the major and minor alleles represent the canonical and non-canonical base at the position -6. However, the low BMD values observed in the three carriers are inconsistent with the lower *SOST* expression levels.

This scenario could be explained by several hypotheses, such as i) the lack of genetic context in the luciferase experiments, which may mask the real effect of this variant, ii) the low effect of the variant observed in *SOST* expression, which, combined with the heterozygosity of the women, might be masked by other mutations or environmental factors, and iii) the allele is recessive and, similar to the situation observed in the van Buchem disease, two copies of a low expressing allele are needed to show the phenotype.

rs17882143: This low-frequency variant showed nominal significant association with LS-BMD, where the minor allele was protective. This variant produces a missense change in the signal peptide of the protein sclerostin. In the functional validation of this mutation, we have been able to verify a decrease in the expression levels of the sclerostin protein both in the extracellular and intracellular spaces, which seems to indicate a defect in a stage previous to protein translocation and secretion. These results are consistent with the phenotype of an HBM woman with this mutation and the protective effect shown by the minority allele in the association study. With these results, we can hypothesize that the rs17882143 variant can cause the establishment of a high BMD. Depending on the background of the carrier, it can become an HBM or only a slight increase in BMD. Nevertheless, we observed that the effect is moderate, therefore the genetic background could mask the rise in BMD completely. In the future, experiments aimed at quantifying the sclerostin amount in patients carrying the mutation could confirm the results shown in this thesis.

SOST proximal promoter: In order to identify the main regulators of the expression of *SOST* in bone cells, we have performed a 4C-seq assay. With this approach, we showed, for the first time, physical interaction between *ECR5* and *SOST* proximal promoter in 3 bone-relevant cell lines. Furthermore, we identified the *SOST* proximal promoter interaction with several putative enhancer elements located between *MEOX1* and *EVT4*. While the regulatory elements appear as non-tissue specific, their interactions with the *SOST* proximal promoter occur as tissue-specific (according to GeneHancer). In the future, it would be interesting to investigate the role of these enhancers in bone-specific *SOST* expression through reporter gene assay and quantification of RNA expression. We also identified a significant interaction between the *SOST* promoter and a regulatory element overlapping exon 18 of *DHX8*, a gene

encoding a spliceosome component. Overall, a picture emerges in which several long-distance enhancers participate in regulating *SOST* in bone cells. Whether they work in an additive way or as alternative redundant enhancers is an interesting question for future research.

6.3. WNT16

Intron 2: We have demonstrated the promoter activity of part of intron 2, and its physical interaction, in bone-related cells, with different putative regulatory regions within *CPED1*. This promoter region contains the variant rs73440215, which is in high LD with the GWAS sentinel SNP and is associated with heel BMD (151). The promoter activity of the C fragment suggests that this variant could be the one responsible for the association with BMD and fracture risk. We found that the C region is expressed in hFOB cells. Therefore, we translated this region into protein and aligned it to all protein sequences with BLAST. Interestingly, the only match we found corresponded to the Wnt16 protein of a microbat. After further analysis, we unexpectedly found that this bat protein is formed by a first exon strikingly similar to the C region. The microbat protein is completed by two more exons that correspond to exons 3 and 4 of the human WNT16. These results confirm that a protein containing the amino acids coded by this previously undescribed human exon exists at least in one other species. The interaction between *CPED1* and *WNT16* that we report is novel and may be bone cell type-specific since it does not appear in the Genenhancer database where different cell lines are included. However, it is still an open question whether the *WNT16* region is regulating *CPED1* expression or vice versa. Previous studies in a mouse model have demonstrated that a deletion of *Wnt16*, including the alternative promoter region, did not affect *CPED1* expression (384). Therefore, the interaction observed here is likely due to a regulatory role of *CPED1* sequences on *WNT16* expression. Based on these results, many exciting works can be started to extend and complement them. These range from the generation of a KO model where the putative regulatory elements of *WNT16* and *CPED1* are eliminated to the characterization of the putative new isoform specific to bone cells.

6.4. HBM family

In the study of the genetic bases of the HBM9 family, we have identified a promising variant in *VAV3* that could be playing an important role in the establishment of HBM.

This finding is consistent with the phenotype of the *VAV3*-KO mouse, which presents high bone mass due to an impaired bone resorption. We have also identified another variant in *SIK3* that could enhance these effects due to its involvement in bone biology, through an impaired osteoblast and osteoclast function. The two variants were present in the two individuals with HBM, but not in the daughter of the proband, with BMD values close -but below- the HBM threshold (near-HBM daughter). From these results our hypothesis is that there could be more than one variant at play to explain the HBM phenotype in the family and that the lack of some of them, including the *VAV3* and *SIK3* mutations, in the daughter only results in high BMD, without reaching HBM levels. Therefore, future work will be necessary to find additional variants to explain the phenotype of the near-HBM daughter. Moreover, functional assays will be necessary to confirm the pathogenicity of the variants in *VAV3* and *SIK3*. If these results are confirmed, *VAV3* might emerge as a novel therapeutic target for the treatment of osteoporosis.

In the present doctoral thesis, I have addressed three GWAs hits found at *loci* of three crucial genes of the Wnt pathway: *DKK1*, *SOST*, and *WNT16*. I performed an in-depth search for variants in the three genes and, for many of them, I conducted a functional validation using *in vitro* approaches. As a result, I have identified several rare or low-frequency variants that showed functional activity and therefore could cause aberrant Wnt signaling activation. Of all the variants studied, none of them showed strong effects. This result is consistent with a picture in which the identified variants contribute, through a sum of small effects, in establishing polygenic diseases such as osteoporosis or some types of HBM. The exploratory approach used to study genetic variants also allowed us to identify unexpected functional regions that were, due to their genomic location, previously unexplored. The studies performed here reinforce the role of regulatory regions in bone biology and highlight that it will be necessary to elucidate the regulators of each gene and their specific time- and cell-dependent role. Importantly, the knowledge acquired could then be translated into new therapeutic targets. Another successful way to find new therapeutic targets is by identifying the gene that causes monogenic skeletal bone diseases. In this line, we

sequenced the exome in a familial case of HBM and identified a variant in *VAV3*, an interesting putative target for an anti-resorptive treatment of osteoporosis. Nevertheless, a functional validation of the variant will be needed in the future to confirm its implication.

CONCLUSIONS

Conclusions from the identification of rare and common variants in three Wnt pathway genes associated with bone mineral density:

- Six common and low frequency variants (*WNT16*: rs55710688, rs2908004, rs142005327, rs2707466, *DKK1*: rs1569198, *SOST*: rs17882143), nominally associated with LS-BMD in the BARCOS cohort of Spanish postmenopausal women, were identified.
- Likewise, the *WNT16* haplotype including the four associated variants plus the GWAS hit from Estrada *et al.* 2012 show nominal association with the LS-BMD.
- Two of the six associated variants (rs55710688 and rs142005327), withstood Bonferroni multiple testing corrections and are located in regulatory regions of *WNT16*.
- The *DKK1* 3'UTR low frequency variant rs74711339 showed significant differences in genotype frequencies between the two extreme BMD groups.
- Four rare variants (the *DKK1* missense variant rs1492268042; the *SOST* TATA box variant rs557054792; the *WNT16* 3'UTR variant rs190011371 and the *WNT16* putative regulatory variant rs113001389) may help determine a low or high BMD phenotype based both on their frequency distribution and on their putative functionality.

Conclusions from the functional characterization of the most interesting variants in *DKK1*, *SOST* and *WNT16*.

- Although a *DKK1* 3'UTR fragment containing variant rs74711339 is involved in transcript expression or stabilization in Saos2 cells, this effect is independent of the variant allele.
- The *DKK1* missense variants p.Tyr74Phe and p.Arg120Leu display a reduced *DKK1* inhibitory capacity, consistent with the HBM phenotype of women bearing these changes.
- The *DKK1* missense variants p.Ala41Thr and p.Ser157Ile, present in the general population, also showed a decrease in the *DKK1* inhibitory capacity that might cause a non-pathological HBM that has not been reported yet.
- For the *DKK1* missense variant p.Ser157Ile the decreased *DKK1* inhibitory capacity is attributable to a reduced secretion of the protein to the extracellular space.

- The region containing 24 BMD-GWA-SNPs, located 350-kb downstream of *DKK1*, interacts both with *DKK1* itself and with the lncRNA gene *LNCAROD*, known to enhance *DKK1* expression in osteoblastic cells.
- The attempt to find an association between Chiari malformation type I, BMD and *DKK1* missense variants was inconclusive due to the small size of the cohort.
- The minor allele of variant rs570754792, located in the extended TATA box motif of *SOST*, displayed a significant decrease in promoter activity.
- The *SOST* missense variant p.Val10Ile showed reduced sclerostin expression both in the intracellular and extracellular compartments consistent with the HBM phenotype of women carrying this variant.
- The *SOST* proximal promoter physically interacts with downstream sequences, in particular with the ECR5 enhancer, several additional enhancers located between *ETV4* and *MEOX1* and a distant region containing exon 18 of *DHX8*.
- Several fragments within *WNT16* intron 2 showed promoter activity. However, the presence of any regulatory activity in the smaller fragment containing variant rs142005327 was discarded.
- Physical interactions between the proximal part of *WNT16* intron 2 and several putative regulatory regions within *CPED1* have been identified.

Conclusions from the identification of genes that could explain Mendelian cases of the HBM phenotype:

- The *SOST* missense variant p.Val10Ile may be in part responsible for the HBM phenotype in an HBM woman.
- A missense variant in *VAV3* co-segregating with HBM in a family could play a central role in the establishment of HBM, consistent with the HBM phenotype of the Vav3- KO mouse.
- In addition, a missense variant in *SIK3* also co-segregating with the phenotype could enhance VAV3 effect.

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ANNEX

OTHER ARTICLES RELATED TO THIS THESIS

1. Martínez-Gil N., Ugartondo N., Grinberg D., Balcells S. Wnt pathway extracellular components and their essential roles in bone homeostasis. To be submitted.
2. Herrera S, Soriano R, Nogués X, Güerri-Fernandez R, Grinberg D, García-Giralt N, Martínez-Gil N, Castejón S, González-Lizarán A, Balcells S, Díez-Pérez A. Discrepancy between bone density and bone material strength index in three siblings with Camurati-Engelmann disease. *Osteoporos Int.* 2017 Dec;28(12):3489-3493. doi: 10.1007/s00198-017-4198-6. Epub 2017 Aug 25. PMID: 28842728.
3. Roca-Ayats N, Martínez-Gil N, Cozar M, Gerousi M, Garcia-Giralt N, Ovejero D, Mellibovsky L, Nogués X, Díez-Pérez A, Grinberg D, Balcells S. Functional characterization of the C7ORF76 genomic region, a prominent GWAS signal for osteoporosis in 7q21.3. *Bone.* 2019 Jun;123:39-47. doi: 10.1016/j.bone.2019.03.014. Epub 2019 Mar 14. PMID: 30878523.

WNT PATHWAY EXTRACELLULAR COMPONENTS AND THEIR ESSENTIAL ROLES IN BONE HOMEOSTASIS

Núria Martínez-Gil, Nerea Ugartondo, Daniel Grinberg, Susanna Balcells

¹ Department of Genetics, Microbiology and Statistics, Faculty of Biology, Universitat de Barcelona, CIBERER, IBUB, IRSJD, Barcelona, Spain.

1. INTRODUCTION

The Wnt pathway (Wingless-type mouse mammary tumor virus integration site) is an ubiquitous pathway involved in a wide variety of cellular processes, and is highly conserved across species. It was first described for its role in carcinogenesis, and later demonstrated to be essential for embryonic patterning and for maintaining adult tissues [1–3]. In the skeletal tissue, the Wnt pathway is involved in the differentiation of osteoblasts, osteoclasts and chondrocytes (Box 1). Therefore, it has a very important role in both skeletal development and bone remodeling (Box 2 and 3). Additionally, it has an essential function in mechanical communication (Box 4).

Historically, the Wnt pathway is categorized into β -catenin-dependent (canonical pathway) and β -catenin independent (non-canonical pathways) (Figure 1). Although some studies postulate that different Wnts activate either the canonical or the non-canonical pathways, it has also been reported that some Wnt ligands (e.g., WNT5A) can act on both pathways [4,5]. In all cases, the Wnt ligands bind to one of the 10 Frizzled family receptors (FZD) and optionally to a co-receptor that can be from the low-density lipoprotein receptor-related protein (LRP) family (LRP5/6) or a transmembrane tyrosine kinase (ROR1/2 or RYK)[6–8]. The FZDs are seven-pass transmembrane G-protein receptors that can transmit signals through dishevelled proteins or through heterotrimeric G proteins [9,10]. Although the canonical pathway has received much attention, there is increasing evidence on the role of the non-canonical pathways in bone and the crosstalk between the pathways [11–13]. The canonical Wnt pathway begins with the formation of a heterotrimeric complex consisting of a FZD, a Wnt ligand and a LRP co-receptor (Figure 1). This binding results in the stabilization and translocation of β -catenin into the nucleus, where it activates the transcription of important target genes. When the pathway is not active, the ‘destructive complex’ phosphorylates and sequesters β -catenin in the cytoplasm for its subsequent degradation in the proteasome. The canonical Wnt pathway is regulated by a series of extracellular inhibitors common to all the Wnt pathways (e.g., Secreted frizzled-related proteins (SFRP) and Wnt Inhibitory factor 1 (WIF-1)) but also by specific canonical Wnt pathway inhibitors that block the LRP5/6 binding (e.g., sclerostin and DKK1) [12,13]. In addition to the canonical pathway, Wnt ligands can activate multiple signaling cascades independent of β -catenin. Within this category the most widely studied are the WNT-planar cell polarity (WNT-PCP) and the WNT-calcium (WNT- Ca^{2+}) pathways (Figure 1) [14–17]. The WNT-PCP pathway begins with the binding of a Wnt ligand, such as WNT5A, to FZD and to ROR1/2 or RYK co-receptors. This binding activates small G proteins, such as Rac and Rho, which are implicated in the establishment of cell polarity and cell migration [18]. In addition, the activation of these pathways may inhibit the canonical Wnt pathway [19,20]. The WNT- Ca^{2+} pathway regulates calcium release from the endoplasmic reticulum in order to control intracellular calcium levels. It begins with the binding of a Wnt ligand, such as WNT5A, to FZD, which in turn activates the phospholipase C (PLC). Cell adhesion, migration and embryonic development are regulated through this pathway [21].

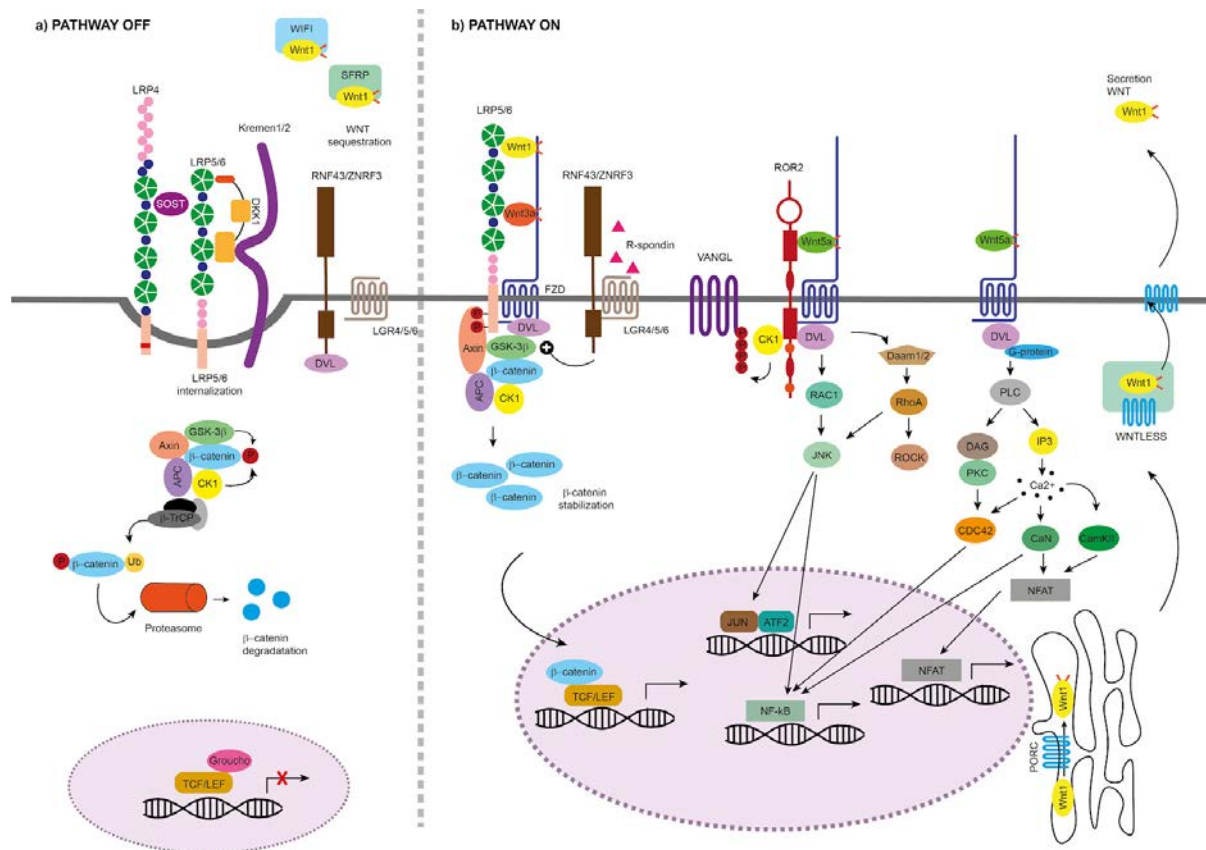


Figure 1. Wnt pathway. Wnt pathway OFF: The Wnt pathway is inactive in absence of Wnt ligands or by the effect of extracellular inhibitors that prevent the activation of the pathway. We can group the extracellular inhibitors into two categories depending on the action they perform: (i) binding to the Wnt ligands and preventing their binding to the membrane receptors, such as SFRP1–5 and WIF-1 and, (ii) interfering with the LRP binding to FZD, such as DKK1 and sclerostin. Due to their mechanism of action, the first type of inhibitors can act on both canonical and non-canonical pathways, while the second type is specific to the canonical Wnt pathway. The inhibition performed by DKK1 and sclerostin is mediated or enhanced by receptors such as Kremen proteins and LRP4 [22,23]. When the canonical pathway is not activated, β -catenin is phosphorylated at three critical residues and sequestered in the cytoplasm by the destruction complex. The destruction complex is formed by scaffold proteins APC and AXIN1; Ser/Thr kinases, CK1 α , ϵ and δ and GSK3 β ; and transcriptional regulators, YAP/TAZ of the Hippo pathway [24]. This phosphorylated β -catenin is polyubiquitinated for its degradation in the proteasome by the complex containing, among others, β -TrCP protein. Thus, it is not available in the nucleus and in its absence a complex is formed by TCF/LEF and TLE/Groucho, inhibiting the transcription of target genes. **Wnt pathways ON:** The binding of a Wnt ligand to FZD/LRP or ROR1/2/Ryk activates the Wnt pathway that will result in a different transcriptional regulation. **Canonical Wnt Pathway ON:** The pathway begins at the cell surface with the formation of a heterotrimeric complex consisting of a Wnt ligand (19 different Wnts), a LRP transmembrane co-receptor (LRP5/6), and a FZD receptor (10 different FZDs). The formation of the LRP-FZD-Wnt complex results in phosphorylation of the LRP5/6 co-receptor by CK1 α and GSK3 β . Then, the DVL (also called DSH) polymerizes and is activated, inhibiting the destruction complex. This produces stabilization and accumulation in the cytoplasm of β -catenin that will translocate to the nucleus. Once there, it displaces the TLE/Groucho repressors forming an active complex with TCF/LEF proteins, that results in the recruitment of coactivators and activation of transcription of genes important for the differentiation and formation of bone such as WISP1 and RUNX2 [12,24]. **Non-canonical Wnt Pathway ON: i) WNT/PCP:** The WNT-PCP pathway begins with the binding of a Wnt ligand, such as WNT5A, to FZD and the co-receptors ROR1/2 or Ryk. Then, DVL is recruited and activated, resulting in the activation of the scaffold protein VANGL. DVL forms a complex with DAMM1, which activates the small GTPases RHOA and RAC1 which in turn activate ROCK and JNK. This leads to rearrangements of the cytoskeleton and/or the induction of transcription through ATF2 and/or NFAT. The activation of the WNT5A-ROR1/2 pathway inhibits the canonical Wnt signaling [19,20]. **ii) WNT/Ca²⁺:** The binding of the ligand to a FZD receptor results in a recruitment and activation of DVL. Then, DVL binds to the small GTPases which activate PLC. This activation leads to the division of PIP2 into DAG and IP3. When IP3 binds to its receptor on the endoplasmic reticulum,

calcium release occurs. This concentration of calcium and DAG through PKC can activate CDC42. Increased intracellular calcium can also activate calcineurin and CaMKII, which in turn can induce the activation of the NFAT transcription factor or NF- κ B [21,25]. **WNT signalling modulators:** The binding of RSPO to a LGR and RNF43/ZNRF3 maintain the Wnt signaling preventing the polyubiquitination and endocytosis of FZD performed by RNF43/ZNRF3 [26]. **WNT production:** Wnt precursors undergo posttranslational modifications such as porcupine-mediated palmitoylation, other lipid modifications and glycosylation in the ER. Then, the transmembrane protein Wntless (Wls) transports the functional Wnt ligands to the plasma membrane via the golgi apparatus. Wnt ligands are secreted from the cell by solubilization, exosome formation, or on lipid-protein particles. Wnt is detached from the cell membrane as components of exosomes or lipid protein particles [27–29]. APC: adenomatous polyposis coli; AXIN1: axis inhibition protein 1; β -TrCP: β -Transducin repeat-containing protein; CAMKII: calmodulin-dependent protein kinase II; CDC42: cell division control protein 42; CK1: casein kinase 1; DAAM1: DVL-associated activator morphogenesis; DVL: disheveled; FZD: Frizzled; GSK3 β : glycogen synthase kinase 3 β ; IP3, inositol 1,4,5 triphosphate, JNK: JUN kinase; LGR: Leucine-rich repeat-containing G-protein-coupled receptor; NFAT: nuclear factor of activated T cells; NF- κ B: nuclear factor kappa B; PKC: protein kinase C; PLC: Phospholipase C; RAC: Ras-related C3 botulinum substrate; RHOA: Ras homolog gene family member A; ROCK: Rho kinase; ROR1/2: bind tyrosine kinase-like orphan receptor 1 or 2; RYK: receptor-like tyrosine kinase; PRCN: Porcupine; RNF43/ZNRF3 : Ring finger protein 43/ Zing and Ring finger 3; RSPO; R-spondin ligand family members; SFRP: Secreted frizzled-related proteins; TCF/LEF: T-cell factor/lymphoid enhancer factor; TLE: Transducin-Like Enhancer of Split proteins; VANGL: Van Gogh-like; YAP/TAZ: Yes-associated protein/transcriptional co-activator with a PDZ-binding domain; WIF1: Wnt Inhibitory factor 1.

Despite the fact that the Wnt pathways are some of the most studied signaling routes, their complexity means that there are still many aspects to discover. To precisely regulate the transcription of Wnt pathway target genes, complex and tight regulation is carried out by extracellular components including ligands (19 Wnt ligands), inhibitors (12 different: sFRPs, Dickkopf (DKKs), sclerostin, among others), co-receptors (LRP, ROR, among others) and receptors (10 FZD). A current challenge is determining the tissue specific and transient modulators of this pathway, for instance in bone tissue, which could be useful as new therapeutic targets. Importantly, many rare and complex diseases are associated with genes from members of the Wnt pathway (Table 1). Since many treatments for bone density diseases target genes that cause rare monogenic bone diseases, a comprehensive understanding of the causal variants and genes is fundamental.

This review aims to describe, summarize and update the findings of all these extracellular modulators in relation to bone homeostasis, with an emphasis on the animal models generated, the diseases associated with each gene and the bone processes in which each member is involved. The review is structured into three main sections: co-receptors, ligands and inhibitors, within which we will review the main members of the Wnt pathway that have an important role in bone homeostasis.

Table 1. Disease or traits caused by mutations in Wnt pathway genes

Disease/Trait	OMIM	Phenotype	Gene	Inheritance	Comment	REF.
Osteogenesis Imperfecta XV	615220	- Low bone mass - Increased bone fragility	WNT1	AR/AD	LoF	[30–32]
Osteoporosis-pseudoglioma syndrome (OPPG)	259770	- Congenital blindness - Severe juvenile-onset OP - Spontaneous fractures	LRP5	AR	LoF	[33,34]
Osteoporosis	615221 166710	- Low bone mass	WNT1 LRP5	AD AD	LoF LoF	[30] [35–37]
Craniodiaphyseal dysplasia	122860	- Massive generalized hiperostosis - Sclerosis, especially involving the skull and facial bones	SOST	AD	LoF and dominant negative	[38]
Sclerosteosis	269500 614305	- Progressive skeletal overgrowth - Syndactyly	SOST LRP4	AR AD, AR	LoF LoF	[39–41] [42,43]
van Buchem disease	239100	- Hyperostosis of the skull, mandible, clavicles, ribs and diaphyses of the long bones , as well as the tubular bones of the hands and feet.	SOST*	AR	*Specific 52kb deletion downstream of SOST	[44]
HBM phenotype: AKA:	144750 607634	- High bone mass, affecting especially the skull and tubular bones	LRP5	AD	GoF	[45–47]
Osteosclerosis; Endosteal hyperostosis; Osteopetrosis type 1, Van Buchem disease type 2	607636	- Low fracture risk	LRP6 SOST DKK1	AD AR AD	GoF LoF LoF	[48] [49] [50]
Robinow syndrome	268310 180700	- Dysmorphic facial features - Hypertelorism - Short-limbed dwarfism - Vertebral segmentation - Short stature-genital -hypoplasia	ROR2	AR	LoF	[51–53]
Pyle's syndrome	265900	- Broadening of the long bones with wide and expanded trabecular metaphyses - Cortical thinning	WNT5A sFRP4	AD AR	LoF	[54] [55]

- Bone fragility and common fractures - Genu valgum - Widening of the ribs and clavicles - Platypondyly					
Oligodontia included: Tooth agenesis, selective 4, 7 and 8	616724	- Absence of one or more teeth	LRP6	AD	LoF [56,57]
	150400		WNT10A	AD/ AR	LoF [58,59]
	617073		WNT10B	AD	LoF [60]
Hand/foot anomalies:					
Cenani-Lenz syndactyly syndrome	212780	- Complex syndactyly of the hands	LRP4	AR	LOF [61]
		- Bony malformations in the forearm and lower extremities			
Isolated bilateral syndactyly		- Fusion of two or more fingers	LRP4	AR	LoF [62,63]
Brachydactyly type B1	113000	- Disproportionately short fingers (hands and feet)	ROR2	AD	GoF [64,65]
Split-hand-foot malformation, isolated form, type 6	225300	- Syndactyly	WNT10B	AR	LoF [66]
		- Median clefts of the hands and feet			
		- Aplasia and/or hypoplasia of the phalanges, metacarpals, and metatarsals.			
Ectodermal dysplasia:					
Ectodermal dysplasia 13	617392	- Severe oligodontia	KRM1	AR	[67,68]
AARRS		- Anomalies of hair and skin			
	276820	- Severe malformations of upper and lower limbs	WNT7A	AR	complete LOF [69]
		- Severely hypoplastic pelvis			
Furhman syndrome		- Abnormal genitalia.			
	228930	- Bowing of the femurs	WNT7A	AR	partial LOF [69]
		- Hypoplasia of the fibula, pelvis, fingers and fingernails			
Santos syndrome		- Cleft lip and palate			
	613005	- Fibular agenesis or hypoplasia	WNT7A		LoF [70]
		- Clubfeet with oligodactyly			
		- Acromial dimples			

- Motion limitations of the forearms and/or hands - Severe nail hypoplasia or anonychia					
Schöpf-Schulz-Passarge syndrome	224750	- Multiple eyelid apocrine hidrocystomas - Palmoplantar keratoderma - Hypotrichosis - Hypodontia - Nail dystrophy	WNT10A	AR	LoF [71]
Odonto-onycho-dermal-dysplasia (OODD)	257980	- Hyperkeratosis and hyperhidrosis of the palms and soles - Atrophic malar patches - Hypodontia and conical teeth - Onychodysplasia - Dry sparse hair	WNT10A	AD, AR	LoF [72]
Skeletal dysplasias:					
Other skeletal dysplasias		- Osteopenia - Bilateral coxa vara deformity - Mild left radial and ulnar bowing - Broadening of metapases - Bilateral shortening of the great toes and thumbs	WNT3A	AR	[73]

OP: Osteoporosis; AARRS: Al-Awadi-Raas-Rothschild syndrome; AR: Autosomal recessive; AD: Autosomal dominant; LoF: Loss-of-function; GoF: Gain-of-function; REF: References

1. CO-RECEPTORS

We define a co-receptor as a cell surface receptor that binds to both a signaling molecule and to a primary receptor, thereby facilitating ligand recognition and initiation of downstream biological processes. Different co-receptors play essential roles in Wnt signaling, both in the canonical and non-canonical pathways. This section includes a subsection entitled LRP4/5/6; a second subsection entitled Kremen, which includes information on Kremen1/2; a third subsection ROR, which includes information related to ROR1/2 and a final subsection LGR, which includes information related to LGR4/5/6. We review the role of these coreceptors on bone remodelling, with special emphasis in mutation causing disease (Figure 2) and in the animal models available (Table 2).

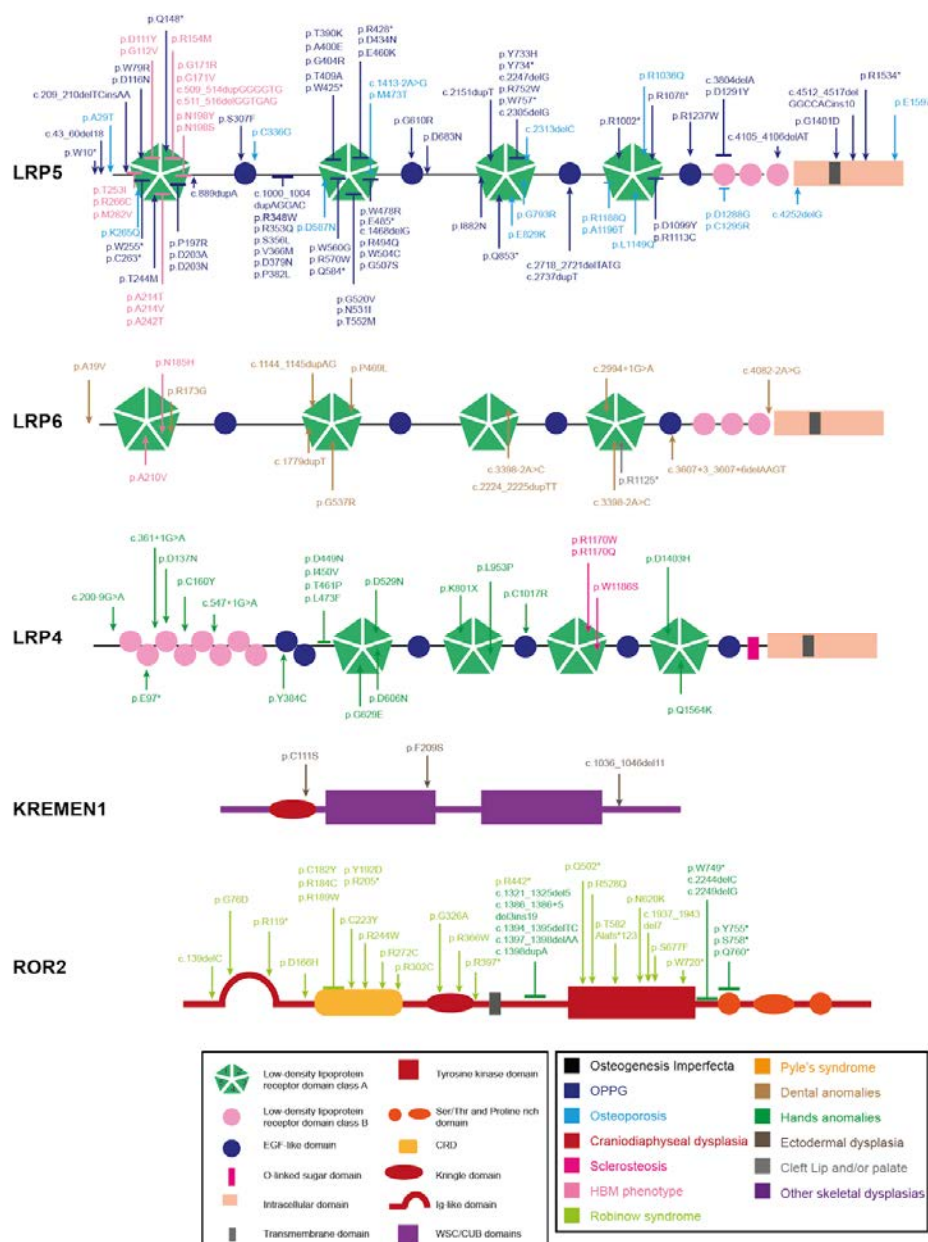


Figure 2. Schematic protein representation of the co-receptors with the mutations causing human skeletal diseases.

Table 2. Wnt pathway (Co)-receptors transgenic mouse models

Gene	Model	Tissue expression	Phenotype			Comments	REF
			B. M	B.F .	B.R .		
Lrp5	KO	Total KO	↓	↓	↓/=	ⁱ ↓ in [74]; = in [75,76]	[74–80]
	cKO-Dermo1-Cre	Embryonic mesenchyme	=	=	-		[80]
	cKO-Ocn-Cre	mature OB	↓	↓	=		[81]
	cKO-α1(I)-Col-Cre	OB	=	=	-		[76]
	cKO Rat 3.6Col1a1-Cre	OB	↓	-	-		[78]
	cKO-Dmp1-Cre	OCy	↓	-	-		[82]
	cKO-Vil1-Cre	intestinal stem cells	↓/=	↓/=	-	ⁱⁱ ↓ in [76] = in [82]	[76,82]
	cKO-Col21-Cre	CD and OB progenitors	↓	-	-		[83]
	KI-A214V or G171V	Total KI	↑	↑	=		[82]
	cKI-Dmp1-Cre A214V or G171V	OCy	↑	↑	=		[82]
	cKI-Prrx1-Cre A214V or G171V	MSC	↑	↑	=		[82]
	cKI-Vil1-Cre A214V	Intestinal stem cells	=	=	=		[82]
	cKI-Vil1-Cre G171V	Intestinal stem cells	↑/=	↑/=	=	ⁱⁱⁱ ↑ in [76]; = in [82]	[76,82]
	cKI-1(I)-Col-Cre G171V	OB	=	=	=		[76]
Lrp6	Tg Rat3.6Col1a1G171V	OB	↑				[84]
	cKI-Ctsk-Cre A214V or G171V	mature OC	↑	↑	↓ ^{iv}	Offtargets in Osteocyte line ^{iv} Only in female mice	[85]
	KO	Total KO	-	-	-	NV	[77,80,86]
	cKO-Dermo1-Cre	Embryonic Mesenchyme	=	=	=		[80]
Lrp6	Ringelschwanz		↓	=	↑	R868W mutation; 3 rd β-propeller	[87,88]
	cKO-Ocn-Cre	mature OB	↓	↓	=		[81]

	cKO-Col2a1-Cre	CD and OB progenitors	↓	-	-		[83]
	KO Lrp5 and Het KO Lrp6	Total KO	↓	-	-	Severe limb abnormalities	[77]
	cKO-Dermo-Cre	Embryonic Mesenchyme	↓	↓	-	NV	[80]
Lrp5&Lrp6	cKO-Ocn-Cre	cKO in mature OB	↓↓	↓	=	NV	[81]
	cKO-Col2a1-Cre	CD and OB progenitors	↓	-	-	NV	[83]
	cKO-Rank-Cre	OC precursors	↓	↓	↓		[89]
	cKO-Ctsk-Cre	mature OC	=	=	=		[89]
	KO	Total KO				NV	[90–92]
	ECD		↓	↑	↑	Only ECD domain	[93]
	cKO-OCN-Cre	mature OB	↑	↑	↓		[90,94]
Lrp4	cKO-Dermo1-Cre	OCy	↑	↑	↓		[94]
	cKO-LysM-Cre	Myeloid lineage cells	=	=	=		[90]
	KI-Lrp4-R1170W	Total KI	↑	↑	=		[95]
	KI-Lrp4-R1170Q	Total KI	↑	↑	=		[96]
	KO	Total KO	-	-	-	NV	[8,97–99]
	Het KO	Heterozygous KO	↑	=	↓		[97]
Ror2	cKO-Rank-Cre	OC	↑	=	↓		[97]
	cKO-Ctsk-Cre	mature OC	↑	=	↓	Only in trabecular bone	[97,100,101]
Krm1	KO	Total KO	=	=	-		[102]
	KO	Total KO	=	=	-	In young mice	[102]
Krm2	KO	Total KO	↑	↑	-	In adult mice	[103]
	Tg Col1a1-Krm2	OB	↓	↓	↑		[103]
Krm1&Krm2	KO	Total KO	↑	↑	=		[102]
	KO	Total KO	↓	↓/≠	↑	↓ [104,105]; = [106]	[104–107]
Lgr4	cKO-LysM	Myeloid cells	↓	=	↑		[106]
Lgr5	KO	Total KO				NV	[108]
Lgr6	KO	Total KO	=	=	=		[109,110]

KO: Knock-out; cKO: conditional KO; KI: Knock-in; cKI: conditional KI; het KO: heterozygous KO
 NV: No viable; OB: osteoblast; OC: osteoclast; Ocy: osteocyte; CD: chondrocyte..

2.1. LRP5

The LRP proteins belong to the low-density lipoprotein receptor (LDLR) family of highly conserved cell surface receptors [111], whose functions include endocytosis (e.g., lipoproteins or proteases) and direct signal transduction (e.g., Bone morphogenic protein (BMP) or Wnt pathway) [112,113]. These transmembrane proteins have a large extracellular domain (ECD) and a small intracellular domain (ICD) separated by a single-pass transmembrane domain. The ECD allows interaction with extracellular proteins (e.g., Wnt ligands or extracellular inhibitors such as DKK1 or sclerostin), and the ICD is in charge of the downstream transmission of signaling events [114].

The most studied LRP family members in relation to bone are LRP5 and LRP6, which share 71% homology at the protein level [115]. Their ECDs consist of four β -propeller domains (class B repeats or YWTD domains) alternating with four epithelial growth factor (EGF) like domains followed by three LDLa domains (class A repeats). Their ICDs include various highly conserved PPPS/TP motifs containing serines and threonines that are phosphorylated upon receptor activation (Figure 2). These phosphorylated sites bind Axin, thereby preventing phosphorylation of β -catenin [7,116,117]. LRP4, also known as MEGF7, has a structure similar to that of LRP5/6 but with some differences. The ECD features eight LDLa domains, four β -propeller domains with six EGF-like repeats and a domain for O-linked oligosaccharide modification. The ICD features two domains: the endocytosis signal NPxY domain responsible for protein internalization, and the PZD-interacting motif at the C-terminal end (Figure 2) [118–120].

The importance of these transmembrane co-receptors in bone was revealed by the description of mutations in *LRP5* that caused two diametrically opposed bone phenotypes: osteoporosis pseudoglioma syndrome (OPPG), and the high bone mass (HBM) phenotype [33,45,46] (Table 1 and Figure 2). The difference between the two types of mutations is that OPPG results from loss-of-function (LoF) mutations causing LRP5 to be unable to activate the canonical Wnt pathway. In contrast, the mutations responsible for the HBM phenotype are gain-of-function (GoF) and cause a loss of affinity for the extracellular inhibitors DKK1 and sclerostin. In this case, LRP5 can no longer be internalized and remains available in the membrane for Wnt pathway activation [34,121–126]. In addition, the LRP5 locus has been associated with bone mineral density (BMD) and risk of fracture in a plethora of studies [127–137].

To understand the effect of LRP5 on bone and the signaling pathways by which it acts, genetically modified mouse models have been generated (Table 2). *Lrp5* total knock-out (*Lrp5*-KO) mouse models and conditional KOs (cKOs) in bone-related cells do not display bone alterations at birth but acquire a decreased BMD during postnatal development due to reduced bone formation [74,75,77–83] (Table 2). On the contrary, global or conditional LRP5 transgenic mice carrying the human LRP5 HBM p.Gly171Val or Ala214Val mutations reproduce the HBM phenotype with high rates of bone formation and better bone quality [82,84] (Table 2). Furthermore, conditional knock-in (cKI) female mice with the HBM mutations present only in osteoclasts, show a reduction in bone resorption [85] (Table 2). Relative to skeletal mechano-responsiveness, *Lrp5*-KO and *Lrp5*-cKO mice have been reported to show severe impairment in later stages of the mechano-transduction signaling cascade, whereas the HBM mice show a higher osteogenic response to mechanical loading *in vivo* than WT mice [78,138,139]. In 2008 Yadav *et al.* proposed that the effect of LRP5 on bone formation and BMD was due to its inhibitory effect on tryptophan hydroxylase 1 and, therefore, on serotonin synthesis in the duodenum, which would otherwise block bone formation by signaling in osteoblasts. Using tissue-specific KO mouse models, the authors postulated that circulating serotonin blocks bone formation through binding to the serotonin receptor 1B in osteoblasts and inhibiting the expression and function of the cAMP response element-binding protein, cyclin and osteoblast activity and function [76,140,141]. However, these hypotheses have not been reproduced, and there is much evidence that LRP5 exerts its effect directly on osteoblastic cells through the Wnt canonical pathway [80–82,142,143].

Similar to LRP5, there is much evidence of a role of *LRP6* in bone. Missense mutations in LRP6 have been described in humans as the cause of tooth agenesis and oligodontia, and recently, HBM [48,56,57] (Table 1 and Figure 2). Interestingly, the mutations causing the HBM phenotype are located exclusively in the first β -propeller domain, and the phenotype of these patients is identical to that of the patients with LRP5-HBM. Moreover, *LRP6* has been associated with heel BMD in three different studies [134–136].

The *Lrp6*-KO mouse model is embryonic lethal with loss of distal limb structures and truncation of the axial skeleton, indicating that *Lrp6* plays a fundamental role during embryogenesis, during which it is widely expressed [77,80,86] (Table 2). Confirmation that *Lrp6* is also involved in postnatal bone metabolism was obtained using cKO mouse models and models with hypomorphic mutations [81,83,87,88] (Table 2). Specifically, the Ringelshwanz (*rs*) mouse with the R886W hypomorphic mutation in the third β -propeller domain showed developmental malformations and delayed ossification, in addition to an increase in bone resorption and decrease in BMD and in the activity of canonical Wnt pathway in osteoblasts [87,88] (Table 2). Through different *in vitro* models, it was determined that this effect was a consequence of the impaired interaction of mutant *Lrp6* with the chaperone *Mesd* and its defective trafficking to the plasma membrane [87,88]. *Lrp6*-cKO mice in the early osteoblast lineage showed reduced BMD due to decreased bone formation [81,83]. In contrast, there was no obvious bone phenotype for *Lrp6*-cKO in embryonic mesenchyme [80] (Table 2).

The double KO and cKO for both co-receptors (*Lrp5+Lrp6*-dKO and *Lrp5+Lrp6*-cdKO) show a more severe phenotype than the single KO and cKO regarding BMD and skeletal development during embryogenesis (Table 2). Many authors agree that there is some redundancy in the functions of these co-receptors, but that they may regulate the Wnt pathway in different time windows (*Lrp6* in early stages and *Lrp5* in the late stages of osteoblast differentiation) and in different skeletal compartments (*Lrp6* in trabecular bone and *Lrp5* in cortical bone) to promote the proper acquisition of BMD. Therefore the expression of the two co-receptors in osteoblasts would be necessary for normal skeletal homeostasis [77,80,81,83,144] (Table 2). More research is needed to better define the tissue distribution and the time at which these two co-receptors act. In this context, a study of *Lrp5+Lrp6*-cKO at early and late stages of osteoclast differentiation showed that there was a low BMD with reduced osteoblast and osteoclast numbers in the early osteoclast model, while the late osteoclast model did not have a bone phenotype [89] (Table 2).

The characterization of all these animal models indicates that *Lrp5* and *Lrp6* exert their function in bone homeostasis mostly through the regulation of bone formation, controlling the differentiation and proliferation of osteoblasts, bone matrix deposition in differentiated osteoblasts and apoptosis of osteocytes. Furthermore, these effects are mainly due to Wnt signaling through the canonical pathway. It has been determined that LRP6 acts not only through the formation of the heterotrimeric complex LRP6-FZD-Wnt, but also through the formation of a complex involving LRP6, Parathyroid hormone (PTH) and PTH receptor 1, which promotes stabilization of β -catenin and thus activates the canonical Wnt pathway [145].

LRP4 has been extensively studied in relation to its role in the neuromuscular junction, where it serves as a co-receptor for motor neuron-derived agrin/Wnt and facilitates acetylcholine receptor clustering on the myocyte [146]. In addition to this function, LRP4 has a very important role in bone homeostasis, where it enhances the inhibitory function of sclerostin in the canonical Wnt pathway [42,90,93,94,147]. *Lrp4* can also enhance other inhibitors of the Wnt pathway such as DKK1 and WISE [93,148].

Missense and splicing mutations in LRP4 have been implicated in several musculoskeletal human diseases depending on the position of the mutation (Table 1 and Figure 2). Mutations in the LDLa, EGF-like domain or in the first and second β -propeller domains cause Cedani-Lenz Syndrome (CLS) [61]; in the central cavity of the third β -propeller domain cause sclerosteosis-2 [42,43]; and in the

fourth β -propeller domain or in the EGF-like domain cause isolated bilateral syndactyly [62,63]. Thanks to functional studies *in vitro*, it has been proven that all these mutations cause a significant decrease in the inhibition of the Wnt canonical pathway by LRP4, this effect being more severe in mutations of sclerosteosis-2. CLS-causing mutations decrease the levels of LRP4 available in the membrane, while this is not observed for the sclerosteosis-causing mutations. Instead, impaired binding between sclerostin and LRP4 has been verified in the mutations causing sclerosteosis, which would explain the severity of the effect [43,61,62]. Interestingly, mutations in the edge of the third β -propeller domain have been found to cause myasthenia gravis, the most common neuromuscular junction pathology. These mutations cause impairment of the MuSK signaling pathway, whereas sclerosteosis-2 mutations have no effect on this pathway, demonstrating the highly position-specific effect of the different LRP4 mutations [149]. Furthermore, like *LRP5/6*, *LRP4* has been associated with BMD in different GWAS studies [130,131,134–136].

Lrp4-KO or null mutant mouse models are not viable due to the absence of neuromuscular junctions and therefore inability to breathe [90–92] (Table 2). In contrast, Lrp4-cKO mouse models in the osteolineage are viable. When the deletion occurs specifically in osteoblasts, or in osteocytes, a high BMD is observed, with high levels of bone formation and reduced bone resorption but without polysyndactyly [90,94] (Table 2). The phenotype is more severe in early osteoblasts, where Lrp4 is more highly expressed, than in osteocytes [94]. In contrast, no bone phenotype is observed in osteoclast-specific KO mice [90] (Table 2). The presence of Lrp4 has been shown to be essential in these animal models for the functioning of sclerostin [90]. In 2017 Xiong *et al.* [150] proposed that reduced bone resorption in Lrp4-cKO in the osteoblast lineage stabilizes the prorenin receptor and as a result increases the production and secretion of the ATP derivative adenosine. Elevated adenosine-A2aR signaling in osteoclast precursors reduces receptor activator of nuclear factor κ B (RANK)-mediated osteoclastogenesis. Furthermore, circulating sclerostin levels are higher in Lrp4-cKO in the osteoblast lineage with unchanged expression of *SOST* per cell, but it is possible that increasing the number of osteocytes with increasing bone formation will also increase the total level of *SOST*. In addition to these models, LRP4-KI mouse models with the R1170W or R1170Q mutations, which cause sclerosteosis in humans, recapitulate the human HBM phenotype associated with this mutation without the presence of syndactyly (Table 2). In addition to high BMD, these mice exhibit high levels of bone formation with unchanged bone resorption and are protected against the effects of anti-sclerostin antibodies and of *SOST* transgenic overexpression [95,96]. Polysyndactyly and other limb deformity problems have been reported in different murine and bovine hypomorphic models of Lrp4 [92,147,148,151–154]. Specifically, the Lrp4 ECD mouse model that does not have the transmembrane or intracellular domains has polysyndactyly and decreased BMD [93] (Table 2).

All these studies on animal models, together with many *in vitro* studies, seem to indicate that Lrp4 exerts its function on the differentiation/function of osteoblasts through the inhibition of the Wnt-induced activation of the canonical pathway.

2.2. ROR

ROR1/2 belong to the evolutionarily conserved Ror-family of receptor tyrosine kinases, which are type I transmembrane protein tyrosine kinases. These receptors contain different motifs including an immunoglobulin-like domain, a frizzled-like cysteine rich domain (CRD), and a kringle domain (KD) in the extracellular region, a transmembrane section, and an intracellular region with a tyrosine kinase domain, two serine/threonine-rich domains, a proline-rich domain and a short C-terminus tail [155–157] (Figure 2). There is some controversy about the activity of these receptors, regarding whether they are true kinases or pseudokinases [158]. ROR1 and ROR2 are structurally very similar, with 58% amino acid identity and high domain similarity. Both bind WNT5A through their CRD domains, and by acting as receptors or co-receptors along with FZD, they activate the non-canonical Wnt signaling pathways [8,158]. The Ror1+Ror2-dKO mouse model revealed that these two co-receptors possess certain redundant functions in skeletal and cardiac development [159,160]. Ror1/2 are widely expressed in

various tissues and organs, such as limbs, heart, lungs, gut, muscles, and skeletal and nervous systems, during embryonic development, while their expression decreases markedly in adult tissues [158]. While *Ror2* is strongly expressed in mature osteoclasts, *Ror1* expression has not been detected in these cells [101]. In addition, *Ror2* has been reported to regulate the length of various organs, including limbs, both through interaction with *Wnt5a* and *Wnt9a* [158,161].

In humans, recessive LoF mutations in *ROR2* are associated with autosomal recessive Robinow syndrome, and dominant GoF mutations are associated with brachydactyly type B [51,52,64,101,155] (Table 1 and Figure 2). Moreover, *ROR2* has been associated with hip BMD in a GWAS study [159].

Ror2-KO mouse models show early lethality and abnormalities characteristic of Robinow patients. The *Ror2*-KO mice exhibit dwarfism, facial abnormalities, short and deformed limbs and tails, dysplasia of lungs and genitals, ventricular septal defects and severe cyanosis [8,97–99] (Table 2). In these mice the bones formed through endochondral ossification are affected, but not those formed by intramembranous ossification. The heterozygous *Ror2*-KO mouse and the *Ror2*-cKO in the osteoclast lineage exhibited a HBM phenotype due to impaired bone resorption only in trabecular bone [97,100,101] (Table 2). The impaired bone resorbing activities of osteoclasts can be restored by the constitutively active form of *RhoA*, showing that this impaired bone resorption is an effect of the non-canonical *Ror2*-*Wnt5a* signaling pathway [97,100,101,162]. Thus, *Ror2* mediated signaling regulates the differentiation and bone resorbing activity of osteoclasts, thereby maintaining bone mass.

2.3. KREMEN

The Kremen family of receptors (*KRM1/2*) are single pass transmembrane proteins that show high affinity for *DKK1* and *DKK2* [163,164]. *KRM* presents an ECD that contains a KD, a carbohydrate binding (WSC) and a CUB domains that are necessary for binding to *DKK* [163,165] (Figure 2). *KRM1/2* can bind to the CRD2 domain of *DKK1* and form the *DKK1*-*KRM*-*LRP5/6* ternary complex, which is internalized, thus removing *LRP5/6* from the cell surface [166,167] (Figure 1). Similar to *LRP4*, Kremen acts by enhancing *DKK1* function, but unlike *LRP4*, Kremen is not essential for *DKK1* to perform its function [163]. While *Krm1* has a more widespread expression pattern, *Krm2* is expressed predominantly in bone [103]. In addition to this effect, *Krm1/2* can activate the Wnt pathway by binding directly to *Lrp6* in the absence of *Dkk1* [103,168,169].

In humans, mutations in *KRM1* affect different protein domains and have been associated with ectodermal dysplasia and oligodontia [67,68] (Table 1 and Figure 2).

Krm1-KO mice and young *Krm2*-KO mice (less than 24 weeks old) are viable without abnormalities and show normal bone formation and bone mass [102], while the adult (24 weeks) *Krm2*-KO mice display higher bone formation and bone mass [102,103] (Table 2). *Krm1*+*Krm2*-dKO are also viable, but show ectopic postaxial forelimb and expanded apical ectodermal ridges and increased bone formation, bone mass and canonical Wnt signaling [102] (Table 2). The dKO mouse phenotype shows similar defects in the limb to those described in the *Dkk1*-KO mutants. In addition, the ectopic growth of digits present in the *Krm1*+*Krm2*-dKO mice is further enhanced by the deletion of a single *Dkk1* allele, but without any additional increase in BMD [102]. In contrast, mice with osteoblast-specific *Krm2* overexpression display a severe osteoporotic phenotype caused by decreased bone formation and increased bone resorption with impaired fracture healing [103,170] (Table 2). Taking all these models together, it seems that *Krm1* and *Krm2* show a certain degree of redundancy in skeletal growth and development and that *Krm2* is important for bone formation during skeletal maintenance/remodeling.

2.4. LGR

The Leucine-rich repeat-containing G protein-coupled receptors (LGRs) 4, 5 and 6 constitute a subfamily of receptors belonging to the G protein coupled receptor (GPCR) superfamily [171]. Structurally, these transmembrane GPCR belong to the class A rhodopsin-like family [172], which have

a characteristic leucine-rich domain (LRR) in the extracellular N-terminal region that is responsible for the ligand interaction [172–175]. All three receptors show some homology, but while LGR4/5 have 17 LRR repeats, LGR6 only has 13 [174]. These receptors bind to R-spondins (RSPOs) causing the formation of the LGR-RSPO-ZNRF/RNF43 complex. This leads to the sequestration of ZNF/RNF43 that causes an increase in membrane-available FDZs, since the transmembrane ubiquitin ligases ZNF/RNF43 are responsible for degrading them [26]. In addition to this agonistic effect on the Wnt pathway, LGR is a target gene for the Wnt pathway, which will produce a positive loop [26]. It has also been reported that LGR proteins mark different adult stem cells and play a very important role in defining progenitor and stem cell behavior [26,176,177]. Specifically, it has been reported that LGR5 is a marker of adult stem cells in the stomach [178], hair follicles [179], and small intestine and colon [176], among others; and LGR6 in taste buds, lung and skin [109,176,178,180].

LGR4, also known as GPR48, is widely expressed in multiple tissues from early embryogenesis to adulthood [181], being abundantly expressed in skeletal, adipose and muscular tissue [104,107,173,182]. Specifically, expression has been observed in osteoblast precursors in *in vitro* osteogenesis, and in osteoclasts in response to RANK ligand (RANKL)-induced osteoclast differentiation. In addition to the Wnt pathway enhancement, LGR regulates a multitude of pathways through its classical G-protein signaling potential [104,183]. In one of these pathways LGR4 acts as a secondary receptor for RANKL, competing with RANK. Furthermore, this LGR4-RANKL binding is inhibited by both osteoprotegerin (OPG) and RSPO1. Through *in vitro* and *in vivo* studies, LGR4 has been shown to inhibit RANKL-induced osteoclast differentiation, survival and function [106].

No rare human diseases associated with LGR4 have been found, but a rare nonsense variant has been linked to osteoporosis in two different association studies [184,185] and common variants are associated with heel BMD [134,136].

Total Lgr4-KO mice are at risk of perinatal death, with approximately 60% of newborn mice dying before day 1. These mice exhibit decreased body weight, size and bone length [104–107] (Table 2). Lgr4-KO mice have a low BMD due to both impaired bone formation and bone resorption. Regarding bone formation, these mice present a dramatic delay in osteoblast differentiation and mineralization during embryonic bone formation, and in postnatal bone remodeling there is a decrease in the kinetic indices of bone formation rate and osteoid formation [104,105]. During bone resorption they present osteoclast hyperactivation with an increase in the number of osteoclasts, surface area, size and bone erosion [104,106] (Table 2), a phenotype that is also observed in Lgr4-cKO mice in monocytes [106] (Table 2). Interestingly, Lgr4 extracellular domain administration abrogated RANKL-induced bone loss in three mouse models of osteoporosis [106]. Thanks to *in vitro* and *in vivo* studies, it has been verified that RSPO signaling has no effect on osteoclast differentiation and that its effect on this is determined by its signaling via RANKL [106,186].

In addition to the evidence from *in vivo* studies, many *in vitro* studies have been carried out demonstrating the positive effect of LGR4 on osteogenic differentiation [105,187,188], adipocyte and myocyte differentiation [105]. Although LGR4 is expressed within bone-marrow-derived mesenchymal stem cells (BMSCs) undergoing osteogenic differentiation, its expression is not correlated with osteogenic markers of *in vitro* differentiation of osteoblasts of BMSCs [189].

LGR5 is also known as GPR49, HG38 and FEX, and is expressed in osteoblast precursors in osteogenesis *in vitro*. Lgr5-KO mice show neonatal lethality (during the first 24 h of life) associated with ankyloglossia characterized by fusion of the tongue to the floor of the mouth, leading to the inability to nurse and subsequent neonatal mortality. Perinatal lethality is due partly to respiratory failure as the result of pressure against the diaphragm from the markedly distended abdomen [108] (Table 2). This phenotype suggests the involvement of LGR5 in craniofacial development.

Regarding LGR6, as with LGR4, a rare variant associated with the risk of postmenopausal osteoporosis has been described [184] along with common variants with heel BMD in a GWAS study

[134]. It is expressed in mouse primary calvarial cells, bone marrow cells, and BMSCs [189]. *In vitro* studies have highlighted the importance of LRG6 in promoting osteogenic differentiation, correlating its expression with typical markers of osteogenic differentiation such as Osterix [189,190]. These results point to LRG6 as a novel marker of osteoprogenitor cells in bone marrow [189]. A recent study shows that the enhancement of the Wnt pathway by LRG6 occurs downstream of the BMP signaling pathway [191]. Despite this, Lgr6-KO mice develop normally and no skeletal phenotype is evident, although Lgr6 is necessary for regeneration of the digit tip bone following amputation in mice [109,110] (Table 2).

2. WNT LIGANDS

Wnt ligands are highly conserved secreted glycoproteins with a molecular weight around 40 KDa. They are formed by 350–400 amino acids, 23–24 of which are conserved cysteine residues. For secretion of an active Wnt ligand, Porc-mediated lipidation by palmitic acid is necessary in a conserved serine residue (Figure 3). Following this it is secreted into the extracellular matrix through Wls binding [27,28,192]. However, some authors have questioned the essential nature of this step [28,193,194].

Although the degree of sequence identity between some Wnt family members is only 18%, it is thought that all Wnt proteins form a similar three-dimensional structure, which is shaped like a fist with a thumb and index finger protrusions [192,193,195]. There are four main regions of concentrated amino acid conservation that map onto the Wnt structure: the tips of the thumb and index finger loops, the core of the N-terminal α -helical domain (NTD), and a large continuous patch in between, facing the exterior. The two main contact points between the Wnt ligand and the Fz receptor are the tips of the thumb and index loops [192,193]. The CRD at the second point of contact with the receptor is similar to that of sFRP and ROR1/2, among others [196]. On the NTD, the lipidation of the conserved serine creates hydrophobic forces with the receptor that, combined with shape complementarity, facilitate binding [193]. Speer *et al.* argued that the lipid is not necessary for this interaction to occur, and thus could be kept outside the cleft [194]. Nevertheless, the importance of the fatty acid binding seems worth noting, as mutations in the residues of the cleft lead to defects in receptor structure, and the presence of the lipid seems to activate the dimerization and activation of the receptor [197]. The thumb loop residues contact with the CRD domain of the FZD receptors with poorer shape complementarity. The other conserved regions of the protein located opposite the FZD binding region bind to co-receptors such as LRP5/6/RYK [192,193,198]. The amino acid differences between Wnt ligands enable their differential binding to receptors. For example, WNT1 binds to the first β -propeller domain of LRP5/6 whereas WNT3a binds to the third β -propeller domain [199].

It has been suggested that the activation of one or another pathway relies on the available receptors [200,201], while other studies suggested that the selection of the pathway depended on the amount of Wnt ligand available [5,202]. A total of 19 Wnt ligands have been identified in humans. Of these, WNT1, WNT3A, WNT4, WNT5A, WNT5B, WNT7A, WNT7B, WNT9A, WNT10A, WNT10B and WNT16 have been extensively studied in relationship to their role in bone.

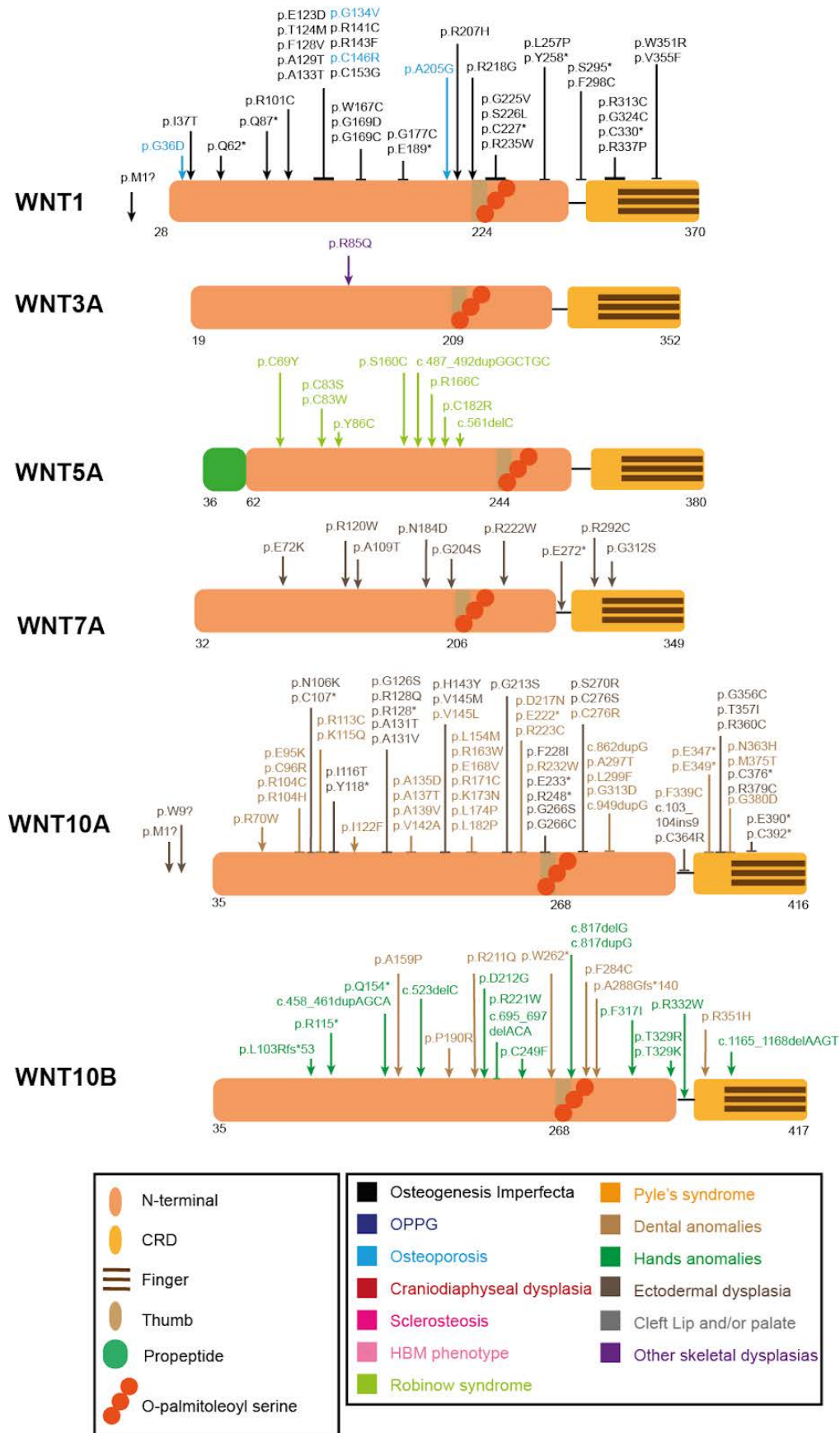


Figure 3. Schematic protein representation of the Wnt ligands with the mutations causing human skeletal diseases.

Table 3. Wnt pathway ligands transgenic mouse models

Gene	Model	Tissue expression	Phenotype			Comments	Ref
			B.M	B.F.	B.R.		
Wnt1	KO	Total KO	-	-	-	NV	[203,204]
	Het KO	Total Het KO	↓	=	=	Mild osteopenia	[203]
	Sway	Hypomorphic	↓	↓	=	G565del.	[205,206]
	cKO-Prrx1-Cre	MSC	↓	↓	↑		[203]
	cKO-Runx2-Cre	OB	↓	-	-		[207]
	cKO-DmpI-Cre	late OB and Ocy	↓	↓	-		[208]
	cKO-Lyz2-Cre	Monocytic lineage (OC included)	=	-	-		[207]
	Tg-Col1a1-tTA	OB, inducible	↑	↑	=		[207]
	Dmp1-Cre-R26 het Wnt1	Overexpression in late OB and Ocy	↑	↑	=	HBM phenotype	[208]
Wnt1 & Lrp5	Tg-Wnt1;Lrp5-KO	OB-targeted inducible overexpression of Wnt1 in LRP5-KO	↑	↑	=	Similar phenotype to LRP5+/+	[207]
Wnt3a	KO	Total KO	-	-	-	NV	[209,210]
Wnt4	KO	Total KO	-	-	-	NV	[211]
	cKO-Prrx1-Cre	MSC	↑	-	-	femoral, in females	[212]
	Tg-Col2.3kb-Wnt4	OB	↑	↑	↓		[213]
Wnt4 & Wnt9a	dKO	Total dKO	↓	-	-	Ectopic cartilage formation	[214]
Wnt5a	KO	Total KO	-	-	-	NV	[215,216]
	Het Wnt5a	Total Het KO	↓	↓	↓	Increased adipogenesis	[97,217]
	cKO-Osx-Cre	OB	↓	↓	↓		[97]
Wnt5b	KO	Total KO	-	-	-		[218]
Wnt7a	KO	Total KO	-	-	-	Abnormal limb development	[219]
Wnt7b	KO	Total KO	-	-	-	NV	[220]
	cKO-Dermo1-Cre	skeletal progenitors	↓	-	-		[221]

	Tg-OSX-Cre;R26	Overexpression in preOB	↑	↑	=	Little bone marrow space	[222]
	Tg-Col1-Cre;R26	Overexpression in mature OB	↑	↑	=	Little bone marrow space	[222]
	Col2-Cre; R26	Overexpression in OB and CD	↑	↑	-	Little bone marrow space	[206]
	Tg-Runx2-Wnt7b	Inducible total overexpression	↑	↑	=		[222]
	Osx-rtTA;tetO-Cre;R26	Inducible overexpression in OB	↑	↑	↑ ⁱⁱⁱ	less than formation	[223]
Wnt9a	KO	Total KO	↓	-	-	NV, shortened long bones	[214]
Wnt9a & Ror1	Ror1hyp/hyp; Wnt9a-KO	Total Wnt9a KO, hypomorphic Ror1	↓	-	-	Deletion of Ror1 3rd exon, containing Ig-like domain	[161]
Wnt9a & Ror2	dKO	Total dKO	↓	-	-	Shortened long bones	[161,214]
Wnt10a	KO	Total KO	↓	↓	-	Impaired dental formation	[224–226]
	Wnt10b-KO	Total KO	↓	↓	-	Early age dependent	[227–229]
Wnt10b	Tg-FABP4-Wnt10b	Overexpression in MSC	↑	-	-	Resistant to obesity and age/hormone-related bone loss	[229]
	Tg-Oc-Wnt10b	Overexpression in OB	↑	↑	=		[228]
	Tg-Oc-Wnt10b	Overexpression in mature OB	↑	↑	↑		[227,228]
	KO	Total KO	↓	=/↓ ^{iv}	↑	in cortical bone ^{iv} ↓ [230]; =[231]	[230–233]
	cKO-Runx2-Cre	OB	↓	-	-	In cortical bone	[231]
Wnt16	cKO-Dmp1-Cre	Ocy	=	-	-		[231]
	CAG-Cre-ER	Tamoxifen-inducible	↓	↓	↑	In cortical bone	[234]
	Tg-2.3Kb rat-Col1a1-Wnt16	OB	↑	=/↑ ^v	=/↓ ^{vi}	^v = [302]; ↑ [235] ^{vi} = [302]; ↓[235]	[302,235]

Tg-Dmp1- Wnt16	OCy	↑	↑	=	[236]
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B.M.: Bone mass; B.F.: Bone formation; B.R.: Bone resorption; KO: Knock-out; cKO: conditional KO; het KO: heterozygous KO; NV: No viable; OB: osteoblast; OC: osteoclast; Ocy: osteocyte; CD: chondrocytes

3.1 WNT1

WNT1, also known as INT1, plays an important part in osteoblastogenesis and can also inhibit osteoclastogenesis and chondrogenesis. WNT1 is expressed in mesenchymal stem cells (MSCs), osteoblasts, monocytes and osteocytes, in which it can activate the canonical Wnt pathway in an autocrine or paracrine way, and also the non-canonical pathways mTORC1 in osteoblasts and JNK in osteoclasts [207,208,227,237]. Activation of the canonical pathway by WNT1 inhibits MSC differentiation into adipocytes, promoting osteoblastogenesis [203,227]. At a later stage, WNT1 activation of mTORC1 in osteoblasts promotes their differentiation and mineralization [208]. Activation of JNK-dependent non-canonical signaling by WNT1 seems to contribute to the suppression of osteoclast differentiation [203]. In adult bone, WNT1 expression can be induced by mechanical loading or physical activity to activate osteoblastogenesis [238–240]. Importantly, the extent of WNT1 induction declines with aging and may underlie the muted bone anabolic response to mechanical loading in aged mice [240].

WNT1 has been linked to bone physiology in humans, as LoF mutations can cause early onset osteoporosis and osteogenesis imperfecta (OI) [30,31,35] (Table 1 and Figure 3). The classification of early onset osteoporosis and OI has been difficult due to the variability of phenotype and genotypes they present, and lately they have been considered to be part of a spectrum rather than two delimited disorders. Moreover, *WNT1* is associated with total-BMD and heel-BMD [130,134–136,232].

Due to the importance of *Wnt1* in early stages of embryonic development of the nervous system, total *Wnt1*-KO mouse models resulted in perinatal death [204,237] (Table 3). The heterozygous *Wnt1*-KO models suffered mild osteopenia but no significantly impaired bone resorption or formation, although osteoblast differentiation and activity were impaired *in vitro* [203]. The hypomorphic *Wnt1* sway mouse model has a better neonatal survival rate than the total *Wnt1*-KO and it recapitulates several phenotypes of OI patients, such as spontaneous fractures and severe osteopenia caused by a decrease in osteoblast activity (Table 3) [205]. Similarly, the *Wnt1*-cKO in MSC [208] and the *Wnt1*-cKO in late osteoblasts and osteocytes [208] also recapitulate the OI phenotype, with impaired osteoblast activity, decreased mineralization and bone formation rate and spontaneous fractures, the latter displaying a milder phenotype, with conserved osteoblast and osteoclast numbers [203,208] (Table 3). In contrast, when *Wnt1* is overexpressed in osteoblasts or osteocytes, a HBM phenotype is obtained [208] (Table 3). Osteoblast number and mineral apposition rate are increased while osteoclast number per bone surface remains similar to controls. Inhibition of mTORC1 in the *Wnt1* GoF model was associated with reduced mineralization and bone formation [208]. The OI phenotype of the hypomorphic *Wnt1* sway mice was also corrected by the mTORC1 activation provoked by *Tsc1*-cKO in late osteoblasts and osteocytes [208]. In a recent study, a comparison between *Wnt1*-cKO in osteoblasts and cKO in monocytes showed the development of general osteoporosis with high bone fragility only in the osteoblast cKO. The effect was gender- and age-independent and independent of LRP5 [207] (Table 3).

3.2 WNT3A

WNT3A can signal through the canonical Wnt pathway and through several non-canonical Wnt pathways including PLC-PKC, PCP, JNK, PI3K-RAC1 and mTORC2 [221,241–244]. Although WNT3A

is not expressed in osteoblasts, it can activate the canonical pathway in them to promote their differentiation (*in vitro*) [245,246]. Because of this feature, Wnt3a is, together with Wnt1, the most used Wnt ligand for the stimulation of canonical Wnt signaling that induces cell proliferation and survival in osteoblasts [247,248]. In uncommitted osteoblast precursors and differentiated osteoblasts, Wnt3a prevents apoptosis through β -catenin-dependent signaling cascades involving Src/ERK and PI3K/AKT [222,245,249]. Wnt3a also activates the non-canonical Wnt/JNK pathway in hMSC. This activation might represent either a feedback mechanism for canonical Wnt signaling regulation or a signaling cascade downstream from Wnt/ β -catenin that induces osteoblast differentiation [242]. Wnt3a treatment does trigger a negative feedback mechanism, as demonstrated by the increase in Wnt antagonists (axin2, DKK2, sFRP2) and the decrease in WNT2B. Important components of the Wnt/PCP pathway such as WNT5A, WNT11, NKD1 and PRICKLE1 are also activated by Wnt3a treatment [242].

In vitro, WNT3A plays an important role in the differentiation of MSCs into osteoblasts by the activation of the canonical pathway and the non-canonical pathway through the activation of PKC δ [221,250]. The expression of alkaline phosphatase (ALP), bone sialoprotein, osteocalcin and osterix is increased by WNT3A [221,248]. These results contradicted those of Boland *et al.* that stated that WNT3A promoted the proliferation of osteoblast precursors in their undifferentiated state, by the inhibition of ALP and bone sialoprotein [245,251]. Liu *et al.* demonstrated that although WNT3A can inhibit osteogenic differentiation, inhibition towards the adipogenic lineage will prevail over inhibition of osteogenic differentiation under lower WNT3A concentrations, thus promoting osteogenic differentiation [252]. WNT3A also inhibits MSC differentiation into chondrocytes [243].

A WNT3A mutation has been described in homozygosis in one patient with a skeletal dysplasia consisting of osteopenia, bilateral coxa vara deformity, mild left radial and ulnar bowing, broadening of metaphases, and bilateral shortening of the great toes and thumbs [73](Table 1 Figure 3). Wnt3a-KO mice were not viable due to several defects in embryonic development [209,210](Table 3).

3.3 WNT4

WNT4 plays a role in the differentiation of osteoblasts through the non-canonical pathways. While expression levels of Wnt4 decrease during the proliferative expansion period of MSC, they significantly increase during the osteoblastic differentiation stage [253]. In addition, BMP-induced osteogenic differentiation is enhanced *in vitro* in human and murine MSC by WNT4 overexpression, which activates the non-canonical p38 MAPK-mediated signaling pathway without activating either the canonical or JNK pathway [254]. WNT4 also inhibits bone resorption and bone-loss-related inflammation [213]. WNT4 has also been reported to play a role in blood vessel development, suggesting that, *in vivo*, WNT4 may promote bone regeneration by promoting MSCs to form a suitable microenvironment to generate an entire bone/bone marrow structure [254].

In humans, the region containing WNT4 and ZBTB40 has been repeatedly reported to be associated with bone mass in different association studies [131,135,255]. *In vitro* studies clearly showed that the knock-down of ZBTB40 in osteoblasts displayed disrupted osteoblast differentiation and mineralization while the WNT4 knock-down did not [212]. *In vivo*, Zbtb40-KO mice showed a reduction in lumbar spine areal BMD and bone volume, but not the decrease in femoral BMD observed in Wnt4-cKO [212] (see below). Therefore, although a role of WNT4 in bone cannot be ruled out, ZBTB40 may also be responsible for the association of this region with bone mass.

Wnt4-KO mice die soon after birth and do not display any skeletal phenotype although a delay in chondrocyte maturation has been observed [211,214] (Table 3). Wnt4-cKO in MSC showed decreased femoral BMD in females [212] (Table 3). The difference in results between the *in vivo* and *in vitro* KO models might reside in the paracrine effect of Wnt4 affecting bone resorption, which would not be observable *in vitro* [212]. On the other hand, overexpression of Wnt4 in differentiated osteoblasts leads to higher BMD and higher osteoblast number and activity [213] (Table 3). Estrogen-deficiency-induced and age-related bone loss were also lower in these Wnt4 overexpressing mice, with higher bone

formation and lower bone resorption. Proinflammatory cytokines typical in estrogen deficiency bone loss were also inhibited under Wnt4 overexpression through the inhibition of the NF κ B in macrophages and osteoclast precursors. Overexpression of Wnt4 partly mitigated the TNF inflammation-caused bone erosion phenotype in TNF+Wnt4 overexpressing mice [213]. Wnt4 suppresses RANKL-induced osteoclast differentiation by inhibiting the binding of the activated RANK to the TNF receptor-associated factor 6 (Traf6) in the osteoclast [213]. Mouse models have demonstrated that although the absence of Wnt4 can be compensated for in bone, its presence provides protection against bone resorption and strengthens bone formation.

3.4 WNT5A

Although WNT5A has been historically classified as a non-canonical Wnt ligand, it has recently been shown that WNT5A can influence multiple Wnt pathways depending on the available FZD receptors [256]. Furthermore, recent studies have demonstrated that WNT5A can both activate and inhibit the canonical Wnt signaling pathway [201,247,256] and that it takes part in both osteoblast and osteoclast differentiation [97,217,257,258]. WNT5A is expressed in MSCs, osteoblasts and osteoclasts [257]. *In vitro* studies showed that in the absence of FZD4 and LRP5, cells responded to WNT5A by inhibiting the WNT3A-mediated canonical pathway [201]. WNT5A can induce β -catenin degradation independently of GSK3 phosphorylation [216] and through the non-canonical WNT/Ca²⁺ pathway, thus inhibiting TCF-mediated transcription downstream of β -catenin stabilization [256,259]. In the presence of FZD4 and LRP5 receptors, however, WNT5A can activate canonical Wnt signaling [201]. It has been suggested that sphingosine-1-phosphate (S1P) promotes the expression of WNT5A and LRP5 in osteoblasts [260], and that WNT5A promotes osteoblast differentiation via LRP5/6 expression in an osterix-dependent manner [217]. Similar to WNT10B, WNT5A inhibits stem cell differentiation into adipocytes, promoting osteoblastogenesis through the non-canonical pathways [261]. Wnt5a treatment stimulated bone nodule formation and ALP expression in hMSCs [257,262]. WNT5A plays an important role in osteoclastogenesis, especially during embryonic development. Osteoblast-lineage cells and osteoclast precursors express WNT5A and ROR2 respectively, and ROR2 signaling in osteoclast precursors enhances RANKL-induced osteoclastogenesis through the JNK/cJun/Sp1 pathway [97,258].

In humans, LoF mutations in *WNT5A* result in the dominant Robinow syndrome [54,247] (Table 1 and Figure 3). On the other hand, WNT5A is highly expressed in synovial tissues in rheumatoid arthritis (RA) patients. Excess WNT5A-ROR2 signaling may contribute to bone loss in this disease, as inhibition of Ror2 signaling suppressed bone loss in rheumatoid arthritis models [97,258].

Wnt5a-KO mouse models show perinatal mortality and a variety of developmental aberrations, partly due to a delay in chondrocyte hypertrophy and skeletal ossification [97,215,263] (Table 3). Osteoblast lineage cells extracted from these mice had impaired mineralization and differentiation [217]. These embryos express increased β -catenin signaling in the distal limb, and *ex vivo* inhibition of the canonical pathway partially recovers the limb phenotype [215,216]. Heterozygous Wnt5a-KO mice are viable, although they show reduced BMD as a result of lower osteoblast and osteoclast number and increased adipogenesis [97]. Wnt5a-cKO in osteoblasts maintained impaired bone formation and osteoclast formation and bone resorption due to suppression of RANK expression in osteoclast precursors [97] (Table 3). In mice, endogenous Wnt5a and Wnt5b regulate endochondral skeletal development by coordinating chondrocyte proliferation [216,263]. The Wnt5a-cKO in chondrocytes showed delayed osteoblast differentiation, suggesting that Wnt5a secreted from chondrocytes regulates osteoblast differentiation separately from the chondrocyte differentiation also necessary for bone formation [263] (Table 3). General overexpression of Wnt5a prior to E7.5 has lethal consequences. After that time point, multiple phenotypes can be obtained due to ectopic Wnt5a expression. Some of these phenotypes can be linked to the inhibition of canonical Wnt signaling and others to its activation. Referring to bone phenotype, Wnt5a overexpression causes delayed bone formation in the developing skull and limbs, suggesting impairment in both endochondral and intramembranous ossification

processes [201]. However, when Wnt5a was overexpressed only in osteoblasts, no differences were detected compared with wild type bone [222] (Table 3).

3.5 WNT5B

WNT5B is linked to bone development, especially MSC differentiation and chondrocyte proliferation [263,264]. WNT5A and WNT5B play redundant roles in the differentiation of mesodermal progenitor cells into MSCs *in vitro*. The inhibition of both ligands is necessary for the impairment of this differentiation. This differentiation is activated by the WNT5/calmodulin pathway [264].

WNT5B has been repeatedly associated with bone mass in GWAS studies [129–131,134–136]. Furthermore, WNT5B has been found upregulated in female to osteoarthritis (OA) patients and *in vitro* in osteoblasts exposed to glucocorticoids through RNA expression microarrays. [265]. [266].

Wnt5b, in coordination with Wnt5a, plays an important role in longitudinal bone growth by regulating chondrocyte proliferation. Both Wnt5b-KO and mice overexpressing Wnt5b in chondrocytes had delayed chondrocyte differentiation, the latter resulting in delayed bone ossification [263], while the skeletal phenotype of Wnt5b-KO mice has yet to be described [218] (Table 3).

3.7 WNT7A

WNT7A can act through the canonical and non-canonical Wnt pathways and plays an important role in limb and craniofacial development during embryogenesis [267,268].

In humans, complete and partial LoF mutations have been described in Al-Awadi-Raas-Rothschild syndrome (AARRS) and in the less severe Fuhrmann syndrome, respectively [69,267]. It is also responsible for the recently described Santos syndrome [70] (Table 1 and Figure 3). These syndromes share a phenotype of ectodermal dysplasias, with severe limb defects among other characteristics [70,267].

Wnt7a-KO mice have impaired limb development, especially affecting digit number and formation [219] (Table 3). The polysyndactyly present in the En1-KO, a Wnt7a suppressor, was restored in En1+Wnt7a-dKO mice. A reduction in Dkk1 expression, typically associated with polysyndactyly, resulted in the restoration of digit number in Dkk1^{dn}+Wnt7a-KO and Dkk1^{dn}+Wnt7a-KO mouse models, whereas other phenotypic characteristics were not restored. These results indicate that both Wnt7a and Dkk1 seem to cooperate to determine digit number [219].

3.8 WNT7B

WNT7B is a potent bone anabolic protein both during embryogenesis and in the postnatal life of mice. Specifically, it markedly increases both the number and function of osteoblasts [222]. WNT7B stimulates bone formation through non-canonical activation of mTORC1 and PKC δ [221,222]. In the developing long bones of the mouse, Wnt7b is enriched in the osteogenic perichondrium flanking the hypertrophic cartilage, but its expression declines in the more mature osteoblasts, indicating transient upregulation of Wnt7b during osteoblast differentiation *in vivo*, regulated by Ihh [206,221,269].

In humans, rare mutations in WNT7B have not been associated with any particular disease. However, some GWAS have reported the association of SNPs in the WNT7B region with heel BMD [134–136]. Additionally, increased WNT7B expression has been reported in the articular cartilage of OA patients [270,271] and in the macrophage-like cells, fibroblastic cells, and vessel walls in the synovium in RA patients, particularly in regions of inflammation [270].

Although total Wnt7b-KO results in perinatal death [220], deletion of Wnt7b in the skeletal osteoprogenitors causes only a modest delay in ossification that is resolved after birth [221,222] (Table 3). This delay is apparently connected to a delay in chondrocyte maturation and a deficit in osterix-

mediated osteoblast differentiation [221]. On the contrary, overexpression of Wnt7b in preosteoblasts, mature osteoblasts or both osteoblasts and chondrocytes resulted in accelerated bone formation and denser bones in mouse models, with little bone marrow space due to its ossification [206,222] (Table 3). This phenotype alleviates with age, probably due to higher bone resorption [222]. Mice overexpressing Wnt7b in mature osteoblasts showed an increase in the amount of osteocalcin and osteoblast number, while the number of osteocytes was similar to the control. Conditional deletion of Raptor in mice overexpressing Wnt7b in preosteoblasts reduced the Wnt7b-induced hyperactivity of osteoblasts, but not their number [222]. Postnatal induction of Wnt7b in mice also resulted in increased mineral density [222]. When Wnt7b was activated in aged mice, the increase in bone formation was higher than the increase in bone resorption, leading to denser bones with increased mineralization. Wnt7b also takes part in the later stages of bone healing, accelerating healing by means of increased mineralization [223]. Therefore, Wnt7b seems to play a role in bone formation but not as much in bone resorption. Wnt7b expression is minimal in the bones of young adult mice, but can be upregulated by mechanical bone loading [240]. This induction decreases with age in both mice and humans [223,240,272].

3.9 WNT9A

The main role of WNT9A in bone is related to chondrocyte regulation and is associated with the Wnt canonical pathway [273,274]. WNT9A is required for appropriate chondrocyte arrangement prior to endochondral ossification [274], and to maintain joint integrity [214]. WNT9A expression decreases in MSCs upon osteoblastic differentiation [245].

Wnt9a-KO mice die at birth and display skeletal abnormalities such as partial joint fusion, shortened long bones, reduced mineralization and chondroid metaplasia [214] (Table 3). The reduction in mineralization in null mice can be explained as a delay in chondrocyte maturation and therefore endochondral ossification. In addition, as signals from the joints are suspected to define the size of the skeletal elements, this would explain why a decrease in Wnt9a in the joints is associated with shorter long bones, through the spatial and temporal modulation of *Ihh* signaling during embryonic development. The phenotypes associated with synovial joints, as well as the delay in chondrocyte maturation, are augmented in Wnt9a+Wnt4 dKO [214] (Table 3). It is possible that the Wnt9a-mediated canonical pathway and the Ror pathway converge to regulate chondrocyte maturation. Ror2+Wnt9a-dKO mice showed a delay in both chondrocyte and osteoblast maturation [161] (Table 3).

3.10 WNT10A

WNT10A plays an early role in osteoblastogenesis, favoring bone formation mainly through the inhibition of adipogenesis. As well as WNT6 and WNT10B, WNT10A suppresses the differentiation of MSC into adipocytes through the canonical Wnt pathway [258,275]. Because of this role, WNT10A is upregulated in the proliferative period of MSCs and its expression decreases upon differentiation into osteoblasts [253]. *In vitro*, demethylation of WNT10A has been seen to inhibit early adipocytic differentiation through the canonical pathway in both MSC and preadipocytes [276].

Mutations in *WNT10A* are associated with ectodermal dysplasias, such as odonto-onycho-dermal dysplasia [277] as well as tooth agenesis due to the important role of WNT10A in odontoblast differentiation [72,278,279] (Table 1 and Figure 3).

Wnt10a-KO mice show a decrease in bone mineralization and impaired bone and dental formation [224–226] (Table 3). Bone volume, mineral apposition rate and bone formation rate were reduced while osteoclast and osteoblast number were similar to that in controls. Interestingly, these mice also showed a decrease in adipocytic differentiation and decrease in adipose tissue in the bone marrow [224]. A Wnt10a-cKO in epithelial cells presented dental defects among other features, although the BMD was not altered [280] (Table 3).

3.11 WNT10B

WNT10B activates the canonical pathway and plays an important role in osteoblastogenesis, inhibiting the expression of adipogenic transcription factors (C/EBP α and PPAR γ) in MSCs while promoting osteoblastogenic transcription factors (RUNX2, DLX5, and Osterix) [227,281]. Although WNT10B does not seem to play such an important role in bone resorption [228,229], osteoclast media including Wnt10b promotes osteoblastogenesis [282]. WNT10B is important in the maintenance and/or renewal of osteoblast progenitor cells [229]. There is evidence about the possible upregulation of Wnt10b expression under mechanical loading of the bone, [283,284] but only under strong stimulation [240]. Wnt10b is expressed in the bone marrow, postnatal growth plate, osteoblastic precursors, and various other stem cell compartments [229].

WNT10B has been implicated in several human diseases [285]. Homozygous missense mutations in WNT10B were demonstrated to cause split-hand/foot malformation with autosomal recessive inheritance [66,247]. Mutations in *WNT10B* are also implicated in dental anomalies and oligodontia [60,286] (Table 1 and Figure 3). In addition, it has been shown that the expression of *WNT10B* correlates with the survival rate in patients with osteosarcoma [287].

Studies in KO and transgenic mice have found that Wnt10b facilitates osteogenesis and increases bone mass [227]. Wnt10b-KO mice have a 25–30% decrease in bone volume fraction, BMD, and trabecular number compared with WT controls [227] (Table 3). A reduction in the number and function of osteoblasts was detected in homozygous and heterozygous Wnt10b-KO mice. Interestingly, Wnt10b-KO animals showed an enhanced trabecular structure at 2–4 weeks of age that was rapidly followed by progressive osteopenia, suggesting that Wnt10b helps to maintain osteoblast precursors in an undifferentiated state and that in its absence, the accelerated differentiation of mesenchymal osteoprogenitors occurs, depleting the stem cell pool [229]. Both osteogenesis and adipogenic centers were affected in this model. Both copies of Wnt10b are required for normal maintenance of adult bone homeostasis and the loss of bone in aged heterozygous animals is as severe as that for Wnt10b-KO mice [229]. Related to that, Wnt10b levels are downregulated in the absence of estrogen in ovariectomized animals, another model of osteoporosis [288]. On the other hand, transgenic overexpression of Wnt10b in mature osteoblasts and bone-marrow-derived mesenchymal precursor cells results in increased bone mass and bone strength [228] (Table 3). Mice overexpressing Wnt10b in MSCs show increased bone mass and are resistant to age-related and hormone-related bone loss (Table 3). Furthermore, these mice are genetically lean, have reduced adiposity and are resistant to obesity [227]. The increased bone mass in mice overexpressing Wnt10b in osteoclasts is caused by increased bone formation due to elevated osteoblast number, while the adipose tissue of the mice is not affected (Table 3). The increase in mandibular bone results in delayed incisor teeth tooth development [227,228].

3.12. WNT16

WNT16 is an important Wnt ligand involved in the regulation of postnatal bone homeostasis. The precise mechanism by which WNT16 acts on the different signaling pathways of WNT has not been fully identified. Different studies postulate that the effect of WNT16 on these pathways is cell-type specific. While there are studies showing that it activates the canonical pathway in MC3T3-E1 preosteoblasts and in Saos2 osteoblast-like cells, WNT16 does not activate this pathway in HEK293 cells [231,289,290]. There is evidence that in chondrocytes, MC3T3-E1 and perivascular stem/stromal cells, WNT16 is also capable of acting through non-canonical pathways (PCP/JNK [291] and JNK [231,292]). Altogether, these results indicate that WNT16 regulates both canonical and non-canonical Wnt targets in osteoblasts [293].

In humans, the genomic region of *WNT16* is one of the most consistent GWAS signals, and has been associated with different skeletal phenotypes, including BMD, bone strength, geometric

parameters, cortical bone thickness and fracture risk [129–133,135–137,232,233,237,294–298]. In addition, the effect of WNT16 has been associated, through various pieces of evidence, with OA. First, the expression of *WNT16* is dramatically upregulated after cartilage injury or in OA onset [299]. Wnt16-deficient mice develop severe OA because of reduced lubricin and increased chondrocyte apoptosis [300]. Using chondrocyte-cKO animal models and overexpression of Wnt16 by intra-articular injection of adenoviral Wnt16 vectors, Tong *et al.* determined that Wnt16 inhibited chondrocyte hypertrophy in both skeletal development and OA pathology, therefore producing positive effects on cartilage and ongoing OA [291]. Contrary to these experiments, Törnqvist *et al.* [301] found that overexpression of Wnt16 in osteoblasts increased the subchondral bone mass but had no impact on OA in young adult female mice.

Wnt16-KO and the osteoblast-specific Wnt16-cKO mouse models show spontaneous fractures as a result of low BMD, low cortical thickness, reduced bone strength, and high cortical porosity, while the trabecular bone volume is not altered [230–233]. Consistent with this, Wnt16 was found to be more highly expressed in cortical bone than trabecular bone [231]. The phenotype of the osteocyte Wnt16-cKO mouse model is modest compared with the early osteoblast Wnt16-cKO [231]. On the other hand, when Wnt16 is overexpressed in osteoblasts and osteocytes, the mouse models show higher BMD and better bone resistance in both trabecular and cortical bone [235,236,302] (Table 3). The difference in effect in the trabecular bone phenotype between the KO and KI mice might be due to the low expression of Wnt16 in the trabecular compartment. Despite the obvious bone phenotype that Wnt16 produces in mouse models, the molecular mechanisms by which Wnt16 regulates bone metabolism are not yet fully understood. Some authors suggest that Wnt16 acts by inhibiting osteoclastogenesis both indirectly, through the increase in OPG, and directly, acting on the osteoclast progenitors by osteoblast-derived Wnt16 [231,303]. Other studies have shown that Wnt16 also exerts an effect on bone formation, increasing both the activity and the number of osteoblasts [230]. Different effects have also been observed in transgenic mouse models overexpressing Wnt16 in osteoblast cells; some do not show differences in bone formation and resorption [302], while others show a large increase in bone formation and a moderate decrease in bone resorption [235,236]. Moreover, Shen *et al.* demonstrated *in vitro* that Wnt16 promotes osteogenic differentiation of human perivascular stem/stromal cells [292]. Taking all these studies into account, it would appear that Wnt16 has a dual anabolic and anti-resorptive effect. Different animal models show that the effect of Wnt16 and estrogen are independent [302], although it has been shown that the expression of Wnt16 in bone varies according to estrogen status [302,304]. Moreover, Wnt16 is a crucial regulator of cortical bone in young adults and old mice [234]. These discoveries make WNT16 a good candidate as a therapeutic target for the treatment of osteoporosis in postmenopausal women. Another interesting potential application of Wnt16 treatment would be in bone loss due to glucocorticoid treatment. In reference to this, studies in animal models with Wnt16 over-expression and glucocorticoid treatment have been performed, with contradictory results. While some report partial protection against glucocorticoid-induced bone loss [305], others report that Wnt16 overexpression does not prevent it [306]. Therefore more studies will be needed to clarify whether WNT16 could be a good therapeutic solution.

2. INHIBITORS

We can group the Wnt inhibitors into two categories depending on the action they perform: (i) interfering with LRP binding to FZD-WNT, such as the DKK protein family, sclerostin and WIF-1, and (ii) binding to the WNT ligands and preventing their binding to the membrane receptors, such as the sFRPs 1–5.

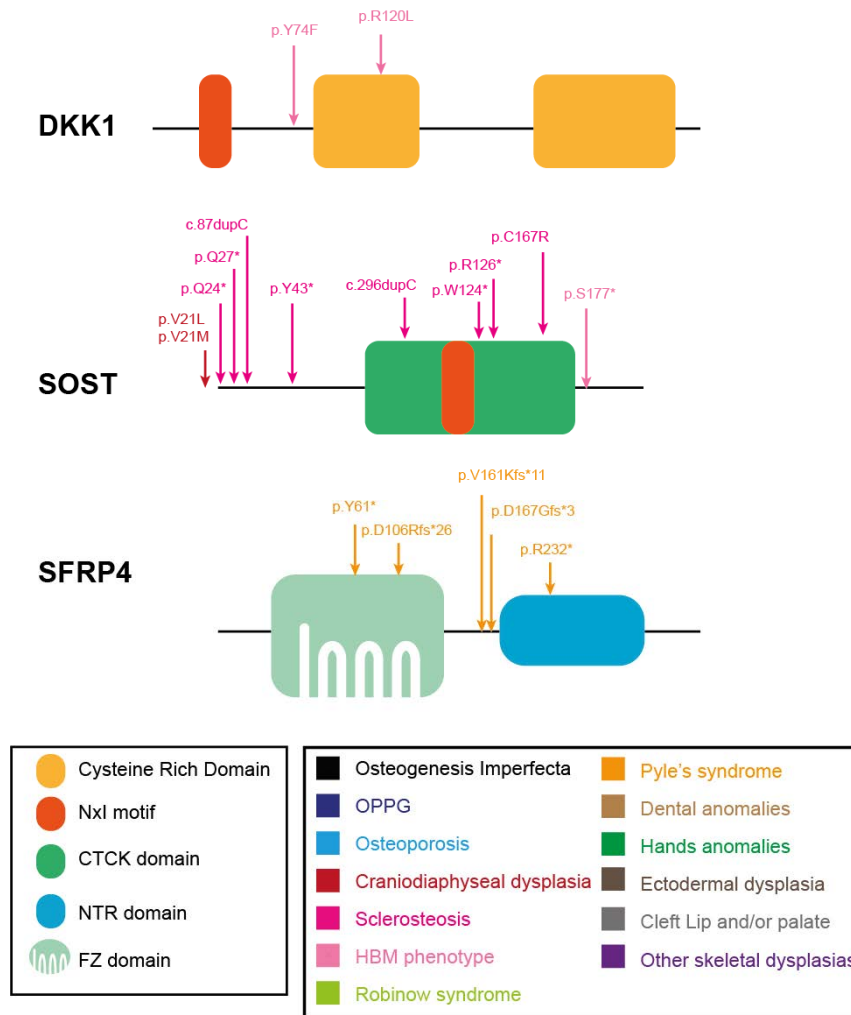


Figure 4. Schematic protein representation of the Wnt inhibitors with the mutations causing human skeletal diseases.

Table 4. Wnt pathway inhibitor transgenic mouse models

Gene	Model	Tissue expression	Phenotype			Comments	Ref
			B.M	B.F.	B.R.		
Dkk1	KO	Total KO	-	-	-	NV	[307]
	Het KO	Heterozygous KO	↑	↑	=		[308,309]
	doubleridge	Hypomorphic	↑	-	-	Deletion of a downstream regulatory region	[309,310]
	Rosa26-CreERT2	Tamoxifen-inducible at 7 weeks	↑	↑	↓	Only the OC number	[311]

	cKO-OSX-Cre	Pre-OB	↑	↑	↓ ⁱ	Only the OC number	[311]
	cKO-Dmp1-Cre	OCy	↑	↑	↓ ⁱ	Only the OC number	[311,312]
	Tg-2.3 Kb rat-Col1a1	OB	↓	↓	=		[313,314]
Wnt3 & Dkk1	dKO - Het Wnt3+ Het Dkk1	Total Het KO	↑	↑	=	NV	[315–317]
Dkk2	KO	Total KO	↓	↓ ⁱⁱ	↑	Major defects in mineralization “Only BFR	[318]
	KO	Total KO	↑↑	↑	=		[319,320]
	cKO-Prx2-Cre	Limb mesenchyme	↑↑	-	=		[321]
	cKO-2.3KbCol1a1-Cre	OB	↑	-	=		[321]
	Dmp1-Cre	OCy	↑	-	=		[321]
Sost	Col10a1-Cre	CD	↑	-	=		[321]
	Tg-Dmp1-SOST	OCy	↓	↓	=		[322,323]
	Tg-Oc-SOST	OB	↓	↓	=		[324]
	Tg-SOST	Total Tg.	↓	↓	=	Insertion of ~158-kb from 3'-end of the <i>DUSP3</i> to <i>MEOX1</i> .	[325]
Sostdc1	KO	Total KO	↑	↑	↓	During the first 1.5 to 3 months of postnatal development	[326]
Sostdc1 & Lrp5	dKO	Total KO	=	-	-		[326]
Sfrp1	KO	Total KO	↑	↑	=	Increased healing	[327–329]
	Tg-Sfrp1	Total overexpression	↓	-	-	Osteopenia	[330]
Sfrp2	KO	Total KO	=	=	=	Syndactyly	[327,331]
Sfrp4	KO	Total KO	↑ ⁱⁱⁱ /↓ ^{iv}	↑ ⁱⁱⁱ	↓ ⁱⁱⁱ	ⁱⁱⁱ in trabecular bone ^{iv} in cortical bone	[55,100,332]
	Tg-Coll1a1-Sfrp4	Overexpression in OB	↓	↓	=		[333]

Sfrp5	KO	Total KO	-	-	-		[334]
Sfrp4 & Ror2	Sfrp4-KO+Ror2-cKO	Total Sfrp4-KO; Ror2-cKO in OC	↑ ^v	-	↓ ^v	in trabecular bone	[100]
Wif-1	KO	Total KO	=	=	=		[335]
	Tg-2.3Col1a-Wif-1	Overexpression in mature OB	=	=	=		[336]

KO: Knock-out; cKO: conditional KO; het KO: heterozygous KO; NV: No viable; OB: osteoblast; OC: osteoclast; Ocy: osteocyte. BFR: Bone formation rate

4.1. DKK

In vertebrates, the Dickkopf (Dkk) family comprises four genes encoding secreted glycoproteins DKK1–4. Members DKK1, DKK2, and DKK4 have a similar structure, with two CRD1/2 (Figure 4) and act through the canonical Wnt pathway, while DKK3 is the most divergent member of the family, with the addition of a soggy domain at the N-terminus and no effect through the canonical Wnt pathway [337,338].

DKK1 is by far the most studied member of the Dkk family due to its crucial role in head morphogenesis [22,339], and specifically in bone tissue, since the GoF mutations HBM-LRP5 actually impair DKK1 binding, thus producing a more active Wnt pathway [34,126]. DKK1 performs its function by inhibiting the canonical Wnt pathway through binding with high affinity to LRP5/6 coreceptors. While it has been shown that DKK1, DKK2, and DKK4 can interact with LRP5/6, DKK3 does not [22,339–343]. The CRD2 domain binds, with high affinity, to the first and third β -propeller domains of the LRP5/6 coreceptors and to KRM1/2, and the NXI domain binds, with moderate affinity, the first β -propeller domains of the LRP5/6 coreceptors [121,199,340,343–346]. As explained in the KRM section, KRM and DKK1 binding facilitates DKK1 inhibition by forming a heterotrimeric LRP5/6-DKK1-KRM1/2 complex that induces rapid internalization of LRP5/6, decreasing the availability of these receptors in the cell membrane [163,164]. However, it can also have an inhibitory effect in the absence of KRM1/2 [163]. DKK1 is detected at low levels in most human tissues except the placenta [347,348], but in a body-wide screen of *Dkk1* expression in young and adult mice, strong expression of *Dkk1* was found to be mainly restricted to bone [313]. This expression is most abundant in bone from young mice and decreases dramatically in adolescent and adult mice [349,350]. *Dkk1* expression has been demonstrated in osteoprogenitors, osteoblasts, osteocytes, and adipocytes [121,308,351,352], but there is controversy over whether the main source is early osteoblasts or mature osteocytes [311,353,354].

DKK1 has been implicated in the pathogenesis of various diseases affecting bone and is a crucial modulator of glucocorticoid-induced [266,355,356] and estrogen-deficiency-induced bone loss [356,357]. Deregulation of DKK1 expression levels is associated with various bone-related disorders such as osteonecrosis of femoral head (ONFH) [358] and Paget disease; diabetes mellitus; autoimmune diseases such as RA, OA, ankylosing spondylitis, and systemic lupus erythematosus; and various types of cancer including multiple myeloma, breast cancer, and prostate cancer [359,360]. Very little data exists on the potential role of *DKK1* in bone mass in humans. To date, no disease-causing mutation has been reported in the Human Gene Mutation Database (HGMD). Recently, we described two mutations (p.Try74Phe and p.Arg120Leu) in patients with the HBM phenotype, which shows a loss of DKK1 inhibitory activity towards the Wnt pathway [50,361]. Nevertheless, the p.Arg120Leu mutation has also been found in patients with unrelated phenotypes such as osteoporosis and anal malformations [362,363]. No SNPs have been found to be associated with BMD or with different bone parameters in several GWAS. However, many SNPs located in a 350 kb-downstream region of *DKK1* have been found to be associated with BMD [129–136,294,298]. Interestingly the *DKK1*-activating long non-coding RNA (*LNCAROD*) is found in that region [364]. A recent study by our group confirmed the physical

interaction between this region enriched in GWAS signals and the *DKK1* promoter, using the circularized chromatin conformation capture (4C) assay [361].

Dkk1-KO mice die shortly after birth and exhibit developmental defects, including head defects and limb dysmorphogenesis [307] (Table 4). In contrast, the heterozygous *Dkk1*-KO mouse, the hypomorphic (doubleridge, *db*) mouse, the tamoxifen-inducible at 7 weeks mouse, and the osteolineage-specific mouse model show an increase in BMD and bone formation [308–312](Table 4). By studying the allelic combination of null or *db* mice (*Dkk1*^{+/db}; *Dkk1*^{-/-}; *Dkk1*^{db/db}; *Dkk1*^{db/-}; *Dkk1*^{-/-}) it was seen that the increase in BMD correlates dose dependently with decreased expression of *Dkk1* [309,310]. In contrast, transgenic *Dkk1* overexpression causes osteopenia with a decrease in the number of osteoblasts and bone formation (Table 4) [313,314]. In dKO mouse models, it was shown that the developmental lethality in the *Dkk1*-KO and head morphogenesis were rescued by reduced *Lrp6* or *Wnt3* expression (Table 4) [310,317]. While all models share an effect on bone formation, some of the generated mouse models show no difference in bone resorption, whereas osteoblast lineage cell-specific or tamoxifen-inducible cKO mice show a significantly reduced number of osteoclasts and a reduced RANKL/OPG ratio [308–311]. In addition to these bone phenotypes, it was found that the response to dynamic tibial loading was augmented in mice lacking *Dkk1*, but that *Dkk1* does not seem to be essential for transducing the response to cyclic loading [240,316,365].

Thanks to *in vitro* and *in vivo* experiments, DKK1 has been confirmed to affect osteoblast function and has been linked to inhibition of the differentiation of osteoblasts and chondrocytes. Moreover, DKK1 also provokes the stimulation of adipogenesis and the activation of osteoclasts, increasing the RANKL/OPG ratio [308,311,313,351,359].

While DKK1 acts as a pure inhibitor of the Wnt canonical pathway, DKK2 can inhibit or activate the canonical Wnt pathway depending on the cellular context [121,163,164,287,366]. DKK2, like DKK1, binds with high affinity to KRM1/2 and LRP5/6 through the CRD2 [121,164,287,366]. *In vitro* studies in HEK293T cells have revealed that in the presence of KRM2, or low levels of LRP5/6, DKK2 behaves like an LRP6 antagonist, preventing the binding of Wnt to the membrane coreceptor. In contrast, in the absence of KRM2 or the presence of high levels of LRP5/6, DKK2 can activate the LRP6 coreceptor independently of DVL [164,366]. The same occurs in the absence of WNT7b, where DKK2 inhibits osteogenesis in culture, and the presence of high WNT7b levels induces terminal osteoblast differentiation [318]. Although the homology between DKK1 and DKK2 is very high, especially in the CRD2 domain, there are some differences in affinity for the LRP5/6 transmembrane coreceptors. While DKK1 has a higher affinity for LRP6, DKK2 has higher affinity for LRP5 [163,341]. Furthermore, the pattern of expression in osteoblasts and the regulation of gene expression are very different. While DKK2 expression is higher in osteoblasts than in osteocytes, that of DKK1 is higher in osteocytes [318].

The *Dkk2*-KO mouse model shows osteopenia, a decreased rate of bone formation, and an increased number of osteoclasts due to the increased RANKL/OPG ratio (Table 4). Through the study of the differentiation of cultures of calvary osteoblasts, it was postulated that *Dkk2* could affect the terminal differentiation of osteoblasts that leads to the formation of mineralized matrices [318].

4.2. Sclerostin and SOSTdc1

Sclerostin (encoded by the *SOST* gene) and SOSTdc1 (SOST domain-containing protein 1; aka SOST1, USAG-1, WISE or ECTODIN) are paralogous secreted proteins and due to their cysteine knot motifs are classified in the DAN family of BMP antagonists [367–369]. Sclerostin and SOSTdc1 share 55% homology, with a very similar C-terminal domain. Both have been described as inhibitors of the BMP pathway and as WNT antagonists [326,370–375].

Sclerostin is a cysteine-rich glycoprotein that acts as a potent antagonist of the Wnt pathway, exerting its effect by blocking LRP5/6 co-receptors [370,371]. To perform this function, sclerostin binds

to LRP4, thereby enhancing its suppressive effect on the canonical Wnt pathway [42,93,94]. The binding of sclerostin to LRP5/6 occurs through the first β -propeller domain, so sclerostin can inhibit the function of the Wnt ligands that bind to this domain (e.g., WNT1) but not those that bind through the third β -propeller domain (e.g., WNT3A) [199]. This function is confirmed by mutations causing the LRP5-HBM phenotype, which show a loss of sclerostin affinity [122,124]. Due to its abundant expression in osteocytes, sclerostin was originally categorized as a protein exclusive to mature osteocytes [376]. Accordingly, the highest levels of sclerostin were found in osteocytes surrounded by mineralized bone and distant from active bone surfaces [377]. However, new studies have reported, through histological examination, that *SOST* transcripts are also found in bone marrow, cartilage, kidney, liver, lung, heart and pancreas [378]. In relation to bone, in addition to osteocytes, sclerostin expression has been found in osteoblasts, osteoclasts, mineralized hypertrophic chondrocytes, odontoblasts, and cementocytes [379]. *SOST* expression has also been found in the developing embryo, where it plays a role in limb patterning, which explains the cases of syndactyly in patients with sclerosteosis [380].

In humans, *SOST* mutations are associated with conditions characterized by excess bone formation: sclerosteosis, van Buchem disease, craniodiaphyseal dysplasia and the HBM phenotype (Table 1 and Figure 4). Indeed, the first evidence of the role of *SOST* in bone was the identification of LoF mutations responsible for sclerosteosis, a very severe autosomal recessive disease, caused by a lack of functional sclerostin [39–41,381]. Furthermore, deletion of a regulatory region 52 Kb downstream of the gene, necessary for the correct expression of *SOST*, has also been found in patients with Van Buchem disease [44]. Van Buchem disease is less severe than sclerosteosis and the difference in severity lies in the fact that in Van Buchem patients there is some expression of the *SOST* gene, whereas in patients with sclerosteosis there is none [382,383]. Moreover, craniodiaphyseal dysplasia is an extremely rare sclerosing bone dysplasia associated with missense mutations in the signal peptide of *SOST* that cause a decrease in its extracellular secretion [38]. Gregson *et al.* [49] also associated mutations in *SOST* with the HBM phenotype. In addition to these diseases, different GWAS studies have shown their association with BMD and the risk of fractures [129–131,133–136,384]. Furthermore, altered expression of the gene has been found under special or pathological conditions. For example, although no sclerostin protein expression has been found in articular cartilage in healthy joint tissue, many studies have reported sclerostin protein expression in articular chondrocytes isolated from osteoarthritic joints or in rheumatic joint diseases. Nevertheless, it is unclear whether sclerostin plays a protective role or whether it mediates disease pathogenesis [385]. Several groups have shown elevated serum sclerostin levels in postmenopausal women [386,387], with aging [388–392], in patients with excess glucocorticoids [393], in multiple myeloma patients correlating with osteoblast function and low survival [394], in prostate cancer and breast cancer [395,396] and in vascular calcifications [397,398].

The importance of the *SOST* gene in the determination of BMD has been demonstrated using animal models. *Sost*-KO mice show a phenotype of high BMD and bone strength with a high number of osteoblasts and increased bone formation that reproduce the phenotype observed in patients with sclerosteosis or Van Buchem disease [319,320] (Table 4). In these mice, no changes were observed in the number of osteoclasts or in bone resorption markers, although an increase in OPG was observed [319]. In order to identify the cell-type specific contributions to HBM of the *Sost*-KO, Yee *et al.* [321] generated different cKOs and observed that despite the fact that osteocytes are the main source of sclerostin, the cKO that best phenocopies the HBM of the *Sost*-KO is the *Sost*-cKO in osteoprogenitor cells of the limb mesenchyme rather than the *Sost*-cKO in early stage osteocytes (Table 4). In contrast, mice that overexpress this gene present a low bone mass, lower bone resistance, a low number of osteoblasts and reduced bone formation [322–325]. In addition, the mouse model with specific overexpression in osteocytes shows high levels of Rankl [322,323] (Table 4). All these data lead to the conclusion that sclerostin is a potent inhibitor of bone formation and that it may also influence the stimulation of bone resorption in some way.

The transcription of the *SOST* gene is finely regulated by a large number of signals. *SOST* expression is controlled by the proximal promoter region of the gene and by a 225-bp bone-specific distal enhancer called Evolutionary conserved region 5 (ECR5) 52 Kb downstream of the gene [399,400]. The proximal promoter region (~ 1.4 kb upstream from the *SOST* gene) contains binding sites to RUNX2, Osterix and SMAD2/3, among others, which promote *SOST* transcription [401–406]. In addition, the proximal promoter contains three estrogen response elements (ERES) that negatively control the expression of *SOST* through estrogen receptor B [407]. Furthermore, it has been determined that this region is subject to epigenetic modifications that regulate its expression, such as methylation (hypermethylated in osteoblasts to hypomethylated in osteocytes) [408,409] or acetylation (deacetylation of H3K9 by Sirt1)[410]. In addition to the proximal promoter, the ECR5 enhancer region regulates the correct expression of *SOST* in bone cells. This region is necessary and sufficient for the correct expression of the gene in osteocytes, although to achieve the high levels of expression that occur, the proximal promoter is also required [400]. The ECR5 region is deleted in patients with Van Buchem disease, and its deletion in mice causes a drastic decrease in *SOST* levels in osteocytes [400]. ECR5 stimulates the expression of *SOST*, in part through the binding of transcription factor Myocyte-enhancer factor 2c (Mef2c) [325,400]. The importance of the Mef2c transcription factor in the enhancing activity of ECR5 was confirmed with the Mef2c-cKO mouse in osteocytes/osteoblasts, which presented HBM and low levels of sclerostin [411]. Besides regulation through the proximal promoter, there is a large number of regulators of the expression of *SOST* in bone whose action is mediated via ECR5. The most relevant negative regulators of *SOST* are: PTH, PTH receptor, cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), mechanical loading, and the IL-6 family of cytokines.

Sclerostin is the first molecular mediator described in osteocytes as responsible for the coupling of mechanical forces to the anabolic response of the skeleton [284,365]. The mechanical loading of bone results in a dramatic reduction in *SOST* expression resulting in increased bone formation, and conversely unloading increases it [365]. This is consistent with the fact that the *Sost*-KO mouse does not lose BMD after unloading [412], while the *SOST* transgenic overexpression mouse shows a reduction in load-induced bone formation [323]. The mechanical loading negatively regulates *SOST* expression by mechanisms that involve prostaglandin E2 production, nitric oxide and periostin expression [413–416]. While *SOST* is absolutely necessary for the anabolic actions of mechanical loading, it appears dispensable for PTH-induced bone gain [399,417]. *SOST* is capable of uncoupling bone formation and bone resorption, inhibiting osteoblast function while stimulating osteoclast function. In addition to these effects, *SOST* has been shown to enhance adipocyte differentiation and promote adipogenesis and adipose hypertrophy via suppression of the Wnt pathway in *in vitro* and *in vivo* experiments [418–420].

Humanized monoclonal antibodies against sclerostin have been developed for the treatment of different bone pathologies such as osteoporosis and OI [421–426]. Antibody treatment against sclerostin stimulates bone formation, decreases bone resorption and increases bone mass and strength while decreasing the risk of fragility fracture in both animals and humans [427–429]. Thanks to positive results in clinical phase 2 and 3 trials for the treatment of postmenopausal osteoporosis [421–424], it is currently approved for the market. The negative aspects associated with anti-sclerostin antibody treatment are that the increase in bone formation is not sustained in the long term and that it is contraindicated in patients with hypocalcemia or a high risk of ischemic heart disease or cerebrovascular disorder, therefore requiring caution before the implementation of treatment [430].

In the case of *SOSTdc1*, many studies, both *in vivo* and *in vitro*, have shown that it acts as an inhibitor of the Wnt pathway in a LRP5/6-dependent manner [326,369,373,431–433]. Despite this, studies in *Xenopus* embryos have shown that *SOSTdc1* can activate or inhibit the Wnt pathway in a context-dependent manner [369]. Furthermore, *SOSTdc1* can also bind to LRP4 [148,434]. Additionally, a mechanism has been described by which the retention of *SOSTdc1* in the endoplasmic reticulum causes a decrease in LRP6 available on the cell surface [435]. Both sclerostin and *Sostdc1* are widely

distributed in the tissues of both the developing embryo and the adult mouse, with *Sostdc1* being the most widely distributed. When expressed in the same organ system, they present non-overlapping expression patterns or different temporal phases, suggesting that these genes have evolved different sub-specializations within the signaling pathways they regulate, as a function of their cellular location [326,380]. For example, while *Sostdc1* is expressed in mesenchymal preosteoblasts during the early period of bone formation (1.5–3 months), at 4 months there is no expression of *Sostdc1* in the osteoblast lineage, while sclerostin is expressed abundantly in both osteoblasts and osteocytes at that time. Hence, *Sostdc1* and *Sost* are differentially expressed in osteoblast cells during temporally distinct phases whereby *Sostdc1* is expressed early and *Sost* later [326].

Sostdc1-KO mice showed a significant transient increase in BMD in the early stages (during the first 1.5 and 3 months) produced by an increase in proliferation and number of osteoblasts (1.5 months), a decrease in the number of osteoclasts and an increase in bone formation rate (2.5 months) [326] (Table 4). In addition, there was dysfunction of the round proliferative chondrocytes, indicating that *Sostdc1* possibly acts as an activator of the Wnt pathway in chondrocytes and as an inhibitor in the osteoblast lineage. A *Sostdc1* spontaneous deficient mouse shown to have severe tooth defects due to the loss of inhibition of the Wnt pathway [326,436–438]. However, another *Sostdc1*-KO model was found to lack any skeletal patterning defects or any obvious limb patterning defects [380] (Table 4). The *Sostdc1* + *Lrp5*-dKO mouse shows normal BMD, thus indicating an interaction between *Lrp5* and *Sostdc1* [326]. Furthermore the *SOST* + *SOSTdc1*-dKO mouse shows a 50% increase in the number of hand defects, suggesting partially redundant and complementary roles for these proteins in limb development [380]. The absence of sclerostin and *Sostdc1* in limbs disrupts the epithelial-mesenchymal communication required for proper limb patterning in these dKO mice [380].

4.3. SFRPs

sFRPs are a family of secreted glycoproteins with a CRD structure in the N-terminus that shares 30–50% similarity with that of FZD receptors and a Netrin-related motif (NTR) in the C terminus [372,439,440] (Figure 4). Thanks to their homology with FZD receptors, sFRPs can bind and sequester Wnt ligands [372] but also FZD receptors [441,442], blocking both canonical and non-canonical Wnt pathways. In the case of direct sFRP-Wnt ligand binding and inhibition, there seems to be a preference for certain combinations. For example, sFRP1 and sFRP2 are able to bind to WNT5A, while sFRP3 and sFRP4 cannot, and sFRP3 can inhibit WNT1 and WNT8 but not WNT3A or WNT5A [443]. This suggests a more complex and specific regulation of the pathway that might also involve the NTR domain and post-translational modifications [372,439,440,443,444]. There are conflicting results as to whether the CRD and NTR domains are sufficient on their own to perform Wnt binding and inhibition [445–447]. These conflicting results might imply that there is a biochemical/functional specificity in sFRP-Wnt interactions in different developmental models [372].

Despite being known as Wnt pathway inhibitors, sFRPs have also been found to activate the pathway. Low concentrations of sFRP1 can stimulate Wnt canonical pathway activation, while inhibiting it at higher concentrations in S2 cells *in vitro* [447]. In HEK and HSG cells, overexpression of Wnt3a and sFRP2 activated the canonical pathway more than Wnt3a overexpression alone [448]. In the case of bone cells, the combination of Wnt3a treatment with sFRP1 or sFRP3 resulted in decreased cell proliferation but increased ALP activity and osteocalcin marker and mineral nodule formation in MSCs that was greater than any of the treatments alone. In this case, osteoblast differentiation was stimulated without the involvement of the Wnt canonical pathway [443,449]. Moreover, sFRPs can also antagonize each other's activity [450], favoring Wnt signaling pathways. Besides, sFRPs can enhance the extracellular transport of Wnt proteins [451], modulate BMP signaling [452] and interact with other receptors or matrix molecules [453]. It was hypothesized that sFRPs may have concentration-dependent, tissue-specific and/or stage-specific effects on MSC differentiation. This would result in small effects on undifferentiated mesenchymal stages that would increase in significance in pre-

osteoblasts or mature osteoblasts *in vitro* [443]. Besides embryonic development, sFRP1–4 have been strongly linked to bone development while sFRP5's involvement in bone has barely been studied [440].

sFRP1 is an important regulator of osteoblast and osteocyte survival *in vitro* [328]. Expression of sFRP1 increases during osteoblast differentiation and peaks in the post-proliferative preosteocytic stage [328]. The higher expression of sFRP1 correlates with the inactivation of the Wnt canonical pathway and acceleration of osteoblast and osteocyte apoptosis *in vitro* and *in vivo* [328,329]. *In vitro* deletion of sFRP1 enhances osteoblast proliferation, differentiation and function, while suppressing osteoblast and osteocyte apoptosis [328]. *In vivo*, Sfrp1-KO mice have an increased bone trabecular area and are protected against age-related bone loss by the slowing down of osteoblast apoptosis and a modest enhancing of osteogenesis and mineralization (Table 4) [328,329]. Chondrogenesis and endochondral bone formation are also affected [454]. Moreover, Sfrp1-KO mice show an advantage in fracture healing due to early bone remodeling. Even though osteogenesis is favored over chondrogenesis, physiologic regulation of Wnt signaling is maintained, possibly thanks to sFRP redundancy, and osteoblast maturation continues as normal [454]. As expected, overexpression of sFRP1 in mice resulted in lower bone formation and osteopenia (Table 4) [330]. Osteoblast activity markers (Propeptide type 1 N-terminal procollagen and OPG) were at similar levels compared to those of the WT but the markers for osteoblast maturation (OC) and bone renovation (RANKL) were lower. Cells derived from Sfrp1 Tg mice had lower ALP activity and mineralized nodule formation. Overexpression of sFRP1 did not affect bone resorption *in vivo* but induced higher osteoclastogenesis *in vitro* [328,330].

On the contrary, overexpression of sFRP2 in MSCs resulted in decreased apoptosis and increased MSC proliferation. Osteogenic and chondrogenic differentiation of the MSCs was inhibited by sFRP2 and could not be retrieved by the addition of recombinant Wnt3a or BMP2. Alfaro et al. proposed that sFRP2 might modulate the expression of Runx during osteogenesis via either the Wnt canonical or BMP pathways [452]. Similarly to sFRP1, sFRP2 negatively affects bone formation *in vitro* but the effects *in vivo* are relatively mild [12]. Sfrp2-KO mice showed defects in digit formation, due to decreased chondrocyte proliferation and delayed differentiation in distal limb skeletal elements [331] (Table 4).

sFRPs show a level of redundancy during embryonic development, as single gene sFRP-KOs showed mild phenotypes that were magnified in the case of double or triple KOs, resulting in embryonic lethality in most cases, such as for Sfrp1+Sfrp2-dKO and Sfrp1+Sfrp2+Sfrp5 triple KO but not Sfrp1+Sfrp5-dKO or Sfrp2+Sfrp5-dKO mice [334,372]. Moreover, Sfrp1+Sfrp2-dKO embryos exhibited craniofacial defects, limb outgrowths, extra digits and a shortened thoracic region [327,334].

sFRP3 expression is also related to the late osteoblast differentiation stage [328,455]. In MSCs, only sFRP3 promoted apoptosis [443,449]. Contrary to sFRP2, which facilitates infiltration and the metastasis of osteosarcomas [258], sFRP3 is downregulated in osteosarcoma patients at the local and systemic levels, [456] and upregulated in bone marrow multiple myeloma samples with advanced lytic bone disease (LBD) as compared to no/limited LBD [457].

sFRP4 is highly expressed in skeletal regions during late embryonic and postnatal developmental stages, in cells of both the osteoblastic and osteoclastic lineages. It is also strongly involved in postnatal skeletal development and aging-related and oxidative stress-related bone loss [332,333,458]. Mutations in sFRP4 are linked to metaphyseal dysplasia or Pyle's disease, characterized by a thinning of the cortical bone, limb deformity and bone fracture [55] (Table 1 and Figure 4). Elevated sFRP4 levels in tumors are associated with osteomalacia, whereby the bone becomes softer and more flexible [440,459]. sFRP4 is also highly expressed in chondrocytes from osteoarthritic patients, where there is a correlation between sFRP4 expression and chondrocyte apoptosis [460].

As sFPR4 alterations are responsible for Pyle's disease, a Sfrp4-KO mouse model was presented as a model for the disease [55]. However, as a single gene KO, Sfrp4-KO mice showed increased trabecular bone mass and decreased cortical bone mass and thickness [55,332] (Table 4). This thinning was due to the activation of the Wnt5a/non-canonical pathway, which induces BMP2 and consequent SOST expression, leading to reduced bone formation [55,100]. Both Rankl and Opg were overexpressed in the cortical bone, with the balance favoring Rankl [55]. Osteocalcin and alkaline phosphatase were strongly expressed in Sfrp4-KO trabecular bone, in which mature osteoclast markers and activity were also decreased whereas osteoblast markers were increased [332]. In aging Sfrp4-KO mice, trabecular and cortical bone density was maintained (compared to younger mice and aged control groups respectively) showing that bone loss under aging and oxidative stress is related to the reactivation of *sFRP4* gene expression [332]. In mice overexpressing Sfrp4 in osteoblasts, a decrease in trabecular bone thickness and volume was detected [333] (Table 4). In contrast to Sfrp4-deficient mice, bone resorption was not affected [333]. In this context, recent results reported by Chen *et al.* reinforce the importance of the non-canonical Wnt pathways in osteoclasts. Comparing Sfrp4-KO and the double KO mouse model Sfr4-KO+Ror2-cKO in osteoclasts these authors determined that although Sfrp4 inhibits both the canonical and non-canonical pathways, the increase in osteoclasts observed is due to activation of the non-canonical pathway Ror2/Wnt/Jnk, overriding the activation of the canonical pathway that would be inhibiting bone resorption [100].

4.4. WIF-1

Wnt inhibitory factor-1 (WIF-1) is an evolutionarily conserved secreted Wnt inhibitor. Even though the inhibitory mechanism is similar to that of sFRPs, WIF-1 does not share sequence similarities with the CRD of the frizzled receptors. Instead, it is composed of five epidermal growth factor-like repeats and one WIF domain that mediates Wnt binding [461,462]. WIF-1 is a downstream target of the Wnt canonical pathway, suggesting that it may act as a negative feedback regulator. For instance, WIF-1 expression is increased during BMP-2 treatment of MSCs as well as during osteoblast differentiation [335,455,462]. Both WIF-1 and sFRP2 are induced in the late phase of osteoblast differentiation [455]. WIF-1 inhibits osteoblastic differentiation, ALP activity and mineralization and enhances adipogenesis. However, osteogenesis inhibition is less efficient than that caused by DKK1 [462].

The most intriguing possible pathogenic alterations of this gene are related to its methylation pattern. A proposed treatment against osteoporosis, Gossypol, was observed to promote bone proliferation through the methylation and inhibition of the WIF-1 promoter [463]. On the other hand, WIF-1 methylation has been associated with bone tumors. WIF-1 is epigenetically silenced in human osteosarcoma cell lines and most primary cultures [335]. However, it has been questioned whether the inhibitory methylation pattern of WIF-1 observed in osteosarcoma tissue might be the cause or a consequence of the tumorigenicity of the cells [335,464]. *In vitro* demethylation restores WIF-1 expression and reduces β -catenin levels and TCF/LEF-dependent transcriptional activity, reducing proliferation in favor of differentiation [335]. On the contrary, tumors that did express WIF-1 showed mature tumor phenotypes, suggesting that WIF-1 demethylation might be a result of the immature/proliferative state of the tumor cells [464]. In addition, mouse Wif-1 is hypomethylated in both normal osteoblasts and osteosarcoma cells, enforcing the hypothesis that there is a different method of regulation behind the control of its expression, such as histone hypoacetylation [464].

Although Wif-1 is highly expressed in the developing and adult skeleton *in vivo*, it does not seem indispensable for its normal development, as Wif1-KO mice have no skeletal abnormalities (Table 4). This might be caused by functional redundancy *in vivo* [335]. Curiously, Wif1-KO mice are more susceptible to spontaneous and radiation-induced osteosarcomas, but not other cancer types [335]. Osteoblast-specific Wif1 overexpression mice have, like Wif1-KO, no bone phenotype, but the overexpression of Wif-1 disrupts stem cell quiescence and self-renewal potency in the osteoblasts of

these mice^[336] (Table 4). Interestingly, this study found that overexpression of Wif-1 induced autocrine induction of Wnt ligands in a compensatory way [336] (Table 4).

BOX 1. SKELETAL CELL INVOLVED IN BONE REMODELLING

Bone remodeling occurs in special units called bone multicellular units (BMU) which are made up of different cell types which are highly spatially and temporally organized. Among these cell types we highlight cells from two different lineages: the bone forming cells and the bone resorbing cells.

– BONE-FORMING CELLS

The osteoblasts are the cells responsible for bone formation and mineralization. They originate from MSC, but may also derive from bone lining cells and potentially from chondrocytes [465,466]. MSCs can also differentiate into chondrocytes, adipocytes, myocytes, and fibroblasts [467,468]. The cell fate depends on the presence of specific stimuli. In the case of the differentiation of MSC to osteoblasts, a process termed osteoblastogenesis (Figure 5), the transcription factors RUNX2 and OSX, play an essential role [469,470]. MSCs will give rise to osteoprogenitor cells that differentiate into osteoblast precursors and finally into mature osteoblasts (Figure 5). In the differentiation process, osteoblasts express a series of specific proteins or osteoblastic markers: Type I collagen, OCN, osteopontin and ALP [471]. They have a cuboidal morphology, with a large and clear nucleus, abundant cytoplasm, enlarged endoplasmic reticulum and Golgi apparatus, and a large number of free ribosomes due to the abundance of proteins they synthesize [472]. Osteoblasts are found in bone surface sites where bone formation is active, where they secrete type I collagen and non-mineralized ECM for subsequent mineralization.

Once differentiated, osteoblasts can undergo apoptosis (50-70%) or mature, becoming osteocytes or lining cells. When the osteoblast is surrounded by the extracellular matrix itself, it becomes an osteocyte. Osteocytes are terminally differentiated bone cells enclosed in the mineralized bone matrix, and they are responsible for mechanotransduction, the control of bone remodeling and calcium homeostasis. They are the most abundant cell type in bone tissue (90% of all adult bone cells) [473], and present a flattened dendritic morphology, with small rough endoplasmic reticulum and Golgi apparatus. They have filopodia, generating an extensive canalicular network that allows communication and the exchange of molecules with neighboring cells [472,474,475]. Unlike osteoblasts, osteocytes do not express much type I collagen or ALP, but instead begin to express other proteins such as sclerostin or DMP1. Osteocytes do not divide and have an average life of 25 years. In addition, inactive osteoblasts can mature into the lining cells that cover the bone surface when there is no active growth. The lining cells have a flattened and elongated structure and regulate the flow of mineral ions from bone extracellular fluid, thus serving as a blood–bone barrier. They retain the ability to redifferentiate to osteoblasts through stimulation by PTH or different mechanical forces [476].

The canonical Wnt pathway stimulates the process of differentiating MSCs into the osteoblastic lineage, suppresses apoptosis in osteoblast precursors before determining the fate of cell differentiation and stimulates preosteoblast replication and matrix mineralization. In addition, the Wnt pathway suppresses adipogenesis and stimulates or inhibits chondrogenesis during bone development or homeostasis [227,276,477–480].

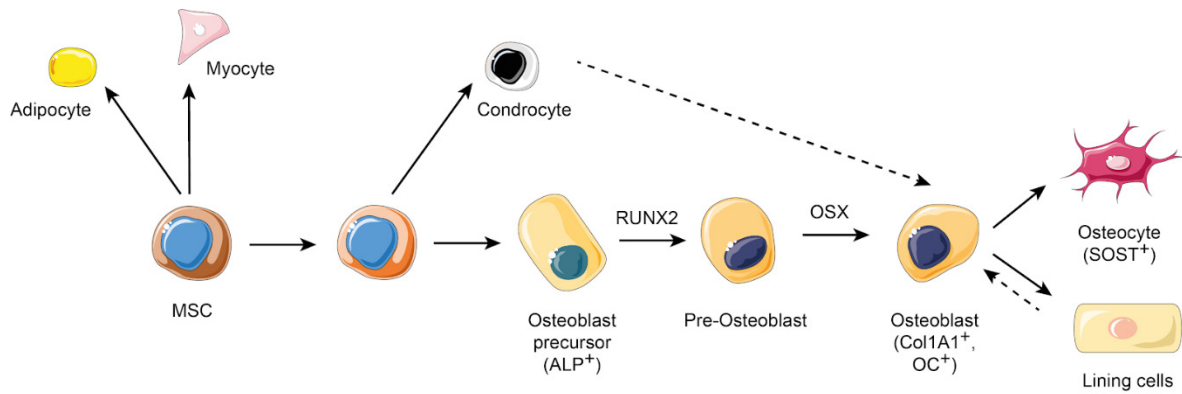


Figure 5. Osteoblastogenesis. MSC cells give rise to mature osteoblasts that differentiate into osteocytes or lining cells. Moreover, osteoblasts can also derive from chondrocytes or lining cells (dashed lines). Some of the biochemical markers for each cell type are highlighted in parentheses. RUNX2 and OSX are two key transcription factors important for the differentiation process. Image created with Servier Medical Art.

– CELLS RESPONSIBLE FOR RESORPTION

The cells responsible for bone resorption are the osteoclasts, large phagocytic multinucleated cells. These cells are the product of the fusion of mononucleated osteoclast precursors derived from the myeloid lineage of hematopoietic stem cells. In the differentiation process, termed osteoclastogenesis (Figure 6), specific molecular signals are necessary for the mononuclear cell differentiation and fusion process. Among these we highlight RANKL, critical for the formation of osteoclasts, and M-CSF, necessary for the proliferation, survival and differentiation of osteoclast precursors and for the cytoskeletal changes necessary for bone resorption. These two molecular signals are expressed by the osteoblasts and osteocytes, while the osteoclast progenitors express the respective receptors M-CSF and RANK. In addition to RANKL and M-CSF, osteoblasts express OPG in response to activation of the Wnt pathway. OPG is a decoy receptor of RANKL, which inhibits the RANKL–RANK interaction to suppress bone resorption [481]. Mature osteoclasts promote structural changes in the cytoskeleton and cell adhesion and are found on the bone surface where the bone is injured, being removed or remodeled. The part of the osteoclast membrane that is in contact with the bone surface presents invaginations that constitute the ruffled border, with many mitochondria and lysosomes that release protons and lysosomal hydrolases during resorption in order to degrade the ECM [482].

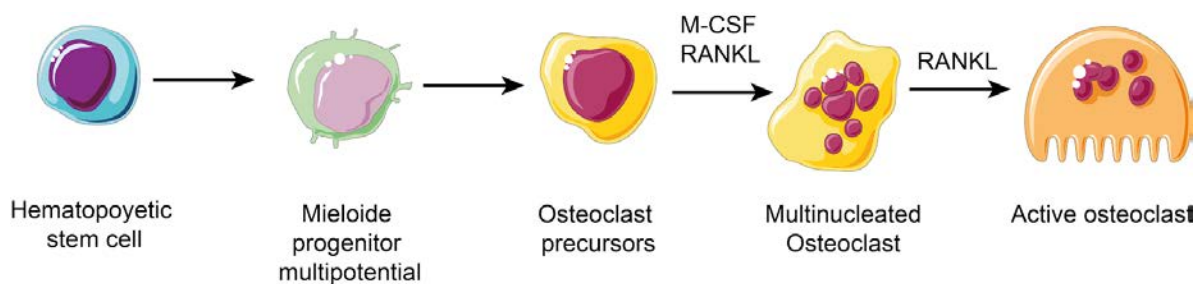


Figure 6. Osteoclastogenesis. Hematopoietic stem cells will give rise to myeloid progenitors that in the presence of M-CSF and RANKL differentiated into active osteoclast. Image created with Servier Medical Art.

BMU: Bone multicellular units; **MSC:** Mesenchymal stem cells; **TF:** Transcription factor; **RUNX2:** Runt related transcription factor 2; **OSX:** Osterix; **OCN:** Osteocalcin; **ALP:** Alkaline phosphatase; **M-CSF:** colony stimulating factor; **ECM:** extracellular matrix; **DMP1:** Dentin matrix acidic phosphoprotein 1; **PTH:** Parathyroid hormone; **M-CSF:** Macrophage colony-stimulating factor 1; **RANKL:** Receptor Activator for Nuclear Factor κ B Ligand; **RANK:** Receptor activator of nuclear factor-kappa B; **OPG:** Osteoprotegerin

BOX 2: BONE REMODELING

The homeostasis of adult bone mass under healthy conditions is maintained through the exquisite balance of bone resorption by osteoclasts and bone formation by osteoblasts. Bone remodeling is the process of renewing bone tissue with the aim of maintaining mineral homeostasis and bone resistance, avoiding the accumulation of microdamage in the bone. Bone remodelling takes place throughout life, but it only has a positive balance until the age of 30, when under normal conditions, between 5 and 10% of the bone is renewed each year [472]. Bone remodeling is finely regulated by different molecular signals and requires extraordinary coordination between the cell types involved. The process consists of five overlapping phases:

1. **Activation:** First, a detection of an initiating remodelling signal (direct mechanical strain or hormones) results in the recruitment and activation of circulating osteoclast precursors. Next, the lining cells on the bone surface are removed to allow osteoclast precursor to bind to the bone matrix. Multiple mononuclear cells fuse to form multinucleated pre-osteoclasts which bind to the bone matrix forming the resorption pit.
2. **Resorption:** A sealing zone is formed between the osteoclast and the bone surface. The plasma membrane of the osteoclast is enlarged, generating a ruffled border with proton pumps (H⁺-ATPase) and chloride channels (CLC-7) that generate an acidic extracellular environment (pH 4.5) to dissolve the bone mineral. Next, cytoplasmic lysosomes release phosphatases (p.e. TRAP), proteases (p.e. CTSK) and matrix metalloproteinases (p.e. MMP9) that allow digestion of the organic matrix; the resorption phase is completed by mononucleated cells after multinucleated cells undergo apoptosis [483–486].
3. **Reversal:** Bone resorption changes to bone formation; the osteoblast lineage cells are recruited and remove bone matrix leftovers, subsequently deposit a first layer of proteins in the resorption pits and finally, form a cement line to enhance osteoblastic adherence [487,488].
4. **Formation:** The reabsorption of the bone mineral matrix results in the release of growth factors and transcription factors (FGF, TGF β , BMPs, interleukins) responsible for the recruitment and differentiation of osteoblasts. The osteoblasts synthesize a new organic matrix called osteoid (especially type I collagen and proteoglycans) and gradually mineralize it by deposition of calcium phosphate crystals [489].
5. **Termination:** 50–70% of osteoblasts enter apoptosis, or become osteocytes or lining cells. The end result of the bone remodeling process is the production of a new osteon.

Bone remodeling takes 120 to 200 days in cortical and trabecular bone, respectively, and three-quarters of this time are intended for the bone formation process.

Because the Wnt pathway has a series of direct and indirect inputs into the control of osteoblastogenesis and osteoclastogenesis, maintaining the proper levels of Wnt activity is critical for bone homeostasis.

TRAP: tartrate-resistant alkaline phosphatase; **CTSK:** cathepsin K; **MMP9:** Matrix metalloproteinase 9; **CLC-7:** Chloride channel 7 alpha subunit; **FGF:** fibroblast growth factors; **TGF β :** Transforming growth factor beta; **BMP:** Bone morphogenetic proteins.

BOX 3. SKELETAL DEVELOPMENT

Bones are formed during embryonic development by two different processes: **intramembranous ossification** and **endochondral ossification**. In both cases, mesenchymal cells first aggregate to form mesenchymal condensations that prefigure the shape of future skeletal elements. In intramembranous ossification, this condensation will lead to the direct differentiation of the MSCs into osteoblasts, which secrete an osteoid that slowly becomes mineralized. The flat bones of the skull are formed through this process. In contrast, in endochondral ossification, condensation will lead to the differentiation of MSCs into chondrocytes that will produce a cartilage template of the future bone, and will then be replaced by bone tissue. Later, the chondrocytes become hypertrophic and undergo apoptosis, allowing

infiltration of blood vessels and osteoblast precursors that differentiate and ossify the structure. A thin layer of cartilage remains between the diaphysis (central portion) and the epiphysis (rounded end) of the bone, forming the epiphyseal plate so that the bone can continue to grow until adulthood, when the epiphyseal plate is completely ossified. The long bones, vertebrae, pelvis, and skull base bones are formed by endochondral ossification. Both processes are regulated at the molecular level by a large number of growth factors and transcription factors, among them various components of the Wnt pathway. For a detailed review of skeletal development and the role of the Wnt pathway in this process, see [490].

MSCs: Mesenchymal stem cells

BOX 4. MECHANOTRANSDUCTION

Bone tissue has the capacity to adapt to mechanical forces such as compression, tension, shear forces and gravity. Mechanotransduction in bone comprises the intracellular signaling pathways that are activated in response to external mechanical forces and will ultimately result in bone formation or resorption (BOX 2). Osteocytes are the main responders to those forces [491].

Within bone, mechanical loading causes a fluid movement from the high-pressure to low-pressure regions, where the osteocytes undergo transient plasma membrane disruption (PMD) that allows for calcium influx into the cell, which in turn initiates mechanotransduction [491–493]. Gap junction proteins like the connexin family and ion channels such as the TRPV4, VGCC and PKD1/2 act as mechanosensors [493]. Both calcium and ATP are important messengers of mechanotransduction [492]. The opening of these channels is followed by the activation of MAPK, ERK and Wnt signaling [12,494].

Several components of the Wnt pathway such as LRP5 and sclerostin are essential for the mechanical response [240,365,495]. Mechanical loading decreases the expression of the Wnt pathway inhibitors sclerostin and DKK1, as well as the RANKL/OPG ratio. As a result, osteoblast differentiation and bone formation are promoted while osteoclastogenesis and bone resorption are reduced. Unloading results in the opposite effect [365,491,493]. Interestingly, both the volume and quality of the bone are increased by mechanical loading (exercise), as the formation of new bone is stimulated on highly strained surfaces while surfaces that experience lower strain remain quiescent. Furthermore, unloading or immobilization has been documented to cause rapid bone loss [365,491,493].

TRPV4: TRP cation channel subfamily V member 4 ; **VGCC:** L-type voltage-gated calcium channel ; **PKD1/2:** polycystin-1/2; **MAPK:** mitogen-activated protein kinase; **ERK:** extracellular signal-regulated kinases.

ABBREVIATIONS

4C: circularized chromatin conformation capture; **ALP:** Alkaline phosphatase; **ARRS:** Al-Awadi-Raas-Rothschild syndrome; **BMD:** Bone mineral density; **BMP:** Bone morphogenic protein; **BMSCS:** Bone marrow derived mesenchymal stem cells; **BMU:** Bone multicellular units; **cAMP:** cyclic adenosine monophosphate; **cKI:** conditional knock-in; **cKO:** conditional knock-out; **CLC-7:** Chloride channel 7 alpha subunit; **CLS:** Cedani-Lenz Syndrome; **CRD:** Cysteine rich domain; **CTSK:** cathepsin K; **DMP1:** Dentin matrix acidic phosphoprotein 1; **ECD:** Extracellular domain; **ECM:** extracellular matrix; **ECR5:** Evolutionary conserved region 5; **EGF:** Epithelial growth factor; **ERES:** Estrogen response elements; **ERK:** extracellular signal-regulated kinases; **FGF:** fibroblast growth factors; **FZD:** Frizzled family receptors; **GoF:** Gain-of-function; **GPCR:** G protein coupled receptor; **HBM:** High bone mass; **HGMD:** Human Gene Mutation Database ; **ICD:** Intracellular domain; **Ig:** Immunoglobulin-like; **KD:** Kringle domain; **KO:** Knock-out; **KRM:** Kremen; **LBD:** Lytic bone disease; **LDLR:** Low-density lipoprotein receptor; **LGR:** Leucine-rich repeat-containing G protein-coupled receptors; **LNCAROD:** DKK1-activating long non-coding RNA; **LoF:** Loss-of-function; **LRP:** Low-density lipoprotein receptor-related

protein; **LRR**: Leucine-rich domain; **MAPK**: mitogen-activated protein kinase; **M-CSF**: colony stimulating factor; **M-CSF**: Macrophage colony-stimulating factor 1; **MEF2C**: Myocyte-enhancer factor 2c; **MMP9**: Matrix metalloproteinase 9; **MSC**: Mesenchymal stem cells; **NTD**: N-terminal α -helical domain; **NTR**: Netrin-related motif **OA**: Osteoarthritis; **OC**: Osteocalcin; **OI**: Osteogenesis Imperfecta; **ONFH**: Osteonecrosis of femoral head; **OPG**: Osteoprotegerin; **OPG**: Osteoprotegerin; **OPPG**: Osteoporosis pseudoglioma syndrome; **OSX**: Osterix; **PKA**: protein kinase A; **PKD1/2**: polycystin-1/2; **PLC**: Phospholipase C; **PRD**: Proline-rich domain; **PTH**: Parathyroid hormone; **RA**: Rheumatoid arthritis; **RANK**: Receptor activator of nuclear factor κ B; **RANK**: Receptor activator of nuclear factor- κ B; **RANKL**: RANK ligand; **RANKL**: Receptor Activator for Nuclear Factor κ B Ligand; **rs**: Ringelshwanz; **RSPO**: R-spondins; **RTK**: Receptor tyrosine kinases; **RUNX2**: Runt related transcription factor 2; **S1P**: Sphingosine-1-phosphate; **SFRP**: Secreted frizzled-related proteins; **TF**: Transcription factor; **TGF β** : Transforming growth factor beta; **TK**: Tyrosine kinase; **TRAF6**: TNF receptor-associated factor 6; **TRAP**: tartrate-resistant alkaline phosphatase; **TRPV4**: TRP cation channel subfamily V member 4; **VGCC**: L-type voltage-gated calcium channel; **WIF-1**: Wnt Inhibitory factor 1; **WNT-Ca $^{2+}$** : WNT-calcium; **WNT-PCP**: WNT-planar cell polarity; **WSC**: carbohydrate binding.

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SHORT COMMUNICATION

Discrepancy between bone density and bone material strength index in three siblings with Camurati-Engelmann disease

S. Herrera¹ · R. Soriano¹ · X. Nogués¹ · R. Güerri-Fernandez¹ · D. Grinberg² · N. García-Giralt¹ · N. Martínez-Gil² · S. Castejón¹ · A. González-Lizarán¹ · S. Balcells² · A. Díez-Pérez^{1,3}

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Abstract

Summary Camurati-Engelmann (CE) is a very rare disease affecting one in every million persons worldwide. It is characterized by an enlargement of long bones. We aimed to assess bone characteristics in three siblings with different tools. Even if there was an excess of bone density, quality seemed to be deteriorated.

Introduction CE disease is a rare monogenic disorder affecting approximately one in every million persons worldwide. It is mainly characterized by a progressive hyperostosis of the periosteum and endosteum of the diaphysis of long bones. Limited data are available about bone characteristics in these patients. In three siblings with CE disease, we aimed to assess bone mineral density (BMD) and trabecular bone score (TBS) by dual-energy X-ray absorptiometry (DXA) and material characteristics at tissue level using bone impact reference point indentation.

Methods Clinical data were collected and a general laboratory workup was performed. At the lumbar spine and hip, BMD and TBS were measured using DXA imaging. Bone material strength index (BMSi) was measured by bone impact microindentation using an Osteoprobe instrument.

Results All three cases had densitometric values consistent with high bone mass (sum of Z-score at the lumbar spine and hip > 4). Hip BMD was extremely high in all three siblings at both total hip and femoral neck, while at the lumbar spine, two of them had normal values but the third again had very high BMD. TBS values were in the normal range. In contrast, BMSi measurements were at low or very low levels, compared with normal controls.

Conclusion Despite strikingly increased BMD and normal microarchitecture, BMSi is affected in patients with CE. Microindentation could be an appropriate tool for assessing bone fragility in these patients. Bone disease in this group of patients requires further study to better understand the underlying regulatory mechanisms and their alterations.

Keywords Camurati-Engelmann · Microindentation · Progressive diaphyseal dysplasia · Rare bone disease

Introduction

Camurati-Engelmann (CE) disease, also known as progressive diaphyseal dysplasia, is a rare genetic disorder affecting approximately one in every million persons [1], although the real prevalence is unknown. It was first described in 1920 by Camurati-Engelmann [2, 3] and later found to have an autosomal dominant inheritance. It is characterized by a progressive hyperostosis of the periosteum and endosteum of the diaphysis of long bones, although in some cases, the cranium and the hip may also be affected [4]. Patients with this condition suffer from bone pain, muscular weakness, a waddling gait, and easy fatigability [5], and may have hematologic systemic manifestations [6]. The diagnostic approach includes clinical and family history, physical findings, and imaging results. Testing for *TGF-β1* mutation has recently been added

✉ A. Díez-Pérez
ADiez@parcdesalutmar.cat

¹ Hospital del Mar Institute of Medical Research, Autonomous University of Barcelona and CIBERFES, Instituto Carlos III, Barcelona, Spain

² Department of Genetics, Microbiology and Statistics, University of Barcelona, IBUB, IDSJD, and CIBERER, Instituto Carlos III, Barcelona, Spain

³ Department of Internal Medicine, Hospital del Mar, P. Maritim 25-29, 08003 Barcelona, Spain

to the available diagnostic tests [7]. Even if there are no specific guidelines for the treatment of this condition, patients are generally prescribed nonsteroidal anti-inflammatory drugs (NSAIDs), losartan, or steroids, and in some cases have been treated with surgery to alleviate complications [8]. Limited data about bone characteristics in these patients and, to our knowledge, no specific information on bone microarchitecture or the mechanical characteristics of tissue are available.

Trabecular bone score (TBS) was developed to estimate the trabecular architecture in the vertebral images obtained by dual X-ray absorptiometry (DXA). More recently, reference point indentation (RPI) emerged as a tool enabling *in vivo* measurements of bone material properties by directly measuring the resistance of cortical bone at the tibia to the penetration of a microscopic probe [9]. Given that bone is the main affected organ in these patients, we aimed to assess bone mineral density (BMD) and TBS by DXA imaging and material characteristics at tissue level using impact reference point indentation in three siblings with CE disease.

Material and methods

We present the case of three siblings previously diagnosed with CE disease who consulted our clinic for specific bone evaluation. All three, a 43-year-old man (case 1) and two women, aged 44 (case 2) and 47 (case 3), respectively, had been diagnosed during childhood. Informed consent was obtained from all individual participants included in the study. Their clinical pictures, bone turnover markers, and radiological and scintigraphic characteristics have been previously reported [10], with elevated bone formation and resorption markers in good correlation with radioisotope uptake. Briefly, case 1 was diagnosed at the age of 8 due to an impaired walk and diaphysis enlargement (Fig. 1). The diagnosis was made by bone biopsy. As a result of his diagnosis, his father and both of his sisters were also diagnosed with the condition. His mother and both of his brothers are healthy. He has developed bilateral hypoacusia due to skull growth, diffuse bone pain, and generalized bone radioisotope uptake. He has undergone two surgeries, an arthrodesis due to lumbar canal stenosis and hip prosthesis due to peri-articular calcifications. He has taken NSAIDs occasionally. Case 2 was also diagnosed early in life and experienced generalized bone pain during childhood. As an adult, she has developed hypoacusia and peripheral facial paralysis. She has been treated with low doses of prednisone (5 mg/day) for the past 10 years and is currently being treated with NSAIDs due to pain secondary to lumbar canal stenosis. Case 3 has also experienced generalized bone pain, for which she takes NSAIDs occasionally, as well as hypoacusia. Both women are pre-menopausal. None of them have ever had a bone fracture.



Fig. 1 Case 1 lower limb radiography

Our cases were compared to healthy controls. These healthy volunteers (no history of fractures, use of potentially bone-acting drugs, or any kind of bone disease) were recruited as a control group from our reference database for microindentation. Controls underwent impact microindentation and DXA as explained below, and demographic data (age, sex, weight, height) were collected.

BMD was measured at the lumbar spine and hip using DXA (Hologic QDR 4500 SR, Hologic, Inc., Bedford, MA, USA). The coefficient of variation of the procedure was 9.8%. TBS was calculated using the iNsight® software (Med.Imaps, Mergnac, France). Values of TBS are considered normal microarchitecture above 1.350, partially degraded between 1.200 and 1.350, and degraded below 1.200. TBS was evaluated in the same measurement regions as used for lumbar spine BMD [11].

Impact microindentation measurements were made with an Osteoprobe® device (Active Life Scientific, Santa Barbara, CA, USA) as previously described [12]. In brief, skin was disinfected using topical chlorhexidine and the anterior face of the mid-shaft of the tibia was anesthetized with subcutaneous mepivacaine. The test probe was inserted perpendicularly through the skin and into the bone. Eight indentations were

performed, separated by 2 mm and each one starting with a 10-N preload force followed by 30 N of triggered force. The same operator then repeated five indentations using the same probe on a reference phantom of polymethyl methacrylate (BMSi-100® Active Life Scientific, Santa Barbara, CA, USA). The indentation units are expressed as bone material strength index (BMSi), calculated as 100 times the ratio of the indentation distance into the calibration material to the bone indentation distance.

Two bone turnover biochemical markers, procollagen type I N propeptide (PINP) and C-terminal telopeptide (CTX-I), were measured by electrochemiluminescence immunoassay (Elecsys 2010®, Roche Diagnostics GmbH, Mannheim, Germany).

Genomic DNA mutation screening of exon 4 of the *TGFBI* gene, where most of the CE mutations are located, was performed for each patient. Primers were designed to amplify this exon and its flanking intronic regions. Polymerase chain reaction (PCR) amplification followed by automatic Sanger sequencing was performed by standard methods.

Statistical analysis

Continuous variables were summarized as mean and standard deviation. Student's *T* test was used for parametric variables and Mann-Whitney *U* test for non-parametric variables. A *p* value of less than 0.05 was considered to indicate significance. All reported *p* values are two-sided. The analysis was performed using Graph Pad Prism (version 7.0) 2017 GraphPad Software®, Inc., USA.

The Ethics Review Board at our institution approved the study protocol.

Results

All patients were found to be heterozygote for mutation: c.652C>T (p.Arg218Cys) in the *TGFBI* gene, the most prevalent mutation in CE patients [13]. All three cases had densitometric values consistent with high bone mass (sum of Z-score at the lumbar spine and hip > 4) [14, 15]. Case 1 had Z-scores of 0.3 (lumbar), 6.2 (femoral neck), and 6.9 (total hip); case 2 had Z-scores of 5.5 (lumbar), 11.0 (femoral neck), and 8.8 (total hip); case 3 had Z-scores of 1.4 (lumbar), 7.3 (femoral neck), and 5.8 (total hip). All these results are summarized in Table 1. Hip BMD was extremely high in all three siblings at both total hip and femoral neck, while at the lumbar spine, two of them had normal values but the third again had very high BMD. TBS values were in the normal range. Turnover markers appeared to be altered, showing both increased bone formation and resorption markers. In contrast, BMSi measurements were at low or very low levels, with a

median value for the three siblings of 76.9 BMSi units compared to 81.8 BMSi units in a group of 29 normal volunteers (5 men and 24 women) recruited from our laboratory (Fig. 2). When comparing lumbar and total hip BMD, CE cases had significantly higher BMD than the controls, although our cases were significantly younger and had a higher proportion of women compared to controls (Table 2). The technique was performed without any complications during or after the procedure.

Discussion

In this series of patients with CE disease, we observed a significantly higher BMD compared to that of healthy volunteers with normal trabecular microarchitecture, along with decreased mechanical properties of bone tissue. To our knowledge, this is the first published description of bone quality measurement with microindentation in this series of CE patients.

Little is known about the bone disease in patients with CE disease. The high BMD values in these three cases are not surprising in the context of a bone dysplasia with hyperostosis and are consistent with previous reports by Whyte [16] and Van Hul [17]. Whyte reported two cases: a 32-year-old patient whose lumbar spine and hip BMD Z-scores were + 7.7 and + 4.4, respectively, and a 60-year-old patient with scores of + 2.6, + 4.4, and − 0.2 in her L1–L4 spine, total hip, and wrist, respectively. In contrast, Bondestam [18] described a child with osteoporosis revealed by DXA and Castro [19] reported on two patients with CE disease, one with osteopenia in the lumbar spine but a + 4 Z-score at femoral neck and another with lumbar osteopenia and normal Z-score at the femoral neck. All three of our cases had an extremely high femoral BMD, showing high levels of bone mass. The normal lumbar BMD observed in two of the cases is likely explained by the disease characteristics, mainly affecting long bones. However, the gross skeletal abnormalities of these patients were clearly distinctive, compared to the structurally normal bones reported in high bone mass syndrome. Although fractures have been reported in CE cases, the true incidence is not established given the rarity of the disease. Nonetheless, a strikingly high rate has not been observed. We might speculate that the high amounts of bone mass observed in our patients represent a compensatory mechanism in response to the remarkable dysplasia with compromised mechanical properties that was measured by impact microindentation. Moreover, the described impairment in fracture healing [20] in these cases perhaps could be linked to these altered bone tissue properties and/or some associated decreased response in the fracture repair mechanisms. BMSi was lower in CE patients compared to controls; due to the small sample, the difference was not statistically significant. Indeed, no firm hypothesis can be

Table 1 Characteristics of the patients

	Case 1: male 43 years old	Case 2: female 44 years old	Case 3: female 47 years old	Normal values
Weight (kg)	86.0	75.0	64.0	
Height (m)	1.95	1.70	1.70	
Lumbar BMD (g/cm ²)	1.088	1.604	1.135	
Lumbar Z-score	0.3	5.1	1.4	
Femoral neck BMD (g/cm ²)	1.698	2.030	1.597	
Femoral neck Z-score	6.2	10.6	7.3	
Total hip BMD (g/cm ²)	2.042	1.981	1.597	
Total hip Z-score	6.9	8.5	5.8	
TBS L1–L4	1431 ^a	1663	1554	> 1350
BMSi ± SD	65.9 ± 15.95	76.9 ± 5.99	79 ± 5.29	
Hemoglobin (g/L)	143	126	128	> 12 female, > 14 male
White blood cells (10 ⁹ /L)	4.75	8.07	4.72	4–11
Bone alkaline phosphate (ng/mL)	87.1	13.4	25.4	< 20
PINP in serum (ng/mL)	156.2	59.13	85.8	< 55
NTX in urine (nMol/mM)	196	NA	NA	< 75
b-CTX in serum (ng/mL)	1.25	NA	1.03	< 0.58
PTH (pg/mL)	32	13	49	14–72
Vitamin D 25 OH (ng/mL)	49	20	34	30–150

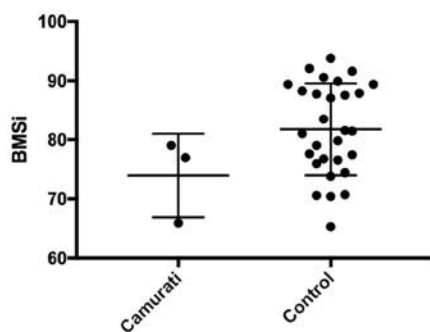
BMSi bone mineral strength index, PTH parathormone, NA not available, TBS trabecular bone strength, BMD bone mineral density, PINP procollagen type I N-terminal propeptide, NTX N-terminal cross-linking telopeptide of type I collagen, b-CTX carboxy-terminal collagen crosslinks

^a Values for L1–L3

derived from our results, and we can only speculate about this discrepancy between bone density and bone indentation properties. Our data also do not allow us to determine the contribution of the turnover increase observed in our cases [10] to this density/tissue discordancy. It is necessary to underline that the interpretation of RPI is still unclear with respect to bone mechanical properties, and thus, it is not possible to make firm conclusions about these results.

Bone disease in CE disease deserves further study. There is an urgent need for specific treatment, not available today,

that prevents the severe complications of the CE disease. Moreover, as in other monogenic diseases, understanding the mechanisms behind this disease may contribute to a better knowledge of the regulatory pathways in bone and could be relevant to other bone conditions. In particular, the striking increase observed in bone density, predominantly in long bones, might lead to developing better treatments to improve cortical bone in peripheral skeleton, precisely where non-vertebral fractures occur in osteoporosis and currently available treatments are less efficacious.

**Fig. 2** Differences in BMSi between CE patients and controls**Table 2** Comparison of Camurati-Engelmann cases and controls

	Camurati-Engelmann (n = 3)	Controls (n = 29)	p value
Age median	44	56	0.0435
Female %	67%	83%	0.0090
BMSi median	76.9	81.4	0.1665
Lumbar BMD median (g/cm ²)	1.135	0.972	0.0335
Total hip BMD median (g/cm ²)	1.981	0.764	0.0004

Conclusion

Bone material strength index is affected in patients with CE disease, regardless of their strikingly increased BMD and normal microarchitecture. Microindentation could be an appropriate tool for assessing bone fragility in these patients. Bone disease in this group of patients requires further study to better understand the underlying regulatory mechanisms and their alterations.

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Compliance with ethical standards Informed consent was obtained from all individual participants included in the study. The Ethics Review Board at our institution approved the study protocol.

Conflicts of interest A D-P owns shares of Active Life Sci, the manufacturer of Osteoprobe. Sabina Herrera, Raquel Soriano, Xavier Nogués, Robert Güerri-Fernandez, Daniel Grinberg, Natalia García-Giralt, Nuria Martínez-Gil, Santiago Castejón, Alba González-Lizarán, and Susana Balcels declare that they have no conflict of interest.

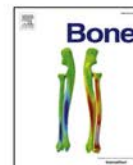
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Full Length Article

Functional characterization of the *C7ORF76* genomic region, a prominent GWAS signal for osteoporosis in 7q21.3

Neus Roca-Ayats^a, Núria Martínez-Gil^a, Mónica Cozar^a, Marina Gerousi^a, Natàlia Garcia-Giralt^b, Diana Ovejero^c, Leonardo Mellibovsky^b, Xavier Nogués^b, Adolfo Díez-Pérez^b, Daniel Grinberg^{a,1}, Susanna Balcells^{a,*,1}

^a Department of Genetics, Microbiology and Statistics, Facultat de Biologia, Universitat de Barcelona, Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), ISCIII, IIBIB, IRSID, Barcelona, Catalonia, Spain

^b Musculoskeletal Research Group, IMIM (Hospital del Mar Medical Research Institute), Centro de Investigación Biomédica en Red en Fragilidad y Envejecimiento Saludable (CIBERFES), ISCIII, Barcelona, Catalonia, Spain

^c National Research Council, Institute of Clinical Physiology, Lecce, Italy

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ABSTRACT

Genome-wide association studies (GWAS) have repeatedly identified genetic variants associated with bone mineral density (BMD) and osteoporotic fracture in non-coding regions of *C7ORF76*, a poorly studied gene of unknown function. The aim of the present study was to elucidate the causality and molecular mechanisms underlying the association. We re-sequenced the genomic region in two extreme BMD groups from the BARCOS cohort of postmenopausal women to search for functionally relevant variants. Eight selected variants were tested for association in the complete cohort and 2 of them (rs4342521 and rs10085588) were found significantly associated with lumbar spine BMD and nominally associated with osteoporotic fracture. *cis*-eQTL analyses of these 2 SNPs, together with SNP rs4727338 (GWAS lead SNP in Estrada et al., *Nat Genet.* 44:491–501, 2012), performed in human primary osteoblasts, disclosed a statistically significant influence on the expression of the proximal neighbouring gene *SLC25A13* and a tendency on the distal *SHFM1*. We then studied the functionality of a putative upstream regulatory element (UPE), containing rs10085588. Luciferase reporter assays showed transactivation capability with a strong allele-dependent effect. Finally, 4C-seq experiments in osteoblastic cell lines showed that the UPE interacted with different tissue-specific enhancers and a lncRNA (*LOC100506136*) in the region.

In summary, this study is the first one to analyse in depth the functionality of *C7ORF76* genomic region. We provide functional regulatory evidence for the rs10085588, which may be a causal SNP within the 7q21.3 GWAS signal for osteoporosis.

1. Introduction

Genome-wide association studies (GWAS) have been successfully used to identify genetic variants associated with complex traits and diseases, such as osteoporosis. In a few cases, the associated SNPs are located within a coding region of a gene, facilitating its functional evaluation. However, the vast majority of associated SNPs lie in non-coding regions, which make it challenging to understand the functional mechanisms underlying the association [1,2]. In addition, it is highly probable that the associated SNPs are in linkage disequilibrium (LD)

with the causal variant.

To date, many GWAS have been performed to find genetic association with bone mineral density (BMD) and osteoporotic fracture [3–12]. BMD is a genetically determined, extensively measured quantitative trait (heritability of 0.5–0.85) and, therefore, a good marker for bone status. Low-trauma fracture, the clinical outcome of osteoporosis, is also heritable, albeit to a lesser extent (heritability of 0.54–0.68) [13]. These GWAS have identified > 500 candidate *loci* [12], although the causal variants remain largely unknown. In addition, all the GWAS findings together only explain a small proportion (~20%) of the total

* Corresponding author at: Departament de Genètica, Microbiologia i Estadística, Facultat de Biologia, Universitat de Barcelona. Av. Diagonal, 643. 08028 Barcelona, Catalonia, Spain.

E-mail address: sbalcells@ub.edu (S. Balcells).

¹ Last co-authors.

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genetic impact on BMD [12]. Some associated *loci* contain genes not previously known to play a role in bone biology, which is the case of *C7ORF76* (AKA *FLJ42280*, currently annotated as transcript variant 6 of gene *SEM1* in GRCh38), in the 7q21.3 genomic region. Several SNPs within this region have been found significantly associated with both lumbar spine (LS) and femoral neck (FN) BMD as well as with osteoporotic fracture in different GWAS and meta-analyses [4,6–8,14–16]. In particular, in the largest meta-analysis carried out so far [7], the lead SNP of this *locus* (rs4727338) was one of the genome-wide top-associated signals. Yet, *C7ORF76* is a poorly studied gene of unknown function and there are other genes within this region that could also be responsible for the associations observed.

In this study, we aimed at elucidating the causality and molecular mechanisms underlying the strong association identified in the *C7ORF76* genomic region. We have deeply re-sequenced the genomic region in two extreme BMD groups of postmenopausal women of the BARCOS cohort and selected some variants to analyse their association in the full cohort. Through a combination of several *in silico* and experimental approaches, we studied a possibly causal SNP located in a regulatory region and demonstrated its functionality.

2. Material and methods

2.1. Study cohort

The BARCOS cohort consisted of 1490 postmenopausal women of Spanish descent from the Barcelona area, monitored at the Hospital del Mar (Barcelona, Spain). Exclusion criteria were any history of bone diseases, metabolic or endocrine disorders, hormone-replacement therapy, or use of drugs that could affect bone mass. BMD of all participants was measured at LS and FN by dual energy X-ray absorptiometry (DXA). The following data were also recorded: age, age of menarche and menopause, number of fractures and anthropometric measures such as weight and height. DNA is available from all samples of the cohort. Details of the cohort and DNA extraction have been described previously [17,18]. Written informed consent was obtained from all patients in accordance with the regulations of the Clinical Research Ethics Committee of Parc de Salut Mar, which approved the study. All experiments and protocols were approved by the Bioethics Committee of Universitat de Barcelona (IRB00003099).

For the re-sequencing of the *C7ORF76* *locus*, two extreme LS-BMD groups were selected from the BARCOS cohort, using the statistical Z-score. The 50 women with the highest Z-score values (from 2.98 to 0.73) were included in the 50-H group and the 50 women with the lowest Z-score values (from −2.41 to −4.26) were included in the 50-L group.

2.2. Re-sequencing

Re-sequencing of the *C7ORF76* (ENSG00000197851; ENST00000356686.1) genomic region was performed in the 100 individuals of the two extreme subgroups of the BARCOS cohort, according to the Z-score. A 28 kb region (chr7:96,108,695–96,136,619; GRCh37), including the *C7ORF76* gene and the 3.8 kb upstream and 2 kb downstream regions of the gene, was amplified in 7 overlapping fragments by Long Range-PCR (Supplementary Table 1). All amplicons were purified and quantified using the Quant-iT PicoGreen dsDNA Reagent and Kit (Life Technologies, Thermo Fisher) before pooling them equimolarly into two groups, one of HBM and another of LBM. Both pools were tagged with a MID adaptor and an emulsion-PCR was carried out prior to massive parallel sequencing at 3600× coverage per pool with Roche's 454 GS Junior System. The massive parallel sequencing was carried out in the Genomics facilities of the Universitat de Barcelona. The raw data obtained were processed to trim the MIDs, using a custom pipeline, and were mapped against the reference genome (GRCh37), using the GS Mapper software (Roche). Mapped

reads were filtered, sorted and indexed using SAMtools [19]. Single Nucleotide Variants (SNVs) and indels were identified using GATK standard hard filtering parameters [20]. The variants were filtered according to the following criteria: coverage ≥ 1000 reads, variants present in $\geq 1\%$ of the reads per pool and low strand bias. The number of reads of a variant was normalised with its coverage and the variants were classified according to minor allele frequency (MAF): Common (MAF $\geq 5\%$), and lower frequency variants (MAF $< 5\%$). The variants were validated either by differential digestion with restriction enzymes or by high resolution melting, using the Light Cycler® 480 ResoLight Dye (Roche).

2.3. In silico functional analyses and motif analysis

In silico functional analyses consisted in annotating the European and Iberian MAF of the variants obtained from dbSNP and 1000 Genomes, when available; predicting the pathogenicity of exonic variants, using SIFT [21], PolyPhen [22] and Mutation Taster [23]; and, for the intronic variants, analysing the DNase I hypersensitivity, histone modifications, transcription factor binding, miRNAs binding, etc. of the regions of interest. All the *in silico* data was obtained from ENCODE [24], International Human Epigenome Consortium [25], The Roadmap Epigenomics Project, FANTOM5 [26], HaploReg [27], RegulomeDB [28], miRTarBase [29], miRdSNP [30], MirSNP [31], BioMart, and Ensembl and UCSC Genome Browser. All variants were analysed with the Variant Effect Predictor from Ensembl, the Variant Annotation Integrator from UCSC, and FuncPred from the National Institute of Environmental Health Sciences. Transcription factor binding sites prediction considering the different alleles of the variants was done using MatInspector [32] and the Bioconductor “motifbreakR” package (<https://bioconductor.org/packages/release/bioc/html/motifbreakR.html>) [33] using default method settings (weighted sum) and a p-value cut-off at 5×10^{-5} for SNPs. Super-enhancer data was obtained from a catalogue of super-enhancers in 86 human cell and tissue samples [34] and from SEDb [35].

2.4. SNV genotyping

Genotyping of 8 selected variants in the complete BARCOS cohort was carried out at LGC Genomics (Hoddesdon, UK). In addition, as BARCOS was included in the replication phase of the meta-analysis by Estrada et al. [7], the genotyping results of the SNP rs4727338 were also available. The genotyping of 6% of the samples was performed in duplicate, as a genotyping quality control, and showed a concordance above 99%.

2.5. Linkage disequilibrium analysis

The Haploview software [36] was used to calculate and represent the degree of linkage disequilibrium between the genotyped common variants using the default parameters.

2.6. Cell culture

The human osteosarcoma cell line Saos-2 was used for luciferase reporter assays and 4C-seq assays. It was obtained from the American Type Culture Collection (ATCC® HTB-85™) and grown in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich), supplemented with 10% Fetal Bovine Serum (FBS; Gibco, Life Technologies) and 1% penicillin/streptomycin (Gibco, Life Technologies), at 37 °C and 5% of CO₂. Human fetal osteoblasts (hFOB) 1.19 cells were used for 4C-seq assays. They were obtained from ATCC (ATCC® CRL-11372™) and grown in DMEM:F12 (1:1) medium without phenol red (Gibco, Life Technologies), supplemented with 10% FBS and 0.3 mg/ml Geneticin (Gibco, Life Technologies), at 34 °C and 5% of CO₂. Human medulla-derived mesenchymal stem cells (MSCs) were also used for 4C-seq

assays. They were kindly provided by Dr. José Manuel Quesada Gómez, from Instituto Maimónides de Investigación Biomédica, Hospital Universitario Reina Sofía, Córdoba, Spain. They were grown in alpha-MEM medium (Gibco, Life Technologies), supplemented with 10% FBS, 1% penicillin/streptomycin and 1 × Glutamax (Gibco, Life Technologies), at 37 °C and 5% of CO₂. Human primary osteoblasts (hOB) were used for eQTL assays. They were obtained from trabecular bone of women who underwent knee replacement due to osteoarthritis and who did not have any other pathology that could affect the bone status. Bony tissue was cut up into small pieces, washed in phosphate buffered saline (PBS; Gibco, Life technologies) to remove non-adherent cells, and placed on a 140 mm culture plate. Samples were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin/streptomycin, 0.4% fungizone (Gibco, Life Technologies) and 100 µg/ml ascorbic acid (Sigma-Aldrich). DNA and RNA extractions were performed at maximum passage 2. HeLa and HEK293 cell lines were obtained from ATCC (ATCC® CCL-2™ and ATCC® CRL-1573™, respectively) and grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO₂.

2.7. Human primary osteoblasts DNA extraction and sequencing

DNA was extracted from cultured hOBs using the Wizard® Genomic DNA Purification Kit (Promega), according to manufacturer's instructions. The concentration of the purified DNA was analysed in a spectrophotometer (Nanodrop). Genotypes for rs4727338, rs10429035, rs12674052, rs4342521, and rs10085588 were assessed by Sanger sequencing using the BigDye® Terminator v3.1 (Applied Biosystems) in the Genomics facilities of Universitat de Barcelona. Primers (Invitrogen, Thermo Fisher) were designed using the Primer3 Input 0.4.0 (Supplementary Table 2).

2.8. Human primary osteoblasts RNA extraction, retrotranscription and qPCR

RNA was extracted from cultured hOBs using the High Pure RNA Isolation kit (Roche), according to manufacturer's instructions. RNA was quantified using a Nanodrop spectrophotometer and retro-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher), according to the specifications of the manufacturer. qPCR was performed using UPL Probes (Roche) on a LightCycler 480 Instrument II (Roche). Expression of *HMBS* was used as a normalizing control, and fold changes (FC) were calculated by relative quantification, using the 2nd derivative method. Primers used to amplify the neighbouring genes of the *C7ORF76* locus are summarised in Supplementary Table 3.

2.9. Amplification of an UPstream regulatory Element (UPE)-derived transcript

UPE-derived transcript was amplified from HeLa, HEK293, human primary osteoblasts and Saos-2 cDNA (200 ng) by PCR, using the following primers: 5'-CACTTTTCAAATCCACCTG-3' and 5'-TGAGAGCTGCTTAGAAATGGAA-3'. PCR products were run in a 2% agarose gel.

2.10. Luciferase reporter constructs and site-directed mutagenesis

The 750 bp-fragment containing the UPE was PCR-amplified from human genomic DNA using the following primers: 5'-CACTTTTCAAATCCACCTG-3' and 5'-TGAGAGCTGCTTAGAAATGGAA-3', and cloned in both orientations using *XhoI* and *KpnI* restriction enzymes in the pGL3-Basic vector (Promega). The minor allele of the rs10085588 (A) was introduced with the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent), following the manufacturer instructions. All the plasmids were validated by Sanger sequencing.

2.11. In vitro luciferase assay

Saos-2 cells were seeded at a density of 3.0×10^5 cells per well in a 6-well plate. After 24 h, they were transfected with 2.2 µg of total DNA per well using FuGENE HD reagent (Promega), according to manufacturer's instructions. Two plasmids were cotransfected in each well: the pGL3-Basic empty or with the UPE fragment cloned upstream of the Firefly Luciferase coding region and the pRL-TK plasmid, containing the Renilla Luciferase gene, in a proportion of 1/10. Forty-eight hours after transfection, cells were rinsed with PBS and lysed. The luciferase activity was measured using a Glomax Multi+ luminometer (Promega), with the Dual-Luciferase® Reporter Assay System reagents (Promega). Each experiment was performed in two biological replicates and was repeated 5 times.

2.12. 4C-seq

4C-seq was carried out at the Functional Genomics Service of the Centro Andaluz de Biología del Desarrollo (Sevilla, Spain). 4C-seq libraries were generated from Saos-2, hFOB 1.19 and hMSCs lines as described previously [37,38]. 4-bp cutters were used as primary (*DpnII*) and secondary (*Csp6I*) restriction enzymes. For each cell line, a total of 1.6 µg of library was amplified by PCR (primers used: CTGGAAGAGTCCCAGGGATC and AATGGAAGAGTGGAGATTCAGG; chr7:96,137,244-96,137,535). Samples were sequenced with Illumina Hi-Seq technology according to standard protocols at the Genomics Service of the Centro Nacional de Investigaciones Cardiovasculares (Madrid, Spain). 4C-seq data were analysed as described previously [39]. Briefly, raw sequencing data were demultiplexed and mapped to the corresponding reference genome (GRCh37). Reads located in fragments flanked by two restriction sites of the same enzyme, in fragments smaller than 40 bp or within a window of 10 kb around the viewpoint were filtered out. 4C-seq data were normalised by the total weight of reads within ± 2 Mb around the viewpoint. The experiments were carried out in one biological replicate.

2.13. Topologically associating domain (TAD) analysis

TAD data on different cell types from Dixon et al. [40] was collected from the 3D Genome Browser (<http://promoter.bx.psu.edu/hi-c/>) [41] and displayed using the UCSC Genome Browser. The 3D Genome Browser was also used to visualise published Hi-C data.

2.14. Statistical methods

Statistical analyses were performed using the R software version 3.4.1. Hardy-Weinberg equilibrium (HWE) was calculated using Chi-square test and p-values < 0.01 were considered significant. Fisher's exact test with Bonferroni correction for multiple testing was used to statistically compare the differences of the genotype frequency of the common variants in each extreme group (HBM and LBM). Linear regression analysis adjusted by years since menopause (YSM) was performed to determine the association between BMD and the genotype of each SNV in the complete BARCOS cohort. Linear regression was also used to assess the association between gene expression levels and genotypes (*cis*-eQTL) in primary osteoblasts. All analyses were performed using the *SNPassoc* package testing the additive, recessive and dominant models. p-values < 0.05 were considered significant. Correction for multiple testing was performed using the Bonferroni's method for the number of SNPs tested.

Relative luciferase units (RLU, i.e. the ratio of the firefly luciferase activity over the Renilla luciferase activity) were calculated for each individual measurement and a one-way blocked ANOVA with TukeyHSD post-hoc test was performed. All the data was ascertained for normality, homoscedasticity and atypical data points and p-values < 0.05 were considered significant.

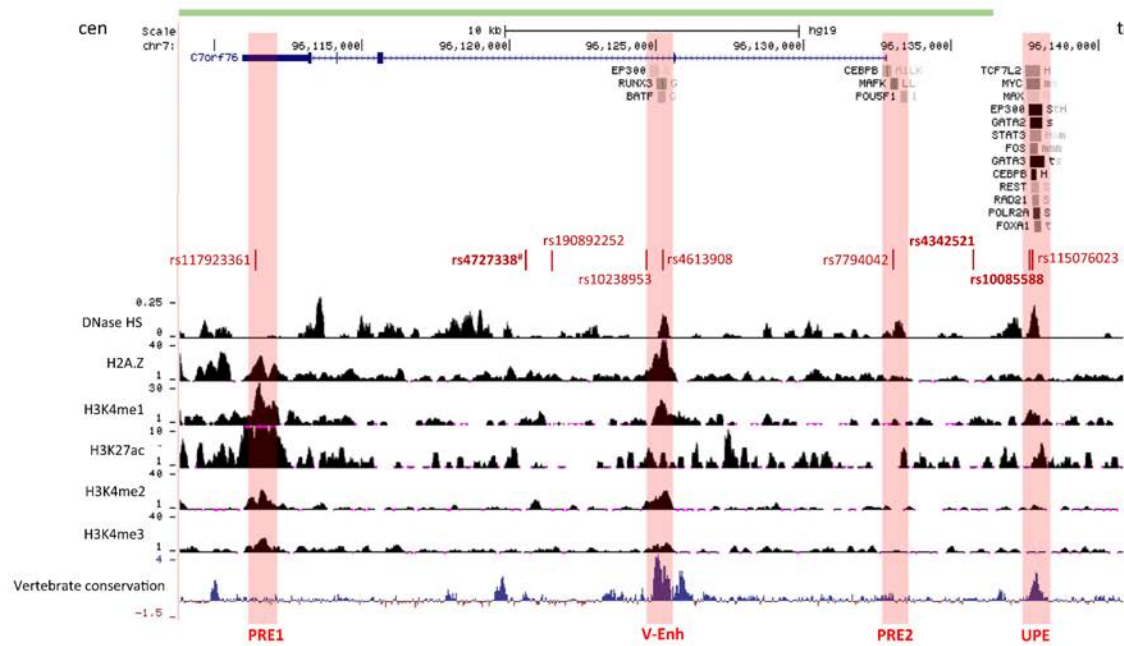


Fig. 1. Schema of *C7ORF76* locus. In green, region re-sequenced in the present study. In dark red, SNPs genotyped in the BARCOS cohort and in bold, SNPs found associated with BMD; # SNP previously genotyped (Estrada et al. [7]). Transcription factor ChIP-seq, DNase HS, H2A.Z, H3K4me1, H3K27ac, H3K4me2 and H3K4me3 ChIP-seq from osteoblasts (ENCODE), and vertebrate conservation are shown. The red boxes represent putative regulatory elements, according to epigenetic marks as well as transcription factors binding. Note that V-Enh corresponds to an enhancer (hs2311) described in VISTA Enhancers database [42]. PRE1 = putative regulatory element 1; PRE2 = putative regulatory element 2; V-Enh = VISTA enhancer hs2311; UPE = 4 kb upstream putative regulatory element.

3. Results

3.1. Re-sequencing of *C7ORF76* locus in extreme BMD groups of the BARCOS cohort and variant functional annotation

We re-sequenced 28 kb of the *C7ORF76* genomic region (chr7:96,108,695-96,136,619; GRCh37) in the 50 women with the highest and 50 women with the lowest LS-BMD of the BARCOS cohort (Fig. 1). Total number and location of single nucleotide variants (SNVs) detected before and after filtering and validating are shown in Table 1. Fifty-one common variants (MAF above 5%) and 59 lower frequency variants (MAF below 5%) were identified. The lower frequency variants were equally distributed between both extreme groups. To better assess the importance of the variants, we explored their functionality using publicly available *in silico* data (transcription factor binding, DNase I hypersensitivity, conservation, miRNAs binding, and histone marks). Twenty-eight variants were found in putative regulatory regions, 4 of which were located in osteoblast regulatory regions (rs9785005, rs10238953, rs4613908 and rs117923361) and 1 of them (rs4613908) was found in an active osteoblast enhancer. One missense variant was also identified but it was predicted to be tolerated by SIFT, to be benign by Polyphen and to be a polymorphism according to MutationTaster. None of the variants was predicted to affect miRNAs binding. Only 1

variant (rs4342521) showed nominal significance between the genotype frequencies of the two extreme groups (Fisher's exact test, $p = 0.0382$). This SNP is located 3 kb upstream of the *C7ORF76* gene and the minor allele (T) was overrepresented in the LBM group.

3.2. Association of variants with BMD in the complete cohort

We selected 8 interesting variants, taking into consideration both frequency and functionality (Fig. 1), including 2 located in a putative regulatory region 4 kb upstream of the *C7ORF76* transcription start site (TSS), which we named UPE, that was not included in the re-sequencing. These 8 variants were genotyped in the complete BARCOS cohort ($n = 1490$) to test their association with BMD and osteoporotic fracture. For all of them, we obtained MAF values similar to those found in the 1000 Genomes database for the European or Iberian populations (Supplementary Table 4). Significant differences were obtained with two of the 5' upstream SNPs, rs4342521 and rs10085588 (Fig. 1), for LS-BMD under additive and recessive models and nominal differences were obtained with the same SNPs for osteoporotic fracture, under additive and dominant models (Table 2). In all cases, the minor allele (T and A, respectively) had a BMD-lowering effect on LS and had a damaging effect on osteoporotic fracture. The two associated SNPs were found to be in linkage disequilibrium with the GWAS hit (rs4727338)

Table 1
Number and location of single nucleotide variants found in this study.

	Raw	Selected by filtering and validating	Coding	Regulatory regions ^a	Putative osteoblast regulatory elements	Active enhancer
Common variants	96	51	0	12	3	1
LFV	24,243	59	1	16	1	0
Total	24,339	110	1	28	4	1

^a Either present in flanking regions, 5'UTR, 3'UTR or introns; LFV: lower frequency variants (MAF < 0.05).

Table 2
Association between common and LFV variants of *C7ORF76* and LS-BMD, FN-BMD, FN-BMD or osteoporotic fracture.

SNP	Genomic position (GRCh37)	Type of variant	p-value LS-BMD			Effect size (β coeff; 95% CI)			p-value FN-BMD			Effect size (β coeff; 95% CI)			p-value osteoporotic fracture			Effect size (OR; 95% CI)		
			A	R	D	A	R	D	A	R	D	A	R	D	A	R	D	A	R	D
rs115076023 ^a	7:96137731 G > A	5'UP	0.00361	0.00343	0.05032	-0.0324 (-0.0541, -0.0108)	-	-	-	-	-	0.03554	0.32373	-	-	-	0.02242	1.51 (1.05, 2.17)	-	-
rs10085588	7:96137674 G > A	5'UP	0.00314	0.00251	0.05233	-0.0332 (-0.0547, -0.0117)	-	-	0.12419	0.11909	0.30848	0.03911	0.39100	0.01974	0.55917	1.00000	0.30987	1.53 (1.06, 2.19)	0.39100	0.01974
rs4342521	7:96136005 G > T	5'UP	0.00314	0.00251	0.05233	-0.0332 (-0.0547, -0.0117)	-	-	0.12453	0.09115	0.36111	0.55917	1.00000	0.30987	0.58472	0.27134	0.18380	0.03911	0.39100	0.01974
rs7794042	7:96132999 C > A	5'UP	0.68070	0.65857	0.63405	-	-	-	0.85483	0.66330	0.89686	0.55917	1.00000	0.30987	0.58472	0.27134	0.18380	0.55917	1.00000	0.30987
rs4613908	7:96125315 G > A	I	0.06164	0.08889	0.15109	-	-	-	0.55060	0.30356	0.86063	0.55917	1.00000	0.30987	0.58472	0.27134	0.18380	0.55917	1.00000	0.30987
rs10238953	7:96124975 T > C	I	0.57370	0.49214	0.69433	-	-	-	0.71522	0.57609	0.83130	0.55917	1.00000	0.30987	0.58472	0.27134	0.18380	0.55917	1.00000	0.30987
rs190892252	7:96121343 A > G	I	0.21722	-	-	-	-	-	0.24861	-	-	0.63625	-	-	0.63625	-	-	0.63625	-	-
rs4727338 ^b	7:96120675 G > C	I	0.00141	0.00207	0.02430	-0.0362 (-0.0592, -0.0132)	-	-	0.06943	0.04540	0.27708	-0.0175 (-0.0345, -0.0004)	-	-	0.07618	0.56459	0.03270	1.50 (1.03, 2.18)	0.07618	0.03270
rs117923361	7:96111486 C > A	3'UTR	0.82861	0.68376	0.87501	-	-	-	0.08217	0.62988	0.08427	0.30507	1.00000	0.22534	0.30507	1.00000	0.22534	0.30507	1.00000	0.22534

Values in bold indicate statistical significance; values in italics indicate nominal significance.

A: Additive model; R: Recessive model; D: Dominant model.

^a SNP found monomorphic in the BARCOS cohort.

^b SNP previously genotyped in the BARCOS cohort (Estrada et al. [7]).

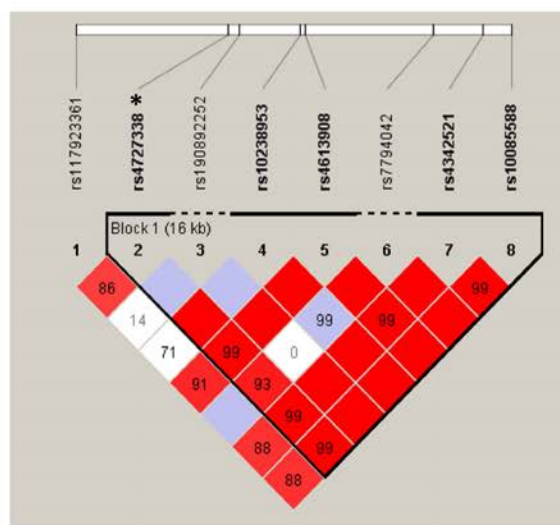


Fig. 2. Linkage disequilibrium plot of *C7ORF76* genotyped variants. *GWAS hit from Estrada et al. [7]. The numbers within the squares and the colour scale both refer to D'/LOD values (with bright red: D' = 1 and LOD ≥ 2; white: D' < 1 and LOD < 2; blue: D' = 1 and LOD < 2 and shades of pink/red: D' < 1 and LOD ≥ 2).

from Estrada et al. [7] (Fig. 2), which was also significantly associated with LS-BMD in the BARCOS cohort (Table 2).

3.3. cis-eQTL analyses

To evaluate the functionality of the associated variants (rs4727338 –GWAS hit, rs4342521 and rs10085588) we first tested them as cis-eQTLs in human primary osteoblasts, a cell type unavailable in GTEx. Of note, we failed to detect *C7ORF76* mRNA expression in these cells. None of the SNPs were found to be eQTLs for *DLX5*, *DLX6*, *DLX6-AS1*, or *SHFM1* (*SEM1* transcript variant 5 in GRCh38), although a tendency was observed for the latter (Table 3). On the contrary, the 3 SNPs were found nominally associated with *SLC25A13* gene expression, where the minor alleles were associated with decreased gene expression. We also tested another SNP of the region (rs10429035) found associated with BMD in a previous GWAS meta-analysis [8] and described in GTEx to be eQTL for *SHFM1* in tibial artery. However, we failed to detect an association of this SNP with gene expression of any of the nearby genes (data not shown).

3.4. Evaluation of the regulatory capability of the UPE

Next, as the associated SNP rs10085588 is located in a putative regulatory region 4 kb upstream of the *C7ORF76* gene (UPE; Fig. 1), we assessed the functionality of the UPE. We performed luciferase reporter

Table 3
cis-eQTL analysis of 3 *C7ORF76* variants associated with BMD.

SNP	p-values				
	<i>DLX5</i>	<i>DLX6</i>	<i>DLX6-AS1</i>	<i>SHFM1</i>	<i>SLC25A13</i>
rs10085588	0.14207	0.53849	0.59125	0.05270	0.03350
rs4342521 ^a	0.09227	0.34167	0.55062	0.05725	0.01442
rs4727338 ^b	0.09227	0.34167	0.55062	0.05725	0.01442

Values in italics indicate nominal significance.

^a p-values are identical as a reflection of the high LD between the 2 SNPs.

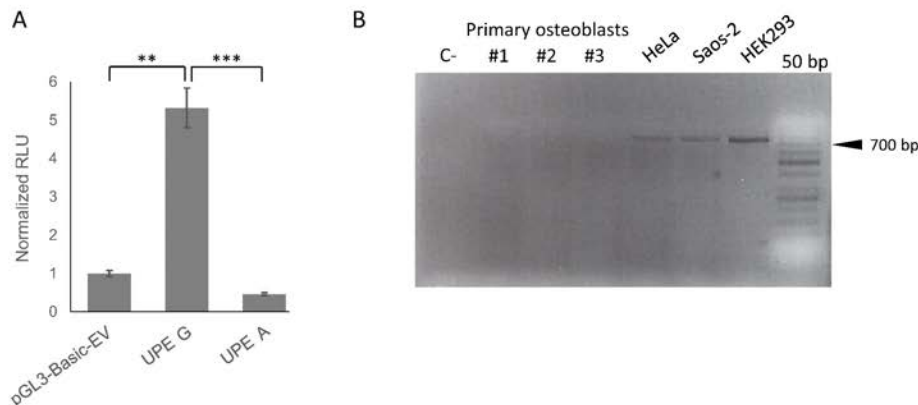


Fig. 3. (A) Luciferase activity of different versions of UPE, containing the G or A allele for the SNP rs10085588, in a forward or inverted orientation, in Saos-2 cells. Results are expressed as mean $\pm$ SD. **p-value < 0.01; ***p-value < 0.001 (B) PCR amplification of UPE from cDNA of different cell types. Expected size: 750 bp.

assays in Saos-2 cells to test UPE activity in both orientations and the effects of the two alleles of rs10085588. As shown in Fig. 3A, the forward UPE construct bearing the major allele (G) showed significantly increased luciferase expression compared to the empty vector (FC: 5.3, $p = 0.0097$), and compared to the construct bearing the minor allele (A) (FC: 11.6, $p < 0.001$). No activity was detected when the UPE was tested in the inverse orientation (data not shown). Results of luciferase assays were consistent with eQTL analyses, in the sense that the minor allele (A) fails to activate transcription and is associated with lower expression of *SLC25A13* in osteoblasts (see above). We also evaluated whether the UPE was transcribed (as occurs in many regulatory regions) by performing RT-PCR in cDNA of human primary osteoblasts, Saos-2, HeLa and HEK293 cells. We were able to amplify the UPE sequence from cDNA of HeLa, HEK293 and Saos-2 cells but we failed to amplify it from different cDNA samples of human primary osteoblasts (Fig. 3B). We also interrogated the FANTOM5 Cap Analysis of Gene Expression (CAGE) dataset [26] for evidence of UPE transcription and we could observe a signal for TSS expression in smooth muscle cells.

3.5. Chromatin interactions from the UPE

Finally, we investigated the possible genomic targets of the UPE by examining the 3D chromatin interactions by 4C-seq in different cell types (MSCs, hFOB 1.19 and Saos-2). We detected interaction between UPE and the genomic region spanning approximately 750 kb on each side of it (Fig. 4), and no other interactions were detected elsewhere in the genome. Notably, we found higher interaction levels with most of the tissue-specific enhancers described in the VISTA browser [42,43], especially in the hFOB 1.19 cell line. We could also detect higher interaction with the long non-coding RNA gene *LOC100506136*, upstream of *C7ORF76*. In addition, we analysed the topologically associated domains (TADs) of the region on different cell types using available Hi-C data [40] and the 3D Genome Browser [41] and we observed that in many cell types, the TAD containing the gene *C7ORF76* spanned from approximately 50 kb upstream of *DYNC111* TSS to approximately 25 kb upstream of *ACN9* TSS (Fig. 4), consistent with the 4C-seq results.

4. Discussion

Different non-coding variants in the *C7ORF76* genomic region have been previously associated with BMD and osteoporotic fracture in different GWAS [4,6–8,14–16] and at this point, deciphering the functionality of this region would be the next logical step for understanding these associations. In this line, we have analysed the *C7ORF76* region in depth, including re-sequencing, testing variants for association in the

BARCOS cohort, and analysing the functionality of the variants and regulatory regions both *in silico* and experimentally. Two upstream variants (rs4342521 and rs10085588) were found significantly associated with LS-BMD and nominally associated with osteoporotic fracture. In addition, both SNPs have been identified as eQTLs of *SLC25A13* in human primary osteoblasts. Moreover, the SNP rs10085588 falls in a regulatory region (UPE) able to stimulate transcription in an allele-dependent manner. This UPE was found to interact with different tissue-specific enhancers and a lncRNA present in the nearby region.

The *C7ORF76* gene is an uncharacterised gene of unknown function without expression data in GTEx. In the current human genome assembly (GRCh38) it is annotated as alternative transcript 6 of *SEM1* (encoding a 26S proteasome complex subunit, whose alternative historical name is *SHFM1* for Split Hand and Foot Malformation 1). However, although RefSeq currently labels it as curated gene, surprisingly few data have been gathered and the real function of *C7ORF76* remains elusive. This is in contrast with the consistent finding of potent GWAS signals for osteoporosis within this gene. Of note, this gene seems to be not expressed in primary osteoblasts, Saos-2, HeLa and SH-SY5Y (data not shown) which suggests that either it might, indeed, not be an osteoblast gene, yet regulate BMD from a different cell type or organ, or, alternatively, other genes in the region could be causal for the association.

Most GWAS variants for complex diseases are located in non-coding regulatory regions (reviewed in Zhang et al. [44]) and several studies have pinpointed the importance of regulatory elements for the susceptibility to osteoporosis [45,46]. Moreover, for some common traits, it has been described that several causal variants exist in a single LD block, located in multiple enhancers that cooperatively influence gene expression (the so called super-enhancers) [34,47]. These enhancer clusters are highly cell type specific. In this respect, although publicly available datasets [34,35] did not consider the *C7ORF76* genomic region to be a super-enhancer, the functional annotation of this region revealed the presence of several putative regulatory elements containing SNPs prone to confer susceptibility to osteoporosis.

To elucidate the molecular mechanisms underlying the strong association, further exploration with high-coverage sequencing to prioritise potentially causal variants was necessary [48]. We performed an extreme-truncate selection of the BARCOS cohort as a discovery phase, prior to genotyping the selected variants in the complete cohort. The efficiency of extreme-truncate selection approach for quantitative trait association studies (e.g. BMD) has been widely proven, as well as its utility for detecting rare variants [6,49,50]. The 2 SNPs found associated in the BARCOS cohort in this study (rs4342521 and rs10085588) were previously found associated with LS-BMD, FN-BMD and

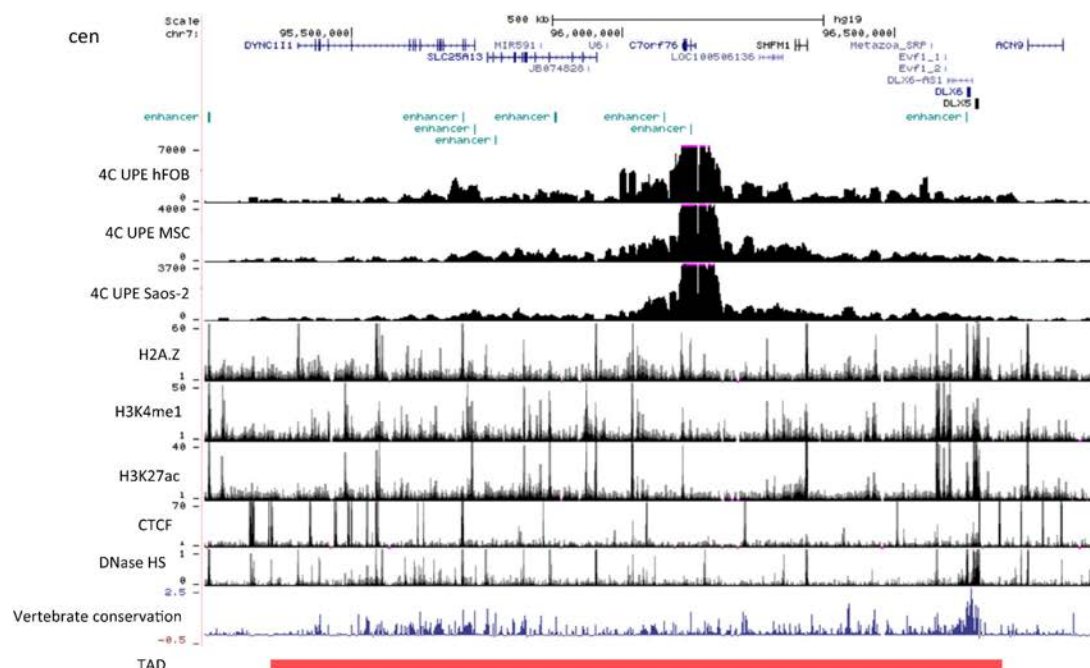


Fig. 4. 4C-seq using UPE as viewpoint in human fetal osteoblast (hFOB) 1.19, mesenchymal stem cells (MSC) and Saos-2 cell line. H2A.Z, H3K4me1, H3K27ac and CTCF ChIP-seq and DNase HS from osteoblasts (ENCODE data), and vertebrate conservation are shown. Experimentally validated active enhancers from VISTA Enhancer Browser [42] are shown in green. In red, topologically associated domain (TAD).

osteoporotic fracture in other GWA studies [4,9]. In contrast, we failed to find association in the rest of the variants interrogated, although other SNPs in this region have been found associated with LS-BMD, FN-BMD, and osteoporotic fracture [6,8,16], as well as with heel BMD [10,12] and total body BMD [11]. Taken together, these association data are consistent with the existence of a large LD block encompassing all the associated variants and the comparatively small sample size of the BARCOS cohort may preclude the detection of some of them.

To further delineate the role of the three associated variants (the two mentioned above and the GWAS SNP in Estrada et al. [7]), we performed *cis*-eQTL analyses in human primary osteoblasts and we detected a nominal association between the minor alleles of the three SNPs and decreased *SLC25A13* gene expression. We also detected a trend for association with decreased expression of *SHFM1*. These results reflect the LD among the SNPs. We failed to find an association between SNP rs10429035 and transcription levels of *SHFM1*, while in GTEx this SNP is described as eQTL for this gene in tibial artery. It could be that it is not eQTL in primary osteoblasts. Alternatively, our limited power ($n = 45$) and the fact that primary cells are not as homogeneous as cell lines may have prevented us to detect it in primary osteoblasts [51].

Out of the 3 associated SNPs, only one lies within a regulatory region (UPE, see Fig. 1), while the others map to sequences lacking regulatory marks. We set out to experimentally study the UPE, which is a conserved region located approximately 4 kb upstream of *C7orf76* TSS, with enhancer marks such as H3K27ac and H3K4me1, as well as a DNase hypersensitivity signal. According to ENCODE ChIP-seq data, many transcription factors bind to UPE, among which RAD21, MYC, POLR2A, and the P300 histone acetyltransferase, known to be involved in transcription regulation, initiation and elongation and in enhancer activity [52,53]. Our results, including luciferase assays, RT-PCR and 4C analyses, indicate that, indeed, the UPE acts as a regulatory element, able to activate transcription of a reporter gene. It is well known that many such elements can be transcribed [54–56], producing non-coding

RNAs (or eRNAs for enhancer-RNAs) and, in this sense, we found UPE to be transcribed in Saos-2 cells.

Our results also provide clear evidence that SNP rs10085588, within the UPE, is itself functional, since we showed that the minor allele (A) abolished luciferase activation, which was otherwise stimulated by the major allele G. We have performed an *in silico* analysis and found predictions that the histone deacetylase HDAC2 may bind the A allele more probably than the G (p-value: 5.30016×10^{-6}), a possible explanation for the reduced expression observed.

The 4C-seq experiments showed that, in the cells included in this study, the UPE interacted with several sites within the TAD where it belongs (see Fig. 4), and nowhere else in the genome. This suggests that the biological function of the GWAS signal should be limited to genes within this TAD, further supported by strong CTCF signals limiting the region. However, interactions with other genomic regions may exist in different tissues or differentiation stages, not tested by us. In this sense and interestingly, a computationally-based characterization of osteoporosis associated SNPs identified an interaction between the GWAS SNP rs4729260 in the *C7orf76* region and the Xq12 genomic region [57], which contains the androgen receptor gene, known to be involved in bone metabolism [58].

Within the TAD, the UPE enhancer interacted with many other tissue-specific enhancers previously identified [42,43], suggesting that they may act cooperatively or redundantly in regulating gene expression. Several of these enhancers have been shown to affect *DLX5/6* gene expression [43]. However, we have not detected strong interaction signals with the *DLX5/6* region and, in our eQTL study in primary osteoblasts, we did not observe any effect of the associated SNPs on *DLX5/6* gene expression. Likewise, we detected modest interactions between the UPE and the *SHFM1* and the *SLC25A13* coding regions, and negligible signals with that of *DYNCH11*. In contrast, we did detect a marked interaction with a lncRNA in the close vicinity of *C7orf76*, namely *LOC100506136*. Interestingly, a recent study found a SNP

within *LOC100506136* to be one of the 2 out of approximately 55,000 genome-wide SNPs in lncRNAs associated with total hip BMD [59]. Another recent publication found a LS-BMD associated signal within *LOC100506136* in a Mexican-Mestizo cohort [60]. These studies suggest that this lncRNA could be involved in osteoporosis pathogenesis.

This work has some limitations including the sample size of the BARCOS cohort, and of the primary osteoblasts used for the eQTL analyses, both of which preclude the detection of variants with smaller effects. In addition, *C7ORF76* was not tested in other bone cells such as osteoclasts. However, these results provide interesting data to understand the functionality of this unexplored region, one of the most frequently found associated with osteoporosis in GWA studies. They also highlight the importance of regulatory variants in bone phenotypes and the usefulness of integrative approaches to uncover their functionality.

5. Conclusions

In summary, this study is the first one to analyse in depth the functionality of the *C7ORF76* genomic region, associated with BMD and osteoporotic fracture in many GWAS. We provide functional regulatory evidence for rs10085588, which may be a causal SNP within this 7q21.3 GWAS signal for osteoporosis.

CRediT authorship contribution statement

Neus Roca-Ayats: Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Núria Martínez-Gil:** Investigation, Formal analysis, Writing - review & editing. **Mónica Cozar:** Investigation, Writing - review & editing. **Marina Gerousi:** Investigation, Writing - review & editing. **Natàlia Garcia-Giral:** Conceptualization, Writing - review & editing. **Diana Ovejero:** Conceptualization, Writing - review & editing. **Leonardo Mellibovsky:** Conceptualization, Writing - review & editing. **Xavier Nogués:** Conceptualization, Writing - review & editing. **Adolfo Díez-Pérez:** Conceptualization, Writing - review & editing. **Daniel Grinberg:** Conceptualization, Funding acquisition, Formal analysis, Supervision, Writing - original draft, Writing - review & editing. **Susanna Balcells:** Conceptualization, Funding acquisition, Formal analysis, Supervision, Writing - original draft, Writing - review & editing.

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Declaration of interests

The authors declare they have no conflict of interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bone.2019.03.014>.

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Supplementary Table 1. Coordinates and primers for the amplification of the *C7ORF76* genomic region by LR-PCR

Fragment	Genomic coordinates (GRCh37)	Primer Fwd	Primer Rev	Product size (bp)
1	7:96,136,619-96,132,152	TTGACCTGAATACTGCCGC	GCCAAATGAATGTGGACAAG	4468
2	7:96,132,302-96,127,711	CACTGCTGGGTCTTAGATTGG	GCATGTGTGCATGATGTTGG	4592
3	7:96,127,863-96,123,028	TGCAAGTTTCCCTCAATTCATC	TCCCTCTCATCTGTGCAACAC	4836
4	7:96,123,158-96,118,311	TTAGGTGAGTAGAAAGCAATGGC	CTGGGTGGCTATAGACCTGAATAG	4848
5	7:96,118,477-96,114,227	GCGGCACTGTGAGAGTACATC	CCTGGTGAAATGGGAACA	4251
6	7:96,114,348-96,110,480	CTGACACTTTGGCAGCACC	GGGATTGTTGAAGCTGACCC	3869
7	7:96,110,702-96,108,695	CAACCATCACAAACCCATAGAC	CCTGAGCAAGTCTCGTAAGTG	2008

Supplementary Table 2. Primers used for genotyping the selected SNPs

SNP	Primer Fwd	Primer Rev
rs4727338	CACATACACTTGACTGTGTTTGGT	GGATTCTGGCTTTGACATCC
rs10429035	TCTTTTGTGTGTTGAGGAAAGG	TCCTGTACGGAACCCCTGACT
rs12674052	GGAAACCCCTGTGTTATTTTCAGC	GGTTGCCCAAGTCACCCAC
rs4342421	TTAAATGTGACCTTTGTACTCAACA	AAATGTCAGAGGATGGTCCAG
rs10085588	TGTTCCAGATGCAAGATGATT	AGTGGAGATTGAGGGGAAT

Supplementary Table 3. Primers used for qPCR assays

Gene	Primer Fwd	Primer Rev
<i>DLX5</i>	CTACAACCGCGTCCCAAG	GCCATTACCATTTCTCACCT
<i>DLX6</i>	ATATATTAGAGAAGAGCGAGGGAGAG	CCCTCTGCAGCCACCTTA
<i>AS-DLX6</i>	TGATTCCTGTATGTATGGCAGCTA	GGTTTTCTTTGTCTCAGCAAT
<i>SLC25A13</i>	AGATGGTTCCGGTCCCACTT	GCAAACGGATCTTGACGATT
<i>SHFM1</i>	GACGACGAGTTTGAAGAGTTCC	CCCAATTATCCTCCAGACA
<i>HMBS</i>	TGCCCTGGAGAAGAATGAAG	CAGCATCATGAGGGTTTTC

Supplementary Table 4. Minor allele frequency (MAF) from 1000 Genomes project: total population (ALL), European population (EUR) and Iberian population in Spain (IBS) and MAF of complete BARCOS cohort.

SNP	Minor allele	ALL	EUR	IBS	BARCOS
rs115076023	A	0.011	0.000	0.000	0.000
rs10085588	A	0.223	0.359	0.360	0.392
rs4342521	T	0.221	0.360	0.360	0.396
rs7794042	A	0.009	0.014	0.005	0.031
rs4613908	A	0.386	0.363	0.332	0.321
rs10238953	C	0.116	0.156	0.220	0.167
rs190892252	G	0.004	0.008	0.009	0.012
rs4727338	C	0.221	0.360	0.360	0.390
rs117923361	A	0.016	0.040	0.014	0.035

