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Review

Genome-wide association studies (GWAS) *vs* functional validation: the challenge of the post-GWAS era

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Abstract

Over the past few years, efforts have been made to determine the variants and genes that may be important to determine bone mineral density (BMD) that, at the same time, are involved in several bone diseases. To achieve this, the approach that has been the most successful of all has been genome-wide association studies (GWAS). In particular, in research on bone biology over 50 different large GWAS or GWAS metanalyses have been published identifying a total of 500 genetic *loci* associated with different bone parameters such as BMD, bone resistance, and risk of fracture. Although the discovery of associated variants is an essential aspect, the functional validation of such variants is equally important to elucidate their effect, as well as the causal correlation they have with genetic disease. Since it is a much more time consuming and tedious aspect it has become the new challenge of this post-GWAS era. Among the genes that have already been studied several Wnt signaling pathway genes have been included, among them, the *SOST* gene that plays a crucial role both determining the BMD of the population and monogenic diseases with elevated bone mass giving rise to a new therapy against osteoporosis. In this review we'll be collecting the main GWAS associated with bone phenotypes, as well as some functional validations undertaken to analyze the associations found in them.

Keywords: Genome-wide association studies. Functional validation. Bone mineral density. Bone diseases.

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GENOME-WIDE ASSOCIATION STUDIES (GWAS)

Over the past few years, genome-wide association studies (GWAS) have been an essential tool to identify what genes are involved in complex diseases (1). These studies consist of establishing an association between the genetic or allelic frequency of millions of SNP (single nucleotide polymorphisms) type markers distributed across the genome and a particular phenotype or disease (2). This approach is the most complete and impartial tool that exists for the particular of complex diseases. Unlike candidate gene association studies, GWAS are a hypothesis-free approximation hypothesis that allows the discovery of new genes or signaling pathways involved in a given phenotype that, up until now, were completely unknown (3). GWAS has been possible thanks to new advances made in high-throughput genome technology, study design, improved statistical analysis, and the possibility of having large biobanks available (4,5). Due to the large number of simultaneous statistical tests performed and, therefore, the statistical corrections made (that require a threshold *p* value of $5x10^{-8}$ to be considered statistically significant at whole genome level, and the small effect each variant presents in complex diseases, extremely large cohorts are required. This has been achieved through metanalyses of the GWAS where different studies have come together to increase the size of the sample (6,7).

Although with the evident success reported, GWAS have 3 main limitations. First, the genetic variants used to validate the association with the particular phenotype are SNP markers (tagSNPs) that are homogeneously distributed across the whole genome with a minor allele frequency (MAF) ≥ 5 % in the population. Therefore, rare variants with possible strong effects in the phenotype are not included in these studies. An attempt has been made to solve this limitation by including variants of less frequency in genotype chips, whole exome/genome sequencing, WES/WGS) and/or using the phenotypic extremes of the cohorts. Second, the success of GWAS largely depends on the size of the sample. Therefore, as commented above, the most widely used strategy today is to establish large consortia including different cohorts from across the world. Therefore, super-cohorts of greater statistical power —but genetically heterogeneous— are obtained in such a way that variants of a specific population are very difficult to find. Third, GWAS report the most statistically relevant SNP called lead SNP. Although this SNP can be the one causing this association, other variants that are in linkage disequilibrium with respect to the lead SNP variant can be responsible too. If the SNP associated is found in a codifying region and involves a change of amino acid, chances are that the SNP will be causal. However, truth is that most lead SNPs can be found in non-codifying regions (96 %) both intronic (41 %) and intergenic (54 %), which complicates the demonstration of their causal roles. Due to their non-coding nature, conducting functional studies of these lead SNPs is truly challenging (8-10). Therefore, these functional studies are still scarce to this date, and establishing the functional basis of the associations found in such analyses is still to be elucidated in this post-GWAS era.

To conduct functionality studies, interdisciplinary approaches are needed including *in silico* analyses (computational approaches) (11,12) —like pathogenicity prediction tools—, *in vitro* studies including, among other, studies of the reporter gene assays (eg, luciferase) (13) and *in vivo* studies of animal models like the zebra fish or mice (14,15).

This review summarizes the main GWAS published to this date using skeletal phenotypes, followed by *in vitro* and *in vivo* studies generated from the first large GWAS metanalysis (16) ever conducted on bone mineral density (BMD) and risks of fracture.

GWAS AND BONES

To conduct GWAS of bone diseases such as osteoporosis, parameters like BMD, and the geometry and microarchitecture of the bone can be taken into consideration. Among these properties, the most widely used and the one that best predicts osteoporotic fracture is BMD that is a quantitative trait measured in a continuous scale using methods like dual-energy X-ray absorptiometry (DXA). It is estimated that BMD is a trait with an approximate heritability between 50 % and 80 %. Similarly, the geometry of the bone shows heritability rates between 30 % and 70 % while bone microarchitecture determined by high-resolution peripheral quantitative computed tomography scan (HR-pQCT) shows heritability rates between 20 % and 80 % (17).

Up until now, over 50 large GWAS have been conducted using bone parameters together with a plethora of GWAS in smaller and more homogenous cohorts. With this over 500 associated *loci* have been identified. Although the percentage of variance explained through GWAS has increased substantially over the past few years thanks to the use of larger cohorts, all these *loci* only explain a small percentage (20 %) of genetic contribution to BMD (18,19). This has created a gap between the variability explained by genetic factors and BMD heritability probably due to overestimating heritability or the fact that other genetic factors like copy number variants (CNV) or epigenetics are not being taken into consideration (20).

All in all, GWAS have yielded significant findings like the association between the *SOST* and *LRP5* genes —that had already been involved in monogenic skeletal disorders— and some skeletal phenotypes or the identification of new genes whose involvement in bone phenotypes was previously unknown (21). Table I

shows some of the most relevant GWAS associated with BMD, most of which have been reported in the GWAS catalog (http:\\ebi.ac.uk/gwas). To narrow it down, only studies with cohorts > 10 000 individuals have been considered.

Many of the GWAS displayed on table I correspond to studies in which large metanalyses have been conducted leaving as a result hundreds of variants in different *loci* associated with skeletal phenotypes. However, most of these studies lack functional approaches.

FUNCTIONAL STUDIES IN THE POST-GWAS ERA

Despite the huge amount of association studies conducted to this date, functional studies have not developed at the same pace. Therefore, only a small fraction (164; 15 %) of the 1051 manuscripts that have cited the first large GWAS metanalysis on bone density (16) included functional studies whether *in vitro* or *in vivo.*

An example of successful functional studies is the characterization of the regulation of the *SOST* gene. This gene codes the sclerostin protein, a canonical Wnt signaling pathway inhibitor (49-51) associated with multiple bone parameters in different association studies across several populations (17,28,33,38,40,43,52,53) (Fig. 1A). Its inhibitory function on bone formation has been widely studied in *in vivo* and *in vitro* models. Currently, antisclerostin antibodies are used to treat bone diseases like osteoporosis or osteogenesis imperfecta (54-59). Therefore, the regulatory factors of the expression of the *SOST* gene are included among the new candidates as a target for the development of new therapies. In humans, *SOST* gene variants have been associated with conditions characterized by an excessive bone formation: sclerosteosis, craniodiaphyseal dysplasia, and the phenotypic trait of high bone mass (60) (Fig. 1B). To these diseases we may add Van Buchem disease. It is due to the deletion of the enhancer element ECR5 of *SOST* situated at the 52 kb region downstream of the gene that is necessary for the proper expression of the *SOST* gene (61) (Fig. 1A). Actually, the transcription of the *SOST* gene is finely regulated by many different signals both through direct regulation on the promoter of the *SOST* gene and through the distal ECR5 regulatory region (62,63) whose physical interaction has been demonstrated in a study recently conducted by our group on bone cells (64) (Fig. 1A). The MEF2C transcription factor is the best described *SOST* regulator in relation to its expression in osteocytes (63,65). The importance of MEF2C in the enhancer effect of ECR5 has been confirmed in the knock-out mouse model of *Mef2c* in osteoblasts/osteocytes that has a high bone mass and low levels of sclerostin (66). Precisely, *MEF2C* is yet another of the most repeated signals in GWAS with bone parameters (16,23,36,37,67-70). Together with MEF2C, HDAC5 has also been described as a negative regulator of the expression of the SOST gene that exerts its function by blocking the association of MEF2C and ECR5 during the differentiation of immature osteocytes (Fig. 1C). Consistent with this, the *HDAC4/5* knock-out mouse model displays low BMD, and high expression of the SOST gene (71-73). Once again, *HDAC4/5* is found among the most repeated *loci* in association studies with bone parameters (18,23,34,39,74) (Fig. 1B).

Another example of how important it is to conduct functional studies of associated regions is the *DKK1 locus.* This is another canonical Wnt signaling pathway inhibitor that plays a crucial role in the morphogenesis of the head (75,76), and bone development (77,78). Currently, no *DKK1* variant has been described causing bone diseases in the HGMD database. Despite of this, our group identified 2 different missense variants in patients with the high BMD phenotype who show a functional loss of their inhibitory ability (13,79). On the other hand, one of these variants has also been found in patients with totally opposed phenotypes like osteoporosis or anal malformations (80,81). Also, we should mention that no GWAS has ever found SNPs in *DKK1* associated with BMD or other bone parameters. However, an association with BMD has been demonstrated in a set of SNPs grouped in a region 350 kb downstream of *DKK1* and 92 kb upstream of *MBL2* (16,18,19,29,33,34,36,37,39,74) (Fig. 2). To distinguish which one of these 2 genes was responsible for this association, a study from our group (13) conducted a 4C chromatin conformation capture using the GWAS signal-rich region as a bait in 3 bone cellular types. This confirmed the physical interaction between this region and the *DKK1* promoter ruling out any interaction with the *MBL2* gene (Fig. 2; lower panel). It is precisely in this region where the *LNCAROD* gene is found, which specifies a DKK1 activator long noncoding (lncRNA), a possible culprit of the association found in the GWAS (82).

One of the most consistent *loci* across different GWAS on BMD is the genomic region situated in 7q31.31 including the *WNT16* gene. This is a very complex *loci,* also including, apart from the *WNT1* gene, the neighboring genes *ING3, FAM3C,* and *CPED1.* The role of the *WNT16* gene determining BMD has been clearly established in functional studies of knock-out mouse models or osteoblast-specific conditional knock-out mice (6,83,84) that, largely, show spontaneous fractures due to low BMD plus reduced cortical thickness and bone resistance. However, evidence has been found on the importance of 3 other neighboring genes in bone metabolism. In the case of the protein coding gene *ING3* (Inhibitor of Growth Family Member 3) —part of the Nucleosome Acetyltransferase of H4 histone acetylation (NuA4 HAT) complex involved in chromatin regulation— it has been found abundantly expressed in bone tissue (85).

Table I. GWAS on bone and genes found with variants associated with skeletal phenotypes

Study	Ancestry	Trait	Sample size	Most relevant *loci*/genes	Brand-new *loci*/genes
Styrkarsdottir *et al.*, 2009 (22)	European	BMD-LS, BMD-FN, OF	15 375	*MARK3, SOST, SP7 (osterix)*	4/9
Rivadeneira *et al.*, 2009 (23)	European	BMD-LS, BMD-FN	19 195	*WLS, CTNNB1, MEPE, STARD3NL, FLJ42280, DCDC5, SOX6, FOXL1, HDAC5, CRHR1, MEF2C*	13/20
Guo *et al.*, 2010 (24)		BMD-Th	11 568	*ALDH7A1*	1/1
Kung *et al.*, 2010 (25)	Asian	BMD-LS, BMD-FN, OF	18 898	*JAG1*	1/1
Hsu *et al.*, 2010 (26)	European	BMD-LS, BMD-FN, FN-AA, WNS, LFN	11 290	*RAP1A, TBC1D8, OSBPL1A*	3/4
Estrada *et al.*, 2012 (16)	European and Asian	BMD-LS, BMD-FN, OF	83 894	*CDKAL/SOX4, CPED1, WNT16, MBL2/DKK1, AXIN1, RPS6KA5, ERC1/WNT5B, FAM210A, FAM9B/KAL1, SOX9, KLHDC5/PTHLH, IDUA, NTAN1, SFRP4, SUPT3H/RUNX2*	32/56
Styrkarsdottir *et al.*, 2013 (27)	European	BMD-LS, BMD-WB, BMD-h, OF	97 315	*LGR4*	1/2
Zhang *et al.*, 2014 (28)	European, Asian, and African American	BMD-LS, BMD-FN, BMD-Th	27 061	*SMOC1, CLDN14*	2/15
Moayyeri *et al.*, 2014 (29)	European, Asian, and North American	BUA, SS, BMD-H	59 242	*TMEM135*	1/7
Zheng *et al.*, 2015 (30)	European	BMD-LS, BMD-FN, BMD-F, OF	561 489	*EN1*	1/36
Styrkarsdottir *et al.*, 2016 (31)	European and Asian	BMD-LS, BMD-h, OF	30 191	*PTCH1*	1/14
Nielson *et al.*, 2016 (32)	European, and North American	BMD-LS, CVF, RVF	42 869	*SLC1A3/RANBP3L*	1/5
Mullin *et al.*, 2017 (33)	European	BUA, SS, OF	16 627	*PPP1R3B, LOC387810, SEPT5/TBX1*	3/8
Kemp *et al.*, 2017 (34)	European	eBMD-H, OF	142 487	*ARID1A, PKN2, TBX15, NGEF, SUSD5, ERC2, BMP2, PLXDC2, BMP5, MEOX2, CREB5, AQP1, CADM1, EMP1, NFATC1, TMEM92, GPC6, BMP4, SMAD3, BMPR2, AXIN2*	153/203
den Hollander *et al.*, 2017 (35)	European, and North American	BSGH, OA	12 784	*MGP, CCDC91*	2/5
Medina-Gomez *et al.*, 2018 (36)	European, African American, and Australian	BMD-WB	66 628	*SLC8A1, PLCL1, SMAD9, ADAMTS5, TOM1L2, TCF7L1, APC, DUSP5, CD44, CCND1, CYP19A1, MAFB, RUNX1, RAI1, ZSCAN25, GRB10, DRG2, ETS2, PSMD13, CSF1*	36/80
Pei *et al.*, 2018 (37)	European, Asian, African American, and Hispanic	BMD-LS, BMD-FN	40 449	*MACROD2, OSBPL2*	2/9
Alonso *et al.*, 2018 (38)	European, and Australian	CVF	10 683	*2q13*	1/1
S. K. Kim 2018 (39)	Europea	DMOe-T, FO	394 929	*RP1L1, PRSS55, MAPT, GPATCH1, SMG6, WNT1, WNT5B*	613/899

(Continues on next page)

Table I (Cont.). GWAS on bone and genes found with variants associated with skeletal phenotypes

Study	Ancestry	Trait	Sample size	Most relevant *loci*/genes	Brand-new *loci*/genes
Trajanoska *et al.*, 2018 (19)	European, North American, Asian, and Australian	OF	562 258	*GRB10/COBL, ETS2, RSPO3*	4/15
Baird *et al.*, 2019 (40)	European, North American, and Australian	DXA-h	15 934	*ASTN2, PTHLH, NKX3-2, FGFR4, GSC/DICER1, HHIP*	6/8
Hsu *et al.*, 2019 (41)	European, North American, and Asian	LFN, AA, WNS, MSFN	18 719	*IRX1/ADAMTS167*	1/4
Morris *et al.*, 2019 (18)	European	eBMD-H, OF	426 824	*DAAM2, WNT7B, WNT2B, COL11A1, SERPINC1, PRKCE, HDAC4, HOXD11, BCL11A, SOX5, TGFBR3, MMP16, EPHA4, MSH6, SEPT11, LRRC1, ADH1B, CTPS1, DNMT3A, MEIS1*	301/518
Pei *et al.*, 2019 (42)	European, North American, and Australian	BMD-H, BMD-WB	209 115	*FBN2, DEF6, TNFRSF19, NFE2L1, SCMH1*	18/56
Styrkarsdottir *et al.*, 2019 (43)	European and Asian	BMD-h, BMD-LS-BA, OF	28 954	*GDF5, ADAMTSL3, BCKDHB, CHRDL2, DYM, CTDSP2*	6/13
Zheng *et al.*, 2019 (44)	European, North American, and Australian	BMD-FN, eBMD-HU	10 584	*B4GLANT3, GALNT1*	2/3
Feng *et al.*, 2020 (45)	European, North American, African American, Asian, and Hispanic	BMD-h, TLM, eBMD-H	11 335	*MC4R*	1/2
Zhang *et al.*, 2020 (46)	European, North American, African American, Asian, and Hispanic	BMD-FN, BLMAL	12 445	*FTO, PPP1CB, TRMT61B, LSAMP, FAM189A2, LOC101928063*	6/26
Surakka *et al.*, 2020 (47)	European	BMD-F	19 705		0/10
Greenbaum *et al.*, 2022 (48)	European,	DMO-CF, DMO-CL	49 487	*IGF2, ZNF423, SIPA1, PED4D, PIGN, TRAF3IP2, NFIB, LYSMD4, MAML2*	9/30

The study is represented by the first author and year. The genes are the study most relevant ones due to their association with skeletal phenotypes and their new finding. AA, axis angle; BLMAL, body lean mass of arms and legs; BMD, bone mineral density; BS, bone size; BSGH, bilateral semi-quantitative grading of the hand; BUA, broadband ultrasound attenuation; CVF, clinically confirmed vertebral fracture; DXA-h, X-ray absorptiometry of the shape of the hip; eBMD, estimated bone mineral density; F, forearm; FN, femoral neck; H, heel; h, hip; HU, heel ultrasound; LFN, length of the femoral neck; LS-BA, lumbar spine-bone area; LS, lumbar spine; MSFN, modular section of femoral neck; OA, osteoarthritis; OF, osteoporotic fracture; RVF, radiographically confirmed vertebral fracture; SS, speed of sound; Th, total hip; TLM, trunk lean mass; WB, whole body; WNS, width of the neck narrow section.

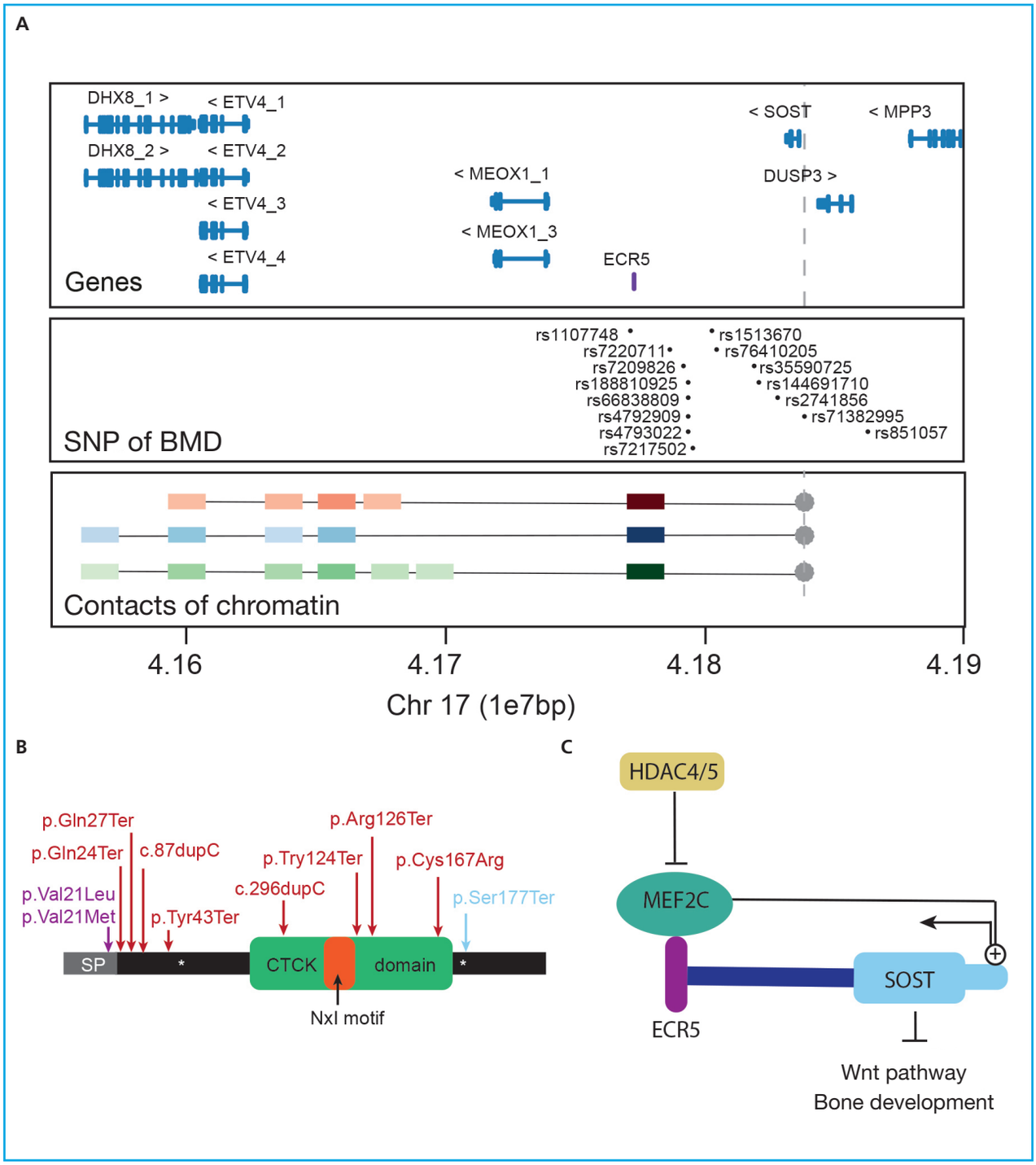

Figure 1. The *SOST* gene. A. Upper panel: *Locus* containing the *SOST* gene and its neighboring genes (GRC37/hg19). In purple, the ECR5 regulatory region. Main panel: SNPs associated with different bone parameters across different GWAS from the GWAS catalogue (https://www.ebi.ac.uk/gwas7). Lower panel: Main results of the 4C clinical trial conducted by Martínez-Gil et al. back in 2021 showing the main interactions of the SOST promoter (used as a bait and indicated with a dot and gray discontinuous). Colored squares show the interactions with color intensity proportional to the intensity of the interaction. Red, blue, and green squares show interactions with mesenchymal stem cells, hFOB cells, and SAOS2 cells, respectively. The units of the genomic scale used (1e7pb) correspond to 10 mega bases ($1x10^7$ base pairs). B. Schematic representation showing of sclerostin protein showing its functional domains and variants responsible for human skeletal conditions. Purple, red, and blue colors show the variants associated with craniodiaphyseal dysplasia, sclerosteosis, and the HBM phenotype variant. CTCK, C/terminal cysteine knot-like. C. Scheme of some of the positive and negative regulators of the expression of the *SOST* gene.

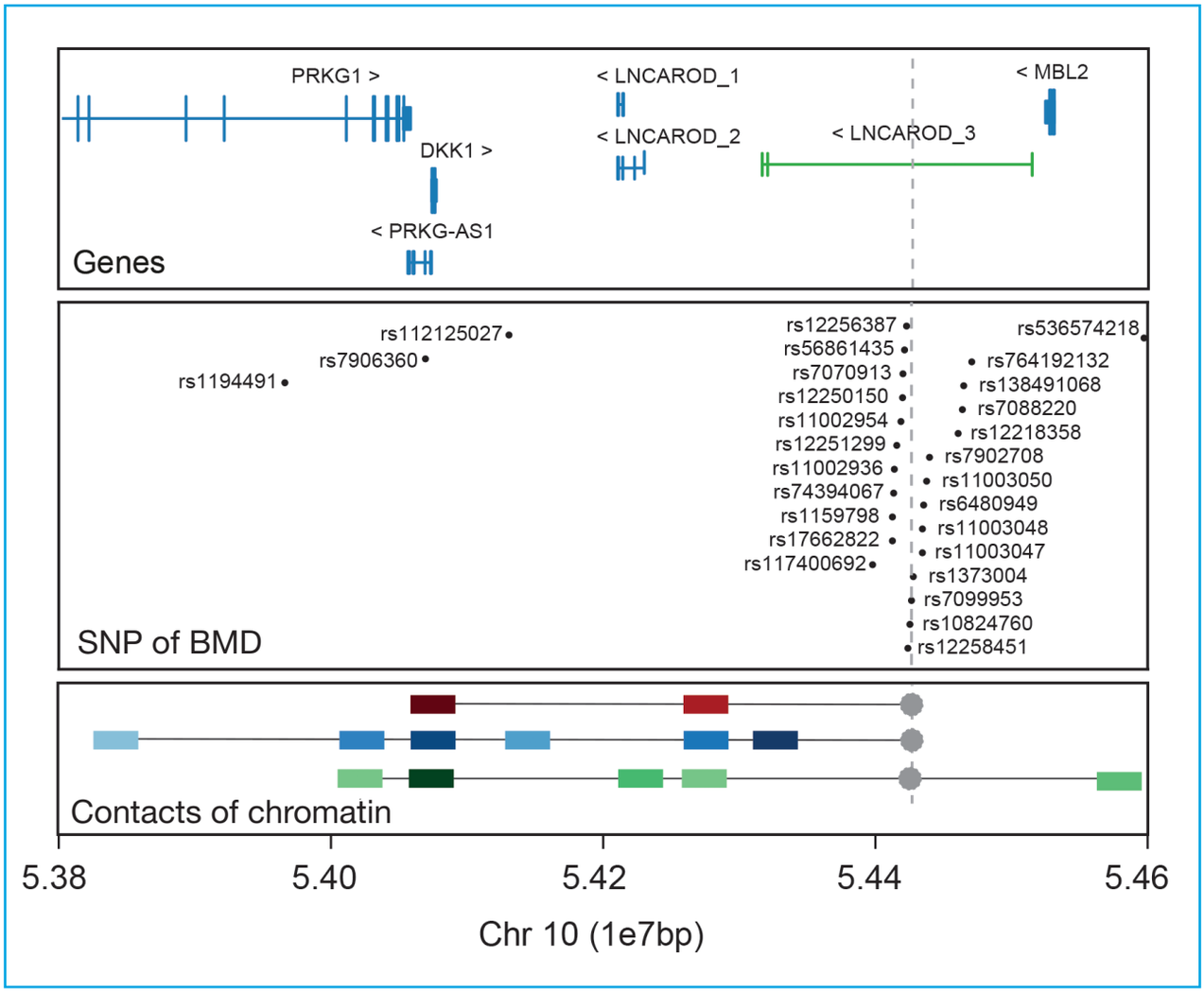

Figure 2. DKK1. Upper panel: *locus* containing the *DKK1* gene and its neighboring genes (GRC37/hg19). In green, the lncRNA *LNCAROD* of GENCODE v32.2 (GRC38/hg18). Main panel: SNPs associated with different bone parameters across different GWAS taken from the GWAS catalogue (https://www.ebi.ac.uk/gwas7). Lower panel: Main results from the 4C clinical trial conducted by Martínez-Gil et al. in 2020 showing the main interactions with the SNP-rich region associated with BMD (used as a bait and indicated with a dot and gray discontinuous line). Colored squares show interactions with color intensity proportional to the intensity of the interaction. Red, blue, and green squares show interactions with mesenchymal stem cells, hFOB cells, and SAOS2 cells, respectively. The units of the genome scale used (1e7pb) correspond to10 mega bases ($1x10^7$ base pairs).

In addition, functional studies of an *in vitro* cellular model of mesenchymal cells knocked-out for *ING3* show osteoblastogenesis damage and stimulation of adipogenic differentiation (86). Regarding the *CPED1* gene (Cadherin Like And PC-Esterase Domain Containing 1), no specific function of this gene has been found in humans or mice. However, in mice, functional studies show that the *Cped1* gene is uniformly expressed in a variety of tissues including bone. Also, different isoforms have been described due to alternative splicing, as well as 3 promoter regions active during osteogenic differentiation (87). To better define its possible role in bone homeostasis, additional functional studies would be needed in *in vitro* cellular or animal models. *FAM3C* (family of sequence similarity 3c) is a cytokine-like growth factor expressed in multiple tissues (88) that plays a very important role in epithelial-mesenchymal transition, and cancer metastasis (89). Its association with bone metabolism has been confirmed with the knock-out mouse model that shows bone structure alterations (88).

Several functional studies have been conducted on the expression regulation of different genes at that region. For example, our group has conducted eQTL studies (expression Quantitative Trait Locus) with primary osteoblasts that show that SNPs located inside the *WNT16* gene regulate the levels of expression of *FAM3C* of those cells (90). Also, in cells of osteoblastic lineage we have seen a physical interaction among different gene enhancers located inside the *CPED1* gene, and the promoter of the *WNT16* gene (91). All this shows the existence of a complex relation among these 4 genes, and suggests the possibility that they are working together. All in all, additional functional studies should be conducted to elucidate the role played by each of these genes, as well as all their possible interactions.

The aforementioned studies reveal the importance of functional studies based on the findings brought by analyzing GWAS. Challenge, now, is in the post-GWAS era. If we keep finding correlations between different variants in GWAS and functional aspects of these variants —*in silico, in vitro* or *in vivo*— we may end up finding new approaches and, therefore, new insights and therapeutic options for associated conditions and disorders.

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