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Multiple mechanisms explain genetic effects at the *CPED1-WNT16* bone mineral density locus

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Abstract

Purpose of review: Chromosome region 7q31.31, also known as the *CPED1-WNT16* locus, is robustly associated with BMD and fracture risk. The aim of the review is to highlight experimental studies examining the function of genes at the *CPED1-WNT16* locus.

Recent findings: Genes that reside at the *CPED1-WNT16* locus include *WNT16*, *FAM3C*, *ING3*, *CPED1*, and *TSPAN12*. Experimental studies in mice strongly support the notion that *Wnt16* is necessary for bone mass and strength. In addition, roles for *Fam3c* and *Ing3* in regulating bone morphology *in vivo* and/or osteoblast differentiation *in vitro* have been identified. Finally, a role for *wnt16* in dually influencing bone and muscle morphogenesis in zebrafish has recently been discovered, which has brought forth new questions related to whether the influence of *WNT16* in muscle may conspire with its influence in bone to alter BMD and fracture risk.

Summary: Rather than a single biological mechanism, multiple genes and tissues may work in tandem to contribute to the spectrum of musculoskeletal phenotypes mapped to the *CPED1-WNT16* locus.

Keywords

Genetics; Osteoporosis; GWAS; BMD; Fracture; WNT signaling

INTRODUCTION

Osteoporosis is a common chronic disease of the skeleton characterized by reduced bone mass and microarchitecture, resulting in increased risk of fragility fractures. Bone mineral density (BMD) is a key indicator for osteoporosis diagnosis and its treatment (1). Genome-

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zebrafish / *wnt16* / Wnt16

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wide association studies (GWAS) represent a powerful approach to better understand the genetic architecture underlying BMD and other osteoporosis-related traits. The identification of the 7q31.31 BMD locus, also known as the *CPED1-WNT16*[1] locus (2), was one of the first examples demonstrating the potential for GWAS to help identify genes critical for bone health. Single nucleotide polymorphisms (SNPs) at this locus were first shown to be associated with BMD in a Korean population in a 2009 study (3). Subsequent genetic studies revealed that the *CPED1-WNT16* locus is robustly associated with BMD and other osteoporosis-related traits including cortical bone thickness, hip geometry, and fracture (3-12). In parallel, mouse studies showed that *Wnt16* is necessary for cortical bone mass and strength (6, 7, 13). The *CPED1-WNT16* locus has been shown to be associated with fracture across multiple studies, including in studies that analyze both vertebral and nonvertebral fracture (5, 11), and in studies of forearm fracture (7). In a study of 1.2 million individuals, the lead SNP at the *CPED1-WNT16* locus (rs2908007) was the most significantly associated with fracture amongst 518 loci identified to be associated with estimated BMD (eBMD) assessed by heel quantitative ultrasound (11). Thus, amongst BMD loci, the *CPED1-WNT16* locus stands out for its robust association with BMD, strong association with fracture, and large body of experimental studies supporting its in-depth study.

Critical aspects regarding the biological mechanism(s) at the *CPED1-WNT16* BMD locus remain incompletely understood. Lead SNPs at GWAS loci are unlikely to be the causal variants, and instead are likely in linkage disequilibrium (LD) with the underlying causal variants (5). Some or even most causal variants likely reside in cis-regulatory elements that alter the expression of one or more protein-coding “target” genes at the locus. As such, the causal variant(s) at the locus, how these variants influence target gene function, and how altered gene function is translated into altered BMD and fracture risk are not fully understood. One complication in determining the underlying biological mechanism at the *CPED1-WNT16* locus is that this locus comprises two signals independently associated with BMD (i.e., they are significant even after genetic linkage is accounted for in conditional analyses) (5, 6). This suggests two different causal variants independently influence BMD (6). It is conceivable that each variant acts through the same or distinct target genes. Given what is known about *Wnt16* function including its necessity for bone mass and strength, *WNT16* is the most likely gene at 7q31.31 to function as a causal gene regulating BMD and fracture (14). However, there is evidence that other genes at the locus such as *FAM3C*, *CPED1*, and *ING3* could have important functions in bone (15-17). This, in concert with recent evidence indicating that BMD variants in regions of open chromatin interact with the promoters for some of these genes (17), suggests that one or more of these genes could mediate the genetic effects at 7q31.31 on BMD and fracture independently of *WNT16*.

Another complication in determining underlying biological mechanisms at 7q31.31 is that not all traits mapped to the locus can be explained by our functional understanding of *WNT16*. For instance, bone and muscle mass are linked, as indicated by the fact that osteoporosis and sarcopenia frequently occur in the same individual, a condition termed osteosarcopenia (18). Prior studies have shown that variants at the *CPED1-WNT16* locus

[1] The following nomenclature is used to indicate genes and proteins for different species:

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exhibit dual associations with BMD and lean mass (Fig 1A), the latter of which is a clinical correlate of muscle mass (19). WNT signaling is critical for embryonic myogenesis (20), and it has been previously speculated that the shared development of muscle and bone might underlie pleiotropic variants that contribute to both tissues (19, 21, 22). A recent study from our lab suggests that *wnt16* exerts dual influence on bone and muscle morphogenesis in zebrafish (23). This brings forth important questions regarding the role of *WNT16* in contributing to pleiotropy at the *CPED1-WNT16* locus, and whether its influence on muscle may help to explain effects on BMD and/or fracture, the latter of which can only partially be explained by BMD (5).

The aim of the review is to highlight experimental studies examining the function of genes at the *CPED1-WNT16* locus, as well as genomic and related studies supporting BMD variants at the locus to act on these genes (Fig 1B). In doing so, we examine three questions: What is the evidence that *WNT16* contributes to genetic effects at 7q31.31? Might genes other than *WNT16* contribute to genetic effects at 7q31.31? Finally, could the influence of *WNT16* on muscle contribute to genetic effects at 7q31.31 on BMD and fracture? Rather than a single biological mechanism, multiple mechanisms may work in tandem to contribute to the spectrum of musculoskeletal phenotypes mapped to the *CPED1-WNT16* locus (Fig 1C).

DOES *WNT16* CONTRIBUTE TO GENETIC EFFECTS AT 7Q31.31?

In this section, we review experimental studies examining the function of *Wnt16* in bone, with a specific focus on mouse studies. For a review of studies examining the relationship between WNT16, WNT signaling, and skeletal homeostasis, the reader is referred to (24).

Wnt16 is necessary for bone mass and strength

WNT16 is a member of the *WNT* gene family encoding secreted signaling molecules that have been implicated in a variety of disease processes, and which have important functions during embryonic development including regulation of proliferation, cell fate, and patterning. The necessity of *Wnt16* for bone mass and strength in mouse was initially established through a series of loss-of-function studies (24). Results were first reported in GWAS that identified BMD associations at the *CPED1-WNT16* locus (6, 7). Analysis of 24-week-old *Wnt16* knockout (KO) mice exhibited reduced total body areal BMD (aBMD), resulting from both reduced total body bone mineral content (BMC) and bone area (6). *Wnt16* KO mice also exhibited reduced cortical cross-sectional area and thickness at the femur midshaft, and reduced bone strength at both the femur and tibia (7).

Loss of *Wnt16* differentially reduces cortical rather than trabecular bone

A subsequent study by Movérare-Skrtic et al. examined underlying mechanisms with respect to the influence of *Wnt16* on cortical bone homeostasis (13). *Wnt16* KO mice had a reduction in cortical cross-sectional area, cortical thickness, and cortical BMC in the long bones compared to the wild-type (WT) mice. Mutant phenotypes were apparent across a range of ages (5-12 weeks of age), suggesting that WNT16 plays a role during skeletal development and maturity. Trabecular bone volume fraction in the distal femur and vertebrae were not significantly altered. Approximately one-third of *Wnt16* KO mice

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exhibited spontaneous tibial fractures. Interestingly, Movérare-Skrtic et al. showed a tibial fracture in a *Wnt16* KO embryo (13), suggesting that some spontaneous fractures can occur before significant weight bearing. Moreover, reduced cortical bone mass and increased fragility in *Wnt16* KO mice was phenocopied in *Wnt16* conditional KO mice in which *Wnt16* was inactivated in the early osteoblast lineage. Finally, addition of recombinant WNT16 suppressed differentiation of osteoclast cultures and increased osteoprotegrin (Opg) expression in osteoblast cultures. Taken together, these studies indicate that inactivation of *Wnt16* reduces cortical but not trabecular bone mass, and support a function of osteoblast-derived WNT16 in suppressing osteoclastogenesis (13).

Does *Wnt16* influence bone resorption and formation?

While some studies indicate that *WNT16* inactivation reduces bone mass and strength primarily by increasing bone resorption (13), others suggest that this also occurs through reduced bone formation (25). In this context, Wergedal et al. found evidence suggesting WNT16 may influence bone size during post-natal growth by positively regulating periosteal bone expansion. In comparison to wildtype siblings, the tibias of *Wnt16* KO mice exhibited reduced cross-sectional area, cortical thickness, and volumetric BMD at 12 weeks of age, with no statistically significant change in marrow area or bone length. In addition, long bone periosteal BFR, MAR, and BFS were reduced by 55%, 32%, and 10%, respectively, whereas TRAP-labeled periosteal surfaces (indicating the presence of osteoclasts) were greater in *Wnt16* KO mice than in wildtype littermates. Moreover, after two weeks of mechanical loading by four-point bending, no measurable increases in tissue volume, tissue cross-sectional area, or periosteal BFR were detected in the loaded tibias of *Wnt16* KO mice as compared to the unloaded contralateral control tibias. These studies suggest that WNT16 may influence cortical bone growth in long bones in part by promoting osteoblast activity and inhibiting osteoclast activity.

WNT16 overexpression increases cortical and trabecular bone mass

Interestingly, whereas inactivation of *Wnt16* specifically reduces cortical rather than trabecular bone mass, multiple studies have shown that overexpression of WNT16 increases cortical and trabecular bone mass. Movérare-Skrtic et al. showed that mice with osteoblast-specific WNT16 overexpression (Obl-Wnt16) displayed a substantial increase in trabecular bone mass, whereas there was only a nonsignificant tendency of increased cortical bone thickness in Obl-Wnt16 mice (26). Additional studies from the Econs lab demonstrated that osteoblast-specific (27) and osteocyte-specific (28) overexpression of human WNT16 increased both cortical and trabecular bone mass and structure in mice. Interestingly, in the studies of Movérare-Skrtic et al., adult (16-week-old) Obl-Wnt16 mice displayed a substantial increase in trabecular bone mass in the absence of significant alterations of bone resorption or bone formation markers (26). One possible explanation is that consequences of WNT16 overexpression on bone formation or resorption occurs prior to the stage of assessment, after which a new steady state for bone turnover is reached (26). A better understanding of the mechanism by which WNT16 overexpression increases bone mass is needed to rationally design therapeutic strategies based on targeting WNT16, and could help explain why osteoblast overexpression of WNT16 was insufficient to prevent bone loss in mice due to glucocorticoid treatment (29) or ovariectomy (19).

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BMD variants may regulate *WNT16* transcription or translation

While the above studies examining *Wnt16* biology support a role for *WNT16* in mediating genetic effects at 7q31.31 on BMD and fracture, causal variants have not been unequivocally identified. A recent study by Chesi et al. generated ATAC-seq and Capture C data sets in mesenchymal stem cell-derived osteoblasts, and used these data to link GWAS variants to gene promoters relevant to BMD loci (17). The authors were able to identify BMD relevant variants that overlapped with open chromatin regions, and determined their interactions with regions of open chromatin and open gene promoters. As part of these studies, Chesi et al. found evidence that two BMD SNPs (rs142005327 and rs2908004, in LD with lead SNP rs3801387) reside in the promotor for *WNT16* and therefore could regulate *WNT16* expression (17). In a different study, Zheng et al. identified a rare variant associated with BMD that appeared to affect DNA accessibility at the *WNT16* promoter (30). These observations support the notion that BMD variants residing in the *WNT16* promoter region could act to alter *WNT16* gene expression, however functional investigations to test this possibility are needed.

Another possibility is that BMD variants regulate WNT16 translation. Hendrickx et al. examined rs55710688, an insertion (–/CCCA) polymorphism that influences the Kozak sequence in the UTR of exon 1 of a WNT16 transcript (NM_016087.2) (10). In a cell-free transcription/translation experiment, this G to C substitution was associated with increased translation efficiency. Based on this work, alterations in BMD could be induced by variation in the Kozak sequence, which leads to abnormal WNT16 translation efficiency.

Summary

In summary, there are multiple lines of evidence that *WNT16* is a bona fide target gene at 7q31.31. Multiple *in vivo* studies indicate that *Wnt16* is necessary for bone mass and strength. Moreover, this influence is apparent in young mice (13, 25), consistent with human genetic studies indicating that genetic effects at the *CPED1-WNT16* locus on BMD are detectable in pediatric populations and thus act early in life (6). Finally, BMD variants have been found in the promoter for WNT16 and have been linked to WNT16 translation efficiency, putting forth testable hypotheses about how genetic variants at 7q31.31 act through WNT16 to influence BMD and fracture.

DO GENES OTHER THAN *WNT16* CONTRIBUTE TO GENETIC EFFECTS AT 7Q31.31?

While a large body of studies has focused on examining the function of *Wnt16* in bone and potential causal variants that might influence *WNT16* expression and/or function, a parallel body of studies has focused on elucidating the biological mechanism underlying the secondary signal at the *CPED1-WNT16 locus*, and whether it is driven by a second gene in the same region. We next review evidence of the potential for four other genes at the 7q31.31 locus with the potential to act as target genes influencing BMD and fracture: *FAM3C* (16), *ING3* (17), *CPED1* (15), and *TSPAN12* (14).

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FAM3C

FAM3C (family with sequence similarity 3, C) belongs to the FAM3 superfamily, which is composed of four members: FAM3A, FAM3B, FAM3C and FAM3D. The encoded protein for *FAM3C* is a predicted secreted factor and has previously been shown to be involved in epithelial-mesenchymal transition (EMT) (31, 32), tumor formation (31), as well as retinal laminar formation (33).

Several loss-of-function studies in *Fam3c* KO mice have been reported (7, 16). In one study, Määttä et al. found that inactivation of *Fam3c* via gene trapping yielded changes in bone morphology and strength. (16). Female *Fam3c* KO mice exhibited reduced trabecular bone volume fraction and increased cortical BMD at 7 months of age. Male *Fam3c* KO mice also exhibited reduced trabecular bone volume fraction and increased cortical BMD at 3 months of age, however at 7 months of age, trabecular bone volume in male *Fam3c* KO mice was increased compared to controls. Three-point bending of tibial shafts revealed that in both sexes the break-point force was significantly reduced in three-month-old KO mice when compared with WT mice. Additionally, bone marrow cultures from *Fam3c* KO mice exhibited accelerated osteogenic differentiation and mineralization *in vitro*. In a follow up study, the same group showed that in differentiating osteoblasts, *Fam3c* knock down resulted in increased alkaline phosphatase expression, whereas overexpression reduced it (34). These findings support the involvement of *FAM3C* in regulating bone mass and strength.

Zheng et al. also reported results in which three separate knockout strategies were employed to inactivate mouse *Fam3c* (7). Using the DEXA and microCT analyses which identified reduced cortical bone thickness and bone strength in *Wnt16* KO mice, the authors failed to observe any skeletal phenotype changes for the three independent *Fam3c* mutant alleles. The sample size was relatively small for each of the cohorts (WT=2; *Fam3c* −/− = 4 for each of the individual cohorts) and thus it is possible that the studies were not sufficiently powered to detect phenotypic changes.

Functional investigations of candidate BMD SNPs that might target *FAM3C* to alter its gene expression or function of the encoded protein have not been reported.

ING3

ING3 is a member of the inhibitor of growth (ING) family consisting of five proteins that have the ability to influence chromatin structure through the recruitment of histone acetyltransferase (HAT) or histone deacetylase (HDAC) protein complexes to methylated lysine residues within nucleosomes (35). Prior studies suggest that *ING3* functions as a transcriptional activator because of its role within the NuA4-Tip60 MYST-HAT complex, which acetylates histones H2A and H4 (35, 36).

Loss-of-function studies in mice have shed light on the developmental function of *Ing3*. Homozygous mutant mice with an insertional mutation in the endogenous *Ing3* locus were embryonic lethal and exhibited severe developmental disorders (37). At embryonic day (E) 10.5, the last time point that homozygous embryos were viable, *Ing3* KO mice were approximately half the size of heterozygous mice (37). Additionally, homozygous mice exhibited developmental defects associated with the prosencephalon (37). These findings

suggest that *Ing3* may also play an important role in embryonic development, specifically of the brain (37).

Recent studies indicate that *ING3* is necessary for osteoblast differentiation. Chesi et al. performed functional studies by knocking down genes at the *CPED1-WNT16* locus in primary human mesenchymal stem cells (17). *ING3* knockdown cells displayed reduced osteoblast differentiation, as indicated by reduced ALP expression and loss of calcium phosphate mineral deposition, and enhanced adipogenic differentiation as shown by aggregation of Oil red O staining and higher expression of the adipogenic transcription factor *C/EBP alpha*. These data suggest *ING3* plays a role in human mesenchymal stem cell differentiation.

There is some evidence that BMD SNPs could regulate *ING3* gene expression. Specifically, using a combination of ATAC-seq and Capture C data in mesenchymal stem cell-derived osteoblasts, Chesi et al. detected interactions between a region containing SNPs rs1861000 and rs3068006 (in LD with the BMD DEXA lead SNP rs13245690) and the *ING3* promoter (17). Additional studies examining whether these SNPs are functional in regulating *ING3* gene expression are warranted. Moreover, further *in vivo* studies are needed to better understand the specific function of *Ing3* in the skeleton.

CPED1

CPED1 (Cadherin-like and PC esterase domain containing 1; also known as *C7orf58*) encodes a protein whose function remains largely unknown. In humans, *CPED1* contains several structural motifs with predicted function: an N-terminal signal for secretion, a cadherin-like domain for membrane localization, and a PC esterase domain (15, 38).

Cped1 appears to be broadly expressed in variety of tissues including in bone. Maynard et al. provide insight into the expression patterns of *Cped1* (15). Using murine models, multiple promoter regions were discovered, and two alternative splicing events were identified with one splice variant skipping exon 3 and the other skipping exons 16 and 17. Exon quantity was found to be variable and dependent on time of osteoblast differentiation. *Cped1* was also found to be uniformly expressed in mouse organs and tissues, but was absent in the monocyte/macrophage RAW264.7 cell line and in circulating leukocytes in the blood. As a result, the authors hypothesized that *Cped1* is expressed in cells that reside in extracellular matrix, whereas circulating cells lack *Cped1* expression. Loss-of-function studies for *Cped1* mouse models and studies examining the specific functions of different *Cped1* transcripts are warranted.

Further evidence in support of a function of *CPED1* in regulating bone comes from prior studies indicating a significant inverse relationship between *CPED1* expression and BMD. Medina-Gomez et al. examined transcript levels derived from iliac bone crest biopsies and their relation to BMD levels in the same individuals (6). Expression levels for a *CPED1* transcript were inversely correlated with total body BMD and skull BMD, suggesting that higher BMD is related to lower expression levels of this *CPED1* transcript.

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Recent studies suggest that BMD SNPs could interact with the *CPED1* promotor. Specifically, Chesi et al. found the *CPED1* promoter interacts with a region containing proxies (rs1861000 and rs3068006) of the BMD lead SNP rs13245690 in mesenchymal stem cell-derived osteoblasts (17). Further studies are needed to test whether these SNPs are functional in regulating *CPED1* gene expression.

TSPAN12

TSPAN12 (Tetraspanin 12) is a member of the tetraspanins, a family of proteins with four transmembrane domains. Many tetraspanins are believed to have a role in cell and membrane compartmentalization due to their interaction with specific proteins and other tetraspanins (39). TSPAN12 forms a complex with FZD4 (Frizzled 4), Norrin (a high affinity ligand for FZD4 that is structurally unrelated to WNTs), and LRP5 (a coreceptor of FZD4). This complex promotes the accumulation of β-catenin to activate LEF/TCF-mediated transcription (40). Junge et al. showed that TSPAN12 specifically regulates Norrin/β-catenin but not Wnt/β-catenin signaling by modulating FZD4 multimerization (40). Because LRP5 functions to regulate bone mass, as well as the fact that TSPAN12 has the ability to interact with LRP5, *TSPAN12* has been suggested as a plausible candidate target gene at the 7q31.31 BMD locus (14). However, while the importance of Wnt/β-catenin signaling in bone has been established, the role of Norrin/β-catenin signaling is less well defined. Thus, further studies are needed to determine the function of Norrin/β-catenin signaling in bone and its relationship to TSPAN12.

Summary

In summary, there is evidence that BMD variants could alter the expression of multiple genes at 7q31.31, and some of these genes have been found to be important for bone morphology and strength *in vivo* (*Fam3c*) and/or osteoblast differentiation *in vitro* (*Fam3c, Ing3*). However, given the lack of *in vivo* loss-of-function studies for some of these genes (*Ing3* and *Cped1*), as well as the fact that the bone phenotypic consequences of gene inactivation in mouse are less severe compared to *Wnt16* (at least for *Fam3c*), the strength of evidence supporting their roles as target genes remains somewhat modest. *In vivo* studies examining the physiological consequences of loss of *Cped1* and *Ing3* on bone morphology and strength, as well as functional investigations of candidate causal variants and their impact on *ING3, CPED1,* and *FAM3C* gene expression, will help to resolve whether the secondary signal at 7q31.31 is due to true allelic heterogeneity whereby multiple alleles act through the same target gene, or if it is driven by a second gene at the locus or even in a distant genomic region.

DOES THE INFLUENCE OF *WNT16* ON MUSCLE CONTRIBUTE TO GENETIC EFFECTS AT 7Q31.31?

Muscle is an organ with a strong influence on bone mass and strength. While coupling between muscle and bone has historically focused on mechanical interactions, it is becoming increasingly appreciated that interactions between these two tissues are much more complex. Such interactions include shared development from somites during embryonic development, as well as paracrine crosstalk and endocrine interactions (41, 42). Here, we review evidence

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supporting the potential for pleiotropic genetic variants at 7q31.31 to act through *WNT16* to influence bone and lean mass.

Variants at the *CPED1-WNT16* locus are associated with pleiotropic effects on BMD and lean mass

Evidence of pleiotropic variants at the *CPED1-WNT16* locus associated with BMD and lean mass (a clinical correlate of skeletal muscle mass) was first identified by Medina-Gomez et al., who performed a bivariate GWAS meta-analysis of total-body lean mass (TB-LM) and total-body less head bone mineral density (TBLH-BMD) regions in 10,414 children (19). The authors identified eight loci harboring variants with pleiotropic effects: *WNT4, GALNT3, MEPE, CPED1/WNT16, TNFSF11, RIN3, PPP6R3/LRP5, and TOM1L2/SREBF1*. The lead variant amongst these eight loci, the *CPED1-WNT16* locus, was the most significantly associated with TB-LM and TBLH-BMD in the bivariate analysis. Importantly, variants were identified to be associated with BMD and lean mass in a pediatric population, highlighting that they operate early in life.

Recently, Peng et al. provided additional evidence supporting the genetic effects at 7q31.31 on lean mass (43). These authors used the conditional false discovery rate (cFDR) methodology to perform a combined analysis of the summary statistics of two large independent GWAS of appendicular lean mass (ALM) and BMD. A total of 156 potential ALM-associated SNPs mapping to 15 chromosomes were identified by cFDR; the top three most significantly associated SNPs were at the *CPED1-WNT16* locus and considered to be replication of the findings of Medina-Gomez et al.

Expression of *WNT16* is correlated with BMD and lean mass

Additional support for a pleiotropic influence of *WNT16* comes from earlier studies by Medina-Gomez et al., which examined transcript levels derived from iliac bone crest biopsies and their relation to BMD and lean mass in the same individuals (6). Expression levels for a *WNT16* transcript was significantly associated with BMD measured for the total body as well as for the skull, legs, total hip, and lumbar spine. In addition, the authors observed a significant correlation with total body lean mass (TBLM, r2 = 0.31) for the *WNT16* transcript. This correlation was of similar magnitude as those for BMD (ranging between 0.25 and 0.31 across several skeletal sites including the total body, skull, legs, total hip and lumbar spine). This suggests that higher expression of *WNT16* is correlated with higher TBLM and BMD. Moreover, the positive relationship between expression of *WNT16* and BMD is consistent with findings from the *Wnt16* KO mice studies.

Wnt16 exerts pleiotropic effects on bone and lean mass in zebrafish

It has been hypothesized that genetic variants with pleiotropic effects on muscle- and bone-related traits might act during the development and growth of both tissues (19, 21, 22). Somites are blocks of paraxial mesoderm that, in vertebrates, divide into dermomyotome and sclerotome--embryonic structures that give rise to skeletal muscle and vertebrae/ribs, respectively. Spine development requires an interaction between the sclerotome and notochord; in zebrafish, the notochord sheath undergoes direct mineralization to form mineralized domains. These mineralized domains form a template for subsequent

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recruitment of sclerotome-derived osteoblasts and formation of mature vertebral bodies (44). A prior study in zebrafish examining the role of *wnt16* in hematopoietic stem cell development showed that it is expressed in developing somites (45), however, the specific somitic compartment (e.g., dermomyotome or sclerotome) was not reported. Recently, our lab examined the function of *wnt16* in spine and muscle development in zebrafish (23). These studies have shed light on a dual role of *wnt16* in influencing spine and muscle morphogenesis, and support the potential for *WNT16* to underlie bone and muscle pleiotropy at the *CPED1-WNT16* locus (23).

We found that *wnt16* signals in parallel in structures adjacent to developing muscle (dermomyotome) and bone (notochord), where it influences the morphogenesis of each tissue. In regard to its expression in developing muscle, we found that *wnt16* is enriched in a dermomyotome-like cell population that co-localizes with cells expressing *pax7a*, a marker of myogenic precursors. In regard to notochord, we found that expression of *wnt16* was not uniformly expressed along the anterior-posterior axis at 22 hours post fertilization, suggesting that *wnt16* expression may propagate in an anterior-to-posterior wave during notochord development as has been seen for *wnt11* (23, 46). At 12 days post fertilization, a time point in which vertebral mineralization has initiated, expression of *wnt16* in the notochord was restricted to cells in the ventral midline of the notochord sheath.

By examining *wnt16* loss-of-function mutants, we found that *wnt16* is necessary for both muscle and spine development. In regard to muscle, at 3 days post fertilization, $wnt16^{-/-}$ mutants exhibited significantly altered myotome morphology. Myotome angle and height were increased in $wnt16^{-/-}$ mutants, while myotome length was reduced. The myotomes in $wnt16^{-/-}$ mutants also exhibited increased elongation along the dorsal-ventral axes and expansion in cross-sectional area. In regard to notochord, $wnt16^{-/-}$ mutant embryos had reduced notochord length and cross-sectional area, indicating that *wnt16* is necessary for notochord elongation and radial expansion. At approximately two weeks post fertilization, $wnt16^{-/-}$ mutant larvae exhibited decreased vertebral size and altered vertebral body shape, appearing more trapezoid-like, indicating that *wnt16* is necessary for notochord sheath mineralization and morphogenesis. Furthermore, $wnt16^{-/-}$ mutant larvae exhibited altered recruitment of osteoblasts to developing vertebrae. Osteoclast formation in $wnt16^{-/-}$ mutant larvae was not obviously affected, suggesting that altered bone morphology was not a consequence of loss of suppression of osteoclastogenesis by Wnt16—the mechanism linked to cortical bone defects in *Wnt16* KO mice (7).

Variants at the *CPED1-WNT16* locus are associated with BMD in both pediatric and adult populations (6). In this context, we showed that morphological abnormalities in muscle and bone in $wnt16^{-/-}$ mutant larvae were mirrored in adults. At 90-days post-fertilization adults, $wnt16^{-/-}$ mutants exhibited reduced centrum volume, increased tissue mineral density, and reduced standard length. Additionally, $wnt16^{-/-}$ mutants exhibited altered myomere shape and size, decreased posterior trunk lean volume, reduced swim bladder lengths, and reduced fineness ratios. Thus, *wnt16* impacts muscle and bone morphology throughout the life course in zebrafish.

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Finally, in order to assess the potential for genes other than *WNT16* to act as causal genes underlying pleiotropic effects on BMD and lean mass, we conducted a reverse genetic screen of genes at 7q31.31. We generated somatic mutants for five genes at the locus: *tspan12, cped1, ing3, fam3c*, and *wnt16*. Somatic mutants for *wnt16* exhibited the most severe alterations in lean tissue mass and morphology. Moreover, germline mutants for *cped1* showed no significant differences in lean tissue mass or morphology. These data suggest that *WNT16* is a gene of major effect on lean mass at the *CPED1-WNT16* locus.

Summary

In summary, there is accumulating genetic evidence that variants at the *CPED1-WNT16* locus have pleiotropic effects on BMD and lean mass. Moreover, recent studies in zebrafish have revealed a function of *wnt16* during muscle and spine morphogenesis. In mouse, *Wnt16* is expressed in somites similar to zebrafish (23). Moreover, *Wnt16* was found to be differentially upregulated in mouse notochord-derived cells during their transition from notochord to nucleus pulposus (47). Thus, it is conceivable that the functions of *wnt16* in muscle and spine morphogenesis could have parallel functions in rodents. Future studies examining this possibility are warranted.

CONCLUSIONS

Since the initial GWAS identifying the 7q31.31 BMD locus and mouse studies showing that *Wnt16* is necessary for bone mass and strength, notable progress has been made in elucidating the underlying biological mechanism. Subsequent experimental studies have mostly reinforced the notion that *WNT16* is a bona fide target gene at the locus. In addition, functions for *Fam3c* and *Ing3* in regulating bone morphology *in vivo* and/or osteoblast differentiation *in vitro* have been discovered, and testable hypotheses have been formed proposing how BMD variants may regulate the expression of genes other than *WNT16*. Finally, an important morphogenetic function for Wnt16 in influencing bone and muscle during embryonic and larval development has recently been discovered. This has brought forth new questions related to the potential for BMD variants to act through developmental processes that affect lifelong bone and muscle mass, and whether the influence of *WNT16* on muscle may conspire with its influence in bone homeostasis to alter fracture risk. It has also brought forth new questions related to whether the multiple independent signals at 7q31.31 originate through contributions of *WNT16* to different biological mechanisms, rather than different causal genes. A better understanding of the function of variants and genes at the locus should help to reveal the identification of causal variant(s) at the locus, how these variants influence target gene function, and how altered gene function is translated into altered lean mass, BMD, and fracture risk.

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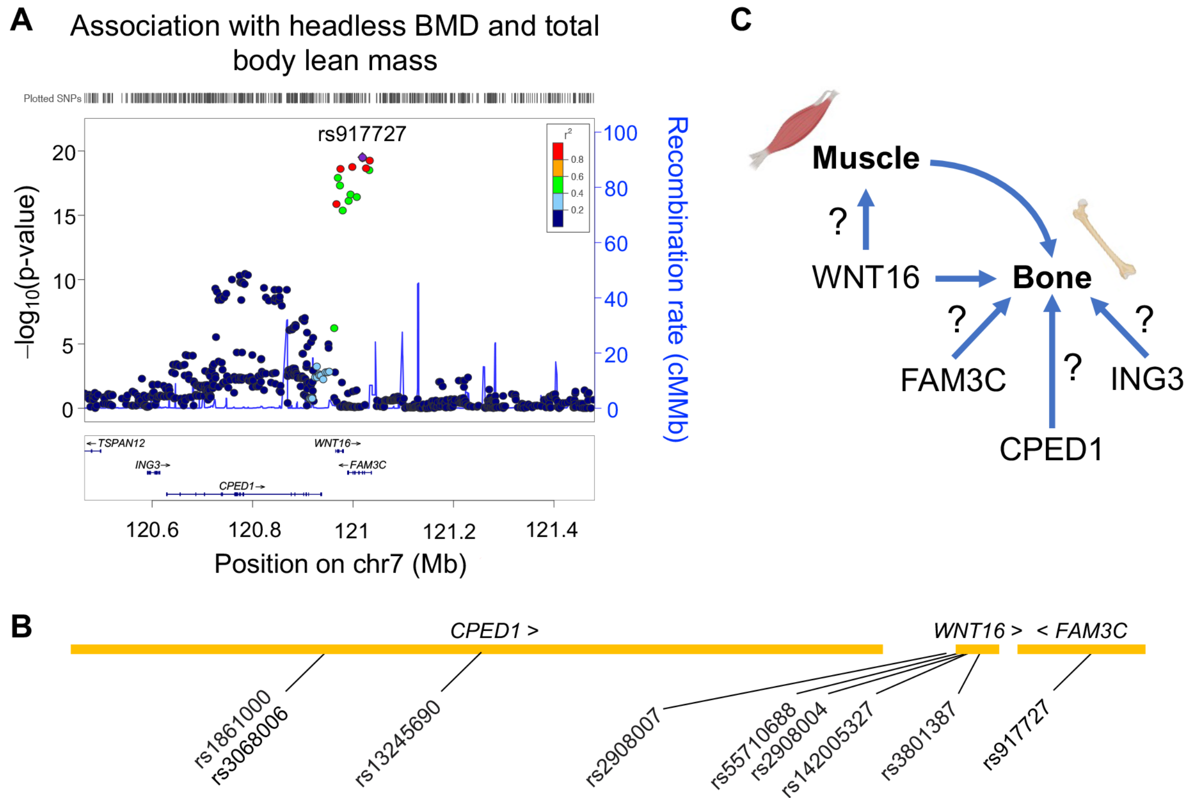

Fig 1. Multiple mechanisms may work in tandem to contribute to the spectrum of musculoskeletal phenotypes mapped to the *CPED1-WNT16* locus.

(A) Regional association plot for headless BMD and total body lean mass at the 7q31.31 locus. Data are from the analysis of (19). Lead SNP rs917727 is represented by the purple diamond. (B) Location of variants related to bone-related traits reviewed in this manuscript. (C) Schematic depicting genes at 7q31.31 with potential influence on bone- and muscle-related traits. Question marks represent relationships in which there is some supporting evidence but for which consensus has not been established. Fig 1 adapts portions of Fig 9 from the following paper: Watson et al., (2022). *wnt16* regulates spine and muscle morphogenesis through parallel signals from notochord and dermomyotome. PLoS Genetics 18:e1010496. The paper of Watson et al., which was published in PLoS Genetics, applies the Creative Commons Attribution 4.0 International (CC BY) license (https://journals.plos.org/plosgenetics/s/licenses-and-copyright). Fig 1C uses images created with Biorender.com.