**Bicc1 is a genetic determinant of osteoblastogenesis and bone mineral density**

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Patient bone mineral density (BMD) predicts the likelihood of osteoporotic fracture. While substantial progress has been made toward elucidating the genetic determinants of BMD, our understanding of the factors involved remains incomplete. Here, using a systems genetics approach in the mouse, we predicted that bicaudal C homolog 1 (Bicc1), which encodes an RNA-binding protein, is responsible for a BMD quantitative trait locus (QTL) located on murine chromosome 10. Consistent with this prediction, mice heterozygous for a null allele of Bicc1 had low BMD. We used a coexpression network–based approach to determine how Bicc1 influences BMD. Based on this analysis, we inferred that Bicc1 was involved in osteoblast differentiation and that polycystic kidney disease 2 (Pkd2) was a downstream target of Bicc1. Knock down of Bicc1 and Pkd2 impaired osteoblastogenesis, and Bicc1 deficiency–dependent osteoblast defects were rescued by Pkd2 overexpression. Last, in 2 human BMD genome-wide association (GWAS) meta-analyses, we identified SNPs in BICC1 and PKD2 that were associated with BMD. These results, in both mice and humans, identify Bicc1 as a genetic determinant of osteoblastogenesis and BMD and suggest that it does so by regulating Pkd2 transcript levels.

Introduction

Osteoporosis is a disease characterized by low bone mass, skeletal fragility, and increased risk of fracture (1). Of the traits intrinsic to bone that influence its strength, bone mineral density (BMD) is one of the strongest predictors of fractures (2). BMD is also highly heritable, with approximately 70% of its variation being attributable to genetic factors. As a result, developing a comprehensive understanding of the genes and pathways that regulate BMD promises to lead to novel therapies aimed at preventing and treating bone fragility.

Genome-wide association studies (GWAS) have significantly expanded the list of known variants and genes that influence BMD (3). A recent meta-analysis of 17 BMD GWAS (Genetic Effects For Osteoporosis [GEFOS] Consortium) involving approximately 83,000 individuals identified 56 robustly significant associations (4). Interestingly, together, the associations explained less than 5% of the total phenotypic variance in BMD, suggesting that bone mass is highly polygenic and that most of the genes influencing BMD remain to be identified.

One strategy that can help fill this knowledge gap is the use of gene discovery in the mouse to inform human BMD GWAS. Several recent studies (5–10) have demonstrated the effectiveness of this strategy. To date, dozens of quantitative trait loci (QTLs) (regions of the genome harboring genetic variation influencing a quantitative trait) affecting BMD have been identified in the mouse (11), and recently the pace of identifying QTL genes has rapidly progressed. This is due in part to the development of systems genetics approaches that integrate global gene expression data into genetics studies (12–14). Systems genetics studies seek to identify genetic variation that impacts gene expression and then use this information to more rapidly identify the subset of variation that contributes to disease (12). For example, by generating global gene expression profiles in a genetic-mapping population, genes whose transcript levels are influenced by local or distant expression QTLs (eQTLs) can be identified on a genome-wide scale (15, 16). Subsequent analyses using tools such as causality modeling can then define the subset of eQTLs that are likely drivers of phenotypic differences (17, 18).

Coexpression network analysis is a distinct systems genetics approach that partitions genes into groups or modules of genes based on coexpression across a set of perturbations, such as differing genetic backgrounds. Genes that are coexpressed often have similar functions or operate in the same pathways (9, 19–21). Thus, it is possible to use coexpression networks to predict the function of novel complex disease genes based on their connections with genes of known function in a network, an approach referred to as “guilty-by-association” (9, 22–24). As a result, a systems genetics analysis that combines eQTL discovery, causality modeling, and coexpression networks can enhance gene discovery as well as provide mechanistic insight into how novel genes influence complex traits, an important step in translating genetic discoveries into an improved understanding of disease biology.

We recently described the genome-wide identification of femoral BMD QTLs in a C57BL/6J x C3H/HeJ (BXH) F2 cross and the identification of candidate genes using eQTL analysis and causality modeling (25). Here, we extend these initial observations and identify bicaudal C homolog 1 (Bicc1) as the gene
Table 1

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* r, Pearson’s correlation between gene expression and femoral BMD.

**Results**

**Bmd43 is a novel QTL.** We previously mapped 9 femoral BMD QTLs in a BXH F2 cross (25). One of the 9, Bmd42, was located at 45.8 cM (104.3 Mbp) on mouse chromosome (Chr) 10. Upon closer inspection of the Chr 10 locus, it appeared that there were at least 2 distinct QTLs, Bmd42 and a second at approximately 28 cM (~65 Mbp) (Figure 1A). Analysis of the linkage data using a 2-QTL model confirmed the independence of the second QTL, which we refer to as Bmd43 (Bmd43, P = 0.005; Bmd43, P = 0.05). Bmd42 and Bmd43 affected BMD only in male F2 mice (Figure 1A). C57BL/6J (B6) alleles at both loci increased femoral BMD relative to C3H/HeJ (C3H) alleles (Figure 1, B and C). B6 alleles at Bmd42 acted to increase BMD in a dominant manner, whereas additive inheritance was observed at Bmd43 (Figure 1, B and C). Bmd43 explained 6.5% of variance, and Bmd43 explained 3.5% of the variance in femoral BMD in males.

A local Bic1 eQTL is predicted to be causal for Bmd43. We used microarray expression data generated on each individual BXH mouse to identify genes overlapping Bmd42 and Bmd43 whose expression correlated with femoral BMD and was regulated by a local eQTL. Expression data on bone were not collected in the BXH F2 mice (25). We previously mapped 9 femoral BMD loci. Of the 109 genes located within the support interval for Bmd42 and Bmd43 that are regulated by a local eQTL (lod ≤2.0) and correlated (P ≤ 0.05) with BMD in BXH F2 male mice, independent of Bmd43 genotype (Figure 1G).

Network edge orienting (NEO) (8), a causality modeling algorithm, was used to predict whether the relationship between each candidate gene and femoral BMD was causal (gene expression caused a change in BMD), reactive (BMD caused the change in gene expression), or independent (no functional connection between the gene and BMD). Of the 9 candidates, only Bicc1 was predicted to be causal for BMD (see Methods) (Table 1 and Figure 1H). Sequencing of the region surrounding the Bic1 microarray probe in B6 and C3H mice did not identify SNPs, excluding the possibility that the Bic1 local eQTL and causality results were due to C3H SNPs overlapping the array probe and altering hybridization to the C3H allele (data not shown).

In addition to adipose, Bic1 was regulated by the same local eQTL in BXH liver, brain, and muscle tissues (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI73072DS1). To determine whether the same local eQTL regulated Bic1 in bone, we used microarray data on bone from the recently described Hybrid Mouse Diversity Panel (HMDP) (9, 27). Bic1 bone expression and 86 SNPs flanking Bic1 in the HMDP were tested for association. Multiple SNPs upstream of Bic1 were significantly (adjusted P > 5.8 x 10⁻⁴) associated with its expression (Figure 1I). As seen in BXH adipose tissue, HMDP strains homozygous for B6 alleles of these SNPs had higher Bic1 expression levels (Figure 1J). Together, these data suggest that genetically regulated differences in the expression of Bic1 are responsible for the effects of Bmd43.

Reduction in Bic1 expression in vitro results in decreased femoral BMD. BIC1 is an RNA-binding protein that is involved in kidney development (28–30). In mice and humans, mutations in Bic1 lead to polycystic kidney disease (PKD) and cystic renal dysplasia, respectively (31, 32). Bic1 has not been previously implicated in bone development. To confirm that Bic1 was responsible for Bmd43, we measured bone mass in mice carrying the juvenile congenital polycystic kidney disease (jcpk) allele of Bic1 (Bic1jcpk). The Bic1jcpk allele is the result of a chlorambucil-induced point mutation in the splice acceptor site of exon 3 (31, 33). This mutation causes exon 3 to be skipped, resulting in a frame shift, the creation of a premature stop codon, and a null Bic1 allele (31). Homozygous Bic1jcpk/jcpk mice develop severe early-onset PKD and die shortly after birth (31). In contrast, young heterozygous Bic1jcpk/+ mice are free of any gross abnormalities (34). Since the difference in Bic1 expression as a function of F2 genotype in the BXH was approximately 50% (Figure 1E), we reasoned that Bic1jcpk/+ mice were an excellent model to validate the causal relationship between Bic1 dosage and BMD. Bic1jcpk/+ mice and wild-type littermates (Bic1 +/+ ) of both sexes (n = 8–15/genotype/sex) were placed on a high-fat diet at 8 weeks of age to replicate the diet used in the BXH F2 cross above, and femoral BMD was measured at 12, 16, and 28 weeks. At 12 and 16 weeks of age, there was no difference in femoral BMD (Figure 2A). However, at 28 weeks of age, a significant (P = 0.001) difference in femoral BMD was observed in male Bic1jcpk/+ mice (Figure 2B). In males, the increase in femoral BMD from 12 to 28 weeks was lower in Bic1jcpk/+ mice (Figure 2C). Additionally, there were no differences in lumbar spine BMD at any time point in either sex (data not shown). Micro-CT analysis of cortical bone.
parameters at the femoral diaphysis in 28-week-old male Bicc1+/jcpk mice revealed no differences in total area (Tt Ar), bone area (Ct Ar), medullary area (Ma Ar), or bone area fraction (Ct Ar/Tt Ar) (Figure 2, D–G). However, cortical thickness (Ct Th) (Figure 2H) and cortical tissue mineral density (TMD) were significantly (P < 0.05) decreased in Bicc1+/jcpk males (Figure 2I). In the trabecular bone compartment, there was a suggestive (P = 0.10) decrease in bone volume fraction (BV/TV) (Figure 2J). This was primarily a function of a decrease (P < 0.05) in trabecular thickness (Tb Th) (Figure 2, K–M). There were also no differences (P > 0.05) in body weight or adiposity that would explain the difference in BMD (Supplemental Figure 2). We did not observe any gross kidney pathologies or increases in kidney size in Bicc1+/jcpk male mice, nor were there differences in serum markers of kidney function (Supplemental Figure 2). The male specificity and magnitude of the BMD difference in Bicc1+/jcpk males (7.4% difference in BMD between F2 genotypes at Bmd43...
ossification \( P = 4.6 \times 10^{-12} \), bone mineralization \( P = 1.5 \times 10^{-5} \), and osteoblast differentiation \( P = 9.6 \times 10^{-7} \). It is also enriched in the homologs of genes implicated in the regulation of human BMD through GWAS \( P < 0.02 \) (Figure 3A and ref. 35). The M6 contained 21 genes that belonged to the gene ontology (GO) category “osteoblast differentiation.” We found that the connections (connectivity was defined by a topological overlap measure [TOM] described in Methods) between Bicc1 and the 21 M6 osteoblast differentiation genes were higher than its connection to all other M6 genes \( (P = 4.7 \times 10^{-5}) \) and to a random set of 910 network genes (equivalent to the total number of M6 genes) \( (P = 7.3 \times 10^{-9}) \) (Figure 3B).

Next, we measured the correlation between Bicc1 expression and the eigengene (a summarized measure of module behavior) for all 21 modules. As expected, Bicc1 was highly correlated with the M6 eigengene \( (r = 0.78; P < 2.2 \times 10^{-16}) \) (Figure 3C). Of the other 20 modules, Bicc1 was also highly correlated with the module 9 (M9) eigengene \( (r = 0.45; P = 2.9 \times 10^{-4}) \) (Figure 3D). This was not unexpected, because like the M6, we have demonstrated that the M9 is specific to cells of the osteoblast lineage (35). Based on its membership in the M6 and its association with the M9, we measured the expression of the 21 genes in the M6 and the 13 genes in the M9 with “osteoblast differentiation” GO annotations in the 15 HMDP strains that displayed the lowest and highest Bicc1 expression. The expression of 31 of the 34 genes was significantly \( (P < 0.05) \) increased in Bicc1 high-expressing strains (Figure 3E), suggesting a positive association between Bicc1 expression and osteoblastogenesis.

Bicc1 is a regulator of osteoblastogenesis. Microarray data from an extensive panel of mouse tissues and cell lines were queried to determine the tissue distribution of Bicc1 (Gene Expression Omnibus [GEO] accession number GSE10246) (http://www.biogps.org) (36, 37). Bicc1 was highly expressed in kidney tissue and in the mIMCD-3 cell line derived from kidney collecting ducts (Supplemental Figure 3), which was expected, based on the known role of Bicc1 in kidney development (31). However, of the 96 samples profiled, Bicc1 expression was highest in primary calvarial osteoblasts (Supplemental Figure 3).

[Figure 1C] and 7.7% difference in BMD between Bicc1 genotypes [Figure 2A]) are consistent with the Bicc1 local eQTL being solely responsible for Bmd43.

Bicc1 shares network connections with genes involved in osteoblast differentiation. A coexpression network generated using bone microarray expression data from 96 mouse inbred strains from the HMDP was queried to predict how Bicc1 affects BMD (27). The network consisted of 13,759 genes partitioned into 21 coexpression modules. Bicc1 was a member of module 6 (M6). M6 contained 910 unique genes, and we have demonstrated that it is enriched for genes (a complete list of M6 genes is provided in Supplemental Table 1) involved in skeletal system development \( (P = 3.0 \times 10^{-19}) \), BICC1 was not expressed in osteoclasts (Supplemental Figure 3).

In an independent study, we measured the expression of the two known isoforms of Bicc1, Bicc1-A, and Bicc1-B, which differ in the absence (Bicc1-A) or presence (Bicc1-B) of exon 21 (31) across a time course of differentiation in primary calvarial osteoblasts. The pattern of expression was similar for both transcripts and was rapidly induced by differentiation \( (P < 0.001 \) for both transcripts) (Figure 4A). At their peak (day 3), Bicc1-A and Bicc1-B were expressed at approximately 275% and 215%, respectively, of the control (undifferentiated) osteoblasts. Their expression decreased on day 4, then increased on day 6, and remained stable through day 14. Semiquantitative RT-PCR
Figure 3

Network analysis predicts that Bicc1 plays a role in osteoblast differentiation. Bicc1 is a member of M6 (A). The strongest (TOM >0.05) connections between M6 genes (nodes connected by lines) are displayed in A. The location of Bicc1 is highlighted and each node is colored based on the gene ontology annotations listed in the top left corner of A. Connections between Bicc1 and M6 genes involved in osteoblast differentiation (M6 osteoblast [OB] genes) were stronger than all other M6 genes and stronger than a random set of genes equivalent to the number of genes in the M6 (B). Bicc1 expression was highly correlated with the M6 (C) and M9 (D) module eigengenes. The expression levels of M6 and M9 osteoblast differentiation genes were higher in HMDP strains with high Bicc1 expression (red and green bars) (E). Data presented in B and E are the means ± SEM. *P < 0.05.
indicated that the overall levels of the 2 transcripts at each time point were approximately equivalent (data not shown).

We next measured markers of osteoblast differentiation in primary calvarial osteoblasts (pools of both sexes) from Bicc1+/+ and Bicc1+/- mice. After culturing in osteogenic media, we found that alkaline phosphatase activity levels and the formation of mineralized nodules were significantly \( (P < 0.05) \) decreased in Bicc1+/- osteoblasts (Figure 4, B and C). We also knocked down Bicc1 in B6 primary calvarial osteoblasts using two siRNAs, each of which targeted both isoforms, to confirm that the impairment in osteoblastogenesis in Bicc1+/- cells was due to cell-autonomous functions of Bicc1. The 2 siRNAs reduced Bicc1 expression by approximately 50% (Bicc1_1) and approximately 70% (Bicc1_2) relative to a scrambled control (SC) (Figure 4D). We observed significant \( (P < 0.05) \) decreases in alkaline phosphatase activity, expression of the osteogenic markers Runx2, Sp7, Col1a1, Bglap (osteocalcin), and Ibsp and mineralized nodule formation in osteoblasts transfected with Bicc1_1 and Bicc1_2 (Figure 4, E–H). These differences displayed a dose-dependent relationship congruent with the extent of Bicc1 knock down. Together, these data indicate that Bicc1 regulates osteoblast differentiation.

We also measured mineralized nodule formation in primary calvarial osteoblasts harvested from individual Bicc1+/+ and Bicc1+/- neonates of both sexes. Both male and female cells from Bicc1+/- mice formed fewer nodules, but there was no difference between the sexes (Figure 4I). Bicc1 expression (both isoforms) was decreased by approximately 25% in female osteoblasts, but this difference was not significant (Figure 4J).

Network analysis and causality modeling identify Pkd2 as a putative downstream target of Bicc1. We reasoned that as an RNA-binding protein, Bicc1 would function as a post-transcriptional regulator of gene expression in osteoblasts. Therefore, we set out to identify putative Bicc1 targets. We first identified the top 15 M6 network genes that were the most strongly connected to Bicc1 (Figure 5A). Next, gene expression...
Figure 5
Network analysis and causality modeling identify Pkd2 as a putative downstream target of Bicc1. Pkd2 was one of the top 15 M6 genes that was most strongly connected with Bicc1 (A). The connection strengths (TOMs, see Methods) are labeled for each Bicc1 gene connection. Pkd2 was the only gene identified from microarray analysis whose expression was significantly (FDR < 0.05) correlated with Bicc1 knock down (B). Bicc1 and Pkd2 expression were correlated in osteoblasts isolated from individual Bicc1+/jcpk and Bicc1+/+ mice of both sexes (C). Bicc1 and Pkd2 expression were correlated in the HMDP bone samples (D). Causality modeling predicted that Bicc1 is upstream of Pkd2 (E).

Microarrays were used to profile primary calvarial osteoblasts after transfection with siRNA SC, Bicc1_1, Bicc1_2, and Bicc1_3 (which was not effective in knocking down Bicc1). From the array data, polycystic kidney disease 2 (Pkd2), one of the M6 genes most strongly connected to Bicc1 (Figure 5A), was the only gene significantly correlated (r = 0.92, P = 3.6 x 10–7, false discovery rate [FDR] = 0.01) with Bicc1 knock down (Figure 5B and Table 2). Pkd2 was particularly interesting, because, as with Bicc1, mutations in Pkd2 lead to PKD (38). Pkd2 encodes for polycystin-2 (PC2), a calcium channel located on primary cilia in kidney epithelium and osteoblasts (38). Bicc1 and Pkd2 expression was also highly correlated in Bicc1+/+ and Bicc1+/jcpk male and female osteoblasts (Figure 5C). As expected, Bicc1 and Pkd2 expression was highly correlated across HMDP bone samples (Figure 5D). Causality modeling predicted that Bicc1 is upstream of Pkd2 (Figure 5E).

Pkd2 knock down impairs osteoblast differentiation. If Bicc1 regulates osteoblastogenesis by controlling Pkd2 levels, then we would expect that Pkd2 knock down would impair osteoblast differentiation. It has already been shown that Pkd2 is expressed in osteoblasts (39), and we found that its level of expression was similar to that of Bicc1 (Supplemental Figure 4). To test this hypothesis, we knocked down the expression of Pkd2 in B6 calvarial osteoblasts. The experiments were performed using 2 unique siRNAs that reduced Pkd2 transcripts by approximately 50% (Figure 6A) and protein levels by approximately 20% (the full, uncut gel is shown in Supplemental Figure 5) 48 hours after transfection. In transfected osteoblasts, we observed significant decreases in alkaline phosphatase activity, the expression of markers of osteoblast differentiation, and mineralized nodule formation (Figure 6, B–F). The defect in osteoblast activity was identical, albeit more severe, to that of Bicc1 knock down. Next, we overexpressed Pkd2 by 2.7-fold (Supplemental Figure 5) in Bicc1+/+ and Bicc1+/jcpk osteoblasts to determine whether Pkd2 could rescue the effects of Bicc1 deficiency on osteoblastogenesis. In Bicc1+/jcpk osteoblasts, mineralized nodule formation...
The second variant was a meta-analysis of CT-derived lumbar spine BMD associated with BMD using data from 2 GWAS meta-analyses. The first was a meta-analysis of CT-derived lumbar spine BMD associated with BMD in MESA. In GEFOSII, 2 SNPs were significantly associated with femoral neck BMD (Figure 7A). These variants were frequent (MAF = 0.025) and located 28 Kbp 5′ and located 52 Kbp 5′ upstream to the 3′ end of both genes. Each variant BICC1 of Pkd2 was evolutionarily conserved (Supplemental Figure 6), with many other SNPs spanning from position=chr10:60377418-60387569). We also determined whether SNP-SNP interactions in BICC1 and PKD2 affected the expression of BICC1, or any regulatory regions (46). One limitation of this approach is that it does not affect expression or in which the change in expression is just downstream of exon 2. Interestingly, rs10740740 and its proxies (r² > 0.95) were the only SNPs 1 Mbp up- or downstream of BICC1 that were also associated (FDR < 0.05) with its expression (Figure 7A). Specifically, rs10740740 was significantly associated with BICC1 expression in skeletal muscle (P = 8.6 × 10⁻¹³), nerve (P = 4.0 × 10⁻¹⁰), artery (P = 6.6 × 10⁻⁸), and skin (P = 1.2 × 10⁻⁶) tissues (data from GTEx) (Figure 7A). The region around rs10740740 was evolutionarily conserved (Supplemental Figure 6), and based on ENCODE data (42), was likely to harbor regulatory elements (http://genome.ucsc.edu/cgi-bin/hgTracks?db=hg18&position=chr10:603677418-60387569). We also determined whether SNP-SNP interactions in BICC1 and PKD2 influenced BMD in the 2 populations, but none of the interactions were statistically significant. In light of functional studies for both genes, these data across a large number of studies, ethnic groups, skeletal sites, and BMD acquisition methods suggest that variation in BICC1 and PKD2 influence variation in human BMD. This evidence is particularly strong for BICC1 in MESA, in which the BMD-associated SNP also influenced BICC1 expression.

### Discussion

New technologies and resources have positioned the mouse as a powerful platform for gene discovery and characterization. Here, we used a multifaceted systems genetics approach to identify Bic1 as the gene responsible for a BMD QTL in the mouse. We also demonstrated that it likely does so by influencing osteoblastogenesis through the regulation of Pkd2. Importantly, we also provide evidence that BICC1 and PKD2 are involved in the regulation of human BMD.

The B6 and C3H strains were first identified by Beamer et al. as having divergent femoral BMD (43). Since this observation, there have been no fewer than 30 QTLs identified that influence the difference in BMD between these strains (11). In addition to Bic1 identified here, Beamer et al. identified the transcript AC084073.22 as the gene most likely responsible for a BMD QTL on mouse Chr 1 using congenic-based fine-mapping and expression analysis (44). Our group recently used the HMDP to identify Axd2 as the gene responsible for an association with BMD on Chr 12 that was coincident with a QTL identified in a BxH F2 cross (9). Other candidate genes, including Pparg (45), Trps1 (7), Wnt9a (25), and Radl (25), have been identified for BxH BMD QTLs using a wide range of approaches.

The systems genetics approach that we used consisted of identifying regulatory variation in Bic1 that was predicted by causality modeling to be upstream and causal for the change in BMD. We believe this strategy represents a powerful paradigm, not only for skeletal traits, but for all complex traits. It is also generalizable, given the significant enrichments that have been observed for complex trait associations located in putative regulatory regions (46). One limitation of this approach is that it will not be successful in cases in which the responsible variant(s) does not affect expression or in which the change in expression is specific to a tissue not investigated. Despite this limitation, our work and that of others illustrates the power of eQTL analysis for gene discovery (25, 47, 48). Most genetic studies stop at gene discovery. By incorporating information from a bone coexpression network (35), we were able to identify connections between Bic1, a gene of unknown function in bone, with genes that have well-defined roles in osteoblasts and, in particular, osteoblastogenesis. Importantly, without knowing

### Table 2

Top 10 genes correlated with Bicc1 knock down in primary calvarial osteoblasts

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<td>-0.82</td>
<td>9.2 × 10⁻⁵</td>
<td>0.33</td>
</tr>
<tr>
<td>Anks3</td>
<td>ILMN_2735877</td>
<td>0.81</td>
<td>1.4 × 10⁻⁴</td>
<td>0.33</td>
</tr>
</tbody>
</table>

* r², Pearson’s correlation with Bicc1 expression.
anything else, this provided us with a testable hypothesis. More importantly, the network not only provided insight into the mechanism, but also identified \textit{Pkd2} as a putative target of \textit{Bicc1} in osteoblasts. We believe this approach is extremely powerful and can obviously be applied for a number of purposes, including the use of networks to infer the function of genes identified in human GWAS.

We demonstrated that the in vivo reduction in \textit{Bicc1} expression results in decreased areal BMD, primarily through decreased cortical thickness and TMD. Osteopenia was only observed at 28 weeks in male mice. The congruency between the male-biased nature of the effects of \textit{Bmd43} and the decreased BMD in \textit{Bicc1}^{+/jcpk} male mice support a model in which the \textit{Bicc1} eQTL is solely responsible for \textit{Bmd43}. However, why \textit{Bicc1} has an effect on BMD only in males is unclear. A male bias in the eQTL is unlikely, at least in adipose tissue, because the eQTL is equally strong in both sexes (Figure 1D). Androgens are known to drive periosteal apposition (49), but we did not see any difference in femoral size in \textit{Bicc1}^{+/jcpk} males, suggesting that sex hormones may not be driving the sex difference. Additionally, there is no evidence that \textit{Bicc1} expression or function is modulated directly by sex hormones. It is interesting that there is an approximately 35% increase in \textit{Bicc1} expression in adipose tissue from BXH mice (Figure 1G) and an approximately 25% increase in expression in male primary calvarial osteoblasts (Figure 4J) (although this difference was not significant). It is possible that this difference underlies the sex specificity. An alternative explanation is that the intrinsic osteoblast defect is present in both sexes, but is only manifested in males as a function of age or other stressors (e.g., a high-fat diet). There was a slight decrease in BMD in C3H homozygous females at \textit{Bmd43} (data not shown) and in \textit{Bicc1}^{+/jcpk} females (Figure 2B). It is possible that this difference becomes more pronounced with age.

The data generated herein suggest that \textit{Bicc1} affects bone mass by perturbing osteoblast function. While available data indicate that \textit{Bicc1} is not likely expressed in other bone cells (Supplemental Figure 3), we cannot rule out the possibility that \textit{Bicc1} impacts bone through one or more cell types other than osteoblasts. We also cannot exclude the possibility that \textit{Bicc1} alters the activity of other cell types indirectly through osteoblasts. Future studies using conditional knockout mice will allow us to test the hypothesis that \textit{Bicc1} impacts bone via osteoblasts in a cell-autonomous manner.

\textit{BICC1} is a 977–amino acid protein that contains 3 RNA-binding K-homology (KH) domains and a C-terminal sterile \(\alpha\) motif (SAM) (28–30). \textit{BICC1} has been shown to bind RNA in vitro (28), and its homolog in \textit{Drosophila} is involved in mRNA localization and translational regulation (30, 50). Here, we demonstrate that \textit{Bicc1} regulates, either directly or indirectly, \textit{Pkd2} transcript levels in osteoblasts. There are a number of observations from other systems that sup-

Figure 6
\textit{Pkd2} regulates osteoblast differentiation and \textit{Pkd2} overexpression rescues the defect in osteoblastogenesis in the absence of \textit{Bicc1}. Transfection of two independent siRNAs targeting \textit{Pkd2} (\textit{Pkd2}_1 and \textit{Pkd2}_2) in primary calvarial osteoblasts reduced \textit{Pkd2} expression 48 hours after transfection (A) and alkaline phosphatase activity (B) and osteogenic gene expression (C) 4 days after transfection. SiRNA transfection also reduced the formation of mineralized nodules (D) 12 days after transfection, as determined by Alizarin red staining (E) and nodule counts (F). Overexpression of \textit{Pkd2} increased nodule formation and rescued impaired nodule formation in \textit{Bicc1}^{+/jcpk} mice (G). Data presented in all panels (except D) are the means ± SEM. \(\#P < 0.10, \ast P < 0.05.\)
port this interaction. First, mutations in both genes cause PKD and malformations in left-right axis formation (29, 31, 51, 52), suggesting that they function in the same or similar pathways. Second, Tran et al. demonstrated that Bicc1 acts as a post-transcriptional regulator of Pkd2 in the pronephros of Xenopus (29).

One of the open questions from our work is how Bicc1 and Pkd2 impact osteoblast activity. Polycystin 1 (PC1) and PC2, encoded by Pkd1 and Pkd2, form a complex on the surface of primary cilia. PC1 acts as a receptor that, when stimulated, activates the PC2 calcium channel. In kidney epithelial cells, this complex is essential for sensing fluid flow in the developing kidney, activates the PC2 calcium channel. In kidney epithelial cells, this complex is essential for sensing fluid flow in the developing kidney, activates the PC2 calcium channel. In kidney epithelial cells, this complex is essential for sensing fluid flow in the developing kidney, activates the PC2 calcium channel. In kidney epithelial cells, this complex is essential for sensing fluid flow in the developing kidney, activates the PC2 calcium channel.

In summary, we have identified Bicc1 as a positive regulator of osteoblast function and BMD using a multifaceted systems genetics approach. Our results suggest that Bicc1 is a key regulator of the PC2 calcium channel in bone. GWAS data also indicate that both genes play a role in the regulation of BMD in humans. This discovery increases our understanding of the genetic factors affecting BMD and highlights the power of systems genetics for complex trait gene discovery and functional characterization.

Methods

Mice. The generation of the BXH F2 cross, its genotyping, and determination of femoral BMD have been previously described in detail (25). The generation and characterization of the HMDP has been described previously (9, 27). Bicc1<sup>−/−</sup> males were mated with C57BL/6J females (The Jackson Laboratory) to generate Bicc1<sup>−/−</sup> and Bicc1<sup>+/−</sup> littersmates. Genotyping for the Bicc1<sup>−/−</sup> allele was performed as previously described (31). For BMD measurements, Bicc1<sup>−/−</sup> and Bicc1<sup>+/−</sup> mice were placed on a high-fat diet (88137; Harlan Teklad) at 8 weeks of age. A small breeding colony of C57BL/6J females (The Jackson Laboratory) was used for the isolation of primary calvarial osteoblasts.
Analysis of bone mass in Bicc1<sup>+/−</sup>jcpk mice. At 12, 16, and 28 weeks of age, BMD of the left femur was measured using a Lunar PIXImus II Mouse Densitometer (Model S1045; GE Medical Systems). Distal femurs were scanned using a vivaCT 40 imaging system (Scanco Medical) to measure trabecular bone volume fraction and microarchitecture of the distal femur and cortical microarchitecture of the femoral midshaft. The samples were scanned completely submerged in 95% ethanol. Scans were performed at an energy level of 55 kVp, an intensity of 145 μA, and an isotropic voxel size of 12.5 μm. Trabecular microarchitecture was evaluated from approximately 200 consecutive slices of the secondary spongiosa. Cortical bone scans were performed at the midpoint of each femur. A total of 50 consecutive slices were scanned.

Human BICC1 and PKD2 data. Two sources of data were used. The first was derived from a genome-wide meta-analysis of femoral neck (LS) and lumbar spine (FN) BMD, conducted as part of the GEFOS Consortium (6). The discovery samples comprised 17 GWAS studies (n = 32,961) from populations across North America, Europe, East Asia (only 1 study with ~1,000 women), and Australia. All studies were approved by their institutional ethics review committees, and all participants provided written informed consent. LS-BMD and FN-BMD were measured in all cohorts using DAXA, following standard protocols. Each study performed single-variant GWAS analysis for FN-BMD and LS-BMD on 2.5 million imputed SNPs using linear regression model (population-based studies) and linear mixed-effects models (family-based studies), with adjustment for sex, age, weight, and ancestral genetic background (8). The imputed genotypes were coded as continuous dosages of the minor allele (additive model) from 0 to 2 to take imputation uncertainty into account. An additive (per-allele) genetic model was applied. A fixed-effects, inverse-variance meta-analysis was performed to combine results from each study. We limited our analyses to SNPs located within BICC1 and PKD2 as well as 60 kb upstream of both genes. SNPs with minor allele frequencies of less than 1% or an imputation quality score/variability ratio of less than 0.3 were excluded from further analyses. A total of 316 SNPs were selected for BICC1 and 71 SNPs for PKD2. To correct for multiple testing, we estimated the number of independent tests of SNPs that were included in the analysis using principal component analysis. We then applied a Bonferroni’s correction to estimate the significant cutoff at P < 4.2 × 10<sup>−6</sup> (≈ 0.05/number of independent tests).

The second source of data was obtained from the MESA GWAS. MESA is an observational cohort of volunteers recruited between July 2000 and August 2002 from 6 field centers in the United States. The study population at baseline consisted of 6,814 men and women aged 45 to 84 years and self-identified as non-Hispanic Whites, Chinese-Americans, African-Americans, or Hispanics who were free of clinical cardiovascular disease. A random sample of MESA participants was selected for BMD measurements. Measurement of BMD in a virtual 10-mm-thick slice of trabecular bone from each vertebra (L2–L5) used software-directed procedures. Participants were genotyped using the Affymetrix Human SNP array 6.0. SNPs were filtered for an SNP level call rate less than 95% and an individual level call rate less than 95%, and monomorphic SNPs were removed. The cleaned genotypic data were deposited with MESA phenotypic data into dbGaP as the MESA SHARE project (study accession number phs000209, http://www.ncbi.nlm.nih.gov/projects/medgen Animal Disease Information Build 36 [dbSNP b126]). We filtered according to an MAF of less than 0.01, a Hardy-Weinberg equilibrium (HWE) P value greater than 1 × 10<sup>−5</sup>, and imputation quality (R<sup>2</sup>) greater than 0.5. In all analyses, we used a basic model including age, sex, study site, and ancestry principal components (PCs) (3 in Whites, 1 in Chinese, 1 in African-Americans, 3 in Hispanics). We corrected for multiple tests using the same procedure as outlined above in GEFOSII, except that LD was assessed independently in each ethnic group, and then the meta-analysis was corrected using the maximum number of tests across the 4 groups using a Bonferroni’s correction.

Transcriptional network analysis. Network analysis was performed using the weighted gene coexpression network analysis (WGCNA) R package (9, 22, 64). A full description of the network used to characterize Bicc1 in this study is provided in ref. 35. To generate the coexpression network, we first calculated Pearson’s correlation coefficients for all gene-gene comparisons across all microarray samples. The matrix of correlations was then converted to an adjacency matrix of connection strengths. The adjacencies were defined as α = |<i>x</i><i>y</i>|<sup>β</sup>, where <i>x</i> and <i>y</i> are the <i>ph</i> and <i>ph</i> gene expression traits. The power β was selected using the scale-free topology criterion previously outlined by Zhang and Horvath (65). Modules were defined as sets of genes with high topological overlap (66). The TOM between the <i>ph</i> and <i>ph</i> gene expression traits was taken as

\[
TOM = \frac{\sum_{\mu \neq j} a_{\mu \mu} a_{\mu j} + a_{jj}}{\min(k_{\text{total}} k_{\text{total}} \sigma_i) + 1 - a_{jj}}
\]  
(Equation 1),

where \(\sum_{\mu \neq j} a_{\mu \mu} a_{\mu j}\) denotes the number of nodes to which both \(i\) and \(j\) are connected, and \(\mu\) indexes the nodes of the network. Principal comm
ponent analysis was used to generate a vector of values (first principal component) that summarized a module behavior. Network depictions were constructed using Cytoscape (67).

**Bicc1 isoform expression analysis.** Using TRIZol reagent (Invitrogen), RNA was isolated from 4 wells per sampling every day from days 0 to 6, followed by every other day until day 14, with a final sampling on day 20. All samples were treated with the DNase DNA-free kit (Applied Biosystems). RNA concentrations were determined spectrophotometrically (A260), and cDNA was synthesized from random primed reverse transcription using an ABI High Capacity Reverse Transcriptase kit (Applied Biosystems). The relative abundance of isoforms A and B Bicc1 cDNA was determined with quantitative PCR (qPCR) using an ABI 7900 with SensiMix Syber Hi-ROX (Bioline) and cycle parameters of 95(10 m), 95C(15 s), 60C(30 s), 72C(30 s).

A forward primer homologous to sequences found in exon 19 (5′-CACG-GAGTGTCTTCCAGCAGCAAG-3′) was used in the determination of both isoforms. For isoform A, a reverse primer homologous to the 3′ end of exon 20 and the 5′ end of exon 22 (5′-GTTTCTACTTAGCTCTGAGATTGC-3′) was used, whereas a reverse primer homologous to the 3′ end of exon 20 and 5′ end of exon 21 (5′-ACAGGTCACAAAATGAGATTGC-3′) was used for isoform B. Note that the reverse primers share a 10-nucleotide sequence at their 3′ ends and that the sequences amplified in both reactions are identical. The 36B4 gene cDNA served as a reference and was amplified using the following primers: forward (5′-ACTGAGATTGGGATATGCGTG-3′) and reverse (5′-TCCTAGACCAGTGTCTGAGCT-3′). Relative quantification of the Bicc1 isoforms was determined by the 2^(-ΔΔCt) method using 36B4 as the reference gene (68).

**Western blot analysis of PC2.** Whole-cell lysates were electrophoresed on NuPAGE gels (Life Technologies) and transferred to nitrocellulose membranes following the manufacturer’s protocol. Blots were incubated with PC2 (H-280) antibody (sc-25749; Santa Cruz Biotechnology Inc.) to detect PC2 and then subsequently stripped and reprobed with anti-β actin (04-1116; Millipore). Signal detection was performed using the Western Lightning Plus-ECL system (PerkinElmer). Films were scanned and images were quantified using Image J software (NIH).

**Characterization of Bicc1 function in osteoblasts.** The details of isolation and culture of primary calvarial osteoblast, siRNA transfection, qPCR, and osteoblast differentiation assays are described in ref. 35. Briefly, primary calvarial osteoblasts were isolated from 3- to 9-day-old neonates using sequential collagenase P digestions. Cells were plated onto 6-well plates at 300,000 cells/2 ml sterile plating media (DMEM, 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin) per well. After 24 hours, confluent cells (day 0) were washed once with Dulbecco’s PBS (DPBS) (Gibco) and plated in sterile differentiation media (DMEM, 10% FBS, 100 μl penicillin, 100 μg/ml streptomycin, 0.1 M ascorbic acid, 1 M B-glycerophosphate). Every 48 hours thereafter, cells were washed once with DPBS, and differentiation media were replaced. After 48 hours, media were replaced with differentiation media (MEM-α, 10% heat-inactivated FBS, 100 μl penicillin, 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, 4 mM B-glycerophosphate) on day 0 and then changed every 2 days thereafter. For siRNA transfection experiments, cells were handled as outlined above, except they were plated at 150,000 cells/well and transfected approximately 24 hours after plating using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer’s directions, with differentiation commencing 72 hours after plating. Stealth Select RNAs siRNAs (Invitrogen) targeting Bicc1 and Pkd2 were used to knock down their expression in calvarial osteoblasts. The sense strands of the duplex siRNA sequences were as follows: Bicc1_1, BICC1MS2S94526; AACAGGAGGAGCACGAGGACUCGUGG, Bicc1_2, BICC1MS2S34735; AAGACAGAACGACUAUCUUCUUGG Bicc1_3, BICC1MS2S34736; UUGCUAGGCACAGACUCAUAAUGGU, Pkd2_1, PKD2MS2S07692; AAACGAGGCGGCAUAGACGAGCUCU and Pkd2_2, PKD2MSS2S07693; AACACCUCCUCGUUCUUACGUCU. The Stealth RNAi Negative Control Duplex (Invitrogen) was used as a scrambled control. qPCR was used to measure gene expression. The following primer sets were used (all sequences 5′-3′): Runx2-F, ACAGTCCCAACTCTCCTGTTG; Runx2-R, TCGTCTGCAAGATGTGC; Sp7-F, TGGCCCAAATCTCAGCAGG; Sp7-R, ACACCCGAGATGTTGGAAG; Col1a1-F, CCAAAGGAAAGACGATCT; Col1a1-R, AGGTCAGCTTGATGACGACT; Bglap-F, GAACAGACAGTCACACACAGC; Bglap-R, GATGGTGCGGCGTGATGAT; Isbp-F, GAGGAGACCTCAAAACGGAAGG; Isbp-R, AGAGACAGACGCAAGCGCA; 36B4-F, ACTGAGATTGGGATATGCGTG; and 36B4-R, TCCTAGACCAGTGTCTGAGCTG. Relative quantification was determined by the 2^(-ΔΔCt) method using 36B4 as the reference gene (68).

**Osteoblast microarray analysis.** Primary calvarial osteoblasts were isolated from B6 mice and transfected with siRNA SC, Bicc1_1, Bicc1_2, and Bicc1_3 (n = 4/treatment for a total of 16 samples). Forty-eight hours after transfection, total RNA was isolated using the QIAGEN RNasy Mini Kit. Microarray expression profiles were generated for all samples using the Illumina MouseWG-6 v1.1 BeadChips at the Center for Public Health Genomics, Genome Sciences Laboratory of the University of Virginia. Biotin-labeled cRNA was synthesized by the total Prep RNA Amplification Kit from Ambion. cRNA was quantified and normalized to 77 ng/μl, and then 850 ng was hybridized to BeadChips. The arrays were scanned, and raw expression values were transformed using the variance stabilizing transformation (VST) algorithm (69) and normalized with the robust spline normalization (RSN) algorithm using the LumiR R package (70). The array data are available in the NCBI’s GEO database (GEO GSE51693).

**Characterization of Bicc1 function in osteoblasts from individual mice.** Calvarial osteoblasts from individual neonates (n = 11–20 individual neonates/sex/genotype) were isolated and differentiated as described above, with the following modifications and additions: PCR on tail DNA was used to genotype as described above and to determine the sex of each neonate as previously described (71). Sequential trypsin-EDTA-collagenase P digestion of individual calvarium was performed in 24-well plates with 0.5 ml of digestion media per well. Isolated osteoblasts from an individual neonate were seeded into a 60-mm dish and allowed to grow for 40 to 48 hours, with a change of plating media approximately 24 hours after seeding. Cells were harvested and plated at 150,000 cells per well of a 6-well dish in 2 ml of plating media and allowed to proliferate for 48 hours, at which time the media were changed. The next day (72 hours after seeding), the cells were washed with 2 ml 1× DPBS per well followed by the addition of 2 ml of differentiation media per well (day 0). The cells were washed and differentiation media replaced every 2 days for 12 days, with alkaline phosphatase activity and mineralization determined on day 13.

**Statistics.** BMDs measured by PIXimus densitometers have a gradient of values along the y-axis that results in gradually lower BMD values, moving from the top of the scan to the bottom (9, 27, 72). Therefore, we analyzed the data using a linear model that included the effects of genotype, weight at sacrifice, and y-axis position of the scan. Weight at sacrifice and y-axis position were significant terms in the model. Least-squares means and the significance of the genotype term were calculated using the lsmeans R package. All other statistical comparisons were performed using a 2-tailed Student’s t test.
Study approval. The animal protocol used in this study was approved by the IACUC of the University of Virginia.

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