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Abstract 17

Osteoporosis, the most common bone disease, is characterized by a low bone mass and 18
increased risk of fractures. Importantly, individuals with the same bone mineral density 19
(BMD), as measured on two dimensional (2D) radiographs, have different risks for 20
fracture, suggesting that microstructural architecture is an important determinant of 21
skeletal strength. Here we took advantage of the rich phenotypic and genetic diversity 22
of the Collaborative Cross (CC) mice. Using microcomputed tomography, we 23
examined key structural parameters in the femoral cortical and trabecular compartments 24
of male and female mice from 34 CC lines. These traits included the trabecular bone 25
volume fraction, number, thickness, connectivity, and spacing, as well as structural 26
morphometric index. In the mid-diaphyseal cortex, we recorded cortical thickness and 27
volumetric BMD. 28

The broad-sense heritability of these traits ranged between 50 to 60%. We 29
conducted a genome-wide association study to unravel 5 quantitative trait loci (QTL) 30
significantly associated with 6 of the traits. We refined each locus by combining 31
information obtained from the known ancestry of the mice and RNA-Seq data from 32
publicly available sources, to shortlist potential candidate genes. We found strong 33
evidence for new candidate genes, including *Rhbd2*, which association to trabecular 34
bone volume fraction and number was strongly suggested by our analyses. We then 35
examined knockout mice, and validated the causal action of *Rhbd2* on bone mass 36
accrual and microarchitecture. 37

Our approach revealed new genome-wide QTLs and a series of genes that have 38
never been associated with bone microarchitecture. This study demonstrates for the first 39
time the skeletal role of *Rhbd2* on the physiological remodeling of both the cortical 40

and trabecular bone. This newly assigned function for *Rhbd2* can prove useful in 41
deciphering the predisposing factors of osteoporosis and propose new investigative 42
avenues toward targeted therapeutic solutions. 43

Author summary 44

In this study, we used the novel mouse reference population, the Collaborative Cross 45
(CC), to identify new causal genes in the regulation of bone microarchitecture, a 46
critical determinant of bone strength. This approach provides a clear advantage in 47
terms of resolution and dimensionality of the morphometric features (versus humans) 48
and rich allelic diversity (versus classical mouse populations), over current practices 49
of bone-related genome-wide association studies. 50

Our genome-wide study revealed 5 loci significantly associated with microstructural 51
traits in the cortical and trabecular bone. We found strong evidence for new candidate 52
genes, in particular, *Rhbd2*. We then validated the specific role of *Rhbd2* on bone 53
mass accrual and microarchitecture using knockout mice. Importantly, this study is 54
the first demonstration of a physiological role for *Rhbd2*. 55

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Introduction 57

Osteoporosis is the most common bone disease in humans, affecting nearly half the US 58
and European population over the age of 50 years. With the globally increasing life 59
expectancy, osteoporosis and related bone fractures are becoming a pandemic health 60
and economic concern. By 2050, the world-wide incidence of hip fractures is expected 61
to increase by 2.5 to 6 fold [1,2]. Importantly, the mortality rate in the 12 months 62
following bone fracture is as high as 20% [3]. Risk of fracture is determined largely by 63
bone density and quality/strength, which are the end result of peak values achieved at 64
skeletal maturity and subsequent age and menopause-related bone loss. Genetic factors 65
have a major role in determining the wide range in the so-called "normal" peak bone 66

mass. Measures of bone status are inherently complex traits, as opposed to Mendelian traits; i.e. they are controlled by the cumulative effect and interactions of numerous genetic loci and environmental factors.

Genome-wide association studies (GWAS), including a large meta-analysis, have identified more than 50 loci associated with bone mineral density (BMD) [4–10]. However, many other genes that were experimentally associated with bone mass were not confirmed by GWAS in human cohorts [11–13]. This suggests that the BMD phenotype does not capture the structural complexity of the bone; there may be other relevant bone phenotypes not yet studied in human GWAS [12], which hitherto have generally relied on areal bone mineral density (aBMD) as the sole bone feature. aBMD measured by dual energy x-ray absorptiometry (DXA) is a two-dimensional projection that cannot measure bone size, individual bone compartments' shape (whether trabecular or cortical) or underlying microstructure, and thus likely conceals important features which are assumed to be controlled by unique genetic determinants. Indeed, there is a growing body of evidence that argues for distinct genetic influences of the cortical and trabecular bone and thus they should be accordingly distinguished [8,9]. A recent GWAS in the Collaborative Cross (CC) mice based on DXA failed to find any heritability of BMD [14], whereas another report based on the same mouse panel showed highly significant heritability levels in most of the cortical and trabecular microstructural parameters measured by micro-computed tomography (μ CT) [15]

Traditional peripheral quantitative CT (pQCT) has the capacity to distinguish between the cortical and trabecular bone compartments, but it lacks the required resolution to detect microstructural differences. A recent report based on high resolution pQCT (HR-pQCT) data in humans, identified two novel bone-related loci,

thus far undetected by DXA and pQCT-based GWAS [9]. Another [16], found strong 91
genetic correlations between 1047 adult participants of the Framingham heart study, 92
therefore indicating that the heritability of bone microstructure constitutes a phenotypic 93
layer which is at least partially independent of DXA-derived BMD. Like HR-pQCT 94
studies in humans, understanding the genetic regulation of bone microstructural 95
parameters using μ CT in small animals is likely to identify genetic factors distinct from 96
those previously identified for DXA-derived traits. 97

The CC mouse panel is designed to provide high resolution analysis of complex 98
traits, with particular emphasis on traits relevant to human health [17,18]. This unique 99
resource currently consists of a growing number of recombinant inbred lines (RIL) 100
generated from full reciprocal breeding of eight divergent strains of mice [19]. In 101
contrast to commonly used laboratory mouse strains, the ancestry of the CC lines 102
includes wild-derived inbred strains that encompass genetic variations accumulated 103
over ~1 million years [20]; more than 50 million single nucleotide polymorphisms 104
segregate in founders of the CC. The high genetic diversity means that QTLs can be 105
mapped using this panel that would have been invisible in a population that involved 106
only classical strains [21,22]. 107

This claim is substantiated in a recent study that identified a genome-wide 108
significant association between *Oxt* (oxytocin) and *Avp* (vasopressin) and skeletal 109
microarchitecture in CC mice [15]. Here, our GWAS in the CC mouse panel identified 110
a novel gene, *Rhbd2*, associated with bone traits and using a specific knockout model 111
we validated its role in the regulation of cortical and trabecular bone structure. This 112
exemplifies the effectiveness and relative ease by which a GWAS with a small CC 113

population can associate a bone-related function to novel genes, and to reveal 114
overlooked key players in skeletal biology. 115

Results 116

CC lines widely differ in bone microarchitecture traits 117

We examined the variation in femoral cortical and trabecular microstructure between 118
34 unique CC lines totaling in 174 mice (71 females and 103 males, with an average of 119
4.25 mice per line). In the trabecular bone compartment we measured bone volume 120
fraction (BV/TV), trabecular number (Tb.N), thickness (Tb.Th), connectivity 121
(Conn.D), and spacing (or separation; Tb.Sp), as well as structural morphometric index 122
(SMI) of the trabecular framework. In the mid-diaphyseal cortex, we recorded cortical 123
thickness (Ct.Th) and volumetric bone mineral density (vBMD). These traits were 124
approximately normally distributed; BV/TV ranged from 0.017 to 0.26 (i.e. 1.7% to 125
26%; mean = 10.2%); Tb.N from 0.52 to 6.11 mm⁻¹ (mean = 2.7 mm⁻¹); Tb.Th from 31 126
to 69 μm (mean = 47 μm); Conn.D from 10.9 to 268.3 mm⁻³ (mean = 104.2 mm⁻³); SMI 127
from 0.6 to 3.3 (mean = 2.3); Tb.Sp from 0.16 to 0.7 mm (mean = 0.33 mm); Ct.Th 128
from 0.14 to 0.29 mm (mean = 0.2 mm); and vBMD from 402.5 to 809.2 mgHA/cm³ 129
(mean = 581.1 mgHA/cm³). μCT images taken from two mice with distinct cortical 130
(Fig. 1A) and trabecular (Fig. 1B) characteristics demonstrate the great variation in 131
bone traits due solely to the genetic background. Color-codes on the graphs in Fig. 2 132
indicate Duncan's least significance range (LSR), which dictates whether the mean 133
value of a line, or a group of lines, for a given trait differs to a degree of at least *P*-value 134
< 0.001 from any other group. LSR allows for a visual representation of the 135
heterogeneity amongst the lines. 136

With 11 distinct groups, vBMD (Fig. 2B) is the most heterogeneous trait, while SMI and Conn.D are the least, with only 3 significantly distinct groups (Fig. 2A). Notably, the heterogeneity of females is greater than that of males for cortical traits but milder for trabecular traits (Figs. S1A1, 2 for males and S1B1, 2 for females).

To examine the inter-dependency between the traits, we assessed the correlation between all the measured parameters, in a pairwise fashion using Pearson's correlation test. The strongest correlation was between BV/TV and Tb.N (Pearson's $r = 0.94$), in line with our previous findings [15], while the weakest was between Tb.N and vBMD ($r < 0.01$). There was also a moderately high correlation between Ct.Th and Tb.Th ($r = 0.61$; and see Table S1). The correlation between sexes for each trait (Table 1) ranged from $r = 0.75$ (Tb.Sp) to $r = 0.20$ (Ct.Th). Body weight (range = 17.4 - 35.0 gr) did not significantly correlate with any of the traits ($r = 0.01$ for Conn.D to $r = 0.19$ for Ct.Th). After separating males from females the correlation slightly increased, yet remained low. Weak correlation was found between weight and Tb.N, SMI, and Ct.Th for females (Pearson's $r = -0.20, 0.23$, and 0.25 respectively), and between weight and Tb.Th and Tb.Sp (Pearson's $r = 0.25$ and -0.25) for males.

Table 1 Heritability, sex correlations, and covariate interactions for trabecular and cortical traits

Trait	H²	logP	H²n	Sex Cor.	Interactions %
BV/TV	0.61	12.43	0.87	0.6695754	-
Tb.N	0.63	13.76	0.88	0.7628464	-
Tb.Th	0.54	9.08	0.83	0.7039887	34.70
Conn.D	0.56	9.90	0.84	0.5188039	-
SMI	0.55	9.43	0.84	0.2984166	26.02
Tb.Sp	0.63	13.45	0.88	0.754563	-
vBMD	0.62	12.15	0.87	0.6268818	53.92
Ct.Th	0.51	7.63	0.82	0.2055684	41.07

H² is the broad-sense heritability (which includes epistatic and environmental influences); logP is the negative 10-base logarithm of the P-value; H²n is the line-mean heritability; Sex Cor. Is the sex correlation of each trait; and interactions % refers to the relative contribution of the cumulative covariate-interactions, which include sex, age, batch, month, season, year, and experimenter (see table S2).

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Table 2 Positions of QTLs associated with trabecular and cortical traits											
QTL	Trait	Chr	logP	99th % thresh	H ² _r	Simulation					
						50% CI (Mb)		90% CI (Mb)		95% CI (Mb)	
						Position	Width	Position	Width	Position	Width
<i>Trl7</i>	BV/T V	11	7.60	4.90	0.69	116.6-116.7	0.12	113.6-118.1	4.50	112.1-118.3	6.40
<i>Trl7</i>	Tb.N	11	6.80	4.84	0.71	116.6-116.87	0.29	114.2-118.35	4.15	112.41-118.68	6.27
<i>Trl8</i>	Tb.Th	4	8.00	4.50	0.61	117.2-117.58	0.32	113.05-125.54	12.49	110.87-126.52	15.65
<i>Trl9</i>	Tb.Sp	5	9.40	6.01	0.78	105.78-106.14	0.35	101.6-109.11	7.54	99.8-110.39	10.62
<i>Crll</i>	Ct.Th	4	8.20	4.80	0.80	9.29-9.72	0.43	4.0-11.7	7.70	3.4-11.8	8.49
<i>Crll2</i>	vBMD	3	9.80	4.93	0.86	97.2-97.4	0.20	94.4-103.1	8.50	93.22-104.3	10.80
Chr = chromosome; logP = negative 10-base logarithm of P value; Sig = genome-wide significance level; 99th % threshold logP = threshold used to define cut-off for QTL peaks (Fig. 4); H ² _r = regional heritability (the proportion to which the locus explains the phenotypic variability). Positions and widths of the simulation-based 50, 90, and 95% CIs are given.											

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While in most lines the traits' correlations were predictive of a given line's rank, 170
in others a less expected pattern was observed; e.g., IL-1513 displayed unusually 171
extreme phenotypes for all trabecular traits and was at the higher end for BV/TV, Tb.N, 172
Tb.Th, and Conn.D and at the lower end for SMI and Tb.Sp, but IL-188 was more 173
discordant between these same traits (Fig. 2 and Table S3), illustrating unexpected co- 174
variation of the traits in the CC. 175

Heritability and confounder-control 176

We quantitated the effects of the covariates sex, age, batch, month, season, year, and 177
experimenter on each trait. Age ranged from 9 (n=6) to 13 (n=9) weeks and the mice 178

were dissected in 20 batches over a three-year course across 8 months during winter, 179
spring and summer, by two experimenters. Whereas age alone had no effect on any 180
trait, sex affected only Ct.Th; batch affected Tb.Th, vBMD, and Ct.Th; month affected 181
Tb.Sp, vBMD, and Ct.Th; season and year affected vBMD and Ct.Th; and Tb.Th and 182
Ct.Th were affected by experimenter. The cumulative effect of the covariates' pairwise 183
interactions was noted for Tb.Th, SMI, vBMD, and Ct.Th. (Table S2). 184

We then estimated the broad-sense heritability (H^2) of each trait among the CC 185
lines, which includes additive and non-additive epistatic effects and gene-environment 186
interactions. The greatest H^2 is seen for Tb.N (0.63, $\log P = 13.76$; where $\log P$ stands 187
for the negative 10-base logarithm of the P value and tests the null hypothesis that the 188
heritability is zero), and the smallest for Ct.Th (0.51, $\log P = 7.63$). 189

We calculated the heritability for the mean values in each line to get a better 190
representation of the percentage of genetic contribution to the phenotypic heterogeneity 191
by incorporating H^2 and the average number of lines [23]. This defines H^2_n , which is 192
directly proportional to H^2 (Methods; Table 1) and ranges between 82 (Ct.Th) and 88% 193
(Tb.N and Tb.Sp). 194

Overall the cortical traits seemed more prone to covariate variation; they were 195
particularly sensitive to sex, batch, and season. This stands in contrast to our previous 196
results [15] where BV/TV, Tb.N, and Conn.D displayed a profound sex effect, although 197
there cortical traits were not measured. This means there is a deeper, complex layer of 198
sex effect dependent upon cooperative environmental and genetical factors which 199
requires further work to fully comprehend. 200

Association analysis for microarchitectural traits highlights 5 QTLs 201

We first measured statistical association between each trait and the founder haplotype at each locus in the genome. Association analyses of the cortical and trabecular traits to the haplotypes segregating in the CC (as defined by the ~70 K MegaMuga SNPs) yielded 5 distinct QTLs. For BV/TV and Tb.N we recognized a marked peak at a locus of length ~0.45 Mb between 116.5 and 116.9 Mb on chromosome 11, with peak logP values of 7.6 and 6.8, which extended above the 99th percentile permutation-threshold by 2.7 and 1.94 logP units, respectively. In Tb.Th, Tb.Sp, Ct.Th, and vBMD we identified different QTLs on chromosomes 4, 5, 4 and 3, with logPs of 8.0, 9.4, 8.2, and 9.8, respectively, above threshold (Fig. 3 and Table 2). To account for false positive results, we kept the false discovery rate (FDR) at 1% for each scan, by employing the Benjamini–Hochberg multiple testing procedure on the logPs of the haplotype associations to the traits. Conn.D and SMI lacked significant peaks above the stringent permuted threshold and thus were not further analyzed (Fig. S2), but Conn.D displayed a borderline peak in a region that matches the peak identified for BV/TV and Tb.N. The 5 QTLs we describe are hereafter referred to as *Trl* (trabecular related locus) 7-9, and *CrI* (cortical related locus) 1-2 (respectively for BV/TV and Tb.N, Tb.Th, Tb.Sp, Ct.Th, and vBMD, and in keeping with our previous report [15] that introduced *Trl* 1-6). The 95% widths of the confidence intervals ranged from 6.4 to 15.6 Mb for the *Trls*, and were between 8.5-10.8 Mb for the *CrIs* (Table 2 and Fig. S3).

We measured the contribution of each CC founder to the QTLs, relative to the wild-derived strain WSB/EiJ (Fig. 4). *Trl*7 is mostly affected by the classic laboratory strains 129S1/SvImJ, NOD/LtJ, and NZO/HiLtJ; notably, the other traits were more strongly driven by the following wild strains: *Trl*8 and *CrI*2 by PWK/PhJ; *Trl*9 by WSB/EiJ; and *CrI*1 by CAST/Ei.

For *Trl7*, at the SNP most adjacent to the QTL peak UNC20471277, we found 226
that the majority of lines with a TT allelic variant (where T refers to the nucleic acid 227
Thymine) mostly congregate at the higher end of the BV/TV and Tb.N values (Mean 228
BV/TV = 17%); lines with a CC variant (where C refers to the nucleic acid Cytosine) 229
are at the lower end (mean BV/TV = 10%); and those with a CT variant are at the 230
intermediate range (Fig. 5). Largely, the more the trait examined is distantly correlated 231
with BV/TV, the less differentiated the CC and TT variants are, at the SNP 232
UNC20471277. This is accentuated in vBMD where there is a weak correlation with 233
BV/TV (Table S1) and leveled CC and TT groups (P value = 0.8 Welch's two sample 234
t-test). 235

Candidate genes identified by merge analysis and RNA-seq 237

To identify the gene most likely driving the skeletal trait, we next performed a merge 238
analysis and RNA-seq analysis and used a scoring system to rank the potential 239
candidates. 240

Merge analysis uses the catalogue of variants segregating in the eight CC 241
founders to impute the genotype dosage of each SNP in each CC line, based on the 242
haplotype reconstruction used for haplotype association [24]. Candidate causal 243
variants, if they exist, would be expected to be more significant (have higher logP 244
values) than the haplotype-based test in the flanking region. We found that *Trl7* had the 245
highest density of polymorphisms (grey and crimson dots in Fig. 6) with merge-logP 246
values above the haplotype logPs (continuous black line in Fig. 6), while *Trl8* and *Cr12* 247
had very few. The merge logP values of the two latter loci congregated more upstream, 248

in accordance with the left-skewness of their respective CI simulations (Fig. 6; Fig. S3). 249

By calculating the relative density of merge logP values which are considerably higher 250

than the haplotype merge logPs -and above the 99th percentile threshold for each scan - 251

at intervals defined by each gene within the QTLs (in meaningful regions; usually 252

between the 50th and 90th CI percentile) we could rank the genes according to their 253

merge analysis results (Table S4). For example, while the proportion of merge analysis 254

SNPs for BV/TV and Tb.N with logPs greater than that of the haplotype scan is 1.4% 255

at the genome-wide scale (as well as at the region spanning the 95% CI, between ~112 256

– 118 Mb), it is 9.4% and ranked 5/36 (for BV/TV) or 14.07% and ranked 3/36 (for 257

Tb.N) at the region in which the gene *Rhbdj2* is situated (~116.5 – 116.6 Mb) (See 258

Table S4 and further discussion below). 259

To strengthen the criteria that classifies potential putative candidate gene as true 260

positives, we analyzed RNA-seq datasets of osteoclasts (Fig. S4) and osteocytes (Fig. 261

S5) made publicly available by Kim *et al.* [25] (Gene Expression Omnibus accession 262

number GSE72846) and St John *et al.* [26] (Gene Expression Omnibus accession 263

number GSE54784). We focused on local maximas that span ~0.5 Mb in and around 264

the peaks suggested by the merge analysis, for each QTL. From the raw count reads we 265

found that *Trl7* had the strongest gene expression differential; e.g while *Mxra7* in the 266

osteoclasts was expressed to a negligible degree (Fig. S4A), it had a strong presence in 267

osteocytes (Fig. S5A), whereas the genes of the other loci had much less prominent 268

differences. This suggests that genes at *Trl7* are differentially expressed between 269

osteocytes and osteoclasts more prominently than in the other loci. 270

For each of the genes with the highest merge analysis scores in *Trl7*, we 271

attributed an *osteoclast* and *osteocyte* RNA-seq (S_{RNA-seq1} and S_{RNA-seq2}, respectively) 272

score based on the following formula: $S_{RNA-seq} = 1 - \frac{r}{n}$; where r is the local gene rank (sorted by the raw read count) and n is the total number of genes at the locus, where $n=10$, if $n>10$. For example, *Ube2o* had an *osteoclast* RNA-seq score of 0.8 (ranked 2nd out of $n>10$ genes) and *osteocyte* RNA-seq score of 0.8. *Rhbd2* had an *osteoclast* RNA-seq score of 0.9 (ranked 1st) and *osteocyte* RNA-seq score of 0.7.

We then summed up the cumulative *MS* (Merge and Sequencing) score for each gene, defined as $MS(i) = \ln(M_{strength}(i)) + (S_{RNA-seq1}(i) + S_{RNA-seq2}(i))$, where $M_{strength}$, $S_{RNA-seq1}$, and $S_{RNA-seq2}$ refer to the merge rank (or strength), osteoclast RNA-seq score, and osteocyte RNA-seq score of a given gene (i), respectively. Our analytical approach and scoring system enabled us to shortlist the most plausible causal genes at the QTLs. In *Trl7*, *Ube2o* had an $MS_{Ube2o} = 2.4 + (0.8 + 0.8) = 4.0$; while *Aanat* scored $MS_{Aanat} = 2.7 + (0 + 0) = 2.7$, and *Rhbd2* scored $MS_{Rhbd2} = 2.6 + (0.9 + 0.7) = 4.2$. In the other loci, the highest ranked genes were *Klf17* and *Kdm4a* for *Trl8*; *Barhl2* and *Zfp644* for *Trl9*; *Asph* and *Gdf6* for *Cr11*; and *Hfe2*, *Acp6*, *Bcl9*, and *Notch2* for *Cr12*.

Because BV/TV is a predominant parameter in bone biology, we first focused on *Trl7*. *Rhbd2* had a merge strength of 14% (the 3rd strongest at the QTL and 2nd at the 50% CI), and a local maxima at the RNA-seq of the osteoclasts. It was located near the haplotype mapping peak, and because it received the highest cumulative *MS* score, *Rhbd2* was retained for validation. The comprehensive list of the genes under the 50, 90, and 95% CI of the QTL, is supplied in table S4.

Validation of the skeletal role of *Rhbd2* in knock-out mice

Femora of male mice ($n=14$) null at *Rhbd2* (on a C57Bl/6J background) were collected, on which we measured the same morphometric traits as above, including BV/TV, Tb.N,

Tb.Th, SMI, Tb.SP, Ct.Th, and vBMD. These were compared to their wild-type (WT) counterparts (n=13), after adjusting for batch, age and weight.

Strikingly, we found that *Rhbd2*^{-/-} mice had a significant bone phenotype. In line with our GWAS data, *Rhbd2*^{-/-} mice displayed a highly significant increase in BV/TV and Tb.N (Fig. 7, 8). As expected, *Rhbd2* KO also affected other microstructural parameters, partly due to the high correlation between the trabecular traits. After adjusting for confounders, we observed a significant difference between KO and WT animals in Tb.Sp (P value < 0.001; uncorrected P value = 0.017), SMI (P value = 0.03; uncorrected P value = 0.156) and Conn.D (P value = 0.008; uncorrected P value = 0.046). Tb.Th and vBMD were not affected by the knockout. Although the cortical compartment did not display a haplotype peak at the vicinity of *Trl7* in the CC animals. However, after adjusting for confounders, we observed a significant difference in Ct.Th between KO and WT bones (P value = 0.01), suggesting that the role of *Rhbd2* is not limited to the trabecular compartment (Fig. 7).

Discussion

Genetic reference population (GRP) are very efficient for the study of complex traits and biological systems, because (i) genotyping is only required once (“genotype once, phenotype many times”, see below), and (ii) replicate individuals with the same genotype can be generated at will allowing for optimal experimental designs [27].

This article is the second to present the results of an ongoing quest to delineate the genetic determinants that govern microstructural bone traits [15]. Here we characterize several key microstructural properties of the mouse femoral bone to assess the extent to which they are heritable; to what environmental perturbations they are

prone; and to identify candidate genes by which they are controlled. Our approach 319
narrowed down a small number of putative candidate genes for 6 of the 8 examined 320
phenotypes. Following merge analysis and RNA-seq, we validated our leading 321
candidate gene, *Rhbd2*, using a knockout model, which confirmed the critical role of 322
Rhbd2 on bone mass accrual and homeostasis. 323

While the heritability rates assessed here - determined to be over 60% for all 324
traits - confirmed our previous findings, the degree to which sex explains the 325
phenotypic variation was very subtle, and appeared only for the cortical traits; this 326
discrepancy may be due to the specific cohort composition used in this study (Table 327
S3), which includes a sex bias due to smaller number of females than males. We found 328
a total of five QTLs in six traits; BV/TV and Tb.N shared one QTL, and Tb.Th, Tb.Sp, 329
vBMD, and Ct.Th yielded one each. Importantly, although bone microarchitecture 330
factors are complex traits, our analyses highlighted no more than two loci for each trait; 331
it is likely that analyzing a larger number of CC lines would result in the identification 332
of further loci. 333

Our analyses yielded three QTLs for the trabecular traits and 2 QTLs for the 334
cortical traits. These are referred to as *Trl7-9*, and *Cr11-2*, respectively. In addition, and 335
in close proximity, to *Rhbd2* (Rhomboid 5 Homolog 2; elaborated below), *Trl7* 336
includes *Ube2o* (Ubiquitin Conjugating Enzyme E2 O), which encodes an enzyme that 337
is an important interactant of SMAD6. Ube2o monoubiquitinates SMAD6, and thereby 338
facilitates the latter to bind BMP 1 receptors [28]. The signal transduction of BMP 1 is 339
in turn limited [29,30], and endochondral bone formation, instead of ossification, is 340
favored. Importantly, 4 week-old SMAD6-overexpressed mice have significantly lower 341
humeral and vertebral BV/TV ratios than their controls [29]. 342

At *Trl8*, *Klf17*, *Kdm4a*, and *Dmap1* are likely putative candidate genes. Since
Klf17 (Kruppel-Like Factor 17) is part of a network that includes BMPs [31] it is more
likely than a nearby gene, *St3gal3* (ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase
3), to affect bone traits, although the latter has a greater merge strength. *Kdm4a* (Lysine
Demethylase 4A) encodes a histone demethylase that promotes the differentiation of
embryonal stem cells (ESCs) to an endothelial fate [32]; endothelial cells are implied
in regulation of bone formation [33]. *Dmap1* (DNA Methyltransferase 1 Associated
Protein) which encodes a DNA-methyl transferase known to regulate obesity
complications, and is differentially methylated in women with polycystic ovary
syndrome [34,35] had the highest meaningful merge density, and it might
epigenetically regulate bone formation as well. *Trl9* includes two genes of interest to
bone biology: *Barhl2* and *Zfp644*. By interacting with caspase3, which is essential for
ossification [36], *Barhl2* (BarH Like Homeobox 2) can inhibit β -catenin activation [37],
and regulate the expression of chordin, a BMP signaling-detrimental protein [38].
Zfp644 (Zinc Finger Protein 644), which encodes a transcription repressor zinc-finger
protein, is upregulated in eight week-old ovariectomized mice following treatment with
estradiol [39], a treatment associated with reduced bone loss [40]. Further support for
the candidacy of *Barhl2* and *Zfp644* is given by the role of *Barhl2* in the development
of amacrine cells [41,42] and the association of *Zfp644* with myopia [43], a condition
speculated to propagate from amacrine cell signaling [44]; interestingly myopia was
linked to reduced postnatal bone mineral content in humans [45] and decreased
expression of BMP 2 and 5 in guinea pigs [46].

The first of two cortical loci, *Cr11* contains as likely candidates the genes *Asph*
and *Gdf6*. *Asph* (Aspartate Beta-Hydroxylase) encodes a protein that has a role in
regulating calcium homeostasis, which may affect bone metabolism [47]. *Gdf6* (Growth

Differentiation Factor 6) is bone morphogenetic protein 13: mice with mutated *Gdf6* 368
exhibit deformed bone formation in various skeletal sites; it is among the earliest known 369
markers of limb joint formation [48], expressed in joints of ankle and knee. In *Gdf6* 370
homozygous mutant mice, bones fuse at the joints early at the segmentation stage [49]. 371
For *Crl2*, we found *Hfe2*, *Bcl9*, *Notch2*, and *Prkab2* as potential candidate genes. *Hfe2* 372
(Hemochromatosis Type 2 (Juvenile)) encodes the BMP co-receptor hemojuvelin 373
which is expressed in skeletal muscles [50] and is responsible for juvenile 374
hemochromatosis, a condition linked to sex hormones depletion and osteoporosis [51]. 375
Bcl9 (B-Cell CLL/Lymphoma 9), the mammalian ortholog of the gene *Legless*, encodes 376
a protein essential to the Wnt/beta-catenin signaling which is important for bone 377
metabolism [52], without which the nuclear localization of β -catenin and myocyte 378
differentiation are compromised [53]. Of note, there are mutual effects between bone 379
and muscle, and accumulating evidence suggest many genes show pleiotropism with 380
respect to muscle strength and bone parameters [54]. *Notch2* encodes a member of the 381
notch protein family, which influence both osteoblasts and osteoclasts [55]; 382
specifically, *Notch2* is associated with the rare Hajdu-Cheney syndrome, that includes 383
severe osteoporosis as one of its main symptoms [56,57]. For this gene, we did not find 384
any significant merge logPs included within its limits. Interestingly, *Sec22b*, an 385
adjacent gene, had the strongest merge logP marks in this locus but no documented link 386
to bone biology. The third-strong gene in terms of merge values was *Prkab2* (Protein 387
Kinase AMP-Activated Non-Catalytic Subunit Beta 2). It encodes an enzyme which is 388
the regulatory subunit of mitogen-activated protein kinase (AMPK). AMPK widely 389
affects bone metabolism [58]. 390

Rhbdf2 is not yet supported by peer-reviewed reports as bearing a relation to 391
bone. Based on its closeness to the *Tlr7* peak (within the 50% CI), its merge strength 392

and RNA-seq in bone cells, we identified *Rhbd2* as a likely causal gene associated with 393
BV/TV and Tb.N. We therefore analyzed the bone phenotype of *Rhbd2*^{-/-} mice, to 394
validate the role of this gene in the modeling of the femoral cortex and trabeculae. 395
Rhbd2 deletion affected all the examined trabecular traits as well as Ct.Th. While the 396
effects on BV/TV and Tb.N were in line with the haplotype mapping, the *Rhbd2* locus 397
did not appear in any of the other traits. This however is expected, because the genetic 398
architecture of the working cohort is such that the assumed contributing variant of 399
Rhbd2 is diluted and compensated, resulting in a QTL detected only for the most 400
affected traits. Noticeably, Tb.Sp differed greatly between the *Rhbd2*^{-/-} and control 401
mice but did not show up at the haplotype mapping; this might be due to (i) the great 402
diversity of the wild-type mice in Tb.Sp, and/or (ii) the need for complete knockout 403
rather than a mere SNP to detect significant changes in Tb.Sp, and/or (iii) the SNPs 404
giving rise to *Trl7* are functioning variants, with differential behavior affecting only 405
BV/TV and Tb.N. A similar interpretation may be valid for the cortical phenotype of 406
the *Rhbd2*^{-/-} mice. Importantly, the significant QTL peak we found in our GWAS for 407
BV/TV and Tb.N ended up revealing a gene that has an important skeletal function in 408
both the trabecular and cortical bone compartments. 409

Rhbd2 encodes the iRhom2 protein, a polytopic membrane protein that is a 410
catalytically inactive member of the rhomboid intramembrane serine proteases 411
superfamily [59]. iRhom2 is necessary in macrophages for the maturation and release 412
of the inflammatory cytokine tumor necrosis factor α (TNF α): it acts in the trafficking 413
of TACE, the protease that releases active TNF α from its membrane-tethered precursor 414
[60,61]. iRhom2 is also implicated in EGF-family growth factor signaling [62–64]. 415
With a recent report of its role in trafficking of another protein, STING, it appears that 416
iRhom2 may have a wider role in regulating membrane trafficking [65]. iRhom2 was 417

also implicated in the regulation of CSF1R (macrophage stimulating factor 1 receptor),
a critical regulator of osteoclasts differentiation and survival [61,66–68]. *In vivo*,
Rhbd2 has been implicated in esophageal cancer, wound healing, bone marrow
repopulation by monocytic cells, and inflammatory arthritis [63,69–71].

Further work will be needed to identify the mechanism by which iRhom2
controls bone homeostasis; a possible direction could involve a positive feedback loop
that leads to differentiation of macrophages to osteoclasts. Indeed, iRhom2 stimulates
the secretion of TNF α by macrophages [68,72]; hyperactivates EGFR [73,74]; and
regulates CSF1R [75,76]. Although *Rhbd2* is expressed in both the osteocyte and
osteoclast lineages, one cannot rule out the possibility that this gene regulates bone
remodeling by virtue of its expression in non-skeletal cells.

In summary, our analyses disclose several putative genes, several of which are
newly linked to a role in bone biology. A confirmation of one such gene, *Rhbd2*,
provides the first conclusive evidence for its effects on bone microstructure.
Importantly, this study is the first demonstration of a physiological role of *Rhbd2*. This
finding prompts future investigations to elucidate the exact mechanism of action of
Rhbd2 and its contribution to osteoporosis in humans.

Materials and Methods

Mice

Mice aged 10 to 13 weeks (male $n = 103$; female $n = 71$), from 34 different CC lines
(average of 5 mice per line) were used in this study. The mice were at inbreeding
generations of 11 to 37, which correspond to 80-99.9% genetic homozygosity,
respectively. The mice were bred and maintained at the small animal facility of the

Sackler Faculty of Medicine, Tel Aviv University (TAU), Israel. They were housed on 441
hardwood chip bedding in open-top cages, with food and distilled water available *ad* 442
libitum, in an identical controlled environment (temperature = $25 \pm 2^\circ\text{C}$; 60% $\leq$ 443
humidity $\leq$ 85%) and a 12-hour light/dark cycle. All experiments protocols were 444
approved by the Institutional Animal Care and Use Committee (IACUC M-13-014) at 445
TAU, which follows the NIH/USA animal care and use protocols. The *Rhbd2* knock 446
out mice and their WT counterparts were bred and maintained at the University of 447
Oxford as approved by license PPL80/2584 of the UK Home Office. 448

Specimen collection 449

Mice were intraperitoneally euthanized with cervical dislocation performed 450
approximately one minute after breathing stops owing to 5% Isoflurane inhalation. The 451
Rhbd2 knock out mice and their WT counterparts were euthanized by inhalation of a 452
rising concentration of carbon dioxide followed by dislocation of the neck. Left femora 453
were harvested and fixed for 24 hours in 4% paraformaldehyde solution, and then stored 454
in 70% ethanol. 455

μ CT evaluation 456

Whole left femora from each mouse were examined as described previously [77] by a 457
 μ CT system (μ CT 50, Scanco Medical AG, Switzerland). Briefly, scans were 458
performed at a 10- μm resolution in all three spatial dimensions. The mineralized tissues 459
were differentially segmented by a global thresholding procedure [78]. All 460
morphometric parameters were determined by a direct 3D approach [79]. Parameters 461
analyzed were determined in the metaphyseal trabecular bone, which included 462
trabecular bone volume fraction (BV/TV; %), trabecular thickness (Tb.Th; μm), 463
trabecular number (Tb.N; mm^{-1}), trabecular connectivity density (Conn.D; mm^{-3}), 464

trabecular structure model index (SMI), and trabecular separation (Tb.Sp; mm). Two
additional parameters are characteristics of the mid-shaft diaphysis section, and include
volumetric bone mineral density (vBMD; mgHA/cm³ [mg Hydroxy-Apatite per cm³])
and cortical thickness (Ct.Th; mm). All parameters were generated and termed
according to the Guidelines for assessment of bone microstructure in rodents using
micro-computed tomography [80].

Genotyping

A representative male mouse from each line was initially genotyped with a high mouse
diversity array (MDA), which consists of 620,000 SNPs (Durrant et al., 2011). After
about two intervals of 4 generations of inbreeding, all the CC lines were regenotyped
by mouse universal genotype array (MUGA, 7,500 markers) and finally with the
MegaMuga (77,800 markers) SNP array to confirm their genotype status [19]. The
founder-based mosaic of each CC line was reconstructed using a hidden Markov model
(HMM) in which the hidden states are the founder haplotypes and the observed states
are the CC lines, to produce a probability matrix of descent from each founder. This
matrix was then pruned to about 11,000 SNPs by averaging across a window of 20
consecutive markers for faster analyses and reduction of genotyping errors [81].

Statistical analyses and data acquisition

All statistical analyses were performed with the statistical software R (R core
development team 2009), including the package happy.hbrem [82].

Heritability and covariate effects. Broad-sense heritability (H^2) was obtained for each
trait by fitting the trait (the independent variable) to the CC line label in a linear
regression model that incorporates relevant covariates (sex, age, batch, month, season,

year, and experimenter). ANOVA test was used to compare a null model (in which all
dependent variables are set to 0) with linear models that fit the covariates and the CC
line labels to the examined trait. Practically, the difference between the residual sum of
squares ($RSS; \sum_1^n (\mu_i - \hat{\mu}_i)^2$) of the covariates model and that of the CC-line labels
can be seen as the net genetic contribution to the trait. Thus, this difference divided by
that of the covariate model gives an estimation of the heritability. Each covariate was
calculated separately, by dividing the RSS difference between the null and full model
with that of the null model. Let F_0 be the model that fits the trait to the covariates; F_1
the model that fits the trait to the covariates and the CC line label; and F_{00} the null
model. Then, employing ANOVA, heritability is:

$$H^2 = (RSS(F_0) - RSS(F_1)) / RSS(F_{00}).$$

Similarly, the effects for each covariate were computed separately, by fitting each in
 F_0 . The covariate effect is thus:

$$(RSS(F_{00}) - RSS(F_1)) / RSS(F_{00}).$$

H^2_n was derived from H^2 according to Atamni *et al.* [23].

Haplotype mapping. Each trait was fitted in a multiple linear regression model to the
probability matrix of descent from each founder, including sex and age as covariates.
The expected trait value from two ancestors, termed the genetic fit, is:

$$\mu_i = \mu + \sum_{s,t} F_{Li}(s,t)(\beta_s + \beta_t) = \mu + \sum_s \sum_t F_{Li}(s,t)\beta_s$$

where μ is a normally distributed trait mean, with sex and age incorporated; $F_{Li}(s,t)$ is
the probability of descent from founders s and t ; and $\beta_s + \beta_t$ is the additive effect of
founders s and t . Because $\sum_s \sum_t F_{Li}(s,t) = 2$ for a diploid organism, the maximum

likelihood estimates $\hat{\beta}_s$ are not independent. Thus, they are expressed here as
differences from the WSB/EiJ founder effect, so that $\hat{\beta}_{WSB} = 0$. Number of members
per line was weighted and integrated in the linear model. ANOVA was then used to
compare this model with a null model where the founder effects are all set to 0; the
resulting F -statistic yielded the significance of the genetic model vs. the null model and
the negative 10-base logarithms of the P values (logP) were recorded.

Permutations of the CC lines between the phenotypes were used to set
significance thresholds levels. Founder effects are the estimates derived from the
multiple linear regression fit above.

Regional heritability (H_r^2) was hereafter computed by ANOVA as in the broad-
sense heritability computation, except that here null linear regression fit was compared
with a genetic linear regression fit with the probability matrix of the founder descent at
the peak QTL as the explanatory variable.

False discovery rate (FDR) was calculated using the p.adjust function in R, with
the method "BH" (Benjamini-Hochberg [83]).

Confidence intervals. Confidence intervals (CIs) were obtained both by simulations and
by the quick method of Li, 2011 [84]. In the simulations, we resampled the residuals of
the original linear regression fit at the peak of each QTL and rescanned 100 intervals
within 7-10 Mb of the original loci to find the highest logP. Accordingly, following
Durrant *et al.* [22], 1000 QTLs were simulated: if $\hat{t}_i$ is a random permutation of the
residuals of fitted genetic model at the QTL peak, and K is a marker interval in a
neighborhood of 3.5 to 5 Mb of the QTL peak L , a set of values for each trait, Z_{iK} is
provided by:

$$Z_{iK} = \hat{t}_i \exp(\hat{\mu} + \sum_s X_{Kis} \hat{\beta}_s). \quad 533$$

Merge analysis. In the merge analysis the eight founder strains are partitioned and 534
merged according to the strain distribution pattern (SDP) of the alleles at the 535
quantitative trait nucleotides (QTN) within a given QTL (formerly obtained by the 536
initial mapping). If we denote the polymorphism as p , then $X_p = 1$ if s has allele a at p , 537
and $X_p = 0$ otherwise [24]. Then, at p , the probability of i to inherit alleles a and b from 538
 s and t , respectively, within L is 539

$$G_{pi}(a, b) = \sum_{s,t} X_p(a, s) X_p(b, t) F_{Li}(s, t). \quad 540$$

This merges the founder strains by p . The expected trait value in the merged strains 541
can now be inferred by 542

$$\sum_{a,b} G_{pi}(a, b) (\beta_a + \beta_b). \quad 543$$

Because this is a sub-model of the QTL model, it is expected to yield higher logP values 544
due to a reduction in the degrees of freedom. Significance was obtained by comparing 545
the merge model with the QTL model. Individual genes were extracted from the Sanger 546
mouse SNP repository (http://www.sanger.ac.uk/sanger/Mouse_SnpViewer). 547

Merge strength. We ranked the list of genes under each QTL according to the density 548
of merge logPs associated with them: only genes that had merge logPs above the 549
haplotype mapping reading, and above the threshold, plus logP=1 were included. We 550
then computed the relative density according to the density of a given gene's merge 551
logPs versus the locus' merge logP density. Let g be the region encompassed by a gene; 552
 l the region encompassed by a QTL; and mp the merge logP values above the haplotype 553

P values plus 1. Then $g_i(mp) = 1$ if at SNP i there exists a mp and 0 otherwise. 554

Similarly $l_j(mp) = 1$ if at SNP j there exists a mp and 0 otherwise. The merge strength 555

(M_{strength}) is therefore: 556

$$M_{\text{strength}} [\%] = 100 * \frac{\sum_i g_i(mp)}{\sum_j l_j(mp)} \%. \quad 557$$

RNA-seq data. RNA-seq data from osteoclasts and osteocytes was obtained from gene 558

expression omnibus (GEO) database (accession numbers GSE72846 and GSE54784) 559

and mapped to the *mus musculus* assembly mm10 using tophat v. 2 [85]. Read counts 560

were then casted on the loci of interest using the R (R Core Team 2015) package 561

GenomicAlignments and raw read counts were taken. For the osteocytes, the data of 562

basal level day 3 was averaged. 563

MS score. Based on the merge analysis and RNA-seq data we ranked each gene 564

according to the score in each category: $MS(i) = \ln(M_{\text{strength}}(i)) + (S_{\text{RNA-seq1}}(i) + S_{\text{RNA-seq2}}(i))$, 565

where MS , M_{strength} , $S_{\text{RNA-seq1}}$, and $S_{\text{RNA-seq2}}$ refer to the merge and sequencing 566

score, merge rank (or strength), osteoclast RNA-seq score, and osteocyte RNA-seq 567

score of a given gene (i), respectively. $S_{\text{RNA-seq1,2}}$ scores were generated according to the 568

formula $S_{\text{RNA-seq}} = 1 - \frac{r}{n}$; where r is the local gene rank (sorted by the raw read count) 569

and n is the total number of genes at the locus, where $n=10$, if $n>10$. 570

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Figure legends 884

Figure 1. μ CT images of trabecular and cortical bone of the femora of representative 885
male CC mice. **(A)** Trabecular bone. Left: IL-2452, Right: IL-1513. **(B)** Cortical 886
bone. Left: IL-785, Right: IL-2689. 887

Figure 2 Trabecular and cortical traits distributions across the CC lines. X-axis is the lines, y-axis is the trait means **A**. From top left, counter-clockwise: BV/TV (%), Tb.N (mm⁻¹), Tb.Th (um), Conn.D (mm⁻³), SMI, and Tb.Sp (mm). **B**. Left, vBMD (mgHA/cm³), right, Ct.Th (mm). Color codes group line(s) which significantly differ from other groups. Lines are ordered inconsistently among the traits, per trait-specific descending order. Refer to Table S3 for more details.

Figure 3 Haplotype association maps for the trabecular and cortical traits. X-axis is the position on the chromosome, y-axis is the -logP value of the association. Lower threshold represents the 95th percentile of 200 simulations, and top represents the 99th percentile. Loci above the 99% cut-off were further investigated. From top to bottom: BV/TV, Tb.N, Tb.Th, Tb.Sp, vBMD, and Ct.Th.

Figure 4. Ancestral effects relative to WSB. Y axis is the strain deviation relative to WSB, x axis is the different strains of the eight CC founders. **(A)** to **(F)**: *Trl7* to *Trl9*, *Crll*, and *Crl2*, respectively.

Figure 5 Traits distribution at the marker UNC20471277 across bearers of homozygous and heterozygous alleles, separated by sex. X-axis is the allelic variation at the marker, y-axis is the trait value.

Figure 6 Merge analysis. Readings below $\log P = 4$ are elided for brevity. X axis is the position on the genome in Mb; y left axis is the $\log P$ score; y right axis is the recombination rate scale; colored bars are genes (note that only strong putative candidate genes are shown.); cyan line is the recombination rate; black continues line is the haplotype test's peak; dashed line is the 99% permutation threshold. **(A) to (F)**: Trl7 of BV/TV, Trl7 of Tb.N, Trl8 of Tb.Th, Trl9 of Tb.Sp, Crl1 of Ct.Th, and Crl2 of vBMD, respectively.

Figure 7 *Rhbd2* knockout versus wildtype for each of the studied traits. Left is KO, right is WT. PV is the confounder-adjusted P value. The unadjusted P value is in brackets. PV = 0 means PV < 0.001.

Figure 8 μ CT images of three-dimensional representative cortical and trabecular bones reconstructions for *Rhbd2* knockout and wildtype. Left: KO, right: WT. **(A)** Trabecular bone. **(B)** Cortical bone. All samples were of male mice, aged 11 weeks.

Supporting information captions

Figure S1 *Trait distributions for Males and Females. A1, A2*: Trabecular and cortical bone, respectively, males. **B1, B2**: trabecular and cortical bone, respectively, females. X-axis is the lines, y-axis is the trait means **A**. From top left, counter-clockwise: BV/TV (%), Tb.N (mm⁻¹), Tb.Th (um), Conn.D (mm⁻³), SMI, and Tb.Sp (mm). **B**: Left, vBMD (mgHA/cm³); right, Ct.Th (mm). Color codes group line(s) which

significantly differ from other groups. Lines are ordered inconsistently among the traits, according to trait-specific descending order.

Figure S2 *Haplotype association maps for the trabecular and cortical traits.* X-axis is the position on the chromosome, y-axis is the $-\log P$ value of the association. Lower threshold represents the 95th percentile of 200 simulations, and top represents the 9th percentile. Loci above the 99% cut-off were further investigated. From top to bottom: Conn.D, SMI.

Figure S3 *Confidence interval simulations.* Loci at a neighborhood of 3-5 Mb around the original locus were simulated by permuting the residual sum of squares of the related phenotype. Maximum $\log P$ was obtained along with its relative position in Mb to the original QTL (histograms, left panels), and with the number of markers from the original QTL (boxplots, right panels). (A) and (B) show simulations results for the BV/TV and Tb.N loci. These determined with high confidence that the peak QTL is responsible for the effect seen in the haplotype scan, thus the narrow CI; (C) to (F) show simulation results for Tb.Th, Tb.Sp, Ct.Th, and vBMD, respectively; note the narrow CI for *Crl2* (vBMD), wide for *Trl8* (Tb.Th), and wider still for *Crtl*(Ct.Th).

Figure S4 *RNA-seq of osteoclasts.* Gene names are on the right of each plot. Green represents plus-stranded genes, black represents minus-stranded genes. Y-axis is the raw expression count, where the negative scale refers to minus-stranded gene count. Each bracket corresponds to a particular gene; left-to-right green (black) brackets fit green (black) top-to-bottom gene-names.

Figure S5 <i>RNA-seq of osteocytes.</i> Gene names are on the right of each plot. Orange	953
represents plus-stranded genes, black represents minus-stranded genes. Y-axis is the	954
raw expression count, where the negative scale refers to minus-stranded gene count.	955
Each bracket corresponds to a particular gene; left-to-right orange (black) brackets fit	956
orange (black) top-to-bottom gene names	957
Table S1 <i>Pearson's correlations between measured μCT traits.</i> Pairwise correlations	958
for each trait are given as Pearson's <i>r</i> . In bold are the 5 most strong correlations.	959
Table S2 <i>Covariates effects. LogP is the negative logarithm of P value.</i> Effects of	960
covariates (i.e., the degree to which each covariate explains the phenotypic	961
difference). Values were determined by regressing the covariates along with the CC	962
lines, and running an ANOVA test. Note the covariates prominent effect on the	963
cortical traits. The batch effect was the strongest, affecting Tb.Th, Ct.Th, and vBMD.	964
	965
Table S3 <i>Trait values per CC line included in this study (for both sexes combined).</i>	966
Trait means for each line, including number of members and standard error (SE). NA	967
means data were not available.	968
	969
Table S4 <i>A comprehensive list of all genes under the 95% confidence-interval (CI)</i>	970
<i>for the QTLs identified in this study.</i> Light blue is the 95% CI region; blue is the 90%	971
CI region; and pink is the 50% CI region. Merge strength column gives the	972
normalized dosage of merge logP values as outlined in the methods section. Gene	973
symbols in bold type are discussed in the manuscript. Genes under the QTLs for the	974
cortical and trabecular traits. Light blue is the 95% CI, blue is the 90% CI, and red is	975
the 50% CI. "Merge Strength" refers to the proportion of merge logP values	976

constricted to the region of the specific gene. Note that for *Trl7* which is common to 977
BV/TV and Tb.N, the average values between the two are provided. 978

Fig. 4

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Fig. 5

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Fig. 6

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Fig. 7

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