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Whole-genome sequencing identifies EN1 as a determinant of bone density and fracture

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Whole-genome sequencing identifies non-coding variants near *ENI* with large effects on bone mineral density

SUPPLEMENTARY INFORMATION

1. Cohorts

Cohort-level characteristics are described in **Supplementary Table 18**. **Supplementary Table 19** describes BMD measurements and covariates used in association testing for all cohorts.

2. Sequencing

Supplementary Table 20 summarizes the data generation method for sequencing-based cohorts. A detailed description is provided below for the whole genome and whole exome sequenced cohorts.

Whole Genome Sequencing

UK10K

ALSPAC and TwinsUK cohorts were sequenced to an average read depth of 6.7x through the UK10K program (www.uk10k.org). Whole genome sequencing was completed at the Wellcome Trust Sanger Institute and Beijing Genomics Institute. Briefly, DNA was sheared and subjected to Illumina paired-end DNA library preparation and sequenced using the Illumina HiSeq platform. Reads were aligned to the GRCh37 human reference, using BWA (v0.5.9-r16).¹ BAM files were further processed to realign around known INDELS, base quality score recalibration, addition of BAQ tags using GATK.

SNV calls were completed using samtools/bcftools (version 0.1.18-r579),² and were then called to produce a VCF file.³ The pipeline to create these calls is available from:

<https://github.com/VertebrateResequencing/vr-codebase/tree/develop>.

Sites were called using the Variant Quality Score Recalibration (VQSR)⁴ and GATK Unified Genotyper was used to recall the sites and alleles discovered by samtools. The VariantRecalibrator within GATK was used to first model the variants, then GATK ApplyRecalibration was applied to assign VQSLOD scores (see http://www.broadinstitute.org/gsa/wiki/index.php/Variant_quality_score_recalibration for more details).

VQSLOD score threshold was set at -0.6804 which improved site concordance with duplicate samples sequenced using high-depth exome sequence methods. Two further thresholds were applied: failure of HWE ($P < 1e-6$) and evidence of batch effects between samples genotyped at Sanger and BGI ($P < 1e-2$). In total, 4.2M variants were removed through these QC criteria. The final set contained over 42M SNVs.

In order to assess the validity of the sequence data, 61 samples were also sequenced using high-depth next generation exome sequencing (depth = 70x), described previously.⁵ 74,621 sites were shared out of the 86,322 sites that were called in the exome data, representing a sensitivity of 86.4% across the 35Mb bait region. We assessed the non-reference discordance rate and found that 0.2% were discordant, suggesting that the resultant dataset had high concordance with high depth exome sequencing.

Whole Exome Sequencing

AOGC

993 samples underwent exome capture and massive parallel sequencing. Sequencing libraries were constructed from 1.6ug genomic DNA using the Illumina TruSeqDNA sample preparation kit. Libraries were combined in pools of six for target capture using the Illumina TruSeq Exome Enrichment Kit. Libraries were assessed pre- and post-capture for both quality and yield using the Agilent High Sensitivity DNA assay and KAPA Library Quantification Kit. Massively parallel sequencing was performed using the Illumina HiSeq2000 to generate 100bp paired-end reads (2x100PE). Either 6 or 12 samples were run per flow cell lane using version 2 or version 3 SBS reagents respectively.

48 Illumina Data Analysis Pipeline software (CASAVA 1.8.2) was used for de-multiplexing and initial base
49 calling and sequence data was aligned to the current build of the human genome (hg19, released February
50 2009) using the Novoalign alignment tool (V2.08.02); sequence alignment files were converted using
51 SAMtools (v0.1.16) and Picard tools (<http://picard.sourceforge.net>) (v1.42) SNPs and indels were called
52 using the Genome Analysis Toolkit (GATK v2.2-3)⁶ including variant recalibration. Variants were
53 annotated using ANNOVAR and Variant Effect Predictor and GERP++ scores.^{7,8}

54 *FHS*

55 DNA samples were constructed into Illumina paired-end pre-capture libraries according to the
56 manufacturer's protocol (Illumina Multiplexing_SamplePrep_Guide_1005361_D) with modifications as
57 described in the *BCM-HGSC Illumina Barcoded Paired-End Capture Library Preparation* protocol.
58 Libraries were prepared using Beckman robotic workstations (Biomek NXp and FXp models). The
59 complete protocol and oligonucleotide sequences are accessible from the HGSC website
60 ([https://hgsc.bcm.edu/sites/default/files/documents/Illumina_Barcoded_Paired-
61 End_Capture_Library_Preparation.pdf](https://hgsc.bcm.edu/sites/default/files/documents/Illumina_Barcoded_Paired-End_Capture_Library_Preparation.pdf)).

62
63 Briefly, 1 µg of DNA in 100ul volume was sheared into fragments of approximately 300-400 base pairs in
64 a Covaris plate with E210 system (Covaris, Inc. Woburn, MA) followed by end-repair, A-tailing and
65 ligation of the Illumina multiplexing PE adaptors. Pre-capture Ligation Mediated-PCR (LM-PCR) was
66 performed for 6-8 cycles of amplification using the 2X SOLiD Library High Fidelity Amplification Mix (a
67 custom product manufactured by Invitrogen). Universal primer IMUX-P1.0 and a pre-capture barcoded
68 primer IBC were used in the PCR amplification. In total, a set of 12 such barcoded primers were used on
69 these samples. Purification was performed with Agencourt AMPure XP beads after enzymatic reactions.
70 Following the final XP beads purification, quantification and size distribution of the pre-capture LM-PCR
71 product was determined using the LabChip GX electrophoresis system (PerkinElmer).

72
73 For the hybridization step, four or six pre-capture libraries were pooled together (~250 ng/sample for a 4-
74 plex and ~166 ng/sample for a 6-plex, totaling 1 µg per pool). These pooled libraries were then hybridized
75 in solution to the HGSC VCRome 2.1 design (42Mb, NimbleGen) according to the manufacturer's protocol
76 *NimbleGen SeqCap EZ Exome Library SR User's Guide (Version 2.2)* with minor revisions. Human COT1
77 DNA and full-length Illumina adaptor-specific blocking oligonucleotides were added into the hybridization
78 to block repetitive genomic sequences and the adaptor sequences. Post-capture LM-PCR amplification was
79 performed using the 2X SOLiD Library High Fidelity Amplification Mix with 14 cycles of amplification.
80 After the final AMPure XP bead purification, quantity and size of the capture library was analyzed using
81 the Agilent Bioanalyzer 2100 DNA Chip 7500. The efficiency of the capture was evaluated by performing
82 a qPCR-based quality check on the four standard NimbleGen internal controls. Successful enrichment of
83 the capture libraries was estimated to range from a 6 to 9 of ΔCt value over the non-enriched samples.

84
85 Library templates were prepared for sequencing using Illumina's cBot cluster generation system with
86 TruSeq PE Cluster Generation Kits. Briefly, these libraries were denatured with sodium hydroxide and
87 diluted to 6-9 pM in hybridization buffer in order to achieve a load density of ~800K clusters/mm². Each
88 library pool was loaded in a single lane of a HiSeq flow cell, and each lane was spiked with 1% phiX
89 control library for run quality control. The sample libraries then underwent bridge amplification to form
90 clonal clusters, followed by hybridization with the sequencing primer. Sequencing runs were performed in
91 paired-end mode using the Illumina HiSeq 2000 platform. Using the TruSeq SBS Kits, sequencing-by-
92 synthesis reactions were extended for 101 cycles from each end, with an additional 7 cycles for the index
93 read.

94 *RS-I*

95 Genomic DNA of RS participants were prepared from blood and fragmented into 200-400 bp fragments
96 using Covaris Adaptive Focused Acoustics (AFA) shearing according to the manufacturer's instructions
97 (Covaris, Inc., Woburn, MA). Illumina TruSeq DNA Library preparation (Illumina, Inc., San Diego, CA)
98 was performed on a Caliper Sciclone NGS workstation (Caliper Life Sciences, Hopkinton, MA), followed
99 by exome capture using the Nimblegen SeqCap EZ V2 kit (Roche Nimblegen, Inc., Madison, WI). This
100 capture targets 44Mb of exonic regions covering 30,246 coding genes, 329,028 exons and 710 miRNAs.

101 Paired-end 2 x100 sequencing was performed on Illumina HiSeq2000 sequencer using Illumina TruSeq V3
102 chemistry.

103

104 Reads were mapped to the human genome hg19 with the BWA algorithm and processed with the Genome
105 Analysis Toolkit (GATK) to recalibrate base quality-scores and perform local realignment around known
106 indels.

107

108 *Variant calling and Quality Control*

109 Target coverage for each sample was computed with the GATK. Single nucleotide variants (SNVs) and
110 small insertions and deletions (indels) were called with the Unified Genotyper module of the GATK and
111 filtered to remove SNVs with annotations indicative of technical artifacts (such as strand-bias, low variant
112 call quality, or homopolymer runs). Samples with fewer than 76% of targeted bases covered to 20x, with an
113 abnormally high number of non-reference alleles or heterozygosity, or with an abnormally low
114 concordance with prior SNP array genotypes (based on the distribution across all samples) were excluded
115 from analysis. Any sample genotype at a site with a genotype quality (GQ) < 20 in the sample was ignored
116 (e.g. set as missing). Variants were annotated with the Variant Effect Predictor.⁷

117 *ESP*

118 The processes of library construction, exome capture, sequencing, and mapping were performed as
119 previously described. Sequencing was performed at the University of Washington (UW) and the Broad
120 Institute of MIT/Harvard (Broad). Single Nucleotide Variants (SNVs) were called using the UMAKE
121 pipeline at University of Michigan, which allowed all samples to be analyzed simultaneously, both for
122 variant calling and filtering. Briefly, we used BAM files summarizing Burrows-Wheeler Alignment (BWA)
123 alignments generated at the UW and the Broad as input.⁹ These BAM files summarized alignments
124 generated by BWA, refined by duplicate removal, recalibration, and indel re-alignment. We excluded all
125 reads that were not confidently mapped (Phred-scaled mapping quality < 20) from further analysis. To
126 avoid PCR artifacts, we clipped overlapping ends in paired reads. We then computed genotype likelihoods
127 for exome targeted regions and 50 flanking bases, accounting for per base alignment quality (BAQ) using
128 SAMtools.⁹ Variable sites and their allele frequencies were identified using a maximum-likelihood model,
129 implemented in glfMultiples.¹⁰ These analyses assumed a uniform prior probability of polymorphism at
130 each site. The final call-set was performed on 6,823 samples.

131

132 We used a support vector machine (SVM) classifier to separate likely true positive and false-positive
133 variant sites, applying SNP quality metrics including allelic balance (the proportional representation of
134 each allele in likely heterozygotes), base quality distribution for sites supporting the reference and alternate
135 alleles, and the distribution of supporting evidence between strands and sequencing cycle, amongst others.
136 We used as the positive training set variants identified by dbSNP or 1000 Genomes and we used variants
137 that failed multiple filters as the negative training set. We found this method to be effective at removing
138 sequencing artifacts while preserving good-quality data, as indicated by the transition-transversion (ti-tv)
139 ratio for previously known and newly identified variant sites, the proportion of high frequency variants
140 overlapping with dbSNP, and the ratio of synonymous to non-synonymous variants, as well as attempts at
141 validation of a subset of sites. A total of 1,908,614 SNVs passed the SVM filter. Among these, genotypes
142 with a corresponding read depth less than 10 were set to missing.

143 *ERF*

144 The exomes of 1,336 individual from the ERF population were sequenced at the Center for Biomics of the
145 Cell Biology department of the Erasmus MC, The Netherlands. The individuals were selected random with
146 the regards to ADHD scores. The sequencing was performed using the Agilent version V4 capture kit on an
147 Illumina HiSeq2000 sequencer using the TruSeq Version 3 protocol. The sequence reads were aligned to
148 the human genome build 19 (hg19) using BWA and the NARWHAL pipeline.^{11,12} The aligned reads were
149 processed further using the IndelRealigner, MarkDuplicates and TableRecalibration tools from the Genome
150 Analysis Toolkit (GATK) and Picard (<http://picard.sourceforge.net>). Genetic variants were called
151 using the Unified Genotyper tool of the GATK. About 1.4 million Single Nucleotide Variants (SNVs) were
152 called. After removing the low quality variants (QUAL < 150), variants with a call rate of < 95% and

153 variants that were out of Hardy Weinberg Equilibrium (HWE; p -value $< 10^{-6}$) and monomorphic variants,
154 and removing individuals with a low call rate ($< 90\%$) we retrieved 540,633 SNVs and 1,301 individuals.
155

156 3. Whole Genome Genotyping

157 All genome-wide genotyping has been previously reported,¹³ except for MrOs and SOF. Genotyping of
158 MrOS and SOF was performed using the Illumina HumanOmni1_Quad_v1-0 B array at the Broad Institute,
159 Cambridge, MA. Genotypes were called using the Illumina's BeadStudio calling algorithm. The sample
160 quality control exclusion criteria were sample call rate $< 97\%$, excessive autosomal heterozygosity, first
161 and second degree relatives, genotypic sex mismatch using X and Y chromosome probe intensities and
162 gross chromosome abnormalities.

163 Imputation of Whole Genome Genotyped Cohorts

164 All samples were imputed using the same reference panel and pipeline. Before imputation, the phased
165 UK10K haplotypes (3,781 samples) were merged with 1000 Genome haplotypes (1,092 samples) using
166 IMPUTE2¹⁴. We excluded singletons in both reference panels before merging. To account for the fact that
167 each panel might contain sites that are not present in the other panel, we imputed the missing variants from
168 each study into the other study and then combined the two reference panels as the union set of sites.¹⁵ A
169 total of 41,992,162 variants were finally presented in the combined 9,746 haplotypes.
170

171 The GWAS data were pre-phased first without a reference panel, using best-guess haplotypes.¹⁶ Then
172 variants from the combined UK10K/1000Genomes reference panel were imputed into the best-guess
173 haplotypes of genome wide genotyped cohorts. IMPUTE2 reports an information (info) metric as a
174 measurement of post-imputation quality. The info metric typically takes values between 0 and 1. A value
175 near 1 indicates a SNV has been imputed with high certainty, whereas a value of 0 means that there is
176 complete uncertainty about the genotypes. The info metric is used to filter poorly imputed SNVs. We
177 applied a cutoff of an info score of 0.4 to all included SNVs in the association analysis. **Supplementary**
178 **Table 21** denotes cohort-level imputation statistics. Separating the phasing and imputation steps in this way
179 is beneficial because most of the computational burden of imputation comes from accounting for the
180 unknown phase of the GWAS samples.

181 4. Association Testing

182 Single Variant Methods

183 Single variant association testing was undertaken using an additive model separately for each BMD site.
184 BMD was defined a forearm (distal 1/3 of radius), lumbar spine (L1-4) and femoral neck, as measured by
185 DXA. Since different DXA machines have known systematic differences in BMD measurements, BMD
186 was standardized within each cohort to have a mean of zero and a standard deviation of one. This also
187 assists in interpretation of data since the effect size of each allele can therefore be measured in standard
188 deviations. BMD was adjusted for age, age-squared, sex and weight. In sex-specific analyses, the sex term
189 was removed.
190

191 The type of software employed for single variant testing for each cohort is shown in **Supplementary Table**
192 **2**. Cohort-specific genomic inflation factors (lambdas) are also shown in **Supplementary Table 2** (the
193 mean lambda was 1.044).
194

195 Single variant tests were undertaken for variants with a $MAF \geq 0.5\%$, using an additive effect of the minor
196 allele at each variant in each cohort. For single-variant tests, statistical significance was declared after
197 taking into account all of the independent tests among variants with $MAF \geq 0.05\%$, as we have described
198 recently.¹⁷ Briefly, to estimate the number of independent SNVs in the UK10K reference panel, we
199 obtained sequence data from chromosome 3 on 2,577,674 genetic variants in 2,432 individuals from the
200 UK10K program. This comprised of 798,175 SNVs with $MAF \geq 0.005$ and the estimated number of SNVs
201 at this threshold, genome-wide was 12,451,530. We resolved these SNVs to 4,268,111 independent tests by
202 calculating the predicted effective number of independent tests and corrected $\alpha = 0.05$ by 4,268,111 to

203 yield a Bonferroni corrected genome-wide significant threshold of 1.2×10^{-8} for single-variant tests at MAF
204 $\geq 0.5\%$.

205 **Single Variant Meta-analysis**

206 Meta-analysis of cohort-level SNV association statistics was undertaken using fixed-effects meta-analysis
207 in GWAMA.¹⁸ Single variant Forest and Locuszoom plots are shown in **Extended Data Fig. 2 and 10**. QQ
208 and Manhattan plots for single variant associations are shown in **Extended Data Figure 1b and 1c**,
209 respectively. QQ and Manhattan plots for variants present in both exome-based and genome-based cohorts
210 are presented in **Extended Data Figure 11a and b**, respectively. Sex-stratified results for novel genome-
211 wide significant variants is provided in **Supplementary Table 22**.

212 **Rare Variant Analysis and Meta-analysis**

213 To test whether variants with low MAF influenced BMD we undertook a region-based collapsing method,
214 the sequence kernel association test (SKAT), to combine information across low-frequency (MAF $\leq 5\%$)
215 and rare (MAF $\leq 1\%$) or rare variants alone. Meta-analysis was conducted using skatMeta,¹⁹ where each
216 cohort provided the necessary intermediate files (skatCohort objects and snpInfo files). Analysis with
217 skatMeta requires providing beforehand the genotypes as input to the skatCohort function. For exome-seq
218 data, the analysis can accommodate genotype data from an entire chromosome; however, for whole
219 genome sequence or imputed data the analysis was not possible due to memory constraints. As a result,
220 genotype data was divided into gene regions, defined as the maximal extent of all isoforms of a gene, with
221 an additional 5kb on either end. This approach provided the flexibility to meta-analyze various subgroups
222 of SNVs across an entire gene, such as coding variants or variants under varying evolutionary constraint.
223

224 Each cohort performed the following:

- 225 1. Converted genotype data to binary IMPUTE2 format using QCTOOL.
- 226 2. Using custom scripts, prepared input files for analysis with skatCohort or skatFamCohort (for
227 family-based cohorts):
 - 228 a. Fetch all SNVs per gene region, where a region is defined as the maximal extent of all gene
229 isoforms plus a predefined flanking region (i.e. 5kb). Due to computational constraints, gene
230 regions greater than 150kb, are broken into multiple non-overlapping smaller regions of at most
231 100kb and at least 50kb.
 - 232 b. For each gene region, prepare files to support analysis with skatCohort and downstream meta-
233 analysis (SNPInfo files, and SNP statistics).
- 234 3. Using custom scripts, execute skatCohort or skatFamCohort per gene-region.
235

236 Cohort-level skatCohort objects were meta-analyzed using skatMeta by use of a custom R script. Within
237 each gene region, windows of 30 SNVs (overlapping by 10 SNVs) were analyzed using skatMeta with
238 default parameters, except for the use of the skatOMeta method and the “liu” p-value method. QQ and
239 Manhattan plots of all skatMeta results are shown in **Extended Data Fig. 7a and 7b**, respectively. These
240 results demonstrate that there were few signals that departed from the line of expectation under the null. All
241 significant findings from skatMeta were driven by single variants whose significance was also seen from
242 single variant testing. For example, **Extended Data Fig. 8a and 8c** shows that the skatMeta genome-wide
243 significant signals at *CPED1* were driven by single SNVs, which were present in several cohorts. These
244 SNVs also achieved genome-wide significance in single variant testing. We also identified a region in
245 *DOCK8* and *HEXB* that achieved genome-wide significance, but arose in only one cohort and we feel
246 requires further replication (**Supplementary Table 17 & Extended Data Fig. 8b**).

247 **Genome-wide suggestive and significant loci from region-based association tests**

248 **Supplementary Table 17** lists all genome-wide significant ($P < 1.2 \times 10^{-8}$) and suggestive loci ($P < 1.2 \times$
249 10^{-6}) for region-based association tests.

250 **Conditional Analyses for Single Variant Associations**

251 Conditional analysis was conducted using GCTAV.0.93.9.²⁰ This method uses an approximate conditional
252 analysis approach from summary-level statistics from the meta-analysis and LD corrections between SNVs
253 estimated from a reference sample. We used UK10K individuals as the reference sample to calculate the

254 LD information of SNVs. The associated regions flanking within 400kb of the top SNVs were extracted
255 and the conditional analyses were conducted within these regions. A stepwise model selection procedure
256 was performed to select independently associated SNVs with a threshold of $P \leq 5 \times 10^{-6}$. Then we
257 conditioned on these independently associated SNVs to seek secondary signals. Quartile-quantile plots for
258 these results are presented in **Extended Data Figure 1**. Conditional analyses of individuals variants
259 presented in **Supplementary Table 6** was conducted using GCTA v 0.93.9 using default parameters.

260 **Relationship between MAF and Effect Size**

261 For each BMD phenotype, genome-wide significant SNVs ($P \leq 1.2 \times 10^{-8}$) were collected and pruned for
262 linkage disequilibrium by first placing variants into 4 discrete MAF bins: [0.005 - 0.01], [0.01 - 0.05], [0.05
263 - 0.1], and [0.1 - 0.5]. For each MAF bin, we used the SNP Annotation and Proxy Search (SNAP)²¹ to
264 calculate the pair-wise correlation (r^2). We retained independent SNVs with the largest effect size by
265 removing all the other SNVs in LD ($r^2 > 0.2$).

266
267 Power for single point tests was calculated using standard approaches. Let N be the sample size, p be the
268 minor allele frequency, β represent the standardized effect of a SNV on a continuous phenotype
269 (standardized so that β is the effect per standard deviation of the phenotype), and let R^2 represent the
270 square of the correlation between a true genotype and a genotype measured with error. The non-centrality
271 parameter of the chi-squared distribution for a single SNV has been shown to be $NCP = 2(N - 1)p(1 -$
272 $p)\beta^2 R^2$.²² We calculated power from a non-central chi-squared distribution for the genome-wide
273 significance threshold of 1.2×10^{-8} and $r^2 = 1$.¹⁷ This was computed for each BMD phenotype and across 4
274 MAF bins (0.005-0.01, 0.01-0.05, 0.05-0.1, and 0.1-0.5).

275 **Fracture Meta-analysis**

276 Fracture was defined as a bone fracture resulting from trauma of any type since even high trauma fractures
277 are strongly associated with risk of osteoporotic fractures. Fractures included were those occurring at any
278 site, except fingers, toes and skull, after age 18. Both incident and prevalent fractures were included.
279 Fractures were verified by either radiographic, casting or clinical reporting.

280
281 All 1,482 genome-wide significant SNVs associated with femoral neck, lumbar spine and forearm BMD
282 were tested for their association with risk of fracture in a sample size of 10,459 cases and 27,581 controls
283 (38,040 total), of which 76.2% of the samples overlap the BMD discovery samples (**Supplementary Table**
284 **23**).

285
286 To obtain association statistics, in each participating study, a logistic regression model (GEE model for
287 family-based studies) adjusted for age, age², sex, height, weight, estrogen/menopause status (women only),
288 ancestral genetic background (PCs) and cohort-specific covariates (such as clinical centers) was applied.
289 The summary effect estimates for fracture risk were computed using fixed-effects inverse variance meta-
290 analysis unless heterogeneity was detected (as defined by $I^2 > 50\%$), where random effects models were
291 also used. To correct for multiple testing, we estimated the effective number of independent SNVs by
292 principal component analyses in the UK10K sequencing dataset and found that 74 principle components
293 explain 97% of the variance in the number of SNVs tested. We then used a Bonferroni correction to
294 estimate the type 1 error. The multiple testing-corrected significant p-value threshold is $P < 0.000676 =$
295 $(0.05/74)$.

296
297 No novel regions achieved genome-wide significance. Accounting for multiple testing for all BMD
298 genome-wide significant SNVs rs4727923 remained nominally significant ($P = 6.73 \times 10^{-4}$; **Supplementary**
299 **Table 4**) which marks the *WNT16* locus, variants at which we have previously described as genome-wide
300 significant for fracture.²³ rs61960954 near the *TNFSF11* locus, was also significant ($P = 3.3 \times 10^{-4}$
301 **Supplementary Table 4**).

302 **Rare Variant Meta-Analysis**

303 **Supplementary Table 17** demonstrates that two regions (DOCK8.1.2761-2790 and DOCK8.1.2421-2450)
304 spanning *DOCK8* on chromosome 9 were genome-wide significant in their association with femoral neck
305 BMD ($P = 1.9 \times 10^{-9}$ and 2.8×10^{-9}). This arose from the analysis of all SNVs with $MAF \leq 1\%$. These regions

306 have low cumulative MAF (summed across the region) of 0.0055 and 0.006, respectively. To assess
307 whether these regions-based signals were driven by multiple rare variants, or just one single variant, we
308 undertook drop-one analysis, which recomputed the test statistic after sequentially removing one SNV from
309 the collapsed region (**Extended Data Fig. 8b**). These analyses showed that signal at region
310 DOCK8.1.2421-2450 was driven by two variants, while DOCK8.1.2761-2790 was driven by one variant.
311 Neither of these regions contained single SNVs that achieved genome-wide significance on their own.
312

313 Next, we tested which cohorts provided this association signal, by sequentially removing one cohort at a
314 time from the test statistic and found that both regions were driven only by signal from the Framingham
315 cohort. While promising, we feel these signals need further follow-up and replication in other cohorts.
316

317 Lastly, we tested whether DOCK8 was expressed in mouse calvarial osteoblasts and found that its
318 expression increased importantly over development (**Extended Data Fig. 4a**).

319 **5. Replication Genotyping and Combined Meta-analysis**

320 For lumbar spine and femoral neck BMD sites, 17 SNPs were selected to genotype in additional 13
321 GENOMOS cohorts for replication analysis, the selection criteria of SNPs were listed in **Supplementary**
322 **Table 24**. Human samples from the 13 GENOMOS cohorts were genotyped on LGC Genomics by KASP
323 genotyping. KASP genotyping assays are based on competitive allele-specific PCR and enable bi-allelic
324 scoring of single nucleotide polymorphisms (SNPs) and insertions and deletions (Indels) at specific loci.).
325 Replication cohorts were selected only if they contained greater than 1,000 individuals prior to genotyping
326 quality control, given then frequency of the variants in the replication panel. Assays are deemed to be
327 working successfully if clusters are distinct and call rates are consistently high. The data is automatically
328 quality control checked on a per SNP basis. No Template Controls (NTCs) are included on each plate to
329 enable the detection of contamination or non-specific amplification. Two SNPs (rs13046645 and
330 chr3:50906922) were found to be monomorphic in initial test GENOMOS cohorts, thus, only 15 SNPs
331 were genotyped. For replication of rs148771817 for forearm BMD, genotyping was undertaken in a single
332 cohort, since this phenotype is rarely collected in population-based cohorts. Genotyping for this SNP was
333 similarly done at LGC Genomics using KASP genotyping in the AOGC cohort, where the age distribution
334 was matched to the discovery cohorts (age cutoff of <80 years was used). Genotyping quality metrics for
335 all variants are provided in **Supplementary Table 25**.
336

337 The genotyping data of the 16 SNPs were formatted into PLINK, the BMD phenotype were adjusted by sex,
338 age, age², weight and standardized to have a mean of zero and a standard deviation of one within each
339 cohort. The association analysis were performed by PLINK in each cohort, then combined analysis of the 9
340 discovery GEFOS cohorts and 13 GENOMOS cohorts of the 16 SNPs were conducted with GWAMA.
341 Cohort-level association results are provided in **Supplementary Table 26**. Meta-analysis results from
342 replication cohorts as well as combined discovery/replication cohorts are provided in **Supplementary**
343 **Table 27**.
344

345 Non-reference discordant rate (NRD) presented in main text and **Supplementary Table 5** was computed as
346 the proportion of non-reference genotypes found to be discordant between compared individuals as
347 compared total measured non-reference genotypes for that variant. For imputed variants, genotype
348 probabilities were converted to hard-calls using a genotype probability of 0.9.

349 **6. Functional Genomics**

350 **Functional Class Enrichment**

351 To ascertain if functional classification using GERP++ scores⁸ enriches for more significant GWAS
352 associations, we computed, for variants that fall below or above a series of GERP++ thresholds the
353 proportion that surpass FDR q-value of 0.05. Prior to FDR analysis, variants from meta-analysis (UK10K
354 + 1KG reference panel SNVs only) were annotated with GERP++ scores and distance to the nearest gene.
355 Variants were then pruned for LD with PLINK using an r^2 of < 0.2, window size of 100kb and step of 20
356 kb. For each SNV in this LD independent set, we selected the single SNV in LD with the lowest p-value,
357 thus yielding a final dataset of LD independent SNVs with lowest p-values. For each GERP++ threshold

358 considered (from 0 to 4, with step of 0.2), we partitioned SNVs into those above (cases) and below
359 (controls) the threshold. To adjust for bias (such as a correlation of GERP++ score to gene distance or
360 MAF) we matched cases to controls using the R package MatchIt (coarsened exact matching algorithm).
361 Subsequent to matching, FDR analysis was conducted on each set of case and control variants, and
362 proportion of variants surpassing FDR q-value of 0.05 was obtained.

363
364 For synonymous and deleterious variants, the above strategy was applied, except only coding variants were
365 partitioned based on whether or not they were synonymous or deleterious. Variant annotations were
366 computed using a local version of Variant Effect Predictor.⁷ Deleterious variants were classified as having
367 the following sequence ontology terms (frameshift_variant, inframe_deletion, inframe_insertion,
368 initiator_codon_variant, missense_variant, splice_acceptor_variant, splice_donor_variant, stop_gained, and
369 stop_lost).

370 **Chromatin Accessibility: DNase I Hypersensitivity Site Correlations**

371 For distal/promoter DHS correlation analyses, we utilized DHS data from ENCODE for 305 cell lines
372 (**Supplementary Table 28**). The distal DHS regions were defined as a peak region from ENCODE DHS
373 data that overlapped the GWAS SNP. For rs148771817 (*WNT16* locus), the peak region was obtained from
374 the “Digital DNase I Hypersensitivity Clusters in 125 cell types from ENCODE” track from the UCSC
375 Genome Browser. For rs188303909, (*EN1* locus), the peak region was obtained from the “Osteoblasts
376 DNase I HS Peaks from ENCODE/Duke” track from the UCSC Genome Browser. Promoter regions were
377 defined as 500 nucleotide flanking the TSS of genes within 500 Kb of the GWAS SNP being considered.
378 Within each of these promoter or distal DHS regions, we obtained the DHS measurements for all
379 overlapping 100nt bins across the 305 cell lines. Correlation analyses between the distal and promoter
380 DHS 100 nucleotide bins was conducted using Pearson correlation in the R statistical software package.
381 For each distal nucleotide bin, the maximal R^2 to across all bins per gene promoter region was reported.
382 Results for *EN1* and *WNT16* loci are shown in **Supplementary Tables 7 & 11**, and **Extended Data Fig. 3**.

383 **ENCODE Analysis of *EN1* variant rs188303909**

384 A genome-wide significant SNV 7kb upstream of *EN1* (rs188303909[T], MAF= 2.0%, effect size from
385 replication cohorts = +0.14 SD, $P_{\text{meta}} = 1.3 \times 10^{-9}$) overlaps a DNase I hypersensitive site (DHS) in
386 osteoblasts bound by CTCF (**Extended Data Fig. 3a**) as well as histone marks. We found evidence of
387 moderate correlation in chromatin accessibility between rs188303909 and the promoter of *EN1* across 305
388 cell types²⁴ (maximum $r^2 = 0.59$, $P = 1.5 \times 10^{-29}$, **Supplementary Table 7, Extended Data Fig. 3a**),
389 suggesting that the DHS overlapping rs188303909 may regulate expression of *EN1*. Hi-C data in human
390 embryonic stem cells further supports an interaction between the rs188303909 DHS and the *EN1* promoter,
391 as both sites lie within the same topologically associated domain (TAD).²⁵ Similarly, chromatin interaction
392 analysis by paired-end tag sequencing (ChIA-PET) data in MCF-7²⁶ cells also supports an interaction
393 between rs188303909 and *EN1* (**Figure 1**).

394 **ENCODE Analysis of *CPED1/WNT16* variant rs148771817**

395 A genome-wide significant within an intron of *CPED1* (rs148771817 [T], MAF= 0.9%, replication effect
396 size= +0.41 SD, $P_{\text{meta}} = 1.1 \times 10^{-11}$) overlaps a DNase I hypersensitive site (DHS) in 14 cell types
397 (**Extended Data Fig. 3b**). We found evidence of moderate correlation in chromatin accessibility between
398 rs148771817 and the promoter of *WNT16* across 305 cell types²⁴ (**Supplementary Table 11, Extended
399 Data Fig. 3b**), suggesting that the DHS overlapping rs148771817 may regulate expression of *WNT16*. Hi-C
400 data in human embryonic stem cells supports an interaction between the rs148771817 DHS and the *WNT16*
401 promoter, as both sites lie within the same topologically associated domain (TAD).²⁵ However, interaction
402 frequency to nearby regions is also elevated (**Supplementary Table 11**).

403 **7. Functional Experiments**

404 **Murine Osteoblast Gene Expression Profiling**

405 Pre-osteoblast-like cells were obtained from neonatal calvaria collected from C57BL/6J mice expressing
406 cyan florescent protein (CFP) under the control of the Col3.6 promoter (pOBCol3.6CFP), using standard
407 techniques.²⁷ pOBCol3.6CFP mice used with permission for this study. These mice were made in an

408 identical fashion to the previously described pOBCol3.6GFPtpz transgenic mice.²⁷ The cells were placed
409 into culture for 4 days in growth media (DMEM containing 10% fetal bovine serum (FBS) and 1X
410 penicillin/streptomycin), removed from culture by trypsin digestions and subjected FACS sorting based on
411 the presence/absence of CFP expression to allow for enrichment of osteoblast lineage cells. Cells
412 expressing CFP were returned to culture, plated at a density of 1×10^4 cells per cm^2 , subjected to an
413 osteoblast differentiation cocktail (α MEM containing 50 $\mu\text{g/ml}$ Ascorbic Acid, 4 mM β -glycerol phosphate,
414 10% FBS and 1X penicillin/streptomycin) and RNA was collected at 2, 4, 6, 8, 10, 12, 14, 16 and 18 days
415 post differentiation. To examine gene expression across osteoblastogenesis, mRNA profiles for each time
416 point were generated by Next Generation High throughput RNA sequencing (RNAseq), using an Illumina
417 HiSeq 2000. Three technical replicates per sample were sequenced. The alignments for abundance
418 estimation of transcripts was conducted using Bowtie version 0.12.9²⁸ using the NCBI m37 transcriptome
419 as the reference for alignments. Among all the possible alignments that had fewer than or equal to three
420 mismatches against reference transcriptome, we only accepted those with the minimum number of
421 mismatches for each of 100bp read (using both '--all' and '--best' options). Expression level per gene was
422 calculated using RSEM version 1.2.0 using the following parameters: --fragment-length-mean 280 and --
423 fragment-length-sd 50 and expression level for each sample was normalized relative to the per sample
424 upper quartile.^{29,30} This data has been submitted to the gene expression omnibus (Accession Number:
425 GSE54461). Results from these experiments for *Dock8* are shown in **Extended Data Fig. 4a**.

426 **Temporal Expression of *En1* in Mouse Osteoblasts and Osteoclasts**

427 Pre-osteoblast-like cells were obtained from neonatal calvaria collected from C57BL/6J mice using
428 standard techniques. The cells were placed into culture for 4 days in growth media (DMEM containing
429 10% fetal bovine serum (FBS) and 1X penicillin/streptomycin) at a density of 2×10^4 cells per cm^2 . The
430 pre-osteoblast cells were subjected to an osteoblast differentiation cocktail (α MEM containing 50 $\mu\text{g/ml}$
431 Ascorbic Acid, 4 mM β -glycerol phosphate, 10% FBS and 1X penicillin/streptomycin) and RNA was
432 collected at 2 and 18 days post differentiation. These cells were independent from the cells used for the
433 RNA-seq experiments and were not subjected to FACS. Bone marrow derived osteoclast precursor cells
434 were isolated from the hind long bones (femur and tibia) of six week old C57BL/6J female mice. In short,
435 bone marrow was flushed from the long bones and marrow stromal cells were plated at a density of 1×10^6
436 cells per cm^2 in α MEM containing 10% FBS, macrophage colony-stimulating factor (25 ng/ml) and
437 RANKL (100 ng/ml). Media was changed at day three post plating and RNA was collected three days
438 after that (day six post plating). Each lane for gene expression represents data from one mouse for the
439 osteoclasts cultures and from one well of cells for the osteoblast cultures. For both the osteoclasts and the
440 osteoblast samples, total RNA was isolated using TriZOL (Life Technologies, USA), as described by the
441 manufacturer, and RNA was treated with DNase to remove any contaminating DNA. For each sample, 500
442 ng of RNA were then converted to cDNA using standard protocols, with random decamers used as primers
443 for the reaction. Expression of *En1*, *Bglap* (osteocalcin) and *Tnfrsf11a* (RANK) was assessed by PCR. For
444 each PCR reaction, 2 μl of cDNA was added to 48 μl of master mix containing 5 μl of 10X PCR Buffer
445 (Clontech), 0.4mM dNTP's, 2 μM of each of a forward and a reverse primer, 1 μl of Taq (Clontech) with a
446 balance of ddH₂O. The PCR was completed using the following cycling conditions: 1 minute (min) hold at
447 94°C and 30 cycles of 94°C for 30 sec and 68°C for 3 min, followed by a hold at 68°C for 3 min. The
448 following primers were used: *En1* forward: TCA AGA CTG ACT CAC AGC AAC CC, *En1* reverse: TTG
449 TCC TGA ACC GTG GTG GTA GAG, *Bglap* forward: CCA TCT TTC TGC TCA CTC TGC TG, *Bglap*
450 reverse: CTT CAA GCC ATA CTG GTC TGA TAG C, *Tnfrsf11a* forward: CCA TCA TCT TCG GCG TTT
451 ACT ACA G and *Tnfrsf11a* reverse: GGA TTA GGA GCA GTG AAC CAG TCG. PCR products were
452 visualized by separating them on a standard 2% agarose gel stained with Ethidium Bromide.

453 **Quantitative Expression of *En1***

454 *iCycler iQ thermal cycler*

455 For osteoblast marker gene expression, total mRNAs were purified from osteoblast cultures. Real time PCR
456 was performed in triplicate using the iCycler iQ thermal cycler and detection system (Applied Biosystems,
457 Carlsbad, CA) following the manufacturer's protocols. Expression of the tested gene was normalized
458 relative to levels of GAPDH. Primers used are: mALP forward: CAC AAT ATC AAG GAT ATC GAC
459 GTG A, mALP reverse: ACA TCA GTT CTG TTC TTC GGG TAC A, mOSX forward: ATG GCG TCC

460 TCT CTG CTT GA mOSX reverse: GAA GGG TGG GTA GTC ATT TG, mBglap forward: GGG CAA
461 TAA GGT AGT GAA CAG mBglap reverse: GCA GCA CAG GTC CTA AAT AGT, mRunx2 forward:
462 TAC AAA CCA TAC CCA GTC CCT GTT T, mRunx2 reverse: AGT GCT CTA ACC ACA GTC CAT
463 GCA, mColl1a1: forward ACT GTC CCA ACC CCC AAA G, Ccoll1a1 reverse: ACG TAT TCT TCC
464 GGG CAG AA. For results see **Extended Data Fig. 4d**. Expression within *sdEn1* is likely due to detection
465 of the non-functional *En1^{Cre}* allele.
466

467 *StepOnePlus Real Time PCR system*

468 Total RNA from whole vertebral bone extract was prepared using TRIZOLreagent (Sigma) followed by
469 RNeasy Mini Kit (Qiagen). The RNA was reverse transcribed into cDNA using cDNAkit (Applied
470 Biosystems) and real-time PCR analysis was performed using custom designed real-time PCR assays and
471 the StepOnePlus Real Time PCR system (Applied Biosystems). Primers used are: forward ACT CAT GGG
472 TTC GGC TAA CG, reverse GAC GGT CCG AAT AGC GTG T, and probe CGG TGG TCA AGA CTG
473 ACT CA. 18S ribosomal RNA (4310893E Applied Biosystems) was included as an internal standard. For
474 results see **Extended Data Fig. 4e**. Expression within *sdEn1* is likely due to detection of the non-functional
475 *En1^{Cre}* allele.

476 **Murine bone histology**

477 *Mouse bone tissue preparation:* Two-month old *En1^{lacZ/+}* mice³¹ were euthanized by CO₂ suffocation and
478 the vertebrae from T13 to S1 were collected as a whole block on ice-cold phosphate-buffered saline (PBS).
479 Soft tissue was gently removed with blunt scissors and a size-11 surgical blade, until the intervertebral
480 discs were visible. The vertebral blocks were then fixed by immersion in 0.25% glutaraldehyde in PBS for
481 90 minutes at room temperature (RT). After several washes with PBS, the vertebrae were decalcified for 4
482 days with EDTA 0.5M pH 7.4 at 4°C (2 changes per day), followed by further washes with PBS. The tissue
483 was then cryoprotected by incubation with a solution of 30% sucrose in PBS overnight at 4°C, and then
484 embedded in OCT compound (Tissue-Tek) using dry-ice-cold isopentane (Sigma).
485

486 *Histochemical methods:* 10-micron sections were collected with a Leica Cryostat on SuperFrost slides
487 (Fisher) and allowed to dry overnight. Sections were stored at -80°C until use. For enzymatic detection of
488 β-galactosidase activity, slides were allowed to reach room temperature (RT) in a closed box, and OCT was
489 washed away for 15 minutes with warm PBS (37°C), followed with several extra PBS washes. The sections
490 were post-fixed 5 minutes with 4% paraformaldehyde (PFA, Electron Microscopy Sciences) in PBS at RT.
491 After PBS washes, the sections were incubated 2x5 minutes with X-gal buffer (2mM MgCl₂, 0.02% NP40
492 and 0.05% deoxycholate in PBS 0.1M pH 7.4) and then overnight at 37°C in X-gal reaction buffer (20
493 mg/ml X-gal, 5mM K₄Fe(CN)₆ and 5mM K₃Fe(CN)₆ in X-gal wash buffer). After PBS rinses, the sections
494 were postfixed 10 minutes in 4% PFA and PBS-rinsed again. At this point, if only X-gal staining was
495 required, the sections were then counterstained with Nuclear Fast Red 0.005% for 15 minutes, serially
496 dehydrated, incubated 3x1 min with xylene, and cover-slipped using DPX mountant (Fisher). If Alkaline
497 Phosphatase (AP) activity was also required, the sections were incubated 2x5 minutes with AP buffer
498 (100mM NaCl, 50mM MgCl₂, 0.1% Tween-20 in Tris-HCl 0.1M pH 8.2) and AP activity was revealed by
499 incubation with Fast Red (2 h at 37°C) following manufacturer instructions (Roche). After PBS rinses, a
500 gelatin/Tris mounting medium was used to coverslip the slides.
501

502 *Imaging:* Z-stacked DIC images were captured of the LacZ and AP double-stained lumbar vertebrae from
503 2-month old *En1^{lacZ/+}* reporter mice at 400x using a Nikon Eclipse Ni-E upright microscope and NIS^{Ar}
504 software.

505 **Mouse Micro-CT**

506 *Mice:* The *En1^{Cre/+}* and *En1^{fllox/+}* mouse strains^{32,33} were maintained in a mixed background and genotyped
507 as described. Male *En1^{Cre/+}* mice were crossed with *En1^{fllox/fllox}* females to generate experimental and control
508 animals.
509

510 *Tissue collection, preparation and imaging:* Lumbar vertebrae from levels L4 to L6 were collected from 4-
511 month old *En1^{Cre/fllox}* (self-deleted conditional *En1* mutants, *sdEn1*; n=5), and compared to *En1^{+fllox}*

512 littermates (control; n=6). All vertebrae were cleaned of soft tissue, fixed in 4% PFA (paraformaldehyde)
513 overnight, washed three times in 1XPBS for 30 mins each, and stored in 70% ethanol at 4°C until scanning.
514 Before scanning, the bones were allowed to reach room temperature. L5 vertebral body starting 100µm
515 from the growth plates were used for trabecular bone micro-computed tomography (microCT, µCT)
516 analysis on Scanco µCT 35 (Scanco Medical, Brüttisellen, Switzerland) system. 6µm voxel size, 55KVp,
517 0.36 degrees rotation step (180 degrees angular range) and a 400ms exposure per view were used for the
518 scans, which were performed in 70% ethanol. The Scanco µCT software (HP, DECwindows Motif 1.6) was
519 used for 3D reconstruction and viewing of images. After 3D reconstruction, volumes were segmented using
520 a global threshold of .4g/c. Directly measured bone volume fraction (BV/TV), thickness (Tb.Th), number
521 (Tb.N) and separation (Tb.Sp), TMD and surface to volume ratio (BS/BV) were calculated for the
522 trabecular bone.

523
524 **Extended Data Figure 6e** presents the microCT parameters for data in **Supplementary Table 29**.
525 Significance between control and *sdEn1* mutants was computed using an unpaired t-test. The thickness map
526 of the 3D image reconstructed microCT image of the LV5 shows the spatial distribution of the mineral
527 density and is presented in **Extended Data Figure 6a**, with morphological characteristics presented in
528 **Extended Data Figure 6b**. **Extended Data Figure 6c and 6d** also presents microCT and morphological
529 characteristics for femur trabecula and cortical bone.

530 **Dynamic and Cellular Histomorphometry**

531 *Mice for skeletal testing and laboratory blinding:* The same animals were used for histomorphometry as
532 MicroCT. Histomorphometry labs (Rower and Ackert) and MicroCT labs (Dahia and Joyner) were blinded
533 to the results of each other's analyses.

534
535 *Sample collection:* Prior to sacrifice, mice received intraperitoneal injections of 10 mg/kg calcein (Sigma
536 C-0875) and 30 mg/kg alizarin complexone (Sigma A-3882) at 7 and 2 days, respectively, prior to sacrifice.
537 The dynamic mineralization activity measurements, which are dependent on being able to detect these dyes
538 in the bone, suffered from a loss of 2 animals in each group due to inadequate uptake of one of the two
539 mineralization dyes. The right hind limb was dissected from the hip, and skin and non-adherent muscle and
540 connective tissues were removed from the bone, without scraping periosteal surfaces. The marrow space
541 was exposed by excising the proximal femur and distal tibial bone leaving with knee joint intact. In
542 addition, the vertebra bodies from L1 through L3 were dissected free of adherent muscle. The samples
543 were placed in 10% formalin at 4°C and shipped to Rowe laboratory. Upon receipt, the samples completed
544 a 3-day exposure to formalin and subsequently were placed in 30% sucrose at -80° C until all the samples
545 had been collected.

546
547 *Tissue sectioning:* Samples were entered into a modification of the fluorescence-based computer-automated
548 dynamic and cellular bone histomorphometry as described by Hong et al.³⁴ The workflow and staining
549 protocols are detailed at the bonebase.org, and is briefly outlined here. Three to four distal femur or
550 vertebral bodies are embedded in the sample mold with the aid spacing device and held in place with
551 layering of the cyro-embedding medium (OCT, ThermoScientific). The ice blocks are positioned on
552 cryostat (Leica CM3050 S) and oriented to cut the multiple bones in the same plane of section. Three 5 µm
553 sections are collected at 100 µm levels using an adhesive cryotape (Cryofilm type IIC(10), Section Lab,
554 Japan.) to capture and maintain the morphology of the section. The tape is bonded to a glass slide
555 (Cryojane, Leica Cat# 39475208) using UV fixation with the sample side exposed. Each slide contains two
556 tapes with all the sections from the Control or *sdEn1* samples as well as spots of fluorescent beads (5-
557 10µm) for subsequent image registration. Three slides per the Control and *sdEn1* samples containing the 3
558 levels of section are processed in a single batch for the staining and imaging steps.

559
560 *Section staining:* In the first step, the samples are incubated for 5 min in a calcein blue solution to identify
561 the accumulated mineral with a fluorescent signal. The slide is cover-slipped with 30% glycerol and
562 imaged for accumulated mineral and the calcein and alizarin complexone (AC) mineralization lines. The
563 second step removes the coverslips and place the slides in a tartrate acid (TRAP) incubation buffer,
564 followed by 5 minutes in the reaction buffer that contains the fluorescent substrate Elf97. The acid
565 conditions remove all the mineralization signals used in the first step. The slides washed in PBS and cover-

566 slipped with 30% glycerol. The TRAP positive cells and registration beads are captured in the second
567 scanning step. The third step removes coverslip and incubates the sections in the alkaline phosphatase (AP)
568 reaction buffer and followed by 10 minutes in the reaction buffer containing fast red substrate. The PBS
569 washed slides are cover-slipped with 30% glycerol/water containing DAPI. The slides are imaged for the
570 red AP signal, DAPI positive nuclei and registration beads. The final step removes the cover slips and
571 stains the slides with toluidine blue to provide visual tissue morphology and identify cartilage proteoglycan
572 of the articular cartilage and growth plate. The slides are cover-slipped again in 50% glycerol/water and
573 imaged from chromogenic signals.

574

575 *Section imaging:* The six slides, each containing 5-6 bone sections, are placed in 3 of the 4 positions of the
576 slide holder and loaded into the slide magazine of the Axioscan Z1 microscope. The software recognizes
577 the bar code of the slide and identifies the individual sections on the slide to create an image file for each
578 section. The operator refines the region of interest (ROI) to be scanned and initiates the scanning
579 procedure using preset exposure times and excitation/emission settings. The light source is the Colibri.2
580 and the image is captured with a high-resolution monochromic digital camera (Zeiss MRm Rev.3). The
581 DAPI, calcein blue and tetracycline sections were excited with the 385nm LED, calcein with the 470nm
582 LED, and the AC and fast red with the 555nm LED. The emission filters used Chroma, #49000ET (LP 400,
583 Emission 460/50) for calcein blue and DAPI, Chroma #49003ET (LP 515, emission 535/30) for calcein, a
584 custom Chroma filter (LP 425, emission 555/30) for tetracycline and Chroma 49005ET (LP 570, emission
585 600/50) for AC and fast red. The toluidine blue stain section uses a tungsten light source and Hitachi HV-
586 F202 camera. Using a Plan-Apochromat 10x/0.45 WD objective, the entire ROI is captured at 100X using
587 an automated image stack compression algorithm.

588

589 *Image Analysis:* The Axioscan generates proprietary multilevel source files that are exported as individual
590 gray scale jpeg files for each filter setting. The files average approximately 65 megapixels per image,
591 which is about 10 pixels per μm^2 and corresponds to the size of a DAPI positive nucleus. A total of 9 gray
592 scale files and 1 color file are generated. A proprietary algorithm was utilized to threshold each fluorescent
593 signal and map it back to the mineralized bone architecture as defined by the calcein blue stain.³⁴ The
594 details of the analysis and the production of measured and calculated data are detailed at bonebase.org.
595 Osteoblastic activity is assessed by a fluorescent AP stain, which is strongly positive in active osteoblasts
596 but still detectable by resting (lining) cell of the bone lineage. The histology measures the proportion of
597 trabecular bone surface (BS) that is positive for AP activity (AP/BS). To control for technical or biological
598 variation in staining activity, the distribution of that signal between bone labeling surfaces (AP_L_BS) and
599 non-labeling surfaces (AP_NL_BS) is calculated. Another measurement that is independent of the total
600 AP activity of a tissue, distributes the proportion of AP activity that is adjacent to mineralizing surfaces
601 (%AP_L) or non-labeling surfaces (%AP_NL). Osteoclastic activity is based on a fluorescent TRAP stain
602 and expressed as the proportion of trabecular bone surface that is TRAP positive (TRAP_BS). The analysis
603 does not account for the size of the individual spots of activity nor the number of nuclei per fluorescent spot.
604 Only activity that resides adjacent to the trabecular surface and not within the marrow space is assessed.
605 The distribution of the TRAP activity to sites of active mineralization (TRAP_L_BS) or osteogenesis
606 (AP_TRAP_BS) versus inactive mineralization (TRAP_NL_BS) has proven to be a useful measurement to
607 discriminate areas of high bone turnover versus bone resorption without an osteogenic response.

608 **Identification of cells belonging to the En1 lineage**

609 *Breeding of mice and sample collection:* Twelve week old $En1^{Cre/+};R26^{LSL-EYFP}$ (*En1* lineage tagged) and
610 $En1^{+/+};R26^{LSL-EYFP}$ (control) mice were sacrificed, the vertebral 1-3 were excised, placed in chilled 10%
611 formalin, packed in wet ice and sent to the Rowe lab for imaging.

612

613 *Tissue process, sectioning and imaging:* The tissues and sectioning protocol was identical to
614 histomorphology including the multiple rounds of imaging and staining. The EYFP signal was captured
615 during the first imaging step using the Colibri.2 470nm LED and the Chroma 49003ET filter (LP 515,
616 Emission 535/30). The EYFP signal from the $R26^{LSL-EYFP}$ allele is very weak in skeletal tissues requiring
617 significant signal enhancement. (**Extended Data Figure 5**)

618 Human EN1 Expression Profiling

619 Human CD14⁺ cells from healthy donors were incubated with 20 ng/ml of human M-CSF (peprotech) for
620 one day to generate OCPs. Osteoclast precursors were incubated with 20 ng/ml of M-CSF and 40 ng/ml of
621 human soluble RANKL for five additional days in α -MEM supplemented with 10 % FBS. Cytokines were
622 replenished every 3 days. On day 6, cells were harvested. mRNA of human osteoclasts (n=5), human
623 macrophages (n=4), 293T cells (fibroblasts, n=4), and human osteoblasts (a kind gift from Dr. Jae Hycuk
624 Shim, Weill Cornell Medical College, n=3) was measured using real-time PCR. EN1 mRNA levels were
625 normalized relative to GAPDH mRNA. Data are shown as mean \pm SEM. *** : $P < 0.001$ by one-way
626 ANOVA. All statistical analyses were performed with Graphpad Prism 5.0 software one-way ANOVA for
627 multiple comparisons (more than two conditions) with posthoc Tukey test. $p < 0.05$ was taken as
628 statistically significant. Results are presented in **Extended Data Fig. 4c**.

629 Mouse and Rat Homologous Regions

630 To test possible conservation between human BMD-associated loci and quantitative trait loci (QTLs) linked
631 to bone related traits in experimental mapping panels in rats and mice, comparative genome data were used.
632 For each region of association to BMD in humans, genes flanking SNV markers showing the strongest
633 evidence of association were searched for homologs in the genome assemblies of the mouse
634 (NCBI37/mm9) and rat (RGSC3.4). For the rat QTL, the corresponding locus was tested for co-localization
635 within boundaries of QTLs for bone related phenotypes fine mapped (circa 4Mb) from the heterogeneous
636 stock (HS).³⁵ For QTL in mice, the peak SNV location was converted to CentiMorgan (cM) distance, as per
637 the Cox-Sex-Average genetic map³⁶ using the freely available Mouse Map Conversion Tool
638 (<http://cgd.jax.org/mousemapconverter/>). All identified mouse QTLs³⁷ mapped within a peak location 5
639 cM either side of the human peak SNV and are listed, along with the strain pair(s) used to identify the
640 original QTL. Results for these analyses are shown in **Supplementary Table 9**.

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801 9. References

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