



Identification of a gene signature associated with elevated bone formation rate in aging mice

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Osteoporosis, a disease of low bone mass that results from bone resorption exceeding bone formation, places individuals at enhanced risk for fracture, disability, and death. There is an urgent and unmet need for novel targets in treating osteoporosis, requiring a better understanding of the endogenous mechanisms regulating bone formation. We reported that deletion of the *Bmpr2* gene in skeletal progenitor cells of mice causes substantially elevated bone mass in young adulthood due to increased bone formation rate (Lowery *et al.*, 2015). As yet unpublished work indicates the age-related decline in bone mass of *Bmpr2* mutant mice is reduced approximately three-fold compared to control mice; quantification of serum bone turnover markers indicates this is caused by a sustained increase in bone formation rate to at least 35 weeks of age with no alteration in bone resorption. Here, we determine the gene signature associated with elevated bone formation rate using genome-wide transcriptome profiling in bones of 35-week-old control and *Bmpr2* mutant mice. Applying stringent criteria comparing the expression data to eight well-accepted housekeeping genes (*Ppib*, *Gapdh*, *Hprt*, *Tbp*, *Ppia*, *Gusb*, *Prkg1*, and *Ywhaz*), we found that, out of 24,980 exon-containing transcripts detected in both genotypes, 334 genes were up-regulated and 310 were down-regulated at least two-fold compared to controls. An additional 704 genes were detected in only one genotype. We refined this putative signature by performing transcriptome profiling in these animals at 55 weeks of age when bone formation rate is no longer elevated. This revealed that, of those genes altered at 35 weeks of age, 461 (71.5%) were either no longer up-regulated or down-regulated in *Bmpr2* mutant mice by 55 weeks of age. Bioinformatic analyses on this refined gene set indicates that elevated bone formation rate in *Bmpr2* mutant mice correlates with enrichment for genes containing binding sites for transcription factors associated with skeletal homeostasis, including FOXP1, SOX2, EGR1, E2F1, KLF4, CNOT3, STAT4, and FOXA1. Further, several genes corresponding with osteoblast differentiation and activity, such as *Pak4* and *Pla2g4a*, the latter of which encodes cytosolic phospholipase A2 and whose deletion causes osteopenia, are up-regulated in *Bmpr2* mutant mice. Collectively, our findings provide insight into the mechanisms regulating age-related bone loss and highlight potential targets for therapeutic modulation of bone mass.

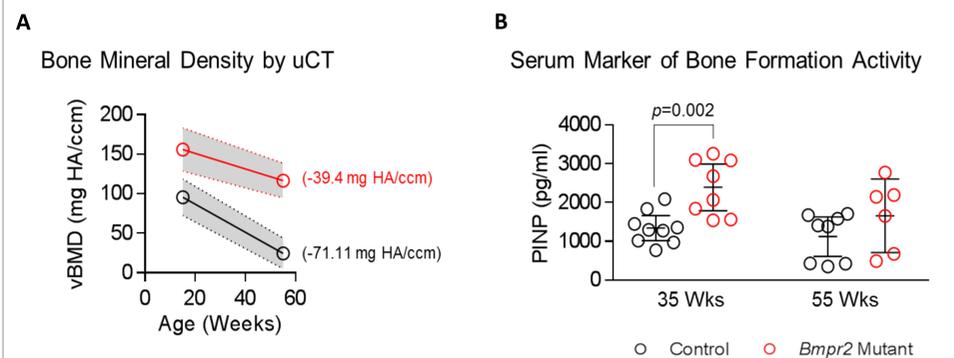
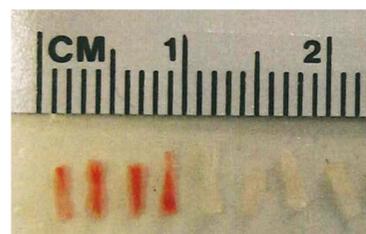


Figure 1. A: *Bmpr2* mutant mice were generated by crossing *Bmpr2^{fl/fl}; Prx1-Cre⁺* males with *Bmpr2^{fl/fl}* females. Volumetric bone mineral density (vBMD) was quantified by micro-CT in females at 15 and 55 weeks of age. Mean decline in mg hydroxyapatite per cubic centimeter for each genotype between 15 and 55 week old cohorts is indicated (mg HA/ccm); gray bars denote 95% confidence intervals. **B:** Quantification of the bone formation marker PINP in sera of control and *Bmpr2* mutant mice using ELISA. Individual samples are represented by circles and group mean by horizontal lines \pm SEM; p values determined by unpaired t test.

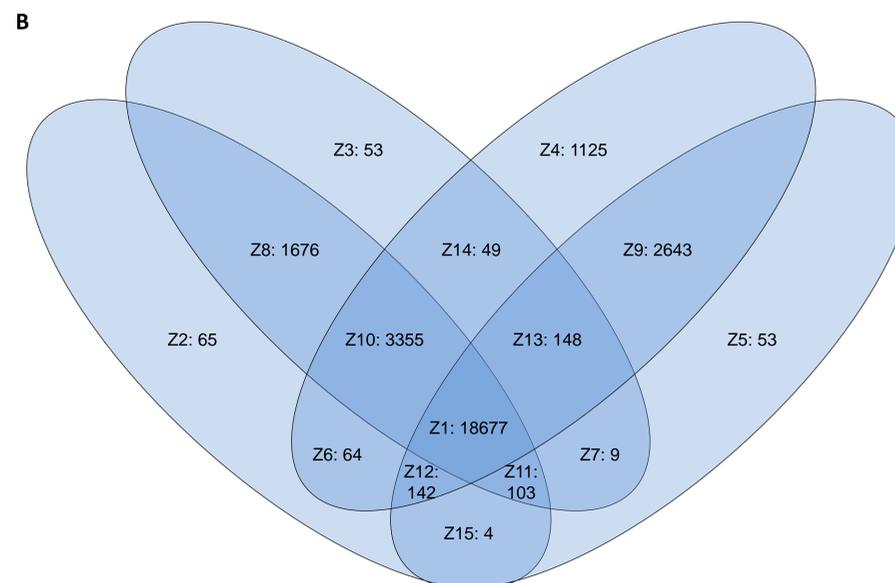
RNA-Seq Workflow:

- 1) Humerii obtained from four each control and *Bmpr2* mutant mice at 35 weeks and 55 weeks of age
- 2) Marrow removed by gentle centrifugation
- 3) Bones homogenized and total RNA collected
- 4) Each genotype pooled at equal RNA amounts per mouse
- 5) Pooled RNA samples shipped to GENEWIZ; quality control performed
- 6) rRNA depletion and library synthesized then sequenced
- 7) Bioinformatics analysis using ENRICH



A

	35 Weeks of Age	55 Weeks of Age
Unchanged	24336 genes	21766 genes
Up-regulated	334 genes	4620 genes
Down-regulated	310 genes	201 genes
Not Detected in Control	349 genes	1387 genes
Not Detected in Mutant	355 genes	104 genes



C

Zones	35 Weeks of Age	55 Weeks of Age
Zone 1: 18677 genes	Unchanged	Unchanged
Zone 2: 65 genes	Up-regulated	Not Detected
Zone 3: 53 genes	Down-regulated	Not Detected
Zone 4: 1125 genes	Not Detected	Up-regulated
Zone 5: 53 genes	Not Detected	Down-regulated
Zone 6: 64 genes	Up-regulated	Up-regulated
Zone 7: 9 genes	Down-regulated	Down-regulated
Zone 8: 1676 genes	Unchanged	Not Detected
Zone 9: 2643 genes	Not Detected	Unchanged
Zone 10: 3355 genes	Unchanged	Up-regulated
Zone 11: 103 genes	Unchanged	Down-regulated
Zone 12: 142 genes	Up-regulated	Unchanged
Zone 13: 148 genes	Down-regulated	Unchanged
Zone 14: 49 genes	Down-regulated	Up-regulated
Zone 15: 4 genes	Up-regulated	Down-regulated

Figure 3: A: Results of RNA-Seq analyses at 35 and 55 weeks of age; expressed relative to control. **B-C:** Comparison of *Bmpr2* mutant results relative to control at 35 and 55 weeks of age represented in Venn diagram (B) and tabular (C) forms.

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SMAD1_18555785_ChIP-Seq_MESCs_Mouse
 DACH1_20351289_ChIP-Seq_MDA-MB-231_Human
 FOXP1_22492998_ChIP-Seq_STRATIUM_Mouse
 SOX2_19030024_ChIP-Seq_MESCs_Mouse
 EGR1_23403033_ChIP-Seq_LIVER_Mouse
 TBP_23326641_ChIP-Seq_C3H10T1-2_Mouse
 SOX2_18692474_ChIP-Seq_MESCs_Mouse
 E2F1_21310950_ChIP-Seq_MCF-7_Human
 RNF1_16625203_ChIP-Seq_MESCs_Mouse
 KLF1_21901194_ChIP-Seq_ERYTHROCYTE_Mouse

ENCODE and ChEA Consensus TFs from ChIP-X

CEBPD_ENCODE
 STAT5A_ENCODE
 NLF_ENCODE
 PBX3_ENCODE
 NFIC_ENCODE
 PML_ENCODE
 FOS_ENCODE
 TAF7_ENCODE
 SP1_ENCODE
 E2F4_ENCODE

Figure 4: A: Example ENRICH analysis on zone 15 genes.

Conclusions, Significance & Future Directions:

- *Bmpr2* mutant mice display high bone mass in young adulthood and reduced age-related bone loss.
- Genome-wide transcriptome profiling of *Bmpr2* mutant bones identified 461 differentially expressed genes associated with increased osteoblast activity.
- The differential gene signature is enriched for genes containing binding sites for transcription factors associated with skeletal homeostasis. Several genes corresponding with osteoblast differentiation and activity are up-regulated in *Bmpr2* mutant mice.
- Collectively, our findings provide insight into the mechanisms regulating age-related bone loss and highlight potential targets for therapeutic modulation of bone mass.
- Future studies will involve functional studies to narrow the gene signature to those that regulate osteoblast function.

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