Rapid *in vivo* screen of genes at BMD-associated loci in zebrafish

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INTRODUCTION: Osteoporosis is a chronic disease with an enormous health burden affecting more than 200 million people worldwide, and which has a strong genetic component. Genome wide association studies (GWAS) have identified hundreds of loci associated with bone mineral density (BMD). Despite the success of GWAS in elucidating the genetic architecture of BMD, the causal "target" genes at most loci remain to be discovered. For most loci, the causal variant likely resides in a non-coding element that acts on a gene(s) at the locus. While functionally annotating genes at GWAS loci in animal knockout models can greatly aid in identifying target genes, this remains daunting due to the large number of candidate genes, and the lack of approaches with sufficient throughput. Previously, our team established a rapid workflow for generating CRISPR-edited F0 "crispant" somatic mutant zebrafish and phenotyping 100s of measures in the adult spine [1,2]. Here, we use these advances to perform an *in vivo* reverse genetic screen of genes that reside at 56 loci which were previously identified in a large GWAS meta-analysis of BMD [3]. We hypothesized that genes at BMD-associated loci would be enriched with novel genes necessary for adult bone mass, morphology, and mineralization.

METHODS: All animal studies were performed following a protocol approved by the University of Washington Institutional Animal Care and Use Committee (IACUC). CRISPR-based gene editing was performed as previously described [1]. Briefly, Cas9:gRNA complexes were injected into wildtype zebrafish embryos (AB) to generate somatic mutants. At 90 days post fertilization (dpf), animals were euthanized and subjected to microCT scanning (Scanco vivaCT40). Vertebrae were segmented and analyzed in FishCuT software as previously described [2]. Standard length was measured in FIJI. PCA and k-means clustering were performed in R. For statistical analysis of FishCuT data, the global test was used as previously described [2]. Standard length was evaluated using a t-test, and dysmorphic features were evaluated using a test of equal of proportions. $p<0.05$ was considered statistically significant.

RESULTS SECTION: We generated somatic mutants for 62 genes, which required the generation and analysis of 1,438 animals (n=720 controls and n=718 mutants). For each animal, we annotated 10 vertebral measures (volume, TMD, and thickness for the centrum, haemal arch, and neural arch, as well as centrum length) in 20 vertebrae (200 measures total per fish), plus standard length, resulting in 289,038 measures examined. We found that somatic mutants for 44 of the 62 genes (69%) had a significant ($p<0.05$) difference for at least one of the 11 measures (Fig 1A). Of the top 10 genes with the most significant p-values, 4 were orthologs of genes with well known roles in bone biology (*tnfrsf11b*, *clcn7*, *axin1*, and *esr1*), whereas 6 had less or no known roles. PCA and K-means clustering revealed somatic mutants grouped into 4 clusters (Fig 1B) which exhibited distinct effects on phenotypic measures and biological directions. For example, somatic mutants for *mfsd5*, which had the most significant p-value in cluster 1, exhibited increased bone and body size as evidenced by significant increases in centrum volume, length, and standard length, as well as haemal arch volume, TMD, and thickness. Somatic mutants for *dusp3a*, which had the most significant p-value in cluster 2, exhibited decreased bone size and mineralization, as evidenced by significant decreases in most volume, thickness, and TMD measures. Finally, somatic mutants for *slx4ip*, which had the most significant p-value of all genes tested, were characterized by increased tissue mineral density, as evidenced by significant increases in centrum, neural arch, and haemal arch TMD.

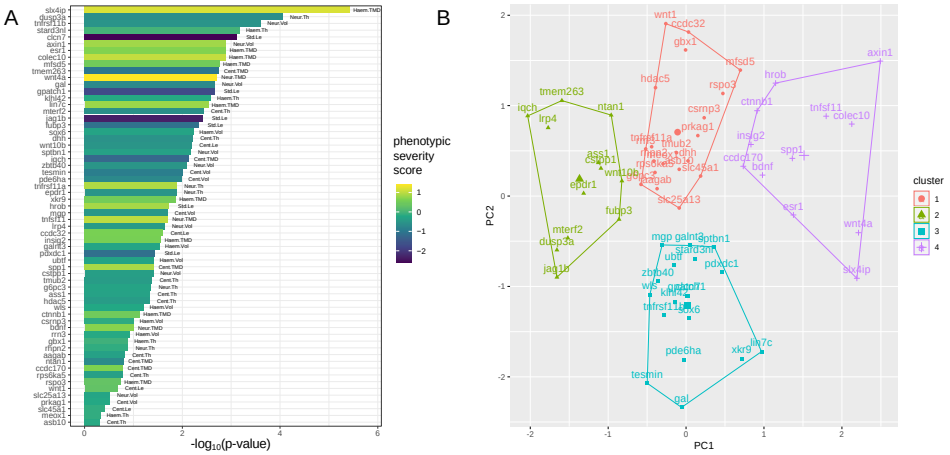

Fig 1. *In vivo* reverse genetic of genes at BMD loci in zebrafish reveals novel skeletal genes and distinct mutant "classes". (A) p-values for measures evaluated in somatic mutants. We assessed p-values for 11 measures; shown is the max p-value, with the corresponding trait listed to the right, for each somatic mutant group. Bars are color-coded by phenotypic severity score, which indicates the severity and biological direction of change in the somatic mutant. (B) Phenotypic clustering of somatic mutants. K-means clustering revealed 4 major clusters, with each cluster comprised of genes with similar somatic mutant phenotypes.

DISCUSSION: In this study we generated adult zebrafish skeletal phenotypes for 62 genes at BMD-associated loci, comprising one of the largest *in vivo* screens of genes at BMD-associated loci conducted to date. Limitations of our screen included (i) variable mutation efficiencies in somatic mutants, (ii) it is conceivable that some somatic mutant phenotypes may not replicate in germline mutants in which the gene is globally knocked out, and (iii) negative results do not conclusively rule out an important gene function in the adult spine. Still, our dataset provides a valuable resource to prioritize individual genes for follow up study. Our results also suggest that somatic mutants cluster into "classes" of mutants with similar effects on phenotypic traits, which might indicate groups of genes that reside within common pathways. Finally, our data suggest that novel cellular and molecular processes may contribute to the adult skeleton. For example, the encoded protein for *SLX4IP*, which resides at the 20p12.2 locus, has been implicated in DNA repair and telomere maintenance [4]. The encoded protein for *DUSP3*, which resides at the BMD-associated locus at 17a21.31, encodes for phosphatase with a preference for phospho-tyrosine substrates and plays an important role in cellular signaling [5]. Finally, the encoded protein for *MFSD5*, which resides at the 12q13.13 locus, has been demonstrated to play a role in molybdate ion transport across cell membrane [6]. Efforts to generate germline zebrafish mutants for these and other genes in our screen are currently underway, which should help to better understand their roles in the skeleton, and in turn, genetic influence on BMD.

SIGNIFICANCE/CLINICAL RELEVANCE: By identifying novel genes that may underlie genetic influence on bone mineral density, this study could lead to new approaches for osteoporosis diagnosis and its treatment.

REFERENCES: 1. Watson CJ, et al., Cell Syst 2020; 2. Hur M, et al., Elife 2017; 3. Estrada K, Nat Genet 2012; 4. Panier et al. Molecular Cell 2019; 5. Pavic et al. FEBS Journal, 2015; 6. Perland et al. PLoS One, 2016.

ACKNOWLEDGEMENTS: Research reported was supported by NIH Award Number AR074417.