Heterozygous Nonsense Variants in *efemp1* Alter Vertebral Characteristics in Adult Zebrafish

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INTRODUCTION: Heritable disorders of connective tissue (HDCT) are a group of heterogenous conditions that can manifest phenotypes in skeletal, ocular, and pulmonary tissues [1]. One such HDCT is Marfan syndrome, an autosomal dominant condition whose clinical phenotypes include disproportionate growth of limb bones, scoliosis, and pes planus [2]. Individuals with Marfan syndrome predominantly carry heterozygous variants in Fibulin 1 (*FBN1*), whose encoded protein makes up extracellular microfibrils [3]. Approximately 25% of *FBN1* mutations cause clinical phenotypes consist of frameshift or nonsense mutations leading to premature termination codons (PTCs) [4]. Recently biallelic and recessive nonsense variants leading to PTCs in *EFEMP1* have been identified in individuals showing Marfan-like characteristics [5–7]. *EFEMP1*, or Fibulin 3 (*FBN3*), is a member of the fibulin family of extracellular matrix glycoproteins. In mice, *Efemp1* is expressed in developing bone and cartilage, and has been found to negatively regulate chondrocyte differentiation in a carcinoma-derived chondrogenic line [8, 9]. The absence of critical animal models needed to assess the *in vivo* consequence of heterozygous and homozygous nonsense mutations in *EFEMP1* severely limits our ability to understand the molecular basis of HDCTs caused by *EFEMP1* mutations. To fill this gap, we have isolated multiple *efemp1* alleles encoding for PTCs in zebrafish and determined changes in adult vertebral characteristics. *We hypothesize that heterozygous and homozygous efemp1 nonsense mutations will result in abnormal vertebral characteristics.*

METHODS: Approval for this project was granted by the University of Washington IACUC. Adult AB zebrafish were sacrificed, and tissues dissected to determine expression of *efemp1*. RNA was extracted using Trizol-chloroform extraction and cDNA synthesis was performed using the SuperScriptIV synthesis kit (Thermo Fisher). To generate *efemp1* mutant alleles, CRISPR-Cas9 gene editing was performed by injecting AB zebrafish embryos with a guide RNA (gRNA, Alt-R system, IDT) targeting exon 4 of *efemp1* complexed with Cas9 protein (Hifi 3xNLS-Cas9, IDT). Somatic founders for germline transmission were bred, F1 progeny were screened for predicted loss-of-function alleles, and multiple founders carrying identical frameshift allele were inbred to create a stable F2 germline *efemp1* mutant. At 3 months of age, adults were sacrificed and subjected to microCT-based phenomic profiling as described in [10].

RESULTS: To determine tissue expression in adult zebrafish, we performed RT-PCR and identified *efemp1* expression at moderate/high expression in skin, swim bladder, heart, eyes, and testes, while expression was low in bone, muscle, and intestine. Based on this data, we wanted to determine whether perturbation of *efemp1* would result in changes in adult vertebra. We used CRISPR-based gene editing to isolate 2 *efemp1* mutant lines with deletions in exon 4: *efemp1 w1014* (ENSDART00000082142.6: c.437_444del; p.(Val147Leufs*60)) and *efemp1 w1016* (ENSDART00000082142.6: c.433_445del; p.(Tyr146Glnfs*25)) (Fig 1A). MicroCT analysis of 90 dpf adult fish revealed significant effects of genotype on bone and body morphology (n= 9-15/group). To determine if variants in *efemp1* affect standard length, we compared the length between wildtype (WT), heterozygous (HET), and homozygous mutant (MUT) animals for each allele. We found no significant genotype:allele interaction, suggesting that the effects of *efemp1* mutations for both alleles were similar. We found significant effects of genotype on standard length, with *efemp1* HETs exhibiting significantly greater length than WT (Fig 1B). We observed significant changes in *efemp1* HET centrum volume and centrum length when compared to WT, suggesting that heterozygous nonsense mutations in *efemp1* significantly increased vertebral size in addition to body length (Fig 1B' and 1B'').

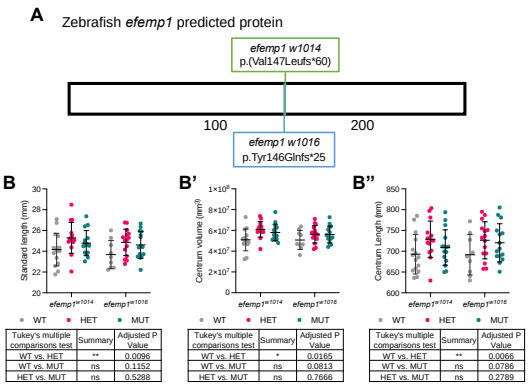

Fig 1. *efemp1* variants cause changes in vertebral characteristics. (A) Schematic of zebrafish *efemp1* protein with location of *efemp1 w1014* and *w1016* variants. (B, B', B'') Measurements of standard length, centrum volume, and centrum length plotted for *efemp1 w1014* and *efemp1 w1016* WT, HET, and MUT fish. The results of Tukey's multiple comparisons test are shown below the plots for each measurement, where * and ** under the summary column indicate significance. Error bars indicate mean and standard deviation.

DISCUSSION: To better understand the spectrum of clinical phenotypes presented within HDCT's we need to identify how variants in disease-causing genes are leading to abnormal protein function. In individuals carrying variants in *EFEMP1,* it remains unclear how heterozygous frameshift or nonsense variants that cause PTCs lead to manifestation of clinical symptoms. In our study, we were interested in investigating whether nonsense mutations in zebrafish *efemp1* would cause changes in vertebral characteristics. We demonstrated that *efemp1* is expressed in zebrafish tissues relevant to human connective tissue conditions, including bone, skin, and heart. In *efemp1* zebrafish models, we found that *efemp1* HET adult fish are significantly longer than controls, which correlates with individuals carrying *EFEMP1* nonsense variants, who are tall in stature. [5, 6] From our microCT analysis, we again noted significant changes in vertebral structures in *efemp1* HET animals. Based on our results, it appears that the heterozygous allelic background causes the most severe phenotypes in adult zebrafish. The potential mechanisms that could be relevant to variants in *EFEMP1* are: (i) haploinsufficiency initiates the pathogenetic sequence, or (ii) the truncated N-terminal peptides encoded by selected nonsense alleles have dominant-negative activity, allowing effective neutralization of function of the WT protein [11]. Future work examining whether overexpression of truncated Efemp1 in a WT background recapitulates MUT phenotypes may help to delineate these possibilities. Our studies demonstrate that HET nonsense mutations in *efemp1* can cause alterations in connective tissue *in vivo*. Based on our work, there is a possibility that individuals carrying heterozygous nonsense variants in *EFEMP1* could present clinical symptoms linked to HDCTs, such as a Marfan-like condition.

SIGNIFICANCE/CLINICAL RELEVANCE: Our study suggests that partial loss of *efemp1* causes significant changes in standard length and vertebral characteristics and *efemp1* positively influences acquisition of bone mass and mineralization in adult zebrafish. Understanding the role of *efemp1* in bone can advance our understanding of genetic influence on HDCTs.

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