The Cys-His-Gly Triplet is Required for Wnt16 Function *In Vivo*

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INTRODUCTION: WNT proteins are secreted morphogens critical to numerous developmental and disease processes. WNT proteins contain a thumb-like domain central to their function as it mediates the addition of an acyl group by the O-acyltransferase Porcupine to WNT and engagement with Frizzled receptors. The thumb-like domain comprises several highly conserved residues, including a Cys-His-Gly triplet whose specific function remains unknown. WNT16 is a member of the WNT protein family associated with genetic risk for osteoporosis, a prevalent and debilitating disease characterized by low bone mineral density and increased fracture risk. Zebrafish are an ideal research model for skeletal diseases because of their shared morphophysiological features with humans, as well as their ease of genetic manipulation, which enables the study of mutations that would be too scientifically or financially risky in mice [1]. Our lab previously showed that *wnt16*$^{-/-}$ (*wnt16*^{w1001}) zebrafish exhibit impaired spinal development and adult vertebral morphology [2]. Here, we tested the hypothesis that the Cys-His-Gly triplet sequence is required for Wnt16 function in zebrafish.

METHODS: These experiments were conducted conforming to protocol approved by the University of Washington Institutional Animal Care and Use Committee (IACUC). We used CRISPR-Cas9 to generate indels at the *wnt16* locus and subsequently isolated *wnt16*^{w1012} mutants containing a nine-base pair deletion in exon 4, resulting in the Cys-His-Gly triplet being deleted. Sanger sequencing revealed that this three amino acid deletion begins at Cys214, corresponding to the tenth conserved cysteine of the WNT family (p.Cys214_Gly216del). At 90 days post fertilization (dpf), ten *wnt16*^{w1012} mutants and wild-type controls were euthanized and scanned using micro-computed tomography (micro-CT) to visualize skeletal structures. FishCuT, a segmentation algorithm developed by our lab for micro-CT-based phenotyping in the zebrafish skeleton, was used to measure volume, tissue mineral density (TMD), thickness, and centrum length for centrums, haemal arches, and neural arches. 2-way ANOVA analyses were performed to compare FishCuT measurements from *wnt16*^{w1012} mutants and *wnt16*$^{-/-}$ zebrafish. Structural protein models were created and superimposed using Mol* Viewer, allowing direct molecular comparison of the protein encoded by *wnt16*^{w1012} and wild-type Wnt16 protein.

RESULTS SECTION: Adult *wnt16*^{w1012} mutants exhibited morphological and mineralization changes in the spine. Analysis of micro-CT scans at 90 dpf revealed significantly reduced centrum lengths ($p<0.0001$), along with a significant increase in neural arch angles ($p=0.0002$) in *wnt16*^{w1012} mutants (n=10/group). Moreover, *wnt16*^{w1012} mutants exhibited significantly reduced centrum volumes ($p=0.0088$) and significantly increased centrum ($p=0.0404$), haemal arch ($p=0.0264$), and neural arch ($p=0.0166$) TMD compared to controls. 2-way ANOVA analyses of *wnt16*^{w1012} and *wnt16*$^{-/-}$ mutants revealed no significant genotype:allele interactions, indicating *wnt16*^{w1012} mutants phenocopy *wnt16*$^{-/-}$ mutants (Fig 1A). Structural modeling of the encoded protein of *wnt16*^{w1012} and superimposition with wildtype Wnt16 revealed conformational changes within the thumb due to the triplet deletion. Specifically, the protein encoded by *wnt16*^{w1012} exhibited alteration of multiple hydrogen bonds and truncation of the antiparallel beta sheets delineating the thumb (Fig 1B, C).

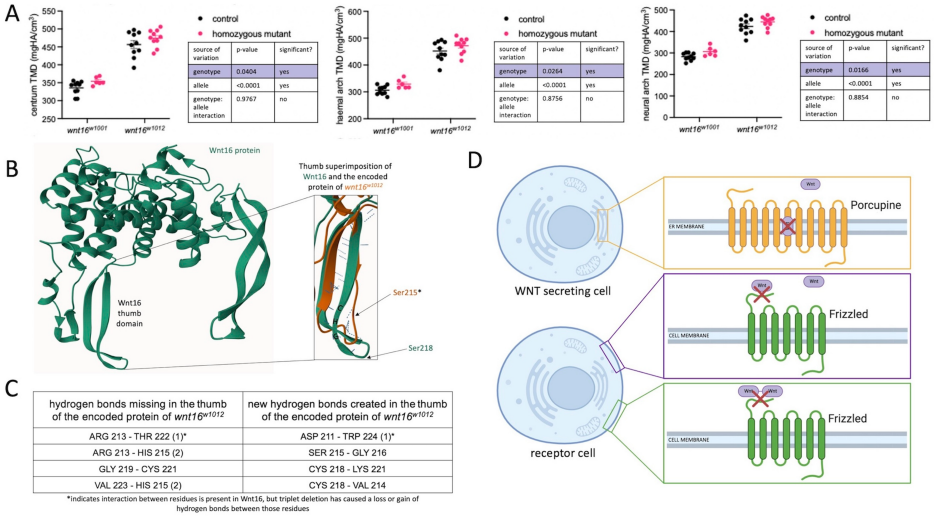

Fig. 1. A. 2-way ANOVA analyses of centrum tissue mineral density (TMD), haemal arch TMD, and neural arch TMD. n=10/group for *wnt16*^{w1001} clutchmate controls and *wnt16*^{w1012}homozygous mutants and clutchmate controls. n=6/group for *wnt16*^{w1001} homozygous mutants. Purple highlight indicates phenotypic features of *wnt16*^{w1001} and *wnt16*^{w1012} that differ significantly from controls. B. (L) Structural model of Wnt16 (green). (R) Thumb superimposition of Wnt16 and the encoded protein of *wnt16*^{w1012} (orange). Blue dotted lines represent hydrogen bonds. "CHG" represents location of the Cys-His-Gly triplet on Wnt16. Arrows indicate location of acyl addition (*Ser215 is equivalent to Ser218 but shifted due to triplet deletion). C. Table showing missing/ created hydrogen bonds within the thumb of the encoded protein of *wnt16*^{w1012}. Numbers in parentheses indicate the number of those specific bonds that are missing or created. D. Proposed mechanisms of action to describe severely decreased Wnt signaling activity. (Top) Triplet deletion prevents recognition of Wnt by Porcupine (located in the endoplasmic reticulum membrane) and/ or acyl addition to the Wnt thumb. (Middle) Severe structural changes in the thumb do not prevent secretion but do prevent recognition of Wnt monomers by Frizzled receptors (located on receptor cell membranes). (Bottom) Triplet deletion promotes Wnt aggregate formation, which prevents recognition by Frizzled receptors.

DISCUSSION: Our *in vivo* studies support prior *in vitro* studies demonstrating the critical role of the thumb in supporting Wnt function. Our results suggest that deletion of the Cys-His-Gly triplet renders Wnt16 non-functional. This is in contrast with prior studies demonstrating that single amino acid substitutions usually diminish, though do not eliminate, Wnt secretion and/or signaling activity [3]. We propose several possible mechanisms by which deletion of the Cys-His-Gly triplet impairs Wnt16 function (Fig 1D). First, truncation of the anti-parallel beta sheets in the thumb and the subsequent hydrogen bond alterations may prevent or significantly reduce recognition of the Wnt16 thumb by Porcupine. Failed acyl addition on the Wnt thumb by Porcupine may prevent Wnt16 from being secreted, thereby reducing Wnt16 activity. Second, structural changes in the thumb may alter Wnt interactions with Frizzled receptors on receptor cells. Wnt16 activity decreases as a result of interrupted signal transduction. Finally, loss of a highly conserved cysteine within the triplet may encourage formation of intermolecular disulfide bonds, creating protein aggregates that prevent recognition by Frizzled receptors, as has been shown for other highly conserved cysteines [4]. These findings provide functional insights for highly conserved residues in WNT proteins which can help interpret genetic variants in WNT genes and may lead to new genetic strategies for manipulating WNT signaling.

SIGNIFICANCE/CLINICAL RELEVANCE: Our findings provide functional insights for highly conserved residues in WNT proteins which can help interpret genetic variants in WNT genes and identify new strategies for manipulating WNT signaling. Given the critical role of WNT16 in mediating genetic risk for osteoporosis, such findings may aid in diagnosing and treating this condition.

REFERENCES: [1] Watson, et al., PLoS Genet 2022. [2] Kwon, et al., Bone 2019. [3] Rios-Esteves, et al., J Biol Chem 2014. [4] MacDonald, et al., J Biol Chem 2014.

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