Mutation in ZDHHC15 Leads to Hypotonic Cerebral Palsy, Autism, Epilepsy, and Intellectual Disability

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Neurol Genet 2021;7:e602. doi:10.1212/NXG.0000000000000602

Abstract

Objective
To determine whether mutations reported for ZDHHC15 can cause mixed neurodevelopmental disorders, we performed both functional studies on variant pathogenicity and ZDHHC15 function in animal models.

Methods
We examined protein function of 4 identified variants in ZDHHC15 in a yeast complementation assay and locomotor defects of loss-of-function genotypes in a Drosophila model.

Results
Although we assessed multiple patient variants, only 1 (p.H158R) affected protein function. We report a patient with a diagnosis of hypotonic cerebral palsy, autism, epilepsy, and intellectual disability associated with this bona fide damaging X-linked variant. Features include tall forehead with mild brachycephaly, down-slanting palpebral fissures, large ears, long face, facial muscle hypotonia, high-arched palate with dental crowding, and arachnodactyly. The patient had mild diminished cerebral volume, with left-sided T2/FLAIR hyperintense perialarial ovoid lesion. We found that loss-of-function mutations in orthologs of this gene cause flight and coordinated movement defects in Drosophila.

Conclusions
Our findings support a functional expansion of this gene to a role in motor dysfunction. Although ZDHHC15 mutations represent a rare cause of neurodevelopmental disability, candidate variants need to be carefully assessed before pathogenicity can be determined.
DHHC domain. deleterious, X-linked variant (p.H158R) that disrupts the core neurodevelopment. We identify deleterious variants within a cohort of cryptogenic cerebral balanced translocation disrupting the gene\(^5\) suggests that Intelligence Scale was reported in association with intellectual disability.\(^2\) This residue zinc\(^15\) (q13.3;cen) translocation disrupting the gene\(^5\) when a balanced t(X;15) (q13.3;cen) translocation disrupting the gene\(^5\) X inactivation was not assessed in this report, the authors were loss of function may not be pathologic. Although X inactivation is not be pathologic. Although X inactivation was not assessed in this report, the authors were unable to detect ZDHHC15 expression in peripheral blood necessitating validation studies of ZDHHC15’s role in neurodevelopment. We identified individuals with predicted deleterious variants within a cohort of cryptogenic cerebral palsy patients who underwent whole-exome sequencing\(^6\) and through gene matching efforts (genematcher.org). We identify functional consequences of these variants and confirm a role in regulating movement in genetic models. Finally, we present clinical information from a patient harboring a verified deleterious, X-linked variant (p.H158R) that disrupts the core DHHC domain.

**Methods**

**Patient Recruitment and Sequencing**

Patients were recruited under the central IRB protocol (\#15-080) approved by the Phoenix Children’s Hospital IRB Committee (\#1). Written informed consents for research were obtained for parents (and assent was obtained for children as appropriate) for families participating in the study. Whole-exome sequencing of patients with cerebral palsy identifying maternally inherited variants p.H158R and p.L13P reported in Jin et al.\(^6\) Depth of coverage for p.H158R at the variant position (X:74649792T<C) is 34 reads and p.L13P (X:74742822A<G) is 30 reads. Other variants were identified through GeneMatcher, and maternally inherited p.K115R was sequenced through GeneDx.

Clinical phenotypes for (p.H158R) were assessed by J.H. and M.C.K. This patient had negative testing for Fragile X, telomeric FISH, chromosomal microarray, and karyotyping. An epilepsy gene panel (Invitae) showed a single heterozygous predicted protein-damaging variant in ALDH7A1 (p.L246E), and WES found a de novo CPM (p.H369R) and X-linked AGTR2 (p.F320L) variant. These variants were thought to be unlikely to represent the underlying cause of his clinical phenotype because ALDH7A1 causes a recessive condition and no second mutation or genomic deletion was detected and the other genes are not predicted to contribute to the patient’s phenotypes.

**Yeasts Complementation Studies**

The BY4742 wild-type (MAT a his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) and pfa3-Δ (MAT a his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pfa3-<:KANMX) yeast strains were obtained from TransOMIC (Huntsville, AL). PFA3, ZDHHC15, and ZDHHC15 p.H158R cDNA sequences were synthesized (GenScript Inc.), confirmed by direct sequencing, and introduced into the p416GPD-URA3 vector using BamHI and EcoRI restriction sites, allowing us to express them under the strong promoter glyceraldehyde-3-phosphate dehydrogenase (GPD). The obtained plasmids were then used to transform yeast using the lithium acetate method.

Yeast cells were grown and labeled in YPD unless treated with dithiothreitol (DTT), in which case they were grown in minimal media. Rapidly growing cells were labeled with 20 μM FM 4–64 (Invitrogen) for 30 minutes and then collected and suspended in fresh media lacking FM 4–64 for 60 minutes. Cells were washed twice with PBS before imaging. For DTT-treated samples, 2 mM DTT was added to cultures at the start of FM 4–64 labeling 2 hours before microscopy. Two millimolar DTT was maintained in the media after the labeling period and in the PBS washes. Images were collected at room temperature with a Zeiss Axio Imager M2 inverted microscope with a ×100 objective, Axioscan 506 monochrome, and NIH Image software.

**Drosophila Movement Assays**

*Drosophila* were reared on a standard cornmeal, yeast, sucrose food as previously reported.\(^6\) Two S' P-element insertions and a deficiency chromosome from Bloomington *Drosophila* stock center (NIH P400D018537) were first outcrossed with balancer stocks for a single generation to replace the 1st chromosome with \(w^+\) and swap the 2nd chromosome balancer to include a larval marker. Experimental genotypes were CG1407\(^{M00131}\)/+ (control), CG1407\(^{pfa3\alpha}\)/+ (control), and CG1407\(^{pfa3\alpha}\)/063-\(G^4\) (compound heterozygote) for larval turning and w\(^1118\)/+ (control), CG1407\(^{pfa3\alpha}\)/063-\(G^4\) (compound heterozygote), and CG1407\(^{pfa3\alpha}\)/Df(2R)X1 (hemizygote) for adult flight analysis.

Larval turning time was defined as the amount of time required to turn onto the ventral surface and initiate forward posttranslation addition of palmitate to cysteine residues by palmitoyl acyltransferases targets proteins to intracellular membrane–associated compartments and to synapses within the CNS.\(^1\) Many palmitoyl acyltransferases contain a 51-residue zinc finger domain with a DHHC motif crucial for enzymatic function. ZDHHHC proteins were implicated in neurodevelopmental disorders (NDDs) when a balanced t(X;15) (q13.3;cen) translocation disrupting the ZDHHC15 gene was reported in association with intellectual disability.\(^2\) This patient showed skewed lyonization, with 100% inactivation of the normal X chromosome. Variants in the related ZDHHC9 have been associated with NDDs\(^3\) including motor delay, coordination difficulties, and cerebral palsy.\(^4\)

However, the finding of normal intelligence (Wechsler Adult Intelligence Scale–III full-scale IQ 111) in a woman with a balanced translocation disrupting the gene\(^5\) suggests that ZDHHC15 loss of function may not be pathologic. Although X inactivation was not assessed in this report, the authors were unable to detect ZDHHC15 expression in peripheral blood necessitating validation studies of ZDHHC15’s role in neurodevelopment. We identified individuals with predicted deleterious variants within a cohort of cryptogenic cerebral palsy patients who underwent whole-exome sequencing\(^6\) and through gene matching efforts (genematcher.org). We identify functional consequences of these variants and confirm a role in regulating movement in genetic models. Finally, we present clinical information from a patient harboring a verified deleterious, X-linked variant (p.H158R) that disrupts the core DHHC domain.

**Glossary**

DTT = dithiothreitol; GPD = glyceraldehyde-3-phosphate dehydrogenase; NDD = neurodevelopmental disorder.
movement after rotation onto the dorsal surface. Time was measured for 48–50 larvae/genotype with 3 trials/larva, and the average time was calculated. A failed trial was when a larva did not move for 30 seconds after rotation onto dorsal surface. Differences in average time were compared with both genetic controls using the paired t test. The number of successes and failures was compared via χ² analysis with 1 degree of freedom and was not significant.

We reanalyzed 9 videos with distance traveled impairments for flight behaviors for 7 seconds after animals were tapped to bottom of vials. The number of attempted flights was counted and categorized by net height. Up: net vertical distance was higher than the start point. Down: net vertical distance below the start point. Up to Down: flight was initially upward, but ended with a net vertical decrease. Center: no net vertical distance change. Movements where the flies jumped in a straight trajectory across the vial with no up or downward movement (i.e., no flight attempt) were excluded. The scorer was blinded to genotype. Comparisons of types of movements/fly between genotypes conducted using 2-tailed, paired t test and overall distribution via χ² analysis with 3 degrees of freedom.

**Data Availability**

Variant information for p.H158R deposited in ClinVar (SCV001468640). Sequencing and clinical descriptions of p.L13P patient described in Jin et al., doi.10.1038/s41588-020-0695-1. Any data not published within the article will be shared by request from any qualified investigator.

**Results**

**ZDHHC15 (p.H158R) Causes Loss of Function**

We investigated 4 patient variants in *Saccharomyces cerevisiae* and found no clear differences in protein abundance between variants and wild type (data not shown). We thus designed a complementation-based assay to discriminate deleterious and benign variants. The yeast ZDHHC15 ortholog, PFA3, encodes a palmitoyl transferase responsible for the palmitoylation of Vac8p, a protein involved in the fusion of vacuolar membranes in yeast. Palmitoylation of Vac8p is important for protein localization to the vacuolar membrane where it performs its function. Consequently, yeast cells lacking PFA3 (pfa3-Δ) show a vacuole fragmentation phenotype when stressed by low glucose or presence of the reducing agent DTT.

We designed a complementation assay where rescue of the vacuole fragmentation phenotype of the pfa3-Δ strain in the presence of DTT was analyzed after introducing a plasmid carrying either yeast PFA3 or human ZDHHC15 variants under the control of the yeast GPD strong promoter. We confirmed that pfa3-Δ cells develop vacuolar fragmentation in the presence of 2 mM DTT and reintroducing either wild type PFA3 or ZDHHC15 rescued this phenotype (Figure 1). In addition, we found that yeast cells expressing ZDHHC15 variants (p.L13P, p.K115R, and p.S330P) were indistinguishable from cells harboring the reference ZDHHC15 allele. However, expressing the ZDHHC15 (p.H158R) variant did not rescue the vacuolar fragmentation phenotype, indicating that this specific change significantly disrupted normal protein function, putatively by disrupting the core DHHC domain (Figure 1A).
The patient is an 18-year-old man with a history of hypotonic cerebral palsy, focal-onset epilepsy, cortical visual impairment, intellectual disability, autism spectrum disorder, anxiety, and aggressive behaviors. Physical examination revealed tall forehead with mild brachycephaly, down-slanting palpebral fissures, large ears, long face, and facial hypotonia. He has a high-arched palate with dental crowding. He has long, slender palms and fingers (arachnodactyly). He is nonverbal and was uncooperative with many portions of the examination.

He was born at 39 weeks gestational age via spontaneous vaginal delivery. There were no complications reported with the pregnancy or delivery, and his neonatal course was unremarkable. He had global developmental delay. Previous cognitive testing established intellectual disability and autism diagnoses, which have been present and nonprogressive throughout his life, although those medical records were not available for review. He first walked independently at age 8 years, but currently requires 2 hands for support. His gait has deteriorated and become crouched from contractures of the gastrocnemius/soleus complex and hamstrings (Video 1).
Gait changes are attributed to unbalanced biomechanical forces related to his hypotonia (no spasticity was apparent) despite previous orthopedic intervention (right pes planus reconstruction, right cavovarus foot reconstruction, and bilateral hamstring lengthening). There is no reported family history of intellectual disability, cerebral palsy, or epilepsy.

His most recent routine EEG demonstrated poorly organized and poorly sustained awake background, and some sharp transients seen over the frontal regions during sleep. Polysomnogram demonstrated increased awakenings after sleep onset and reduced sleep efficiency, but no evidence of sleep disordered breathing, nocturnal movement disorders, or potentiation of epileptiform activity. Brain MRI was remarkable for mild cerebrum sulci widening, corpus callosum foreshortening, and a left lateral periatrial white matter ovoid hyperintensity (Figure 2B–E). On laboratory testing, only transient hyperammonemia (attributed to valproic acid use) was noted. His seizures are currently well controlled on valproic acid and lamotrigine. He is receiving speech, physical, and occupational therapies.

**ZDHHC15 Regulates Motor Control in a Genetic Model**

We assessed the role of **ZDHHC15** in motor control using the functional ortholog in *D. melanogaster* (CG1407, DIOPT 12/15). We found an increase in average time to execute a coordinated axial twisting task in mutant larva (13.4 vs 10.3 and 9.6 seconds, p < 0.008, t test, Figure 3). We also examined movement strategies used by adult *Drosophila* in a negative geotaxis flight task. We characterized movement types by direction of flight path and net upward/downward travel. Wild-type flies often use upward flights to reach the top of the assay container. Mutant flies were less likely to initiate flights (2.4 and 2.2 vs 3.5 movements/fly, p < 0.04, t test). There was a change in the ratio of movements for the most severely impaired genotype compared with both the genetic control (p < 0.001) and the compound heterozygote (p < 0.05, χ² test). This was attributable to a decrease in upward flight (1.7 vs 1.0 upward flights/fly, p < 0.02 t test). This suggests defects in coordinating and initiating movement as well as for effective flight. This is consistent with the decreased distance traveled in a locomotor assay we reported previously. Together, this shows a role of **ZDHHC15** in the regulation of multiple motor activities across development in our genetic model.

**Discussion**

We report a patient with a hemizygous X-linked mutation in the **ZDHHC15** gene (p.H158R) with a mixed neurodevelopmental phenotype that included cerebral palsy, intellectual disability, autism spectrum disorder, and epilepsy. As cerebral palsy is a clinical description, not a specific pathogenic mechanism, the diagnosis of cerebral palsy was retained after identifying a genetic etiology in recognition of the expanding genetic landscape of cerebral palsy identifying it as a neurodevelopmental feature analogous to intellectual disability, autism, and epilepsy.

We used yeast and fly models to verify loss of function of the (p.H158R) variant and a role of this gene in coordinated motor tasks. We conducted the current studies in part to clarify whether **ZDHHC15** mutations could lead to neurodevelopmental disorders. Previous work has shown that mutations within the conserved DHHC domain in **ZDHHC9** impair protein function, and we show here that a similar disruption at residue 158 of **ZDHHC15** also leads to a mixed neurodevelopmental disorder. However, most of the missense variants we detected in **ZDHHC15** did not alter function. This has important implications for interpretation of these variants in the context of clinical sequencing studies, as borderline in silico prediction scores often pose problems in interpretation. This is particularly challenging for X-linked variants, which are typically inherited from an unaffected mother. The impact of heterozygous balanced translocations disrupting **ZDHHC15** is currently unresolved. Possible explanations include variability in patterns of X inactivation across tissues or individuals as has been recently reported epistatic influences.
ZDHHC15 regulates PSD95 palmitoylation and trafficking, with knockdown in rat neuron cultures reducing dendrite outgrowth and excitatory synapse maturation. Future work may enhance our understanding of the biological effects of ZDHHC15 mutations by cataloging other proteins that undergo palmitoylation by this enzyme, particularly in the developing brain. A palmitoylation profile could catalog protein posttranslational modification targets more comprehensively. This could represent an important first step toward the aim of ultimately developing targeted therapies.

Acknowledgment
The authors are grateful to the patients and their families for their gracious support of this work. These studies were supported by NIH 1R01NS106298 (M.C.K.), the Cure CP Foundation, and NIH R00HL143036-02 to S.C.J. The authors acknowledge Cathy Stevens for contributing information on variant p.K115R. They appreciate the Phoenix Children’s Hospital clinicians for their expert care including Korwyn Williams, MD, PhD, Wendy Bernatciavious, MD, David Shafron, MD, Laura Wilner, MD, and Emily Andrisevic, MD.

Study Funding
NIH 1R01NS106298 (to M.C.K), the Cure CP Foundation, and NIH R00HL143036-02 (to S.C.J).

Disclosure
The authors report no disclosures relevant to the manuscript. Go to Neurology.org/NG for full disclosures.

Publication History
Received by Neurology: Genetics September 25, 2020. Accepted in final form May 3, 2021.

Appendix

| Name            | Location                                      | Contribution                                |
|-----------------|-----------------------------------------------|---------------------------------------------|
| Jennifer Heim, MD | Phoenix Children’s Hospital and University of Arizona College of Medicine, Phoenix, AZ | Neuroimaging interpretation                |
| Patricia Cornejo, MD | Phoenix Children’s Hospital and University of Arizona College of Medicine, Phoenix, AZ | Neuroimaging interpretation                |
| James Liu, BS  | Arizona State University, Tempe, AZ | Conceptual design and analysis of yeast studies |
| Aris Huang      | Arizona State University, Tempe, AZ | Conceptual design and analysis of fly studies |
| Andrew Musmacker | Phoenix Children’s Hospital, Phoenix, AZ | Analysis of fly studies                     |
| Sheng Chih Jin, PhD | Department of Genetics, Washington University, St. Louis, MO | Conceptual design of genomic sequencing |
| Kaya Bilgувар, MD | Department of Genetics, Yale University, New Haven, CT | Intellectual contributions for genomic sequencing |
| Sergio R. Padilla-Lopez, PhD | Phoenix Children’s Hospital and University of Arizona College of Medicine, Phoenix, AZ | Conceptual design and intellectual contributions to yeast studies |
| Michael C. Krue, MD | Phoenix Children’s Hospital, University of Arizona College of Medicine, Arizona State University, Phoenix, AZ | Intellectual contributions to paper draft and revisions and overall data interpretation |

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