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

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## 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 periatrial 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.

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# Glossary

**DTT** = dithiothreitol; **GPD** = glyceraldehyde-3-phosphate dehydrogenase; **NDD** = neurodevelopmental disorder.

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. ZDHHC 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.

# 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.

## Yeast 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, Axiocam 506 monocamera, and NIH Image software.

## *Drosophila* Movement Assays
*Drosophila* were reared on a standard cornmeal, yeast, sucrose food as previously reported.[6] Two 5′ P-element insertions and a deficiency chromosome from Bloomington *Drosophila* stock center (NIH P40OD018537) 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 $CG1407^{MI00131}/+$ (control), $CG1407^{063-G4}/+$ (control), and $CG1407^{MI00131/063-G4}$ (compound heterozygote) for larval turning and $w^{1118}/+$ (control), $CG1407^{MI00131/063-G4}$ (compound heterozygote), and $CG1407^{MI00131}/Df(2R)X1$ (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

**Figure 1** Yeast Complementation Studies With Human *ZDHHC15* Variants
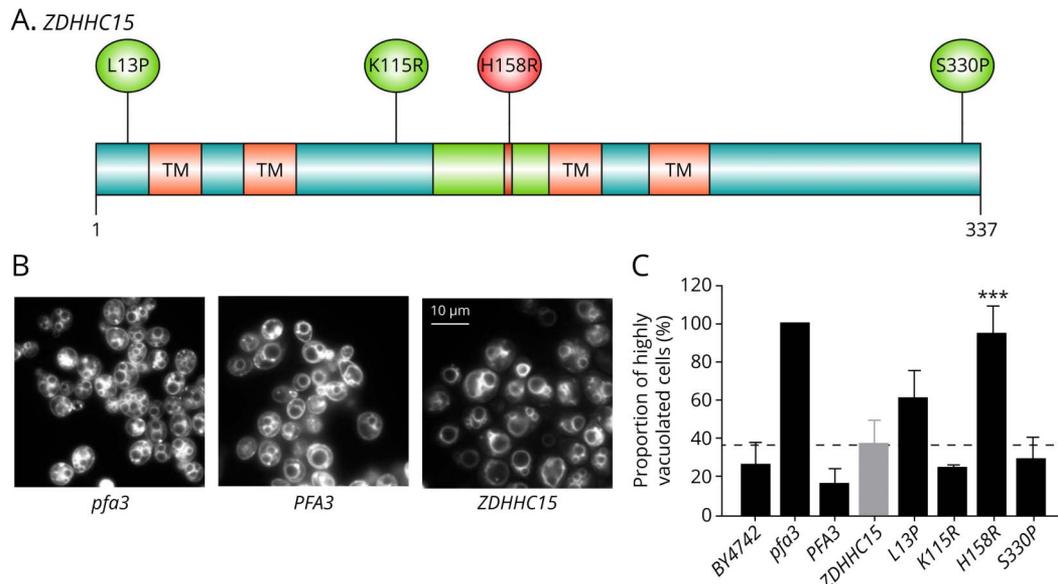


(A) Human ZDHHC15 protein depicting transmembrane, DHHC-CRD domains and the position of missense variants detected in individual cases. (B) Representative images of FM-464 vacuolar membrane staining in pfa3 yeast strain transformed with empty vector (*pfa3*), yeast *PFA3* (*PFA3*), or human *ZDHHC15* (*ZDHHC15*) after 2-hour incubation with 2 mM DTT. (C) Quantification of the number of cells with more than 6 vacuoles after incubation with DTT. ***Indicates significance determined by 2-tailed t test $p < 0.0001$ compared to ZDHHC15 control (grey bar). DTT = Dithiothreitol.

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 $\chi^2$ analysis with 1 degree of freedom and was not significant.

We reanalyzed 9 videos with distance traveled impairments[6] 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 $\chi^2$ 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.,[6] 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.[7] 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).
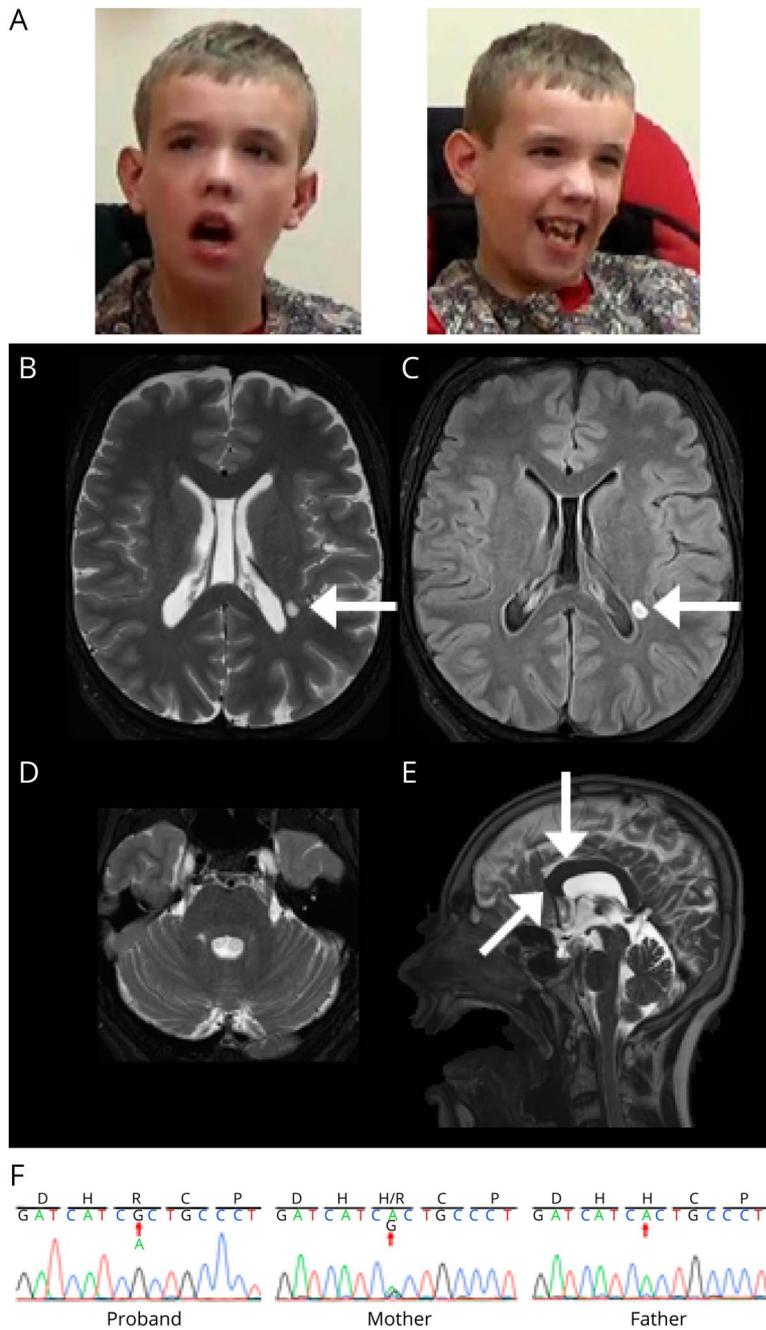
(A) Dysmorphic facial features include mild brachycephaly, down-slanting palpebral fissures, large ears, long face, and facial hypotonia. (B–E) Brain MRI at age 17 years. Axial T2 (B) and axial FLAIR (C) images demonstrate mild symmetric widening of the cerebral sulci and a small ovoid focus of T2/FLAIR signal prolongation in the left lateral periatrial white matter (white arrow). Axial FLAIR (D) image at the posterior fossa level demonstrates symmetric widening of the cerebellar folia. Sagittal T2 (E) image at midline shows a smaller-than-expected craniofacial ratio. In addition, the corpus callosum is mildly thickened and foreshortened (white arrow). All these findings have been stable over time on serial MRI. (F) Sanger traces of the proband, mother, and father confirming A to G base change inherited from the mother.

## *ZDHHC15* (p.H158R) Clinical Summary

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 muscle hypotonia (Figure 2A). 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).

## Figure 3 Larval Turning and Adult Flight Defects Caused by Loss-of-Function Mutations in the ZDHHC15 *Drosophila* Ortholog
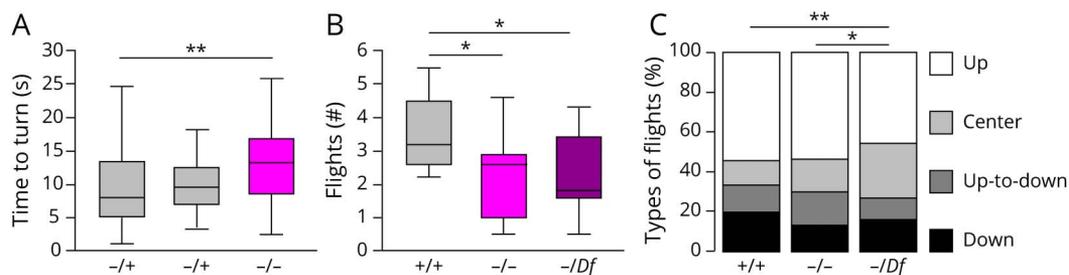


(A) Time to turn is increased in compound heterozygotes compared with either heterozygote alone. (B) Number of flights normalized to number of flies in assay is decreased in compound heterozygous and hemizygous flies. (C) Types of movements used by each genotype. These could be up, center (no net change), up to down (initial up trajectory but net down movement), or down; center movements took place over the entire assay vial and were not limited to the bottom. *-/Df* flies had a significant change in types of flights compared with +/+ ($p < 0.001$) or −/− ($p < 0.05$). N = 48–50 larvae per genotype and 72-100 adult flies per genotype across 9 trials. *$p < 0.05$, **$p < 0.008$ by *t* test (A, B) or $\chi^2$ (C).

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 cerebral 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 ($p < 0.05$, $\chi^2$ 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.[6] 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[6] 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,[8] 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[9] or epistatic influences.

ZDHHC15 regulates PSD95 palmitoylation and trafficking, with knockdown in rat neuron cultures reducing dendrite outgrowth and excitatory synapse maturation.[10] 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.

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

## Appendix Authors

| Name | Location | Contribution |
| --- | --- | --- |
| Sara A. Lewis, PhD | Phoenix Children's Hospital and University of Arizona College of Medicine, Phoenix, AZ | Design, data collection, statistics of fly studies, and drafted and revised the manuscript for intellectual content |
| Somayeh Bakhtiari, PhD | Phoenix Children's Hospital and University of Arizona College of Medicine, Phoenix, AZ | Bioinformatics analysis, GeneMatcher patient recruitment, and record collection |

## Appendix *(continued)*

| Name | Location | Contribution |
| --- | --- | --- |
| Jennifer Heim, MD | Phoenix Children's Hospital and University of Arizona College of Medicine, Phoenix, AZ USA | Treating clinician for the patient and collected descriptions |
| 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 Bilguvar, 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. Kruer, 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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