The American Journal of Human Genetics, Volume *106*

Supplemental Data

Loss-of-Function Variants in *PPP1R12A*: From Isolated Sex Reversal to Holoprosencephaly Spectrum and Urogenital Malformations

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Supplemental Note: Case Reports

Individual 1: She was born at term to a 19-year-old mother by Cesarean delivery and weighed 3.23 kg (50^{th} centile). The pregnancy was complicated by maternal hypertension, pre-eclampsia, diabetes, and exposure to alcohol and marijuana at approximately 8 weeks gestation. She had syntelencephaly/middle interhemispheric variant (MIHV) of HPE, polymicrogyria, and Chiari I malformation identified on brain MRI. Other medical problems included intellectual disability, attention deficit hyperactivity disorder (ADHD), and seizures. Sanger sequencing of the four most common genes associated with HPE, *SHH* (MIM: 600725), *ZIC2* (MIM: 603073), *SIX3* (MIM: 603714), and *TGIF* (MIM: 602630), failed to identify any detectable pathogenic variants. Trio exome sequencing revealed a heterozygous *de novo* variant in *PPP1R12A,* NM_002480.3:c.2033_2034del p.(Ser678*), which was confirmed by Sanger sequencing.

Individual 2: She was a 6-year-old female with semilobar HPE and agenesis of the corpus callosum identified on MRI. Other medical problems included myoclonus, intellectual disability, and syndactyly of the toes. Sanger sequencing of *SHH, SIX3, ZIC2,* and *TGIF* failed to identify detectable pathogenic variants. Trio exome sequencing revealed a heterozygous *de novo* variant in *PPP1R12A,* NM_002480.3:c.1415C>G p.(Ser472*), which was confirmed by Sanger sequencing.

Individual 3: Prenatally, he had a fetal ultrasound and MRI which demonstrated agenesis of the corpus callosum and colpocephaly as well as pyelectasis and intrauterine growth restriction. Chromosome analysis of amniocytes showed 46,XY Normal male. A Cesarean delivery occurred at 36 2/7 weeks for breech presentation and oligohydramnios. Significant findings on newborn evaluation included low birth weight of 2.04 kg (3-10^{th} centile), decreased subcutaneous fat, mild facial asymmetry, ulnar drift at the wrist and fisting, upper back kyphosis, and grade 1 hydronephrosis with renal asymmetry detected by ultrasound. Findings at age 5.5 years were grossly unchanged apart from additional behavioral issues including ADHD and defiance. A chromosome microarray analysis (CMA) was arr(1-22normal. Trio

exome sequencing through GeneDx revealed a heterozygous *de novo* splice site variant in *PPP1R12A,* NM_002480.3: c.793-1G>A.

Individual 4: Prenatally, an 11-week antenatal ultrasound showed fetal acrania with exencephaly, hypertelorism, flattened facial profile with non-visualization of the nasal bone, omphalocele, echogenic bowel, and non-visualization of the lumbosacral spine. Fetal growth was normal. The parents were of Chinese descent, non-consanguineous and with an unremarkable family history. The pregnancy was interrupted and limited pathological assessment of the fetus and placenta at 12 weeks gestation revealed the presence of a partial cranial vault with scant white-grey tissue and spinal column. Chorionic villus and fetal neural, renal, gastrointestinal, and cardiac tissues were histologically examined and unremarkable. Maternal serum folate and vitamin B12 levels were within normal range. CMA on products of conception was arr(1-22,X)x2 normal female. *UPD11* testing and *CDKN1C* sequencing obtained due to the presence of an omphalocele on ultrasound showed no abnormalities. Trio exome sequencing identified a heterozygous *de novo* frameshift variant in *PPP1R12A,* NM_002480.3:c.223_224delAC p.(Thr75Cysfs*8).

Individual 5: He was a 3-year-old male with ambiguous genitalia at birth. Physical exam revealed micropenis, chordee, scrotal hypospadias, bilateral cryptorchidism, and a uterus on ultrasound. No other birth defects were reported. Additionally, serum anti-Müllerian hormone levels were below normal range. Karyotype was 46,XY Normal male. Developmentally, he was reported appropriate for age and had an unremarkable head CT scan. Trio exome sequencing by GeneDx identified a *de novo* heterozygous frameshift variant in *PPP1R12A* NM_002480.3: c.2739_2740delCT p.(Leu914Argfs*14).

Individual 6: He was a 6-year-old male found on fetal ultrasound, at 19 weeks of gestation, to have an encephalocele at the posterior parietal region and colpocephaly. The pregnancy was complicated by chronic maternal hypertension, type II diabetes mellitus controlled with insulin, and intrauterine growth restriction. At birth, he was noted to have thrombocytopenia requiring platelet transfusion. He had ventriculoperitoneal shunt insertion for hydrocephalus and encephalocele repair shortly after birth. Postnatal MRI revealed dysgenesis of the corpus callosum, absent septum pellucidum, Chiari

malformation, cortical dysplasia/polymicrogyria and grey matter heterotopia. Neurological examination was notable for global developmental delay, intellectual disability, autistic features, appendicular hypotonia with foot pronation requiring supra malleolar orthosis (SMO) braces bilaterally, and an unsteady gait. He sat at 1 year, walked at 3 years, and continued to have difficulties with expressive language with limited speech. He had minor dysmorphic facial features including short upslanting palpebral fissures, low-set ears, and micrognathia. Other significant features included short stature, patent ductus arteriosus, and ophthalmologic abnormalities including strabismus, astigmatism, hyperopia, and alternating esotropia. The genitourinary abnormalities included glandular hypospadias and chordee which required surgical correction. Karyotype was 46,XY Normal male and trio exome sequencing by GeneDx identified a heterozygous nonsense *de novo* variant in *PPP1R12A* NM_002480.3:c.1510C>T p.(Arg504*).

Individual **7:** He was a 7-year-old male with genitourinary malformations including hypospadias, cryptorchidism, and a uterus. The prenatal and early medical history is unknown, as he was adopted out. Karyotype was 46,XY Normal male. He had generalized developmental delay, seizures and brain MRI showed microcephaly and leukomalacia along with opacified left tympanic cavity and mastoid air cells. Facial dysmorphism was noted including long face, large prominent ears, ptosis, and a small pointed nose. Other abnormalities included, 5th finger clinodactyly, and blind shallow rectal cleft. Further investigation revealed delayed bone age, bilateral rod and cone dysfunction with decreased vision, and latent nystagmus. Singleton exome sequencing by GeneDx revealed a heterozygous nonsense variant in *PPP1R12A,* NM_002480.3:c.2573G>A p.(Trp858*).

Individual 8**:** She was a 45-year-old female with typical female external genitalia and a 46,XY Normal male karyotype. Pelvic ultrasound identified a small uterus didelphys. She had a history of a bilateral gonadectomy in childhood; however, the pathologist did not specify ovarian or testicular tissue on report. She had primary amenorrhea and was over 6 feet tall. *SRY* and *NR5A1* gene sequencing were unremarkable. There were no reported neurological issues, developmental delay, or other malformations.

Singleton exome sequencing performed at GeneDx identified a heterozygous frameshift in *PPP1R12A* NM_002480.3:c.2073dupA p.(Ser692Ilefs*2).

Individual 9: She was a 9-month-old phenotypic female with a 46,XY Normal male karyotype, and had external genitalia notable for clitoral hypertrophy (0.5 cm in diameter), urogenital sinus (UGS), vaginal opening and posterior fusion of the labia majora. No uterus was identified on pelvic ultrasound. Prenatal history was unremarkable with spontaneous vaginal delivery at 39 weeks. Biochemical workup was not consistent with congenital adrenal hyperplasia. Neuroimaging was not indicated at that time. Trio exome sequencing performed via a German health care project for rare diseases revealed a heterozygous *de novo* nonsense variant in *PPP1R12A* NM_002480.3:c.2698C>T p.(Arg900*).

Individual 10: He was a 2-year-old phenotypic male with 46,XY Normal male karyotype, evaluated by clinical genetics due to grade 2 hypospadias and cryptorchidism. He was born premature at 27 weeks. A Fallopian tube without mention of an attached gonad was identified during surgical repair of a right inguinal hernia. Abdominal ultrasound showed a uterus. Physical exam was notable for short stature, macrocephaly, triangular face, long palpebral fissures, ptosis, small mouth and wide nasal tip. He had global developmental delay and brain MRI was normal. A CMA was normal. Trio exome sequencing by GeneDx revealed a likely pathogenic heterozygous maternally inherited variant in *SCN8A,* NM_01491.3:c.2424dupT p.(Pro809Serfs*13) which was identified in a clinically unaffected sib, as well as a heterozygous *de* novo variant in *PPP1R12A,* NM_002480.3:c.960dupA p.(Glu321Argfs*6).

Individual 11**:** She was a adult female with 46,XY gonadal dysgenesis, alopecia totalis, obesity and acanthosis nigricans. Diagnostic laparoscopy at age 6 identified two streak gonads (abdominal on the right and inguinal on the left) which were resected, with rudimentary Fallopian tubes, a vaginal opening, and no uterus. She has had normal development and brain MRI was normal. At age 30, she is doing well on hormone replacement therapy. Trio research genome sequencing identified a heterozygous *de novo* variant in *PPP1R12A*, NM_002480.3:c.1189delA p.(Thr397Hisfs*42) which was confirmed by Sanger sequencing.

Individual 12: She was evaluated by genetics due to discordance between the chromosome sex (46,XY) on cell free non-invasive prenatal testing and the phenotypic sex as identified on fetal ultrasound showing a female external genitalia. Postnatally, she was noted to have jejunal and ileal atresia. Surgery revealed an aberrant mesenteric blood supply, normal-appearing ovaries with Fallopian tubes and a uterus. CMA was arr(1-22)x2,(X,Y)x1 normal male. An ultrasound at 1 year showed normal kidneys and confirmed the presence of a uterus, but did not identify gonads, suggesting gonadal degeneration. Examination at age 2 showed a clitoris, posterior labial fusion, increased labial rugation and pigmentation, and mild gynecomastia. Her growth parameters were normal. She had strabismus, bilateral epicanthus inversus, right esotropia, abnormal auricles, bilateral 5th digit clinodactyly, and spoon-shaped toenails. She had developmental delay and autism spectrum disorder. Brain MRI has not been done. Luteinizing hormone and FSH was within normal range, and anti-müllerian hormone and testosterone was lower than normal range for a male with a 46,XY karyotype. Research genome sequencing identified a heterozygous *de novo* variant in *PPP1R12A*, NM_002480.3:c.681dupT (p.Lys228Ter) which was confirmed by Sanger sequencing.

Supplemental Material and Methods

Brain: mouse *in situ* hybridization. Genes that regulate forebrain patterning and play a role in HPE pathogenesis are expected to be expressed in the prosencephalic neural folds that give rise to the forebrain during primary neurulation.[1] Therefore, we conducted *in situ* hybridization (ISH) on mouse embryos at gestational day (GD) 8.25. This stage is representative of early neurulation and within the critical period for development of HPE.[2, 3] Studies were conducted in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The protocol was approved by the University of Wisconsin-Madison School of Veterinary Medicine Institutional Animal Care and Use Committee (protocol

number G005396). CD-1 mice (Mus musculus) were purchased from Charles River and C57BL/6J mice from The Jackson Laboratory. Timed-pregnancies were established as previously described.[4] Embryos were dissected at GD8.25 and fixed overnight in 4% paraformaldehyde. *In situ* hybridization (ISH) was conducted on whole C57BL/6J embryos or 50 µm sections cut from CD-1 embryos with a vibrating microtome in the transverse plane along the anterior-posterior axis. ISH was conducted as previously described.[5]

Urogenital: mouse and human immunostaining. Human lower urinary tracts were obtained from the Joint MRC / Wellcome (MR/R006237/1) Human Developmental Biology Resource (www.hdbr.org) under an approved University of Wisconsin-Madison IRB protocol (2016-0449). C57BL/6J mouse lower urinary tracts were obtained under a University of Wisconsin-Madison approved ACUC protocol (protocol number G005396). Tissues were embedded in paraffin and stained with antibodies against PPP1R12A (Thermo Scientific PA579857, 1:250), PPP1R12B (Sigma HPA024171, 1:250), CDH1 (BD Transduction Labs 610181, 1:250) and with DAPI (nuclei) using an established method.[6] Mouse results are representative of three independent mice per group and human results are representative of one per group.

DNA Sequence and Analysis Methods

National Institutes of Health: DNA samples from study participants underwent exome sequencing at the National Intramural Sequencing Center (NISC) as previously described.[7] The mean read depth for each sample was 79.8. Copy number variation (CNV) prediction from exome data was done using the XHMM (eXome-Hidden Markov Model) caller.[8] We used

GATK to generate the depth of coverage statistics required for XHMM from the BAM files of our HPE cohort and a control set. GATK output was then run through the XHMM pipeline, generating a VCF file containing each predicted CNV. We then annotated each CNV for genes contained and cytogenetic region using Annovar. All probands were first searched for four common genes known to cause HPE: *SHH* (MIM: 600725) on 7q36, *ZIC2* (MIM: 603073) on 13q32, *SIX3* (MIM: 603714) on 2p21, and *TGIF* (MIM: 602630) on 18p11.3 using Sanger sequencing as recommended.[9] With the goal of new gene discovery, minimizing false positives, and sacrificing sensitivity, the discovery cohort was filtered with stringent criteria including *de novo* inheritance in genes intolerant of variation,[10] variant absence in the ExAC database,[10] and Combined Annotation-Dependent Depletion (CADD) scores above 20.[11] Variants that met these criteria were considered deleterious. A total of 101 trios affected by holoprosencephaly (proband, father, and mother) were sequenced.

Technical University of Munich: DNA was extracted from peripheral blood using the Gentra Puregene Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Trio exome sequencing (ES) was performed using a Sure Select Human All Exon 60 Mb V6 Kit (Agilent) and a NovaSeq 6000 (Illumina) as previously described.[12] Mitchondrial DNA was derived from off-target exome reads as previously described.[13] Reads were aligned to the human reference genome (UCSC Genome Browser build hg19) using Burrows-Wheeler Aligner (v.0.7.5a). Detection of single-nucleotide variants and small insertions and deletions (indels) was performed with SAMtools (version 0.1.19). ExomeDepth was used for the detection of copy number variations (CNVs).[14] For the analysis of *de novo,* autosomal dominant and mitochondrial variants, only variants with a minor allele frequency (MAF) of less than 0.1% in the in-house

database of the Helmholtz center Munich containing over 18,000 exomes were considered. For the analysis of autosomal recessive and X-linked variants (homozygous, hemizygous or compound heterozygous) only variants with a MAF of less than 1.0% were considered.

Children's National Hospital: A cohort of 300 samples, belonging to 94 families with a variety of syndromic or isolated DSD conditions was sequenced. Whole genome sequencing at an average 30x coverage was performed on a HiSeqX instrument at the Baylor facility under the Gabriella Miller Kids First Initiative XO1 mechanism (https://commonfund.nih.gov/kidsfirst/x01projects). Targeted search for exonic variants in *PPP1R12A* analysis was performed using both the Genoox platform (https://www.genoox.com/) and the Broad Institute's *Seqr* software (https://www.seqr.broadinstitute.org/).

GeneDx: Limited availability of detailed commercial practices regarding exome processing and analysis. Test info sheet for XomeDx accessible through their public website for review (https://www.genedx.com/).

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