

Biallelic Variants in *OTUD6B* Cause an Intellectual Disability Syndrome Associated with Seizures and Dysmorphic Features

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Ubiquitination is a posttranslational modification that regulates many cellular processes including protein degradation, intracellular trafficking, cell signaling, and protein-protein interactions. Deubiquitinating enzymes (DUBs), which reverse the process of ubiquitination, are important regulators of the ubiquitin system. *OTUD6B* encodes a member of the ovarian tumor domain (OTU)-containing subfamily of deubiquitinating enzymes. Herein, we report biallelic pathogenic variants in *OTUD6B* in 12 individuals from 6 independent families with an intellectual disability syndrome associated with seizures and dysmorphic features. In subjects with predicted loss-of-function alleles, additional features include global developmental delay, microcephaly, absent speech, hypotonia, growth retardation with prenatal onset, feeding difficulties, structural brain abnormalities, congenital malformations including congenital heart disease, and musculoskeletal features. Homozygous *Otud6b* knockout mice were subviable, smaller in size, and had congenital heart defects, consistent with the severity of loss-of-function variants in humans. Analysis of peripheral blood mononuclear cells from an affected subject showed reduced incorporation of 19S subunits into 26S proteasomes, decreased chymotrypsin-like activity, and accumulation of ubiquitin-protein conjugates. Our findings suggest a role for *OTUD6B* in proteasome function, establish that defective *OTUD6B* function underlies a multisystemic human disorder, and provide additional evidence for the emerging relationship between the ubiquitin system and human disease.

Whole-exome sequencing (WES) has proven powerful for discovering new genes associated with genetically heterogeneous disorders, including global developmental delay, intellectual disability, and seizures.^{1–4} In our clinical WES cohort of roughly 9,000 unrelated individuals, the majority of whom have neurologic manifestations and belong to the pediatric age group, we identified two unrelated individuals with biallelic variants in *OTUD6B* (MIM: 612021) with overlapping clinical features. Through

GeneMatcher,⁵ we subsequently identified 10 additional individuals, yielding a total of 12 affected subjects from 6 unrelated families (Figure 1). We obtained written informed consent from all study participants in accordance with protocols approved by institutional review boards at Baylor College of Medicine (USA), University of Kiel (Germany), National Research Center (Egypt), CHU de Nantes (France), and Makassed Islamic Charitable Hospital (Jerusalem).

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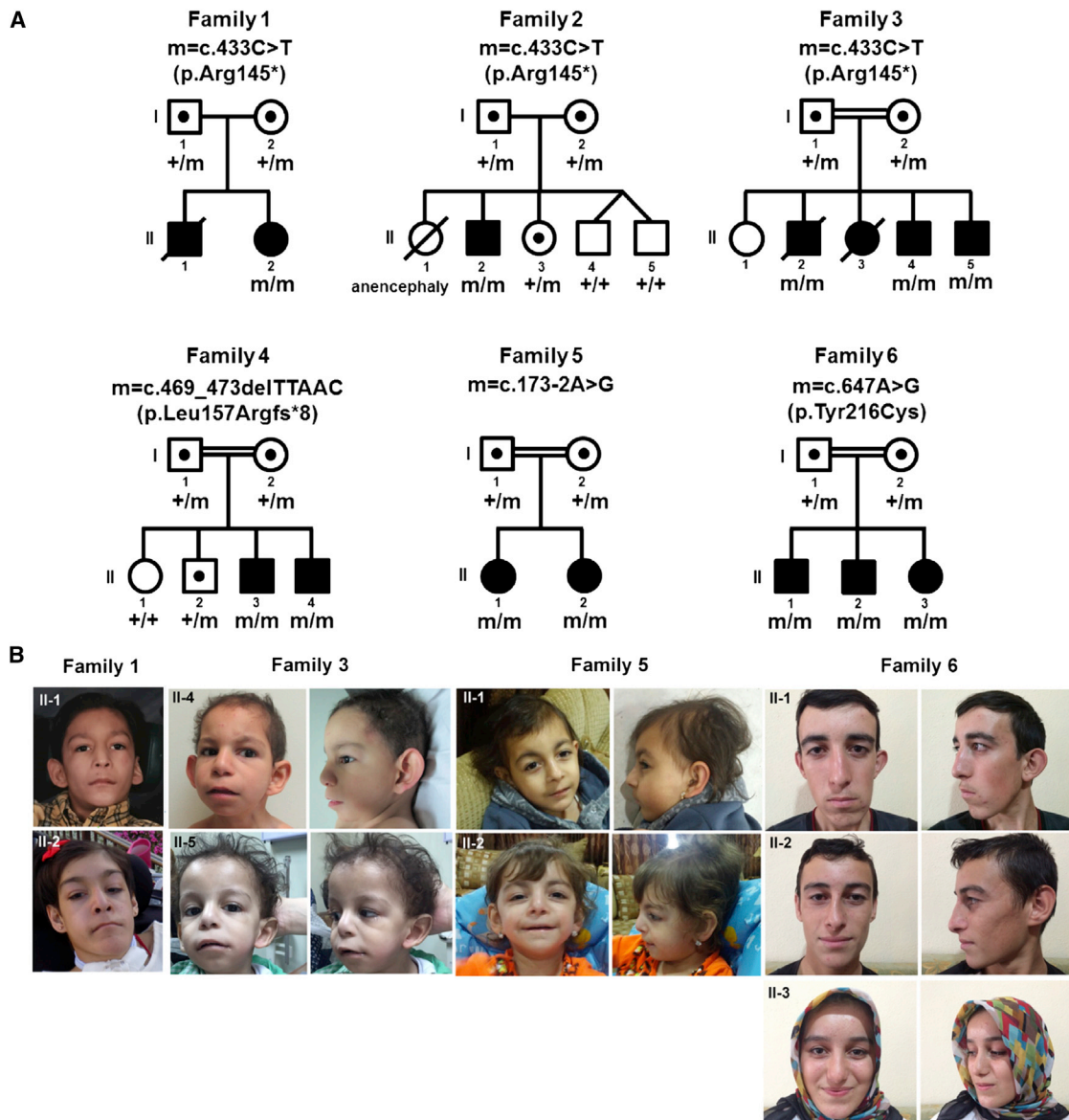


Figure 1. Pedigrees and Clinical Features of Families with Pathogenic Variants in *OTUD6B*

(A) Families with biallelic variants in *OTUD6B*. Allelic status is given below each tested individual. Symbols are as follows: filled, affected; empty, unaffected; dotted, heterozygous carrier; hash, deceased.

(B) Facial photos of affected individuals from families 1, 3, 5, and 6.

A summary of clinical findings is provided in [Table 1](#), detailed case summaries can be found in the [Supplemental Note](#), and pedigrees and photos are available in [Figure 1](#). Of the six families with variants in *OTUD6B*, two were non-consanguineous and four consanguineous. The cohort comprised eight males and four females, ages 3 to 20 years. All subjects exhibited intellectual disability. Nine subjects (75%) were severely impaired with absent speech and significant global developmental delay, and three subjects had only mild to moderate intellectual disability without delay in language or motor development. All subjects presented with seizures, mostly of the generalized tonic-clonic type, but with varying degrees of severity and frequency (from one episode to daily, refractory epilepsy). Other seizure types noted were absence, myoclonic, and atonic

seizures. In nine subjects, particularly those with severe intellectual disability, additional recurrent features included severe microcephaly (Z-scores between -2.8 and -6.5), hypotonia, gross motor delay, and growth retardation. Intra-uterine growth restriction (IUGR) was observed in seven pregnancies (58%). Nine subjects had feeding difficulties, which were evident as early as the neonatal period and required gastrostomy tube feeds for three subjects. Six subjects never walked independently. One subject (II-2 in family 1) with congenital spastic quadriplegia is able to walk short distances but is largely wheelchair dependent. One subject (II-2 in family 2) who did attain walking subsequently lost this ability due to severe and progressive spasticity. Among 12 subjects with neuroimaging studies, 6 showed structural brain abnormalities (50%) including

Table 1. Clinical Features of Subjects with Biallelic OTUD6B Variants

	Family 1	Family 2	Family 3			Family 4		Family 5		Family 6		
	II-2	II-2	II-2	II-4	II-5	II-3	II-4	II-1	II-2	II-1	II-2	II-3
Gender	female	male	male	male	male	male	male	female	female	male	male	female
Ethnic origin	Hispanic	Hispanic/Italy	Egypt	Egypt	Egypt	Syria	Syria	Palestine	Palestine	Turkey	Turkey	Turkey
Age at last examination	18 years, 2 months	17 years, 2 months	13 years (died same year)	8 years, 1 month	3 years	9 years, 5 months	3 years, 11 months	5 years, 4 months	3 years, 2 months	20 years, 7 months	16 years, 6 months	14 years, 8 months
Intellectual disability	severe	severe	severe	severe	severe	severe	severe	severe	severe	moderate	mild	moderate
Speech delay	no words	no words	no words	no words	no words	no words	no words	no words	no words	no delay	no delay	no delay
Seizure (onset)	+ (12 months)	+ (15 months)	+ (NA)	+ (NA)	+ (NA)	+ (17 months)	+ (3 years)	+ (7 months)	+ (2 years)	+ (18 months)	+ (7 years)	+ (8 years)
IUGR	+	+ (poor fetal activity)	+	+ (poor fetal activity)	+	+	+	-	-	-	-	-
Weight, kg (z-score)	28 (-8.9)	47.6 (-2.2)	15 (-2.2) (at 10 years)	12 (-4.5) (at 5 years)	9 (-3.5)	15.5 (-1.6) (at 5 years 2 months)	15 (-1)	15.6 (-1.4)	10 (-3.4)	67 (-0.33)	66 (0.27)	54 (0.27)
Height, cm (z-score)	129.5 (-5.2)	137 (-4.9)	103 (-5) (at 10 years)	90 (-3.8) (at 5 years)	80 (-3.7)	107 (-0.6) (at 5 years 2 months)	93 (-2.5)	100 (-2.1)	87 (-2.0)	176 (-0.1)	171 (-0.5)	159 (-0.4)
OFC, cm (z-score)	47.9 (-4.7)	52 (-2.8)	44.5 (-6.5) (at 10 years)	44 (-5.3) (at 5 years)	43 (-5.3)	46.5 (-3.5) (at 5 years 2 months)	45 (-4.3)	45 (-4.5)	44 (-4.3)	55.7 (-0.35)	54.5 (-0.59)	54.3 (0.38)
Hypotonia	+	+	+	+	+	+	+	+	+	-	-	-
Gross motor delay	congenital quadriplegic, mostly in wheelchair	walked at 3 years 9 months but now non-ambulatory	could sit, non-ambulatory	sits, non-ambulatory	does not sit, non-ambulatory	walked 4 years 6 months	sits, non-ambulatory	crawled at 3 years; stands and walks only with support	does not crawl or pull to stand, non-ambulatory	no delay	no delay	no delay
Feeding difficulties	+	+	+	+	+	+	+	+	+	-	-	-
Others	FTT, chronic constipation, G-tube feeds	FTT, chronic constipation and diarrhea, G-tube feeds	FTT	FTT	FTT	FTT	FTT, G-tube feeds					
Additional neurologic features	congenital quadriplegia, ataxia, spasticity	cerebral palsy, ataxia, spastic quadriplegia	autism spectrum disorder	autism spectrum disorder	autism spectrum disorder	-	-	-	-	-	-	-

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Table 1. Continued

	Family 1	Family 2	Family 3			Family 4		Family 5		Family 6		
	II-2	II-2	II-2	II-4	II-5	II-3	II-4	II-1	II-2	II-1	II-2	II-3
Brain imaging	normal	prominent perivascular spaces, hypoplastic corpus callosum, generalized white matter volume loss, gliosis in bilateral parietal lobes	mild fronto-parietal cortical changes	mild fronto-parietal cortical changes, short corpus callosum	mild fronto-parietal cortical changes	normal	cortical and white matter atrophy	mild dilatation of lateral ventricles mainly occipital horns, deep interhemispheric fissure/hypoplastic corpus callosum	normal	normal	normal	normal
Physical Features												
CHD	NA	normal echo	PS, ASD	normal echo	PS, ASD, VSD	NA	VSD	NA	TOF, ASD	NA	NA	NA
Other congenital malformations	sacral dimple	spina bifida occulta, cryptorchidism	–	cryptorchidism	–	cryptorchidism	sacral dimple cryptorchidism	–	–	–	–	–
Head/Neck	displaced posterior hair whorl	brachycephaly, sparse hair	short neck, sloping shoulders	short neck, sloping shoulders	short neck, sloping shoulders	–	–	flat occiput, sparse hair	flat occiput	wide forehead, narrow long asymmetric face	wide forehead, narrow long face	narrow long face
Eyes	arched eyebrows, long eyelashes, long palpebral fissures	arched eyebrows, long down-slanting palpebral fissures, ptosis	long palpebral fissures	long palpebral fissures	long palpebral fissures	long palpebral fissures	–	arched eyebrows, deep-set eyes, long eyelashes	deep-set eyes, long eyelashes	down-slanting palpebral fissures	–	–
Nose	long nose, hypoplastic alae, high nasal bridge, long philtrum	prominent nasal bridge, short columella, small alae, smooth long philtrum	broad root and prominent nasal bridge, smooth long philtrum	broad root and prominent nasal bridge, smooth long philtrum	broad root and prominent nasal bridge, smooth long philtrum	–	–	slightly long philtrum	slightly long philtrum	tubular nose	–	–
Mouth/Chin	high arched palate, cupid bow, dental crowding	thin upper lip, downturned mouth corners, high arched palate, mild retrognathia	very thin upper lip, retrognathia	very thin upper lip, retrognathia	very thin upper lip, retrognathia	–	–	thin upper lip	thin upper lip	high arched palate, dental malocclusion, narrow chin	high arched palate, narrow chin	high arched palate
Ears	–	posteriorly rotated ears, bilateral pits	large protruding low-set ears	large protruding low-set ears	large protruding low-set ears	large ears	large ears	large ears	large ears	prominent, dysplastic ears	–	–

(Continued on next page)

Table 1. Continued

	Family 1		Family 2		Family 3		Family 4		Family 5		Family 6		
	II-2	II-2	II-2	II-2	II-4	II-5	II-3	II-4	II-1	II-2	II-1	II-3	
Scoliosis	+	+	+	+	-	-	-	+	+	-	-	-	
Extremities	broad thumbs, tapered fingers, long first toes, joint contractures, brachydactyly of other toes, hallux valgus	broad thumbs, bulbous finger tips, forearm asymmetry, feet turned outward, joint contractures overriding toes, planovalgus	broad thumbs, clubbed fingers, clubfoot	broad thumbs, hyperextensibility of interphalangeal joints, overriding toes	broad thumbs, hyperextensibility of interphalangeal joints, overriding toes	broad thumbs, hyperextensibility of interphalangeal joints, overriding toes	broad thumbs and first toes, fetal pads	broad thumbs	bilateral toe syndactyly	bilateral toe syndactyly	arachnodactyly	hyperextensibility of elbows	arachnodactyly, hyperextensibility of elbows

Abbreviations are as follows: IUGR, intrauterine growth restriction; OFC, occipitofrontal circumference; NA, not available; CHD, congenital heart disease; PS, pulmonic stenosis; VSD, ventricular septal defect; ASD, atrioseptal defect; TOF, tetralogy of Fallot; FTT, failure to thrive. For weight and height, data were obtained from last examination, unless otherwise noted. Z-scores were calculated using the CDC charts for children ages 2–20. For head circumference, z-score was calculated based on published data.³⁸

cortical changes/white matter volume loss (n = 5), abnormal corpus callosum (n = 3), and dilatation of lateral ventricles (n = 1) (Figure S1).

Dysmorphic features were noted in all individuals. Shared facial features included large/protruding ears (n = 8), long philtrum (n = 7), thin upper lip (n = 6), long palpebral fissures (n = 6), high arched palate (n = 5), prominent/high nasal bridge (n = 5), retrognathia (n = 4), and arched eyebrows (n = 3). Abnormalities of fingers and/or toes were also common (n = 10), including broad thumbs (n = 6), overriding toes (n = 3), bulbous finger tips/fetal pads (n = 2), syndactyly (n = 2), arachnodactyly (n = 2), and brachydactyly (n = 1). Other shared features included progressive scoliosis (n = 5), hyperextensible joints (n = 4), joint contractures/clubfoot (n = 3), and sacral dimple/spina bifida occulta (n = 3). Four subjects had congenital heart defects including atrioseptal defect (n = 3), ventricular septal defect (n = 2), pulmonic stenosis (n = 2), and tetralogy of Fallot (n = 1). Four of eight males (50%) had either unilateral or bilateral cryptorchidism.

Not included in the study cohort are two additional deceased affected subjects (II-1 in family 1 and II-3 in family 3) for whom DNA samples were unavailable (Figure 1). Subject II-1 in family 1 had intellectual disability, intractable seizures, poor growth, sacral dimple, progressive scoliosis, and bilateral toe polydactyly. He died at age 12 years due to complications associated with Lennox-Gastaut syndrome. Subject II-3 in family 3 had a congenital heart defect, dysmorphic facial features, and delayed milestones. She died at age 2 years due to recurrent pulmonary infections. Based on the significant overlap of clinical features with those in our study cohort, we suspect that these individuals also carried biallelic pathogenic variants in *OTUD6B*.

A summary of molecular findings is provided in Figure 1 and Table 2. Four homozygous variants in *OTUD6B* (GeneBank: NM_016023.3; ClinVar: SCV000492514, SCV000492515, SCV000492516, SCV000492517)—one nonsense, one frameshift, one splice variant, and one missense variant—were identified by WES or whole-genome sequencing (family 6) and subsequently confirmed by Sanger sequencing. Consistent with a recessive mode of inheritance, the unaffected parents in all of the families were heterozygous for the respective familial variants, and all tested unaffected siblings (in families 2 and 4) were either heterozygous for the variant or homozygous for the reference allele. The available affected individuals in families 1, 2, and 3 shared the same homozygous nonsense variant, c.433C>T (p.Arg145*), located in exon 4 (Figure 2). This variant is annotated in dbSNP 147 (rs368313959) and has a maximum subpopulation allele frequency in the Latino population of 0.0026 (5/1,898 alleles) in the Exome Aggregation Consortium (ExAC) database and 0.00079 (25/31,718) in the Genome Aggregation Database (gnomAD) (Table 2). Therefore, this variant is significantly enriched in our cohort (6 of 12 alleles from 6 families versus 5 of 1,898 reference alleles from the Latino

Table 2. Variants in OTUD6B

	Families 1,2,3	Family 4	Family 5	Family 6
Genomic position (hg19)	8: 92,090,611	8: 92,090,647–92,090,651	8: 92,083,364	8: 92,090,825
Exon/Intron	exon 4	exon 4	intron 1	exon 4
Nucleotide change	c.433C>T	c.469_473delTTAAC	c.173–2A>G	c.647A>G
Protein change	p.Arg145*	p.Leu157Argfs*8	–	p.Tyr216Cys
Allele Frequency in ExAC				
Total	8/43,688 = 0.00018	not present	not present	not present
Latino	5/1,898 = 0.0026	–	–	–
African	1/4,386 = 0.000023	–	–	–
South Asian	2/8,864 = 0.000023	–	–	–
Number of homozygotes	none	–	–	–
Allele Frequency in gnomAD				
Total	32/239,902 = 0.00013	not present	not present	not present
Latino	25/31,718 = 0.00079	–	–	–
African	1/22,484 = 0.000044	–	–	–
South Asian	1/25,966 = 0.000039	–	–	–
Number of homozygotes	none	–	–	–
SIFT	–	–	–	deleterious
PolyPhen2	–	–	–	probably damaging

Variant nomenclature is based on GenBank: NM_016023.3. Abbreviations are as follows: ExAC, Exome Aggregation Consortium; gnomAD, Genome Aggregation Database.

population in ExAC, $p = 6.295 \times 10^{-12}$; Fisher's exact test). The calculated prevalence of individuals who are homozygous for this variant based on random mating would therefore be approximately 1/145,000 in the Latino population. This variant is not present in the homozygous state in ExAC or gnomAD, but this locus was covered in only 949 and 15,859 Latino individuals in these databases, respectively. Of note, families 1 and 2 were reported to be non-consanguineous, and both are of Hispanic descent. Illumina HumanExome-12v1 (cSNP) array data of the affected subjects from these families showed that *OTUD6B* lies in a small stretch of absence of heterozygosity (AOH) (5.4 Mb and 7.4 Mb, respectively) in chromosome 8. Also, AOH is seen in multiple chromosomes in these subjects, suggesting that AOH regions likely occurred through identity-by-descent. The affected subjects in families 4, 5, and 6 carried a homozygous frameshift variant (c.469_473delTTAAC [p.Leu157Argfs*8]), a homozygous splice variant (c.173–2A>G), and a homozygous missense variant (c.647A>G [p.Tyr216Cys]), respectively. These variants were not reported in ExAC or gnomAD.

OTUD6B maps to chromosome 8q21.3 and encodes a member of the ovarian tumor (OTU) domain-containing subfamily of deubiquitinating enzymes (DUBs). It consists of seven exons that encode a 323-amino acid protein (Figures 2A and 2B). The predicted protein contains several coiled coil (CC) domains and a conserved OTU domain, which is associated with cysteine protease activity. The predicted catalytic residues that function together at the cen-

ter of the enzyme's active site are Asp185, Cys188, and His307; the C-loop, variable loop, and H-loop are the predicted ubiquitin-binding regions (Figures 2B and 2D). The location of the variants identified in our cohort in relation to the gene structure is shown in Figure 2. The truncating variants c.433C>T and c.469_473delTTAAC are predicted to lead to nonsense-mediated decay of the mRNA molecule. The c.173–2A>G variant affects an intronic splice acceptor site and is predicted to cause exon 2 skipping and introduction of a premature termination codon. This change is also predicted to result in nonsense-mediated decay of the mRNA molecule. The c.647A>G (p.Tyr216Cys) variant affects a highly evolutionarily conserved tyrosine residue in the OTU domain and is predicted to be deleterious by SIFT and PolyPhen-2 prediction tools (Figure 2C and Table 2). This tyrosine residue is also highly conserved among other OTU domain-containing enzymes and is located between the C-loop and variable loop (Figure 2D). Notably, subjects with predicted loss-of-function alleles (families 1 to 5) have a very severe clinical presentation while subjects with the missense variant (family 6) have a milder phenotype, suggestive of a hypomorphic allele (Table 1).

The International Mouse Phenotyping Consortium (IMPC) is generating a knockout mouse strain for every protein-coding gene in the mouse genome and is employing a standardized, broad-based adult and embryonic phenotyping of the knockout mice to identify gene-to-phenotype associations.^{6,7} A C57BL/6N mouse

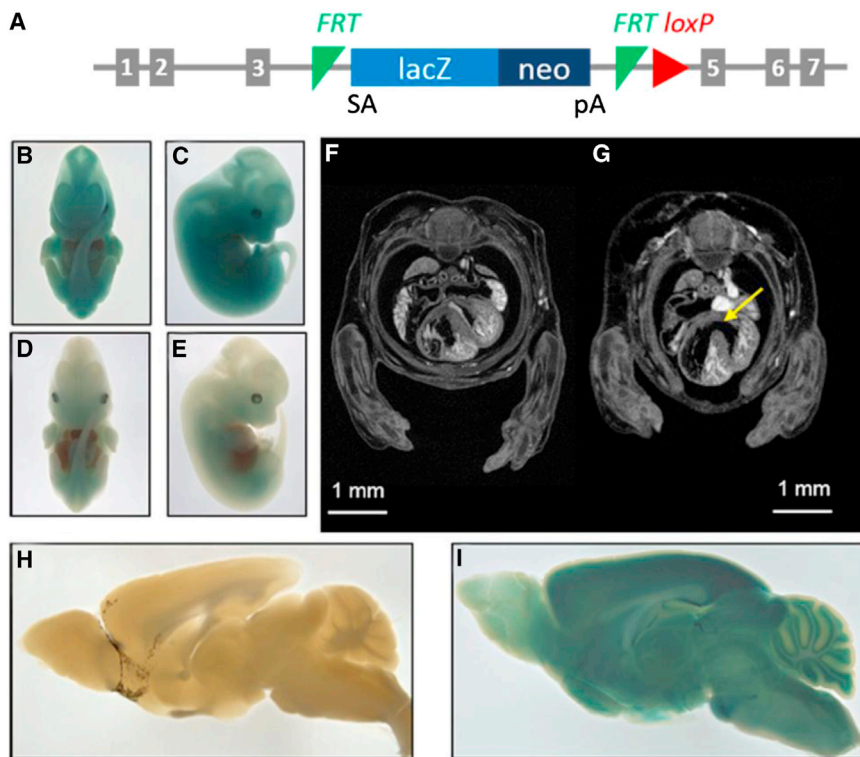


Figure 3. Phenotypic Effects of *Otud6b* Deficiency in Mice

(A) Schematic of the *Otud6b*^{tm1b} knockout allele. Exon 4 is deleted and β-galactosidase (*lacZ*) is expressed following splicing to exon 3 (SA = splice acceptor, pA = polyadenylation signal).

(B–E) *LacZ* expression, as determined by X-gal staining, from the *Otud6b*^{tm1b} allele is widespread in E12.5 *Otud6b*^{tm1b/tm1b} (B, C) and *Otud6b*^{tm1b/+} (D, E) embryos.

(F and G) Representative μCT image of E14.5 wild-type (F) and *Otud6b*^{tm1b/tm1b} knockout (G) embryos. The ventricular septal defect in the *Otud6b*^{tm1b/tm1b} knockout embryo is indicated (arrow).

(H and I) Representative X-gal staining images of wild-type (H) and *Otud6b*^{tm1b/+} (I) adult brains (>50 days of age).

subjects had failure to thrive, and eight had small stature (Table 1). Furthermore, μCT imaging of *Otud6b*^{tm1b/tm1b} embryos showed ventricular septal defects (VSDs) in 80% of hearts (3 of 3 E14.5 hearts with a VSD; 1 of 2 E18.5 hearts with a VSD) (Figures 3F, 3G, and S4). Blood obscured imaging of the heart in the E18.5 *Otud6b*^{tm1b/tm1b} embryo in which a VSD could not be confirmed. Importantly, VSDs are not a common feature of the C57BL/6N inbred background. Only one VSD has been observed in 150 C57BL/6N embryos analyzed (0.67% incidence). Consistent with the high incidence of VSDs in *Otud6b*^{tm1b/tm1b} mouse embryos, four human subjects in our study cohort had congenital heart defects, two of whom specifically had VSDs (Table 1).

Because homozygotes were sub-viable, heterozygous (*Otud6b*^{tm1b/+}) knockout male and female mice (n = 8 for each sex) were placed into the adult IMPC phenotyping pipeline, and values were compared to C57BL/6N controls. No significant differences in adult phenotypes were reported between *Otud6b*^{tm1b/+} mice and wild-type controls (using the IMPC suggested p value of 1E-4). However, using the *lacZ* expression reporter in the *Otud6b* knockout allele, human disease-relevant expression patterns for *Otud6b* were identified in *Otud6b*^{tm1b/+} adult mice. Expression of *lacZ* was observed in several organ systems including the cardiovascular system (heart and aorta), digestive system (large intestine and stomach), nervous system (spinal cord, peripheral nervous system, pituitary gland, and the brain), and musculoskeletal system (skeletal muscle and cartilage) (data not shown; available on the IMPC website). As reported by the IMPC, within the brain *lacZ* reporter

expression was near ubiquitous, localizing to several anatomical locations including the olfactory bulb, cortex, cerebellum (granular and Purkinje layers with weak *LacZ* staining in the white matter), brain stem, hypothalamus, midbrain (inferior and superior colliculus), hippocampus (CA1/2/3 and subiculum), striatum, and thalamus (Figures 3H, 3I, and S2E). The widespread expression pattern of *Otud6b* in adult mice is consistent with a previous study showing *Otud6b* gene expression in multiple tissues.¹⁰ Furthermore, it is consistent with the multiple organ systems affected in the described subjects, including the cardiovascular system (congenital heart defects), nervous system (cognitive disability, seizures, structural brain abnormalities, hypotonia, feeding and digestion issues), musculoskeletal system (scoliosis, abnormalities of the fingers/toes), and digestive system (feeding and digestion issues).

Post-translation modification by ubiquitin is an essential process that occurs in almost all cellular tissues in humans and other eukaryotes. Ubiquitin becomes covalently linked to target proteins by an isopeptide bond involving the ubiquitin C-terminal glycine and ε-amino group of a lysine within the target. Ubiquitination is mediated via the sequential action of a three enzyme conjugation pathway involving an E1-activating enzyme, an E2 conjugating enzyme, and an E3 ligase enzyme.^{11,12} Ultimately, the attachment of ubiquitin, or chains of ubiquitin, to a protein substrate creates a signal for the protein's fate that is primarily associated with degradation via the 26S proteasome but may also result in changes in cellular location, protein activity, and interaction with other proteins.^{13–15}

Ubiquitination is a reversible process countered by the action of DUBs, which are proteases that specifically cleave ubiquitin isopeptide linkages from protein substrates. Therefore, DUBs can regulate ubiquitin-dependent processes. The human genome encodes approximately 90

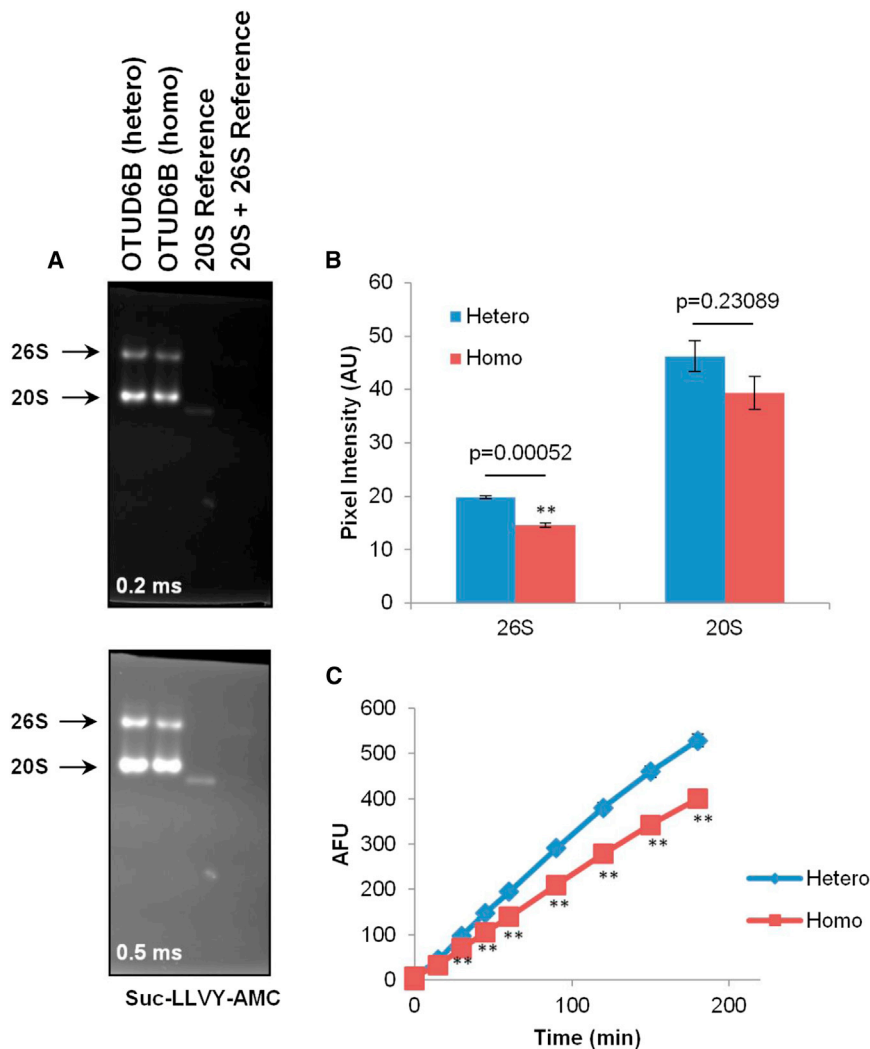


Figure 4. PBMCs from Individual Carrying the Homozygous c.469_473delTTAAC *OTUD6B* Deletion Exhibit Decreased Chymotrypsin-like Proteasome Activity

(A) Forty micrograms of whole-cell extracts of PBMCs derived from subjects with the heterozygous (hetero) or homozygous (homo) c.469_473delTTAAC *OTUD6B* deletion were separated by native PAGE, as indicated. Size controls in this experiment consisted of purified blood-derived 20S (20S reference) and a combination of 20S and 26S complexes (reference 20S + 26S) for 600 kDa and 1.2 MDa, respectively. Chymotrypsin-like proteasome activity was examined by incubating the gels with 0.1 mM of Suc-LLVY-AMC for 20 min at 37°C. Two exposure times (0.2 and 0.5 ms) at 360 nm are shown. One representative experiment of three is shown.

(B) Quantification of the 20S and 26S proteasome fluorescent bands from PBMCs with the hetero or homo c.469_473delTTAAC *OTUD6B* deletion by densitometry using the ImageJ 1.48v software. Shown is the mean pixel intensity calculated from three independent experiments following an exposure time of 0.3 ms. **p < 0.001 (Student's t test).

(C) Ten micrograms of whole-cell lysates from PBMCs carrying the hetero or homo *OTUD6B* deletion were incubated in quadruplicates with 0.1 mM Suc-LLVY in a final volume of 100 μ L in non-denaturing conditions on 96-well plates. The fluorescence activity emerging from the release of AMC was measured at 360 nm every 15 min for the first hour, and then every 30 min for the last two hours. **p < 0.01 (Student's t test).

DUBs, which are classified into five subfamilies by their sequence diversity: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific peptidases (USPs/UBPs), Josephin or Machado-Joseph disease (MJD) proteins, JAMM (Jab1/MPN domain-associated metalloisopeptidase) domain proteins, and lastly, the OTU-domain containing subfamily to which *OTUD6B* belongs. Several members of the OTU subfamily have been shown to regulate important signaling pathways including NF- κ B signaling, interferon signaling, p97-mediated processes, and the DNA damage response.^{16–21}

Given the potential role of *OTUD6B* in removing ubiquitin and/or ubiquitin chains from protein substrates, we reasoned that it may regulate the ubiquitin-proteasome system (UPS). To address the contribution of *OTUD6B* to the UPS, peripheral blood mononuclear cells (PBMCs) from subjects carrying a heterozygous or a homozygous c.469_473delTTAAC (p.Leu157Argfs*8) *OTUD6B* deletion (family 4; subjects I-1 and II-3, respectively) were analyzed for their proteasome content. To this end, proteasome complexes were separated on non-denaturing (native)-PAGE and visualized by their capacity to hydrolyze a Suc-

LLVY-AMC fluorogenic substrate. As shown in Figure 4A, both of the 20S and 26S complexes from PBMCs of the homozygous subject exhibited weaker fluorescence intensity than those from the heterozygous subject, indicating that chymotrypsin-like activity was reduced in this sample. Densitometric analysis of the proteasome bands indicated that the intensity of the chymotrypsin-like activity of the 26S proteasome complexes from the homozygous subject was reduced by 20% when compared to those from the heterozygous subject (Figure 4B). Likewise, the activity of the 20S complexes in the homozygous sample was lower than that detected in the heterozygous sample, albeit not significantly. To further confirm these differences, the chymotrypsin-like activity was directly measured in whole-cell extracts by exposing them to 0.1 mM of the Suc-LLVY peptide for various periods of time. As shown in Figure 4C, the 26/20S complexes from the homozygous subject exhibited a decreased capacity to hydrolyze the Suc-LLVY substrate when compared to the same analysis on PBMCs from the heterozygous subject. This difference was statistically significant at all time points. This is in agreement with the data of our above-described, in-gel

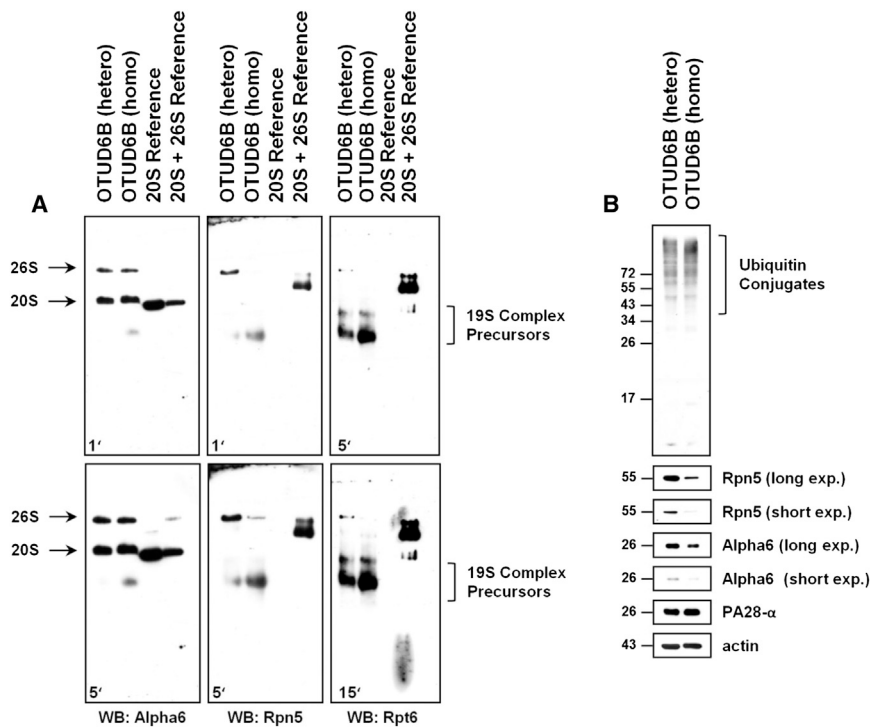


Figure 5. The Homozygous c.469_473delTTAAC *OTUD6B* Deletion Results in Decreased Amounts of 26S Proteasomes and Increased Accumulation of Ubiquitin-Protein Conjugates in PBMCs

(A) Forty micrograms of whole-cell extracts from PBMCs from subjects with the hetero or homo c.469_473delTTAAC *OTUD6B* deletion were resolved on native PAGE and subjected to western blotting using antibodies to Alpha6 (clone MCP20, Enzo Life Sciences), Rpn5 (clone H-9, Santa Cruz Biotechnology), and Rpt6 (clone p45-110, Enzo Life Sciences), as indicated. Controls consisted of 20S and 20S + 26S references, as described in Figure 4.

(B) Ten micrograms of protein lysates were separated by 15% SDS-PAGE followed by western blotting using antibodies specific for ubiquitin (ref #Z0458, DAKO GmbH), Rpn5 (clone H-9, Santa Cruz), Alpha6 (clone MCP20, Enzo Life Sciences), and PA28- α (K232/1, laboratory stock). Equal protein loading was ensured by probing the membranes with the anti-actin monoclonal antibody.

activity assays and therefore confirms that the homozygous c.469_473delTTAAC *OTUD6B* deletion compromises the chymotrypsin-like proteasome activity in PBMCs.

We next tested whether such impaired activity was due to a decreased amount of proteasome complexes in these samples. Protein-cell lysates derived from PBMCs from the heterozygous and homozygous subjects were resolved on native PAGE and subjected to western blotting to detect proteasome subunits: proteasome subunit alpha 6 (PSMA6 or Alpha6), proteasome 26S subunit, non-ATPase 12 (PSMD12 or Rpn5), and proteasome 26S subunit, ATPase 5 (PSMC5 or Rpt6). As illustrated in Figure 5A, probing the membrane with the Alpha6 antibody showed two strongly staining bands in both samples corresponding to the 20S and 26S proteasome complexes in these cells. Strikingly, the incorporation of the Rpn5 and Rpt6 subunits into 26S proteasomes was substantially reduced in the homozygous subject, as shown by decreased intensity of the Rpn5 and Rpt6 bands at the position of the 26S proteasome. These data strongly suggest that the c.469_473delTTAAC *OTUD6B* homozygous deletion is associated with a 26S proteasome assembly defect. In an attempt to determine whether the c.469_473delTTAAC *OTUD6B* deletion was a dominant or recessive genetic trait, the 26S proteasome populations from both of the heterozygous and homozygous *OTUD6B* subjects were then compared to those of *OTUD6B* wild-type individuals. To this end, proteasome complexes derived from PBMCs of four healthy blood donors were characterized using native-PAGE and western blotting, as described above. As expected, all four wild-type *OTUD6B* donors exhibit full incorporation of the Rpt6 and Rpn5 subunits in 26S pro-

teasome complexes (Figure S5), confirming that the assembly pathway of the 19S regulatory particle was not compromised in these cells. To enable reliable comparison between wild-type, heterozygous, and homozygous *OTUD6B* subjects, our 26S reference was used as standard for normalization of the Rpn5 signal intensities emerging from cellular 26S proteasomes between experiments on different gels. Interestingly, our densitometric analysis showed that the relative amounts of Rpn5 in the 26S proteasome complexes was reduced by about 65% and 90% in the *OTUD6B* heterozygous and homozygous subjects, respectively, when compared with those of the *OTUD6B* wild-type controls (Figure S5), indicating that impaired proteasome assembly begins with the disruption of either of the two *OTUD6B* copies. This notion is fully in line with the observation that 19S precursor complexes accumulate in both of the heterozygous and homozygous subjects (Figure 5A) but not in wild-type controls (Figure S5). The lack of visible phenotype in the *OTUD6B* heterozygous subject suggests the existence of a compensation mechanism.

To further evaluate the functional significance of the c.469_473delTTAAC *OTUD6B* deletion, we next analyzed the cellular content of the ubiquitin-modified proteins in PBMCs in both of the heterozygous and homozygous subjects by SDS-PAGE. As shown in Figure 5B, the ubiquitin-protein conjugates accumulated much stronger in the homozygous sample than in the heterozygous one. This finding is in agreement with our previous observation that 26S proteasomes from the homozygous subject are defective. Interestingly, our SDS-PAGE analysis further confirmed a decrease in the total amount of the Rpn5

19S subunit and to a lesser extent of the Alpha6 subunit in PBMCs from the homozygous subject. By contrast, the steady-state expression level of the proteasome activator PA28- α remains unaffected in these cells. Interestingly, comparing both of the heterozygous and homozygous subjects with wild-type controls showed that the loss of one *OTUD6B* copy is sufficient to dramatically affect protein homeostasis, as evidenced by the accumulation of ubiquitin-protein conjugates in the *OTUD6B* heterozygous subject (Figure S6). Importantly, the increased amount of ubiquitin-modified proteins in subjects with the *OTUD6B* heterozygous and homozygous variant was paralleled by a substantial reduction of the steady-state expression level of the Rpt6 subunit in both of these samples (Figure S6). However, the expression level of the Rpn5 subunit was decreased in the homozygous *OTUD6B* subject but not in the heterozygous one, as determined by western blotting. Altogether, these data suggest that the c.469_473delTTAAC *OTUD6B* deletion results in proteasome malfunction whose severity proportionally increases with the number of affected alleles.

In this work, we identified the homozygous c.469_473delTTAAC *OTUD6B* deletion as a new member of the growing family of genetic alterations specifically affecting proteasome function.^{22–26} To the best of our knowledge, the alteration described in this paper is the first one occurring outside the genes encoding the proteasome maturation protein (POMP) or any of seven alpha and/or beta proteasome subunits. The cellular loss-of-function phenotype of *OTUD6B* in the homozygous subject was characterized by a substantially reduced incorporation of 19S subunits into 26S proteasomes (Figure 5A) as well as decreased steady-state expression levels of 26S proteasome subunits (Figure 5B). Accordingly, this was accompanied by a drop of the chymotrypsin-like activity (Figure 4) and a concomitant accumulation of ubiquitin-protein conjugates (Figure 5B). It remains unclear whether the defect in 26S proteasome assembly detected in our native PAGE analysis is the cause or the consequence of the overall decreased expression of the proteasome subunits observed in SDS-PAGE. The accumulation of 19S precursor complexes in the homozygous subject suggests that the 19S subunits are not a rate-limiting factor for the formation of 26S proteasome complexes. It is therefore highly likely that the decreased steady-state levels of the proteasome subunits may reflect a higher susceptibility of these subunits to degradation due to their failure to incorporate into 26S proteasomes.

The precise mechanisms by which *OTUD6B* affects 26S proteasome assembly are ill defined and open unexpected avenues to understanding the regulation of protein homeostasis by the UPS. Given the described role of *OTUD6B* as a DUB, one can speculate that the formation of 26S complexes is a process regulated by ubiquitin modification. This notion would be in agreement with previous studies reporting the ubiquitination of various proteasome subunits including Rpn10, Rpn13, or Rpt5.^{27–29} As such, it is

conceivable that any impaired de-ubiquitination of these subunits might impact proteasome assembly and/or function. In this matter, further experiments will attempt to address the capacity of *OTUD6B* to remove ubiquitin from proteasome subunits.

Interestingly, by transmission electron microscopy, we identified abnormal cytoplasmic inclusions in lymphocytes from subject II-2 in family 2 (Figure S7). We speculate that these inclusions represent accumulation of protein substrates due to an imbalance in ubiquitination/deubiquitination activities in these cells. Although this study was performed in only one subject and only in lymphocytes, it raises the possibility that the inclusions might serve as a biomarker for this syndrome.

Many DUBs have been implicated in human diseases such as neurodegeneration, inflammation, infection, and cancer. For example, pathogenic variants in the ubiquitin-specific protease genes *USP9X* (MIM: 300072) and *USP27X* (MIM: 300975) cause X-linked intellectual disability (MIM: 300919, 300968, 300984). De novo loss-of-function variants in *USP7* (MIM: 602519) cause a neurodevelopmental disorder.³⁰ Trinucleotide repeat expansion in *ATXN3* (MIM: 607047) causes Machado-Joseph disease (MIM: 109150), a neurologic disorder characterized by ataxia, spasticity, and ocular movement abnormalities. Heterozygous truncating variants in *A20/TNFAIP3* (MIM: 191163) cause familial Behcet-like auto-inflammatory syndrome (MIM: 616744) and biallelic pathogenic variants in *OTULIN* (MIM: 615712) cause autoinflammation, panniculitis, and dermatosis syndrome (MIM: 617099). Germline variants in *BAP1* (MIM: 603089) and *CYLD* (MIM: 605018) can cause tumor predisposition syndromes (MIM: 614327, 132700). Maternal germline variants and de novo events in the human OTU domain-containing enzyme *ALG13* (MIM: 300776) cause early infantile epileptic encephalopathy 36 (MIM: 300884). Similarly, many ubiquitin ligase genes have been implicated in human diseases.^{31–37} For example, defects in the *UBR1* (MIM: 605981), *MID1* (MIM: 300552), *MID2* (MIM: 300204), and *CUL4B* (MIM: 300304) have been associated with neurodevelopmental disorders.^{31–34} Overall, this demonstrates the importance of the ubiquitination/deubiquitination process in human cells and that its disruption can lead to disease.

In summary, we describe a severe intellectual disability syndrome caused by biallelic loss-of-function variants in the deubiquitinating enzyme gene *OTUD6B*. This syndrome is characterized by cognitive dysfunction with absent speech, seizures, microcephaly, hypotonia, growth retardation, dysmorphism, and other variable features. We also describe a milder phenotype in humans with mild to moderate intellectual disability, seizures, and dysmorphic features caused by a homozygous missense variant in *OTUD6B* that is presumed to represent a hypomorphic allele. *Otud6b* knockout mice display several of the features seen in human subjects with *OTUD6B* loss-of-function variants and demonstrate the power of

the International Mouse Phenotyping Consortium as a resource for functional validation of novel disease genes. Finally, *in vitro* analyses implicate OTUD6B in proteasome function, thereby strengthening the notion that dysregulation of the ubiquitin system is causative of human disease.

Supplemental Data

Supplemental Data include Supplemental Note, seven figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.ajhg.2017.03.001>.

Consortia

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Web Resources

ClinVar, <https://www.ncbi.nlm.nih.gov/clinvar/>
Clustal Omega, <http://www.ebi.ac.uk/Tools/msa/clustalo/>
dbSNP, <http://www.ncbi.nlm.nih.gov/projects/SNP/>
ExAC Browser, <http://exac.broadinstitute.org/>
gnomAD Browser, <http://gnomad.broadinstitute.org/>
Growth Charts, http://www.cdc.gov/growthcharts/clinical_charts.htm
HPC, <https://hpc.uni.lu/>

International Mouse Phenotyping Consortium, <http://www.mousephenotype.org/>
OMIM, <http://www.omim.org/>
UniProt, <http://www.uniprot.org/>

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