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Supplemental Data

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

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Supplemental Data

Detailed clinical summaries

Family 1

Subject II-2 from Family 1 is an 18 year old female who was born to non-consanguineous Hispanic parents at 37 weeks gestational age by caesarean section (C-section) following a pregnancy complicated by intrauterine growth restriction (IUGR). She had congenital quadriplegia, jaundice, and feeding difficulties postnatally necessitating a 10 day admission to the neonatal intensive care unit (NICU). A sacral dimple and dysmorphic features were noted at that time. Her subsequent development was delayed. She sat independently at 9 months, walked at 18 months, and has not acquired language skills to date. She had onset of seizures at 1 year of age and was ultimately diagnosed with Lennox-Gastaut refractory epilepsy. At her most recent follow-up visit at 18 years of age, she had significant feeding problems including dysphagia and gastroesophageal reflux disease (GERD) that had required a Nissen procedure and gastrostomy-tube (G-tube) feeds and resulted in failure to thrive. Medical concerns included refractory seizures, respiratory insufficiency secondary to scoliosis requiring tracheostomy, recurrent infections, anemia, irregular menses, hypothyroidism, and bilateral hearing loss. On exam, she was noted to be nonverbal, minimally ambulatory, and had severe intellectual disability. Height was -5.2SD, weight was -8.9SD, and occipitofrontal circumference (OFC) was -4.7SD, consistent with global growth failure and microcephaly. She had long eyelashes, arched brows, long palpebral fissures, a displaced posterior hair whorl, a long nose, hypoplastic alae, high nasal bridge, long philtrum, high-arched palate, cupid bow, and dental crowding. Additional physical exam findings included abnormal tone and ataxia with abnormal posturing, severe kyphoscoliosis, multiple joint contractures, tapered fingers, long great toes with hallux valgus, brachydactyly of the 2^{nd} – 4^{th} toes, varus of the 5^{th} toe, and

high arches. Family history was notable for unaffected healthy parents and a similarly affected brother who died at age 12 years due to complications associated with Lennox-Gastaut syndrome.

Whole exome sequencing detected a homozygous c.433C>T (p.Arg145Ter) variant in *OTUD6B* in subject II-2. This variant was found as a heterozygous change in both parents. A sample was not available for testing of the deceased brother.

Family 2

Subject II-2 from Family 2 is a 17 year old male who was born to non-consanguineous parents of Hispanic and Italian descent at 36 weeks by spontaneous vaginal delivery following a pregnancy complicated by IUGR, poor fetal activity, and oligohydramnios. Postnatal concerns included irritability, feeding difficulties, lack of a rooting reflex, a high pitched cry, hypotonia of the arms with hypertonia of the legs, and presence of spina bifida occulta. Subsequent development was delayed. He sat independently at 18 months, walked independently at 3 years 9 months, and has acquired no language skills to date. He had onset of seizures at 15 months, including tonic-clonic seizures and staring spells. Athetoid movements were also noted in early childhood. At his most recent evaluation at 17 years of age, he had significant persistent feeding difficulties and GERD causing failure to thrive and requiring G-tube feeds. He had a vagal nerve stimulator for refractory epilepsy. He was partially ventilator dependent and had a history of obstructive sleep apnea, asthma, renal tubular acidosis, bilateral retractile testes, neurogenic bladder, sensorineural hearing loss, and growth hormone deficiency. Recurrent infections had prompted a diagnosis of hypogammaglobulinemia. On physical exam, he was nonverbal, non-ambulatory, and severely intellectually disabled. Height was -4.9SD, weight was -2.2SD, and OFC was -2.8SD, consistent with growth failure and microcephaly. On physical exam he had athetosis and abnormal tone including both hyper- and hypotonia. He was brachycephalic, had sparse scalp hair, full and arched brows, long and down-slanting palpebral fissures, mild ptosis, infraorbital

creases, posteriorly rotated ears with bilateral pits, a long nose with a convex nasal ridge, long smooth philtrum, high arched palate, thin upper lip, and mild retrognathia. Severe scoliosis was noted, as were joint contractures, broad thumbs, bulbous fingertips, and overlapping toes. Family history was notable for asymptomatic parents and three healthy siblings; one additional sister was reported to have died shortly after birth from anencephaly.

MRI of the brain demonstrated leukodystrophy, prominent Virchow-Robin spaces, hypoplastic corpus callosum, generalized white matter volume loss, gliosis in the bilateral parietal lobes, and mega cisterna magna. Whole exome sequencing performed on subject II-2 detected a homozygous c.433C>T (p.Arg145Ter) variant in *OTUD6B*. This variant was found as a heterozygous change in both parents and one healthy sibling. The other two healthy siblings were homozygous for the reference allele.

Family 3

Subject II-2 from Family 3 was a 13 year old male who was born to consanguineous parents of Egyptian descent by C-section at term following a pregnancy complicated by IUGR. Postnatally, he had a poor cry, weak movement, and feeding difficulties. He was diagnosed with congenital heart anomalies including pulmonic stenosis and an atrioseptal defect. His subsequent development was severely delayed. He learned to sit independently but not walk. He was seen for follow-up at age 10 years, at which time his height was -5SD, weight was -2.2SD, and OFC was -6.5SD, consistent with growth failure and microcephaly. At his most recent follow-up at 13 years of age, he carried a diagnosis of autism and of epilepsy with generalized tonic-clonic seizures. He had a history of persistent feeding difficulties. On exam, he was non-ambulatory, nonverbal, and severely intellectual impaired. Notable features included long palpebral fissures, large protruding and low set ears, a broad nasal root and prominent nasal bridge, long flat philtrum, thin upper lip, retrognathia, short neck, and sloping shoulders. He also had hypotonia, scoliosis, clubfoot, broad thumbs, and clubbed fingers. Subject II-2 died at 13 years of age

from recurrent pulmonary infections. Family history was significant for three affected siblings, including one sister (subject II-3) who died at 2 years of age also from recurrent pulmonary infections secondary to failure to thrive, and two brothers ages 8 years (subject II-4) and 3 years (subject II-5) at last follow-up. Both subjects II-4 and II-5 shared a strikingly similar phenotype with subject II-1, although subject II-4 did not have a known congenital heart defect, and both subjects II-4 and II-5 had the additional findings of hyperextensible interphalangeal joints and overriding toes.

Brain imaging demonstrated mild frontoparietal cortical changes in subject II-2 that were also seen in subjects II-4 and II-5. Whole exome sequencing detected a c.433C>T (p.Arg145Ter) variant in *OTUD6B* that was found to be homozygous in all three affected brothers and heterozygous in both parents. A sample was not available for testing of the deceased younger sister or the healthy older sister.

Family 4

Subject II-3 from Family 4 is a 9 year old boy born at 36 weeks by spontaneous vaginal delivery to consanguineous parents of Syrian ancestry following a pregnancy complicated by IUGR. He had onset of seizures at 17 months of age, and also had early developmental delay with independent ambulation achieved at 4.5 years of age. Subject II-3 was seen for follow-up at age 5 years, at which time his height was -0.6SD, weight was -1.6SD, and OFC was -3.5SD, consistent with microcephaly. At his most recent visit at age 9 years, he was nonverbal and had severe intellectual disability. He had a diagnosis of retinopathy with an abnormal electroretinogram (ERG). He had feeding difficulties causing failure to thrive. Notable features included hypotonia, long palpebral fissures, large ears, broad thumbs and first toes, and persistent fetal pads. Family history was remarkable for a similarly affected younger brother (subject II-4), also born by spontaneous vaginal delivery at 38 weeks gestational age. Subject II-4 had hypotonia and feeding difficulties in the neonatal setting requiring G-tube placement. He also had an interventricular septal defect, bilateral cryptorchidism, and sacral dimple. His motor and speech

development were delayed and he had one episode of seizure at 3 years of age. At his most recent follow-up visit at 3 years and 11 months, he had a diagnosis of hypothyroidism. On exam, he was nonverbal and non-ambulatory with severe intellectual disability. Height was -2.5SD, weight was -1SD, and OFC was -4.3SD consistent with short stature and microcephaly. He had large ears, was hypotonic, and had scoliosis.

Brain imaging performed on subject II-3 was normal while subject II-4 had cortical and white matter atrophy. Whole exome sequencing detected a homozygous c.469_473del (p.Leu157Argfs*8) variant in *OTUD6B* in both affected brothers. This variant was found as a heterozygous change in both parents and in one healthy sibling. The other healthy sibling is homozygous for the reference allele.

Family 5

Subject II-1 from Family 5 is a 5 year old female who was born to consanguineous Palestinian parents by spontaneous vaginal delivery at 38 weeks following an unremarkable pregnancy. There were no neonatal concerns however her development was subsequently delayed. She learned to sit independently at 24 months and crawl at 3 years. She had onset of myoclonic seizures at 7 months of age. At her most recent follow-up at 5 years of age, she could stand and walk with assistance, recognize her parents, was socially engaging, and was babbling. Height was -2.1SD, weight was -1.4SD, and OFC was -4.5SD. On exam, she had generalized hypotonia and was noted to have myoclonic movements. Notable features included sparse hair, a flat occiput, arched eyebrows, deep set eyes, long eyelashes, large ears, long philtrum, thin upper lip, scoliosis, and syndactyly of the 2nd and 3rd toes bilaterally. Family history was remarkable for unaffected parents and a similarly affected 3 year old younger sister (subject II-2) with significant phenotypic overlap. Subject II-2, however, had a history of more severe developmental delay and the additional finding of complex congenital heart disease including tetralogy of Fallot and a small atrioseptal defect.

Brain imaging performed on subject II-1 showed mild dilatation of the lateral ventricles, a deep interhemispheric fissure with hypoplasia of the corpus callosum, whereas brain CT performed on subject II-2 was normal. Whole exome sequencing detected a c.173-2A>G variant in *OTUD6B* that was found as a homozygous change in both affected siblings and as a heterozygous change in both parents.

Family 6

Subject II-1 from Family 6 is a 20 year old male who was born to consanguineous Caucasian parents at full term by normal spontaneous vaginal delivery. There were no neonatal complications and no early developmental delays. He had seizure onset at 18 months, with generalized tonic-clonic seizures occurring initially in the context of fever and subsequently during sleep, often with clustering. Learning problems emerged in primary school, and the subject learned to read and write but did not progress past 8th grade. At his most recent follow-up visit at 20 years of age, he had moderate intellectual disability. Height was at the 45th centile, weight at the 37th centile, and OFC at the 25th centile, consistent with normal stature and head circumference. He had a long narrow face with facial asymmetry, broad forehead, down-slanting palpebral fissures, prominent dysplastic and anteriorly angled ears, a tubular nose, high arched palate, occlusion anomalies of the teeth, a narrow chin, and arachnodactyly. Family history was remarkable for asymptomatic parents and two similarly affected siblings including a 16 year old brother (subject II-2) and a 14 year old sister (subject II-3). Both subjects II-2 and II-3 from family 6 were noted to have hyperextensibility of the elbow, which was not seen in the eldest brother.

Brain imaging studies performed on subjects II-1, II-2, and II-3 were normal. Whole-genome sequencing detected a c.647A>G (p.Tyr216Cys) variant in *OTUD6B* that was found as a homozygous change in all affected siblings and as a heterozygous change in both parents.

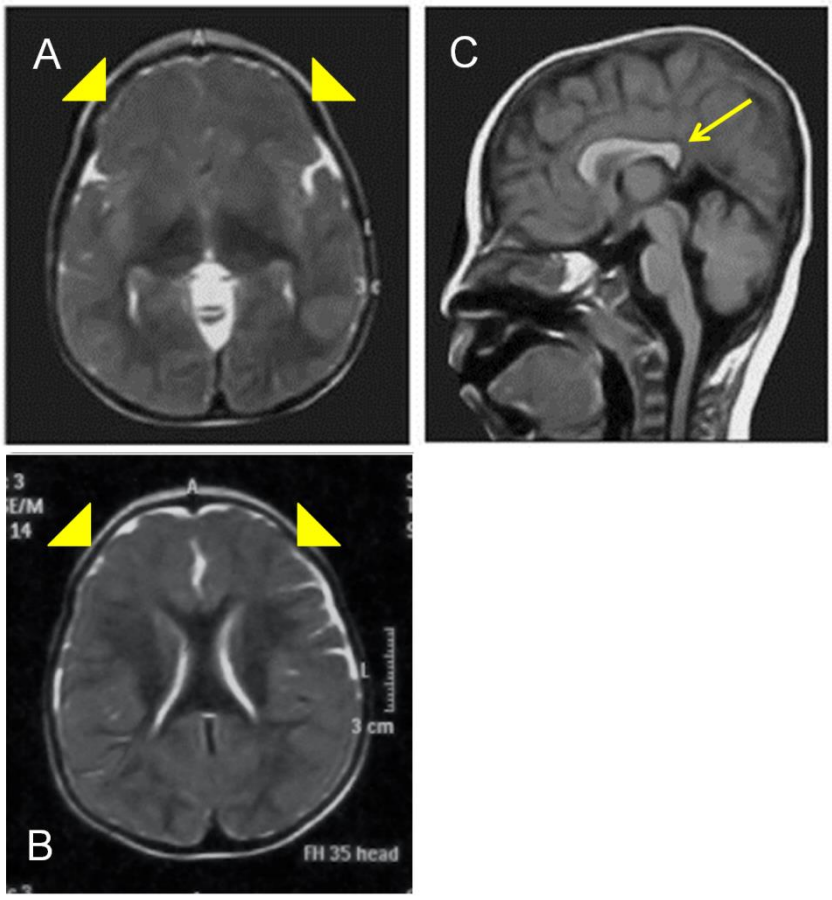

Figure S1. Brain structural abnormalities in Subject II-4 from Family 3.

Brain MRI images at age 1 year 11 months. Arrowheads indicate mild frontoparietal cortical changes **(A & B)** and the arrow indicates short corpus callosum **(C)**.

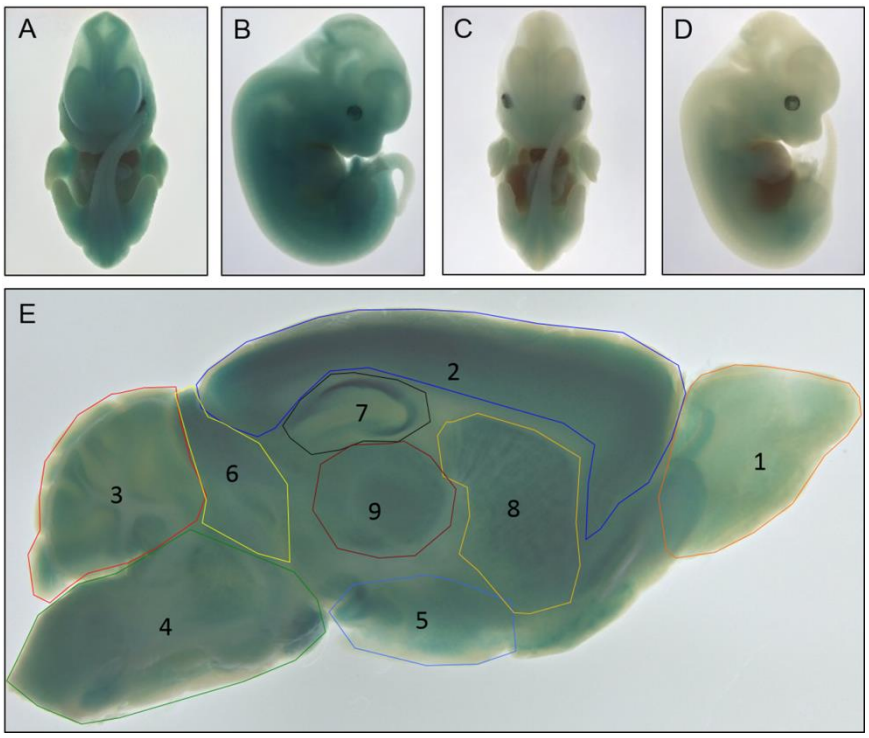

Figure S2. Otud6b expression in the mouse embryo and adult brain. (A-D) LacZ expression, as determined by X-gal staining, from the *Otud6b*^{tm1b} allele is widespread in E12.5 *Otud6b*$^{tm1b/tm1b}$ (A & B) and *Otud6b*$^{tm1b/+}$ (C & D) embryos. Enlarged images of the same embryos in Figure 3 B-E. **(E)** A representative X-gal staining image of an *Otud6b*$^{tm1b/+}$ adult brain (> 50 days of age). Expression of the lacZ reporter localized to several anatomical locations including (1) olfactory bulb, (2) cortex, (3) cerebellum, (4) brain stem, (5) hypothalamus, (6) midbrain, (7) hippocampus, (8) striatum, and (9) thalamus.

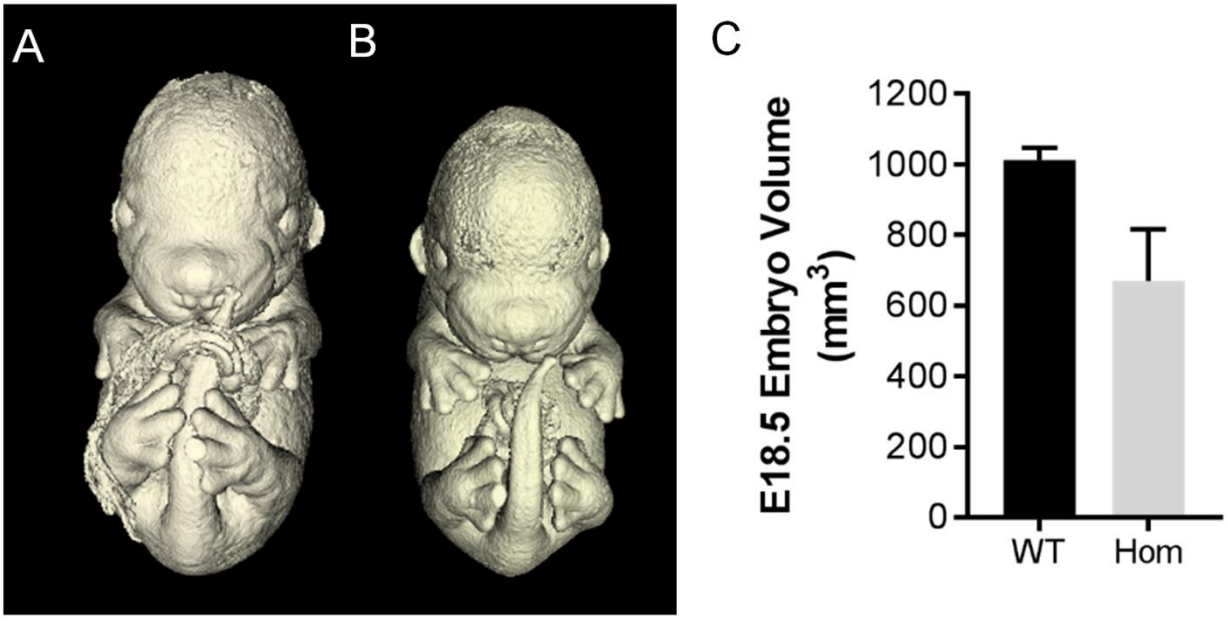

Figure S3. *Otud6b* deficiency reduces mouse embryo volume. (A & B) Representative μCT images of E18.5 wild-type (A) and *Otud6b*$^{tm1b/tm1}$ (B) embryos. **(C)** Analysis of E18.5 wild-type (WT) and *Otud6b*$^{tm1b/tm1b}$ knockout embryo (Hom) μCT images reveals a reduction in knockout embryo volume (N = 2 for each group).

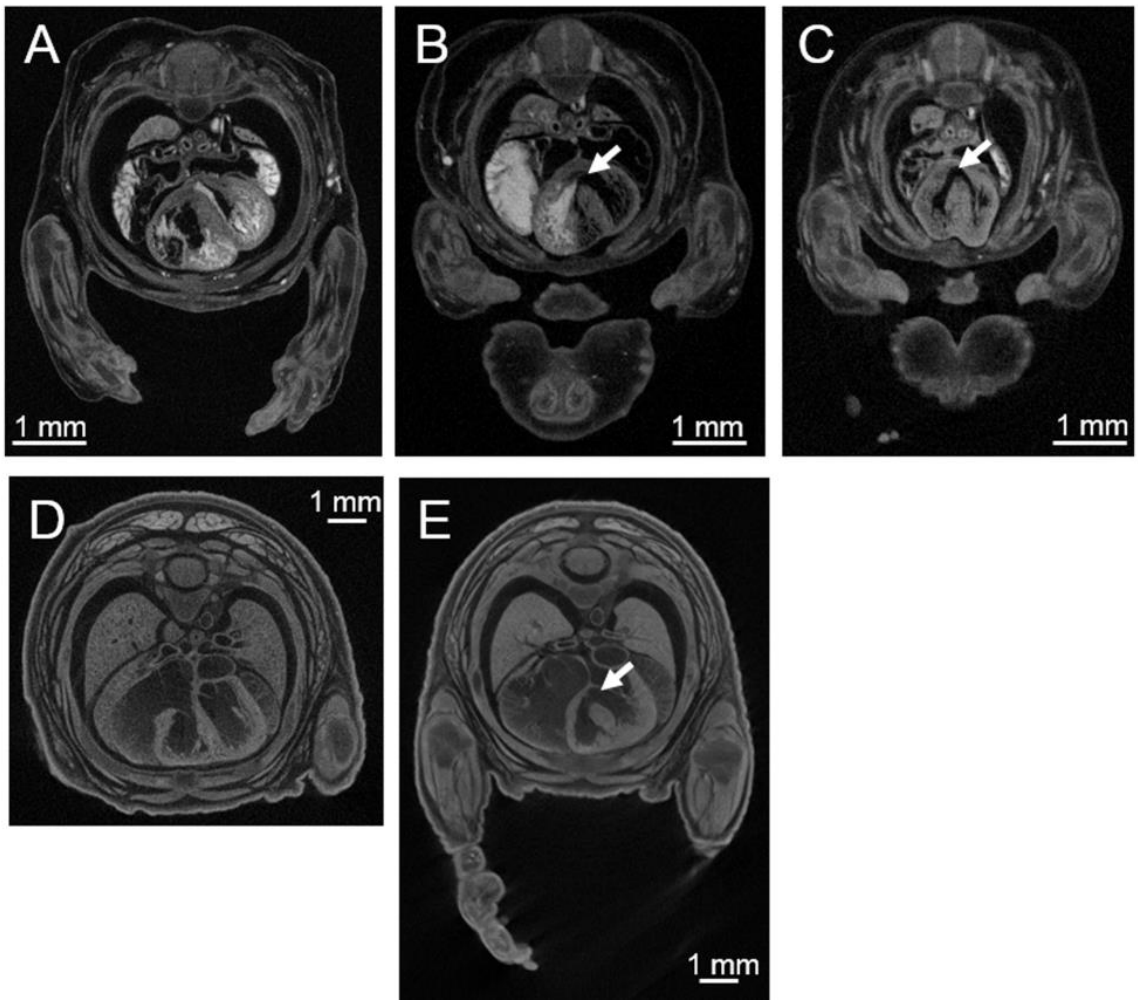

Figure S4. Ventricular septal defects in *Otud6b* knockout embryos. μCT image of E14.5 wild-type **(A)** and *Otud6b*$^{tm1b/tm1b}$ knockout **(B & C)** embryos and E18.5 wild-type **(D)** and *Otud6b*$^{tm1b/tm1b}$ knockout **(E)** embryos. The ventricular septal defect in the *Otud6b*$^{tm1b/tm1b}$ knockout embryo is indicated (arrow).

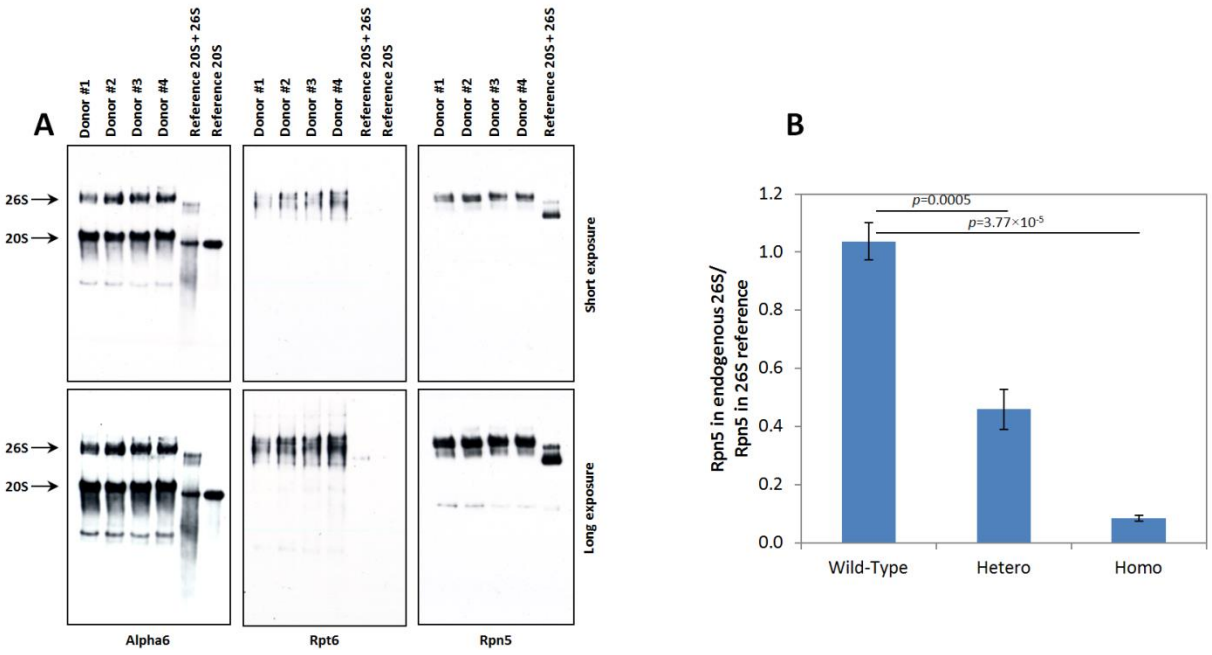

Figure S5. Wild-type *OTUD6B* are more efficient than *OTUD6B* heterozygotes and homozygotes at incorporating the Rpn5 subunit into 26S proteasomes. (A) Forty micrograms of whole-cell extracts of PBMC derived from four healthy blood donors were resolved by native-PAGE and subjected to western-blotting using antibodies specific for Alpha6, Rpt6 and Rpn5, as indicated. Two exposure times (short, upper panel and long, lower panel) are shown. **(B)** The intensity of the Rpn5 bands located in the region of 26S proteasomes from the native-PAGE experiments depicted in Figures 5 A and S5 A were quantified by densitometry (ImageJ 1.48v) and normalized to the 26S reference (200 ng) of the same gel. Shown is the ratio of Rpn5 in endogenous 26S proteasome/Rpn5 in 26S reference for the wild-type *OTUD6B* subjects as well as for subjects heterozygous or homozygous for the c.469_473delTTAAC *OTUD6B* deletion.

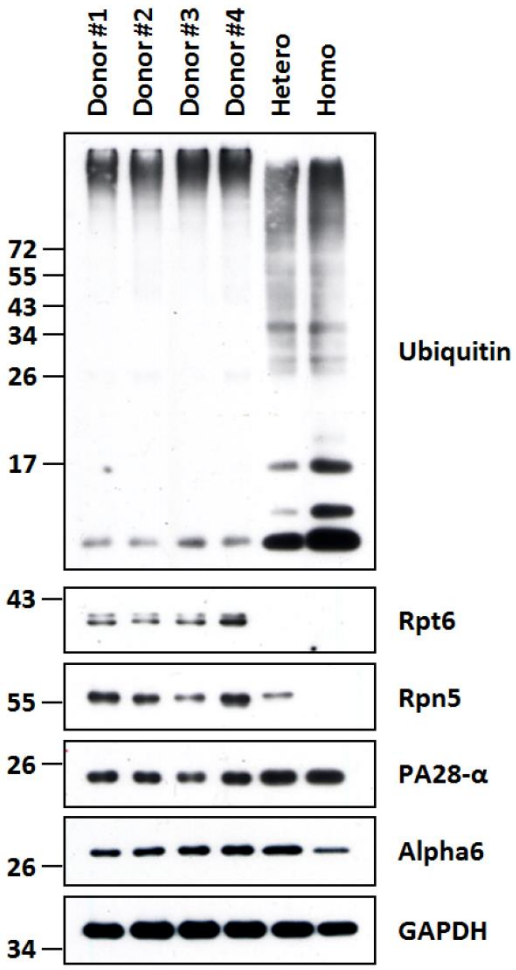

Figure S6. Both *OTUD6B* heterozygotes and homozygotes show proteasome impairment as well as disturbed protein homeostasis. Ten micrograms of protein lysates of PBMC derived from four healthy blood donors or from subjects with the hetero or homo c.469_473delTTAAC *OTUD6B* deletion were separated by SDS-PAGE prior to western-blotting using antibodies specific for ubiquitin (DAKO), Rpt6 (clone p45-110, Enzo Life Sciences), Rpn5 (clone H-3, Santa Cruz Biotechnology, Inc.), PA28-α (K232/1, laboratory stock) and Alpha6 (clone MCP20, Enzo Life Sciences), as indicated. Equal protein loading between samples was ensured by probing the membrane with anti-GAPDH antibody.

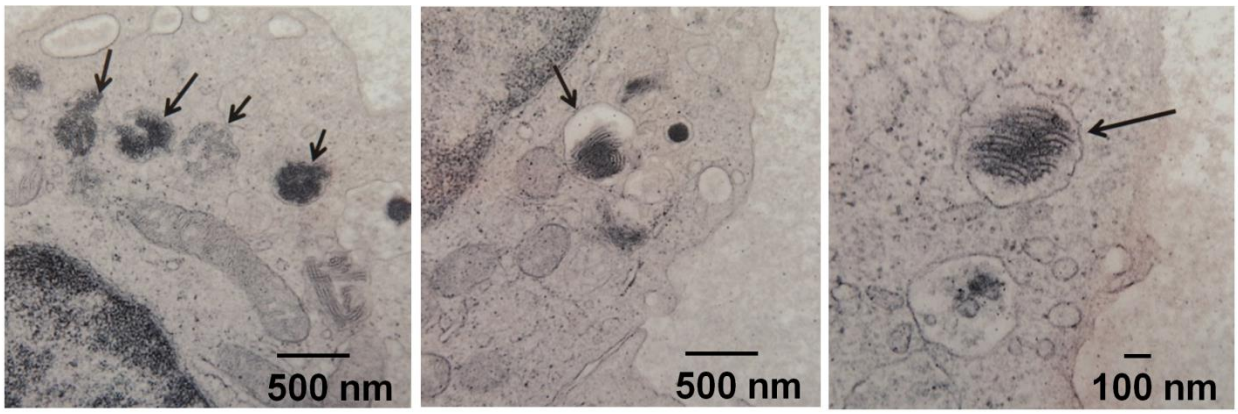

Figure S7. Accumulation of abnormal cytoplasmic inclusions in lymphocytes of Subject II-2 from Family 2. Transmission electron microscopy images of lymphocytes showing an accumulation in the cytoplasm of abnormal inclusions with a tubular configuration (arrows).

Table S1. *Otud6b* genotypes observed after birth.

Genotype	No. observed*	No. expected	Test score (χ^2, *P* value)†
Otud6b$^{+/+}$	32	32	
Otud6b$^{tm1b/+}$	63	64	28.14, < 1E-5
Otud6b$^{tm1b/tm1b}$	2	32	

***** Assumes *Otud6b*$^{+/+}$ mice were observed at the expected frequency and a 1:2:1 segregation ratio. † Chi-square goodness-of-fit tests (2 D.F.) tested for statistical differences between the observed and expected number of progeny.