

## Biallelic *TBCD* Mutations Cause Early-Onset Neurodegenerative Encephalopathy

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We describe four families with affected siblings showing unique clinical features: early-onset (before 1 year of age) progressive diffuse brain atrophy with regression, postnatal microcephaly, postnatal growth retardation, muscle weakness/atrophy, and respiratory failure. By whole-exome sequencing, we identified biallelic *TBCD* mutations in eight affected individuals from the four families. *TBCD* encodes TBCD (tubulin folding co-factor D), which is one of five tubulin-specific chaperones playing a pivotal role in microtubule assembly in all cells. A total of seven mutations were found: five missense mutations, one nonsense, and one splice site mutation resulting in a frameshift. In vitro cell experiments revealed the impaired binding between most mutant TBCD proteins and ARL2, TBCE, and  $\beta$ -tubulin. The in vivo experiments using olfactory projection neurons in *Drosophila melanogaster* indicated that the *TBCD* mutations caused loss of function. The wide range of clinical severity seen in this neurodegenerative encephalopathy may result from the residual function of mutant TBCD proteins. Furthermore, the autopsied brain from one deceased individual showed characteristic neurodegenerative findings: cactus and somatic sprout formations in the residual Purkinje cells in the cerebellum, which are also seen in some diseases associated with mitochondrial impairment. Defects of microtubule formation caused by *TBCD* mutations may underlie the pathomechanism of this neurodegenerative encephalopathy.

Microtubules, cylindrical structures composed of rows of  $\alpha/\beta$ -tubulin heterodimers, are essential components of all eukaryotic cells and are involved in a number of functions such as cell division, morphology, polarization, migration, and intracellular transport.<sup>1,2</sup> Their unique feature of growth (polymerization) and shrinkage (depolymerization) is known as microtubule dynamics.<sup>3,4</sup>  $\alpha/\beta$ -tubulin heterodimers are formed with the aid of tubulin-specific chaperones comprised of five tubulin folding co-factors (TBC): TBCA, TBCB, TBCC, TBCD, and TBCE.<sup>5,6</sup>  $\alpha$ - and  $\beta$ -tubulin monomers initially bind with TBCB and TBCA, respectively, and are passed to TBCE and TBCD, respectively, and form complexes together with TBCC and ARL2.<sup>5,7–9</sup>

Of the five TBC genes, only mutations in *TBCE* (GenBank: NM\_003193.4; MIM: 604934) have been found

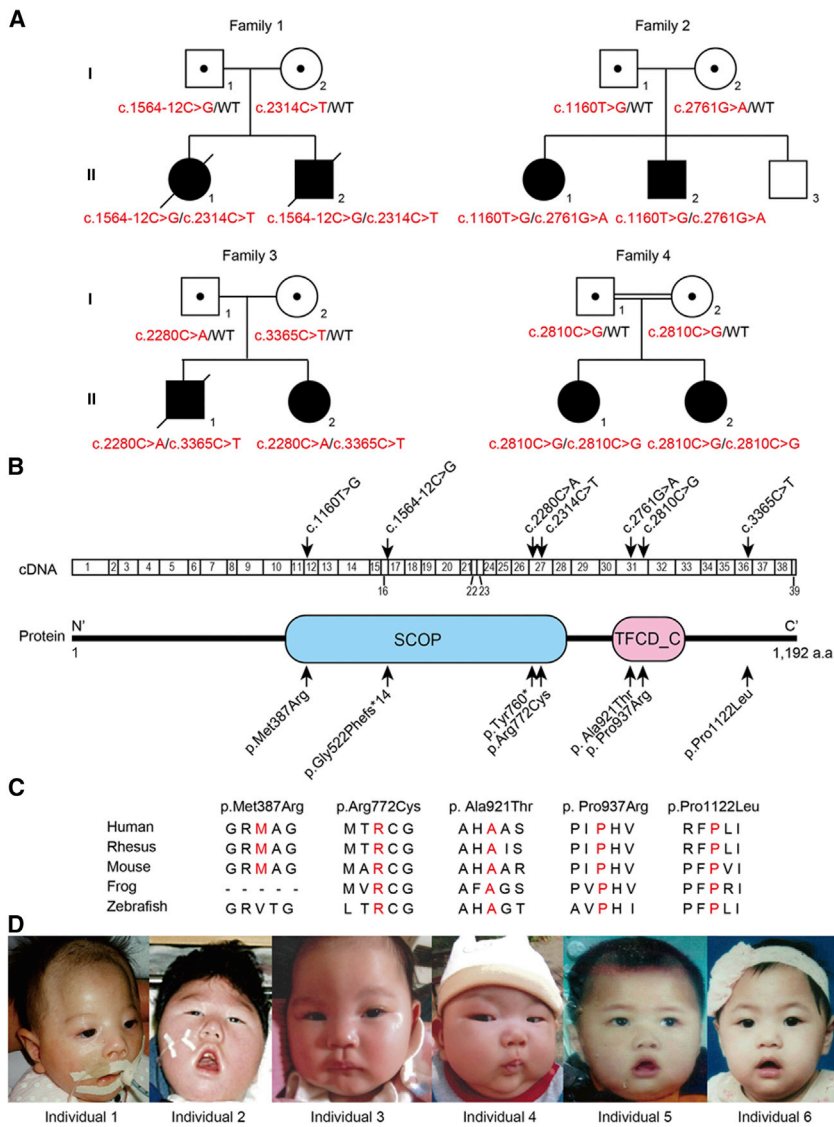
to be associated with an autosomal-recessive hypoparathyroidism-retardation-dysmorphism syndrome (MIM: 241410).<sup>10</sup> This is characterized by congenital hypoparathyroidism, microcephaly, hypoplasia of the corpus callosum, intellectual disability, growth failure, and dysmorphic features.<sup>11</sup> It is also associated with a similar disease with bone dysplasia, Kenny-Caffey syndrome (MIM: 244460).<sup>10</sup> Here, we report biallelic mutations in *TBCD* (GenBank: NM\_005993.4; MIM: 604649) encoding TBCD and the resulting mutant phenotypes in humans.

In this study, eight affected individuals from two Japanese families, a Chinese family, and an Israeli family were investigated (Figure 1A). The Israeli family was consanguineous, but the others were not. Genomic DNA was extracted from peripheral leukocytes using Quick-Gene-610L (Fujifilm) or formalin-fixed paraffin-embedded

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**Figure 1. Familial Pedigrees and *TBCD* Mutations**

(A) The pedigrees of four families with biallelic *TBCD* mutations. The mutations are shown in red. Affected and unaffected individuals are shown as black and white symbols, respectively. Affected individual identities are as follows: individuals 1 (II-1 in family 1), 2 (II-2 in family 1), 3 (II-1 in family 2), 4 (II-2 in family 2), 5 (II-1 in family 3), 6 (II-2 in family 3), 7 (II-1 in family 4), and 8 (II-2 in family 4).

(B) *TBCD* cDNA and *TBCD* protein structures. The p.Met387Arg and p.Arg772Cys variants fall within the SCOP domain, and p.Ala921Thr and p.Pro937Arg within the TFCD\_C domain. The seven variants found in the cDNA and protein are indicated by arrows. Functional domains of the *TBCD* protein were predicted by the SMART program.

(C) The evolutionarily conserved amino acids in the missense variants. The conserved amino residues are marked in red.

(D) The facial features of the affected individuals. From left to right, individual 1 at 3 months, individual 2 at 3 years, individual 3 at 4 months, individual 4 at 3 months, individual 5 at 1 year, and individual 6 at 1 year.

Genome, HGVD, and our in-house exome database ( $n = 575$ ). All of the missense variants were predicted to be pathogenic by PolyPhen-2 and MutationTaster. In addition, four altered amino residues due to missense mutations were located within the functional domains (Figure 1B). Five of the amino residues altered by the missense mutations were evolutionarily conserved (four from zebrafish to human and one from mouse to human) (Figure 1C).

brain tissue using QIAamp DNA FFPE tissue kit (QIAGEN) (for individual 1: II-1 in family 1 in Figure 1A) according to the manufacturer's instructions. Blood samples or brain tissue from affected individuals and their families were collected after obtaining written informed consent. The study protocol was approved by the institutional review board of Yokohama City University School of Medicine. To identify the genetic cause of the disease, we performed whole-exome sequencing as described previously.<sup>12</sup> The presence of affected siblings in the families implies an autosomal-recessive mode of inheritance (Figure 1A). Therefore, we focused on genes with homozygous or compound heterozygous variants and identified biallelic *TBCD* mutations in all probands in the four families (Figure 1A and Tables S1–S3). Using Sanger sequencing, we confirmed that the biallelic mutations perfectly co-segregated with the disease status (Figure S1). A total of seven mutations were found in *TBCD*: five missense, one nonsense, and a splicing mutation (Table 1). These variants were unreported or extremely rare ( $\leq 0.0002$ ) in ExAC, EVS, 1000

The intronic mutation (c.1564–12C>G) resulted in an 11-bp insertion of intronic sequence between exons 16 and 17, which was confirmed at the cDNA level to cause a frameshift (p.Gly522Phefs\*14) subjected to nonsense-mediated mRNA decay (Figure S2). This genetic evidence strongly indicates that biallelic *TBCD* mutations may cause this neurodegenerative encephalopathy.

The clinical data of all affected individuals are shown in Table 2 and the Supplemental Note. In brief, affected individuals with biallelic *TBCD* mutations showed an early onset of symptoms (before 1 year of age). The first symptoms varied among the families but were similar in affected siblings from the same family: neonatal asphyxia in family 1, decreased movement and hypotonia at 1 month of age in family 2 and at 5 months in family 3, and seizures at 9–11 months in family 4. The affected individuals commonly had progressive clinical courses with muscle weakness, absent visual tracking, and postnatal microcephaly. More than half of the affected individuals had postnatal growth failure, seizure, respiratory failure,

**Table 1. Summary of *TBCD* Mutations**

Mutation	Amino Acid Change	Position on chr17 (hg19)	dbSNP141	PolyPhen-2 (Score)	MutationTaster (Score)	ExAC	1000 EVS	Genomes	HGVD	In-house (n = 575)
c.1160T>G	p.Met387Arg	80,767,595	no	possibly damaging (0.733)	disease causing (0.996)	no	no	no	no	0
c.1564–12C>G	p. Gly522Phefs*14	80,851,411	no	NA	NA	no	no	no	no	0
c.2280C>A	p.Tyr760*	80,882,834	no	NA	disease causing (1.000)	no	no	no	no	0
c.2314C>T	p.Arg772Cys	80,882,868	rs181969865	probably damaging (0.972)	disease causing (1.000)	no	no	0.0002	no	0
c.2761G>A	p. Ala921Thr	80,887,056	no	probably damaging (1.000)	disease causing (1.000)	no	no	no	no	0
c.2810C>G	p.Pro937Arg	80,887,105	no	probably damaging (1.000)	disease causing (1.000)	no	no	no	no	0
c.3365C>T	p.Pro1122Leu	80,896,008	no	probably damaging (1.000)	disease causing (1.000)	0.00003645	no	no	no	0

The description of the mutation was based on GenBank: NM\_005993.4. Abbreviations are as follows: ExAC, Exome Aggregation Consortium; EVS, Exome Variant Server; HGVD, Human Genetic Variation Database; NA, not applicable. Data of minor allele frequencies were examined in May 2016.

regression, optic nerve atrophy, hypotonia, and muscle atrophy. In addition, two affected individuals had variable blood pressure and two other individuals showed urinary incontinence, one of whom also had fecal incontinence, suggesting an involvement of the autonomic system in some affected individuals. Six affected individuals in three of the families became immobile and five required respiratory management. Three of the eight affected individuals were deceased by 20 years of age. Most affected individuals had muscle weakness and atrophy. A muscle biopsy at 12 days of age (corrected gestational age: 5 days) in individual 2 (family 1, II-2) revealed the following myopathic changes: (1) variation in moderate muscle fiber size, ranging from 5 to 15  $\mu\text{m}$  in diameter (Figure 2A); (2) numerous type 2C fibers; (3) atrophy of type 1 muscle fibers (Figure 2B and 2C); and (4) dot-like staining in the myofiber cytoplasm after the NADH-tetrazolium reductase reaction (Figure 2D). The neuromuscular junction was not included in the section. No evidence of neuropathy was observed. These findings may reflect the immaturity of muscle fibers. However, it was not possible to confirm this because only limited samples were available. Interestingly, four affected individuals had episodes of elevated creatine kinase (CK). The elevation of CK was noticed only at birth (individual 1, 1,338 IU/L; individual 2, 1,345 IU/L) in family 1, and at several time points in individual 3 (max. 285 IU/L at 7 months) and individual 4 (max. 752 IU/L at 11 months) (Table 2, Supplemental Note). We believe that elevated CK was caused by asphyxia and bone fracture in family 1 and by frequent episodes of afebrile seizures in family 2. However, the actual triggers remain unclear. Brain imaging studies from three affected

individuals indicated progressive brain atrophy involving the cerebrum, corpus callosum, cerebellum, and brain stem (Figures 3 and S3). As early as 3 months of age, atrophy in the frontal lobe and enlarged ventricles (especially in the frontal horn of the lateral ventricles) were detected. Despite the flat and expressionless faces, downturned corners of the mouth, and open mouths, we did not see any specific dysmorphic features (Figures 1D and S4).

Interestingly, some clinical features overlapped between biallelic *TBCD* and *TBCE* mutations: early-onset (<1 year) intellectual disability, postnatal growth failure, hypoplastic corpus callosum, and microcephaly are common in both diseases, while intrauterine growth retardation and hypoparathyroidism were not seen in individuals with *TBCD* mutations (Table S4).<sup>11,13</sup>

TaqMan assays revealed that *TBCD* was ubiquitously expressed in all tissues, but highly expressed in brain, heart, and skeletal muscle in the fetus and adult (Figure S5). The expression pattern was consistent with the affected organs, including the central nervous system and skeletal muscles, in affected individuals with *TBCD* mutations.

An autopsy was performed on individual 1 (family 1, II-1). Although no malformation of the internal organs was detected (Table S5), striated muscle volume, including the iliopsoas, was severely decreased and the diaphragm was extremely thin. The brain and spinal cord were generally atrophic. In the cerebrum, there was severe atrophy of the cortex and white matter and the lateral ventricles were markedly dilated (Figure 2E). The corpus callosum was rudimentary, although the basal ganglia and thalamus were generally well formed. Both the anterior and posterior limbs of the internal capsules were severely atrophic.

**Table 2. Genetic and Clinical Information of the Affected Individuals with TBCD Mutations**

Family ID	Family 1		Family 2		Family 3		Family 4	
Ethnicity	Japanese		Japanese		Chinese		Israel	
Mutations and Variations	c.1564–12C>G (splicing), c.2314C>T (p.Arg772Cys)		c.1160T>G (p.Met387Arg), c.2761G>A (p. Ala921Thr)		c.2280C>A (p.Tyr760*), c.3365C>T (p.Pro1122Leu)		c.2810C>G (p.Pro937Arg), c.2810C>G (p.Pro937Arg)	
Sex	female	male	female	male	male	female	female	female
Individual ID	II-1	II-2	II-1	II-2	II-1	II-2	II-1	II-2
Individual number	individual 1	individual 2	individual 3	individual 4	individual 5	individual 6	individual 7	individual 8
Pregnancy	hydramnion	hydramnion	normal	normal	normal	normal	normal	normal
Age of onset	at birth	at birth	1 month	1 month	5 months	5 months	11 months	9 months
Initial symptom(s)	asphyxia, bone fracture	asphyxia, bone fracture	paucity of movement	hypotonia	hypotonia, regression	hypotonia, regression	febrile seizure	generalized seizure
Age of evaluation	4 years	19 years	9 years	5 years	2 years	3 years	17 years	16 years
Growth failure <sup>a</sup>	yes	yes	yes	yes	yes	yes	no	no
Seizure	no	no	West syndrome	cataplexy	GTS	GTS	GTS/GTCS	GTS/GTCS
Onset age of seizure	ND	ND	4 months	6 months	6 months	1 years	11 months	9 months
Respiratory failure	yes	yes	yes	yes	yes	no	no	no
Regression	ND	ND	yes	yes	yes	yes	yes	yes
Visual tracking	no	no	no	no	no	no	no	no
Optic nerve atrophy	no	yes	yes	yes	yes	no	no	no
Postnatal microcephaly	yes	yes	yes	yes	yes	yes	yes	yes
Ataxia	ND <sup>b</sup>	ND <sup>b</sup>	yes	yes	no	no	no	no
Hypotonia	yes	yes	yes	yes	yes	yes	no	no
Muscle weakness	yes	ND <sup>b</sup>	yes	yes	yes <sup>c</sup>	yes <sup>c</sup>	yes	yes
Muscle atrophy	yes	yes	yes	yes	no	no	yes	yes
Fasciculation <sup>d</sup>	no	no	yes	yes	no	no	no	no
Urinary incontinence	ND	yes <sup>e</sup>	no	no	no	no	yes	no
Fecal incontinence	no	no	no	no	no	no	yes	no

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Family ID	Family 1		Family 2		Family 3		Family 4	
	no	no	no	no	no	no	no	no
Disturbed sweating	no	no	no	no	no	no	no	no
Blood-pressure variability	ND	no	moderate	moderate	ND	ND	no	no
Elevation of CK	yes	yes	yes	yes	no	no	no	no
Prognosis	immobile with ventilation and deceased at 4 years (respiratory failure)	immobile with ventilation and deceased at 19 years (anaphylactic reaction)	immobile with ventilation	immobile with ventilation	immobile with ventilation and deceased at 4 years (pneumonia)	immobile	walking, moderate intellectual disability	walking, moderate intellectual disability

Abbreviations are as follows: ND, no data; GTs, generalized tonic seizure; GTCS, generalized tonic clonic seizure; CK, creatine kinase.

<sup>a</sup>Judged as height < -2 SD.

<sup>b</sup>Not assessed because individual was immobile.

<sup>c</sup>Floppy and almost immobile.

<sup>d</sup>Fasciculation in tongue.

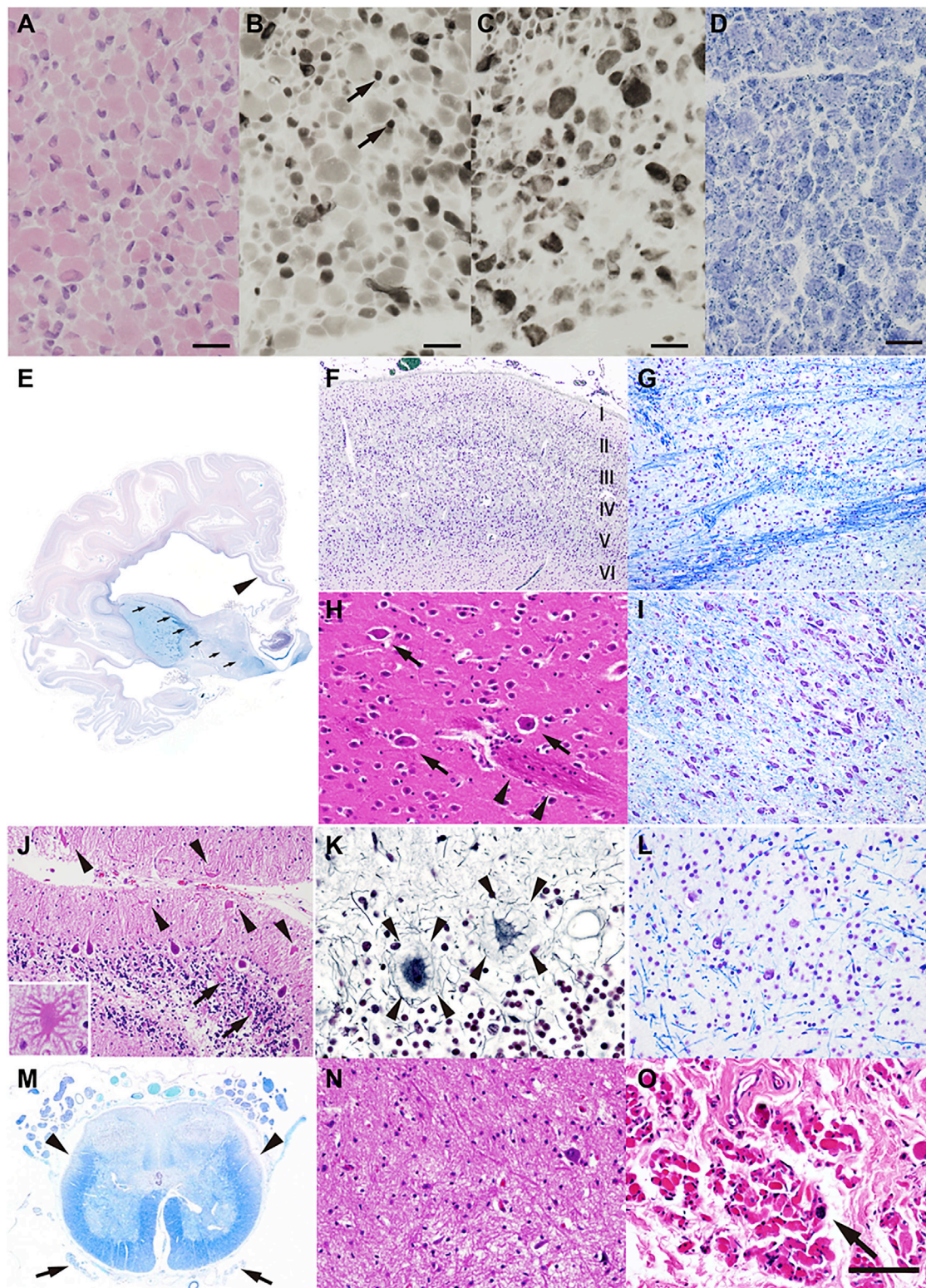
<sup>e</sup>Neurogenic bladder.

Histopathological examination revealed systemic laminar necrosis in the cerebral cortex, although a normal six-layer structure was observed with no cytologically abnormal neurons, such as dysmorphic neurons or balloon cells (Figure 2F). Furthermore, the hippocampus, thalamus, pontine nucleus (Figure 2G), globus pallidus, and inferior olivary nucleus had severe neuronal loss and gliosis. Many globular structures of unknown origin were observed in the internal segment of the globus pallidus. Purkinje and granule cells in the cerebellum were severely depleted.

These histological abnormalities might be ascribable, at least in part, to intrapartum asphyxia, in which the pattern of neuronal injury may be almost comparable to that of “full-term” severe asphyxia.<sup>14</sup> However, in this individual, other anatomical regions susceptible to asphyxial injury in term infants, including the putamen (Figure 2H), quadrigeminal bodies, substantia nigra (Figure 2I), and dorsal vagal nucleus, had only mild gliosis without apparent neuron loss. Additionally, in the cerebellum, many residual Purkinje cells had focal swellings of the dendrites (cactus/asteroid bodies) (Figure 2J) and some had halo-like amorphous structures around the cell bodies (somatic sprouts) (Figure 2K). Neuronal depletion was severe in the dentate nucleus (Figure 2L). In addition, the brainstem lower motor neuron (LMN) nuclei, including the oculomotor and hypoglossal nuclei, had severe neuronal loss and associated gliosis. In the spinal cord, the lateral corticospinal tracts and dorsal column showed severe degeneration with myelin pallor (Figure 2M). The anterior horns showed severe loss of the LMNs and associated gliosis, which was somewhat more apparent in the lateral nuclear group (Figure 2N). The spinal anterior roots were severely atrophic, whereas the posterior roots were preserved. The diaphragm was severely degenerated with fibrosis and scattered pyknotic nuclear clumps (Figure 2O).

We constructed homology-modeled structures of human *TBCD* using the Protein Homology/analogy Recognition Engine v.2.0 (Phyre2) server,<sup>15</sup> because no experimental structure is available. The resultant models had  $\alpha$ -solenoid repeats with pairs of antiparallel alpha helices, which have a deformable nature. We also employed an in silico detector of an  $\alpha$ -solenoid repeat based on an amino acid sequence, Alpha-rod Repeat Detector 2 (ARD2)<sup>16</sup> to verify the modeled structures. The results by ARD2 were consistent with those by the Phyre2 prediction (data not shown).

In the structures obtained, the side chains of Met387 and Ala921 are buried in the hydrophobic cores of  $\alpha$  solenoid repeat (Figure S6A). Therefore, the p.Met387Arg and p.Ala921Thr variants might affect the folding of the  $\alpha$ -solenoid repeat. The methylene part of the Arg772 side chain is part of a hydrophobic core and the guanidium group is exposed to the protein surface in the modeled structure (Figure S6A). Thus, the p.Arg772Cys variant might also affect at least the folding structure. Because Pro937 and Pro1122 were predicted to reside within a loop between antiparallel  $\alpha$  helices and be exposed to a protein surface



**Figure 2. Histopathological Findings in an Individual with Biallelic *TBCD* Mutations**

(A–D) Histology of a muscle biopsy from individual 2 at the age of 12 days (corrected gestational age: 5 days). Shown are (A) hematoxylin and eosin staining, (B) myosin ATPase (pH 4.2), (C) myosin ATPase (pH 10.5), and (D) NADH-tetrazolium reductase. The arrows indicate type 1 fibers. Scale bars represent 20  $\mu$ m.

(E) The cerebral cortex of individual 1 showing systemic laminar necrosis, which is more marked in the temporal lobe. The white matter is also atrophic and associated with marked dilatation of the lateral ventricles. The corpus callosum (arrowhead) and posterior limb of the internal capsule (arrows) are thin. The basal ganglia and thalamus are generally well formed.

(F) The frontal cortex has a normal six-layer architecture. Laminal necrosis is evident in the middle cortical layers.

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(Figure S6A), the p.Pro937Arg and p.Pro1122Leu variants might affect the loop structure and/or interactions with other molecules, although no functional data are available. TBCD functions as a component of the tubulin chaperone multiprotein complex (Figure S6B).<sup>17</sup> Therefore, all of the variants described here have the potential to impair complex formation.

Next, we examined protein-protein interactions between TBCD (wild-type and mutant) and other complex components ( $\alpha/\beta$ -tubulin, TBCE, TBCC, and ARL2) by co-immunoprecipitation. The open reading frames of human *TBCD*, *TBCE*, *TBCC* (GenBank: NM\_003192.2), and *ARL2* (GenBank: NM\_001667.3) were amplified using human cDNA as a template purchased from Clontech. The PCR products were cloned into the Gateway pDONR221 vector (Life Technologies) to create the entry clones, and each sequence was confirmed by Sanger sequencing. The QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) was used to create mutant constructs. To create the transient mammalian expression vectors, each entry clone was used for LR recombination with pcDNA3.1/nV5-DEST (for N' V5-tagged TBCC or TBCE), pcDNA-DEST53 (for N' GFP-fused TBCD), or pcDNA-DEST40 (for C' V5-6xHis-tagged ARL2). Wild-type TBCD interact with ARL2, TBCE, and  $\beta$ -tubulin (Figure 4). No apparent binding was observed between TBCD and  $\alpha$ -tubulin or TBCD and TBCC (data not shown); therefore, we focused on TBCD binding with ARL2, TBCE, and  $\beta$ -tubulin. As for the binding with ARL2, TBCD mutants p.Met387Arg almost completely, and p.Arg772Cys, p.Ala921Thr, p.Pro1122Leu, and p.Tyr760\* partially, lost binding capacity compared with wild-type TBCD. p.Pro937Arg retained its binding capacity (Figure 4A). Mutant TBCD showed similar impaired binding to TBCE (Figure 4B). All TBCD mutants showed decreased binding to endogenous  $\beta$ -tubulin (Figure 4C). These experiments suggest that all the investigated *TBCD* mutations may impair the proper formation of microtubule chaperone complexes.

Finally, to investigate the possible effects of disease-associated *TBCD* mutations in vivo, we took advantage of mosaic analysis with a repressible cell marker (MARCM) in *Drosophila*, which allowed us to analyze gene function in vivo at single-cell resolution.<sup>18</sup> We expressed mem-

brane-tethered GFP (mouse CD8::GFP, mCD8-GFP) to visualize the neuronal morphology of olfactory projection neurons (PNs) in the intact brain (Figure 5).<sup>19,20</sup> The axons of DL1 PNs elongate and form axonal branches at the calyx of the mushroom body and the lateral horn. The dendrites of PNs target a single glomerulus (DL1 glomerulus) in the antennal lobe, the first olfactory center in the *Drosophila* brain (Figures 5A and S7 for wild-type axon and dendrites, respectively). As we have previously reported,<sup>21</sup> PNs homozygous for *tbcd<sup>1</sup>* mutations (*tbcd<sup>1</sup>* PNs), in an otherwise largely heterozygous animal, had shorter axons and/or reduced axonal branches (Figures 5B–5D). Next, we carried out a MARCM-rescue experiment, in which we cell autonomously expressed cDNA of wild-type human *TBCD* in *tbcd<sup>1</sup>* PNs and found that overexpression of human *TBCD* significantly suppressed the axonal phenotypes of *tbcd<sup>1</sup>* PNs (Figure 5E). In addition, the dendritic phenotype of *tbcd<sup>1</sup>* PNs also tended to be suppressed by human *TBCD* overexpression (Figure S7). These results suggest that the function of TBCD in neuronal morphogenesis is partly conserved between *Drosophila* and humans.

To test the effect of disease-associated *TBCD* mutations, we performed MARCM-rescue experiments with human *TBCD* carrying disease-associated mutations. To avoid the positional effect that causes unexpected transgene expression differences, we utilized the PhiC31-integrase system to generate *UAS* transgenic animals and all the *UAS* transgenes were inserted in an *attP2* landing site.<sup>22</sup> Human *TBCD* bearing a disease-associated mutation was less able to suppress the axonal phenotype of *tbcd<sup>1</sup>* PN compared with wild-type human *TBCD* (Figure 5E). A similar tendency was also observed in PN dendrites (Figure S7). These results indicate that the *TBCD* disease-associated mutations we tested cause loss of function of the TBCD protein.

In this study, we have reported four families with early-onset neurodegenerative encephalopathy associated with biallelic *TBCD* mutations. Core clinical features include early-onset progressive diffuse brain atrophy with regression, postnatal microcephaly, postnatal growth delay, muscle weakness/atrophy, and respiratory failure. Our in vitro and in vivo studies showed that seven *TBCD* mutations are likely to lead to loss of function of the TBCD protein. Interestingly, the clinical course was very similar

(G) The pontine nucleus with severe neuronal loss and gliosis. Note that both the longitudinal and transverse fibers are degenerative, and longitudinal fibers are almost unrecognizable.

(H) The putamen has a normal composition of medium spiny and large aspiny neurons (arrows). The putamino-pallidal fibers are evident (arrowheads).

(I) Substantia nigra is well preserved in this individual.

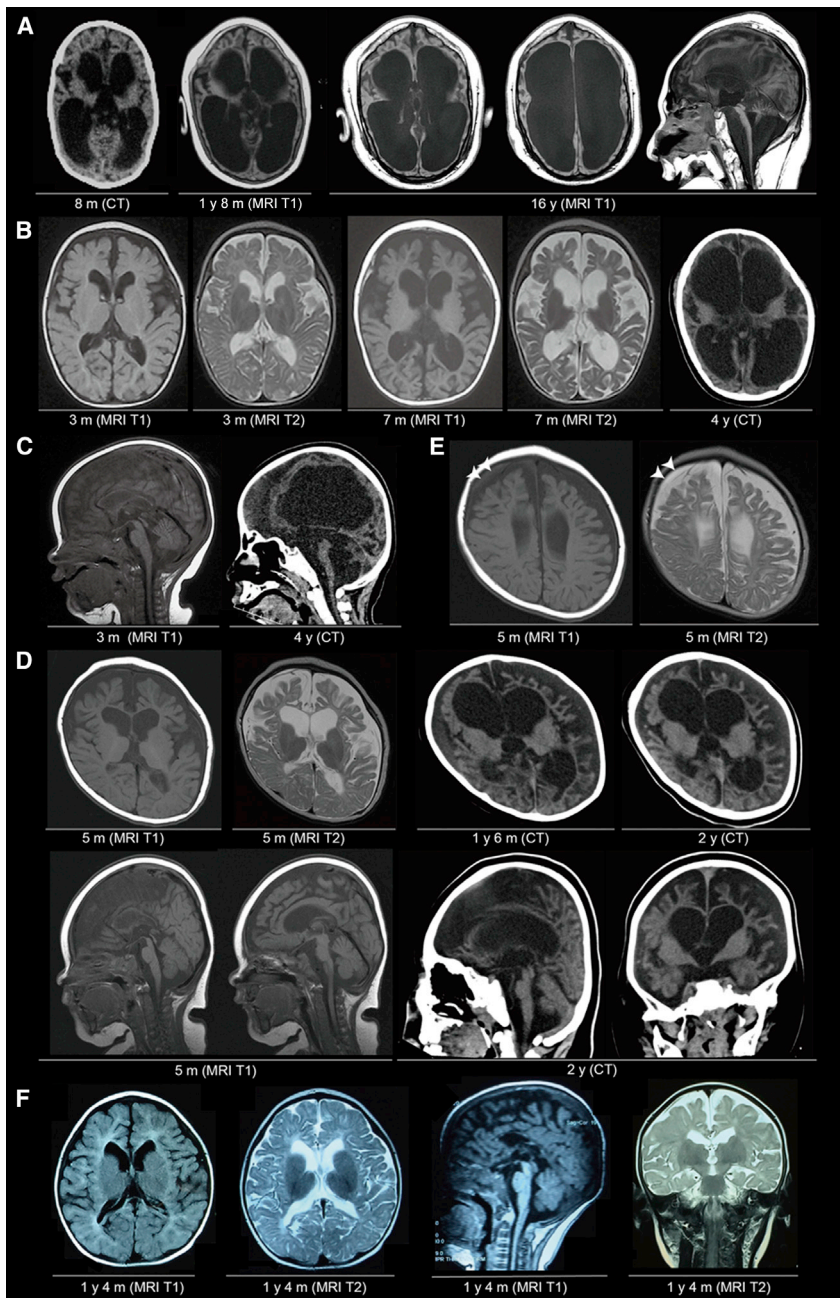
(J–L) Loss of Purkinje and granule cells is evident in the cerebellum. The residual Purkinje cells show torpedoes (J, arrows) and asteroid bodies/cactus (J, arrowheads and inset), as well as halo-like amorphous structures (somatic sprouts) (K, arrowheads). Neuron loss is also apparent in the dentate nucleus (L).

(M) Anterior horn volume is reduced in the lumbar cord. The anterior nerve roots are severely atrophic (arrows), whereas the posterior roots are almost preserved. Degeneration of the lateral corticospinal tracts (arrowheads) and posterior column is also evident.

(N) The lumbar anterior horn with severe loss of the lower motor neurons and associated gliosis.

(O) Severe degeneration of the muscle fibers in the diaphragm, with fibrosis and scattered pyknotic nuclear clumps (arrow).

Shown are Klüver-Barrera staining (E, F, G, I, L, M), hematoxylin and eosin staining (H, J, N, O), and Bodian staining (K). Scale bars represent 1 cm (E), 500  $\mu$ m (F), 200  $\mu$ m (G, I), 100  $\mu$ m (H, L, N, O), 160  $\mu$ m (J), 50  $\mu$ m (J inset and K), and 1.5 mm (M).



### Figure 3. Neuroimaging of Affected Individuals with Biallelic *TBCD* Mutations

(A) The neuroimages of individual 2 at 8 months (computed tomography: CT), 1 year 8 months and 16 years (T1-weighted MRI). Severe, progressive enlargement of bilateral ventricles, thin brain stems, and severe cerebellar atrophy are shown.

(B) The axial brain paired T1- and T2-weighted MRI images of individual 3 at 3 months and 7 months and CT at 4 years (right).

(C) The sagittal images of individual 3. T1-weighted MRI at 3 months (left) and CT at 4 years (right). Thinned corpus callosum and enlargement of the great cistern can be seen on the MRI (left).

(D) The serial images from individual 4. From upper left to upper right, axial T1-weighted MRI and T2-weighted MRI at 5 months, CT at 1 year 6 months, and CT at 2 years. Gyral pattern and myelination seemed to be consistent with age, whereas the progressive atrophy of the cerebral cortex (predominantly in the frontal cortex) and enlargement of the ventricles is remarkable. From lower left to lower right, two sections of sagittal T1-weighted MRI at 5 months, sagittal and coronal sections from CT at 2 years.

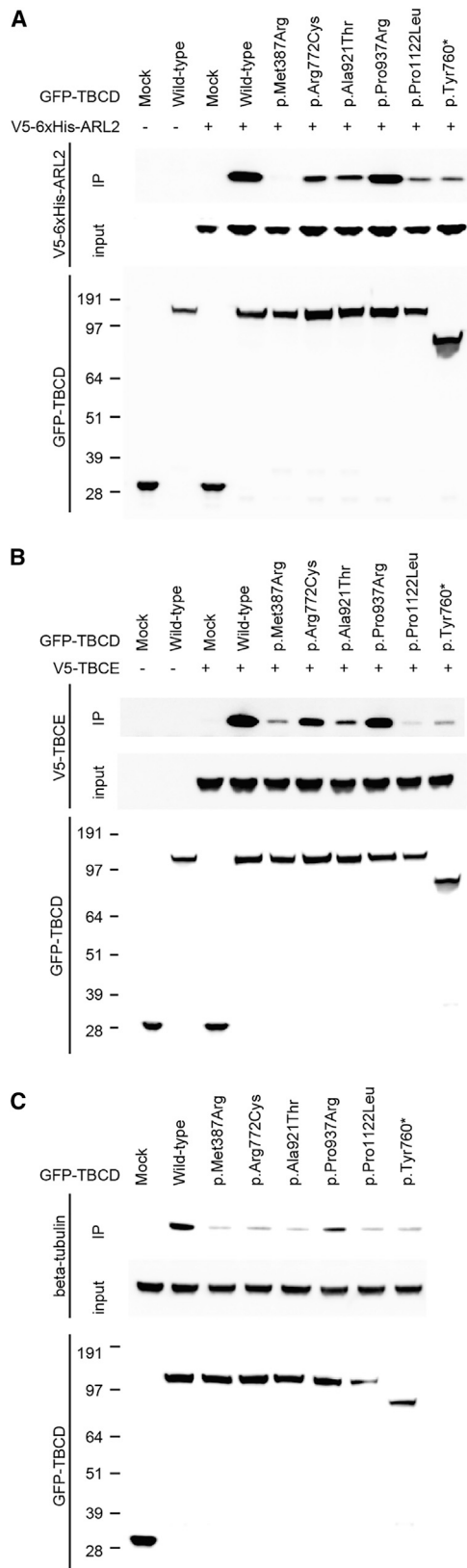
(E) T1- and T2-weighted MRI images of individual 4 at 5 months. The white arrowheads indicate a chronic subdural hematoma.

(F) T1- and T2-weighted MRI images of individual 6 at 1 year 4 months.

We neuropathologically examined an autopsied individual (individual 1) in whom the original lesions caused by *TBCD* mutations could have been masked by asphyxial effects. However, we found the following pathological findings that could not be easily explained by asphyxia: (1) neuronal loss of dentate nuclei of the cerebellum and LMN in the brainstem and anterior horn of the spinal cord and (2) somatic sprout and cactus formations in cerebellar Purkinje cells. Neuronal loss in the dentate nuclei and LMN may also be caused by asphyxia.<sup>23</sup> However, regions that are known to be susceptible to asphyxia, such as the putamen and quadrigeminal bodies,<sup>14</sup> were well preserved in this affected individual. Therefore, the disproportionately severe loss of LMNs in the brainstem, spinal cord, and the cerebellar dentate nucleus may arise from lesions caused by *TBCD* mutations, rather than asphyxia. In addition, cactus formations are usually observed in Menkes disease and mitochondrial diseases,<sup>24–26</sup> and somatic sprouts have been reported in Menkes disease and spinocerebellar ataxia type 31,<sup>27–29</sup> but not in asphyxia. Notably, mitochondrial dysfunction is also associated with Menkes disease.<sup>30</sup>

within individual families, but differed between families. This might be partly explained by the specific mutations in each family. The siblings in family 4 with a homozygous missense mutation (c.2810C>G [p.Pro937Arg]) had a milder phenotype, possibly because binding of the altered *TBCD* protein with  $\beta$ -tubulin was only mildly affected. In more severe clinical cases (such as the deceased individuals in families 1 and 3), affected individuals carried truncating and missense variants that significantly impaired *TBCD* binding to ARL2, TBCE, and  $\beta$ -tubulin (Table S6). This indicates that biallelic *TBCD* mutations may lead to a wide range of clinical severities depending on the level of residual protein function resulting from the specific *TBCD* mutation.





**Figure 4. Intermolecular Associations between TBCD and Its Binding Partners**

(A and B) The molecular interaction between human TBCD and ARL2 (A) or TBCE (B). GFP-fused wild-type TBCD (159 kDa) or its mutants were transiently co-overexpressed with V5-6xHis-tagged ARL2 (22 kDa) or V5-tagged TBCE (60 kDa) in HEK293T

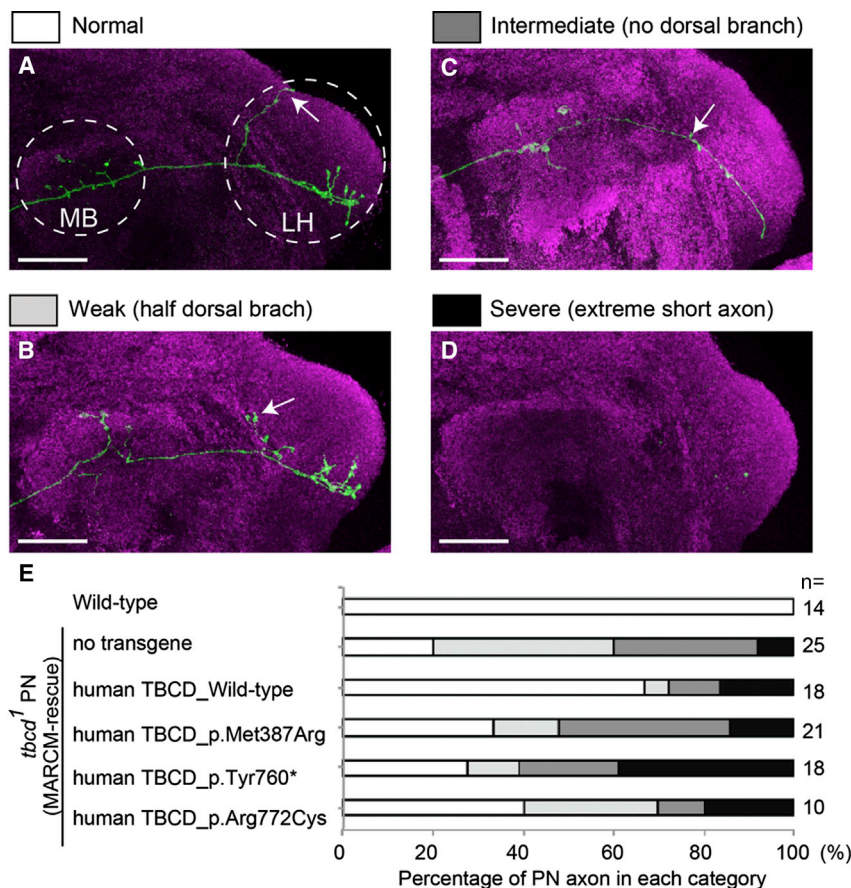
Mitochondria are essential organelles providing energy to all eukaryotic cells. Most mitochondria are newly produced in the soma and are transported to the distal end of axons and dendrites.<sup>31–33</sup> In neurons, mitochondria are distributed unevenly through microtubule trafficking, depending on local energy demand.<sup>33–36</sup> Microtubules are essential for long-distance axonal transport,<sup>32,37</sup> and impaired mitochondrial transport results in neuronal degeneration.<sup>33,38–40</sup> Reduced levels of TBCD protein affect microtubule network formation and impair microtubule transport in *tbcd<sup>1</sup>* PNs.<sup>41</sup> Furthermore, TBCD is also required for axon and dendrite maintenance in PNs.<sup>41</sup> Therefore, TBCD depletion may result in an abnormal microtubule network and abnormal microtubule trafficking in the human brain. Consequently, energy supply to neuronal cells is reduced, causing neuronal degradation. The formation of cactus and somatic sprouts might reflect structural and metabolic abnormalities resulting from disrupted mitochondrial transport in neuronal cells caused by TBCD depletion.

In conclusion, we have described an early-onset neurodegenerative encephalopathy caused by biallelic *TBCD* mutations. We performed in vitro and in vivo experiments which revealed that the described *TBCD* mutations probably cause loss of function of the TBCD protein. We also investigated a possible genotype-phenotype correlation, particularly with regard to disease severity. Our knowledge of human *TBCD* mutations is currently limited, and therefore more genetic and clinical studies are required to better understand the clinical consequences of *TBCD* mutations.

cells using the X-treme GENE9 DNA transfection kit (Roche). At 48 hr after transfection, the cells were collected and lysed with lysis buffer (25 mM Tris-HCl [pH 7.5], 100 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, and protease inhibitors). GFP-fused TBCD proteins were immunoprecipitated using a rabbit polyclonal anti-GFP antibody (Abcam cat# ab6556, RRID: AB\_305564) and Dynabeads Protein-G (Thermo Fisher Scientific). Input and immunoprecipitated samples (IP) were immunoblotted with mouse monoclonal anti-GFP (Roche) and anti-V5 antibodies (Thermo Fisher Scientific cat# R960-25, RRID: AB\_2556564).

(C) The binding of human TBCD and endogenous  $\beta$ -tubulin (50 kDa). Wild-type GFP-fused TBCD or its mutants were transiently overexpressed in HEK293T cells. GFP-fused TBCD proteins were immunoprecipitated with a rabbit polyclonal anti-GFP antibody and immunoblotted with mouse monoclonal anti-GFP or anti  $\beta$ -tubulin antibodies (Chemicon International cat# MAB3408). Horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch) was used as the secondary antibody. Immunodetection was performed using Supersignal West Dura Extended Duration Substrate (Thermo Fisher Scientific) on a ChemiDoc Touch Imaging system (Bio-Rad Laboratories).

At least three independent experiments were performed to confirm the consistency of results for TBCD binding with ARL2, TBCE, or  $\beta$ -tubulin. Mock indicates only GFP protein (27 kDa). “+” and “-” denote the presence and absence of V5-tagged proteins (or only V5-tag with the size of 1 kDa, which could not be detected due to its small molecular weight), respectively.



**Figure 5. Human *TBCD* with Disease-Associated Mutations Impaired Neuronal Morphology**

(A–D) The representative axonal morphologies of *Drosophila* olfactory projection neurons. The axons of DL1 single-cell clones for wild-type *Drosophila* (A) show the typical branching pattern at the calyx of the mushroom body (MB) and lateral horn (LH). Axons of *tbcd<sup>1</sup>* *Drosophila* PN show branching and elongation defects (B–D). The dorsal branch at the LH is often affected (arrow in A–C). Axons cannot reach the MB in the severe class (no axon is visible in D). PN morphologies and Bruchpilot (presynaptic marker) are shown in green (anti-CD8 antibody) and magenta (nc82), respectively. Scale bars represent 25  $\mu$ m.

(E) Quantification of MARCM-rescue experiments. Each gray scale corresponds to the colors in (A)–(D) above. n indicates the number of MARCM clones examined. Because the number of clones we can observe in the *Drosophila* brain cannot be controlled, we analyzed a variable number of clones (minimum n = 10). Details for the generation of transgenic flies, MARCM clone induction, immunohistochemistry of fly brains, image processing, and quantification methods are available upon request.

## Supplemental Data

Supplemental Data include supplemental note, seven figures, and six tables and can be found with this article online at <http://dx.doi.org/10.1016/j.ajhg.2016.08.005>.

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## Web Resources

ExAC Browser (accessed May 2016), <http://exac.broadinstitute.org/>  
 GATK, <https://www.broadinstitute.org/gatk/>  
 GenBank, <http://www.ncbi.nlm.nih.gov/genbank/>  
 Human Genetic Variation Database (HGVD), <http://www.genome.med.kyoto-u.ac.jp/SnpDB/>  
 NHLBI Exome Sequencing Project (ESP) Exome Variant Server, <http://evs.gs.washington.edu/EVS/>  
 Novoalign, <http://www.novocraft.com/main/page.php?s=novoalign>  
 OMIM, <http://www.omim.org/>  
 Picard, <http://broadinstitute.github.io/picard/>  
 RRID, <https://scicrunch.org/resources>  
 SMART, <http://www.smart.embl-heidelberg.de/>

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