

Supplemental Data

Case Reports

Individuals 1 and 2 are sisters from the same non-consanguineous healthy family. **Individual 1** was an 8-year-old girl born at full term without prenatal complications. Her birthweight was 2.75 kg. At 3 months of age, she began to develop generalized tonic-clonic seizures (GTCS) during sleep. One and a half months later, myoclonic seizures and focal seizures with impaired consciousness occurred every day. Electroencephalogram (EEG) showed generalized spike and spike-slow waves. An episode of status epilepticus occurred at 6 months of age. EEG at that time showed hypsarrhythmia and detected partial seizures, primarily originating from the left side. She was treated with sodium valproate, levetiracetam, and topiramate from 3 months of age. However, the seizures failed to be controlled. At the age of 8 months, the seizures were reduced after a combination with nitrazepam. One year later, she stopped taking the drug without medical advice. A ketogenic diet was tried, but without a satisfactory response. The current seizure frequency is less than 10 episodes a year. Brain MRI at age of 5 years showed mild white matter lesions, slightly enlarged left lateral ventricle and small corpus callosum. She exhibited global developmental delay and severe intellectual disability. At the last examination at the age of 8 years, she could not raise head, turn over, sit independently, crawl or walk upright. She could make sounds, such as laughing and screaming, but could not speak. Neurological examination revealed spastic quadriplegia, athetoid movement and hypertonia. Her height was 101cm (<-3SD), weight was 13.8kg (<-3SD), and head circumference was 46cm. Esotropia was observed. Facial dysmorphisms included ocular hypertelorism, low nose bridge, glazed eyes and big mouth.

Trio exome sequencing analysis identified compound heterozygous variants in *SEC16A*

[NM_014866.2: c.2460delT (p.E821Sfs*9) and c.4606C>G (p.L1536V)] in this individual.

Except for *SEC16A*, the variants in the remaining genes (Supplemental Table S4) are all heterozygous, which is inconsistent with their known inheritance patterns, and these variants were all inherited from the patients' phenotypically unaffected parents. Therefore, no additional genomic variants that could account for the severity of symptoms were found in Family 1.

Individual 2 was a 6-year-old girl. She was born prematurely at 35 weeks with a birth weight of 2.3 kg. At 3 months of age, she also developed GTCS during sleep and it occurred about once a week. 3 months later, epileptic spasms appeared, manifested as nodding, knee flexion, hip flexion, and flexion and lifting of both upper limbs. The seizures occurred repeatedly at intervals of several seconds and lasted for several minutes. EEG showed hypsarrhythmia and generalized spike-slow waves and epileptic spasms were detected. Then, she was diagnosed with infantile spasms. Myoclonic seizures and focal seizures with impaired consciousness were occasionally observed. Sodium valproate and topiramate were taken from the age of 3 months but failed to control seizures. At the age of 7 months, the seizures were reduced with a combination with nitrazepam and vigabatrin. Same as Individual 1, she discontinued the medication one year later. After that, she developed a maximum of 2 seizures per year, but each consciousness loss lasted about two hours. Brain MRI at the age of 3 months showed mild white matter lesions and agenesis of the corpus callosum. Echocardiography at 34 days of age revealed ventricular septal defect, patent foramen ovale and tricuspid regurgitation. At 3 years of age, left cardiac enlargement was observed. She was found to have a cataract in the right eye at the age of 2 years. She exhibited global developmental delay and severe intellectual disability. At the last examination at the age of 6 years, she failed to raise head, turn over, sit independently, crawl or

walk upright. She could make sounds but was non-verbal. Physical examination showed spastic quadriplegia, athetoid movement and hypotonia. Her height was 91cm (<-3SD), weight was 11kg (<-3SD), and head circumference was 43cm (<-3SD). Esotropia was observed. Facial dysmorphisms included ocular hypertelorism, blepharophimosis, low nose bridge, glazed eyes, big mouth and small ears.

Individual 3 was a 2-year-old boy born at full term with no abnormality at birth assessment. The birth weight was 2.75 kg. 7 months after birth, he started to experience epileptic spasms characterized by nodding and flexion and abduction of the limbs. The seizures occurred in clusters with more than 30 times per cluster. The frequency was one cluster per day at the beginning and then aggravated to 3-4 clusters per day. Myoclonic seizures and focal seizures with impaired consciousness were also present. EEG at 7 months of age showed hypsarrhythmia and epileptic spasms were detected. EEG at 9 months of age showed generalized spike-slow waves. He was diagnosed with infantile spasms. A polytherapy of topiramate and vigabatrin was administered at 7 months of age. The seizures were well-controlled since 9 months of age. Adrenocorticotrophic hormone was also tried at 7 months of age, but with poor response. Brain MRI was found to be normal. Echocardiography showed patent foramen ovale. He was presented with global developmental delay and moderate intellectual disability. Mild regression in motor ability was observed after seizure onset. At the last examination at the age of 2 years, he could not walk upright and could only speak simple words. Physical examination showed a height of 89cm, a weight of 12kg, and a head circumference of 48cm. There was no facial dysmorphism.

Individual 4 was a 4-year-old girl born at full term with normal perinatal conditions. At 12 months of age, seizures occurred after vaccination, characterized by frequent nodding, sudden forward leaning, flexion and rigidity of both upper limbs lasting for several seconds. The

seizures were more common in the middle of the night. EEG showed hypsarrhythmia and generalized spike-slow waves and epileptic spasms were also monitored. The patient was diagnosed with infantile spasms. At the age of 12 months, she was treated with sodium valproate and levetiracetam which failed to reduce seizures. At 1 year and 6 months, levetiracetam was discontinued, and vigabatrin was taken. She was seizure-free since 2 years of age. Brain MRI was found to be normal. She presented with global developmental delay and mild intellectual disability. At the last examination at the age of 4 years, she failed to walk independently and had feeble speech. Physical examination showed a height of 93cm (<-3SD), a weight of 15kg, and a head circumference of 48cm. She had no facial deformity.

Individual 5 was a male who passed away at the age of 15 years. There were no reported complications during pregnancy or birth. He was diagnosed with Pervasive Developmental Disorder and was enrolled in special education classes. Developmental records indicated a mild intellectual disability (IQ 75). He exhibited significant behavioral challenges, including Attention-Deficit/Hyperactivity Disorder (ADHD) and severe temper tantrums. At the age of 9 years, poor gross motor coordination was noted. Six days before death, he was hospitalized with diabetic ketoacidosis due to a previously unknown diabetes and obesity, dehydration, and acute kidney failure. His condition rapidly worsened, with neurological decline, rhabdomyolysis, and elevated urea levels. In the final days of life, he experienced nasal bleeding, airway obstruction, and respiratory failure, ultimately leading to cardiac arrhythmia and death.

Postmortem examination revealed a muscular body habitus despite a body mass index (BMI) of 40. There was marked organ enlargement: the liver weighed 3.2 kg and showed fat accumulation (steatosis) without fibrosis; the pancreas, kidneys, and lungs were also enlarged, raising

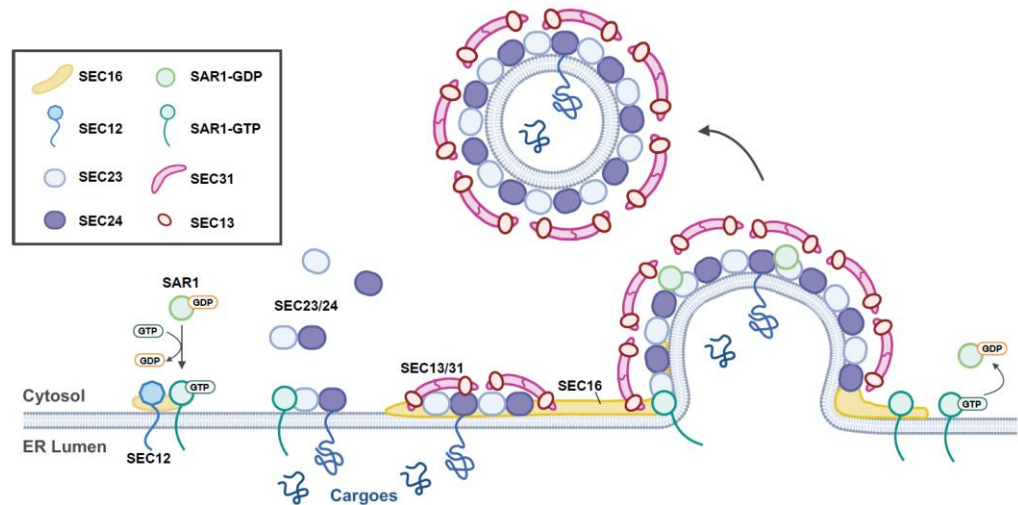
suspicion of a possible overgrowth disorder. The brain was enlarged but structurally normal, though signs of herniation were present and may have contributed to death.

Individual 6 is an 8-year-old female with global developmental delay, significant speech and language impairment, and a structural brain abnormality. Developmental delays were noted from infancy. She began walking independently later than average at 20 months, but subsequently demonstrated improvement in gross motor skills, with some developmental catch-up over time. In contrast, speech and language development remained markedly impaired. At the age of 4.5 years, she was only able to form 2-3 word phrases, and by the age of 6 years, her expressive language remained limited to isolated words, without full sentence formation. Her brain MRI revealed periventricular nodular heterotopia. Dental findings show abnormal tooth enamel, necessitating dental crowns.

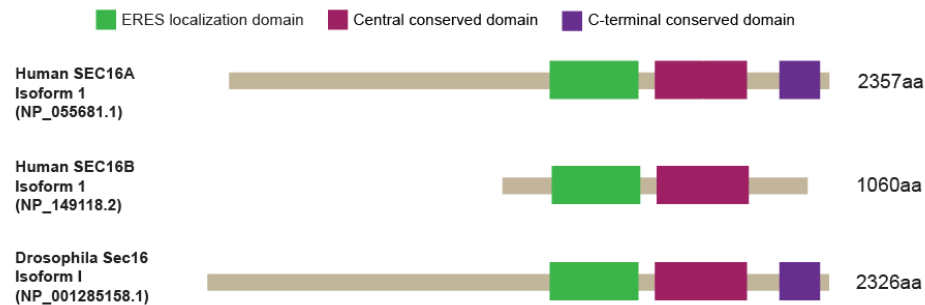
Supplemental Figures

Figure S1. Validation of *Drosophila Sec16* reagents

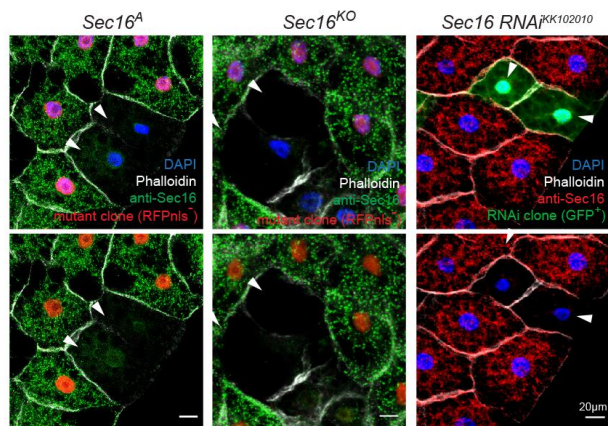
A COPII assembly at ERES



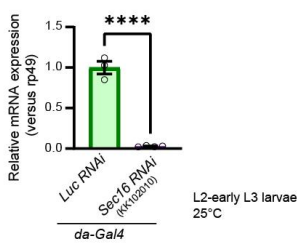
B Fly Sec16 has conserved protein domains



C Antibody validation of mutant and RNAi



D qPCR validation of *Sec16* RNAi



E Ubiquitous knockdown

GAL4	RNAi	KK102010
Tub-GAL4		Lethal
Act-GAL4		Lethal
da-GAL4		Lethal

(A) Schematic of COPII coat assembly at ER exit sites (ERES). Adapted from (McCaughey and Stephen, 2018) (1) Created with BioRender.com.

(B) Schematic showing the domain organization of human SEC16A, SEC16B, and *Drosophila* Sec16 protein domains.

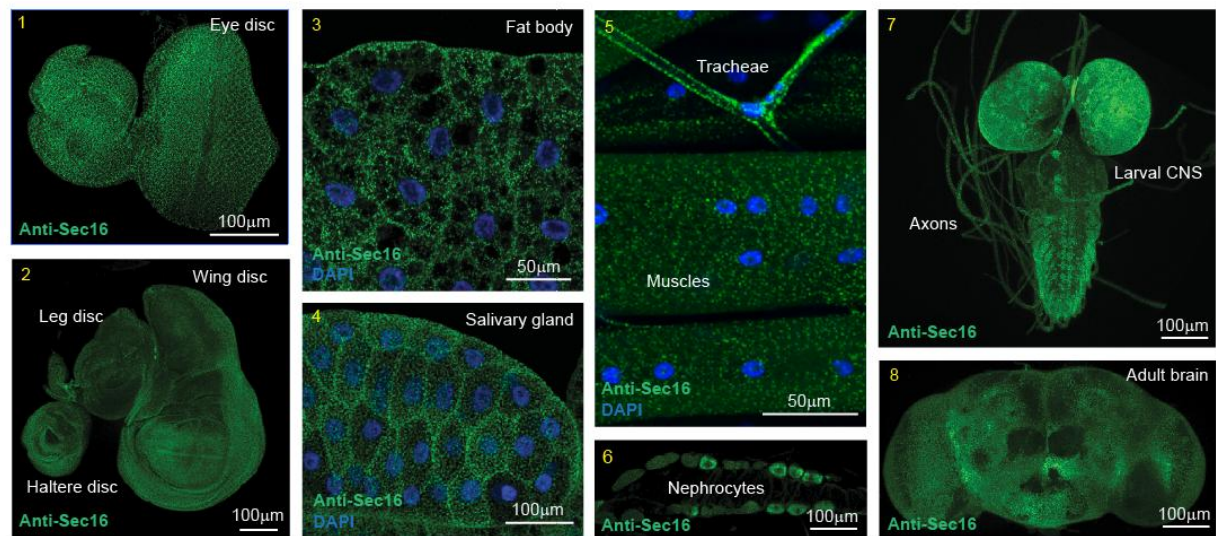
(C) Antibody validation of *Sec16* mutant alleles and RNAi. *Sec16^{KO}* and *Sec16^A* mutant clones are labeled as negative for nuclear RFP (red). *Sec16* RNAi clones are labeled as positive for GFP (green). Fat body cells of L3 larvae are stained with anti-Sec16 antibody (green in mutant clone images, red in RNAi clone images). The Sec16 signal is significantly reduced in the mutant or RNAi clone cells compared with the surrounding control cells. Nuclei are labeled with DAPI (blue). Cell boundaries are visualized by phalloidin staining (gray). At least three larvae per genotype were dissected for imaging. Scale bars, 20µm.

(D) Quantitative RT-PCR analysis showing knockdown of *Sec16* mRNA in *da-GAL4>UAS-Sec16 RNAi^{KK102010}* larvae (surviving to L2 to early L3 stage). The mRNA levels are reduced to <5% of controls (*da-GAL4>UAS-Luciferase RNAi*). Each dot represents one independent replicate per genotype. Sample sizes n=3 replicates for *Luc RNAi* and n=4 replicates for *Sec16 RNAi*. Welch's t test, **p<0.01, mean ± SEM.

(E) Ubiquitous knockdown of *Sec16* causes early lethality. Three different ubiquitous GAL4 drivers were crossed with *UAS-Sec16 RNAi^{KK102010}*.

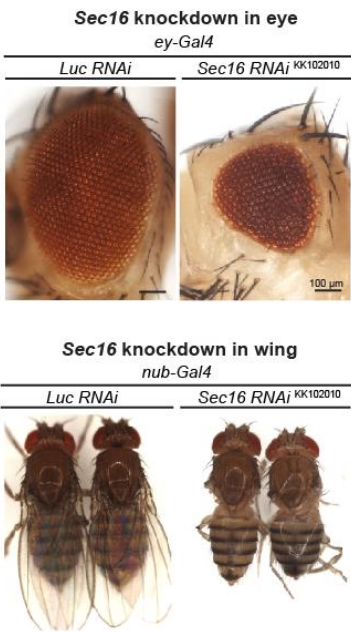
Figure S2. Sec16 is presented and required in most tissues

A Sec16 is presented in most tissues



B Tissue specific knockdown of Sec16 in different tissues (GAL4>UAS-RNAi at 29°C)

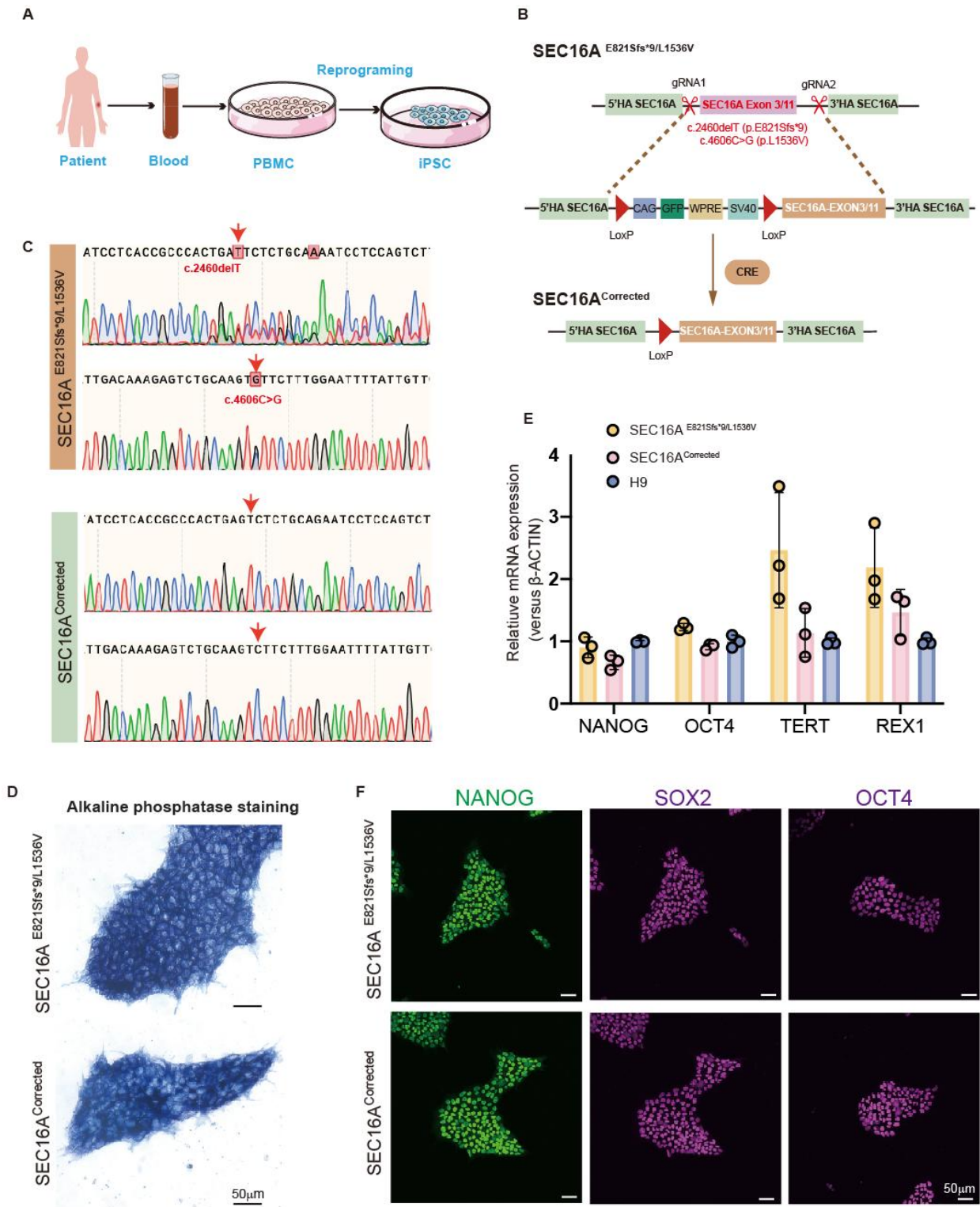
Tissue	GAL4	Expression pattern	RNAi ^{KK102010}	RNAi ^{HMJ21252}
Nervous system	elav-GAL4 (II)	pan-neuron, embryo to adult	pupae lethal, some escapers (uncoordinated)	lethal
	elav-GAL4 (III)		>80% survived to adults, with behavioral phenotypes	lethal
	nSyb-GAL4	pan-neuron, strong in mature neurons	lethal	lethal
	repo-GAL4	pan-glia, embryo to adult	lethal	lethal
Eye	ey-GAL4	developing and adult eye	pupae lethal, few escapers with small eye	lethal
	GMR-GAL4	developing and adult eye	lethal	lethal
	rh1-GAL4	late pupae and adult outer photoreceptors	viable	viable
Wing	nub-GAL4	embryo to adult wing disc, ventral thoracic disc	pupae lethal, escapers with no wings	lethal
	MS1096-GAL4	embryo to adult wing disc	pupae lethal, escapers with no wings	lethal
Fat body	SPARC-GAL4	embryonic to adult fat body	lethal	lethal
	Cg-GAL4	embryonic to adult fat body	lethal	lethal
Muscle	Mef2-GAL4	embryonic to adult muscle	lethal	lethal
	Mhc-GAL4	embryonic to adult muscle	lethal	lethal



(A) Immunofluorescence staining with an anti-Sec16 antibody showing Sec16 expression in multiple larval and adult tissues, including imaginal discs (1, eye disc; 2, leg, wing, and haltere discs), fat body (3), salivary gland (4), tracheae and muscles (5), nephrocytes (6), larval central nervous system (CNS) (7), and adult brain (8). Sec16 signal is shown in green. Nuclei are labeled with DAPI (blue) in panels 3-5. Scale bars as indicated. At least three larvae were dissected for imaging.

(B) Summary of tissue-specific *Sec16* knockdown phenotypes using the GAL4/UAS system (*GAL4s>UAS-Sec16 RNAi*^{KK102010} or *UAS-Sec16 RNAi*^{HMJ21252}). Representative images show that *Sec16* knockdown in the developing eye using *ey-GAL4* causes a severely reduced eye phenotype, whereas knockdown in the developing wing using *nub-GAL4* causes a no-wing phenotype. At least 5 flies per genotype were sampled for imaging.

Figure S3. Establishment of iPSC lines derived from Individual 1, and the correction of the *SEC16A* variants



(A) Schematic of the generation of induced pluripotent stem cell (iPSC) lines derived from the peripheral blood mononuclear cells (PBMCs) obtained from Individual 1.

(B) Strategy for the CRISPR/Cas9-based correction of the *SEC16A* variants in the patient-derived iPSCs (*SEC16A*^{E821Sfs*9/L1536V}) to generate the isogenic control iPSCs (*SEC16A*^{Corrected}). Two guide RNAs (gRNA1 and gRNA2) and donor homology arms (HA) flanking exons 3 and 11 were used for targeted repair. The corrected allele retains LoxP sites for optional Cre-mediated recombination.

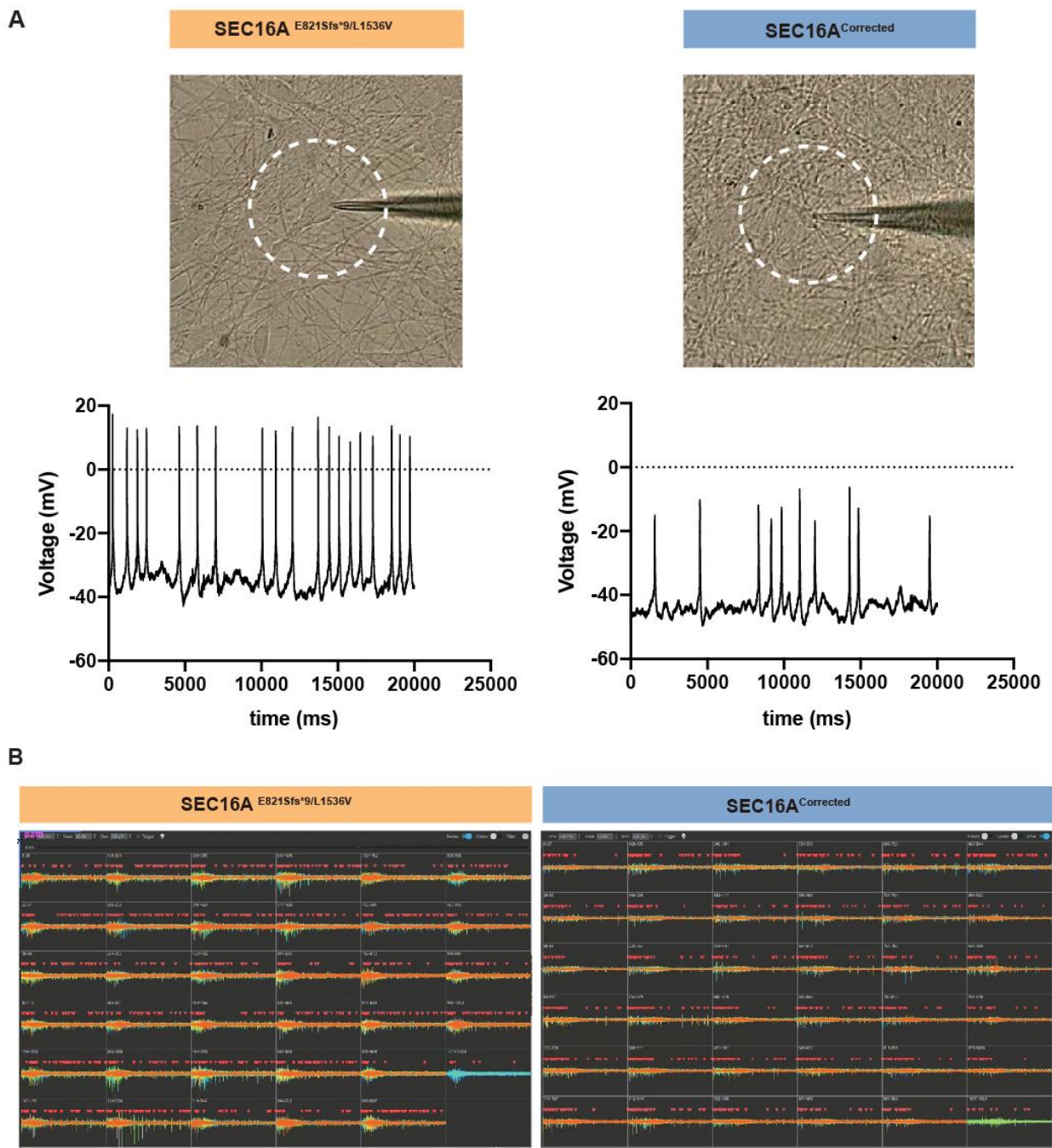
(C) Sanger sequencing confirming the correction of the biallelic *SEC16A* variants in *SEC16A*^{E821Sfs*9/L1536V} iPSCs.

(D) Alkaline phosphatase staining of iPSC colonies confirming the undifferentiated pluripotent state of the iPSC lines. Scale bars, 50µm.

(E) Quantitative RT-PCR analyses showing robust expression of pluripotency markers (NANOG, OCT4, TERT, and REX1) in the iPSC lines, with no significant differences in the mRNA expression levels of these markers among three lines. H9 human embryonic stem cells (WA09, WiCell Research Institute, Madison, WI, USA) were included as a reference control. mRNA levels were normalized to β-ACTIN. Each dot represents one independent experimental replicate, with three replicates analyzed for each marker in each iPSC line. Data are presented as mean ± SD.

(F) Immunofluorescence staining showing expression of the pluripotency markers NANOG, SOX2, and OCT4 in *SEC16A*^{E821Sfs*9/L1536V} and *SEC16A*^{Corrected} iPSC colonies. Scale bars, 50µm.

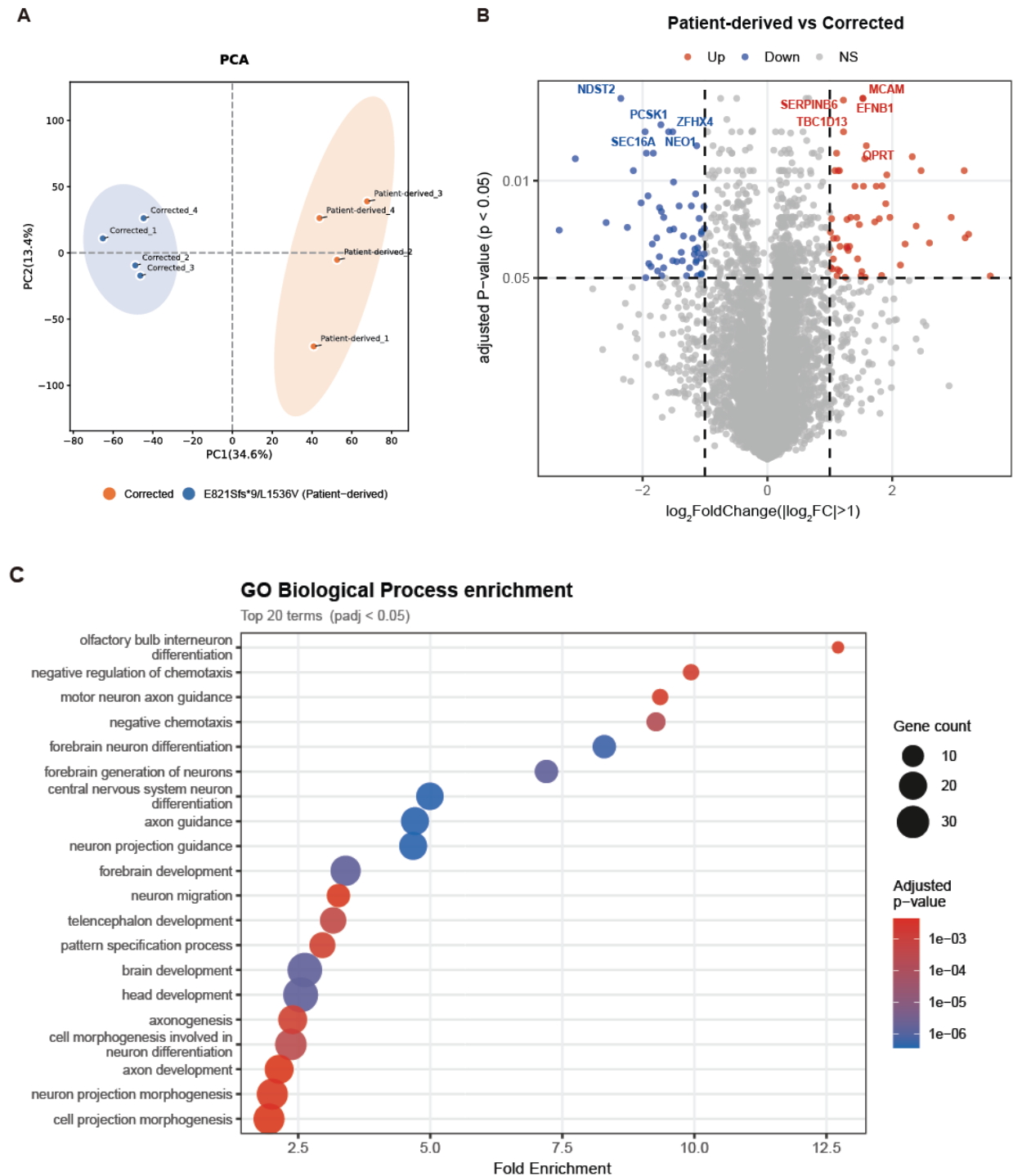
Figure S4. SEC16A variants lead to abnormal firing activity in the patient iPSC-derived neurons



(A) Representative whole-cell patch-clamp recordings from iPSC neurons at DIV38 (Day In Vitro). Patch electrode placement is shown, with the recorded soma outlined by a white dashed circle. Spontaneous action potential traces indicate that both lines are electrophysiologically active at this stage.

(B) Representative multi-electrode array recordings from SEC16A^{E821Sfs*9/L1536V} and SEC16A^{corrected} iPSC neuron cultures at DIV38 show spontaneous network activity across individual electrodes, with spike events marked by red dots. Colored traces are shown at higher magnification in Figure 5C.

Figure S5. Proteomic analyses show reduced SEC16A levels in patient iPSC-derived neurons

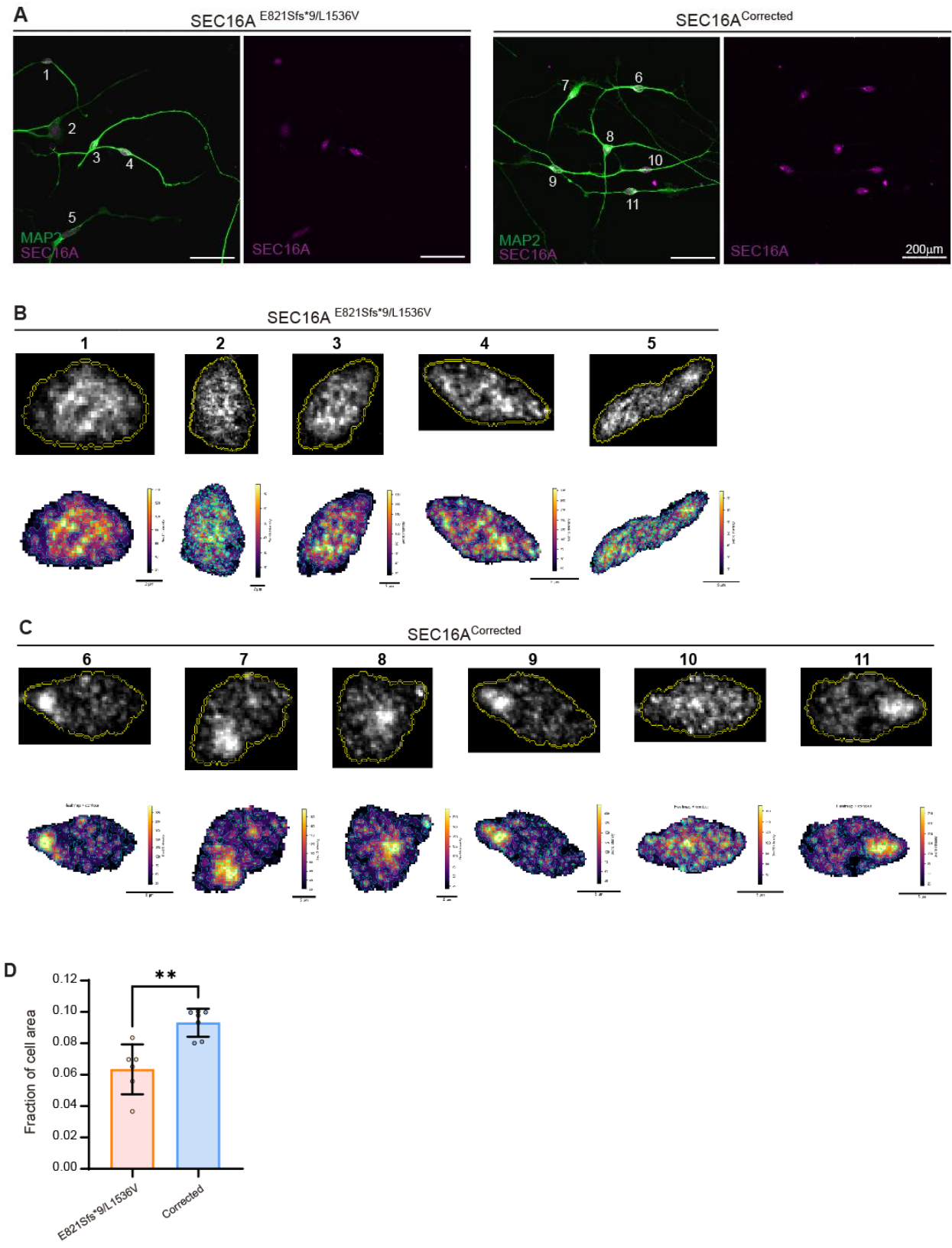


(A) Principal component analysis (PCA) of proteomic data. PCA shows clustering of SEC16A^{E821Sfs*9/L1536V} (Patient-derived, orange, n=4) and SEC16A^{corrected} (blue, n=4) iPSC-derived neurons based on global protein expression profiles. PC1 and PC2 account for 34.6% and 13.4% of the total variance, respectively. The clear separation between groups indicates distinct proteomic signatures.

(B) Volcano plot of differentially expressed proteins in SEC16A^{E821Sfs*9/L1536V} versus SEC16A^{Corrected} neurons. The plot displays log₂ fold change (x-axis) versus statistical significance (y-axis). Significantly upregulated proteins (red, log₂FC > 1 and p-value < 0.05) and some significantly downregulated proteins (blue, log₂FC < -1 and p-value < 0.05) are highlighted.

(C) Gene Ontology (GO) Biological Process enrichment analysis of differentially expressed proteins in SEC16A^{E821Sfs*9/L1536V} neurons. The dot plot shows the top 20 significantly enriched GO Biological Process terms (adjusted p value < 0.05), ranked by fold enrichment on the x axis. Circle size represents the number of genes associated with each term, and circle color indicates the adjusted p value (FDR), with red corresponding to more significant enrichment.

Figure S6. SEC16A protein subcellular distribution is altered in patient iPSC-derived neurons

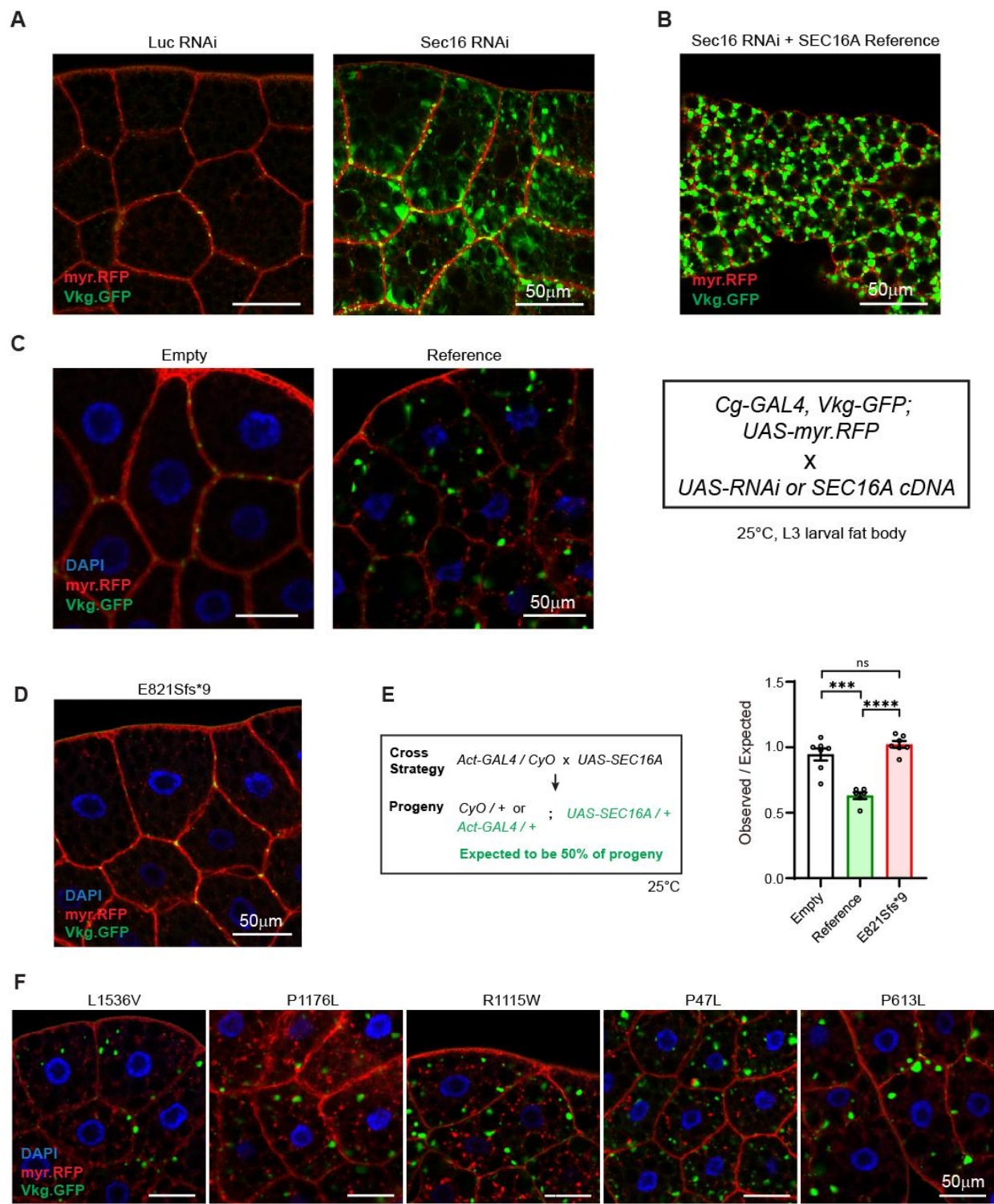


(A) Representative confocal images of iPSC-derived neurons stained for SEC16A (magenta) and MAP2 (green), with MAP2 marking the neuronal outline. Scale bars, 200 μ m.

(B-C) Neuronal somata were segmented from confocal images containing DAPI, MAP2, and SEC16A channels and analyzed in Python. Neuronal nuclei were identified from the DAPI signal and classified as neuronal when their perinuclear region contacted the MAP2-positive mask. Soma regions were then defined from the SEC16A signal following denoising, background subtraction, thresholding, and morphological filtering, and only SEC16A-positive connected components overlapping neuronal nuclei were retained for analysis. For visualization, each soma was cropped and rendered as a false-color SEC16A intensity heat map for SEC16A^{E821Sfs*9/L1536V} (B) and SEC16A^{corrected} (C) iPSC neurons (analysis details are described in Supplemental Methods). Numbered cells in (A) correspond to the soma crops shown in (B) and (C); cells 1-5 are from SEC16A^{E821Sfs*9/L1536V} neurons and cells 6-11 are from SEC16A^{corrected} neurons. Scale bars, 2 μ m for cells 1, 2, 3, 7, and 8; 5 μ m for cells 4, 5, 6, 9,10, and 11.

(D) Quantification of SEC16A distribution was performed by thresholding the SEC16A signal within each soma at the 90th percentile of the within-soma intensity distribution and expressing the area occupied by this highest-intensity signal as a fraction of the total soma area. Values were averaged across cells within each image (2-14 cells per image), and each dot represents one image. Group comparisons were performed using these image-level values. Sample size n=6 images for SEC16A^{E821Sfs*9/L1536V} neurons and n=7 images for SEC16A^{Corrected} neurons. Welch's t test, **p<0.01, mean $\pm$ SD.

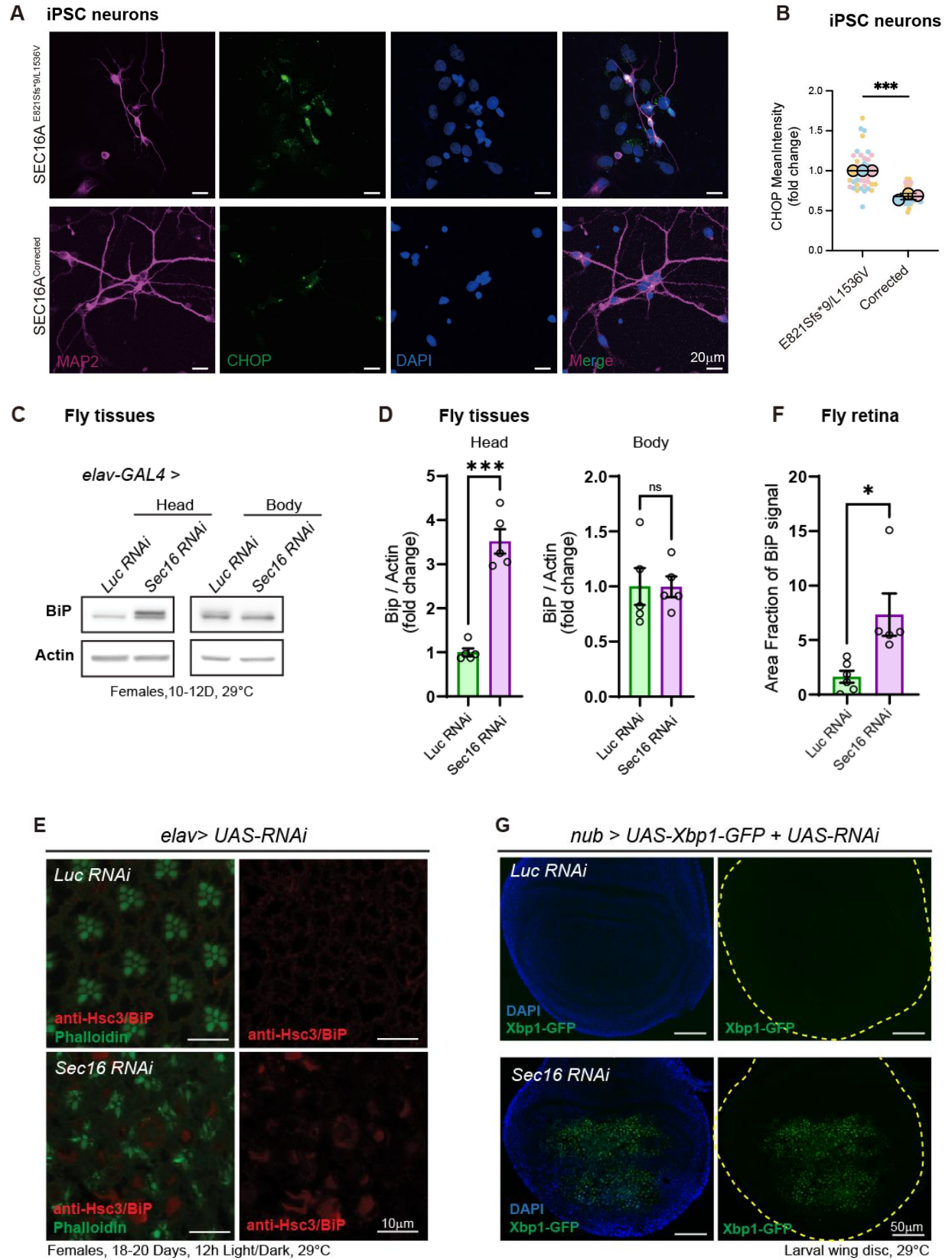
Figure S7. Proper *Sec16/SEC16A* dosage is required for secretion of Collagen IV



(A-D, F) Representative images of larval fat body cells expressing UAS constructs driven by *Cg-GAL4*. In control samples (*UAS-Luc RNAi* and *UAS-Empty*), Collagen IV/Vkg.GFP (green) is efficiently secreted and shows minimal intracellular accumulation. *Sec16* knockdown caused intracellular accumulation of Collagen IV (A), and this phenotype was not rescued by co-expression of *SEC16A* reference cDNA (B). Overexpression of *SEC16A* reference cDNA also induces intracellular accumulation of Collagen IV (C), whereas the *SEC16A* p.(E821Sfs*9) variant does not (D). Additional *SEC16A* missense variants are shown in (F). Plasma membranes are labeled with myr.RFP (red), and nuclei with DAPI (blue, in C, D and F). At least three larvae per genotype were dissected for imaging. Scale bars, 50µm.

(E) Ubiquitous overexpression of *SEC16A* reference cDNA is toxic and significantly reduces the eclosion rate, whereas *SEC16A* p.(E821Sfs*9) variant does not. Heterozygous *Act-GAL4/CyO* flies were crossed to *UAS-SEC16A* transgenic flies; based on Mendelian inheritance, 50% of progeny are expected to carry both the GAL4 driver and the UAS transgene. The graph shows the Observed/Expected fraction of *Act-GAL4>UAS-SEC16A* progeny raised at 25°C. Each dot represents one independent cross, each containing more than 100 flies collected for analysis. Sample sizes n=7 for Empty, n=6 for Reference, and n=7 for E821Sfs*9. Welch's t test, ****p<0.0001, ***p<0.001, ns: not significant, mean ± SEM.

Figure S8. Patient iPSC-derived neurons exhibit elevated ER stress



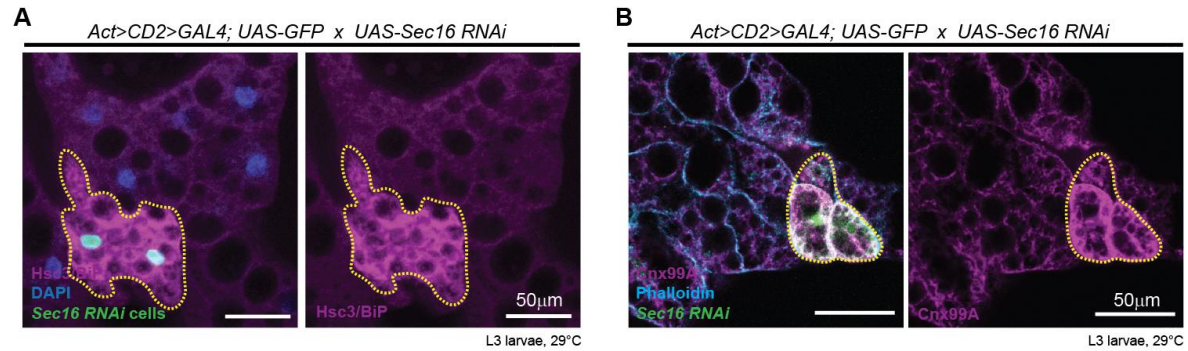
(A-B) Representative confocal images of iPSC-derived neurons stained for CHOP (green) and MAP2 (magenta). Nuclei are labeled with DAPI (blue). SEC16A^{E821Sfs*9/L1536V} neurons show increased CHOP signal compared with SEC16A^{corrected} neurons. Scale bars, 20µm. For quantification, small dots represent individual cells and large dots represent the mean of each experimental replicate, with colors indicating the three replicates. Values are normalized to the mean of SEC16A^{E821Sfs*9/L1536V} neurons within each replicate. The total sample size n=42 for SEC16A^{E821Sfs*9/L1536V} neurons and n=47 for SEC16A^{Corrected} neurons. Statistical significance was determined on raw values using Welch's t test, ***p<0.0001. Data are presented as mean ± SEM of the three experimental means.

(C-D) Immunoblot analysis of BiP in lysates from adult fly heads and bodies with neuron-specific *Sec16* knockdown. BiP was probed first, then the membrane was stripped and reprobed for Actin; BiP signals were normalized to Actin. The increase in BiP is obvious in the head but not in the body as less than 5% of the cells in body are neurons. Each dot represents one biological replicate, Sample size n=5 for each genotype. Welch's t test, ***p<0.001, ns: not significant, mean ± SEM.

(E-F) Confocal images of adult fly retina stained for BiP (red) and phalloidin-labeled rhabdomeres (green), with quantification of BiP signal (fraction of area above intensity threshold; see Supplementary Methods). Neuron-specific *Sec16* knockdown (*elav>Sec16 RNAi*) increases BiP expression and disrupts rhabdomere morphology compared with controls (*elav>Luc RNAi*), indicating photoreceptor degeneration. Each dot represents one retina (one image per retina). Sample sizes n=6 images for *Luc RNAi* and n=5 images for *Sec16 RNAi*. Welch's t test, *p<0.05, mean ± SEM. Scale bars, 10µm.

(G) Representative confocal images of larval wing discs expressing the Xbp1-GFP ER stress reporter (green), together with either *UAS-Sec16 RNAi* or *UAS-Luciferase RNAi* (control RNAi). Knockdown of *Sec16* induces Xbp1-GFP expression, consistent with non-canonical *Xbp1* splicing and activation of the IRE1-XBP1 branch of the unfolded protein response. Nuclei are labeled with DAPI (blue). At least three larvae per genotype were dissected for imaging. Scale bars, 50µm

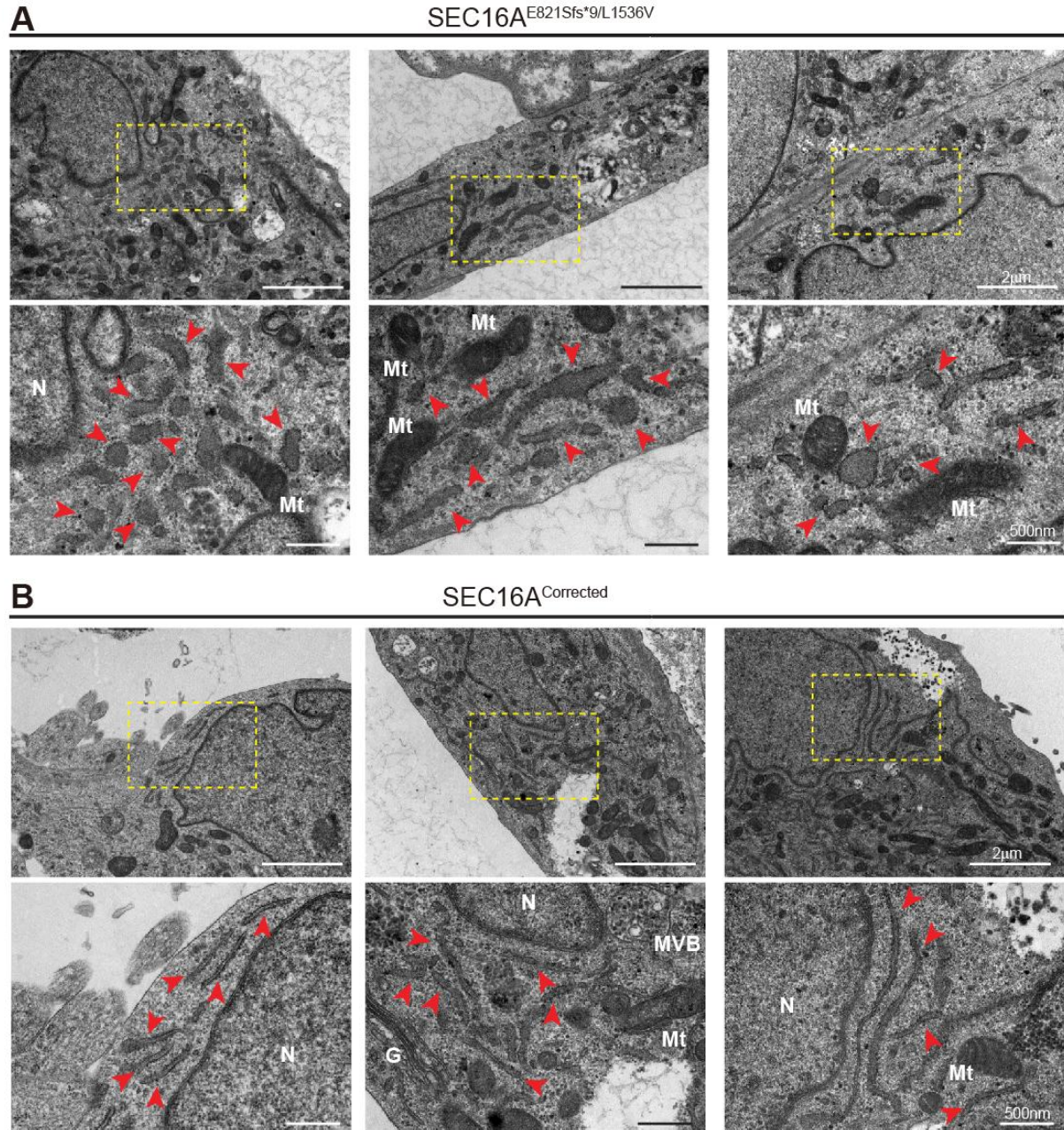
Figure S9. Cell-autonomous upregulation of ER chaperones in *Sec16 RNAi* clones



(A) Random Flp-out clones expressing *Sec16 RNAi* were generated in fly fat body and marked by GFP. Hsc3/BiP staining (magenta) is increased in *Sec16 RNAi*-positive cells (green) compared with surrounding non-expressing cells, indicating that BiP upregulation is primarily cell-autonomous. Nucleus labeled by DAPI (blue). At least three animals per genotype were dissected for imaging. Scale bars, 50 µm.

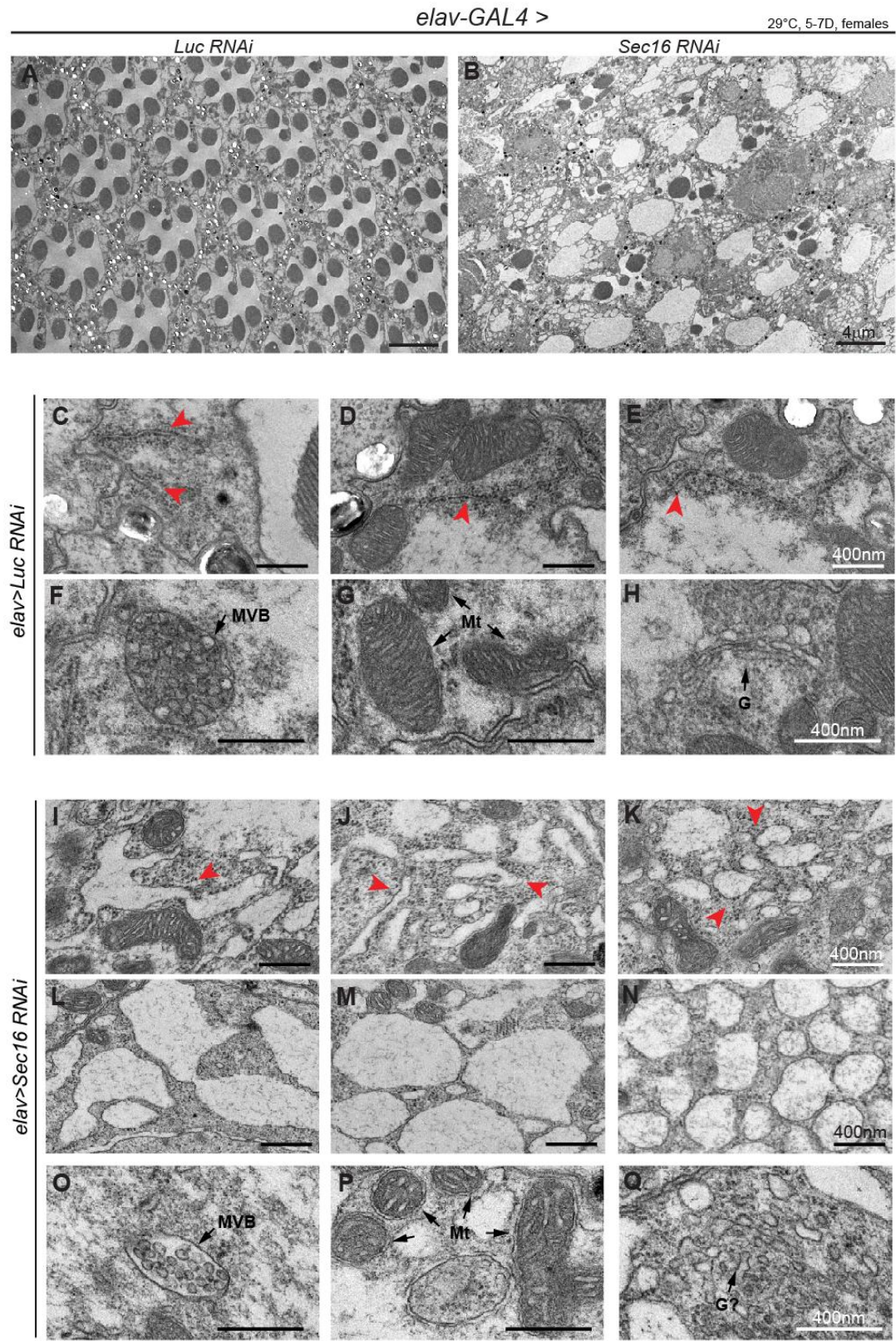
(B) Analysis of the ER luminal chaperone Cnx99A in random Flp-out *Sec16 RNAi* fat body clones. *Sec16 RNAi*-expressing cells are marked by GFP (green). Cnx99A staining (magenta) is elevated in the RNAi-positive clone compared with surrounding cells. Cell boundaries are visualized by phalloidin staining (cyan). At least three animals per genotype were dissected for imaging. Scale bars, 50 µm.

Figure S10. ER morphological defects in patient iPSC-derived neurons



Representative TEM images of iPSC-derived neurons. Rough ERs identified by ribosome attachment are indicated by red arrowheads. Higher-magnification views of the regions outlined by yellow dashed rectangles are shown. SEC16A^{E821Sfs*9/L1536V} neurons (A) exhibit increased ER volumes compared with SEC16A^{corrected} neurons (B). Mt, mitochondria; G, Golgi apparatus; N, nucleus; MVB, multivesicular body.

Figure S11. ER morphological defects in fly photoreceptors with *Sec16* knockdown in neurons



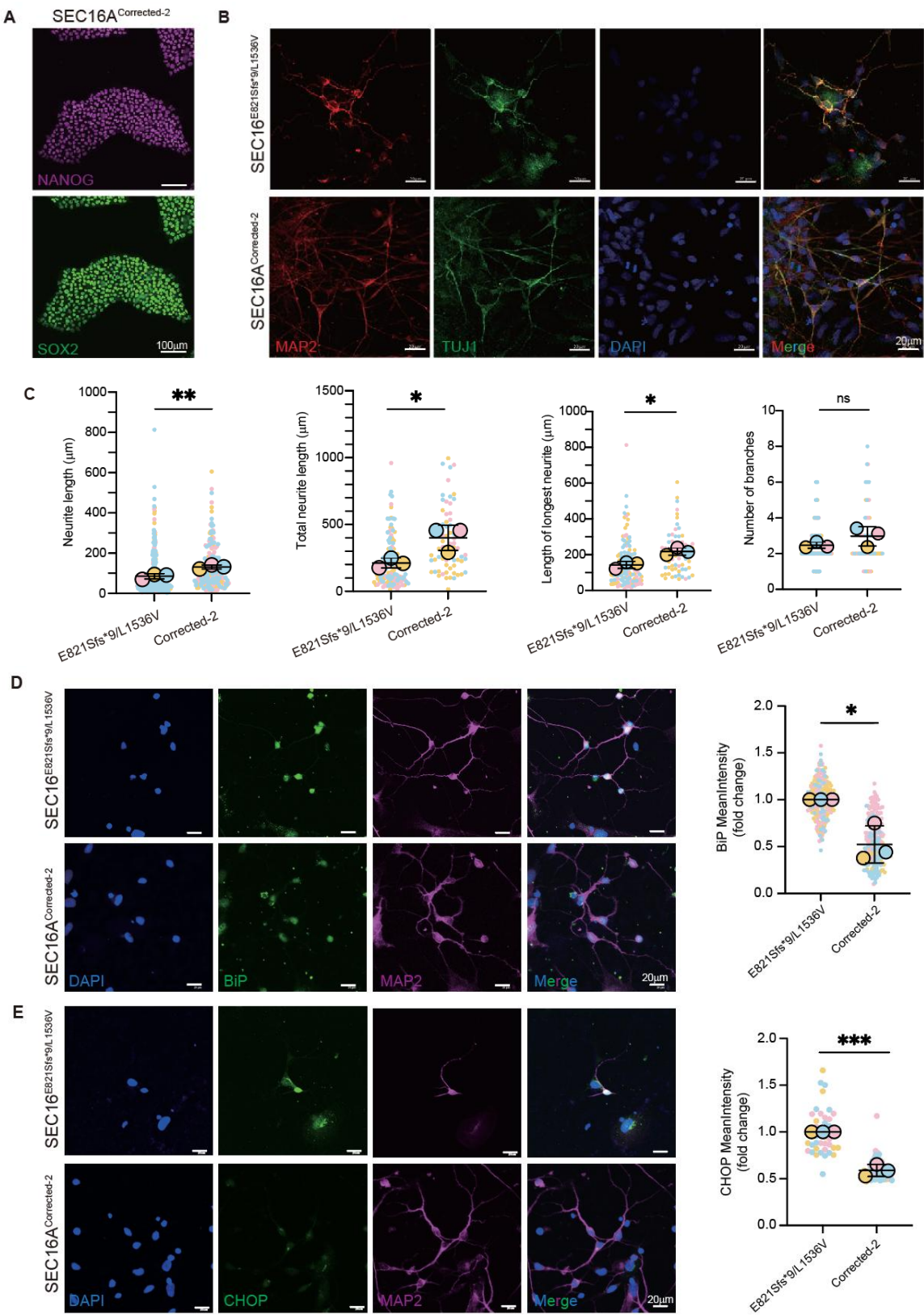
Representative TEM images of fly retina. Retinas from three flies per genotype were examined.

(A and B) Overall views of fly photoreceptors. In control *elav>Luc RNAi* flies, photoreceptor ultrastructure is well organized, whereas *elav>Sec16 RNAi* flies show severe loss of photoreceptors and numerous morphological defects, including loss of rhabdomeres and extensive vacuolization. Scale bars, 4μm.

(C-H) Higher-magnification views of organelles in control photoreceptors, including rough ER (C-E, red arrowheads), a multivesicular body (F), mitochondria (G), and Golgi apparatus (H). MVB, multivesicular body; Mt, mitochondria; G, Golgi. Scale bars, 400nm.

(I-Q) Higher-magnification views of organelles in *elav>Sec16 RNAi* photoreceptors, including putative rough ER with dilated lumens (I-K, red arrowheads), large vacuolar structures of unclear identity (L-N), a multivesicular body (O), mitochondria (P), and putative Golgi apparatus (Q). MVB, multivesicular body; Mt, mitochondria; G, Golgi. Membrane organization of intracellular organelles appears abnormal in *Sec16 RNAi* photoreceptors. Scale bars, 400nm.

Figure S12. An independent corrected line of iPSC neurons validates the rescue of morphological abnormalities and ER stress phenotypes



(A) Validation of pluripotency in an independent SEC16A^{Corrected-2} iPSC line.

Immunofluorescence staining shows expression of the pluripotency markers NANOG (magenta) and SOX2 (green). Scale bars, 100µm.

(B) Neuronal differentiation outcomes in SEC16A^{E821Sfs*9/L1536V} and SEC16A^{Corrected-2} iPSC lines.

The SEC16A^{Corrected-2} line shows rescue of the abnormal neuronal morphology and impaired neurite outgrowth phenotypes. Immunofluorescence staining for neuronal markers MAP2 (red) and TUJ1 (green). Nuclei are labeled with DAPI (blue). Scale bars, 20µm.

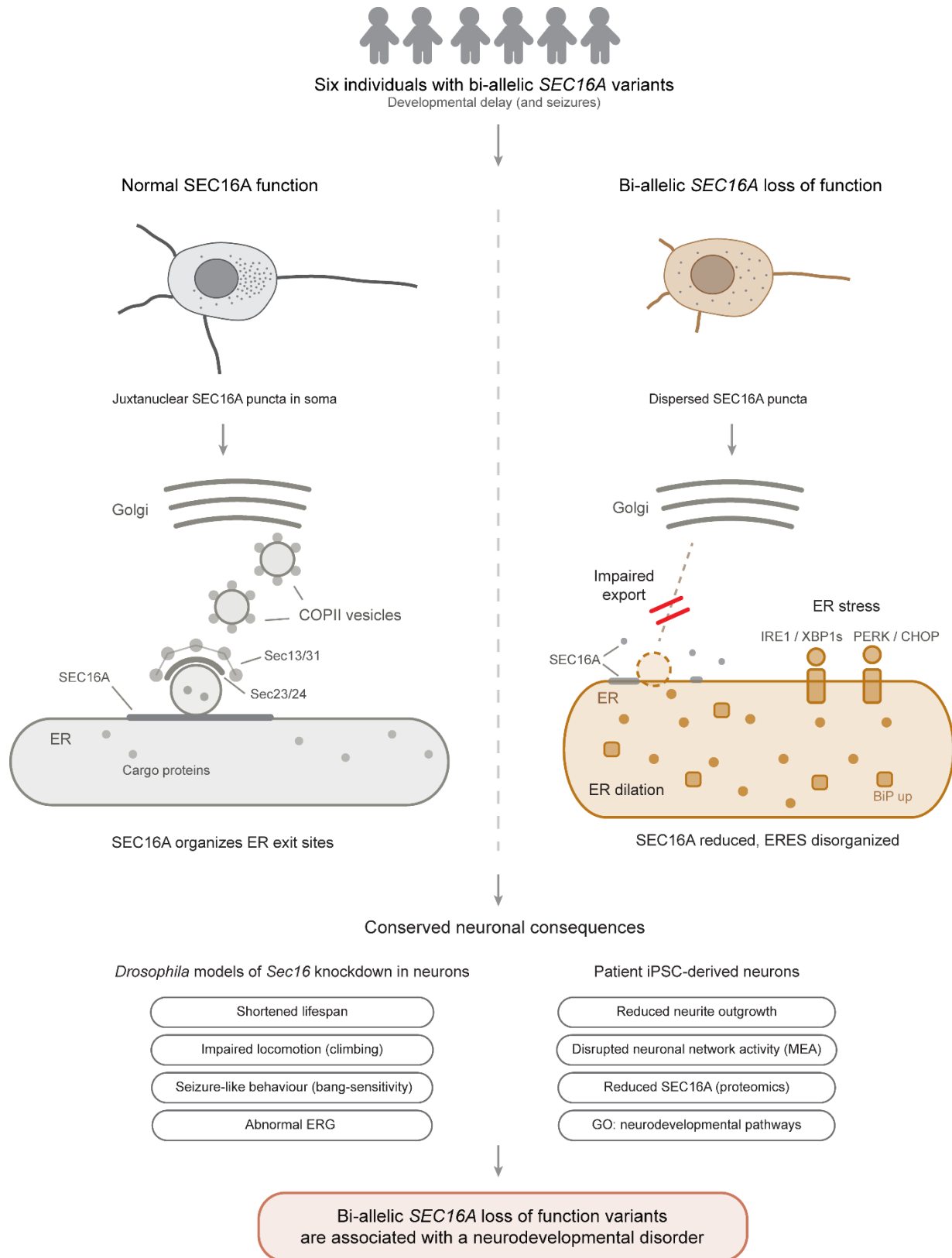
(C) Quantification of neurite morphology in SEC16A^{E821Sfs*9/L1536V} and SEC16A^{Corrected-2} iPSC neurons. Small dots represent individual neurites (neurite length) or individual cells (total neurite length, length of the longest neurite, and number of branches), and large dots represent the mean of each experimental replicate, with colors indicating the three replicates. Sample size: Neurite length, n=348 and 189; Total neurite length, n=133 and 64; Length of the longest neurite, n=131 and 64; Number of branches, n=138 and 65; for SEC16A^{E821Sfs*9/L1536V} and SEC16A^{Corrected-2} neurons respectively. Statistical significance was determined on raw values using Welch's t test, **p < 0.01, *p<0.05, ns: not significant. Data are presented as mean ± SD of the three experimental means.

(D) Representative images of BiP (green) in the SEC16A^{E821Sfs*9/L1536V} and SEC16A^{Corrected-2} iPSC neurons, co-stained with neuronal marker MAP2 (magenta). Nuclei are labeled with DAPI (blue). Scale bars, 20µm. For quantification, small dots represent individual cells and large dots represent the mean of each experimental replicate, with colors indicating the three replicates. Sample size n=40 for SEC16A^{E821Sfs*9/L1536V} and n=70 for SEC16A^{Corrected-2} neurons. Statistical significance was determined on raw values using Welch's t test, ***p<0.001. Data are presented

as mean $\pm$ SD of the three experimental means.

(E) Representative images of CHOP (green) in the SEC16A^{E821Sfs*9/L1536V} and SEC16A^{Corrected-2} iPSC neurons, co-stained with neuronal marker MAP2 (magenta). Nuclei are labeled with DAPI (blue). Scale bars, 20 μ m. For quantification, small dots represent individual cells and large dots represent the mean of each experimental replicate, with colors indicating the three replicates. Sample size n=42 for SEC16A^{E821Sfs*9/L1536V} and n=83 for SEC16A^{Corrected-2} neurons. Statistical significance was determined on raw values using Welch's t test, *p<0.05. Data are presented as mean $\pm$ SD of the three experimental means.

Figure S13. Working model of *SEC16A*/*Sec16* loss of function in neurons



SEC16A localizes to juxtannuclear punctae and organizes ER exit sites (ERES) to support COPII-mediated ER export. Bi-allelic SEC16A variants reduce SEC16A levels, disperse its puncta and disorganize ERES, impairing ER export and causing ER dilation and ER stress with activation of the unfolded protein response and increased BiP levels. These defects are accompanied by conserved neuronal phenotypes in *Drosophila* and patient iPSC-derived neurons, supporting an association between bi-allelic *SEC16A* loss-of-function variants and a neurodevelopmental disorder.

Supplemental Methods

Generation of the *Sec16*^{KO} allele

Two gRNAs targeting the start and end of the first common exon of *Sec16* (gRNA1, gagctggttccgctggagccggg; gRNA2, cacgctcctcgggaggacgacgg) were cloned into the pCFD5-white vector (Addgene #112645) and integrated into the VK33 docking site by ϕ C31-mediated transgenesis (82), using *w*⁺ as a red-eye selection marker. Transgenic flies were crossed to *nos-Cas9* flies, and F1 females carrying both gRNAs and Cas9 were crossed to flies carrying an X chromosome balancer. Approximately 30 stocks were established from individual F2 females. Stocks showing homozygous lethality were screened by PCR across the target region using genomic DNA from heterozygotes, followed by Sanger sequencing and Inference of CRISPR Edits (ICE) analysis (v3.0, Synthego; accessed 9/23/2021). One allele carrying a 7 bp deletion near gRNA1 and a 3 bp deletion near gRNA2, which introduces a premature stop codon, was designated *Sec16*^{KO}.

Generation of *Drosophila* fat body *Sec16* mutant clones

To generate Flp-FRT recombination *Sec16* mutant clones, females heterozygous for *Sec16* alleles with FRT sites (*y w Sec16*^A *FRT19A/FM7C Kr>GFP* or *y w Sec16*^{KO} *FRT19A/FM7C Kr>GFP*) were crossed to males carrying Flp recombinase (*Ubi-mRFP.nls hsFlp FRT19A/Y*). Embryos (12 ± 12 hours) underwent heat shock (37°C for 1 hour) to induce Flp recombinase expression, catalyzing mitotic recombination between *FRT19A* sites. This produced daughter cells homozygous for either the *Sec16* mutant allele (unmarked) or wild-type alleles (marked by *Ubi-mRFP.nls*). Flies were maintained at 25°C until third-instar larval stage (L3). Larvae with the genotype of *Sec16*^{allele} *FRT19A / Ubi-mRFP.nls, hsFlp, FRT19A* were selected by absence of *Kr>GFP* and dissected for analysis.

Generation of patient-derived and variant-corrected iPSC lines

The SEC16A^{E821Sfs*9/L1536V} hiPSC line was generated from peripheral blood mononuclear cells (PBMCs) isolated from Individual 1 by Gemple (Shanghai, China), and pluripotency was confirmed. To correct the c.2460delT variant, two guide RNAs targeting the 5'UTR and intron 3-4 flanking exon 3 were cloned into the pX458 vector (Addgene #48138) and co-nucleofected with a homologous donor containing corrected *SEC16A* exon 3 (c.2460T) fused to a CAG promoter-driven eGFP expression cassette flanked by loxP sites (SEC16A-exon3-CAG-eGFP-loxP), using the Neon system (Invitrogen). GFP⁺ clones with successful replacement of mutant exon 3 were isolated and subsequently transfected with pCAG-Cre (Addgene #13775) to excise the eGFP cassette. GFP⁻ clones were selected and confirmed by Sanger sequencing. The c.4606C>G p.(L1536V) variant was then corrected in this line using a similar locus-specific strategy. Two independently corrected clones (SEC16A^{Corrected} and SEC16A^{Corrected-2}) were isolated. All iPSC lines were validated by Sanger sequencing and shown to retain pluripotency by immunofluorescence and qPCR for pluripotency markers.

Alkaline Phosphatase (AP) Staining

Cells were cultured in a 24-well plate until they reached approximately 60-80% confluency. Then the culture medium was aspirated, and the cells were gently washed 1-2 times with phosphate-buffered saline (PBS). The cells were fixed in 4% Paraformaldehyde in PBS for 2-5 minutes at room temperature (RT). After fixation, the fixative was aspirated, and the cells were washed thoroughly 2-3 times with PBS/DPBS (5 minutes per wash) to eliminate all fixative traces. Following the final wash and aspiration, freshly prepared AP Staining Solution

(Beyotime, #C3206) was added to fully cover the cells. Samples were incubated in dark at RT, protecting the reaction from light. Color development was monitored every 5-15 minutes. When optimal staining intensity was observed, the staining solution was aspirated, and the cells were washed 2-3 times with PBS to halt the reaction. Finally, the cells were covered with PBS and immediately examined using bright-field microscopy.

2D neuronal differentiation

iPSCs were dissociated into single cells using TrypLE and plated on Matrigel-coated 12-well plates at 70,000 cells/well in mTeSR medium supplemented with 10 μ M Y27632. At ~95% confluency, neural induction was initiated by switching to a 1:1 mix of DMEM/F12 (Gibco) and Neurobasal (Gibco), supplemented with 1 $\times$ GlutaMAX (Gibco #35050061), 2 mM L-glutamine (Gibco #A2916801), 0.5 $\times$ NEAA (Gibco #11140050), 0.5 $\times$ N2, 5 μ g/mL insulin (Sigma #19278), 0.5 $\times$ B27 (Gibco #17504044), 1 μ M dorsomorphin (Selleck #S7840), and 10 μ M SB431542 (Selleck #S1067). Cells were maintained in this medium for 12 days to form neural epithelia, then dissociated with 0.5 mM EDTA and Accutase (STEMCELL Technologies #07920), split at a 1:3 ratio, and replated on poly-L-ornithine/laminin-coated plates in neural induction medium. The medium was then switched to IMP+A medium with daily changes until the neurons matured 45 days later. Fully differentiated neurons were subjected to downstream assays.

Patch Clamp Electrophysiology

Neurons were seeded on poly-L-ornithine and laminin-coated coverslips. Extracellular (bath) solution matching physiological ionic conditions (e.g., 140mM NaCl, 5mM KCl, 2mM CaCl₂, 1mM MgCl₂, 10mM HEPES, pH 7.4) and intracellular (pipette) solution (e.g., 140mM KCl, 2mM MgCl₂, 10mM EGTA, 10mM HEPES, pH 7.2) were filtered (0.22μm) and oxygenated. Cells plated on glass coverslips were transferred to a recording chamber perfused with extracellular solution at 22-37°C. Borosilicate glass pipettes (1.5mm OD) were pulled (tip diameter: 0.5-5μm), fire-polished, filled with intracellular solution (avoiding bubbles), mounted in a holder with Ag/AgCl wire contact, and grounded via a bath electrode. Pipette offset potential was zeroed, and resistance (typically 36MΩ for whole cell) measured before approaching the cell surface under slight positive pressure to prevent clogging. Upon contact, positive pressure was released, and gentle suction was applied to establish a gigaseal (>1GΩ), monitored via current response to -5 mV test pulses. For whole-cell configuration, the membrane patch was ruptured through strong suction or a voltage "zap", and successful transition to the whole-cell mode was confirmed by the appearance of slow capacitance transients. Following membrane rupture, capacitance and series resistance were compensated by 70-80% to optimize recording conditions. All recordings were performed in current-clamp configuration. Current protocols (including step pulses and ramps) were applied during acquisition, with signals filtered at 2-10 kHz and sampled at ≥ 20 kHz. Pharmacological agents were bath-perfused to modulate ion channels regulating action potential properties. Recordings were terminated by slow pipette withdrawal, discarding data if the seal resistance dropped below 1GΩ. Critical considerations included vibration control (air tables), solution grounding, series resistance maintenance (<20MΩ), and rigorous contamination prevention.

High-Content Imaging (HCI)

HCI was used to acquire images for neurite morphology analysis. Neurons derived from SEC16A^{E821Sfs*9/L1536V} and SEC16A^{Corrected} iPSCs were seeded onto poly-D-lysine and laminin-coated 24-well plates. A density of 5,000-10,000 cells per well was used to ensure sufficient cell growth and a suitable density for synaptic analysis without overcrowding. The plates were incubated in a humidified incubator with 5% CO₂ at 37°C and the cells were maintained in neuron-specific culture media, with media changes every day. For staining, neurons were fixed with 4% paraformaldehyde in PBS for 15-20 min, then washed with PBS. Cells were permeabilized with 0.1% Triton X-100 for 10 min, and then washed with PBS 3 times in PBS (5 min each). Cells were subsequently incubated with primary antibody against anti-Rabbit TUJ1 (Abcam, #ab18207) at 4°C overnight, followed by Alexa Fluor-conjugated secondary antibodies. Nuclei were labeled with DAPI and the cells were washed again before imaging. HCI equipment set up: 405 nm for DAPI excitation, 488 nm for Alexa Fluor 488 with an exposure time of 500ms. For data analysis, images were transferred to ImageJ. Cell bodies and synaptic puncta were segmented, and synaptic numbers were quantified. Length measurements were performed, and cellular morphology was analyzed. Statistical analyses are indicated for each experiment.

Transmission Electron Microscopy (TEM) of iPSC-derived neurons

Cells were cultured in 3 cm glass-bottom dishes. The supernatant was aspirated and cells were fixed overnight at 4°C in the dark using an electron microscopy (EM) fixative solution. The samples were then washed three times in PBS (10min each) on a shaker, followed by fixation with 1% OsO₄ at RT for 30 min (prepared at a 1:1 ratio with water, adding water first and then

OsO₄). The samples were then rinsed three times with water (10min each) and dehydrated through a graded ethanol series (30%, 40%, 70%, 80%, 95%, and 100%, 10min per step). To ensure complete dehydration, the samples were treated twice with anhydrous sodium sulfate (Na₂SO₄, 15min each). Subsequently, resin infiltration was performed, and the samples were left overnight. The embedded samples were carefully dissolved from the substrate using hydrofluoric acid and prepared for sectioning. After embedding, ultrathin sections were cut following standard protocols and stained with uranyl acetate and lead citrate. Finally, the samples were imaged and analyzed using a transmission electron microscope.

Western Blot

Western blotting for *Drosophila* samples was performed as previously described with modifications (2). Fly adult heads and bodies were separated and homogenized on ice in RIPA buffer (ThermoFisherScientific, #89900) supplemented with EDTA and protease inhibitor cocktail, using 100µl buffer per 10 heads or 150µl buffer per 10 bodies. Body lysates were further diluted 1:2 before use. Lysates were mixed with Laemmli buffer (Bio-Rad, #1610747) and heated, then separated by SDS-PAGE on 4%-20% Mini-PROTEAN TGX precast gels (Bio-Rad, #4561094), and transferred to PVDF membranes (Millipore). Membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with horseradish peroxidase (HRP)-or IRDye-conjugated secondary antibodies. Signals were detected using Supersignal chemiluminescent substrate (ThermoFisherScientific, #34580) for HRP-conjugated secondary antibodies and imaged with a Bio-Rad Chemidoc MP Imaging System. Primary antibodies were rabbit anti-GRP78 (BiP) (1:2000, StressMarq Biosciences, #SPC-180) and mouse anti-Actin (1:2000, EMD-Millipore, #MAB1501R). Secondary antibodies were goat anti-Rabbit HRP

(1:5000, Abcam, #ab6721) and donkey anti-Mouse IRDye 680LT (1:10000, LI-COR Biosciences, #926-68022).

Quantitative RT PCR (qRT-PCR)

For fly tissues, qPCR was performed following the protocol previously described (3), with minor modifications. Total RNA was reverse transcribed using All-In-One 5X RT MasterMix (abm, #G592). PCR amplification was carried out using iTaq Universal SYBR Green Master Mix (Bio-Rad, #1725120) on a Bio-Rad C1000 Touch Thermal Cycler.

For human cells, total RNA was isolated from all samples using the RNeasy Mini Kit (Qiagen). Complementary DNA (cDNA) was synthesized from the extracted RNA using SuperScript III Reverse Transcriptase (Invitrogen, #18080044) with random hexamer primers (Invitrogen). Quantitative PCR was subsequently performed using GoTaq qPCR Master Mix (Promega) on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Primers are listed in Supplemental Table S3.

Analysis of SEC16A subcellular distribution in iPSC-derived neurons

Confocal images (DAPI/DNA, Alexa Fluor 488/MAP2, Cy5/SEC16A; 0.207 $\mu\text{m}/\text{pixel}$) were analyzed in Python 3.11.15 using NumPy 2.4.6, SciPy 1.17.1, scikit-image 0.26.0, tifffile 2026.3.3 and Matplotlib 3.10.9. The SEC16A channel was then denoised with a small Gaussian filter ($\sigma = 0.10 \mu\text{m}$) and background-subtracted using 5th-percentile intensity. Neuronal cell-body (soma) regions were defined in three steps: (i) DAPI stained nuclei were segmented with an

adaptive Otsu threshold (`skimage.filters.rank.otsu`, 51-px window) followed by hole-filling, removal of sub-nuclear debris and a shape-based (distance-transform) watershed to separate touching nuclei; (ii) a nucleus was classified as neuronal when its perinuclear region (the nucleus dilated by $0.6\ \mu\text{m}$) contacted the MAP2-positive mask (MAP2 thresholded at $0.5 \times$ its Otsu value) by ≥ 5 px, so that nuclei embedded in the MAP2 network were retained; and (iii) because SEC16A diffusely fills the cell body, the soma itself was taken directly from the SEC16A signal. SEC16A was thresholded at $0.4 \times$ its Otsu value, opened with a $1.0\ \mu\text{m}$ disk to trim thin processes and inter-cell bridges, and small fragments ($< 30\ \mu\text{m}^2$) were removed. Each SEC16A connected component was kept whole as one soma only if it overlapped a neuronal nucleus, yielding 82 somata (Patient-derived: 35; Corrected: 47). Within each soma, the intracellular SEC16A distribution pattern was quantified by a high-intensity aggregate descriptor obtained by lightly smoothing SEC16A ($\sigma = 0.5\ \mu\text{m}$), thresholding at the 90th percentile of the within-soma intensity and labelling its 4-connected components (`scipy.ndimage.label`), from which we took the area of the largest component as a fraction of the soma and the number of high-intensity components. As thresholding at the 90th percentile fixes the high-intensity area at $\approx 10\%$ of the soma for every cell, these measures capture whether the brightest $\sim 10\%$ of pixels coalesces into a single connected region or fragment into many. For visualization, each soma was cropped to its bounding box and its SEC16A signal rendered as a false-colour heat map (Matplotlib `inferno`). This was overlaid with eight iso-intensity contour lines evenly spaced between the 20th and 98th within-soma intensity percentiles, displaying each cell as a topographic map of its SEC16A distribution. Group differences (Patient-derived vs Corrected) were assessed per image (each field mean as one observation; Patient-derived $n=6$, Corrected $n=6$).

Fly retina staining

For fly retina dissection, fly heads were dissected in PBS and fixed in 4% formaldehyde in PBS overnight at 4 °C. On the following day, retinæ were dissected in PBS and fixed for an additional 30min in 4% formaldehyde/PBS. Samples were then washed with PBS containing 0.3% Triton X-100 (PBT). To remove eye pigments, retinæ were incubated in 10 mg/ml sodium borohydride (Sigma-Aldrich) in PBS for 20min at RT. Retinæ were subsequently blocked in 10% normal goat serum (NGS) in PBT for 1h at RT and incubated with primary antibodies overnight at 4 °C. On the third day, retinæ were washed three times with 0.3% PBT and incubated with secondary antibodies overnight at 4 °C. After three additional washes in 0.3% PBT, samples were prepared for imaging. The following antibodies were used: rat anti-Elav (1:500; Developmental Studies Hybridoma Bank, #7E8A10), mouse anti-BiP (1:1000; gift from Dr. Hyung Don Ryoo), rabbit anti-mCherry (1:100, GeneTex, #GTX128508), Alexa Fluor 488-conjugated phalloidin (1:250, Invitrogen, #A12379), anti-rat Alexa Fluor 633 (1:400; Jackson ImmunoResearch), anti-rabbit Alexa Fluor 568 (1:400; Jackson ImmunoResearch), and anti-mouse Alexa Fluor 568 (1:400; Jackson ImmunoResearch). Specimens were mounted using Vectashield mounting medium with DAPI (Vector Laboratories). Images were acquired using a STELLARIS confocal microscope (Leica) and processed with ImageJ. For quantification of the BiP signal, pixel intensity distributions were first calculated for all images in the control group. The 99th percentile (P99) value of the intensity histogram was extracted from each control image, and the mean P99 value across all control images was defined as a fixed threshold (T) for signal segmentation. This threshold was applied uniformly to all images using Fiji, and pixels with an intensity $\geq T$ were classified as positive. The percentage of the image area occupied by positive pixels was calculated and used as the quantitative metric.

LC-MS/MS analysis

Two sample groups (SEC16A^{E821Sfs*9/L1536V} and SEC16A^{Corrected}) were each analyzed in four replicates (n=4 per group, 8 samples in total). Peptides were separated on a Vanquish Neo UHPLC system at 2.5µL/min using a 6.9-min gradient of 4-25% B (0-4min), 25-35% B (4-5.8min) and a wash to 99% B (5.8-6.9min), with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). Eluting peptides were analyzed on a Thermo Scientific Orbitrap Astral mass spectrometer in positive, peptide mode. MS1 spectra were acquired in the Orbitrap at 240,000 resolution over 380-980 *m/z* (normalized AGC 500%, maximum injection time 5ms). Data-independent acquisition (DIA) MS/MS was performed on the Astral analyzer across 380-980 *m/z* using 2 *m/z* isolation windows without overlap (300 scan events per 0.6s cycle) with higher-energy collisional dissociation at a normalized collision energy of 25%, recording fragment ions over 150-2000 *m/z* (normalized AGC 500%, maximum injection time 3ms). All spectra were acquired in profile mode.

Supplemental References

1. McCaughey, J., and Stephens, D.J. 2018. COPII-dependent ER export in animal cells: adaptation and control for diverse cargo. *Histochem Cell Biol* 150:119-131.
2. Ma, M., Ganapathi, M., Zheng, Y., Tan, K.L., Kanca, O., Bove, K.E., Quintanilla, N., Sag, S.O., Temel, S.G., LeDuc, C.A., et al. 2024. Homozygous missense variants in YKT6 result in loss of function and are associated with developmental delay, with or without severe infantile liver disease and risk for hepatocellular carcinoma. *Genet Med* 26:101125.
3. Ravenscroft, T.A., Janssens, J., Lee, P.T., Tepe, B., Marcogliese, P.C., Makhzami, S., Holmes, T.C., Aerts, S., and Bellen, H.J. 2020. Drosophila Voltage-Gated Sodium Channels Are Only Expressed in Active Neurons and Are Localized to Distal Axonal Initial Segment-like Domains. *J Neurosci* 40:7999-8024.