Supplemental Data

CHARACTERIZATION OF THE PROPERTIES OF A NOVEL MUTATION IN VAPB IN FAMILIAL ALS

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Experimental Procedures

Two step mutagenesis PCR- In the first step, an internal primer containing the targeted mutant base in the centre was used with the 5' VAPB primer, the PCR product of which was purified and used as a mega primer for the second round of PCR. In the second PCR, the mega primer was used with VAPB 3' primer to produce the full length VAPB containing correspondent nucleotide change, which were then cloned into either GFP or RFP vector as mentioned. Full length wild-type VAPB was used as template for both rounds of PCR. The sequences of internal primers used to generate T46I and P56S were ACGTGGTGCTGTAATCTTCACCTTAAAAC and GATTCCGCTGTTGGACCTCACACAGTACCT, respectively.

DNA extraction from tissue samples- DNA was extracted from the aqueous phase of TRIZOL homogenates (22) using 0.5 ml of BEB buffer (4M guanidine thiocyanate, 50 mM NaCl, 1M Tris) After centrifuging for 30 min at 12000g, the upper phase was transferred to a fresh tube and DNA was then precipitated by adding isopropanol (0.4 ml). The sample was centrifuged for 15 min at 12000g at 4°C to pellet DNA. After a 70% ethanol wash and air dry, DNA was resuspended in water for use.

Scanning electron microscopy and eye histology- Eyes were fixed in 3% glutaraldehyde, in 0.1M sodium cacodylate buffer pH 7.3, for 2-3 h. The samples were then washed 3 times with 0.1M sodium cacodylate buffer for 10 min. Specimens were then post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 45 min, prior to being washed three times in 0.1M sodium cacodylate buffer for 10 min. The samples were then dehydrated in 50%, 70%, 90% and 100% normal grade acetone for 10 min each, then two more times in acetone for 10 min. Dehydrated samples were then critical point dried, mounted on aluminium stubs, sputter coated with gold palladium, and viewed in a Hitachi 4700 FESEM. Areas of interest were photographed on black and white negative films. For sections of adult fly eyes, adult heads were fixed overnight at room temperature in 37% formaldehyde, ethanol and acetic acid (10:85:5), dehydrated in ethanol, embedded in paraffin for vertical semi-thin sections and then stained with haematoxylin-eosin.

Microscopy and morphological quantification- To acquire the 2D images for processing, the bodies were photographed using a digital camera attached to an Olympus stereomicroscope. The same magnification was set for all genotypes and so the units of measurement were all equal. Flies were rendered unconscious to limit their movement and then laid on their side atop a white background so the eyes could be easily and consistently located. For comparison between genotypes, samples were processed simultaneously and imaged using identical microscopic acquisition parameters. The experimenter was blinded to genotypes during both imaging and analysis.

Immunocytochemistry staining for endogenous VAPB in NSC-34 cells- Cells were fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature and permeabilised with 0.1% (vol/vol) Triton X-100 in PBS for 15 minutes. After washing with PBS, the cells were then incubated for 30 minutes in blocking buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 2% bovine serum albumin, 1% glycine, 10% goat serum, and 0.1% Triton X-100). VAPB antibody provided by Dr. Lev was used for primary staining (1:3000) and Alexa Fluor 594-anti rabbit IgG (Invitrogen) was used as secondary antibody, both of which took place at room temperature for 1 hour.

Caspase inhibition assay- COS-7 cells were pre-treated with 10 μM of either zVAD (BioVision) or zATAD (BioVision) one hour before transfection with GFP-VAPBs. Caspase inhibitors were continuously present until the cells were harvested 48 hours after transfection. Annexin V staining and quantification were carried out as described in the paper.

Legends of Supplemental Figures

FIGURE S1. Distribution of GFP-VAPB in N2a and NSC-34 cells. Cells were fixed 48 hours after transfection with GFP-VAPB constructs. Nuclei were detected with DAPI staining (blue). The scale bar is 20 μm.

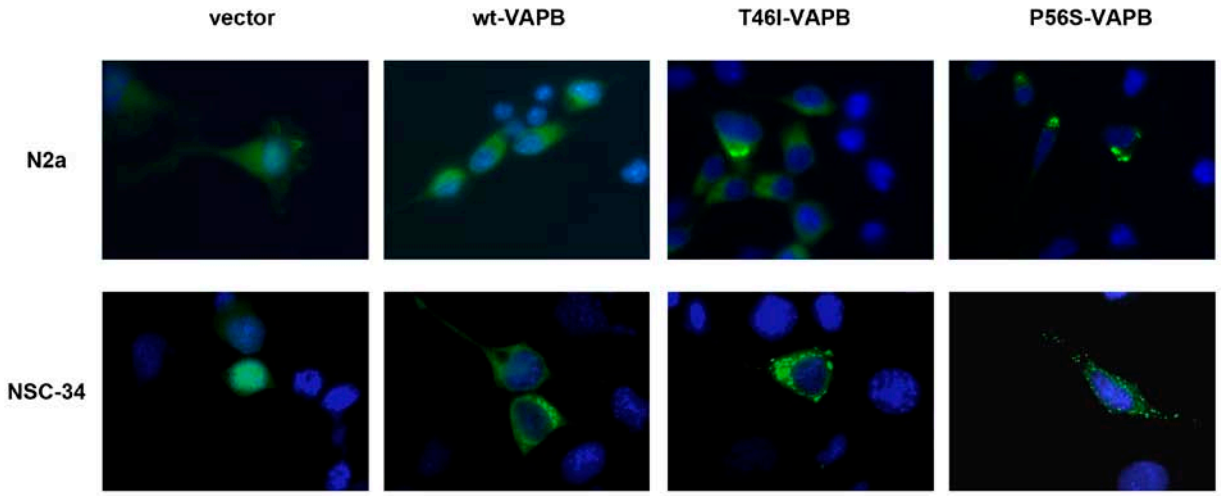

FIGURE S2. The time course induction of phosphorylated eIF2alpha. The time course of induction of phosphorylated eIF2alpha following TG and TN treatments in N2a and NSC-34 cells are shown. Cells were harvested after the indicated treatment period and lysed in RIPA buffer. Endogenous phosphorylated eIF2alpha was detected by immunoblotting using actin as a loading control.

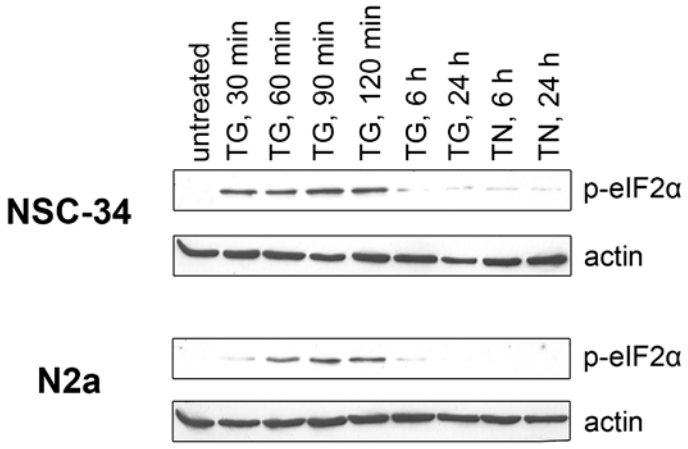

FIGURE S3. The staining of endogenous VAPB in cells adjacent to GFP-VAPB expressing cells. NSC-34 cells were fixed and stained for endogenous VAPB three days after GFP-VAPB transfection. The exogenous VAPB is shown in green whereas endo/exogenous VAPB stained by VAPB and Alexa Fluor 594 secondary antibodies is shown in red. Nuclei were stained by DAPI (blue) in the merge image. The scale bar is 20 μm.

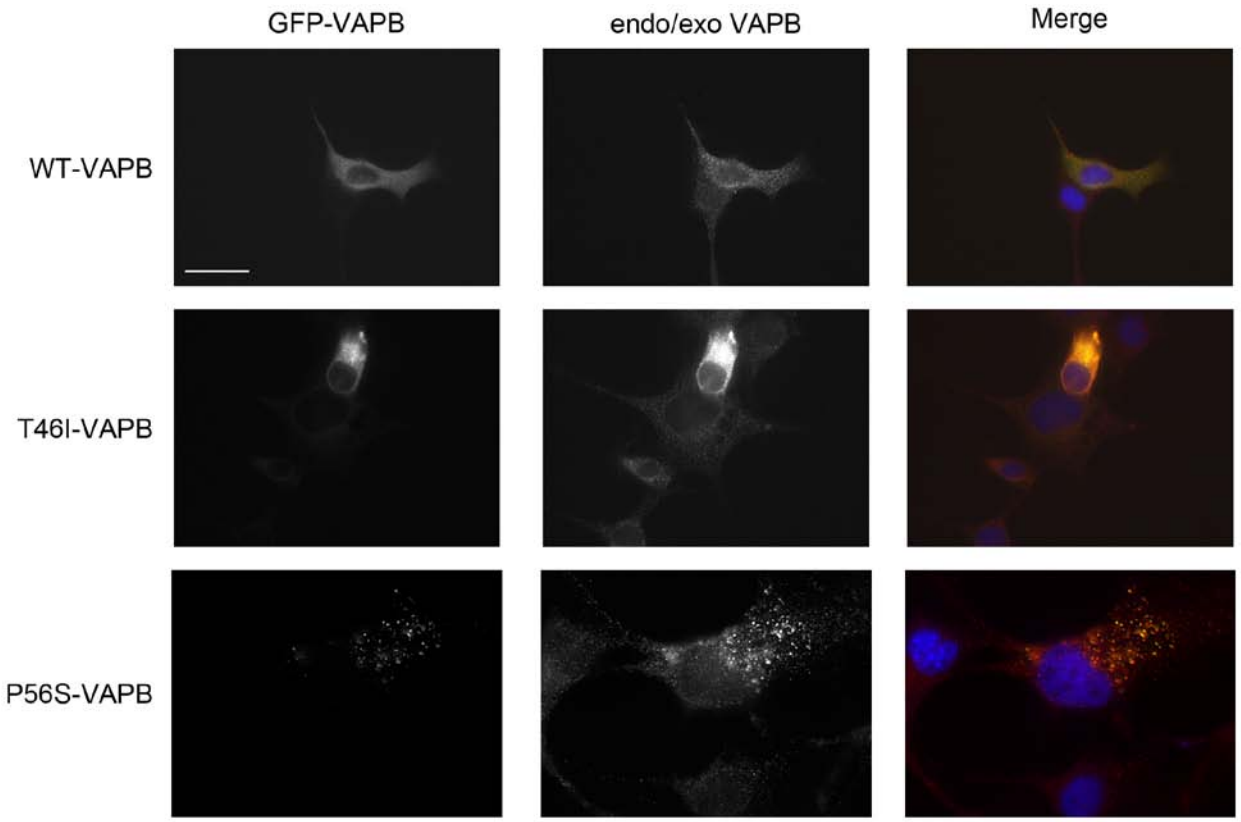

FIGURE S4. Detection of UPS activity in VAPB expressing cells. The UPS reporter, GFP-CL1, was transfected into NSC-34 cells transiently (A) or permanently (B) expressing VAPB. The number of GFP-CL1 positive cells are shown (Mean ± SEM) with proteasome inhibitor (PSI)-treated cells used as a positive control.

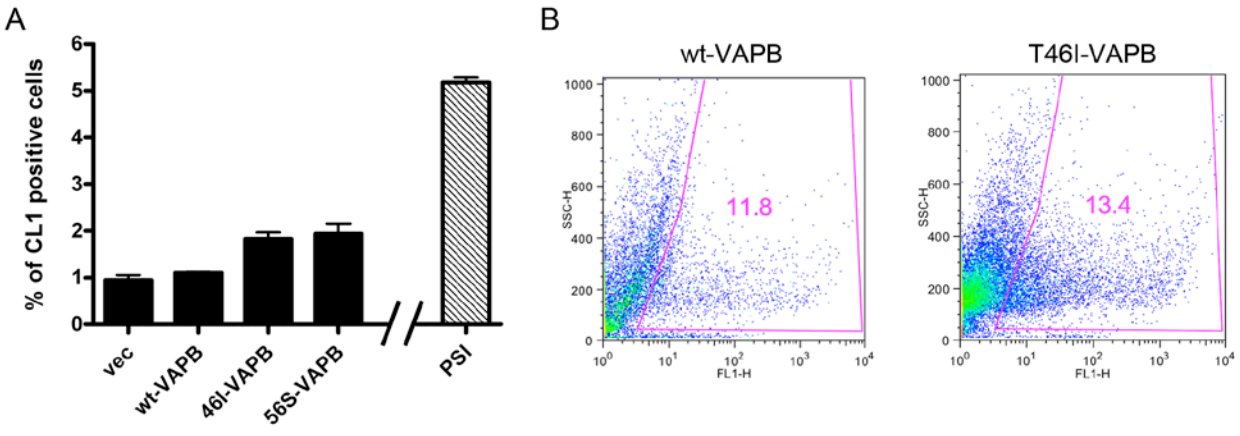

FIGURE S5. The effect of caspase inhibitors on apoptosis induced by GFP-VAPB. COS-7 cells were transfected with GFP-VAPB in the presence of either a pan-caspase inhibitor (zVAD) or caspase-12 inhibitor (zATAD) and were harvested and stained for annexin V two days after transfection. The histograms show the number of annexin V positive cells that are also GFP positive.

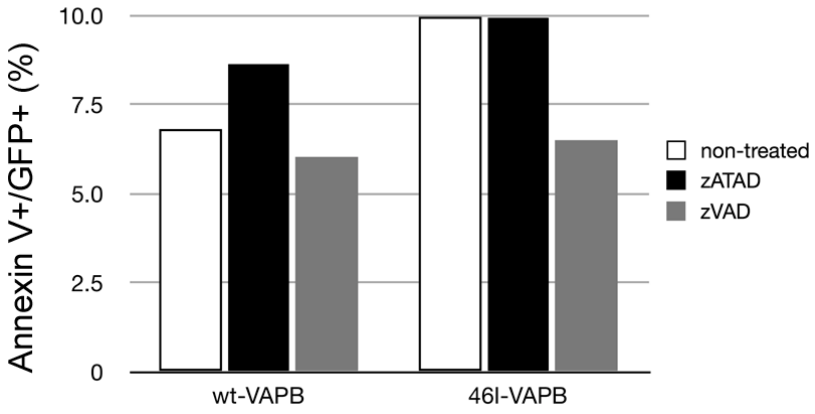

FIGURE S6. Effects T48I-DVAP on ER fragmentation. Transgenic expression of T48I-DVAP in larval neurons induces ER fragmentation as shown by staining with an antibody specific for Boca, an ER marker. A-C, Neuronal cell bodies of control larval brains stained with an anti-DVAP (red) and anti-Boca (green) antibodies. D-F, Brains of T48I-DVAP transgenic larvae stained with the same antibodies as in A-C. Scale bars are 20 μm.

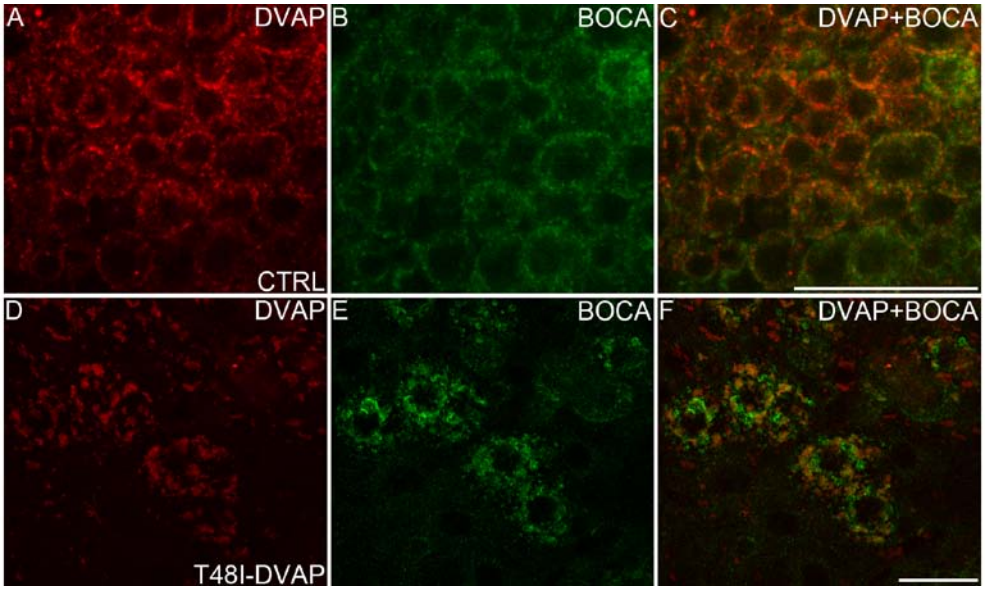

TABLE S1. Primers used for VAPB sequencing

Exon	forward primer (5' to 3')	reverse primer (5' to 3')	size of PCR product (bp)
1	CCCCGCCTTTTTGTAAAACT	CTCCGTCCTTCCAGCACT	224
2	CTTTCATCCATTGGCATGTTAA	CTATCATCTTCTTCTGCTACAC	391
3	GGCGTCCATCCTAAGACATA	GATAAAGTACAAACCTCCTTGA	362
4	CATCAGGGCTTTCTCATTAAG	AAGGCCCACTTATTTGCTGA	350
5	CGTTTGGTGTTTGCTGAGAA	AGGGGACAAGAGCTTCAACA	519
6	TCCAACACTGGGCATAAACA	TGGATCCACCAATCCAATTT	300

TABLE S2. Primers used for SNP analysis

SNP	forward primer (5' to 3')	reverse primer (5' to 3')	RE used for genotyping
rs6026230	ACTAGAAGGGAAACTGCATGAG	GCACTTAGCACTATCTGCAATG	HhaI (NEB)
rs6026256	CTCCATCCAAGAAGGAGCTG	CACGATGCTATGACCACCAG	BsmAI (NEB)
rs2234489	TTGTTCAGTGGTGGGCCTGTC	TCCGGTTTTGCCTCCTTCCAC	Tsp509I (NEB)
rs6100067	GGCATCATCTTCTTCCTTTG	ACATTGATCACACTGGCTTG	MspI (NEB)
rs2234487	GGCGTCCATCCTAAGACATA	GATAAAGTACAAACCTCCTTGA	MaeIII (Roche)
rs1802459	AGGTCCAAGTCTGAGCCTGA	CATACTGTCAGCTGAAGACAAC C	Hpy188III (NEB)