LEGENDS TO SUPPLEMENTARY FIGURES AND TABLES

Supplementary Fig 1. VAPB-P56S aggregates do not colocalize with cellular organelles

A-D) Representative images of HeLa cells transfected with HA-VAPB-P56S, fixed and processed for immunofluorescence using antibodies against HA (green) and trans-Golgi marker γ-adaptin (A), cis-Golgi marker GM130 (B), lysosomal marker LAMP1 (C) or early endosomal marker EEA1 (D) (red). Enlargement of the boxed region in the merged images is shown at right. Note that VAPB-P56S aggregates do not colocalize with any of the tested markers.

E) Redistribution of VAPB-P56S mutant proteins to the ER. Representative images of HeLa cells transfected with HA-VAPB-wt or HA-VAPB-P56S incubated at 37°C (control), 15°C or 20°C for 3 hours, treated with 5μg/μl Brefeldin A (BFA), which inhibits intracellular transport or 10μM Nocodazole (Noco), which disrupts the microtubule network, for 30 minutes, fixed and processed for immunofluorescence using antibodies against HA. Note that in contrast to HA-VAPB-P56S expressing cells at 37°C, HA-VAPA-P56S partially localizes to the ER at 20°C.

Supplementary Fig 2. VAPB-P56S aggregates do not colocalize with chaperone, proteasome, autophagy and ER stress markers

A-D) Representative images of HeLa cells transfected with HA-VAPB-P56S or GFP-Htt(Q74), fixed and processed for immunofluorescence using anti-HA (A,C) and proteasome markers anti-ubiquitin (A,B) or anti-vimentin (C,D) antibodies. While Htt(Q74) colocalizes with proteasomal components and leads to intracellular redistribution of vimentin, VAPB-P56S aggregates do not stain for ubiquitin or effect the vimentin filament network in the cell.

E-F) Representative images of HeLa cells transfected with HA-VAPB-P56S, fixed and processed for immunofluorescence using anti-HA (E,F) and ER stress markers anti-Grp78/BiP (E) and anti-phospho-eIF-2alpha (F) antibodies. Note that the ER-stress markers are not influenced by VAP-P56S aggregates, in contrast to studies using mutant Htt (Waelter et al., 2001).

G) COS-1 cells transfected with HA-VAPB-wt and HA-VAPB-P56S were incubated for 24 hours with (+MG132) or without (-MG132) proteasome inhibitors, harvested (lysates (L)) and solubilized with Triton-X100, fractionated in supernatant (S) and pellet (P) under non-reducing (-DTT) or reducing (+DTT) conditions and analyzed by immunoblotting using anti-HA antibodies. Note that treatments with proteasome inhibitors increases the VAPB-P56S levels in the soluble fraction

H-I) Representative images of HeLa cells transfected with HA-VAPB-P56S or GFP-Htt(Q74) incubated for 24 hours with proteasome inhibitor MG132 (1μM), fixed and processed for immunofluorescence using antibodies against HA (H) and vimentin (H,I).

J-K) Representative images of HeLa cells transfected with HA-VAPB-P56S, fixed and processed for immunofluorescence using antibodies against HA (J,K) and autophagy markers Atg8/LC3 (J) and Atg12 (K). Note that VAP-P56S aggregates are not labeled by the autophagy markers, in contrast to mutant Htt.

Supplementary Fig 3. shRNA knockdown of VAPA and VAPB in primary hippocampal neurons

A) Knockdown of endogenous VAPA and VAPB-levels by different siRNA-oligos. HeLa-cells were transfected with siRNA-oligos for 4 days and protein samples were analyzed by Western Blot with anti-VAPA (#04), anti-VAPB (#00) and anti-actin antibodies.

B-E) Representative images of primary neurons co-expressing ß-galactosidase and pSuper (B,D) or VAPA-shRNA#4 (C) or VAPB-shRNA#1 (E) for 6 days and stained for ß-galactosidase and endogenous VAPA (B,C) or VAPB (D,E). Enlargement shows VAP staining in dendrites.

F) Quantification of VAPA and VAPB-levels in dendrites of knockdown neurons. Per image, VAP-levels of two untransfected and two transfected dendrite sections were measured using Metamorph software, ~5 images per shRNA-construct were measured. Differences were analysed using the Student's T-test. *** $p<0.0005$, * $p<0.05$.

Supplementary Fig 4. Model of mutant VAPB leading to motor neuron degeneration

A) Structure of the complex formed between the MSP domain of VAPA (in light gray mixed ribbon/sticks representation) and the FFAT motif-containing peptide originating from ORP1 (in yellow sticks representation; PDB ID 1Z9O). The S-shaped d1-d2 loop and the C-terminal half of the e-strand of VAPA MSP are represented as sticks (β-strand nomenclature according to (Kaiser et al., 2005)). Hydrophobic residue side chains of VAPA MSP laying beneath the d1-d2 loop and the d1-strand are represented as sticks and colored in green. Oxygen, nitrogen, and carbon atoms are colored in red, blue, and gray/yellow, respectively. The hydrogen-bonding network involving the d1-strand, e-strand, Asn57 of VAPA MSP, and the C-terminal moiety of the FFAT motif-containing peptide is highlighted by yellow dashed lines.

B) Model for the formation of P56S mutant VAPB aggregates. Wild type VAPB undergoes dimerization mediated by the coiled-coil domain (black) and the C-terminal transmembrane domain containing the GxxxG motif (grey). The P56S change induces local conformation changes in the MSP homology domain (red) which results in self-association of mutant MSP domains between neighboring mutant VAPB molecules. The recruitment of wild type VAPB to the mutant VAPB complexes is mediated by the transmembrane domain (blue dashes) and the interaction with the FFAT-motif is disrupted by the P56S mutation.

C) We propose a mechanistic model in which the P56S mutant VAPB expression leads to motor neuron degeneration through two overlapping pathways. The mutation induces a local conformational change of the MSP domain, which leads to aberrant self-association and aggregation and disrupts the binding to FFAT-containing proteins. In addition, the mutation causes recruitment of wild type VAPA and VAPB in aggregates thereby lowering the concentrations of wild type VAPs in the ER, leading to a further impairment of normal VAP function. This dominant negative effect may further abolish the mistargeting of FFAT-motif containing proteins and induce the formation of larger tubular aggregates. In the long term, mutant aggregates may influence membrane trafficking and further disrupt the mislocalization of FFAT-motif containing lipid binding proteins, which together may cause abnormal transport and lipid biosynthesis leading to motor neuron degeneration.

Supplementary Table 1. FFAT motif containing proteins as binding partners of bio-HA-VAPB in HeLa cells identified by mass spectrometry

Identified protein (VAPB)	MW (Da)	% cover	Pept. uniq	Pept. total	NCBI GI number
OSBPL3 / ORP3	102130	28.3	19	23	17389382
OSBPL6 / ORP6	107379	7.3	4	4	14210532
OSBPL9 / ORP9	63059	7.9	3	3	19684104
PITPNM1 / NIR2	135844	6.9	6	6	12667436
Identified protein (VAPB-P56S)	**MW (Da)**	**% cover**	**Pept. uniq**	**Pept. total**	**NCBI GI number**
No FFAT motif containing proteins were identified					

The table shows FFAT motif containing proteins identified with a significant Mascot score in the pull down with streptavidin beads from extracts of HeLa cells co-expressing biotin ligase BirA and bio-HA-VAPB or bio-HA-VAPB-P56S. The list is corrected for background proteins which were identified in a control pull-down from HeLa cells expressing BirA only. For each identified protein, the list is filtered for duplicates and shows only the hits with most identified peptides. Abbreviations used in the table to indicate the identified proteins: OSBPL, oxysterol binding protein-like; ORP, OSBP related protein; PITPNM, phosphatidylinositol transfer protein, membrane-associated; NIR, N-terminal domain-interacting receptor

Movie 1. Photobleaching of ER localized GFP-VAPB

HeLa cells expressing GFP-VAPB were imaged 1 day after transfection. A 4 by 4 μm region of the cell was bleached by high laser power and the fluorescent recovery was monitored over 300 seconds. Time interval is 1.50 sec.

Movie 2. Photobleaching of GFP-VAPB-P56S aggregates

HeLa cells expressing GFP-VAPB-P56S were imaged 1 day after transfection. A 4 by 4 μm region of the cell was bleached by high laser power and the fluorescent recovery was monitored over 300 seconds. Time interval is 1.50 sec.

Movie 3. Photobleaching half an area of a GFP-VAPB-P56S cluster

HeLa cells expressing GFP-VAPB-P56S were imaged 1 day after transfection. Half the area of a VAPB-P56S aggregate was bleached by high laser power and the fluorescent recovery was monitored over 300 seconds. Time interval is 0.5 sec.