Supplementary materials

SQSTM1^{L341V} variant that is linked to sporadic ALS exhibits impaired association with MAP1LC3 in cultured cells

Masahisa Nozaki, Asako Otomo, Shun Mitsui, Suzuka Ono, Ryohei Shirakawa, YongPing Chen, Yutaro Hama, Kai Sato, XuePing Chen, Toshiyasu Suzuki, Hui-Fang Shang, Shinji Hadano

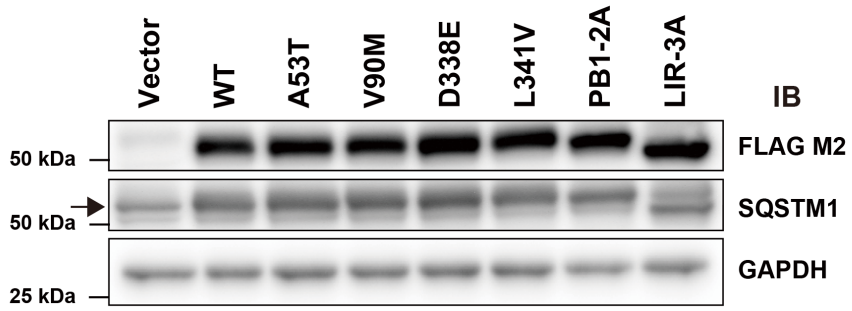

Fig. S1. Expression levels of SQSTM1_WT and its mutant/variant in PC12 cells. PC12 cells were transfected with FLAG-tagged pCIneo_SQSTM1 constructs. Expression levels of SQSTM1_WT and its mutant/variant were analyzed by Western blotting using anti-FLAG M2 and anti-SQSTM1 (p62-C) antibodies (IB: immunoblotting). Arrow indicates the position of endogenous SQSTM1. GAPDH was used for a loading control.

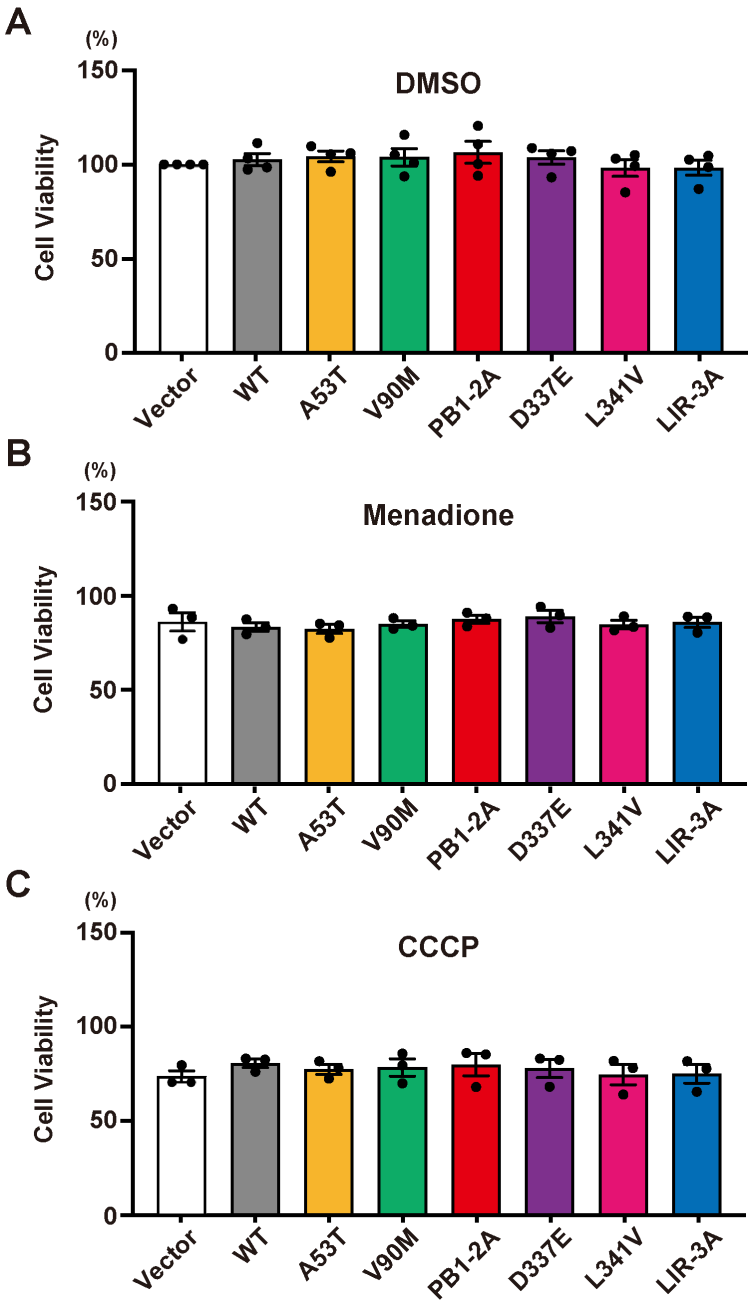

Fig. S2. Effects of overexpression of wild-type SQSTM1 or its mutant/variant on viability in PC12 cells under either normal or stressed conditions. After 24 h of transfection with FLAG-tagged SQSTM1_WT or mutant/variant-expressing construct, PC12 cells were incubated under following conditions: (**A**) non-treated (DMSO), (**B**) 10 μM of menadione for 4 h and (**C**) 750 μM of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) for 10 h. Alamar Blue Reagent was used to assess the cell viability. PB1 mutants/variants include A53T and V90M variations identified in ALS patients, and PB1-2A (K7A/D69A) artificial mutation. LIR mutants/variants include D337E and L341V variations identified in ALS patients, and LIR-3A (D335A/D336A/D337A) artificial mutation. Values for the cell viability represent mean percentage relative to control cells that are transfected with pCIneo-vector (Vector) under non-treated conditions [± S.E.M.; n = 3 ~ 4 (biological replicates)]. Individual data points are also shown. Statistical significance was evaluated by one-way ANOVA with Tukey's multiple comparison test.

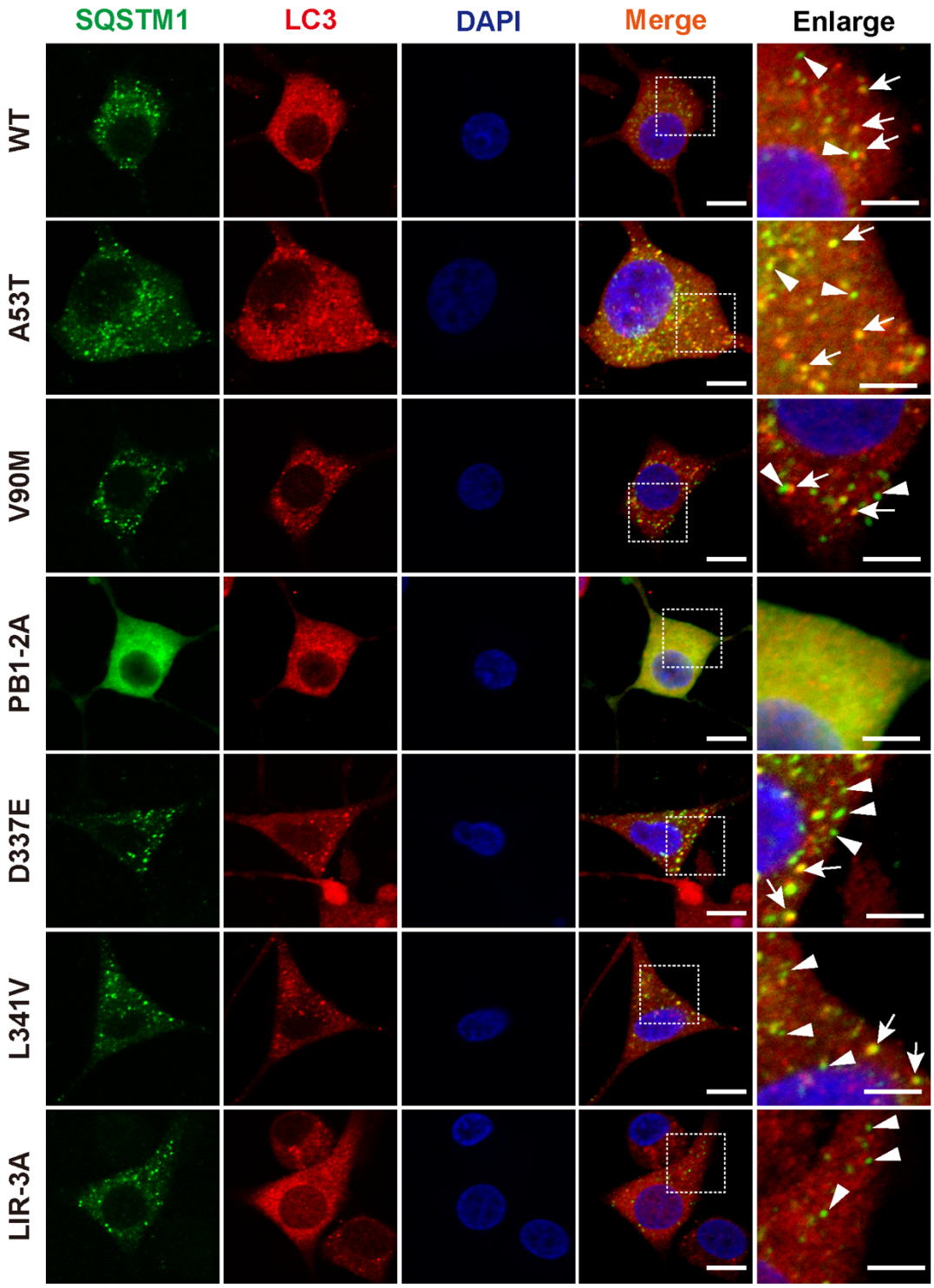

Fig. S3. Subcellular distribution of SQSTM1 mutants in PC12 cells. FLAG-tagged SQSTM1_WT (WT), PB1-domain ALS-linked variants (A53T and V90M), PB1-domain artificial mutant (PB1-2A), LIR ALS-linked variants (D337E and L341V) and LIR artificial mutant (LIR-3A) were expressed in PC12 cells, and their subcellular distributions were analyzed. Images for SQSTM1 and LC3 were obtained by staining with anti-FLAG M2 and anti-LC3 antibodies, respectively. Nuclei were counterstained with DAPI. High-resolution magnified images are shown on the right. Arrows and arrowheads indicate LC3-positive and -negative SQSTM1-bodies, respectively. Scale bars for merged and enlarged images indicate 20 μm and 10 μm, respectively.

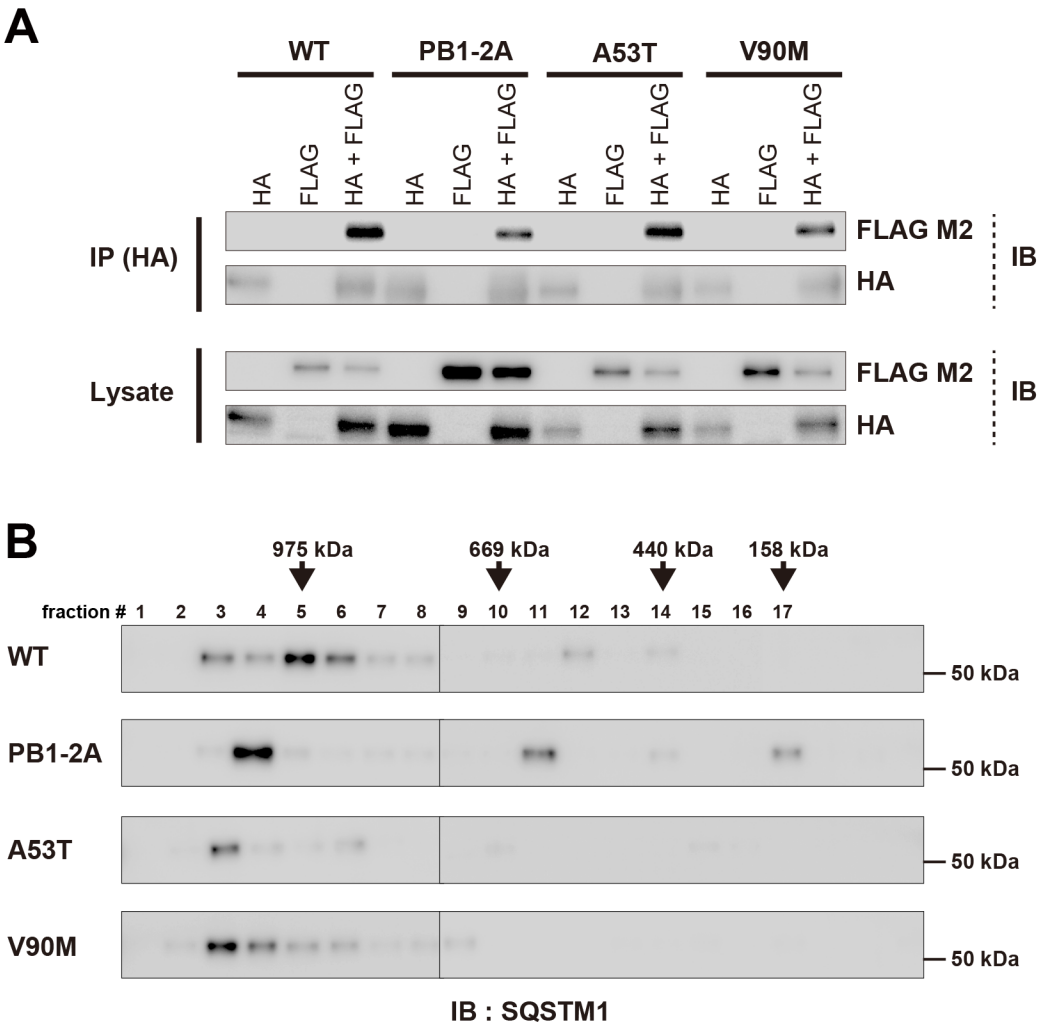

Fig. S4. Analysis of self-oligomerization of the SQSTM1 PB1 mutants. (**A**) Co-immunoprecipitation analysis. COS-7 cells expressing HA-tagged SQSTM1 and FLAG-tagged SQSTM1 were immunoprecipitated by monoclonal anti-HA antibody (IP: immunoprecipitation). Both precipitated samples and cell lysates were analyzed by Western blotting using anti-FLAG M2 and anti-HA antibodies (IB: immunoblotting). (**B**) Gel filtration analysis of the SQSTM1 proteins. SQSTM1 purified from transformed BL21 *E. coli* were applied to the Superose6 10/300 column, and fractions were analyzed by Western blotting using anti-SQSTM1 (p62-C) antibody (IB: immunoblotting). Arrows indicate the positions of molecular mass markers.

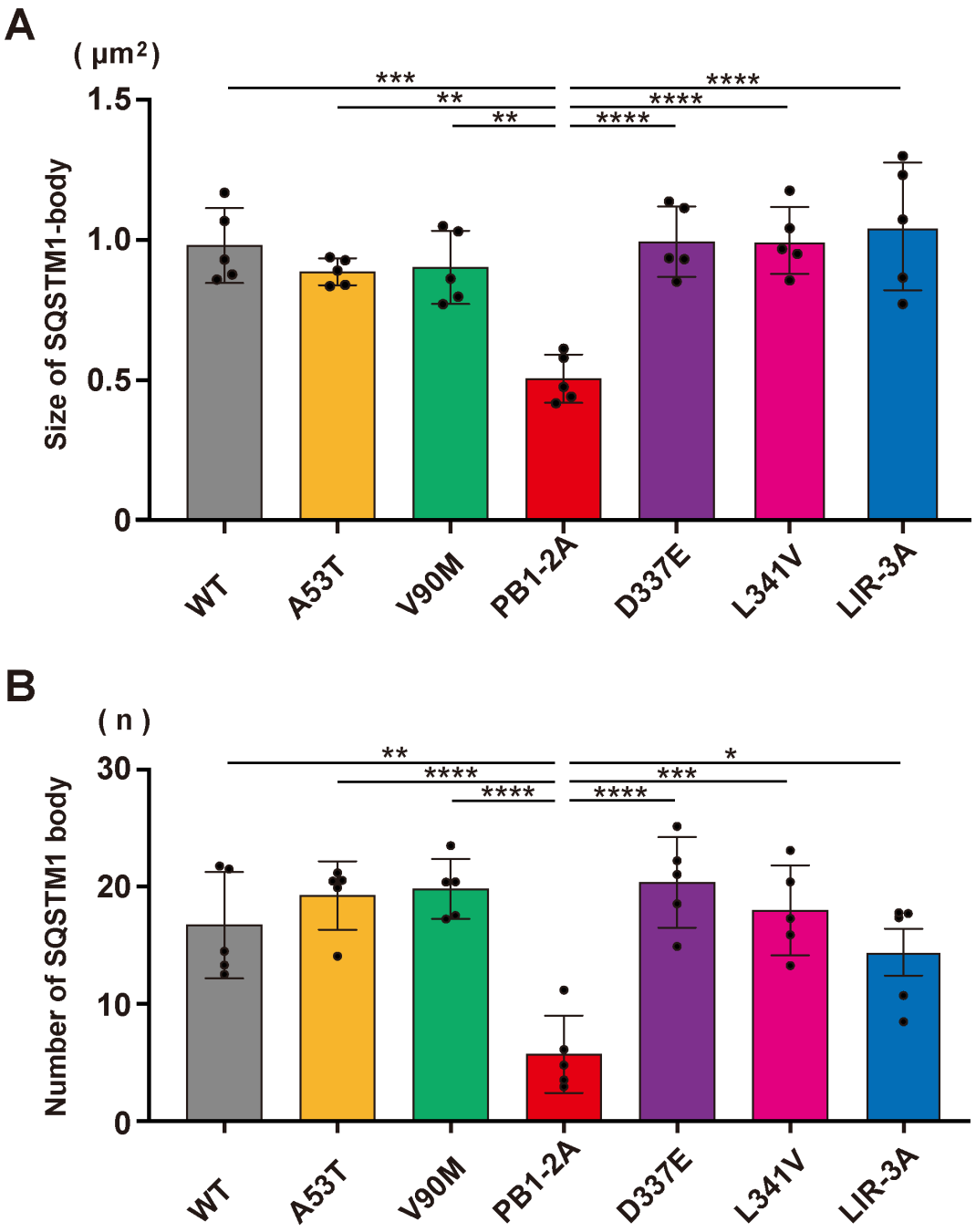

Fig. S5. Quantitative analysis of the size and number of SQSTM1-positive bodies in *Sqstm1* deficient mouse embryonic fibroblast. *Sqstm1* deficient mouse embryonic fibroblasts (*Sqstm1*-KO MEFs) were transfected with pCIneoFLAG-SQSTM1 constructs. The size of each SQSTM1-body in each cell was measured. We also counted and normalized the number of SQSTM1-body with the adjusted area of cell body (per 1,000 μm^2). Mean values of the size [± S.D.; n = 5 biological replicates (total number of cells = 26 ~ 28)] (**A**) and the number [± S.D.; n = 5 biological replicates (total number of cells = 21~ 22)] (**B**) for "SQSTM1-body" are shown. Statistical significances were evaluated by one-way ANOVA with Tukey's multiple comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).