

Supporting Information

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SI Materials and Methods

FTLD-U Mouse Model. The frontotemporal lobar degeneration with ubiquitin (+) inclusions (FTLD-U) mouse model carried transgenic full-length mouse TDP-43 cDNA under the transcription control of an 8.5-kb promoter region of the CaMKII gene (1). Genotyping by PCR and Southern blotting was used to identify the transgene-positive mice of the founders and their progenies, and Western blotting and immunohistochemistry were used to check the expression of the transgene in specific regions, in particular the hippocampus and cortex. The assays of behavioral performances and pathological features of the homozygous (+/+) TDP-43 Tg mice were done as described before (1). Mice were bred at the animal facility of the Institute of Molecular Biology (IMB), Academia Sinica, Taiwan. Experimental procedures for handling the mice followed the guidelines of the IMB. The animals were housed in a room maintained on a 12-h/12-h light/dark cycle (light on at 7:00 AM).

Drug Treatment. For the behavioral tests, WT and TDP-43 Tg (+/+) male mice of 2 mo of age were injected intraperitoneally with rapamycin (10 mg/kg) three times a week for a period of 4 mo. Water maze tests were carried out first at the age of 2 mo to ascertain impairment of the learning/memory of the Tg mice, and then at the age of 3 mo, to examine the effect of rapamycin on cognition. The rotarod tests for the alleviation of motor function loss by rapamycin were carried out at the age of 6 mo.

The possible rescues of motor dysfunction of 6-mo-old Tg mice by spermidine, carbamazepine, tamoxifen, and rapamycin were also examined. Spermidine powder (Sigma) was dissolved in water, whereas carbamazepine and tamoxifen (Sigma) were dissolved in DMSO with a stocking concentration of 250 mg/mL. All working solutions were prepared freshly before use with a final concentration of 5 mg/mL. TDP-43 Tg (+/+) mice of 6 mo of age were injected intraperitoneally with rapamycin (10 mg/kg) or spermidine (50 mg/kg) (2) or carbamazepine (50 mg/kg) (3). Tamoxifen administration (50 mg/kg) (4) was carried out by s.c. injection. All drugs were given to the mice three times a week for 1 mo. The rotarod tests were carried out right after the treatment with each of the above autophagy enhancers.

Morris Water Maze Task. For assessment of spatial learning, the Morris water maze task was used as described previously (5). Animals were subjected to four trials per session and two sessions a day. A complete test consisted of six sessions in 3 d. The average time spent by individual mice to reach the hidden platform in the water was recorded as escape latency. After the hidden platform tasks, the visible platform tasks were also performed to exclude the differences in vision, swim speed, and movement among the different mice.

Rotarod Test. The rotarod tests were performed as described before (1). In brief, the mice were placed on a rod rotating at 20 rpm and the time taken for them to fall from the rod was measured. If a mouse stayed on the rod until the end of the 2-min trial, a time of 120 s was recorded.

Immunohistochemistry. Immunohistochemistry analysis of adult brains from FTLD-U mice was performed as described previously (1). For immunohistostaining, brain sections were stained with anti-TDP-43 (Gene Tex), anti-caspase-3 (Cell Signaling) or antineuron-specific nuclear protein (NeuN) (Millipore), and then with biotin-conjugated secondary antibodies (Millipore) followed

by detection with 3,3' diaminobenzidine (DAB) (Millipore). Hematoxylin was used to locate the nuclei.

For immunofluorescence staining, coronal brain sections were stained with anti-LC3 antibody (Novus Biologicals). DAPI staining was used to locate the nuclei. All sections were examined in a laser scanning confocal microscope (LSM 510; Carl Zeiss).

Fluorometric Assay of Caspase-3 Activity. The hippocampus and cortex were homogenized in lysis buffer (100 mM Hepes, pH 7.4, 0.1% (wt/vol) CHAPS, 1 mM EDTA, 10 mM DTT, and 1 mM PMSF) and then lysed by freezing and thawing three times with liquid nitrogen and 37 °C water bath (6). Following centrifugation at 11,500 × g for 5 min, 50 μL supernatant was added to 150 μL of reaction buffer (25 mM Hepes, 1 mM EDTA, 0.1% (wt/vol) CHAPS, 10% (wt/vol) sucrose, 3 mM DTT, pH 7.5) (7), which was supplied with 50 μM of 7-amino-4-methylcoumarin, N-acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide (Ac-DEVD-AMC). After incubation at 37 °C for 1 h, fluorescence was measured using the excitation wavelength of 380 nm and emission wavelength of 460 nm. The caspase-3 activity was measured as change in fluorescence units per microgram of proteins.

Western Blotting Analysis of Brain Extracts. For analysis of the expression levels of different proteins, extracts were prepared from the hippocampus and cortex of the cerebrum of male WT and TDP-43 Tg mice 6 to 7 mo of age. For analysis of soluble proteins, tissues were homogenized in RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% (vol/vol) Igepal CA-630, 2 mM EDTA, pH 8.0, 1 mM Na₃VO₄, 20 μg/mL pepstatin A, 20 μg/mL leupeptin, 20 μg/mL aprotinin, 1 mM PMSF, 50 mM NaF). Extracts were then analyzed by Western blotting with anti-TDP-43 (Gene-Tex), anti-caspase-3 (Cell Signaling), anti-LC3 (Novus Biologicals), antiphospho-p70 S6 kinase (Thr389) (Cell Signaling), anti-p70 S6 kinase (Cell Signaling), anti-p62 (Progen), and antitubulin (Millipore) as the probes. After hybridizations, blots were incubated at room temperature with the appropriate secondary antibodies and Western Lightning Plus-ECL (PerkinElmer). For quantitative analysis, relative intensities of the bands were normalized against that of tubulin and expressed as means ± SEM.

For analysis of insoluble proteins, tissues were dissected and sequentially extracted with buffers of increasing strengths as previously described (1, 8). In brief, the forebrains were extracted sequentially at 5 mL/g (volume/weight) with low-salt (LS) buffer (10 mM Tris, pH 7.5, 5 mM EDTA, 1 mM DTT, 10% (wt/vol) sucrose, and a mixture of protease inhibitors), high-salt Triton X-100 (TX) buffer [LS + 1% (vol/vol) Triton X-100 + 0.5 M NaCl], myelin flotation buffer [TX buffer containing 30% (wt/vol) sucrose], and Sarkosyl (SARK) buffer [LS + 1% (wt/vol) N-lauroyl-sarcosine + 0.5 M NaCl]. The SARK-insoluble materials were further extracted in 0.25 mL/g urea buffer (7 M urea, 2 M thiourea, 4% [wt/vol] 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate, 30 mM Tris, pH 8.5). The urea soluble proteins were then analyzed by Western blotting.

GFAP Staining and TUNEL Assay. The preparation of brain sections was as described previously (1). For anti-GFAP staining, mouse monoclonal anti-GFAP (Millipore) and Alexa Fluor 488-conjugated goat antimouse antibodies (Invitrogen) were used. For TUNEL assay, cortex brain sections from FTLD-U mice were

fixed with 4% (wt/vol) formaldehyde solution and stained following the protocols of the DeadEnd fluorometric TUNEL system (Promega), followed by incubation with DAPI. Quantitative analysis was assessed by TissueQuest software.

Motor Neuron Counting. Preparation of lumbar cord sections and Nissl staining were as described previously (9). Motor neurons were identified using the following criteria: (i) The presence of a large nucleolus located within the nucleus surrounded by light blue-staining cytoplasm and (ii) a cell soma area over $100 \mu\text{m}^2$. Ranges in motor neuron soma areas are used to distinguish

gamma (γ) motor neurons (from 100 to $250 \mu\text{m}$) from alpha (α) motor neurons (from 250 to $1,100 \mu\text{m}$).

Statistical Analysis. All data are reported as the mean \pm SEM for Morris water maze task; escape latency was analyzed by repeated-measures analysis of variance (ANOVA) with Bonferroni test for multiple comparisons (10). Other independent experiments with multiple groups were compared with each other by one-way ANOVA followed by post hoc Tukey's test or with Student's *t* test for two-group comparisons. Differences were considered statistically significant at $P < 0.05$, as indicated by the asterisks.

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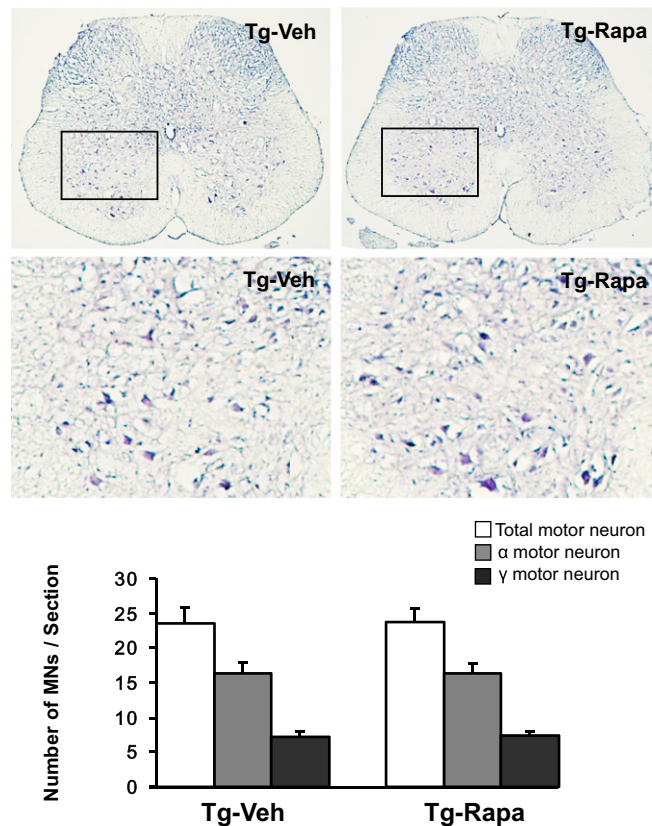


Fig. S1. Histological analysis of motor neurons in FTLD-U mice. Microscopic view of Nissl-stained sections showing the lumbar spinal cord of 6-mo-old TDP-43 Tg mice with 4 mo of rapamycin or vehicle treatment. Numbers of motor neurons were quantified with respect to the alpha (α) and gamma (γ) types (1). Boxed areas in the single anatomical level of the spinal cords (Upper) representing one ventral horn of each section are magnified for better visualization (Lower). Note the similarity of staining patterns between the two groups. (Bars, $100 \mu\text{m}$.) Below, histogram representing the numbers of motor neurons per lumbar spinal cord section. For each mouse, >10 sections from L3 to L5 of the lumbar cord were counted. Data represent the mean \pm SEM of three independent experiments ($n = 3$ male mice per group).

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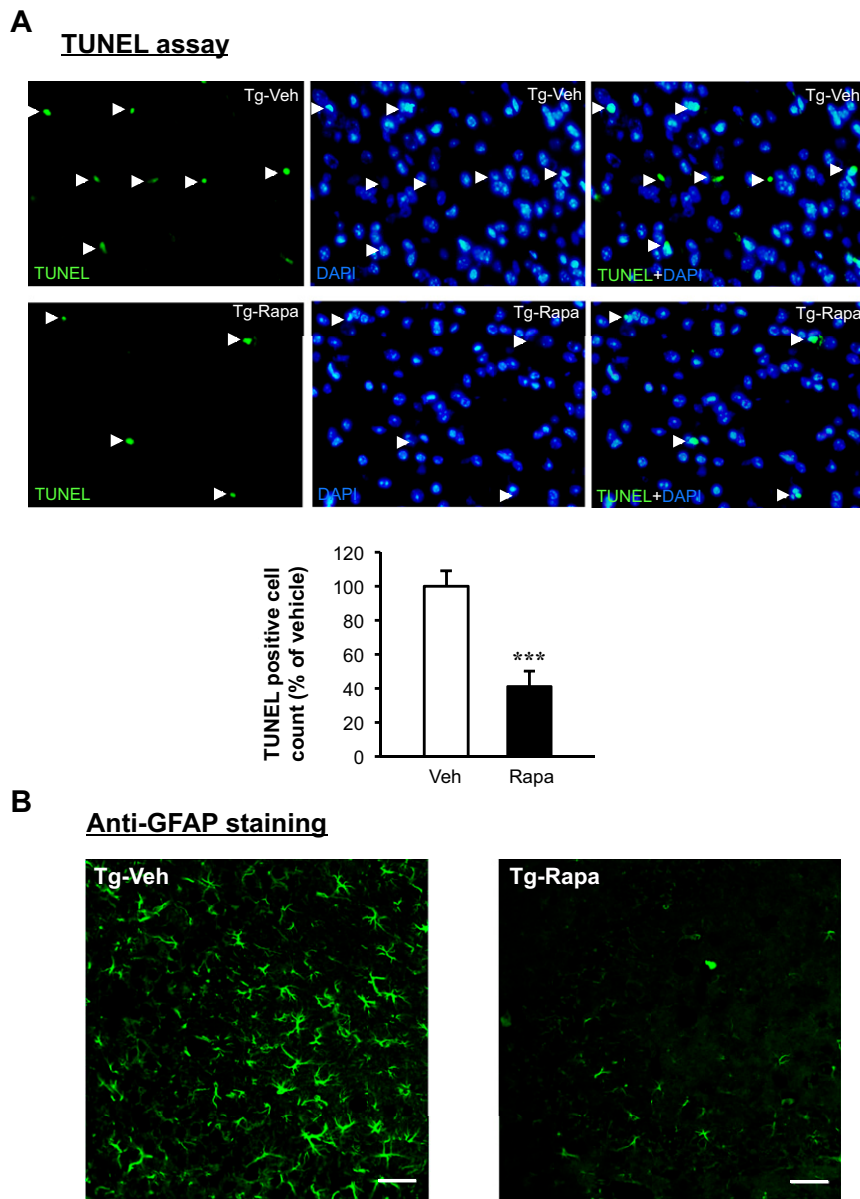
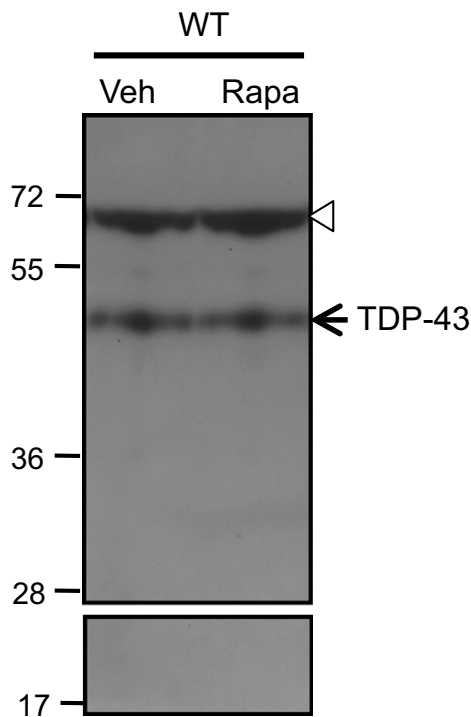


Fig. S2. TUNEL assay and anti-GFAP staining of the forebrains of FTLD-U mice treated with rapamycin. (A) *Top* six panels, representative of immunofluorescent images showing the detection by TUNEL assay of apoptotic nuclei (green, *Left* two panels) in the forebrains of 6-mo-old FTLD-U mice with rapamycin (*Bottom* row) or vehicle treatment (*Top* row). Nuclei were labeled with DAPI (blue, *Middle* two panels). Quantitative analysis as measured by tissue cytometry is shown in the *Bottom* histogram. Results represent the mean \pm SEM of three independent experiments ($n = 3$ mice per group). (Bars, 50 μm .) $*P < 0.0001$. (B) Representative images of anti-GFAP staining of the forebrain sections of 6-mo-old FTLD-U mice treated with rapamycin or vehicle for 4 mo ($n = 3$ mice per group). (Bars, 50 μm .)

A
Urea fraction



B
TDP-43/ Nuclei

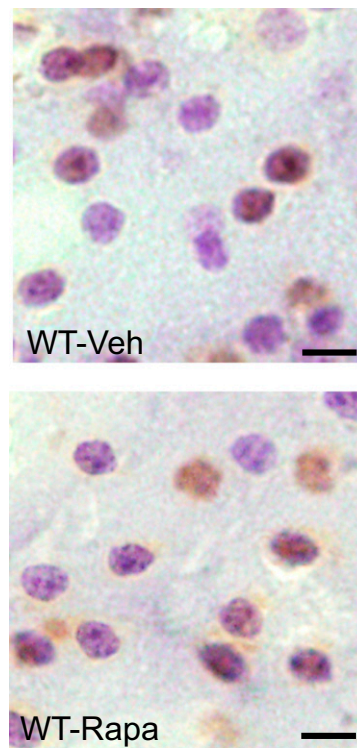


Fig. S3. Western blot and immunohistochemical analyses of TDP-43 in the forebrains of the FVB-WT mice. (A) Western blot analysis of TDP-43 in the urea-soluble fraction of the forebrain extract from FVB-WT mice with or without rapamycin treatment. The arrow points to the unmodified form of TDP-43 and the triangle is an anti-TDP-43 hybridizing band of unknown origin. The result is representative of three independent experiments ($n = 3$ mice per group). (B) Representative patterns of staining of TDP-43 and nuclei of brain sections from 6-mo-old FVB-WT mice treated with rapamycin or vehicle for 4 mo. Sections were immunohistochemically stained for detection of TDP-43 (brown) and nuclei (blue). Note the absence of cytosolic TDP-43 (+) inclusions in either set of samples. (Bars, 10 μm .) Data represent the mean \pm SEM of three independent experiments ($n = 3$ mice per group).