

Supplementary Materials

A conserved MTMR lipid phosphatase increasingly suppresses autophagy in brain neurons during aging

**Tibor Kovács, Janka Szinyákovics, Viktor Billes, Gábor Murányi, Virginia B. Varga,
Annamária Bjelik, Ádám Légrádi, Melinda Szabó, Sára Sándor, Enikő Kubinyi, Cecília
Szekeres-Paracky, Péter Szocsics, János Lőke, Jun Mulder, Balázs Gulyás, Éva Renner,
Miklós Palkovits, Károly Gulya, Zsófia Maglóczky and Tibor Vellai**

Supplementary Figures S1 to S8

Supplementary Tables S1 to S6

Figure S1.

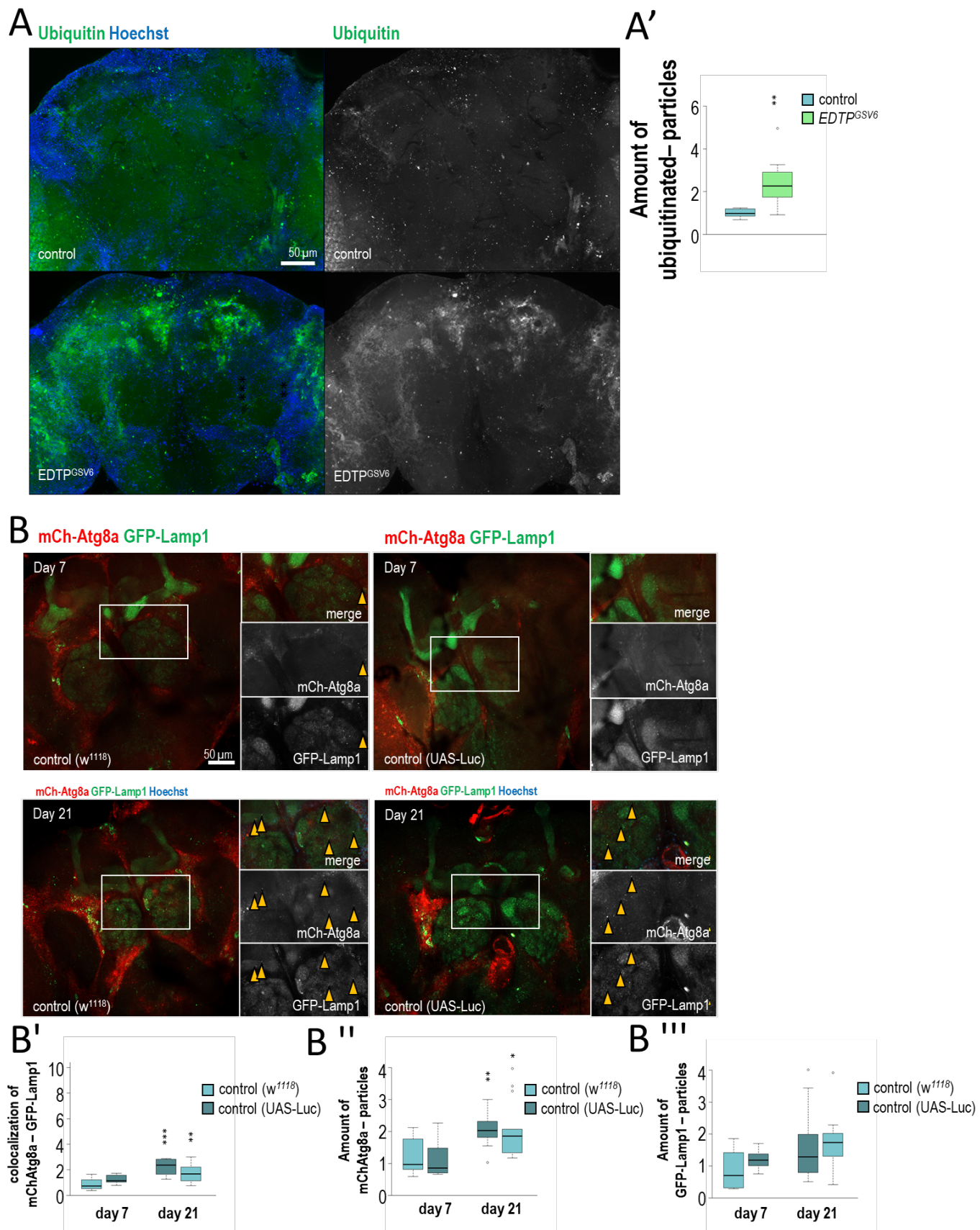


Figure S1. The amount of autolysosomes increases with age in brain neurons. (A-A') Relative amount of ubiquitinated structures in EDTP-overexpressing (*EDTP^{GSV6}*), 21-day-old animals maintained at 29°C. The amount of ubiquitin-positive structures (green) elevates in response to EDTP hyperactivity. Hoechst staining (blue) indicates nuclei. **(B-B''')** Co-localisation of mCherry-Atg8a (autophagic structures) and GFP-Lamp1 (lysosomal compartments) reporters in animals at the stage of 7 and 21 days. More co-labelled structures (yellow arrows) are visible in old animals (**B'**). On the plot, the boxes represent the most typical 50% of the samples, the line indicates the median, upper and lower whiskers show remaining 25%-25% of the samples. Circles mark outliers. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ at each comparison with day 1. For statistics, see the Materials and Methods.

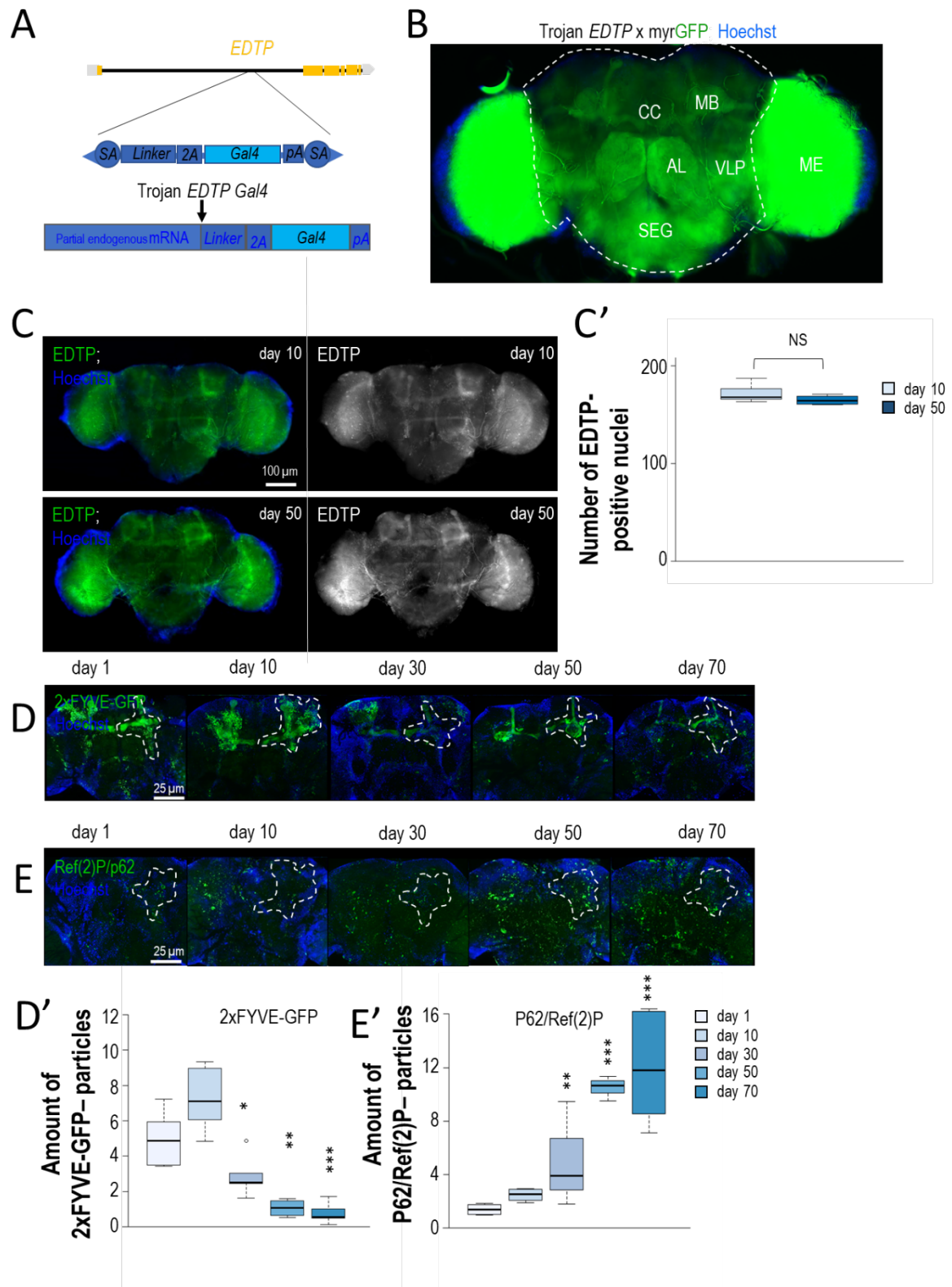
Figure S2.

Figure S2. Expression of a Trojan *EDTP*-GFP gene trap reporter system in the *Drosophila* brain. (A) Structure of the Trojan-*EDTP*-*Gal4* gene trap system used in this study. A *Gal4* driver, which activates a *UAS*-GFP reporter, was inserted into the first intron of *EDTP* gene,

and serves as an artificial exon containing a STOP codon (*i.e.*, *GFP* transcription is controlled by the endogenous *EDTP* regulatory sequences; *UAS-myr-eGFP* was expressed). **(B)** Expression of a Trojan-*EDTP-Gal4* driver in the brain. Dashed line indicates the midbrain, where relative *EDTP* levels were measured. AL, antennal lobe; MB, mushroom body; CC, central complex; ME, medulla; VLP, ventrolateral protocerebrum; SEG, subesophageal ganglion. Medullas were excluded from the analysis. **(C)** The amount of cells expressing *EDTP* does not change with age. *UAS-GFP.nls* (nuclear localization signal-tagged GFP) was driven by Trojan-*EDTP-Gal4*. Left: fluorescent images, right: the corresponding uncoloured versions. **(C')** Quantification of cells expressing *EDTP* in the brain at different stages of adulthood (10 vs. 50 days). *EDTP* expression is increased during ageing in the area of mushroom body (MB). **(D-D')** The amount of 2xFYVE-GFP-positive structures is lowered in old samples relative to young ones. **(E-E')** Ref(2)P levels increase in the MB in old animals compared with young ones. On the plot, the boxes represent the most typical 50% of the samples, the line indicates the median, upper and lower whiskers show remaining 25%-25% of the samples. Circles mark outliers. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ at each comparison with day 1. For statistics, see the Materials and Methods. Flies were maintained at 25°C.

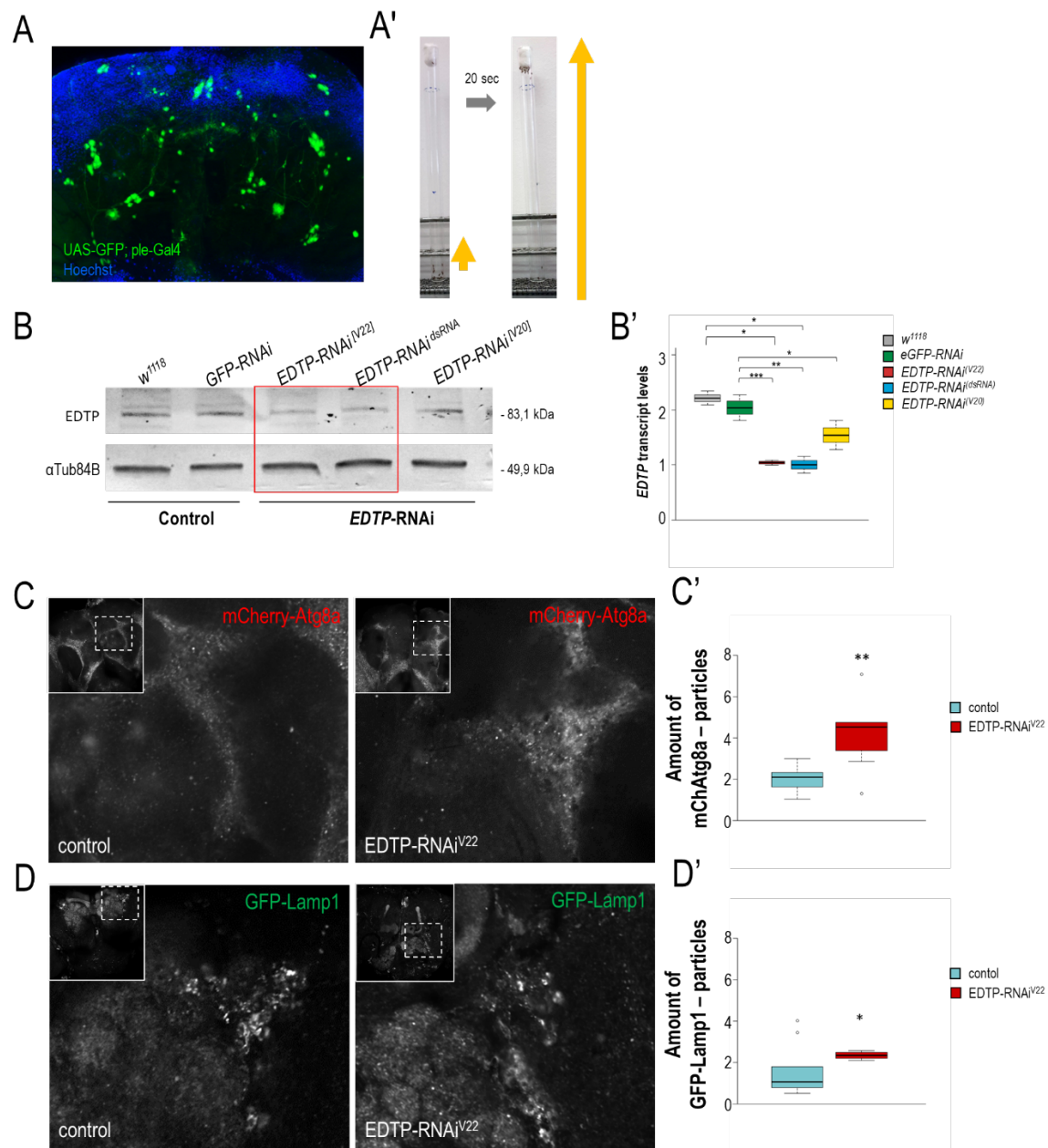
Figure S3.

Figure S3. *EDTP* downregulation in dopaminergic neurons. (A) Expression of a *ple-Gal4* driver in dopaminergic neurons. The system restricts *EDTP* downregulation to dopaminergic neurons only (green). Hoechst staining (blue) indicates nuclei. (A') Climbing assay used in this study. Animals were first collected at the bottom of a 25 cm long glass vial by tapping, then allowed to climb up on the wall (negative geotaxis). The number of animals reaching the top within 20 sec was determined. (B) Downregulation of *EDTP* by three different RNAi constructs. Western blot analysis showing that *V22* and *dsRNA* RNAi constructs (red frame) work effectively, so they were used further in this study. (B') Quantification of *EDTP* transcript levels in brain samples isolated from staged adults. (C-D') *EDTP* downregulation increases the

amount of mCherry-Atg8a- and GFP-Lamp1-positive structures in animals at the stage of 21 days. Animals were maintained at 29°C. (C' and D') Quantification of mCherry-Atg8a- and GFP-Lamp1-positive structures in brain samples. On the plot, the boxes represent the most typical 50% of the samples, the line indicates the median, upper and lower whiskers show remaining 25%-25% of the samples. Circles mark outliers. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ at each comparison with day 1. For statistics, see the Materials and Methods.

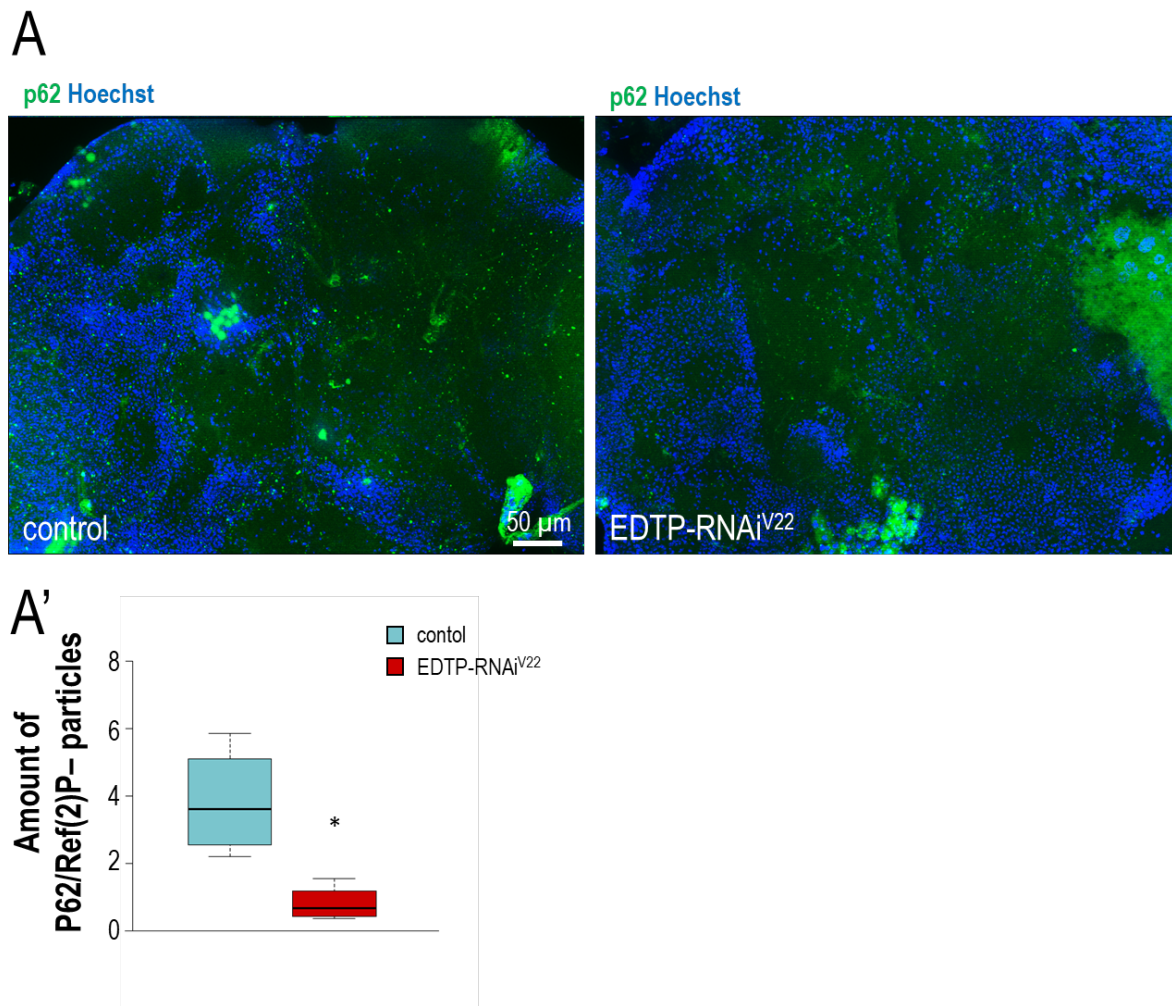
Figure S4.

Figure S4. *EDTP* downregulation lowers the amount of Ref(2)P-positive structures. The amount of Ref(2)P-positive structures decreases in animals at the stage day 21 compared with control at the same stage. Animals were maintained at 29°C, an anti-Ref(2)P antibody was used for labelling. Hoechst staining (blue) indicates nuclei. Scale bars represent 50 μ m. On the plot, the boxes represent the most typical 50% of the samples, the line indicates the median, upper and lower whiskers show remaining 25%-25% of the samples. Circles mark outliers. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ at each comparison with day 1. For statistics, see the Materials and Methods.

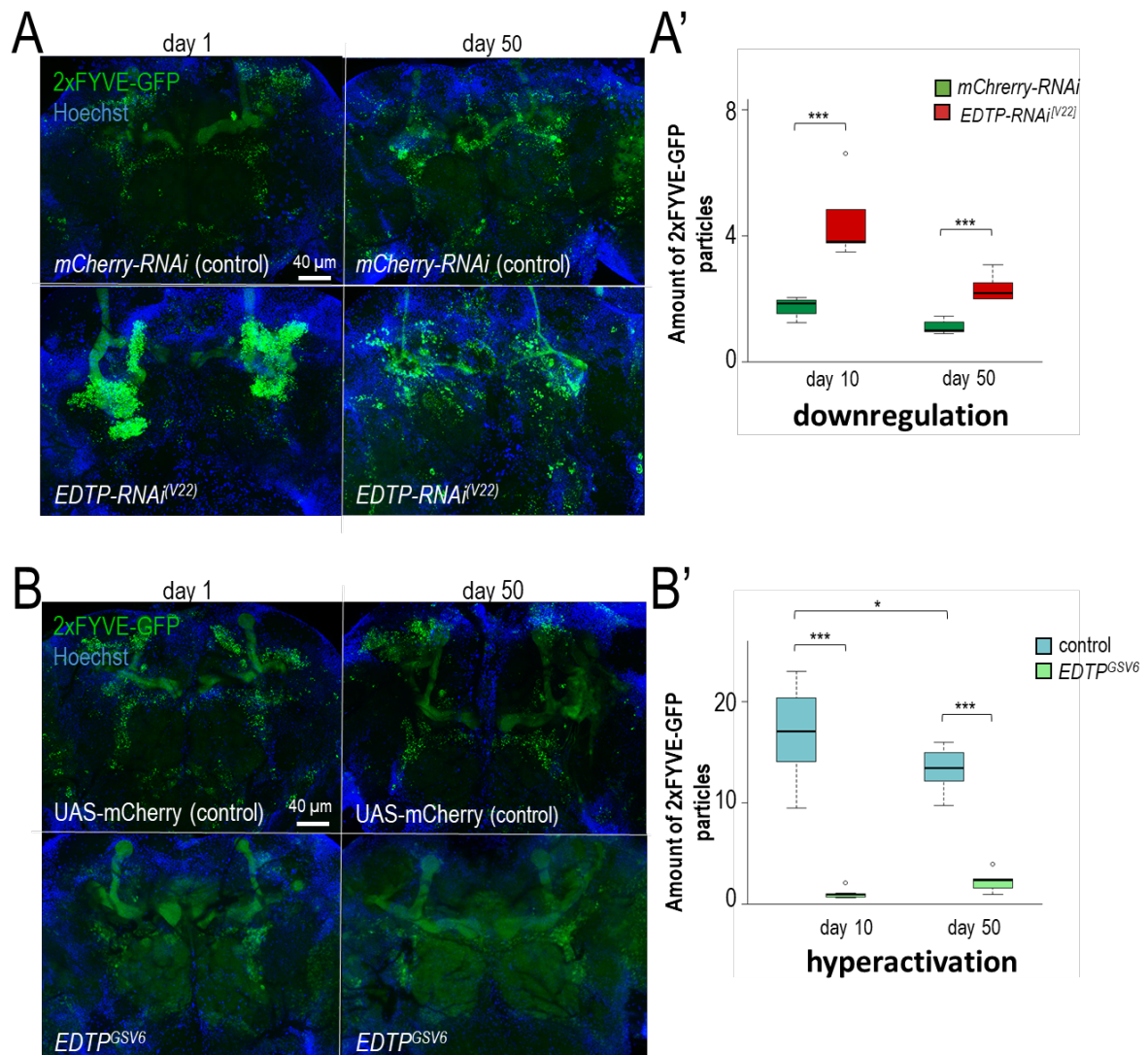
Figure S5.

Figure S5. EDTP activity influences PI3P accumulation. (A) Fluorescent images displaying control (up) and *EDTP* downregulated (bottom) samples at the adult ages of day 10 (left) and 50 (right). (A') Quantification of relative amounts of PI3P-positive structures. (B) Fluorescent images showing control (up) and *EDTP*-overexpressing (bottom) samples at the adult age of day 10 (left) and 50 (right). (B') Quantification of relative PI3P area ratio. In panels A to B, RNAi constructs were driven by *App-Gal4*, green foci indicate 2xFYVE-GFP-labelled PI3P-positive structures, Hoechst staining (blue) indicates nuclei. Scale bars represent 40 μ m. On the plot, the boxes represent the most typical 50% of the samples, the line indicates the median, upper and lower whiskers show remaining 25%-25% of the samples. Circles mark outliers. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ at each comparison with day 1. For statistics, see the Materials and Methods.

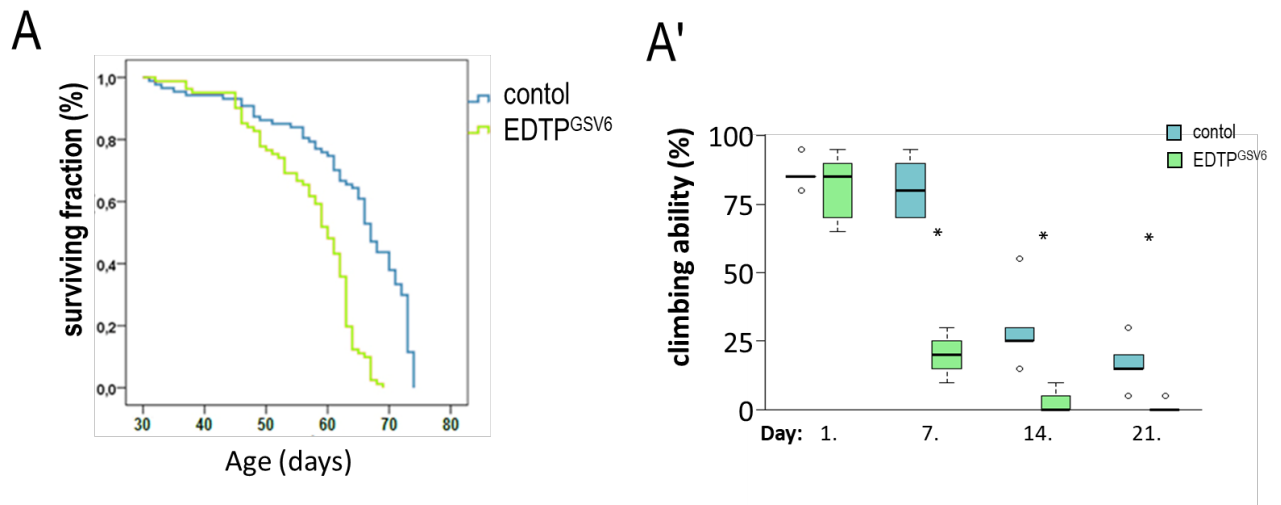
Figure S7.

Figure S7. EDTP hyperactivity limits lifespan and decreases climbing ability. (A) Animals over-expressing *EDTP* in the brain from the adult stage of 30th day live shorter than control. Kaplan-Meier lifespan curves of control and EDTP-overexpressing (*EDTP^{GSV6}*) animals. (A') The ability of animals to climb up on the wall of a glass vial becomes decreased in response to EDTP hyperactivity in brain neurons. On the plot, the boxes represent the most typical 50% of the samples, the line indicates the median, upper and lower whiskers show remaining 25%-25% of the samples. Circles mark outliers. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ at each comparison with day 1. For statistics, see the Materials and Methods.

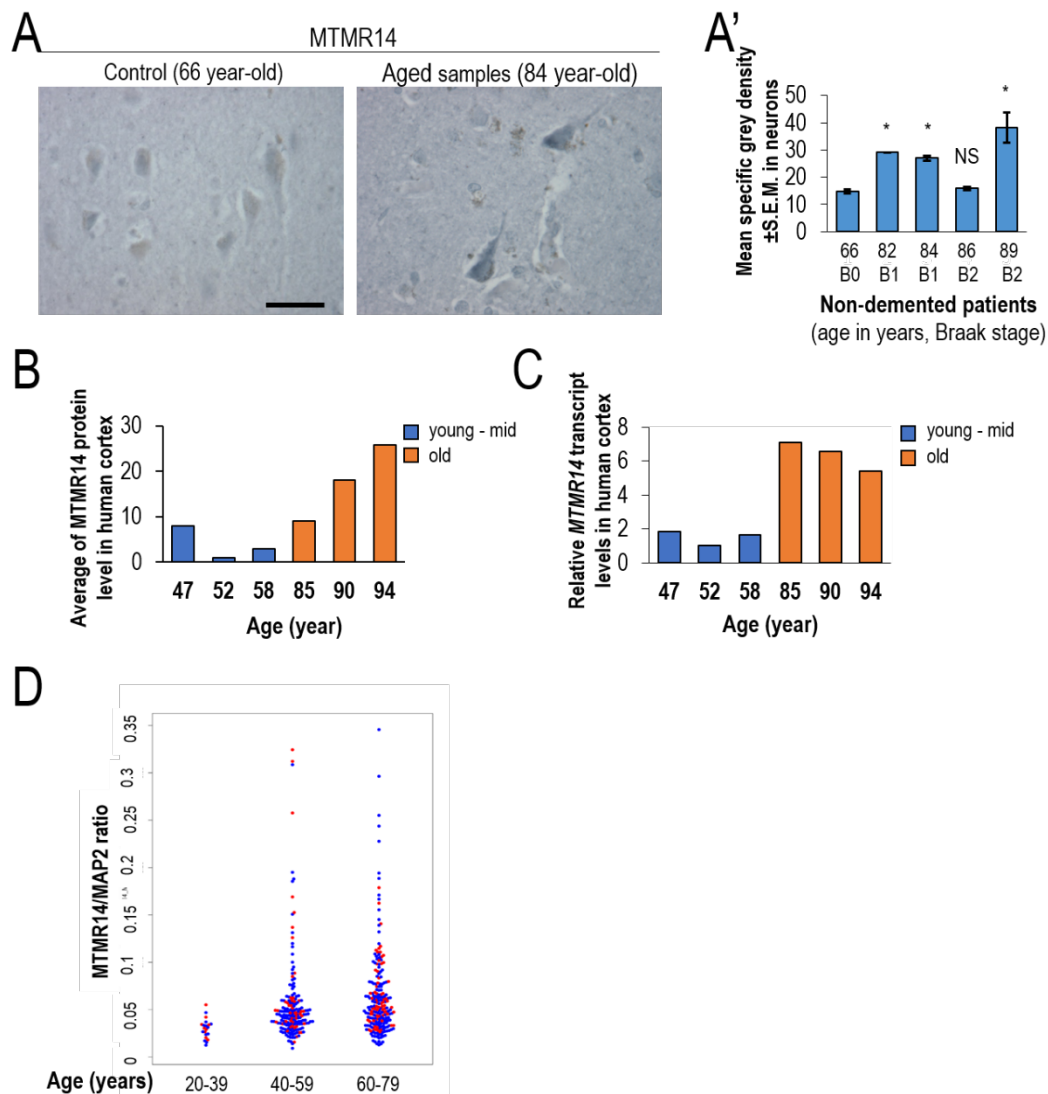
Figure S7.

Figure S7. MTMR14 accumulates in brain neurons at higher levels at advanced ages compared with younger stages. (A) Representative light microscopic immunohistochemical images from a 66-year-old male subject (left) and a 84-year-old male subject (right) demonstrate the accumulation of MTMR14 immunoreactivity as a function of age. Bar indicates 20 μ m. (A') Quantification of MTMR14 immunoreactivities. Bars represent specific mean density values \pm S.E.M.), *: $P < 0.001$ (mean specific grey density values that are significantly increased over the value of the 66 year-old individual). For statistics, see Table S4. (B) MTMR14 protein levels increase during ageing in human cortex. GAPDH was used as an internal control (western blot). (C) *MTMR14* transcript levels in human cortex increases with age (qPCR analysis). For statistics, see Table S5 and Materials and Methods. (D) The ratio of *MTMR14:MAP2* transcript levels was determined. Data were extracted from GTExPORTAL version 7 (<https://gtexportal.org/home/>). Input of 408 RNAseq samples (combined frontal

cortex, cortex and cingulate cortex) was grouped into 3 age groups (20-39, 40-59 and 60-79 years). MTMR14 expression levels were normalized to neuronal content, based on *MAP2* expression. Created beeswarm plots show male (blue) and females (red) dots. Groups and mean RNAseq data: 20-39 years, 0.0298; 40-59 years, 0.0573; 60-79 years, 0.065. Tukey was performed to find significant changes (Tukey multiple comparisons of means, 95% family-wise confidence level). Differences between groups: I. 40-59 years – 20-39 years, 0.027476363 (diff), 0.001779765 (lwr), 0.05317296 (upr), $P < 0.0328198$; II. 60-79 years – 20-39 years 0.035202536 (diff), 0.009747328 (lwr), 0.06065774 (upr), $P < 0.0035359$; III. 60-79 years – 40-59 years 0.007726173 (diff), 0.003638182 (lwr), 0.01909053 (upr), $P < 0.2471079$.

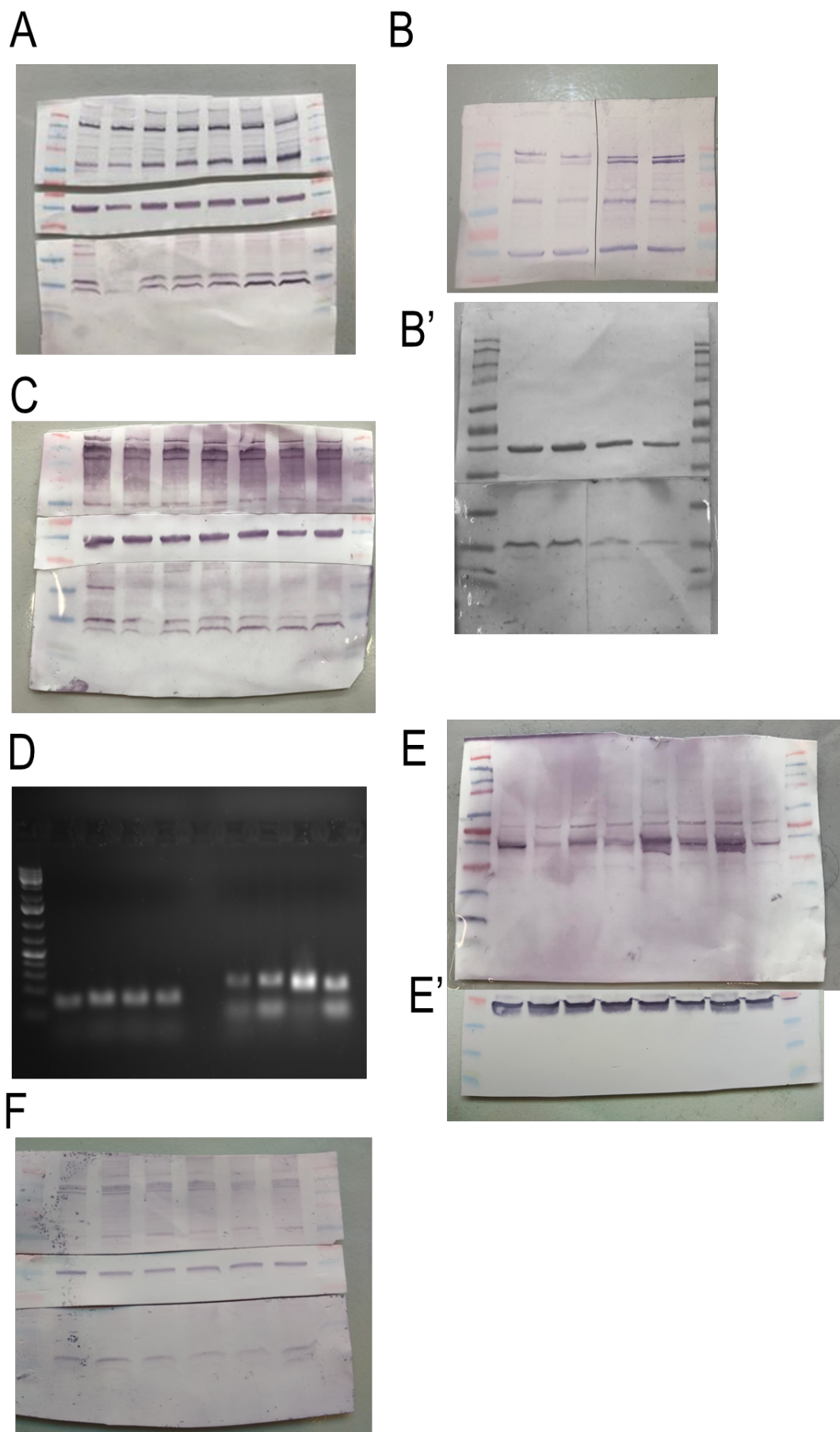


Figure S8. Original images of western blot and PCR results. The following images represent the original images of the western blot and PCR results: **A)** Figure 1 C, **B)** and **B')** Figure 4 C, **D)** Figure 4 D and E and **E'** Figure 6 C, **F)** Figure S. 3. After blotting, the membranes were cut into several parts because different proteins were labelled with different antibodies.

Supplementary Tables

Table S1. Statistics for lifespan data.

Genotype (crossing)	Number of flies examined	Number of repeats (vials)	Mean lifespan (days)	±S.E.M. (days)	Log Rang value	Mean survival Mann-Whitney U-test <i>P</i> value
for Figure 4C to C'						
ple-Gal4 x <i>eGFP</i> -RNAi (V22)	85	4	51.835	1.974	vs. <i>w¹¹¹⁸</i> <i>P</i> =0.018	
ple-Gal4 x <i>EDTP</i> -RNAi (V22)	176	5	55.312	1.389	vs. <i>w¹¹¹⁸</i> <i>P</i> =0.0001 vs. <i>eGFP</i> - <i>RNAi</i> <i>P</i> =0.017	vs. <i>w¹¹¹⁸</i> <i>P</i> =0.0007 vs. <i>eGFP</i> - <i>RNAi</i> <i>P</i> =0.0457
for Figure 4D to D'						
ple-Gal4 x <i>eGFP</i> -RNAi (V22)	185	5	48.346	1.454	vs. <i>w¹¹¹⁸</i> <i>P</i> =0.018	
ple-Gal4 x <i>EDTP</i> -RNAi (V22)	179	5	50.41	1.561	vs. <i>w¹¹¹⁸</i> <i>P</i> =0.002 vs. <i>eGFP</i> - <i>RNAi</i> <i>P</i> =0.0001	vs. <i>w¹¹¹⁸</i> <i>P</i> =0.0001 vs. <i>eGFP</i> - <i>RNAi</i> <i>P</i> =0.0489

ple-Gal4 x <i>EDTP</i> -RNAi (dsRNA)	134	4	51.441	1.263	vs. <i>w¹¹¹⁸</i> <i>P</i> =0.015 vs. <i>eGFP</i> - <i>RNAi</i> <i>P</i> =0.001 vs. <i>EDTP</i> - <i>RNAi(V22)</i> <i>P</i> =0.241	vs. <i>w¹¹¹⁸</i> <i>P</i> =0.0003 vs. <i>eGFP</i> - <i>RNAi</i> <i>P</i> =0.5104 vs. <i>EDTP</i> - <i>RNAi(V22)</i> <i>P</i> =0.62

Table S2. Clinical and vital characteristics of human subjects studied for SQSTM1/p62 immunoreactivity.

Subjects	Age (year)	Post-mortem delay (min)	Sex	Braak stage
Non-demented aged persons	77 ± 8	653 ± 478		
95/307	73	330	F	1
95/339	81	1335	F	1
96/030	68	630	F	2
96/049	86	315	F	1

Tissue samples were obtained from the Netherlands Brain Bank (Project 598/2009), Netherlands Institute for Neuroscience, Amsterdam. Averaged data are expressed as mean ± S.D. M: male, F: female.

Table S3. MTMR14 Immunoreactivity-specific optical intensity values in human cortical tissues.

Sample	counted cells	cortical tissues
SKO20/Br38	33	22.02 ± 8.77
SKO7/Br38	31	18.71 ± 6.68
SKO19/Br38	32	19.16 ± 5.43
SKO11/Br38	30	22.53 ± 8.31
SKO16/Br38	30	19.12 ± 5.97
SKO18/Br38	31	25.30 ± 8.17
statistical analysis (merged) form results of upper samples		
YOUNG	96	20.01 ± 7.15
OLD	91	22.35 ± 7.91*

Data are expressed as means of anti-MTMR14 immunofluorescence relative intensity values ±S.D. *: P=0.034 (independent samples t-test).

Table S4. Clinical characteristics of human subjects studied for MTMR14 immunoreactivity.

Subjects	Age (year)	Post-mortem delay (hour)	Sex	Category
SKO20/Br38	27	4-5 hours	M	YOUNG
SKO7/Br38	55	3-4 hours	M	YOUNG
SKO19/Br83	61	3 hours	F	YOUNG
SKO11/Br38	72	3 hours	M	OLD
SKO16/Br38	77	2-3 hours	M	OLD
SKO18/Br38	85	4-5 hours	M	OLD

Table S5. Clinical characteristics of human subjects studied for MTMR14 fluorescent microscopy, qPCR and Western blot analyses.

Subjects	Age (year)	Post-mortem delay (hour)	Sex	Category
Or F120 right	47	6 hours	M	MID
Or E965 right	52	8 hours	M	MID
Or F216 right	58	6 hours	F	MID
Or E692 left	85	8 hours	F	OLD
Or D259 left	90	4-5 hours	M	OLD
Y E249 left	94	4 hours	M	OLD

Table S6. Individual dog samples for determining age-associated *MTMR14* expression levels in the prefrontal cortex. For [Figure 6C](#), transcript levels were determined by qRT-PCR.

Table of individual dogs		
Breed	Age (years)	Category
golden retriever	17	OLD
small münsterlander	17	OLD
border collie	14	OLD
labrador	14	OLD
labrador	14	OLD
labrador	13	OLD
beagle	3	YOUNG
beagle	3	YOUNG
beagle	3	YOUNG
beagle	3	YOUNG
beagle	3	YOUNG

beagle	3	YOUNG
beagle	3	YOUNG
boxer	1	YOUNG
border collie	1	YOUNG