Science Advances
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Supplementary Materials for

Neuronal C/EBPβ/AEP pathway shortens life span via selective GABAnergic neuronal degeneration by FOXO repression

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

Figure S1

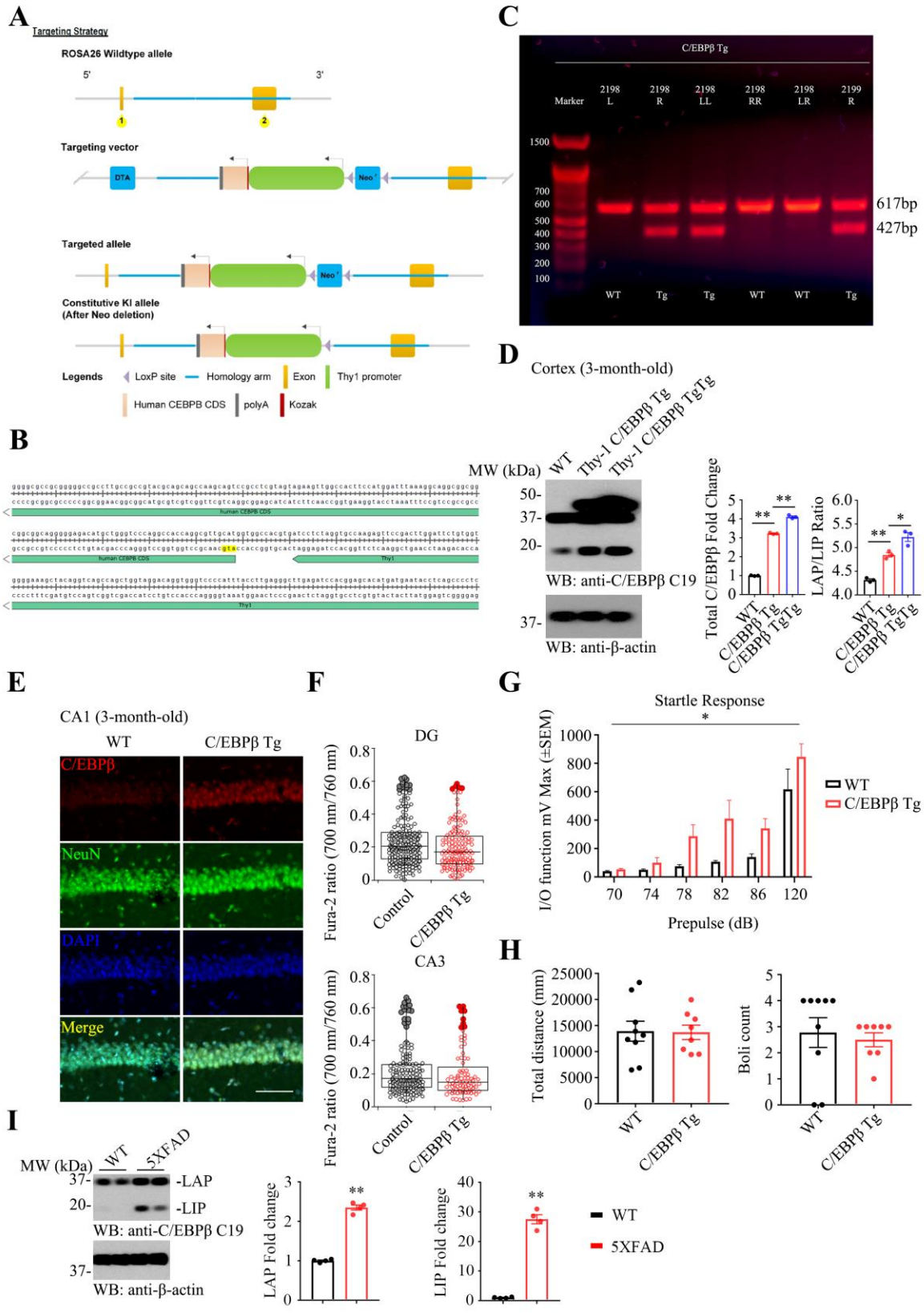

A
Targeting Strategy
ROSA26 Wildtype allele
5'
3'
1
2
Targeting vector
DTA
Neo
Targeted allele
Constitutive KI allele
(After Neo deletion)
Legends
LoxP site
Homology arm
Exon
Thy1 promoter
Human CEBPB CDS
polyA
Kozak
B
C
C/EBPβ Tg
Marker
2198 L
2198 R
2198 LL
2198 RR
2198 LR
2199 R
1500
700
600
500
400
300
200
100
617bp
427bp
WT
Tg
Tg
WT
WT
Tg
D
Cortex (3-month-old)
MW (kDa)
WT
Thy-1 C/EBPβ Tg
Thy-1 C/EBPβ TgTg
50-
37-
20-
WB: anti-C/EBPβ C19
37-
WB: anti-β-actin
Total C/EBPβ Fold Change
LAP/LIP Ratio
WT
C/EBPβ Tg
C/EBPβ TgTg
E
CA1 (3-month-old)
WT
C/EBPβ Tg
C/EBPβ
NeuN
DAPI
Merge
F
DG
Fura-2 ratio (700 nm/760 nm)
Control
C/EBPβ Tg
CA3
G
Startle Response
I/O function mV Max (±SEM)
Prepulse (dB)
WT
C/EBPβ Tg
H
Total distance (mm)
Boli count
I
MW (kDa)
WT
5XFAD
37-
-LAP
20-
-LIP
WB: anti-C/EBPβ C19
37-
WB: anti-β-actin
LAP Fold change
LIP Fold change
WT
5XFAD

Fig S1. Characterization of Thy1-C/EBPβ transgenic mice.

A. Schematic diagram of the targeting strategy and allele. **B.** Key sequence of the transgenic gene, Thy1 promoter and human C/EBPβ CDS are connected with a Kozak motif. **C.** Genotyping of the C/EBPβ Tg mice, WT=617 bp band, Tg=427 bp band. **D.** WB verification of human C/EBPβ expression in Thy1 C/EBPβ Tg mice brains. Human C/EBPβ MW ~ 45 kDa, mouse C/EBPβ ~ 37 kDa. Total C/EBPβ change and the LAP/LIP ratios were calculated from quantification by immunoblots, Data are represented as mean ± s.e.m., n=3 per group. *p<0.05, **p<0.01; two-way ANOVA and Bonferroni's post hoc test. **E.** Colocalization of C/EBPβ and NeuN in the CA1 region of 3-month-old mice. C/EBPβ (Red), NeuN (Green), DAPI (Blue), Scale bar, 100 μm. **F.** No significant difference in $[Ca^{2+}]_i$ in DG or CA3 determined by two-photon Ca^{2+} imaging in acute brain slice loaded with Fura-2. The box plots indicate first, median, and third quatiles as well as outliers. *N*=194 and 148 (WT control), 138 and 84 cells (C/EBPβ Tg) pooled from 6 animals each, $p>0.05$ and 0.5, $t(330)=1.91$ and $t(230)=0.67$ for DG and CA3, respectively, two-tailed Student's *t*-test. **G.** I/O function of Prepulse Inhibition (PPI) showed that C/EBPβ Tg mice were increased in hearing sensitivity vs. WT mice (mean ± s.e.m.; n = 8 mice per group; unpaired t test with Welch's correction). **H.** Open field test, no difference between WT and C/EBPβ Tg mice in total distance and boli count (mean ± s.e.m.; n = 8 mice per group; unpaired t test with Welch's correction). **I.** 12-months-old 5XFAD and its WT littermate brain tissue were used to detect C/EBPβ by WB. both C/EBPβ LAP and LIP were upregulated by Aβ in AD mouse model. Data are represented as mean ± s.e.m., n=4 per group. **p<0.01; two-tailed Student's t-test.

Figure S2

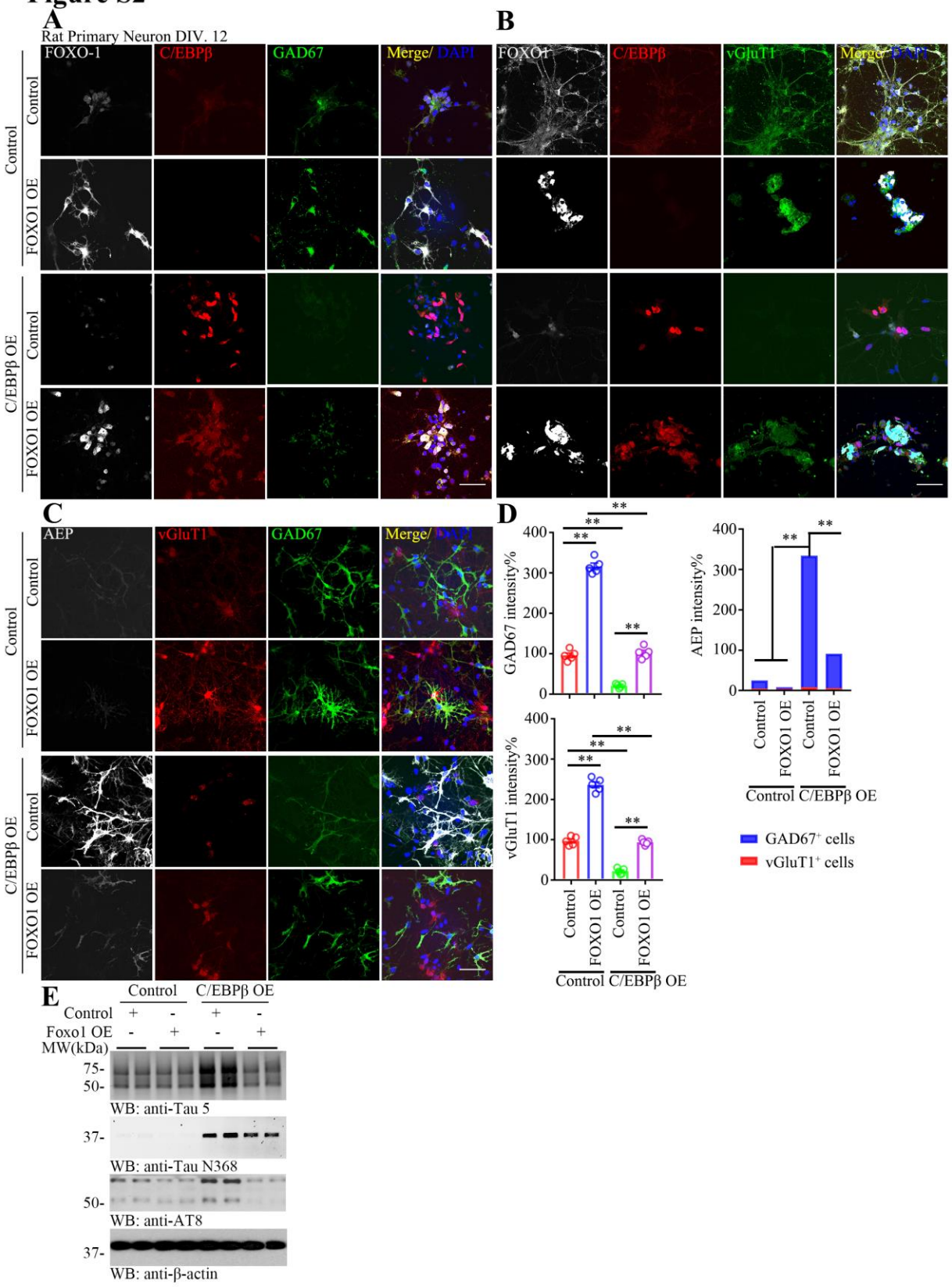

A
Rat Primary Neuron DIV. 12
FOXO-1
C/EBPβ
GAD67
Merge/ DAPI
Control
FOXO1 OE
C/EBPβ OE
B
FOXO1
C/EBPβ
vGluT1
Merge/ DAPI
C
AEP
vGluT1
GAD67
Merge/ DAPI
D
GAD67 intensity%
vGluT1 intensity%
AEP intensity%
Control
FOXO1 OE
Control C/EBPβ OE
GAD67+ cells
vGluT1+ cells
E
Control
C/EBPβ OE
Foxo1 OE
MW(kDa)
75-
50-
WB: anti-Tau 5
37-
WB: anti-Tau N368
50-
WB: anti-AT8
37-
WB: anti-β-actin

Fig S2. C/EBPβ overexpression selectively augments AEP levels in GABAnergic neurons

A-B. C/EBPβ overxpression decreases Glutamatergic or GABAnergic neurons, which is reversed by FOXO1 overexpression. Rat primary neurons (DIV. 12) were infected with FOXO1 or C/EBPβ lentivirus for 72 h. Neurons were fixed and permeabilized, cells were incubated with FOXO1, C/EBPβ, vGlut1 and GAD67 antibodies. vGlut1 marked the Glutamatergic neurons, GAD67 marked GABAnergic neurons. The nuclei were stained with DAPI. Control=Vector virus (Scale bar: 50 μm). **C.** C/EBPβ selectively augments AEP in GABAnergic versus Glutamatergic neurons. Rat primary neurons (DIV. 12) were infected with FOXO1 or C/EBPβ lentivirus for 72 h. Neurons were fixed and permeabilized, cells were incubated with AEP, vGlut1 and GAD67 antibodies. vGlut1 marked the Glutamatergic neuron, GAD67 marked GABAergic neuron. The nuclei were stained with DAPI. Control=Vector virus (Scale bar: 50 μm.). **D.** Quantification of GAD67 and vGlut1 fluorescent intensities induced by C/EBPβ and FOXO1 overexpression (Left, mean ± s.e.m.; n = 6; **, $p < 0.01$; two-way ANOVA and Sidak's multiple comparison test). Quantification of AEP fluorescent intensities in neurons induced by C/EBPβ and FOXO1 overexpression, percentage of Glutamatergic or GABAnergic neurons also showed. (Right, mean ± s.e.m.; n = 6; **, $p < 0.01$; unpaired t test with Welch's correction). **E.** Immunoblotting from C/EBPβ overexpressed neurons. WB showed the cleavage of Tau N368 in neurons by active AEP and tau phosphorylation induced by C/EBPβ or FOXO1 overexpression.

Figure S3

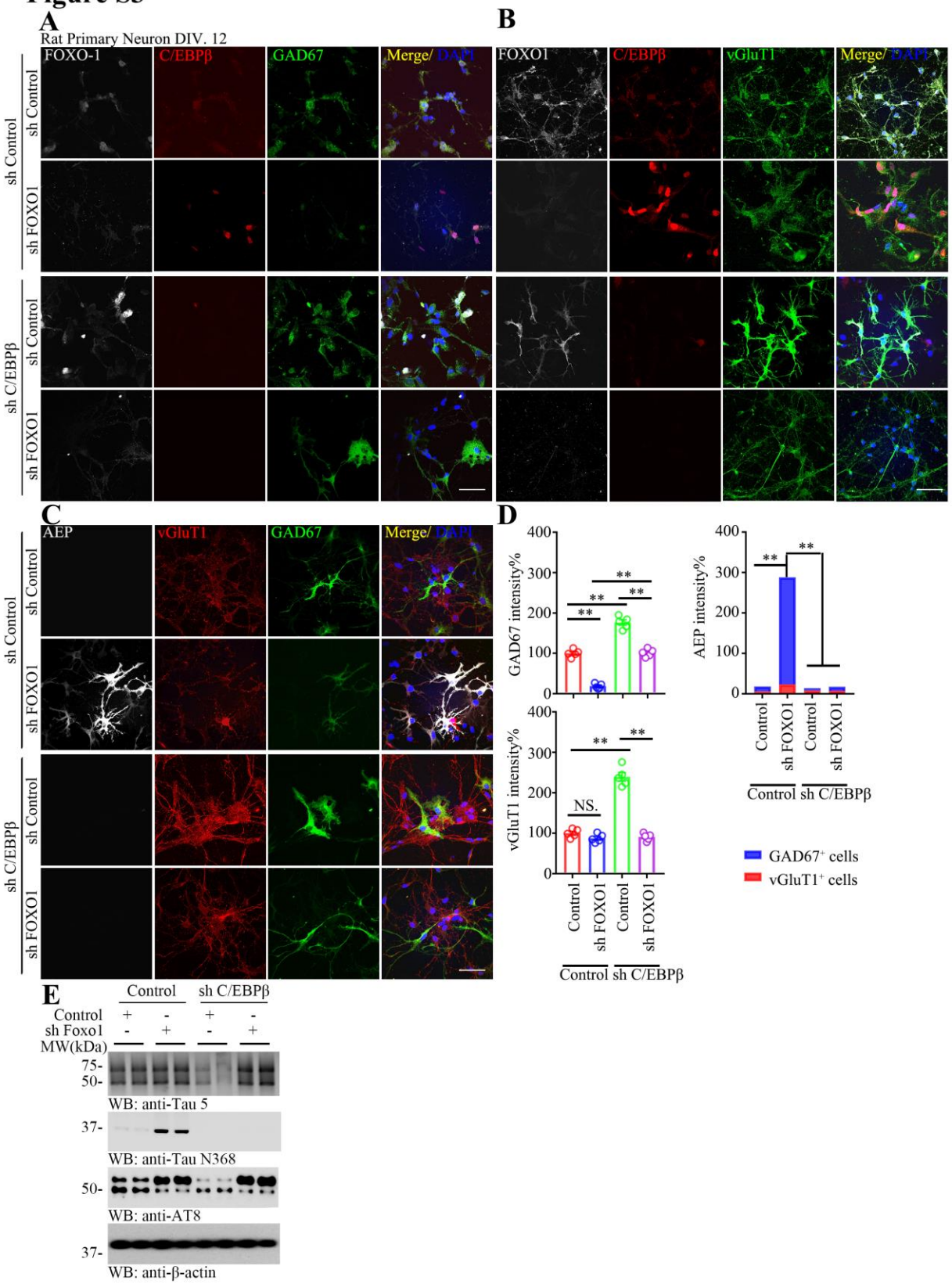

A
Rat Primary Neuron DIV. 12
FOXO-1
C/EBPβ
GAD67
Merge/ DAPI
sh Control
sh FOXO1
sh C/EBPβ
B
FOXO1
C/EBPβ
vGluT1
Merge/ DAPI
C
AEP
vGluT1
GAD67
Merge/ DAPI
D
GAD67 intensity%
vGluT1 intensity%
AEP intensity%
NS.
Control
sh FOXO1
sh C/EBPβ
GAD67+ cells
vGluT1+ cells
E
Control
sh C/EBPβ
sh Foxo1
MW(kDa)
75-
50-
37-
WB: anti-Tau 5
WB: anti-Tau N368
WB: anti-AT8
WB: anti-β-actin

Fig S3. FOXO1 deletion increases C/EBPβ and AEP levels in GABAnergic neurons.

A-B. Immunofluorescent co-staining. Knocking down FOXO1 and C/EBPβ regulated GAD67 and vGlut1 expression in Glutamatergic or GABAergic neurons. Rat primary neurons (DIV. 12) were infected with sh-FOXO1 or sh-C/EBPβ lentivirus for 72 h. Neurons were fixed and permeabilized, and cells were incubated with FOXO1, C/EBPβ, vGlut1 and GAD67 antibodies. vGlut1 marked the Glutamatergic neurons, GAD67 marked GABAergic neurons. The nuclei were stained with DAPI. Control=Vector virus (Scale bar: 50 μm). **C.** Knocking down FOXO1 selectively increases AEP expression in GABAnergic versus Glutamatergic neurons, which is abolished by depletion of C/EBPβ. Rat primary neurons (DIV. 12) were infected with sh-FOXO1 or sh-C/EBPβ lentivirus for 72 h. Neurons were fixed and permeabilized, cells were incubated with AEP, vGlut1 and GAD67 antibodies. vGlut1 marked the Glutamatergic neurons, GAD67 marked GABAergic neurons. The nuclei were stained with DAPI. Control=Vector virus (Scale bar: 50 μm). **D.** Quantification of GAD67 or vGlut1 fluorescent intensities induced by C/EBPβ knock down (Left, mean ± s.e.m.; n = 6; **, $p < 0.01$; two-way ANOVA and Sidak's multiple comparison test). Quantification of AEP fluorescent intensities in neurons infected with sh-FOXO1 or sh-C/EBPβ, percentage of Glutamatergic or GABAnergic neurons also showed. (Right, mean ± s.e.m.; n = 6; **, $p < 0.01$; unpaired t test with Welch's correction). **E.** WB showed the cleavage of Tau N368 by active AEP and tau phosphorylation induced by C/EBPβ or FOXO1 knock down.

Figure S4

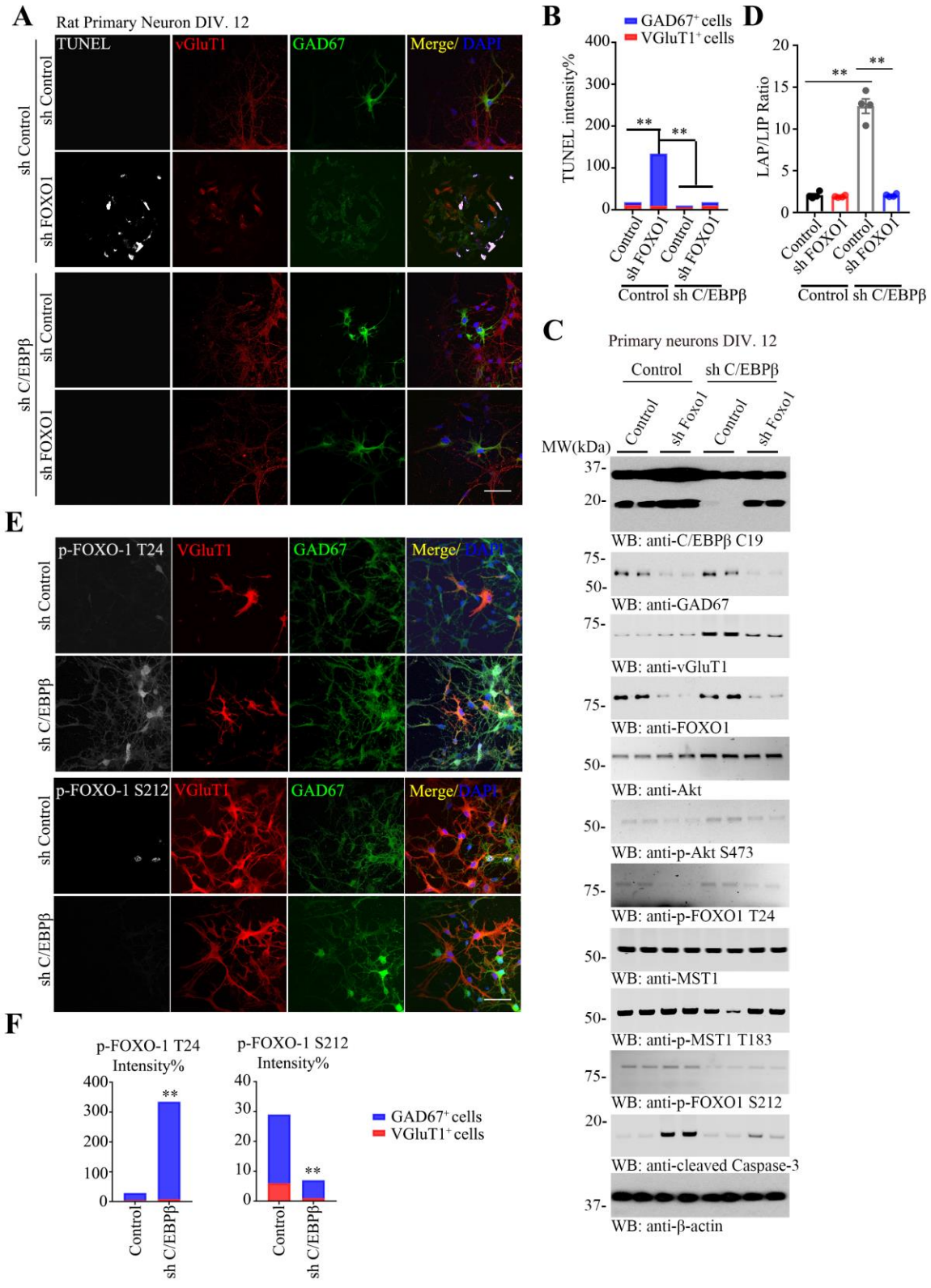

A
Rat Primary Neuron DIV. 12
TUNEL
vGluT1
GAD67
Merge/ DAPI
sh Control
sh FOXO1
sh C/EBPβ
B
GAD67+ cells
VGluT1+ cells
TUNEL intensity%
Control
sh FOXO1
sh C/EBPβ
D
LAP/LIP Ratio
C
Primary neurons DIV. 12
MW(kDa)
Control
sh Foxo1
WB: anti-C/EBPβ C19
WB: anti-GAD67
WB: anti-vGluT1
WB: anti-FOXO1
WB: anti-Akt
WB: anti-p-Akt S473
WB: anti-p-FOXO1 T24
WB: anti-MST1
WB: anti-p-MST1 T183
WB: anti-p-FOXO1 S212
WB: anti-cleaved Caspase-3
WB: anti-β-actin
E
p-FOXO-1 T24
VGluT1
GAD67
Merge/ DAPI
p-FOXO-1 S212
F
p-FOXO-1 T24 Intensity%
p-FOXO-1 S212 Intensity%
GAD67+ cells
VGluT1+ cells

Fig S4. Knocking down neuronal C/EBPβ protects GABAnergic neuronal degeneration induced by FOXO1 depletion.

A. C/EBPβ knockdown inhibits GABAergic neuronal apoptosis. Rat primary neurons (DIV. 12) were infected with sh-FOXO1 or sh-C/EBPβ lentivirus for 72 h. Neurons were fixed and permeabilized. After TUNEL staining, cells were incubated with vGlut1 and GAD67 antibodies. After incubated with 2 secondary antibodies conjugated with Alexa Fluor^{TM}-488 or Cy5 for 1 h respectively, neurons were analyzed under a confocal microscope. vGlut1 marked the Glutamatergic neurons, GAD67 marked GABAnergic neurons. The nuclei were stained with DAPI. Arrows indicate the merged part, respectively. Control=Vector virus (Scale bar: 50 μm). **B.** Quantification of TUNEL-positive neurons induced by FOXO1 or C/EBPβ knockdown, percentage of Glutamatergic or GABAergic neurons also showed. (mean ± s.e.m.; n = 6; ** $p < 0.01$; unpaired t test with Welch's correction). **C.** Immunoblotting analysis from neurons infected with virus expressing of sh-C/EBPβ. C/EBPβ knockdown promoted Akt activation and FOXO1 T24 phosphorylation and inhibited MST1 activation and FOXO1 S212 phosphorylation. Western blot was conducted with various indicated antibodies. **D.** The LAP/LIP isoform ratios were calculated from quantification by immunoblots of C, Data are represented as mean ± s.e.m., n=4 per group. **$p<0.01$; two-way ANOVA and Bonferroni's post hoc test. **E.** Depletion of C/EBPβ mediates p-FOXO1 status. C/EBPβ knockdown promoted FOXO1 T24 phosphorylation in GABAnergic other than Glutamatergic neurons. Rat primary neurons (DIV. 12) were infected with sh-vector or sh-C/EBPβ lentivirus for 72 h. Neurons were fixed and permeabilized, and cells were incubated with p-FOXO1, vGlut1 and GAD67 antibodies. After incubated with 3 secondary antibodies conjugated with Alexa Fluor^{TM}-488, -594 or -Cy5 for 1 h, respectively, neurons were analyzed under a confocal microscope. vGlut1 marked the Glutamatergic neurons, GAD67 marked GABAnergic neurons. The nuclei were stained with DAPI. Arrows indicate the merged part, respectively. Control=Vector virus (Scale bar: 50 μm). **F.** Quantification of p-FOXO1 T24 or p-FOXO1-S212-positive neurons induced by C/EBPβ knockdown, percentage of Glutamatergic or GABAergic neurons also showed. (mean ± s.e.m.; n = 6; **, $p < 0.01$; unpaired t test with Welch's correction).

Figure S5

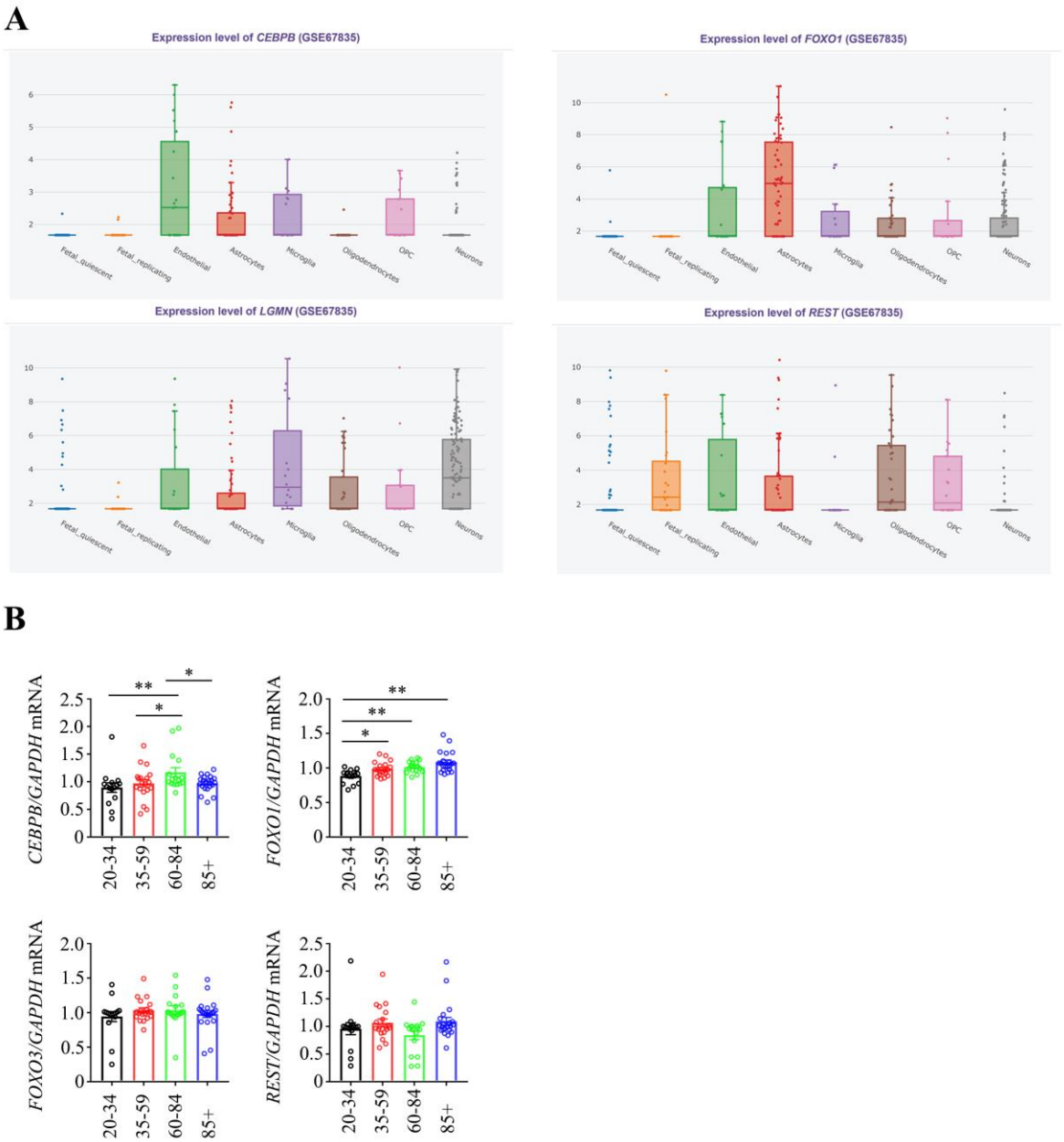


Fig S5. Analysis of *CEBPB, FOXO1, LGMN,* and *REST* genes expression in the brain

A. Single cell RNA-seq of different cell types from the human brain. (Data set ID: GSE67835, online program http://www.alzdata.org/single_RNAseq1.php). Shown the expression levels of *CEBPB*, *FOXO1*, *LGMN*, and *REST* in different brain cell types. **B.** Expression of *CEBPB*, *FOXO1*, *FOXO3*, and *REST* in different ages of individuals (20-34 years old, n=15; 35-59 years old, n=19; 60-84 years old, n=16; 85+ years old, n=21) in the cohorts (Data set ID: GDS5204 &GDS707). Data are represented as mean ± s.e.m.. *p<0.05, **p <0.01, two-way ANOVA and Bonferroni's post hoc test.

Figure S6

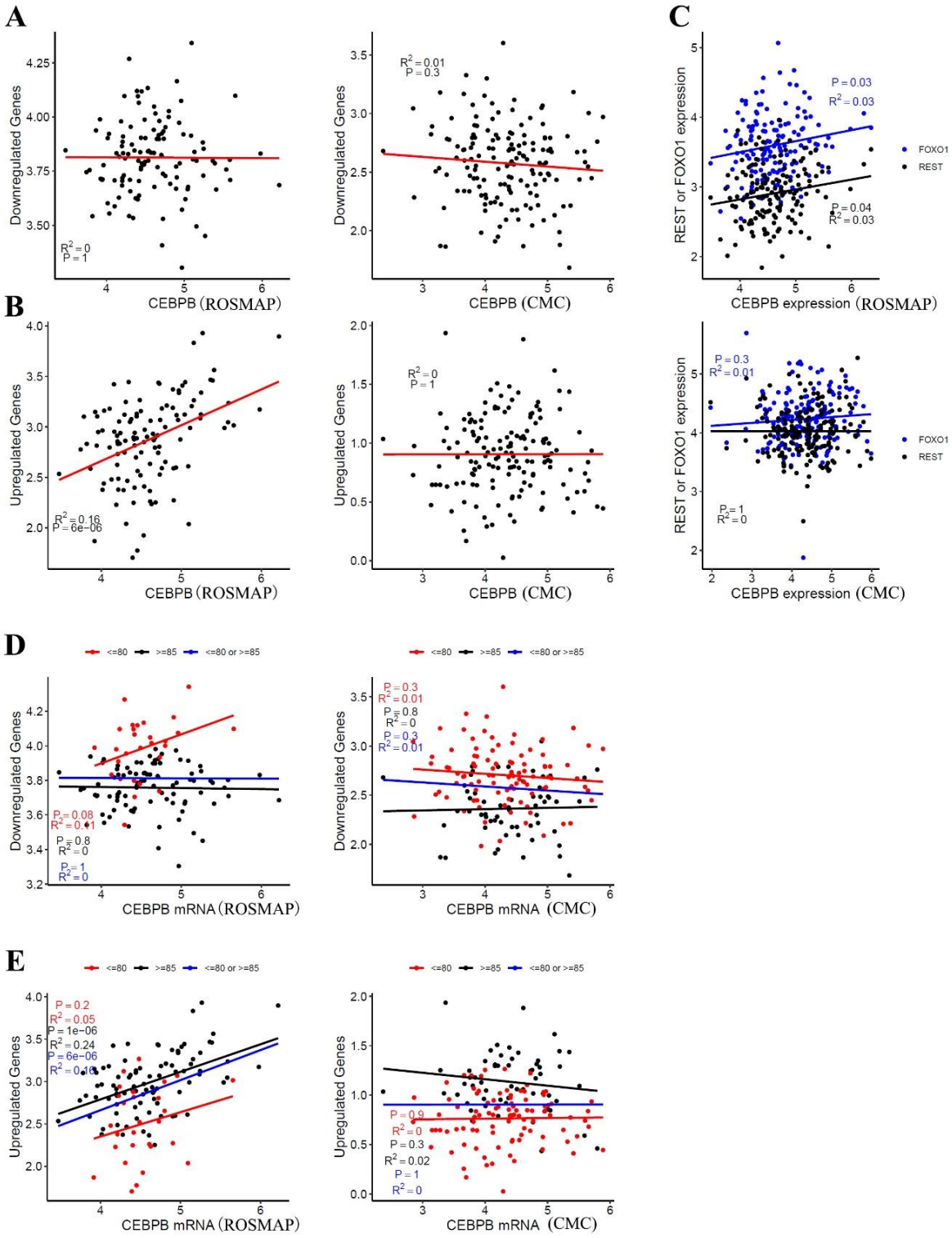

A
B
C
D
E
Downregulated Genes
Upregulated Genes
CEBPB (ROSMAP)
CEBPB (CMC)
REST or FOXO1 expression
CEBPB expression (ROSMAP)
CEBPB expression (CMC)
FOXO1
REST
<=80
>=85
<=80 or >=85
CEBPB mRNA (ROSMAP)
CEBPB mRNA (CMC)

Fig S6. Partitioning of the ageing human population for analysis of gene expression in the brain related to C/EBPβ

A. Expression of genes downregulated in individuals with extended longevity (≥85 versus ≤80 years old) in the ROSMAP (n = 117 individuals) and CMC (n = 155 individuals) cohorts. There is no correlation to levels of *CEBPB* mRNA. Linear regression analysis of the mean expression of all downregulated genes, p values derived by t-tests of the regression line slopes. **B.** Expression of genes upregulated in individuals with extended longevity (≥85 versus ≤80 years old) in the ROSMAP (n = 117 individuals) and CMC (n = 155 individuals) cohorts. *CEBPB* mRNA is significantly associated with upregulated genes in ROSMAP, but insignificant in CMC. Linear regression analysis of the mean expression of all downregulated genes, p values derived by t-tests of the regression line slopes. **C.** Borderline significant associations with *REST* and *FOXO1* in ROSMAP, but not significant in CMC. **D.** Expression of genes downregulated in individuals with different ages (≥85 or ≤80 years old) in the ROSMAP (n = 117 individuals), and CMC (n = 155 individuals) cohorts. **E.** Expression of genes upregulated in individuals with different ages (≥85 or ≤80 years old) in the ROSMAP (n = 117 individuals) and CMC (n = 155 individuals) cohorts. *CEBPB* mRNA is significantly associated with upregulated genes in ROSMAP with extended longevity.

Figure S7

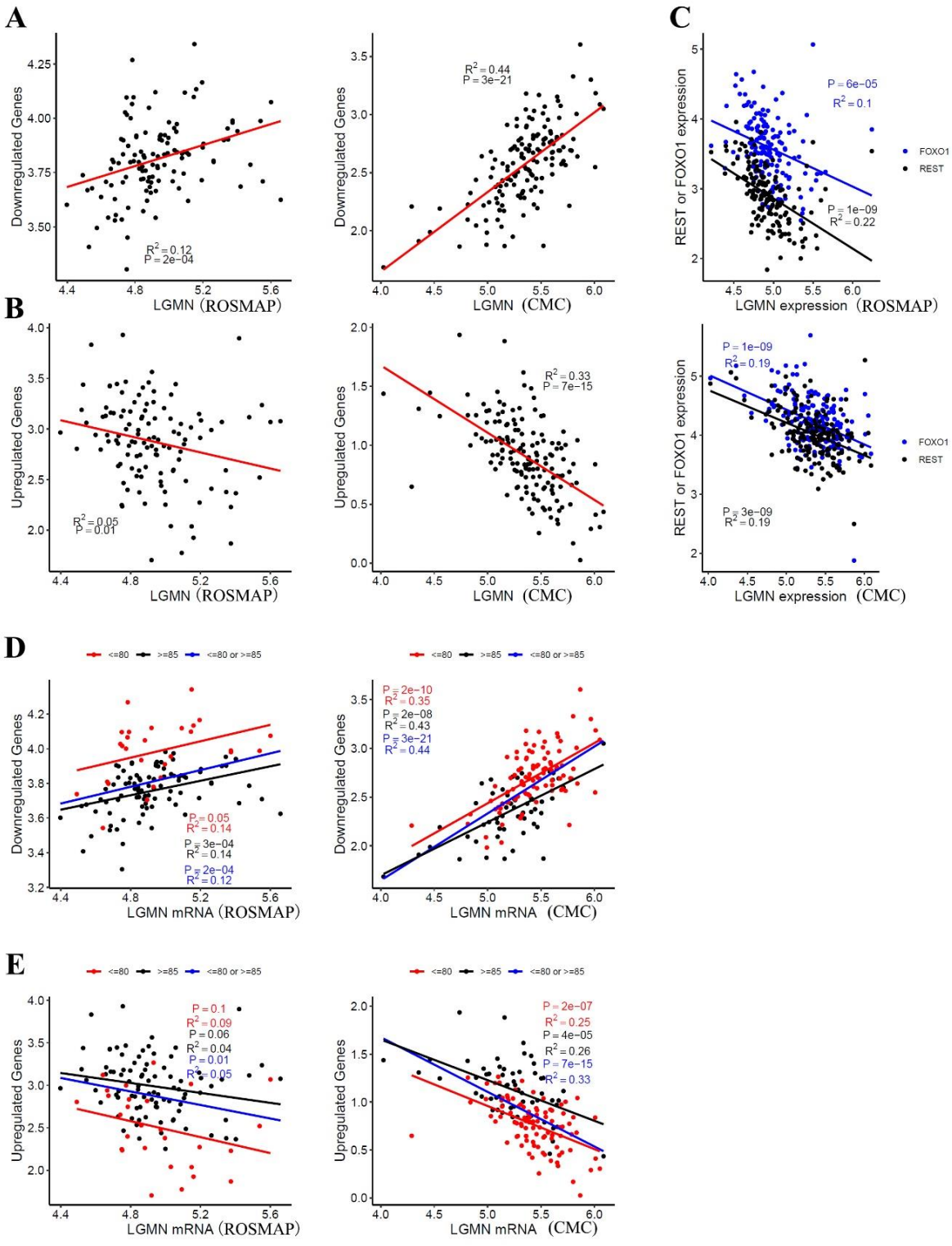
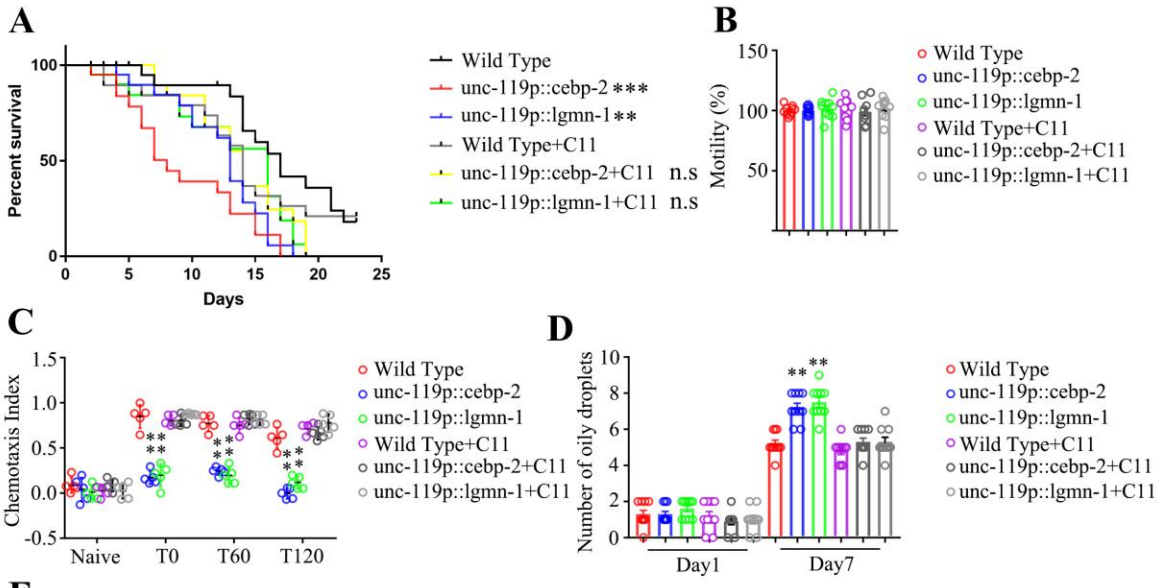

A
B
C
D
E
Downregulated Genes
Upregulated Genes
LGMN (ROSMAP)
LGMN (CMC)
R2 = 0.12
P = 2e−04
R2 = 0.44
P = 3e−21
R2 = 0.05
P = 0.01
R2 = 0.33
P = 7e−15
REST or FOXO1 expression
LGMN expression (ROSMAP)
LGMN expression (CMC)
P = 6e−05
R2 = 0.1
P = 1e−09
R2 = 0.22
P = 1e−09
R2 = 0.19
P = 3e−09
R2 = 0.19
FOXO1
REST
<=80
>=85
<=80 or >=85
LGMN mRNA (ROSMAP)
LGMN mRNA (CMC)
P = 0.05
R2 = 0.14
P = 3e−04
R2 = 0.14
P = 2e−04
R2 = 0.12
P = 2e−10
R2 = 0.35
P = 2e−08
R2 = 0.43
P = 3e−21
R2 = 0.44
P = 0.1
R2 = 0.09
P = 0.06
R2 = 0.04
P = 0.01
R2 = 0.05
P = 2e−07
R2 = 0.25
P = 4e−05
R2 = 0.26
P = 7e−15
R2 = 0.33

Fig S7. Partitioning of the ageing human population for analysis of gene expression in the brain related to AEP

A. Expression of genes downregulated in individuals with extended longevity (≥85 versus ≤80 years old) in the ROSMAP (n = 117 individuals) and CMC (n = 155 individuals) cohorts. There is significant association with downregulated in ROSMAP and CMC related to levels of *LGMN* mRNA. Linear regression analysis of the mean expression of all downregulated genes, p values derived by t-tests of the regression line slopes. **B.** Expression of genes upregulated in individuals with extended longevity (≥85 versus ≤80 years old) in the ROSMAP (n = 117 individuals) and CMC (n = 155 individuals) cohorts. *LGMN* mRNA is significant association with upregulated in CMC, but only borderline significant in ROSMAP. Linear regression analysis of the mean expression of all downregulated genes, p values derived by t-tests of the regression line slopes. **C.** Significant associations with *REST* and *FOXO1* in ROSMAP and CMC. **D.** Expression of genes downregulated in individuals with different ages (≥85 or ≤80 years old) in the ROSMAP (n = 117 individuals) and CMC (n = 155 individuals) cohorts. *LGMN* mRNA is in significant association with downregulated genes in ROSMAP and CMC. **E.** Expression of genes upregulated in individuals with different ages (≥85 or ≤80 years old) in the ROSMAP (n = 117 individuals) and CMC (n = 155 individuals) cohorts. *LGMN* mRNA is in significant association with upregulated in ROSMAP and CMC. There is evidence for a consistent association between *LGMN* expression and *DEGs*, *REST*, and *FOXO1*.

Figure S8



E

Alignment of AEP in different species

					Gene	Organism
NP_001008530.1	183	VFYIEACESGSMMNH-LPDNINVYATTAANPRESSYACYYDE-------K	224		LGMN	H.sapiens
XP_001148301.1	183	VFYIEACESGSMMNH-LPDNINVYATTAANPRESSYACYYDE-------K	224		LGMN	P.troglodytes
XP_001092047.1	183	VFYIEACESGSMMNH-LPDNINVYATTAANPRESSYACYYDE-------K	224		LGMN	M.mulatta
XP_537355.2	183	VFYIEACESGSMMRH-LPSNINVYATTAANPSESSYACYYDE-------K	224		LGMN	C.lupus
NP_776526.1	183	VFYIEACESGSMMNH-LPPDINVYATTAANPRESSYACYYDE-------Q	224		LGMN	B.taurus
NP_035305.1	185	VFYIEACESGSMMNH-LPDDINVYATTAANPKESSYACYYDE-------E	226		Lgmn	M.musculus
NP_071562.2	185	VFYIEACESGSMMNH-LPDDIDVYATTAANPNESSYACYYDE-------E	226		Lgmn	R.norvegicus
XP_421328.1	183	VFYIEACESGSMMNH-LADNINVYATTAANPRESSYACYYDD-------E	224		LGMN	G.gallus
NP_999924.1	185	VFYVEACESGSMMKP-LPVDINVYATTAANPDESSYACYYDE-------A	226		lgmn	D.rerio
NP_506137.1	196	TFYLEACESGSMFEEVLRSDMDIYAISAANSHESSWGTFCEN-------D	238		lgmn-1	C.elegans
NP_188656.1	200	VIYVEACESGSMFEGILKKNLNIYAVTAANSKESSWGVYCPESYPPPPSE	249		DELTA-VPE	A.thaliana
NP_001005720.1	183	VFYIEACESGSMMNH-LPNNINVYATTAANSHESSYACYYDE-------K	224		lgmn	X.tropicalis

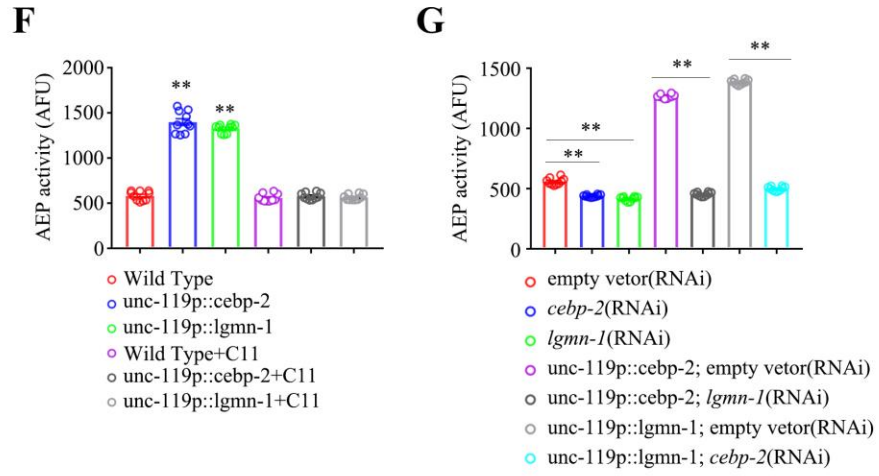


Fig S8. AEP (LGMN-1) inhibitor C11 reverses the short lifespan induced by neuronal overexpression of *lgmn-1* or *cebp-2*.

A. AEP inhibitor compound #11 **(**C11) reverses the short lifespan induced by neuronal overexpression *lgmn-1* or *cebp-2*. n = 30 worms per group. p <0.0001, log-rank test, ** p<0.01, *** p<0.001 vs. N2. **B.** C11 does not affect whole animal locomotion. Day 2 worms were transferred to M9 liquid and thrashing rate was assessed. Shown are mean motility scores for the first 30 s. n=10 worms per group. Mann–Whitney U-test with multiple testing correction by Holm's method. **C.** C11 restores cognitive function. C11 treatment reversed the defective positive butanone association scores induced by neuronal overexpression of *lgmn-1* or *cebp-2*. For each time point (T0 = 0, T60 = 60, and T120 = 120 min after conditioning), the CI values of [unc-119p::cebp-2 + C11] and [unc-119p::lgmn-1 + C11] are similar to those of wild type. unc-119p::cebp-2 and unc-119p::lgmn-1 show impaired learning and memory compared with wild type. Data were analyzed by two-way ANOVA and Sidak's multiple comparison test, represented of five independent experiments. (n = 14-20 worms per experiment). Color circles represent individual CIs. **D.** C11 decreases age-associated oily droplet accumulation between the pharyngeal bulbs induced by neuronal overexpression of *lgmn-1* or *cebp-2*. Data are represented as mean ± s.e.m. (days 7; two-way ANOVA and Sidak's multiple comparison test; ** p < 0.01 vs. N2). **E.** Alignment of AEP protein sequences in different species shows that the protease enzymatic domain is highly conserved. **F.** Quantification of C11's inhibitory effect in AEP protease activities. Day 2 worm's protein lysates were collected for AEP activity assay. Data are represented as mean ± s.e.m., two-way ANOVA and Sidak's multiple comparison test; ** p < 0.01 vs. N2. **G.** Quantification of RNAi's inhibitory effect in AEP protease activities. Day 2 worm's protein lysates were collected for AEP activity assay. Data are represented as mean ± s.e.m., two-way ANOVA and Sidak's multiple comparison test; ** p < 0.01.

Figure S9

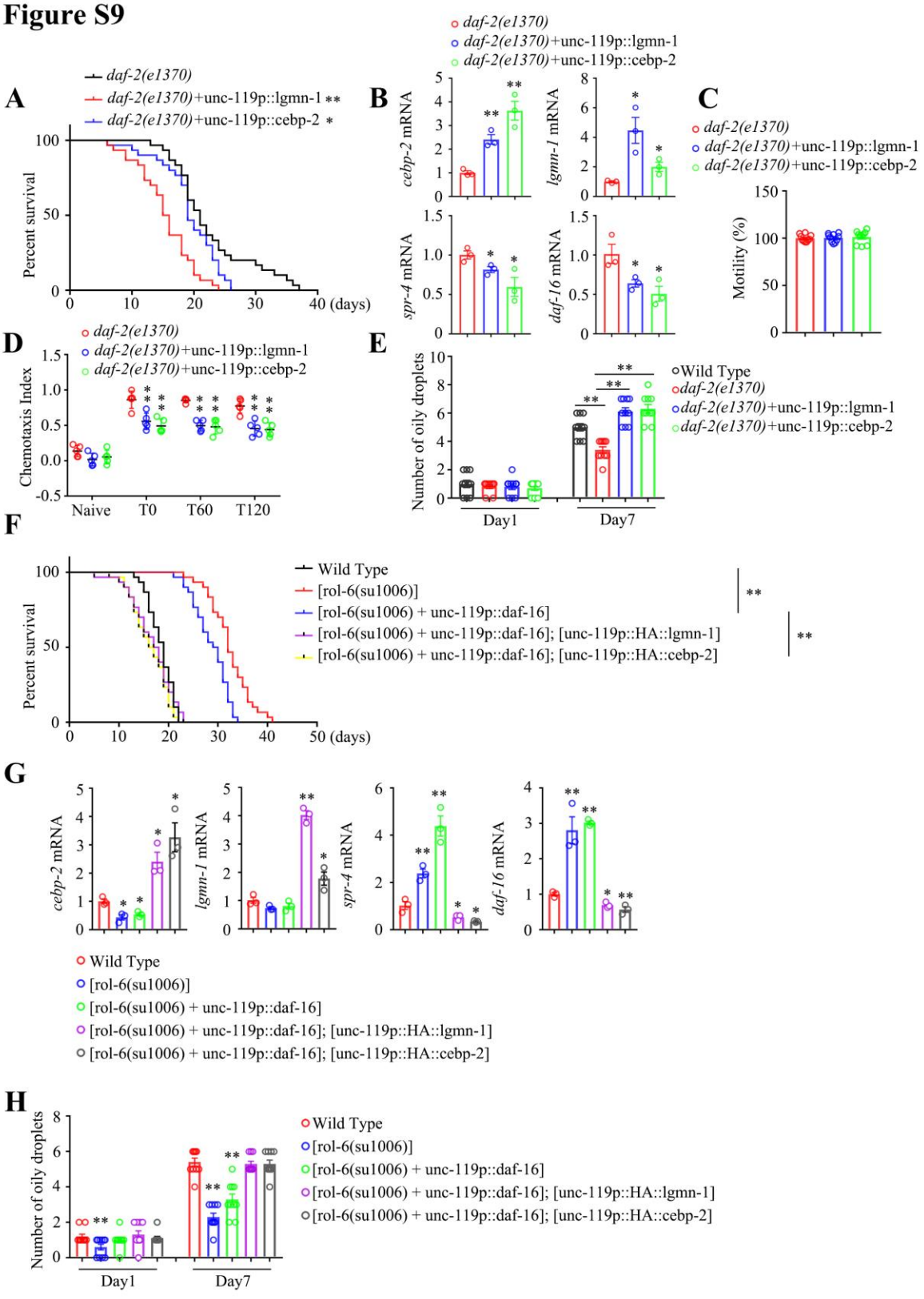

A
daf-2(e1370)
daf-2(e1370)+unc-119p::lgmn-1 **
daf-2(e1370)+unc-119p::cebp-2 *
Percent survival
40 (days)
B
daf-2(e1370)
daf-2(e1370)+unc-119p::lgmn-1
daf-2(e1370)+unc-119p::cebp-2
cebp-2 mRNA
lgmn-1 mRNA
spr-4 mRNA
daf-16 mRNA
C
Motility (%)
D
Chemotaxis Index
Naive
T0
T60
T120
E
Number of oily droplets
Day1
Day7
Wild Type
F
Percent survival
50 (days)
Wild Type
[rol-6(su1006)]
[rol-6(su1006) + unc-119p::daf-16]
[rol-6(su1006) + unc-119p::daf-16]; [unc-119p::HA::lgmn-1]
[rol-6(su1006) + unc-119p::daf-16]; [unc-119p::HA::cebp-2]
G
cebp-2 mRNA
lgmn-1 mRNA
spr-4 mRNA
daf-16 mRNA
H
Number of oily droplets
Day1
Day7

Fig S9. Neuronal *lgmn-1* or *cebp-2* overexpression shortens the lifespan extended by loss of function of *daf-2* or overexpression of *daf-16*.

A. Neuronal *lgmn-1* or *cebp-2* overexpression shortens the lifespan extended by loss of function of *daf-2*. n = 30 worms per group. p <0.0001, log-rank test, * p<0.05, ** p<0.01 vs. *daf-2(e1370)*. **B.** *cebp-2, lgmn-1, spr-4* and *daf-16* mRNA levels in worms of *daf-2(e1370)* and Neuronal *lgmn-1* or *cebp-2* overexpression *daf-2(e1370)*. Transcript levels were determined by qRT–PCR and normalized to *daf-2(e1370)* controls. Mean ± s.e.m., n = 3. *P < 0.05; **P<0.01 by one-sided Student's T test. **C.** Motility assay. Neuronal *lgmn-1* or *cebp-2* overexpression did not affect the locomotion of *daf-2(e1370)*. Day 2 worms were transferred to M9 liquid and thrashing rate was measured. Shown are mean motility scores for the first 30 s. n=10 worms per group. Mann–Whitney U-test with multiple testing correction by Holm's method. **D.** Chemotaxis assay. Neuronal *lgmn-1* or *cebp-2* overexpression reduced the improvement in the positive butanone association induced by loss of function of *daf-2*. For each time point (T0 = 0, T60 = 60, and T120 = 120 min after conditioning), [daf-2(e1370); unc-119p::lgmn-1] and [daf-2(e1370); unc-119p::cebp-2] showed impaired learning and memory compared with *daf-2(e1370)*. Data were analyzed by two-way ANOVA and Sidak's multiple comparison test, represented of five independent experiments. (n = 14-20 worms per experiment). Color circles represent individual CIs. **E.** Oily droplet assay. Neuronal *lgmn-1* or *cebp-2* overexpression increased age-associated oily droplet accumulation induced in *daf-2(e1370)* worms. Wild Type= N2 line. Data are represented as mean ± s.e.m. (days 7; two-way ANOVA and Sidak's multiple comparison test; ** p < 0.01 vs. daf-2(e1370)). **F.** Neuronal *lgmn-1* or *cebp-2* overexpression shortened the lifespan extended by *daf-16* overexpression. To conduct these experiments we used the strain GB332 [muIs131 [unc-119p::GFP::daf-16 + rol-6(su1006)] which overexpresses GFP tagged DAF-16 in all neurons, and also carries the transgene marker *rol-6(su1006)*. Our true "wild type" control is actually WB141 which contains *rol-6(su1006)*. Note that WB141 (red line) has a much longer lifespan than wild type (strain N2; black line). This extended lifespan is likely due to the restricted locomotion of *rol-6(su1006)* which "rolls in place" rather than moves around searching for food, and thus is subjected to "caloric restriction". The relevant comparisons are between the overexpression of DAF-16 (plus *rol-6*) as a blue line, and [overexpression of DAF-16 (plus *rol-6*); unc-119p::lgmn-1] as a purple line, or [overexpression of DAF-16 (plus *rol-6*); unc-119p::cebp-2] as a yellow line. n = 30 worms per group. p <0.0001, log-rank test, ** p<0.01. **G.** *cebp-2, lgmn-1, spr-4* and *daf-16* mRNA levels in worms list in F. Transcript levels were determined by qRT–PCR and normalized to Wild Type controls. Mean ± s.e.m., n = 3. *P < 0.05; **P<0.01 by one-sided Student's T test. **H.** Oily droplet assay. Neuronal *lgmn-1* or *cebp-2* overexpression increased age-associated oily droplet accumulation induced by *daf-16* overexpression. The relevant comparisons are between the green and purple or black bars. Data are represented as mean ± s.e.m. (days 7; two-way ANOVA and Sidak's multiple comparison test; ** p < 0.01 vs. N2).

Figure S10

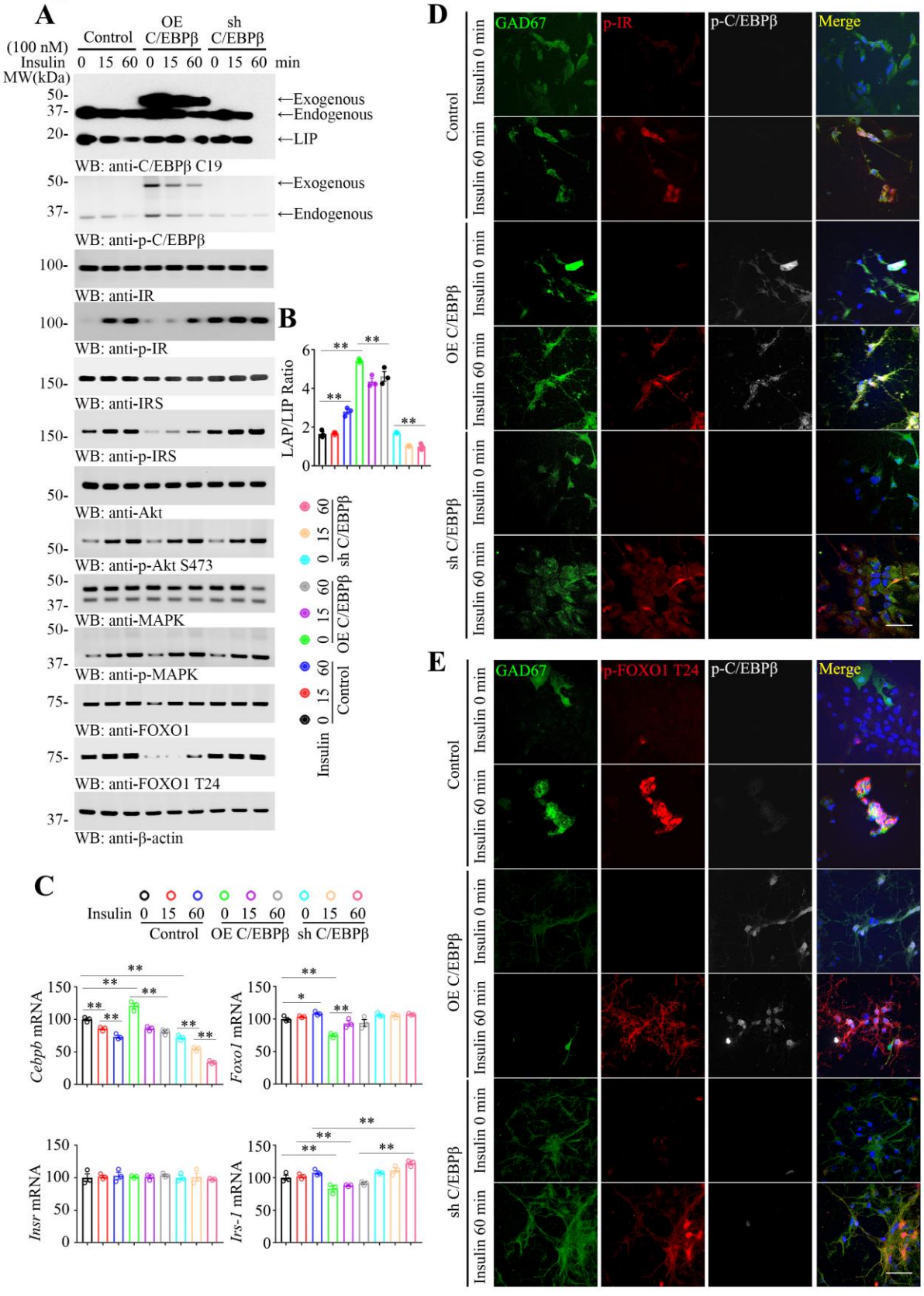
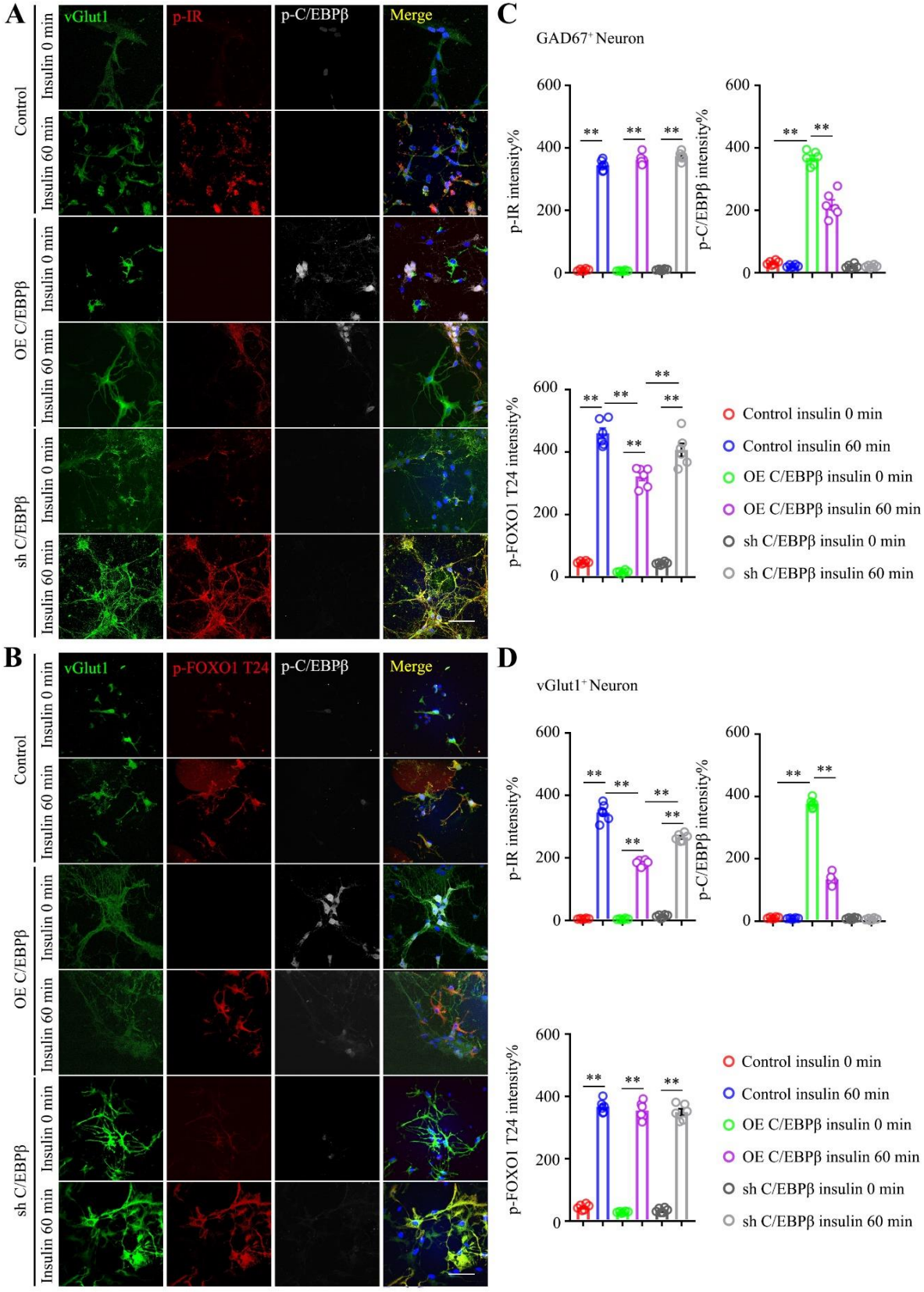

A
Control
OE C/EBPβ
sh C/EBPβ
(100 nM) Insulin 0 15 60 0 15 60 0 15 60 min
MW(kDa)
50-
37-
20-
←Exogenous
←Endogenous
←LIP
WB: anti-C/EBPβ C19
50-
37-
←Exogenous
←Endogenous
WB: anti-p-C/EBPβ
100-
WB: anti-IR
100-
WB: anti-p-IR
150-
WB: anti-IRS
150-
WB: anti-p-IRS
50-
WB: anti-Akt
50-
WB: anti-p-Akt S473
50-
37-
WB: anti-MAPK
50-
37-
WB: anti-p-MAPK
75-
WB: anti-FOXO1
75-
WB: anti-FOXO1 T24
37-
WB: anti-β-actin
B
LAP/LIP Ratio
Insulin 0 15 60 Control
0 15 60 OE C/EBPβ
0 15 60 sh C/EBPβ
C
Insulin 0 15 60 0 15 60 0 15 60
Control OE C/EBPβ sh C/EBPβ
Cebpb mRNA
Foxo1 mRNA
Insr mRNA
Irs-1 mRNA
D
GAD67
p-IR
p-C/EBPβ
Merge
Control
OE C/EBPβ
sh C/EBPβ
Insulin 0 min
Insulin 60 min
E
GAD67
p-FOXO1 T24
p-C/EBPβ
Merge
Control
OE C/EBPβ
sh C/EBPβ
Insulin 0 min
Insulin 60 min

Fig S10. C/EBPβ mediates insulin signaling in neurons, modulating p-FOXO1 T24 in GABAnergic neurons.

A-C. C/EBPβ overexpression diminishes insulin-induced p-IRS and p-FOXO1 T24. Rat primary neurons (DIV. 12) were infected with vector, overexpression or sh-C/EBPβ lentivirus for 72 h. without/with 100 nM insulin treatment for 0, 15, 60 min, respectively. Control=Vector virus. Neurons were harvested for WB with various indicated antibodies (A) or real-time PCR (C). Overexpression of C/EBPβ represses *Foxo1, Irs-1* mRNA expression. Data are represented as mean ± s.e.m., n=3 per group. *p<0.05, **p <0.01, two-way ANOVA and Bonferroni's post hoc test. The LAP/LIP isoform ratios were calculated from quantification by immunoblots of A (B), Data are represented as mean ± s.e.m., n=3 per group. **p<0.01; two-way ANOVA and Bonferroni's post hoc test. **D.** C/EBPβ overexpression does not affect insulin-induced IR phosphorylation in GABAnergic neurons. Neurons were analyzed under a confocal microscope. GAD67 marked the GABAergic neurons. The nuclei were stained with DAPI. Control=Vector virus (Scale bar: 50 μm). **E.** C/EBPβ overexpression inhibits insulin-induced p-FOXO1 T24 in GABAnergic neurons. Rat primary neurons (DIV. 12) were infected with vector, overexpression or sh-C/EBPβ lentivirus for 72 h. without/with 100 nM insulin treatment for 0, 15, 60 min, respectively. Neurons were fixed and permeabilized, cells were incubated with p-FOXO1 T24, p-C/EBPβ and GAD67 antibodies. After incubated with 3 secondary antibodies conjugated with Alexa FluorTM-488, -594 or -Cy5 for 1 h, respectively, neurons were analyzed under a confocal microscope. GAD67 marked GABAnergic neurons. The nuclei were stained with DAPI. Control=Vector virus (Scale bar: 50 μm).

Figure S11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vGlut1
p-IR
p-C/EBPβ
Merge
Control
OE C/EBPβ
sh C/EBPβ
Insulin 0 min
Insulin 60 min
B
vGlut1
p-FOXO1 T24
p-C/EBPβ
Merge
C
GAD67+ Neuron
p-IR intensity%
p-C/EBPβ intensity%
p-FOXO1 T24 intensity%
D
vGlut1+ Neuron
Control insulin 0 min
Control insulin 60 min
OE C/EBPβ insulin 0 min
OE C/EBPβ insulin 60 min
sh C/EBPβ insulin 0 min
sh C/EBPβ insulin 60 min

Fig S11. Quantification of C/EBPβ overexpression's effect on insulin-induced p-FOXO1 T24 in neuron.

A, C/EBPβ overexpression inhibits insulin-induced p-IR in Glutamatergic neurons. Neurons were analyzed under a confocal microscope. vGluT1 marked the Glutamatergic neurons. The nuclei were stained with DAPI. Control=Vector virus (Scale bar: 50 μm). **B,** C/EBPβ overexpression does not affect insulin-induced FOXO1 T24 phosphorylation in GABAnergic neurons. Neurons were analyzed under a confocal microscope. vGlut1 marked the Glutamatergic neuron. The nuclei were stained with DAPI. Control=Vector virus (Scale bar: 50 μm). **C.** Quantification of $GAD67^+$ neuron p-C/EBPβ, p-IR and p-FOXO1-T24 intensity with treatment in Fig. S10 D-E. (mean ± s.e.m.; n = 6; **, $p < 0.01$; two-way ANOVA and Sidak's multiple comparison test). **D.** Quantification of $vGlut1^+$ neuron p-C/EBPβ, p-IR and p-FOXO1-T24 intensity with treatment in Fig. S11 A-B. (mean ± s.e.m.; n = 6; **, $p < 0.01$; two-way ANOVA and Sidak's multiple comparison test).

Figure S12

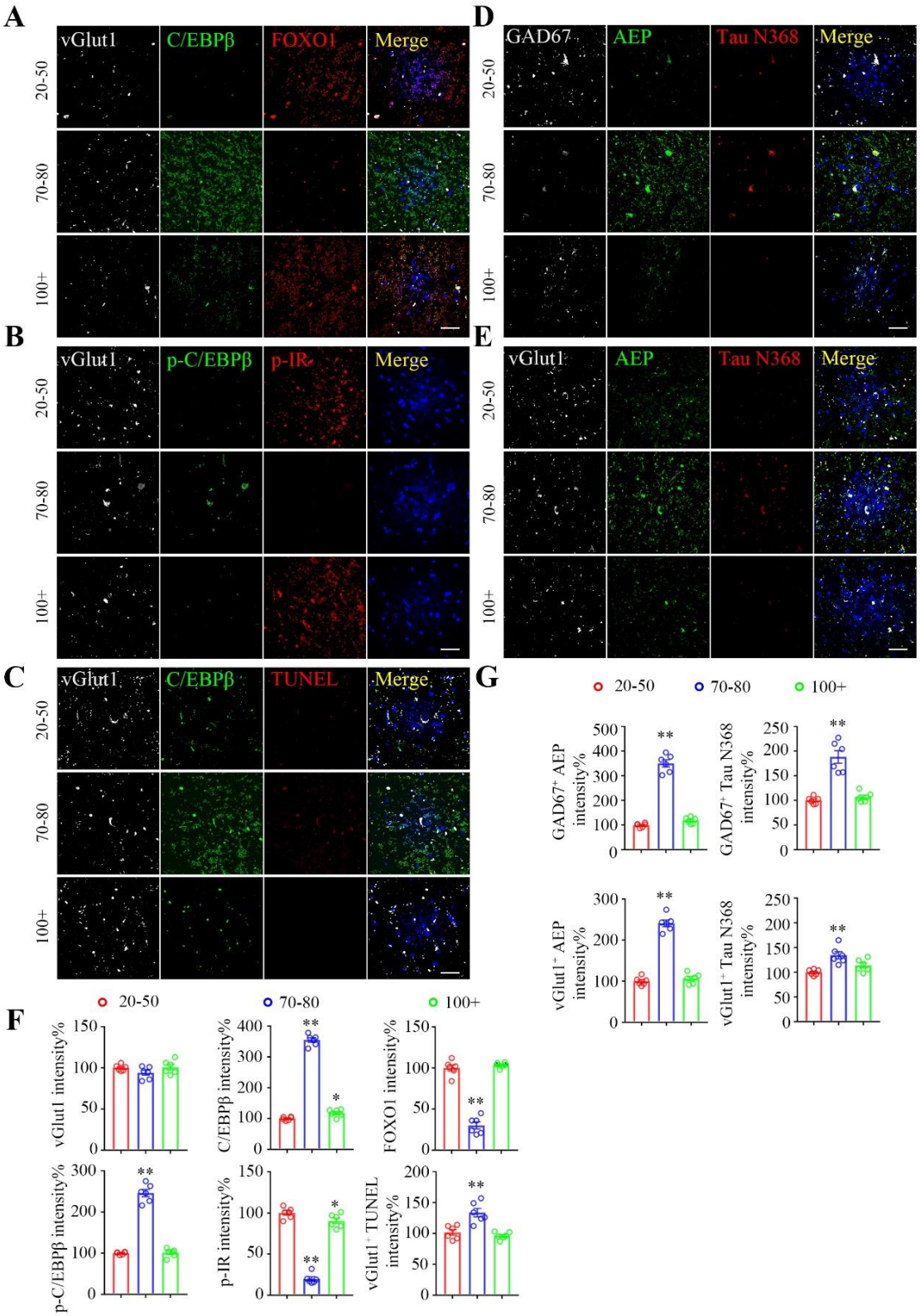
A
vGlut1
C/EBPβ
FOXO1
Merge
20-50
70-80
100+
B
vGlut1
p-C/EBPβ
p-IR
Merge
C
vGlut1
C/EBPβ
TUNEL
Merge
D
GAD67
AEP
Tau N368
Merge
E
vGlut1
AEP
Tau N368
Merge
F
20-50
70-80
100+
vGlut1 intensity%
C/EBPβ intensity%
FOXO1 intensity%
p-C/EBPβ intensity%
p-IR intensity%
vGlut1+ TUNEL intensity%
G
GAD67+ AEP intensity%
GAD67+ Tau N368 intensity%
vGlut1+ AEP intensity%
vGlut1+ Tau N368 intensity%

Fig S12. C/EBPβ mediates insulin signaling in neurons, and promotes tau N368 cleavage by AEP

A-C. Immunofluorescent co-staining on human brain sections. Colocalization of C/EBPβ, FOXO1, p-C/EBPβ, p-IR, and TUNEL in $vGlut1^+$ neurons from the different ages' human cerebral cortexes. Confocal immunofluorescence microscopy was performed in human cerebral cortex. Scale bar, 40 μm. The image shown is representative of immunofluorescence labeling performed in 3 individuals. **D&E.** Colocalization of AEP and Tau N368 in $vGlut1^+$ or $GAD67^+$ neurons from the different ages' human cerebral cortexes. Confocal immunofluorescence microscopy was performed in human cerebral cortex. Scale bar, 40 μm. The image shown is representative of immunofluorescence labelling performed in 3 individuals. **F.** Quantification of A-C, Data are represented as mean ± s.e.m., n=6 per group. **p <0.01 vs. 20-50 group; Mann–Whitney U-test with multiple testing correction by Holm's method. **G.** Quantification of D&E, Data are represented as mean ± s.e.m., n=6 per group. *p<0.05, **p <0.01 vs. 20-50 group; Mann–Whitney U-test with multiple testing correction by Holm's method.

Figure S13

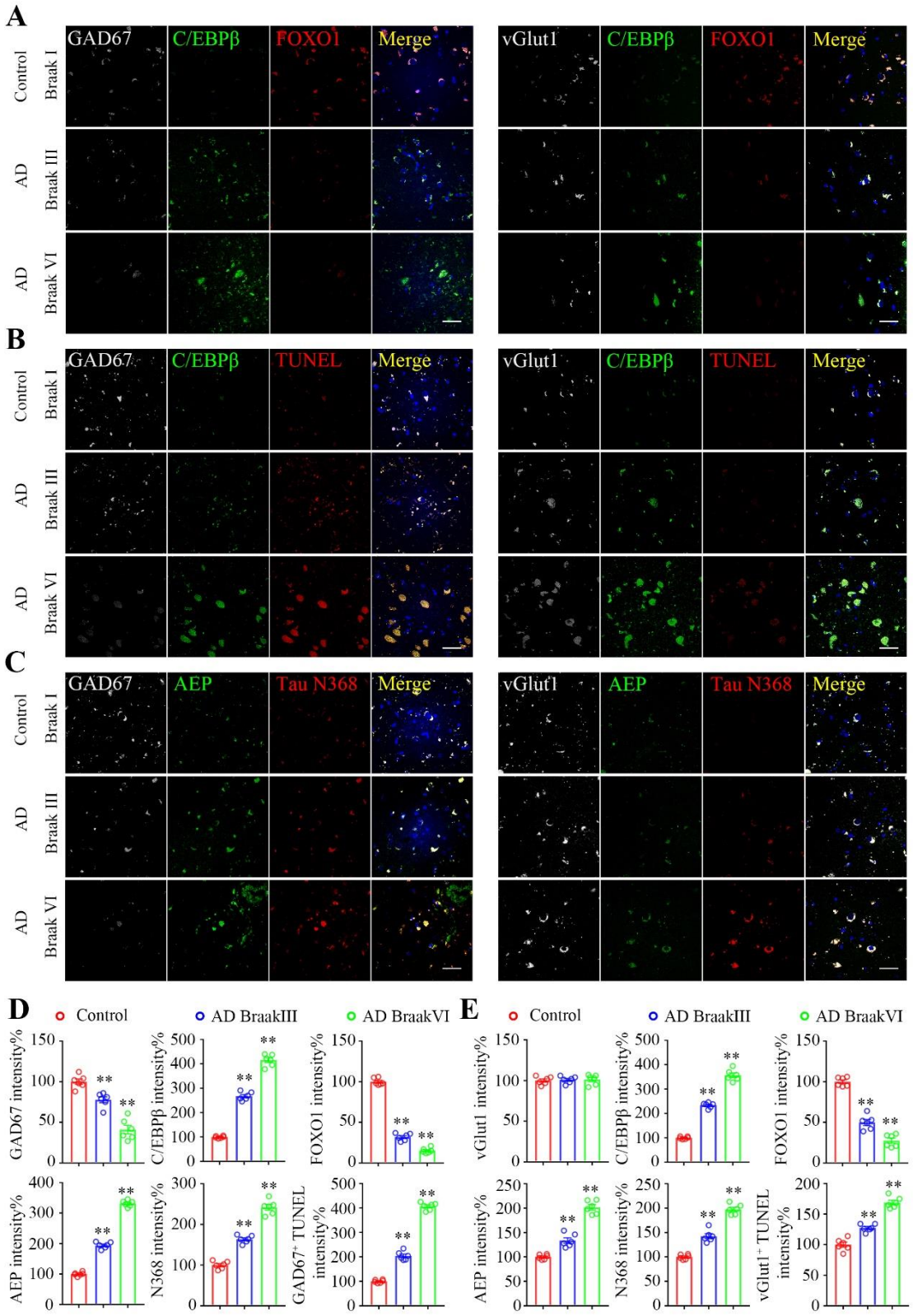
A
GAD67
C/EBPβ
FOXO1
Merge
vGlut1
Control
Braak I
AD
Braak III
Braak VI
B
TUNEL
C
AEP
Tau N368
D
E
Control
AD BraakIII
AD BraakVI
GAD67 intensity%
C/EBPβ intensity%
FOXO1 intensity%
vGlut1 intensity%
AEP intensity%
N368 intensity%
GAD67+ TUNEL intensity%
vGlut1+ TUNEL intensity%

Fig S13. C/EBPβ, FOXO1, and AEP in AD pathologies.

A-C. Immunofluorescent analysis of C/EBPβ, FOXO1, AEP, Tau N368, and TUNEL in $GAD67^{+}$ or $vGlut1^{+}$ neurons of the different Braak stages in human AD patients' cerebral cortexes. Confocal immunofluorescence microscopy was performed in human cerebral cortex. Control=Normal ageing. Scale bar, 40 μm. The image shown is representative of immunofluorescence labelling performed in 3 individuals. **D&E.** Quantification of A-C, Data are represented as mean ± s.e.m., n=6 per group. **p <0.01 vs. control group; Mann–Whitney U-test with multiple testing correction by Holm's method.

Figure S14

A

CEBPB NP_005185.2

Sequence ID: Query_195459 Length: 345 Number of Matches: 1

Range 1: 272 to 331 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
57.8 bits(138)	9e-16	Compositional matrix adjust.	29/60(48%)	41/60(68%)	0/60(0%)

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Query  18   DDYSTKRKRNNEAVNRTRQKKRQEENDTAEKVDELKKENETLERKVEQLQKELSFLKEMF  77
            D+Y  +R+RNN AV ++R K +     +T  KV EL  ENE L++KVEQL +ELS L+ +F
Sbjct  272  DEYKIRRERNNIAVRKSRDKAKMRNLETQHKVLELTAENERLQKKVEQLSRELSTLRNLF  331
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B

CEBPG NP_001797.1

Sequence ID: Query_34229 Length: 150 Number of Matches: 1

Range 1: 55 to 128 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
70.9 bits(172)	6e-22	Compositional matrix adjust.	34/74(46%)	54/74(72%)	0/74(0%)

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Query  10   SEPREDDEDDYSTKRKRNNEAVNRTRQKKRQEENDTAEKVDELKKENETLERKVEQLQKE  69
            S P + + D+Y  +R+RNN AV ++R K +Q+  DT ++V++LK+ENE LE K++ L KE
Sbjct  55   SSPMDRNSDEYRQRRERNNMAVKKSRLKSKQKAQDTLQRVNQLKEENERLEAKIKLLTKE  114

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C

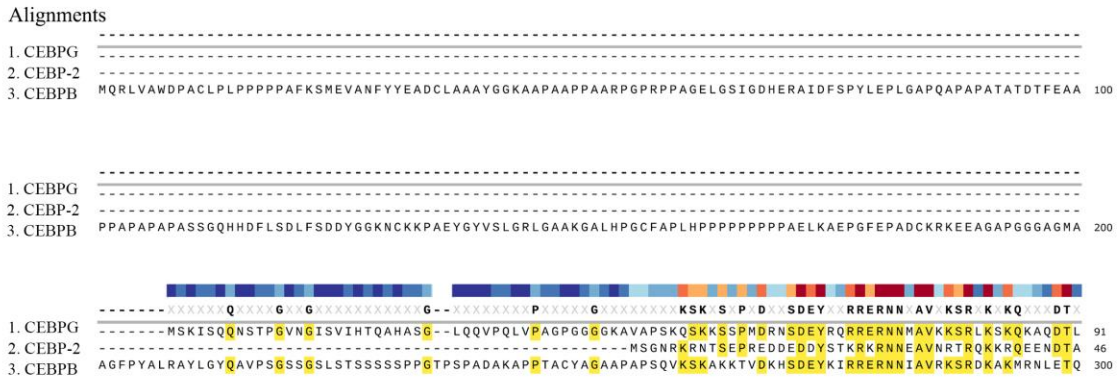

Fig S14. Alignments of C. elegans CEBP-2 and human C/EBPγ and C/EBPβ proteins.

A. Blasting CEBP-2 (NP_871835.1) with C/EBPβ (NP_005185.2) showed 48% amino acid (a. a.) identity with the human C/EBPβ. **B.** Blasting CEBP-2 (NP_871835.1) with C/EBPγ (NP_001797.1) showed 46% amino acid (a. a.) identity with the human C/EBPγ. **C.** Protein sequence alignments of C. elegans CEBP-2 and human C/EBPβ and C/EBPγ showed there is not much difference between C/EBPβ and C/EBPγ.

Table S1. Human Brain tissue information

Case	Primary Neuropathologic Diagnosis	Secondary Neuropathologic Diagnosis	PMI (hr)	Age at Onset	Age at Death/Bx	Duration (years)	ApoE	Race/Sex	Braak Score
1	Control		4.5		70		E3/3	hm	I
2	Control		6		75		E3/3	wf	I
3	Control		7		74		E3/3	wf	II
4	AD	Microinfarcts	78		77		E3/4	wm	III
5	AD	NFT-Braak stage III	6		91		E3/3	wf	III
6	AD		20	50	57	7	E3/4	bf	III
7	AD	LBD-amygdala	5.5	~48	69	~21	E4/4	wf	VI
8	AD	Infarct	9	57	77	21	E4/4	wm	VI
9	AD		5	52	60	8	E4/4	bm	VI
10	Control		4.5		20		E4/4	bf	0
11	Control		31		40		E3/4	wm	0
12	Control		6.5		46		E3/3	wf	0
13	Control		6.5		53		E4/4	bm	II
14	Control		17		57		E3/3	bf	I
15	Control		8		60		E3/4	bf	I
16	Control				72			wf	II
17	Control				74			wf	II
18	Control				76			wf	I
19	Control		6		91		E3/3	wf	III
20	Control		15.5		92		E3/3	wf	III
21	Control		5.5		94		E3/3	wm	II
22	Control				101			wf	III
23	Control				103			wf	IV
24	Control				103			wf	IV

Table S2. Key reagents list

REAGENT or RESOURCE	SOURCE	IDENTIFIER
AEP (11b7) mouse monoclonal antibody	Colin Watts Lab	NA
Akt antibody	Cell signaling	#9272
Bim (C34C5) antibody	Cell signaling	2933S
C/EBPβ (C19) antibody	Santa Cruz	SC-150
C/EBPβ (H-7) antibody	Santa Cruz	sc-7962
Cleaved caspase 3 antibody	Cell signaling	#9661
FoxO1 antibody	Cell signaling	#9462
FoxO3a (D19A7) antibody	Cell signaling	#12829
GABA antibody	Abcam	ab17413
GAD67 (F-6) antibody	Santa Cruz	sc-28376
GFP (B-2) antibody	Santa Cruz	sc-9996
Histone H3 (D2B12) antibody	Cell signaling	#4620
Insulin Receptor β (4B8) antibody	Cell signaling	#3025
IRS-1 (C-20) antibody	Santa Cruz	SC-559
Legumain (D6S4H) antibody	Cell signaling	#93627
MAPK 1/2 antibody	Sigma	ABS44
mCherry (E5D8F) antibody	Cell signaling	#43590
Mouse Legumain/Asparaginyl Endopeptidase antibody	R&D Systems	AF2058
MST1/Krs2 antibody	Sigma	07-061
NeuN (E4M5P) antibody	Cell signaling	#94403
phospho-Akt (Ser473) antibody	Sigma	05-1003
Phospho-C/EBPβ (Thr235) antibody	Cell signaling	#3084
Phospho-FoxO1 (Thr24)/FoxO3a (Thr32) antibody	Cell signaling	#9464
Phospho-FOXO1/FOXO3 (Ser207, Ser212) antibody	Thermofisher	44-1230G
Phospho-Insulin Receptor β (Tyr1146) antibody	Cell signaling	#3021
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody	Cell signaling	#4370
Phospho-Tau (Ser202, Thr205) Monoclonal Antibody (AT8)	Thermofisher	MN1020
p-IRS1 Tyr608 mouse/Tyr612 human antibody	Sigma	09-432
p-MST1 T183 antibody	Abcam	ab79199
REST antibody	Thermofisher	22242-1-AP
Tau Monoclonal Antibody (TAU-5) antibody	Thermofisher	AHB0042
Tau N368 antibody	Ye lab	NA
VGLUT 1 antibody	Synaptic Systems	135011
β-actin antibody	Sigma	A1978
Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 594 Conjugate)	Cell signaling	8889S
Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 488 Conjugate)	Cell signaling	4412S
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Thermofisher	D1306
Pentylenetetrazole(PTZ)	Caymanchemical	18682
Human Insulin ELISA Kit	Sigma	RAB0327

Supporting file 1. The sequence of targeting vectors of C/EBPβ Tg mice.
Mouse genomic fragments containing homology arms (HAs) were amplified from bacterial artificial chromosome (BAC) clone by using high fidelity Taq, and were sequentially assembled into a targeting vector together with recombination sites and selection markers.