# Supporting Information

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### SI Materials and Methods

**Generation of PR61/B′δ-null mice.** We isolated *Ppp2r5d* genomic sequences from a λGEM11 phage genomic library (Promega). A targeting vector was constructed by PCR on the phage genomic DNA using 5′- GAATTCATCGATTCAGAGCTGCCACAGGAC -3′ (forward primer) and 5′-GAGCTCATCGATGCCTTTGCCAGGAGAC-3′ (reverse primer) to obtain a 4.4 kb fragment consisting of 1 kb of exon 15 and 3.4 kb downstream sequence, which was cloned into the *Cla*I restriction site of the pBluescript112-SK-neo-tk vector. A second PCR with 5′-GAAGTCGACACTGATCCTTGTGTCTCTAC-3′ (forward primer) and 5′-GAAGTCGACGGTCGCTTGTTGGATGATG -3′ (reverse primer) resulted in a 1.5 kb fragment consisting of intron 2 sequence and 47 bp of exon 3, which was cloned into the *Sal*I restriction site of the pBluescript112-SK-neo-tk vector already containing the 4.4 kb fragment that is transcribed with a phosphoglycerate kinase promotor. In the resulting targeting construct a neomycin cassette of 1.2 kb replaced 3.9 kb of the *Ppp2r5d* gene consisting of 146 bp of exon 3, exons 4 to 14 and 45 bp of exon 15 (Fig. S1*A*). The targeting vector was linearized with *Xho*I in the pBluescript backbone before electroporation in the ES cell line E14 [129P2/OlaHsd]. G418-resistent clones were isolated and analyzed by PCR with 5′-CCACCTCTTCCTCGCCTGTT-3′ (5′ flanking primer) and 5′-TCTCCTCCAGCTCGTTCAGA-3′ (neomycin primer) (Fig. S1*A*). Genomic DNA was isolated from PCR-positive ES cell clones and further analyzed by Southern blotting (see further). The positive recombinant ES cell line 129 Ola/Hsd was injected into C57BL/6 blastocysts, which were transferred to the uterus of a pseudopregnant NMRI foster mother using standard techniques. The resulting male chimeric mice were tested for germline transmission by mating with C57BL/6 females and heterozygotes were interbred to generate PR61/B′$\delta^{-/-}$ (KO) and PR61/B′$\delta^{+/+}$ (WT) mice. To discriminate between WT, heterozygous and homozygous KO mice, a double PCR was performed on tail genomic DNA using 5′-TACCACACGCTGTCTTCATC-3′ (located in intron 2) as the forward primer and 5′-CACAGCACTGGCGTAGCTTC-3′ (located in exon 4) and 5′-TCTCCTCCAGCTCGTTCAGA-3′ (neomycin primer) as the reverse primers. This resulted in a 700 bp WT fragment and a 500 bp fragment for the mutated allele. All protocols involving mice were in compliance with the KU Leuven university animal care and usage committee.

**Southern Blotting of PCR-Positive ES Cells.** 10 μg of genomic DNA was digested using *Bam*HI and *Bcl*I, subjected to agarose gel electrophoresis and transferred to Hybond-N+ (Amersham Biosciences) by capillary transfer. Membranes were prehybridized for 30 min in Rapid-hyb buffer (Amersham Biosciences) and hybridized overnight at 65 °C with [$^{32}$P] labeled probes. Probes were generated by PCR on genomic DNA using 5′-CTAAGAAGCCACGCTCA AC-3′ (forward primer) and 5′-AGTTCCCAAGGCAGCCTGGC-3′ (reverse primer) to obtain a 5′ flanking probe, and by PCR on the pBluescript-neo-TK vector using 5′-CAACAGACAAT CGGCTGCTCTGATGC-3′ (forward primer) and 5′-GATAGAAGGCGATGCGCTGCGAT CG -3′ (reverse primer) to obtain a neomycin probe. Probes were labeled using the random primed labeling kit (Roche).

**Generation of TKO Mice.** Heterozygous mouse line 5 expressing one copy of human Tau TT4$^{+/-}$ transgene (FVB background) under the thy-1 promoter ('htau40-5' or 'H5') (1) was bred with PP2A$_{T61\delta^{-/-}}$ mice (C57BL6/129 background), resulting in a TKO mouse (TT4$^{+/-}$/PP2A$_{T61\delta^{-/-}}$) in a mixed background (FVB/C57BL6/129) as follows: PP2A$_{T61\delta^{-/-}}$ male mice were crossed with TT4$^{+/-}$ female mice. TT4$^{+/-}$/PP2A$_{T61\delta^{+/-}}$ males were then bread with PP2A$_{T61\delta^{-/-}}$ females and vice versa, resulting in TT4$^{+/-}$/PP2A$_{T61\delta^{-/-}}$ mice. Primers for genotyping are described in ref. 1 and above. All mice used for this study are females of 10 months of age and were compared with age and gender-matched WT, PR61/B′$\delta^{-/-}$ KO or TT4$^{+/-}$ transgenic mice.

**Behavioral Analyses.** ***Hot plate test.*** Pain reflexes in response to a thermal stimulus were measured using a hot plate (2) with a constant temperature of 55 °C, as measured by a built-in digital thermometer with an accuracy of 0.1 °C and verified by a surface thermometer. Mice were placed on the hot plate (25.4× 25.4 cm), which was surrounded by a transparent acrylic cage (19 cm tall, open top). The latency to respond with either a hindpaw lick, hindpaw flick, or jump (whichever came first) was measured to the nearest 0.1 sec. If a mouse did not respond within 30 sec, the test was terminated and the mouse removed from the hot plate. Animals were tested one at a time and were not habituated to the apparatus prior to testing. Each animal was tested only once ($n = 6$ for each condition).

***Adhesive removal test.*** For the adhesive removal test (3), a 0.8 cm round Avery label was placed on the forehead, out of sight for the mice. To remove this adhesive stimulus, mice raised both forelimbs and swiped off the stimulus within a 60 sec trial, after which the sticker was removed by the experimenter. The latency to remove the stimulus was noted and the average of three trials was taken into account ($n = 6$ for each condition).

***Freeze frame test (2).*** These experiments were performed in a standard fear conditioning chamber (30.5 × 24.1 × 21 cm) with a grid floor (4.8 mm diameter rods, spaced 1.6 cm apart) connected to a current shocker (Med Associates, Inc.). The training (conditioning) consisted of a single trial: after a 60 sec baseline period, mice were exposed to three consecutive tone-footshock rounds (tone, 30 sec, 3,000 Hz, 90 dB; footshock, 2 sec, 0.5 mA; intertrial interval, 2 min; tone and footshock coterminated at the end of the training block). Ninety seconds after the third shock, mice were returned to their home-cages. The next day, contextual fear conditioning was assessed by returning the mice to the conditioning chamber and measuring freezing behavior during a five-minute period. Afterwards the mice were tested for cued fear conditioning. Two minutes after being placed in a novel chamber, created by covering the floor and all sidewalls with white plexiglass (except the front wall required for monitoring), the conditioning stimulus was presented continuously for 3 min. Freezing was quantified automatically using a video-based conditioned fear testing system, FreezeFrame (Actimetrics Software). Freezing was defined as the lack of any movement except that required for respiration. The software package allowed for simultaneous visualization of the animals' behavior and adjustment of a "freezing threshold" that defined the behavior as freezing or not freezing. Total freezing was summed over the length of the trial. Freezing was presented as the percent time spent freezing (time spent freezing/total time × 100; $n = 6$).

***Object recognition test (4).*** Briefly, mice ($n = 6$) were habituated individually for five minutes in a Plexiglas open-field box (52 × 52 × 40 cm) with black vertical walls and a translucent floor

dimly illuminated by a lamp placed underneath the box. The next day the animals were placed in the same box and submitted to a 10-minute acquisition trial. During this trial mice were placed individually in the open field in the presence of object A (pepper or flask), and the time spent exploring object A (when the animal's snout was directed toward the object at a distance < 1 cm) was measured. During a 10-minute retention trial, which was performed 24 hr later, a novel object (object B: pepper or flask) was placed together with the familiar object (object A) in the open field. The time the animal spent exploring the two objects was recorded. The recognition index (RI), defined as the ratio of the time spent exploring the novel object over the time spent exploring both objects was used to measure nonspatial memory.

***Rotarod and beam walk tests.*** Rotarod and beam walk tests were performed as described (5).

**Immunofluorescence of Differentiated PC-12 Cells.** PC-12 cells (ATCC) were grown on coverslips in 12-well plates and treated with 100 ng/ml Nerve Growth Factor (Sigma) for a week to induce differentiation. Cells were fixed in 4% para-formaldehyde (PBS) for 15 min at RT. Following fixation, cells were permeabilized in 0.2% Triton X-100 (PBS) for 15 min and blocked in 1.5% BSA (PBS) for 30 min. Incubation with the primary antibodies was done in the same blocking solution for 45 min at RT. After three washes in PBS, incubation with the Alexa488 or 594-conjugated secondary antibodies (Molecular Probes) was done for 45 min again in the same blocking buffer. When appropriate, Alexa594-conjugated phalloidin (Molecular Probes) was included in the secondary antibody mix. After three washes in PBS and one wash in water the cells were mounted in DAPI-containing mounting medium (Molecular Probes). Images were captured on a confocal laser scanning microscope (LSM510, Carl Zeiss Inc.) equipped with a 63x/1.4 Plan-Apochromat oil immersion objective.

**Kinase Assays.** CDK5 and GSK3β were immunoprecipated from approx. 500 μg brain stem lysate using 5 μg of CDK5 (C-8) or total GSK3β antibody (6) according to the procedure described in (7). IPs were washed three times with kinase buffer (100 mM Tris.HCl pH 7.4, 50 mM $MgCl_2$, 5 mM EDTA, 50 μM NaF, 5 μM $Na_2VO_3$, 5 mM DTT) and resuspended in 30 μl. After threefold dilution with 10 mM Tris.HCl pH 7.4, 5 mM DTT, 10 μl of these diluted immunoprecipitates were incubated for the indicated times in a total volume of 60 μl with 10 μg Histone IIA (Sigma) as a substrate for CDK5 or with 5 μg of the GSK3β substrate Phospho-Glycogen synthase peptide 2 (Upstate) in the presence of 5 μCi [γ $^{32}$P] ATP (0.5 mM) and 25 mM $MgCl_2$. CDK5 specificity was checked with 10 μM Roscovitin (Alexis Biochemicals). 5 μl aliquots of these assay mixtures were spotted onto P81 phosphocellulose in 75 mM phosphoric acid at 0 °C, and washed three times for 10 min each in 75 mM phosphoric acid and once in 95% ethanol. The squares were air dried and counted in a scintillation counter (Beckman).

**Phosphatase Assays.** GST fusion proteins of PR55/Bα and PR61/B'δ were expressed and purified from COS cells (8) and eluted using 10 mM reduced glutathione, 50 mM Tris-HCl pH 8.0. Samples were dialyzed overnight against phosphatase buffer (25 mM Tris.HCl pH 7.4, 5 mM DTT) at 4 °C and concentrated by Speedvac. Subsequently phosphatase activity was measured with [$^{32}$P]-labeled phosphorylase *a* as substrate (9) for equilibration of activity. To isolate hyperphosphorylated P301L tau, one hemisphere of the brain of terminally aged transgenic mice was quickly homogenized in six volumes of boiling buffer (100 mM MES pH 6.8, 1 mM β-mercaptoethanol, 5 mM EDTA, protease inhibitors) without phosphatase inhibitors. Samples were boiled at 95 °C for 10 min, cooled in an ice-bath and centrifuged at 20,000 × *g*, 30 min, 4 °C. Heat-stable hyperphosphorylated tau represents the majority of protein in the supernatant (5). Carefully equilibrated amounts of different PP2A isoforms, prepared as described above ($PP2A_{T55\alpha}$ and $PP2A_{T61\delta}$), or purified from rabbit skeletal muscle ($PP2A_{T55\alpha}$ and $PP2A_D$) (9) were incubated with 5 μl of hyperphosphorylated tau in a total volume of 38.5 μl phosphatase buffer for different times at 30 °C. At each time point equal aliquots were taken out and the reaction was stopped with 2x SDS/PAGE sample buffer. Subsequently, immunoblotting was performed with HT-7, AT8 and AT180 antibodies to check the amount and phosphorylation status of tau. Alternatively, 1 mg/ml of bacterially produced hT40 tau was phosphorylated with recombinant CDK2/cyclinA (10) in the presence of 30 μM [γ-$^{32}$P] ATP, 20 mM $MgCl_2$ for 120 minutes up to a level of about 1 mole/mole. Subsequently, the preparation was boiled, cooled on ice, supplemented with Brij up to 0.03% and passed over a Sephadex G25 equilibrated in 0.03% Brij, 20 mM Tris.HCl pH 7.5 to remove the nonreacted ATP. This [$^{32}$P]-labeled tau was then used as substrate in a phosphatase assay in which the liberated phosphate was measured over time as TCA soluble radioactivity (9). His-tagged p35 was expressed in BL21-pLys cells and purified on $Ni^{2+}$ Pentadentate Chelator Sepharose beads (Affiland) using standard procedures. Phosphorylation with recombinant CDK2/cyclin A and removal of unincorparated ATP was essentially the same as described for recombinant tau, except that after phosphorylation the kinase assay mixture was not boiled. Dephosphorylation of p35 with equal amounts (1 unit/ml) of several purified phosphatases (as equilibrated versus phosphorylase *a*) was evaluated via autoradiography following SDS/PAGE. Pilot experiments ensured that under these conditions dephosphorylation still occurred in the linear range of the reaction.


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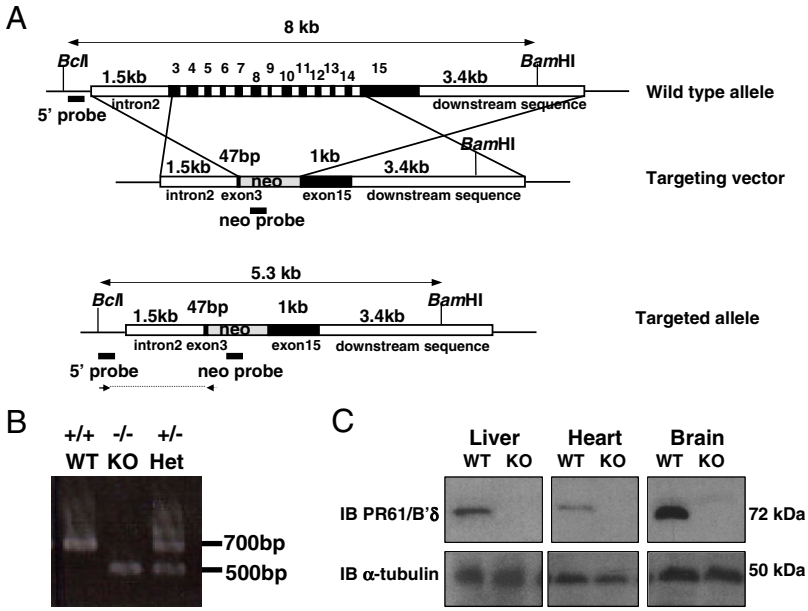



**Fig. S1.** Generation of PR61/B′δ-null mice. (*A*) Schematic overview of the wild-type (WT) allele (top), targeting construct (middle) and targeted allele (bottom). In the targeting vector a neomycin cassette of 1.2 kb replaces 3.9 kb of wild-type sequence (*SI Materials and Methods*). The Southern blot probes (5′ probe and neo probe) and the sizes of the *Bcl*I/*Bam*HI restriction enzyme fragments detected with those probes are indicated. The arrows indicate the approximate positions of the PCR primers initially used for ES cell genotyping. Because exons 1 and 2 and part of exon 3 are not targeted, a polypeptide consisting of the 50 N-terminal amino acids of the PR61/Bδ protein might still be produced. (*B*) PCR genotyping of tail genomic DNA was performed with three primers (*SI Materials and Methods*): a forward primer in intron 2, a reverse primer in exon 4, and a reverse primer in the neomycin cassette to generate a 500 bp fragment for the targeted allele and a 700 bp fragment for the WT. (*C*) Western blot analysis of equal amounts of the indicated tissue extracts from WT and KO mice using a PR61/B′δ-specific antibody.

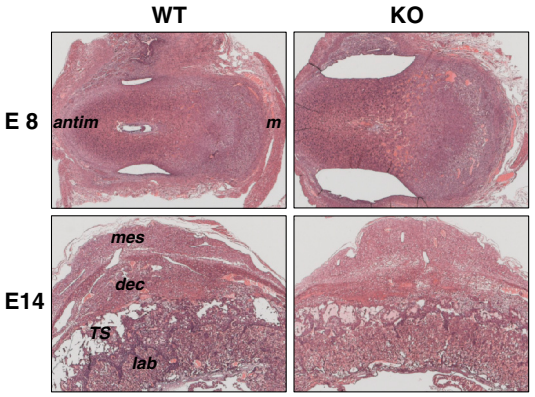



**Fig. S2.** Placental morphology of PR61/B′δ KO mice. Placental sections made at embryonic day 8 (top) and 14 (bottom) of pregnant females from WT × WT (left) and KO × KO (right) matings did not reveal any major morphological abnormalities (antim = anti-mesodermial side; *m* = mesodermial side; mes = mesometrial triangle; dec = decidua; TS = trophospongium; lab = labyrinth)

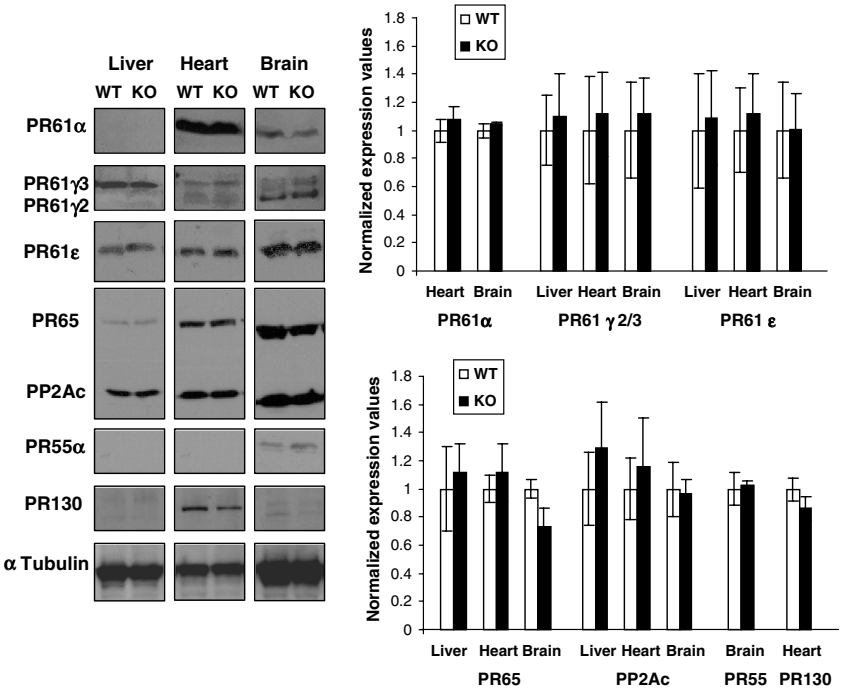
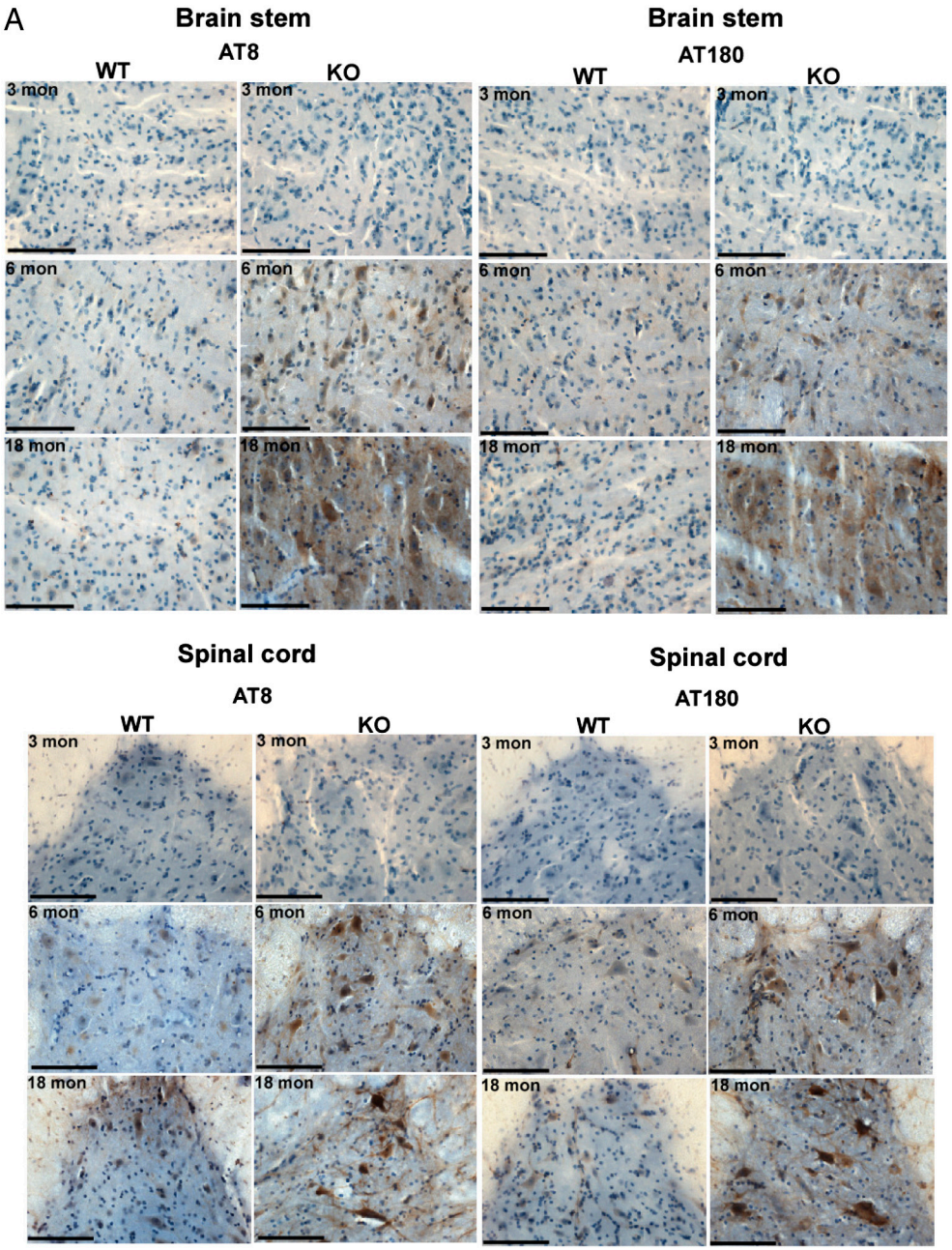



**Fig. S3.** Expression analysis of PP2A subunits in PR61/B'δ-null mice. Representative Western blots (left) of total tissue extracts from three-month-old WT and KO mice using the antibodies indicated. Quantifications (right) were performed on at least two independent blots or dilution series using ImageQuant software. This analysis was repeated several times on organs originating from different PR61/B'δ-null individuals, with similar results.


A
Brain stem
AT8
WT
KO
3 mon
6 mon
18 mon
Brain stem
AT180
WT
KO
3 mon
6 mon
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Spinal cord
AT8
WT
KO
3 mon
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Spinal cord
AT180
WT
KO
3 mon
6 mon
18 mon

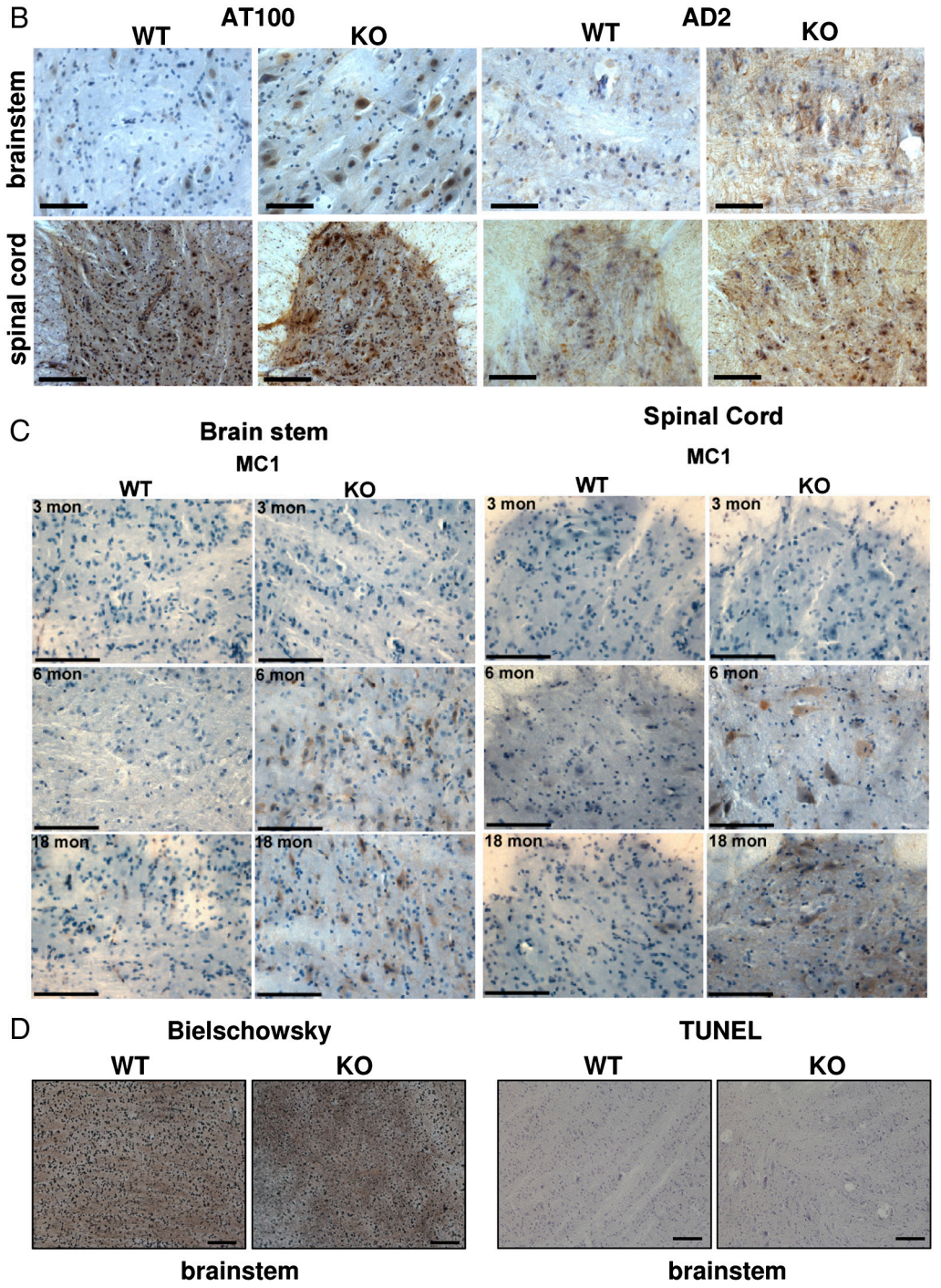


**Fig. S4.** Immunohistochemical stainings of brain sections with phosphorylation- and conformation-dependent tau antibodies, CongoRed/X34 and TUNEL. (*A*) Age-related hyperphosphorylation of tau in brainstem and cervical region of spinal cord of PR61/B′δ-null mice. Immunohistochemistry with AT8 and AT180 antibodies showed increased tau phosphorylation in the brain stem of PR61/B′δ KO mice as compared to age-matched WT mice, progressing with age. Similar results were obtained for AT8 and AT180 immunostainings of the dorsal horn of the cervical region of the spinal cord. Scale bars: 100 μm. (*B*) Tau hyperphosphorylation at AT100 and AD2 epitopes in brainstem and spinal cord of six-month-old KO mice. Immunohistochemistry with AT100 and AD2 detected clear positive neurons at six months in the brain stem (B.S) and spinal cord (Sp.C) (only cytoplasmic stainings were taken into account for quantification). The number of positive neurons did not increase with age even at 18 months in the KO mice (not shown). Scale bars: 100 μm. (*C*) Altered, pretangle conformation of tau in brain stem and the cervical region of the spinal cord of PR61/B′δ-null mice. Immunohistochemistry with the MC1 antibody, recognizing an altered tau conformation, in PR61/B′δ KO mice as compared to age-matched WT mice in brain stem and dorsal horn of the cervical region of the spinal cord. Scale bars: 100 μm. (*D*) Absence of NFT pathology in 18-month-old KO mice. Brains stained for Bielschowsky silver did not reveal any tangles; this was further confirmed with CongoRed/X34. Likewise, TUNEL stainings did not show any apoptotic neurons. Scale bars: 100 μm.

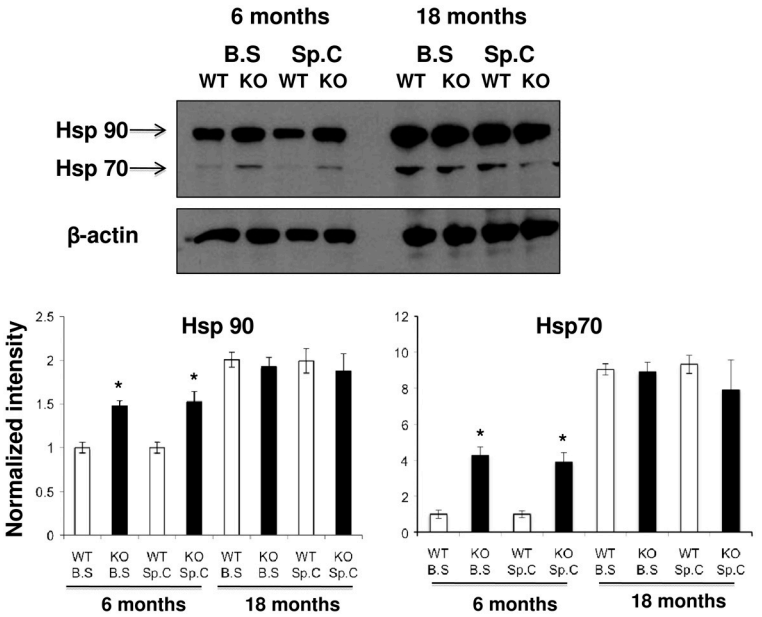


**Fig. S5.** Expression of heat shock proteins in brain stem and spinal cord of PR61/B'δ KO mice. Representative Western blot of brain stem and spinal cord extracts of age-matched KO and WT mice, developed with the indicated antibodies. ImageQuant software was used for quantitation of the bands of at least two independent blots.

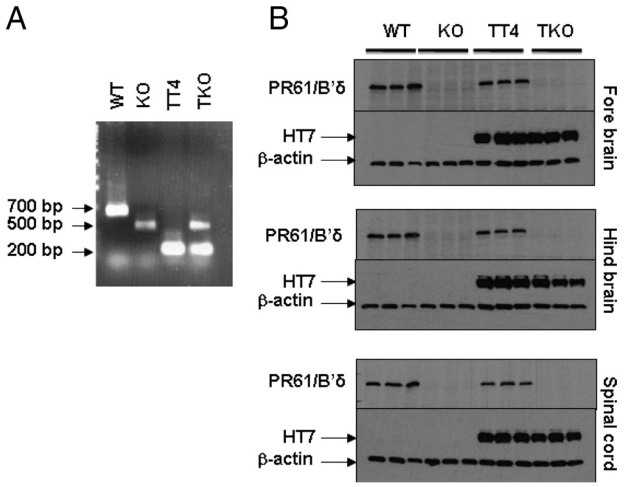


**Fig. S6.** Generation of PR61/B' δ-null x TT4 crossings. (*A*) PCR genotyping of WT, KO, TT4, and TKO tail genomic DNA with specific primers for TT4 and KO. A detailed protocol for the generation of TKO mice can be found in *SI Materials and Methods*. (*B*) Western blots with PR61/B'δ and HT-7 (recognizing human tau) antibodies on total protein homogenates from fore brain, hind brain, and spinal cord of three independent WT, KO, TT4, or TKO individuals. β-actin served as a loading control.

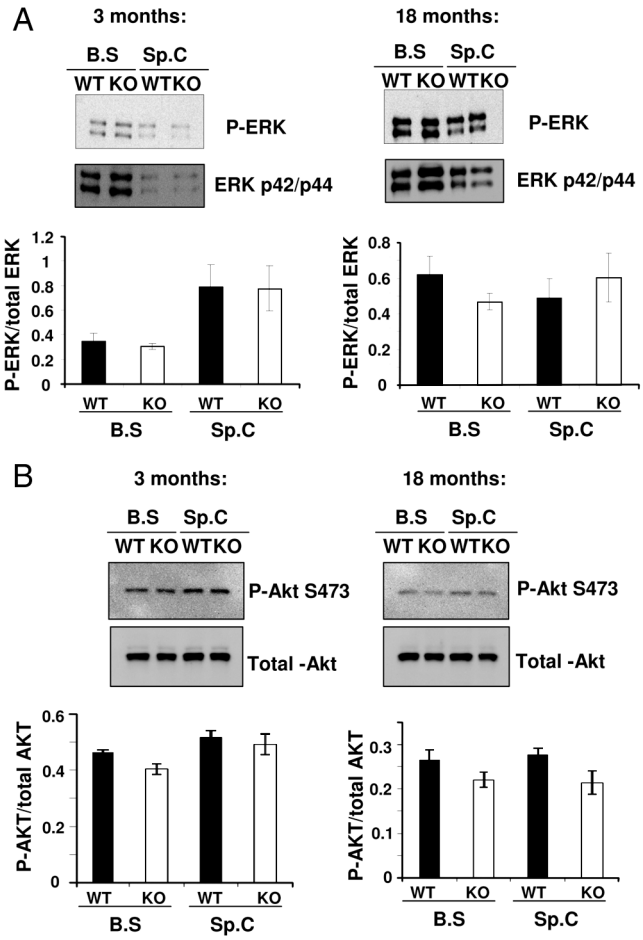


**Fig. S7.** Status of ERK (*A*) and Akt/PKB kinases (*B*) in brain stem and spinal cord of three- and 18-month-old PR61/B′δ KO mice. Representative Western blot of brainstem (B.S) and spinal cord (Sp.C) extracts of age-matched KO and WT mice ($n = 5$), developed with the indicated antibodies. ImageQuant software was used for quantitation of the bands of at least two independent blots.

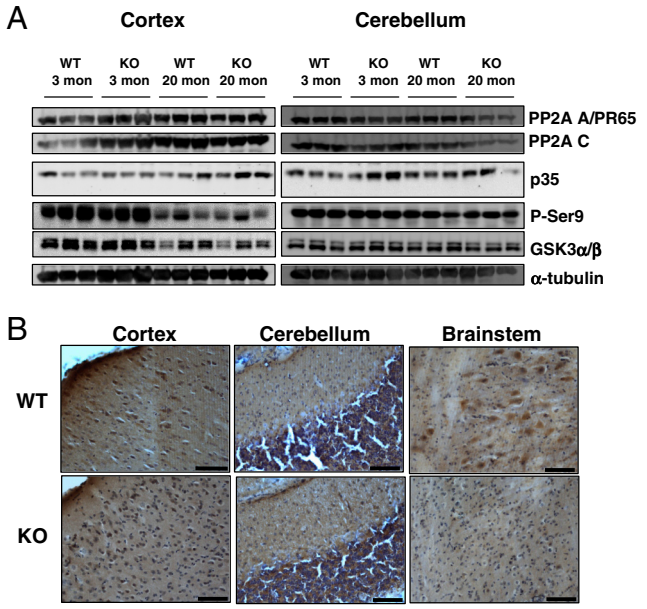


**Fig. S8.** CDK5 activator p35 expression and GSK-3β phospho-Ser9 status in cortex and cerebellum of three- and 20-month-old PR61/B′δ KO mice. (*A*) Representative Western blot of cortex and cerebellum extracts of age-matched KO and WT mice ($n = 3$ for each condition), developed with the indicated antibodies. While p35 expression is hardly detectable in brain stem and spinal cord of the KO mice (Fig. 6), cortex and cerebellum of the KO mice show normal p35 expression levels. Likewise, no significant differences in GSK-3β Ser9 phosphorylation were observed in these extracts between aged-matched WT and KO samples. (*B*) IHC staining of comparable WT and KO sections (six-month-old mice) of cortex, brain stem, and cerebellum with p35 antibody. Scale bar: 100 μM.

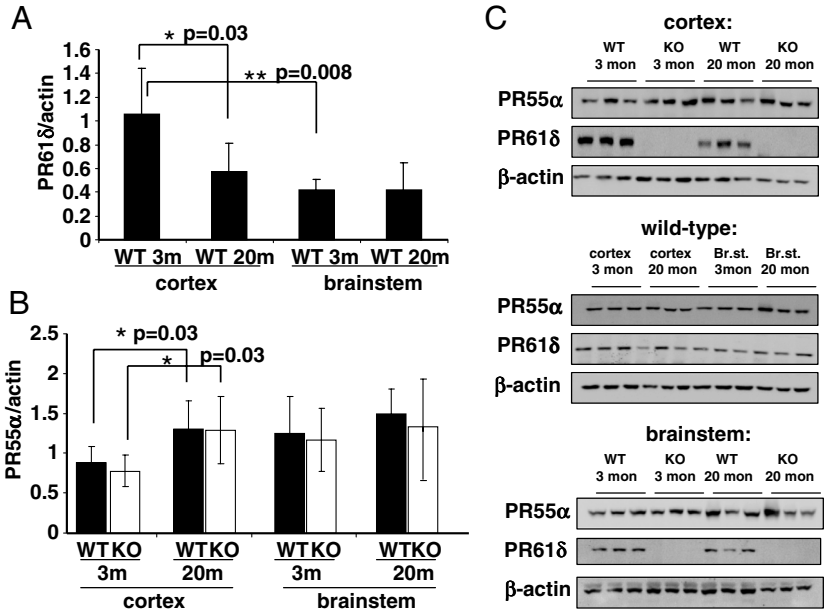


**Fig. S9.** Temporal and spatial expression patterns of PR55/Bα and PR61/B′δ in WT and KO mice. Brain stem and cortex extracts of three independent mice of each condition (three- and 20-month-old WT or KO mice) were subjected to Western blot analysis with the indicated antibodies. Quantifications were made of the relevant bands for PR61/B′δ (*A*) and PR55/Bα (*B*) relative to the β-actin loading control from the blots shown in *C*.

**Table S1. Antibodies used in this study**

| NAME | ANTIGEN/EPITOPE | SPECIES | BLOCKING | DILUTION | SOURCE |
|---|---|---|---|---|---|
| PR61/B′α | peptide to PR61α subunit | rabbit | milk | 1/500 | Dr. B.A. Hemmings |
| PR61/B′γ 2-3 | peptide to PR61γ subunit | rabbit | milk | 1/500 | Dr. B.A. Hemmings |
| PR61/B′ϵ | peptide to PR61ϵ subunit | rabbit | milk | 1/1000 | Dr. B.A. Hemmings |
| PR61/B′δ | peptide to PR61δ subunit | rabbit | milk | 1/1000 | (11) |
| PR55/Bα | peptide to PR55α subunit | rabbit | milk | 1/500 | Calbiochem |
| PR130/B″α1 | recombinant $PR130_N$ | rabbit | milk | 1/1000 | (7) |
| PR65/A | recombinant PR65/Aα | mouse | milk | 1/2500 | Dr. S.M. Dilworth |
| $PP2A_C$ | catalytic subunit of PP2A | mouse | milk | 1/2500 | Dr. S.M. Dilworth |
| α-tubulin | α-tubulin | mouse | milk | 1/10,000 | Sigma |
| β-actin | β-actin | mouse | milk | 1/50,000 | Sigma |
| β-actin | β-actin | rabbit | milk | 1/5000 | Sigma |
| AT-8 | tau phospho-Ser202/Thr205 | mouse | BSA | 1/200 | Innogenetics |
| AT-100 | tau phospho-Thr212/Ser214 | mouse | BSA | 1/1000 | Pierce |
| AT-180 | tau phospho-Thr231 | mouse | BSA | 1/200 | Innogenetics |
| AD2 | tau phospho-Ser396/Ser404 | mouse | BSA | 1/1000 | BioRad |
| MC-1 | tau AA5-15/312-322, conformation-dependent | rabbit | milk | 1/1000 | Dr. P. Davies |
| Tau-5 | tau phospho-independent | mouse | milk | 1/1000 | Calbiochem |
| HT-7 | tau phospho-independent | mouse | milk | 1/1000 | Innogenetics |
| Tau-46 | tau phospho-independent | mouse | BSA | 1/100 (IF) | Cell Signaling Technology |
| HSP70 | Hsp70 | mouse | milk | 1/1000 | Stressgen |
| HSP90 | Hsp90 | mouse | milk | 1/1000 | Stressgen |
| Total GSK3β | peptide to GSK3β | rabbit | BSA | 1/1000 (IP) | (6) |
| Total GSK3α/β | peptide to GSKα/β | rabbit | milk | 1/1000 (IB) | Cell Signaling Technology |
| phospho-GSK3β | phospho-Ser 9 | rabbit | BSA | 1/1000 | Cell Signaling Technology |
| CDK5 (C-8) | CDK5 C terminus | rabbit | milk | 1/500 | Santa Cruz Biotechnology |
| CDK5 (C-19) | p35 and p25 C terminus | rabbit | milk | 1/500 | Santa Cruz Biotechnology |
| ERK p42/p44 | total ERK | rabbit | BSA | 1/1000 | Cell Signaling Technology |
| phospho-ERK | phospho-Thr202/Tyr204 | rabbit | BSA | 1/1000 | Cell Signaling Technology |
| Akt/PKB | total Akt | rabbit | BSA | 1/1000 | Cell Signaling Technology |
| phospho-Akt | phospho-Ser473 | rabbit | BSA | 1/1000 | Cell Signaling Technology |