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Supplemental Information

Age-Dependent Decrease in Chaperone Activity Impairs MANF Expression, Leading to Purkinje Cell Degeneration in Inducible SCA17 Mice

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SUPPLEMENTAL FIGURES AND LEGENDS:

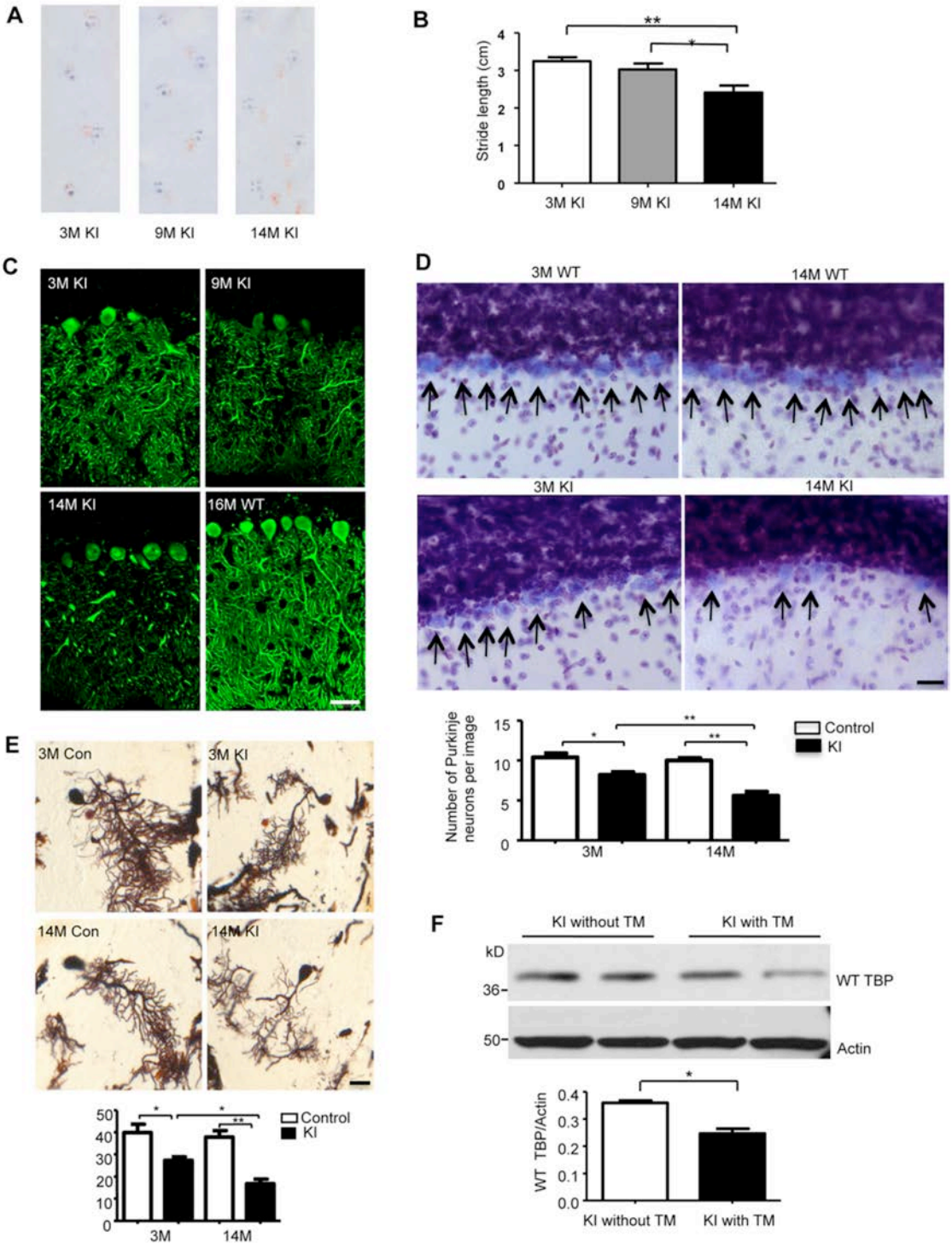

Figure S1. Foot printing assay and characterizing Purkinje cell degeneration

(A-B) Foot printing assay of gait performance of TBP105Q inducible KI mice injected with tamoxifen at 3, 9, and 14 months of age to induce the expression of mutant TBP (n=6, * P<0.05; ** P<0.01). (C) High magnification (40X) images showing degeneration of Purkinje cells in TBP05Q inducible KI mice at different ages (3M, 9M, and 14M) compared with 16-month-old wild type mice. (D) Nissl staining result of Purkinje cells (arrows) from WT and SCA17 KI mice at 3 and 14 months of age. Quantification data of Purkinje cell numbers are also presented (* P<0.05, ** P<0.01, n=6). (E) Golgi staining result of Purkinje cells from differently aged KI mice and controls. Quantification of dendritic branches from Golgi staining is also presented (* P<0.05, ** P<0.01, n=15). (F) TBP105Q inducible KI mouse cerebellum with tamoxifen (TM) induction showing a reduction in wild type TBP level. The ratio of WT TBP to actin is also quantified (* P<0.05). Scale bars in (C, D, F): 20 μm. Data are represented as mean ± SEM.

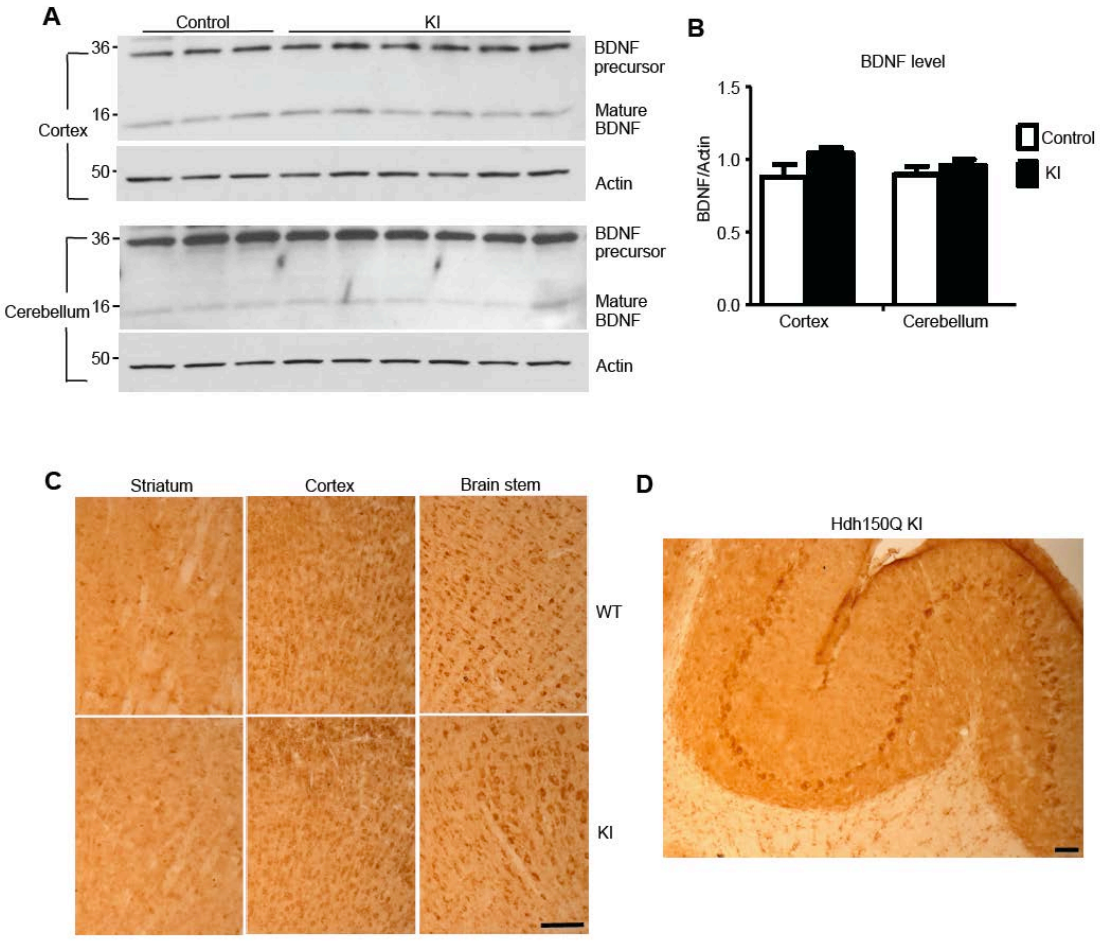

Figure S2. Expression levels of BDNF and MANF in the mouse brain

(A) Western blot analysis of BDNF level in the cortex and cerebellum of TBP105Q inducible KI mice. (B) The ratios of BDNF to actin on western blots showed no significant differences between KI and control mice. (C) No obvious changes in MANF immunostaining intensity were seen in the striatum, cortex, and brain stem between KI mice and WT mice at 14 months of age. (D) The enrichment of MANF in the Purkinje cell layer of cerebellum remains in a Huntington's disease knock-in (HdhQ150) mouse at the age of 12 months. Scale bars: 40 μm. Data are represented as mean ± SEM.

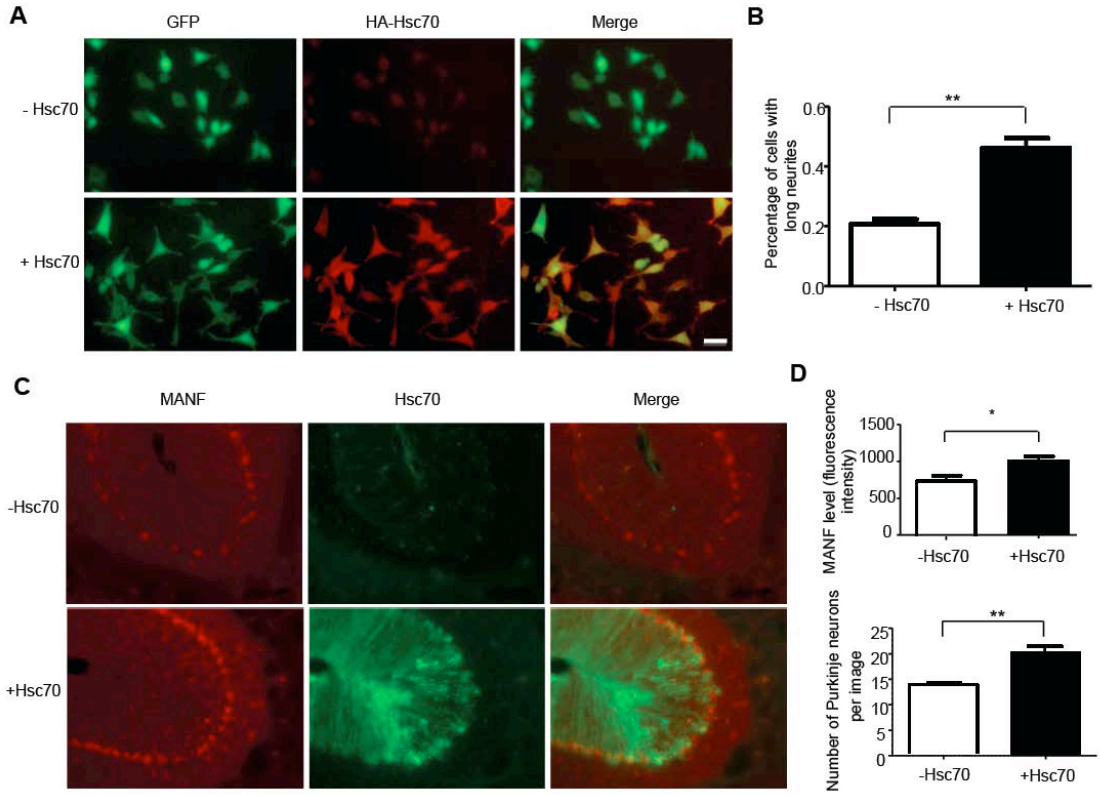

Figure S3. Protective effect of Hsc70 on mutant TBP toxicity

(A) Representative images (20X) showing that overexpression of Hsc70 increased the neurite outgrowth of TBP105Q PC12 cells in response to NGF. Scale bar: 10 μm. (B) The percentage of PC12 cells containing neurites longer than two cell body diameters (n=10, ***P*<0.01) (C) Immunofluorescent staining images (20X) of SCA17 KI cerebellum infected (+) or uninfected (–) with Hsc70 adenovirus. (D) Quantitative assessment of MANF fluorescence intensity and the number of Purkinje cells in SCA17 KI cerebellar sections (n=9, **P*<0.05, ***P*<0.01). Data are represented as mean ± SEM.

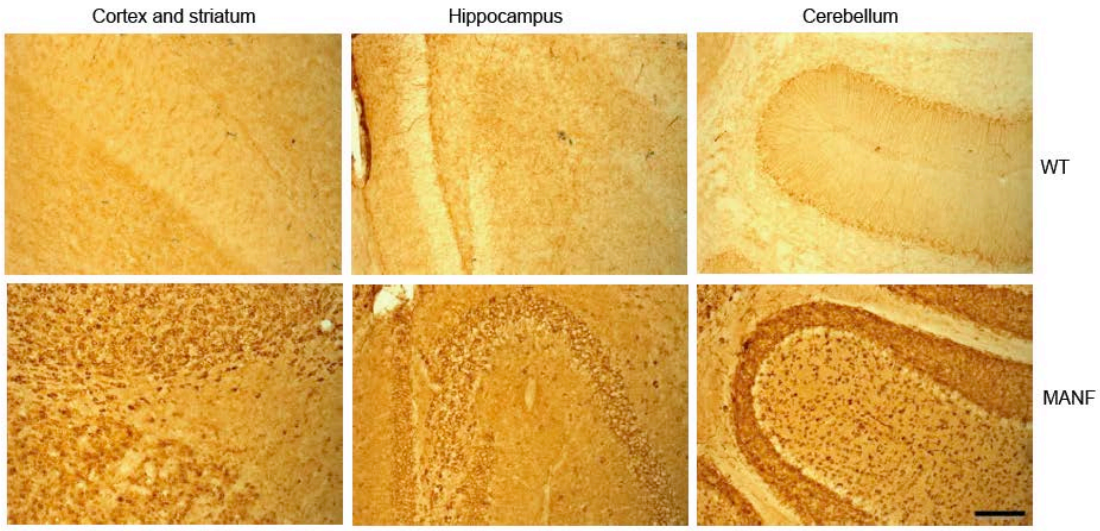

Figure S4. Immunostaining of transgenic MANF in different brain regions of MANF transgenic (TG) mice

Immunohistochemistry using HA antibody confirmed ubiquitous expression of HA tagged MANF in different brain regions of TG mice, such as the cortex, striatum, hippocampus and cerebellum. The brain of wild-type (WT) mice showed negative staining result. Scale bar: 50 μm.

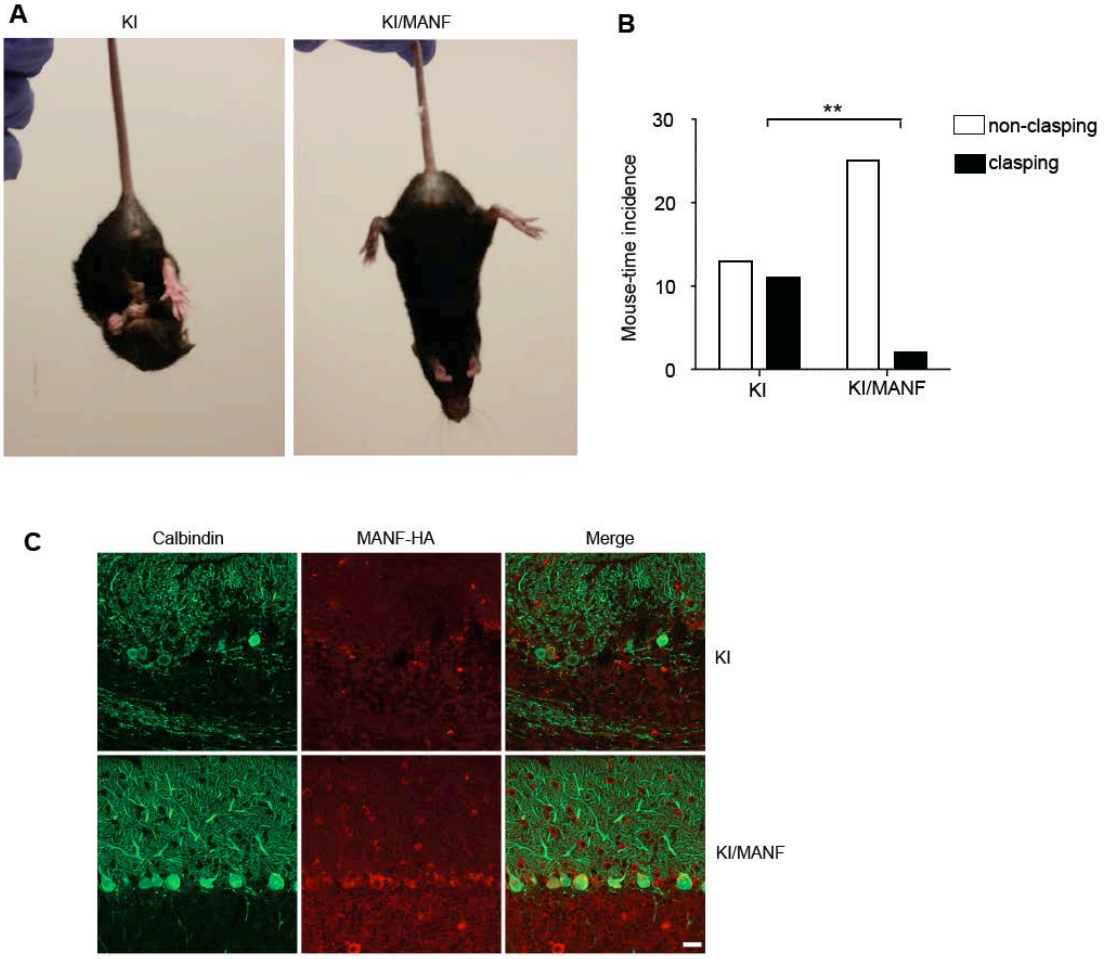

Figure S5. Alleviating clasping phenotype by transgenic MANF in KI/MANF mice.

(A) Photos of SCA17 KI mice showing the clasping phenotype, which was not seen from KI/MANF mice. (B) Quantification and analysis of the clasping phenotypes using Chi-square test (**P < 0.01). (C) High magnification images of the cerebellum sections of KI and KI/MANF mice at the age of 6 months, The brain sections were doubly immunostained with antibodies to calbindin and the HA epitope. Scale bar: 20 μm.

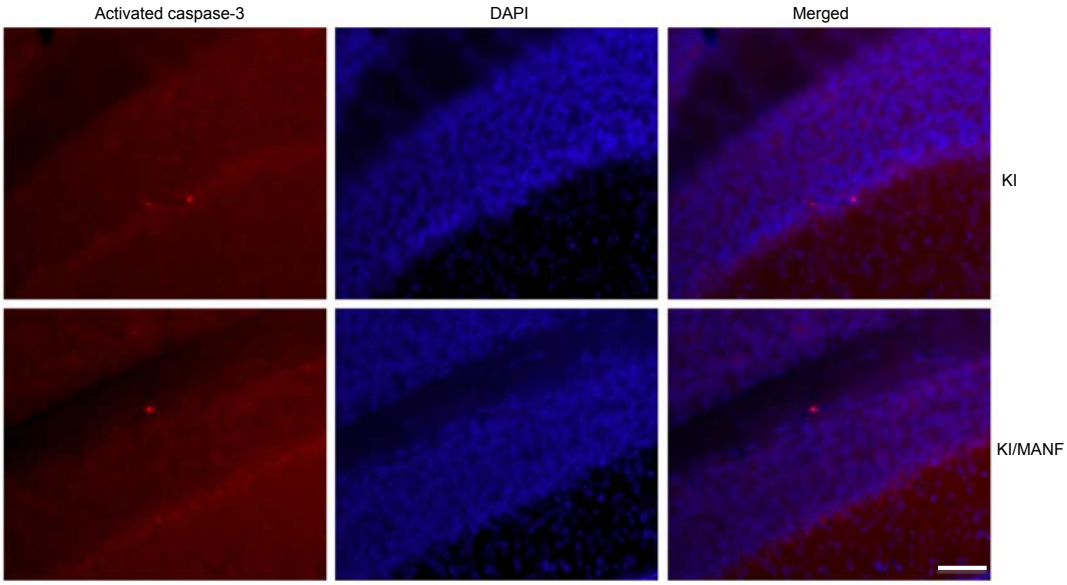

Figure S6. Immunofluorescent staining the cerebellum of SCA17 KI and KI/MANF mice with an antibody to the activated form of caspase-3

No significant difference was seen between SCA17 KI and KI/MANF cerebellar tissues. Scale bar: 50 μm.

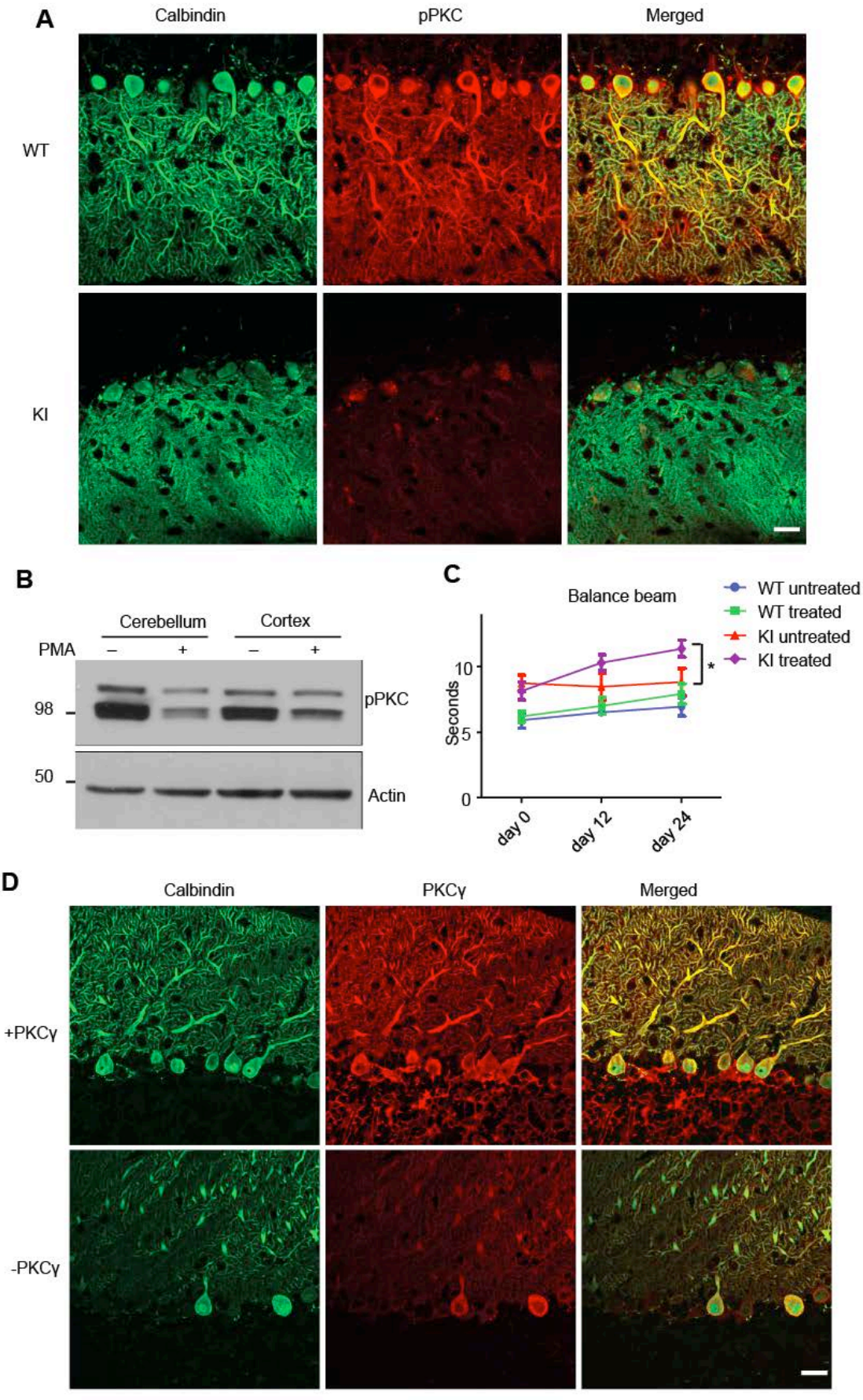
A
Calbindin
pPKC
Merged
WT
KI
B
Cerebellum
Cortex
PMA
–
+
–
+
98
pPKC
50
Actin
C
Balance beam
WT untreated
WT treated
KI untreated
KI treated
Seconds
10
5
0
*
day 0
day 12
day 24
D
Calbindin
PKCγ
Merged
+PKCγ
-PKCγ

Figure S7. Decreased PKC signaling underlies dendritic defects and motor coordination impairment in SCA17 KI mice

(A) Immunofluorescent staining images (40X by confocal microscope) of WT and TBP105Q inducible KI cerebellum. Calbindin antibody was used to reveal Purkinje cells, and pPKC antibody was used to stain phosphorylated PKC. Scale bar: 20 μm. (B) Western blotting analysis of the brain lysates from WT mice that were injected with (+) or without (-) the PKC phosphorylation modulator PMA. (C) Balanced beam test of WT and SCA17 KI mice before PMA injection and 12 or 24 days after the first PMA injection. Mice injected with corn oil only were used as a control (n=6 per group, $*P < 0.05$). (D) Immunofluorescent staining images (40X by confocal microscope) of SCA17 KI mouse cerebellum that was injected with (+) or without (–) adenoviral PKCγ. Scale bar: 20 μm. Data are represented as mean ± SEM.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES:

Antibodies and plasmids

Primary antibodies from commercial sources used in this study include: 1C2 (Millipore/Chemicon, MAB1574), 1TBP18 (QED bioscience, 70102), calbindin (Millipore/ Chemicon), Hsc70 (Santa Cruz, 7298), Hsp90 (Cell Signaling, 4877), Hsp70 (Cell Signaling, 4872), Hsp40 (Cell Signaling, 4871), Actin (Sigma, A5060), MANF (LSBio, C53208, B2688; Abcam, ab67271), GFP (BD Living Colors, 632376), XBP1 (Santa Cruz, 7160), Flag (Cell Signaling, 2368), BDNF (Santa Cruz, 546), p62/ SQSTM1 (Sigma, P0068), LC3B (Sigma, L7543), HA (Cell Signaling, 2367), PKCγ (Santa Cruz, 211), and phosphorylated PKC (Cell Signaling, 9379). Rabbit polyclonal antibody EM192 against mouse TBP was generated as described before (Friedman et al., 2007). All secondary antibodies were purchased from Jackson Immunoresearch.

Hsc70 plasmid was generated by cloning Hsc70 cDNA from GST-Hsc70 plasmid (Yang et al., 2009)(forward: 5'-ATG GCT AGC ATG TCT AAG GGA CCT GC-3'; reverse: 5'-TAC CCG CGG TTA ATC CAC CTC TTC AAT G-3'), and inserting Hsc70 cDNA into pRK vector at BamHI and EcoRI restriction sites. The sequence encoding HA tag has been previously engineered into pRK vector so that it is linked to the C-terminus of Hsc70. XBP1u, and XBP1s plasmids were provided by Dr. David Ron. TBP13Q and TBP105Q plasmids were generated as described before (Friedman et al., 2007).

Mouse behavior tests

The body weight of mice was measured every two days. The motor function of mice was assessed by rotarod test (Rotamex, Columbus Instruments) every six days. Prior to the initial test, mice were trained on the rotarod at the speed of 5 rpm for 10 minutes for 3 consecutive days. During the test, the rotarod was set to accelerate from 0 rpm to 40 rpm, with the increment of 0.1 rpm per second. Each mouse was subjected to three trials, and the time it stayed on the rotarod was recorded automatically by the machine. The average time of three trials was used to evaluate the motor activity of the mice.

For foot printing assay, red ink was applied to the forepaws of tested mice, and black ink to the hindpaws. The mice were allowed to walk through a narrow tunnel with white paper placed at the bottom. Stride lengths were calculated by measuring the distances between forepaws of each step.

For the balance beam test, mice were trained for 2 days to walk the entire length of a 0.6 cm (approximately 1″) wide × 80 cm long wooden beam that was suspended 50 cm above the floor. On each trial the mouse was released onto the end of the beam and required to run down the entire beam and into the dark box. Each mouse was tested, and each session was the average of 3 trials. The time for a mouse to cross to the end was recorded.

Cell culture

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotencin B. PC12 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% horse serum, 5% fetal bovine serum, 100 U/ ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotencin B. The generation of stable PC12 cell lines was described previously (Friedman et al., 2007, Shah et al., 2009).

Viral vector and generation

To generate MANF lentivirus, MANF cDNA was used for PCR cloning with primers (forward: 5'-ATG GAT CCA GGA TGT GGG CTA CGC-3'; reverse: 5'-ATG AAT TCC AGA TCA GTC CGT GCG-3'). PCR product was in-frame fused with F2A-ECFP fragment and inserted into pFUGW lentiviral vector. Viral PKCγ and Hsc70 were expressed via adenoviral vectors. Lentiviral MANF and GFP viruses were produced at The Viral Vector Core at Emory University and adenoviral PKCγ and Hsc70 viruses were obtained from Vector Biolabs.

Stereotaxic injection and quantification of neuronal degeneration

For virus injection into the mouse cerebellum, mice were anesthetized with a 2.5% solution of Avertin (Sigma, T48402) at 0.15 ml per 10 g body weight intraperitoneally. The mice were then stabilized on a stereotaxic instrument (David Kopf Instruments). The location for injection was determined according to the distance from bregma: anterior-posterior=-6.3 mm, medial-lateral=±1.7 mm, dorsal-ventral = -1.5 mm. Small holes were drilled on the skull, and a 30-gauge Hamilton microsyringe was used to deliver the virus. The delivery speed was set to 200 nl per minute. The microsyringe was kept for 5 minutes after injection, and retracted in 10 minutes. The wound was sutured, and Meloxican as well as penicillin/streptomycin was applied. After surgery the mice were placed on heated blankets to recover from anesthetic.

The neurite outgrowth assay and MTS assay of stably transfected TBP105Q PC12 cells have been described in previous studies (Friedman et al., 2007, Shah et al., 2009). Briefly, after plating the cells, MANF lentivirus (10^9/ml titer) was added into culture medium at 1:1000 dilution. For the neurite outgrowth assay, 1 day after infection, PC12 cells were treated with 100 ng/ml nerve growth factor in culture medium for 2 days. For MTS assay, 2 days after infection, PC12 cells were treated with 2 μM staurosporine in culture medium for 12 hours.

For immunostaining of mouse brains, mouse brains were cut into 40 μm sagittal sections in a cryostat and mounted on glass slides. For Nissl staining, brain slices were immersed in cresyl violet solution overnight, dehydrated by alcohol, and cleared by xylene. For Golgi stain, mouse brains were incubated in 3% potassium dichromate in the dark for

4 days with daily changes, and then transferred to 2% silver nitrate in the dark for 2 days. Purkinjec cells were also labeled by an antibody to calbindin in immunofluorescent staining. Purkinje neuron images were taken by Olympus FV1000 confocal microscope. To quantify Purkinje neuron numbers in an unbiased manner, NIH ImageJ software was used as described before(Bowman et al., 2007). Briefly, the "threshold" of calbindin-stained images (10X or 20X) was adjusted to highlight the soma of Purkinje neurons over background noise. The same setting has been applied to all images analyzed, and the number of Purkinje somas were counted. Molecular layer thickness of Purkinje cells was measured along the third, fourth and ninth folia half way down the preculminate, using the "spatial calibration" tool in Openlab software. To perform Sholl analysis, calbindin-stained images by confocal microscope were converted to 8-bit greyscale. NIH ImageJ software with "sholl analysis" plugins was used to count the number of neurite crossings, with the starting radius set to the radius of the Purkinje soma, and the ending radius set to the total length of Purkinje neurons.

Western blot, immunohistochemistry and immunoprecipitation

Methods for western blot and immunohistochemistry were described previously (Huang et al., 2011). For immunoprecipitation, transfected 293 cells were homogenized in NP-40 buffer (50 mM NaCl, 50 mM Tris-HCl pH8.0, 0.1% Triton X-100, 0.5% NP-40). Protein concentration was determined by BCA protein assay kit (Thermo Scientific, 23227), and 300 μg of protein was used for one experiment. The protein was first pre-cleared with

protein A-agarose for 1 hour, and then incubated with primary antibody overnight. The next day, protein A-agarose was added into the mixture and kept for 1 hour incubation. The precipitated antibody-protein complexes were used for western blotting.

Luciferase protection assay

The method for luciferase protection assay and related reagents used in our study were adopted from a previous study (Thulasiraman and Matts, 1998). To prepare mouse brain lysate, mouse cortex and cerebellum tissues were homogenized in lysis buffer (0.25 M sucrose, 15 mM Tris-HCl pH8.0, 60 mM KCl, 15 mM NaCl, 5 mM EDTA, 1 mM EGTA, and 2 mM ATP) with a glass homogenizer. The homogenate was first centrifuged at 5000 RPM for 10 minutes at 4℃. The supernatant was collected and centrifuged again at 16000 RPM for 30 minutes at 4℃. The supernatant was saved for experiments. To perform luciferase protection assay, 20 μg of brain lysate was either added with 3 μl of the chaperone inhibitor PU-H71 (10 mM, Sigma) as a negative control or without any inhibitors. The lysate was kept at 37℃ for 90 minutes to allow PU-H71 to inhibit chaperone function. After that, the lysate was mixed with recombinant luciferase and other additions (cold mix, creatine phosphate, and creatine phosphokinase) for incubation at 42℃ for 40 minutes. Finally, the luciferase activity was determined by a microplate reader (Synergy H4, BioTek).

RNA isolation, reverse transcriptase PCR and real time PCR

RNA from mouse cortex and cerebellum was isolated by RNeasy Lipid Tissue Mini Kit (QIAGEN, 74804). RNA concentration was determined by Synergy H4 reader, and equal amount of RNA was used for cDNA synthesis with SuperScript III First-Strand synthesis system (Invitrogen, 18080-051). For semi-quantitative PCR, rTaq, reaction buffer and dNTP used are all from TaKaRa, and primers used are listed below.

For TBP, forward: 5'-TAC TCC ACA GCC TAT TCA GAA CAC C-3'; reverse: 5'-AAT GGA AGA GTT GTG GGG TCT GG-3'. For MANF, forward: 5'-ATG GAT CCA GGA TGT GGG CTA CGC-3'; reverse: 5'-ATG AAT TCC AGA TCA GTC CGT GCG-3'. For Actin, forward: 5'-TGA GAC CTT CAA CAC CCC AG-3'; reverse: 5'-GTG GTG GTG AAG CTG TAG CC-3'

For real time PCR, RealMasterMix 2.5× from 5 Prime was used. The PCR reaction was performed in Mastercycler realplex (Eppendorf). Primers used are listed below. For MANF, forward: 5'-ATT GAC CTG AGC ACA GTG GAC CTG-3'; reverse: 5'-TTC AGC ACA GCC TTT GCA CAT CTC-3'. For Actin, forward: 5'-TCA CTG TCC ACC TTC CAG CAG ATG-3'; reverse: 5'-CTC AGT AAC AGT CCG CCT AGA AGC-3'

Promoter transcriptional activity assay

Mouse MANF promoter (-300 bp to 0 bp) was isolated from mouse genomic DNA by PCR with forward primer 5'-TAG GTA CCC CAA CAT GGC GAC C-3' and reverse primer 5'-ATC AAG CTT CCT CCT CAG CCG TCT C-3'. Promoter with XBP1 binding site deletion was created by PCR with the same forward primer and a different reverse primer 5'-ATC AAG CTT GAT GTT GCC CAG GAG C-3'. The isolated promoters were inserted into the pGL4.14 reporter construct (Promega) using HindIII and KpnI restriction sites. Lipofectamine (Invitrogen) was used to transfect the luciferase reporter together with other indicated plasmids into HEK293 cells. Two days after transfection, cells were collected, and luciferase reporter assay was performed using ONE-Glo Luciferase Assay System (Promega). Luciferase intensity was measured by Synergy H4 microplate reader.

Chromatin immunoprecipitation (ChIP)

HEK293 cells were transfected with TBP (13Q or 105Q) and XBP1s plasmids and collected 2 days after transfection. Cells were fixed with 1% formaldehyde for 10 minutes at 37℃, and transferred to SDS lysis buffer. DNA was sheared by 10 seconds × 10 sonication. After that, ChIP was performed according to the manual of ChIP assay kit (Millipore, 17-295). Flag antibody (Cell Signaling, 2368) was used to pull down XBP1s. Primers used for semi-quantitative PCR are listed as follows. Forward: 5'-ACT CTC TAG GTC CCA GAC AGC AGC-3'; reverse: 5'-ATG GGC TGG AAC AGA AAC CTG AG-3'.

Purification of recombinant MANF protein

Mouse MANF cDNA was cloned into pET-28a vector containing His tag. The vector was transformed into XL1-Blue competent cells, and MANF production was induced by incubating the cells with IPTG for 1 hour at 37℃. The cells were lysed in lysis buffer (5mM imidazole, 500mM NaCl, 20mM Tris-HCl, pH 8.0, 20mM Betamercaptoethanol, 1mM PMSF) by sonication, and then mixed with Ni-NTA beads (QIAGEN) at 4℃ for 2 hours. The beads with lysate were loaded to Poly-Prep Chromatography column (BIO-RAD), and washed with washing buffer (15mM imidazole, 500mM NaCl, 20mM Tris-HCl, pH 8.0, 20mM Betamercaptoethanol, 0.1% NP40, 1mM PMSF) three times. MANF was finally eluted in elution buffer (400mM imidazole, 500mM NaCl, 20mM Tris-HCl, pH 8.0, 20mM Betamercaptoethanol, 0.1% NP40, 1mM PMSF), and concentrated using Amico Ultra-4 centrifugal filters (Millipore). MANF aliquots, dissolved in PBS, were kept at -80℃.

SUPPLEMENTARY REFERENCES:

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