Supplemental information

Loss of Hap1 selectively promotes striatal degeneration in Huntington disease mice

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Supplemental Methods

Antibodies and plasmids

The following antibodies were previously designed and produced by our laboratory: guinea pig anti-Hap1 antibody EM77 (1), which detects N-terminal Hap1, and mouse anti-huntingtin mEM48 (2), which detects the amino acid VA residues after the polyQ and polyproline repeats in exon1 of human Huntingtin (HTT). All other antibodies were obtained from commercial sources: polyQ (1C2, Millipore, MAB1574), HTT (2166, Millipore, MAB2166), Tubulin (Sigma, T6557), Vinculin (Sigma, V9131), RFP (MBL, PM005), NeuN (Cell Signaling, D440), GFAP (Millipore, AB5804), IBA1 (WAKO, 019-19741), DARPP32 (Abcam, ab40801), Flag (Sigma, F3165), Rhes (GeneTex, GTX85428), β-actin (Cell signaling technology, 4967L), GAPDH (Invitrogen, 43700), SUMO1 (Abcam, ab5316), MEK (Cell signaling technology, 4694) and Connexin 43 (Cell signaling technology, 3512).

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Mouse genotyping

Genotyping of mice was performed using genomic DNA extracted from the mouse tails with the following PCR primers: *Cas9*, forward (5′-AAG GGA GCT GCA GTG GAG TA -3′) and reverse (5′-CCG AAA ATC TGT GGG AAG TC-3′); *Huntingtin* (forward (5′-ACT GCT AAG TGG CGC CGC GTA G-3′) and reverse (5′-GAC GCA GCA GCG GCT GTG CCT G-3′) ; *Hap1*, (forward: 5′-TTT TTC TGG GGA GCA TAC GTC-3′ and reverse: 5′-ATC CGT TAT CCC AGG GTC TGA-3′); *Cre*, forward (5′-GCG GTC GGC AGT AAA AAC TAT C-3′) and reverse (5′-TGT TTC ACT ATC CAG GTT ACG G-3′). All mice were maintained in Emory University's Division of Animal Resources (DAR) facility in a 12 hrs light/dark room in accordance with IACUC and DAR policies.

Stereotaxic surgery

Surgical procedures were performed in accordance with the guidelines for the Care and Use of Laboratory Animals and biosafety procedures at Emory University. Stereotaxic surgery was performed following the protocol described previously (1). Mice were anesthetized with 1.5% isoflurane inhalation and stabilized on a stereotaxic instrument (David Kopf Instruments). Meloxicam was injected (5 mg/kg) as an analgesic. Hair surrounding the surgical site was removed and the site was disinfected thoroughly before injection. At the surgical site, the skin was cut open and the location of the bregma was identified. The injection site location was determined based on the distance from the bregma. A small hole was drilled into the skull, and viruses (10^{13} particles/ml) were injected into the region of interest using the following coordinates. For striatum

injection: anteriorposterior = +0.55 mm, medial-lateral = ± 2 mm, dorsal-ventral =−3.5 mm. For hypothalamus injection: anteriorposterior = −1.5 mm, medial-lateral = ± 0.35 mm, dorsal-ventral =−5.5 mm. For cortex injection: anteriorposterior = +0.65 mm, medial-lateral = ± 1 mm, dorsal-ventral = −2.0 mm. To deliver the virus at a speed of 200 nl/min, a 30-guage Hamilton microsyringe was used. The microsyringe was left in the injection site for 10 min before it was withdrawn slowly. Post-surgery, mice were placed on a heated blanket for recovery and monitored for 5 consecutive days.

Cell culture

N2a cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 μg/mL penicillin, 100 units/mL streptomycin. Cell cultures were incubated at 37ºC with 5% CO_2. At 80% confluency, cells were transfected with 1 μg /well of DNA in a 6-well plate using lipofectamine 2000 (Invitrogen) for 48 hours.

Immunohistochemistry

Mice were anesthetized and perfused intracardially with 0.9% saline solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.2. Isolated mouse brains were fixed for 24 hours in 4% paraformaldehyde in 0.1M PB and then transferred into 30% sucrose for 48 hours at 4°C. After sinking, the brains were then sectioned at 30 μm by freezing microtome and stored in 0.1% NaN_3 in phosphate-buffered saline.

For immunohistochemistry, tissue sections were blocked in 4% normal goat serum in 0.3% Triton X-100/1X PBS for 30 min, followed by incubation of primary antibodies overnight at 4°C. After 3 rounds of 1X PBS wash, tissue sections were incubated with

secondary antibodies. Sections were developed with the Avidin-Biotin Complex Kit (Vector ABC Elite, Burlingame, CA, USA) and Sigma Fast DAB (Millipore Sigma).

Western blot

Cells or brain tissues were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0, 0.1% SDS, 0.5% DOC, and 1% Triton X-100) with freshly-added protease inhibitor cocktail. Samples were heated at 100 °C for 10 min to denature the protein. Proteins were separated by SDS-PAGE electrophoresis and then transferred onto a nitrocellulose membrane. The membrane was then blocked with 5% milk in 1X PBS. After rinsing 3 times in PBS, the membrane was incubated with primary antibody in 3% BSA/1X PBS at 4°C overnight. The membrane was then incubated with HRP-conjugated secondary antibodies in 5% milk/1X PBS for 1 hour at room temperature. After three rounds of 1X PBS wash, protein bands were developed with ECL Prime (GE Healthcare).

Immunoprecipitation

Brain tissues were homogenized in 1% NP40 lysis buffer (150 mM NaCl, 1% NP-40, 2 mM EDTA, 50 mM Tris, pH 8.0, 1 mM PMSF) with protease inhibitor. Approximately 800 µg brain homogenates were incubated with primary antibody overnight at 4°C. Protein A-beads (20 µl), after pre-blocking, were then added into the mixture and kept for 1 h with rocking at 4°C. The beads were collected by centrifugation and boiled with 1×SDS loading buffer for western blotting analysis.

Fractionation of cytosolic and membrane fractions

Fractionation was performed as described previously (3). Briefly, the striatum from 8-month-old WT, HD-KI and HD-KI/Hap1-KO mice were homogenized in soluble lysis buffer (10 mM Tris pH 7.4, 1% Triton-X 100, 150 mM NaCl, 10% glycerol) with protease inhibitor and phosphatase inhibitor cocktails and lysed on ice for 1 h. About 50 µl homogenate was saved as input. Samples were then centrifuged at 4°C at 15,000 x g for 20 min and the supernatant was used as cytosolic fraction. The pellet was washed with soluble lysis buffer and served as the membrane fraction, which was resuspended with 2% SDS buffer followed by the sonication for 20 seconds. Fractions were analyzed by SDS-PAGE and Western blotting with antibodies to MEK (cytosolic protein) and connexin-43 (membrane protein).

Statistical Analysis.

Data were analyzed using the Prism 8 (GraphPad) software. Two-tailed Student's t test was normally used to compare two groups and one-way ANNOVA followed with Tukey's multiple comparisons test was performed for comparison of more than two groups. All quantification data were presented as mean ± SEM. A P value of less than 0.05 was considered statistically significant.

Supplemental references

- 1. Sheng G*, et al.* (2006) Hypothalamic huntingtin-associated protein 1 as a mediator of feeding behavior. *Nat Med* 12(5):526-533.

- 2. Wang CE*, et al.* (2008) Suppression of neuropil aggregates and neurological symptoms by an intracellular antibody implicates the cytoplasmic toxicity of mutant huntingtin. *J Cell Biol* 181(5):803-816.
- 3. Ochaba J, Morozko EL, O'Rourke JG, & Thompson LM (2018) Fractionation for Resolution of Soluble and Insoluble Huntingtin Species. *J Vis Exp* (132) doi: 10.3791/57082.

A

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ATG CGC CCG AAA GAG CAG GTG CAG AGC GGT GCG GGA GAC GGG ACA GGG TCG GGG GAC CCA GCA GCA GGC ACC CCC ACG ACC CAG CCT
 M   R   P   K   E   Q   V   Q   S   G   A   G   D   G   T   G   S   G   D   P   A   A   G   T   P   T   T   Q   P

GCA GTT GGT CCC GCT CCG GAG CCC TCG GCG GAG CCC AAA CCT GCT CCA GCG CAG GGA ACC GGG TCC GGA CAA AAA TCA GGA TCC CGA
 A   V   G   P   A   P   E   P   S   A   E   P   K   P   A   P   A   Q   G   T   G   S   G   Q   K   S   G   S   R
                                                                                  T3
ACC AAG ACA GGA AGC TTT TGT CGG TCC ATG ATC ATT GGT GAT TCG GAC GCA CCA TGG ACC CGC TAC GTA TTC CAG GGG CCT TAC GGT
 T   K   T   G   S   F   C   R   S   M   I   I   G   D   S   D   A   P   W   T   R   Y   V   F   Q   G   P   Y   G
                                                                                  T2
CCC CGG GCC ACT GGC CTG GGC ACT GGA AAG GCC GAG GGA ATC TGG AAG ACA CCA GCC GCG TAC ATC GGC CGG AGG CCC GGC GTG TCC
 P   R   A   T   G   L   G   T   G   K   A   E   G   I   W   K   T   P   A   A   Y   I   G   R   R   P   G   V   S

GGC CCT GAG CGT GCG GCG TTT ATT CGA GAG CTG CAG GAA
 G   P   E   R   A   A   F   I   R   E   L   Q   E
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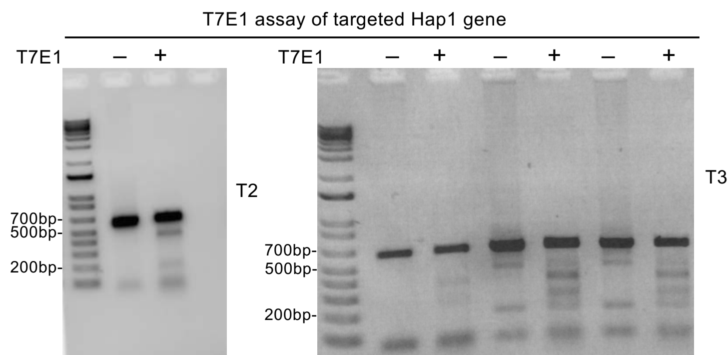
B



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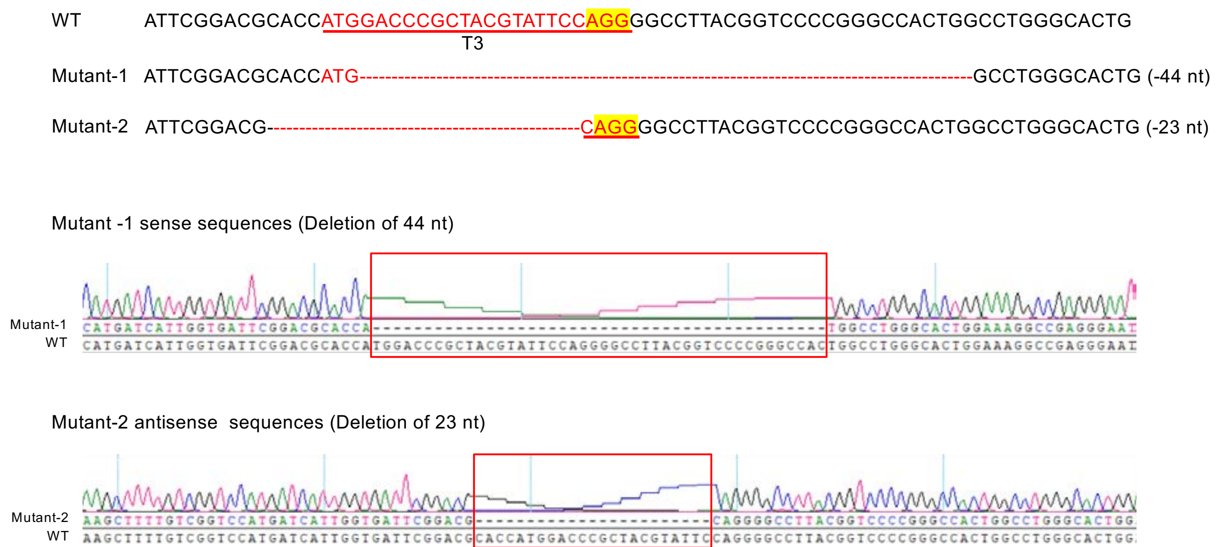

Fig. S1. CRISPR/Cas9 targeting endogenous Hap1 in mice. (*A*) DNA sequences encoding N-terminal Hap1 and the targeted sequences (red) by two gRNAs (T2 and T3) (*B*) T7E1 assays verified that AAV-Hap1 gRNA injection could cause mutations in the *Hap1* gene in the mouse brain. (*C*) DNA sequencing analysis showed that Hap1 gRNA/Cas9 can generate small deletions in the targeted region in the *Hap1* gene.

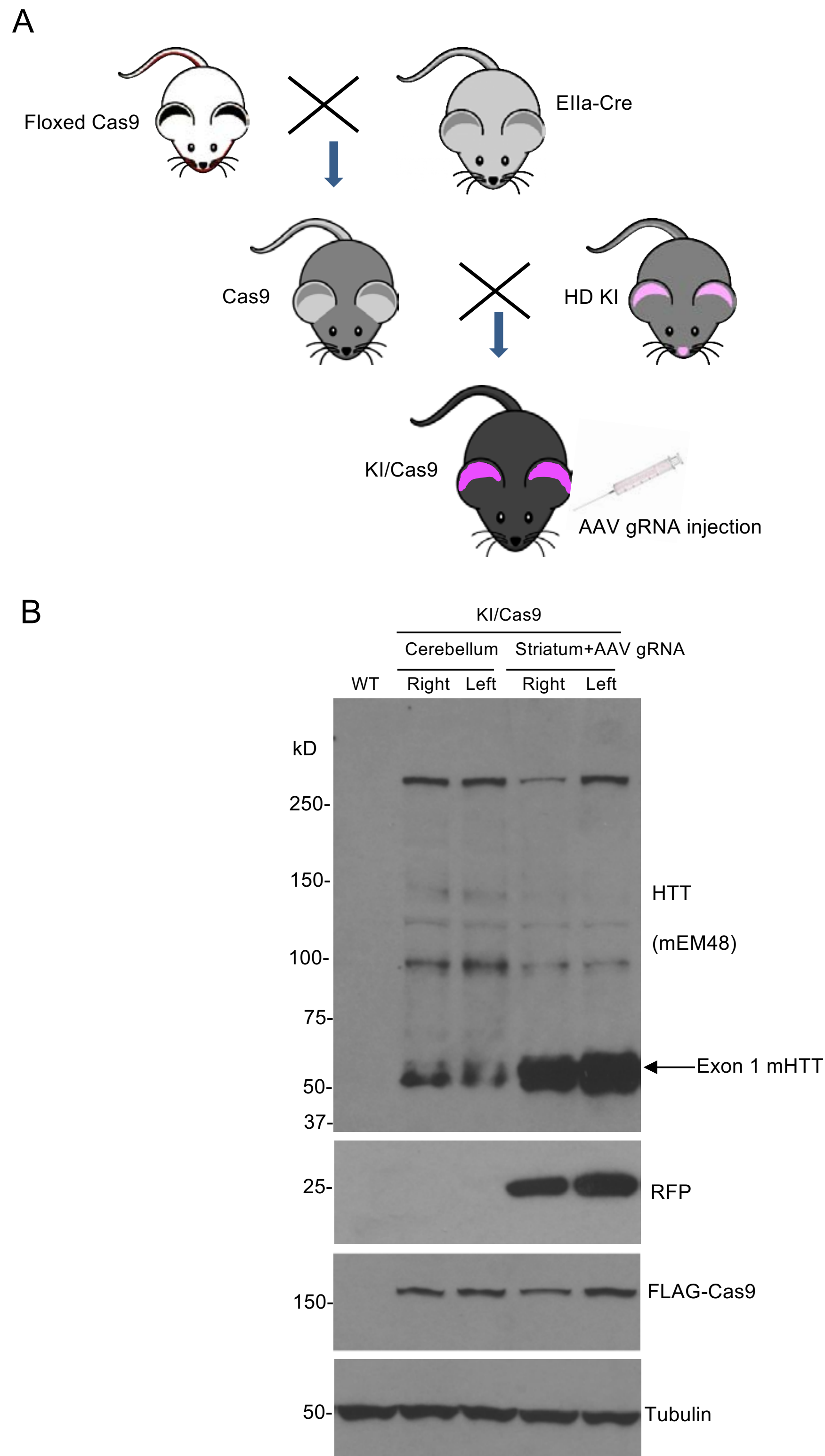

Fig. S2. Generation of KI/Cas9 mice. (*A*) Conditional Cas9 transgenic mice were crossed with EIIa-Cre transgenic mice to generate Cas9 mice in which the loxp sites in the transgenic *Cas9* gene were removed by Cre recombinase to activate Cas9 expression. Cas9 mice were then crossed with HD140Q knock-in (HD KI) mice to yield KI/Cas9 mice that express both Cas9 and mutant HTT. KI/Cas9 mice were used for stereotaxic injection of AAV-gRNA to deplete a specific gene in the selective brain region. (*B*) Western blotting of 3-month-old KI/Cas9 mouse brain tissues after injection of AAV-control gRNA viruses, which also express RFP, into the striatum. Note that mEM48 strongly reacts with a small N-terminal mutant HTT (arrow), which is equivalent to exon1 HTT. RFP represents AAV-gRNA expression, and transgenic Cas9 with FLAG is recognized by anti-FLAG. WT mouse striatum served as a control.

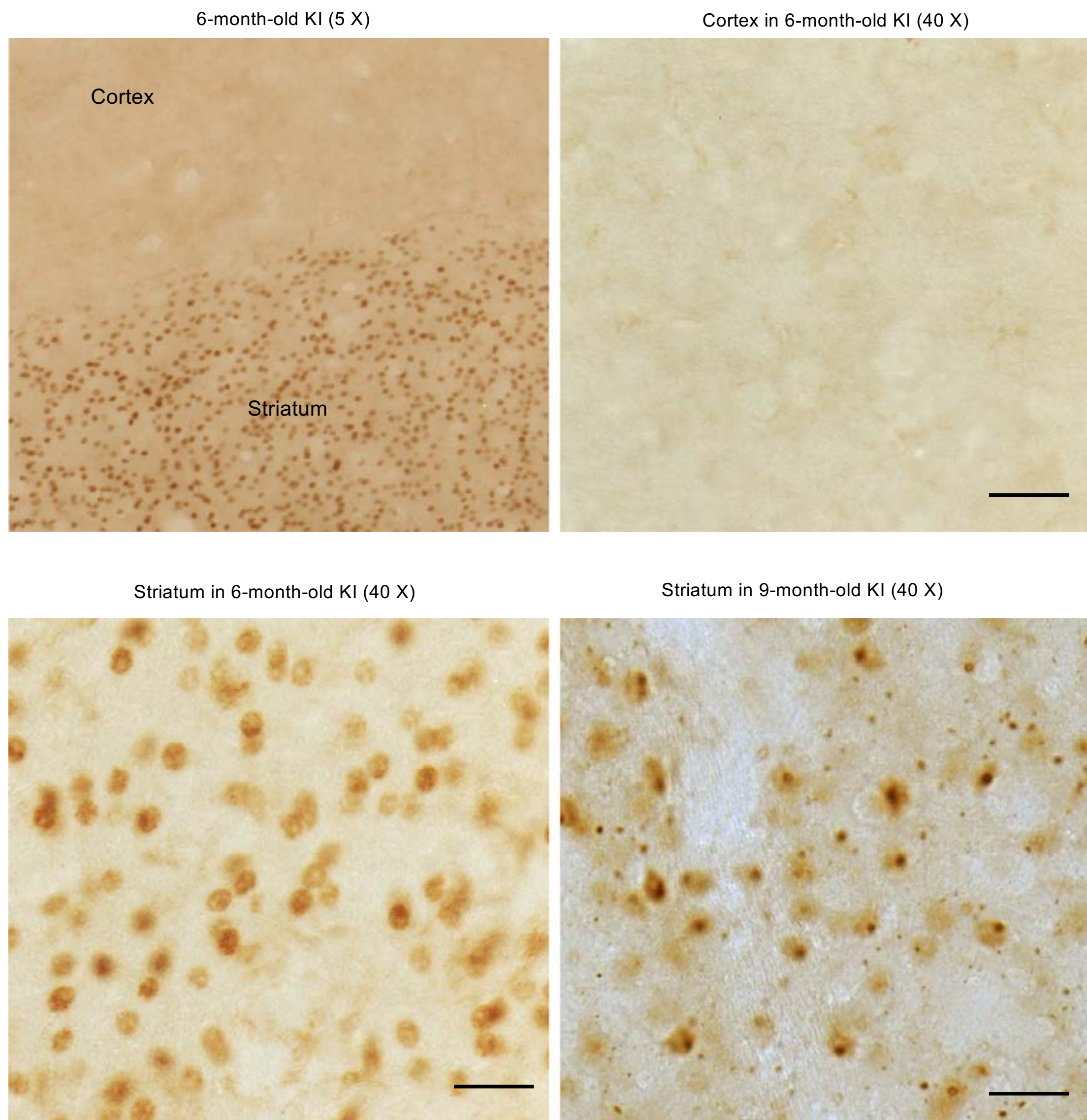

Fig. S3. Selective accumulation of mutant HTT in the striatum of HD KI mice. mEM48 immunostaining reveals the enrichment of nuclear HTT in the striatum in HD KI mouse. Mutant HTT is accumulated in the nucleus of striatal neurons in young HD KI mouse at 6 months of age and becomes aggregated in an older HD KI mouse at 9 months of age. Scale bars: 20 μm.

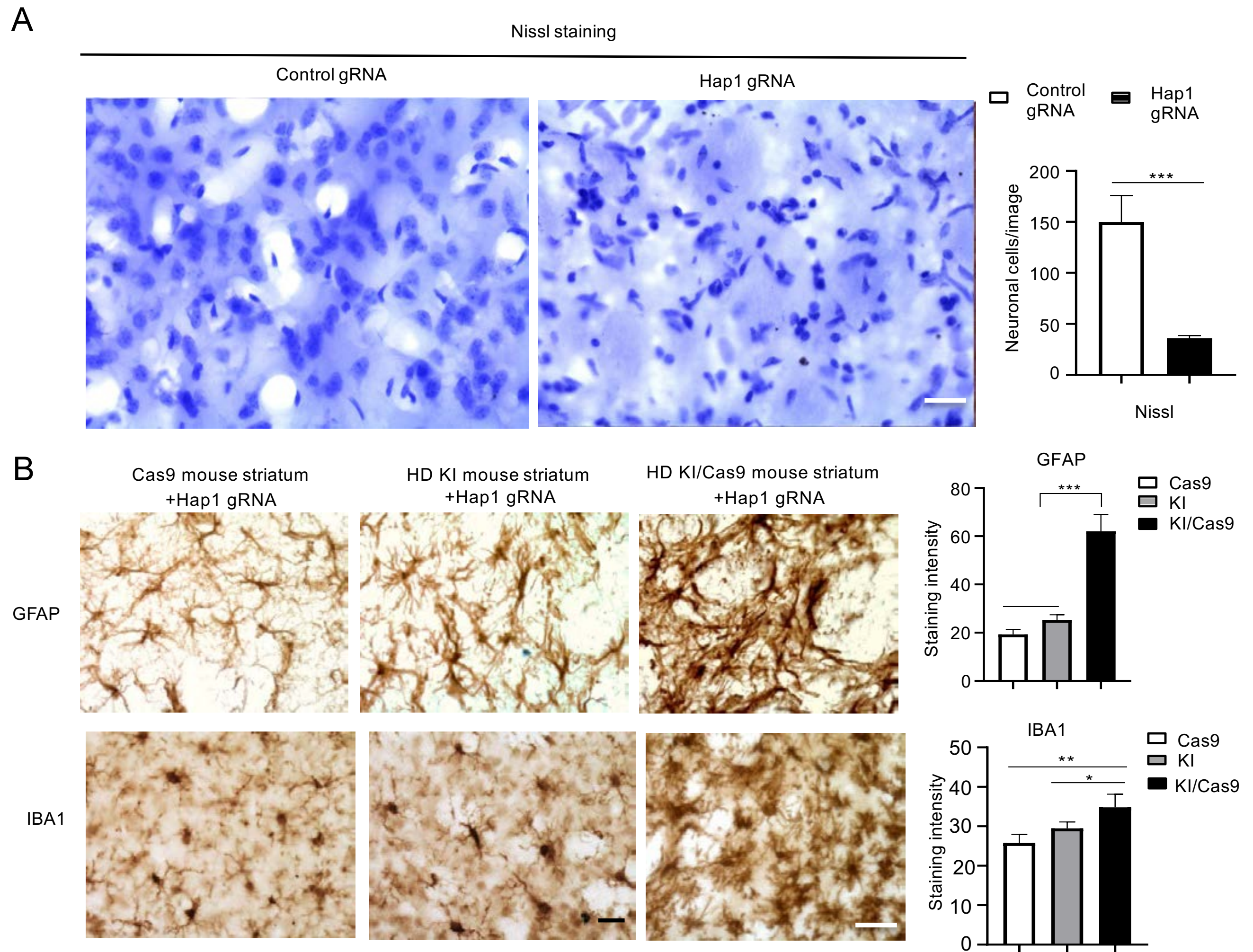

Fig. S4. Loss of Hap1 in HD KI striatum reduced cell numbers and increased reactive gliosis. (*A*) Nissl staining of AAV-control gRNA- or AAV-*Hap1* gRNA-injected striatum in HD KI/Cas9 mouse brain showing the reduced density of neuronal cells of large size and increased density of glial cells of small size. The number of large-sized neuronal cells were counted per image (40X) and presented (right panel). *** P<0.001. (*B*) Immunostaining with antibodies to the astrocytic marker GFAP and microglial marker IBA1 showing the increased reactive glial cells when Hap1 was depleted by AAV-*Hap1* gRNA. Right panels are the density of reactive astrocytes (GFAP) and microglia (IBA1) in the AAV-*Hap1* gRNA-injected striatum of Cas9, KI, or KI/Cas9 mice. For quantification in this figure, we counted 20 images from 4 animals per group to obtain the above data for statistical analysis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bars: (*A, B*) 10 μm.

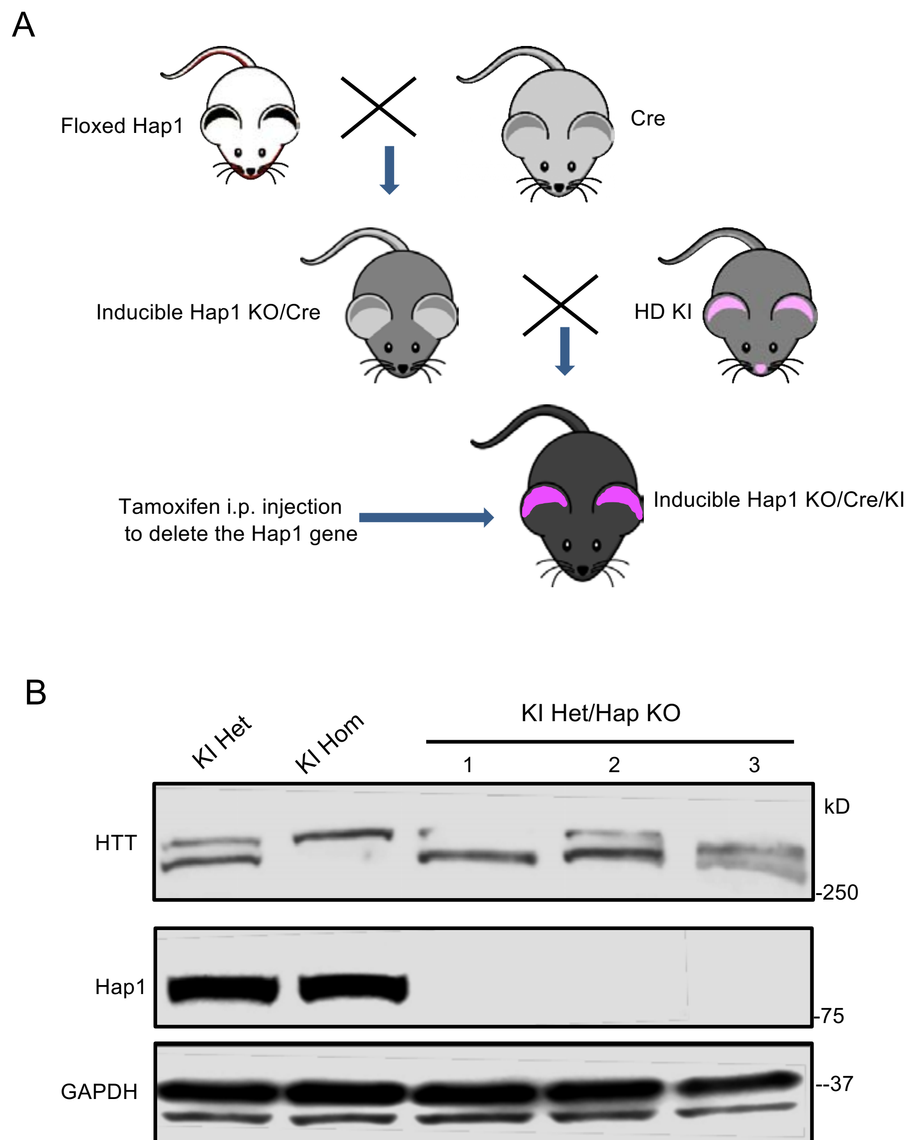

Fig. S5. **Generation of tamoxifen-induced Hap1 knockout (Hap1 KO) mice that also express mutant HTT.** (*A*) Conditional Hap1 KO mice, in which exon 1 of the *Hap1* gene was flanked by two loxP sites, were crossed with transgenic mice that express Cre-ER driven by the chicken β actin promoter/enhancer coupled with the CMV immediate-early enhancer to generate inducible Hap1 KO mice in which Cre-mediated recombination occurs after tamoxifen-administration. These mice were then crossed with HD KI mice to produce inducible Hap1 KO/Cre/KI mice. After intraperitoneal (i.p.) injection of tamoxifen, the *Hap1* gene is deleted in KI/Hap1 KO mice. (*B*) Western blotting analysis of three 9-month-old KI/Hap1 KO mouse brains showing the depletion of Hap1 in the KI/Hap1 KO mouse brains. HTT was probed with an antibody (2166) that can recognize both normal and polyQ-expanded HTT. Heterozygous and homozygous HD KI mouse striatum served as controls.

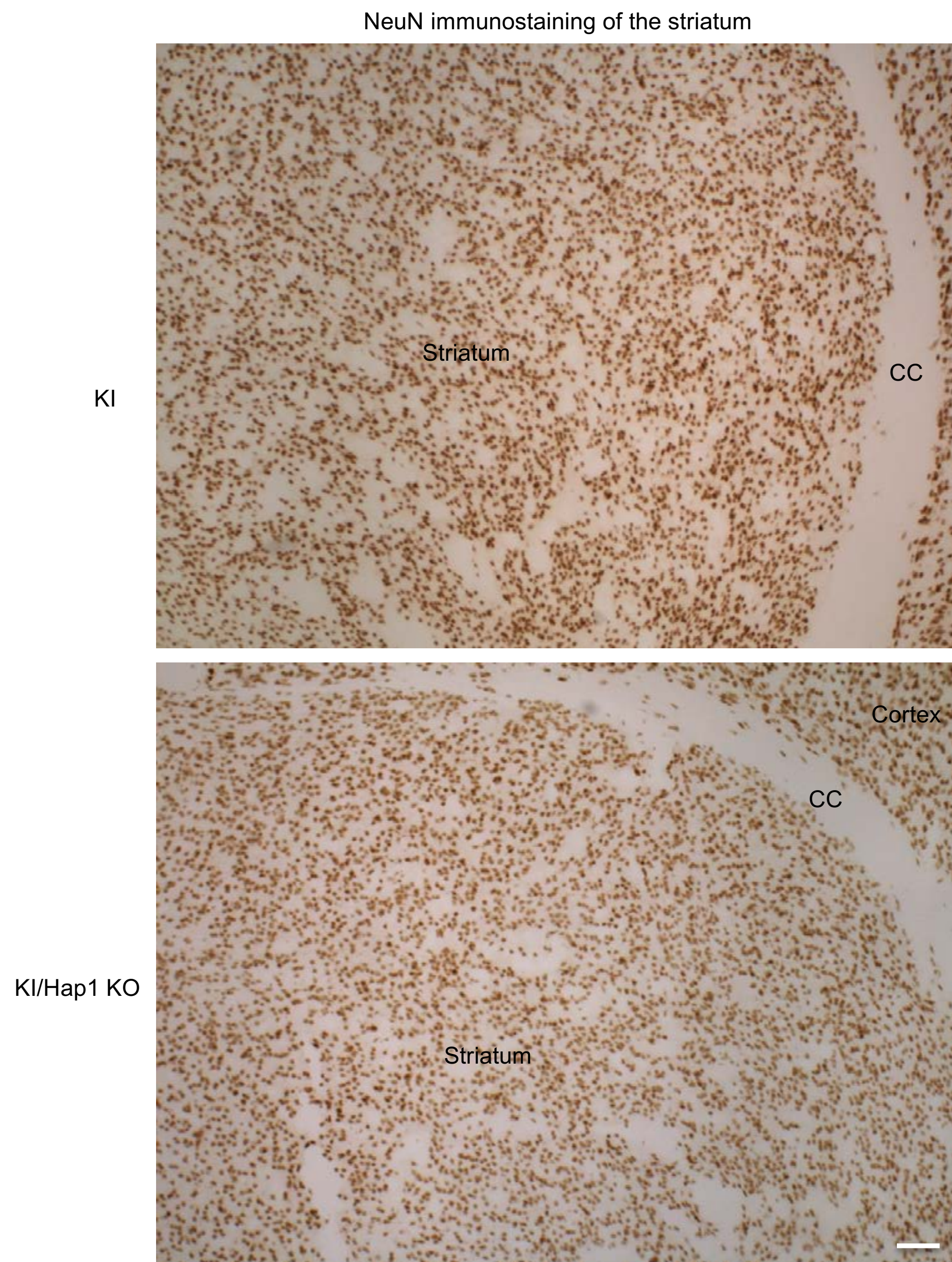

Fig. S6. NeuN staining of the striatum of HD KI and KI/Hap1 KO mice after tamoxifen injection. The mice at 9 months of age were examined. Note that NeuN staining in the striatum shows no obvious difference between KI and KI/Hap1 KO mice. Scale bar: 50 μm.

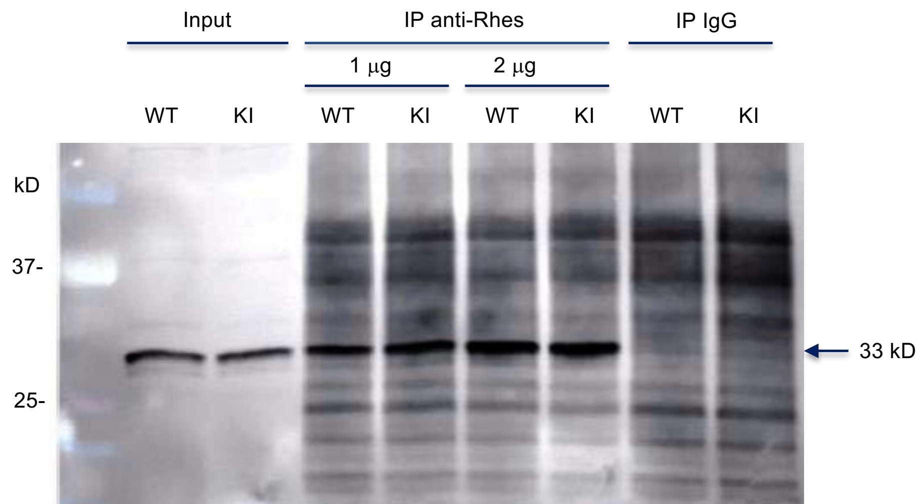

Fig. S7. Immunoprecipitation of Rhes from the mouse striatum. Different amounts of anti-Rhes (1 and 2 μg) were used to immunoprecipitate Rhes from wild type and KI mice at age of 9 months. IgG immunoprecipitation served as a negative control. Note that Rhes at 33 kD was precipitated by anti-Rhes.