SUPPLEMENTAL FIGURES AND FIGURE LENGENDS

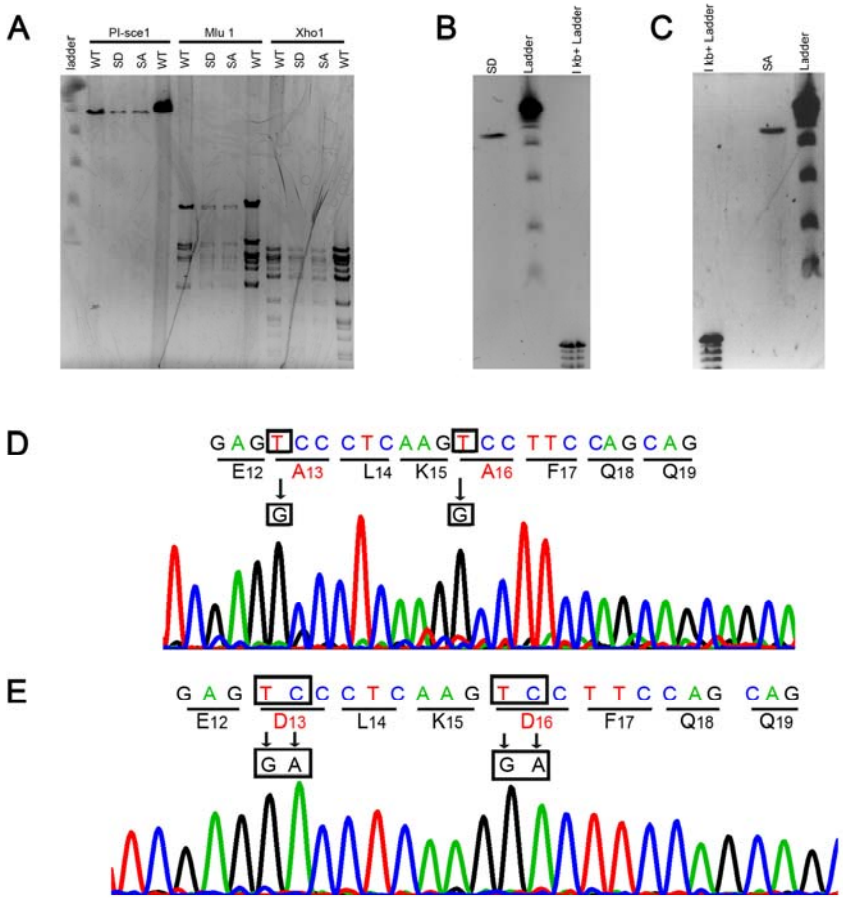
Supplemental Figure S1. Characterization of SD and SA BACs and Confirmation of SD and SA Mutations in Transgenic Mice.

(A) Restriction enzyme mapping on a pulse-field gel was used to generate DNA fingerprints of wild type RP11-866L6 BAC (baseline unmodified human htt BAC) and SD and SA BAC. The BAC DNA were digested with three rare cutting restriction enzymes, PI-SceI, Mul, and Xho I, and restriction fragments were separated on a pulse-field gel. This analysis did not reveal any new or missing fragments in SD or SA BACs compared to the WT BAC, suggesting no unwanted rearrangement or deletions in these modified BACs.

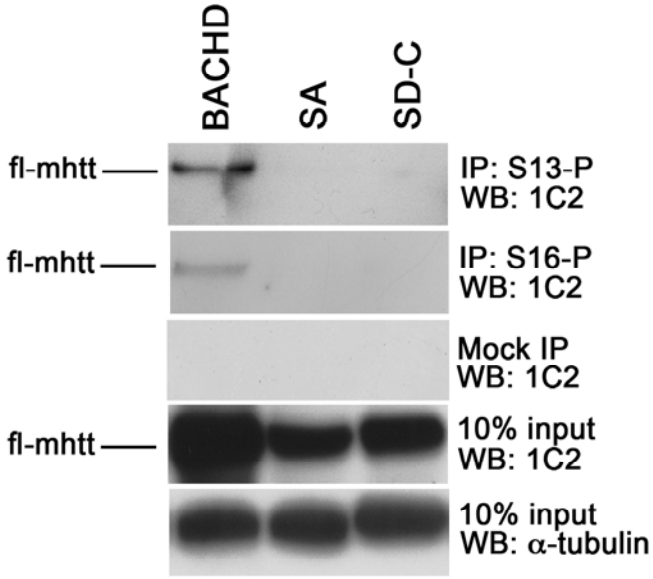
(B and C) Cesium chloride centrifugation method (Gong and Yang, 2005) was used to purify SD and SA BACs. PI-Sce-I linearized SD BAC **(B)** and SA BAC **(C)** are separated by pulse-field gel electrophoresis to demonstrate that they are intact and free of a substantial amount of degraded BAC DNA fragments. These intact SD and SA BAC DNA were used for pronuclear microinjection to generate transgenic founders.

(D and E) Tail DNA was prepared from SD and SA transgenic mice, and the NT17 region was amplified by PCR and followed by genomic DNA sequencing. SA mice contain the double TCC (encoding serine) to GCC (encoding alanine) mutations at serines 13 and 16 **(D)**, whereas SD mice contain doubled TCC to GAC (encoding aspartate) mutations at the two serines **(E)**.

Supplemental Figure S1.



Supplemental Figure S2. Fl-mhtt is Phosphorylated at Serine 13 and Serine 16 in BACHD Mouse Brain. Immunoprecipitation were performed using purified anti-S13-P and anti-S16-P antibodies with soluble cortical extracts prepared from 1-month old BACHD, SA, and SD-C mice. Ser13 and Ser16 phosphorylated species of fl-mhtt can be detected only in BACHD mice but not in SD and SA mice by western blot analysis probed with the 1C2 antibody. 10% input loading and Mock IP (IP with no antibody) controls of BACHD, SA, and SD-C cortex soluble protein extracts are indicated with the α-tubulin and 1C2 antibodies.



Supplemental Figure S3. Monoclonal Antibody 4H7H7 Preferentially Recognizes Disease-Associated Polyglutamine Expansions.

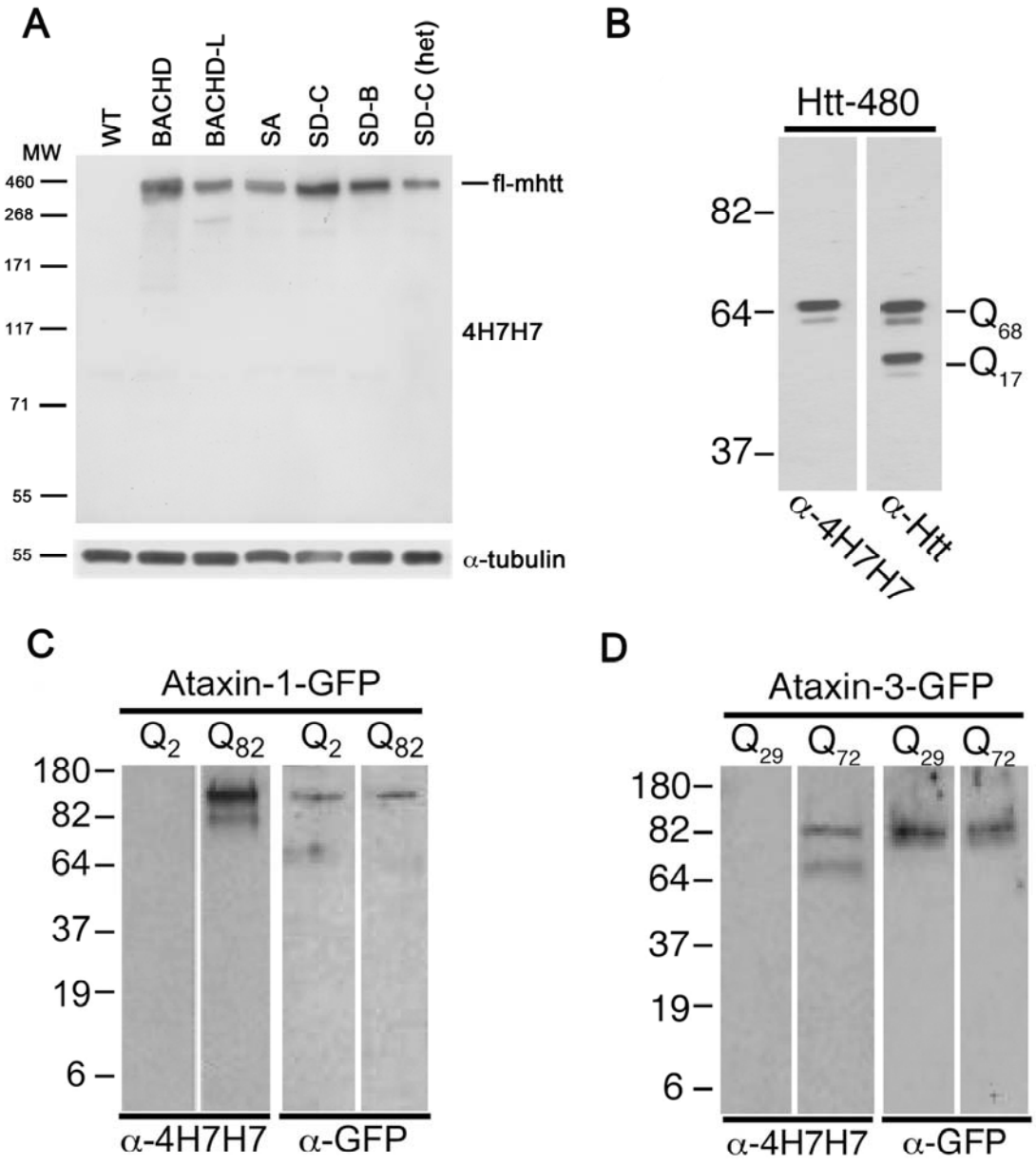
(A) Western blot analysis using 4H7H7 of 1-month wild type, BACHD, BACHD-L, SA, SD-B/C, and SD-C (het) mice demonstrates that the 4H7H7 antibody is specific for fl-mhtt, and does not recognize endogenous wild type htt. Notice that the 4H7H7 antibody recognizes both the SA and SD forms of fl-mhtt from LDS-solublized SA, SD-B, and SD-C cortical lysates.

(B) Comparable amounts of extracts of COS-7 cells expressing a 480 amino acid N-terminal fragment of huntingtin (htt) with either wild type (Q_{17}) or disease-associated (Q_{68}) polyglutamine expansions were mixed together and blotted with 4H7H7 or an antibody against htt (mAb4C10).

(C) Extracts of HEK293 cells expressing Ataxin-1 GFP with either wild type (Q_2) or disease-associated (Q_{82}) polyglutamine expansions were blotted with 4H7H7 or an antibody against GFP.

(D) Extracts of HEK293 cells expressing Ataxin-3 GFP with either wild type (Q_{29}) or disease-associated (Q_{72}) polyglutamine expansions were blotted with 4H7H7 or an antibody against GFP. 4H7H7 selectively recognizes mutant forms of the htt, Ataxin-1, and Ataxin-3 proteins although comparable amounts of proteins were loaded. A second band appears below full-length mutant ataxin-1 and ataxin-3 in lanes blotted with 4H7H7 that is approximately the predicted molecular weight of these proteins after cleavage of the GFP moiety.

Supplemental Figure S3.



Supplemental Figure S4. Subcellular Fractionation of BACHD, SA, and SD-C Cortical Extracts Do Not Reveal Any Major Difference in Fl-mhtt Localization or 1C2 Fragmentation Pattern.

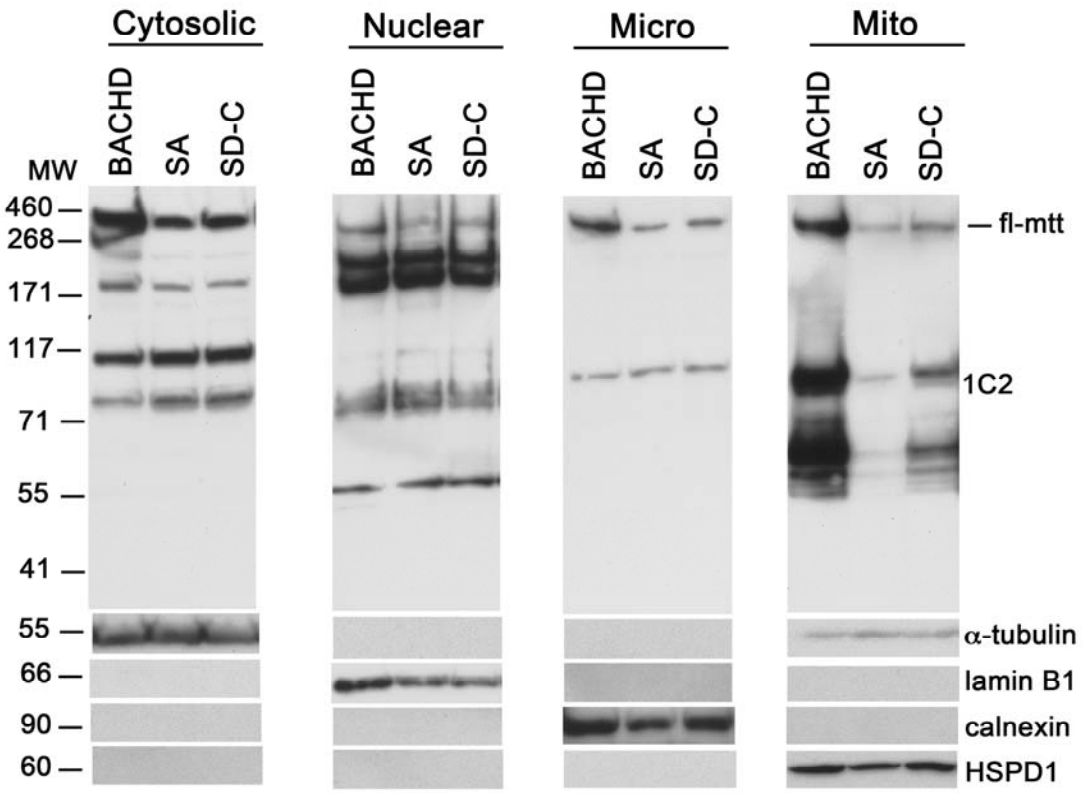
(1) Cytosolic Fraction. Western blot analysis of cytosolic fractions from 1-month old (1m) BACHD, SA, and SD-C cortical homogenates probed with anti-1C2 and anti-α-tubulin as a loading control and localization marker.

(2) Nuclear Fraction. Western blot analysis of nuclear fractions from 1m BACHD, SA, and SD-C cortical homogenates probed with anti-1C2 and anti-lamin B1 as a loading control and localization marker**.**

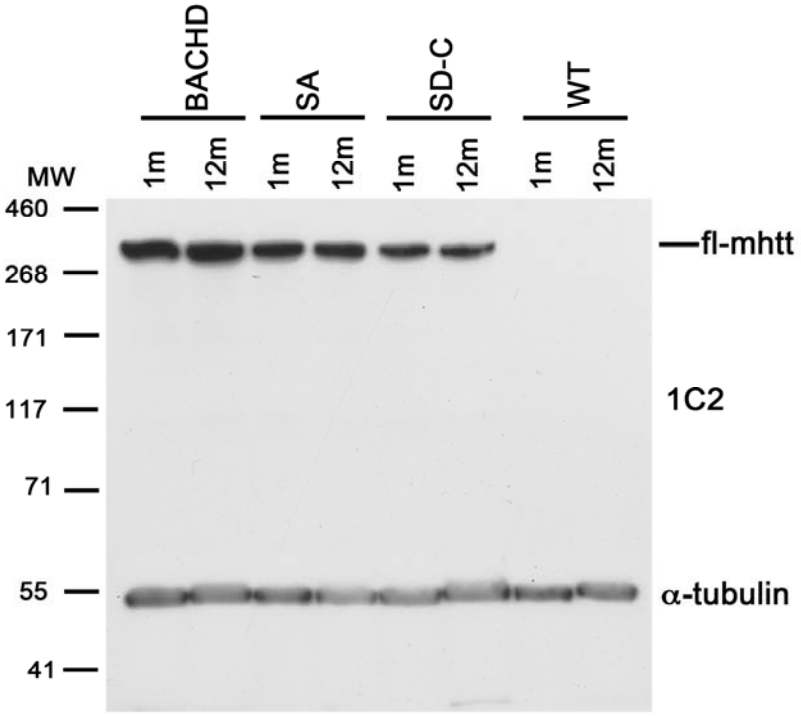
(3) Microsomal Fraction. Western blot analysis of microsomal fractions from 1m BACHD, SA, and SD-C cortical homogenates probed with anti-1C2 and anti-calnexin as a loading control and localization marker.

(4) Mitochondrial Fraction. Western blot analysis of mitochondrial fractions from 1m BACHD, SA, and SD-C cortical homogenates probed with anti-1C2 and anti-HSPD1 (Hsp60) as a loading control and localization marker.

Supplemental Figure S4.

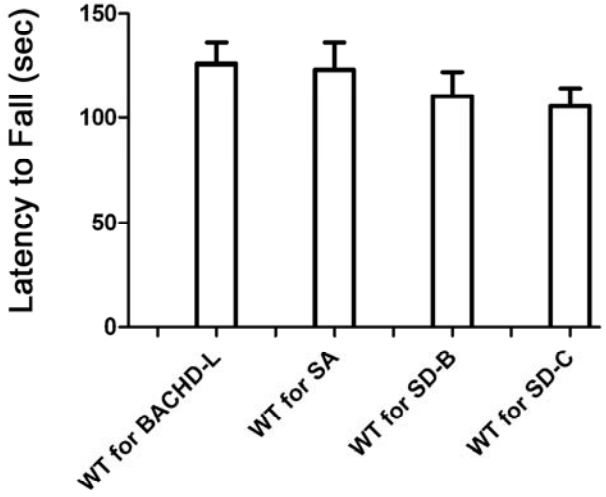


Supplemental Figure S5. Soluble Fl-mhtt Protein Levels in BACHD, SA, and SD-C Mice Do Not Change Between 2- and 12-Months of Age. Western blot analysis with anti-1C2 reveals that the BACHD, SA, and SD-C mice express fl-mhtt protein levels that are comparable between 2 and 12 months of age. These blots also did not reveal any aberrant increase in the generation of soluble mhtt polyQ fragments in the SD or SA mice compare to the BACHD mice. The same blot was probed with anti-α-tubulin for the loading control.



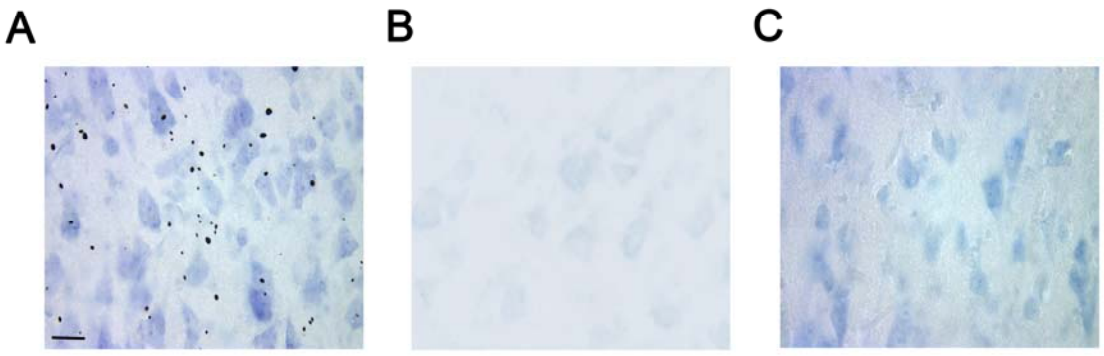
Supplemental Figure S6. Wild Type Mice from BACHD-L, SA, SD-B, and SD-C Demonstrate No Significant Differences in Their Rotarod Performance at 6-Month Age.

This is a control comparison to ensure that the Rotarod performance in BACHD, SD, and SA genotypes cannot be attributed to significant differences in their WT performance. One-way ANOVA with Fisher's LSD *post hoc* test was applied to analyze Rotarod performance of wild type control mice from different genotypes at 6 months of age. There is no significant difference across all lines ($F_{(3,38)}$ =1.292, p=0.285 and p>0.05 in all pairwise comparisons.



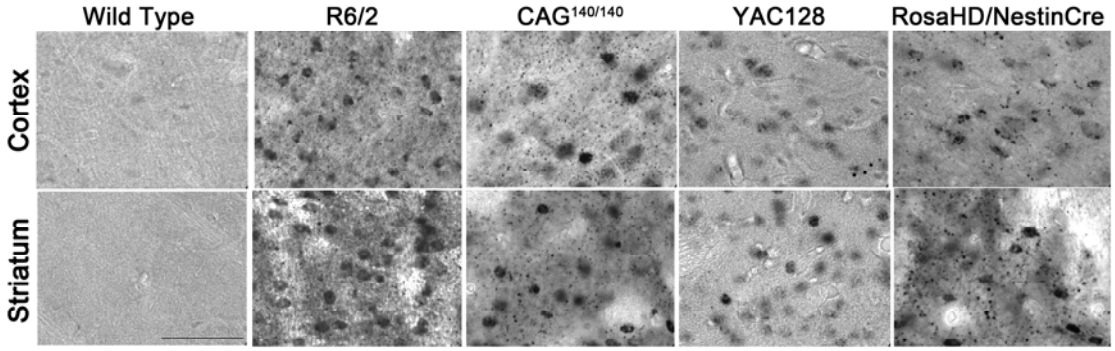
Supplemental Figure S7. 4H7H7-Immunoreactive Neuropil Aggregates in HD cortex.

(A) Light micrograph showing typical spherical and elongated, ellipsoidal neuropil aggregates in a section from the middle frontal gyrus of a grade 2/3 HD case, age 54 F, with CAG repeat of 45/20; no staining was seen without formic acid treatment **(B)** or in another neurodegenerative disease control **(C),** a formic acid-treated section of a case of early onset familial AD, age 46 F. Scale bar represents 20 μm.

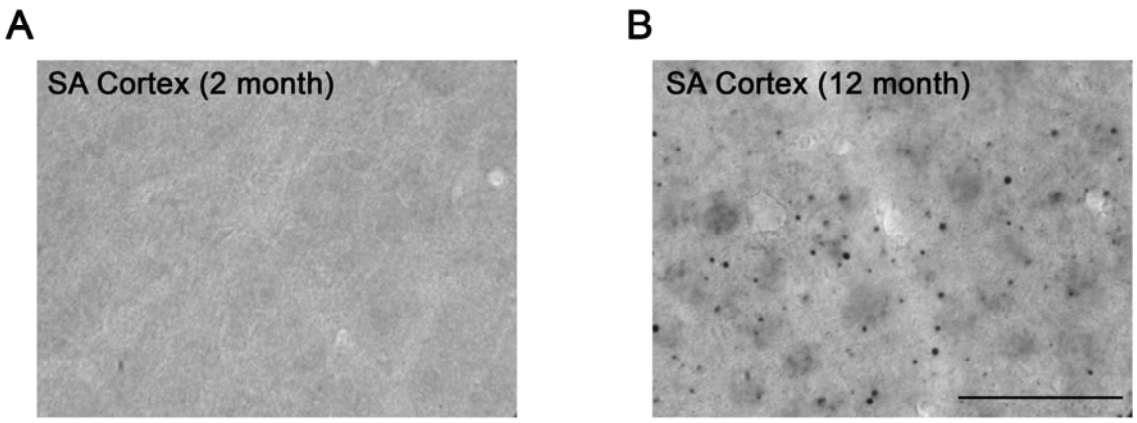


Supplemental Figure S8. 4H7H7-Immunostainig of Aggregates in N-terminal Fragment and Full-length Mutant Htt Mouse Models of HD.

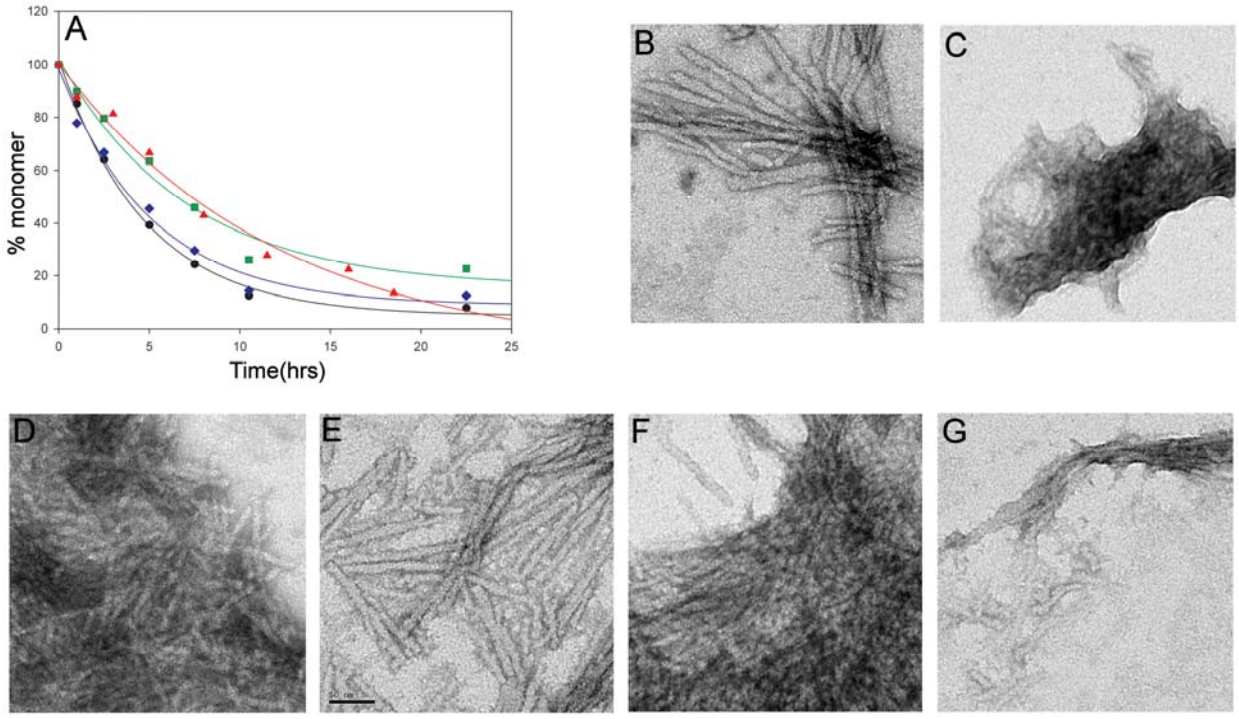
Immunohistochemical staining with antigen retrieval using the biotinylated monoclonal antibody 4H7H7 shows no appreciable staining in wild type cortex or striatum at 12 months of age (left two panels). 4H7H7 staining can readily detect neuropil and nuclear aggregates in the cortex and striatum of multiple HD mouse models at ages known to accumulate such aggregates. R6/2 mice (3 month age) had the most nuclear inclusions and neuropil aggregates both in the cortex and striatum. The other fragment mouse model, RosaHD/NestinCre (12 month age), had multiple nuclear aggregates and numerous neuropil aggregates. CAG140 knock-in mice at 4-month age already had heavily nuclear accumulation and neuropil aggregates. YAC128 mice (12 month age) had accumulation of nuclear and neuropil aggregates, though it had the least amount of staining in all the four mouse lines. Thus, immunohistochemistry with antigen retrieval using 4H7H7 antibody can readily detect both nuclear and neuropil polyQ mhtt aggregates in the brain of multiple HD mouse models. Scale bar represents 50 μm.



Supplemental Figure S9. Progressive Accumulation of 4H7H7 Immunoreactive Mutant Htt Aggregates in SA Mice. While 4H7H7 does not detect mhtt aggregates in WT control littermates at either 2 or 12 months of age (data not shown), it can readily detect such aggregates in the cortex of SA mice at 12-months **(B)** but not at 2-months **(A)**. Thus, the accumulation of 4H7H7 positive polyQ htt aggregates is a progressive process as observed in HD as well as other mouse models of HD. Scale bar represents 50 μm.



Supplemental Figure S10. Aggregation studies of single SD mutants. (A) Kinetics (HPLC based sedimentation assay) of aggregation of NT17-$Q_{37}P_{10}K_2$ peptides: WT NT17 sequence,10.6 μM (●, R^2 = 0.9969); S13D, 11.6 μM (♦, R^2 = 0.9873); S16D, 10.5 μM (■, R^2= 0.9752); SD double mutant,10.0 μM (▲, R^2 = 0.9863**). (B-G)** Electron microscope images of the final aggregates of the S13D **(B-D)** and S16D **(E-G)** single mutants isolated after 30 hrs aggregation time. These aggregates exhibit a mixture of mature fibrils and short protofibrillar intermediates. Scale bar represents 50 nm.



SUPPLEMENTAL RESULTS

Characterization of the novel 4H7H7 monoclonal antibody for detecting soluble fl-mhtt and mhtt aggregates in BACHD patients and HD mice:

To examine the formation of htt aggregates in BACHD, SA, and SD mice, we decided to test different anti-htt and anti-polyQ antibodies to identify ones that may be sensitive in detecting mhtt aggregates in mouse brains. This approach was taken because the original anti-htt antibody used to detect htt aggregates, EM48, revealed a relatively low abundance of mhtt aggregates in BACHD brains (Gray et al., 2008). Although other anti-polyQ antibody (e.g., 1C2) can detect mhtt aggregates in the HD patients, their utility in HD mouse models are limited due to the relatively high background in the wild type mice (Osmand et al., 2006). In this study, we found that the new anti-expanded-polyQ monoclonal antibody, 4H7H7, appears to be a robust reagent that can detect mhtt aggregates in HD patients and in a variety of HD models while it does not exhibit high background staining in wild type controls at all.

(1) Western blot analyses to determine 4H7H7 can selectively recognize expanded polyQ epitope in mutant huntingtin as well as other expanded polyQ proteins.

Similar to other anti-polyQ antibodies such as 1C2 and 3B5H10, the mouse monoclonal 4H7H7 antibody preferentially binds to mutant proteins with pathogenic expansions of the polyQ repeat (Brooks et al., 2004). Western blots with 4H7H7 antibody can successfully detect the full-length mutant htt allele from cortical extracts of 1m BACHD, BACHD-L, SA, SD-B/C, and SD (het) mice, but not the wild type endogenous huntingtin (Figure S3A). Likewise, 4H7H7 is able to detect mhtt N-terminal fragments with 68 polyglutamines but not 17 polyglutamines

(Figure S3B), and can also detect mutant ataxin-1 with 82 polyglutamines (Figure S3C) and ataxin-3 with 72 polyglutamines (Figure S3D).

(2) Immunohistochemical Staining with 4H7H7 Can Detect Mutant Huntingtin Aggregates in the Brains of HD Patients and a Variety of HD Mouse Models.

We next examined whether 4H7H7 antibody could uncover polyQ epitope buried in aggregated mhtt and thereby allow sensitive detection of nuclear inclusions (NI) and nuclear aggregates (NA). As in HD and HD mouse models. Using this antibody to stain cortical sections from HD postmortem brains with an antigen retrieval protocol (Osmand et al., 2006; see Supplementary Experimental Procedures), we found that 4H7H7 antibody could detect mhtt NIs and NAs (Figure S7A), and this staining pattern was not detected in HD brains without antigen retrieval (Figure S7B) nor in control non-HD brains with antigen retrieval (Figure S7C). Similar results were obtained in a variety of HD mouse models expressing either mhtt N-terminal fragments or fl-mhtt. As shown in Figure S8, no 4H7H7 positive mhtt aggregates were detected in wild type control mice up to 1 year of age, but abundant aggregates could be readily detected in the cortex and striatum of mutant HD mice, including R6/2 mice (Davies et al., 1997), RosaHD/Nestin-Cre mice (Gu et al., 2005), CAG140 knock-in mice (Menalled et al., 2003), and YAC128 mice (Slow et al., 2003). Since 4H7H7 antibody combined with an antigen retrieval protocol could readily detect mhtt aggregates in HD mice and HD patients, we used this method to examine the accumulation of mhtt polyQ aggregates in BACHD, SA, and SD mice (Figures 5 and S9, refer to description in the Results section)

SUPPLEMENTAL METHODS

Restriction Fingerprint Mapping of Engineered SD and SA BACs and Generation of BAC Transgenic Mice. To ensure the modified SD and SA BACs do not contain any unwanted rearrangements or deletions during homologous recombination (Yang et al., 1997; Yang et al., 1999; Gong and Yang, 2005, Yang and Gong, 2005), we performed restriction enzyme digestion of the modified BACs to compare with that of the parental wild type htt BAC (RP11-866L6; Gray et al., 2008). Briefly, SD, SA, and WT htt BAC DNA was digested with restriction enzymes PI-Sce I, Mlu I, Xho-I and AscI, Not I, Pac I at 37 °C overnight and separated on pulse-field gel electrophoresis (Figure S1; data not shown). The patterns of the restriction fragments with different digestions (*i.e*. restriction fingerprints) are very similar among the SD, SA, and WT htt BACs, suggesting no gross rearrangement or deletion is present in these BACs (Yang et al., 1999; Yang and Gong, 2005). The presence of S13,16D and S13,16A mutations were confirmed by PCR amplification of the modified NT17 region (see PCR primers and conditions below) and sequencing. BAC DNAs were prepared according to our established protocol (Gong and Yang, 2005). The purified SD and SA BAC DNA were free of small degradations as confirmed by pulse-field gel electrophoresis (see Figures S1B and S1C). The SD and SA BAC DNA was linearized with PI-Sce I enzyme (Gong and Yang, 2005) and microinjected into fertilized FvB one cell embryo at UCLA Transgenic Facility.

PCR Primers and Conditions for Genotyping and Sequencing of Genomic DNA from SD, SA and BACHD Mice.

Genotyping of SD, SA, and BACHD mice from tail genomic DNA was performed using the published PCR primers and conditions from the previous BACHD study (Gray et al., 2008).

We routinely used the following primers for genotyping purposes: (1) amplifying the Htt Intron I (N-htt5': GAG CCA TGA TTG TGC TAT CG; N-htt3': AGC TAC GCT GCT CAC AGA AA); (2) the 5' UTR and exon 1 region including NT17 and polyQ domains (Q-htt5': CCT CCC TTA CCA TGC AGT CCC G; Q-htt3': TTGCTGCTGTTGGAAGCTT); (3) and amplifying the C-terminal of human htt gene (C-htt5': GCA ACG TGC GTG TCT CTG ; C-htt3': GAG CAG GCT TTG GGA ACA) regions were used for genotyping. PCR conditions are the following: initial 3 minute denaturation at 94 ºC, followed by 30 cycles of 94 ºC denaturation for 1 minute, 55 ºC annealing for 1 minute, and 72 ºC for 1 minute. This is followed by a 10-minute incubation at 72 ºC. For PCR through the polyQ region we add Q solution (Invitrogen) in PCR reaction buffer.

To confirm that the SD and SA mutations are both present in the established and maintained mouse lines, we also used the strategy of PCR amplification of tail genomic DNA using the Q-htt5' and Q-htt3' primers and the PCR conditions described above. The PCR product is then sequenced using the Q-htt5' primer to confirm the presence of SD and SA mutations in appropriate mouse lines (see Figures S1D and S1E).

Assessment of Mixed CAA-CAG Repeat Size in Mutation Huntingtin in SD and SA BACHD Mice.

The CAA-CAG repeat length in SD and SA BACHD mice were determined from tail genomic DNA using our established protocol for BACHD mice (Gray et al., 2008). Briefly, purified tail genomic DNAs were prepared using Puregene Kit A (Qiagen) and the CAA-CAG repeat region was PCR amplified using Q-htt5' and Q-htt3' primers (see above) and subjected for automatic sequencing (Applied Biosystems 3730XL) at Laragen (Los Angeles). The CAA-CAG repeat

numbers were calculated using the GeneMapper software.

Preparation of Brain Tissue Lysates, and Immunoprecipitation, and Western Blotting.

Brain protein extracts were prepared as previously described (Gray et al., 2008). Briefly, mouse brains were dissected in ice-cold 100 mM PBS and homogenized in modified RIPA buffer supplemented with Complete Protease Inhibitor Mixture tablets and Phos-Stop Phosphatase Inhibitor tablets (Roche) using 10 strokes from a Potter-Elvehjem homogenizer followed by centrifugation at 4°C for 15 min at 16,000*g*. Protein concentrations of soluble whole cell lysates were determined using the Bio-Rad Protein Assay (Bio-Rad).

Cortical extracts (0.5 mg) from 1m BACHD, SA, and SD-C mice were subjected to immunoprecipitation with anti-S13-P and anti-S16-P (1:500) using Protein G Dynabeads (Invitrogen). Anti-S13-P and Anti-S16-P antibodies were provided by J.S. Steffan and L.M. Thompson, whom have confirmed the specificity of the antibodies using immunoblots against phosphorylated NT17 peptides (Thompson et al., in press). Immunoprecipitated proteins were washed, eluted with NuPAGE LDS loading buffer, and 10% input, mock IP (no antibody), and immunoprecipitates were analyzed by SDS-PAGE and Western blot analysis.

We used the published Western blotting protocols in the current study (Gray et al., 2008). Briefly, protein samples were prepared for loading by heating in NuPAGE LDS buffer (Invitrogen) for 10 min at 70°C. Proteins (40-μg) were resolved on 3-8% Tris-acetate NuPAGE gels (Invitrogen) using Tris-acetate running buffer, followed by a wet-transfer onto polyvinylidene difluoride (PVDF) or nitrocellulose membranes using NuPAGE transfer buffer (Invitrogen). Immunoblots were probed with anti-Htt 2166 (Abcam, mAb2166, 1:3000), 1C2 (Millipore, MAB1574, 1:3000), S13-P (J. Steffan and L. Thompson, UC Irvine, 1:1000), mAb

4H7H7 (1:3000 dilutions , S. Finkbeiner), and α-tubulin (Sigma, 1:10,000). Secondary antibodies used for Western analysis are goat anti-mouse-HRP and goat anti-rabbit HRP (Biorad). Chemilluminescence detection was accomplished using ECL Plus Western Blotting Detection reagents (GE Healthcare). Densitometric values from scanned Western blot film were obtained using ImageQuantTL software (GE Healthcare).

Biochemical Characterization of the Epitope Recognized by Mouse Monoclonal 4H7H7 Antibody.

HEK293 cells were transfected with mammalian expression plasmids encoding wild type or mutant ataxin-1-GFP or ataxin-3-GFP. Extracts were blotted with 4H7H7 (1:5000) or anti-GFP (1:1000) and then HRP-linked secondary antibodies (1:5000-1:10,000) as described. Blots were developed by enhanced chemillumescence and autoradiography.

Subcellular Fractionation of BACHD, SD and SA Brain Extracts.

Subcellular fractionation of 1-month old cortices of BACHD, SA, SD-C and respective wild type control littermate mice (n=3 per genotype) were carried out as previously described (Cox et al., 2006). Briefly, mouse brains were dissected in ice-cold 100 mM PBS and homogenized in 4-8 volumes of 250 mM sucrose, 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM DTT, 1mM PMSF, 25 μg ml^{-1} Spermine and Spermidine (250-STMDPS buffer) in a pre-chilled dounce glass tube with a Teflon pestle for 2 minutes. The nuclei and unbroken cells were pelleted at 800*g* for 15 minutes, and the resultant supernantant was further processed for isolation of mitochondrial, cytosolic, and microsomal fractions. The nuclei pellet was resuspended in 250-STMDPS and subjected to a sucrose gradient by layering above 2 M sucrose, 50 mM Tris-HCl (pH 7.4), 5 mM

$MgCl_2$, 1 mM DTT, 1 mM PMSF, 25 μg ml^{-1} Spermine and Spermidine (2-STMDPS buffer), followed by differential centrifugation at 80,000*g* for 35 minutes. To extract the nuclear proteins, the pure nuclei were resuspended in 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.5 M NaCl, 0.2 mM EDTA, and 20% glycerol (NE buffer), followed by gentle rocking at 4°C for 30 minutes, lysing by 10 passages through an 18-gauge needle, and centrifugation at 9,000*g* for 30 minutes. The resultant supernatant is the nuclear fraction. The mitochondria fraction was obtained by spinning the initial homogenate supernatant at 6,000*g* for 15 minutes, resuspension of the pellet in 10 volumes of 250-STMDPS buffer, followed by another centrifugation at 6,000*g* for 15 minutes. The mitochondrial pellet was resuspended in 10 mM HEPES (pH 7.9), 1 mM DTT, and 1 mM PMSF (HP buffer), incubated on ice for 30 minutes, sonicated to lyse the mitochondria, harvested at 9,000*g* for 30 minutes, resuspended and incubated in 20 mM Tris-HCl (pH 7.8), 0.4 M NaCl, 15% glycerol, 1 mM DTT, 1 mM PMSF, and 1.5% Triton-X-100 (ME buffer) for 30 minutes, followed by microcentrifugation at 9,000*g* for 30 minutes. The resultant supernatant is the mitochondrial fraction containing mitochondrial membrane proteins. A cytosolic fraction was obtained using the supernatant from the initial mitochondrial isolation by ultracentrifugation at 100,000*g* for 1 hour. The resultant supernatant is the pure cytosolic fraction, and the pellet is the microsomal fraction. The microsomal pellet was resuspended in ME buffer, incubated for 1 hour with gentle rocking, followed by microcentrifugation at 9,000*g* for 30 minutes. The resultant supernatant is the microsomal fraction. Markers for cytosol (α-tubulin, Sigma, 1:10,000), nuclei (lamin B1, Abcam,1:1000), microsomes (calnexin, Abcam, 1:3000), and mitochondria (HSPD1, Abcam, 1:10,000) were blotted against extracts from subcellular fractions to verify fractionation purity and correct subcellular location.

Immunohistochemistry with Biotinylated Mouse Monoclonal 4H7H7 Antibody.

Immunohistochemical detection of polyQ mhtt aggregates in the brain sections using biotinylated 4H7H7 antibody was performed using an established protocol (Osmand et al., 2006). Briefly, brain sections 35 μm thick were cut on a cryostat and washed with 0.01 M PBS 3x10 min., followed by one wash with water. Sections then treated with 90% formic acid 3x10 min. After quickly rinsing three times in water, sections were washed 2 x 10 min. in PBS, followed by treatment with 1% sodium borohydride (w/vol.) in PBS for 30 min, followed by 3x10 min. washes in PBS (or until no bubbles remained). Brain sections were moved into 0.01 M PBSTx (0.01 M PBS, 0.04% Triton X-100) and washed for 10 min, 30 min, and then 10 min. Sections were then incubated with biotinylated 4H7H7 antibody (25 ng/ml) overnight at 4 °C with gentle shaking. The next day, sections were washed in PBSTx 6 x 10 min, followed by incubation in biotinylated anti-mouse IgG (1: 2,000; Vector Lab) for 2 hours. Brain sections were then washed with PBST x 6 x 10 min, and incubated with Streptavidin-HRP (TSA BIOTIN System, PerkinElmer) diluted 1:100 with 1% normal goat serum in PBSTx for 30 min. Sections were then washed 3x10min. in PBSTx, followed by incubating with TSA diluted 1:100 with amplification diluents for 10 min. After washing with PBSTx 3 x 10 min, sections were incubated with avidin-biotinylated peroxidase (ABC "Elite" reagent, Vector Lab) for 2 hours. Sections were then washed three times in PBS and incubated in Vector SG Substrate (Vector Lab) until the color developed properly (usually within 5 min). Sections were quickly removed from SG substrate and washed three times in PBS, rinsed once in water, and mounted on Superfrost plus slides. Sections were imaged sing a Zeiss Axioscope 2 microscope with an AxioCamHRc camera.

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