

## Progressive phenotype and nuclear accumulation of an amino-terminal cleavage fragment in a transgenic mouse model with inducible expression of full-length mutant huntingtin

Yuji Tanaka,<sup>a,1</sup> Shuichi Igarashi,<sup>a,2</sup> Masayuki Nakamura,<sup>a,2</sup> Juliette Gafni,<sup>b</sup> Cameron Torcassi,<sup>b</sup> Gabrielle Schilling,<sup>c,3</sup> Danielle Crippen,<sup>b</sup> Jonathan D. Wood,<sup>a,4</sup> Akira Sawa,<sup>a,d</sup> Nancy A. Jenkins,<sup>e</sup> Neal G. Copeland,<sup>e</sup> David R. Borchelt,<sup>c,5</sup> Christopher A. Ross,<sup>a,d,f,\*</sup> and Lisa M. Ellerby<sup>b,\*</sup>

<sup>a</sup>Division of Neurobiology, Department of Psychiatry, Johns Hopkins University School of Medicine, CMSC 8-121, 600 North Wolfe Street, Baltimore, MD 21287, USA

<sup>b</sup>The Buck Institute for Age Research, Novato, CA 94945, USA

<sup>c</sup>Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

<sup>d</sup>Department of Neuroscience, and Program in Cellular and Molecular Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 2120, USA

<sup>e</sup>Mouse Cancer Genetics Program, NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702, USA

<sup>f</sup>Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD, USA

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**Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized behaviorally by chorea, incoordination, and shortened lifespan and neuropathologically by huntingtin inclusions and neuronal degeneration. In order to facilitate studies of pathogenesis and therapeutics, we have generated a new inducible mouse model of HD expressing full-length huntingtin (Htt) using a tetracycline-regulated promoter. In double transgenic mice Htt was**

**expressed widely in the brain under the control of the tet-transactivator (tTA) driven by the prion promoter PrP (in the absence of doxycycline). Mice expressing full-length mutant Htt, but not full-length normal Htt, displayed a progressive behavioral phenotype, consisting of slowed and irregular voluntary movements, gait ataxia, tremor and jerky movements, incoordination, and weight loss, with a shortened lifespan. Neuropathology included prominent intranuclear inclusions in cortex and striatum as well as cytoplasmic aggregates. This phenotype is very similar to the phenotypes of previous transgenic mice expressing N-terminal fragments of mutant Htt. The current HD-transgenic mice had nuclear accumulation of Htt, particularly an approximately 60-kDa fragment, which appears to represent an N-terminal cleavage product. This fragment is smaller than calpain or caspase-derived cleavage products of Htt, but it is comparable to a product, termed cp-A, which accumulates in nuclei of cells in a previously described cell model. This new mouse model may be useful in the future for pathogenic and preclinical therapeutic studies related to HD. The data suggest that proteolytic processing could be a part of the pathogenesis of HD, potentially representing an attractive therapeutic target.**

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\* Corresponding authors. C.A. Ross is to be contacted at Johns Hopkins University School of Medicine, CMSC 8-121, 600 North Wolfe Street, Baltimore, MD 21287, USA. L.M. Ellerby, The Buck Institute for Age Research, Novato, CA 94945, USA.

E-mail addresses: caross@jhu.edu (C.A. Ross),  
lellerby@buckinstitute.org (L.M. Ellerby).

<sup>1</sup> Current address: Department of Neuropsychiatry, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama City, Okayama 700-8558, Japan.

<sup>2</sup> Current address: Department of Neuroscience, Brain Research Institute, Niigata University, I Asahimachi, Niigata 951-8585, Japan.

<sup>3</sup> Current address: Leibniz Institute for Age Research—Fritz-Lipmann-Institute e.V. (FLI), Beutenbergstr.11, D-07745 Jena.

<sup>4</sup> Current address: Academic Neurology Unit, University of Sheffield, Western Bank, Sheffield, S10 2TN, UK.

<sup>5</sup> Current address: Santa Fe Health Alzheimer's Research Center, Department of Neuroscience, McKnight Brain Institute, University of Florida, 100 Newell Drive, Rm. L1-100H, PO Box 100244, Gainesville, FL 32610-0244, USA.

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### Introduction

Huntington's disease (HD) is a neurodegenerative disorder caused by expansion of a CAG repeat in the HD gene, coding for polyglutamine in the huntingtin protein (Htt) (Brinkman et al., 1997; Duyao et al., 1993; Huntington's Disease Collaborative Research Group, 1993; Ross et al., 1997; Stine et al., 1993). Onset

is usually in mid-adult life but can range from childhood to old age, depending on the length of the CAG repeat expansion. The threshold for the disease is 36 repeats or above, and longer expanded repeats result in earlier onset. The clinical features of HD include movement disorder, cognitive disorder, and behavioral changes, progressively worsening over time, and leading to death. The movement disorder consists of involuntary movements, such as chorea and dystonia, and also progressive incoordination, bradykinesia, and rigidity. Weight loss is often a feature of the phenotype (Djousse et al., 2002). Neuropathologically, HD is characterized by selective neuronal degeneration in the caudate and putamen, as well as by overall brain atrophy (Vonsattel et al., 1985). A pathological hallmark of the disease consists of intranuclear inclusion bodies, which can be labeled with antibodies to the Htt protein, to expanded polyglutamine repeats, or to ubiquitin (Davies et al., 1999).

HD is one of several neurodegenerative disorders caused by expanding polyglutamine repeats. All of the disorders involve neuronal degeneration in selective regions of the brain, distinct in each disease, but with considerable overlap (Ross, 1995). The disease gene products show no homology except for the polyglutamine expansions, leading to the hypothesis that expanded polyglutamine is directly toxic to neurons. While the mechanisms of cell death are incompletely understood, they likely involve largely gain of function mechanisms, mediated in part by translocation of the mutant polyglutamine stretch into the cell nucleus and alterations of gene transcription (Cha, 2000; Ross, 2002; Sugars and Rubinsztein, 2003). Of the polyglutamine diseases, dentatorubral and pallidoluysian atrophy (DRPLA) is most similar to HD in its clinical features and neuropathology (Ross et al., 1997). Study of the different polyglutamine diseases in parallel is likely to lead to elucidation of shared mechanisms.

Generation of mouse models has played an important role in the study of HD pathogenesis (Menalled and Chesselet, 2002; Rubinsztein, 2002). Transgenic mouse models made using an N-terminal fragment of Htt containing the expanded polyglutamine repeat have yielded a severe behavioral phenotype, with abnormal movements, incoordination, ataxia, and weight loss, progressing to early death (Mangiarini et al., 1996; Schilling et al., 1999a). The intranuclear inclusions were originally discovered in the transgenic mouse models and then later identified in human patient material (Davies et al., 1997; DiFiglia et al., 1997). Transgenic mice, like HD patients, have both intranuclear inclusions and also neuritic and cytoplasmic aggregates. Transgenic and knock-in mouse models have been made expressing full-length Htt with an expanded repeat. To date, these have generally had a less severe behavioral phenotype but have a more restricted neuropathology, including nuclear translocation of mutant Htt (Lin et al., 2001; Menalled et al., 2002; Slow et al., 2003; Wheeler et al., 2000). The transgenic models made with the N-terminal fragment of Htt have generally had more widespread pathology than seen in human cases. A transgenic model with inducible expression of an N-terminal fragment of Htt has also been generated, making it possible to show that there is at least partial reversibility of both the behavioral and neuropathologic phenotype when expression of mutant Htt is switched off (Yamamoto et al., 2000).

Several lines of evidence suggest the possibility that proteolytic cleavage of Htt may contribute to the pathogenesis of HD. The mouse models made with an N-terminal fragment generally have a more severe behavioral and pathologic phenotype than models expressing full-length Htt. In cell model experiments, transfection of N-terminal fragments generally gives greater cell toxicity than

transfection of full-length mutant Htt (Cooper et al., 1998; Martindale et al., 1998; Saudou et al., 1998). Most cell model experiments have suggested that translocation of the N-terminal fragment into the nucleus enhances toxicity (Peters et al., 1999; Ross, 2002; Saudou et al., 1998). In addition, N-terminal fragments of Htt have been detected in human postmortem tissue and mouse models, though the length and nature of the fragments have been difficult to characterize precisely (DiFiglia et al., 1997; Gafni and Ellerby, 2002; Goldberg et al., 1996; Kim et al., 2001; Mende-Mueller et al., 2001; Sun et al., 2002; Wellington et al., 2002; Yu et al., 2003). By contrast, other careful examinations of Htt have not found any evidence for cleavage (Wheeler et al., 2000), and it has been suggested that mutant Htt may even be resistant to proteolytic cleavage (Dyer and McMurray, 2001).

Htt can be cleaved in several places by caspase enzymes (Goldberg et al., 1996; Wellington et al., 2002). Caspase cleavage may contribute to toxicity (Ellerby et al., 1999; Wellington et al., 2000). The caspase cleavage sites are located between amino acids 513 and 586. Caspase enzymes that can cleave Htt include caspase-2, -3, -6, and -7 (Hermel et al., 2004; Wellington et al., 1998, 2000). Htt can also be cleaved in several places by calpains (Kim et al., 2001; Gafni and Ellerby, 2002), which may also contribute to toxicity (Gafni et al., 2004). The major calpain sites are located between amino acids 469 and 536 (Gafni and Ellerby, 2002; Gafni et al., 2004), though other smaller fragments have been described after ischemic injury (Kim et al., 2003). Calpains defined as capable of cleaving Htt include calpains-1 and -2.

In addition, putative N-terminal fragments of Htt smaller than those which would be predicted to arise by caspase or calpain cleavage have been detected in human HD postmortem brain material (DiFiglia et al., 1997) and in at least one knock-in mouse model (Li et al., 2000; Zhou et al., 2003). However, the length and nature of these fragments have been difficult to establish exactly. In a recent cell model study in which a full-length Htt construct with an expanded repeat was inducibly expressed in NG108 cells, at least two smaller cleavage products were detected. The fragments were termed cp-A and cp-B and suggested to arise from sequential cleavage events in the approximate range of amino acids 100–240, with the smaller fragment entering the nucleus, and contributing to the formation of nuclear inclusions and possibly cell toxicity (DiFiglia, 2002; Lunke et al., 2002).

In the present study, we sought to generate a new mouse model of HD expressing full-length Htt using an inducible promoter so that expression can be controlled in order to facilitate studies of pathogenesis and therapeutics. In addition, we sought to determine whether there might be proteolytic cleavage of Htt in this model and the nature of the fragments that might be generated.

## Materials and methods

### *Transgene and other plasmid construction*

The pTet-splice vector (Life Technologies, Inc.) was digested with *SalI* and *HindIII*, and an SKH linker containing a *BspEI* site was ligated into the cut pTet-splice vector. The construct was then digested with *BspEI*. Full-length cDNA of Htt containing 23 consecutive glutamines was excised from a wild-type full-length Htt construct with *NotI*, and ligated into the *BspEI*-cut pTet-splice vector (designated as pTet-splice-FL23Q-with-no-tag). To create a myc tag, N-terminal Htt fragments containing N-terminal myc tag

and the first 171 amino acids with either 23Q or 148Q (myc-N171-23Q or myc-N171-148Q) were amplified by PCR using N-myc-FL-U-primer (CAGGTCGACGCCACCATGGAACAAA-AACTCATCTCAGAAGAGGATCTGAATATGGGACCCTGGA-AAAGCTGA) and Xho-Cla-L-primer (CTGATCGATCTC-GAGCTGTAACCTTGGAAGAT). A Htt polyglutamine repeat expansion with 148 consecutive glutamines was generated using a method that introduced a CAA interruption (Peters and Ross, 1999) in order to attempt to reduce polymerase slippage during cell division. The amplified DNA fragments containing N-terminal myc tag and 23 or 148 repeats of CAG were subcloned into the pTet-splice vector after digestion with *SalI* and *ClaI* (designated as pTet-N171-23Q or pTet-N171-148Q). These constructs were digested with *XhoI* and the resulting DNA fragments containing N-terminal myc tag and 23 or 148 repeats of CAG were ligated into the pTet-splice-FL23Q-without-tag after digestion with *XhoI*. These resulting constructs of full-length Htt with 23 or 148 repeats of CAG were designated as iFL23Q or iFL148Q. Plasmids were linearized and purified for injection.

Stop constructs designated as pTet-N111-23Q and pTet-N111-148Q were also made by site-directed mutagenesis with the following primers: (CTGACAATATGTGAAAATAAGTGGCA-CAGTCTGTCAGAAATTC) and (GAATTTCTGACAGACTGT-GCCACTTAGTTTTTCACATATTGTCAG). PCR was performed using 50 ng DNA, 5.0  $\mu$ l  $10\times$  *Pfu* buffer (Stratagene), 0.2 mM dNTPs (Roche Molecular Biochemicals), 125 ng each of forward and reverse primers (Integrated DNA Technologies), 5.0% DMSO (Sigma), and 1.0  $\mu$ l *Pfu* polymerase (Stratagene) at 96°C for 1 min, 18 cycles at 96°C for 50 s, 55°C for 1 min and 68°C for 24 min, and 68°C for 7 min. Plasmids were DpnI (Stratagene)-treated, transformed into XL1-Blue Supercompetent Cells (Stratagene) and purified using the Qiagen Plasmid Mini Kit. Mutations, CAG repeat length, and construct integrity were confirmed by DNA sequencing.

The MoPrP.tTA vector was generated by cutting out the tTA open reading frame contained within the pT.tTAk vector (Life Technologies, Inc) using *HindIII* and *SpeI*. The fragment was blunted with Klenow and *XhoI* linkers were ligated. This product was digested with *XhoI* and ligated into *XhoI* digested MoPrP.Xho vector (Borchelt et al., 1996). Vectors containing insert were mapped by restriction endonuclease digestion and orientation of the tTA open reading frame within the vector was verified by directional PCR amplification (sense primer GCTTAAT-GAGGTCGGAATCGAAGG; antisense primer GTGGATA-CCCCCTCCCCAGCCTAGACC). The same PCR strategy was used in genotyping transgenic animals. Prior to injection of C3B6F2 embryos, vector DNA was digested with *NotI* and chromatographed in agarose gel to separate carrier plasmid from vector elements (~15 kb). The vector elements were purified from the gel and injected as previously described (Schilling et al., 1999a). Two lines have been generated, termed lines 6 and 46. Most of the experiments done to date have involved crossbreeding with line 6.

#### Western blot analysis

Mouse brain tissue was homogenized (Teflon/glass) in 16 volumes ice-cold 0.25 M sucrose/buffer (50 mM triethanolamine, pH 7.5, 25 mM KCl, 5 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl), and a cocktail of protease inhibitors

(Complete Mini; Boehringer Mannheim). Proteins were separated in 7.5% polyacrylamide gels, and transferred onto nitrocellulose membrane (Schleicher and Schuell). Blots were blocked in 5% non-fat dry milk-phosphate-buffered saline (PBS) and probed with a polyclonal *c-myc* antibody (1:1000, Upstate), 1C2 antibody (1:1000, Chemicon) or N-terminal Htt antibody AP194 (peptide antibody, amino acids 1–17, 1:500) overnight. After washing in 5% milk-PBS, the blots were visualized by ECL Western blotting detection reagent (Amersham Pharmacia Biotech).

#### Rotarod testing

Eight mice from PrP-tTA-6/iFL148Q-69 Dox(–) were compared with eight age-matched mice from PrP-tTA-6/iFL23Q-1 Dox(–). Four, six-, or eight-month-old mice were tested. The mice were tested on a Rotarod device (Rotamex 4/8, Columbus Instruments International). The speed of the rod was set to increase from 10 to 40 rpm over a 10-min period. The interval for the mice to fall from the rod was measured in four trials per day over a 4-day period. The mice were given at least 8-min recovery time.

#### Immunohistochemistry

Transgenic mouse brains from 10-month-old mice were fixed with PLP (4% paraformaldehyde, 75 mM D/L-lysine (Sigma), 10 mM sodium *m*-periodate (Sigma) in PBS pH 7.4). Cut sections were permeabilized in methanol–peroxide for 10 min. The sections were washed and blocked in 5% normal goat serum for 45 min and incubated in an N-terminal Htt antibody (AP360, 1:5000 dilution), GFAP (1:500, Sigma), or monoclonal 1C2 antibody (1:500) overnight. Washed sections were incubated in secondary antibody in a dilution of 1:200 for 3 h. Washed sections were then incubated in ABC reagent (Vectastain-Elite ABC kit, Vector Laboratories) for 45 min and developed with DAB–peroxide for 5 min.

#### Nuclear and cytosolic fractions

Mouse brain tissue from 7-month-old mice was homogenized (Teflon/glass) in 16 volumes ice-cold 0.25 M sucrose/buffer (50 mM triethanolamine, pH 7.5, 25 mM KCl, 5 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl, and a cocktail of protease inhibitors (Complete Mini; Boehringer Mannheim)). The homogenate was centrifuged at 800  $\times$  g for 15 min at 4°C, and the pellets were re-suspended in 10 ml of the 0.25 M sucrose/buffer and mixed with 20 ml of 2.3 M sucrose/buffer. The mixture was layered on top of 10 ml of 2.3 M sucrose/buffer and centrifuged at 124,000  $\times$  g for 1 h at 4°C. The pellets were resuspended in the 0.25-M sucrose/buffer and washed by centrifugation at 800  $\times$  g for 30 min. The final pellets were designated as the nuclear fractions. The supernatants from the initial centrifugation were centrifuged at 100,000  $\times$  g for 1 h at 4°C. The resulting supernatants were taken as the cytosolic fractions. Proteins were separated in 6% polyacrylamide–SDS gels and transferred onto nitrocellulose membrane (Schleicher and Schuell). Blots were blocked in 5% non-fat dry milk-PBS and probed with a polyclonal AP194 antibody at a dilution of 1:2000 overnight. Polyclonal TATA-binding protein (1:1000; sc-204) or monoclonal  $\beta$ -tubulin (1:1000; Sigma) was used to evaluate fractionation. After washing in 5% milk-PBS, the blots were visualized by ECL Western blotting detection reagent (Amersham Pharmacia Biotech).

### Treatment of cell lysates with recombinant caspase or calpain

Cell pellets from transgenic tissue or 293T transfected cells were homogenized in NP-40 lysis buffer (0.1% NP-40, 50 mM HEPES, pH 7.4, 250 mM NaCl, 5 mM EDTA) or RIPA (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% SDOC, 1% NP-40, protease inhibitor (Complete Mini, Roche Molecular Biochemicals)). The supernatant was collected following centrifugation. Samples were prepared by treating Htt 23Q or 148Q transfected cell lysates in NP-40 lysis buffer with *m*-calpains (calpain-2; Sigma) incubated 5 min at 30°C or recombinant caspase-3 (kind gift of Guy Salvesen) incubated 60 min at 37°C. Protein lysates were resolved on a 4–12% polyacrylamide gel, transferred to a PVDF membrane, and probed with polyclonal Htt antibody AP194 and monoclonal polyglutamine antibody 1C2. Immunoblots were developed with a peroxidase-conjugated secondary antibody and enhanced chemiluminescence.

## Results

The constructs used for inducible expression in this mouse model are shown in Fig. 1. An N-terminal myc tag was introduced into a full-length Htt construct with 148 consecutive glutamines in order to facilitate identification of N-terminal Htt cleavage fragments. This construct was cloned into the pTet-splice vector, and lines of tet-op huntingtin mice were generated, using standard transgenic technology (Schilling et al., 1999a,b), incorporating the transgene encoding either mutant (148Q) or normal (23Q) huntingtin.

In order to drive expression throughout the brain with a regional distribution comparable to that of our previous model made using the N-terminal fragment of Htt (Schilling et al., 1999a), a line of mice was also generated expressing the tet-transactivator (tTA) under the control of the prion promoter (PrP-tTA), using a strategy similar to our previous models using this promoter (Schilling et al., 1999a,b). The inducible HD mouse model involves crossbreeding the PrP-tTA mice with the TetO-Htt mice. Expression of the transgene is controlled using the tetracycline analogue doxycycline (500 mg/L in the drinking water). Fig. 2 shows inducible

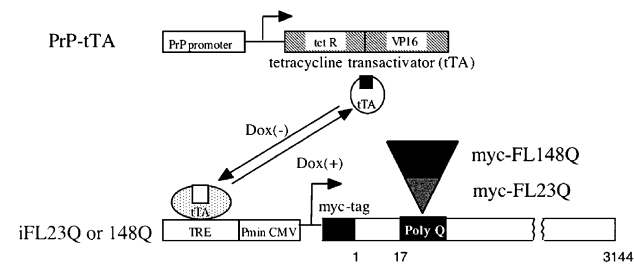


Fig. 1. The Tet-Off gene expression regulatory system. The Tet-Off system is regulated through tTA, a fusion protein of the Tet repressor (TetR), and VP16 activation domain. Expression of the transgene is induced when doxycycline (Dox) is removed (“induced” conditions). Conversely, in the presence of doxycycline, tTA is prevented from binding to the tet-responsive element (TRE) and expression of the transgene is inhibited. The full-length human Htt cDNA including 23Q or 148Q was cloned into the pTet-splice vector (see Materials and methods). The mouse prion protein promoter (PrP) targets transgene expression to most brain neurons except cerebellar Purkinje cells.

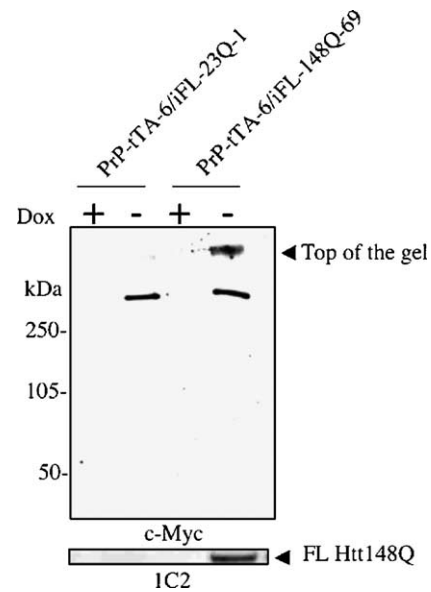


Fig. 2. Confirmation of transgene expression by Western blot analysis in induced or uninduced conditions. Mice were kept in uninduced conditions in the presence of Dox until birth of offspring, at which time Dox was discontinued in order to induce transgene expression. The Western blots probed with the myc antibody show expression levels of the transgene at 5 months of age. Left margin, protein molecular mass. Dox+, uninduced conditions; Dox–, induced conditions. PrP-tTA-6/iFL23Q-1, mice crossed with PrP-tTA mouse line 6 and iFL 23Q mouse line 1. PrP-tTA-6/iFL148Q-69, mice crossed with PrP-tTA mouse line 6 and iFL 148Q mouse line 69. Expression of mutant Htt can be confirmed with either c-Myc antibodies to the epitope or 1C2 antibodies to the polyglutamine expansion (lower panel).

expression of normal Htt or mutant Htt, using the antibody to the myc epitope. Similar inducible expression could be demonstrated using the AP194 antibody to Htt (Schilling et al., 1999a) or the 1C2 antibody to the expanded polyglutamine stretch (Fig. 2). Expression of the transgene was substantially suppressed in the presence of doxycycline, and expression can be induced by withdrawal of doxycycline. Full-length mutant Htt had slightly slower migration in the gel compared to full-length normal Htt, consistent with the retardation caused by the expanded polyglutamine tract. Levels of expression appeared comparable (Fig. 2). The mice expressing mutant but not normal Htt showed aggregated material at the top of the gel.

For the study of the behavioral phenotype, double transgenic (PrP-tTA crossed to TetO-Htt) mice were maintained off doxycycline after weaning. Mice expressing full-length mutant Htt, but not full-length normal Htt, displayed a progressive behavioral phenotype, consisting of slowed and irregular voluntary movements, gait ataxia, tremor and jerky movements, incoordination, and progressive weight loss. The behavioral phenotype was strikingly similar to that of our previous mouse model made with the N-terminal fragment of Htt (Schilling et al., 1999b). The phenotype was progressive and led to early death of the animals, as shown in Fig. 3. Lifespans ranged about 7–10 months. Toward the end of the lifespan, the mice showed a hunched abnormal posture when walking and limb clasp behavior when suspended by the tail (Fig. 4).

The progressive movement disorder could be quantified using the Rotarod as shown in Fig. 5. Mice appeared normal and were

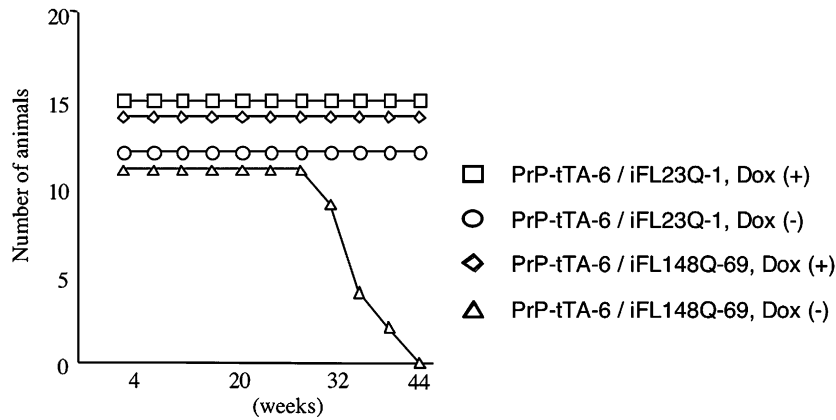


Fig. 3. Reduced lifespan in transgenic mice expressing full-length mutant Htt. Mice expressing full-length normal Htt (PrP-tTA-6/iFL23Q-1, Dox(-)) or in uninduced conditions (Dox(+)) showed normal lifespans. Mice expressing full-length mutant Htt (PrP-tTA-6/iFL148Q-69, Dox(-)) had shorter lifespans, dying at about 8–11 months of age.

able to walk normally on the Rotarod at 4 months of age. Beginning at 6 months of age they had awkward gait and fell off the Rotarod earlier than controls. This ataxia was progressive, and at 8 months of age the behavior was clearly very abnormal.

In subsequent generations, the phenotype appeared to have a later onset (about a year, possibly with slower progression) apparently due in part to a contraction in the CAG repeat length to 112 repeats, and in part to lower expression of tTA. We have an additional line under control of the mouse prion promoter (line 46), as well as a line of PrP-tTA mice (*Prn-tTA-F959*) which has expression driven by the hamster rather than the mouse prion promoter, and which has stable expression of tTA (Tremblay et al., 1998). Furthermore, we have crossed the Htt mice with mice expressing tTA under the control of the CaMKII promoter (Mayford et al., 1996), and preliminary data indicate that these double transgenics also have comparable levels of expression, in this case restricted to the forebrain, as would be expected. In addition we have conducted a series of injections to generate new lines expressing the construct with 148Q. These additional models will be the subjects of future studies.

Upon neuropathologic observation of the initial cohorts of the PrP-tTA-6/iFL148Q-69 mice, there was mild to moderate atrophy of the entire brain. Histological examination using Nissl stain showed no evidence of abnormal brain development. Examination using antibodies to the N-terminus of Htt (Fig. 6) demonstrated intranuclear inclusions in cerebral cortex, hippocampus, striatum, and cerebellar granule cells. In addition, there were cytoplasmic aggregates found in some cortical and striatal neurons as detected

by the 1C2 antibody (Fig. 6). The PrP-tTA-6/iFL148Q-69 mice (Fig. 7) have ventricular enlargement (approximately 30% increase) and reactive astrocytosis (as detected by increased GFAP label).

We conducted Western blot analysis in order to determine whether we could detect a cleavage fragment of Htt. Since previous studies suggested that this fragment might be enriched in neuronal nuclei, we separated homogenates into cytoplasmic and nuclear fractions. As can be seen in Fig. 8, mice expressing full-length Htt with a normal polyglutamine repeat showed full-length Htt in the cytoplasm with none detectable in the nuclear fraction. By contrast, the mice expressing mutant Htt had a different pattern. There was full-length Htt in the cytoplasm, again migrating slightly slower than normal full-length Htt. However, in the nucleus, in addition to a small amount of full-length Htt, there was a band running at a much lower position in the gel, approximately 60 kDa. This could be detected either with antibodies to N-terminal Htt epitopes (Fig. 8A) or with antibodies to the myc epitope tag (Fig. 8B). As can be seen in Fig. 8B, the level of human mutant huntingtin is approximately equal to endogenous Htt in the double transgenics and nuclear fractions are free of cytoplasmic markers such as tubulin. In some blots fainter bands could be detected at higher positions in the gel (at about 120 kDa in the gel shown). We have conducted preliminary time course studies of the presence of the small fragment. It was most clearly detected between the ages of 5 and 7 months, during the early and middle stages of the behavioral phenotype, but could be detected less strongly later near the end stage of the behavioral phenotype.

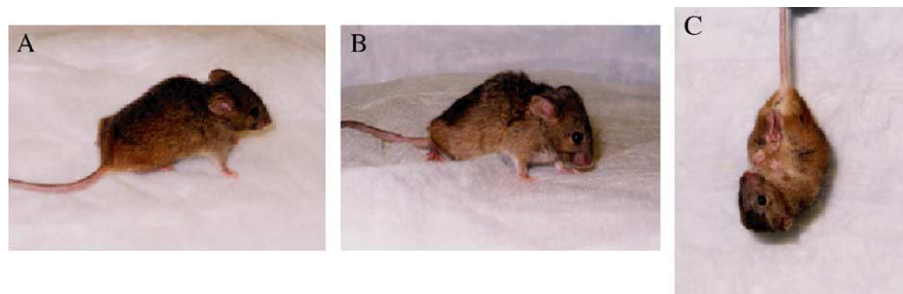


Fig. 4. Representative photographs of an end-stage mouse from PrP-tTA-6/iFL148Q-69 at 9 months. (A) Mice expressing full-length mutant Htt were smaller and had hunched backs. (B) Neurological abnormalities included hypokinesia, loss of coordination, abnormal gait, and tremors. (C) In addition, fore- and hindlimb claspings were detected.

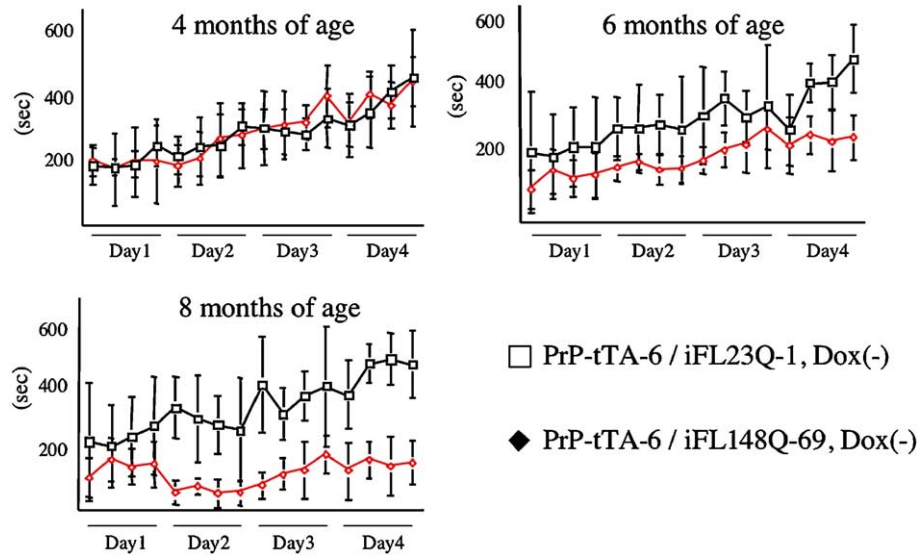


Fig. 5. Mice expressing mutant Htt showed motor dysfunction and a progressive phenotype. After 6 months of age, mice expressing full-length mutant Htt (PrP-tTA-6/iFL148Q-69, Dox(-)) showed impaired performance on the Rotarod test. Mice expressing wild-type Htt (PrP-tTA-6/iFL23Q-1, Dox(-)) showed normal motor function.  $n = 8$  in every trial. Error bars, standard deviation.

In order to compare the length of the fragment detected in the mice with previously described caspase or calpain cleavage fragments of Htt, in vitro cleavage experiments were conducted (Fig. 9). Full-length Htt with 148 glutamines (using the same construct as expressed in the mice) was expressed in 293T cells. When cell extracts were treated with either recombinant caspase-3 or calpain-2 in vitro, analysis by Western blot showed an Htt

fragment at 88 kDa for the caspase-derived Htt cleavage product and fragments at 85 kDa and 80 kDa for the calpain cleavage products. The caspase or calpain cleaved Htt clearly migrated more slowly in the gel than the fragment from the transgenic mice (either nuclear or in whole lysates). Cleavage of Htt by the other caspase isoforms known to cleave Htt also produced fragments larger than the fragment seen in the mice (data not shown); by contrast, the fragment in the mice was of similar size to a fragment produced from a construct with the N-terminal 111 amino acids of Htt containing 148 glutamines. Fig. 9 (lower panel) shows that the cp-A-like fragment is reactive to the 1C2 antibody, which is selective for expanded polyglutamine. Thus, the fragment must derive from mutant huntingtin rather than endogenous huntingtin.

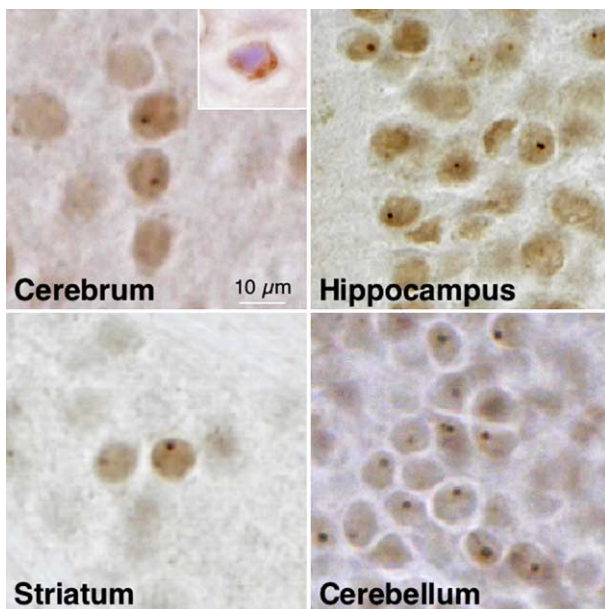


Fig. 6. Pathology in mutant mice. Frozen/free-floating sections from mutant mice (PrP-tTA-6/iFL148Q-69, Dox(-)) were immunostained with AP360 antibody, which was made to the amino-terminus of Htt. The mice expressing full-length mutant Htt showed intranuclear aggregates in cerebral cortex, hippocampus, striatum, and cerebellar granule cells. In addition, Htt protein accumulated diffusely in nuclei in these regions. Cytoplasmic aggregates were also detected in the cerebral cortex (panel inset) and striatum (data not shown) with the 1C2 antibody.

## Discussion

Our results indicate that we have developed an inducible mouse model of HD expressing full-length Htt with an expanded polyglutamine repeat. The model has a progressive behavioral phenotype, leading to death. Aspects of the phenotype, including abnormal movements, incoordination and ataxia, weight loss, and hindlimb clamping when suspended by the tail, continue to progress to an end stage, in which the animals have a hunched back, are smaller than controls, and are hypo-active. The constellation of features of the behavioral phenotype is strikingly similar to that in our previous mouse model expressing an N-terminal fragment of Htt (N171) also driven by the prion promoter (Schilling et al., 1999a). Neuropathologically, the mice have intranuclear inclusions and cytoplasmic aggregates, again similar to the previous model.

The distribution of the inclusions and other aggregates is more widespread than is typically seen in HD patients, with inclusions prominent not only in cortex and striatum, but also in hippocampus, brain stem, and cerebellum. There may be two reasons for this. First, our constructs have a very long polyglutamine repeat, and HD patients with longer polyglutamine repeats also tend to have more widespread pathology. Second, expression is driven not

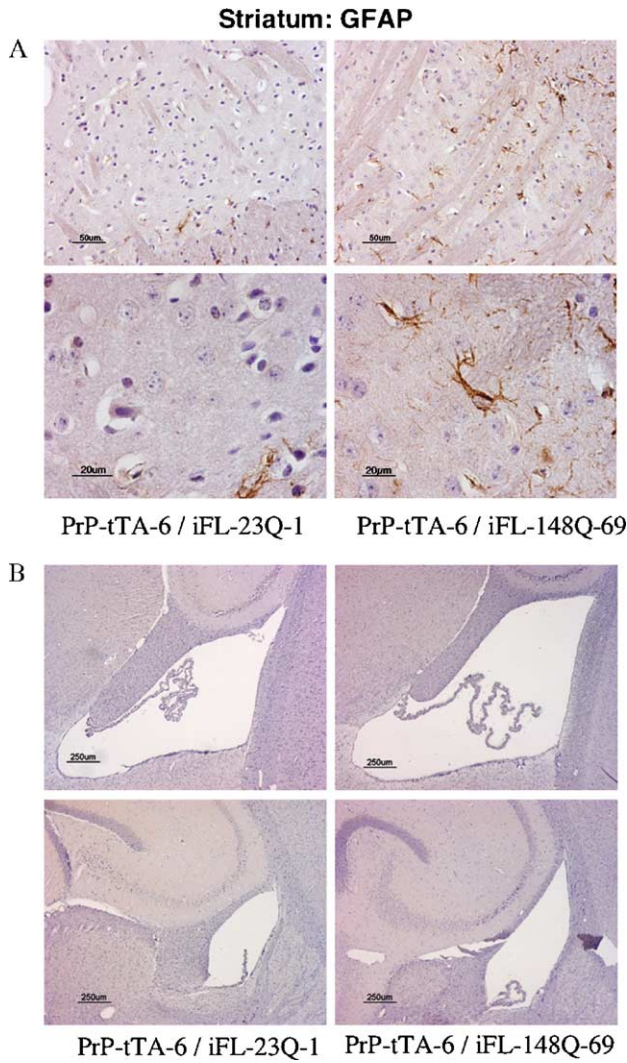


Fig. 7. Ventricular enlargement and reactive astrocytosis in mice expressing mutant Htt. (A) Sections from mutant mice (PrP-tTA-6/iFL148Q-69, Dox(-)) or control (PrP-tTA-6/iFL23Q-1) were immunostained with GFAP antibody. Quantitation of GFAP immunoreactivity in the striatum and cortex indicated a 40% increase in GFAP staining in the mice expressing mutant Htt. (B) Sections from mutant mice (PrP-tTA-6/iFL148Q-69, Dox(-)) or control (PrP-tTA-6/iFL23Q-1) were examined for ventricular enlargement. Mice expressing mutant Htt had ventricular enlargement of about 30%. Two representative sagittal sections in the striatum are shown.

by the Htt promoter but by the prion protein promoter. In our previous model expressing the N-terminal 171 amino acids with 82 glutamines, we also saw a rather widespread distribution of pathology.

Another difference between our model and human HD is the relative lack of marked neuronal cell death in the striatum in this mouse model. There is increased GFAP labeling, characteristic of astrogliosis, and ventricular enlargement, consistent with neurodegeneration, but there does not appear to be massive neuronal loss in the striatum. In this respect, our model is similar to other genetic mouse models of HD, including both models made with N-terminal fragments, such as N171 and R6/2, and other models expressing full-length Htt (Yu et al., 2003). While there has been some degree of degeneration and astrogliosis in several of these models, none of them reproduces the massive selective neuronal

cell death of up to 95% of medium spiny neurons that can be seen in advanced HD. The reasons for this are uncertain but may have to do with expression levels and the relatively short lifespan of the mouse. By contrast, models made using viral expression of mutant Htt, which can achieve higher levels of expression, appear to have been able to reproduce greater degrees of cell death (de Almeida et al., 2002).

Of considerable potential interest is our observation of an N-terminal fragment of Htt in this mouse model. While the issue of cleavage of Htt has received considerable attention, it has been difficult to study in vivo. Thus, our model may provide a useful tool for future study. A fragment of comparable size (considering the different CAG repeat lengths) has been seen in a knock-in HD model (Li et al., 2000). In our model, we see a striking enrichment of the N-terminal mutant Htt fragment in the nucleus in the mice expressing Htt with the expanded repeat, and not in the mice expressing Htt with a normal polyglutamine repeat. This localization is consistent with the studies described in the introduction, suggesting that nuclear localization of an N-terminal fragment of

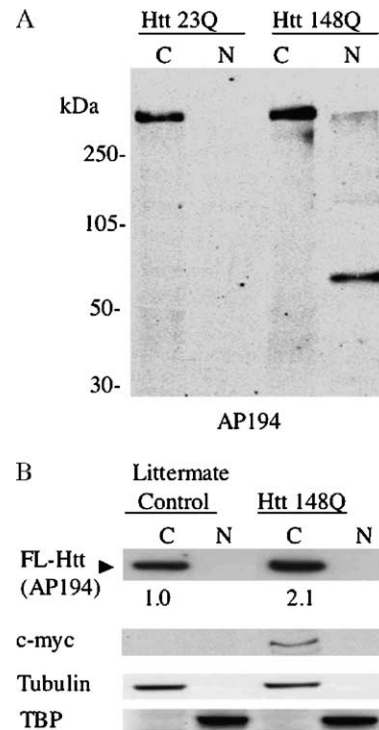


Fig. 8. (A) N-terminal fragments of Htt in mice expressing full-length mutant Htt. A cleavage band, about 60 kDa, was detected in mice expressing full-length mutant (148Q) Htt in nuclear fractions with AP194 antibody, affinity-purified antibody against amino acids 1–17. By contrast, no cleavage band was found in mice expressing normal (23Q) Htt. C, cytosol fractions; N, nuclear fractions; 23Q, PrP-tTA-6/iFL23Q-1 Dox(-); 148Q, PrP-tTA-6/iFL148Q-69 Dox(-). Left margin, protein molecular mass. Our nuclear preparation is free of cytosolic proteins such as tubulin and actin. Analysis of several ER marker proteins demonstrates that our preparations have about 5% mixture with ER proteins. (B) Levels of expression of huntingtin in double transgenic 148Q mice compared to littermate controls. Detected with an N-terminal Htt antibody (AP194), which recognizes both human and mouse Htt, expression in double transgenics is about twice (2.1 to 1.0) that in controls suggesting the transprotein is expressed at levels approximately equal to endogenous. Blots were probed with c-myc. Tubulin and TBP levels demonstrate relative purity nuclear and cytoplasmic fractions.

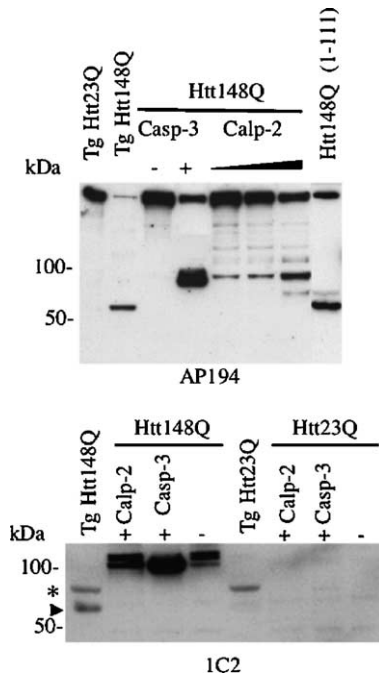


Fig. 9. Caspase-3 or calpain cleavage of full-length Htt148Q generates fragments different from N-terminal fragment accumulated in mice expressing full-length mutant Htt, PrP-tTA-6/iFL148Q-69 Dox(-). Upper panel: Western blot analysis of total extracts from mice expressing full-length normal (23Q) and nuclear extract from mutant (148Q) Htt (12 months) and 293T cells expressing Htt148Q (upper panel) probed with N-terminal Htt polyclonal antibody (AP194). Cellular extracts from 293T cells were treated with recombinant caspase-3 or calpain-2 (or no protease indicated with - sign). The Western blot demonstrates that the 60-kDa fragment that accumulates in the HD-transgenic mice is smaller than the cleavage products of cell lysates expressing Htt148Q treated with recombinant caspase-3 or calpain-2, and similar in size to a fragment (the upper band is endogenous Htt) from a construct encoding the first 111 amino acids with 148 glutamines, comparable to the cp-A fragment of Lunkes et al. (2002). Lower panel: Western blot analysis of nuclear extract from mice expressing full-length normal (23Q) and mutant (148Q) Htt (12 months) and 293T cell expressing Htt23Q and Htt148Q probed with monoclonal 1C2 antibody shows similar results, indicating that the fragment is derived from the mutant protein (asterisk notes non-specific band).

mutant Htt enhances toxicity. We believe that the fragment accumulates with age in nucleus in the PrP-tTA-6/iFL-148Q-69 mice, while any fragment generated in the PrP-tTA-6/iFL-23Q mice does not. This is consistent with reports that fragments containing the expanded repeat have a longer half-life and accumulate in cells to a much greater extent than fragments with a normal polyglutamine repeat (Kaytor et al., 2004). This is also consistent with our observations of a cleavage product of atrophin-1, another polyglutamine disease protein, in a mouse model of the disease DRPLA (Schilling et al., 1999b).

Limitations of the sensitivity of our antibody detection make it difficult to define definitively the time course of the existence of the fragment compared to the time course of the phenotype. We can detect the fragment as a band in the soluble fraction on a Western blot around 5 months, during the early appearance of the phenotype. We detected the cleavage band prior to the appearance of inclusions, consistent with the idea that fragments of mutant Htt are more likely to aggregate and form inclusions than the full-

length protein. It was present in the soluble fraction later in the course as well. Detailed time course studies were limited by the later onset in the later generations. Additional studies are planned using crossbreeding to line 46 (also expressing tTA under control of the mouse prion promoter) and line F959, expressing tTA under the control of the hamster prion promoter (Tremblay et al., 1998). In addition, the levels of expression of Htt in the forebrain, crossing the TetO-Htt mice with CaMK2-tTA mice, are comparable to the levels achieved in the current study, indicating that this is also likely to be a suitable model for future studies. We are also conducting a new round of injections in order to generate additional lines.

As shown by our *in vitro* studies, the length of the fragment that we detect in the nuclei of cells in our mouse model is significantly smaller than the caspase fragments that can be generated from full-length Htt with the same length polyglutamine repeat. Similarly calpain, which also can cleave Htt, yields fragments comparable to the caspase fragments and different from the fragment in our mouse model. The site of cleavage is unknown, but the presence of some full-length huntingtin in the nucleus raises the possibility that cleavage could occur at least in part in the nucleus.

In a cell model study using full-length huntingtin inducibly expressed in a neuronal-like cell line (NG 108) (DiFiglia, 2002; Lunkes et al., 1998, 2002), a fragment termed cp-A was detected, comparable in size to the fragment we see in our mouse model (taking into the account the lengths of the CAG repeat in the two constructs). Interestingly, they detected another fragment, which they termed cp-B. However, the localization of the two fragments differed. Cp-A was present in the nucleus, while the cp-B was predominantly localized to cytoplasm. They suggested there might be sequential cleavage of Htt, initially yielding cp-B, and then cp-A, with the cp-A fragment translocated to the nucleus, and most relevant to toxicity. We believe our *in vivo* results resemble these cell culture results and suggest that our nuclear fragment may be comparable to the cp-A fragment (Lunkes et al., 2002). There may be sequential or parallel cleavage of Htt, perhaps including caspase or calpain cleavage, finally resulting in the N-terminal fragment that accumulates in the nucleus.

The presence of an N-terminal fragment in this HD mouse model is reminiscent of our previous study of a DRPLA-transgenic mouse model (Schilling et al., 1999b). In this model we expressed full-length atrophin-1 directly under the control of the prion promoter, and we were able to detect an N-terminal fragment in nuclear but not cytoplasmic fractions. The fragment of atrophin-1 seen in the DRPLA-transgenic mice is likely to be shorter than the previously described atrophin-1 caspase fragments. We recently conducted studies with atrophin-1 constructs, which suggest that the cleavage event produces a fragment of atrophin-1 that contains a functional NLS but deletes a functional NES, thereby concentrating the fragment abnormally in the nucleus. In a cell culture model, transfection of a comparable fragment of mutant atrophin-1 causes enhanced cellular toxicity (Nucifora et al., 2003). There is also evidence for cleavage of ataxin-3 (Goti et al., 2004) in a mouse model of SCA3. Thus, we would propose that proteolytic processing, perhaps by both caspases and non-caspase enzymes, may be a common feature of polyglutamine diseases.

Once the N-terminal fragment of Htt with the expanded polyglutamine repeat is present in the nucleus, what might be its role in toxicity? We and others have proposed that there might be abnormal interactions with nuclear transcription machinery resulting in abnormal gene transcription, leading to toxicity.

Toxic N-terminal fragments of Htt may undergo abnormal interactions with transcription factors, leading to cell dysfunction and death (Dunah et al., 2002; Jiang et al., 2003; Nucifora et al., 2001; Shimohata et al., 2002; Steffan et al., 2000; Sugars et al., 2004; Wyttenbach et al., 2001). Atrophin-1 can also interact abnormally with transcription factors, leading to toxicity (Nucifora et al., 2001). The N-terminal myc tag on our Htt construct may facilitate the identification of other protein interactors with Htt.

In summary, we have created a new transgenic mouse model inducibly expressing full-length Htt with an expanded polyglutamine repeat. The model had, in its initial cohort, a progressive behavioral phenotype leading to death, quite similar to previously described transgenic mouse models expressing N-terminal Htt fragments. Neuropathologically, it has intranuclear inclusions and other aggregates. The ability to turn on and turn off expression of full-length mutant Htt may facilitate studies of pathogenesis and of reversibility of the pathogenic process. The detection of an N-terminal fragment enriched in the nucleus will facilitate the study of the role of proteolytic processing of Htt and interference with gene transcription in HD pathogenesis. Proteolytic processing may make an especially attractive therapeutic target.

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