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Sp1 Regulates Human *Huntingtin* Gene Expression

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Abstract Huntington's disease (HD) is a hereditary neurodegenerative disorder resulting from the expansion of a polyglutamine tract in the huntingtin protein. The expansion of cytosine–adenine–guanine repeats results in neuronal loss in the striatum and cortex. Mutant huntingtin (HTT) may cause toxicity via a range of different mechanisms. Recent studies indicate that impairment of wild-type HTT function may also contribute to HD pathogenesis. However, the mechanisms regulating HTT expression have not been well defined. In this study, we cloned 1,795 bp of the 5′ flanking region of the human *huntingtin* gene (*htt*) and identified a 106-bp fragment containing the transcription start site as the minimal region necessary for promoter activity. Sequence analysis reveals several putative regulatory elements including Sp1, NF-κB, HIF, CREB, NRSF, P53, YY1, AP1, and STAT in the *huntingtin* promoter. We found functional Sp1 response elements in the *huntingtin* promoter region. The expression of Sp1 enhanced *huntingtin* gene transcription and the inhibition of Sp1-mediated transcriptional activation reduced *huntingtin* gene expression. These results suggest that Sp1 plays an important role in the regulation of the human *huntingtin* gene expression at the mRNA and protein levels. Our study suggests that the dysregulation of Sp1-mediated huntingtin transcription, combining with mutant huntingtin's detrimental effect on other Sp1-mediated downstream gene function, may contribute to the pathogenesis of HD.

Keywords Huntingtin · Sp1 · Transcription · Huntington disease

Abbreviation
HD Huntington's disease

Introduction

Huntington's disease (HD) is a hereditary neurodegenerative disorder resulting from the expansion of a polyglutamine tract of the huntingtin protein (The Huntington's Disease Collaborative Research Group 1993). The expansion of cytosine–adenine–guanine (CAG) repeats located near the 5′-end in exon 1 of the HD gene led to neuronal loss in the striatum and cortex (Reiner et al. 1988). The disorder is manifested when there are more than 35 CAG repeats (Kremer et al. 1994). The mutant HTT is predominantly present in the nucleus, whereas wild-type HTT is mainly distributed in the cytoplasm (DiFiglia et al. 1995; Kegel et al. 2002; Landles and Bates 2004). Neuronal intranuclear inclusions aggregated by mutant huntingtin are characteristics of HD (Scherzinger et al. 1999; Ross and Poirier 2004). The mutant huntingtin has been shown to cause

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intracellular dysfunction such as transcription dysregulation (Dunah et al. 2002; Li et al. 2002; Cui et al. 2006), activation of proteases (Kim et al. 2001; Gafni and Ellerby 2002; Sun et al. 2002), protein misfolding (Cui et al. 2006; Strand et al. 2007), and synaptic dysfunction (Morton et al. 2001; Modregger et al. 2002; Smith et al. 2005). However, some studies showed that neuronal intranuclear inclusions were not associated with neuronal death, indicating that soluble mutant huntingtin may also play an important role (Saudou et al. 1998; Jiang et al. 2006). The soluble mutant huntingtin exerts a detrimental influence on transcription since many proteins with polyglutamine tracts could serve as transcription factors (Chan et al. 2002; Luthi-Carter et al. 2002; Sipione et al. 2002; Sugars and Rubinsztein 2003; Alba and Guigo 2004; Borovecki et al. 2005). The interactions with soluble mutant huntingtin result in the changes of transcription response and cell survival (Steffan et al. 2000; Jiang et al. 2006). Recent studies indicate that impairment of wild-type huntingtin function may also contribute to HD pathogenesis (Cattaneo et al. 2001).

In addition to its role in axonal transport (McGuire et al. 2006), postsynaptic signaling (Sun et al. 2001), endocytosis (Walling et al. 1998), and pro-survival (Dragatsis et al. 2000; Rigamonti et al. 2000; Zhang et al. 2006), huntingtin affects gene transcription regulation (Zuccato et al. 2003). Transcriptional dysregulation has been implicated in HD patients (Borovecki et al. 2005). Mutant Huntingtin has been reported to inhibit the expression of the brain-derived neurotrophic factor (BDNF), a crucial survival factor for striatal neurons. The downregulation of BDNF expression is mediated by the altered binding activity of Repressor Element-1 Silencing Transcription Factor/Neuron-Restrictive Silencer Factor (REST/NRSF). Wild-type huntingtin retains REST/NRSF in the cytoplasm and prevents it from going into the nucleus. Decreased wild-type huntingtin in HD causes the nuclear accumulation of REST/NRSF, thereby repressing the transcription of BDNF (Zuccato et al. 2003; Rigamonti et al. 2007). Sp1 is another major transcription factor involved in HD. Many housekeeping and tissue-specific genes contain functionally important Sp1 binding sites. Sp1 contains three Cys-His zinc finger motifs (Dynan and Tjian 1983; Letovsky and Dynan 1989), and its C-terminal domain interacts with other transcription factors in a synergistic manner to control gene expression in a temporal and spatial manner (Li et al. 1991). Sp1 is required for normal embryonic development, and Sp1-null embryos have severe developmental abnormality and die at an early embryonic stage (Marin et al. 1997; Bouwman et al. 2000). Mutant huntingtin inhibits Sp1-mediated gene transcription (Dunah et al. 2002; Li et al. 2002). However, the notion has been questioned by the fact that the reduction of Sp1 is neuroprotective in HD transgenic mice (Qiu et al. 2006). These studies indicated that the actual role of Sp1 involved in HD pathogenic mechanism remains elusive.

To investigate the molecular mechanism by which the human *huntingtin* gene transcription is regulated, we cloned and functionally analyzed the *huntingtin* gene promoter. We identified functional Sp1 response elements in the huntingtin promoter region, and Sp1 upregulates *huntingtin* gene expression at the mRNA and protein levels. Our study demonstrates that huntingtin is one of the target genes of Sp1 signaling and that huntingtin transcription is regulated by Sp1.

Materials and Methods

Primers and Plasmids

The 5′ flanking regions of the human *huntingtin* gene were amplified by PCR from human BAC DNA clone RP11-1069C14 (BACPAC Resources Center, CHORI, Oakland, CA, USA). Seven fragments covering the 5′ flanking region of the *huntingtin* gene from −1,573 bp upstream to +222 bp downstream of the transcription start site at +1 (adenine) were amplified by PCR and inserted in front of the luciferase reporter gene (Luc) in the pGL3-Basic expression vector (Promega, Madison, WI, USA). Primers were designed to include restriction enzyme digestion sequence at the 5′-end that is the same as the cloning sites of pGL3-Basic. To construct the longest promoter plasmid pHtt-A, the primers Htt-1573*Xho*I (5′-ccgctcgagctcaagaaaagggaggctactgc) and Htt+147r*Hin*dIII (5′-cacaagcttgcacggcagtccccgga) were used to amplify the −1,573- to +147-bp region of the *huntingtin* gene using BAC DNA from the clone RP11-1069C14 as template. This fragment was then cloned into pGL3-Basic at the *Xho*I and *Hin*dIII sites. To construct the fragment pHtt-B, the primers Htt-1573*Xho*I and Htt-788r*Hin*dIII (5′-cacaagcttggccagagccatactcac) were used to amplify the −1,573- to −788-bp region. This fragment was cloned into pGL3-Basic at the *Xho*I and *Hin*dIII sites. To construct the fragment pHtt-C, the primers Htt-1101*Xho*I (5′-ccgctcgagtgcttctcgctgcactaatcac) and Htt+147r*Hin*dIII were used to amplify the −1,101- to +147-bp region. To construct plasmids pHtt-D, pHtt-F, and pHtt-G, the following pairs of primers were used: Htt-465*Xho*I (5′-ccgctcgagtgcc taatgtccccgtc) and Htt+222r*Hin*dIII (5′-cacaagctttgctgctg gaaggact); Htt-79*Xho*I (5′-caactcgagctagggctgtcaatcatgct) and Htt+147r*Hin*dIII; Htt+27*Xho*I (5′-caactcgaggacgggtc caagatggac) and Htt+147r*Hin*dIII. To generate plasmid pHtt-E, pHtt-D was cut with *Sma*I. After purification, the *Sma*I sites on the vector and pHtt-D, respectively, were ligated by T4 DNA Ligase (New England BioLabs, Inc.) to construct the plasmid containing the −183- to +222-bp region. The human *huntingtin* promoter region and all inserts of constructed plasmids were confirmed by restriction enzyme digestion check and sequenced by an automatic fluorescence-

based DNA Sequencer (ABI PRISM DNA analyzer; Applied Biosystems, CA, USA). Computer-aided sequence analysis was performed with the SeqMan software (DNASTAR, Inc., Madison, WI, USA).

Cell Culture, Transfection, and Luciferase Assays

HEK293, N2a, and SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1 mmol/L of sodium pyruvate, 2 mmol/L of L-glutamine, and 50 U of penicillin and 50 μg of streptomycin (Invitrogen, Carlsbad, CA, USA). All cells were cultured at 37°C in an incubator supplemented with 5% CO_2. Cells were plated onto 24-well plates 24 h prior to transfection and cultured to approximately 70% confluence before transfection. Cells were transfected with 0.5 μg of DNA per well using calcium phosphate transfection methods. The pCMV-Luc plasmid was also co-transfected to normalize for the transfection efficiency of various luciferase reporter constructs. After 48 h of transfection, cells were harvested and lysed with 75 μL 1X passive lysis buffer (Promega) per well. *Firefly* luciferase activities and *renilla* luciferase activities were measured using the dual-luciferase reporter assay system (Promega). The *firefly* luciferase activity was normalized to the *renilla* luciferase activity and expressed as relative luciferase units to reflect the promoter activity.

5′-RACE Assay

Rapid amplification of 5′ complementary DNA ends (5′-RACE)-PCR (Liu et al. 2011) was performed to determine the transcription initiation site of *huntingtin*. Total RNA was extracted from SH-SY5Y cells with TRI reagent following the manufacturer's protocol (Sigma). Two reverse primers, 5′-cacaagctttgctgctggaaggact and 5′-cacaagcttgcacggcagtc cccgga, corresponding to +207 to +222 and +131 to +147 of the 5′ untranslated region, were synthesized.

The 5′-RACE-PCR was carried out according to the FirstChoice®RLM-RACE Instruction Manual (Ambion, Foster, CA, USA). The PCR product was cloned into pcDNA4 vector at the *Bam*HI and *Hin*dIII sites. The plasmids were analyzed on a 2% agarose gel and were further sequenced.

Gel Shift Assay

Gel shift assay (GSA) or electrophoretic mobility shift assay was performed as previously described (Wang et al. 2011). To make Sp1-enriched nuclear extract, HEK293 cells were transfected with the pCGN-Sp1 expression vector and lysed in hypotonic buffer for subcellular fractionation (Cai et al. 2008). Probe oligonucleotides were labeled with IR700 Dye (LI-COR Biosciences) and annealed to generate double-stranded probes at a final concentration of 0.1 pmol/μL. For competition studies, nuclear extract was first incubated with 10× (1 pmol) or 100× (10 pmol) of unlabeled competition oligonucleotides for 10 min. Then, 0.1 pmol of the labeled probe was added and the samples incubated for 20 min at 22°C. For the supershifting assay, rabbit anti-Sp1 polyclonal antibody Sp1-ab2 (Active Motif, Carlsbad, CA, USA) was added to the gel shift reaction. The sequences of the oligonucleotides were: consensus SP1: 5′-attc gatcggggcggggcgagc; mutant SP1: 5′-cccttggtgggttgggggcc taagctgcg; HttSp1-wt1: 5′-cacggccccgccccgtccat; HttSp1-wt2: 5′-tcctgccccgcccccagcct; HttSp1-wt3: 5′-cagccgccc cgcccctcagc; HttSp1-wt4: 5′-atcgccccgccccgccccgtct cgccccgcccctca; HttSp1-wt5: 5′-ctgtgccccgccccggcctc; HttSp1-wt6: 5′-tgcagtcccgccccgtcccttcctcgtcccgcctcgccgcg; HttSp1-wt7: 5′-ggggcaggggcgggctggtt; HttSp1-wt8: 5′-ccgtgccgggcgggagaccg. The GSA samples were analyzed on a 4% non-denaturing polyacrylamide gel and the gel scanned using the Odyssey scanner (LI-COR Biosciences) at a wavelength of 700 nm.

Mithramycin A Treatment

Mithramycin A (Sigma) can selectively inhibit Sp1-mediated transcriptional activation. For the luciferase assay, HEK293 cells were co-transfected with pHtt-A, pHtt-C, pHtt-D, pHtt-E, and pHtt-F plasmids and pCMV-Luc, respectively, using calcium phosphate transfection methods. Twenty-four hours after transfection, cells were treated with mithramycin A at 125 nmol/L for 12 h. Control cells were treated with vehicle solution, methanol. Cell lysates were assayed for luciferase activity. For immunoblotting and RT-PCR, HEK293 cell was treated with 125 nmol/L mithramycin A for 48 h and then lysed for protein and RNA extraction.

Quantitative RT-PCR

Total RNA was extracted from cells using TRI reagent (Sigma). ThermoScript reverse transcriptase (Invitrogen) was used to synthesize the first-strand cDNA from an equal amount of the RNA sample according to the manufacturer's instructions. The synthesized cDNA templates were further amplified by Platinum *Taq* DNA polymerase (Invitrogen) in a 20-μL reaction volume. Thirty-five cycles of PCR were performed to cover the linear range of the PCR amplification. The *huntingtin* gene-specific primers 5′-agcagcagcag-cagcagcagcagcagcaa and 5′-atctgactctgcgtcatcactgcac were used to amplify a 344-bp fragment of the *huntingtin* gene coding region. β-Actin mRNA levels were used as an internal control. Gene-specific primers 5′-ggacttcgagcaagagatgg and 5′-gaagcatttgcggtggag were applied to amplify a 462-bp

fragment of the β-actin gene. The products were run on a 1% agarose gel. Gel images were captured using the GelDoc-It^{IM} imaging System (UPLAND, CA, USA) and quantified using quantified with the Kodak Image Analysis.

Immunoblot Analysis

Cell lysates were resolved by 6% Tris–Tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting analysis performed as previously described (Qing et al. 2008). Cells were lysed with RIPA-Doc buffer containing 50 mM Tris–HCl (pH 7.2), 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and protease inhibitor cocktail Complete (Roche Molecular Biochemicals, Indianapolis, IN, USA). For Western blot analysis, samples were loaded onto 6% Tris–Tricine gels and transferred to polyvinylidene fluoride membranes. Membranes were blocked for 1 h in phosphate-buffered saline (PBS) containing 5% non-fat dried milk followed by overnight incubation at 4°C in primary antibodies diluted in the blocking medium. Huntingtin was detected using the anti-huntingtin antibody, and β-actin was also detected as a loading control using the β-actin antibody AC-15 (Sigma). The membranes were rinsed in PBS with 0.1% Tween-20 and incubated with IRDye 800CW-labeled goat anti-mouse antibodies in PBS with 0.1% Tween-20 at 22°C for 1 h and visualized on the Odyssey system (LI-COR Biosciences).

Results

Identification of the Human *Huntingtin* Gene Promoter and its Transcription Start Site

The human *huntingtin* gene contains 67 exons spanning 169,280 bp on chromosome 4 (4p16.3). It encodes a 339-kDa protein of 3,144 amino acids (Fig. 1a). Huntingtin is widely expressed throughout the body in neuronal and non-neuronal cells. To study the transcriptional regulation of the human *huntingtin* gene expression, a 5′ flanking region of the human *huntingtin* gene was cloned from human BAC DNA clone RP11-1069C14 (CHORI), and the region spanning 1,795 bp was sequenced (Fig. 1b). 5′-RACE-PCR was performed to identify the transcription start site of the human *huntingtin* gene. An outer primer and an inner primer, located +59 and −20 bp upstream of the translation start site ATG, were used for the 5′-RACE assay. The primer extension assay yielded a 149-bp DNA product (Fig. 1c). The DNA product was cloned into the pcDNA4 vector (Fig. 1d). DNA sequencing indicates that the major transcription start site is located 163 bp upstream of the translation start site ATG (Fig. 1e). This transcription initiation site is designated as +1 and begins with adenine. Sequence analysis shows that the human *huntingtin* gene has a complex transcriptional unit. Computational analysis of the promoter region applying MatInspector2.2 software (Genomatrix, Oakland, CA, USA) reveals that the 5′ flanking region contains several putative regulatory elements, such as Sp1, NF-κB, HIF, CREB, NRSF, YY1, AP1, and STAT (Fig. 1b).

Functional Analysis of the Human *Huntingtin* Gene Promoter

To investigate the transcriptional regulation of the human *huntingtin* gene, a series of nested deletions of the 5′ flanking fragments were subcloned into a promoterless luciferase reporter plasmid pGL3-Basic. The expression of luciferase in cells transfected with pGL3-Basic relies on the insertion and proper orientation of a functional promoter upstream of the luciferase gene. The pGL3-Basic vector lacking a eukaryotic promoter and enhancer sequences upstream of the luciferase reporter gene has negligible luciferase expression. Seven fragments covering different lengths from −1,573 to +222 bp of the 5′ flanking region of the human *huntingtin* gene were amplified by PCR and subcloned into pGL3-Basic vector according to the restriction enzyme cutting sites as described in "Materials and Methods" (Fig. 2a). The constructs were verified by gel analysis and sequencing (Fig. 2b).

To investigate whether the 5′ flanking region contains the promoter of the human *huntingtin* gene, the deletion plasmids were co-transfected into HEK293 cells along with plasmid pCMV-Luc, which served as an internal transfection efficiency control. Luciferase activity was measured to reflect promoter activity. Plasmid pGL3-Basic (vector) was served as the negative control (Fig. 2c). Plasmid pHtt-A contains a 1,720-bp segment of the 5′ flanking from −1,573 to +147 of the *huntingtin* gene upstream of the luciferase reporter gene. Luciferase assay indicated that pHtt-A has a significant promoter activity in HEK293 cells (105.60±1.77 relative luciferase units, RLU) compared with control pGL3-Basic (Fig. 2c). This indicates that the 1,720-bp fragment contains the functional promoter region of the human *huntingtin* gene.

A plasmid (pHtt-C) containing the sequence from −1,101 to +147 bp displayed a similar promoter activity to pHtt-A, 82.76±1.37 RLU in HEK293 cells (Fig. 2c). Further deletion of 636 bp in the 5′ region (pHtt-D) significantly reduced the promoter activity (26.78±0.70 RLU), and the plasmid containing an additional deletion of 282 bp (pHtt–E) still had significant promoter activity (17.60±0.35 RLU) in HEK293 cells compared with the pGL3-Basic vector ($p<0.001$; Fig. 2c).

To identify the minimal 5′ flanking region required for promoter activity, several additional deletion plasmids were

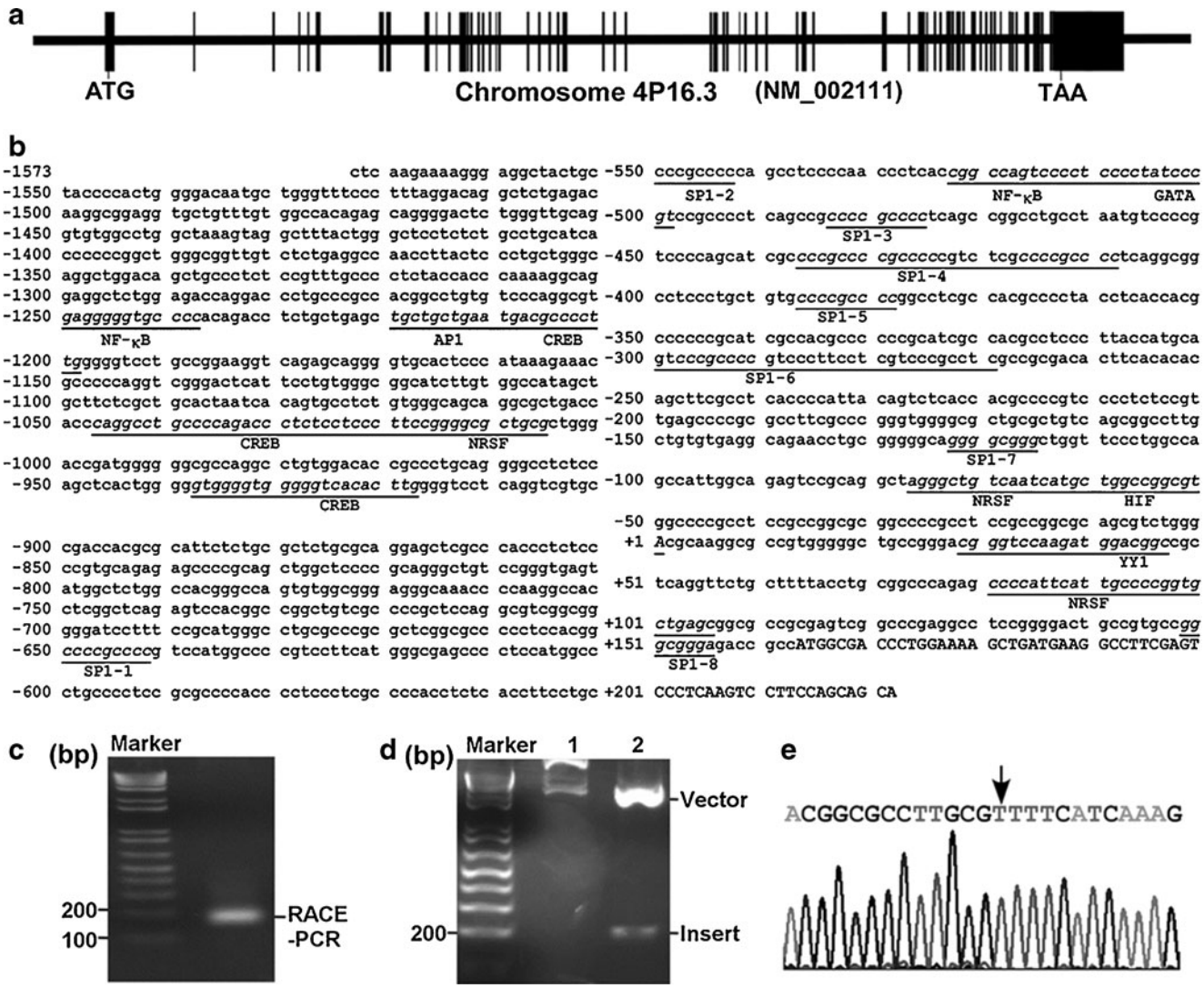

Fig. 1 Sequence features of the human *huntingtin* gene promoter. **a** The genomic organization of human *huntingtin* gene on chromosome 4. The *vertical lines* and *bars* represent exon. The *Huntingtin* gene consists of 67 exons. ATG is the translation start codon and TAA is the stop codon. **b** The nucleotide sequence of the human *huntingtin* gene promoter. A 1,795-bp fragment of the 5′ flanking region of the human *huntingtin* gene was cloned from the human BAC genomic DNA by the primer walking strategy. The adenine +1 represents the transcription start site. The putative transcription factor binding sites are *underlined in italics*. **c** RNA was isolated from SH-SY5Y cells and treated with CIP to remove free 5′-phosphates from DNAs and other RNA. Then it was treated with tobacco acid pyrophosphatase and ligated to a 45-bp RNA adapter using T4 RNA ligase. A random-primed reverse transcription reaction and nested PCR followed. A 149-bp PCR product was generated and analyzed on a 2% agarose gel. **d** The 5′-RACE product was cloned into pcDNA4 and the digested samples analyzed on a 2.0% agarose gel. **e** The plasmid containing the 149-bp 5′-RACE product was sequenced. The *arrow* points to the 5′-end of the 5′-RACE product insert in the plasmid, which corresponds to the transcription start site

generated. Plasmid pHtt-E, containing 405 bp from −183 to +222 bp from the transcription start site, had significant promoter activity (17.60±0.35 RLU) in HEK293 cells (Fig. 2c). Deletion of 104 bp from the plasmid pHtt-E to generate the plasmid pHTT-F resulted in an increase of luciferase activity, 97.46±3.25 RLU (Fig. 2c). Further additional deletion of 106 bp from pHtt-F abolished its luciferase activity, and there was no difference between pHtt-G and pGL3-Basic ($p>0.05$). Plasmid pHtt-B (−1573 to −788 bp) containing no transcription stat site in the insert, similar to pGL3-basic, had no promoter activity. These data showed that pHtt-F contains the sequence of nucleotides from −79 to +147 bp necessary for basal transcription of the *huntingtin* gene.

The Human *Huntingtin* Gene Promoter Contains Sp1 Binding Sites

Transcription factor binding site search reveals that the 1,795-bp 5′ flanking region of the human *huntingtin* gene

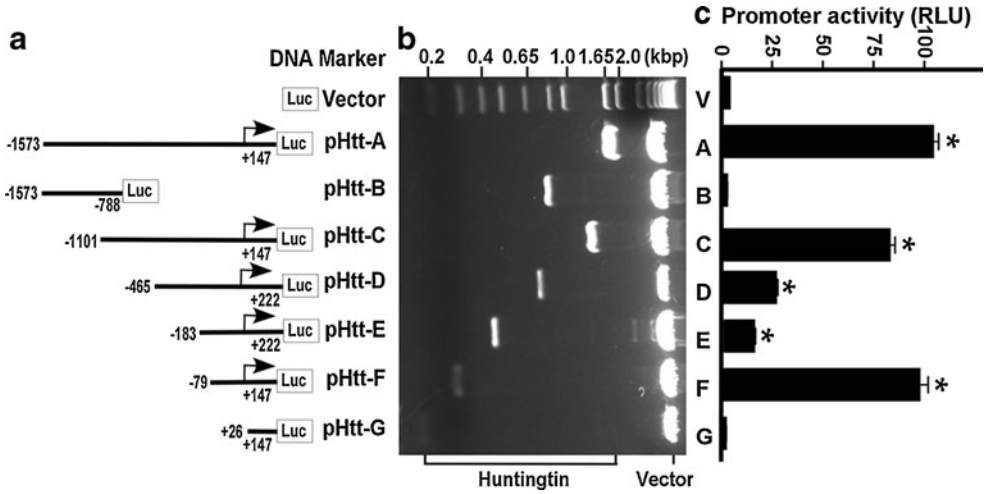

Fig. 2 Deletion analysis of the human *huntingtin* gene promoter. **a** Schematic diagram of a series of the human *huntingtin* deletion promoter constructs in pGL3-Basic vector. *Arrow* shows the direction of transcription. *Numbers* represent the end points of the human *huntingtin* inserts in the plasmid. **b** The deletion plasmids were confirmed by restriction enzyme digestion, and the digested samples were analyzed on a 1.5% agarose gel. The vector size is 4.8 kb. Huntingtin promoter fragment sizes range from 0.121 to 1.72 kb. The sequence of the inserts was further confirmed by sequencing. **c** The plasmid constructs were co-transfected with pCMV-Luc into HEK293 cells. The cells were harvested 48 h after transfection and the luciferase activity measured with a luminometer and expressed in relative luciferase units (RLU). The pCMV-Luc luciferase activity was used to normalize for transfection efficiency. The values represent the means±SEM. $N=3$, $^*p<0.001$ by analysis of variance with the post hoc Newman–Keuls test

contains 12 putative SP1 binding sites at position bp −645 (Sp1-1), −545 (Sp1-2), −480 (Sp1-3), −430 and −413 (Sp1-4), −382 (Sp1-5), −295 and −274 ((Sp1-6), −120 (Sp1-7), and +151 (Sp1-8) (Fig. 1b). To determine whether the elements are functional Sp1 binding sites, electromobility shift assay was carried out. Double-stranded oligonucleotides containing a consensus Sp1 binding element (attcgatcg **GGGCGG**ggcgagc) were synthesized and end-labeled as a probe for GSA. A shifted protein–DNA complex band was observed after incubating the labeled consensus SP1 probe with nuclear extract (Fig. 3a, lane 2). The intensity of this shifted band was reduced using a 10X unlabeled SP1 consensus competition oligonucleotide, and the shifted band disappeared by the addition of a 100X SP1 consensus oligonucleotide (Fig. 3a, lanes 3 and 4). Excessive mutant Sp1 oligonucleotides with the binding site mutations had no competitive effect on the shifted band (Fig. 3a, lanes 5 and 6). The addition of the 10X unlabeled huntingtin-Sp1 probe Htt-SP1-wt6 markedly decreased the signal of the shifted band, and the 100X probe completely abrogated the band (Fig.3a, lanes 7 and 8). A supershift analysis was conducted to further confirm the Sp1 element in the htt-Sp1-wt6 oligonucleotides. A supershift band was detected after the incubation of the anti-Sp1 antibody with the Htt-Sp1-wt6 (Fig. 3a, lane 9). The supershifted bands were competed away by the further addition of a 100X unlabeled htt-Sp1-wt6 oligonucleotide (Fig. 3a, lane 10). These data clearly indicated that Htt Sp1-wt6 of the human *huntingtin* promoter contains a Sp1 bind element.

To further determine whether other putative Sp1 sites contain the Sp1 binding element, we used Htt-Sp1-wt6 as a positive control, and an excess of unlabeled huntingtin-Sp1-wt1, 2, 3, 4, 5, 7, or 8 oligonucleotides were used for competition assays. All oligonucleotides were able to abolish the Sp1 shifted band (Fig. 3b, c, lanes 6–10). Taken together, the results clearly demonstrated that the human huntingtin promoter contains multiple Sp1 binding sites.

Sp1 Upregulates the Human *Huntingtin* Gene Promoter Activation and Enhances Huntingtin Gene Expression

To determine whether Sp1 affects *huntingtin* gene transcription, the *huntingtin* promoter luciferase activity was measured in cells overexpressing Sp1. The Sp1 expression plasmid pCGN-Sp1 or empty vector was co-transfected with either the human huntingtin promoter plasmid or the control plasmid into HEK293 cells. Co-transfection of pHTT-A with pCGN-Sp1 resulted in a significant increase in the luciferase activity by 165.32±0.86% in HEK293 cell ($p<0.001$). Sp1 expression had no effect on luciferase activity in control cells ($p>0.05$; Fig. 4a). Similar results were also obtained in neuroblastoma cell N2a (data not shown). The results showed that SP1 expression augmented the transcriptional activation of the human *huntingtin* promoter.

To further examine whether SP1 affects the endogenous gene expression, a quantitative RT-PCR method was conducted to measure the endogenous *huntingtin* mRNA levels. Sp1 expression markedly increased the endogenous mRNA

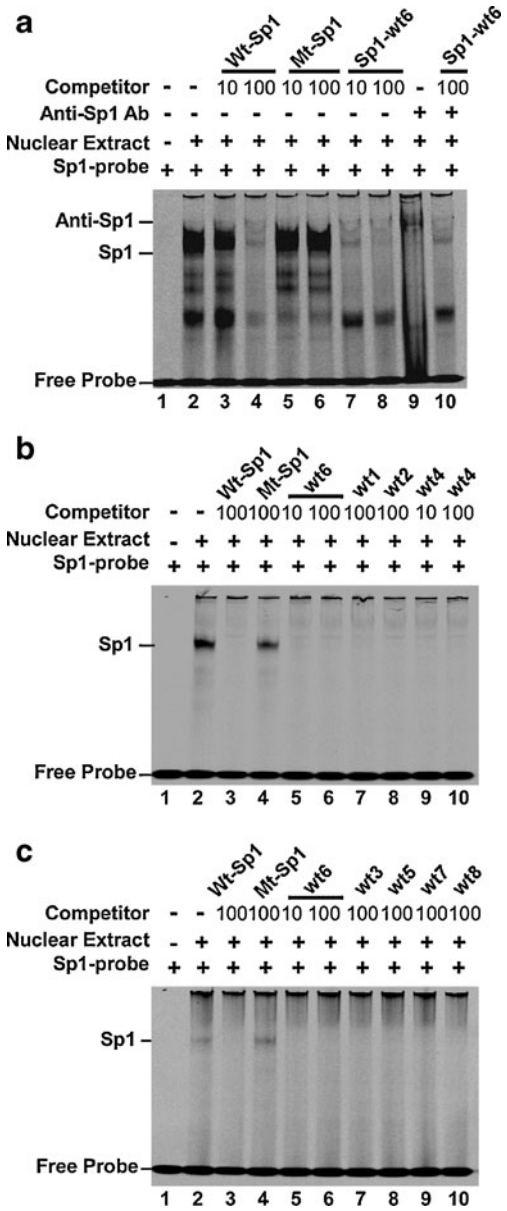

Fig. 3 Identification of Sp1 binding elements in the *huntingtin* gene promoter by GSA. GSA was performed as described in "Materials and Methods." **a** *Lane 1* was labeled human consensus Sp1 probe only. *Lane 2* was a shifted DNA–protein complex formed between the labeled Sp1 with nuclear extracts. Competition assays were carried out by further applying different concentrations of unlabeled competition oligonucleotides, consensus wild-type Sp1 (*lanes 3* and *4*), mutant Sp1 (*lanes 5* and *6*), and homologous huntingtin-Sp1-wt6 (*lanes 7* and *8*). *Lane 9* showed supershifted band with the anti-Sp1 antibody. The supershifted and shifted bands were eliminated by the further addition of unlabeled htt-Sp1-wt6 oligonucleotides (*lane 10*). **b** Consensus Sp1 oligonucleotide probes were further used for individual Sp1 binding site gel shift competition assays: no competition (*lane 2*), competition with consensus SP1 (*lane 3*), mutant Sp1 (*lane 4*), and huntingtin Sp1-wt6 (*lanes 5* and *6*), -wt1 (*lane 7*), -wt2 (*lane 8*), -wt4 (*lanes 9* and *10*). **c** Consensus Sp1 oligonucleotide probes were further used for individual Sp1 binding site gel shift competition assays: no competition (*lane 2*), competition with consensus Sp1 (*lane 3*), mutant SP1 (*lane 4*), and huntingtin SP1-wt6 (*lanes 5* and *6*), -wt3 (*lane 7*), -wt5 (*lane 8*), -wt7 (*lane 9*), and -wt8 (*lane 10*)

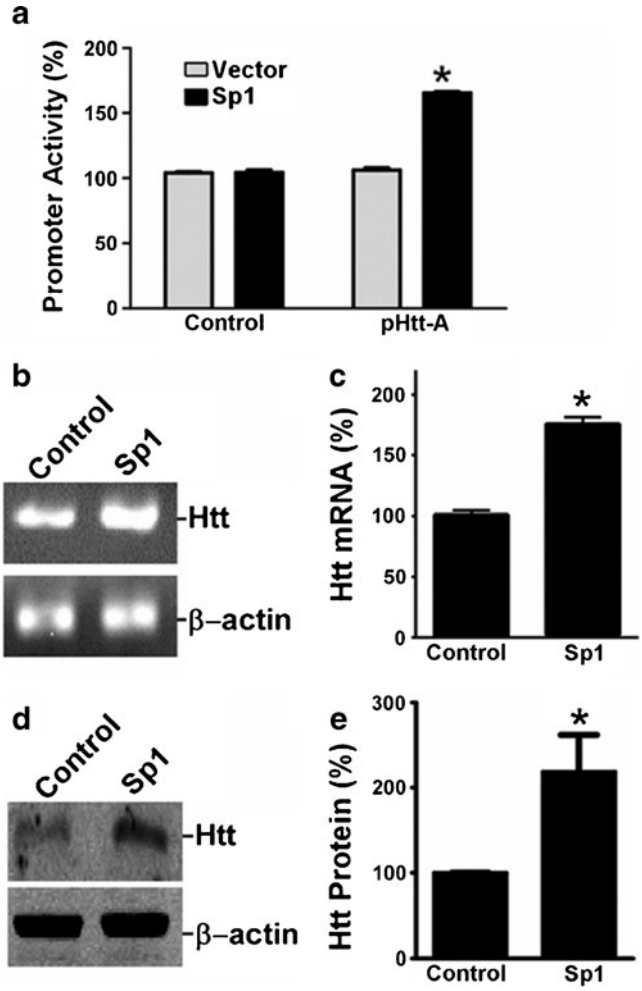

Fig. 4 Sp1 facilitates huntingtin transcriptional activation. **a** Transcriptional activation of the huntingtin promoter is potentiated by SP1. The huntingtin promoter plasmid pHtt-A containing Sp1 binding sites was co-transfected with Sp1 expression plasmid pCGN-Sp1 into HEK293 cells. Sp1 expression markedly increased the pHtt-A promoter activity and had no significant effect on the empty vector control plasmid. The values represent the means±SEM. $N=3$, $^*p<0.001$. **b** An increase of endogenous huntingtin mRNA levels was detected in HEK293 cells with transfected pCGN-Sp1. The mRNA levels of huntingtin were determined by quantitative RT-PCR and normalized against the levels of β-actin. The DNA gel represents RT-PCR products analyzed on a 2% agarose gel. **c** Quantification of huntingtin and β-actin mRNA level by ImageJ software. Data are presented as the mean±SEM. $N=3$, $^*p<0.001$. **d** Co-transfection with pCGN-Sp1 in HEK293 cell results in an enhancement in huntingtin protein expression. Lysates from the cells co-transfected with pCGN-Sp1 were analyzed by Western blot using mouse anti-huntingtin monoclonal antibody; β-actin was used to control for protein loading. **e** Quantification of huntingtin and β-actin protein level in HEK293 cell by Li-COR Imager. Data are presented as the mean±SEM. $N=3$, $^*p<0.001$

level of the *huntingtin* gene to 177.73±2.70% ($p<0.001$; Fig. 4b). Similarly, Western blot analysis confirmed the increase of huntingtin protein level resulted from Sp1 expression. The protein level of huntingtin in HEK293 cell was elevated by SP1 expression by 218.84±43.14% relative

to the control ($p<0.005$; Fig. 4c). Taken together, these data demonstrated that SP1 increases *huntingtin* gene expression via its transcriptional regulatory effect on the *huntingtin* gene promoter.

Mithramycin A Inhibits *Huntingtin* Gene Expression

Mithramycin A selectively inhibits Sp1-mediated transcriptional activation (Christensen et al. 2004). To investigate whether inhibiting Sp1-mediated transcriptional activation of the human *huntingtin* promoter had an effect on the huntingtin gene expression, we first examined the effect of mithramycin A on *huntingtin* promoter activity. HEK293 cells transfected with plasmids pHTT-A were treated with mithramycin A for 12 h. Mithramycin A treatment led to a significant inhibition of *huntingtin* promoter activity (41.44 ±8.16%) compared with the control ($p<0.001$; Fig. 5a). The data indicated that the SP1 response elements mediated the inhibitory effect of mithramycin A on the human *huntingtin* promoter activation.

Quantitative RT-PCR and Western blot analysis were carried out to detect the endogenous levels of huntingtin mRNA and protein in HEK293 cells. Cells were exposed to mithramycin A (125 nM) for 24 h before total RNA and protein extraction. β-Actin expression served as a loading control. Mithramycin A treatment resulted in a marked decrease of the levels of endogenous huntingtin mRNA in HEK293 cells (51.90±2.61%, $p<0.005$; Fig. 5b, c). Similarly, huntingtin protein level in HEK293 cells treated with mithramycin A was significantly reduced to 51.98± 0.86% (Fig. 5d, e). These data suggest that the inhibition of SP1 signaling by mithramycin A negatively regulates *huntingtin* gene transcription and expression.

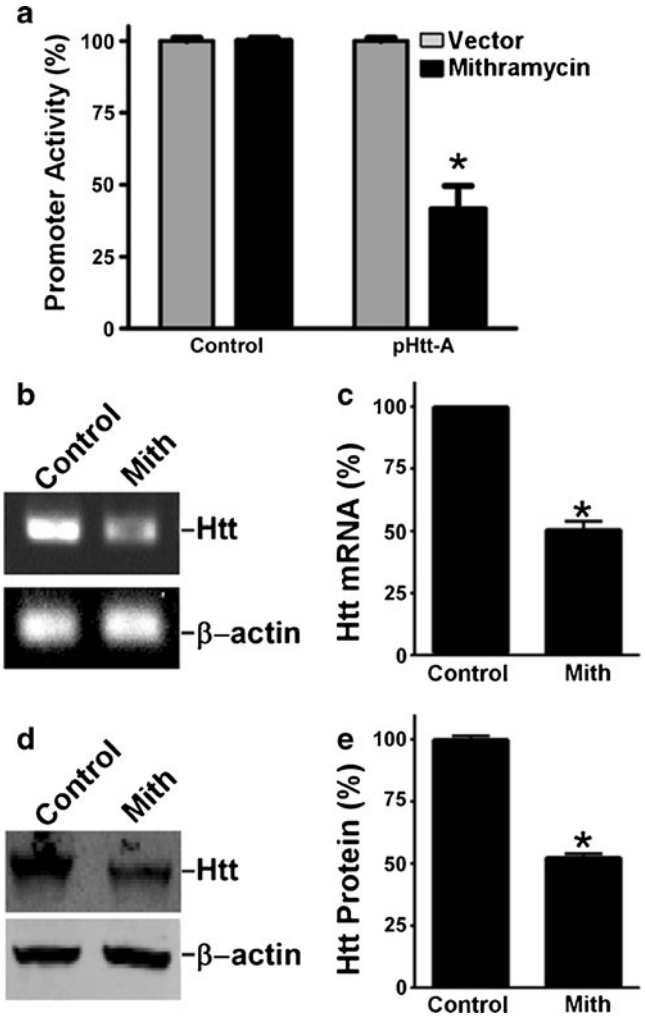

Fig. 5 Inhibition of huntingtin transcription by mithramycin A. **a** Inhibition of the human huntingtin promoter activity by mithramycin A. The huntingtin promoter constructs pHtt-A and pCMV-Luc were co-transfected into HEK293 cells, and the transfected cells were then treated with mithramycin A at 125 nmol/L or vehicle solution for 12 h. Cells were harvested at the same time for the luciferase assay. Luciferase activities were determined by the dual luciferase assay, and pCMV-Luc luciferase activity was used for transfection efficiency normalization. The values represent the mean±SEM. $N=3$, $^*p<0.001$ by analysis of variance with the post hoc Newman–Keuls test. **b** HEK293 cells were exposed to mithramycin A at 125 nmol/L for 24 h. Total RNA was extracted. The mRNA levels of huntingtin were determined by quantitative RT-PCR and normalized against the levels of β-actin. **c** Quantification of huntingtin and β-actin mRNA level in HEK293 cell by ImageJ software. Data are presented as the mean± SEM. $N=3$, $^*p<0.005$ by analysis of variance with the post hoc Newman–Keuls test. **d** Cell lysates from mithramycin A-treated HEK293 cells were analyzed by immunoblotting with anti-huntingtin antibody; β-actin was used as the control for protein loading. **e** Quantification of huntingtin and β-actin protein level HEK293 cell by Li-COR Imager. The values represent the mean±SEM. $N=3$, $^*p<0.001$ by analysis of variance with the post hoc Newman–Keuls test

Discussion

The huntingtin protein is ubiquitously expressed, but has the highest expression in the brain and testes (Sharp and Ross 1996). Many of the functions implicated on huntingtin are related to its mutant form, which is the primary cause of Huntington's disease. Since wild-type huntingtin was presumed to be irrelevant in the pathological mechanisms seen in HD, substantially less is known about the role of Htt under normal cellular functions. In this study, we examined the regulatory mechanisms underlying Htt expression. We cloned and functionally characterized the human Htt gene promoter. We identified the minimal promoter region required for *huntingtin* expression and also identified many putative Sp1 *cis*-binding elements within the Htt promoter. We found that Sp1 binding to the Htt promoter increases *Htt* gene transcription.

Previous studies have found that wild-type Htt may be involved in regulating gene transcription (Zuccato et al. 2003), protein trafficking (McGuire et al. 2006), facilitating endocytosis of membrane-associated protein (Walling et al. 1998), and transducing pro-survival signals with anti-

apoptotic effects (Dragatsis et al. 2000; Rigamonti et al. 2000; Zhang et al. 2006). There are substantial differences between the mutant and wild-type Htt protein, which could partially explain their functional differences. The N-terminus of Htt typically contains 11–24 glutamine repeats in normal individuals. However, in HD patients, the number of glutamine repeats expands to more than 27 repeats, resulting in a protein with an abnormally long polyglutamine tract in its N-terminus (Group T.H.s.D.C.R 1993). The expanded glutamine repeats render the mutant Htt protein to easily aggregate together and induced neurotoxic changes (DiFiglia et al. 1995; Gutekunst et al. 1999; Li et al. 2001; Nucifora et al. 2001).

Moreover, unlike the mutant form, wild-type Htt is distributed through the cytoplasm of the neuron and interacts with numerous proteins. Immunolabeling and immunoprecipitation experiments indicated that wild-type Htt associates with vesicular membrane proteins, indicating that Htt has a role in protein trafficking (Rubinsztein and Carmichael 2003; Goehler et al. 2004). While the wild-type Htt is mainly localized in the cytoplasm, the mutant Htt is mainly localized in the nucleus (Kegel et al. 2002; Landles and Bates 2004). The underlying reason is unclear. A previous study showed that the N-terminus of Htt has the ability to shuttle between the cytoplasm and the nucleus (Cornett et al. 2005). The N-terminus of mutant Htt seems to lack nuclear export sequences which may explain its accumulation in the nucleus. The wild-type Htt protein is also more accessible to posttranslational modifications such as ubiquitination, phosphorylation, and sumoylation (Humbert et al. 2002; Steffan et al. 2004; Luo et al. 2005). Presumably, this reflects the diverse subcellular distribution of Htt which enables it to elicit certain functions that would not be feasible in the aggregated mutant Htt. Conceivably, both the loss of function in the wild-type htt protein and the toxic gain of function in the mutant Htt protein could contribute to the pathologies observed in HD patients.

Htt appears to exert a neuroprotective effect in cultured primary neurons and in transgenic mice. A previous report by Leavitt et al. (2001) showed that diminished wild-type Htt functions contribute to HD pathogenesis. Furthermore, wild-type Htt has been found to play regulatory roles in prenatal neuronal development, as well as promoting the survival of neurons in adult forebrains. The observation that Htt plays key roles in brain development and neuroprotection raises the possibility that subtle loss of wild-type Htt may compromise neuronal integrity and result in HD. Indeed, pathological examinations of HD mouse and human brains have suggested that neuronal dysfunction accompanied by decreased wild-type Htt levels occurs long before neuronal loss (Hodgson et al. 1999; Usdin et al. 1999; Hilditch-Maguire et al. 2000). The loss of wild-type huntingtin function results in the inhibition of some protein expressions such as BDNF and NeuroD (Marcora et al. 2003), a helix–loop–helix transcription factor that is crucial for the development of the hippocampus and pancreatic islets (Liu et al. 2000; Huang et al. 2002).

The transcription factor Sp1 has been implicated in the pathogenesis of HD. The inhibition of Sp1-mediated transcription caused by mutant-type huntingtin has been previously reported (Dunah et al. 2002; Li et al. 2002). The mutant Htt has been found to hinder the Sp1-mediated transcription of target genes including dopamine D2 receptor and preproenkephalin (Li et al. 2002; Chen-Plotkin et al. 2006). This indicated that maintaining Sp1-mediated gene transcription is important for preventing HD. However, a previous report by Qiu et al. (2006) showed that reduced Sp1 DNA binding activity is neuroprotective in HD-modeled mice. In summary, the relationship between Sp1 and Htt and how this affects HD pathogenesis is not clear.

Our findings suggest that Sp1 is essential for *huntingtin* expression. We found that there are no functional differences between neuronal and non-neuronal cells in terms of Sp1-mediated Htt transcription. Higher-than-normal Sp1 levels have previously been reported in HD cases, but the underlying mechanism is unclear (Chen-Plotkin et al. 2006; Qiu et al. 2006). Given the anti-apoptotic effects of wild-type Htt, it is possible that the elevated Sp1 levels are there to promote the expression of wild-type Htt. This compensatory increase in Sp1-mediated Htt expression may alleviate cellular toxicity induced by aggregated mutant Htt. Furthermore, wild-type Htt was previously found to inhibit the activation of caspase 3, the key effector molecule in the extrinsic cell death cascade (Leavitt et al. 2001).

Polyglutamine-expanded proteins such as mutant huntingtin have large interaction domains that could aberrantly bind glutamine-rich transcription factors such as Sp1, leading to neurodegeneration (Nucifora et al. 2001). For example, BDNF is known to be critical of maintaining cell viability. BDNF is produced and secreted by a specific population of cells in the central nervous system. Wild-type Htt has been found to affect BDNF synthesis and release in cultured cells and in mice. The overexpression of wild-type Htt promotes BDNF synthesis and release in primary neuronal cultures, whereas transgenic mice with reduced wild-type Htt levels have diminished BDNF activity. The proximal promoter region of BDNF also contains Sp1 *cis*-elements. The mechanism by which wild-type Htt promotes BDNF activity is not known, but the mutant Htt has been demonstrated to reduce Sp1-dependent BDNF transcription, thereby leading to neuronal death in HD models (Luthi-Carter et al. 2002).

In conclusion, we demonstrated that *huntingtin* gene transcription is regulated by SP1 signaling. Future studies will be needed to determine the interaction of SP1 and *huntingtin* in HD pathogenesis. A better understanding of

huntingtin transcription and SP1 signaling in HD will open up avenues for developing therapies for HD and related disorders.

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Conflict of Interest The authors declare no conflict of interest.

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