CONTEXT-DEPENDENT DYSREGULATION OF TRANSCRIPTION BY MUTANT HUNTINGTIN

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Running Title: Transcriptional dysregulation by huntingtin

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Huntington’s disease (HD) is an adult-onset neurodegenerative disease caused by expansion of a polyglutamine (polyQ) tract in the N-terminal region of huntingtin (htt). Although the precise mechanisms leading to neurodegeneration in HD have not been fully elucidated, transcriptional dysregulation has been implicated in disease pathogenesis. In HD, multiple N-terminal mutant htt fragments smaller than the first 500 amino acids have been found to accumulate in the nucleus and adversely affect gene transcription. It is unknown whether different htt fragments in the nucleus can differentially bind transcription factors and affect transcription. Here, we report that shorter N-terminal htt fragments, which are more prone to misfolding and aggregation, are more competent to bind Sp1 and inhibit its activity. These effects can be reversed by Hsp40, a molecular chaperone that reduces the misfolding of mutant htt. Our results provide insight into the beneficial effects of molecular chaperones and suggest that context dependent transcriptional dysregulation may contribute to differential toxicity of various N-terminal htt fragments.

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disorder resulting from expansion (> 37 repeats) of a polyglutamine (polyQ) repeat in the N-terminal region of huntingtin (htt), a 350 kDa protein of unknown function. Proteolytic cleavage of full-length htt, which is predominantly cytoplasmic, generates N-terminal htt fragments that accumulate abnormally and form inclusions over time in neuronal nuclei (1). The nucleus is thought to be a primary site of polyQ toxicity, as blocking the nuclear entry of htt suppresses its ability to cause cell death (2) while targeting htt to the nucleus by the addition of a nuclear localization signal (NLS) causes a more severe phenotype (3-5).

In the nucleus, mutant htt abnormally interacts with a number of transcription factors (6). Accordingly, the nuclear pathology observed in HD is thought to be largely due to transcriptional dysregulation (7, 8). In fact, mRNA levels are altered for specific genes in HD mouse and cell models as well as in postmortem human HD brain (7-11). Mutant htt has a higher affinity than normal htt for certain transcription factors, such as Sp1 (12-15), and these aberrant interactions can functionally deactivate transcription factors by titrating them away from their normal DNA binding sites (12-16).

Biochemical analysis of HD knockin mice that express a 150-glutamine repeat in
the endogenous mouse htt (17) revealed the presence of multiple N-terminal htt fragments smaller than the first 508 amino acids in the nucleus (18), consistent with the notion that cleavage of mutant htt is a key event in HD pathology (19,20). Since htt protein length can influence its cellular toxicity and ability to cause neurodegeneration in HD cellular models and transgenic mice (21, 22), it is important to know whether nuclear N-terminal htt fragments of different length can differentially affect gene transcription. Understanding this issue will help elucidate the mechanisms underlying the context-dependent neuropathology observed in various HD mouse models.

In this study, we examined the interaction between mutant htt and the transcription factor Sp1. We demonstrate that this interaction is affected by htt protein context and further show that the ability of different N-terminal htt fragments to affect the activity of an Sp1-dependent promoter is context-dependent. In each case, shorter htt fragments, which are more likely to become misfolded, produced the greater inhibitory effect. The ability of these htt fragments to bind Sp1 and to reduce its activity was decreased by Hsp40 expression, suggesting that protein misfolding plays a key role in this process.

Experimental Procedures

Plasmids—cDNA constructs encoding exon-1 htt (1-67 amino acids) plus different glutamine (Q) repeats in the pRK5 eukaryotic expression vector were generated in our previous study (23). The 72Q repeat encoded stably by CAA/CAG mix repeats (24) was introduced into our previous htt constructs (25) to produce N-terminal mutant htt (1-212 or 1-508 amino acids) with an expanded polyQ domain (72Q-212 and 72Q-508). The nuclear localization sequence (NLS) of the SV large T antigen (PKKKRKV) was linked to the N-terminus of these htt proteins to generate NLS-72Q-67, NLS-72Q-212, and NLS-72Q-508. The nerve growth factor receptor (NGFR) promoter from the luciferase reporter construct (11) was placed into the multiple cloning site of pDsRed-Express-1 vector (Clontech) to generate the NGFR-DsRed reporter construct. Full-length human Hsp40 in the pRK-HA vector, which contains a C-terminal HA epitope, was generated in our previous study (26).

Antibodies—The rabbit polyclonal antibody (EM48) and mouse monoclonal antibody (mEM48) to htt were generated using the first 256 amino acids of human htt as described previously (18, 27). 1C2, a mouse antibody to polyQ, was obtained from CHEMICON International, Inc. For Sp1 immunoprecipitation, we used rabbit antibodies to Sp1 (S9809, Sigma) and (sc-59, Santa Cruz Biotechnology). Mouse antibody to the HA epitope (12CA5, Cell Signaling) was used to detect transfected Hsp40.

Cell Culture and Transfection—Human Embryonic Kidney (HEK) 293T cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, 100 µg/ml penicillin, and 100 µg/ml streptomycin. NIH 3T3 and Sp1 (-/-) cells, both of which were derived from mouse fibroblasts, were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum, 100 µg/ml penicillin, and 100 µg/ml streptomycin. All cells were incubated at 37°C in a humidified 5% CO2 atmosphere. cDNAs were transfected into 70-80% confluent HEK293T, NIH 3T3, and Sp1 (-/-) cells for 24-48 h using Lipofectamine (Invitrogen) following the manufacturer’s protocol. Transfection efficiencies for NIH 3T3 and Sp1 (-/-) cells were equal as
determined by visualization of transfected red fluorescent protein.

**Immunoprecipitation**—For immunoprecipitation of endogenous Sp1 from cultured cells, HEK293T cells in a 6-well plate were transfected with 1 µg/well htt cDNA for 24 h using Lipofectamine (Invitrogen). The transfected cells were collected and lysed in 1 ml of RIPA lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1 mM EDTA [pH 8.0], 1 mM EGTA [pH 8.0], 0.1% SDS, 0.5% DOC, 1% Triton X-100, 1mM PMSF, protease inhibitor cocktail [1,000X; P8340; Sigma]) at 4°C on a rocking platform for 30 min. The lysates were spun at 500 RPM for 10 min. The supernatant was pre-cleared with 50% Protein A-Sepharose (Sigma) slurry on a rocking platform at 4°C for 1 h and centrifuged at 10,000 x g for 15 min at 4°C. The supernatant (1 µg/µl of 300 µl) was then subjected to immunoprecipitation with anti-Sp1 as described above. Immunocomplexes were resolved by SDS-PAGE and detected by western blotting with anti-Sp1 and mEM48 or 1C2.

**Immunocytochemistry**—Immunostaining of cultured cells was done as described previously (12). Cells were plated in 12-well plates at ~70-80% confluency and transfected with 1 µg plasmid DNA/well for 24-48 h. At the end of the transfection, cells were fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.2% Triton X-100 in PBS and 3% bovine serum albumin (BSA) for 1 h, and incubated with primary antibody in PBS with 3% BSA overnight at 4°C. After washes 3 times with PBS, the cells were incubated with secondary antibodies conjugated with either FITC or rhodamine (Jackson Immunoresearch Laboratories) in PBS containing 3% BSA and Hoechst dye (1 µg/ml) for 30 min at 4°C. At the end of incubation, the cells were washed 3 times of 10 minutes each with PBS. A Zeiss fluorescent microscope (Axiovert S100) and a 3CCD camera video system (Dage-MTI Inc.) were used to capture images with different optical filters. The captured images were stored and processed using Adobe Photoshop software.

**Western blotting**—Western blotting was performed using polyacrylamide Tris-
Glycine gels (Invitrogen). Proteins were transferred to nitrocellulose membranes and blocked at room temperature for 1 h with 5% milk in PBS. Membranes were washed 3 times (10 min/each time) in PBS and incubated with primary antibodies in PBS and 3% BSA overnight at 4°C. Membranes were again washed 3 times (10 min/each time) in PBS, blocked at room temperature for 1 h with 5% milk in PBS and incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immunoresearch Laboratories). Immunoreactive bands were visualized using ECL plus chemiluminescence kits (Amersham Biosciences).

**Transcription reporter assays**—To examine the effect of different fragments of intranuclear htt on the NGFR promoter, we transfected 70–80% confluent HEK293T cells in a 12-well plate for 48 h with pRK vector, NLS-72Q-67, NLS-72Q-212, or NLS-72Q-508 (1 µg/well) and NGFR DsRed reporter constructs (0.33 µg/well). After transfection for 48 h, cells were scraped from the plate and suspended in sterile 1X PBS (500 µl/well). A total volume of 100 µl (50 µl of the cell suspension and 50 µl of 1X PBS) was aliquot in duplicate into a black polystyrene 96-well assay plate (Corning Inc.). Relative fluorescent units (R.F.U.) were measured with a fluorescence plate reader (FluoStar Galaxy, BMG Labtechnologies) set at 544 nm excitation and 590 nm emission. All results are expressed as means ± SE of at least 3 independent experiments. Statistical analysis was performed using Student’s t-test when comparing two groups or using one-way analysis of variance followed by Dunnett’s multiple comparison post test when comparing three or more groups. Statistical significance was considered to be $P < 0.05$.

Western blot analysis of transfected HEK293T cells with mEM48 was also performed to verify the expression of transfected htt.

**Results**

**Generation and Expression of N-terminal Htt Constructs**—To study whether protein length can alter the ability of htt to affect gene transcription, we used three N-terminal htt constructs encoding exon-1 htt (72Q-67), htt amino acids 1-212 (72Q-212) or 1-508 (72Q-508) with a 72Q repeat. These proteins cover the range of N-terminal htt fragments that can accumulate in the nucleus in brains from HD repeat knockin mice (18). Since transient transfection leads to the cytoplasmic localization of the majority of transfected htt in cultured cells, we added the SV40 nuclear localization sequence (NLS) to the N-terminus of each htt fragment to ensure its nuclear localization (Fig. 1A). In this way, any differences in the effects of the N-terminal htt fragments (NLS-72Q-67, NLS-72Q-212, and NLS-72Q-508) in the nucleus are likely due to their protein context. Western blot analysis of transfected htt revealed the expected molecular weight and equal expression of these htt fragments (Fig. 1B), and nuclear localization of each htt fragment was confirmed by immunostaining (Fig. 1C).

**Sp1 Preferentially Associates with Shorter N-terminal Htt fragments Both in vitro and in vivo**—Htt has been shown to interact with the transcriptional activator Sp1, and this interaction is enhanced by polyQ expansion (12, 14). However, the effect of htt protein length on its binding to Sp1 has not been examined. We used immunoprecipitation to evaluate the interactions of different htt fragments with Sp1 in cells. We have found that aggregated htt can be co-precipitated with Hsp70 but not Sp1 (12, 25). Thus, we focused on the interaction of soluble mutant htt with Sp1. We transfected HEK 293T cells with NLS-72Q-67, NLS-72Q-212, or
NLS-72Q-508, immunoprecipitated endogenous Sp1, and examined the precipitates by western blotting with mEM48 and anti-Sp1 (Fig. 2). While the expression levels of these htt fragments are equivalent, a greater amount of shorter N-terminal fragments of htt were precipitated with Sp1 than longer fragments. Among the three N-terminal htt fragments tested, the shortest one (NLS-72Q-67) bound Sp1 the most. Although NLS-72Q-67 also formed aggregates that remained in the stacking gel (Fig. 2), only the soluble form of this protein was precipitated with Sp1. This result is consistent with previous reports (12, 14, 30) that soluble but not aggregated mutant htt associates with Sp1. Taken together, protein context regulates the ability of soluble htt to interact with Sp1.

To test if the context-dependent interaction of N-terminal htt with Sp1 in transfected cells also occurs in HD mouse brain, we examined three different HD mouse models that express N-terminal or full-length mutant htt. In brain cortex from 4-week-old R6/2 mice that express human exon-1 htt (1-67 amino acids) with a 150Q repeat (28), both soluble and aggregated htt were detected in nuclear fractions. Another soluble band at ~125 kD was also detected and may reflect a misfolded or oligomeric species of htt. Both soluble species of htt were precipitated with Sp1 but htt aggregates were not (Fig. 3A). We next examined 8-week-old N171-82Q HD transgenic mice that express the first 171 amino acids of human htt with an 82Q repeat (29). The transgenic htt was also precipitated with Sp1 (Fig. 3B). It is difficult to compare the amounts of precipitated htt from R6/2 and N171-82Q transgenic mice because of different transgene expression levels. Thus, we focused on HD knockin mice that express full-length htt with 150Q from the endogenous mouse locus (17) and can accumulate degraded N-terminal htt fragments over time (18). We used the crude P1 fraction from the cortex of HD knockin mice at 2-12 months of age and performed western blotting with 1C2, as it can sensitively detect N-terminal fragments of mutant htt in HD knockin mouse brain. This crude fraction contains nuclear proteins and full-length htt as well as N-terminal htt fragments, allowing us to examine whether full-length or N-terminal htt preferentially interact with transcription factors in the brain. We then immunoprecipitated endogenous Sp1 and examined the precipitates by western blotting with 1C2. N-terminal htt fragments are not as abundant as full-length htt in the input for immunoprecipitation, perhaps because they are unstable or become aggregates soon after degradation of full-length mutant htt. However, N-terminal htt fragments are clearly preferentially co-precipitated with Sp1 as compared to full-length htt. More importantly, Sp1 binding to N-terminal htt fragments, but not full-length htt, increased with age (Fig. 3C). Since HD repeat knockin mice show age-dependent behavioral phenotypes (17), the age-dependent increase in the association of Sp1 with N-terminal htt fragments is likely to contribute to transcriptional dysregulation and neurological symptoms in this HD mouse model.

Shorter N-terminal Htt Fragments Are More Competent to Suppress Sp1 Activity—We next wanted to determine if the context-dependent interactions of htt with Sp1 are related to changes in Sp1-mediated gene expression. Like many other Sp1-dependent promoters, the p75 nerve growth factor receptor (NGFR) promoter lacks TATA and CAAT elements and is GC-rich. The activity of the NGFR promoter is dependent on Sp1 (31) and inhibited by mutant htt (9, 12). Thus, we used the NGFR promoter as a tool to examine the effect of different htt
fragments on Sp1-mediated transcription. To do so, we used the NGFR promoter to drive a DsRed reporter so that red fluorescence intensity can be used to quantify the activity of the NGFR promoter (Fig. 4A). We cotransfected HEK 293T cells with NGFR-DsRed and Sp1 or PRK vector. Cotransfection of Sp1 increased DsRed reporter expression ~10-fold as compared to the vector control (Fig. 4B). To ensure that this reporter activity is indeed dependent on Sp1, we also examined Sp1-/− mouse fibroblast cells (32). In the absence of Sp1, DsRed expression was reduced to background levels. By contrast, DsRed expression was readily detected in NIH 3T3 mouse fibroblast cells (Sp1+/+) in which the normal Sp1 level is present (Fig 4B). Thus, the NGFR-DsRed reporter construct is a good tool to examine Sp1-dependent gene transcription.

Next, we transfected HEK 293T cells with NGFR-DsRed and NLS-72Q-67, NLS-72Q-212, or NLS-72Q-508 to examine if htt protein length is important for its ability to affect Sp1 activity. All three htt constructs decreased DsRed expression but NLS-72Q-67 had the strongest inhibitory effect (Fig. 4C). Since western blots showed that the htt fragments were expressed at equivalent levels (Fig. 4D), these results support the idea that the protein context of htt influences the ability of N-terminal htt fragments to decrease Sp1-mediated gene expression.

Protein Misfolding Is Important for the Context-Dependent Effects of N-terminal Htt on Sp1-One possible explanation for the stronger effect of shorter N-terminal htt fragments on Sp1 activity is that protein misfolding plays a role in this process. Shorter htt fragments are more likely to become misfolded or aggregated (21, 27) as evidenced by the inverse correlation between protein length and aggregation for NLS-72Q-67, NLS-72Q-212 and NLS-72Q-508 (Fig. 4D). In the cell, proper protein folding is maintained by molecular chaperones while clearance of misfolded proteins is mediated by the ubiquitin-proteasome system (UPS) (33-35). It is also likely that the nuclear environment is more favorable for mutant htt to become misfolded, as both chaperone (36) and proteasome (18) activities have been reported to be lower in the nucleus than the cytoplasm. To test this idea, we focused on exon-1 htt because of its propensity to misfold and aggregate. We transfected HEK 293 cells with exon-1 htt containing either a 20Q (20Q-67) or 120Q (120Q-67) repeat and visualized transfected htt by immunocytochemistry using two antibodies. The first antibody (EM48) recognizes both soluble and aggregated htt (27) while the second antibody (1C2) is specific to polyQ domains and does not recognize aggregated forms of mutant htt (18, 37). Both 20Q-67 and 120Q-67 were localized to the cytoplasm but 120Q-67 also formed aggregates that were intensely labeled by EM48. As expected, 1C2 antibody labeled soluble 120Q-67 but not aggregates (Fig. 5). 1C2 also weakly labeled 20Q-67 because it has a shorter polyQ stretch. To visualize mutant htt in the nucleus, we used NLS-72Q-67 for transfection. NLS-72Q-67 was distributed mostly in the nucleus, and its diffuse nuclear localization was clearly revealed by EM48 staining. However, 1C2 did not recognize soluble mutant htt in the nucleus, though it was able to label diffuse mutant htt in the cytoplasm (Fig. 5A). We have shown that HEK293 cells cotransfected with mutant htt and Hsp40 express both proteins and that Hsp40 can reduce polyQ aggregation (26). Even in the cells transfected with both NLS-72Q-67 and Hsp40, 1C2 still primarily labeled the cytoplasmic htt despite the intense nuclear staining of mutant htt by EM48 (Fig. 5B). Since western blots show 1C2 immunoreactive bands of soluble
mutant htt in the nucleus (Fig. 3 and ref. 18), these results support the idea that mutant htt in the nucleus possesses a conformation that prevents 1C2 immunoreaction in immunocytochemistry. If misfolding of nuclear htt contributes to its effect on Sp1, factors that reduce htt misfolding might also decrease its ability to interact with Sp1 and suppress Sp1 activity. To test this idea, we used the molecular chaperone Hsp40 which can effectively suppress htt misfolding and aggregation (25, 26). Western blots confirmed the expression of NLS-72Q-67 and reduction in htt aggregation by Hsp40 transfection (Fig. 6A). Importantly, transfected Hsp40 decreased the amount of soluble htt precipitated with Sp1 (Fig. 6A). We then examined the effect of Hsp40 on the activity of NGFR-dsRed in htt transfected HEK 293T cells. Compared to the control htt (NLS-20Q-67) transfection, mutant htt (NLS-72Q-67) inhibited the transcriptional activity of the NGFR promoter. However, Hsp40 expression reversed this inhibition (Fig. 6B). In the absence of NLS-72Q-67, Hsp40 expression had no significant effect on the NGFR promoter as compared to vector expression alone (Fig. 6C), suggesting that Hsp40 selectively reduces mutant htt-mediated impairment on Sp1-dependent gene transcription. Taken together, htt misfolding is important for its ability to interact with Sp1 and reduce Sp1 activity at specific promoters. Factors that prevent htt misfolding can therefore restore normal Sp1 function.

Discussion

In the present study, we show that Sp1 interacts with multiple N-terminal fragments of htt in the nucleus in both transfected cells and HD mouse brain. Importantly, these interactions are regulated by htt protein context. Shorter htt fragments bound more Sp1 than longer fragments and also suppressed the transcriptional activity from the NGFR promoter, which is known to be regulated by Sp1 (31). Thus, consistent with previous microarray studies showing that increased htt length reduces the number of polyQ-induced gene changes in HD mice (38), analysis of Sp1 and its transcriptional activity in our studies suggests that smaller N-terminal htt fragments may have a stronger effect on transcriptional activity than larger htt fragments. The context-dependent effect of mutant htt on transcription factors may contribute to different neurological symptoms seen in HD mice expressing different forms of mutant htt. Finally, htt misfolding appears to underlie its ability to bind Sp1 and reduce Sp1-dependent activity of the NGFR promoter and Hsp40, which prevents htt misfolding, deters this process. This observation provides further insight into the protective effects of molecular chaperones in HD (39). In support of this idea, chaperones are also found to reduce the interaction of exon-1 mutant htt with TATA box-binding protein TBP (16).

The findings in the present study suggest a model in which small htt fragments, after entering the nucleus, undergo a conformational change, interact with Sp1, and directly disrupt Sp1 function in gene expression. In HD repeat knock-in mice that show an age-dependent increase in accumulation of mutant N-terminal htt fragments and their formation of nuclear aggregates (17, 18), the association of N-terminal fragments of mutant htt with Sp1 in the brain is also increased over age (Fig. 3C). Moreover, reduction of the expression of some Sp1-mediated genes has been reported to become more severe over time in HD mouse models (40). Since only soluble mutant htt binds Sp1, shorter htt fragments may bind Sp1 and prevent Sp1 binding to promoter DNA before they form aggregates.
It should be noted that Sp1 mediates the expression of a large number of genes and is reported to be up-regulated in some models of HD (13, 41). It is known that Sp1 expression is induced by oxidative stress in neurons (42). Mutant htt probably causes oxidative stress to increase Sp1 levels, which may also activate some genes that can trigger cellular pathological pathways under certain conditions. However, growing evidence has shown that mutant htt inhibits the binding of Sp1 to DNA promoters and suppresses the transcription of certain Sp1-mediated genes (12-15). Our data further demonstrate that this inhibitory effect is context-dependent. Since mutant htt aberrantly interacts with multiple transcription factors (6), it is possible that these interactions are also context-dependent and that protein context and conformation are key aspects of suppression of selective transcription factors by mutant htt. Likewise, context-dependent inhibition of transcription could contribute to the selective or specific neuropathology in HD.
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**FOOTNOTES**

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The abbreviations used are: HD, Huntington disease; Htt, huntingtin; polyQ, polyglutamine; Hsp, heat shock protein; WT, wild type; RFP, red fluorescent protein; NLS, nuclear localization sequences; NGFR, nerve growth factor receptor; HEK-293, human embryonic kidney 293; IP, immunoprecipitation.

**Figure Legends**

**Figure 1. Expression of N-terminal htt fragments.** (A) Diagrammatic representation of htt cDNA expression constructs used in this study. The SV40 NLS was fused to the N-terminus of each protein. The names of the constructs indicate the number of glutamines in the polyQ repeat and the amino acid length of each protein (67, 212, and 508) not including the polyQ tract. (B-C) Western blot (B) and immunostaining (C) of N-terminal htt constructs. HEK
293T cells were transfected with NLS-72Q-67, NLS-72Q-212, or NLS-72Q-508 for 48 h followed by western blotting or immunostaining respectively using anti-htt antibody mEM48 (upper panel). For immunostaining, nuclei were labeled with Hoechst (lower panel). Bracket indicates the stacking gel in which aggregated htt is present.

**Figure 2. Shorter N-terminal htt fragments have increased association with Sp1.** HEK 293T cells were transfected with NLS-tagged N-terminal htt fragments for 24 h. Endogenous Sp1 was precipitated with rabbit antibody to Sp1 (Anti-Sp1), and rabbit pre-immune serum (pre) served as a control. The immunoprecipitates (IP) were examined with mEM48 in western blots (upper panel). The same blots were re-probed with antibody to Sp1 (lower panel). Note that NLS-72Q-67 bound more Sp1 in the IP than longer htt fragments. Only soluble htt, but not aggregated htt (bracket), was precipitated with Sp1.

**Figure 3. Sp1 associates with N-terminal htt in vivo.** (A) Immunoprecipitation of endogenous Sp1 in the brain cortex of 4-week-old R6/2 HD mice. Soluble htt and an ~125 kD mEM48 immunoreactive band, which may correspond to misfolded or oligomeric mutant htt, were precipitated with Sp1. Aggregated htt (bracket) was not detected in the IP. (B) Immunoprecipitation of Sp1 (lower panel) and in the brain cortex of 8-week-old N171-82Q HD mice. Transgenic mutant htt (upper panel) was also seen in Sp1 immunoprecipitates. (C) Immunoprecipitation of Sp1 from the brain cortex of 2, 7, and 12-month-old HD knockin mice. At all ages analyzed, full-length mutant htt (arrowhead) could be detected along with multiple N-terminal htt fragments but N-terminal htt fragments were more enriched in the immunoprecipitates (IP). Also note the age-dependent increase in the association of N-terminal htt with Sp1. The above western blots were probed with htt antibody, either mEM48 (A) or 1C2 (B-C), and the same blots were re-probed with antibody to Sp1 (lower panel). WT, wild-type mouse brain.

**Figure 4. Context-dependent suppression of the activity of the NGFR promoter by mutant htt.** (A) Schematic structure of the NGFR-DsRed reporter construct showing Sp1 binding sites (GC box) in the NGFR promoter. (B) NGFR-DsRed reporter activity is dependent on the presence of Sp1. Cotransfection of Sp1 with NGFR-DsRed in HEK 293T cells for 48 h increases NGFR promoter activity ~10-fold compared to vector (left graph). NGFR-DsRed expression is reduced to background levels in Sp1 +/- mouse fibroblasts (right graph). NIH 3T3 cells (Sp1 +/+ ) mouse fibroblasts served as the positive control. ** P<0.01 (C) HEK 293T cells were cotransfected with NGFR-DsRed and NLS-tagged N-terminal htt fragments or empty vector for 48 h. The transcriptional activity of the NGFR promoter is expressed as relative fluorescent units (R.F.U.). The data are presented as means ± SE of 3 independent experiments. ** P < 0.01 as compared to vector. (D) Western blot analysis of the expression of transfected N-terminal htt in HEK 293T cells used for measuring the activity of the NGFR-DsRed reporter. Note that shorter htt fragments form more aggregates (bracket) than longer fragments.

**Figure 5. Nuclear localization promotes misfolding of mutant htt.** (A) HEK293 cells were transfected with N-terminal htt (20Q-67, 120Q-67 or NLS-72Q-67). Transfected cells were immunostained with the mouse antibody (1C2) to polyQ (green) and rabbit antibody (EM48) to htt (red). Nuclei were labeled with Hoechst (blue). 1C2 antibody does not recognize
aggregated htt (middle panel) and soluble htt in the nucleus (lower panel). Note that 1C2 staining of NLS-72Q-67 is restricted to the cytoplasm, though the nuclear localization of htt is seen with EM48 staining. (B) Immunostaining of NLS-72Q-67 in HEK293 cells that were also transfected with Hsp40 for 48 h. Note that 1C2 still primarily reacts with transfected htt in the cytoplasm.

**Figure 6. Htt misfolding reduces Sp1 activity.** (A) Western blot analysis of the expression of transfected N-terminal htt, Hsp40, and the immunoprecipitation of endogenous Sp1 with htt in HEK 293T cells. Cells were transfected with htt (NLS-72Q-67 or NLS-20Q-67) and Hsp40 or PRK vector (+vector) for 48 h. Note that Hsp40 reduces htt aggregation (bracket). HEK 293T cells cotransfected with NLS-72Q-67 and either empty vector or Hsp40 for 48 h were used for immunoprecipitation of endogenous Sp1. The immunoprecipitates (IP) were examined by western blotting with anti-htt (upper panel) and anti-Sp1 (lower panel). Immunoprecipitation using preimmuno-serum (Pre) served as a control. Hsp40 expression reduces the interaction of htt with Sp1. (B) Hsp40 reverses the inhibitory effect of NLS-72Q-67 on the activity of NGFR-DsRed. HEK 293T cells were cotransfected with NGFR-DsRed, htt (NLS-20Q-67 or NLS-72Q-67), and Hsp40 or PRK vector for 48 h. NGFR-DsRed activity is expressed as relative fluorescent units (R.F.U). The data are presented as means ± SE of 3 independent experiments. ** P < 0.01. (C) NGFR-dsRed activity (mean±S.E, n=4-6) in HEK 293T cells transfected with PRK vector alone or vector plus Hsp40 (Hsp40+vector).
Fig. 2

|     | Input | Pre | Anti-Sp1 |
|-----|-------|-----|----------|
| NLS-72Q-67 | NLS-72Q-212 | NLS-72Q-508 | NLS-72Q-67 | NLS-72Q-212 | NLS-72Q-508 |

kD

| 250 | 250 |
|-----|-----|
| 200 |     |
| 150 |     |
| 100 |     |
| 75  |     |
| 50  |     |
| 37  |     |

Sp1
Fig. 4

A

NGFR p75 promoter

DsRed

GC Box

B

- Vector
- + Sp1

Sp1 ++/
Sp1 -/-

R. F. U.

C

- Vector
- + NLS-72Q-212
- + NLS-72Q-67
- + NLS-72Q-508

R. F. U.

D

Vector
NLS-72Q-67
NLS-72Q-212
NLS-72Q-508

kD
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