

## SUPPLEMENTAL INFORMATION

### MATERIALS AND METHODS

**Generation of Httex1p plasmids.** The generation of each plasmid is summarized in Table SI. Complete or truncated Httex1p (without the proline-rich region) plasmids encoding either a normal range or expanded polyQ tract with alternating CAG/CAA repeats were fused in frame with GFP at the C-terminus (1-17-97QP-GFP, 1-17-103Q-GFP, 1-17-25QP-GFP and 1-17-25Q-GFP) in pcDNA3.1 (Invitrogen) as described previously (83, 84). All constructs below are fused in frame to GFP and are subcloned into pcDNA3.1 unless otherwise indicated. The proline-rich region is designated as “proline”. 1-17aa-proline-GFP (1-17-P-GFP), was constructed in pBS- (Stratagene) first by digesting NLS-proline-GFP (NLS-P-GFP) (83) with *PstI* and *BamHI* to drop out the proline-rich region, and ligating it into pBS- digested with *PstI* and *BamHI* to yield pBS-proline. pBS-proline was then digested with *HindIII* and *PstI* and ligated to annealed oligonucleotides ER5 and ER6 (Supplementary Table II) which encode the 1<sup>st</sup> 17 aas of Htt (mutated to delete the *HindIII* site (underlined) present at the end of the 1<sup>st</sup> 17 aa) containing *HindIII* and *PstI* ends. pBS-1-17-P was then digested with *HindIII* and *BamHI* and the insert subcloned into 1-17-103QP-GFP (previously digested with *HindIII* and *BamHI* to drop out 1-17-103Q leaving only pcDNA3.1 GFP) to yield 1-17-P-GFP.

The proline-GFP (P-GFP) plasmid was constructed by digesting pBS-1-17-P with *HindIII* and *PstI*, and ligating it with annealed oligonucleotides ER7 and ER8 (Table II) encoding methionine with *HindIII* and *PstI* ends. The pBS-P was then digested with *HindIII* and

*Bam*HI to subclone into 1-17-103QP-GFP as above to yield P-GFP in pcDNA3.1. The 1-17aa-GFP (1-17-GFP) construct was generated by digesting 1-17-103QP-GFP with *Hind*III and *Bam*HI (to drop out 1-17-103Q leaving only pcDNA3.1 GFP), and subcloning annealed oligonucleotides ER11 and ER12 (Supplementary Table II) which encode the 1<sup>st</sup> 17 aas with *Hind*III and *Bam*HI ends.

Constructs were also generated with a FLAG epitope tag fused in frame to the C-terminus instead of the GFP tag to yield both 1-17-97QP-FLAG and 1-17-103Q-FLAG by digesting 1-17-97QP-GFP and 1-17-103Q-GFP with *Bam*HI and *Xba*I and ligating with annealed oligonucleotides FLAG #1 and #2 (supplementary Table II) encoding the FLAG tag with *Bam*HI and *Xba*I ends. 1-17-97QP-FLAG and 1-17-103Q-FLAG were digested with *Hind*III to remove the 1<sup>st</sup> 17 aas and ligated with annealed oligonucleotides Met#1 and #2 (Supplementary Table II) encoding methionine with *Hind*III ends to yield 97QP-FLAG and 103Q-FLAG.

To generate 97QP-GFP and 103Q-GFP, the FLAG tag constructs 97QP-FLAG and 103Q-FLAG were digested with *Bam*HI and *Xba*I to drop out the FLAG and subcloned into pcDNA3.1-GFP as described above.

mMDH-1-17-97QP-GFP was generated using annealed oligonucleotides, mMDH #1 and #2 (Supplementary Table II) encoding the rat malate dehydrogenase mitochondrial targeting signal with *Kpn*I and *Nco*I ends. 1-17-97QP-GFP was digested with *Kpn*I and *Bam*HI and subcloned into *Kpn*I and *Bam*HI digested pBSKS+. pBSKS+ 1-17-97QP was

digested with *KpnI* and *NcoI* and ligated with annealed oligonucleotides mMDH #1 and mMDH #2 to generate pBSKS+ mMDH-1-17-97QP. This plasmid was digested with *KpnI*, blunt-ended and digested with *BamHI* (dropping out mMDH-1-17) and ligated with 1-17-97QP-GFP digested with *HindIII*, blunt-ended, and digested with *BamHI* (dropping out 1-17) to yield mMDH-1-17-97QP-GFP in pcDNA 3.1.

To generate mMDH-1-17-25QP-GFP, pBSKS+ mMDH-1-17-97QP was digested with *NcoI* and *BamHI* to drop out the 1-17-97QP and ligated with 1-17-25QP from pcDNA3.1 1-17-25QP-GFP digested with *NcoI* and *BamHI* to yield pBSKS+ mMDH-1-17-25QP. This plasmid was digested with *KpnI*, blunt-ended, and digested with *BamHI* (to drop out mMDH-1-17-25QP) and ligated in frame to GFP generated from pcDNA3.1 1-17-25QP-GFP that was digested with *HindIII*, blunt-ended and digested with *BamHI* to ultimately generate mMDH-1-17-25QP-GFP.

Httex1p was also fused to a nuclear localization signal (NLS) to generate NLS-2-17-97QP-GFP. Previously described 25Q and 97Q containing Htt exon I DNA fragments (83) cloned in pBS- were used for PCR amplification to introduce a short NLS signal at the N-terminus. PCR primers, complementary to N-terminal Htt sequence prior to polyQ repeat, were designed in such a way that the forward primer contained the NLS signal and a *KpnI* restriction site. PCR products were used for replacement of the N-terminal Htt fragment using unique *KpnI* and *HindIII* restriction sites (see Supplementary Table II for sequence of forward and reverse primers). Successful replacement was verified by

sequencing and pBS-NLS-2-17-97QP-GFP was subcloned into pcDNA3.1 digested with *KpnI* and *XbaI* to yield NLS-2-17-97QP-GFP.

To generate ptmtNLS-1-17-97QP-GFP, annealed oligonucleotides encoding a point mutant, non-functional nuclear localization signal (mutation of a lysine to a threonine) as described in (16) with *KpnI* and *NcoI* ends were used. pBSKS+ 1-17-97QP was digested with *KpnI* and *NcoI* and ligated with annealed oligonucleotides ptmtNLS #1 and #2 (Supplementary Table II) to generate pBSKS+ ptmtNLS-1-17-97QP. This was then digested with *KpnI* and *BamHI* to drop out ptmtNLS-1-17-97QP and subcloned into pcDNA 3.1 GFP (generated from pcDNA3.1 1-17-97QP-GFP digested with *KpnI* and *BamHI*) to yield ptmtNLS-1-17-97QP-GFP.

**Western analysis of Htt peptide expression in immortalized striatal neurons and differentiated PC12 cells.** All Httex1p constructs were verified by sequencing and by transient transfection into PC12 and St12.7 mammalian cells. Immortalized striatal neurons (St12.7) (85) and rat adrenal pheochromocytoma (PC12) cells in 6-well plates were transiently transfected with Httex1p plasmids (1 $\mu$ g) using Lipofectamine 2000 (Invitrogen). St12.7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) and 10% fetal bovine serum (FBS) at 33 $^{\circ}$  and PC12 cells were grown in DMEM with 10% horse serum (HS) and 5% FBS at 37 $^{\circ}$  (all reagents from Invitrogen). PC12 cells were plated on collagen (BD Biosciences) coated dishes and were differentiated 24 hours post-transfection using 50ng/mL NGF (Harlan Bioproducts, Indianapolis) in DMEM with 1% HS. 48 hours post-transfection, cells were lysed with 200 $\mu$ L of Lysis buffer (20mM

HEPES-KOH, 100mM NaCl, 0.1% Triton-X-100, and 10% glycerol) containing protease inhibitors [10 $\mu$ g/mL leupeptin, 10 $\mu$ g/mL aprotinin and 0.1mM PMSF (phenylmethylsulfonyl fluoride)] and cells were collected and placed on ice. The cell lysates were sonicated for 10 seconds at speed 2 (Fisher Scientific sonic dismembrator 60). Bradford assays were performed to determine protein concentrations and equivalent amounts of each protein were loaded on a 10% SDS-polyacrylamide gel. The gels were transferred overnight to Immobilon-P membrane (Millipore) by standard Western blot wet transfer methods. The immobilon membrane was blocked for 1hour in 4% Fraction V BSA (United States Biochemical) in 1X Tris-buffered saline/Tween 20, and incubated for 1 hour in antibody  $\alpha$ -GFP (Clontech) at 1:200 dilution, washed 3X, and incubated with donkey  $\alpha$ -rabbit horseradish peroxidase-conjugated antibodies (Amersham Biosciences) at 1:5000 for 45 minutes. Immunoreactive bands were detected using supersignal west pico chemiluminescent substrate (Pierce).

## SUPPLEMENTARY INFORMATION

### FIGURE AND TABLE LEGENDS

**Supplementary Figure 1: Western analysis of Httex1p constructs.** To evaluate expression of Httex1p polypeptides, Httex1p constructs were transiently transfected into both (A) St12.7 and (B) differentiated PC12 cells, and 48 hours post-transfection, cells were lysed and sonicated. Equivalent protein amounts were run on a 10% SDS-PAGE gel. Western blot analysis using  $\alpha$ -GFP is shown. In some lanes of ST12.7 lysates, multiple bands are observed and at least one could represent a GFP cleavage product. GFP alone shows no co-localization with mitochondria, Golgi or ER, therefore, cleaved GFP would not influence the % association with these organelles. Similar localization results are observed for ST12.7 and PC12 cells.

**Supplementary Table I: Httex1p plasmids in pcDNA3.1.** Description of mammalian expression constructs encoding specific regions of Httex1p domains in pcDNA3.1. Constructs were synthesized using double-stranded oligonucleotides (Table II).

**Supplementary Table II: DNA sequence of each oligonucleotide and primer used for Httex1p plasmid generation.**

**Supplementary Table III: Analysis of cytosolic  $\text{Ca}^{2+}$  loads over time in PC12 transfected cells.** To measure the total  $[\text{Ca}^{2+}]_i$  load evoked by a glutamate challenge, we

evaluated changes in  $[Ca^{2+}]_i$  by integral analysis of the area under the curve of fura-2 ratios over time. Two different areas were considered: (1) T5-25 that indicates the  $[Ca^{2+}]_i$  changes occurring during the glutamate exposure, and (2) T25-45 that provides information about the  $[Ca^{2+}]_i$  changes occurring in the washout period. The table shows average integral values, standard deviation (SD), standard error (SEM), and sample size (number of cells) for each indicated construct. Statistical analysis was performed by t-student test comparing for each construct transfected and untransfected (ctrl) cells.

## SUPPLEMENTARY TABLES

**Supplementary Table I:**

<u>Htt Plasmid</u>	<u>Cloning Strategy in pcDNA3.1 vector</u>
1-17-25Q(97Q)P-GFP	Described previously (83, 84).
1-17-25Q(103Q)-GFP	Described previously (83, 84).
1-17-P-GFP	Annealed oligonucleotides encoding the 1 <sup>st</sup> 17 aas (mutated to delete the <i>Hind</i> III site (underlined) present at the end of the 1 <sup>st</sup> 17 aas) with <i>Hind</i> III and <i>Pst</i> I ends were ligated into pBS-Proline previously generated (supplementary information). pBS-1-17-P was then digested with <i>Hind</i> III and <i>Bam</i> HI and the insert subcloned into pcDNA-GFP generated from digesting pcDNA 3.1 1-17- 97QP-GFP with <i>Hind</i> III and <i>Bam</i> HI to delete the insert.
P-GFP  This construct has two extra aas from the <i>Pst</i> I site, therefore is actually M-L-Q-Proline-rich region-GPF.	Annealed oligonucleotides encoding methionine with <i>Hind</i> III and <i>Pst</i> I ends were ligated into pBS- Proline digested with <i>Hind</i> III and <i>Pst</i> I to generate pBS- M-P-GFP. Prolines were then subcoloned into pcDNA3.-GFP generated from digesting pcDNA3.1 1 1-17-97QP-GFP with <i>Hind</i> III and <i>Bam</i> HI.
1-17-GFP	Annealed oligonucleotides encoding the 1 <sup>st</sup> 17 aas with <i>Hind</i> III and <i>Bam</i> HI ends were ligated into pcDNA3.-

	GFP as described above.
<p>97QP(103Q)-GFP</p> <p>This construct has three additional aas from the 1<sup>st</sup> 17 aas [M-A-S-F-97QP(103Q)-GFP, represents Δ 14aa).</p>	<p>Annealed oligonucleotides encoding methionine with <i>Hind</i>III ends were used to generate 97QP-FLAG and 103Q-FLAG (see supplementary information). M-97QP-FLAG and M-103Q-FLAG were digested with <i>Bam</i>HI and <i>Xba</i>I to drop out the FLAG and GFP was inserted (generated from digesting pcDNA3.1 1-17-103QP-GFP with <i>Bam</i>HI and <i>Xba</i>I).</p>
mMDH-1-17-97(25)QP-GFP	mMDH-1-17-97(25)QP-GFP was generated using annealed oligonucleotides encoding the rat malate dehydrogenase mitochondrial targeting signal(41) with <i>Kpn</i> I and <i>Nco</i> I ends.
NLS-2-17-97QP-GFP	Previously described 25Q and 97Q containing Httex1p DNA fragments (83) cloned in pBS- were used for PCR amplification to introduce a short NLS signal at the N-terminus. PCR primers, complementary to N-terminal Htt sequence prior to polyQ repeat, designed in such a way that the forward primer contained the NLS signal and a <i>Kpn</i> I restriction site. PCR products were used for replacement of the N-terminal Htt fragment using unique <i>Kpn</i> I and <i>Hind</i> III restriction sites (see supplementary information for primer

	<p>sequences). pBS- NLS-2-17-97QP-GFP was subcloned into pcDNA3.1 digested with <i>KpnI</i> and <i>XbaI</i> to yield NLS-2-17-97QP-GFP.</p>
<p>ptmtNLS-1-17-97QP-GFP</p>	<p>Annealed oligonucleotides encoding a point mutant, non-functional nuclear localization signal (mutation of a lysine to a threonine) as described in (16) and with <i>KpnI</i> and <i>NcoI</i> ends were subcloned into pcDNA3.1-GFP (generated from digestion of pcDNA3.1 1-17-97QP-GFP with <i>KpnI</i> and <i>BamHI</i>) to yield ptmtNLS-1-17-97QP-GFP.</p>

**Supplementary Table II: DNA sequence of each oligonucleotide and primer used for Httex1p plasmid generation.**

Oligo	Sequence
ER5	5'-AGCTTATGGCGACCCTGGAAAAGCTGATGAAGGCCTTCGAGTCCCTCAAAA <u>AGTTTC</u> CCTGCA-3'
ER6	5'-GGAAACTTTTGAGGGACTCGAAGGCCTTCATCAGCTTTTCCAGGGTCGCCATA-3'
ER7	5'-AGCTTGCGGCCCGCATGCTGCA-3'
ER8	5'-GCATGCGGCCGCA-3'
ER11	5'-AGCTTATGGCGACCCTGGAAAAGCTGATGAAGGCCTTCGAGTCCCTCAAAAAGTTTCCTGG-3'
ER12	5'-GATCCCAGGAAACTTTTGAGGGACTCGAAGGCCTTCATCAGCTTTTCCAGGGTCGCCATA-3'
FLAG #1	5'-GATCCATGGATTACAAGGATGACGACGATAAGTAAT-3'
FLAG #2	5'-CTAGATTACTTATCGTCGTCATCCTTGTAAATCCATG-3'
Met #1	5'-AGCTTGCGGCCCGCATGGCA-3'
Met #2	5'-AGCTTGCCATGCGGCCGCA-3'
mMDH #1	5'- CATGCTGTCCGCTCTCGCCCGTCCTGTCCGGTGCCGCTCTCCGCCGACGCTTCAGCACTTCAGGCCAGA ACAATGC-3'
mMDH #2	5'- CATGGCATTGTTCTGGCCTGAAGTGCTGAAGCTGCGGCGGAGAGCGGCACCGACAGGACGGGCGAGA GCGGACAGCATGGTAC-3'
NLS forward	5'-TAAGGTACCATGGGTCCAAAGAAGAAGCGTAAGGCGACCCTGGAAAAGCTG-3'
NLS reverse	5'-CAAGAAGCTTTTGAGGGACTCGAAGCC-3'
ptmtNLS #1	5'-CATGCCTCCAAAACTAAGAGAAAGGTAGACGTCGC-3'
ptmtNLS #2	5'-CATGGCGACGTCTACCTTTCTCTTAGTTTTGGAGGCATGGTAC-3'

**Supplementary Table III: Analysis of cytosolic Ca<sup>2+</sup> loads over time in PC12**

**transfected cells.**

	TRANS				CTRL			Trans % of CTRL			CTRL vs AVG CTRL		
	T5-25	T25-45	T5-45		T5-25	T25-45	T5-45	T5-25	T25-45	T5-45	T5-25	T25-45	T5-45
<b>1-17-25QP</b>	22.149	12.075	33.719	<b>mean</b>	17.913	8.353	25.853	123.645	144.565	130.424	100.000	100.000	100.000
	19.930	17.711	34.872	<b>SD</b>	20.983	17.472	36.224	111.258	212.035	134.882	117.135	209.172	140.112
	3.523	3.131	6.164	<b>SE</b>	1.983	1.651	3.423	19.668	37.483	23.844	11.068	19.765	13.239
	NS	NS	NS	<b>t-Test</b>									
<b>1-17-97QP</b>	23.380	9.261	32.097	<b>mean</b>	10.394	5.026	15.135	224.937	184.264	212.069	100.000	100.000	100.000
	27.264	19.473	43.509	<b>SD</b>	15.107	16.651	29.810	262.304	387.460	287.466	145.344	331.296	196.957
	4.746	3.390	7.574	<b>SE</b>	1.272	1.402	2.510	45.661	67.448	50.041	12.240	27.900	16.587
	<b>*P=0.002</b>	<b>§ P=0.044</b>	<b>° P=0.002</b>	<b>t-Test</b>									
<b>M-97QP</b>	17.027	10.223	<b>26.808</b>	<b>mean</b>	12.338	7.685	19.680	138.000	133.026	136.219	100.000	100.000	100.000
	16.934	26.236	40.108	<b>SD</b>	14.826	13.684	26.424	137.248	341.385	203.801	120.161	178.061	134.267
	2.994	4.638	7.090	<b>SE</b>	1.140	1.053	2.033	24.262	60.349	36.027	9.243	13.697	10.328
	NS	NS	<b>P=0.029</b>	<b>t-Test</b>									
<b>M-103Q</b>	7.905	5.448	13.118	<b>mean</b>	8.194	5.519	13.482	96.470	98.704	97.301	100.000	100.000	100.000
	7.738	6.902	14.113	<b>SD</b>	9.284	8.324	17.207	94.430	125.044	104.679	113.305	150.808	127.630
	1.824	1.627	3.326	<b>SE</b>	1.087	0.974	2.014	22.257	29.473	24.673	13.261	17.651	14.938
	NS	NS	NS	<b>t-Test</b>									
<b>1-17-</b>	13.783	6.503	19.917	<b>mean</b>	10.571	5.642	15.959	130.379	115.250	124.796	100.000	100.000	100.000
	13.745	11.998	22.958	<b>SD</b>	16.432	15.045	29.444	130.020	212.641	143.851	155.438	266.651	184.493
	3.240	2.828	5.411	<b>SE</b>	1.526	1.397	2.734	30.646	50.120	33.906	14.432	24.758	17.130
	NS	NS	NS	<b>t-Test</b>									
<b>1-17-P</b>	13.945	10.251	24.196	<b>mean</b>	12.717	4.049	16.766	109.655	253.150	144.312	100.000	100.000	100.000
	16.305	12.612	28.017	<b>SD</b>	18.330	14.558	28.180	128.214	311.454	167.104	144.136	359.499	168.072
	3.741	2.893	6.428	<b>SE</b>	1.709	1.357	2.628	29.414	71.452	38.336	13.441	33.523	15.673
	NS	NS	NS	<b>t-Test</b>									
<b>P</b>	10.765	1.102	11.585	<b>mean</b>	10.982	3.563	14.250	98.028	30.941	81.297	100.000	100.000	100.000
	11.836	4.933	14.696	<b>SD</b>	11.900	10.350	20.379	107.775	138.471	103.131	108.361	290.522	143.014
	3.283	1.368	4.076	<b>SE</b>	1.184	1.030	2.028	29.891	38.405	28.603	10.782	28.908	14.230
	NS	NS	NS	<b>t-Test</b>									
<b>mMDH</b>	11.116	7.825	18.554	<b>mean</b>	11.463	6.865	18.003	96.978	113.978	103.059	100.000	100.000	100.000
	12.289	8.155	19.530	<b>SD</b>	13.790	12.993	25.005	107.203	118.788	108.483	120.305	189.255	138.893
	2.620	1.739	4.164	<b>SE</b>	1.254	1.181	2.273	22.856	25.326	23.129	10.937	17.205	12.627
	NS	NS	NS	<b>t-Test</b>									
<b>NLS</b>	7.458	4.448	11.691	<b>mean</b>	6.028	3.844	9.708	123.727	115.705	120.432	100.000	100.000	100.000
	9.444	10.602	18.612	<b>SD</b>	6.614	5.500	10.875	156.684	275.798	191.727	109.729	143.068	112.022
	1.696	1.904	3.343	<b>SE</b>	0.516	0.429	0.849	28.141	49.535	34.435	8.568	11.172	8.747
	NS	NS	NS	<b>t-Test</b>									

Supplementary Figure 1: Western analysis of Httex1p constructs.

