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Essential sequence of the N-terminal cytoplasmic localization-related domain of huntingtin and its effect on huntingtin aggregates

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Huntington's disease (HD) is caused by abnormal CAG repeat expansion in the 5′-end of the Huntingtin (*HTT*) gene. In addition to the canonical C-terminal full-length huntingtin (htt) nuclear export signal, a cytoplasmic localization-related domain (CLRD) in the N-terminus of htt has recently been reported. Here, we analyzed this domain by introducing deletion and substitution mutations in a truncated N-terminal htt protein and subsequently monitored htt expression, aggregation and subcellular localization by immunocytochemistry and Western blot analysis. We demonstrated that $Htt_{4\text{-}17}$ was the essential sequence for htt cytoplasmic localization. We also found that the subcellular distribution of htt was altered when $Htt_{1\text{-}17}$ was mutated to contain amino acids of different charges, suggesting a structural requirement of $Htt_{1\text{-}17}$ for the cytoplasmic localization of htt. Deletion of the first three amino acids did not affect its association with mitochondria. We observed that defective cytoplasmic localization resulted in a reduction of total htt aggregates and increased nuclear aggregates, indicating that the subcellular distribution of the protein might influence the aggregation process. These studies provide new insight into the molecular mechanism of htt aggregation in HD.

huntingtin, aggregates, cytoplasmic localization related domain, mitochondria, polyglutamine

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Huntington's disease (HD) is an autosomal dominant neurodegenerative disease characterized by motor abnormalities, cognitive deficits and neuropsychiatric symptoms. The abnormal expansion of polyglutamine (polyQ) repeats (>36) is believed to be the underlying cause of the disease and contributes to the formation of nuclear and cytoplasmic aggregates [1,2]. However, the precise relationship between huntingtin (htt) aggregates and pathogenesis remains controversial.

Normally, htt is mainly located in the cytoplasm of cells [3] and it associates with microtubules and proteins involved in transcription, cell signaling and intracellular transport [2,4]. In mitochondria of the mouse brain, mutant htt fragments are more stable than full-length wild-type htt fragments. In addition, the interaction between the N-terminal mutant htt and mitochondria disrupts the association of microtubule-based transport proteins with mitochondria [5]. Thus, disrupted mitochondrial functions are likely to be important in HD and the exact pathological mechanism of this dysfunction requires elucidation.

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The objectives of the present study are: (i) to identify the essential sequence of the N-terminal cytoplasmic localization-related domain (CLRD) of htt and to determine its association with mitochondria; (ii) to demonstrate the effects of cytoplasmic localization on the aggregation of mutant pathological htt.

1 Materials and methods

1.1 Plasmid constructs

Oligonucleotides encoding the SV40 nuclear localization sequence (NLS) were subcloned into the *Eco*R I-*Bam*H I sites of the pEGFP-N1 vector to create the plasmid NLS-GFP. Plasmids expressing various constructs of htt were constructed by inserting the corresponding oligonucleotides between the *Hin*d III-*Eco*R I sites of NLS-GFP.

The complete exon 1 of *HTT*, with or without mutation of the 4th amino acid, leucine (L), to arginine (R) was amplified by PCR using the following primers: $Htt_{Ex1P(20Q/59Q)}$-GFP with primers HD1 and HD2; $Htt_{Ex1P(L4R, 20Q/59Q)}$-GFP with primers HD3 and HD2 (Table 1). These fragments were then fused in frame with the N-terminus of eGFP between the *Eco*R I-*Bam*H I sites in pEGFP-N1.

1.2 Cell culture

Chinese hamster ovary (CHO), human embryonic kidney 293T (HEK293T) and mouse neuroblastoma (N2a) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, USA) at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. The medium was supplemented with 10% fetal bovine serum (FBS, Invitrogen, New York, USA), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin.

1.3 Immunocytochemistry

Transient transfection of plasmids was performed in 24-well dishes containing cover slips using Lipofectamine™ 2000 transfection reagent (Invitrogen). Fixed cells were then stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma, New York, USA) for 5 min, and then mounted on microscope slides. Confocal Microscopy (Leica TCS SP5 X, Wetzlar, Germany) was performed to detect the subcellular localization of htt and its aggregates.

To stain mitochondria, ER and Golgi, anti-cytochrome C (Biosource, New York, USA), concanavalin A (Con A, Molecular Probes, New York, USA) and anti-golgi 58K (Abcam, Cambridge, UK) were used respectively. Confocal Microscopy was then performed to detect co-localization with htt.

1.4 Western blot analysis

HEK293T and N2a cells were lysed in SDS sample buffer. Protein concentrations of the cell lysates were determined using a BCA Protein Assay kit (Pierce, Rockford, USA). Lysates were then subjected to SDS-PAGE (12%) electrophoresis and electroblotted onto Immobilon Polyvinylidene Difluoride membranes (Millipore, Billerica, MA, USA). The blots were incubated with anti-GFP antibody (1∶1000; Santa Cruz Biotech, California, USA) and anti-GAPDH (1∶10000) overnight at 4°C. After incubation with appropriate HRP-conjugated secondary antibody (BioRad, California, USA) at 1∶10000 for 1 h, the blots were visualized using the ECL plus detection kit (Amersham, Uppsala, Sweden).

1.5 Statistical analysis

Data are expressed as mean±SD (n=3) and were analyzed using SPSS 16.0 software (SPSS Inc., Chicago, IL). Statistical significance (P<0.05) was assessed using Student's t-test. For each experiment, three wells, each with 500 cells, were counted.

2 Results

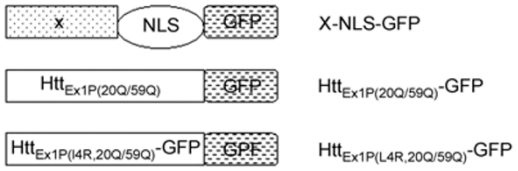
2.1 $Htt_{4\text{-}17}$ is essential for cytoplasmic targeting

Htt contains a signal sequence that mediates its export from the nucleus to the cytoplasm and the first 17 amino acids of htt are critical to the cytoplasmic localization of htt [6]. Here, we analyzed the essential sequence within $Htt_{1\text{-}17}$ that is responsible for the cytoplasmic targeting of htt, using sequential truncation of amino acids from both ends of $Htt_{1\text{-}17}$. To study the subcellular distribution of $Htt_{1\text{-}17}$, we added an NLS and GFP to the C-terminus of $Htt_{1\text{-}17}$. All the primers used to engineer the constructs are shown in Table 1, the schematic structures are shown in Figure 1. Forty-eight hours after transfection of CHO cells with various constructs, we found that $Htt_{1\text{-}17}$-NLS-GFP was primarily localized in the cytoplasm (Figure 2A), which is consistent with a previous report [6]. Deletion of the first 3 amino acids did not change this cytoplasmic localization (Figure 2B), suggesting that the first 3 amino acids were not essential for the cytoplasmic targeting of htt. However, deletion of one more amino acid from either the N-terminus or the C-terminus of $Htt_{4\text{-}17}$ led to a marked shift of the GFP signal from the cytoplasm to nucleus (Figure 2C and D), which was similar to the NLS-GFP control (Figure 2E). These results suggested that $Htt_{4\text{-}17}$ was essential for its normal localization in the cytoplasm. These observations were confirmed in HEK293T cells (Figure S1B and C).

Comparison of the first 17 amino acids of htt among different species indicated that this region was highly conserved, with only one variation at Position 4 (L in higher species and M in lower species) (Table 2). We thus investigated whether replacement of leucine (L) at Position 4 with methionine (M) in $Htt_{1\text{-}17}$ affected its subcellular distribution.

Table 1 Primers for constructs

Primer name	Primer number	Sequence (5′→3′)
NLS	1	gccgaattcccgccaccatgccaaaaaagaagagaaaggtagtggatcc
	2	ccaggatccactacctttctcttctttttggcatggtggcgggaattc
1-17	3	agcttatggcgaccctggaaaagctgatgaaggccttcgagtccctcaagtccttccg
	4	aattcggaaggacttgagggactcgaaggccttcatcagcttttccagggtcgccata
4-17	5	agcttatgctggaaaagctgatgaaggccttcgagtccctcaagtccttccg
	6	aattcggaaggacttgagggactcgaaggccttcatcagcttttccagcata
5-17	7	agcttatggaaaagctgatgaaggccttcgagtccctcaagtccttccg
	8	aattcggaaggacttgagggactcgaaggccttcatcagcttttccata
4-16	9	agcttatgctggaaaagctgatgaaggccttcgagtccctcaagtcccg
	10	aattcgggacttgagggactcgaaggccttcatcagcttttccagcata
L4R(1-17)	11	agcttatggcgacccgagaaaagctgatgaaggccttcgagtccctcaagtccttccg
	12	aattcggaaggacttgagggactcgaaggccttcatcagcttttctcgggtcgccata
L7R(1-17)	13	agcttatggcgaccctggaaaagcgaatgaaggccttcgagtccctcaagtccttccg
	14	aattcggaaggacttgagggactcgaaggccttcattcgcttttccagggtcgccata
L4M(1-17)	15	agcttatggcgaccatggaaaagctgatgaaggccttcgagtccctcaagtccttccg
	16	aattcggaaggacttgagggactcgaaggccttcatcagcttttccatggtcgccata
M8L(1-17)	17	agcttatggcgaccctggaaaagctgctgaaggccttcgagtccctcaagtccttccg
	18	aattcggaaggacttgagggactcgaaggccttcagcagcttttccagggtcgccata
HD1	19	gcgcgaattcatggcgaccctggaaaagctgatg
HD2	20	cgggatccctcggtcggtgcagcggctcc
HD3	21	gcgcgaattcatggcgacccgagaaaagc



Sequence for X

X	X-NLS-GFP	X sequence
1-17	1-17-NLS-GFP	MATLEKLMKAFESLKSF
4-17	4-17-NLS-GFP	MLEKLMKAFESLKSF
5-17	5-17-NLS-GFP	MEKLMKAFESLKSF
4-16	4-16-NLS-GFP	MLEKLMKAFESLKS
L4R(1-17)	L4R(1-17)-NLS-GFP	MATREKLMKAFESLKSF
L7R(1-17)	L7R(1-17)-NLS-GFP	MATLEKRMKAFESLKSF
L4M(1-17)	L4M(1-17)-NLS-GFP	MATMEKLMKAFESLKSF
M8L(1-17)	M8L(1-17)-NLS-GFP	MATLEKLLKAFESLKSF

Figure 1 Schematic presentation of Htt_{Ex1P} constructs. M represents the start sequence of 4-17-NLS-GFP, 5-17-NLS-GFP and 4-16-NLS-GFP.

We found that this replacement did not change its cytoplasmic localization (Figure 3A and B). We also replaced M at Position 8 with L, and this replacement did not change the cytoplasmic localization of htt either (Figure 3C). These observations are consistent with the fact that L and M are both hydrophobic amino acids and this replacement does not disrupt the alpha-helical structure of Htt_{1-17}, as has been shown by CD-spectroscopy, point mutations and crystallography [7,8]. However, when we mutated the hydrophobic, neutral L into the hydrophilic, charged amino acid arginine (R) at either Position 4 or 7, GFP localized extensively in the nucleus (Figure 3D and E), suggesting that the structural changes induced by the mutations abolished the cytoplasmic targeting of htt. These results further supported the critical role of Htt_{4-17} in the cytoplasmic targeting of htt.

2.2 Deletion of Htt_{1-3} does not affect the association of htt with mitochondria

Recent studies suggested that the first 17 amino acids of htt might also have roles in the association of htt with mitochondria and the endoplasmic reticulum (ER) and, in the presence of polyQ expansion, might contribute to mitochondrial dysfunction [5,7,9]. Thus, we studied whether the sequential deletion of Htt_{1-17} affected its association with the ER, Golgi apparatus, and mitochondria. Htt_{1-17}-NLS-GFP was mainly co-localized with mitochondria instead of the ER or Golgi apparatus, and deletion of the first 3 amino acids did not significantly alter this co-localization (Figure 4). Both NLS-GFP and GFP provided satisfactory negative controls, as shown in the supplementary data (Figure S2).

2.3 CLRD modulates Htt aggregation

To investigate the effects of the CLRD on htt aggregation, we inserted exon 1 of htt (Htt_{Ex1P}) into the pEGFP-N1 vector and transfected HEK293T cells with this construct. We observed that wild-type $Htt_{Ex1P(20Q)}$-GFP was extensively localized in the cytoplasm of cells, whereas the mutant $Htt_{Ex1P(L4R,20Q)}$-GFP was distributed in both the nucleus and

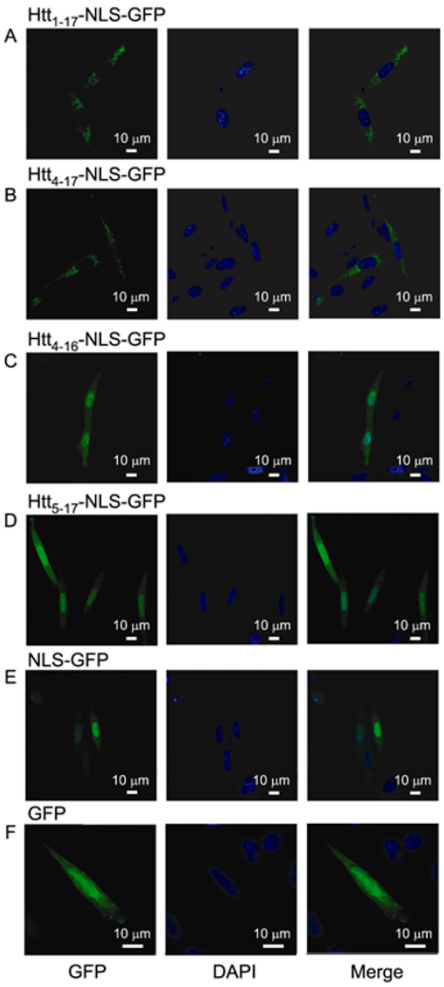


Figure 2 Subcellular distribution of various Htt_{1-17} constructs. Plasmids containing Htt_{1-17}-NLS-GFP (A), Htt_{4-17}-NLS-GFP (B), Htt_{5-17}-NLS-GFP (D), Htt_{4-16}-NLS-GFP (C), NLS-GFP (E) and GFP (F) were transfected into CHO cells. Cells were then fixed, stained with DAPI and visualized by Confocal Microscopy 48 h post-transfection.

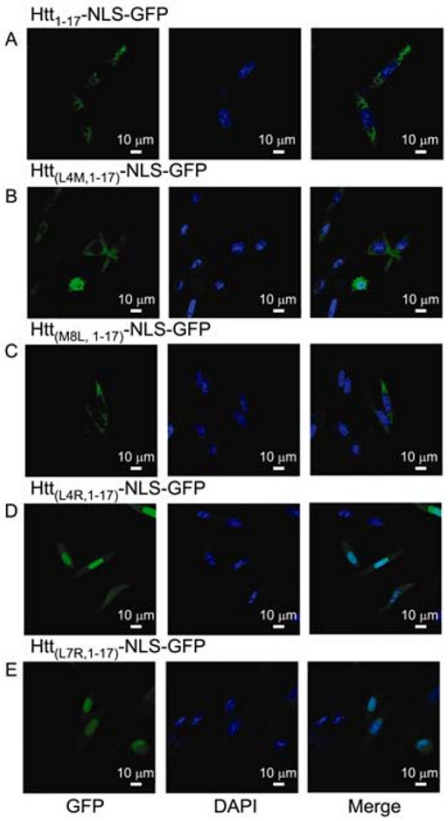


Figure 3 Effects of mutations on the subcellular distribution of Htt_{1-17}. Plasmids containing Htt_{1-17}-NLS-GFP (A), $Htt_{(L4M,1-17)}$-NLS-GFP (B), $Htt_{(M8L,1-17)}$-NLS-GFP (C), $Htt_{(L4R,1-17)}$-NLS-GFP (D) or $Htt_{(L7R,1-17)}$-NLS-GFP (E) were transfected into CHO cells. Confocal Microscopy was used to visualize the cellular localization of htt constructs 48 h after transfection. The localization of the NLS-GFP and GFP controls was the same as that in Figure 2E and F.

Table 2 Comparison of the first 17 amino acids of Htt among various species

Species	The first 17 amino acids of huntingtin
Human	$_{1}$MATLEKLMKAFESLKSF$_{17}$
Mouse	$_{1}$MATLEKLMKAFESLKSF$_{17}$
Fugu rubripes	$_{1}$MATMEKLMKAFESLKSF$_{17}$
Zebrafish	$_{1}$MATMEKLMKAFESLKSF$_{17}$

L and M show that there is only one variation at Position 4 (L in higher species and M in lower species) after comparison.

cytoplasm (Figure 5A and B), which was consistent with the above results of Htt_{1-17}-NLS-GFP and $Htt_{(L4R,1-17)}$-NLS-GFP. When Htt_{Ex1P} with a 59 polyQ expansion was examined, we found many Htt_{Ex1P} aggregates in the transfected cells (Figure 5C and D). In case of $Htt_{Ex1P(59Q)}$-GFP, the htt aggregates were located mainly in the cytoplasm (Figure 5C), whereas the L4R mutation led to more intranuclear htt aggregates (Figure 5D). These results suggested that the CLRD of htt regulated the intracellular trafficking of both normal and pathogenic htt and that loss of its cytoplasmic localization activity caused more intranuclear htt aggregates.

Quantitative analysis indicated that disruption of the CLRD in $Htt_{Ex1P(59Q)}$-GFP by the L4R mutation decreased the overall number of htt aggregates, in addition to inducing more nuclear aggregates (Figure 6A and B). The reduction of $Htt_{Ex1P(59Q)}$-GFP aggregation because of the L4R mutation was confirmed by Western blot analysis (Figure 6C). In addition, we confirmed these results in mouse neuroblastoma (N2a) cells, as shown in Figures S3–5.

3 Discussion

Htt protein contains a classical nuclear export signal sequence at the C-terminus and a CLRD at the N-terminus [6,10]. Because full-length htt is often cleaved at Position 513, 552 or 586 by caspases [11], the CLRD was assumed to be more important for the cytoplasmic localization of htt. The CAG expansion that causes HD is also located at the N-terminus; therefore, the CLRD might also have a role in

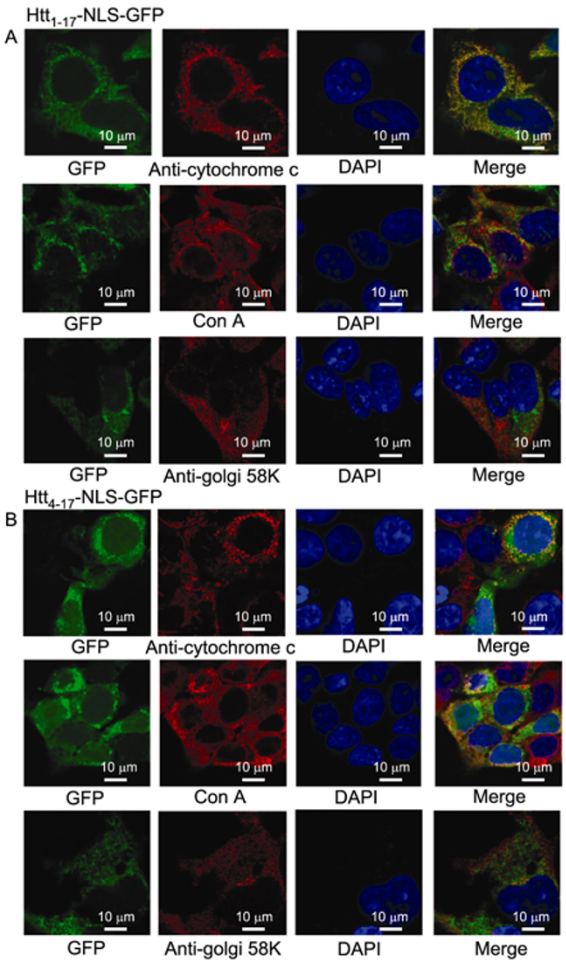


Figure 4 Mitochondrial localization of Htt_{1-17}-NLS-GFP (A) and Htt_{4-17}-NLS-GFP (B) constructs. Plasmids containing Htt_{1-17}-NLS-GFP or Htt_{4-17}-NLS-GFP were transfected into HEK293T cells, followed by triple fluorescence staining 48 h post transfection. Anti-cytochrome c, Con A, anti-Golgi and DAPI were used as markers of mitochondria, ER, Golgi and nucleus, respectively.

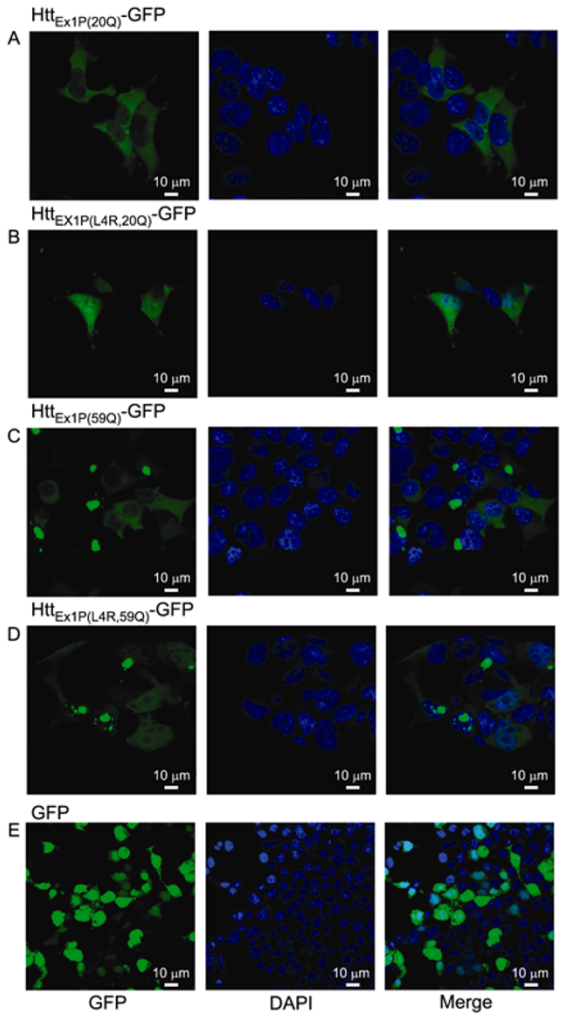


Figure 5 Subcellular localization and aggregation of various Htt_{Ex1P}-GFP proteins. The exon 1 of htt (Htt_{Ex1P}) including a normal number (20) or an expanded number (59) of polyQ repeats was inserted into pEGFP-NI and then transfected into HEK293T cells. Double fluorescence microscopy with DAPI and GFP was carried out 48 h after transfection.

the pathogenesis of HD.

Using a sequential truncation technique, we demonstrated for the first time that Htt_{4-17} was the essential sequence for htt cytoplasmic localization, a result consistent with the hypothesis that the export of N-terminal truncated htt is mediated by the first 17 amino acids of htt [6]. Whether this sequence is a nuclear export signal or just a CLRD is still controversial. It was first demonstrated by Cornett *et al*. [6] that the first 17 amino acids are involved in nuclear export of N-terminal htt fragments. However, the exact mechanism of this nuclear export pathway is still not entirely understood, except for the interaction with the translocated promoter region (Tpr) of the nuclear pore protein, which was verified by GST pull down assays and immunoprecipitation [6]. Htt_{4-17} is an amphipathic alpha helix membrane association signal, as shown by both CD-spectroscopy and crystallography, supporting its role as a CLRD [8,9]. Our results suggest that the first 3 amino acids are not required for htt cytoplasmic targeting, but they do not exclude the possibility that they have another function. Aiken *et al*. [12] showed that both T3D and T3A mutations reduced the neurodegeneration caused by expanded Htt_{Ex1p}. We also found that mutation of a single hydrophobic, neutral amino acid, Leucine, to the hydrophilic, charged amino acid, Arginine, within this signal sequence disrupted its cytoplasmic targeting function, indicating a structural requirement for cytoplasmic targeting. The strict conserved property of this sequence among species during evolution also suggested its importance.

We demonstrated that the first 17 amino acids of htt are mainly localized to mitochondria. In addition, Htt_{1-17} has been described previously to act as a membrane association signal [7]. It is possible that mutation of $Htt_{Ex1P(20Q)}$-GFP to $Htt_{Ex1P(L4R,\ 20Q)}$-GFP may have altered the N-terminal frag-

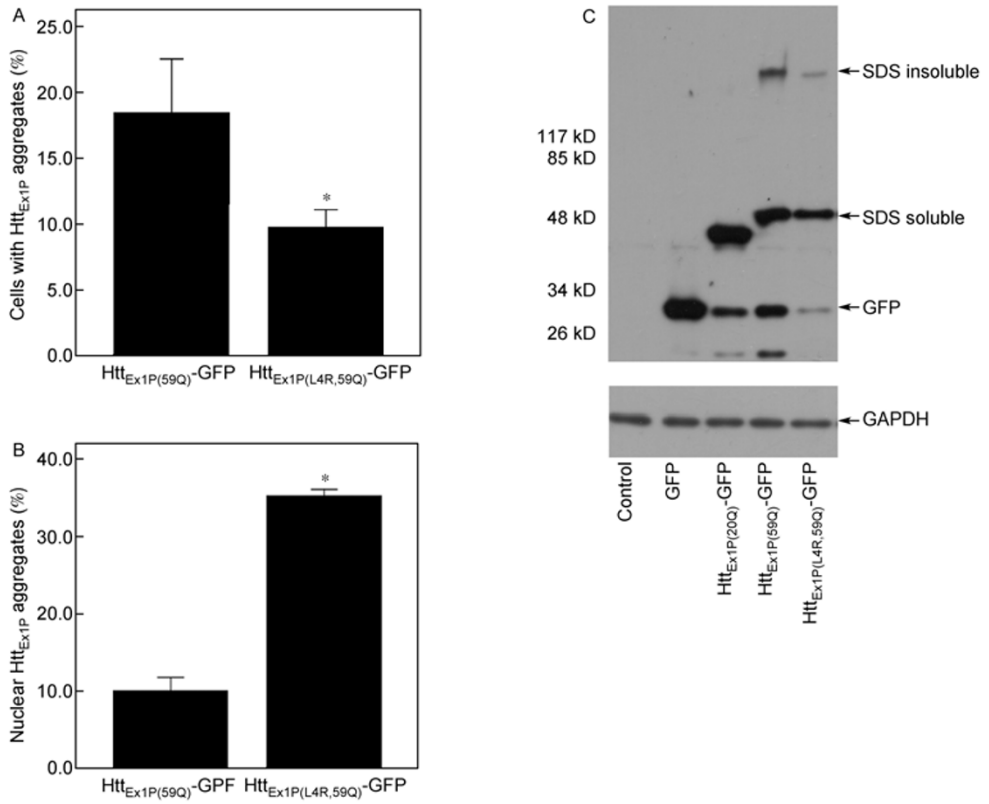


Figure 6 Effects of L4R mutation on aggregation of pathological htt. Htt$_{Ex1P(59Q)}$-GFP and Htt$_{Ex1P(L4R,59Q)}$-GFP plasmids were transfected into HEK293T cells. Double fluorescence microscopy with DAPI and GFP was carried out 48 h after transfection. The GFP aggregates were quantified and are presented as the percentage of cells showing GFP aggregates relative to the total number of cells (A) and as the number of nuclear aggregates relative to the total number of aggregates (B). The experiment was performed three times, and statistical analysis showed that the difference reached significance (*, P<0.05). Panel (C) shows Western blot analysis of cell lysates, developed with anti-GFP. The SDS-insoluble aggregates in the stacking gel and the SDS-soluble fraction in the separating gel are shown. GAPDH blots are included as loading controls.

ment's association with the nuclear or mitochondrial membrane, allowing free diffusion between the cytoplasm and nucleus. Studies investigating the effects of mutant htt in cell culture and animal models reveal mitochondrial changes, including decreased Ca^{2+} buffering capacity, loss of its membrane potential, and reduced expression of oxidative phosphorylation enzymes [9,13]. Structural changes in mitochondria, altered electron transport and increasing brain lactate levels have indicated mitochondrial abnormalities in HD pathophysiology [14]. Our future work will focus on how Htt$_{1-17}$ co-localizes with mitochondria and will include attempts to identify compounds that decrease the interaction between mutant htt and mitochondria to improve mitochondrial function.

Our data show that cytoplasmic localization affects the aggregates caused by the abnormal polyQ expansion. We observed that the loss of cytoplasmic localization activity by mutation analysis resulted in a reduction in the total number of htt aggregates and increased the numbers of nuclear aggregates, suggesting a role in modulating the htt aggregation process. How the CLRD regulates htt aggregation remains elusive.

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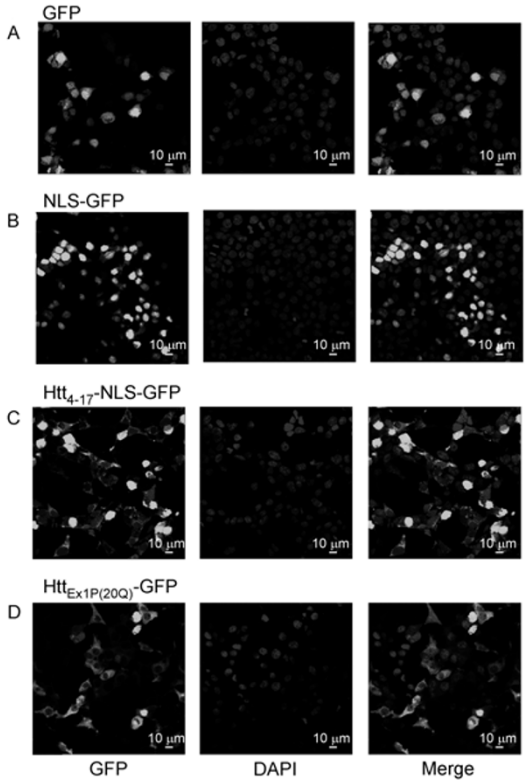

Figure S1 Comparison of subcellular localization between various Htt related proteins in HEK293T cells. C shows that $Htt_{4\text{-}17}$ was localized in the cytoplasm in HEK293T cells, with NLS-GFP as a control (B). This was a transient expression experiment; therefore, some cells showed strong fluorescence while others showed weak fluorescence. $HttEx1P_{(20Q)}$-GFP also led to a cytoplasmic distribution of the protein (D), with GFP as a control (A).

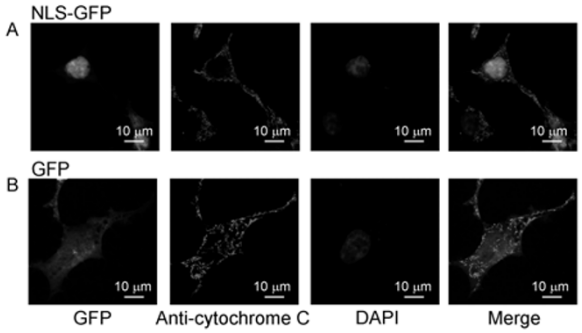

Figure S2 Mitochondrial localization of NLS-GFP and GFP constructs. Plasmids containing NLS-GFP or GFP were transfected into HEK293T cells, followed by anti-cytochrome C staining 48 h post-transfection. Anti-cytochrome C and DAPI were used as markers of mitochondria and the nucleus, respectively. Neither NLS-GFP nor GFP co-localized with mitochondria, in contrast to the data shown in Figure 4.

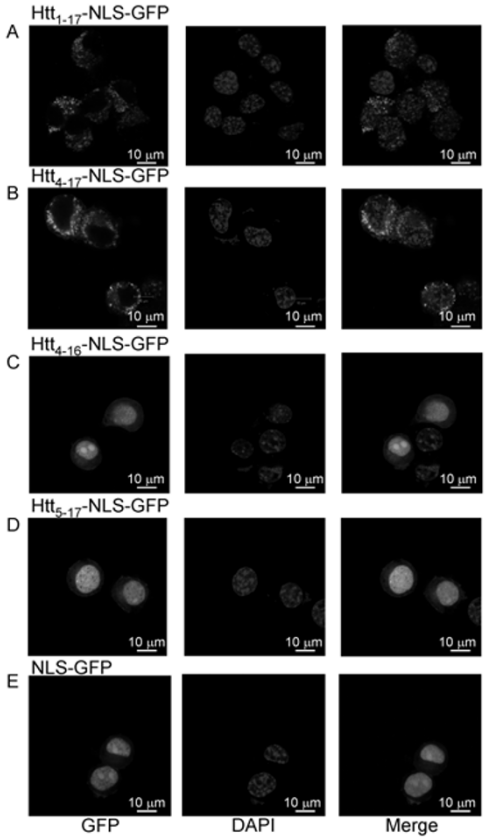

Figure S3 Subcellular distribution of various Htt_{1-17} constructs. Plasmids containing Htt_{1-17}-NLS-GFP, Htt_{4-17}-NLS-GFP, Htt_{5-17}-NLS-GFP, Htt_{4-16}-NLS-GFP or NLS-GFP were transfected into N2a cells. The cells were fixed, stained with DAPI and visualized by confocal microscopy 48 h post-transfection.

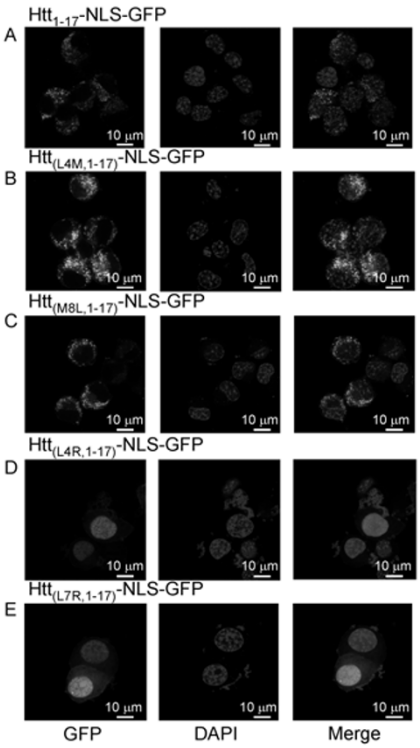

Figure S4 Effects of mutations on the subcellular distribution of Htt_{1-17} in N2a cells. Plasmids containing Htt_{1-17}-NLS-GFP, $Htt_{(L4M,1-17)}$-NLS-GFP, $Htt_{(M8L,1-17)}$-NLS-GFP, $Htt_{(L4R,1-17)}$-NLS-GFP or $Htt_{(L7R,1-17)}$-NLS-GFP were transfected into N2a cells. Confocal microscopy was used to visualize the cellular localization of Htt constructs 48 h after transfection.

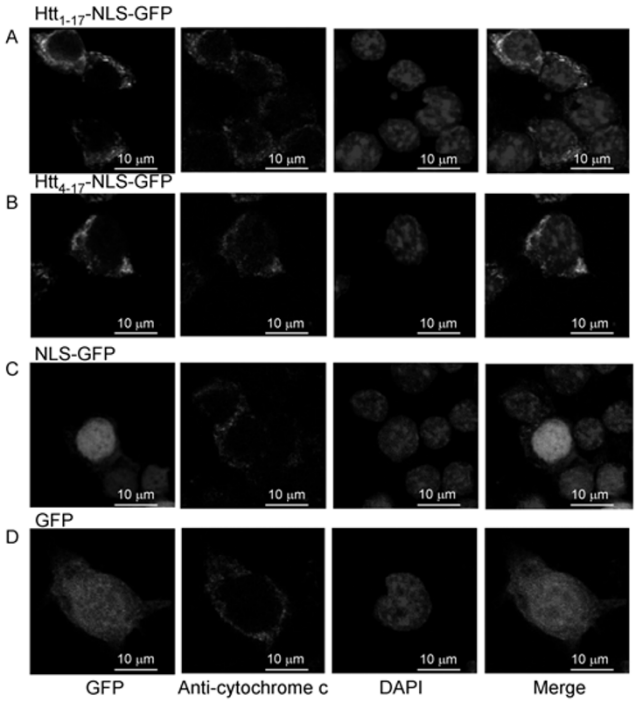

Figure S5 Mitochondrial localization of various Htt_{1-17} constructs. Plasmids containing Htt_{1-17}-NLS-GFP, Htt_{4-17}-NLS-GFP, NLS-GFP or GFP were transfected into N2a cells, followed by fluorescence staining 48 h post transfection. Anti-cytochrome c and DAPI were used as markers of mitochondria and the nucleus, respectively.