FAT10 Protein Binds to Polyglutamine Proteins and Modulates Their Solubility*§

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Expansion of polyglutamine (pQ) chain by expanded CAG repeat causes dominantly inherited neurodegeneration such as Huntington disease, dentatorubral-pallidolysian atrophy (DRPLA), and numbers of other spinocerebellar ataxias. Expanded pQ disrupts the stability of the pQ-harboring protein and increases its susceptibility to aggregation. Aggregated pQ protein is recognized by the ubiquitin proteasome system, and the enzyme ubiquitin ligase covalently attaches ubiquitin, which serves as a degradation signal by the proteasome. However, accumulation of the aggregated proteins in the diseased brain suggests insufficient degradation machinery. Ubiquitin has several functionally related proteins that are similarly attached to target proteins through their C terminus glycine residue. They are called ubiquitin-like molecules, and some of them are similarly related to the protein degradation pathway. One of the ubiquitin-like molecules, FAT10, is known to accelerate protein degradation through a ubiquitin-independent manner, but its role in pQ aggregate degradation is completely unknown. Thus we investigated its role in a Huntington disease cellular model and found that FAT10 molecules were covalently attached to huntingtin through their C terminus glycine. FAT10 binds preferentially to huntingtin with a short pQ chain and completely aggregates huntingtin with a long pQ chain. In addition, ataxin-1,3 and DRPLA proteins were both positive for FAT10, and aggregation enhancement was observed upon FAT10 knockdown. These findings were similar to those for huntingtin. Our new finding will provide a new role for FAT10 in the pathogenesis of polyglutamine diseases.

Accumulation of aggregated proteins is one of the key events in the pathogenesis of neurodegenerative disorders (1). The accumulation is usually irreversible, and the causative proteins aggregate to form inclusion bodies that can be detected microscopically. Every neurodegenerative disease has unique inclusion bodies, such as the neuronal nuclear inclusions in polyglutamine diseases, Lewy bodies in Parkinson disease, and neurofibrillary tangles in Alzheimer disease, and the presence of these inclusion bodies characterizes the pathological state. Aggregation clearance mitigates the symptoms of neurodegeneration in animal models, indicating that this acceleration of aggregate degradation could be one of the therapeutic targets (2).

In the diseased brain, aggregated proteins are often ubiquitylated, indicating that an abnormal conformational change leads to activation of the ubiquitylation machinery, which is supposed to accelerate their degradation (3). During the ubiquitylation process, the critical step is performed by ubiquitin ligases, which detect abnormal conformation of substrates and attach a ubiquitin molecule to a lysine side chain of the substrate via an isopeptide bond (4). The fact that ubiquitylated proteins are still widely found in the diseased brain indicates that the degradation machinery is overwhelmed by the presence of aggregated proteins (5).

Although ubiquitin is the best-understood post-translational modifier, there are several other proteins that modify cellular targets by a pathway different from that of ubiquitin. They are classified as ubiquitin-like proteins (ubls),2 which include SUMO, NEDD-8, Fubl, FAT10, Atg-8, Atg-12, ubl-5, IsG-15, and urm1 (6). Although they exhibit only modest primary amino acid sequence homology to ubiquitin, they share a similar structural fold called the ubiquitin fold. The ubls are also translated with C-terminal extensions that are processed to expose a diglycine motif, which is essential for covalent bond formation with the target substrates. In most cases, the attachment of ubls to substrates acts as a regulatory and not a degradation signal for the proteasomes. However, a few ubls have been reported to act as degradation signals independent of ubiquitin (7). Ubl function is thought to be essential for cellular homeostasis, and ubl dysfunction plays an important role in cancer genesis (8), viral diseases (9), cardiovascular diseases (10), and neurodegeneration. Besides the well studied SUMO and NEDD-8, the functions and substrates of the other seven ubls are not well understood (11, 12).

Polyglutamine (pQ) diseases are a set of dominantly inherited neurodegenerative disorders caused by the expansion of CAG repeats in the causative gene (13, 14). The CAG repeat is translated to polyglutamine, and the expanded polyglutamine

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§ The abbreviations used are: ubl, ubiquitin-like protein; pQ, polyglutamine; Htt, huntingtin; DRPLA, dentatorubral-pallidolysian atrophy; FRET, fluorescence resonance energy transfer; NES, nuclear export signal; NLS, nuclear localization signal; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.

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tract destabilizes the conformation of the causative protein. This conformational change is so powerful that a simple over-expression of polyglutamy1 proteins, such as the exon-1 fragment of huntingtin, in cultured cells or transgenic animals can recapitulate protein aggregation in vitro. Thus, this system is often used to study the basic mechanisms of protein aggregation and degradation (15). It has been reported that SUMO can bind to huntingtin, a product of the gene responsible for Huntington disease, a polyglutamy1 disease, and exacerbate the disease phenotype in vivo (16); however, the role of ubls in neurodegeneration has not been elucidated.

In an attempt to find ubls that can modify huntingtin exon-1, we screened various ubls and identified FAT10. Here, we report that FAT10 binds to polyglutamy1 proteins and stabilizes them.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—The huntingtin (Htt)-exon-1 constructs used in this study have been described previously (17). FAT10, Fubl, Isg15, ubl-5, ubiquitin, and urml cDNAs were cloned from a human cDNA library using standard PCR techniques and subcloned into p3×FLAG-CMV-14 vector (Sigma). FAT10 C-terminal diglycine deletion was achieved by PCR. N-terminal HA-tagged inserts were generated by PCR and subcloned into p3×FLAG-CMV-10 vector by placing a stop codon before the C-terminal FLAG sequence (Sigma). Full-length ataxin-1, ataxin-3, and DRPLA cDNAs were inserted into pcDNA4 Myc-His vector (Invitrogen, Carlsbad, CA). The sequences of all the constructs used were confirmed by direct sequencing of both strands.

**RNAi**—Control (AM4611) and FAT10 siRNAs (siRNA IDs 120463, 120464, and 120465) were purchased from Applied Biosystems (Mannheim, German). The plasmids were transfected into HEK293 (Clontech, Mountain View, CA). The plasmids were transfected into HEK293 cells in a 1:1 ratio, and cell extracts were obtained by lysing cells in 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, and Complete (Roche Applied Science). Fluorometric spectroscopy was performed using Spectramax Gemini XS (Molecular Devices, Sunnyvale, CA) at wavelengths 460–550 nm. Relative FRET value was determined by using Equation 1.

\[
\frac{ex425em_{525}}{ex425em_{476}}(acceptor\_wt) - \frac{ex425em_{525}}{ex425em_{476}}(acceptor\_\Delta GG)
\]

(Eq 1)

**Immunocytochemistry**—Cells were fixed in 4% paraformaldehyde and incubated with primary antibody at 4 °C overnight, followed by 1-h incubation at room temperature with Alexa 488- or Alexa 546-labeled secondary antibodies (Molecular Probes, Eugene, OR) and 50 μg/ml bisbenzimide (Sigma) or 0.5 μM SYTOX orange (Molecular Probes) for nuclear staining. Images were acquired using an LSM-510 confocal microscope system (Carl Zeiss, Oberkochen, Germany).

**Quantitative RT-PCR**—Total RNA was purified with TRIzol (Invitrogen), and reverse transcription was carried out with ReverTra Ace (Toyobo, Japan). Quantitative PCR was performed with the HT-7900 system (Applied Biosystems, Foster City, CA) using the Assay ID Hs00197374_m1 probe set for human FAT10. HuGAPDH (Applied Biosystems) was used as a control.

**MTS Assay**—Cell viability was measured using the colorimetric CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) with a SPECTRAMAXplus plate reader at an optical density of 490 nm (Molecular Devices, Sunnyvale, CA).

**RESULTS**

**FAT10 Binds to Htt-exon-1 through Its C-terminal Glycines**—First, we attempted to identify ubls that could bind to Htt-exon-1-GFP. To that end, we overexpressed N-terminal FLAG-tagged ubls and Htt-exon-1-GFP bearing different lengths of glutamine tracts. Empty vector and ubiquitin served as negative and positive controls, respectively. As shown in Fig. 1A, FAT10 clearly showed FLAG-positive bands that were larger in molecular weight than the original Htt-exon-1 (Fig. 1A). These bands disappeared when the C-terminal diglycine was deleted (FAT10ΔGG), and GFP lacking Htt-exon-1 did not show a similar band (supplemental Fig. S1A). We next pulled down FLAG-FAT10 from the lysate and confirmed the binding between two molecules (supplemental Fig. S1B). In both FAT10 and in FAT10ΔGG, the GFP-immunoprecipitated fraction contained FLAG-positive low molecular weight bands and supposedly non-covalently bound FAT10. This band was also present when GFP alone was cotransfected with FAT10 (supplemental Fig. S1A), which suggests that overexpressed FAT10 can bind covalently to Htt-exon-1 and non-covalently to GFP-Htt-exon-1. To further investigate the precise binding manner of FAT10 to Htt-exon-1, we generated C-terminal, His8-tagged Htt-exon-1-
GFP constructs and overexpressed them in HEK293 cells. After cell lysis, Htt-exon-1 was pulled down by Ni\(^{2+}\)/H11001-chelated resin and blotted with anti-human-FAT10 antibody (antibody evaluation in supplemental Fig. S1C). The result clearly showed that the bands observed in the FAT10 blot and those in the IC2 and GFP blots completely overlapped (Fig. 1B), and monomeric FAT10 was absent in the pulled down fraction. Finally, to eliminate the possibility that our anti-human FAT10 antibody was directly binding to Htt-exon-1-GFP, we transfected the same constructs into mouse Neuro2A cells and pulled down the Htt-exon-1-GFP. No signal, other than nonspecific bands, was detected with the anti-FAT10 antibody (supplemental Fig. S2A). These results confirmed that endogenous FAT10 covalently binds to Htt-exon-1 both in the cytoplasm and in the nucleus.

The Htt-exon-1 proteins extracted from FAT10-overexpressing cells were larger than the original Htt-exon-1, and the bands detected by the endogenous antibody had the same molecular weight as the original Htt-exon-1. This discrepancy indicates that Htt-exon-1 normally incorporates a single FAT10 molecule, whereas under overexpression; additional FAT10 can be incorporated to Htt-GFP.

**An Evaluation System for Quantitative FAT10 Binding**—We next examined the binding between FAT10 and ubiquitin. Because both FAT10 and ubiquitin have C-terminal diglycine motifs, a potential binding between the two molecules was expected. To facilitate unidirectional binding, we generated acceptors by deleting the diglycine motif from HA-tagged constructs. The binding donor was 3×FLAG-tagged ubiquitin or FAT10. These were coexpressed in HEK293 cells, immunoprecipitated with anti-HA antibody, and blotted with HA or FLAG antibodies (Fig. 2A). The result clearly showed that one FAT10 molecule covalently bound to one ubiquitin molecule. The FAT10-FAT10 interaction was mediated by covalent or non-covalent bonding, with a preference for the latter. Ubiquitin formed polyubiquitin chains with both ubiquitin and FAT10 (Fig. 2B). These results again indicate that the band shift observed in Fig. 1A is due to additional FAT10 molecules binding to the first FAT10 and that FAT10 binds to Htt-exon-1 directly and not through the ubiquitin chain.

**FAT10 Prefers Huntington with Short Glutamine Stretches**—We then tried to develop a method to quantify the binding of FAT10 to Htt-exon-1. To this end, we used the ubiquitin-FAT10 interaction as a positive control for FRET. As shown in Fig. 2C, we detected positive FRET signals between YFP-tagged ubiquitin and CFP-tagged FAT10 molecules; this finding was comparable to the binding data obtained from the immunoprecipitation experiment (Fig. 2A). Using this system, FRET between YFP-tagged Htt-exon-1 and CFP-tagged FAT10 was measured. As shown in Fig. 2D, the FRET signal decreased as the number of glutamines increased. This indicates that the binding between FAT10 and Htt-exon-1 is dependent on the length of the pQ tract.

**FAT10 Binds to Huntingtin in Both the Cytoplasm and Nucleus to Facilitate Its Degradation**—FAT10 exists in the cytoplasm and enhances substrate degradation. Our overexpression experiment confirmed this localization (supplemental Fig. S1C). To determine whether FAT10-bound Htt-exon-1 was prone to degradation through the ubiquitin proteasome system, we transfected nuclear export signal (NES)- or nuclear localization signal (NLS)-tagged Htt-exon-1 GFP into HEK293 cells and incubated the cells with...
Regardless of the cellular localization, proteasome inhibition resulted in an increase in the amount of FAT10-positive Htt-exon-1, especially with long stretches of glutamine (Fig. 3A). Thus, FAT10 binds to Htt-exon-1 both in the cytoplasm and the nucleus and enhances its degradation by the proteasome.
FAT10 Does Not Associate with Aggregated Huntingtin—The “gel top aggregates” (denoted by asterisk) seen in Figs. 1A and 3A were negative for FAT10, although they were positive for GFP or ubiquitin. To test if aggregated Htt-exon-1 had FAT10 immunoreactivity, we captured the aggregated Htt-exon-1 using a filter retardation assay and incubated it with anti-FAT10 antibody. The aggregates were positive for GFP or ubiquitin but not FAT10 (Fig. 3B). Moreover, in vivo-formed inclusion bodies positive for ubiquitin did not show any signal for FAT10 (Fig. 3C). These results strongly indicate that highly aggregated Htt-exon-1 was FAT10 free.

FAT10 Stabilizes Huntingtin and Provides Protection from Its Toxicity—We next examined the significance of the FAT10-Htt-exon-1 interaction. First, we co-expressed Htt-exon-1 with wild-type FAT10, which did not affect the amount of soluble or aggregated Htt-exon-1 (Fig. 4A). Then, we knocked down FAT10 by 80% and transfected Htt-exon-1 (supplemental Fig. S2, B and C). This slightly decreased the amount of soluble Htt-exon-1 and significantly increased aggregated Htt-exon-1. To measure the level of FAT10 binding after RNAi, we pulled down His-tagged Htt-exon-1 expecting to see decreased FAT10 binding. Surprisingly, only 20% decrease in FAT10-Htt-exon-1 binding was observed, suggesting that Htt-exon-1 preferably incorporates FAT10 even when the amount of endogenous FAT10 is very low. In addition, huntingtin pulldown assays before and after RNAi did not reveal any differences in ubiquitylation or fragmentation patterns (Fig. 4C). Taken together, the results suggest that the amount of FAT10 was abundant enough to bind to every single overexpressed huntingtin molecule and

![FIGURE 4](image_url)
that huntingtin becomes readily insoluble when the FAT10 concentration is low.

To see if huntingtin stabilization by FAT10 was beneficial for the cells, we performed MTS assay in FAT10-knockdown and Htt-overexpressed cells. As shown in Fig. 4D, significant decrease in cell viability upon FAT10 knockdown was observed when Htt with a toxic length of polyglutamines was overexpressed.

**FAT10 Binds to Various Polyglutamine Proteins and Affects Their Aggregation Property**—We next tested if FAT10 binding was specific to Htt-exon-1. To this end, we transfected ataxin-1, ataxin-3, and DRPLA into cells expressing FAT10 siRNA and performed pulldown assays with the anti-Myc antibody. All tested polyglutamine proteins were positive for FAT10 (Fig. 5A), and inhibition of FAT10 expression resulted in an increase in polyglutamine protein aggregation (Fig. 5B). Thus, FAT10 binds to and stabilizes not only htt-exon-1 but also ataxin-1, ataxin-3, and DRPLA. Moreover, FAT10 overexpression with these pQ proteins did not alter the expression level of either soluble or insoluble pQs, which was consistent with the result from Fig. 4A, suggesting that a very small amount of FAT10 was enough for stabilizing overexpressed pQ proteins.

**DISCUSSION**

Polyubiquitylation through the 48th lysine of ubiquitin serves as a degradation signal, which is recognized by proteasome subunits (18). In neurodegeneration, the presence of ubiquitylated protein aggregates in the postmortem brain suggests that the proteasome machinery is not effective enough to degrade all the abnormal aggregates; as a result, the remaining toxic aggregates cause neuronal damage. Thus, enhanced degradation of aggregated proteins is a potential therapeutic target in neurodegenerative disorders (19). To achieve this goal, we need to have a better understanding of the aggregation and stabilization processes of the aggregate-prone proteins. There are several types of post-translational modifications, such as phosphorylation, acetylation, ubiquitylation, and modification partly because they are not well understood but mostly because they may play an important role in conjunction with substrate ubiquitylation (20). FAT10 serves as a ubiquitin-independent signal for proteasomal degradation under cytokine-induced conditions (7), but its involvement in aggregate-prone proteins has not been reported. Furthermore, the ubl-aggregate interaction has been described in only two reports to date (16, 21).

FAT10 was first found as a ubl after sequencing the human major histocompatibility complex class I locus (22). FAT10 is known to be up-regulated upon interferon and TNF-α stimulation (23), and its ablation results in an abnormality in the lymphocyte population (24). Like ubiquitin, FAT10 is activated by UBA6 and conjugated to the target through its C-terminal diglycine motif (25, 26). Previous reports indicate that FAT10 is a ubiquitin-independent proteasomal degradation signal (7), but the degradation relies on NUB1L (27). FAT10 is also involved in p53 regulation (28, 29) and chromosomal stability (30); other functions of FAT10 are emerging.

We used the Htt-exon-1 overexpression system to recapitulate the in vitro aggregation process and screened various ubls for potential binding to Htt. The results demonstrate the direct binding of FAT10 to Htt. This is interesting, because, although ubiquitin seems to prefer cytoplasmic Htt, FAT10 binds to nuclear Htt as well (Fig. 1B). This suggests that FAT10, in addition to UPS and UHRF-2, may function as a dominant pQ modifier and degradation signal in the nucleus (17).

According to our FRET evaluation system and the blot from supplemental Fig. S2B, FAT10 prefers Htt with short stretches of Q (Fig. 2D). As seen in Fig. 3A, FAT10-modified Htt is prone to degradation by the proteasome, and completely aggregated Htt lacks any FAT10. In addition, FAT10 knockdown results in increased aggregates. From these results, we can conclude that FAT10 stabilizes soluble Htt by facilitating their interaction with the proteosome. This function is similar to that of SUMO. However, SUMO competes with ubiquitin to modify Htt (16); whereas, FAT10 seems to modify Htt in a non-competitive manner, because FAT10 overexpression (not shown) or inhibition (Fig. 4C) did not alter the ubiquitylation level of Htt. FAT10 knockdown resulted in only a modest amount of FAT10-modified Htt (Fig. 4B). This may indicate that FAT10 preferably modifies soluble overexpressed Htt over other proteins. The filter retardation assay data in Fig. 3B show that FAT10-free HttS are insoluble.

As observed in Fig. 4C, FAT10 knockdown doesn’t seem to reduce FAT10-associated pQs significantly. This is possibly due to very high efficiency of FAT10 incorporation to soluble pQs, thus FAT10-unbound pQ can be readily insoluble. This is supported by the result shown in Fig. 3B that FAT10 knockdown leads to increased amount of aggregates. The same phenomenon is seen in the case of other pQs in Fig. 5A.

There remains a question about the modified lysine residue. Htt-exon-1-GFP is a quite small protein but still contains many lysine residues. In the case of ubiquitin, we observed high molecular weight smears suggesting that the modification may occur at any lysine residue and that polyubiquitylation may also occur. However, in the case of FAT10, this modification seems by ubls. We were interested in studying ubl-mediated protein modification partly because they are not well understood but mostly because they may play an important role in conjunction with substrate ubiquitylation (20).
to be achieved by a single molecule, according to the predicted molecular weights. Although ataxin-1, ataxin-3, and DRPLA contain similar polyglutamine stretches, they lack sequence homology; thus, the question as to which lysine residue is modified remains to be solved (31–33).

To our knowledge, this is the first study to report that FAT10 can modify various polyglutamine proteins. FAT10 knock-out in rodents results in a minimal phenotype (24), suggesting that this molecule may not play an important role in protein degradation in the normal healthy state. However, our findings suggest that FAT10 may assist the proteasome degradation system in parallel with the ubiquitylation system.

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