Degradation of mutant huntingtin via the ubiquitin/proteasome system is modulated by FE65

Wan Ning Vanessa CHOW, Hon Wing LUK, Ho Yin Edwin CHAN and Kwok-Fai LAU

School of Life Sciences, Faculty of Science, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong

An unstable expansion of the polyglutamine repeat within exon 1 of the protein Htt (huntingtin) causes HD (Huntington’s disease). Mounting evidence shows that accumulation of N-terminal mutant Htt fragments is the source of disruption of normal cellular processes which ultimately leads to neuronal cell death. Understanding the degradation mechanism of mutant Htt and improving its clearance has emerged as a new direction in developing therapeutic approaches to treat HD. In the present study we show that the brain-enriched adaptor protein FE65 is a novel interacting partner of Htt. The binding is mediated through WW–polyproline interaction and is dependent on the length of the polyglutamine tract. Interestingly, a reduction in mutant Htt protein level was observed in FE65-knockdown cells, and the process requires the UPS (ubiquitin/proteasome system). Moreover, the ubiquitination level of mutant Htt was found to be enhanced when FE65 is knocked down. Immunofluorescence staining revealed that FE65 associates with mutant Htt aggregates. Additionally, we demonstrated that overexpression of FE65 increases mutant Htt-induced cell death both in vitro and in vivo. These results suggest that FE65 facilitates the accumulation of mutant Htt in cells by preventing its degradation via the UPS, and thereby enhances the toxicity of mutant Htt.

Key words: FE65, huntingtin (Htt), Huntington’s disease (HD), protein degradation, toxicity, ubiquitination.

INTRODUCTION

HD (Huntington’s disease) is an autosomal dominant neurodegenerative disease caused by an unstable expansion of the glutamine-encoding CAG trinucleotide repeats (>35) in exon 1 of Htt (also known as IT15) that encodes Htt (huntingtin) [1]. Htt is a ubiquitously expressed 348 kDa protein with little sequence homology with other proteins [2,3]. In spite of its ubiquitous expression, mutant Htt exerts its toxicity primarily in striatal and cortical neurons [3]. Cellular and animal models of HD implicate that accumulation of N-terminal mutant Htt proteolytic fragments plays an important role in the pathogenesis of HD [4–8]. It is generally believed that the mutant Htt fragments are responsible for the perturbation of a number of cellular processes, including transcriptional regulation, mitochondrial metabolism, axonal transport, neuronal interaction and circuitries at the corticostriatal synapse, and the UPS (ubiquitin/proteasome system) [3]. The preferential accumulation of mutant Htt, but not wild-type, fragments is accounted by the lower susceptibility of mutant Htt towards degradation. Mutant Htt is primarily removed by both the UPS and lysosome, and the process can be modulated by post-translational modifications including phosphorylation and acetylation, as well as interaction with other proteins such as co-chaperone CHIP (carboxy terminus of heat-shock protein 70-interacting protein), BAG1 (Bcl-2-associated athanogene 1) and gp78 [9–14]. Given the toxicity of mutant Htt fragments, understanding the degradation mechanism of mutant Htt and improving its clearance are essential to developing therapeutic tools to treat HD.

FE65 is a brain-enriched developmentally regulated adaptor protein that mediates assembly of multimolecular complexes [15]. It possesses three protein interaction domains, including an N-terminal WW domain and two C-terminal PTB (phosphotyrosine binding) domains. The WW domain typically recognizes proline-rich sequences [16]. Known FE65 WW domain-binding proteins include Mena (mammalian enabled) [17], the c-Abl tyrosine kinase [18], the ionotropic P2X2 (purinergic receptor P2X2, ligand-gated ion channel 2) receptor subunit [19] and Nek6 [NIMA (never in mitosis gene a)-related kinase 6] [20]. PTB domains were found to bind with the canonical NPXY sequence or the reduced NXXY sequence [21]. The first PTB domain of FE65 has been reported to interact with the transcription factor CP2/LS/PBP [22], the histone acetyltransferase Tip60 (HIV-1 Tat-interactive protein of 60kDa) [23] and LRP (low-density lipoprotein receptor-related protein) [24]. On the other hand, the second PTB domain interacts with the intracellular domain of APP (amyloid precursor protein) [25,26]. It was also reported recently that the PTB2 domain binds to Dextras1 (dexamethasone-induced ras-related protein 1) which lacks an NPXY motif [27]. The wide spectrum of FE65-interacting partners underscores the roles of FE65 in various cellular processes. It is therefore important to identify the full complement of FE65-binding proteins.

There are several lines of evidence to suggest that Htt is a possible interacting partner of FE65. First, both FE65 and Htt are translocated between the cytosol and nucleus when bound to different interacting proteins or at different stages of proteolytic processing [3,15,28–31]. Secondly, FE65 is abundantly expressed in the striatum and the cerebral cortex of adult brain which are regions that are most affected in HD [3,32]. Thirdly, the PRR (proline-rich region) at the N-terminus of Htt is known to be responsible for mediating interaction with WW domain-containing proteins [2]. As FE65 possesses a WW domain and
has been reported to interact with PRR-containing proteins such as Mena [17], it is possible that FE65 also binds with Htt via WW domain–PRR interaction. Moreover, in a high-throughput yeast two-hybrid screen using recombinant Htt as bait, an isoform of FE65 was identified as a Htt-interacting protein [33], which further supports our speculation on the possible interaction between FE65 and Htt. In fact, we demonstrate in the present study that FE65 interacts with the N-terminus of Htt in a polyQ (polyglutamine)-dependent manner. Intriguingly, FE65 was found to stabilize mutant Htt by preventing it from degradation mediated by UPS, and thereby enhances the toxicity of mutant Htt in both cell and Drosophila models of HD. All of the experiments, except the interaction assays, were performed in both CHO (Chinese hamster ovary) and HEK (human embryonic kidney)-293 cells with similar results.

EXPERIMENTAL

Cell culture and transfection

CHO and HEK-293 cells were cultured in Ham’s F-12 medium and DMEM (Dulbecco’s modified Eagle’s medium) low glucose (Invitrogen) respectively, both of which contain 10% (v/v) FBS (fetal bovine serum) supplemented with 2 mM glutamine (Invitrogen), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen) [34]. For plasmid transfection, cells were transfected using FuGENETM 6 (Roche) according to the manufacturer’s instructions. Knockdown experiments were conducted by transfecting FE65 and control non-targeting siRNAs (short interfering RNAs; Dharmacon, Thermo Scientific) using Fugene 6 (Roche) according to the manufacturer’s instructions. For RNA interference, cells were transfected with siRNA using LipofectAMINE™ RNaiMAX (Invitrogen). To assay the effect of UPS on Htt degradation, the cells were treated with 2.5 μM MG132 (Merck Chemicals) or DMSO vehicle for 16 h.

Drosophila strains

Drosophila were raised on cornmeal medium supplemented with dry yeast. The full-length UAS-flHtt128Q line was a gift from Professor Juan Botas (Baylor College of Medicine, Houston, TX, U.S.A.) [35], whereas the UAS-APBB1-HA [APBB1 amyloid beta (A4) precursor protein-binding B1] is the official gene name of FE65; stock number 29882 and gmr-GAL4 (stock number 1104) lines were obtained from Bloomington Drosophila Stock Centre (Bloomington, IN, U.S.A.).

Plasmids

Mammalian expression constructs of native and Myc-tagged FE65 were as described previously [36,37]. Myc-tagged FE65ΔWW, FE65ΔPTB1 and FE65ΔPTB2 were generated by deleting amino acids 255–294, 374–532 and 518–710 of wild-type FE65 respectively. Full-length wild-type Htt (fHtt23Q) and mutant Htt (fHtt113Q) carrying 23 and 113 glutamines in the polyQ tract respectively were gifts from Professor Marcy E. MacDonald (Harvard Medical School, Boston, MA, U.S.A.). FLAG-tagged wild-type (23Q) and mutant (83Q) Htt1–550 were generated by cloning a PCR fragment of the full-length Htt construct between the sites BamHI and EcoRI of the pcMV-Tag2B vector (Stratagene). The primers used were 5′-ACCGGATCCATGGCAGGGTCAGATG-3′ and 5′-CAAGATATCTGAGGTCAGATG-3′. A stop codon was introduced at the end. Htt deletion mutants HttΔ1–550, HttΔ1–550–23Q, HttΔ1–550–23Q and HttΔ1–550–23Q were generated by PCR using HttΔ1–550–23Q as template. HttΔ1–171–23Q/83Q were generated by removing an XhoI fragment from the respective HttΔ1–550 constructs. All truncated Htt constructs carried an N-terminal FLAG tag. Renilla luciferase construct phRL-TK was purchased from Promega.

Antibodies

Myc-tagged FE65 was immunoprecipitated and detected by anti-Myc 9B11 antibody (Cell Signaling Technology) as described previously [27,37]. Mouse (M2, Sigma) and rabbit (Cell Signaling Technology) anti-FLAG antibodies were used to immunoprecipitate and/or detect the FLAG tag at the N-terminus of Htt constructs. Endogenous and native FE65 were immunoprecipitated and detected by an anti-FLAG antibody (Cell Signaling Technology) and an anti-FLAG antibody (E20; Santa Cruz Biotechnology). Anti-Htt antibody (MAB2166) was purchased from Millipore for endogenous Htt immunoprecipitation and detection. α-Tubulin and ubiquitin were detected by anti-α-tubulin antibody DM1A (Sigma) and anti-ubiquitin (P4D1) antibody (Cell Signaling Technology) respectively.

Co-immunoprecipitation

HEK-293 cells were transfected with either FLAG-tagged HttΔ1–550 or FLAG-tagged HttΔ1–550 and Myc-tagged FE65. Cells were harvested in ice-cold cell lysis buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100 and Complete™ protease inhibitor (Roche)] as described previously [27]. Myc-tagged FE65 or FLAG-tagged Htt was immunoprecipitated from cell lysates using 9B11 anti-Myc or M2 anti-FLAG antibody for 16 h at 4°C. Protein A–Agarose (Sigma) was then used to capture the antibody at 4°C for 2 h. The immunoprecipitates were then washed three times with ice-cold lysis buffer. Proteins in the immunoprecipitates were subjected to analysis by SDS/PAGE (10% gel) and Western blotting. Myc-tagged FE65 and FLAG-tagged HttΔ1–550 were detected by 9B11 anti-Myc and rabbit anti-FLAG antibodies respectively. Co-immunoprecipitations of HttΔ1–550–23Q and Myc-tagged FE65 deletion mutants, and FLAG-tagged HttΔ1–550 deletion mutants and Myc-tagged FE65 were performed similarly.

For endogenous co-immunoprecipitation, FE65 or Htt was immunoprecipitated from untransfected HEK-293 cell lysates by an anti-FE65 or anti-Htt antibody. The immunoprecipitates were analysed by Western blotting using anti-FE65 and anti-Htt antibodies.

Indirect immunofluorescence staining

Transfected cells were grown on glass coverslips for 2 days. Subsequently, cells were fixed with 4% (w/v) paraformaldehyde in PBS for 10 min, permeabilized in 0.1% Triton X-100 in PBS for 30 min for blocking and stained with primary antibody at room temperature (25°C) for 1 h. FLAG-tagged Htt was detected by M2 anti-FLAG antibody. FE65 was detected by a polyclonal antibody. After washing, cells were incubated with donkey anti-mouse Alexa Fluor 488 or donkey anti-goat Alexa Fluor 594 in the dark for 1 h. Nuclei were stained by DAPI (4′,6-diamidino-2-phenylindole; Sigma). Coverslips were mounted on to microscope slides using fluorescence mounting medium (Dako) and observed under an inverted fluorescence microscope (Olympus).

Cell-based ubiquitination assay

Briefly, cells were co-transfected with either non-targeting or FE65 siRNA, followed by HttΔ1–171–83Q. At 48 h post-transfection,
cells were harvested in ice-cold cell lysis buffer. FLAG-tagged Htt1–550–83Q was immunoprecipitated from cell lysates using M2 anti-FLAG antibody. The level of ubiquitination of Htt1–550–83Q was analysed by Western blotting using an anti-ubiquitin antibody.

**Cell viability assay**

Cell viability assay was performed as described previously [38,39]. Briefly, cells grown in 24-well plates were co-transfected with the indicated Htt and FE65 constructs with luciferase reporter phRL-TK. At 48 h post-transfection, cells were harvested in Glo Lysis Buffer (Promega) and luciferase activity was measured using a Luciferase Assay System (Promega) according to the manufacturer’s instructions. The viability of the transfected cells is reflected by the luciferase activity due to rapid degradation of luciferase upon the death of transfected cells.

**Pseudopupil assay**

A pseudopupil assay was performed as described previously [40]. The *Drosophila* adult eye is composed of approximately 800 ommatidia, and each ommatidium contains eight photoreceptor neurons. The pseudopupil assay is a method designed to measure the integrity of adult photoreceptor neurons, and has been widely used to monitor Htt-induced neurodegeneration in adult *Drosophila* [41]. *Drosophila* were raised at 26 °C. Three hundred ommatidia obtained from 15 adult flies (0–1 day post-eclosion) from three independent crosses were used to calculate the average number of rhabdomeres per ommatidium.

**Statistical analyses**

Statistical analyses were performed using unpaired Student’s *t* test. Significance is indicated between different treatments as *P* < 0.05, **P** < 0.005 and ***P*** < 0.001. Error bars show the S.D. in Figure 4 and the S.E.M. in Figure 5.

**RESULTS**

**FE65 interacts with Htt via its WW domain in a polyQ-dependent manner**

To test whether FE65 interacts with Htt, we performed co-immunoprecipitation by either transfecting FLAG-tagged Htt57–550–23Q alone or with Myc-tagged FE65 into HEK-293 cells. FE65 was immunoprecipitated by an anti-Myc antibody. Western blotting revealed that Htt57–550–23Q co-immunoprecipitated with FE65 in Htt57–550–23Q and FE65-transfected cells, but not in cells transfected with Htt57–550–23Q alone (Figure 1A, left-hand panel). To further confirm the interaction, we performed reverse co-immunoprecipitation of Htt57–550–23Q using anti-FLAG antibody. Again, FE65 was detected in the immunoprecipitate (Figure 1A, right-hand panel). These indicate that FE65 is an interacting partner of Htt.

To demonstrate an *in vivo* interaction between FE65 and Htt, we performed endogenous co-immunoprecipitation in untransfected HEK-293 cells by anti-Fe65 antibody. Endogenous Htt co-immunoprecipitated with endogenous FE65 (Figure 1B, upper panels). The endogenous interaction of FE65 and Htt is further validated by reverse co-immunoprecipitation of Htt (Figure 1B, lower panels). These indicate that FE65–Htt interaction occurs physiologically.

Next, we asked which domain of FE65, the WW domain, PTB1 domain or PTB2 domain, is responsible for the interaction. Co-immunoprecipitation was performed using Myc-tagged FE65 deletion mutants. Cells were co-transfected with Htt57–550–23Q and full-length FE65, FE65ΔWW, FE65ΔPTB1 or FE65ΔPTB2. FE65 and the deletion mutants were immunoprecipitated by anti-Myc antibody. As shown in Figure 1(C) (upper panels), Htt57–550–23Q co-immunoprecipitated in cells transfected with full-length FE65, FE65ΔPTB1 and FE65ΔPTB2. However, the interaction was markedly reduced in the immunoprecipitation with FE65ΔWW. Similarly, less FE65ΔWW mutant was pulled down from the immunoprecipitation of Htt57–550–23Q as compared with the other deletion mutants (Figure 1C, lower panels). These provide evidence that the WW domain of FE65 is required for FE65–Htt interaction.

To determine the region of Htt required for FE65–Htt interaction, we generated several deletion mutants of Htt. The PRR of Htt is composed of two stretches of polyP (polyproline) sequence (amino acids 41–51 and 69–78, numbered using a normal polyQ length of 23). We therefore cloned three deletion mutants with the first polyP sequence deleted (Htt57–550–23Q), the whole PRR deleted (Htt57–550–23Q) or only the second polyP sequence of the PRR remaining (Htt57–550–23Q) (Figure 1D, lower panel). Co-immunoprecipitation was performed by co-transfecting FE65 and Htt57–550–23Q or various Htt deletion mutants to HEK-293 cells. Upon immunoprecipitation of FE65 by anti-Myc antibody, all except Htt57–550–23Q were pulled down (Figure 1D, upper panel). The result indicates that while the first 57 amino acids carrying the polyQ sequence and the first polyP stretch are not necessary for FE65–Htt interaction, the absence of the whole PRR would abolish the interaction. However, the interaction with FE65 is restored when residues 68–80, a 13-amino-acid long region encompassing the second polyP stretch, is present (Figure 1D, bottom panel). We confirmed this observation by immunoprecipitating Htt from the cell lysates, and FE65 did not interact with Htt57–550–23Q (Figure 1D, upper panel). The current findings suggest that the WW domain of FE65 binds to the second polyP stretch of Htt.

It has been reported that Htt with different polyQ lengths displays differential binding affinity to some of its interacting partners [2,42]. We therefore sought to determine whether FE65–Htt interaction is polyQ-dependent. To do this, we immunoprecipitated FE65 from Htt57–550–23/83Q and FE65 co-transfected cells and the immunocomplexes were blotted for Htt. Interestingly, FE65 was found to display higher affinity with Htt57–550 with expanded polyQ (Figure 1E, upper panels). Similar result was observed in the immunoprecipitations of Htt (Figure 1E, lower panels).

**Loss of FE65 destabilizes mutant Htt and promotes its ubiquitination**

HD is characterized by an abnormal accumulation of mutant Htt fragments, which perturbs normal cellular functions and leads to cell death [3]. Enhancing mutant Htt removal could ultimately ameliorate HD pathology. To this end, a number of Htt-interacting proteins including IKK (IkB kinase), co-chaperone CHIP, BAG1 and gp78 were identified as regulators of Htt clearance [10,12–14]. As we have shown in the present study that FE65 binds to Htt, we enquired whether FE65 modulates Htt protein level. To do this, we knocked down FE65 in Htt57–550–23/83Q-transfected cells and the protein lysates were analysed by Western blotting 48 h post-transfection. Immunoblotting revealed that FE65 knockdown has no obvious effect on the wild-type Htt protein level. In contrast, substantial reduction of the mutant Htt protein level was observed in FE65-depleted cells (Figure 2A), suggesting that FE65 acts...
Figure 1  FE65 interacts with Htt

(A) Co-immunoprecipitations were performed from HEK-293 cells transfected with Htt1–550-23Q or Htt1–550-23Q and FE65 (left-hand panel), and FE65 or FE65 and Htt1–550-23Q (right-hand panel). FE65 was immunoprecipitated by the 9B11 antibody against the Myc tag, whereas Htt was immunoprecipitated by the M2 antibody against the FLAG tag. − and + refer to the absence or presence of anti-Myc or anti-FLAG in the immunoprecipitations. Immunoprecipitated FE65 was detected by an anti-Myc antibody and Htt was detected by an anti-FLAG antibody. (B) Endogenous FE65–Htt complex was detected by immunoprecipitating either FE65 or Htt by an anti-Myc or anti-FLAG antibody from untransfected HEK-293 cells and probing for Htt or FE65. − and + refer to the absence or presence of anti-FE65 or anti-Htt antibody in the immunoprecipitations. (C and D) The FE65 WW domain interacts with the second polyP stretch of Htt1–550-23Q. Co-immunoprecipitations were performed from HEK-293 cells either transfected with Htt1–550-23Q and FE65, FE65 ΔWW, FE65 ΔPTB1 or FE65 ΔPTB2 (C), and FE65 and Htt1–550-23Q, Htt57–550–23Q, Htt81–550–23Q or Htt1–550 Δ41-67aa–23Q (D) using anti-Myc and anti-FLAG antibodies. FE65 and its deletion mutants were detected by an anti-Myc antibody, whereas Htt and its deletion mutants were detected by an anti-FLAG antibody. The bottom panel of (D) shows the schematic diagram of the Htt deletion mutant constructs. Amino acid numbering using a normal polyQ length of 23. (E) FE65 binds more strongly to Htt with expanded polyQ. Co-immunoprecipitations were performed from HEK-293 cells transfected with Htt1–550-23Q/83Q and FE65. FE65 and Htt were immunoprecipitated by anti-Myc and anti-FLAG antibodies respectively. Immunoprecipitates were blotted for FE65–Myc and FLAG–Htt. Molecular mass is shown on the left-hand side in kDa. IP, immunoprecipitation; UT, untransfected.

© The Authors Journal compilation © 2012 Biochemical Society
Degradation of mutant huntingtin is reduced by FE65

Figure 2  Loss of FE65 facilitates mutant Htt degradation via the UPS

(A) FE65 knockdown reduces Htt1–171-83Q protein level. HEK-293 cells were co-transfected with Htt1–171-23Q/83Q and control or FE65 siRNA. Htt protein levels in transfected cell lysate were analysed by Western blotting using an anti-FLAG antibody against the N-terminal FLAG tag on Htt. Htt1–171-83Q protein level decreases, whereas Htt1–171-23Q is not affected in FE65-knockdown cells. (B) The UPS is involved. HEK-293 cells co-transfected with Htt1–171-83Q and control or FE65 siRNA were incubated with 2.5 μM MG132 or DMSO vehicle for 16 h. Htt destabilization promoted by FE65 knockdown was diminished when the proteasome was inhibited. (C) Loss of FE65 promotes Htt1–171-83Q ubiquitination. Transiently expressed Htt1–171-83Q was immunoprecipitated from control or FE65-knockdown cells by an anti-FLAG antibody. Immunoprecipitates were resolved by SDS/PAGE (6% gel) and subjected to Western blotting. Ubiquitinated Htt was shown by an anti-ubiquitin antibody. Input lysates were immunoblotted for FLAG–Htt1–171-83Q, total polyubiquitinated proteins, FE65 and α-tubulin. Similar result was observed in CHO cells (results not shown). Molecular mass is shown on the left-hand side in kDa. IP, immunoprecipitation; (Ub)n, polyubiquitinated proteins; (Ub)n-Htt, polyubiquitinated Htt.

selectively on pathogenic Htt. A previous report implicated that loss of FE65 causes destabilization of p53 via the UPS [43]. We wondered whether the destabilization of mutant Htt in FE65-knockdown cells was also mediated by the UPS. To test this hypothesis, the proteasome inhibitor MG132 was added to cells co-transfected with Htt1–171-83Q and control or FE65 siRNA. The Htt level was compared after 16 h of MG132 treatment. We found that the reduction in mutant Htt level in FE65-depleted cells was attenuated upon proteasome inhibition (Figure 2B). This observation supports our notion that the UPS is involved in the destabilization of Htt in FE65-depleted cells. We further investigated the effect of FE65 knockdown on the ubiquitination of Htt using a cell-based ubiquitination assay. Briefly, mutant Htt was immunoprecipitated from control and FE65 knockdown cells. The input lysates and immunoprecipitates were immunoblotted for both Htt and ubiquitin. As shown in Figure 2(C), loss of FE65 increased the ubiquitination level of mutant Htt, whereas the level of total polyubiquitinated proteins, as indicated by the lysates, was not altered. This is in line with our finding that loss of FE65 destabilizes mutant Htt specifically via the UPS.

FE65 associates with Htt aggregates

A number of proteins involved in mutant Htt degradation and/or ubiquitination have been implicated to co-localize with mutant Htt aggregates [12,13,44]. We wondered whether FE65 also localized to Htt aggregates. To check this, cells were co-transfected with Htt1–171-83Q and FE65 and processed for immunofluorescence staining 48 h post-transfection using anti-FLAG and anti-FE65 antibodies respectively. We also transfected cells with either Htt1–171-83Q or FE65 alone in parallel to study their distribution pattern. The results show that mutant Htt, when transfected alone, formed prominent aggregates around the nucleus (Figures 3A–3C). On the other hand, singly-transfected FE65 was found to localize in both nuclei and cytoplasm as reported previously (Fig-
FE65 enhances the toxicity of mutant Htt in cells and in a Drosophila model of HD

Because loss of FE65 destabilizes mutant Htt and promotes its ubiquitination (Figure 2), we sought to determine the effect of FE65 on the toxicity of mutant Htt. We employed a viability assay that requires co-transfection of luciferase together with Htt and FE65 constructs. The principle of the assay is that luciferase is only expressed in transfected cells, and when the transfected cells die, luciferase would be rapidly degraded [38,39]. Thus the luciferase reading could serve as an indicator of the viability of the transfected cells. Full-length wild-type (flHtt23Q) and mutant (flHtt113Q) Htt constructs were used in this assay. It has been reported previously that overexpression of FE65 induces apoptosis [45], which is in line with our observation that FE65 reduces cell viability (Figure 4A). On the other hand, as expected, mutant Htt exerts prominent deleterious effect on cell viability, whereas wild-type Htt does not alter cell viability significantly. Importantly, the viability of mutant Htt-transfected cells further decreased when FE65 was co-transfected (Figure 4A).

However, the data in Figure 4(A) could not exclude the possibility that the combined effect of FE65 and mutant Htt is additive. Therefore, we employed the FE65ΔWW mutant, which cannot interact with Htt, in the cell viability assay. As shown in Figure 4(B), FE65 and FE65ΔWW had similar effects on cell viability. This suggests that, in the absence of mutant Htt, the effect of FE65 on cell viability is not associated with its WW domain. Importantly, the magnitude of decrease in cell viability in FE65 and mutant Htt co-transfected cells was significantly higher than that in FE65ΔWW and mutant Htt. These data indicate that FE65 exerts a synergistic effect on the toxicity of mutant Htt, and FE65–Htt interaction is essential to such an effect.

We further analyzed the effect of FE65 on Htt toxicity in a Drosophila model of HD. The extent of neurodegeneration was evaluated by counting the number of rhabdomeres in ommatidia of adult eyes of full-length mutant Htt flies (flHtt128Q) and flHtt128Q and FE65 double transgenic flies. Consistent with the cell model result, flHtt128Q and FE65 double transgenic flies displayed lower number of rhabdomeres per ommatidium than flHtt128Q single transgenic flies (Figure 5), which indicates that FE65 enhances the toxicity of mutant Htt in vivo.

DISCUSSION

In the present study, we have demonstrated that FE65 interacts with the N-terminus of Htt by several cell-based assays. We also confirmed that FE65 interacts with full-length Htt by detection of endogenous FE65–Htt complex in untransfected cell lysate (Figure 1). Further characterization revealed that FE65–Htt interaction is mediated by the WW domain of FE65 and the PRR of Htt. This is consistent with the current understanding that the WW domain typically binds to proline-rich sequence [2,42]. In fact, it has been reported that Htt interacts with a family of WW domain proteins including HYP (Htt-interacting protein) A/FBP-11 (formin-binding protein 11), HYPB and HYPC via the PRR [33]. On the other hand, a number of FE65 WW domain-binding partners such as Mena and c-Abl tyrosine kinase carry proline-rich sequences [16]. Furthermore, we successfully identified that residues 68–80 on Htt, a 13-amino-acid-long region (QPPPPPPPPPPGP) encompassing the second polyP stretch, are crucial to binding with FE65. This is in line with the structural analysis of the FE65 WW–Mena complex performed by Meiyappan et al. [46], which provided evidence that the general recognition motif of FE65 WW domain is the sequence PPXPP.

Increasing evidence shows that the accumulation of N-terminal mutant Htt proteolytic fragments is crucial to HD pathogenesis [4–8]. It is speculated that the expanded polyQ tract renders Htt protein less prone to degradation, and more susceptible to proteolytic processing, leading to the accumulation of N-terminal toxic fragments [3]. Although targeting the Htt proteolytic pathway could be one of the approaches to alleviate HD pathology, improving mutant Htt clearance emerges as a feasible therapeutic strategy. Thus understanding the molecular mechanism of Htt degradation is of great importance. We have shown in the present study that loss of FE65 causes a reduction in the mutant Htt protein level, whereas the protein level of wild-type Htt is not...
affected (Figure 2). As shown earlier, FE65 binds more tightly to Htt with expanded polyQ (Figure 1E). The different binding affinities of wild-type and mutant Htt to FE65 might explain why loss of FE65 affects primarily the stability of mutant Htt.

There are two main routes for the clearance of Htt: the UPS and macroautophagy (generally referred to as autophagy) [3,9,10]. Of note, UPS inhibition has been demonstrated to be more critical to the accumulation of N-terminal mutant Htt than autophagy inhibition in knock-in mouse models of HD [47]. The finding underscored the importance of the UPS in mutant Htt clearance, and gave insight into developing an effective treatment. We showed in the present study that the reversion of mutant Htt accumulation in FE65-depleted cells is UPS-dependent, as evident from MG132 treatment (Figure 2B). In fact, the ubiquitination level of mutant Htt was found to be enhanced in FE65-knockdown cells (Figure 2C). Our findings implicate a critical role of FE65 in the stabilization of proteins via the UPS. Still, there remains a big unanswered question: how does FE65 mediate the stabilization and ubiquitination of mutant Htt? As FE65 functions by preventing positive regulators of Htt degradation, thereby reducing neurotoxicity of mutant Htt. Additionally, the brain-enriched tissue distribution of FE65 may explain, at least in part, why HD primarily affects brain area despite the ubiquitous expression of Htt throughout the body [32,51]. It is therefore worthwhile to examine FE65 levels in HD compared with normal individual brains, and the role of FE65 in HD pathogenesis should not be neglected.

Previously, Nakaya et al. [43] showed that loss of FE65 results in destabilization of p53 in osmotically stressed cells via the UPS. The findings of the present study further confirms the role of FE65 in the stabilization of proteins via the UPS. Still, there remains a big unanswered question: how does FE65 mediate the stabilization and ubiquitination of mutant Htt? As FE65 has been reported to compete with X11 and Dab1 (disabled homologue 1) for binding with APP, it is possible that FE65 functions by preventing positive regulators of Htt degradation such as E3 ubiquitin ligase gp78, co-chaperone CHIP and BAG1 from accessing Htt [36,52]. Another well-studied regulatory mechanism of Htt clearance via the UPS is phosphorylation at Ser13 and Ser16 [10]. In this regard, FE65 is known to interact with a number of kinases including c-Abl tyrosine kinase and Nek6 [20,37]. It is therefore possible that FE65, through its interaction with kinases, modulates phosphorylation of Htt. Although the
detailed mechanism remains to be identified, the present study reinforces the role of FE65 in stabilization of proteins via the UPS. In summary, our results show that FE65 interacts with Htt and facilitates the accumulation of mutant Htt by preventing its clearance via the UPS, which ultimately leads to enhanced toxicity in cell and Drosophila models of HD.

AUTHOR CONTRIBUTION
Wan Ning Vanessa Chow, Ho Yin Edwin Chan and Kwok-Fai Lau conceived the study and designed the experiments. Wan Ning Vanessa Chow and Hon Wing Luk performed the experiments. Wan Ning Vanessa Chow, Ho Yin Edwin Chan and Kwok-Fai Lau analysed the data and wrote the paper.

ACKNOWLEDGEMENTS
We thank Professor Juan Botas (Baylor College of Medicine, Houston, TX, U.S.A.) for the full-length UAS-Ih/t1228 Drosophila line and Professor Marcy E. MacDonald (Harvard Medical School, Boston, MA, U.S.A.) for full-length wild-type Htt (iHtt13Q) and mutant Htt (iHtt13QD) constructs.

FUNDING
This work was supported by the Research Grant Council Hong Kong, [grant number CUHK grant number CA11156] and the Chinese University of Hong Kong direct grant scheme [grant number 2030404] and the United College endowment fund research grant [grant number CA11156].

REFERENCES
Degradation of mutant huntingtin is reduced by FE65


Received 15 December 2011/20 February 2012; accepted 22 February 2012
Published as BJ Immediate Publication 22 February 2012, doi:10.1042/BJ20112175

© The Authors Journal compilation © 2012 Biochemical Society