UNIVERSITY OF CALIFORNIA, SAN DIEGO

Mutant Huntingtin Exon-1 Proteins Form trans-Dimers/Oligomers as Detected by Bimolecular Fluorescence Complementation

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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The Thesis of Eugene Kiwo Han is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

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Chapters 1 through 5 will be submitted for publication to the Journal of Biological Chemistry. Along with Chapters 1-5, additional figures that will contribute to the complete manuscript are currently being prepared for submission.

The thesis author was the co-primary investigator and author of this paper. Co-authors of the manuscript include Professor Chengbiao Wu, as the first co-author, Xiaobei Zhao, and Dr. William Mobley.

ABSTRACT OF THE THESIS

Mutant Huntingtin Exon-1 Proteins Form trans-Dimers/Oligomers as Detected by Bimolecular Fluorescence Complementation

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Eugene Kiwo Han

Master of Science in Biology

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Professor Chengbiao Wu, Chair

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Toxic oligomers of mutant Huntingtin (mHtt) proteins have been implicated in the pathogenesis of Huntington's disease (HD). To examine how oligomerization of mHtt is initiated within cells, we have developed a novel set of bimolecular fluorescence complementation (BiFC) probes to evaluate dimerization/oligomerization of Huntingtin protein by live cell imaging. Expression of either the wild-type Huntingtin exon 1 GFP

(wtHttPolyQ25-GFP) or the mutant Huntingtin exon 1 GFP (mHttPolyQ97-GFP) was revealed by the GFP signal. By splitting mCherry, we were able to further differentiate between the diffuse signals associated with monomeric Htt proteins and aggregated Htt proteins that may implicate the early developments of inclusion bodies or insoluble oligomers associated with HD pathogenesis. As expected, we detected dimerization between mHttPoly97 proteins. To our surprise, we also found that wtHttPolyQ25 formed dimers. Moreover, we discovered that dimerization of wtHttPolyQ25 or mHttPoly97 proteins occurred in a *trans*- but not in a *cis*-manner. Our findings suggest that mHtt could potentially form hetero-dimers and sequester wtHtt, thereby disrupting its normal function.

INTRODUCTION

Polyglutamine diseases are neurodegenerative disorders associated with the formation of an abnormally long polyglutamine (polyQ) tract (Margolis and Ross, 2001) in disease proteins. A major polyQ disease, Huntington's disease (HD) is caused by the excessive number of CAG codon repeats in exon 1 of the HD gene, which leads to the expansion of the polyQ tract in Huntingtin (Htt), the protein product encoded by the *htt* gene (The Huntington's Disease Collaborative Research Group, 1993). Although the wild-type *htt* gene contains 7-36 glutamine repeats in the polyQ tract region located near the N-terminus of the Htt protein, mutant Huntingtin proteins (mHtt) generated in HD have polyQ tracts exhibiting 37 or more glutamines (The Huntington's Disease Collaborative Research Group, 1993). The aggregation of mHtt containing excessive polyglutamine repeats is a major characterization of HD and is suspected to play a significant role in HD pathogenesis (Gray et al., 2008). Furthermore, it has been shown that the expanded CAG repeats in exon 1 of the *htt* gene in transgenic mice are sufficient to cause neurodegenerative symptoms highly akin to those of HD (Mangiarini et al., 1996).

Although the aggregation of mHtt corresponding to a longer polyQ tract is a general hallmark of HD, it has been reported that the main contributor to mHtt aggregation may not be the polyQ site per se, but the N-terminal 17 amino acids (N17) located directly upstream of the polyQ sequence (Tam et al., 2009). However, in vivo studies examining HD pathogenesis utilizing mutant Huntingtin-exon 1 to model HD in transgenic mice and mammalian cell lines have supported the notion that the extensive

polyglutamine tract is the primary culprit for Htt aggregation (Mangiarini et al., 1996). While the nuances between the prominence of the N17 amino acids and the polyQ tract in respect to Htt aggregation remain unresolved, it is evident that Huntingtin-exon 1 is a region of interest when examining Htt interaction.

It has been noted that mHtt exists as monomers, soluble oligomers, and insoluble inclusion bodies (IBs) (Takashi et al., 2008). Although many studies concur that IBs formed by mHtt aggregation have a prominent role in the pathology of HD, there is still some debate as to whether or not the formation of inclusion bodies are detrimental. Some studies have reported that IBs are highly toxic, advocating that the insoluble aggregation of Htt result in neuronal damage and cell death (Becher et al., 1998; Davies et al., 1997; DiFiglia et al., 1997; Ordway et al., 1997). However, more recent studies have shown that the formation of IBs compensatory mechanism of curtailing the spread of the soluble Htt monomers and oligomers, which may be more toxic than its insoluble counterpart (Arrasate et al., 2004; Bence et al., 2001; Takashi et al., 2008; Taylor et al., 2003).

The uncertainty of the role of IBs in relation to toxicity necessitates a more extensive study on the formation of Htt aggregation. Previous research concerning this issue has established two novel methods of examining Htt aggregation: 1) Detecting oligomerization between Htt monomers via bimolecular fluorescence complementation (BiFC) using a split-Green Fluorescent Protein (GFP) system (Lajoie and Snapp, 2010). 2) Utilizing the specific interaction between a tetra-cysteine (TC) tag and cell permeable biarsenical dye, ReAsH to distinguish monomeric Htt protein from oligomers in live cells (Ramdzan et al., 2010).

The split-GFP BiFC assay incorporates the fusion of wtHtt or mHtt to one half of GFP, either 157-GFP (amino acids 1-157 of GFP) or 238-GFP (amino acids 158-238 of GFP) in order to obtain a detectable signal in transfected cells (Lajoie and Snapp, 2010). Thus, if a pair of Htt containing each of the split-GFP fragments, 157-GFP and 238-GFP were to directly interact or were within close proximity, the two halves of the split-GFP would irreversibly reconstitute into a single, complete GFP that becomes detectable by fluorescence microscopy. The set of experiments conducted using the split-GFP BiFC method not only revealed that mutant Huntingtin-exon 1 formed IBs; but also demonstrated that monomeric wild-type Huntingtin-exon 1 can undergo dimerization, and more importantly are capable of being sequestered into large IBs formed by mutant Huntingtin-exon 1 aggregation (Lajoie and Snapp, 2010).

However, a shortcoming of the split-GFP BiFC assay lies in its inability to visually distinguish between the monomeric Htt and insoluble IBs since visualization of IBs is facilitated through the forced increases in soluble oligomeric mHtt exon 1 which can only be detected by the irreversible binding of the split-GFP fragments (Lajoie and Snapp, 2010). Furthermore, sequestration of wtHtt by mHtt derived IBs was merely suggested, but not proved, through the co-localization of the green and red fluorescence in N2a cells transiently co-transfected with wild-type Huntingtin exon 1 (Q23) conjugated to full length GFP, green signal and mutant Huntingtin exon 1 (Q145) conjugated to full length mCherry, red signal (Lajoie and Snapp, 2010).

Contrary to the split-GFP BiFC assay, the method involving a tetra-cysteine (TC) motif inserted into several regions flanking the polyQ sequence of Htt differentiates

monomeric proteins from oligomers based on the ability of cell permeable biarsenical dye, ReAsH to bind the TC motif and consequently increase fluorescence 50,000 fold (Ramdzan et al., 2010). Monomeric Htt containing a TC tag that is fused to fluorescent protein, Cerulean would be accessible to ReAsH for fluorescent detection. Thus, exhibiting both cyan and red fluorescent signals from the Cerulean fluorescent marker and the ReASH biarsenical dye, respectively. However, higher level oligomers of the Cerulean fused Htt proteins would bury the TC motif, preventing ReAsH-TC interactions and resulting in no detectable red signal (Ramdzan et al., 2010). Although the absence of the ReAsH signal due to a buried TC tag in Htt oligomers implicate the formation of IBs and higher level oligomers, the presumption made from these results are visually indirect.

The previous studies exploring the role of mHtt aggregation have provided valuable insight into the pathology of HD. However, a method of visually probing for insoluble IBs while clearly marking both monomeric and oligmeric Htt has yet to be established. Thus, we have developed a novel BiFC assay with the aim of visually differentiating between monomeric and oligomeric Htt *in vivo* and the capacity to visually detect Htt oligomers via the presence of a positive signal upon direct interaction, qualities absent from the split-GFP BiFC and ReASH-TC motif methods respectively. This novel GFP split-mCherry BiFC assay may allow one to investigate the dynamics of mHtt aggregation within neurons and have the potential to contribute to the effort to clarify the exact role of IBs in HD pathology.

The thesis author was the co-primary investigator and author of the Introduction. Co-authors of the manuscript include Professor Chengbiao Wu, as the first co-author, Xiaobei Zhao, and Dr. William Mobley.

CHAPTER 1

Design and validation of GFP-mCherry Probes

The bimolecular fluorescence complementation assay (BiFC) is a powerful tool to detect protein-protein interaction by live cell imaging. The split-GFP system of BiFC has been used to detect oligomerization of Htt exon 1 previously (Lajoie and Snapp, 2010). However, the method does not allow tracking of the dynamics between diffused Htt signals and Htt aggregates. Our new approach will utilize a unique GFP split-mCherry BiFC system (Fan et al., 2008), in which GFP is used to monitor the expression of the GFP-tagged Htt construct(s), while the appearance of mCherry reveals dimerization/oligomerization of these expressed proteins as depicted in Figure 1A. This particular version of the BiFC assay targets both the wild-type Huntingtin exon1 protein (wtHtt25) and mutant Huntingtin exon1 protein (mHtt97), which contain 25 and 97 glutamines in the polyQ tract of Htt exon 1 respectively.

We obtained the GFP-tagged the wild-type Huntingtin exon1 and mutant Huntingtin exon 1 expression vectors (wtHttQ25 ex.1-GFP and mHttQ97 ex.1-GFP) from Dr. J. Frydman of Stanford University. As BiFC probes require the reconstitution of the split fluorescent marker for fluorescent emission, a pair of specific constructs was designed for wtHttQ25 ex.1-GFP as well as mHttQ97 ex.1-GFP. The red fluorescent protein, mCherry was split into two fragments: MCS159 for the N-terminal 1-159 amino acids and MCS160 for the C-terminal 160-239 amino acid segment (Figure 1A). For the initial experiments, each of the two MCS fragments was cloned into the C-terminus of GFP to generate the pairing probes: Fragment 1 and 2 for the wtHttQ25 ex.1-GFP. The

construct pair wtHttQ25 ex.1-GFP-MCS159 and wtHttQ25 ex.1-GFP-MCS160 were designated as Htt25 ex.1 F.1 and F2, respectively. Similarly, Fragment 1 and 2 for the mHttPolyQ97 ex.1 constructs: mHttPolyQ97 ex.1-GFP-MCS159: mHttPolyQ97 ex.1-GFP-MCS160 were designated as Htt97 ex.1 F1 and F2 herein (Figure 1A).

The expression of these new probes were examined by transfecting these constructs either alone (F1, F2) or in pair (F1+F2) into HEK293FT cells using Lipofectamine2000. The backbone vectors, Htt25 ex.1-GFP and Htt97 ex.1-GFP were also expressed in HEK293FT cells as comparison. In addition, expression of GFP alone or mCherry alone was also examined as controls. 48-72 hrs post transfection, cell lysates of different transfections as well as cell lysates from non-transfected cells were harvested and separated on 4-12% SDS-PAGE. The blot was probed with an antibody to GFP. As shown in Figure 1B, no band was recognized in untransfected cell lysate, GFP migrated as a 27kD band.

As expected, the GFP specific antibody failed to recognize mCherry. Re-probing the blot with an antibody specific to mCherry did reveal the presence of mCherry protein with a molecular mass of 27 kD (data not shown). Htt25 ex.1-GFP was predicted to be ~37kD, but migrated as ~46 kD band (Figure 1B). Htt25 ex.1 F.1 migrated at ~55 kD; Htt25 ex.1 F2: ~46 kD, co-transfection of Htt25 ex.1F1 and F2 yielded two bands corresponding to 55 and 46 kD, respectively (Figure 1B). The molecular weight of Htt97 ex.1-GFP was predicted to be 44 kD, but migrated at ~52 kD instead. Htt97 ex.F1 migrated as a 62 kD band and F2 as ~57 kD (Figure 1B). Co-transfection of Htt97 ex.1 F1 and F2 gave rise to two bands that corresponded to 62 and 57 kD (Figure 1B). Based

on these results, we concluded that the new BiFC probes, Htt25 ex.1 F1 and F2, Htt97 ex.1 F1 and F2, were sufficiently expressed in HEK293FT cells.

The thesis author was the co-primary investigator and author of Chapter 1. Co-authors of the manuscript include Professor Chengbiao Wu, as the first co-author, Xiaobei Zhao, and Dr. William Mobley.

Figure 1

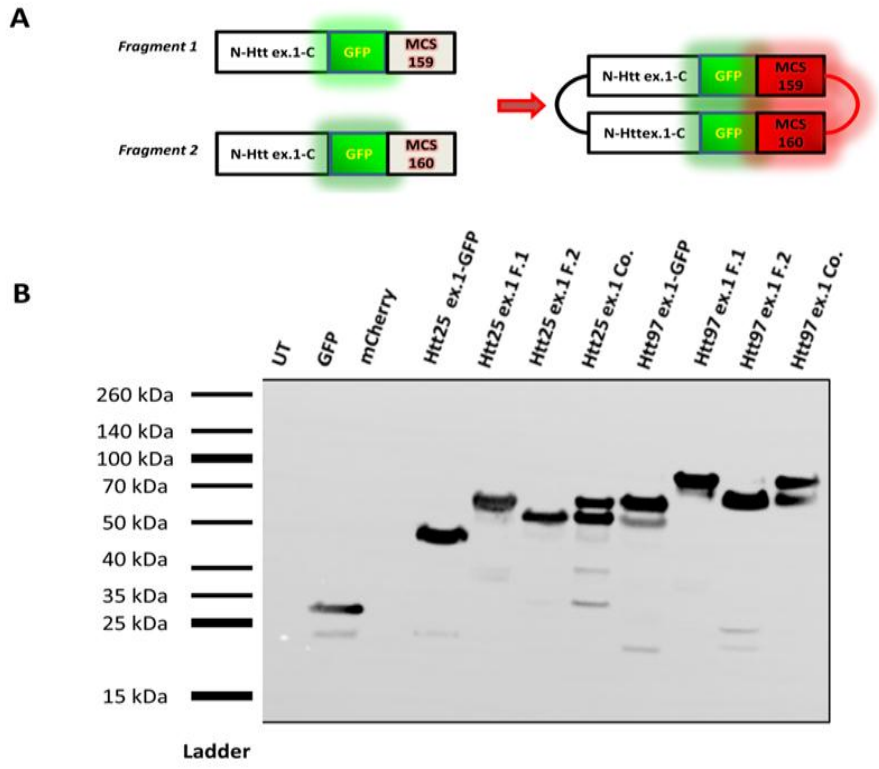

FIGURE 1: A, *Cis*-configuration schematic of original complementing fragments for the GFP-Split mCherry BiFC assay. B, Western blot of F.1 and F.2 of Htt 25 ex.1 and Htt 97 ex.1 with mouse anti-GFP Ab.

CHAPTER 2

Huntingtin exon 1 protein does not form cis-dimers

We next examined if the split-mCherry BiFC probes gave rise to fluorescent signals by live cell imaging as designed. Each of the probe pairs for Htt25 ex.1 (F1, F2) and Htt97ex.1 (F1, F2) were transfected into PC12 cells either alone or in pair. Transfection with GFP or mCherry was also carried out as controls. 48-72 hrs post transfection, cells were examined using a Leica DMI6000 inverted microscope equipped with a 100x objective. We first used GFP and mCherry-transfected cells to establish the parameters (exposure time, fluorescent intensity etc) at which there was no bleach through. The FITC and Texas Red channels set under a 300 ms exposure restriction were used to detect green and red fluorescence respectively. However, DIC images were taken using a 100 ms exposure restriction in order to identify viable samples. Figure 2A showed that a cell expressing GFP was only visible in the FITC channel, but not visible in the Tex Red channel, similarly a cell expressing mCherry was yielded only red signal (Tex Red) but no green signal (FITC). DIC images for these two cells were also captured and shown (Figure 2A).

As expected, expression of either Htt25 ex.1 probes (F1 or F2) showed diffused GFP signals throughout the cytoplasm (Figure 2B). Co-expression of Htt25 ex.1 F1 and F2 gave rise to the same results (Figure 2B). In all cases, we did not detect any credible signal(s) in the Tex Red channel. However, we did occasionally see green aggregates in the nucleus when Htt25 ex.1 F1 and F2 were co-expressed (Figure 2B). These green aggregates were not visible in the Tex Red channel.

Expression of Htt97 ex.1 F1 or F2, for most part, yielded diffused GFP signals throughout the cytoplasm (Figure 2C). Frequently, similar to its parental vector, mHttPolyQ97 ex.1-GFP (a.k.a Htt97 ex.1), had a strong tendency to form large aggregates when overexpressed, specifically cytoplasmic aggregates (Top panel in Figure 2C). Again, the green aggregate was not seen in the Tex Red channel. Co-transfection of Htt97 ex.1 F1 and F2 showed signals within the cytoplasm with either diffused or aggregate pattern. However, the aggregates did not result in complementation of split-mCherry and failed to give rise red signal in the Tex Red channel (Figure 2C). We speculated that Htt ex.1 may dimerize/oligomerize under a *trans*-configuration, but not a *cis*-formation as we originally designed.

The thesis author was the co-primary investigator and author of Chapter 2. Co-authors of the manuscript include Professor Chengbiao Wu, as the first co-author, Xiaobei Zhao, and Dr. William Mobley.

Figure 2

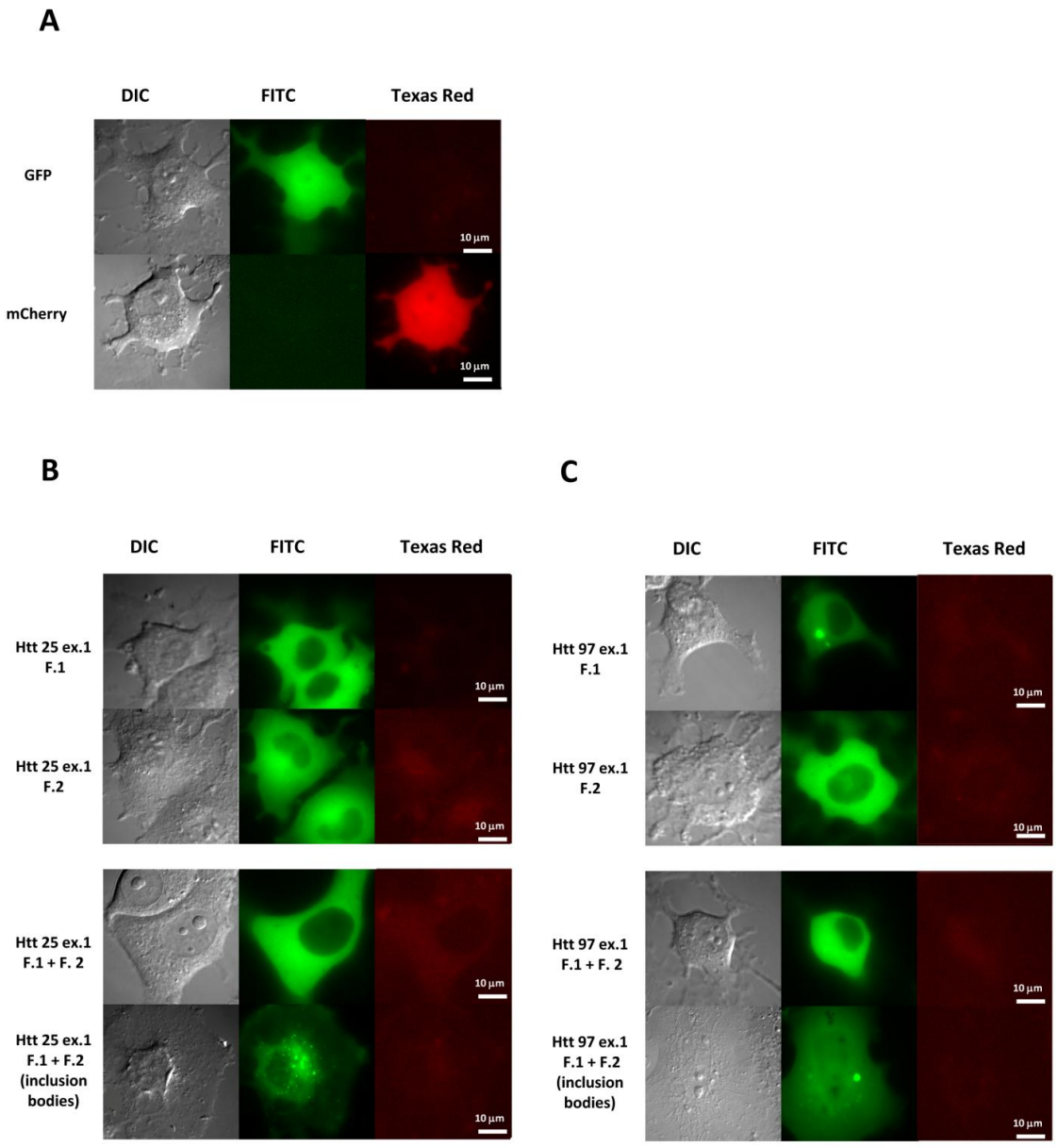

FIGURE 2: A, Images of PC12 cells transiently transfected with GFP (FITC) and mCherry (Texas Red) controls. B,C images of PC12 cells singly transfected with F.1 or F.2 of the Htt25 ex.1 and Htt97 ex.1 samples as well as the co-transfection with F.1 and F.2 for both the Htt 25 ex.1 and Htt97 ex.1 samples.

CHAPTER 3

Huntingtin exon 1 proteins form trans-dimers

To investigate if mutant Htt exon 1protein products formed trans-dimers prior to oligomerization, Fragment 2 for Htt97 ex.1 sample was re-designed by moving the MCS160-239 fragment from the C-terminus to the N-terminus of Htt 97 ex.1. The new fragment, thus denoted as Fragment 2N (Htt97 ex.1 F.2N), was constructed using a two-step PCR protocol and was confirmed by sequencing. A start codon (ATG) was introduced to the beginning of the MCS 160. Htt25 ex.1 F2N was constructed in a similar fashion (Fig. 3A).

To confirm the identity and expression levels of these new F.2N fragments by Western blotting with an antibody specific to GFP, transient expression experiments were performed similar to those described in Figure 1B. Expression of Htt25 ex.1 F.1 and Htt97 ex.1 F1 were observed to be consistent with those in Figure 1B. Expression of Htt25 ex.1 F.2N yielded three bands: 40, 46, and 55 kD (Figure 3B). We determined that the 55 kD was the full length of Htt25 ex.1 F.2N and the two lower bands, each marked by an arrow to be unspecific bands, possibly resulting from cellular processing of the transfected constructs or the transcription of the DNA construct at an alternative start site. Co-transfection of Htt25 ex.1 F1 with Htt25 ex.1 F1 confirmed the presence of an additional band of ~ 60 kD that corresponded to Htt25 ex.1 F1 (Figure 3B).

The predominant band for Htt97 ex.1 F2N was ~ 70 kD as expected (Figure 3B). Co-transfection of F.1 and F.2N for Htt97 ex.1 (Lane Htt97 ex.1 Co in Fig. 3B) seems to

be less equivalent. Interestingly, when comparing the expression levels of both constructs in the co-transfected samples between Figure 1B and Figure 3B, it appears that only Htt97 ex.1 F.2N has a lower expression level when co-transfected with Htt97 ex.1 F.1, compared to being singly transfected. It is unclear as to what exactly is the reason for such results, but it is possible that the results seen were a consequence of the transient transfection per se.

With the identities of the new F.2N constructs confirmed, images of PC12 cells singly transfected with each of the F.2N constructs were taken under the same conditions used during the examination of the original fragments, F.1 and F.2. As expected, under the FITC channel the images of the single transfection of Htt25 ex.1 F.2N showed a diffuse green fluorescence, while Htt97 ex.1 F.2N produced both diffused and punctuate pattern (Figure 3C). But red signals in the Texas Red images were not seen in either sample (Fig. 3C). These F.2N single transfections would serve as the negative controls for PC12 cells co-transfected with both F.1 and F.2N for the wild-type and mutant Htt exon 1 samples.

Figure 3

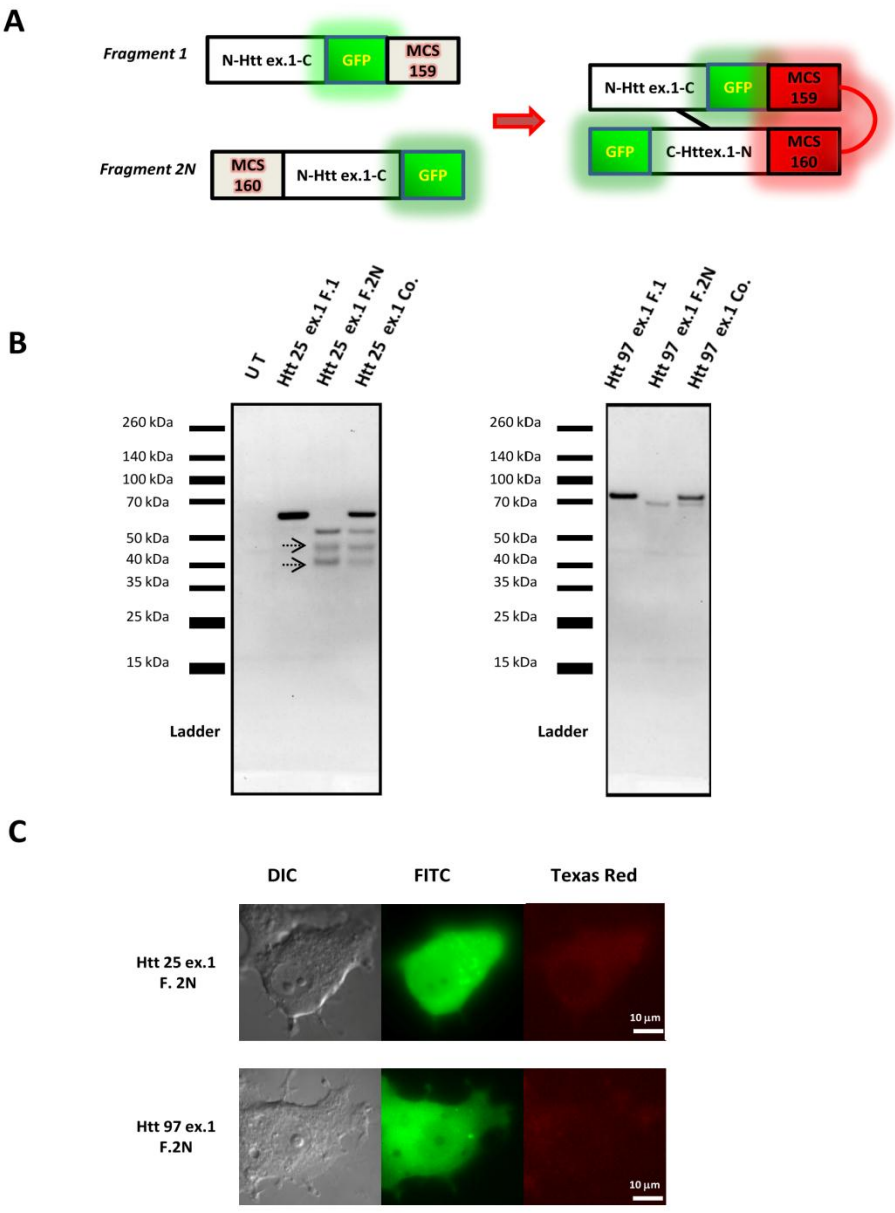

FIGURE 3: A, trans-confirguration schematic of F.1 and F.2N of Htt BiFC probes. B, western blot of F.1 for Htt25 ex.1 and Htt97 ex.1 samples with new F.2N for Htt 25 and Htt97 BiFC probes. Probed using rabbit anti-GFP antibody. C, Images of PC12 cells singly transfected with Htt 25 ex.1 F.2N or Htt 97 ex.1 F.2N.

Contrary to the previously conducted co-transfections with F.1 and F.2, the new co-transfected cells for wild-type Huntingtin exon 1 (Htt25 ex.1 F.1+2N) and mutant Huntingtin exon 1 (Htt97 ex.1 F.1+F.2N) produced detectable red signals in the form of punctas in both the wild-type and mutant samples (Fig. 4A, B). It is clear when examining the lower panel of images in Figure 4 A and B that wild-type co-transfection (Htt25 F.1+F.2N) and mutant co-transfection (Htt97 F.1+F.2N) samples exhibiting punctate patterns under the FITC channel also exhibited similar structures under the Texas Red channel. The detection of such puncta signals, which could be characterized as insoluble aggregates or IBs, suggests that the new BiFC probes were indeed functional. However, what was more serendipitous was the sign of positive red signals in cells that did not exhibit any punctas under the FITC channel (Fig. 4A,B lower panels). This finding could indicate the early formation of IBs or perhaps the initial aggregation of Htt that give rise to higher level insoluble IBs.

In addition to imaging, a series of in vitro crosslinking assay were conducted in order to ensure that F.1 and F.2N interaction of the Htt25 and Htt97 samples were due to the interaction of Htt and not the attached fluorescent tags. For this novel assay, HEK 293 cells were transiently transfected with GFP or remained untransfected (UT). Each of the cell lysate samples, transfected and untransfected alike, were either left untreated or treated with 0.1 mM of disuccinimidyl glutarate (DSG), a cell-membrane permeable chemical crosslinker. Western blotting of HEK 293 cells treated or untreated with DSG showed that monomeric GFP was not subject to aggregation in the presence of DSG (Fig. 4C). This implicates that the aggregation detected as punctas under the FITC and Texas

Red channels of transfected cells are due to interactions facilitated by Htt rather than the GFP incorporated into each probe.

Another set of crosslinking assays using the same conditions as the experiment in Figure 4C was done in order to determine if the fragments did indeed carry the capacity to interact and form higher level oligomers. For this experiment, equal volumes of cell lystates of HEK 293 cells singly transfected with Htt25 ex.1 F.1 or Htt97 ex.1 F.1 were treated with DMSO vehicle or DSG. Although DMSO treatment did not lead to higher level oligomerization in the wild-type and mutant F.1 fragments, in vitro DSG treatment of Htt25 ex.1 F.1 and Htt97 ex.1 F.1 cell lysate resulted in the formation of higher level oligomers. Htt25 ex.1 F.1, normally expressed at approximately 60 kDa prominently formed aggregates that were near the 120 kDa mark when treated with 0.1 mM DSG, which may be indicative of Htt25 ex.1 F.1 dimerization (Left blot, Fig. 4D). Similarly, Htt97 ex.1 F.1 lysate treated with 0.1 mM DSG also exhibited some level of dimerization, as Htt97 ex.1 F.1, which is approximately 70 kDa formed aggregates near the 140 kDa mark (Center blot, Fig. 4D).

In tandem with the results from these crosslinking assays, a third crosslinking assay was conducted to investigate the possibility of mHtt to form dimers with wtHtt. To our surprise, a 1:1 mixture of Htt25 ex.1 F.1 and Htt97 ex.1 F.1 cell lysates formed an aggregate near the 130 kDa mark upon treatment with 0.1 mM DSG (Right blot, Fig. 4D). Despite these results, whether this higher level oligomer is due to dimerization between the two fragments or dimerization of each individual fragment remains uncertain without further confirmation. An important implication of these findings is that mHtt

may interfere with normal cellular function of wtHtt by forming hetero-dimmers/hetero-oligomers with wtHtt.

The thesis author was the co-primary investigator and author of Chapter 3. Co-authors of the manuscript include Professor Chengbiao Wu, as the first co-author, Xiaobei Zhao, and Dr. William Mobley.

Figure 4

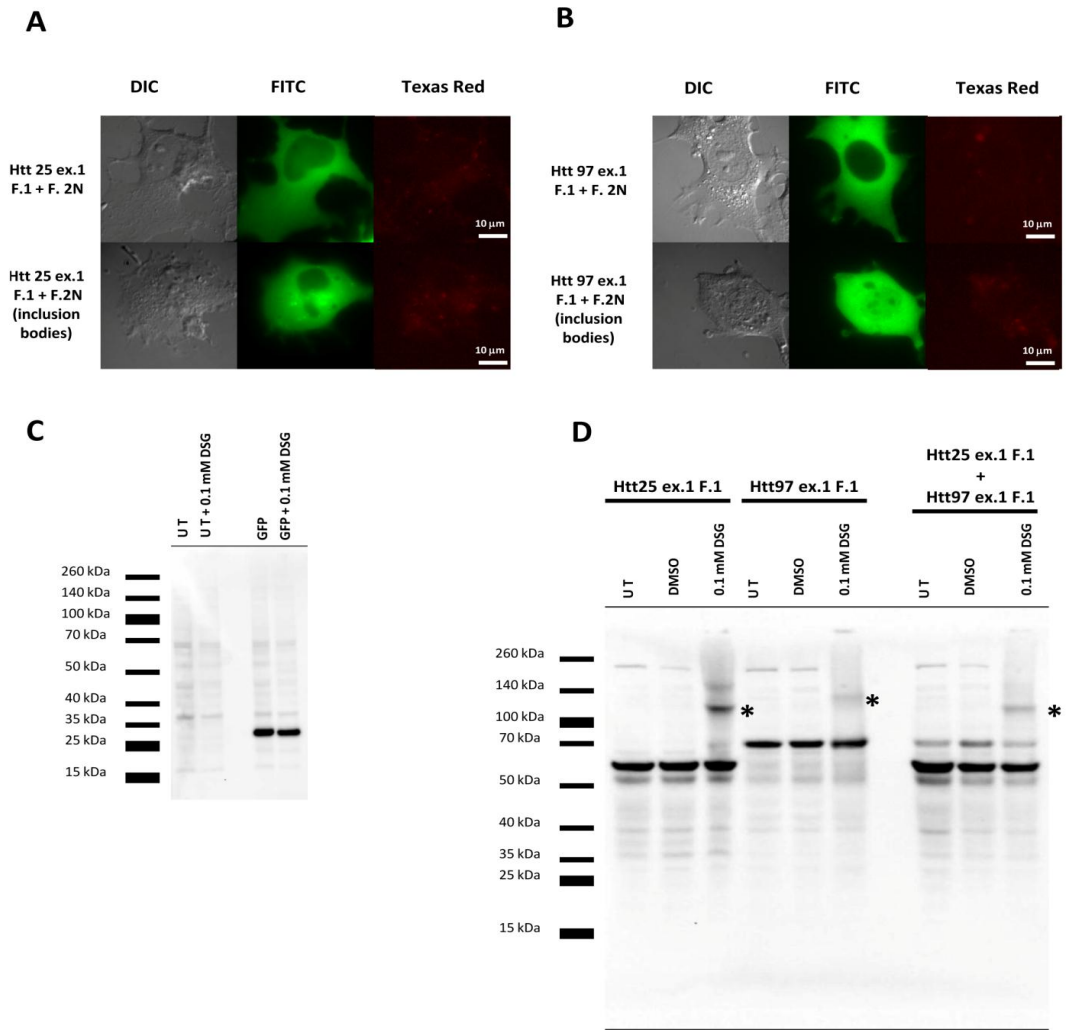

FIGURE 4: A, Images of PC12 cells co-transfected with Htt 25 ex.1 F.1 or F.2N produces clear red signals. Signals of early dimerization (top panel, Texas Red); signals of early dimerization along with signals of inclusion body detected under FITC (bottom panel, Texas Red) B, Images of PC12 cells co-transfected with Htt 97 ex.1 F.1 and F.2N produces similar positive signals as seen in A. Signals of early dimerization (top panel, Texas Red); signals of early dimerization along with signals of inclusion body detected under FITC (bottom panel, Texas Red). C, In vitro DSG crosslinking assay with HEK293 cells transfected with monomeric GFP or untransfected treated or untreated with DSG crosslinker. Mouse anti-GFP Ab. D, In vitro DSG crosslinking assay with HEK293 transfected with Htt25 ex.1 F.1 or Htt97 ex.1 F.1. Htt25 ex.1 F.1 and Htt97 ex.1 F.1 mix conducted in vitro with equal volumes of cell lysate. Mouse anti-Htt Ab.

CHAPTER 4

Mismatch Pairing of Huntingtin protein Exon 1 leads to trans-dimerization

Based on the results of the crosslinking assay involving dimerization between Htt25 ex.1 F.1 and Htt97 ex.1 F.1, further co-transfections for fluorescent imaging were conducted to further investigate if mHtt is capable of sequestering wtHtt. For this live imaging experiment, Htt25 F.1 was co-transfected with Htt97 F.2N and Htt25 F.2N was co-transfected with Htt97 F.1. Similar to the F.1 and F.2N co-transfections, the mismatching pairing reveal punctas detected under the Texas Red channel (Fig. 5A). The aggregation between wtHtt and mHtt detected by these probes may elucidate the role of mHtt in HD pathology.

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Figure 5

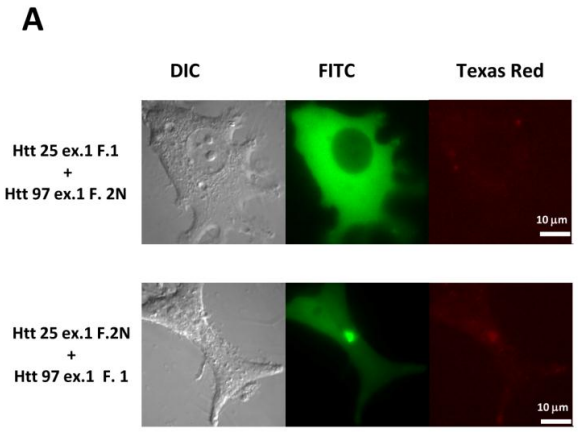

FIGURE 5: A, Images of PC12 cells co-transfected with Htt 25 ex.1 F.1 and Htt 97 ex.1 F.2N (top panel) or co-transfected with Htt 25 ex.1 F.2N and Htt 97 ex.1 F.1 (bottom panel).

CHAPTER 5

Discussion

With the advantages inherent with this newly developed GFP split-mCherry BiFC probes, we have discovered that both the wtHtt25 ex.1 and the mHtt97 ex.1 formed *trans*-dimers, but not *cis*-dimers. As expected, we detected dimerization between mHtt97 ex.1 proteins. To our surprise, we also found that wHttQ25 formed dimers. Moreover, we discovered that dimerization of wHttQ25 or mHtt97 proteins occurred in a *trans*- but not in a *cis*-manner. Our findings suggest that mHtt could potentially form hetero-dimers/oligomers with wtHtt and sequester wtHtt to prevent it from normal cellular function.

In addition to these findings, we speculate two potential models for IBs formation via trans-dimerization. Given the design of the BiFC probes, it is possible that the two fragments, F.1 and F.2N interact in a completely overlapping configuration thereby facilitating the direct interaction of the N-terminus and C-terminus of Htt, which would be indicative of the projected trans-dimers that we speculate are prominent in HD (Fig.6A). However, it is also probable that the corresponding fragments facilitate an incomplete overlap of wtHtt or mHtt, while keeping the direct interaction between the N-terminus and C-terminus (Fig.6B). Both models are possible, and without further investigation it is uncertain if one of the two models takes precedence, or if both interactions are present under physiological conditions.

Figure 6

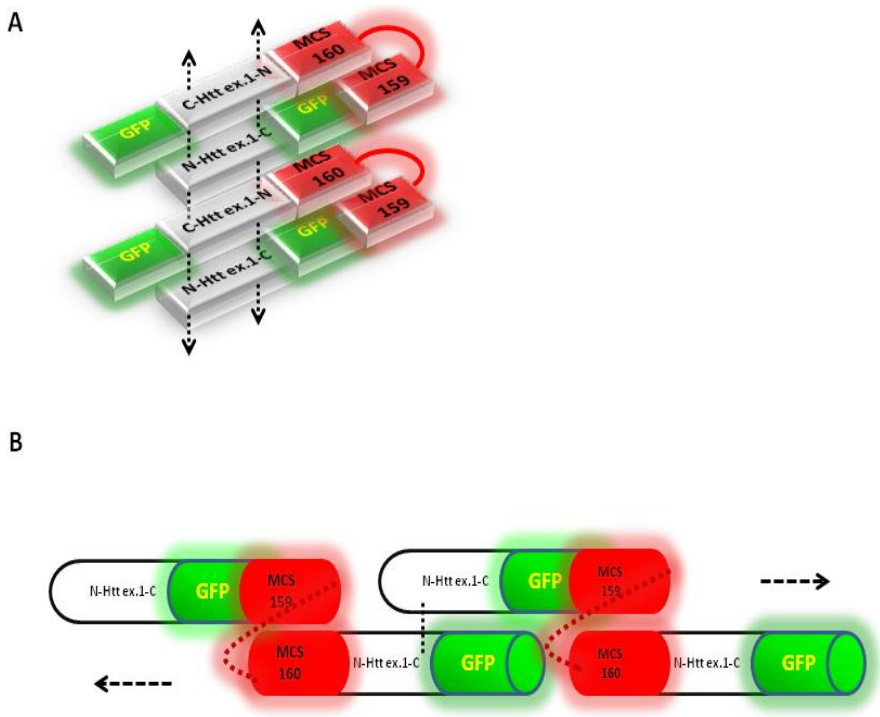

FIGURE 6: A, Schematic of possible arrangement of BiFC probes that align under a stacking configuration involving direct interaction between the C-terminus and N-terminus of Htt. B, Schematic of an alternative arrangement of BiFC probes that aggregate in an elongating configuration involving the same C-terminus to N-terminus interaction.

The novel GFP split-mCherry BiFC probes we have developed for investigating Htt interaction has two unique capabilities that provide certain experimental advantages compared to the previous methods of exploring Htt aggregation. The first of these advantages lie in ability of the split-mCherry probes to distinguishing between monomeric Htt and the formation of dimers which may give rise to insoluble IBs; a function that is unavailable using the split-GFP BiFC probes. Secondly, unlike the TC-

ReASH interaction based fluorescence which visually inferred the oligomerization of Htt due to the absence of a positive fluorescent signal, the novel split-mCherry BiFC method detects dimers and higher level oligomers that form under a trans-formation.

It would be possible to visualize the growth or perhaps even the degradation of the marked IBs in spatial and temporal manner recording of transfected samples. Not only could this help determine the degree of oligomerization that takes place under specified conditions, but we could use this method in assessing the possibility of turnover or degrading process of wtHtt and mHtt. Another route for investigation using this method of BiFC could be evaluating the ratio of monomeric Huntingtin exon 1 to IBs if a quantitative standard could be implemented in cell lines or neurons alike. Moreover, with this new assay, it is possible to move from PC12 cells and apply these constructs into neurons in order to mimic the physiological setting that would be apparent in HD. Despite challenges in efficiency, transfections of hippocampal or cortical neurons isolated from mouse models of HD such as the BACHD mice could be a route for future experiments in order to examine the localization of possible aggregates or evaluate other areas of interest related to Htt interaction.

With the use of the information gained from a new BiFC assay, new insight on the formation of IBs and associated cytological processes can assist in clarifying the pathological role of inclusion bodies in HD. Furthermore, the implications regarding Htt turnover rate gained from future time course experiments may open new opportunities for a series of investigations examining the pathway of Htt degradation regulated by

chaperonin, TriC. In tandem with evidence of TriC preventing significant aggregation of Htt by blocking the N17 sequence (Tam et al., 2009), the evidence gained from these future experiments could be used to present a more complete view of the TriC-Htt interactions and the progression of HD.

The thesis author was the co-primary investigator and author of Chapter 5. Co-authors of the manuscript include Professor Chengbiao Wu, as the first co-author, Xiaobei Zhao, and Dr. William Mobley.

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