MECHANISMS OF POLYGLUTAMINE EXPANDED HUNTINGTIN INDUCED TOXICITY

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Huntington's Disease (HD) belongs to the CAG repeat family of neurodegenerative diseases and is characterized by the presence of an expanded polyglutamine (polyQ) repeat in the huntingtin (htt) gene product. PolyQ-expanded htt accumulates within large aggregates in various subcellular compartments, but are more often localized within the nucleus. The sequestration of proteins essential to cell viability may be one mechanism that accounts for toxicity generated by polyQ-expanded proteins. Nuclear inclusions containing polyQ-expanded htt recruit the transcriptional cofactor, CREB-binding protein (CBP). PolyQ toxicity appears to involve alterations of gene transcription and reduced neuronal cell viability. In the HT22 hippocampal cell line, we found that toxicity within individual cells induced by polyQ-expanded htt was associated with the localization of the mutant htt within either nuclear or perinuclear aggregates. However, in addition to CBP recruitment, we found that CBP ubiquitylation and degradation can be selectively enhanced by polyQ-expanded htt. Thus, selected substrates may be directed to the ubiquitin/proteasome-dependent protein degradation pathway (UPP) in response to polyQ-expanded htt within the nucleus. While both the polyQ domain and the histone acetyltransferase domain (HAT) of CBP have been found to interact with polyQ-

expanded htt, deletion of either domain does not affect its enhanced degradation in the presence of polyQ-expanded htt in HT22 cells. Thus, enhanced degradation of CBP in cells expressing polyQ-expanded htt may not involve a direct interaction between CBP and htt. It seems likely specific enzymes in the UPP may be activated by htt and selectively target proteins such as CBP for degradation.

Since molecular chaperones are found in the aggregates containing polyQ-expanded proteins, misfolding of polyQ-expanded proteins may play a key role in polyglutamine disease pathogenesis. In a number of some studies, HDJ-2, a member of DnaJ family molecular chaperones, was found to reduce aggregation and toxicity induced by polyQ-expanded proteins. In contrast, we show that HDJ-2 is unable to rescue aggregate formation of polyQ-expanded htt in transfected HEK293 fibroblast cells, nor is it recruited into these aggregates in vivo in a HD transgenic mouse model. Thus, molecular chaperone effects on polyQ-expanded protein induced toxicity could be cell-type specific or influenced by the developmental state of the culturable cells. These factors must be considered in any attempts to use chaperones as potential therapeutic targets in polyglutamine diseases.

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PREFACE

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Chapter II – Jiang, H., Nucifora, F.C., Jr., Ross, C.A. and DeFranco, D.B. (2003) Cell death triggered by polyglutamine-expanded huntingtin in a neuronal cell line is associated with degradation of CREB-binding protein. Hum Mol Genet, 12, 1-12.

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LIST OF ABBREVIATIONS

AD --- Alzheimer's disease

ALS --- Amyotrophic lateral sclerosis

APP --- Amyloid precursor protein

AR --- Androgen receptor

BDNF --- Brain derived neurotrophic factor

CFTR --- Cystic fibrosis transmembrane conductance regulator

CBP --- CREB-binding Protein

CN --- Caudate nucleus

CNS --- Central nervous system

CTNF --- Ciliary neurotrophic factor

DFF --- DNA fragmentation factor

CREB --- cAMP response element binding protein

CRE --- cAMP response element

CREM --- cAMP responsive element modulator

DAPI --- 4',6-diamidino-2-phenylindole dihydrochloride

DAB --- Diaminobenzidine

D/N --- Dominant negative

DRPLA --- Dentatorubral pallidoluysian atrophy

E1 --- Ubiquitin activating enzyme

E2 --- Ubiquitin carrier protein (ubiquitin conjugating enzyme)

E3 --- Ubiquitin ligase

E4 --- Ubiquitin chain assembly factor

EM --- Electron microscopy

GFP --- Green fluorescent protein

GR --- Glucocorticoid receptor

HAP1 --- Huntingtin-associated protein1

HAT --- Histone acetyltransferase

HD --- Huntington's disease

HIP1 --- Huntingtin-interacting protein 1

Hprt --- Hypoxanthine phosphoribosyltransferase gene

Hsp --- Heat shock protein

Htt --- Huntingtin

Htt-polyQ --- Polyglutamine-expanded huntingtin

IIF --- Indirect immunofluorescence

IP --- Immunoprecipitation

IT15 --- *interesting transcription 15*

JNK --- c-Jun NH_2-terminal kinase

Mdm2 --- Mouse double minute 2

NGF --- Nerve growth factor

NFT --- Neurofibrillary tangles

NI --- Nuclear inclusion

NLS-BP --- Bipartite nuclear localization signal

PD --- Parkinson's disease

PI --- Propidium iodide

PARP --- Poly(ADP-ribose) polymerase

PKCγ --- Protein kinase C γ isoform

PolyQ --- Polyglutamine

PSP --- Progressive supranuclear palsy

RTS --- Rubinstein–Taybi syndrome

SAHA --- Suberoylanilide hydroxamic acid

SBMA --- Spinobulbar muscular atrophy (Kennedy's Disease)

SCA --- Spinocerebellar ataxia

TBP --- TATA-box binding protein

TSA --- Trichostatin

TUNEL --- Tdt-mediated dUTP-biotin nick end labeling

UPP --- Ubiquitin proteasome pathway

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CHAPTER I: INTRODUCTION

I. NEURONAL CELL DEATH

1. Apoptosis and Necrosis

In 1972, Kerr et al. described and compared two types of cell death, necrosis vs. apoptosis in liver cells (1). Necrosis generally occurs in response to acute injury. Necrotic cells swell and burst, releasing cellular components to the extracellular space and causing inflammation to surrounding tissues (2). In contrast to necrosis, apoptosis has its unique morphological features such as cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation (2). Apoptotic cells release no harmful material to the extracellular space and therefore restrict cell death only within themselves (2). Table 1 shows the major differences between apoptosis and necrosis. Among those features, apoptosis requires new gene expression and protein synthesis, which indicates that it is a well-programmed intrinsic suicide process (also see later for details).

Apoptosis, also named Programmed Cell Death occurs in many organisms. It is both a physiological phenomenon that occurs during normal development and induced by various disease conditions. In this chapter, only apoptosis in neuronal systems will be discussed.

Table 1: Comparison between apoptosis and necrosis Adapted from (2)

	Necrosis	Apoptosis
Cytoplasmic organelles	Swelling of all cytoplasmic compartments due to altered transmembrane ion influx	Cytosol condensation without changes in ion influx
Nucleus	Nuclear membrane rupture	Membrane blebbing, condensation of nucleus
chromatin	Loose aggregates of chromatin	Internucleosomal DNA cleavage, DNA fragmentation and condensation
ATP	Depletion of cellular energy (ATP)	ATP dependent
Macromolecular synthesis	Decreased RNA and protein syntheses	New RNA and protein syntheses dependent
Affect on surrounding cells	Affects surrounding cells by releasing toxic cellular components.	Death within scattered individual cells
	Passive process	Active process

Neuronal apoptosis can be induced by a variety of stimuli such as neurotropic factor withdrawal, oxidative stress, DNA-damage agents, neurotransmitter release, irradiation and oxidative stress (2). In fact, the same types of stimuli may cause both apoptosis and necrosis dependent on the dose and duration of the damaging stimuli (3). For example, low dose of NMDA activation may induce neuronal apoptosis while high dose may cause necrosis (4, 5). In models of both acute neuronal injuries such as stroke and chronic neurodegenerative disease such as Parkinson's Disease (PD), neuronal cell death may exhibit features of either apoptosis or necrosis or a combination of both (6, 7).

In addition to utilizing the morphological changes specific to apoptosis, there are several ways to identify the presence of apoptosis. One is the Tdt-mediated dUTP-biotin nick end labeling (TUNEL). TUNEL labels the 3' end of single or double stranded cleaved DNA, which may represent DNA fragmentation in the apoptotic process (2). Another way is to detect ladder-type DNA fragmentation by gel electrophoresis that results from DNA cleavage at the internucleosomal linker region (2). However, both the TUNEL staining and DNA electrophoresis can not reliably distinguish apoptosis from necrosis as they can be positive in some cells with necrosis (2, 8). False positive and negative results may occur under certain circumstances (2). Therefore, to confirm the presence of apopotosis, a combination of multiple methods must be used which often target molecular markers of apoptois (9) (see below).

2. Apoptotic proteins and apoptotic pathways

Apoptosis is a complicated process and involves multiple pathways. While numerous proteins have been shown to play a role in apoptosis, several groups of proteins are most important. For example caspases are a family of cysteine proteases that cleave their substrates with signature specificity after aspartic acid residues (10). They are apoptotis-specific proteases. There are more than ten members of the caspase family that form an apoptotic cascade to execute apoptosis in respond to death signals (Figure 1) (11). They can be divided into two categories: upstream initiator caspases and downsteam executioner caspases. Initiator caspases are activated in response to apoptotic stimulti and cleave other caspases (11). For example, caspase 9 is an initiator caspase that can be activated by the death signals through a relay initiating from mitochondria (see later). Activated caspase 9 in turn cleaves downstream caspases. For example, it cleaves procaspase 3 after a specific aspartic acid residue into a short form that is the active form of caspase 3 (12). Caspase 3 is an example of downstream executioner caspases that cleaves cellular protein substrates in various subcellular compartments. Activated caspase 3 may then translocate into the nucleus and cleave substrates such as lamin A, poly(ADP-ribose) polymerase (PARP) and DNA fragmentation factor (DFF) 40 and 45 (10). The active forms of DFF have DNase activity. DFF 40 and 45 act on DNA causing DNA fragmentation and chromatin condensation which are the hallmarks of apoptosis (10). The importance of caspase 3 in neuronal apoptosis is demonstrated through genetic studies. Caspase-3 knockout mice exhibited hyperplasia and disorganized cellular deployment in the brain (13).

Another important family of proteins involved in neuronal apoptosis is the Bcl-2 family proteins. They regulate apoptosis by affecting cytochrome c release from mitochondria. Cytochrome c release to the cytosol activates pro-caspase 9 and then initiates an apoptotic cascade. Bcl-2 and Bcl-$_{XL}$ are important anti-apoptotic proteins, while Bak, Bim, Bad and Bax are pro-apoptotic (10). Bcl-2 is a negative regulator of cytochrome c release and therefore is anti-apoptotic. Transgenic mice that overexpress Bcl-2 showed increased brain size resulting from decreased apoptosis (14, 15). In contrast, Bax is a pro-apoptotic Bcl-2 family protein. Bax knock-out mice show significantly decreased apoptosis of synapse-bearing neurons whose survival are neurotrophic factor-dependent (16). In addition to caspases and Bcl-2 family proteins, many other proteins also play important roles in regulating apoptosis such as transcription factors that discussed later (also see Figure 1).

Neuronal apoptosis can be triggered by both intracellular and extracellular signals. As shown in Figure 1, extracellular apoptotic signals convey through a FAS/FADD/Caspase-8 pathway to activate caspase 3. Intracellular signals activate caspase-3 through a Cytochrome C/Apaf-1/Caspase-9 pathway (10).

3. Apoptosis and transcription

Apoptosis generally is dependent on new gene transcription that results in new mRNA and protein synthesis. Apoptosis can be blocked by inhibitors of macromolecular synthesis such as actinomycin D, an inhibitor to transcription and cycloheximide, an

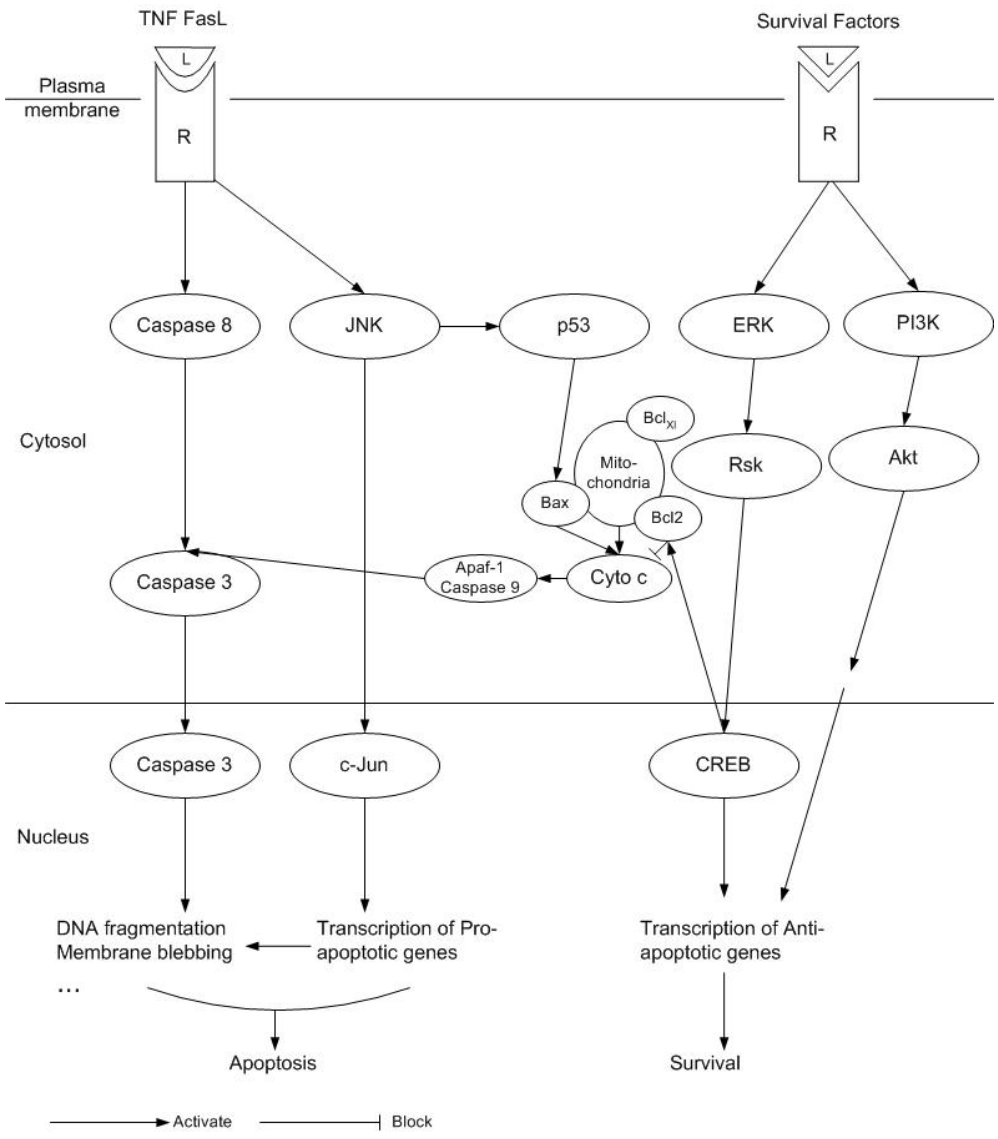

Figure 1: Some important pathways regulating apoptosis

Apoptosis is regulated by multiple pathways. The two main apoptotic pathways acting through the activation of caspase cascade are the FAS/FADD/Caspase-8 death receptor dependent pathway and Cytochrome c/Apaf-1/Caspase-9 mitochondria dependent pathway. These pathways are also regulated by other signaling cascades or proteins such as the JNK pathway, the ERK pathway and p53.

inhibitor to protein synthesis (17). For example, Sakhi et al. showed that the glutamate analogue kainic acid induced apoptosis of adult rat central nervous system where p53 transcription is elevated. Cycloheximide treatment to these rats resulted in both an inhibition of apoptosis and p53 synthesis (18). Gobble et al. showed that moderate amount of irradiation caused apoptosis to rat cortical neuron that was blocked by cycloheximide treatment (3) (also see later).

What are the genes that are activated during apoptosis? The activity of some transcription factors are increased during apoptosis which results in the new synthesis of "apoptotic genes" such as DNA damage responsive genes and DNA repair proteins (3). For example, the c-Jun NH_2-terminal kinase (JNK) pathway may mediates apoptosis in a transcription dependent manner (2). JNK is activated upon phosphorylation by upstream kinases, and it in turn phosphorylates and activates c-jun. c-jun is a transcriptional factor and mediates the transcription of several apoptotic genes such as cyclin D1 (19). p53 is also a transcription factor that can activate many apoptotic related genes (20). One target of p53 is Bax, which plays an important role in regulating mitochondrial cytochrome c release to promote apoptosis (20).

In addition to proteins that have pro-apoptotic functions, those that are anti-apoptotic also play important roles in neuronal survival and death. Among these proteins are also some transcription factors. For example, cAMP response element binding protein (CREB) is a well-known transcription factor that has important roles on neuronal function (21). It binds to the cAMP response element located within the upstream DNA sequences of a

variety of genes. Many of these genes are involved in neuronal function such as neuropeptides/neurotransmitters and neuronal growth factors (21). Of particular interest to neuronal apoptosis is one of the CREB regulated genes that encodes the Bcl-2 protein, which is a key anti-apoptotic factor that regulates the mitochondrial dependent apoptotic pathway in a cell (14). Riccio et al. used a sympathetic neuron model and demonstrated that the increase of neuronal survival by neurontrophins was due to the expression of certain prosurvival genes. The transcription of these genes including Bcl-2 is dependent on CREB family transcription factors (22).

4. Apoptosis during neuronal development and under neuropathological conditions

The study of apoptosis in brain development primarily focuses on the synapse-bearing neurons. It is well believed that apoptosis in these neurons occurs in order to facilitate appropriate neuron-target cell innervation (10). Presynaptic neurons that can not find a target cell will undergo apoptosis. Target cells once innervated secrete limited amounts of neurotrophic factors called neurotrophins, such as nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF). Presynaptic neurons that are not exposed to neurotrophins at specific times during their development will die through apoptotic pathways. This mechanism ensures that the correct amount of neurons will survive to provide precise target cell innervation (2, 23).

Apoptosis in the developing brain occurs in pre-synaptogenic neurons (i.e. neuronal precursor cells and immature neurons) as well. Apoptosis in these neurons facilitates

appropriate brain morphogenesis and the removal of damaged neurons (10, 23). In the previous section, the function of some important apoptosis related proteins have been discussed such as caspase 3 and Bcl-2.

Neuronal cell death also occurs under neuropathological conditions, either acute injury such as stroke or chronic neurodegenerative diseases (2). However, as apoptosis is clearly identified in brain development by both morphological and molecular methods, evidence for neuronal apoptosis under various disease conditions are not very clear. Both necrosis and apoptosis may be present simultaneously, and be related to the amount of toxic stimuli (3, 9). Morphological changes associated with brain injury are often not quite typical of apoptosis (9). Apoptosis in the brain of some neurodegenerative diseases will be discussed in detail in the following section.

II. NEURODEGENERATIVE DISEASES

1. Diseases

Table 2 lists some of most common neurodegenerative diseases. Among them, Alzheimer's disease (AD) and Parkinson's disease (PD) affect millions of elderly people severely and significantly influences the quality of life of the patients and their families. Huntington's disease (HD) is one of polyglutamine-related neurodegenerative diseases and affects 1/10,000 people in the United States (24).

Table 2: A list of most common neurodegenerative diseases Adapted from (10, 24, 25)

Diseases	Disease onset	Prevalence in US	Genetic Features	Gene	Sites of degeneration	Symptoms	Inclusions and Subcellular sites	Toxic protein
Alzheimer's (AD)	senior 10% over 65	4 million	Sporadic & genetic	APP Presenilin1 & 2	Neocortex and hippocampus	Memory loss, dementia impairement in other forms of cognition and behavior	Extracellular plagues Intracellular tangles	Aβ 42kd Tau
Parkinson's (PD)	Senior 1-2% over 65	1 million	Sporadic & genetic	a-synuclein Parkin UCHL1 PRNP	Dopaminergic neurons in the substantia nigra	Muscle rigidity, bradykinesis and resting tremor	Cytoplasmic Lewy bodies	α-Synuclein
Huntington's (HD)	30-40	30,000	genetic	Huntingtin (htt)	Striatum and cortex	Progressive chorea, rigidity and dementia	Nuclear and Cytoplasmic aggregates	htt
Amyotrophic lateral sclerosis (ALS)	40-	20,000	Sporadic & genetic	SOD	Upper and lower motor neurons	Muscle atrophy and fatal paralysis	Bunina body	SOD
Progressive supranuclear palsy (PSP)	Mid-age to eldly	15,000		Tau	Striatum, cerebellum	Early falls and abnormal eye movement	Cytoplasmic tangles (NFT)	Tau
Prion		400	Sporadic & genetic	prion		Psychiatric and behavior abnormalities	Prion plaque	PrP^{sc}

2. Common mechanisms underlying the pathogenesis of various neurodegenerative diseases

Each neurodegenerative disease is well distinguished from the others in several aspects. For example, each of them has distinct symptoms based on the sites of pathogenesis in brain. AD mainly affects neocortex and hippocampus which results in cognition and behavior abnormalities with little effect on motor function even at very late stages of the disease (24). Compared to AD, PD and HD both affect CNS on motor functions at early stages of the diseases. PD primarily affects dopaminergic neurons in the substantia nigra and causes mainly motor dysfunction such as muscle rigidity, bradykinesis and resting tremor at early stages of disease (24). HD is a definite genetic disease and its pathology initiates from caudate nucleus striatal neurons which causes an opposite effect on motor function compared to that of PD. HD patients suffer progressive chorea at the beginning the disease, but later also develop rigidity and dementia resulting from massive damage to numerous brain areas (26).

Despite all the differences, it is increasingly apparent that all of the neurodegenerative diseases share common features at both the pathological and molecular levels (24, 27, 28). A better understanding of these similarities may enable researchers to develop effective therapies to treat these diseases. Some of these similarities are discussed below.

2.1 Aggregates: As shown in Table 2, an important feature of many neurodegenerative diseases is the presence of intracellular and/or extracellular aggregates that contain specific abnormal proteins in each disease. For example, in AD brains, both extracellular

plaques and intracellular neurofibrillary tangles (NFT) are detected (25). The extracellular plaques contain mostly the 42kD Aβ protein, which is an aberrant proteolytic product of amyloid precursor protein (APP). The NFT also contain microtubular tau protein (25). In PD, cytoplasmic Lewy's bodies containing α-synuclein are detected in the affected brain area (28). As in polyglutamine diseases, intracellular aggregates containing specific proteins encoded by specific diseases genes are considered as a hallmark of this group of diseases (27). Aβ, α-synuclein and polyglutamine proteins have been found to form compact β-sheet rich structures which are insoluble in the cells (27).

Many questions remain about protein aggregates in neurodegenerative diseases. For example, how do aggregates form? Aggregates containing the disease-proteins such as Aβ and α-synuclein also recruit ubiquitin, other components of Ubiquitin Proteasome Pathway (UPP) and molecular chaperones (29-31). These observations suggest that a failure of the cell to refold or degrade toxic proteins may result in their accumulation into insoluble aggregates. Toxic proteins in the aggregates may recruit critical factors such as transcriptional factors (32). Recruitment of components of UPP into aggregate may inhibit the normal functioning of the protein degradation pathway (33).

There is still much debate about the role of these aggregates in toxicity. Some studies show that aggregates are intrinsically toxic (33, 34), while other studies suggest that aggregation may serve to sequester disease protein and lower the exposure of toxic

surfaces to the cell (35). Therefore, aggregation may also be beneficial. A detailed discussion of this issue will follow in later sections.

2.2 Proteolysis: In some neurodegenerative diseases, proteolysis of disease proteins from their longer precursors is also likely to play a role in neuronal toxicity (27, 36). In AD, the 42 kD Aβ protein is a proteolytical cleavage product of an amyloid precursor protein (APP) (36). APP is a transmembrane protein and is not toxic. APP can be processed by several secretases. Insoluble 42kD Aβ protein is obtained after cleavage of APP by γ-secretases followed by its translocation to the extracellular space and the formation of tangles (37). Several polyglutamine disease proteins including huntingtin, androgen receptor (AR) and ataxin 1 also are likely to be more toxic when they are cleaved into their truncated forms (38-40). The relationship between polyQ protein truncation and toxicity will be discussed in later sections.

2.3 Selectivity: In several of these diseases, such as PD and HD, toxic proteins only affect a certain subpopulation of neurons (27). The reason why certain types of neurons are particularly prone to pathology in these specific cases is far from understood.

2.4 Apoptosis and other mechanisms: Studies from different neurodegenerative diseases reveal that apoptosis may occur in the affected brain areas (2). However, the evidence from in vivo studies is not quite strong. For example, results from TUNEL staining of PD postmortem brain revealed a large variability (9). Some studies showed that about 1% of nigra cells were positive, while other studies showed no TUNEL

positive cells (9). This variability can be explained by the limitation of techniques of the TUNEL assay. Another explanation of negative or low percentage of apoptosis found in PD postmortem brain is that as the chronic disease process takes years to develop, few neurons may be undergoing apoptosis at any time point that is examined (2, 9). Bax and caspase 3 activation have also been found in PD postmortem brains although only in a few neurons (i.e. <3%) (41). Evidence of apoptosis in postmortem brains of in AD and HD are not quite clear as well (42, 43). However, in vitro assays such as cell transfection are able to demonstrate the presence of apoptosis at the molecular levels with more simplicity. Both the FAS/FADD/Caspase-8 pathway and mitochondria Cytochrome c/Apaf-1/Caspase-9 pathway have been shown to play roles in HD, PD and AD (9, 42, 43). Detailed discussion of apoptosis in HD will follow in the next section. Stressful stimuli including oxidative stress, excitatory toxicity, mitochondrial dysfunction and lack of sufficient neruotrophic factors may also play a role in triggering neuronal apoptosis (2).

3. Protection from toxicity in neurodegenerative diseases: Some therapeutic strategies are being developed to target common mechanisms of these neurodegenerative diseases.

3.1 Targeting aggregates directly: In a Tet-regulated conditional HD transgenic mouse model, reversal of aggregate formation is followed by the improvement of symptoms (44). This study suggests that blocking the expression of aggregates may be beneficial. Congo red has been shown to block aggregation formation and reduce toxicity (45). Other ways to target aggregates directly include improving the correct-folding of these

toxic proteins by overexpression of molecular chaperones (29); or promoting the degradation of the aggregates through the activation the UPP (27).

3.2 Targeting cellular proteins that are recruited by the aggregates: Many transcriptional factors such as Sp1 and CREB-binding protein (CBP) have decreased functions as a result of aberrant interactions with polyQ proteins (46, 47). Recovering the functions of these important factors may be helpful to reduce neuronal toxicity induced by aggregation (28, 32). As will discuss more detailed later in chapter II, recovery of CBP activities by its overexpression or restoring its histone acetyltransferase activity has been shown to reduce toxicity in several in vivo and in vitro HD models (47, 48).

3.3 Targeting proteolysis and translocation of toxic proteins: Aβ is a cleavage product of APP that moves from the cytosolic membrane to the extracellular space where it forms tangles. Futhermore, although full length huntingtin protein is cytoplasmic, the truncated form translocates to the nucleus to aggregate. Therefore, blocking truncation and translocation may be a useful way to block aggregation and toxicity in some of these diseases (28). For example, Wellington et al. showed that htt protein with a mutation at the putative caspase 3 cleavage sites was resistant to cleavage and had reduced toxicity in neuronal or nonneuronal cells (49).

3.4 Targeting apoptosis: Apoptosis may be the final outcome of affected neurons in all of these diseases. Directly targeting apoptosis may also be an efficient treatment (28). For example, there have been some studies that used caspase inhibitors to rescue neuronal

toxicity in different neurodegenerative diseases (9, 50, 51). See the later sections for details.

III. POLYGLUTAMINE DISEASES

1. Introduction:

Polyglutamine diseases are a group of at least 9 neurodegenerative diseases, whose disease-proteins all contain an expanded polyglutamine tract (Table 3). HD is caused by a CAG expansion in the huntingtin (htt) gene. Kennedy's Disease is caused by a similar mutation in androgen receptor gene. In Dentatorubral pallidoluysian atrophy (DRPLA), the disease gene is atrophin 1. Finally there are 5 types of SCAs (1, 2, 3, 6, 7) caused by mutations in ataxin genes (27). Recently a new CAG expansion was identified at the TATA-box binding protein (TBP) gene in the disease SCA17 (52). In addition to the expanded CAG repeat, these diseases share many common features (discussed below).

2. Genetics:

PolyQ diseases are all dominantly inherited diseases with the exception of Kennedy's Disease (SBMA), which is X-chromosome linked recessive inherited. All others are autosomal.

2.1 Polymorphism of CAG repeat and its relevance to disease phenotype:

The CAG repeat has a polymorphism in the normal population. In normal individuals, these genes all bear a normal range of CAG expansion. Generally, there is a threshold of

Table 3: Polyglutamine diseases Adapted from (42, 53)

Polyglutamine Repeat Diseases	Disease protein	Normal glutamine repeat range	Pathological glutamine repeat range	truncation	Physiological functions	Molecular weight (kDa)	Nuclear localization signal
Huntington's disease (HD)	Huntingtin (htt)	6-35	36-121	YES	Unknown	348	No
Spinobulbar muscular atrophy (SBMA) (Kennedy's Disease)	Androgen receptor (AR)	9-36	38-62	YES	Known, ligand-dependent transcription factor	99	Yes
Dentatorubral pallidoluysian atrophy (DRPLA)	Atrophin-1	6-35	49-88	YES	Unknown	124	No
Spinocerebellar ataxia type 1 (SCA1)	Ataxin-1	6-44	39-82	NO	Unknown	87	Yes
Spinocerebellar ataxia type 1 (SCA2)	Ataxin-2	15-31	36-63	N/A	Unknown	90	No
Spinocerebellar ataxia type 1 (SCA3)	Ataxin-3	12-40	55-84	YES	Unknown	42	No
Spinocerebellar ataxia type 1 (SCA6)	Ca^{2+} channel subunit	4-18	21-33	N/A	Unknown	large	No
Spinocerebellar ataxia type 1 (SCA7)	Ataxin-7	4-35	37-306	N/A	Unknown	95	Yes
Spinocerebellar ataxia type 1 (SCA17)	TBP	-42	47-	N/A	Transcription factor	42	Yes

the number of CAG repeats, only above which will give rise to disease phenotype (27). Listed in Table 3, we can see that threshold in most of these diseases is in the range of 35-45 consecutive CAGs in the repeat (27). Although the exact reason why this range will cause the disease is not known, some data suggest that a certain number of consecutive glutamine residues cause aberrant conformational changes in the disease proteins (54, 55). The number of the glutamine residues in the disease proteins also conspicuously impacts the age of disease onset and progression: the higher the number, the earlier the disease onset and the more severe the symptoms (28).

2.2 Disease proteins: Proteins in each of these diseases with a normal range of glutamine repeats does not have a clear physiological function except for the AR in Kennedy's Disease (SBMA) and TBP in SCA17. AR is a ligand-dependent transcription factor. After binding with hormone it translocates from the cytoplasm to the nucleus and initiates transcription of some genes (56). TBP is a critical protein in the basal transcription machinery (52). It binds to the TATA box sequence of promoter region of genes which is one of the key steps required to initiate transcription. Disease proteins with normal lengths of CAG repeat in other diseases do not have a clear function. Therefore, whether these proteins lose normal functions when expanded with a long CAG repeat is difficult to be determined. In some following sections, whether a loss-of-normal function or a gain-of-toxic function of these proteins contributes to the pathogenesis of polyglutamine disease (especially HD) will be discussed in detail.

3. Pathology:

3.1 Selectivity of subpopulation of neurons: In nearly all patients with polyglutamine diseases, the mutant protein is expressed throughout the central nervous system (CNS). However, some regions of the brain tissues are more susceptible to the expression of mutant protein than others. Furthermore, in affected areas, neurons are more prone to die than glia cells(26, 57). For example, in early stages of HD, striatal and cortical neurons are most affected (26). In SCA1, cerebellar Perkinje neurons are the first and mostly affected (57). The reasons for this selectivity are not clear yet, but probably depend on the function of each specific protein, their normal function in the affected areas and other interacting proteins.

3.2 Aggregates (inclusions): Intracellular inclusions or aggregates have been considered as a pathological hallmark of polyglutamine diseases (53).

3.2.1 Subcellular localization of polyQ aggregates: For proteins such as ataxin-1 which is normally a nuclear protein, mutant ataxin-1 forms nuclear aggregates in the affected neurons (58). For proteins like htt whose full-length wide type version is entirely cytoplasmic in the adult brain, aggregates are found in all three compartments of an affected neuron i.e. nucleus, perikaryon and processes in affected areas (38). Except for SCA2, in which mainly cytoplasmic aggregates are observed (59), all other polyglutamine disease proteins form nuclear aggregates (53). Nuclear aggregates may

have an impact on some nuclear functions such as transcription (32), while aggregates in neuronal processes may block functions such as neurotransmitter transport (60).

3.2.2 Proteins trapped in aggregates: Intracellular aggregates of polyQ diseases are stained positive by antibodies to each specific disease protein or a certain fragment of it (53). They also show positive staining by antibodies against the expanded glutamine tract but not the normal range of glutamine residues (e.g. 1C2 antibody) (61). The fact that these aggregates stain positive for ubiquitin indicates that they contain ubiquitylated proteins (62). Polyglutamine aggregates also contain some proteasome subunits and molecular chaperones (29). Many transcription factors such as TBP and CBP were found to be localized in the aggregates which results from the interaction of these proteins with the expanded polyQ tract (32). Some proteins involved in apoptosis are found to be in the aggregates as well. For example, caspase 8 has been shown to be recruited into aggregates of HD patient postmortem brains (51). Entrapment of proteins into aggregates can be a temporary or permanent process. For example, in a HD cell model, molecular chaperones such as Hsp70 exhanges between the aggregates and soluble components of the cell, while TBP remains in the aggregates once it is recruited (63) . Clarifying what proteins are in the polyQ aggregates and why they are recruited is beneficial to understand the pathogenesis as well as to develop new therapeutics.

3.2.3 Structure of polyQ aggregates: PolyQ-containing aggregates which can be seen under the light microscope are usually SDS-insoluble (53). The formation of the insoluble, amyloid-like fibril aggregates has been proposed as a nucleation-dependent

process (64). As shown in Figure 2A, an unstructured monomer transiently forms a β-sheet structure. This β structured monomer acts a nucleation centre and soon recruits other monomers to form oligomers. Oligomer formation is the rate-limiting step of aggregation formation (64). Oligomers will finally form highly ordered β-sheet structured aggregates (64, 65). Biophysical analyses such as x-ray fiber diffraction studies, circular dichroism and computerized modeling have proposed several models for the β-structured monomer as shown in Figure 2B (64). For example, as shown in panel f, Perutz et al. proposed a β-helix structure of 20 Q residues per turn (54). This makes a total 40 Q residues which is a critical number for aggregation formation in vivo of polyQ diseases. This study also showed that Aβ, which makes of the extracellular tangle in AD and Sup35 (i.e. yeast prion protein) which makes prion aggregates, share same structural features with polyQ (54). Another recent study using a mutational analysis of the modified synthetic polyQ peptides suggested a structure shown in panel e. This model proposed that polyQ monomer forms a compact β-sheet structure with alternating elements of extended chain and turn (55).

3.2.4 Roles of aggregates in toxicity: The most intriguing issue about polyQ aggregation regards the toxicity of these aggregates. Nuclear inclusions containing expanded polyQ proteins were thought to contribute to neuronal damage and degeneration in polyQ repeat diseases (53). For example, formation of aggregates precedes the symptoms in a HD mouse model (66). Reversal of aggregate formation also reverses the symptoms in a conditional HD mouse model (44). Bucciantini et al. showed that aggregation of two non-disease-associated proteins is inherently highly cytotoxic (34). However results obtained

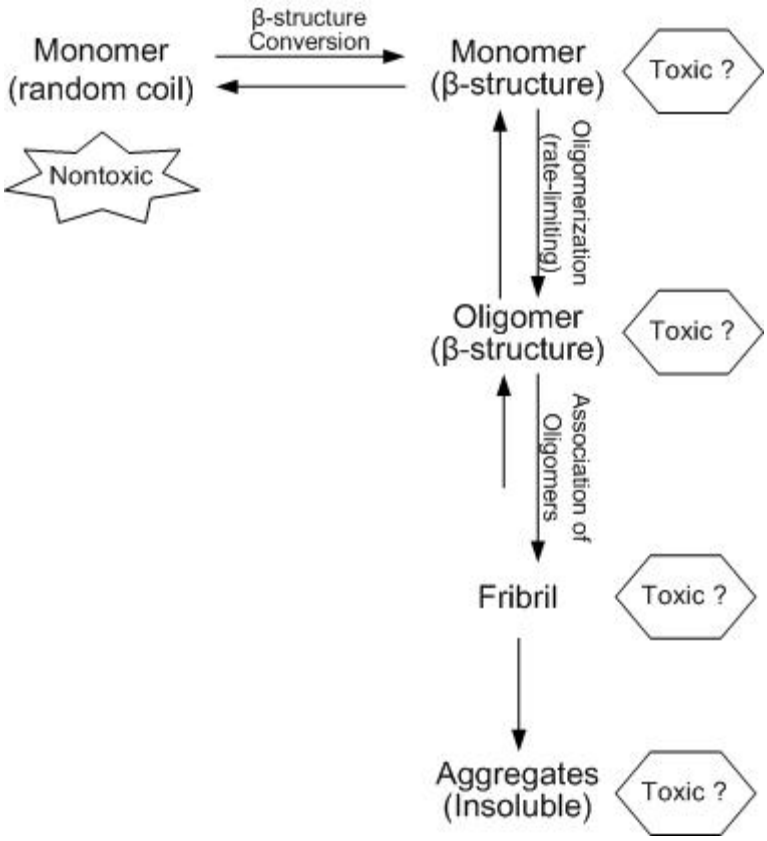

Figure 2A: PolyQ aggregation process Adapted from (64, 65)

PolyQ aggregation is proposed as a nucleation-dependent process (64). PolyQ-expanded proteins such as polyQ-expanded htt are not toxic when they present as monomer of random coiled structure. However, these monomers tend to have a conformation change to β-structured monomers (i.e. β-sheet, also see Figure 2B). These β-structured monomer then oligomerized into β-structured oligomers. Oligomerization is the rate limiting step of polyQ aggregate formation. Then, oligomers form the final insoluble aggregates. This step is fast. Whether the large insoluble polyQ aggregates trigger cell death is still the topic of much debate. β-structured monomers and oligomers may also be toxic although they do not form visible aggregates under light microscope (64, 65).

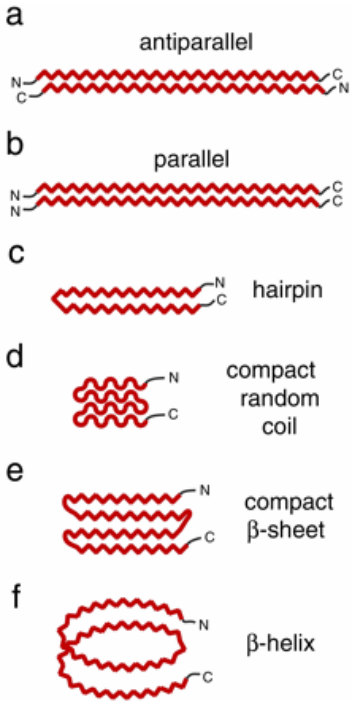

Figure 2B: Structure models of mutant polyQ monomer

From Ross, C.A., Poirier, M.A., Wanker, E.E. and Amzel, M. (2003) Polyglutamine fibrillogenesis: the pathway unfolds. *Proc Natl Acad Sci U S A,* 100**,** 1-3. (64)

Proposed models of PolyQ-expanded protein monomers: a. antiparallel poly-zipper; b. parallel poly-zipper; c. β-hairpin; d. compact random coil; e. compact β-sheet; f. β-helix (64). For detailed explanation of e and f, see text.

in a number of in vitro and in vivo models of polyQ repeat diseases have led to a re-examination of the relationship between aggregates and toxicity (35, 58, 67-69). In transfected primary striatal neurons, accumulation of polyQ-expanded htt within nuclear aggregates was enhanced by agents such as BDNF and ciliary neurotrophic factor (CTNF), or expression of Bcl-$_{XL}$, yet neuronal toxicity was reduced (35). Furthermore, cotransfection of a dominant-negative ubiquitin-conjugating enzyme with polyQ-expanded htt reduced aggregation but led to increased toxicity (35). In a transgenic mouse model of SCA-1, mutation of the self-association domain of polyQ-expanded ataxin-1 blocked its aggregation, but did not affect its ability to cause degeneration of cerebellar Perkinje cells (58). One way to explain these controversies is that microscopic detectable large stable aggregates may not be toxic. The toxic forms might be intermediates such as β-sheet monomers and small oligomers (65). This issue will be further discussed in Chapter III.

4. Molecular mechanisms underlying polyQ expanded protein induced neuronal toxicity

4.1 Loss or gain of function: The presence of an expanded polyglutamine tract in each specific gene product may induce toxicity by a loss of its normal function or a gain of toxic function of the polyQ-expanded protein (70). Because most of these diseases are autosomal dominantly inherited diseases, the first studies to examine whether a loss of function plays a role in polyQ diseases came from genetic analysis. Does the number of copies of the gene affect the severity of the disease? This proves to be true for DRPLA

and SCA6 (71-74). For example, a homozygote SCA6 patient showed an earlier age of onset and more severe symptoms than his heterozygote sister who had the same CAG repeat length in the ataxin-6 gene (71). However, a similar phenomenon does not apply to HD. Heterozygote HD patients have no improvement in phenotype compared to homozygotes (70). This indicates at least a simple loss of normal huntingtin function is not enough to induce a neurodegenerative phenotype (70). Therefore, a gain-of-function is thought to be the main cause of HD (70).

A polyglutamine peptide itself is sufficient to induced toxicity in vitro and in vivo (70). For example, a GFP tagged with a CAG expansion has been used in many cell culture studies and shown to be toxic (51). An in vivo model showed that mouse bearing hypoxanthine phosphoribosyltransferase gene (Hprt), with an artificially introduced expanded polyglutamine tract cause neurological symptoms and early death (75). Therefore, a gain of function by the presence of polyQ can induce a neurodegenerative disease phenotype. However, the importance of a loss of normal protein function can not be evaluated at the present time, especially because the functions of most polyQ disease proteins are not known yet (70) . In DRPLA and SCA6, loss-of-function is certain to have an important physiological function (70-72). A detailed discussion concerning this issue in HD pathogenesis will be presented in the next section.

4.2 Proteolysis: As mentioned earlier, several polyglutamine disease proteins including hungtintin, AR and ataxin 1 (38-40) were found to be more toxic when they are cleaved into a truncated form. Why are truncated forms more toxic and what enzymes are

involved in the proteolysis of full length proteins? It has been suggested that shorter forms of the protein more readily form oligomeric nuclei by relieving steric restrictions (76, 77). It is also reasonable to imagine that shorter forms of protein are easier to translocate into the nucleus (78, 79) and accumulate there to form nuclear aggregates. Several types of enzymes may be involved in the proteolysis of huntingtin, such as caspase 3/6 (49) and aspartic endopeptidases (80). In HD, the cleavage process is of particular interest because full-length htt protein is a large protein of 348 kD. The proteolysis process in HD and its importance will be discussed in the next section.

4.3 Aspects of polyglutamine induced toxicity:

4.3.1 Transcriptional activity: Because many transcription factors or cofactors contain short polyglutamine repeats or polyglutamine and proline-rich regions (81), several studies have examined whether their association with polyQ repeat proteins can impact gene transcription (32). For example, crucial transcriptional factors for neuronal survival such as TBP, Sp1, cofactor CBP have been found to interact with polyQ repeat proteins and, as a result, have their transcriptional regulatory activity reduced (46, 47, 82, 83). The reduction may be due to them being recruited into the aggregates such as CBP or interacting with a soluble form of polyQ protein such as Sp1 (46, 47). The main part of this thesis study focuses on the interference of CBP function in a HD cell culture model. Therefore, a very detailed introduction and discussion of this issue will follow in Chapter II.

4.3.2 Ubiquitin-proteasome degradation pathway (UPP): UPP is the main pathway for protein degradation and elimination of unwanted or toxic proteins in the cytoplasm (84). The UPP may attempt to degrade polyQ-proteins, but a failure to do so may result in polyQ aggregation. Several studies show that the presence of aggregates significantly inhibits UPP activity (33, 85). Some experiments addressed in chapter II of this dissertation study will provide new evidence concerning the effect on UPP and consequently UPP targeted protein by polyQ expanded htt.

4.3.3 Molecular chaperones: Molecular chaperones may be functioning in at least two aspects of polyglutamine diseases. First, they may be involved in the folding of polyQ proteins. If the folding is not successful, this may lead to the misfolded protein accumulation (62). Polyglutamine containing protein may also cause a cellular stress that induces the expression of some molecular chaperones (62). It has been suggested that the imbalance between the limited chaperones activity and progressive build-up of toxic polyQ proteins leads to the onset of these diseases (65). Chapter III of this dissertation will describe experiments that address the roles of molecular chaperones in a cellular model of HD.

4.3.4 Apoptosis: Morphological studies of the dying neurons in the polyglutamine diseases do not support clear apoptotic features. Electron microscopy (EM) examination of affected neurons in HD transgenic mice and patients' brains show neither apoptosis nor necrosis characteristics (86). However, many in vitro and in vivo studies show that caspases activation plays an important role in polyQ induced cell death. Caspases are

activated by the overexpression of polyQ-expanded diseases proteins. For example, caspase 8 activation has been shown in a HD cell culture model (87) and even detected in HD patient postmortem brain (51). Caspase 1 activation is detected in a HD mouse model (50). In all these cases, inhibition of the activation of these caspases can prolong the survival and improve the symptoms of transgenic mice. Wild-type full-length huntingtin, may directly act on the inhibition of caspase activation (70). For example, it has been shown that normal htt prevents caspase-3 activity in striatal neurons (88). In addition, caspases may have other functions. For example, as previously mentioned in the proteolysis section, caspase 3 may be an enzyme that cleaves the full length htt into the truncated form (49).

4.4 Selectivity of neuronal toxicity: Polyglutamine diseases proteins are expressed throughout the central nervous system. However, only certain subpopulations of neurons are affected (26). For example, in HD, only striatal and cortical neurons show pathological changes, although at every late stage other areas are also affected (89). In SCA1, only neurons in spinal cord and cerebellum are affected (57). Unfortunately there is no good explanation for selectivity so far. There are several possibilities. First, normal disease protein have specific function in the areas affected. For example, Htt has been shown to indirectly upregulate the transcription of BDNF in cortical neurons, which is an crucial neurotrophic factor released from cortex to the striatum (90). Therefore, striatal neurons are affected as a consequence of reduced BDNF expression in cortex. Second, each polyQ disease protein specifically interacts with other proteins such as apoptotic proteins and chaperones which may be enriched in the specific areas. The dysfunction of

these proteins may lead to specific toxicity (70). The third possibility, in HD or other polyQ diseases involving proteolysis of full length disease proteins, is that the proteolysis may be a cell-type specific process. A good example for this hypothesis comes from HD transgenic mouse model. Mouse bearing full-length polyQ-htt gene show selective neuronal toxicity in striatum, and truncated polyQ-htt is found in the striatum (91). However in other studies, transgenic mice bearing only N-terminal of htt with expanded CAG show toxicity in many areas of the brain with no selectivity (92). These results suggest that some proteins involved in the proteolytic process have specific expression pattern in the affected area in vivo.

IV HUNTINGTON'S DISEASE (HD)

1. Introduction:

Huntington's Disease is named for Dr. George Huntington, who first described this hereditary disorder in 1872 (26). 30,000 americans are affected by HD and another 150,000 run the risk of developing the disease (26). This is much more than that the number of people affected by other polyglutamine diseases. HD is also one of most well-studied polyglutamine diseases. Research on HD has provided many insights into the common mechanisms underlying the pathogenesis of polyglutamine diseases. This dissertation work focuses on some of mechanisms of HD. Therefore, a detailed introduction on HD will be presented here.

The gene causing HD was discovered in 1993 (93). The mutation that causes the disease is an expansion of CAG triplet repeats with polymorphism within *interesting transcription 15* (*IT15*) gene located on Chromosome 4p16.3 (93). The gene product of *IT15* is named huntingtin (htt), which is composed of 3114 amino acids (26). The CAG repeat in the coding sequence encodes a polyglutamine repeat within the N-terminal domain of the protein. Normal individuals have 6-35 consecutive CAG repeats in their htt gene (42). Expansion of htt from 36 to 41 glutamines is associated with an increased risk for the disease, whereas lengths of 42 and over invariably cause HD (42). HD is an autosomal dominantly inherited disease, with offspring of their affected parents having at least a 50% chance of inheriting the disease (26)

HD patients normally start to show symptoms between the ages of 35-45. It is a progressive neurodegenerative disease. The average course of the disease is about 17 years (89). Patients initially present with both cognitive dysfunction and motor disturbances such as chorea. They generally die from choking, infection or heart failure (89). The number of Q residues in the polyQ tract of htt in the patients is the most important factor in determining the severity of disease (70). Furthermore the age of symptom onset is inversely related to the number of CAG repeats in the huntingtin gene (38). For example, in juvenile cases of HD, the number of CAG repeat is sometimes beyond 100 (42). The severity of the symptoms is also positively related to the number of CAG repeats (70).

In normal individuals, the 348kD wild-type huntingtin protein is expressed throughout the body (94, 95). While htt has a higher expression in the brain, there is no evidence that it's expression is further elevated in the striatal and cortical neurons which are the affected areas in HD patients (94, 95). The wild-type htt protein is a cytoplasmic protein in the adult nervous system (94, 95). The function of normal htt has not been firmly established yet. Some of the proposed functions of htt will be discussed in the "underlying mechanisms of HD" section.

2. Pathology:

2.1 Gross pathology and neuropathological classification: The earlier and most affected areas of HD are the striatum and cortex (89). In striatum, the medial paraventricular portions of the caudate nucleus (CN), the tail of the CN and the dorsal part of the putamen present the earliest neuronal loss (89). The striatal medium spiny GABAergic neurons, but not the glia cells are the targets of degeneration (89). However, at very late stage of HD, degeneration affects most of the brain areas, rendering about 95% of neuronal loss (89). Classically, the severity of the diseases is classified into grades 0-4 by Vonsattel et al. (89). Patients of Grade 0 are clinically diagnosed, but without microscopically neuronal loss in striatum. In patients of grade 1, caudate nucleus has a 50% of neuronal loss with moderate fibrillary astrocytosis. However there is no gross atrophy. From grade 2, gross atrophy of the striatum becomes more evident with more neuronal loss and astrocytosis. At grade 4, patients show extensive neuronal loss that is not only confined to the striatum. For example, caudate nucleus shows 95%

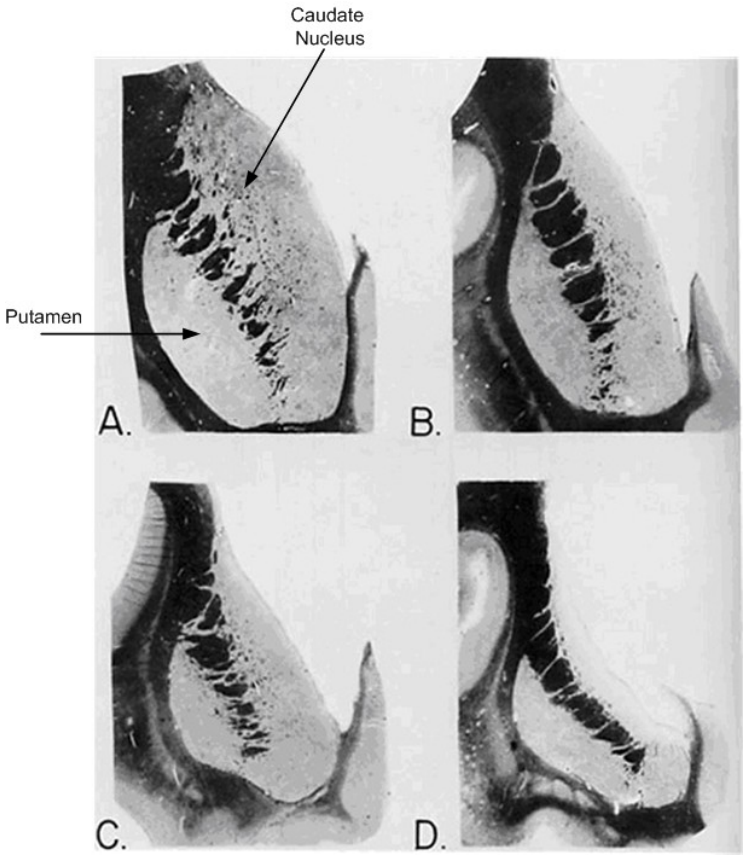

Figure 3: Striatum of HD patients at different disease stages

From Vonsattel, J.P., Myers, R.H., Stevens, T.J., Ferrante, R.J., Bird, E.D. and Richardson, E.P., Jr. (1985) Neuropathological classification of Huntington's disease. *J Neuropathol Exp Neurol,* **44,** 559-77. (89)

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Striatum is the earliest and most affected areas in HD. A-D shows the atrophy of striatum including the caudate nucleus and the putamen regions of HD patient postmortem brains at different stages of the disease. A: Stage 0-1; B: Stage 2; C: Stage 3; D: Stage 4.

neuronal loss at stage 4. Other brain region such as hippocampus is severely affected as well. Figure 3 shows the severity of atrophic striatum at CN and putamen in affected HD patients at different stages (89).

2.2 Aggregates: As mentioned above, intracellular aggregation is the most important microscopical characteristic of the polyglutamine diseases including HD (53). An understanding of polyQ protein aggregation and its prevention is a critical step for therapeutic strategies. Aggregates were first detected in HD patients' brain in 1997 by Difiglia et al. (38). In this study, some aggregates were stained positive with an antibody against the N-terminus of htt. These aggregates are detected both in the nucleus of striatal and cortical neurons (nuclear inclusion - NI) and within "dystrophic neurites". Some of these aggregates are also ubiquitin-positive. The frequency of subcellular sites of aggregates is closely correlated with the onset of the disease and the number of CAG repeats. In juvenile patients who have more than 100 CAG repeat, nuclear aggregates are more frequently seen than those in neurites (38). As in HD patients' brain, although polyQ-expanded htt shows expression throughout the brain, exhibiting the same pattern as wide type huntingtin (94, 95), aggregates are only confined to the striatum and cortex, the two most affected areas in HD even at late stages of the disease with brain atrophy in other areas(38, 96). However, the frequency of aggregates seen in cortex is higher than that seen in the striatum, although striatum is more affected than cortex (38).

3. Underlying mechanisms of HD pathogenesis:

3.1 Loss or gain of function?

The phenotypes of htt knockout mice models have been carefully reviewd by Cattaneo et al. (70). The mouse homologue of htt is called hdh. Mice that have both alleles of hdh knocked-out exhibit embryonic lethality before embryonic day 8.5, at the stage of gastrulation (97, 98). This result indicates that wild type hdh plays an important role in embryogenesis. However, heterozygote hdh knockout mice which only have a single copy of hdh gene appear to be normal without neurodegenerative symptoms (36, 97, 99). These results strongly support the idea that a gain-of-toxic-function by the polyQ expanded htt is the main cause of HD pathogenesis (70). Another study that supports this idea showed that a single copy of full-length hdh with an expanded CAG repeat can substitute for the wild-type hdh during embryonic neurogenesis (100). In contrast, in a conditional hdh knockout mouse model, inactivation of both alleles of hdh in adult mouse brain causes neurodegeneration (101) which strongly supports the idea that loss-of-function may also play a role in the HD pathogenesis. Therefore, hdh-knockout models suggest a role for both an gain-of-function and loss-of-function of polyQ-expanded htt in HD (70).

Do any human mutations implicate a pathological effect of htt loss of function? Wolf Hirschhorn Syndrome is a genetic disorder caused by a partial chromosomal deletion of the short arm from one copy of chromosome 4 (i.e. 4p16.3) (102), where the htt *IT15* gene is located . Patients with this syndrome show several growth retardation and mental

defects (102). The gene lost in the chromosomal region which is responsible for these symptoms is not known yet. However, these patients do not show symptoms of HD although one allele of IT 15 is deleted, suggesting that a loss-of-function of normal htt protein can not count for the pathogenesis for HD (103). As mentioned in the above section, heterozygotes patients with one allele of htt gene and an expanded CAG repeat do not show more severe symptoms and earlier onset than homozygote patients (70). This fact provides additional evidence to support that the hypothesis of a gain-of-toxic-function of polyQ-expanded htt protein is crucial to induce neuronal toxicity.

3.2 Loss-of-normal-function

As mentioned above, wide type htt clearly plays a role in embryonic development. Normal htt is a cytoplasmic protein, but it has been found to be loosely associated with endoplasmic reticulum, microtubules, mitochondria and synaptic vesicles. This suggests that normal huntingtin may be involved in vesicle trafficking (104). Recently, it has been shown that wild type htt may have other functions that contribute to the selectivity and neuronal dysfunction of HD. Zuccato et al. showed that full-length wild type htt can indirectly upregulate the transcription of BDNF in cortical neurons, BDNF is a neurotrophic factor critical for the survival of striatal neurons (90). In contrast, the transcription of BDNF is reduced by the full-length htt with an expanded polyQ tract (90). Some other studies show that wild-type htt has anti-apoptotic function through caspase inhibition. It functions downstream of Bcl-2 and upstream of caspase 3. Truncated forms of htt without an expanded polyglutamines does not have such a function (88). Some in vivo evidence also support a role for wild type htt in apoptosis. In

a conditional mouse model generated by Dragatsis et al, double-knockout of hdh alleles in adult mouse shows apoptosis in cortical and striatal neurons (101). In addition, the function of htt interacting proteins may also provide clues about normal function of htt. For example, htt interacting proteins HAP1 (Huntingtin-associated protein1) and HIP1 (Huntingtin-interacting protein 1) are also found to be associated with vesicles, supporting the role of normal htt in vesicle trafficking (70). HIP1, which interacts with wild type but not mutant htt, is a pro-apoptotic protein suggesting that normal htt blocks apoptosis through HIP inhibition (105).

3.3 Gain-of-toxic-function: aggregates in the nucleus and neuronal processes

Neurons expressing polyQ expanded htt exhibit neuronal aggregates, while those expressing only the normal range of Q residues never cause aggregates. The formation of aggregates is thought to bring new toxic functions to the affected neurons (53). Aggregation has been detected in numerous in vivo and in vitro systems including patients' brain, transgenic mice' brain, transgenic fly nervous system especially the photoreceptor neurons, primary neurons from mice and cell culture systems (38, 47, 48, 66). PolyQ aggregates are found in the nucleus, the cytoplasm and neuronal processes – axon and dendrites (53). Therefore, aggregates may induce toxicity in the nucleus, cytosol and the neuronal processes.

In the nucleus, the presence of aggregates interferes with gene transcription. PolyQ-expanded htt interacts with several crucial transcription factors and co-factors such as CBP, TBP and Sp1 and decrease their functions as transcriptional factors or cofactors.

Therefore the gene expression profile is significantly altered by polyQ-expanded htt (106). In the introduction of chapter II, the polyQ induced transcription deficiency will be carefully reviewed.

Aggregates of polyQ expanded htt in both the nucleus and processes often show recruitment of ubiquitin, other components of UPP and molecular chaperones (65). It has been shown that aggregates inhibit the UPP, leading to insufficient degradation of other proteins (33). In the introduction of Chapter II, the dysfunction of UPP by polyQ-expanded protein will be reviewed in detail.

Aggregates of polyQ expanded htt may also cause degeneration of neuronal processes, especially axons (60, 91). By examining of amount and sites of aggregates in HD patients, the presence of aggregates containing the N-terminal htt in the axons has been suggested to induce the degeneration of the corticostriatal pathway in earlier stages of HD (60). Other studies showed that the degeneration of neuronal processes induced by aggregates occurs before any evidence of cell death seen from the nucleus (107, 108). These results emphasize the possibility of axonal dysfunction prior to the appearance of an obvious nuclear defect. The presence of aggregates in neuronal processes may block neurotransmitter transport or uptake by synaptic vesicles (91, 107).

As aggregates are found to contain mostly truncated forms of htt with expanded polyglutamine, the proteolysis process of full length htt clearly is a critical step for the gain-of-toxic function of htt mutant phenotype in HD. Full-length htt with an expanded

polyglutamine tract is cleaved by certain proteases to form the truncated form, which then forms aggregates. In turn, the truncated and polyQ expanded htt may recruit wild type htt into the aggregates (70). The ability of certain subpopulation of neurons to cleave full-length htt may provide some basis for selectivity of neurodegeneration (91, 109). The truncation process may also partially explain why HD patients do not show symptoms before disease onset although they carry the mutant htt gene from the very beginning. Transgenic mice with full-length mutant htt gene have a much later disease onset and longer life span than those with a truncated mutant htt gene (106). This is presumably because truncation is a progressive process. Once truncation is completed, the aggregation process is thought to begin. In vivo, both normal and HD patients show truncated htt fragment, either with a normal or an expanded polyQ repeats (110). Therefore, htt cleavage is a normal event. Importantly the short N terminal fragment of htt forms aggregates and induces toxicity only when it includes an expanded polyQ tract.

The next question concerning the truncation process is what enzymes cleave htt. A few possibilities have been proposed. The first candidate is caspase 3. Full-length htt protein has several caspase 3 cleavage sites including the two active sites at amino acids 513 and 552 (49). In cell culture models, mutation on the htt caspase cleavage sites reduced toxicity (49). The size of htt fragments resulting from caspase 3 cleavage seems to be much larger that those found in vivo (111). Other studies suggest that calpain, another class of proteases, may further cleave these large fragments into small ones (111). Another candidate is aspartic endopeptidases, which may cleave htt into different fragments in the nucleus and the cytosol. After this cleavage, htt fragments may be

processed by UPP, or if they are not degraded, form nuclear or cytoplasmic aggregates (80). Since full-length htt does not contain a putative nuclear localization signal (53), the truncation may also facilitate the nuclear translocation of polyQ expanded htt.

3.4 Apoptosis

Regardless of whether neuronal toxicity is induced by a gain or loss of function of htt, affected neurons receive many inputs of cell death such as oxidative stress, excitotoxity, mitochondrial dysfunction and loss of neurotrophins. Affected neurons appear to undergo cell death including apoptosis. As mentioned above, some evidence supports the view that apoptosis occurs in affected neurons of HD. Caspase 1, caspase 8, HIP are activated to induce toxicity in HD models (50, 51, 87, 105). Inhibition of caspase activation by inhibitors may improve the symptoms in HD transgenic mice and prolong their life span (50). In addition, proapoptotic signal transduction pathways, such as JNK pathway, have been reported to be activated by polyQ expanded htt (112). The significance of these studies is that the blockage of apoptosis may be a promising strategy for therapeutics. Figure 4 summarizes how both the gain and loss of function mutations within htt could finally lead to cell death in affected neurons.

4. Therapeutics perspectives of HD

The targets of HD therapeutics are very similar to those discussed in the neurodegenerative disease section. Here, I will just summarize what have been done in HD mice and patients.

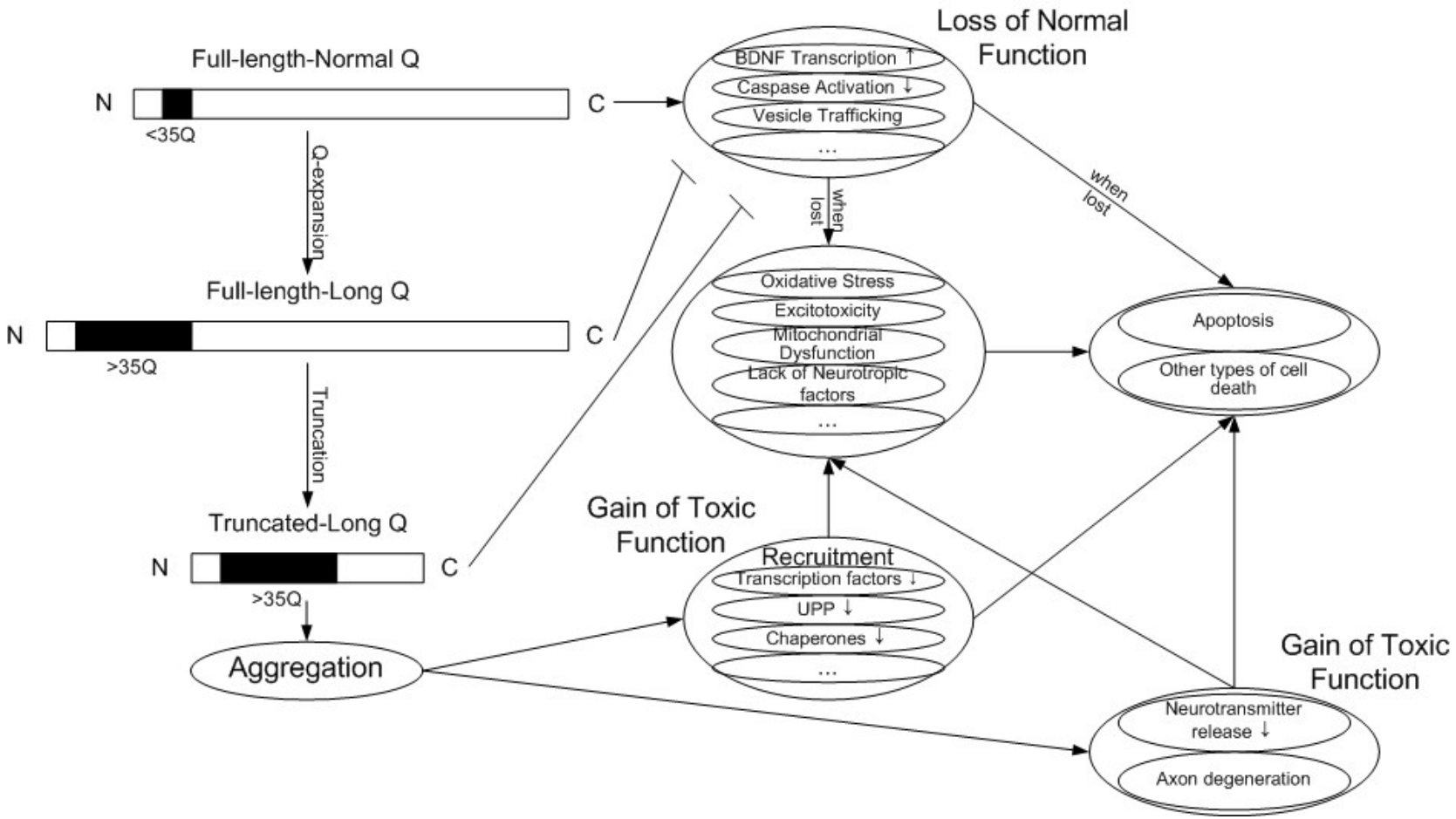

Figure 4: Mechanisms of HD pathogenesis

Legends of Figure 4: PolyQ-expanded htt induced neuronal toxicity is a result of a combination of a loss-of-normal function of full-length wild type protein and a gain-of-toxic-function of truncated protein with an expanded polyQ tract. Loss-of-function of wild type htt can lead to the downregulation of BDNF transcription and inteference of vesicle trafficking. Gain-of-toxicity can result in the recruitment of important cellular proteins into polyQ-htt containing aggregates such as transcritpional factors and components of the UPP. Both the loss and gain of functions can lead to disrupted neuronal functions in both the nucleus and in neurites, and finally result in neuronal death.

Although many reagents are effective in reducing polyQ induced toxicity in cell culture and lower organisms such as flies, various treatments on transgenic mice show only a limited improvement on symptoms and life span by 10-20 percent (113). Here are some examples: gutamate antagonists have been used to counter excitotoxicity (114). Caspase inhibitors have been used to prevent apoptosis (50). Transglutaminase has been used to prevent aggregation (115), while congo red is used to reverse aggregation (45). In patients, some neuroprotective agents have been used in clinical trials (113). Riluzole and remacemide which are glutamate antagonists and coenzyme Q10 may have an effect on motor function improvement, but these minor effects do not appear to be statistically significant (113). Although there is still a long way for the final goal of curing HD, more and more drug targets are being identified based on exploring the mechanisms of polyQ induced neurodegeneration.

CHAPTER II

DEGRADATION OF CREB-BINDING PROTEIN INDUCED BY POLYGLUTAMINE EXPANDED HUNTINGTIN

I. INTRODUCTION

1. Polyglutamine diseases and transcription deficiency

There have generally been two ways to examine whether transcription is compromised in polyglutamine diseases (106). First researchers have assessed the function of specific transcriptional factors. The main reason is that a number of proteins regulating transcription contain a polyQ region (such as CBP), Q-rich region (such as Sp1) or proline-rich region (such as p53) (32). Interactions between Q residues may interrupt the normal function of the proteins, and could result in their recruitment into aggregates in polyQ diseases proteins (32). This is very reasonable hypothesis since even wild type proteins such as atrophin or full-length htt with a normal range of Q tracts can be sequestered into these aggregates (70). As discussed below (Table 4), a number of important transcriptional factors and cofactors are found to interact with polyQ disease proteins, leading to the disruption of their functions (106).

Recent studies went on to look at gene transcription mainly by microarray analysis (106). A few microarray analyses have been done to look at the changes of mRNA levels in several polyglutamine diseases. These data reveal that some genes are similarly impacted

by polyQ in a context-independent manner (116). For example, SNFK1, a protein involved in histone phosphorylation has a consistent decreased level in transgenic mice of HD, DRPLA, SCA7 and SBMA, suggesting a potential role for histone regulation and gene transcription in polyQ diseases (116). Other genes that are consistently affected are those encoding neurotransmitter receptors, neurotransmitter and neuropeptides and signal transducing enzymes. (116). Many of the affected genes in polyQ disease microarray analysis contain binding sites within their promoters or enhancers for factors such as Sp1 and CREB, which are known to be affected in polyQ disease models (106) (see below).

1.1 Transcription factors affected by polyQ disease protein

Table 4 lists some of the well-known transcriptional factors or cofactors that are affected by one or more polyglutamine diseases. They include transcriptional factors such as Sp1, coactivators such as CBP and TAFII130 and proteins in the basal transcriptional machinery such as TBP. Some of them such as CBP and TBP are recruited into the polyQ aggregates, indicating they interact with insoluble polyQ disease proteins (47). Some such as p53 and Sp1 are not recruited into the aggregates, indicating they interact with a relatively soluble fraction of polyQ expanded proteins, including "microaggregates" that are not detectable under the light microscope (46, 82). The putative interaction domains are mostly the polyQ, Q-rich or proline-rich regions of the transcription factors (32). Recently, proteins such CBP and pCAF have been suggested to interact with polyQ disease proteins via their histone acetyltranferase domains (HAT) (48). These interactions interfere with the transcriptional activation function of these proteins. Some of these transcriptional factors preferentially interact with the disease protein with an expanded

polyQ tract (32). These tighter interactions normally decrease their activity (32). As mentioned above, some of genes regulated by these factors are crucial for neuronal survival (106).

Sp1 and CBP are the two most well-studied transcription factors that are affected in polyglutamine diseases. Here I will briefly discuss Sp1 as an example of how interference in the function of a transcriptional factor could cause toxicity in polyQ disease. In the next section, CBP will be particularly emphasized. Sp1 is a transcription factor that binds to the promoter DNA region of many genes such as dopamine D2 receptor (46). Sp1 binds more tightly with polyQ expanded htt in vivo (i.e. patients) and in vitro (cell culture) (46). However, Sp1 is not recruited into the aggregates, indicating that it interacts with the soluble form of htt-polyQ (46, 117). In cell culture models, overexpression of Sp1 can rescue the toxicity induced by polyQ-expanded htt including a neurite extension defect (117). Sp1 dependent transcription is decreased by polyQ-expanded htt. This result is supported by the in vivo evidence that dopamine D2 receptor expression is significantly lowered in the striatum of HD patients at early stages of the disease (46, 117).

1.2 CREB-binding protein (CBP) and polyQ induced neuronal toxicity

Effects of polyQ disease proteins on CBP, a transcriptional coactivator, have received considerable attention, given the established role for CBP and its target transcription factors (e.g. CREB) in neuronal cell survival (21). The major goal of this thesis is to elucidate the mechanism of the interruption of CBP function by polyQ-

Table 4: Transcription factors and cofactors affected by polyglutamine diseases Adapted from (32, 106)

Transcriptional factor	Nuclear function	polyQ diseases	Interaction domain	Differential binding with short or long CAG	Recruitment into the aggregates	Transcription affected	Target genes that are affected	Effect on polyQ induced toxicity
CBP	Transcriptional coactivator/HAT	HD, DRPLA, SCA3, SBMA	polyQ HAT	more tightly with mutant	Yes	Yes	Genes regulated by creb	Yes
p300	Transcriptional coactivator/HAT	HD	N/A	N/A	No	Yes by decreasing HAT activity	N/A	N/A
Sp1	Transcriptional factor	HD	C-terminus	more tightly with mutant	No	Yes	Dopamine D2 receptor	Yes
TBP	Basal transcriptional machinery	HD, SCA3	polyQ		Yes	N/A	N/A	N/A
p53	Transcriptional factor	HD	N/A	more tightly with mutant	No	Yes	N/A	N/A
TAFII130	Transcriptional coactivator	HD, SCA3	Q-rich	NO	Yes	Yes	CBP/SP1 regulated genes	Yes
PQBP-1	Putative transcriptional corepressor	SCA1	N/A	Yes	Yes	Reduce Pol II phosphorylation	N/A	Yes
CtBP	Transcriptional co-repressor	HD	N/A	N/A	N/A	Yes	N/A	N/A
p/CAF	HAT	HD	HAT	Yes	N/A	N/A	N/A	N/A

expanded htt. Therefore, I will provide a thorough introduction of CBP including its structure and functions. I will also review published data concerning how its function is interrupted in polyglutamine diseases.

1.2.1 CBP (CREB-binding protein): structure and functions

CBP is so named because it was first identified as the transcriptional coactivator for CREB. CBP, a large protein of 265kD, and its closely-related homolog p300 are transcriptional co-activators that can influence multiple functions including cell growth, proliferation, differentiation and apoptosis (118). As shown in Figure 5, CBP/p300 have interaction domains with hundreds of proteins (118).

Although CBP can be divided into several domains, only some of them which are related to this study will be highlighted here. For example, CBP interacts with CREB via the KIX CREB-binding domain, which also binds to other transcriptional factors (118). CBP binds with Mouse double minute 2 (Mdm2), a human E3 ubiqitin-ligase of UPP through the first Zinc finger domain. This interaction is important for degradation of p53 by Mdm2 (119). The HAT domain in the middle region of CBP carries its histone acetyltransferase activity (118). The glutamine/proline-rich (QP) domain near the C-terminus associates with other coactivators including HAT proteins P/CIP and SRC-1 (118). Both N- and C-terminal regions of p300/CBP have transcription activation activity when they are fused to a heterologous DNA binding domain (120). In addition,

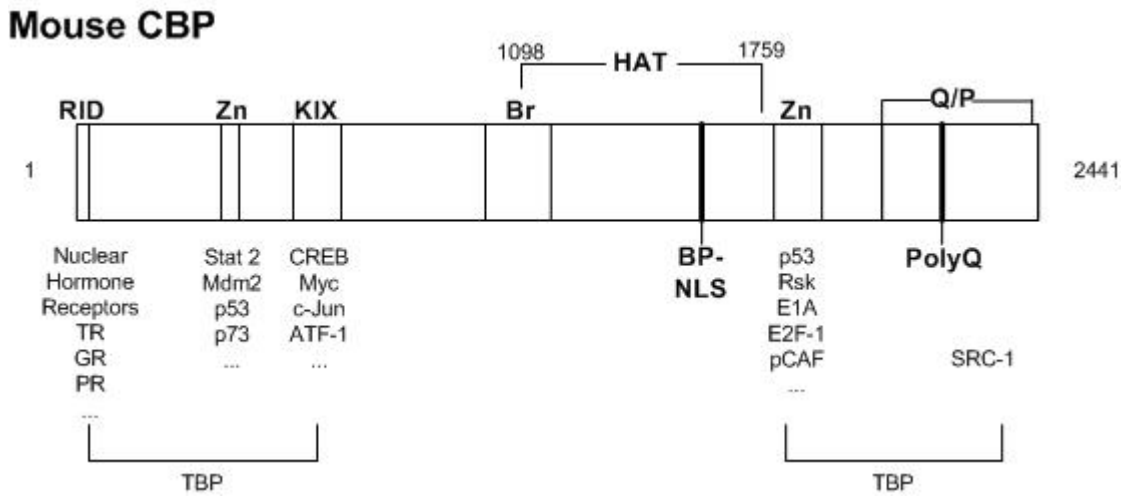

Figure 5: Functional domains of CBP/p300 Adapted from (118, 121)

CBP/p300 have multiple functional domains with the ability to bind with numerous proteins. RID: Receptor binding domain. Q/P: Glutamine/Proline rich. BP-NLS: Biparate nuclear localization signal. See text for details.

there is a bipartite nuclear localization signal (NLS-BP) located in the middle region of CBP/p300 (118). CBP/p300 could implement their transcriptional coactivator activity by three different mechanisms as shown in Figure 6 (118). In one case, CBP/p300 acts as a bridge to connect certain transcriptional factors such as CREB with the basal transcriptional machinery (i.e. RNA polymerase II, TBP and other TAFs). In some cases, transcription of certain genes requires the coordination of multiple cofactors. Thus CBP/p300 could also act as a scaffold to recruit several other proteins including proteins with HAT activities. Finally the HAT activity of CBP/p300 could directly affect transcription (122, 123). HAT proteins are able to transfer an acetyl group to the ε-amino group of a lysine residue of the histone molecule. The acetylation level of chromatin-associated histones plays a critical role in regulating transcription (118). High acetylation levels of histones represent an active transcription region of the chromatin. In contrast, low acetylation marks repression of transcription in that region (28). Therefore, proteins such as CBP/p300, SRC-1 and pCAF that have intrinsic HAT activities can directly activate transcription by remodeling chromatin structure. Histone deacetylases have opposite functions to that of HATs. The balance between these two types of enzymes plays an important role in transcription regulation. As a result, abnormal histone acetylation is involved in diverse human diseases including leukemia, solid tumors, and Rubinstein–Taybi Syndrome (RTS, (28). In addition to nucleosomal histones, CBP/p300 can modify the acetylation level of other transcriptional factors as well in order to regulate their activities (120).

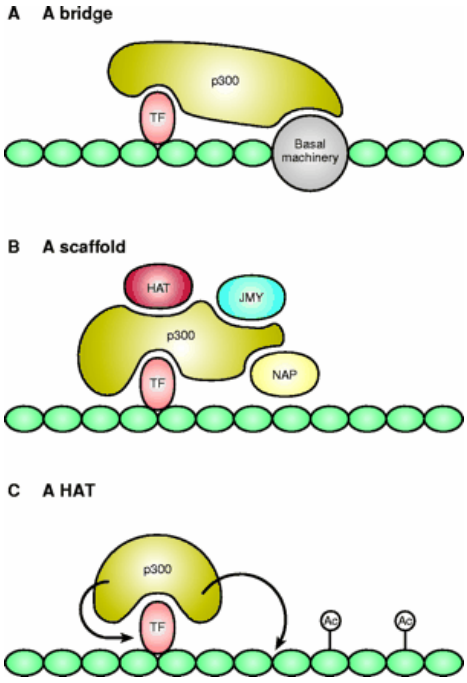

Figure 6: Models of CBP/p300 function as a transcription co-activator

From Chan, H.M. and La Thangue, N.B. (2001) p300/CBP proteins: HATs for transcriptional bridges and scaffolds. *J Cell Sci,* **114,** 2363-73 (118) Copyright of The Company of Biologists LTD, 2001

CBP/p300 may function as a transcription co-activator by three distinct mechanisms: In model A, CBP/p300 act as a bridge to connect the basal transcriptional machinery with transcription factors such as CREB to activate gene transcription. In model B, when the transcription of certain genes required the coordination of transcription factors and multiple co-factors, CBP/p300 act as a scaffold to recruit all of these factors. In model C, CBP/p300 utilize their intrinsic HAT activity to acetylate chromatin and transcriptional factors to regulate transcription (118). Although this figure depicts the models for p300, similar mechanisms apply to CBP as well.

Based on the fact that CBP/p300 have numerous functions in a cell, it is very possible that their amount is limited (121). Mutations within a single copy of the CBP gene have been detected in RTS (124) and some types of acute leukemia (125). RTS is an autosomal-dominant disease with mental retardation, skeletal abnormalities and a high incidence of neoplasia (124). Most RTS patients are found to have only one normal CBP allele. In CBP knockout mice, homozygotes are embryonic lethal, while heterozygotes may be embryonic lethal or have other developmental deficiencies with some symptoms similar with RTS patients (126, 127). These in vivo results indicate that CBP levels are limiting under physiological circumstances and may be involved in multiple pathways (118).

1.2.2 CBP and polyglutamine diseases:

CBP is recruited into the polyQ aggregates in cultured cells, primary neurons, transgenic mice and postmortem brains of multiple polyglutamine diseases including HD, DRPLA, SBMA and SCA3 (47, 128). This suggests that the interruption of CBP function is a common mechanism underlying the pathogenesis of polyglutamine diseases.

Nucifora et al. found that endogenous and transfected CBP are recruited into both nuclear and cytoplasmic aggregates of cultured cells expressing an htt construct with an expanded CAG repeat (Htt-N63-148Q). CBP is localized to the aggregates of HD patients' brains as well. In rat primary cortical neurons, CBP effects on CREB are impaired, leading to reductions in CRE-dependent transcription and toxicity (47). The importance of this

reduction in CBP activity in expanded polyQ toxicity was revealed by experiments which showed a rescue of toxicity by CBP overexpression (47).

What specific function of CBP is disrupted by this aberrant interaction and how can these findings lead to therapeutics of polyglutamine diseases? In another pioneering paper by Steffan et al., it was found that the HAT activities of CBP, p300 and pCAF are significantly reduced by polyQ expanded proteins as measured by acetyl-H4 and acetyl-H3 levels. This leads to toxicity. Mostly importantly, in an htt-polyQ transgenic fly model, which showed degeneration in flies' photoreceptor neurons, treatment with histone deacetylase inhibitors, which increases the acetylation level of chromatin, rescued polyQ induced toxicity (48).

These two studies along with others show that the interruption of CBP function is critical in polyQ induced toxicity. In the two studies mentioned above, two domains of CBP are suggested to interact with polyQ-expanded huntingtin. The paper by Nucifora et al. emphasized the polyQ domain of CBP which localizes at the C-terminus (47). In this domain, CBP differs from most other transcriptional factors including p300 and Sp1 in that it contains a short polyglutamine repeat region. In human, there are 18 consecutive Q residues. In mouse, there are 15 (47). p300 and Sp1 only have Q-rich regions (47). In the paper, it was shown that a CBP mutant in which the polyQ region is deleted (CBPΔQ) was not recruited into the aggregates formed by htt-polyQ, and was not co-precipitated with htt-polyQ protein (47). The paper by Steffan et al. showed that the HAT domain of CBP and pCAF is the domain that interacts with the htt-polyQ (48). The region

containing the HAT activity of CBP is not exactly defined. It covers several domains, and may span from amino acid 1098 to 1758 of CBP (Figure 5) (120). In the study by Steffan et al, they used a GST pull down assay to assess the ability of different fragments of CBP to bind with polyQ (120). As shown in Figure 7, the fragment of amino acids 1459-1877 of CBP has the strongest affinity with a htt exon1-51Q protein (48). The fragment covers part of HAT domain. In addition, for pCAF which is another HAT protein, its c-terminal region including the HAT was also co-immunoprecipitated with the htt-polyQ protein (48). In contrast, the C-terminal fragment of CBP which covers the polyQ domain did not show binding with htt-polyQ (48). They concluded that it is the HAT domain of CBP that interacts with polyQ expanded huntingtin. Therefore, htt-polyQ may interact with CBP through its polyQ domain or the HAT domain.

2. Polyglutamine diseases and ubiquitin-proteasome pathway (UPP)

Degradation of proteins in cells is mostly controlled by two pathways. The first is the vacuolar pathway such as through lysosomes, endosomes and endoplasmic reticulum. (84). For example, the lysosomes primarily degrade membrane proteins and extracellular components that are engulfed into the cell by endocytosis (62). The second is the ubiquitin-proteasome pathway (UPP). Although it was only identified 20 years ago, it has been found to be responsible for the degradation of most cellular proteins. Therefore, this pathway plays vital roles in numerous cellular processes (84). Caspases were identified as a way to partially degrade certain proteins. They cleave after aspartate residues of the

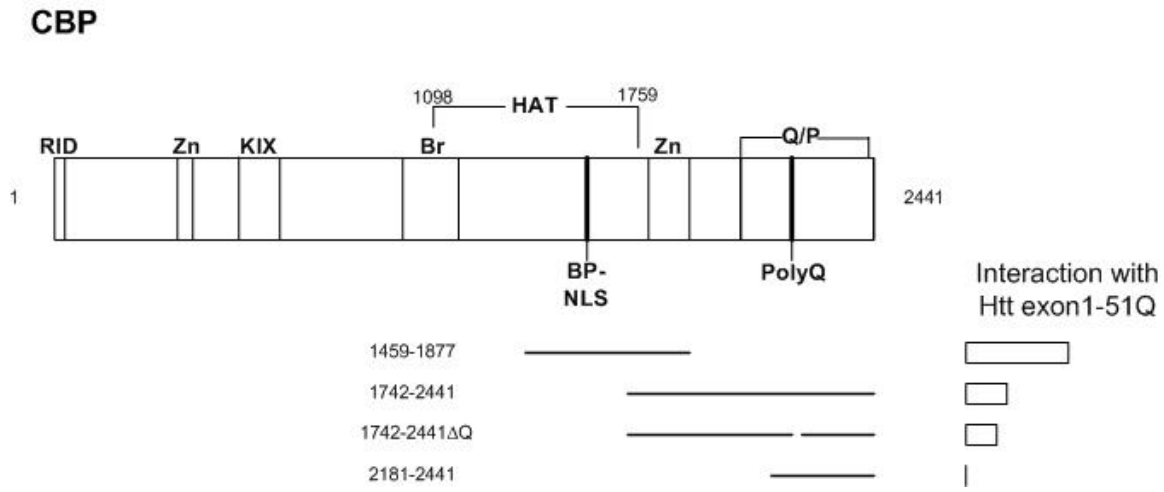

Figure 7: The HAT domain of CBP interacts with polyQ-expanded htt in vitro

Adapted from (48)

GST pull-down assays performed by Steffan et al. showed that amino acids 1459-1877 of CBP has the highest affinity for the htt exon1-51Q protein by GST pull-down assay (48). This fragment of CBP contains part of the HAT domain. In contrast, Nucifora et al. (47) found that the fragment containing the polyQ domain of CBP (amino acids 2181-2441) did not bind with the htt exon1-51Q protein in GST pull-down assay.

substrate (129). Caspases are mostly involved in signal transduction pathways that are crucial for apoptosis. As mentioned earlier, in the caspase cascade, caspases are cleaved by their upstream caspases and after activated cleavage downstream caspases or other targeting proteins. Similar to caspases, calpain-like proteases also function to cleave their substrates and partially degrade them (130). For example, calpains cleave lamin and other proteins which results in apoptotic morphology under certain circumstances (130). In addition, caspases and calpains may also function to cleave proteins outside the apoptotic signaling pathways. As mentioned earlier, both types of proteases may be able to cleave full-length htt protein into truncated and toxic fragments (111).

2.1 Ubiquitin-Proteasome Pathway (UPP): process, components and general functions

UPP contains multiple components and degrades targeted proteins in a multi-step manner. The whole process mainly involves two distinct stages, polyubiquitylation of the target protein, followed by its degradation by the proteases in the 26S proteasome (Figure 8)

In order for a protein to be degraded by the UPP, it must be tagged with a special polypeptide called ubiquitin, which serves as a marker for the recognition of proteasome (84). Ubiquitin is a polypeptide of 76 amino acids, which is well conserved in various species (84). Ubiquitin is attached to the protein for degradation by a covalent bond

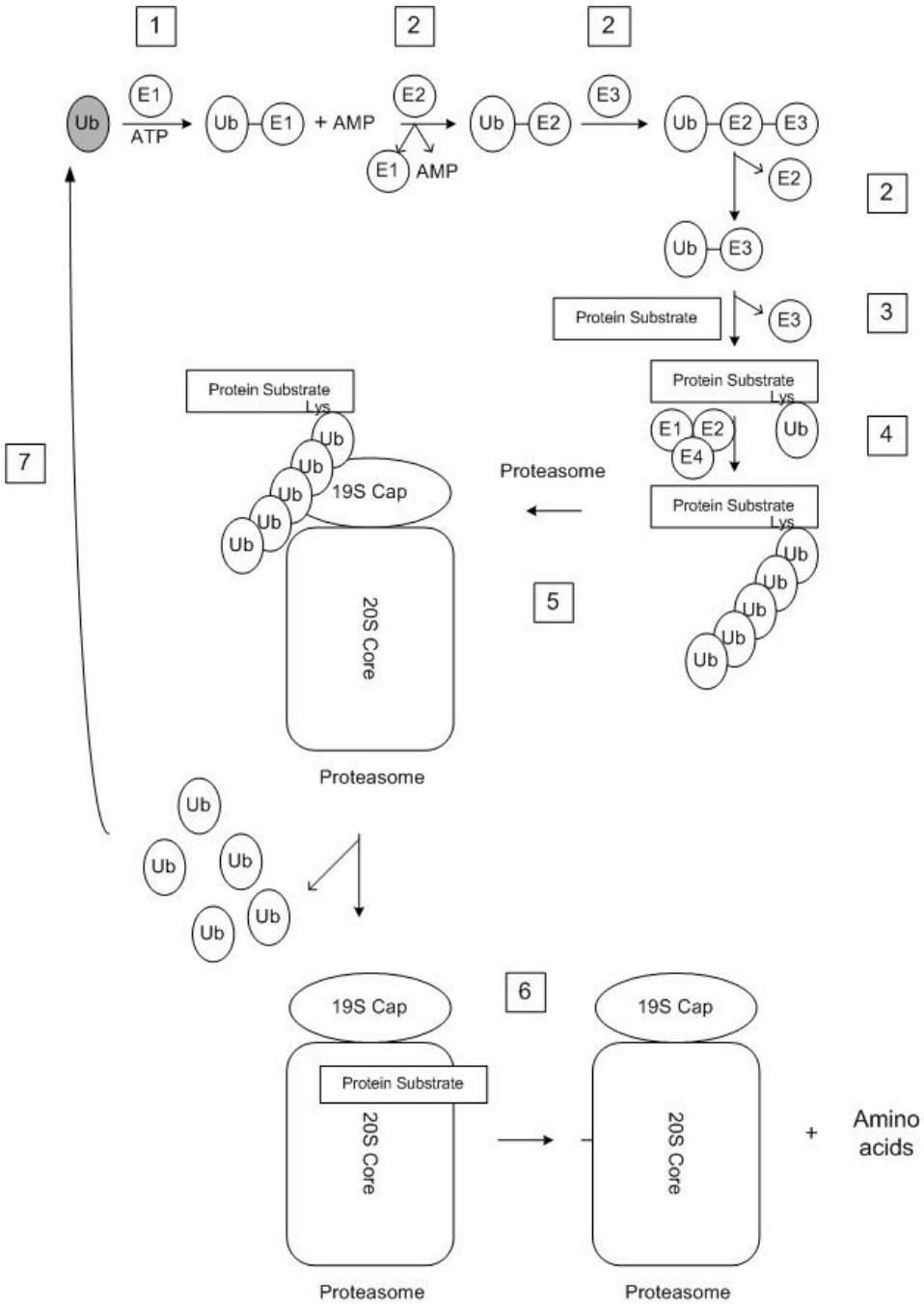

Figure 8: Schematic representation of ubiquitin proteasome pathway (UPP) for protein degradation

Legends of Figure 8: The process of protein degradation via UPP involves the following steps:

1) Ubiquitin molecules are activated by an E1 ubiquitin-activating enzyme;

2) Activated ubiquitin molecules are transferred to an E3 ubiquitin ligase by an E2 ubiquitin conjugation enzyme;

3) E3 tranfers ubiquitin molecules to the protein substrate;

4) Polyubiquitin chain formation is catalyzed by an E4 (ubiquitin chain assembly factor) along with E1 and E3 enzymes.

5) The polyubiquitin chain serves as a tag for the protein substrate to be recognized by the 19S cap of the proteasome;

6) The protein substrate is degraded by the 20S catalytic core of the proteasome to single amino acids;

7) Ubiquitin moleculars are recycled.

See text for details.

between an ε-NH2 group within an internal lysine residue of the protein. However, the preparation of this attachment requires another three enzymes, E1, E2 and E3 (84). At the very first step, ubiquitin must be activated by E1, ubiquitin-activating enzyme. Ubiquitin is activated to a high-energy thiolester on the glycine residue of its C-terminus by E1 enzymes (84). After being activated, an E2, so called ubiquitin-carrier protein or ubiquitin conjugating enzyme, transfers the active ubiquitin to an E3 (84). E3 enzymes are called ubiquitin ligase, and bind either directly or indirectly to some specific proteins targeted for degradation (84). Therefore, the activated ubiquitin is attached to the protein which is recruited by E3. After the first ubiquitin molecule is attached to the protein, multiple ubiquitin molecules are added to the first ubiquitin on its own lysine 48. The step of polyubiquitin chain formation is catalyzed by E4 enzymes (ubiquitin chain assembly factor) along with E1 and E2 enzymes (131). The polyubiquitin chain serves as a marker for the recognition of 26S proteasome and subsequent degradation (84).

There is only one human E1 gene, whose deletion is lethal (84). There are two isoforms of E1 that function either in the cytosol or the nucleus (84). There are more than a hundred E2s have been identified in mammalian cells. They define some specificity for the proteins that are being degraded (84). However, most of selectivity comes from E3 enzymes. There are hundreds of E3s in mammalian cells. Each of them can only bind with one or a few proteins and target them to the proteasome (62). There are several families of E3s, such as E3α and E6-AP. Degradation of a protein by the proteasome may require ubiquitylation at more than one lysine residues, and ubiquitylation of a protein may not necessarily lead to degradation. Usually, the site of lysine residues for ubiquitin

attachment are different for purposes of degradation versus internalization and endocytosis (84).

The 26S proteasome is composed of a 19S recognition cap and a 20S catalytic core. The 19S recognition cap recognizes and binds the polyubiquitin chain and unfolds the protein before sending it to the catalytic core (132). The 20S core is composed of six catalytic sites, each of which also contains multiple subunits. They carry out three well-studied proteolytic functions including the trypsin-like, chymotrypsin-like and post-glutamylpeptidylhydrolytic activities. All these enzymes work together to degrade proteins to small peptides (133), which are in turn rapidly hydrolyzed to amino acids by cytosolic peptidases (62). Proteasomal inhibitors can target the 20S catalytic core to inhibit the activity of UPP, therefore block general protein degradation. For example, compounds like lactacystin, MG132 and ALLN are widely used in research studies regarding the functions of UPP (33).

Disruption of the UPP leads to a variety of diseases including cancers and neurodegenerative diseases. The disruption can be caused by the loss-of function of a component of the UPP; or a mutation of a protein which leads to its insufficient degradation and excessive deposition. Accelerated degradation of certain protein targets of the UPP has also been found (84). Most frequently, in a disease state, more than one of these circumstances may occur simultaneously.

2.2 UPP and polyglutamine diseases

As mentioned previously, the primary reason prompting researchers to study the roles of UPP in neurodegenerative diseases is that insoluble aggregates present in these diseases are usually ubiquitylated (62) (Table 2). Many types of proteins could be the targets for UPP degradation such as the proteins damaged by oxidants, incomplete proteins and free subunits of multmeric protein complexes (62). Of particular importance for polyglutamine diseases is misfolded proteins that are caused by polyQ mutations. An expanded polyglutamine tract within the disease proteins causes its aberrant folding which ultimately leads to its aggregation. The recruitment of ubiquitin and other proteasomal subunits (29, 62, 65) into polyQ containing aggregates indicates that UPP may be recruited in an initial effort to degrade these misfolded proteins. This is confirmed by other in vitro and in vivo assays. For example, treatment of proteasome inhibitors leads to more aggregates in both SCA1 and HD cell culture models (29). Both wild-type and mutant ataxin-1 can be ubiquitylated in vitro. However, the mutant can not be degraded by the proteasome as fast as that of the wild-type (29). Similar results were found in HD cell model as well (85). In a conditional HD transgenic mouse model, the reversal of aggregation leads to improvement of symptoms (44). However, the elimination of aggregates is dependent on the normal proteasome function, as lactacystin, a proteasomal inhibitor blocked aggregate clearance (134). Taken together, these data suggest that the polyglutamine expanded proteins undergo degradation through the UPP. However, despite of the effort of UPP, it is still unable to degrade the misfolded polyQ proteins adequately which finally leads to aggregation.

Recruitment of some of the components of UPP into the aggregates may deplete them and ultimately inhibit its normal ability to degrade other cellular proteins. This could contribute to cellular toxicity. Aggregation of polyQ-expanded proteins within cells has been found to reduce proteasomal function and contribute to toxicity (33, 85, 135). For example, Bence et al. showed that presence of either cystic fibrosis transmembrane conductance regulator (CFTR) or htt-polyQ aggregates impaired UPP activity in the cell, leading to insufficient degradation of a coexpressed unstable GFP construct (33). Another study shows that proteasomal function is increased in nuclear fraction of cells expressing htt-polyQ aggregates, while that of the cytoplasmic fraction is decreased (85). Overall, the presence of aggregates does influence normal UPP function which is harmful to the cells.

In the majority of this thesis study, we used a hippocampal neuronal cell line (i.e. HT22 cells) to examine the effects of polyQ-expanded htt on CBP localization and expression. As observed in other cell culture systems, the subcellular localization of expanded polyQ htt aggregates was an important determinant in its effects on HT22 cell toxicity. While these cells were useful in confirming the recruitment of endogenous CBP into expanded-polyQ htt aggregates, they also revealed unique effects of htt on CBP accumulation. Loss of CBP function associated with polyQ-expanded htt resulted from either CBP recruitment into nuclear or large perinuclear inclusions or its degradation, and was shown to be directly related to toxicity as assessed in single cells by TUNEL assays. Furthermore, the selective enhancement of CBP ubiquitylation in HT22 cells expressing polyQ-expanded htt demonstrates that UPP may be mobilized to specific substrates when

cells are challenged with disease-causing polyQ-expanded proteins. Some preliminary experiments were performed to examine mechanisms that may underlie the selectivity of CBP degradation.

II MATERIAL AND METHODS

1. Plasmids

The htt (i.e. Htt-N63-19Q and Htt-N63-99Q) and CBP expression vectors (without Flag tag) used in these studies have been described previously (136). The HA-tagged ubiquitin plasmid (137) was obtained from Dirk Bohmann (University of Rochester). The full-length Flag-CBP plasmid was obtained from Dr. Richard Goodman (Oregon Health Sciences University).

Flag-CBP-ΔQ was constructed from the full length Flag-CBP plasmid by the following procedures. The aim of this cloning is to substitute the 45 nucleotides (CAG/CAA) which encode 15 consecutive glutamine residues with an EcoRI site "GAATTC". This polyQ domain is located at the C-terminus of CBP protein. Two PCR products were made before and after the "polyQ" site. The PCR product to the N-terminus is made from two primers: 5'CCAGCAGCAGATCCAGCATCGC3' and 5'CCGGAATTCGTGCTGTAGCAGCTGTCT3' (underlined is the EcoRI site). The PCR product to the C-terminus is made from two primers: 5'CCGGAATTCAATAGTGCCAGCTTGGCC3' (underlined is the EcoRI site) and

5’CTCTAGCATTTAGGTGACACTA3’. The two PCR products were cut with SgrAI / EcoRI and EcoRI / NotI respectively. Then they were ligated with the large fragment digested from SgrAI / NotI of the original full-length Flag-CBP plasmid. Flag-CBP-ΔQ was then sequenced to confirm the correct sequence.

Flag-CBP-ΔHAT was constructed from an in-frame deletion of the nucleotides encoding amino acids 1446-1866. To do this, the full length Flag-CBP vector was digested with BspE1/SgrAI. The large fragment from this digestion was ligated back itself to complete the construction of the Flag-CBP-ΔHAT construct.

2. Cell culture and transfection

Mouse HT22 cells were maintained in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA, USA) and penicillin/streptomycin (BioWhittacker, Walkersville, MD, USA). Mouse neuroblastoma 2a (N2a) cells (American-type culture collection, ATCC), Manassas, VA, USA) were maintained in minimum essential medium Eagle (ATCC) supplemented with 10% Vitacell FBS (ATCC) and penicillin/streptomycin. Cells were grown on coverslips in 6-well plates (Corning Incorporated Life Sciences, Acton, MA, USA) to ~50% confluency at the time of transfection for IIF. For Western blot experiments, cells were growing on 60 mm plates. Transfections were performed using lipofectamine with cells maintained in serum free medium as recommended by the supplier (Invitrogen). Following 5 hours of exposure to the DNA–lipofectamine mixture, cells were refed with

medium containing 10% FBS. Following an additional 24–48 h incubation, cells were fixed and processed for indirect immunofluorescence (IIF). Lactacystin (Sigma Chemicals, St Louis, MO, USA), when used, was added 18 h after transfection to attain a final concentration of 5 μM. In this case, cells were harvested after additional 24 h incubation.

3. Antibodies and IIF

Cells grown on glass coverslips were fixed and permeabilized using 2% paraformaldehyde (Sigma) and 0.1% Triton X-100 (ICN Biomedicals, Costa Mesa, CA, USA) in PBS, pH 7.4 for 10 minutes. Fixed cells were incubated with primary antibodies diluted in PBS plus 10% goat serum at appropriate concentrations for 1–1.5 h at 37°C. The rabbit anti-c-myc A-14 antibody (Santa Cruz Biologicals, Santa Cruz, CA, USA) was used at a 1:200 dilution, the mouse anti-CBP C-1 antibody (Santa Cruz Biologicals) at 1:50-1:100, mouse anti-Sp1 1C6 antibody (Santa Cruz Biologicals) at 1:50 and mouse anti-ubiquitin P4D1 antibody (Santa Cruz Biologicals) at 1:100. Following primary antibody incubation, cells were then washed and incubated with Rhodamine anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at 1:250 and FITC anti-mouse IgG (Chemicon International, Temecula, CA, USA) at 1:400, and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma) at 1:1000 for 1 h at 37°C before mounting. Coverslips were mounted onto slides using vectashield-mounting medium (Vector laboratories, Burlingame, CA, USA). IIF staining was examined using conventional fluorescence microscope. Approximately 100 transfected cells were counted

in each experiment. Data are presented as mean±SEM of at least three separate experiments with statistical analysis performed using Student's *t*-test.

4. Western blot analysis and immunoprecipitation (IP)

Protein extracts from transfected HT22 cells were prepared by lysis in buffer containing 50 mM Tris–Cl (pH 7.5), 2 mM EDTA, 100 mM NaCl, 1% NP-40 and a protease inhibitor cocktail (1:1000) (Sigma). Following a 5 min centrifugation of the extract at 14 000 *g* at 4°C, detergent-soluble supernatant and detergent-insoluble pellet fractions were collected. The insoluble fraction was solubilized with an equal volume of 2**x**SDS–sample buffer (1**x**=62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 20% β-mercaptoethanol, 2% SDS) prior to loading onto gels while the soluble fraction was adjusted to a final concentration of 1**x** in SDS–sample buffer prior to loading. Unless specifically indicated, identical amounts of total protein were loaded per well. Western blot analysis was performed essentially as described previously (138). Primary antibodies used were the mouse anti-CBP C-1 antibody (Santa Cruz Biologicals) at 1:100 dilution, the BuGR2 anti-GR monoclonal antibody (Affinity Bioreagents, Goldon, CO, USA) at 1:2000 dilution, the mouse anti-c-Myc 9E10 antibody (Santa Cruz Biologicals) at 1:200 dilution, the mouse anti-flag antibody (Sigma) at 1:1000 dilution, the mouse anti-actin C-2 antibody (Santa Cruz Biologicals) at 1:500 dilution, the mouse anti-Mdm2 SMP14 antibody (Santa Cruz Biologicals) at 1:500 dilution, or the goat anti-lamin B M-20 antibody (Santa Cruz Biologicals) at 1:100 dilutions. Western blots were developed using

an enhanced chemiluminescence detection system (Perkin Elmer Life Science, Boston, MA, USA).

For IP experiments, HT22 cells were growing on 100mm plates. Cells were transfected with an htt vector (either 19Q or 99Q) and an HA-tagged ubiquitin vector at 1:1 ratio with total DNA amount of 18μg in each plate by lipofectamine. Cells were harvested approximately 48 hours after transfection. To obtain enough protein for IP, usually three identical 100mm plates of transfected cells were collected together as one sample. As described previously (139), cells were lysed using lysis buffer containing 50mM Tris-Cl (pH. 8.8), 5mM EDTA, 2% SDS, 10mM dithiothreitol, 100μM Sodium orthovanadate, 10 mM sodium fluoride, 5mM N-Ethylmaleimide and proteasome inhibitors. Cell lysates were then diluted using dilution buffer containing 20 mM Tris-Cl, 150mM NaCl, 2mM EDTA, 100μM Sodium Orthovanadate, 10 mM sodium fluoride, 5mM N-Ethylmaleimide and proteasome inhibitors. Then the lysates were further homogenized by passing the extract through a 22-gauge needle three times to shear the DNA. The extract was centrifuged for 10 min at 14,000 × g at 4°C, and the resulting supernatant was incubated for at 4°C overnight with 20 μl of protein A/G-agarose (sc-2003; Santa Cruz Biotechnology, Inc.), which prior to this step, has been incubated with 5μl rabbit anti-CBP A-22 antibody for each sample. Immunoprecipitates were washed three times by high salt washing buffer containing 20mM Tris-Cl, 500mM NaCl, 2mM EDTA, 2mM dithiothreitol, 10 mM sodium fluoride, 5mM N-Ethylmaleimide and proteasome inhibitors. After each wash, the mixture was centrifuged at top speed for 1-2 minutes to obtain the protein-antibody-agarose beads precipitates. The protein-antibody complex is

eluted in 1X SDS-Sample Buffer and following SDS-PAGE, subjected to Western blot analysis to detected HA-tagged ubiquitin using the HA antibody, or endogenous CBP or GR. GR immunoprecipitation was performed using the CBP-depleted extracts in which a BuGR2 anti-GR monoclonal antibody was used.

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5. Cell viability—TUNEL assay

TUNEL staining was performed using an *In Situ* Cell Death Detection Kit, Fluorescein (Roche Molecular Biochemicals, Indianapolis, IN, USA). According to the manufacturer's protocol, forty-eight hours after transfection, cells grown on coverslips were fixed using 4% paraformaldehyde in PBS at room temperature for 30 min. Cells were then washed and permeabilized with 0.1% Triton X-100 in PBS on ice for 2 min. Prior to TUNEL staining (1 h at 37°C under conditions recommended by the supplier), cells were incubated with a rabbit anti-c-myc antibody for 1–1.5 h at 37°C. At last, cells were incubated with a Rhodamine anti-rabbit antibody and DAPI as described in the IIF section.

6. Cell viability—luciferase assay

HT22 cells were transiently co-transfected with 0.05 μg of a pCMV-luciferase plasmid (140) and 1 μg of Htt-N63-19Q or 99Q plasmid in 35 mm tissue culture plates. Cells were harvested and lysed 48 h following transfection in Luciferase Cell Culture Lysis Buffer (Promega, Madison, WI, USA). 20 μl of supernatant collected after centrifugation

of the lysate were used to measure luciferase activity using the Luciferase Assay Reagent (Promega) as described previously (140). Relative light units were normalized in each sample to total protein concentration.

III RESULTS

1. Expression of polyQ-expanded htt in HT22 cells

The mouse HT22 hippocampal neuronal cell line has provided a useful model of neuronal cell death and is particularly sensitive to oxidative stress (141-143). Since oxidative stress may contribute to the demise of vulnerable neurons in HD (144), we used HT22 cells for studies of toxicity upon expression of transfected polyQ-expanded htt. HT22 cells were therefore transfected with truncated htt protein that contains 63 N-terminal amino acids and either 99 (Htt-N63-99Q) or 19 (Htt-N63-19Q) consecutive glutamine residues. Figure 9 shows the schematic diagrams of the two constructs used. Htt-N63-99Q, but not Htt-N63-19Q, protein expressed in a variety of transfected neuronal and non-neuronal cell lines forms aggregates and induces toxicity (136). As will be shown below, this dependence on polyQ repeat length for aggregate formation and toxicity (136, 145) is also observed in HT22 cells (Figures 10 and 11). Note that both constructs encode truncated htt proteins, as Htt-full-length-99Q protein showed neither aggregates nor toxicity in transfected N2a cells (136). When Htt-N63-99Q protein was visualized in transfected HT22 cells by IIF four distinct staining patterns were observed (Figure 10).

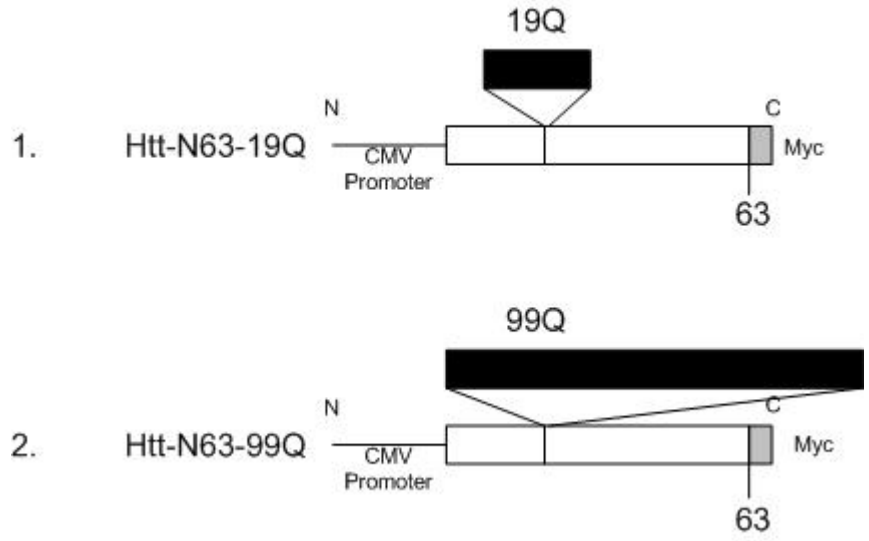

Figure 9: Schematic representation of Htt-N63-19Q/99Q constructs

Construct 1 (Htt-N63-19Q) encodes an N-terminal truncated form of huntingtin protein which contains the N-terminal 63 amino acids, a 19Q tract within the 63 amino acids and a c-myc epitope at its C-terminus. Construct 2 (Htt-N63-99Q) encodes the same protein as construct 1, except that it contains a 99Q tract within the protein in place of the 19Q tract.

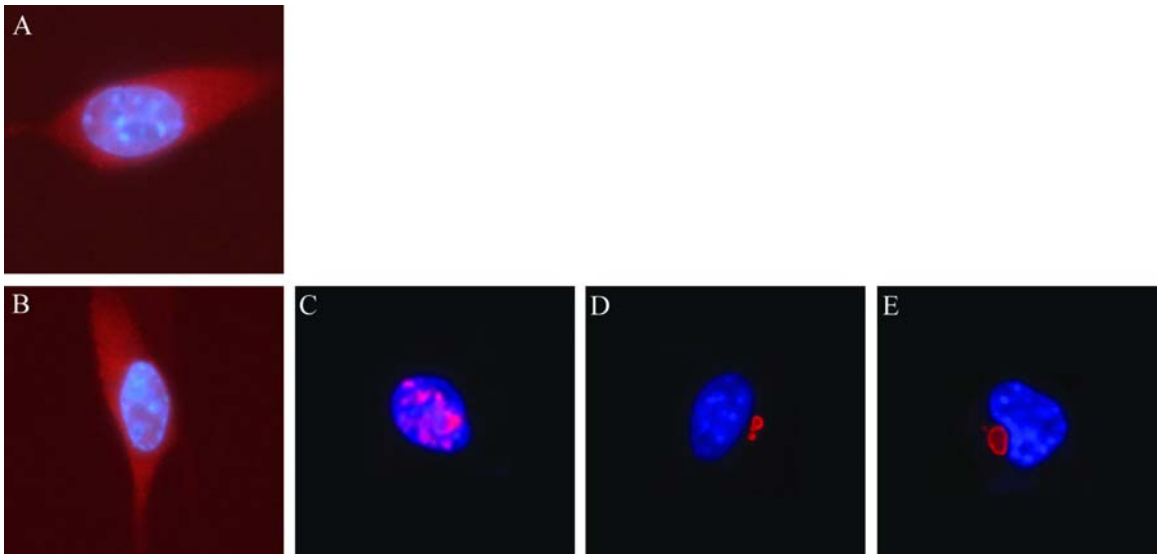

Figure 10: Expression of Htt-N63-19/99Q in HT22 cells

Transfected HT22 cells were co-stained with a rabbit anti-c-myc antibody to reveal Htt-N63-99Q protein in red and by DAPI to reveal nuclei in blue. Overlap of Htt-N63-99Q with nuclei is shown in each panel. Panel A shows that Htt-N63-19Q is expressed in cells as diffused cytoplasmic staining. Panels B–E show the expression patterns of Htt-N63-99Q. Htt-N63-99Q may be expressed in a cell as diffused cytoplasmic staining (B), nuclear aggregates (C), cytoplasmic aggregates (D) or perinuclear aggregates (E).

In the majority of HT22 cells (typically 35–40%) Htt-N63-99Q was found in large nuclear aggregates (Figure 10C), while cytoplasmic aggregates (Figure 10D), diffuse cytoplasmic staining (Figure 10B) and large perinuclear aggregates were also observed (Figure 10E). Perinuclear aggregates have been observed previously in 293 cells expressing mutant htt protein (135) and are defined by their localization, size (i.e. larger than nucleoli), and their association with distorted nuclear morphology. These diverse staining patterns of a polyQ-expanded protein are not unique to HT22 cells and have been observed in many other transfected cell lines (136, 145).

Numerous methods have been used to examine the cellular toxicity induced by polyQ expanded proteins such as TUNEL, propidium iodide (PI) and tryphan blue staining (42). To more precisely quantify cell death in transfected cell populations, some methods have been applied that rely on the reduction in protein levels or activity (e.g. GFP (47), firefly luciferase (146)) expressed from cotransfected plasmids. To verify that polyQ-expanded htt is indeed inducing cell death in HT22 cells, cells were cotransfected with a luciferase reporter plasmid and Htt-N63-19Q or Htt-N63-99Q expression plasmids. The principle of using luciferase assay to test toxicity is based on the fact that the degradation of luciferase is accelerated in dying cells (146). As shown in Figure 11, luciferase activity in HT22 cells expressing Htt-N63-99Q was only 10% of that recovered in cells expressing Htt-N63-19Q, indicating 99Q expression cells exhibit significant toxicity comparing to 19Q. Luciferase assay has also been applied in another study to demonstrate the polyQ-expanded huntingtin induced toxicity (45). Therefore, in HT22 cells Htt-N63-99Q protein

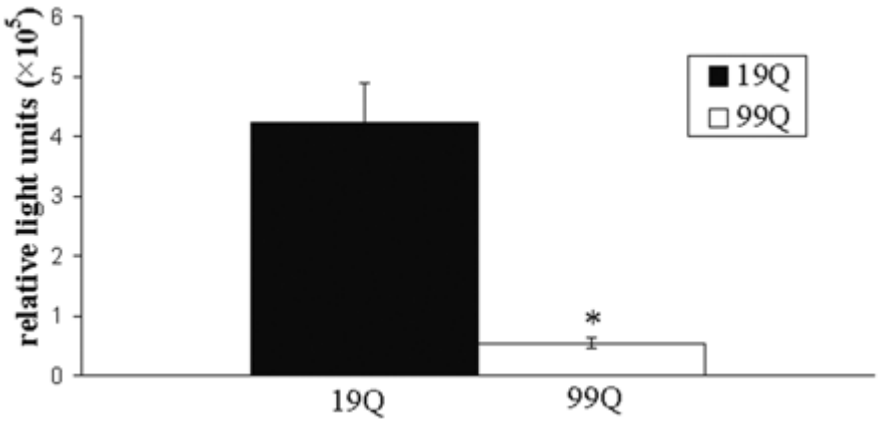

Figure 11: Cell death induced by Htt-N63-99Q

Viability of HT22 cells was assessed by measuring luciferase activity derived from a CMV-luciferase reporter plasmid co-transfected (at a 1:20 ratio) with either Htt-N63-19Q or Htt-N63-99Q plasmids. Data shown are average (±SEM) of four independent experiments (*P<0.05).

shows both the aggregation feature and cellular toxicity, indicating it is a valid in vitro system to study HD.

2. Distinct responses of CBP to polyQ-expanded htt in HT22 cells

As mentioned in the Introduction, various transcription factors and cofactors are recruited into aggregates containing polyQ-expanded protein (47, 63, 82, 128). Recruitment of the CBP transcriptional coactivator into polyQ repeat protein aggregates appears to play an important role in in vitro toxicity and is a characteristic of vulnerable brain tissue in HD and mouse models of the disease (47). CBP is a nuclear protein that exhibits diffused nuclear staining in the cell. We used a mouse anti-CBP antibody to examine endogenous expression of CBP. The specificity of the antibody in IIF assay is shown in Figure 12. In HT22 cells that are transfected with Htt-N63-99Q, endogenous CBP is recruited into nuclear aggregates containing Htt-N63-99Q protein (Fig. 13 A–D). Endogenous CBP is also recruited into perinuclear Htt-N63-99Q protein aggregates (Fig.14 E–H). In cells expressing aggregated cytoplasmic Htt-N63-99Q protein, endogenous CBP exhibits a uniform diffuse nuclear staining pattern (Fig. 14 A–D). This apparently normal nuclear staining pattern of CBP is observed in all cells expressing cytoplasmic Htt-N63-99Q protein that is not visibly aggregated (data not shown). In non-transfected HT22 cells or transfected cells expressing Htt-N63-19Q, CBP also shows diffuse nuclear staining (data not shown). Surprisingly, in addition to the recruitment of endogenous CBP into expanded polyQ htt protein aggregates, we noticed many cells where CBP staining was diminished (Fig. 13 E–H). As summarized in Figure 15, in ~55% of HT22 cells

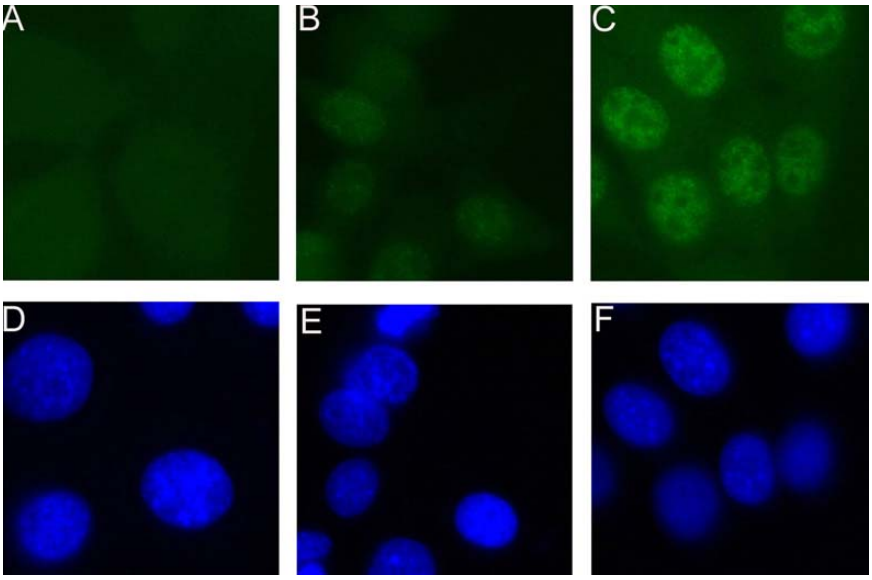

Figure 12: Specificity of CBP antibody

HT22 cells were stained with a mouse anti-CBP antibody to reveal endogenous CBP in green, as shown in C. To examine the specificity of this antibody, HT22 cells were stained without primary antibody as shown in A. Cells were also stained with anti-CBP antibody which was previously incubated with corresponding blocking peptide at 1:50 ratio, as shown in B. The same dilution of FITC anti-mouse secondary antibody was used in all the experiments. Nuclei were co-stained blue by DAPI as shown in D to F. Preincubation of CBP antibody with the blocking peptide blocked the diffused nuclear staining pattern of CBP in HT22 cells (comparing B with C).

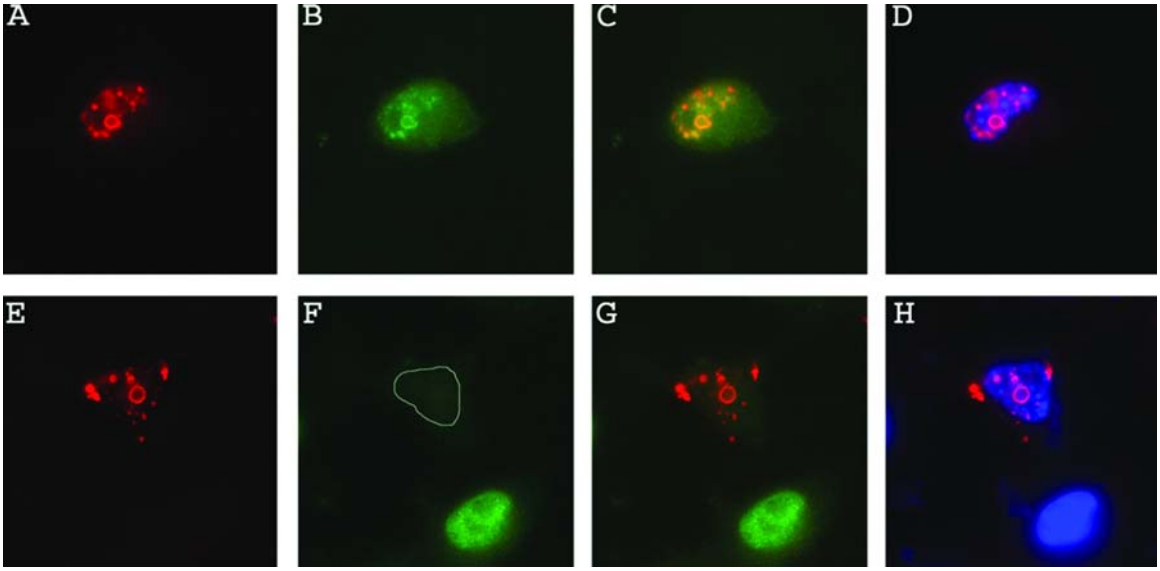

Figure 13: Localization and expression of endogenous CBP in HT22 cells containing Htt-N63-99Q nuclear aggregates

Transfected HT22 cells were co-stained with a rabbit anti-c-myc antibody to reveal Htt-N63-99Q protein in red (A, E) and a mouse anti-CBP antibody to reveal endogenous CBP in green (B, F). Areas of overlap between CBP and Htt-N63-99Q are shown in yellow (C, G). Htt-N63-99Q overlap with nuclei stained blue by DAPI is shown in D and H. Panels A–D show recruitment of endogenous CBP into Htt-N63-99Q nuclear aggregates. Panels E–H illustrate the loss of endogenous CBP that is observed in some (i.e. 55%) HT22 cells expressing Htt-N63-99Q nuclear aggregates.

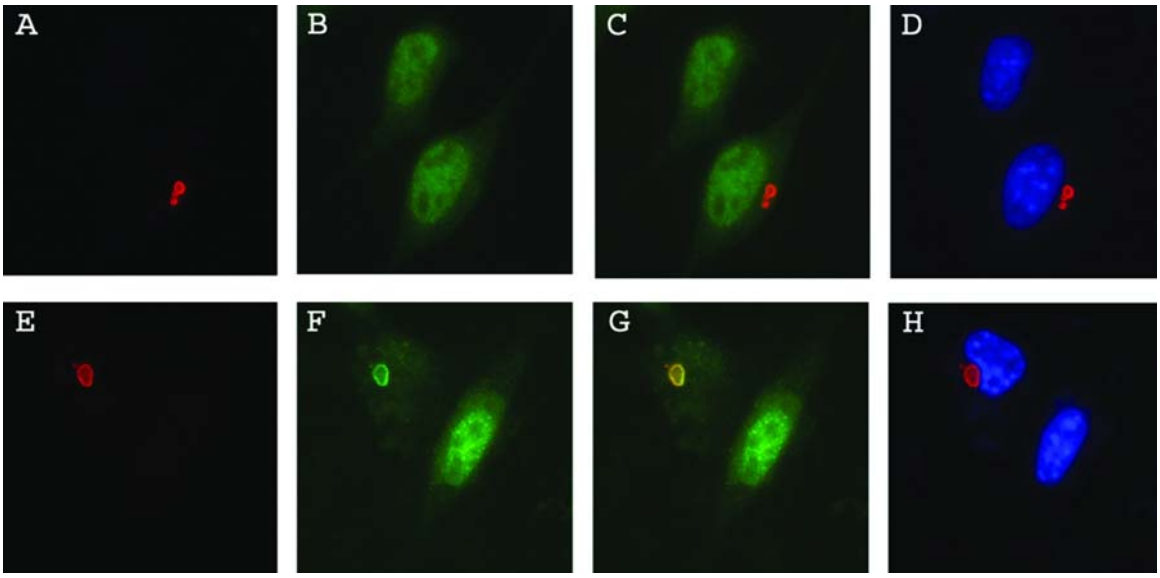

Figure 14: Localization of endogenous CBP in HT22 cells containing Htt-N63-99Q within cytoplasmic and large perinuclear aggregates

Transfected HT22 cells were co-stained with a rabbit anti-c-myc antibody to reveal Htt-N63-99Q protein in red (A, E) and with a mouse anti-CBP antibody to reveal endogenous CBP in green (B, F). Areas of overlap between CBP and Htt-N63-99Q are shown in yellow (C, G). Htt-N63-99Q overlap with nuclei stained blue by DAPI is shown in D and H. Panels A–D show that endogenous CBP maintains normal diffuse nuclear staining in a cell containing Htt-N63-99Q cytoplasmic aggregates. In H, the upper cell contains a perinuclear Htt-N63-99Q aggregate that 'pushes' and deforms the adjacent nucleus. CBP is recruited into the Htt-N63-99Q perinuclear aggregate (F).

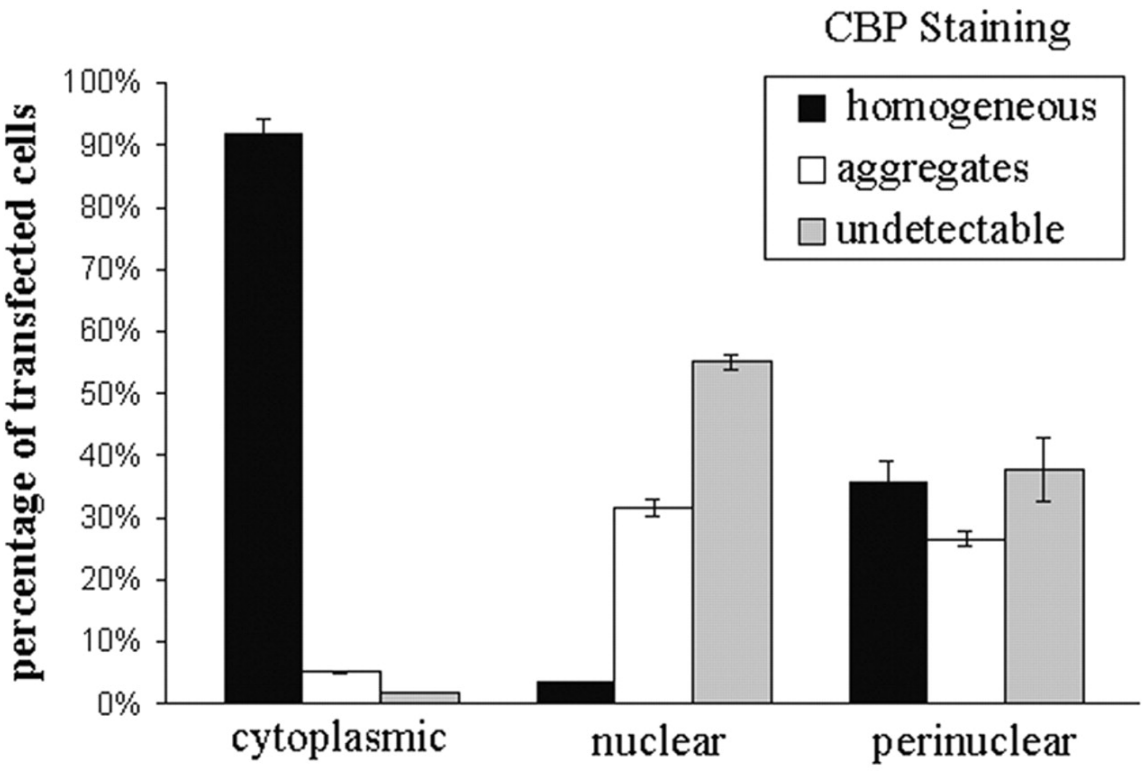

Figure 15: Quantitative analysis of endogenous CBP expression in HT22 cells containing Htt-N63-99Q aggregates.

Data shown are average of four independent experiments. Approximately 100 cells containing Htt-N63-99Q aggregates were counted in each experiment. In 95% of HT22 cells containing Htt-N63-99Qnuclear aggregates, CBP is no longer homogeneously distributed throughout the nucleus but either recruited into aggregates (40%) or no longer visible (55%). In contrast, CBP maintains its normal diffuse nuclear staining in cells containing cytoplasmic Htt-N63-99Q aggregates.

expressing nuclear aggregates of Htt-N63-99Q, CBP staining was undetected. Endogenous CBP staining was also dramatically reduced in ~40% of transfected HT22 cells containing perinuclear Htt-N63-99Q protein aggregates. Under our IIF conditions, CBP was detected in 98% of HT22 cells expressing diffuse cytoplasmic Htt-N63-99Q protein, non-transfected cells or those expressing Htt-N63-19Q protein (data not shown). The apparent loss of endogenous CBP was also observed in ~30% of transiently transfected N2a neuroblastoma cells expressing Htt-N63-99Q (data not shown) and thus is not a property unique to HT22 cells.

In order to test the selectivity of Htt-N63-99Q induced effects on CBP, we examined the localization of endogenous Sp1 transcription factor in transfected HT22 cells. Previous studies in N2a cells showed that the diffuse nuclear localization of Sp1 is maintained in transfected cells expressing polyQ expanded htt protein (47). As shown in Figure 16 A–H, the diffuse nuclear staining pattern of Sp1 in HT22 cells is not altered upon expression of Htt-N63-99Q protein, irrespective of the localization of htt protein. There is no evidence of loss of Sp1 staining even in cells with a very condensed nucleus (data not shown). We also examined the localization of ubiquitin and the HDJ-2 molecular chaperone in Htt-N63-99Q transfected HT22 cells. As expected from previous studies in other cell lines and tissues (29, 147-149), both ubiquitin (Figure 17 A–H) and HDJ-2 (data not shown) were recruited into Htt-N63-99Q nuclear aggregates. Thus, the apparent loss of CBP that results from expression of polyQ-expanded htt within nuclear or perinuclear aggregates is selective and not characteristic of either another htt-interacting

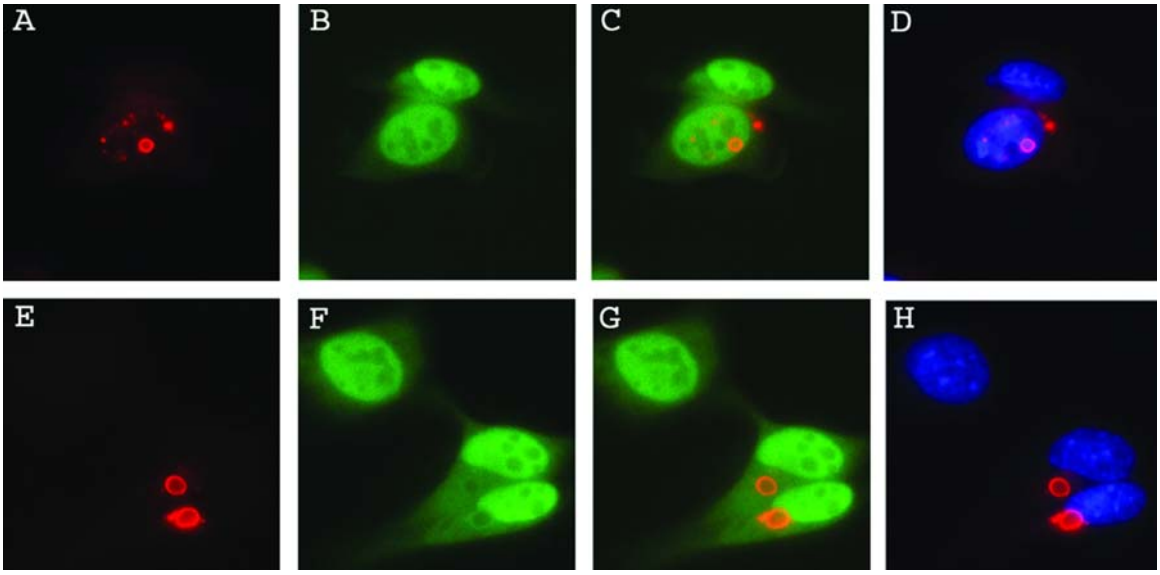

Figure 16: Localization of endogenous Sp1 in HT22 cells containing Htt-N63-99Q nuclear or cytoplasmic aggregates

Transfected HT22 cells were co-stained with a rabbit anti-c-myc antibody to reveal Htt-N63-99Q protein in red (A, E) and a mouse anti-SP1 antibody to reveal endogenous SP1 in green (B, F). Areas of overlap between SP1 and Htt-N63-99Q are shown in yellow (C, G). Htt-N63-99Q overlap with nuclei stained blue by DAPI is shown in D and H. Panels A–D show that endogenous SP1 maintains its normal diffuse nuclear staining pattern in cells containing Htt-N63-99Q nuclear aggregates. Panels E–H show that endogenous SP1 maintains its normal diffuse nuclear staining in cells containing cytoplasmic Htt-N63-99Q aggregates.

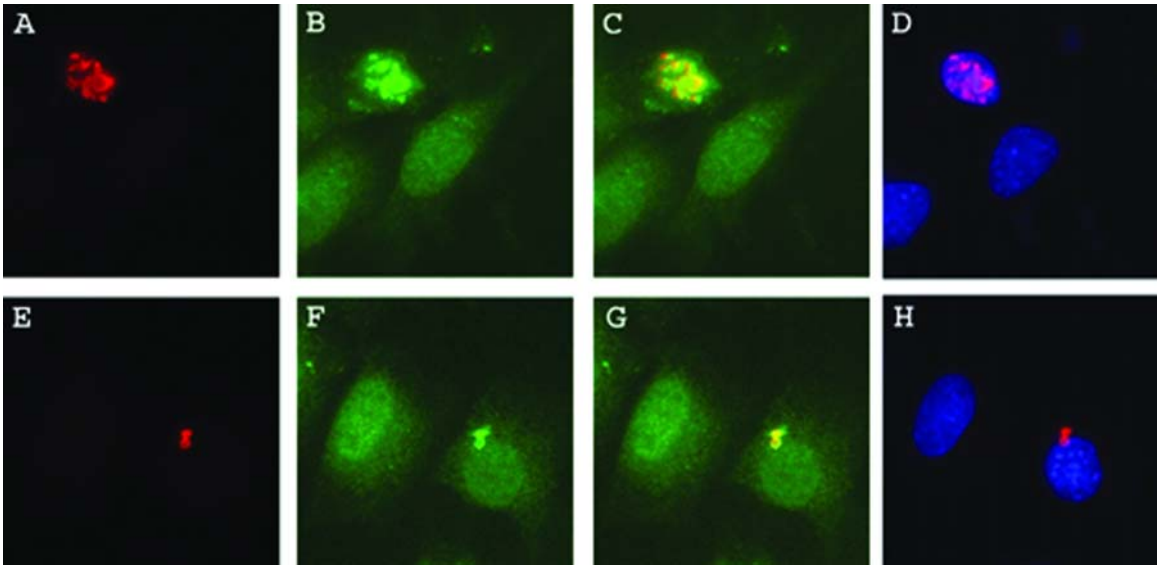

Figure 17: Localization of ubiquitin in HT22 cells containing Htt-N63-99Q nuclear or cytoplasm aggregates

Transfected HT22 cells were co-stained with a rabbit anti-c-myc antibody to reveal Htt-N63-99Q protein in red (A, E) and with a mouse anti-ubiquitin antibody to reveal endogenous ubiquitin in green (B, F). Areas of overlap between ubiquitin and Htt-N63-99Q are shown in yellow (C, G). Htt-N63-99Q overlap with nuclei stained blue by DAPI is shown in D and H. Panels A–D show that ubiquitin is recruited into Htt-N63-99Q nuclear aggregates. Panels E–H show that some ubiquitin is recruited into cytoplasmic Htt-N63-99Q aggregates, although the majority of ubiquitin remains within the nucleus.

transcription factor (i.e. Sp1) (46, 117), or other proteins recruited into polyQ-expanded htt aggregates.

Although these preliminary results are partially similar with other published work, such as the recruitment of CBP into aggregates of polyQ-expanded htt, several points drew our attention for further analysis. For example, we found that CBP expression is affected only by the nuclear or perinuclear aggregates, but not the cytoplasmic aggregates (Figures 13 to 15). In the paper by Nucifora et al, they found that in most cells which showed cytoplasmic aggregates, transfected CBP was entirely recruited into cytoplasmic aggregates as well (47). The advantage of our study is that we examine the expression of endogenous CBP, and therefore may provide data that is biologically more relevant. This differential effect on CBP by the localization of aggregates prompted us to study the correlation between CBP defect and nuclear toxicity, as shown below.

3. HT22 cell toxicity is associated with the formation of nuclear aggregates containing polyQ-expanded htt

The loss of CBP function has been found to be a major factor in the cell death induced by polyQ-expanded proteins (47, 82, 106). In HT22 cells, the subcellular localization of Htt-N63-99Q has an impact on CBP recruitment or degradation (see above). In order to examine whether CBP localization has an impact on Htt-N63-99Q induced toxicity, we used a TUNEL assay to stain transfected HT22 cells. As show in Figure 18 and summarized in Figure 19, 80% of transfected HT22 cells expressing Htt-N63-99Q

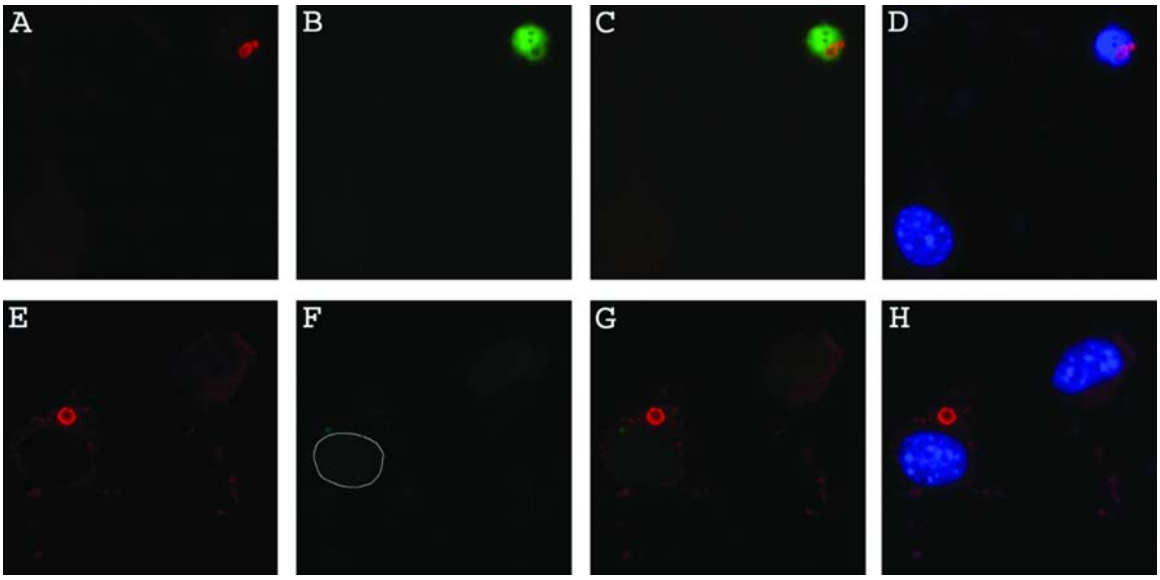

Figure 18: Toxicity in HT22 cells expressing Htt-N63-99Q aggregates

Transfected HT22 cells were co-stained with a rabbit anti-c-myc antibody to reveal Htt-N63-99Q protein in red (A, E) and with a TUNEL assay kit to detect DNA damage in green (B, F). Areas of overlap between CBP and Htt-N63-99Q are shown in yellow (C). Htt-N63-99Q overlap with nuclei stained blue by DAPI is shown in D and H. HT22 cells expressing nuclear Htt-N63-99Q aggregates are positive in TUNEL assay (C) while cells containing cytoplasmic Htt-N63-99Q aggregates are negative in TUNEL assay (G).

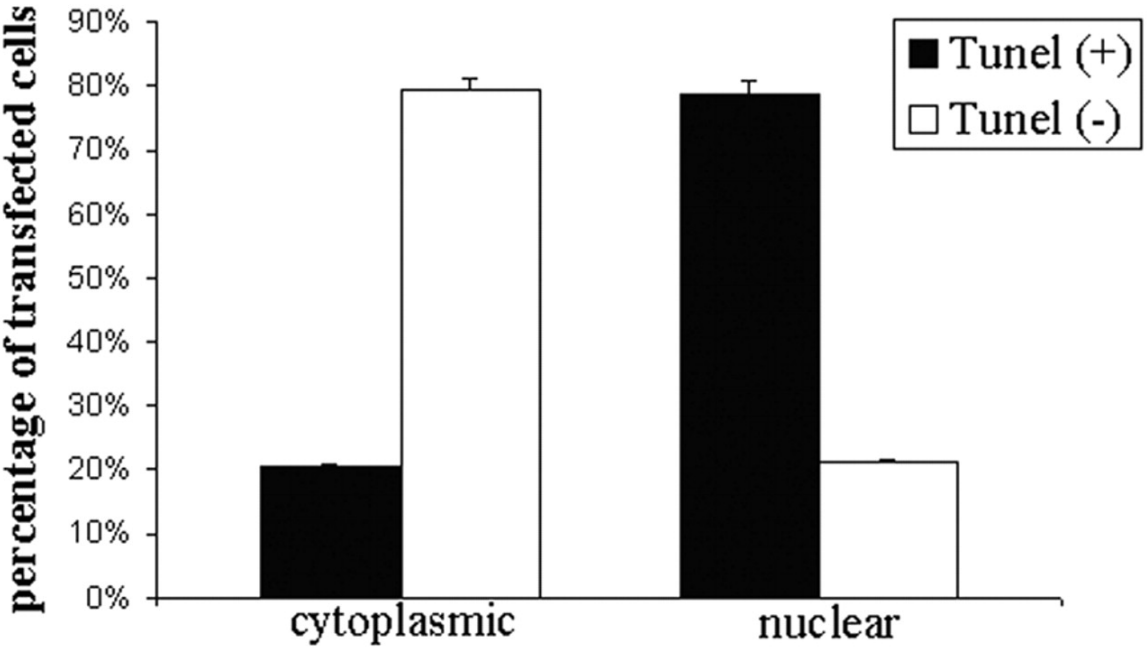

Figure 19: Quantitative analysis of cell death by TUNEL assay in HT22 cells expressing Htt-N63-99Q aggregates.

Data are from the average of four independent experiments. Approximately 100 cells containing Htt-N63-99Q aggregates were counted from randomly selected fields in each experiment.

nuclear aggregates were TUNEL-positive, while only 20% of the cells containing cytoplasmic aggregates of Htt-N63-99Q were TUNEL-positive. These results are consistent with other published studies examining polyQ-expanded htt in transfected cells where the extent of cellular toxicity was correlated with nuclear localization of polyQ-expanded htt (78, 150). However, our results are unique in demonstrating at a single cell level a relationship between the loss of CBP function and cell toxicity. Therefore, along with the luciferase assay (Figure 11), two independent assessments of cell death reveal Htt-N63-99Q-induced toxicity in HT22 cells, either at the level of individual cells (Figure 18 and 19) or transfected cell populations.

4. PolyQ-expanded htt selectively enhances CBP engagement with the UPP

Another interesting point is that we detected a loss of CBP staining is some cells containing nuclear or perinuclear aggregates. We asked the question whether this loss of CBP reflects its enhanced degradation. Given the important role of UPP in polyglutamine diseases, we suspected that the loss of CBP staining may be related to the altered UPP function in the presence of Htt-N63-99Q.

Despite the variability in both Htt-N63-99Q localization and the response of CBP (Figure 15), we were able to detect a reduction in transfected CBP levels by Western blot analysis of HT22 cell populations expressing Htt-N63-99Q but not Htt-N63-19Q (Figure 20). Note that the decreased CBP level can be explained in at least three ways. A few previously published papers suggest that loss of CBP as the result of recruitment of CBP

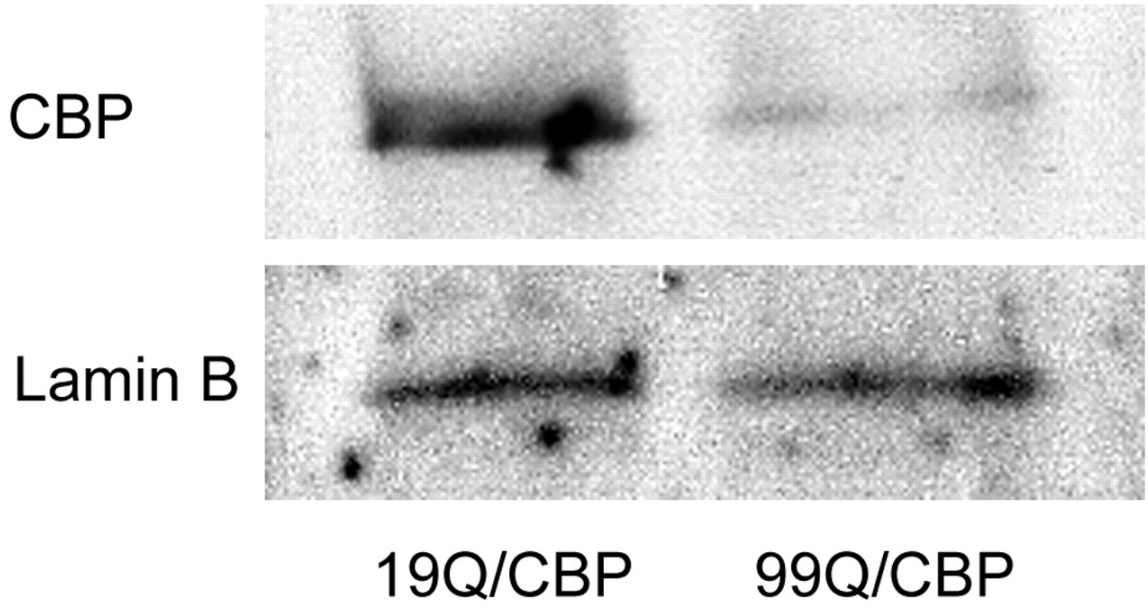

Figure 20: Reduction of soluble CBP levels in HT22 cells expressing Htt-N63-99Q

Soluble protein extracts were prepared from HT22 cells transfected with a CBP expression plasmid, Htt-N63-99Q or Htt-N63-19Q expression plasmids, and then subjected to Western blot analysis to detect CBP (top panel). Stripped blots were probed with an anti-lamin B (bottom panel) antibody to provide an internal control for protein loading. Blot shown is representative of three separate transfection experiments where reductions in CBP levels (normalized to lamin B expression) in Htt-N63-99Q expressing cells, relative to those expressing Htt-N63-19Q, varied from 40 to 60%.

into insoluble aggregates, which therefore reduces the levels of soluble level of CBP (47). We are unable to detect any CBP in the insoluble fraction of the cell lysates, in which insoluble Htt-N63-99Q can be detected and do not favor this interpretation (see later Figure 24). Alteratively CBP expression may be inhibited at the transcription level. This appears to be unlikely since the level of CBP mRNA may be slightly increased when coexpressed with a polyQ-expanded htt protein (128). Therefore, in our HT22 cell system, the Western blot data support the IIF analysis, suggesting that selective CBP loss detected within individual cells is due to its enhanced degradation.

Since CBP may be a substrate for proteasome-mediated degradation (151), we examined whether proteasome inhibition would reduce CBP loss observed in some HT22 cells expressing nuclear Htt-N63-99Q. Effects of proteasome inhibitors on the loss of CBP in cells expressing polyQ-expanded htt might also rule out CBP synthesis as a target of mutant htt. As shown in Figure 21 and quantified in Figure 22, treatment of HT22 cells with the irreversible proteasome inhibitor, lactacystin, significantly reduced the fraction of cells that exhibited reduced CBP expression. In this particular experiment, 40% of cells expressing nuclear aggregates of Htt-N63-99Q exhibited CBP loss in the absence of lactacystin treatment (Figure 22). Since the percentage of cells with nuclear aggregates of Htt-N63-99Q was not affected by lactacystin, the decrease in cells showing CBP loss is accompanied by an increase in cells exhibiting diffuse nuclear staining of CBP (Figure 22). These results are compatible with previous studies (135) showing lactacystin effects on polyQ-expanded htt aggregation, since lactacystin treatment did affect Htt-N63-99Q nuclear aggregation, leading to an increase in the fraction of cells containing

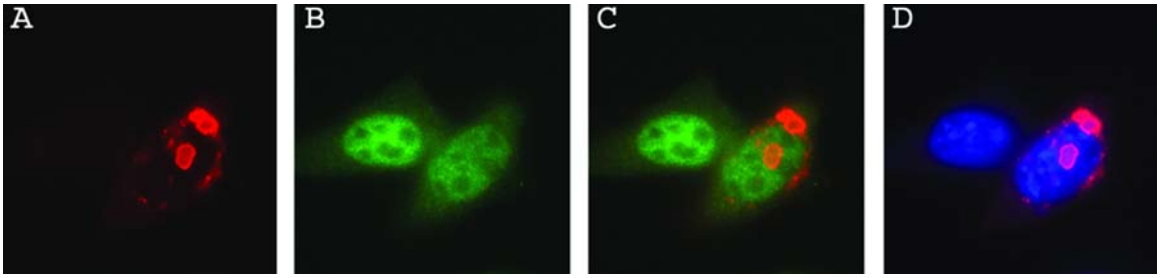

Figure 21: Lactacystin reduces endogenous CBP loss in HT22 cells expressing Htt-N63-99Q nuclear aggregates

Transfected HT22 cells were co-stained with a rabbit anti-c-myc antibody to reveal Htt-N63-99Q protein in red (A) and with a mouse anti-CBP antibody to reveal endogenous CBP in green (B). Areas of overlap between CBP and Htt-N63-99Q are shown in yellow (C). Htt-N63-99Q overlap with nuclei stained blue by DAPI is shown in D. Endogenous CBP maintains its normal diffuse nuclear staining in cells expressing Htt-N63-99Q nuclear aggregates upon treatment of cells with lactacystin, a proteasome inhibitor.

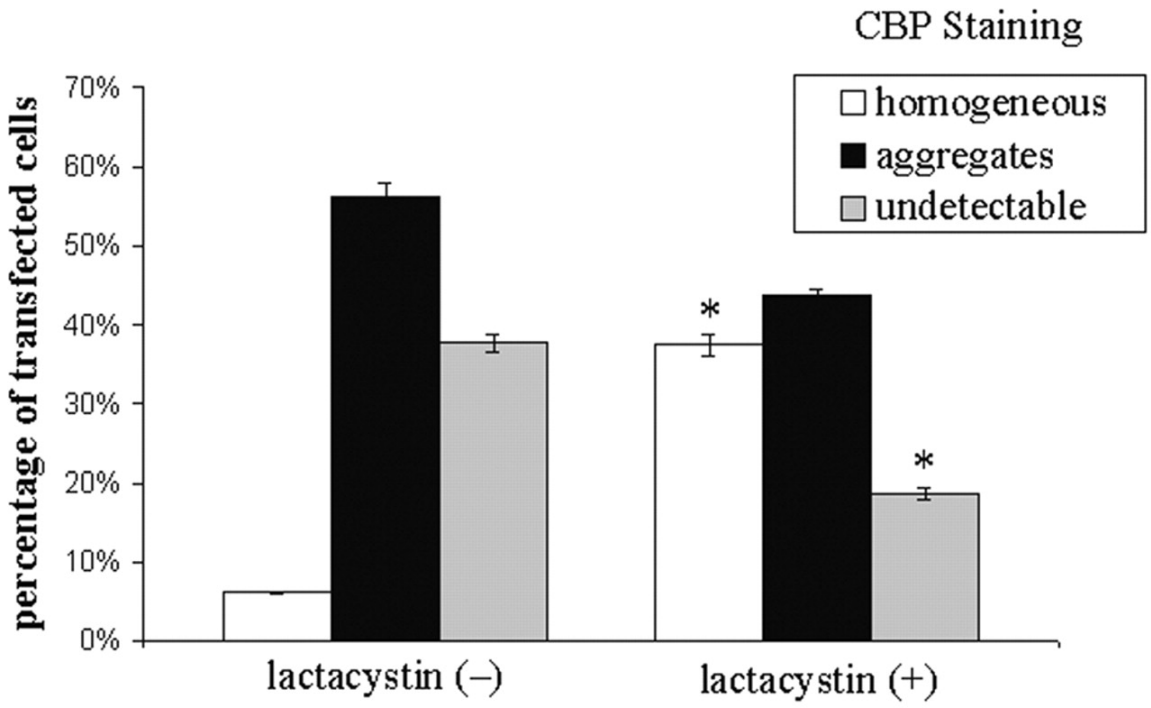

Figure 22: Quantitative analysis of lactacystin effects on endogenous CBP localization in HT22 cells containing Htt-N63-99Q nuclear aggregates

Data are from the average of three independent experiments. Approximately 50 cells containing Htt-N63-99Q nuclear aggregates were counted from randomly selected fields in each experiment. Approximately 35% of cells containing nuclear aggregates show homogeneous CBP staining (i.e. open bars) upon the addition of lactacystin compared to ~5% of such cells not treated with lactacystin (*P<0.05). In addition, the amount of HT22 cells exhibiting CBP loss (i.e. gray-filled bars) is reduced from 40 to 20% upon addition of lactacystin (*P<0.05).

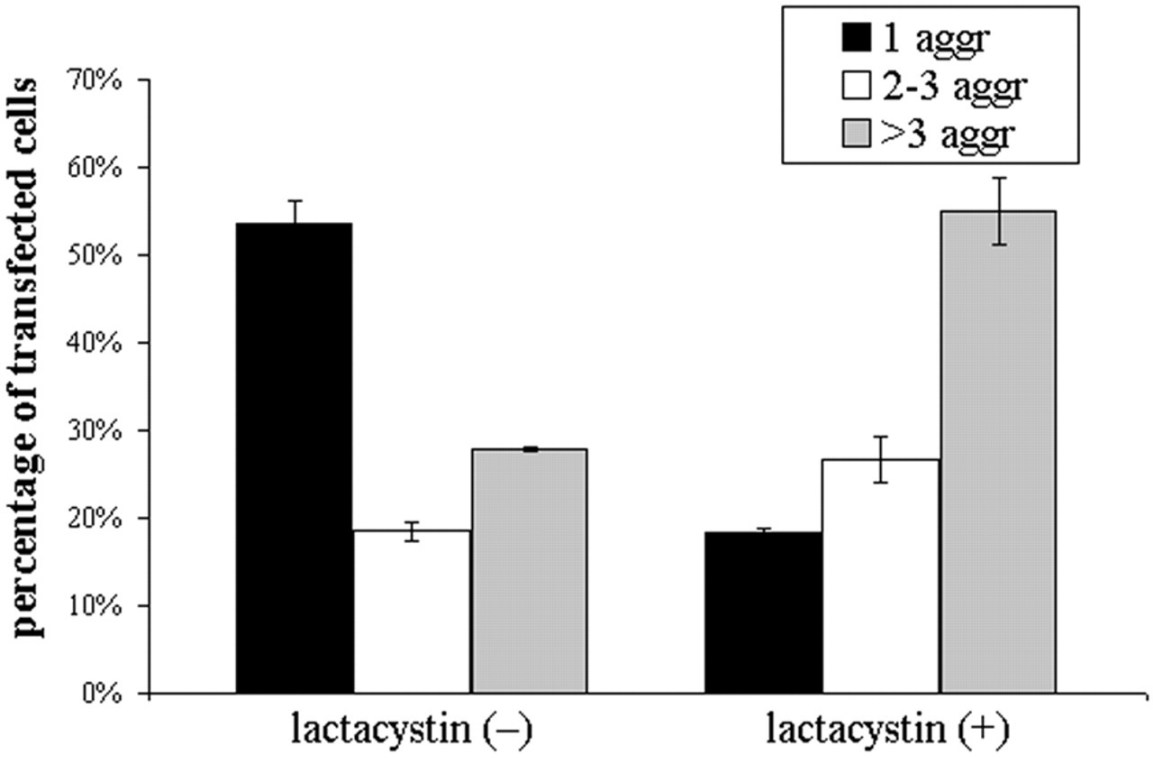

Figure 23: Quantitative analysis of aggregate formation in lactacystin-treated HT22 cells containing Htt-N63-99Q nuclear aggregates

Data are from the average of three independent experiments. Approximately 50 cells containing Htt-N63-99Q nuclear aggregates were counted from randomly selected fields in each experiment.

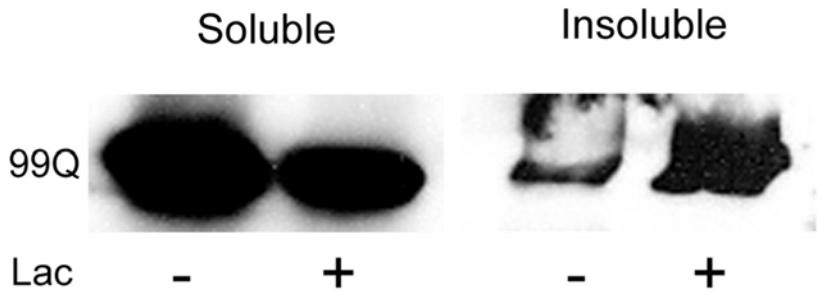

Figure 24: Lactacystin treatment reduces solubility of Htt-N63-99Q in HT22 cells

Detergent-soluble and -insoluble fractions were prepared from either untreated or lactacystin-treated (5 μM, treated 18 h after transfection for 24 h) HT22 cells transfected with an Htt-N63-99Q expression plasmid. Equivalent amounts of total soluble protein and cell equivalents of insoluble protein were separated by SDS–PAGE and subjected to Western blot analysis to detect Htt-N63-99Q protein. Insoluble Htt-N63-99Q protein was detected in the stacking gel. This blot is representative of three separate transfection experiments. The expression level of Htt-N63-19Q protein was not changed by lactacystin treatment (data not shown).

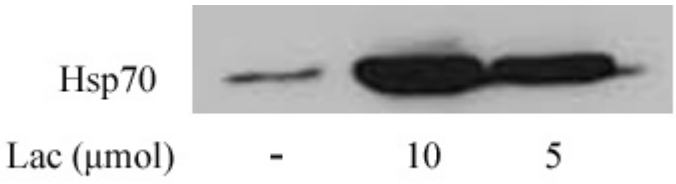

Figure 25: Induction of Hsp70 by lactacystin in HT22 Cells

HT22 cells growing on 60mm plates were treated with lactacystin of 10μmol (lane 2) or 5 μmole (lane 3) or ddH_2O (lane 1) for 18 hours. Cells were then collected and Hsp70 levels were detected by Western blot analysis. Consistent with other published data, Lactacystin treatment leads to activation of Hsp70.

multiple (i.e. >3) nuclear aggregates (Figure 23). This increase in Htt-N63-99Q aggregation by lactacystin treatment was also revealed by Western blot analysis, which also showed a corresponding reduction in soluble Htt-N63-99Q (Figure 24 left) accompanied with more 99Q protein in the insoluble fraction (Figure 24 right). The effectiveness of lactacystin in altering proteasome function was also confirmed by the induction of heat shock protein 70 (Hsp70) in lactacystin-treated HT22 cells (Figure 25). Hsp70 induction is a hallmark of proteasome inhibition in a number of cell types (152).

The recovery of CBP expression upon lactacystin treatment of HT22 cells expressing nuclear Htt-N63-99Q suggests that CBP recruitment to the UPP may be enhanced as a consequence of polyQ-expanded htt expression. Since proteasome targeting requires ubiquitin modification, we examined whether increased CBP ubiquitylation accompanied Htt-N63-99Q expression. Endogenous CBP ubiquitylation was assessed using IP from extracts prepared following co-transfection of HT22 cells with HA-tagged ubiquitin and Htt-N63 plasmids. Gels used for this Western blot analysis were loaded to attain equivalent amounts of total CBP levels/lane (Figure 26A, top panel) in order to aid in comparison of CBP ubiquitylation. As shown in Figure 26A (bottom panel), endogenous CBP ubiquitylation was enhanced in HT22 cells expressing Htt-N63-99Q, but not Htt-N63-19Q protein. Importantly, detection of ubiquitylated CBP in Htt-N63-99Q transfected cells did not require the pharmacological inhibition of proteasome function, which on its own leads to increased accumulation of ubiquitylated CBP (Fig. 26A, bottom panel). In the absence of htt proteins, lactacystin treatment is able to induce ubiquitylation

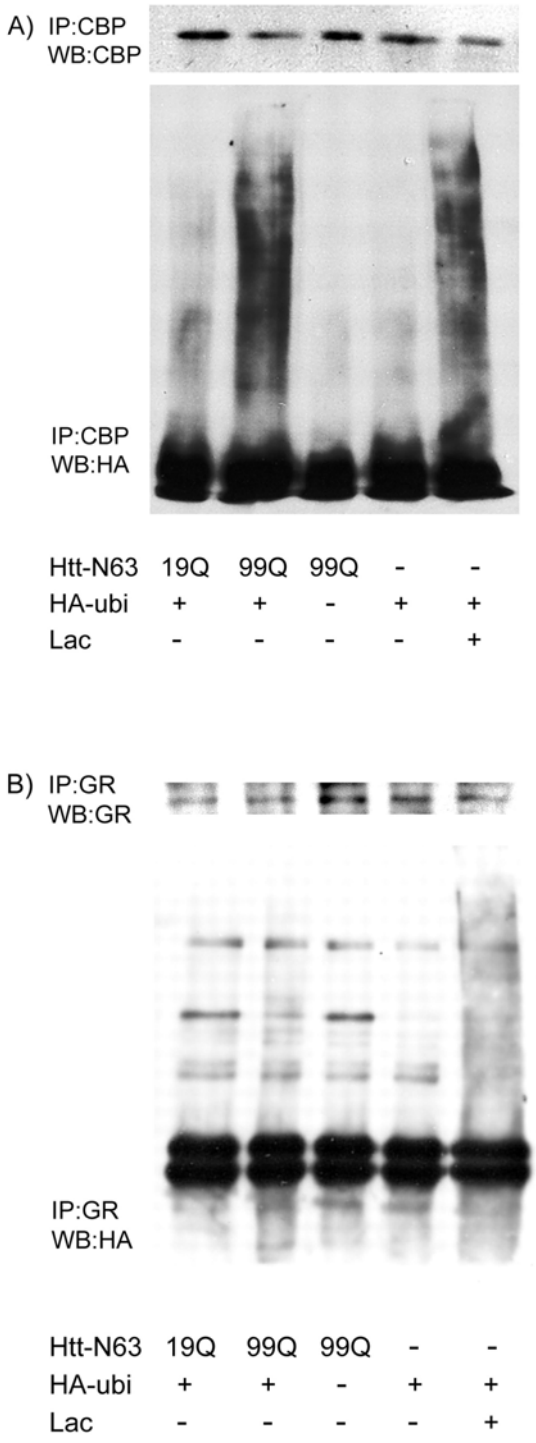

Figure 26: Selective enhancement of endogenous CBP ubiquitylation in Htt-N63-99Q expressing HT22 cells

Legends for Figure 26: HT22 cells were transfected and an HA-tagged ubiquitin expression plasmid and either Htt-N63-19Q or Htt-N63-99Q expression plasmids. Where indicated, 5 μM lactacystin was added to transfected cultures for 24 h. Soluble extracts from transfected cells were subjected sequentially to an immunoprecipitations with first, an anti-CBP antibody (A) and then an anti-GR (B) antibody. Immunoprecipitated proteins were subjected to Western blot analysis to detected HA-tagged ubiquitylated proteins (A and B), or endogenous CBP (A) or GR (B) in the identical samples on parallel blots.

of endogenous CBP in HT22 cells, indicating that CBP may be degraded through UPP under normal conditions. In order to reveal whether Htt-N63-99Q effects on CBP were selective, we examined glucocorticoid receptor (GR) ubiquitylation in the same extracts used for analysis of CBP ubiquitylation. GR is a substrate for the UPP in HT22 cells, as revealed by the increased accumulation of ubiquitylated receptor upon lactacystin treatment (Figure. 26B). This corroborates previously published work from our laboratory where proteasome inhibition by MG132 treatment of HT22 cells was found to increase GR ubiquitylation (139). However, unlike CBP, GR ubiquitylation is not affected by Htt-N63-99Q (Fig. 26B). Thus, the enhancement of CBP ubiquitylation by Htt-N63-99Q is selective and does not represent an overall increase in ubiquitin conjugation or proteasome inhibition.

5. Mechanisms underlying the selectivity of enhanced CBP degradation

Why is CBP selected by the UPP for accelerated degradation in the presence of Htt-N63-99Q? There may be two possibilities. First of all, specific domains of CBP may bind to polyQ-expanded htt and recruit CBP to the UPP. As mentioned earlier, both polyQ domain of CBP and its HAT domain are candidates for this interaction. Secondly, the selectivity of CBP degradation may be due to the activation of specific E2 ubiquitin carrier proteins or E3 ubiquitin ligases, both of which function to target proteins to the UPP.

5. 1 The effect of Htt-N63-99Q on expression of CBP deletion mutants

As previously mentioned a number of transcriptional factors or cofactors have Q-rich regions such as Sp1 and p300 (81). The mouse CBP also contain a polyQ region in which there is 15 consecutive Qs (47). As shown in Figure 16, Sp1 is not recruited into the aggregates, nor does it display any loss-of-staining. Nucifora et al. also showed that a polyQ-deletion mutant of CBP is not recruited into the aggregates. Therefore, we first wanted to examine whether deletion of the polyQ domain of CBP has an effect on its degradation.

To test this possibility, we first used the same CBPΔQ construct as the one used in the paper by Nucifora et al. Before using this mutant, we have sequenced both the wide-type CBP and CBPΔQ constructs. Results indicate that the only difference between the two construct is the deletion of 15Q tract in the mutant. As shown in Figure 27A, the level of CBPΔQ is still decreased when cotransfected with 99Q versus 19Q htt (lane 3 and lane 4), suggesting that the degradation of CBP is not Q-domain dependent.

The CBPΔQ construct from Nucifora used in these initial studies is not a tagged version, therefore the transfected CBP can not be distinguished from endogenous CBP. Differences of CBP levels that can be detected are critically dependent on the transfection efficiency. To eliminate this problem, we used a flag tagged wild-type CBP construct and its counterpart Flag-CBPΔQ mutant. In the latter one, the 15 consecutive CAG repeats in the sequence were substituted with an EcoRI site. As shown in the Western blot of Figure 27B, which is probed with an anti-flag antibody, there is a significant decrease of Flag-

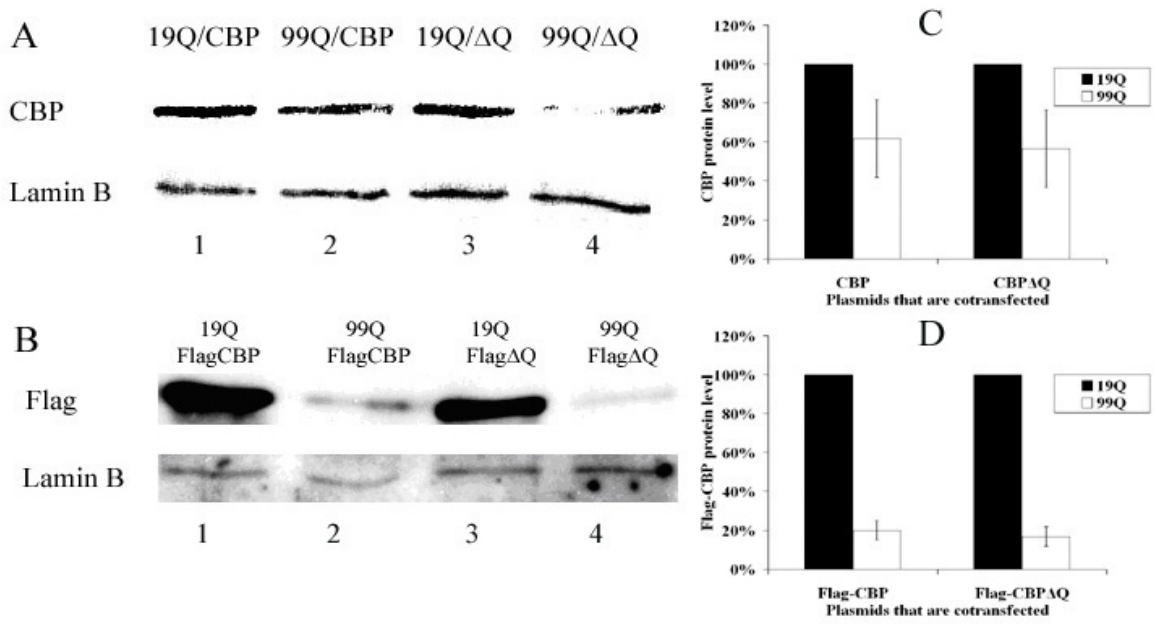

Figure 27: Degradation of CBP is independent of its polyQ domain

In A, soluble protein extracts were prepared from HT22 cells transfected with a CBP (untagged) or CBPΔQ expression plasmid with Htt-N63-99Q or Htt-N63-19Q expression plasmid, and then subjected to Western blot analysis to detect total cellular CBP (top panels). Stripped blots were probed with an anti-lamin B (bottom panels) antibody to provide an internal control for protein loading. Decrease CBP levels in the presence of 99Q instead of 19Q (i.e. lanes 1 and 2) is not altered when the polyQ domain of transfected CBP is deleted (i.e. lanes 3 and 4). In B, a Flag-CBP or a Flag-CBPΔQ construct was transfected with either Htt-N63-19Q or 99Q. Western blots were probed with an anti-flag antibody. The results are similar with those from panel A. Furthermore, when tagged versions of the constructs were used, the decrease of CBP or CBPΔQ level is more obvious than with untagged versions where endogenous CBP is also detected in Western blots. . Right panels (C.D) showed quantitative analysis, in which data were collected from three independent experiments.

CBP level when it co-expressed with Htt-N63-99Q (lane 1 and lane 2). A similar decrease is observed for Flag-CBPΔQ expression. This figure clearly indicated that the degradation of the CBP is not simply dependent on its polyQ domain, which may be important for binding with Htt-N63-99Q. Since Nucifora et al. showed that CBPΔQ is not recruited into the aggregates, we still need to confirm whether Flag-CBPΔQ is recruited into 99Q aggregates in our system by IIF. If not, it may suggest that the polyQ domain is responsible for recruitment but not degradation.

Next, we set out to examine whether the degradation of CBP is dependent upon its HAT domain. Since the HAT domain is quite large, we selected only a partial HAT deletion according to the results from Steffan et al, as shown in Figure 7. The region that has the strongest affinity for polyQ expanded htt mutant is the fragment starting from amino acids 1459 to 1877. Therefore, we made an in-frame deletion mutant from the Flag-CBP in which about the same region is deleted (amino acids 1446-1866). As the preliminary data shown in Figure 28, expression of Flag-CBPΔHAT (about 219kD) is still reduced when coexpressed with 99Q. Therefore, this region is still not required for increased degradation of CBP in the presence of 99Q. It has been reported that a well-conserved bipartite nuclear localization signal localizes within the deleted region (120). However, our IIF staining study showed that Flag-CBPΔHAT maintained the diffused nuclear staining in HT22 cells (data not shown).

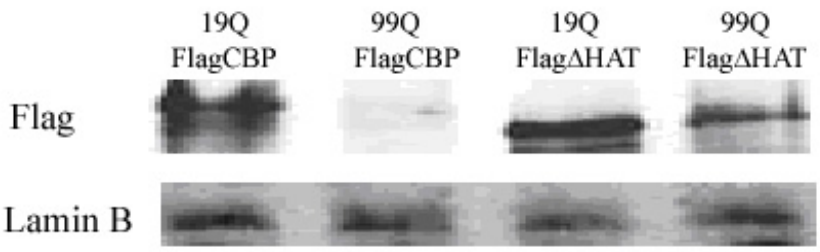

Figure 28: Degradation of CBP is independent of its HAT domain

A Flag-CBP or a Flag-CBPΔHAT construct was transfected with either 19Q or 99Q plasmid construct, and then subjected to Western blot analysis to detect CBP by an anti-flag antibody (top panel). Stripped blots were probed with an anti-lamin B (bottom panel) antibody to provide an internal control for protein loading. Comparing lane 3 and 4, decrease of CBP level in the presence of 99Q instead of 19Q is not altered when the amino acids 1446-1866 (which consists of part of the HAT domain) of transfected CBP is deleted. This result is preliminary and needs to be repeated.

5.2 Does Mdm2 play a role in polyQ-induced degradation of CBP?

Mdm2 is an E3 ubiquitin ligase that has been linked to the CBP/p300 family of coactivators (119). It is reasonable to suspect that Mdm2 may be the E3 that targets CBP for degradation for the following reasons. There is an Mdm2-binding site on the N-terminus of CBP. Furthermore Mdm2, p53 and CBP function as a complex to increase the degradation of p53 (119). We hypothesize that Mdm2 may be the E3 that targets CBP for degradation. If it is true, Mdm2 may be activated and accelerates the degradation of CBP in Htt-N63-99Q expressed HT22 cells. To test these hypotheses, we co-transfected HT22 cells with CBP (not tagged), Mdm2 and either 19Q or 99Q plasmids. As the shown in Figure 29, in cells transfected with 19Q, overexpression of Mdm2 reduced CBP levels to 23% (lane 1 and 3). In cells transfected with 99Q, Mdm2 had a smaller effect on CBP level (45%). These preliminary results suggest that in normal cells (i.e. cells expressing 19Q), Mdm2 may be the specific E3 for CBP and that polyQ-expanded htt may block its activity. However, this result is preliminary and needs to be repeated. We still can not assess whether Mdm2 plays a role in CBP degradation in polyQ expressed cells. The use of a dominant-negative mutant of Mdm2 may aid these studies.

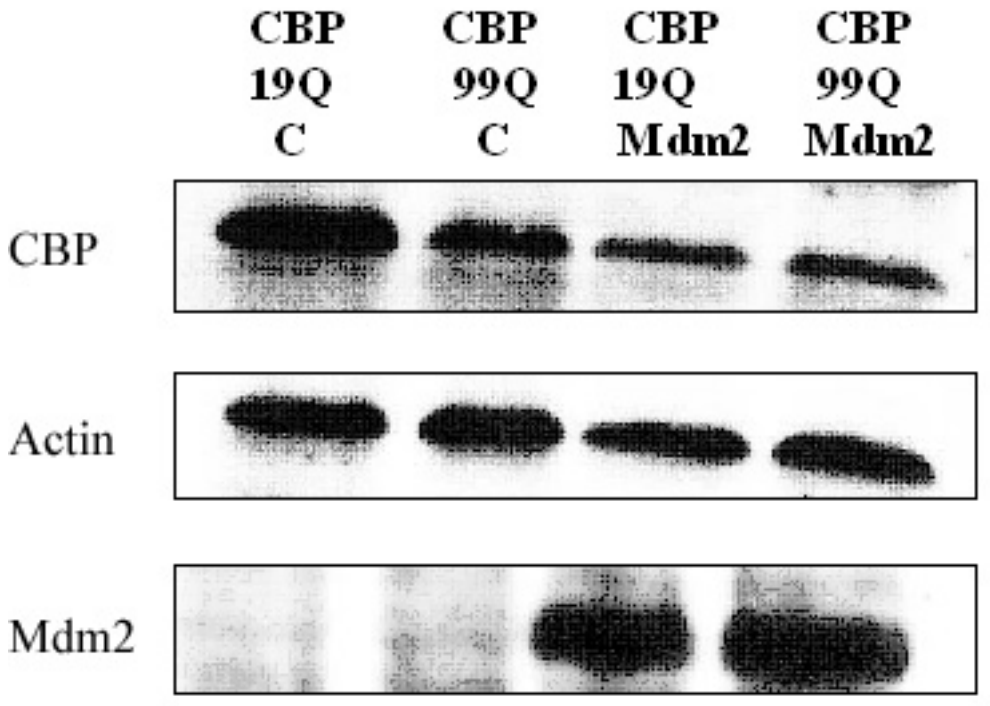

Figure 29: The effect on CBP level by the overexpression of Mdm2 in cells transfected with htt constructs

HT22 cells were transciently transfected with CBP (untagged), control or Mdm2 and either Htt-N63-19Q or 99Q plasmic constructs, and then subjected to Western blot analysis to detect CBP by an anti-CBP antibody (top panel). Stripped blots were probed with an anti-lamin B (middle panel) antibody to provide an internal control for protein loading. The blot is also reprobed with anti-Mdm2 to confirm the expression as shown in the bottom panel. In lanes 1 and 2, CBP level is decreased in the presence of 99Q comparing to 19Q (58%). Comparing lane 1 and 3, overexpression of Mdm2 decreased the level of CBP at the presence of 19Q (23%). Comparing lane 2 and 4, overexpression of Mdm2 had a smaller effect on CBP level at the presence of 99Q (45%).

IV DISCUSSION

1. CBP is a target of polyQ-expanded proteins

A number of studies have implicated the CBP coactivator as a selective target of polyQ-expanded proteins inclusion htt, atrophin-1, AR and ataxin 3 (47, 82, 120, 128). CBP is essential for neuronal cell survival through its impact on various transcription factors with established prosurvival effects in neurons, such as CREB (47, 82, 118, 121, 128). The most exciting evidence to support the relevance of CREB function in HD is from a recent study using a conditional mouse model containing a double knockout of CREB and cAMP-responsive element modulator (CREM) in postnatal forebrain. Mice with a CREB/CREM double knockout showed progressive neurodegeneration in the dorsolateral striatum and the hippocampus, reminiscent of Huntington disease (21). Upon the recruitment of CBP into nuclear aggregates containing polyQ-expanded protein, CBP function is disrupted, leading to reductions in transcription from CREB target genes (47, 149). Evidence of a decrease in CREB mediated transcription is obtained from two types of analyses. This first uses CRE reporter assays in cell culture systems, where CRE-mediated transcription was shown to be decreased (149). In addition to CBP, other transcriptional factors such as TAFII130 may also be responsible for the decrease of CRE-dependent transcription (46, 83). Therefore the disruption of CREB mediated transcription may result from the dysfunction of multiple proteins. PolyQ-expanded protein effects on CRE-dependent genes have also been examined in vivo. Some cAMP responsive genes such as somatostatin and corticotrophin releasing hormone are found to

have reduced expression in early HD brain (153, 154). Furtherfore a few microarray analysis from cell culture or transgenic mice have identified a number of candidate CREB-responsive genes whose expression is altered by polyQ-expanded htt (149). The fact that CBP overexpression (47, 128) or elevations in cAMP (149) could rescue polyQ-expanded htt or androgen receptor-mediated toxicity provides strong support for the notion that CBP can be limiting for neuronal cell survival. Although most studies have indicated recruitment of CBP into insoluble aggregates as the basis for its disrupted function, it may also be possible that CBP interacts with the soluble polyQ expanded proteins.

Along with other coactivators, CBP exerts its affects on transcription through the recruitment of histone acetyltransferases (48). The relevance of this CBP activity for neuronal cell survival is suggested by the observed inhibition of polyQ protein-dependent toxicity when histone acetylation is restored upon inhibition of histone deacetylases (48). Histone deacetylases such as trichostatin (TSA) and suberoylanilide hydroxamic acid (SAHA) are promising targets for drug development of HD and other polyglutamine diseases (28, 48).

2. HT22 cell toxicity is associated with nuclear localization of polyQ-expanded htt

Nuclear localization of polyQ-expanded proteins appears to be important for triggering neuronal cell death (35, 58, 78, 155), although in some cases polyQ-expanded proteins localized within the cytoplasm can also contribute to toxicity (59, 156, 157). In the HT22 hippocampal cell line, we find that toxicity within individual cells induced by polyQ-expanded htt, as revealed by a TUNEL assay, is associated with either nuclear or

perinuclear aggregates. HT22 cells that contain cytoplasmic polyQ-expanded htt aggregates exhibit minimal signs of cell death under our culture conditions. Likewise, CBP loss or its recruitment into polyQ-expanded htt aggregates only occurs in HT22 cells containing nuclear or perinuclear aggregates. These results suggest that, as in other cell types examined (47), disruption of CBP function in an important determinant of polyQ-expanded htt toxicity.

3. PolyQ-expanded htt can enhance CBP processing by the UPP

While CBP recruitment into polyQ-expanded htt aggregates is one mechanism that serves to limit its function, we show here that loss of CBP protein, probably via degradation, could provide another mechanism that contributes to polyQ-expanded htt toxicity. In previous studies, CBP was found to co-localize with polyQ-expanded disease proteins in nuclear aggregates (47, 82, 128). Recruitment of CBP into polyQ-expanded protein aggregates has also been observed in brain tissue from polyQ repeat disease patients and transgenic mouse models of these diseases (47, 82, 128). While CBP is also recruited into polyQ-expanded htt protein aggregates in HT22 cells, CBP degradation appears to accompany the formation of nuclear htt aggregates in a relatively large fraction of HT22 cells (i.e. up to 40%). The loss of CBP staining was also observed in another neuroblastoma cell line (i.e. N2a cells) expressing htt nuclear aggregates and thus is not strictly a property of HT22 cells. Our results do not definitively exclude the possibility of polyQ-expanded htt effects on CBP gene expression, but two independent assessments of UPP action on CBP (see below) support a role for enhanced degradation contributing to CBP loss.

Aggregation of polyQ-expanded proteins within cells has been found to reduce proteasome function and contribute to toxicity (33, 85). However, we find that in many cells expressing htt aggregates, CBP expression is reduced, owing in part to its degradation by the UPP. The recovery of CBP expression by proteasome inhibition in polyQ-expanded htt expressing cells supports this notion. Thus in addition to acting to block proteasome activity through the recruitment of essential proteasome components into nuclear aggregates (33, 85), polyQ-expanded proteins may also specifically enhance proteasome-dependent degradation of select substrates (e.g. CBP). This possibility could reflect increased activity of some component of the UPP or altered conformation of polyQ-bound CBP leading to engagement of the ubiquitylating enzymes or the proteasome. In fact, we find increased CBP ubiquitylation in HT22 cells expressing polyQ-expanded htt. Thus, some fraction of CBP appears to be more effectively engaged with the UPP in cells expressing polyQ-expanded htt. CBP degradation was observed in cells that express Htt-N63-99Q nuclear aggregates, although we cannot rule out the possibility that enhancement of proteasome activity can accompany the accumulation of toxic polyQ-expanded proteins that are not visibly associated with aggregates (35, 58). Furthermore, the toxic form of polyQ-expanded htt may be an unfolded intermediate (34) that alters the conformation of associated CBP and thereby enhances its targeting to the UPP prior to the formation of visible aggregates. For example, Gines S et al. reported that decrease of CRE-signaling in striatum of a HD mouse model occurred preceding aggregate formation (158).

Expression of polyQ-expanded htt can have a positive impact on protein degradation through an enhancement of proteasome subunit expression (159) or stimulation of

endosomal-lysosomal activity (160). In differentiated N2a neuroblastoma cells, ubiquitin conjugation can be detected on both normal and polyQ-expanded htt, although proteasome-mediated degradation of the ubiquitylated polyQ-expanded htt is selectively impaired (85). Thus, it is conceivable that the UPP could be activated in cells expressing polyQ-expanded htt but restricted in its action towards substrates (e.g. CBP) that are efficiently targeted to proteasomes following their ubiquitylation. While there is a reduction (i.e. ~40%) in soluble proteasome activity in differentiated N2a cells expressing htt with an expansion containing 150 glutamines, proteasome activity in a pelleted fraction is dramatically enhanced (i.e. ~10-fold) upon expression of this polyQ-expanded htt (85). Within an organelle (i.e. the nucleus) where proteasome action may typically be limited (161), any recruitment of proteasome subunits could enhance the degradation of potential substrates, prior to the eventual limitation of proteasome function through their sequestration into polyQ protein aggregates.

Not all nuclear proteins may share sensitivity to presumed activation of the UPP by expanded polyQ htt, as exhibited in our studies by the apparent lack of effects of polyQ-expanded htt nuclear aggregates on Sp1 localization and expression. PolyQ-expanded htt effects on Sp1 function do not require UPP involvement, but rather are due to a sequestration of Sp1 from productive interactions with both its cognate DNA recognition sites and the TAFII130 transcription factor (46). Furthermore, the enhancement of CBP ubiquitylation is selective since polyQ-expanded htt did not affect ubiquitylation of another proteasome substrate in HT22 cells, i.e. the GR protein. Since pharmacological inhibition of the proteasome increased both GR and CBP ubiquitylation in HT22 cells, the lack of enhanced GR ubiquitylation in cells expressing polyQ-expanded htt demonstrates

that proteasome function is not uniformly inhibited upon expression of polyQ-expanded htt. In a transgenic mouse model of SCA1, turnover of the protein kinase C delta isoform was increased in Purkinje cells of the cerebellum (162). In contrast, cerebellar Purkinje cell expression of the mGluR1 metabotropic glutamate receptor subunit protein was not affected in the SCA1 mice (162). Thus, selective enhancement of protein degradation may be a feature of other polyglutamine diseases and contribute at various levels to disease progression.

Although previous studies showed that either the polyQ domain of CBP or its HAT domain interact with polyQ-expanded htt (48), we are unable to detect an effect of those domains on the degradation of CBP by using corresponding deletion mutants. Thus, neither the polyQ nor HAT domain alone may be sufficient for binding to polyQ expanded htt. Other domains or a combination of more than one domain may be required. Alternatively it is possible that it is not the interaction of CBP and polyQ-expanded htt, but rather selective activation of the E3 ligase who targets CBP to UPP which triggers enhanced CBP degradation. As shown by our data in Figure 29, Mdm2 may be involved in the degradation of CBP under normal conditions. Therefore, the CBP-Mdm2 interaction domain may be important for the degradation of CBP induced by polyQ-expanded htt. This possibility could be tested by examining htt effects on CBP mutants lacking this domain.

Although nuclear dysfunction is devastating to cells expressing polyQ-expanded proteins, deficits of cytoplamic and neruite functions may also contribute to toxicity. For example, Sapp et al. showed that in lower grade HD patients, at the time that no nuclear aggregates

were detected in the striatum, mutant htt had accumulated in both the cytosol and axons of extensive striatal and cortical areas (60). This finding suggests that dysfunctions in axons may be an early event in HD pathogenesis. Several other studies also showed that neruite outgrowth and synaptic plasticity were affected before detectable nuclear changes in polyQ-expanded htt expressed cells (163). The cytoplasmic and synaptic dysfunctions can be induced by several mechanisms such as the presence of cytoplamic aggregates and decreased transcription of synaptic protein genes (164). It may also result from the enhanced degradation of related proteins in the presence of polyQ-expanded proteins through UPP, which has been shown to degrade some synaptic proteins. For example, human Siah proteins, which are putative E3 ligase, were shown to trigger degradation of the synaptic protein synaptophysin (165). Furthermore, the synaptic vesicle protein Ves1-1S/Homer-1a has an increased level and accumulates in vesicles upon proteasome inhibition (166). Therefore, altered UPP function on protein degradation in the presence of polyQ-expanded proteins may not be restricted to only nuclear proteins. As previously mentioned, in a SCA-1 transgenic mouse model, there is an obvious reduction of PKCγ that is normally located in cytoplasm or the dendrites of Perkinje cells (162). This reduction may primarily result from its enhanced degradation since only a minor decrease of PKCγ mRNA levels is observed. This degradation is also selective since mGluR1 protein levesl are not reduced (162). Morton et al. reported that complexin II, a cytoplasmic protein involved in neurotransmitter release, was depleted in both a HD transgenic mouse model and HD postmortem patient's brains (167). The reduction of protein level is not accompanied by the decrease of its mRNA level in the mice, suggesting that enhanced degradation of complexin II could be triggered by polyQ-

expanded htt. In contrast, complexin I protein levels were not changed (167). Therefore, it will be also interested to examine whether the loss of PKCγ in the SCA1 model and complexin II in the HD model was also due to the enhanced ubiquitylation and selective degradation through the UPP, as what we have seen for CBP.

Cell culture and transgenic models of polyQ repeat diseases have been informative and provided important insights into the molecular mechanisms contributing to neuronal cell death in these diseases. However, given the protracted time course of polyQ repeat disease progression in humans, there are likely to be many biochemical changes that occur over many years that contribute to the selective neuronal cell vulnerability that is characteristic of these diseases. The UPP is clearly critical to the cell function at many levels and therefore requires precise regulation to maintain its appropriate action. As shown in this report, opposing effects of polyQ-expanded htt on proteasome action can ultimately have comparable detrimental effects on neuronal cell survival. The ubiquitin–proteasome pathway could therefore be involved at many levels during the progression of chronic neurodegenerative diseases.

CHAPTER III

RESCUE OF POLYGLUTAMINE EXPANDED HUNTINGTIN BY MOLECULAR CHAPERONES

I. INTRODUCTION

1. Molecular chaperones

Molecular chaperones are proteins that can facilitate protein folding and prevent or reverse protein misfolding or aggregation (65). They mainly function in two stages during protein metabolism. First, they bind to the nascent polypeptides which have not emerged from the ribosome. The binding of molecular chaperones facilitates their proper folding into higher order structures while avoiding improper aggregation (62). Molecular chaperones also play multiple roles in maintaining proper functioning of mature proteins. For example, when cells are under stress, molecular chaperones function to refold denatured proteins and avoid aggregation (62). When chaperones are unable to implement these functions, they can accelerate degradation of the denatured or aggregated proteins through their connection to the UPP. In addition, chaperone proteins are involved in other cellular process such as hormone function through regulating the translocation of certain nuclear receptors [reviewd by (62)].

Two well-understood families of chaperones are DnaK and DnaJ families that are conserved among species from eukaryotic bacteria to mammals. Hsc70/Hsp70 are

members of DnaK family chaperones in mammalian cells. Hsc70 is the constitutive form and Hsp70 (heat shock protein 70) the inducible form of this protein family. Hsp70 has intrinsic ATPase activity and binds with the unfolded peptides mostly through hydrophobic interactions (168). The HDJ-1 (Hsp40) and HDJ-2 are mammalian members of DnaJ family proteins. They are cochaperones of DnaK family proteins and regulate their ATPase activities (65). Figure 30 shows a simplified representation of how an Hsp70/Hsp40 interaction cycles facilitate the folding of unfolded polypeptide in an ATP-dependent manner. The mechanism also applies to the process of refolding denatured proteins. Binding of Hsp40 with Hsp70 stimulates the ATPase activity of Hsp70. The ADP-bound form of Hsp70 has an increased affinity for unfolded protein (169). Hsp40 also binds to hydrophobic peptides and targets them to Hsp70 (170). ADP-bound Hsp70 aids in the appropriate folding of bound-peptides. Once nucleotide exchange factors facilitate the replacement of ADP with ATP (such as Bag-1), the folded peptide is released, and Hsp70 and Hsp40 resume a new reaction cycle (170).

2. Molecular chaperones and polyglutamine diseases

A number of neurodegenerative diseases involve deposition of mutant protein formed extra or intracellular aggregates (28), suggesting inappropriate folding of the disease proteins. In addition, affected neurons are under multiple cellular stresses. Therefore, molecular chaperones can perform various functions that limit neurodegenerative

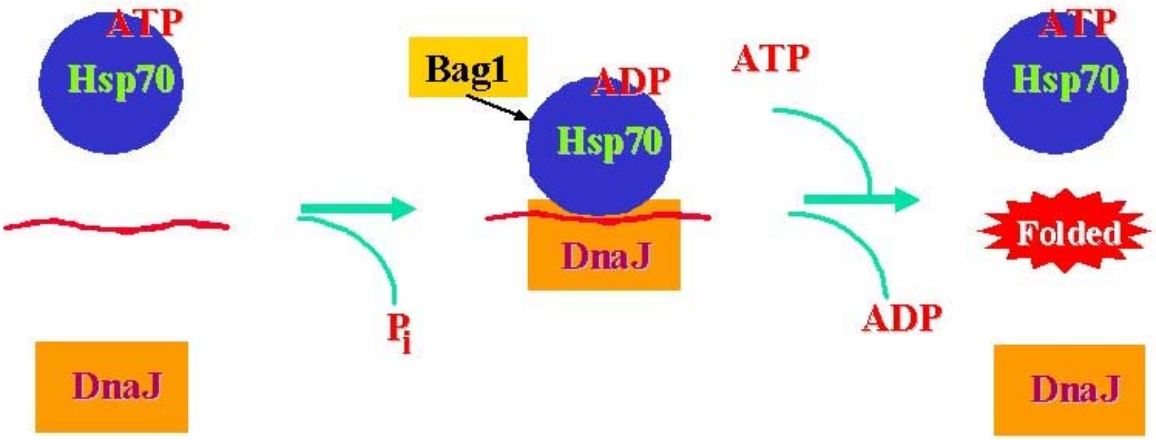

Figure 30: Hsp70/DnaJ reaction cycle

DnaK family molecular chaperones have ATPase activatity. The ADP bound state of Hsp70 has an increased affinity with the unfolded polypeptides and fold them. Once ADP is exchanged by ATP on Hsp70, the well-folded peptides are released. DnaJ family molecular chaperones can stimulate the ATP hydrolysis of DnaK and therefore facilitate peptide folding. Nuclear exchange factors such as Bag-1 indicated in this figure have opposite functions.

Table 5: Possible functions of molecular chaperones in neurodegenerative diseases

Adapted from (62)

Prevent aggregation of mutant proteins such as polyQ-expanded htt
Prevent aggregation and promote refolding of damaged proteins under cellular stress
Promote the solubility of aggregated proteins including those that are trapped by the aggregated proteins
Sort abnormal proteins for ubiquitylation and degradation if they can not be well-folded
Suppress apoptotic programs
Facilitate correct folding and glycosylation of membrane and secreted proteins

conditions. As reviewed by Sherman and Goldberg, Table 5 summarizes the possible roles chaperones may play to prevent aggregation and improve cellular survival (62). The roles of molecular chaperones in polyglutamine diseases were initially studied by Cumming et al. (29). They found that overexpression of HDJ-2 was able to reduce the formation of NI containing polyQ-expanded atxin-1 in tranfected Hela cells. Furthermore HDJ-2 was recruited into the nuclear aggregates of SCA-1 patients' brains (29). Since this study, numerous studies have tested the roles of DnaJ family and other chaperones in different polyglutamine diseases. Subsequently, more chaperones proteins have been examined for their roles in polyglutamine diseases in different disease models including neuronal and nonneuronal cell lines, transgenic mice and patients' brains (65). Despite the general consensus that molecular chaperones are involved in reducing the aggregation and toxicity of polyQ proteins, results are very variable (Table 6). For example there is variability in chaperones rescue within different cell lines. Furthermore there is often a dissociation of chaperone rescue of aggregation versus toxicity (Table 6). To confirm the value of chaperones in rescuing polyQ induced toxicities, additional evidence has been obtained from in vivo assays, especially from double transgenic models. Some of these results are summarized in Table 7 by Sakahira H et al. (65). For example, in a polyQ transgenic Drosophila eye model, a P-element directed screen identified dHdj-1 and dTPR2 (both contain J-domain) as suppressors of polyQ toxicity in Drosophila retinal neurons. (171). In a SCA-1 double transgenic mouse model, overexpression of inducible Hsp70 chaperone suppresses neuropathology and improves motor function in mice without effect on aggregation (172). These in vivo studies confirm that molecular chaperones are promising targets for therapeutic strategies for polyglutamine diseases.

Table 6: A summary of chaperone rescue of polyQ aggregation and toxicity in polyglutamine repeat diseases in cell culture and transgenic mice

PolyQ disease (protein-polyQ)	Results of chaperones rescue of polyQ induced toxicity in vitro	Reference
SCA1 (ataxin1/polyQ)	HDJ-2 colocalized into polyQ aggregate in mouse and cell culture. HDJ-2 rescues aggregation in cell culture. (Hela cells)	(29)
SBMA (AR-polyQ)	HDJ-2 is colocalized into polyQ aggregates and rescues aggregation (Hela cells)	(173)
SBMA (AR-ployQ)	Hsc70/HDJ-1 combination or hsc70 alone rescue aggregation and apoptosis. HDJ-2 is unable to rescue aggregation and apoptosis (N2a cells)	(147)
HD (htt-polyQ)	HDJ-2 increase aggregation in a certain cell type (Cos7 cells but not PC12 cells)	(174)
HD (htt-polyQ)	HDJ-2 and hsc70 colocalize with polyQ aggregates in transgenic mice. Only HDJ-1 and/or hsc70 rescue polyQ aggregation and toxicity. (N2a cells)	(85)
SCA3 (ataxin3-polyQ)	HDJ-1, HDJ-2, Hsp70 colocalized into the polyQ aggregate in patient brains. HDJ-1 rescues aggregation and toxicity in cell culture. HDJ-2 rescues aggregates but not toxicity (Cos7cells and PC12 cells)	(148)

Table 7: Effects of molecular chaperones on polyQ aggregation and toxicity in vivo

Adapted from (65)

Chaperones	Organisms	PolyQ disease	Aggregation	Toxicity	Domain of mutation	References
Hsp70	Mouse	SCA-1	no change	decrease		(172)
dHdj-1	*Drosophila*	PolyQ	no change	decrease		(171)
dTPR2	*Drosophila*	PolyQ	no change	decrease		(171)
dHsp70	*Drosophila*	SCA-3	no change	decrease		(68)
dHsp70 (D/N)	*Drosophila*	SCA-3	n.d.	increase	ATP-binding domain	(68)
dHdj-1	*Drosophila*	SCA-3	n.d.	decrease		(175)
dHdj-1 (D/N)	*Drosophila*	SCA-3	n.d.	increase	J-domain deletion	(175)
dHdj-1 (D/N)	*Drosophila*	SCA-3	n.d.	increase	Substrate binding domain	(175)
dHsp70 + dHdj-1	*Drosophila*	SCA-3	no change under LM; but more soluble fraction by Western blot	decrease more		(175)
dHdj-1	*Drosophila*	HD	n.d.	decrease		(175)
dHdj2	*Drosophila*	HD	n.d.	no change		(175)
dHdj-1	*Drosophila*	SCA-1	more compact	decrease		(176)

n.d., not determined; D/N, dominant-negtive

However, as shown in higher organisms, molecular chaperones appear to rescue toxicity but not necessarily on aggregation of polyQ-expanded proteins (Table 7). In this part of the dissertation, we have examined chaperone function in both in vivo and in vitro models of HD and confirmed that chaperone overexpression is not always sufficient for rescue of NI formation.

II. MATERIAL AND METHODS

1. Plasmids

The plasmid p6R flag-HDJ-2/HSDJ encodes the full-length human HDJ-2 that is tagged with a flag epitope at its N-terminus. The plasmids pcDNA3.1-Htt-N63-19/99Q-myc-His have been decrible in the methods section of chapter II.

2. Cell culture, transfection, Western blot analysis and IIF

Human HEK 293 fibroblast cells were maintained in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% FBS (Atlanta Biologicals) and penicillin/streptomycin (BioWhittacker). Transfection was performed using the lipofectamine reagent (Invitrgen) as decribed in Chapter II. Western blot analysis and IIF were performed as decribed in Chapter II. Mouse anti-Hsp70 antibody and rabbit anti-Hsp40 (HDJ-1) antibody were purchased from Stressgen. Mouse anti-HDJ-2/DNAJ Ab-1 (clone KA2A5.6) was purchased from Neomarkers.

3. Animal model and immunohistochemistry of brain sections

HD transgenic mice express Htt-N171-82Q, which contains the N-terminal 171 amino acids with an 82Q expansion. Brains were obtained from transgenic mice that were 4-5 months old. Control mice of the same age were killed, sectioned and examined at the same time. After dissection, brains were treated with PFA, sectioned at 30-40 μm coronal plane and stored at -20°C. For brain staining, sections from whole brains were washed with PBS and then incubated with primary antibody diluted in PBS supplemented with donkey serum at 4°C overnight. The next day, sections were washed with PBS and then incubated with secondary antibodies followed by avidin-biotin complex at room temperature for 2 and 1.5 hours, respectively. Finally, the sections were reacted with diaminobenzidine (DAB) (Sigma) for 10 minutes and then hydrogen peroxide (Sigma). The reaction was terminated by washing with fresh buffer. All the sections were mounted and examined under light microscopy. The mouse anti-htt antibody is made against the N-terminal 17 amino acids of htt and was provided by Dr. Christopher Ross of John Hopkins University.

III. RESULTS:

1. Overexpression of HDJ-2 does not reduce the formation of aggregates containing polyQ-expanded htt in HEK 293 cells

We first selected human HEK293 embryonic kidney cell line to assess the ability of HDJ-2 to rescue aggregation of polyQ expanded htt. Overexpressed Htt-N63-99Q in

transiently transfected HEK293 cells exhibit primarily three patterns: diffuse cytoplasmic staining, nuclear aggregates and cytoplasmic aggregates. Htt-N63-19Q consistently displayed diffused cytoplasmic staining in transfected cells.

As HDJ-2 was shown to reduce aggregation of ataxin-1 in Hela cells (29), we first examined whether it could also decrease aggregation of Htt-N63-99Q in our cell system. Therefore, we cotransfected a flag-HDJ-2 construct with Htt-N63-99Q into HEK 293 cells and examined Htt-N63-99Q aggregates. As shown in Figure 31 and summarized in Figure 32A, Flag-HDJ-2 did not reduce the number of Htt-N63-99Q expressing cells containing aggregates, although HDJ-2 was recruited into the aggregate in a majority of the 99Q aggregate containing cells (Figure 32B). To exclude that the inability of HDJ-2 to rescue aggregation was due to its inadequate expression, we performed the following experiments. As shown in Figure 32B, we confirmed that Flag-HDJ-2 is expressed in 96% of the cells expressing 99Q. An elevation of the ratio of HDJ-2 to 99Q DNA to 5:1 in transfection experiments was still unable to reduce the aggregation. We also perform Western blots to show that the total HDJ-2 amount is increased about 1.8 folds (Figure 33). Given the 10-20% transfection efficiency, this amount of HDJ-2 increase represents only a minimum estimation of overexpression within individual cells. Therefore, we conclude that overexpression of HDJ-2 can not rescue the formation of aggregates containing 99Q in HEK293 cells. This result is consistent with other published papers which showed that HDJ-2 can not rescue polyQ-expanded huntingtin aggregates in neuronal and nonneuronal cell lines (Table 7) (85, 174). We also tested other chaperones such as Hsp70 and HDJ-1. Neither can rescue polyQ-htt aggregation (data not shown).

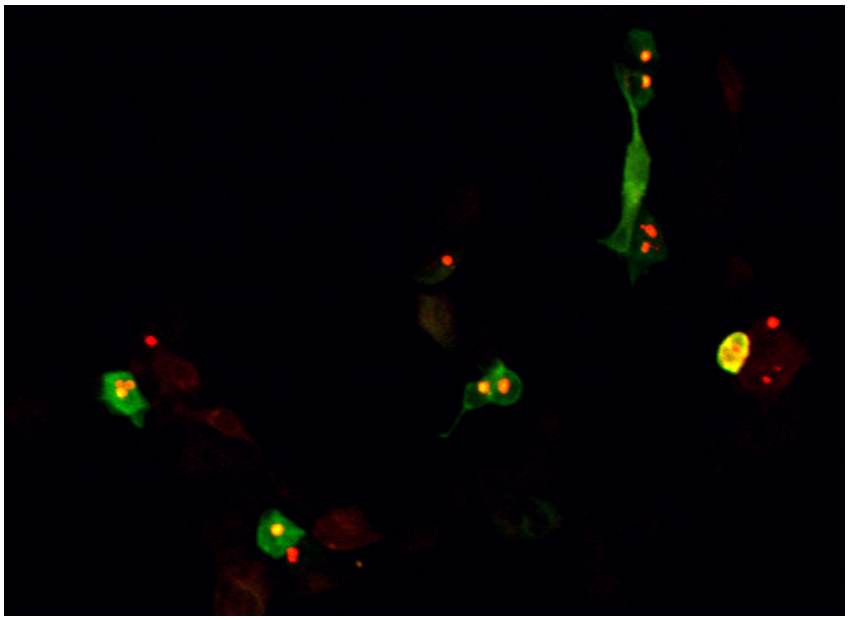

Figure 31: Colocalization of Htt-N63-99Q protein with cotransfected flag-HDJ-2 in HEK293 cells

HEK293 cells were cotransfected with 1 µg of the Htt-N63-99Q DNA and 5µg of flag-HDJ-2. Cells were processed for IIF using a rabbit anti-c-myc and a mouse anti-flag antibody, and then examined by confocal fluorescence microscopy. Red staining shows the Htt-N63-99Q protein, while green staining shows the HDJ-2 protein. The areas of overlap are depicted in yellow. In the lower three cells, although HDJ-2 is overexpressed, aggregates containing 99Q does not disappear. Note that HDJ-2 can be recruited into these aggregates.

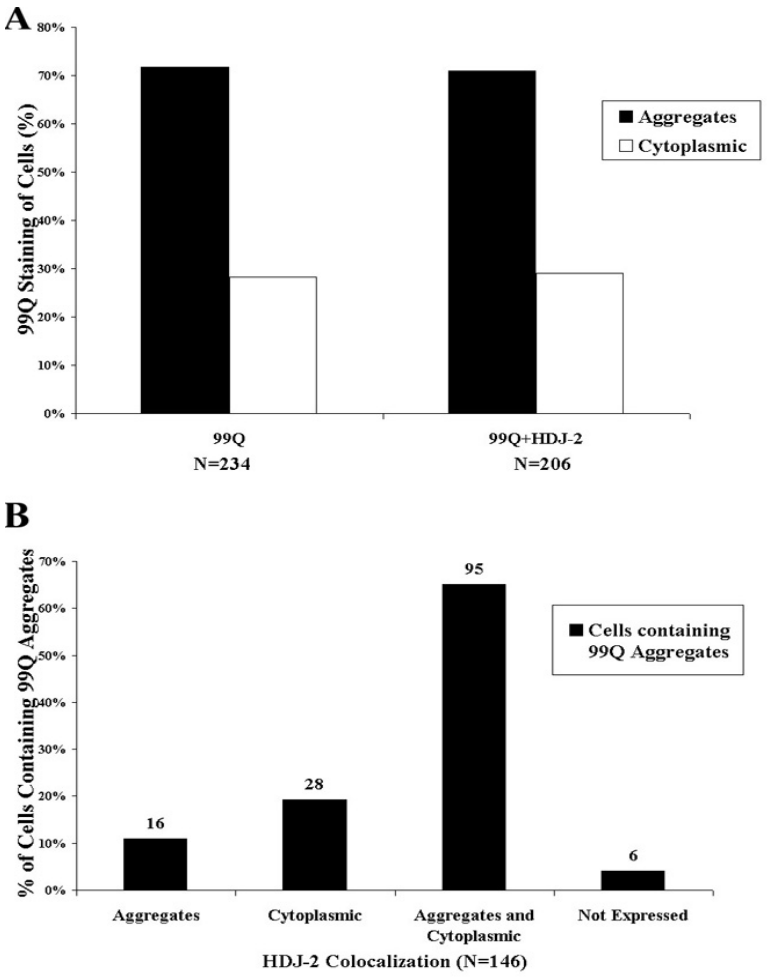

Figure 32: Overexpression of HDJ-2 does not rescue 99Q aggregate formation

Cells from randomly selected fields were counted following IIF. Panel A (left columns) shows the percentage of cells containing aggregates or with cytoplasmic staining when 1µg Htt-N63-99Q was transfected. The right columns show the percentage of cells containing aggregates when 1µg 99Q and 5µg flag-HDJ-2 were cotransfected. There is no difference between the two groups. Panel B shows the percentage of cells with different staining patterns when 99Q and HDJ-2 were cotransfected (panel right columns). Note that when 99Q is present within aggregates, most of the cells also express HDJ-2 in the aggregates. These experiments have been repeated at least three times.

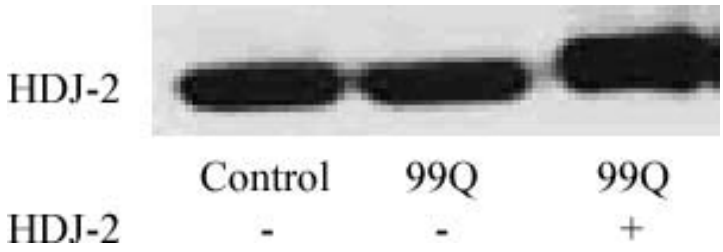

Figure 33: Overexpression of HDJ-2 in cells cotransfected with Htt-N63-99Q

HEK 293 cells were transfected with control or Htt-N63-99Q with HDJ-2 plasmid vectors. Western-blot analysis was performed to detect HDJ-2 level using an anti-HDJ-2 antibody. The level of total HDJ-2 is increased about 1.8 folds upon the transfection of flag-HDJ-2 (comparing lanes 2 and 3).

2. Molecular chaperone recruitment into aggregates containing Htt-N63-99Q in transfected cells

Although HDJ-2 was unable to rescue polyQ-htt aggregate formation, it was recruited into the aggregates in 80% of cells expressing 99Q aggregates (Figures 31 and 32B). This prompted us to examine expression of endogenous HDJ-2 and other chaperones including HDJ-1 and Hsc70 in cells expressing 99Q aggregates. As shown in Figure 34, endogenous HDJ-2 is also recruited into nuclear aggregates. Thus, the recruitment of HDJ-2 to 99Q aggregates in vitro is not sufficient to ensure rescue of aggregates. In contrast to the recruitment of HDJ-2, 99Q aggregates in transfected HEK293 cells do not recruit endogenous HDJ-1. HDJ-1 remains predominantly cytoplasmic and seems to be excluded form the aggregates with minimal colocalization. Hsc70 localization changes slightly in cells expressing 99Q, as some Hsc70is recruited to the nuclear aggregates. However, the major fraction of Hsc70 remains in the cytoplasm.

3. Molecular chaperone recruitment into aggregates containing polyQ-expanded huntingtin in a transgenic mouse model of HD

HDJ-2 and Hsc70 were detected in the ataxin-1 aggregates of cerebellum Perkinje cells in both patients' brain and a transgenic mouse model (29). Therefore, we examined whether molecular chaperones are recruited into aggregates formed in a HD transgenic mouse model. The mouse model we used is derived from the R6/2 strain. These mice bear a

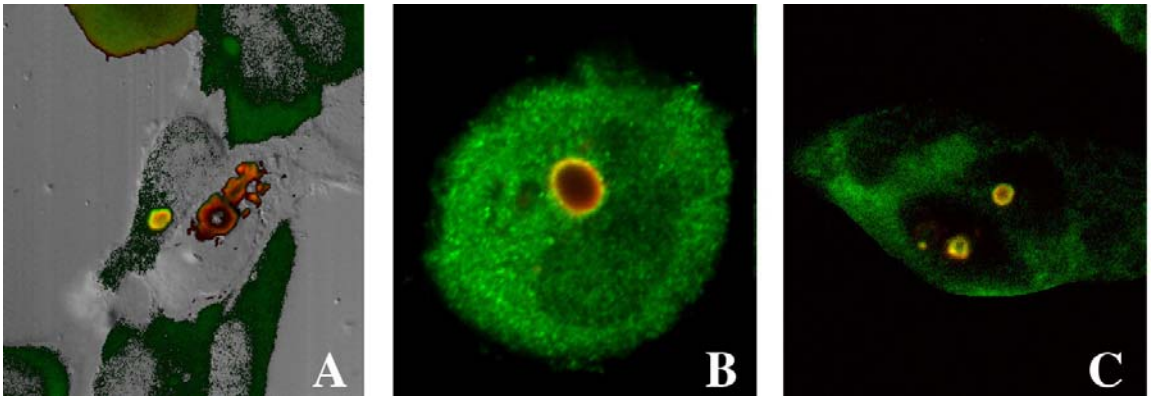

Figure 34: Recruitment of endogenous HDJ-2, HDJ-1 and Hsc70 into Htt-N63-99Q aggregates. HEK293 cells were transfected with 2.5µg of Htt-N63-99Q DNA and then costained with rabbit anti-c-myc and mouse anti-HDJ-2 antibodies (panel A), mouse anti-HDJ-1 (panel B), or rat anti-Hsc70 (panel C). Cells were examined under confocal fluorescence microscopy. The red shows Htt-N63-99Q protein, while the green shows endogenous HDJ-2, HDJ-1 or Hsc70. The areas of overlap are depicted in yellow. Panel A is a triple overlay of red, green immunofluorescence and differential interference contrast microscopy (DIC). Endogenous HDJ-2 is recruited into 99Q-containing aggregates. HDJ-1 localizes predominately within the cytoplasm and is only minimally recruited into the 99Q containing aggregates. Hsc70 is partially recruited into 99Q-containing aggregates.

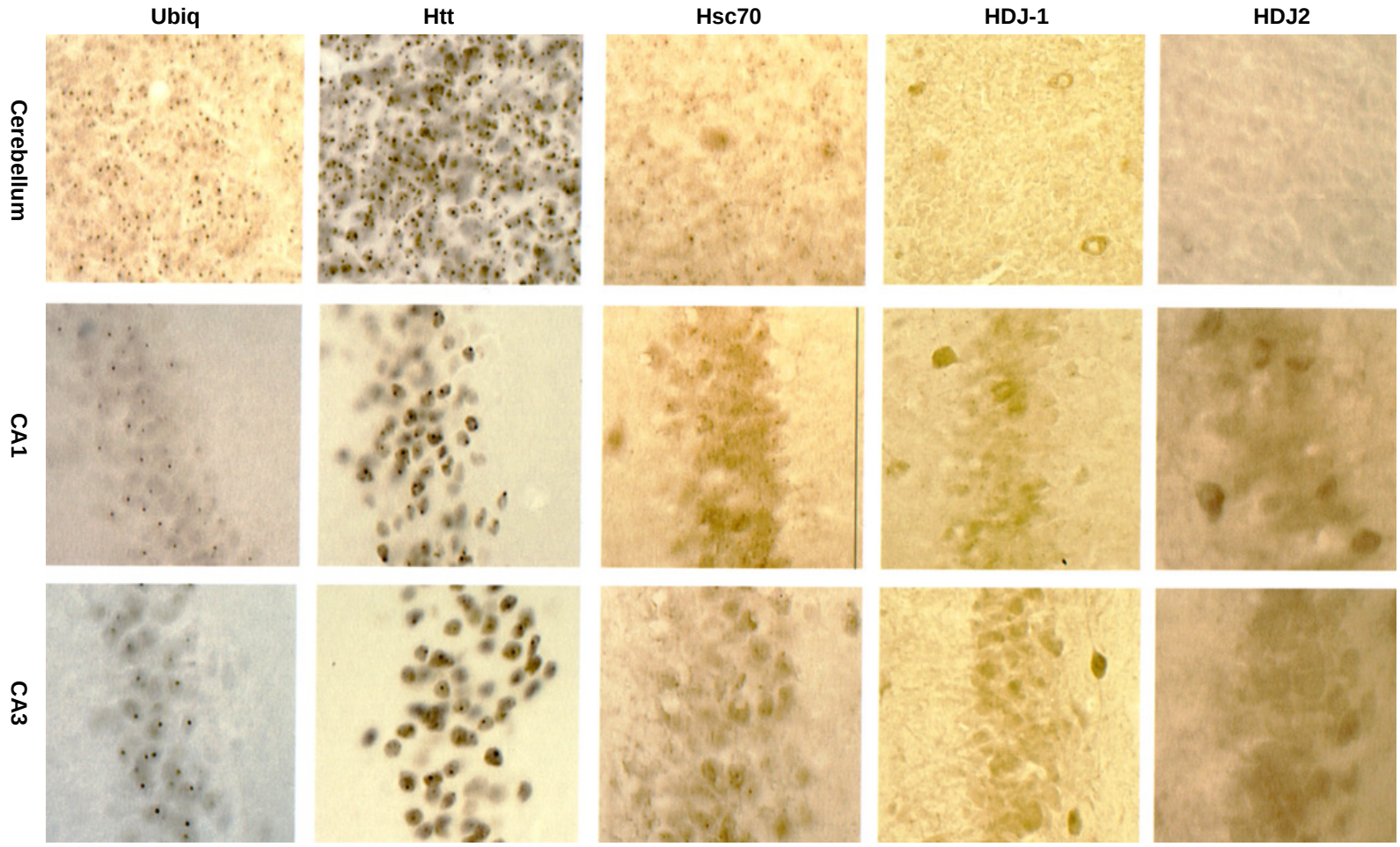

Figure 35: Hsc70, HDJ-1 and HDJ-2 expression in the brain of HD transgenic mice bearing Htt-N171-82Q (R6/2 strain).

Legends of Figure 35: Brain sections from transgenic mice bearing Htt-N171-82Q were stained with antibodies to either ubiquitin, the N terminus of human huntingtin, Hsc70, HDJ-1 or HDJ-2. Certain areas which are most affected by the mutant protein are shown here (i.e. cerebellum, CA1 and CA3 fields of the hippocampus). Note that neither HDJ-1 nor HDJ-2 was recruited into the htt-containing aggregates. Some recruitment of Hsc70 was observed. No aggregates were seen in control mice brain sections stained with antibodies against ubiquitin, huntingtin or Hsc70 (Data not shown).

transgene encoding the N terminal 171 amino acids of htt within which there is an 82 polyQ tract (Htt-N171-82Q). The control mice bear an 18Q instead (Htt-N171-18Q). This mouse line has been well studied and the 82Q mice showed HD like phenotypes (92). NI were also detected in multiple sites of the brain including cortex, hippocampus and at a late stage, striatum (92). The Htt-N171-82Q mice at 4-5 months of age show neurological deficits such as tremor, loss of weight and uncoordination. This line of mice normally die at 5-6 months of age (92).

Before examining chaperone recruitment, we first looked at brain regions that contain aggregates by using an anti-htt antibody and an anti-ubiquitin antibody, respectively in 4-month old mice. The most obvious regions with aggregates are cerebral cortex, hippocampus (CA1 and CA3) and cerebellum. As shown in Figure 35, aggregates containing 82Q were detected in these regions that also contain ubiquitin. Therefore we stained these regions with antibodies against individual chaperones. Unlike what was reported for SCA1 (29), HDJ-2 is not recruited into aggregates although HDJ-2 was well-stained in the areas examined as diffused cytoplasmic staining. To exclude the possibility that our staining technique was unable to detect HDJ-2 in the aggregates, we obtained the same SCA-1 mouse tissue as published by Cumming et al.(29). We indeed detected HDJ-2 in aggregates containing polyQ-expanded ataxin-1 using exactly the same antibody and staining procedures (data not shown). Therefore, we concluded that at least in the HD mouse brain, HDJ-2 is not recruited into aggregates containing polyQ-expanded htt at late stages of disease progression.

In addition to HDJ-2, we also examined the localization of HDJ-1 and Hsc70. As shown in Figure 35, HDJ-1 is not recruited into htt aggregates as well. However, we did detect recruitment of Hsc70 into aggregates in some cells. HDJ-1, HDJ-2 and Hsp70 were detected in aggregates in a SCA-3 mouse model as well (148). Comparing the SCA1 and SCA-3 mouse models with our HD model, it suggests that molecular chaperones may not react uniformly to different polyQ-expanded proteins in vivo. There is still no report of any chaperone recruitment into the aggregates of HD patients' brains.

IV DISCUSSION

1. Limitation of in vitro assays of polyQ-expanded protein aggregation and toxicity

As shown in Table 6, there is substantial viability of chaperone rescue of polyQ induced aggregation and toxicity. These differences may result from at least two reasons. The first is the inconsistency of cell lines used. Even in the same study, different cell lines may exhibit different abilities of chaperone rescue (Table 6). This may be due to variations of levels of endogenous chaperones or chaperone partners. For example, in some cell line tested, only an overexpression of both a member of DnaK protein and a member of DnaJ protein can rescue aggregation (85, 147). In addition, each cell type could be subject to different "stressful" conditions. The second inconsistency is the length of Q residues in the protein and their context. PolyQ repeat length has been shown to be positively correlated with aggregate formation and toxicity. The length of the truncation of htt also has an effect on aggregation and toxicity (145). Therefore, these factors may both affect on chaperone rescue in various in vitro assays. In our study, we used Htt-N63-99Q.

Another construct such as Htt-N171-82Q may be differentially sensitive to chaperones rescue.

2. Dissociation of polyQ-induced aggregation and toxicity

Despite the inconsistency concerning chaperone rescue in various in vitro models, studies using double transgenic flies and mice do suggest that molecular chaperones may function to reduce toxicity (Table 7) (65). These results suggest that molecular chaperones should be considered as potential targets for therapeutic drugs to treat polyQ diseases. However, as shown in Table 7, in mammals, rescue of toxicity often is not accompanied by reduction of aggregation (65). We found that in HEK293 cells overexpression of HDJ-2 can not rescue aggregation. It may be possible that HDJ-2 can rescue toxicity without apparent effects on aggregation. I have tried to measure toxicity using the luciferase assay described in the previous chapter (data not shown). The results showed that overexpression of chaperones did not rescue Htt-N63-99Q induced toxicity, as luciferase activity was increased in both 19Q and 99Q expressing cells at the same ratio. However, other methods to test toxicity may be useful. Alternatively, the inability of HDJ-2 to rescue both aggregation and toxicity in our HEK 293 cells may be due to the recruitment of HDJ-2. As reported by Bonini et al., proteins that physically interact with polyQ-expanded proteins in their polyQ transgenic fly model failed to rescue cells from toxicity (177). Therefore, the lack of recruitment of HDJ-2 into aggregates in our HD mouse model can not rule out the possibility that HDJ-2 may function to reduce toxicity in vivo.

In addition to polyglutamine diseases, Hsp70 can also reduce α-synuclein induced toxicity in a transgenic fly model of PD without a corresponding reduction of its aggregation (178). Thus, what are the bases for the dissociation of aggregation and toxicity? One possibility is that chaperones change the solubility of polyQ-expanded proteins without distinguishable morphological changes of aggregates as detected by light microscopy (177). For example, in a polyQ transgenic fly or a yeast model, overexpression of chaperones rescue polyQ induced toxicity, while no change is observed on aggregation by light microscopy. However, using Western blot and a filter-trap assays, the SDS-soluble fraction of polyQ expanded protein was found to be significantly increased by overexpression of Hsp70 and Hsp104, respectively (24, 177). By solublizing the polyQ-expanded poteins, other polyQ protein interacting proteins may also become more soluble in the presence of chaperones and resume their normal functions. CBP and Sp1 are good candidates for proteins whose function may be restored by chaperones. Therefore, two experiments could be performed to test these possibilities. The first is to compare the solubility of Htt-N63-99Q in the presence or absence of molecular chaperones using Western blot or filter trap assays. The second is to examine whether the activity of CBP is increased upon overexpression of chaperones.

3. Mechanisms underlying chaperones rescue of polyQ-expanded protein induced toxicity

As shown in Figure 2A, the physical forms of polyQ-expanded proteins that are toxic may not be restricted to amyloid-like aggregates (24, 65). Other intermediate forms may

be more toxic than large soluble aggregates. For example, β-structured monomer and oligomers may trap other interacting proteins more efficiently than large aggregates (24, 65). Molecular chaperones may function at multisteps during the folding of polyQ-expanded proteins to reduce cellular toxicity (24, 65).

In addition to their roles in the folding process, molecular chaperones may participate in other pathways to reduce cellular stress (Table 5). Bag-1 and Chip are proteins that interact with Hsp70 (179). Both can target Hsp70-bound protein intermediates to the UPP for degradation. In this way, Hsp70 can also eliminate certain misfolded proteins. Therefore different Hsp70 interacting proteins can sort misfolded proteins into folding or the degradation pathways (179). In addition, Hsp70 has also been reported to have anti-apoptotic functions through either c-Jun NH_2-terminal kinase (JNK) dependent or independent mechanisms (180). It has been reported that the JNK pathway is activated by polyQ-expanded htt and contributes to apoptosis in a hippocampal cell line (112). Hsp70 can inhibit JNK activation through increasing the JNK phosphatase activity and thus repress apoptosis (62), which has been also reported in an in vitro model of HD (181). Hsp70 does not need its chaperone acitivity to inhibit JNK activation since an Hsp70 mutant deficient in substrate binding still functions by this mechanism (180). In a JNK-independent manner, Hsp70 may function at the apoptosome level to block apoptosis (182). The apoptosome is a large complex containing cytochrome c, dATP, Apaf-1 oligomer and procaspase 9 in which procapase 9 is activated (12). Activated caspase 9 then cleaves and activates caspase 3. It has been reported that Hsp70 binds to Apaf-1 and thus inhibits the recruitment of procapase 9 into the apoptosome to block the

activation of caspase 9 and caspase 3. The chaperone activity of Hsp70 is required in this process (182). This apoptosome-dependent anti-apoptotic function of Hsp70 may also play a role in its protection against polyglutamine and other neurodegenerative diseases.

Given the abilities of molecular chaperons to rescue the toxicity in various in vivo models of neurodegenerative diseases, molecular chaperones may be one of the most promising targets for development of drugs to treat these diseases.

CHAPTER IV:

SUMMARY AND CONCLUSION

As discussed in the first chapter, the mechanism underlying pathogenesis of HD is very complicated. This dissertation study has added to the mechanisms in several aspects.

I. TRANSCRIPTION DEFICIENCY - CBP

In previously published papers, the loss of CBP function in polyglutamine diseases was normally explained as due to its recruitment into aggregates containing polyQ expanded proteins (47, 82, 128). As shown in Chapter II of this study, we showed another mechanism that may contribute to loss of CBP function, which is through enhanced degradation. This conclusion is supported by results showing increased ubiquitylation of CBP in the presence of 99Q (Figure 26) and the recovery of CBP levels by the treatment of htt-polyQ expressing cells with a proteasomal inhibitor (Figures 21 and 22). However, we have only detected this phenomenon in an immortalized cell line. In order to confirm the relevance of CBP degradation in vivo, we should next examine primary neurons and transgenic mouse brain tissues. Considering the long and complicated process of pathogenesis in HD mouse, CBP loss may be temporary. In addition, we should further identify other mechanisms underlying selective CBP degradation in HT22 cells expression polyQ-expanded htt.

Transcriptional deficiency is one of most important mechanisms underlying the pathogenesis of polyglutamine diseases. Among numerous transcription factors and

cofactors that are involved, CBP is the best-studied. Most importantly, it is promising to target this mechanism for therapeutic strategies based on its effects on toxicity of manipulating the HAT activity. Histone deacetylase inhibitors have been shown to reduce polyQ-expanded htt induced toxicity in transgenic Drosophila eye (48). Interestingly some of these inhibitors are even in clinical trials for the treatment of other diseases such as SAHA in cancer treatment (48).

II. IMPAIRMENT OF UPP IN HD

The UPP no doubt plays important roles in polyglutamine diseases and other neurodegenerative diseases. It was suggested that the general inhibition of UPP by polyQ-expanded proteins contributes to toxicity. Based on the results of accelerated CBP degradation through UPP in HT22 cells expressing Htt-N63-99Q, we suggested that impairment of UPP may not only be restricted to its inhibition. UPP can be more activated as well and target specific proteins, such as CBP for degradation. This activation can be temporally restricted. For example, at the early stage of the disease, in the attempt to eliminate aggregation, activated UPP may be involved in the degradation of other important proteins. At later stages of the disease, recruitment of UPP components into insoluble aggregates would eventually reduce its normal function. Different effects on UPP can be spatially regulated as well. For example, the UPP may be activated in the aggregates and inhibited elsewhere (85). Since the degradation of certain proteins is the result of activation of some E2, E3 or proteasome subunits, there are multiple levels at which the UPP could be affected by polyQ-expanded proteins. In addition, as discussed in Chpter II, degradation of proteins in the cytoplasm and synapse

may also be important for cellular toxicity, especially as synaptic loss is found in early stages of HD (60).

III. MOLECULAR CHAPERONES

As discussed in chapter III, molecular chaperones play multiple roles to reduce cellular toxicity under different stressful conditions. They are shown to be beneficial for the reduction of toxicity in polyglutamine diseases as well. In contrast to the initial paper concerning ataxin-1 by Cumming et al. (29), we did not detect HDJ-2 rescue of Htt-N63-99Q aggregation and toxicity in HEK293 cells, nor did we found the recruitment of HDJ-2 into aggregates of our HD mouse model. These discrepancies could result from the inconsistency of in vitro assays, the dissociation between aggregation and toxicity, and methods used to examine insolubility of the polyQ-expanded proteins in the aggregates. In our in vivo and in vitro assays, individual chaperone may respond differently to the same cellular stress, such as the difference in recruitment of endogenous HDJ-1 and HDJ-2 in HEK293 cells transfected with Htt-N63-99Q.

IV. CONNECTING MOLECULAR CHAPERONES WITH UPP IN POLYGLUTAMINE DISEASES

When cells are under stress, such as heat shock, the levels of several groups of proteins are increased. These proteins include some molecular chaperones and some components of the UPP (62). Unfolded or misfolded polypeptides will either be folded into their native form or be degraded such as through UPP. Therefore these unwanted polypeptides will not cause toxicity to the cells (62). For example, Hsp70 and some of its associated

proteins serve as hinges to connect the chaperones machinery with UPP (179). Given the important roles of both chaperones and UPP in the polyglutamine diseases, it is reasonable to imagine that the link between them may have an intriguing impact on the folding and degradation of polyQ-expanded proteins and other proteins in these diseases.

REFERENCES:

1. Kerr, J.F., Wyllie, A.H. and Currie, A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer,* **26,** 239-57.
2. Sastry, P.S. and Rao, K.S. (2000) Apoptosis and the nervous system. *J Neurochem,* **74,** 1-20.
3. Gobbel, G.T. and Chan, P.H. (2001) Neuronal death is an active, caspase-dependent process after moderate but not severe DNA damage. *J Neurochem,* **76,** 520-31.
4. Ankarcrona, M., Dypbukt, J.M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S.A. and Nicotera, P. (1995) Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. *Neuron,* **15,** 961-73.
5. Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P. and Lipton, S.A. (1995) Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci U S A,* **92,** 7162-6.
6. Buettner, G.R. (1993) The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. *Arch Biochem Biophys,* **300,** 535-43.
7. Loo, D.T., Copani, A., Pike, C.J., Whittemore, E.R., Walencewicz, A.J. and Cotman, C.W. (1993) Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. *Proc Natl Acad Sci U S A,* **90,** 7951-5.
8. Fukuda, K., Kojiro, M. and Chiu, J.F. (1993) Demonstration of extensive chromatin cleavage in transplanted Morris hepatoma 7777 tissue: apoptosis or necrosis? *Am J Pathol,* **142,** 935-46.
9. Tatton, W.G., Chalmers-Redman, R., Brown, D. and Tatton, N. (2003) Apoptosis in Parkinson's disease: signals for neuronal degradation. *Ann Neurol,* **53 Suppl 3,** S61-70; discussion S70-2.
10. Nijhawan, D., Honarpour, N. and Wang, X. (2000) Apoptosis in neural development and disease. *Annu Rev Neurosci,* **23,** 73-87.
11. Friedlander, R.M. (2003) Apoptosis and caspases in neurodegenerative diseases. *N Engl J Med,* **348,** 1365-75.
12. Adams, J.M. and Cory, S. (2002) Apoptosomes: engines for caspase activation. *Curr Opin Cell Biol,* **14,** 715-20.
13. Kuida, K., Zheng, T.S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P. and Flavell, R.A. (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature,* **384,** 368-72.
14. Martinou, J.C., Dubois-Dauphin, M., Staple, J.K., Rodriguez, I., Frankowski, H., Missotten, M., Albertini, P., Talabot, D., Catsicas, S., Pietra, C. *et al.* (1994) Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. *Neuron,* **13,** 1017-30.

15. Zanjani, H.S., Vogel, M.W., Delhaye-Bouchaud, N., Martinou, J.C. and Mariani, J. (1996) Increased cerebellar Purkinje cell numbers in mice overexpressing a human bcl-2 transgene. *J Comp Neurol,* **374,** 332-41.
16. Deckwerth, T.L., Elliott, J.L., Knudson, C.M., Johnson, E.M., Jr., Snider, W.D. and Korsmeyer, S.J. (1996) BAX is required for neuronal death after trophic factor deprivation and during development. *Neuron,* **17,** 401-11.
17. Martin, D.P., Schmidt, R.E., DiStefano, P.S., Lowry, O.H., Carter, J.G. and Johnson, E.M., Jr. (1988) Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J Cell Biol,* **106,** 829-44.
18. Sakhi, S., Bruce, A., Sun, N., Tocco, G., Baudry, M. and Schreiber, S.S. (1994) p53 induction is associated with neuronal damage in the central nervous system. *Proc Natl Acad Sci U S A,* **91,** 7525-9.
19. Herber, B., Truss, M., Beato, M. and Muller, R. (1994) Inducible regulatory elements in the human cyclin D1 promoter. *Oncogene,* **9,** 2105-7.
20. el-Deiry, W.S. (1998) Regulation of p53 downstream genes. *Semin Cancer Biol,* **8,** 345-57.
21. Mantamadiotis, T., Lemberger, T., Bleckmann, S.C., Kern, H., Kretz, O., Martin Villalba, A., Tronche, F., Kellendonk, C., Gau, D., Kapfhammer, J. *et al.* (2002) Disruption of CREB function in brain leads to neurodegeneration. *Nat Genet,* **31,** 47-54.
22. Riccio, A., Ahn, S., Davenport, C.M., Blendy, J.A. and Ginty, D.D. (1999) Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. *Science,* **286,** 2358-61.
23. Roth, K.A. and D'Sa, C. (2001) Apoptosis and brain development. *Ment Retard Dev Disabil Res Rev,* **7,** 261-6.
24. Muchowski, P.J. (2002) Protein misfolding, amyloid formation, and neurodegeneration: a critical role for molecular chaperones? *Neuron,* **35,** 9-12.
25. Shastry, B.S. (2003) Neurodegenerative disorders of protein aggregation. *Neurochem Int,* **43,** 1-7.
26. Petersen, A., Mani, K. and Brundin, P. (1999) Recent advances on the pathogenesis of Huntington's disease. *Exp Neurol,* **157,** 1-18.
27. Ross, C.A. (2002) Polyglutamine pathogenesis: emergence of unifying mechanisms for Huntington's disease and related disorders. *Neuron,* **35,** 819-22.
28. Taylor, J.P., Hardy, J. and Fischbeck, K.H. (2002) Toxic proteins in neurodegenerative disease. *Science,* **296,** 1991-5.
29. Cummings, C.J., Mancini, M.A., Antalffy, B., DeFranco, D.B., Orr, H.T. and Zoghbi, H.Y. (1998) Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA1. *Nat Genet,* **19,** 148-54.
30. McNaught, K.S., Shashidharan, P., Perl, D.P., Jenner, P. and Olanow, C.W. (2002) Aggresome-related biogenesis of Lewy bodies. *Eur J Neurosci,* **16,** 2136-48.
31. Lopez Salon, M., Pasquini, L., Besio Moreno, M., Pasquini, J.M. and Soto, E. (2003) Relationship between beta-amyloid degradation and the 26S proteasome in neural cells. *Exp Neurol,* **180,** 131-43.

32. McCampbell, A. and Fischbeck, K.H. (2001) Polyglutamine and CBP: fatal attraction? *Nat Med,* **7,** 528-30.
33. Bence, N.F., Sampat, R.M. and Kopito, R.R. (2001) Impairment of the ubiquitin-proteasome system by protein aggregation. *Science,* **292,** 1552-5.
34. Bucciantini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., Taddei, N., Ramponi, G., Dobson, C.M. and Stefani, M. (2002) Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature,* **416,** 507-11.
35. Saudou, F., Finkbeiner, S., Devys, D. and Greenberg, M.E. (1998) Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. *Cell,* **95,** 55-66.
36. Temussi, P.A., Masino, L. and Pastore, A. (2003) From Alzheimer to Huntington: why is a structural understanding so difficult? *Embo J,* **22,** 355-61.
37. Lichtenthaler, S.F., Beher, D., Grimm, H.S., Wang, R., Shearman, M.S., Masters, C.L. and Beyreuther, K. (2002) The intramembrane cleavage site of the amyloid precursor protein depends on the length of its transmembrane domain. *Proc Natl Acad Sci U S A,* **99,** 1365-70.
38. DiFiglia, M., Sapp, E., Chase, K.O., Davies, S.W., Bates, G.P., Vonsattel, J.P. and Aronin, N. (1997) Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science,* **277,** 1990-3.
39. Wellington, C.L., Ellerby, L.M., Hackam, A.S., Margolis, R.L., Trifiro, M.A., Singaraja, R., McCutcheon, K., Salvesen, G.S., Propp, S.S., Bromm, M. *et al.* (1998) Caspase cleavage of gene products associated with triplet expansion disorders generates truncated fragments containing the polyglutamine tract. *J Biol Chem,* **273,** 9158-67.
40. Welch, W.J. and Diamond, M.I. (2001) Glucocorticoid modulation of androgen receptor nuclear aggregation and cellular toxicity is associated with distinct forms of soluble expanded polyglutamine protein. *Hum Mol Genet,* **10,** 3063-74.
41. Tatton, N.A. (2000) Increased caspase 3 and Bax immunoreactivity accompany nuclear GAPDH translocation and neuronal apoptosis in Parkinson's disease. *Exp Neurol,* **166,** 29-43.
42. Hickey, M.A. and Chesselet, M.F. (2003) Apoptosis in Huntington's disease. *Prog Neuropsychopharmacol Biol Psychiatry,* **27,** 255-65.
43. Raina, A.K., Hochman, A., Ickes, H., Zhu, X., Ogawa, O., Cash, A.D., Shimohama, S., Perry, G. and Smith, M.A. (2003) Apoptotic promoters and inhibitors in Alzheimer's disease: Who wins out? *Prog Neuropsychopharmacol Biol Psychiatry,* **27,** 251-4.
44. Yamamoto, A., Lucas, J.J. and Hen, R. (2000) Reversal of neuropathology and motor dysfunction in a conditional model of Huntington's disease. *Cell,* **101,** 57-66.
45. Sanchez, I., Mahlke, C. and Yuan, J. (2003) Pivotal role of oligomerization in expanded polyglutamine neurodegenerative disorders. *Nature,* **421,** 373-9.
46. Dunah, A.W., Jeong, H., Griffin, A., Kim, Y.M., Standaert, D.G., Hersch, S.M., Mouradian, M.M., Young, A.B., Tanese, N. and Krainc, D. (2002) Sp1 and TAFII130 transcriptional activity disrupted in early Huntington's disease. *Science,* **296,** 2238-43.

47. Nucifora, F.C., Jr., Sasaki, M., Peters, M.F., Huang, H., Cooper, J.K., Yamada, M., Takahashi, H., Tsuji, S., Troncoso, J., Dawson, V.L. *et al.* (2001) Interference by huntingtin and atrophin-1 with cbp-mediated transcription leading to cellular toxicity. *Science,* **291,** 2423-8.
48. Steffan, J.S., Bodai, L., Pallos, J., Poelman, M., McCampbell, A., Apostol, B.L., Kazantsev, A., Schmidt, E., Zhu, Y.Z., Greenwald, M. *et al.* (2001) Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in Drosophila. *Nature,* **413,** 739-43.
49. Wellington, C.L., Singaraja, R., Ellerby, L., Savill, J., Roy, S., Leavitt, B., Cattaneo, E., Hackam, A., Sharp, A., Thornberry, N. *et al.* (2000) Inhibiting caspase cleavage of huntingtin reduces toxicity and aggregate formation in neuronal and nonneuronal cells. *J Biol Chem,* **275,** 19831-8.
50. Ona, V.O., Li, M., Vonsattel, J.P., Andrews, L.J., Khan, S.Q., Chung, W.M., Frey, A.S., Menon, A.S., Li, X.J., Stieg, P.E. *et al.* (1999) Inhibition of caspase-1 slows disease progression in a mouse model of Huntington's disease. *Nature,* **399,** 263-7.
51. Sanchez, I., Xu, C.J., Juo, P., Kakizaka, A., Blenis, J. and Yuan, J. (1999) Caspase-8 is required for cell death induced by expanded polyglutamine repeats. *Neuron,* **22,** 623-33.
52. Nakamura, K., Jeong, S.Y., Uchihara, T., Anno, M., Nagashima, K., Nagashima, T., Ikeda, S., Tsuji, S. and Kanazawa, I. (2001) SCA17, a novel autosomal dominant cerebellar ataxia caused by an expanded polyglutamine in TATA-binding protein. *Hum Mol Genet,* **10,** 1441-8.
53. Ross, C.A. (1997) Intranuclear neuronal inclusions: a common pathogenic mechanism for glutamine-repeat neurodegenerative diseases? *Neuron,* **19,** 1147-50.
54. Perutz, M.F., Finch, J.T., Berriman, J. and Lesk, A. (2002) Amyloid fibers are water-filled nanotubes. *Proc Natl Acad Sci U S A,* **99,** 5591-5.
55. Thakur, A.K. and Wetzel, R. (2002) Mutational analysis of the structural organization of polyglutamine aggregates. *Proc Natl Acad Sci U S A,* **99,** 17014-9.
56. Takeyama, K., Ito, S., Yamamoto, A., Tanimoto, H., Furutani, T., Kanuka, H., Miura, M., Tabata, T. and Kato, S. (2002) Androgen-dependent neurodegeneration by polyglutamine-expanded human androgen receptor in Drosophila. *Neuron,* **35,** 855-64.
57. Humbert, S. and Saudou, F. (2002) Toward cell specificity in SCA1. *Neuron,* **34,** 669-70.
58. Klement, I.A., Skinner, P.J., Kaytor, M.D., Yi, H., Hersch, S.M., Clark, H.B., Zoghbi, H.Y. and Orr, H.T. (1998) Ataxin-1 nuclear localization and aggregation: role in polyglutamine-induced disease in SCA1 transgenic mice. *Cell,* **95,** 41-53.
59. Huynh, D.P., Figueroa, K., Hoang, N. and Pulst, S.M. (2000) Nuclear localization or inclusion body formation of ataxin-2 are not necessary for SCA2 pathogenesis in mouse or human. *Nat Genet,* **26,** 44-50.
60. Sapp, E., Penney, J., Young, A., Aronin, N., Vonsattel, J.P. and DiFiglia, M. (1999) Axonal transport of N-terminal huntingtin suggests early pathology of corticostriatal projections in Huntington disease. *J Neuropathol Exp Neurol,* **58,** 165-73.

61. Stevanin, G., Trottier, Y., Cancel, G., Durr, A., David, G., Didierjean, O., Burk, K., Imbert, G., Saudou, F., Abada-Bendib, M. *et al.* (1996) Screening for proteins with polyglutamine expansions in autosomal dominant cerebellar ataxias. *Hum Mol Genet,* **5,** 1887-92.
62. Sherman, M.Y. and Goldberg, A.L. (2001) Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. *Neuron,* **29,** 15-32.
63. Kim, S., Nollen, E.A., Kitagawa, K., Bindokas, V.P. and Morimoto, R.I. (2002) Polyglutamine protein aggregates are dynamic. *Nat Cell Biol,* **4,** 826-31.
64. Ross, C.A., Poirier, M.A., Wanker, E.E. and Amzel, M. (2003) Polyglutamine fibrillogenesis: the pathway unfolds. *Proc Natl Acad Sci U S A,* **100,** 1-3.
65. Sakahira, H., Breuer, P., Hayer-Hartl, M.K. and Hartl, F.U. (2002) Molecular chaperones as modulators of polyglutamine protein aggregation and toxicity. *Proc Natl Acad Sci U S A,* **99 Suppl 4,** 16412-8.
66. Davies, S.W., Turmaine, M., Cozens, B.A., DiFiglia, M., Sharp, A.H., Ross, C.A., Scherzinger, E., Wanker, E.E., Mangiarini, L. and Bates, G.P. (1997) Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. *Cell,* **90,** 537-48.
67. Kim, M., Lee, H.S., LaForet, G., McIntyre, C., Martin, E.J., Chang, P., Kim, T.W., Williams, M., Reddy, P.H., Tagle, D. *et al.* (1999) Mutant huntingtin expression in clonal striatal cells: dissociation of inclusion formation and neuronal survival by caspase inhibition. *J Neurosci,* **19,** 964-73.
68. Warrick, J.M., Chan, H.Y., Gray-Board, G.L., Chai, Y., Paulson, H.L. and Bonini, N.M. (1999) Suppression of polyglutamine-mediated neurodegeneration in Drosophila by the molecular chaperone HSP70. *Nat Genet,* **23,** 425-8.
69. Cummings, C.J., Reinstein, E., Sun, Y., Antalffy, B., Jiang, Y., Ciechanover, A., Orr, H.T., Beaudet, A.L. and Zoghbi, H.Y. (1999) Mutation of the E6-AP ubiquitin ligase reduces nuclear inclusion frequency while accelerating polyglutamine-induced pathology in SCA1 mice. *Neuron,* **24,** 879-92.
70. Cattaneo, E., Rigamonti, D., Goffredo, D., Zuccato, C., Squitieri, F. and Sipione, S. (2001) Loss of normal huntingtin function: new developments in Huntington's disease research. *Trends Neurosci,* **24,** 182-8.
71. Matsumura, R., Futamura, N., Fujimoto, Y., Yanagimoto, S., Horikawa, H., Suzumura, A. and Takayanagi, T. (1997) Spinocerebellar ataxia type 6. Molecular and clinical features of 35 Japanese patients including one homozygous for the CAG repeat expansion. *Neurology,* **49,** 1238-43.
72. Lerer, I., Merims, D., Abeliovich, D., Zlotogora, J. and Gadoth, N. (1996) Machado-Joseph disease: correlation between the clinical features, the CAG repeat length and homozygosity for the mutation. *Eur J Hum Genet,* **4,** 3-7.
73. Sato, K., Kashihara, K., Okada, S., Ikeuchi, T., Tsuji, S., Shomori, T., Morimoto, K. and Hayabara, T. (1995) Does homozygosity advance the onset of dentatorubral-pallidoluysian atrophy? *Neurology,* **45,** 1934-6.
74. Sobue, G., Doyu, M., Nakao, N., Shimada, N., Mitsuma, T., Maruyama, H., Kawakami, S. and Nakamura, S. (1996) Homozygosity for Machado-Joseph disease gene enhances phenotypic severity. *J Neurol Neurosurg Psychiatry,* **60,** 354-6.

75. Ordway, J.M., Tallaksen-Greene, S., Gutekunst, C.A., Bernstein, E.M., Cearley, J.A., Wiener, H.W., Dure, L.S.t., Lindsey, R., Hersch, S.M., Jope, R.S. *et al.* (1997) Ectopically expressed CAG repeats cause intranuclear inclusions and a progressive late onset neurological phenotype in the mouse. *Cell,* **91,** 753-63.
76. Scherzinger, E., Sittler, A., Schweiger, K., Heiser, V., Lurz, R., Hasenbank, R., Bates, G.P., Lehrach, H. and Wanker, E.E. (1999) Self-assembly of polyglutamine-containing huntingtin fragments into amyloid-like fibrils: implications for Huntington's disease pathology. *Proc Natl Acad Sci U S A,* **96,** 4604-9.
77. Scherzinger, E., Lurz, R., Turmaine, M., Mangiarini, L., Hollenbach, B., Hasenbank, R., Bates, G.P., Davies, S.W., Lehrach, H. and Wanker, E.E. (1997) Huntingtin-encoded polyglutamine expansions form amyloid-like protein aggregates in vitro and in vivo. *Cell,* **90,** 549-58.
78. Hackam, A.S., Singaraja, R., Wellington, C.L., Metzler, M., McCutcheon, K., Zhang, T., Kalchman, M. and Hayden, M.R. (1998) The influence of huntingtin protein size on nuclear localization and cellular toxicity. *J Cell Biol,* **141,** 1097-105.
79. Lunkes, A. and Mandel, J.L. (1998) A cellular model that recapitulates major pathogenic steps of Huntington's disease. *Hum Mol Genet,* **7,** 1355-61.
80. Lunkes, A., Lindenberg, K.S., Ben-Haiem, L., Weber, C., Devys, D., Landwehrmeyer, G.B., Mandel, J.L. and Trottier, Y. (2002) Proteases acting on mutant huntingtin generate cleaved products that differentially build up cytoplasmic and nuclear inclusions. *Mol Cell,* **10,** 259-69.
81. Gerber, H.P., Seipel, K., Georgiev, O., Hofferer, M., Hug, M., Rusconi, S. and Schaffner, W. (1994) Transcriptional activation modulated by homopolymeric glutamine and proline stretches. *Science,* **263,** 808-11.
82. Steffan, J.S., Kazantsev, A., Spasic-Boskovic, O., Greenwald, M., Zhu, Y.Z., Gohler, H., Wanker, E.E., Bates, G.P., Housman, D.E. and Thompson, L.M. (2000) The Huntington's disease protein interacts with p53 and CREB-binding protein and represses transcription. *Proc Natl Acad Sci U S A,* **97,** 6763-8.
83. Shimohata, T., Nakajima, T., Yamada, M., Uchida, C., Onodera, O., Naruse, S., Kimura, T., Koide, R., Nozaki, K., Sano, Y. *et al.* (2000) Expanded polyglutamine stretches interact with TAFII130, interfering with CREB-dependent transcription. *Nat Genet,* **26,** 29-36.
84. Schwartz, A.L. and Ciechanover, A. (1999) The ubiquitin-proteasome pathway and pathogenesis of human diseases. *Annu Rev Med,* **50,** 57-74.
85. Jana, N.R., Zemskov, E.A., Wang, G. and Nukina, N. (2001) Altered proteasomal function due to the expression of polyglutamine-expanded truncated N-terminal huntingtin induces apoptosis by caspase activation through mitochondrial cytochrome c release. *Hum Mol Genet,* **10,** 1049-59.
86. Turmaine, M., Raza, A., Mahal, A., Mangiarini, L., Bates, G.P. and Davies, S.W. (2000) Nonapoptotic neurodegeneration in a transgenic mouse model of Huntington's disease. *Proc Natl Acad Sci U S A,* **97,** 8093-7.
87. Gervais, F.G., Singaraja, R., Xanthoudakis, S., Gutekunst, C.A., Leavitt, B.R., Metzler, M., Hackam, A.S., Tam, J., Vaillancourt, J.P., Houtzager, V. *et al.*

(2002) Recruitment and activation of caspase-8 by the Huntingtin-interacting protein Hip-1 and a novel partner Hippi. *Nat Cell Biol,* **4,** 95-105.
88. Rigamonti, D., Bauer, J.H., De-Fraja, C., Conti, L., Sipione, S., Sciorati, C., Clementi, E., Hackam, A., Hayden, M.R., Li, Y. *et al.* (2000) Wild-type huntingtin protects from apoptosis upstream of caspase-3. *J Neurosci,* **20,** 3705-13.
89. Vonsattel, J.P., Myers, R.H., Stevens, T.J., Ferrante, R.J., Bird, E.D. and Richardson, E.P., Jr. (1985) Neuropathological classification of Huntington's disease. *J Neuropathol Exp Neurol,* **44,** 559-77.
90. Zuccato, C., Ciammola, A., Rigamonti, D., Leavitt, B.R., Goffredo, D., Conti, L., MacDonald, M.E., Friedlander, R.M., Silani, V., Hayden, M.R. *et al.* (2001) Loss of huntingtin-mediated BDNF gene transcription in Huntington's disease. *Science,* **293,** 493-8.
91. Li, H., Li, S.H., Johnston, H., Shelbourne, P.F. and Li, X.J. (2000) Amino-terminal fragments of mutant huntingtin show selective accumulation in striatal neurons and synaptic toxicity. *Nat Genet,* **25,** 385-9.
92. Schilling, G., Becher, M.W., Sharp, A.H., Jinnah, H.A., Duan, K., Kotzuk, J.A., Slunt, H.H., Ratovitski, T., Cooper, J.K., Jenkins, N.A. *et al.* (1999) Intranuclear inclusions and neuritic aggregates in transgenic mice expressing a mutant N-terminal fragment of huntingtin. *Hum Mol Genet,* **8,** 397-407.
93. (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group. *Cell,* **72,** 971-83.
94. Sharp, A.H., Loev, S.J., Schilling, G., Li, S.H., Li, X.J., Bao, J., Wagster, M.V., Kotzuk, J.A., Steiner, J.P., Lo, A. *et al.* (1995) Widespread expression of Huntington's disease gene (IT15) protein product. *Neuron,* **14,** 1065-74.
95. Trottier, Y., Devys, D., Imbert, G., Saudou, F., An, I., Lutz, Y., Weber, C., Agid, Y., Hirsch, E.C. and Mandel, J.L. (1995) Cellular localization of the Huntington's disease protein and discrimination of the normal and mutated form. *Nat Genet,* **10,** 104-10.
96. Maat-Schieman, M.L., Dorsman, J.C., Smoor, M.A., Siesling, S., Van Duinen, S.G., Verschuuren, J.J., den Dunnen, J.T., Van Ommen, G.J. and Roos, R.A. (1999) Distribution of inclusions in neuronal nuclei and dystrophic neurites in Huntington disease brain. *J Neuropathol Exp Neurol,* **58,** 129-37.
97. Duyao, M.P., Auerbach, A.B., Ryan, A., Persichetti, F., Barnes, G.T., McNeil, S.M., Ge, P., Vonsattel, J.P., Gusella, J.F., Joyner, A.L. *et al.* (1995) Inactivation of the mouse Huntington's disease gene homolog Hdh. *Science,* **269,** 407-10.
98. Nasir, J., Floresco, S.B., O'Kusky, J.R., Diewert, V.M., Richman, J.M., Zeisler, J., Borowski, A., Marth, J.D., Phillips, A.G. and Hayden, M.R. (1995) Targeted disruption of the Huntington's disease gene results in embryonic lethality and behavioral and morphological changes in heterozygotes. *Cell,* **81,** 811-23.
99. Zeitlin, S., Liu, J.P., Chapman, D.L., Papaioannou, V.E. and Efstratiadis, A. (1995) Increased apoptosis and early embryonic lethality in mice nullizygous for the Huntington's disease gene homologue. *Nat Genet,* **11,** 155-63.

100. White, J.K., Auerbach, W., Duyao, M.P., Vonsattel, J.P., Gusella, J.F., Joyner, A.L. and MacDonald, M.E. (1997) Huntingtin is required for neurogenesis and is not impaired by the Huntington's disease CAG expansion. *Nat Genet,* **17,** 404-10.
101. Dragatsis, I., Levine, M.S. and Zeitlin, S. (2000) Inactivation of Hdh in the brain and testis results in progressive neurodegeneration and sterility in mice. *Nat Genet,* **26,** 300-6.
102. Battaglia, A., Carey, J.C. and Wright, T.J. (2001) Wolf-Hirschhorn (4p-) syndrome. *Adv Pediatr,* **48,** 75-113.
103. Housman, D. (1995) Gain of glutamines, gain of function? *Nat Genet,* **10,** 3-4.
104. Velier, J., Kim, M., Schwarz, C., Kim, T.W., Sapp, E., Chase, K., Aronin, N. and DiFiglia, M. (1998) Wild-type and mutant huntingtins function in vesicle trafficking in the secretory and endocytic pathways. *Exp Neurol,* **152,** 34-40.
105. Hackam, A.S., Yassa, A.S., Singaraja, R., Metzler, M., Gutekunst, C.A., Gan, L., Warby, S., Wellington, C.L., Vaillancourt, J., Chen, N. *et al.* (2000) Huntingtin interacting protein 1 induces apoptosis via a novel caspase-dependent death effector domain. *J Biol Chem,* **275,** 41299-308.
106. Sugars, K.L. and Rubinsztein, D.C. (2003) Transcriptional abnormalities in Huntington disease. *Trends Genet,* **19,** 233-8.
107. Li, H., Li, S.H., Yu, Z.X., Shelbourne, P. and Li, X.J. (2001) Huntingtin aggregate-associated axonal degeneration is an early pathological event in Huntington's disease mice. *J Neurosci,* **21,** 8473-81.
108. Parker, J.A., Connolly, J.B., Wellington, C., Hayden, M., Dausset, J. and Neri, C. (2001) Expanded polyglutamines in Caenorhabditis elegans cause axonal abnormalities and severe dysfunction of PLM mechanosensory neurons without cell death. *Proc Natl Acad Sci U S A,* **98,** 13318-23.
109. Hodgson, J.G., Agopyan, N., Gutekunst, C.A., Leavitt, B.R., LePiane, F., Singaraja, R., Smith, D.J., Bissada, N., McCutcheon, K., Nasir, J. *et al.* (1999) A YAC mouse model for Huntington's disease with full-length mutant huntingtin, cytoplasmic toxicity, and selective striatal neurodegeneration. *Neuron,* **23,** 181-92.
110. Wellington, C.L., Ellerby, L.M., Gutekunst, C.A., Rogers, D., Warby, S., Graham, R.K., Loubser, O., van Raamsdonk, J., Singaraja, R., Yang, Y.Z. *et al.* (2002) Caspase cleavage of mutant huntingtin precedes neurodegeneration in Huntington's disease. *J Neurosci,* **22,** 7862-72.
111. Kim, Y.J., Yi, Y., Sapp, E., Wang, Y., Cuiffo, B., Kegel, K.B., Qin, Z.H., Aronin, N. and DiFiglia, M. (2001) Caspase 3-cleaved N-terminal fragments of wild-type and mutant huntingtin are present in normal and Huntington's disease brains, associate with membranes, and undergo calpain-dependent proteolysis. *Proc Natl Acad Sci U S A,* **98,** 12784-9.
112. Liu, Y.F. (1998) Expression of polyglutamine-expanded Huntingtin activates the SEK1-JNK pathway and induces apoptosis in a hippocampal neuronal cell line. *J Biol Chem,* **273,** 28873-7.
113. Young, A.B. (2003) Huntingtin in health and disease. *J Clin Invest,* **111,** 299-302.
114. Kieburtz, K. (1999) Antiglutamate therapies in Huntington's disease. *J Neural Transm Suppl,* **55,** 97-102.

115. Karpuj, M.V., Becher, M.W., Springer, J.E., Chabas, D., Youssef, S., Pedotti, R., Mitchell, D. and Steinman, L. (2002) Prolonged survival and decreased abnormal movements in transgenic model of Huntington disease, with administration of the transglutaminase inhibitor cystamine. *Nat Med,* **8,** 143-9.
116. Luthi-Carter, R., Strand, A.D., Hanson, S.A., Kooperberg, C., Schilling, G., La Spada, A.R., Merry, D.E., Young, A.B., Ross, C.A., Borchelt, D.R. *et al.* (2002) Polyglutamine and transcription: gene expression changes shared by DRPLA and Huntington's disease mouse models reveal context-independent effects. *Hum Mol Genet,* **11,** 1927-37.
117. Li, S.H., Cheng, A.L., Zhou, H., Lam, S., Rao, M., Li, H. and Li, X.J. (2002) Interaction of Huntington disease protein with transcriptional activator Sp1. *Mol Cell Biol,* **22,** 1277-87.
118. Chan, H.M. and La Thangue, N.B. (2001) p300/CBP proteins: HATs for transcriptional bridges and scaffolds. *J Cell Sci,* **114,** 2363-73.
119. Grossman, S.R., Perez, M., Kung, A.L., Joseph, M., Mansur, C., Xiao, Z.X., Kumar, S., Howley, P.M. and Livingston, D.M. (1998) p300/MDM2 complexes participate in MDM2-mediated p53 degradation. *Mol Cell,* **2,** 405-15.
120. Yuan, L.W. and Giordano, A. (2002) Acetyltransferase machinery conserved in p300/CBP-family proteins. *Oncogene,* **21,** 2253-60.
121. Vo, N. and Goodman, R.H. (2001) CREB-binding protein and p300 in transcriptional regulation. *J Biol Chem,* **276,** 13505-8.
122. Bannister, A.J. and Kouzarides, T. (1996) The CBP co-activator is a histone acetyltransferase. *Nature,* **384,** 641-3.
123. Ogryzko, V.V., Schiltz, R.L., Russanova, V., Howard, B.H. and Nakatani, Y. (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell,* **87,** 953-9.
124. Petrij, F., Giles, R.H., Dauwerse, H.G., Saris, J.J., Hennekam, R.C., Masuno, M., Tommerup, N., van Ommen, G.J., Goodman, R.H., Peters, D.J. *et al.* (1995) Rubinstein-Taybi syndrome caused by mutations in the transcriptional co-activator CBP. *Nature,* **376,** 348-51.
125. Borrow, J., Shearman, A.M., Stanton, V.P., Jr., Becher, R., Collins, T., Williams, A.J., Dube, I., Katz, F., Kwong, Y.L., Morris, C. *et al.* (1996) The t(7;11)(p15;p15) translocation in acute myeloid leukaemia fuses the genes for nucleoporin NUP98 and class I homeoprotein HOXA9. *Nat Genet,* **12,** 159-67.
126. Yao, T.P., Oh, S.P., Fuchs, M., Zhou, N.D., Ch'ng, L.E., Newsome, D., Bronson, R.T., Li, E., Livingston, D.M. and Eckner, R. (1998) Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. *Cell,* **93,** 361-72.
127. Tanaka, Y., Naruse, I., Maekawa, T., Masuya, H., Shiroishi, T. and Ishii, S. (1997) Abnormal skeletal patterning in embryos lacking a single Cbp allele: a partial similarity with Rubinstein-Taybi syndrome. *Proc Natl Acad Sci U S A,* **94,** 10215-20.
128. McCampbell, A., Taylor, J.P., Taye, A.A., Robitschek, J., Li, M., Walcott, J., Merry, D., Chai, Y., Paulson, H., Sobue, G. *et al.* (2000) CREB-binding protein sequestration by expanded polyglutamine. *Hum Mol Genet,* **9,** 2197-202.

129. Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W. and Yuan, J. (1996) Human ICE/CED-3 protease nomenclature. *Cell,* **87,** 171.
130. Villa, P.G., Henzel, W.J., Sensenbrenner, M., Henderson, C.E. and Pettmann, B. (1998) Calpain inhibitors, but not caspase inhibitors, prevent actin proteolysis and DNA fragmentation during apoptosis. *J Cell Sci,* **111 (Pt 6),** 713-22.
131. Hatakeyama, S. and Nakayama, K.I. (2003) U-box proteins as a new family of ubiquitin ligases. *Biochem Biophys Res Commun,* **302,** 635-45.
132. Adams, J. (2003) The proteasome: structure, function, and role in the cell. *Cancer Treat Rev,* **29 Suppl 1,** 3-9.
133. Kisselev, A.F., Akopian, T.N., Woo, K.M. and Goldberg, A.L. (1999) The sizes of peptides generated from protein by mammalian 26 and 20 S proteasomes. Implications for understanding the degradative mechanism and antigen presentation. *J Biol Chem,* **274,** 3363-71.
134. Martin-Aparicio, E., Yamamoto, A., Hernandez, F., Hen, R., Avila, J. and Lucas, J.J. (2001) Proteasomal-dependent aggregate reversal and absence of cell death in a conditional mouse model of Huntington's disease. *J Neurosci,* **21,** 8772-81.
135. Waelter, S., Boeddrich, A., Lurz, R., Scherzinger, E., Lueder, G., Lehrach, H. and Wanker, E.E. (2001) Accumulation of mutant huntingtin fragments in aggresome-like inclusion bodies as a result of insufficient protein degradation. *Mol Biol Cell,* **12,** 1393-407.
136. Cooper, J.K., Schilling, G., Peters, M.F., Herring, W.J., Sharp, A.H., Kaminsky, Z., Masone, J., Khan, F.A., Delanoy, M., Borchelt, D.R. *et al.* (1998) Truncated N-terminal fragments of huntingtin with expanded glutamine repeats form nuclear and cytoplasmic aggregates in cell culture. *Hum Mol Genet,* **7,** 783-90.
137. Musti, A.M., Treier, M., Peverali, F.A. and Bohmann, D. (1996) Differential regulation of c-Jun and JunD by ubiquitin-dependent protein degradation. *Biol Chem,* **377,** 619-24.
138. Yang, J. and DeFranco, D.B. (1994) Differential roles of heat shock protein 70 in the in vitro nuclear import of glucocorticoid receptor and simian virus 40 large tumor antigen. *Mol Cell Biol,* **14,** 5088-98.
139. Wang, X., Pongrac, J.L. and DeFranco, D.B. (2002) Glucocorticoid receptors in hippocampal neurons that do not engage proteasomes escape from hormone-dependent down-regulation but maintain transactivation activity. *Mol Endocrinol,* **16,** 1987-98.
140. Yang, L., Guerrero, J., Hong, H., DeFranco, D.B. and Stallcup, M.R. (2000) Interaction of the tau2 transcriptional activation domain of glucocorticoid receptor with a novel steroid receptor coactivator, Hic-5, which localizes to both focal adhesions and the nuclear matrix. *Mol Biol Cell,* **11,** 2007-18.
141. Li, Y., Maher, P. and Schubert, D. (1997) A role for 12-lipoxygenase in nerve cell death caused by glutathione depletion. *Neuron,* **19,** 453-63.
142. Tan, S., Sagara, Y., Liu, Y., Maher, P. and Schubert, D. (1998) The regulation of reactive oxygen species production during programmed cell death. *J Cell Biol,* **141,** 1423-32.
143. Stanciu, M., Wang, Y., Kentor, R., Burke, N., Watkins, S., Kress, G., Reynolds, I., Klann, E., Angiolieri, M.R., Johnson, J.W. *et al.* (2000) Persistent activation of

ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. *J Biol Chem,* **275,** 12200-6.
144. Browne, S.E., Ferrante, R.J. and Beal, M.F. (1999) Oxidative stress in Huntington's disease. *Brain Pathol,* **9,** 147-63.
145. Martindale, D., Hackam, A., Wieczorek, A., Ellerby, L., Wellington, C., McCutcheon, K., Singaraja, R., Kazemi-Esfarjani, P., Devon, R., Kim, S.U. *et al.* (1998) Length of huntingtin and its polyglutamine tract influences localization and frequency of intracellular aggregates. *Nat Genet,* **18,** 150-4.
146. Coombe, D.R., Nakhoul, A.M., Stevenson, S.M., Peroni, S.E. and Sanderson, C.J. (1998) Expressed luciferase viability assay (ELVA) for the measurement of cell growth and viability. *J Immunol Methods,* **215,** 145-50.
147. Kobayashi, Y., Kume, A., Li, M., Doyu, M., Hata, M., Ohtsuka, K. and Sobue, G. (2000) Chaperones Hsp70 and Hsp40 suppress aggregate formation and apoptosis in cultured neuronal cells expressing truncated androgen receptor protein with expanded polyglutamine tract. *J Biol Chem,* **275,** 8772-8.
148. Chai, Y., Koppenhafer, S.L., Bonini, N.M. and Paulson, H.L. (1999) Analysis of the role of heat shock protein (Hsp) molecular chaperones in polyglutamine disease. *J Neurosci,* **19,** 10338-47.
149. Wyttenbach, A., Swartz, J., Kita, H., Thykjaer, T., Carmichael, J., Bradley, J., Brown, R., Maxwell, M., Schapira, A., Orntoft, T.F. *et al.* (2001) Polyglutamine expansions cause decreased CRE-mediated transcription and early gene expression changes prior to cell death in an inducible cell model of Huntington's disease. *Hum Mol Genet,* **10,** 1829-45.
150. Peters, M.F., Nucifora, F.C., Jr., Kushi, J., Seaman, H.C., Cooper, J.K., Herring, W.J., Dawson, V.L., Dawson, T.M. and Ross, C.A. (1999) Nuclear targeting of mutant Huntingtin increases toxicity. *Mol Cell Neurosci,* **14,** 121-8.
151. Lonard, D.M., Nawaz, Z., Smith, C.L. and O'Malley, B.W. (2000) The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. *Mol Cell,* **5,** 939-48.
152. Bush, K.T., Goldberg, A.L. and Nigam, S.K. (1997) Proteasome inhibition leads to a heat-shock response, induction of endoplasmic reticulum chaperones, and thermotolerance. *J Biol Chem,* **272,** 9086-92.
153. De Souza, E.B., Whitehouse, P.J., Folstein, S.E., Price, D.L. and Vale, W.W. (1987) Corticotropin-releasing hormone (CRH) is decreased in the basal ganglia in Huntington's disease. *Brain Res,* **437,** 355-9.
154. Timmers, H.J., Swaab, D.F., van de Nes, J.A. and Kremer, H.P. (1996) Somatostatin 1-12 immunoreactivity is decreased in the hypothalamic lateral tuberal nucleus of Huntington's disease patients. *Brain Res,* **728,** 141-8.
155. Perez, M.K., Paulson, H.L., Pendse, S.J., Saionz, S.J., Bonini, N.M. and Pittman, R.N. (1998) Recruitment and the role of nuclear localization in polyglutamine-mediated aggregation. *J Cell Biol,* **143,** 1457-70.
156. Becker, M., Martin, E., Schneikert, J., Krug, H.F. and Cato, A.C. (2000) Cytoplasmic localization and the choice of ligand determine aggregate formation by androgen receptor with amplified polyglutamine stretch. *J Cell Biol,* **149,** 255-62.

157. Hackam, A.S., Hodgson, J.G., Singaraja, R., Zhang, T., Gan, L., Gutekunst, C.A., Hersch, S.M. and Hayden, M.R. (1999) Evidence for both the nucleus and cytoplasm as subcellular sites of pathogenesis in Huntington's disease in cell culture and in transgenic mice expressing mutant huntingtin. *Philos Trans R Soc Lond B Biol Sci,* **354,** 1047-55.
158. Gines, S., Seong, I.S., Fossale, E., Ivanova, E., Trettel, F., Gusella, J.F., Wheeler, V.C., Persichetti, F. and MacDonald, M.E. (2003) Specific progressive cAMP reduction implicates energy deficit in presymptomatic Huntington's disease knock-in mice. *Hum Mol Genet,* **12,** 497-508.
159. Ding, Q., Lewis, J.J., Strum, K.M., Dimayuga, E., Bruce-Keller, A.J., Dunn, J.C. and Keller, J.N. (2002) Polyglutamine expansion, protein aggregation, proteasome activity, and neural survival. *J Biol Chem,* **277,** 13935-42.
160. Kegel, K.B., Kim, M., Sapp, E., McIntyre, C., Castano, J.G., Aronin, N. and DiFiglia, M. (2000) Huntingtin expression stimulates endosomal-lysosomal activity, endosome tubulation, and autophagy. *J Neurosci,* **20,** 7268-78.
161. Rivett, A.J. (1998) Intracellular distribution of proteasomes. *Curr Opin Immunol,* **10,** 110-4.
162. Skinner, P.J., Vierra-Green, C.A., Clark, H.B., Zoghbi, H.Y. and Orr, H.T. (2001) Altered trafficking of membrane proteins in purkinje cells of SCA1 transgenic mice. *Am J Pathol,* **159,** 905-13.
163. Lee, J.A., Lim, C.S., Lee, S.H., Kim, H., Nukina, N. and Kaang, B.K. (2003) Aggregate formation and the impairment of long-term synaptic facilitation by ectopic expression of mutant huntingtin in Aplysia neurons. *J Neurochem,* **85,** 160-9.
164. Sipione, S., Rigamonti, D., Valenza, M., Zuccato, C., Conti, L., Pritchard, J., Kooperberg, C., Olson, J.M. and Cattaneo, E. (2002) Early transcriptional profiles in huntingtin-inducible striatal cells by microarray analyses. *Hum Mol Genet,* **11,** 1953-65.
165. Wheeler, T.C., Chin, L.S., Li, Y., Roudabush, F.L. and Li, L. (2002) Regulation of synaptophysin degradation by mammalian homologues of seven in absentia. *J Biol Chem,* **277,** 10273-82.
166. Ageta, H., Kato, A., Fukazawa, Y., Inokuchi, K. and Sugiyama, H. (2001) Effects of proteasome inhibitors on the synaptic localization of Vesl-1S/Homer-1a proteins. *Brain Res Mol Brain Res,* **97,** 186-9.
167. Morton, A.J. and Edwardson, J.M. (2001) Progressive depletion of complexin II in a transgenic mouse model of Huntington's disease. *J Neurochem,* **76,** 166-72.
168. Zhu, X., Zhao, X., Burkholder, W.F., Gragerov, A., Ogata, C.M., Gottesman, M.E. and Hendrickson, W.A. (1996) Structural analysis of substrate binding by the molecular chaperone DnaK. *Science,* **272,** 1606-14.
169. Cheetham, M.E. and Caplan, A.J. (1998) Structure, function and evolution of DnaJ: conservation and adaptation of chaperone function. *Cell Stress Chaperones,* **3,** 28-36.
170. Hartl, F.U. and Hayer-Hartl, M. (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science,* **295,** 1852-8.
171. Kazemi-Esfarjani, P. and Benzer, S. (2000) Genetic suppression of polyglutamine toxicity in Drosophila. *Science,* **287,** 1837-40.

172. Cummings, C.J., Sun, Y., Opal, P., Antalffy, B., Mestril, R., Orr, H.T., Dillmann, W.H. and Zoghbi, H.Y. (2001) Over-expression of inducible HSP70 chaperone suppresses neuropathology and improves motor function in SCA1 mice. *Hum Mol Genet,* **10,** 1511-8.
173. Stenoien, D.L., Cummings, C.J., Adams, H.P., Mancini, M.G., Patel, K., DeMartino, G.N., Marcelli, M., Weigel, N.L. and Mancini, M.A. (1999) Polyglutamine-expanded androgen receptors form aggregates that sequester heat shock proteins, proteasome components and SRC-1, and are suppressed by the HDJ-2 chaperone. *Hum Mol Genet,* **8,** 731-41.
174. Wyttenbach, A., Carmichael, J., Swartz, J., Furlong, R.A., Narain, Y., Rankin, J. and Rubinsztein, D.C. (2000) Effects of heat shock, heat shock protein 40 (HDJ-2), and proteasome inhibition on protein aggregation in cellular models of Huntington's disease. *Proc Natl Acad Sci U S A,* **97,** 2898-903.
175. Chan, H.Y., Warrick, J.M., Gray-Board, G.L., Paulson, H.L. and Bonini, N.M. (2000) Mechanisms of chaperone suppression of polyglutamine disease: selectivity, synergy and modulation of protein solubility in Drosophila. *Hum Mol Genet,* **9,** 2811-20.
176. Fernandez-Funez, P., Nino-Rosales, M.L., de Gouyon, B., She, W.C., Luchak, J.M., Martinez, P., Turiegano, E., Benito, J., Capovilla, M., Skinner, P.J. *et al.* (2000) Identification of genes that modify ataxin-1-induced neurodegeneration. *Nature,* **408,** 101-6.
177. Bonini, N.M. (2002) Chaperoning brain degeneration. *Proc Natl Acad Sci U S A,* **99 Suppl 4,** 16407-11.
178. Auluck, P.K., Chan, H.Y., Trojanowski, J.Q., Lee, V.M. and Bonini, N.M. (2002) Chaperone suppression of alpha-synuclein toxicity in a Drosophila model for Parkinson's disease. *Science,* **295,** 865-8.
179. Cyr, D.M., Hohfeld, J. and Patterson, C. (2002) Protein quality control: U-box-containing E3 ubiquitin ligases join the fold. *Trends Biochem Sci,* **27,** 368-75.
180. Mosser, D.D., Caron, A.W., Bourget, L., Meriin, A.B., Sherman, M.Y., Morimoto, R.I. and Massie, B. (2000) The chaperone function of hsp70 is required for protection against stress-induced apoptosis. *Mol Cell Biol,* **20,** 7146-59.
181. Merienne, K., Helmlinger, D., Perkin, G.R., Devys, D. and Trottier, Y. (2003) Polyglutamine expansion induces a protein-damaging stress connecting heat shock protein 70 to the JNK pathway. *J Biol Chem,* **278,** 16957-67.
182. Beere, H.M., Wolf, B.B., Cain, K., Mosser, D.D., Mahboubi, A., Kuwana, T., Tailor, P., Morimoto, R.I., Cohen, G.M. and Green, D.R. (2000) Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. *Nat Cell Biol,* **2,** 469-75.

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Figure 3: Striatum of HD patients at different disease stages

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July 21, 2003

Haibing Jiang
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412-648-1945

Dear Mr. Jiang:

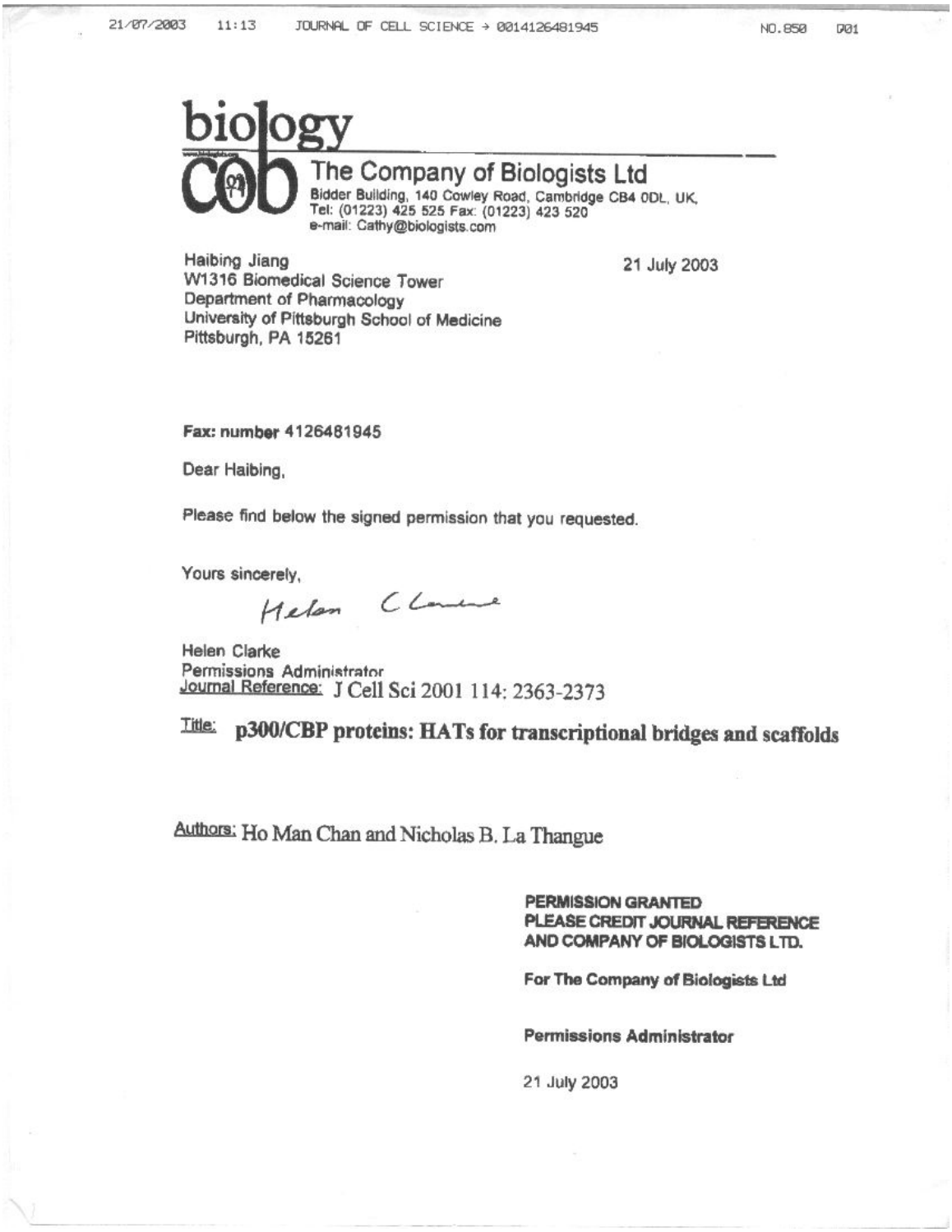
I am writing in response to your letter of July 17, 2003 requesting permission to use illustrations from "Neuropathological Classification of Huntington's Disease" which was published in the *Journal of Neuropathology & Experimental Neurology* (44:559-77, 1985). You have my permission to use figure(s) #2 in your work in "Mechanisms of Polyglutamine Expanded Huntington Induced Toxicity" to be published by the University of Pittsburgh. The following statement should appear in your legend crediting the JNEN: "Reproduced with permission from the Journal of Neuropathology and Experimental Neurology".

Sincerely,

Michael Noel Hart, M.D.
Editor-in-Chief

MNH/mlk

Figure 6: Models of CBP/p300 function as transcription co-activators



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