INVESTIGATING THE MECHANISM OF PROMOTER-SPECIFIC
N-TERMINAL MUTANT HUNTINGTIN-MEDIATED
TRANSCRIPTIONAL DYSREGULATION

by

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Table of Contents

List of Tables

List of Figures

Abstract

Huntington's disease (HD) is a neurodegenerative disorder caused by the inheritance of one mutant copy of the *huntingtin* gene. Mutant huntingtin protein (mHtt) contains an expanded polyglutamine repeat region near the N-terminus. Cleavage of mHtt releases an N-terminal fragment (N-mHtt) which translocates, and accumulates in the nucleus. Nuclear accumulation of N-mHtt has been directly associated with cellular toxicity. Decreased transcription is among the earliest detected changes that occur in the brains of HD patients and is consistently observed in all animal and cellular models of HD. Transcriptional dysregulation may trigger many of the perturbations that occur later in disease progression and an understanding of the effects of mHtt may lead to strategies to slow the progression of the disease. Current models of N-mHtt-mediated transcriptional dysregulation suggest that abnormal interactions between N-mHtt and transcription factors impair the ability of these transcription factors to associate at N-mHtt-affected promoters and properly regulate gene expression. We tested various aspects of these models using two N-mHtt-affected promoters in *in vitro* transcription assays and in two cell models of HD using techniques including overexpression of known N-mHtt-interacting transcription factors, chromatin immunoprecipitation, promoter deletion and mutation analyses and *in vitro* promoter binding assays. Based on our results and those in the literature, we proposed a new model of N-mHtt-mediated transcriptional dysregulation centered on the presence of N-mHtt at affected promoters. We concluded that simultaneous interaction of N-mHtt with multiple binding partners within the transcriptional machinery would explain the gene-specificity of N-mHtt-mediated transcriptional dysregulation, as well as the observation that some genes are affected early in disease progression while others are affected later. Our model explains why alleviating N-mHtt-mediated transcriptional dysregulation through overexpression of N-mHtt-interacting proteins has proven to be difficult and suggests that the most realistic strategy for restoring gene expression across the spectrum of N-mHtt affected genes is by reducing the amount of soluble nuclear N-mHtt.

List of Abbreviations and Symbols Used

24OHC	24S-hydroxycholesterol
3-NP	3-nitroproprionic acid
aa	amino acid
AAL	arginosuccinate acid lyase
Ach	Acetylcholine
ANOVA	analysis of variance
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
BiFC	bimolecular fluorescence complementation
BMI	basal metabolic index
bp	base pair
BSA	bovine serum albumin
C	carboxy
C/EBP	CCAAT-enhancer-binding protein
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
cDNA	complementary DNA
CER	cytoplasmic extraction reagent
ChIP	chromatin immunoprecipitation
CMV	human immediate early cytomegalovirus
coIP	co-immunoprecipitation
CRE	CREB response element
CREB	cAMP response element binding protein

CTBP	C-terminal binding protein
DARPP	dopamine- and cAMP-regulated neuronal phosphoprotein
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DLR™	Dual-Luciferase Reporter
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ETC	electron transport chain
FBS	fetal bovine serum
FDU	fast-digest unit
FRET	Förster resonance energy transfer
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
HAP1	huntingtin-associated protein 1
HBSS	Hank's balanced salt solution (supplemented)
HD	Huntington's disease
HDAC	histone deacetylase
Hdh	mouse huntingtin gene
HEK	human embryonic kidney

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA reductase
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HRP	horseradish peroxidase
Htt	huntingtin protein
IgG	immunoglobulin G
IP	immunoprecipitation
KA	kainic acid
LB	Luria broth
LTR	long terminal repeat
luc+	luciferase mRNA
MALDI-TOF	matrix-assisted laser desorption/ionization-time-of-flight
MD	myotonic dystrophy
mHtt	mutant huntingtin protein
MPT	mitochondrial permeability transition
mRNA	messenger RNA
MSN	medium spiny neuron
mUb	monoubiquitination
N	amino
N171	N-terminal 171 aas of huntingtin protein
N548hd	immortalized ST14A cells overexpressing N-terminal 548 aa Htt with 128Q
N548wt	immortalized ST14A cells overexpressing N-terminal 548 aa Htt with 15Q
NCoR	nuclear receptor co-repressor protein
NES	nuclear export signal

NFkB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGFR	nerve growth factor receptor
NII	neuronal intranuclear inclusion body
NLS	nuclear localization signal
NMDA	N-methyl-D-aspartic acid
N-mHtt	N-terminal mutant huntingtin protein
NRSF	neuron-restrictive silencing factor
nt	nucleotide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterase
PFA	paraformaldehyde
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PI	preimmune serum
PIC	preinitiation complex
PMSF	phenylmethanesulfonylfluoride
PolII	RNA polymerase II
polyQ	polyglutamine
ppENK	preproenkephalin
PVDF	polyvinylidene fluoride
QA	quinolinic acid
qPCR	quantitative polymerase chain reaction
RAP30	RNA Polymerase II associating protein 30
RAP74	RNA Polymerase II associating protein 74

rATP	ribosyladenosine triphosphate
rCTP	ribosylcytidine triphosphate
REST	repressor element silencing transcription factor
rGTP	ribosylguanosine triphosphate
RNA	ribonucleic acid
Rnase	ribonuclease
rNTP	ribonucleotide triphosphate
RT	reverse transcriptase
rUTP	ribosyluracil triphosphate
SAHA	suberoylanilide hydroxamic acid
SDS-PAGE	sodium dodecyl sulphate pholyacrylamide gel electrophoresis
SFM	serum-free media
shHtt	plasmid driving short hairpin RNA complementary to huntingtin
shNeg	empty plasmid used as a control for shRNA experiment
shRNA	short hairpin RNA
Sp1	specificity protein 1
Sp3	specificity protein 3
SSRI	selective serotonin reuptake inhibitor
ST14A	immortalized embryonic day 14 rat striatal cells
StHdh	stable cell line derived from striatal cells of Hdh knock-in mice
TAF	TBP-associated factors
TAF4	TBP-associated factor 4 (aka TFII130)
TBP	TATA box binding protein

TBS	tris buffered saline
TBS-T	tris buffere saline with tween 20
TE	tris EDTA
TF	transcription factor
TFIIA	general transcription factor IIA
TFIIB	general transcription factor IIB
TFIID	general transcription factor IID
TFIIE	general transcription factor IIE
TFIIF	general transcription factor IIF
TFIIH	general transcription factor IIH
TG2	transglutaminase-2
TK	herpes simian virus thymidine kinase
Tpr	nuclear pore protein traslocated promoter region
tRNA	transfer RNA
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
UPS	ubiquitin-proteasomal system
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
YAC	yeast artificial chromosome
YT	yeast tryptone

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1.1 Clinical Characteristics and Management of Huntington's Disease

1.1.a Symptoms of Huntington's Disease

Huntington's disease (HD) is a late onset neurodegenerative disorder, characterized by motor, cognitive, and psychiatric impairments (Walker, 2007). HD is recognized primarily as a movement disorder and was originally named in reference to its characteristic motor impairments. Dr. George Huntington termed the disorder Huntington's chorea, from the Greek word "chorea" meaning 'dance', which described the clonic spasms affecting the voluntary muscles, keeping the patient in a "continual jigger" (Huntington, 1872). The movement disorder can be divided into early and late phases. Early in the disease, symptoms present in the form of excessive involuntary (chorea) and uncontrolled voluntary movement. Impairments resulting from the excessive movement include reduced manual dexterity, problems with swallowing, speech, and balance (Hayden *et al.*, 1981; Young *et al.*, 1986; Thompson *et al.*, 1988). As the disease progresses, the excessive movement is often replaced by an inability to initiate, as well as a slowness of execution of movement. Motor symptoms at this stage of HD include bradykinesia, rigidity in the musculature, and dystonia (Bates *et al.*, 2002; Garcia Ruiz *et al.*, 2002; Mahant *et al.*, 2003).

Although diagnosis is often made based on abnormal movement, cognitive and psychiatric disturbances are common throughout HD. Cognitive deficits affect the broadly defined "executive functions" including attention, perception, memory, language,

planning and organizational skills (Watkins *et al.*, 2000; Bates *et al.*, 2002; Lemiere *et al.*, 2004). Psychiatric disturbances in HD are highly variable between patients. The most common psychiatric disturbance observed in HD patients is depression. Depression in HD has been reported at ~40% lifetime incidence in one study (Shiwach, 1994), which is much higher than the 8% lifetime incidence in the general population (Canada, 2002). Other psychiatric symptoms commonly reported include agitation, irritability, apathy, anxiety, paranoia, and obsessive-compulsive disorder (Kirkwood *et al.*, 2001; Paulsen *et al.*, 2001; Anderson *et al.*, 2010). It is important to note that the symptomatic profile of HD is different for every patient, as both the presence and degree of the symptoms described vary among individuals.

1.1.b Progression of HD

A clearly defined, linear progression of the symptoms of HD does not exist. The symptoms that a HD patient will develop, as well as the relative onset and severity of those symptoms vary among individuals. Because of this, the progression of HD is grouped into three broadly defined stages, early, middle and late, which reflect the patient's quality of life (Rosenblatt *et al.*, 1999). Early stage HD includes the diagnosis of disease onset and the gradual development of HD symptoms. Although chorea is the most visible and recognizable symptom in HD, and is most often the basis for diagnosis of HD symptom onset, cognitive and psychiatric abnormalities can often be detected prior to the onset of motor impairments (Brandt *et al.*, 2002; Duff *et al.*, 2007). In a study of 1766 HD patients, it was determined that 48% were diagnosed as symptomatic based on motor symptoms, while 20% had psychiatric onset, and 13% had cognitive onset (Orth *et*

al., 2010). Symptomatic diagnosis of HD generally does not usually occur until mid-life.

The median age of onset has been reported to be between 35 and 55 years of age in

different studies (Newcombe, 1981; Adams *et al.*, 1988; Roos *et al.*, 1993). After

symptom onset, the disease progresses, increasing the severity of symptoms for 15-20

years until death of the patient (Foroud *et al.*, 1999). During the early stage of HD

progression, the majority of patients are self-sufficient, and can maintain a job, manage

finances, drive and supervise children (Beglinger *et al.*, 2010). The middle stage of HD

is characterized by the worsening of the motor disorder and includes the loss of ability to

grasp objects, an increased propensity to fall, as well as intellectual decline (Kirkwood *et*

al., 2001). Patients in the middle stage of HD usually remain self-sufficient but usually

can no longer work or bear sole responsibility for their family (Rosenblatt *et al.*, 1999).

Loss of independence occurs in late-stage HD. Patients become bed-ridden and require

full time assistance. Analysis of mortality data in the United States has determined that

the leading causes of death in HD patients attributable to the disease are pneumonia,

nutritional deficiencies, choking, and complications from mental disorders (Lanska *et al.*,

1988a; Lanska *et al.*, 1988b).

1.1.c Pathology of HD

Huntington's disease is classified as a neurodegenerative disease. The original

characterization of brain atrophy in HD patients revealed that neurodegeneration occurred

almost exclusively in the caudate and putamen regions of the neostriatum (Vonsattel *et*

al., 1985). A grading scale, termed the Vonsattel scale, characterizing the degree of

pathology in the brains of HD patients was created through post-mortem analysis of HD

patients at various stages of the illness (Vonsattel *et al.*, 1985). The rating scale

categorizes the pathology from a score of 0 to 4 based on the progressive atrophy and

neurodegeneration in the caudate-putamen, globus pallidus and nucleus accumbens.

Quantification of cell number in the caudate nucleus using cresyl-violet staining

demonstrated a 50% reduction in neurons in grade 1 patients and a 95% reduction in the

number of neurons in grade 4 patients compared to control (Vonsattel *et al.*, 1985). More

recently, post-mortem analysis comparing the atrophy in brains of 7 HD patients to 12

age-matched controls showed that although the volumes of the caudate and putamen had

the largest percentage reduction (approximately 50% loss of neurons relative to controls),

the volume of the entire cerebral cortex was significantly lower in HD patients compared

to controls (Halliday *et al.*, 1998). In the subcortex, in addition to the caudate and

putamen, the volume of the internal and external pallidus, but not the thalamus was

decreased relative to controls. No differences were detected in white matter volume in

any region of the brain (Halliday *et al.*, 1998). Although not characterized to the same

degree as striatal and cortical neurodegeneration, neuronal atrophy has also been detected

in the cerebellum (Jeste *et al.*, 1984; Ruocco *et al.*, 2006), and hypothalamus (Kremer *et

al.*, 1990; Douaud *et al.*, 2006). Despite the widespread atrophy in HD, degeneration of

the caudate and putamen regions of the striatum occurs at earlier stages of the disease

than other brain regions (Vonsattel *et al.*, 2008).

Within the basal ganglia, neurodegeneration does not occur homogeneously with

respect to neuronal subtype. The corpus striatum is made up of medium spiny neurons

(MSNs), and aspiny interneurons (Fig. 1.1). Aspiny neurons are subdivided into

cholinergic and GABAergic interneurons based on the neurotransmitter they produce,

A
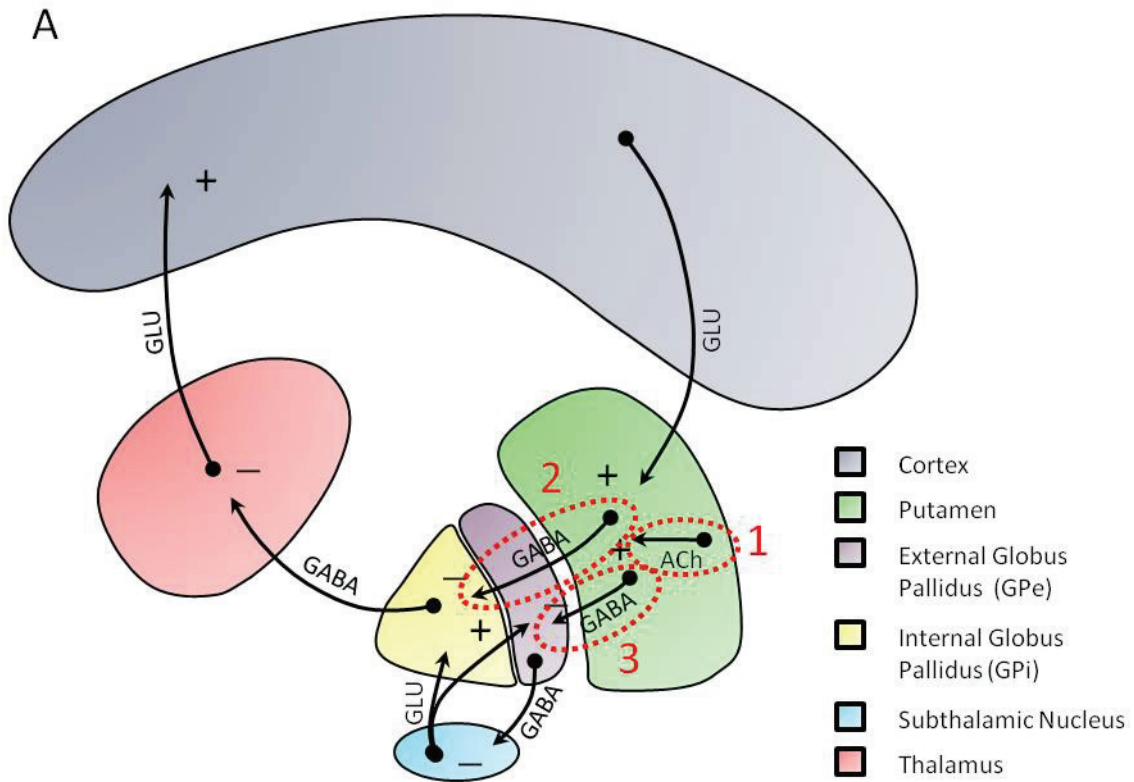
B

	Neuron	Innervates	Neurotrans.	Dopamine Receptor Subtype	Cell Fate in HD
1	Aspiny Interneuron	Putamen	Ach/GABA	D_2	Spared
2	Medium Spiny	GPi	GABA + Substance P	D_1	Degenerate Later
3	Medium Spiny	GPe	GABA + Enkephalin	D_2	Degenerate Earlier

Figure 1.1. The neuronal subtypes of the neostriatum are involved in regulating movement, and are differentially affected in HD. **(A)** The direct and indirect movement pathways of the basal ganglia. + indicates excitatory synaptic input; - indicates inhibitory synaptic input. **(B)** Comparisons between aspiny interneurons, and the two subtypes of medium spiny neurons which are highlighted in red and numbered 1-3 in part A. Modified from (Graybiel, 2008).

package and release. MSNs are GABAergic, but are subdivided into two classes based on the neurotransmitters they co-package, the dopamine receptor subtype they express and where they project. One type of MSN innervates the internal segment of the globus pallidus (termed the direct movement pathway). The other type of MSN innervates the external globus pallidus (termed the indirect movement pathway) (Gerfen, 1992). MSNs projecting to the internal globus pallidus produce substance P and dynorphin in addition to GABA, and express dopamine D_1 receptors. MSNs projecting to the external globus pallidus express enkephalin in addition to GABA and express the D_2 subtype of the dopamine receptor (Kawaguchi, 1997). The first characterizations of cell-specific degeneration within the basal ganglia in HD identified the loss of MSNs and the sparing of aspiny interneurons (Graveland *et al.*, 1985). It was later determined that the two subpopulations of MSNs in the basal ganglia were also subjected to cell-specific neurodegeneration. MSNs belonging to the direct movement pathway that produce substance-P and express D_1 receptors are spared early in the disease, when MSNs that innervate the indirect pathway, produce enkephalin and express D_2 receptors are more susceptible to degeneration (Richfield *et al.*, 1995).

Although the defining features of HD are neurological, peripheral changes occur during the progression of the disease. The most prominent and well characterized peripheral pathology in HD is weight loss. Significant weight loss and an inability to gain weight occur in patients suffering from HD (HSG, 2006; Robbins *et al.*, 2006). Weight loss occurs early in HD progression, as evidenced by a study performed examining the body mass index (BMI) of 361 fully independent HD patients diagnosed as symptomatic within the previous four years. At this early stage of HD, BMI was

significantly lower in both men and women compared to sex- and age-matched controls (Djousse *et al.*, 2002). Weight loss in HD occurs despite the fact that patients have an increased appetite and increase caloric intake relative to unaffected individuals (Trejo *et al.*, 2004) refuting the idea that weight loss in HD is due to disease-related swallowing impairments.

Another peripheral change observed in HD is imbalanced endocrine regulation. HD patients have higher circulating levels of corticosteroids (Markianos *et al.*, 2005) and lower levels of testosterone and lutenizing hormone (Van Raamsdonk *et al.*, 2007). It is possible that decreased hormone levels may result from imbalances in pituitary regulation casting doubt on the inclusion of these findings as peripheral changes. Post-mortem analyses of HD patients have documented testicular atrophy characterized by decreased production of spermatocytes and spermatids as well as thicker seminiferous tubules when compared to age-matched controls (Van Raamsdonk *et al.*, 2007). Therefore, while the most visible pathology in HD is the striatal-specific neurodegeneration, HD patients experience effects of the disease throughout the body.

1.1.d Treatment of HD

There is currently no cure for HD. Pharmacological treatment of the disease is symptomatic in nature and has been met with limited success. The only therapeutic agent approved specifically for use in HD is tetrabenazine, a dopamine-depleting agent, which is used to reduce chorea (Jankovic & Beach, 1997; 2006). Tetrabenazine inhibits the packaging of monoamines into vesicles (Pettibone *et al.*, 1984) and antagonizes dopamine D_2 receptors (Reches *et al.*, 1983). In addition to tetrabenazine, neuroleptics

have been used to limit chorea in HD patients because of their ability to antagonize dopamine D_2 receptors. An evidence based review summarized findings of randomized and non-randomized clinical trials as well as open-label trials reporting on the efficacy of antipsychotic drugs in the treatment of HD (Bonelli & Wenning, 2006). Tiapride was the only one of four randomized, double-blind clinical trials reviewed that reported a positive outcome for improving motor symptoms in HD, however, a separate study using tiapride two years prior showed no effect. Non-randomized and open label trials have suggested that haloperidol, fluphenazine, perphenazine, pimozide, clozapine, olanzapine and thiopropazate are able to improve motor impairments in HD.   In addition to D_2 agonists, a number of NDMA-receptor antagonists have been investigated for their ability to control impaired movement. Riluzole, ketamine and milacemide were shown to have no effect on motor impairment in HD.

Once the excessive choreic movement is replaced by Parkinson's-like bradykinesia in the late stages of HD, dopamine replacement therapies, such as those used to treat Parkinson's disease, have been employed. Only case studies have been published outlining the successes of this approach. The dopamine precursor levodopa was shown to improve motor function in four HD patients. Bradykinesia and rigidity were improved and three of the patients continued to be responsive to levodopa treatment at a two year follow-up (Reuter *et al.*, 2000). A separate case study reported improvement in tremor, finger and hand dexterity, and postural stability in a 38 year-old HD patient following levodopa treatment. This patient remained responsive to levodopa treatment at the one-year follow up (Racette & Perlmutter, 1998). Pramipexole, a dopamine D_2 receptor family agonist, was administered to a 34 year-old HD patient that

had been suffering from symptoms for more than 10 years. This patient was unresponsive to levodopa, but showed markedly decreased rigidity that resulted in the patient no longer requiring a wheelchair following the administration of pramipexole (Bonelli *et al.*, 2002).

Motor dysfunction is the most prominent symptom of HD, however, depression and cognitive impairments that result in decreased functional ability have been reported by patients to be of greater impact on quality of life (Ho *et al.*, 2009). Case studies have described improvement in mood and cognitive ability following treatment using a number of different approaches. Three-week treatment with imipramine (Whittier *et al.*, 1961), and two-week treatment with mirtazapine (Bonelli, 2003) resulted in improved mood in HD patients. A larger study examining 26 HD patients showed that four-week administration of the serotonin-norepinephrine reuptake inhibitor venlafaxine showed marked improvement in mood as determined by increased scores in two rating scales used to categorize depression (Holl *et al.*, 2010). Agitation and irritability were ameliorated following administration of the selective serotonin reuptake inhibitor (SSRI) fluoxetine (De Marchi *et al.*, 2001). Irritability and obsessive compulsive behaviour were improved in three of six patients treated with a combination treatment of lithium and haloperidol (Leonard *et al.*, 1975). Improvement was not observed using lithium or haloperidol alone. Obsessive compulsive disorder has also been successfully treated using the SSRI sertraline (Patzold & Brune, 2002). Despite the incidence of psychosis in HD, combined with the fact that antipsychotics are used to target movement disorder in the disease, very little has been published regarding the use of antipsychotics for treatment of psychosis in HD. Case studies have reported success in treating psychotic

symptoms of HD using the atypical antipsychotics quetiapine (Seitz & Millson, 2004), aripiprazole (Lin & Chou, 2008), and risperidone (Madhusoodanan & Brenner, 1998). A drug or therapy capable of slowing or stopping the progression of HD does not currently exist. Symptomatic therapy is the only treatment option available to clinicians, and this is hampered by the absence of a common therapeutic strategy due to the lack of clinical trials involving HD patients. Currently, the treatment of HD is handled on a patient-by-patient basis.

Several potential therapies are being investigated in ongoing or planned phase III clinical trials. The neuroleptic drugs olanzapine, tetrabenazine and tiapride (NCT00632645), the nutritional supplement creatine (NCT00712426), and the antioxidant coenzyme Q10 (NCT00608881) are all being independently administered in an attempt to improve the total functional capacity score in HD patients. The antihistamine latrepirdine (NCT01085266) is being investigated in another phase III clinical trial with the hypothesis that it will be able to improve cognitive deficits in HD.

1.2   Genetics of HD

1.2.a Discovery of the HD Gene

When Dr. George Huntingtin characterized HD in 1872, he described the hereditary nature of the disease as being confined within families as an "heirloom" from generations past (Huntington, 1872). He also inferred the autosomal dominant nature of HD, noting that if the offspring of an affected individual did not develop the disorder, future generations would be free from the disease. Using restriction fragment length polymorphism analysis in combination with Southern blotting of genetic material isolated

from an American and a large, extended Venezuelan HD family, the location of the gene

responsible for HD was narrowed to the short arm of chromosome 4 (Gusella *et al.*,

1983). Using exon trapping, which is a technique enabling the identification of exon

sequences in unknown DNA regions, hybridization probes were created and used to

screen cDNA libraries for the gene within the short arm of chromosome 4. A gene

named *Interesting Transcript 15* (since renamed *huntingtin*) was identified that encoded a

transcript of 10,366 nts, corresponding to a protein of 3,144 amino acids (aas) with a

predicted size of 348 kDa (HDCRG, 1993). *Huntingtin* has 67 exons spanning a locus of

180 kb (Ambrose *et al.*, 1994). Shortly after the discovery of the gene, a second

huntingtin transcript of 13.7 kb was identified (Lin *et al.*, 1993). The difference in size

was due to an extended 3' untranslated region in the 13.7 kb transcript. The extended

13.7 kb transcript is the predominant transcript expressed in neuronal tissue, while the

smaller 10.3 kb fragment is the prominent transcript in intestine, liver and lung, however

both transcripts have been detected in all tissues examined (Lin *et al.*, 1993; Ambrose *et

al.*, 1994). The *huntingtin* gene contains a polymorphic CAG repeat region in exon 1.

Both sized transcripts encoded by *huntingtin* are translated and the resulting huntingtin

protein (Htt) has a polyglutamine (polyQ) repeat region in the N-terminal region.

Sequencing of chromosomes from HD patients and unaffected individuals revealed that

98% of the 173 normal chromosomes from unaffected individuals analyzed contained

between 11 and 24 CAG repeats, two had between 25-30 CAG repeats and one each had

33 and 34 repeats. Of the 74 chromosomes from HD patients analyzed, 30 had between

42 and 47 CAG repeats and 44 had greater than 48 CAG repeats. The absence of overlap

in CAG repeat length in the *huntingtin* gene between HD patients and unaffected family

members implicated the CAG repeat region as the critical area in the gene causing HD and defined affected from unaffected individuals based on a range of CAG repeat lengths. To further examine the relationship between CAG repeat length and development of HD, a study examined more than 1000 HD patients and determined that HD patients possessed between 36 and 121 CAG repeats with a median of 44 CAG repeats. The majority of the 1595 control subjects had between 10 and 29 CAG repeats, with a median of 18 CAG repeats. Twelve controls had between 30 and 35 repeats, and rather surprisingly, one had 37 and one had 39 CAG repeats (Kremer *et al.*, 1994). The overlap in CAG repeat length between controls and diagnosed HD patients raised doubt over the threshold CAG repeat length causing the disease. It has since been determined that individuals with a CAG repeat length between 36 and 39 are designated as having 'reduced penetrance' of HD as it is not certain, based on CAG repeat length, whether the individual will develop HD. A recent clinical study followed 204 people who had a *huntingtin* allele with a CAG repeat number between 36 and 39. They determined that an individual possessing 36-39 CAG repeats had a 40% and 30% chance of being free of symptoms of HD at 65 and 75 years of age, respectively (Quarrell *et al.*, 2007).

Following the discovery of the *huntingtin* gene, the regional mRNA and protein distribution was quantified to determine the possible role of altered levels of *huntingtin* gene product as the cause of HD. *Huntingtin* mRNA is expressed throughout the brain and in all peripheral tissues examined (Strong *et al.*, 1993), and mRNA expression was shown to be unchanged in HD (Landwehrmeyer *et al.*, 1995). Likewise, Htt is widely expressed with the highest levels of Htt expression in the brain. High levels of Htt were also detected in the lung, testes and ovaries. Htt is expressed throughout the brain, and is

not enriched in the basal ganglia (Li *et al.*, 1993; Sharp *et al.*, 1995). Consistent with

huntingtin mRNA expression, Htt expression is not decreased in HD (Li *et al.*, 1993).

Taken together, these findings would suggest that the symptoms of HD result from

alteration in protein function resulting from polyQ expansion and not because of altered

protein expression.

In addition to determining if an individual will develop HD, the number of CAG

repeats also influences the age of onset of HD. A larger number of CAG repeats

correlates with earlier onset of HD. CAG repeat length alone, however, accounts for only

about 50% of the variation in age of symptom onset (Andrew *et al.*, 1993; Stine *et al.*,

1993). Environmental factors and genetic polymorphisms have also been shown to

influence the age of symptom onset (Wexler *et al.*, 2004; Li *et al.*, 2006; Gayan *et al.*,

2008). It has also been suggested that a longer CAG repeat in the *huntingtin* gene

correlates with a more rapid progression of symptoms (Brandt *et al.*, 1996; Penney *et al.*,

1997; Rosenblatt *et al.*, 2006; Ravina *et al.*, 2008). A number of studies, however, have

reported no correlation between the number of CAG repeats in the mutant allele and the

clinical progression or the rate of decline in HD patients (Kieburtz *et al.*, 1994; Claes *et

al.*, 1995; Squitieri *et al.*, 2002). In summary, the discovery of the *huntingtin* gene

revealed that CAG repeat expansion, with the exception of those possessing a 'reduced

penetrance' copy of the gene, is the causal factor in HD. In addition to determining

whether an individual will develop HD, CAG repeat length influences the age of disease

onset and potentially disease severity. Symptoms of HD are not the result of altered

huntingtin gene transcription, or Htt protein expression suggesting that pathogenesis

results from an abnormal function of the transcript or protein, likely in combination with additional cell-specific factors.

1.2.b Impact of polyQ Expansion on Htt Function

HD results from the inheritance of one mutant copy of the *huntingtin* gene. Following the discovery of the *huntingtin* gene, it was hypothesized that a development of the disease could result either from haploinsufficiency, or from a gain of function resulting from polyQ expansion in the mutant protein. Situations where Htt function is decreased or lost can provide information regarding whether the pathogenesis in HD results from a loss of wild-type Htt function. Loss of part of chromosome 4, which contains the *huntingtin* gene, occurs in patients who have Wolf-Hirschhorn syndrome. Symptoms of Wolf-Hirschhorn disease include craniofacial deformations, mental retardation, muscular hypotrophy, seizures and heart defects (Battaglia *et al.*, 2008). Although symptoms of Wolf-Hirschorn disease are severe, they do not resemble those seen in HD. This suggests that the loss of one copy of *huntingtin* does not lead to the development of HD symptoms. A knock-out mouse model was created to gain insight into the function of *huntingtin*. Mice heterozygous for the *huntingtin* deletion showed no HD-like phenotype. This suggests that expression of one copy of *huntingtin* is sufficient to perform all of the functions required of Htt. Deletion of both copies of *huntingtin* in the mouse model led to death at embryonic day 8.5 suggesting a critical role for Htt in development (Duyao *et al.*, 1995). These results demonstrate that at least one copy of *huntingtin* is necessary for survival through embryogenesis, however, only one copy of the gene is required to support all functional requirements of the protein. Since

individuals homozygous for the mutant *huntingtin* allele survive embryogenesis and live normally until symptom onset, it would indicate that mutant Htt (mHtt) is capable of performing the role required of wild-type Htt at least during development.

Animal models of HD that overexpress *huntingtin* provide evidence for the toxic gain of function hypothesis of HD. Several transgenic models of HD express two wild-type copies of the *huntingtin* gene in addition to overexpressing a fragment or a full-length copy of the mutant *huntingtin* gene. The two most studied overexpression HD animal models are the R6/2 and the YAC128 mouse lines. The R6/2 mouse model of HD was generated by overexpressing exon 1 of the human *huntingtin* gene with 144 CAG repeats (Mangiarini *et al.*, 1996). Overexpression of the N-terminal fragment of mHtt (N-mHtt) in R6/2 mice leads to a progression of HD-like symptoms including development of neuronal intranuclear inclusions (from birth), motor impairments (starting at 35 days of age), failure to gain weight and brain atrophy (observed at 90 and 60 days, respectively), and premature death (at approximately 14 weeks) (Stack *et al.*, 2005). The YAC128 mouse model of HD was generated through expression of a yeast artificial chromosome (YAC) containing the full-length human *huntingtin* gene containing 128 CAG repeats. YAC128 mice exhibit cognitive deficits (starting at 2 months), hyper- followed by hypokinetic movement impairments (observed 3 and 6 months of age, respectively), decreased striatal brain weight (detected at 9 months), striatal and cortical volume loss (observed at 9 and 12 months, respectively), and striatal neurodegeneration (observed at 12 months) (Slow *et al.*, 2003; Van Raamsdonk *et al.*, 2005b). Despite the fact that both of these HD mouse models contain two copies of *huntingtin* with a non-pathogenic CAG repeat length, they develop a phenotype that

resembles HD. Although the loss of wild-type Htt function may lead to certain symptoms of HD, these examples demonstrate that the toxic gain of function relating to polyQ expansion in mHtt causes changes in brain function and suggests that the same is true in HD.

1.3 Cleavage of Huntingtin

Htt has a number of caspase and calpain cleavage sites in the N-terminal region of the protein. Wild-type Htt can be cleaved *in vitro* at aas 513 and 552 by caspase-3, at aa 586 by caspase-6, and by caspase-2 at aa 552 (Hermel *et al.*, 2004). Wild-type Htt can also undergo calpain-mediated cleavage which releases an N-terminal fragment smaller than caspase-mediated cleavage fragments (Gafni & Ellerby, 2002). N-terminal fragments of wild-type Htt corresponding to the size produced by caspase-3-mediated cleavage have been detected in human striatal, cortical and cerebellar brain tissue (Kim *et al.*, 2001). Activation of calcium-dependent calpains mediate cleavage of wild-type Htt in cultured striatal cells (Goffredo *et al.*, 2002). These studies collectively suggest that cleavage of Htt is a natural process. Expansion of the polyQ region of Htt increases the activity of caspase enzymes, increases the rate of Htt cleavage, and consequently increases the amount of detectable N-mHtt in the cell. Caspase-2 and -3-mediated cleavage of Htt both increase in a polyQ length-dependent manner *in vitro* (Goldberg *et al.*, 1996; Hermel *et al.*, 2004). The expression of mHtt in mouse neuroblastoma cells led to increased activation of caspase-3 and caspase-6 compared to cells expressing wild-type Htt (Wang *et al.*, 1999), and caspase-6 activity is increased in the YAC128 mouse model of HD as well as in human HD patients (Graham *et al.*, 2010).

A number of studies have correlated decreased caspase activity and caspase-mediated cleavage with a reduction in the severity of HD symptoms suggesting that cleavage of mHtt may be a pivotal step in the progression of HD. Co-transfection of a catalytically inactive caspase-2, -6 or -7 mutant with full length Htt containing 138 polyQ repeats reduced N-mHtt-mediated cell death in rat striatal cells (Hermel *et al.*, 2004). Daily administration of minocycline in 6 week-old R6/2 mice inhibited caspase-1 and caspase-3 activity, reduced the cleavage of N-mHtt, delayed the decline of motor performance and increased the life span of the animals (Chen *et al.*, 2000). In an effort to more directly link cleavage of Htt with symptoms of HD, Gafni *et al.* (2004) mutated two known calpain cleavage sites on a 1200 aa fragment of N-mHtt and transfected them into HEK293T cells. Cells overexpressing un-cleavable N-mHtt were more resistant to cellular toxicity than cells transfected with unaltered N-mHtt (Gafni *et al.*, 2004). Prevention of caspase-6-mediated cleavage of mHtt has been shown to improve symptoms of HD. Mice genetically engineered to overexpress a copy of the *huntingtin* gene with 120 CAG repeats that was resistant to caspase-6-mediated cleavage had reduced brain atrophy, larger striatal volume and improved motor function as measured by rotorod performance compared to mice expressing mHtt which was susceptible to caspase-6-mediated cleavage (Graham *et al.*, 2006). These experiments demonstrate that caspase-mediated cleavage of mHtt is directly related to many of the symptoms that develop in HD including motor impairment and neurodegeneration.

1.4 Nuclear Translocation of N-mHtt

Following cleavage, the N-terminal fragment of Htt can localize to the nucleus (Wheeler *et al.*, 2000; Sawa *et al.*, 2005; Havel *et al.*, 2009). N-mHtt has been detected in the nuclei of human striatal and cortical cells (Gutekunst *et al.*, 1999), as well as in the neuronal nuclei of R6/2 mice (Meade *et al.*, 2002), mice stably expressing the N-terminal 171 aas of Htt with 82 glutamines (Schilling *et al.*, 2004), and in YAC128 mice (Van Raamsdonk *et al.*, 2005a). PolyQ expansion has been directly tied to nuclear accumulation. In a mouse model of HD that can be induced to express full-length Htt with either 23 or 148 glutamines, N-terminal Htt was detected in the nuclear fraction of the mutant but not the wild-type mice (Tanaka *et al.*, 2006). Despite the fact that wild-type Htt can be cleaved by caspases and calpains, evidence suggests that polyQ expansion in mHtt leads to increased cleavage, translocation to and accumulation in the nucleus.

To determine directly if the presence of N-mHtt in the nucleus is related to neuronal dysfunction, N-mHtt and a modified version of N-mHtt in which the calpain cleavage site was eliminated were transfected in HEK293T cells. Cells transfected with the calpain-resistant form of N-mHtt had less cleaved N-mHtt, less N-mHtt in the nucleus, and were more resistant to thapsigargin-induced apoptosis than cells expressing the N-terminal fragment that was susceptible to calpain-mediated cleavage (Gafni *et al.*, 2004). Caspase-6 mediated cleavage fragments of mHtt were present in the nuclei of immortalized and primary striatal cells (Warby *et al.*, 2008). A separate study demonstrated that mutation of mHtt to eliminate the caspase-6 cleavage site rescued the HD phenotype in YAC128 mice (Graham *et al.*, 2006). Taken together, these studies

suggest that eliminating the formation of the fragment of N-mHtt that translocates to the nucleus can halt development of the disease in a model of HD. Conversely, caspase-2 and -3 cleaved fragments of mHtt were localized to the perinuclear region of immortalized and primary striatal cells (Warby *et al.*, 2008), and elimination of caspase-2- or -3-mediated cleavage were unable to alter the progression of symptoms in YAC128 mice (Graham *et al.*, 2006) suggesting that eliminating the formation of fragments of N-mHtt that do not translocate to the nucleus has no effect on the disease course. The addition of a nuclear localization signal (NLS) to N-mHtt and subsequent expression in N2a cells led to an increase in N-mHtt in the nucleus, and a 111% increase in cell death compared to N-mHtt without the NLS. If the NLS was switched for a nuclear export signal (NES), the amount of N-mHtt in the nucleus decreased and cell death was 57% lower compared to N-mHtt without the NES (Peters *et al.*, 1999). Together, these experiments demonstrate that N-mHtt in the nucleus influences cellular dysfunction and toxicity.

PolyQ expansion results in increased caspase activity and formation of N-mHtt. Formation of N-mHtt enables nuclear translocation, which correlates with cellular dysfunction. Although the formation of N-mHtt appears to be required for nuclear translocation and accumulation, the mechanism by which this occurs is not clear. There is a functional NES near the carboxy-terminal end of Htt. HeLa cells were transiently transfected with vectors expressing green fluorescent protein (GFP) fused with one of five separate 50 aa fragments of Htt that were each believed to contain active NESs. Of these putative NESs, only the one located at aa 2404 was an active NES, and mutation of the lucine residue at aa 2404 of Htt to serine led to increased GFP in the nucleus of the

cells, suggesting that this mutation compromised NES activity (Xia *et al.*, 2003).

Cleavage of full length Htt into an N-terminal fragment would release the NES located at aa 2404, which would favour nuclear accumulation of Htt. Since Htt cleavage increases proportionally with polyQ length, it is not surprising that N-mHtt accumulates in the nucleus whereas wild-type Htt does not.

Hackam *et al.* (1998) reported that mHtt entry into the nucleus was dependent on fragment size. HEK293 cells were transfected with cDNA coding for various sized mHtt fragments. mHtt consisting of the 40, 151 and 224 N-terminal aas was detected in the nucleus, however the 436, 989 and 771 N-terminal aa fragments were dispersed between the nuclear and perinuclear space. The largest N-terminal fragments, at 1597, 1955 and 10366 aas in size were located exclusively in the cytoplasm (Hackam *et al.*, 1998). They hypothesized that N-mHtt diffuses passively into the nucleus and that this diffusion requires the N-terminal fragment to be less than 40 kDa. Although this theory suggests how cleavage of mHtt could lead to translocation of N-mHtt, it does not propose how N-mHtt accumulates in the nucleus. If fragments less than 40 kDa can passively diffuse into the nucleus, it would be predicted that these same fragments could passively exit the nucleus. The nuclear pore protein translocated promoter region (Tpr) is involved in nuclear export (Cornett *et al.*, 2005). PolyQ expansion reduced interactions between N-mHtt and Tpr relative to the interaction between the N- terminus of wild-type Htt and Tpr. Decreasing Tpr expression in the cell through RNA interference, or mutation of mHtt to eliminate mHtt-Tpr interaction both resulted in increased nuclear accumulation of N-mHtt. Together these studies demonstrate that the cleavage of Htt to a size that can passively enter the nucleus, the loss of a functional NES, and the polyQ-mediated

decreased interaction with a nuclear export protein favour accumulation of N-mHtt, but not N-terminal fragment or full length wild-type Htt, in the nucleus.

1.5   Soluble and Insoluble mHtt: Toxic or Protective?

1.5.a N-mHtt Aggregates

The formation of insoluble N-mHtt aggregates in the nuclei of cells called neuronal intranuclear inclusion bodies (NIIs) is one of the hallmarks of HD pathology. NIIs were first observed upon examination of brain sections of R6/2 mice (Davies *et al.*, 1997). Since that discovery, the presence of NIIs has been well documented in human HD patients (DiFiglia *et al.*, 1997), and in both rat and mouse models of HD (Davies *et al.*, 1999; Schilling *et al.*, 1999; von Horsten *et al.*, 2003). Aggregates immunoreactive to Htt antibody have also been detected in perinuclear (Martindale *et al.*, 1998; Waelter *et al.*, 2001) and cytoplasmic regions of the cell (DiFiglia *et al.*, 1997; Li *et al.*, 1999a). With the discovery that the presence of NIIs correlates more closely with disease severity than the presence of cytoplasmic aggregates (Hoffner *et al.*, 2005), the focus on aggregates in HD has centered on NIIs. Htt can form aggregates *in vitro* following proteolytic cleavage of the protein, and the propensity to aggregate is polyQ length-dependent (Scherzinger *et al.*, 1997).

Prior to the discovery of aggregates in HD, Max Perutz showed that synthetic extended polyglutamine stretches adopted β-sheet conformations (Perutz *et al.*, 1994). β-sheets from multiple monomers could interact through hydrogen bonding between main chain and side chain amide and carboxy groups and form what were termed polar zippers.

He hypothesized that the formation of polar zippers may favour abnormal interactions with polyQ-containing transcription factors, and that this interaction may be responsible for disease pathology. When NIIs were first observed in R6/2 mice, it was believed that since their formation preceded the onset of symptoms, aggregation of N-mHtt was the cause of the HD pathology (Davies *et al.*, 1997).

To determine directly if the presence of polyQ aggregates in cellular nuclei were toxic, a series of synthetic peptides were created containing 20 or 42 glutamines (20Q and 42Q, respectively) flanked by two lysines on either side, and an N-terminal fluorescein group. These peptides were induced to aggregate *in vitro* then added to cell culture medium, where they were taken up by Cos-7 and PC-12 cells. The presence of Q20 and Q42 aggregates in the cytoplasm of Cos-7 and PC-12 cells did not decrease cell viability. When Q20 and Q42 aggregates were targeted to the nuclei of the cells by incorporating a NLS into the synthetic peptides, approximately 60% of cells died (Yang *et al.*, 2002). The fact that nuclear localization was required, and that death occurred in the absence of soluble polyQ supported the hypothesis that N-mHtt-containing NIIs were the toxic mediator of HD progression. Two drawbacks of this experiment were that the peptide used did not contain any aas that flank the polyQ repeat such as those found in N-mHtt, and that the Q20 peptide, which was as small as the average length of polyQ in Htt in the unaffected population, was as toxic to cells as the Q42 fragment. Further study showed that the correlation between formation of NIIs and cellular toxicity was inconsistent. NIIs have been detected in a higher percentage of cortical cells than striatal cells, and at earlier time points in cortical cells than in striatal cells in multiple studies despite the fact

that striatal cells are more likely to die than cortical cells, and undergo neuronal death at earlier disease stages than cortical cells (Gutekunst *et al.*, 1999; Kuemmerle *et al.*, 1999).

As more animal models of HD were created and characterized, it was observed that many of these models developed NIIs in brain tissue well before symptom onset or neuropathological changes (Wheeler *et al.*, 2000; Wheeler *et al.*, 2002). In contrast, a knock-in model of HD that expressed 140 CAG repeats in exon 1 of the endogenous mouse *huntingtin* gene had a severe behavioural phenotype long before NIIs were detectable (Menalled *et al.*, 2003). The YAC128 mouse model of HD develops symptoms of the disease in the absence of NIIs, whereas mutant YAC128 mice that express a shorter fragment of Htt with the same number of glutamine repeats form NIIs but do not develop symptoms of HD (Slow *et al.*, 2005). These results suggest that the formation of NIIs does not correlate with the onset or even the eventual development of symptoms of HD.

1.5.b Soluble N-mHtt is Toxic

As evidence accumulated that NIIs were not toxic, some investigators argued that NIIs might actually be protective. Using a technique that allowed tracking of individual neurons, Arrasate *et al.* (2004) showed that the formation of aggregates predicted improved survival of neurons rather than cellular death (Arrasate *et al.*, 2004). They observed that in striatal cells transiently transfected with exon 1 of human *huntingtin* with 47 CAG repeats, cells forming aggregates were significantly less likely to die than cells that did not. Transfecting cells with a chaperone protein that bound directly to N-mHtt and increased oligomerization of the protein decreased cellular toxicity

(Behrends *et al.*, 2006). Mutation of the calpain-2 cleavage site on Htt led to increased aggregation and decreased cellular toxicity in HEK293 cells transiently transfected with human mHtt (Ratovitski *et al.*, 2009). All of these studies demonstrated that driving mHtt into an insoluble state decreased the likelihood of neuronal death.

From these observations, it was hypothesized that the soluble form of N-mHtt was the toxic form of the protein, and the protective nature of N-mHtt aggregates resided in their ability to decrease the concentration of soluble N-mHtt in the cell and in the nucleus. To test this hypothesis, a dominant-negative mutant of a ubiquitin-conjugating enzyme required for NII formation was co-transfected with N-mHtt into striatal cells. Inactivation of the ubiquitin-conjugating enzyme led to a decrease in the formation of NIIs and an increase in the percentage of cells that succumbed to apoptosis, suggesting that the NIIs were inhibiting cell death (Saudou *et al.*, 1998). Another study deleted aas 2-13 of Htt, which are required for nuclear aggregation. This deletion blocked the formation of NIIs, increased nuclear entry of N-mHtt and led to a dramatic increase in cellular toxicity of N-mHtt (Atwal *et al.*, 2007). These experiments provide evidence that aggregation of N-mHtt in NIIs is a protective mechanism that decreases intracellular concentrations of soluble N-mHtt.

1.6 Cellular Effects of mHtt

HD is a late-onset disorder. The majority of patients that inherit a mutant copy of the *huntingtin* gene will live symptom free for upwards of 35 years (Newcombe, 1981; Adams *et al.*, 1988; Roos *et al.*, 1993) despite the fact that Htt expression is detected as early as the blastocyst stage of embryogenesis (Jeong *et al.*, 2006). The cleavage of

mHtt, accumulation of N-mHtt in the cytoplasm and translocation of N-mHtt to the nucleus likely occur during this period. As levels of soluble mHtt and its N-terminal cleavage product accumulate in the cell, many aspects of cellular function are affected prior to the development of the motor, psychological and cognitive symptoms that clinically define HD. Expression of mHtt has been shown to be correlated with dysfunction of a number of important cellular functions including protein degradation, autophagy, apoptosis, mitochondrial function, energy metabolism, intra- and intercellular signaling, production and release of neurotrophic support, and transcription. Some of these changes occur earlier than others. Some of the changes may result directly from expression of mHtt, while others may result as secondary effects of other cellular changes. Understanding the relationship between expression of mHtt and the cellular changes, and how changes in these cellular functions impact the health of the cell is important for determining how these changes may be interrelated and whether one might be causal to others.

1.6.a Proteasomal Degradation

The fact that mHtt accumulates into insoluble complexes suggests that cellular processes meant to remove misfolded protein from the cell may be impaired. Proteins destined for degradation via the ubiquitin-proteasomal system (UPS) are tagged with multiple ubiquitin groups by three ubiquitin-conjugating enzymes. Poly-ubiquinated proteins are then degraded by the 26S proteasome leaving free aas, short peptide chains, and poly-ubiquitin chains (reviewed in Hershko & Ciechanover, 1998). Ubiquitinated mHtt has been detected in human HD patients (Sieradzan *et al.*, 1999), in overexpression

and knock-in mouse models of HD (Bennett *et al.*, 2007), and in cell models of HD (Jana *et al.*, 2001) indicating that mHtt is targeted for degradation in cells by the proteasomal pathway. Using an inducible cell model of HD, it was shown that all components of the proteasome co-localized with perinuclear inclusion bodies. Inhibition of proteasomal function led to accumulation of aggregates in the same cells, suggesting that the proteasomal pathway functions to reduce the number of aggregates (Waelter *et al.*, 2001). Mammalian proteasomes are unable to cleave long polyQ protein tracts (Venkatraman *et al.*, 2004). A 20Q peptide flanked by two lysine residues on either side was cleaved once, after the initial glutamine residue, releasing a 19Q peptide from the proteasome. Using a 35Q peptide flanked by 15 aas, this study demonstrated that the 15 flanking aas were completely degraded, but a long polyQ fragment remained intact. These studies suggest that, in attempting to degrade N-mHtt, the proteasome could become blocked by long, non-degradable polyQ peptide fragments, or that the proteasome may be releasing toxic polyQ fragments lacking flanking peptides back into the cell. There is evidence that polyQ peptide fragments lacking flanking amino acids are more toxic than polyQ-containing peptides (Marsh *et al.*, 2000). To test whether the presence of N-mHtt impaired the degradation of non-Htt proteins by the UPS, HEK293 cells were stably transfected with a GFP protein fused to a C-terminal aa sequence that targets a protein for degradation by the UPS, enabling quantification of UPS function based on the half-life of GFP fluorescence. Cells transiently transfected with exon 1 of human *huntingtin* containing 103 CAG repeats (expressing 103Q N-mHtt fragment) had significantly higher fluorescence than cells transfected with a 23Q N-terminal Htt fragment (Bence *et al.*, 2001). In addition, in cells transfected with 103Q, those that contained aggregates

had lower UPS activity, and there was a correlation between the size of the aggregates and the degree of UPS inhibition. Although these experiments demonstrate that mHtt can inhibit the UPS, debate remains as to whether the UPS is inhibited in HD and if UPS inhibition contributes to the HD pathology. A study examining R6/2 mice showed that levels, as well as activity, of the 20S and 26S proteasome subunits were unaltered in the HD mice compared to wild-type controls at 4, 8 and 13 weeks of age (Bett *et al.*, 2006). Another study showed that in a conditional mouse model in which exon 1 of *huntingtin* containing 94 CAG repeats was overexpressed, proteasomal activity was actually increased in the presence of N-mHtt (Diaz-Hernandez *et al.*, 2003). Although mHtt is ubiquitin-tagged for degradation and may have the potential to inhibit the proteasome based on its extended polyQ repeat, whether mHtt impairs the UPS or whether this potential impairment causes further pathology in cells expressing mHtt is uncertain.

1.6.b Autophagy

Autophagy is another cellular degradation process that disposes of proteins that are damaged or require turnover (reviewed in Todde *et al.*, 2009). Autophagy has been shown to regulate levels of both wild-type and mutant Htt. Levels of N-terminal Htt increased following inhibition of autophagy, and decreased following stimulation of autophagy (Qin *et al.*, 2003). Overexpression of wild-type or mutant Htt increased levels of the lysosomal enzyme cathepsin and induced autophagy, and Htt co-localized with cathepsin in vacuoles in a polyQ length-dependent manner (Kegel *et al.*, 2000).

Analysis of the formation of autophagic vacuoles and their fusion with the lysosome was performed in animal models of HD as well as using lymphoblasts from HD

patients to determine how expression of mHtt altered autophagy (Martinez-Vicente *et al.*, 2010). It was determined that expression of mHtt resulted in a decreased cargo load in autophagic vesicles without inhibiting the formation of autophagosomes or the delivery of their contents to the lysosome. The authors hypothesized that this decrease in the ability of autophagosomes to take up cargo targeted for degradation led to an oberserved increase in lipid droplets in cells expressing mHtt, and consequently, to mitochondrial impairment.

To clarify its role in HD pathogenesis, various methods have been used to modify the autophagy pathway and determine the effect on HD symptoms. One study showed that mutant, but not wild-type Htt, is acetylated at a lysine residue that targets the protein for autophagic degradation in a mouse model of HD and in human brain tissue. Inhibition of acetylation at this residue led to an accumulation of mHtt and increased toxicity in neuronal cells, whereas increased acetylation of mHtt decreased toxicity (Jeong *et al.*, 2009). Intrastriatal delivery of a protein that selectively targeted mHtt for chaperone-mediated autophagy reduced the amount of soluble and aggregated mHtt in R6/2 mice, which resulted in decreased striatal atrophy, increased body weight and prolonged life span (Bauer *et al.*, 2010). These studies show that the autophagic degradation pathway regulates levels of Htt, and that increasing autophagy-regulated degradation of mHtt can improve symptoms of HD. It is not clear, however, if mHtt inhibits the autophagy system, and whether dysfunction in autophagy negatively affects the cell in a manner apart from clearance of mHtt.

1.6.c Apoptosis

Apoptosis is the process of programmed cellular death. Apoptosis is primarily regulated by the activity of members of the caspase and bcl family of proteins (Reviewed in Wyllie, 2010). Because caspase activity has been shown to increase in HD, and because the most prominent pathology in HD is neuronal cell death, the role of apoptosis in the disease has been an area of interest. Striatal and hippocampal neurons were positive for TUNEL staining, which detects fragmented DNA and is considered an indicator of apoptosis, in transgenic mice expressing full-length human Htt with 89 CAG repeats, but not in wild-type controls (Reddy *et al.*, 1998). This was contradicted by a study that showed no difference in the amount of DNA damage in transgenic mice expressing full length *huntingtin* with 144 CAG repeats compared to wild-type animals (Menalled *et al.*, 2000).

Studies have attempted to correlate the expression and activity of proteins involved in the apoptotic pathway with the presence of mHtt. The expression of N-mHtt in neuronal cells led to increased caspase-3 activity, and induced apoptosis (Li *et al.*, 2000). Expression of the apoptotic proteins N-myc and C-myc were not different in striatal neurons in post-mortem brain samples from HD patients when compared to controls (Ferrer & Blanco, 2000). A different study showed that the apoptotic proteins Fas and its ligand Fas-L, which are involved in extracellular activation of the apoptotic pathway, were decreased in brains from grade 3 HD patients (Ferrer *et al.*, 2000a).

One of the difficulties in studing mechanisms of cell death in HD is that neurodegeneration is not observed in the majority of animal models of the disease. One animal model that does experience neurodegeneration is the YAC128 model of HD. A

number of studies have reported an increase in apoptosis in YAC mice, or primary striatal neurons from those mice, expressing mHtt relative to controls following glutamatergic excitation (Tang *et al.*, 2005; Shehadeh *et al.*, 2006; Fernandes *et al.*, 2007). Although these studies do not directly link mHtt expression with apoptosis, they suggest that apoptosis may be important in mediating the pathogenic effects resulting from direct alterations in other cellular function. Overall, however, the conflicting studies reflect the lack of consensus regarding whether apoptosis occurs more frequently in the presence of mHtt, and the role apoptosis plays in the progression of HD.

1.6.d Mitochondrial Dysfunction

The theory that mitochondrial dysfunction is involved in the progression of HD followed from the observation that striatal administration of the mitochondrial inhibitors 3-nitroproprionic acid (3-NP) or malonate produced age-dependent neurodegeneration similar to that seen in HD (Beal *et al.*, 1993; Bossi *et al.*, 1993). Administration of these compounds to mice less than 6 weeks-old had no effect on neuronal health. Striatal administration of malonate produced lesioning in 4 month- and 12 month-old mice, and striatal administration of 3-NP produced lesioning in mice aged between 7-14 weeks, which was more severe when administered to animals aged over 16 weeks. Moreover, the activity of mitochondrial complexes II, III and IV was shown to be decreased in striatal tissue from HD patients relative to age-matched controls (Gu *et al.*, 1996). This tissue, however, was from late stage HD patients with significant striatal degeneration and cell loss. Analysis of grade 1 HD patients showed no difference in the activities of mitochondrial complexes I-IV relative to controls (Guidetti *et al.*, 2001). Mitochondrial

complexes I-IV had similar activities in transgenic mice expressing full-length *huntingtin* with 48 or 89 CAG repeats, and it has been separately reported that activity of the mitochondrial complexes is unaltered in Hdh knock-in and N171 overexpression mouse models (Browne & Beal, 2004). These studies argue against the hypothesis that mHtt-mediated impairments in the mitochondrial electron transport chain (ETC) lead to the pathology observed in HD, but suggests that dysfunction in mitochondrial energy regulation may be the secondary effect of a separate action of mHtt.

In addition to the pivotal role in energy production, mitochondria contribute to the regulation of calcium levels within cells. Mitochondria utilize the proton gradient created by the ETC to drive the exchange of calcium into the organelle from the cytosol. Release of calcium from mitochondria is driven primarily by a sodium/calcium exchanger (reviewed in Saris & Carafoli, 2005). Calcium overload in the cell can lead to opening of the mitochondrial permeability transition (MPT) pore, which has been shown to be involved in cell death following ischemia/reperfusion injury and has been hypothesized to be involved in neuronal death in neurodegenerative diseases (reviewed in Pivovarova & Andrews). In a study that examined mitochondria isolated from the lymphoblasts of HD patients, it was shown that these mitochondria had a lower membrane potential, and depolarized at lower calcium concentrations than mitochondria from control patients (Panov *et al.*, 2002). Similarly, mitochondria isolated from neuronal cells of transgenic HD mice expressing full-length *huntingtin* with 72 CAG repeats had lower membrane potential, depolarized at lower calcium concentrations and had decreased capacity for calcium uptake compared to wild-type mice (Panov *et al.*, 2002). A separate study showed that mitochondria isolated from striatal neurons in R6/2

and YAC128 HD mice were better able to handle increased calcium loads compared to controls, while the Hdh 150 mouse model of HD had no difference in mitochondria calcium handling compared to wild-type mice (Oliveira *et al.*, 2007). These studies present evidence suggesting that the presence of mHtt can lead to imbalances in mitochondrial calcium regulation, however, conflicting evidence from different animal models suggests that further experimentation is required to determine whether this is a direct effect of mHtt.

To investigate how mHtt might directly inhibit mitochondrial function, Orr *et al.* (2008) showed that mHtt associates with mitochondria in cortical cells isolated from a knock-in mouse model of HD, but not in mitochondria isolated from wild-type mice, and that N-terminal fragments are present within mitochondrial fractions from HD but not wild-type mice (Orr *et al.*, 2008). In contrast to previous studies suggesting that the presence of neuropil aggregates physically impeded mitochondria from travelling along axons, this study demonstrated that soluble N-mHtt impeded axonal trafficking by interfering with the association of mitochondrial trafficking protein to the mitochondria. The consequence of the mHtt interaction with mitochondria and the subsequent impairment in mitochondrial trafficking was an increase in the number of degenerated mitochondria in nerve terminals, and lower levels of synaptic ATP in the HD knock-in mice. A separate study demonstrated that expression of mHtt causes an imbalance in mitochondrial fission/fusion homeostasis, resulting in increased mitochondrial fragmentation, which can trigger apoptosis (Song *et al.*, 2011). The authors showed that mHtt has a higher affinity for the mitochondrial fission protein DRP1 than wild-type Htt in YAC128 mice and in tissue from human HD patients. Expression of a dominant

negative DRP1 protein increased transport, motility and velocity of mitochondria, and reduced mitochondrial fragmentation and cell death in neurons transiently expressing exon 1 of human Htt with 97 CAG repeats relative to 17 CAG repeat controls. These results provide support for the theory that mHtt directly impairs mitochondria which results in altered energy metabolism, synaptic activity, and imbalanced mitochondrial fission/fusion homeostasis.

Two therapeutic strategies aimed at improving mitochondrial function in the presence of mHtt have emerged. Creatine is a nitrogen-containing compound important for regenerating cellular ATP levels. Creatine can be synthesized endogenously, but is also a natural occurring compound that can be obtained through diet. Creatine has been shown to play an important role in neuronal function (reviewed in Andres *et al.*, 2008). Creatine administration in mice overexpressing the N-terminal 171 aas of human Htt with 82 glutamines improved motor dysfunction, decreased weight loss and brain atrophy, and prolonged life span of the animals (Andreassen *et al.*, 2001). It was suggested in these studies that the neuroprotective properties of creatine could reside in its ability to inhibit MPT pore opening and calcium imbalance, however this was not demonstrated and others have shown that creatine treatment does not affect the MPT balance (Brustovetsky *et al.*, 2001). Coenzyme Q10 resides in mitochondria and is directly and extensively involved in mediating electron transfer in the electron transfer gradient (reviewed in Lenaz *et al.*, 2007). Administration of coenzyme Q10 has been shown to inhibit the MPT-mediated release of calcium from the mitochondria (Papucci *et al.*, 2003), and delay the onset of symptoms, including motor dysfunction, weight loss, and cerebral atrophy in the R6/2 mouse model of HD (Ferrante *et al.*, 2002).

Despite the evidence that mHtt can interact with mitochondria and inhibit mitochondrial function, debate continues regarding the impact that mHtt has on mitochondrial function *in vivo*. Improvement in the symptoms of HD following molecular intervention or drug therapy has not been attributed directly to improved mitochondrial function or the inhibition of a mHtt effect on mitochondria.

1.6.e Excitotoxicity

Excitotoxicity refers to the death of neurons following a toxic level of excitatory input. The idea that HD is caused by excitotoxicity originated based on observation that striatal administration of the glutamate agonist kainic acid (KA) in rats led to a pattern of neurodegeneration and neurochemical imbalance reminiscent of that seen in of HD (Coyle & Schwarcz, 1976). It was hypothesized that elevated glutamatergic activity at the dendrites of MSNs, either due to increased cortical input, increased sensitivity of post-synaptic NMDA receptors, or decreased neurotransmitter clearance leads to selective striatal neurodegeneration in HD (Fan & Raymond, 2007). Support of the excitotoxicity hypothesis from animal models of HD has been mixed. Striatal injection of the NMDA receptor agonist quinolinic acid (QA) caused a greater degree of striatal cell death in transgenic mice expressing full length Htt with 72 glutamines compared to wild-type mice (Zeron *et al.*, 2002). Striatal QA injections in rats overexpressing N-terminal Htt with 51 glutamines, as well as in R6/2 mice did not result in more severe lesioning than their wild-type littermates in fact, R6 mice appear to be resistant to QA (Hansson *et al.*, 1999; Winkler *et al.*, 2006).

Analysis of NMDA receptor expression in models of HD also showed conflicting results. mRNA levels for the NR1 subunit of the NMDA receptor did not change in 4, 8 or 12 week-old R6/2 mice, but mRNA and protein levels of NR2A and NR2B subunits progressively decreased (Luthi-Carter *et al.*, 2000). A later study in R6/2 mice confirmed that NR2A mRNA expression was decreased at both early and late disease stages but that NR2B expression was unaltered (Ali & Levine, 2006). A study examining YAC128 mice showed increased radioligand binding to the NMDA receptor that did not correlate with increased overall receptor expression, but did correlate with increased NMDA receptors at synapses (Benn *et al.*, 2007), suggesting that YAC128 mice have increased glutamatergic activity. NMDA receptor activity was tested in several HD mouse models, and NDMA receptor currents were shown to be increased in both presymptomatic and symptomatic R6/2 mice as well as in YAC72 mice compared to wild-types (Cepeda *et al.*, 2001).

If overactivity of NMDA receptors was responsible for excitotoxic cell death then we would predict that NMDA receptor antagonists, if administered pre-symptomatically, prior to excitotoxic insult, would slow or prevent the development of HD symptoms. Administration of a tryptophan metabolite N-(2-N,N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carobxamide hydrochloride to N171-82Q HD mice increased survival by 30%, improved motor dysfunction, and decreased striatal atrophy (Zadori *et al.*, 2011). However, a number of NMDA receptor antagonists have been studied in clinical trials and case studies and have shown very little efficacy (reviewed in Bonelli *et al.*, 2004).

Recent evidence suggests that activation of synaptic NMDA receptors may be protective to striatal cells expressing mHtt, but that excitation of extra-synaptic NMDA receptors mediates cellular toxicity (Okamoto *et al.*, 2009). Extra-synaptic NMDA receptor activation led to decreased inclusion formation and decreased the activity of the neuroprotective CREB-PGC-1α signalling cascade. Administration of low-dose memantine selectively antagonized extra-synaptic NMDA receptors, decreased striatal atrophy, and recovered motor impairments in YAC128 mice. Using electrophysiology, a follow-up study confirmed that increased NMDA activity in cells expressing mHtt was due to activation of extra-synaptic NMDA receptors (Milnerwood *et al.*, 2010). The authors demonstrated that low-dose memantine treatment increased CREB phosphorylation and increased motor activity in YAC128 mice. Taken together, these studies provide evidence that selective antagonism of extra-synaptic NMDA receptors may provide a means for decreasing cell death of striatal neurons.

1.6.f Neurotrophic Support

Neurotrophic factors are signaling molecules that support neuronal growth. Decreased neurotropic factor signaling is detrimental to neurons and has been shown to be involved in various pathological conditions and diseases (reviewed in Twiss *et al.*, 2006). Although members of the glial-derived neurotrophic factor and ciliary neurotrophic factor families have been implicated in HD (Alberch *et al.*, 2004), much of the recent research into neurotrophin involvement in the disease has centered around brain-derived neurotrophic factor (BDNF).

Supporting the theory that decreased cortico-striatal signaling is important to the progression of HD, decreased cortical BDNF mRNA levels have been documented in human HD patients (Zuccato *et al.*, 2008) as well as in young R6/2 and YAC72 mice (Hermel *et al.*, 2004; Zuccato *et al.*, 2005). Decreased striatal BDNF receptor levels have also been seen in presymptomatic HD patients (Zuccato *et al.*, 2008). Other findings, in human HD patients or using animal models of HD, that have failed to show impairments in cortico-striatal BDNF signaling, or striatal-specific decreases in BDNF production contradict the importance of decreased neurotrophic support to the progression of HD. Decreased striatal, but not cortical, BDNF levels have been shown in human HD tissue (Ferrer *et al.*, 2000b) and in R6/1 mice (Spires *et al.*, 2004) and no changes in BDNF levels in both the striatum and cortex were reported in studies using Q109/109, and older R6/1 and R6/2 mice (Canals *et al.*, 2004; Borrell-Pages *et al.*, 2006; Seo *et al.*, 2008). This contradictory data suggests further research is required to elucidate the true role of BDNF signaling in the progression of HD.

Despite the controversy regarding relative levels of BDNF and its receptors, studies that have manipulated BDNF expression provide support for the neurotrophic-deficiency hypothesis of HD. Changes in gene expression in both heterozygous and homozygous cortex-specific BDNF knockout mice were more similar to the pattern of altered gene expression in HD compared to those observed in R6/2 or 3-NP-treated mouse models of HD (Strand *et al.*, 2007). When R6/1 mice were crossed with heterozygous BDNF knockout mice, the progeny (BDNF$^{+/-}$ expressing N-mHtt) had more severe motor deficits and earlier symptom onset than either R6/1 or BDNF$^{+/-}$ mice (Canals *et al.*, 2004). Efforts targeted at replenishing BDNF levels have also provided

support that deficiencies in neurotrophic support contribute to the symptoms of HD. Transgenic overexpression of BDNF in R6/1 mice increased BDNF mRNA and protein expression in the striatum and cortex, and improved motor coordination, reversed brain weight loss as well as cortical and striatal atrophy (Gharami *et al.*, 2008). Transgenic overexpression of BDNF in YAC128 mice improved motor dysfunction, reversed cognitive deficits, prevented brain atrophy and striatal neurodegeneration and restored transcription levels of a subset of mHtt-affected genes (Xie *et al.*, 2010). Although it is not clear whether, or to what degree BDNF mRNA and protein levels are altered in cortical and striatal neurons, the strongest evidence linking neurotrophins to the symptoms of HD suggests that decreasing BDNF expression exacerbates symptoms of HD, and increasing BDNF levels in mouse models of HD can improve the symptoms of the disease.

1.6.g Cholesterol Biosynthesis

Cholesterol is essential for neuronal health. Cholesterol is required for axon myelination, cell membrane composition, the formation of lipid rafts which help to structure synapses, and cholesterol metabolites are necessary for signal transduction (reviewed in Pfrieger, 2003; Katsuno *et al.*, 2009). Cholesterol cannot cross the blood brain barrier, and therefore must be synthesized and metabolized within the brain (Martin *et al.*, 2010). Because dietary cholesterol does not enter the brain, mHtt-mediated dysfunction in the synthesis or metabolism of cholesterol would be expected to have a more severe effect on the brain than other organs in the body. The expression of mHtt in an inducible cell model of HD led to a reduction in cholesterol synthesis, and cholesterol

levels in the brains of 5 and 10 week-old R6/2 mice, and 10 month-old YAC128 mice were decreased relative to wild-type mice (Valenza *et al.*, 2005; Valenza *et al.*, 2007a). Although cholesterol levels were increased in striatal cells and in primary striatal cell cultures from YAC72 mice, this increase was attributed to decreased intracellular cholesterol trafficking in the presence of mHtt (Trushina *et al.*, 2006).

Impairment in the cholesterol production pathway in the presence of mHtt has also been demonstrated through characterization of the enzymes and precursors involved in cholesterol synthesis. Decreased levels of sterol precursors required for neuronal cholesterol synthesis have been shown in YAC128, R6/2 and Hdh Q111/111 mice at early time points in disease progression (Valenza *et al.*, 2007a; Valenza *et al.*, 2007b; Valenza *et al.*, 2010). Activity of the cholesterol synthesis enzyme HMG-CoA-Reductase is also decreased in YAC128 and R6/2 mice (Valenza *et al.*, 2007a; Valenza *et al.*, 2007b). Lastly, levels of the brain cholesterol metabolite 24S-hydroxycholesterol (24OHC) were decreased in 13 week-old R6/2 mice, 10 month-old YAC46, YAC72, YAC128, 12 month-old Hdh Q7/111, and Hdh Q111/111 mice (all symptomatic ages), as well as in 21 month-old transgenic HD rats expressing 1962 bp N-terminal *huntingtin* with 51 CAG repeats compared to wild-type levels (Valenza *et al.*, 2010). Quantification of the 24OHC cholesterol metabolite using MRI in pre-symptomatic and symptomatic human HD patients showed a significantly lower amount of metabolite in symptomatic, but not pre-symptomatic HD patients compared to controls (Leoni *et al.*, 2008). Analysis of caudate volume in these patients revealed a correlation between plasma levels of 24OHC and the caudate volume, suggesting that the brain cholesterol metabolite could serve as a bio-marker for neurodegeneration. Compelling evidence exists from animal

models and human patients that the cholesterol production and metabolism pathway in the brain is impaired in HD. Although studies performed in aged animals are unable to provide evidence that imbalances in the cholesterol pathway are a primary effect resulting from the present of mHtt, studies performed in younger animals and pre-symptomatic patients suggest it may be the case.

1.6.h Altered mRNA Expression

The presence of mHtt leads to altered expression of many genes, however the majority remain unaffected. This demonstrates that mHtt impairs transcription in a gene-specific manner. Altered transcription was first observed in a study that demonstrated that dopamine receptor D_2 mRNA was decreased in the caudate and putamen in tissue from grade 0-3 HD brains relative to unaffected individuals (Augood *et al.*, 1997). This study, unlike a similar previous one, showed that in addition to overall decreased mRNA expression, the number of copies of D_2 mRNA per cell was also decreased, confirming that the result was not a consequence of cell death. With the development of animal models of HD and microarray technology, the ability to examine the extent to which mHtt alters transcription increased. Reinforcing the theory that mHtt decreases mRNA levels in a gene-specific manner, a microarray study on 6 and 12 week-old R6/2 mice revealed that expression of ~1.7% and 1.2%, respectively, of the more than 6000 genes analyzed were altered in these R6/2 mice relative to wild-types (Luthi-Carter *et al.*, 2000). The genes that were decreased in these mice outnumbered those that were increased by 3-1. The genes that were increased belonged primarily to the inflammatory response pathway, while those that decreased belonged to signal transduction, ion

channel, transcription, metabolism, and cell structure pathways. Gene-expression studies were performed on the R6/1 model of HD following behavioural analysis. Gene expression changes occurred prior to the onset of motor dysfunction, and did not change significantly over time (Hodges *et al.*, 2008). Microarray studies have also demonstrated selective gene expression changes at early time points in the YAC128 mouse model (Becanovic *et al.*, 2010).

Cell models of HD were used to demonstrate that mHtt alters transcription in a cell autonomous manner, and not as a consequence of altered cellular signaling. Microarray data from immortalized striatal cells expressing N-mHtt in an inducible manner showed changes in mRNAs involved in lipid metabolism, vesicle trafficking and transcription (Sipione *et al.*, 2002). Importantly, this study showed that transcriptional dysregulation could be detected within 24 h following induction of N-mHtt expression, supporting the hypothesis that transcriptional dysregulation is a direct effect of N-mHtt.

The studies described examined global changes in gene expression that occur in the presence of mHtt. Since the striatal region of the brain is more susceptible to the toxic effects of mHtt, examination of cell-specific gene expression changes was performed to determine if a link might exist between transcription and later changes that occur in HD. A microarray survey was performed on brains from 44 HD brains ranging from grade 0 to 4. The greatest degree of alteration in mRNA levels in HD patients occurred in the caudate nucleus, compared to the cerebellum, and two cortical areas (Hodges *et al.*, 2006). Of the nearly 10,000 genes analyzed, 21% had altered levels in the caudate compared to 1% in the cerebellum, 3% in the motor cortex and 0% in the prefrontal association cortex. As the grade of pathology increased, the degree of mRNA

expression changes for those genes downregulated in the caudate became more pronounced. These experiments support the theory that mHtt can decrease transcription in a gene-specific manner, that expression of a greater number of genes is affected in the striatum than in other regions of the brain in HD, and that some changes in mRNA expression occur early in the disease process, while others are not detected until later on.

Not only does transcriptional dysregulation occur early in the progression of the disease, but many of the genes whose expression is altered can be connected to previously described cellular changes. The transcriptional co-activator PGC-1α regulates the expression of many mitochondrial genes (Lin, 2009). PGC-1α mRNA expression is decreased in presymptomatic HD patients, and overexpression of PGC-1α improved mitochondrial dysfunction in an HD cell model, and was neuroprotective R6/2 mice (Cui *et al.*, 2006). HMG-CoA reductase is the rate-limiting enzyme in the synthesis of cholesterol (DeBose-Boyd, 2008). Microarray analysis of an inducible striatal cell model of HD revealed that HMG-CoA reductase mRNA was decreased shortly after induction of mHtt expression (Sipione *et al.*, 2002), which was confirmed in 6 week-old R6/2 mice and striatal tissue from grades 1 and 2 HD patients (Valenza *et al.*, 2005). Decreased expression of the rate-limiting enzyme in cholesterol synthesis early in disease progression could lead to the decreased cholesterol levels that have been observed in the presence of mHtt (Valenza *et al.*, 2005; Valenza *et al.*, 2007a). The theory that decreased BDNF trophic support of striatal cells leads to their dysfunction and death is based, in part, on the observation that BDNF transcription is impaired in the presence of mHtt (Zuccato *et al.*, 2001). These experiments demonstrate that many of the theories

regarding how the presence of mHtt alters cellular function can be traced back to transcriptional dysregulation.

1.7 Regulation of Gene-Expression and Mechanisms of N-mHtt-Mediated Transcriptional Dysregulation

Alteration of steady-state mRNA levels occurs early in the progression of HD. Transcriptional changes have been detected in presymtomatic human patients, at early time points in animal models, and immediately following induction of mHtt expression in cell models. Altered gene-expression is most prevalent in the striatum, which is one of the most severely affected tissues in HD. Cellular toxicity is correlated with the presence of N-mHtt in the nucleus, suggesting that mHtt is most detrimental to the cell when in the nucleus. Whereas some models of HD do not develop NIIs, or undergo neurodegeneration, striatal-specific decreases in gene expression are common across all animal models of HD as well as in humans. The presence of N-mHtt in the nucleus and its effect on gene expression may prove to be a gateway to the later effects of HD. If this is true, understanding the effect of N-mHtt on gene-specific transcription may provide a target to restoring transcription, which could slow or stop the progression of HD.

Gene expression is regulated on several levels. DNA is packaged into a highly ordered chromatin which can physically impede the access of proteins necessary to transcribe DNA to RNA. Gene-specific transcription factors bind to DNA in a sequence-specific manner and influence the stability of general transcription factors near the transcription start site, either through direct binding or via interaction with co-activator or co-repressor complexes. The general transcription factors are involved in the assembly of the transcription complex culminating in the recruitment of RNA polymerase II (PolII).

The general transcription machinery is required for the synthesis of all gene transcripts (Watson, 2003).

N-mHtt has been implicated in disrupting transcription at each of the three levels described. The majority of the mechanisms proposed to explain N-mHtt-mediated transcriptional dysregulation are based on known interactions between N-mHtt and transcriptional proteins. Table 1.1 lists the known Htt-interacting proteins sorted by their cellular processes. To date, 84 proteins are known to interact with mHtt and affect cellular processes that include aggregation, apoptosis, energy metabolism, proteolysis, cellular signaling, cellular trafficking, and transcription. Of these interacting proteins, 30 (~36%) are proteins whose principle functions are directly related to transcription. Many transcription factors contain polyQ repeat regions which enable them to interact with other protein possessing polyQ repeats, such as Htt. In most cases, polyQ expansion within Htt increases the affinity of these interactions, as predicted by Perutz (Perutz *et al.*, 1994). The increased affinity of N-mHtt for transcription factors may enable N-mHtt to sequester interaction partners from their site of action, or mHtt may be recruited by its interaction partner into the transcriptional machinery at affected promoters directly inhibiting transcription. Each level of regulation, and the potential mechanisms of N-mHtt-mediated transcriptional dysregulation at each of the levels will be discussed.

1.7.a Regulation of Chromatin Folding

The DNA in a cell is packaged into chromatin. Chromatin is a highly ordered structure whose primary purpose is to condense DNA to a size that will physically fit within the nucleus and regulate gene expression. This condensed structure must allow

Table 1.1. Known Htt interaction partners

Cellular Process	Gene	Name	Function	Reference
Trafficking				
	HIP14	zinc finger, DHHC-type containing 17	Palmitoyl acetyltransferase	Goytain *et al.,* 2008
	PACSIN1	protein kinase C and casein kinase substrate in neurons 1	Endocytosis, vesicular transport	Modregger *et al.*, 2002
	DLG4	discs, large homolog 4 (Drosophila) (aka PSD-95)	Synaptic scaffolding	Sun *et al.*, 2001
	SH3GL3	SH3-domain GRB2-like 3	Endocytosis, vesicle recycling	Ringstad *et al.*, 1997
	DNAL1	dynein, axonemal, light chain 1	Trafficking	HDBase, 2004b
	PFN2	Profiling	Regulates actin polymerization	Goehler *et al.*, 2004
	DCTN1	dynactin 1	Highway for vesicle, protein transport	Caviston *et al*., 2007
	APA2A	adaptor-related protein complex 2, alpha 2 subunit	Involved in endocytosis	Faber *et al.*, 1998
	TPR	translocated promoter region	Nuclear export of mRNA and protein	Cornett *et al.*, 2005
	HIP1R	huntingtin interacting protein 1 related	Actin/Clathrin binding	Chopra *et al.*, 2000
Signaling				
	CALM1	Calmodulin	Ca-binding regulatory protein	Bao *et al.*, 1996
	TRIP10	thyroid hormone receptor interactor 10	Cdc42-related signaling	Holbert *et al.*, 2003
	GRB2	growth factor receptor-bound protein 2	Growth Factor Receptor signaling	Liu *et al.*, 1997
	TPH	Tryptophan Hyrdoxylase	Seratonin synthesis	HDBase, 2004b
	FICD	FIC domain containing (aka HYPE)	RhoGTPase signaling	Faber *et al.*, 1998
	PDE1A	Phosphodiesterase 1A, calmodulin-dependent	Second messenger signaling	HDBase, 2004b
	GRAP	GRB2-related adaptors proteins	Couples tyrosine-kinase to Ras	HDBase, 2004b
	RASA1	RAS p21 protein activators	EGF receptor signaling	Liu *et al.*, 1997
	ITPR1	inositol 1,4,5-triphosphate receptor, type 1	Receptor that mediates calcium release	Tang *et al.*, 2003
	MAP3K10	mitogen-activated protein kinase kinase kinase 10	Involved in JNK signalling pathway	Liu *et al.*, 2000
	CRMP1	collapsin response mediator protein 1	Signalling during neuronal development	Goehler *et al.*, 2004
	GPRASP2	G protein-coupled receptor associated sorting protein 2	Regulates GPCRs	Goehler *et al.*, 2004

	UBC	ubiquitin C	Regulates cell-cycle signaling	Steffan *et al.*, 2004
	IKBKAP	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein	Scaffold protein, regulates kinases involved in proinflammatory signaling	Goehler *et al.*, 2004
	SHC1	SHC (Src homology 2 domain containing) transforming protein 1	Involved in signal transduction	HDBase, 2004a
	EGFR	epidermal growth factor receptor	Tyrosine-Kinase receptor	Liu *et al.*, 1997
Proteolysis				
	UBE2K	Ubiquitin conjugating enzyme (aka HIP2)	Protein degradation	Kalchman *et al.*, 1996
	DNAJB2	DnaJ (Hsp40) homolog, subfamily B, member 2	Proteasomal delivery	Westhoff *et al.*, 2005
	PSMD8	proteasome (prosome, macropain) 26S subunit, non-ATPase, 8	Protein degradation	Faber *et al.*, 1998
Transcription				
	TCERG1	transcription elongation regulator 1 (aka CA150)	Regulates transcriptional elongation, pre-mRNA splicing	Holbert *et al.*, 2001
	CREBBP	CREB binding protein	Transcriptional co-activator, histone acetylation	Steffan *et al.*, 2000
	CTBP1	C-terminal binding protein 1	Transcriptional repressor	Kegel *et al.*, 2002
	CEBPA	CCAAT enhancer binding protein alpha	Transcriptional activator	Chiang *et al.*, 2007
	GTF2F2	General transcription factor IIF polypeptide 2 (RAP30)	Binds with RAP74 to form TFIIF	Zhai *et al*., 2005
	PRPF40A	PRP40 pre-mRNA processing factor 40 homolog A (S. cerevisiae) (aka HYPA)	Pre-mRNA splicing	Faber *et al.*, 1998
	SETD2	SET domain containing 2 (aka HYPB)	Transcriptional activator, histone methyltransferase	Faber *et al.*, 1998
	PRPF40B	PRP40 pre-mRNA processing factor 40 homolog B (aka HYPC)	Pre-mRNA splicing	Faber *et al.*, 1998
	NCOR1	nuclear receptor corepressor 1	Transcriptional repressor, histone deacetylase	Boutell *et al.*, 1999
	NEUROD1	NEUROD1 neurogenic differentiation 1	Transcriptional activator	Marcora *et al.*, 2003
	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	Transcriptional regulator	Takano & Gusella, 2002

SP1	Sp1 transcription factor	Transcriptional activator, DNA binding protein	Li *et al.*, 2002
TAF4	TAF4 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 135kDa	Subunit of TFIID, interacts with CREB	Dunah *et al.*, 2002
CHD3	chromodomain helicase DNA binding protein 3	Histone deacetylase	Goehler *et al.*, 2004
TBP	TATA box binding protein	Binds directly to TATA box. Component of TFIID. Involved in DNA melting.	Schaffar *et al.*, 2004
p53	tumor protein p53	Binds DNA directly, can arrest cell cycle as well as initiate apoptosis	Steffan *et al.*, 2000
REST	RE1-silencing transcription factor	Transcriptional repressor. DNA binding protein	Zuccato *et al.*, 2003
HAP1	huntingtin-associated protein 1	Vesicular trafficking, endocytosis, transcription	Li *et al.*, 1998
HIP1	huntingtin interacting protein 1	Endocytosis, pro-apoptotic, transcription	Chopra *et al.*, 2000
SAP30	SIN3-associated polypeptide 30	Member of HDAC complex	Boutell *et al.*, 1999
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Glycolysis, transcription	Burke *et al.*, 1996
PQBP1	PolyQ binding protein 1	Activator/repressor protein	Waragai *et al.*, 1999
HYPM	Chromosome x open reading frame 27	DNA binding	Faber *et al.*, 1998
SYMPK	Symplekin	Polyadenylation	Faber *et al.*, 1998
OPTN	Optineurin	Interacts with TFIIA	Hatula & Peranen, 2000
PIAS4	protein inhibitor of activated STAT, 4	Transcriptional repressor	Goehler *et al.*, 2004
MED31	Mediator complex subunit 31	Transcriptional co-activator	Goehler *et al.*, 2004
KAT2B	K(lysine) acetyltransferase 2B	Transcriptional regulator. Binds p300/CBP	Steffan *et al.*, 2001
SUMO1	SMT3 suppressor of mif two 3 homolog 1 (S. cerevisiae)	Transcriptional repressor	Steffan *et al.*, 2004
SIN3A	SIN3 homolog A, transcription regulator (yeast)	Transcriptional regulator	Steffan *et al.*, 2000
Aggregation			
GIT1	G protein-coupled receptor kinase interacting ArfGAP 1	Facilitates aggregation	Goehler *et al.*, 2004
PAK1	p21 protein (Cdc42/Rac)-activated kinase 1	Facilitates aggregation	Luo *et al.*, 2008

Apoptosis				
	TGM2	transglutaminase 2	Apoptosis	Chun *et al.*, 2001
	AKT1	v-akt murine thymoma viral oncogene homolog 1	Pro-survival; Suppresses apoptosis	Humbert *et al.*, 2002
	ESRRBL1	intraflagellar transport 57 homolog (Chlamydomonas)	Pro-apoptotic	HDBase, 2004a
	CASP1	caspase 1, apoptosis-related cysteine peptidase	Apoptosis	Wellington *et al.*, 1998
	CASP2	caspase 2, apoptosis-related cysteine peptidase	Apoptosis	Hermel *et al.*, 2004
	CASP3	caspase 3, apoptosis-related cysteine peptidase	Apoptosis	Wellington *et al.*, 1998
	CASP6	caspase 6, apoptosis-related cysteine peptidase	Apoptosis	Hermel *et al.*, 2004
	CASP7	caspase 7, apoptosis-related cysteine peptidase	Apoptosis	Hermel *et al.*, 2004
	CASP8	caspase 8, apoptosis-related cysteine peptidase	Apoptosis	Hermel *et al.*, 2004
Energy				
	ECH1	enoyl CoA hydratase 1, peroxisomal	Fatty acid oxidation	HDBase, 2004b
	PDK2	pyruvate dehydrogenase kinase, isozyme 2	Regulating metabolism	Stelzl *et al.*, 2005
Other				
	MAGEA3	Melanoma antigen, family A, 3	Unknown	Faber *et al.*, 1998
	EIF2C2	eukaryotic translation initiation factor 2C, 2	miRNA silencing	Savas *et al.*, 2008
	MTSS1	Metastasis supressor 1	Unknown	Goehler *et al.*, 2004
	TUBG1	tubulin, gamma 1	Unknown	Hoffner *et al.*, 2002
	TUBB	tubulin, beta	Microtubule scaffolding	Hoffner *et al.*, 2002
	PCM1	pericentriolar material 1	Anchors mictrotubules to centrosomes	HDBase, 2004a
	UTP14A	UTP14, U3 small nucleolar ribonucleoprotein, homolog A (yeast)	Ribosome biogenesis	Goehler *et al.*, 2004
	XRCC6	X-ray repair complementing defective repair in Chinese hamster cells 6	DNA repair	Goehler *et al.*, 2004
	F8A	coagulation factor VIII-associated (intronic transcript) 1	Unknown	Peters & Ross, 2001
	HSPA4	heat shock 70kDa protein 4	Chaperone protein	Cornett *et al.*, 2005
	FEZ1	fasciculation and elongation protein zeta 1	Axonal guidance	Goehler *et al.*, 2004
	CBS	Cystathionine B-synthase	Generation of cysteine	Boutell *et al.*, 1998

transcription factors to access the DNA and generate mRNA as the cell requires. At the highest level, DNA is folded into either heterochromatin or euchromatin (Fedorova & Zink, 2008). Heterochromatin is a condensed structure that is almost completely inaccessible to transcriptional proteins. Heterochromatin is considered silent and is not transcribed. Euchromatin is a more accessible chromatin structure, and can undergo rearrangements to facilitate or inhibit protein access to DNA. The functional unit of euchromatin is the nucleosome, which is composed of a combination of four histone proteins. Histones can be modified, which alters chromatin structure and influences transcription (Strahl & Allis, 2000; Saha *et al.*, 2006). Nucleosomes can also be physically shifted on the DNA with the use of ATP-dependent histone remodelling proteins (Saha *et al.*, 2006).

Histone monoubiquitination is a more poorly understood histone modification, but one that has been shown to be altered in the presence of mHtt. Monoubiquitination (mUb) of histone H2A was increased in the brains of 8 week-old R6/2 mice relative to wild-type animals (Kim *et al.*, 2008). The altered H2A ubiquitination was not attributable to dysfunction in the proteasomal pathway, but was likely due to an increased interaction between histones and the histone modifier Bmi-1. They showed that mUb H2A association was increased at genes exhibiting decreased transcription in the presence of mHtt in a cell model of HD, as well as in the brains of R6/2 mice. In contrast, mUb of H2B correlates with higher levels of transcription. mUb of H2B was decreased in R6/2 mice, resulting in a decreased presence of these histones at HD-affected genes (Kim *et al.*, 2008). In a separate attempt to correlate histone modifications in HD directly to gene expression changes observed, Anderson *et al.* (2008) mapped the chromosomal location

of genes altered in human HD patients detected using blood samples and post-mortem brain tissue. They showed that changes in gene expression could be related to coordinated repression or activation over large chromosomal areas, which supports the hypothesis that alterations in histone acetylation or methylation cause the gene expression changes seen in HD (Anderson *et al.*, 2008).

Studies in yeast and *Drosophila* models of HD demonstrated that histone deacetylase (HDAC) inhibitors could ameliorate polyQ-mediated toxicity (Hughes *et al.*, 2001; Steffan *et al.*, 2001). Following this finding, various HDAC inhibitors have been tested in animal models of HD. Suberoylanilide hydroxamic acid (SAHA) and sodium butyrate treatment increased histone acetylation and improved motor impairment in the R6/2 mouse model of HD. Sodium butyrate, but not SAHA, decreased striatal neurodegeneration (Ferrante *et al.*, 2003; Hockly *et al.*, 2003). When administered after symptom onset in the N171-89Q mouse model of HD, phenylbutyrate increased histone acetylation, decreased histone methylation, prolonged survival and attenuated brain atrophy but had no effect on motor dysfunction (Gardian *et al.*, 2005). Treatment of R6/2 mice with the HDAC inhibitor 4b following symptom onset led to improved motor function, increased gene expression of a large percentage of a panel of genes known to be affected in HD, and decreased neurodegeneration. Changes in life span were not reported (Thomas *et al.*, 2008). These studies show that histone modifications are altered in the presence of mHtt, that mHtt can directly interact with proteins capable of modifying histones, and that treatments targeting histone modification can ameliorate the HD phenotype.

1.7.b Gene-Specific Transcription Factors

Once nucleosomes have been rearranged, transcriptional activators can bind in a sequence-specific manner and initiate the recruitment of the basal transcription machinery. As these interactions are sequence-specific, the interactions between transcription factors and the promoter provide the greatest level of selective regulation of different genes. Some activators interact directly with the general transcription factor TFIID to initiate assembly of the transcriptional machinery, however in many cases transcriptional activators are aided by a co-activator complex which mediates the assembly of the transcriptional machinery by binding directly to TFIID, TFIIB or PolII (Naar *et al.*, 2001).

Premised on the observation that many of the genes downregulated in HD are controlled by the DNA-binding transcriptional activator Sp1, the role of Sp1 in N-mHtt mediated transcriptional dysregulation has been extensively studied. Co-immunoprecipitation (coIP) was used to show that N-mHtt interacts with Sp1 *in vitro*, in HEK cells transiently expressing N-mHtt, and in human caudate tissue from HD patients (Li *et al.*, 2002; Chen-Plotkin *et al.*, 2006). Chromatin immunoprecipitation (ChIP) has demonstrated that Sp1 occupancy at the N-mHtt-affected Nerve Growth Factor Receptor (NGFR) promoter was decreased in PC12 cells transiently expressing N-mHtt (Li *et al.*, 2002), and at the N-mHtt-affected dopamine D_2 receptor promoter in human HD brains (Chen-Plotkin *et al.*, 2006). These studies led to the hypothesis that N-mHtt binds Sp1 and inhibits its association with Sp1-regulated genes. This theory was contradicted by evidence from electromobility shift assays, which showed that overall Sp1 binding to its recognition sequence in the presence of nuclear proteins in R6/2 mice was actually

increased (Chen-Plotkin *et al.*, 2006). Additionally, reducing levels of Sp1 in R6/2 and N171-87Q mice by breeding them with heterozygous Sp1 knock-out mice decreased neurodegeneration, increased life expectancy and restored dopamine D_2 receptor mRNA levels (Qiu *et al.*, 2006). While these studies indicate that N-mHtt interacts with Sp1 and that levels of Sp1 do not decrease in the presence of N-mHtt, it is unclear how N-mHtt interaction with Sp1 leads to gene-specific transcriptional dysregulation, as many Sp1-regulated genes are not affected in HD. It is also unclear whether increased or decreased Sp1 binding at the DNA mediates transcriptional dysregulation in HD.

The most studied transcriptional repressor in HD is the repressor element silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF). In 2003, it was reported that REST, which is normally restricted to the cytoplasm, is enriched in the nucleus in cells expressing N-mHtt and that transcription of REST-regulated promoters was decreased in the presence of N-mHtt. It was shown that wild-type Htt interacts with REST and that polyQ expansion decreases this interaction (Cattaneo, 2003). It was proposed that the decreased interaction between N-mHtt and REST allows REST to translocate to the nucleus and repress transcription. As demonstrated by ChIP, REST DNA binding is increased, and mRNA levels of REST-regulated genes are decreased in the presence of N-mHtt. Additionally, expression of a dominant-negative REST protein, consisting of only the DNA-binding domain, alleviated N-mHtt-mediated transcriptional repression of the three REST-regulated genes tested (Zuccato *et al.*, 2007). An oligonucleotide decoy for REST reduced REST occupancy, and restored transcription of REST-regulated gene expression (Soldati *et al.*, 2011). One issue with the argument that decreased wild-type Htt binding to REST is responsible for decreased transcription

of REST-regulated genes is that decreased gene expression of REST-regulated genes has been observed in many cellular and animal models of HD that involve overexpression of N-mHtt and no reduction in levels of wild-type Htt (Zuccato *et al.*, 2001; Luthi-Carter *et al.*, 2002; Zuccato *et al.*, 2005; Pang *et al.*, 2006). One study addressed this issue, and claims that increased REST occupancy at affected promoters is not due to decreased N-mHtt/REST interaction in the cytoplasm, but to an Sp1-mediated increase in transcription of REST mRNA and subsequent protein expression (Ravache *et al.*, 2010). They showed that REST mRNA levels increased in the R6/2 mouse model of HD, that transcription of the REST promoter was increased in cells transiently expressing N-mHtt, and that this increase was mediated by Sp1 and Sp3. This experiment fits with the argument presented in the previous paragraph stating that increased Sp1 activity is involved in the pathogenesis of HD. The mechanism of REST-mediated transcriptional dysregulation in HD remains unclear, however most studies agree that REST-mediated gene transcription is decreased in the presence of N-mHtt, and that this results from increased binding of REST to the promoters of affected genes.

1.7.c Co-Activators

Co-activators can assist in initiating transcription by performing one of two functions. Some co-activators open up chromatin folding by means of histone acetyltransferase activity. Others act to bridge gene-specific transcription factors with the general transcription machinery and help stabilize TFIID at the transcription start site (reviewed in Featherstone, 2002). CREB binding protein (CBP) is a transcriptional co-activator that functions both as an acetyltransferase protein and as a bridge to the general

transcription complex (Chan & La Thangue, 2001), and has been shown to interact with mHtt (Steffan *et al.*, 2000).

CBP was detected in NIIs in human HD brain tissue leading to the hypothesis that recruitment of CBP into aggregates leads to impaired CBP-dependent transcription (Nucifora *et al.*, 2001). Two studies have correlated decreased CBP with an increased severity of the disease in mice expressing N-mHtt suggesting a link between CBP activity and symptoms of HD. One study crossed N171-82Q mice with heterozygous knockout CBP mice, and showed that the progeny (N171-82Q/CBP$^{+/-}$) had a decreased life expectancy compared to N171-82Q mice with normal levels of functional CBP (Klevytska *et al.*, 2010). The second study examined single cells transfected with mHtt and showed that CBP levels decreased along with histone acetylation and resulted in cellular toxicity (Jiang *et al.*, 2006).

Other studies have refuted the idea that CBP is sequestered into NIIs and suggest instead that interactions between soluble mHtt and CBP lead to functional changes in CBP activity. The localization of CBP within striatal and cortical nuclei was not different between HdhQ150 or N171-82Q mice and wild-type controls (Yu *et al.*, 2002). NIIs in striatal and cortical nuclei in these animals did not contain CBP, and western blotting confirmed that CBP protein levels were unchanged. Transcription of a CBP-dependent reporter plasmid was decreased in PC12 cells inducibly expressing exon 1 of *huntingtin* with 74 CAG repeats (Cong *et al.*, 2005). An interaction between N-mHtt and CBP was detected at the time when transcription was decreased, however CBP was not detected in N-mHtt aggregates. The interaction between mHtt and CBP occurs within the histone acetyltransferase domain on CBP (Steffan *et al.*, 2001), suggesting that mHtt binding to

CBP would impair its ability to actetylate histones. A separate study, however, reported that transcriptional dysregulation of a CBP-dependent reporter plasmid occured earlier (6 h) than altered histone acetyltransferase activity of CBP (5d) following induction of N-mHtt expression (Cong *et al.*, 2005). Although these studies do not resolve whether lower levels or decreased function of CBP leads to transcriptional dysregulation, N-mHtt interacts with CBP and CBP-dependent transcription is impaired in the progression of HD.

1.7.d General Transcription Machinery

After chromatin has been unfolded, and trans-acting transcription factors have bound along with any required co-activator complexes, the basal transcription machinery must be assembled at the transcription start site. Assembly of the basal transcriptional machinery occurs in a step-wise manner which is nucleated by the binding of TFIID (reviewed in Orphanides *et al.*, 1996; Hampsey, 1998). TFIID recruits TFIIA, which stabilizes the interaction between TFIID, the DNA, and any associated co-activators. TFIID also recruits TFIIB, which is essential for the subsequent recruitment of the TFIIF/PolII complex and its placement at the transcription start site. TFIIF is composed of two subunits, RAP30 and RAP74. TFIIF chaperones PolII within the nucleus, binds to the DNA with the aid of TFIIB, and stabilizes the preinitiation complex (PIC). TFIIF is also thought to increase the efficiency of transcriptional elongation by reducing PolII pausing following the initiation of transcription. Following the incorporation of TFIIF and PolII to the promoter, TFIIE is recruited. TFIIE acts as a scaffold for TFIIH, and also stimulates TFIIH activity. TFIIH phosphorylates the C-terminal tail of PolII, and

possesses helicase activity that allows it to unwind the DNA so that PolII can move along the DNA in the 3' direction. At this point, the structure is considered to be a fully formed PIC. The transcriptional machinery has been isolated as an intact complex. This intact PIC was termed a holoenzyme complex. There has been evidence that both the sequential assembly pathway and the holoenzyme pathway are utilized *in vivo* (Thomas & Chiang, 2006).

The general transcription factor TFIID is composed of TBP and a complement of general TBP-associated factors (TAFs). Staining of cortical tissue of human HD patients showed that TBP accumulates in insoluble aggregates in the cytoplasm (van Roon-Mom *et al.*, 2002). Experiments performed using cortical tissue from N171-82Q mice, and striatal tissue from R6/2 mice revealed normal distribution of TBP compared to wild-type mice, and TBP was not detected in N-mHtt aggregates (Yu *et al.*, 2002). A direct interaction between TBP and N-mHtt has been demonstrated using coIP *in vitro*, where it was determined that N-mHtt can recruit TBP into N-mHtt aggregates. It was determined that an interaction between soluble N-mHtt and TBP mediated this recruitment (Schaffar *et al.*, 2004). TBP function is inhibited by N-mHtt *in vitro* by restricting TBP binding to DNA. Using an *in vitro* transcription assay containing purified transcription factors it was shown that N-mHtt-mediated transcriptional repression of an Sp1-dependent promoter could be recovered by increasing the presence of TFIID in the assay (Zhai *et al.*, 2005). The fact that TBP binding to DNA is decreased in the presence of N-mHtt and that transcriptional repression could be recovered using excess TFIID seems to suggest that the N-mHtt/TBP interaction results in decreased function of TFIID. Whether this functional inhibition results from sequestration of TBP into insoluble

aggregates, if N-mHtt binding to TBP alone is sufficient to inhibit function, or if both occur, remains unclear.

Using the *in vitro* transcription assay described in the previous paragraph, it was also shown that N-mHtt-mediated repression of Sp1-dependent transcription could be alleviated with the addition of excess TFIIF (Zhai *et al.*, 2005). They showed that N-mHtt directly interacted with the RAP30 subunit of TFIIF, specifically in the region between aas 40 to 60 of the protein, which is the region of RAP30 used to associate with RAP74. They determined that overexpression of RAP30 in primary striatal cells transiently expressing N-mHtt alleviated transcriptional repression of the dopamine D_2 receptor promoter, and decreased cellular toxicity. Lastly, using ChIP they showed that RAP30 binding to the D_2 promoter was decreased in R6/2 mice compared to wild-type controls. From this experiment it was hypothesized that in binding to RAP30, N-mHtt inhibits the formation of TFIIF which decreases the amount of TFIIF available for formation of transcriptional complexes.

It is unlikely that any one of these theories provides the answer for all of the dysregulation in gene expression in HD. N-mHtt interacts with a large number of proteins, and each of those interactions could have a functional outcome. Some important questions that must still be answered are how the mechanism of N-mHtt-mediated transcriptional dysregulation differs for different genes, and why interaction with a specific protein does not impair transcription of all genes regulated by that protein.

1.8 Overall Objectives

The overall goal of my research was to contribute to our understanding of the mechanism of N-mHtt-mediated transcriptional dysregulation, which occurs to the greatest extent in the cellular population that experiences the highest rates of neuronal dysfunction and neurodegeneration in HD. Although changes occur in many areas of cellular function during the progression of HD, transcriptional dysregulation is among the earliest detectable changes and HD-related changes in other aspects of cellular function may originate with altered gene expression. It is known that levels of soluble N-mHtt in the nucleus correlate with the severity of HD (Peters *et al.*, 1999; Benn *et al.*, 2005). Importantly, decreased cleavage of mHtt eliminates neurodegeneration and reduces motor dysfunction (Graham *et al.*, 2006), whereas increased translocation of N-mHtt to the nucleus increases the toxicity in cellular models of HD (Peters *et al.*, 1999). As early alterations in transcription may precipitate other dysfunctions within the cell, understanding the interaction between nuclear N-mHtt and factors that control transcription may slow or even stop the progression of HD.

I specifically chose to determine if 1) N-mHtt can directly decrease transcription independent of chromatin folding or higher-ordered DNA structure, 2) N-mHtt can impair transcription independent of *huntingtin* mRNA, 3) N-mHtt is able to repress transcription independent of intercellular signaling, 4) if N-mHtt affects transcription driven by the human immediate-early cytomegalovirus (CMV) and herpes simian virus thymidine kinase (TK) promoters, 5) transcriptional repression of a specific promoter is dependent on gene-specific DNA binding elements, 6) increased expression of known N-mHtt-interacting transcription factors can restore transcription, 7) N-mHtt sequesters

transcription factors from the promoter, or if N-mHtt is recruited to the transcriptional

machinery, 8) mHtt expression levels influence transcriptional dysregulation, or the

ability to recover transcriptional dysregulation. In formulating and performing

experiments to achieve these research objectives, my aim was to determine which, if any,

of the current models of N-mHtt-mediated transcriptional dysregulation are applicable to

transcriptional dysregulation of both the CMV and TK promoters.

Chapter 2:

Materials and Methods

2.1 *In Vitro* Transcription Assay

2.1.a *Preparation of Recombinant N-mHtt*

Recombinant N-mHtt was prepared using pET-N171-23Q and pET-N171-89Q plasmids, which contained cDNA encoding the 171 N-terminal aas of human Htt with 23 and 89 glutamines in the N-terminal polyQ repeat region, respectively. Plasmids were electroporated into electrocompetent BL-21 *E. coli* cells and grown on LB plates [(20 g/L LB Broth (Sigma), 15 g/L Bacto Agar (BD)] containing 50 μg/ml kanamycin (Sigma). Individual clones of pET-N171-23Q and pET-N171-89Q cells were grown overnight in 2xYT broth [31 g/L 2xYT Microbial Medium (Sigma)] containing 50 μg/ml kanamycin at 37°C shaking at 250 rpm. In the morning, 50 μl of each overnight culture was inoculated into 50 ml of 2xYT broth containing 50 μg/ml kanamycin. The cells were allowed to grow at 37°C shaking at 250 rpm until the absorbance at $\lambda 600$ was between 0.6 and 0.8. Isopropyl β-$_D$-1 thiogalactopyranoside (Invitrogen) was added to a final concentration of 1 mM and allowed to grow at 37°C and shaken at 250 rpm for 2 h. The cultures were subjected to centrifugation at 4000 x *g* for 5 m and the supernatant was discarded. The cell pellets were resuspended in 8 ml of binding buffer [5 mM imidazole (Sigma), 500 mM NaCl (Sigma), 20 mM Tris-HCl (pH 8.0) (Sigma), 25 mM β-mercaptoethanol (BioRad), 0.1% (v/v) NP-40 (Sigma), 1 mM PMSF (Sigma)]. Lyzozyme (8 mg) (Sigma) was added to the solution and the tubes were incubated on ice for 30 m. The cells were sonicated for 6 x 10 s pulses with 10 s between each pulse at a

Tip Limit of 6 using a Sonifier 250 (Branson). The lysates were passed through an 18 G

needle 4 times. The homogenate was subjected to centrifugation at $1.88 \times 10^4 \times g$ for 30

m at 4°C. The supernatant containing recombinant protein was removed and stored on

ice prior to purification.

To purify the recombinant protein, Nickel-NTA columns were prepared by

adding 1.5 ml of Ni-NTA agarose (Invitrogen) into 10 ml Poly-Prep™ Chromatography

Columns (BioRad) and subjected to centrifugation at 850 x g for 1 m. The supernatant

was removed and the Ni-NTA resin was resuspended in 6 ml of H_2O. The tubes were

subjected to centrifugation for 1 m at 850 x g and the supernatant was removed and

discarded. The Ni-NTA resin was washed 3 times with 6 ml of binding buffer, with a 1

m centrifugation at 850 x g in between each wash. The N171-23Q- and N171-89Q-

containing supernatants were added to the prepared Ni-NTA columns and incubated at

room temperature for 1 h while rotating. The columns were subjected to centrifugation at

850 x g for 1 m. The supernatant was removed and stored, and the resin was washed

three times with 8 ml wash buffer [50 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl

(pH 8.0), 25 mM β-mercaptoethanol, 0.1% (v/v) NP-40, 1 mM PMSF]. The supernatant

at the end of each wash step was stored for future analysis to ensure that Ni-bound

recombinant protein was not lost during the wash steps. The remaining Ni-bound protein

was eluted in 5 ml elution buffer [400 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl

(pH 8.0), 25 mM β-mercaptoethanol, 0.1% (v/v) NP-40, 1 mM PMSF] and collected as 1

ml fractions. The first two 1 ml elutions, which contained the highest concentration of

recombinant protein, were desalted using Zeba Desalt Spin Columns (Pierce) according

to manufacturer instructions and stored at -80°C.

Recombinant N171-23Q and N171-89Q were quantified following sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of recombinant proteins and silver staining. A 2 μl volume of each desalted protein fraction was fractionated alongside known concentrations of bovine serum albumin (BSA) on a 7.5% gel in 1x running buffer [25 mM Tris-HCl, 192 mM glycine (MP Biomedicals) and 0.1% (w/v) SDS (Fisher Scientific)]. The gel was fractionated for 20 m using 90 V of force, and then the voltage was increased to 125 V until the dye front reached the bottom of the gel. To visualize the fractionated proteins, the gel was subjected to silver staining (Switzer *et al.*, 1979). The gel was soaked while shaking in 50% (v/v) methanol (Fisher Scientific) overnight. The following day the gel was rehydrated in RNase-free H_2O for 2 x 30 m washes. The gel was dehydrated in a 1 h wash in 50% (v/v) methanol. After the 1 h wash, the gel was rinsed with RNase-free H_2O. The gel was soaked in staining solution [19 mM NaOH (BDH), 207 mM NH_4OH (Sigma), 0.4 mM $AgNO_3$ (Sigma)] for 90 m. The gel was washed twice in RNase-free H_2O for 5 m. The gel was soaked in developing solution [0.005% (w/v) citric acid (Sigma) and 0.019% (v/v) formaldehyde (Fisher Scientific)] until bands on the gel were visible. The reaction was terminated by placing the gel in stop solution [10% (v/v) acetic acid (Fisher Scientific), 45% (v/v) methanol]. A standard curve of optical density versus amounts of BSA standards was constructed and used to determine the concentration of recombinant N171-23Q and N17189Q present. In addition, the relative purity of each sample was judged by the number and abundance of proteins observed in each sample.

2.1.b Preparation of Promoter Template

The positive control provided by Promega with the *in vitro* transcription assay consisted of the 825 bp of the CMV promoter 5' to the transcription start site and a 363 bp coding sequence. The 1188 bp fragment had previously been digested from the 3.8 kb pMK11 plasmid and inserted into pUC18 plasmid using *EcoR*I and *BamH*I restriction enzymes. Linear templates for *in vitro* transcription assays were produced by restriction digest of the CMV promoter from pUC18-CMV plasmid according to Double-Digest™ protocols (Fermentas) for the specific combination of enzymes. The 3' end of the DNA template was released from the plasmid using *Bam*HI. The 5' end of -825, -223 and -68 bp CMV promoter fragments were released by digestion using *EcoR*I, *Nco*I, and *Hinc*II, respectively. Reactions were incubated at 37°C overnight. Digestion reactions were resolved on an agarose gel and gel extracted using the GenElute™ Gel Extraction Kit (Sigma).   The product was ethanol precipitated by adding 1/10 volume of 3 M sodium acetate [pH 5.2 (Sigma)] and 2x volumes of 100% ethanol (Fisher Scientific). The sample was incubated on ice for 15 m and subjected to centrifugation at $1.6 \times 10^{4} \times g$ for 20 m at 4°C. The supernatant was discarded and the pellet was rinsed gently with ice cold 75% ethanol and subjected to centrifugation at $1.6 \times 10^{4} \times g$ for 5 m at 4°C. The wash step was repeated once more.   Trace amounts of ethanol wash were removed using a 1 ml syringe and a 30 G needle. The tube was incubated at room temperature with the lid open for 10 m to allow for evaporation of any remaining supernatant and then the pellet was resuspended in 1x TE buffer (Sigma). The CMV promoter fragments were quantified on an agarose gel using MassRuler™ Express Forward DNA Ladder Mix (Fermentas). A standard curve was created by plotting the intensity of the bands in the

ladder mix, as determined using Kodak 1D imaging software, against their known masses. The mass of the CMV promoter fragments loaded on the agarose gel was determined by extrapolation from the standard curve based on the band intensity of the restriction fragments.

2.1.c In Vitro *Transcription Assay*

In vitro transcription reactions were prepared by combining DNA template (126 fmol), recombinant N171-23Q or N171-89Q (1 pmol), MgCl₂ [6 mM (Promega)], HeLa Nuclear Extract [8 U (Promega)], and transcription buffer [20 mM HEPES (pH 7.9) (Sigma)], 100 mM KCl (BDH Chemicals), 200 μM EDTA (Fluka), 500 μM DTT (Sigma), and 20% (v/v) glycerol (Sigma)] in a 23 μl reaction. The reaction was preincubated for 30 m at 30°C. Two μl of rNTP mix consisting of 400 μM each of rCTP, rATP, and rUTP, 16 μM rGTP (rNTPs from Promega), and 130 nM α-^{32}P rGTP [10 μCi specific activity (Perkin-Elmer)] was added to the reaction. The reaction was incubated at 30°C for 30 m and transcription was halted with the addition of 175 μl of stop solution [300 μM Tris-HCl [pH 7.4 (Sigma)], 300 μM sodium acetate (Sigma), 0.5% (w/v) SDS, 2 mM EDTA, 3 μg/ml tRNA (Invitrogen)].

The RNA generated in *in vitro* reactions was purified by phenol:chloroform extraction. Phenol:chloroform:isoamyl alcohol (25:24:1) (Invitrogen) was added at a volume equal to the reaction product and the stop solution. The tubes were vortexed for 30 s and subjected to centrifugation at 1.28 x 10^4 x *g* for for 6 m. The upper aqueous phase was removed and placed in a new eppendorf tube. Ethanol was added to a final concentration of 33% and incubated overnight at -20°C. The following day, the solution

was subjected to centrifugation at 1.6×10^4 x g for 20 m. The supernatant was discarded, and the pellet was rinsed in a volume of 75% ethanol equal to the volume of ethanol used the previous day. The tube was subjected to centrifugation at 1.6×10^4 x g for 5 m. The supernatant was discarded, and the tube was pulse spun to collect any remaining supernatant at the bottom of the tube. The remaining supernatant was collected using a syringe and 30 G needle and the tube was left open to the air for 5 m to evaporate any remaining ethanol.   The RNA was resuspended in loading dye [98% (v/v) formamide (Fisher Scientific), 10 mM EDTA, 0.1% (w/v) xylene cyanol (Sigma), 0.1% (w/v) bromophenol blue (Sigma)]. Samples were resolved on a 4% polyacrylamide/8 M urea gel for 2.5 h at 50°C, using 2.5×10^3 V of force, and 125 W of power. The gel was transferred to a piece of filter paper (Whatman) and dried for 5 m at 50°C. The gel was rinsed with H_2O to remove the crystallized urea from the gel, which was heated again at 50°C until dry. The gel was exposed to a storage phosphor screen (Amersham Biosciences) overnight in an exposure cassette and developed using the STORM imaging system.

2.2   Cell Culture Manipulations

2.2.a Cell Growth Conditions

Immortalized striatal cells from embryonic day 14 rats (ST14A), as well as derivatives stably expressing the N-terminal 548 aas of human Htt with 15 (N548wt), or 128 (N548hd) glutamine repeats (gifts from Dr. Elena Cattaneo), were cultured at the permissive temperature of 33°C with 5% CO_2 and 95% air. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with sodium

pyruvate (0.11 g/L) (Gibco), glutamine (0.29 g/L) (Gibco), HEPES (238.3 g/L) (Gibco), penicillin-streptomycin (10,000 U) (Gibco) and 10% (v/v) fetal bovine serum (FBS) (Gibco). Between passages, cells were washed in Hank's Balanced Salt Solution (HBSS) (Gibco) supplemented with sodium pyruvate (0.11 g/L), sodium bicarbonate (3.7 g/L) (Gibco), penicillin-streptomicin (10,000 U) and HEPES (238.3 g/L). Cells were removed from plates using TrypLE (Gibco). Cells were not used after passage number 10.

StHdh Q7/7, Q7/111 and Q111/111 cells were purchased from the Coriell Institute for Medical Research. Cells were cultured at the permissive temperature of 33°C with 5% CO_2 and 95% air. Cells were initially grown in DMEM supplemented with glutamine (0.29 g/L), penicillin-streptomycin (10,000 U) and 10% (v/v) FBS. Twenty-four hours after reviving frozen cells, the media was replaced with growth media described above supplemented with 400 µg/ml geniticin (Invitrogen). Between passages, cells were washed in 1x PBS Solution (Sigma). Cells were removed from plates using TrypLE. Cells were considered to be at passage number 7 following revival, as indicated by the Coriell Institute, and were used until passage 14.

Prior to seeding for experiments, cells were counted using a hemocytometer. An aliquot of cells was diluted 1:1 with 0.4% (w/v) Trypan Blue Solution (Sigma). The solution was pipetted onto the hemocytometer and the number of cells in each of four 1 mm^2 squares was averaged. The volume in each 1 mm^2 square was 100 nl given a depth of 100 µm between the cover slip and the counting surface. The concentration of cells was obtained by dividing the average number of cells per square by the volume of the square (100 nl) and multiplying by the dilution factor. This concentration was converted to cells per ml by multiplying by the conversion factor 1 x 10^4.

2.2.b Preparation of Cell Culture Plates

All cells were grown on poly-D-lysine coated plates. Cell culture plates were incubated in 0.01% (w/v) poly-D-lysine (Sigma) for 1 h at 37°C. Each well was washed twice with 1x PBS and the plates were left uncovered in the cell culture hood overnight to dry. Plates were stored at 4°C prior to use. If coverslips were required for an experiment, a prepared coverslip was placed into each well of a 4-well plate prior to poly-D-lysine coating.

2.3 Western Blotting

2.3.a Collection of Cell Lysates

ST14A, N548wt, and N548hd cells were grown to confluence in a 25 cm^2 flask. Cells were washed with 5 ml of 1x PBS solution, scraped into 2 ml of 1x PBS solution using a cell-scraper, and collected into a 15 ml conical falcon tube. The tubes were subjected to centrifugation at 100 x g for 3 m and the supernatant was discarded. The pellet was resuspended in 1 ml of 1x PBS buffer and transferred to a 1.5 ml eppendorf tube. The cell pellets were collected after centrifugation at 100 x g for 3 m. The supernatant was discarded. The pellet was resuspended in lysis buffer [10 mM Tris-HCl (pH 8.0), 140 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100 (Sigma), 0.1% (w/v) sodium deoxicolate (Sigma), 1 mM PMSF, Complete Mini Tab Protease Inhibitor Cocktail (Roche)] and stored at -80°C. Protein concentration was determined using the Protein Assay Dye-Reagent (Bio-Rad) according to the microassay procedure using 2 µl of each sample, and known amounts of BSA to create a standard curve. Samples were

analyzed in duplicate and averaged. Readings were taken using an EL_X-800 Universal Microplate Reader (Bio-Tek) at the 595 nm wavelength. The mass of protein in the unknown sample was determined by extrapolating from the standard curve using the light emission detected at 595 nm.

2.3.b Protein Fractionation & Immunodetection

Proteins were fractionated using SDS-PAGE on a 7.5% gel as described in section 2.1.a. Following SDS-PAGE, fractionated protein was transferred to polyvinylidene fluoride (PVDF) membrane (Fisher Scientific) on ice in 1x transfer buffer [124 mM Tris Base (Roche), 1 M glycine, 20% (v/v) methanol] for 2 h at 100 V of force. The membrane was blocked in 5% skim milk diluted in 1x TBS-T [500 mM NaCl, 20 mM Tris-HCl, 0.1% Tween-20 (Sigma)] for 1 h with shaking. Following blocking the membrane was incubated with primary antibody (Table 2.1) in blocking buffer overnight at 4°C with shaking. The following day the membrane was washed 5 x 10 m in 1x TBS-T. The membrane was then incubated in horseradish peroxidase (HRP)-conjugated secondary antibody diluted in blocking buffer for 2 h with shaking. The membrane was washed 5 x 10 m in 1x TBS-T. Protein-bound primary antibody on the membrane was detected using ECL Plus™ kit (Amersham) as directed. Signal was detected using the STORM imaging system. Following primary antibody detection the membranes were stained using amido black to verify that lanes contained equal amounts of total protein. Membranes were incubated in amido black staining solution [0.1% (w/v) amido black (Sigma), 11% (v/v) acetic acid (Fisher Scientific), 55% (v/v) ethanol] for 10 m. The

Table 2.1. Antibodies used for western blotting and immunoprecipitation

Protein	Antibody	Company	Dilution
Htt	MAB2166	Millipore	1/2000
RAP30	ab28180	Abcam	1/2000
RAP74	ab28179	Abcam	1/2000

membrane was then destained in 3 x 5 m washes in destain solution [90% (v/v) ethanol, 7% (v/v) acetic acid].

2.4 Quantification of Cell Number and Transfection Efficiency

Cells were plated (5 x 10^4 cells per well) onto poly-D-lysine-coated coverslips in 4-well plates (Nunc) and grown overnight at 33°C. The following morning the cells were washed twice with 500 μl of serum-free media (SFM) [DMEM supplemented with sodium pyruvate (0.11 g/L), glutamine (0.29 g/L), HEPES (238.3 g/L)]. pEGFP-N1 plasmid DNA [100 ng (BD Biosciences)] was combined with 4 μl PLUS reagent (Invitrogen) and SFM to a volume of 25 μl per well. After a 15 m incubation at room temperature, 1 μl (per well) of Lipofectamine 2000 (Invitrogen) and 24 μl (per well) of SFM were added to the reaction tube. The mixture was incubated for 15 m at room temperature and then added to cells at 50 μl per well. Cells were incubated at 33°C for 4 h when 40 μl of FBS and 160 μl SFM were added to each well. The following day, the cells were washed with 500 μl of 1x PBS and incubated at room temperature for 10 m in 500 μl of filtered 4% paraformaldehyde (PFA) (Marivac) solution diluted in 100 mM NaPO$_4$ buffer [pH 7.4 (Sigma)]. The PFA solution was removed and the coverslips were washed 3 x 5 m in 1x PBS. PFA-fixed cells were incubated in 500 μl of 2 ng/ml Hoechst stain solution (Sigma) diluted in 1x PBS for 15 m at room temperature. Coverslips were washed 3 x 5 m in 1x PBS.

To select a representative sample of each coverslip for quantification, four different locations on each coverslip were photographed at 400x magnification. Locations were chosen on the radii of each coverslip extending to 0°, 90°, 180° and 270°.

Each location was photographed under both ultraviolet (UV) light and green fluorescence. The number of Hoechst-positive and GFP-positive cells in each photograph were summed. The number of GFP-positive cells was divided by the number of Hoechst-positive cells for each coverslip to calculate transfection efficiency. Eight coverslips per cell-type were analyzed. In performing all experiments involving cell lines, n represents independent biological replicates as opposed to independent technical replicates. For each replicate, individual cell counts were performed and individual transfection mixes were prepared. A one-way ANOVA followed by Bonferroni post-hoc testing was performed to determine statistical significance between ST14A, N548wt and N548hd cells with regards to total number of cells present, as well as transfection efficiency.

2.5 Quantification of Total Protein

ST14A, N548wt and N548hd cells were grown on poly-D-lysine-coated 4-well plates (5×10^4 cells per well) and grown overnight at 33°C. The following day the cells were transfected according to the transfection protocol described in section 2.4. Cells were washed with 500 μl of 1x PBS (Sigma) and incubated in 100 μl of 1x Passive Lysis Buffer for 15 m at room temperature on a shaker. Cells were scraped and collected into 0.5 ml eppendorf tubes. Samples were frozen and thawed 3x in a dry ice/ethanol bath and subjected to centrifugation at 1.6×10^4 x g for 30 s at 4°C to collect cell debris at the bottom of each tube. Quantification of the protein in the supernatant was then performed using Protein Assay Dye-Reagent (Bio-Rad) as described in section 2.3.a.

2.6 Preparation of Plasmids for Promoter Activity and Overexpression Experiments

2.6.a Preparation of Electrocompetent InvF' E. coli

InvF' *E. coli* was grown on LB agar plates and incubated overnight at 37°C. The following day, a colony was selected and grown in 25 ml of 2xYT broth and grown overnight at 37°C shaking at 250 rpm. The following morning, 5 ml of the overnight culture was subcultured in 500 ml of 2xYT broth. The 500 ml culture was grown at 37°C shaking at 250 rpm until it had reached an absorbance reading between 0.5-1.0 at λ600. The flask was chilled on ice for 30 m, and the contents were divided into 4 centrifuge bottles. The bottles were subjected to centrifugation at 4000 x *g* for 15 m at 4°C and the supernatant was discarded. The pellet in each bottle was resuspended in 250 ml of 1 mM HEPES [pH 7.0 (Sigma)], subjected to centrifugation at 4000 x *g* for 15 m at 4°C, and the supernatant was discarded. The pellet in each bottle was resuspended in 125 ml of 1 mM HEPES (pH 7.0) and the contents were combined into 2 bottles. The bottles were subjected to centrifugation at 4000 x *g* for 15 m at 4°C and the supernatant was discarded. The pellet in each bottle was resuspended in 125 ml of 1 mM HEPES (pH 7.0) and the contents were combined into 1 bottle. The bottle was subjected to centrifugation at 4000 x *g* for 15 m at 4°C and the supernatant was discarded. The pellet was resuspended in 20 ml of 1% glycerol. The bottle was subjected to centrifugation at 4000 x *g* for 15 m at 4°C and the supernatant was discarded. The pellet was resuspended in 3 ml of 1% glycerol and divided into 40 μl aliquots. The cells were stored at -80°C. For each batch of cells prepared, the number of transformants per microgram of pUC18 DNA was determined to quantify viability and transfection efficiency of the cells.

2.6.b Generation of CMV Promoter Fragments

Polymerase chain reaction (PCR) was performed to generate the promoter sequences for cloning. pCMV-luc plasmid, which contained the CMV promoter, was used as template for the PCR reaction. The CMV For, -297 CMV, and -99 CMV sense primers in conjunction with the CMV Rev antisense primer (Table 2.2) were used to generate CMV promoter fragments spanning the 779, 297 and 99 bp 5' to the transcription start site (-779 CMV, -297 CMV, and -99 CMV, respectively). Sense and antisense primer (500 nM) were combined with 100 ng DNA template, 500 μM dNTP mix (dATP, dGTP, dTTP, dCTP nucleotide mixes from Fermentas), 1x PCR Buffer, 1x Q Solution, and 2 U Hot Start Taq Polymerase Enzyme (Buffers and Taq contained in Qiagen Hot Start Taq PCR Kit). The PCR reaction conditions consisted of a 15 m incubation at 95°C followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 m. The reaction was completed with a 10 m extension at 72°C. The PCR reaction products were resolved in agarose gels in 0.5 x TBE [44.5 mM Tris, 44.5 mM Boric acid, 1 mM EDTA and 0.25 μg/ml ethidium bromide] and visualized under UV illumination. The desired PCR product was gel extracted using GenElute™ Gel Extraction Kit (Sigma). The resulting sample was ethanol precipitated as described in section 2.1.b and resuspended in 8 μl of H_2O.

2.6.c Cloning

The ethanol precipitated/resuspended PCR product was ligated into the pGEM®-T vector. Resuspended PCR product (7 μl) was combined with 50 ng of

Table 2.2 - Primers used for PCR and qPCR. * D = Deletions; M = Mutations; IV = *in vitro* Transcription Assay; P = *in vitro* Promoter Binding

Primer Name	Orientation	Sequence	Uses *
CMV For	sense	ctcgagccagtgccaagctgat	D, M, IV
-297 CMV	sense	gcccagtacatgaccttacggg	D, IVP
-99 CMV	sense	aaatgtcgtaataaccccgccc	D
Luciferase	antisense	caggaaccagggcgtatc	D, M
CMV Rev	antisense	cacaggacgggtgtggtc	IV
-278/266	sense	acgacgtacgaagccttggcagtacatct	D
-278/266	antisense	gcttcgtacgtcgtaaggtcatgtac	D
-172/161	sense	acgacgtacgaagcgagtttgttttggcacca	D
-172/161	antisense	gcttcgtacgtcgtggggtggagacttgga	D
-149/136	sense	acgacgtacgaagcgtctccaccccatt	D
-149/136	antisense	gcttcgtacgtcgtgagtcaaaccgctat	D
-109/98	sense	acgacgtacgaagcaatgtcgtaataacc	D
-109/98	antisense	gcttcgtacgtcgttgattttggtgccaaaa	D
-109/71	antisense	gcttcgtacgtcgtgttattacgacattttg	D
-38/20	sense	acgacgtacgaagcgctcgtttagtgaaccgtcaga	D
-38/20	antisense	gcttcgtacgtcgtcctcccaccgtacacgccta	D
-14/1	sense	acgacgtacgaagctcagatctggtacccag	D
-14/1	antisense	gcttcgtacgtcgtctaaacgagctctgctta	D
Luc ChIP For	sense	ctaccgtggtgttcgtttcc	Q
Luc ChIP Rev	antisense	accgggaggtagatgagatg	Q
Biotin Luc Rev	antisense	ctttatgttttggcgtcttcc	P

pGEM-T Vector, 1x T4 Ligation Buffer and 3 U T4 Ligase (all from Promega) and incubated overnight at 4°C. The ligation reaction was ethanol precipitated as described in section 2.1.b and resuspended in 5 µl of H$_2$O. The 5 µl pGEM-T/insert ligation was electroporated into InvF' electrocompetent *E. coli* cells. A 500 µl culture (in 2xYT broth) of electroporated cells was grown at 37°C in an incubator shaking at 250 rpm for 1 h. At the end of 60 m, 100 and 200 µl volumes of the culture were spread on LB plates containing 50 µg/ml carbenicillin (Sigma) and 25 mg/ml X-gal (Promega). Plates were incubated overnight at 37°C. pGEM-T colonies positive for insert presence were selected based on X-gal blue/white screening. Colonies were picked and inoculated in 3 ml of 2xYT broth containing 50 µg/ml carbenicillin. Cultures were grown overnight at 37°C shaking at 250 rpm. The following day, plasmids were isolated using GenElute^{TM} Plasmid Miniprep Kit (Sigma) according to the kit protocol.

Ligation using the pGEM-T vector system could result in DNA insertion in multiple orientations. The orientation of the DNA insert was determined by restriction digest using a restriction enzyme that cut within the DNA insert and an enzyme that cut within the pGEM-T vector. pGEMT-T plasmids containing an insert in the desired orientation were digested with *Mlu*I (Fermentas) and *Bgl*II (Fermentas) to release the promoter fragment. Digestion reactions were carried out in 1x Fast Digest Green Buffer (Fermentas) using 1 FDU each of *MluI* and *BglII* in a 10 µl reaction volume. The tubes were incubated at 37°C for 30 m. Digestion products were resolved on an agarose gel, excised, gel purified, ethanol precipitated, and resuspended as described above. Resuspended promoter product was quantified using agarose gel electrophoresis as described in section 2.1.b and the promoter was ligated into *Mlu*I/*Bgl*II digested pGL3-

Basic vector in a 5:1 molar ratio using T4 ligase (Promega) in 1x T4 ligase buffer (Promega). The ligation reaction was incubated overnight at 4°C. The ligation reaction was ethanol precipitated, resuspended and electroporated into InvF' electrocompetent *E. coli* cells as described above. Individual colonies were selected and grown overnight in 2xYT broth containing 50 μg/ml carbenicillin. Plasmid was isolated from the overnight cultures using the GenElute™ Plasmid Miniprep kit as per kit protocol. Isolated plasmids were digested with *Mlu*I/*Bgl*II to verify insert presence and size. All plasmids were sequenced by The Center for Applied Genomics (www.tcag.ca) prior to use to verify the insert sequence.

Plasmids used in this study that were not generated in the lab include pEGFP-N1 (U55762)(BD Biosciences), pRL-TK (AF025846)(Promega), pET-N171-23Q and pET-N171-89Q (gifts from Dr. Xiao-Jiang Li)(Li *et al.*, 2002), pCMV-luc (a gift from Dr. Mark Nachtigal), pCDNA-RAP30 and pCDNA-RAP74 (gifts from Dr. Dimitrius Kranic) and pProEx-Hta-TBP (a gift from Dr. Ulrich Hartl)(Schaffar *et al.*, 2004).

2.7 Promoter Activity Assays

Cells were grown and transfected as described in section 2.4. Twenty-four hours after transfection, promoter activity was measured using the DLR™ Assay System (Promega). RNase-free conditions were maintained throughout the procedure. Cells were collected and prepared as described in section 2.5. Firefly luciferase activity was measured by adding 10 μl of soluble sample to 50 μl of Luciferase Assay Substrate and quantifying the amount of light produced in the enzymatic reaction using a 20/20^n luminometer (Turner Biosystems). Following quantification of firefly luciferase activity,

50 μl of Stop & Glo reagent was added to the reaction and *Renilla* luciferase activity was measured using the 20/20^n luminometer. Total protein was quantified following the dual reporter assay as described in Section 2.5 and firefly luciferase activity and *Renilla* luciferase activity values were normalized to total protein.

2.8   Quantification of Luciferase mRNA

2.8.a RNA Extraction

Following the dual reporter assay and protein quantification, 200 μl of TRIzol® (Invitrogen) was added to each of the sample tubes. The tubes were vortexed and incubated at room temperature for 5 m, and 40 μl of chloroform (Invitrogen) was added to the samples. The tubes were shaken gently and left to incubate at room temperature for 3 m. The tubes were subjected to centrifugation at 1.2 x 10^4 x *g* for 15 m at 4°C. The supernatants were placed in fresh eppendorf tubes along with 100 μl of isopropyl alcohol and incubated at room temperature for 10 m. The samples were subjected to centrifugation at 1.2 x 10^4 x *g* for 10 m at 4°C, and the supernatants were removed and discarded.   The pellets were washed with 200 μl of 75% ethanol, the tubes were subjected to centrifugation at 1.2 x 10^4 x *g* for 5 m at 4°C, and the supernatants were discarded. The tubes were pulse-spun and the remaining supernatant was removed using a 1 mL syringe and a 30 G needle. The tubes were left open to the air for 2 m to allow any remaining ethanol to evaporate. The pellets were resuspended in 10 μl of H$_2$O and incubated at 55°C for 10 m. Following recovery of RNA, DNase I digestion was performed to eliminate all contaminating CMV reporter plasmid DNA present in the sample. Plasmid DNA was digested using 1 U of RQ1 RNase-Free DNase (Promega) in

1x reaction buffer (Promega) in a 10 μl reaction volume. Following a 10 m incubation at 37 °C, the enzyme was heat inactivated via addition of 1 μl of RQ1 DNase Stop Solution (Promega) and a 10 m incubation at 65 °C.

2.8.b Reverse Transcription

RNA was quantified using spectrophotometry. cDNA was prepared by combining 500 ng of total RNA with 500 ng Oligo(dT)$_{12-18}$ Primer (Invitrogen) and 10 mM dNTP mix in a 13 μl reaction. The samples were heated at 65°C for 5 m and then incubated on ice for a minimum of 1 m. Each tube was split into equal aliquots, one was used to produce the cDNA and one was used as a 'minus' reverse transcriptase (RT) negative control. Reactions were performed according to the Superscript III Reverse Transcriptase (Invitrogen) protocol, in 1x First-Strand buffer, 50 mM DTT, and 20 U RNase OUT (Invitrogen). To the +RT tubes, 100 U Superscript III was added. An equal volume of H$_2$O was added to the -RT samples. The sample tubes were incubated at 50°C for 1 h and then at 70°C for 15 m to inactivate the enzyme. Sample tubes were stored at -20°C.

2.8.c. Quantitative PCR (qPCR)

The qPCR reactions were performed using the LightCycler® DNA Master SYBR Green I kit (Roche) using 2 μl of RT reaction, 4 mM MgCl$_2$, and 500 nM primer mix (indicated in Table 2.2). The reactions were carried out for 45 cycles of 10 s at 95°C, 5 s at 58°C and 10 s at 72°C in a LightCycler (Roche). Once the reaction was completed, the samples were separated on an agarose gel and visualized using UV

illumination. The PCR product was excised and recovered as described in section 2.6.b.

The concentration of recovered qPCR product (in copies/μl) was determined using F1

fluorimetry and a series of λ DNA standards. A dilution series was created using known

copy numbers of the desired qPCR product. For future qPCR reactions, 2 μl of unknown

cDNA or -RT sample was used in the PCR reaction and performed in parallel with qPCR

samples containing 2 μl of each of the standards. The cycle number at which each

standard reaction crossed the fluorimetry threshold was used to create a standard curve.

The number of copies of the desired product in the unknown sample was determined by

extrapolating from the standard curve.

2.9 Linker-Scanning Mutagenesis

A series of 14 bp regions of the CMV promoter were selected for mutation.

Primers were designed complementary to the 10-15 bp 5' (for the antisense primer) and

3' (for the sense primer) to the mutation site. A 14 bp linker containing a nonsense DNA

sequence including a restriction site for *BsiW*I was added at the 5' end of both primers.

The regions of the CMV promoter 5' and 3' to the desired mutation were PCR amplified

using the primers listed in table 2.2 along with the CMV primer (in combination with the

antisense mutation primer) and the CMV Luc R primer (in combination with the sense

mutation primer). PCR amplification was performed using a 95°C initiation step for 15

m, followed by 35 cycles consisting of a 95°C denaturation for 30 s, a 58°C annealing

step for 30 s, and a 60 s extension at 72°C. The PCR reaction was completed with a 10 m

extension at 72°C. The PCR products were collected and ligated into the pGEM-T vector

as described in sections 2.6.b and 2.6.c. Ligation products were electroporated into InvF'

electrocompetent *E. coli* cells, and InvF' colonies positive for insert were collected as described in section 2.6.c. Samples were digested with *BsiW*I (which cut within the mutation) and an enzyme that cut in the pGEM-T multiple cloning site to free the promoter fragment. Digested products were run on an agarose gel, excised and collected as described in section 2.1.b. The fragments of the CMV promoter extending in the 3' and 5' direction from a mutated site were combined into one tube. The combined sample was ethanol precipitated as described in section 2.1.b and resuspended in 8 µl H_2O. The upstream and downstream fragments were ligated in 1x ligation buffer and 2 U T4 ligase overnight at 4°C. The ligation product was used as a template for PCR amplification of the CMV promoter. PCR reactions (using 2 µl of ligation product) were performed using the CMV and CMV Luc R primers (Table 2.2) and the PCR conditions described above. PCR product was recovered, ligated into pGEM-T vector, grown in *E. coli*, and mini-prepped as described in sections 2.6.b and 2.6.c. The presence of the CMV promoter in the plasmid, and the presence of the mutation in the correct location in the CMV promoter were verified by restriction digest. The promoter was excised from pGEM-T using *Bgl*II and *Mlu*I enzymes and sub-cloned into pGL3-Basic as described in section 2.6.c. The presence of the desired mutations was verified using sequencing analysis performed by The Centre for Applied Genomics.

2.10   ChIP Assay

2.10.a Growth, Transfection and Harvesting of Cells

For each cell line used (N548wt, N548hd), 1.5×10^6 cells were plated into 2 poly-D-lysine-coated 100 mm plates and grown overnight. The following day the cells

were transfected with 12 μg of pGL3-CMV plasmid and 1.5 μg of pEGFP-N1 plasmid. The plasmid mix was diluted in 720 μl of PLUS reagent and SFM to a total volume of 4.4 mL. The mix was incubated for 15 m at room temperature and then mixed with 180 μl of Lipofectamine and 4.32 mL of SFM. Following a second 15 m room temperature incubation the mixtures were added to the plates. After 4 h in the 33°C incubator, 1.2 mL of FBS and 4.8 mL SFM were added to each plate. The following day the plates were washed twice with 15 ml of 1x PBS and then incubated at room temperature in 15 ml of 1x PBS with 1% (v/v) formaldehyde (Fisher Scientific) with shaking for 10 m. After 10 m glycine was added to a final concentration of 125 mM and the plates were shaken for an additional 5 m. Each plate was washed with 15 ml of 1x PBS, scraped into 5 ml of 1x PBS and collected in a 15 ml falcon tube. Plates containing the same cell lines were combined into one falcon tube. The tubes were subjected to centrifugation at 1.6×10^4 x g for 3 m and the supernatants were discarded. The pellets were resuspended in 1 ml of ChIP Lysis Buffer [10 mM Tris-HCl (pH 8.0), 140 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) sodium deoxicolate] with 1 mM PMSF and one-half of a Complete Mini Tab protease inhibitor. Lysates were homogenized for a 10 s pulse using a tissue homogenizer. At this point 50 μl of each tube was saved as an 'input' sample.

2.10.b Immunoprecipitation

Rec-Protein G Sepharose™ (Invitrogen) was prepared by spinning 300 μl in a 1.5 mL eppendorf tube for 30 s at 1.6×10^4 x g. The supernatant was discarded and the pellet was resuspended in 1 ml of ChIP lysis buffer. The tube was subjected to centrifugation for 30 s at 1.6×10^4 x g. The pellet was resuspended in 1 ml of ChIP lysis

buffer and the tube was rotated slowly for 10 m at room temperature. The tube was subjected to centrifugation for 30 s at 1.6 x 10^4 x g and the supernatant was discarded. The pellet was resuspended in 1 ml of 5% BSA and rotated for 1 h at room temperature. The tube was subjected to centrifugation at 1.6 x 10^4 x g for 30 s and the supernatant was discarded. The pellet was washed 3x with 1 ml of ChIP lysis buffer and the final pellet was resuspended in 500 μl of ChIP lysis buffer. After 50 μl of prepared protein G sepharose was added to each sample, tubes were rotated for 1 h at 4°C. The tubes were subjected to centrifugation at 1.6 x 10^4 x g. The supernatants were split into 2 tubes. Primary antibody (5 μg) was added to the reaction tube and rabbit preimmune serum (5 ul) was added to the negative control sample. The tubes were placed on a rotisserie shaker overnight at 4°C.

The following day, 50 μl of prepared protein G sepharose was added to each sample and the tubes were rotated for 2 h at 4°C. The tubes were subjected to centrifugation for 5 m at 1.6 x 10^4 x g. The supernatant (the unbound fraction) was removed and stored at -20°C. The pellet was washed 3x using wash 1 [10 mM Tris-HCl (pH 8.0), 140 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) sodium deoxicolate], wash 2 [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% (w/v) sodium deoxicolate, 0.5% (v/v) NP-40 , 250 mM LiCl], and 1x TE buffer. The pellets were resuspended in 1 ml of the appropriate wash solution and the tubes were rotated for 10 m at room temperature. The tubes were subjected to centrifugation for 2 m at 1.6 x 10^4 x g and the supernatants were removed. After the 1x TE wash, the pellets were resuspended in 1 ml of 1x TE buffer and 50 μl was saved to verify using western blotting that the desired protein was captured. The tubes were subjected to centrifugation at 1.6 x 10^4 x g

for 2 m and the supernatants were removed. The pellets were resuspended in 100 μl 1x

TE buffer with 20 μg RNase and incubated at 37°C for 30 m. The volume in the tubes

was increased by addition of 1 ml of 1x TE and the tubes were vortexed and rotated for

10 m at room temperature. The samples were subjected to centrifugation at 1.6 x 10^4 x g

and the supernatants were discarded. The pellets were resuspended in 250 μl of elution

solution [1% (v/v) SDS, 0.1 mM $NaHCO_3$ (EM Science)], vortexed and rotated at room

temperature for 15 m. The samples were subjected to centrifugation for 5 m at 1.6 x 10^4

x g. The supernatant (bound fraction) was collected for each tube. The remaining pellet

was resuspended in 250 μl of elution solution, vortexed and rotated at room temperature

for 45 m. The tubes were subjected to centrifugation at 1.6 x 10^4 x g for 5 m and the

supernatant was combined with that from the previous spin. NaCl (200 μM final) was

added to each sample as well as to the input samples obtained the previous day. The

samples were incubated at 65°C overnight. The following day, 450 μl of H_2O was added

to the input samples. Proteinase K buffer (Promega) and 20 μg of proteinase K

(Promega) were added to all tubes. The samples were vortexed and incubated for 1 h at

50°C. The pGL3-CMV reporter plasmid present in the samples was isolated using the

GenElute™ Plasmid Miniprep kit (Sigma) according to the kit instructions.

2.10.c Quantification

The eluted sample recovered from the miniprep reaction was used as a template

for qPCR using Luc ChIP For and Luc ChIP Rev primers (Table 2.1) as described in

section 2.8.c. Quantification of the qPCR results was performed by expressing the

amount of plasmid recovered using the specific antibody as a percentage of plasmid

present in the input reaction, and then normalizing that amount to the amount of plasmid recovered using non-specific IgG antibody (as a percentage of plasmid present in the input reaction).

2.11   *In Vitro* Promoter Binding Experiment

2.11.a Generation of Promoter

A fragment of the CMV promoter spanning 297 bp upstream of the transcription start site and 75 bp downstream of the transcription start site was generated by PCR amplification. The pGL3-CMV plasmid was used as template for PCR reactions using the -297 CMV and Biotin Luc Rev primers (Table 2.2). PCR reactions were performed as described in section 2.6.b. Product from the PCR reaction was purified using the GenElute™ PCR Clean-Up Kit (Sigma) according to manufacturer protocol. The product was eluted in 50 μl elution buffer. The concentration of biotinylated -297 CMV promoter fragment was determined as described in section 2.1.b.

2.11.b Nuclear Extraction of Cellular Proteins

Nuclear proteins were isolated from N548wt and N548hd cells using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). Cells were grown to confluence in 75 cm^2 flasks, washed with 10 ml HBSS solution and lifted from the flask surface using 4 ml TrypLE. The cells were placed in a 15 ml Falcon tube and subjected to centrifugation at 500 x *g* for 5 m. The supernatant was discarded and the pellet was resuspended in 1 ml of ice cold 1x PBS and moved to a 1.5 ml eppendorf tube. The tubes

were subjected to centrifugation at 500 x g for 3 m at 4°C, and the supernatant was discarded. The pellet was resuspended in 500 μl of cytoplasmic extraction reagent (CER) I (with 1 mM PMSF) and vortexed for 15 s. Tubes were incubated on ice for 10 m. CER II (27.5 μl) was added and the tubes were vortexed twice for 5 s each time with a 1 m incubation on ice in between. Tubes were subjected to centrifugation at 1.6 x 10^4 x g for 5 m at 4°C and the supernatant (the cytoplasmic fraction) was saved. The pellet was resuspended in 250 μl of nuclear extraction reagent (with 1 mM PMSF added). Tubes were vortexed for 15 s followed by a 10 m incubation on ice. The 15 s vortex followed by 10 m incubation on ice was repeated for a total of 4 times. The tubes were subjected to centrifugation at 1.6 x 10^4 x g for 10 m at 4°C. The supernatant (soluble nuclear fraction) was collected and stored at -80°C.

2.11.c Preparation of Dynabeads

Streptavadin-coated M-280 Dynabeads® (Invitrogen) were resuspended in their vial and 150 μg (15 μl) per reaction was moved to a 1.5 ml eppendorf tube. The eppendorf tube was placed on a MPC®-S Magnetic Particle Concentrator (Dynal Biotech) for 2 m to isolate the magnetic beads. The supernatant was removed and the pellet was resuspended in a volume of wash buffer [1x TE Buffer (pH 7.5), 1 M NaCl] + 0.003% (v/v) NP-40 equal to the original volume of Dynabeads collected. The tube was placed on the magnet for 2 m and the supernatant was discarded. The wash step was repeated twice more for a total of three washes. The final pellet was resuspended in wash buffer + 0.003% NP-40, at a volume equal to that withdrawn originally.

2.11.d Binding of -297 CMV DNA to Dynabeads

Prepared Dynabeads® were divided into reaction tubes at a volume of 15 μl per tube. Three pmol of -297 CMV was combined with wash buffer + 0.003% NP-40 to a total volume of 15 μl and added to the reaction tubes. The tubes were placed on a rotisserie shaker for 30 m at room temperature. The tubes were placed on the magnet for 2 m and the supernatant was discarded. The beads were washed with wash buffer, resuspeded in 150 μl blocking buffer [100 mM KCl, 20 mM HEPES (pH 7.6) (Sigma), 5 mM MgCl$_2$ (Sigma), 2 mM EDTA, 60 mg/ml casein (Sigma), 5 mg/ml polyvinylpyrrolidine (Sigma), 2.5 mM DTT] and incubated at room temperature on the rotisserie shaker for 15 m. The samples were washed 3 times with 400 μl transcription buffer [100 mM KCl, 20 mM HEPES (pH7.6), 5 mM MgCl$_2$, 2 mM EDTA]. The final pellet was suspended in 15 μl transcription buffer to a concentration of 10 mg/ml, and the tubes were stored on ice.

2.11.e Binding of Cellular Nuclear Extract to -297 CMV

Nuclear extract from N548wt and N548hd cells was combined with binding buffer [100 mM KCl, 20 mM HEPES (pH 7.6), 5 mM MgCl$_2$, 2 mM EDTA, 2.5 mM DTT, 0.05% (v/v) NP40, 30 ng/μl poly dI:dC] to a final volume of 50 μl and added to the Dynabead-bound -297 CMV promoter. The tubes were incubated at room temperature on the rotisserie shaker for 30 m. The tubes were placed on the magnet for 2 m and the supernatant was removed and stored as the unbound fraction. The pellets were washed 3 times with 400 μl reaction wash buffer [100 mM KCl, 20 mM HEPES (pH 7.6), 5 mM MgCl$_2$, 2 mM EDTA, 2.5 mM DTT, 0.05% NP-40] and the supernatants were collected

for analysis. The final pellet was suspended in 20 µl of Laemmli Sample Buffer

(BioRad).

2.11.f Visualization of Promoter-Bound N-mHtt

The protein in the unbound fraction and in the third wash of the *in vitro*

promoter binding experiment was precipitated. A volume of trichloroacetic acid (Sigma)

equal to ¼ of the total sample volume was added to each of the tubes. The samples were

incubated on ice for 10 m and subjected to centrifugation for 10 m at 1.6 x 10^4 x *g* at 4°C.

The supernatant was discarded and the pellet was washed 3 times with 200 µl of acetone,

with a 5 m centrifugation step at 1.6 x 10^4 x *g* at 4°C in between each wash. The tubes

were incubated at 95°C for 3 m with caps open to dry the pellets, which were then

resuspended in 20 µl Laemmli Sample Buffer. The resuspended unbound and wash #3

samples as well as the bound fraction (from 2.11.e) and 2 µg of N548wt and N548hd

nuclear extract were loaded onto a 7.5% SDS-PAGE gel and Western Blotting was

performed according to section 2.3.b. MAB2166 was the primary antibody used, at a

dilution of 1:2000. Anti-mouse secondary was used at a dilution of 1:5000.

2.12   shRNA-Mediated Knockdown of Htt

N548wt and N548hd cells were plated and grown as described in section 2.4.

The following day, cells were transfected with a mix containing 200 ng of pGL3-CMV

plasmid, 50 ng of pRL-TK plasmid, 50 ng of pEGFP-N1 plasmid and either 30 nM

plasmid driving the expression of RNA complementary to the sequence between

nucleotides 413-438 of human *huntingtin* mRNA (shHtt) or empty plasmid (shNeg) as

described in section 2.4. The cells were grown for 48 h following transfection, at which

point cell lysates were collected and the DLR™ Assay was performed as described in

section 2.7. Protein was quantified (as described in section 2.5) and 5 µg of protein from

each reaction condition was fractionated using SDS-PAGE. Western blotting was

performed as described in section 2.3 using antibody MAB2166 (Table 2.1).

Chapter 3:

N-terminal Mutant Huntingtin Protein Represses Transcription

In Vitro and *Ex Vivo*

3.1 Introduction

Steady-state mRNA levels of specific genes have been shown to decrease in human HD patients, as well as in animal and cellular models of HD (Luthi-Carter *et al.*, 2000; Sipione *et al.*, 2002; Hodges *et al.*, 2006; Hodges *et al.*, 2008; Becanovic *et al.*, 2010). Some of these transcriptional changes occur early in the progression of the disease, while other changes occur later (Luthi-Carter *et al.*, 2000; Ali & Levine, 2006; Becanovic *et al.*, 2010). Later changes in gene expression may result from compensatory mechanisms, while the earliest changes are more likely a direct result of the presence of N-mHtt.

Expression of mHtt has been shown to inhibit mitochondrial function (Gu *et al.*, 1996; Panov *et al.*, 2002), intercellular signaling (Zeron *et al.*, 2002; Zuccato *et al.*, 2008), protein degradation (Bence *et al.*, 2001; Heng *et al.*, 2010), and cholesterol homeostasis (Valenza *et al.*, 2007b). An imbalance in any one of these cellular functions would be expected to cause secondary changes within the cell, which could include changes in gene expression. For example, mice engineered to have chronically active glutamatergic signaling, mirroring the excitotoxicity model of HD, exhibited changes in gene expression that reflect some of the changes observed in the presence of mHtt (Wang *et al.*, 2010). Similarly, increased production of reactive oxygen species due to mHtt-mediated mitochondrial dysfunction has been shown to produce secondary changes in

gene expression (Gibson *et al.*, 2010). Since many cellular changes occur following

expression of mHtt, and because it is not clear if transcriptional dysregulation occurs in

response to other changes in the cell, it is important to determine whether N-mHtt directly

inhibits transcription.

Several theories have been proposed that attempt to explain the mechanism of

N-mHtt-mediated transcriptional dysregulation. The predominant theory states that N-

mHtt interacts directly with transcription factors to inhibit their function (Cha, 2007).

Another theory suggests that N-mHtt alters nucleosome dynamics resulting in impaired

chromatin folding, which leads to decreased gene expression (Stack *et al.*, 2007). It has

also been proposed that N-mHtt has a lower affinity for repressive transcription factors

than wild-type Htt and that decreased interaction between N-mHtt and these proteins

allows the repressor transcription factors to translocate to the nucleus and decrease

transcription (Zuccato *et al.*, 2007).

The possibility also exists that mutant *huntingtin* mRNA, and not protein,

mediates transcriptional dysregulation. The *huntingtin* transcript forms stem-loop

structures characteristic of microRNA secondary structure (de Mezer *et al.*, 2011). Some

polyQ-expansion disorders, including myotonic dystrophy (MD) and fragile X-associated

tremor ataxia syndrome, are believed to be caused by increased association of RNA

binding proteins with the mutant transcript of the disease-causing gene, leaving those

RNA binding proteins unable to perform their normal splicing functions (reviewed in

Licatalosi & Darnell, 2006). CAG repeat expansion in a *Drosophila* model of

Spinocerebellar ataxia 3, another CAG repeat expansion disorder, has been shown to

cause neurodegeneration in a CAG repeat length-dependent manner, independently of

translation of the mRNA transcript (Li *et al.*, 2008), and CAG repeats have been shown to bind RNA splicing factors in a repeat length-dependent manner (Yuan *et al.*, 2007). Although the symptoms and proposed mechanisms of pathogenesis in MD and Fragile X-associated tremor ataxia syndrome are not common to HD, the fact that CAG repeat expansion in mRNA can be toxic, and other polyQ expansion disorders involve toxic mRNA suggest that it is important to determine if N-mHtt is toxic in the absence of *huntingtin* mRNA. Human patients and all cellular and animal models of HD express both *huntingtin* mRNA and protein, making it impossible to determine conclusively which is the disease-causing agent. Although it would be possible to express *huntingtin* mRNA that could not be translated into protein, this has yet to be attempted. Expression of recombinant mHtt in an *in vitro* transcription assay in the absence of *huntingtin* mRNA can separate the pathogenic effects of the protein from those of the mRNA.

The work in this chapter was performed to fulfill two goals. The first was to determine if N-mHtt could decrease transcription independent of *huntingtin* mRNA, chromatin folding, or the various cellular changes that occur following expression of mHtt, and whether transcriptional repression reflected a deleterious gain-of-function of mHtt. The second goal of the chapter was to establish the appropriate conditions and methods to study N-mHtt-mediated transcriptional dysregulation in a cellular model of HD. The CMV promoter was selected as a model promoter for studying N-mHtt-mediated transcriptional dysregulation. The CMV promoter was selected because it is highly active (Invitrogen), well characterized, and initiates transcription from a defined transcription start site (Meier & Stinski, 1996). Since CMV promoter activity is

controlled by eukaryotic transcription factors, information obtained could be applied to endogenous genes that are regulated in a similar manner.

Studying the effects of overexpression of mHtt-interacting proteins on transcriptional dysregulation required a cellular model of HD. Many cellular models have been used to study transcriptional dysregulation in HD (reviewed in Sipione & Cattaneo, 2001). These models can be broadly divided into those that express mHtt transiently and those that stably express mHtt. Despite the fact that mHtt expression begins during embryonic development, HD is a late onset disorder. Therefore, a disadvantage of transient expression models is their failure to mirror the prolonged exposure of neurons to mHtt. A second disadvantage is that transient transfection allows for a limited amount of plasmid to be delivered to the cell, and inclusion of plasmid driving the expression of mHtt reduces amount or number of additional reporter or expression plasmids that can be included in an experiment. An advantage of transient expression models is that changes observed following transient expression of mHtt are likely to be direct effects of the mutant protein, and not the result of secondary changes due to prolonged exposure to mHtt. Transient transfection models have been used to study the cellular distribution (Cooper *et al.*, 1998) and aggregation properties (Scherzinger *et al.*, 1999) of mHtt. Transient transfection also allows for flexibility with respect to the size of fragment expressed, as well as the cell types used. This advantage has helped shed light on the influence that the size of the N-terminal mHtt fragment expressed has on the changes it produces (Cooper *et al.*, 1998; Hackam *et al.*, 1998), as well as how one N-mHtt fragment can have cell-specific effects (Gomez *et al.*, 2006).

Cell models stably expressing mHtt were generated to model continuous cellular exposure observed in the disease state. Cell lines that stably express mHtt can be further subdivided by the size of mHtt expressed. Cellular models of HD express either full-length mHtt, or an N-terminal fragment of mHtt. While full-length expression of mHtt models endogenous expression in HD, the formation and nuclear accumulation of N-mHtt have been correlated with disease-related cellular toxicity (Peters *et al.*, 1999; Graham *et al.*, 2006). Additionally, animal models overexpressing an N-terminal fragment of mHtt exhibit behavioral changes, decreased brain volume, aggregation, increased glutamate signaling, altered lipid metabolism, and transcriptional changes similar to those observed during the progression of HD, suggesting that N-mHtt is sufficient for producing and studying these pathogenic effects observed in the disease (Li *et al.*, 2005).

Yet another way to divide cell models is in regards to the manner in which protein expression is driven. Some cells drive expression of mHtt or N-mHtt using the *huntingtin* promoter, modelling endogenous Htt expression. Others use a viral promoter to overexpress the protein. Viral promoters are used for overexpression because they are highly active promoters, leading to a high level of protein expression. Many of the cellular changes that occur during the progression of HD are believed to be related to the level of mHtt expression (de Almeida *et al.*, 2002; Wang *et al.*, 2009). The late-onset nature of HD may be due to the gradual accumulation of mHtt over a long period of time. The artificially high level of mHtt expression obtained using a viral promoter may accelerate both the accumulation of mHtt and the changes induced by mHtt, and mask the timeline in which they occur. An advantage of viral mHtt expression is that the high

level of mHtt expression produced using viral promoters may amplify the changes that result from mHtt expression, making them easier to detect and study. A drawback, however, is that the changes observed following viral expression of mHtt may be more difficult to reverse, making it more difficult to study the mechanism through which mHtt effects those changes.

Some of the stable cell lines used to study HD are the StHdh, ST14A, HC2S2 and PC12 cells lines cell lines. The StHdh cells were derived from the striatal cells of a knock-in mouse model of HD (Trettel *et al.*, 2000). This line is the most genetically accurate cell model of HD, as the cells express two copies of full-length Htt with either 7 or 111 glutamine repeats, and expression of the protein is controlled by the human *huntingtin* promoter. In contrast, ST14A, HC2S2 and PC12 cell lines are overexpression models of HD that use viral promoters to express full-length or N-terminal mHtt in addition to their endogenous wild-type Htt. ST14A cells and their derivatives are rat striatal cells stably expressing the N-terminal 548 aas of human Htt with either 15 or 128 glutamine repeats, in addition to the two endogenous copies of Htt (Rigamonti *et al.*, 2000). Expression of N548 aa Htt in these cells is driven by the 5' LTR viral promoter. The PC12 cell model was derived from immortalized rat pheochromocytoma cells and stably expresses exon 1 of *huntingtin* containing either 20 or 150 CAG repeats, driven by the viral CMV promoter (Li *et al.*, 1999b). PC12 cells have also been stably transfected to express full-length *huntingtin* with 16, 48 or 89 CAG repeats driven by an unnamed viral promoter, in addition to the two endogenous wild-type copies (Song *et al.*, 2002). HC2S2 cells are rat neuroblastoma cells that can be induced to differentiate into neurons, and overexpress exon 1 of human *huntingtin* with either 28 or 74 CAG repeats (Dong *et*

al., 2011). Expression of this fragment is driven by the CMV promoter. The disadvantage of overexpression models is that they cannot be used to model the loss-of-function aspects of HD.

For the purposes of this chapter, immortalized rat striatal cells (ST14A cells) (Cattaneo & Conti, 1998) and derivative lines expressing N-terminal Htt were selected. Striatal neurons have been extensively studied with respect to gene expression, and show significant differences in expression of specific genes in all models of HD and in post-mortem analysis of HD human brain (Hodges *et al.*, 2006). Thus, striatal cells are a logical choice to study N-mHtt-mediated transcriptional dysregulation. N548wt and N548hd derivatives of ST14A cells stably express the N-terminal 548 aas of human Htt with either 15 or 128 glutamines, respectively. The 548 aa N-terminal fragment approximates the predominant cleavage fragment of full-length Htt (Hermel *et al.*, 2004). ST14A, N548wt and N548hd cells were examined to determine if they are an appropriate model for studying N-mHtt-mediated repression of CMV transcription.

3.2 Results

In vitro transcription assays were performed to determine whether addition of N-mHtt could directly decrease transcription driven by CMV promoter. Restriction digest of a plasmid containing a portion of the CMV promoter was performed to generate a linear DNA template for *in vitro* transcription assays. The DNA template spanned -297 to +1 relative to the +1 transcription start site of the CMV promoter and contained a synthetic 363 bp coding region. HeLa nuclear extract, which contains transcription factors, DNA-binding proteins and PolII necessary for transcription, was combined with

DNA template and non-radioactive rATP, rCTP, rTTP and rGTP ribonucleotides, and α-^{32}P-labeled rGTP. Reactions contained purified recombinant N-terminal human Htt spanning the first 171 aas with 23 (N171-23Q) or 89 (N171-89Q) glutamines, or no added N-terminal Htt. Following 30 m of transcription, RNA was isolated and fractionated on a denaturing urea-polyacrylamide gel, transferred to filter paper and dried. Incorporation of α-^{32}P-labeled rGTP during transcription allowed detection of transcripts generated in the *in vitro* transcription assay using autoradiography. Based on the predicted transcription start site, a 363 nt transcript was expected as the product of the *in vitro* reaction. There were several transcripts produced that differed in size by only one or a few bps. It appeared that these transcripts were produced in equal amounts relative to each other in all conditions tested. The addition of recombinant N171-23Q did not change the relative amount of transcript produced by the CMV promoter compared to reactions without added recombinant N-terminal human Htt (Fig. 3.1). The amount of transcript produced in the presence of recombinant N171-89Q appeared to be lower than the product generated in the presence of N171-23Q or in the absence of added recombinant Htt. In four replicates of the experiment, the amount of transcript produced in the presence of N171-89Q was lower than in the control reactions each time. These experiments suggest that N-mHtt with an expanded polyQ repeat is capable of directly decreasing transcription driven by a linear DNA fragment of the CMV promoter, supporting the use of CMV as a model promoter for studying the mechanism of N-mHtt-mediated transcriptional dysregulation. To determine whether CMV transcription was impaired by N-mHtt using a quantitative assay, and to study transcription in an environment more
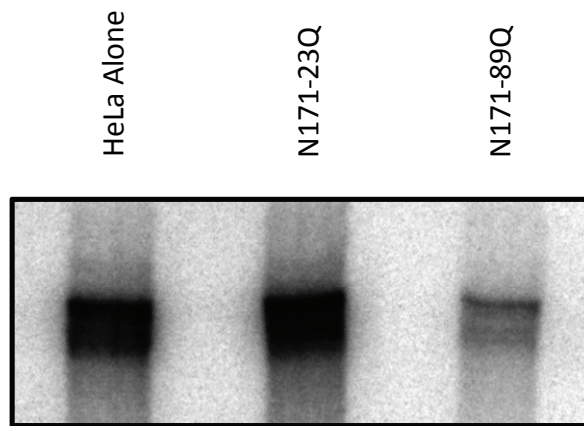
Figure 3.1. Recombinant N-mHtt decreased transcription driven by the CMV promoter. An *in vitro* transcription assay was performed containing HeLa nuclear extract with no added recombinant Htt (HeLa alone) or with HeLa nuclear extract supplemented with recombinant N-mHtt consisting of the N-terminal 171 aa and either 23 (N171-23Q) or 89 (N171-89Q) glutamines. RNA reaction products were fractionated on a denaturing polyacrylamide gel and the detectable transcripts of approximately 363 nts are shown.

conducive to overexpression of protein, CMV promoter construct activity was tested in a cellular model of HD.

ST14A cells and derivatives were selected as a model to study the gain-of-function of N-mHtt. Since ST14A, N548wt and N548hd cells express two copies of endogenous Htt, differences in gene expression between cell lines cannot be attributed to a loss of wild-type Htt. Any changes observed in the N548hd cells would logically be a result of a novel function resulting from polyglutamine expansion in the N-terminal fragment of huntingtin protein or a dominant-negative effect of the N-terminal fragment on wild-type Htt. We observed that ST14A cells were bigger and more elongated than either the N548wt or N548hd cells (Fig. 3.2). N548wt and N58hd cells had a similar size and round appearance. This would suggest that the process of stably transfecting the N548 cells with N-terminal Htt, or the expression of the N-terminal fragment of Htt, regardless of polyQ length, caused changes in the N548 cell lines that altered morphology.

Western blotting was performed using antibody MAB2166 raised against aa 180 to 810 of human Htt to determine the relative expression of Htt in ST14A, N548wt and N548hd cells (Fig. 3.3). No rat Htt was detected in ST14A, N548wt or N548hd cell lysates. The study that originally characterized the N548 cell lines stated that rat Htt was detected using MAB2166 following a longer film exposure (Rigamonti *et al.*, 2000). The level of rat Htt expression relative to the level of overexpressed N-terminal Htt combined with the method of signal detection likely contributed to the inability to detect rat Htt in these cells. Several MAB2166-immunoreactive bands were present in N548wt and N548hd lysates. The predominant immunoreactive bands were 75 kDa and 100 kDa,
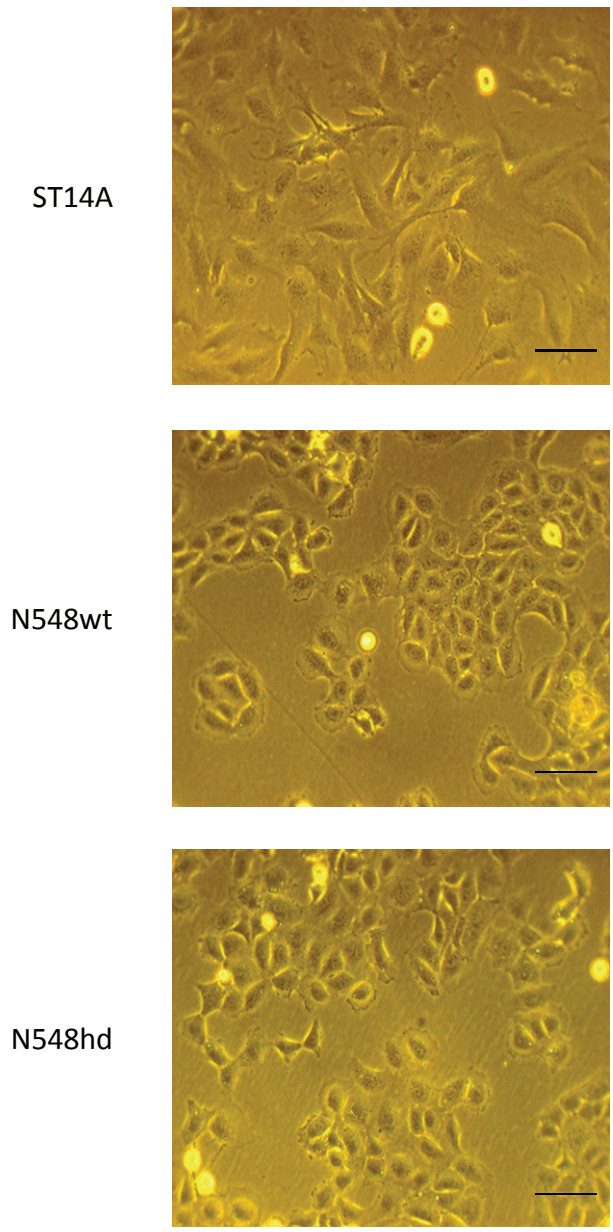
ST14A

N548wt

N548hd

Figure 3.2. Expression of N-terminal 548 aa of human Htt led to morphological changes in cells. Bright field photographs of ST14A, N548wt and N548hd cells were taken at 320x magnification. Scale bar equals 50 μm.
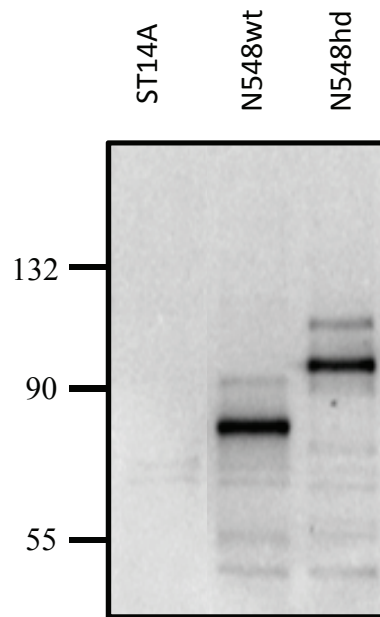
Figure 3.3. The N-terminal portion of Htt was expressed at approximately equal levels in N548wt and N548hd cells. Western blotting was performed using cell lysates from ST14A, N548wt and N548hd cells. The blot was probed using an antibody specific to the N-terminal region (aa 181-810) of Htt. Numbers on the left indicate relative mobility of molecular weight size markers in kDa.

respectively, as estimated based on relative mobility to molecular weight markers. The estimated size of immunoreactive bands was very similar to the size of 72 kDa and 115 kDa reported in the original description of N548wt and N548hd cells, respectively (Rigamonti *et al.*, 2000). The estimated size of the major MAB2166-immunoreactive bands in both cases was higher than the predicted molecular weight of the N-terminal 548 aa fragment of Htt with 15 or 128 contiguous Q in the polyQ repeat, which was calculated to be 59 and 74 kDa, respectively.

The differences between the apparent and predicted molecular weight of N-mHtt fragments in N548 cell lines could have resulted from CAG repeat instability, post-translational modification of the N-terminal fragment or anomalous migration due to secondary structure not blocked by SDS, reducing agents and heat. Expansion and contraction of the CAG repeat during somatic cell division has been demonstrated in animal models of HD (Ishiguro *et al.*, 2001). Western analysis of the cell lysates was repeated at intervals throughout this study and no change in the relative size of the MAB2166-immunoreactive bands occurred (data not shown). This suggests that despite the fact that the absolute size of N-terminal Htt in N548wt and N548hd cells differed slightly from the literature, the difference in size between the N-terminal fragments in the two cell lines reflected a difference in polyQ length approximately equal to that originally described, and the polyQ length of the fragments did not change throughout the duration of the experiment. Together, western analyses demonstrated that N-terminal Htt was highly expressed at approximately equivalent concentrations in N548wt and N548hd cells.

The DLR™ Assay is an indirect measure of transcriptional activity. There are a number of steps between transfection of the cells with a plasmid containing a promoter and the luciferase reporter and measurement of luciferase activity, which could be impacted by expression or overexpression of N-mHtt (Fig. 3.4). To ensure that the activity of a transiently transfected reporter plasmid could be compared in different cell lines and related to the expression and length of the polyQ repeat, it was important to confirm that luciferase activity was directly related to the amount of luciferase mRNA produced. Following transfection of the pGL3-CMV plasmid into cells, the CMV promoter drives expression of the 1653 bp *luc+* mRNA transcript. This transcript is then translated into firefly luciferase enzyme. Firefly luciferase generates light as a by-product of a reaction that cleaves beetle luciferin to produce oxyluciferin. The amount of light generated is assumed to be directly related to the amount of *luc+* transcript, which is assumed to be directly dependent on the strength of the promoter of the reporter plasmid. If expression of N-mHtt decreased cell survival, transfection efficiency, translation efficiency, protein stability or enzymatic activity of the firefly luciferase protein, the apparent luciferase activity would be decreased. Therefore, we tested whether there was a difference in cell survival, protein concentration, transfection efficiency, and we then measured levels of *luc+* mRNA in ST14A, N548wt and N548hd cells.

N-mHtt has been shown to decrease cell viability in several cell culture models (Wyttenbach *et al.*, 2001; Carmichael *et al.*, 2002; Gong *et al.*, 2008; Leon *et al.*, 2010), however decreased viability was not seen in ST14A cells and the N548 derivative cell lines when grown at their permissive temperature (Rigamonti *et al.*, 2000). If transfection efficiency and cell survival are not different among ST14A cells and the
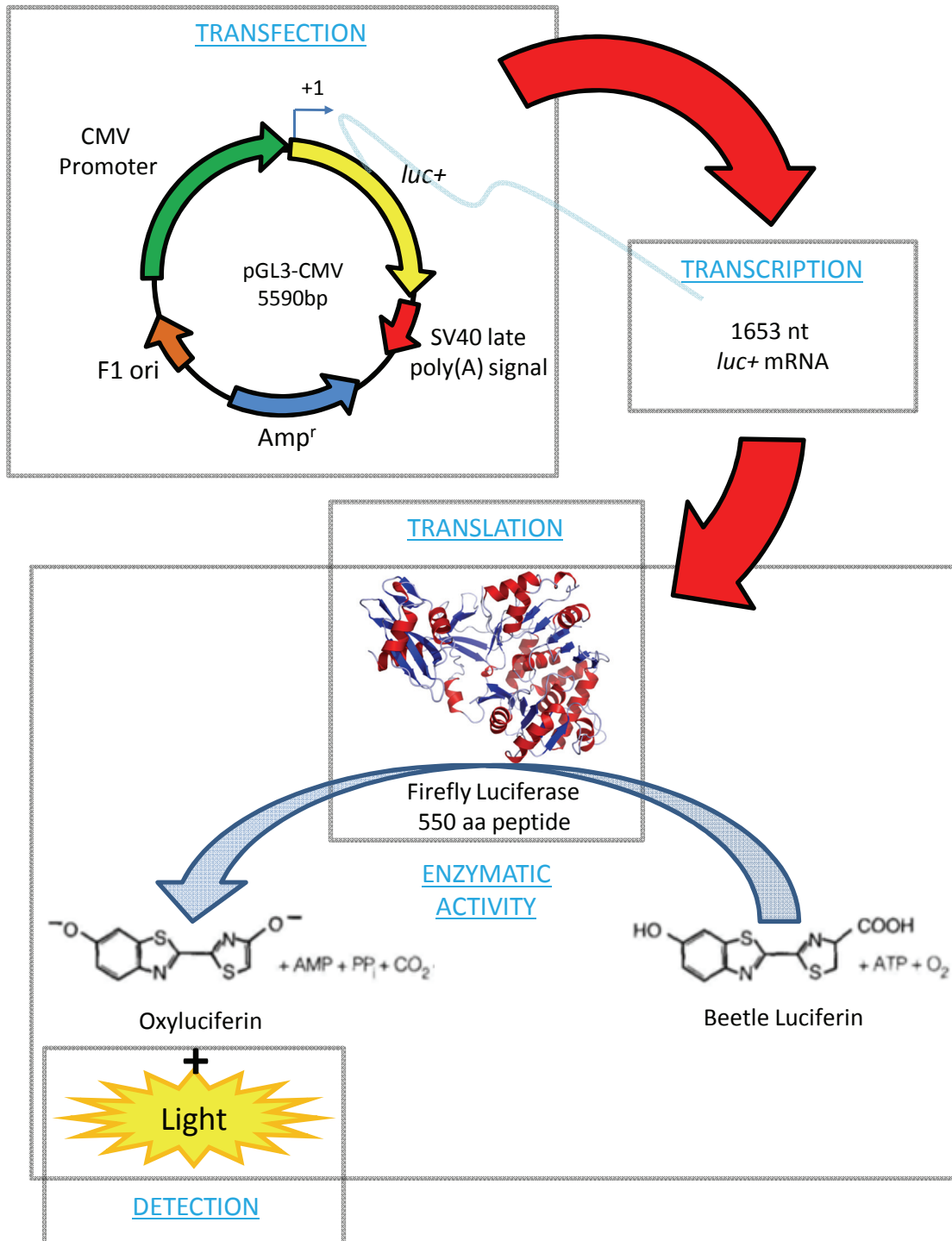
Figure 3.4. Diagram of the luciferase reporter assay. Transcriptional activity of the luciferase reporter plasmid is measured indirectly via the light-generating enzymatic activity of the firefly luciferase protein.

N548 derivative cell lines, then differences in luciferase activity would assumed to be a result of differences in transcription, translation, or protein degradation between the cell lines. To ensure that cell viability was not different among the three cell lines, 5 x 10^4 ST14A, N548wt and N548hd cells were seeded on coverslips. The following day, the cells were transfected with a plasmid driving production of GFP and allowed to grow for another 24 h. Hoechst staining was performed and the number of cell nuclei were counted to quantify the number of surviving cells. Fewer ST14A cells were present after 48 h of growth than the number present for both N548wt and N548hd cells (Fig. 3.5). Since it did not appear that cell death was occurring, the decreased number of ST14A cells may have been due to a slower growth rate in ST14A cells than in either N548 cell line. Theoretically, because there are fewer ST14A cells when the luciferase assay would be performed, equal transcriptional activity per ST14A cell would result in significantly less total luciferase activity relative to N548wt and N548hd cells.

N-mHtt has been shown to alter protein processing and degradation pathways (Bence *et al.*, 2001; Shibata *et al.*, 2006). The apparent relationship between the transcriptional strength of the CMV promoter and the detectable luciferase activity may have been affected if N-mHtt impaired protein production or stability. ST14A, N548wt and N548hd cells were plated and transfected with plasmid driving GFP expression the following day. Twenty-four hours after transfection, the cells were collected and the total protein in the cell lysates was quantified. The amount of total protein was decreased in N548hd cells compared to ST14A cells demonstrating that N-terminal Htt with an extended polyQ repeat region decreased total protein in cell lysates (Fig. 3.6). Expression of N-terminal Htt in N548wt cells did not decrease total protein, as there was
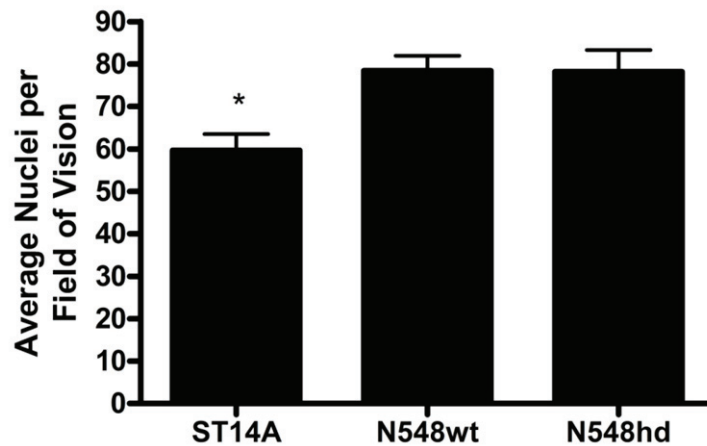
Figure 3.5. ST14A cells were present at lower numbers than N548wt or N548hd cells at the time of the luciferase assay. Fifty thousand ST14A, N548wt and N548hd cells were seeded and grown on coverslips for 48 h. The cells were formaldehyde-fixed and Hoechst staining was performed to visualize nuclei as an indicator of the number of cells present. The number of Hoechst-positive cells at four locations per coverslip were averaged. * $P <$ 0.05 relative to N548wt and N548hd cells as determined by one-way ANOVA followed by Bonferroni post-hoc testing. N=8 for each cell type.
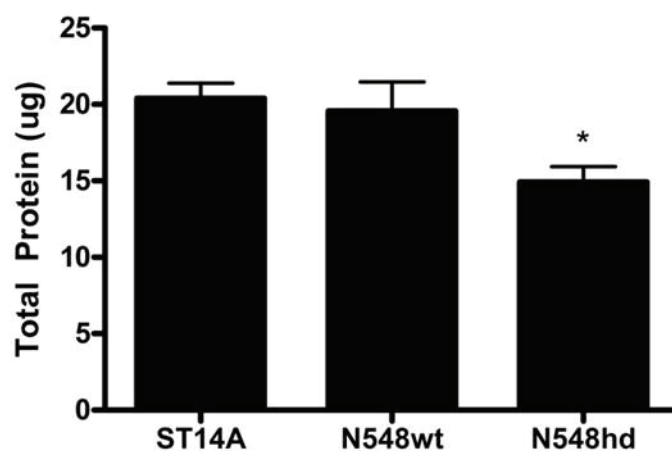
Figure 3.6. Expression of N-mHtt reduced total protein levels in N548hd cells relative to ST14A but not N548wt cells. An equal number of ST14A, N548wt and N548hd cells were plated and grown for 48 h. Cell lysates were collected and total protein levels were quantified using a Bradford assay. $*P < 0.05$ relative to ST14A cells as determined by one-way ANOVA followed by Bonferroni post-hoc test. N=8 per cell type.

no difference in total protein between ST14A and N548wt cells. There was no difference in the amount of total protein between N548wt and N548hd cells. Despite the fact that there were fewer ST14A cells than N548wt and N548hd cells (Fig. 3.5), ST14A cells expressed a larger amount of protein per cell. Since the relationship between protein levels and cell number was different between ST14A cells and N548wt or N548hd cells, total protein could be used to estimate cell number between N548wt and N548hd cells but not for comparisons between ST14A cells and either of the N548 cell lines.

To ensure that the presence of N-mHtt did not alter the ability of cells to take up and express luciferase encoded by mammalian expression vectors, transfection efficiency was determined in ST14A, N548wt and N548hd cells. Cells were transfected with a plasmid driving production of GFP. Twenty-four hours after they were transfected, the cells were fixed using formaldehyde. Hoechst staining was performed, and the number of GFP- and Hoechst-positive cells were counted to determine the number of transfected (GFP-positive) cells as a percentage of total (Hoechst-positive) cells (Fig. 3.7). Transfection efficiency was approximately 30% in ST14A, N548wt, and N548hd cells (Fig. 3.8). N-terminal Htt with or without an extended polyQ repeat did not affect the ability of cells to take up or express plasmid-encoded GFP.

To determine if transcription driven by the CMV promoter was decreased in the presence of N-mHtt in striatal cells, a 772 bp fragment of the CMV promoter 5' to the transcription start site was cloned into the pGL3-Basic luciferase reporter plasmid. ST14A, N548wt and N548hd cells were transfected with the pGL3-CMV reporter plasmid, and luciferase activity was measured 24 h later. CMV-driven luciferase activity was significantly decreased in N548hd cells compared to ST14A and N548wt cells
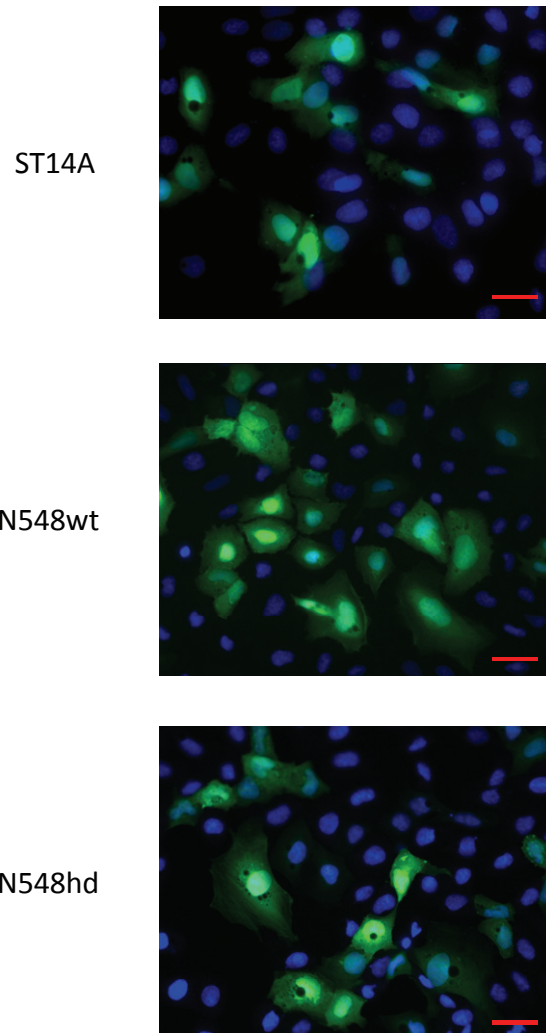
ST14A

N548wt

N548hd

Figure 3.7. ST14A, N548wt and N548hd cells expressed GFP following lipofectamine transfection of pEGFP-N1. Cells were fixed 24 h after transfection, stained with Hoechst and photographed at 400x magnification. Green cells represent transfected cells. Blue staining represents cell nuclei. Scale bar equals 40 μm.
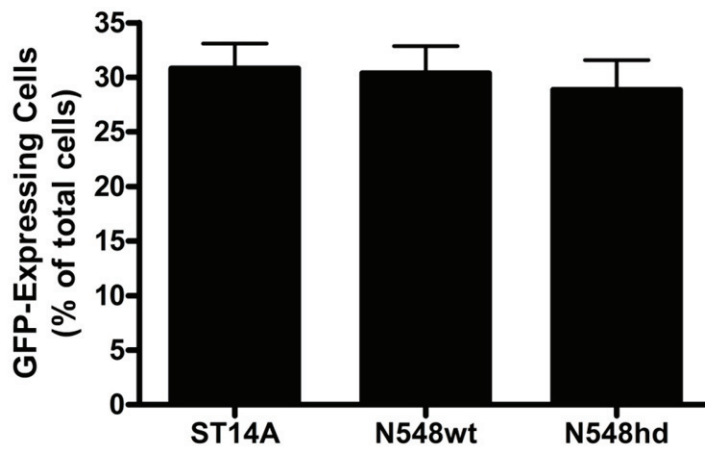
Figure 3.8. Expression of the N-terminal region of Htt did not affect transfection efficiency in N548wt or N548hd cells. Cells were transfected with a plasmid driving expression of GFP protein and fixed 24 h later. Hoechst staining was performed to visualize nuclei, and the number of GFP- and Hoechst-positive cells were tabulated at four locations per coverslip. A one-way ANOVA was performed to test statistical significance using an α value of 0.05. N=6 per cell type.

(Fig. 3.9). No difference in activity was observed between ST14A and N548wt cells. N-terminal Htt with a non-pathogenic length of polyQ repeat region did not affect CMV-driven luciferase activity. N-terminal Htt with an expanded polyQ region decreased CMV activity. To determine if the decreased luciferase activity was directly related to the amount of luciferase mRNA (*luc+*) produced, RNA was extracted from the cell lysates following the luciferase assay. Reverse transcription followed by qPCR was performed to determine the number of copies of *luc+* in ST14A, N548wt and N548hd cell lysates. *Luc+* mRNA levels were significantly decreased in N548hd cells compared to N548wt cells (Fig. 3.10A). There was no difference in *luc+* mRNA levels between N548hd and ST14A cells. To determine the relationship between transcription of *luc+* mRNA and luciferase activity, CMV-driven luciferase activity from figure 3.9 was normalized to *luc+* mRNA copy number (Fig. 3.10B). The luciferase activity generated per copy of *luc+* mRNA was significantly greater in ST14A cells compared to N548wt and N548hd cells. There was no difference in luciferase activity generated per copy number of *luc+* mRNA between N548wt and N548hd cells (Fig. 3.10B) demonstrating that there was a similar relationship between CMV-driven transcription of *luc+* mRNA and the luciferase activity in N548wt and N548hd cells but that the luciferase activity per copy of *luc+* mRNA was significantly greater in ST14A cells relative to that observed in either type of N548 cell line. In summary, N548wt and N548hd cells are morphologically similar, have the same growth and survival characteristics, have no differences in total protein levels or transfection efficiency and have a similar relationship between transcription of *luc+* mRNA and luciferase activity. In contrast, we observed differences in the relationship between transcription and luciferase activity, morphology,
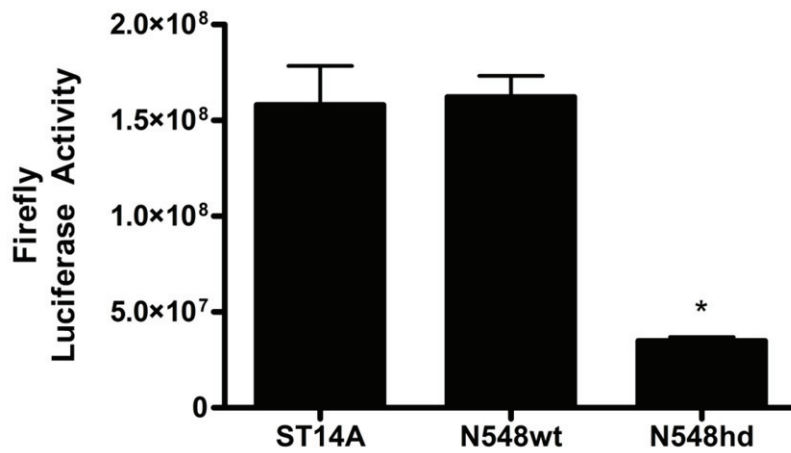
Figure 3.9. CMV-driven luciferase activity was decreased in the presence of N-mHtt. ST14A, N548wt and N548hd cells were transfected with a firefly luciferase reporter plasmid driven by the CMV promoter. Cell lysates were collected 24 h post-transfection and a DLR™ Assay was performed. * $P < 0.05$ relative to ST14A and N548wt cells as determined by a one-way ANOVA followed by a Bonferroni post-hoc test. N=4 per cell type.
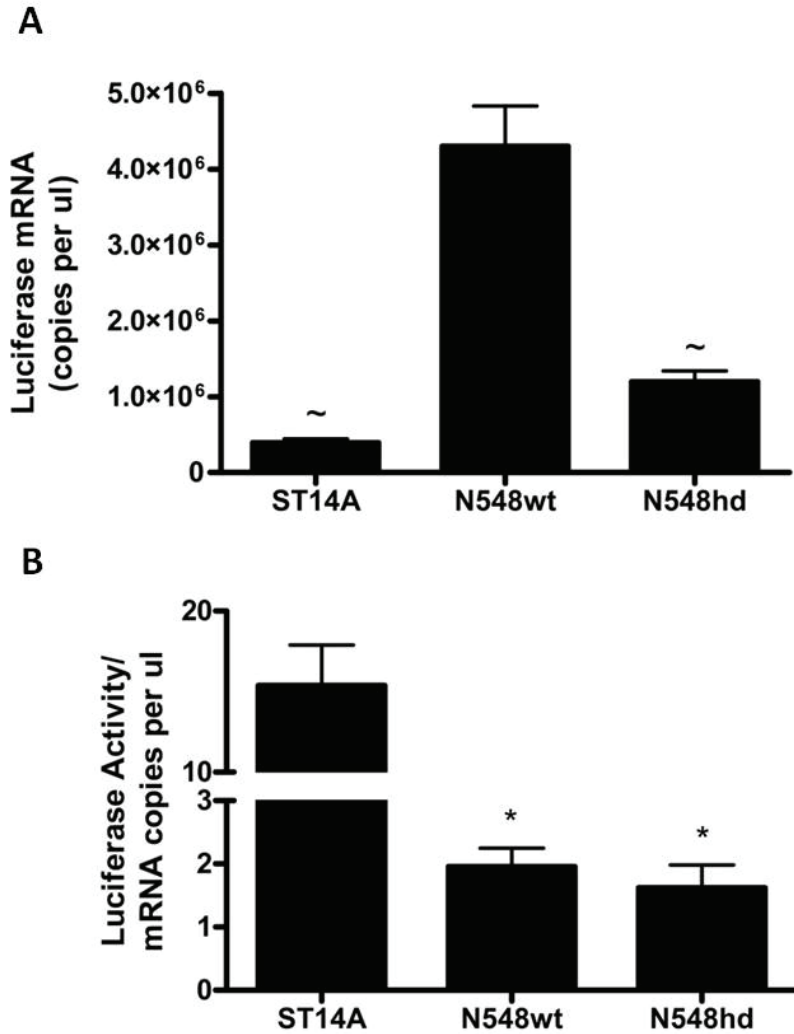
Figure 3.10. The relationship between luciferase activity and *luc+* mRNA copy number was similar in N548wt and N548hd cells but not in ST14A cells. **(A)** mRNA was extracted from cell lysates in CMV activity experiment (Fig 3.9) and quantified. **(B)** Luciferase activity was normalized to *luc+* mRNA for each replicate. ~$P < 0.05$ relative to N548wt, * $P < 0.05$ relative to ST14A as determine by a one-way ANOVA followed by a Bonferroni post-hoc test. N=4 per cell type.

and cell number at the time of the luciferase assay between ST14A and either N548wt or N548hd cells. Based on these observations, it was determined that N548wt cells expressing the N-terminal 548 aas of human Htt with 15 glutamines were the appropriate control for studying the effect of N-mHtt with a pathological length of polyQ on CMV transcription in N548hd cells.

The DLR™ Assay correlates the activity of an experimental promoter with the activity of a control promoter within the same cell (Promega, 2011). The purpose of the control promoter is to negate variability due to differences in cell viability or transfection efficiency between cell lines and between replicates. The TK promoter is commonly used as a control promoter in the DLR™ Assay (Promega, 2011). N548wt and N548hd cells were transfected with a TK reporter plasmid driving expression of *Renilla* luciferase, and *Renilla* luciferase activity was measured 24 h later. *Renilla* activity was significantly decreased in N548hd cells compared to N548wt cells (Fig. 3.11). Normalization of firefly luciferase to *Renilla* luciferase activity did not accurately represent the effect of N-mHtt on CMV-dependent transcription (Data not shown). Instead, we chose to normalize CMV-dependent firefly luciferase and TK-dependent *Renilla* luciferase activity to total protein levels. When normalized to total protein, both CMV activity and TK activity were significantly lower in N548hd cells than in N548wt cells (Fig. 3.12). The activity of the CMV and TK promoters in N548hd cells relative to the activity of the same promoter in N548wt cells was calculated to determine the magnitude of the N-mHtt-mediated decrease in transcriptional activity for both promoters. Despite the large difference in overall activity of the two promoters, there was no difference in the proportion of the N-mHtt-mediated decrease in transcriptional
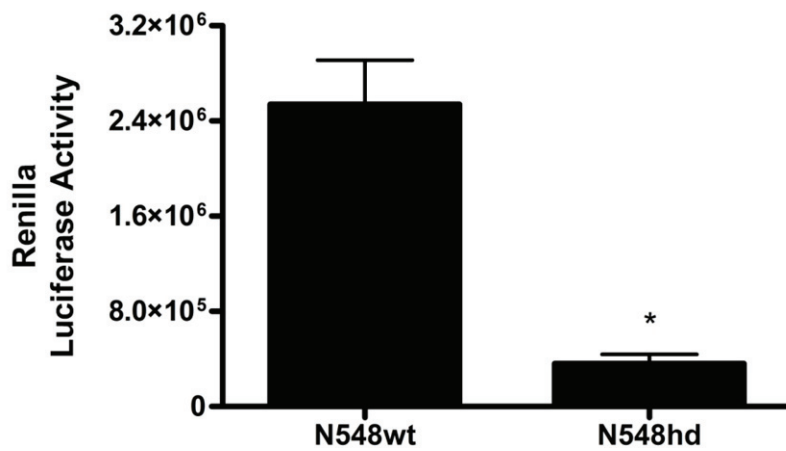
Figure 3.11. Luciferase activity driven by the TK promoter was decreased in N548hd cells. *Renilla* luciferase-expressing reporter plasmid driven by the TK promoter was transfected into N548wt and N548hd cells. *Renilla* activity was measured 24 h later. * $P <$ 0.05 relative to N548wt cells as determined by a two-tailed *t*-test. N=8 per cell type.
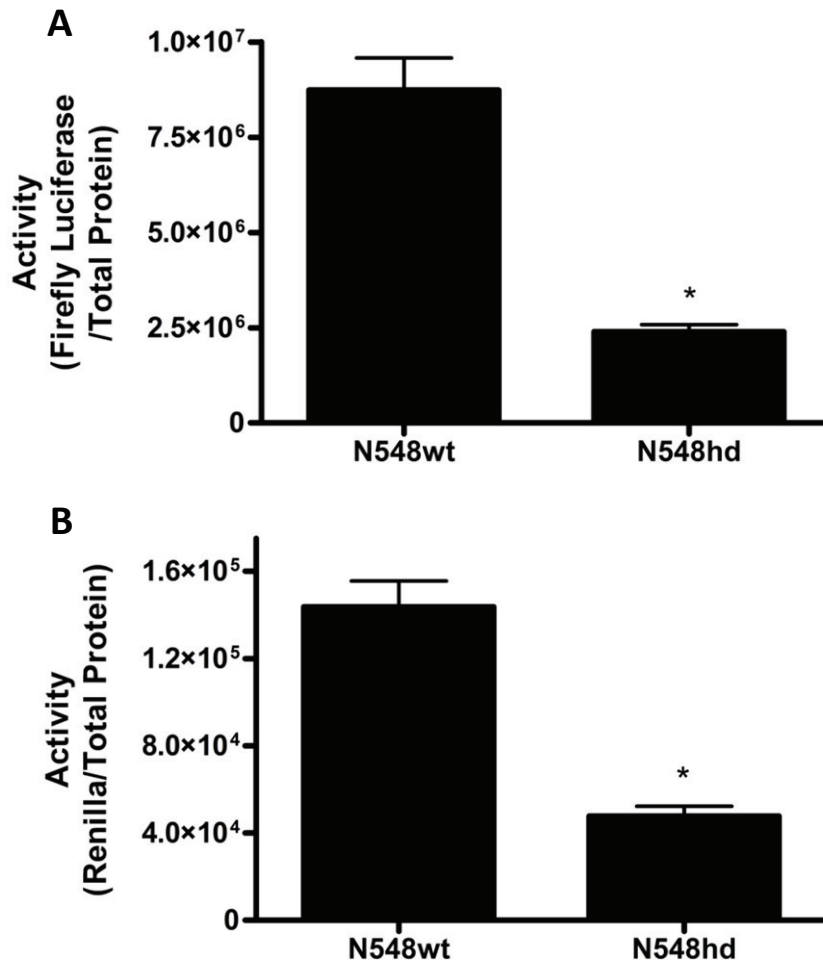
Figure 3.12. CMV and TK activity were decreased in N548hd cells. CMV-driven firefly luciferase activity **(A)** and TK-driven *Renilla* luciferase activity **(B)** were normalized to total protein in cell lysates. * $P < 0.05$ relative to N548wt as determine by two-tailed *t*-test. N=8 per cell type.

activity between the CMV and TK promoters (Fig. 3.13). In the presence of N-mHtt, the activity of both the CMV and TK promoters decreased by ~70% relative to the activity of each promoter in N548wt cells. The ability to measure the activity of the CMV and TK promoters expressed simultaneously allowed us to study the mechanism of N-mHtt-mediated transcriptional dysregulation on two promoters in a common environment.

3.3 Discussion

In vitro transcription assays were performed to investigate the nature and mechanism of N-mHtt-mediated transcriptional dysregulation. Specifically, we wanted to determine whether N-mHtt protein could directly impair expression of specific genes because of the possibility that transcriptional dysregulation is a secondary consequence of N-mHtt-mediated changes in other cellular functions. For example, the neurotrophic theory of HD suggests that mHtt impairs cortical BDNF signaling causing altered expression of some genes in striatal neurons. BDNF is produced in cortical cells and anterogradely transported where it is delivered to striatal cells (Altar *et al.*, 1997). Cortical production and delivery of BDNF has been shown to be impaired in the presence of mHtt (Gauthier *et al.*, 2004; Zuccato *et al.*, 2008). BDNF signaling has been shown to stimulate CREB-mediated transcription (Arthur *et al.*, 2004), which suggests that transcriptional dysregulation in striatal cells could be a secondary result of decreased cortico-striatal BDNF signaling. There is also evidence that mitochondrial dysfunction could lead to impaired transcription. 3-NP-induced mitochondrial inhibition causes elevations in transglutaminase-2 (TG2) activity, which has been attributed to the
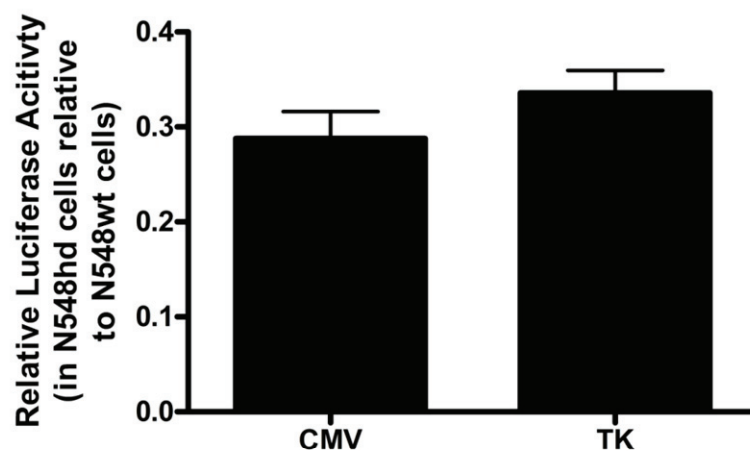
Figure 3.13. Transcription driven by the CMV and TK promoters was equally affected by N-mHtt. Protein-normalized CMV and TK activity in N548hd cells from Figure 3.12 was normalized to the activity driven by the same promoter in N548wt cells. A two-tailed *t*-test was performed to test for a significant difference between the groups. N=8 per cell type.

increased generation of reactive oxygen species (Lesort *et al.*, 2000). Mitochondrial

dysfunction and increased production of reactive oxygen species have been well

characterized in HD (Reviewed in Chen, 2011) and elevated TG2 activity in HD has also

been reported (Lesort *et al.*, 1999). A recent study hypothesized that elevated TG2

activity led to decreased transcription of several genes in HD (McConoughey *et al.*,

2010). TG2 was shown to bind to the *cytochrome C* and *pgc-1α* promoters in a cell

model of HD. Pharmacological and siRNA-mediated inhibition of TG2 reversed

transcriptional dysregulation of both promoters in these cells.

In the present study, addition of recombinant N171-89Q decreased the amount

of 363 nt product generated by the CMV promoter, relative to the amount of product

generated in the presence of recombinant N171-23Q, or in the absence of added

recombinant protein in *in vitro* transcription assays. This suggested that N-mHtt was able

to directly decrease transcription of the CMV promoter, in the absence of intracellular

signaling, mitochondrial dysfunction, elevated levels of reactive oxygen species or as a

secondary consequence of other cellular changes induced by N-mHtt. Other studies have

supported the hypothesis that transcriptional dysregulation of specific genes is a direct

effect of N-mHtt. Primary striatal neurons expressing N-mHtt following transduction

with recombinant lentivirus, and mice selectively expressing mHtt in striatal tissue

undergo transcriptional changes similar to those observed in mice that express N-mHtt

throughout all neuronal phenotypes in the brain, suggesting that mHtt-mediated

transcriptional dysregulation of some genes is cell-autonomous (Runne *et al.*, 2008;

Thomas *et al.*, 2011). In support of these findings, adenoviral vector-mediated siRNA

knockdown of N-mHtt led to a partial recovery of ppENK and DARPP transcript levels

in striatal neurons of R6/1 mice (Rodriguez-Lebron *et al.*, 2005). This suggests that striatal N-mHtt contributes to the intracellular gene expression changes that occur in this mouse model of HD. Moreover, cell models that inducibly express N-mHtt have been used to determine the timeline of cellular changes that follow expression of N-mHtt. Transcriptional dysregulation occurs early after the induction of N-mHtt expression and precedes mitochondrial dysfunction and impairments in neurite outgrowth (Wyttenbach *et al.*, 2001; Sugars *et al.*, 2004; Weiss *et al.*, 2009). Although impaired BDNF signaling, or increased reactive oxygen species resulting from mitochondrial dysfunction may lead to impaired transcription in striatal cells, decreased CMV transcription observed in the presence of N171-89Q in the *in vitro* assay as well as the studies referenced above suggest that transcriptional dysregulation can occur independently of changes in other cellular functions.

In addition to providing evidence that impairment of CMV transcription was a direct effect of N-mHtt, *in vitro* transcription assays also provided insight into the mechanism of N-mHtt-mediated transcriptional dysregulation. It has been argued that the mechanism of N-mHtt-mediated transcriptional dysregulation involves impaired histone modification, resulting in more condensed chromatin folding. Decreased histone acetylation, a post-translational modification that results in a more condensed chromatin structure, has been documented in humans HD patients (Stack *et al.*, 2007), N171-82Q and R6/2 mouse models (Sadri-Vakili *et al.*, 2007; Stack *et al.*, 2007) as well as transiently transfected, stably transfected and inducible cell models of HD (Jiang *et al.*, 2006; Sadri-Vakili *et al.*, 2007). Increased histone methylation was shown in human HD patients (Ryu *et al.*, 2006) and in N171-82Q and R6/2 mouse models (Stack *et al.*, 2007),

and alterations in histone ubiquitation were reported in R6/2 mice as well as in stable and inducible HD cell models (Kim *et al.*, 2008; Bett *et al.*, 2009). One question regarding a chromatin-centered explanation for N-mHtt-mediated transcriptional dysregulation is how histone modification, and therefore impaired chromatin regulation, can explain gene-specific transcriptional dysregulation. The chromatin-centered explanation of gene dysregulation has been supported by a study that analyzed existing microarray data from human HD patients, and reported a correlation between genes exhibiting altered transcription and their chromosomal location, suggesting that impaired regulation of chromatin folding can explain some of the gene-specific alterations reported (Anderson *et al.*, 2008). Additionally, drug treatments that decrease chromatin folding, making DNA more accessible to transcription factors, have been shown in several independent studies to increase transcription and improve the disease phenotype in models of HD (Gardian *et al.*, 2005; Giorgini *et al.*, 2008; Thomas *et al.*, 2008). Therefore, although N-mHtt has demonstrated effects on chromatin structure, *in vitro* transcription assays, using a linear fragment of the CMV promoter that had not been previously exposed to histone proteins demonstrated that N-mHtt can affect transcription in the absence of chromatin and chromatin remodelling. N-mHtt may alter histone modification and chromatin folding *in vivo*, however the results from the *in vitro* assays suggest that transcriptional dysregulation can occur independently of chromatin structure.

Htt has been shown to interact with a large number of proteins, including various gene-specific transcription factors and members of the general transcription machinery [Table 1.1, reviewed in (Li & Li, 2004)]. The affinity of Htt for many of its interaction partners is altered following polyQ expansion (Li & Li, 2004). Some interactions have

increased affinity while others have decreased affinity following polyQ expansion. The impact of polyQ expansion on the binding affinity of Htt with interacting partners highlights the divide between gain-of-function and loss-of-function hypotheses for N-mHtt-mediated transcriptional dysregulation. Some loss-of-function theories of transcriptional dysregulation propose that a role of wild-type Htt is to bind repressor transcription factors and withhold them from the nucleus. PolyQ expansion results in a loss of this function, freeing repressor proteins to bind to promoters and inhibit transcription. This has been demonstrated for the repressor proteins REST (Zuccato *et al.*, 2003; Zuccato *et al.*, 2007; Shimojo, 2008; Marullo *et al.*, 2010) and CtBP (Kegel *et al.*, 2000). Other loss-of-function theories suggest that a role of wild-type Htt is to act as a transcriptional co-activator. Htt has been shown to lose its ability to bind to and regulate transcriptional activation mediated by NFκB (Marcora & Kennedy, 2010) and liver X receptor (Futter *et al.*, 2009) following polyQ expansion. Loss-of-function models would predict similar levels of CMV transcription in the presence of N171-89Q relative to CMV transcription in the absence of recombinant Htt when tested in *in vitro* transcription assays, because the addition of a protein lacking a function would not be expected to decrease the level of promoter activity supported by transcription-competent HeLa extract. Since CMV transcription was decreased in the presence of N171-89Q in the *in vitro* assay, our data supported a gain-of-function, rather than a loss-of-function, mechanism for N-mHtt-mediated transcriptional repression of the CMV promoter.

The ST14A cell line and its N548wt and N548hd derivative cell lines were selected to investigate the mechanism of N-mHtt-mediated transcriptional dysregulation. A striatal cell line was chosen because striatal cells display the highest levels of

transcriptional dysregulation of all neuronal cells, and are the most vulnerable brain cells with regards to neuronal dysfunction and eventual neurodegeneration. Cells expressing the 548 aa fragment of Htt were chosen because the 548 aa fragment length approximates the N-terminal fragments generated through caspase-mediated cleavage of full-length Htt *in vivo*. The 548 aa fragment translocates to the nucleus and causes cellular toxicity. Studies that have employed these cell lines have compared N548hd cells to both N548wt and the parental ST14A cell lines (Zuccato *et al.*, 2003; Zuccato *et al.*, 2007), N548hd cells to ST14A cells (Wang *et al.*, 2005; Zhang *et al.*, 2006), and N548hd cells to N548wt cells (Ermak *et al.*, 2009). Our goal was to determine an appropriate control for the effect of N-mHtt on transcription. ST14A cells represent striatal cells in a wild-type state, with two normal copies of the *huntingtin* gene. N548wt cells are able to control for the high level of N-terminal Htt expressed under the control of the viral LTR promoter, and are better able to isolate the impact of overexpression of N-terminal Htt with an expanded polyQ. In the present study, the cell lines were examined for similarities and differences in morphology, growth rate, transfection efficiency, total protein level per cell number, and the relationship between production of *luc+* mRNA from reporter CMV plasmids and the resulting luciferase activity. The N548hd and N548wt cells were morphologically similar, grew and survived at a similar rate, and were transfected at equal efficiency. Most importantly, there was a linear relationship between CMV-driven transcription of reporter plasmids and quantifiable luciferase activity for both the N548wt and N548hd cells. While N548wt and N548hd cells were similar in all regards, ST14A cells differed from one or both cell lines with respect to several characteristics. ST14A cells were morphologically larger than both N548wt and N548hd cells. Quantification of

cell numbers revealed fewer ST14A cells than N548wt or N548hd cells after plating the

same number of cells and growing to confluence. Despite the fact that cells were grown

to full confluence, cell lysate collected from ST14A cells contained significantly more

protein than N548hd lysates. Normalizing luciferase activity to *luc+* mRNA levels

revealed a discrepancy in the relationship between transcription of the reporter plasmid

containing a CMV promoter and luciferase activity in ST14A cells compared to both

N548wt and N548hd cells. Together, this collection of data suggested that ST14A cells

were fundamentally different than N548wt and N548hd cells and, therefore, were not an

appropriate control for studying N-mHtt-mediated transcriptional dysregulation. To

determine the effect of polyQ expansion within the context of N-terminal Htt on

transcription, we compared activity in N548wt and N548hd cells.

The purpose of normalization is to ensure that the measurement of the

independent variable in an experiment is not adversely affected by global cellular

changes or experimental error. The pRL-TK plasmid, which uses the TK promoter to

drive *Renilla* luciferase expression, has been used to normalize firefly luciferase activity

in studies of N-mHtt-mediated transcriptional dysregulation (Liang *et al.*, 2009). The TK

promoter, in fact, is recommended in the DLR™ Assay protocol for internal

normalization (Promega, 2011). Prior to using the pRL-TK plasmid to normalize CMV

activity in N548wt and N548hd cells, its activity was tested in these cells. *Renilla*

activity driven by the TK promoter was decreased in N548hd cells relative to N548wt

cells. Since transfection efficiency and cell growth were similar between N548wt and

N548hd cells, the decreased *Renilla* activity in N548hd cells was attributed to decreased

transcription driven by the TK promoter. TK activity was affected by expression of the

N-terminus of mHtt in N548hd cells and therefore the pRL-TK plasmid could not be used to normalize luciferase activity.

While ideally we would have liked to internally normalize every promoter activity assay, we have not yet found a plasmid-encoded promoter that does not have significantly lower promoter activity in N548hd cells compared to N548wt cells. Previous studies examining the effect of N-mHtt on promoter activity have normalized experimental promoter reporter activity to activity of *Renilla* luciferase or β-galatosidase activity driven by the TK (Liang *et al.*, 2009), SV40 (Godavarthi *et al.*, 2009), EF-1α (Wyttenbach *et al.*, 2001), and CMV (Yohrling *et al.*, 2003) promoters. The activity of the promoter used for normalization in these studies was not reported, therefore it is assumed that it was unaffected by the presence of N-mHtt or that the relative effect of N-mHtt on the experimental reporter was greater than that of the internal control. The activity of the SV40, CMV and TK promoters has been described in primary striatal cells and in PC12 cells transiently transfected with exon 1 of *huntingtin* containing 25 or 109 CAG repeats (Chiang *et al.*, 2005). Activity driven by the SV40 and TK promoters was decreased in the presence of N-mHtt in both cell types, however CMV activity was not affected (Chiang *et al.*, 2005). This study indicated that the effect of N-mHtt on promoter activity is influenced by the cellular context. This supports our finding that selection of a promoter for normalization in DLR™ assays requires preliminary analysis within each cell type tested.

One normalization technique that is independent of transcriptional activity and has been used for the analysis of N-mHtt-mediated transcriptional repression is normalizing to total protein (Bae *et al.*, 2005; Rigamonti *et al.*, 2007). A disadvantage of

this method is that it does not normalize for differences in transfection efficiency. Although transfection efficiency may vary slightly between replicates, overall transfection efficiency was not different between N548wt and N548hd cell lines, minimizing the importance of using a normalization method that corrects for transfection efficiency. Normalizing to total protein does correct for variability in cell plating, and acts as a control to ensure cell survival of each replicate. We chose to normalize CMV-driven luciferase activity to the amount of total protein in each cell lysate.

Since TK-driven *Renilla* activity was decreased in N548hd cells relative to N548wt cells, the TK promoter can also be studied to understand the mechanism of N-mHtt-mediated transcriptional dysregulation. Luciferase activity driven by the TK promoter was nearly two orders of magnitude lower than activity driven by the CMV promoter in N548wt cells. Despite the discrepancy in the relative strength of the two promoters, transcription driven by the CMV and TK promoters was decreased by approximately the same magnitude in the presence of N-mHtt. This suggested that the ability of N-mHtt to inhibit transcription, as well as the degree to which transcription was inhibited at a specific promoter was not simply a function of the transcriptional activity of that promoter. The ability to study the CMV and TK promoters in the same cells and under the same conditions will provide insight into whether a common mechanism of N-mHtt-mediated transcriptional dysregulation applies to multiple affected promoters.

Chapter 4:

N-mHtt is Present in Promoter-Bound Protein Complexes

and Overexpression of General Transcription Factors Does Not Recover

N-mHtt-Mediated Transcriptional Repression

4.1 Introduction

Decreased expression of a subset of genes is a characteristic feature of HD (Hodges *et al.*, 2006). The work presented in Chapter 3 demonstrated that transcription driven by the CMV promoter was directly decreased in the presence of N-mHtt in *in vitro* transcription assays, and that polyQ expansion within the N-terminal fragment of mHtt that accumulates in the nucleus decreased transcription driven by the CMV and TK promoters in a cell culture model of HD. The work presented in Chapter 3 also established the CMV and TK promoters as model promoters for studying the mechanism of N-mHtt-mediated transcriptional dysregulation.

Numerous mechanisms have been proposed to explain how N-mHtt impairs transcription. The majority of the mechanisms proposed for a direct, gain-of-function N-mHtt-mediated impairment of gene expression are based on an interaction between N-mHtt and specific proteins involved in the regulation of transcription (Li & Li, 2004; Cha, 2007). Some suggest that an abnormal interaction between nuclear N-mHtt and transcription factors and co-activators results in recruitment of such protein into NIIs, reducing the amount of soluble transcription factor able to perform its functional role in transcription (Steffan *et al.*, 2000). Some studies suggest that soluble N-mHtt interacts with specific transcription factors (Chiang *et al.*, 2005; Chen-Plotkin *et al.*, 2006),

inhibiting the protein from binding to DNA and performing its function without reducing soluble levels of the transcription factor. One study suggested that N-mHtt itself acts as a DNA-binding protein (Benn *et al.*, 2008), inhibiting transcription factors from associating with the promoter and, therefore, acting as a direct transcriptional repressor. The work in this chapter attempted to determine if transcriptional repression of the CMV and TK promoters was caused by some, or any, of the proposed mechanisms. We also sought to test the effect of overexpressing transcription factors previously shown to physically associate with mHtt and that were proposed to be involved in the mechanism of transcriptional dysregulation.

N-mHtt-mediated transcriptional dysregulation was examined from two perspectives. The first perspective examined was how the DNA sequence of a N-mHtt-affected promoter contributes to its own susceptibility to transcriptional dysregulation. Since certain genes have decreased expression in the presence of N-mHtt while most do not, and the expression level of a specific gene is intimately tied to the strength of its promoter, the promoter of a N-mHtt-affected gene may hold information regarding the mechanism of promoter-specific transcriptional dysregulation. Work performed in this chapter attempted to determine the specific region of the N-mHtt-affected CMV promoter necessary for impaired transcription in the presence of N-mHtt. The CMV promoter was also examined to determine if transcriptional activation, or lack thereof, mediated by a gene-specific transcription factor binding site of a protein known to interact with N-mHtt was responsible for N-mHtt-mediated transcriptional dysregulation of the CMV promoter.

The second perspective examined used knowledge of previously identified N-mHtt-interacting transcriptional proteins, as well as information obtained following examination of the CMV promoter to attempt to determine how N-mHtt inhibits transcription of the CMV and TK promoters. N-mHtt has been shown to bind directly to RAP30, a member of the general transcription factor TFIIF, and overexpression of RAP30 was able to restore transcriptional repression of a 75 bp promoter fragment of the dopamine D_2 receptor in primary striatal cells transiently transfected with N-mHtt (Zhai *et al.*, 2005). This study hypothesized that N-mHtt binds to and sequesters RAP30, reducing the amount of functional TFIIF available for recruitment into transcriptional complexes. To test this hypothesis, experiments were performed to determine if TFIIF binding to the CMV promoter was decreased in N548hd cells, and whether overexpression of the components of TFIIF could alleviate transcriptional repression. The general transcription factor TBP has been identified in NIIs in humans (van Roon-Mom *et al.*, 2002) and in aggregates *in vitro*, which led to a decreased TBP association with DNA (Schaffar *et al.*, 2004). These studies suggest that polyQ expansion of N-mHtt results in an increased association with TBP, decreasing the amount of TBP available for the formation of transcriptional complexes. To test whether this hypothesis can explain transcriptional repression of the CMV or TK promoters, TBP was overexpressed in the presence of N-mHtt.

Lastly, in addition to testing whether hypothesized mechanisms of N-mHtt-mediated transcriptional dysregulation apply to impaired transcription of the CMV and TK promoters, the work in this chapter attempted to provide new information regarding

whether N-mHtt impairs transcription by sequestering proteins or by directly associating at the DNA.

4.2 Results

To localize the region of the CMV promoter necessary for N-mHtt-mediated transcriptional repression, a promoter deletion analysis was performed. Templates incorporating the 68, 268 and 772 bp of the CMV promoter 5' to the transcription start site (-68 CMV, -268 CMV, -772 CMV) with a 363 bp coding sequence 3' to the transcription start site were produced via digestion of the pUC18-CMV plasmid using restriction enzymes. The -772, -268 and -68 CMV promoter templates were used to generate product in *in vitro* transcription assays as a preliminary non-quantitative assessment of different regions of the CMV promoter (Fig. 4.1). Product generated using the -268 CMV promoter appeared to be increased relative to product generated using the -772 CMV promoter, suggesting that repressor elements were present in the deleted region. The product generated using the -68 CMV promoter was barely detectable, suggesting that activator elements reside in the region between 69 and 268 bp 5' to the transcription start site. The addition of 1 pmol of N171-23Q to the reaction appeared to cause a slight increase or no change in transcription driven by the -772 CMV promoter (Fig. 4.1, and data not shown). Addition of N171-23Q to the reaction containing the -268 CMV promoter did not appear to alter transcription (Fig. 4.1, and data not shown). These results suggest that the 171 aas of human Htt with a non-pathogenic length of glutamines did not inhibit transcription driven by the CMV promoter. Addition of 1 pmol of N171-89Q appeared to decrease transcription driven by the -772 and -268 CMV promoters
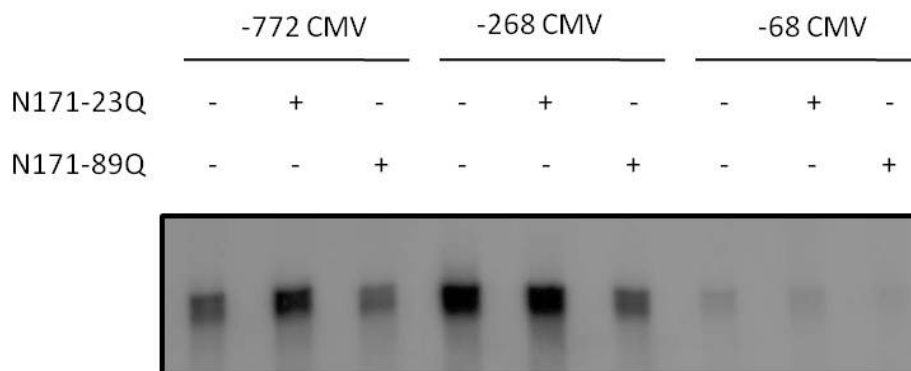
Figure 4.1. The -268 CMV promoter had high activity and decreased transcription in the presence of N-mHtt *in vitro*. CMV promoter fragments consisting of 772 (-772 CMV), 268 (-268 CMV) and 68 (-68 CMV) bp immediately upstream of the CMV transcription start site were used to drive transcription of a 363 nt mRNA product in an *in vitro* transcription assay. Recombinant protein consisting of the N-terminal 171 aas of human Htt containing either 23 (N171-23Q) or 89 (N171-89Q) glutamines was added to the *in vitro* assay to determine the effect of N-mHtt on CMV-driven transcription. ^{32}P-labeled mRNA products were fractionated on a denaturing polyacrylamide gel and exposed to film. The 363 nucleotide products are shown above.

relative to transcripts produced by those promoters in the presence of N171-23Q, or in the absence of added recombinant N-Htt (Fig. 4.1). Due to the fact that transcription generated using the -68 CMV promoter was barely detectable, determining the impact of N171-23Q and N171-89Q on the ability of this promoter fragment to drive transcription was not possible. The *in vitro* promoter deletion experiment suggested that strong activator elements reside in the -69 to -297 region of the CMV promoter, and that the addition of N171-89Q appeared to decrease transcription driven by the smallest active fragment of the CMV promoter. Since product generated in the *in vitro* transcription assay was not quantified, the CMV promoter deletion fragments were tested in the N548 cell model.

Transcription driven by the CMV promoter was decreased in N548hd cells relative to N548wt cells (Fig. 3.12). To determine if a specific region of the CMV promoter mediated transcriptional dysregulation in the presence of 548 aa N-mHtt in striatal cells, and to quantify the effect of CMV promoter deletion, fragments of the CMV promoter were generated by PCR amplification and were cloned into pGL3-Basic luciferase reporter plasmids. Reporter plasmids containing fragments of the CMV promoter spanning 772, 297 and 99 bp 5' to the transcription start site (-772 CMV, -297 CMV, and -99 CMV) were transfected into N548wt and N548hd cells. Activity driven by the -297 CMV promoter was significantly lower than activity produced by the -772 CMV promoter (Fig. 4.2), suggesting that activator elements reside within the region between 297 and 772 bp on the CMV promoter. Further deletion to 99 bp dramatically reduced CMV activity compared to the -297 CMV promoter, suggesting that additional activator elements are contained between 100 and 297 bp on the CMV promoter.
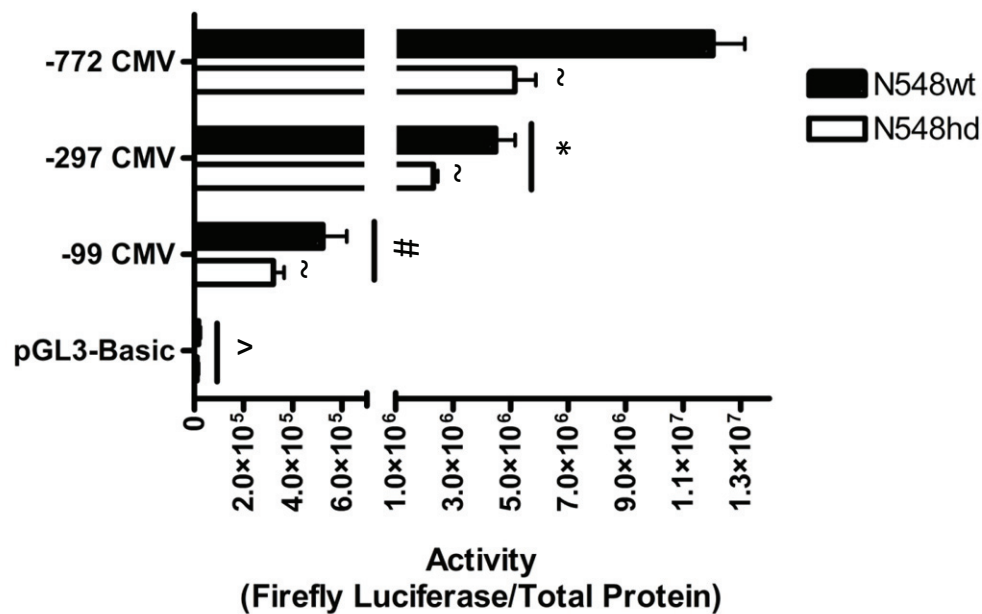
Figure 4.2. Transcription driven by the -99 CMV promoter was inhibited by N-mHtt. The -772 CMV promoter was sequentially deleted to 297 (-297 CMV) and 99 (-99 CMV) bp upstream of the transcription start site and promoters were inserted into pGL3-Basic reporter plasmid. Activity of these plasmids in N548wt and N548hd cells is shown normalized to total protein. * $P < 0.05$ relative to -772 CMV. # $P < 0.05$ relative to -772 CMV and -297 CMV. ^ $P < 0.05$ relative to -772 CMV, -297 CMV and -99 CMV ~ $P < 0.05$ relative to N548wt cells as determined by two-way ANOVA followed by a Tamhane's T2 post-hoc test for unequal variance to analyze effect of promoter deletion, and one-tailed t-test to analyze cell-type effect. N=8 per data set.

Activity of the -99 CMV promoter was significantly higher than the background activity produced by empty pGL3-Basic plasmid, which indicated that deletion of the CMV promoter to the 99 bp proximal to the transcription start site did not completely eliminate transcriptional activity. Examining N-mHtt-mediated transcriptional dysregulation of the CMV promoter, activity of the -772, -297 and -99 CMV promoters was significantly lower in N548hd cells relative to N548wt cells. Activity of the pGL3-Basic control plasmid was not significantly different in N548hd cells compared to N548wt cells, suggesting that N-mHtt-mediated repression of luciferase activity was specific to the CMV promoter. Deletion of regions of the CMV promoter lowered activity but did not alleviate N-mHtt-mediated transcriptional dysregulation of the CMV promoter. It appeared that the N-mHtt-mediated repression was localized to the region within 99 bp of the transcription start site.

It has been hypothesized that the mechanism of gene-specific N-mHtt-mediated transcriptional dysregulation involves N-mHtt interacting with and sequestering gene-specific transcription factors. If transcription driven by the CMV promoter relied on any of these co-activators, co-repressors, or gene-specific transcription factors, functional impairment of that protein resulting from its interaction with N-mHtt could explain the decreased CMV activity observed in N548hd cells. To determine if repression of CMV transcription was mediated by interaction between N-mHtt and one or more gene-specific transcription factors, the CMV promoter was examined for the presence of putative binding sites for the transcriptional proteins identified in Table 1.1. MatInspector online software, provided by Genomatix software suite, was used to analyze the CMV promoter. The region spanning 297 bp 5' to the transcription start site was chosen for analysis.

Although promoter deletion analysis of the CMV promoter suggested that the region of the promoter between 100 and 297 bp was not required for N-mHtt-mediated transcriptional repression of CMV activity, this region was included in the promoter analysis as it has not, yet, been determined whether N-mHtt inhibits CMV transcription through interaction with one, or multiple transcription factors. Since the 100 to 297 bp region of the CMV promoter was responsible for a large degree of transcriptional activity, this region was included in the promoter analysis. The -297 CMV promoter contained three putative binding sites for nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), three CREB-response element (CRE) sites, one Sp1 binding site, one binding site for CCAAT-enhancer binding protein (C/EBP) and a TBP binding site (Fig. 4.3). Binding sites for REST and p53 were not detected in the analysis. Involvement of the co-activator and co-repressor complexes including CBP, C-terminal binding-protein (CTBP), nuclear co-repressor-1 (NCoR), and Mediator could not be determined using this analysis as they do not directly bind the CMV promoter DNA.

If N-mHtt impaired the activation of transcription mediated by proteins that interact with one or more of the candidate binding sites, we predicted that removal of the binding site would alleviate N-mHtt-mediated repression of CMV transcription. The putative binding sites for transcription factors known to interact with N-mHtt situated within the 297 bp of the CMV promoter upstream of the transcription start site identified in Figure 4.3 were eliminated using linker scanning mutagenesis. The candidate sequences were replaced with a 14 bp DNA sequence that did not contain a transcription factor binding site, which eliminated individual or multiple transcription factor binding sites while maintaining the relative spacing of the remaining elements in the CMV
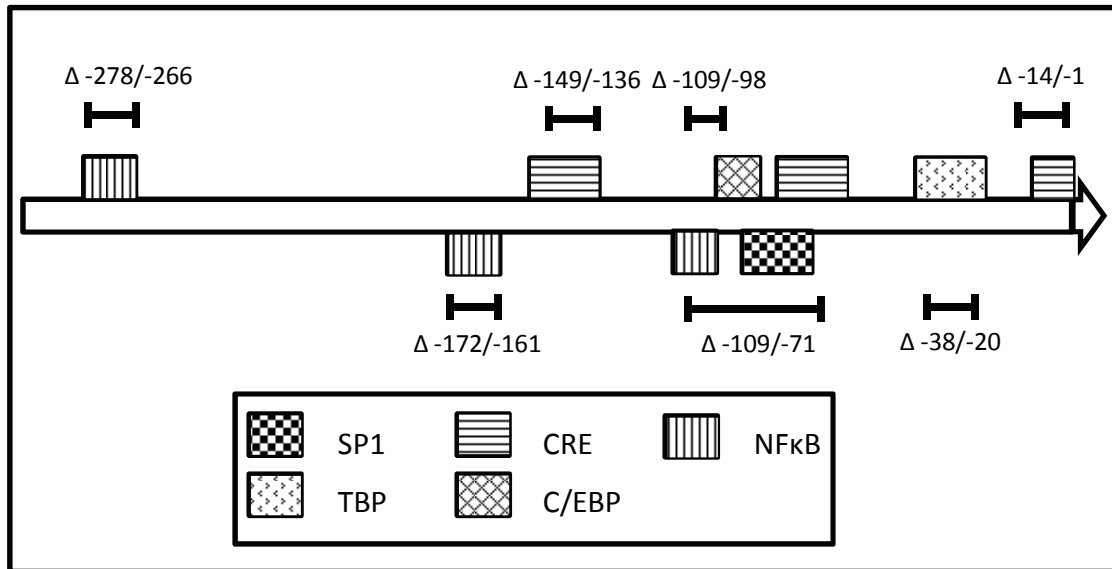
Figure 4.3. Putative binding sites for transcription factors of interest on the -297 CMV promoter. Binding sites for Sp1, TBP, CREB, C/EBP, and NFκB present on the CMV promoter within the 297 bp upstream of the transcription start site are shown. Bars indicate the region of the promoter mutated using linker scanning mutagenesis, while the numbers indicate the location of these mutations, relative to the transcription start site.

promoter. The altered promoter sequences created via insertion of the linker sequence were sequenced to verify the recognition element was replaced by the linker and that no new transcription factor binding site had been created during plasmid engineering.

Using linker scanning deletion mutants, the activity of the CMV mutants was tested in N548wt and N548hd cells. Mean activity of all of the CMV promoter mutants except Δ -38/-20 remained unchanged relative to the original CMV promoter (Fig. 4.4). In contrast, mutation of the TATA box located between 20 and 38 bp 5' to the transcription start site (Δ -38/-20) led to a significant decrease in CMV promoter activity. This suggested that TBP was involved in activation of CMV promoter activity, whereas the recognition elements for NFκB, CREB, C/EBP and Sp1 did not contribute significantly to CMV activity. Activity of every mutant promoter tested was significantly lower in N548hd cells relative to N548wt cells, indicating that no site tested was solely responsible for N-mHtt-mediated repression of the CMV promoter. Eliminating DNA binding sites within the smallest affected region of the CMV promoter for transcription factors implicated in N-mHtt-mediated transcriptional dysregulation did not relieve N-mHtt-mediated repression of CMV transcription, suggesting that gene-specific transcription factors were not the sole mediators of N-mHtt-mediated transcriptional dysregulation of the CMV promoter.

Promoter deletion experiments examining the DARPP-32 (Gomez *et al.*, 2006), adenosine A2A receptor (Chiang *et al.*, 2005), and PDE10A (Hu, 2007) promoters reported similar results to those obtained using the CMV promoter, as the minimally active promoter fragment tested for each of the promoters had decreased activity in the presence of N-mHtt. The fact that N-mHtt was able to impair transcription driven by the
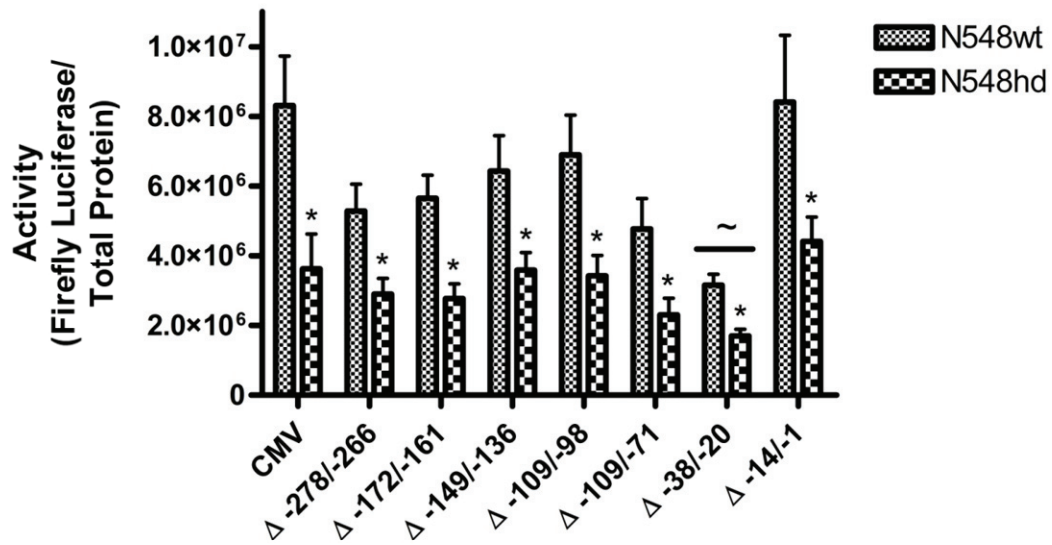
Figure 4.4. Mutation of specific DNA sequences did not relieve N-mHtt-mediated transcriptional repression of the CMV promoter. The DNA sequences on the CMV promoter indicated (relative to the transcription start site) were mutated using linker scanning mutagenesis and inserted into pGL3-basic reporter plasmid. Activity of these plasmids in N548wt and N548hd cells is shown normalized to total protein. $*$ $P < 0.05$ relative to activity of the same plasmid in N548wt cells, $\sim P < 0.05$ relative to wild-type CMV promoter as determined by a two-way ANOVA followed by a Bonferonni post-hoc test. One-tailed t-test performed to analyze cell effect within each mutation. N=8 per data set.

minimally active fragment of multiple promoters, and that a gene-specific transcription

factor could not be implicated in repression of CMV transcription suggested that N-mHtt

impaired transcription by interfering with the assembly or function of the general

transcription machinery. Based on the study by Zhai *et al.* (2005) suggesting that N-mHtt

inhibits the formation and function of TFIIF through interaction with RAP30, we

hypothesized that overexpression of RAP30 would restore TFIIF levels and increase

transcription driven by model promoters including the CMV and TK promoters. cDNA

coding for full-length human RAP30 was cloned into the pcDNA mammalian expression

vector and was co-transfected with CMV and TK reporter plasmids. To control for the

presence of the pcDNA plasmid in the cells, pcDNA plasmid lacking RAP30 cDNA was

used as an empty vector control. CMV and TK activity were assayed 24 h following

transfection. Analysis of activity demonstrated that expression of human RAP30 in

N548wt cells did not significantly increase CMV transcription (Fig. 4.5A). RAP30

expression in N548hd cells also did not alter CMV activity. In contrast, overexpression

of RAP30 increased TK activity significantly in N548wt cells relative to empty vector

control (Fig. 4.5B). Two possibilities exist to explain this result. RAP30 may have been

the limiting factor in transcription driven by the TK promoter, and thus, by increasing the

amount of the limiting factor, TK transcription was increased in N548wt cells. The

second possibility is that N-terminal Htt in N548wt cells may have been repressing TK

activity through inhibition of RAP30 function. By increasing the amount of functional

RAP30 in N548wt cells, this inhibition was alleviated and TK activity was increased. In

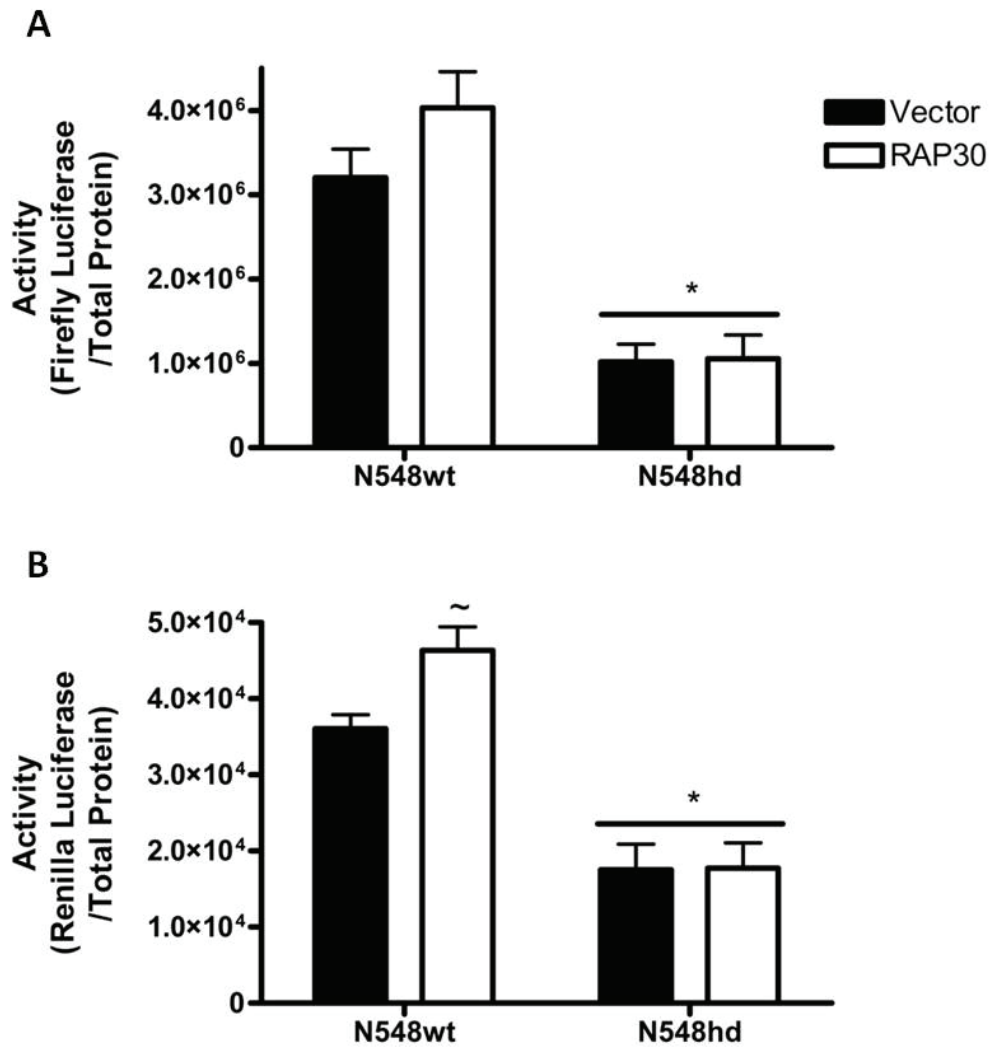the presence of N-mHtt, expression of RAP30 in N548hd cells did not increase

Figure 4.5. Overexpression of RAP30 protein did not recover N-mHtt-mediated transcriptional repression. N548wt and N548hd cells were transfected with reporter plasmids driven by either the CMV (**A**) or TK (**B**) promoters and either an empty expression plasmid or one driving production of human RAP30 protein. Luciferase activity was normalized to total protein. *$P < 0.05$ relative to N548wt as determined by a two-way ANOVA. Two-tailed *t*-tests were performed to determine cell-specific RAP30 effect. N=8 per data set.

transcription driven by the TK promoter, suggesting that N-mHtt inhibited the mechanism by which RAP30 increased TK activity in N548wt cells.

To ensure that transfection with the pcDNA vector containing RAP30 cDNA led to increased expression of RAP30 in the cells, western blotting was performed using an antibody raised against recombinant full-length human RAP30. An immunoreactive band at a molecular weight of approximately 30 kDA, corresponding to the predicted size of RAP30, was detected 24 h after transfection of cells with pcDNA-RAP30 plasmid but not after transfection with pcDNA control (Fig. 4.6), despite the fact that the antibody used was predicted by the manufacturer to react with rat RAP30. Based on the predicted size of the detected protein only in cells transfected with the RAP30 expression vector, it appeared that the antibody detected recombinant human but not endogenous rat RAP30. Endogenous RAP30 may have been expressed at relatively low levels compared to the overexpressed human RAP30. The membrane was stained with amido black to ensure equal loading. Western blotting results suggested that transfection with pcDNA-RAP30 plasmid resulted in increased expression of RAP30 protein, however the increased RAP30 expression did not increase CMV or TK activity in N548hd cells. Levels of N-mHtt in N548hd cells are much higher than levels of mHtt generated under control of the *huntingtin* promoter. It is possible that the level of RAP30 produced using the pcDNA-RAP30 expression vector was not sufficient to compensate for an interaction between N-mHtt and RAP30 in N548hd cells.

The hypothesis that N-mHtt binds to and sequesters RAP30 from promoters predicts that the amount of RAP30 associated with DNA would be reduced in the presence of N-mHtt. To test this hypothesis, ChIP was performed to quantify RAP30
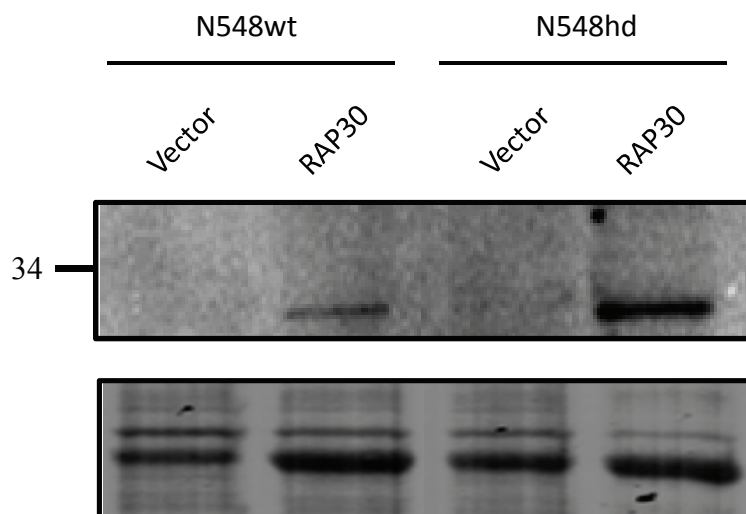
Figure 4.6. Transfection with pcDNA vector containing human RAP30 cDNA led to RAP30 expression in N548wt and N548hd cells. N548wt and N548hd cells were transfected with the pcDNA mammalian expression vector containing cDNA coding for human RAP30 (RAP30) or empty vector control (Vector). Western blotting was performed using an antibody specific to human RAP30. Amido black staining is shown below. Number on left of blot indicates relative mobility of molecular weight size markers in kDA.

association with the CMV promoter in N548wt and N548hd cells. N548wt and N548hd

cells were transfected with the -772 CMV reporter plasmid, which was shown to be

highly active in both N548wt and N548hd cells, but have decreased activity in N548hd

cells relative to N548wt cells (Fig. 3.12). The following day, the cells were incubated in

formaldehyde to stabilize protein-protein and protein-DNA interactions. The cells were

collected, and antibody specific to RAP30 was used to precipitate the protein along with

all protein and DNA associated with it. qPCR was performed to determine the amount of

CMV reporter plasmid in the immunoprecipitated sample as an indirect measure of

RAP30 association with the CMV promoter. The amount of DNA detected in the

immunoprecipitated fraction was expressed as a percentage of the DNA in the total lysate

and was then normalized to the amount of CMV precipitated using non-specific IgG

antibody. The presence of N-mHtt in N548hd cells did not decrease RAP30 directly

bound to, or in complexes bound to the CMV promoter (Fig. 4.7A). The amount of CMV

promoter associated with RAP30, however, was not enriched relative to the negative

control in either N548wt or N548hd cells, indicated by a fold PI value of less than 1.0.

Western blotting was performed to confirm the specificity of the immunoprecipitation. A

RAP30-immunoreactive band was present in the sample immunoprecipitated with

RAP30-specific antibody from both N548wt and N548hd cells, and RAP30-

immunoreactive bands were absent from the non-specific IgG sample in both cell types

(Fig. 4.7B).

A previous study demonstrated that the ability to detect RAP30 binding at the

hsp70 promoter was lost following activation of transcription (Lebedeva *et al.*, 2005).

Polytene chromosome staining and ChIP analysis were performed to detect the binding of
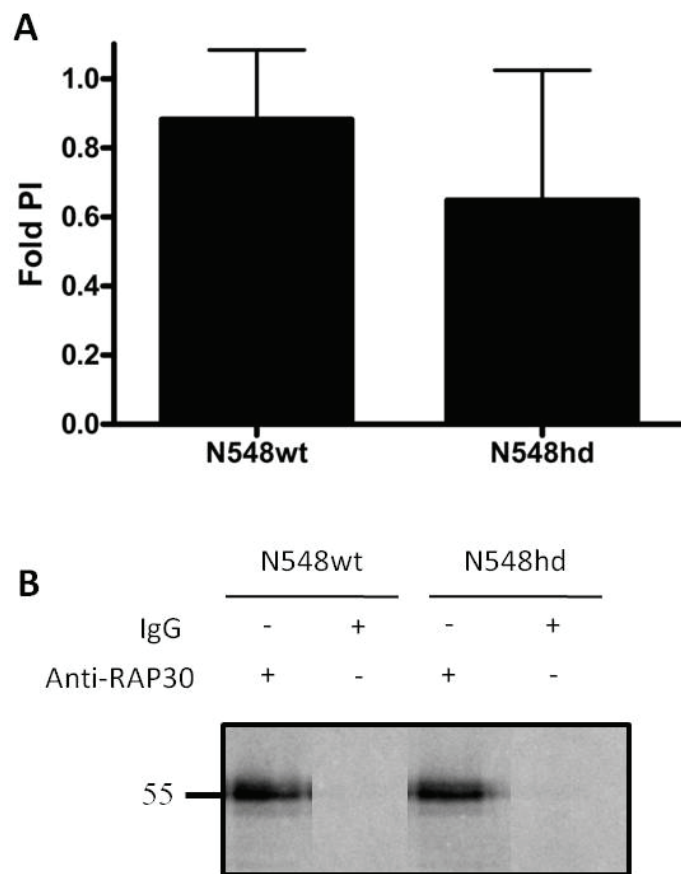
Figure 4.7. RAP30 protein was not specifically detected at the
CMV promoter. **(A)** ChIP was performed using protein from
N548wt and N548hd cells transfected with reporter plasmid driven
by the CMV promoter. Data was expressed as the amount of CMV
reporter plasmid precipitated using RAP30 antibody as a
percentage of the total amount of CMV in the cell lysate,
normalized to the amount of CMV reporter plasmid precipitated
using non-specific IgG antibody as a percentage of the total
amount of CMV in the cell lysate. **(B)** Protein precipitated using
RAP30-specific, and non-specific IgG antibody was fractionated
using SDS-PAGE and probed with a RAP30-specific antibody.
The number on the left represents the relative mobility of a
molecular weight size marker in kDa. Statistical significance was
analyzed by a two-tailed *t*-test with a significance level of 0.05.
N=3 for each cell type.

a number of transcription factors, including RAP30, at the *Drosophila hsp70* gene. RAP30 was determined to be associated with the *hsp70* promoter in the absence of heat shock, but absent from the promoter following activation of *hsp70* expression using heat-shock. Similar results were observed for TBP, TFIIB and several TAFs, but not for PolII, TFIIH, and Mediator, among others. The researchers hypothesized that either RAP30 was absent from the promoter following activation, or that RAP30 was only transiently present at the active promoter and this transient interaction could not be detected using the techniques employed. The CMV promoter is a highly active promoter, and it is possible that RAP30 was not detected because of this high activity. The study referenced did not indicate whether RAP30 would be expected to be present at a less active promoter, however RAP30 has been successfully detected at several gene promoters using ChIP (Cojocaru *et al.*, 2008). ChIP was repeated using N548wt and N548hd cells transfected with a HPRT reporter plasmid to determine if RAP30 association was reduced in the presence of N-mHtt. The HPRT promoter was less active in N548wt and N548hd cells compared to the CMV promoter, and HPRT activity was decreased in N548hd cells relative to N548wt cells (Fig. 4.8A). RAP30 association with the HPRT promoter was enriched in the antibody-specific sample relative to immunoprecipitation with non-specific IgG antibody (Fig. 4.8B). The amount of RAP30 associated with the HPRT promoter was not decreased in N548hd cells relative to N548wt cells. Based on ChIP analysis, RAP30 was not sequestered from HPRT as an HD-affected promoter in the presence of N-mHtt. If N-mHtt physically binds to RAP30 in N548hd cells, this interaction did not inhibit RAP30 from binding to the HPRT promoter.
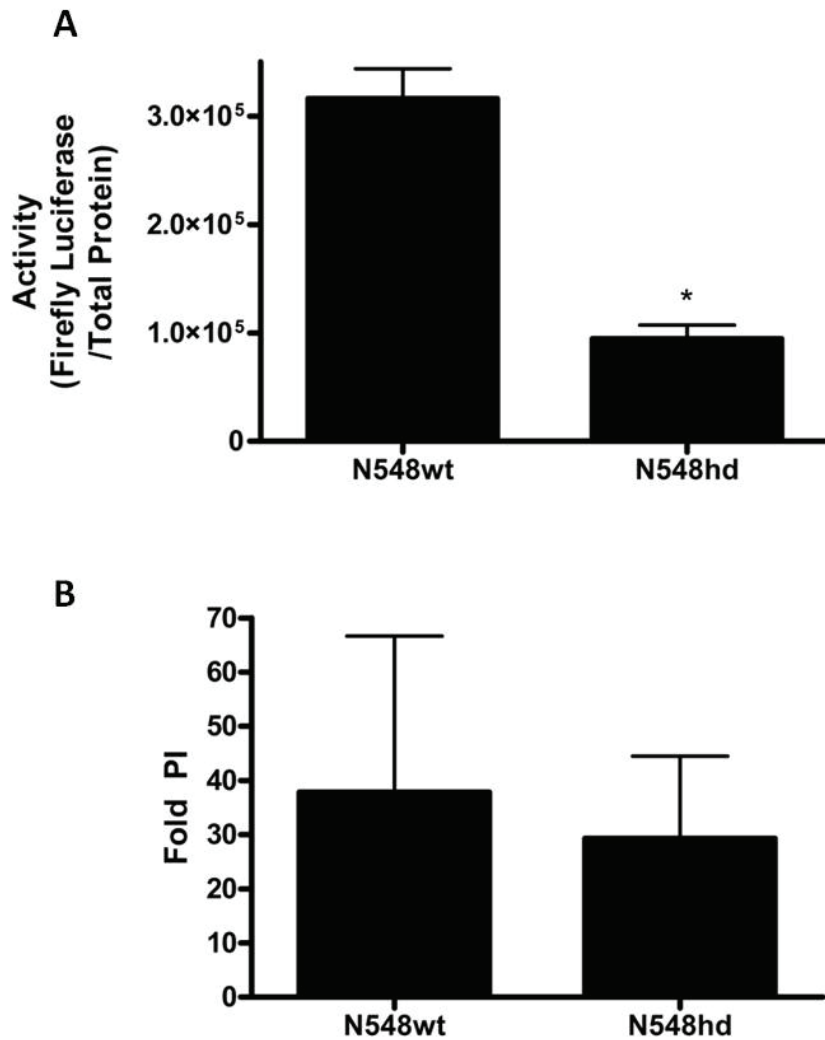
Figure 4.8. RAP30 protein was not sequestered from the N-mHtt-affected HPRT promoter. **(A)** Reporter plasmid driven by the HPRT promoter was transfected into N548wt and N548hd cells. Firefly luciferase activity was normalized to total protein in cell lysates. **(B)** ChIP was performed using protein from N548wt and N548hd cells transfected with reporter plasmid driven by the HPRT promoter. Data is expressed as the amount of HPRT reporter plasmid precipitated using RAP30 antibody as a percentage of the total amount of HPRT in the cell lysate, normalized to the amount of HPRT reporter plasmid precipitated using non-specific IgG antibody as a percentage of the total amount of HPRT in the cell lysate. * $P < 0.05$ as determined by a two-tailed t-test with a significance level of 0.05. N=6 for each cell type in **(A)**; N=3 for each cell type in **(B)**.

In the study performed by Zhai *et al.* (2005), it was shown that N-mHtt interacts with aas 40-60 of RAP30. We hypothesized that if RAP74 and N-mHtt were competing for the same binding region on RAP30, overexpression of RAP74 would shift the competition to favour formation of TFIIF, restoring transcription in N548hd cells. cDNA coding for full-length human RAP74 was cloned into the pcDNA mammalian expression vector and was co-transfected with CMV and TK reporter plasmids. Empty pcDNA plasmid was used to control for the presence of plasmid in the cells. CMV and TK activity were assayed 24 h following transfection. CMV activity did not change in N548wt cells in the presence of increased RAP74 expression (Fig. 4.9A). Similarly, expression of human RAP74 in N548hd cells did not increase transcription driven by the CMV promoter. In contrast, overexpression of RAP74 increased TK activity by almost 50% following transfection with the RAP74 expression vector (Fig. 4.9B). The increased TK activity in N548wt cells was similar to what was seen following overexpression of RAP30 in N548wt cells (Fig. 4.5). Similar to what was observed using the CMV promoter, TK activity was unaffected by increased RAP74 in N548hd cells. Taken together, these results suggest that the capacity exists for TK activity to increase in the presence of excess RAP30 and RAP74. The presence of N-mHtt in N548hd cells appeared to inhibit this increased TK activity.

To ensure that transfection with the pcDNA vector containing RAP74 cDNA led to increased expression of RAP74 in the cells, western blotting was performed using an antibody raised against recombinant full-length human RAP74. RAP74 was predicted to migrate to ~74 kDa. A band of ~70 kDA was detected 24 h after transfection of cells with pcDNA-RAP74 plasmid but not after transfection with pcDNA control (Fig. 4.10),
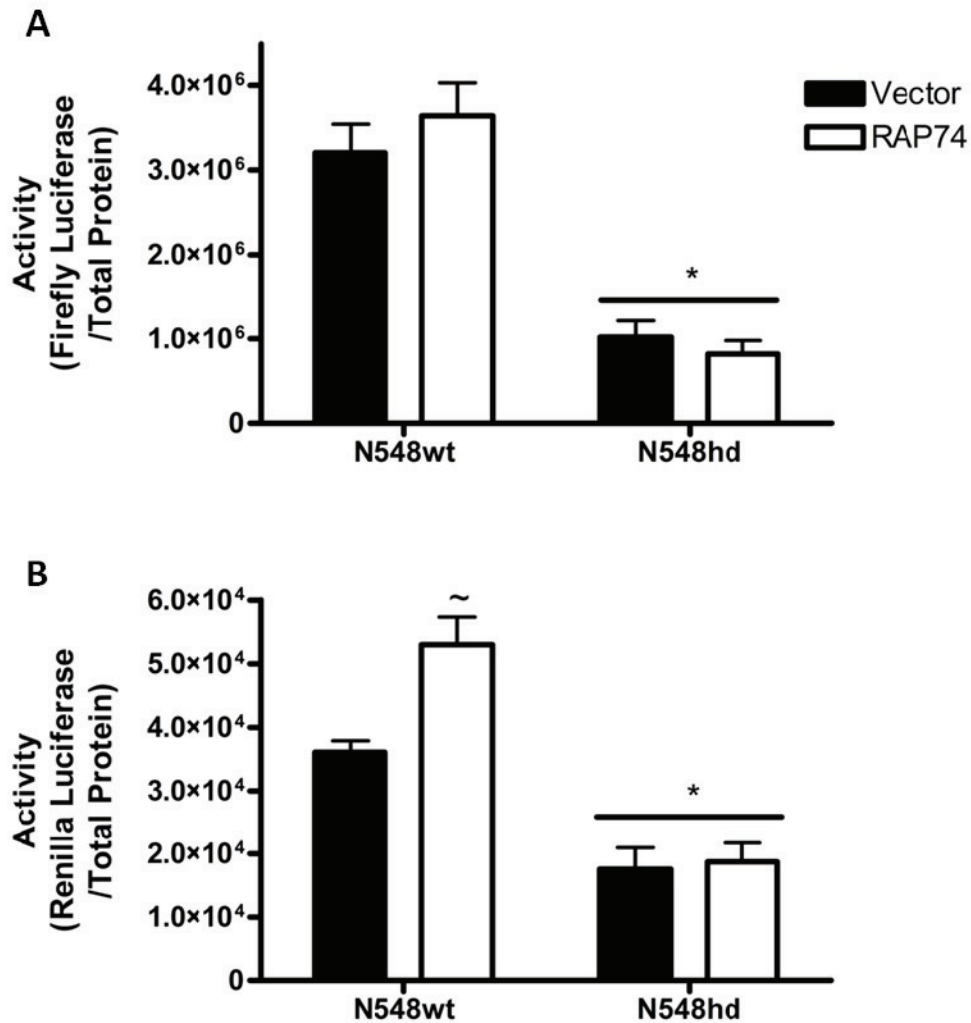
Figure 4.9. Overexpression of RAP74 protein did not recover N-mHtt-mediated transcriptional repression. N548wt and N548hd cells were transfected with reporter plasmids driven by either the CMV **(A)** or TK **(B)** promoters and either an empty expression plasmid or one driving production of human RAP74 cDNA. Luciferase activity was normalized to total protein. $*P < 0.05$ relative to N548wt as determined by a two-way ANOVA. Two-tailed *t*-tests were performed to determine cell-specific RAP74 effect. N=8 per data set.
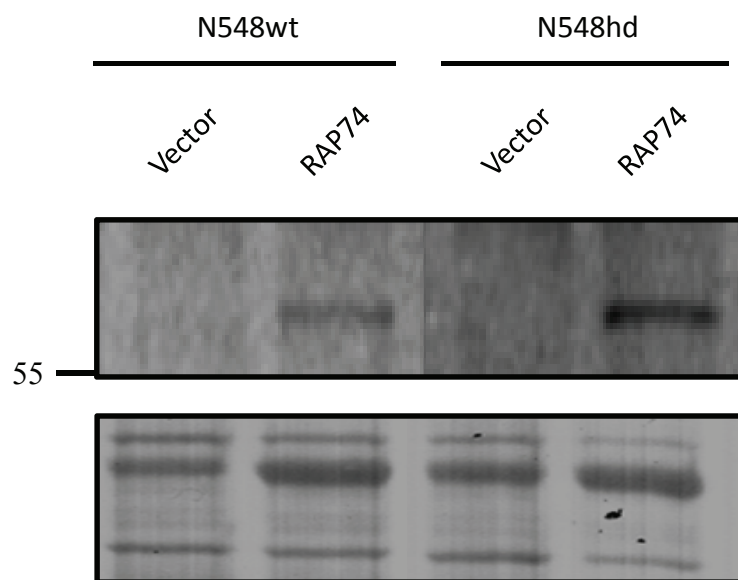
Figure 4.10. Transfection with pcDNA vector containing human RAP74 cDNA led to increased RAP74 expression in N548wt and N548hd cells. N548wt and N548hd cells were transfected with the pcDNA mammalian expression vector containing cDNA coding for human RAP74 (RAP74) or empty vector control (Vector). Western blotting was performed using an antibody specific to human RAP74. Amido black staining is shown below the blot. The number to the left of the blot represents the relative mobility of a molecular weight size marker in kDa

despite the fact that the antibody used was predicted to react with rat RAP74 by the manufacturer. The membrane was stained with amido black to ensure equal protein loading. Based on the predicted size of the detected band only in cells transfected with the RAP74 expression vector, it appeared that the antibody detected recombinant human but not endogenous rat RAP74. Similar to the conclusion reached in figure 4.6, endogenous RAP74 may have been expressed at relatively low levels compared to the overexpressed human RAP74. Western blotting results suggest that transfection with pcDNA-RAP74 plasmid resulted in increased expression of RAP74 protein, however the increased RAP74 expression did not increase CMV or TK activity in N548hd cells. As was theorized following expression of RAP30 protein, it is possible that the level of RAP74 produced using the pcDNA-RAP74 expression vector was not sufficient to compete with N-mHtt for association with RAP30 in N548hd cells.

If N-mHtt competes with RAP74 for association with RAP30 for formation of TFIIF, interaction between N-mHtt and RAP30 at an affected promoter would be expected to decrease the amount of RAP74 associated with that promoter. ChIP was performed to quantify RAP74 association with the CMV promoter in N548wt and N548hd cells. Immunoprecipitation was performed using an antibody specific to RAP74. ChIP revealed that RAP74 association with the CMV promoter was not different between N548wt and N548hd cells (Fig. 4.11A). Similar to figure 4.7A, the amount of CMV promoter associated with RAP74 was not enriched relative to the negative control in N548wt cells, and was only slightly enriched in N548hd cells. ChIP was repeated in cells transfected with a HPRT reporter plasmid to determine if RAP74 association was reduced at a less active N-mHtt-affected promoter in N548hd cells relative to N548wt cells.

Figure 4.11. RAP74 protein was not sequestered from N-mHtt-affected promoters in N548hd cells. **(A)** ChIP was performed using protein from N548wt and N548hd cells transfected with reporter plasmid driven by the CMV promoter. Data is expressed as the amount of CMV reporter plasmid precipitated using RAP74 antibody as a percentage of the total amount of CMV in the cell lysate, normalized to the amount of CMV reporter plasmid precipitated using non-specific IgG antibody as a percentage of the total amount of CMV in the cell lysate. **(B)** ChIP was performed using protein from N548wt and N548hd cells transfected with reporter plasmid driven by the HPRT promoter. Data is expressed as described in **(A)**. Statistical significance was analyzed by a two-tailed *t*-test with a significance level of 0.05. N=3 for each cell type. **(C)** Protein precipitated using RAP74-specific, and non-specific IgG antibody was fractionated using SDS-PAGE and probed with a RAP74-specific antibody. The number on the left represents the relative mobility of a molecular weight size marker in kDa.
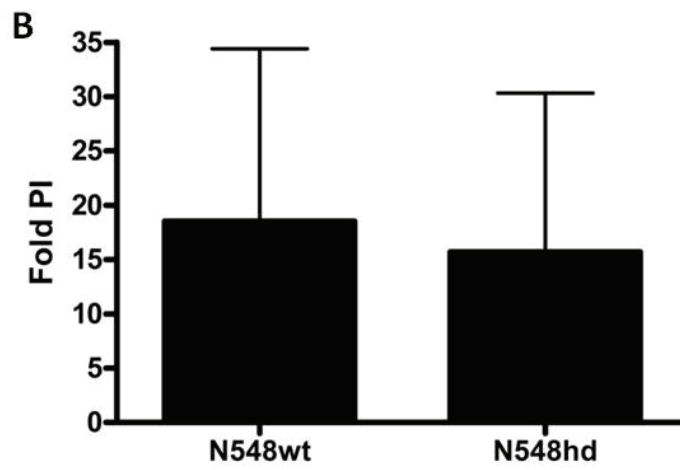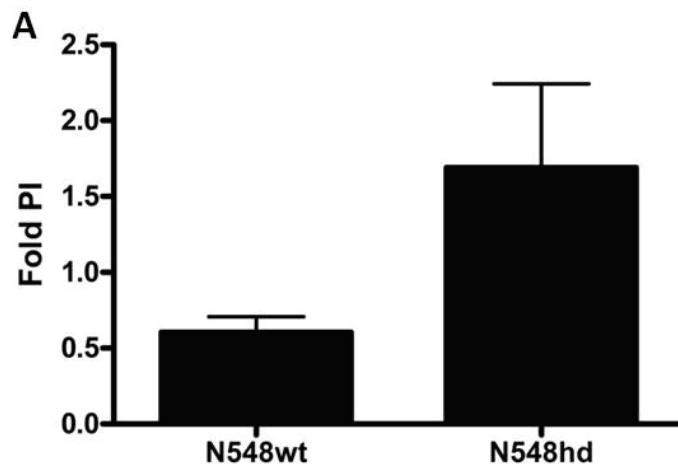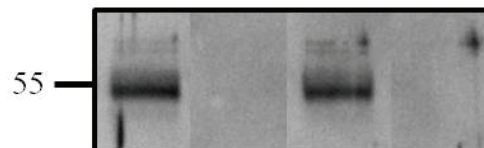
RAP74 association with the HPRT promoter was enriched in the antibody-specific sample relative to immunoprecipitation with non-specific IgG antibody (Fig. 4.11B). The amount of RAP74 associated with the HPRT promoter was not decreased in N548hd cells relative to N548wt cells.

Western blotting was performed to confirm the specificity of the immunoprecipitation. A RAP74-immunoreactive band was present in the sample immunoprecipitated with RAP74-specific antibody from both N548wt and N548hd cells, and RAP74-immunoreactive bands were absent from the non-specific IgG sample in both cell types (Figure 4.11C). Even though there was a high degree of variability in the results obtained using ChIP, these results suggested that N-mHtt did not inhibit the association of RAP74 with a promoter that exhibited decreased transcriptional activity in N548hd cells. If N-mHtt physically binds to RAP30 in N548hd cells, this interaction did not inhibit the association of TFIIF at the HPRT promoter.

N-mHtt-mediated transcriptional repression of the CMV promoter was not alleviated through overexpression of individual components of TFIIF. Although association of RAP30 and RAP74 with the CMV promoter could not be determined, association of RAP30 and RAP74 with the N-mHtt-affected HPRT promoter was not impaired in the presence of N-mHtt. This suggested that if N-mHtt binds to RAP30 in N548hd cells, it inhibits the function, and not the formation of TFIIF or the association of TFIIF in promoter-bound complexes. We hypothesized that overexpression of both RAP30 and RAP74 may increase CMV transcription in the presence of N-mHtt by increasing the amount of functional TFIIF in the cell. Mammalian expression vectors containing cDNA encoding human RAP30 and RAP74, or empty vector control were co-

transfected into N548wt and N548hd cells along with reporter plasmids driven by CMV and TK promoters. Co-expression of human RAP30 and RAP74 did not affect CMV activity in N548wt cells, and also failed to recover N-mHtt-mediated repression of CMV transcription in N548hd cells (Fig. 4.12A). In contrast, co-expression of RAP30 and RAP74 doubled TK activity in N548wt cells relative to empty vector control (Fig. 4.12B). The activity of the TK promoter in N548hd cells was similar between cells transfected with TFIIF and cells transfected with empty pcDNA vector. This suggests that the presence of N-mHtt in N548hd cells inhibited the increased functional TFIIF from alleviating N-mHtt-mediated transcriptional repression of the TK promoter. Increased TFIIF did not recover N-mHtt-mediated transcriptional repression of the CMV or TK promoters in N548hd cells. Because N548hd cells are an overexpression model of HD, the significantly higher expression of N-mHtt relative to that which would be produced under the control of the *huntingtin* promoter may have prohibited the alleviation of N-mHtt-mediated transcriptional repression through overexpression of TFIIF.

Results obtained using ChIP suggested that if N-mHtt associated with RAP30, this association occurred at N-mHtt-affected promoters. To determine if N-mHtt was present in the complement of proteins that associate with promoter DNA, an *in vitro* DNA-binding assay was performed using the CMV promoter. A biotin-tagged 297 nt fragment of the CMV promoter was generated using PCR amplification and was attached to streptavadin-coated magnetic beads. The bead-bound CMV promoter was incubated with nuclear extract isolated from N548wt and N548hd cells. Following three washes to remove weak or non-specific interacting partners, the protein complexes that remained
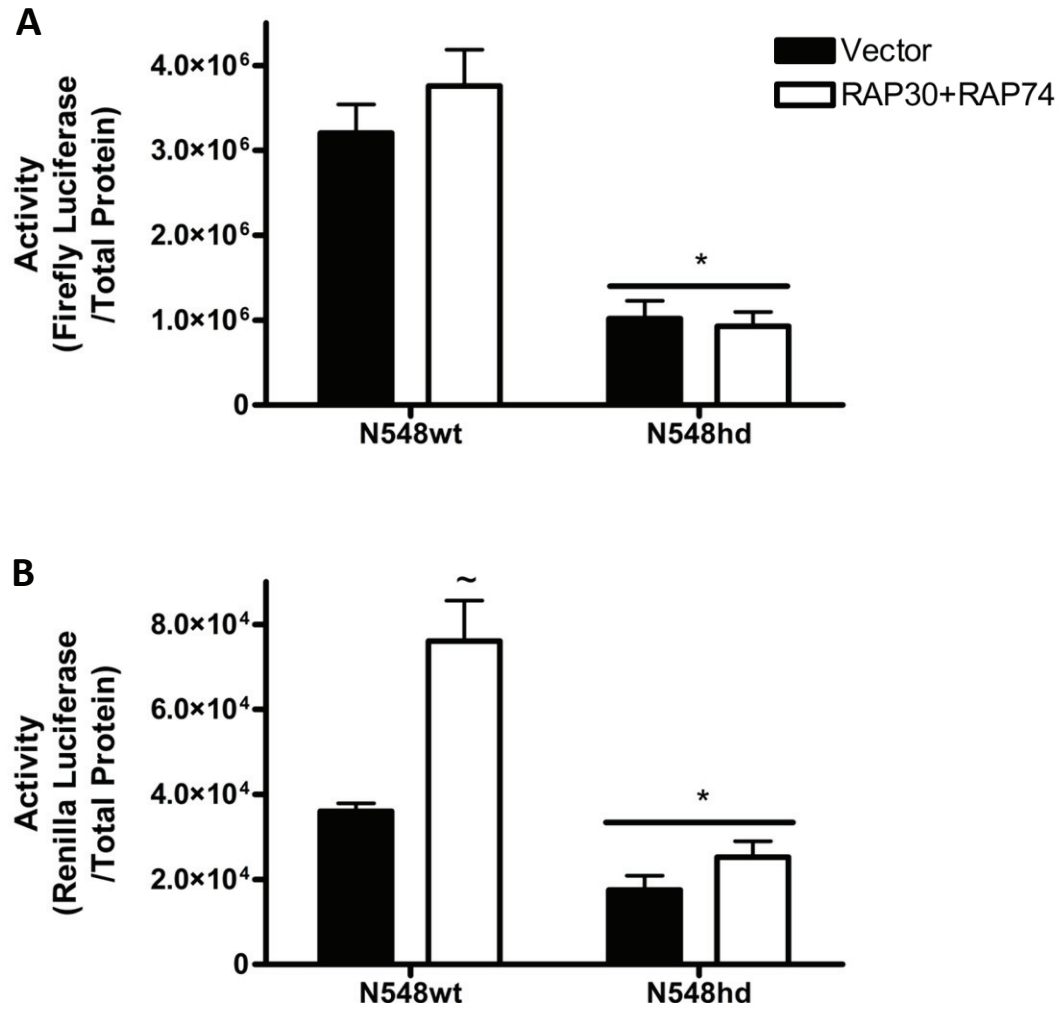
Figure 4.12. Overexpression of RAP30 and RAP74 protein together did not recover N-mHtt-mediated transcriptional repression. N548wt and N548hd cells were transfected with reporter plasmids driven by either the CMV **(A)** or TK **(B)** promoters and either an empty expression plasmid or ones driving production of human RAP30 and RAP74 cDNA. Luciferase activity was normalized to total protein. *$P < 0.05$ relative to N548wt. ~$P < 0.05$ relative to vector control within cell type as determined by a two-way ANOVA followed by a Bonferroni post-hoc test. Two-tailed t-tests were performed to determine cell-specific RAP74 effect. N=8 for each data set.

directly bound to the CMV promoter were eluted and fractionated on a denaturing

polyacrylamide gel. Western blotting was performed using the Htt-specific MAB2166

antibody. Bands at the size predicted for N-terminal Htt in N548wt and N548hd cells

were detected in the input samples (Fig. 4.13). N-terminal Htt from both N548wt and

N548hd nuclear extracts was detected in the complement of proteins bound to the -297

CMV promoter. No Htt was detected in the control reaction that contained magnetic

beads lacking CMV promoter DNA following incubation with N548hd nuclear extract.

MAB2166 detects bands of lower molecular weight than the predominant N548wt and

N548hd bands (Fig. 3.3). The faint MAB2166-immunoreactive band observed in

samples containing magnetic beads lacking CMV promoter DNA following incubation

with N548wt extract comigrated with a lower molecular weight protein than that

observed in the input lanes. Since MAB2166-immunoreactive bands were detected in the

presence of -297 CMV DNA and not in the absence of CMV promoter DNA, it was

concluded that N-terminal Htt binding was enriched in the presence of the -297 CMV

promoter. No Htt was detected in the supernatant collected following the third wash of

the protein-bound DNA, which confirms that the protein detected in the bound fraction

was bound specifically with high affinity to the -297 CMV promoter. The results from

this experiment suggested that N-mHtt was able to bind directly to the -297 CMV

promoter, or to associate with protein complexes present at the -297 CMV promoter,

supporting the hypothesis that N-mHtt can impair transcription without sequestering

proteins from an affected promoter.

Promoter deletion experiments using several different N-mHtt-affected

promoters have shown that transcription driven by the smallest fragment tested was
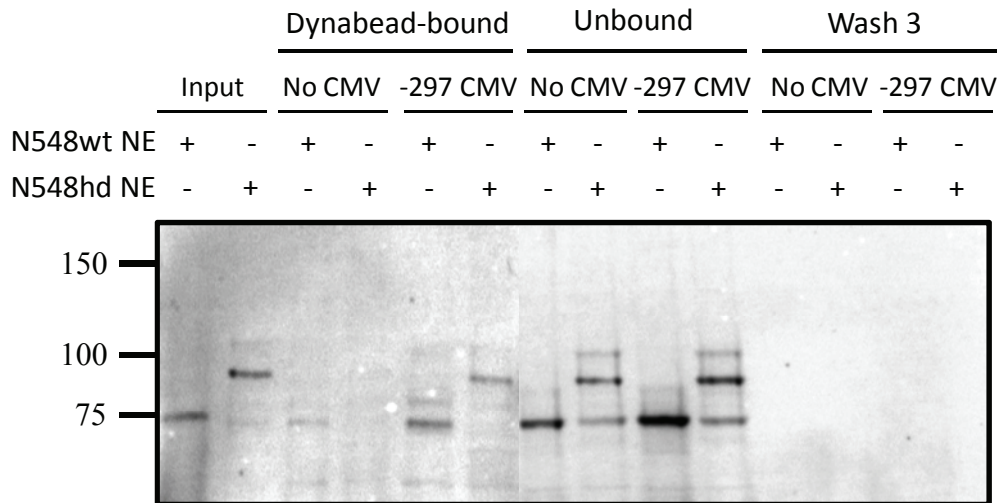
Figure 4.13. N-terminal Htt was present in the protein complement that bound to the -297 CMV promoter. Nuclear extract isolated from N548wt and N548hd cells was incubated with -297 CMV promoter attached to magnetic beads (-297 CMV) or to magnetic beads with no attached DNA (No CMV). After a 30 m incubation the reactions were washed three times and the protein complexes that remained bound were collected. The protein from the bound and unbound fractions, as well as the protein in the third wash were fractionated on a SDS-PAGE gel. MAB2166 antibody, which is specific to N-mHtt, was used for western blotting. Numbers on the left of the figure represent relative mobility of molecular weight size markers in kDa.

decreased in the presence of N-mHtt [Fig. 4.2, (Chiang *et al.*, 2005; Gomez *et al.*, 2006; Hu, 2007)]. N-mHtt was detected in the complement of proteins associated with the -297 CMV promoter. These studies, taken together, suggest that N-mHtt may interact with one or more members of the general transcription machinery at the promoter and impair its proper function. TBP fits the profile of a protein involved in formation of the general transcription machinery that has been demonstrated to interact with N-mHtt (van Roon-Mom *et al.*, 2002; Schaffar *et al.*, 2004). If TBP was involved in transcriptional repression of either the CMV or TK promoters in N548hd cells, we hypothesized that increasing TBP expression could alleviate transcriptional repression by 1) increasing the abundance of a N-mHtt binding partner in the cell and reducing the amount of N-mHtt available to integrate into transcriptional complexes and repress transcription and 2) increasing the pool of TBP available for incorporation into functional PICs. Together, we predicted that increasing TBP would restore transcription.

Plasmid driving the expression of full-length human TBP cDNA, or a control expression vector that did not contain TBP cDNA was co-transfected into N548wt and N548hd cells along with CMV and TK reporter plasmids. Cell lysates were collected 24 h later, and CMV- and TK-driven luciferase activities were measured. Expression of human TBP did not alter transcriptional activity driven by the CMV promoter, relative to empty vector control in either N548wt or N548hd cells (Fig. 4.14A). In contrast, expression of TBP in both N548wt and N548hd cells significantly increased TK activity compared to the empty vector control (Fig. 4.14B). Increased TK activity in the presence of excess TBP suggested that TBP was a limiting factor for transcription of TK in N548wt and N548hd cells. Recovery of TK activity, but not CMV activity, in N548hd
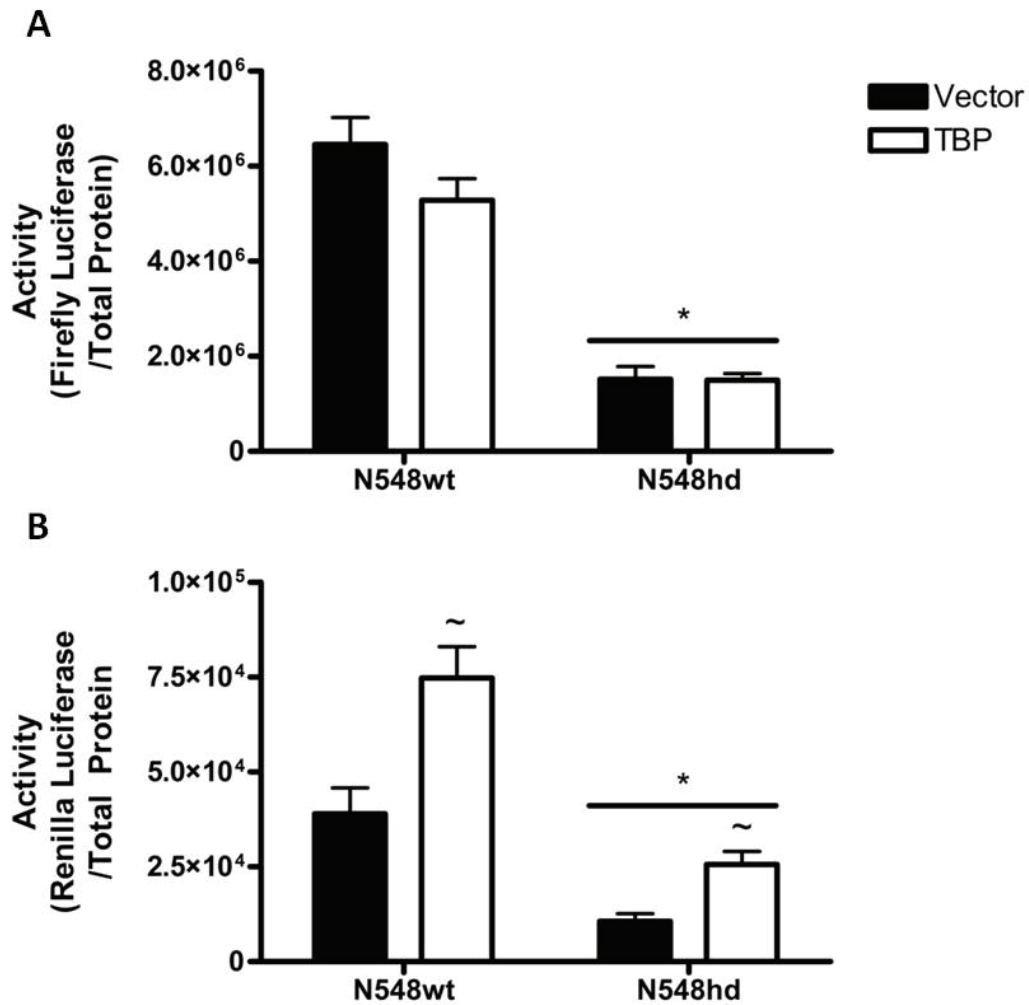
Figure 4.14. TBP overexpression increased transcription driven by the TK but not the CMV promoter in N548hd cells. N548wt and N548hd cells were transfected with CMV **(A)** and TK **(B)** reporter plasmids and either an empty expression plasmid, or one driving production of TBP cDNA. Luciferase activity was normalized to total protein. * $P < 0.05$ relative to N548wt cells. ~ $P < 0.05$ relative to empty vector control within cell type as determined by two-way ANOVA followed by Bonferroni post-hoc analysis. Two-tailed t-tests were performed to analyze effect of TBP expression within cell type. N=8 per data set.

cells following expression of TBP suggests that different mechanisms were involved in transcriptional dysregulation of the CMV and TK promoters. These results suggest that the N-terminal Htt inhibited the ability of TBP to activate transcription driven by the TK promoter, but that TBP was not involved in repression of CMV activity.

Overexpression of N-mHtt-interacting proteins was unsuccessful in alleviating transcriptional repression of the CMV promoter in N548hd cells. The N548 cell lines are an overexpression model of HD in which expression of N-mHtt is driven by the viral LTR promoter, which results in levels of the N-terminal fragment of Htt that are significantly greater than than physiological expression of Htt (Fig. 3.3). These artificially high levels of N-mHtt result in robust transcriptional dysregulation, however, the large amount of N-mHtt in N548hd cells may preclude the possibility of blocking the effect of nuclear Htt and alleviating transcriptional repression. To test this theory, short hairpin RNA (shRNA) was used to reduce the amount of N-mHtt in N548hd cells in an attempt to restore transcription. A vector driving transcription of a shRNA sequence complementary to nt 413-436 of *huntingtin* mRNA (shHtt) or empty vector (shNeg) was transfected into N548wt and N548hd cells. Cell lysates were collected 48 h following transfection, and 5 μg of the total protein was fractionated on a denaturing polyacrylamide gel. Western blotting was performed using an antibody raised against aas 181-810 of human Htt. Transfection of N548wt cells with shHtt led to a reduction in the amount of N-terminal Htt in the cell lysate (Fig. 4.15). Transfection of N548hd cells with shHtt also appeared to reduce the amount of N-mHtt in the total protein (Fig. 4.15). The band detected below the N-terminal Htt-specific band did not differ in size between lysates from N548wt and N548hd cells, suggesting that this band was unrelated to N-
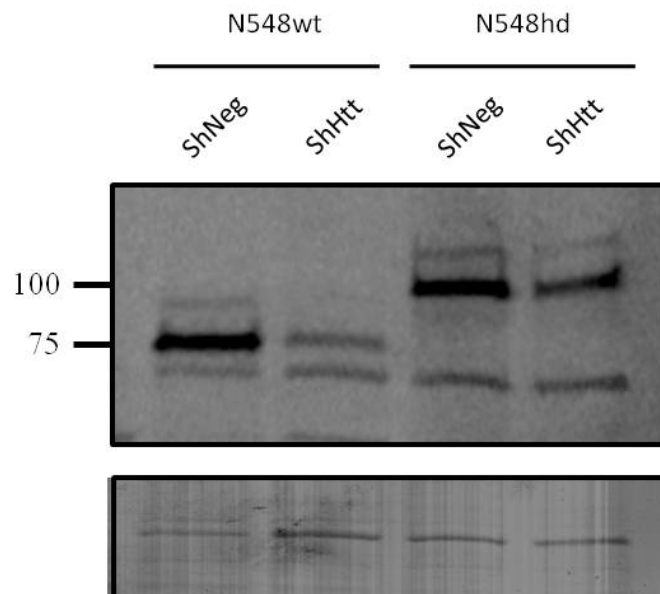
Figure 4.15. N-mHtt levels were decreased using shRNA. N548wt and N548hd cells were transfected with a plasmid driving expression of shRNA complementary to nt 413-436 of *huntingtin* mRNA (shHtt) or an empty vector control (ShNeg). Cell lysates were collected and western blotting was performed using an antibody specific to Htt. Amido staining of the membrane is shown below. Numbers on the left represent the relative mobility of molecular weight size markers in kDa.

terminal Htt. The intensity of this cross-reactive band did not decrease in samples where shHtt was expressed, suggesting that the knockdown was specific to Htt. Following antibody detection, the membrane was stained with amido black to ensure equal protein loading among samples. Expression of shHtt reduced the amount of protein in N548wt and N548hd cells 48 hours after transfection. Expression of N-terminal Htt was not eliminated altogether, and it appeared that there was a greater degree of reduction in N548wt cells than in N548hd cells. Both of these observations could be attributed to the rate of degradation of N-terminal Htt in N548wt and N548hd cells. If the rate of degradation of preexisting protein was decreased in N548hd cells relative to N548wt cells, the levels of N-mHt would be greater in N548hd cells than N-terminal Htt in N548wt 48 h after shRNA treatment. Since the proteasomal degradation pathway has been shown to be inhibited by mHtt (Bence *et al.*, 2001), protein turnover would be expected to occur more slowly in N548hd cells.

To determine if shRNA-dependent N-mHtt reduction recovered transcriptional dysregulation, CMV and TK reporter plasmids were co-transfected with shHtt or shNeg vector into N548wt and N548hd cells. Cell lysates were collected 48 h post-transfection and a DLR™ Assay was performed. Expression of shHtt did not change CMV activity in N548wt or N548hd cells (Fig. 4.16A). In contrast, knockdown of N-terminal Htt led to a statistically significant increase in transcription driven by the TK promoter in N548wt cells (Fig. 4.16B). Expression of shHtt also significantly increased TK activity in N548hd cells. Increased TK activity in N548wt cells following Htt knockdown suggests that the N-terminal 548 aas of Htt with 15 glutamines inhibited transcription in N548wt cells, acting as a less toxic version of the fragment expressed in N548hd cells. Although

A

B
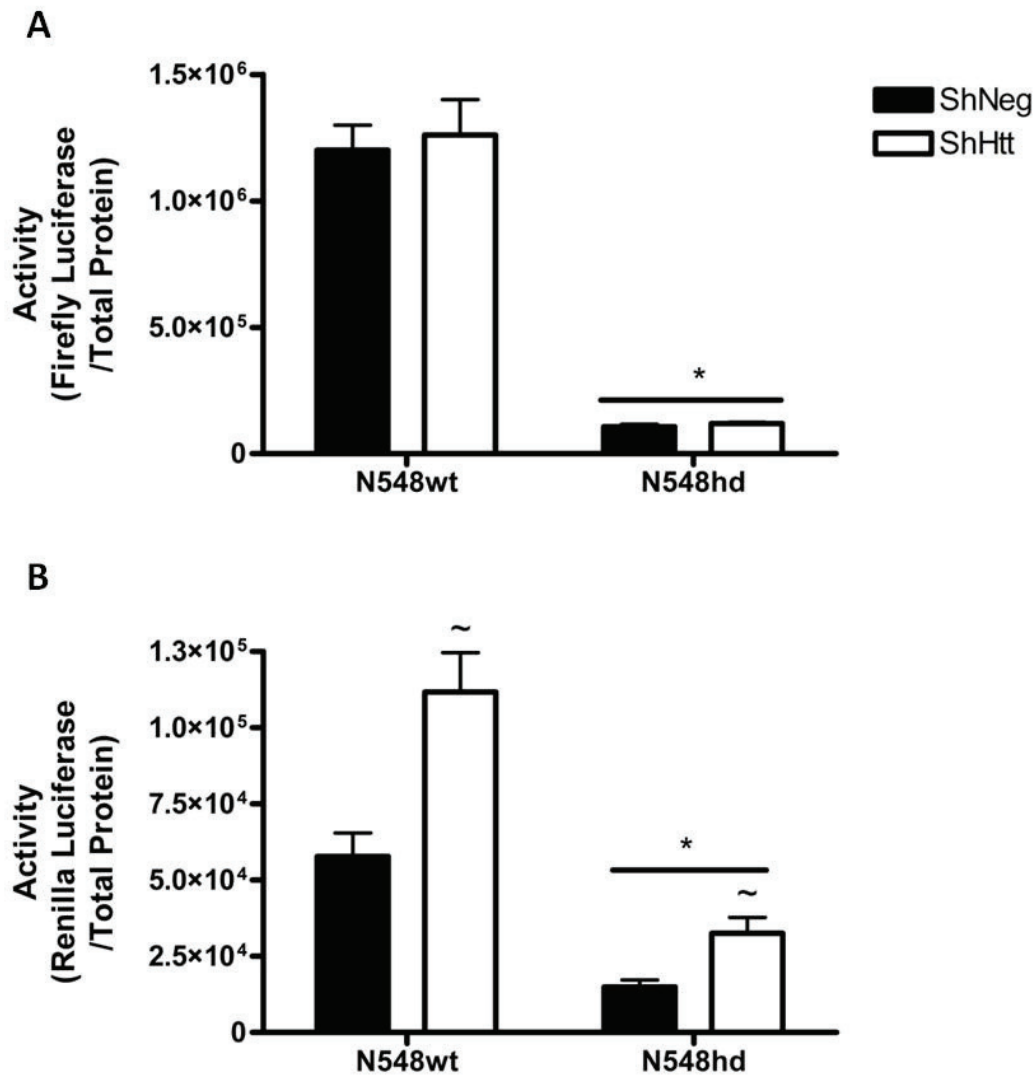
Figure 4.16. shRNA-mediated reduction of N-mHtt increased transcription driven by the TK but not the CMV promoter in N548hd cells. N548wt and N548hd cells were transfected with CMV **(A)** and TK **(B)** reporter plasmids and either plasmid driving production of a *huntingtin*-specific shRNA (shHtt) or empty vector control (shNeg). Luciferase activity was normalized to total protein. $*P < 0.05$ relative to N548wt cells. $\sim P < 0.05$ relative to shNeg within cell type as determined by a two-way ANOVA followed by a Bonferroni post-hoc test. Two-tailed *t*-tests were performed to determined cell-specific effect of knock-down. N=6 per data set.

huntingtin-specific shRNA decreased protein expression, it did not eliminate Htt expression completely. Recovery of transcription of TK, but not CMV activity in N548hd cells suggested that these two N-mHtt-affected promoters had different thresholds of susceptibility with respect to the amount of nuclear N-terminal Htt present. The level of N-terminal Htt expression that remained following shRNA-mediated knockdown was sufficient to fully inhibit transcription of the CMV but not the TK promoter.

Overexpression of N-mHtt interacting proteins, or partial knockdown of the N-terminus of Htt did not fully block the effect of N-terminal Htt on transcription of the CMV promoter due to the high level of N-mHtt expression in N548hd cells. Since the ability to overcome transcriptional repression at the CMV promoter could be strongly influenced by the amount of N-mHtt expressed, a more appropriate cellular model for studying the recovery of N-mHtt-mediated transcriptional dysregulation is one that expresses Htt at endogenous levels. We obtained StHdh cells that express Htt under the control of the *huntingtin* promoter. StHdh cells are immortalized striatal cells derived from embryonic day 14 *huntingtin* knock-in mice, which express two copies of the mouse *huntingtin* gene. To create an accurate genetic model of HD, exon 1 of the mouse *huntingtin* gene was replaced with exon 1 of human *huntingtin* with 7 or 111 CAG repeats. Therefore the three cell lines express two copies of wild-type *huntingtin*, one wild-type and one mutant copy of *huntingtin*, and two mutant copies of *huntingtin* (StHdh Q7/7, Q7/111 and Q111/111, respectively). There is a significant difference in expression of Htt between N548 cell lines and the StHdh cell lines. Htt transgene expression was readily detected by western blot following fractionation of 5 μg of total

protein isolated from N548 cells (Fig. 3.3 and 4.14) using MAB2166 antibody. In contrast, expression of Htt in StHdh Q7/7, Q7/111 and Q111/111 cells was only detected by western blot after fractionation of 60 μg of total protein from cell lysate using antibody MAB2166 (Trettel *et al.*, 2000).

To test the activity of CMV and TK promoters in the presence of physiological levels of Htt, reporter plasmids driven by the CMV and TK promoters were transfected into StHdh Q7/7, Q7/111 and Q111/111 cells. Cell lysates were collected 24 h post transfection and DLR™ Assays were performed to measure promoter activity. CMV activity was significantly lower in StHdh Q7/111 and Q111/111 cells compared to Q7/7 cells (Fig. 4.17A). There was no significant difference in CMV activity between StHdh Q7/111 and Q111/111, suggesting that the absence of wild-type Htt did not negatively affect CMV-driven transcription. Moreover, expression of one or two copies of mHtt reduced CMV promoter activity by approximately the same percentage of the level observed in the absence of mHtt. There was no difference in activity between Q7/7, Q7/111 and Q111/111 cells transfected with the TK reporter plasmid (Fig. 4.17B). Therefore, transcription driven by the CMV, but not the TK promoter, was decreased in Q7/111 and Q111/111 cells. The differential susceptibility of the CMV and TK promoters to mHtt-mediated transcriptional repression reflected the results obtained using shRNA knockdown of N-mHtt in N548hd cells (Fig. 4.10), which suggested that transcription driven by the CMV promoter was more sensitive to the level of mHtt expressed in a cell. Just as reducing the level of N-mHtt in N548hd cells reduced the transcriptional repression of the TK promoter but not the CMV promoter, the lower
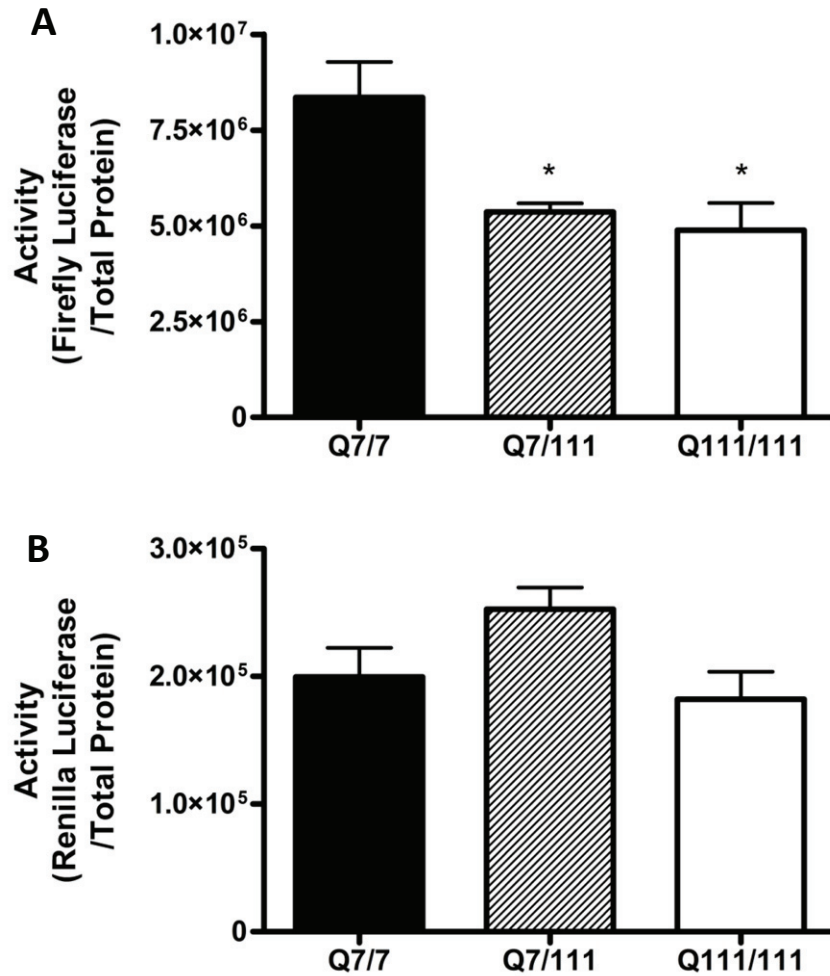
Figure 4.17. CMV but not TK promoter activity was decreased in StHdh Q7/111 and Q111/111 compared to Q7/7 cells. Reporter plasmids driven by the CMV **(A)** and TK **(B)** promoters were transfected in StHdh Q7/7, Q7/111, and Q111/111 cells. Cell lysates were collected 24 h post-transfection and a DLR™ Assay was performed. $*P < 0.05$ relative to StHdh Q7/7 as determined by a one-way ANOVA followed by a Bonferroni post-hoc test. N=8 for each data set.

endogenous expression of mHtt in Q7/111 and Q111/111 cells was sufficient to inhibit CMV activity but not TK activity. As TK activity was not decreased in the presence of mHtt in StHdh cells, only the CMV promoter was used to study the mechanism of mHtt-mediated transcriptional inhibition in the StHdh cells.

To determine if components of TFIIF could recover transcriptional repression caused by expression of mHtt under control of the *huntingtin* promoter, RAP30 and RAP74 were overexpressed individually and in combination along with the CMV reporter plasmid. Expression of human RAP30 in Q7/7 cells did not alter CMV-driven transcription (Fig. 4.18A). RAP30 expression in StHdh Q7/111, and Q111/111 cells did not increase CMV transcription, as CMV activity remained significantly lower in these cells than in the Q7/7 cells. Expression of RAP74 in Q7/7 cells led to a significant reduction in CMV activity (Fig. 4.18B). This was an unexpected result, and it is not clear how RAP74 overexpression might decrease luciferase activity. A previous study demonstrated that RAP74 overexpression was toxic to primary striatal neurons (Zhai *et al.*, 2005), however, the decreased CMV-driven luciferase activity in StHdh Q7/7 cells did not result from increased cell death (data not shown). Expression of human RAP74 did not increase or decrease CMV activity in Q7/111 or Q111/111 cells. CMV activity was significantly lower in Q7/111 cells but not in Q111/111 cells relative to CMV activity in Q7/7 cells although there was no difference in CMV activity between Q7/111 and Q111/111 cells. Co-expression of human RAP30 and RAP74 did not affect CMV activity in StHdh Q7/7 cells (Fig. 4.18C) suggesting that excess RAP74 but not functional TFIIF was deleterious to CMV activity. Expression of RAP30 and RAP74 in

Figure 4.18. Overexpression of the components of TFIIF did not recover N-mHtt-mediated transcriptional repression. StHdh Q7/7, Q7/111 and Q111/111 cells were transfected with reporter plasmids driven by the CMV promoter and either an empty expression plasmid or ones driving production of human RAP30 **(A)**, RAP74 **(B)**, or both RAP30 and RAP74 **(C)** protein. Luciferase activity was normalized to total protein. $*P < 0.05$ relative to StHdh Q7/7 cells. $\sim P < 0.05$ relative to vector control within cell type as determined by a two-way ANOVA followed by a Bonferroni post-hoc test. Two-tailed t-tests were performed to determined cell-specific effect of knock-down. N=8 for each data set.

A

B

C
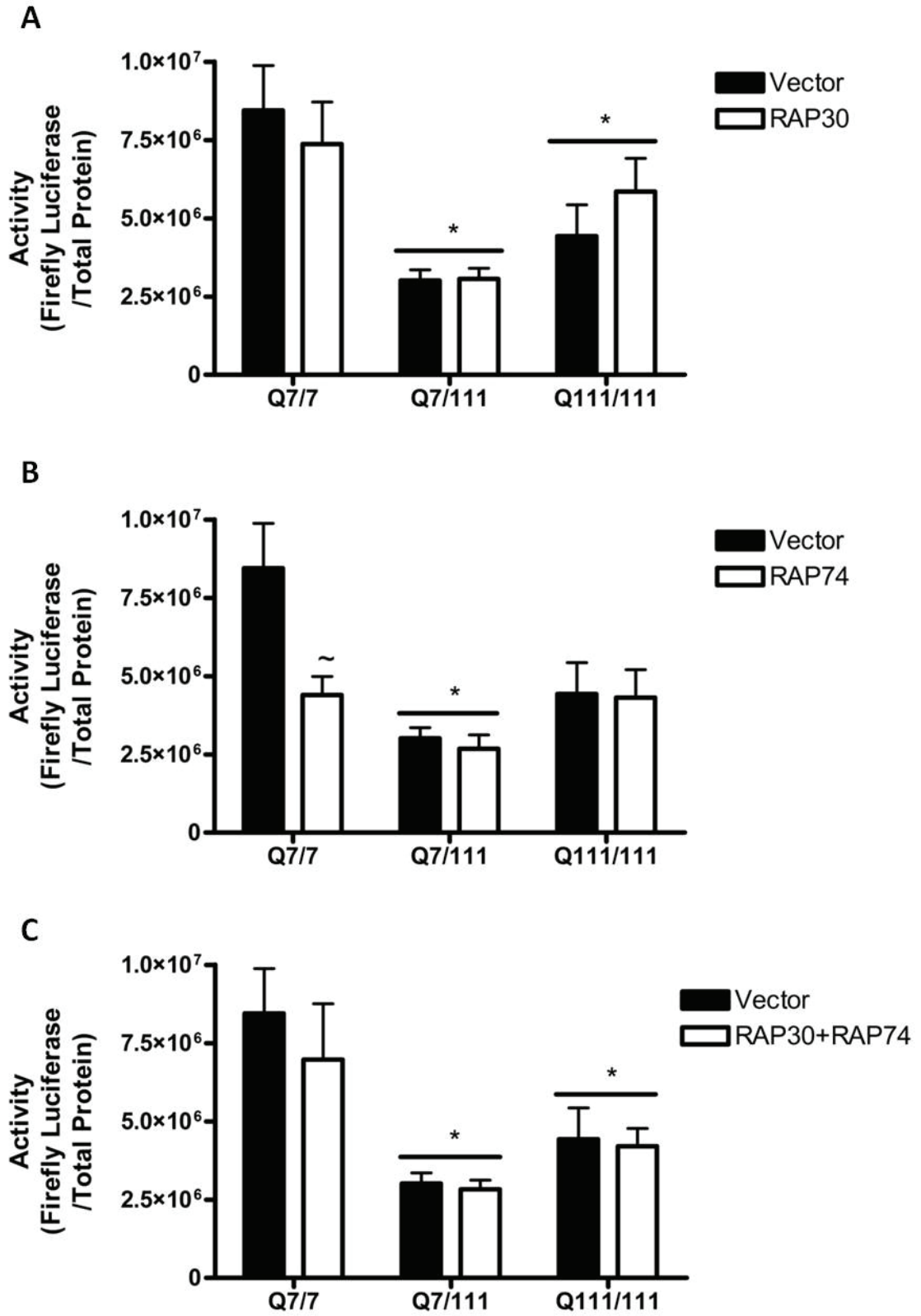
Q7/111 and Q111/111 cells did not alleviate mHtt-mediated repression of transcription driven by the CMV promoter. Increased expression of RAP30 and RAP74, either alone or in combination did not significantly increase CMV transcription in the presence of mHtt. These results refute the hypothesis that the lack of recovery of N-mHtt-mediated repression of the CMV promoter in N548hd cells was due to abnormally high expression of N-mHtt. Previous experiments used coIP to demonstrate that N-mHtt can physically interact with RAP30 (Zhai *et al.*, 2005). As such, the results in this work suggest that either mHtt did not block the activity of RAP30 in N548wt and N548hd cells or the interaction between mHtt and the components of the basal transcription machinery could not be prevented by increasing the amount of RAP30.

TBP has been shown to interact with transiently expressed N-mHtt in neuroblastoma cells (Schaffar *et al.*, 2004). Expression of human TBP in N548hd cells did not recover N-mHtt-mediated repression of CMV promoter activity. To determine if the lack of recovery in N548hd cells was due to the fact that levels of N-mHtt in N548 cells are much greater than Htt expression under the control of the *huntingtin* promoter, human TBP was expressed in the StHdh cell lines. StHdh Q7/7, Q7/111 and Q111/111 cells were transfected with vector driving expression of full-length human TBP cDNA or empty vector control, along with CMV reporter plasmid. Cell lysates were collected 24 h later and a DLR™ Assay was performed. TBP overexpression in Q7/7 cells did not significantly alter CMV activity (Fig. 4.19), suggesting that TBP was not limiting with respect to transcription of the CMV promoter in StHdh cells. TBP expression in Q7/111 and Q111/111 cells did not significantly increase CMV transcription, and CMV activity
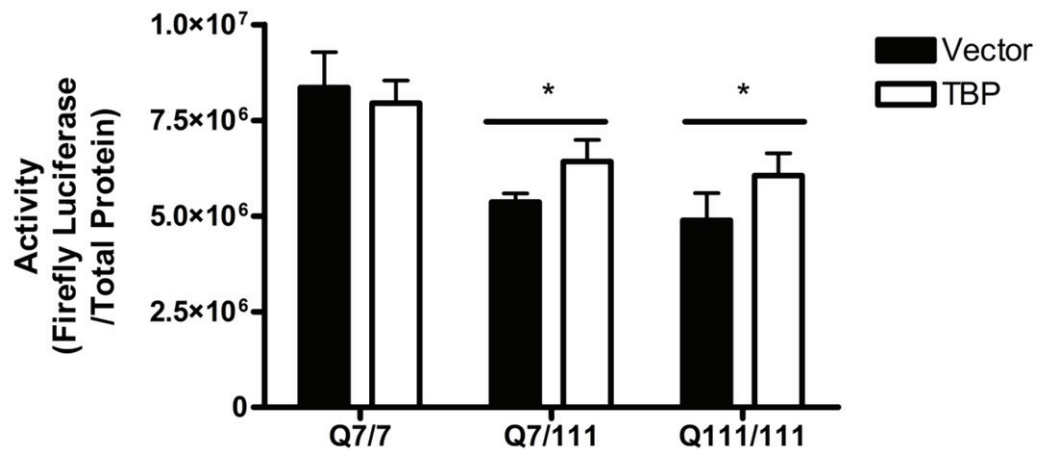
Figure 4.19. TBP overexpression did not recover N-mHtt-mediated transcriptional repression. StHdh Q7/7, Q7/111, and Q111/111 cells were transfected with a CMV reporter plasmid and either an empty expression plasmid, or one driving production of TBP cDNA. Luciferase activity was normalized to total protein. * $P < 0.05$ relative to StHdh Q7/7 cells as determined by a two-way ANOVA followed by a Bonferroni post-hoc test. Two-tailed t-tests were performed to determined cell-specific effect of knock-down. N=8 per data set.

remained significantly lower in Q7/111 and Q111/111 cells than in Q7/7 cells. The lack of a recovery of CMV transcription in Q7/111 and Q111/111 cells suggested that either TBP does not interact with mHtt in these cells, or that the interaction occurs in a manner that cannot be overcome by increased expression of the interacting protein.

4.3 Discussion

A promoter deletion experiment was performed using fragments of the CMV promoter in *in vitro* transcription assays to identify the specific region of the promoter necessary for N-mHtt-mediated transcriptional repression. Transcription driven by the smallest active fragment of the CMV promoter, -297 CMV, appeared to be decreased in the presence of recombinant N171-89Q. The smaller CMV fragment, -68 CMV, was not sufficiently active to determine if transcription was impaired in the presence of N-mHtt.

Reporter plasmids containing CMV promoter deletion fragments of similar sizes to those tested in the *in vitro* transcription assay were transfected into N548wt and N548hd cells to localize the specific region of the CMV promoter required for N-mHtt-mediated transcriptional repression using a more sensitive and quantitative assay. Although the sequential deletion of the CMV promoter resulted in progressive reduction in CMV activity in both N548wt and N548hd cells, promoter activity of the smallest CMV promoter fragment was significantly decreased in N548hd cells relative to N548wt cells. N-mHtt, therefore, exerted its negative effect on the minimally active promoter fragment, which included the 99 bp 5' to the transcription start site. The results from the CMV promoter deletion analysis reflect the findings from other promoter deletion experiments performed using N-mHtt-affected promoters. The smallest fragments of the

DARPP (160 bp) (Gomez *et al.*, 2006) and PDE10A (69 bp) (Hu, 2007) promoters tested in N548wt and N548hd cells, and the smallest fragment of the adenosine A2A receptor promoter (105 bp) (Chiang *et al.*, 2005) tested in PC12 cells inducibly expressing exon 1 of human *huntingtin* with either 25 or 109 CAG repeats had decreased transcription in the presence of N-mHtt. In addition a 75 bp fragment of the dopamine D_2 receptor promoter had reduced activity in primary striatal cells transiently expressing N-mHtt (Dunah *et al.*, 2002). Taken together, these results suggest that N-mHtt inhibits transcription by interacting with proteins that bind near, or at, the transcription start site.

To test whether N-mHtt inhibited CMV transcription by interfering with activation mediated by gene-specific transcription factors, linker-scanning mutagenesis was performed to eliminate putative transcription factor binding sites on the CMV promoter. Although promoter deletion suggested that the region of the CMV promoter required for N-mHtt-mediated transcriptional repression was limited to the 99 bp upstream from the transcription start site, it was also possible that repression of CMV transcription in the presence of N-mHtt was the summative result of transcriptional activation mediated by the contributions of multiple individual transcription factors. Since the region between 100 and 297 bp upstream of the transcription start site on the CMV promoter was responsible for a large degree of CMV activity, it was included in the promoter analysis. Eliminating a C/EBP binding site on the argininosuccinate acid lyase (AAL) promoter transiently co-transfected with a vector expressing exon 1 of human *huntingtin* with 25 or 109 CAG repeats under the control of the CMV promoter in PC12 cells alleviated N-mHtt-mediated transcriptional repression (Chiang *et al.*, 2007). In the present study, putative binding sites for several transcription factors previously shown to

interact with N-mHtt were identified and eliminated from the CMV promoter using linker-scanning mutagenesis. The activity of all CMV promoter mutants remained lower in N548hd cells relative to N548wt cells. N-mHtt-dependent impairment of transcriptional activation was not mediated through any of the transcription factor binding sites tested. One of the linker-scanning mutants eliminated a putative C/EBP binding site. Elimination of the C/EBP site did not alleviate N-mHtt-mediated repression of the CMV promoter. This suggested that, although N-mHtt has been shown to be capable of inhibiting C/EBP-mediated transcriptional activation, this mechanism did not account for repression of CMV activity. Comparison of this finding to that obtained using the AAL promoter also highlights the promoter-specific nature of N-mHtt-mediated transcriptional repression. The results from CMV promoter deletion and mutation experiments demonstrated that N-mHtt inhibited transcriptional activity of the CMV promoter driven by the core promoter region and that the contribution of a single gene-specific transcriptional activator previously implicated in N-mHtt-mediated transcriptional dysregulation was not responsible for this transcriptional repression. These results, in combination with the observation that N-mHtt inhibited transcription driven by the smallest active fragment of numerous promoters, suggests that N-mHtt may interact with the core transcriptional machinery to impair transcription.

TFIIF (RAP30/74) is a member of the general transcription machinery. RAP30 has been shown to interact with N-mHtt (Zhai *et al.*, 2005). Reduced promoter activity of the CMV or TK promoters was not compensated by overexpression of RAP30 in N548hd cells. RAP30 occupancy was decreased at the dopamine D_2 promoter in R6/2 mice and overexpression of RAP30 increased transcription driven by a 75 bp fragment of the D_2

promoter in primary striatal neurons transiently transfected with N-mHtt. To determine if N-mHtt decreased RAP30 occupancy at the CMV promoter, as proposed by Zhai *et al.* (2005), ChIP was performed on N548wt and N548hd cells transfected with a CMV reporter plasmid. The CMV reporter plasmid was not enriched in the complement of DNA associated with RAP30. Previous findings demonstrated that the ability to detect RAP30 association with the *hsp70* promoter using ChIP was lost following heat shock-induced activation of the *hsp70* promoter suggesting that RAP30 promoter association may be labile and difficult to detect at highly active promoters, which would include the CMV promoter (Lebedeva *et al.*, 2005). ChIP was performed to examine RAP30 association with the less active HPRT promoter. Although RAP30 association with the less active HPRT was readily measured using ChIP, RAP30 binding was not altered in N548hd cells relative to N548wt cells suggesting that a N-mHtt interaction with RAP30 did not restrict association of RAP30 to active promoters in general. RAP30 binding was shown to be unaltered at the GAPDH promoter in R6/2 mice relative to wild-type controls (Zhai *et al.*, 2005) despite the fact that GAPDH expression is decreased in multiple brain regions in R6/2 mice (Luthi-Carter *et al.*, 2002). Zhai *et al.* (2005) used GAPDH ChIP analysis as a control assuming that mHtt did not affect GAPDH expression. The results from our experiments in combination with previous studies suggest that, although N-mHtt may impede RAP30 association with some affected promoters, altered RAP30 association does not occur at all N-mHtt-affected promoters.

One study suggested that RAP74 associates with aas 40-60 of RAP30 to form TFIIF (Zhai *et al.*, 2005), while another study suggested that the interaction between RAP30 and RAP74 involves aas 16-30 of RAP30 (Tan *et al.*, 1995). N-mHtt was shown

to interact with RAP30 in the region between aas 40-60 (Zhai *et al.*, 2005). If RAP74 associates with RAP30 via aas 40-60, N-mHtt interaction with RAP30 would inhibit the formation of TFIIF. If RAP74 associates with RAP30 via aas 16-30, RAP30 could theoretically interact simultaneously with both RAP74 and N-mHtt. If RAP74 interacts at aas 40-60 of RAP30, we hypothesized that overexpression RAP74 would compete with N-mHtt for RAP30 binding and increase the amount of functional TFIIF available to promote transcription. Overexpression of RAP74 did not alleviate transcriptional repression of the CMV or TK promoters in N548hd cells. Additionally, any interaction between N-mHtt and RAP30 did not inhibit RAP74 association with the HPRT promoter, as would have been predicted based on the shared interaction site on RAP30 for N-mHtt and RAP74. These findings suggest that N-mHtt interaction with RAP30 does not inhibit formation of TFIIF. N-mHtt might inhibit the function of TFIIF without inhibiting its formation or translocation to the promoter.

In an attempt to increase the pool of functional TFIIF available for recruitment into transcriptional complexes, RAP30 and RAP74 were simultaneously overexpressed and measurement of CMV and TK promoter activity was performed. Concurrent RAP30 and RAP74 overexpression did not increase CMV or TK activity in N548hd cells relative to controls. We did not find any evidence that N-mHtt altered association of TFIIF with N-mHtt-affected promoters.

Assuming N-mHtt interacts with RAP30 and does not inhibit the association of RAP30 in a TFIIF complex with an N-mHtt-affected promoter, we predicted that N-mHtt would be present in the protein complexes associated with an N-mHtt-affected promoter. To test this prediction, a fragment of the CMV promoter consisting of the 297 bp

upstream of the transcription start site was incubated with nuclear protein isolated from N548wt and N548hd cells in an *in vitro* binding assay. N-terminal Htt from both N548wt and N548hd cells was detected in the complement of proteins bound specifically to the CMV promoter. The presence of Htt from N548wt nuclear extract at the CMV promoter suggested that the association with transcriptional proteins at promoters occurred via the N-terminal fragment of Htt. However, we could not quantify the relative affinity between the CMV promoter and N-terminal Htt in this assay to determine how polyQ expansion influenced N-terminal Htt/promoter affinity. The association of the N-terminus of wild-type Htt with promoter DNA may or may not occur *in vivo*. Cleavage of wild-type Htt generates a fragment of ~548 aas but the concentration of the fragment in nucleus is not known. In contrast, N-mHtt readily accumulates in the nucleus (Sun *et al.*, 2002). The Htt fragment overexpressed in N548wt cells, therefore, may be a less toxic form of N-mHtt with respect to transcription and may not represent the conditions achieved in human HD striatal neurons.

Although we could show that N-terminal Htt was associated with a DNA promoter complex, we could not directly determine how N-mHtt associated with the DNA. It is possible that N-mHtt interacted directly with the CMV promoter DNA or associated indirectly with DNA via protein-protein interaction. ChIP has been used to demonstrate N-mHtt association with the PGC-1α promoter in StHdh cells and in HD knock-in mice (Cui *et al.*, 2006). In this study, it was inferred that N-mHtt associates with chromatin. ChIP is capable of identifying DNA and non DNA-bound proteins in complexes associated with DNA sequences (Masternak *et al.*, 2000; Goardon *et al.*, 2006; Roth *et al.*, 2008). A single report suggested that both N-terminal wild-type Htt and N-

mHtt associate directly with DNA *in vivo* and that this association occurred in a polyQ-dependent manner (Benn *et al.*, 2008). The results in our experiments obtained using protein/promoter complex capture agree with previous findings using alternative techniques that suggest that N-mHtt may bind directly to promoter DNA, to DNA-binding proteins that associate with the promoter, or it may integrate into DNA-bound protein complexes.

Soluble N-mHtt has been shown to interact with TBP (Schaffar *et al.*, 2004), which is a component of the general transcription factor TFIID (Thomas & Chiang, 2006). Despite the fact that some promoters do not have a consensus TATA box (Smale, 1997), TBP is present at the core promoter and involved in the assembly of all promoter complexes, including recently characterized complexes that do not contain TFIID (Kaufmann & Smale, 1994; Tokusumi *et al.*, 2007; Anish *et al.*, 2009). To determine if TBP was involved in N-mHtt-mediated repression of the CMV or TK promoter, TBP was overexpressed in N548 cell lines. TBP overexpression did not increase transcriptional activity of the CMV promoter in N548hd cells. Overexpression of TBP did, however, recover transcriptional repression of the TK promoter in N548hd cells. This result implied that N-mHtt interfered with the ability of TBP to activate transcription driven by the TK promoter, but that either functional TBP was not limiting or that the level of TBP following overexpression was insufficient to alleviate the effect of N-mHtt at the CMV promoter, and suggests that different mechanisms exist for transcriptional dysregulation of different promoters.

The lack of recovery of CMV activity following overexpression of TBP and TFIIF could have been related to the level of N-mHtt expression in N548hd cells and the

relative sensitivity of different promoters to N-mHtt concentrations. shRNA-mediated knockdown of N-terminal Htt was performed in N548 cells to determine if transcription of the CMV and TK promoters could be increased following reduction of N-terminal Htt expression. shRNA-mediated knockdown decreased but did not eliminate N-mHtt in N548hd cells. shRNA-mediated reduction in the concentration of N-mHtt led to an increase in transcription driven by the TK but not the CMV promoter. Results produced following TBP overexpression and shRNA knockdown of N-mHtt identified the TK promoter as being less sensitive to the deleterious effects of N-mHtt than the CMV promoter. These results suggest that differential susceptibility of promoters to N-mHtt-mediated transcriptional dysregulation is dependent on the concentration of N-mHtt. Moreover, recovery of transcription using overexpression of N-mHtt-interacting proteins may be dependent on the sensitivity of a promoter to the concentration of N-mHtt.

To study mHtt-mediated transcriptional dysregulation in a cell model that expressed Htt via the *huntingtin* promoter, CMV and TK activity were measured in StHdh Q7/7, Q7/111, and Q111/111 cells. Importantly, these cells are genetically accurate models of HD and have levels of Htt expression equivalent to those found in human neurons. Transcription driven by the CMV, but not the TK promoter, was decreased in Q7/111 and Q111/111 cells relative to activity in Q7/7 cells. This result supported the findings obtained following shRNA-mediated knockdown in N548hd cells, suggesting that TK activity is less sensitive than CMV promoter activity to levels of N-mHtt.

Increased expression and accumulation of mHtt, as well as increased nuclear accumulation of N-mHtt in animal models of HD have been correlated with earlier

disease onset and increased severity, suggesting that the symptoms of the disease are directly associated with the concentration of mHtt (Benn *et al.*, 2005; Graham *et al.*, 2006). Caspase-mediated cleavage of mHtt has been shown to positively regulate caspase activity, suggesting that the concentration of nuclear N-mHtt increases as the disease progresses (Graham *et al.*, 2010). N-mHtt-mediated transcriptional dysregulation occurs in a time-dependent manner. Some genes exhibit decreased transcription earlier in the disease while others are not affected until later in disease progression (Becanovic *et al.*, 2010)(Fig. 4.20). Later changes may be due to compensatory changes in gene expression, or they could be direct effects of the mutant protein. Assuming that both early and late changes in gene expression can be directly caused by mHtt, genes that are unaffected early in disease progression but exhibit decreased transcription at later stages may be more resistant to the concentration of N-mHtt than genes affected earlier in HD progression. In this sense, the differential susceptibility of the CMV and TK promoters to the concentration of N-mHtt may model early- and late-affected promoters, respectively.

In addition to N-mHtt concentration-dependent gene expression changes, transcriptional dysregulation in HD also occurs in a gene-specific manner. In microarray analysis of R6/2 mice, 99.8% of more than 6000 genes analyzed were unaffected in late stages of the disease, relative to wild-type mice (Luthi-Carter *et al.*, 2000)(Fig. 4.20). A separate study measured BrU incorporation into RNA in rat cells as an indicator of global transcription (Hoshino *et al.*, 2004). Less BrU was detected in cells expressing N-mHtt than in cells expressing a wild-type fragment of Htt. The result, the authors concluded, indicated that there was a predominant decrease in global transcription in the presence of
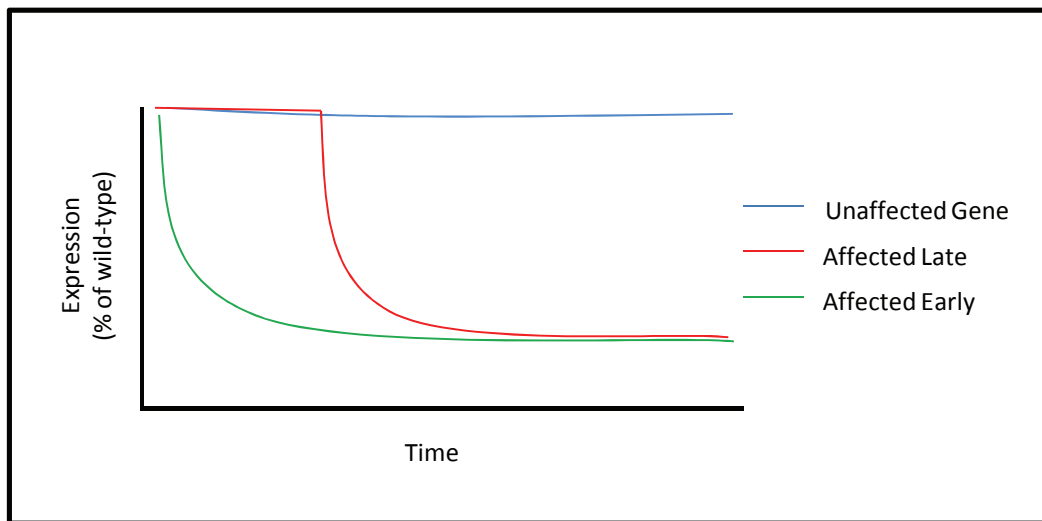
Figure 4.20. Gene expression changes in the presence of mHtt. mRNA expression of genes that are decreased in HD can occur early (green line) or late (red line) in disease progression, while expression of many genes does not change at all.

N-mHtt. It is possible that transcription of all genes is altered to some degree, and the limitations of how quantification experiments such as qPCR and microarray analyses are performed restrict the ability to detect the most subtle gene expression changes. Regardless of which of these interpretations is correct, the fact remains that at late stages of HD transcription of certain genes is more affected than transcription of other genes.

DNA is either silenced or permissive based on whether it is packaged as heterochromatin or euchromatin (Fig. 4.21). Because N-mHtt does not silence transcription, it appears that it affects the relative activity of promoters. Synthesis of mRNA transcripts from genes packaged as euchromatin follows chromatin remodelling, which exposes DNA to transcriptional proteins, the formation of a functional PIC at the transcription start site, and its subsequent escape from the promoter (Fig. 4.21). The formation of a functional PIC requires the stepwise recruitment of general transcription factors which is regulated by the transcription factors, co-activator, and co-repressor complexes specific to a promoter (Naar *et al.*, 2001; Thomas & Chiang, 2006). Once a PIC is formed, PolII is activated and released from the transcription start site where it will pause before either aborting transcription or committing to elongation (Nechaev & Adelman, 2011)(Fig. 4.21). N-mHtt could directly inhibit transcription by interfering with the assembly of the transcriptional machinery, the stability of PICs at the transcription start site, the probability of promoter escape or the kinetics of elongation (Fig. 4.21).

The current models that attempt to explain the gain-of-function mechanism of N-mHtt-mediated transcriptional dysregulation are predicated on known N-mHtt interaction partners. The aggregation model was synthesized following the observation of N-mHtt-
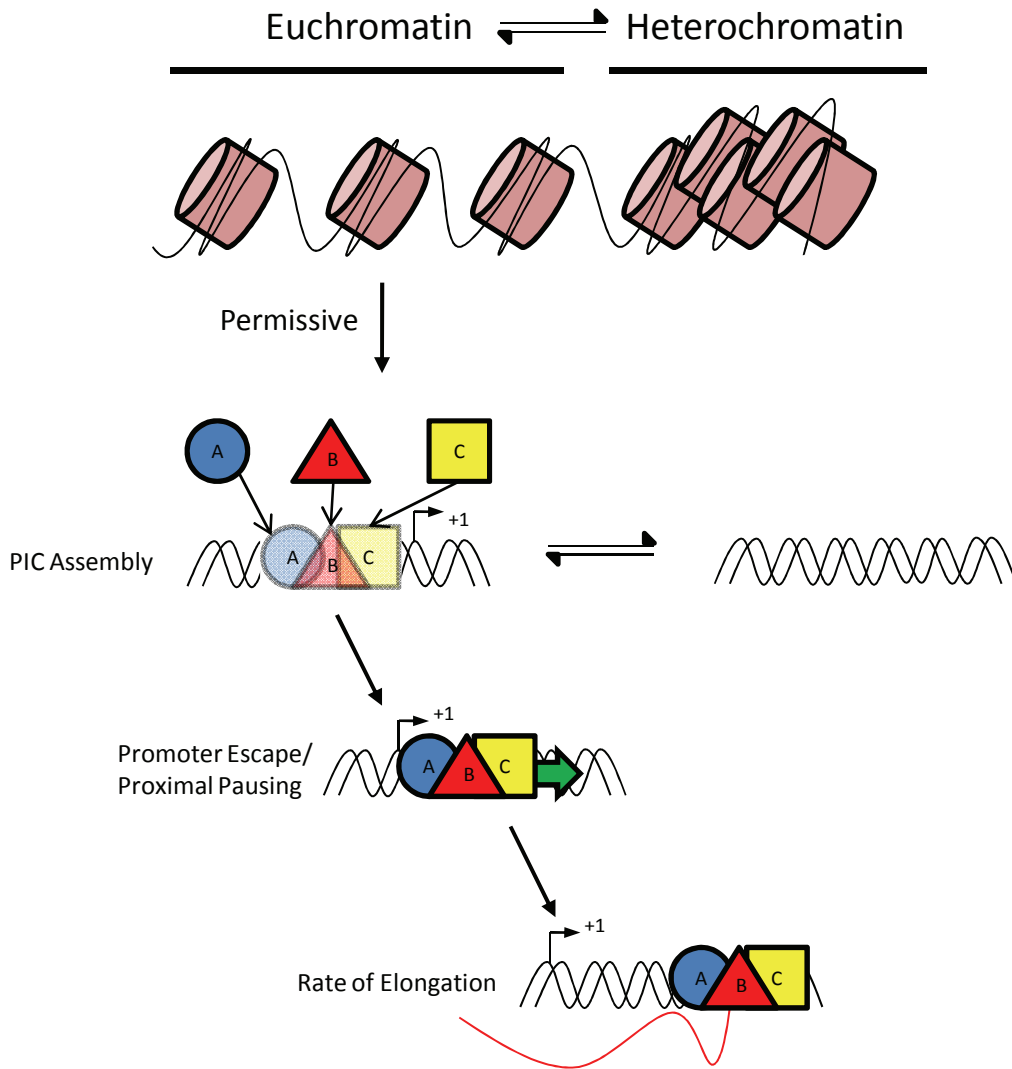
Figure 4.21. Steps in which regulation of gene expression can occur. DNA is packaged as silenced heterochromatin, or permissive euchromatin. DNA structured as euchromatin is either inactive or permissive for active transcription. The production of a mRNA transcript relies on the sequential recruitment of the general transcription machinery, followed by release of PolII from the start site, proximal pausing and then either abortive transcription or committment to elongation. N-mHtt may interfere with any or all of these steps. A, B, C represent hypothetical members of the general transcriptional machinery

interacting with specific proteins and localization of those proteins to NIIs. The

transcription factors PPAR gamma (Chiang *et al.*, 2011), HSF1 (Chiang *et al.*, 2009),

NF-Y (Yamanaka *et al.*, 2008), C/EBP (Chiang *et al.*, 2007), CBP, TBP (Matsumoto *et*

al., 2006), and p53 (Bae *et al.*, 2005) have been identified in NIIs. Transcription

regulated by these numerous proteins has also been shown to decrease in the presence of

N-mHtt, leading to the hypothesis that N-mHtt decreases the amount of soluble

transcription factor in the nucleus by recruiting it into NIIs (Fig**.** 4.22A). By decreasing

the soluble pool of a specific transcription factor in the nucleus, the frequency that a PIC

relying on that specific transcription factor is formed would decrease as well. A

reduction in the frequency of PIC formation would translate to a decrease in overall

transcription for that gene.

The soluble Htt model was based on the observation that the soluble form of N-

mHtt interacts with transcription factors such as TBP (Schaffar *et al.*, 2004) and Sp1

(Dunah *et al.*, 2002). Association of Sp1 with promoters of Sp1-regulated genes that

have decreased transcription in the presence of N-mHtt is lower, and Sp1-dependent

transcription is impaired in the presence of mutant protein (Dunah *et al.*, 2002; Li *et al.*,

2002; Chen-Plotkin *et al.*, 2006). These findings led to the hypothesis that interaction

with soluble N-mHtt impairs the ability of transcription factors to associate with

promoters and perform their transcriptional function (Fig 4.22B).

Although the studies describing the presence of transcription factors in

aggregates predicted that recruitment into aggregates decreased soluble transcription

factor levels in the nucleus, none of these studies demonstrated that soluble levels of the

transcription factors in question were limiting. Soluble levels of TBP, CBP and Sp1 were

A Aggregated N-mHtt Model
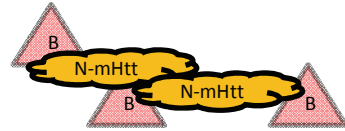
•N-mHtt sequesters B into NIIs
•Less B available for transcription
•Fewer PICs formed that rely on B
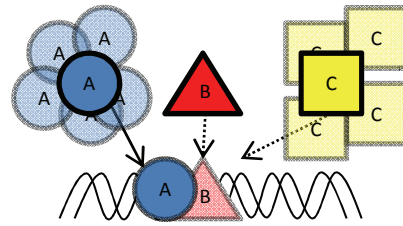
B Soluble N-mHtt Model

•Soluble N-mHtt binds B
•B/N-mHtt does not let B associate at promoter
•Fewer PICs formed that rely on B
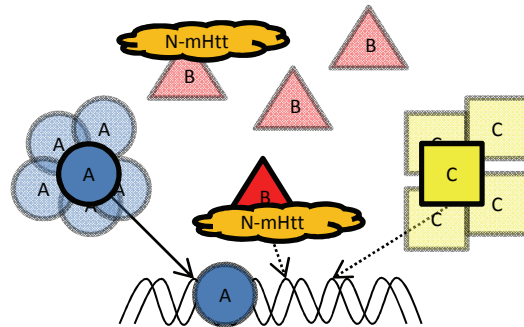
OR

•Soluble N-mHtt binds B
•N-mHtt inhibits B from recruiting C into PIC
•Fewer PICs formed that rely on B
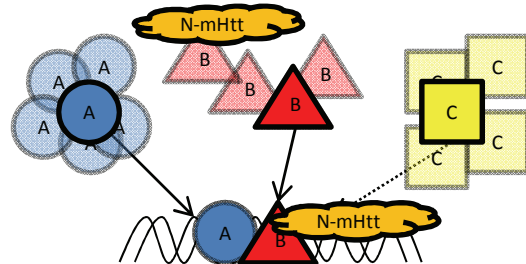
Figure 4.22. Models of N-mHtt-mediated transcriptional dysregulation. **(A)** NIIs recruit transcription factors, decreasing soluble levels available for transcription. **(B)** Soluble N-mHtt interacts with a soluble transcription factor and impairs its ability to associate in PICs, or to recruit subsequent transcriptional machinery. A, B, C, represent hypothetical transcription factors.

not decreased in a group of mouse models of HD at times when transcriptional dysregulation could be detected (Yu *et al.*, 2002). The presence of NIIs within striatalcells in R6/2 mice did not lead to altered transcription when compared to cells that did not contain NIIs (Sadri-Vakili *et al.*, 2006). Together, these studies suggest that aggregates do not reduce soluble levels of transcription factors or correlate with impaired transcription.

Both soluble and aggregation models of transcriptional dysregulation predict that N-mHtt restricts the association of a specific transcription factor at N-mHtt-affected promoters, as has been demonstrated for both Sp1 and RAP30 (Dunah *et al.*, 2002; Zhai *et al.*, 2005; Chen-Plotkin *et al.*, 2006). A limitation of the theories suggesting that restriction of RAP30 or Sp1 association from promoters, however, is that they do not account for transcription of all genes regulated by the protein in question. For example, RAP30 is a member of the general transcription machinery and is assumed to be involved in the formation of PICs for all genes. RAP30 association was not decreased at the N-mHtt-affected HPRT promoter in N548hd cells N548wt cells (Fig. 4.8) or at the N-mHtt-affected GAPDH promoter in the present of transiently transfected N-mHtt (Zhai *et al.*, 2005). Despite the fact that Sp1 binding is decreased at Sp1-regulated N-mHtt-affected promoters, Sp1 occupancy has been shown to increase at Sp1-regulated, non-N-mHtt-affected promoters (Chen-Plotkin *et al.*, 2006). Given that interaction between N-mHtt and Sp1 or RAP30 does not lead to reduced association with all promoters regulated by those proteins, the aggregation and sequestration models claiming that reduced promoter interaction of transcription factors mediates N-mHtt-mediated transcriptional impairments appear to be unable to explain important aspects of gene dysregulation.

Both aggregate-mediated and soluble-N-mHtt-mediated sequestration theories suggest that N-mHtt association with specific protein reduces the pool of functional protein available for transcription (Fig. 4.22). These theories would predict that overexpression of a N-mHtt-interacting protein would increase the pool of functional protein and restore transcription at promoters regulated by that protein. Despite the large number of studies that have characterized N-mHtt interacting partners, few experiments have attempted to overcome N-mHtt-mediated transcriptional repression through overexpression of these interacting partners. The study that formed the basis for attempting to recover transcriptional repression of the CMV and TK promoters through overexpression of TFIIF demonstrated that overexpression of RAP30 restored transcription of a 75 bp fragment of the dopamine D_2 receptor promoter in striatal cells transiently expressing the N-terminal 480 aas of Htt with 68 glutamines (Zhai *et al.*, 2005). This appears to be the only study in the literature that successfully restored N-mHtt-mediated transcriptional repression through overexpression of a single transcription factor. Transcription driven by the CREB-regulated PGC-1α promoter did not increase following overexpression of either CREB or TAF4, however simultaneous overexpression of these proteins restored activity in the presence of N-mHtt (Cui *et al.*, 2006). Likewise, transcriptional repression of the 75 bp fragment of the dopamine D_2 receptor promoter was restored following overexpression of both Sp1 and TAF4 when individual overexpression of the proteins was unsuccessful (Dunah *et al.*, 2002). The lack of literature demonstrating alleviation of N-mHtt-mediated transcriptional repression following overexpression of individual N-mHtt-interacting partners suggests that restoring transcription is not as simple as replenishing the levels of a single interacting

protein in the cell. It is possible that these experiments have been attempted, as was done in this thesis, but that it is difficult to alleviate transcriptional repression of N-mHtt-affected promoters by overexpressing individual N-mHtt-interacting proteins and, as such, these studies have not been published. The observation that overexpression of multiple transcription factors has successfully alleviated transcriptional repression when individual overexpression did not suggests that there are synergistic effects of overexpression of multiple proteins to block effects of mHtt on transcription.

The sequestration models of N-mHtt-mediated transcriptional dysregulation propose that aberrant N-mHtt interactions occur away from the promoter, and would predict that N-mHtt would be absent from the complement of proteins associated with a HD-affected promoter. N-mHtt in N548hd nuclear extract was associated with the N-mHtt-affected CMV promoter. N-terminal Htt from N548wt nuclear extract was also detected at the CMV promoter, suggesting that a normal function of Htt may involve its interaction with or at gene promoters. Both wild-type Htt and N-mHtt have been shown to bind directly to the DNA (Benn *et al.*, 2008). This study proposed that polyQ expansion increased DNA binding and led to impairments in the proper recruitment and association of transcription factors. A specific DNA-binding sequence for N-mHtt could not be determined in the study by Benn *et al.* (2008), and the pattern of DNA binding for mHtt was not correlated with the pattern of gene expression changes observed in the StHdh cell model of HD, making it difficult to interpret how direct association with the DNA would translate to gene-specific transcriptional dysregulation. Another study detected N-mHtt at the N-mHtt-affected PGC-1α promoter (Cui *et al.*, 2006). They suggested that N-mHtt might interact with promoter-bound transcriptional complexes and

inhibit their function rather than their formation. The presence of N-mHtt at N-mHtt-affected promoters does not support models where transcription factors are sequestered from DNA.

The soluble and aggregated models of N-mHtt-mediated transcriptional dysregulation made two predictions: 1) Levels of N-mHtt-interacting proteins were limiting within the nucleus and 2) N-mHtt would sequester these proteins from the promoter. Our data highlights the fact that current models of transcriptional dysregulation do not adequately explain gene-specific N-mHtt-mediated transcriptional dysregulation. The current models also fail to account for the N-mHtt time-dependent changes in gene expression related to disease progression and the possibility that N-mHtt is capable of interacting simultaneously with multiple proteins. These shortcomings suggest that an extension of current models, or a brand new model is required. Any new model of N-mHtt-mediated transcriptional dysregulation must be able to explain the elements of gene-specificity characteristic of HD, as well as how decreased gene expression occurs early in disease progression for some genes, and later in disease progression for others.

Using the assumption that N-mHtt associates at the promoter, and is capable of interacting simultaneously with multiple interaction partners, we propose a new model that attempts to explain promoter-specific and time-dependent N-mHtt-mediated transcriptional dysregulation. Certain assumptions need to be clarified prior to developing our model. First, it is assumed that at the amount of soluble N-mHtt in the nucleus increases during the progression of HD. Second, it is assumed that interactions between N-mHtt and its interaction partners occur in a reversible manner with fixed

association and dissociation constants. Based on these constants, it would be assumed that the amount of an N-mHtt-interaction partner associated with N-mHtt would be dependent on the concentrations of both N-mHtt and its interaction partner. Lastly, this model assumes that interaction between N-mHtt and transcription factors does not impair the ability of those proteins to translocate to the promoter or interact with other binding partners.

This model predicts that N-mHtt associates at a promoter and inhibits the function and not the formation of the PIC. Functional inhibition of transcriptional machinery could result from aborted transcription, impaired promoter escape, or from a decreased rate of elongation (Fig. 4.21). According to our model, every promoter has a probability that a given PIC will contain N-mHtt. The higher the probability of inclusion of N-mHtt at a specific promoter, the more likely that transcription driven by that promoter will be impaired in the presence of N-mHtt. The probability that N-mHtt will be present in a transcriptional complex at a promoter is a function of the probability that N-mHtt will be recruited to the promoter, and the probability that N-mHtt will remain at a promoter (Fig. 4.23).

The probability that N-mHtt will be recruited to a promoter by an individual binding partner depends on the probability that N-mHtt will be associated with that binding partner and the probability that the binding partner will translocate to the promoter (Fig 4.24). Assuming constant concentrations of N-mHtt and the binding partner at a specific time, the probability that N-mHtt will be associated with a binding partner is dependent on the association constant of the interaction. The probability that the binding partner will bind to a specific promoter is dependent on the role that specific

Probability$_{(N\ recruited\ to\ DNA)}$

Probability$_{(N\ remains\ @DNA)}$
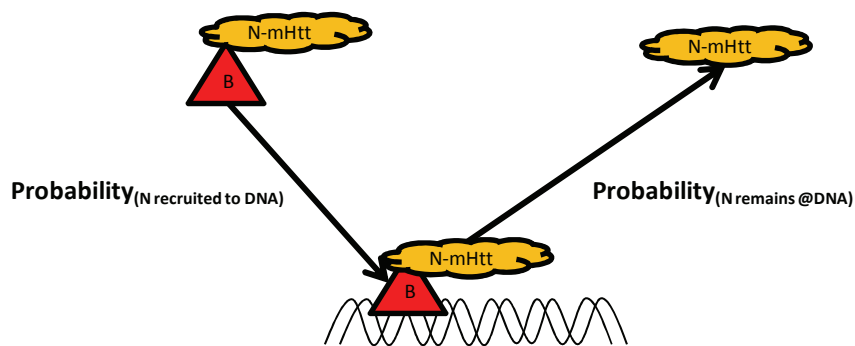
Probability$_{(N\ in\ PIC)}$ = (**Probability**$_{(N\ recruited\ to\ DNA)}$) **x** (**Probability**$_{(N\ remains\ @DNA)}$)

Figure 4.23. The factors influencing the probability that N-mHtt will be in a PIC. The probability that N-mHtt will be in a PIC is equal to the probability that N-mHtt will be recruited to the promoter multiplied by the probability that N-mHtt will remain at the promoter. N represents N-mHtt. B represents a hypothetical N-mHtt interacting partner
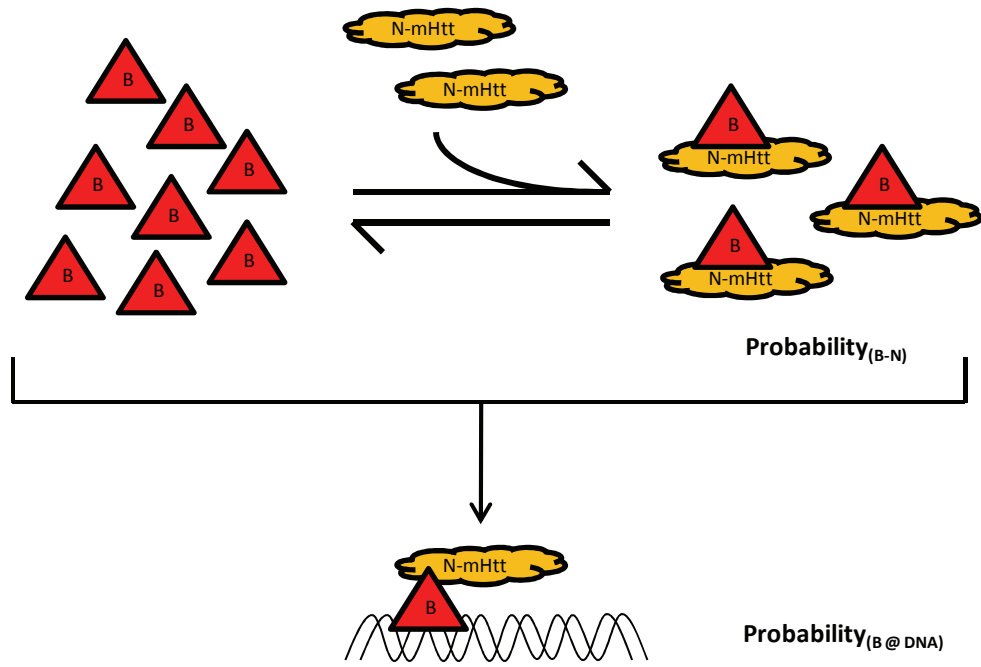
$$\text{Probability}_{(B \text{ recruits N to DNA})} = (\text{Probability}_{(B @ DNA)}) \times (\text{Probability}_{(B\text{-}N)})$$

Figure 4.24. The interaction between N-mHtt and a hypothetical N-mHtt interacting partner (B). The probability that B recruits N-mHtt to a promoter is dependent on the probability that B interacts with N-mHtt and the probability that B will associate with the promoter. N refers to N-mHtt, N-B refers to a N-mHtt/B interaction

transcription factor plays in the regulation of that promoter in a cell context-dependent rate. For example, a protein that has a 20% chance of being associated with N-mHtt and is involved in 100% of transcriptional events at a promoter will recruit N-mHtt to the promoter 20% of the time. The probability that N-mHtt will be recruited to a promoter increases proportionally with the number of N-mHtt interaction partners that regulate the activity of that promoter.

Because N-mHtt binds reversibly to its interaction partners, our model assumes that recruitment of N-mHtt to a promoter does not necessarily ensure that it will remain associated with the transcriptional machinery. Although recruitment to a promoter relies on interactions with individual binding partners, once at the transcriptional machinery N-mHtt is more likely to be in the company of multiple interaction partners that can tether it with higher affinity to the PIC. Our model predicts that the probability that N-mHtt will remain at the promoter is a function of the number of N-mHtt-interacting partners at the promoter. If each binding partner has a specific affinity defining its interaction with N-mHtt, and multiple proteins are able to bind to N-mHtt simultaneously, the energy retaining N-mHtt in a transcriptional complex will be a combination of the individual affinities. Consequently, the higher the combined affinities holding N-mHtt in a PIC, the more energy required to remove it and the more likely it will remain at the promoter (Fig. 4.25).

According to this model, a gene that is regulated by many N-mHtt-interacting proteins will be more susceptible to transcriptional dysregulation at a given concentration of N-mHtt than a gene regulated by fewer N-mHtt-interacting proteins. With more N-mHtt-interaction partners able to bind to the promoter, there is a higher probability that
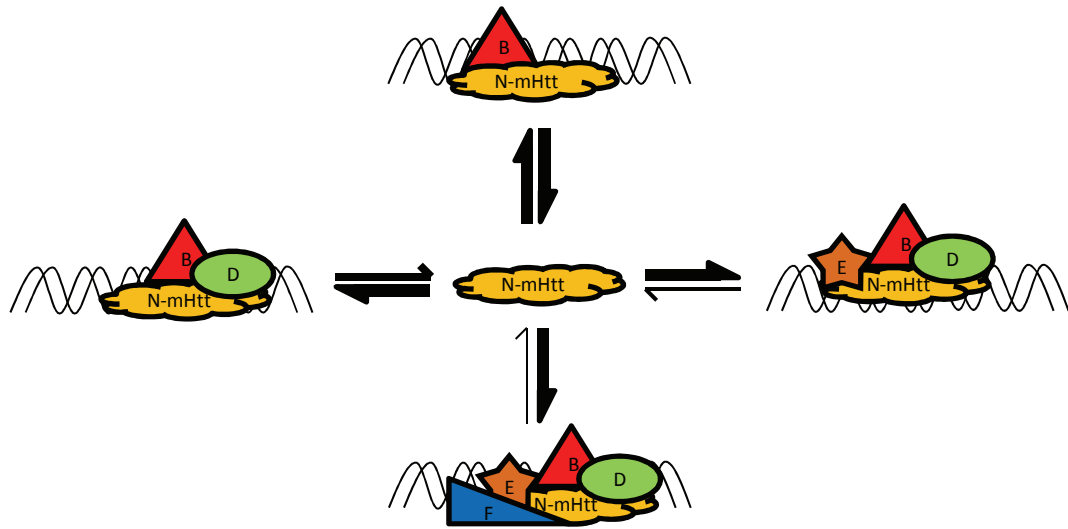
$$\text{Probability}_{\text{(N-mHtt stays in complex)}} \, \alpha \; \text{Affinity}_{\text{(Protein B for N)}} + \text{Affinity}_{\text{(Protein D for N)}} +$$
$$\text{Affinity}_{\text{(Protein E for N)}} + ... + \text{Affinity}_{\text{(Protein x for N)}}$$

Figure 4.25. The probability that N-mHtt remains in PICs increases with the number of N-mHtt-interacting proteins at a promoter. Assuming that proteins can associate simultaneously with N-mHtt, the tendency for N-mHtt to remain in a PIC will increase proportionally with the number of interacting proteins that can retain it in a PIC. N represents N-mHtt. B, D, E, F are hypothetical N-mHtt-interacting transcription factors.

N-mHtt will be recruited to the promoter, and because more N-mHtt-interaction partners can bind to that promoter there is a higher chance that N-mHtt will be retained in a PIC once recruited. This characteristic of the model provides the framework to explain the gene-specificity observed in HD.

The probability that a specific N-mHtt-interacting protein will be bound to N-mHtt is a function of the concentration of N-mHtt in the cell at a given point in time. According to our model, the probability that N-mHtt will be present in a PIC increases directly with the concentration of N-mHtt in the cell. Based on this principle, a promoter that may be unaffected at low concentrations is more likely to be affected at higher concentrations. Since it is assumed that the concentration of N-mHtt in the nucleus increases with disease progression, a promoter that is unaffected at an early stage of the disease is more likely to be affected at a later stage.

In addition to its ability to explain gene-specificity as well as early and late gene expression changes, this model provides an explanation as to why genes regulated by a specific N-mHtt-interacting protein are not globally affected by N-mHtt. In this model, the function of a N-mHtt-interacting transcription factor is irrelevant to its role in mediating transcriptional dysregulation. The role of a N-mHtt-interacting protein in this model is simply as a means to recruit and retain N-mHtt in a PIC. Whether a gene regulated by CBP is or is not affected in the presence of N-mHtt depends on the other N-mHtt-interacting proteins that are involved in the regulation of that promoter.

One final strength of this model is that is provides an explanation for why overexpression of N-mHtt-interacting proteins has had minimal success in restoring transcription at N-mHtt-affected promoters. The more N-mHtt-interacting proteins that

are involved in regulating the expression of a promoter, the less likely that overexpression of one of those N-mHtt-interacting proteins will alleviate transcriptional repression of that promoter. This is because the interacting proteins that are not overexpressed remain able to recruit N-mHtt to the promoter with the same probability. To use a simple example, let us examine two hypothetical promoters. One (P1) is regulated by one N-mHtt interacting protein and the second (P2) is regulated by four N-mHtt interacting proteins. The probability of any of the interacting proteins recruiting N-mHtt to the promoter is 10%. Based on conditional probability theory, this would result in a 10% probability of N-mHtt being recruited to P1 [P1 = 10%], and a 34% probability of N-mHtt being recruited to P2 [P2 = 10% + 9% + 8.1% + 7.3%] (Ross, 2007). We will assume that overexpression of one of the N-mHtt-interacting proteins decreases the likelihood of that protein recruiting N-mHtt to a promoter by 25%. This would reduce the probability of N-mHtt being recruited to P1 to 7.5%, and would reduce the probability of N-mHtt being recruited to P2 to 33%. In one case, the probability of recruiting N-mHtt decreased by 25%, and in the second case the probability of recruiting N-mHtt decreased by 5%. In this hypothetical model, you would have to reduce the probability that of each of the four N-mHtt-interacting proteins able to recruit N-mHtt to P2 from 10% to less than 3% each just to match the original 10% probability of N-mHtt being recruited to P1.

This model of N-mHtt-mediated transcriptional dysregulation makes several predictions. It predicts that N-mHtt will be associated with promoters of affected genes. This has already demonstrated, however the role N-mHtt plays once associated with transcriptional complexes remains unknown. The model predicts that N-mHtt interacts

with multiple binding partners simultaneously. This is a possibility due to the fact that N-mHtt interacts with its different binding partners using different interaction domains (Harjes & Wanker, 2003). Assuming that interaction of one protein does not sterically hinder association with others, it is conceivable that multiple interactions could occur simultaneously. Our model predicts that genes that are affected earlier in the progression of HD will be regulated by more N-mHtt interacting proteins than those affected later in the progression of HD. Lastly, our model predicts that decreasing the amount of soluble N-mHtt will be the most effective strategy for alleviating transcriptional dysregulation at the most severely affected promoters.

Chapter 5:

Conclusion

Based on our observation that N-mHtt associates with N-mHtt-affected promoters, a better understanding of how N-mHtt associates with protein complexes at the core promoter would provide insight into how N-mHtt impairs transcription. The components of the general transcription machinery were originally fractionated using ion exchange chromatography (Matsui *et al.*, 1980). Fractionation of soluble nuclear extract from cells expressing N-mHtt using this technique would localize N-mHtt to specific biochemical fractions reflecting association with groups of proteins and, based on fractionation characteristics, indicate the relative affinity of N-mHtt for subcomplexes of the general transcription factors. Given that the relative orientation of subcomplexes in the PIC is known, this would provide information on the relative location and protein co-associations of N-mHtt. Subsequent analysis of the fractions containing N-mHtt could be performed using techniques such as MALDI-TOF mass spectrometry. Comparison of fractions known to contain N-mHtt formed in the presence and absence of N-mHtt would reveal whether the presence of N-mHtt in the transcriptional machinery alters the relative composition of those complexes.

The fundamental assumption of the model proposed in this thesis is that N-mHtt is capable of interacting simultaneously with multiple binding partners, which would promote retention of N-mHtt in promoter bound protein complexes. The recently developed technique combining bimolecular fluorescence complementation (BiFC) and Förster resonance energy transfer (FRET) could provide insight into dynamic interactions

of multiple N-mHtt-interacting proteins (Kwaaitaal *et al.*, 2010). In BiFC, a fluorescent protein is divided in two and one half of the protein is attached to each candidate binding partner. When the two binding partners interact, the two portions of the fluorescent protein combine to form the intact protein and fluorescence can be detected following excitation of the molecule (reviewed in Kerppola, 2008). FRET involves attaching two different fluorescent proteins to two candidate interaction partners. The emission spectrum of one protein stimulates the fluorescence of the second protein if they are in close enough proximity to each other (reviewed in Zhang & Allen, 2007). By combining BiFC and FRET, the emission spectrum created when N-mHtt and one binding partner interact could be used to stimulate a fluorescein group tethered to a second N-mHtt binding partner. Detection of fluorescence in the emission spectrum elicited by the acceptor molecule would provide evidence that N-mHtt and two of its binding partners were present within close proximity.

Another important step in validating the model would be to test the predictions it makes using an N-mHtt-affected promoter. Several experiments presented in Chapter 4 could be repeated with a slightly different approach to directly test the model proposed. The model predicts that the susceptibility of a promoter to N-mHtt-mediated transcriptional dysregulation is directly related to the number of N-mHtt interacting proteins that regulate the activity of that promoter. It is assumed that deletion of portions of the CMV promoter, as was performed in Figure 4.2, would reduce the number of N-mHtt interacting proteins that could bind to the promoter. With a lower number of binding sites for N-mHtt interacting proteins, recovery of transcriptional repression through overexpression of N-mHtt-interacting proteins or through shRNA-mediated

knockdown of N-mHtt using the -99 CMV promoter may be more easily achieved than what was observed using the -772 CMV promoter. Mutation of putative binding sites for transcription factors known to interact with N-mHtt (Fig. 4.4) was performed to test whether a single N-mHtt interacting protein was responsible for transcriptional repression. As the model proposed predicts that transcriptional repression results from the combined contribution of multiple N-mHtt-interacting proteins, the elimination of multiple transcription factor binding sites would be expected to have a greater impact on the recovery of transcription in N548hd cells than mutation of single sites.

Provided that N-mHtt-mediated transcriptional dysregulation is related to the probability that N-mHtt is present in transcriptional complexes at the promoter, the key to restoring transcription at affected promoters would rest on decreasing the probability that N-mHtt is recruited to promoters. Decreasing the probability that N-mHtt is recruited to promoters could be accomplished by decreasing the frequency that N-mHtt is chaperoned to the DNA through overexpressing N-mHtt-interacting transcription factors. As described earlier, the ability to alleviate repression of transcription by way of protein overexpression is inversely proportional to the number of N-mHtt-interacting proteins that regulate the activity of a promoter. A more efficient approach than overexpression of intact N-mHtt-interacting proteins would be to overexpress fragments of interacting proteins that are able to associate with N-mHtt, but are unable to integrate into transcriptional complexes. N-mHtt has been shown to utilize distinct regions to associate with different proteins. p53 (through its C-terminal domain) and PACSIN1 (through an SH3 domain) interact with the poly-proline domain of N-mHtt (Steffan *et al.*, 2000; Modregger *et al.*, 2002), polyQ-binding protein (likely through polar aa-rich regions)

interacts with the polyQ region of N-mHtt (Imafuku *et al.*, 1998; Waragai *et al.*, 1999) and HAP1 (using aas 323-416) interacts between aas 171-230 of N-mHtt (Li *et al.*, 1995; Li *et al.*, 1998). Expression of only the domains of these proteins that interact with N-mHtt would reduce the frequency that fully intact, functional versions of these proteins could bind to and recruit N-mHtt to promoters. Additionally, the association of these fragments with interaction domains in N-mHtt would also reduce the frequency that other transcriptional proteins that associate with these regions of N-mHtt could bind and recruit N-mHtt to promoters. These N-mHtt interacting molecules may act as dominant-negative regulators of the toxic function of N-mHtt. Combining these modules with NESs or sequences that target N-mHtt for degradation might be attempted.

An alternative to decreasing the probability that N-mHtt will be present at the DNA by reducing its recruitment to promoters is to reduce the nuclear concentration of N-mHtt available for recruitment to the DNA. Based on the model of transcriptional dysregulation proposed in this thesis, decreasing the concentration of N-mHtt in the nucleus would be the most efficient approach to improving gene transcription at all N-mHtt-affected promoters. Reducing the amount of N-mHtt would theoretically decrease the probability that every N-mHtt-interacting protein would escort N-mHtt to promoters, since it would decrease the proportion of each N-mHtt-interacting protein associated with N-mHtt. There has been considerable effort made in trying to reduce the synthesis, cleavage or nuclear accumulation of mHtt with varying degrees of success. Expression of mHtt can be decreased using siRNA technology. Using miRNA complementary to the CAG repeat region of *huntingtin* mRNA, but containing several mismatched bp, led to a 30-fold greater knockdown of mutant *huntingtin* mRNA than wild-type mRNA in cells

derived from HD patients (Hu *et al.*, 2010). Phosphorylation of mHtt decreases cleavage of mHtt and reduces the nuclear accumulation of N-mHtt. Phosphorylation of mHtt at serine 421 was inversely proportional to the amount of cleaved, nuclear N-mHtt detected in cells transiently transfected with mHtt (Warby *et al.*, 2009). Serum- and glucocorticoid-induced kinase (Rangone *et al.*, 2004) and the serine/threonine kinase Akt (Humbert *et al.*, 2002) have been shown to phosphorylate Htt at serine 421, providing targets for manipulating the production of N-mHtt. The fusion of a polyQ-binding protein domain, which selectively targets the expanded polyQ region of mHtt, with a domain targeting the construct for chaperone-mediated autophagy resulted in a reduction in the amount of soluble N-mHtt and an improvement in phenotype in R6/2 mice (Bauer *et al.*, 2010).

Overall, the proposed experiments provide a means of testing the assumptions that underlie the model proposed in this thesis, as well as testing the predictions made by the model. Most importantly, should these proposed experiments validate elements of our model, the experiments outlined above would provide the most effective means, according to the mechanism of our model, of alleviating transcriptional dysregulation in the hopes of interfering with the cascade of changes that occur in HD. It is our hope and belief that recovery of transcription will halt the cascade of changes that occurs in HD, and slow the progression of the disease.

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