Role of Huntingtin Phosphorylation at Serine 421 in Huntington Disease

by

SIMON CHRISTOPHER WARBY

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ABSTRACT

Huntington Disease (HD) is a lethal neurodegenerative disorder that results from polyglutamine-expansion in the huntingtin protein. Despite the widespread tissue expression pattern of huntingtin, there is selective degeneration of specific neurons populations in the brain. The regulation of huntingtin by posttranslational modifications, such as phosphorylation, is not well understood. The objective of this thesis was to conduct molecular and cell biological studies to determine whether huntingtin was regulated by phosphorylation and what role this might play in the disease.

I found that huntingtin is phosphorylated on serine-421 (S421) by the pro-survival signalling protein kinase Akt (PKB) in the brain under normal physiological conditions. There are differences in the basal endogenous phosphorylation state of huntingtin in different regions of the brain and regions more susceptible in the disease have lower levels of S421 phosphorylation. Consistent with the hypothesis that S421 phosphorylation protects against the toxicity of polyglutamine-expanded huntingtin, S421 phosphorylation is reduced in the presence of the HD mutation.

Furthermore, I discovered significant relationships between the phosphorylation status of huntingtin and pathological processes in the disease, including the cleavage and nuclear localization of polyglutamine-expanded huntingtin. The S421 site is close to the proteolytic domain containing the caspase cleavage sites (aa500-600) and phosphorylation at S421 reduces the cleavage of huntingtin.

The nuclear localization of huntingtin fragments is also known to be important in the disease. I directly assessed whether phosphorylation at S421 alters the nuclear localization of huntingtin using subcellular fractionation and immunofluorescent techniques. Phosphorylation at S421 reduces the nuclear localization of both full length huntingtin and huntingtin fragments. Further, the 1-586aa huntingtin fragment is specifically trafficked and concentrated in the nucleus, providing a functional link between cleavage and nuclear localization that may be mediated by S421 phosphorylation.

Phosphorylation at S421 is a dynamic means of regulating huntingtin and is implicated in HD because it is reduced by the polyglutamine-expansion and modifies key pathogenic processes such as the cleavage and nuclear localization of huntingtin. The integration of signalling pathways on huntingtin and the phosphatase regulation of huntingtin phosphorylation are enticing therapeutic possibilities for modulating the pathogenesis of HD.

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LIST OF ABBREVIATIONS

A2a	adenosine 2A receptor
aa	amino acid
Akt	protein kinase Akt, also known as protein kinase B (PKB)
BAC	bacterial artificial chromosome
bp	base pair
C3R	caspase 3 resistant
C6R	caspase-6 resistant
CB1	cannabinoid 1 receptor
CMMT	Centre for Molecular Medicine and Therapeutics
CMV	cytomegalovirus
CNS	central nervous system
CR	caspase resistant
CT	computed tomography
D1-2	dopamine receptor 1-2
DNA	deoxyribonucleic acid
DRPLA	dentatorubral-pallidoluysian atrophy
ENK	enkephalin
ER	endoplasmic reticulum
ex vitro	experiments performed with immunopurified and purified reagents
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Gpe	globus pallidus external
Gpi	globus pallidus internal
GST	glutathione S-transferase
HAP	huntingtin-associated protein
HD	Huntington disease
Hdh	murine HD homolog
HDL	Huntington disease-like disorder
HDAC	histone de-acetylase
HEAT	repeat sequence found in huntingtin, elongation factor 3, protein phosphatase 2A, TOR1
HIP	huntingtin-interacting protein
htt	huntingtin protein
HunMAD	huntingtin membrane-associated domain
IA	intermediate allele
in vitro	experiments performed in cultured cells
in vivo	experiments performed in/from mouse tissue
InsP3R	inositol tri-phosphate receptor
JPH3	junctophilin 3
MAPK	mitogen-activated protein kinases
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MTOC	microtubule organizing center
NES	nuclear export signal
NII	neuronal intranuclear inclusion
NLS	nuclear localization signal

NMDA	N-methyl-D-aspartic acid
NRSF	neuron restrictive silencing factor
nt	nucleotide
PAT	palmitoyl acyl-transferases
PACSIN	protein kinase C and casein kinase substrate in neurons-1
PET	positron emission topography
PDK1	pyruvate dehydrogenase kinase
PGD	preimplantation genetic diagnosis
PMI	post mortem interval
polyP	poly-proline
polyQ	poly-glutamine
PP	phosphatase
PrP	prion protein
pS421	phospho-serine 421 of huntingtin
PSD	post-synaptic density
REST	repressor element-1 transcription
SBMA	spinal bulbar muscular atrophy
SCA1	spinocerebellar ataxia type 1
SGK	serum and glucocorticoid-inducible protein kinase
SNc	substantia nigra compacta
SNP	single nucleotide polymorphism
SNpr	substantia nigra pars reticulata
SP	substance P
STN	subthalamic nucleus
SUMO	small ubiquitin-related modifier
TBP	tata-binding protein
TOR	target of rapamycin
TPR	translocated pore promoter
UCHL1	ubiquitin carboxy-terminal hydrolase L1
WT	wildtype
YAC	yeast artificial chromosome

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Cheers.

CHAPTER 1

Introduction

Huntington disease (HD) is a fatal neurodegenerative disorder that is dominantly inherited and currently has no effective treatment or cure. The disease is caused by an expansion in the CAG tract of the HD gene, resulting in polyglutamine-expansion of the huntingtin protein. The onset of the disease generally does not occur until midlife, often after presymptomatic HD allele carriers have children, and have potentially passed the HD allele on to the next generation.

1.1 CLINICAL FEATURES OF HD

1.1.1 Pre-Clinical Stage

Prior to clinical onset, individuals at-risk for developing HD are healthy and free of detectable signs or symptoms of the disease. There exists a pre-clinical stage, however, (often referred to as 'pre-symptomatic' phase) where patients may have subtle signs involving cognitive deficits and neuropathological changes detected by MRI (Walker, 2007). The clinical phase of the disease follows with a relentless progression of motor, cognitive and psychiatric symptoms (Figure 1.1).

1.1.2 Motor Symptoms

A distinguishing clinical feature of the disease are the unique involuntary writhing movements of the head, neck and arms known as Huntington's Chorea. Other motor symptoms such as abnormalities in eye movements, fine motor control, and gait (Folstein et al., 1986; Kremer et al., 1992; Di et al., 1993; Lasker and Zee, 1997) often accompany or precede (Kirkwood et al., 1999) chorea. The involuntary chorea subsides as the disease progresses, as does the ability to perform voluntary movements (bradykinesia leading to dystonia and rigidity) (Harper, 2005). These motor deficits inevitably lead to difficulties in speaking (dysarthria) and swallowing (dysphagia) (Walker, 2007). Although motor abnormalities are typically used to define the onset of disease in a neurological exam, the sequence and presentation of HD symptoms can be quite variable from person to person, even within families (Friedman et al., 2005; Gomez-Esteban et al., 2006). Specific psychiatric and cognitive impairments often precede the motor onset (Paulsen et al., 2001b).

1.1.3 Psychiatric Symptoms

Psychiatric symptoms resulting in personality changes are often associated with the disease (Shiwach, 1994; Paulsen et al., 2001a; Thompson et al., 2002). These changes may occur prior to onset (Kirkwood et al., 2002) and may range from changes in mood including irritability and apathy (Craufurd et al., 2001; Anderson and Marshall, 2005), to extreme manifestations that resemble schizophrenia (Caine and Shoulson, 1983) or major affective disorders (Folstein and Folstein, 1983). Rates of depression and suicide are high in HD patients relative to the general population (Hayden et al., 1980; Shiwach, 1994; Almqvist et al., 1999; Slaughter et al., 2001; Paulsen et al., 2005; Robins Wahlin, 2006).

1.1.4 Cognitive Symptoms

A decline in specific aspects of cognitive function is frequently observed (Lawrence et al., 1996; Lawrence et al., 1998), with progression to more severe and widespread abnormalities (Bamford et al., 1995; Foroud et al., 1995; Jason et al., 1997; Snowden et al., 2001; Ward et al., 2006). Early deficits include reduced performance on frontal executive tasks (Sprengelmeyer et al., 1995; Ho et al., 2006; Beste et al., 2006), memory function (Bylsma et al., 1990; Brandt et al., 2005; Montoya et al., 2006) and language abilites (Ho et al., 2002; Teichmann et al., 2006; Yoon et al., 2006). Many behavioural difficulties later in the disease are believed to be the result of cognitive impairments such as reduced thinking speed, further memory impairments, difficulties in sequencing activities appropriately, loss of visiospatial awareness, and fixed thinking (Bourne et al., 2006).

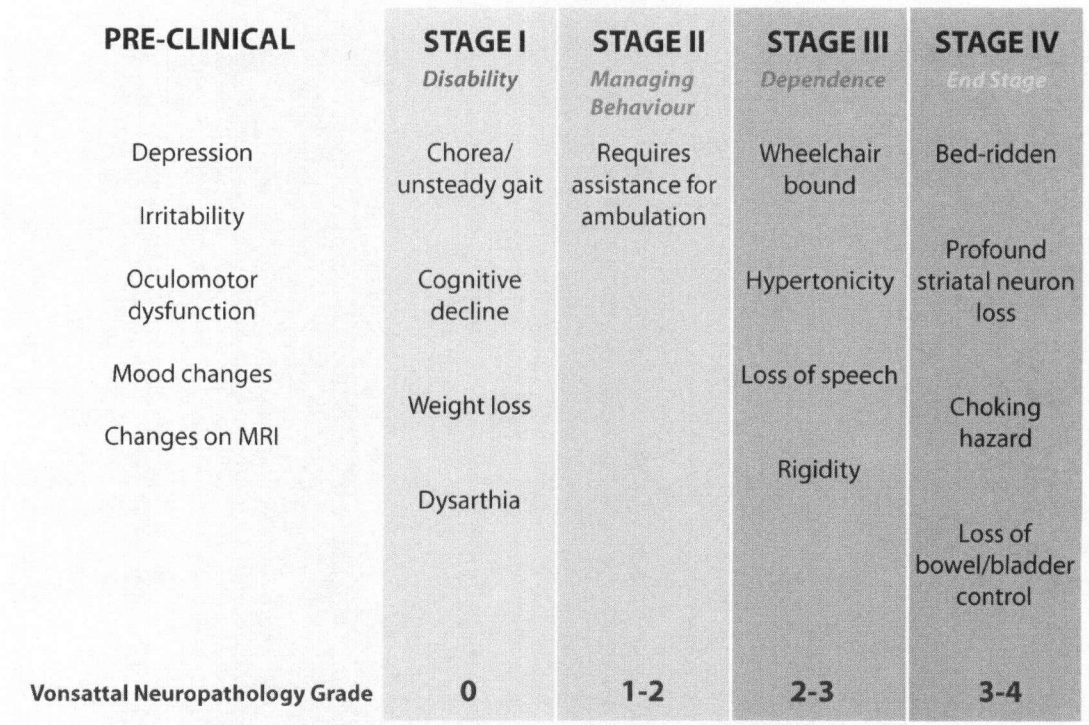

Figure 1.1: Progression of Signs, Symptoms and Neuropathology in HD.
Subtle pre-clinical changes are observed in HD patients prior to the appearance of overt motor symptoms that are used to define the clinical onset of the disease. Vonsattal neuropathology grade refers to (Vonsattel et al., 1985).

1.1.5 Duration

The duration of HD, from disease onset to death, averages ~20 years (Foroud et al., 1999). However, there are differences in duration depending on the age at onset of the disease. In a large study of 2068 HD patients, juvenile HD patients and late-onset patients had a significantly shorter duration than patients with onset in mid-life (20-49 years of age) (Foroud et al., 1999). Previous studies had also found the shortest disease duration in the late-onset cases (Roos et al., 1993). Juvenile HD cases tend to have the most variability in disease duration, and males tend to have a shorter duration than females (Foroud et al., 1999).

1.1.6 Other Features

Weight loss, sleep disturbances and testicular degeneration are 'non-classical' symptoms associated with HD. The reduction of body weight in HD patients occurs early (Djousse et al., 2002) and without intervention becomes profound later in the disease (Stoy and McKay, 2000; Hamilton et al., 2004; Walker and Raymond, 2004; Robbins et al., 2006). Early loss in weight, despite an increased appetite (Sanberg et al., 1981; Morales et al., 1989), may be due to defects in metabolism (Pratley et al., 2000), perhaps resulting from hypothalamic neuron loss or

dysfunction (Kremer and Roos, 1992; Petersen and Bjorkqvist, 2006). Later in the disease, increased metabolism due to chorea and dystonia and eventually dysphagia likely also contribute.

Sleep and circadian rhythm disturbances are observed in HD patients (Morton et al., 2005) and increase with disease progression (Hansotia et al., 1985). The reduced amount and quality of sleep (Wiegand et al., 1991) may be due to the dysfunction or loss of neurons in the hypothalamus (Kremer and Roos, 1992; Kassubek et al., 2004b; Petersen and Bjorkqvist, 2006).

Testicular degeneration has also been observed in HD patients and mouse models of the disease (Leavitt et al., 2001; Papalexi et al., 2005; Van Raamsdonk et al., 2005b). Affected HD patients have a significant reduction in the number of germ cells as well as abnormal seminiferous tubule morphology (Van Raamsdonk et al., 2007). Prior to the onset of the disease however, HD allele carriers appear to have normal fertility (Shokeir, 1975; Mastromauro et al., 1989; Pridmore and Adams, 1991) suggesting that testicular changes occur as part of the degenerative phenotype of the disease.

1.1.7 Juvenile HD

In a subset of HD cases (2-10%), the disease begins prior to 21 years of age (Hayden MR, 1981; van Dijk et al., 1986; Siesling et al., 1997; Nance and Myers, 2001). In contrast to adult onset HD, which generally occurs in mid-life, the clinical presentation of juvenile HD differs and is more severe. Juvenile onset HD initially features progressive rigidity, rapid intellectual deterioration, motor dysfunction and/or seizures (Nance and Myers, 2001; Yoon et al., 2006; Gonzalez-Alegre and Afifi, 2006). Disease progression is much more rapid in juvenile-onset HD and in general, leads to shorter duration (Gonzalez-Alegre and Afifi, 2006).

1.1.8 Summary

HD is a disease that involves all members of a family including HD allele carriers, individuals at risk, and spouses (Figure 1.2). Although many drug trials are underway (Di Prospero and Fischbeck, 2005; Scatena et al., 2007; Bonelli and Hofmann, 2007; Kimura et al., 2007), there is currently no effective therapy to slow progression or delay the age of onset of HD (Borrell-Pages et al., 2006). Current treatments are palliative and focus on the reduction of positive motor

symptoms and depression, often at the expense of motor and psychiatric side-effects (Truant et al., 2006b).

Positive motor symptoms (particularly chorea and dystonia) are the most distinguishing features and are most often used to define the onset of the disease. However, negative motor features (rigidity), cognitive impairment, and psychosocial well-being have the greatest impact on quality of life and lead to greater overall disability for the patient (Mayeux et al., 1986; Helder et al., 2001; Mahant et al., 2003). HD is usually fatal within 20 years of onset, generally due to pneumonia, heart failure and/or malnutrition (Hayden MR, 1981; Truant et al., 2006b).

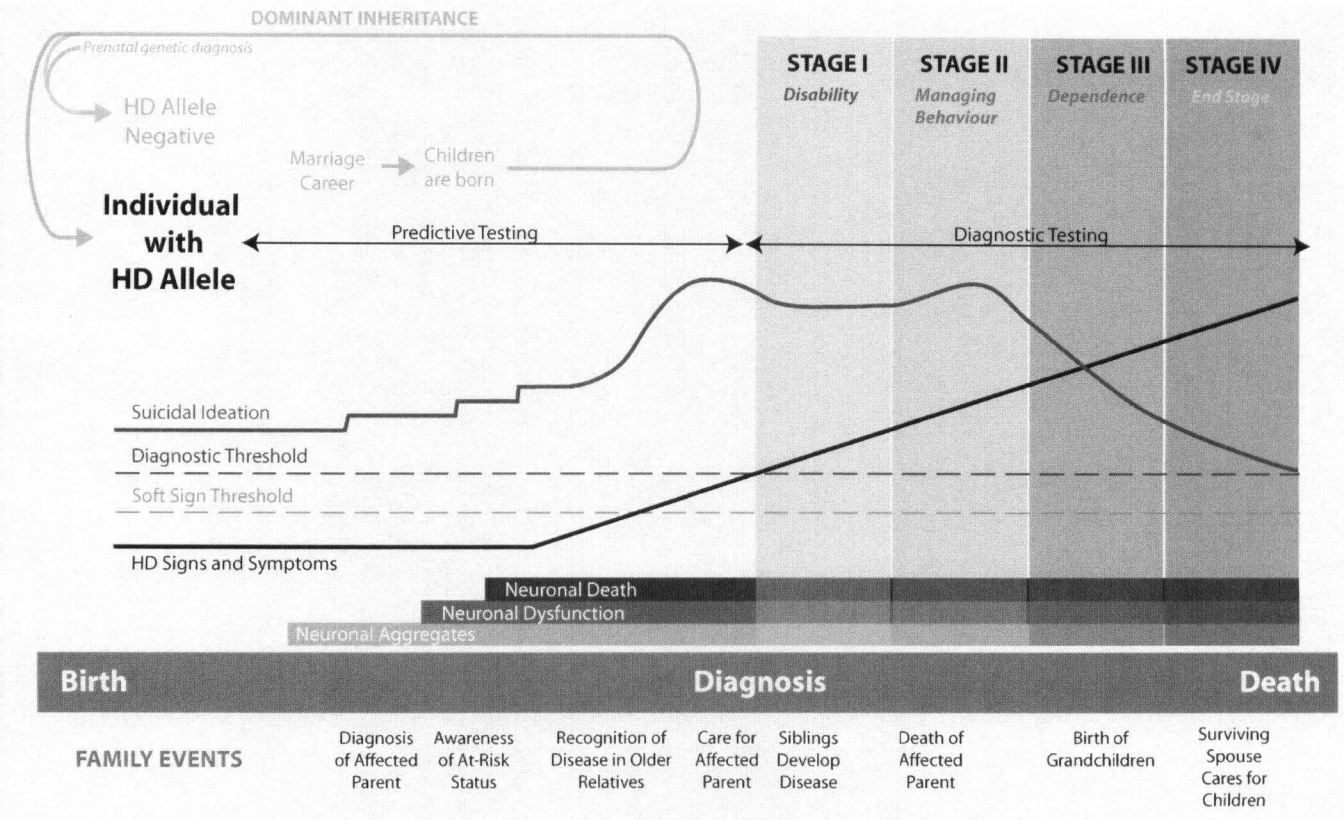

Figure 1.2: Natural History of HD.
Hypothetical timeline of important events affecting an HD family from the perspective of an individual carrying an HD allele (not drawn to scale). For most HD patients, the majority of their lifetime is symptom free. However, for most adult-onset cases, the disease begins after marriage, career building and parenthood have begun. The HD allele is frequently passed on to the next generation prior to the onset of the disease in the parents (indicated by orange cycle). Pre-implantation genetic diagnosis (PGD) and prenatal screening, accompanied by genetic counselling, are means of preventing the transmission of the HD allele. Figure adapted and reprinted from (Walker, 2007), with permission.

1.2 NEUROPATHOLOGY

Although the mutant huntingtin protein is expressed at similar levels in all brain regions (Aronin et al., 1995; Landwehrmeyer et al., 1995; Trottier et al., 1995a), many of the clinical features of HD result from dysfunction and loss of specific neuronal subpopulations primarily in the basal ganglia of the brain. Within the basal ganglia, neurons within the caudate and putamen of the striatum are most severely affected.

1.2.1 Structure and Function of the Basal Ganglia

In the most general sense, the basal ganglia receives information from the cortex, filters and processes this information in a basal ganglia circuit, and returns the processed signal to the cortex. Excitatory input from various regions of the cortex enters the basal ganglia circuit through connections to the striatum. The striatum passes inhibitory signals to the globus pallidus and substantia nigra, which act on the thalamus to modify excitatory signals sent back to the cortex.

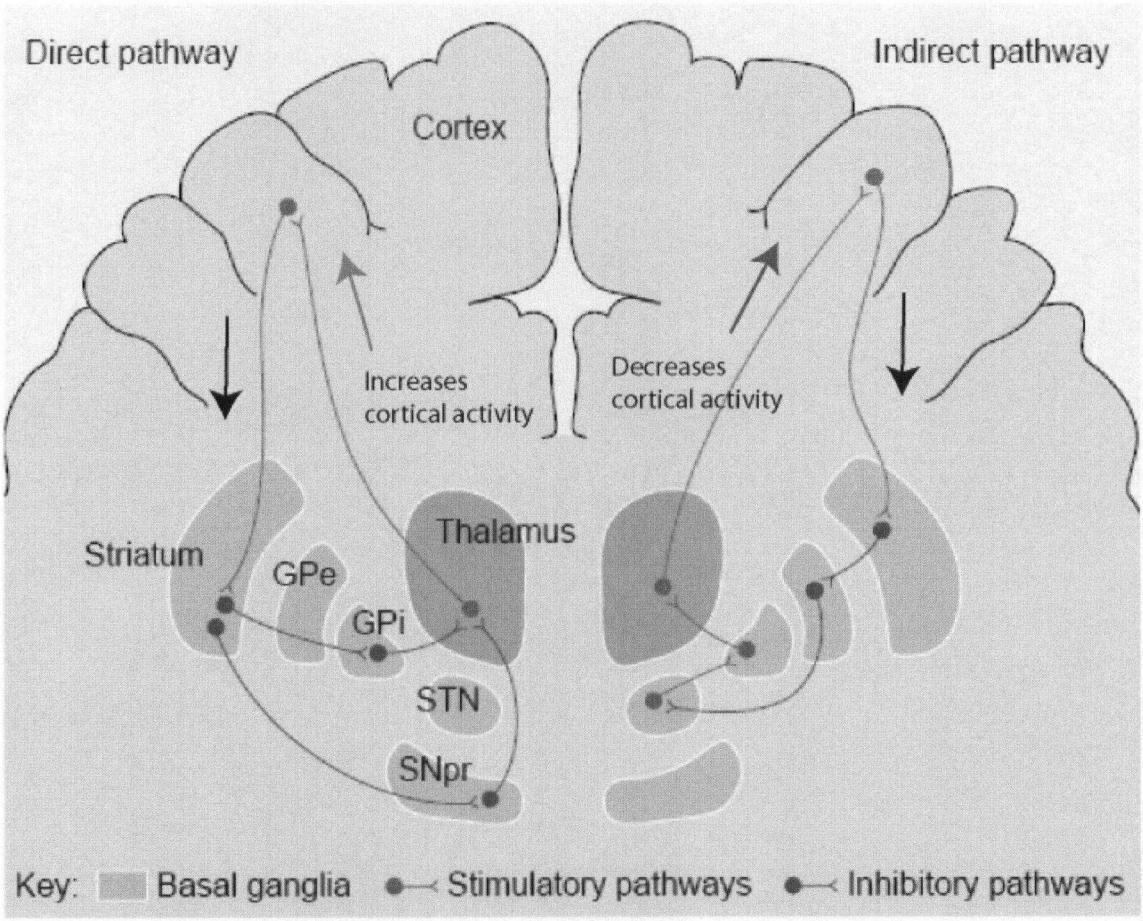

Figure 1.3: Direct and Indirect pathways of the Basal Ganglia
The direct pathway (left) has medium spiny inhibitory projections from the striatum to the substantia nigra pars reticulata (SNpr) and the internal segment of the globus pallidus (GPi). The indirect pathway (right) projects to the GPi via the external segment of globus pallidus (GPe) and the subthalamic nucleus (STN). The direct and indirect pathways have opposing effects on the thalamus, which projects back to the cortex. Reprinted from (Andrews and Brooks, 1998) with permission.

The basal ganglia circuit is broken down into the direct and indirect pathways (Figure 1.3). Each pathway utilizes a different circuit and involves a different set of neurotransmitters. The net result of the direct pathway is to increase cortical activity while the indirect pathway reduces cortical activity.

1.2.2 Pathology in the Striatum

The striatal degeneration in HD is principally a progressive degeneration of the gamma-aminobutyric acid (GABA)-releasing medium-spiny neurons that project from the striatum (Vonsattel et al., 1985) (Figure 1.4). The striatal neuropathological changes that occur have been classified into 5 pathological stages or grades (Vonsattel et al., 1985; Myers et al., 1988) and

tend to occur in a caudal to rostral pattern (Hedreen and Folstein, 1995). In late stages of the disease, there is a profound loss of neurons in the striatum and generalized atrophy of the cortex, resulting in gross enlargement of the lateral ventricles.

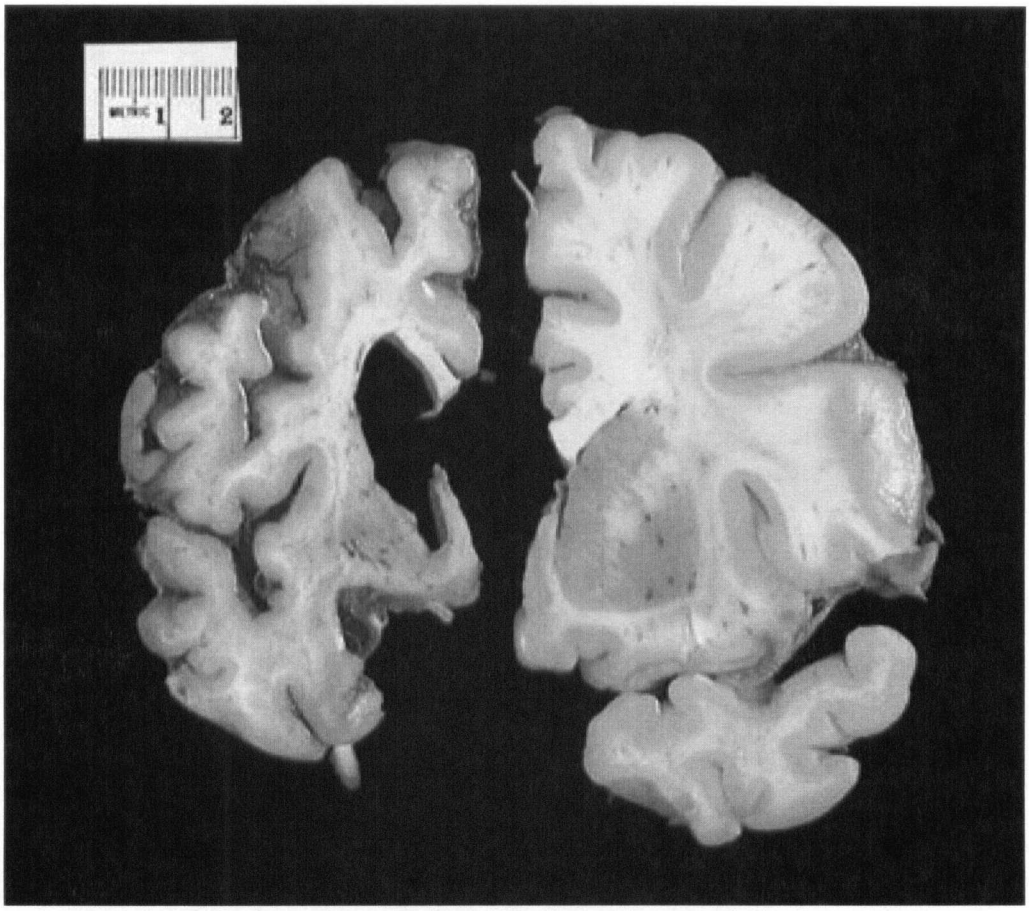

Figure 1.4: Atrophy and neuron loss in the HD brain
Late stage HD brain (left) has severe atrophy of the striatum and cortex relative to the unaffected control brain (right). Reprinted with permission from the Harvard Brain Tissue Resource Center.

The pattern of neurodegeneration and receptor changes that occur in the basal ganglia follow a specific sequence (Glass et al., 2000) (Figure 1.5). The initial GABAergic subpopulation of neurons to degenerate are those of the indirect striatal pathway that express enkephalin and are enriched in the dopamine receptor D2 (Reiner et al., 1988). It is the initial loss of neurons in this indirect pathway (and therefore *loss* of thalamic-cortical *inhibition*) that is believed to cause the choreiform movement disorder characteristic of HD (Mitchell et al., 1999). Later in the disease, loss of neurons in the direct pathway, composed of GABAergic neurons expressing substance P,

suppresses movement and results in hypoactivity and dystonia. Neuropathological changes occur prior to clinical manifestations of the disease, as significant neuropathological changes have been observed by MRI in 'pre-clinical' HD patients including reduced basal ganglia volume (Aylward et al., 1994; Aylward et al., 1996; Thieben et al., 2002) (Aylward et al., 2004; Kipps et al., 2005).

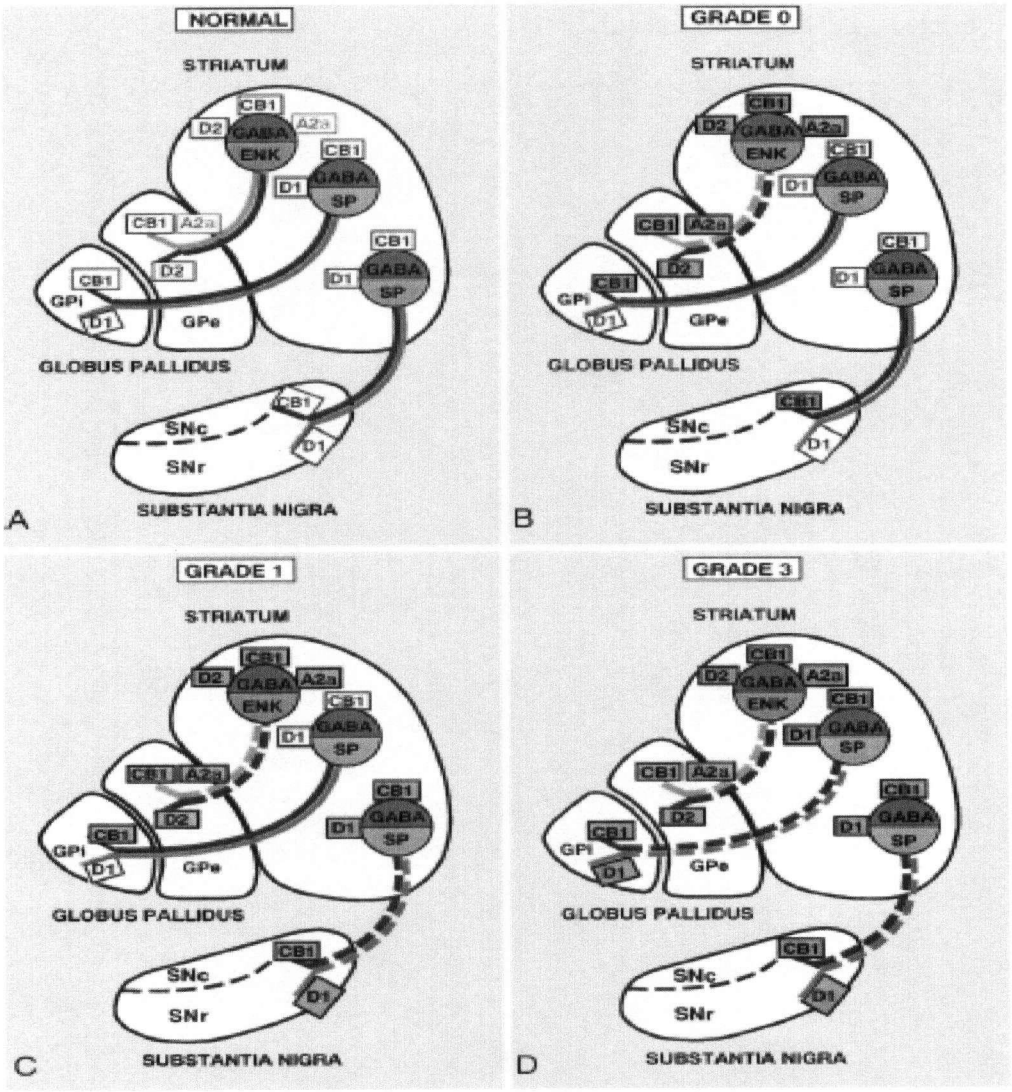

Figure 1.5: Progression of selective HD neuropathology in the Basal Ganglia
(A) In the normal brain, there are three sub-populations of GABAergic medium-sized spiny striatal projection neurons that express different neurotransmitters and receptors. In early stages of HD (B), the indirect pathway projection to the GPe is the first subset of neurons to lose receptor expression and degenerate. Medium spiny projection neurons of the direct pathway subsequently degenerate in later stages of the disease (C and D). Abbreviations: Globus pallidus internal (GPi) and external (GPe), Substantia Nigra pars compacta (SNc) and reticulata (SNr), Gamma-aminobutyric acid (GABA), enkephalin (ENK), substance P (SP), dopamine receptor 1 (D1) and 2 (D2), cannabinoid receptor 1 (CB1), adenosine 2A receptor (A2a). Adapted and reprinted from (Glass et al., 2000), with permission.

Variability in the psychiatric and cognitive symptoms of HD has been associated with the variability in the selective degeneration of specific subpopulations of medium spiny neurons in the striatum. The striatum is organized into two major neurochemical compartments: striosomes (or 'patch') and matrix (Holt et al., 1997). Neuroanatomical studies have shown that the striosomes and matrix are functionally connected to different regions of the cortex. Striosomes are linked to limbic circuitry, and the matrix is interconnected with sensorimotor and associative cortical circuitry (Graybiel et al., 1994). As predicted by the patch/matrix model of the basal ganglia, there is a significant association between the selective loss of GABAa receptors in the striosomes and mood dysfunction in HD patients (Tippett et al., 2007).

1.2.3 Neuroimaging Studies and Extra-Striatal Degeneration

Neuroimaging studies that utilize a variety of techniques such as MRI, PET, and CT have been used extensively to characterize the neuropathology in HD (Andrews and Brooks, 1998; Rosas et al., 2004; Laihinen and Halsband, 2006), with specific interest in its use for clinical trials (Aylward et al., 2003; Paulsen et al., 2006). Initially, neuroimaging studies focused on atrophy of the basal ganglia (Aylward et al., 2000; Rosas et al., 2001), but these studies have also found significant volume decreases in the cortex, hippocampus, cerebral grey and white matter, and global atrophy of the brain, even at early stages of the disease (Rosas et al., 2003; Kassubek et al., 2004a; Kassubek et al., 2004b; Rosas et al., 2005). Extrastriatal degeneration may be important in the clinical features of HD, as cognitive changes have been correlated with atrophy of the frontal cortex in HD patients (Bamford et al., 1995; Thiruvady et al., 2007).

1.2.4 Neuronal Intranuclear Inclusions

Neuronal intranuclear inclusions (NIIs) (Figure 1.6) were first observed in transgenic mouse models of HD (Davies et al., 1997; Scherzinger et al., 1997). Subsequently, NIIs were observed in HD brains (DiFiglia et al., 1997; Scherzinger et al., 1997; Becher et al., 1998) and cultured cells (Li and Li, 1998; Cooper et al., 1998). These NIIs contain polyglutamine-expanded huntingtin and ubiquitin (DiFiglia et al., 1997), and may have the capacity to sequester and deplete many other proteins (Preisinger et al., 1999; Swayne and Braun, 2007).

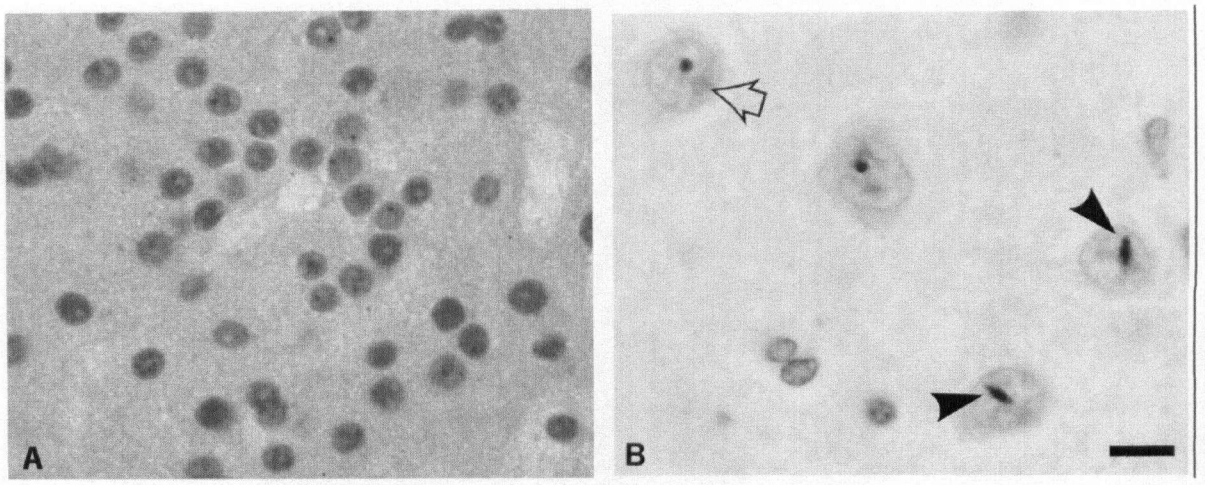

Figure 1.6: Neuronal intranuclear inclusions (NIIs) in the brains of mice and human HD patients.
(A) NIIs stain as dark EM48 inclusions inside the stained nucleus of striatal neurons from a YAC128 mouse brain (R Graham). **(B)** Cortical neurons from an HD patient with 74 CAG repeats shows NIIs stained for ubiquitin. (B) is adapted from (Becher et al., 1998), with permission.

A classically held view of HD pathogenesis is that these nuclear inclusions cause neurodegeneration and HD symptomatology (Davies et al., 1998; Wanker, 2000). However, several lines of recent evidence suggest that soluble huntingtin containing the HD mutation, and not the inclusions, are the toxic species (Slow et al., 2006):

i) Although nuclear inclusions are present in post-mortem human HD brain, there is dissociation between nuclear inclusions and the selective pattern of striatal neuron loss observed in HD. Within the striatum, nuclear inclusions are predominantly observed in spared interneurons, with few or no inclusions found within the medium spiny neurons that are known to degenerate (Kuemmerle et al., 1999).

ii) The presence of inclusions and the onset of neurological signs and symptoms does not correlate in full-length huntingtin mouse models of the disease (Slow et al., 2003; Menalled et al., 2003). These data demonstrate that neuropathology can occur in the absence of inclusion formation and argues against inclusions being the toxic moiety.

iii) Therapeutic trials that have shown improvements in mouse models of HD have done so without altering number of inclusions (histone de-acetylase (HDAC) inhibitors (Hockly et al., 2003; Ferrante et al., 2003)) or in some cases, increasing the number of inclusions (tissue transglutaminase depletion (Mastroberardino et al., 2002)).

iv) The shortstop mouse expresses a fragment of huntingtin (exon 1 and 2) and develops extensive NIIs throughout the brain but does not develop any other pathological features of the disease (Slow et al., 2005). This disassociation is inconsistent with inclusions being the causative agent in HD.

v) Alternatively, inclusions may serve a protective function by sequestering toxic molecules. In one experiment, neurons transfected with polyglutamine-expanded huntingtin were monitored for inclusions and cell death over time and surprisingly, the survival rates of neurons that formed inclusions was better than those that did not (Arrasate et al., 2004). This suggests that inclusions may improve neuron survival, possibly by sequestering mTOR and promoting autophagy of toxic huntingtin (Ravikumar et al., 2004).

1.2.5 **Neuroglia**

In addition to neuron loss, the striatum of HD brains displays several markers of inflammation and neurotoxicity in the neuroglia. Activated microglia are known to release neurotoxic cytokines and are observed in the striatum of early grade HD brains (Messmer and Reynolds, 1998; Sapp et al., 2001). The microglial activation is enriched in the striatum and correlates with the severity of the disease (Pavese et al., 2006). Reactive astrocytes are also observed in the striosomal compartments of symptomatic (Vonsattel et al., 1985) and pre-symptomatic (Hedreen and Folstein, 1995) brains. It is not clear whether glial activation is primary or secondary to other causes of neural dysfunction and what role it plays in degeneration.

1.3 GENETICS

1.3.1 **The HD Gene**

The human gene that is mutated in HD was identified in 1993 using linkage analysis (Huntington's Disease Collaborative Research Group, 1993). The HD locus is located on chromosome 4p16.3 and contains 67 exons across 180kb of genomic sequence (Ambrose et al., 1994). Due to differential 3' polyadenylation, the human HD gene encodes two mRNA products of 10.3kb and 13.6kb (Lin et al., 1993). The gene product is expressed throughout the body

(Sharp et al., 1995) although the larger transcript is predominantly expressed in the brain while the smaller transcript is highly expressed in other tissues (Lin et al., 1993).

1.3.2 The HD Mutation

HD is monogenic and dominantly inherited (Harper, 1993; Harper, 2005). The mutation is an expansion in the number of repeated CAG codons in exon 1 of the HD gene. The normal alleles in the HD gene generally contain between 10 and 26 CAG codon repeats (Table 1.1) (mean is ~18 CAGs in western populations (Andres et al., 2003)). Individuals carrying anything less than 36 repeats will not be affected with HD (Langbehn et al., 2004). A CAG tract expanded beyond 35 CAG repeats results in HD. Alleles with 36-39 repeats have a reduced risk of developing the disease (Rubinsztein et al., 1996; Brinkman et al., 1997; McNeil et al., 1997), while >40 CAG repeats are fully penetrant (Kremer et al., 1994). The majority of HD cases have between 40-55 CAG repeats (Figure 1.7).

Table 1.1: CAG sizes for alleles in the HD gene.

	CAG SIZE
Normal Allele	7 - 26
Intermediate Allele	27 - 35
HD Allele	
Reduced Penetrance	36 - 39
Full Penetrance	40 - 60
Juvenile Onset	60 +

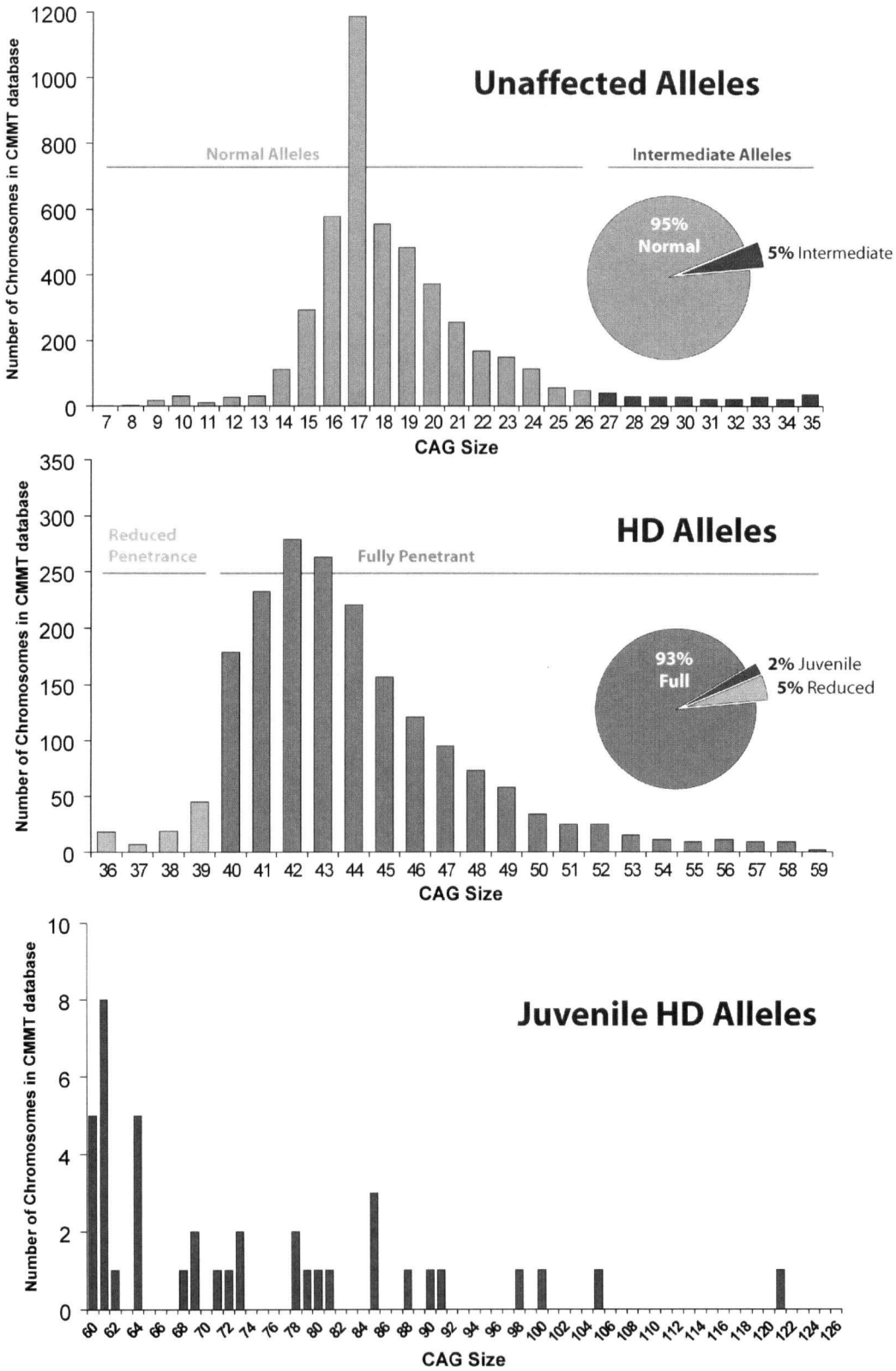

Figure 1.7: Distribution of CAG allele sizes in HD database at the Centre for Molecular Medicine and Therapeutics (CMMT)

The frequency of each CAG size for normal (n=4490 chromosomes), intermediate (n=256), reduced penetrance (n=89), fully penetrant (n=1829) and juvenile HD alleles (n=41). Percentage of each allele type is given in the inset pie-charts.

1.3.3 HD Gene Product

The huntingtin protein is a large soluble protein of 3,144aa (~350-kDa) that contains a stretch of CAG-encoded polyglutamines in the N-terminal domain. The expansion of the CAG tract therefore results in an increased number of polyglutamines in huntingtin. The disease does not result from altered expression of huntingtin (Sharp et al., 1995; Stine et al., 1995), nor is expression of the CAG-expanded RNA transcript sufficient to cause disease (Goldberg et al., 1996a). The expression patterns of wildtype and polyglutamine-expanded huntingtin are similar and ubiquitous in the adult (Li et al., 1993; Ide et al., 1995; Persichetti et al., 1995; Jou and Myers, 1995) and developing brain (Bhide et al., 1996) and thus does not explain the selective regional pathology. Evidence therefore suggests that the underlying mechanism of disease pathogenesis is altered interaction or posttranslational modification resulting from polyglutamine expansion.

1.3.4 CAG Size and Age of Onset

A strong inverse correlation exists between the size of the CAG tract and the age of onset (based on motor symptoms) of HD (Andrew et al., 1993; Brinkman et al., 1997; Langbehn et al., 2004) (Rubinsztein et al., 1993; Duyao et al., 1993; Craufurd and Dodge, 1993; Stine et al., 1993) (Trottier et al., 1994) and is seen in HD patients from all ethnic groups examined thus far (Simpson et al., 1993; Novelletto et al., 1994; Ashizawa et al., 1994; Yapijakis et al., 1995; Masuda et al., 1995; Soong and Wang, 1995; Atac et al., 1999; Maat-Kievit et al., 2002; Wang et al., 2004). Overall, the CAG size accounts for up to 60-75% of the total variation in the age of onset but the relationship is complex and changes with CAG size (Brinkman, 2001; Andresen et al., 2006). Lower CAG tract sizes have more variability in the age of onset and are more influenced by other modifying factors.

The majority of adult onset HD cases have between 40-55 CAG repeats (Kremer et al., 1994) with a typical age of onset of 30-50 years. Individuals with $\geq$ 60 repeats inevitably develop juvenile-onset HD (Telenius et al., 1993; Trottier et al., 1994). The rate of progression increases with higher CAG sizes (Marder et al., 2002; Mahant et al., 2003; Rosenblatt et al., 2006). Large CAG sizes, resulting in a younger age of onset, are associated with increased dystonia and less chorea, while the converse is true in older onset cases (Mahant et al., 2003). Early cognitive deficits also tend to be related to CAG size (Foroud et al., 1995; Jason et al., 1997). Interestingly, no correlation has been found between the CAG size and the psychiatric symptoms

of HD (Zappacosta et al., 1996; Weigell-Weber et al., 1996) although other genetic factors appear to contribute to the psychiatric features of HD as these tend to run in families (Lovestone et al., 1996; Tsuang et al., 2000).

Homozygotes for HD polyglutamine-expanded alleles are rare and do not have a significantly earlier age of onset than heterozygotes of the same CAG size (Wexler et al., 1987; Myers et al., 1989; Kremer et al., 1994; Durr et al., 1999; Squitieri et al., 2003). However, some investigators have observed altered phenotype and accelerated clinical progression in homozygotes (Squitieri et al., 2003).

1.3.5 Intermediate Alleles

Individuals carrying intermediate alleles (IA, 27-35 CAG repeats) in the HD gene are not at risk for developing HD. However, due to intergenerational instability in the CAG tract (Leeflang et al., 1995; Giovannone et al., 1997), the offspring of IA carriers are at risk for inheriting >35 repeats and developing HD later in life (Semaka et al., 2006). Intergenerational expansion of CAG from IA to HD alleles are termed 'new mutations' for HD. IA are not rare, as they are found in 1-5% of individuals in some populations (Goldberg et al., 1995; Maat-Kievit et al., 2001). The risk of HD for offspring of IA carriers is not well defined (Chong et al., 1997), but the occurrence of new mutations for HD is more common than once believed, and mutational flow analysis has suggested that up to 10% of HD cases are the result of expansions from IA carriers (Falush et al., 2000).

1.3.6 Prevalence

HD affects both sexes with equal frequency and has a prevalence in Europe and North America of 5-10 cases per 100,000 individuals (Squitieri et al., 1994). The prevalence is much lower in Africa, China, Japan and in some pockets of Europe such as Finland (Harper, 1992).

1.3.7 Relationship to Other Polyglutamine Diseases

There are at least 9 neurodegenerative disorders resulting from simple DNA repeat expansions (Table 1.2) (Gatchel and Zoghbi, 2005). Nine of these genetic disorders, including HD, result from elongation of CAG repeats leading to expression of different polyglutamine-expanded proteins (Riley and Orr, 2006).

The family of polyglutamine-expansion disorders share many common features with HD. All are dominantly inherited (with the exception of SBMA which is X-linked) and show somatic and germline repeat size instability leading to anticipation, particularly with paternal transmission. All have progressive neuronal dysfunction and neurodegeneration usually beginning in mid-life and prominently featuring motor dysfunction (Zoghbi and Orr, 2000). Each disease shows a strong inverse relationship between CAG length and age of onset of the disease (Gusella and MacDonald, 2000) and results in death 10-20 years after onset (Riley and Orr, 2006). The similarities in disease characteristics and genetic etiology suggest that these disorders have similar mechanisms of pathogenesis leading to neurodegeneration (Gusella and MacDonald, 2000).

There are also crucial differences between the polyglutamine-expansion disorders. Each of the CAG expansion disorders results from mutations in different genes encoding proteins that are widely expressed, yet lead to degeneration of a unique subset of neurons (Gatchel and Zoghbi, 2005). Aside from the CAG tract, these mutant proteins show no sequence or structural similarity to each other and each disorder results in a unique pattern of neuropathology and clinical symptoms (Gusella and MacDonald, 2000; La Spada and Taylor, 2003). The mechanism of degeneration is proposed to be a toxic gain of function of each polyglutamine-expanded protein, as simple loss of function may contribute to disease characteristics, but is not sufficient to cause the degenerative characteristics of each disease (Morfini et al., 2005).

Table 1.2: Diseases caused by polyglutamine (CAG) repeat expansions
Adapted from (Riley and Orr, 2006)

Disease	Gene Locus	Normal Allele	Disease Allele	Protein	Phenotype
DRPLA	12q	6–36	49–84	Atrophin-1	Epilepsy, Ataxia, Dementia
HD	4p16.3	6-27	36–121	Huntingtin	Motor, Psychiatric, Cognitive
SBMA	Xq11-12	6-39	40-63	Androgen Receptor	Proximal muscle atrophy
SCA1	6p22-23	8-44	39-83	Ataxin-1	Ataxia
SCA2	12q23-24	13-33	32-77	Ataxin-2	Ataxia
SCA3/MJD	14q24-31	12-40	54-89	Ataxin-3	Ataxia
SCA6	19p3	4-18	19-33	CACNA1A	Ataxia
SCA7	3p12-21	4-35	37-306	Ataxin-7	Ataxia, Retinal degeneration
SCA17	2q13	29-42	47-55	TATA-BP	Ataxia

1.3.8 **HD Phenocopies**

There are several diseases that have clinical and pathological phenotypes that are similar to HD but do not result from CAG-expansion of the HD gene. One study conducted after the discovery of the mutation in the HD gene found 12 phenocopy cases in a cohort of 1,022 patients (1.2%) (Andrew et al., 1994). Another found 3 cases out of 310 (1%) (Persichetti et al., 1994). Understanding the mechanism underlying phenocopy disorders may highlight specific pathways that function in HD pathogenesis. It is not yet clear whether these phenocopies result from mutations in proteins that perform similar functions to huntingtin or interfere with normal huntingtin function. In addition, the etiology of many HD-like conditions has not yet been determined (Rosenblatt et al., 1998; Vuillaume et al., 2000; Bauer et al., 2002; Keckarevic et al., 2005; Costa et al., 2006).

1.3.8.1 HDL1 (PRNP)

The locus for Huntington disease-like 1 (HDL1, OMIM 603218) was originally isolated to chromosome 20p (Xiang et al., 1998) and later linked to 8 extra octapeptide repeats (8 repeats of 24 bp each for a total of 192bp insertion) in the prion protein (PrP) gene, *PRNP* (Laplanche et al., 1999; Moore et al., 2001). This prion disease has an early-onset and is slowly progressive with an autosomal dominant pattern of inheritance and a wide range of clinical features that overlaps with HD. Other mutations in this locus are also known to result in more classic Creutzfeldt-Jakob disease (Goldfarb et al., 1991; van Gool et al., 1995).

1.3.8.2 HDL2 (JPH3)

Huntington disease-like 2 (HDL2, OMIM 606438) is autosomal dominant and is clinically and neuropathologically indistinguishable from HD (Stevanin et al., 2003; Walker et al., 2003). HDL2 is the result of a CAG/CTG expansion in the junctophilin-3 (JPH3) gene found on 16q24.3 (Holmes et al., 2001; Margolis et al., 2001). The expanded repeats lie in an alternatively spliced region and it is unclear whether they are translated into protein. Junctophilin-3 is believed to function in junctional membrane structures and calcium regulation (Walker et al., 2003). Mice lacking junctophilin-3 show impaired motor coordination (Nishi et al., 2002).

HDL2 accounts for a small proportion of HD-like cases (Stevanin et al., 2002) but interestingly, is more common among South African Blacks where the prevalence of HDL2 is as high as HD (Margolis et al., 2005).

1.3.8.3 HDL3

The term 'Huntington disease-like 3' (HDL3, OMIM 604802) is tentatively being used to describe an autosomal recessive, progressive HD-like disorder that was originally linked to 4p15 in a single consanguineous family (Kambouris et al., 2000; Bohlega et al., 2001). However, the mode of inheritance and quality of the data that links this disorder to chromosome 4 have been questioned (Lesperance and Burmeister, 2000).

1.4 HD MOUSE MODELS

HD has been modeled in a variety of biological systems including mice, drosophila, pigs, worms and yeast (Wang and Qin, 2006; Marsh and Thompson, 2006). Genetic mouse models are the most extensively studied and the most useful and applicable for the development of therapeutics. HD mouse models are numerous and contain a variety of different *HD* constructs.

1.4.1 Full-Length Huntingtin Mouse Models

The YAC mouse model of HD is a transgenic mouse containing the full-length genomic human HD gene with its endogenous promoter elements, providing appropriate developmental and tissue-specific expression of the huntingtin protein (Hodgson et al., 1999). The YAC128 mouse contains the human HD gene with 120 CAG repeats and is characterized by progressive behavioural and cognitive abnormalities, motor dysfunction and selective neuronal loss similar to HD in humans (Figure 1.8) (Slow et al., 2003; Van Raamsdonk et al., 2005a; Van Raamsdonk et al., 2005c). The HD phenotype in the YAC mice is modulated by the expression level of the polyglutamine-expanded huntingtin protein; increased expression leads to an earlier age of onset, more rapid progression, increased striatal volume loss and nuclear accumulation of huntingtin (Graham et al., 2006b).

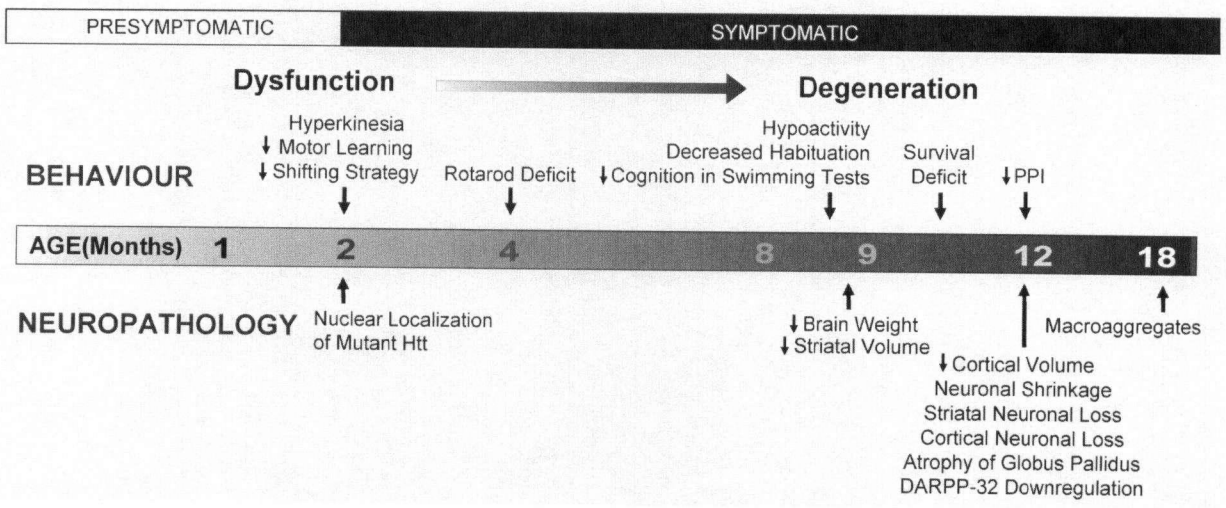

Figure 1.8: Timeline of pathological changes in the YAC128 mouse model of HD.
Adapted from Van Raamsdonk, PhD thesis, with permission.

The caspase-resistant YAC mouse strains contain variants of the YAC128 that express the full length, polyglutamine-expanded genomic human HD gene but have a single point mutation to prevent caspase cleavage at specific locations. They were generated to determine the importance of specific cleavage events in the disease. The transgenic huntingtin expressed in the caspase-3 resistant (C3R) mice cannot be cleaved at aa552 while caspase-6 resistant mice (C6R) express huntingtin that cannot be cleaved at aa586. Blocking cleavage at aa552 had little effect on the HD phenotype as the caspase-3 resistant (C3R) mice develop neuropathological and behavioural phenotypes indistinguishable from the YAC128. In the C6R mice, however, the development of pathological phenotypes is prevented (Graham et al., 2006a). This important and surprising finding suggests that cleavage of huntingtin at aa586 is a crucial process in the pathogenesis of HD.

In addition to the YAC128 mouse, there are other full length models including the BAC mouse (unpublished), a high-expressing CMV-driven cDNA mouse (Reddy et al., 1998) and four knock-in models of HD (Shelbourne et al., 1999; Wheeler et al., 2000; Lin et al., 2001; Menalled et al., 2002). The knock-in mice are accurate genetic models of the disease in the murine huntingtin gene (rather than human huntingtin in the YAC and BAC lines), but in general tend to manifest milder motor or neuropathological phenotypes without overt neurodegeneration (Menalled, 2005).

1.4.2 Truncated Huntingtin Mouse Models

There are a number of mouse models that over-express a fragment of huntingtin containing an expanded polyglutamine tract. In general, these truncated models recapitulate general features of polyglutamine toxicity, rather than the specific characteristics of adult-onset HD. The R/6-2 line is the most commonly studied model, and expresses exon 1 (67aa) of the human gene with ~144 CAG repeats (Sathasivam et al., 1999). The R/6-2 line develops early motor and general neurodegenerative features, with onset around 6 weeks and survival until 12-16 weeks (Mangiarini et al., 1996).

Other truncated models include the N171 mouse (Schilling et al., 1999; Schilling et al., 2001), the conditional exon 1 model (Yamamoto et al., 2000), and the NSE mouse (Laforet et al., 2001). Truncated mouse models, with the exception of the shortstop mouse, tend to demonstrate early behavioural and neuropathological phenotypes with widespread inclusions. The shortstop mouse is notable because it does not develop a pathological phenotype despite the presence of inclusions in the brain (Slow et al., 2005). The transgenic protein expressed in the shortstop mouse, which has the same 25kb endogenous promoter as the YAC128, is truncated after exon 2.

1.5 FUNCTION OF HUNTINGTIN

Several approaches have been used to understand the function of huntingtin. First, any function described for the protein must be consistent with its localization, both in the body and in the cell. Second, "function" can be described as the *molecular function* (i.e. discrete actions the protein can perform) which for huntingtin, includes (but is not limited to) scaffolding other proteins involved in transcription and trafficking. Furthermore, huntingtin function can be described in a broader sense to include *biological processes*, such as development, neuroprotection and excitotoxicity.

1.5.1 Tissue Expression

The huntingtin protein is widely expressed in the body with high expression in the brain (Li et al., 1993; Gutekunst et al., 1995; Landwehrmeyer et al., 1995; Sharp et al., 1995) and testis (Van Raamsdonk et al., 2007). Huntingtin is present in both neurons and glia (Landwehrmeyer et al., 1995), and the regional distribution of huntingtin in the brain, and even within cell types of the striatum, does not correlate with the selective neuropathology (Vonsattel and DiFiglia, 1998).

1.5.2 Subcellular Localization

Huntingtin is broadly distributed at the subcellular level as it is found throughout the cytoplasm (DiFiglia et al., 1995; Trottier et al., 1995a; Jones, 1999), the nucleus (Hoogeveen et al., 1993; De Rooij et al., 1996; Saudou et al., 1998; Dorsman et al., 1999; Wheeler et al., 2000; Tao and Tartakoff, 2001; Kegel et al., 2002; Martin-Aparicio et al., 2002; Lloret et al., 2006; Jeong et al., 2006) and is particularly abundant in the perinuclear region (Sapp et al., 1997; Hoffner et al., 2002).

Huntingtin is associated with membranes (Kim et al., 2001; Kegel et al., 2005; Suopanki et al., 2006) including caveolae (Trushina et al., 2006), clathrin coated vesicles (DiFiglia et al., 1995), and other endosomal compartments (Velier et al., 1998). Huntingtin also associates with microtubules (Kegel et al., 2002; Hoffner et al., 2002) and other organelles such as the endoplasmic reticulum (ER) and golgi (Rockabrand et al., 2007). Unfortunately the widespread regional expression and subcellular localization of huntingtin does not help define a single precise function in any specific compartment of the cell. Rather, it suggests huntingtin may have a number of functions.

1.5.3 Huntingtin is a Scaffold That Binds Other Proteins

Huntingtin interacts with numerous proteins (Figure 1.9). At least 42 protein interactors have been identified using a combination of yeast2hybrid, GST-pulldown, co-immunoprecipitation and affinity chromatography (Goehler et al., 2004). The details of specific interactions have been reviewed elsewhere in this thesis and the literature (Li and Li, 2004; Goehler et al., 2004). Many interacting proteins interact with the N-terminal domain of huntingtin and many of these interactions are altered by polyglutamine-expansion.

However, the functions of many of these interacting proteins have not been well characterized. One approach to understanding their functions is to cluster protein interactors into larger interacting networks. These interacting networks consist of proteins that are involved in the regulation of transcription, protein synthesis and turnover, intracellular transport, and cell signalling. Collectively, the interaction data suggests that huntingtin has a role in many biological processes and may do so by functioning as a scaffolding protein. These biological processes may not necessarily be related to each other, as the only feature in common is the scaffolding abilities of huntingtin. A survey of huntingtin interacting proteins suggests that

huntingtin facilitates the transfer of information between cell compartments through its interaction with other proteins.

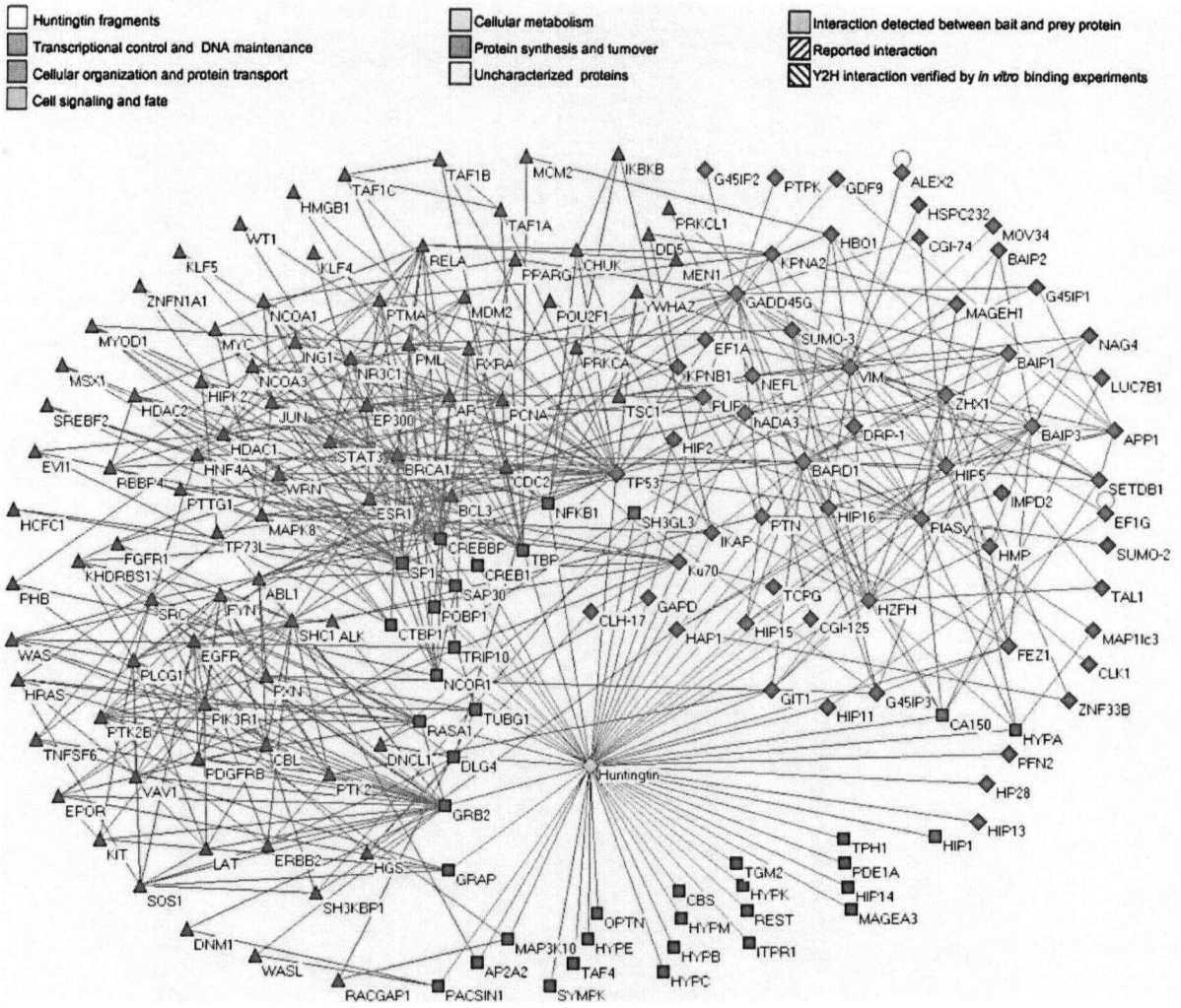

Figure 1.9: Protein interaction network for huntingtin.
Huntingtin interacts with a large number of proteins that can be clustered into functional groups. This network diagram was assembled using Y2H studies and data from HRPD, MINT and BIND databases. Reprinted from (Goehler et al., 2004) with permission.

1.5.4 Transcriptional Regulation

Several lines of evidence suggest that huntingtin plays both a direct and indirect role in regulating gene transcription as part of its normal function. Altered gene transcription is also observed in the presence of polyglutamine expansion.

1.5.4.1 Gene expression changes in HD

In human HD brain, changes in the expression of many genes have been observed in early stages of the disease (grade 0-2), with the greatest number of changes occurring in the striatum (Hodges et al., 2006). Many studies have also demonstrated that the expression of polyglutamine-expanded huntingtin results in selective changes in gene expression in cultured cells (Sipione et al., 2002) and in mouse models of HD (Luthi-Carter et al., 2000; Spektor et al., 2002; Chan et al., 2002a; Luthi-Carter et al., 2002a; Luthi-Carter et al., 2002b; Zucker et al., 2005; Desplats et al., 2006). Interestingly, expression of polyglutamine-expanded huntingtin in cell models of HD has been shown to alter the mitogen-activated protein kinase (MAPK) signalling pathway and therefore suggest this may be a viable target for therapeutic intervention (Apostol et al., 2006). However, gene expression changes in the presence of polyglutamine-expanded huntingtin may simply be a reaction to other pathological processes and is not direct evidence that wildtype huntingtin regulates normal gene expression.

1.5.4.2 Polyamine Regulation of Transcription

Huntingtin contains structural features, such as a polyglutamine and several polyproline tracts that suggest a more direct role in the regulation of transcription. Numerous well characterized transcription factors and transcriptional co-activators such as CREB-binding protein (CBP) and TATA binding protein (TBP) contain polyglutamine domains that appear to play a role in their transcriptional activity (Cha, 2000). Polyglutamine and polyproline domains alone are sufficient to activate transcription *in vitro* (Tanaka and Herr, 1994) and their activity increases with increasing tract length (Gerber et al., 1994). Thus, the polyglutamine-expansion of huntingtin might confer a direct 'gain-of-function' transcriptional activity that is important in the pathogenesis of HD.

1.5.4.3 Nuclear Shuttling

Nuclear shuttling of proteins involved in transcription is a commonly observed mechanism of regulating their activity. Huntingtin has the ability to shuttle in and out of the nucleus (Tao and Tartakoff, 2001). The mechanism of nuclear transport of huntingtin is somewhat unique as it is transported in a Ran GTPase-independent manner, unlike many nuclear shuttle proteins (Cornett et al., 2005). Evidence suggests that regulation of this shuttling involves the nuclear pore protein Tpr (Cornett et al., 2005) and GAPDH/Siah1 (Bae et al., 2006). Importantly, the nuclear export

of huntingtin is impaired when the polyglutamine tract is expanded (Cornett et al., 2005), suggesting that mutant huntingtin is selectively retained in the nucleus.

A cytoplasmic retention signal (Rockabrand et al., 2007) and nuclear export signal (Xia et al., 2003) are present in huntingtin, which regulate the cytoplasmic localization of huntingtin. The presence of a nuclear localization signal (NLS) in huntingtin (Bessert et al., 1995) is controversial, and it is not clear what specifically regulates its nuclear entry. Huntingtin could also be facilitating the nuclear import/export of other transcription factors and co-activators/repressors.

1.5.4.4 Interaction with Gene Specific Transcription Factors and Co-Activators/Repressors

Numerous laboratories have demonstrated that huntingtin is involved in transcription by interacting with cofactors that bind to the promoters of specific genes and specific transcription factors such as CBP, SP1, p53, TBP and others (For review, see (Cha, 2000; Sugars and Rubinsztein, 2003; Okazawa, 2003; Truant et al., 2007)). Polyglutamine-expanded huntingtin can impair gene transcription either by altered interaction with these specific transcription factors or by sequestering them into inclusions. Evidence from HD mouse models, however, argues that the gross levels of available CBP, TBP and SP1 are not significantly reduced by sequestration into inclusions (Yu et al., 2002; Tallaksen-Greene et al., 2005).

One example of altered transcriptional regulation due to the HD mutation results from the altered interaction between huntingtin and repressor element-1 transcription/neuron restrictive silencer factors (REST/NRSFs) (Figure 1.10). Huntingtin binds REST and retains it in the cytoplasm, thereby preventing its repression of neuron restrictive silencer element (NRSE)-containing genes. This NRSE DNA consensus sequence is found in neuron specific genes such as brain derived neurotrophic factor (BDNF). Polyglutamine-expansion of huntingtin decreases its ability to bind REST, and causes repression of neuron specific genes (Zuccato et al., 2001; Zuccato et al., 2003).

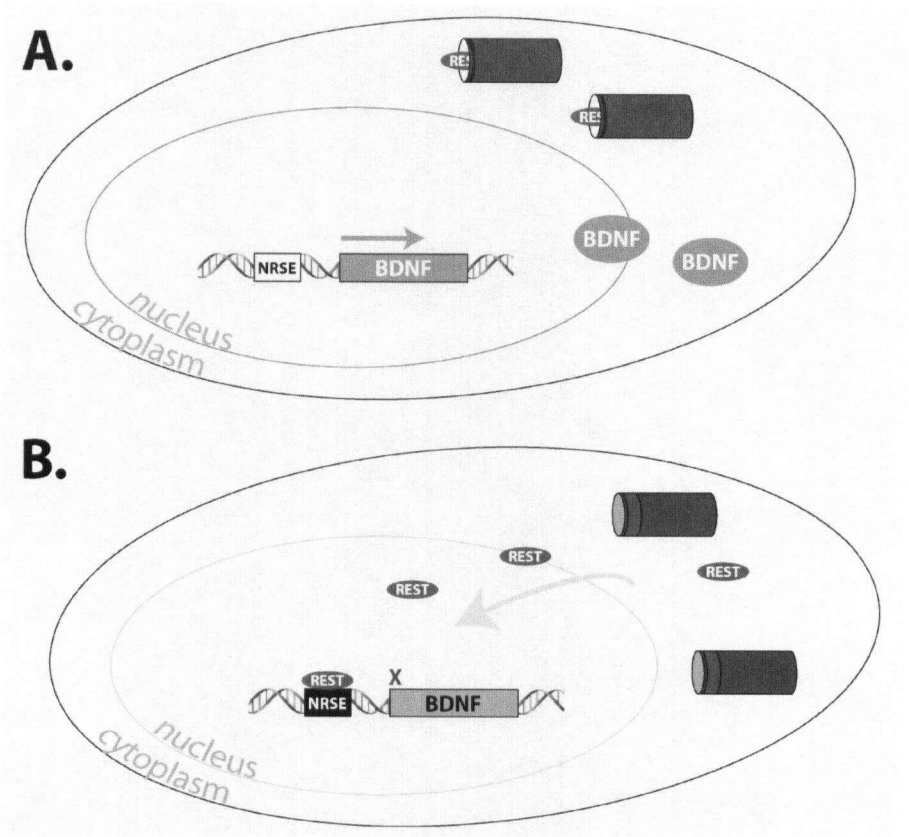

Figure 1.10: Huntingtin binds REST and regulates the expression of NRSE-containing genes.
Simplified schematic showing the role of huntingtin in the expression of neuron-specific genes. (**A**) Wildtype huntingtin (red cylinder) binds REST/NRSF (blue) and retains it in the cytoplasm. This allows the expression of NRSE-containing genes, such as BDNF, which are important for the survival of striatal neurons. (B) Polyglutamine-expanded huntingtin binds REST poorly, allowing it to enter the nucleus, bind the NRSE and repress transcription. Repression occurs through the recruitment of histone deactylases (HDACs) involved in 'closing' chromatin and preventing transcription.

1.5.4.5 Histone Regulation

Histone acetylation plays a crucial role in the regulation of gene transcription. In general, acetylation of lysine residues of histones leads to transcriptional activity while methylation of lysine and arginine residues represses transcription. Histone deactylases (HDACs) are a group of enzymes that catalyze the de-acetylation of histones and therefore lead to transcriptional repression. Polyglutamine-expansion of huntingtin alters histone acetyl-transferase activity leading to the hypo-acetylation of histones in models of HD (Igarashi et al., 2003; Sadri-Vakili and Cha, 2006). Huntingtin specifically interacts with HYPB, a histone H3 lysine 36-specific methyltransferase believed to be important in histone regulation (Sun et al., 2005). Furthermore, HDAC inhibitors have shown to have some protective effects against polyglutamine-expanded huntingtin (Steffan et al., 2001; Hockly et al., 2003; Ferrante et al., 2003; Gardian et al., 2005; Ryu et al., 2006).

1.5.5 **Trafficking**

Multiple lines of evidence suggest that huntingtin functions directly or indirectly in the intracellular transport of proteins (Charrin et al., 2005). Huntingtin is itself transported retrogradely by fast axonal transport in the rat sciatic nerve (Block-Galarza et al., 1997) but many studies have suggested that huntingtin functions to regulate the transport of other proteins.

Huntingtin interacts with several proteins involved in intracellular transport or endocytosis, including huntingtin-associated protein 1 (HAP1) (Li et al., 1995; Block-Galarza et al., 1997; Li and Li, 2005), huntingtin-interacting protein 1 (HIP1) (Kalchman et al., 1997; Wanker et al., 1997; Metzler et al., 2001; McGuire et al., 2006), HIP1- related protein (HIP1R) (Seki et al., 1998; Chen and Brodsky, 2005), protein kinase C and casein kinase substrate in neurons-1 (PACSIN1) (Modregger et al., 2000; Modregger et al., 2002), and postsynaptic density 95 (PSD95) (Sun et al., 2001). In addition, huntingtin interacts with, (Singaraja et al., 2002) and is modified by (Yanai et al., 2006), huntingtin-interacting protein 14 (HIP14), a palmitoyl transferase (PAT) involved in sorting proteins from the Golgi (Huang et al., 2004). Indeed, huntingtin has been found to colocalize with clathrin in membranes of the trans-Golgi network and in cytoplasmic vesicles (Velier et al., 1998; Strehlow et al., 2006).

As would be expected for proteins involved in axonal or organelle transport, huntingtin interacts with microtubules (Gutekunst et al., 1995; Tukamoto et al., 1997; Hoffner et al., 2002). In lysates from both lymphoblasts and brain, huntingtin co-immunoprecipitates with β-tubulin, although this association is not influenced by the size of the polyglutamine tract of huntingtin (Tukamoto et al., 1997; Hoffner et al., 2002). In a fraction of cells, huntingtin was found to co-localize with γ-tubulin at the centrosome/microtubule organizing center (Hoffner et al., 2002) Expression of polyglutamine-expanded huntingtin disrupts centrosome organization (Sathasivam et al., 2001).

Many studies have directly examined the effect of manipulating huntingtin expression levels on transport function in different model systems. In axoplasm preparations from the *loligo* giant squid axons, the presence of expanded polyglutamine peptides inhibits fast axonal transport (Szebenyi et al., 2003). In *Drosophila*, the reduction of the endogenous huntingtin ortholog results in axonal transport defects in larval nerves and degeneration of photoreceptors in adult fly eyes (Gunawardena et al., 2003). In primary cultures of cortical neurons, manipulating the

expression level of huntingtin altered the microtubule based transport of BDNF-containing vesicles (Gauthier et al., 2004), which can be rescued by HDAC inhibitors (Dompierre et al., 2007). Reduced expression of huntingtin, or the expression of polyglutamine expanded huntingtin, resulted in a reduction in the post-golgi trafficking of specific BDNF alleles (del et al., 2006). Finally, expression of full-length polyglutamine expanded huntingtin in transgenic mice has been shown to impair vesicular and mitochondrial trafficking in neurons (Trushina et al., 2004).

1.5.6 **Development**

Huntingtin function is required for normal development as homozygous inactivation of the mouse HD homolog, *Hdh,* results in impaired gastrulation and embryonic lethality between day 7.5 and 10.5 (Zeitlin et al., 1995; Nasir et al., 1995; Duyao et al., 1995; Dragatsis et al., 1998). Mice hemizygous for *Hdh* have morphological alterations in the basal ganglia (O'Kusky et al., 1999) and partial inactivation (greater than 50% reduction) of *Hdh* similarly yields a malformed central nervous system and perinatal lethality (White et al., 1997). Evidence suggests an important early developmental role for huntingtin in extra-embryonic tissues (tissues that support the embryo development but do not become part of the embryo) (Dragatsis et al., 2000) for the regulation of pattern formation (Woda et al., 2005). Interestingly, the primary defect appears to be loss of nutritive transport from extraembryonic tissue, rather than in the embryo itself (Dragatsis et al., 1998).

Huntingtin seems to play a specific role in neurogenesis (White et al., 1997) but this function may not be performed in the neurons themselves (Reiner et al., 2001) as it is not involved in the neuronal differentiation of embryonic stem (ES) cells (Metzler et al., 1999). However, huntingtin is required for normal hematopoiesis (Metzler et al., 2000).

Polyglutamine-expansion does not critically disrupt the developmental functions of huntingtin, as homozygous HD mutations in humans are not lethal (Durr et al., 1999; Squitieri et al., 2003), and polyglutamine-expanded huntingtin can rescue *Hdh*-null mice from embryonic lethality (Hodgson et al., 1996; Leavitt et al., 2001).

1.5.7 Neuroprotection

Aside from its developmental role, huntingtin also plays an essential role in the adult nervous system, as the conditional inactivation of the *Hdh* gene in adult mouse forebrains leads to neurodegeneration and early mortality (Dragatsis et al., 2000).

The expression of wildtype huntingtin protects against numerous toxic stressors in cultured neurons (Rigamonti et al., 2000; Rigamonti et al., 2001; Ho et al., 2001) and neurons in the mousebrain (Zhang et al., 2003; Leavitt et al., 2006), including partial protection from the toxic effects of polyglutamine-expanded huntingtin (Leavitt et al., 2001; Van Raamsdonk et al., 2005b; Van Raamsdonk et al., 2006). Furthermore, wildtype huntingtin expression is decreased during disease progression in the R6/2 model of HD and following brain injury (Zhang et al., 2003). Various mechanisms could underlie the protection afforded by wildtype huntingtin, including its ability to inhibit caspase 9 (Rigamonti et al., 2000; Rigamonti et al., 2001), caspase 3 (Zhang et al., 2006) or regulate BDNF expression (Zuccato et al., 2003).

1.5.8 Intracellular Calcium and Excitotoxicity in HD

There is much evidence to support the excitotoxicity hypothesis of HD which argues that susceptible neurons of the striatum degenerate as the result of improper cytosolic Ca^{2+} handling (For review, see (Sieradzan and Mann, 2001; Bezprozvanny and Hayden, 2004)). Neurons of the striatum receive abundant excitatory inputs from the cortex which activate ionotropic glutamate receptors and allow Ca^{2+} to flow into the cytoplasm. Mismanaged cytosolic Ca^{2+} is cytotoxic and overstimulation of these glutamate receptors can lead to cell death (Zeron et al., 2001; Hanson et al., 2004).

Specifically, much attention has been directed towards the N-methyl-D-aspartate (NMDA) receptors, as toxic doses of NMDA agonists result in HD-like pathology of the brain (McGeer and McGeer, 1976) and increased NMDA current has been shown in several HD mouse models (Levine et al., 1999; Zeron et al., 2002; Li et al., 2004; Shehadeh et al., 2006; Fan and Raymond, 2007). Huntingtin, through interaction with other proteins such as huntingtin associated protein 1 (HAP1) (Gervais et al., 2002; Tang et al., 2003), has been implicated in altered mitochondrial Ca^{2+} regulation (Gu et al., 1996; Tabrizi et al., 1999), function of NMDA receptors (Zeron et al., 2002; Luthi-Carter et al., 2003; Li et al., 2003b; Zeron et al., 2004) and the type 1 inositol 1,4,5-trisphosphate receptor (InsP3R1) (Tang et al., 2004; Tang et al., 2005) of the endoplasmic

reticulum (summarized in (Bezprozvanny and Hayden, 2004)). Recently, altered NMDA trafficking has been implicated in NMDA receptor dysfunction in the YAC mouse model of HD (Fan et al., 2007).

1.6 STRUCTURAL FEATURES OF HUNTINGTIN

The level and regional pattern of huntingtin expression does not easily explain the neuron-selective degeneration that occurs in HD. Therefore, it must be a primary, secondary or tertiary structural property of the polyglutamine-expanded protein that dictates the selective degeneration.

1.6.1 Homology

The importance of huntingtin function is suggested by the high degree of evolutionary conservation (80%) of the *HD* gene in vertebrates (Cattaneo et al., 2005). The murine ortholog, *Hdh*, is 86% identical at the nucleotide level and 90% identical at the protein level to human huntingtin (Lin et al., 1994; Barnes et al., 1994). As with the human gene, the mouse *Hdh* also has two transcripts (Lin et al., 1994; Casanova et al., 1996).

The polyglutamine (Q) tract size, however, is not well conserved and only polymorphic in humans. The polyglutamine tract first appears in fish (4Q) (Baxendale et al., 1995; Karlovich et al., 1998) and is much smaller in mice (7Q) (Lin et al., 1994) than in the miniature pig (17-18Q) (Matsuyama et al., 2000) and the average tract size in humans (18Q) (Myers, 2004).

HD orthologs appear to be less well conserved in invertebrates, as the *Drosophila melanogaster* ortholog has many differences to the human gene: the *Drosophila* ortholog encodes a larger protein (3583aa), contains fewer exons (29) and lacks a polyglutamine tract. In all, there are five regions in the Drosophila homolog with 20-50% conservation to the human HD gene (Li et al., 1999). HD orthologs appear to be present in *Dictyostelium discoideum*, but not in the older fungi, *Saccharomyces cerevisiae* (Cattaneo et al., 2005). Evolutionarily older homologs appear to have non-neuronal functions in lower chordates (Kauffman et al., 2003), and the gene appears to have been lost in some later animals, such as *Caenorhabditis elegans* (Holbert et al., 2003).

Within the human genome, there are no paralogs with a high degree of similarity to huntingtin. The HD gene does contain several tandemly repeated elements in non-coding regions, but these repeats are extremely common throughout the genome and do not suggest functional similarity to other genes (Warby et al., 2006). Some domains in huntingtin that are found in other proteins include the polyglutamine (Figure 1.11) and polyproline tracts and the HEAT repeats.

1.6.2 Polyglutamine Tract

The function of a polyglutamine tract is not entirely understood, although it is clear that polyglutamine-expansion results in new protein properties. Expansion of polyglutamine tracts beyond 35-40 residues seems to cause a phase change to occur where the protein adopts new conformations and properties. Smaller polyglutamine tracts (<30) appear to adopt a random coil formation, while expanded glutamines are believed to act as polar zippers that can assemble β-strands of proteins into sheets (Perutz, 1996; Poirier et al., 2005). The phase change of huntingtin is supported by the demonstrations that some antibodies, such as 1C2, only detect expanded polyglutamine tracts (Trottier et al., 1995b). The polyglutamine tract size clearly modifies huntingtin interaction with several proteins (Li and Li, 2004).

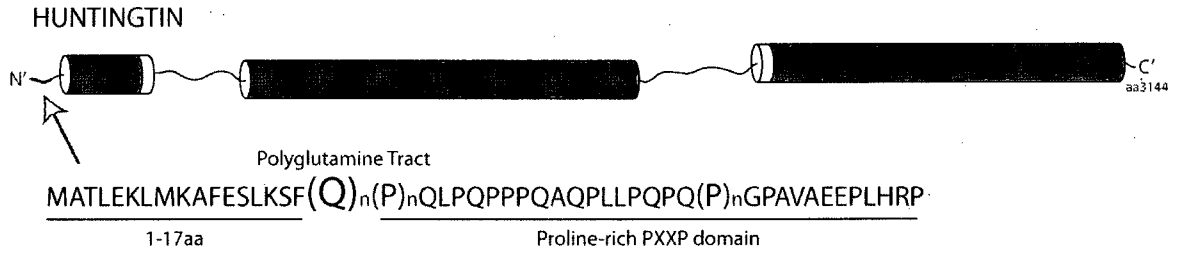

Figure 1.11: Polyglutamine and polyproline domains are located in the N-terminus of huntingtin

Interestingly, the polyglutamine tract of huntingtin is not essential for its function, as mice lacking the polyQ in Hdh, develop normally and without gross phenotypic differences from wildtype littermates (Clabough and Zeitlin, 2006).

1.6.3 Proline-Rich Region

Huntingtin contains a proline-rich region immediately adjacent to the polyglutamine tract. The polyP stretch is conserved in higher vertebrates although its function is not clear. It may function as a binding domain or modify the solubility of the protein (Steffan et al., 2004).

Although there are no data to suggest that the length of the proline tract may modify features of the disease in humans (Vuillaume et al., 1998), the polyproline tract profoundly alters the toxicity of polyglutamine-expanded huntingtin in yeast (Duennwald et al., 2006a; Duennwald et al., 2006b) (Bhattacharyya et al., 2006; Dehay and Bertolotti, 2006). The proline-rich region is important for posttranslational modification on the N-terminus of huntingtin (Steffan et al., 2004), likely through altered protein-protein interactions.

1.6.4 HEAT repeats

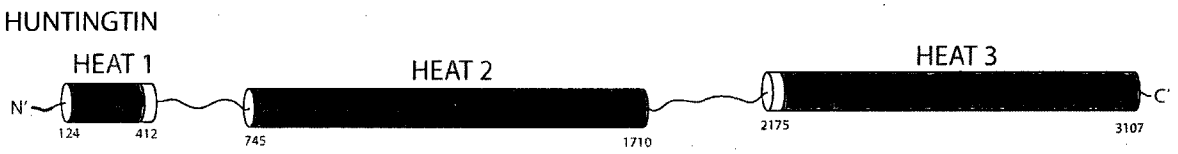

Figure 1.12: HEAT repeats domains in Huntingtin are composed of multiple HEAT repeats stacked in a solenoid structure.
Huntingtin is predominantly composed of HEAT repeats, which are clustered into three domains. From the amino acid prediction, it is not clear if the last repeat of HEAT 1 and the first repeat of HEAT 3 are actually HEAT repeats (indicated in pink). Structural predictions suggest that the domains, composed of multiple coiled and stacked HEAT repeats (red cylinders) would have a tertiary structure that is further coiled but is shown here in a linear depiction for simplicity.

The HEAT repeats appear to be the single most important defining feature of huntingtin protein structure (Figure 1.12). Huntingtin contains multiple HEAT repeat domains (Andrade and Bork, 1995; Takano and Gusella, 2002) which are involved in protein-protein interactions (Neuwald and Hirano, 2000). The HEAT domain (named for its presence in huntingtin, elongation factor 3, regulatory A subunit of protein phosphatase 2A and target of rapamycin 1 (TOR1)) is roughly 40 amino-acids and well conserved in huntingtin homologs. There are three main clusters of heat repeats, although the number of functionally active repeats is not clear. The current structural model of full length huntingtin is of a single flexible superhelical solenoid, entirely composed of folded HEAT repeats with a continuous hydrophobic core (Figure 1.13) (Li et al., 2006). As with other HEAT repeat proteins, this structural model is consistent with huntingtin functioning as a scaffold protein.

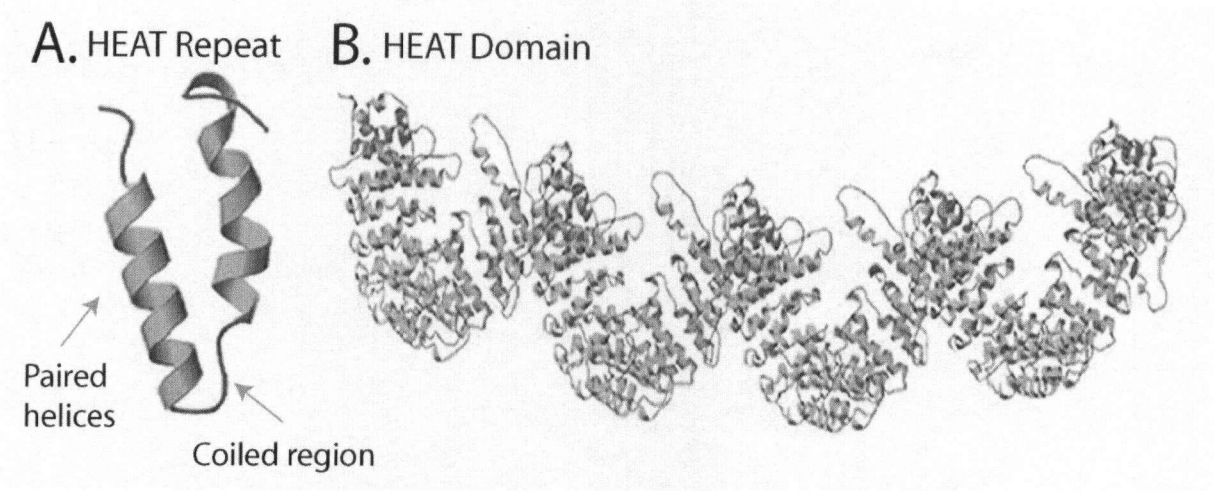

Figure 1.13: Secondary and tertiary structure of HEAT repeats
(A) A single HEAT repeat is a helix-turn-helix structure. The length of the helices (cyan) and intervening coil regions (red) can vary. (B) Model of the tertiary structure of a hypothetical HEAT repeat protein similar to huntingtin. The tight superhelix that is formed would have a hydrophobic core, but many coiled regions are potentially flexible and would be exposed on the outside of the protein. Adapted from (Li et al., 2006).

1.6.5 **HunMAD Domain**

The huntingtin membrane-associated domain (HunMAD) is composed of the first 17aa of the protein (aa1-17) and appears to be critically important for the subcellular localization of the huntingtin protein (Figure 1.14) (Truant et al., 2006a). These N-terminal 17 amino acids have also been found to interact with the nuclear pore protein 'translocated pore promoter' (TPR) and mediate the export of huntingtin from the nucleus (Cornett et al., 2005). The interaction with TPR is reduced with polyglutamine-expansion of huntingtin and suggests that impaired nuclear export plays a role in the disease (Cornett et al., 2005). Alternatively, the hunMAD domain has been proposed to act as a cytoplasmic retention signal, specifically regulating its association with mitochondria (Rockabrand et al., 2007), and may be regulated by SUMOylation sites within this domain (Steffan et al., 2004).

1.6.6 **NLS/NES**

Huntingtin was originally proposed to have a nuclear localization signal (NLS) near its N-terminus (Figure 1.14) (Bessert et al., 1995). The putative NLS peptide (PIRRKGKEK) is found at aa1182 in huntingtin and is highly homologous to an NLS consensus sequence. When this peptide was fused to a β-gal reporter, it mediated the nuclear translocation of the reporter

(Bessert et al., 1995). Subsequent studies, however, were not able to confirm the function of the NLS (Hackam et al., 1999b; Xia et al., 2003).

Although the presence of a nuclear localization signal (NLS) in huntingtin is controversial, evidence suggests that huntingtin contains a functional nuclear export signal (NES) in its C-terminus between aa2397-2406 (IIISLARLPL) (Xia et al., 2003). This NES is strictly conserved among huntingtin homologs. Notably, the NES lies C-terminal of the proteolytic domain and would therefore be cleaved away from the N-terminal polyglutamine tract following caspase proteolysis of huntingtin.

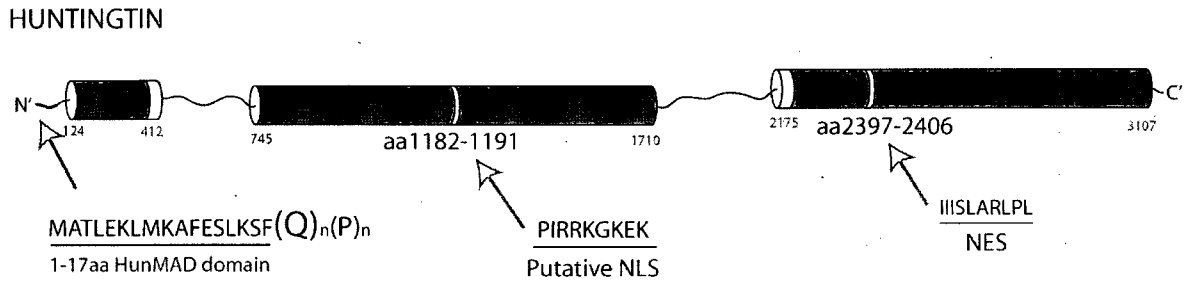

Figure 1.14: Huntingtin membrane associated domain (HunMAD), a nuclear export signal (NES) and a putative nuclear localization signal (NLS) are features of huntingtin

In addition to these above features that comprise its primary and secondary structure, the huntingtin protein can undergo several posttranslational modifications that modify its secondary and tertiary structure and potentially regulate its function. There are over 200 known types of posttranslational modifications, but only a few are reversible and known to be important for the dynamic regulation of proteins. Phosphorylation is the most well studied reversible posttranslational modification of proteins, and the potential phosphorylation of huntingtin is of great interest for understanding huntingtin function in health and disease.

1.7 PHOSPHORYLATION AND POLYGLUTAMINE DISEASE

1.7.1 Phosphorylation – What is it?

Phosphorylation is a posttranslational modification where a phosphate (PO_4) group is added to a protein or small molecule. The process of enzymatic phosphorylation is ubiquitous and well

studied. Phosphorylation is a reversible, covalent modification of a substrate protein that induces a conformational change within the molecule. The process is an important means of regulating proteins by altering their enzymatic activity, binding to other proteins, or subcellular location.

Phosphorylation often occurs on multiple distinct sites on a protein and the effect of phosphorylation on protein function can be dramatic. In some cases, a the activity of a protein can be switched on or off by the addition of a phosphate group (s) (Figure 1.15). The phosphorylation reaction is catalyzed by a class of enzymes called 'kinases'. In eukaryotes, there are two families of kinases, defined by whether they catalyze the addition of phosphate on serine/threonine or tyrosine residues. Dephosphorylation is performed by 'phosphatases'.

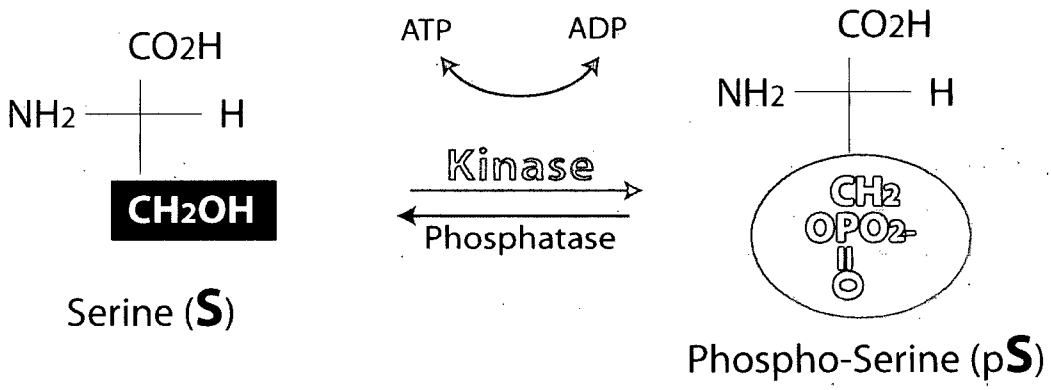

Figure 1.15: Phosphorylation of a substrate on a serine by transfer of the terminal phosphate group of ATP by a kinase.
Phosphorylation may also occur on threonines and tyrosines. Dephosphorylation is the removal of the phosphate from the substrate and is catalyzed by a phosphatase.

An important concept that arises out of the discovery of protein phosphorylation is the idea of signal transduction pathways and signalling cascades. Activation of a single kinase molecule can result in the phosphorylation of many substrates and amplification of a biological signal. Phosphorylation is a crucial biological process and plays a key role in many normal and pathological processes, including neurodegenerative disease.

1.7.2 Role of Phosphorylation in Neurodegenerative Disease

The aberrant regulation of proteins by altered phosphorylation is important in the pathogenesis of several disorders including Alzheimer disease (Gong et al., 2005; Avila, 2006) and other polyglutamine-expansion disorders. For example, the phosphorylation of atrophin-1 by JNK kinase is believed to be reduced by the polyglutamine-expansion that causes DRPLA (Okamura-Oho et al., 2003). As well, the androgen receptor (AR), which is expanded in Spinal and Bulbar Muscular Atrophy (SBMA; Kennedy's disease), is phosphorylated by both the MAP kinase and Akt signalling pathways. MAPK phosphorylation is increased by the polyglutamine-expansion in the AR and increases its toxicity in cultured cells (LaFevre-Bernt and Ellerby, 2003). Akt also phosphorylates AR and reduces androgen binding, ligand-dependent nuclear translocation and toxicity of polyglutamine-expanded AR in cell cultures (Palazzolo et al., 2007).

In vivo evidence has demonstrated that phosphorylation plays a central role in the pathology of spinocerebellar ataxia type 1 (SCA1) (Heintz, 2003; Paulson, 2003). SCA1 results from polyglutamine expansion in the ataxin-1 protein, causing upper motor neuron and cerebellar degeneration in humans (Orr and Zoghbi, 2001). Transgenic mice that model SCA1 express human ataxin-1 with an expanded polyglutamine tract (ataxin-1[82Q]) and develop cerebellar pathology and ataxia at 12 weeks of age (Burright et al., 1995).

Ataxin-1 contains a crucial phosphorylation site at S776 that is phosphorylated by the serine/threonine kinase Akt when in the nucleus of purkinje cells of the cerebellum (Chen et al., 2003b). Importantly, transgenic mice that were generated to contain polyglutamine-expanded but unphosphorylatable ataxin-1 (ataxin-1(82Q)S776A) did not develop the symptoms or pathology of the ataxin-1(82Q) mice (Emamian et al., 2003). Preventing phosphorylation on S776 prevented the disease in this mouse model of SCA-1.

The phosphorylation of ataxin-1 by Akt permits its interaction with several forms of the multifunctional regulatory protein 14-3-3. This interaction slows its degradation (Chen et al., 2003b), allowing ataxin-1 (and in the case of SCA-1, polyglutamine-expanded ataxin-1) to persist in the nucleus of cerebellar Purkinje cells and result in the pathology of SCA-1 (Figure 1.16).

The phosphorylation of ataxin-1 being crucial in the pathogenesis of SCA1 demonstrates two key points:

i) Protein context is important in polyglutamine-expansion disorders – the clinical features of SCA1 are the result of properties of the ataxin-1 protein and not simply due to the over-expression of expanded polyglutamines.

ii) Within these polyglutamine-containing proteins, domains outside the polyglutamine tract can regulate the toxicity of polyglutamine-expansion

Because the phosphorylation of ataxin-1 plays such a profound role in the pathology of SCA1, and emerging data is linking phosphorylation to pathological processes in other polyglutamine diseases (Mushegian et al., 2000; Morfini et al., 2006), it bolsters interest in exploring the Akt phosphorylation of huntingtin *in vitro* and *in vivo* and its relationship to the pathogenesis of HD.

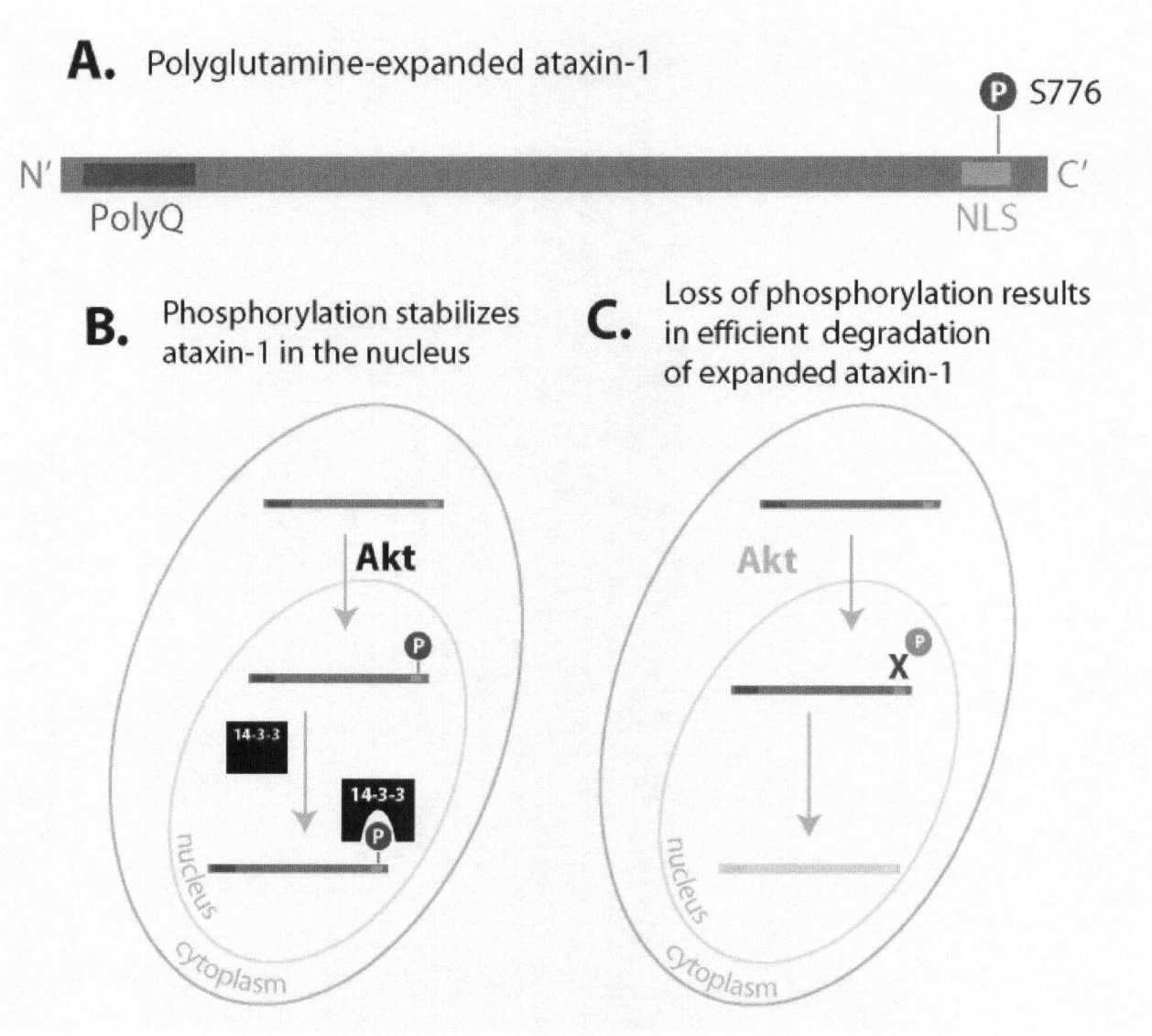

Figure 1.16: Role of ataxin-1 phosphorylation in the pathogenesis of SCA-1
(A)The phosphorylated S776 residue is located distal to the polyglutamine tract in ataxin-1 but has a crucial role in the pathogenesis of SCA1. (B) Phosphorylation by Akt at S776 results in binding to 14-3-3, which prevents the degradation of nuclear ataxin-1. Polyglutamine-expanded ataxin-1 is thus allowed to persist in the nucleus and results in degeneration. (C) Preventing the phosphorylation at S776 permits the efficient degradation of ataxin-1 and prevents pathogenesis in a mouse model of SCA1.

1.7.3 **Huntingtin Phosphorylation**

At the beginning of this graduate study in 2001, nothing was known about the phosphorylation of huntingtin other than it was potentially a phosphoprotein. Dr Frederic Saudou presented preliminary data to suggest that huntingtin was phosphorylated in cultured cells in response to IGF stimulation, although the kinase(s) and site(s) of phosphorylation were unknown. This was an important finding because it suggested that phosphorylation could be a powerful regulator of activity of both the wildtype and polyglutamine-expanded forms of huntingtin. The possibility

of huntingtin being phosphorylated in response to survival signalling pathways was particularly intriguing given the hypothesized protective role of wildtype huntingtin.

Knowing that huntingtin was potentially a phosphoprotein, bioinformatic analysis of primary amino acid sequence revealed that huntingtin was likely phosphorylated at multiple sites. Several sequences in huntingtin match the consensus sequence of many kinases, but there was no experimental confirmation of huntingtin phosphorylation, let alone any knowledge about the function of huntingtin phosphorylation. Bioinformatics tools suggested that the consensus sequence around serine S421 (S421) was a strong candidate for phosphorylation by Akt (PKB). S421 is in the N-terminus and the closest candidate to the caspase proteolysis domain (see below) and was therefore selected for study (Figure 1.17).

It was later demonstrated by the Saudou laboratory that huntingtin could be phosphorylated on this same site, S421, by stimulation of cultured cells with insulin growth factor (IGF) (Humbert et al., 2002). The IGF stimulation protected cells from the toxic effects of a fragment (1-480aa) of polyglutamine-expanded huntingtin and this effect was dependent on the phosphorylation at S421. Furthermore, they found that the Akt/PKB pathway mediated this effect and that Akt was capable of phosphorylating huntingtin at S421 in transfected cells.

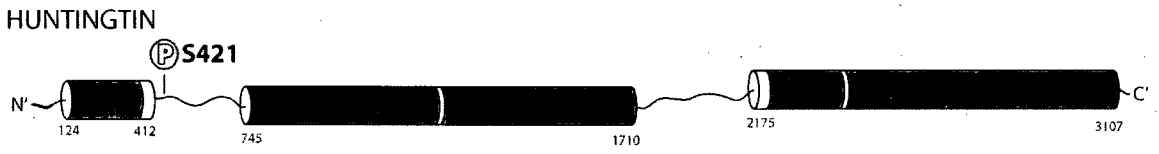

Figure 1.17: Schematic of huntingtin phosphorylated at S421

What remained unknown was whether this phosphorylation occurs *in vivo* under physiologically-normal conditions, whether it could play a role in the pathogenesis of HD, and if and how phosphorylation at S421 regulated the molecular function of huntingtin.

1.7.4 Roles of the Akt Pathway

The Akt pathway is well studied and serves numerous functions in the cell (Figure 1.18) (Blume-Jensen and Hunter, 2001). Understanding the impact of Akt on its other substrates may enlighten the understanding of huntingtin S421 phosphorylation. A prominent outcome of Akt

activation is cell survival (Frebel and Wiese, 2006). At the molecular level, Akt phosphorylation of substrates has multiple effects including regulating proteolytic cleavage, subcellular localization, or protein-protein interactions.

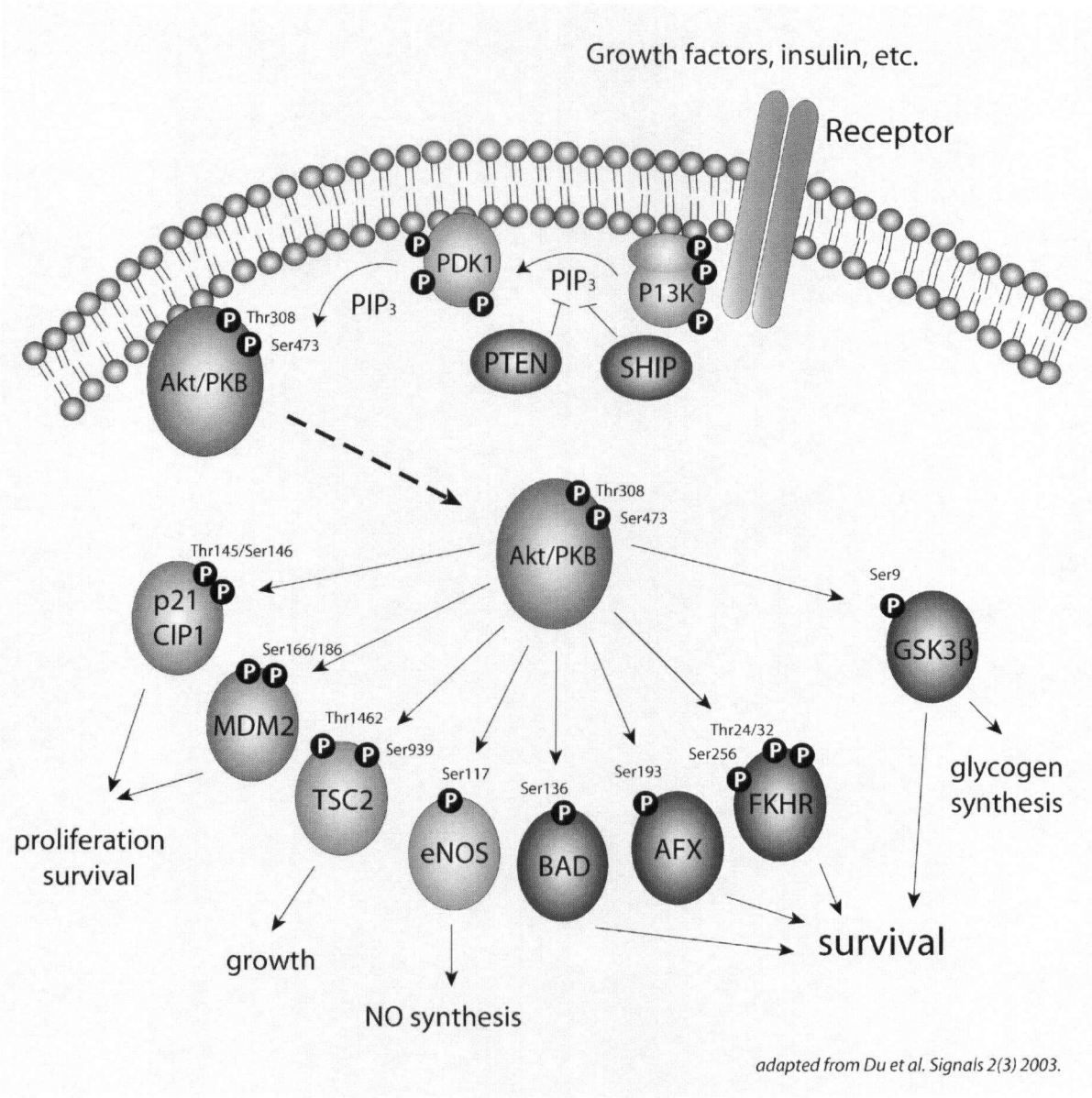

Figure 1.18: The Akt signalling pathway
The Akt kinase phosphorylates a variety of substrates that have an affect on multiple biological processes such as proliferation and cell survival.

1.7.4.1 Phosphorylation and Proteolytic Cleavage

There are many examples where phosphorylation regulates the proteolytic cleavage of its substrate (Tozser et al., 2003). Phosphorylation of the substrate may increase (Parker et al.,

1984; Warrener and Petryshyn, 1991; Watanabe and Kobayashi, 1995; Vecchi et al., 1998; LaFevre-Bernt and Ellerby, 2003; Santhamma et al., 2004; Hornig and Uhlmann, 2004) or decrease (Fujimoto et al., 1995; Barkett et al., 1997; Cardone et al., 1998; Walter et al., 1999; Lim et al., 2000; Krippner-Heidenreich et al., 2001; Desagher et al., 2001; Nicolas et al., 2002; Hoon et al., 2003; Choi et al., 2003; Rametti et al., 2004; Fluhrer et al., 2004) its cleavage and in some cases, substrates are selectively phosphorylated only after cleavage (Walter et al., 1998; Sebbagh et al., 2001). In the case of pro-caspase 9, cleavage and activation of the caspase itself is inhibited by Akt phosphorylation at S196 (Cardone et al., 1998).

1.7.4.2 Phosphorylation and Subcellular Localization

Phosphorylation of substrates can regulate protein function by altering their subcellular localization. Many Akt substrates (Brunet et al., 1999; Biggs, III et al., 1999; Kops et al., 1999) are translocated out of the nucleus when phosphorylated. There are many examples of co-regulation between phosphorylation and palmitoylation, another posttranslational modification important for subcellular localization (Soskic et al., 1999; Hawtin et al., 2001; Dorfleutner and Ruf, 2003; Ponimaskin et al., 2005). The nuclear/cytoplasmic localization of huntingtin is important for its toxic (Hackam et al., 1999a) and protective functions (Zuccato et al., 2003) and phosphorylation may ultimately regulate the movement of huntingtin in the cell.

In addition, the Akt-regulated localization of arfaptin-2 has been shown to be important for survival in a cellular model of HD. Preventing the phosphorylation of arfaptin-2 resulted in its altered subcellular localization, and increased toxicity caused by polyglutamine-expanded huntingtin (Rangone et al., 2005).

1.7.4.3 Phosphorylation and Protein-Protein Interactions

There are many examples of protein-protein interactions being regulated by phosphorylation. Many Akt substrates have phosphorylation-dependent interactions (Yamada et al., 2005; Urschel et al., 2005; Wanzel et al., 2005). Akt phosphorylation of ataxin-1 regulates its interaction with 14-3-3 and when ataxin-1 is polyglutamine-expanded, this interaction contributes to the pathogenesis of SCA1 (Chen et al., 2003b). Huntingtin has many interacting partners and in many cases the binding domains of these interactions are unknown.

1.8 OTHER POSTTRANSLATIONAL MODIFICATIONS OF HUNTINGTIN

In addition to phosphorylation, huntingtin undergoes many other posttranslational modifications. These posttranslational modifications may modulate the cleavage, interactions, and/or subcellular distribution of huntingtin within the neuron. Ultimately, these processes likely play an important role in the pathogenesis of HD, either by modulating polyglutamine-expanded huntingtin toxicity, endogenous function of huntingtin, or removal of polyglutamine-expanded huntingtin from the neuron.

1.8.1 Huntingtin Proteolysis

Although not classically considered a posttranslational modification, cleavage of huntingtin is a crucial process in both the normal and pathological functions of huntingtin, and is therefore a posttranslational modification of protein function. In addition to being processed in the proteosome (Jana et al., 2001) and in autophagy/lysosome systems (Qin et al., 2003; Ravikumar et al., 2004), huntingtin is a substrate for many enzymes which cleave proteins, including caspases, calpains and aspartyl peptidases (Table 1.3). Many cleavage sites are clustered together into a "proteolysis domain" between aa500-600 (Figure 1.19).

Table 1.3: Cleavage sites of huntingtin

Cleavage Site	Sequence	Protease	Reference
CASPASES			
513	DSVD	Caspase-3	(Goldberg et al., 1996b; Wellington et al., 1998; Wellington et al., 2000; Kim et al., 2001)
530	DEED	silent	(Wellington et al., 1998; Wellington et al., 2000)
552	DLND	Caspase 2 & 3	(Goldberg et al., 1996b; Wellington et al., 1998; Wellington et al., 2000; Kim et al., 2001)
586	IVLD	Caspase 6	(Wellington et al., 1998; Wellington et al., 2000)
589	DGTD	silent	(Wellington et al., 1998; Wellington et al., 2000)
CALPAINS			
437	VLS	unknown	(Gafni and Ellerby, 2002; Gafni et al., 2004)
469	LTA	unknown	(Gafni and Ellerby, 2002; Gafni et al., 2004)
536	SSS	unknown	(Gafni and Ellerby, 2002; Gafni et al., 2004)
ASPARTYL PROTEASES			
104-114		CpA	(Lunkes et al., 2002)
146-214		CpB	(Lunkes et al., 2002)

Proteolysis of normal and expanded huntingtin protein is observed in normal and HD brains and results in the formation of N-terminus fragments containing the polyglutamine tract (Kim et al., 2001; Wellington et al., 2002). Transfection of polyglutamine-expanded huntingtin into cells results in the cleavage of huntingtin and can induce cell death in cultured cells and primary neurons (Li et al., 2000; Cha, 2000; Yu et al., 2003). Inhibiting the cleavage of huntingtin, either by inhibiting proteases or mutating the site of cleavage, protects against the toxicity of polyglutamine-expanded huntingtin in cells (Martindale et al., 1998; Wellington et al., 2000; Gafni et al., 2004; Bizat et al., 2005) and mouse models of HD (Ona et al., 1999; Chen et al., 2000; Graham et al., 2006a).

However, *in vitro* and *in vivo* evidence also indicate that not all fragments of huntingtin are toxic. The toxicity of polyglutamine expanded huntingtin is context dependent; different sized huntingtin fragments result in different pathology in several mouse models of HD (Yu et al., 2003; Slow et al., 2005; Graham et al., 2006a). The R6/2 mouse model of HD expresses a short (exon 1) fragment of huntingtin with expanded polyglutamines and suffers brain atrophy, behavioural deficits and decreased survival (Mangiarini et al., 1996). In contrast, the shortstop mouse also expresses a short polyglutamine-expanded fragment of huntingtin but does not result in pathology (Slow et al., 2005), indicating that not all polyglutamine-expanded fragments are toxic.

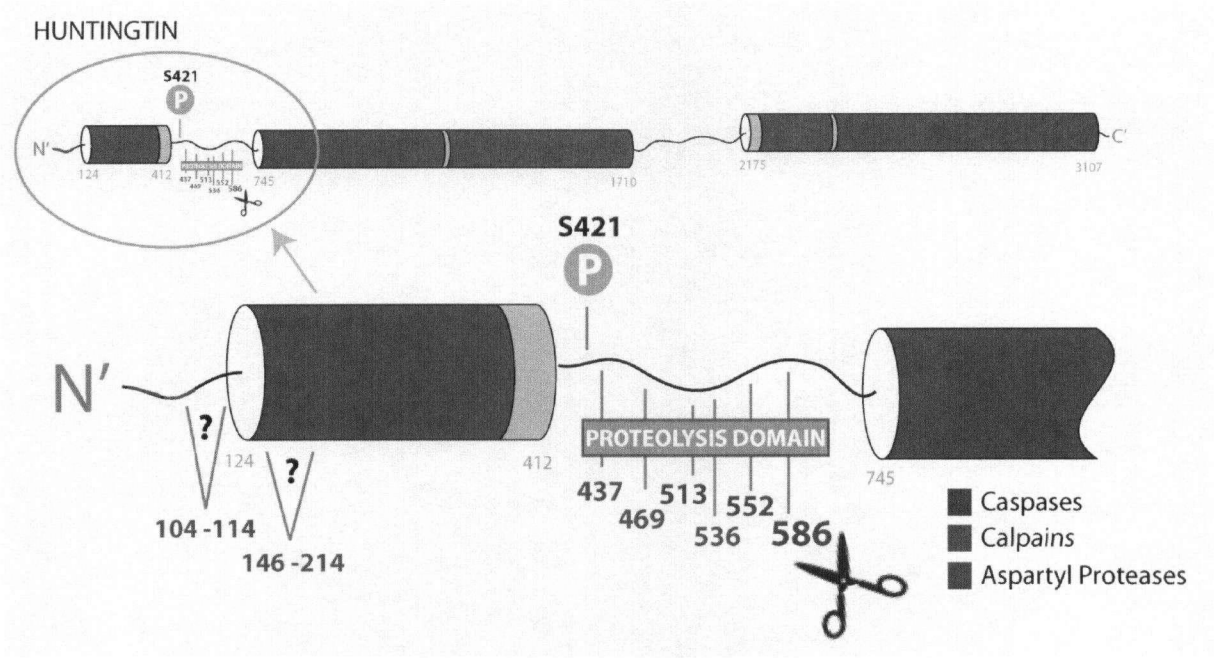

Figure 1.19: Schematic of cleavage sites in huntingtin

Studies of YAC mouse lines expressing caspase cleavage-resistant huntingtin have illustrated the importance of *specific* cleavage events in the pathogenesis of HD. While the YAC128 and C3R mice have progressive neurodegeneration and behavioural deficits, the C6R mice that are resistant to caspase-6 cleavage at aa586 are protected from developing this HD-like pathology (Table 1.4) (Graham et al., 2006a). Furthermore, neurons from the C6R mice are protected from a variety of neurotoxic stressors such as quinolinic acid (QA), NMDA and staurosporine. This suggests that cleavage of polyglutamine-expanded huntingtin at aa586 is a crucial requirement for pathology to occur and blocking this cleavage enhances the neuroprotective properties of huntingtin function.

Table 1.4: Caspase resistant (CR) YAC mice

Mouse	Sites Blocked	Pathology?
YAC128	None	YES
YAC128-**C3R**	513, 530, 552	YES
YAC128-**C6R**	586, 589	**No**

It is clear that the metabolic processing of huntingtin is a crucial rate-limiting step in the pathogenesis of HD but the molecular mechanisms that regulate the cleavage of huntingtin remain unknown.

1.8.2 **Palmitoylation**

Palmitoylation regulates the subcellular trafficking and function of proteins in neurons (el-Husseini and Bredt, 2002; Smotrys and Linder, 2004; Huang and El-Husseini, 2005; Resh, 2006). The palmitoylation reaction is the reversible addition of the lipid palmitate to cysteine residues. Palmitoyl acyl-transferases (PATs) are the class of enzymes that transfer palmitate to a substrate, while palmitoyl thioesterases catalyze the reverse reaction. The addition of palmitate increases protein hydrophobicity and results in the targeting of the substrate to lipid domains.

Huntingtin is palmitoylated on cysteine 214 (Figure 1.20) by a PAT known as huntingtin interacting protein-14 (Hip14, also known as DHHC-17) (Huang et al., 2004). The polyglutamine-expansion of huntingtin results in a reduced interaction with HIP14 (Singaraja et al., 2002) and reduced palmitoylation (Yanai et al., 2006). Blocking the palmitoylation of huntingtin disrupts its association with membranes and increases its toxicity, suggesting that reduced palmitoylation of polyglutamine-expanded huntingtin contributes to disease pathology.

There are many examples of co-regulation between palmitoylation and phosphorylation of a substrate (Soskic et al., 1999; Hawtin et al., 2001; Dorfleutner and Ruf, 2003; Ponimaskin et al., 2005) and it is possible that phosphorylation and palmitoylation of huntingtin modulate one another and ultimately the subcellular localization of huntingtin.

1.8.3 **Ubiquitination**

Ubiquitination is a multifunctional modification of proteins that involves the transfer of ubiquitin, a 76 amino acid protein, to specific lysine residues on a substrate from a series of ubiquitin ligases. Proteins are targeted for degradation through the ubiquitin-proteosome system (UPS), one of the major cellular pathways controlling protein degradation in eukaryotic cells, by poly-ubiquitination. Mono-ubiquitination, on the other hand, is believed to be a marker for endocytosis of membrane proteins. Ubiquitination of proteins, therefore, is important for the regulation of protein stability, activity and trafficking (Aguilar and Wendland, 2003; Schnell and Hicke, 2003; Patrick, 2006).

Huntingtin is ubiquinated (Kalchman et al., 1996; Sieradzan et al., 1999) (Figure 1.20) and can be degraded through the UPS. HIP2 (human ubiquitin-conjugating enzyme hE2-25K) interacts with huntingtin and is believed to be the enzyme mediating its ubiquitination (Kalchman et al., 1996). Proteosome dysfunction is believed to play a role in HD (Petrucelli and Dawson, 2004; Valera et al., 2005) although this data is still controversial (Bowman et al., 2005).

Interestingly, a specific single nucleotide polymorphism (SNP) in the ubiquitin carboxy-terminal hydrolase L1 (UCHL1) gene has been shown to modify the age of onset of HD (Naze et al., 2002; Metzger et al., 2006). UCHL1 is an abundant neuron-specific deubiquitinating enzyme and carriers of the S18Y variant had a delayed age of onset. This human clinical data is consistent with increased ubiquitination having a protective effect in the disease.

1.8.4 **SUMOylation**

As the name implies, the small 'ubiquitin'-related modifier (SUMO) family of proteins are structurally and mechanistically similar to ubiquitin. SUMOylation and ubiquitination both occur on lysine residues. They can have distinct regulatory functions in many proteins and in some cases, SUMO seems to function to block ubiquitination (Seeler and Dejean, 2003). SUMO attachment is covalent and reversible, and catalyzed by a family of ligases similar to the ubiquitin-ligases (Dohmen, 2004).

The discovery of SUMO modification is relatively recent (several groups independently discovered SUMO (Meluh and Koshland, 1995; Matunis et al., 1996; Okura et al., 1996; Boddy et al., 1996; Shen et al., 1996) so it is also termed 'GMP', 'PIC', 'sentrin', 'SMT3' and 'UBL1'). One interesting feature of this modification is that, unlike phosphorylation, it seems that only a small proportion of SUMO substrates are actually SUMOylated at any one time (Johnson, 2004).

Huntingtin is believed to be SUMOylated at lysines in its N-terminus (K6 and K9) (Steffan et al., 2004) (Figure 1.20). In cultured cells and in a *Drosophila* model of HD, the SUMOylation of huntingtin exon-1 fragment resulted in enhanced stability of the toxic protein and enhanced toxicity. Mutations that block both the ubiquitination and SUMOylation of these N-terminal lysines seem to reduce the toxicity, suggesting that the negative effects of SUMOylation on cell

survival are more than simply the loss of function of ubiquitination (Steffan et al., 2004). The detailed mechanisms of regulation of ubiquitination and SUMOylation is unknown.

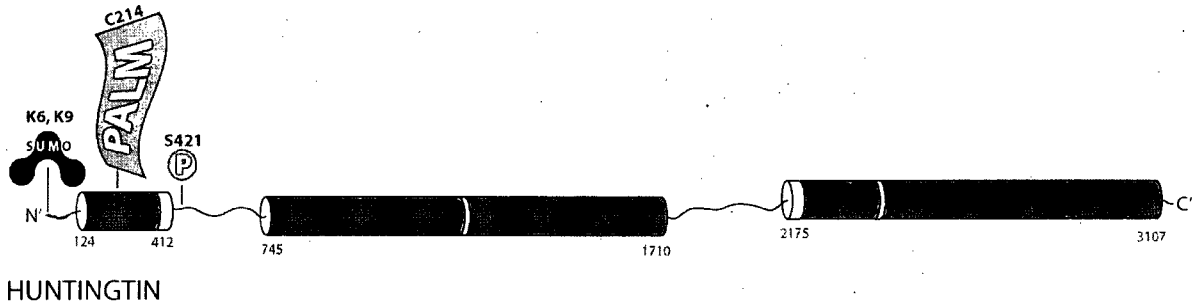

Figure 1.20: Schematic of sites of huntingtin palmitoylation, ubiquitination, and SUMOylation
Huntingtin is palmitoylated by HIP14 at C214 and SUMOylated at K6 and/or K9.

1.8.5 Phosphorylation-Dependent Prolyl Isomerization

Peptidyl-prolyl bonds can exist in either a cis or trans conformation and this transition is catalyzed by peptidyl-prolyl cis/trans isomerases (Gothel and Marahiel, 1999). Pin-1 is a phosphorylation-dependent isomerase, as it contains a WW motif and binds and isomerases specific pSer/Thr-Pro residues. Importantly, isomerization of pSer/Thr-pro residues into a cis formation can block further processing by phosphatases and kinases (Lu et al., 2002; Wulf et al., 2005). Aberrant Pin-1 activity has been implicated in a variety of diseases (Lu, 2004).

Initial data suggests that huntingtin could be modified by Pin-1. Huntingtin contains multiple S/T proline motifs in its N-terminus that, if phosphorylated, could be targets for Pin-1, although no single site has yet been confirmed. Both wildtype and polyglutamine-expanded huntingtin have been shown to interact by co-immunoprecipitation and GST-pulldown with Pin-1 (Michelazzi and Persichetti, pers comm). This potential posttranslational modification adds an additional layer of regulation and complexity to huntingtin function.

1.9 THESIS HYPOTHESIS AND OBJECTIVES

Currently, no treatment is available to effectively treat or prevent the neurodegeneration in HD. A major challenge is to identify the initial changes in the brain that cause neural dysfunction and degeneration to occur. Clearly, the expression pattern of huntingtin does not explain the

selective pathology. Protein context is import for polyglutamine-expansion pathology and the clinical features of HD must therefore be dictated by structural or biochemical properties of the huntingtin protein.

The huntingtin protein is a large multifunctional protein that is transported through many compartments in the cell and has many protein interacting partners. What remains unknown is how huntingtin subcellular localization, protein interactions and cleavage are regulated.

Posttranslational modification, particularly phosphorylation, is a common and important means of regulating proteins and we therefore hypothesize that:

> *Huntingtin phosphorylation at S421 is dynamic and regulated and is important in the molecular function of the protein. S421 phosphorylation imparts a conformational change in huntingtin that is important for its regulated interaction with other proteins, subcellular localization and cleavage by proteases. Ultimately, the molecular function of huntingtin phosphorylation at S421 may modulate the protective properties of wildtype huntingtin and the toxic characteristics of polyglutamine-expanded huntingtin.*

The primary objective of this thesis was to characterize the phosphorylation of huntingtin at S421 and understand what role it might play in the pathogenesis of HD. The specific goals of this thesis were to:

1. Characterization huntingtin phosphorylation at S421.

Bioinformatic analysis of the huntingtin protein suggests that it contains several serine and threonine residues that match consensus sequences for phosphorylation sites, including the serine at aa421 (S421). *Is huntingtin phosphorylated at S421 under normal physiological conditions in brain? What kinase phosphorylates S421? Under what conditions is it phosphorylated?*

2. Determine if S421 phosphorylation is altered by polyglutamine-expansion of huntingtin.

The polyglutamine tract is located in the N-terminus of huntingtin and polyglutamine expansion is believed to impart a dramatic conformational change of the protein. *Does*

polyglutamine-expansion alter the basal phosphorylation state at S421 in cells? Is the basal phosphorylation state of huntingtin altered by polyglutamine expansion in the YAC mouse brain? Does polyglutamine expansion alter the signalling pathways responsible for phosphorylation at S421 and/or does it alter the specific interaction between the kinase/phosphatase and huntingtin?

3. Determine if S421 phosphorylation alters the cleavage of huntingtin.

Phosphorylation regulates the cleavage of many substrates. *Does phosphorylation at S421 regulate the cleavage of huntingtin under normal physiological conditions? After cellular stress? Does S421 phosphorylation reduce the caspase-6 cleavage at the crucial 586aa residue known to be important in the pathogenesis of HD?*

4. Determine if S421 phosphorylation alters the subcellular localization of huntingtin.

Huntingtin has a broad distribution in the cell and shuttles between the nucleus and the cytoplasm. Nuclear localization of huntingtin is believed to be important in the disease. *Does S421 phosphorylation influence the subcellular localization of huntingtin? Is phosphorylation altered when full length or fragments of huntingtin are in the nucleus? Does phosphorylation regulate the nuclear shuttling of huntingtin?*

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CHAPTER 2

Huntingtin phosphorylation on serine 421 is significantly reduced in the striatum and by polyglutamine expansion *in vivo*

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Preface

I designed and performed all of the *in vivo* experiments and some of the *in vitro* experiments in this manuscript. I developed the techniques for huntingtin immunoprecipitation and quantitative immunoblotting necessary for the pS421 assay. I performed the bioinformatics and data analysis for all experiments, generated the figures, and wrote the manuscript.

The initial concept for the experiments described in this chapter was conceived in collaboration with Ed Chan, who directed the generation and characterization of the pS421-specific antibody and collected the *in vitro* data in Figure 2.5.

For the cloning and *in vitro* data collection, I received the following assistance: Roshni Singaraja performed the site directed mutagenesis at S421 and Christina Tognon (Dr. Poul Sorensen's Lab, CFRI) provided the MSCV viral backbone from which I performed all of the cloning of huntingtin phosphorylation mutants. Lu Gan and Martina Metzler performed the Akt co-immunoprecipitation (Figure 2.3B). Susan Crocker and Harry Robertson performed the Akt blots in R6/2 tissue (Figure 2.6C).

2.1 INTRODUCTION

The neurodegeneration in Huntington disease (HD) is caused by a dominantly inherited mutation that results in an expanded polyglutamine tract in the huntingtin protein. The expanded polyglutamine tract confers cytotoxic properties to huntingtin (Li et al., 2000; Cha, 2000; Yu et al., 2003) and compromises some of its endogenous functions (Rigamonti et al., 2000; Cattaneo et al., 2001; Zuccato et al., 2003; Thompson, 2003; Cornett et al., 2005). Although the mutant huntingtin protein is expressed at similar levels in all brain regions (Aronin et al., 1995; Landwehrmeyer et al., 1995; Trottier et al., 1995a), there is regional selectivity and a temporal pattern to the neuropathology in HD. The molecular basis underlying this specificity is poorly understood. Striatal neurons are the first to degenerate and show the most severe neurodegeneration (Vonsattel et al., 1985), while cortical neurons are often also affected at later stages of the disease (Halliday et al., 1998). Currently, no therapeutic strategies are available to effectively slow the neurodegeneration in HD and a major challenge is to identify factors in the aging brain that create a local environment permissive to this neurodegeneration.

Several recent reports have demonstrated that huntingtin undergoes numerous posttranslational modifications such as phosphorylation (Humbert et al., 2002; Rangone et al., 2004), ubiquitination (Kalchman et al., 1996), SUMOylation (Steffan et al., 2004), palmitoylation (Huang et al., 2004) and proteolysis (Mende-Mueller et al., 2001; Kim et al., 2001; Wellington et al., 2002; Goffredo et al., 2002; Lunkes et al., 2002). Although it still remains to be definitively demonstrated, these processes likely play an important role in the pathogenesis of HD, either by modulating polyglutamine-expanded huntingtin toxicity, endogenous function of huntingtin, or removal of polyglutamine-expanded huntingtin from the neuron. Phosphorylation by Akt (also known as PKB) plays a central role in the pathology of another polyglutamine expansion disorder, spinocerebellar ataxia type 1 (SCA1) (Emamian et al., 2003; Chen et al., 2003b) and for this reason, we wished to explore the Akt phosphorylation of huntingtin *in vitro* and *in vivo* and its relationship to the pathogenesis of HD.

It has been demonstrated *in vitro* and in cultured cells that huntingtin can be phosphorylated on serine-421 (S421) by Akt and the closely related serum- and glucocorticoid-inducible kinase (SGK) (Humbert et al., 2002; Rangone et al., 2004). In primary striatal neurons, the toxicity of a

transfected amino-terminal fragment of polyglutamine-expanded huntingtin could be attenuated by the co-transfection of Akt or SGK. This attenuation was dependent on S421 phosphorylation, as there was no protection if huntingtin was genetically modified at S421 to render it non-phosphorylatable. In addition, a modification that mimics constitutive phosphorylation at S421 of this polyglutamine-expanded huntingtin fragment also rendered it non-toxic (Humbert et al., 2002).

Although the phosphorylation of huntingtin by Akt is protective and can prevent polyglutamine-expanded huntingtin toxicity in cell culture, its role in the pathogenesis of HD remains unclear. Several lines of indirect evidence suggest that huntingtin phosphorylation might be altered in the disease state. Given the profound effect of phosphorylation of expanded huntingtin on its toxicity in cell culture, we sought to determine whether phosphorylation at S421 is altered by the polyglutamine expansion *in vivo*. We report here that huntingtin is phosphorylated at S421 under normal physiological conditions in brain tissues, that the striatum has a low level of endogenous huntingtin phosphorylation, and that phosphorylation of polyglutamine-expanded huntingtin is reduced in HD.

2.2 MATERIALS AND METHODS

2.2.1 Antibodies and reagents

Immunoblotting was performed with antibodies against htt (mAb 2166 Chemicon), human specific htt (HD650 - (Slow et al., 2003)), anti-Akt (BD Pharmingen), anti-pAkt rabbit polyclonal (P-SER472/473/474 - BD Pharmingen) and Actin (Sigma). Experiments with the BD Pharmingen antibodies were repeated with pAkt and total Akt antibodies from Cell Signalling and produced equivalent data. Purified Akt1/PKBalpha (Upstate) was used for *in vitro* phosphorylation and Lambda Protein Phosphatase (gamma-PPase - New England Biolabs) or PP2A (Upstate) was used to dephosphorylate htt. The 1212aa (pRcCMV-3949-15Q) htt constructs have been described previously (Wellington et al., 2002). Serine-421 substitution was created using a site-directed mutagenesis approach as previously described (Wellington et al., 2000) and confirmed by sequencing. The mutagenic primers used were: S421A-F: GAAGCCGTAGTGGGGCTATTGTGGAAC; S421A-R:

GTTCCACAATAGCCCCACTACGGCTTC. The cloning primers used were: Xho-F: CAAGGTTACAGCTCGAGCTCTATAAG; Kpn-R: CAAATACTGGTTGTCGGTACCGTCTAAC.

The mutations were first generated in the htt fragments containing 3840 nucleotides (1-1212aa) and were subsequently subcloned to generate the serine mutation in full-length htt.

2.2.2 Generation of anti-pS421-htt antibody

Rabbits were immunized with peptide RSG(pS)IVELIAGC conjugated to KLH (complete Freund's adjuvant) followed by repeated boosts (incomplete Freund's adjuvant). Serum was collected 49 days after the initial immunization and screened in ELISA assays. Serum displaying strong immune response was used for double affinity purification. Serum was passed over a column containing the peptide RSGSIVELIAGC and the flow-through was affinity-purified on a column containing RSG(pS)IVELIAGC phospho-peptide.

2.2.3 Cell culture and Tissue Lysis

HeLa and HEK293 cells were grown at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS (GIBCO). Transient transfection of the cells was performed with Fugene (Roche) following the manufacturer's standard instructions. Briefly, cells were transfected in a 6 well dish (1 ug DNA + 3 uL Fugene reagent) and then harvested 24 hours later.

To harvest the cultured cells, SDP+ lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Igepal, 40 mM NaF, 1 mM PMSF, 2 mM sodium vanadate, 1X protease inhibitors (Roche)) was added to the plates and left on ice for 5 minutes. The cells were scraped, sonicated briefly and centrifuged at 10,000 RCF for 15 min at 4°C. To generate the brain lysates, FVB wildtype or YAC transgenic mice were sacrificed with CO_2 and the brain was isolated and homogenized with a Dounce homogenizer in SDP+ lysis buffer. Samples were left on ice for 10 minutes, sonicated briefly and centrifuged at 10,000 RCF for 15 min at 4°C. Protein concentration was assessed with the Bradford assay (BioRad). Human samples were from the frontal cortex of a 68-year-old male. The YAC18Q (line 212) (Hodgson et al., 1999) and YAC128Q (line 53) (Slow et al., 2003) mouse models contain the full-length genomic human HD gene with its endogenous promoter elements, providing appropriate developmental and tissue specific expression of the htt protein (Slow et al., 2003). R6/2 mice, which express a truncated N-terminal fragment of huntingtin (Mangiarini et al., 1996) were sacrificed with sodium

pentobarbital. The tissue was extracted, snap frozen in liquid nitrogen and stored in the -80 freezer until it was processed. The tissue was homogenized using a motorized Teflon pestle (Sigma) in 0.32M Sucrose with Complete Mini (Roche) protease inhibitors and PMSF.

Immunopurification of htt was performed on Sepharose G beads pre-bound with mAb2166 or HD650. Lysates were incubated with the beads for two hours at 4 °C and then washed with SDP+. Samples were separated on a 3-8% Tris-acetate or 4-12% bis-Tris gel (Invitrogen) and then transferred to PVDF membrane for immunoblotting. Densitometry of immunoblots was performed with a Bio-Rad Multi-Fluor-S and/or Quantity 1 software. Densitometry data were normalized and groups compared by student's T-test. For *in vitro* kinase reactions, immunoprecipitates were washed, resuspended in assay dilution buffer (20mM Mops pH 7.2, 25mM β-glycerophosphate, 5 mM EGTA, 18 mM MgCl2, 5 mM sodium vanadate and 1 mM DTT), and incubated with 0.5 units of purified Akt at 30 °C for 1 hour. For *in vitro* phosphatase reactions, immunoprecipitates were washed, resuspended in phosphatase buffer (20 mM Hepes pH 7.6, 1 mM MnCl2, 100 ug/mL BSA and 1 mM DTT) and treated with PP2A or Lamda phosphatase for 30 minutes at 30 °C.

For co-immunoprecipitation, brains from 6 month old wild-type, YAC18Q, or YAC128Q transgenic mice were isolated and homogenized in homogenization buffer (10 mM Hepes-KOH pH 7.4, 0.83 mM Benzamidine, 0.25 mM PMSF, 0.5 ug/ml leupeptin, 0.5 ug/ml aprotinin) followed by centrifugation at 800 *g* for 5 min at 4 °C. The supernatant was isolated and centrifuged at 205,000 x g for 30 min at 4 °C. The protein concentration was measured in the supernatant and after pre-clearing, 1 mg protein was incubated with 5 uL of a mouse anti-Akt antibody (5G3, Cell Signaling) or normal mouse IgG in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 ug/ml aprotinin, 1 ug/ml leupeptin, 1 mM $NaVO_4$, 1 mM β-glycerophosphate, 2.5 mM sodium-pyrophosphate, PefaBloc, 2.5 mM NaF for 4 h at 4°C. After incubation, samples were washed three times in PBS. Bound proteins were eluted in SDS-PAGE sample buffer and processed for Western blot analysis. Immunoprecipitation of Akt was verified with a rabbit polyclonal antibody (Cell Signaling) and co-immunoprecipitation of htt was demonstrated with the mouse monoclonal anti-htt antibody 2166 (Chemicon).

2.3 RESULTS

2.3.1 Generation of the Huntingtin Phosphorylation-Specific Antibody

Using the peptide sequence [RSG(pS)IVELIAGC] corresponding to phosphorylated S421 of human huntingtin, we generated a phosphorylation-specific polyclonal antibody. We first validated the specificity of this phospho-antibody using lysates from HeLa cells transfected with truncated constructs containing amino acids 1-1212 of human huntingtin with 15 polyglutamines. From these lysates, huntingtin was immunoprecipitated and then immunoblotted using the phospho-huntingtin antibody (Figure 2.1). Interestingly, the phospho-huntingtin antibody detected the truncated huntingtin fragment in resting, untreated cells indicating that huntingtin is normally phosphorylated to significant levels. As expected, the intensity of the pS421-huntingtin signal increased when the immunoprecipitate was pre-incubated with purified active Akt. Conversely, the pS421-huntingtin signal was completely removed when the immunoprecipitate was pre-incubated with recombinant bacteriophage protein phosphatase.

We also confirmed that the phospho-specific antibody does not detect huntingtin with a serine to alanine (S421A) mutation that prevents its phosphorylation. To control for protein loading, the immunomembrane was reprobed for total huntingtin. We conclude that the pS421-huntingtin antibody specifically detects phosphorylation at S421 and that a portion of huntingtin is phosphorylated under normal conditions in HeLa cells.

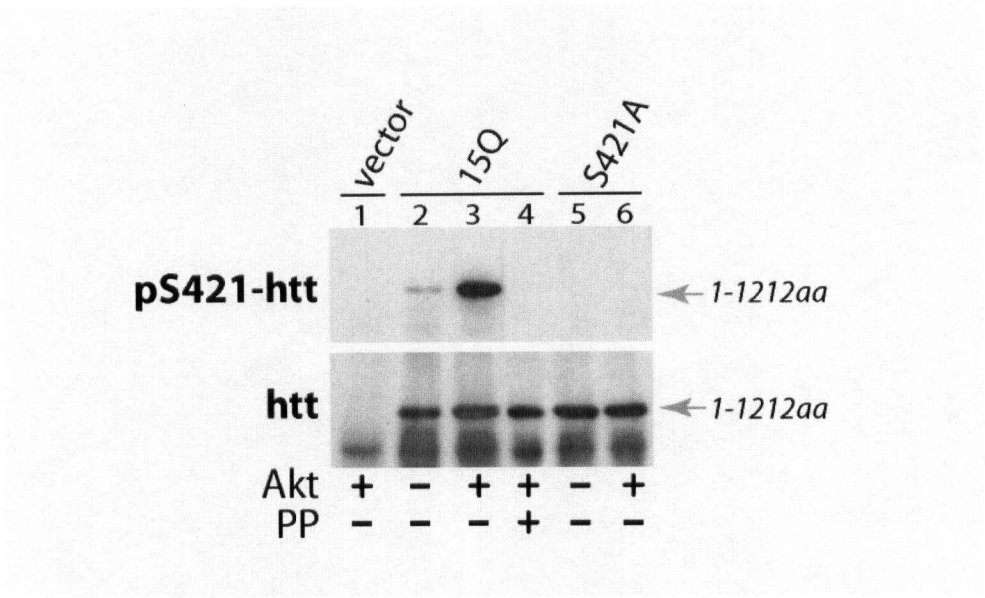

Figure 2.1: Huntingtin (htt) is phosphorylated at S421 in cultured cells
The anti-pS421-huntingtin antibody specifically detects huntingtin only when phosphorylated at S421. Huntingtin constructs (1-1212aa, lanes 2-6) were transiently transfected and immunoprecipitated from HeLa cells. Immunoprecipitates were treated with purified Akt or lamda protein phosphatase (PP) as indicated and then separated on a polyacrylamide gel and probed with the pS421-huntingtin antibody or mAb2166. Lane 2 demonstrates that the truncated huntingtin fragment is endogenously phosphorylated in these human cells. The phosphorylation status is increased by Akt treatment (lane 3) and eliminated by lambda phosphatase treatment (lane 4), demonstrating the specificity of the antibody. Furthermore, constructs with serine-421 mutated to an unphosphorylatable alanine (S421A) are not detected by the antibody (lane 5), even when treated with purified active Akt (lane 6). Immunoblotting against huntingtin with mAb2166 on the same membrane (lower panel) confirms expression of the constructs.

2.3.2 Huntingtin is Phosphorylated at S421 Under Normal Physiological Conditions in Brain Tissues

We adapted our immunoprecipitation-immunoblot protocol described above to determine if endogenous full-length huntingtin is normally phosphorylated at S421 in brain tissues. Our initial attempts at detecting phosphorylation of huntingtin in normal human brain samples proved difficult and we speculated that perhaps the modification was labile and especially sensitive to post mortem interval (PMI) (Li et al., 2003a). To favour our ability to detect phosphorylation, we obtained a human frontal cortex sample with the shortest PMI available (3.5 hours). Endogenous phosphorylation could be detected in extracts derived from this tissue with extended exposures of the immunoblot to film (Figure 2.2A, lane 1). Incubation of the immunopurified huntingtin from this sample with Akt increased phosphorylation of huntingtin (lane 2).

Our analysis was extended to mouse brain samples in order to directly investigate the relationship between PMI and huntingtin phosphorylation. A mouse was sacrificed and then given a controlled PMI of 2 hours before harvesting of brain tissue from the skull cavity. Similar to findings from the human brain sample, phosphorylation on endogenous huntingtin could be detected upon long exposures of the pS421 immunoblot to film, and this phosphorylation was increased by the addition of purified Akt (lane 3 and 4). In fresh (zero PMI) mouse tissue, the signal was stronger but could still be increased by the addition of purified Akt to the sample. In all samples, huntingtin phosphorylation could be removed completely upon incubation of mouse brain extract with recombinant protein phosphatase 2A (Figure 2.2B). In our experiments, an equivalent amount of total huntingtin was always confirmed by reprobing the membrane with mAb 2166.

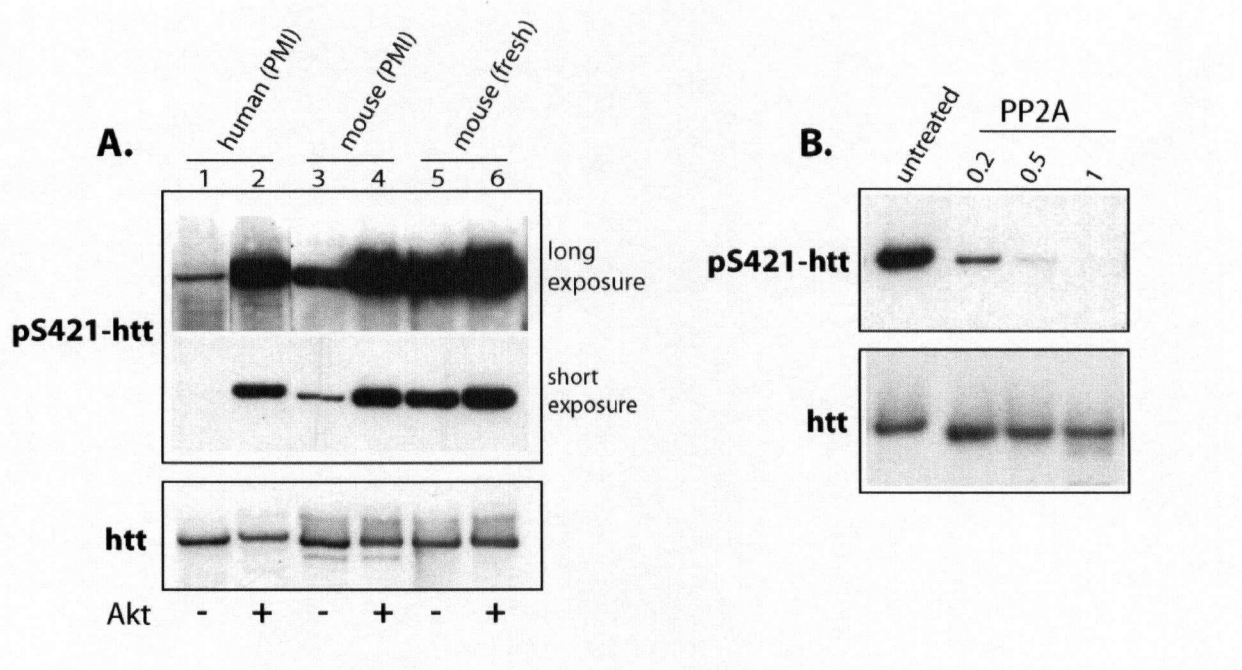

Figure 2.2: Endogenous huntingtin (htt) is phosphorylated at S421 *in vivo* in human and mouse brain
(A) Full length huntingtin was immunoprecipitated from human and mouse brain and probed with the phospho-*specific* antibody (for short or long exposure time, upper two panels). As a control for loading, the membrane was stripped and reprobed for total huntingtin with mAb2166 (bottom panel). The pS421-huntingtin antibody detects endogenously phosphorylated huntingtin in human frontal cortex with a 3.5 hour post mortem interval (PMI) (lane 1), whole mouse brain with a 2 hour PMI (lane3), and freshly prepared whole mouse brain (lane 5). The addition of purified Akt enzyme to the lysates increased the phosphorylation signal in each of these samples (lane 2, 4, and 6).
(B) The mammalian PP2A dephosphorylates pS421. Full-length huntingtin was immunoprecipitated from fresh mouse brain and then treated with purified PP2A. Dose of the PP2A is indicated in units.

Our data indicate that huntingtin is phosphorylated at S421 under normal physiological conditions, although this phosphorylation does not appear to be maximal. Furthermore, our data indicate that phosphorylation at this site is labile and is rapidly removed after death in vivo, presumably by endogenous phosphatases.

2.3.3 Huntingtin Interacts with Akt

The huntingtin amino acid sequence flanking serine-421 matches the Akt substrate motif RXRXX(S/T)p (Scheid and Woodgett, 2001; Yaffe et al., 2001). A multiple sequence alignment of vertebrate huntingtin orthologs demonstrates that the phosphorylated serine and two key arginine residues of the Akt motif are present in human huntingtin and conserved in species as evolutionarily distant as zebrafish (Figure 2.3A).

To assess whether Akt and huntingtin interact *in vivo*, we performed co-immunoprecipitation experiments using whole mouse brain from wildtype (FVB) or YAC transgenic mice expressing human huntingtin with a polyglutamine tract of 18 or 128 repeats (Figure 2.3B). Lysates were immunoprecipitated with antibodies against Akt, and then immunoblotted for bound huntingtin. These experiments demonstrate that Akt and huntingtin co-immunoprecipitate in wildtype, YAC18 and YAC128 mouse-brains and are consistent with the phosphorylation of huntingtin by Akt *in-vivo*. The reverse immunoprecipitation experiment (immunoprecipitating huntingtin and blotting for Akt) was inconclusive because of non-specific binding of huntingtin to the beads (data not shown). Interestingly, huntingtin and Akt appear to have co-immunoprecipitated to a similar degree from YAC18 and YAC128 mice, indicating that the protein-protein interaction between huntingtin and Akt is not greatly disrupted by polyglutamine expansion.

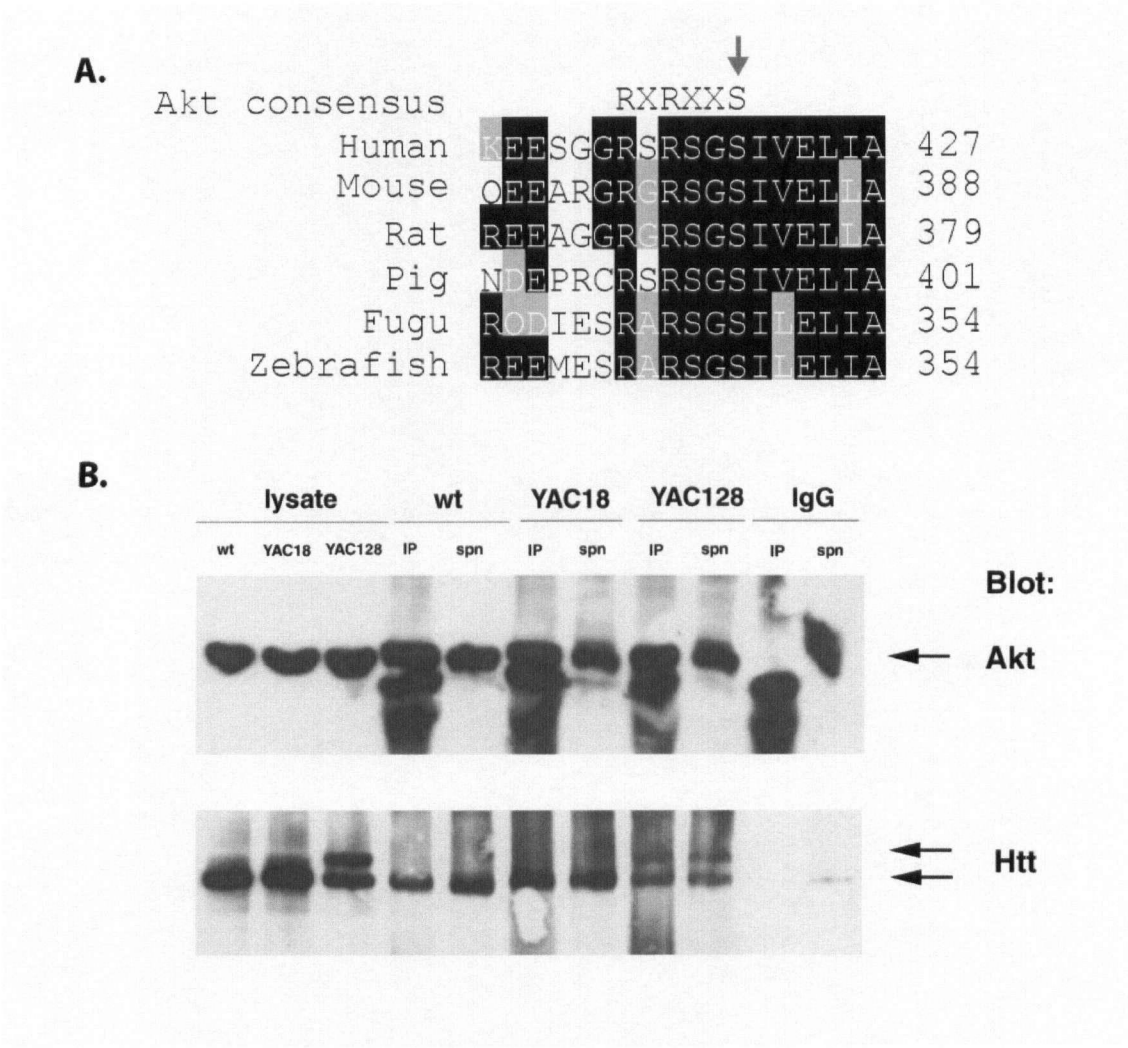

Figure 2.3: Huntingtin(htt) interacts with Akt
(A) The region surrounding huntingtin S421 matches the Akt substrate consensus sequence RXRXX(S/T) (where X is any amino acid) and is highly conserved in a multiple sequence alignment of huntingtin orthologs. S421 is indicated with an arrow.
(B) Huntingtin co-immunoprecipitates with Akt in brain lysate from wild-type and YAC transgenic mice overexpressing full-length huntingtin with 18 and 128 glutamine repeats. Proteins in brain lysate from wild-type and YAC transgenic mice were immunoprecipitated with an antibody against total pan-Akt. Immunoprecipitated proteins were analyzed by immunoblotting with anti-Akt and anti-huntingtin antibodies.

2.3.4 Levels of Huntingtin Phosphorylation are Lowest in Regions that Degenerate in HD

The striatum is the region of primary degeneration in HD while the cerebellum remains relatively unaffected (Vonsattel et al., 1985). To explore whether the degree of huntingtin phosphorylation might be related to this regional specificity, we next determined if there were differences in the amount of huntingtin phosphorylation in different areas of the normal brain. Huntingtin was immunoprecipitated from extracts of striatal, cortical and cerebellar tissues

derived from thirteen wildtype mouse brains at 3 months of age. The samples were immunoblotted with the pS421 antibody and then reprobed with mAb 2166 to derive phospho-huntingtin/total huntingtin ratios in each region (Figure 2.4A). The amount of specific huntingtin phosphorylation was 45% less in the striatum relative to the cerebellum (ANOVA $P < 0.001$, Tukey's post hoc striatum vs cerebellum $P < 0.001$). Relative to the striatum, cortical pathology in HD is delayed and more variable in HD patients (Vonsattel et al., 1985; Aylward et al., 1996) and accordingly, we found the level of huntingtin phosphorylation in the cortex was intermediate and variable in mouse brain.

To determine why there may be regional differences in the phospho-huntingtin/huntingtin ratio, we next assessed regions of the brain for differences in the activation of Akt. Samples from wildtype mouse brain (N=9) were immunoblotted using an antibody for residue S473 of Akt that has been shown to be an effective marker for Akt activation state (Alessi et al., 1996). Densitometry of pAkt and Akt immunoblot signals was used to compute the activated (pAkt)/total Akt ratio (Figure 2.4B). The regional differences in the Akt ratios resemble regional differences in phospho-huntingtin ratios: the ratio was significantly lowest in the striatum, slightly higher in the cortex and cerebellum had the highest Akt activation state (ANOVA $P < 0.0001$). Our data indicate that the regional variability in phospho-huntingtin parallels regional differences in Akt activation status and this pattern inversely correlates with regions most affected by neurodegeneration in HD.

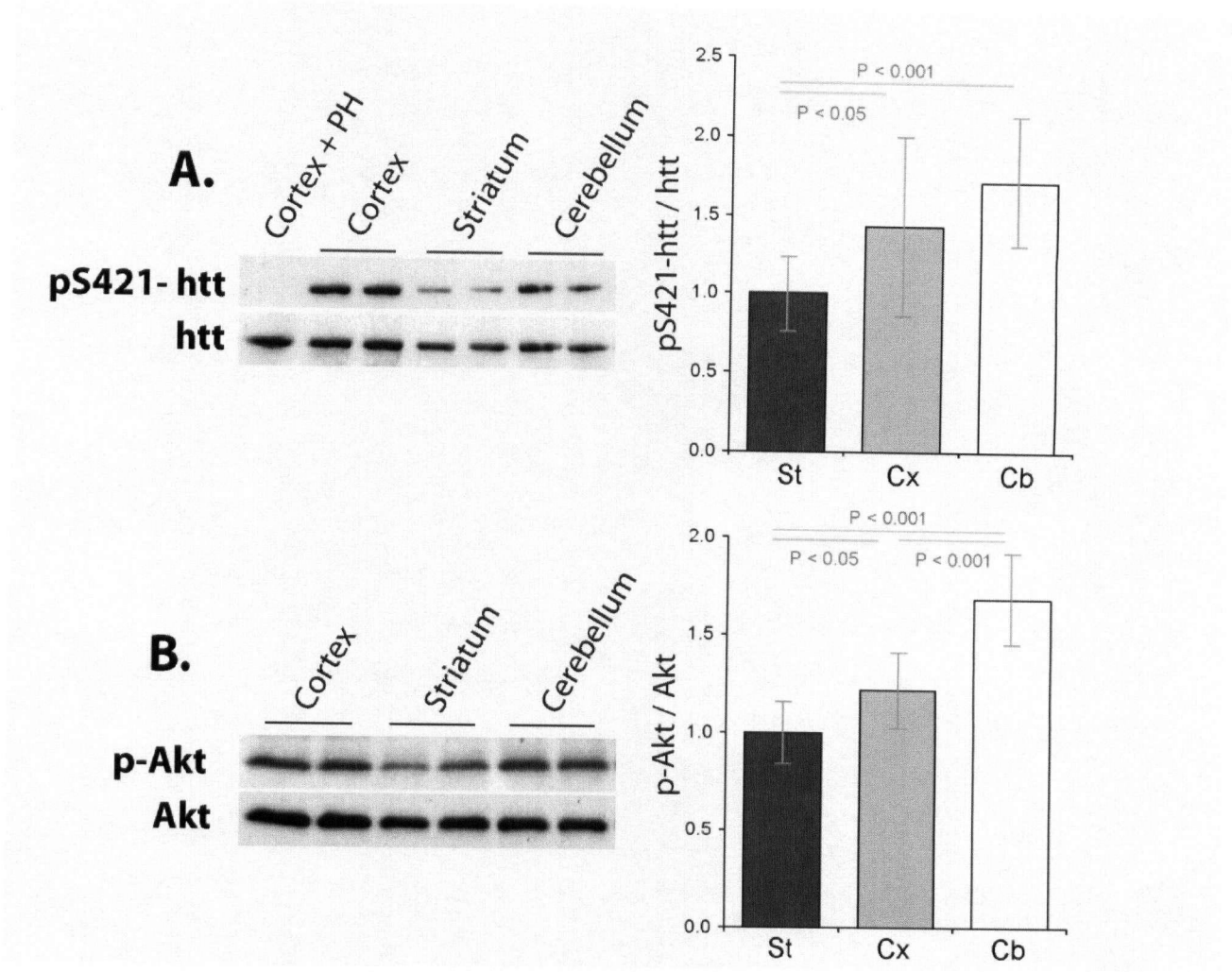

Figure 2.4: Huntingtin (htt) phosphorylation is significantly less in the striatum than the cerebellum in normal mice

(A) Striatal (St), cortical (Cx), and cerebellar (Cb) samples from 3 month old wildtype mice were blotted with the pS421-huntingtin antibody and then reprobed for total huntingtin to obtain a relative ratio of phosphorylated huntingtin. A representative immunoblot from two mice is shown on the left. The treatment of the sample with lambda phosphatase (PH) is a control to confirm the specificity of the phospho-antibody (first lane). The phospho-huntingtin ratio was obtained by quantifying the band intensity of the pS421 signal divided by the mAb2166 (total huntingtin) intensity on the film. The chart on the right is the mean ratio (normalized to striatum) and standard deviation of 13 wildtype mice. The ratio of pS421-huntingtin/huntingtin is lowest in the striatum, the primary region of degeneration of HD, and highest in the cerebellum, a brain region spared from pathology in the disease (ANOVA $P < 0.001$, Tukey's post hoc St vs Cx $P < 0.05$, St vs Cb $P < 0.001$, Cx vs Cb $P = 0.21$).

(B) Regional differences in the activation of Akt reflect the regional differences in pS421. Nine wildtype mice were blotted for activated (P-Ser 472/473/474) pan-Akt and total pan-Akt. The activated Akt/Akt levels were significantly lowest in the striatum, highest in the cerebellum and intermediate in the cortex (ANOVA $P<0.0001$, Tukey's post hoc St vs Cx $P < 0.05$, St vs Cb $P < 0.001$, Cx vs Cb $P < 0.001$).

2.3.5 Polyglutamine Expansion in Huntingtin Decreases Phosphorylation

Having confirmed the interaction between Akt and huntingtin, and determined that huntingtin phosphorylation is present in human and mouse brain, we sought to determine whether the

phosphorylation of huntingtin was altered as a result of the HD mutation. Phosphorylation has been shown to decrease polyglutamine-expanded huntingtin toxicity (Humbert et al., 2002) but we wanted to address whether the expansion also affected its ability to become phosphorylated. HEK293 cells were transiently transfected with constructs that express the N-terminal region of huntingtin (1-1212) containing either 15Q or 128Q. Huntingtin was immunoprecipitated and immunoblotted with the pS421 antibody and then reprobed for total huntingtin. Phosphorylation of huntingtin containing 128Q was consistently lower than 15Q huntingtin (Figure 2.5A). Densitometry indicates the phospho-huntingtin to huntingtin ratio was reduced by 50% on huntingtin 128Q when compared to huntingtin 15Q (T-test $p<0.005$).

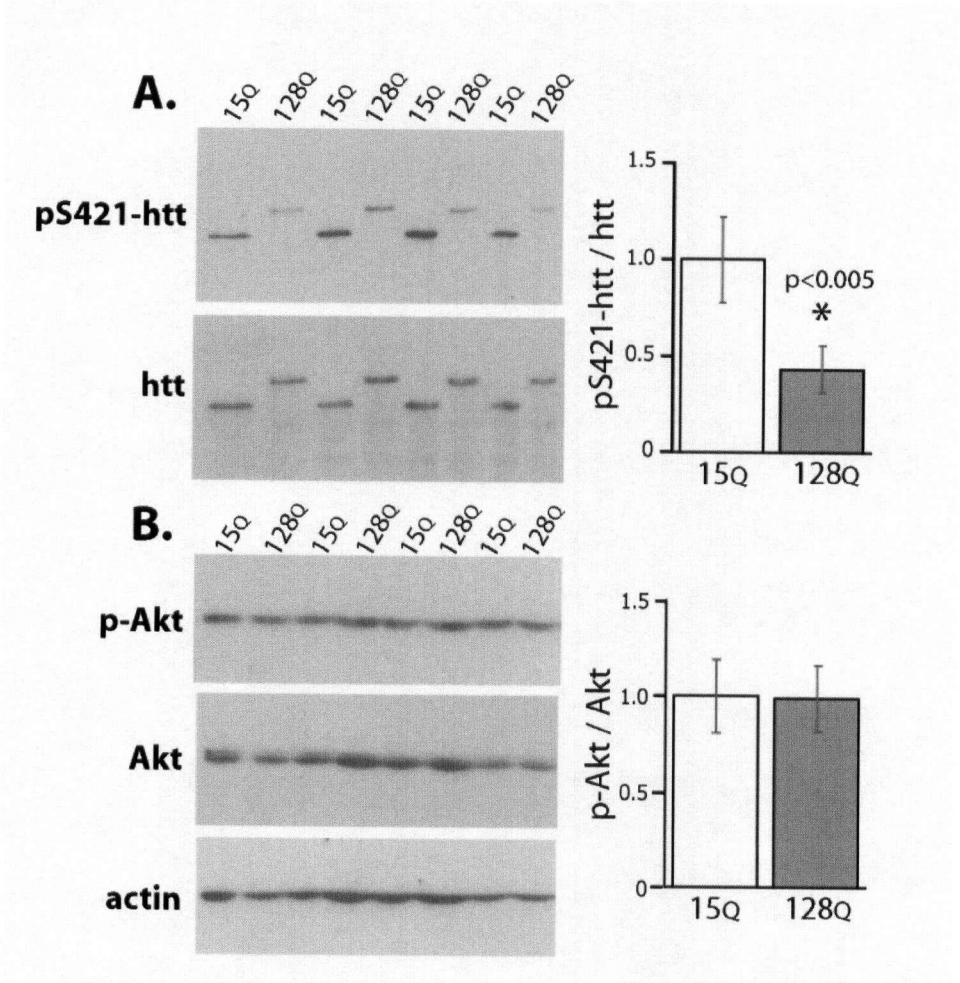

Figure 2.5: Phosphorylation of polyglutamine-expanded huntingtin (htt) is reduced *in-vitro*
(A) HEK293 cells overexpressing 1-1212aa huntingtin with 15Q or 128Q were immunoprobed with the pS421-huntingtin antibody against phospho-huntingtin and then with mAb2166 for total huntingtin. Densitometry was performed and the ratio of phosphorylated huntingtin/total huntingtin calculated and graphed. Phosphorylation of 128Q huntingtin was reduced by at least 50% relative to 15Q huntingtin in these cells. (N=4, t test $p<0.005$).
(B) In these same HEK293 cells expressing 15Q or 128Q, the ratio of activated Akt/ total Akt was not altered. Samples were first probed for pAkt and then reprobed for total pan-Akt and actin (N=4).

To assess whether the phosphorylation of expanded huntingtin was also reduced *in vivo*, we used whole brain lysates derived from 6-8 month old YAC transgenic mice expressing either 18Q or 128Q full length huntingtin. To avoid the confounding effects of the endogenous mouse huntingtin, human huntingtin expressed from the transgene was specifically immunoprecipitated using a human-specific huntingtin antibody (HD650). Phosphorylation of immunoprecipitated huntingtin was then detected using the pS421-huntingtin antibody. Consistent with the cell culture experiments, the phosphorylation of 128Q huntingtin was reduced compared to 18Q full-length huntingtin (Figure 2.6A). Our quantitative analysis of 9 mice per genotype demonstrated a 50% reduction of specific phosphorylation of 128Q compared to 18Q huntingtin (T-test $p=0.016$).

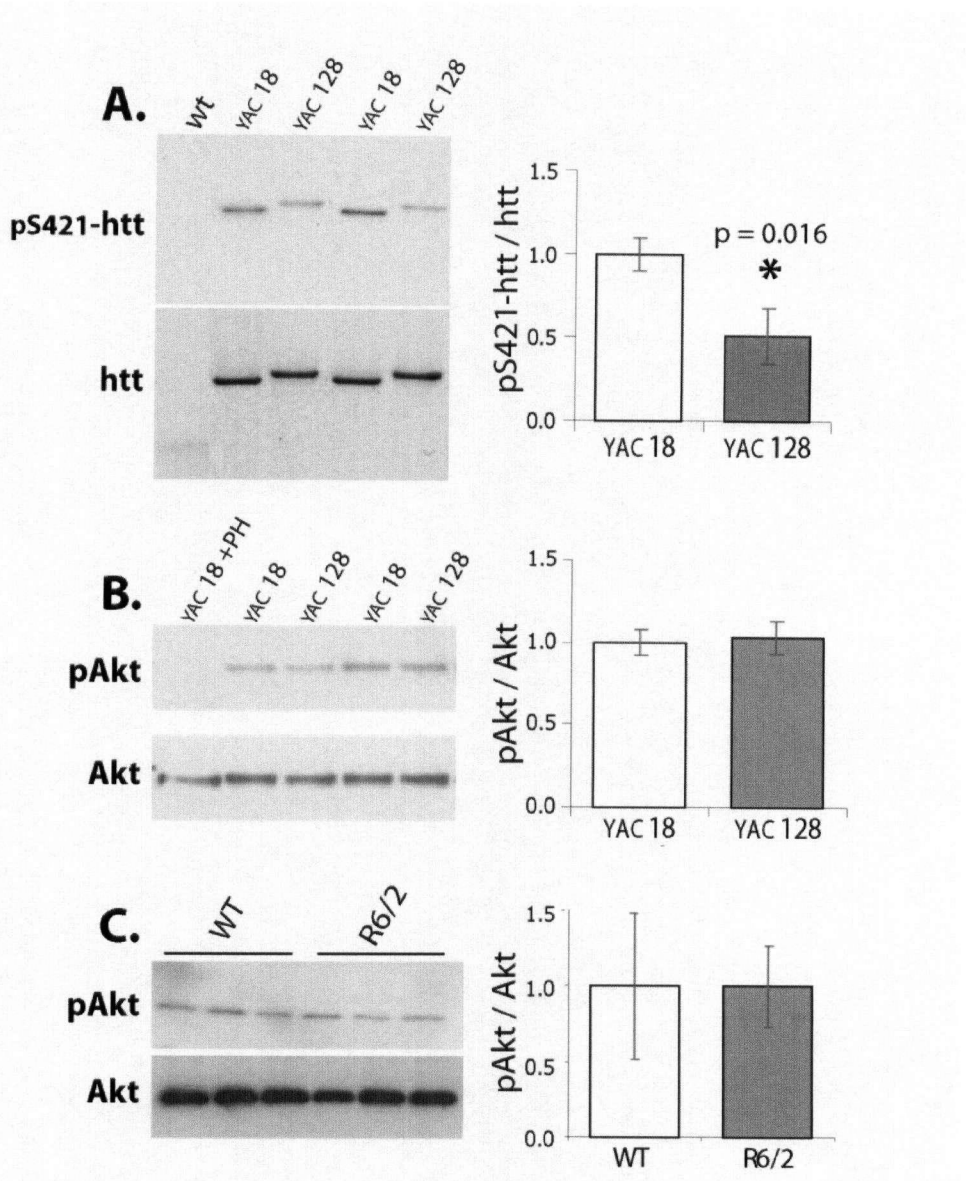

Figure 2.6: Phosphorylation of polyglutamine-expanded huntingtin (htt) is reduced *in-vivo* and not due to altered Akt activation

(A) Phosphorylation of expanded huntingtin is reduced by 50% compared to wildtype huntingtin in YAC transgenic mice. Lysates from whole brain of YAC18 vs YAC128 mice (N= 9 mice) were immunoprecipitated with the human specific HD650 antibody to separate the human transgene from the endogenous mouse huntingtin homolog. Lysates from wildtype mice were used to demonstrate the specificity of the human huntingtin antibody. (wt control, lane 1). The immunoprecipitates were separated on an SDS-polyacrylamide gel, probed with the pS421-huntingtin antibody and then reprobed with the antibody to total huntingtin. Similar to the cell culture experiments, the ratio of the phospho huntingtin to total huntingtin is significantly reduced in lysates from the YAC128 mice vs YAC18 mice (t-test, p=0.016).

(B) The activation of Akt does not differ between YAC18 and YAC128 mice. Lysate from YAC18 and 128 were probed for pAkt and then reprobed for total pan Akt. The lysate in the first lane (+PH) was pre-treated with Lamda phosphatase as a control to remove phosphorylation. Quantification of samples from 9 mice shows no differences in the Akt activation state between YAC18 and 128.

(C) Akt activation is not altered in R6/2 mice relative to wildtype (WT) littermates. Based on immunoblot analysis the ratio of pAkt/total pan Akt is similar in WT and R6/2 mice at 4 weeks of age (N=3).

2.3.6 Akt Activity Levels are Unaltered in the YAC Model of HD

We next wanted to determine whether differences in Akt activity might underlie the decreased phosphorylation of polyglutamine-expanded huntingtin. We analyzed the pAkt/Akt ratio in the same cell lysates and observed no significant differences between cells expressing either the 15Q or 128Q N-terminal huntingtin fragment (Figure 2.5B). As such, the reduced phosphorylation does not appear to be caused by a generalized decrease in Akt activity in cells expressing expanded huntingtin.

Consistent with the experiments in transfected cells, there was no difference in the Akt activation state of these YAC18Q and 128Q mice based on immunoblot analysis (Figure 2.6B). We further assessed the Akt activity in the R6/2 HD mouse model, which over-expresses the N-terminal fragment (exon1) of the huntingtin protein containing an expanded polyglutamine tract, but lacking the S421 site. In agreement with the results obtained from the YAC model, the pAkt/Akt ratio was unaltered in pre-symptomatic (4 week old – Figure 2.6C) and symptomatic (10 week old – data not shown) R6/2 mice.

2.4 DISCUSSION

In the present study, we have shown that the phosphorylation of huntingtin at S421 is present in brain and altered as a result of the HD mutation. Using a novel phospho (S421)-specific antibody, we find that a portion of huntingtin is phosphorylated under normal physiological conditions in mouse and human brain. This phosphorylation is very labile and huntingtin is rapidly dephosphorylated in brain tissue during a post-mortem interval. We find that some, but not all, of the cellular huntingtin in brain is phosphorylated at S421, indicating that this is a dynamic means of regulating the huntingtin protein.

The amount of phospho-S421 huntingtin varied between major regions of the wildtype mouse brain and negatively correlates with the pattern of degeneration seen in HD. The ratio of phospho-huntingtin to total huntingtin was least in the striatum, intermediate and variable in the cortex, and greatest in the cerebellum. In part, the regional differences in the phosphorylation state appear to be due to regional differences in the activation of Akt as it had a similar regional specificity. Phosphorylation of polyglutamine-expanded huntingtin has been shown to reduce its

toxicity (Humbert et al., 2002), indicating that the low level of striatal phosphorylation may predispose it to degeneration and may provide part of the answer to the long standing question as to why this disease has a regional neuropathology despite widespread expression of huntingtin in the CNS.

Phosphorylation by Akt is known to play an important role in the pathogenesis of another polyglutamine expansion disorder, spinocerebellar Ataxia type 1 (SCA1). SCA1 results from polyglutamine expansion in the ataxin-1 protein, causing upper motor neuron and cerebellar degeneration in humans. Phosphorylation of ataxin-1 by Akt is required in the pathogenesis of SCA-1 (Chen et al., 2003b) and preventing the phosphorylation of polyglutamine-expanded ataxin-1 reduces its toxicity in a mouse model of SCA1 (Emamian et al., 2003). The profound effect of Akt phosphorylation in ataxin-1, and the previous demonstration that genetic modifications that mimic phosphorylation of S421 of mutant huntingtin can prevent toxicity in striatal cell cultures (Humbert et al., 2002), provide a strong impetus for the further study of Akt phosphorylation in HD. Consistent with previous reports that a transfected Akt construct is capable of phosphorylating huntingtin in cell cultures (Humbert et al., 2002), we find that these two proteins stably interact in brain tissues, indicating that the regulation of huntingtin by Akt is a real and important phenomenon *in vivo*.

In addition to Akt, it has been shown recently that serum and glucocorticoid-induced kinase (SGK) is capable of phosphorylating huntingtin at S421 (Rangone et al., 2004). Transfection of SGK into cell cultures results in increased phosphorylation of huntingtin and protects against expanded-polyglutamine toxicity. Akt and SGK are both activated by PDK1, have homologous catalytic domains, share a similar substrate consensus sequence (Casamayor et al., 1999), and are believed to share many cellular functions (Rangone et al., 2004). SGK has been implicated in stress signalling following brain injury (Imaizumi et al., 1994) and cell volume changes (Leong et al., 2003) and is likely increased in HD patients as a response to degenerative processes already underway. The stress-induced regulation of SGK argues that it is a response to injury and not part of the pathogenesis of the disease. For this reason, we chose to focus on the Akt pathway and, more specifically, the phosphorylation of huntingtin itself.

There are many conflicting lines of indirect evidence to suggest that the phosphorylation of huntingtin at S421 may be altered in HD. Some data indicate that Akt is proteolytically

processed in grade 3 and 4 HD patient brain tissues (Humbert et al., 2002), presumably leading to its inactivation and decreased levels of phosphorylated huntingtin. Others have found increased expression of SGK in late stage HD patient brains (Rangone et al., 2004), suggesting increased huntingtin phosphorylation. Similarly, Akt activity is constitutively elevated in the Q111/Q111 murine knock-in model of HD (Gines et al., 2003). Considering that the increased phosphorylation of polyglutamine-expanded huntingtin reduces its toxicity (Humbert et al., 2002), we ultimately sought to determine whether expanded polyglutamines alter phospho-S421 directly.

We find that the S421 phosphorylation of polyglutamine-expanded huntingtin is reduced in cell culture and a mouse model of HD relative to non-expanded huntingtin. In both cells over-expressing expanded huntingtin and in the brains of 6 month old YAC mice expressing expanded human huntingtin, the ratio of phosphorylated huntingtin to total huntingtin was reduced by 50%. Unfortunately, because huntingtin is rapidly dephosphorylated during the PMI, a comparative examination of phosphorylation status in human brain tissue is not feasible at this time. However, the data from the YAC mice argues that the reduction in phosphorylation occurs early in the disease. At 6-8 months of age, YAC128 demonstrate behavioural dysfunction just prior to overt striatal degeneration (Slow et al., 2003). This significant and early reduction in phosphorylation may contribute to the neuronal dysfunction that precedes selective neuronal degeneration in HD.

Interestingly, decreased phosphorylation of mutant huntingtin was independent of changes in Akt activity as demonstrated using brain extracts of YAC HD mice and transfected cells. These data indicate that polyQ expansion in huntingtin interferes with its ability to be phosphorylated by Akt. However, we also find that the co-immunoprecipitation of huntingtin and Akt from YAC18 and YAC128 mice was similar, indicating that mutant huntingtin interacts with Akt generally to the same degree as wildtype huntingtin. Therefore, the inhibitory effect of polyQ expansion on huntingtin phosphorylation appears to involve aberrant protein conformation as opposed to altered kinase-substrate interaction.

The normal levels of Akt activity in the YAC128 HD mice are interesting in contrast to the Q111/Q111 HD knock-in mouse model, where there is increased Akt activity in primary neuron cultures and brain tissues from 2 to 18 months of age (Gines et al., 2003). We also examined the

R6/2 mouse, which overexpresses the exon 1 fragment of the HD gene (and therefore lacks the S421 site) containing expanded polyglutamines (Mangiarini et al., 1996). Similar to the YAC model, Akt levels in R6/2 were unaltered at 4 weeks (pre-symptomatic) and 10 weeks (symptomatic) of age compared to wildtype littermates. Although the knock-in model of HD is a genetically accurate model of HD (Lin et al., 2001), it does not result in overt striatal degeneration or decreased lifespan, and clearly has the least severe phenotype of these three mouse models. The increased Akt activity in the knock-in may protect against the degenerative processes (Gines et al., 2003) through the phosphorylation of the polyglutamine-expanded huntingtin.

Both the full-length YAC transgenic model, which exhibits specific degeneration of the striatum and an HD-like phenotype, and the truncated huntingtin R6/2 model, which has brain atrophy and a significantly decreased lifespan, lack elevated Akt activation and huntingtin phosphorylation and thus are susceptible to the effects of expanded huntingtin. Comparison of these mouse models suggests that increasing Akt activation and huntingtin phosphorylation may be an effective means of preventing striatal degeneration.

In the current study, we find that huntingtin phosphorylation is present *in vivo* and altered early in the pathogenesis of HD. Huntingtin is phosphorylated on S241 in mouse and human brain under normal physiological conditions. This phosphorylation could play an important regulatory role for the huntingtin protein both in its wildtype and polyglutamine-expanded forms. The endogenous pattern of huntingtin phosphorylation is lowest in the striatum relative to the cortex and cerebellum of wildtype mice and may predispose the striatum to degeneration.

Most importantly, the S421 phosphorylation is reduced in cell culture and brains of YAC transgenic mice when huntingtin is polyglutamine expanded and as such, the decreased phosphorylation would be predicted to increase the toxicity of mutant huntingtin in dysfunctional but metabolically active neurons. The decreased phosphorylation is seen early in the disease, prior to overt neuropathology in the YAC mice, and may therefore contribute to pathogenesis. Given that decreased phosphorylation of polyglutamine expanded huntingtin is linked to increased toxicity *in vitro*, restoring or increasing the phosphorylation of expanded huntingtin may be an attractive therapeutic strategy for HD.

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CHAPTER 3

Huntingtin phosphorylation at S421 reduces cleavage at aa586 by caspase-6

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Preface

I conceived, designed and analyzed the data for all of the experiments in this chapter. The manuscript and figures were prepared by me. I developed all of the techniques, reagents, and conducted all of the experiments with the exceptions noted below.

Technical work was performed with assistance from a technician hired with funds from a grant written by me (HD Society of Canada): Crystal Doty was trained by me specifically on the assays I developed and assisted in cell culture and immunoblotting. The neo-586 antibodies were developed by Rona Graham, Anita Kwok and Youzhou Zhang. Characterization of neo-586 was performed by Rona Graham and myself. Rona Graham and Roshni Singaraja provided intellectual and technical advice.

3.1 INTRODUCTION

Huntington disease (HD) results from polyglutamine-expansion in the N-terminal of the huntingtin protein (Borrell-Pages et al., 2006; Truant et al., 2006b). The disease is dominantly inherited and results in progressive and selective neurodegeneration that leads to motor, cognitive and psychiatric dysfunction. The first neurons to be affected in the progression of the disease are the medium sized GABA-ergic spiny neurons of the striatum (Vonsattel et al., 1985; Myers et al., 1988). HD is one of many polyglutamine-expansion disorders, each presenting a unique set of clinical and neuropathological features dictated by the protein context of the polyglutamine expansion (Zoghbi and Orr, 2000). In the case of HD, the selective pathology is not explained simply by the presence of polyglutamine-expanded huntingtin, as there is a poor correlation between the amount or distribution of huntingtin expression and vulnerable regions of the brain (Sharp et al., 1995; Fusco et al., 1999). This evidence suggests the neuropathological features of HD must be dictated by other properties of the huntingtin protein.

Huntingtin is cleaved by numerous proteases including caspases (Goldberg et al., 1996b; Wellington et al., 1998; Wellington et al., 2000; Hermel et al., 2004), calpains (Kim et al., 2001; Gafni and Ellerby, 2002; Bizat et al., 2003; Kim et al., 2003; Gafni et al., 2004) and several lyososomal endopeptidases (Lunkes et al., 2002; Kim et al., 2006). Caspase-cleavage of huntingtin occurs at very specific cleavage sites in the N-terminal region of huntingtin. The cleavage occurs immediately after amino acid (aa) 513 (caspase-3), 552 (caspase-2 and -3) and 586 (caspase-6) (Goldberg et al., 1996b; Wellington et al., 1998).

Cleavage of huntingtin at aa552 has been observed in normal brains, and HD brains before the onset of the disease, leading to the hypothesis that generation of these fragments is a normal physiological event (Kim et al., 2001; Wellington et al., 2002). In the case of polyglutamine-expanded huntingtin, these cleavage events produce fragments containing the HD mutation. The caspase-cleavage of huntingtin is a critical step in the disease pathogenesis as it has previously been demonstrated that blocking all caspase cleavage sites of huntingtin leads to protection against polyglutamine-expanded toxicity in cell culture (Wellington et al., 2000).

Further characterization of huntingtin fragments provides evidence to suggest that the pathogenesis of HD is dependent on the generation of very specific cleavage fragments.

YAC128 mice, which express full-length polyglutamine-expanded human huntingtin, generate multiple cleavage fragments of huntingtin and develop an HD-like cognitive dysfunction, motor deficits and selective striatal degeneration in an age, expression-level, and CAG-length dependent fashion (Slow et al., 2003; Van Raamsdonk et al., 2005a; Van Raamsdonk et al., 2005c; Graham et al., 2006b). Similarly, the caspase-3-resistant (C3R) YAC128 mouse develops pathology and behavioural deficits even though cleavage at aa552 and aa513 is prevented (Graham et al., 2006a). In sharp contrast, blocking cleavage at aa586 in the caspase-6-resistant (C6R) mice completely blocks the HD-like pathology seen in the YAC128 (Graham et al., 2006a). The specific cleavage of huntingtin at aa586, and not aa552 or aa513, appears to be a crucial and rate limiting step in the development of HD in a mouse model of the disease. It is not clear however, what factors regulate the generation of the 1-586aa fragment or how this fragment results in selective neurodegeneration.

There are numerous examples of phosphorylation regulating the proteolytic cleavage of its substrate (Tozser et al., 2003). Phosphorylation of the substrate may increase (Parker et al., 1984; Warrener and Petryshyn, 1991; Watanabe and Kobayashi, 1995; Vecchi et al., 1998; LaFevre-Bernt and Ellerby, 2003; Santhamma et al., 2004; Hornig and Uhlmann, 2004) or decrease cleavage (Fujimoto et al., 1995; Barkett et al., 1997; Cardone et al., 1998; Walter et al., 1999; Lim et al., 2000; Krippner-Heidenreich et al., 2001; Desagher et al., 2001; Nicolas et al., 2002; Hoon et al., 2003; Choi et al., 2003; Rametti et al., 2004; Fluhrer et al., 2004) and in some cases, substrates are selectively phosphorylated only after cleavage (Walter et al., 1998; Sebbagh et al., 2001).

Huntingtin is phosphorylated on serine-421 (S421) by the pro-survival signalling protein kinase Akt (PKB) and this has been shown to be protective against the toxicity of polyglutamine-expanded huntingtin in cell culture (Humbert et al., 2002). Previously, we have shown that huntingtin phosphorylation at S421 is present under normal physiological conditions, found at lower levels in susceptible regions of the brain, and is reduced in the presence of polyglutamine-expansion (Warby et al., 2005). The evidence suggests that phosphorylation of huntingtin may play a role in the disease. However, the molecular function of phosphorylation at S421 remains unknown.

Using mass spectrometry, several other phosphorylation sites in huntingtin have been identified (Schilling et al., 2006). One of these phosphorylation sites, S536, lies on a putative calpain cleavage site and when mutated (S536A), blocks cleavage at aa536 (Schilling et al.,

2006). Huntingtin is also phosphorylated on another residue, serine 434 (S434) by Cdk5 which reduces huntingtin cleavage at aa513 by caspase 3 (Luo et al., 2005). There is a clear precedent for phosphorylation modulating the proteolysis of huntingtin, but it is not clear what role it plays in the generation of the crucial 1-586aa fragment.

In order to investigate the influence of phosphorylation of huntingtin at S421 on the cleavage at aa586, we used phospho-huntingtin mutants and a cleavage-site specific huntingtin antibody to demonstrate that there is a relationship between phosphorylation at S421 and cleavage of huntingtin by proteases. Specifically, the cleavage at aa586 by caspase-6 is increased when S421 phosphorylation is blocked, and decreased when S421 phosphorylation is enhanced by over-expression of active Akt.

3.2 MATERIALS AND METHODS

3.2.1 Tissue Culture and DNA constructs

Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS + L-glutamine (Gibco). All lines were incubated at 37°C and 5% CO2 except for ST14A cells, which are derived from the rat primordial striatum at day E14 and proliferate at 33°C (Cattaneo and Conti, 1998; Ehrlich et al., 2001).

Transient cell transfections were performed in 293, COS , N2A and BOSC23 cells with Fugene 6 (Roche) following the manufacturer's standard instructions. Briefly, cells were transfected in 6 well (1 ug DNA + 3 uL Fugene reagent) or 10cm dishes (8ug DNA + 24uL Fugene reagent) and then harvested 18 hours later. The DNA constructs used for transient transfection were either 1-3144aa full length huntingtin (pci-10366nt-15Q or pci-10366nt-138Q) or 1-1212aa (pci-3949nt-15Q or pci-3949nt-138Q). Some of these constructs contained site directed mutations at the S421 phosphorylation site (see below). As well, when indicated, a DNA construct containing constitutively active Akt (myr-Akt, Upstate #21-151) was used to transfect cells and increase the S421 phosphorylation of huntingtin.

ST14A cells were infected with MSCV virus to stably express full length huntingtin. The MSCV viral vectors contained full length huntingtin (10366-15Q and 10366-138Q) with site directed mutations at S421 (see below) to modulate the phosphorylation status of huntingtin. The BOSC23 cell line was transiently transfected with the MSCV-htt vectors along with the

gag/pol and env cofactors to generate the viral supernatant used to infect ST14 cells following the manufacturer's instructions for MSCV vectors (Clontech #K1062-1). ST14 cells stably expression the MSCV virus containing huntingtin constructs were selected and maintained using puromycin (900 ug/ml).

3.2.2 Site Directed Mutagenesis for S421

Site directed mutagenesis was used to modulate the phosphorylation status of huntingtin at S421. The S421 phosphorylation mutants were generated by changing the serine (S) at aa421 to either an alanine (A) or aspartic acid (D). Huntingtin with a wildtype S421 is highly phosphorylated under normal physiological conditions in cultured cells and mouse brain (Warby et al., 2005). Changing S421 to an alanine (S-A) prevents phosphorylation at this site while changing to an aspartic acid (S-D), with a large acidic side-chain, mimics constitutive phosphorylation (pseudo-phosphorylation). These S421 substitutions were created using a site-directed mutagenesis approach as previously described (Wellington et al., 2000) using the following primers: S421A-F: GAAGCCGTAGTGGG**GCT**ATTGTGGAAC; S421A-R: GTTCCACAAT**AGC**CCCACTACGGCTTC; S421D-F: GAAGCCGTAGTGGG**GAT**ATTGTGGAAC; S421D-R: GTTCCACAAT**ATC**CCCACTACGGCTTC. Integrity of the constructs was confirmed by sequencing.

3.2.3 Cell Lysis and Immunoprecipitation

To harvest cultured cells, an SDS lysis procedure was used. Cells are released from the plates by trypsinization or scraping and washed once with ice cold PBS. One volume of 1% SDS/TEEN (50mM Tris pH 7.5, 1mM EDTA, 1mM EGTA, 150mM NaCl, 20% SDS, 1X Complete protease inhibitors (Roche), 1mM sodium orthovanadate, 0.8mM PMSF, 0.005mM zVAD) was added and left on ice for 5min. Four volumes of 1% TritonX-100/TEEN (50mM Tris pH 7.5, 1mM EDTA, 1mM EGTA, 150mM NaCl, 20% TritonX-100, 1X Complete protease inhibitors (Roche), 1mM sodium orthovanadate, 0.8mM PMSF, 0.005mM zVAD) was added and left on ice for 10 min. The sample was passed through a 22.5 gauge needle to shear the DNA and spun down at 14,000rpm, 4°C, 15min to pellet the debris. Bradford assay (BioRad) was used to determine the protein concentration of the supernatant.

Following cell lysis, immunopurification of huntingtin was performed when indicated to purify huntingtin. Sepharose-G beads were washed and pre-bound with the anti-huntingtin BKP1 monoclonal antibody (5ug) for 1-2 hours. Lysates (250ug to 2mg) were incubated with the beads for two hours, rotating at 4 °C and then washed three times with SDP+.

3.2.4 Cleavage Assays

To determine the effect of phosphorylation on proteolysis we examined cleavage of huntingtin in *ex vitro* (cell free conditions with purified reagents) and *in vitro* (in cultured cells) conditions. Instead of using the SDS lysis procedure described above samples for the cleavage assays were lysed in SDP+ lysis buffer (50mM Tris pH 8.0, 150mM NaCl, 1% Igepal (NP40), 40mM NaF, 1mM PMSF, 2mM sodium orthovanadate, and 1X protease inhibitors (Roche)). SDP+ was added to the plates and left on ice for 5 minutes. The cells were scraped, sonicated briefly (3 x 10s on ice) and centrifuged at 10,000 RCF for 15 min at 4°C. Protein concentration was assessed with the Bradford assay (BioRad).

E*x vitro* experiments were conducted by purification of huntingtin from cell lines and treatment with purified caspases. The advantage of these types of experiments with purified reagents is that the active protease is known, and it eliminates the influence of other (unknown) proteins. Phosphorylation mutants were purified from transfected COS, N2A, ST14, or 293 cells and proteolysis assessed by immunoblot analysis following treatment with purified recombinant caspases. Following immunoprecipitation, huntingtin was treated with proteases in caspase assay buffer (50mM Hepes pH 7.4, 100mM NaCl, 0.1% NaCl, 0.1% CHAPS, 1mM EDTA, 10% glycerol and 10mM DTT) for 1 hour at 37 degrees. Cleavage products were detected by immunoblotting with anti-huntingtin antibodies. The molecular weight of the cleavage products and differential detection with different anti-huntingtin antibodies was used to identify the cleavage bands.

In vitro experiments involved treatment of cells to induce cleavage of huntingtin described previously (Wellington et al., 1998). The advantage of these experiments is that cleavage occurs under physiological conditions in cultured cells. To induce apoptosis and huntingtin cleavage, N2A, COS, ST14 or HEK 293 cells were treated with 35uM tamoxifen for 1 h at 37 °C. Cell lysates were generated and cleavage products were detected by immunoblotting for huntingtin.

3.2.5 Immunoblotting

Immunoblots for huntingtin were conducted using mAb2166 (Chemicon 4C8), 1C2 (Chemicon 1574) and pS421 antibodies. The mAb2166 antibody epitope is aa443-457 of huntingtin (Cong et al., 2005), while 1C2 selectively detects polyglutamine-expanded huntingtin (Trottier et al., 1995b). The pS421 antibody specifically detects huntingtin only when phosphorylated on S421 (Warby et al., 2005). The anti-huntingtin Bkp1 monoclonal antibody (aa1-17) was used to immunoprecipitate huntingtin.

The neo586 polyclonal antibody was generated from the ^{583}IVLD^{586} epitope found at the caspase-6 cleavage site in huntingtin at aa586. The neo586 antibody specifically detects huntingtin only when the aa586 epitope (IVLD) is exposed following cleavage by caspase-6. The detailed characterization of the neo586 antibody is described elsewhere (Chapter 4).

Standard procedures were used for immunoblotting. Briefly, sample preparation involved denaturing the lysates or immunoprecipitates in LDS Sample Buffer (Invitrogen) or SDS gel loading buffer and heating for 10mins at 70°C. For huntingtin immunoblots, a 3-8% tris-acetate gel (Invitrogen) was run with tris acetate buffer (50mM Tricine, 50mM Tris base, 3.5mM SDS, pH 8.25) for 1 hour at 200V. Transfers were performed onto Immobilon-FL membranes at 30V for 1-2 hours using transfer buffer (25mM Bicine, 25mM BisTris, 1.025mM EDTA, 10% MeOH, pH 7.2). Blocking was performed with 5% milk or 5% BSA depending on the primary antibody.

All immunoblots used fluorescently labeled secondaries, Immobilon-FL membranes and the Licor Odyssey immunoblotting system. Reliable quantitation relies on clean, crisp separation of bands that are of moderate exposure. For statistical analysis of immunoblotting, groups were compared based on the numerical densitometry results from Licor Odyssey software (v2.0). A t-test was performed with 2 groups, one-way ANOVA performed for more than 2 groups. P values <0.05 were considered to be statistically significant.

3.3 RESULTS

3.3.1 Phosphorylation at S421 Reduces Stress-Induced Cleavage of Huntingtin

To determine the effect of huntingtin phosphorylation on its proteolysis, we first examined the stress-induced cleavage of huntingtin. Untreated ST14 cells stably expressing full length human huntingtin have low levels of endogenous huntingtin cleavage. However, cellular stress induced by tamoxifen treatment results in the cleavage of huntingtin in a time dependent manner (Figure 3.1). Multiple cleavage fragments are detected from both 15Q (Figure 3.1A, left) and 138Q (Figure 3.1B, left) full length huntingtin by immunoblotting following tamoxifen treatment.

To test the effect of huntingtin phosphorylation at S421 on cleavage, ST14 cells expressing full length polyglutamine-expanded huntingtin with different states of phosphorylation were stressed with tamoxifen. Huntingtin with a serine at aa421 (S421) is phosphorylated in cells under normal physiological conditions (Warby et al., 2005). An alanine (S421A) substitution at aa421 prevents phosphorylation at this site. A serine to aspartate (S421D) substitution however, replaces the polar CH3-OH side chain with an acidic side chain that mimics the structure of a constitutively phosphorylated serine.

Following tamoxifen treatment, more cleavage occurred of the non-phosphorylated (S-A) huntingtin than either the phosphorylated (S421) or pseudophosphorylated (S-D) huntingtin. The number and intensity of the cleavage bands detected by immunoblotting is increased when the phosphorylation at S421 is blocked by a point mutation.

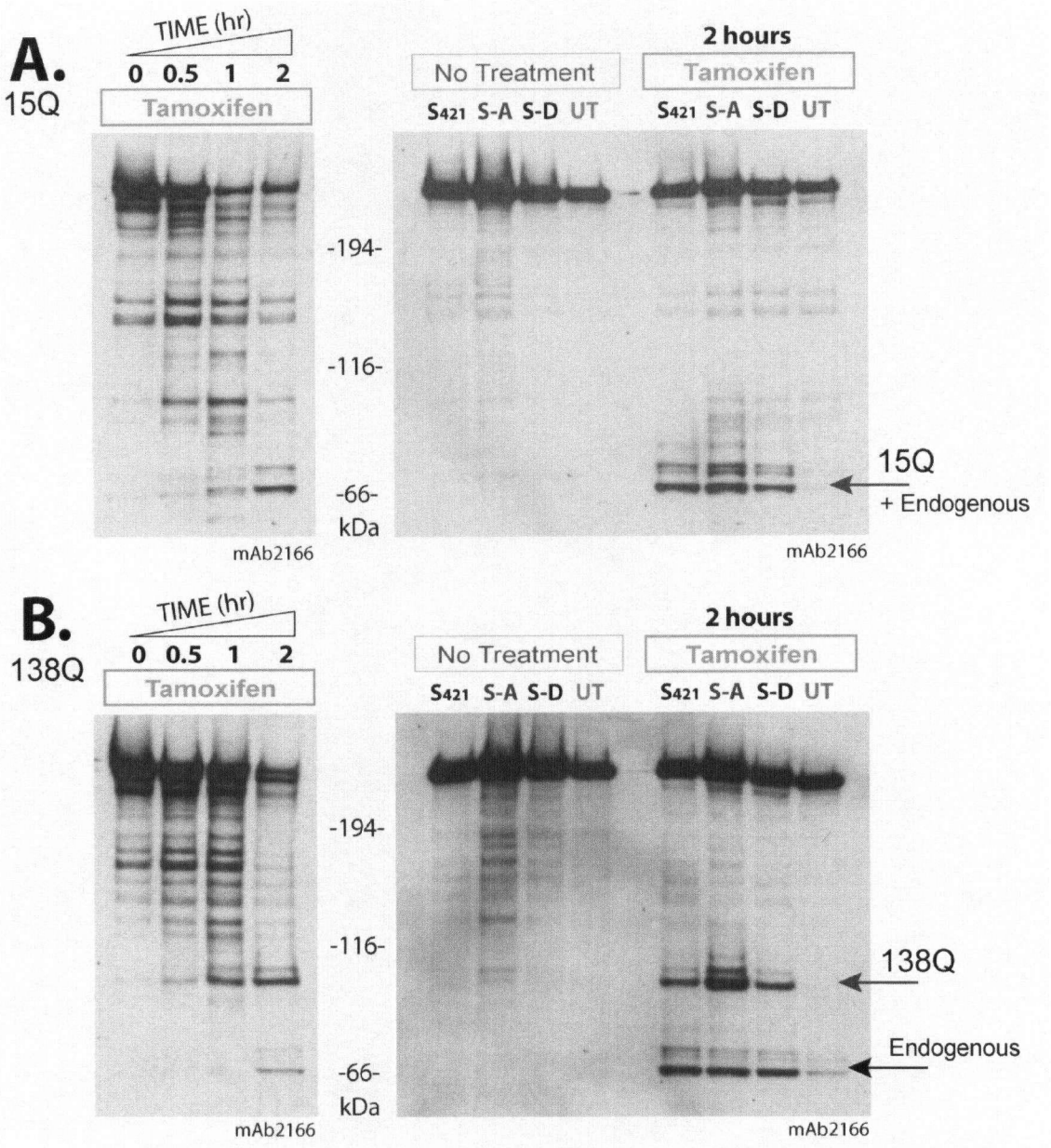

Figure 3.1: Blocking S421 phosphorylation increases the stress-induced cleavage of huntingtin.
Tamoxifen induces the cleavage of 15Q (A) and 138Q (B) full length huntingtin in ST14 cells in a time dependent manner (left A & B). 2 hours of 35uM Tamoxifen was sufficient to produce many small molecular-weight cleavage fragments that are detected by the 2166 antibody. Phosphorylation status of huntingtin alters the amount of huntingtin cleavage (right A & B). Following a 2 hour treatment of tamoxifen, phosphorylated (wildtype serine (S421) and pseudophosphorylated (SD) huntingtin is cleaved less than non-phosphorylated huntingtin (SA). As expected, the major cluster cleavage band (blue arrows) has a higher molecular weight in the 138Q relative to 15Q. (UT = untransfected control).

3.3.2 Phosphorylation at S421 Reduces Endogenous Cleavage of Huntingtin

To determine whether phosphorylation at S421 altered the endogenous cleavage of huntingtin, we used COS cells which have a very low level of endogenous huntingtin expression and when transfected with huntingtin, readily cleave huntingtin without stimulation. COS cells were

transfected with a DNA construct expressing 1-1212aa huntingtin fragment containing expanded polyglutamines (128Q) and the endogenously phosphorylated (S421) or non-phosphorylated (S-A) forms at S421. Consistent with the stress-induced cleavage in ST14 cells, the endogenous cleavage of huntingtin in COS cells is increased in the non-phosphorylated (S-A) relative to phosphorylated constructs (Figure 3.2). Quantification of immunoblots from 6 experiments reveals an average of 38% decrease in the intensity of the cleavage band from cells transfected with phosphorylated huntingtin. The exact cleavage sites and proteases involved in the generation of this endogenous ~70kDa cleavage fragment generated in the untreated COS cells is unknown, although the size of the fragment suggests that it is smaller than the aa586 fragment. It is possible that any aa586 fragments generated in these cells are further processed into smaller fragments.

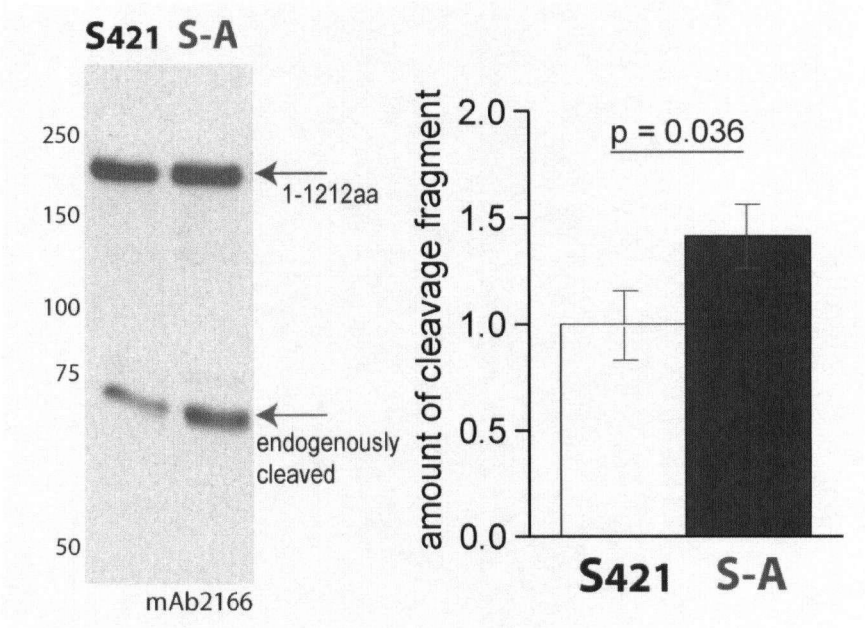

Figure 3.2: Blocking S421 phosphorylation increases the endogenous cleavage of huntingtin in COS cells.
COS cells were transfected with 1-1212aa huntingtin with a point mutation at S421 to block phosphorylation (S-A). In the absence of cell stress, transfected COS cells generate a ~70kDa cleavage fragment. The cleavage of unphosphorylated (S-A) is significantly greater (n=6, t-test p=0.036) than the cleavage of the endogenously phosphorylated construct (S421). Huntingtin was detected by immunoblotting with mAb2166.

3.3.3 Blocking S421 Phosphorylation increases Huntingtin Cleavage by Caspase-6

To determine the effect of phosphorylation at S421 specifically on the caspase-6 cleavage of huntingtin, *ex-vitro* experiments were conducted using purified huntingtin and caspase-6.

Phosphorylated (S421), or non-phosphorylated (S-A) huntingtin was immunopurified from transfected N2A cells and then cleaved with purified recombinant caspase-6 (Figure 3.3). Huntingtin with a serine at 421 (wildtype) is phosphorylated under normal physiological conditions in N2A cells (data not shown). Under these conditions, purified caspase-6 enzyme cleaved the non-phosphorylated (S-A) huntingtin twice as efficiently as S421 phosphorylated huntingtin (S421).

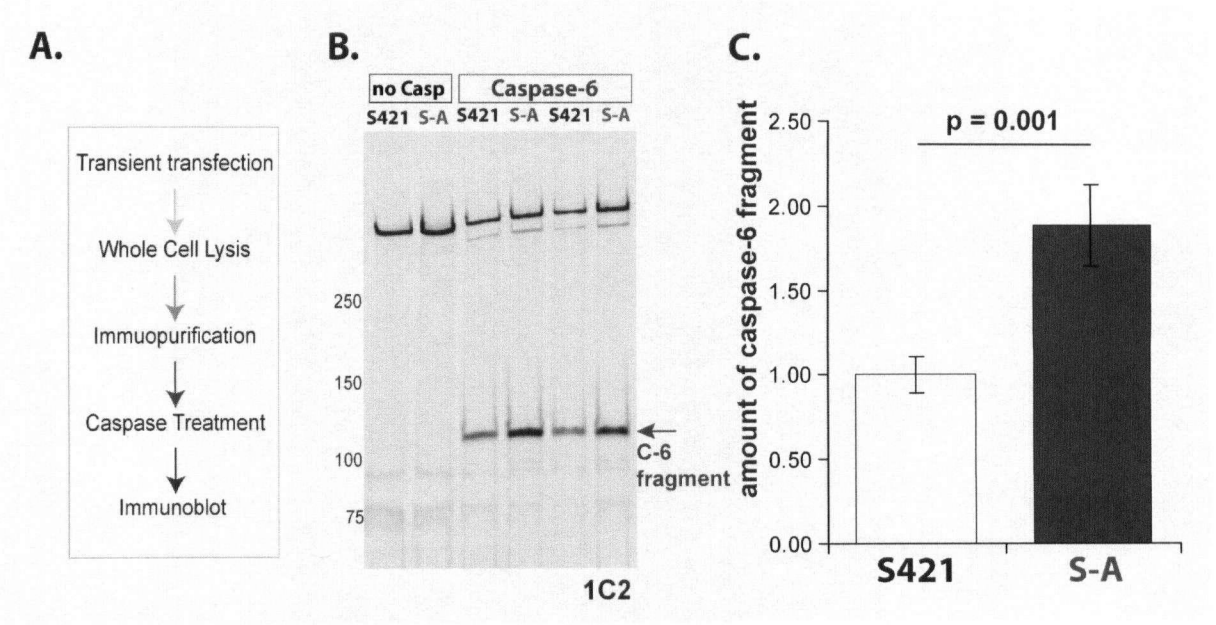

Figure 3.3: Blocking S421 phosphorylation increases the cleavage of huntingtin by caspase-6.
(A) Full length, polyglutamine-expanded huntingtin was expressed and immunopurified from N2A cells and then cleaved with purified caspase-6 *ex vitro* to test the ability of the enzyme to cleave phosphorylated (S421) and non-phosphorylated (S-A) huntingtin. **(B)** Immunoblot with 1C2 antibody specifically detects polyglutamine-expanded huntingtin and demonstrates that caspase-6 cleavage results in a ~115kDa fragment that is consistent with 1-586aa. **(C)** Quantification of experiments demonstrates that preventing phosphorylation at S421 (S-A) was sufficient to significantly increase cleavage of huntingtin derived from N2A cells by purified caspase-6 (n=4, t-test p = 0.001).

3.3.4 Increasing Phosphorylation at S421 Reduces Cleavage by Caspase-6

To increase the amount of S421 phosphorylation, COS cells were co-transfected with active Akt and huntingtin. To determine the effect on the caspase-6 cleavage, huntingtin was immunoprecipitated, treated with purified recombinant caspase-6, and immunoblotted for huntingtin and S421-phosphorylated huntingtin (Figure 3.4). The amount of caspase-6 cleavage fragment detected by immunoblotting with 1C2 was found to be inversely correlated with the amount of S421 detected by the pS421 specific antibody. Huntingtin S421 co-transfected with active Akt had the highest phosphorylation state, and generated the least amount of caspase-6 cleavage fragment.

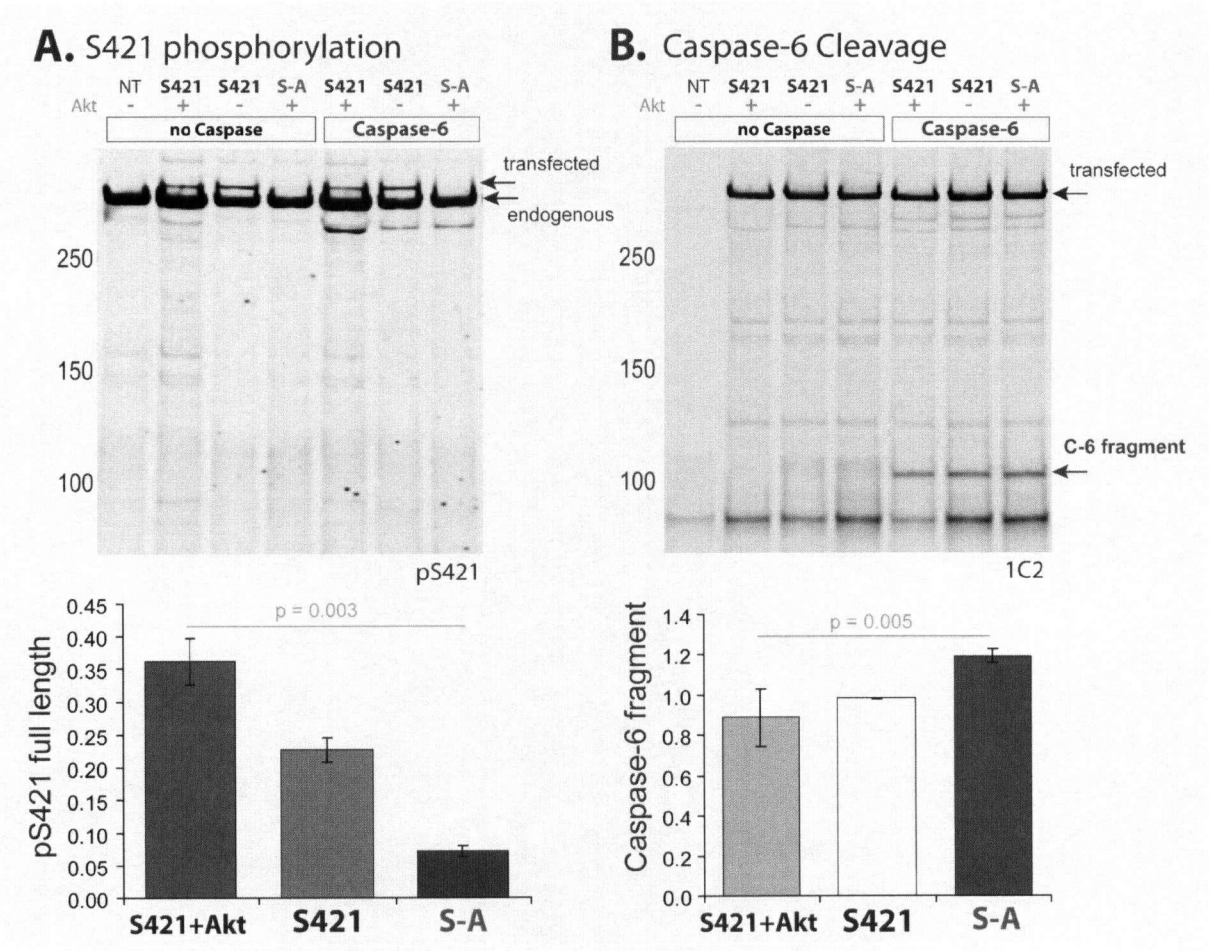

Figure 3.4: Increasing S421 phosphorylation decreases aa586 cleavage of huntingtin.
Full length, polyglutamine-expanded huntingtin was expressed and immunopurified from COS cells and then cleaved with purified caspase-6 *ex vitro* to test the ability of the enzyme to cleave non-phosphorylated (S-A), endogenously phosphorylated (S421), and S421 phosphorylation increased by the expression of active Akt (S421+Akt). **(A)** Control immunoblot with the pS421 (S421 phosphorylation specific) antibody demonstrating that transfected huntingtin (S421) is endogenously phosphorylated and significantly increased by the co-transfection of active Akt (n=2, anova p = 0.003). **(B)** Increasing the S421 phosphorylation (S421+Akt) significantly decreases the intensity of the cleavage band produced by caspase-6 treatment (n=4, anova p = 0.005).

3.3.5 S421 Phosphorylation reduces the generation of the 1-586aa fragment

To specifically determine if caspase-6 treatment was cleaving huntingtin at aa586, a neo586 antibody was generated against the caspase-6 recognition site in huntingtin at ^{583}IVLD^{586}. This polyclonal antibody does not detect huntingtin unless specifically cleaved by caspase-6 to reveal this IVLD epitope at aa586. Further characterization of this antibody is described elsewhere (Chapter 4). Full length huntingtin was immunoprecipitated from ST14 cells stably expressing pseudophosphorylated (S-D) or non-phosphorylated (S-A) huntingtin. The cleavage band

generated on the immunoblot for both neo586 and mAb2166 is the same, and of a molecular weight that is consistent with the 1-586aa fragment (Figure 3.5). For both antibodies, caspase-6 treatment generated more 1-586aa fragment from the S-A fragment than the S-D. This is again consistent with phosphorylation at 421 reducing the cleavage of huntingtin at aa586 by caspase-6.

Interestingly, further processing appears to have occurred of the 1-586aa fragment as smaller molecular weight bands are apparent with both neo586 and mAb2166. The pattern of processing is different between the S-A and S-D forms, suggesting that S421 phosphorylation may be important in modulating further processing of these fragments. It is not clear from these experiments however, what proteases may be responsible for this further processing, and warrants future investigation.

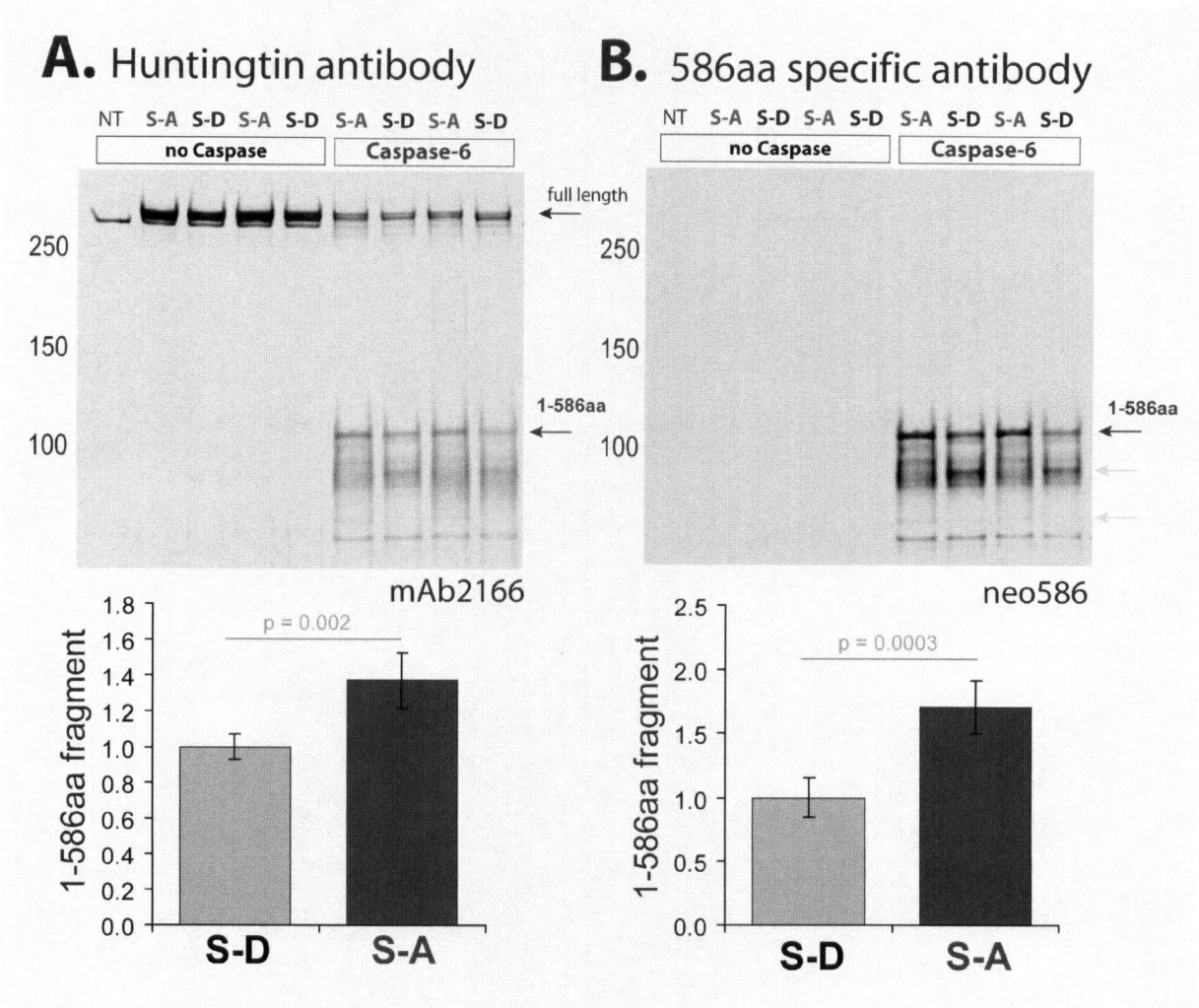

Figure 3.5: The neo586 antibody specifically detects huntingtin cleaved by caspase-6 and demonstrates that 1-586aa cleavage is increased when S421 phosphorylation is blocked.
Huntingtin was immunopurified and treated with caspase-6 from ST14 cells stably expressing non-phosphorylated (S-A) or pseudophosphorylated (S-D) huntingtin. **(A)** Caspase-6 treatment results in a cleavage fragment consistent in molecular weight with the 1-586aa fragment. Immunoblotting for anti-huntingtin mAb2166 demonstrates that 1-586aa cleavage is increased when S421 is prevented (S-A) (n=4, t-test, p = 0.002). **(B)** The neo-586 antibody, raised against the aa586 epitope in huntingtin (IVLD), specifically detects huntingtin after caspase-6 treatment. The generation of 1-586aa is significantly enhanced (n=4, t-test, p = 0.0003) when S421 phosphorylation is blocked (S-A). Both antibodies suggest that further processing of the 1-586aa fragment appears to be possible and differentially affected by S421 phosphorylation status (yellow arrows).

3.4 DISCUSSION

The relationship between huntingtin cleavage fragments and pathology is dependent on the generation of specific cleavage events or pathways. While some HD mouse models expressing truncated huntingtin have pathological changes (Mangiarini et al., 1996; Schilling et al., 1999), others do not (Slow et al., 2005). Furthermore, studies of the caspase-resistant YAC mice suggest that cleavage at the aa586 site (rather than cleavage at 513 or 552) is crucial in the pathogenesis of the disease. Characterization of the C6R mice, in which the cleavage at aa586 is blocked, resulted in three important findings: i) pathological features of the disease are prevented, ii) nuclear translocation of huntingtin is delayed, and iii) neurons are protected from excitotoxicity and staurosporine-induced neuronal death (Graham et al., 2006a). For this reason, we wanted to understand what factors may regulate huntingtin cleavage specifically at aa586.

Our results show that stress-induced and endogenous cleavage of huntingtin, including the specific cleavage at aa586 by caspase-6, are reduced by phosphorylation at S421. Blocking S421 phosphorylation of huntingtin by a point mutation (S-A) increases the cleavage, while increasing S421 phosphorylation by expression of active Akt, reduces the cleavage at aa586. We hypothesize that conformational change in the structure of huntingtin due to S421 phosphorylation may limit the binding of caspase-6 to huntingtin or access to the IVLD cleavage site at aa586.

Huntingtin is a large protein (~350kDa) and although its structure is not entirely understood, huntingtin is composed almost entirely of HEAT repeats which are predicted to form "barrel" or "solenoid" type structures (Andrade and Bork, 1995; Takano and Gusella, 2002; Li et al., 2006). A HEAT repeat is a single helix-turn-helix, and multiple HEAT repeats are interspersed by flexible coiled domains and stacked to form a HEAT domain with a solenoid structure with a hydrophobic core. There are three major clusters of HEAT repeats, forming three major HEAT domains in huntingtin, which are separated by intervening sequence of 300-500aa. Both the caspase cleavage sites and the S421 phosphorylation residue fall in the intervening sequence between HEAT domain 1 and 2. It is possible that S421 phosphorylation alters the orientation of HEAT domain 1 and 2 and allows/restricts access to the neighboring caspase cleavage sites in

the intervening sequence. Although our data suggests that S421 phosphorylation does not block cleavage completely, it may act in concert with other phosphorylation sites such at S434 (Luo et al., 2005) or other modifications in this intervening sequence to achieve this effect.

The modulation of specific cleavage events is important in other neurodegenerative diseases such as Alzheimer disease (AD). Amyloid precursor protein (APP) is cleaved by beta secretase and the gamma secretase complex to produce the amyloid beta peptide (Aβ) that can vary in length between 39-42 amino acids. Alteration in the cleavage by the gamma secretase complex at amino acid position 40 to 42 is important in the pathogenesis of AD, as Aβ42 exists as an abnormally folded fibrillar form that comprises the amyloid plaques found in the brains of AD patients (Hardy and Selkoe, 2002; Goedert and Spillantini, 2006).

In addition to reducing huntingtin cleavage at aa586, the stress-induced and endogenous cleavage data suggests that cleavage at other sites is also modified by S421 phosphorylation. We speculate that huntingtin is processed in specific proteolytic pathways as part of its normal function. There are multiple caspase, calpain and aspartyl protease sites in huntingtin and it is not clear in what order cleavage occurs and what effect specific cleavage events may have on the subcellular localization or function of specific fragments. Previous studies have suggested that huntingtin cleavage products are selectively associated with the nucleus, although the function of this selective localization is not understood (Kegel et al., 2002; Sawa et al., 2005c). It is clear, however, that cleavage at aa586 is a requirement for HD pathogenesis to occur in the YAC model of HD (Graham et al., 2006a). Further studies are needed to understand cleavage pathways and the role that posttranslational modifications, such as S421 phosphorylation, may serve to modify this sequence of cleavage events.

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CHAPTER 4

Huntingtin 1-586aa fragment is localized in the nucleus and altered by huntingtin phosphorylation at S421

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Preface

The experiments in this chapter were conceived and designed by me. I analyzed the data, prepared the figures and wrote the manuscript. I developed all of the techniques, reagents, and conducted all of the experiments with the exceptions noted below.

Preparation of samples for immunofluorescence and immunoblotting was assisted by Crystal Doty who was hired and trained by me with funds from a grant written by me (HD Society of Canada). Cheryl Wellington, in collaboration with Merck Frosst, developed the neo-513 and neo-552 antibodies which were characterized by Cheryl Wellington and myself (Wellington et al., 2002). The neo-586 antibodies were developed by Rona Graham, Anita Kwok and Youzhou Zhang. Characterization of neo-586 was performed by Rona Graham and myself. Rona Graham has provided considerable intellectual input regarding caspase-6 biology and C6R mice. Roshni Singaraja and Jeff Carroll also provided intellectual and technical advice.

4.1 INTRODUCTION

Huntington disease (HD) is an inherited neurodegenerative disorder that results from polyglutamine-expansion in the N-terminal of the huntingtin protein (Borrell-Pages et al., 2006; Truant et al., 2006b). YAC128 mice, which express full-length polyglutamine-expanded human huntingtin, generate multiple cleavage fragments of huntingtin and develop an HD-like cognitive dysfunction, motor deficits and selective striatal degeneration in an age, expression-level, and CAG-length dependent fashion (Slow et al., 2003; Van Raamsdonk et al., 2005a; Van Raamsdonk et al., 2005c; Graham et al., 2006b). Studies in mice which express polyglutamine-expanded huntingtin that are resistant to cleavage by caspases has suggested that specific cleavage events are critical in the disease pathogenesis. The caspase-3-resistant (C3R) YAC128 mouse develops pathology and behavioural deficits similar to the YAC128 even though cleavage at aa552 and aa513 is prevented (Graham et al., 2006a). However, in stark contrast, blocking cleavage at aa586 in the caspase-6-resistant (C6R) mice completely blocks the HD-like pathology seen in the YAC128 (Graham et al., 2006a). It is not clear what factors confer toxicity to the aa586 cleavage event relative to cleavage at other caspase sites.

Huntingtin is phosphorylated on serine-421 (S421) by the pro-survival signalling protein kinase Akt (PKB) (Humbert et al., 2002; Warby et al., 2005). S421 phosphorylation has been shown to both reduce the cleavage of huntingtin at aa586 (Chapter 3) and protect against the toxicity of polyglutamine-expanded huntingtin fragments in cell culture (Humbert et al., 2002).

Several lines of evidence have suggested that the nuclear localization of huntingtin is important in the pathogenesis of HD (Ross, 1997; Saudou et al., 1998; Peters et al., 1999; Schilling et al., 2004; Benn et al., 2005). Nuclear accumulation of mutant huntingtin is seen in human brain (DiFiglia et al., 1997; Sapp et al., 1997; Becher et al., 1998; Gourfinkel-An et al., 1998; Martin-Aparicio et al., 2002), cell culture (Hackam et al., 1998; Martindale et al., 1998; Cooper et al., 1998) and mouse models of HD (Hodgson et al., 1999; Schilling et al., 1999; Davies et al., 1999; Wheeler et al., 2000; Slow et al., 2003). Furthermore, the nuclear localization of huntingtin occurs earliest and to the greatest extent in the striatum relative to other brain regions in the YAC128 mouse model of the disease (Van Raamsdonk et al., 2005a) while the C6R mice, which

are protected from pathology, have delayed nuclear translocation of huntingtin by EM48 staining (Graham et al., 2006a). However, data thus far suggests that toxicity in HD depends on the translocation of *specific* soluble fragments of huntingtin into the nucleus, rather than the presence of nuclear inclusions, as the size and number of nuclear inclusions does not correlate with cytotoxicity in cell models (Saudou et al., 1998; Arrasate et al., 2004) or the shortstop mouse model of HD, where extensive inclusions are present but do not cause neurodegeneration (Slow et al., 2005). Full length and fragments of huntingtin are found in the nucleus, and huntingtin is believed to shuttle in and out of the nucleus (Tao and Tartakoff, 2001; Cornett et al., 2005; Bae et al., 2006). Interestingly, studies from lymphoblasts (Sawa et al., 2005b) and fibroblasts (Kegel et al., 2002) have suggested that huntingtin cleavage products are selectively associated with the nucleus.

The different huntingtin cleavage fragments may have specific 'cellular itineraries' which could influence their pathogenicity. In order to examine the subcellular localization of the huntingtin 1-586aa fragment generated by caspase-6 cleavage, we have generated a neo-epitope antibody that specifically detects huntingtin only when cleaved at aa586 (neo586). Over-expression of a 1-586aa fragment in cultured cells results in a diffuse cytoplasmic staining with neo586. Surprisingly, we find that the 1-586aa fragment generated endogenously by cleavage in the cell is highly enriched in the nucleus. Caspase-6 is also found to be enriched in the nucleus or perinuclear region, and may be the subcellular site of huntingtin cleavage aa586. Furthermore, we wanted to determine if huntingtin phosphorylation at S421 may impact the nuclear localization of huntingtin. We find that blocking S421 phosphorylation increases the nuclear localization of both full length and 1-586aa huntingtin. Regulation of this nuclear translocation may be an important mechanism to regulate this caspase-6 cleavage of huntingtin at aa586.

4.2 MATERIALS AND METHODS

4.2.1 Tissue Culture and DNA constructs

Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS + L-glutamine (Gibco). All lines were incubated at 37°C and 5% CO2 except for ST14A cells, which are derived from the rat primordial striatum at day E14 and proliferate at 33°C (Cattaneo and Conti, 1998; Ehrlich et al., 2001). Cultured primary striatal neurons were dissected from 6-

10 newborn FVB pups (P0-1) and cultured for 9-10 days (Zeron et al., 2002) before fixing and staining the cells for immunofluorescence. Mouse brain samples from Figure 4.7 were from 3 month old FVB wildtype mice. Whole brain samples were lysed in a dounce homogenizer following the nuclear/cytoplasm separation protocol outlined below.

Transient cell transfections were performed in COS or 293 cells with Fugene 6 (Roche) following the manufacturer's standard instructions. Briefly, cells were transfected in 6 well (1 ug DNA + 3 uL Fugene reagent) or 10cm dishes (8ug DNA + 24uL Fugene reagent) and then harvested 18 hours later. The DNA constructs used for transient transfection were either 1-3144aa full length huntingtin (pci-10366nt-15Q or pci-10366nt-138Q), 1-1212aa (pci-3949nt-15Q or pci-3949nt-138Q) or caspase fragments (pci-1-513aa, pci-1-552aa, pci-1-586aa). Some of these constructs contained site directed mutations at the S421 phosphorylation site or the caspase cleavage domain (see below).

4.2.2 Site Directed Mutagenesis for S421 and aa586 Cleavage Mutants

Site directed mutagenesis was used to modulate the phosphorylation status of huntingtin at S421. The S421 phosphorylation mutants were generated by changing the serine (S) at aa421 to either an alanine (A) or aspartic acid (D). Huntingtin with a wildtype S421 is highly phosphorylated under normal physiological conditions in cultured cells and mouse brain (Warby et al., 2005). Changing S421 to an alanine (S-A) prevents phosphorylation at this site while changing to an aspartic acid (S-D), with a large acidic side-chain, mimics constitutive phosphorylation (pseudo-phosphorylation). These S421 substitutions were created using a site-directed mutagenesis approach as previously described (Wellington et al., 2000) using the following primers: S421A-F: GAAGCCGTAGTGGG**GCT**ATTGTGGAAC; S421A-R: GTTCCACAAT**AGC**CCCACTACGGCTTC; S421D-F: GAAGCCGTAGTGGG**GAT**ATTGTGGAAC; S421D-R: GTTCCACAAT**ATC**CCCACTACGGCTTC. Integrity of the constructs was confirmed by sequencing.

DNA Constructs (1-1212aa = 1-3949nt) containing site directed mutations at both the S421 phosphorylation site and caspase cleavage domain were generated to specifically examine the role of phosphorylation on S421 on cleavage at aa586. Due to low amounts of 1-586aa fragment generated and/or low sensitivity of the neo586 antibody, it is difficult to detect endogenously

generated 1-586aa fragment. To enhance the ability to detect the 1-586aa fragment, other cleavage sites in the caspase cleavage domain were blocked by site directed mutagenesis to prevent further processing of the 1-586aa fragment. The 4C construct can only be cleaved at aa586, as four other caspase cleavage sites (aa513, aa530, aa552, and aa589) are blocked by changing the P1 aspartate at each cleavage site to an alanine, as described previously (Wellington et al., 2000). The 5C construct is a control construct in which all caspase sites are blocked (aa513, aa530, aa552, aa586, and aa589) and therefore cannot be cleaved at aa586.

4.2.3 Immunofluorescence Microscopy

Immunofluorescence microscopy was performed in cultured cells grown on coverslips using standard procedures. Briefly, cells were fixed by MeOH fixation (2 mins with ice-cold MeOH) followed by permeabilization in PBS containing 0.3% Triton X-100 for 30 min. Cells were washed, and then blocked in 4% normal goat serum (Gibco) in PBS for 1h at room temperature, followed by incubation with the primary antibody in 2% normal goat serum in PBS for 2h at room temperature or overnight at 4°C. Cells were washed 3x with 1% BSA in PBS, and then incubated in the secondary antibody (Goat anti Mouse Alexa 594 (red): 1/800 or Goat anti Rabbit Alexa 488 (green): 1/800) in 2% normal goat serum in PBS for 2h at room temperature. Cells were washed again 3x in PBS before mounting; DAPI staining (1:10,000 in PBS from 10mg/mL stock in 70% ethanol, SIGMA) was included in the first wash (room temperature, 5min). Fluoromount-G (Southern Biotech Cat. #0100-01) was used to mount coverslips on slides. Immunofluorescence was detected using a laser confocal microscope (BioRad) or conventional immunofluorescence microscopy (Zeiss) with a CCD camera (Princeton Instruments Inc.). Images were captured using Metamorph software and stored as separate JPG or TIFF files for each channel.

4.2.4 Nuclear / Cytoplasmic Separation

A basic subcellular fractionation method was used to separate cellular lysates into a supernatant and pellet that roughly correspond to cytoplasmic and nuclear compartments of the cell. The technique used has been described elsewhere (Clabough and Zeitlin, 2006). Briefly, cultured cells were harvested by trypsinization and washed once with ice cold PBS. Cells were resuspended in cell lysis buffer (5mM PIPES pH 8.0, 85mM KCl, 0.5% NP40, 1X Roche complete protease inhibitor, 1mM sodium orthovanadate, 800uM PMSF and 5μM zVAD) and left on ice for 30mins. The pellet containing the crude nuclear compartment is isolated by

centrifugation (4min, 8200g, 4°C) and the supernatant retained as the cytoplasmic compartment. The nuclear pellet was washed with PBS and then lysed in nuclear lysis buffer (10mM EDTA, 50mM Tris-HCl pH8, 1% SDS, 1X Roche complete protease inhibitor, 1mM sodium orthovanadate, 800uM PMSF and 5μM zVAD) for 30mins on ice. An equal volume of 1% TritonX-100 in TEEN buffer (50mM Tris pH 7.5, 1mM EDTA, 1mM EGTA, 150mM NaCl, 20% TritonX-100, 1X Complete protease inhibitors (Roche), 1mM sodium orthovanadate, 0.8mM PMSF, 0.005mM zVAD) was added, mixed by vortexing and left on ice for an additional 5mins. The nuclear lysate was then sonicated to shear DNA and protein concentration quantitated by the Bradford Assay (BioRad).

4.2.5 Immunoblotting

Immunoblots for huntingtin were conducted using mAb2166 (Chemicon 4C8), 1C2 (Chemicon 1574) and pS421 antibodies. The mAb2166 antibody epitope is aa443-457 of huntingtin (Cong et al., 2005), while 1C2 selectively detects polyglutamine-expanded huntingtin (Trottier et al., 1995b). The pS421 antibody specifically detects huntingtin only when phosphorylated on S421 (Warby et al., 2005). The anti-huntingtin Bkp1 monoclonal antibody (aa1-17) was used to immunoprecipitate huntingtin.

The neo586 polyclonal antibody was generated from the ^{583}IVLD^{586} epitope found at the caspase-6 cleavage site in huntingtin at aa586. The neo586 antibody specifically detects huntingtin only when the aa586 epitope (IVLD) is exposed following cleavage by caspase-6. The detailed characterization of the neo586 antibody is described elsewhere (Chapter 4). Characterization of the neo552 and neo513 antibodies, which specifically detect huntingtin when the aa552 and aa513 epitopes are exposed by cleavage, has also been described in detail previously (Wellington et al., 2002).

Standard procedures were used for immunoblotting. Briefly, sample preparation involved denaturing the lysates or immunoprecipitates in LDS Sample Buffer (Invitrogen) or SDS gel loading buffer and heating for 10mins at 70°C. For huntingtin immunoblots, a 3-8% tris-acetate gel (Invitrogen) was run with tris acetate buffer (50mM Tricine, 50mM Tris base, 3.5mM SDS, pH 8.25) for 1 hour at 200V. Transfers were performed onto Immobilon-FL membranes at 30V for 1-2 hours using transfer buffer (25mM Bicine, 25mM BisTris, 1.025mM EDTA, 10% MeOH, pH 7.2). Blocking was performed with 5% milk or 5% BSA depending on the primary antibody.

All immunoblots used fluorescently labeled secondaries, Immobilon-FL membranes and the Licor Odyssey immunoblotting system. Reliable quantitation relies on clean, crisp separation of bands that are of moderate exposure. For statistical analysis of immunoblotting, groups were compared based on the numerical densitometry results from Licor Odyssey software (v2.0). A t-test was performed with 2 groups, one-way ANOVA performed for more than 2 groups. P values <0.05 were considered to be statistically significant.

4.3 RESULTS

4.3.1 Characterization of the neo586-IVLD Antibody

In order to further investigate the generation and localization of the huntingtin aa586 fragment, a 586-IVLD-epitope specific antibody (neo-586) was generated (Figure 4.1A). IVLD is the amino-acid epitope that is exposed when huntingtin is cleaved specifically at aa586. The IVLD cleavage site is unique to huntingtin, as no other IVLD substrates for caspase-6 are known (Table 4.1). To demonstrate that the neo-586 antibody only detects huntingtin when cleaved at aa586, COS cells were transfected with DNA constructs expressing huntingtin fragments of various lengths. The neo-586 antibody robustly detects the transfected aa586 fragment, but not the aa552 or aa513 fragments (Figure 4.1B). Accordingly, the neo-552 and neo-513 antibodies, which are generated against epitopes that are exposed by huntingtin cleavage at aa552 and aa513, also specifically and robustly detect their corresponding fragments but not any other huntingtin fragment (Figure 4.1B). The specificity of the neo-552 and neo-513 have been further characterized previously (Wellington et al., 2002; Sawa et al., 2005a). Over-expression of each of the huntingtin caspase fragments (1-513aa, 1-552aa, 1-586aa) resulted in a general, diffusely cytoplasmic pattern of subcellular localization by immunofluorescence, irrespective of the fragment size (Figure 4.1B and C).

A.

Antibody	*Epitope*	*In vitro cleavage*
neo-586	**IVLD**	Caspase-6
neo-552	CSDPAM**DLND**	Caspase-2/3
neo-513	CHTLQA**DSVD**	Caspase-3

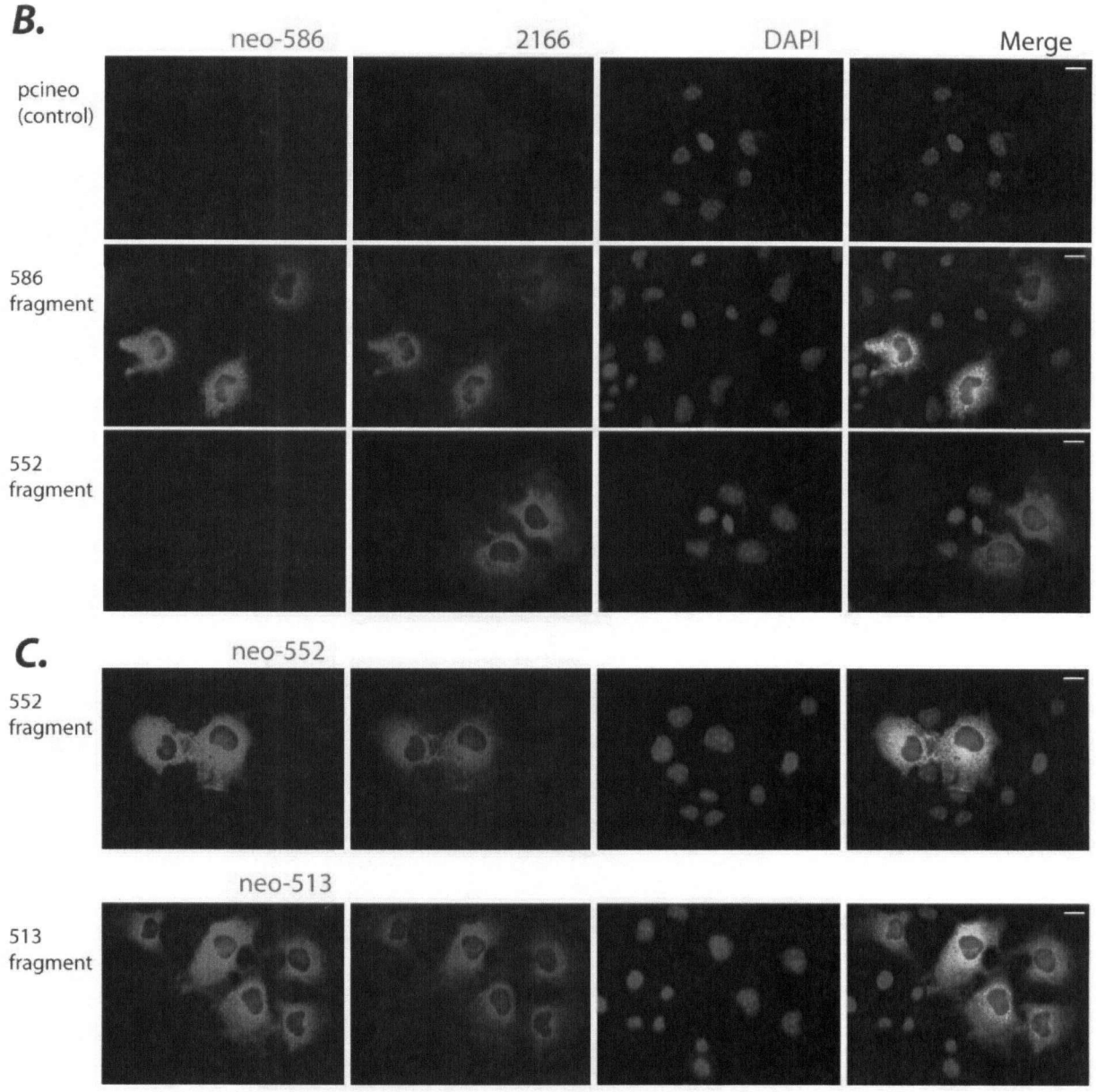

Figure 4.1: The neo-586 antibody specifically detects the huntingtin 1-586aa fragment
(A) Description of the three cleavage sites and neo-epitope antibodies used in this study. The caspase recognition sequence is indicated in bold. **(B)** COS cells, which have a low level of endogenous huntingtin, were transfected with aa552 or aa586 fragment. The neo-586 antibody only detects huntingtin with the specific IVLD epitope at aa586 and not other fragments of huntingtin. Nuclei of cells are indicated by DAPI stain. **(C)** Huntingtin552 neo-epitope antibody specifically detects only the aa552 fragment and huntingtin513 neo-epitope antibody only detects aa513 fragments, as described elsewhere (Wellington et al., 2002). 63x magnification, scale bar is 20uM.

Table 4.1: Caspase-6 substrates and epitopes
The caspase-6 cleavage recognition epitope for each substrate is listed, when known. Huntingtin is the only known caspase-6 substrate with an IVLD recognition sequence. The substrates listed below with 'unknown' epitopes do not contain IVLD in there protein coding sequence. *Data courtesy R.Graham.*

CASPASE-6 SUBSTRATE	EPITOPE	REFERENCE
Amyloid Precursor Protein (APP)	VEVD	(Pellegrini et al., 1999; Galvan et al., 2006)
AP-2α	DRHD	(Nyormoi et al., 2001)
Caspase-3	IETD	(Srinivasula et al., 1996; Thornberry et al., 1997; Xanthoudakis et al., 1999; Van de et al., 1999; Allsopp et al., 2000)
Caspase-6	TETD	(Van de et al., 1999)
	TEVD	(Srinivasula et al., 1996; Talanian et al., 1997)
Caspase-8	VETD	(Thornberry et al., 1997)
	VEVD	(Cowling and Downward, 2002)
CREB binding protein (CBP)	unknown	(Rouaux et al., 2003)
Desmin	VEMD	(Chen et al., 2003a)
Desmoplakin	unknown	(Aho, 2004)
Emerin	unknown	(Columbaro et al., 2001)
NOS3 (eNOS)	unknown	(Tesauro et al., 2006)
Focal Adhesion Kinase (FAK)	VSWD	(Gervais et al., 1998)
Guanylate Cyclase 1 alpha	unknown	(Payne et al., 2003)
Huntingtin	**IVLD**	(Wellington et al., 2000; Graham et al., 2006a)
Keratin 14	unknown	(Aho, 2004)
Keratin 15	VEMD	(Badock et al., 2001)
Keratin 17	VEMD	(Badock et al., 2001)
Keratin 18	VEVD	(Caulin et al., 1997; Aho, 2004)
Lamin A/C	VEID	(Takahashi et al., 1996; Srinivasula et al., 1996; Orth et al., 1996a; Ruchaud et al., 2002)
Notch1	EEED	(Cohen et al., 2005)
	ANRD	
	DITDHMD	
	CLLD	
NFkβ	VFTD	(Levkau et al., 1999)
Nuclear mitotic apparatus protein (NuMA)	unknown	(Hirata et al., 1998)
PARP	unknown	(Orth et al., 1996b; Miyashita et al., 1998)
Periplakin	TVAD	(Aho, 2004; Kalinin et al., 2005)
Plectin	unknown	(Aho, 2004)
SATB1	VEMD	(Galande et al., 2001)
Tau	VMED	(Horowitz et al., 2004)
	VSED	(Guo et al., 2004)
Topoismoerase I	PEDD	(Samejima et al., 1999)
	EEED	
Vimentin	IDVD	(Byun et al., 2001)
5' Lipoxygenase	IQFD	(Werz et al., 2005)

4.3.2 **Detection of Endogenously Generated Fragments cleaved at aa586**

We next used the neo-epitope specific antibodies to examine the subcellular localization of endogenously generated huntingtin fragments by immunofluorescence. In contrast to the over-expressed huntingtin caspase fragments, the endogenously generated fragments each have a markedly different localization (Figure 4.2). In untreated ST14 cells, which have a moderate level of endogenous huntingtin expression, neo-586 antibody staining is found almost exclusively in the nuclear compartment. In contrast, neo-552 staining is found at the perinuclear region and to a lesser degree, diffusely in the cytoplasm.

Previous studies have demonstrated that huntingtin is associated with microtubules (Hoffner et al., 2002) and to determine whether the perinuclear staining of neo-552 was associated with the centrosome, we co-stained for gamma-tubulin. Neo-552 staining was observed around, but not overlapping with gamma tubulin (Figure 4.3), suggesting the 552 fragment is not directly associated with the centrosome. In contrast to the unique staining patterns of neo-586 and neo-552, no detectable staining is observed with neo-513. The lack of staining with the neo-513 antibody could be due to either a low abundance of endogenously generated 1-513aa fragments or lower sensitivity of the neo-513 antibody. The neo-513 readily detects a transfected 1-513aa fragment, suggesting that the endogenous aa513 fragment is less abundant. Taken together, these data suggest that endogenously generated aa586 fragments are specifically identified in the nucleus, while aa552 fragments condense in the perinuclear region.

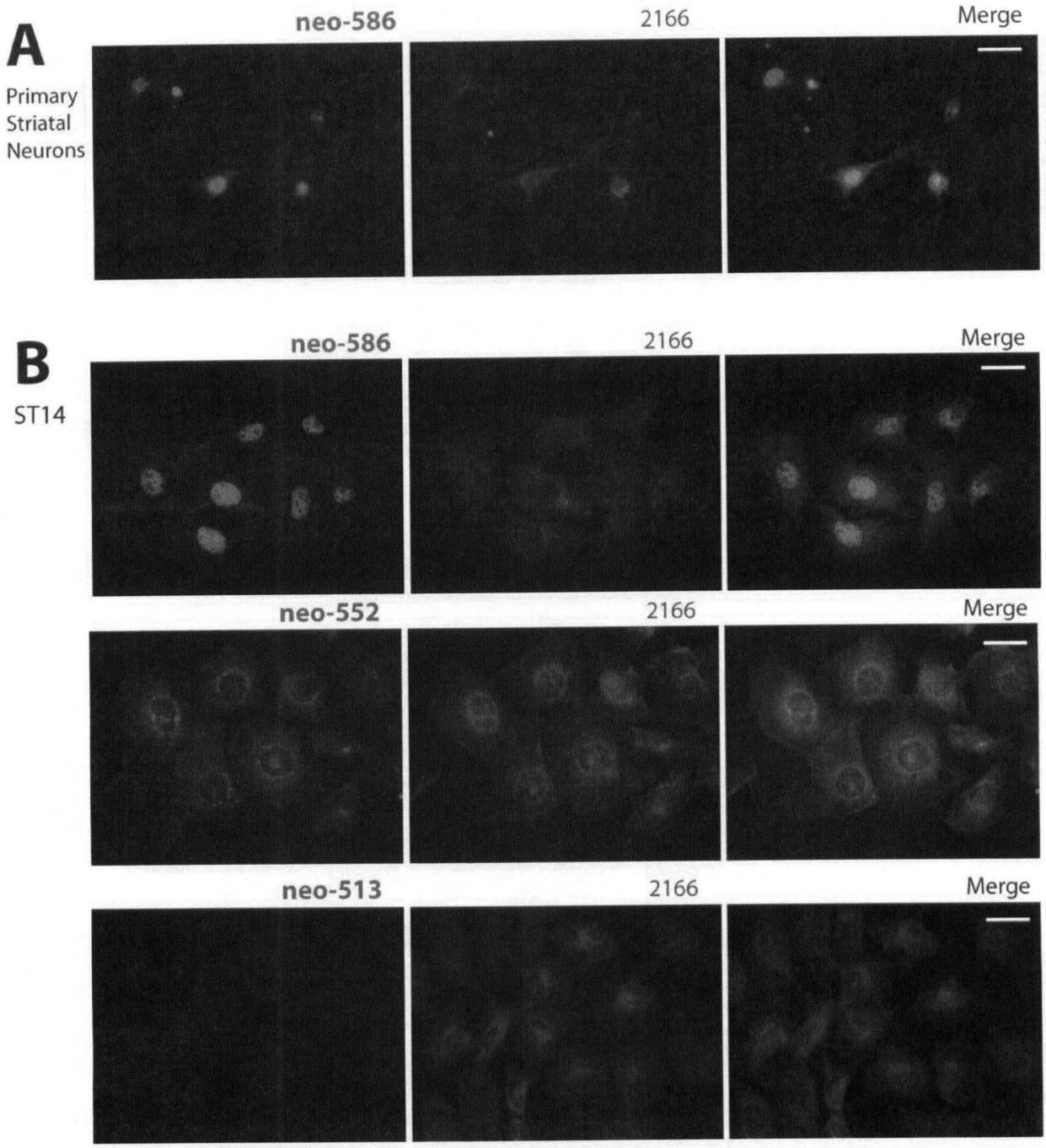

Figure 4.2: Endogenous neo-586 staining is nuclear
Neo-586 staining is concentrated in the nucleus of untreated and untransfected primary striatal neurons **(A)** and ST14 cells **(B)**. In contrast, neo-552 staining is perinuclear and cytoplasmic. Comparatively, neo-513 shows a low level endogenous staining. These endogenously generated fragments have distinctly different patterns of subcellular localization from each other, suggesting that they are either trafficked differently or generated in different sites within the cell. 100X magnification, scale bar is 20uM.

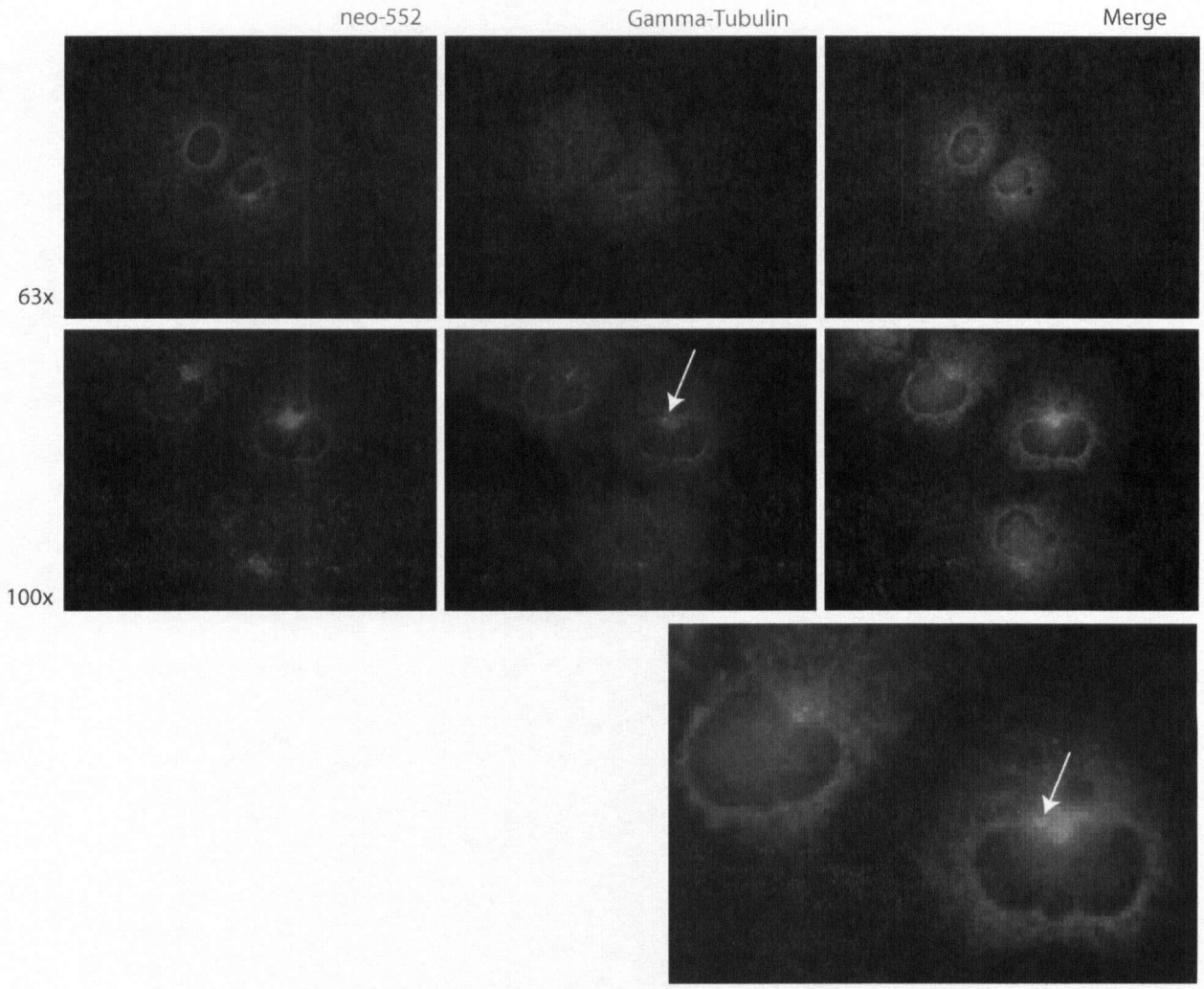

Figure 4.3: Lack of co-localization between gamma-tubulin and huntingtin 1-552aa fragment.
The neo-552 antibody (green) detects the 1-552aa fragment around the nucleus but does not co-localize with the gamma-tubulin (red), indicating that, unlike caspase-6 (see Figure 4.5) it is not directly located at the MTOC/centrosome (white arrow). DAPI is blue in the merged image. Bottom panel is an enlargement of the merged 100x image to show the MTOC is distinctly red and does not overlap with the green neo552 staining.

4.3.3 Endogenous Caspase-6 Staining

Given the extensive localization of neo-586 staining in the nucleus, we wished to determine if caspase-6 was located in close proximity to the nucleus. Punctate staining for the caspase-6 (p20) antibody, which detects both the pro- and active forms of caspase-6, was found both in the nucleus and cytoplasm of untreated ST14 cells (Figure 4.4A). Staining for the active caspase-6 (p10) antibody, which only detects the active caspase, was punctate and nuclear. Interestingly, the active caspase-6 staining was only seen in a very small subset of untreated

cells, but when seen, was very distinct. The staining for both caspase-6 and active caspase-6 was the same in primary striatal neurons (Figure 4.4B) as ST14 cells (Figure 4.4A).

Treating ST14 cells with a kinase-inhibitor (staurosporine) resulted in an increase in aa586 fragment generation, as indicated by increased nuclear neo-586 staining (Figure 4.5A). Similarly, there is an increase in caspase-6 staining in these treated cells (Figure 4.5B). In addition, staining with the caspase-6 antibody suggests that staurosporine treatment induces caspase-6 to translocate from the cytoplasm to the perinucleus and nucleus. Notably, a focus of perinuclear caspase-6 staining was observed in treated cells. To determine whether these distinct foci of staining were associated with the MTOC, we co-stained for gamma-tubulin (Figure 4.6). The perinuclear localization of caspase-6 distinctly co-localizes with gamma-tubulin, suggesting increased caspase-6 is trafficking to the centrosome following staurosporine treatment.

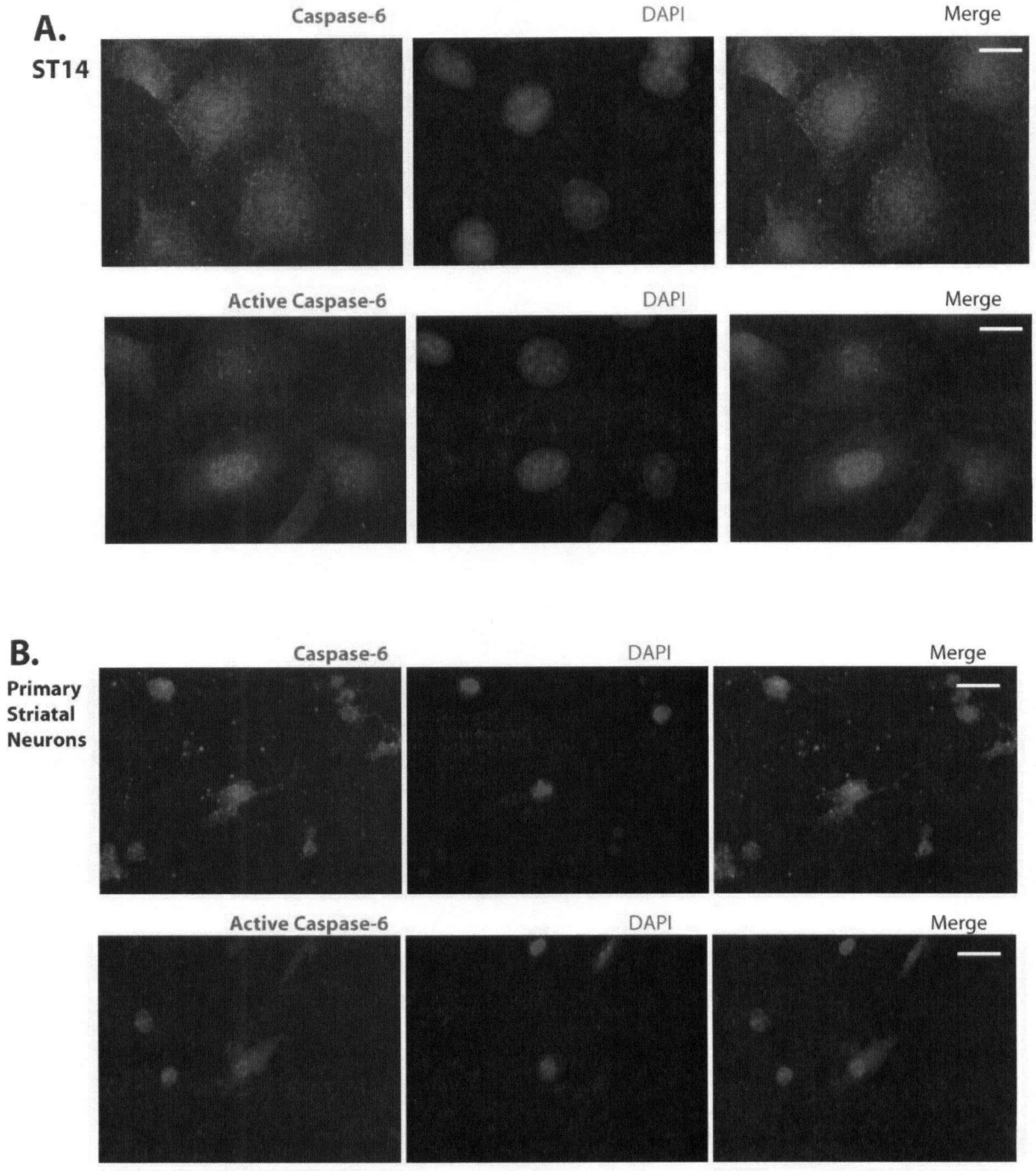

Figure 4.4: Endogenous caspase-6 staining is cytoplasmic and concentrated in the nucleus
In untreated ST14 cells **(A)**, total (pro-form and active forms) caspase-6 (sc-15381) staining is punctate in the nucleus and cytoplasm. The active caspase-6 fragment (sc-9761) however, is exclusively nuclear. Only a small subset of untreated ST14 cells show staining for active caspase-6, the remainder have little or no staining. A similar staining pattern is seen in primary striatal neurons (B). 100X magnification, scale bar is 20uM.

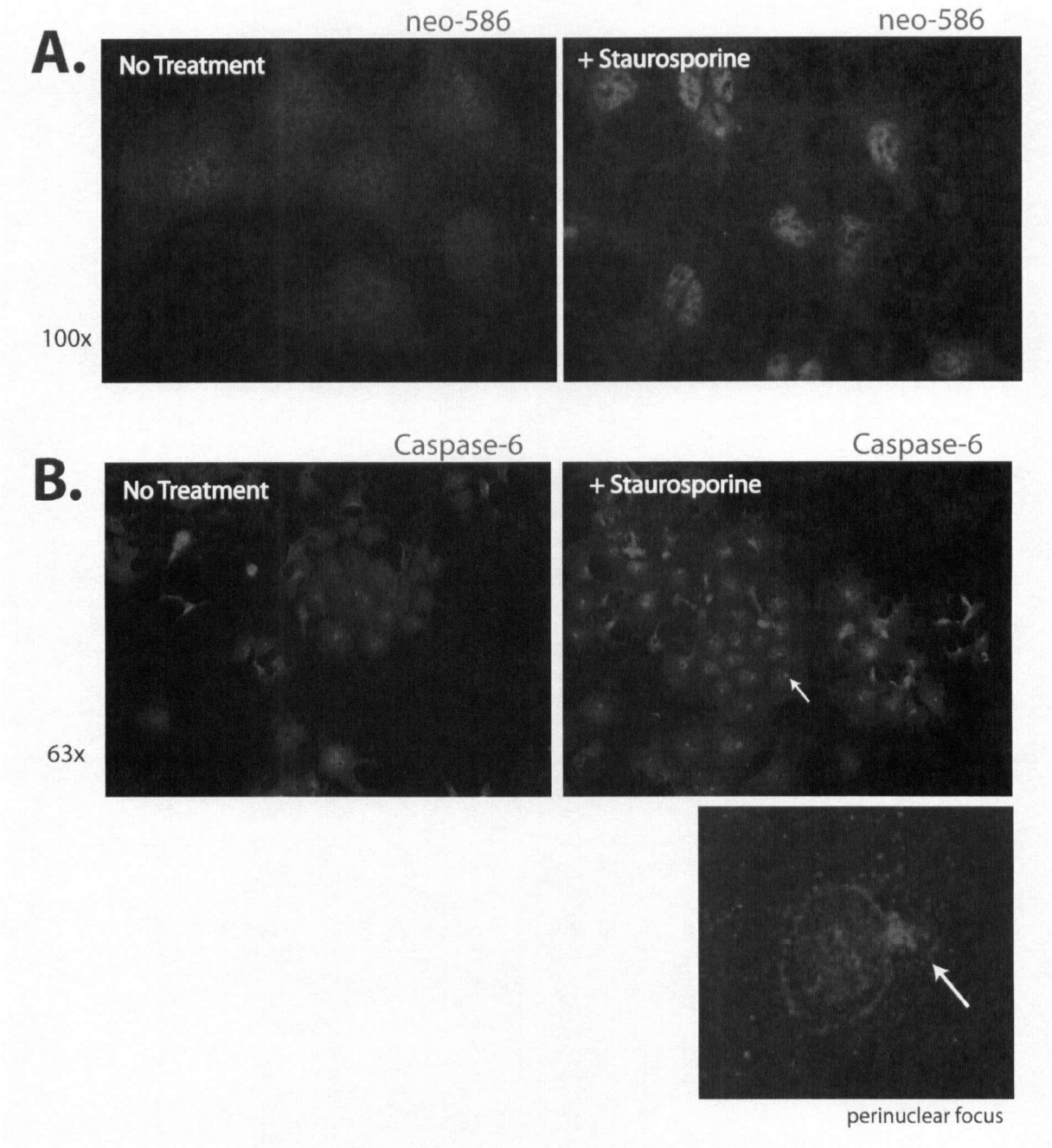

Figure 4.5: Kinase-inhibition increases neo-586 and caspase-6 staining
(A) Staurosporine treatment increases the intensity of nuclear neo-586 staining in ST14 cells. Representative pictures from cells +/- staurosporine treatment taken at the same exposure setting. **(B)** Caspase-6 staining is increased in the nucleus and the perinuclear region following staurosporine treatment. Specifically, a perinuclear focus of staining is apparent (white arrow in the enlarged image).

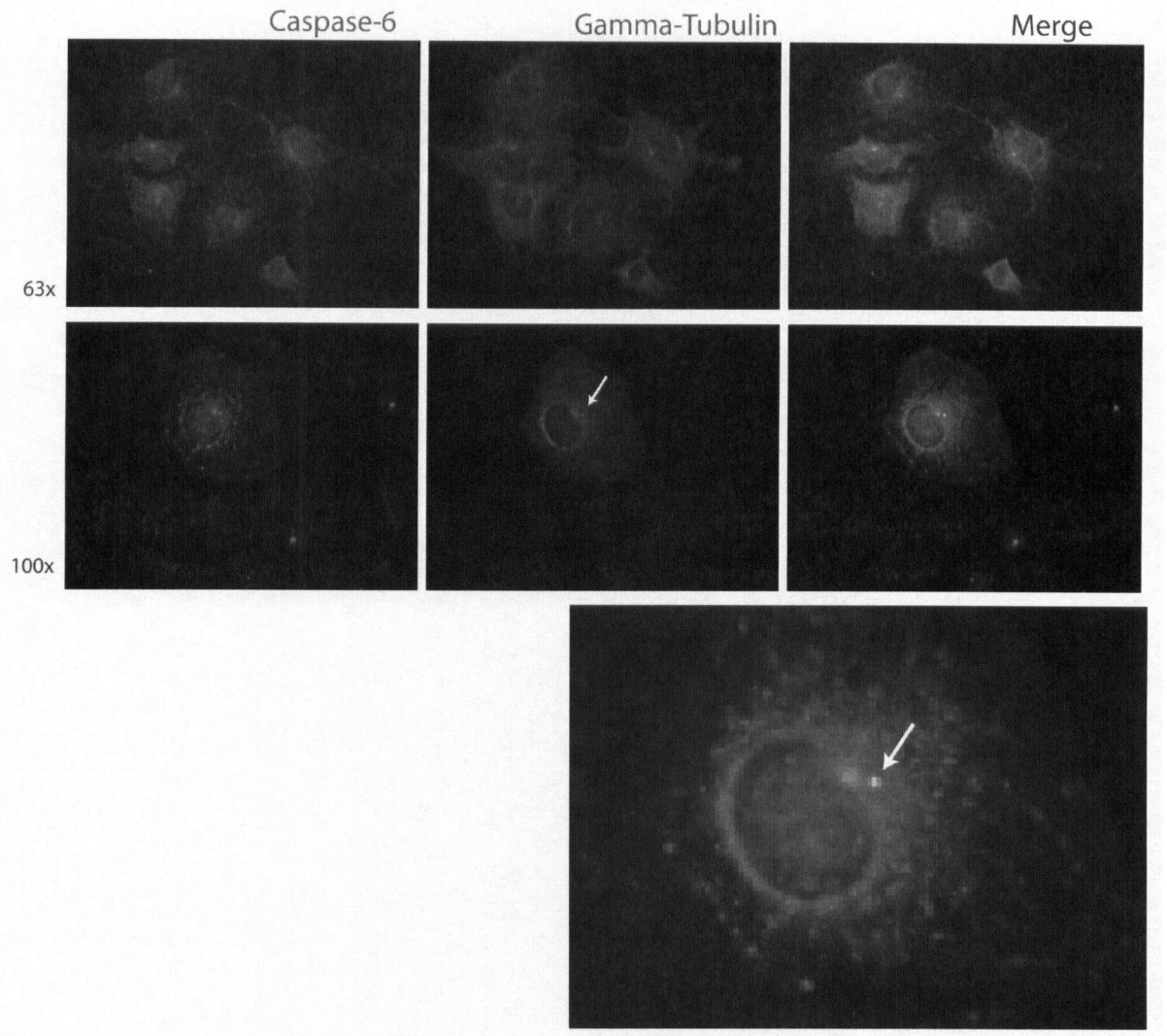

Figure 4.6: Caspase-6 staining at the MTOC/centrosome.
The focussed caspase-6 co-localization with gamma-tubulin staining increases following staurosporine treatment in ST14 cells. Staining for total caspase-6 (pro-forms and active) is green. Gamma-tubulin (red) is a marker for the microtubule organizing center (MTOC) at the centrosome (white arrow). This co-localization suggests that activated caspase-6 is translocated to the centrosome following activation. DAPI is blue in the merged image. Bottom panel is an enlargement of the merged 100x image to show the MTOC is distinctly yellow as a result of overlap with green caspase-6 staining.

4.3.4 Nuclear Localization of Huntingtin

Numerous studies have suggested that a portion of cellular huntingtin is found in the nucleus (De Rooij et al., 1996; Sapp et al., 1997; Hackam et al., 1998; Dorsman et al., 1999; Tao and Tartakoff, 2001; Kegel et al., 2002; Martin-Aparicio et al., 2002; Xia et al., 2003; Cornett et al., 2005; Sawa et al., 2005d; Bae et al., 2006; Jeong et al., 2006). To determine whether there is a relationship between the nuclear localization of huntingtin and the phosphorylation status at S421, we isolated huntingtin from the nuclear and cytoplasmic compartments and then calculated

the amount of S421 phosphorylation as a ratio of the total huntingtin by immunoblots. Whole brain lysates from untreated 3 month old wildtype mice were separated into nuclear and cytoplasmic extracts and immunoprobed with pS421-specific and total huntingtin (mAb2166) antibodies. For full length huntingtin, the intensity ratio of pS421/2166 is on average 4 times greater for the cytoplasmic extracts than the nuclear extract, indicating that S421-phosphorylated huntingtin is more likely to be found in the cytoplasm than the nucleus (Figure 4.7A). The cytoplasmic extract is enriched for S421-phosphorylated huntingtin relative to the nuclear compartment. If there were no relationship between the S421 phosphorylation and nuclear localization, the ratio would be expected to be equal for both compartments.

To confirm this relationship between S421 phosphorylation and nuclear localization of huntingtin, 293 cells were transfected with S421 phosphorylation mutants. The S421 (wildtype) huntingtin is endogenously phosphorylated (Warby et al., 2005), S-D is pseudo-phosphorylated with a serine to aspartate mutation that mimics the conformational state of phosphorylation, and S-A substitutes an unphosphorylatable alanine for the serine at 421. Using the ratio of huntingtin in nuclear/cytoplasmic extracts from these cells, we find that non-phosphorylated (S-A) full length huntingtin is more likely to be found in the nucleus than the phosphorylated (S) or pseudophosphorylated (S-D) huntingtin. S421 phosphorylated huntingtin was more likely to be found in the cytoplasm (Figure 4.7B). From this data, it is not possible to determine if phosphorylation either prevents the nuclear entry or facilitates nuclear export.

Taken together, data from untreated wildtype mouse brain and transfected cells suggest that huntingtin phosphorylated at S421 is more likely to be found in the cytoplasm than the nucleus. Given that neo-586 fragments are found in the nucleus and that S421 phosphorylation reduces nuclear huntingtin, we next determined if phosphorylation at S421 alters the nuclear localization of the aa586 fragment.

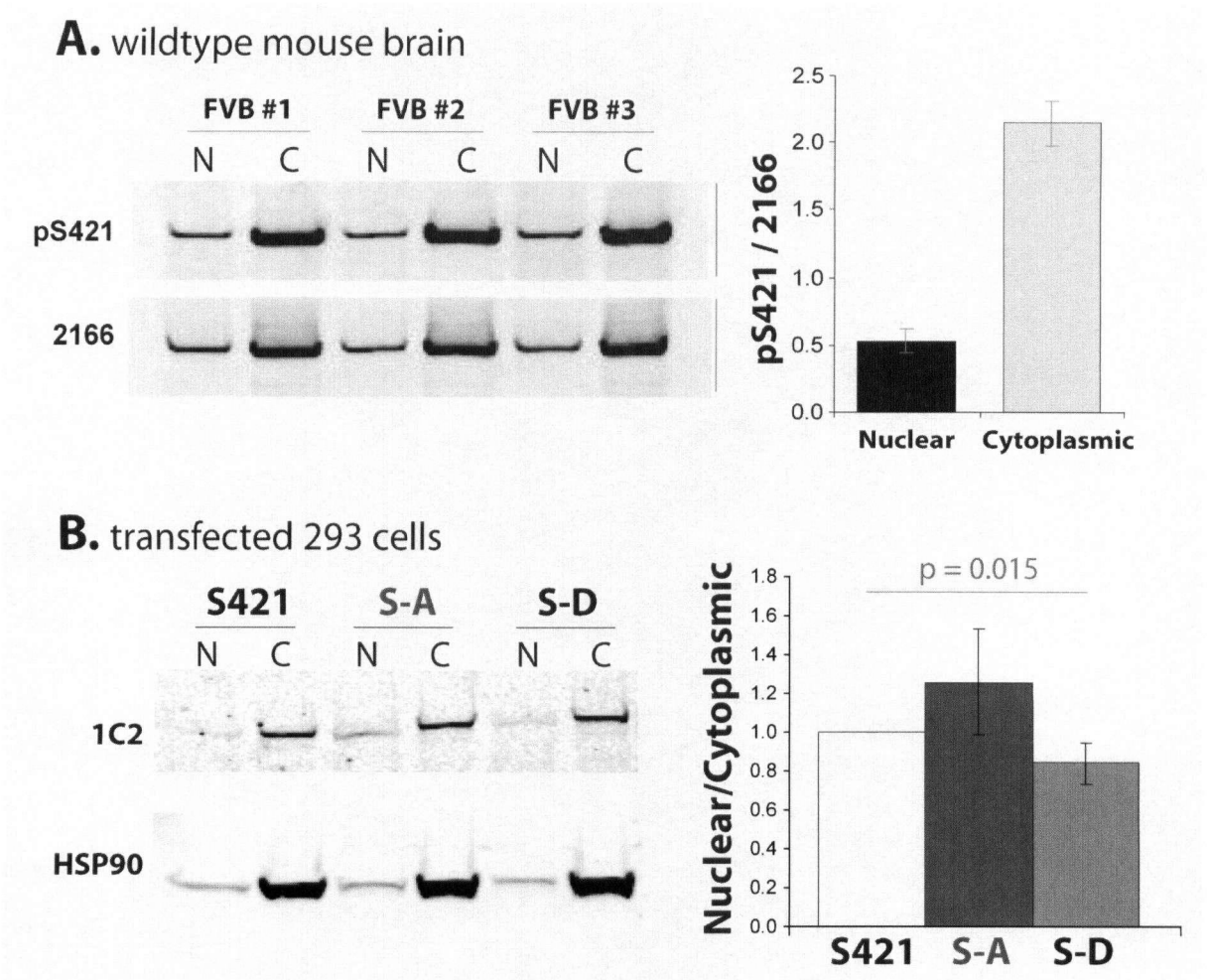

Figure 4.7: Nuclear localization of huntingtin is decreased by phosphorylation at S421.
(A) Whole brain samples from 3 untreated wildtype mice (FVB strain, mouse 1, 2 and 3) were collected and separated into nuclear and cytoplasmic fractions. The ratio is calculated to control for antibody affinity and inter-mouse variability. The ratio of pS421-huntingtin to total huntingtin is significantly less in the nuclear fraction (N) than cytoplasmic fraction (C), suggesting that there is less phosphorylated huntingtin in the nucleus than the cytoplasm. **(B)** 293A cells were transfected with 138Q full-length huntingtin constructs containing mutations at aaS421. Nuclear and cytoplasmic extracts from these cells were immunoblotted with 1C2 for polyglutamine-expanded huntingtin. A greater proportion of non-phosphorylated (S-A) huntingtin was found in the nucleus component than the endogenously phosphorylated (S421) or pseudophosphorylated (S-D) constructs. HSP90 is predominantly a cytoplasmic protein and HSP90 staining control was performed to demonstrate the efficiency of the separation of nuclear and cytoplasmic compartments (~5% contamination of cytoplasmic proteins in the nuclear isolate). Quantification of immunblot results were normalized to S421 and compared by two way ANOVA ($p = 0.015$).

4.3.5 Nuclear Localization of aa586 Fragments

To determine if the nuclear localization of the aa586 fragment is influenced by phosphorylation, we transfected COS cells with 1212aa huntingtin and performed nuclear/cytoplasmic extraction to separate proteins from these subcellular compartments (Figure 4.8). The level of aa586 generated by endogenous cleavage is difficult to detect by immunoblotting, so to increase the

amount of aa586 fragment to perform quantitative assessment, 4C constructs were used. Caspase cleavage of 4C constructs is blocked at all sites except aa586, preventing the further caspase processing and allowing for concentration of aa586 cleavage fragments to aid in their detection. To clearly identify the 1-586aa fragment, both a caspase-6 cut control (sample treated with recombinant caspase-6 *ex-vitro*) and the neo-586 antibody was used. As a control, the 5C-S421 construct has all caspase cleavage sites blocked by point mutations, including the aa586 cleavage site. As expected, the 5C construct is not cut by caspase-6 and is not detected by the neo-586aa antibody (Figure 4.8A).

Transfection of 1-1212aa fragments with the 4C caspase mutation (allowing aa586 cleavage) into COS cells results in endogenously generated 1-586aa huntingtin fragments. Importantly, the subcellular fractionation of these cell lysates into nuclear and cytoplasmic components demonstrates that the endogenously generated 1-586aa is highly enriched in the nucleus, as detected by both Bkp1 monoclonal and neo586 (Figure 4.8A). This is consistent with the immunofluorescence experiments showing enriched nuclear 1-586aa with neo586 antibody.

To determine the effect of S421 phosphorylation on the nuclear localization of the endogenously generated 1-586aa fragment, S421 phosphorylation mutants were transfected into COS cells. Nuclear and cytoplasmic compartments were separated and the 1-586aa cleavage band detected by immunoblot with Bkp1 monoclonal and neo586. Quantification of the 1-586aa fragment demonstrates that blocking the S421 phosphorylation (S-A) increases the amount of nuclear 1-586aa fragment relative to phosphorylated (S421) and pseudophosphorylated (S-D) huntingtin (Figure 4.8B).

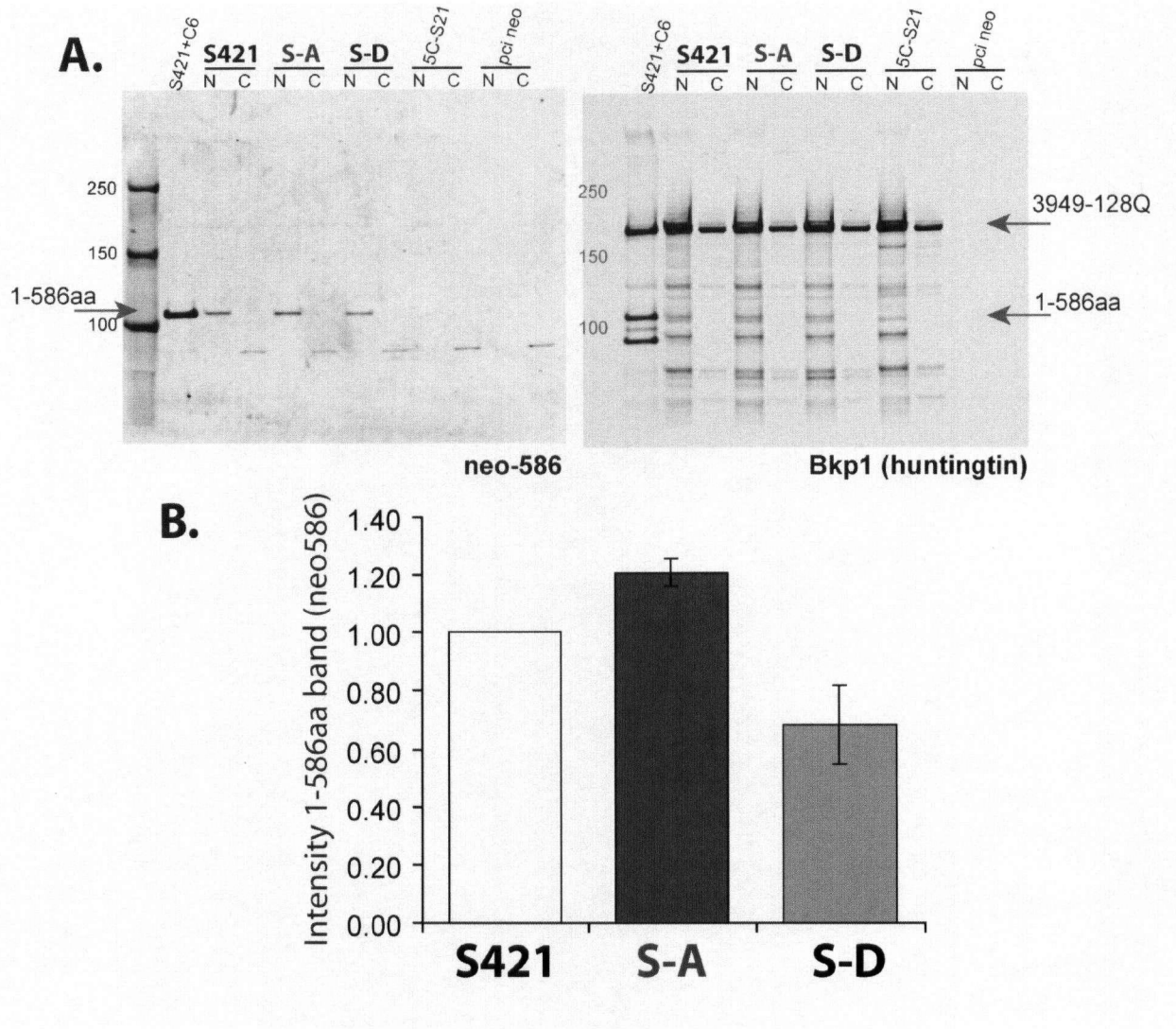

Figure 4.8: Nuclear localization of 1-586aa fragment is decreased by phosphorylation at S421
(A) Representative immunoblots from COS cells transfected with 3949-128Q-4C constructs (blue arrow), lysed and immunoblotted for total huntingtin (BKP1) or the 1-586aa fragment (neo-586aa). Each sample was separated into nuclear (N) and cytoplasmic (C) components. S421+C6 is a caspase-6 cut control to identify the 1-586aa fragment (red arrow). The 4C construct is mutated at all caspase cleavage sites except for aa586 and is used to prevent further processing of the 1-586aa fragment. The 5C construct is a control mutated at aa586 and does not generate the 1-586aa fragment. Pci-neo lane is untransfected cells to control for background bands in the immunoblot. **(B)** Quantification the neo-586aa immunoblots reveals increased nuclear 1-586aa fragment from non-phosphorylated (SA) huntingtin.

4.4 DISCUSSION

Examination of the subcellular localization of huntingtin caspase fragments reveals a crucial difference between endogenously generated and over-expressed huntingtin caspase fragments. Using neo-epitope antibodies that specifically detect fragments cleaved at caspase sites, we find that over-expression of huntingtin caspase fragments (1-513aa, 1-552aa, and 1-586aa) all result in a similar diffuse cytoplasmic localization. These over-expressed proteins do not appear to be specifically targeted to any subcellular compartment in the cell and accumulate in the cytoplasm.

The endogenously generated fragments (cleaved in the cell from longer huntingtin constructs), however, had a different subcellular localization. The endogenous 1-586aa fragment from both wildtype and polyglutamine-expanded huntingtin was highly enriched in the nucleus of primary striatal neurons and ST14 cells. In contrast, the endogenous 1-552aa staining was highly enriched in the perinuclear region. This is consistent with previous reports of 1-552aa fragments preferentially associating with membranes (Kim et al., 2001) and localizing to the perinuclear region (Sawa et al., 2005e). Although we could cleanly detect over-expressed pre-cleaved 1-513aa fragment, we could not detect endogenously generated 1-513aa.

These data suggest that the trafficking of endogenously generated huntingtin fragments is specific to each fragment size. One important limitation of many of the current truncated HD mouse models (Mangiarini et al., 1996; Schilling et al., 1999; Slow et al., 2005) is that the size of the huntingtin fragments have been selected based on artificial genetic criteria (ie protein fragment contained in a particular subset of exons, convenient restriction sites for DNA cloning, random gene truncation) rather than authentic cleavage sites within the huntingtin protein. The clear difference between the trafficking and localization of the different fragments, particularly between 'pre-cleaved' fragments and endogenous cleavage events, highlights the importance of both the exact size of the cleavage fragment and how the fragment is generated. Truncated models of HD may not be modeling pathological processes of the disease because these 'pre-cleaved' fragments do not enter the correct cellular pathways and are not trafficked properly. The shortstop mouse, for example, expresses exon 1 and 2 of the HD gene and has extensive inclusions, but unexpectedly, does not have a pathological phenotype (Slow et al., 2005).

The specific subcellular localization of endogenously generated 1-586aa and 1-552aa suggests that these fragments are either specifically trafficked to these locations *after* cleavage, or that huntingtin cleavage *only occurs* at specific locations in the cell. The transient transfection of huntingtin caspase fragments does not result in the same localization as endogenously cleaved fragments, suggesting that cleavage only occurs at specific locations.

To test whether cleavage may occur at specific locations in the cell, we examined the subcellular localization of caspase-6. Immunostaining for caspase-6 pro- and active- forms revealed a punctate pattern in both the cytoplasm and the nucleus of ST14 cells and primary striatal neurons. Active caspase-6 staining was concentrated in the nucleus, a finding that is consistent with previous reports (Guo et al., 2004; Narkilahti and Pitkanen, 2005) and the nuclear localization of the neo-586 fragment of huntingtin. Stimulation of these cells with staurosporine, a kinase inhibitor, resulted in a dramatic enrichment of caspase-6 in the nucleus. In addition, staurosporine treatment increased the amount of activated caspase-6 and induced the translocation of caspase-6 to the perinuclear region, including its association with the microtubule organizing center (MTOC) at the centrosome. Interestingly, huntingtin is found at the centrosome and is known to interact with microtubules (Hoffner et al., 2002)(Tukamoto et al., 1997; Sathasivam et al., 2001) and other studies have shown that caspase-6 also cleaves Tau, a microtubule stabilizing protein (Guo et al., 2004). Many caspase-6 substrates are nuclear or cleaved in the nucleus (Table 4.1) and it is possible that caspase-6 may regulate the microtubule transport and nuclear localization of many substrates by cleavage, both during normal cellular functioning and apoptosis. This is consistent with the caspase-6 mediated cleavage of huntingtin at aa586 taking place in the perinuclear region or in the nucleus.

The differential targeting of specific fragments to the nucleus suggests that cleavage of huntingtin is important in the function of the protein, rather than simply a pathway for degrading the protein. However, the order of huntingtin cleavage events that occur under normal physiological conditions is not entirely clear. At least two models are feasible: the shorter aa552 fragment could be generated from the longer aa586 fragment and exported from the nucleus, or alternately, aa552 and aa586 cleavage could represent parallel, rather than sequential, cleavage events (Figure 4.9).

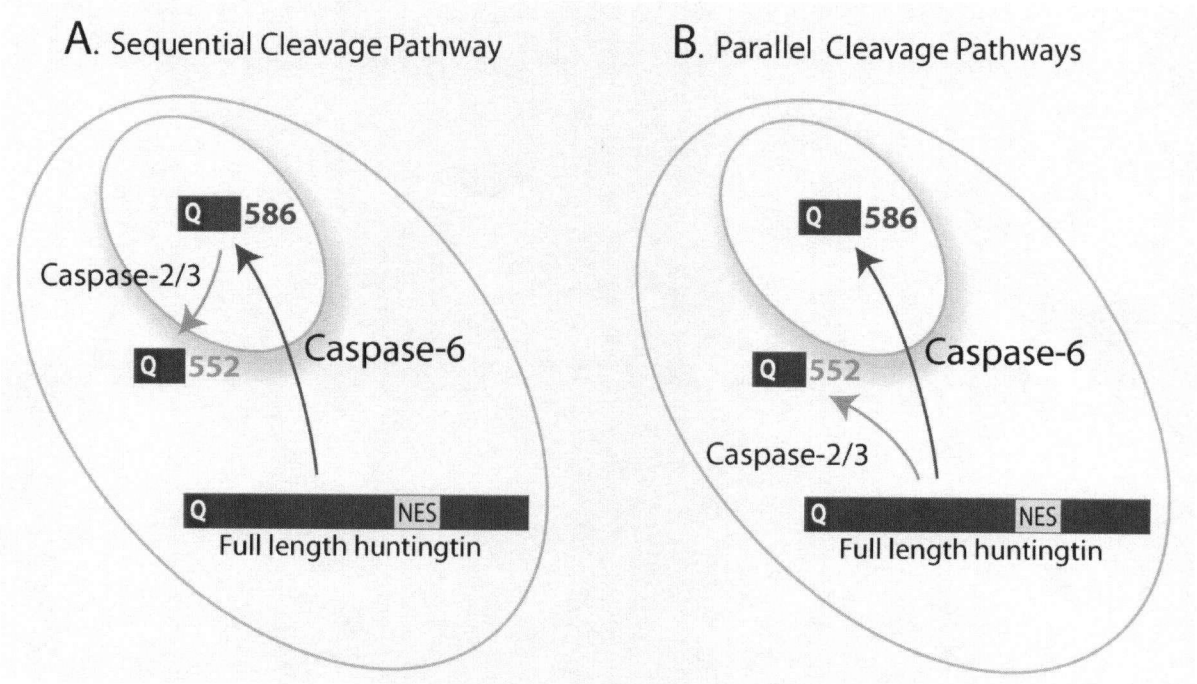

Figure 4.9: Hypothesized huntingtin cleavage pathways
(A) In the sequential pathway, cleavage occurs first at aa586 at or around the nucleus and the smaller, perinuclear aa552 fragment results from the caspase cleavage and nuclear export of this fragment. Alternatively, **(B)** cleavage at aa586 may be mutually exclusive and result in different subcellular localizations. Current data favours the second hypothesis **(B)**.

Many lines of evidence are consistent with huntingtin shuttling in and out of the nucleus as part of its normal function (Tao and Tartakoff, 2001; Xia et al., 2003; Cornett et al., 2005). Although the presence of a putative nuclear localization signal (NLS) in huntingtin is controversial (Bessert et al., 1995; Hackam et al., 1999b; Xia et al., 2003), huntingtin appears to contain a nuclear export signal (NES) in its C-terminus between aa2397-2406 (Xia et al., 2003). The N-terminal 17 amino acids have also been found to interact with the nuclear pore protein 'translocated pore promoter' (TPR) and mediate the export of huntingtin from the nucleus (Cornett et al., 2005). The interaction with TPR is reduced with polyglutamine-expansion of huntingtin and suggests that impaired nuclear export plays a role in the disease (Cornett et al., 2005). This 1-17aa domain of huntingtin acts as a cytoplasmic retention signal, specifically regulating its association with mitochondria (Rockabrand et al., 2007), and could be regulated by SUMOylation in this domain (Steffan et al., 2004). As well, huntingtin, GAPDH and Siah1 form a ternary complex that facilitates the nuclear translocation of N-terminal 1-171aa (N171)

fragments of huntingtin (Bae et al., 2006). The role of this huntingtin/GAPDH/Siah1 complex on the nuclear translocation of full length huntingtin, or endogenously generated fragments of huntingtin is not clear.

Importantly, full length huntingtin is almost entirely composed of HEAT repeats (Andrade and Bork, 1995; Takano and Gusella, 2002; Li et al., 2006) which may play a role in the regulation of its nuclear shuttling. HEAT repeats regulate protein/protein interaction and in proteins from the importin family, HEAT repeats are intimately involved in nuclear transport (Chook and Blobel, 1999; Cingolani et al., 1999). The HEAT repeats are likely the primary determinants of huntingtin structural conformation and phosphorylation at S421 may modulate this structure, impacting both the ability of caspase-6 to cleave at aa586 (Chapter 3) and the ability of huntingtin to associate with the nucleus.

We have shown previously that huntingtin phosphorylation at S421 modifies the aa586 cleavage of huntingtin and wanted to determine if S421 phosphorylation influences the localization of full length and 1-586aa huntingtin. In untreated wildtype mouse brain tissue, we find that S421 phosphorylated full-length huntingtin is enriched in the cytoplasm relative to the nuclear compartment. As well, in cells transfected with full length huntingtin, preventing the phosphorylation at S421 increased the amount of full length huntingtin found in the nucleus. This is consistent with, and provides a mechanism for a previous study showing that S421 phosphorylation of a 1-480aa fragment of polyglutamine-expanded huntingtin reduces its toxicity and the number of intranuclear inclusions (Humbert et al., 2002). Given that the aa586 cleavage event may occur in or around the nucleus, we speculate that phosphorylation may reduce the amount of full length huntingtin that is trafficked to the nucleus and is available for caspase-6 cleavage. Consistent with this, we find that blocking S421 phosphorylation reduces the amount of nuclear 1-586aa fragment.

The current findings are consistent with the hypothesis that full-length huntingtin is trafficked to the nucleus/perinuclear region where is it is cleaved by caspase-6. We find that both 1-586aa fragments and active caspase-6 are concentrated in the nucleus, and this localization is enhanced by cell stress such as tamoxifen. Nuclear localization of huntingtin fragments may also be enhanced by a selective impairment of nuclear export as a result of polyglutamine-expansion (Cornett et al., 2005). The nuclear localization of huntingtin has been implicated in the pathogenesis of the disease in human brains and mouse models of the disease and is delayed in

C6R mice that are protected from disease (Graham et al., 2006a). The present data suggests that phosphorylation at S421 reduces both the nuclear localization of huntingtin, and the cleavage of huntingtin by caspase-6, resulting in a reduction in the number of aa586 fragments in the nucleus.

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CHAPTER 5

Discussion

5.1 SUMMARY OF FINDINGS

The objective of this thesis was to conduct molecular and cell biological studies to determine whether huntingtin was regulated by phosphorylation and what role this might play in the disease.

The first specific goal was to confirm and characterize huntingtin phosphorylation at S421. To do this, we created several reagents including a huntingtin phospho-S421-specific antibody and huntingtin constructs with point mutations at the S421 phosphorylation site. We find that huntingtin is specifically phosphorylated at S421 in brain under normal physiological conditions. Through interaction studies, we find that huntingtin interacts with Akt, and in agreement with other studies (Humbert et al., 2002), that this kinase readily phosphorylates huntingtin *in vitro*. We also find differences in the basal endogenous phosphorylation state of huntingtin in different regions of the brain. The striatum, the primary site of pathology in HD, has a much lower endogenous basal phosphorylation state than less susceptible regions of the brain.

The second specific goal was to determine if S421 phosphorylation is altered by polyglutamine-expansion of huntingtin. We find that an expanded polyglutamine tract results in lower levels of basal S421 phosphorylation in transfected cells and in the brains of YAC mice. The decreases in polyglutamine-expanded huntingtin phosphorylation at S421 in mouse brain were observed at 8 months, prior to overt neurodegeneration in the striatum of these mice. Decreased phosphorylation of polyglutamine-expanded huntingtin has subsequently been confirmed in cultured cells from the knock-in model of HD (Pardo et al., 2006). We also wanted to determine whether the activation of the Akt signalling pathway was altered in the cell culture and YAC model of HD. Using antibodies against the active form of Akt, we did not find alterations, despite the change in S421 phosphorylation of huntingtin. This suggests that polyglutamine-expansion alters the specific interaction between huntingtin and Akt, rather than altering the activation of the Akt pathway.

The third and fourth specific goals were to determine the function of huntingtin phosphorylation at S421. Determining a specific function for S421 phosphorylation proves to be a challenging task considering the complex and multiple functions of huntingtin, and lack of a defined

functional assay. However, we were able to determine significant relationships between the phosphorylation status of huntingtin, its cleavage by proteases, and its subcellular localization. The S421 site is close to the proteolytic domain containing the caspase cleavage sites (aa500-600) and phosphorylation at S421 reduces the cleavage of huntingtin. Data from the C6R mice have demonstrated that a specific cleavage event, cleavage at aa586 by caspase-6, is a crucial rate limiting step in HD pathogenesis. We therefore wanted to specifically assess the impact of S421 phosphorylation on aa586 cleavage. In *ex vitro* and *in vitro* studies, we find that the cleavage of aa586 is reduced when huntingtin is phosphorylated.

The nuclear localization of huntingtin fragments is known to be important in the disease and we wanted to directly assess whether phosphorylation at S421 alters the nuclear localization of huntingtin. Using subcellular fractionation and immunofluorescent techniques, we find that phosphorylation at S421 reduces the nuclear localization of both full length huntingtin and huntingtin fragments. Furthermore, caspase-6 is also concentrated in the nucleus and the S421 phosphorylation of huntingtin may mediate its nuclear localization where it is cleaved by caspase-6 to generate the 1-586aa fragment. These findings provide an important functional link between cleavage and nuclear localization that may be mediated by S421 phosphorylation.

5.2 ROLE OF PS421 IN THE MOLECULAR FUNCTION OF HUNTINGTIN

How does S421 phosphorylation fit in with the current understanding of huntingtin function?

Phosphorylation is a rapid, reversible, covalent modification capable of altering the conformation of the huntingtin protein. Integrating the role of phosphorylation into the structural properties of huntingtin is crucial to deciphering its molecular functions.

5.2.1 Phosphorylation Modifies the Structure of Huntingtin

The molecular functions of huntingtin are ultimately dictated by the conformation of the protein. There are at least three lines of evidence that suggest that huntingtin has a great deal of structural flexibility:

i) Differential detection by various antibodies depending on the polyglutamine size (ie 1C2 antibody) and its subcellular location (ie EM48 antibody) (Trottier et al., 1995b; Trettel et al., 2000; Ko et al., 2001).

ii) Polyglutamine-expansion alters the three-dimensional structure of huntingtin such that proteins that interact with huntingtin outside the polyglutamine tract may have altered binding (Li and Li, 2004)

iii) There have been great difficulties in obtaining a crystal structure for huntingtin, which is consistent with it being an intrinsically flexible protein (Li et al., 2006).

There is the opportunity, therefore, for phosphorylation to have a large impact on the flexible framework of the huntingtin protein.

5.2.1.1 Huntingtin is a Flexible Solenoid

Huntingtin is almost entirely composed of HEAT repeats (Andrade and Bork, 1995; Takano and Gusella, 2002; Li et al., 2006) which are the single most important determinant of its physical structure (Kobe et al., 1999). HEAT repeats are helix-turn-helix structures, interspersed by flexible coiled domains and stacked to form a solenoid structure with a hydrophobic core. HEAT repeat proteins assume different conformations in different functional states and in general, mediate important protein-protein interactions involved in cytoplasmic and nuclear transport (Neuwald and Hirano, 2000).

Huntingtin is considered an 'AAA class' of HEAT repeat. The protein with the greatest degree of sequence similarity with a known crystal structure is the PR65/A structural subunit of PP2A (Figure 5.1B) (Andrade et al., 2001). PR65/A is composed of 15 consecutive HEAT repeats, whereas huntingtin is estimated to have up to 36 HEAT repeats dispersed into three domains and interspersed with coiled domains (Figure 6.1).

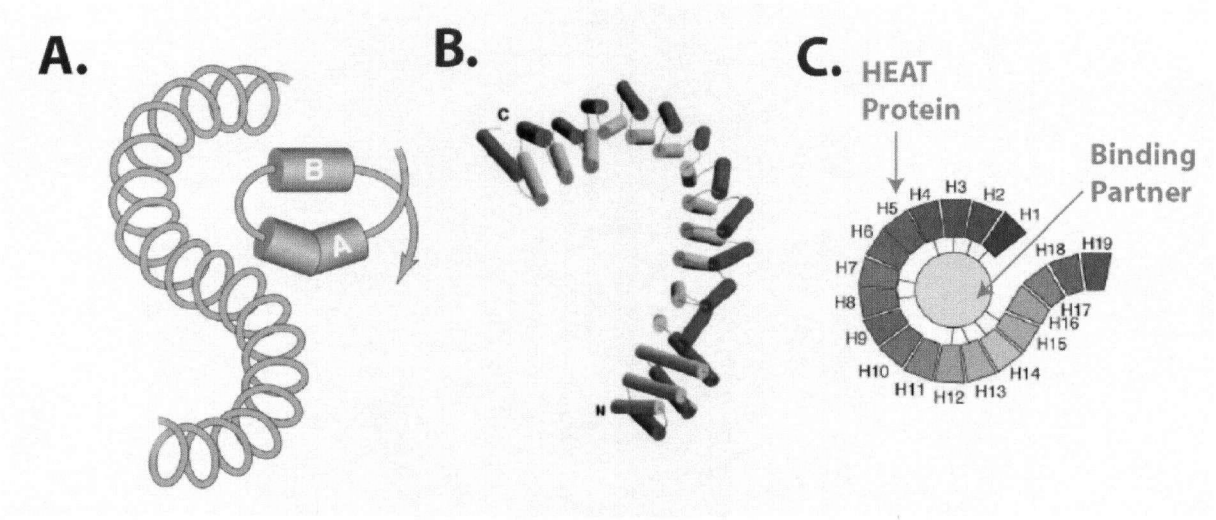

Figure 5.1: Flexible solenoid structure of HEAT proteins
(A) A single HEAT repeat is composed of two helices that stack to form a 'spring' like structure.
(B) Structure of the HEAT repeat-containing PR65/A subunit of protein phosphatase 2A (PP2A) closely related to huntingtin. PR65/A and huntingtin are both in the AAA class of HEAT repeats that form a solenoid shape with a hydrophobic core. (C) The HEAT repeat domain of Importin-B (H1 – H19) mediates interaction with other proteins by wrapping around the binding partner and interacting at multiple surfaces. Adapted from (Andrade et al., 2001), (Stewart, 2003) and (Conti et al., 2006), with permissions.

5.2.1.2 Posttranslational modifications may modify HEAT orientation

Solenoid conformations of HEAT repeats are believed to be intrinsically flexible and small changes in the relative orientation of successive HEAT coils would result in large changes in the helicoidal pitch of each HEAT domain (Conti et al., 2006). Further changes could be induced by hinge-like movements between HEAT domains. Phosphorylation is a crucial regulatory mechanism for many HEAT repeat proteins, such as members of the importin family and the adenomatous polyposis protein, (APC) (see below). Huntingtin phosphorylation at sites in the coiled domains between HEAT domains, such as at S421, could therefore have a profound effect on the overall protein conformation. Both S421 and the 'caspase protease domain' (aa500-600) fall in a region predicted to be a coiled domain between HEAT domain 1 and 2.

5.2.2 Phosphorylation and Protein-Protein Interactions

Phosphorylation mediates many types of protein-protein interactions at discrete binding domains (Pawson and Nash, 2003). HEAT-repeat proteins, however, bind proteins using a variety of different interaction surfaces (Conti et al., 2006). The flexibility of HEAT repeat structures are believed to accommodate different binding partners through an 'induced-fit' type of mechanism with each HEAT repeat representing a single turn in a tightly wound spring (Figure 5.1A) (Conti et al., 2006). As opposed to 'modular binding domains' (such as SH2 and SH3) which bind

discrete linear peptide motifs, the 'solenoid binding domains' (HEAT, ANK, TPR, ARM) provide a structural framework that mediate protein-protein interactions based on surface-exposed residues and arrangement of inter-repeat packing (Kobe et al., 1999). The karyopherins are a HEAT repeat containing family of proteins that are believed to bind other proteins by wrapping around them, rather than interacting at a simple recognition interface (Figure 5.1C) (Conti et al., 2006). The principle of HEAT repeat proteins functioning as scaffolding proteins is consistent with the observation that huntingtin binds many protein interactors, although the domains of interaction in huntingtin is not well understood (Li and Li, 2004)

No S421 phosphorylation-specific interactions with huntingtin have been identified thus far. However, the complicated interface between HEAT repeat domains and protein interactors may make the detection of S421 phosphorylation-specific interactions more difficult than traditional interactions mediated by discrete binding domains. Further studies are required.

5.2.3 **Phosphorylation and Cleavage**

Phosphorylation at S421 reduces the cleavage of huntingtin at the caspase proteolysis domain (aa500-600). S421 and the proteolysis domain both lie in between HEAT domains, and phosphorylation presumably restricts access to the cleavage site. Several cleavage events are decreased, including the cleavage of aa586. Studies of the caspase-resistant mice have demonstrated that, in the presence of polyglutamine-expanded huntingtin, cleavage at aa586 is required for pathogenesis (Graham et al., 2006a).

However, evidence suggests that huntingtin cleavage at these caspase sites is important for regulating the *normal function* of huntingtin, rather than a pathological metabolic degradation of the protein:

i) Cleavage fragments are specifically trafficked to different regions of the cell

ii) Cleavage is modulated by posttranslational modifications, such as phosphorylation at S421

iii) Cleavage occurs under non-pathological conditions (in normal brains and prior to pathology in HD brains (Wellington et al., 2002) and in untreated cells (Chapter 4))

iv) Both wildtype and polyglutamine-expanded huntingtin are cleaved (Wellington et al., 2002)

Our *in vitro* studies (Chapter 4) show that cleavage at specific sites is intimately linked to trafficking within the cell, and therefore suggest that specific fragments perform specific functions. The 1-586aa fragment is found in the nucleus, and may therefore have nuclear functions including transcriptional regulation or transport of co-factors into the nucleus. The perinuclear staining of the 1-552aa fragment is distinctly different, and it may function to retain co-factors in the perinuclear region or cytoplasm.

Full-length huntingtin may simply be an inactive form of smaller, active molecules. Trafficking of the full-length protein is crucial however, and may be regulated by phosphorylation that precedes nuclear localization/cleavage events. Transfection of pre-cleaved fragments of huntingtin is not sufficient to reproduce these functions, however, as the trafficking of pre-cleaved fragments is clearly different than endogenously generated fragments.

5.2.3.1 HEAT Repeats and Cleavage

HEAT domains and S421 phosphorylation may regulate both access to cleavage sites and release of cleaved fragments. The coiled regions between the HEAT repeats can be pushed out of the hydrophobic core where they may be susceptible to proteolytic cleavage. The hydrogen bonding and hydrophobic core of the HEAT repeat structure has been shown to hold full length soluble huntingtin together as a single molecule, despite digestion by trypsin at several sites along the molecule (Li et al., 2006). In other words, tryptic digest of the protein can cleave between amino acids at multiple sites, but the full length soluble molecule can remain intact, being held together by interactions believed to be mediated by the HEAT repeats (Li et al., 2006).

This is an important consideration because the *cleavage* and the *release* of the fragment therefore do not have to occur simultaneously. Cleavage may occur some time previous, or even in a different cellular compartment, than the release of the fragment. Phosphorylation may not only impact the cleavage of huntingtin, as we have shown, but may regulate the release of the fragments which perform different functions.

5.2.4 Phosphorylation and Nuclear Localization

Huntingtin is believed to shuttle in and out of the nucleus and the current data suggests that phosphorylation is involved in the regulation of this movement. There are many examples of

phosphorylation regulating nuclear transport (Moorhead et al., 2007). HEAT repeat proteins from the importin family are intimately involved in nuclear transport (Chook and Blobel, 1999; Cingolani et al., 1999) where phosphorylation is a major mechanism of regulation (Jans et al., 2000; Poon and Jans, 2005).

Huntingtin phosphorylation at S421 is reduced when located in the nucleus. However, the mechanism of this subcellular regulation remains unknown. It is not clear whether phosphorylation prevents nuclear import or facilitates nuclear export, perhaps through interaction with the NES and/or the cytoplasmic retention signal in the HunMAD domain (aa1-17). The data also suggests that there is a relationship between the nuclear localization and specific cleavage events.

Active caspse-6 is found in and around the nucleus. The caspase-6 cleaved 1-586aa fragment is found in the nucleus. It is plausible, therefore, that the caspase-6 cleavage of huntingtin occurs in or around the nucleus. This is supported by the observation that transiently transfected huntingtin 1-586aa caspase fragments are not trafficked to the nucleus appropriately. The nuclear delivery of huntingtin would therefore be an important step in the process of cleaving huntingtin at aa586, which has been shown to be crucial to the development of HD pathogenesis. S421 phosphorylation of huntingtin reduces its nuclear localization, and under this hypothesis, would be a mechanism for the observation that S421 phosphorylation also reduces huntingtin cleavage at aa586.

5.2.5 The BDNF paradox – Huntingtin as a "Moonlighting Protein"

Huntingtin has tissue-specific functions. The regulation of NRSE-containing genes, such as BDNF, is an example. Huntingtin regulates the expression of BDNF by binding REST (NRSF), a transcriptional repressor, and retaining it in the cytoplasm, thereby allowing BDNF transcription to occur. This is clearly an essential function in neuronal cells that require BDNF expression. However, huntingtin is widely expressed throughout the body (in tissues that should *repress* expression of BDNF and other neuron-specific genes regulated by REST) where presumably it does *not* perform this function. In fact, REST is widely expressed in non-neuronal tissues but is down-regulated in neuronal precursors and eliminated in post-mitotic neurons (Chen et al., 1998; Jones and Meech, 1999). The paradox therefore, is that huntingtin presumably binds REST in post-mitotic neurons (where REST expression is low to non-existent)

to allow BDNF expression, and does not bind REST in other tissues (where REST expression is high) to allow repression. It is not clear what prevents REST/huntingtin binding in non-neuronal tissues. Despite the paradox, the BDNF story illustrates the fact that huntingtin must have different functions in different tissues.

The multifunctional nature of huntingtin places it within a special class of 'moonlighting proteins' that does not fit the paradigm of 'one protein – one function' (Jeffery, 1999). With proposed roles as a scaffolding protein involved in transport, transcription, and signalling, as well as clearly different roles in different tissues during development, haematopoietic stem cells, and cells expressing and transporting BDNF, huntingtin is a versatile molecule. Although the molecular functions of huntingtin may be unified by the idea that huntingtin binds proteins and moves (or inhibits movement) across intracellular compartments, the larger scale biological processes affected by these functions may be very diverse and difficult to clearly describe. Even opposing biological processes such as cell survival and apoptosis may share similar fundamental molecular processes.

Moonlighting proteins, such as huntingtin, need a large amount of regulation to perform multiple and complicated functions. Posttranslational modifications such as phosphorylation and palmitoylation may be keys to the switch in function for huntingtin, similar to the Cystic Fibrosis Transmembrane Conductance Regulator protein (CFTR), where phosphorylation can result in a dramatic change in function (Schwiebert et al., 1999). In the case of intrinsically unstructured proteins (IUPs), the same protein can have opposing functions depending on its regulatory state (Tompa et al., 2005).

5.2.6 Huntingtin as a Signalling Molecule

The phosphorylation by Akt places huntingtin in an important signalling pathway that is consistent with many known functions for huntingtin, both at the level of its molecular function and the biological processes affected by its function.

The molecular function of huntingtin involves interaction with many proteins, including proteins involved in transcriptional control, and movement between different compartments in the cell. The structural features and cleavage of huntingtin play an integral role in these molecular functions. The biological processes altered by these molecular functions may be complex,

because its molecular function (binding proteins and mediating transport) may involve multiple proteins with mutually exclusive or even opposing functional effects. It may be easier therefore to decipher the precise molecular functions of huntingtin phosphorylation, such as cleavage, subcellular localization and protein binding, than to decipher the role of huntingtin in biological processes such as cell survival and proliferation.

Nevertheless, the S421 phosphorylation of both wildtype and polyglutamine-expanded huntingtin seem to play a role in cell survival (Figure 5.2). During development, huntingtin is required for the extraembryonic transport of nutrients required for the proliferation, growth and survival of the developing nervous system (Dragatsis et al., 2000). In striatal neurons, phosphorylation reduces its nuclear localization, cleavage at aa586, and the toxicity of polyglutamine-expanded huntingtin fragments.

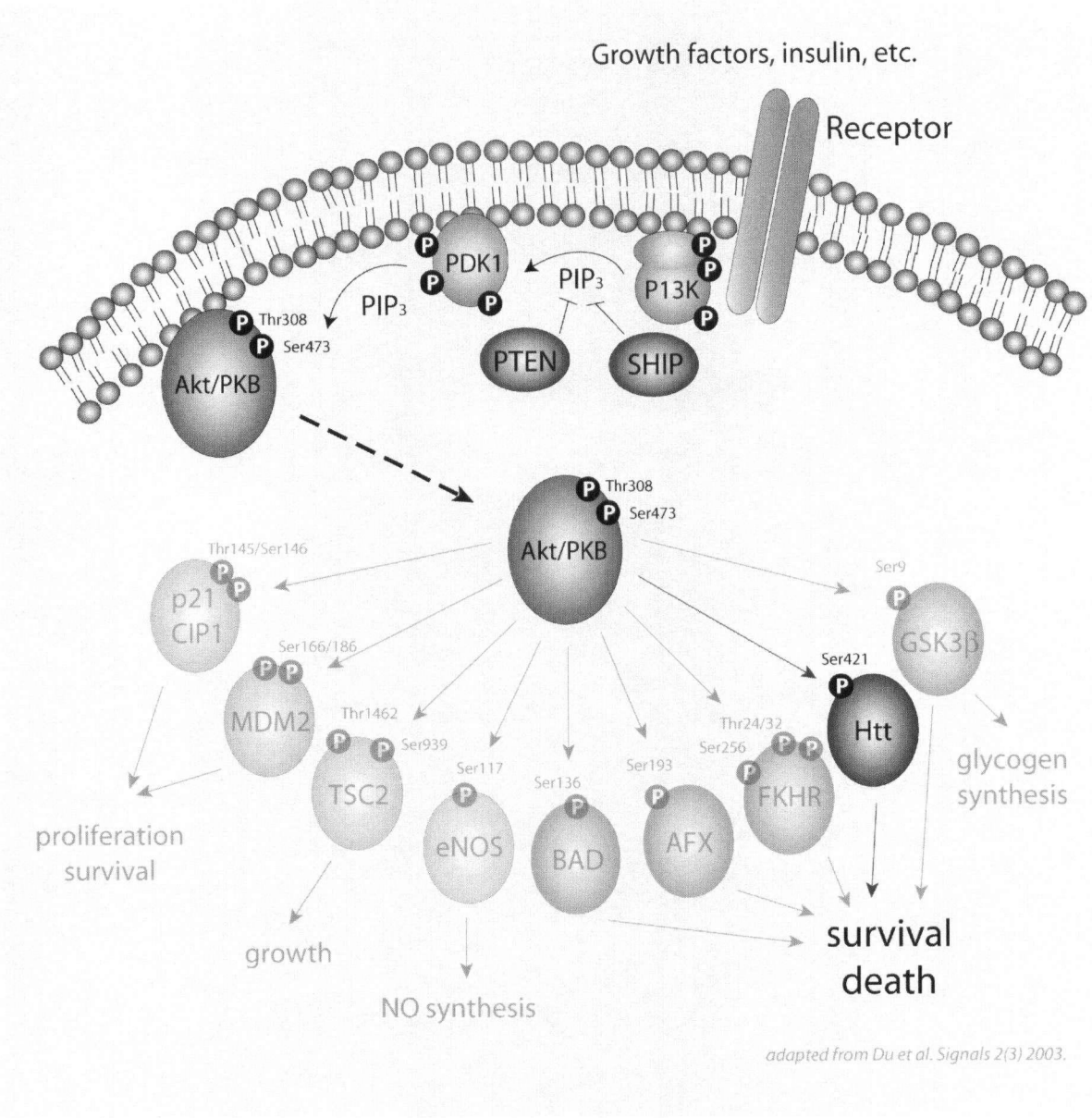

Figure 5.2: Huntingtin as a signalling molecule in the Akt pathway

5.2.7 Akt Pathway Dysregulation

There are conflicting results over whether the Akt pathway is altered in the course of HD. In YAC mice, R6/2 mice, and cell culture models of HD, we did not detect gross changes in Akt pathway activation, despite the observation of reduced S421 phosphorylation of huntingtin (Warby et al., 2005).

Using similar methods, other studies have inconsistently found both increases and decreases in the Akt pathway activation in HD models and HD patients. In a lentiviral-injected rat model of

HD, decreased levels of both total and activated Akt were observed prior to the onset of neurodegeneration (Colin et al., 2005). However, in the knock-in model of HD, Akt activation is increased and can be reduced to normal activation levels by MK801, arguing that these Akt increases are due to NMDAR activation (Gines et al., 2003). In lymphoblasts from HD patients, the total Akt protein levels are twice that of controls, although the amount of activated Akt is unchanged, resulting in a lower ratio of total/active Akt in these peripheral tissues (Colin et al., 2005). In late stage HD patient brains, Akt was cleaved by caspase-3 into a smaller form, suggesting a loss of Akt function in these tissues (Humbert et al., 2002; Colin et al., 2005). The conflicting results may be due to secondary and variable effects that results in altered survival signalling after degeneration has begun.

5.2.7.1 SGK

Huntingtin has also been shown to be phosphorylated at S421 by serum- and glucocorticoid-inducible kinase (SGK) (Rangone et al., 2004). SGK is closely related to Akt functionally and mediates the protective benefits of IGF-1 signalling similar to Akt (Humbert et al., 2002). In mouse models and human HD brains, SGK levels are increased in the presence of polyglutamine-expanded huntingtin. The SGK increase is presumably in response to the stress of polyglutamine-expanded huntingtin and appears to be mediated through the MAPK/p38 pathway.

The pattern of Akt and SGK dysregulation in HD is confounded by differences in HD models and secondary protective effects. What is clear, however, is that the regulation of huntingtin is complex and may depend on the interplay between kinases and phosphatases on several sites of phosphorylation.

5.3 OTHER SITES OF PHOSPHORYLATION IN HUNTINGTIN

In the last few years, various phosphorylation sites in huntingtin have been identified using a combination of biochemical methods and mass spectrometry analysis (Figure 5.3):

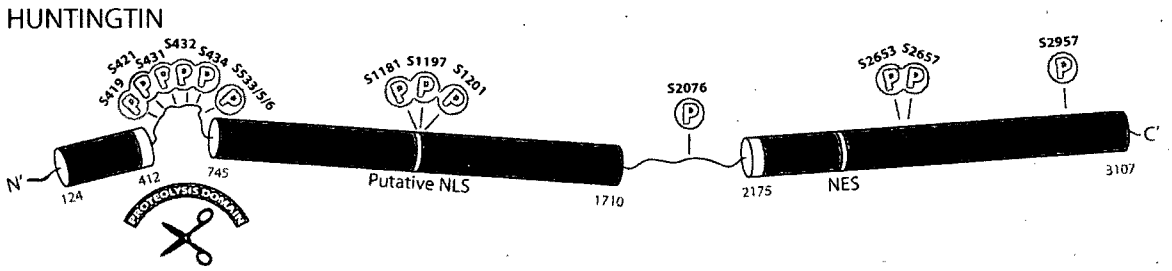

Figure 5.3: Schematic of identified phosphorylation sites in huntingtin
This figure illustrates the position of phosphorylation sites that have been identified by biochemical and mass spectrometry studies (see text). Interestingly, all sites identified so far have been phosphorylated serines.

5.3.1 Serine 434

Huntingtin phosphorylation at S434 by cyclin-dependent kinase 5 (Cdk5) has been confirmed *in vitro* and *in vivo* in one study (Luo et al., 2005). S434 phosphorylation has been shown to attenuate the caspase-3 mediated cleavage of huntingtin at aa513 and attenuate the toxicity of a polyglutamine-expanded 1-588aa fragment in neuroblastoma cells. The effect of S434 phosphorylation on aa586 cleavage has not been examined, although it would be expected to work in a manner similar to S421 phosphorylation. S421 and S434 are adjacent in the protein and it is plausible that these phosphorylation sites integrate signalling from multiple pathways (Akt and Cdk5) to perform similar regulatory functions on huntingtin.

5.3.2 Mass Spectrometry Analysis

Mass spectrometry (MS) analysis is a powerful and unbiased method for detecting phosphorylation sites *in vitro* (cells) and *in vivo* (tissue from an animal) (Ong and Mann, 2005; Goshe, 2006). Analytical MS techniques generally have a low sensitivity for detecting phosphorylated residues, and false positives (less than 1%) are unlikely compared to false negative results, which may be common (Raggiaschi et al., 2005).

We conducted a preliminary MS analysis of full length huntingtin to determine additional sites of phosphorylation (Table 5.1). Wildtype murine huntingtin was immunopurified from mouse brain for this study. Following the tryptic digest of murine huntingtin, the two phosphorylated peptides were detected with certainty:

Table 5.1: Phosphorylation sites detected by mass spectrometry in wildtype mousebrain.
Huntingtin was immunoprecipitated using mAb2166 from whole brain lysate. Several potential phosphorylation sites exist in each peptide (S or T). 'Human site' gives the aa with the highest probability of phosphorylation based on motif searching, and then translated from mouse to human aa numbering. Kinase predictions made with Scansite and NetPhoK.

Peptide from mouse *Hdh*	Human site	Kinase prediction
AALP**S**L**T**NPP**S**L**S**PIR	1181	cdc2/cdk5
A**S**DP**S**PA**T**PD**SES**VIVAMER	2950 or 2952	unknown

Another group has recently conducted an extensive MS analysis of huntingtin (Schilling et al., 2006). Human huntingtin was overexpressed and N-terminally tagged with myc for immunopurification from 293 cells. The following serine phosphorylation sites were confirmed by tandem mass spectrometry (Table 5.2):

Table 5.2: Phosphorylation sites detected by mass spectrometry in 293 cells.
Cells were transfected with myc-tagged human huntingtin (Schilling et al., 2006).

Serine Site	Predicted Kinase
421	Akt/SGK
533/5/6	*unknown*
1181	CDC2/CDK5
1201	CDC2/CDK5
2076	ERK1
2653	ERK1
2657	*proline-directed kinase*

Further, a large scale phosphoproteomics screen using MS analysis has identified further sites of phosphorylation in huntingtin (Table 5.3). This study assessed phosphorylation of all proteins in the proteome of HeLa cells following EGF stimulation (Olsen et al., 2006).

Table 5.3: Phosphorylation sites detected by mass spectrometry in HeLa cells.
Cells were unstimulated or treated with EGF for 5-60 minutes to determine the change in phosphorylation status (Olsen et al., 2006).

Serine Site	Predicted Kinase	Effect of EGF on phosphorylation
419	CaMK2/PKB	decreasing
421	PKB/CKI/CKII/CaMK2	decreasing
431	unknown	decreasing
432	unknown	decreasing
434	CKI/GSK3	decreasing
1197	GSK3	no change
1201	CK1/CDK2/ERK	no change

Interestingly, this study separated nuclear and cytoplasmic fractions prior to MS analysis. Phosphorylation was only detected on huntingtin in the cytoplasmic fraction (Olsen et al., 2006). Another large scale study using similar methods, but focussed exclusively on the nuclear fraction, also did not detect huntingtin as a phosphorylated protein in the nucleus (Beausoleil et al., 2004). These data are consistent with the hypothesis that phosphorylation reduces the nuclear localization of huntingtin.

Other smaller scale phospho-proteomic analyses have been conducted using a variety of methods but did not detect phosphorylated huntingtin residues (Amanchy et al., 2005; Thelemann et al., 2005). Curated online phosphorylation resources currently provide no further information (Diella et al., 2004; Peri et al., 2004).

5.3.3 Serine 536

Following the huntingtin-specific MS analysis, phospho-mutants of the S536 phosphorylation site were generated to examine the effect of phosphorylation at this site on the cleavage and toxicity of huntingtin (Schilling et al., 2006). It was concluded from this study that S536 phosphorylation by an unknown kinase results in the inhibition of calpain cleavage at S536.

One major caveat of this study is that although MS data determines that it is phosphorylated, the authors could not conclusively show which of the serine residues in the phosphorylated peptide (S533/5/6) was actually phosphorylated. None of these sites match a known kinase consensus sequence. The conclusion that S536 is the site of phosphorylation is based solely on the S536D mutation reducing cleavage at aa536. It is not surprising that mutation of the terminal residue for

cleavage site (S536D) blocks cleavage, and therefore this is not evidence that this is a site of phosphorylation. Mutation of S533 or S535 did not alter cleavage at S536. Which of the three sites (S533, S535 or S536) is actually phosphorylated and the function of this phosphorylation is unclear and requires further study.

5.3.4 Signal Integration at the Huntingtin Phospho-Domain

The clustering of multiple phosphorylation sites in close proximity to each other (S419/421/431/432/434) suggests they comprise a phospho-domain in huntingtin. This phospho-domain, as well as the caspase protease domain (aa500-600), falls in sequence between two major solenoid-shaped HEAT repeat domains. Phosphorylation at either S421 or S434 inhibits cleavage events in the caspase protease domain, suggesting that all phosphorylation events in this phospho-domain may have similar regulatory effects on huntingtin. As well, EGF stimulation of HeLa cells resulted in decreased phosphorylation of all of S419/421/431/432/434 (Olsen et al., 2006), suggesting these sites may be regulated in a coordinated manner. The structural angle between these major HEAT domains could be altered by phosphorylation and would be predicted to significantly impact protein interactions and access to proteolytic cleavage domain. Further structural analysis of this region would be useful.

Furthermore, multiple phosphorylation events in the aa419-434 phospho-domain of huntingtin may have an additive effect. Many proteins are phosphorylated at multiple sites, modulating variables such as the intensity and duration of a phosphorylation response, and leading to complex regulatory mechanisms (Cohen, 2000; Holmberg et al., 2002; Yang, 2005). The protein kinase MAPK-activated protein kinase-2 (MAPKAP-K2), for example, requires phosphorylation at multiple sites to reach maximal activity (Ben-Levy et al., 1995). It is possible that multiple phosphorylation events in the phospho-domain of huntingtin are required for maximal effect on cleavage and nuclear localization of the huntingtin protein.

The phospho-domain of huntingtin could therefore be an integration point of several signalling pathways. The phosphorylation sites appear to be phosphorylated by different kinases (and perhaps dephosphorylated by different phosphatases) that are activated by different cell signalling pathways. Three kinases that act on the 419-434 phospho-domain have been identified so far (Akt, SGK and CDK5) but consensus sequence predictions suggest several other

candidate kinases. This integration point is consistent with huntingtin being a multifunctional 'moonlighting protein'.

5.4 S421 PHOSPHORYLATION AS A THERAPEUTIC TARGET

The data in this thesis provide evidence that huntingtin phosphorylation at S421 may be a useful target for therapeutics because it modifies two events believed to be crucial in the disease process – huntingtin cleavage at aa586 and its nuclear localization. Furthermore, in striatal primary cultures (Humbert et al., 2002) and a lentiviral-injected rat model of HD (Pardo et al., 2006), blocking phosphorylation enhances, and pseudophosphorylating reduces, the toxicity and inclusion-forming potential of a polyglutamine-expanded fragment (1-480aa) of huntingtin. Finally, we know that S421 phosphorylation is reduced in the presence of polyglutamine-expansion *in vivo* (Warby et al., 2005) and therefore presents the opportunity to therapeutically restore or increase the normal basal level of huntingtin phosphorylation.

Phosphatase inhibitors are emerging as a useful class of drugs for a variety of diseases (McCluskey et al., 2002) and may offer a promising method of increasing the phosphorylation at huntingtin S421. Phosphatase inhibitors, particularly naturally occurring inhibitors, tend to be non-specific and so the development of small molecule inhibitors of specific phosphatases has been a focus for the treatment of diseases such as asthma, diabetes and cancer (Sakoff and McCluskey, 2004). For HD, the effective use of phosphatase inhibitors requires knowledge of the phosphatases that act on huntingtin S421.

5.5 PHOSPHATASE REGULATION OF S421

5.5.1 PP2B/Calcineurin

We first identified PP2B as a phosphatase that efficiently dephosphorylates huntingtin *ex vitro* (Warby et al., 2005). Further studies have demonstrated that PP2B/calcineurin co-localizes with huntingtin during Ca+-induced activation and dephosphorylates S421 *in vitro* (Pardo et al., 2006). The Ca+-induced activation of PP2B resulting in a reduction in S421 phosphorylation is consistent with the excitotoxic theory of HD pathogenesis.

FK506 is an immunosuppressant drug that acts through the inhibition of PP2B (Dumont, 2000). FK506 increases the S421 phosphorylation *in vitro* and *in vivo* and can protect cultured cells from the toxicity of polyglutamine expanded huntingtin (Pardo et al., 2006). This study demonstrates that FK506 can be delivered to the brain of mice, effectively increases the S421 phosphorylation of huntingtin, and is promising initial work to *suggest* that FK506 protects against polyglutamine-expanded huntingtin toxicity in mice (although this has not yet been demonstrated) (Pardo et al., 2006).

Other studies, however, have had opposite results with PP2B inhibitors in mouse models of HD. In the R6/2 mouse, treatment with the PP2B inhibitors FK506 and cyclosporin A resulted in an accelerated phenotype (Hernandez-Espinosa and Morton, 2006). The implication of this study is not clear, however, as the R6/2 mouse expresses a truncated exon 1 fragment of huntingtin that does not contain S421, suggesting that phosphorylation of the polyglutamine-expanded huntingtin (rather than wildtype huntingtin) may be necessary for protection.

Interestingly, four dual specificity phosphatases (MKP1, MKP3, CPG21, and TD1) were found to be upregulated in response to polyglutamine-expanded huntingtin toxicity in PC12 cells (Wu et al., 2002). In this PC12 cell model of HD, protein tyrosine phosphatase inhibitors delayed cell death. No effect was seen with the general serine/threonine inhibitor okadaic acid (Wu et al., 2002).

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CHAPTER 6

Future Directions

The data presented in this thesis suggest numerous avenues of future investigation. This includes molecular and genetic studies ultimately aimed at identifying therapeutic targets for HD.

6.1.1 Huntingtin Phosphodomain (aa419-434) Studies

The data suggest that multiple phosphorylation sites are clustered in a phospho-domain (aa419-434) that serves as an integration point for many signalling pathways that regulate huntingtin function. The functional S421 assays developed in this thesis suggest that this domain regulates the cleavage and nuclear localization of huntingtin. Future studies that assess the phospho-domain as a whole (i.e. multiple mutations at S419/421/431/432/434) may find a much more powerful regulatory effect. Further data and reagents are needed to test whether these sites work synergistically, and whether priming a specific site is required before phosphorylation at nearby sites. The established assays could be re-tested with various mutations within this huntingtin phospho-domain.

6.1.2 Phosphorylation and Other Sites of Cleavage

One focus of this thesis work has been to specifically examine the affects of S421 phosphorylation on the cleavage of huntingtin at aa586. The data suggests that this is not the only site of cleavage regulated by S421, and cleavage at aa552 and aa513 may also be altered. Immunofluorescence data suggests that the 1-552aa and 1-513aa fragments are cleaved at a different subcellular location and/or are trafficked differently than 1-586aa. Further studies are needed to understand the relationship between these cleavage events. *Is cleavage at these sites sequential (ie aa586, followed by aa552 and aa513)? Are these mutually exclusive cleavage pathways (ie cleavage at aa586 or aa552 or aa513)?* The aa552 and aa513 cleavage pathways could be alternate, and therefore beneficial, cleavage pathways that reduce aa586 cleavage. Future biochemical studies should address the effect of phosphorylation on aa552 and aa513 cleavage. In addition, cleavage at several calpain and aspartyl protease cleavage sites may follow cleavage at caspase sites and are also potentially modified by phosphorylation at the aa419-434 phosphodomain.

6.1.3 Phosphatase Regulation

Initial data suggest that huntingtin interacts with several phosphatases. These interactions will be crucial for regulating the phosphorylation of huntingtin and are important potential targets of

therapeutic intervention. Further biochemical and functional characterization of these interactions are required. In addition, specific phosphatases that are enriched in the nucleus and the striatum are also interesting theoretical candidates for the following reasons:

Many phosphatases are enriched in the nucleus where they dephosphorylate key substrates involved in nuclear processes (Moorhead et al., 2007). An alternate explanation to reduced nuclear phosphorylation of huntingtin is that S421 is targeted specifically by nuclear phosphatases, such as the nuclear isoforms of PP1 (Jagiello et al., 2000; Tran et al., 2004). The interaction of nuclear-specific phosphatases and huntingtin is unexplored and could be an interesting avenue of investigation that fits well with what is currently known about huntingtin function.

The striatal enriched tyrosine phosphatase (STEP, PTPN5) (Braithwaite et al., 2006) is an interesting candidate phosphatase for huntingtin for several reasons. First, although no phosphorylated tyrosines in huntingtin have been described, bioinformatic tools predict several sites of tyrosine phosphorylation. Tyrosine phosphorylation is an unexplored mechanism of huntingtin regulation. Second, many tyrosine phosphatases are found to have dual activities on serines/threonines (Alonso et al., 2004) and STEP may dephosphorylate S421 directly. Third, the expression pattern of STEP is very similar to the pattern of neuropathology seen in HD. The mRNA expression of STEP (Lombroso et al., 1991), the deficits of basal S421 phosphorylation (Warby et al., 2005) and degeneration in HD (Van Raamsdonk et al., 2005a) are greatest in the striatum (specifically the medium-spiny neurons), somewhat less in the cortex and absent in cerebellum. If STEP is a phosphatase acting on huntingtin, the expression pattern of STEP may be a crucial determinant of the pattern of pathology in HD.

6.1.4 Huntingtin as a HEAT Repeat Protein

Understanding the HEAT repeat structure of huntingtin is a crucial and underrepresented area of investigation in HD. Huntingtin is structurally a complicated molecule that is almost entirely composed of solenoid-shaped HEAT repeats. This fact is almost completely ignored by studies utilizing fragments of huntingtin. A great deal of information about huntingtin can be inferred through understanding other HEAT repeat proteins and more effort is required to examine the structural biology of huntingtin. The HEAT repeat structure of huntingtin will interact with

posttranslational modifications of the protein and studies in each of these fields will inform each other.

6.1.5 Protein Interactions

Huntingtin is largely composed of HEAT repeats that are believed to mediate its protein-protein interactions. Phosphorylation within the aa419-434 phospho-domain, including phosphorylation at S421, might be expected to modify these interactions. However, S421-dependent interactions with huntingtin have not yet been identified. Simplified binding assays, such as yeast-2-hybrid, may not sufficiently capture the complex binding interactions mediated by HEAT repeats and may require more specific co-immunopurification studies. As well, the effects of multiple phosphorylation sites, rather than a single site, may be required for altered binding. It could be expected that altered protein-protein interactions would mediate the effects of phosphorylation on cleavage and nuclear localization and are important areas of future investigation.

6.1.6 Other Phosphorylation Sites of Huntingtin

Bioinformatic and mass spectrometry analysis suggest that huntingtin contains many other sites of phosphorylation outside the aa419–434 region. Clusters of phosphorylation at aa1181-1201 and aa2553-2657 may comprise other regulatory phosphodomains that are completely unexplored. Assays performed in this thesis can serve as a framework for these future studies.

Specifically, phosphorylation at S1181 is of particular interest because it falls within a putative NLS domain. Previous studies have reported conflicting results over the function of this NLS (Bessert et al., 1995; Hackam et al., 1999b; Xia et al., 2003), perhaps because its regulation by phosphorylation was not considered at that time.

6.1.7 Phosphorylation and Other Posttranslational Modifications

Multiple types of posttranslational modifications often work in concert to regulate protein function (Yang, 2005; Seet et al., 2006). Often one posttranslational modification may regulate the amount of change, the direction of change or work in opposition to other modifications on the same substrate. There are many examples of co-regulation between palmitoylation and phosphorylation of a substrate (Soskic et al., 1999; Hawtin et al., 2001; Dorfleutner and Ruf, 2003; Ponimaskin et al., 2005) and it is possible that phosphorylation and palmitoylation of huntingtin at C214 (Yanai et al., 2006) modulate one another and ultimately the subcellular localization of huntingtin.

6.1.8 Genetic Modifiers of Posttranslational Modifications

Modifying the function of proteins that are integral to the pathogenesis of HD should modify characteristics of the disease for HD patients. Single nucleotide polymorphisms (SNPs) are common in the human genome and some are known to modify the function or expression of proteins. If a specific protein is important in the pathogenesis of HD, then polymorphisms that modify its function should alter the disease presentation. This testable hypothesis, using standard gene-association studies, is an important test of a protein's functional significance in HD pathogenesis.

The age of onset of the disease is a critical disease characteristic that is known to be modified by genetic factors. Age of onset is highly dependent on the CAG-repeat size, however, a great deal of heritable variability exists, especially in the incomplete penetrance (36-39) and lower range of affected CAG sizes (40-45). Important functional SNPs therefore should be associated with changes in the age of onset of HD. Characterized SNPs that could function to modify phosphorylation of huntingtin are present in Akt (Matsubara et al., 2001), CDK (Pellegata et al., 2006), PI3-kinase (Campbell et al., 2004), PTPs (Di et al., 2002; Criswell et al., 2005), and IGF1 (Walenkamp et al., 2005).

6.1.9 APC as a Functional Analog to Huntingtin

What can we learn from proteins that seem to perform similar functions to huntingtin?

Other authors (Truant et al., 2007) have pointed out similarities between huntingtin function and proteins operating the Wnt signalling pathway. Wnt signalling is important for developmental gene regulation and occurs through a series of interactions starting at the cell surface, leading into the nucleus and back again (Gordon and Nusse, 2006). The adenomatous polyposis protein, APC (Nathke, 2004) is regulated by Wnt signalling and has many similarities to huntingtin:

i) Structurally, APC is a large protein (310kDa) composed of ARM/HEAT repeats (Peifer et al., 1994) and NES domains (Truant et al., 2007) similar to huntingtin.

ii) APC functions as a scaffolding protein and is involved in a variety of biological processes

iii) Cleavage and release of N-terminal fragments of APC are important in its function and are linked to disease

iv) APC interacts with the cytoskeleton and shuttles in and out of the nucleus

In the Wnt signalling pathway, APC binds beta-catenin, a transcriptional regulator, in the nucleus and mediates its export to the cytoplasm where it is degraded (Rubinfeld et al., 1996). Functionally, this is analogous to the regulation of transcriptional regulators, such as REST, by huntingtin. Interestingly, the phosphorylation of APC plays a key role in its function (Rubinfeld et al., 1996; Ikeda et al., 2000; Fodde et al., 2001) and nuclear localization (Zhang et al., 2001). The speed and energetic efficiency of phosphorylation make it useful as a signalling mechanism. A signalling molecule can be involved in multiple rounds of signalling, or even within multiple circuits, without requiring protein synthesis or degradation. Together, huntingtin and APC could be grouped into a class of large scaffolding proteins containing HEAT/ARM repeats that function as signalling molecules and may have similar cellular itineraries.

6.2 CONCLUSION

Posttranslational modification of huntingtin is the primary means of regulating its cellular function. Phosphorylation, palmitoylation, cleavage, and other modifications dictate how huntingtin is moved in the cell, how it binds to other molecules, and when it is degraded. Phosphorylation at S421 is a dynamic means of regulating huntingtin and is implicated in HD because it is reduced by the polyglutamine-expansion and modifies the cleavage and nuclear localization of huntingtin. The findings from the characterization of S421 phosphorylation of huntingtin suggest that posttranslational modifications of huntingtin can modify crucial pathogenic processes and open new therapeutic possibilities for HD.

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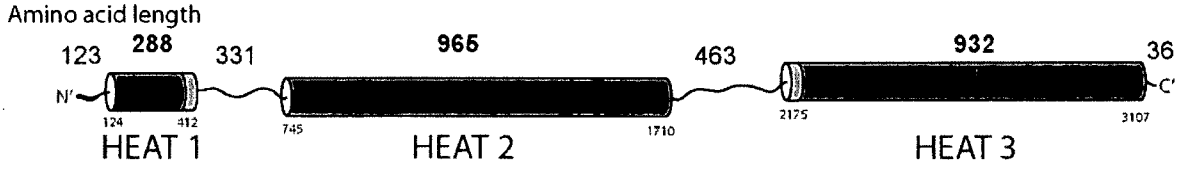
APPENDIX A HEAT Repeat Domains of Huntingtin

A.



B.

	aa_start	aa_end	SEQUENCE	Score	Gap	Domain
1	124	162	QKLLGIAMELFLLCSDDAESDVRMVADECLNKVIKALMD	1510	**43**	**1**
2	205	243	RPYLVNLLPCLTRTSKRPEESVQETLAAAVPKIMASFGN	1990	**4**	**1**
3	247	285	DNEIKVLLKAFIANLKSSSPTIRRTAAGSAVSICQHSRR	1590	**2**	**1**
4	287	317	QYFYSWLLNVLLGLLVPVEDEHSTLLILGVL	1010	**1**	**1**
5	318	355	LTLRYLVPLLQQQVKDTSLKGSFGVTRKEMEVSPSAEQ	1620	**17**	**1**
6	372	412	HNVVTGALELLQQLFRTPPPELLQTLTAVGGIGQLTAAKEE	960	**333**	**1**
7	745	783	EYPEEQYVSDILNYIDHGDPQVRGATAILCGTLICSILS	1250	**20**	**2**
8	803	841	TFSLADCIPLLRKTLKDESSVTCKLACTAVRNCVMSLCS	1500	**4**	**2**
9	845	883	SELGLQLIIDVLTLRNSSYWLVRTELLETLAEIDFRLVS	1020	**21**	**2**
10	904	942	KLQERVLNNVVIHLLGDEDPRVRHVAAASLIRLVPKLFY	1930	**42**	**2**
11	984	1025	RIYRGYNLLPSITDVTMENNLSRVIAAVSHELITSTTRALTF	1370	**112**	**2**
12	1137	1176	VPMVEQLFSHLLKVINICAHVLDDVAPGPAIKAALPSLTN	1260	**113**	**2**
13	1289	1327	GKCVEEILGYLKSCFSREPMMATVCVQQLLKTLFGTNLA	1030	**98**	**2**
14	1425	1463	RLFEPLVIKALKQYTTTTCVQLQKQVLDLLAQLVQLRVN	1370	**30**	**2**
15	1493	1531	EAIIPNIFFFLVLLSYERYHSKQIIGIPKIIQLCDGIMA	1100	**3**	**2**
16	1534	1575	RKAVTHAIPALQPIVHDLFVLRGTNKADAGKELETQKEVVVS	1280	**35**	**2**
17	1610	1648	RQIADIILPMLAKQQMHIDSHEALGVLNTLFEILAPSSL	1280	**24**	**2**
18	1672	1710	QLWISGILAILRVLISQSTEDIVLSRIQELSFSPYLISC	1350	**465**	**2**
19	2175	2210	LARVSGTVQQLPAVHHVFQPELPAEPAAYWSKLNDL	970	**1**	**3**
20	2211	2230	FGD.AALYQSLPTLARALAQY	1110	**1**	**3**
21	2231	2268	LVVVSKLPSHLHLPPEKEKDIVKFVVATLEALSWHLIH	1170	**116**	**3**
22	2384	2422	SGVPAFLTPLLRNIIISLARLPLVNSYTRVPPLVWKLGW	1110	**54**	**3**
23	2476	2514	VLVTQPLVMEQEESPPEEDTERTQINVLAVQAITSLVLS	1000	**284**	**3**
24	2798	2836	DDTAKQLIPVISDYLLSNLKGIAHCVNIHSQQHVLVMCA	1430	**139**	**3**
25	2975	3013	ARVVARILPQFLDDFFPPQDIMNKVIGEFLSNQQPYPQF	1250	**55**	**3**
26	3068	3107	SPWVAAILPHVISRMGKLEQVDVNLFCLVATDFYRHQIEE	1160	**37**	**3**
	3144					

Figure 6.1: Predicted AAA_Class HEAT repeats in human huntingtin.
(A) Schematic of HEAT repeat domains in huntingtin. The length (aa) of each domain and intervening sequence is indicated. (B) REP tool predictions of each HEAT repeat location. Score indicates the likelyhood of forming a domain, higher score is better. GAP is the distance (aa) to the next HEAT repeat. HEAT domains are hypothesized based on the clustering of the HEAT repeats.

APPENDIX B Methods

B.1 REAGENTS AND SAMPLES

B.1.1 Tissue Culture

Cell lines used during the course of the thesis work are listed in Table 6.1. Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS + L-glutamine (GIBCO). All lines were incubated at 37°C and 5% CO2 except for ST14A cells, which proliferate at 33°C and are differentiated at 37°C (Cattaneo and Conti, 1998; Ehrlich et al., 2001).

Table 6.1: Cell lines

NAME	SOURCE	CELL TYPE
ST14A	Rat	Primordial (day 14) striatal neuron
293A	Human	Kidney
COS-1	African Green Monkey	Kidney
HeLa	Human	Cervix; epithelial; adenocarcinoma
N2A	Mouse	Brain/neuroblast
Primary Striatum	FVB Mouse	Striatal
CHO	Chinese Hamster	Ovary
3T3	Mouse	Fibroblast, embronal
HBL-100	Human	Breast epithelial
LA-9	Mouse	Fibroblast
MCF7	Human	Mammary Gland
BHK-21	Hamster	Kidney
MDA-MB 231	Human	Breast adenocarcinoma
MDA-MB 453	Human	Breast carcinoma
NT2	Human	Fibroblast, embronal, carinoma
SH-SY5Y	Human	Neuroblastoma
PC12	Rat	Adrenal gland; pheochromocytoma
Rat2	Rat	
BOSC23		MSCV Viral packaging cell line

B.1.2 Mouse Lines

All *in vivo* work, including YAC and wildtype mice, was done with mice from the FVB strain. Mice were housed either at the CMMT or the Wesbrook Building at UBC.

The YAC18Q (line 212) and YAC128Q (line 53) mouse models contain the full-length genomic human HD gene with its endogenous promoter elements, providing appropriate developmental and tissue specific expression of the huntingtin protein (Slow et al., 2003). Cognitive, behavioural and neuropathological features of the YAC128 mouse model have been studied extensively (Slow et al., 2003; Van Raamsdonk et al., 2005a; Van Raamsdonk et al., 2005c). The use of mice for research was approved in UBC animal care certificate A04-0073.

B.1.3 Antibodies

Table 6.2 and Table 6.3 list the antibodies used during the course of the thesis work. Western immunoblotting against Akt and pAkt were performed with both BD Pharmingen and Cell Signaling antibodies and found to produce equivalent data.

Table 6.2: Commercial Antibodies

1° ANTIBODY	SUPPLIER	CATALOG	SOURCE	EPITOPE	WORKING DILUTION
Actin	Chemicon	mAb150IR	mouse mAb		1:5000
Actin (AC40)	Sigma	A4700	mouse mAb	C-term Actin	
Huntingtin	Chemicon	mAb2166	mouse mAb	aa443 - 457	1:2000
Huntingtin (EM48)	Chemicon	mAb5374	mouse mAb	aa1-256 (no polyQ)	
Huntintingtin (1C2)	Chemicon	mAb1574	mouse mAb	homopolymeric Q	
Huntingtin (pS421)	Rockland	600-401-433	rabbit pAb	aa416-474	
pAkt (S473)	BD	BD559029	rabbit pAb	P-SER472/473/474	1:833
pAkt (S473)	Cell Signaling	CS4051	mouse mAb	pSER473	
pAkt (T308)	Stressgen	KAP-PK007	rabbit pAb	pT308 specific	
Akt-1	BD	BD559028	rabbit pAb	Akt-1 aa466-479	1:600
Akt-1	Cell Signaling	5G3 2966	mouse mAb	Akt-1 aa140-480	
GAPDH	Chemicon	mAb374	mouse mAb		1:10,000
Caspase 6 p20	Santa Cruz	sc15381	rabbit pAb	human aa24-113	1:500
Caspase 6 active	Cell Signaling	9761	rabbit pAb	C6 cleaved @ asp162	
Caspase 3	Santa Cruz	SC7272	mouse mAb	aa1-277	1:1000
Caspase 2	Santa Cruz	SC625	rabbit pAb	human C2 C-term	1:500
γ-Tubulin	Abcam	11316	mouse mAb	aa38-53	
HA	Covance		mouse mAb	YPYDVPDYA	1:1000
REST	Upstate	07-579	rabbit pAb	human aa801-1097	
GM130	BD	610822	mouse mAb	rat aa869-982	
GSK-3B	Cell Signaling	9332	rabbit pAb	human	
pSGK	Upstate	36-002	rabbit pAb	around S255/T256	
SGK	Upstate	26066	rabbit pAb	aa399-412	
Ubiquitin	Upstate	07-375	rabbit pAb	bovine ubiquitin	
pS/T Akt Substrate	Cell Signaling	9611	rabbit pAb	K/RXK/RXXS/T	
PARP	Cell Signaling	CS9542	rabbit pAb	caspase site in PARP	1:1000
β-Tubulin	Sigma	T4026	mouse mAb	rat brain tubulin	1:2000

Table 6.3: Antibodies generated in the Hayden Lab

1° ANTIBODY	SOURCE	EPITOPE	REFERENCE	WORKING DILUTION
pS421	rabbit pAb	RSG(pS)IVELIAGC	(Warby et al., 2005)	1:2000
BKP1mono	mouse mAb	aa1-17	(Kalchman et al., 1996)	1:2000
BKP1poly	rabbit pAb	aa1-17	(Kalchman et al., 1996)	1:2000
HD650	mouse mAb	human specific @ aa650	(Slow et al., 2003)	1:2000
Hap 1	rabbit pAb	aa595-609	(Chan et al., 2002b)	1:3
Hip 14	rabbit pAb	aa49-60	(Singaraja et al., 2002)	1:2000
neo513	mouse mAb	CHTLQADSVD	(Wellington et al., 2002)	1:1000
neo552	mouse mAb	CSDPAMDLND	(Wellington et al., 2002)	1:1000
neo586	mouse mAb	IVLD	(Chapter 3)	1:1000

B.1.4 Generation of anti-pS421-huntingtin antibody

Rabbits were immunized with peptide RSG(pS)IVELIAGC conjugated to KLH (complete Freund's adjuvant) followed by repeated boosts (incomplete Freund's adjuvant). Serum was collected 49 days after the initial immunization and screened in ELISA assays. Serum displaying strong immune response was used for double affinity purification. Serum was passed over a column containing the peptide RSGSIVELIAGC and the flow-through was affinity-purified on a column containing RSG(pS)IVELIAGC phospho-peptide. This procedure resulted in an antibody that specifically detects huntingtin only when phosphorylated on S421.

B.1.5 Generation of the neo-Epitope Huntingtin Antibodies

The neo586 polyclonal antibody was generated from the ^{583}IVLD^{586} epitope found at the caspase-6 cleavage site in huntingtin at aa586. Characterization of the neo552 and neo513 antibodies has been described in detail previously (Wellington et al., 2002).

B.2 DNA CONSTRUCTS AND CLONING

B.2.1 DNA Constructs

The plasmids containing various cDNA constructs used during the course of the thesis work are listed in Table 6.4 and Table 6.5. DNA was generated from these constructs in bacteria (usually DH5α or STBL2 bacterial lines) using standard methods.

Table 6.4: Huntingtin DNA Constructs

ID	PolyQ	Mutation	nt	aa	Vector	Reference
1955	15, 128	-	1-1955	1-548	pcineo	(Goldberg et al., 1996b)
3949	15, 128	-	1-3949	1-1212	pcineo	(Wellington et al., 2002)
3949-4C	128	-	1-3949	1-1212	pcineo	(Chapter 4)
	128	S421A	1-3949	1-1212	pcineo	(Chapter 4)
	128	S421D	1-3949	1-1212	pcineo	(Chapter 4)
3949-5C	128	-	1-3949	1-1212	pcineo	(Chapter 4)
	128	S421A	1-3949	1-1212	pcineo	(Chapter 4)
	128	S421D	1-3949	1-1212	pcineo	(Chapter 4)
10366-HA	15,128	-	1-10366	1-3144	pcineo	(Chapter 3)
	15,128	S421A	1-10366	1-3144	pcineo	(Chapter 3)
	15,128	S421D	1-10366	1-3144	pcineo	(Chapter 3)
MSCV-10366	15	-	1-10366	1-3144	MSCVpuro	(Chapter 3)
	128	-	1-10366	1-3144	MSCVhygro	(Chapter 3)
	15,128	S421A	1-10366	1-3144	MSCVpuro	(Chapter 3)
	15,128	S421D	1-10366	1-3144	MSCVpuro	(Chapter 3)
1-513aa	15,128	-	1-1853	1-513	pcineo	(Wellington et al., 2002)
1-552aa	15,128	-	1-1969	1-552	pcineo	(Wellington et al., 2002)
1-586aa	15,128	-	1-2071	1-586	pcineo	(Chapter 4)

Table 6.5: Non-huntingtin DNA constructs

ID	Mutation	Vector	Reference
Akt-myr	myr (constitutively active)	pUSEamp	Upstate
Akt-DN	dominant negative	pUSEamp	Upstate
Caspase-6		pCMVsport6	Mamalian Genome Collection
CMV-LacZ		pCMVsport6	Invitrogen
CMV-pcineo	(empty vector)	pCMVsport6	Invitrogen
gag/pol	(MSCV packaging)		Clontech
env	(MSCV packaging)		Clontech

B.2.2 Site Directed Mutagenesis of Huntingtin at pS421

Site directed mutagenesis was used to generate S421 phosphorylation mutants: changing S421 to an alanine (S421A) renders huntingtin unphosphorylatable while changing to an aspartic acid (S421D), with a large acidic side-chain, mimics constitutive phosphorylation (pseudo-phosphorylation). These S421 substitutions were created using a site-directed mutagenesis approach as previously described (Wellington et al., 2000) and confirmed by sequencing.

The mutations were first generated in the huntingtin fragments containing the Xho1-Kpn1 fragment of huntingtin and then subcloned into full length huntingtin vectors. For S421 phosphorylation mutants, the mutagenesis primers (AGT–GCT = S-A and AGT–GAT = S-D) and cloning primers are listed in Table 6.6.

Table 6.6: Primers used for site directed mutagenesis at S421
Mutations sites at S421 in the mutagenesis primers are highlighted

Cloning Primers	
Xho-F:	CAAGGTTACAGCTCGAGCTCTATAAG
Kpn-R:	CAAATACTGGTTGTCGGTACCGTCTAAC
Mutagenesis Primers	
S421A-F:	GAAGCCGTAGTGGG**GCT**ATTGTGGAAC
S421A-R:	GTTCCACAAT**AGC**CCCACTACGGCTTC
S421D-F:	GAAGCCGTAGTGGG**GAT**ATTGTGGAAC
S421D-R:	GTTCCACAAT**ATC**CCCACTACGGCTTC

B.2.3 Generation of Huntingtin pS421 Vectors

B.2.3.1 MSCV Viral Vectors

MSCV viral vectors infect murine cells and were generated to infect ST14A cell lines. The site-directed Xho1-Kpn1 fragment containing the phosphorylation mutations was cloned into MSCVpuro vectors (Clontech). Linkers were used to assist cloning: Not1 and Mlu1 linkers at the EcorR1 and BglII sites in the vector respectively. The quadruple ligation involved the following fragments: MluI/XhoI (htt N-terminal containing 15Q or 128Q), XhoI/KpnI (containing the phosphorylation mutants), KpnI/NotI (containing the C-terminal of huntingtin) and NotI/MluI (the MSCVpuro vector backbone). All constructs were cloned into the MSCVpuro vector, except for 10366-128-S421 which was originally cloned into MSCVhygro.

B.2.3.2 3949 Cleavage Mutants

Constructs (1-3949nt) containing both the cleavage and phosphorylation mutations were generated to examine the role of phosphorylation on S421on cleavage at 586aa. The cleavage mutants were used to enhance the cleavage at aa586: the 4C constructs can only be cleaved at aa586, while the 5C cannot be cleaved at any caspase site. The 1-3949nt +4C or +5C constructs

were generated by ligating the Bsu36I fragment (containing the 4C and 5C region) from 3949-pfrt into 3949-pCIneo and performing site directed mutagenesis at S421 (see primers above).

B.2.3.3 Full-length 10366-HA tagged

Full length huntingtin containing the S421 phosphorylation mutants (S421A, S421D) were generated for transient transfection of cultured cells. The PshA1/XmaI fragment of huntingtin containing S421 (from MSCV vectores) was cloned into 10366-15Q and -128Q in pcineo.

B.2.4 Generation of Stable ST14 Cell Lines

For the purpose of protein production and biochemical studies, ST14A cells were infected with MSCV virus to stably express full length huntingtin containing the S421 phosphorylation mutations. First, viral supernatant is produced by transient transfection (CaPO4 precipitation method) of the viral components into BOSC23 cells plated in 10cm culture dishes. Along with the MSCV vectors, the gag/pol and env packaging vectors need to be transfected. The viral supernatant (media from the B23 cells) were harvested at 24, 48 and 72 hours, filtered (0.22um) and frozen immediately at -80°C. The media was replaced (DMEM +10%FBS) on the cells after each harvest.

To infect the ST14A cells, cell growth media was replaced with viral supernatant + 1uL polybrene for 6-10 hours. Fresh media was added for 24 hours, then replaced again with fresh media containing selection (Puro selection: 2 days 1.5 ug/ml Puro, G418 selection: 5-7 days; 900 ug/ml, Hygro selection: 2-3 days, 300 ug/ml). Cells were harvested to check for protein expression 3 days later.

B.3 CELL LYSIS AND TISSUE PREPARATION

B.3.1 Transient Transfection

The majority of transient cell transfections were performed with Fugene 6 (Roche) following the manufacturer's standard instructions. Briefly, cells were transfected in 6 well (1 ug DNA + 3 uL Fugene reagent) or 10cm dishes (8ug DNA + 24uL Fugene reagent) and then harvested 18 hours later. Calcium phosphate (CaPO4) precipitation transfections were also performed, usually on the 293 or BOSC23 cell lines at 40-50% confluency. For each 6 well dish, the pre-transfection

mixture (2ug DNA, 45uL ddH20, 5uL 2.5M CaCl2) was added dropwise to 50uL 2XBES pH7.0 and then added dropwise to the cells and left for 5-6 hours. Transfection was replaced with normal growth media and cells were harvested 24-72 hours later.

B.3.2 Cell Lysis

B.3.2.1 Cultured Cells

To harvest the cultured cells, SDP+ lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Igepal, 40 mM NaF, 1 mM PMSF, 2 mM sodium vanadate, 1X protease inhibitors (Roche) and 5uM zVAD) was added to the plates and left on ice for 5 minutes. The cells were scraped, sonicated briefly (3 x 10s on ice) and centrifuged at 10,000 RCF for 15 min at 4°C. Protein concentration was assessed with the Bradford assay (BioRad) using 1mL volumes.

B.3.2.2 Fresh Brain Lysates

To generate fresh brain lysates, FVB wildtype or YAC transgenic mice were sacrificed with CO_2 and the brain was isolated and homogenized with a Dounce homogenizer in SDP+ lysis buffer. Samples were Dounce homogenized for 15 seconds on ice and then remained on ice for an additional 10 mins. Samples were sonicated briefly (3 x 10s on ice) and centrifuged at 10,000 RCF for 15 min at 4°C. Protein concentration was assessed with the Bradford assay (BioRad) using 1mL volumes..

B.3.2.3 Frozen Brain Lysates

Frozen tissue was used for human, R6/2 and occasionally YAC or wildtype brains. To generate lysates from frozen tissue, a motorized tissue homogenizer was used. Samples were homogenized immediately from a frozen state for 10s and kept on ice. Samples remained on ice for an additional 10 mins and were further processed as per fresh samples. Control human samples (Figure 2.2) were from the frontal cortex of a 68-year-old male. The R6/2 mouse samples (Figure 2.5), which express a truncated N-terminal fragment of huntingtin (Mangiarini et al., 1996) were sacrificed with sodium pentobarbital. The tissue was extracted, snap frozen in liquid nitrogen and stored in the -80 freezer until it was processed. The tissue was homogenized using a motorized Teflon pestle (Sigma) in 0.32M Sucrose with Complete Mini (Roche) protease inhibitors and PMSF.

B.4 IMMUNOPRECIPITATIONS

B.4.1 Huntingtin Immunoprecipitation

Immunopurification of huntingtin was performed to purify and isolate huntingtin (along with its bound protein interactors). Sepharose G beads (25uL) were washed and pre-bound with anti-huntingtin mAb2166, BKP1mono, or the human huntingtin-specific HD650 (roughly 2ug antibody) for 1-2 hours. Lysates (250ug to 2mg) were incubated with the beads for two hours, rotating at 4 °C and then washed three times with SDP+.

B.4.2 Akt Immunoprecipitation

For co-immunoprecipitation studies with Akt (Figure 2.1), brains from 6 month old wild-type, YAC18Q, or YAC128Q transgenic mice were isolated and homogenized in homogenization buffer (10 mM Hepes-KOH pH 7.4, 0.83 mM Benzamidine, 0.25 mM PMSF, 0.5 ug/ml leupeptin, 0.5 ug/ml aprotinin) followed by centrifugation at 800 *g* for 5 min at 4 °C. The supernatant was isolated and centrifuged at 205,000 x g for 30 min at 4 °C. The protein concentration was measured in the supernatant and after pre-clearing, 1 mg protein was incubated with 5 uL of a mouse anti-Akt antibody (5G3, Cell Signalling) or normal mouse IgG in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 ug/ml aprotinin, 1 ug/ml leupeptin, 1 mM $NaVO_4$, 1 mM β-glycerophosphate, 2.5 mM sodium-pyrophosphate, PefaBloc, 2.5 mM NaF for 4 h at 4°C. After incubation, samples were washed three times in PBS and prepared for immunoblot analysis.

B.5 IMMUNOBLOTTING

Standard procedures were used for most immunoblotting. Briefly, sample preparation involved denaturing the lysates or immunoprecipitates in Invitrogen nupage LDS Sample Buffer (NP0007) or SDS gel loading buffer and heating for 10mins at 70°C. For proteins of smaller molecular weight (30 – 150kDa), such as Akt and caspases, either 4-12% bis-tris (Invitrogen) or standard 10% polyacrylamide gels were used. The gels were run for 1-2 hours at 200V. Transfers were performed onto Immobilin-P or Immobilon-FL membranes at 30V for 1-2 hours in the Invitrogen gel boxes using Towbin transfer buffer (25mM Tris, 192mM glycine, 10%

MeOH) or Invitrogen transfer buffers. Blocking was performed with 5% milk or 5% BSA depending on the primary antibody.

B.5.1 Huntingtin Immunoblotting and Allelic Separation

Huntingtin is a large protein that is prone to aggregation and irregular formations, and therefore has a tendency to run irregularly on a normal acrylamide gel. In order to achieve clean and sharp resolution of huntingtin for quantitative immunoblotting, numerous gel systems with different running conditions and chemistries were tested. For full-length huntingtin, a 3-8% tris-acetate gel (Invitrogen) gave vastly superior results to even large (30cm) linear gradient acrylamide gels. One hour at 200V is sufficient to clearly separate polyglutamine-expanded (128Q) from wildtype (18Q) huntingtin. Transfers were also performed using the Invitrogen system, using Immobilon-P or Immobilon-FL PVDF membranes and Invitrogen transfer buffers.

B.5.2 Quantitative Immunoblotting

Quantitation was originally performed using standard HRP-conjugated secondaries, ECL, film and Quantity 1 software from BioRad. Subsequently (after 2005), all immunoblots were used with fluorescently labeled secondaries, Immobilon-FL membranes and the Licor Odyssey immunoblotting system. Reliable quantitation relies on clean, crisp separation of bands that are of moderate exposure (not over/underexposed). For statistical analysis of immunoblotting, groups were compared based on the numerical densitometry results from either BioRad Quantitiy 1 or Licor Odyssey software. A t test was performed with 2 groups, one way ANOVA performed for more than 2 groups. P values <0.05 were considered to be statistically significant.

B.6 MOLECULAR ASSAYS

B.6.1 pS421 Assay

To determine the basal S421 phosphorylation status of huntingtin from cell cultures and brain tissues, huntingtin is first immunopurified and then immunoblotted with the pS421-specific antibody. Immunopurification of huntingtin (see above) in SDP+ lysis buffer prior to the immunoblot allows for a clean blot with a strong pS421 signal, allowing for accurate quantitation. Immunopurification of huntingtin with either mAb2166, BKP1 monoclonal or HD650 gave similar results. Quantitation of pS421 from straight lysate is not recommended.

The pS421 status can be compared between treatments (YAC18 vs YAC128 for example) by quantitative immunoblotting by comparing the intensity of the pS421 band as a ratio to the total huntingtin (probed with mAb2166 or other total huntingtin antibody). In order to separate the transgenic (human) huntingtin from the endogenous mouse *Hdh* gene product, the human-specific HD650 was used for the immunoprecipitation.

B.6.2 Kinase and Phosphatase Assays

Kinase and phosphatase treatment was used to modify the phosphorylation status of huntingtin. Purified Akt1/PKBalpha (Upstate) was used for *in vitro* phosphorylation and Lambda Protein Phosphatase (gamma-PPase - New England Biolabs) or PP2A (Upstate) was used to dephosphorylate huntingtin. For *in vitro* kinase reactions, immunoprecipitates were washed, resuspended in assay dilution buffer (20mM Mops pH 7.2, 25mM β-glycerophosphate, 5 mM EGTA, 18 mM MgCl2, 5 mM sodium vanadate and 1 mM DTT), and incubated with 0.5 units of purified Akt at 30 °C for 1 hour. For *in vitro* phosphatase reactions, immunoprecipitates were washed, resuspended in phosphatase buffer (20 mM Hepes pH 7.6, 1 mM MnCl2, 100 ug/mL BSA and 1 mM DTT) and treated with PP2A or Lamda phosphatase for 30 minutes at 30 °C.

B.6.3 Cleavage Assay

To determine the effect of phosphorylation on proteolysis we examined cleavage of huntingtin in *ex vitro* (cell free conditions with purified reagents) and *in vitro* (in cultured cells) conditions.

B.6.3.1 Ex vitro

E*x vitro* experiments were conducted by purification of huntingtin from cell lines or mouse brain and treatment with purified caspases. The advantage of these types of experiments with purified reagents is that the active protease is known, and it eliminates the influence of other (unknown) proteins. Phosphorylation mutants were purified from transfected COS, N2A, ST14, or HEK293 cells and proteolysis assessed by immunoblot analysis following treatment with purified calpains (μ and m) and caspases (1, 2, 3 and 6). Huntingtin was purified using BKP1 monoclonal and then treated with proteases in caspase assay buffer (50mM Hepes pH 7.4, 100mM NaCl, 0.1% NaCl, 0.1% CHAPS, 1mM EDTA, 10% glycerol and 10mM DTT) for 1 hour at 37 degrees. Cleavage products were detected by immunoblotting with anti-huntingtin antibodies. The molecular weight of the cleavage products and differential detection with different anti-huntingtin antibodies was used to identify the cleavage bands. Additionally, modifying the

phosphorylation status of wildtype huntingtin was performed directly by using purified kinases (Akt) or phosphatases (PP2A or Lamda phosphatase) (see above).

B.6.3.2 In vitro

In vitro experiments involved treatment of cells to induce cleavage of huntingtin described previously (Wellington et al., 1998). The advantage of these experiments is that cleavage occurs under physiologically relevant conditions that include factors in the cell milieu that could influence the metabolism of huntingtin. To induce apoptosis and huntingtin cleavage, N2A, COS, ST14 or HEK 293 cells were treated with 10nM staurosporine or 35uM tamoxifen for 1 h at 37 °C. Cell lysates were generated and cleavage products were detected by immunoblotting for huntingtin. Physiological modulation of phosphorylation was achieved by co-transfection of active (myristoylated) or dominant-negative Akt constructs prior to treatment and cleavage.

B.6.4 Nuclear / Cytoplasmic Separation

Two methods were used for crude nuclear / cytoplasmic separation of lysates: NE-PER kit (Roche) and the EDBC- method. The NE-PER kit uses buffers and a protocol supplied by the manufacturer. The EDBC method is based on previously published methods (Erin Clabough, (Clabough and Zeitlin, 2006)). Cells were harvested by trypsinization, lysed in Cell Lysis Buffer (5mM PIPES pH 8.0, 85mM KCl, 0.5% NP40) containing protease inhibitors (Roche complete protease inhibitor, sodium orthovanadate (1mM), PMSF (800μM) and zVAD (5μM)), and incubated on ice for 30 min. Samples were spun down (4min, 8200g, 4°C) to pellet crude nuclei and supernatant (cytoplasmic fraction) removed to a fresh tube. The crude nuclear pellet was washed once in ice cold PBS and respun (4min, 8200g, 4°C). Supernatant was discarded and the nuclear pellet lysed in Nuclear Lysis Buffer (10mM EDTA, 50mM TRIS-HCL pH8, 1% SDS) for 30 mins on ice. After incubation, an equal volume of 1% Triton X-100 in TEEN buffer was added and vortexed. Nuclear samples were then sonicated (3x2s) to shear DNA. Bradford assay was used to determine protein concentration of the lysate.

B.7 IMMUNOFLUORESCENCE TECHNIQUES

Immunofluorescence microscopy was performed in cultured cells that were grown on coverslips. Cells were first washed in PBS before fixation. One of two fixation methods were used depending on the primary antibody: Paraformaldehyde (4% (w/v) for 5min) or MeOH (2 mins

with ice-cold MeOH). Many antibodies produced similar staining with both fixation methods, however some antibodies (neo-586, neo-552) only stained appropriately with MeOH fixation. Following fixation, cells were permeabilized in PBS containing 0.3% Triton X-100 for 30 min. Cells were washed, and then blocked in 4% normal goat serum (GIBCO)in PBS for 1h at RT, followed by incubation with the primary antibody in 2% normal goat serum in PBS for 2h at RT or overnight at 4°C. Cells were washed 3x with 1% BSA in PBS, and then incubated in the secondary antibody (Goat anti Mouse Alexa 594 (red): 1/800 or Goat anti Rabbit Alexa 488 (green): 1/800) in 2% normal goat serum in PBS for 2h at RT. Cells were washed 3x PBS before mounting; DAPI staining (1:10,000 in PBS from 10mg/mL stock in 70% ethanol, SIGMA) was included in the first wash (RT 5min). Fluoromount-G (Southern Biotech Cat. #0100-01) was used to mount coverlips on slides. Immunofluorescence was detected using a laser confocal microscope (Biorad) or conventional immunofluorescence microscopy (Zeiss) with a CCD camera (Princeton Instruments Inc.). Images were captured using Metamorph software and stored as separate JPG or TIFF files for each channel.

B.8 BIOINFORMATICS

The prediction of potential phosphorylation sites was performed by submitting huntingtin protein sequence (P42858) to PROSITE (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_prosite.html) and NetPhos (Blom et al., 1999) (http://www.cbs.dtu.dk/services/NetPhos/).

HEAT repeat predictions were performed using submitted to the REP tool (huntingtinp://www.embl-heidelberg.de/~andrade/papers/rep/search.html). REP searches for repeats such as Ankyrin, Armadillo, HAT, HEAT, HEAT_AAA, HEAT_ADB, HEAT_IMB, Kelch, Leucin Rich Repeats, PFTA, PFTB, RCC1, TPR, and WD40 (Andrade et al., 2000).

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