Nuclear Localization of the Huntingtin Protein

Huntingtin Nuclear Localization: Current Insights into Mechanism and Regulation

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ABSTRACT

Huntington's Disease brains display a striking accumulation of huntingtin in the nucleus of striatal and cortical neurons, suggesting that nuclear functions may be key to the onset of cellular pathogenesis. Entry to the nucleus is tightly regulated by a family of import receptors called karyopherins that limit their binding to proteins bearing specific nuclear localization signals (NLS). Although huntingtin is primarily cytoplasmic in healthy neurons, it is also found in the nucleus at low levels and contains a nuclear export signal (NES), suggesting that shuttling to and from the nucleus is part of the protein's normal function. Indeed, recent publications from our lab (Atwal *et al.* 2007 and 2011, and Munsie *et al*. 2011) describe huntingtin's ability to enter the nucleus in response to cell stress, and localize to cofilin-actin rods. We have identified an active NLS near the amino terminus of huntingtin between amino acids 174-207, which is recognized by both karyopherin β2 and β1. While functional in ST*Hdh* and NIH 3T3 cells, the huntingtin NLS lacks activity in HEK 293 and MCF-7 cell lines. This surprising observation suggests that additional levels of regulation exist amongst cell types. We have isolated a shorter sequence within the NLS that localizes to membrane structures throughout the cell (such as the plasma membrane and vesicles). This thesis explores several putative secondary regulatory mechanisms, such as, nuclear export activity, transcriptional dependence, palmitoylation and acetylation. However, the most promising mechanism thus far is masking of the NLS by HAP1 and HMGB1. The functions of these proteins, in vesicular trafficking and autophagy/apoptosis, respectively, may offer further insight into huntingtin's nuclear function. By understanding the underlying regulatory mechanisms of huntingtin nuclear import, we hope to gain further insight into why huntingtin accumulates in the nucleus of specific neurons in HD, and whether or not this mislocalization directly contributes to disease.

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ABBREVIATIONS

8OHdG	8-hydroxydeoxyguanosine
ADP	Adenosine-diphosphate
AFP	Aequorea Victoria
AHI1	Abelson helper integration site 1
AMP	Adenosine-monophosphate
ATP	Adenosine-triphosphate
BAC	Bacterial Artificial Chromosome
Bcl-2	B-cell Lymphoma 2
BDNF	Brain Derived Neurotrophic Factor
CAG	Cytosine-Adenine-Guanine
CBP	cyclic AMP response element-binding protein (CREB) binding protein
CD Spectroscopy	Circular Dichroism Spectroscopy
CRM1	chromosome region maintenance 1
CtBP	Carboxyl-terminal Binding Protein
DIC	Differential Interference Contrast
DMAT	2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DRPLA	Dentatroubo-pallido-luysion Atrophy
EDTA	Ethylenediaminetetraacetic Acid
ER	Endoplasmic Reticulum
eYFP	enhanced Yellow Fluorescent Protein
F-actin	Filamentous Actin
FBS	Fetal Bovine Serum
FLIM	Fluorescence Lifetime Imaging Microscopy
FRET	Förster Resonance Energy Transfer
GFP	Green Fluorescent Protein
G-protein	Guanine Nucleotide Binding Protein
H3	Histone 3
HAP1	Huntingtin Associated Protein 40
HAP40	Huntingtin Associated Protein 40
HAT	Histone Acetyl Transferase
HCL	Hydrochloric Acid
HD	Huntington's Disease
HD	Huntington's Disease Gene
HDAC	Histone Deacetylase
HDF	Hereditary Disease Foundation
Hdh	Huntington's Disease Homolog (murine)

HEAT	Huntingtin, Elongation factor 3, Protein Phosphatase 2A, mTOR
HEK293	Human Embryonic Kidney Cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HIC	Human I-mfa domain-containing protein
HIP1	Huntingtin Interacting Protein 1
HIP14	Huntingtin Interacting Protein 14
HIV	Human Immunodeficiency Virus
HMGB1	High Mobility Group Box 1
hnRNPA1	heterogeneous nuclear Ribonucleoprotein A1
HTT	Huntingtin Gene
Hu	Human
IFI16	Gamma-interferon-inducible Protein 16
InsP3R1	Inositol 1,4,5-triphosphate receptor type-1
iPS	induced Pluripotent Stem Cells
IT15	Interesting Transcript 15
IVS	Intervening Sequence
Kapβ	Karyopherin β
KAT	Lysine Acetyl Transferase
KDAC	Lysine Deacetylase
KIF17	Kinesin Family Member 17
Lamp1	Lysosomal-Associated Membrane Protein 1
LC3	microtubule associated protein 1 light chain-3
mCer	mCerulean
MEF	Mouse Embryonic Fibroblast
mRNA	messenger RNA
MS	Mass Spectrometry
MSN	Medium Spiny Neuron
N17	The amino-terminal 17 amino acids of huntingtin
NCoR	Nuclear Corepressor
NES	Nuclear Export Signal
NF-kappaB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear Localization Signal
NMDA	N-methyl-D-aspartate
NPC	Nuclear Pore Complex
NXF1	Nuclear RNA Export Factor 1
PACSIN1	Protein Kinase C and Casein Substrate in Neurons Protein 1
P-bodies	Processing Bodies
PBS	Phosphate Buffered Saline
PCAF	P300/CBP Associated Factor
PCM1	Pericentriolar Material 1
PCR	Polymerase Chain Reaction

PKC	Protein Kinase C
PP2A	Protein Phosphatase 2A
PSD-95	Post Synaptic Density 95
PVDF	Polyvinylidene fluoride
PY-NLS	proline-tyrosine nuclear localization signal
Q	Glutamine
RanGAP	Ran-GTPase Activating Protein
RanGDP	Ran-Guanine diphosphate
RanGEF	Ran-Guanine nucleotide exchange factor
RanGTP	Ran-Guanine triphosphate
Rb	Retinoblastoma Protein
REST	Repressor Element 1 Silencing Transcription Factor
REV	Regulator of Viron Expression
RFLP	Restriction Fragment Length Polymorphism
RhoGEF	Rho Guanine Nucleotide Exchange Factor
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SBMA	Spinal Bulbar Muscular Atrophy
SCA	Spinocerebellar Ataxia
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
shRNA	short hairpin RNA
siRNA	small interfering RNA
Sp1	Specificity Protein 1
SREBP-2	Sterol Regulatory Element Binding Protein 2
ST*Hdh*	Transformed Striatal Progenitor Cells from Humanized Exon1 Knock-in mice
SV40	Simian Vacuolating Virus 40
TBP	TATA-binding protein
TBS	TRIS Buffered Saline
TBST	TRIS Buffered Saline + Tween 20
TCSPC	Time-correlated single-photon counting
UPLC	Ultra Performance Liquid Chromatography
UPR	Unfolded Protein Response
WD40	Tryptophan-Aspartic Acid 40 Domain
YAC	Yeast Artificial Chromosome

DECLARATION OF ACADEMIC ACHIEVEMENT

All experimental content within this thesis is the work of the author: Carly R. Desmond

CHAPTER 1

INTRODUCTION

1.1 Huntington's Disease

1.1.1 Huntington's Disease: Symptoms and Neuropathology

Huntington's Disease (HD) is an autosomal dominant neurodegenerative disorder affecting as many as 1 in 10 000 individuals in North America (Harper 1992). The symptoms and hereditary nature of the disease were first described in 1872 by George Huntington, after whom the disease is named. A third generation doctor, George Huntington had access to decades of medical records. Using this information, he successfully mapped the dominant inheritance pattern of the disease, years before the advent of modern genetics.

HD is characterized by three primary clinical features: involuntary motor movement, impaired cognitive function and emotional imbalance. The disease was originally named "Huntington's Chorea" (a latin word meaning "to dance"), in reference the dance-like movements originating from the extremities of HD patients. While this is certainly the most immediately recognizable feature of HD, it tends to be proceeded years before by cognitive and emotional changes, making early-stage diagnosis quite difficult (Paulsen et al. 2001). As HD progresses, involuntary movement can affect almost every muscle in the body. Even simple tasks, such as swallowing, become a great challenge. Thus, although HD itself is not fatal, patients typically succumb to complications associated with the disease, such as choking, pneumonia and heart disease, 15-20 years after onset. Sadly, due to the psychiatric impact of HD and high rates of clinical depression, suicide is also prevalent amongst patients (Lanska et al. 1988). At this time, there are no

treatments available to prevent or even slow the progression of the disease. Some drugs are capable of partially managing the symptoms, however, these treatments have no long-term effect on disease outcome.

Throughout the course of HD, neurodegeneration is most prominent within the striatum, a central region within the forebrain that includes the caudate nucleus and putamen (Vonsattel et al. 1985). By later stages, significant tissue loss is also observed in the cerebral cortex, thalamus and cerebellum (de la Monte, Vonsattel, and Richardson 1988; Vonsattel et al. 1985). Within the striatum, medium spiny neurons (MSN) are found to be selectively vulnerable to HD neurodegeneration (Graveland, Williams, and DiFiglia 1985). This neuronal subtype makes up over 95% the striatal population. In a normal, healthy brain, two pathways feed through the striatum to communicate with the motor cortex: the direct pathway (with a net-excitatory effect) and indirect pathway (with a net-inhibitory effect) (Crossman 2000). When HD causes dysfunction and eventual loss of MSNs within the striatum, the indirect pathway becomes impaired (Andre et al. 2011). This leads to overstimulation of the motor cortex and involuntary movement. The remaining cognitive and psychiatric symptoms of HD are likely caused by overall dysfunction of the basal ganglia. This brain region, which encompasses the striatum, is critical for learning new tasks and processing emotions (Arsalidou, Duerden, and Taylor Epub 2012).

1.1.2 The Genetics of Huntington's Disease

Huntington's disease is autosomal dominant, meaning that it affects both males and females equally, and that only one copy of the mutant gene is necessary for disease pathogenesis. HD is caused by a cytosine-adenine-guanine (CAG) trinucleotide repeat expansion in the IT15 (interesting transcript 15) gene, later renamed the *HTT* or *HD* gene (The Huntington's Disease Collaborative Research Group 1993). These mutations result in an excessively long polyglutamine tract in the translated protein, huntingtin (Persichetti et al. 1995).

IT15 was the first disease-associated gene to be mapped to a specific chromosome within the human genome. Using technology that was novel at the time, researchers employed Restriction Fragment Length Polymorphism (RFLP) screening as a tool to identify genetic markers that track with the disease in HD affected families (Gusella et al. 1983). In particular, they acquired thousands of samples from two small communities in *Lake Maracaibo, Venezuela*, a population with the highest incidence of HD in the world. It is thought that the mutant gene first emerged in this region in the 1800's, resulting in 18000 disease positive descendants, 14000 of which are still living in Lake Maracaibo today (Wexler et al. 2004). Thanks to the generosity of these communities, the location of the HD mutation was narrowed down to the short arm of chromosome 4 (Gusella et al. 1983).

The mapping of the gene in 1983 meant that individuals could now be tested for HD pre-symptomatically. However, it was not until 1993 that the gene was actually

sequenced and the exact nature of the mutation was known (The Huntington's Disease Collaborative Research Group 1993). Even in healthy individuals, the HD gene contains a CAG tract. Normal repeat numbers fall between 6 and 35 CAGs (Stine et al. 1993). HD is fully penetrant at tract lengths exceeding 40 repeats, but displays reduced penetrance between 35-40 (McNeil et al. 1997). People with tract lengths between 27-35 do not develop HD themselves, but have a greater chance of passing on a pathogenic repeat length to their offspring, due to a phenomenon called "genetic anticipation"(Trottier, Biancalana, and Mandel 1994; Duyao et al. 1993). This refers to the germ-line expansion of CAG tracts, more commonly during spermatogenesis than oogenesis, due to genetic instability of the CAG region.

The length of the CAG tract is inversely correlated with the age of disease onset (Andrew et al. 1993), such that patients with longer repeat lengths are afflicted with HD earlier in life. If the CAG tract exceeds 60 repeats, the mutation produces a juvenile phenotype; however, this version of the disease is extremely rare and is considered symptomatically and pathologically distinct from the adult cases (Nance and Myers 2001). Typical age-of-onset for HD is midlife, around 40 years of age. However, if the juvenile cases are also considered, diagnosis can be made in patients as young as two and as old as 80-90 (Myers 2004).

Although CAG repeat length determines when patients will develop the disease, the closer to the disease threshold the tract falls, the less predictive it becomes. At borderline repeat lengths, genetic modifiers and environmental factors play a significant

role in disease pathogenesis. Genetic modifiers are defined as genes that negatively or positively impact the expression of other genes, or the function of their protein products. Polymorphisms within genetic modifiers can alter the pathological effects of a disease from one patient to the next (Kearney 2011). Like most forms of illness, environmental factors also have the ability to impact HD pathogenesis. Although the disease cannot be prevented, healthy lifestyle choices such as exercising, avoiding smoking and managing stress are all associated with later age-of-onset and better management of symptoms (van Dellen, Grote, and Hannan 2005).

1.2 CAG Triplet Repeat Diseases

HD belongs to a family of nine CAG triplet repeat diseases that includes, spinocerebellar ataxia types 1, 2, 3 (Machado Joseph disease), 6, 7 and 17, as well as dentatorubro-pallido-luysion atrophy (DRPLA) and spinal bulbar muscular atrophy (SBMA). These diseases are caused by a triplet repeat expansion in the coding region of nine individual affected genes and all produce a neurodegenerative phenotype at mid-life. However, the regions of the brain and types of neurons that are affected in each of the diseases are unique (He, Lin, and Qin 2010). For example, HD causes neurodegeneration predominantly within the striatum of the central nervous system, whereas SBMA affects the motor neurons in the spinal cord and brainstem (Brooks and Fischbeck 1995). The variability of pathological phenotypes within the CAG triplet repeat disease family suggests that the resulting expanded polyglutamine tract is not

toxic in itself, but rather that each disease is dependent on the protein context of the mutation. For this reason, it is unlikely that any one treatment method will be suitable for all polyglutamine diseases. Understanding the normal and pathological functions of each of the affected proteins will be essential to the discovery of suitable drug targets.

1.3 The Huntingtin Protein

1.3.1 Huntingtin Expression and Localization

Huntingtin is a large, 350 kDa protein, spanning 67 exons. Despite its size, huntingtin bears no significant homology to any other protein of known function. Although HD manifests primarily as a neurodegenerative disease, the huntingtin protein is ubiquitously expressed throughout the body, with the highest levels being found in the central nervous system and testis (Li et al. 1993; Sharp et al. 1995).

Within the cellular environment, the localization of huntingtin is diverse and provides few clues in regard the protein's potential function. In a healthy neuron, huntingtin is predominantly cytoplasmic, where it localizes to the endoplasmic reticulum and Golgi, as well as endocytic, lysosomal and plasma membranes (DiFiglia et al. 1995; Atwal et al. 2007; Velier et al. 1998). Fractionation and immunofluorescence studies have also found huntingtin within the nucleus, although at considerably lower levels (De Rooij et al. 1996; Hoogeveen et al. 1993). The identification of a nuclear export signal (NES) towards huntingtin's carboxyl terminus (Xia et al. 2003), and more recently at the

extreme amino terminus (Maiuri et al. *in press*), suggest that huntingtin shuttles to and from the nucleus (See Figure 1).

HD post-mortem tissues reveal that mutant huntingtin forms nuclear and cytoplasmic aggregates in the brain (Huang et al. 1998). Although these aggregates were at first considered toxic, more recent studies have shown that their presence is not limited to those brain regions specifically affected by the disease, and that neurons containing aggregates may actually be less susceptible to neurodegeneration (Kuemmerle et al. 1999). This suggests that the formation of aggregates is a protective mechanism meant to sequester and clear misfolded huntingtin, and that the soluble protein is the true pathogenic species.

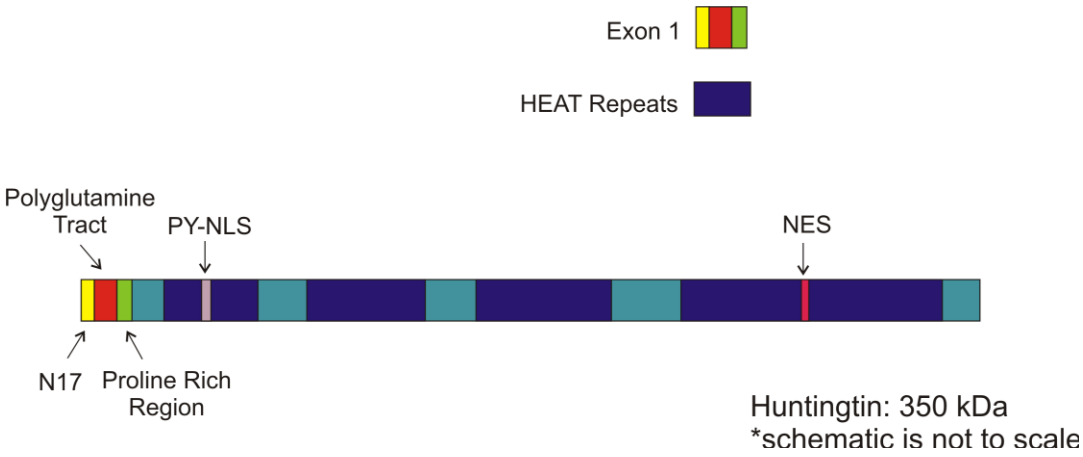

Figure 1: *Schematic of the Huntingtin Protein*. Schematic depicting selected domains of the huntingtin protein. N17 is the first 17 amino acids of huntingtin responsible for targeting the protein to the endoplasmic reticulum. The N17 sequence is also a CRM1-dependent nuclear export signal (NES). The HEAT repeat regions, polyglutamine tract, proline rich region, carboxyl-terminal NES and novel PY-NLS are also identified.

1.3.2 Huntingtin Structure

For many years, the structure of huntingtin remained a mystery. An inability to crystalize the protein and its lack of homologous domains prevented structure analysis or prediction. However, bioinformatic analysis and more recently electron microscopy image reconstruction, strongly suggest that huntingtin is made up almost entirely of HEAT repeats (Andrade and Bork 1995; Li et al. 2006).

Named after several proteins known to exhibit the domain (Huntingtin, Elongation factor 3, Protein Phosphatase 2A, mTOR), HEAT repeats are approximately 40 amino acid long helix-turn-helix motifs, that when repeated, stack into a larger solenoid structure (Andrade et al. 2001). Many HEAT repeat proteins are known for their ability to mediate protein-protein interactions, and thus tend to act as scaffolding proteins, bringing together proteins and other biological molecules, such as membranes and DNA. The strong alpha helical content of HEAT repeats and their ability to stack into tertiary structures confer HEAT repeat proteins with a high degree of flexibility (Kappel et al. 2010). Studies examining the dynamics and energetics of PP2A (protein phosphatase 2A) show that HEAT repeat proteins are capable of forming an almost endless number of conformations and have the ability to hold and release energy, like an elastic or spring (Groves et al. 1999; Grinthal et al. 2010). These proteins easily adapt to mechanical stress and may take on different functions in different cellular environments. Huntingtin is already known to have many interaction partners, suggestive of a scaffolding function. However, in light of these analyses, it is also possible that huntingtin's function is

controlled by the conformation of the HEAT repeats, whereby stretching and bending of the repeats in reaction to mechanical stress alters the protein's accessible interaction domains.

Huntingtin's polyglutamine tract is found within the protein's very first exon. This region is not predicted to be incorporated into the HEAT repeats (HDF Bioinformatics Report). Exon1 consists of three functionally important domains. The polyglutamine tract begins at amino acid 18 of huntingtin, and is flanked by the N17 domain and proline-rich region. N17 is named in reference to its amino acid length and position at huntingtin's extreme amino terminus. The proline-rich domain consists of two stretches of pure proline (11 and 10 amino acids in length) separated by a proline rich , but mixed intervening sequence. The structure of exon1 was previously predicted using bioinformatics and CD spectroscopy, but has now been determined experimentally by X-ray crystallography (Atwal et al. 2007; Kim et al. 2009). N17 forms an amphipathic alpha helix that extends into the beginning of the polyglutamine tract. Within the normal glutamine repeat range (17 glutamines were included in the crystal structure) the polyglutamine region does not form secondary structure, but rather exists as a random coil. The first section of the proline-rich region, containing pure prolines, was found to adopt a proline helix, but the structure analysis loses resolution within the mixed proline region (Kim et al. 2009). Several studies have attempted to assess the structural changes that occur within the polyglutamine region and its surrounding domains when mutated by expansion. Although there are conflicting reports, most

studies suggest that glutamine tracts in the pathogenic range become more structured, alternating between random coil and beta-sheet conformations (Nagai et al. 2007).

1.4 Animal Models of Huntington's Disease

Throughout the years, chemical, cellular and transgenic/knock-in animal models have all provided valuable insight into the mechanisms and pathology of Huntington's Disease. Before the discovery of the IT15 gene, HD was first modeled in rodents by administration of glutamate analogs such as Kainic Acid and Quinolinic Acid (Schwarcz et al. 1984; Schwarcz and Kohler 1983). These compounds produce motor symptoms, neurochemical changes, and neurodegenerative phenotypes similar to those observed in HD patients (Beal et al. 1986). These similarities appear to be caused by a hyper-sensitivity of striatal neurons to excitotoxicity (Brustovetsky et al. 2004). Medium spiny neurons within the striatum receive excitatory glutamatergic signals from the cerebral cortex via NMDA (N-methyl-D-aspartate) receptors. Activation of these receptors causes a rapid influx of Na^{+} and Ca^{2+}, setting off a signaling cascade that results in the release of Ca^{2+} from the endoplasmic reticulum (Fan and Raymond 2007). Excitotoxicity occurs when activation of the NMDA receptors is sustained over long periods. A build-up of cytosolic calcium leads to detrimental effects such as oxidative stress and impaired mitochondrial function, which eventually cause neurodegeneration (Mark et al. 2001).

To date, a variety of animal models have been used to study HD. Although this primarily constitutes mouse models, other animals such as Drosophilia melanogaster,

zebrafish, sheep and non-human primates have been, or are in the process of being, developed. The mouse models of HD fall under two categories: transgenic and knock-in. The first transgenic mouse model was produced using only the first exon of huntingtin and polyglutamine tracts as long as 144 repeats (Mangiarini et al. 1996). This model was followed by a 1-171 Q82 fragment mouse a few years later (Schilling et al. 1999). There are also two full-length huntingtin transgenic models, YAC128 (with 128 glutamines) and BACHD (97 glutamines) (Gray et al. 2008; Slow et al. 2003). In general, the small fragment models display more widespread atrophy, but less actual neuronal loss compared to the full-length models, and develop pathology much earlier in the mouse's lifespan. Most recently, the first fully humanized transgenic mouse (Hu97/18) was created, where mutant huntingtin is expressed transgenically on a *Hdh*(-/-) knock-out background (Southwell et al. 2012). These mice closely match the phenotypes of the BACHD model with the same number of glutamines (Gray et al. 2008).

Multiple knock-in models of HD have also been generated. *Hdh*Q92 and *Hdh*Q111, were designed using a humanized exon1 inserted into the endogenous murine gene (*Hdh*) and promoter (Wheeler et al. 1999). Less severe than the full-length transgenic models, these mice have little to no observed atrophy or degeneration by the end of the mouse's normal lifespan (Wheeler et al. 2000). However, they do have other symptoms of HD including motor and cognitive deficits. When all of the mouse models are considered, there seems to be an underlying theme that the more closely they match the true genetics of the disease, the more subtle the effects. While full-length,

and particularly knock-in, models are excellent for understanding the steps of pathogenesis and for the discovery and design of prodromal treatments, some researchers still choose to work with short fragment models due to their faster, and more robust phenotypes.

The most recent models of HD include exon1 non-human primates, as well as full-length transgenic sheep (Yang et al. 2008; Jacobsen et al. 2010). The large brains and longer lifespans of these animals more closely mimic the human disease. As inconvenient as these models are in terms of study duration, the information they provide will be invaluable to the future study of HD and the development of novel treatments.

Although animal models have been a staple for all genetic diseases, new technologies are changing the research landscape. Recent breakthroughs in stem cell research have allowed the development of induced pluripotent stem cell (iPS) lines from real HD patient tissues (Zhang et al. 2010). These lines, which can be differentiated into a variety of cell types, will be instrumental in the screening of novel therapeutic agents and in furthering our understanding of mutant huntingtin function.

1.5 Huntingtin Function in Health and Disease

1.5.1 General Cellular Functions of the Huntingtin Protein

Huntington's Disease is monogenetic. A single mutation in a single gene is all that is required to cause this age-associated neurodegenerative disease. However,

understanding the pathological impact of the huntingtin protein, or even its normal function, continues to challenge the field.

On a broad scale, huntingtin appears to possess two major functions: it is required for embryonic development and acts to suppress apoptosis. Several mouse models have been developed that either abolish the expression of huntingtin, or express only a small amino-terminal fragment of the protein (Zeitlin et al. 1995; Duyao et al. 1995; Nasir et al. 1995). In both cases, the mice were unable to progress past the gastrulation stage of development; the embryos became increasingly disorganized and were reabsorbed between days 8.5-10.5. This is thought to be due to a requirement for huntingtin during brain development and neurogenesis. Mice expressing less than 50% of the normal level of huntingtin have profoundly malformed brains, and are either still-born or die shortly after birth (White et al. 1997). However, when huntingtin knockout embryonic stem cells are implanted into a normal blastocyst to produce a chimera, the mice do develop normally, although the huntingtin knockout cells are only maintained in select areas of the brain to adulthood (Dragatsis, Efstratiadis, and Zeitlin 1998; Reiner et al. 2001). These studies reveal that huntingtin expression is strictly required in the extraembryonic tissues, and that it may participate in the transport and delivery of nutrients between the mother and the developing embryo.

A recent study shows that huntingtin is also required for the orientation of the mitotic spindle during cell division (Godin et al. 2010). During neurogenesis, progenitor cells divide along the apical surface of the ventricular zone (Tabata, Yoshinaga, and

Nakajima 2012). The orientation of these cells can influence their future differentiation. Cells that divide vertically on the apical surface have a tendency to continue on in proliferative division, while cells that divide horizontally, or in an intermediate position, are more likely to differentiate into neurons and migrate away from the ventricular zone (Farkas and Huttner 2008). When huntingtin expression was knocked-down in the brains of mice on embryonic day 14.5 and analyzed on day 16.5, there was a distinct reduction in the number of cells that divided vertically, and an increase in the number that divided at horizontal or intermediate angles to the apical surface (Godin et al. 2010). This may explain why mice expressing less than 50% of normal huntingtin have such disorganized brains structures, and why huntingtin knockout cells fail to populate specific regions of the brain in chimeric mice (White et al. 1997; Reiner et al. 2001). Only cells expressing the adequate levels of huntingtin may be capable of normal differentiation and migration away from the ventricular zone at the correct developmental time period.

In an adult, huntingtin has a pro-survival, anti-apoptotic function. Conditional inactivation of the *Hdh* gene in the forebrain of mice after birth leads to adult onset neurodegeneration, motor dysfunction and early mortality similar to HD (Dragatsis, Levine, and Zeitlin 2000). This suggests a critical function of huntingtin in protecting neurons from cell death as we age. Indeed, overexpression of wild-type huntingtin has been found to reduce the toxicity of mutant huntingtin in vivo (Leavitt et al. 2001), and

primary neurons from transgenic YAC18 mice expressing an extra copy of huntingtin are protected from excitotoxicity and apoptosis (Leavitt et al. 2006).

The more specific biological roles of huntingtin are still under investigation. In line with its potential function as a scaffolding protein, the activities of huntingtin within the cell appear to be diverse. Therefore, although I cannot cover all of huntingtin's possible functions here, I will discuss those that are best characterized and have the most direct connections to my research. These include vesicular trafficking, the cell stress response, transcriptional regulation and synaptic function.

1.5.2 The Role of Huntingtin in Vesicular Trafficking

Vesicular trafficking is a broad term used to describe the movement of endocytic, lysosomal, synaptic and transport vesicles along the cellular cytoskeleton. Early on, huntingtin was found to associate with vesicular membranes by subcellular fractionation and to co-localize with clathrin coated vesicles at the ER and plasma membrane (DiFiglia et al. 1995; Velier et al. 1998). Wildtype huntingtin participates in fast axonal trafficking in both the retrograde and anterograde direction, moving vesicles and organelles, such as mitochondria, throughout the cell; this function is believed to be impaired by polyglutamine expansion of the huntingtin protein (Block-Galarza et al. 1997; Trushina et al. 2004). In Drosophila melanogaster, both knockdown of huntingtin and expression of a mutant huntingtin fragment impairs axonal transport (Gunawardena et al. 2003). Two domains within huntingtin's amino terminus (N17 and a segment of the protein

between amino acids 172-372) are capable of mediating membrane association. In 2007, our lab published a paper describing N17's ability to interact with the ER, late endosomes and autophagic vesicles (Atwal et al. 2007). The downstream region between amino acids 172-372 selectively interacts with acidic phospholipids (Kegel et al. 2005).

Several protein interaction partners of huntingtin also participate in the mediation of vesicular trafficking. Huntingtin interacting protein 1 (HIP1) associates with clathrin coated pits, and is involved in receptor mediated endocytosis through an interaction with clathrin, α-adaptin A and α-adaptin C (Metzler et al. 2001; Waelter et al. 2001; Legendre-Guillemin et al. 2005). Endocytosis at the synapse produces endocytic vesicles that are typically transported through the axon towards the cell body. For short-range transport, within the axon terminal, these vesicles move along the actin cytoskeleton, however, for long range transport, they must be transferred to microtubules (Lanzetti 2007; Brown 1999). Huntingtin may mediate this switching event via an interaction with HAP40 (Huntingtin Associated Protein 40), which recruits huntingtin to Rab5 positive, F-actin associated, early endosomes (Pal et al. 2006; Peters and Ross 2001). Mutant huntingtin impairs F-actin to microtubule switching, reducing the long-range movement of endocytic vesicles through the axon (Pal et al. 2008).

The first protein found to interact with huntingtin, called huntingtin associated protein 1 (HAP1), acts as an adaptor between huntingtin and the microtubule motor proteins dynein (for retrograde movement) and kinesin (for anterograde movement) (Li

et al. 1995; Li, Gutekunst et al. 1998; McGuire et al. 2006; Engelender et al. 1997). HAP1 interacts directly with the p150glued subunit of dynactin, which is a co-factor of dynein required for the transport of membranes (Engelender et al. 1997), as well as directly with kinesin (McGuire et al. 2006). In line with huntingtin's pro-survival function, wildtype huntingtin and HAP1 are required for the transport and delivery of brain derived neurotrophic factor (BDNF) from the cortex to the striatum (Gauthier et al. 2004). This process is believed to be impaired by mutant huntingtin, as reduced BDNF levels are observed in the striatum of HD brains (Ferrer et al. 2000).

The association of huntingtin with HAP1 is also an important mediator of ciliogenesis. Primary cilia are antenna-like structures that are considered beacons for cellular signaling due to their high local concentration of surface receptors (such as the Wnt and G-protein coupled varieties) (Goetz and Anderson 2010; Zaghloul and Brugmann 2011). HAP1 and huntingtin are both critical to the trafficking of pericentriolar material 1 (PCM1) to the base of the cilium (Keryer et al. 2011). This protein mediates the targeting of proteins to the centriole that are necessary for the promotion of ciliogenesis (Vladar and Stearns 2007). Knockdown of huntingtin or HAP1 impairs cilia formation, while expression of mutant huntingtin causes aggregation of PCM1 and the formation of abnormally long and inappropriately directed cilia (Keryer et al. 2011).

Other huntingtin interacting proteins may promote transport along the actin cytoskeleton. Optineurin (otherwise known as Huntingtin Interacting Protein 7) binds

to the actin associated molecular motor myosin VI and participates in post-Golgi trafficking (Sahlender et al. 2005).

1.5.3 Huntingtin and the Cell Stress Response

Cellular aging is driven by the accumulation of damaged biological molecules and is associated with increased cell stress (Kourtis and Tavernarakis 2011). Huntingtin participates in multiple stress response pathways, including the unfolded protein response (UPR) and oxidative stress response. Huntingtin's relationship to these pathways may explain why wildtype huntingtin is protective in neurons, and why the biggest risk factor for HD is aging.

Proteins that are destined for secretion or are localized to membranes are post-translationally modified and folded within the ER. Disruptions of Ca^{2+} homeostasis, glucose starvation and oxidative stress can all lead to a build-up of unfolded proteins within the ER lumen (Xu, Bailly-Maitre, and Reed 2005). ER resident stress sensing proteins can activate a series of signaling events designed to prevent misfolded proteins from entering the cell. In three steps, UPR halts translation of new proteins, triggers membrane expansion mechanisms to increase the volume of the ER, and up-regulates the transcription of genes involved in the clearance and folding of misfolded polypeptides (Walter and Ron 2011; Schuck et al. 2009). In cases of prolonged ER stress, cells will undergo apoptosis and be eliminated.

Several studies suggest that wildtype huntingtin participates in the ER stress response. Huntingtin knockout cells display abnormal ER structure and impaired ER membrane trafficking, suggesting that it may be required for the physical expansion of the ER during stress (Hilditch-Maguire et al. 2000; Omi et al. 2005). Alternatively, expression of mutant huntingtin is associated with increased staining of ER-markers (Trettel et al. 2000) in ST*Hdh*Q111/Q111 cells and upregulated expression of UPR target genes such as BiP and Chop in patient samples (Carnemolla et al. 2009). Mutant ST*Hdh* Q111/Q111 cells are also more susceptible to apoptosis compared to wildtype cells when treated with chemicals that induce ER stress, such as tunicamycin (Lajoie and Snapp 2012). These experiments all suggest a possible loss-of-function of mutant huntingtin in dealing with ER stress.

Data from our lab demonstrates that huntingtin's N17 domain is responsible for targeting the protein to the ER (Atwal et al. 2007). During ER stress, N17 becomes phosphorylated at serines 13 and 16, leading to huntingtin's release from the ER membrane and subsequent entry into the nucleus (Atwal et al. 2011). We hypothesized that huntingtin acts as an ER stress sensor that shuttles between the two compartments.

In addition to ER stress, HD is also associated with high levels of oxidative stress (Browne and Beal 2006). All types of biological macromolecules, including nucleic acids, proteins, carbohydrates and lipids, are susceptible to damage if an excess of reactive oxygen species (ROS) is allowed to accumulate in the cell. Evidence of oxidative stress is apparent in HD patient brain tissue, which has high levels of the stress marker, 8-

hydroxydeoxyguanosine (8OHdG) (Browne et al. 1997). This nucleic acid modification is also detected in patient blood samples and has been found to track with disease progression (Hersch et al. 2006; Long et al. 2012).

In response to oxidative stress or heat shock, our lab has demonstrated that huntingtin localizes to cofilin-actin stress rods within the nucleus (Munsie et al. 2011). The precise function of these rods remains unclear. Bundling of F-actin and saturation with cofilin rapidly inhibits the constant formation and breakdown of actin called "actin-treadmilling"(Nishida et al. 1987; Bamburg et al. 2010). Treadmilling is a major energy draw of the cell, thus the current hypothesis is that cofilin-actin stress rods form as a method of freeing up ATP for more immediate cellular functions (Bernstein et al. 2006). In mutant huntingtin expressing cells, the cofilin-actin nuclear rods that form are longer and fewer than in normal cells and persist after the stress is removed (Munsie et al. 2011). This impairment of rod formation and clearance is associated with increased cell death in tissue culture assays.

Further highlighting the importance of huntingtin to the cell stress response pathways, p53 response element sequences are found within the promoter of the IT15 (HD) gene (Feng et al. 2006). Although p53 is best known as a tumor suppressor and thus an activator of apoptosis, it can also respond to cell stress by activating autophagy, a process in which huntingtin is known to play an important role. Deletion of the polyglutamine tract in knock-in mouse models leads to a hyperautophagic phenotype and increased longevity (Clabough and Zeitlin 2006; Zheng et al. 2010). Thus, a potential

function of huntingtin may be to promote autophagy and prevent apoptosis in the face of cell stress, particularly within post-mitotic neurons.

1.5.4 Huntingtin: A Transcriptional Regulator?

Within the nucleus, huntingtin may act as a regulator of transcription. This is most clearly suggested by the observation that mutant huntingtin expression is associated with altered transcriptional profiles for a wide-range of genetic targets (Cha 2000). Other proteins with known functions in transcription have glutamine rich regions similar to huntingtin, including specificity protein 1 (Sp1) and cyclic AMP response element-binding protein (CREB) binding protein (CBP) (Suske 1999; Johannessen, Delghandi, and Moens 2004). The polyglutamine tract behaves as a protein-protein interaction domain within these transcriptional activators, producing a scaffold for the formation of transcriptional complexes. Other polyglutamine disease proteins, such as TBP of SCA17, atrophin-1 of DRPLA and the androgen receptor of SBMA, are also involved in functions related to transcription (Nakamura et al. 2001; Yazawa et al. 1995; La Spada et al. 1991). TATA-binding protein (TBP) binds directly to DNA to build transcription factor complexes at TATA promoter elements whereas atrophin-1 is a nuclear receptor corepressor involved in recruitment of histone deacetylases to chromatin, allowing transcriptional repression (Greenblatt 1992; Wang and Tsai 2008). The androgen receptor is activated by testosterone, leading to nuclear entry and modulation of gene expression (Bennett et al. 2010). Chromatin immunoprecipitation

analysis indicates that huntingtin occupies gene promoters, and has a preference for p53 consensus elements (Benn et al. 2008). Huntingtin indirectly modulates the transcription of brain derived neurotrophic factor (BDNF), which is required for the long-term survival of particular neuronal populations, including the medium spiny neurons of the striatum (Zuccato et al. 2003). Normal huntingtin upregulates the transcription of BDNF by sequestering its transcriptional repressor, repressor element 1 silencing transcription factor (REST), in the cytoplasm. Mutant huntingtin has reduced affinity for REST, leading to a reduction in the expression of BDNF. Huntingtin is known to interact with other transcriptional co-repressors, such as C-terminal binding protein (CtBP) and nuclear corepressor (NCoR) (Kegel et al. 2002; Yohrling et al. 2003).

1.5.5 The Synaptic Functions of Huntingtin

Early on, it was reported that huntingtin localizes to neuronal synapses, and synaptic dysfunction is a possible mechanism of HD pathogenesis (Velier et al. 1998; Usdin et al. 1999). Huntingtin interacts directly with the critical synaptic regulator post synaptic density 95 (PSD-95), which links huntingtin to NMDA and kainite receptors at the surface of the dendrites (Sun et al. 2001). Huntingtin also interacts with PACSIN1 through its poly-rich region (Modregger et al. 2002). This protein is thought to be involved in the induction of membrane curvature during the formation of synaptic vesicles and has also been implicated in linking vesicles to actin remodeling proteins at the synapse (Plomann, Wittmann, and Rudolph 2010; Wasiak et al. 2001). In

presymptomatic HD brains, PACSIN1 is absent from the synaptic space (Modregger et al. 2002). Huntingtin also stimulates the palmitoylation activity of huntingtin interacting protein 14 (HIP14) (Huang et al. 2011). Palmitoylation is an lipid based post-translational modification that targets protein to membranes, and is found in a multitude of synaptic proteins (Young et al. 2012).

As described previously, glutamate excitotoxicity is proposed to contribute to neurodegeneration in HD (Beal et al. 1986). Neuronal plasticity and transmission mediated though NMDA receptors are disrupted in HD transgenic mice before visible neurodegeneration or motor dysfunction (Milnerwood et al. 2006). Mutant huntingtin expression in YAC128 mice causes an increase in the expression of extrasynaptic NMDA receptors within the striatum (Milnerwood et al. 2010). These receptors, as opposed to those located within the synapse, are associated with pro-death signaling and are activated by glutamate spillover at the synaptic cleft (Kaufman et al. 2012; Soriano and Hardingham 2007). Therefore, increased expression and activation of these receptors could explain the enhanced susceptibility of the striatum to excitotoxicity in HD brains.

1.5.6 Is Huntington's Disease Caused by a Gain-of-Function or Loss-of-Function?

There has been much debate as to whether HD is caused by a gain or loss of function of the huntingtin protein. Perhaps the most convincing argument in support of a gain-of-function mechanism is that heterozygous *Hdh* +/- knock-out mice are phenotypically normal and do not develop neurodegeneration (Duyao et al. 1995).

Furthermore, there are rare examples of individuals who are born with a chromosomal breakage of chromosome 4 that results in loss of huntingtin expression from one allele (Zellweger et al. 1975). Although this genetic abnormality is responsible for another disease called Wolf-Hirshhorn syndrome, it is not associated with late-onset neurodegeneration.

Nonetheless, there are other factors to consider which suggest that HD cannot be labelled strictly a gain-of-function or loss-of-function disorder. Although 50% of the normal expression of huntingtin is sufficient for development and the protein's adult biological functions, post-natal inactivation of the *Hdh* gene in mice leads to late- onset neurodegeneration similar to HD (Duyao et al. 1995; Dragatsis, Levine, and Zeitlin 2000). Indeed, many of the described normal functions of huntingtin are associated with a potential mechanism of dysfunction in the disease. Two theories exist to explain these observations: A gain-of-function which, for example, increases huntingtin's affinity for a particular interaction partner, may manifest as a loss-of-function if these interactions impact the protein's normal activities. Alternatively, a true loss-of-function may occur, but be limited to processes of the aging brain. If huntingtin is critical for cell stress response pathways, 50% activity of the protein may be sufficient in early life, but inadequate as we age and the levels of cell stress increase. Such a situation is not sufficiently represented in the *Hdh* +/- knockout mice, since they have a comparatively short life-span and minimal stress compared to a human being.

1.6 Huntingtin Localization and Disease

1.6.1 Cell-Type Specific Nuclear Accumulation of the Huntingtin Protein

One of the most reliable markers of HD progression, in both mouse models of the disease and human patient brain tissue, is an increase in nuclear huntingtin staining, which is particularly visible using the antibody EM48 (Van Raamsdonk et al. 2005; Wheeler et al. 2000; Hoogeveen et al. 1993; Sapp et al. 1997). Designed using an epitope common to the wildtype and mutant protein (1-256 with a polyglutamine tract deletion), EM48 recognizes both the normal and pathogenic forms of huntingtin by western blot (Gutekunst et al. 1999). However, by immunocytochemistry, the antibody highlights nuclear and aggregated huntingtin almost exclusively. This is believed to be due to a conformational change or unmasking of huntingtin's amino-terminus, allowing EM48 recognition in the nuclear, but not the cytoplasmic compartment (Wheeler et al. 2000). Both knock-in and transgenic full-length mouse models of HD display progressive nuclear EM48 staining (Van Raamsdonk et al. 2005; Gray et al. 2008; Wheeler et al. 2000). This effect is both cell-type and polyglutamine length dependent, such that nuclear EM48 staining is seen primarily in the striatum and cerebral cortex, and occurs earlier in mice with more numerous CAG repeats (Wheeler et al. 2000; Van Raamsdonk et al. 2005).

1.6.2 Nuclear Huntingtin and the Cleavage Hypothesis

Progressive nuclear accumulation of mutant huntingtin has been reported in many studies; however, it remains controversial as to whether or not this population is full-length, or small, amino-terminal cleavage products. Thus far, only amino-terminal antibodies have been capable of detecting nuclear huntingtin by immunofluorescence (Wheeler et al. 2000; DiFiglia et al. 1997). Therefore, the common belief was that cleavage of huntingtin precedes nuclear targeting. However, this theory was challenged when Wheeler *et al*. (2000) reported nuclear localization of full-length huntingtin protein by fractionation prior to the formation of visible aggregates. Although they also described an inconsistency of localization between amino-terminal and carboxyl-terminal huntingtin antibodies by immunocytochemistry, these differences could be resolved if the samples were analyzed by fractionation and western blot. Just as the EM48 antibody preferentially recognizes nuclear huntingtin in the cellular context, carboxyl-terminal antibodies are associated more strongly with the protein's cytoplasmic form, making it impossible to detect a cleavage event by immunocytochemistry alone.

Despite these discrepancies, many studies have identified the presence of small amino-terminal fragments of huntingtin using alternative techniques, such as western blot (Mende-Mueller et al. 2001). However, it appears that nuclear translocation occurs before, and not after, the cleavage process (Warby et al. 2008). In 2006, Graham *et al* generated a caspase-6 resistant YAC128 transgenic mouse model by mutating huntingtin's putative caspase-6 cleavage site. Blocking this site was protective against

mutant huntingtin toxicity and found to prevent further proteolytic cleavage of the protein at other, more amino-terminal sites. Later, it was discovered that full-length huntingtin enters the nucleus before it is cleaved by caspase-6, which also translocates to the nuclear compartment (Warby et al. 2008). After this initial event, subsequent processing of huntingtin becomes accelerated. Interestingly, expression of huntingtin fragments corresponding to those that would be generated by caspase-6 cleavage does not result in the same localization patterns as if the fragments were generated endogenously (Tebbenkamp et al. 2011). This may be related to the observation that huntingtin fragments in exon1 or 1-171 transgenic mouse models do eventually localize to the nucleus, but do not show the same cell-type specificity of the full-length models (Meade et al. 2002; Schilling et al. 1999). The normal localization patterns and cellular functions of the full-length protein are likely required to trigger the events that lead to nuclear accumulation within the affected neuronal population.

1.6.3 Modifying Huntingtin Nuclear Localization Alters Toxicity

Since nuclear accumulation of huntingtin correlates strongly with the onset of pathogenesis, several studies have set out to understand if this is a causal relationship or just a consequence of other toxic mechanisms. When mutant huntingtin fragments, such as exon 1 (1-81) or 1-171 were fused to an exogenous nuclear export signal to prevent nuclear accumulation, toxicity of the fragments was inhibited (Peters et al. 1999; Saudou et al. 1998). Alternatively, if polyglutamine expanded exon1 is fused to a

nuclear localization signal to accelerate nuclear translocation, the toxicity of the fragments is enhanced (Peters et al. 1999). Similar to these tissue culture assays, addition of a nuclear localization signal to huntingtin 1-171 in a transgenic mouse model also increases the fragment's pathogenesis (Schilling et al. 2004). Compared to mice without the NLS, the 1-171-NLS mice had shorter lifespans and reduced ability to perform motor related tasks.

1.6.4 Normal Functions Associated with Nuclear Localization of Huntingtin

Despite the association between nuclear accumulation of huntingtin and toxicity, there are still many normal and non-pathogenic reasons for huntingtin to enter the nucleus. As previously described, huntingtin has a role in the cell stress response (Atwal et al. 2007; Atwal and Truant 2008; Munsie et al. 2011). Phosphorylation of huntingtin at serines 13 and 16 during stress leads to release from the ER and translocation to the nucleus. Within the nucleus, huntingtin localizes to phospho-specific nuclear puncta and cofilin-actin rods (Atwal et al. 2011; Munsie et al. 2011). Treatment of tissue culture cells with chemical agents that elicit DNA damage or block RNA transcription (cisplatin and high concentrations of actinomycin D) causing cell stress, also trigger nuclear translocation of endogenous wildtype huntingtin (Tao and Tartakoff 2001). This effect is found to be dependent on the integrity of the cytoskeleton. Treatment with nocodazole to block microtubule polymerization also prevents huntingtin nuclear entry.

As described previously, huntingtin has been implicated in the regulation of transcription, but also RNA biogenesis. Huntingtin knockout cells have abnormally shaped nucleoli, the cellular organelle required for the transcription and assembly of ribosomal RNA (Hilditch-Maguire et al. 2000). Also, two transcription factors (HYPB and N-CoR) that have been shown to bind huntingtin, become mislocalized to the cytoplasm when the huntingtin protein is absent, indicating that it is required for regulation of these proteins (Hilditch-Maguire et al. 2000).

Normal nuclear targeting of huntingtin may be most critical during early development. Post-fertilization, but pre-implantation, huntingtin localizes strongly to the nuclei of proliferating embryonic mouse cells (Jeong et al. 2006). However, by the 8 cell stage, huntingtin has already begun its translocation towards the cytoplasm, and localization becomes almost exclusively cytoplasmic by the time the blastocyst has formed.

It is likely that the nuclear localization of huntingtin is tightly regulated in both development and in adults. Transgenic and knock-in mice generated in different strain backgrounds show subtle alterations in the timing of huntingtin's nuclear accumulation during the onset of pathogenesis (Van Raamsdonk et al. 2007; Lloret et al. 2006). This suggests that huntingtin nuclear localization is sensitive to genetic modifiers and that misregulation of pathways that affect huntingtin nuclear import may lead to disruptions in the timing and execution of nuclear functions. This could explain the cell-type specificity of the disease. For example, if one of huntingtin's nuclear functions involves

the inhibition of apoptotic pathways, misregulation of these events could be extremely detrimental to non-proliferating cells, such as neurons, but of little consequence in other cellular populations. Also, if nuclear localization of huntingtin is required for early developmental pathways, could sustained nuclear accumulation in an adult neuron be harmful? Ongoing research from the Alzheimer's research community suggests that repression of cell-cycle in adult neurons is critical to their function and health, and that loss of this regulation can cause neurodegeneration (Herrup 2010). Perhaps nuclear accumulation of huntingtin is causing activation of developmental pathways long repressed. Together with the evidence that phosphorylation, and interaction partners of huntingtin can alter its nuclear entry, it is possible that loss of this regulation, and inappropriate nuclear entry has serious consequences.

1.7 Nuclear-Cytoplasmic Transport

1.7.1 General Mechanisms of Nuclear Import and Export

Nuclear entry is a tightly regulated process in eukaryotic cells. A double membrane encasing the nucleus called the nuclear envelope, acts as a physical barrier between the delicate DNA and the cellular processes of the cytoplasm (Watson 1955). To allow nuclear entry and exit of select proteins, the nuclear membrane is coated in large macromolecular protein channels called nuclear pore complexes. Even with an 8-fold symmetry, more than 30 different proteins, called nucleoporins, are required to form the nuclear pore complex (Stoffler, Fahrenkrog, and Aebi 1999). Some of these proteins

are intra-membrane proteins used to form the barrel of the nuclear pore complex, while others span the pore creating a diffusion barrier between the two compartments. The nuclear pore complex is often referred to as the gatekeeper of the nucleus, due to its inherent selectivity (Ribbeck and Gorlich 2001).

Proteins and other molecules smaller than 50-60 kDa may enter the nucleus by passive diffusion (Terry, Shows, and Wente 2007). However, proteins beyond this size limit require the assistance of active transport mechanisms (Ribbeck and Gorlich 2001). Nuclear transport receptors, called karyopherins, bind to pathway specific sequences on the surface of their respective cargo (Radu, Blobel, and Moore 1995). These sequences, named nuclear localization signals (NLS) or nuclear export signals (NES), depending on their intended direction of transport, allow the karyopherin family to identify which proteins should be granted or denied access to the nucleus.

The karyopherin β family is comprised of over 20 members in mammalian cells (Chook and Blobel 2001). These proteins all share a common HEAT repeat rich structure, but recognize their own specific set of substrates (Chook and Blobel 1999). Although relatively large, karyopherin β proteins are flexible, and have the ability to interact strongly with the hydrophobic nucleoporins within the nuclear pore complex (Conti, Muller, and Stewart 2006; Bayliss, Littlewood, and Stewart 2000). These interactions guide karyopherin β through the nuclear pores along with their associated cargo (Iovine, Watkins, and Wente 1995).

The directionality of nuclear transport is controlled by the RanGTP/RanGDP gradient (Figure 2) (Gorlich et al. 1996). In the nucleus, chromatin associated RanGEF (guanine nucleotide exchange factor) is responsible for converting all incoming RanGDP to RanGTP (Bischoff and Ponstingl 1991). Alternatively, in cytoplasm, RanGAP (GTPase activating protein) stimulates the intrinsic GTPase activity of Ran, thus maintaining high concentrations of RanGDP in the cytoplasmic compartment (Bischoff et al. 1994; Corbett et al. 1995). When karyopherin β family members enter the nucleus in complex with a protein substrate, they are quickly bound by RanGTP, which triggers a conformational change in karyopherin β, initiating cargo release (Chook and Blobel 1999). Once the karyopherin β-RanGTP complex exits the nucleus, RanGAP stimulates the conversion of RanGTP to RanGDP. This event promotes disassociation of the complex, allowing the entire process to begin again (Floer, Blobel, and Rexach 1997). Nuclear export is essentially an inversion of nuclear import. Binding of substrates to karyopherin proteins that mediate nuclear export, such as CRM1 (chromosome region maintenance 1) is enhanced by RanGTP in the nucleus and disassociated by GTP hydrolysis in the cytoplasm (Askjaer et al. 1998; Floer and Blobel 1999).

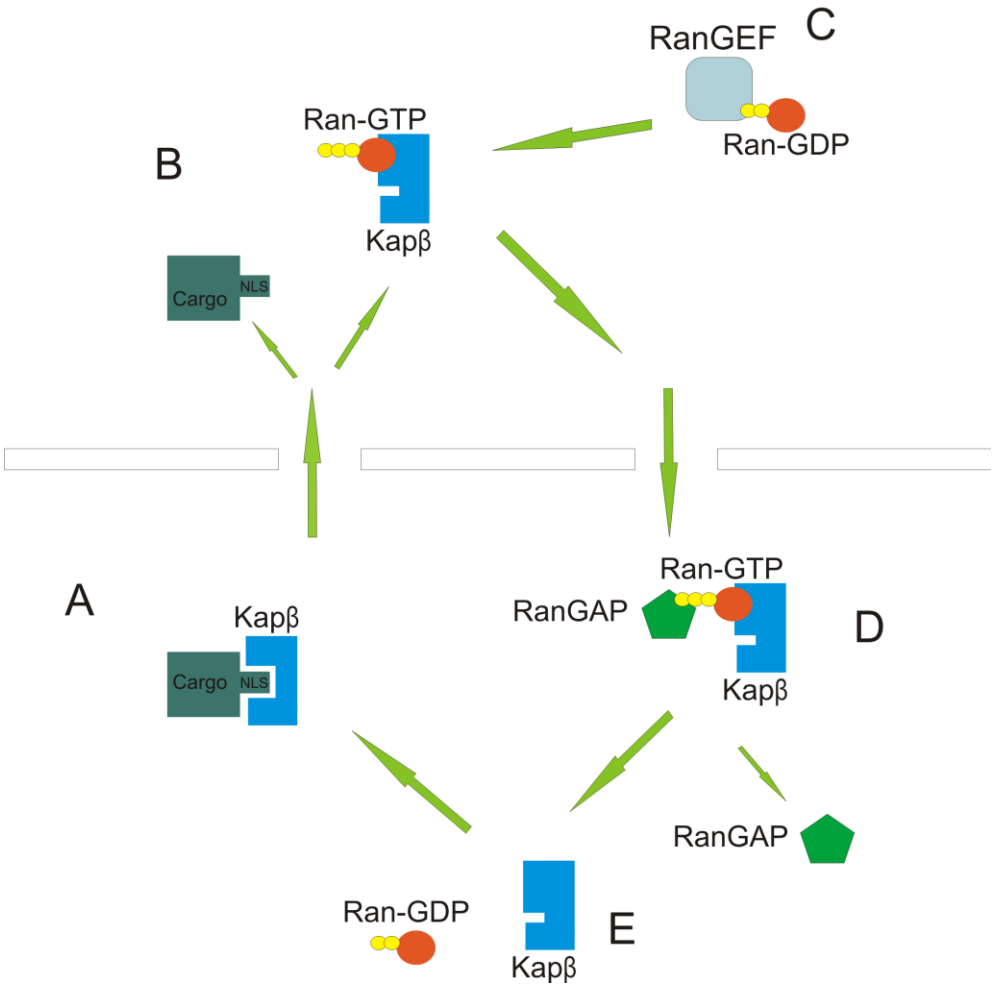

Figure 2: *General Mechanism of Nucleocytoplasmic Transport* Nuclear transport is initiated by the binding of the karyopherinβ (Kap β) protein to the NLS bearing substrate (A). Kapβ then mediates the import of the substrate into the nucleus. Once in the nucleus, the Kapβ- cargo complex encounters a high concentration of RanGTP, which binds to Kapβ creating a conformational change that initiates cargo release (B). The high concentration of RanGTP is maintained by RanGEF (Guanosine Exchange Factor) that replaces GDP bound to Ran with GTP (C). Kapβ-RanGTP exits the nucleus, where it encounters RanGAP (GTPase Activating Protein) which activates the hydrolysis of RanGTP to GDP (D), thus releasing Kapβ and re-starting the import process (E).

1.7.2 Nuclear Localization Signals

To date, an NLS consensus has been derived for two nuclear import pathways: the classical karyopherin α/β pathway, and karyopherin β2. Most nuclear localization signals bind directly to the karyopherin β subunit. However, classical NLSs utilize an adaptor protein, called karyopherin α, for sequence recognition and cargo binding (Gorlich et al. 1995). Despite these unique properties, the "classical NLS" was so named due to its history of being the first nuclear import mechanism described, and the first to have a defined consensus (Dingwall and Laskey 1991; Lange et al. 2007). Karyopherin α/β-type NLSs fall under two categories: monopartite and bipartite (Fontes, Teh, Jans et al. 2003). The mono-partite classical NLS follows the sequence K-K/R-X-K/R, where X represents any amino acid. Bipartite NLSs, alternatively, contain two basic rich sequence elements separated by approximately 10-12 amino acids [(K/R)(K/R)- $X_{(10-12)}$-$(K/R)_{3/5}$, i.e 3 out of 5 residues must be a lysine or arginine] (Kalderon et al. 1984; Lange et al. 2007). Although many bipartite classical NLS sequences do follow this consensus, linker regions as large as 29 amino acids have been described (Lange et al. 2010). Other NLS sequences bind karyopherin β1 directly, however, a defined consensus has yet to be identified for this avenue of import (Truant and Cullen 1999).

The nuclear import consensus of another transport receptor, karyopherin β2, has also been described (Lee et al. 2006). Dubbed the PY-NLS, this sequence contains an R/K/H-$X_{(2-5)}$-PY motif 8-13 amino acids down downstream from either a hydrophobic or basic rich region. Karyopherin β2 binds its substrates directly, without the need for an

adaptor protein (Pollard et al. 1996). Commonly, although by no means exclusively, nuclear import substrates of karyopherin β2 share functions related to the processing and transport of mRNA (Lee et al. 2006).

1.7.3 Regulatory Mechanisms of Nuclear Import: Examples from the Literature

The most direct mechanism of regulating the import and export of proteins is by the simple presence or absence of an NLS or NES. However, many, more intricate, secondary mechanisms exist that allow the temporal regulation of nuclear import throughout the lifetime of the cell. The most common method of inhibiting nuclear import is by intra- and inter-molecular masking of the NLS sequence. The p50 subunit of NF-kappaB exists in a larger, precursor form in the cytoplasm (p105). However, phosphorylation of NF-kappaB leads to degradation of the p105/50 carboxyl-terminus, exposing the NLS to karyopherin α/β (Henkel et al. 1992; Oeckinghaus and Ghosh 2009). This is an example of intramolecular masking. The nuclear import of other proteins, such as the HIV regulator REV, are inhibited intermolecularly. REV has been shown to bind HIC (Human I-mfa domain-containing protein) in the cytoplasm, masking its NLS and preventing nuclear entry (Gu et al. 2011).

Nuclear transport can also be modulated by post-translational modifications such as phosphorylation. These modifications can either enhance or inhibit the nuclear translocation of substrates. SV40 large T-antigen is an example of a protein that's nuclear import is enhanced by phosphorylation when a serine adjacent to the NLS is

modified (Fontes, Teh, Toth et al. 2003). The altered conformation leads to increased association of the import sequence with its cognate receptor. Alternatively, when lamin B2 is phosphorylated by PKC at a site adjacent to its NLS, import is inhibited (Hennekes et al. 1993).

Cleavage of proteins within the cytoplasm or nucleus is also an effective way of regulating nuclear import and export. SREBP-2 (sterol regulatory element binding protein-2) is an example of a transcription factor that only translocates to the nucleus when cleaved. SREBP-2 is a transmembrane protein at the ER that senses low intracellular cholesterol, which induces cleavage, liberating its amino-terminal fragment at the cytoplasmic surface of the ER (Brown and Goldstein 1997). This fragment enters the nucleus via a karyopherin β1-direct mechanism, where it activates the transcription of genes involved in cholesterol and lipid biosynthesis (Nagoshi et al. 1999).

1.8 Thesis Rationale

The nuclear import and accumulation of huntingtin appears to be closely tied to the pathogenic progression of Huntington's Disease. Unrestricted diffusion into the nuclear compartment is strictly limited to those proteins smaller than 50-60 kDa, therefore, large proteins, such as huntingtin (350 kDa) require the assistance of the active transport mechanisms previously described. In order to better understand both the normal functions of huntingtin, and the pathological changes that occur throughout the disease, we sought to identify the pathway responsible for huntingtin's nuclear

import. Since nuclear accumulation does not occur in every cell type, and nuclear entry of normal huntingtin is limited to particular cellular events (such as during cell stress and early development), it is likely that secondary mechanisms of regulation are in place that control the timing of nuclear entry. If this is true, these processes could be targets of drug design, as a means of limiting the nuclear entry of huntingtin during aging. Therefore, beyond describing the basic mechanism of nuclear import, the goal of my thesis has also been to explore a range of possible regulatory mechanisms.

CHAPTER 2

EXPERIMENTAL PROCEDURES

With permission, some of the methods in this chapter were modified from:

Desmond, C.R., Atwal, R.S., Xia, J., Truant R. (2012) **Identification of a Karyopherin β1/β2 Proline-Tyrosine Nuclear Localization Signal in Huntingtin Protein**. Journal of Biological Chemistry. 287 (47):39626-33

and

Atwal, R. S., C. R. Desmond, N. Caron, T. Maiuri, J. Xia, S. Sipione, and R. Truant. (2011) **Kinase inhibitors modulate huntingtin cell localization and toxicity**. *Nat Chem Biol* 7 (7):453-60

2.1 Plasmid Constructs

All heterologous GFP-βGalactosidase fusions were created using the phm830 cloning vector (Sorg and Stamminger 1999; Xia et al. 2003). Synthetic DNA oligonucleotides were generated bearing overhangs at either end corresponding to SacII and XbaI restriction sites (MOBIX, McMaster University). These oligonucleotides were then annealed to produce double stranded DNA and ligated into digested phm830 vector. A similar cloning strategy was implemented for mCherry-M9M (EcoRI, BamHI) and mCerulean-Htt NLS (EcoRI, SacII), along with the various NLS and intervening sequence (IVS) mutants. The base cloning vectors for these constructs were p-mCherry-C1 and p-mCerulean-C1 (BD Biosciences/Clontech).

The cDNA for HAP1B (mouse) was supplied by Open Biosystems. The gene was amplified by PCR and cloned into p-mCherry-C1 (EcoRI, Acc651). HAP1A (mouse) is the same as HAP1B apart from unique carboxyl-terminal sequences; therefore mCherry-HAP1A was generated from mCherry-HAP1B. The nucleotide sequence corresponding to amino acids 579-629 of HAP1B was removed by inverse PCR and a new SacII site was introduced. The carboxyl-terminal sequence of HAP1A (amino acids 579-599) was generated as a synthetic oligonucleotide with SacII and Acc651 overhangs and cloned into the truncated mCherry-Hap1B construct. The SacII site was then removed by inverse PCR.

Human HMGB1 cDNA was obtained from Origene, amplified by PCR, and cloned into p-mCherry-C1 using XhoI and Acc651.

Htt 1-465-eYFP was generated by deletion of the sequence encoding amino acids 466-586 from Htt 1-586-eYFP, cloned as previously described (Atwal et al. 2007) . This product was created by performing inverse PCR using primers flanking the region of deletion. The resulting PCR product was then phosphorylated at the 5' OH using T4 Polynucleotide Kinase (New England Biolabs) followed by blunt end ligation to re-circularize the vector. Full or partial deletion of the NLS sequence was achieved by the same method. Point mutations of the NLS sequence were introduced to Htt 1-465-eYFP by either Quikchange II XL site-directed mutagenesis (Stratagene) or the inverse PCR method. The inverse PCR method is similar to the deletion method described above, but alternatively the point mutation is introduced as an overhang on the forward primer.

The point mutations in Htt 1-465 K178R-eYFP and Htt 1-465 KK177/178RR-eYFP were also generated by the inverse PCR method.

eYFP-LC3 was a generous gift of A.Yamomoto. Ataxin-7 (228-619)-eYFP was generated previously by Jillian Taylor.

mCherry-karyopherinβ2(541-890) was created by PCR of the karyopherin β2 fragment and ligation into the p-mCherry-C1 vector using the restriction sites SmaI and XhoI. mCherry-karyopherinβ1(256-876) was cloned in the same way, except using the

restriction sites XhoI, and SacII. All restriction enzymes and PCR polymerases were purchased from New England Biolabs unless otherwise stated.

2.2 Tissue Culture

Conditionally immortalized ST*Hdh* Q7/Q7 striatal progenitor cells (a kind gift from M.E. MacDonald) were originally derived from a wild-type (7 glutamine) knock-in transgenic HD mouse containing a humanized Exon 1 and G418 resistance cassette (White et al. 1997; Trettel et al. 2000). To maintain the expression of the transgene and of the SV40 large T antigen, the cells were grown under G418 selection at 33°C in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Invitrogen). The HEK293 and MCF-7 cell lines were grown at 37°C (5% CO_2) in alpha minimal essential media containing 10% FBS. NIH 3T3 cells were cultured in DMEM + 10% FBS at 37°C (5% CO_2).

2.3 Microscopy

All widefield imaging was performed using a Nikon TE200 inverted widefield epifluorescence microscope and plan apochromat 60X oil immersion objective (Nikon, Japan). The imaging platform controlling the scope was NIS elements 3.1. All filter sets and dichroic filters were from Semrock (Rochester, NY) and the filter wheel was from Sutter Instruments (Novato, CA). The light source was a 175W Xenon lamp (Sutter Instruments), with ND2 or ND4 filters. Images were acquired using a Hamamatsu Orca ER digital camera (Hamamatsu Photonics, Japan). The confocal imaging was performed

using a Leica DMI6000B with a Leica TCS SP5 scanner with acoustic optical beam splitter and a 100 mW 488 nm argon ion laser line. The objective used was a 63× 1.3NA DIC glycerol HCX Plan-Apochromat and the detector was a 2× steady state photo multiplier tube. The microscope was controlled using Leica Application Suite Advanced Fluorescence software. This SP5 microscope and software were also used for FRET-FLIM imaging. Fluorescent lifetime of the mCerulean donor fluorophore was quantified using time-correlated single-photon counting (TCSPC). Photons were generated by exciting the samples with a Chameleon Ultra (Coherent Inc.) two-photon laser, then collected and analyzed using the integrated SPC-830 TCSPC system and software (Becker and Hickl GmbH). Fluorescence Lifetime decay curves were generated for every pixel of the microscope image. The Becker and Hickl FLIM plug-in was used to produce the fluorescence lifetime histograms and pseudo-colour images.

2.4 Transfections and Quantitative Analysis

Both the ST*Hdh* Q7/Q7 and the HEK293 cells were transfected with either Exgen 500 (Fermentas) or Turbofect (Fermentas) and imaged approximately 20-24 hours post transfection. To determine the percent nuclear fluorescence, approximately 50-100 cells were imaged per construct and quantified using Simple PCI (Compix). To ensure unbiased data collection, the cells were co-transfected with mCherry, and cells were selected under the red channel before being imaged in the green channel, in a method of unbiased imaging data acquisition described elsewhere (Voss, Demarco, and Day

2005). Nuclear and cytoplasmic intensities were collected by manually defining the nuclear and cytoplasmic regions in each image, and then collecting blank areas of equal size to represent the image background. The intensities of the defined regions were then measured using the Simple PCI measurement tool. Percent Nuclear Fluorescence was calculated using the equation % nuclear fluorescence = [(nuclear fluorescence - background)/(whole cell fluorescence - background)] X 100.

2.5 Statistical Analysis

All statistical analysis was performed using the SigmaPlot 11.0 software. Determination of statistical significance was calculated as deemed most appropriate by the software, where for normal data it employed the student's T test, and for non-normal data, the Whitney-Mann method.

2.6 Immunofluorescence

Cells were grown in glass bottom dishes and transfected 24 hours before fixation (if applicable). All samples were fixed using 4% paraformaldehyde in PBS (Sigma-Aldrich) and permeabilized using 0.5% Triton-X 100 with 2% FBS in PBS. Samples to be stained with phospho-N17 were fixed and permeabilized in ice cold methanol. All samples were blocked with 2% FBS in PBS for 1 hour. Primary antibodies were diluted into antibody dilution solution (1% FBS, 0.02% Tween 20 in PBS) and incubated for 4 hours at room temperature, or overnight at 4°C on the rocker. After 3, 15 minute washes in blocking buffer, the samples were incubated with suitable secondary antibodies in antibody

dilution solution for 45 minutes, followed by extensive washing in PBS. The following antibodies and concentrations were used: Huntingtin (N-18) goat polyclonal (1:100, Santa Cruz) with Alexa Fluor 488 donkey anti-goat (1:100), Lamp1 (E-5) mouse monoclonal (1:150, Santa Cruz) with Alexa Fluor 594 goat anti-mouse (1:200), HMGB1 rabbit polyclonal (1:100, Abcam) with Alexa Fluor 488 goat anti-rabbit , HAP1 (1B6) mouse monoclonal (1:100, Abcam) with Alexa Fluor 488 chicken anti-mouse, phospho-N17 rabbit polyclonal (1:500) generated by New England Peptide as previously described (Atwal et al. 2011). Phosopho-N17 was used with Alexa Fluor 488 donkey anti-rabbit. All secondary antibodies were purchased from Molecular Probes.

2.7 NLS Peptide Affinity Chromatography

Wildtype and mutant (KK177/178AA) huntingtin NLS peptides were diluted in ddH_20 to create 20 mg/mL stocks. The peptides were diluted to a final concentration of 1 mg/mL in 500 µl peptide coupling buffer (50 mM TRIS, 5 mM EDTA-Na; pH 8.5) and then coupled to 30 µl of resin as per the manufacturer's instructions (Thermo Scientific). After the coupling reaction, the resin was equilibrated in 500 µl of Binding Buffer (0.1 M NaCl, 10 mM HEPES pH 7.6, 10% Glycerol). ST*Hdh* Q7/Q7 lysates were prepared in 1% NP-40 lysis buffer (0.15 M NaCl, 0.05 M TRIS pH 8, 1% NP-40) and diluted into 500 µl of Binding Buffer. One confluent plate was lysed per affinity column. The coupled resin was mixed end-over-end with diluted lysate at 4°C before loading into a 200 µl pipette tip column stuffed with glass wool. The columns were washed four times with 200 µl of

Binding Buffer and then 60 µl volumes of 0.2 M, 0.3 M and 0.4 M NaCl in 10 mM HEPES pH7.6 and 10% glycerol. The bound proteins were eluted in 60 µl of 0.5 M NaCl. All affinity chromatography was conducted at 4°C. Equal amounts of each elution were analyzed by western blot for the presence of either karyopherin β2 or HMGB1.

2.8 Peptide Pull-downs: Batch Method

WT NLS and KK177/178AA peptides were diluted to a concentration of 1 mg/mL in coupling buffer (0.1 M NaCl, 10 mM HEPES pH 7.4, 10% Glycerol). 250 µl of each diluted peptide was combined with 30 µl of equilibrated Sulfolink beads and incubated end-over-end for 2 hours at room temperature. The beads were washed 3 times with coupling buffer and then blocked with 500 µl of 50 mM cysteine-HCL in coupling buffer (end-over-end for 15 minutes, then 30 minutes standing on bench). 50 µl of lysate, from a 10cm confluent ST*Hdh* cell lysate (200 µl total) was diluted with 100 µl of coupling buffer and combined with the peptide coated beads. The samples were incubated at room temperature for 1 hour, washed 3 times with 500 µl of coupling buffer and then eluted in 60 µl of elution buffer (0.5 M NaCl, 10 mM HEPES, 10% Glycerol, 0.1% SDS) by end-over-end agitation for 10 minutes at room temperature. 40 µl of each elution was sent to be analyzed by mass spectrometry analysis.

2.9 Mass Spectrometry (MS) Analysis

All mass spectrometry was conducted at University of Western Ontario Mass Spectrometry Laboratory. MS analysis was performed on a Micromass QToF Global, mass spectrometer equipped with a Z-spray source operating in the positive ion mode with the following parameters: Data range, MS Survey: 400-1800; Data range, MS/MS: 50-1800; Cone Voltage: 100V; Source Temperature: 80°C; Nano-ESI, DDA Mode. UPLC was via a Waters nanoUPLC. Data were acquired using MassLynx 4.1, and PLGS 2.2.5.

2.10 SDS PAGE and Western Blot Analysis

All samples were run on 12% SDS-polyacrylamide gels, transferred to PVDF membrane (PALL) and blocked with 5% (w/v) milk in TBST (25 mM Tris, 150 mM NaCl, 2 mM KCl pH 7.4 + 0.1% Tween 20). The primary antibodies used for immunoblotting were anti-karyopherin β2 (D45, Abcam) at 1/2000, anti-HMGB1 (ab18256, abcam) at 1/2000, anti-AFP polyclonal (Living Colors) at 1/2500, anti-actin (20-33, Sigma, Aldrich) at 1/7500 and anti-HAP1 (mouse specific antibody) at 1/1500. The HAP1 antibody was a kind gift of S.H Li and X.J Li. Rabbit anti-mouse HRP conjugated secondary antibody (ab97046, Abcam) was used at 1/25000. Goat anti-rabbit HRP conjugated secondary (ab97051, Abcam) was used at 1/40000. Blots were developed with Immobilon Western Chemiluminescent Substrate (Millipore) and imaged on a DNR Microchemi western blot imager. Some older blots were developed on film.

2.11 Circular Dichroism Spectroscopy

Huntingtin NLS Peptides (NH2-LYKEIKKNGAPRSLRAALWRFAELAHLVRPQKCRPY-COOH) were purchased from New England Peptide and reconstituted in PBS (10 mM NaH_2PO_4, 0.14 M NaCl, 1 mM EDTA, pH 7.4). The peptides were further diluted in PBS to 0.25 mg/ml for CD Spectroscopy analysis in an Aviv CD spectrometer model 215 (Aviv Instruments, NJ, USA). The data was collected at 25°C in millidegrees and then converted to mean residue ellipticity [Θ]λ in degree/cm^2/dmole through the formula (Θ/molarity) X (no. of residues) X pathlength. After the spectrum was generated, the numbers were fed into the online CD spectroscopy analysis software, K2D Neural Network (Greenfield 2006). This tool allows for interpretation of CD spectra by systematically comparing them to a large library of spectra from other proteins or peptides of known structure.

2.12 Membrane Pellet Extraction

10 cm tissue culture dishes of HEK293 cells were transfected with equal amounts of mCerulean, mCerulean-IVS and mCerulean-IVS L195A,L198A. After 24 hours of expression, the plates were washed and scraped into PBS. The cells were then pelleted by centrifugation at 1000 rpm for 5 minutes. The pellet was then resuspended in 200 µl of 1% NP-40 lysis buffer (0.15 M NaCl, 0.05 M TRIS pH 8, 1% NP-40) supplemented with fresh 1X protease inhibitor cocktail and vortexed in bursts for 1 minute. (Roche) The lysate was incubated on ice for 15 minutes, vortexed again and then centrifuged for 15

minutes at 14000 rpm. The supernatant (the soluble fraction) was then removed and the pellet was resuspended in 200 µl of lysis buffer. The pellet (the membrane fraction) and the supernatant were both sonicated 3 times, 8 pulses each time, with 30 second rest periods (duty cycle 10%, output control 4). The samples were then centrifuged for 3 minutes at 14000 rpm and the final supernatants (of both the membrane and the soluble fractions) were collected.

2.13 Chemical Treatments

ST*Hdh* cells expressing with GFP-Htt NLS-βGalactosidase and GFP-M9 NLS-βGalactosidase for 24 hours were subjected actinomycin D (Sigma-Aldrich) treatment for 4 hours at 5 µg/mL. To observe the localization of endogenous huntingtin in the presence of actinomycin D, the cells were treated with at a range of concentrations indicated in Figure 14 for 3-4 hours. After treatment, the cells were fixed using 4% paraformaldehyde and either imaged directly, or subjected to immunofluorescence.

ST*Hdh* cells expressing Htt 1-465-eYFP or Ataxin 7 (228-619)-eYFP were treated with 10 nM leptomycin B (Sigma-Aldrich) for 2 hours in serum free media. The cells were then fixed and imaged.

2.14 Co-Immunoprecipitation

Transfected huntingtin fragments (either in 1-465 or GFP-βGalactosidase context) and mCherry-HAP1B (co-transfected or alone) were expressed for 24 hours in HEK293 cells.

The plates were lysed using 200 µl of 1% NP-40 lysis buffer supplemented with 1X protease inhibitor cocktail. 0.1 µg of anti-Aequorea victoria fluorescent protein polyclonal antibody (Clontech) was added to each lysate and incubated overnight at 4°C with end-over-end agitation. 40 µl of protein A agarose beads (Sigma) were added to each sample, as well as 500 µl of PBS, and incubated for 1-3 hours at room temperature. The beads were then washed 3 times with 500 µl of PBS buffer followed by elution of the sample by boiling in 40 µl of PBS buffer with 20 µl 3X SDS running buffer.

2.15 Toxicity Assays

ST*Hdh* Q7/Q7 cells were transfected with 3µg of Htt 1–171 Q138–eYFP or Htt1–171 (Q142) S13A S16A–eYFP plasmids in triplicate using Turbofect (Fermentas). Seventeen hours before analysis, the medium was replaced on the transfected dishes and subjected to either no treatment, 2 µM DMAT or 4 µM BMS-345541. The dishes were treated with ethidium homodimer III (1 µM) (Biotium) dead cell stain 2 hours before analysis, which was performed at 48 hours. To ensure that all the dead cells were appropriately counted, the medium was removed while the adherent cells were trypsinized, and then the same medium was used to resuspend the remaining culture. Flow cytometry was completed on the Beckman Coulter EPICS ALTRA. The flow cytometer recorded the percentage of transfected cells from each dish as well as the percentage of transfected cells also positive for ethidium homodimer III stain (percent toxicity = (transfected and dead cells/total transfected cells) × 100, where green and red

staining marked transfected and dead cells, respectively). 100 000 cells were counted per sample.

CHAPTER 3

IDENTIFICATION OF A KARYOPHERIN β1/β2 PROLINE-TYROSINE NUCLEAR LOCALIZATION SIGNAL IN HUNTINGTIN PROTEIN

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All experimental work was conducted by the author: Carly R. Desmond

3.1 Abstract

Among the known pathways of protein nuclear import, the karyopherin β2/ transportin pathway is only the second to have a defined nuclear localization signal (NLS) consensus. Huntingtin, a 350 kDa protein, has defined roles in the nucleus, as well as a CRM1/exportin–dependent nuclear export signal, however the NLS and exact pathway of import have remained elusive. Here, using a live cell assay and affinity chromatography, we show that huntingtin has a karyopherin β2-dependent PY-NLS in the amino–terminus of the protein. This NLS is comprised of three consensus components: a basic charged sequence, a downstream conserved arginine and a proline-tyrosine (PY) sequence. Unlike the classic PY-NLS, which has an unstructured intervening sequence between the consensus components, we show that a beta sheet structured region separating the consensus elements is critical for huntingtin NLS function. The huntingtin PY-NLS is also capable of import through the importin/karyopherin β1 pathway, but was not functional in all cell types tested. We propose that this huntingtin PY-NLS may comprise a new class of multiple import factor–dependent NLSs with an internal structural component that may regulate NLS activity.

3.2 Introduction

One of the most fundamental differences between a eukaryotic and prokaryotic cell is the evolution of the nucleus, which provides an isolated and protected environment for the cell's genetic information. Nuclear import and export of proteins is strictly controlled, allowing for more complex functions and thus providing greater opportunity for biological diversity (Chook and Blobel 2001). Proteins smaller than 50-60 kDa may enter the nucleus by passive diffusion (Terry, Shows, and Wente 2007), but larger proteins must be accompanied into the nucleus through one of at least 14 nuclear transport pathways. These pathways are often used for smaller proteins as well, since they provide more rapid and strict control over import and export than is possible by diffusion alone (Cullen 2003). Facilitated diffusion across the nuclear pores is mediated by nuclear import proteins termed karyopherins or importins (Ribbeck and Gorlich 2001). Despite identification of every importin/karyopherin family member in the mammalian proteome, alternative mechanisms for nuclear entry that do not rely on these pathways also exist (Wagstaff and Jans 2009). The first isolated and best understood nuclear import pathway is mediated by karyopherin β1 via the adaptor protein karyopherin α. In this pathway, karyopherin α recognizes a well characterized classical NLS consensus consisting of either one basic rich cluster (monopartite) or two clusters separated by an intervening sequence (bipartite). These NLSs have now been identified in thousands of nuclear proteins (Lange et al. 2007). Multiple isoforms of

karyopherin α are expressed differentially across cell types, allowing tissue specific regulation of NLS function (Marfori et al.).

A second NLS consensus has been defined for karyopherin β2 mediated import (Chook and Suel 2010; Lee et al. 2006). The karyopherin β2 type NLS, or "PY-NLS" has an invariant proline-tyrosine (PY) sequence, a conserved arginine, and a basic or hydrophobic sequence 8-13 amino acids upstream of the PY (Chook and Suel 2010). This class of NLS has been described as unstructured, and assumes a binding conformation only when interacting with the inner helical face of the HEAT-rich karyopherin β2 protein (Suel, Gu, and Chook 2008). The PY-NLS is often found in proteins involved in mRNA trafficking (Lee et al. 2006) and has recently been described as a key regulator of import to the neuronal primary cilium (Dishinger et al. 2010). Compared to typical karyopherin α/β1-dependent NLSs, the PY-NLS is relatively rare, although an increasing number have been identified since the discovery of its consensus.

Huntingtin is a HEAT (huntingtin, EF3, PP2A, mTor) -rich protein of 350 kDa that has known functions in both the nucleus and the cytoplasm, and shuttles between the two compartments (Truant, Atwal, and Burtnik 2007). Previously, we described a typical consensus CRM1-dependent, leptomycin B-sensitive NES in the carboxyl-terminus of huntingtin. Although several putative sequences were tested, a typical karyopherin α/β1 type NLS was not found (Xia et al. 2003). This led to the hypothesis that huntingtin itself may be capable of nuclear import, since HEAT-repeats are a common structural element between huntingtin and the karyopherin family. However, this prediction was

later disproved when we noted by fluorescence recovery after photobleaching (FRAP), that a fragment of huntingtin between amino acids 81 and 586 containing few HEAT repeats was still capable of active nuclear transport (Atwal et al. 2007).

Here, we identify an active NLS sequence between amino acids 174-207 of huntingtin that interacts with karyopherin β2 both in a novel live cell assay and by affinity chromatography. The huntingtin NLS contains all of the necessary epitopes to be recognized by karyopherin β2: an upstream basic region, or "B" site, and a downstream arginine-proline-tyrosine (R-X_5-PY) "A" site (Chook and Suel 2010). However, the space between the "A" and "B" sites of this sequence far exceeds the length limits defined by the consensus. Furthermore, a functional redundancy appears to exist whereby karyopherin β1 is also capable of mediating import through the same NLS motif. CD spectroscopy and mutational analysis has identified a proline flanked structured intervening sequence (IVS) between the "A" and "B" sites of the huntingtin NLS that is critical to its function. This sequence may also provide clues regarding regulation of huntingtin nuclear import. In isolation, the IVS exhibits independent targeting to the cytoplasm. Coupled with the observation that huntingtin NLS activity varies substantially amongst cell types, we predict that the IVS has the ability to negatively regulate huntingtin NLS function in a cell-type specific manner by a unique competitive or allosteric mechanism.

3.3 Results

3.3.1 Huntingtin Contains a Highly Conserved PY- NLS Sequence Defined by Amino Acids 174-207

An NLS consensus for karyopherin β2 mediated nuclear transport has been elucidated (Lee et al. 2006). Termed the PY-NLS, this sequence is defined by an R-X_5-PY motif, 8-13 amino acids downstream from either a hydrophobic or basic rich region (Figure 3A). We noted the presence of a strictly conserved arginine and downstream PY motif within huntingtin 81-586, a region previously shown by our lab to exhibit nuclear import activity (Figure 3B). In our first attempt to delineate the boundaries of the NLS, we identified a hydrophobic sequence that closely matched the rules of the consensus, falling only 8 amino acids upstream from the PY (LWRFAEL). When tested, however, this fragment failed to demonstrate NLS activity (data not shown). We next proceeded to examine sequences further upstream, and located a basic rich region (KEIKK) 27 amino acids away from the PY. To assess whether or not this fragment possessed NLS activity we implemented a classic live cell *in vivo* NLS assay, in which huntingtin fragments were fused to the reporter proteins GFP and βGalactosidase, creating a triple fusion larger than 120 kDa (Sorg and Stamminger 1999). Since this far exceeds the diffusion limit of the nuclear pore, and neither GFP, nor βGalactosidase have an endogenous NLS, nuclear import is only achieved only by fusion of an active NLS sequence. Activity was assayed by quantification of the percent nuclear fluorescence compared to the total cell

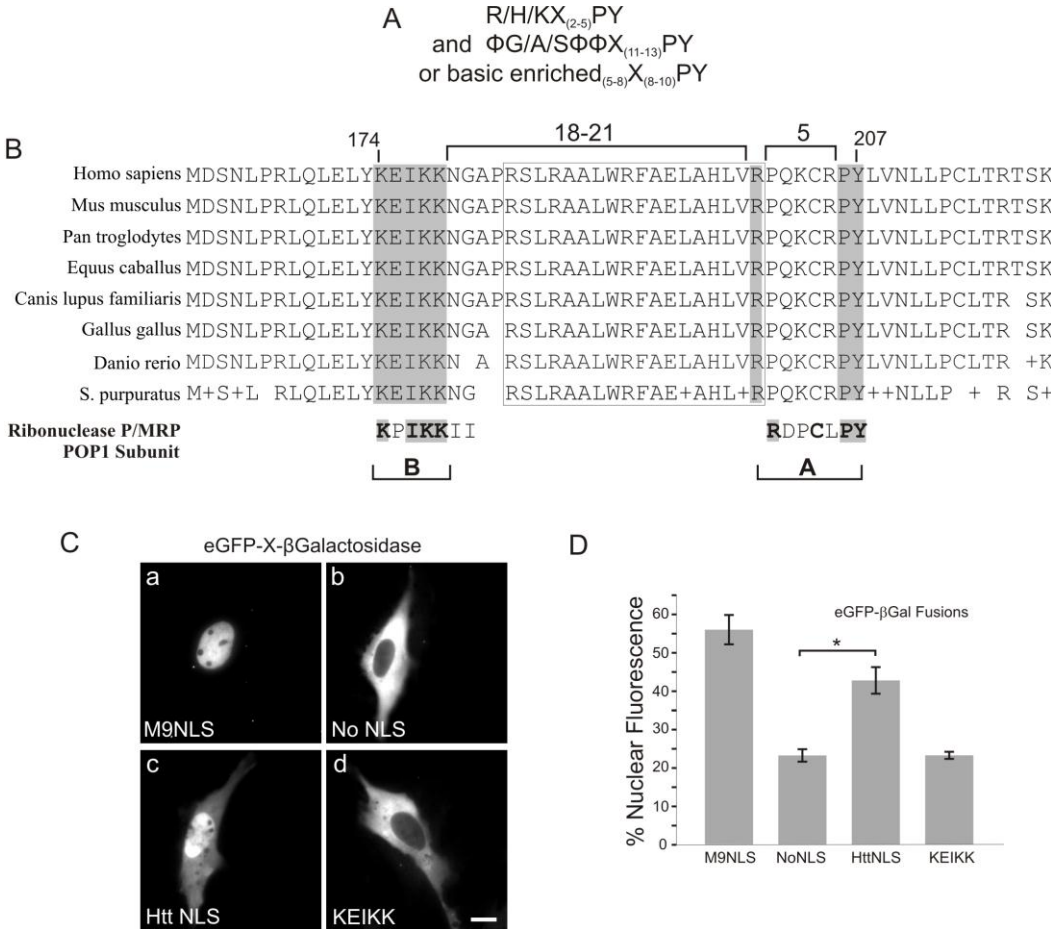


Figure 3: *Huntingtin Amino Acids 174-207 Contains Elements of a Karyopherin β2 Basic PY Nuclear Localization Signal*. The defined consensus for karyopherin β2-type NLSs is shown in (A), alongside sequence alignments of the huntingtin NLS displaying full conservation of the basic rich KEIKK region, arginine 200 and proline-tyrosine PY 206/207 (boxed in grey) in (B). The 18 amino acid intervening sequence flanked by proline turns, highlighted by a boxed outline, is also conserved. Below, the PY-NLS of ribonuclease P/MRP POP1 subunit is shown, with highly similar "A" and "B" sites. (C), Huntingtin 174-207 was cloned in frame with GFP and βGalactosidase, a >120 kDa reporter for live cell expression. Sample images of the huntingtin NLS as a GFP-βGalactosidase fusion, compared to no NLS, M9 hnRNPA1 NLS, and the KEIKK sequence. (D), quantification of data in (C), n= approximately 50 cells quantified per construct. Constructs expressed in *STHdh* cells. *=P value <0.01. Bars represent standard error. Scale bars = 10 µm.

fluorescence within a population of 50-100 transfected cells per data set. In ST*Hdh* cells, nuclear import activity of the PY-NLS sequence was found to be dependent upon the KEIKK basic sequence located upstream (Figure 3B-D) in addition to the PY region (Figure 3C,D). The KEIKK sequence alone was not sufficient to mediate nuclear entry of the reporter (Figure 3C, panels c versus d). Thus, the nuclear localization sequence of huntingtin strictly requires amino acids 174-207, falling outside the length limits of the defined PY-NLS consensus (Figure 3A) (Lee et al. 2006). However, despite this key difference, the sequence still contains individual elements of the consensus that are strikingly similar to other PY-NLS's; particularly, those motifs found within the POP1 subunit of P/MRP ribonuclease (Figure 3B).

Using the same GFP-βGalactosidase fusion assay in ST*Hdh* cells, we next explored the relative importance of individual NLS residues to import activity. Mutation of the PY residues at 207/208 had no effect, whereas mutation of lysines 177 and 178 within the basic rich region abrogated nuclear import activity (Figure 4A, panels a-c, quantified in Figure 4B). All residues were converted to alanines. We examined similar mutations in the basic region and PY residues in the context of huntingtin 1-465 as a carboxyl-terminal eYFP fusion (Figure 4C). In this context, KK177/178AA mutation reduced, but did not eliminate nuclear entry, whereas mutation of the PY residues had no effect (Figure 4C, panels b,c). Similar results have previously been observed when PY mutations were made to the hnRNPA1 M9 NLS (Lee et al. 2006). Complete abrogation of

nuclear entry was observed upon deletion of the entire NLS sequence from 174-207 (Figure 4C, panel d). However, the same loss of nuclear import could be achieved by

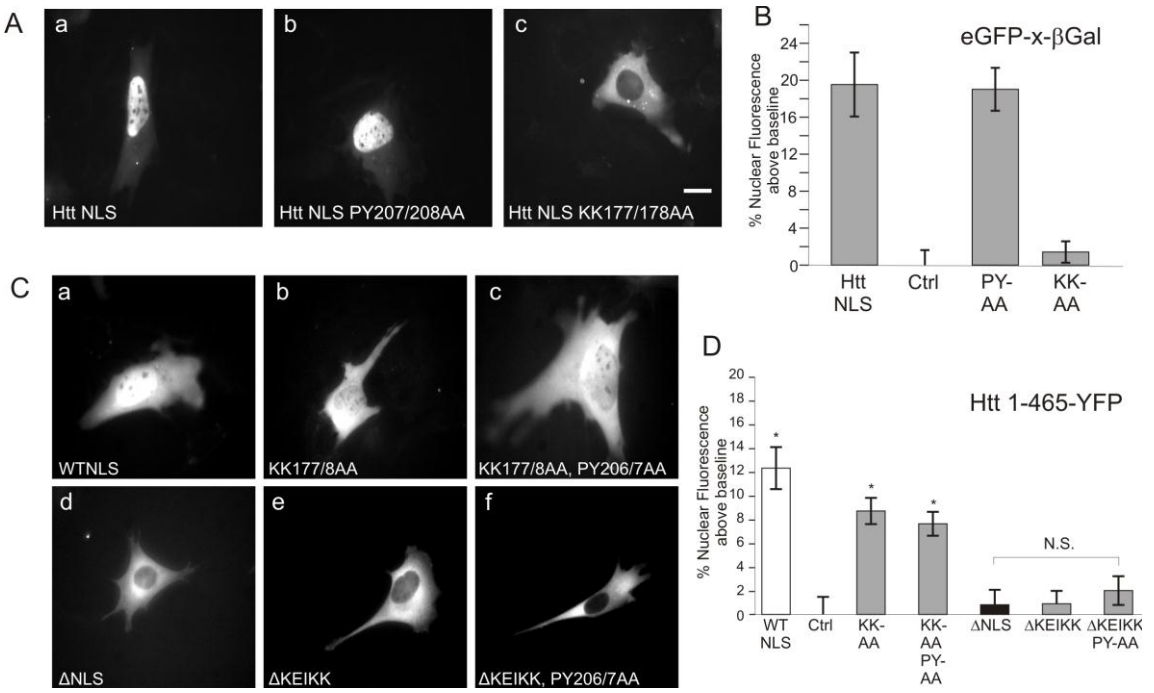


Figure 4: *The Huntingtin PY-NLS Basic Region is Critical for NLS Activity in vivo.* (A), Positive control of GFP-Htt NLS-βGal, compared to PY207/208AA and KK177/178AA mutants, quantified in (B). Ctrl is GFP-βGalactosidase, which has been quantified and then set as baseline to 0. (C), (D), analysis of huntingtin NLS mutants in the [Q17]1-465-eYFP context. n= approximately 50 cells quantified per construct. *=P value <0.01. Bars represent standard error. Scale bars = 10µm.

deletion of the KEIKK basic rich sequence alone (Figure 4C, panels e,f). Although the KEIKK sequence is clearly necessary for NLS activity, it is not sufficient to mediate nuclear import in a heterologous nuclear transport assay, indicating that this is not a typical basic karyopherin α/β1 –type NLS (Figure 3C, panel d) To ensure that the

observed changes to NLS activity after mutation are not due to gross alterations of expression, we quantified the average exposure time in each of our data sets. Although the exposures varied greatly from image to image in all our data sets, the average fluorescence intensity did not change appreciably, and showed no correlation with high or low levels of nuclear import (data not shown).

3.3.2 Huntingtin PY-NLS activity is Cell-Type Specific and Employs Multiple Nuclear Import Pathways

During the course of these experiments we observed that the huntingtin PY-NLS sequence exhibited low nuclear import levels in human embryonic kidney HEK293 cells (Figure 5A, B). This was despite the detection of similar levels of karyopherin β2 by western blotting (data not shown). Thus, for unknown reasons, we had found a non-permissive cell line for huntingtin NLS nuclear entry. To determine if karyopherin β2 is capable of mediating nuclear transport of the huntingtin NLS in live cells we attempted to drive nuclear entry in the HEK293 cell line. A fragment of karyopherin β2 (residues 541-890) lacking the RanGTP/GDP binding domain (18) was fused to mCherry and over-expressed in the HEK293 cells (Figure 5C). Without the Ran interaction domain, we predicted that karyopherin β2 would be incapable of cargo release once across the nuclear pore complex. Co-expression of karyopherin β2(541-890) with GFP-huntingtin NLS- βGalactosidase resulted in nuclear entry of the reporter. However, it did not promote nuclear entry of GFP-βGalactosidase alone (Figure 5C, panels a-d versus e-h).

Consistent with the earlier work in ST*Hdh* mouse cells, this nuclear entry could be abrogated with the KK177/178AA mutation (Figure 5C, panels i-l, quantified in 5D). As further confirmation that the huntingtin NLS interacts with karyopherin β2, we

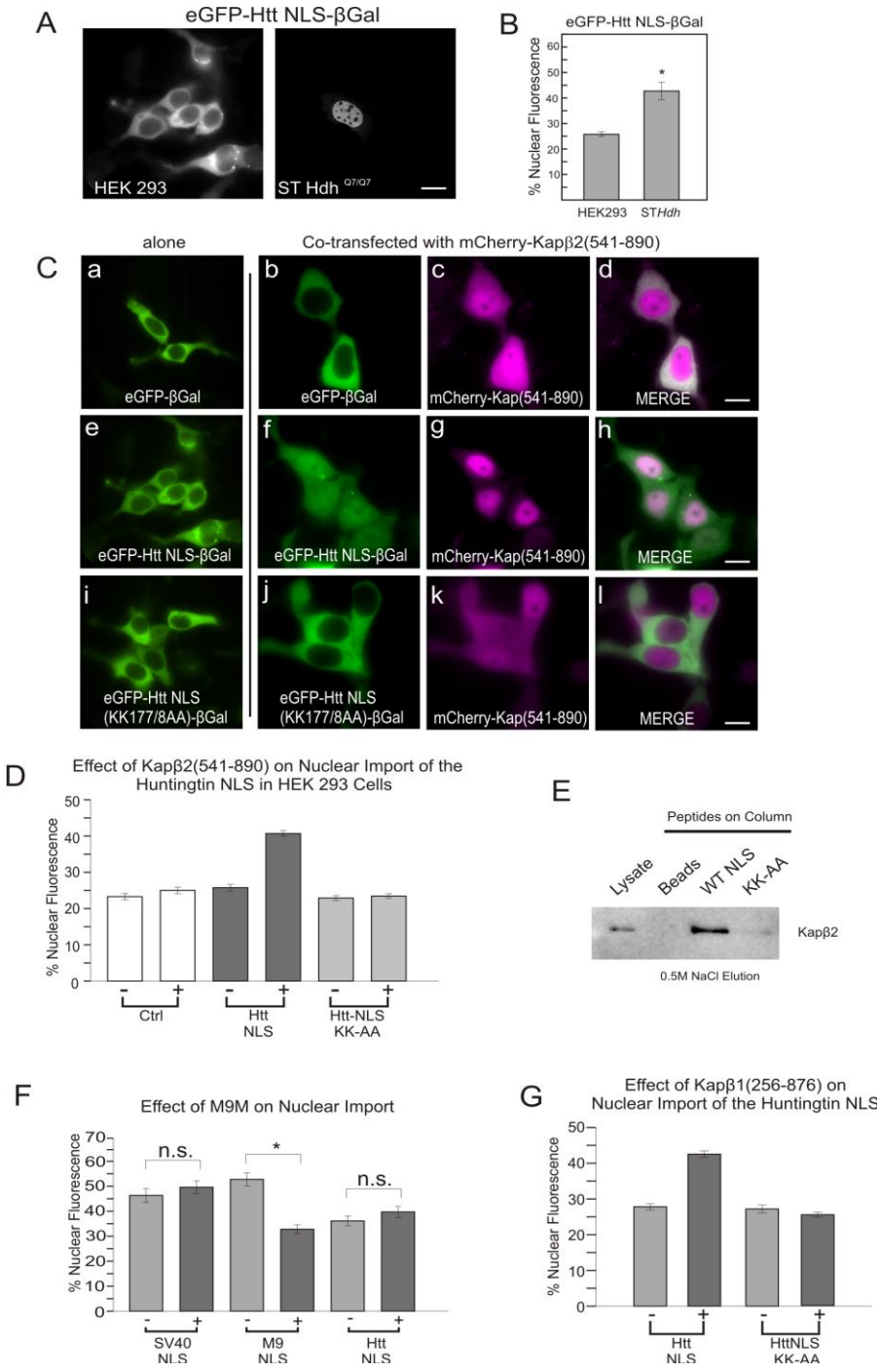


Figure 5: *The Huntingtin PY-NLS Utilizes the Karyopherin β2/ β1 Import Pathways***.** (A), comparison of huntingtin NLS-mediated nuclear entry in human HEK293 versus mouse ST*Hdh* cells, quantified in (B). (C), over-expression of mCherry-karyopherin β2 (541-890) (ΔRan binding) restores wild-type NLS activity in HEK 293 cells (C, panels f-h), but not KK177/178AA mutant NLS (C, panels j-l). (D), quantification of (C). (E), affinity chromatography of ST*Hdh* cell lysates on huntingtin NLS peptide coupled columns. Column elutions at 0.5M NaCl were separated by SDS PAGE and western blotted for karyopherin β2 (anti-Kapβ2,D45). Lysate lane contains 1/20th of column input. Beads indicates the beads alone column. (F), comparison of the SV40 NLS, M9 NLS and huntingtin NLS in response to over-expression of M9M peptide competitor to karyopherin β2. (G), over-expression of karyopherin β1 256-876 (ΔRan binding) can increase nuclear entry via the huntingtin NLS, but not a KK177/178AA mutant NLS. All NLSs are in GFP-βGalactosidase context in ST*Hdh* cells unless otherwise stated. For all quantification, n=approximately 50 cells per construct. *= P value <0.01. Bars represent standard error. Scale bars = 10μm.

performed affinity column chromatography of ST*Hdh* cell lysate using huntingtin NLS and mutant (KK177/178AA) peptides. After washing the columns with increasing concentrations of NaCl (up to 0.4M), the bound proteins were eluted at 0.5M NaCl. Karyopherin β2 was found to associate with the wild-type huntingtin NLS peptide, but not the KK177/178AA mutant. The beads alone did not interact with karyopherin β2 (Figure 5E). Together these findings suggest that huntingtin NLS activity can be mediated by karyopherin β2. To assess the specificity of huntingtin's NLS for the karyopherin β2 pathway, we tested the effect of over-expressing a karyopherin β2 NLS inhibitor, M9M (Figure 5F). M9M is a synthetic chimera of the "A" and "B" sites from two different PY-NLS sequences that produces a motif with higher than physiological affinity for karyopherin β2. This increased affinity prevents M9M's release from karyopherin β2, even in the presence of RanGTP in the nucleus (Cansizoglu et al. 2007). In our live cell

assay, mCherry-M9M over-expression inhibited nuclear import of the hnRNPA1 M9 karyopherin β2-dependent PY-NLS, but had no effect on the huntingtin PY-NLS, or the classical SV40 NLS (Figure 5F).

The observation that M9M does not inhibit nuclear import of the huntingtin NLS suggests that it may not be restricted to recognition by karyopherin β2 alone. To test this hypothesis, we over-expressed a similar Ran-independent fragment of karyopherin β1 (Vetter et al. 1999) in the HEK293 assay and found that it could also restore huntingtin NLS activity. This occurred without the need to over-express a karyopherin α adaptor protein (Figure 5G). This suggests that aside from being capable of nuclear entry via karyopherin β2, the huntingtin NLS can also utilize the karyopherin β1 pathway, but via a karyopherin β1 direct mechanism, and not the classical α/β1 interactions.

3.3.3 The Huntingtin PY-NLS Contains an 18-mer Beta Sheet Sequence that is Critical for NLS Activity

A unique aspect of the huntingtin PY-NLS is the 27 amino acid intervening sequence between the basic region and the proline/tyrosine of the "A" site defined for PY-NLSs. An 18 amino acid sequence within this region is flanked by two proline residues (Figure 3B) suggesting it may be a structured element lying between two proline turns. To test this hypothesis, we synthesized a synthetic peptide corresponding to the 18 amino acid sequence and performed CD-spectroscopy at physiological salt concentrations with a

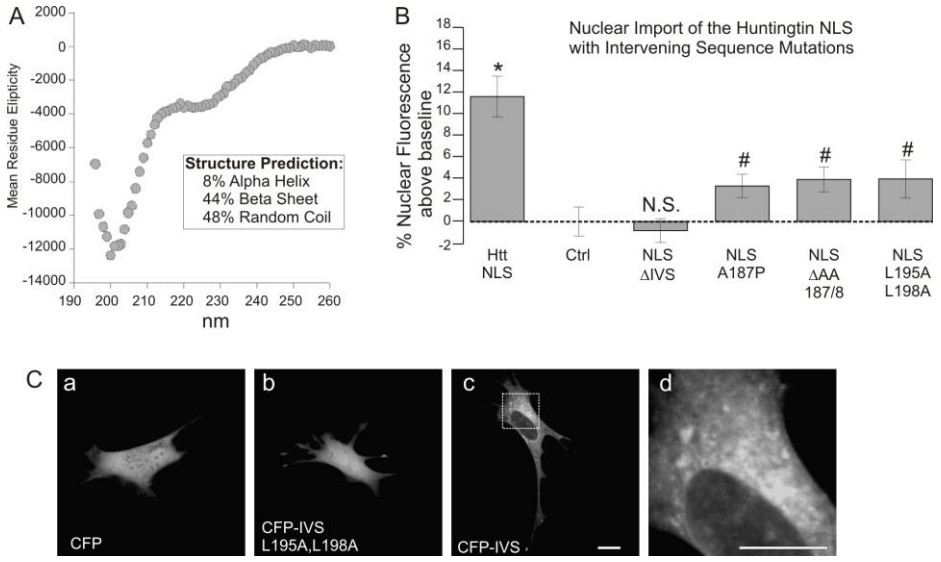


Figure 6: *The Huntingtin NLS 18 Amino Acid Intervening Sequence Has a Beta sheet Structure and Cytoplasmic Targeting Activity.* (A), CD-Spectroscopy of the huntingtin IVS peptide: NH2-PRSLRAALWRFAELAHLVRP-COOH, showing 44% beta sheet characteristics. (B), effect of IVS mutants on huntingtin NLS activity in the context of eGFP-X-βGalactosidase. Note the single proline substitution A187P, ΔAA(187,188) deletion and double leucine substitution L195A, L198A. (C), comparison of nuclear localization of CFP alone (panel a), CFP-IVS (panel c) and CFP-IVS L195A, L198A (panel b) in ST*Hdh* cells. Leucines 195 and 198 are critical for targeting to cytoplasm. Area in panel c is magnified in panel d to highlight targeting to cytoplasmic structures. N=approximately 70 cells per construct. #= p values which are statistically significant compared to the wild-type huntingtin NLS ($P<0.01$). *= p values which are statistically significant compared to Ctrl ($P<0.01$). N.S is non-significant compared to control. Bars represent standard error.

protein concentration of 0.25 mg/ml. (Figure 6A). Using a neural 2D network algorithm (Greenfield 2006), the secondary structure was calculated to be 44% beta sheet. Similar results were seen when the entire PY-NLS 174-207 peptide was assayed (data not shown). When an A187P or ΔAA(187,188) mutation was made at the centre of the IVS (Figure 6B), NLS activity was lost in context of GFP-βGalactosidase, despite the fact that these mutations fall at a considerable distance from the consensus elements. This indicates that structure is critical to huntingtin NLS nuclear import activity.

3.3.4 The Huntingtin PY-NLS Intervening Sequence has Cytoplasmic Targeting Activity

In order to determine if the structured PY-NLS intervening sequence has intrinsic biological activity, or is only behaving as a spacer element, we fused it to an mCerulean blue fluorescent protein for expression in live cells. When expressed alone, mCerulean blue (CFP) localizes to both the cytoplasm and the nucleus since its molecular weight falls below the diffusion limit of the nuclear pore complex (NPC) (Figure 6C, panel a). AFP proteins can even be trimerized in tandem and still diffuse across the NPC (Seibel et al. 2007). Fusion of the IVS to CFP only increases the molecular weight by a few kilo Daltons, therefore, it would be expected to display the same diffuse nuclear and cytoplasmic localization. However, in ST*Hdh* cells, this fusion was found to target the cytoplasm. In order to establish that the targeting of the CFP-IVS fusion was not solely due to insolubility of the small peptide, we created a L195A, L198A double mutation of similar hydrophobicity. However, the interaction appeared to be specific, as the

mutation led to a loss of cytoplasmic targeting (Figure 6C, panels a-d). Since the L197A, L198A mutation disrupted cytoplasmic targeting, we anticipated that it might allow functional separation of the counteractive cytoplasmic targeting of the intervening sequence and the nuclear targeting of the NLS. However, even this structurally subtle mutation inhibited the activity of the NLS (Figure 6B). We noted that the hydrophobic residues and spacing in the IVS sequence could potentially fit the consensus of a CRM1-dependent nuclear export signal (Bogerd et al. 1996) However, the sequence appeared to be to targeted to specific cytoplasmic structures (Figure 6C, panel d), inconsistent with an NES, and localization was not affected by leptomycin B treatment (data not shown). Thus, the huntingtin PY-NLS intervening sequence appears to have independent targeting capacity to cytoplasmic factors, an activity that may sterically regulate the PY-NLS functional interactions with karyopherins.

3.4 Discussion

The defined PY-NLS consensus was derived *in vitro* by establishing important interaction residues between NLSs and karyopherin β2 by x-ray co-crystal and isothermal calorimetry studies (Lee et al. 2006). We have discovered a PY-NLS in the huntingtin protein that is both highly evolutionarily conserved, and active in heterologous fusion nuclear import assays in live cells. Although components of the huntingtin PY-NLS conform to the sequence requirements of the consensus, including the A site (R/H/$KX_{(2-5)}$-PY) and the B site (the upstream basic rich region) (Lee et al. 2006) it diverges in terms of the spacing of these epitopes. While the consensus defines 8-10 amino acids between the PY motif and the basic rich region, the intervening sequence of the huntingtin PY-NLS is 27 amino acids (from the proline-tyrosine to the upstream basic region). CD spectroscopy analysis reveals another significant inconsistency with typical PY-NLSs. While typical PY-NLSs are found in unstructured regions, it appears that the huntingtin PY-NLS contains structured elements within its IVS. These two important findings define the huntingtin NLS as a novel sub-type of structured PY-NLS. To fit the binding pocket of karyopherin β2 or β1, this longer PY-NLS may require the formation of secondary structure to align the critical epitopes for import factor interaction in 3D space. This is consistent with the ability of the consensus elements of the PY-NLS to bind karyopherin β2 in an energetically independent manner (Suel, Gu, and Chook 2008).

HEK 293 cells do not permit huntingtin PY-NLS function, despite the presence of karyopherin β2 and β1. This suggests that there may be an additional factor in this cell line that competitively inhibits nuclear entry by binding to the PY-NLS, possibly through the intervening sequence. Consistent with this hypothesis, the intervening sequence has independent cytoplasmic targeting ability *in vivo*. These data present a caveat for the interpretation of studies that use HEK293s as a model system to examine huntingtin nuclear import and toxicity. If huntingtin is able to activate or repress nuclear entry through the IVS it may explain how huntingtin nuclear localization is regulated in different cell types throughout the body. Some differentiated cells may restrict the ability of huntingtin to enter the nucleus after development. Indeed, classic studies of huntingtin localization in human brain have shown a neuronal cell-type variance in huntingtin nuclear localization (Hoogeveen et al. 1993; Sapp et al. 1997).

Consistent with the concept of an additional class of karyopherin β1 and β2-dependent regulated NLSs, recently, the HIV-1 Rev protein was shown to import into the nucleus by both karyopherin β1 –direct (Truant and Cullen 1999), and karyopherin β2 pathways, with a binding factor that regulates these NLS-transport factor interactions (Gu et al. 2011). Other proteins, such as the c-jun oncoprotein, have been seen to interact with both karyopherin β1 and β2 as well as other import factors *in vitro*, via a basic stretch of amino acids, but without the PY epitope of a karyopherin β2-dependent NLS (Nikolaev, Cochet, and Felenbok 2003; Waldmann, Walde, and Kehlenbach 2007). In the case of C-jun, regulation of the karyopherin β1/ β2 NLS was demonstrated to

depend upon steric hindrance by homo-dimerization (Malnou et al. 2007). The mRNA export factor, NXF1 contains a PY-NLS, but this signal can enter the nucleus through redundant pathways that include karyopherins β1, β2, importin alpha and importins 4 and 11 (Zhang et al. 2011).

Huntingtin has an essential and critical role in neurogenesis during development, and a role at the mitotic spindle apparatus in cell division (Zhang et al. 2011; Kamei et al. 1999; Sato et al. 2011). Thus, it is likely that the nuclear import of such a critical factor has evolved to not rely solely on one import pathway. Furthermore, by utilizing a karyopherin β1 direct pathway, the huntingtin NLS would not depend on the karyopherin α adaptor family, and thus not be limited by their cell-type specific expression (Kamei et al. 1999). Consistent with the karyopherin β2 import factor pathway for huntingtin nuclear entry, in mouse brain, karyopherin β2 is highly expressed in regions of neurogenesis and circadian clock regulation (Sato et al. 2011). Disruption in circadian rhythms have been noted in both HD patients, and HD mouse models (Petersen, Hult, and Kirik 2009).

Our mutational analysis in the 1-465 fragment of huntingtin concludes that this PY-NLS sequence is the only NLS within the amino-terminus of huntingtin. This fragment contains the expanded polyglutamine tract and comprises regions seen in proteolytic fragments of huntingtin implicated in HD pathology (Mende-Mueller et al. 2001; Leavitt et al. 2001; Gafni et al. 2004). The identification of huntingtin intervening sequence

binding proteins, and their possible regulation of huntingtin nuclear entry, are the subject of further study.

CHAPTER 4

DIRECT REGULATION OF HUNTINGTIN NLS ACTIVITY: AN EXPLORATION OF POTENTIAL MECHANISMS

The data described in this chapter is currently unpublished.
All experimental work was conducted by the author: Carly R. Desmond

4.1 Huntingtin NLS Activity May Be Regulated By the Intervening Sequence

The huntingtin NLS is regulated directly by at least two nuclear transport receptor pathways: karyopherin β1 and β2. Even with this redundancy in place, the huntingtin NLS fails to mediate nuclear import of the eGFP-βGalactosidase reporter when expressed in the HEK293 cell line, despite moderate activity in ST*Hdh* cells (Figure 5A,B). The ability to import other karyopherin β2 and β1 substrates, such as the M9 NLS and SV40 NLS, and confirmed of expression of both karyopherins by western blot, suggest that these pathways are neither inhibited nor absent in HEK293 cells. The implication instead is that the huntingtin NLS is responsive to a secondary mechanism of regulation, independent of import receptor binding, that controls huntingtin's ability to enter the nuclear compartment. Even in ST*Hdh* cells, where the huntingtin NLS shows marked activity, the nuclear import of eGFP-Htt NLS-βGalactosidase is still moderate compared to other import substrates that use the karyopherin β2 pathway, such as the M9 NLS (Figure 3C,D). While activity is altogether absent in HEK293s, it may also be impaired or regulated in ST*Hdh* cells, although at reduced levels.

As a follow-up to these experiments, the activity of eGFP-Htt NLS-βGalactosidase was tested in additional cell lines to search for possible trends. NLS activity in NIH 3T3 cells resembled that of ST*Hdh*, however, MCF-7 cells produced that same inhibited

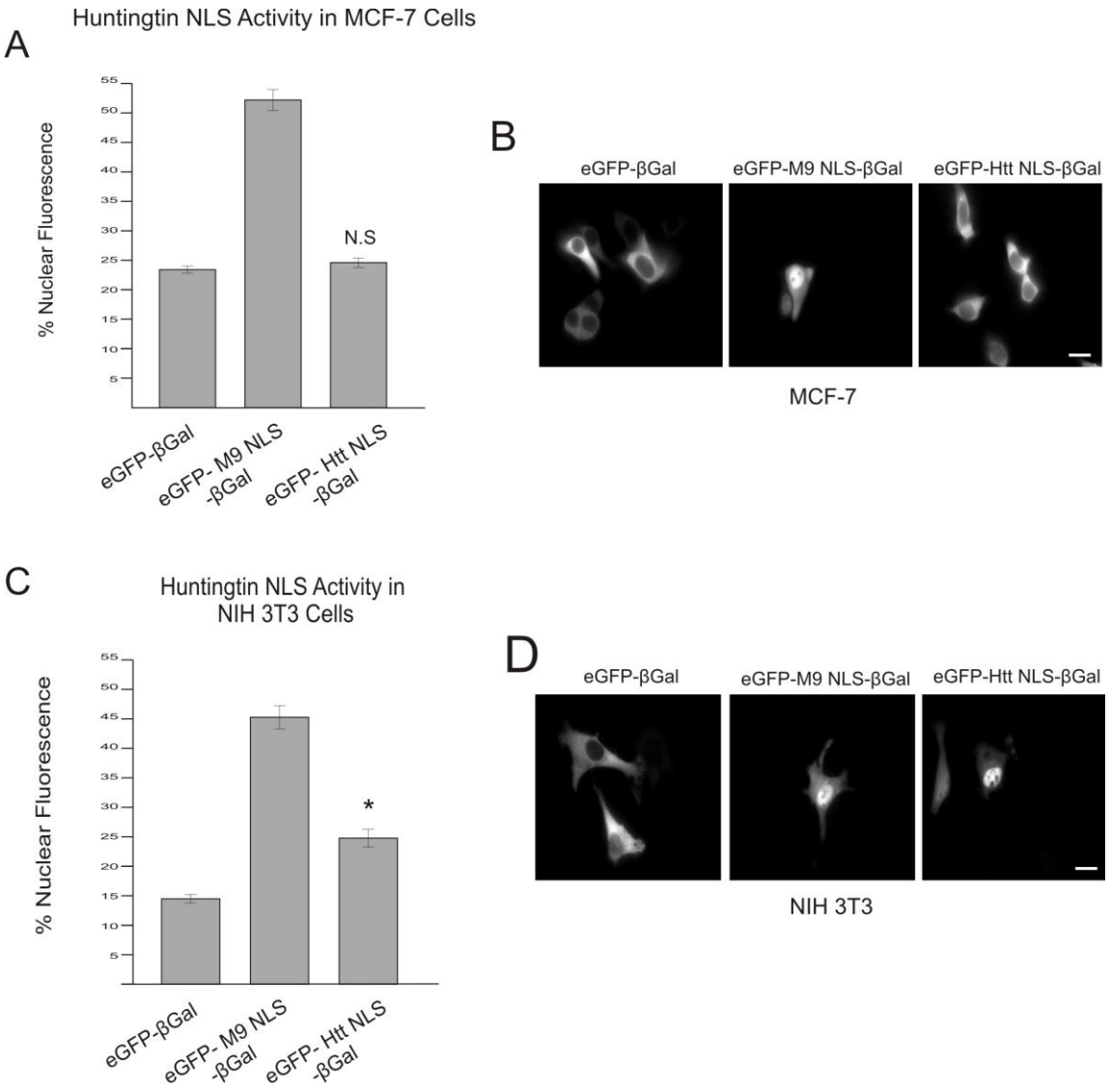


Figure 7: *Huntingtin NLS Activity in MCF-7 and NIH 3T3 Cells*. Huntingtin NLS activity in the context of the fluorescent reporter eGFP-βGalactosidase, in MCF-7 and NIH 3T3 cells, is quantified in A and C respectively. The nuclear fluorescence/import activity of the positive control (M9 NLS) and negative control (eGFP-βGalactosidase) are quantified for comparison. Representative images are shown in B and D. n= approximately 50 cells quantified per construct. Significance was measured relative to eGFP-βGal. *=P value <0.01. N.S indicates non-significant values. Bars represent standard error. Scale bars = 10 µm.

phenotype as HEK293s (Figure 7). There are two possible explanations for these cell-line specific discrepancies: First, both the ST*Hdh* and NIH 3T3 cell lines are of mouse origin, while the HEK293 and MCF-7 cells are human. It is possible that factors which affect the activity of the NLS are expressed at different levels, or have different functions, in mouse versus human cells, offering stricter control over the activity of the NLS in the human cell lines. The second possible explanation is that the nuclear import of huntingtin is affected by cellular morphology. Both ST*Hdh* and NIH 3T3 cells have a fibroblast-like phenotype, consisting of a flat and expansive cytoplasm rich in cytoskeletal components, such as microtubules and actin (See Figure 7D). The HEK293 and MCF-7 cell lines, alternatively, possess a more compact cellular morphology, and make less contact with the bottom of the tissue culture dish (See Figure 7B).

Although the huntingtin NLS contains the rudimentary elements of a PY-NLS, its excessive length, and structured intervening sequence (IVS), are unlike any previously characterized karyopherin β2 substrates (Figure 3A,B and 6A,B) (Lee et al. 2006). Even though the IVS is contained within the limits of the NLS sequence, it appears to have its own preferences for cellular localization. The IVS is sufficient to mediate targeting of a fluorophore to the cytoplasm, suggesting that may compete with the nuclear import activity of the NLS (Figure 6C). Like a tug-of-war, the levels of cytoplasmic elements such as proteins or lipids that enhance the cytoplasmic localization of the IVS may also contribute to the overall activity of the NLS. It is therefore possible that the IVS and its

interacting partners are responsible for the cell-line specific differences in huntingtin NLS activity.

The structure of the intervening sequence is required for full function of the huntingtin NLS. Mutations that alter the structural properties of the IVS, even slightly, reduce binding of the NLS to karyopherin β2 and limit the ability of the sequence to mediate nuclear entry. This effect is readily observed when IVS mutations are introduced to eGFP-Htt NLS-βGalactosidase and expressed in ST*Hdh* cells (Figure 6B). However, these observations are quite different when the same constructs are expressed in a normally non-permissive cell line, such as HEK293s. Disrupting the structure of the IVS by mutation actually enhances the activity of the NLS in these cells (Figure 8A,B). When expressed alongside karyopherin β2 (541-890), the import of these constructs is improved even further (Figure 8C). These results are consistent with the hypothesis that the intervening sequence participates in the regulation of huntingtin NLS activity. The cytoplasmic targeting of the IVS is likely dependent, at least partially, on the sequence's structural features. If the reason the NLS lacks activity in HEK 293 and MCF-7 cells is due to increased repression of the NLS though the IVS, relief of this repression would, theoretically, trigger nuclear entry. Even though IVS mutations also decrease the affinity of the huntingtin NLS for karyopherin β2, this effect may be minimal compared to their direct impact on interaction partners of the IVS itself. This may explain why the same mutations that disrupt NLS activity in ST*Hdh* cells boost activity in HEK293s.

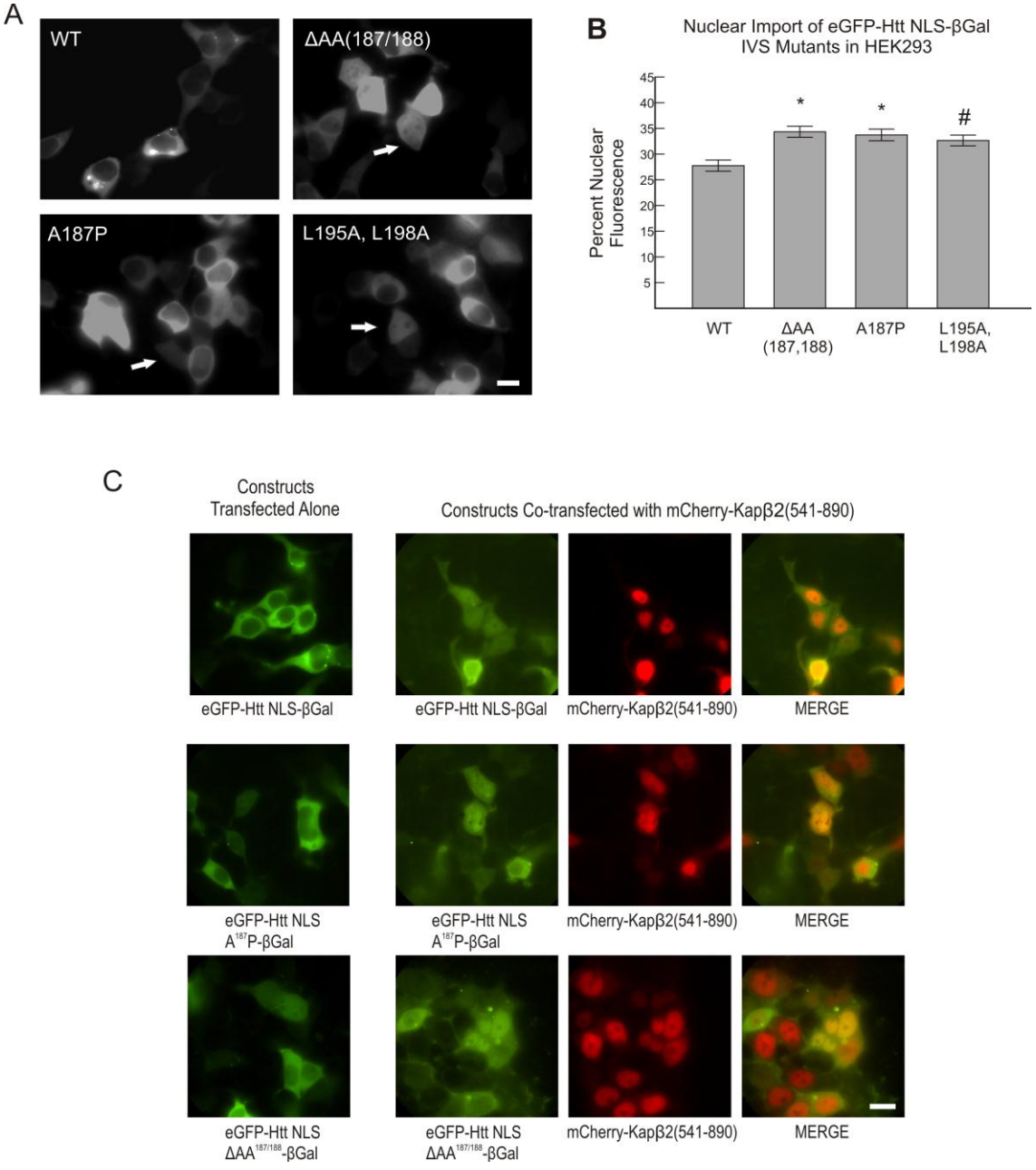


Figure 8: *Huntingtin NLS Activity is Partially Rescued in HEK293 Cells by Mutation of the Intervening Sequence (IVS).* eGFP-Htt NLS-βGal and three IVS mutants [ΔAA(187/188), A187P and L195A,L198A] were transfected in HEK293 cells; images are shown in A. The percent nuclear import activity of each construct in the HEK293 cell line is quantified in B. Nuclear import activity was further enhanced by co-expression of the IVS mutants with mCherry-Karyopherin β2 (541-890) (C). n= approximately 80 cells quantified per construct. Significance was measured relative to eGFP-Htt NLS-βGal (WT). * = P value <0.001 and # = P value 0.002. Bars represent standard error. Scale bars = 10 µm.

If the intervening sequence controls the activity of the huntingtin NLS, overexpression of the domain in isolation should partially relieve the repression of huntingtin nuclear import in larger contexts. When huntingtin 1-465-eYFP is co-expressed with mCerulean-IVS, we observed a slight increase in the construct's nuclear localization (Figure 9A). However, when the same experiment was completed with a larger fragment of huntingtin (1-586-eYFP), the results were not significant (Figure 9B). The cellular localization of these construct differs slightly, even under normal conditions. While 1-465-eYFP displays diffuse nuclear and cytoplasmic localization in almost every transfected cell, 1-586-eYFP has a more variable phenotype, with a far higher percentage of cells having only cytoplasmic localization of the construct. It is possible that overexpression of the IVS does not alter the localization of huntingtin 1-586 due to other levels of regulation that override the activity of the NLS.

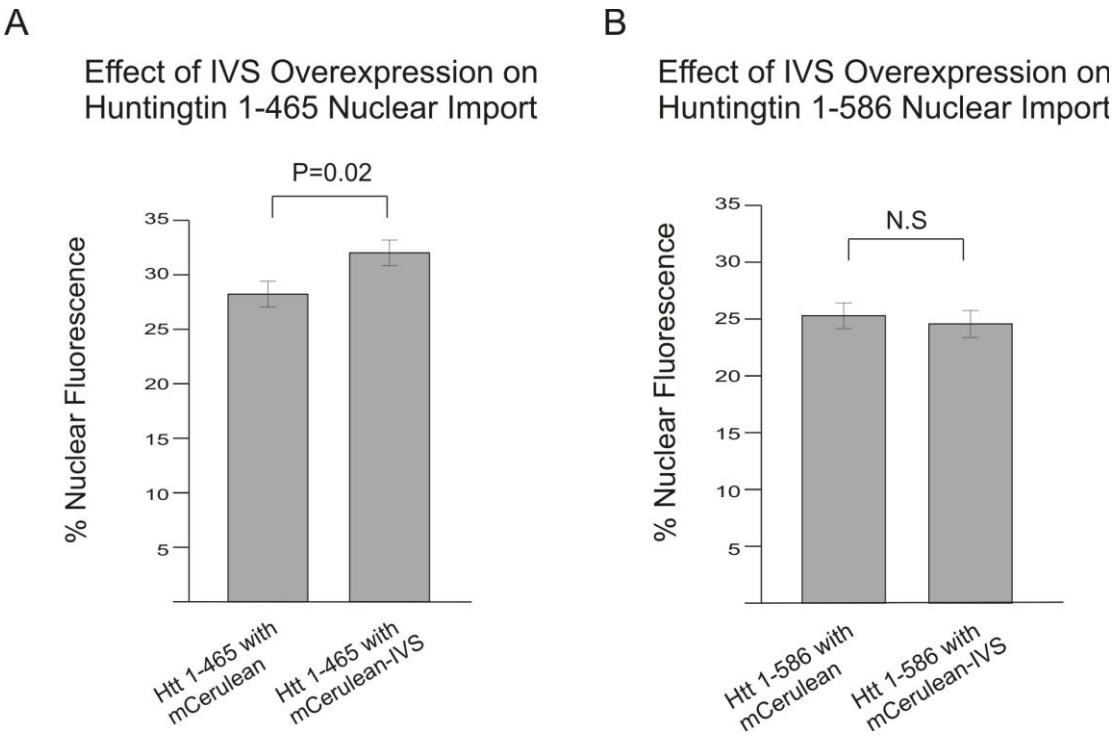


Figure 9: *Overexpression of mCerulean-IVS Increases the Nuclear Localization of Huntingtin 1-465-eYFP, but not Huntingtin 1-586-eYFP*. Htt 1-465-eYFP and Htt 1-586-eYFP were co-expressed in ST*Hdh* cells with mCerulean alone or mCerulean-IVS. The percent nuclear fluorescence of the huntingtin fragments are shown in A and B respectively. n= approximately 50 cells quantified per construct. The significant value (P=0.02) is indicated. N.S indicates values that are not significant relative to the mCerulean control. Bars represent standard error.

4.2 The Intervening Sequence Targets the Huntingtin NLS to Membranes, but is not Abundantly Associated with Autophagic or Lysosomal Vesicles.

Overexpression of mCerulean-IVS in HEK293 cells, and extraction of the membrane fraction after NP-40 lysis, followed by western blot analysis, indicates that the IVS sequence has preference for the membrane fraction (Figure 10A). In contrast to mCerulean alone, or the L195A, L198A mutant (which causes loss of cytoplasmic targeting, Figure 6C), the levels of the mCerulean-IVS construct are higher in the insoluble "pellet" fraction of the lysate. In an attempt to narrow down whether the NLS has affinity for membranes and vesicles associated with particular cellular functions, we assessed the co-localization of mCerulean-IVS with the autophagic and lysosomal markers, LC3 (microtubule associated protein 1 light chain-3) and LAMP1 (Lysosomal-associated membrane protein 1) respectively (Figure 10B,C). Co-localization of mCerulean-IVS with eYFP-LC3 was assessed by co-expression in ST*Hdh* cells (Figure 10B), while LAMP1 localization was highlighted by immunofluorescence (Figure 10C). Although a few small points of co-localization could be identified, overall the markers showed little consistency with the localization of mCerulean-IVS.

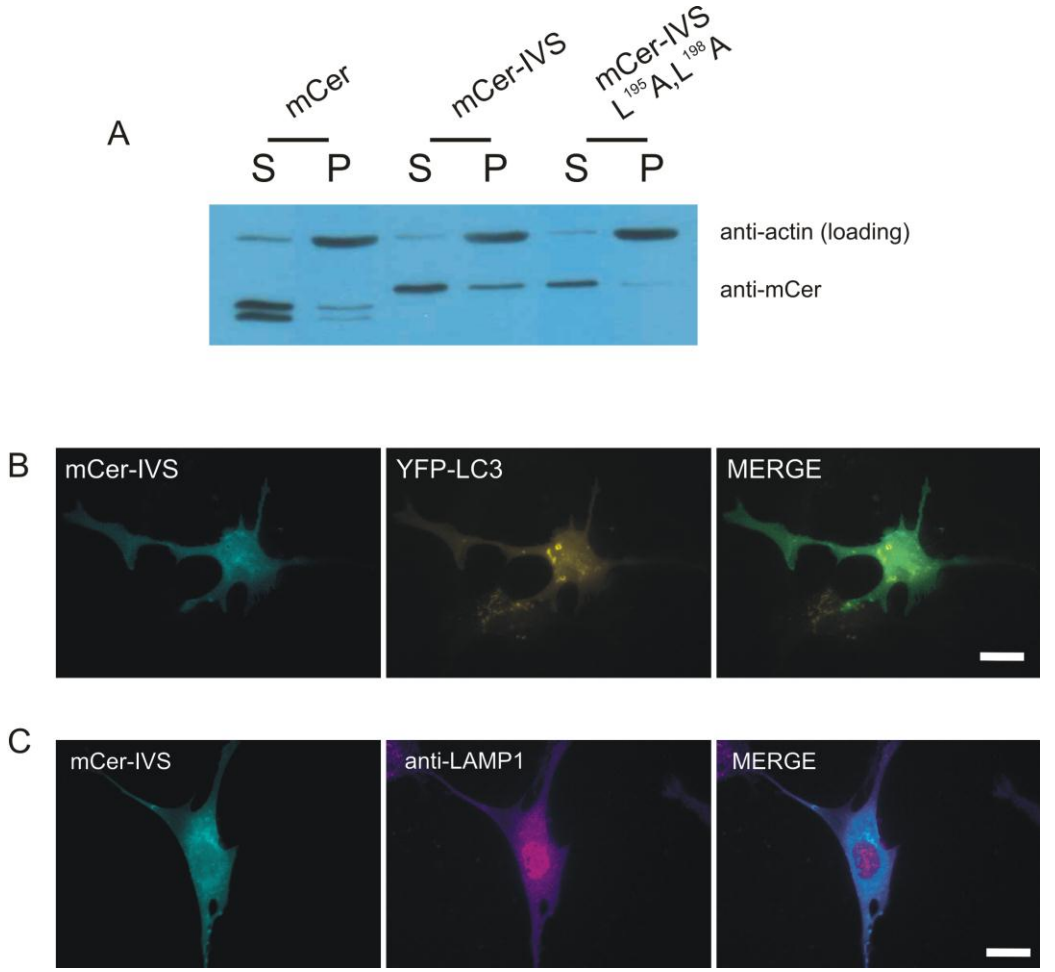


Figure 10: *The Intervening Sequence Mediates Targeting to Membranes, but is not Abundantly Associated with Endosomes or Lysosomal Vesicles.* Membrane (P for pellet) and Soluble (S) lysate fractions from HEK293 cells transfected with mCerulean, mCerulean-IVS and mCerulean-IVS L195A,L198A were analyzed by western blot (A). Blots were probed with anti-AFP (to visualize mCerulean) and the loading control anti-actin. mCerulean-Htt NLS was expressed in ST*Hdh* cells alongside YFP-LC3 (B) or alone and stained for endogenous LAMP1 by immunofluorescence (C). LC3 is a marker of endocytic vesicles and LAMP1 is a marker of lysosomal vesicles. Images were taken at 60X on a widefield microscope. Scale bars represent 10 µm.

4.3 Overexpression of the Intervening Sequence Triggers the Formation of Cellular Projections or "Neurites"

Overexpression of the IVS produced some unexpected phenotypes in ST*Hdh* cells. Besides highlighting membranes and vesicular structures, the intervening sequence also initiated a morphology change in approximately 5-10% of the transfected cells. Within this small population, the cytoplasm appears compact near the nucleus, and long branching projections are formed, similar to ST*Hdh* cells that have undergone differentiation into neurons (Figure 11). Although it is unclear what relationship the intervening sequence has to cellular morphology, it is yet another unique aspect of the domain's function that suggests that it could impact NLS activity and nuclear import of huntingtin as a whole.

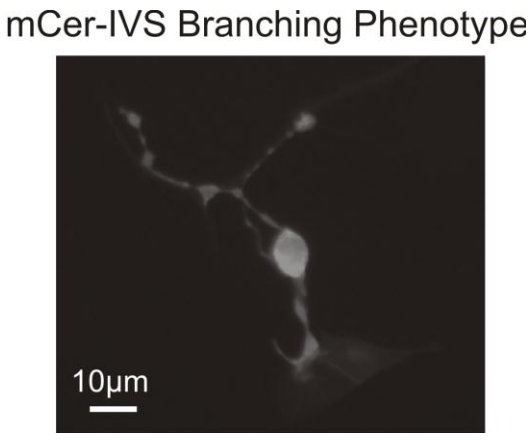


Figure 11: *Overexpression of the Intervening Sequence Generates a Neurite Branching Phenotype*. mCerulean-IVS was overexpressed in ST*Hdh* cells. A 60X widefield image with prominent projections and a compact nucleus is shown. Phenotype is visible in approximately 10% of transfected cells. Scale bar represents 10 µm.

4.4 Cytoplasmic Targeting of the Intervening Sequence is Not Mediated by a CRM1 Nuclear Export Signal.

Computational secondary structure analysis predicts that the IVS forms an alpha helix, and not a beta sheet as suggested by CD spectroscopy (Figure 6A). Nuclear export signals, which typically fall within alpha helical domains, are loosely defined by the consensus L-X(2,3)-[LIVFM]-X(2,3)-L-X-[LI], where X represents any amino acid (Bogerd et al. 1996; Guttler et al. 2010). The huntingtin NLS contains a region within the intervening sequence that closely, although not entirely, matches the consensus for CRM1 mediated nuclear export (Figure 12A). Leptomycin B is a chemical that enters the binding pocket of CRM1 and forms a covalent bond, permanently inhibiting CRM1 nuclear export activity (Kudo et al. 1998). When cells expressing mCerulean-IVS were treated with leptomycin B, no change to the cytoplasmic localization was observed (data not shown). Therefore, if the IVS does have export activity, it is not the primary reason why the sequence targets the cytoplasm so strongly. We hypothesized that a potential NES may be more active in larger fragments of huntingtin, and therefore opted to treat Htt 1-465-eYFP with leptomycin B, alongside the positive control ataxin-7 (228-619), which contains a CRM1-type nuclear export signal (Figure 12B). Although a small, but statistically significant, increase in nuclear localization was observed for 1-465 in the presence of leptomycin B, it was minor compared to the altered localization of the positive control (Figure 12B). After this experiment was completed, a nuclear export signal was identified and characterized within the first 17 amino acids of huntingtin

(Maiuri et al. *in press*). It is likely that the small increase in nuclear localization observed with leptomycin B treatment can be attributed to this NES, and not to any activity of the intervening sequence. However, in attempt to rule out this possibility, mutations were introduced to mCerulean-IVS to abolish the putative NES sequence (L195A,L198A) (Figure 12C). Although this does cause a complete loss of cytoplasmic targeting, as would be expected if the intervening sequence was a nuclear export signal, the putative NES sequence alone (lwrfaelahl) failed to behave as an NES in isolation (i.e no predominantly cytoplasmic localization was observed) (Figure 12C). This suggests that the loss of targeting caused by the L195A,L198A mutation is not caused by a loss of a CRM1 interaction, but rather inhibition of a cytoplasmic protein-protein (or protein-membrane) interaction that depends on the leucine side-groups. In retrospect, it would have been valuable to also test the sequence lraalwrfaelahl, in case the NES was misidentified in the first fragment. However, most of the data thus far indicates that the IVS does not have true NES activity.

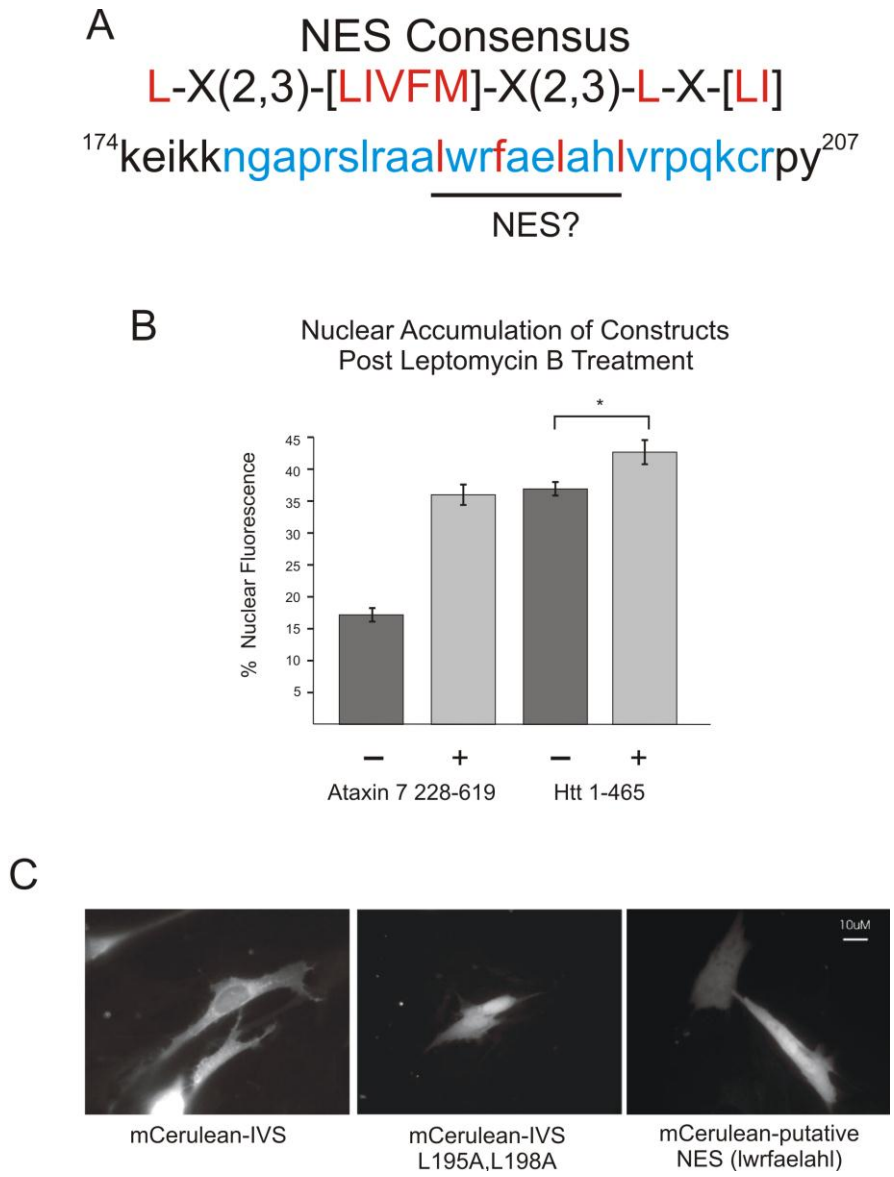


Figure 12: *Cytoplasmic Targeting of the Intervening Sequence is not Mediated by an Embedded Nuclear Export Signal.* The loose consensus for nuclear export signals is shown in (A), alongside the huntingtin NLS sequence. The intervening sequence is highlighted in blue and the consensus elements are highlighted in red. The percent nuclear accumulation of Htt 1-465-eYFP and the positive control Ataxin-7 (228-619)-eYFP in ST*Hdh* cells were quantified in the absence and presence of 10 nM leptomycin B (B). * = P values < 0.01. mCerulean-IVS localization is compared to mCerulean-IVS L195A,L198A (a mutation of the putative NES sequence) and mCerulean-putative NES (isolation of the predicted NES sequence) in (C). 60X widefield images. Scale bar represents 10 µm.

4.5 Cytoplasmic Targeting of the Intervening Sequence is not Mediated by Palmitoylation

Palmitoylation is a reversible post-translational modification that involves the attachment of a C16 carbon saturated fatty acyl chain to cysteine residues via a thioester linkage. This attachment is mostly mediated by palmitoyl-acyl transferase proteins, and can be reversed through the action of protein palmitoyl thioesterases. The hydrophobicity of a protein is enhanced by palmitoylation, which commonly causes an increased association with membranes (Charollais and Van Der Goot 2009). Because palmitoylation is reversible, it is an effective way of temporarily changing a protein's localization during a variety of signaling events (Young et al. 2012).

Thus far, only one palmitoylation site has been identified within huntingtin at cysteine 214 (C214), just seven amino acids downstream from the huntingtin NLS (Yanai et al. 2006). This site is thought to be palmitoylated by huntingtin-interacting protein 14 (HIP14), and modification is reduced on polyglutamine expanded huntingtin (Yanai et al. 2006; Huang et al. 2011). Palmitoylation of C214 reduces mutant huntingtin toxicity in overexpression assays and is therefore considered beneficial and necessary to huntingtin's normal function (Yanai et al. 2006). C204 was tested, but not detected to be a site of palmitoylation in these studies. However, due to the distinctly membranous localization of the intervening sequence in our assays, we did not want to rule out the possibility of C204 palmitoylation without further experimental evidence. Therefore, we introduced a palmitoylation resistant mutation to cysteine 204 (C204S) in the context of

mCerulean-IVS and expressed the fusion in ST*Hdh* cells. We did not observe any changes to the localization of mCerulean-IVS C204S, indicating that palmitoylation of the cysteine residue is not responsible for targeting the fragment to the cytoplasm (Figure 13).

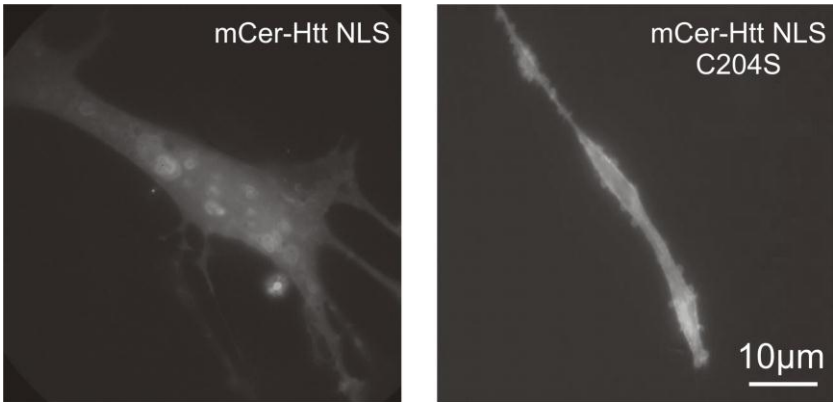


Figure 13: *Palmitoylation of Cysteine 204 does not Direct Membrane Targeting of the Huntingtin NLS.* ST*Hdh* cells expressing mCerulean-Htt NLS and mCerulean-Htt NLS C204S (palmitoylation resistant mutant) are shown. 60X widefield images. Scale bar represents 10 µm.

4.6 Active Transcription is not Required for Huntingtin NLS Activity

The first NLS sequence found to interact with karyopherin β2, and the same sequence used to generate the PY-NLS consensus by X-ray crystallography, was the M9 NLS from the mRNA binding protein hnRNPA1 (heterogeneous nuclear ribonuclear protein A1) (Siomi and Dreyfuss 1995; Pollard et al. 1996; Lee et al. 2006). It has since been discovered that a large fraction of the NLS sequences recognized by karyopherin β2 are involved in either the processing or transport of RNA (Lee et al. 2006).

Interestingly, a dependence on transcriptional activity has been linked directly to the activity of some PY-NLS sequences, such as the M9 NLS and the NLS of HuR (human antigen R) (Michael, Choi, and Dreyfuss 1995; Izaurralde et al. 1997; Fan and Steitz 1998). Inhibiting RNA polymerase II with actinomycin D blocks nuclear translocation of these NLS sequences, even when expressed out of their normal protein context. We hypothesized that huntingtin's PY-NLS may also be regulated by transcription. ST*Hdh* cells transfected with either eGFP-Htt NLS-βGalactosidase, or the positive control eGFP-M9 NLS-βGalactosidase, were treated with actinomycin D at a concentration of 5 μg/mL. Although the M9 NLS responded to the treatment appropriately, with reduced nuclear import of the reporter, there was no statistically significant change to the localization of the huntingtin NLS (Figure 14A). Even though active transcription is not required for function of the huntingtin NLS, these experiments do not rule out the possibility that huntingtin has an RNA associated function. When ST*Hdh* cells were treated with lower concentrations of actinomycin D (0 μg/mL, 0.025 μg/mL, 0.05 μg/mL and 0.1 μg/mL) and the localization of endogenous huntingtin was probed by immunofluorescence, the nuclear targeting of huntingtin increased in a concentration dependent manner (Figure 14B). If huntingtin participates in the export and cytoplasmic transport of mRNA, it is possible that a shortage of newly polymerized mRNA transcripts could cause an accumulation of huntingtin in the nuclear compartment. However, there are alternative explanations that could account for this change in localization. Huntingtin is known to become phosphorylated at N17 and re-locate to the nucleus in response to cell stress.

Therefore, high concentrations of actinomycin D, which beyond inhibiting RNA polymerase may induce cell stress, could increase the level of huntingtin within the nucleus (Figure 14C). This altered localization was apparent when cells treated with actinomycin D were probed for endogenous huntingtin using a phospho-specific antibody, however, since actinomycin D intercalates DNA at promoters, it is possible that the only effect we observe is a loss of more direct targeting of huntingtin to these sections of DNA [Figure 14C modified from (Atwal et al. 2011)].

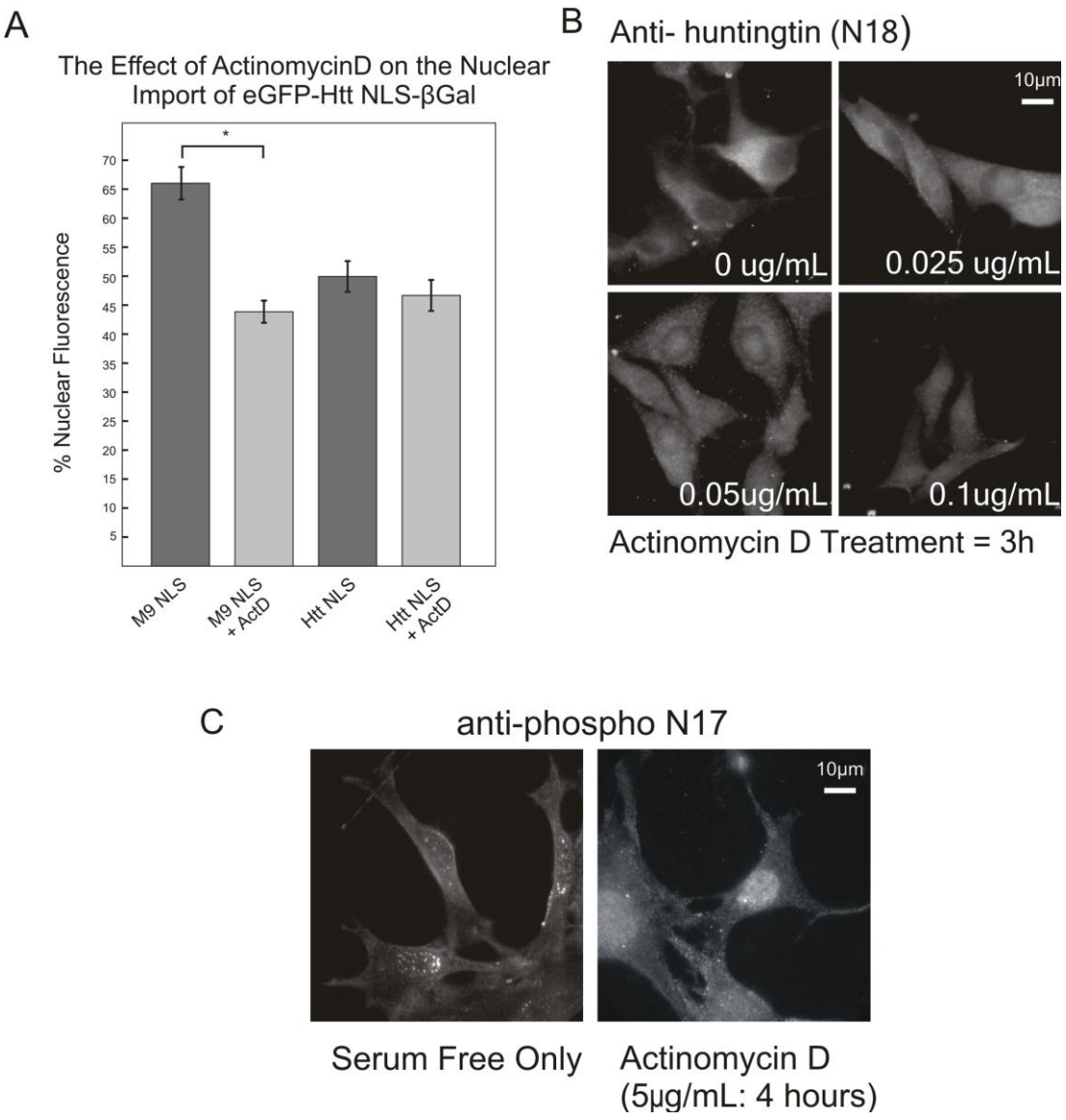


Figure 14: *Transcriptional Inhibition with Actinomycin D Does Not Disrupt Huntingtin NLS Activity.* eGFP-Htt NLS-βGal and the positive control eGFP-M9 NLS-βGal were expressed in ST*Hdh* cells. Quantification of the percent nuclear import of the constructs with and without addition of actinomycin D (ActD) is shown in (A). *= P values <0.01. n=approximately 60 cells. (B) ST*Hdh* cells were treated with the indicated concentrations of actinomycin D for 3 hours then fixed and stained for endogenous huntingtin (N18). (C) ST*Hdh* cells were treated with high concentrations of actinomycin D (5 µg/mL) for 4 hours and stained with anti-phospho N17. 60X widefield images. Scale bars represent 10 µm.

4.7 Huntingtin Localization is Not Altered by Acetylation

Acetylation is a post-translational modification most commonly associated with the biological function of histones (Allfrey, Faulkner, and Mirsky 1964). HATs (histone acetyl transferases) catalyze the covalent linkage of acetyl groups (CH_3C0) to charged amino groups of lysine side-chains. Alternatively, HDACs (Histone Deacetylases) are responsible for reversing this chemical reaction. In histones, eliminating the positive charge of lysine residues by acetylation causes a decreased affinity for negatively charge DNA. As a result, acetylated histones tend to be associated with regions of active transcription (Sterner and Berger 2000).

Although acetylation was first characterized on histones, it is now apparent that many "HATs" and "HDACs" function in the cytoplasm as well, to modify other protein substrates (Sadoul et al. 2011). This has led to the introduction of new nomenclature, which more accurately refers to HATs and HDACs as Lysine Acetyl-transferases (KATs) and Lysine Deacetylases (KDACs). Like phosphorylation, acetylation can have a variety of effects on protein function, from altering protein-protein interactions to influencing protein stability and targeting.

Several acetylation sites were recently identified within huntingtin 1-612 using mass spectrometry analysis. These experiments confirmed the location of two previously reported acetylated amino acids (K9 and K444), while identifying three novel sites at K178, K236 and K345 (Cong et al. 2011; Jeong et al. 2009). The K178 acetylation site falls within the basic rich region of huntingtin's PY-NLS. Mutation of KK177/178AA

in the context of eGFP-Htt NLS-βGalactosidase abolishes nuclear import activity of the NLS and reduces its activity in Htt 1-465-eYFP.

In the past, acetylation of NLS sequences has been found to both up-regulate and down-regulate nuclear import, depending on the protein studied. The acetyl-transferase PCAF becomes autoacetylated within its NLS, causing increased nuclear localization (Blanco-Garcia et al. 2009). PCAF is also responsible for the acetylation of retinoblastoma protein (Rb) within its NLS during development, which also enhances nuclear localization (Pickard, Wong, and McCance 2010). However, in other cases, acetylation can also be disruptive to nuclear localization. Both IFI16 and Histone 3 (H3) are acetylated within their NLS sequences, inhibiting nuclear import activity (Li et al. 2012; Blackwell et al. 2007). Intuitively, loss of NLS activity by acetylation of critical lysine residues (neutralizing their charge) offers a more likely scenario; the distribution of positively charged lysines across an NLS is one of the most important determining factors for sequence recognition by the karyopherin β family.

To test if acetylation regulates activity of the huntingtin NLS, the acetylation resistant mutation K178R was introduced to huntingtin 1-465-eYFP and expressed in ST*Hdh* cells. Neither the K178R mutation, nor the more severe mutation KK177/178RR, altered the nuclear import activity of the huntingtin 1-465 fragment, either in the positive or negative direction (Figure 15A). The same K178R mutation was tested in the context of eGFP-Htt NLS-βGalactosidase as well, but it also had no observable effect on NLS activity (Figure 15B). Since the original identification of the K178 acetylation site

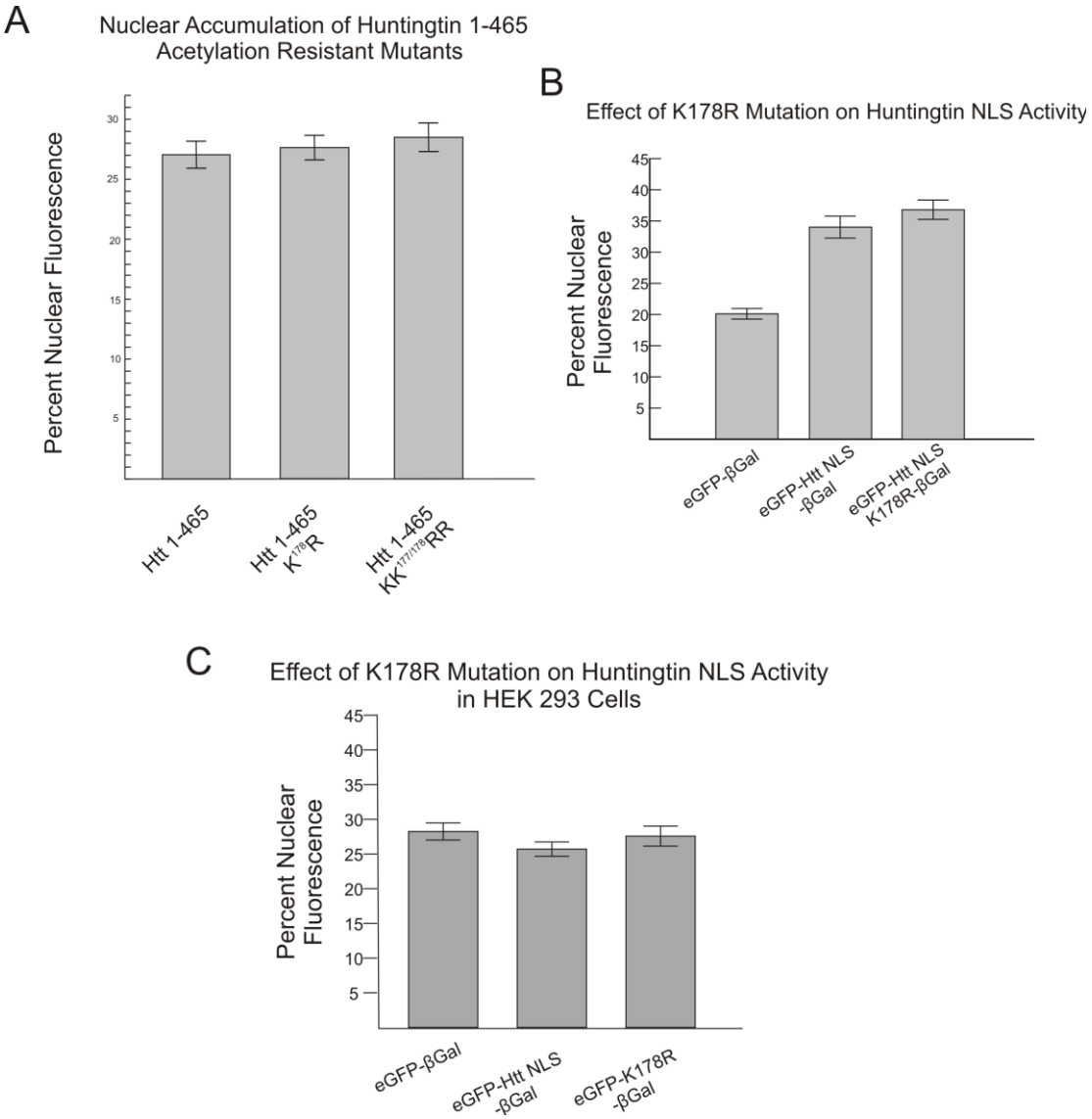


Figure 15: *Acetylation Resistant Mutation of the Huntingtin NLS Does Not Alter NLS Activity*. The nuclear import of Htt 1-465-eYFP is compared to the acetylation resistant K178R and KK177/178RR mutants in ST*Hdh* cells (A). The K178A mutation was also introduced to the huntingtin NLS in the context of eGFP-βGal. The percent nuclear import of these constructs in ST*Hdh* cells and HEK293 cells are shown in (B) and (C) respectively. No significant values were found. n=approximately 50 cells per data set.

was performed using extracts of transfected HEK293 cells (Cong et al. 2011), and because this was the cell-line in which we observed the strongest "repression" of NLS activity, the nuclear import of the eGFP-Htt NLS K178R-βGalactosidase construct was also tested in the HEK293 cell line. However, resistance to acetylation did not serve to "activate" the NLS (Figure 15C). In this context, the mutation had no effect in either of the cell lines tested.

4.8 The Huntingtin NLS Intervening Sequence Interacts with Huntingtin Associated Protein 1 (HAP1)

Isolated by yeast-two-hybrid, huntingtin associated protein 1 (HAP1) was the first protein found to interact with huntingtin (Li et al. 1995). Although the original fragment of huntingtin used in this analysis was 1-230, the HAP1 binding region was later narrowed down to amino acids 171-230 (Bertaux et al. 1998). The observed localization of HAP1 is similar to huntingtin, as it localizes predominantly to the cytoplasm, particularly to microtubules and membrane structures such as the endoplasmic reticulum, endosomes and lysosomes (Gutekunst et al. 1998). HAP1 is a mediator of vesicular trafficking, one of the major functional areas that huntingtin is proposed to participate in (Block-Galarza et al. 1997). It interacts with p150glued, a major subunit of dynactin, linking both itself and likely huntingtin to the retrograde motor protein dynein (Engelender et al. 1997). HAP1 also interact with kinesin light chain, a subunit of the anterograde microtubule motor kinesin, suggesting that HAP1 is

involved in transport in both directions (McGuire et al. 2006). It has been proposed that HAP1's function is to act as an adaptor between huntingtin and the microtubule transporters, allowing huntingtin to behave as a scaffold for the recognition of vesicular cargo (Pardo et al. 2010).

Hap1 is expressed almost exclusively in the central nervous system (Li et al. 1995; Li, Hosseini et al. 1998). Originally, it was proposed that HAP1 could be the link between huntingtin and the cell-type specific toxicity of the disease. However, further analysis appears to suggest that those brain regions most severely affected by HD express the lowest levels of HAP1, at least within the brain. It was instead hypothesized that HAP1 could have a protective role (Fujinaga et al. 2004).

In mice, there are two HAP1 isoforms, HAP1A and HAP1B (Li et al. 1995). These proteins are identical aside from two unique tail regions beginning at amino acid 578. In humans, there is only one HAP1 isoform that is most similar to HAP1A (62% identical) (Li, Hosseini et al. 1998). The functions and protein interaction partners of the two mouse isoforms are mostly, but not entirely, the same. Both isoforms interact with huntingtin; however, only HAP1A has been shown to interact with the ER associated receptor InsP3R1 (Tang et al. 2003). HAP1A has also been shown to promote neurite outgrowth when overexpressed in PC12 cells (Li et al. 2000). This phenotype is strikingly similar to the branching phenotype caused by overexpression of mCerulean-IVS in our ST*Hdh* cell line (Figure 11).

We had hypothesized that the targeting of the intervening sequence to the cytoplasm may be facilitated by a protein-protein interaction. The HAP1 binding domain was already known to be contained within amino acids 171-230, the same region that contains the huntingtin NLS sequence (174-207). Furthermore, the localization of overexpressed HAP1 in tissue culture cells closely resembles the membrane targeting and abnormal loop-like structures that form with mCerulean-IVS overexpression (Li et al. 2000) (See Figure 13 for similar phenotype). Therefore, we sought to determine if the intervening sequence of the huntingtin NLS is sufficient for binding to HAP1.

The first isoform of HAP1 cloned for use in our lab was HAP1B, therefore, it was the subtype of HAP1 used in the earliest experiments. When mCerulean-IVS was co-expressed with mCherry-HAP1B in ST*Hdh* cells, we observed strong co-localization at the HAP1 rich cytoplasmic puncta (Figure 16A). Similar cytoplasmic structures, named stigmoid bodies, have been observed in the past with HAP1 overexpression alone (Li, Gutekunst et al. 1998). To prove that the IVS and HAP1 interact directly, we performed FRET-FLIM microscopy on cells expressing the mCerulean-IVS and the eYFP-HAP1B constructs. We observed a strong shortening of the lifetime of the mCerulean fluorophore within the stigmoid bodies where the two proteins showed the most robust co-localization (Figure 16B). This effect is most clearly demonstrated in the pseudocoloured lifetime images.

The interaction between HAP1 and the intervening sequence was also followed-up with more traditional biochemical experiments. Huntingtin 1-465-eYFP and

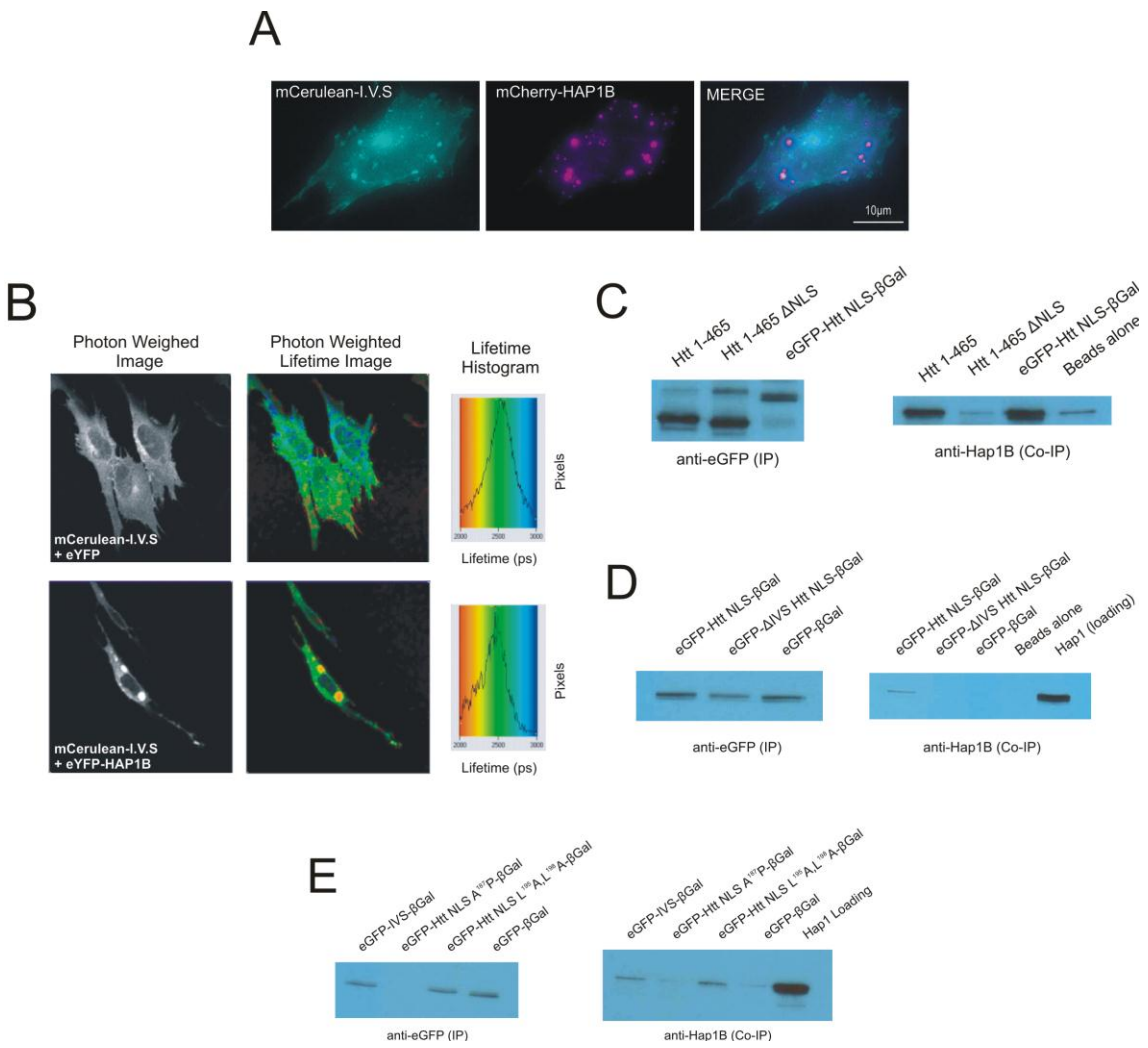


Figure 16: *The Huntingtin NLS Intervening Sequence Interacts With HAP1.* The co-localization of mCerulean-IVS with mCherry-HAP1B in ST*Hdh* cells is shown in (A). All images were acquired by widefield microscopy and then deconvolved. FRET/FLIM analysis of the interaction between the IVS and HAP1 was performed (B). Both the photon weighted mCerulean-IVS image and the photon weighted lifetime image are shown. The photon weighted lifetime image assigns a colourmetric scale to the image based on the lifetime at each pixel. Note the reduced lifetime of mCerulean-IVS at the large HAP1 containing inclusions indicating high FRET. Co-immunoprecipitation experiments were also completed (C, D, E). In (C), an interaction between Htt 1-465-eYFP and mCherry-HAP1B is demonstrated, whereas binding is equal to beads alone when the NLS is deleted (Htt 1-465 ΔNLS-eYFP). mCherry-HAP1B is also immunoprecipitated by eGFP-Htt NLS-βGal, but only when the intervening sequence is

present (D). mCherry-HAP1B interacts with eGFP-IVS-βGal alone (E), however the interaction is not disassociated by the L195A,L198A mutation, which abolishes IVS mediated targeting to the cytoplasm. eGFP-Htt NLS A187P-βGal co-immunoprecipitation was also attempted, however, the anti-eAFP antibody failed to pull-down the NLS construct, likely due to low expression. All constructs were expressed in HEK293 cells. Anti-eAFP (a pan specific *Aequorea victoria* fluorescence protein antibody) was used to immunoprecipitate the eYFP and eGFP constructs. In each experiment, (C-E) the amount of each construct precipitated is visualized the first panel and the co-immunoprecipitated HAP1 is shown in the second western blot.

huntingtin 1-465 ΔNLS-eYFP, as well as eGFP-Htt NLS-βGal were expressed in HEK293 cells, alongside mCherry-HAP1B. AFP specific antibodies that recognized both YFP and GFP were used to immunoprecipitate the huntingtin fragments. Western blot analysis was then used to assess the level of protein pulled down for each construct and the quantity of mCherry-HAP1B co-immunoprecipitated. HAP1 was observed to bind to both huntingtin 1-465 and eGFP-Htt NLS-βGal (Figure 16C). Although low levels of HAP1 co-immunoprecipitated with Htt 1-465 ΔNLS-eYFP, it was consistent with those levels observed when the co-immunoprecipitation was performed with beads alone, indicating that this interaction was not specific. To prove that the intervening sequence is necessary to mediate the interaction between huntingtin and HAP1, the experiment was repeated with eGFP-ΔIVS Htt NLS-βGal. As predicted, this construct failed to interact with mCherry-HAP1B (Figure 16D). Lastly, we repeated the co-immunoprecipitation to determine if the IVS is sufficient to mediate HAP1 interaction and if mutations that disrupt the structure or targeting of the intervening sequence effect the formation of the complex. HAP1 was efficiently co-immunoprecipitated by eGFP-IVS-βGal (Figure

16E). Although eGFP-Htt NLS A187P-βGal did not express well enough to complete the experiment, eGFP-Htt NLS L195A,L198A-βGal (which completely abolished targeting of the IVS to the cytoplasm) was still capable of interacting with HAP1. This was later confirmed by overexpression and co-localization experiments in ST*Hdh* cells (Figure 17). Thus, although HAP1 does interact with the intervening sequence, it is not the sole mechanism that facilitates the cytoplasmic targeting of the IVS sequence.

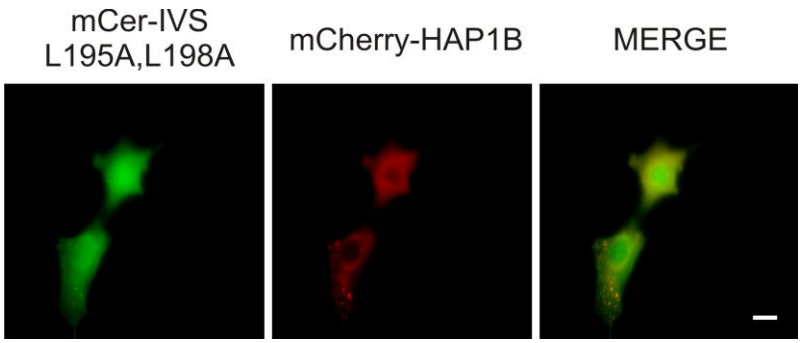


Figure 17: *Mutations that Disrupt Membrane Targeting of the Intervening Sequence Do Not Inhibit HAP1 Co-Localization*. mCerulean-IVS L195A,L198A was expressed with mCherry-HAP1B. Scale bar = 10 μm.

4.9 HAP1 Partially Disrupts the Nuclear Import of eGFP-Htt NLS-βGalactosidase

To determine if HAP1 could be capable of regulating the activity of the huntingtin NLS by inhibiting karyopherin binding and nuclear import, the reporter eGFP-Htt NLS-βGalactosidase was co-expressed with mCherry-HAP1A. After adjusting the values to account for the baseline nuclear import of the negative control (eGFP-

βGalactosidase), the nuclear import activity of the huntingtin NLS was found to be reduced almost by half when co-expressed with HAP1 (Figure 18A,B).

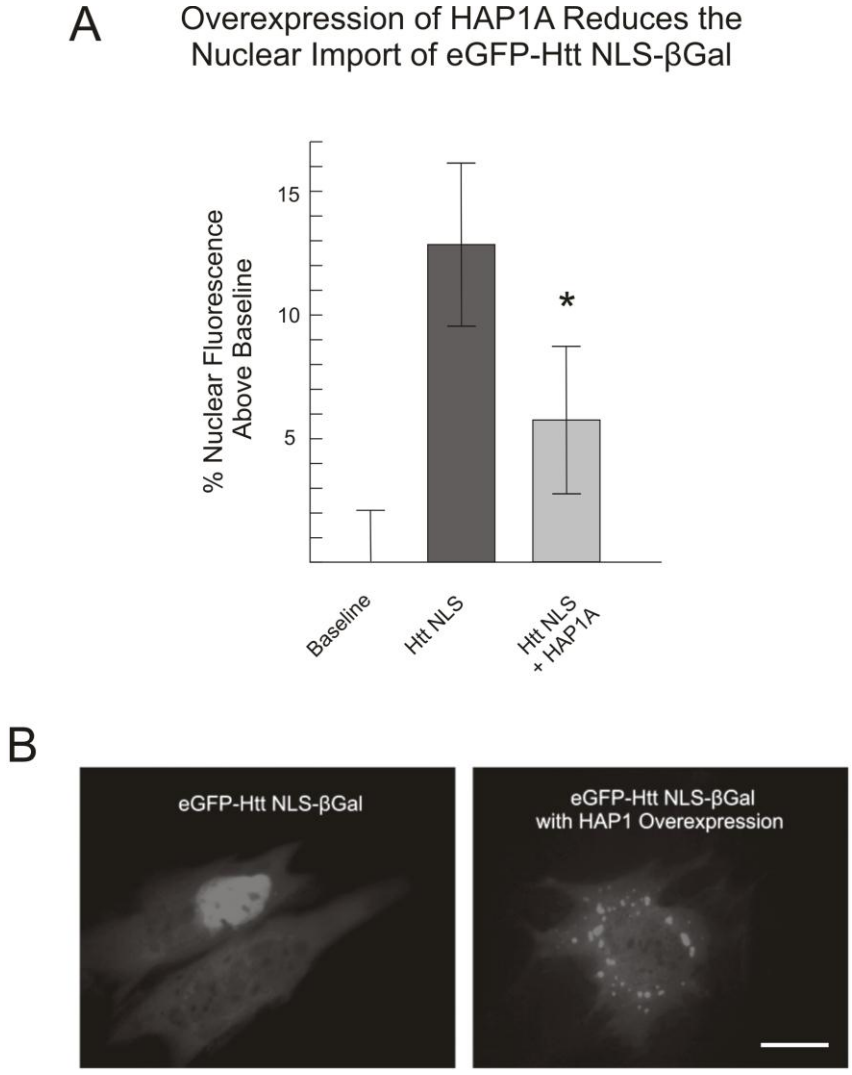


Figure 18: *HAP1 Partially Disrupts the Nuclear Import of eGFP-Htt NLS-βGalactosidase.* The nuclear import of eGFP-Htt NLS-βGal alone, and when co-expressed with mCherry-HAP1A in ST*Hdh* cells is quantified (A). Values were subject to baseline subtraction, where baseline is equal to the nuclear import of the negative control eGFP-βGal. Representative images are shown in (B) *= P values < 0.01 compared to eGFP-Htt NLS-βGal. n= approximately 50-60 cells per data set.

Although HAP1 was capable of altering the localization of the huntingtin NLS in isolation, it failed to have the same impact on the endogenous protein (data not shown). This may be due to the low stress environment of tissue culture cells and minimal basal levels of nuclear import of the full-length protein.

4.10 HAP1 Expression and Localization Does Not Differ Between ST*Hdh* and HEK293 Cell Lines

We have hypothesized that the reason huntingtin NLS activity differs between ST*Hdh* cells and HEK293 cells may be due to altered expression of regulatory proteins. Given that HAP1 binds the NLS intervening sequence, and can reduce huntingtin NLS activity in the context of eGFP-βGalactosidase, we sought to determine if there are differences in either HAP1 expression or localization in ST*Hdh* versus HEK293 cells. Both immunofluorescent staining of endogenous HAP1 and western blot analysis was performed. The localization of HAP1 between the two cell lines was mostly consistent, although HAP1 localization in ST*Hdh* cells appears to be more closely associated with microtubules (Figure 19A). However, this is likely due to differences in cellular morphology and cytoskeletal composition. The western blot analysis did not reveal any obvious expression differences (Figure 19B). Although actin was chosen as a loading control, it became apparent that its levels are not consistent between cell lines.

However, in a similar experiment conducted at the same time, equivalent actin loading was observed, suggesting that the samples are evenly loaded (note levels in Figure 21B).

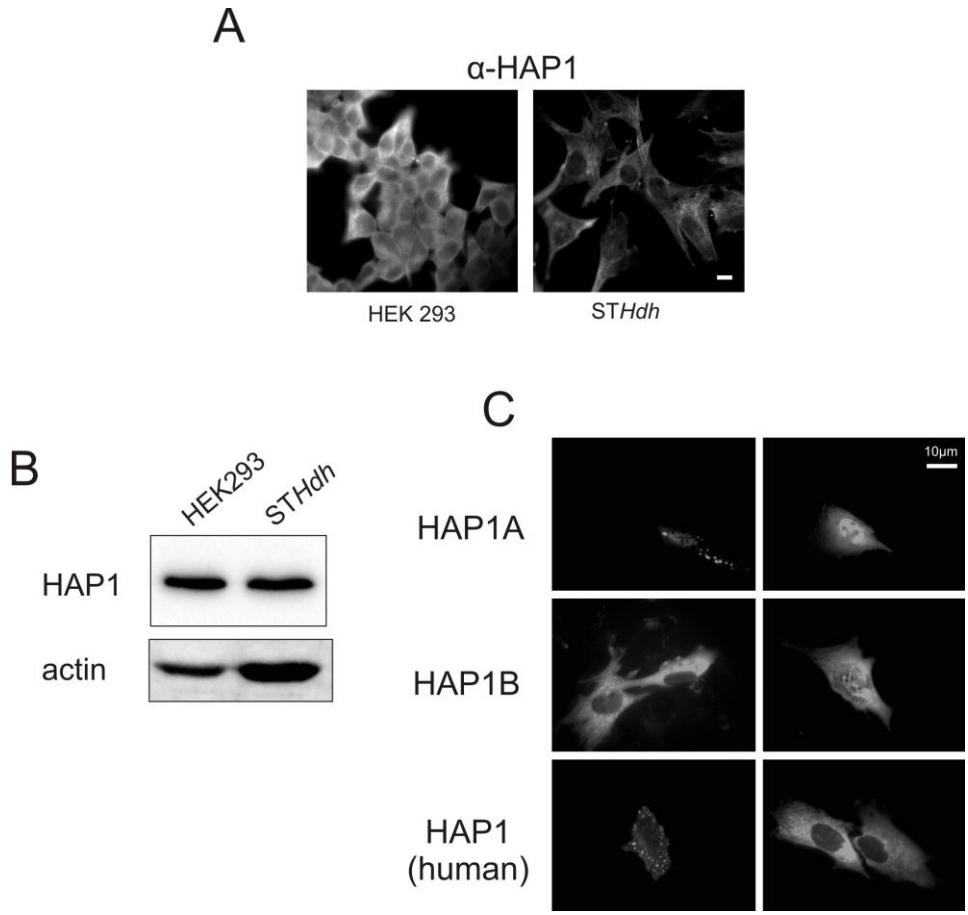


Figure 19: *HAP1 Localization and Expression is Similar in HEK293 and STHdh Cells.* ST*Hdh* and HEK293 cells lines were stained for endogenous HAP1 (A). The levels of HAP1 were compared between the two cell lines in (B). Although actin was chosen as a loading control, there appears to be a difference in its expression between the two cell lines. This difference is also visible in western blots for HMGB1, suggesting that the lanes are evenly loaded (see Figure 21B). (C) Overexpression phenotypes of mCherry-HAP1A (mouse), mCherry-HAP1B (mouse), and mCherry-HAP1 (human) in ST*Hdh* cells. For mCherry-HAP1A and mCherry-HAP1B, multiple images are shown to display the different localizations (cytoplasmic/nuclear and cytoplasmic puncta). For HAP1 (human), only cytoplasmic localization was observed, with and without the formation of puncta. Images are 60X widefield. Scale bar = 10 μm.

Interestingly, although endogenous HAP1 localization is not strikingly different between human HEK293 cells and mouse ST*Hdh* cells, when mCherry fusions of both the mouse isoforms and human isoform are expressed in ST*Hdh* cells, they do behave somewhat differently. Both of the mouse isoforms (HAP1A and HAP1B) have similar phenotypes. They can localize strongly to the nucleus or the cytoplasm (although never exclusively the nucleus), with the majority of the cells having cytoplasmic puncta (Figure 19C). However, the human isoform is rarely found within the nuclear compartment (Figure 19C). It is not known if these differences can be seen with the endogenous protein, since the immunofluorescence experiments showed no major differences in our cell lines. It is therefore possible that these localizations are simply artifacts from the overexpression of the HAP1.

4.11 The Huntingtin NLS Interacts with High Mobility Group Protein B1 (HMGB1)

To identify possible protein interaction partners of the intervening sequence, we performed peptide pull-downs with ST*Hdh* cell lysate and had the elutions analysed by mass spectrometry. The results of mass spectrometry are typically reported as "number of peptide hits". One of the top hits for the wild-type NLS peptide, that did not appear in either the beads alone or the mutant NLS KK177/178AA peptide elutions, was high mobility group box 1 (HMGB1) (Figure 20A).

HMGB1 is a small (30 kDa), predominantly nuclear, multi-functional protein. Two DNA binding domains (Box A and Box B) within its structure mediate an interaction with

chromatin (Stros 2010). HMGB1 induces DNA bending to promote the assembly of transcriptional complexes at the start sites of specific genes (McCauley et al. 2007). Although this was HMGB1's first defined function, it is now known to be involved in a diverse range of cellular processes in both the nucleus and the cytoplasm.

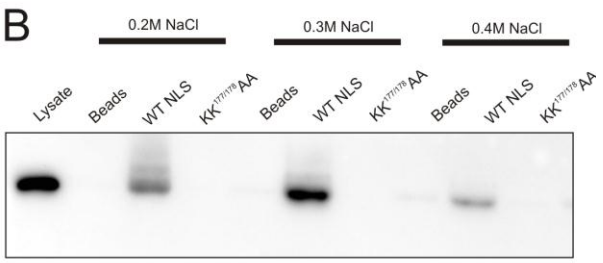
One of the most interesting aspects of HMGB1 biology, with respect to huntingtin function, is its proposed role in the oxidative stress response. HMGB1 is capable of binding the tumor suppressor and pro-apoptotic protein p53 in the nucleus, as well as the pro-autophagic protein Beclin-1 in the cytoplasm (Livesey et al. 2012; Kang et al. 2010). During periods of cell stress and accumulating reactive oxygen, HMGB1 translocates to the cytoplasm, where it binds Beclin-1 and breaks apart its association with Bcl-2, leading to sustained autophagy (Kang et al. 2010). HMGB1 itself may act as a redox sensor in the cell, detecting and responding to high levels of reactive oxygen (Tang et al. 2010). HMGB1 contains three cysteines within its sequence that are susceptible to oxidation: C23, C45 and C106. Under relatively mild oxidative stress conditions, C23 and C45 form a disulfide bond, which upregulates autophagy by promoting HMGB1's interaction with Beclin-1. Under very severe stress, which cannot be resolved by autophagic mechanisms, C109 also becomes oxidized. This version of HMGB1 is associated with the onset of apoptosis. When programmed cell death is initiated, p53 translocates to the nucleus. This limits the cytoplasmic localization of HMGB1, thus reducing autophagic flux and promoting cell death (Livesey et al. 2012).

Since HMGB1 interacts with the huntingtin NLS in peptide pull-downs, we sought to reproduce the interaction by affinity chromatography. Using Sulfolink beads coupled to wildtype huntingtin NLS peptides, or mutant KK177/178AA peptides, lysates were run over small columns and eluted with increasing concentrations of NaCl. We confirmed the presence of a highly specific interaction between HMGB1 and the wildtype NLS, that was absent from the mutant KK177/178AA column (Figure 20B). It is likely that HMGB1 requires the basic rich region for interaction with huntingtin. The dependence on K177 and K178 for interaction is interesting, since K178 has been identified as a site of acetylation (Cong et al. 2011). If acetylation abolishes K178's charge, it may have a similar effect as mutating the residue to an alanine. Therefore, it is a possibility that acetylation of the basic rich region of huntingtin's NLS could alter its ability to interact with HMGB1 under different cellular conditions.

To determine if the huntingtin NLS and HMGB1 interact in live cells, mCerulean-Htt NLS was overexpressed in ST*Hdh* cells and the localization of endogenous HMGB1 was highlighted by immunofluorescence. Some co-localization of the two proteins was observed at the periphery of the nucleus, as well as at various puncta in the cytoplasm (Figure 20C). However, to increase the cytoplasmic association of HMGB1 and the huntingtin NLS, oxidative stress conditions may be required.

A

BEADS ALONE	WT NLS	KK177/178AA
tropomyosin 1, alpha	tropomyosin 1, alpha	Atp5b
beta-tropomyosin	vimentin	gamma-actin
protymosin alpha	tropomyosin 4	vimentin
SET translocation	Atp5b	tropomyosin 1, alpha
tropomyosin 3, gamma	tropomyosin 3, gamma	gamma-actin-like protein
calreticulin	alpha-cardiac actin	tropomyosin 3, gamma
vimentin	high mobility group 1 protein	basic transcription factor 3 isoform B
alpha-actin	basic transcription factor 3 isoform B	Nucleolin
unnamed protein	Nucleolin	nascent polypeptide associated complex alpha polypeptide
ribosomal protein L5	calreticulin	Myh9 protein



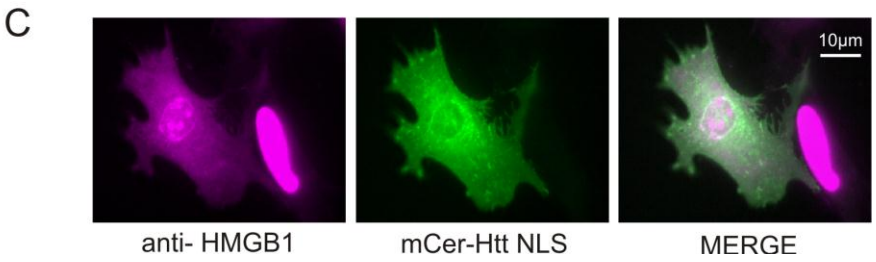


Figure 20: *The Huntingtin NLS Interacts with HMGB1.* Peptide pull-downs from ST*Hdh* cell lysates were performed using beads alone, WT NLS peptide and KK177/178AA mutant NLS peptide. The top protein interaction partners, as identified by mass spectrometry, are shown in (A). Note the association of HMGB1 with the WT NLS, but not KK177/178AA. This interaction was confirmed by affinity chromatography (B). Images of overexpressed mCerulean-Htt NLS and endogenous HMGB1 immunofluorescence are shown in (C). 60X widefield images. Scale bars represent 10 µm.

4.12 HMGB1 Expression and Localization Does Not Differ Between ST*Hdh* and HEK293 Cell Lines

As was done for HAP1, we compared the localization and expression level of HMGB1 between the HEK293 and ST*Hdh* cells, to determine if differences between the cell lines could be responsible for altered huntingtin NLS activity levels. However, like HAP1, HMGB1 localization was the same in both HEK293 and ST*Hdh* cells, where it was found to be almost exclusively nuclear (Figure 21A). The level of HMGB1 in the two cell lines was also the same (Figure 21B). As was explained previously, actin was used inappropriately as a loading control between the two samples. In both the HAP1 and HMGB1 western blots, however, the same actin levels were observed in HEK293s and ST*Hdh* cells, suggesting that the loading of the samples was even (See figure 19B for comparison).

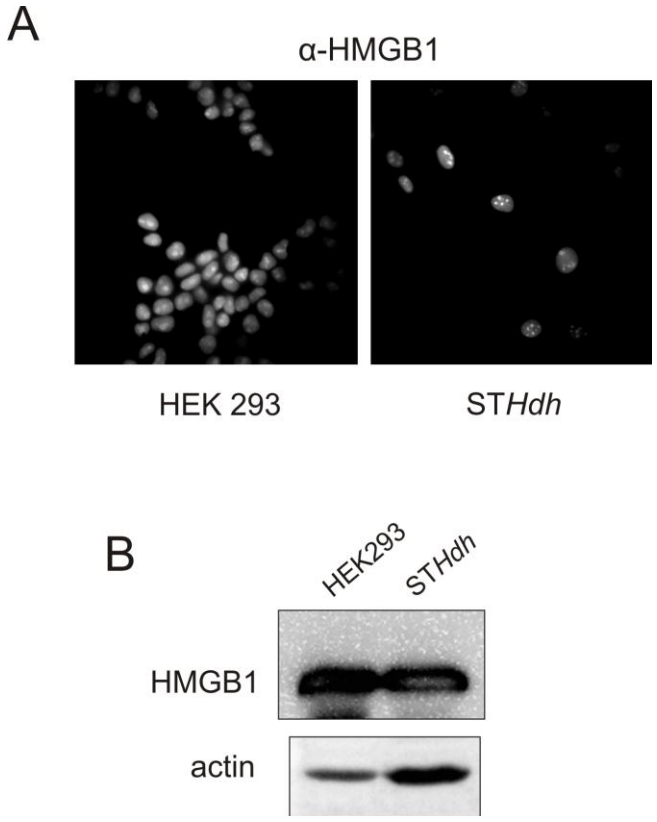


Figure 21: *HMGB1 Localization and Expression is Similar in HEK293 and* ST*Hdh Cells.* ST*Hdh* and HEK293 cells lines were stained for endogenous HMGB1 (A). The levels of HMGB1 were compared between the two cell lines in (B). Actin was used as a loading control, but its expression was found to differ between HEK293 and ST*Hdh* cells. However, the observed difference is reproduced in Figure 19B, and suggesting that the loading was indeed equal. Images are 60X widefield.

CHAPTER 5

DISCUSSION

Figure 22, contained within this chapter was modified from work completed by the author (Carly R. Desmond) for the publication:

Atwal, R. S., C. R. Desmond, N. Caron, T. Maiuri, J. Xia, S. Sipione, and R. Truant. (2011) **Kinase inhibitors modulate huntingtin cell localization and toxicity**. *Nat Chem Biol* 7 (7):453-60

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5.1 Overview

Over the course of my PhD studies, I have worked towards creating a better understanding of normal huntingtin function through the identification and characterization of huntingtin's mechanism of nuclear entry. I have, additionally, explored a range of possible regulatory mechanisms that may impact the protein's nuclear localization at the level of the NLS sequence.

In summary, we have discovered a PY-NLS nuclear localization signal towards the amino terminus of huntingtin that is recognized by the nuclear transport receptor karyopherin β2, as well as karyopherin β1. While this sequence is similar to other PY-NLSs, it exceeds the spacing requirements of the published consensus and requires the formation of a secondary structure for optimal function. Previously identified PY-NLS sequences have only been isolated in unstructured regions (Lee et al. 2006). The unique attributes of huntingtin's NLS suggest the existence of a new class of PY-NLS sequences, which depend on structure to form a stable interaction with karyopherin β2.

The sequence between the PY-NLS consensus elements, we have since labeled the "intervening sequence" or IVS, has been found to mediate cytoplasmic targeting of the huntingtin NLS, particularly to membranes and vesicles. We hypothesized that the intervening sequence may counterbalance the activity of the NLS through the binding of proteins or membranes in the cytoplasm, inhibiting karyopherin β2 or β1's interaction with the huntingtin NLS. We identified multiple cell lines in which the huntingtin NLS was inactive (HEK293 and MCF-7), suggesting that the import of the protein could be

differentially regulated amongst cell-types, possibly due to differences in expression or localization of proteins or membranes that bind the IVS. In an effort to identify mechanisms that regulate huntingtin NLS activity, we explored a diverse set of possibilities, from a potential nuclear export signal, to post-translational modification, to masking of the NLS sequence via protein interaction partners. These experiments provide a sense of direction and preliminary data for future studies of huntingtin nuclear import.

5.2 The Interplay between Huntingtin Nuclear Import and Export Mechanisms

Although this thesis has focused mainly on those factors that govern huntingtin nuclear import directly through the NLS sequence, there are other aspects of huntingtin biology that are known to affect the protein's nuclear localization. For example, one of the most robust methods of altering huntingtin's cellular localization is by modifying the phosphorylation status of N17. In two publications from our lab, we describe the ability of huntingtin to interact with the ER via the N17 sequence, which anchors huntingtin to the cytoplasm under steady state conditions (Atwal et al. 2007). However, during cell stress, huntingtin becomes phosphorylated at serines 13 and 16, leading to a change in N17's alpha helical structure, causing huntingtin to release from the ER (Atwal et al. 2011). This free and soluble pool of huntingtin readily enters the nucleus.

Aside from very early development, wildtype huntingtin does not accumulate substantially within the nuclear compartment (Jeong et al. 2006; Hoogeveen et al. 1993).

Its timely export is likely critical to its function, and may be especially important when huntingtin contains the toxic polyglutamine expansion. To mediate this nuclear export, huntingtin contains two nuclear export signals. One NES is located towards the carboxyl-terminus of the protein (amino acids 2397-2404), and the second, most recently identified by our lab, is found within the N17 domain (Xia et al. 2003; Maiuri et al. *in press*). Both sequences are leptomycin B sensitive, indicating that they require CRM1 for activity. In co-immunoprecipitation experiments, CRM1 was found to interact with wildtype N17-eYFP expression constructs, but not with the phospho-mimetic N17 S13D, S16D-eYFP (Maiuri et al. *in press*). Therefore, although phosphorylation of S13, S16 releases huntingtin from the ER and increases its nuclear localization, huntingtin dephosphorylation at these sites may be required before the N17 NES can be recognized by CRM1 for export.

It is still unclear why huntingtin requires two nuclear export sequences. This may be a method of controlling the rate of nuclear export compared to nuclear import. However, it is also possible that conformational changes conferred by various states of post-translational modification could occlude one or both of the NES sequences during specific signaling events. Also, if the cleavage hypothesis holds true, the carboxyl-terminal NES of huntingtin may be removed during specific cellular events, such as in response to apoptotic stimuli. This would leave only the amino-terminal N17 NES available for export of huntingtin fragments.

5.3 Other Potential Functions of the Huntingtin Nuclear Localization Signal

It was recently discovered that huntingtin participates in the formation of cilia (Keryer et al. 2011). When huntingtin expression is reduced by siRNA knockdown in ST*Hdh* tissue culture cells, the number of cells that produce primary cilia is drastically reduced. Huntingtin was reported to localize to the base of the cilium, in a manner that is dependent on HAP1. Our lab has now found that huntingtin is also capable of entering the cilium (work by Tanya Woloshansky for Maiuri et al. *in press*). While S13, S16 phosphorylated huntingtin localizes to the base of the cilium, only the dephosphorylated protein is found within the cilial body.

Import of proteins into cilia is very similar to import into the nucleus. The base of the cilium is composed of non-membrane bound nuclear pore complex proteins, that restrict cilial entry (Kee et al. 2012). A karyopherin β2 PY-NLS is now known to mediate the movement of the kinesin-2 motor KIF17 into cilia (Dishinger et al. 2010). Given that huntingtin can also be found within cilia, its PY-NLS may perform dual function as a nuclear import and cilial import sequence. The requirement of HAP1 for proper localization of huntingtin to the base of the cilium is also intriguing. Thus far, there are no reports of HAP1 within cilia, therefore, it is most likely that it is involved in bringing huntingtin to the base of the cilia, and possibly shielding its cilial import until huntingtin becomes appropriately dephosphorylated.

5.4 Huntingtin Nuclear Import and its Relationship to Toxicity: Recent Mouse Model Data

With the identification of the NLS, we may finally address the question of whether or not nuclear import of mutant huntingtin is required for toxicity. Several mouse models have already been generated that hint at the importance of the nucleus as a site of toxicity.

When the phosphorylation sites at S13 and S16 were identified and discovered to indirectly mediate huntingtin nuclear entry, it became a logical target of drug discovery (Atwal et al. 2011). However, the relationship between the phosphorylation status of N17, its nuclear localization, and toxicity are still somewhat unclear. The laboratory of William Yang produced a BACHD transgenic mouse in which serines 13 and 16 were mutated to either aspartic acid to mimic the effect of phosphorylation, or to alanines, to make the residues phospho-resistant (Gu et al. 2009). While blocking phosphorylation with the alanine mutations was neither beneficial nor detrimental to the toxicity of the transgenic polyglutamine expanded full-length huntingtin, phospho-mimicry was found to be protective. The S13D, S16D mutation prevented the onset of a neurodegenerative phenotype. It prohibited any measurable atrophy in the striatum or cerebral cortex of the mice, as well as the onset of motor or psychiatric dysfunction. These findings are consistent with more recent experiments that show that increasing huntingtin phosphorylation by therapeutic intervention is also beneficial. Post-mortem HD brain tissues and HD mouse models have decreased levels of the ganglioside GM1 (Desplats et

al. 2007; Maglione et al. 2010). Infusing the brains of YAC128 transgenic HD mice with GM1 has been found to increase S13,S16 phosphorylation and to reverse existing motor abnormalities (Di Pardo et al. 2012).

We have also attempted to assess the impact of phosphorylation on huntingtin toxicity, but instead using a tissue culture model and overexpressed huntingtin fragments. ST*Hdh* cells were transfected with Htt 1-171 Q138 or Htt 1-171 Q142 S13A,S16A and treated with kinase inhibitors that either decrease (DMAT) or increase (BMS) phosphorylation of N17 (Figure 22). In contrast to the GM1 treatments, increasing huntingtin 1-171 Q138 phosphorylation was not protective in our experiments. However, this may be due an inability to overcome the toxicity of small fragments, which is enhanced compared to the toxicity of full-length protein expressed in BACHD and YAC128 mouse models. Alternatively, inhibiting the phosphorylation of N17 with DMAT was found to increase Htt 1-171 Q138 toxicity. This effect was not due to toxic properties of the kinase inhibitor itself, since DMAT treatment had no impact on the phospho-resistant Htt 1-171 Q142 S13A,S16A construct. This is somewhat confusing given that we would expect inhibition of phosphorylation to encourage an unphosphorylated state similar to S13A,S16A. However, we must remember that that constitutive phospho-resistance and kinase inhibition are not equal and may have slightly different effects.

It is interesting to consider what these studies reveal about the relationship between huntingtin nuclear import and toxicity. Data from our lab suggests that huntingtin phosphorylation promotes nuclear import of huntingtin by inhibiting its ER

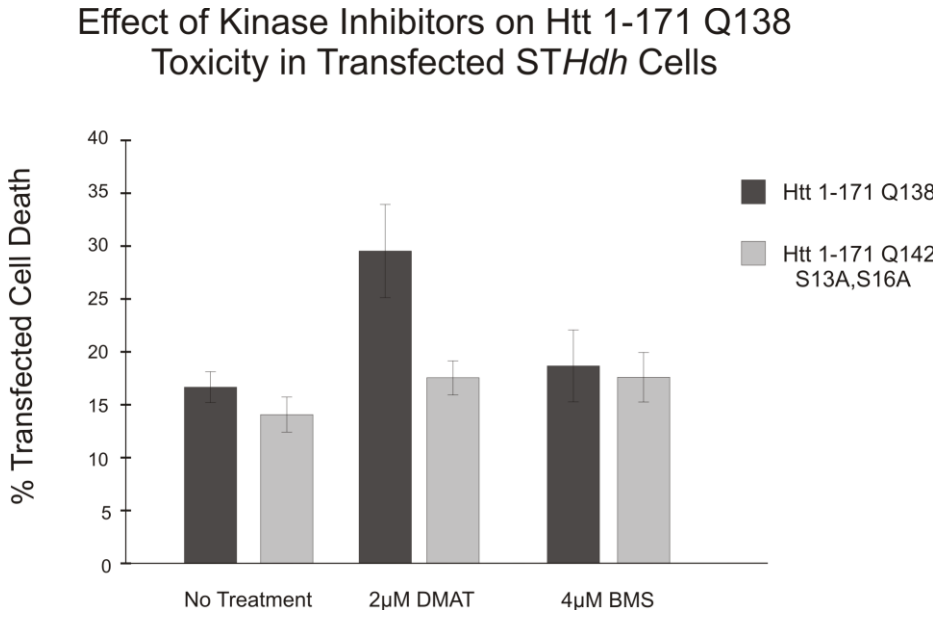

Figure 22: *Inhibition of N17 Phosphorylation Increases Huntingtin 1-171 Q138 Toxicity in Transfected* ST*Hdh Cells*. ST*Hdh* cells were transfected with Htt 1-171 Q138 or Htt 1-171 Q142 S13A,S16A and treated with 2 μM DMAT (CK2 kinase inhibitor) or 4 μM BMS (IKKβ kinase inhibitor). % cell death was measured by staining with ethidium homodimer III (dead cell stain) and performing flow cytometry to count the total transfected cells, and total transfected + dead (% transfected cell death = (transfected + dead / total transfected) X 100). Bars represent standard deviation.

targeting, as well as the activity of the NES. And even though many studies report a relationship between nuclear accumulation of mutant huntingtin and disease progression, constitutive phosphorylation of S13 and S16, is none-the-less protective (Gu et al. 2009). Simple nuclear targeting of huntingtin is therefore likely not sufficient

to produce toxicity, although it is possible it is still required. Additional levels of post-translational modification or sub-nuclear localization may be necessary for pathogenesis. For example, phosphorylated S13, S16 huntingtin targeted to nuclear puncta may be beneficial, but huntingtin that is dephosphorylated while inside the nucleus may become toxic.

An inability to regulate nuclear import of huntingtin may explain why short fragment mouse models of the disease produce such severe phenotypes. Exon1 transgenic mice (R6/2) exhibit pathology as early as 5-6 weeks after birth (Mangiarini et al. 1996). YAC128 full-length transgenic mice do not show symptoms until 8-12 weeks, and the knock-in model *Hdh* Q111/Q111, not until around 96 weeks (Slow et al. 2003; Wheeler et al. 2002). The reason for this may be due to differences in how small fragments versus full-length huntingtin enter the nucleus. Small fragments, such as exon1, that are below the diffusion limit of the nuclear pores, can enter the nucleus by passive diffusion. These fragments do not contain the 174-207 PY-NLS sequence, and are thus unresponsive to regulatory mechanisms that act on the NLS in larger contexts. It would therefore take longer for enough full-length huntingtin to enter the nucleus by legitimate means, than for small fragments to simply diffuse inside, leading to more rapid disease onset. This effect is especially well demonstrated by R6/2 exon1 mouse models with extremely long polyglutamine repeats (greater than 300) (Morton et al. 2009; Dragatsis et al. 2009). Although repeat length is inversely correlated with age of disease onset in humans and in R6/2 mice, very long repeats appear to cause a reversal

of huntingtin toxicity in this small fragment model (Andrew et al. 1993; Dragatsis et al. 2009; Morton et al. 2009). The reason for this may be simpler than it appears. The long polyglutamine tract may be no less toxic than shorter sequences within the pathogenic range. However, the extreme length added to exon1 in this context may prevent or delay the fragment's nuclear accumulation, easing pathogenesis (Dragatsis et al. 2009).

The flip-side of this argument is that the toxicity of mutant huntingtin can also be enhanced by increasing nuclear import, rather than simply decreased by inhibiting it. A BACHD transgenic mouse was recently created with a full deletion of the N17 domain (Dr. William Yang, *personal communication*). Without N17, anchoring to the ER is abolished, and huntingtin import is greatly enhanced. This produces an extremely severe neurodegenerative phenotype, more severe than even the traditional BACHD model. The heightened toxicity of BACHD ΔN17 has other implications as well. Both BACHD ΔN17 and BACHD S13D,S16D are expected to accumulate more strongly in the nucleus, and yet the two models have completely opposite effects on toxicity. This further reaffirms the importance of phosphorylation status and post-translational modifications to the toxicity of nuclear huntingtin.

While phosphorylation of N17 is clearly important during aging, it has no major impact on huntingtin's developmental function. All three BACHD transgenes (S13A,S16A; S13D,S16D and ΔN17) are capable of rescuing the embryonic lethal phenotype of huntingtin knockout mice (Gu et al. 2009; Dr. William Yang, *personal communication*). Instead, regulation of nuclear import during development may depend

more heavily on the nuclear localization signal itself. Strong nuclear accumulation of huntingtin is observed during very early embryogenesis, indicating that the huntingtin NLS is active during this stage (Jeong et al. 2006). Therefore, while N17 is not required, an intact nuclear localization signal may be critical.

Now that we have characterized huntingtin's PY-NLS, we are in a better position to study the direct impact of nuclear import on both the normal cellular functions of huntingtin and on disease pathogenesis. Although we have made several attempts to measure the toxicity of NLS mutants in the context of huntingtin fragments in live cells, the assays were not sensitive enough to detect differences. If huntingtin nuclear localization is triggered by cell stress or events associated with aging, the levels of toxicity observed in animal models or HD patients may be difficult to reproduce in a tissue culture environment. To obtain the most high quality data, it is important that NLS mutants are studied in the context of full-length huntingtin in an HD mouse model. In collaboration with the laboratory of Dr. William Yang, we are hoping to generate a BACHD transgenic mouse model with deletion of the basic rich region of the huntingtin NLS. This particular mutation is preferred due to its ability to abolish the activity of the NLS sequence, without disturbing the binding region of HAP1 within the intervening sequence. A transgenic mouse model is the best choice for this particular project because disruption of nuclear import in knock-in models would likely severely impact the normal activity of huntingtin, and possibly interfere with its developmental functions. The BACHD model will allow us to test both the relationship of nuclear import

to toxicity, as well as its importance to development, with the aid of genetic rescue experiments. If mutant NLS BACHD mice, on an *Hdh*-/- background are viable, they will be an unexpected, yet valuable tool to study the adult nuclear functions of the huntingtin protein. However, in the event that mutation of the NLS impacts huntingtin function too severely, the traditional three copy BACHD transgenic will still provide information about the pathological implications of inhibiting nuclear import.

5.5 Huntingtin Nuclear Import: A Drug Target?

If mutation of the huntingtin NLS in the context of the BACHD model proves to be protective, altering the rate of huntingtin nuclear import may be an appropriate therapeutic strategy for the treatment of HD. Although targeting karyopherin β2 or β1 function with small molecules could be an effective means of limiting huntingtin nuclear import, it would also affect the vast number of alternative substrates that are imported through these pathways. However, the huntingtin NLS's unique characteristics may make it possible to inhibit huntingtin nuclear import without disrupting the nuclear localization of other proteins. One possible strategy is to perform a high-content screen to identify small molecules that specifically inhibit the nuclear import of the reporter eGFP-Htt NLS-βGal, but do not affect other karyopherin β2 or β1 substrates. These molecules could theoretically impact the NLS in a number of ways: they may target the structure of the intervening sequence, limiting huntingtin's ability to bind to the nuclear import receptors, or they could act indirectly, through other pathways of NLS regulation.

5.6 Regulation of Huntingtin NLS Activity: Current Insights and Future Work

Our current understanding of the mechanisms that affect huntingtin NLS activity is still very limited. Thus far, we have explored a range of potential events, such as post-translational modification (palmitoylation and acetylation), nuclear export activity, RNA association and shielding of the NLS by binding of HAP1 or HMGB1.

We conducted several experiments to determine if the intervening sequence contains a CRM1-type nuclear export signal. Although mutation of key leucines in the putative NES sequence did prevent targeting of the IVS to the cytoplasm, consistent with NES activity, the mCerulean-IVS construct did not relocate to the nucleus with leptomycin B treatment. Furthermore, when the putative NES sequence alone was fused to mCerulean, it was not sufficient to localize the fluorophore to the cytoplasm. We later noted the presence of another leucine a few amino acids upstream of the sequence we chose that could also fit the consensus of an NES. Although this sequence should be tested for NES activity before any conclusions are made, at this time, there is insufficient evidence to suggest that the intervening sequence mediates nuclear export through the CRM1 pathway. Instead, it appears that cytoplasmic targeting of the IVS occurs because of its interaction with membranes or cytoplasmic membrane proteins. However, even if this is true, and even if there is no CRM1-type NES, it does not fully rule out the possibility of nuclear export activity. Other PY-NLS sequences, such as the M9 NLS, have been found to mediate both import and export through a non-CRM1

dependent mechanism. However, this activity is associated with the ability of the M9 NLS to directly export mRNA, which does not appear to be a functional capability of the huntingtin NLS in our experiments.

Nuclear import of huntingtin may not be influenced directly, at the level of the NLS, by transcription; however, it is possible that huntingtin still has an RNA related function. Recent publications from the Tanese Laboratory, have described a role of huntingtin in the repression of mRNA translation within P-bodies, and an involvement in dendritic mRNA transport (Savas et al. 2008; Savas et al. 2010). So far, they have isolated two mRNAs that are transported with the assistance of huntingtin: BDNF and β-actin (Ma et al. 2010; Ma et al. 2011). This process requires huntingtin and HAP1, as well as the microtubule motor proteins kinesin and dynein (Ma et al. 2011). Since the HAP1 binding site falls within the intervening sequence of huntingtin's NLS, regulation of mRNA transport may still have some effect on NLS activity. However, huntingtin's localization to P-bodies seems to depend on upstream domains as well, such as the polyglutamine tract and the proline-rich region (Savas et al. 2008). An impairment in the transport of BDNF mRNA could explain the BDNF deficiency observed in the striatum of HD patients (Ferrer et al. 2000). It may also explain huntingtin's contribution to synaptic function, since mRNA transport and localized translation are important for synaptic plasticity and neuronal function in the brain (Kiebler and Bassell 2006).

We also examined post-translational modification as a possible method of NLS regulation. Although there was no evidence to suggest that either palmitoylation or

acetylation contribute to the activity of the NLS, there are a few additional experiments that could be conducted in regards to the acetylation site at lysine 178. In the Cong *et al.* (2001) paper that describes the identification of this acetylation site, the authors attempted to generate an antibody towards the modified site. However, due to low titre issues, they were unable to obtain a functional antibody. Instead, they performed semi-quantitative mass spectrometry to look for differences between the acetylation of wildtype and polyglutamine expanded huntingtin fragments. Their data suggested that wildtype huntingtin is acetylated more easily than mutant huntingtin. If a suitable antibody is generated to the lysine 178 acetylation site, we may be able to re-address the question of whether or not modification of this site alters nuclear import of huntingtin. A comparison of the acetylation levels of nuclear versus cytoplasmic endogenous huntingtin after cellular fractionation would allow us to see if there is a difference in the acetylation status of lysine 178 in either of these compartments. However, in the meantime, it may be useful to revisit our current KK177/178RR acetylation resistant mutation in the context of 1-612, in order to be more consistent with the fragment used in the Cong *et al.* study. Perhaps smaller fragments are not modified in overexpression assays.

The two most promising potential mechanisms of regulation we have explored are the protein-protein interactions of the huntingtin NLS with HAP1 and HMGB1. Substantial data now shows that HAP1 binds within the intervening sequence, while HMGB1 likely requires the full NLS. Our current hypothesis is that the binding of either

of these proteins could conceal the NLS, reducing the sequence's ability to interact with the nuclear import receptor karyopherin β2 (or β1). To test this, it will be necessary to perform competition assays with recombinant purified protein, on NLS peptide coupled columns. In live cells, gene silencing techniques could be used to lower the expression of both HAP1 and HMGB1. If these proteins have an inhibitory effect on the huntingtin NLS, we will expect to see a stronger nuclear accumulation of endogenous huntingtin by immunofluorescence in these assays. Similar experiments could be conducted with HMGB1 knockout MEF cell lines.

As a potential regulator of huntingtin NLS activity, HAP1 is an interesting and attractive candidate. HAP1 has been identified as a genetic modifier of Huntington's disease age-of-onset (Metzger et al. 2008). A homozygous threonine (T) to methionine (M) substitution at amino acid 441 of HAP1 is capable of delaying HD onset by an average of 8 years. This polymorphism causes HAP1 to interact more strongly with huntingtin. In tissue culture assays, the HAP1 M441 isoform was found to be capable of redistributing mutant huntingtin fragments (1-208) from the nucleus to the cytoplasm. This led to a reduction in the toxicity of the fragment. These experiments are consistent with our hypothesis that HAP1 masks the huntingtin NLS, but also implies that the reduced nuclear localization of huntingtin due to increased HAP1 interaction may positively impact huntingtin pathology in patients carrying the M441 polymorphism.

HAP1's interaction with the intervening sequence of huntingtin's NLS may also explain the unusual "neurite branching" phenotype that is induced by overexpression of

mCerulean-IVS, or mCerulean-NLS in tissue culture cells. Mouse HAP1A, which is most similar to the human isoform of HAP1, has been found to localize to the synapse and neuritic growth cones, and overexpression in PC12 cells causes the formation of cellular projections (Li et al. 2000). Several of HAP1's interaction partners are involved in neuritogenesis, however in vastly different capacities. HAP1 interacts with a protein called Duo that has RhoGEF activity and is thought to participate in actin remodeling, a critical mechanism for neurite outgrowth (Colomer et al. 1997). Another binding partner, called Abelson helper integration site 1 (AHI1) contains several WD40 domains, which are protein-protein interaction domains that are often involved in cytoskeleton assembly and vesicular trafficking (Sheng et al. 2008). Alternatively, HAP1 is also known to interact with the transcription factor NeuroD, which controls the expression of genes required for cellular differentiation into neurons (Marcora, Gowan, and Lee 2003). It is possible that overexpression of huntingtin's intervening sequence is having a stimulatory effect on these HAP1 associated proteins, leading to neurite outgrowth.

One of huntingtin's primary cellular functions is to protect neurons from apoptotic cell death. Overexpression of wild-type huntingtin is protective against apoptotic stimuli and conditional knockout of huntingtin leads to a neurodegenerative phenotype (Leavitt et al. 2006; Dragatsis, Levine, and Zeitlin 2000). However, the connection between huntingtin and these apoptotic pathways is still being elucidated. In the face of stress, cells can respond in two ways: they can initiate apoptosis, or they can attempt to deal with the stress by undergoing macroautophagy. Macroautophagy

(referred to as autophagy from here on) is a process through which the cell is able to rapidly break-down biological molecules to eliminate unnecessary proteins and generate ATP (Wirawan et al. 2011). A constant balancing act between stress signaling pathways determines whether the cell will continue with its attempt to alleviate the cell stress through autophagy or enter the cell death cascade of apoptosis.

Many proteins have dual roles in the signaling of both of these mechanisms. Huntingtin's function may involve regulating the decision process between autophagy and apoptosis. Aside from being an anti-apoptotic protein, huntingtin has also been described as pro-autophagic. However, all of this appears to depend heavily on huntingtin's polyglutamine tract. When the normal mouse polyglutamine tract of 7 glutamines is deleted in a knock-in mouse model, the mice become hyper-autophagic, and as a result, have longer lifespans than even normal mice (Zheng et al. 2010). However, when the polyglutamine tract is expanded, as is the case in Huntington's Disease, the result is neurodegeneration, likely due to neurons that are hyper-apoptotic. This effect is not just observed in the brain. HD patients have a reduced risk of all types of cancer, possibility as a result of a higher rate of apoptosis (Turner, Goldacre, and Goldacre 2012). One observation from HD patients and mouse models is that that the ATP/ADP ratio is reduced (Seong et al. 2005). This may be due to increased rates of apoptosis, and decreased autophagy. When the levels of ATP were measured in fibroblast cells taken from the ΔQ/ΔQ mice, they were found to have elevated ATP compared to their wild-type counterparts, and to enter senescence earlier (Clabough

and Zeitlin 2006). Even in the presence of a copy of polyglutamine expanded huntingtin, one copy of ΔQ huntingtin is capable of enhancing the longevity of Q140 knock-in mice, as well as providing protection against the motor and behavioural deficits associated with the disease (Zheng et al. 2010). Although the evidence is thus far only anecdotal, patients who enhance autophagy naturally, through forms of extreme exercise such as marathon running, tend to have delayed onset of HD.

Huntingtin's interaction with HMGB1 may provide a mechanism through which huntingtin participates in the autophagy versus apoptosis decision making process. As described, HMGB1 has the ability to sense oxidative stress and to respond by either enhancing autophagy or allowing p53 signaling to take over, thus initiating the apoptotic cascade (Kang et al. 2010; Livesey et al. 2012). During severe cell stress, p53 translocates to the nucleus where it activates the transcription of proteins involved in programmed cell death (Haupt et al. 2003). HMGB1 and p53 have been found to interact in both the cytoplasm and the nucleus (Livesey et al. 2012). In p53 knock-out cells, HMGB1 localization to the cytoplasm is increased, and autophagy is also enhanced. This may be similar to what occurs, mechanistically, when a cell stress event is not serious enough to induce substantial accumulation of p53 in the nucleus. However, when p53 does localize to the nucleus, it may also sequester HMGB1, simultaneously reducing autophagy and upregulating apoptosis. Huntingtin is also capable of sensing and responding to cell stress events. The phosphorylation of serines 13 and 16 during stress causes increased translocation of huntingtin to the nucleus (Atwal et al. 2011). It is

possible that huntingtin binds HMGB1 in the nucleus and facilitates its export to the cytoplasm. Since HMGB1 has been found to bind within the huntingtin NLS sequence, it presents an interesting mechanism by which masking of huntingtin's nuclear import signal may increase the protein's nuclear export, and prevent subsequent re-entry.

5.7 Conclusions

This thesis describes huntingtin's ability to enter the nucleus through the karyopherin β2 and β1 pathways, as well as an exploration of possible secondary regulatory mechanisms of the PY-NLS. Nuclear entry is a normal function of the huntingtin protein. However, excessive translocation from the cytoplasm to the nuclear compartment in striatal and cortical neurons is a pathogenic hallmark of HD. Moving forward, it will be interesting to see what impact mutation of the NLS sequence in transgenic mouse models will have on both the normal functions of huntingtin and on the onset of disease. Small molecules that restrict or slow the nuclear accumulation of huntingtin may have therapeutic benefit; however, finding ways to manipulate nuclear import will require further knowledge of the normal nuclear functions of huntingtin and of its associated regulatory pathways. None-the-less, this thesis offers the foundation necessary to guide future studies within this exciting new branch of HD research.

CHAPTER 6

CONTRIBUTIONS TO PUBLIC EDUCATIONAL RESOURCES

The following articles were written for, and published on, HDBuzz. This website is an educational resource for Huntington's Disease patients and their families where current research is written about and discussed in plain language.

www.hdbuzz.net

6.1 Stem-cell neurons make the right connections

Written by: Carly Desmond

Edited by: Dr. Jeff Carroll

New work suggests that neurons made from stem cells can replace adult neurons better than we thought – at least in mice whose brains have been injured with a toxin. How likely is this to help HD patients – is cell replacement possible in a more chronic condition?

HD and the loss of brain cells

Huntington's disease is caused by ***neurodegeneration***, or loss of brain cells called *neurons*. Early in HD, this cell loss particularly affects *neurons* in a part of the brain called the **striatum**. Even within the striatum, one type of *neuron* called a 'medium spiny *neuron*' is most susceptible to degeneration. These *neurons* make up 96% of the striatum, so losing them is bad news for this part of the brain.

The symptoms of Huntington's disease reflect this distinctive pattern of cell loss. The striatum helps to control our body's movement and emotions, as well as performing cognitive tasks, such as learning, multi-tasking and problem solving. These are all areas reported by patients to be affected by Huntington's disease.

The problem with *neurodegenerative* diseases like HD is that once vulnerable *neurons* like the medium spiny *neurons* are lost, they do not grow back. As far as we understand now, once they're gone, they're gone.

The brain can cope with loss

The TRACK-HD project, led by Professor Sarah Tabrizi, used *MRI* scans to reveal a noticeable loss of brain tissue early in the course of the disease. This progression of *neuron* loss is seen even before symptoms are reported by the patients.

In one way, this is bad news — the brains of HD mutation carriers are shrinking even before they experience symptoms that we call 'HD'. But another way of looking at this is very hopeful — despite the fact that most *neurons* cannot regenerate, the brain **can** still show remarkable flexibility to compensate and maintain normal function.

Since degeneration of the medium spiny *neurons* in the striatum causes the symptoms of Huntington's disease, one approach to treatment would be to replace the *neurons* that have been lost.

Cell replacement in brain diseases

While this may sound a little like science fiction, it might be more feasible than once thought. For treatment of Parkinson's disease, this approach has already been undertaken with moderate success.

Parkinson's disease is caused by degeneration of a tiny but important set of *neurons* that produce a brain chemical called '*dopamine*'. Loss of these cells causes tremors, stiffness and poor coordination.

In clinical trials, cells from fetal tissue have been transplanted into the brains of patients with Parkinson's Disease, resulting — in some cases — in marked improvements in their movement abnormalities and overall health.

However, compared to Huntington's Disease, treating Parkinson's by cell replacement therapy is relatively easy. Since loss of *dopamine* in Parkinson's Disease brains causes its symptoms, all that is needed to treat the disease is replacement of the *dopamine* source. To have a beneficial effect, it only matters that transplanted cells are able to grow and release *dopamine*.

Unfortunately, this is not the case in Huntington's disease. Medium spiny *neurons* in the striatum have many intricate connections with other *neurons* in the brain. The medium

spiny *neurons* are required to both receive information from these other regions, as well as pass the information along.

Not surprisingly, this process can get pretty complicated, as the formation of connections between *neurons* starts when we're developing in the womb and continues throughout our lives. These connections form and reform based on genetics and experience.

So, we might not expect that replacing medium spiny *neurons* would repair the devastating effects of Huntington's disease, because it's unlikely that replacement cells would be able to re-form the specific connections with other cells in the brain.

Proof of concept in a mouse brain

Interested in testing this this cell replacement idea, a team at the University of Wisconsin lead by Su-Chun Zhang recently injected replacement cells into mice whose striatum had been damaged. They found that the transplanted cells are capable of forming new connections in an adult mouse brain, and even more importantly, that these connections can improve the movement abnormalities of the mouse model.

The cells transplanted into the brains of mice were generated from human embryonic *stem cells*. Human embryonic *stem cells* are obtained from early stage *embryos*

discarded after *in vitro fertilization* (*IVF*) procedures. These cells can develop into every cell type in the human body, including *neurons* and other brain cells.

The advantage using embryonic *stem cells*, as opposed to obtaining *neurons* from fetal tissues, is the ability of *stem cells* to continuously regenerate, creating a more consistent source of tissue.

Sonic hedgehog: the secret to spiny *neurons*?

What makes one cell type different from another is the collection of proteins that they produce, which allows the cells to take on different shapes and functions.

For example, a *neuron*, which transmits impulses that allow us to think and move, has a very different role in the body from the cells that line our intestines and absorb nutrients. To make a *neuron* from a stem cell, the action of proteins called ***transcription* factors** make the stem cell gradually more specialized. *Transcription* factors work by turning on some genes, while turning other genes off.

Zhang's team treated the human embryonic *stem cells* with a *transcription factor* called 'Sonic Hedgehog' or a chemical that mimics its affects, and triggered the cells to transform into *neurons*. These man-made *neurons* looked like mature medium spiny *neurons* — the specific cells lost early on in Huntington's disease.

Replacing *neurons* in the mouse brain

In the past, before the genetic basis for Huntington's disease was understood, researchers would model the disease in mice by treating them with a neurotoxin called *Quinolinic acid*.

Quinolinic acid treatment doesn't cause HD, but it causes medium spiny *neurons* in the striatum to die, which also happens in HD.

These days, mouse models are much more advanced — they carry *CAG repeat* mutations in the mouse huntingtin gene or an extra copy of a mutant huntingtin gene. These genetic mouse models experience physical and behavioural symptoms similar to the real disease.

In their recent work, Zhang's group used *Quinolinic acid* to mimic the cell loss in Huntington's Disease, and then replaced the lost cells by injecting the medium spiny *neuron*-like cells they generated from human embryonic *stem cells*.

They were happy to discover that not only did the newly formed *neurons* grow in the brains of the mice, but they were also able form the correct connections with surrounding tissues. When the movement functions of the mice were tested, they showed moderate improvement in symptoms.

Hopes and limitations

This work is hopeful, as it suggests that transplanted *neurons* have more capacity to form functional connections than previously predicted. That means that cell-based therapy for Huntington's disease could be a real possibility in the future.

However, it must be noted that this study uses a *Quinolinic acid* treated mouse model, where the other, unaffected *neurons* are healthy. That's not the case in a real HD-affected brain. The formation of healthy connections may be more difficult in HD patients.

Furthermore, the mice used in this study received radiation treatment to disable the immune system, meaning that they will not reject the transplanted tissues. Although the brain is usually protected from our immune systems, there is still a risk of rejection of the transplanted tissue after surgery. So if this were tried in human patients, they'd likely need to take drugs to suppress the immune system, putting them at risk of developing serious infections.

With cell-based therapy, especially using tissues generated from embryonic *stem cells*, there is also a risk of uncontrolled cell growth — which could result in cancer. There are real questions about how we stop the transplanted cells from growing when they're done replacing the dead cells.

Clearly, cell replacement treatments will need to be further refined before any clinical trials can take place. But this new piece of work suggests that new *neurons* might be more flexible than we previously thought.

6.2 Phosphodiesterase inhibitors: new HD drugs entering trials soon

Written By: Carly Desmond

Edited By: Dr. Jeff Carroll

At this year's HD therapeutics conference, Pfizer Pharmaceuticals and the CHDI Foundation announced big plans for new HD drugs that target something called 'phosphodiesterases'. What's so exciting about these new drugs, and what's the timeline for bringing them to people?

Phospho-di-what?

Most people have probably heard of the drug Viagra. However, many would be surprised to discover that the “little blue pill” was originally developed as a treatment for high blood pressure, and only during clinical trials was it discovered to have some — ahem — unexpected side-effects.

Viagra is just one of a series of drugs called ***phosphodiesterase*** **inhibitors** (pronounced foss-foe-die-EST-er-ayze), which have been used to treat a range of ailments such as heart disease and asthma. These drugs all work in similar ways, but have very different

effects in the body. They are so variable, in fact, that select *phosphodiesterase* inhibitors are now being investigated for the treatment of HD.

To understand how *phosphodiesterase* inhibitors might benefit HD, we must first learn a little about phosphodiesterases themselves and how they are used by our brains.

Neurons need to connect

Our *neurons* make it possible for us to think and move by forming many connections with other surrounding *neurons*, which each have distinct roles in creating and delivering messages to our body. One *neuron* can form many thousands of connections.

Messages are passed from one *neuron* to another through chemical signals called neurotransmitters. Like a relay race, when one *neuron* sends out a neurotransmitter to another *neuron*, it sets off a series of events that activate the receiving *neuron*, and prepare it to pass the message along.

Neurotransmitters are called 'first messengers' because they're the first message to arrive, signaling that another *neuron* has fired off a message. Inside *neurons* there are 'second messengers', including the chemicals **cyclic AMP** and **cyclic GMP**, which change the receiving *neuron*'s behavior in response to the first message.

This process could be compared to a mailman attempting to deliver a letter. When he knocks on the door, it is answered by a child, so the mailman asks the child to pass the

message along to his mother. In this example, the mailman is the neurotransmitter (or first messenger), who passes the message along from outside the house, and it is the child (or second messenger) who passes the message along to his mother from the inside.

The second messengers cyclic AMP and cyclic GMP are critical to brain function. One of the ways they work is by activating and turning off genes through their interaction with '*transcription* factors'.

So, even though a burst of neurotransmitter is very brief, by changing the levels of cyclic AMP and cyclic GMP inside the cell, it can leave a lasting imprint on a *neuron* by interacting with *transcription* factors and turning genes on or off.

To grow and learn, *neurons* need to be shaped and moulded according to the messages they receive. Signaling by second messengers is very important for everyday learning and memory. Genes turned on by increased concentrations of second messengers cause connections with other *neurons* to be strengthened or lost. This flexibility in the strength of connections between *neurons* enables the formation of new memories and the learning of new tasks.

Having the right levels of cyclic AMP and cyclic GMP is obviously very important. *Neurons* that are not able to properly receive and interpret signals will lose their connections, which can cause them to die.

What about phosphodiesterases in the brain?

And so finally, we reach the important task of phosphodiesterases. Phosphodiesterases deactivate cyclic AMP and cyclic GMP by breaking apart their chemical structure.

Because phosphodiesterases dampen second messenger signals, drugs that block phosphodiesterases -*phosphodiesterase* inhibitors — allow the buildup of more cyclic AMP and cyclic GMP, strengthening their message.

Normally, having phosphodiesterases active in our brains is a good thing — too much cyclic AMP and cyclic GMP message would lead to over-stimulation of *neurons*. And when it comes to chemicals in the brain, we must always maintain a fine balance.

The striatum, garbled messages and new hope

In HD mouse models, researchers have found that the levels of cyclic AMP in the striatum are lower than in normal mice. That may explain why this brain region is particularly sensitive to the effects of Huntington's disease.

Although neurotransmitters may be sending the correct messages to vulnerable cells of an HD-affected brain, low levels of second messengers might mean those cells can't interpret the information correctly.

A team of scientists at CHDI, led by Dr Vahri Beaumont, are very interested in measuring neuronal communication. Rather than waiting around for *neurons* to die, they reason, it's better to design tests for changes in how *neurons* talk to one another.

Working with specialists in measuring the communications between *neurons*, Beaumont and her team have developed tests that accurately measure the communications between *neurons*. Having established these tests, they've shown that communication between *neurons* is distinctly altered in HD brains, especially in the striatum — the region of the brain most vulnerable in HD.

Their consistent finding is that vulnerable *neurons* in the striatum of HD mice are 'restless' and too excitable.

New human drug trial on the way

To try and combat this hyper-excitability, CHDI launched a collaboration with Pfizer, the international pharmaceutical giant. Pfizer has well developed drugs that work as *phosphodiesterase* inhibitors, including Viagra, so they have a lot of experience which could prove useful for solving this problem.

One of Pfizer's *phosphodiesterase* inhibitor drugs, called TP-10, blocks a particular form of *phosphodiesterase* found at higher levels in parts of the brain that are vulnerable in HD.

When HD mice were treated with TP-10, the results were very encouraging. Not only did the researchers observe an overall benefit to the motor skills of the mice, but they also saw less *neuron* loss in the striatum.

At the annual HD *Therapeutics* Conference, Pfizer and CHDI announced their joint efforts to testing TP-10 and a related drug in people. They are currently finishing animal studies, and plan several pilot studies in people in 2012 and 2013.

These preliminary studies are important to ensure that the drugs get where we think they should, and that they do what they're supposed to do when they get there. If all goes as planned, look for a 6 month human study in late 2013 designed to try and prove that these drugs work in HD patients.

This is a very exciting development. CHDI and Pfizer have done an enormous amount of work in animals to prove that this drug does interesting things. They've also laid out a short but sensible pathway to clinical testing, to see if the drug is what we all hope for — an effective treatment for HD.

6.3 Lithium with a twist gets second chance for HD

Written By: Carly Desmond

Edited By: Dr. Ed Wild

Lithium treatment of Huntington's disease is given a second chance, as new, safer dosing methods enter the drug development pipeline. Will long-term treatment with an old therapeutic be capable of preventing or slowing neurodegeneration in HD patients?

A brief history of lithium

Lithium is a soft, silver-white metal. As one of earth's natural elements, it belongs to a group of only 100 or so chemical building blocks that make up everything around us.

Lithium's earliest documented medical use dates back to the late 1800's; however, it was over 50 years before its more common therapeutic application was discovered. Administering small doses of the metal proved successful at smoothing over the emotional highs (mania) and lows (depression) suffered by people with bipolar disorder — echoing the ancient Greek tradition of soaking in mineral baths rich in lithium to

soothe psychiatric mania. To this day, lithium remains one of the most effective treatments for serious mood disorders available.

Although lithium has been approved for clinical use in most countries since the early 1960's, how it works has remained a mystery. Only now are scientists beginning to understand how lithium acts on the brain at the molecular level. As it turns out, some of the chemical and biological processes known to go wrong in *neurodegenerative* diseases like Huntington's disease can also be altered by lithium.

Why might lithium be helpful?

As HD progresses, degeneration occurs mostly in two parts of the brain called the striatum and cortex of the brain. The striatum is deep in the brain, while the cortex is the crinkly surface. Among other things, the striatum and cortex work closely together to control mood and movement.

Within the striatum, a very specific type of cells called **medium spiny *neurons***, are particularly susceptible to the disease. Medium spiny *neurons* cells are activated when a transmitter chemical called ***glutamate*** lands on *receptor* molecules on the cell surface. In HD, these receptors develop an increased sensitivity to *glutamate*, sending the medium spiny *neurons* into a state of over-stimulation.

This over-stimulation can trigger a process called ***excitotoxicity***, where chemicals within the *neuron* are inappropriately released, causing an avalanche of harmful effects. If the accumulated damage is too severe, the *neuron* will die. *Excitotoxicity* is one of the most established working theories of *neurodegeneration* for HD.

Where does lithium come in to this? Well, treatment with lithium has been found to block *excitotoxicity* in animal models. Even better, multiple studies have now shown that lithium is actually capable of shielding *neurons* from cell death, and possibly even encouraging their regeneration.

This is not the first time that researchers have considered treating Huntington's disease patients with lithium. Clinical studies were conducted as far back as the 1970's, with negative results — lithium didn't help. However, in retrospect, the trials suffered from one significant pitfall: all the patients were already deep into disease progression before receiving their first treatment.

Prevention is better?

Today, our understanding of lithium's biological effects have changed. We are more interested in its potential value as a preventative drug, rather than to treat existing symptoms.

Over the past few years, several studies in mouse models of HD have investigated the long-term benefits of lithium treatment. Instead of waiting until the mice were already sick, they were given the drug from a young age. The results were encouraging. These studies suggest that lithium has the ability to slow *neurodegeneration* and its associated symptoms in animal models.

Lithium's downside

Nonetheless, a significant hurdle stood in the way of testing long-term lithium treatment in humans — namely, the potential for serious side-effects.

Drug experts say lithium has a very narrow 'therapeutic window'. This means that patients require constant monitoring and blood tests to ensure they are receiving the proper dosage. It's difficult to keep the right level of lithium in the blood, and too much lithium can cause serious complications. Side-effects range from mild, such as tremor, confusion and nausea, to severe neurological deficits.

Another potentially bigger downside is that long-term treatment with lithium, even at a therapeutic level, can lead to major health issues such as reduced kidney function, forcing discontinuation of treatment. This would be a huge problem for HD patients, who might be required to take lithium for decades on end.

A new spin on an old drug

To overcome the current obstacles, a new lithium drug (NP03) and delivery system has been developed by Medesis Pharma. NP03 is a combination of lithium-citrate (a traditional lithium compound) and a new drug delivery system called Aonys®.

How is NP03 different from 'normal' lithium? Well, every cell in the human body is held together by fatty molecules called lipids. If cells were houses, lipids would be the bricks in the walls. In chemistry, some molecules are called '*hydrophilic*' (meaning water-loving) or '*hydrophobic*' (water-hating). Lipids are long molecules that are water-loving at one end, and water-fearing at the other. So any drug that wants to get into a cell needs to cross both a *hydrophobic* and *hydrophilic* barrier.

NP03 eases lithium's transition because the lithium is attached to lipids that can blend with the lipid 'wall' of cells. This means that more lithium is absorbed by the cells, and less drug is needed in order to achieve the same effect. NP03 may offer better control in long-term treatment with low-dose lithium, reducing its potential for side-effects.

NP03 tested in HD mice

In a recent publication from the laboratory of Dr. Michael Hayden at the Centre for Molecular Medicine and *Therapeutics* in British Columbia, Canada, evidence has been presented for the long-term application of NP03 treatment in a Huntington's disease mouse model.

The mice used in the study, called *YAC128*, produce a human version of the mutant HD gene, alongside the mouse's normal two copies. At about 3 months old, the mice develop movement symptoms similar to those seen in HD patient, and visible *neurodegeneration* occurs by 9 months.

To test if NP03 had the same *neuroprotective* properties as traditional lithium, the mice were treated with the drug from 2 months of age onwards — before the first symptoms surfaced.

The results were very encouraging. Mice given NP03 had significantly improved motor control compared to untreated HD mice. The striatum and its medium spiny *neurons* were spared from degeneration. Even more exciting was that, despite such a long treatment period, there were no observed adverse side-effects from the NP03.

One of the most devastating aspects of HD is that it is a genetic disease, affecting generation upon generation within families. But when it comes to preventative medicines, this might actually be a major advantage. A unique opportunity exists to identify individuals who will develop HD years in advance, and to stop the disease before the onset of symptoms.

A new start for lithium?

In the end, we must be cautious about how studies conducted in mouse models are interpreted. There is no guarantee that NP03's therapeutic benefit will carry over to actual Huntington's disease patients, or that it won't have unexpected side-effects.

The goal of any treatment is for the benefits to outweigh the risks. In an attempt to tip the balance, NP03 takes an old drug, already approved for use in humans, and simply aims to make it safer. If all goes well, it shouldn't be long before extended low-dose lithium treatment is ready for testing in people with the HD mutation.

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