

**THE INTERACTOME AT THE N17 DOMAIN  
OF HUNTINGTIN**

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HUNTINGTIN**

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**ABSTRACT:**

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by a polyglutamine expansion in the huntingtin protein. Recent research demonstrates that post-translational modifications of huntingtin could be an important determinant of mutant huntingtin's toxicity in HD. In particular, phosphorylation at residues serine 13 and 16 within the first 17 amino acids of huntingtin (N17), have been shown to be critical modulators of mutant huntingtin's toxicity and localization, and can be triggered by stress. This project aims to study how phosphorylation within N17 alters the interactome at this site and what physiological stress results in the nuclear localization of N17 and huntingtin. The initial search to identify potential interactors was conducted through an affinity chromatography assay using a wild type striatal cell line derived from knock in mouse model of HD. Fluorescent lifetime imaging microscopy (FLIM) to determine Förster resonance energy transfer (FRET), co-immunoprecipitation and co-immunofluorescence assays were then used to validate real interactors of N17. Analysis from this project has validated two previously unidentified interactors of N17; SET, a small nucleo-oncoprotein, and vimentin, a type 3 intermediate filament. Both interactors have suggested two potentially novel roles for N17 within huntingtin, in cell cycle regulation and intermediate filament dynamics. Finally, smart screening techniques using stress-inducing compounds reveal that the sensitivity of N17 to stress and its subsequent nuclear localization can be attributed in part to activation of oxidative stress pathways. Data shown here can be expanded upon to elucidate how huntingtin function and response to cell stress are regulated and mediated via N17 and potentially how this mechanism is disrupted within HD.

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## ABBREVIATIONS:

<b>AMP-PNP</b>	adenylyl 5'-( $\beta,\gamma$ -imido)diphosphate
<b>CDK</b>	Cyclin dependent kinase
<b>CRM1</b>	Chromosome Region Maintenance 1
<b>Co-IF</b>	Co-immunofluorescence
<b>Co-IP</b>	Co-immunoprecipitation
<b>DMSO</b>	Dimethyl Sulfoxide
<b>DTT</b>	Dithiothreitol
<b>ER</b>	Endoplasmic reticulum
<b>FLIM</b>	Fluorescence Lifetime Imaging Microscopy
<b>FRET</b>	Förester Resonance Energy Transfer
<b>GABA</b>	Gamma Aminobutyric Acid
<b>HD</b>	Huntington's Disease
<b><i>Hdh</i></b>	Huntingtin disease homolog
<b>HEAT</b>	Huntingtin Elongation factor 3, regulatory A subunit of protein phosphatase 2A TOR1
<b><i>HTT</i></b>	Huntingtin disease gene
<b><i>IT15</i></b>	Interesting Transcript 15
<b>I<sub>2</sub>PP2A</b>	Inhibitor 2 of protein phosphatase 2A
<b>LC-MS/MS</b>	liquid chromatography mass spectrometry
<b>M8P</b>	Methionine 8 to proline
<b>MOC</b>	Mander's Overlap Coefficient
<b>N17</b>	Amino acids 1-17 of huntingtin
<b>NES</b>	Nuclear export sequence
<b>N17PO<sub>4</sub></b>	N17 phosphorylated at serine residues 13 and 16
<b>NLS</b>	Nuclear localization sequence
<b>PACSIN</b>	Protein Kinase C and Casein Kinase 2 Substrate in Neurons 1
<b>PCC</b>	Pearson's Correlation Coefficient
<b>PPR</b>	Polyproline Rich Region
<b>S13,16A</b>	Serine 13 and 16 to alanine
<b>S13,16D</b>	Serine 13 and 16 to aspartic acid
<b>SH3</b>	SRC Homology 3 Domain
<b>Tpr</b>	Translocated promoter region
<b>UPR</b>	Unfolded protein response

## **1.0 BACKGROUND AND INTRODUCTION**

### **1.1 Huntington's Disease**

#### **1.1.1 A Brief History:**

Huntington's disease is as an autosomal dominant neurodegenerative disease. Historically, the earliest comprehensive description of the disease and its symptoms has been attributed to George Huntington whose paper in 1872 titled 'on chorea' described in great detail key characteristics of Huntington's Disease (HD) (Huntington, 2003). It encapsulated three generations worth of work, gathered by his father and grandfather before him, which discussed various well known adult-onset symptoms and the pattern of inheritance. (Bates, 2005). Though George Huntington did provide the basis for the genetic inheritance associated with the disease, it wasn't recognized that the disease followed an autosomal dominant pattern of inheritance until after the rediscovery of Gregor Mendel's work (Bates, 2005; McComas, 1914).

Many years later, with the advent of genome mapping techniques, projects were underway to identify the gene that was the source of the disease. Partly due to the efforts of Nancy Wexler and her interest in a large family of HD carriers in Venezuela (Wexler et al., 1987), in 1983 the HD gene was mapped to the short arm of chromosome 4 (Gusella et al., 1983). Finally in 1993, when both the technology and the resources were available the HD gene was linked to the 'interesting transcript' 15 (*IT15*) (now designated the *HTT*), where they identified a CAG trinucleotide repeat that varied from the normal population (11-34 repeats) and known HD patients (44 to 62) (The Huntington's Disease Collaborative Research Group, 1993).

### **1.1.2 A Monogenetic Disease**

After 1993, HD was identified as a monogenetic neurodegenerative disease and one of four trinucleotide repeat disorders identified at the time (Bates, 2005; The Huntington's Disease Collaborative Research Group, 1993). Today, it is one of nine polyglutamine diseases that affect varying regions of the brain and yet all share a critical pathogenic threshold number of repeats (Truant et al., 2006). Within HD, the pathogenic threshold occurs at polyglutamine lengths greater than 40, under 35 is considered normal, and the range of 35-40 is known as 'incomplete penetrance' (Rubinsztein et al., 1996).

Repeat length has shown to have an inverse correlation with the age of onset whereby individuals with longer repeats show earlier onset of symptoms, but repeat length is only partially responsible for age of onset (Gusella & MacDonald, 1995; Snell et al., 1993). The typical age of onset is during middle age (around 40 years), although a subset of individuals do experience juvenile onset HD which occurs at ages younger than 20. The inverse correlation between repeat length and age of onset is best exemplified in patients with juvenile onset HD, since they have repeat lengths of 50 or higher and this correlates with a very early age of onset ranging from 2-20 years (Andrew et al., 1993; Imarisio et al., 2008). Even with an inverse co-relation seen between repeat length and age of onset, there is variability in age of onset that exists within a given length of expansion. Study of siblings and family members of the HD kindred found around Lake Maracaibo in Venezuela have revealed that around 40% of the variability in the age of onset is due to genetic factors while the remaining 60% is from the environment

suggesting that even if CAG tract length is known there can be no accurate prediction for the age of onset (Wexler et al., 2004).

Sporadic HD or occurrence of HD in the founder member of a HD in a family is hypothesized to occur through a process known as genetic anticipation (Zuhlke et al, 1993). Genetic anticipation within HD is believed to occur due to the instability of trinucleotide repeat sequences during meiosis, but the mechanism through which expansion occurs is still an area of continuous study (Pearson et al., 2005). During the study of affected parent and child pairs within a given HD family, it was seen that there was a greater risk for CAG expansion when transferred paternally (Duyao et al., 1993; Zuhlke et al., 1993). As such, it is hypothesized that genetic anticipation is more prevalent in spermatogenesis than in oogenesis (Mangiarini et al., 1997). Genetic anticipation also in part contributes to the higher prevalence of HD in populations where individuals have trinucleotide repeat lengths close to the pathogenic threshold (Harper, 1992) and provides a basis for the origin of HD.

As can be seen with the above works, the new understanding for the genetic basis of HD generated still more new questions that arose. One of those questions was how a single genetic mutation causes the variable phenotypic profiles.

### **1.1.3 Neuropathological and Clinical Features**

HD symptoms include a mix of cognitive, psychiatric and motor abnormalities and have a rate of incidence as high as 1 in 6000 (Nehl et al., 2001). Patients often experience behavioural and mood disorders before the onset of any motor symptoms, these often include symptoms such as depression, aggression and irritability (Nehl et al., 2001; Van

Duijn et al., 2007). Motor symptoms that follow vary, and can include chorea, a hallmark trait of the disease, but also other extremes such as bradykinesia (a slowness of movement) and akinesia (an inability to initiate movement) (Roos, 2010). The cognitive symptoms often start off as an inability to focus, plan or organize and as the disease progresses, dementia develops during later stages (Paulsen et al., 2001; Roos, 2010). Secondary features often experienced by patients include insidious weight loss and sleep disorders (Arnulf et al., 2008; Aziz et al., 2008). As the disease manifests, patients eventually succumb to death 15-20 years after the age of onset. Causes of death include suicide, pneumonia and choking due to dysphagia (Lanska et al., 1988; Sorensen & Fenger, 1992; Walker, 2007).

The earliest and most pronounced changes within HD occur within the neostriatum where there is a significant reduction in the neuronal cell population and size of the striatum, particularly the caudate nucleus and to a lesser extent the putamen (Vonsattel et al., 1985). Atrophy within the striatum follows closely with disease progression, and the degree of atrophy is used to stratify the five neuropathological stages of HD. The stages range from 0 to 4, with stage 0 defined as presymptomatic and grade 4 as late stage HD. By stage 4 there can be as much as 95% neuronal loss within striatum (Aylward et al., 2000). During stages 3 and 4 neuronal atrophy is also noted outside the striatum including layers V and VI of the cerebral cortex (Hedreen et al., 1991). Gross neuronal atrophy is also accompanied with fibrillary astrogliosis (Hedreen et al., 1991) and loss of white matter tracts in regions connecting cortical and subcortical areas of the brain (De la Monte et al., 1988; Rosas et al., 2006).

Another hallmark feature of the disease is the presence of ubiquitinated aggregates that include both intraneuronal and intranuclear inclusions. The formation of inclusions is common to the field of neurodegeneration, but the formation and toxicity of these aggregates in the context of the disease is often highly controversial and has been shown to have both deleterious and beneficial effects (DiFiglia et al., 1997; Truant et al., 2008). It is worth noting however that these aggregates often appear in the pre-symptomatic stages and don't follow the pattern of neurodegeneration seen within HD (Gutekunst et al., 1999).

Although some of these neuropathological features (such as accompanying astrogliosis and protein aggregation) do appear to be common for most neurodegenerative diseases, within HD their cumulative effects target very specialized regions and tracts of the brain. The basal ganglia-thalamo-cortical pathway experiences the greatest dysfunction in HD and is the root cause of many of the motor abnormalities experienced by patients. Within the striatum, GABAergic (gamma aminobutyric acid) medium-sized spiny neuronal population are the most vulnerable to atrophy within HD, while interneurons (aspiny) and large spiny neuronal populations are relatively spared (Reiner et al., 1988).

Currently no effective therapeutic option exists for patients to combat or prevent the onset of the disease. With the use of both genetic and diagnostic tools today massive international efforts, such as PREDICT-HD and TRACK HD, are underway to study pre-symptomatic or pre-manifest individuals to find, study and test efficacy of potential therapeutic drugs and targets (Tabrizi et al., 2011). As a result, HD research is leading the

way for all other neurodegenerative disorders, not only because it is a monogenic disorder but also because the field has a well defined target; the protein huntingtin.

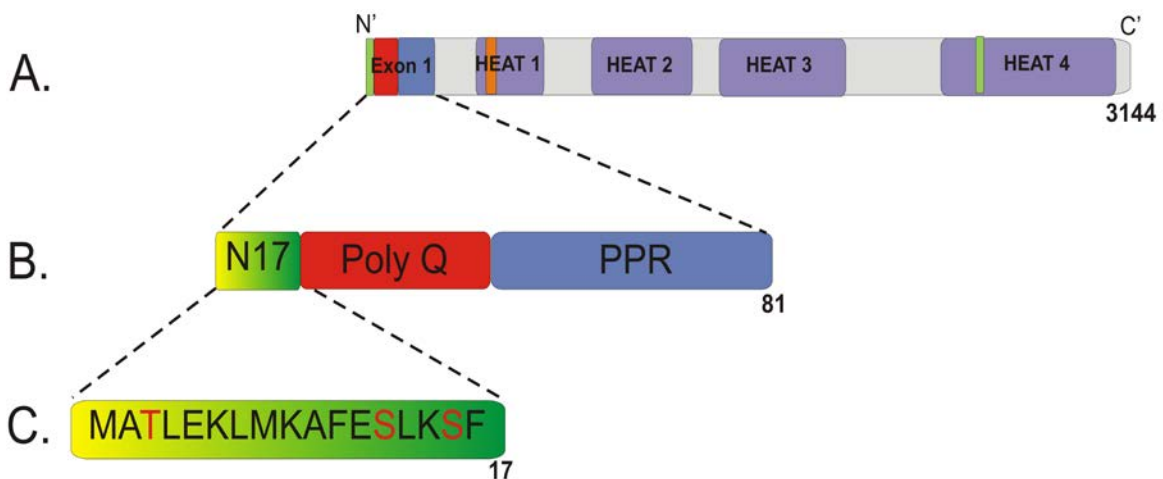
## **1.2 The Huntingtin Protein**

The trinucleotide repeat mutation identified on *HTT* (huntingtin) gene translates to a polyglutamine expansion found at the amino terminus of the protein huntingtin. This protein is a large soluble protein containing 3144 amino acids and is 348kDa in size with little or no sequence similarity with other known proteins. It is found ubiquitously both within the cell and the human body with the highest levels of expression within the central nervous system and testes (Cattaneo et al., 2005). Homologs of huntingtin with a polyglutamine tract exist as far back as sea urchin *Strongylocentrotus purpuratus*, with increasing lengths of the polyglutamine tract the closer one gets in the phylogenetic tree to humans (Tartari et al., 2008). Study of huntingtin homology suggests that huntingtin acquired the polyglutamine region in parallel with increased complexity and development of an organism's nervous system (Cattaneo et al., 2005; Z. Li et al., 1999). Therefore the polyglutamine region within huntingtin may be important for the development of a specialized nervous system and is one of many domains within huntingtin that have relevance to huntingtin function.

### **1.2.1 Structural Features and Cellular Localization of Huntingtin**

Huntingtin is a multi-domain protein. It contains 4 clusters of a domain known as HEAT (Huntingtin Elongation factor 3, regulatory A subunit of protein phosphatase 2A, TOR1) repeats (Andrade & Bork, 1995) shown on **Figure 1A**. HEAT repeats are known sites of protein-protein interaction (Cattaneo et al., 2005). HEAT repeats clusters contain

sequences approximately 40 amino acids that form an  $\alpha$ -helix-turn- $\alpha$ -helix structure and gives huntingtin a solenoid like structure (W. Li, Serpell, Carter, Rubinsztein, & Huntington, 2006; Takano & Gusella, 2002). In addition, at the far amino terminus of the protein we find the polyglutamine tract and a region adjacent to it known as a polyproline rich (PPR) region. The polyglutamine region has been hypothesized to form a polar zipper like structure (Perutz et al., 1994) and promotes binding to other transcription factors (which are abundant in polyglutamine tracts) to initiate or enhance transcription



**Figure 1: A diagrammatic image of Huntingtin.**

**A.** Full length huntingtin contains 4 clusters of HEAT repeats (shown in purple). Huntingtin also contains two nuclear export sequences (shown in green) at the carboxyl terminus and the amino terminus, and a nuclear import sequence (shown in orange). **B.** The first 81 amino acids of the amino terminus contains a sequence known as N17 (the first 17 amino acids) which also works as a nuclear export sequence, the polyglutamine (Poly Q) tract (which is expanded in HD) and a PolyProline Rich region (PPR). **C.** The N17 region contains a sequence of 17 amino acids in which the sites of phosphorylation have been highlighted in red.



(Rubinsztein et al., 1996). The PPR tract is also another known site of protein-protein interaction and binds to proteins that contains specialized domains such as the SH3 (SRC Homology 3 Domain) domain found with known interactor PACSIN1 (protein kinase C and casein kinase 2 substrate in neurons 1) (Modregger et al., 2002; Williamson, 1994). Huntingtin also has a nuclear localization signal (NLS) and two nuclear export signals (NES). At the amino terminus of the protein, we find that huntingtin contains both a chromosome region maintenance-1 (CRM1) dependent nuclear export signal (NES) located within residues 1-17 (Maiuri et al, 2013) and a proline-tyrosine NLS that works through both karyopherin  $\beta$ 1 and  $\beta$ 2 located at residues 171-204 (Desmond et al., 2012). In addition, we find a second CRM1 dependent export signal at the carboxyl terminus of the protein (Xia et al., 2003). These signals, shown in **Figure 1A**, work to regulate normal huntingtin translocation into and out of the nucleus, a function which could be disrupted in the HD, contributing to the accumulation of intranuclear inclusions (Desmond et al., 2013; Zheng, Li et al., 2013).

Huntingtin can also be post-translationally modified and cleaved. Known post translational modifications of huntingtin include phosphorylation (Desmond et al., 2013; Zheng et al., 2013), SUMOylation (Steffan et al., 2004) acetylation (Cong et al., 2011), and palmitoylation (Cong et al., 2011). Phosphorylation in particular has been shown to be a key regulator of huntingtin localization and toxicity (Atwal et al., 2011; Gu et al., 2009). Additionally, huntingtin cleavage occurs by both caspases (Graham et al., 2006) and caplains (Gafni et al., 2002) and is hypothesized to contribute to the formation of

amino-terminal aggregates of mutant huntingtin within HD (Havel et al., 2011; Sathasivam et al., 2013).

To add complexity to uncovering what function of huntingtin is perturbed within HD and responsible for pathogenesis, huntingtin is also localized to numerous cellular organelles; including the nucleus, endoplasmic reticulum, and cilia (Atwal et al., 2007; Desmond et al., 2013; Maiuri et al., 2013). It is also found at both the soma and synapse of neuronal cells (Trottier et al., 1995). The nuclear localization of huntingtin has also shown to be relevant to neurotoxicity, as improper localization of huntingtin to the nucleus has been implicated in neurodegeneration and the formation of nuclear inclusions (Havel et al., 2009; Saudou et al., 1998).

The conformational changes of huntingtin depend on its cellular localization. This is best illustrated when antibodies directed to various sites (or post translational modifications) of huntingtin show different localization (DiFiglia et al., 1997; Ko et al., 2001). For example, the intraneuronal aggregates, a characteristic feature of HD, were only visualized with the use of amino terminal epitope antibodies (Gutekunst et al., 1999). Therefore huntingtin reveals different epitopes depending on the cellular compartment in which it resides in, and these conformational changes and post translational modifications regulate its activity.

## **1.2.2 Huntingtin is a Pleiotropic Protein**

### **1.2.2.1 Huntingtin is Vital for Development**

Huntingtin is a multifunctional protein and a large multidomain protein which is expressed ubiquitously both within the cell and the human body. One of the critical

processes in which huntingtin has been characterized has been neurogenesis. In 1995, two independent labs showed that targeted disruption or knockout of the mouse huntingtin homolog causes embryonic lethality of mice around day 8.5 of gastrulation (Nasir et al., 1995; Zeitlin et al., 1995). In addition, polyglutamine expanded huntingtin or mutant huntingtin doesn't appear to affect huntingtin function in development. This was best exemplified when mice that are nullizygous for wild type huntingtin were rescued from embryonic lethality through the introduction of mutant huntingtin at endogenous levels (White et al., 1997). Studies of HD patients homozygous for the mutant allele also point to the same conclusion, as they show no obvious developmental abnormalities (Wexler et al., 1987). The use of histological techniques to study huntingtin and mutant huntingtin in developing mouse and human brains has revealed that wild type and mutant huntingtin expression patterns appear to be similar, owing further proof to the hypothesis (Bhide et al., 1996). Finally expression levels of (wild type or mutant) huntingtin are critical to regulate normal development of mice. Auerbach *et al.* showed that transgenic mouse models expressing reduced levels of huntingtin had severe developmental defects including increased ventricle volume and reduced size at birth (Auerbach et al., 2001). These findings reveal that huntingtin also has neuroprotective effects in development and that a minimum amount of huntingtin is essential for proper neurological development. Therefore it appears that huntingtin has a real and vital role in human and mouse neurogenesis and that this function is preserved even in the presence of polyglutamine expansion.

### 1.2.2.2 Huntingtin, Microtubules and its Role in Mitosis

Huntingtin is involved in microtubule dynamics. Huntingtin associates with microtubule structures and microtubule associated proteins (Hoffner et al., 2002; Tukamoto et al., 1997). Study of potential huntingtin interactors through affinity purification mass spectrometry analysis within wild type and HD mouse models have pulled out several tubulin isoforms (Shirasaki et al., 2012). In addition, regulation of aggregate formation in the presence of mutant huntingtin is also dependent on an intact microtubule network, as use of microtubule-destabilizing compounds disrupts formation of intranuclear inclusions (Muchowski et al., 2002). Finally, immunohistological staining for neurofilament and tubulin isoforms has revealed a reduction in these proteins in the cortex of presymptomatic HD patients (Diprospero et al., 2004) which might have downstream effects on disease progression.

The interaction of huntingtin with microtubules might be important for vesicular trafficking and cell cycle regulation. Huntingtin has been shown to bind directly to motor proteins that direct vesicular trafficking along microtubules, including dynein, dynactin (Caviston et al., 2007) and kinesin (Morfini et al., 2009). Trushina *et al.* further elaborated upon this connection when they showed that mutant huntingtin disrupts the normal function of huntingtin in vesicular trafficking, by increasing its propensity to aggregate, thereby sequestering huntingtin and associated motor proteins (Trushina et al., 2004). Huntingtin has also co-localized to spindle fibers during prophase to anaphase, and to the spindle midzone during telophase (Atwal et al., 2011; Godin et al., 2010). This localization works in concert with the interaction of huntingtin with dynein and dynactin,

which are also required components of spindle fiber formation during cell division (Merdes et al., 2000). Furthermore, short interfering RNA (siRNA) mediated knockdown of huntingtin expression in mouse neuronal cells results in spindle misorientation (a process which is required in determining cell fate during differentiation) and might be relevant for the role of huntingtin in development (Godin et al., 2010). The localization of huntingtin to very specialized structures during cell division and its interaction with motor proteins makes a strong case for a functional relationship between huntingtin and microtubule dynamics within neurons, and potentially disruption of the latter in HD.

### **1.2.2.3 Huntingtin in Stress Response**

Study of stress response and how it is enhanced by aging in HD can help answer another big question within the field: why does HD primarily afflict individuals during middle age? Stress response pathways slowly deteriorate with age, and so the combined effect of this reduced efficiency along with defective huntingtin function and accumulation of mutant huntingtin could lead to an inability of neurons to combat stress as an individual ages. This particular mechanism might not be distinct to HD, but could also be relevant to other late-onset neurodegenerative diseases (Morimoto, 2008).

Various stress-inducing agents have been applied to HD models and have been shown to result in a huntingtin stress response, suggesting that huntingtin could be important for specific stress response pathways. Huntingtin has been implicated in oxidative stress (Melkani et al., 2013; Sajjad et al., 2013), heat shock stress response (Bersuker et al., 2013; L. Munsie et al., 2011), and unfolded protein response (UPR) or

ER (endoplasmic reticulum) stress pathways (Atwal et al., 2007; Omi et al. 2005). These forms of stress regulate the localization of huntingtin fragments and modulate the ability of mutant huntingtin to form inclusions (Atwal et al., 2007; Wyttenbach et al., 2000). The role of huntingtin in cell stress response and its potential disruption within HD could explain why HD afflicts individuals during adulthood, where mature neurons are more vulnerable to metabolic stresses (Desmond et al., 2013; Shankar, 2010).

In addition to the aforementioned functions, huntingtin has also been shown to be important for transcriptional regulation (Benn et al., 2008; Hodges et al., 2006), energy metabolism (Browne & Beal, 2004) and synaptic transmission (Mandal et al., 2011) to name a few. Some of these functions have been determined through huntingtin interactome screens using yeast two hybrid or co-immunoprecipitation assays which have identified hundreds of potential huntingtin interactors (Harjes & Wanker, 2003; Kaltenbach et al., 2007). Together the multifunctional, multidomain and promiscuous nature of huntingtin gleaned so far have promoted the idea that huntingtin might in fact act as a scaffold protein (MacDonald, 2003). Evidence from work on HD model systems draw us back to the site of the mutation to understand which of these roles is relevant to disease.

### **1.3 The Amino-terminus of Huntingtin**

The HTT gene is made of 67 exon sequences (The Huntington's Disease Collaborative Research Group, 1993), but exon 1 is of particular interest. Exon 1 translates to a tract within huntingtin that contains the polyglutamine tract flanked by two domains; N17 (amino acids 1-17) at polyglutamine domain's amino terminus,

and on its carboxyl terminus resides a PPR tract which is made up of two stretches of repeating 10 and 11 proline residues with a short intervening sequence. This 81 amino acid fragment (commonly referred to as exon1) and longer fragments of huntingtin are often used as model systems instead of full length huntingtin because the size of huntingtin makes it difficult to study and isolate as a whole (Cattaneo et al., 2005). In addition, huntingtin cleavage leads to accumulation of amino-terminal huntingtin fragments within HD patients (Graham et al., 2006; Mende-Mueller et al., 2001), so these fragments have been used to mimic disease pathology.

### **1.3.1 Exon 1 and Mouse Models**

There are several reasons why exon 1 has been of interest to the HD field. First, the polyglutamine tract is found within it, rendering it an obvious starting point for further investigation. In addition, staining of post-mortem HD patient's brains has revealed the presence of intranuclear inclusions made up of amino-terminal fragments of huntingtin (DiFiglia et al., 1997; Sathasivam et al., 2013). Within HD mouse models proteolysis of huntingtin results in accumulation of neuronal aggregates composed of huntingtin amino terminal fragments (Landles et al., 2010). Finally, transgenic mice expressing a human huntingtin exon 1 with an expanded CAG tract, referred to as R6/2, developed phenotypic symptoms analogous to those seen in HD patients (Mangiarini et al., 1996). Although this model did seem to reproduce some of the abnormal motor defects seen in HD, regionally specific neurotoxicity, such as severe degeneration striatal neurons, was not observed. This suggests there are other factors at play that might be specific to the intra or intermolecular interactions of huntingtin.

### 1.3.2 Polyglutamine Flanking Regions and Relevance to Neurodegenerative Diseases

Huntington's disease is one of nine CAG trinucleotide disorders; which includes HD, spinobulbar muscular atrophy, dentatorubral-pallidoluysian atrophy and the spinocerebellar ataxias 1,2,3,6,7 and 17 (Truant et al., 2006). Although these disorders share some inherent similarities, such as the inverse relationship between length of polyglutamine tract and age of onset (Duennwald et al., 2006), each of these diseases shows a distinct neuropathology whereby each disease most prominently affects specialized regions of the brain (Truant et al., 2006). The distinct neuropathology within these diseases has not been shown to correlate with higher expression of pathogenic proteins in these areas, so it is important to understand the roles these proteins play within the neuronal populations that are selectively degenerated with each respective disease, and the relative contribution of the polyglutamine tract and adjacent domains (La Spada & Taylor, 2003).

Towards understanding the protein's contribution to polyglutamine expansion-induced neurotoxicity some insightful work has been conducted within *Saccharomyces cerevisiae*. Within this model organism, it was shown that regions on either end of the polyglutamine tract (hereafter referred to as flanking regions) can affect the toxicity of the protein (Duennwald et al., 2006). The addition of a PPR tract and the placement of a tag in close proximity to an expanded polyglutamine tract could alter the toxicity seen within the yeast revealing the importance of the flanking tracts. Other groups have also gone on to show that flanking regions affect aggregate formation within mammalian cell models and can sometimes reverse the ability of an expanded polyglutamine tract to promote



aggregation (Bulone et al., 2006; Crick et al., 2013; Lakhani et al., 2010; Nozaki et al., 2001). Recently work by Caron *et. al.* via the use of a FRET (Förster Resonance Energy Transfer) sensor, has shown that polyglutamine region can act as a flexible hinge, whereby polyglutamine lengths below the pathogenic threshold allow for the flanking regions to interact (Caron et al., 2013). Furthermore proteins such as PACSIN1 were required to mediate the intramolecular interactions between the flanking sites (Caron et al., 2013).

Overall, the cumulative efforts of the work summarized above suggest that protein context and protein interactions are important determinants of neurotoxicity that is induced by polyglutamine expansion in these diseases.

#### **1.4 Focus on N17**

The N17 domain is the first 17 amino acids that precede the polyglutamine tract within amino acids 1-81 of huntingtin (shown in **Figure 1C**). The N17 region within huntingtin has been of great interest within the field of HD for various reasons. The foremost rationale for studying N17 is due to its sequence conservation within a wide variety of vertebrate species (Atwal et al., 2007; Rockabrand et al., 2007). The preservation of the sequence implies an important evolutionary role of this region for the function of huntingtin.

In addition N17, a polyglutamine flanking sequence, has been shown to regulate the structure of the huntingtin fragment 1-81. This regulation of this structure is critical because it is believed to play a role in aggregation kinetics and aberrant protein-protein interactions which can contribute to disease pathology (Thakur et al., 2009). X-ray

crystallography studies have revealed that the 1-81 huntingtin fragment has variable structural conformations, and that these conformations were shown to be dependent upon the flanking regions (Kim et al., 2009). Finally, molecular modelling simulations predict that N17 regulates the ability of the polyglutamine tract to form globular oligomeric aggregates (Thakur et al., 2009) and secondly that an expanded polyglutamine tract can disrupt the hydrophobic interaction of N17 region with the PPR rich region exposing the amino-terminus region and inducing aggregation (the region located at the carboxy-terminus of the polyglutamine tract) (Dlugosz & Trylska, 2011). Finally, the structure of N17 has been identified as an amphiphatic alpha helix, the relevance of which is critical to both the localization and function of the huntingtin protein (Atwal et al., 2007; Dlugosz & Trylska, 2011; Thakur et al., 2009).

#### **1.4.1 Known functions of N17 within huntingtin**

When expressed alone or within the large context of amino acids 1-81 of huntingtin, N17 acts a cytoplasmic retention signal (Atwal et al., 2007; Rockabrand et al., 2007) and even with the addition of a strong nuclear localization sequence it still retains a primary cytoplasmic distribution (Steffan et al., 2004). The disruption of its amphiphatic alpha helical structure by the introduction of a methionine to proline mutation at residue 8 (M8P) causes a change in localization and relocation to the nucleus (Atwal et al., 2011; Atwal et al., 2007). Nuclear localization is believed to be a critical step in mutant huntingtin-induced pathogenesis and one of the hallmark pathological features of the disease is the presence of intranuclear aggregates of mutant huntingtin. Therefore the significance of these findings is twofold: the ability of N17 to retain huntingtin in the

cytoplasm is structure-specific and it may be relevant for the ability of mutant huntingtin to cause neurotoxicity within HD.

The N17 region targets huntingtin to membrane structures and this is dependent on the amphiphatic alpha helix structure of the N17 domain (Atwal et al., 2007). N17 has membrane interactions with the endoplasmic reticulum, Golgi, and mitochondria (Atwal et al., 2007; Michalek et al., 2013; Rockabrand et al., 2007). The interaction of N17 with the membrane was shown to occur at a tilt angle ~75 degrees to the membrane (almost lying parallel to the surface) through the use of solid state nuclear magnetic resonance showing that this region works as an anchor for the huntingtin protein within membrane structures (Michalek et al., 2013).

Finally it was recently shown that N17 is a CRM1 (chromosome region maintenance 1) nuclear export signal (NES) (Maiuri et al., 2013; Zheng et al., 2013). Leptomycin B was used to show that wild type N17 fragments showed a predominantly nuclear localization upon addition of the treatment when it was otherwise cytoplasmic within a striatal mouse knock-in cell model of HD (Maiuri et al., 2013). These findings were further exemplified in both human cell lines as well as primary mouse neuronal cultures, whereby mutations at critical residues within the NES worked to increase nuclear accumulation and altered aggregation formation (Zheng et al., 2013).

Nucleocytoplasmic shuttling of huntingtin is critical to the pathogenesis of the disease since it is hypothesized that the nuclear aggregates found with HD causes transcriptional dysregulation and contributes to the neurotoxicity (Havel et al., 2009). As a result we see that N17 plays an integral part in export of huntingtin from the nucleus:

moreover post-translational modifications at N17 can also modulate huntingtin nuclear localization.

#### **1.4.2 Phosphorylation of N17 and its effect on toxicity**

N17 is subject to post-translational modifications such as SUMOylation (Steffan et al., 2004), ubiquitination (Cong et al., 2011), acetylation and phosphorylation, all of which have been shown to regulate the toxicity of huntingtin (Ehrnhoefer et al., 2011; Pennuto et al., 2009), but only phosphorylation within N17 has shown to abate and reverse HD like symptoms.

Phosphorylation is a highly regulated process within cells and is essential in various signal transduction pathways. Within HD, mutant huntingtin is hypophosphorylated, and this has previously been linked to neurotoxicity (Atwal et al., 2011; Pardo et al., 2012). The N17 region has three sites of phosphorylation; threonine at position 3 (T3) (Aiken et al., 2009) and serines at positions 13 and 16 (S13,16) (Gu et al., 2009). Phosphorylation at serines sites 13 and 16 is known to regulate the translocation of huntingtin from the cytoplasm to the nucleus (Atwal et al., 2011). Addition of stress-inducing agents, such as dithiothreitol (DTT) and tunicamycin, triggers the unfolded protein response pathway (Bicknell et al., 2010) and alters the localization of N17 from ER to the nucleus (Atwal et al., 2011). In addition, mutations that mimicked phosphorylation at S13,16 correlated with decreased neurotoxicity within HD cell models and prevented the onset of HD-like symptoms in mice (Atwal et al., 2011; Gu et al., 2009). Phosphorylation of S16 is also shown to be relevant for the generation of amino-terminal fragments aggregating within the nuclei of striatal neurons (Havel et al., 2011). Finally phosphorylation, which can be

modulated by the use of small molecule drugs, has been shown to abrogate and even remarkably reverse HD-like phenotypes in HD mice models (Pardo et al., 2012).

Therefore, we see that phosphorylation at S13,16 within N17 has several functional consequences for huntingtin. Firstly, we find that these phosphorylation events can regulate the localization of huntingtin within neurons, which is similarly seen by the structure-disrupting mutants (M8P). Secondly, S13 and S16 phosphorylation affect neurotoxicity and aggregation formation, providing mechanistic insight into two hallmark pathological features of HD. Furthermore, these phosphorylation events can be modulated by the use of specific cellular interactors or small molecular inhibitors or drugs (Atwal et al., 2011; Luo et al., 2005; Thompson et al., 2009). Thus phosphorylation might act as a mechanistic trigger for huntingtin function within neurons and that this might be important to the pathogenesis of HD (Greiner & Yang, 2011).

In order to gain further insight into the pathway where stress and phosphorylation trigger translocation of N17 translocation from the ER to the nucleus, it becomes important to understand what other proteins are involved in this mechanisms and how this process is regulated. The next step to build upon the work done thus far would be to identify what interactors cause this phosphorylation event or are recruited during phosphorylation, and also to determine how these events are triggered within neurons and what stresses can cause these events.

### **1.5 Experimental Rationale: The Search for N17 interactors**

The significance of N17 in regulation of huntingtin function and toxicity, combined with past success in gaining greater understanding of protein's cellular functions with the

use of interactome studies, has prompted the search for N17 interacting partners (Harjes & Wanker, 2003; Li & Li, 2004). Since the pathway through which phosphorylation at N17 occurs is relatively unknown, my project is also directed towards uncovering the triggers of N17 phosphorylation at serine residue 13,16 and nuclear localization.

Previous interactors of N17 noted in the literature, including a nuclear pore protein known as Tpr protein (Cornett et al., 2005) and a chaperone protein TRiC (Tam et al., 2009), have helped identify new roles or pathways for both N17 and huntingtin. With similar goals in mind, this project starts off with affinity chromatography columns and use of biochemical and cell imaging assays to identify new N17 interactors. Furthermore, in an effort to understand the mechanisms through which stress triggers phosphorylation at N17 and translocation of huntingtin to the nucleus, cell stress screening experiments have been conducted with the use of N17 and N17 mutants.

We hypothesize that post-translational modifications act as switches to alter the interactome of the N17 region of huntingtin, which in turn results in a functional change of the huntingtin protein within neuronal cells. Within Huntington's disease, the steric hindrance caused by polyglutamine expansion in mutant huntingtin might deter the N17 region's ability to interact with its modifiers during events of stress and therefore contribute to the disease phenotype.

### **1.5.1 Experimental Aims:**

Aim 1: To identify a list of interactors at the N17 that are phospho-dependent and independent through the use of protein affinity chromatography with synthetically developed peptides and liquid chromatography mass spectrometry (LC-MS/MS).

Aim 2: To validate the list developed from the LC-MS/MS results using methods such as co-immunofluorescence (co-IF), co-immunoprecipitation (co-IP), Förster Resonance Energy Transfer (FRET).

Aim 3: To study the ability of the N17 domain to react to physiologically relevant stress events by use of smart screening techniques whereby the localization of N17 and N17 mutants will be imaged upon addition of stress-inducing treatments.

## 2.0 MATERIALS AND METHODS

### 2.1 Plasmid Constructs:

All constructs were created in the Clontech peGFP1 vector backbone with a kanamycin selective marker amended to include varying fluorescent proteins. Constructs that had a yellow fluorescent protein (YFP) vector including N17 wild type and N17 variants including a methionine to proline at residue 8 (M8P), serines 13 and 16 to aspartic acid (S13,16D), and serine 13 and 16 to alanine (S13,16A) were all created as described elsewhere (R. S. Atwal et al., 2011). SET was amplified out of a Genecopia vector (product ID: Z5816) with addition of overhangs for BglII and Hind III to be cut and cloned into CFP variant vector known as mTurquoise 2 created by Goedhart *et al.* (Goedhart et al., 2012) with the multiple cloning site located at the carboxyl terminus. Vimentin was also cloned similarly, it was amplified out of a vimentin-LSSmOrange vector from Addgene (Plasmid number 37134) with SalI and BamHI overhangs. The amplified fragment was then ligated into p-mCerulean C1 vector (BD Biosciences/Clontech). The 14-3-3 zeta-RFP construct was created by a past student.

### 2.2 Cell Culture and Transfection

All experiments were conducted in conditionally immortalized mouse-derived striatal cells referred to as *STHdh*<sup>Q7/Q7</sup> (a kind gift from M.E. McDonald). The cells were extracted from transgenic mice expressing a wild type (7 glutamines) exon 1 of humanized huntingtin as described by Cattaneo *et al.* (Cattaneo & Conti, 1998). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and with G418 selection marker at 33°C with 5% CO<sub>2</sub>.



When dishes were at around 70% confluency they were transfected with TurboFect (Thermo Scientific) transfection reagent. Reagent volumes and concentrations were followed as per manufacture's recommendation. DNA and TurboFect were left to incubate for 20 minutes in serum free DMEM at room temperature before being added directly to the dish.

### **2.3 Protein Affinity Chromatography Assay**

When 10 cm dishes of *STHdh*<sup>Q7/Q7</sup> were fully confluent they were lysed as follows. Cells were first washed with phosphate buffered saline solution (PBS), scraped off the dish in PBS solution, and spun down at 3000 rpm for 5 minutes. From this point on everything was done at 4°C and all buffers were supplemented with protease (Roche) and phosphatase inhibitors (PhosphoSTOP, Roche). The supernatant was discarded and the first buffer (10mM Tris pH 7.9, 1.5mM MgCl<sub>2</sub>, 10mM KCL, 0.5mM DTT and 0.2mM PMSF) was added on at 2 times the volume of the pellet. Cell pellet and solution were dounced. Pellet was spun down at 3000 rpm for 10 minutes. Supernatant was then added to make up 1 x solution using 10x buffer B (0.3M Tris 7.9, 1.4M KCl, 30mM MgCl<sub>2</sub>) and was labelled cytosolic fraction. Pellet was taken and resuspended in 1 x pellet volume of buffer C (20mM Tris 7.9, 25% glycerol, 0.42M NaCl, 1.5mM MgCl<sub>2</sub>, 0.2mM EDTA, 0.5mM PMSF, 0.5mM DTT) by douncing. Solution was spun end over end for 30 minutes and then centrifuged at 12 000 rpm for 30 minutes. Supernatant was labelled as the nuclear fraction. The pellet was resuspended in 1x volume of pellet in buffer E (50mM Tris 7.9, 25% glycerol, 0.5mM EDTA, 5mM MgCl<sub>2</sub>, 5mM DTT, 0.2mM PMSF), dounced and centrifuged at 12 000 rpm for 10 minutes. Final supernatant was added to

nuclear fraction solution. All lysate was then dialyzed overnight into coupling buffer solution (0.1M NaCl, 10mM Hepes pH 7.4 and 10% glycerol). Concentrations of cytoplasmic and nuclear fractions were measured by Bradford.

Columns were prepared as follows: synthetically made peptides of N17 wild type (Genescript), M8P (Genescript), S13,16pS (New England Peptide), S13pS (New England Peptide), S16pS (New England Peptide) were diluted from stock to 0.25mg/mL into coupling buffer using 500 $\mu$ L per column. Sulfo-resin beads (30 $\mu$ L per column) were washed and then covalently coupled to peptides as per manufactures instructions (Thermo Scientific). Columns were prepared by added glass wool to a 200 $\mu$ L plastic pipette tip and adding on 500 $\mu$ L of coupled bead and peptide. The column then had 3 volume's worth of 50mM L-cysteine and 3 washes with 200 $\mu$ L of coupling buffer. Then 200 $\mu$ L of cytoplasmic lysate diluted to 1.3mg/mL in coupling buffer per column, followed by three 200 $\mu$ L washes of coupling buffer and eluted using 60 $\mu$ L of elution buffer 1 (0.5mM NaCl, 10mM Hepes pH7.4 and 10% glycerol). The column was washed 3 times again, and 200 $\mu$ L of nuclear fraction lysate at a concentration of 1.3mg/mL was added. Again it was washed 3 times with coupling buffer and then eluted into 60 $\mu$ L of elution buffer 1. Finally 60 $\mu$ L of elution buffer 2 (0.1% SDS in elution buffer 1) was added and the column was discarded.

15 $\mu$ L of each elution sample were run on a 12% SDS-PAGE gel, transferred to a PVDF membrane. Membrane was blocked in 5% milk with (Tris-buffered saline with Tween 20) TBS-T was blotted for  $\beta$ -tubulin (E7, DSHB) at 1:500 concentration overnight and then anti goat anti mouse conjugated to horse radish peroxidase (HRP) (Sigma

Aldrich, A9917) secondary at 1:5000. Blot was developed with Immobilon Western Chemiluminescent Substrate (Millipore) and imaged on film.

Remaining elutions were sent off for liquid chromatography mass spectrometry mass spectrometry (LC MS/MS) analysis at the University of Western Ontario Biological Mass Spectrometry Lab. Mass spectrometry was performed on a Waters, QToF Global mass spectrometer equipped with a Z-spray (ESI) source and run in positive ion mode. Instrument was run in DDA mode. MS Acquisition Software: MassLynx 4.1 (Waters) for the digested samples. Waters nanoAcquity was used for LC, using a standard one hour run. All sample preparation procedures including trypsinization, desalting and vacuum concentration were followed as listed on their website.

#### **2.4 Co-immunoprecipitation and Western Blot**

Co-immunoprecipitation was conducted using GFP-Trap (ChromoTek) magnetically charged beads. When 10 cm dishes of *STHdh<sup>Q7/Q7</sup>* were around 70% confluent they were transfected with appropriate constructs and allowed to express 16-24 hours.

With BMS 345541 treatment cells were transfected at 40% confluency, treatment was added 24 hours post-transfection. BMS 345541 treatment was added at a 10 $\mu$ M concentration directly into the media and left on the dish for 16 hours before use of the dish for further analysis. One dish was used per column.

Cells were then lysed using 100 $\mu$ L lysis buffer (10mM Tris/Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40) and the beads were equilibrated as per manufactures instructions. Beads and lysate were incubated at 4°C end-over-end for 2 hours. Beads

were then magnetically separated from the solution and 20 $\mu$ L of solution was then removed to be used as unbound fraction and remaining solution was discarded. Beads were then washed and magnetically separated with two rounds of 500  $\mu$ L wash buffer (10mM Tris/Cl pH: 7.5, 150mM NaCl, 0.5mM EDTA) and then one round of 500 $\mu$ L high salt wash buffer (10mM Tris/Cl pH 7.5, 500mM NaCl, 0.5mM EDTA). The bound solution was eluted off the beads into 60 $\mu$ L of 2x SDS-Sample buffer and boiled off at 95°C for 10 minutes.

Samples were run on two 12% SDS-PAGE gels (30 $\mu$ L loaded per lane), transferred to PVDF membrane and blocked in 5% milk in TBS-T for an hour before addition of antibodies. Primary antibodies used include anti-Aequorea victoria fluorescent protein at 1: 2500 (rabbit polyclonal antibody, Clontech), anti-14-3-3 zeta at 1:1000 (Abcam, Ab51129), anti-SET at 1:1000 (Abcam, Ab85389) and anti-vimentin at 1:1000 (DSHB, AMF-17b), which were diluted in blocking solution and left on a rocker overnight at 4°C. Secondary antibodies were then applied and diluted in blocking solution. Secondary antibodies used include goat anti mouse HRP (Sigma Aldrich, A9917) 1: 5000 or 1:2500 (with anti-vimentin primary) and goat anti-rabbit HRP at 1:50,000 (Abcam, ab97051). Blots were then developed using Immobilon Western Chemiluminescent Substrate (Millipore) and were imaged with use of DNR Microchemi bio-imager.

## **2.5 Co-immunofluorescence**

Cells were grown on a 2.5 cm glass bottom dish. When cells were around 70% confluent they were fixed and permeabilized with ice cold methanol at -20°C for 12

minutes. If cells were transfected with a construct an alternate fixation method was employed where 4% paraformaldehyde (PFA) was applied for 20 minutes at room temperature, then 3 washes with PBS and finally permeabilization with methanol for 12 minutes at  $-20^{\circ}\text{C}$ . Dishes are then washed in PBS and blocked in 2% FBS in PBS for at least half an hour before addition of primary antibodies. Antibody solutions were diluted in 0.02% Tween 20 in 5% FBS in PBS and left overnight at  $4^{\circ}\text{C}$  with primary antibodies. Antibodies used include anti-14-3-3 zeta at 1:100, 14-3-3beta at 1:75, anti-wild type N17 (N7wt) (rabbit polyclonal, in house) at 1:200, anti-phospho-N17 (N17-PO<sub>4</sub>) at 1:200 (rabbit polyclonal, in house), anti-SET at 1: 100, N17wt conjugated to Alexa Fluor 488 at 1:25 and N17-PO<sub>4</sub> conjugated to Alexa Fluor 488 at 1:25. Primary antibodies were removed and dishes were blocked again for half hour before addition of secondary antibodies for 1 hour in room temperature. Secondary antibodies used included Alexa Fluor 488 donkey anti rabbit at 1:500, Alexa Fluor 594 goat anti mouse 1:500 and Alexa Fluor 594 chicken anti rabbit at 1:500 (or 1:400 with SET primary). Nuclear stains include Hoechst 33258 (Sigma) diluted to 1 $\mu\text{g}/\text{mL}$  in PBS added on for 10 minutes and DRAQ5 (Biostatus) diluted to 5 $\mu\text{M}$  in PBS for 20 minutes both at room temperature.

Dishes were viewed on a Nikon TE200 inverted widefield epifluorescent microscope and images were taken using a Hamamatsu Orca camera using a 60x oil immersion objective. Pearson's Correlation coefficient (PCC) and Mander's Overlap coefficient (MOC) were measured with the use of NIS elements 4.1 software.

## 2.6 FLIM-FRET assay

Cells were grown to 60-70% confluency on glass bottom 35 mm dishes before transfection and allowed to express 16 hours before use in assay. Transfected dishes were taken out of media and placed into Hanks Saline HEPES buffer before imaged.

FLIM-FRET images were performed on an inverted confocal microscope with a laser scanner (Leica TCS SP5). The images were taken using a 63x glycerol immersion NA 1.4 Plan apochromat objective. The images were analyzed using Leica application suite advanced fluorescence software. For FRET donor fluorophores were excited using a Chameleon Ultra two-photon laser. Donor and acceptor pairs included eYFP and mRFP, mCer and eYFP, and mTurq2 and eYFP respectively. The mTurq2 was treated as mCer and was excited at a wavelength of 820nm and eYFP was excited at 920nm as these were described as the optimal excitation wavelength for a two photon system (Chen & Periasamy, 2004). Fluorescent lifetime was collected and quantified using a time-correlated single photon counting (TCSPC) using the SPC-830 system and software. Photons were collected for 60 seconds, and image size (256x256 pixels) and scanning speed (400Hz) were kept constant.

Lifetime values of entire cells were found using the ImageJ. Percent FRET efficiency was calculated based on the equation  $\text{FRET (\%)} = 1 - (\tau_{\text{FRET}}/\tau_{\text{Donor}}) * 100$  where  $\tau$  is lifetime. Statistical significance was determined with the use T-test by Sigma Plot program version 11.0.

## 2.7 Cell Stress Assay and Image Analysis

Stable *STHdh*<sup>Q7/Q7</sup> expressing N18-eYFP constructs and variant including M8P-YFP, S13,16A-YFP and S13,16D were plated on a glass bottom 96 well dish (Ibidi) 24 hours before addition of treatment. Each drug treatment was added to the appropriate concentration in wild type *STHdh*<sup>Q7/Q7</sup> media with FBS. Treatments added included (into each well per construct): 1%, 2% and 5% dimethyl sulfoxide (DMSO); 1mM, 3mM and 5mM dithiothreitol (DTT); 1µg/µl, 3µg/µl and 5µg/µl tunicamycin; 100mM, 400mM and 600mM hydrogen peroxide; 1µM, 2µM and 4µM A23187 (Sigma); 1mM, 2mM and 4mM adenylyl 5'-(β,γ-imido)diphosphate (AMP-PNP); and finally 0.031mM, 0.063mM and 0.124mM cadmium chloride all acquired from Sigma-Aldrich.

Cells were first rinsed in pre-warmed PBS before addition of treatments. Treatments were added on for 1 hour before fixation. After elapsed time dishes were rinsed once again in pre-warmed PBS and 4% PFA solution was added and left to incubate for 20 minutes in the 33°C incubator. Upon fixation, cells were rinsed in PBS solution 3 times for 5 minutes each. Hoechst dye (as stated above) was added and left to incubate for 10 minutes before plate was imaged.

Images of YFP and Hoechst dye (to identify the nucleus) were taken on Nikon TE200 inverted widefield epifluorescent microscope using a Hamamatsu Orca camera with a 40x air objective. The multipoint system on NIS elements 4.1 software with pre-assigned co-ordinates was applied and set for 10 images per well in two channels.

Images were then analyzed using a percent nuclear pipeline created on CellProfiler which identified cell and nucleus, and quantified amount of YFP intensity

found within the nuclear and cytoplasmic compartment. The calculation for % nuclear was done as described elsewhere (Maiuri et al., 2013). Statistical analysis of the output data was conducted using student t-test function on excel.



### 3.0 RESULTS

#### 3.1 Affinity Chromatography List and Validation

An affinity chromatography method was employed to identify a list of potential N17 interactors and how they depend on the structure or the phosphorylation status of N17. This assay was performed using synthetic peptides of wild type (N17), structurally disrupted N17 (M8P), phosphorylated N17 at serine 13 (S13pS), serine 16 (S16pS) and serine 13,16 (S13,16pS) as the bait and whole cell lysate from wild type cells as the prey. Elutions were then sent out for mass spectrometry analysis and the list of proteins generated was scanned for proteins of interest based on literature searches.

The list of potential interactors chosen for further investigation is shown in **Figure 2A** and includes 14-3-3 isoforms, SET- $\beta$  and vimentin. The table is divided into the fractionation components (cytoplasmic and nuclear) as well as a final elution (which required the use of a denaturing agent). When comparing the cytoplasmic fraction only SET- $\beta$  is found to have a preference for phosphorylated peptides of N17, and binds both S13pS and S16pS. Vimentin binds to M8P, while 14-3-3 isoforms zeta and beta bind to both wild type and phosphorylated peptides of N17. Within the nuclear fraction, SET- $\beta$  and 14-3-3 beta bind non-specifically to all peptides, while vimentin binds both wild type and M8P. Finally within the final elution, SET- $\beta$  binds to only wild-type N17, vimentin binds to phosphorylated S13,16pS, unphosphorylated N17 and M8P, while 14-3-3 eta binds to all phosphorylated peptides of N17.

A subset of the elutions was used for western blot analysis to verify the specificity of the columns.  $\beta$ -tubulin was used as a positive control (a previously known binding

partner). In **Figure 2B** we see that  $\beta$ -tubulin did not bind to the beads alone (negative control) column, but varying levels of  $\beta$ -tubulin were found bound to the peptide columns.

Since application of any given technique to search for protein-protein interactions has a background level of non-specific false positives that are distinct to the technique employed (Howell et al., 2006) it is always best to proceed with caution and reaffirm potential bait and prey protein interactions using alternate *in vitro* and *in vivo* techniques. As such, validation and analysis of the chosen potential N17 interactors were conducted through the use of co-immunofluorescence (co-IF), co-immunoprecipitation (co-IP) and fluorescent lifetime imaging microscopy to determine Förster resonance energy transfer (FLIM-FRET) assays. Each technique provides its own level of sensitivity and specificity in aiding with the identification and validation of protein-protein interactions. Co-IF displays co-localization (not direct interaction) between endogenous proteins at a single time point. FLIM-FRET allows for live cell analysis and the cellular localization of a direct protein-protein interaction, but does so in the context of over-expressed protein products and their tags. Finally, co-IP provides an *in vitro* method for analysis of protein-protein interaction (both direct and within a complex) but lacks the ability to detect weak and transient interactors as the technique involves cellular lysis and therefore disrupts the homeostatic environment within the cell. Therefore, use of all three techniques will provide a broader means to study the interactions.

### 3.2 Analysis of interaction between 14-3-3zeta and N17

14-3-3's have been cited previously as interactors of huntingtin in interactome studies (Omi et al., 2008; Rong et al., 2007; Shirasaki et al., 2012). These proteins are involved in numerous cellular processes including nucleocytoplasmic shuttling, the regulation of enzymatic activity, and as a moderator of protein-protein interactions (Bridges & Moorhead, 2005). 14-3-3 zeta (14-3-3 $\zeta$ ) in particular has been shown to modulate the aggregation dynamics of amino terminal polyglutamine expanded huntingtin fragments (Omi et al., 2008). For these reasons 14-3-3s were chosen to start off the validation of the affinity chromatography results.

Study of 14-3-3zeta (14-3-3 $\zeta$ ) and its interaction with N17 was initiated by co-immunofluorescence experiments. A 14-3-3 $\zeta$  antibody and antibodies targeting wild type N17 (N17WT) and phosphorylated (at serine residues 13 and 16) N17 (N17PO<sub>4</sub>) of endogenous huntingtin were applied to *STHdh*<sup>Q7/Q7</sup> wild type cells. Mander's overlap (MOC) and Pearson's correlation (PCC) coefficients were used as means of quantifying co-localization; where values of 1 with both coefficients are considered perfect co-localization (Zinchuk & Grossenbacher-Zinchuk, 2009). As can be seen in **Figure 3**, there are high MOC and PCC values in the cytoplasm and high MOC value but low PCC values in the nucleus. This implies that there is a high level of co-localization between the proteins within the cytoplasm, but due to diffuse staining of both proteins within the cytoplasm these values may not be significant.

Next, co-IP analysis of wild type mouse striatal cells expressing N17-YFP (wild type N17 tagged with yellow fluorescent protein) showed that 14-3-3 $\zeta$  does not bind N17

and that the interaction is not promoted even when N17 is phosphorylated. As can be seen in **Figure 4** 14-3-3 $\zeta$  was unable to be pulled down with N17. This interaction could be promoted even by use of compounds that induced phosphorylation such as BMS-345541 (Atwal et al., 2011).

The final methodology with which this interaction has been studied was FLIM-FRET. The two constructs used were 14-3-3 $\zeta$  tagged with a red fluorescent protein (RFP) that acted as the acceptor molecule and N17-YFP that acted as the donor. From **Figure 5** it can be seen that % FRET efficiency between N17-YFP and 14-3-3 $\zeta$ -RFP was slightly higher than N17-YFP and RFP alone (negative control). This shift wasn't significant and therefore would imply that there was no robust FRET observed between 14-3-3 $\zeta$ -RFP and N17-YFP.

Therefore none of the assays showed any indication of an interaction 14-3-3 $\zeta$  and N17. Further analysis will be required to deduce if this potential interaction is in fact non-existent or if alternate conditions exist to promote it.

### **3.3 FLIM-FRET reveals vimentin and N17 interaction**

Vimentin is a type III intermediate filament and as such is a member of the cytoskeleton network of proteins (Fuchs & Weber, 1994). It was chosen for two reasons: firstly, because huntingtin has already been shown to interact with other cytoskeletal proteins such as actin and microtubules (Hoffner et al., 2002; Munsie et al., 2011) and secondly, because vimentin has been highlighted in the literature as a potential regulator of huntingtin aggregation (Bauer et al., 2012).

Co-immunofluorescence was conducted using a mCerulean tagged vimentin (mCer-Vim) along with immunostaining of N17 and N17PO<sub>4</sub>. MOC and PCC values were calculated for a set of images. **Figure 6** shows a high MOC within the nucleus but a negative PCC, while within the cytoplasm we see a low MOC and a low positive value for PCC. These trends were seen in both N17 and N17PO<sub>4</sub> alike. This information reveals that there is little or no co-localization between vimentin and N17.

Next co-immunoprecipitation assays were performed with lysate of wild type cells transfected with different YFP tagged constructs of N17, phospho-resistant N17 constructs S13,16A (serine 13,16 to alanine mutation) and N17 phospho mimetic S13,16D (serine 13 and 16 to aspartic acid). The negative control once again was YFP alone. All the constructs (including the negative control) pulled down vimentin (**Figure 7**). Therefore the co-IP proves inconclusive since the blot suggests that vimentin binds to the N17 fragments but, also to the fluorescent tag alone.

The final assay employed for studying the interaction between N17 and vimentin was FLIM-FRET, where mCer-Vimentin acted as the donor vector and N17-YFP acted as the acceptor. **Figure 8** shows that there is a shift in % FRET efficiency between the negative control and that this shift is significant ( $p < 0.002$ ). The FLIM-FRET results therefore suggest that an interaction between vimentin and N17 might exist. This interaction was unable to be detected by either co-IP or co-IF potentially because either only a subset of the vimentin population interacts with N17 or it is a transient interaction.

### 3.4 SET and N17PO<sub>4</sub> co-localization dependent upon cell cycle

SET is a nucleoprotein around 39kDa in size and is also otherwise known as I<sub>2</sub><sup>PP2A</sup> (inhibitor 2 of protein phosphatase 2A). It is a potent and specific inhibitor of protein phosphatase 2A (Li et al., 1996) which has previously been suggested to regulate N17 phosphorylation (Atwal et al., 2011). SET has also been noted in other neurodegenerative diseases and is involved in neuronal apoptosis as well as the hyperphosphorylation of tau within Alzheimer's disease (Arnaud et al., 2011; Maderira et al., 2005)

A co-IF was conducted using antibodies against SET and N17 or N17PO<sub>4</sub>. Initial results using MOC and PCC values proved inconclusive (data not shown). Upon further analysis, it appears that SET co-localizes with N17PO<sub>4</sub> in a cell-cycle dependent fashion. As shown in **Figure 9**, this co-localization is seen within the earlier stages of mitosis, during prophase. Here we see that both N17PO<sub>4</sub> and SET are localized to centrosomes. In addition, during anaphase it appears that SET localizes to the spindle midzone (Glotzer, 2009), at which point it no longer co-localizes with N17PO<sub>4</sub>.

The SET and N17 co-localization seen by co-IF was not verified by the co-IP or the FLIM-FRET assays. In the co-IP assay SET was not pulled down with N17 or any of the N17 constructs (**Figure 10**). In addition, FLIM-FRET showed no robust FRET between donor mTurquoise2 tagged to SET (mTurq2-SET) and N17-YFP as shown by lack of significant increase in FRET efficiency in **Figure 11**. The failure of these assays to show supporting evidence could be due to two factors: firstly because the mitotic cell population within a single dish is negligible compared to whole cell population and secondly because neither the phospho-mimics nor N17 alone are sufficient for binding.

New assays will be required to examine this observation further and will be discussed later on.

### **3.5 H<sub>2</sub>O<sub>2</sub>, AMP-PNP, A23187 and CdCl<sub>2</sub> compounds triggers N17 nuclear localization**

The final aim of my project was to study N17 cellular localization in the presence of various stress inducing agents. *STHdh*<sup>Q7/Q7</sup> cells stably expressing wild type N17 tagged to YFP, N17 mutants such as M8P-YFP (acting as a negative control), S13,16A-YFP (phospho resistant) and S13,16D (phospho mimic) were used and imaged. These constructs were used to see if physiological stresses that affect N17 could also affect mutants that were either structurally dysfunctional, unable to be phosphorylated or were phospho-mimics.

The treatments used include adenylyl 5'-(β,γ-imido) diphosphate (AMP-PNP), dimethyl sulfoxide (DMSO), tunicamycin, dithiothreitol (DTT), hydrogen peroxide, A23187 (a calcium ionophore), and cadmium chloride. AMP-PNP is a non-hydrolyzable analog of ATP used as an inhibitor of kinesin and fast axonal transport (Leopold et al., 1992). Tunicamycin and DTT have previously been shown to induce ER stress (Bicknell et al., 2010) and are relevant for the study of how N17 responds to ER stress and its subsequent localization. DMSO has also been used previously to induce cellular stress and alter N17 localization (Atwal et al., 2007). A23187 (a calcium ionophore), cadmium chloride (a toxic heavy metal) and hydrogen peroxide have been shown to be inducers of oxidative stress (Bagchi et al., 2000; Petersen et al., 2000) which are relevant since ER resident proteins (such as N17) are highly sensitive to oxidative stress (Van der Viles et

al., 2003). The output used to analyze stress was the percent (%) nuclear staining of the YFP tagged N17 constructs as it has been shown previously that N17 moves from the ER to the nucleus upon induction of stress (Atwal et al., 2007).

**Figure 12** highlights all the relevant findings sorted according to treatment and cell type. After an hour within the various treatments significant increases in nuclear localization can be seen in the N17-YFP stably expressing cell line with the addition of 400mM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), 1uM and 4uM A23187, all concentrations of AMP-PNP and cadmium chloride (CdCl<sub>2</sub>). Similar to N17-YFP, S13,16A-YFP expressing stable cell line also had a significant increase in nuclear localization with the addition of cadmium chloride treatment. No other significant changes were noted suggesting that cell lines expressing constructs other than wild type N17 were not as responsive to stress, or at least not within the time span of the hour allotted.



#### 4.0 DISCUSSION

N17 has been shown to be an important domain in the normal physiological function of huntingtin. It is involved in the nucleocytoplasmic shuttling of huntingtin within neurons. Furthermore, phosphorylation of N17 has shown to be a critical component in regulating the pathogenesis of mutant huntingtin within HD mouse and cell models (Atwal et al., 2011; Gu et al., 2009; Pardo et al., 2012). Due to the significance of such findings, there has been greater interest in N17 and how a small 17-amino acid domain can regulate the function of a protein 3144 amino acids in length.

This project aimed to gain a greater understanding of N17 and its role in modifying huntingtin function by searching for interactors which could identify novel pathways for huntingtin regulation. Furthermore, the study of physiological stresses that trigger N17 translocation to the nucleus was pursued due to newly acquired evidence indicating that N17 could be important for huntingtin and its role in cell stress response (Atwal et al., 2007; Xiao et al., 2013). Characterizing the triggers of N17 phosphorylation and its subsequent nuclear localization might reveal the basis of N17 hypophosphorylation within mutant huntingtin and therefore has relevance to disease pathology.

This work identified three potential interactors; 14-3-3 $\zeta$ , SET- $\beta$  and vimentin. Two of these interactors (14-3-3 $\zeta$  and vimentin) have been identified previously as regulators of huntingtin function, but it was unknown if that interaction involved N17. Furthermore use of a cell stress screening technique has identified a subset of compounds to which N17 displays a stress induced phenotype.

#### **4.1 Lack of conclusive evidence to support 14-3-3 $\zeta$ interaction with N17**

14-3-3s are small (~30kDa) cell signalling molecules that comprise around 1% of the total soluble protein mass in the brain (Berg et al., 2003). There are seven identified isoforms ( $\beta$ ,  $\gamma$ ,  $\epsilon$ ,  $\eta$ ,  $\zeta$ , and  $\tau/\theta$ ) in mammals that form hetero- and homodimeric structural units (Fu et al., 2000). 14-3-3 proteins have often been shown to be components of multi molecular signalling complexes which are responsible for intracellular signal transduction pathways (Bridges & Moorhead, 2005). Most often the interactions with their ligands have been dependent upon phosphorylation, but interactions that are independent of phosphorylation have also been identified (Yaffe & Smerdon, 2004). These proteins form a homodimeric or heterodimeric complex to facilitate binding, and often induce a conformational change in their ligand protein which propagates downstream cell signaling pathways (Bridges & Moorhead, 2005). Due to the reasons highlighted, 14-3-3's seemed a reasonable starting point for validation of the affinity chromatography results.

The culmination of all the techniques used to verify 14-3-3 $\zeta$  interaction with N17 have suggested that its interaction with N17 is either rare, transient (less than the 30s required for time-domain FLIM-FRET) (Lleres et al., 2007), requires the use of a cell signalling event or simply does not occur. The FLIM-FRET data did not result in FRET between both proteins, and co-IP revealed an inability of 14-3-3 $\zeta$  to be pulled down with N17. Although the co-IF did show high values of MOC and PCC in the cytoplasm it might be irrelevant since both proteins are abundant and show a diffuse staining within the cytoplasm, therefore high levels of overlap might just be a consequence of these facts.

It is also plausible, due to the large quantities of 14-3-3's within neurons (Berg et al., 2003), that 14-3-3's have been isolated as interactors of huntingtin because of its abundance within these cells. In addition, since 14-3-3's are known to form complexes in hetero or homodimer pairs, the use of more than a single isoform of 14-3-3 might be required to induce binding with N17 (Bridges & Moorhead, 2005). It is also possible that the interaction of 14-3-3 $\zeta$  requires specific structural conformations within huntingtin, similar to the binding of PACSIN1 with both PPR and N17 (Caron et al., 2013), and might therefore need longer fragments of huntingtin to support binding. Omi *et al.* has shown that 14-3-3 $\zeta$  binds to polyglutamine expanded huntingtin fragment 1-81 (Omi et al., 2008), and so we could ask if N17 is necessary to promote this binding.

Some of the drawbacks or disadvantages of the methods applied during this project can be addressed in future work by the use of alternative techniques. For example the time restriction found in FLIM-FRET can be addressed by use of frequency domain-FLIM. Use of this technique would not have the lag due to the collection time of photons (used to calculate lifetime) since detection of photons is conducted in analog mode, and therefore overcomes the temporal restrictions found in time domain-FLIM (Buranachai et al., 2008). Additionally, a multicolor bimolecular fluorescent complementation (bi-FC) method can be applied to study potential of multiple 14-3-3 isoforms forming a complex with N17 and also to visualize weak and transient interactions since complementation of the fluorophore in this assay permanently links the interactors (Kerppola, 2008). Briefly the method involves the use of tags consisting of fragments of fluorescent proteins that once complemented (by the interaction of their fused protein counterparts) results in

formation of a complete fluorophore. Fragmented fluorophores do not emit light until the fragments unite thus revealing the interaction between the fused proteins. This technique was tried earlier but could not to be optimized due to excess background and false positive fluorescent complementation, although there is hope that in time this methodology can become an invaluable tool if such background could be reduced or eliminated.

#### **4.2 A potential transient interaction between vimentin and N17**

Vimentin is a member of the intermediate filament family and is used as a marker of cells of mesenchymal origin, therefore the presence of vimentin in *STHdh*<sup>Q7/Q7</sup> cells was unexpected as they are neurons and not of the same origin (Ivaska et al., 2007). However, since the discovery of vimentin there has been increasing evidence of their presence in cells of neuronal lineage, within early differentiating neurons of rodents (Bignami et al., 1982) as well as mature neurons in patients with AD (Levin et al., 2009). In the latter paper, it was believed that the vimentin was found within the neurons to act as a damage-response mechanism (Levin et al., 2009), and it has also been seen to be vital for astrocyte response to neurotrauma (Pekny et al., 1999). Paradoxically, lack of vimentin within astrocytes has also shown to improve regenerative capabilities (Pekny & Lane, 2007). Thus the role of vimentin within the central nervous system is still not fully understood.

FLIM-FRET analysis of vimentin and N17 interaction suggests that a unique interaction between N17 and vimentin exists. This will have to be explored in greater depth as other methodologies were unable to see such an interaction, suggesting it might

be rare or transient and therefore unable to be visualized by either co-IF or co-IP. Co-IP could be re-attempted with the use of crosslinking compounds in an attempt to stabilize the interaction for further analysis. In addition, use of compounds to stabilize microtubules could prove useful. Previous work has shown that stabilizing microtubule networks using compounds such as taxol results in increased phosphorylation and stabilization of the vimentin intermediate filament network (Clarke & Allan, 2002; Vilalta et al., 1998). Since vimentin and intermediate filament networks are intrinsically dynamic taxol, and other similar treatments that stabilize these networks, could potentially result in an enhanced association between N17 and vimentin.

This interaction might be relevant to understanding huntingtin function in the cell stress response as vimentin fibers are associated with mechanotransduction signalling, mechanical stress (specifically shear stress) and are also as a component of aggresomes (Guo et al., 2013; Kopito, 2000). Similar to AD, the presence of vimentin and its interaction with huntingtin might be part of a mechanism involved in response to traumatic brain injury (Levin et al., 2009) or in reaction to the presence of intraneuronal aggregates. As such, it would be fascinating to study vimentin and N17 interaction in conditions of mechanical stress, possibly with the use of micro-ferromagnetic beads as has been described previously (Guo et al., 2013).

#### **4.3 SET and N17 co-localization is dependent upon phosphorylation and cell cycle**

The SET proteins were first described within a SET-CAN chromosome translocation event in acute myeloid leukemia (Adachi et al., 1994) and since then two splicing variants have been identified;  $\alpha$  and  $\beta$  with unknown differences in function

(Brennan et al., 2000). SET is widely expressed within most cell types and has been shown to localize primarily within the nucleus (Adachi et al., 1994). SET is a nucleoncoprotein but has also been cited as a regulator of transcription (Kim et al., 2012) and cell cycle. SET acts at the growth phase 2 to mitosis ( $G_2/M$ ) phase transition by regulating cyclin B-cyclin dependent kinase (CDK) 1 activity (Canela et al., 2003), and also at the growth phase 1 to synthesis phase ( $G_1/S$ ) transition through its interaction with cyclin dependent kinase inhibitor  $p21^{Cip1}$  and its substrate cyclinE-CDK2 (Estanyol et al., 1999). Furthermore, SET has a defined role in meiosis where it is essential for proper sister chromatid segregation possibly modulated by inhibition of protein phosphatase 2A and is also noted to localize to the centromeres (Chambon et al., 2013).

Both the co-IP and FLIM-FRET results imply that there is little or no interaction between N17 and SET. In opposition, the co-IF results suggest a potential interaction between endogenous huntingtin and SET could exist in a manner dependent upon both the cell cycle stage and the phosphorylation of N17. Staining of SET appeared to be associated with spindle fiber elongations and huntingtin has previously shown to localize to these locations as well (Godin et al., 2010). The caveat of co-localization analysis is that only a maximum of ~200 nm resolution can be achieved. Molecules are much smaller and therefore a high level of co-localization merely implies a sharing of volumetric space, but does not prove direct interaction. This short fall can be overcome by conducting co-localization analysis on images using super-resolution microscopy which can achieve resolutions as high as 20-30nm (Huang, Wang, Bates, & Zhuang, 2008). In addition, the co-IP can be further optimized to study this interaction by arresting

cells at G<sub>2</sub>/M phase transition and increasing the population of cells in early mitosis at which time there may be an interaction between SET and huntingtin. This can be then taken a step further with the aid of cross linking agents to isolate the complex at specific cell cycle check points, which upon isolation could be sent off for mass spectrometry analysis. The final challenge to overcome would be to find an accurate means of replicating phosphorylation within N17 as phospho-mimics are artificial. Hence, agents that promote phosphorylation might be of aid to help promote N17 and SET interaction.

Lack of significant FRET between mTurq2-SET and N17-YFP could be due to inability to replicate phosphorylation or an inability to identify transfected cells that were also progressing through mitosis. In an attempt to correct for the latter obstacle alternate assays to study FRET such as sensitized emission FRET and cell imaging time course experiments were attempted (data not shown). Unfortunately over-expression of SET over an extended time period is not an option as it appears to halt cell cycle progression (Canela et al., 2003) and appears to be toxic to the cells when co-expressed with N17.

It is tempting to postulate the meaning behind a potential interaction of SET and N17. We already know that the localization of S13,16 phosphorylated huntingtin changes during the stages of the cell cycle (Atwal et al., 2011), and studies using antibodies directed to other phosphorylated sites within huntingtin have also shown the same (Godin et al., 2010). Both these observations suggest a huntingtin function within the cell cycle. This role has not been characterized fully as it is considered a normal function of huntingtin (and disease relevance is yet to be found). It is possible that SET's interaction with N17 could be relevant to huntingtin and its function within mitosis. The ability of

SET to inhibit protein phosphatase 2A might be responsible for promoting phosphorylation of N17 during cell cycle check points. Only further work and characterization of this interaction will reveal the answers.

#### **4.4 Oxidative stress triggers N17 nuclear localization**

Aging is a major determinant for the diagnosis of neurodegenerative diseases (Finkel & Holbrook, 2000). Aging is also associated with an inability to combat cell stress and increased evidence of cellular stress events (Fagnoli et al., 1990). As a result, the impaired ability to tackle stress and increased incidence of stress is hypothesized to contribute to the development of adult-onset neurodegenerative diseases such as Alzheimer's and Parkinson's (Andersen, 2004; Welch & Gambetti, 1998). Within HD, understanding how stress contributes to aging could explain why individuals have an adult onset symptomatology.

Previously it has been noted that huntingtin is involved in cell stress responses, such as heat shock, cold shock and ER stress (Atwal et al., 2007; Munsie & Truant, 2012; Wytenbach et al., 2000). Furthermore, stress-inducing agents such as tunicamycin, dithiothreitol (DTT), adenosine triphosphate (ATP) depletion and cold shock can result in N17 translocation to the nucleus (Atwal et al., 2007). Using N17 nuclear translocation as an indicator of cell stress, smart screening was conducted to evaluate which physiological stresses elicit a more pronounced cell stress phenotype.

When looking closely at all the compounds that resulted in significant nuclear localization of N17 we find that activation of oxidative stress pathways are a common underlying theme. Cadmium chloride is a toxic heavy metal, and similar to other heavy



metal compounds (such as iron and copper), it has been shown to promote the production of reactive oxygen species and elevate the levels of lipid peroxidation (Bagchi et al., 2000). A23187 a calcium ionophore was used to evaluate the effect of calcium induced neurotoxicity. This compound disrupts the calcium ion gradient by transporting  $\text{Ca}^{2+}$  ions across the lipid bilayers of the cell, resulting in an influx of calcium from the extracellular environment and also out of the ER (Reed & Lardy, 1972). It also induces oxidative stress (Petersen et al, 2000). We see similar results with the use of hydrogen peroxide, a well-known inducer and marker of oxidative stress, where a mid-level dosage of peroxide resulted in a significant increase in nuclear N17.

The most surprising result was AMP-PNP, which has not been cited to have any involvement in oxidative stress pathways and might simply be due to cellular dysfunction. AMP-PNP is a non-hydrolysable analog of ATP and is often used as an inhibitor of kinesin (Fort et al., 2011) and therefore also used to inhibit fast axonal transport (Leopold, et al, 1992). Due to the ability of ATP to selectively bind to kinesin it is possible that anterograde transport within cells is disrupted leading to build up of vesicles, membrane proteins, and other components of fast axonal transport, similar to what is seen within kinesin motor mutants (Hurd & Saxton, 1996). Since this is the only compound that causes this particular disruption, it is less convincing as a potential instigator of cell stress and N17 translocation, therefore further work will be required to validate and understand this result.

Additional treatments such as use of DTT and tunicamycin are known to cause ER stress and activate unfolded protein response pathways (Bicknell et al., 2010). These

treatments have resulted in N17 nuclear localization in the past, but on a time scale that extends beyond the one hour time scale used here, which allows for sufficient buildup of unfolded proteins to elicit a response.

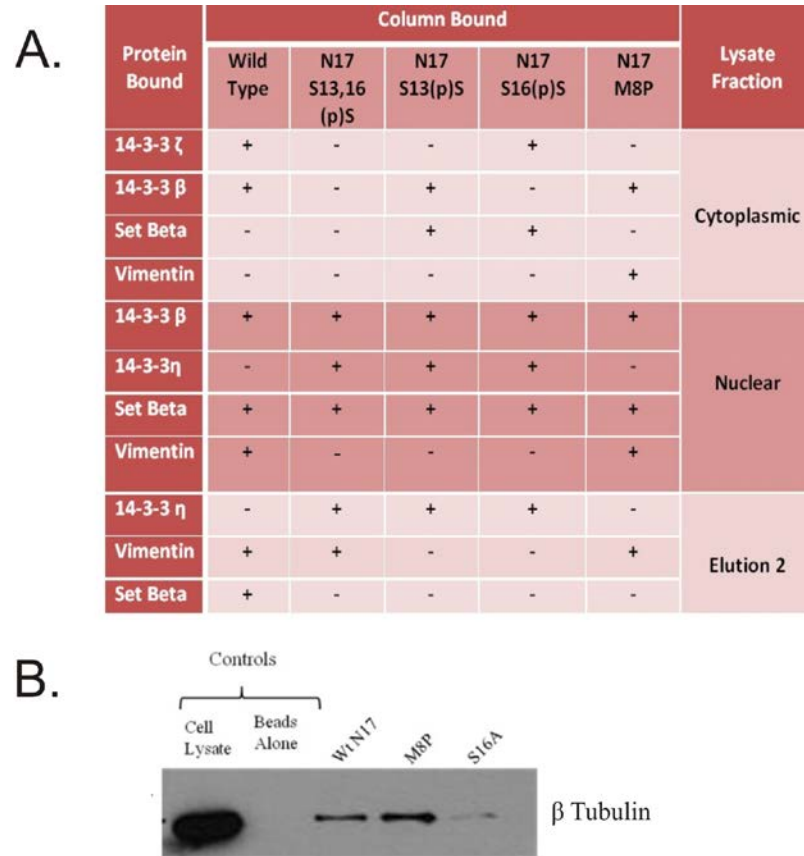
To expand upon and further cement this theory this screen should be repeated first using an oxidative marker to monitor the levels of oxidative stress, and secondly with the use of antioxidant compounds to study if the effects on N17 nuclear localization can be reduced or abated entirely. Similar work done by Peterson *et al* (Petersen et al., 2000) has shown that this approach could be relevant. Here they showed that A23187, added to rat embryonic striatal cells, induced cell toxicity (both through apoptosis and necrosis), and addition of antioxidant species reduced its cytotoxic effects (Petersen et al., 2000).

Oxidative stress as a cause of neuronal cell death has long been postulated within the field of neurodegeneration and HD. Examples of oxidative stress can be seen by the presence of increased reactive oxygen species within Alzheimer's (AD), Parkinson's (PD) and amyotrophic lateral sclerosis (ALS) patients as well as the reduced activity of antioxidant enzymes (Sayre et al., 2001). In fact, oxidative damage is already noted within HD patients and hypothesized to contribute to the etiology of HD (Browne et al., 1999; Melkani et al., 2013). Furthermore, we know that N17 is found on the surface of the ER, and ER proteins are extremely sensitive to oxidative cell stress (Van der Vlies et al., 2003). In conclusion, the results outlined with the cell stress assay show that N17 might be important in the regulation of huntingtin and its response to oxidative stress.

#### **4.5 Conclusions**

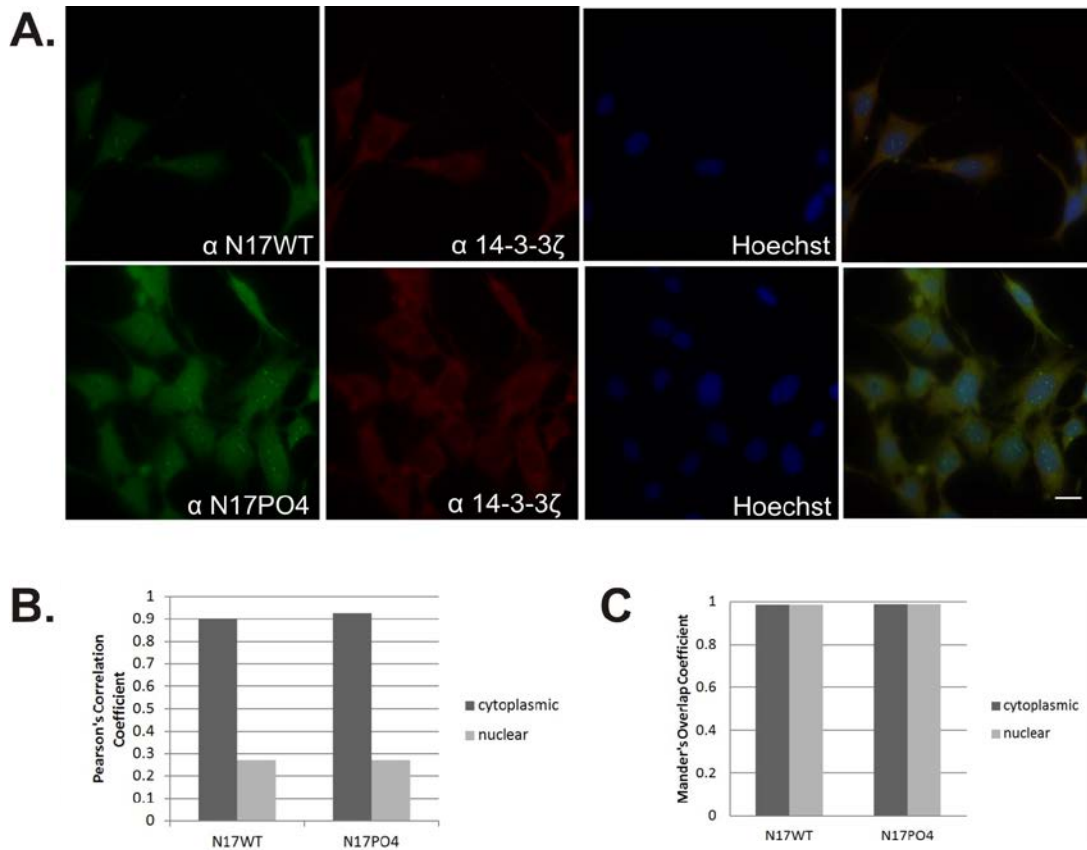
This project aimed to characterize the function of N17 in cell stress and study of its potential protein-protein interactions to gain a greater understanding of N17 and its role within the context of huntingtin and HD. Study of the N17 interactome using affinity chromatography has revealed two potential novel interactors of N17; vimentin and SET. Huntingtin is a multifunctional protein and these interactors exemplify this feature of the protein, as they could be applicable to two separate and distinct functions for huntingtin; within cell cycle regulation and architecture of intermediate filament networks. In addition, through the use of smart screening techniques it was shown that N17 nuclear localization (an indicator of cell stress) is largely dependent on the activation of oxidative stress pathways. Future work will now be required to expand on the preliminary investigations put forth here and to study if these interactions and functions have pathological consequences within HD.

## 5.0 FIGURES



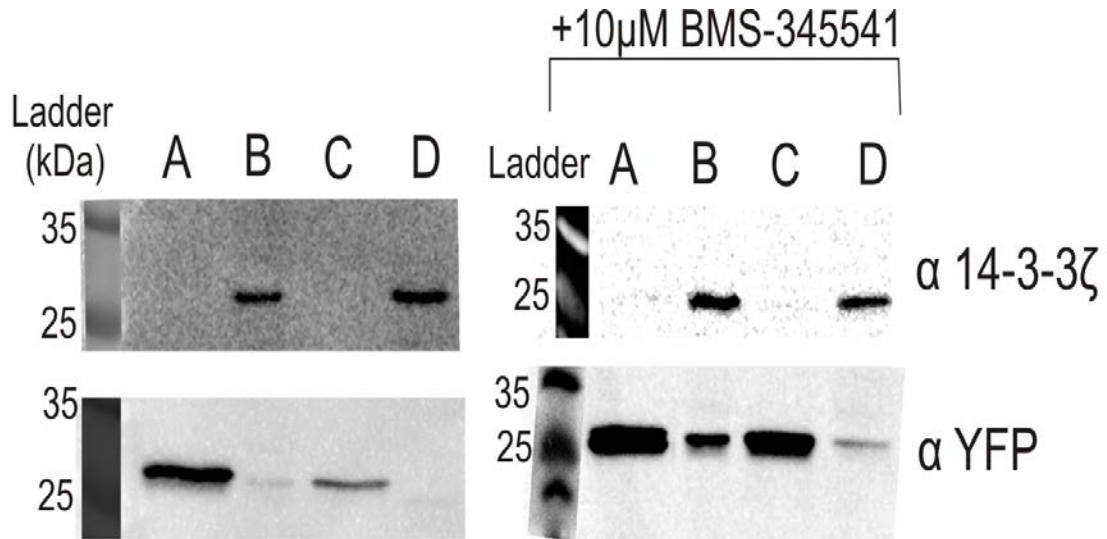
**Figure 2: Affinity chromatography list of potential N17 interactors.**

**A.** Mass spectrometry results from affinity chromatography columns. ‘+’ indicates the presence of the protein within the respective fractions (cytoplasmic elution, nuclear elution or elution 2). **B.** A western blot for beta-tubulin for the first elution off the affinity chromatography columns using wild type N17, methionine 8 to proline mutated N17 (M8P) and serine 16 to alanine mutated N17 (S16A) peptides was conducted. Notice in both S16A and the beads alone (acting as negative control) lanes that little or no band is visible when compared to the column rows wild type, M8P or S16A.



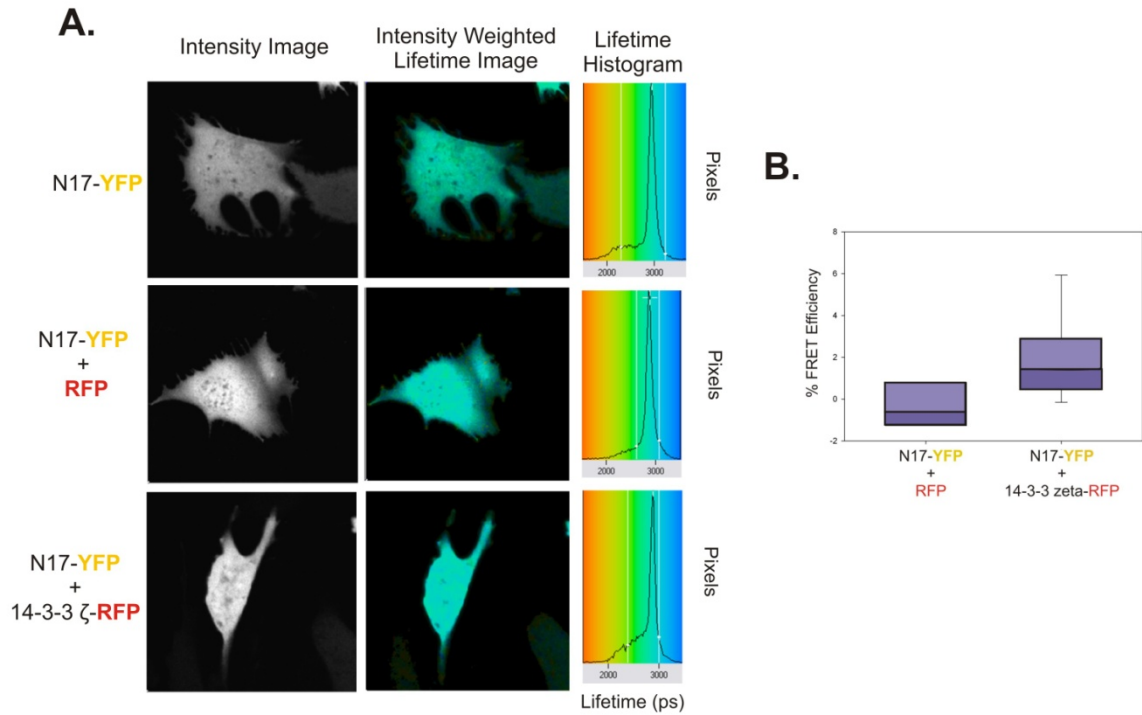
**Figure 3: Co-localization between 14-3-3 zeta and N17**

**A.** Co-immunofluorescence images in *STHdh*<sup>Q7/Q7</sup> of 14-3-3 zeta with amino acids 1-17 of endogenous huntingtin (N17WT) or with phosphorylated N17 (N17PO<sub>4</sub>). Cells were also stained with hoechst to identify the nucleus. Scale bar represent 10μm **B.** Pearson's correlation coefficient and **C.** Mander's overlap coefficient for a series of co-IF images (n=25) within the nuclear and cytoplasmic compartments of the cells are displayed.



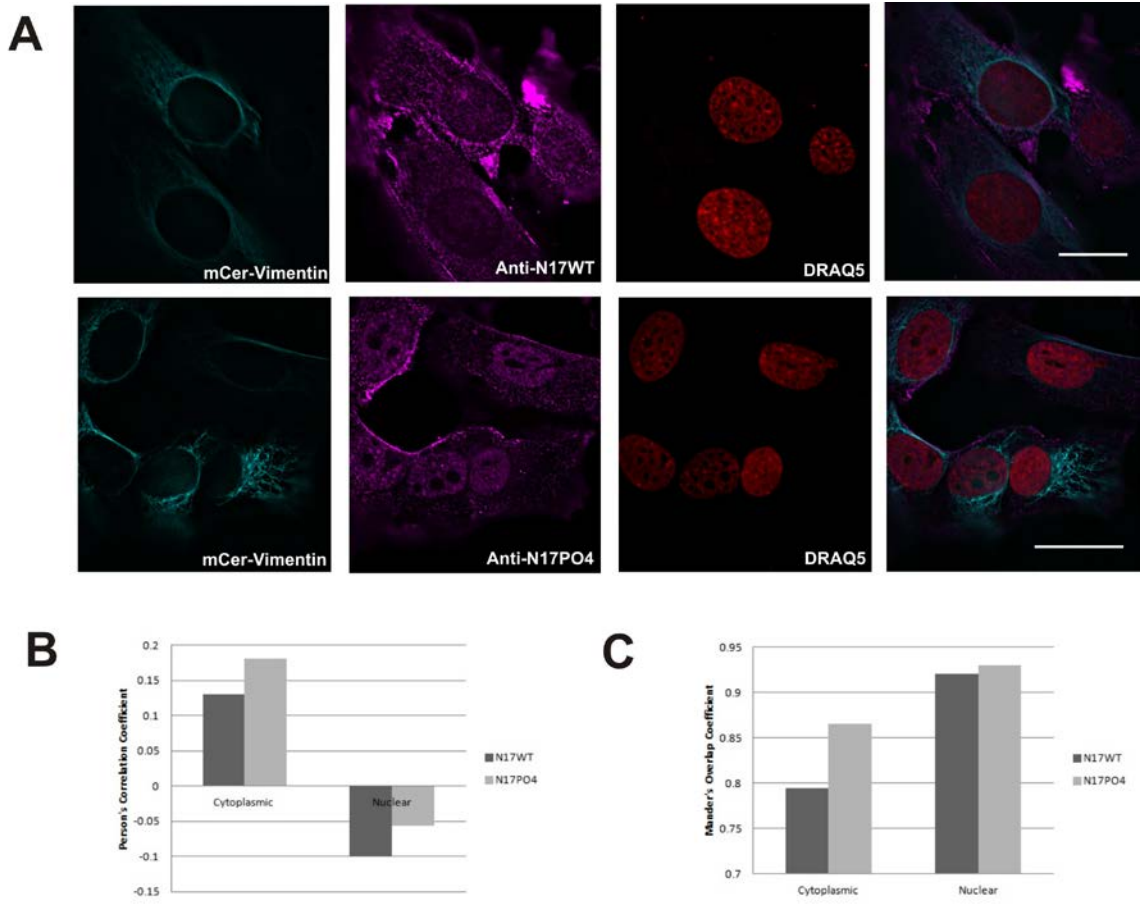
**Figure 4: Co-IP results reveal 14-3-3 zeta is not pulled down with N17.**

*STHdh<sup>Q7/Q7</sup>* cells transfected with N17-YFP were pulled down by YFP and then blotted for 14-3-3 zeta and YFP. Row A is the negative control and is lysate from cells transfected with YFP alone. Row B is the input, row C is the bound lysate and lane D is the unbound lysate. The blots on the right were lysate from cells that were treated with a compound (BMS-34554) which has previously been shown to increase levels of phospho N17 (R. S. Atwal et al., 2011). As can be seen 14-3-3 zeta was unable to be pulled down even with the presence of compounds that stimulate N17 phosphorylation.



**Figure 5: FLIM-FRET between 14-3-3zeta-RFP and N17-YFP**

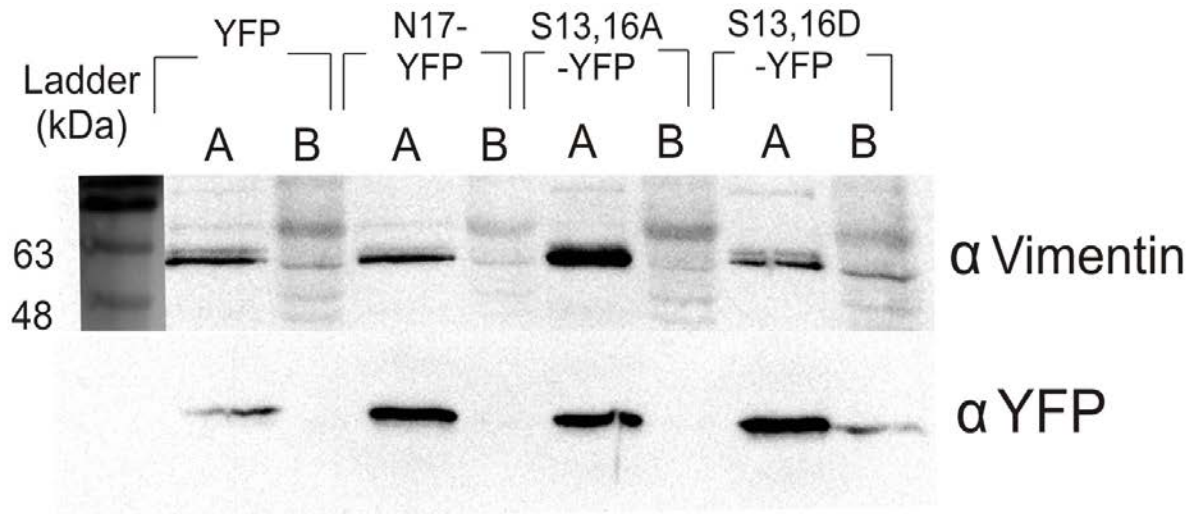
**A.** Cells were transfected with N17-YFP, N17-YFP and RFP (negative control) and 14-3-3 $\zeta$ -RFP and N17-YFP respectively. Their respective fluorescent lifetime histograms are presented to the right from 1500- 3500 picoseconds (ps). **B.** Box and whisker plot for percent (%) FRET efficiency where the boxes are 1 standard deviation from the mean and the whiskers are 2 standard deviations from the mean.



**Figure 6: No co-localization between vimentin and N17**

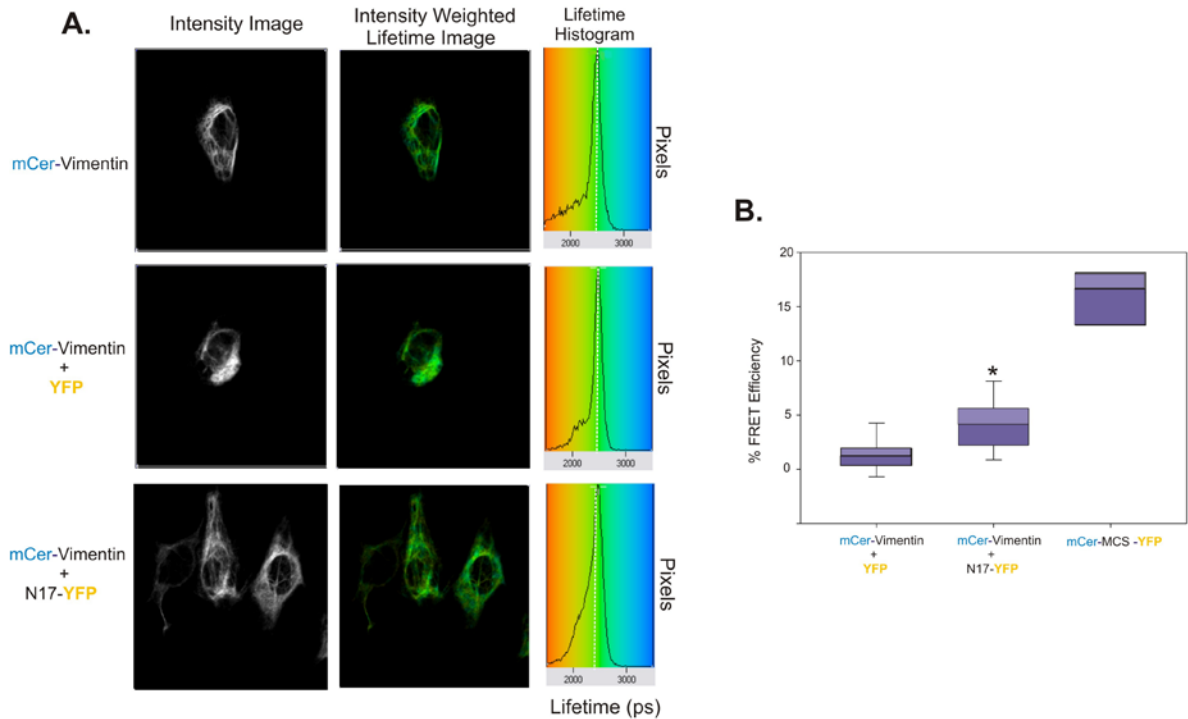
*STHdh*<sup>Q7Q7</sup> were transfected with mCer-vimentin and then fixed and permeabilized before addition of N17 (N17WT) and phosphorylated N17 (N17PO4) antibodies. Draq5 stain was used to identify the nucleus. Images were taken on a 60x oil objective. Scale bar represent 10 $\mu$ m. **B.** Mean Pearson's correlation and **C.** Mander's overlap coefficients for a series of images (n=25) suggest that there is no co-localization between mCer-vimentin and endogenous huntingtin, and this is independent of the phosphorylation state of N17.





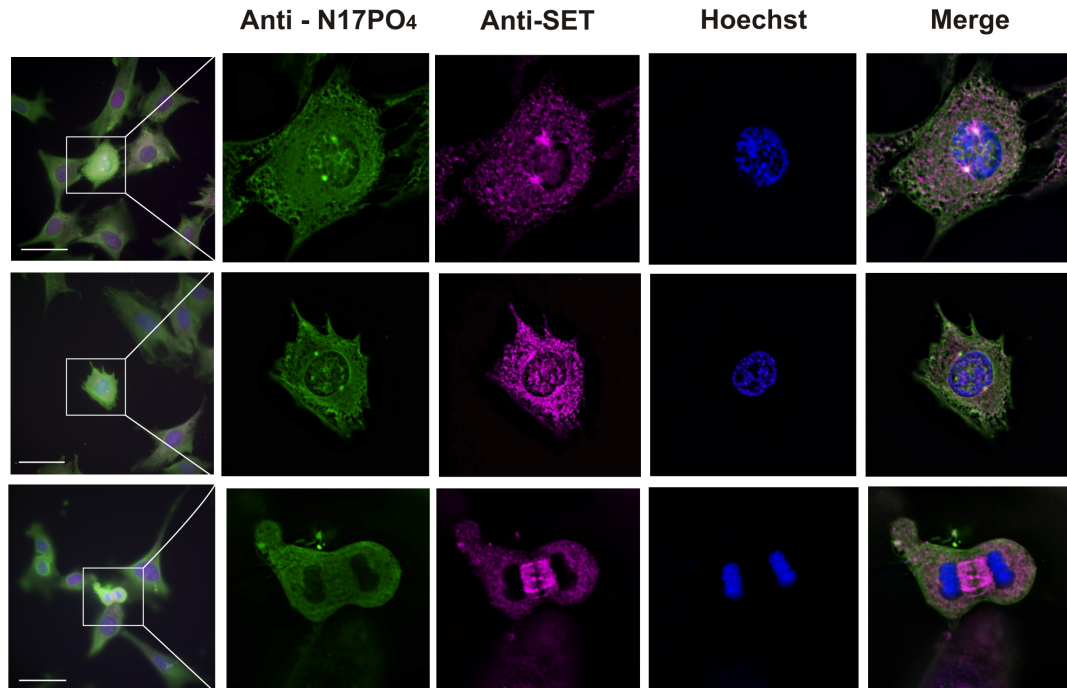
**Figure 7: N17-YFP and vimentin Co-IP assay results in non-specific pull down**

*STHdh*<sup>Q7/Q7</sup> cells transfected with various N17 constructs including wild type (N17), serine 13,16 to alanine (S13,16A) and serine 13,16 to aspartic acid (S13,16D) and YFP (the negative control). Bound elution was created by pulling down with YFP which was then run on a gel and blotted for vimentin and YFP as described in the methods section. Lane A represents the bound lane and lane B the unbound. As can be seen vimentin is pulled down by all lanes.



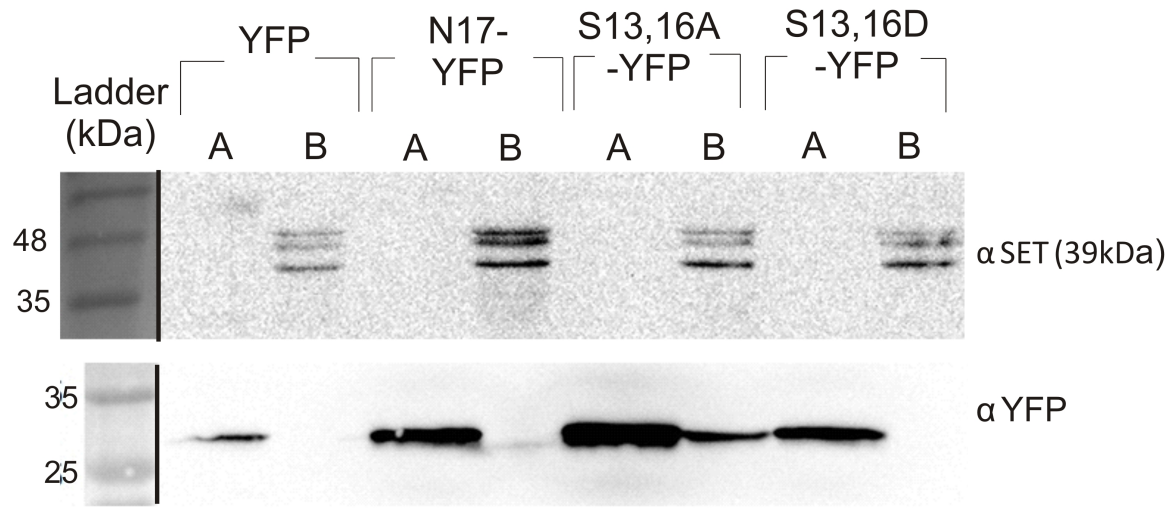
**Figure 8: Significant FRET is observed between mCer-Vimentin and N17-YFP**

**A.** Cells were transfected with mCer-vimentin, mCer-vimentin with YFP (negative control) and mCer-Vimentin with N17-YFP and mCer tagged to YFP (positive control mCer-MCS-YFP) respectively. Their representative fluorescent lifetime histograms are presented to the right from 1500-3500 picoseconds (ps). **B.** Box and whisker plot for percent FRET efficiency where the boxes are 1 standard deviation from the mean and the whiskers are 2 standard deviations from the mean. Asterisk (\*) represents significant ( $p < 0.002$ ) change in percent FRET efficiency between mCer-Vimentin with YFP and mCer-Vimentin with N17-YFP using student t-test ( $n=12$ ).



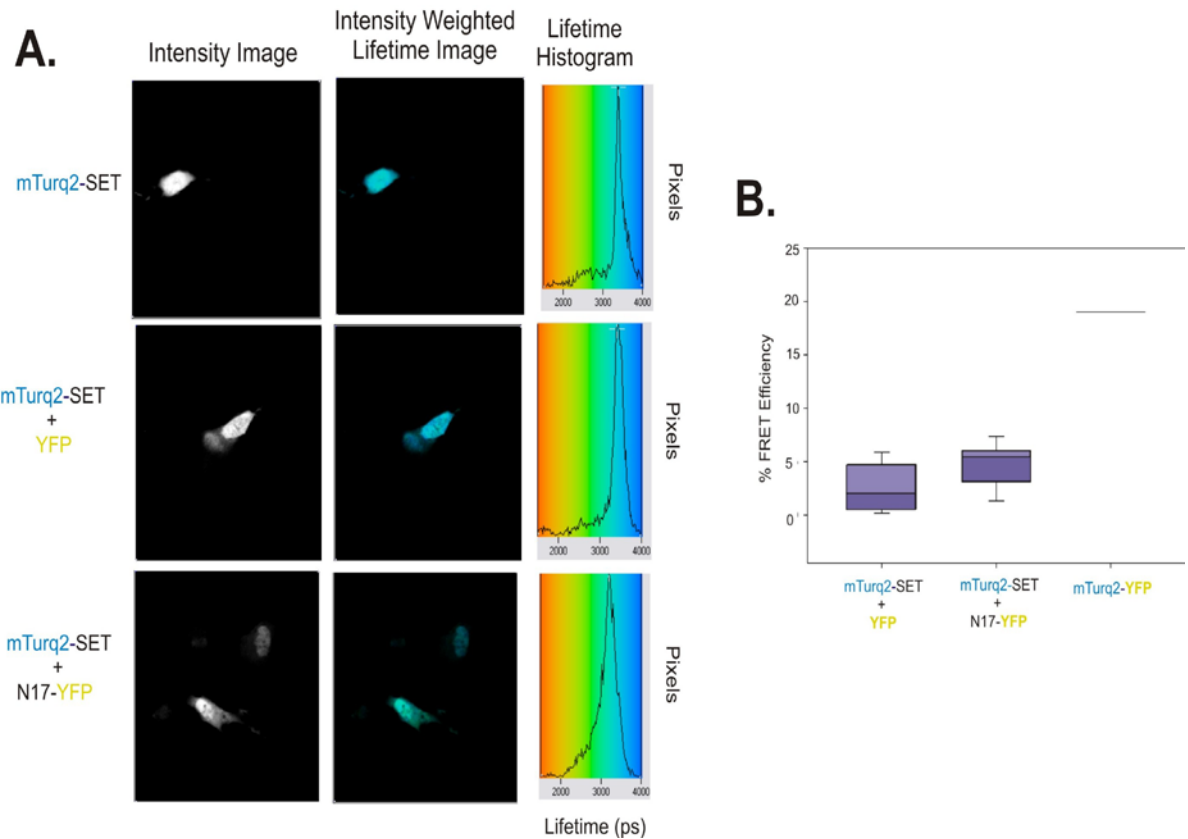
**Figure 9: Co-localization between SET and N17PO<sub>4</sub> is cell cycle dependent.**

Wild type cells were fixed and stained for endogenous SET and S13,16 phosphorylated N17 (N17PO<sub>4</sub>) conjugated to Alexa 488 to stain for endogenous phosphorylated huntingtin. Hoechst dye was used to stain the nucleus. Scale bars represent 20µm. Here it can be seen that both huntingtin and SET are both found within the centrosomes during prophase and no longer co-localize during anaphase where huntingtin appears disperse and SET localizes to spindle fibers in the spindle midzone. Images were taken at 60x magnification and were deconvolved.



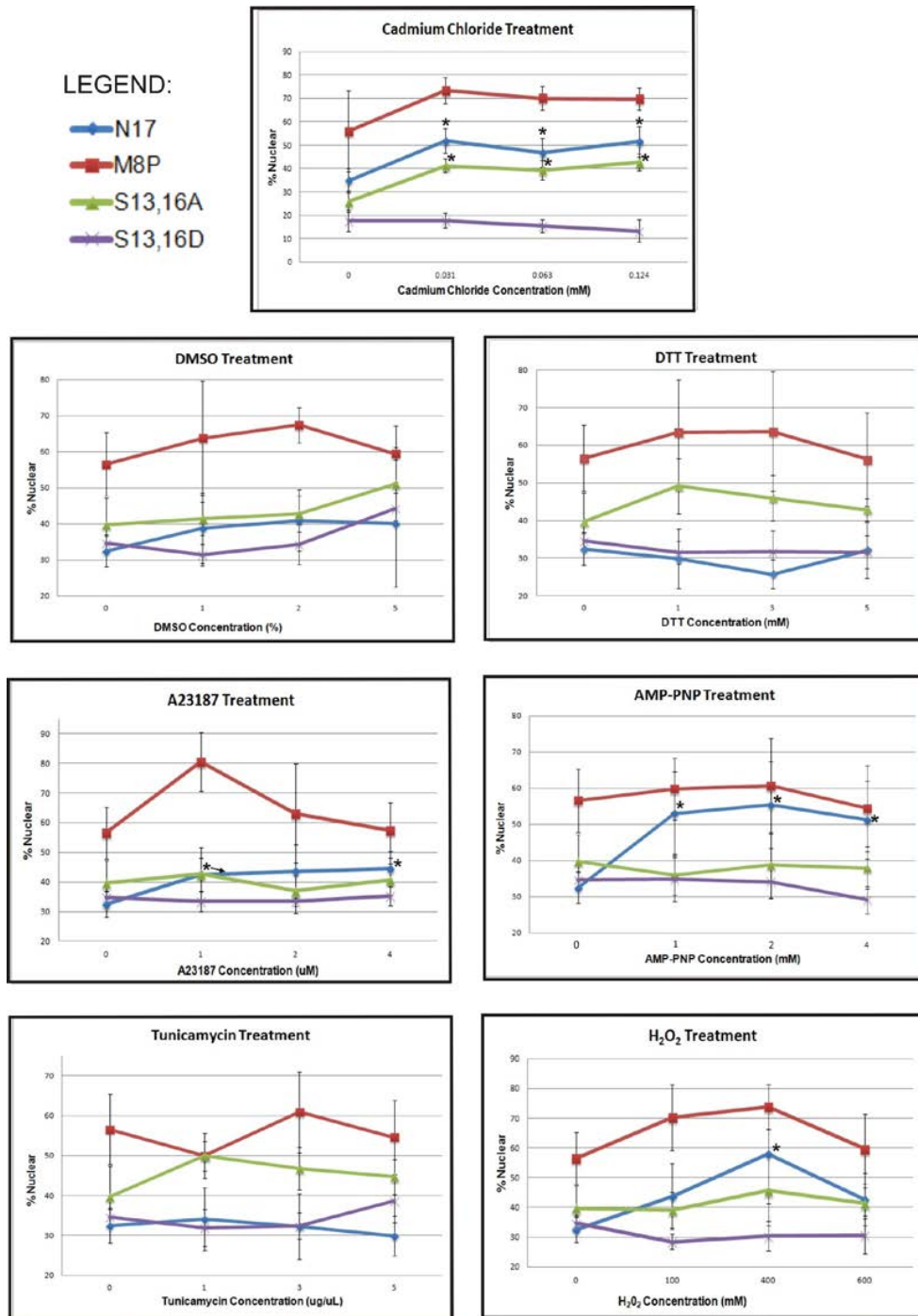
**Figure 10: Co-immunoprecipitation assay results of SET and N17**

*STHdh*<sup>Q7/Q7</sup> cells transfected with various N17 constructs including wild type (N17), serine 13,16 to alanine (S13,16A) and serine 13,16 to aspartic acid (S13,16D) and YFP (the negative control). Bound elution was created by pulling down with YFP and this was then run on a gel and blotted for SET and YFP as described in the methods section. Lane A represents the bound lane and lane B the unbound. SET is not found in any of the bound lanes above.



**Figure 11: FLIM-FRET between mTurq2-SET and N17-YFP**

**A.** Cells were transfected with mTurquoise2-SET (mTurq2-SET), mTurq2-SET and YFP (negative control), mTurq2-SET and N17-YFP and mTurq2 tagged to YFP (positive control mTurq2-MCS-YFP) respectively. Their respective fluorescent lifetime histograms are presented to the right from 1500- 4000 picoseconds (ps). **B.** Box and whisker plot for % FRET efficiency where the boxes are 1 standard deviation from the mean and the whiskers are 2 standard deviations from the mean. No significant increase in percent FRET efficiency was observed (n=9).



**Figure 12: Cell stress assay reveals N17-YFP’s sensitivity to select stress inducing compounds** Wild type *STHdh* cells stably expressing wild type N17 and N17 mutants

including methionine 8 to proline (M8P), methionine 8 to alanine (M8A), serine 13,16 to alanine (S13,16A) and serine 13, 16 to aspartic acid (S13,16D) tagged to YFP were plated on a 96 well dish. Cells were then incubated for an hour in treatment in the given concentrations. Percent nuclear (% nuclear) values generated by quantifying intensity of YFP staining within the nucleus were used as an indicator of cell stress. Significant results are represented by (\*) and are generated using a student t-test ( $p < 0.001$ ) by comparing treatments with control (a concentration of 0) for each cell type. AMP-PNP; adenylyl 5'-( $\beta,\gamma$ -imido)diphosphate, DMSO; Dimethyl sulfoxide, DTT; Dithiothreitol,  $H_2O_2$ ; hydrogen peroxide

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