PHOSPHORYLATION OF HUNTINGTIN N17 DOMAIN IS CELL CYCLE REGULATED AND BECOMES DYSREGULATED IN HUNTINGTON'S DISEASE

PHOSPHORYLATION OF HUNTINGTIN N17 DOMAIN IS CELL CYCLE REGULATED AND BECOMES DYSREGULATED IN HUNTINGTON'S DISEASE

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TITLE: Phosphorylation Of Huntingtin N17 Domain Is Cell Cycle Regulated and Becomes Dysregulated in Huntington's disease
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ABSTRACT

Huntington's disease (HD) is a genetic disorder caused by a polyglutamine tract expansion in the huntingtin protein. This unstable expansion causes degeneration of cortical and striatal neurons thus emphasizing studies of post-mitotic neurons. In addition to neurological symptoms, HD is also known to affect peripheral areas of the body which makes studying the developmental aspect necessary for a thorough understanding of the disease. Huntingtin is required for proper mitotic spindle formation and orientation. Our group has previously shown that phosphorylation at critical serine residues, 13 and 16 of huntingtin causes localization to various mitotic structures such as centrosomes, spindle microtubules, and the cleavage furrow suggesting that post-translational modifications of huntingtin play a role in the cell cycle. Here we use biophotonic and biochemical techniques to observe the role of phosphorylated huntingtin in the cell cycle. In mouse striatal cells, we observed phosphorylated huntingtin in different stages of mitosis and saw increased phosphorylation and differential localization of huntingtin as mitosis progressed. We also observed an increase in phosphorylation levels as the cell cycle progressed with immunoblotting assays. These findings can be used to identify huntingtin dependant phases of the cycle which may have relevance to the role of huntingtin during development. Furthermore, the role of huntingtin at the mitotic spindle may translate to other microtubule-related functions with relevance to DNA integrity checkpoints.

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DECLARATION OF ACADEMIC ACHIEVEMENT

Shreya Patel performed all the experiments and data analysis except for those stated below:

- Nicholas Caron performed all immunofluorescent experiments and created figures 4, 5, and 12.

ABBREVIATIONS

αMEM	Alpha minimal essential medium
µL	Microliter
AD	Alzheimer's disease
BAC	Bacterial Artificial Chromosome
BDNF	Brain derived neurotrophic factor
CC-3	Phospho mitotic protein
DMAT	2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HAP1	Huntingtin-associated protein 1
HD	Huntington's disease
Hdh	Huntingtin disease homolog
hTERT	Human telomerase reverse transcriptase
HTT	Huntingtin disease gene
IT15	Interesting Transcript 15
HEAT	Huntingtin, Elongation factor 3, subunit of protein phosphatase 2A (PP2A) and TOR1 – P13 kinase target of rapamycin
mRNA	Messenger ribonucleic acid
MRI	Magnetic resonance imaging
MSN	Medium spiny neurons
N17	The first 17 amino acids of huntingtin

N17S13PS16P	The first 17 amino acids of huntingtin with serine 13 and 16 phosphorylated
NP-40	Nonidet-P40
PBS	Phosphate buffered saline
Phospho-huntingtin	Phosphorylated huntingtin at amino acids 13 and 16
PI	Propidium iodide
PVDF	Polyvinylidene fluoride
REST	Repressor element 1 silencing transcription factor
S421p	Phosphorylated serine 421 on the huntingtin protein
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	shRNA – Short hairpin RNA
siRNA	Small interfering ribonucleic acid
SV40	Simian virus 40
ST*Hdh*$^{Q7/Q7}$	Wildtype immortalized striatal mouse cell line that produces huntingtin from both alleles that contains 7 consecutive glutamine residues in the polyglutamine tract
ST*Hdh*$^{Q7/Q111}$	Heterozygous immortalized striatal mouse cell line that produces 2 types of huntingtin. One allele produces wild-type huntingtin with 7consecutive glutamine residues in the polyglutamine tract. The other allele produces mutant huntingtin with 111 consecutive glutamine residues in the polyglutamine tract.
ST*Hdh*$^{Q111/Q111}$	Homozygous mutant immortalized striatal mouse cell line that produces mutant huntingtin from both alleles that contains 111 consecutive glutamine residues in the polyglutamine tract.
TBS	Tris buffered saline
TBST	Tris buffered saline with tween 20
WT	Wild-type

CHAPTER 1.0: GENERAL INTRODUCTION

1.1.1 Huntington's disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder with incidence as high as 1:5500 individuals (Evans *et al*., 2013). HD is characterized by loss of efferent medium spiny neurons (MSN) in the striatum, accompanied by the degeneration of cortical neurons in the cerebral cortex at a later stage in the disease (Zuccato *et al*., 2010; Gosh *et al*., 2013). HD was first fully described in America in 1872 by a third generation doctor, Dr. George Huntington, in a paper called *On Chorea*. HD is characterized by three primary clinical features: involuntary motor movement, cognitive decline, and personality changes. Originally named "Huntington's chorea" in reference to the dance-like movements that are the most noticeable symptoms of patients, HD actually presents earlier with subtle symptoms such personality changes and cognitive decline, making early-stage diagnosis difficult (Paulsen *et al*. 2001; Imarisio *et al*., 2000). In the later stages, patients experience progressive dementia and deterioration in overall health and quality of life. Simple tasks such as swallowing become challenging. Eventually, patients succumb to complications such as pneumonia, choking, and heart disease approximately 15-20 years after disease onset. Although disease-modifying treatments are underway for HD, there is currently no effective cure for it (Bates *et al*., 2014). A limited number of drugs are capable of providing some relief to the symptoms; however, these drugs do not provide long-term effects on the disease outcome.

1.1.2 Neuropathology

The pathology of HD is brain-specific, with cell loss and atrophy most prominent in the striatum and cerebral cortex. The striatum is comprised of the caudate nucleus and putamen, which are integral to the basal ganglia. The basal ganglia are situated in the base of the forebrain and play a central role in voluntary motor control, learning new tasks and processing emotions (Vonsattel *et al*. 1985, Arsalidou *et al*., 2013). The MSNs make up 95% of the striatal neuron population and are the most vulnerable to neurodegeneration in HD. In healthy brains, two pathways are involved in communication between the striatum and the motor cortex: the direct pathway (excitatory effect) and indirect pathway (inhibitory effect) (Crossman, 2000). With progressive loss of MSNs in the striatum, the indirect pathway becomes impaired in HD, leading to overstimulation of the motor cortex and resulting in involuntary movement (Kim *et al*., 2014; Bates *et al*., 2014). This insult to the basil ganglia is most likely responsible for the cognitive and psychiatric symptoms of HD. Vogela *et al*. provide evidence that dysfunction occurring in neurons prior to atrophy of critical brain structures in the later stages of HD is responsible for the early symptoms of HD. These observations are supported by evidence from studies in mouse models. Although there is no validated model that accurately replicates HD pathology entirely, there are models that exhibit some of the major hallmarks of HD. Knock-in mouse models genetically mimic the appropriate symptoms, and behavioural phenotypes (Levine *et al*., 2004). In addition, advanced imaging techniques such as magnetic resonance imaging (MRI) are

being employed in HD patients to detect early changes in the brain prior to the onset of symptoms (Tabrizi *et al*., 2012). These studies not only indicated cell loss in the obvious regions, such as the striatum and cortex, but also the white matter of the brain. Post mortem tissues from advanced HD patients exhibit reduced brain weight by as much as 25-30%. To assess the severity of the degeneration from HD, cases are grouped into five different severity levels (0-4). Grade 0 is the least severe in which post mortem brain tissue appears indistinguishable from a normal brain. Grade 1 is characterized by neuronal loss and astrogliosis of about 50% of the head, and occasionally tail, of the caudate nucleus. Cases with severe gross striatal atrophy are classified into Grades 2 and 3. Lastly, Grade 4 is the most severe form of HD with extreme striatal neuronal loss as high as 95% (Zuccato *et al*., 2012).

1.1.3 Genetics of Huntingtin's disease

HD is an autosomal dominant inherited disorder, which affects both sexes with equal incidence and severity, as far as has been determined at this point. This disease is caused by an expanded cytosine-adenine-guanine (CAG) triplet repeat in exon 1 of the *IT15* gene, now renamed the huntingtin gene (*HTT*), found on the short arm of chromosome 4 (The Huntington's Disease Collaborative Research Group 1993). At repeats 40 and above, the mutation is fully penetrant and invariably leads to disease onset. Individuals with 36-39 repeats have incomplete penetrance of HD, meaning they may or may not develop the disease at some point in their lives. Typically, individuals

unaffected by the disease possess 36 or less CAG repeats; however, recent evidence suggests late-onset of the disease in patients with 30-35 repeats (Gosh and Tabrizi, 2013). Multiple studies have shown an inverse correlation between the length of CAG repeats and the age of disease onset though there is enormous variability in onset, especially at shorter repeat lengths (Andrew *et al*., 1993; Duyao *et al*., 1993). In general, individuals with a large number of repeats develop symptoms at an earlier age and possess a higher risk of transmitting the disease to their offspring (Andrew *et al*., 1993). The repeat length only explains 40-50% of the variance in age of onset, additional factors such as the environment and paternal inheritance can also influence the age of onset. Paternal inheritance is the transmission of a longer CAG repeat and earlier symptom onset, to the next generation by way of the father. This is due to "genetic anticipation", as CAG repeats are not stable and the chances of expansion are more frequent during spermatogenesis (Tabrizi *et al*., 2012; White *et al*., 1997). Extremely large repeats are often associated with Juvenile HD where age of onset is before the age of 20 years old (Zuccato *et al*., 2012).

1.1.4 Peripheral pathology

In addition to neurological symptoms, areas outside of the brain are also affected in HD patients. These peripheral symptoms that not necessarily directly associated with changes in brain function include: activation of immune cells, severe weight loss,

deficiencies in the endocrine system, and skeletal-muscle wasting (Van der Burg *et al*., 2011; Sturrock and Leavitt, 2010; Tai *et al*., 2007) **(Figure 1)**.

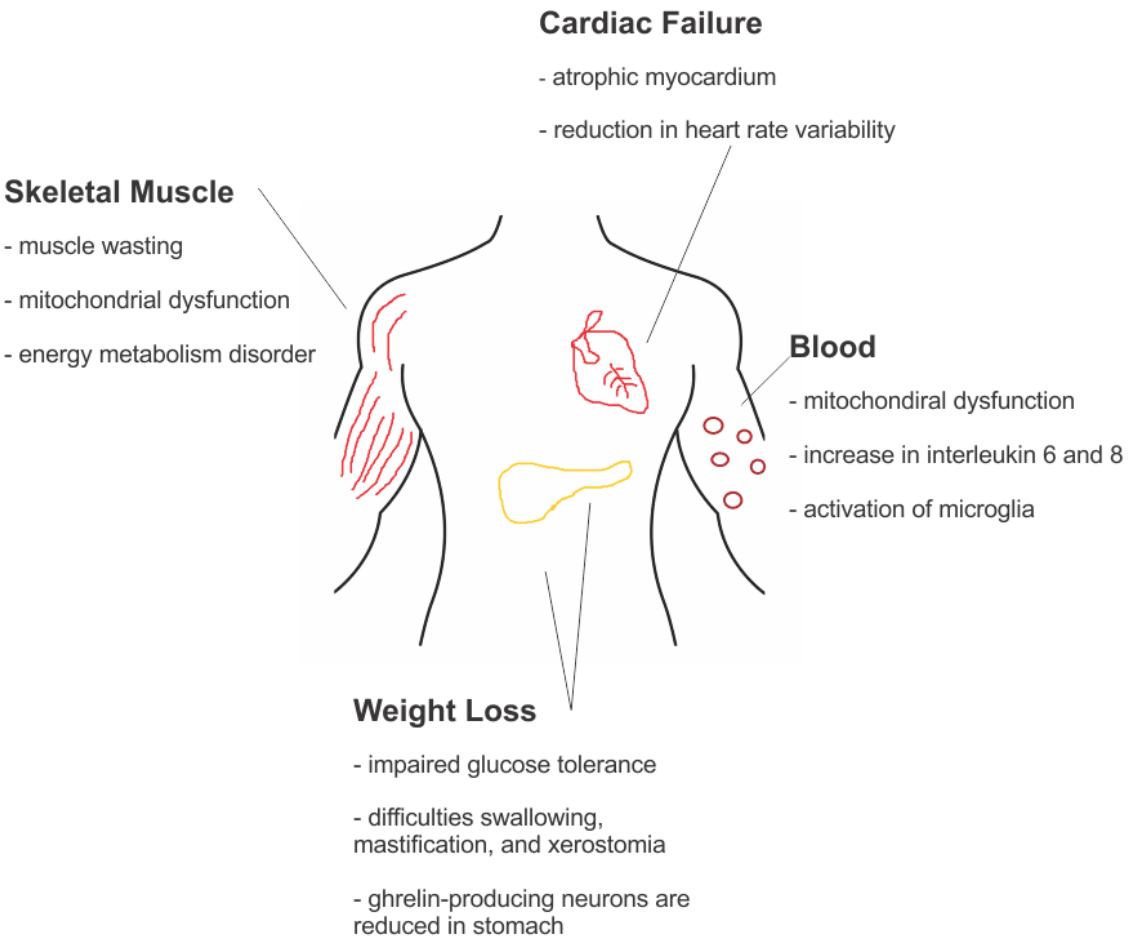

Figure 1 Overview of peripheral dysfunctions in patients with Huntington's disease.

Mitochondrial defects observed in the lymphocytes are indicative of the immune system being compromised in HD patients (Kwan *et al*., 2012). Blood samples from HD patients showed increased levels of inflammation markers such as chemokines and cytokines (Wild *et al*., 2008; Trabizi *et al*., 2012; Björkqvist *et al*., 2008). With the

replacement of peripheral immune system by carrying out a bone marrow transplant from a wild-type mouse into two lethally irradiated transgenic mouse models of HD (YAC128 and BACHD), a modest rescue of cytokine levels were observed (Kwan *et al*., 2012). This supports the hypothesis that mutant huntingtin affects various metabolic functions in the body and HD is not merely a disease of the brain. Altered gene expressions of haematocytes, caspase activity, and mitochondrial function have all been shown to be disrupted in blood samples from HD patients. These samples suggest a noticeable increase in interleukin 6 and 8 indicating immune activation (Tai *et al*., 2007; Almeida *et al*., 2008; Panoy, Lund, and Greenamry, 2005). *In vitro* post-mortem studies and *in vivo* imaging also revealed that microglia are activated in premanifest and manifest HD. Supporting this, a study by Sapp *et al*., showed more frequent activation of microglia with increasing grade of striatal pathology of the striatum and cortex (Sapp *et al*., 2001). The distribution of the microglia to regions of neurodegeneration in HD suggests that presence of these cells is related to the disease. Immune activation and altered inflammatory signaling may contribute to the many other peripheral phenotypes of HD, such as weight loss and muscle wasting (Björkqvist *et al*., 2008; Sangberg, 1981).

Weight loss is a progressive and common peripheral abnormality observed in patients with HD. There is limited knowledge about the mechanism underlying weight loss in HD patients. Various studies have reported that this phenotype is not secondary to hyperactivity or anorexia, but results from an increased metabolic rate (Farrer and Yu,

1985; Stoy and McKay, 2002, Farrer and Meaney, 1985). In fact, patients with a higher body mass index were found to have a slower progression of the disease (Sanberg *et al.*, 1981). This may suggest that weight loss may serve as a valuable target for therapeutic intervention. Furthermore, minor weight loss is observed in presymptomatic gene carriers and ends with extreme cachexia in the advanced stages of the disease (Stoy and McKay, 2002). The majority of studies regarding weight loss have been conducted in transgenic mouse models, and organs that are critical for weight regulations (Trejo *et al*., 2005). The pancreas has been shown to be affected in both patients and mouse models as they develop impaired glucose tolerance. In a study conducted by Lalic *et al*., a decrease in insulin sensitivity and impairment in glucose secretion capacity was observed in patients with HD compared to controls. Studies conducted in mouse models of HD have shown pancreatic islet cells to be atrophic leading to defects in insulin, and glucagon production. Comparably, the islet cells in HD patients were observed to be of normal size; however, transcriptional dysfunction in the islet cells may explain the tendency to develop glucose intolerance. Another contributing factor to weight loss is that patients with HD suffer from features such as defects in swallowing, inability to masticate (chew), and xerostomia (dry mouth) (Robbin, Ho and Barker, 2006; Mochel *et al*., 2007; Bjorkqvist *et al*., 2005; Myers *et al*., 1991). Studies conducted in the R6/2 transgenic mouse model suggest that the number of ghrelin-producing neurons is reduced in the stomach and thus effecting appetite. Along with the digestive system, adipocytes have also been hypothesized to be affected in HD. In white adipose tissue,

expression of fat-storage genes and hormones adipokine, leptin, and adiponectin are impaired in mouse models (Myers *et al*., 1991).

Another factor contributing to weight loss, which is unrelated to changes in cellular metabolism, is skeletal muscle wasting. Although the muscles in HD patients are hyperactive as a consequence of hyperkinesia (Lodi *et al*., 2000; Ribchester *et al*., 2004), the skeletal muscles suffer from substantial muscle wasting separate from this phenomena. A possible observation supporting this hypothesis is the presence of mutated huntingtin in myocytes (Strand *et al*., 2005; Arenas *et al*., 1998). Similar to the effects seen in the brain, it has been shown in R6/2 mouse models that the formation of inclusion bodies in myocytes disrupts gene expression (Ciammola *et al*., 2006; Turner, Cooper and Schapira, 2007). Huntingtin interacts with a number of transcription factors, such as peroxisome proliferator-activated receptor-γ coactivator (PGC-1α, a regulator of mitochondrial biogenesis and function), which is highly expressed in slow twitch skeletal muscles. A reduction in PGC-1α was observed in HD transgenic mice aludding to the dysfunctions observed in HD (Cui *et al*., 2006; Chaturvedi *et al*., 2009). Studies conducted in skeletal muscle cell cultures from patients showed evidence of apoptosis, abnormal cristae, and cytochrome c release (Turner *et al*., 2007). In addition to the dysfunctions observed in the cellular metabolism of myocytes, cardiomyocytes in particular are affected.

Cardiac failure is one of the leading causes of death in HD patients as approximately 30% patients succumb to it (Lanska *et al*., 1988). Cardiac failure may be associated with the effects of mutant huntingtin expression in cardiomyocytes. For instance, the myocardium has been observed to be atrophic and cardiac output is reduced by 50% in the R2/6 transgenic mouse model. Furthermore, expression of cardiomyocyte-specific genes in polyglutamine fragments with 83 CAG repeats in wild-type mice leads to autophagy and aggregate formation all of which induces heart failure (Bar *et al*., 2008; Mihm *et al*., 2007). These limited experimental data suggest that further studies of cardiac tissues of HD patients could serve to be helpful in understanding the physiological mechanism of this phenotype. Another possible mechanism underlying cardiac failure is that the autonomic nervous system (ANS) may contribute to this phenomenon. Studies conducted by Bar *et al*., revealed that cardiac autonomic nervous system dysfunction in HD affected both the sympathetic and parasympathetic branch of the ANS. The studies provided evidence of low modulation of autonomic cardiovagal activity in mid stage HD patients by showing a reduction in heart rate variability at rest and during deep sleep respiration (Mihm *et al*., 2007). These conditions are known to further influence the susceptibility of the patients to cardiovascular dysfunctions such as cardiac arrhythmias.

1.2 THE HUNTINGTIN PROTEIN

1.2.1 Huntingtin structure and domains

Huntingtin is a large, 348 kDA protein composed of 3144 amino acids that is expressed in all tissues of the body, with highest levels in the brain and testes. The most heavily studied region of huntingtin is exon 1, which contains the polyglutamine domain - the region of pathogenic expansion in HD. The polyglutamine tract is flanked on the amino-terminal end by a 17 amino acid, membrane targeting domain, termed N17, and on the carboxy-terminal end by a proline-rich region **(Figure 2)**. The huntingtin protein is required for human development and normal brain function. Huntingtin with a polyglutamine expansion greater than 36 in exon 1 is known as the mutant huntingtin protein (Davies *et al*., 2008; Zheng and Diamond, 2012).

Downstream of the polyglutamine region are protein scaffolding domains called **H**untingtin, **E**longation factor 3, protein phosphatase 2**A**, and m**T**OR1 (HEAT) repeats. There are a total of 88 HEAT repeats in the huntingtin protein that are approximately 40 amino acids in length each **(Figure 2)**. The repeat sequences give an overall super-helical structure to the protein, allowing it to serve as a mechanical scaffold for other proteins (Ross and Tabrizi, 2011). In addition, the structure allows the redistribution of mechanical stress along the molecule, permitting communication of interacting partners of the protein with distant parts within the protein (Grinthal, 2010). Members of the HEAT repeat family share a number of features. For instance, these proteins possess

homologous regions that form alpha-helical topology and occur in consecutive units within each protein. All members of the family are large in size and are often part of cytoplasmic transport complexes (Ross and Tabrizi, 2011).

The N17 region of huntingtin is a highly conserved sequence, suggesting its critical role in protein function. N17 forms an amphipathic alpha helical structure that targets huntingtin to the endoplasmic reticulum (ER) and late endosomes (Atwal *et al*., 2007). In the event of ER stress, or the mutation of a specific hydrophobic methionine to a proline (M8P), the alpha-helical structure of N17 is disrupted. As a result, when expressed in the context of mutant huntingtin, this mutation decreases the occurrence of aggregates and increases the toxicity of the protein (Atwal *et al*, 2011). Huntingtin has functions in the nucleus and cytoplasm, shuttling between the two compartments. These functions include critical cellular processes such as intracellular transport, regulation of transcription factors, inhibition of programmed cell death, and embryonic development (Zuccato *et al*., 2010; Zeitlin *et al*., 1995; Auerbach *et al*., 1995; Li *et al*., 2004). Our group has recently identified an active nuclear import signal recognized by karyopherin β2 and a nuclear export signal mediated by chromosomal region maintenance 1 (CRM-1) (Desmond *et al*., 2012; Maiuri *et al*., 2013). Expansion of the polyglutamine tract of huntingtin inhibits the ability of the protein to properly carry out the above mentioned process as well as affecting its ability to be exported from the nucleus (Saudou *et al*., 1998).

The N17 region of huntingtin is subject to various post-translational modifications such as phosphorylation, SUMOylation, acetylation, and ubiquitination (Zheng and Diamond 2012; Mitchison, 1972). In the N17 region, phosphorylation of two serine residues at positions 13 and 16 have been identified as crucial regulators of cellular localization, structure of N17, and the toxicity of mutant huntingtin (Atwal *et al*., 2011; Ehrnhoefer *et al*., 2011; Di Pardo *et al*., 2012) **(Figure 2)**.

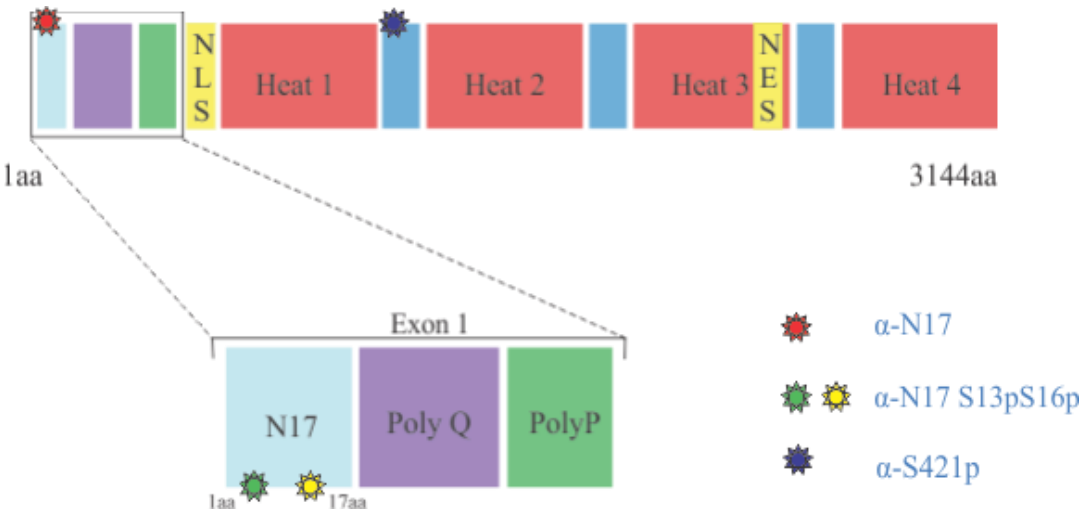

Figure 2 Schematic of the huntingtin protein. Full length huntingtin contains 4 clusters of HEAT repeats shown in red (not to scale). Huntingtin is a large 350 kDa protein consisting of 3144 amino acids. The amino terminus of the protein consists of, the N17 region shown in light blue (containing the first 17 amino acids), the polyglutamine (PolyQ) tract (which is expanded in HD), and a PolyProline Rich region (PolyP). Major sites of phosphorylation are in the exon 1 of huntingtin at serines 13 and 16. Serine 421 is also a critical phosphorylation site that is depicted outside of exon 1. Antibodies and the epitopes they recognize are represented by stars. The carboxyl-terminus contains a leucine-rich nuclear export signal (NES) and a nuclear import signal in the N17 region.

The Yang group has shown that when serines 13 and 16 were mutated to phospho-resistant alanines (S13AS16A) in the context of full-length, polyglutamine expanded huntingtin, mice expressing these mutations in trans via bacterial artificial chromosome

(BACHD mice) exhibited HD-like symptoms. However, a phospho-mimicking mutation to aspartic acids (S13DS16D) resulted in protection against the toxic effects of the mutant protein. Phosphorylation of these residues is also critical during cellular division (Godin *et al*., 2010; Roffler-Tarlov *et al*., 1996). In mitotic cells, phosphorylated huntingtin is observed at the mitotic centrosome, and microtubule spindles (Atwal *et al*., 2011). Furthermore, mutant huntingtin is known to be hypo-phosphorylated in comparison to wild-type huntingtin (DiPardo *et al*., 2012). High content screening using a kinase inhibitor library found that certain compounds could be used to modulate N17 phosphorylation, and that these compounds had consequences in terms of protein toxicity (Atwal *et al*., 2011). The results from this study revealed DMAT, a casein kinase 2 inhibitor, reduced huntingtin phosphorylation, while BMS-345541, an IKKβ inhibitor, increased phosphorylation, implicating the role of these kinases in huntingtin function.

1.2.2 Huntingtin function

Huntington's disease is a monogenic disease, but is complicated by the challenge of understanding the normal function of the protein. Huntingtin is a multi-functional protein with roles during development that differ from its roles in adult organisms. The involvement of huntingtin in development, cell division, vesicular and axonal trafficking, stress response, and transcriptional regulation has been shown to be crucial for cell health (Zuccato *et al*., 2010; Ghosh *et al*., 2013; Warby *et al*., 2013; Andrew *et al*., 2007). These critical functions are disrupted by expansion of the polyglutamine tract.

1.2.3 Huntingtin is vital for development

Huntingtin is known to be a protein that is essential for development. Studies in huntingtin knock-out mice have shown that the absence of huntingtin leads to embryonic lethality and defects in all three germ layers (Lumsden *et al*., 2007). Two independent labs demonstrated that the inactivation or knockout of the mouse huntingtin homolog (*Hdh*), causes embryonic lethality of mice at age 8.5 of gastrulation (Nasir *et al*., 1995; Zeitlin *et al*., 1995). Interestingly, polyglutamine expanded huntingtin does not seem to affect huntingtin function during the developmental stage (White *et al*., 1997). Mice homozygously expressing of huntingtin with an expanded polyglutamine tract at normal levels developed normally, but those expressing mutated huntingtin that was reduced to 60% of normal levels developed an abnormal nervous system and died shortly after birth. This is consistent with the idea that normal levels of huntingtin are required from at least one allele for normal development (Dragatsis, Efstratiadis, and Zeitlin 1998; Reiner *et al*. 2001). Also in support of this are studies in HD patients homozygous for the mutant allele, who showed no developmental abnormalities (Wexler *et al*., 1987). It is also important to mention that the expression levels of huntingtin (normal or polyglutamine expanded) are critical for normal development in murine models. An unexpected mutation made while generating a transgenic mouse resulted in a decrease in huntingtin levels to about two thirds than normal. These mice exhibited defects that included reduced size at birth and enlargement of the ventricle. This led to the conclusion that huntingtin levels below fifty

percent resulted in central nervous system (CNS) defects in mice (Auerbach *et al*., 2001). Therefore, these studies support the hypothesis that huntingtin is critical and required for proper neurological development.

1.2.4 The eukaryotic cell cycle

The cell cycle is a highly regulated process, the primary function of which is to precisely duplicate and segregate one parent cell into two genetically identical daughter cells. The typical eukaryotic cell cycle is divided into two major stages - mitosis and interphase, which together take the course of approximately 24 hours. Mitosis (nuclear division) lasts up to one hour and involves the segregation of daughter chromosomes followed by cytokinesis (cellular division). A greater amount of time is spent in interphase, where chromosomes are de-condensed, cell growth occurs, and DNA replication occurs in preparation for cellular division (Zheng and Diamond, 2012; Zhu, 1999). The cell continuously grows in interphase, which consists of three phases: DNA replication in the S phase, G_1 (gap between M phase and S phase), and G_2 (the gap between S phase and M phase) as represented in **Figure 3**.

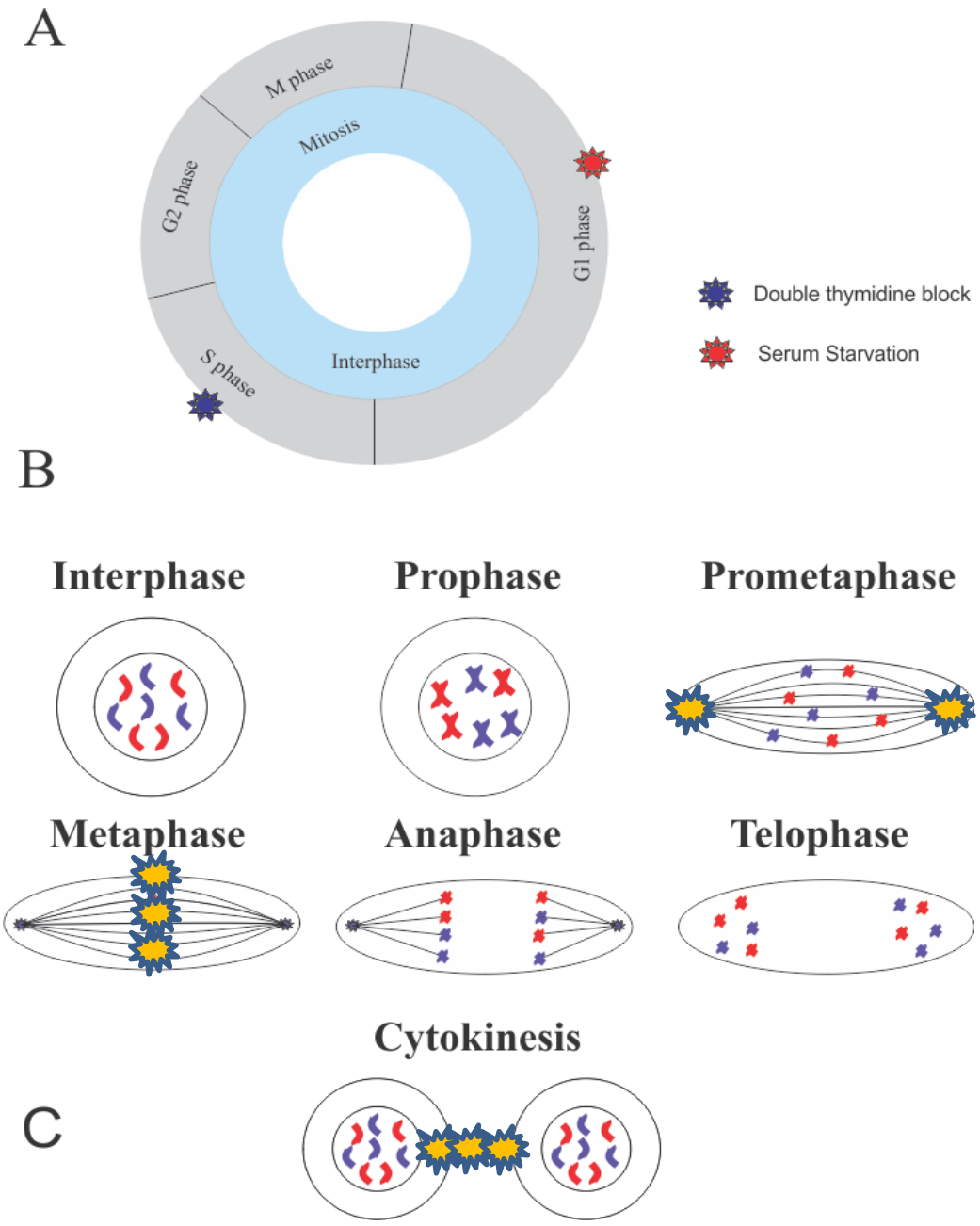

Figure 3 Huntingtin and the eukaryotic cell cycle. The huntingtin protein is represented by yellow stars at stages in the cell cycle where it has known to be involved. **(A)** The duration of the cell cycle is approximately 24 hours. Cells pass through a series of stages: gap phases (G_1 and G_2); an S (for synthesis) phase, where genetic material is duplicated; and an M phase, in which mitosis separates the genetic material and produces two identical daughter cells. Double thymidine block arrests the cells in early S phase and serum starvation synchronizes the cells in G_0/G_1 phase. **(B)** The 5 stages of mitosis are: prophase, prometaphase, metaphase, anaphase, and telophase. Cytokinesis is the final stage of the cell cycle where the cytoplasm divides forming two identical daughter cells from the parent cell.

The activation of cyclin/cyclin dependant kinase (cdk) complexes is the main driving force that determines the transition between one phase and another in the cell cycle. Post mitotic cells typically enter, and stay, in the G_0 resting phase; however, upon the activation of cyclin D/cdk4, 6 complex, re-entry from G_o to G_1 phase is initiated. If there are insufficient growth factors to proceed to the$_1$ next stage, cyclin D expression ceases instantly and cell division will halt in early G phase. The transition from G_1 to early S phase is dependent upon the activation of cyclin E/cdk2 complex. Under insufficient environmental conditions, the presence of cyclin A is crucial in determining if a cell can enter quiescence or result in cell death in its absence (Herrup and Yang, 2007).

Mitosis is divided into five stages: prophase, prometaphase, metaphase, anaphase and telophase. In prophase, spindle fibres appear and chromosomes decondense and later attach to the formed spindles in prometaphase. The chromosomes next become aligned at the mid-plate in metaphase. This is followed by anaphase, where the chromosomes move to the opposite poles of the mitotic spindle. The final stage of mitosis, telophase, is a reversal of the events that occur in prophase. The spindles disappear, nuclear membrane reforms, and chromosomes are grouped at either end of the poles. Cytokinesis is the final stage of cellular division where constriction of the cytoplasm occurs and the parent cell divides into two genetically identical daughter cells **(Figure 3A).**

1.2.5 Importance of huntingtin in cellular division, association with microtubules and mitotic structures

A recent study showed that huntingtin is required for the orientation of the mitotic spindle during cellular division. The study showed that knocking-out huntingtin expression in embryonic day 14.5 mouse brains, the number of cells dividing vertically decreased. In contrast, the number of cells dividing at intermediate angles or horizontally increased (Godin *et al*., 2010). Proper cell orientation of cell division is particularly important during neurogenesis, progenitor cells divide along the apical surface of the ventricular zone. Cells that divide horizontally or in an intermediate position are likely to differentiate into neurons. However, cells that divide vertically proliferate and continue to divide (Farkas and Huttner 2008). This further supports the hypothesis huntingtin is required for proper neurogenesis and brain development.

Huntingtin is involved in microtubule dynamics, and is found associated with microtubule structures and microtubule associated proteins (Hoffner *et al*., 2002; Tukamoto *et al*., 1997). Identification of huntingtin interactors by mass spectrometry analysis and affinity purification assays in wild-type HD mouse models revealed multiple tubulin isoforms (Shirasaki *et al*., 2012). Furthermore, aggregate formation in the presence of mutant huntingtin is dependent on an intact microtubule network and can be disrupted by the addition of microtubule-destabilizing compounds (Muchowski *et al*., 2002). The interaction of huntingtin with microtubules may be attributed to vesicular trafficking as the protein has been shown to bind directly to various motor proteins such

as dynein (for retrograde movement), dynactin, and kinesin (for anterograde movement) (Caviston *et al*., 2007; Morfini *et al*., 2009). Post translational modifications of huntingtin, such as phosphorylated serine 421, affect the association of this protein with microtubule associated proteins and the directionality of axonal transport (Colin *et al*., 2008). Short interfering RNA (siRNA) mediated knockdown of huntingtin expression in mouse neuronal cells resulted in incorrect spindle orientation. Incorrect spindle orientation may lead to dysfunctions in development, as spindle orientation has been shown to be essential for determining cell fate during differentiation (Godin *et al*., 2010). The localization of huntingtin to specialized mitotic structures and its interaction with microtubules, suggests huntingtin may play a functional role in cell division which may be disrupted in the context of HD, and contribute to dysfunction.

1.2.6 Huntingtin and neurodegeneration

Classically, neurons are thought to lack the ability to divide and are terminally differentiated. However, some reports have suggested that isolated neurons *in vitro* have the ability to re-enter the cell cycle without dividing (Maricich, Soha, Trenkner, Herrup, 1997; Migheli *et al*., 1999). It is also hypothesized that post-mitotic neurons re-entering the cell cycle are susceptible to apoptosis. A study by Migheli *et al*. provides evidence, using mouse models, that once terminally differentiated cells receive a cell division signal, there is a switch to an alternative effector pathway that leads to cell death. Consistent with this study, a number of neurodegenerative diseases have been

linked to dysregulated re-entry into mitosis leading to an altered cell cycle. For instance, studies in the field of Alzheimer's disease (AD) have shown cytoskeleton alternations, hyper phosphorylation of tau, and re-emergence of select cyclins in post-mitotic neurons, which are all features of cycling cells (Maricich, Soha, Trenkner, Herrup, 1997). Using immunohistochemistry, Nagi *et al*., showed that the expression of cyclin B in AD patients results in the bypass of the G_1/S checkpoint and cells become arrested in G_2. As a result, neurons arrested in G_2 phase are not capable of dedifferentiation, and eventually die via an apoptotic pathway (Nagy, Esiri, Cato, and Smith, 1997). Furthermore, a recent study showed using carbon-14 dating evidence of neurogenesis and the integration of new striatal neurons in adult humans (Ernest *et al*., 2014). The idea of post-mitotic neurons re-entering the cell cycle has led researchers to investigate the roles of the huntingtin protein in mitotic structures.

The huntingtin protein is believed to be tightly regulated during the eukaryotic cell cycle. Huntingtin is not restricted to differentiated neurons and is found in dividing cells where it is shown to be associated with the centrosomes, and microtubules (Atwal *et al*., 2011; Godin *et al*. 2010; Auerbach *et al*., 2001). For correct segregation of chromosomes, the orientation and position of the mitotic spindle is critical. The dynein/dynactin complex is a central player in spindle pole organization and huntingtin has already been shown to facilitate the dynein/dynactin-mediated transport of organelles along microtubules in neuronal cells (Caviston *et al*., 2010; Pagano *et al*.,

2004). As previously discussed, the N17 region is critical to the function of huntingtin, and of particular importance is the phosphorylation state of serine residues 13 and 16. A recent paper by Atwal *et al*., showed, using an antibody to phospho-N17 huntingtin (anti-N17S13pS16p), that endogenous full-length huntingtin localized to mitotic structures including: condensed chromatin, centrosomes and mitotic spindle, and the cell cleavage furrow **(Figure 3B)**. In HD mouse-derived striatal cells, polyglutamine-expanded huntingtin is hypo-phosphorylated at serines 13 and 16 (Atwal *et al*., 2011). Treatment with the ganglioside GM1 was able to restore phosphorylation in these neurons and restored normal motor function in a YAC mouse model of HD (Di Pardo, *et al*., 2012). Similarly, residue S421 is hypo-phosphorylated in mutant cells resulting in dysregulated transport of secretory vesicles, contributing to neurodegeneration. Furthermore, huntingtin plays a crucial role in axonal transport as it is required for the trafficking of brain derived neurotrophic factor (BDNF) containing vessicles. The trafficking of these secretory vesicles has been shown to be involved in striatal cell survival. Any dysfunction in this transport can lead to neurodegeneration (Colin *et al*., 2008; Zuccato *et al*., 2001).

PROJECT RATIONALE:

Post-translational modifications of huntingtin are linked, and critical, to the cytoskeleton and mitotic machinery. Evidence from the literature and observations from our own group led us to examine post-translational modifications, particularly phosphorylation of huntingtin in the context of cell division. Our work has shown that huntingtin post-translational modifications are differentially regulated throughout the cell cycle, and at subregions of the mitotic spindle. In addition, the cell cycle appears to be altered in mouse striatal cells expressing mutant huntingtin and in fibroblasts derived from HD patients. Dysregulation in the cell cycle is detrimental to neuronal health and plays a role in neurodegeneration. Understanding the role of huntingtin at the mitotic spindle may translate its other microtubule-related functions with relevance to Huntington's disease.

CHAPTER 2.0 : MATERIALS AND METHODS

2.1.1 Antibodies

Primary antibodies

Sources of primary antibodies and dilutions were as follows:

Mouse anti-GAPDH (Abcam)
Western blot 1:7500

Rabbit monoclonalanti-N17huntingtin unmodified (In-House - McMaster University) – epitope: amino acids 9-17 of N17
Western blot 1:2500
Immunofluorescence: 1:250

Rabbit anti-N17S13PS16P huntingtin (In-House - McMaster University) – epitope: amino acids 9-17 of N17 with serines 13 and 16 phosphorylated
Western blot 1:2500
Immunofluorescence: 1:250

Rabbit anti-N17pan huntingtin – epitope: the first 8 amino acids of N17
Western blot 1:2500
Immunofluorescence: 1:250

Mouse monoclonal anti-CC-3 (Abcam)
Western blot 1:2500

Rabbit polyclonal anti-huntingtin S421p (Michael Hayden - Department of Medical Genetics, University of British Columbia)
Western blot 1:1000
Immunofluorescence: 1:100

Mouse monoclonal anti-β-tubulin (University of Iowa, Developmental Studies Hybridoma Bank E7)
Immunofluorescence: 1:200

Secondary antibodies

The following conjugated secondary antibodies were incubated 1 hr at room temperature diluted 1/500 in antibody dilution solution: Alexa488 donkey anti-rabbit, Alexa488 goat anti-rabbit, Alexa594 chicken anti-rabbit (Molecular Probes/Life Technologies)
Goat anti-rabbit HRP (Abcam) 1:50000

Rabbit anti-mouse HRP (Abcam) 1:10000

Goat anti-mouse HRP (Biorad) 1:10000

Alexa488 donkey anti-rabbit (Molecular Probes) 1:500

2.1.2 General solutions

Tris buffered saline (TBS): 10mM Tris, 150mM NaCl, and pH 7.4

TBST: TBS with 0.05% Tween 20 (Sigma)

Phosphate buffered Saline (PBS): 10mMNaH2PO4, 0.14M NaCl, 1mM EDTA, pH 7.4

Tris- Glycine transfer buffer: 25 mM Tris, 192 mM glycine, 10% methanol

2mM Thymidine Solution: 2mM Thymidine in PBS

Tris-glycine SDS electrophoresis buffer: 0.125 M, Tris 1.25 M, Glycine 0.5 % SDS

2.1.3 Cell culture

Conditionally immortalized ST*Hdh*$^{Q7/Q7}$ striatal progenitor cells were originally derived from a wild-type (7 glutamine) knock-in transgenic HD mouse containing a

humanized Exon 1 and G418 resistance cassette (Trettel *et al*., 2000). The cells were a kind gift from Dr. Marcy Macdonald (MGH/Harvard). The cells were grown under G418 selection at 33°C with 5% CO2 in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS) to maintain the expression of the SV40 large T antigen.

Primary human fibroblasts from a 51 year old male with HD (Coriell catalogue # GM01061, CAG lengths are not known) and a 54 year old unaffected female (Coriell catalogue #GM02149, CAG lengths are not known) were cultured in Minimal Essentials Medium Eagle (MEM, Life Technologies) supplemented with 15% FBS.

2.1.4 Immunofluorescence

Cells were grown to 70% confluency in a 2.5 cm glass bottom and fixed and permeabilized with ice cold methanol at -20˚C for 12 minutes. Cells were washed twice in 1xPBS and blocked in 2% FBS in PBS for a minimum of 30 minutes. Primary antibodies, anti-N17, anti-N17S13pS16p, anti-S421, anti-β-tubulin, and Hoechst 33258, were diluted in antibody solution (0.02% Tween 20 in 5% FBS in PBS) in the mentioned concentrations above. They were applied to cells and were left to incubate at room temperature for a minimum of four hours or left overnight at 4˚C. Primary antibodies were then removed from the dishes and a second block was applied for a minimum of 30 minutes before the addition of secondary antibodies. Secondary antibodies left to incubate in the dishes for 45 minutes to one hour at room temperature. Hoechst 33258

(Sigma), a nuclear stain, was diluted to 1µg/mL in PBS and applied to the cells for 10 minutes.

Widefield fluorescence images were captured using a Nikon TE200 inverted epifluorescent microscope. Images were captured using a Hamamatsu Orca camera equipped with a 60x oil immersion objective camera (Hamamatsu Photonics, Japan).

2.1.5 Synchronization treatments

Thymidine block- Arresting cells in early S phase

For ST*Hdh* cell lines, cells were grown in DMEM in 10 cm dishes to 40% confluency. Cells were washed twice with 1x PBS and DMEM was added + 2mM Thymidine for 19 hours (first block). After the first block, thymidine was removed by washing the cells twice with 1xPBS and fresh DMEM was added for 9 hours to release the cells. After releasing the cells in early S phase, DMEM +2mM Thymidine was added for 15 hours (second block) **(Figure 3A)**. Proceeding the second block, thymidine was removed by washing the cells twice with 1xPBS and released with the addition of fresh DMEM.

Serum Starvation – Arresting cells in G_0/G_1 phase

Cells were grown in DMEM in 10 cm dishes to 60% confluency. Cells were washed twice with 1xPBS and DMEM was added without FBS. After 72 hours, the cells were

restimulated with DMEM with 10% FBS where they were synchronized and released in the G_1 phase of the cell cycle **(Figure 3A)**

2.1.6 Protein extraction and western blotting

ST*Hdh*$^{Q7/Q7}$, ST*Hdh*$^{Q7/Q111}$, ST*Hdh*$^{Q111/Q111}$ were cultured and synchronized as mentioned previously. Cells were extracted from the plates using a rubber scraper, pelleted and washed with 1xPBS. They were then resuspended in fresh NP-40 lysis buffer with phosphatase and protease inhibitors and allowed to incubate for 15 minutes on ice. Cells were then spun at 14000xg for 15 minutes and the protein fraction (supernatant) was carefully collected.

Supernatants were denatured in 1x SDS with DTT for 10 minutes at 100 ˚C. Equal amounts of protein were loaded into a 4-12% Tris-HCl polyacrylamide precast gels (Bio Rad) and electroblotted to a polyvinylidene fluoride (PVDF) membrane. After the gel was transferred onto a nitrocellulose membrane, blotting was performed with the SNAP i.d.® 2.0 Protein Detection System (EMD Millipore) using 0.5% milk in Tris-Buffered Saline (TBS) -Tween-20 (Sigma) according to the user manual. The following antibodies were incubated in 0.5% milk TBS-T: anti-huntingtin N17 (1: 2500), anti-huntingtin N17S13pS16p (1:2500), anti-huntingtin S421p (1:1000), anti- CC-3 (1:2500), and anti GAPDH (1:7500) followed by the addition of an appropriate HRP-conjugated secondary antibody. The protein on the membrane was visualized by Immobilon western

chemiluminescence HRP substrate (ECL) and bands were quantified using Image J software normalized to the control.

2.1.7 Flow Cytometry

Media was aspirated from STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells cultured in 10 centimeter plates and washed with 1x PBS. 5 mL of 0.25% trypsin in EDTA (Life Technologies) was added for 2 minutes. Cells were harvested and centrifuged at 1500 rpm for 5 minutes. They were permeabilized and fixed in ice cold methanol slowly to avoid aggregation and put in -20˚C for 12 minutes. Cells were then incubated in anti-N17 and anti-N17S13pS16p conjugated to Alexa 488 and Cy5. Propidium iodide was added for 10 minutes. Cells were sieved using a 35-µm nylon mesh cell strainer prior to flow cytometry analysis. The cells were analyzed using the BD LSR II flow cytometer equipped with the 488nm Blue laser, for the excitation of Alexa 488; detection filter 530/30, and 561 nm Yellow-Green laser for the excitation of Propidium Iodide; detection filter 610/20. Data acquisition (10^5 events per sample) was performed on BD FACS Diva software (version 6.1.2) and analyses were performed using FlowJo version 9.7.6 software.

CHAPTER 3.0: RESULTS

3.1.1 Huntingtin phosphorylation increases during mitosis

The localization of endogenous full-length huntingtin at the centrosome in resting cells has previously been reported by our group (Atwal *et al*., 2010). Using immunofluorescence imaging, we observed an increase in phosphorylation of the huntingtin protein during mitosis. In order to assess the phospho-modifications of huntingtin during different stages of mitosis, an antibody recognizing the huntingtin N17 domain phosphorylated at serine residues 13 and 16 (anti-N17S13pS16p), and anti-S421p (recognizes phosphorylated serine 421) was used. Wild-type mouse striatal cells, ST*Hdh*^{Q7Q7}, that were undergoing mitosis showed a higher fluorescence intensity compared to surrounding non-mitotic cells **(Figure 4B, C)**. To investigate this observation further, we compared the staining intensity of interphase cells to that of cells undergoing mitosis for a panel of antibodies against huntingtin. As shown in **Figure 4A**, antibodies against total huntingtin (anti-N17 pan huntingtin), which recognizes phosphorylated and unphosphorylated regions of the huntingtin amino terminus, and anti-MW8 (recognizes the last 7 residues of the carboxyl-terminus in exon 1 of huntingtin) were used and we observed a modest increase in the total levels of huntingtin. One caveat of this is that the observed increase could be a consequence of rounded cells and not due to the increase in total huntingtin levels. In contrast, a robust

increase in fluorescence intensity was observed in mitotic cells with antibodies anti-N17S13pS16p and anti-S421p **(Figure 4B, C, and quantified Figure 4F)**.

3.1.2 Phosphorylation of huntingtin increases with DNA content

Immunofluorescence experiments conducted were limited to a small population of cells. In order to assess the aforementioned phenomenon in a large number of cells, we used flow cytometry to measure phosphorylated N17 huntingtin using anti-N17S13pS16p antibody staining, unphosphorylated N17 huntingtin using anti-N17, and total DNA content using propidium iodide (PI) staining - indicating G_2 and mitotic cells. **Figure 5** demonstrates a minimal increase in staining intensity of the anti-N17 antibody in cells with a higher DNA content. In contrast, the anti-N17S13pS16p antibody staining showed a robust shift with increased DNA content, aligning with the immunofluorescence results. These flow cytometric analyses indicate that cells with increased DNA content have higher levels of phosphorylated huntingtin protein. These results also address caveats from our previous immunofluorescence experiments. The flow cytometric experiments confirm that increase in anti-N17 is due to increased levels of the total huntingtin protein and not rounded mitotic cells. We have also shown an increase in phospho-huntingtin in mutant huntingtin by immunofluorescence **(Figure 4E)** and flow cytometry **(Figure 5 bottom panel)**. However, the intensity of anti-N17S13pS16p in cells expressing mutant huntingtin is to a smaller extent than the wild-

type. From these results, we can conclude that the phosphorylation of huntingtin at S13, S16, and S421 are upregulated in mitotic cells.

3.1.3 Aberrant cell cycle synchronization in cells expressing mutant huntingtin

Although we observed modest increases in phospho-N17 during mitosis in mouse striatal cells expressing mutant huntingtin, the literature indicates that the mutant protein is hypo-phosphorylated at serines 13 and 16. Therefore, it was important to investigate any irregularities the cell cycle of HD cell models. Furthermore, since our immunofluorescence and flow cytometry results consistently showed the upregulation of phospho-N17 in mitotic cells, we wished to examine this effect further in a large population of cells through western blotting. To observe the changes in phosphorylation during the different phases of the cell cycle, we synchronized the cells in S phase of the cell cycle using high concentrations of thymidine resulting in a negative feedback on nucleotide biosynthesis. Differential interference contrast microscopy (DIC) imaging showed an increase in the number of mitotic cells, which were identified by rounded cells between 12 and 24 hours in ST*Hdh*$^{Q7/Q7}$ cells **(Figure 6A)**. Cell lysates collected post-synchronization at 0, 3, 6, 12, 24, and 30 hours showed a peak in the amount of a phospho-mitotic marker, anti-CC-3 **(Quantification in Figure 10)**, at 12 and 24 hours **(Figure 7 Top)**. These results were consistent with the observations from the DIC images. Consistent with our immunofluorescence and flow cytometric data, we also detected a robust increase in anti-N17S13pS16p levels as the cell cycle progressed. The

N17S13pS16p band volume of the western blot was quantified and adjusted to reflect the differences in loading controls. The results are shown graphically in **Figure 8** and indicate there is an effect of time on the phosphorylation levels of huntingtin. A normality test (Shapiro-Wilk) was conducted on the data from 0 to 30 hours (P=0.207). A one-way ANOVA test was conducted ($p<0.001$) followed by a pairwise multiple comparison, Holm-Sidak test, with a significance level of 0.05. The difference in N17S13pS16p levels between time points was also statistically significant.

In contrast to the wild-type cells, we noticed inconsistencies in phospho-huntingtin levels in the mouse striatal cells expressing mutant huntingtin. Furthermore, anti-CC-3 patterns highly varied between trials, indicating the inability of mutant huntingtin to re-enter the cell cycle reliably **(Figure 7 Bottom, Quantification in Figure 11)**. The anti-N17S13pS16p profile patterns gradually and consistently increased from 0-30 hours in ST*Hdh*$^{Q7/Q7}$, but were very unpredictable in ST*Hdh*$^{Q7/Q111,}$ and ST*Hdh*$^{Q111/Q111}$ cells. The N17S13pS16p band volume of the western blot was quantified and adjusted to reflect the differences in loading controls. The results are shown graphically in **Figure 9** and indicate there is no statistical significant difference between time points. From these data, we concluded that there is no effect of time on phosphorylation and the HD cell model exhibited failure to re-initiate the cell cycle post synchronization, thus indicating an aberrant cell cycle.

3.1.4 Differential localization of huntingtin at mitotic structures

Our lab has previously shown that phosphorylated huntingtin at residues 13 and 16 is present at various mitotic structures (Atwal *et al*., 2010). As S421 is another important site of huntingtin phosphorylation, we sought to investigate the localization of huntingtin phosphorylated at this residue during mitosis by immunofluorescence in human fibroblasts using antibodies against N17S13pS16p and S421p. By assessing the staining patterns of these antibodies at different stages of the cell cycle, we observed an increase in signal intensity of anti-N17S13pS16p and anti-S421p at the centrosomes, as shown in **Figure 12**. Interestingly, we noted a differential localization of the anti-S421p, relative to anti-N17S13pS16p, to the mitotic spindle during metaphase and anaphase. Furthermore, during telophase and cytokinesis, anti-S421p is distinctly observed at the mid-plate at the cleavage furrow. These results suggest that the phosphorylated residues are modified differently throughout the course of the cell cycle.

CHAPTER 4.0: DISCUSSION

In this study, we investigated the potential role of huntingtin during the cell cycle by observing the localization of phospho-modified huntingtin during mitosis. Post translational modifications at the amino terminus of huntingtin have been associated with decreased neurotoxicity in HD cell and animal models, as well as influencing the function of huntingtin during vesicular trafficking (Colin *et al*., 2008). Our initial findings, which revealed the differential localization of huntingtin and phospho-modified huntingtin at mitotic structures, led us to further investigate the role of huntingtin in mitosis (Atwal *et al*., 2010). Utilizing biophotonic and biochemical techniques, we investigated the changes in phospho-huntingtin levels and localization in wild-type and mutant huntingtin-expressing cell lines.

The differential localization of the various phospho-modifications of huntingtin identified in our study suggests that huntingtin could have multiple roles at mitotic structures and may play a key role in cell cycle through interaction with different proteins within the mitotic machinery. This is supported by a study by Godin *et al*., confirming that huntingtin localizes to the spindle pole, and RNAi-mediated knockdown of huntingtin leads to incorrect spindle orientation (Godin *et al*, 2010). Huntingtin has also been demonstrated to regulate mammary stem cell differentiation through its role at the mitotic spindle poles (Elias *et al*., 2014). In fibroblast cultures derived from R6/2 Huntington's disease transgenic mice and HD patients, multiple centrosomes, and high

frequency of aneuploidy was evident (Sathasivam *et al*., 2001). Disorganization of the centrosome, followed by a disruption in the cell cycle could account for the observed phenotypes including multi-nucleic cells. Although the role of huntingtin in mitosis is not clearly established, huntingtin knock-down experiments demonstrated that huntingtin is critical for proper positioning and orientation of the mitotic spindle (Elias *et al*., 2014). Our data clearly shows that different huntingtin phospho-modifications occur at different mitotic apparatus throughout the cell cycle.

Consistent with our observation that phospho-huntingtin localizes to microtubule-based structures such as spindle fibres during mitosis, previous studies have defined huntingtin having multiple links to microtubules, molecular motors and vesicles (Atwal *et al*., 2011; Godin *et al*., 2010; Hoffner *et al*., 2002; Engelender *et al*., 1997). Huntingtin has an established role in the transport of brain-derived neurotrophic factors in neuronal axons and acts as a scaffolding protein along with Huntingtin associated protein1 (HAP1) and dynactin. Using a yeast two hybrid assay, HAP1 was found to bind with huntingtin in a polyglutamine length-dependent manner. Furthermore, HAP1 was found to interact with microtubule associate proteins pericentriolar material-1 (PCM1) and dynactin, thus linking huntingtin to the cytoskeleton (Engelender *et al*., 1997). The association of huntingtin to cytoskeletal elements strongly suggest that there may be a link between huntingtin and the mitotic apparatus.

As mentioned previously, post translational modifications affect huntingtin binding to microtubule associated proteins. Phosphorylation of serine 421 affects the directionality of axonal transport, where huntingtin recruits kinesin to the dynactin complex and in turn increases axonal transport (Colin *et al*., 2008). In our study we found evidence that phosphorylation of different huntingtin residues influences its localization at different stages of mitosis. For instance in metaphase of the cell cycle, huntingtin phosphorylated at serine 421 was concentrated at the centrosomes and cleavage furrow, whereas, we observed huntingtin phosphorylated at serines 13 and 16 concentrated mainly at the centrosome. This indicates that the phosphorylation of huntingtin at different residues is distinct from one another which may lead to the differential localization at mitotic structures.

Our work in the current study provides further crucial evidence for understanding the role of huntingtin in mitosis. This work not only identified the increase in phospho-modified huntingtin during mitosis, but also suggested an aberrant cell cycle in murine cells expressing mutant huntingtin. Although results from our immunoblotting assays revealed a gradual increase in phospho-huntingtin from S-phase to mitosis in wild type cells, inconsistent levels of phospho-huntingtin were observed from trial to trial. These recurring inconsistencies in the mutant cell line (ST*Hdh*$^{Q111/Q111}$) led us to conclude that they simply cannot be synchronized, as seen by the phospho mitotic protein anti-CC-3, and experience dysfunctional regulation of their cell cycle.

Cells expressing mutant huntingtin cannot be synchronized

We synchronized both the normal and mutant cell lines in G_0/G_1 phase of the cell cycle utilizing serum starvation methods. Serum starvation deprives cells of essential nutrients to proliferate, thus forcing them into quiescence (Keyomarsi *et al*., 1991). Inconsistent phospho-huntingtin levels in our mutant cell line led us to believe that perhaps our synchronizing protocol may be inducing excessive stress to the cells and contributing to our varying results. We decided to employ a double thymidine block protocol which synchronizes cells in early S phase. In this technique, very high concentrations of thymidine result in a negative feedback on nucleotide biosynthesis. A low supply of nucleotides leads to a halt in DNA replication, leaving a large population of cells stranded in S phase (Bostock *et al*., 1971; Shedden and Cooper, 2002). Although we were able to increase our protein yields, as well as minimize stress by using a double thymidine block, we still obtained inconsistent phospho-huntingtin levels during mitosis in cells expressing mutant huntingtin, further supporting our theory that cells expressing mutant huntingtin cannot be synchronized due to their aberrant cycling.

There is quite a bit of indirect evidence that suggests that the irregularities in the eukaryotic cell cycle may play a role in the pathogenesis of HD. Consistent with our results, a recent study was able to identify, using a cell model of HD (ST*Hdh*$^{Q111/Q111}$), a delay in progression of the mutant cell line in the S and G_2-M phase compared to the control (Roshan *et al*., 2009). Additionally, Das *et al.* observed reduced miRNA expression in HD models in the late S phase and G_2-M, which may be contributing to

increased cell death (Das *et al*., 2015). MicroRNA, a class of small non-coding RNA molecules that function in transcriptional and post translational regulation, is associated with neuronal differentiation in mice. Specifically, mircoRNA-124, a brain specific microRNA, plays a critical role in the regulation of neurodegeneration and neuronal differentiation (Roshan *et al*., 2009). In HD, the expression of microRNA-124 has been found to be repressed in both mouse models and human patients. Decreased expression of microRNA-124 results in the dysfunction of RE1-Silencing Transcription Factor (REST). Dysregulated translocation of REST to the nucleus leads to transcriptional downregulation of critical trophic factor BDNF, resulting in neuronal damage (Shimojo, 2008; Johnson and Buckley, 2009; Wang *et al*., 2006).

Mutant huntingtin-associated aberrant cell cycle may be due to defective DNA damage response

In comparison to somatic cells, neurons are prone to higher levels of oxidative stress, which can often result in DNA damage. Cell cycle machinery plays a key role in quiescent cells during the DNA damage response. Although evidence suggests that terminally differentiated neurons have the ability to reinitiate the cell cycle, this re-initiation does not necessarily lead to neuronal proliferation but instead to apoptosis (Kruman, 2004; Zhu, Raina, and Smith, 1999). The ataxia telangiectasia mutated (ATM) protein, a member of the PI3kinase family, becomes activated during DNA damage, and is critical in inducing cell cycle checkpoints during DNA repair. Herrup *et al*. showed that

the suppression of ATM mitigated cell cycle re-entry and apoptosis, and ATM is required for apoptosis in post-mitotic neurons that experienced oxidative stress. Neurons from mice deficient of ATM were shown to be resistant to apoptosis resulting from DNA damage, indicating that ATM is critical for removal of DNA damaged neurons (Herrup and Yang, 2007). This remains true in humans as ATM-deficient patients have increased incidence of DNA single-strand breaks. A recent paper reported elevated levels of ATM in cells derived from HD mice as well as brain tissue from patients. Inhibition of ATM by knockdown or chemical inhibitors abrogated the toxic effects of mutant huntingtin in transgenic drosophila models and rat striatal neurons respectively (Lu *et al*., 2014). It is therefore possible that the effect of mutant huntingtin on ATM activity could be contributing to the cell cycle dysregulation we have seen in our study.

Another crucial player that may be influencing cell cycle in mutant huntingtin-expressing cells is the tumour suppressor protein, p53, which is involved in DNA repair, cell cycle arrest, and apoptosis. In 2000, Steffan *et al*. determined that full length huntingtin co-precipitated with p53 *in vitro*. Furthermore, huntingtin with a pathogenic polyglutamine expansion interfered with the p53-regulated promoters, p21WAF1/CIP1 and MDR-1, *leading* the authors to conclude that expanded huntingtin repressed transcription factors causing neuronal defects and eventually cell death in HD (Steffan *et al*., 2000). In addition to this, p53 binds to the huntingtin gene via p53-responsive elements *in vivo* and *in vitro*. Through this, the p53 protein is able to regulate huntingtin expression at the transcriptional level. Feng *et al*. showed that the activation of p53

with γ-irradiation increases huntingtin gene expression that is triggered with DNA damage in the striatum of the mouse brain. These results provide strong evidence of the p53 protein regulating the huntingtin gene. It is also worth noting that ATM directly phosphorylates p53. The dysregulation of ATM, as observed in neurodegeneration, in turn leads to the ineffective functioning of p53 (Canman *et al*., 1998). Phosphorylation of p53 induced by DNA damage has been shown to correlate with transcription of downstream p53 target genes. These results provide strong evidence of the p53 protein regulating the huntingtin gene. Thus, it is possible that the reciprocal interaction between huntingtin and p53 may affect the regulation of the cell cycle.

In HD we also know that there are pathways involved in DNA repair mechanisms. The inactivation of *MLH1* (the human homolog of the E. coli DNA mismatch repair gene *mutL*) in humans resulted in the instability of the CAG repeat (Dragileva *et al*., 2009). Further support to this is from recent genome-wide genetic screen which identified *MLH1* and other variants in DNA repair genes to play a role in modifying HD progression (*Lee et al*., 2015).

SV40 T antigen-immortalized cells disrupt cell cycle checkpoints by inhibiting p53 activation

We have conducted the majority of our experiments in mouse striatal cell lines that are immortalized with simian virus 40 (SV40) T antigen, a class of DNA tumour virus. Once cells undergo transformation with SV40, they become capable of continued

proliferation and are able to survive in a wide range of environmental conditions that would normally not be possible. Earlier literature described defective karyotypes in rodent and human cells infected with SV40 T antigen (Wolman *et al*., 1964). SV40 is dependent on the machinery of the host cell. In order to successfully replicate, SV40 inactivates tumour suppressor proteins, pRb and p53, causing re-entry into the cell cycle at inappropriate times (Bocchetta *et al*., 2008). Consistent with these findings, human fibroblasts transformed with the SV40 T antigen exhibited altered mitotic checkpoint control, chromosome aberrations, and increased incidence of aneuploidy. These characteristics pose limitations on analyzing various cellular functions, primarily those related to cell cycle regulation. Inconsistent results in our research were indicative of a dysfunctional cell cycle and from this we can conclude that cell lines transformed with a T antigen are not appropriate models of HD research. Based on this evidence, we would recommend that future experiments should be conduct in human telomerase reverse transcriptase (hTERT)-immortalized fibroblasts that have been shown to possess functional cell cycle checkpoints and remain diploid (Yudoh *et al*., 2001; Lee; 2004). Furthermore, it has been shown recently that trans-differentiation of fibroblasts into functional neurons can be accomplished with cell lineage-specific transcription factors. This would be a beneficial tool as it may potentially allow us to obtain and compare results from cycling (hTERT) and non-cycling (derived-neurons) cells (Xue *et al*., 2013).

Using these cell lines, we can conduct future experiments that involve observing the differences or irregularities in cellular division between normal and HD cell models. We can conduct experiments involving live-cell imaging to note any key characteristic differences between wild-type and mutant cell lines. We can also test if the number of aneuploid cells increase or decrease by adding a phosphorylation promoting agent, such as GM1. To extend our understanding of the role of huntingtin at mitotic structures during cellular division, it would be beneficial to explore which other proteins co-localize in these regions. Based on these results, we can tease out a potential role that huntingtin may play at these critical structures. We can extrapolate these findings and identify the role of huntingtin in other cytoskeletal functions that are crucial to neuronal health.

5.0 CONCLUSION

Although Huntington's disease is a systemic disorder there is little focus in the literature about the developmental aspects. Furthering our knowledge on peripheral pathology and examining huntingtin in the context of the cell cycle may aid in providing us insight to the mechanisms underlying the disease. In this study, we found changes in huntingtin localization and phosphorylation levels in cells expressing normal and mutant huntingtin during mitosis. We showed that the cell cycle is dysregulated in HD cell models compared to normal. It is likely that the hyper-activation of ATM in the context of mutant huntingtin upon DNA damage results in altered cell cycle checkpoints. Importantly, from the inconsistencies we incurred in our studies with the ST*Hdh* cell we can conclude that they are not suitable models of HD as they alter cell cycle checkpoints. The importance of post-translational modifications and their association to the mitotic machinery is crucial. Future work should focus on elucidating the mechanistic role of huntingtin at the mitotic structures using physiologically relevant models of HD.

6.0 FIGURES

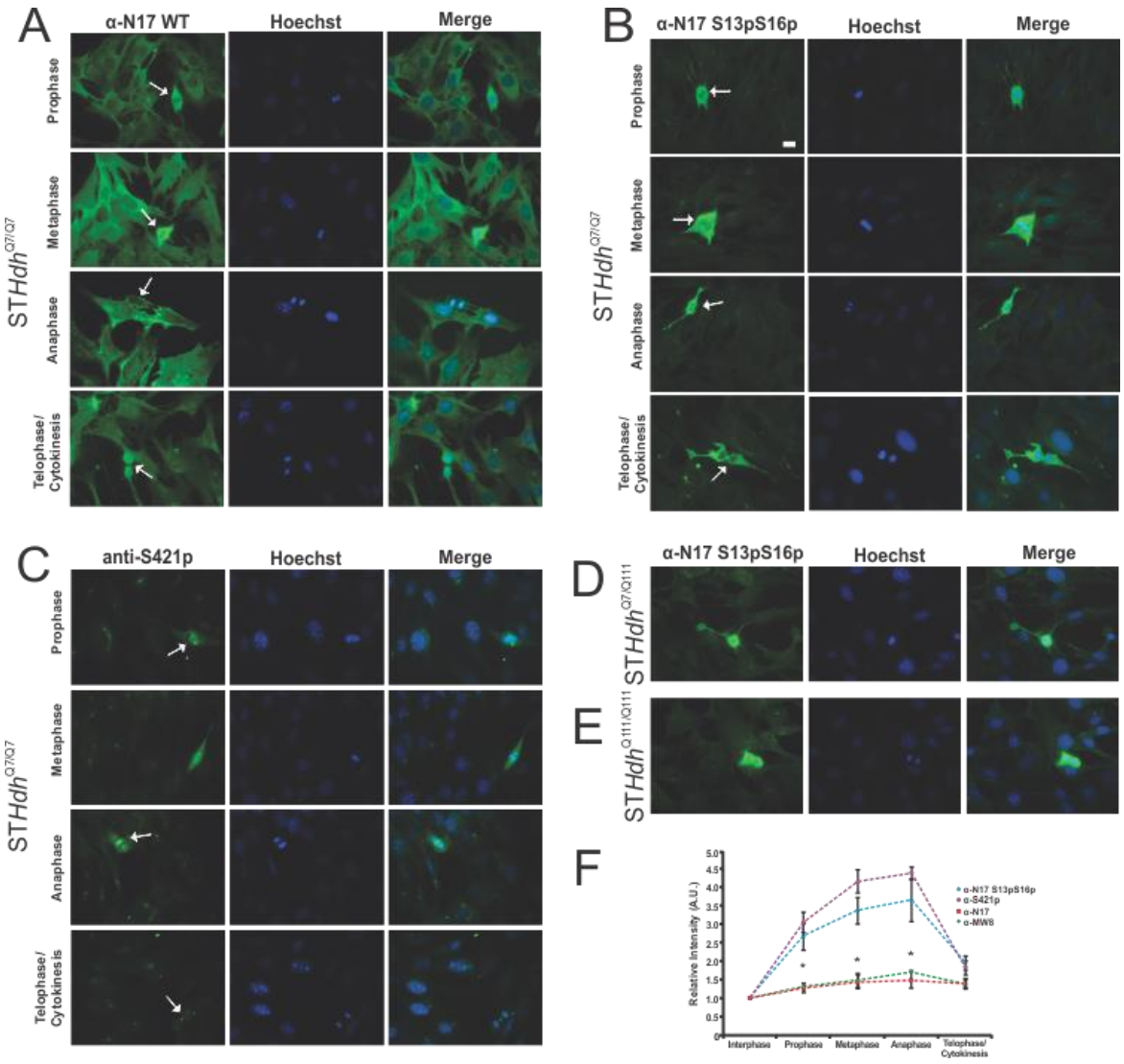

Figure 4 Immunofluorescence imaging of mouse striatal cells against huntingtin specific antibodies. (A) Immunofluorescence imaging of STHdh$^{Q7/Q7}$cells stained with anti-N17. This antibody recognizes unphosphorylated regions of huntingtin measuring total levels of huntingtin in the cell. An increase in intensity of cells undergoing mitosis is observed compared to the surrounding, non-dividing cells. Mitotic cells are represented with white arrows. **(B)** Immunofluorescence imaging of STHdh$^{Q7/Q7}$ stained with anti-N17S13pS16p and **(C)** anti-421p. As total levels of huntingtin are increasing in mitotic cells **(A)**, there is an even greater increase in intensity of phosphorylated huntingtin at serines 13 and 16 **(B)**. Mitotic cells are represented with white arrows. **(D)** Images of ST*Hdh*$^{Q7/Q111}$ and **(E)** STHdh$^{Q111/Q111}$ stained with anti-N17S13pS16p. **(F)** Relative intensity of images from STHdh$^{Q7/Q7}$ quantified. Experiment was conducted by Dr. Nicholas Caron.

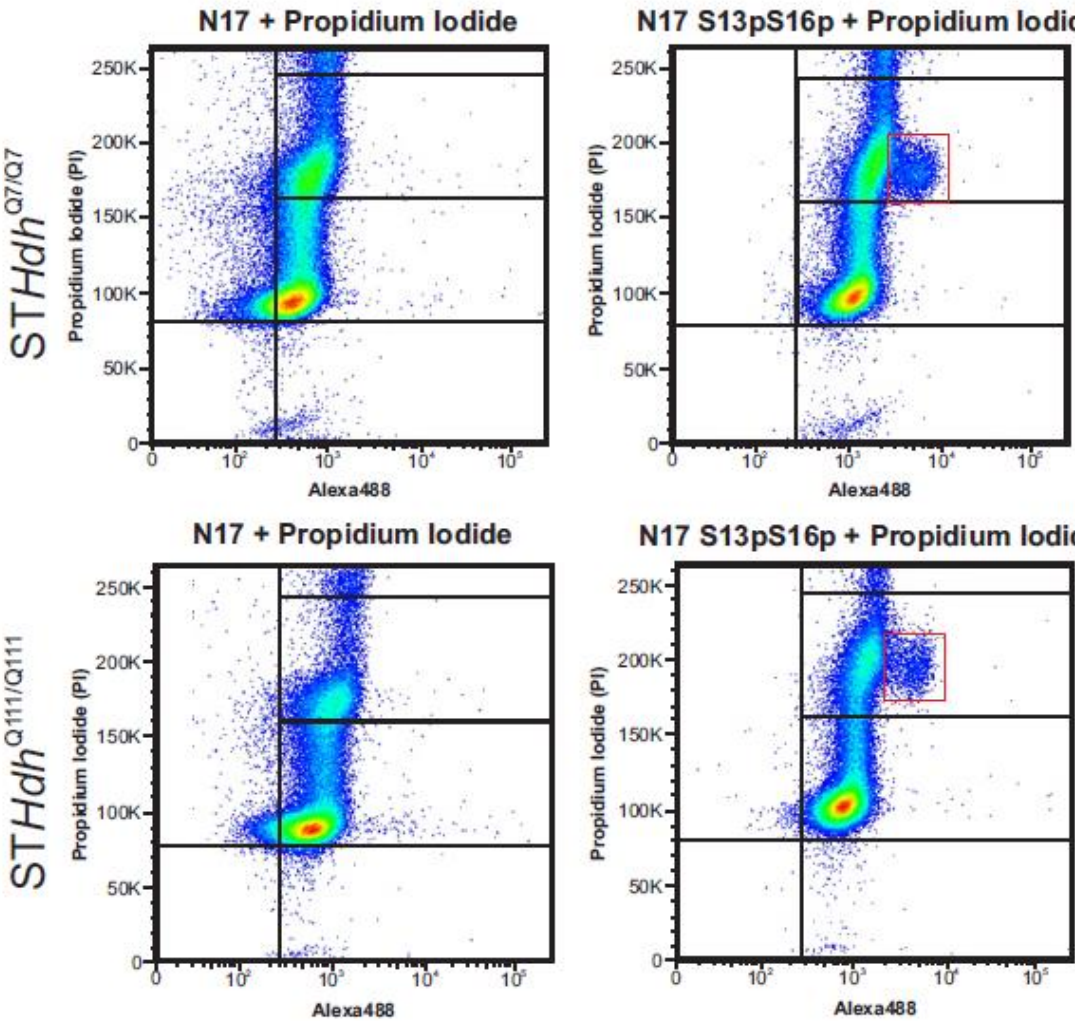

Figure 5 Flow cytometric analysis of wild-type (ST*Hdh*$^{Q7/Q7}$) and mutant (ST*Hdh*$^{Q111/Q111}$) mouse striatal cells. This figure illustrates a minor increase in the staining intensity of anti-N17S13pS16p antibody in cells with a higher DNA content, represented by the propidium iodide. The cryptogram in the bottom panel shows a similar but less robust increase in the staining of anti-N17S13pS16p in ST*Hdh*$^{Q111/Q111}$ (n=1).Experiment was conducted by Dr. Nicholas Caron.

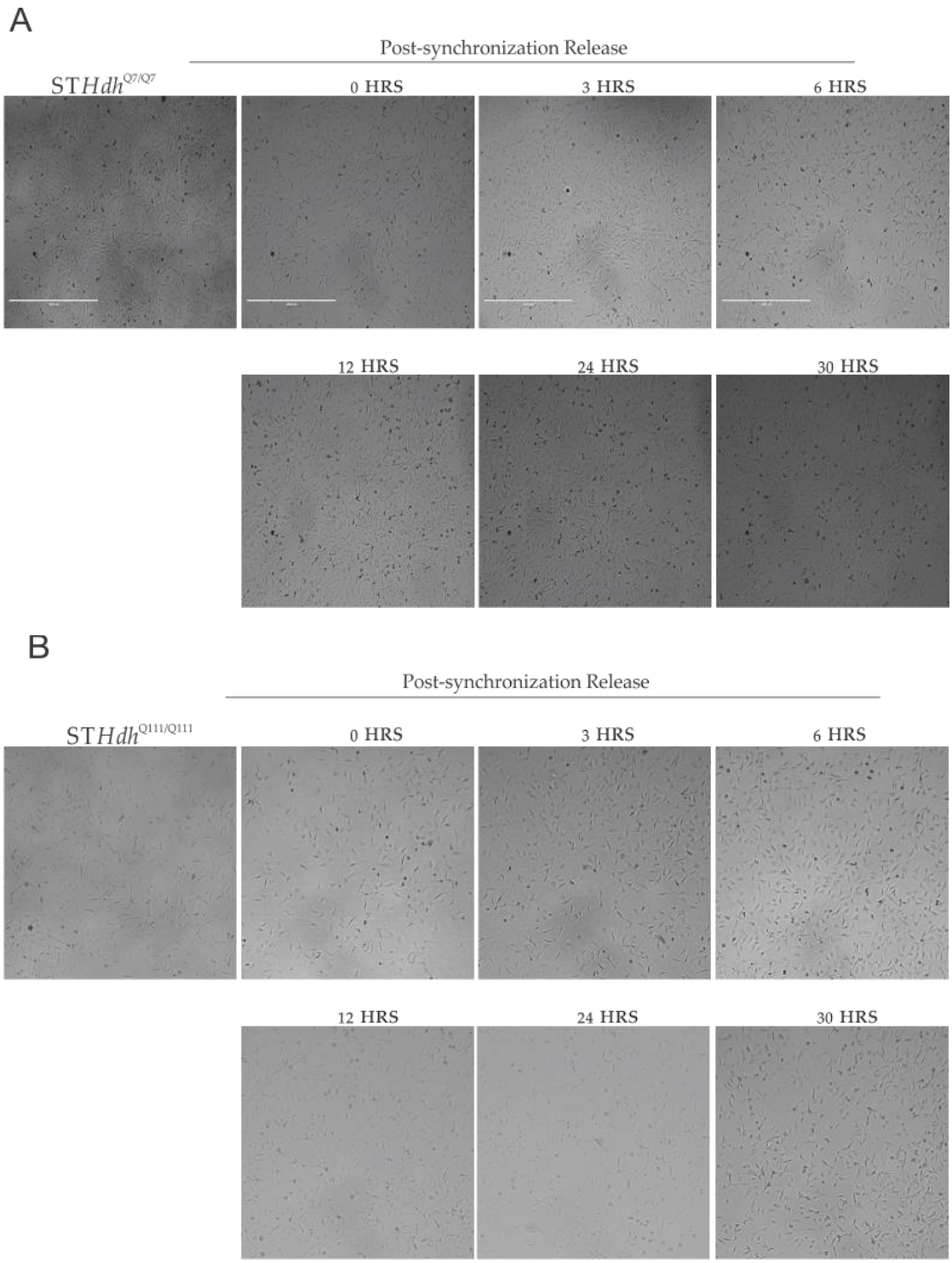

Figure 6 DIC images of cells prior to protein extraction. (A) This figure shows DIC images captured throughout the cell cycle time-course in ST*Hdh*$^{Q7/Q7}$ cells and **(B)** ST*Hdh*$^{Q111/Q111}$. An increase in the number of mitotic cells, which were identified by rounded cells, between 12 and 24 hours in ST*Hdh*$^{Q7/Q7}$ cells. In ST*Hdh*$^{Q111/Q111}$ **(B)** there is an inconsistence change in the number of mitotic cells when compared to ST*Hdh*$^{Q7/Q7}$ **(A)**, indicating an inability to be synchronized.

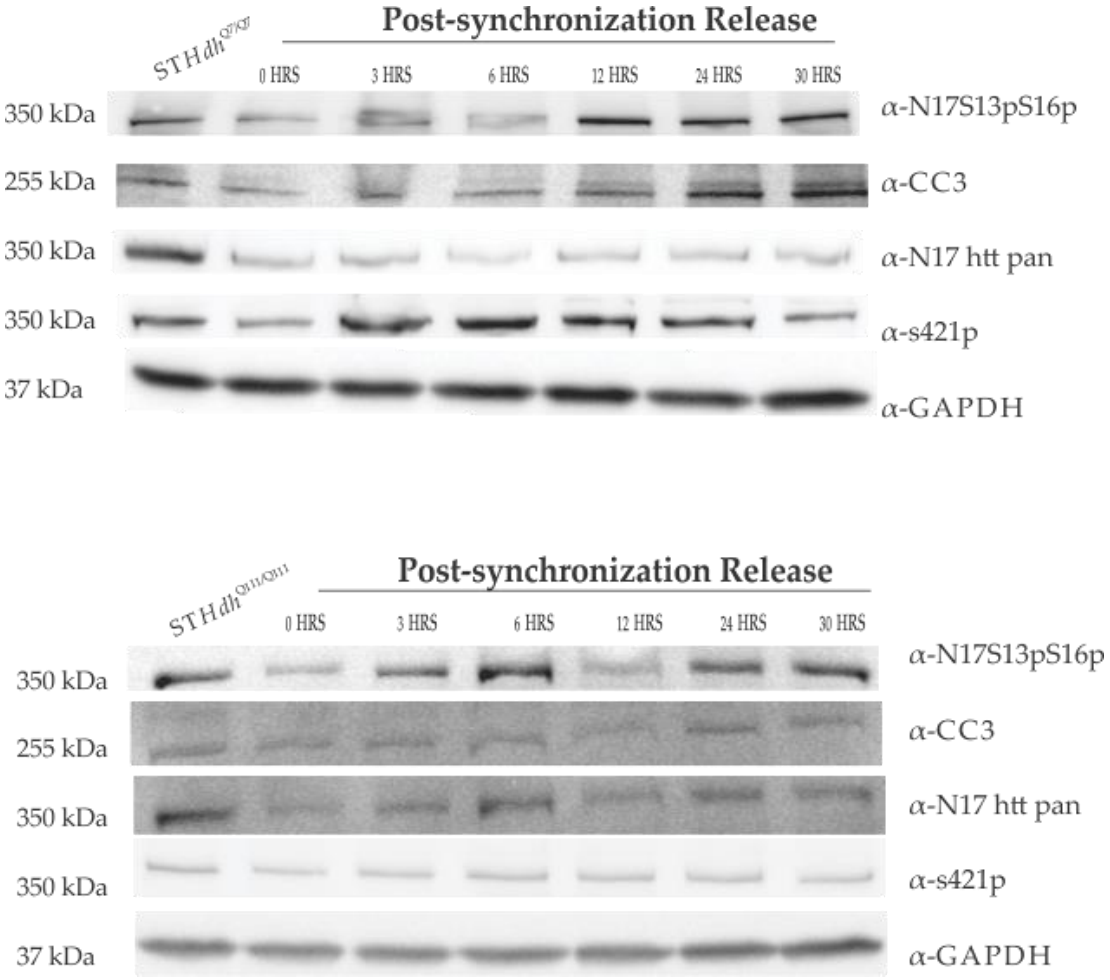

Figure 7 A time-course immunoblotting assay showing changes of phospho-specific huntingtin antibodies. (A) This figure represents SDS-PAGE gels throughout the course of 30 hours from ST*Hdh*$^{Q7/Q7}$ and ST*Hdh*$^{Q111/Q111}$ lysates post synchronization. **(Top)** In ST*Hdh*$^{Q7/Q7}$, gels blotted with anti-N17S13pS16p show an increase in signal from 0 to 30 hours, indicating changes in phosphorylation as the cell cycle progresses. When probed with a mitotic phospho protein (anti-CC3), a difference in signal intensity is observed at time points 12 and 24 hours indicating cycling of the cells. Anti-N17 was used to show total huntingtin and it increases modestly throughout the course of 30 hours. **(Bottom)** In ST*Hdh*$^{Q111/Q111}$, SDS-PAGE gels blotted with anti-N17S13pS16p, show an irregular and inconsistent pattern compared to the wild-type.

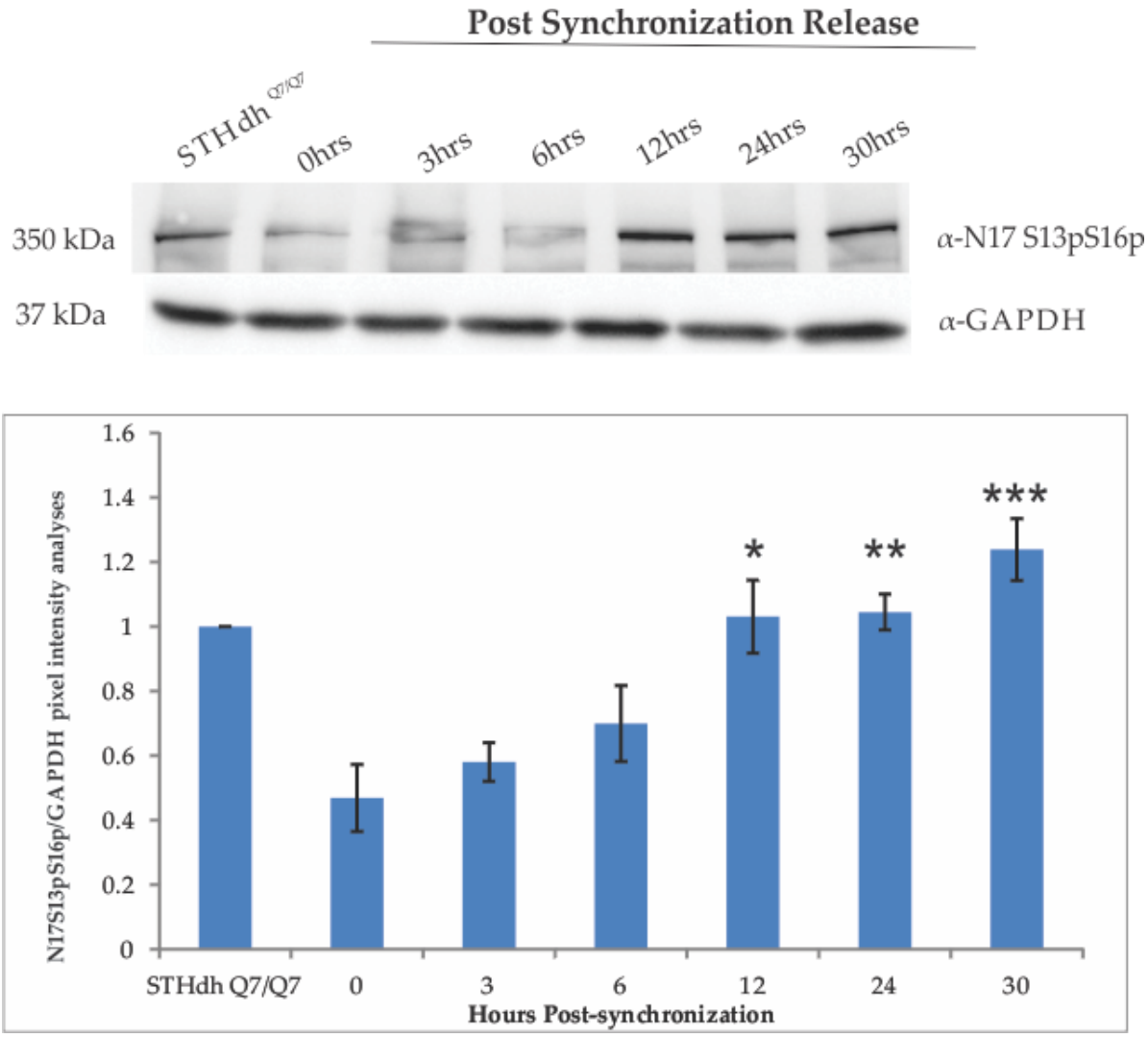

Figure 8 Quantification of anti-N17S13pS16p post-synchronization in wild-type cells. The anti-N17S13pS16p band volume of the western blot was quantified and adjusted to reflect the differences in loading controls. A normality test (Shapiro-Wilk) was conducted on the data from 0 to 30 hours (P=0.207). A one-way ANOVA test (p=<0.001) showed there is an effect of time on phospho levels. A pairwise multiple comparison, Holm-Sidak test, was conducted where p<0.05. There was a statistical difference in anti-N17S13pS16p levels between 0 and 30 hours (p=0.001), 0 and 24 hours (p=0.019), 0 and 12 hours (p= 0.021), 3 and 30 hours (p=0.006), and 6 and 30 hours (p=0.027). One star indicates the data is statistically different from one time point, two stars indicate the data is statistically different from 2 time-points, and three stars indicate the data is different from 3 time-points.

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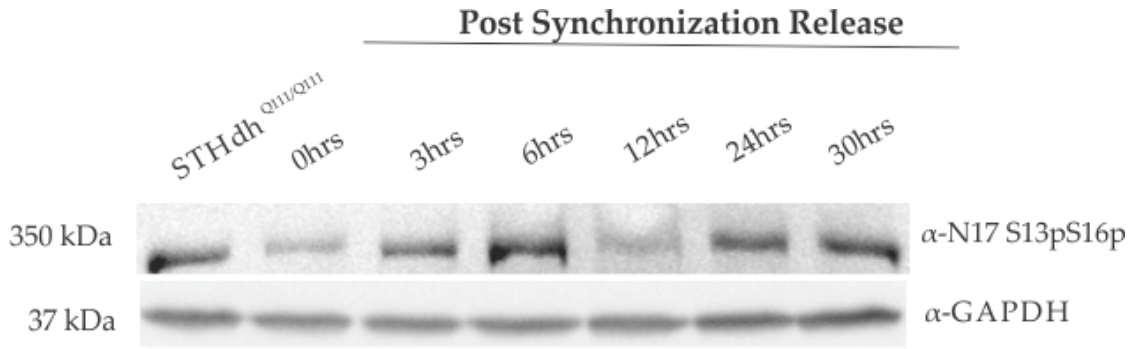

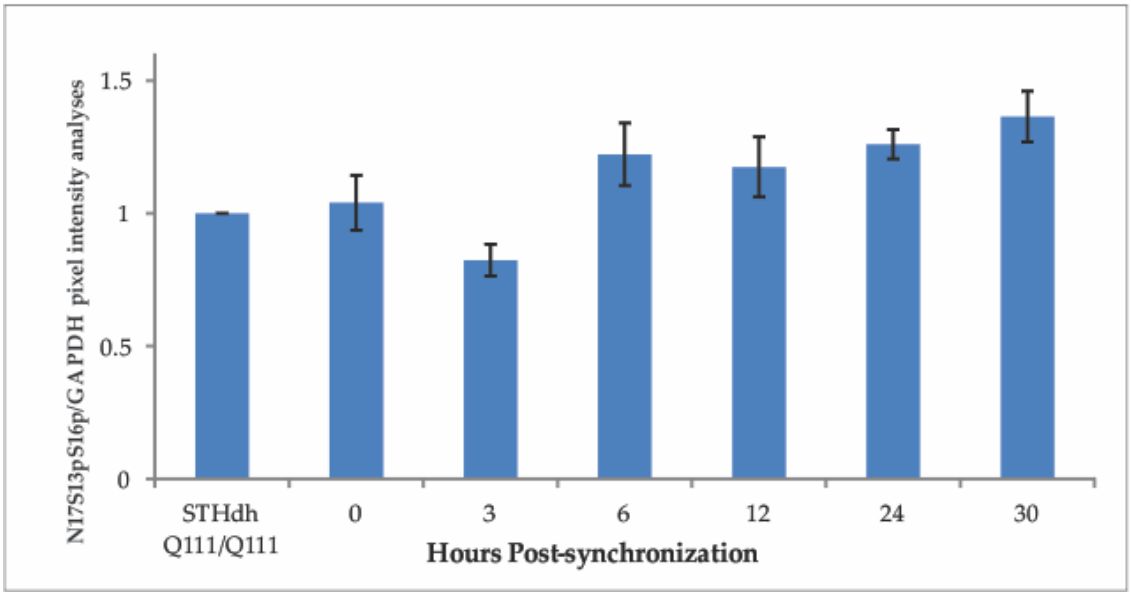

Figure 9 Quantification of anti-N17S13pS16p post-synchronization in mutant cells. The band volume analysis of the western blot from four separate trials shows the changes in phospho-huntingtin levels over 30 hours in ST*Hdh*$^{Q111/Q111}$ cells. A normality test (Shapiro-Wilk) was conducted where p=0.196. A one-way ANOVA test showed there was no statistically significant difference observed between 0 and 30 hours.

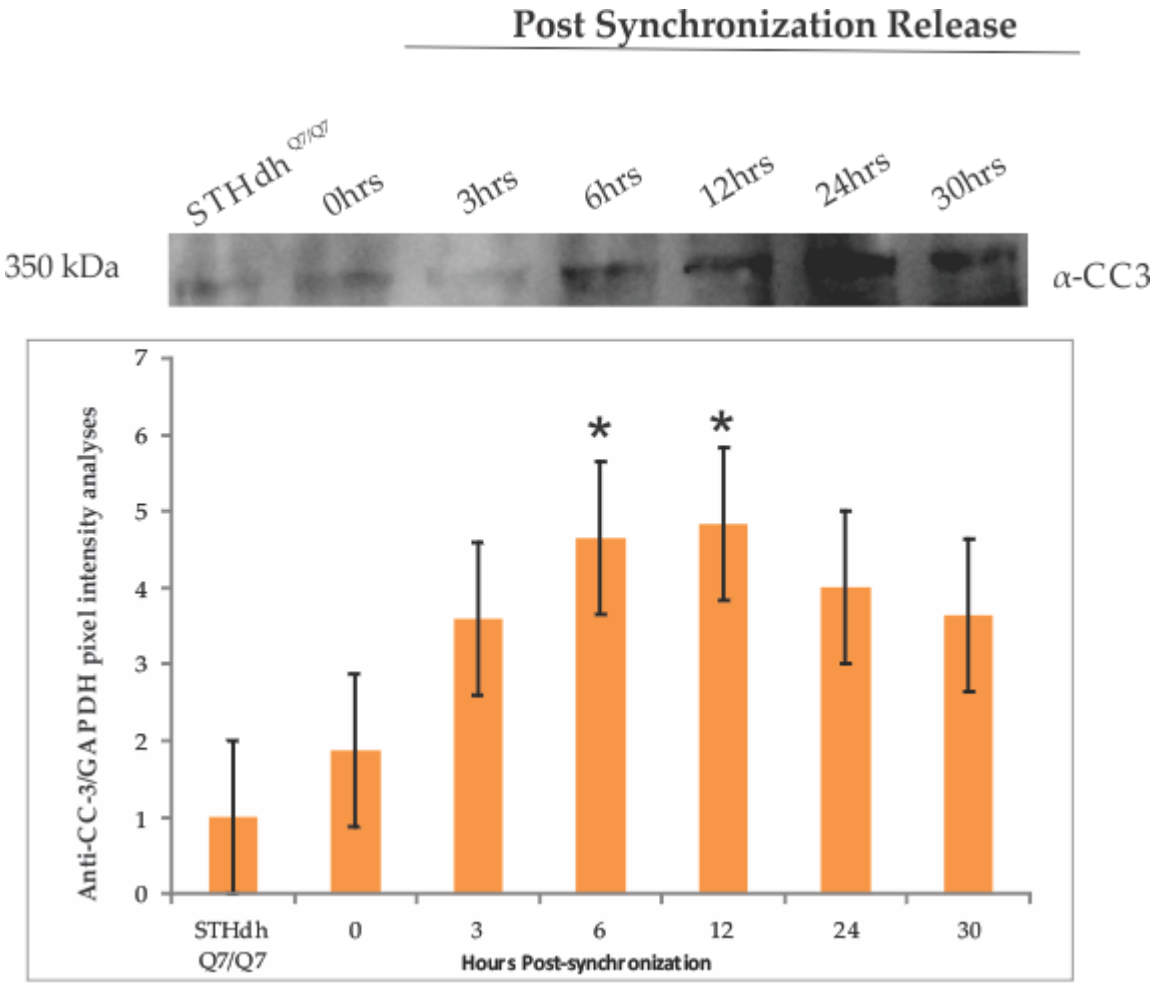

Figure 10 Quantification of anti-CC-3 post-synchronization in wild-type cells. The anti-CC-3 band volume of the western blot was quantified and adjusted to reflect the differences in loading controls. A normality test (Shapiro-Wilk) was conducted on the data from 0 to 30 hours (P=0.244). A one-way ANOVA test (p=<0.001) showed there is an effect of time on the levels of phospho mitotic protein anti-CC-3. A pairwise multiple comparison, Holm-Sidak test, was conducted where p<0.05. There was a statistical difference in anti-CC-3 levels between 0 and 6hours (p=0.011), 0 and 12 hours (p=0.017).

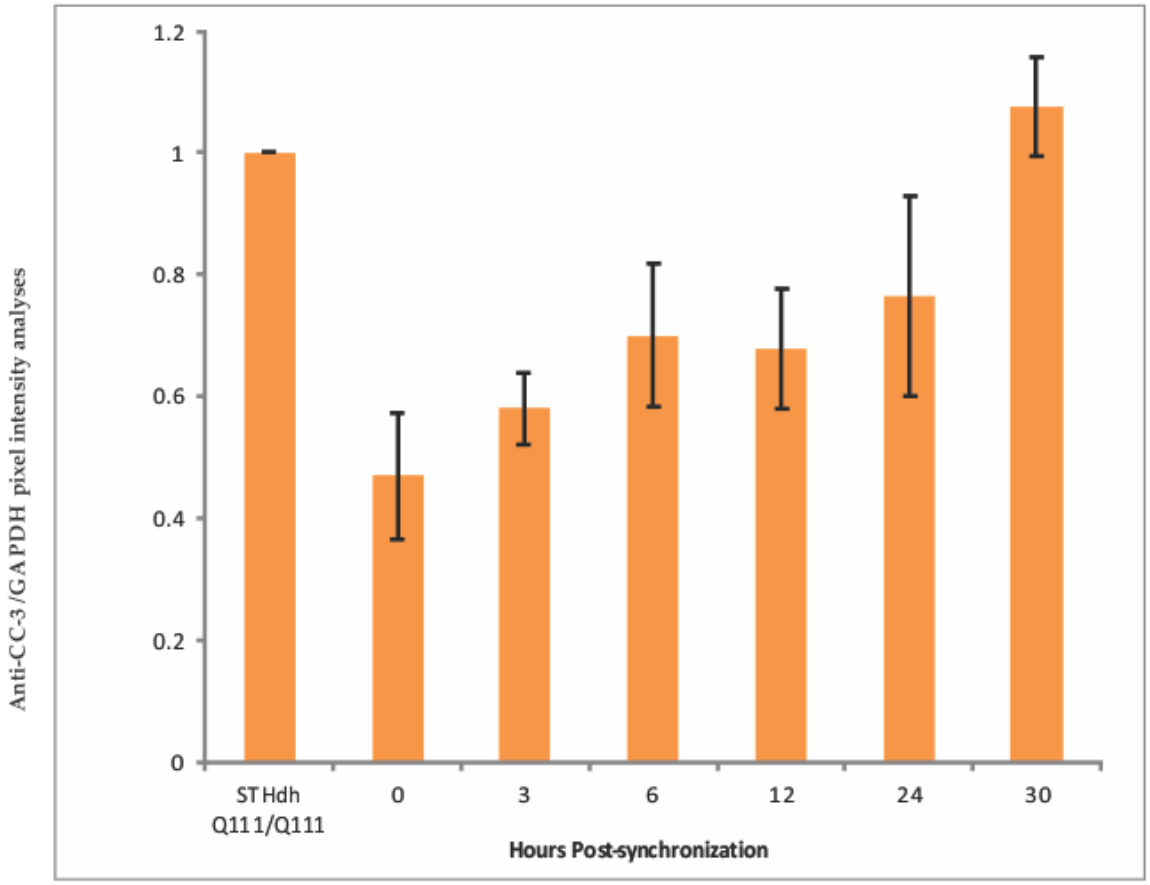

Figure 11 Quantification of anti-CC-3 post-synchronization in mutant cells. The band volume analysis of the western blot from three separate trials shows the changes in phospho mitotic protein, anti-CC-3, levels over 30 hours in ST*Hdh*$^{Q111/Q111}$ cells. A normality test (Shapiro-Wilk) was conducted where p=0.183. A one-way ANOVA test showed there was no statistically significant difference observed between 0 and 30 hours.

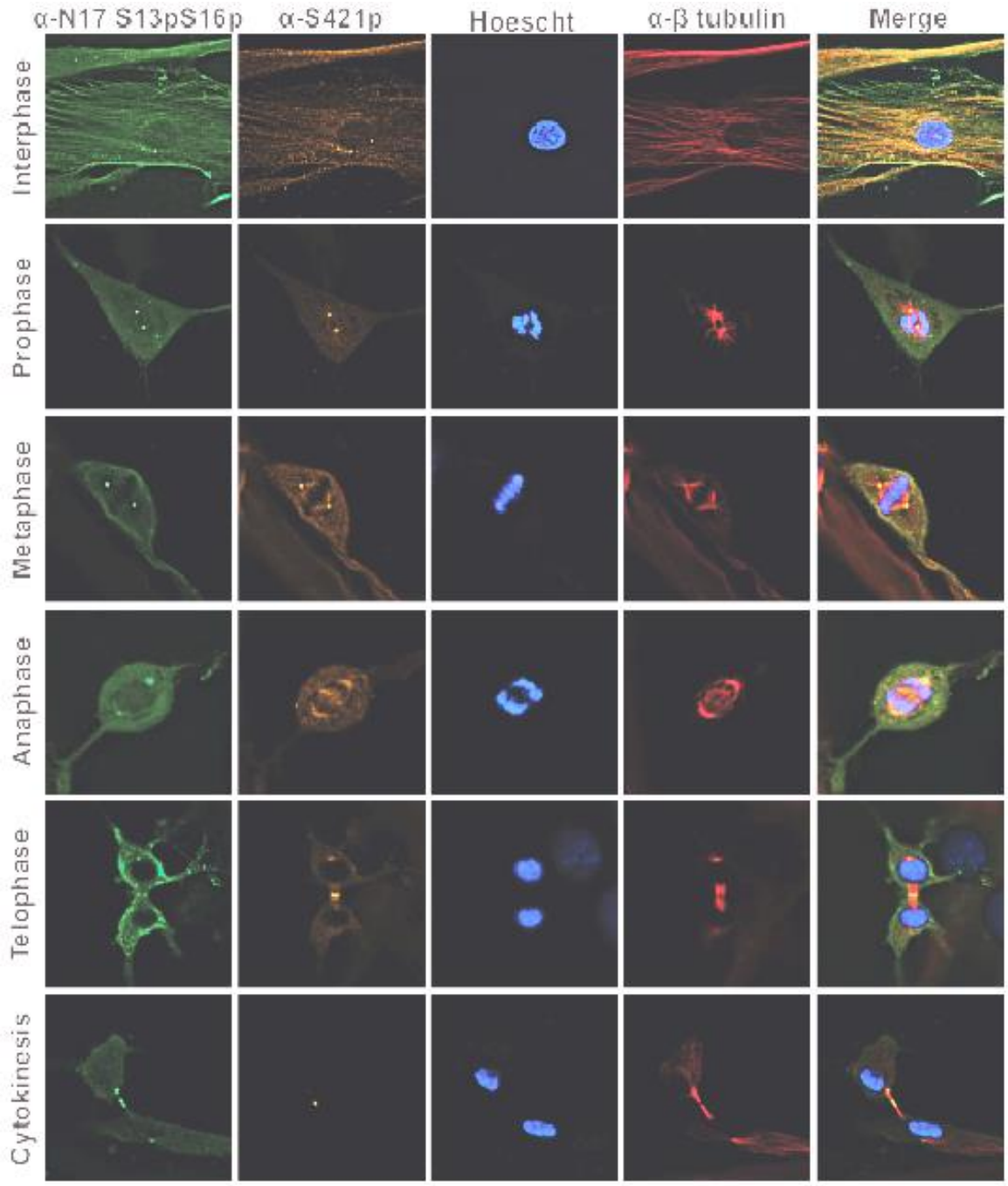

Figure 12 Differential localization of huntingtin during mitosis in fibroblasts derived from HD patients. Immunofluorescence imaging in fibroblasts, a more physiologically relevant cell line, confirm results observed in ST*Hdh*$^{Q7/Q7}$. Phosphorylated huntingtin at serines 13 and 16 are located at the centrosomes of the mitotic spindle in the far left images. Huntingtin modified at serine 421 highlights not only the centrosomes but also the midplate during cell division. Experiment was conducted by Dr. Nicholas Caron.

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