Primary Cilia Dynamics, Morphology and Acetylation are Abnormal in Huntington's Disease Cell Models

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Abstract

The primary cilium is a singular signaling organelle found on most mammalian cell types. Dysfunction of the primary cilium or associated structures form a group of genetic disorders called ciliopathies. Recently, Huntington's disease (HD), a monogenetic neurodegenerative disorder, was classified, at least in part, as a ciliopathy. How the primary cilium contributes to the pathogenesis of HD is the focus of this work. We demonstrate that huntingtin localization to the basal body or primary cilium is dependent on the phosphorylation status of serine residues 13 and 16. Furthermore, we demonstrate that, compared to controls, HD cell models have an increased number of cells with a primary cilium and that these cells have higher presence of huntingtin within the ciliary compartment. The primary cilia that form in HD cell lines demonstrate abnormal dynamics and morphology with bulging tips, characteristic of defective retrograde trafficking. We also demonstrate that alpha tubulin acetyltransferase 1 (αTAT1) expression and localization is increased in the primary cilium of HD cell lines. Subsequently, the primary cilium of HD cell lines are highly acetylated when compared to controls. These data support that primary cilia structure, ciliogenesis and ciliome are altered in HD.

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List of Abbreviations

αTAT-alpha tubulin acetyltransferase

5HTr6-serotonin receptor 6

ATP-adenosine triphosphate

BBS-Bardet-Biedl syndrome

CAG-polyglutamine

cAMP-cyclic adenosine monophosphates

CK1□-casein kinase 1

CLS-cilial localization signal

CoA-coenzyme A

CPC-ciliary pore complex

CSF-cerebral spinal fluid

DNA-deoxyribonucleic acid

Dvl2-Dishevelled 2

FGF-fibroblast growth factor

GCN5-general control of amino-acid synthesis 5

GPCR-G-protein coupled receptors

HAP-huntingtin associated protein

HDAC6-histone deacetylase 6

HD-Huntington's disease

HEAT-huntingtin, elongation factor 2, subunit of protein phosphatase 2A, TOR1

Hef1-human enhancer of filamentation 1

HIP-huntingtin interacting protein

HIPPI-huntingtin interacting protein 1 protein interactor

Htt-huntingtin

IFT-intraflagellar transport

IT15-interesting transcript 15

JATD-Jeune asphyxiating thoracic dystrophy

KAP-kinesin associated protein

kDa-kilodalton

KIF-kinesin like protein

MCHR1-melanin concentrating hormone receptor 1

N17-amino terminal 17 amino acids of huntingtin

NES-nuclear export signal

NLS-nuclear localization signal

NPC-nuclear pore complex

Odf2-outer dense fiber 2

OSM1-osmotic avoidance abnormal 1

PCM1-periocentriolar material 1

PC-polycystin

PCP-planar cell polarity

PDGFα-platelet derived growth factor alpha

PKD-polycystic kidney disease

Plk2-polo-like kinase 2

PTMs-post-translational modifications

PY-NLS-proline-tyrosine nuclear localization signal

RanGTP-Ras-related nuclear protein-guanosine triphosphate

SEPT2-septin 2

SHH-Sonic hedgehog

SSR3-somatostatin receptor 3

ST*Hdh* –Striatal progenitor cells

TSA-trichostatin A

WT – Wild type

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Declaration of Academic Achievement

Tanya Woloshansky performed all experiments and data analysis.

- Dr. Randy Atwal created the huntingtin fragments corresponding to amino acids 1-586 with phosphomimetic or alanated mutations.

Chapter 1: Introduction

1.1 The Primary Cilium

"Centralgeissel", meaning central flagella, was the first description provided by Swiss anatomist Karl Wilhelm Zimmerman, in 1898, to define solitary cilia on mammalian cells. (Zimmerman, 1898). While Zimmerman did postulate a sensory function for this novel organelle, it was largely ignored by the scientific community for almost a century, regarded as a vestige of evolution. Since the organelle first appears during development, it was renamed the primary cilium in 1968 (Sorokin, 1968). Clinical significance was attributed to primary cilia in 2000, when it was discovered that the causative proteins of Polycystic Kidney Disease (PKD) localized there (Pazour, G., et al. 2000). Considerable investigation has since been devoted to the primary cilium, as it is key in the pathogenesis of a vast number of human diseases such as polycystic kidney disease, Bardet-Biedl syndrome and Joubert Syndrome.

The primary cilium is a singular, non-motile, hair-like extension from the apical surface of the cell into the extracellular environment. By extending into the extracellular space, the primary cilia utilizes membrane receptors to recognize extracellular ligands, thus initiating signaling that is relayed to the cell body. While the primary ciliome (protein content of the cilia) varies between cell types, membrane receptors initiating Wnt (Corbit et al. 2007), Platelet derived growth factor alpha (PDGFα) (Schneider et al. 2007), Sonic hedgehog (SHH) (Rohatgi et al. 2007), hippo cascades (Habbig, et al. 2011), integrin (Praetorious et al. 2004) and fibroblast growth factor (FGF) (Leick et al. 2001) signaling pathways are commonly found within the primary cilium's membrane. In order to properly relay these signals, the primary cilium has developed an evolutionarily conserved structure, a modified nuclear pore, to exclude most cellular proteins – allowing entry only to proteins key to its function.

1.1.1 Structure

The primary cilium is 200-300nm wide and between 2-10µm long (Han et al. 2010). Mechanical integrity of the primary cilia is provided by an axoneme – a microtubule scaffold composed of 9 microtubule doublets arranged in a ring, termed "9+0" arrangement. Each doublet is composed of two subfibers: a longer outer tubule, designated 'A' tubule, composed of 13 tubulin filaments and an inner 'B' tubule containing only 9 filaments (Peterson et al. 2012). Nexin linkages tether peripheral microtubule doublets to each other, (Palmblad et al. 1984) occurring every 86nm along the microtubule (Meeks et al. 2000). In order to develop the axoneme structure, the primary cilium develop from a basal body – a modified centriole composed of nine microtubule triplets arranged in a barrel. Once the centriole attaches to the apical plasma membrane, it is known as a basal body.

Basal body ultrastructure differs from a centriole with the addition of striatal rootlets (Hagiwara et al. 2000), a basal foot and transition fibers (Dawe et al. 2007). Striatal rootlets are necessary for structural integrity and long term survival of the primary cilium (Yang et al. 2006). Under the control of planar cell polarity (PCP) proteins, the basal foot establishes basal body and polarization of the cilium (Kunimoto et al. 2012). Transitional fibers were originally assumed only to attach and stabilize the basal body to the plasma membrane. However, transitional fibers are necessary in docking intraflagellar transport (IFT) (Deane et al. 2001) particles. As well, various proteins, such as septin 2 (SEPT2), are targeted to transition fibers (Hu et al. 2010). Finally, the transition fibers radiate from the basal body in a wing-like manner, excluding particles with a diameter larger than 60nm (Anderson et al. 1972).

As protein synthesis does not occur in primary cilia, an additional means of entry is necessary to traffic proteins larger than diffusion will allow. While the primary cilium lacks a membrane separating the ciliary compartment from the cytosol, a transition zone exists which allows entry to only a subset of cellular proteins. The transition zone is composed of the transitional fibers and the ciliary necklace (Hammond et al. 2008). The

ciliary necklace is composed of several parallel strands of cytoskeletal linkages attached to the membrane (Cordier et al. 1979). In order to bypass the physical barrier of the transition zone, it was hypothesized that the primary cilium may employ transport mechanisms similar to the nucleus. With concern to nuclear import, a steep ras-related nuclear (Ran) protein gradient exists with elevated levels of RanGTP in the nucleus with lower levels in the cytosol (Gorlich et al. 2003). In the cytosol, importin α forms a heterotrimer with importin β and the cargo protein, which transverses the nuclear pore (Pemberton et al. 2005). Within the nucleus, increased RanGTP levels dissociate the heterotrimer and the importin proteins are recycled back to the cytoplasm (Pemberton et al. 2005). Recently, it was discovered that not only does a Ran gradient exist across the primary cilium transition zone but it is crucial to import of KIF17, a kinesin-II motor (Dishinger et al. 2010), into the primary cilium. Similar to the nuclear localization signal (NLS), cilia localization signal (CLS) was discovered in the carboxyl terminus of KIF17, crucial for interaction with importin β. Importin proteins interact via FG repeats on nucleoporin proteins, allowing them to shuttle cargo through the nuclear pore complex (NPC) meshwork. With the demonstration that several different nucleoporins localize to the basal body (Kee et al. 2012), it is currently hypothesized that a modified NPC, termed ciliary pore complex (CPC), operates active transport into the primary cilium. Analogous to the nucleus, primary cilia maintains a specific ciliome through active transport across the CPC or through passive diffusion of smaller proteins.

Adjacent to the transition zone, a depression of the plasma membrane occurs which surrounds the base of the primary cilium. Known as the ‘ciliary pocket’, this depression is an endocytic domain characterized by clathrin coated pits (Ghossoub et al. 2010) used to recycle materials from the primary cilium. The ciliary pocket may also serve as docking point for Golgi-derived vesicles transporting ciliary proteins. In photoreceptor cells, it has been shown that Golgi-derived vesicles delivered IFT proteins to the periciliary membrane (Sedmak et al. 2010). In addition to vesicle trafficking, the ciliary pocket connects the primary cilium to the actin cytoskeleton, possibly aiding in the positioning the organelle (Benmerah, 2013).

Plasma membrane composition of the ciliary pocket differs from that which ensheathes the body of the primary cilium. In line with the role of transmitting extracellular cues, the primary cilium's membrane is studded with specific membrane receptors and ion channels. Comparable to other regions of the membrane enriched with receptors, the lipid composition of the ciliary membrane is similar to that of a lipid raft with high levels of sterols, sphingolipids and glycolipids (Tyler et al. 2009).

Lacking vesicles and protein synthesis, the primary cilium requires a trafficking system necessary for the movement of proteins from the transition zone to the distal tip (Nauli et al. 2003). Intraflagellar transport (IFT), a near universally conserved process, is crucial for the assembly, maintenance and disassembly of the organelle. Two complexes composed of the 19 IFT proteins, IFT A (retrograde) and IFT B (anterograde), interact with molecular motors, adaptor and cargo proteins to deliver materials along the cilia's length (Gerdes et al. 2009). Molecular motors from the Kinesin-2 family, specifically kinesin-II and KIF17, associate with IFT B complex to move cargo away from the cell body towards the distal end of the cilium (Green, 2010). Kinesin II is composed of two motor subunits, KIF3a (90kDa) and Kif3b (85kDa), and an additional 100kDa kinesin associated protein (KAP) subunit performing protein-protein interactions (Cole, 2003). KIF17, a homolog of *Caenorhabditis elegans* OSM3 (osmotic avoidance abnormal 3), localizes mainly to middle and distal sections of the primary cilium (Pedersen et al. 2008). Knockouts of either Kif3a or Kif3b abolish primary ciliogenesis while knockouts of KIF17 simply result in shortened primary cilia (Gerdes et al. 2009). The IFT A complex associates with dynein-2, which orients towards the cell body from the tip of the primary cilium. Through this retrograde motion, the IFTA-dynein-2 complex removes turnover material and resorbs the primary cilium. Dynein-2 knockouts result in primary cilium with tips bulging with accumulating turnover products (Rajagopalan et al. 2009).

IFT proteins are not exclusive to the primary cilium, as they are often involved in trafficking throughout the rest of the cell. Additionally, IFT proteins are common culprits for ciliopathies, where mutants often abolish ciliogenesis or alter primary cilia structure. For example, mutations in IFT80, a protein found in IFT complex B, results in Jeune

asphyxiating thoracic dystrophy (JATD), as it alters SHH signaling (Beales et al. 2007). Ciliopathies caused by IFT proteins tend to be particularly severe as both primary and motile cilia are affected.

1.1.2 Functions of primary cilia

Relaying cues from the extracellular environment is the defining function of the primary cilium however, the translation of such signals and resultant behaviour is dependent on cell type and the primary ciliome. Densely studded along the primary cilium's membrane are receptors responsible for initiating several signaling pathways. Activation of these signaling factor families allows the primary cilium to be involved in a number of cellular activities such as migration (Schneider et al. 2010), differentiation (Ezratty et al. 2011), apoptosis, division (Christensen et al. 2008), homeostasis (Schneider et al. 2005), intracellular calcium regulation (Praetorius et al. 2001), neurogenesis (Han et al. 2008), and cell polarity. Though best known for the coordination of multiple signaling pathways, primary cilia are also able to sense photo, mechanic (Nauli et al. 2003) and osmotic (Liu et al. 2003) stimuli.

Signal transduction is dependent on the positioning of receptors and downstream effector molecules such as G-protein coupled receptors (GPCR), cyclic nucleotides (cAMP), calcium secondary messengers and ion-gated channels. A subset of GPCR, exclusive to the membrane of primary cilia, contain a C-terminal motif (RVxP) that is necessary for this localization (Berbari et al. 2008). Somatostatin receptor 3 (SSR3), serotonin receptor 6 (5HTr6) and melanin concentrating hormone receptor 1 (MCHR1) all contain the RVxP and localize only to the primary cilium – making them useful for visualizing the primary cilium. Different cell types contain a different subset of ciliary proteins thus specializing its role in each cell. For example, MCHR1 is found in brain regions involved in feeding and energy balance, such as the hippocampus (Berbari et al. 2008)

Translating the primary cilium's cellular function to organ or systemic level is complicated. However, primary cilia are necessary for proper brain (Spassky et al. 2008), heart (Clement et al. 2009) and bone development (Haycraft et al. 2008). Specifically, the primary cilium has been implicated in cognitive processes such as learning and memory (Einstein et al. 2010), likely by promoting neurogenesis of hippocampal neurons. The importance of proper primary cilium function is highlighted through its involvement in the maintenance of tissues and organs. While the effect of primary cilia on kidney and eye maintenance is well documented, implications for primary cilia in neurodegeneration (Jackson, 2012), bone/joint deterioration (Morcuendo et al, 2010), skin/hair maintenance, etc., (Exratty et al. 2011) have recently arisen. Since primary cilia are found on most cell types, it is likely that disruption in cilia signaling will affect many organs and tissues.

1.1.3 Ciliogenesis

Primary cilia are post-mitotic structures, generally assembled in G1 phase of the cell cycle and maintained through G0 and S phase. Disassembly of the primary cilium occurs before the cell cycle, as the basal body must be recycled to serve a centriole during division (Seeley and Nachury, 2010).

Following the cell cycle, cilia formation or ciliogenesis is initiated once the centrioles have divided. A primary cilium can only be nucleated by the mother centriole, as the daughter centriole is still structurally and functionally immature (Ishikawa et al. 2005). For example, the mother centriole develops distal appendages, composed of proteins such as outer dense fiber 2 (Odf2), ninein, ε-tubulin and centriolin (Ishikawa et al. 2005), required for basal body docking. As the basal body begins migrating towards the apical plasma membrane, it traffics along the actin-myosin network (Boisvieux et al. 1990). A Golgi-derived vesicle attaches to the migrating basal body, near the distal appendages (Moser et al. 2010). Depending on cell type, extension of the axoneme from the basal body through IFT can occur during migration or after docking to the membrane (Dawe et al. 2007). Nearby vesicles fuse with the migrating vesicle-basal body complex,

providing more membrane material to grow with the extending axoneme. Eventually, the growing vesicle fuses with the plasma membrane and the distal appendages of the basal body attach to surrounding cytoskeleton to form the transition zone.

What guides the basal body during migration is not fully understood. Actin and myosin are key in both migration and docking of the basal body to the membrane as treatment with inhibitors (e.g. cytochalasin D) prevent ciliogenesis (Boisvieux et al. 1990). Additionally, members of the Wnt Planar cell polarity pathway such as Fuzzy, Inturned (Park et al. 2006) and Disheveled (Park et al. 2008) are employed in docking the basal body to the membrane. Once docked, IFT begins or continues to extend the axoneme, which will then undergo post-translational modifications (PTMs) by various enzymes.

The Wnt signaling pathway is also involved in the disassembly of the primary cilium. To enter the cell cycle, centrioles must be regenerated to serve as organizing centers for the mitotic spindle. Cell cycle activators casein kinase 1 epsilon (ck1ε) is involved in the phosphorylation of Disheveled 2 (Dvl2) (Bryja et al. 2007). Once phosphorylated, Dvl2 can interact with another cell cycle activator polo-like kinase 2 (plk1) to stabilize a complex between Aurora A kinase and human enhancer of filamentation (Hef1) (Lee et al. 2012). Aurora A kinase, also known for promoting and participating in mitosis (Marumoto et al. 2005), localizes to the basal body and promotes cilia disassembly (Pugacheva et al. 2007). Aurora-A kinase, in association with HEF1, when activated phosphorylates histone deacetylase 6 (HDAC6), which will remove acetyl groups from α tubulin – necessary for disassembly (Pugacheva et al. 2007). Dismantling the axoneme is likely performed by IFT, but another mechanism could be responsible as it has been observed that entire axonemes are shuttled into the cytoplasm prior to disassembly (Rieder et al. 1979).

1.1.4 Acetylation

Once IFT has extended the scaffold of the axoneme, individual tubulin subunits are subject to PTMs. PTMs help to determine the protein interacting partners at the primary cilium's axoneme (Bulinski, 2009). Additionally, some PTMs are thought to increase half-life and stability of the axoneme (El Zein et al., 2009). Tubulin in primary cilia is modified through glutamylation (Lee, 2012), glycylation (Dossou, 2007), detyrosination (Wheatley, 1996) and acetylation (Pugacheva, 2007). Acetylation has been particularly helpful to the study of primary cilia, as immunofluorescence using anti-acetylated tubulin antibodies are commonly used to identify it. While other ciliary PTMs occur in the C-terminal region of tubulin proteins, acetylation occurs on lysine 40, an amino terminal residue which localizes within the lumen of the microtubules. Recently, the major N-acetyltransferase of alpha tubulin was discovered (Shida et al. 2010).

In 2010, a novel N-acetyltransferase was discovered that is universally and exclusively conserved in ciliated organisms (Shida et al. 2010). Named alpha tubulin acetyltransferase 1 (αTAT1), the protein was found not only to be necessary for lysine 40 acetylation but also for normal primary cilium dynamics. Structurally, αTAT1 is similar to a histone acetylating protein, general control of amino-acid synthesis 5 (GCN5), which activates transcription by promoting protein binding to DNA (Friedmann et al. 2012). αTAT1 contains a folded core composed of 5 beta sheets and 1 alpha helix, which is structurally similar to the active site of GCN5. Within the core are two highly conserved amino acids, aspartate 157 and cysteine 120, crucial for αTAT1's catalytic activity as mutations in either residue abolish acetyltransferase activity (Friedmann et al. 2012). It has been suggested that the catalytic mechanism of αTAT1 involves either aspartate 157 or cysteine 120 to act as a general base to deprotonate the lysine residue. The resultant neutral lysine side chain can perform a nucleophilic attack on the bound acetyl-CoA molecule (Taschner et al. 2012).

In recent years, acetylation sites have been discovered on numerous proteins, fueling curiosity to the function of this modification (Choudhary et al. 2009). To test

whether acetylation is necessary for the stability of microtubules, one group generated αTAT1 knockout mice (αTAT1-/-). The αTAT1 knockouts were successful as acetylated tubulin staining was abolished from primary cilia or axonal microtubules. Interestingly, after treatment with nocodazole, a compound which interferes with the polymerization of microtubules, fibroblast cells from αTAT1 knockout mice retained some polymerized microtubules whereas cells from WT mice did not (Kalebic et al. 2013). From this work, it appears that αTAT1 is the main lysine 40 acetyltransferase for alpha tubulin and acetylation may decrease stability of microtubules.

Another publication from the same group suggested that αTAT1 may destabilize microtubules independent of its acetyltransferase activity. Overexpression of αTAT1 was associated with destabilized microtubules using live cell movies where cells were transfected with α tubulin or a protein specific to the end of the primary cilia, end binding protein 1 (Kalebic et al. 2013). In both cases, transfection of αTAT1 resulted in higher primary cilium dynamics by increasing growth and tubulin exchange at the end of microtubules. Additionally, this group also reported that these effects were present even with a catalytically null mutant of αTAT1 (Kalebic et al. 2013), suggesting that other domains within the protein may be affecting the stability of microtubules.

1.1.5 Motile Cilia

Primary cilia are structurally similar to motile cilia, another hair-like organelle that are found on the surface of cells. Motile cilia are found on terminally differentiated tissue in the upper respiratory tract, oviduct, digestive system, cerebral ventricles and embryonic node (Satir et al. 2008). Through synchronous and directional beating, motile cilia are able to propel fluids (Brennen et al. 1977) down aforementioned passages to help clear mucous, determine right-left body symmetry, move ovules down fallopian tubes and maintain cerebral spinal fluid flow. While similar in structure, primary cilia tend to be 2-10μm long whereas motile cilia vary from 50-200um (Salathe, 2007). In contrast to the singular primary cilia, hundreds of motile cilia can be found on a single cell.

Although structurally similar, motile cilia require an additional microtubule pair and accessory proteins to carry out the beating mechanism. An additional microtubule doublet is located in the center of the nine microtubules doublet barrel characteristic of the primary cilium. Termed a "9+2 arrangement", the central microtubule doublet rotates to initiate the beat (Omoto et al. 1999). Radial spoke proteins attached to the central microtubule doublet transduce the motion to the peripheral doublets, by interacting with associated dynein arm proteins (Palmblad et al. 1984). Inner dynein arm and outer dynein arm protein complexes are necessary for motile ciliary beating (Goodenough et al. 1985). Each complex is composed of a combination of heavy, medium and light chain proteins. ATP hydrolysis occurs at the location of the heavy chain proteins, crucial for the motility of the cilia (Pazour et al. 2006). Primary ciliary dyskinesia (PCD), a rare condition marked by respiratory infections, situs inversus, hearing loss and decreased fertility, is commonly caused by mutation in dynein arm proteins (Olbrich, 2002). PCD is an example of a ciliopathy, a pathology caused by irregularities in the cilia or associated structures, where motile cilia are solely responsible. Ciliopathies are often caused by genes affecting both primary and motile cilia.

1.1.6 Ciliopathies

Ciliopathies are a broad class of rare, genetic diseases that affect the structure or function of the cilia. Rather than localizing to the primary cilium, most disease causing cilial proteins are found at the base, basal body, and transition zone or associated structures. Since primary cilia are found on all mammalian tissues, ciliopathies often exhibit overlapping symptoms and effect multiple organ systems. Common ciliopathic symptoms include retinitis pigmentosa (Estrada-Cuzcano et al. 2012), renal, hepatic and pancreatic cystic disease (Torres et al. 2006), polydactyl (Merrill et al. 2009), situs inversus (Olbrich et al. 2002), cognitive impairment (Einstein et al. 2010), skeletal defects (Kolpakova-Hart et al. 2007), obesity (Davenport et al. 2007) and brain defects (Bandano et al. 2006). Primary ciliopathies attracted attention with the discovery that polycystin 1

(PC1) and polycystin 2 (PC2), the causative proteins for some polycystic kidney diseases (PKD), which localize around the basal body (Pazour et al. 2000). Intracellular calcium concentration is regulated by PC1 and PC2, as the proteins sense flow through mechanosensation or flow-induced bending of the primary cilium (Nauli et al. 2003). Knockout studies for PC1 and PC2 demonstrate altered calcium regulation and cyst formation. PKD is unique as a ciliopathy as symptoms are generally contained to renal structures. Oftentimes, symptoms are widespread throughout the body as primary cilia across various cell types can share crucial proteins.

Bardet-Biedl Syndrome (BBS), a "model" ciliopathy, is systemic and marked with developmental and degenerative phenotypes which affect 1:16,000 (Zaghloul et al., 2009). Common symptoms of BBS include polydactyl, mental retardation, developmental delay, retinal degeneration and renal cystic disease. (Davis et al. 2012). BBS is caused by mutations in one of 12 different genes that are highly conserved in ciliated organisms (Nachury et al. 2007). Protein products of the causative genes localize to the axoneme, basal body and pericentriolar area. Of the 9 BBS proteins, 7 complex to form the BBSome, which has been implicated in IFT assembly and turnaround (Wei et al. 2012), as well as localization of G-coupled protein receptors to the primary cilia in neurons (Berbari et al. 2008). Symptoms can vary depending on which of the causative genes are affected.

As interest in the primary cilium increased over the last decade, it is unsurprising that many diseases have been classified as ciliopathies. In addition, numerous diseases have been redefined to include cilia in the pathogenesis although other, independent defects exist. Intense interest in primary ciliary dynamics and signaling in many cancers followed the discovery that several cancers do not assemble the organelle. Breast (Yuan et al. 2010), pancreatic (Hassounah et al. 2013), ovarian (Egeberg et al. 2012) and other cancer cell populations showed decreased populations of cells with a primary cilium. Furthermore, it was demonstrated that cancer cell populations for ovarian and chondrocytes had increased activation of the SHH signaling pathway – an activator of cell proliferation. (Ho et al. 2012). Additionally, an increase of Wnt signaling was observed in

pancreatic cancers, which also demonstrated decreased percentage of cells with a primary cilium (Hassounah et al. 2013). Altogether, the primary cilium may regulate signaling pathway activation and its loss may contribute to ectopic signaling pathway activation and cancer pathogenesis.

Huntington's disease (HD) is a dominant, genetic, neurodegenerative disease marked by loss of the striatum, cerebral cortex and other neuronal populations. HD may be classified as a "part" ciliopathy as defects have been observed in the ciliogenesis and structure of the primary cilium in animal and cellular models (Keryer et al. 2011). Huntingtin, the causative protein of HD, and huntingtin associated protein 1 (HAP1), an adaptor protein which binds to dynactin p150 subunit, are crucial for transporting periocentriolar material 1 (PCM1) in ciliogenesis (Keyer et al. 2011). If PCM1 fails to localize near the basal body, lower incidence of primary cilia formation occurs and those that do are shorter in length. Furthermore, in HD mouse models, an increase in PCM1 localization to the form basal body was observed, coupled with an increase in the percentage of cells in a population with a primary cilium. Finally, the primary cilia found on HD cell models were, on average, about 1µm longer than those in control populations (Keyer et al. 2011).

Primary cilia aside, mouse models demonstrate a role for motile cilia in the pathogenesis of HD as well. In the cerebral ventricles of HD mouse models, cerebral spinal fluid (CSF) flow was disturbed as motile cilia were disoriented and unorganized (Keryer et al. 2011). As a result, the migration of neuroblasts from the subventricular region was affected, decreasing adult neurogenesis at the olfactory bulb. Thus, HD is an example of ciliopathy that affects both primary and motile cilia.

Often, proteins implicated in ciliopathies can be found at a single cilium structure. Huntingtin, however, can be found at three. Huntingtin is involved in trafficking proteins to the primary cilium but it is also found at the basal body and within the cilium's compartment (Maiuri et al. 2013). In fact, phosphorylation of 2 key serine residues (13 and 16) within the highly conserved amino terminal 17 amino acid region

(N17) determines huntingtin's location in regard to the primary cilium. If both serine residues are phosphorylated, huntingtin localizes to the basal body (Maiuri et al. 2013). Upon removal of the two phosphates, huntingtin is able to move within the compartment of the primary cilium. The role that huntingtin is playing within the cilium has yet to be elucidated and it is unknown whether dysfunctional ciliary signaling occurs in HD. The purpose of this work is to explore the aberrations in the primary cilium's structure or function that are observed in HD and to identify other proteins that may be involved.

1.2 Huntington's Disease

In 1872, a young scientist by the name of George Huntington first characterized Huntington's disease (HD) in his paper "on chorea" (Huntington, 1872) . Chorea, defined as involuntary, jerky dance-like movements is one of the characteristic symptoms of HD. Patients suffer through a multitude of motor, cognitive and psychological symptoms. Symptom onset generally initiate around middle age at 35-45 years old. Currently, no effective treatment or cure is available and patients pass away around 15-20 years after diagnosis.

1.2.1 Huntingtin Gene

HD is a monogenic, autosomal dominant disorder caused by a polyglutamine (CAG) expanded repeat in huntingtin. Discovered in 1993, huntingtin is product of the interesting transcript 15 (IT15) gene located on chromosome 4 (MacDonald et al. 1993). In the amino terminal region of the protein lies a polyglutamine tract of between 7-35 repeats in healthy individuals. When the polyglutamine tract exceeds the threshold of 36 glutamine repeats, the individual is affected. Patients typically exhibit incomplete penetrance of disease until the polyglutamine tract extends to 40 repeats (Langbenh et al.

2004). Juvenile HD is observed in patients with 60 or more repeats, which is marked with exacerbated symptoms and early onset (less than 20 years of age) (Nance et al. 2001).

HD pathology is readily observable at organ, cellular and subcellular levels. Neuronal degeneration occurs primarily in the striatum and deep layers of the cerebral cortex (neuronal layers V and VI) (Reiner et al. 1988). As the disease progresses, the globus pallidus, thalamus, hypothalamus and white matter diminish as well (Ciarmiello et al. 2006).

1.2.2 Structure of Huntingtin

Huntingtin is ubiquitously expressed in all tissues but enriched in both neurons and testes (Engelender et al. 1997). Composed of 3,144 amino acids, Huntingtin is a multidomain protein with a molecular weight of 350kDa (Martin et al. 1986). The first 17 amino acids, which are highly conserved, form an alpha helix which is the site of PTMs such as acetylation and phosphorylation (Atwal et al. 2007). Phosphorylation on serine 13 and 16 of the N17 region is been sufficient to reverse HD symptoms in mouse models (De Pardo et al. 2012). The N17 region is also necessary for cellular localization as it contains a nuclear export signal (NES) and targets huntingtin to specific membranes (Atwal et al. 2007).

Immediately downstream of the N17 region lies the polyglutamine tract, which acts as a flexible hinge bringing the two flanking regions, N17 and a polyproline tract, into close proximity (Caron et al. 2013). When the polyglutamine tract exceeds 36 repeats, it displays multiple, rigid conformations which reduce the flexibility of the tract. Adjacent to the polyglutamine tract lies the polyproline region. Associated with stabilizing the structure of the amino terminal region, the polyproline tract also serves as an interaction site for many of huntingtin's binding partners (Kim et al. 2010). Following the polyproline region lies huntingtin's proline-tyrosine nuclear localization signal (PY NLS), found between amino acids 174-207. (Desmond et al. 2013) To assist in nuclear-

cytoplasmic shuttling, huntingtin contains an additional NES, which is found in the C-terminus around amino acid 2400 (Xia et al. 2003).

A considerable percentage of the C-terminus of huntingtin form huntingtin, elongation factor 3, a subunit of protein phosphatase 2A and TOR1 (HEAT) repeats (Li et al. 2006). HEAT repeats consist of approximately 40 amino acids which form hydrophobic α helices that assemble into an extended superhelix (Takano et al. 2002). Several interacting partners, such as HAP1 (Engelender et al. 1997), Hip1 (Wanker et al. 1997) and Hip14 (Huang et al., 2011) associate with huntingtin's HEAT repeats. While it is unknown how many HEAT repeats exist in huntingtin, bioinformatics techniques estimate somewhere around 36 (Huang et al. 2011).

1.2.3 Huntingtin Localization and Function

The huntingtin protein is crucial in embryonic development and a number of cellular processes. Mouse homozygous knock-out studies for huntingtin alleles result in death on day 7.5 of embryonic development. (Cattaneo 2001). Huntingtin's importance is also underlined by the number of functions it undertakes within the cell. Although additional functions are likely to be uncovered, to date huntingtin is implicated in regulation of transcription (i.e. bone derived neurotrophic factor or BDNF) (Hebb et al. 2004), facilitating vesicular transport (Velier et al. 1998), actin remodeling and stress response (Munsie et al. 2011), inhibition of apoptosis (Saudou, 1998), excitotoxicity protection (Zeron et al. 2001), and promoting protein-protein interaction through scaffolding and endocytosis (Velier et al. 1998). To carry out such a wide range of functions, huntingtin localizes to several organelles within the cell including the nucleus (Saudou et al. 1998), the endoplasmic reticulum (Atwal et al. 2007), the Golgi (Strehlow et al. 2007), endocytic vesicles (DiFiglia et al. 1995), the primary cilium (Maiuri et al. 2013), the plasma membrane (Kegel et al. 2005) and the mitochondria (Orr et al. 2008).

1.3 Project Rationale

Investigation into huntingtin's role in the primary cilium was initiated when it was discovered that huntingtin's PY-NLS is recognized by transportin, which mediates its transfer into the nucleus (Desmond et al. 2013). Recognition of a PY-NLS on other proteins by transportin was implicated in mediating cilial entry (Dishinger et al. 2010). Therefore, transportin may be moving huntingtin into the primary cilium to perform an unknown role.

Huntingtin localization to the primary cilium in a manner dependent on phosphorylation at serine 13 and 16. We therefore hypothesize that huntingtin may function at the primary cilium and that this function is disrupted in mutant huntingtin expressing cells.

Chapter 2: Materials and Methods

2.1 Fusion Proteins:

1-586 Q17 huntingtin YFP

1-586 Q17 EE huntingtin YFP

1-586 Q17 AA huntingtin YFP

*The preceding three fragments were created by a past lab member, as described.

5HTr6-eGFP (kind gift from Dr. Mykytyn)

Pmkate2-αTAT1 (Addgene)

2.2 Primary Antibodies

Mouse anti-acetylated tubulin (Covance) 1/50 (IF)

Rabbit anti-gamma tubulin (Abcam) 1/200 (IF)

The following N17 antibodies were generated by a previous lab member, as described (Atwal et al. 2011).

Rabbit anti-N17 WT (New England Peptides): 1/200 (IF)

Rabbit anti-N17 S13PS16P (New England Peptides): 1/200 (IF)

Anti-αTat1 (Sigma): 1/500 (WB)

2.3 Secondary Antibodies (AlexaFluor, Molecular Probes):

Goat anti-mouse 594 nm: 1/500 (IF)

Goat anti-rabbit 488 nm: 1/1000 (IF)

Goat anti-rabbit horse radish peroxidase-conjugated secondary antibody: 1/10,000 (WB)

2.4 Solutions

Antibody dilution solution: 2% fetal bovine serum (FBS), 0.5% Tween 20 in phosphate buffered saline (PBS)

Blocking solution: 2% FBS in PBS

NP-40 lysis buffer: 150nM NaCl, 50mM Tris-HCl 1% NP-40, pH 8.0

Permeabilization solution: 2% FBS, 0.5% Triton X-100 in PBS

PBS - 10mM NaH_2PO_4, 0.14M NaCl, 1mM EDTA, pH 7.4

Sodium dodecyl sulfate (SDS) running buffer – 25mM Tris, 0.192M Glycine, 0.1% (v/w) SDS

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

TBST: TBS with 0.05% Tween 20

2.5 Cell Culture

Mouse ST*Hdh*^{Q7Q7}, ST*Hdh*^{Q7Q111}, and ST*Hdh*^{Q111Q111} cell lines, (a generous gift from Dr. Marcy MacDonald, Harvard), progenitor cells from striatum, were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 33˚C with 5% CO_2 and atmospheric O_2. In order to ensure clonal selection, 0.3mg/mL G418 (Gibco) was added to the cell's growth medium.

Two lines of human derived fibroblast cell lines were used. One fibroblast cell line was derived from an unaffected female where both huntingtin alleles had CAG tracts that were less than 36 repeats (Coriell, catalog number 02149). The other fibroblast cell line was from an affected female who had one mutant huntingtin allele with 70 CAG repeats and a second allele with only 15 CAG repeats (Coriell, catalog number 21756).

Fibroblast cell lines were grown in Eagle's Minimum Essential Medium with Earle's salts (Invitrogen) supplemented with 2mM L-glutamine (SOURCE) and 15% fetal bovine serum (Invitrogen).

For imaging purposes, cells were grown in a 2cm tissue culture dish with a cover slip bottom. If grown for passaging of or for protein extraction from cells, 10cm dishes were used.

2.6 Immunofluorescence Analysis

Paraformaldehyde fixation: Cells were washed twice in PBS, before being fixed in 4% paraformaldehyde for 20 minutes. After being washed in PBS, the cells were then permeabilized in 0.5% Triton X-100 with 2% FBS in PBS at 4˚C for 15 min. Cells were washed again in PBS before three incubations in blocking solution, each lasting for 15 min. Primary antibodies were diluted as indicated above in antibody solution and incubated for 2 hrs at room temperature or overnight at 4˚C. Cells were subject to blocking solution again, 3 times for 15 min. Secondary antibodies were diluted in antibody solution before applied to cells for 1 hr. Following this, cells were washed in PBS 3 times for 10 min.

Methanol fixation: Cells were washed twice in PBS before being fixed and permeabilized on ice cold methanol at -20˚C for 15 min. Cells were washed twice and placed in blocking solution for 3 intervals of 15 min. All steps following this are the same as stated for paraformaldehyde fixation.

2.7 Protein Extraction

All cells were grown on 10 cm dishes up to 90% confluency. Cells were washed twice in PBS before being collected with a rubber scraper. Cells were precipitated via centrifuge using at the speed of 1200g for 5 min. The pellet was then resuspended in

NP40 lysis buffer with protease inhibitors (Sigma Aldrich). Samples were placed and ice and vortexed every 2 min for the next 10 min. Samples are then placed in the centrifuge and precipitated at 14000g for 10 min at 4˚C. Afterwards, the supernatant was collected.

2.8 SDS Page and Western Blot

Equal amounts of each protein samples were run on a 12% SDS-polyacrylamide gel, transferred to PVDF membrane (PALL), and blocked with 5% (w/v) milk in TBST. The membrane was blotted with anti- αTat1 at 1/500, overnight at 4°C. Rabbit anti-mouse HRP-conjugated secondary antibody (ab97046; Abcam) was applied at 1/1000 for 2 hr at room temperature. Blots were developed with Immobilon Western Chemiluminescent Substrate (Millipore) and imaged on a DNR Microchemi Western blot imager.

2.9 Drug treatments

Compounds DMAT and BMS-345541 were used at concentrations optimized previously (Atwal et al. 2011).

2.10 Microscopy

The Nikon TE200 inverted widefield epifluorescence microscope using a 60X oil immersion Pan fluor objective (Nikon, Japan) was used for imaging fixed cells and live cell movies. The light source was a 175W Xenon Lamp with ND2 or ND4 neural density filters (Sutter Instruments). Filter sets and dichroic filters used were obtained from Semrock (Rochester, NY) in a Sutter Instruments filter wheel (Novato, CA). Images were acquired using a Hamamatsu Orca ER digital camera (Hamamatsu Photonics). NIS Elements version 4.0 (Nikon, USA), was used for image acquisition. ImageJ (NIH) was utilized for image analysis.

2.11 Live Cell Imaging

Live cell visualization was done using the Delta T4 heated stage, lid and objective system (Bioptechs). Cells were seeded and treated in 0.17 mm delta T dishes (Bioptechs). Cells were maintained at 33°C using the heated stage and objective and visualized at 100× plan apochromat oil N.A1.3. Images were recorded once every 60 s for 2 hr.

2.12 Statistical Analysis

All statistical analysis was performed using SigmaPlot 11.0. Determination of statistical significance was **calculated** as a Student's *t* test.

Chapter 3: Results

3.1 N17 Regulates Huntingtin Localization to the Primary Cilium

To determine if huntingtin localizes within the primary cilium, endogenous localization was examined in both unmodified and phosphorylated (serine 13 and 16 in N17 region) states. Primary cilia on from ST*Hdh* cells were visualized via acetylated α tubulin antibodies (Figure #2, panels a, d, g, and k). Cells were co-stained with antibodies against unmodified (Figure #2, panels h, k, i, l) or phosphorylated N17 (Figure #2, panels b, c, e, f). Localization of unmodified huntingtin was found within the ciliary compartment whereas phosphorylated N17 staining appeared restricted to the basal body.

To further analyze huntingtin localization, an N-terminal 586-amino acid fragment of huntingtin with a normal polyQ tract (17 repeats) was fused to YFP (1-586 Huntingtin YFP) and expressed in ST*Hdh* cells. Cells were stained with antibodies against acetylated α tubulin to identify cilia. In this case, WT exogenous huntingtin was localized only to the basal body, not within the primary cilium (Figure 3, row 1). To test if phosphorylation alters huntingtin localization in this context, phosphomimetic and alanine substitution mutants were expressed. Serine 13 and 16 of the 1-586 Huntingtin fragment were both changed to either glutamate (phosphomimetic) or alanine (Atwal et al. 2011). Glutamate's negative charge mimics that of a phosphorylated serine. Alanine is used to mimic unphosphorylated serine as both have a neutral charge. Similar to the endogenous phosphorylated huntingtin staining, the phosphomimetic mutant localized only to the base of the primary cilium, at the basal body (Figure 3, row 2). The fragment with alanine substitutions, to mimic unphosphorylated huntingtin, failed to localize to any primary cilia structures (Figure 3, row 3). While phosphomimetic mutants demonstrate the same localization as the staining of the phosphorylated antibody, the WT huntingtin fragments were unable to enter primary cilia as IF with N17 unphosphorylated antibody predicted. Therefore, results from protein overexpression of the 1-586 huntingtin fragments cannot add further evidence to the IF finding that unmodified huntingtin is able to enter the compartment of the primary cilium but not phosphorylated huntingtin.

3.2 Kinase Inhibition Does Not Significantly Alter Huntingtin Localization

Two pathways that alter huntingtin N17 phosphorylation were inhibited. DMAT (2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole), a specific inhibitor of casein kinase 2 (CK2), removes the phosphate groups on serine 13 and 16 of huntingtin, which should amplify its signal in the primary cilia. To contrast this, BMS-35541, a IkB inhibitor which increases the phosphorylation of huntingtin, was applied. Cells were incubated in BMS (10mM), DMAT (10mM) or serum free medium for four hours before fixation and immunofluorescence with acetylated α tubulin antibody and a conjugated antibody raised against unmodified N17. After quantification, there was no significant difference between drug treated and control cells (Figure 4).

3.3 Increased Huntingtin Observed in the Primary Cilium in Some HD Models

Hypophosphorylation of mutant huntingtin occurs in HD at serine 13 and 16. Since only unmodified huntingtin appears to enter the primary cilia, HD cells may have increased huntingtin localization within the primary cilium. To test this hypothesis, ST*Hdh*^{Q7Q7}, ^{Q7Q111} and ^{Q111Q111} cells were immunostained with anti-acetylated α tubulin before undergoing direct immunofluorescence with a conjugated antibody raised against unmodified N17 (Figure 5). As hypothesized, huntingtin localization to primary cilia in ST*Hdh*^{Q7Q111} and ^{Q111Q111} was significantly higher than ST*Hdh*^{Q7Q7}. Therefore, increased mutant huntingtin in the primary cilium could result from hypophosphorylation.

To ensure that the increased huntingtin observed in mutant ST*Hdh* cell lines was not an artifact of the immortalized cell line, the same experiment was carried out in primary human fibroblasts. Strikingly, huntingtin localization into the ciliary compartment did not differ significantly between patient and control cells (Figure 6). This result demonstrates that increased huntingtin localization in mutant cell types could be

due to altered biology of the immortalized cell line or a finding specific to neuronal derived cells.

3.4 Number of Cells in a Population With a Primary Cilium is Increased in HD Models

To determine if huntingtin could play a role in ciliogenesis, primary cilia formation was assayed in the ST*Hdh* cell models. All three striatal cell types ST*Hdh* ^{Q7Q7}, ^{Q7Q111} and ^{Q111Q111} were quantified for the presence of primary cilia. Cells were cultured in serum free media (to arrest cells) for 24 hours prior to fixation. Cells were then co-stained with acetylated α tubulin antibody and a γ tubulin antibody to mark the basal body. There was a significant increase in the percentage of cells with primary cilia in ST*Hdh*^{Q111Q111} compared to wild type ST*Hdh*^{Q7Q7} cells (Figure 7). Therefore, huntingtin's role in regulating the number of primary cilia could be altered in the presence of mutant huntingtin.

To confirm that the increase of ciliogenesis was not a product of immortalized cell lines, primary human fibroblast cells were assayed. Human fibroblast cells were cultured in media with serum before fixation, followed by co-staining with acetylated α tubulin antibody and a γ tubulin antibody. Similar to the ST*Hdh* cell lines, fibroblasts derived from the HD patient had a higher percentage of cells with primary cilia compared to the control (Figure 8).

3.5 Altered Primary Cilium Dynamics and Morphology Observed in HD Cell Models

Since primary cilia on HD cells are longer on ST*Hdh*^{Q7Q111} and ^{Q111Q111} than ST*Hdh*^{Q7Q7} (Keyer et al. 2011), further investigation into the dynamics of the primary cilium was performed. ST*Hdh*^{Q7Q7}, ^{Q7Q111} and ^{Q111Q111} were transfected with 5HTr6-eGFP fusion protein to mark the primary cilium. After 48 hour incubation time, primary cilium dynamics were assayed in live cells. While primary cilia on ST*Hdh*^{Q7Q7} appeared mainly

static, with no branching, shrinking or breaking, primary cilia on ST*Hdh*^{Q7Q111} and ^{Q111Q111} had gross abnormalities. ST*Hdh*^{Q7Q111} and ^{Q111Q111} commonly exhibited large bulges at the tips of primary cilia as well as branching and excessive elongation/shrinking. Growing and shrinking of primary cilia was quantified by measuring the cilium length every 10 min and calculating the absolute value of the change from previous length. Significant differences in dynamics of primary cilia were recorded between ST*Hdh*^{Q7Q7} and ST*Hdh*^{Q7Q111} cells only. Thus, in heterozygous HD ST*Hdh* cell models, primary cilia are more dynamic phenotype than WT ST*Hdh* cell models.

In order to quantify cilia abnormalities visualized in live cells, 5HTr6-eGFP was transfected into all three ST*Hdh* cell lines before fixation. Cells were co-stained with γ tubulin, to identify primary cilia. Bulges observed at the tips of primary cilia were quantified by measuring the width of the ciliary tip and subtracting the average width of the stalk. Tips of primary cilium found in ST*Hdh* cell types^{Q7Q111} and ^{Q111Q111} demonstrated significant bulging when compared to ST*Hdh*^{Q7Q7}. This bulging tip phenotype suggests that retrograde trafficking may be defective in HD cell models.

3.6 Primary Cilia on HD Cells Show Increased Acetylated Tubulin Levels

Due to altered primary cilium dynamics observed in HD cell models, the level of acetylation, which may be a marker of stability, was tested. ST*Hdh* ^{Q7Q7}, ^{Q7Q111} and ^{Q111Q111} cells were immunostained with acetylated α tubulin antibodies and the intensity of staining was analyzed using ImageJ. Acetylated tubulin levels observed in ST*Hdh* ^{Q7Q111} were significantly higher than in ST*Hdh*^{Q7Q7} cell types (Figure 11). Thus, altered primary dynamics observed in HD cell models could result from hyperacetylation of primary cilia.

3.7 Whole Cell Extracts of HD Cells Reveal αTAT1 Levels are Increased

Increased acetylation of primary cilia in HD models could be due to increased αTAT1 expression. To examine endogenous levels of αTAT1, immunoblotting was performed on protein samples from HD patient and control fibroblast cells, and the ST*Hdh* cell lines. The Western blot, using an antibody against αTAT1, demonstrated in both ST*Hdh* and fibroblast HD models had amplified signal compared to WT models (Figure 12). Therefore, increased acetylation of primary cilia in HD models could result from increased αTAT1 expression.

3.8 Levels of αTAT1 Fusion Protein are Higher in the Primary Cilium of HD Cells

Increased αTAT1 protein levels could result in increased acetylation of ciliary tubulin. To examine whether higher levels of αTAT1 localize to the primary cilia in HD cell models, pmkate2-αTAT1 fusion proteins were expressed in ST*Hdh* cells. Cells were then stained with acetylated α tubulin antibody to mark primary cilia. In accordance with acetylated tubulin levels, a significant increase of αTAT1 protein expression was also observed in primary cilia of both heterozygous STH*dh* cell models ($p = 0.039$) relative to WT STH*dh* cells (Figure 13). Thus, increased expression and localization of αTAT1 could result in hyperacetylation of primary cilium in vitro.

Overexpression of the pmkate2-αTAT1 construct was also attempted in the primary fibroblast cell line. Similar to huntingtin, αTAT1 expression levels are higher in neurons, with relatively low expression in other cell types, as demonstrated by Western blot. However, once transfected, fibroblasts expressing αTAT1 were not viable, especially control cells. Cytotoxicity of the pmkate2- αTAT1 occurred in ST*Hdh*^{Q7Q7} as well but to a lower extent.

3.9 Leptomycin B Does Not Alter the Number of Cells In a Population With a Primary Cilium or The Localization of Huntingtin Within the Ciliary Compartment

Primary cilium import uses proteins and mechanisms similar to the nucleus. Little is known about exporting proteins from the primary cilia; however, export may occur through chromosome regional maintenance 1 (Crm1), a nuclear export protein. Huntingtin has two NES' which could mediate its removal from the primary cilia via crm1. Leptomycin B inhibits crm1 mediated export from the nucleus through a covalent modification of a key cysteine residue. (Kudo et al. 1998) The three ST*Hdh* cell lines were incubated in 10uM Leptomycin B for 2 hr before fixation with methanol. Cells were then co-stained with anti-acetylated α tubulin and an N17 antibody directly conjugated to Alexa 488 fluorophore. In ST*Hdh*^{Q7Q7}, Leptomycin B treatment did not change huntingtin localization within the primary cilium. In ST*Hdh*^{Q7Q111} and ST*Hdh*^{Q111Q111}, a significant decrease of huntingtin in the primary cilium was observed (Figure 5). Thus, in HD cell models, Leptomycin B appears to decrease huntingtin localization.

Chapter 4: Discussion and concluding remarks

4.1 Huntingtin Localizes into Primary Cilia with Unmodified N17 Region

Huntingtin appears to localize to the ciliary compartment only if unmodified at serine residues 13 and 16. Phosphorylation at these crucial serine residues target huntingtin to centrioles, thus it is reasonable that it would also target it to the basal body, a modified centriole. As well, the N17 region of huntingtin has previously been identified as a targeting sequence, directing the protein to the membrane of the ER. Therefore, the phosphorylated form may be targeted to the basal body and at appropriate times, phosphates are removed from the N17 region and huntingtin is directed into the primary cilia compartment or ciliary membranes.

The presence of phosphorylated huntingtin at the base of the primary cilium was confirmed by the transfection of phosphorylation mimetic mutants. However, neither alanine substituted nor wild type amino terminal constructs were able to enter the ciliary compartment. Given the large size of huntingtin, transfection of a fluorescently tagged full length construct is difficult. Transfections of fragments are often used to bypass this problem but they may not reflect the exact behavior of the full length protein. Since the carboxy-terminal portion of huntingtin is involved in many protein-protein interactions and scaffolding, this region could bind a protein that contains a CLS, shuttling both proteins into the primary cilia. Alternatively, it is possible that huntingtin's NLS is indeed a CLS but requires regions downstream of 174-220 to promote entry. In the case of KIF17, the NLS also acts as a CLS but it alone is insufficient to shuttle into the compartment of the primary cilium (Dishinger et al. 2010). Finally, there are other cases where a known ciliary protein, confirmed by immunofluorescence, cannot enter the primary cilia when overexpressed as a fusion protein (Dishinger et al. 2010).

4.2 Huntingtin May Be Involved in the Regulation of Primary Ciliogenesis

Using the assay to quantify the number of primary cilia with acetylated α tubulin and γ tubulin (to identify the basal body), both HD cell models had a significantly higher percentage of cells in a population with a primary cilium when compared to control. Although very little is published regarding huntingtin's actions in the primary cilium, this finding is in agreement with current literature. It is hypothesized that mutant huntingtin concentrates PCM1 at the basal body, which increases the number of cells with a primary cilium. (Keryer et al. 2011).

A higher percentage of cells with primary cilia could result in a multitude of downstream effects. One may speculate that signaling pathways could be amplified since primary cilia concentrate membrane receptors and effector molecules. In HD cell models, the dynamics and morphology of primary cilia are aberrant (discussed below), which could also affect the ability of the primary cilium to properly initiate signaling pathways. If defective, the increased number of primary cilia may still result in diminished or aberrant signaling. Diminished activation of ciliary signaling could result in a number of defects, including increased apoptosis, which is consistent with attenuated SHH initiation (Elamin et al. 2010). Part of mutant huntingtin's toxicity is due to promotion of apoptosis in striatal neurons. In the presence of mutant huntingtin, increased phosphorylation of p53 initiates a cascade that results in the activation of pro-apoptotic genes (Grison et al. 2011). Loss of SHH signaling activates p53-dependent apoptosis in the nervous tissue of zebra fish (Prykhozhij et al. 2010). Of course, the network of proteins interacting with p53 is complex and these processes are likely independent. Nonetheless, performing a signaling assay to determine levels of SHH, Wnt and other transduction pathways initiated at the primary cilium may reveal aberrations and insight to HD pathogenesis.

4.3 Morphology and Huntingtin Localization into Primary Cilia Are Altered in HD Cells

In ST*Hdh*^{Q7Q111} and ST*Hdh*^{Q111Q111} cell models, it appeared that a high percentage of huntingtin localized to the compartment of the primary cilium. Since phospho-huntingtin appears excluded from the primary cilium, it seems logical that HD models may have increased huntingtin presence as hypophosphorylation of serine residues 13 and 16 is characteristic of the disease. Promoting phosphorylation of the N17 region of huntingtin is beneficial and can prevent or even reverse symptoms of disease (Di Pardo et al. 2012). Therefore, the increased huntingtin localization as a result of the disease may be toxic and promoting dysfunction. Indeed, altered primary cilia morphology is observed in HD, including increased length and bulging tips (Figure 9).

In both heterozygous and homozygous ST*Hdh* mutants, the majority of primary cilia contained a bulge at the tip or along the shaft. Defective retrograde trafficking by knocking out dynein 2 results in a similar phenotype with bulging tips (Merrill et al. 2009). In addition, some BBS mutants, which are involved in IFT, have cilia with bulging tips filled with protein and vesicles (Shah et al. 2008). Hence, mutant huntingtin models may have defective retrograde trafficking in primary cilia and defective protein turnover.

Primary cilia from HD ST*Hdh* cell lines were more dynamic than WT controls. Our live cell assay compared ciliary length between frames to measure cilial growing/shrinking. ST*Hdh*^{Q7Q111} cells were more dynamic compared to the other two types, although we cannot infer that it is due to the corresponding increased acetylation. Microtubules may be destabilized as a result of increased αTAT1 presence in the primary cilium as the presence of the enzyme has been demonstrated to increase microtubule dynamics in vitro (Kalebic et al. 2013). Therefore, it is hard to speculate the exact cause of the increased ciliary dynamics demonstrated by HD STH*dh* cell models without further investigation (described below). Increased tubulin dynamics in HD cell models could have clinical significance as growing/shrinking of microtubules requires ATP (Bershadsky et al. 1981). As HD models exhibit decreased ATP, this phenotype may be especially burdensome (Moshel et al. 2012).

Immortalized ST*Hdh* cells and the primary fibroblast cells differed in terms of huntingtin localization to the primary cilium. Where ST*Hdh* demonstrated a significant increase in huntingtin staining in the cilium of HD cells compared to WT, primary fibroblasts did not. The patient's primary fibroblasts did have a slight increase compared to the control but this was not significant. Several reasons may account for this discrepancy aside from aberrant biology of the ST*Hdh* cell line. First, since huntingtin is enriched in neuronal cells, the overall expression of huntingtin is higher in ST*Hdh* cells when compared to human fibroblasts. This feature could make detecting differences between cell types easier in neuronally derived cell lines. Also, the patient fibroblasts contain a mutant huntingtin allele with 60 CAG repeats, where the ST$Hdh^{Q7/Q111}$ cell line has 111 CAG repeats. In HD, larger repeat lengths correspond to exacerbated symptoms. For example, in the assay developed to determine the percentage of cells in a population with a primary cilium, a larger difference existed between the controls and HD cell lines of ST*Hdh* versus the difference observed between the HD patient and control in the fibroblasts. Increased ciliogenesis in ST*Hdh* HD models compared to the WT control could relate to the fact that the ST*Hdh* huntingtin allele contains almost double the number of repeats. Thus, primary fibroblast cells from juvenile patients, having much longer repeat lengths, with very severe symptoms may exhibit a significant difference between mutant and control huntingtin localization.

4.4 Primary Cilia in HD Models Have Increased αTAT1 and Acetylation Levels

αTAT1's discovery in 2010 was exciting to researchers interested in the primary cilia as it is necessary for the acetylation of ciliary axonemes in mice. Originally it was expected that the primary cilia of HD cells would have decreased acetylated α tubulin as it has been reported that HD brains are hypoacetylated (Dompierre et al. 2010). As described above, primary cilia of ST*Hdh* HD models had higher acetylation levels than the WT STHdh^{Q7Q7}. This discrepancy may be accounted for because the antibody that the authors used was for a different isoform of α tubulin. The authors used an antibody

against α tubulin 1a, whereas the primary cilium is enriched with isoforms 1b and 2. Recent work has also suggested that an inverse relationship may exist between acetylation levels of primary cilia and cytoplasmic microtubules (Berbari 2013).

Since the primary cilia of HD cells had higher levels of acetylation, it was predicted that αTAT1 localization in cilia would be increased. From whole cell extracts, immunoblots against αTAT1 produced a stronger signal in both HD ST*Hdh*'s and fibroblast cells compared to WT controls. From this data, it could be hypothesized that acetylated α tubulin levels would be higher in HD models, which is contrary to current literature on the subject. However, the presence of mutant huntingtin could affect the activity of αTAT1. Four lysine residues on αTAT1 increase catalytic activity if acetylated. While αTAT1 can acetylate itself, other acetyltransferases may help regulate activity. An enzymatic activity assay should be performed to determine if αTAT1 from HD and WT cell lines have similar catalytic rates.

Another caveat to the quantification of αTAT1 via fusion protein localization is the lack of an appropriate control. For other organelles, the fluorescent vector with an empty multiple cloning site (in this case pmkate2) is transfected as a control. Studying the primary cilia is difficult as fluorescent proteins can simply diffuse across the transition zone into the cilia. To confirm results, immunofluorescence with a conjugated αTAT1 antibody should be performed.

4.5 Primary Ciliogenesis and Huntingtin Localization to Cilia Was Not Affected by Leptomycin B

It was hypothesized that Leptomycin B would increase localization of huntingtin to the primary cilium's compartment. No change was observed in ST*Hdh*^{Q7Q7} and a significant decrease was observed in the case of both STHdh$^{Q7Q111 \text{ and } Q111Q111}$. The decrease of huntingtin presence may have been due to its sequestering into the nucleus.

Leptomycin B also did not alter the percentage of cells in a population with primary cilia. Leptomycin B appeared to deplete huntingtin from the primary cilium in STHdh$^{Q7Q111 \text{ and } Q111Q111}$ without affecting the number of primary cilia present in a population. From finding this, it can be suggested that huntingtin is not required to maintain the primary cilium.

4.6 Small Molecular Kinase Inhibitors Do Not Alter Huntingtin Localization Within Primary Cilia

Huntingtin's N17 region is crucial for proper subcellular targeting to organelles. Relating this to the primary cilium, it was hypothesized that altering the phosphorylation status of serine residues 13 and 16 using small molecules DMAT and BMS-345541 would modify huntingtin's cilial localization. Particularly, CK2 inhibition through DMAT promotes the unmodified form of N17 – which should allow entry within the ciliary compartment. DMAT treatment was not successful in promoting huntingtin's presence in the primary cilium and may result from CK2-dependent targeting to the base of the primary cilia. Nephrocystin, a causative protein for one of the renal cystic diseases, requires phosphorylation of 3 serine residues by CK2 before recognition by the protein which transports it to the cilia (Schermer et al. 2005). Thus, phosphorylation could be necessary for huntingtin accumulation at the basal body before removal of phosphates required for ciliary entry.

While the BMS-345541 treated cells demonstrated lower huntingtin cilial content in comparison to the control, the difference was not significant. Since this experiment was performed in ST*Hdh*^{Q7Q7}, which produce stable primary cilia, the exchange of huntingtin from the primary cilium to the cytosol could be very low. Thus, throughout the 4 hour incubation period, BMS-345541 could have been unable to affect the huntingtin content of primary cilia that were already formed.

Finally, due to the exclusivity of the primary cilium, several conditions for huntingtin or an external signal may be necessary to cross the transition zone. Protein

entry and export mechanisms to the primary cilium remain uncertain. Until further research has been performed, it is difficult to speculate the requirements governing huntingtin entry into the primary cilium.

4.7 Future Directions

Huntingtin accumulation in the stalk of primary cilia was more common in cells that also demonstrated huntingtin accumulation in the nucleus. Huntingtin localizes within the nucleus under cell stress conditions (Atwal et al. 2011), thus cell stress may also trigger huntingtin into primary cilia. Heat shock experiments, subjecting cells to an elevated temperature (i.e. 42°C) for 20-30 minutes would be ideal. Longer incubations at increased temperatures result in the disassembly of primary cilia (Prodromou et al. 2012).

Live cell movies revealed that primary cilia found in STH*dh*^{Q7Q111} and STH*dh*^{Q111Q111} cells may be more dynamic than in the STH*dh*^{Q7Q7} cells however, results were not significant due to the number of cilia assayed. Future assays to test dynamics of the primary cilium`s axoneme should be performed using alpha tubulin fusion proteins. Tubulin fusion proteins would be better because it would reveal exactly what is occurring at the backbone of the cilia rather than the membrane, as assayed with 5HT6-GFP. Furthermore, point mutations at lysine 40, the site of acetylation in alpha tubulin monomers, could be introduced to assess whether acetylation affects the dynamics of the primary cilium.

The relationship between αTAT1 and huntingtin should also be investigated since the former is involved throughout the cell and changes within its association with huntingtin may affect the whole cell. STH*dh*^{Q7Q111}, but not STH*dh*^{Q111Q111}, had significantly higher levels of acetylated tubulin within the primary cilium when compared to STH*dh*^{Q7Q7} even though both STH*dh*^{Q7Q111} and STH*dh*^{Q111Q111} both had higher levels of αTAT1 in the western blot. An enzyme assay of αTAT1 purified from WT and HD cell models may reveal different enzyme activity levels. As well, measuring αTAT`s mRNA

levels using RT-PCR in both WT and HD cells may help explain the increased levels of αTAT in HD cell types.

In order to ensure that the bulging phenotype is not a product of protein overexpression, it would be beneficial to demonstrate a similar phenotype through immunofluorescence. Usually primary cilia are visualized with an acetylated α tubulin antibody, which accentuates the axoneme. This antibody would fail to highlight protein accumulation as tubulin monomers are deacetylated prior to depolymerization (Piperano et al. 1987). Therefore, immunofluorescence with an antibody specific to a GPCR specific to the primary cilium would be important to confirm these findings. Additionally, it would be ideal if these results were tested in primary HD cell types to ensure that this result is not an adaptation acquired by the immortalized cells.

4.8 Concluding Remarks

Huntingtin performs numerous functions throughout the cell, and yet, the list continues to grow. It appears that WT huntingtin regulates primary ciliogenesis and that this ability is attenuated in mutant huntingtin models, as an increase in the number of cells with a primary cilium is observed. Conversely, mutant huntingtin appears to negatively impact primary cilium morphology, possibly by increasing its presence in the ciliary compartment. Additionally, it appears that the primary cilium is highly dynamic in HD cell models, which may stem from the amplified αTAT1 expression and its localization to primary cilia, producing hyperacetylated ciliary axonemes. How the observed defects in primary cilia translate from our cellular models to symptoms and pathogenesis of HD is unknown. However, future research should assay signal transduction pathways in HD models to confirm that these structural and dynamic defects are affecting primary cilia function.

Chapter 5: Figures

Figure 1

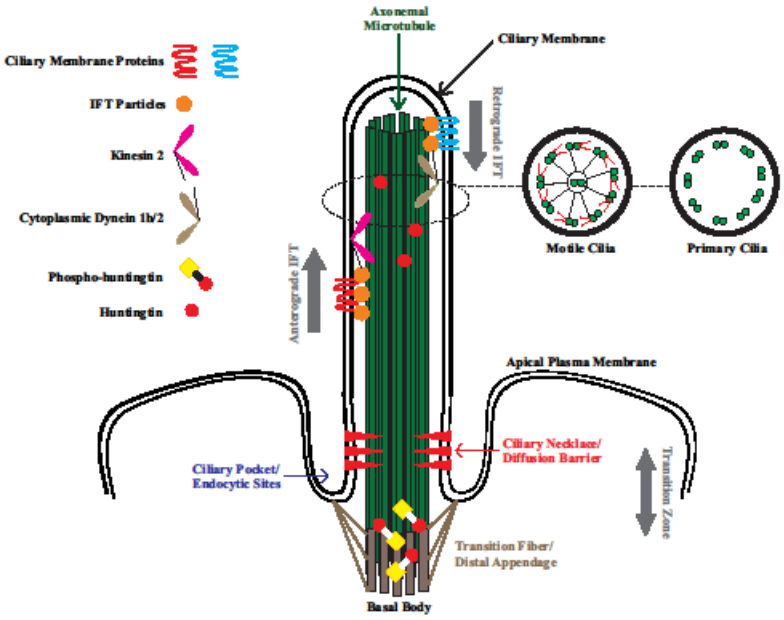

Figure 1: Schematic of the Structure of Primary Cilia. The axoneme of the primary cilium extends from the basal body and is composed of 9 microtubule doublets. The basal body is anchored to the plasma membrane through transition fibers. Distal to the transition fibers lies the ciliary necklace, which helps ensure only necessary proteins are able to enter the primary cilium's compartment. Intraflagellar transport complexes, attached to molecular motors kinesin and dynein, move proteins and other materials necessary for cilia maintenance along microtubules. When phosphorylated at serine 13 and 16, huntingtin appears to associate with basal body and is not found within the primary cilium. If serine 13 and 16 are unmodified, huntingtin is able to move into the compartment of the primary cilium. Adapted from Green et al., (2010) with permission.

Figure 2

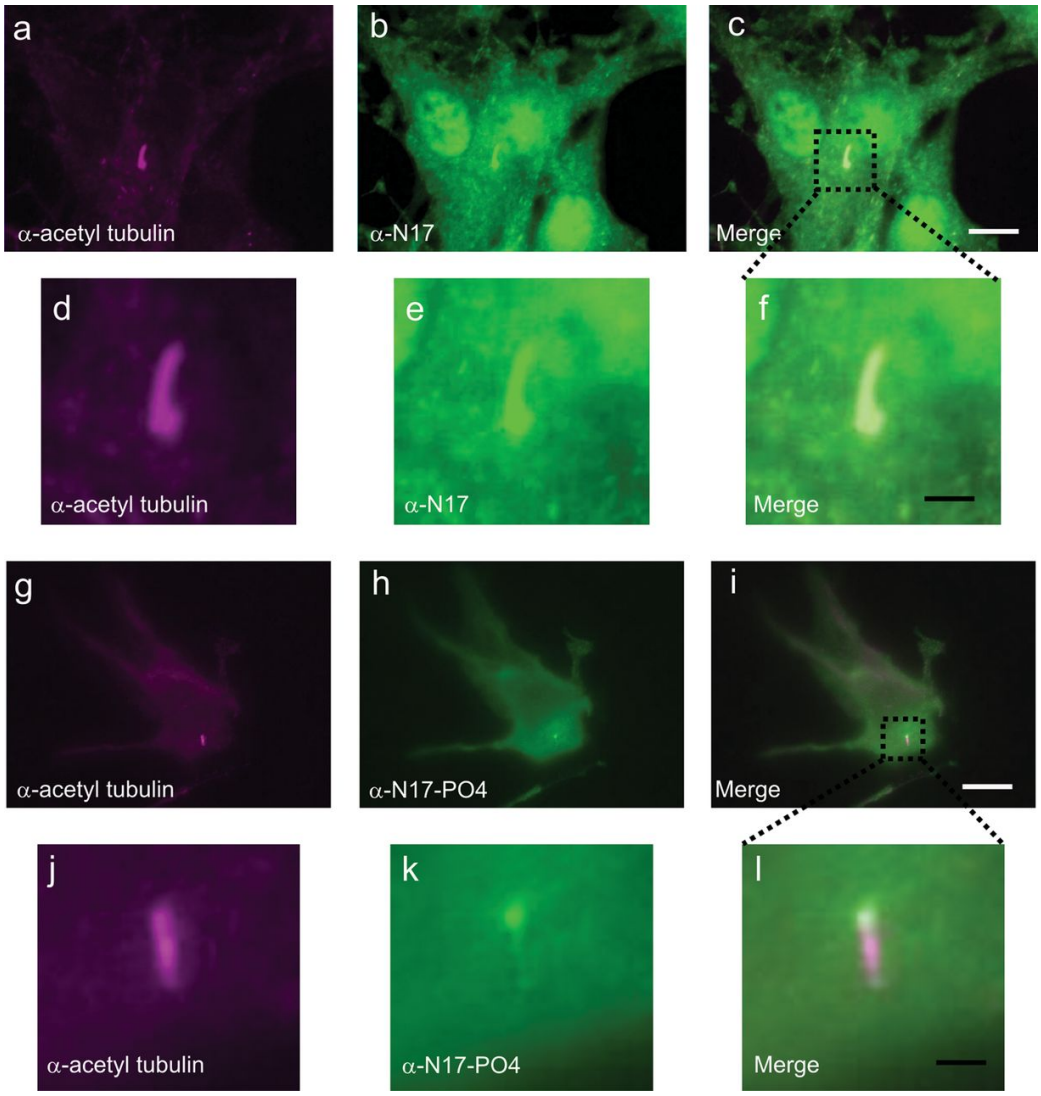
a
α-acetyl tubulin
b
α-N17
c
Merge
d
α-acetyl tubulin
e
α-N17
f
Merge
g
α-acetyl tubulin
h
α-N17-PO4
i
Merge
j
α-acetyl tubulin
k
α-N17-PO4
l
Merge

Figure 2: Phosphorylation Status of Huntingtin's Amino Terminal Specifies Localization between Basal Body and Ciliary Compartment. ST*Hdh*^{Q7Q7} cells were immunostained against acetylated alpha tubulin to visualize primary cilia (a,d,g,j). Next, cells were co-stained with an antibody specific to endogenous huntingtin that is phosphorylated N17 (h,k,I,l) or unmodified N17 (b,c,e,f). Images demonstrate unmodified endogenous huntingtin enters the primary cilium whereas huntingtin phosphorylated at N17 is confined to the basal body. White scale bars are 10µm and black scale bars are 2µm. Used with permission from Maiuri et al., 2013.

Figure 3

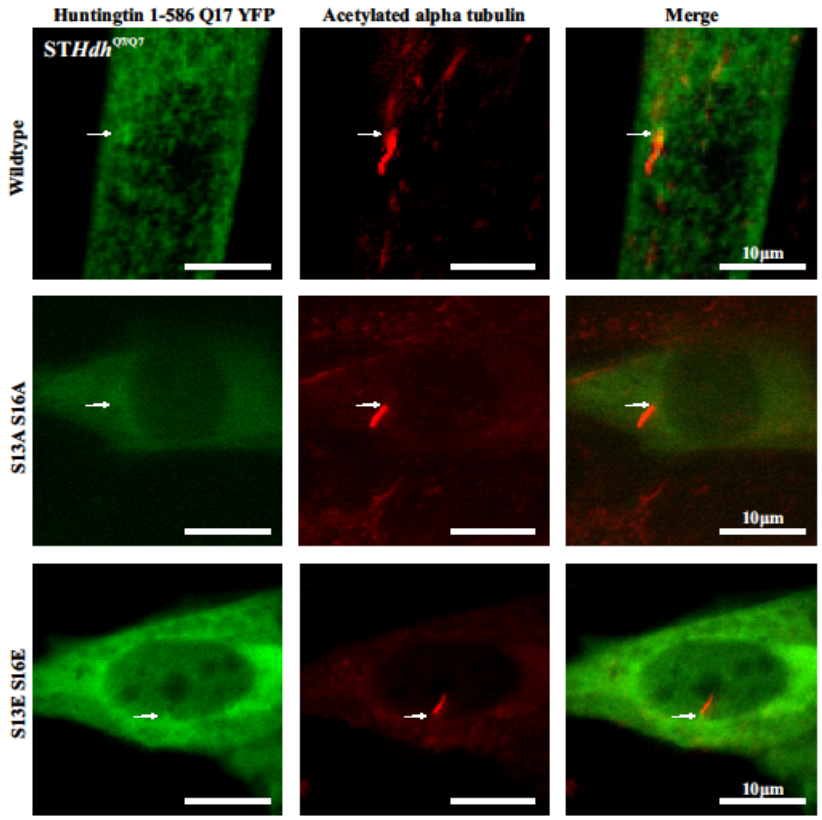
Huntingtin 1-586 Q17 YFP
Acetylated alpha tubulin
Merge
STHdh Q7/Q7
Wildtype
S13A S16A
S13E S16E
10µm
10µm
10µm

Figure 3: Transfection with the 1-586 Fragments Verify Localization of Phosphorylated Huntingtin at the Basal Body. ST*Hdh*$^{Q7/Q7}$ cells were transfected with amino terminus fragments of huntingtin consisting of the first 586 amino acids. WT, phosphomimetic (glutamate) substitution or alanine point mutations were produced at serine 13 and 16 in the 1-586 huntingtin fragment. Once fixed, cells were stained with an antibody against acetylated α-tubulin. As exemplified by the first row, transfection of 1-586 Huntingtin-YFP fragments localize only to the basal body. Examination of row two demonstrates that the 1-586 huntingtin fragment with alanine substitutions fails to localize to any ciliary structures. The third row demonstrates that the phosphomimetic fusions of 1-586 huntingtin-YFP, localized to the basal body.

Figure 4

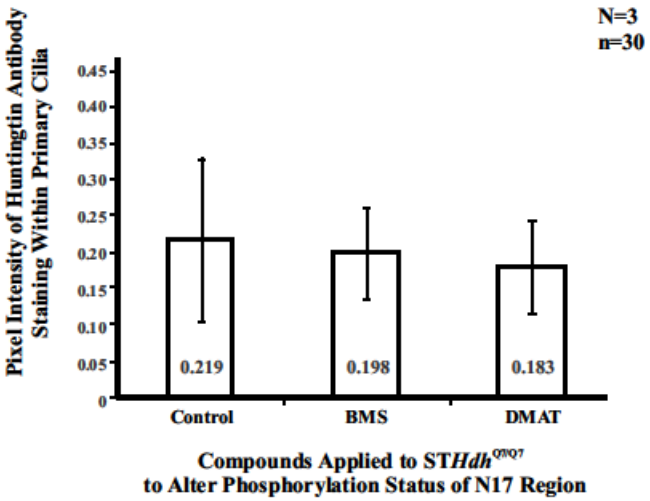
N=3
n=30
Pixel Intensity of Huntingtin Antibody Staining Within Primary Cilia
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
0.219
0.198
0.183
Control
BMS
DMAT
Compounds Applied to STHdhQ7/Q7 to Alter Phosphorylation Status of N17 Region

Figure 4: Kinase Inhibition Through Small Molecules Fail to Alter N17 Huntingtin Localization to Primary Cilia. ST*Hdh*$^{Q7/Q7}$ cells treated with BMS or DMAT (both at 10μM for 4 hours) compounds before being fixed and co-stained against acetylated α-tubulin and unmodified endogenous N17. No significant difference was observed (*p values were 0.76 for DMAT vs. control and 0.85 for BMS vs. control, N=3) between the control and BMS/DMAT treated cells when comparing huntingtin localization into the primary cilium. N=3. Error bars represent standard deviation.

Figure 5

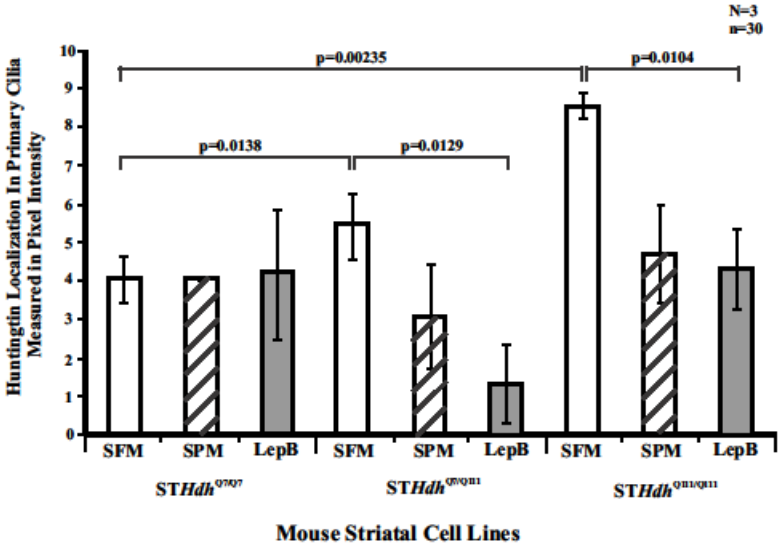
N=3
n=30
p=0.00235
p=0.0104
p=0.0138
p=0.0129
Huntingtin Localization In Primary Cilia
Measured in Pixel Intensity
0
1
2
3
4
5
6
7
8
9
10
SFM
SPM
LepB
SFM
SPM
LepB
SFM
SPM
LepB
STHdhQ7/Q7
STHdhQ9/Q111
STHdhQ111/Q111
Mouse Striatal Cell Lines

Figure 5: Huntingtin Localization into the Primary Cilium is increased in HD Cell Models but not with Application of Leptomycin B. ST*Hdh*$^{Q7/Q7}$, ST*Hdh*$^{Q7/Q111}$ and ST*Hdh*$^{Q111/Q111}$ were each culture in regular media for 24 hours before being transferred to one of three conditions. Cells were subject to a 2 hour incubation in serum free media (SFM), serum positive media (SPM) or a 5uM Leptomycin B treatment. Cells were then fixed and co-stained for acetylated α-tubulin and unmodified endogenous N17 antibody directly conjugated to a 488 fluorophore. Cells were then analyzed by ImageJ to determine huntingtin localization under the three conditions. There was a significant difference in unmodified endogenous huntingtin localization between ST*Hdh*$^{Q7/Q7}$ and ST*Hdh*$^{Q7/Q111}$ (*p value = 0.0138, N = 3) as well as between ST*Hdh*$^{Q7/Q7}$ ST*Hdh*$^{Q111/Q111}$ (*p value = 0.00235, N=3). Leptomycin B treated cells showed unmodified N17 endogenous huntingtin localization was also significantly decreased in both ST*Hdh*$^{Q7/Q111}$ (*p value = 0.0129, N=3) and ST*Hdh*$^{Q111/Q111}$ (*p value = 0.0104, N=3), but not ST*Hdh*$^{Q7/Q7}$ (p value = 0.931). Error bars represent standard deviation.

Figure 6

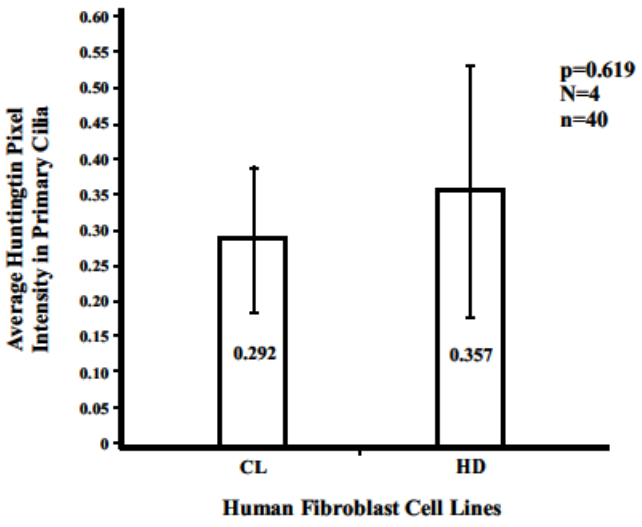
Average Huntingtin Pixel Intensity in Primary Cilia
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
0.292
0.357
p=0.619
N=4
n=40
CL
HD
Human Fibroblast Cell Lines

Figure 6: Huntingtin Localization to Primary Cilia in Human Fibroblasts.
Fibroblasts from an HD patient (1 expanded allele with 60 repeats) and an environment control (CL) were assayed for endogenous huntingtin, with unmodified N17 antibody, localization into the primary cilium. Similar to ST*Hdh* cells, after fixation cells were immunostained against acetylated α-tubulin and unmodified endogenous N17 antibody directly conjugated to a 488 fluorophore. Pixel intensity analysis with ImageJ showed no significant difference between localization of huntingtin to CL or HD (p value = 0.619). Error bars represent standard deviation.

Figure 7

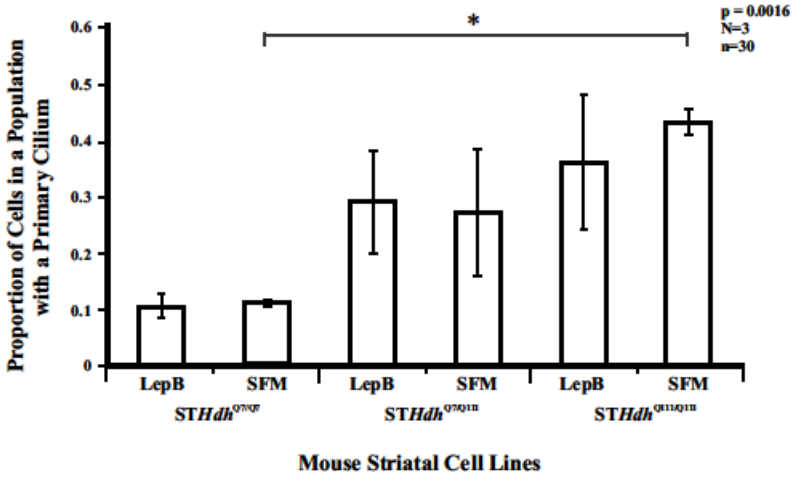
p = 0.0016
N=3
n=30
*
Proportion of Cells in a Population with a Primary Cilium
0.6
0.5
0.4
0.3
0.2
0.1
0
LepB
SFM
LepB
SFM
LepB
SFM
STHdhQ7/Q7
STHdhQ7/Q111
STHdhQ111/Q111
Mouse Striatal Cell Lines

Figure 7: HD Cell Models Have a Higher Percentage of Primary Cilia in a Population of Cells, which is not affected by Leptomycin B Treatment. ST*Hdh*$^{Q7/Q7}$, ST*Hdh*$^{Q7/Q111}$ and ST*Hdh*$^{Q111/Q111}$ were cultured and fixed before they were immunostained for acetylated α-tubulin and γ tubulin antibodies. After quantification, HD cell models demonstrated a significant increase in the number of cells in a population with a primary cilium. Between ST*Hdh*$^{Q7/Q7}$ and ST*Hdh*$^{Q111/Q111}$, the *p value is 0.0016. Between ST*Hdh*$^{Q7/Q7}$ and ST*Hdh*$^{Q7/Q111}$, the difference was not significant.

Alternatively, application of Leptomycin B for 2 hours at 5uM did not affect the number of primary cilia formed in any of the three cell types.

Figure 8

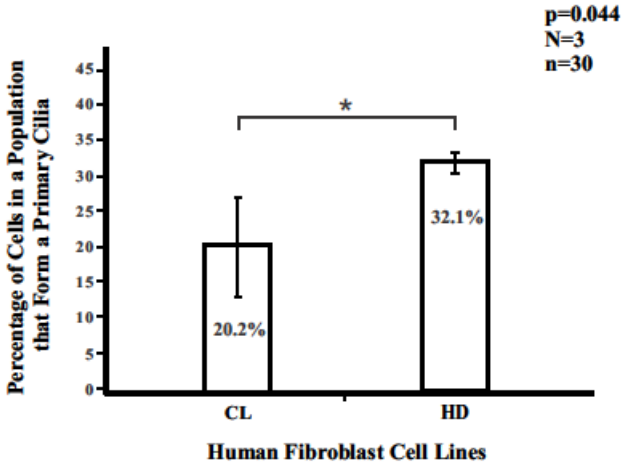
p=0.044
N=3
n=30
*
Percentage of Cells in a Population that Form a Primary Cilia
45
40
35
30
25
20
15
10
5
0
20.2%
32.1%
CL
HD
Human Fibroblast Cell Lines

Figure 8: Percentage of Human Fibroblast Cells that Form Primary Cilia. Similar to the assay above, using acetylated α tubulin and γ tubulin antibodies to identify primary cilia, a significant difference was observed between WT (CL) fibroblasts and mutant (HD) fibroblasts. (*p=0.044, N=3). Error bars represent standard deviation.

Figure 9

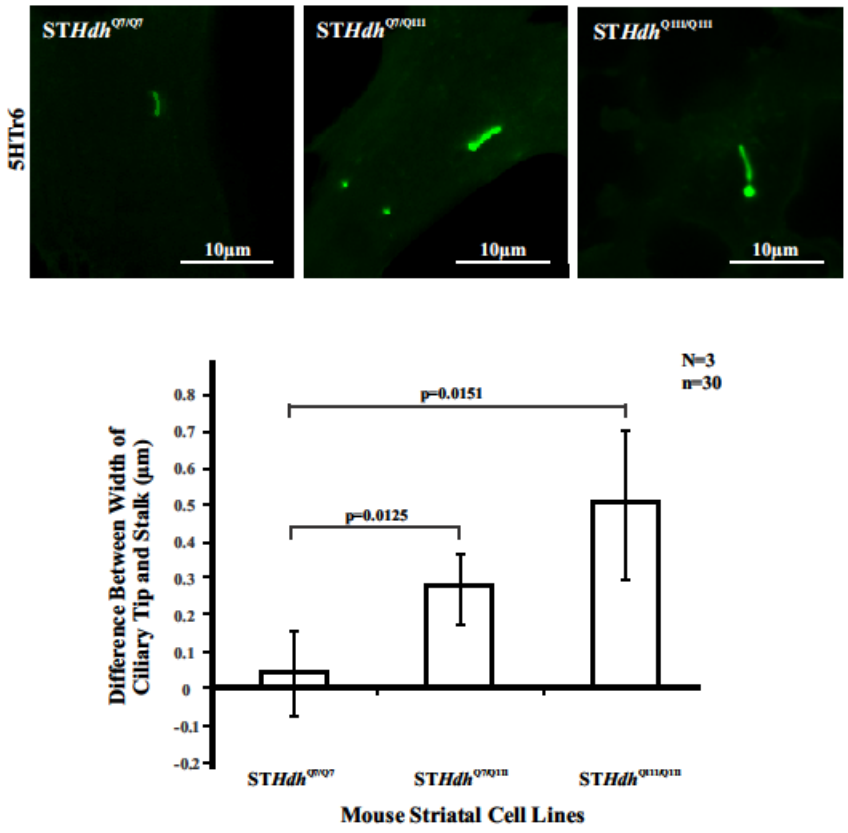
5HTr6
STHdh Q7/Q7
STHdh Q7/Q111
STHdh Q111/Q111
10µm
10µm
10µm
N=3
n=30
p=0.0151
p=0.0125
Difference Between Width of Ciliary Tip and Stalk (µm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
STHdh Q7/Q7
STHdh Q7/Q111
STHdh Q111/Q111
Mouse Striatal Cell Lines

Figure 9: Primary Cilia conformation in ST*Hdh* cells. ST*Hdh*$^{Q7/Q7}$, ST*Hdh*$^{Q7/Q111}$ and ST*Hdh*$^{Q111/Q111}$ cells transfected with 5HTr6-eGFP to reveal different conformations of primary cilia. In order to quantify the bulging tip phenotype exhibited by ST*Hdh*$^{Q7/Q111}$ and ST*Hdh*$^{Q111/Q111}$ cells, width of primary cilium stalk was subtracted from the width of the bulge at the tip. From this quantification, it was determined that ciliary tip morphology of ST*Hdh*$^{Q7/Q7}$ from ST*Hdh*$^{Q7/Q111}$ (*p value = 0.0125) and ST*Hdh*$^{Q111/Q111}$ (*p value = 0.0151). The difference between ST*Hdh*$^{Q7/Q111}$ and ST*Hdh*$^{Q111/Q111}$ (p value = 0.11). Error bars represent standard deviation.

Figure 10

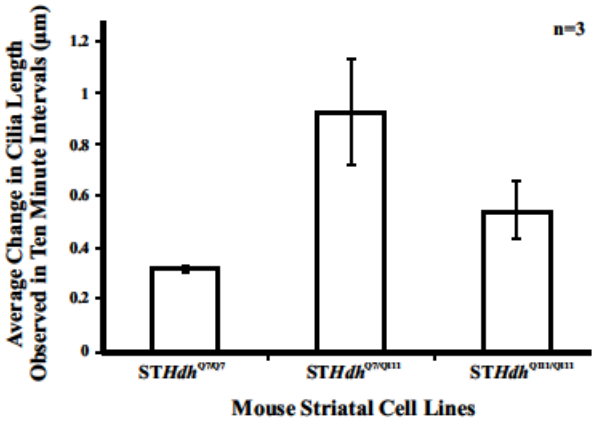
n=3
Average Change in Cilia Length Observed in Ten Minute Intervals (µm)
1.2
1
0.8
0.6
0.4
0.2
0
STHdhQ7/Q7
STHdhQ7/Q111
STHdhQ111/Q111
Mouse Striatal Cell Lines

Figure 10: Primary Cilia dynamics are increased in HD ST*Hdh* cells. ST*Hdh*$^{Q7/Q7}$, ST*Hdh*$^{Q7/Q111}$ and ST*Hdh*$^{Q111/Q111}$ cells transfected with 5HTr6-eGFP to reveal different conformations of primary cilia. To quantify the dynamics, live cell movies were performed over a period of 2 hours. In ten minute intervals, the change in length was recorded from the previous interval in ImageJ. There were not enough trials to determine statistical significance.

Figure 11

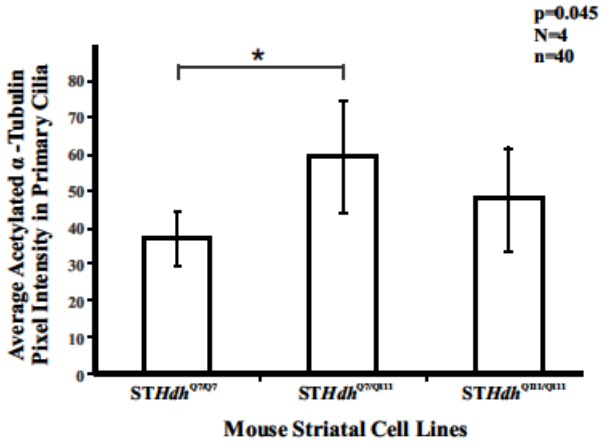
p=0.045
N=4
n=40
*
Average Acetylated α-Tubulin Pixel Intensity in Primary Cilia
0
10
20
30
40
50
60
70
80
STHdhQ7/Q7
STHdhQ7/Q111
STHdhQ111/Q111
Mouse Striatal Cell Lines

Figure 11: Primary Cilia of HD ST*Hdh*'s are hyperacetylated. ST*Hdh*$^{Q7/Q7}$, ST*Hdh*$^{Q7/Q111}$ and ST*Hdh*$^{Q111/Q111}$ cells were fixed immunostained for endogenous acetylated α-tubulin before quantification. Through ImageJ quantification, it was determined that the primary cilia of ST*Hdh*$^{Q7/Q111}$ cells have significantly higher levels of acetylation when compared to ST*Hdh*$^{Q7/Q7}$ (*p-value = 0.045, N= 3). Additionally, while ST*Hdh*$^{Q111/Q111}$ levels appear to be higher than ST*Hdh*$^{Q7/Q7}$, this difference is not significant. Error bars represent standard deviation.

Figure 12

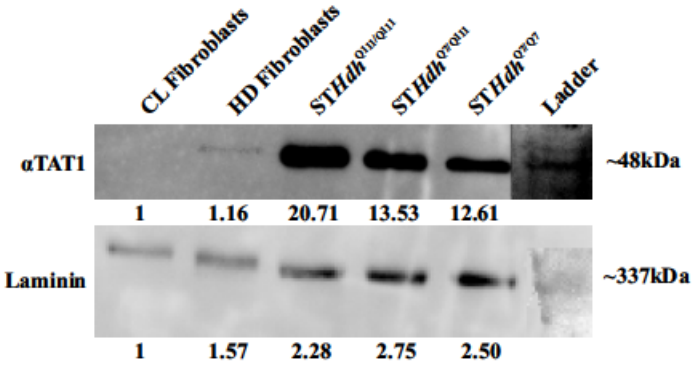
CL Fibroblasts
HD Fibroblasts
STHdh Q111/Q111
STHdh Q7/Q111
STHdh Q7/Q7
Ladder
αTAT1
~48kDa
1
1.16
20.71
13.53
12.61
Laminin
~337kDa
1
1.57
2.28
2.75
2.50

Figure 12: Amplification of αTAT1 Expression in ST*Hdh* Mutant Cells. Using whole ST*Hdh* cell protein extractions, immunoblotting was performed with a αTAT1 antibody. Laminin was used as a loading control.

Figure 13

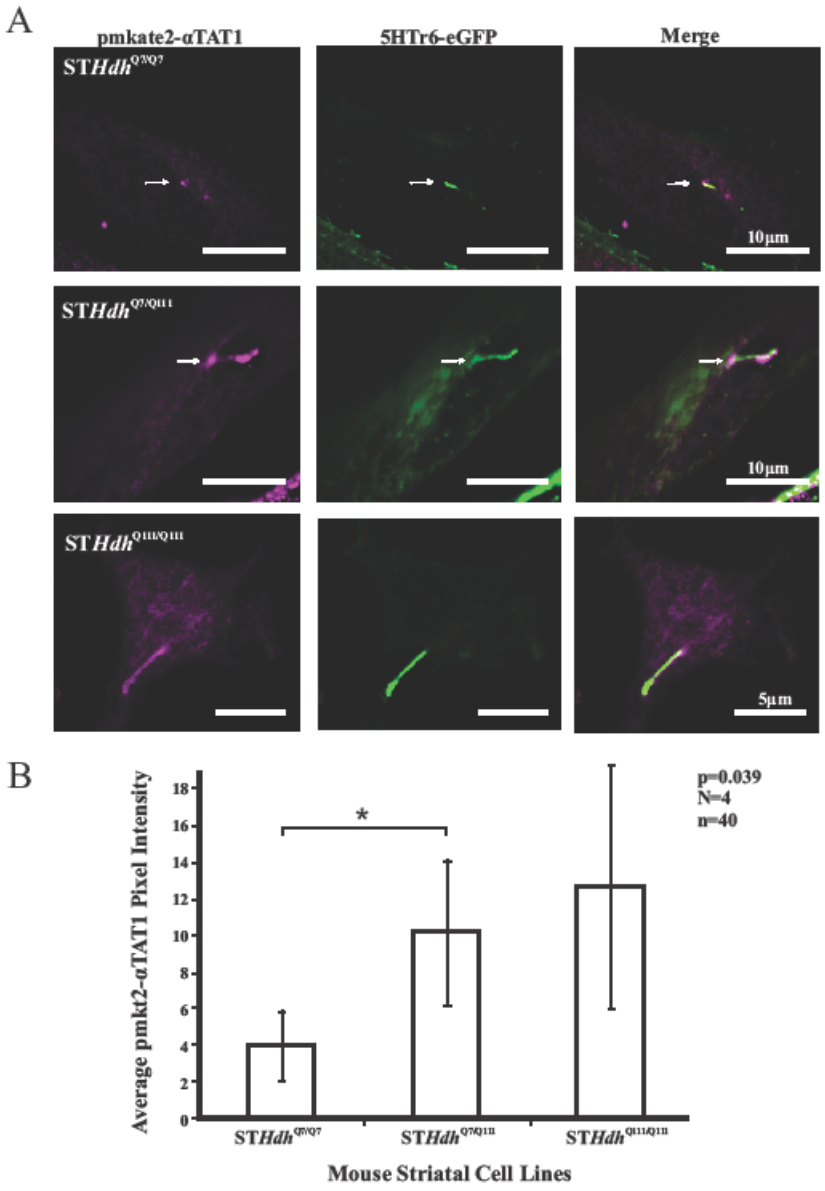
A
pmkate2-αTAT1
5HTr6-eGFP
Merge
STHdh Q7/Q7
STHdh Q7/Q111
STHdh Q111/Q111
10µm
10µm
5µm
B
Average pmkt2-αTAT1 Pixel Intensity
0
2
4
6
8
10
12
14
16
18
*
p=0.039
N=4
n=40
STHdh Q7/Q7
STHdh Q7/Q111
STHdh Q111/Q111
Mouse Striatal Cell Lines

Figure 13: αTAT1 demonstrates increased Localizes to Primary Cilium of HD ST*Hdh* Cell Lines. ST*Hdh*$^{Q7/Q7}$, ST*Hdh*$^{Q7/Q111}$ and ST*Hdh*$^{Q111/Q111}$ cells co-transfected with pmkate2-αTAT1 and 5HTr6-GFP to reveal increased αTAT1 localization to primary cilium of ST*Hdh*$^{Q7/Q111}$ and ST*Hdh*$^{Q111/Q111}$. Expression in the primary cilium was quantified with ImageJ, revealing a significant increase between αTAT1 localization in ST*Hdh*$^{Q7/Q111}$ and ST*Hdh*$^{Q7/Q7}$ (*p value = 0.039, N = 4). No significant difference was observed between the αTAT1 primary cilia localization between *STH*dh^{Q7Q7} and *STH*dh^{Q111Q111}. The arrows mark the primary cilium. Error bars represent standard deviation.

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