Huntingtin N17 Domain is a Reactive Oxygen Species Sensor Regulating Huntingtin Phosphorylation and Localization

Huntingtin N17 Domain is a Reactive Oxygen Species Sensor Regulating Huntingtin Phosphorylation and Localization

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Abstract

The huntingtin N17 domain is the master regulator of huntingtin intracellular localization. N17 is post-translationally modified, and phosphorylation of N17 serines 13 and 16 facilitate the stress dependent nuclear translocation of huntingtin by inhibiting CRM1 binding and nuclear export. In Huntington's disease (HD), mutant huntingtin is hypo-phosphorylated and increasing N17 phosphorylation has been shown to be protective in HD mouse models. N17 phosphorylation is therefore a valid therapeutic sub-target of huntingtin. The ER stresses that have been previously characterized to affect huntingtin phosphorylation are broad, likely activating a plethora of response pathways. Thus, in this study, we sought to define a specific stress that could affect huntingtin phosphorylation and nuclear localization. Here we show that huntingtin localization and phosphorylation can be specifically affected by reactive oxygen species (ROS). We identify a highly conserved methionine at position 8 (M8) as the specific sensor of oxidative species within N17 and show the capacity of oxido-mimetic M8 point mutations to affect N17 structure, localization and phosphorylation. We also define a specific molecular mechanism whereby N17 oxidation promotes membrane dissociation, thus increasing kinase accessibility and subsequent phosphorylation. These results define a precise molecular mechanism for the normal biological regulation of huntingtin phosphorylation by oxidative signalling. This ability of huntingtin to sense ROS levels at the ER provides a link between age-associated stress and altered huntingtin function. It suggests that ROS stress due to aging may be a critical molecular trigger of HD that could explain the age-onset nature of disease.

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Abbreviations

α-pN17	Polyclonal antibody to huntingtin phosphorylated at S13 and S16
3-NP	3-nitropropionic acid
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic glutamate receptor
APE1	Apurinic/apyrimidinic endonuclease
ATM	Ataxia-telangiectasia mutated protein
BACHD	Transgenic bacterial artificial chromosome HD mouse model expressing full-length huntingtin with a 97 CAG repeat
CD	Circular dichroism
CK2	Casein kinase 2
CRM1	Chromosome region maintenance protein 1
DMEM	Dulbecco's modified eagle's medium
DTT	Dithiothreitol
E5AE12A	Glutamic acid to alanine mutation at N17 positions 5 and 12.
ER	Endoplasmic reticulum
Ero1	ER oxidoreductin 1
ETC	Electron transport chain
FBS	Fetal bovine serum
FEN1	Flap endonuclease 1
H_2O_2	Hydrogen peroxide
HAP-1	Huntingtin-associated protein 1
HAP40	Huntingtin associated protein of 40kDa
HD	Huntington's Disease
Hdh	Knock-in HD mouse model containing a human version of huntingtin exon 1 carrying a 111 CAG repeat
HEAT	Huntingtin, Elongation factor 3, Protein phosphatase A, TOR1

hMTH1	human MutT homolog 1
hTERT	Human telomerase reverse transcriptase
HTT mRNA	Huntingtin Mrna
KA	Kainite glutamate receptor
M1	Methionine 1
M1L	Methionine to leucine mutation at N17 position 1
M8AS13AS16A	Reduced-mimetic, phospho-resistant N17 mutant
M8	Methionine 8
M8L	Methionine to leucine mutation at N17 position 8
M8P	Methionine to proline mutation at N17 position 8
M8Q	Methionine to glutamine mutation at N17 position 8
M8QS13AS16A	Oxido-mimetic, phospho-resistant N17 mutant
MEM	Minimum essential medium
MSNs	Medium spiny neurons
MsrA	Methionine sulfoxide reductase A
N17	A domain of huntingtin comprised of the first 17 amino acids of the protein
Neil1	Nei-like glycosylase 1
NES	Nuclear export signal
NMDA	N-methyl-D-asparate glutamate receptor
N-mHtt	Amino terminal mutant huntingtin
$O_2^{\bullet -}$	Superoxide anion radical
OGG1	Oxoguanine glycosylase 1
OH•	Hydroxide radical
Oxido-mimetic	Point mutant mimicking oxidized methionine
PAR	Poly-ADP-ribose
PBS	Phosphate-buffered saline

PDI	Protein disulfide isomerase
Phospho-N17	N17 phosphorylated at S13 and S16
R/62	HD mouse model expressing an amino terminal huntingtin fragment with a 144 CAG repeat
Reduced-mimetic	Point mutant mimicking reduced methionine
ROS	Reactive oxygen species
RPE1	Retinal pigment epithelial cells
S13	Serine 13
S16	Serine 16
S421	Serine 421
SDS	Sodium dodecyl sulfate
ST*Hdh*$^{Q7/Q7}$	Wild-type striatal mouse cell line
ST*Hdh*$^{Q7/Q111}$	Knock-in heterozygous huntingtin striatal mouse cell line expressing 1 huntingtin allele with a 111 CAG repeat
ST*Hdh*$^{Q111/Q111}$	Knock-in homozygous mutant huntingtin striatal mouse cell line expressing 2 huntingtin alleles with 111 CAG repeats
TBST	Tris buffered saline with tween 20
UPR	Unfolded protein response
UVA	Ultra-violet A
XRCC1	X-ray repair cross-complementing protein 1
YAC128	Transgenic yeast artificial chromosome HD mouse model expressing full-length huntingtin with a 128 CAG repeat
YFP	Yellow fluorescent protein

Declaration of Academic Achievement

The material presented here is a representation of the following publication:

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Laura DiGiovanni performed all the experiments and data analysis except for MATLAB intensity quantification in Figure 1B which was performed by Andrew Mocle.

1.0 Introduction

1.1 Huntington's Disease and Huntingtin Protein

1.1.1 Huntington's Disease

1.1.1.1 Genetics

Huntington's disease (HD) is a monogenic, autosomal dominant, neurodegenerative disorder. HD is caused by a CAG repeat expansion in the first exon of the *huntingtin* gene, found on the short arm of chromosome 4. This CAG expansion results in a polyglutamine expansion in the huntingtin protein (1). HD occurs when the number of glutamine repeats exceeds the critical threshold of 40. However, disease may also occur with incomplete penetrance at glutamine repeats between 36 and 39 (2-4). HD is an age onset disorder that typically manifests during adulthood in the fourth or fifth decade. The primary determinant of HD age of onset is the number of glutamine repeats. Expansion length is inversely correlated with age of onset (5-8), meaning earlier onset of disease generally occurs with longer repeat lengths. This relationship is exemplified by cases of juvenile HD, where repeat lengths of 50 or higher correlate with disease onset in childhood (9). However, the inverse correlation between expansion length and HD age of onset shows extreme variation, particularly at low glutamine repeats, suggesting that there are additional genetic and environmental modifiers of disease. This is supported by intra-family studies within the Venezuelan HD kindreds, the largest and most characterized HD population in the world, which determined that approximately 40% of the variance observed in HD onset age is attributed to genetic factors while the remaining 60% is due to environmental differences (10).

De novo instances of HD may also occur due to genetic anticipation. Genetic anticipation is a phenomenon where the severity of an inherited disease increases with successive generations. In HD, this increase in severity is associated with progressive lengthening of the CAG repeat over generations, due to meiotic instability of pathogenic CAG repeats (5, 11). There is a greater instance of genetic anticipation through paternal inheritance, rather than maternal, indicating spermatogenesis as a source of CAG expansion (5, 11). Expansion of near pathogenic CAG lengths in parental gametes may result in inheritance of pathogenic repeat lengths in subsequent generations, thus generating *de novo* instances of HD.

There are many genetic systems in place to model HD. R6/2 mice, expressing an amino terminal mutant huntingtin fragment with 144 CAG repeats, demonstrate early and severe HD pathology (12). However, there is debate over how representative amino terminal huntingtin fragments are of the full-length protein. Transgenic yeast artificial chromosome (YAC128), and bacterial artificial chromosome (BACHD) HD mice models have also been generated that express full-length mutant huntingtin with 128 and 97 CAG repeats respectively (13, 14). A knock-in HD mouse model (*Hdh*), containing a human version of huntingtin exon 1 carrying 111 CAG repeats, is also commonly used (15). From the *Hdh* mouse model, a striatal cell line, ST$Hdh^{Q111/Q111}$, has been generated by isolating and transforming mouse striatal cells using T-antigen. An unmodified *Hdh* striatal cell line, ST$Hdh^{Q7/Q7}$, is commonly used as a wild-type control line and is useful in the study of normal huntingtin biology (16).

1.1.1.2 Pathophysiology

HD occurs at an incidence of 1:5500 individuals within the western population (17). Early symptoms of disease, typically before onset of brain atrophy, include weight loss, circadian rhythm dysregulation, personality changes and depression (18-23). As the disease advances, symptoms of motor dysfunction (termed chorea, a hallmark of HD), dementia and general cognitive decline begin to develop progressively (24, 25). Eventually, individuals with HD succumb to aspiration pneumonia, stemming from their difficulty swallowing, suicide or heart disease (26-28).

HD is a characterized by progressive atrophy of neurons in the striatum and cerebral cortex of the brain (29, 30). The striatum is made up of the caudate nucleus and the putamen, and is situated in the basal ganglia. These regions experience significant reduction in cell population and volume in HD. As the striatum functions in voluntary motor control, learning and emotion processing, loss of neurons in this area are believed to contribute to the development of HD motor and psychiatric symptoms (31). The striatal neuronal population is comprised primarily of GABAergic medium spiny neurons (MSNs), which are the cell type preferentially degenerated in HD (29). In addition to the striatum, cortical neurons of the cerebral cortex, involved in efferent connections with the striatum, are also heavily affected at later stages of disease (30). Atrophy of the globus pallidus, thalamus, subthalamic nucleus, substantia niagra, hypothalamus and cerebellum have also been reported (32, 33).

An additional hallmark of HD pathology is the presence of mutant huntingtin inclusion bodies. Inclusion bodies containing amino terminal mutant huntingtin (N-mHtt) fragments have been found in the neurons of HD patient brains. Tissue from patients at more severe stages of HD has an increased number of aggregates (34). Furthermore, brain slices from transgenic mice expressing an N-mHtt fragment, R6/2 mice, show huntingtin inclusions present throughout the brain (12). This evidence established the concept of mutant huntingtin aggregation as mechanistic in HD pathology. However, more recent evidence has put this hypothesis into question, and suggests that mutant huntingtin aggregation may actually be protective in disease.

Tracking of single cultured neurons, transfected with a fluorescently tagged N-mHtt fragment, over time actually showed that the formation of aggregates was beneficial to cell survival. The study determined an inverse correlation between inclusion size and cell death, where larger aggregates were associated with longer survival time (35). This finding was supported subsequent study of an N-mHtt fragment containing a point mutation which abolished inclusion formation, yet greatly increased toxicity (36). In light of this evidence, previous studies showing the presence of N-mHtt fragment inclusions in post mortem HD brains (34) may in fact be interpreted to suggest a protective role of aggregation in HD. The visualized aggregate containing neurons may have been the healthy, surviving population of cells. In comparison to the atrophied neurons no longer present, the visualized neurons may have survived due to their ability to form aggregates (37). This rational suggests that it is actually the soluble form of mutant huntingtin which is pathogenic.

1.1.2 The Huntingtin Protein

1.1.2.1 Huntingtin Structure and Localization

Beyond the causal role of mutant huntingtin in HD, the wild-type huntingtin protein itself plays an integral role in cell biology. Understanding more about the normal function of huntingtin in the cell may help to elucidate the mechanism of mutant huntingtin pathology. Huntingtin is a large, 348kDa protein that is ubiquitously expressed throughout the body, with the highest levels of expression occurring in the brain and the testes (38, 39). Within the cell, huntingtin is localized to the cytoplasm and nucleus and has been shown to associate with a number of organelles including the endoplasmic reticulum (ER), golgi apparatus, mitochondria and primary cilium (36, 40-43). Within the nucleus, huntingtin localizes to the mitotic spindle, centrosome, cleavage furrow and cofilin-actin stress rods through direct interaction with actin and microtubules, discussed later (44-47).

Huntingtin is a multi domain protein containing 4 clusters of HEAT (Huntingtin, Elongation factor 3, protein phosphatase 2A, TOR1) repeat sequences. HEAT repeats are helix turn helix structures that act as sites of protein-protein interactions (48, 49). The HEAT repeat sequences in huntingtin contribute to the overall solenoid structure of the protein (50, 51). As huntingtin has no known catalytic activity, the abundance of protein-protein interaction domains in huntingtin, as well its large size, suggests that huntingtin functions as a scaffold protein. Multiple sequence alignment of eight representative chordate huntingtin homologs has shown that the size and predicted HEAT repeat

structure of huntingtin have been conserved through 500 million years of evolution (50), further indicating their importance in huntingtin function.

The polyglutamine tract, as previously discussed, is a repeating length of glutamine residues found at the amino terminal of the protein and is responsible for disease when expanded to a pathogenic length. The polyglutamine tract is not as highly conserved as huntingtin HEAT repeats, implying it may be involved in additional levels of regulation or function in higher vertebrate species (50). The polyglutamine tract is flanked on the amino end by the N17 domain (36, 52), discussed in detail later, and on the carboxyl end the polyproline rich region (53).

Intramolecular interactions between huntingtin sub-domains have also been shown to influence huntingtin structure. Intramolecular interaction between the N17 domain and polyproline region has been shown to occur in live cells using Förster resonance energy transfer (54). This interaction is facilitated by the polyglutamine tract, which acts as a flexible domain that is able to fold back on itself, bringing the N17 domain and polyproline region into proximity. The flexibility of the polyglutamine domain is influenced by post-translational modification of N17 as well as polygltamine expansion. Amino terminal regions of huntingtin have also been shown to interact with carboxyl terminal regions through immunoprecipitation of huntingtin fragments from cells (55). This was confirmed through electron microscopy and cross linking mass spectrometry studies which showed that the N17 domain is able to fold back to carboxy terminal regions of huntingtin, forming a spherically shaped solenoid (51).

Huntingtin contains several known localization signals. At the carboxyl terminus of the protein there is a chromosome region maintenance protein 1 (CRM1) dependent nuclear export signal (NES) (56); at positions 174-206 there is an importin beta1/beta2 dependent nuclear and ciliary localization signal (57); and the N17 domain, discussed in depth later, is a ER lipid-binding domain and additional CRM1 NES (36, 52, 58, 59). Huntingtin localization is dependant on N17 post-translational modifications, which include phosphorylation (60, 61), acetylation (60) and SUMOylation (62, 63). Specifically, phosphorylation of serines 13 (S13) and 16 (S16) are especially influential on N17 localization and are discussed in depth later.

1.1.2.2 Huntingtin Functions

1.1.2.2.1 Development

Huntingtin plays a critical role in embryonic development. In mice, targeted disruption or knockout of the huntingtin gene resulted in embryonic lethality at day 7.5 gastrulation (64, 65). The timing of lethality implicates huntingtin function in neurulation and development of the anterior-posterior axis (64). This function of huntingtin is not impaired by polyglutamine expansion, as huntingtin knockout mice were rescued from embryonic lethality by expression of mutant huntingtin (66). This conclusion is supported by study of homozygous HD patients which develop fully without any apparent abnormalities (67). As mutant huntingtin is fully functional in its embryonic role, the development of HD later in life suggests that huntingtin has a secondary function which becomes active in adulthood. It is in this secondary function that mutant huntingtin is

likely dysfunctional. Finally, the level of huntingtin expression is also important for normal embryonic development. Mice models with <50% of normal huntingtin levels were able to develop past day 7.5 gastrulation, however they developed other developmental defects later in embryogenesis (68).

1.1.2.2.2 Microtubule Association, Vesicular Transport and Mitosis

Huntingtin is known to associate with microtubules and microtubule structures. Huntingtin was isolated from cell lysate using an *in vitro* tubulin-affinity column, indicating the ability of huntingtin to bind, although not necessarily directly, microtubules (69). Using electron microscopy, huntingtin was also seen assembled on microtubules in cells. This is supported by immunostaining which showed co-localization of huntingtin with microtubules in the cytoplasm, particularly at the perinuclear and centrosome regions (46). Proteins involved in microtubule-based transport were also enriched in huntingtin containing complexes identified by affinity-purification mass spectrometry (70). The ability of huntingtin to associate with microtubules facilitates its cellular functions in vesicular trafficking, endocytosis and mitosis.

Huntingtin's role in vesicular trafficking is to act as a scaffold between the cytoskeleton and motor proteins. Huntingtin has been found in vesicle-enriched cell fractions, as well as localized to vesicle membranes, using immunofluorescence. This indicates the ability of huntingtin to associate with vesicles (71). Huntingtin has also been shown to associate with microtubule motor proteins dynein, dynactin (72) and kinesin (73) using yeast two-hybrid assays. This association is mediated by huntingtin-associated

protein 1 (HAP-1) (74), which interacts with both huntingtin and microtubule motor proteins, dynactin or kinesin, to mediate a connection between huntingtin and the microtubule network (75, 76). Huntingtin also provides a molecular switch between anterograde and retrograde transport through phosphorylation of huntingtin serine 421 (S421) (77). Huntingtin phosphorylated at S421 recruits kinesin to the dynactin complex to promote anterograde transport. Conversely, when S421 is unmodified, kinesin is not recruited and vesicles undergo retrograde transport. Vesicular transport is dysregulated in HD as expression of mutant huntingtin promoted the aggregation and sequestration of motor complexes (78).

Huntingtin interaction with microtubule motor proteins also facilitates its role in endocytosis, where huntingtin and dynein function to position endosomes and lysosomes on the cytoskeleton. Cells depleted of huntingtin or dynein had dispersed localization of early and recycling endosomes, as well as lysosomes. Correct localization was restored through expression of huntingtin in depleted cells (79). This evidence suggests a role of huntingtin as a scaffold between endosomal vesicles and cytoskeletal structures. Huntingtin is recruited to endosomes by Huntingtin associated protein of 40kDa (HAP40). This recruitment forms a complex between huntingtin, HAP40 and Rab5, a GTPase regulator of endocytosis, on the early endosome (80). HAP40 is upregulated in HD patient fibroblasts and in ST*Hdh*$^{Q111/Q111}$ striatal cells. Artificial overexpression of HAP40 caused a reduction of early endosomal motility through displacement from microtubules (80). Huntingtin is also implicated in endosome recycling, where it is required for activation of Rab11, a GTPase involved in recycling endosomes to the

plasma membrane. In huntingtin knockout cells, nucleotide exchange activity on Rab11 was significantly reduced (81). This process is impaired in HD as cells expressing mutant huntingtin had abnormally large vesicle structures, indicating inhibited endosome recycling (82).

The interaction of huntingtin with microtubule motor proteins also suggests a role of huntingtin in cell division. The dynactin motor complex is involved in spindle formation during division and acts to focus microtubule minus ends at spindle poles (83-85). Huntingtin also localizes to spindle fibers during division, as well as the cleavage furrow and centrosome (44). SiRNA knockdown of huntingtin results in defective spindle orientation (45). This suggests that huntingtin functions in cell division, possibly through its role in microtubule dynamics and association with the dynactin motor complex.

1.1.2.2.3 Stress Response

Huntingtin function is critical to several stress response pathways, including the ER stress response. Huntingtin has been shown to be important for ER health as siRNA knockdown of endogenous huntingtin dramatically disrupts the structure of the ER network (86). Furthermore, increased expression of ER stress markers has been seen in post mortem HD patient brain tissue (87), as well as in the striatum and cortex of an HD mouse model (88). Huntingtin is able to anchor to the ER membrane through the N17 domain and, in response to temperature, tunicamycin and dithiothreitol (DTT) induced unfolded protein response (UPR), is seen to dissociate from the ER and translocate to the

nucleus (36) where it localizes to puncta structures (44). This pathway is discussed in depth later.

Upon heat shock induced UPR, huntingtin also localizes to cofilin-actin rods within the nucleus (47). Cofilin, an actin binding protein involved in normal actin treadmilling, saturates actin upon conditions of stress to produce cofilin-actin rods (89). The formation of these rods releases ATP involved in actin treadmilling, functioning to rapidly increase ATP availability for use in the cell stress response (90). This process is defective in the context of mutant huntingtin, which causes the formation of persistent rods and inhibits actin dynamics from resuming (47). This may indicate an inability of HD cells to properly respond to stress, resulting in a persistent stress response.

Huntingtin has also been shown to form cytosolic puncta, termed huntingtin stress bodies, in response to heat shock stress (91). These huntingtin stress bodies were associated with early endosomes and function to arrest early-to-late and early-to-recycling endosome fusion. The mechanism by which huntingtin contributes to this arrests is unknown. Similar to cofilin-actin rods, the formation of huntingtin stress bodies in response to stress is proposed to rapidly increase ATP availability by arresting vesicular trafficking (91). Cells expressing mutant huntingtin are also defective in their ability to clear huntingtin stress bodies upon stress relief, again indicating defective stress response in HD (91).

What is still not yet fully understood is what triggers these huntingtin functions in response to cell stress. The inducers of the UPR, used in the studies above, are broad

stresses that trigger global protein misfolding, therefore likely activating a plethora of response pathways. Thus, a specific signaling mechanism linking the stress state of the cell to huntingtin has not been identified.

1.1.3 The N17 domain

1.1.3.1 N17 Structure and Function

A key regulator of huntingtin localization and function is the N17 domain, comprised of the first 17 amino acids at the amino terminus of the protein (36, 52). The importance of N17 to huntingtin biology is indicated by the high degree of N17 sequence conservation across vertebrate species (36). N17 is also suggested to have a critical role in disease pathogenesis. HD mice expressing mutant huntingtin lacking the N17 domain have more accelerated and overt disease pathology than mice expressing normal mutant huntingtin (92). Also, N17 has been shown to mediate polyglutamine aggregation in mutant huntingtin (93).

N17 is an amphipathic alpha helix (36, 58) and functions both as an NES (59) and ER membrane anchor (36, 58) to target huntingtin to the cytoplasm under normal conditions. 3D NMR has shown that N17 is able to peripherally interact with membranes through its hydrophobic face, which inserts into lipid bilayers at a 77-degree angle (58). N17 membrane association is sensitive to cell stress and is affected by temperature (36) and heat shock (91). The hydrophobic face of N17 is also a CRM1 dependent NES (59). Huntingtin localization is dependent on N17 alpha helicity as elimination of N17 structure, through introduction of a methionine to proline (M8P) mutation at the center of

the helix, disrupts N17 cytoplasmic targeting and leads to a diffuse cellular localization (36).

Huntingtin localization is also dependent on N17 post-translational modifications, which include phosphorylation (60, 61), acetylation (60) and SUMOylation (62, 63). Phosphorylation of N17 S13 and S16 is particularly influential on huntingtin localization as it inhibits N17 NES function by preventing interaction with CRM1 (59). This inhibition results in huntingtin nuclear accumulation and localization to nuclear puncta (44). Phosphorylation of N17 occurs under conditions of stress. Immunofluorescence of phosphorylated N17 showed that this phosphorylation and huntingtin nuclear retention were triggered by various cell stresses including the UPR (36, 44). At the single molecule level, N17 phosphorylation triggers a conformational change in huntingtin that relies on the flexibility of normal polyglutamine tracts, allowing interaction with distal domains in huntingtin (51, 54, 55).

The stress-dependent regulation of N17 phosphorylation is not currently understood. Data from our lab (manuscript in preparation) shows that the kinase responsible for S16 phosphorylation is casein kinase 2 (CK2), which only acts on N17 following phosphorylation of S13. The kinase responsible for S13 phosphorylation is still under study. CK2 is a promiscuous kinase and is not regulated by upstream elements (94, 95), indicating that control of N17 phosphorylation occurs through alternative means. Thus, the molecular trigger of N17 phosphorylation in response to cell stress remains to be elucidated.

1.1.3.2 N17 Phosphorylation is a Therapeutic Sub-target for HD

N17 phosphorylation is dysregulated in HD and is therefore of clinical interest. Mutant huntingtin is hypo-phosphorylated at S13 and S16 (44) in heterozygous ST*Hdh*$^{Q7/Q111}$ cells. The cause of mutant huntingtin hypo-phosphorylation is not clear, although one possibility is inaccessibility of N17 to kinases due to polyglutamine steric hindrance. ST*Hdh*$^{Q111/Q111}$ cells have also been shown to have lower resting levels of ATP inversely correlated with polyglutamine expansion length (96). This was also seen in mice with a deleted polyglutamine tract which had significantly higher ATP levels than that of normal polyglutamine length mice (97).

Furthermore, restoration of mutant huntingtin phosphorylation, through various methods, has been shown to modulate disease pathology. In HD mouse models, phospho-mimetic aspartic acid mutants of S13 and S16 were shown to abolish disease pathogenesis (98). Also, N17 S13S16 phosphorylation induced with GM1 ganglioside treatment was seen to attenuate mutant huntingtin toxicity and restore normal motor function in HD mice (99). Thus, N17 phosphorylation at S13 and S16 has been established as a potential therapeutic sub-target for HD.

1.2 Oxidative Stress

1.2.1 ROS and Redox Signalling

Reactive oxygen species (ROS) is a term used to describe a family of reactive molecules and free radicals derived from molecular oxygen. Biologically relevant ROS include superoxide anion radical ($O_2^{\bullet -}$) formed through a one electron reduction of

oxygen, hydrogen peroxide (H_2O_2) often formed through enzyme catalyzed dismutation of $O_2^{\bullet -}$, and the hydroxyl radical ($OH^{\bullet}$) formed through metal ion decomposition of H_2O_2 (100). ROS have typically been viewed strictly as accidental by-products of cellular metabolism implicated in aging and disease. However, it is becoming increasingly apparent that ROS can in fact be purposely made by the cell to act as messengers in cell signalling and protein regulation. The basic principle behind redox signalling is that specific oxidation prone residues, predominantly sulfur containing cysteine and methionine, can be rapidly and reversibly oxidized by low physiological levels of ROS (101). Oxidation of these residues can result in protein structure and polarity changes, which may have significant influence on protein functionality, interactions or localization. Not all ROS are equally implicated in signalling processes, and some are found to be more associated with cellular damaging effects. Among the characterized ROS, H_2O_2 is most commonly implicated in signalling pathways due to its membrane permeability, lower reactivity and longer half-life. Its importance is supported by the presence of enzymes specifically used in its generation, as well as its relatively high cellular concentration in comparison to other ROS (102).

Specificity of redox signalling is achieved through co-localization of the oxidant source and the intended target. This is an essential aspect of redox signalling, given the general reactivity and innate instability of ROS, to prevent off target oxidation (103). The cellular effect of ROS is also dependent on its physiological concentration. ROS have been shown to have duel counteracting effects in both proliferation and apoptotic signalling dependent on their cellular concentration (104, 105).

ROS are generated in a number of cellular compartments. Mitochondria are perhaps the largest producers of ROS in the cell. In order to generate ATP, electrons are shuttled down the electron transport chain (ETC) where they are eventually used to reduce molecular oxygen into water. During this process it is possible for electron leakage to occur, resulting in the generation of an $O_2\bullet^-$ through one electron reduction of molecular oxygen (106). The luminal compartment of the ER is also a major site of ROS production in the cell. The formation of covalent disulfide crosslinks between cysteine residues, known as oxidative protein folding, occurs within the ER and requires the use of molecular oxygen as an electron acceptor. Disulfide bond formation is catalyzed by oxidases, such as ER oxidoreductin 1 (Ero1), which triggers the oxidation of the thioredoxin protein disulfide isomerase (PDI). Oxidized PDI is then able to oxidize substrate cysteine residues to mediate oxidative folding. Because Ero1 uses molecular oxygen as an electron acceptor, a necessary by-product of this process is H_2O_2, where one equivalent is formed for each disulfide bond catalyzed by Ero1 (107). This generates an oxidative environment within the ER lumen. In addition to the Ero1-PDI machinery, the ER also contains NADPH oxidases, a family of membrane bound enzymes whose primary function is to generate H_2O_2 by transferring electrons from NADPH to molecular oxygen (108). It has been well characterized that reductive or oxidative insults can disrupt ER homeostasis and trigger the UPR (109, 110). It has also been shown however, that the reverse is also true and ER stress triggers elevated cytoplasmic ROS as part of its signal cascade (111).

1.2.2 Methionine Sulfoxidation

Methionine is one of the major targets of ROS, and can be reversibly oxidized to methionine sulfoxide through addition of a free oxygen onto the sulfur group (112). Further oxidation to methionine sulfone may also occur, but only at high, non-physiological or pathophysiological levels of oxidation (113). Methionine sulfoxide is more rigid and polar than that of methionine and thus has the ability to impose structural and functional changes in certain proteins (114). Not all methionines are equally susceptible to oxidation, and different methionines within the same protein can be affected by different oxidants (115-117). Surrounding acidic residues tend to increase susceptibility to ROS, as do alanines, threonines and serines (117).

Methionine oxidation is a reversible process and its reduction is catalyzed by the thioredoxin-dependent enzyme, methionine sulfoxide reductase A (MsrA). MsrA localizes subcellularly to the cytoplasm (118), mitochondria (118, 119) and nucleus (120). MsrA is also differentially expressed throughout the body and is found to be particularly abundant in the liver, kidney, heart and brain, indicating that methionine oxidation may play a prominent role within the cells of these tissues (121, 122). Bacteria and yeast cells with knocked-out MsrA have increased sensitivity to oxidative stress (123, 124), and MsrA overexpression significantly increased lifespan in yeast cells and *Drosophila* (124, 125). MsrA knockout mice also displayed lower survival rates, as well as an ataxia-like tip-toe walking behaviour (126). Ataxia motor dysfunctions are attributed to cerebellar based abnormalities, and therefore indicate a role of MsrA within these cell types, as well

as the involvement of oxidative stress in ataxia (126). Additionally, MsrA expression was increased in response to prolonged, low dose ultra-violet A (UVA) and H_2O_2 exposure (127).

1.2.3 Oxidative Stress and Huntington's Disease

Although ROS are normal members of cell homeostasis, conditions of oxidative stress can occur when there is an imbalance between the levels of ROS produced and the anti-oxidant capacity of the cell. This condition of high intracellular ROS can result in non-specific oxidative damage of cellular components including lipid peroxidation, protein damage and DNA damage. Increased oxidative stress is associated with aging, and levels of oxidative stress have been shown to be particularly elevated within the brain due to the high level of oxygen consumption needed for oxidative metabolism, the primary source of neuron energy (128). Oxidative stress has specifically been seen in the context of HD. Elevated levels of multiple oxidative stress markers, such as malondialdehyde, superoxide dismutase, glutathione peroxidase and glutathione reductase, have been reported in HD patient blood (129-134). In particular, 8-hydroxydeoxyguanosine, a marker of oxidative DNA damage, has been extensively studied and has been shown to be elevated in post-mortem HD patient brain tissue and blood plasma, as well as in the striatum of HD mice (135, 136). Additionally, several processes related to cell redox have been shown to be dysregulated in HD including mitochondrial function, iron homeostasis and glutamate excitotoxicity.

Mitochondrial dysfunction has been extensively studied in HD. Initial interest was triggered by the use of ETC complex II inhibitors, 3-nitropropionic acid (3-NP) and malonate, to replicate the physiological symptoms of HD in animal models (137). Both mutant and wild-type huntingtin have also been shown to bind the mitochondrial outer membrane, indicating a possible role of huntingtin in mitochondrial function (42). However, attempts to specifically determine if mitochondria are dysfunctional in HD have shown conflicting evidence. High lactate concentrations have been found in the cortex and basal ganglia of HD patient brains using nuclear magnetic resonance spectroscopy (138). This indicates to a reliance on anaerobic respiration and impairment of the ETC in HD. Complexes II and III of the ETC have also been shown to have decreased activity in human HD brains (139), however no impairment of these complexes was observed in ST*Hdh*$^{Q111/Q111}$ cells (140). Studies in HD patient lymphoblast and YAC128 mouse brain mitochondria, show a lower membrane potential and calcium load in the context of HD (141). Contrastingly, another study using striatal cells from YAC128 mice found no evidence of altered mitochondrial respiration, calcium uptake capacity or membrane potential (142). It is therefore uncertain if mitochondria are in fact dysfunctional in HD, and if so, whether this dysfunction is mechanistic in HD.

Iron dysregulation within the central nervous system has also been reported in HD. Iron is an essential element in cells due to its ability to transfer electrons by transitioning between its ferrous and ferric forms (143). However, this redox ability also poses a potential for toxicity by catalysing the conversion of H_2O_2 into free oxygen radicals through Fenton chemistry (144). Intracellular iron levels are therefore tightly

regulated in the cell though receptor mediated endocytosis between transferrin bound extracellular iron and transferrin receptors on the cell surface (143). Iron overload has been reported in the brains of HD patients, R6/2 mice and ST*Hdh*$^{Q111/Q111}$ cells (16, 145-147). Dysregulation of transferrin receptor expression has also been reported, although conflictingly, where decreased transferrin levels were shown in the cortex and striatum of R6/2 mice (145), while elevated levels were observed in ST*Hdh*$^{Q111/Q111}$ cells (16). It is possible that altered iron homeostasis may contribute to the elevated ROS levels observed in HD. Given the previously discussed role of huntingtin in endocytosis and vesicular trafficking, and the endocytic pathway of iron uptake, it is possible that huntingtin functions in regulating intracellular iron levels. Dysregulation of this process by mutant huntingtin could contribute to the iron overload seen in HD. Although, as with mitochondrial dysfunction, oxidative stress through iron overload may also only be a secondary result of other disease causing pathways.

Finally, glutamine excitotoxicity, and subsequent oxygen radical generation, has also been shown in HD. MSNs receive input from glutamatergic neuron projections from the thalamus and cortex. These projections secrete glutamine, an excitatory neurotransmitter, which activates NMDA (N-methyl-D-asparate), AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic) and KA (kainite) glutamate receptors on the MSN membrane (148). These glutamate receptors are membrane-spanning channels which are permeable to Na^+, K^+ and Ca^{2+} ions upon stimulation (149). Glutamate-receptor binding stimulates a signal cascade involving the Ca^{2+} dependent release of arachidonic acid (150, 151) and subsequent generation of ROS through ETC uncoupling

(152, 153). Excitotoxicity is a process that occurs from excessive activation of receptors by neurotransmitters, such as glutamate, and has been observed in HD. Glutamate excitotoxicity in HD is evidenced by loss of glutamate receptor expression, and decreased glutamate-NMDA receptors binding, in the striatum of post mortem HD patient brain tissue (154, 155). Aberrant glutamate signalling may be caused by increased glutamate release from cortical projections, or impaired clearance of glutamate from the synaptic cleft by glial cells (156, 157). Regardless of the mechanism, ROS production through excessive glutamate receptor activation may contribute to the elevated levels of oxidative stress observed in HD.

Anti-oxidants, such as XJB-5-131 (158) and curcumin (159), have shown protective effects in HD animal models, suggesting that oxidative stress may be mechanistic in HD pathogenesis. This is a particularly intriguing hypothesis as accumulation of oxidative damage over time could explain the delayed onset and progressive nature of HD. Although oxidative stress is elevated in HD, and multiple pathways related to redox have been shown to be dysregulated, a specific mechanism connecting oxidative stress and disease pathology has not been found. It is not clear whether increased oxidative load contributes to HD, or is merely a result of other disease causing pathways. A recent natural compounds screen from our lab (manuscript in preparation) pulled out several anti-oxidant hits that were found to affect the phosphorylation state of N17. Given the protective effects of N17 phosphorylation on HD pathology (98, 99), this evidence suggests a connection may exist between cell redox and huntingtin pathology through N17 phosphorylation.

1.3 Project Rationale

We were interested in learning more about the biological role of wild-type huntingtin in the cell stress response. We previously reported that N17 phosphorylation and nuclear retention could be triggered by various cell stresses at the ER (36). We also previously reported that N17 membrane association could be affected by temperature (36) and heat shock (91). However, these stresses trigger global protein misfolding and were therefore used as broad tools that likely activate a plethora of response pathways. In this study, we sought to determine a specific stress that could affect huntingtin phosphorylation and nuclear localization. In particular, we were interested in age-associated stresses, given the typical late onset of HD. Oxidative stress was identified as a promising candidate, given its association with age, as well as the protective effects of anti-oxidants, such as XJB-5-131 (158) and curcumin (159), in HD animal models. A natural compounds screen from our lab (manuscript in preparation) also pulled out several anti-oxidant hits that were found to affect the phosphorylation state of N17. This evidence suggests a connection between cell redox and huntingtin through N17 phosphorylation and gave us cause to explore the role of ROS stress in N17 phosphorylation. Additionally, the stress dependent phosphorylation of N17 by a promiscuous unregulated kinase, CK2, (94, 95) led us to suspect that regulation of N17 phosphorylation occurs through modification of the N17 domain itself. We hypothesized that oxidative post-translational modification of N17 may regulate phosphorylation by CK2, and thus provide a molecular link between cellular stress and huntingtin phosphorylation and nuclear retention.

2.0 Materials and Methods

2.1 Tissue Cell Culture

T- antigen immortalized mouse ST*Hdh*$^{Q7/Q7}$ (a kind gift from M.E. MacDonald) were grown as previously described. hTERT-immortalized retinal pigment epithelial cells (RPE1s) (ATCC) were cultured at 37˚C in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies) containing 10% fetal bovine serum (FBS) (Life Technologies), 0.26% $NaHCO_3$ (Bioshop), and 0.01mg/mL hygromycin B (Invitrogen). Primary wild-type human fibroblast cells (Coriell) containing 21/18 CAG repeats were cultured at 37˚C in Minimum Essential Media medium (MEM) containing 15% FBS and 1% Glutamax. These are all wild-type cell lines, expressing non-mutant huntingtin, and were used to study the basic biology of huntingtin outside of disease.

2.2 Immunofluorescence Analysis

Cells were washed with phosphate-buffered saline (PBS) then fixed and permeabilized with ice-cold methanol at -20 °C for 12 min. Cells were washed three times with PBS in 5 min intervals at room temperature then blocked with 2% FBS in PBS three times in 12 min intervals at room temperature. Primary phospho-N17 antibody (New England Peptide) directly conjugated to Alexa 488 was diluted 1:15 in antibody solution (2% FBS, 0.02% (v/v) Tween-20 in PBS) and incubated overnight at 4 °C. Alternatively, primary anti-huntingtin protein antibody MAB2166 (Millipore) was diluted 1:250 and applied as described above. MAB2166 incubation was followed by a blocking with 2% FBS in PBS for 30 min and secondary antibody Alexa Fluor Cy5 goat anti-

mouse (Molecular Probes) incubation at 1:500 for 45 min. Finally, washes in PBS were performed as previous, and nuclei were counterstained with Hoechst dye 33258 (Life Technologies) for 15 min at room temperature prior to imaging in PBS.

To directly conjugate the primary phospho-N17 antibody to Alexa Fluor 488, the phospho-N17 antibody was incubated with 1µl Alexa Fluor 488 carboxylic acid succinimidyl ester (Life Technologies) per 10µL antibody and 5% $NaHCO_3$ overnight at 4°C on a rotator. The solution was then run through a Sephadex bead (Amersham Pharmacia Biotech AB) column and collected to the dye front.

Cells were treated with 3-NP (Sigma), prepared in in PBS at pH 8.0 and diluted in DMEM, for 1 hour at 37°C. Image intensity was calculated using MATLAB in 40 images over 4 trials. Images were thresholded to the cell using the Otsu method and average intensity was calculated per image. Nuclear puncta were also quantified using an open source speckle counting pipeline in Cell Profiler (www.cellprofiler.org). The number of puncta per cell was counted in >500 cells over 4 trials.

2.3 CD Spectroscopy

Circular dichroism (CD) was performed on an Aviv CD spectrometer model 215. Peptides were obtained from Genscript or New England Peptide and used as 0.25 mg/mL solutions in phosphate buffer (10 mM NaH_2PO_4, 0.14 M NaCl, 1 mM EDTA, pH 7.4). Solutions were scanned between 260nm and 195nm and maintained at 25°C. Triplicate scans were averaged and secondary structure determination was done using the

CONTILL algorithm against a database of 56 proteins, 43 soluble and 13 membrane proteins.

2.4 Plasmid Constructs

Double-stranded synthetic DNA oligonucleotides were generated (McMaster MOBIX facility) encoding the first 17 amino acids (WT or M8Q, M8P and M8QS13AS16A mutants) of huntingtin containing a Kozac consensus translation start site at the start methionine and 5' AgeI and 3' SalI sites. Annealed oligonucleotides were cloned between AgeI/SalI sites of peYFPN1 (BD Biosciences/Clontech) containing a peYFPC1 multiple cloning site to create Htt N17-eYFPN1 plasmids. Subsequent M8L, M8AS13AS16A and E5AE12A N17 mutant peYFPNI plasmids were created using the Q5 site-directed mutagenesis kit from New England Biolabs as directed. All DNA manipulation enzymes were purchased from New England Biolabs. All plasmid constructs were verified by nucleotide sequencing (McMaster MOBIX facility).

2.5 Transfection and Nuclear Localization Analysis

ST*Hdh*$^{Q7/Q7}$ cells were transfected using Turbofect (Fermentas) and imaged approximately 24 hours after transfection. Cells were fixed with 4% (w/v) paraformaldehyde in PBS at 33˚C for 20 min and nuclei were counterstained with Hoechst dye for 15 min at room temperature prior to imaging in PBS. Leptomycin B (Sigma) treated cells were incubated at 10ng/mL for 30 min. Percent nuclear fluorescence was calculated in approximately 100 cells per construct over 3 trials using ImageJ. Nuclear and whole cell areas were defined using Hoechst staining and YFP fluorescence

respectively. YFP fluorescence intensity was calculated for nuclear and whole cell areas and percent nuclear fluorescence was calculated using the equation: percentage nuclear fluorescence = (nuclear intensity/whole cell intensity) x 100.

2.6 Microscopy

Microscopy was performed on a Nikon TiEclipse epifluorescent inverted microscope using a Plan Apochromatic 60x oil immersion objective and Orca-Flash 4.0 digital camera (Hamamatsu). Widefield microscopy was performed using a LED Lumencor Spectra Light Engine, dichroic filters from Semrock and filter wheel from Sutter Instruments. Confocal imaging was performed using 405nm and 555nm laser lines and C2 confocal head. All image acquisition was done using NIS-Elements Advanced Research version 4.1.1 64-bit acquisition software (Nikon).

2.7 Immunoprecipitation, SDS-Page and Western Blot Analysis

ST*Hdh*$^{Q7/Q7}$ cells were transfected with yellow fluorescent protein (YFP) fusion proteins as described above. 24 hours post-transfection, cells were lysed in NP40 lysis buffer (150mM NaCl, 50mM Tris pH 8.0, 1% NP40) containing 1X protein and phosSTOP inhibitor cocktails (Roche). Supernatants were incubated with Living Colors GFP polyclonal antibody (Clontech), or without antibody as a control, for 15 min on ice followed by overnight rotation at 4˚C. Protein A agarose beads (Sigma) in NP40 lysis buffer were added for 1 hour with rotation at 4˚C and were subsequently washed with PBS. Purified proteins, and unaltered supernatant diluted 1:10, were resolved on a 12% Sodium dodecyl sulfate (SDS) polyacrylamide gel and electroblotted to a polyvinylidene

membrane. Membranes were blocked with 5% non-fat dry milk in Tris buffered saline with tween 20 (TBST) for 1 hour followed by overnight incubation with the phospho-N17 antibody (1:2500). Rabbit anti-mouse HRP-conjugated secondary antibody (1:50000) (Abcam) was incubated for 45min. Bands were visualized using Immobilon Western Chemiluminescent Substrate (Millipore) and Microchemi (DNR Bio-Imaging Systems).

2.8 Pearson Correlation

Transfection and fixation of ST*Hdh*$^{Q7/Q7}$ was performed as described above. Co-transfection of YFP fusion proteins with mCherry (BD Biosciences), or counterstaining with ER tracker red (Molecular Probes), was performed prior to fixation to visualize the cells soluble phase or ER respectively. Pearson correlation quantification between mCherry (red) and YFP (green) channels was done per cell using the Coloc2 ImageJ plugin on defined ROI. Every cell was represented as its own N for each condition. 50 cells were quantified for each condition over 3 trials and graphed on a box-whisker plot. The box represents 25%-75% of data and the line indicates the data median. The whiskers represent 5%-95% of data and outliers were removed if they fell outside 2 standard deviations from the mean.

2.9 Statistics

All statistical analysis was done using the Real Statistics Excel (Microsoft) add-on. If data passed Shapiro-Wilk normality assumptions groups were compared using a Student's t-test. If data was not normal groups were compared using the Mann-Whitney method.

3.0 Results

3.1 Oxidative stress promotes N17 phosphorylation

We first assessed the effect of oxidative stress on endogenous wild-type human huntingtin phosphorylation and localization. We used a dose-response immunofluorescence assay in human retinal epithelial (RPE1) cells, a wild-type model, immortalized by human telomerase reverse transcriptase (hTERT) catalytic subunit. hTERT immortalized cells were used, as opposed to T-antigen transformed cells, as they preserved tumor protein p53 functionality. This was important given the role of p53 in huntingtin transcriptional regulation (160), as well as its own function in responding to oxidative stress (161). Intracellular ROS was induced using 3-NP, a mitochondrial complex II inhibitor that elevates intracellular ROS levels (162). Huntingtin phosphorylation was assessed through immunofluorescence with a previously validated, affinity purified, phospho-specific polyclonal antibody to huntingtin S13 and S16 phospho epitope (α-pN17) (44) directly conjugated to a fluorescent dye. Direct conjugation of α-pN17 allowed phosphorylated N17 to be fluorescently labelled in a 1:1 ratio, permitting accurate intensity quantification. Dosing cells with 0.1mM, 1mM or 10mM 3-NP resulted in a significant increase in both levels of phospho-N17 (Figure 1A, B), at 0.1mM and 1mM 3-NP, and the number of nuclear phospho-puncta, at 0.1mM, 1mM and 10mM 3-NP (Figure 1A, C). Thus, endogenous huntingtin phosphorylation and nuclear localization is responsive to ROS.

3.2 Methionine is a redox sensor in N17

Because we suspected that regulation of N17 phosphorylation occurred through modification of the N17 domain itself, we next looked for residues in N17 which could be post-translationally modified through oxidation. Huntingtin N17 sequence alignment across vertebrate species revealed conservation of methionine 8 (M8) in mammals, and evolution of a second methionine at position 4 in some reptile and bird species (Figure 2). Interestingly, both methionine residues are lost in the sea lancelet (*Branchiostoma floridae*) and owl limpet (*Lottia gigantean).* As discussed previously, methionine residues are susceptible to oxidation and can be reversibly oxidized to methionine sulfoxide through addition of a free oxygen onto the sulfur group (112). N17 M8 specifically has been shown to be susceptible to oxidation and is able to affect huntingtin small fragment aggregate properties (163). Additionally, NMR has revealed that residues 4 and 8 lay in the same face of the N17 helix and are adjacent to the ER membrane-aqueous interface when N17 is membrane bound (58). The conservation of methionines in N17 suggested that they might have a functional role in N17, possibly by acting as sensors of oxidative species given their ability to be post-transitionally modified through oxidation. The positioning of methionine residues along the ER membrane-aqueous interface of the N17 helix also indicated that the location of methionine within the N17 helix, and the proximity to the ER membrane, may also be important for this function.

We analyzed synthetic N17 peptide structure by circular dichroism (CD) spectroscopy under either reducing conditions, with DTT, or oxidizing conditions, with

H_2O_2 (Figure 3). Reducing and oxidizing conditions imposed contrasting shifts in N17 CD spectra, with oxidation promoting a more helical structure (Figure 3). The untreated N17 peptide was likely a mix of the reduced and oxidized forms, due to baseline atmospheric oxidation, and the resulting CD spectra was an average of both species. Treatment with H_2O_2 or DTT pushed the population of N17 peptide to either fully oxidized or fully reduced, and the resulting spectra reflected the structure of one species only. The response of N17 structure to oxidizing and reducing conditions indicated that N17 contained a redox sensitive residue which was capable of inducing a profound structural change in N17. As structural change is often indicative of functional consequence, the effect of oxidation on N17 alpha helical structure also suggested that oxidation may have a regulatory role in N17 function.

We next tested whether M8 oxidation had an effect on N17 localization. Recombinant N17 constructs, M8Q-YFP and M8L-YFP, were generated using glutamine and leucine point mutations to mimic the structure and polarity of oxidized (oxido-mimetic) and reduced (reduced-mimetic) N17 M8 respectively. These species were shown to be structurally representative using CD spectroscopy, where M8L and M8Q synthetic peptides showed similar alpha helical structure to that of N17 (Figure 4). Transfection and quantification of nuclear fluorescence in ST*Hdh*$^{Q7/Q7}$ cells showed that oxido-mimetic M8Q-YFP significantly increased nuclear targeting compared to M8L-YFP and N17Wt-YFP (Figure 5A, B). As previously discussed, the hydrophobic face of N17 is defined as a CRM1-dependent NES (59), which is disrupted by phosphorylation at S13 and S16 to promote nuclear retention. The increased nuclear accumulation of oxido-

mimetic M8Q therefore suggested that it was hyper-phosphorylated in comparison to N17Wt, supporting the hypothesis that oxidation of N17 M8 promotes S13 S16 phosphorylation.

As N17 contains two possible redox sensitive residues, methionine 1 (M1) and M8, it was also necessary to identify which was responsible for inducing the change seen in N17 alpha helicity under oxidizing conditions. CD spectroscopy was first attempted on synthetic N17 peptide with the start methionine removed. No structure was observed however, as seen in comparison to an M8P peptide in which the substituted proline completely abolishes alpha helical structure (Figure 6). This suggested that the start methionine is not cleaved off post-translationally, as has been previously reported (61), and that the start methionine is important to N17 structure. CD spectroscopy was instead performed, as described in Figure 3, on synthetic N17 peptides containing leucine point mutations at positions 1 (M1L) or 8 (M8L). In this way, only one redox target was available in each peptide. Both M1L and M8L mutants responded to redox conditions, with weaker effect than wild-type sequence (Figure 7A-C), indicating that both methionine residues were likely contributing to redox sensing. However, mutation of M8 alone was sufficient to alter N17 localization (Figure 5).

3.3 N17 M8 oxidation does not directly impair nuclear export

Although it was possible that the observed nuclear accumulation of M8Q was due to hyper-phosphorylation, it was also possible that M8 oxidation directly impaired nuclear export independently of S13 S16 phosphorylation. To address this, we assessed nuclear

export of recombinant oxido-mimetic and reduced-mimetic N17 constructs using leptomycin B to impair export from the nucleus and quantify the resulting nuclear accumulation. In the context of phospho-resistant S13 and S16 alanine mutants, N17 with either M8A (M8AS13AS16A) or M8Q (M8QS13AS16A) mutation was still responsive to leptomycin B, and hence contained a functional NES (Figure 8A, B). These data establish that M8 oxidation does not directly affect the NES activity of N17. Interestingly, a striking difference was also noted between the localizations of M8Q (Figure 5A panel c) and M8QS13AS16A (Figure 8, panel e). The absence of M8QS13AS16A nuclear accumulation further indicates involvement of serine phosphorylation in M8Q nuclear localization and supports the hypothesis that N17 oxidation indirectly inhibits nuclear export by promoting N17 phosphorylation.

3.4 N17 M8 oxidation directly promotes N17 phosphorylation

Using a quantitative pull-down assay, we directly showed that M8 oxido-mimetic mutation resulted in enhanced N17 phosphorylation. We performed western blot analysis of N17Wt-YFP, M8Q-YFP and M8L-YFP recombinant protein following YFP-immunoprecipitation from ST*Hdh*$^{Q7/Q7}$ cells. N17 moieties were assessed for their level of phosphorylation using western blot analysis with α-pN17, normalizing to α-YFP as an internal loading control (Figure 9A). Oxido-mimetic M8Q-YFP was shown to be phosphorylated approximately 2.5 times more than N17Wt-YFP (Figure 9B). Thus, oxidation of N17 M8 is sufficient to promote phosphorylation.

3.5 Increased membrane dissociation promotes N17 phosphorylation

M8 oxidation alters the structure (Figure 3), and promotes the phosphorylation (Figure 9), of N17. As M8 is positioned along the ER membrane-aqueous interface within the N17 helix (58), the structural change induced through M8 oxidation may affect association of N17 with the ER membrane. We therefore hypothesized that methionine oxidation disrupts N17 anchoring to the ER membrane, enhancing the solubility and kinase accessibility of the protein. To test that solubility modulated N17 phosphorylation, we compared the phosphorylation of recombinant E5AE12A and M8P N17 mutants, which have previously been shown to have vesicular and un-targeted localizations respectively (36). We first validated the targeting of these mutants using Pearson's correlation with mCherry or ER Tracker™ dye (Figure 10A, B). YFP was used as a control for both maximum correlation (with mCherry) and minimum correlation (with ER tracker). E5AE12A-YFP showed poor correlation to mCherry (below 0.4) and high correlation with ER tracker (above 0.9), indicating poor solubility. In comparison, M8P-YFP showed poor correlation to the ER tracker signal, but a very high correlation to mCherry. Therefore, M8P-YFP is highly soluble, while E5AE12A-YFP is not.

We then compared these two mutants in a similar assay as in Figure 9 and could note a significantly higher phosphorylation of M8P-YFP, but significantly lower modification of E5AE12A-YFP (Figure 10C, D). The elevated phosphorylation of soluble N17, and the reduced phosphorylation of membrane bound N17, indicates that the degree of membrane association affects N17 phosphorylation. Taken together with

previous evidence, the increase in N17 phosphorylation from both oxidation and solubility suggests that M8 oxidation acts to promote N17 phosphorylation by increasing solubility and accessibility to kinases.

3.6 Total huntingtin levels increase with oxidative stress

Using a similar 3-NP dose-response immunofluorescence assay as in Figure 1, we also assessed the effect of oxidative stress on total levels of endogenous huntingtin. Total huntingtin was detected using secondary immunofluorescence with MAB2166, an antibody whose epitope is downstream of N17 and thus unaffected by phosphorylation. Qualitative assessment of 3-NP dosed 1° wild-type human fibroblast (Figure 11A) and RPE1 (Figure 11B) cells, showed elevated levels of total huntingtin. The increase in total huntingtin appears to be weaker than the previously observed increase in huntingtin phosphorylation (Figure 1A, B), although further replicates and quantification is needed to validate this observation. Therefore, the increase in huntingtin phosphorylation is likely primarily due to promoted phosphorylation of pre-existing huntingtin, rather than because of an increased amount of total huntingtin available. However, as the 3-NP treatment period was relatively short, 1 hour, the effect of total huntingtin levels on huntingtin phosphorylation may be more significant with prolonged or chronic stress where it may act as an additional level of regulation.

4.0 Discussion

We were interested in learning more about the biological role of wild-type huntingtin in the cell stress response, given that the stress dependent phosphorylation of N17 S13 and S16 has been established as a potential therapeutic sub-target for HD (98, 99). The N17 domain is critical for normal huntingtin function as it mediates the stress dependent localization of huntingtin between the ER membrane and the nucleus. N17 is an amphipathic alpha helix (36, 58) and functions both as a NES (59) and ER membrane anchor (36) to target huntingtin to the cytoplasm under normal conditions. N17 phosphorylation disrupts N17 NES functionality and promotes huntingtin localization to nuclear puncta (44, 59), a location where huntingtin presumably functions in a stress response capacity. What is not fully understood is what could trigger this phosphorylation in response to cell stress. The stresses which have been previously characterized to affect huntingtin phosphorylation trigger global protein misfolding, likely activating a plethora of response pathways. Therefore, in this study, we sought to determine a specific molecular trigger of huntingtin phosphorylation and nuclear localization.

Here, we have identified oxidative stress as a specific stress that can affect huntingtin phosphorylation and nuclear localization, and have defined a direct mechanistic relationship between the two through oxidation of N17 M8. We show that ROS stress promotes huntingtin phosphorylation and localization to nuclear puncta. Using CD spectroscopy on synthetic N17 peptides we show that N17 alpha helicity is sensitive to M8 redox state and demonstrate the capacity of M8 oxido-mimetic mutation

to affect N17 nuclear targeting and phosphorylation using recombinant mutant N17 fragments. We also define a specific molecular mechanism whereby N17 oxidation promotes membrane dissociation, thus increasing kinase accessibility and subsequent phosphorylation. This work has defined a novel function of huntingtin as a ROS sensor.

4.1 Huntingtin has a normal biological function as a ROS sensor

Here, we show that huntingtin has a normal biological function as a ROS sensor, specifically through direct oxidation of N17 M8. In support of this novel role of huntingtin and the importance of M8 in N17 biology are observations from our sequence alignment of N17 (Figure 2). Many bird and reptile species, which are historically considered to have high basal metabolic rates and long lifespans respectively, have evolved a second methionine at position 4 in addition to the one found at position 8. In contrast, largely immobile species, such as the owl limpet and sea lancelet, have no methionines in N17 outside of M1. The correlation between N17 methionine number and species metabolism and lifespan supports the concept that methionines present in N17 are involved in sensing oxidative stress. It may be that species with high ROS loads have evolved additional methionine sensors in N17 for increased regulation or sensitivity to oxidative stress. Huntingtin M8 oxidation has also previously been seen to affect huntingtin small fragment overexpression aggregate properties (163), suggesting M8 oxidation also affects the properties of mutant huntingtin in the disease context. Similarly, in Parkinson's disease, alpha synuclein has been shown to be oxidized at methionines (164). Interestingly, like huntingtin, alpha synuclein contains a small alpha helical lipid

interaction domain (165), suggesting a similar method of regulation by oxidation may be occurring. As adult stem cell neurogenesis is triggered by ROS signaling (166), deficits in neurogenesis within the striatal interneuron population of adult HD brains (167) is also consistent with the role of huntingtin as an oxidative stress sensor and suggests dysfunction of this role may be occurring in HD.

We suggest a model in which huntingtin trafficking between the ER membrane and nuclear puncta is controlled by increased ROS levels in the cytoplasm and ER (Figure 12). We hypothesize that huntingtin, anchored to membranes by N17, can sense elevated ROS levels through oxidation of methionines in N17, primarily M8. This oxidation induces an increase in N17 alpha helicity and, as suggested by the solubility assays (Figure 10), promotes release of huntingtin from membranes. Previous NMR data has shown M8 is located very near the ER membrane-aqueous interface (58), and is presumably accessible by reactive oxygen atoms. Increased huntingtin solubility enhances kinase accessibility, resulting in increased levels of phosphorylated huntingtin. Dissociation of huntingtin from the ER membrane also allows nuclear entry mediated by a downstream PY-NLS (57). Phosphorylation of N17, which we have previously shown to regulate nuclear export by inhibiting CRM1 interaction (59), allows huntingtin nuclear accumulation and localization to phospho-puncta (44).

4.2 A potential role for oxidative stress in HD pathology

This model is consistent with the nuclear accumulation of mutant huntingtin inclusion bodies seen in HD (34). We propose that M8 oxidation is still able to enhance

the solubility and nuclear translocation of mutant huntingtin, however export from the nucleus is impaired. Because mutant huntingtin is hypo-phosphorylated (44), the observed nuclear accumulation is likely due to the altered conformation of the polyglutamine expansion preventing CRM1 interaction, not enhanced phosphorylation as in the wild-type pathway. It is possible that steric hindrance from the polyglutamine expansion also prevents kinase accessibility which results in the observed hypo-phosphorylation of mutant huntingtin. This explanation is consistent with a previous study which showed that polyglutamine expanded huntingtin, rendered fully soluble through M8P mutation, increased cell toxicity and nuclear accumulation (36). This further indicates that the soluble form of mutant huntingtin contributes to nuclear accumulation and toxicity. It also implies that the protective effects of anti-oxidants in HD models (158, 159) function by lowering the oxidative environment of the cell. This reduces the oxidation, and therefore solubility and toxicity, of mutant huntingtin. We suggest that soluble, non-phosphorylated, nuclear mutant huntingtin is the toxic species in HD which accumulates in the nucleus due to its altered conformation preventing CRM1 binding, which is consistent with other past studies (35, 168).

4.3 Possible mechanisms of increased total huntingtin with oxidative stress

In addition to quantifying elevated huntingtin phosphorylation in response to 3-NP induced oxidative stress, we have also qualitatively described an increase in total huntingtin levels under oxidative conditions (Figure 11). The increase in total huntingtin appeared to be weaker than the increase in phosphorylated huntingtin (Figure 1),

indicating that elevation of huntingtin phosphorylation is likely to be primarily due to promoted phosphorylation of pre-existing huntingtin, rather than an increased amount of huntingtin available. Nevertheless, elevation of total huntingtin levels by oxidative stress suggests that oxidative regulation of total huntingtin levels may be a secondary level of control. It may be that under our manufactured conditions of oxidative stress, which were relatively short at 1 hour, regulation primarily occurs through modification of already present huntingtin. However, under different conditions of oxidative stress, possibly long term chronic stress, regulation of total huntingtin levels may become more prominent. Elucidating these two mechanisms will require further study.

There are a number of mechanisms by which oxidative stress could regulate total huntingtin levels. Firstly, is through transcriptional regulation of the huntingtin gene by p53. P53 is a transcription factor that is activated by numerous cell stresses, including oxidative stress, to transcriptionally activate downstream target genes involved in the cell stress response, cell cycle arrest or apoptosis (161). The huntingtin gene contains p53 response elements and huntingtin mRNA and protein levels are elevated following p53 activation with gamma irradiation (160). It is possible that p53, activated by oxidative stress, initiates huntingtin transcription. However, following p53 activation, quantitative real-time PCR analysis showed only a 1.4x fold-increase of huntingtin mRNA levels after 8 hours (160). Therefore, it is likely that no change in huntingtin levels would be seen after only 1 hour, as were our conditions. Thus, the increase in total huntingtin levels following oxidative stress seen by us was likely not due to transcriptional activation by p53.

The second possible mechanism of total huntingtin regulation by oxidative stress is through translational regulation. Huntingtin has been shown to associate with its own mRNA (*HTT* mRNA), exclusively of other mRNA's, in a complex with RNA binding proteins and translation factors (169). This association was independent of ribosomes, suggesting that huntingtin associated *HTT* mRNA is not undergoing translation. It is possible that huntingtin is able to regulate its own expression level through translational regulation of *HTT* mRNA. The binding of huntingtin to *HTT* mRNA may act to suppress *HTT* mRNA translation under normal cell conditions, while oxidation of huntingtin during stress could release it from the complex and allow translation to occur. This stress dependent translation of pre-existing *HTT* mRNA could be used to elevate total huntingtin levels in a relatively rapid manner compared to the previously described transcriptional regulation mechanism. To explore this hypothesis, it would be interesting to see if the huntingtin–*HTT* mRNA association is sensitive to ROS.

Finally, regulation of total huntingtin levels by oxidative stress could also be occurring through modification of huntingtin degradation. Multiple studies have suggested that autophagy may be involved in the normal processing of huntingtin in cells. Blocking autophagy using 3-methyladenide raised the levels of exogenously expressed wild-type (170) and polyglutamine expanded (171) amino terminal huntingtin fragments, indicating that huntingtin may be in a constant state of degradation under normal conditions. Therefore, it may be that total huntingtin levels are increased following oxidative stress due to inhibition of huntingtin degradation. However, it is important to note that the studies documenting huntingtin autophagy (170, 171) all employed

overexpression models of exogenous amino terminal protein fragments. These fragments are likely toxic to cells, even when not containing a poly-glutamine expansion, and therefore their degradation may not be representative of physiologically conditions. Study of endogenous huntingtin autophagy has not been performed but would strengthen the evidence surrounding autophagic regulation of huntingtin levels.

4.4 Oxidative stress sensing by huntingtin could trigger its function in DNA damage repair and subsequent toxicity in HD

The nuclear translocation of huntingtin in response to ROS, described here, is complimentary and consistent with recent evidence from Maiuri et al. that identified huntingtin as a DNA damage response protein (manuscript in preparation). Maiuri et al. showed that, in response to DNA damage caused by oxidative stress, huntingtin co-localizes with base excision repair proteins X-ray repair cross-complementing protein 1 (XRCC1), apurinic/apyrimidinic endonuclease (APE1) and poly-ADP-ribose (PAR) at nuclear puncta similar to the phospho-N17 nuclear puncta described here. Huntingtin was also shown to co-immunoprecipitate with XRCC1 and APE1, as well as other DNA damage response proteins flap endonuclease 1 (FEN1) and ataxia-telangiectasia mutated (ATM), in a ROS-dependent manner. This ROS dependent co-localization and interaction of huntingtin with DNA damage response proteins strongly suggests a role of huntingtin as an oxidative DNA damage response protein. It also suggests that the ROS-induced phospho-N17 puncta studied here are sites of oxidative DNA damage that are undergoing active repair. Taken together, the role of huntingtin as an oxidative stress sensor in the

cytoplasm and DNA damage response protein in the nucleus, suggests that the purpose of huntingtin cytoplasmic oxidative stress sensing is to trigger nuclear translocation in preparation of oxidative DNA damage repair.

There are many lines of evidence to suggest that oxidative DNA damage repair is dysregulated in HD, indicating the relevance of this pathway to disease. Excessive accumulation of DNA damage has been observed in HD models and patient samples (172-176). Furthermore, recent evidence from Maiuri et al. directly showed that HD cells are deficient in their ability to repair oxidative DNA damage using an alkaline comet assay, indicating impairment of this pathway by mutant huntingtin. The role of huntingtin in DNA damage repair, and the impairment of this role upon polyglutamine expansion, suggests that accumulation of oxidative DNA damage could be a pathogenic mechanism of HD. The contribution of oxidative DNA damage and subsequent repair mechanisms to disease has been examined by multiple groups.

4.4.1 DNA damage response genes are genetic modifiers of HD age-onset

CAG repeat length is the primary determinant of HD age of onset, where glutamine repeats exceeding the critical threshold of 40 cause disease at an age inversely correlated with expansion length (5-8). However, as discussed previously, this inverse correlation shows extreme variation, particularly at low glutamine repeats, indicating other genetic and environmental modifiers in disease pathogenesis. A recent genome wide association study, that looked to identify genetic modifiers of HD age-onset, suggests that these genetic modifiers are almost exclusively related to DNA damage repair and

oxidative stress (177). The majority of genes which showed significant association with age at HD motor onset were involved in DNA damage, mitochondrial function and peroxisome function. This suggests that ROS and DNA damage are the critical determinants of HD age of onset.

4.4.2 ATM recruits' huntingtin to DNA damage sites and contributes to HD pathology

The DNA damage response protein ATM, has also been shown to contribute to HD progression. ATM is a serine/threonine kinase which becomes activated by double stranded DNA breaks, single stranded DNA breaks, chromatin reorganization and oxidative stress (178-181). ATM acts within the early stages of the DNA damage response to phosphorylate and activate other response proteins at the site of DNA damage. In the event of extensive and irreparable DNA damage, ATM can also initiate apoptosis through activation of p53 (182). ATM signalling is persistently elevated in cells derived from HD mice and brain tissue from HD patients (183), consistent with reports of elevated and delayed clearance of DNA damage markers in HD cells (176). Furthermore, knockdown and pharmacological inhibition of ATM has been shown to protect HD cell, mouse and fly models against disease-associated behavioral deficits, neuropathology and cell death (183). The modulation of HD pathology by ATM indicates that ATM activity is likely mechanistic in HD. The functional relationship between ATM and huntingtin was recently identified by Maiuri et al, where ATM was shown to co-localize with phosphorylated huntingtin at nuclear puncta and sites of DNA damage. Huntingtin

recruitment to DNA damage sites was sensitive to ATM inhibition, indicating that ATM kinase activity is necessary for huntingtin function in DNA damage repair.

The role of ATM in recruiting huntingtin to sites of DNA damage, taken together with the effect of ATM knockdown and inhibition on HD pathology, further suggests that the function of huntingtin in DNA damage repair is relevant to disease. It is possible that mutant huntingtin impairs the repair of DNA, leading to the accumulation of damage and persistent ATM activation observed in disease. The observed benefits of ATM inhibition may therefore be due to inhibition of mutant huntingtin recruitment to DNA damage sites.

4.4.3 Repair of oxidative DNA damage contributes to disease through somatic expansion

Somatic expansion of the inherited mutant huntingtin CAG tract has been shown to contribute to HD pathology. Somatic expansion refers to the lengthening of the huntingtin CAG repeat from that which was initially inherited. Accumulation of somatic expansion with age has been proposed as a molecular trigger of disease, where the initial inherited expansion provides the biological setting for somatic expansion to occur, while the actual accumulation of somatic expansion to a pathogenic length over time is what triggers disease (184). Significant somatic expansion has been detected in the striatum, the region most affected in disease, of post-mortem HD patient brains (185). The hypothesis that HD onset is triggered by accumulation of somatic expansion is consistent with the observation that clinically relevant inherited allele lengths, 37-44, do not generate an obvious phenotype in mouse models (186). The synthetic polyglutamine

repeats, in excess of 100, used in mice to elicit disease phenotype may in fact mimic the somatically expanded huntingtin seen in patients at age onset, essentially skipping the premanifest stage of the disease during which time expansion takes place.

Somatic expansion has been reported by several groups to occur through removal of oxidized base lesions by DNA repair pathways. Knockdown of base excision repair enzymes oxoguanine glycosylase 1 (OGG1) (184, 187, 188) and nei-like glycosylase 1 (Neil1) (189), and mismatch repair enzymes MutL, MutS and MutY (188, 190), have been shown to inhibit somatic expansion of the huntingtin gene in HD mice. XJB-5-131, a mitochondrial-targeted scavenger of reactive oxygen species which reduces the number of oxidative DNA lesions, was shown to prevent somatic expansion (184). In addition, these studies also correlate inhibition of somatic expansion with delayed onset of HD (184). The attenuation of disease pathology through both inhibition of DNA damage repair mechanisms and prevention of oxidative DNA damage, demonstrates the relevance of oxidative DNA damage to disease. The role of huntingtin as a DNA damage response protein also suggests that mutant huntingtin may contribute to its own somatic expansion by dysregulating base excision repair and mismatch repair mechanisms.

4.4.4 3-NP toxicity may occur through oxidative DNA damage

Additional evidence that accumulation of oxidized DNA lesions contributes to HD pathology was shown through studies of human MutT homolog 1 (hMTH1), a hydrolase which degrades oxidized purine NTPs, thereby preventing their incorporation into DNA. Transgenic expression of hMTH1 was shown to protect against 3-NP induced

neurodegeneration in mice (191). In addition to the use of 3-NP as an inducer of intracellular ROS (162), 3-NP was also commonly used to replicate the physiological symptoms of HD in animal studies before the generation of genetic disease models (137). The induction of HD-like symptomology using a mitochondrial inhibitor initiated the long-held hypothesis that mitochondrial dysfunction is instrumental in HD pathology. However, the protection against 3-NP toxicity through removal of oxidized NTPs suggest that mitochondrial dysfunction may be an intermediary step in 3-NP toxicity, which ultimately replicates HD pathology by inducing excessive oxidative DNA damage.

The roles of huntingtin in ER ROS sensing and DNA damage repair are likely tightly linked. We propose that huntingtin acts as a sensor of oxidative stress in the cytoplasm, with nuclear localization as a response, to trigger its involvement in oxidative DNA damage repair in the nucleus. The above evidence suggests an importance of oxidative DNA damage, and therefore oxidative stress sensing, in HD pathology.

4.5 Age associated ROS stress may be a critical molecular trigger of Huntington's disease

The role of huntingtin as an oxidative stress sensor presents an interesting connection between age related increases in oxidative stress and HD age of onset. We speculate that the recognition and response of huntingtin to oxidative stress is a secondary function which normally becomes active later in life with increasing ROS load. In the case of mutant huntingtin, the triggering of this secondary function by ROS may initiate HD by prompting mutant huntingtin nuclear accumulation and resulting pathology, likely

involving disruption of DNA damage repair and somatic expansion of the huntingtin gene. In this way, age-associated ROS could be a molecular trigger of disease and provide a link between altered function of huntingtin and onset of HD pathogenesis. This suggests that modeling HD using ROS stress, such as the historic 3-NP disease model of striatal degeneration, may provide a more accurate model of human disease than the extreme synthetic polyglutamine repeat lengths commonly used in mice models.

The role of huntingtin as an oxidative stress sensor may also contribute to tissue specificity of HD. Brain and cardiac tissues are regions of high ROS in the body and are also regions of pathology in HD. Neurons, the primary affected cell type in HD, are particularly vulnerable to ROS due to their large dependence on oxidative phosphorylation for energy. It is estimated that approximately 10,000 oxidative DNA lesions occur per neuron per day (192). Cardiac failure is historically a major cause of death in HD (26), and the substantial amount of metabolism generated ROS in cardiac tissue further indicates a correlation between tissue oxidative stress levels and HD regional toxicity. In these regions of high ROS, it may be that huntingtin function as an oxidative sensor is more prominent, and therefore sensitive to mutant huntingtin dysfunction toxicity. We therefore suggest that ROS stress due to aging is a critical molecular trigger of HD and may be a contributing factor in explaining the age-onset and tissue specificity of HD.

5.0 Conclusion

This study explores the basic biology of wild-type huntingtin in relation to oxidative stress, while considering the potential role of this process in disease pathogenesis. We have defined a novel function of huntingtin as an oxidative stress sensor and provide a specific molecular mechanism of ROS sensing through oxidation of M8 in N17. We show that ROS stress results in enhanced phosphorylation and localization of huntingtin to nuclear puncta. These results have led to a modification of earlier mechanistic models of huntingtin localization, in which we now establish that M8 sulfoxidation is what triggers huntingtin ER release, allowing the soluble protein to be modified at S13 and S16. It also provides supporting evidence for huntingtin function in the DNA damage response, a function that has been strongly implicated in disease pathogenesis. The elucidation of this mechanism is important as it provides a link between age-associated increases in ROS, altered function of huntingtin and onset of pathogenesis, suggesting that ROS stress may be critical molecular trigger of age-onset HD.

6.0 Figures

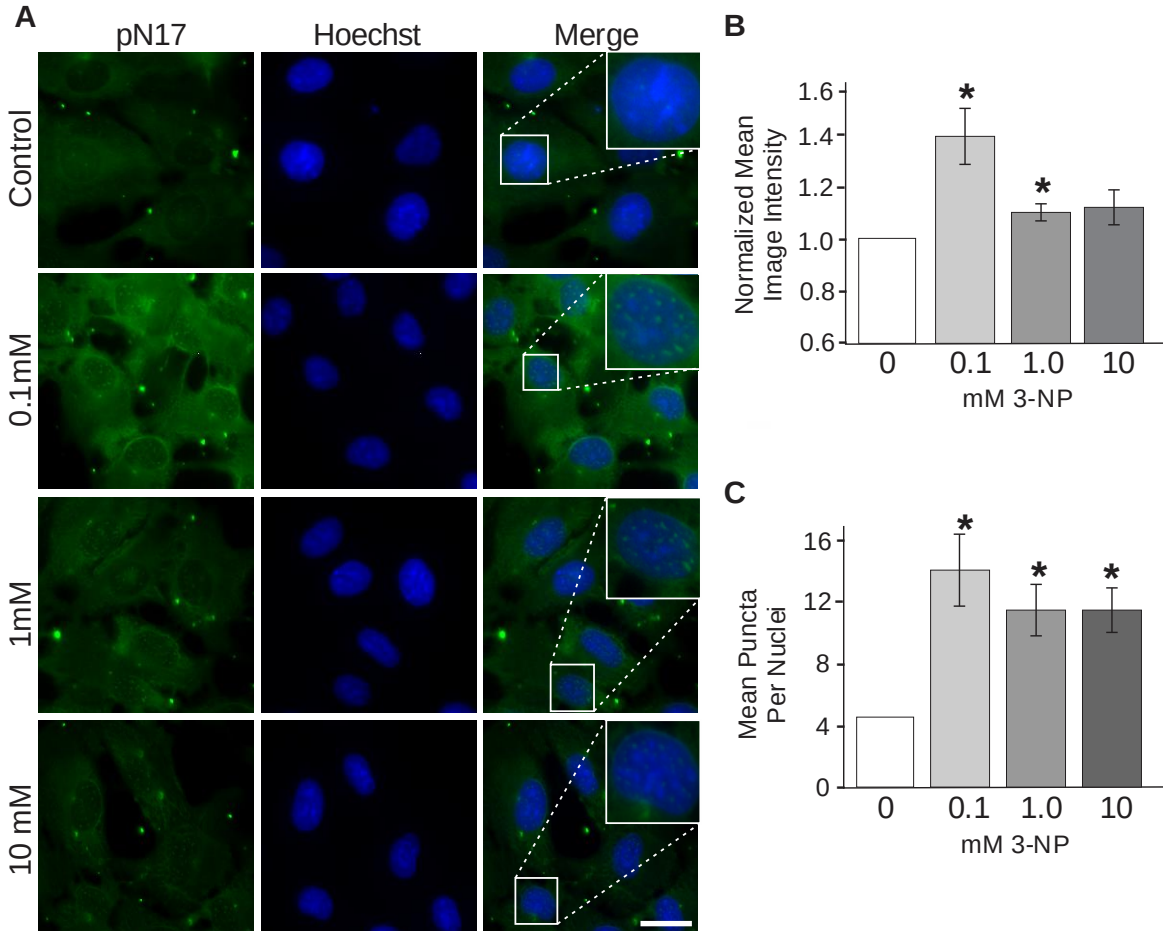

Figure 1. Huntingtin N17 phosphorylation levels and nuclear puncta formation increase in response to oxidative stress. (A) Immunofluorescence of 3-NP treated RPE1 cells with Alexa-488 conjugated α-pN17. (B) Per image intensity quantification of thresholded images and (C) per nucleus puncta quantification of α-pN17 channel. 40 images and >500 cells measured per condition. N=4. Error bars represent standard error. Scale bar is 10μm.

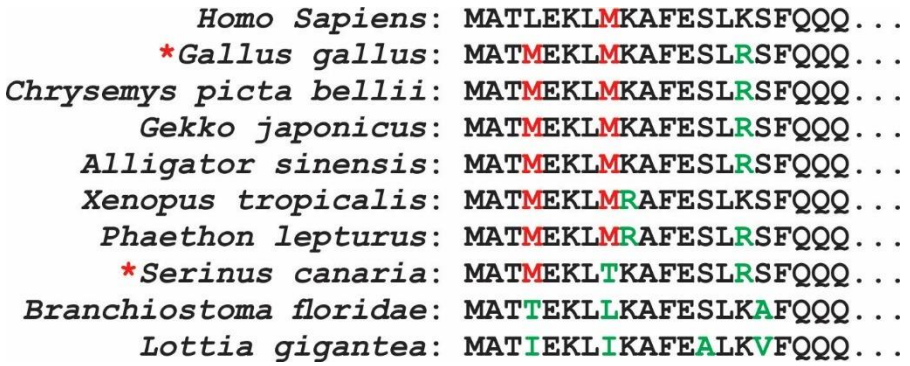

Figure 2. Alternative methionine residues are conserved in huntingtin N17. (A) BLAST sequence alignment between huntingtin species. Methionine's shown in red, other altered residues shown in green. * Indicates many similar species containing this sequence.

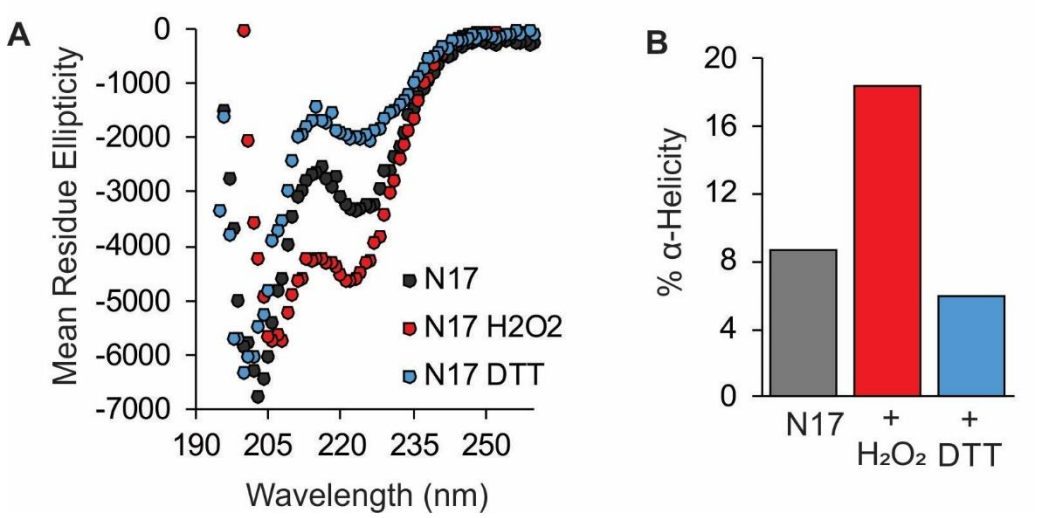

Figure 3. Oxidation and reduction alters α-helical content of huntingtin N17 peptides. (A) CD spectroscopy data of N17 synthetic peptide treated with 250μM H_2O_2 or 1mM DTT. (B) Percent α-helical content calculated using the Contill algorithm.

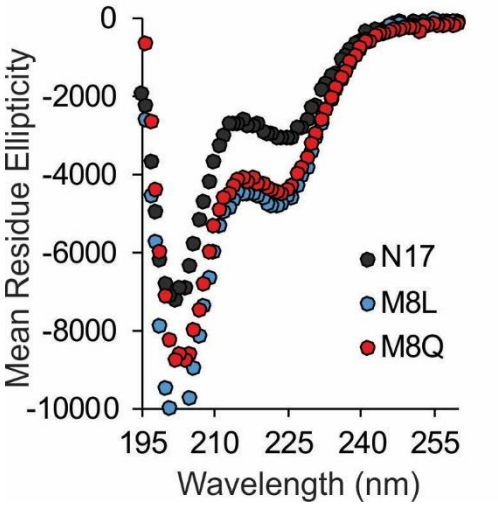

Figure 4. M8Q and M8L N17 mutant moieties are structurally similar to N17. CD spectroscopy data of N17, M8L and M8Q synthetic peptides.

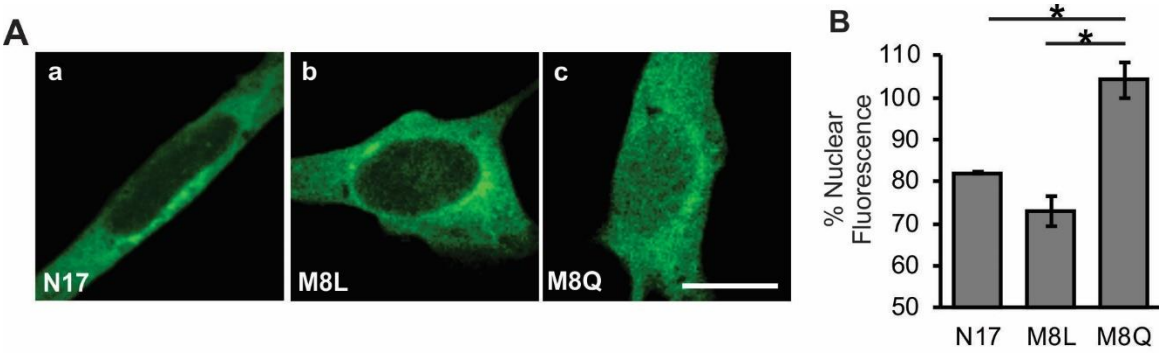

Figure 5. Mimetic-oxidation of N17 methionine 8 influences N17 nuclear targeting.

(A) Representative confocal images of transfected (a) N17 (b) M8L and (c) M8Q YFP mutant moieties in ST*Hdh*$^{Q7/Q7}$ cells. (B) Quantification of nuclear targeting. 70-100 cells were observed for each construct. N=3. * indicated p values <0.05. Scale bar is 10μm.

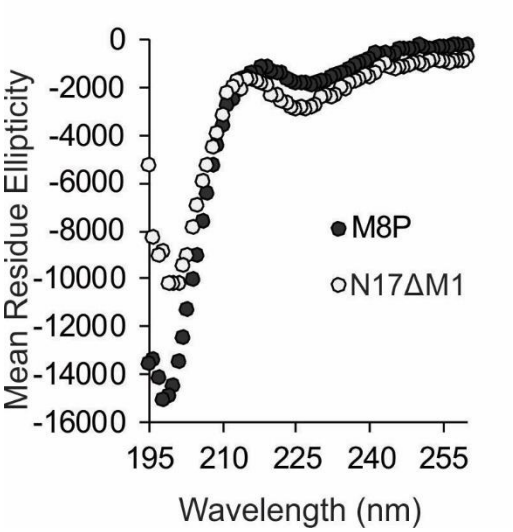

Figure 6. Start methionine is needed for N17 α-helicity. CD spectroscopy data of M8P and N17ΔM1 synthetic peptides.

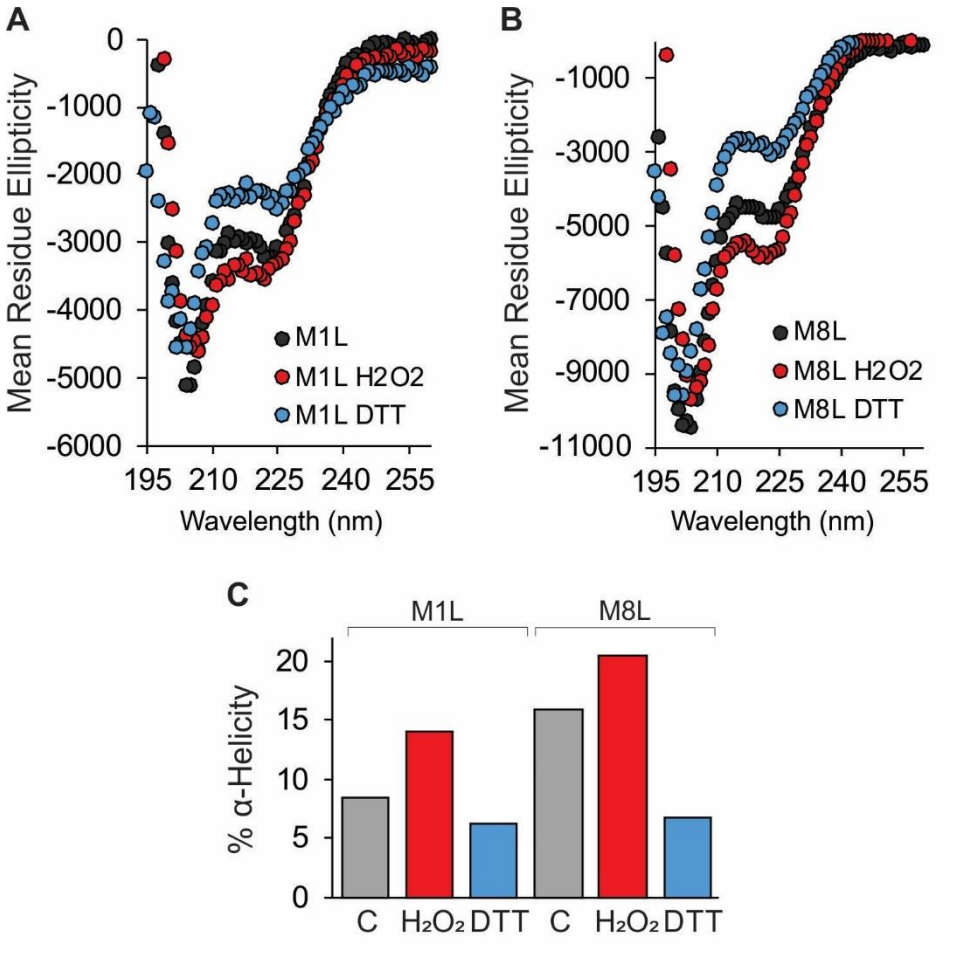

Figure 7. Oxidation of N17 methionines 1 and 8 influences N17 structure. CD spectroscopy data of (A) M1L and (B) M8L synthetic peptides treated with 250μM H2O2 or 1mM DTT. (C) Percent α-helical content calculated using the Contill algorithm.

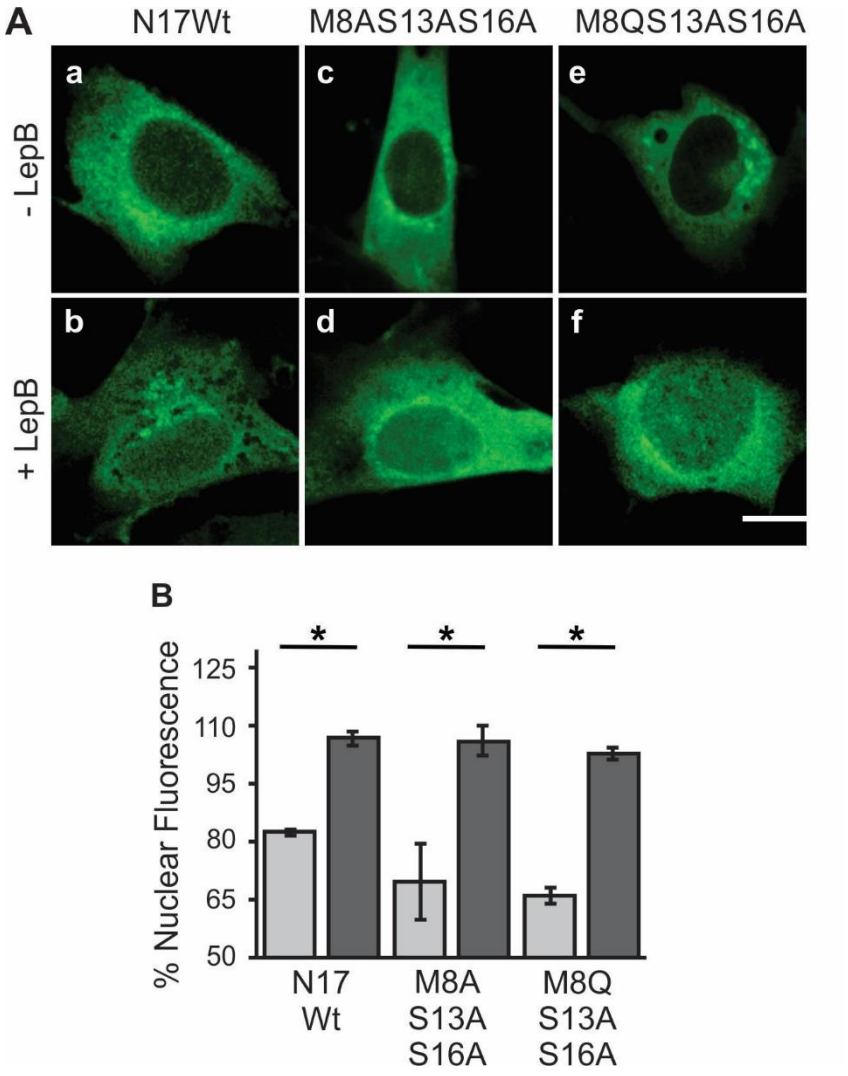

Figure 8. Oxidation of N17 does not directly impair nuclear export. (A) Representative confocal microscopy images of transfected (a, b) N17 (c, d) M8AS13AS16A and (e, f) M8QS13AS16A imaged after 30min treatment with either vehicle (a, c, e,) or 10 ng/mL leptomycin B (b, d, f) in ST*Hdh*$^{Q7/Q7}$ cells. (B) Quantification of nuclear targeting minus (grey) or plus (black) leptomycin B. 40-100 cells were observed for each construct. N=3, * indicated p values <0.05. Scale bar is 10μm.

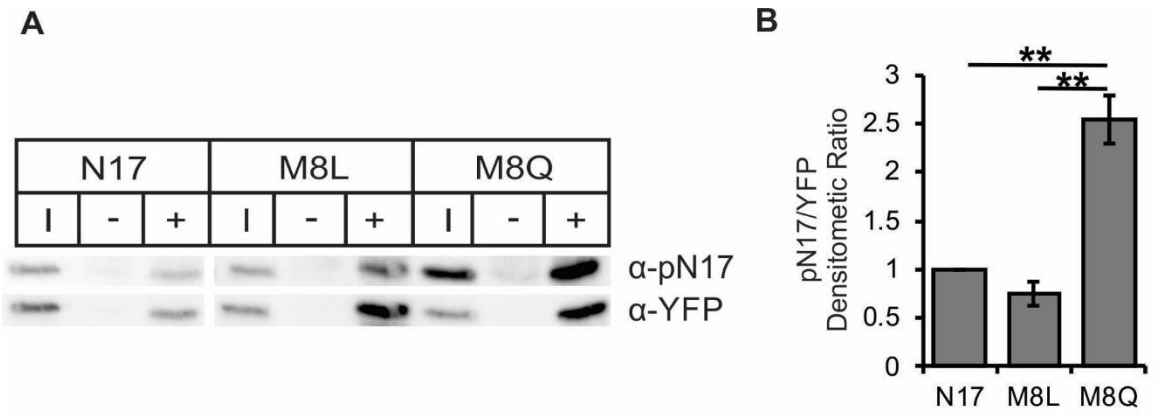

Figure 9. Mimetic-oxidation of N17 methionine 8 promotes phosphorylation.

Western blot analysis of N17-YFP mutant moieties M8L and M8Q, immunoprecipitated from expressing ST*Hdh*$^{Q7/Q7}$ cells. (A) Immunoprecipitation with α-YFP antibody and detection with α-pN17 and α-YFP. I, - and + indicate input, no α-GFP and α-GFP (B) Pixel intensity quantification of α-pN17 to α-GFP internal control. Error bars represent standard error. N17 and M8Q N=6, M8L N=3, ** indicated p values <0.005.

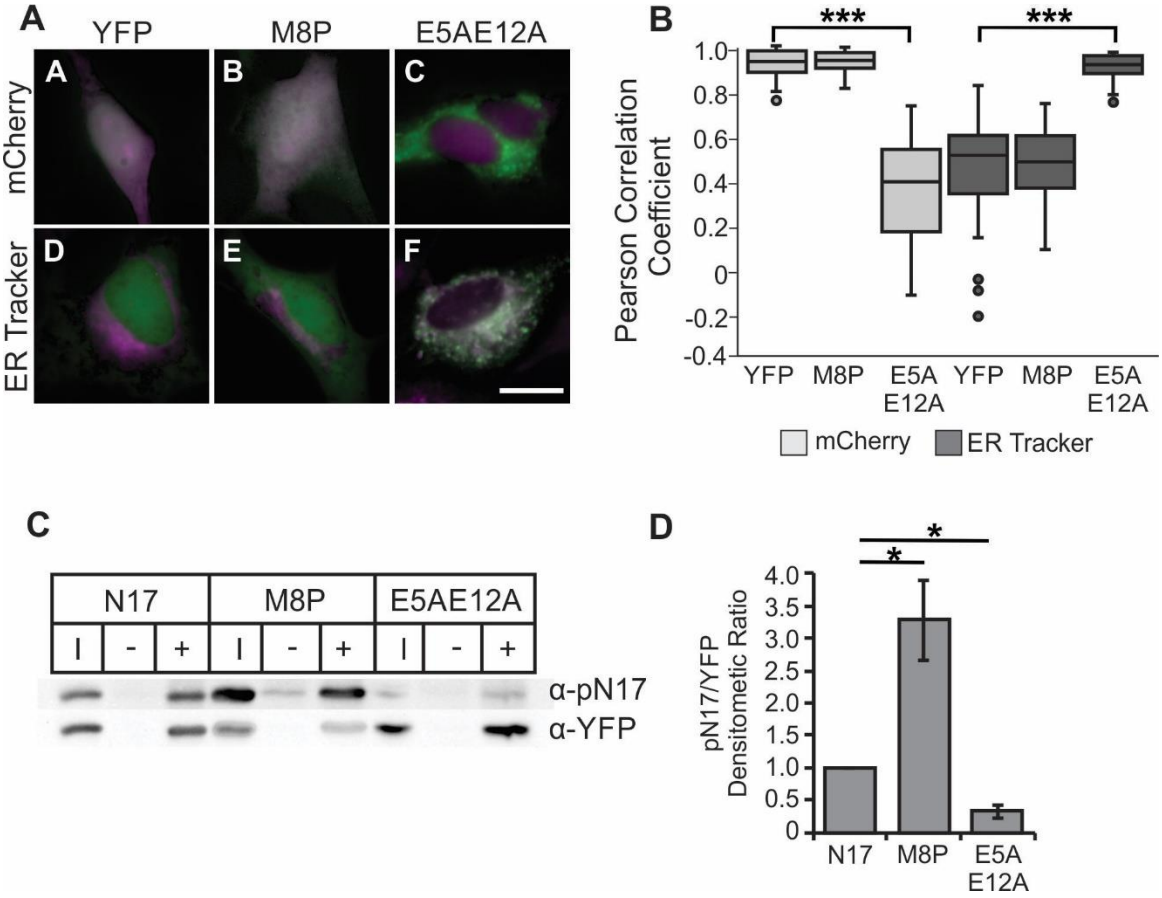

Figure 10. N17 solubility affects phosphorylation. (A) Representative images of YFP (a, d), M8P (b, e) and E5AE12A (c, f) expressing ST*Hdh*$^{Q7/Q7}$ cells with either mCherry (a-c) or ER tracker red (d-f). (B) Pearson Correlation between YFP, M8P or E5AE12A and mCherry or ER tracker. Black line represents median values, boxes encompass 25%-75% of data and whiskers indicate 5%-95% of data. *** indicated p values< 0.001. (C) Immunoprecipitation with α-YFP and western blot analysis of ST*Hdh*$^{Q7/Q7}$ cells expressing N17, M8P and E5AE12A. Detection with α-pN17 and α-YFP. I, - and + indicate input, no α-YFP and α-YFP. (D) Pixel intensity quantification of α-pN17 to α-YFP internal control. N=3, * indicated p values <0.05.

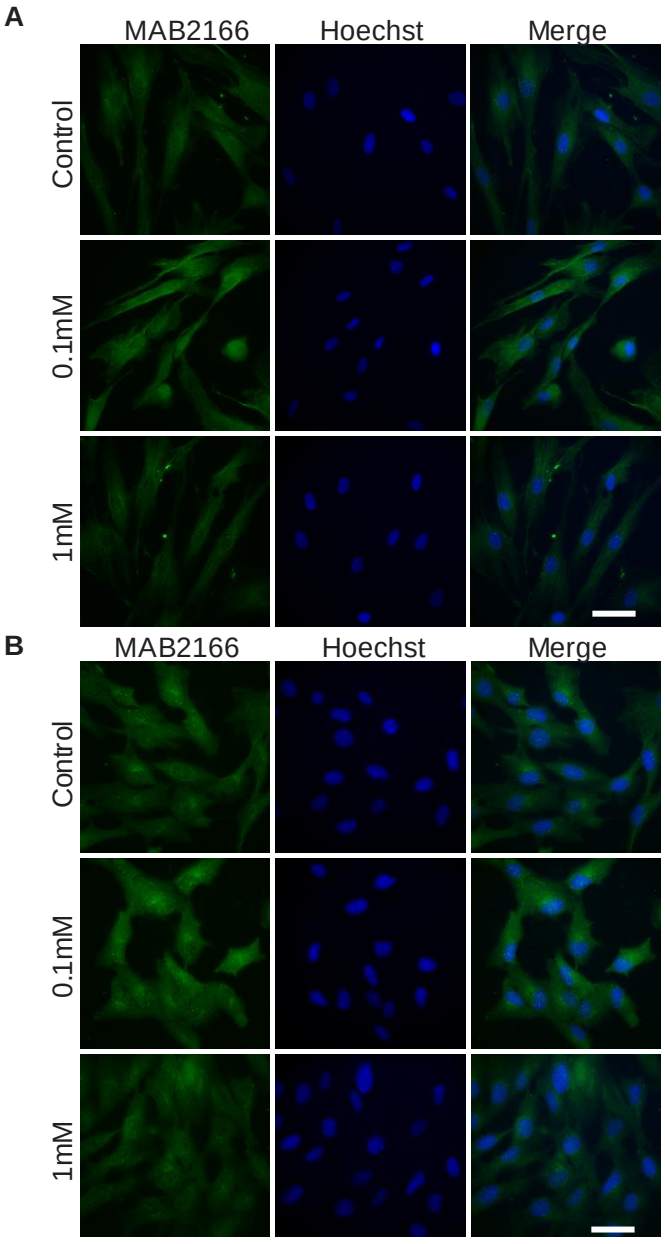

Figure 11. Total huntingtin levels increase in response to oxidative stress.

Representative immunofluorescence images of 3-NP treated (A) primary wild-type fibroblast and (B) RPE1 cells with Alexa-488 conjugated α-pN17. Scale bar is 20μm.

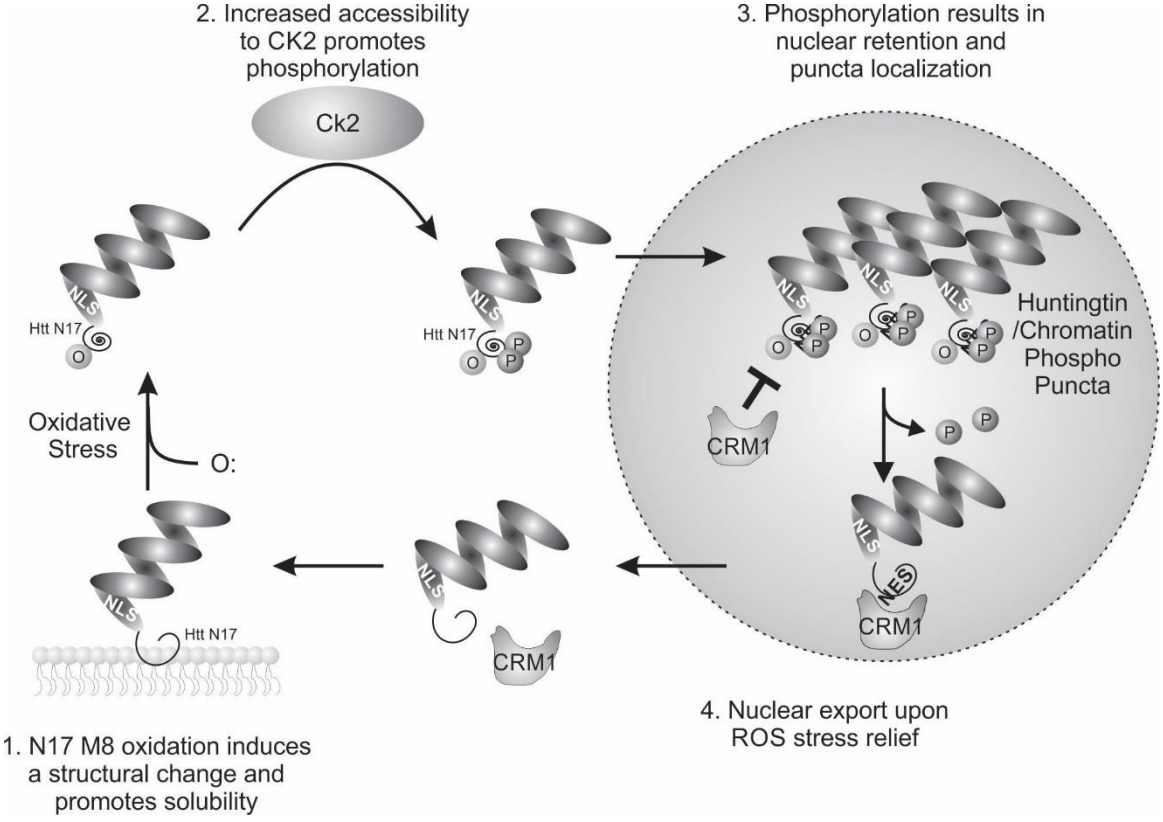

Figure 12. Model of the role of N17 oxidation in huntingtin stress response. Upon high levels of reactive oxygen species, direct oxidation of N17 M8 induces a change in N17 alpha helicity, promoting release from the ER outer lipid. This enhanced solubility increases the accessibility of N17 to kinases, thereby enhancing N17 phosphorylation. Soluble huntingtin then translocates into the nucleus through its downstream nuclear localization signal. Phosphorylated N17 is unable to bind the CRM1 nuclear export factor and huntingtin localizes to nuclear chromatin-dependent puncta. N17 de-phosphorylation upon stress relief results in huntingtin nuclear export via CRM1 binding to the N17 NES.

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