

Cargo Recognition Failure Underlies Macroautophagy Defects in Huntington's Disease

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Abstract

Defects in protein and organelle degradation may be involved in the pathogenesis of Huntington's disease (HD), as macroautophagy can protect against polyglutamine toxicity by attenuating the accumulation of mutant huntingtin protein. HD cells provide the first instance in which autophagic vacuoles form at normal or even increased rates and are adequately degraded by lysosomes, but these vacuoles are "empty" and appear to fail to engulf their normal cytosolic cargo. This impaired cargo recognition results in increased lipid droplets and blocked mitochondrial turnover in HD cells, which could contribute to toxicity and damage. These findings suggest that autophagy activators may have a limited beneficial effect in HD, while interventions to improve autophagic recognition of cargo may provide a promising therapeutic direction.

INTRODUCTION

Huntington's disease (HD), described by George Huntington in 1872, is an autosomal dominant neurodegenerative disorder characterized by cognitive decline, psychiatric disturbances, and motor dysfunction (Vonsattel and DiFiglia, 1998). HD affects approximately 3–10 per 100,000 people. Most patients exhibit HD symptoms between the ages of 35 and 50 and die 10–15 years after the onset of symptoms. The major pathological feature of HD is a loss of medium spiny neurons (MSNs) in the striatum caudate nucleus and putamen (Vonsattel and DiFiglia, 1998). Neuronal loss, together with decreased levels of neurotransmitters and associated enzymes, is also observed in other areas of the brain, including the cerebellar cortex, thalamus, and cerebellum. To date, there is no cure for HD, and most treatments only help alleviate some movement and psychiatric symptoms associated with the pathology.

The genetic cause of HD is a highly polymorphic CAG trinucleotide repeat expansion in exon 1 of the huntingtin gene (*HTT*) on chromosome 4, which encodes the ubiquitously expressed huntingtin protein (htt). These expanded CAG repeats produce a series of glutamine residues known as polyglutamine (polyQ) tracts. The length of polyQ tracts in normal human subjects is polymorphic but below a threshold of 35. PolyQ repeats >40 are invariably associated with the disease, with a strong inverse correlation between repeat number and the age of onset. An intermediate number (36–40) of CAG repeats leads to a slower progression of the pathology as a result of the incomplete penetrance of the mutant allele. An unaffected parent with an "intermediate" number of repeats (36–40) may pass on a copy of the gene with an increased CAG repeat that generates fully penetrant HD with an earlier age of onset and more severe clinical courses in successive generations, a phenomenon known as genetic anticipation.

Mammalian htt is an approximately 350-kDa protein composed of several subdomains (Bano *et al.*, 2011). At the N-terminus, the polyQ stretch encoded by the CAG repeats may function as a membrane association signal. Following the polyQ stretch is a polyproline sequence that stabilizes protein conformation, and three main clusters of HEAT repeats, which are essential for the binding with interacting proteins. In addition, htt includes a range of consensus sites for post-translational modifications, including proteolytic cleavage, phosphorylation, and sumoylation.

While *HTT* was identified as the genetic cause for HD over two decades ago by the Huntington's Disease Collaborative Research Group (1993), and was the first gene identified to cause a widespread neurological disorder, the normal functions of htt protein remain unclear. Htt interacts with a number of proteins involved in gene transcription, intracellular trafficking, synaptic complexes, the plasma membrane, and cytoskeleton and mitochondrial function. Consistently, mutant htt (mHtt) is widely thought to cause cytotoxicity by affecting these intracellular processes (Borrell-Pagès *et al.*, 2006), although there is also abundant evidence for toxic gain of function due to mHtt, as detailed below. In neurons, htt protein has been detected in the nucleus, mitochondria, Golgi, and endoplasmic reticulum, and can be found in the soma, dendrites, and synapses.

Htt is ubiquitously expressed in various types of cells during embryonic development and at high levels in mature postmitotic neurons in adult human brain. Complete suppression of htt expression in mice leads to embryonic lethality as a result of increased apoptosis,

while heterozygous knockout animals exhibit severe cognitive deficits associated with neuronal loss in the subthalamic nucleus of the basal ganglia (Nasir *et al.*, 1995; Zeitlin *et al.*, 1995), although there is little reason at this point to suggest that htt deletion leads to the sort of neurotoxicity that occurs with mHtt. HD pathogenesis may result from a combination of increased gain-of-function of the mHtt, together with a loss-of function of wild-type htt, largely due to the function of the N-terminal polyglutamine region that can form a polar zipper structure able to bind transcription factors (Bano *et al.*, 2011).

A histopathologic hallmark of HD is polyQ-containing protein inclusions in the cytoplasm and nucleus. Htt protein with CAG repeats above the threshold adopts a non-native conformation highly prone to self-associate into high-molecular-weight, stable aggregates. These aggregates are enriched in truncated htt polyglutamine containing-proteolytic fragments, and are associated with the selective loss of striatal medium spiny neurons in HD patients. The mechanisms responsible for the toxicity of these proteolytic products remain elusive. Htt protein can be cleaved within the N-terminal region by multiple proteases, including caspases, calpains, and some matrix metalloproteinases (MMPs), a process to which it is more susceptible as the length of the polyQ tract increases (Bano *et al.*, 2011). These cleavage products may combine with other proteins to form inclusion bodies and aggregates in the neuronal cytoplasm and nuclei. The aggregates further cause proteasome dysfunction and transcriptional dysregulation. However, in some circumstances, intracellular aggregates can sometimes represent a protective effort by sequestering toxic soluble protein species and thus provide beneficial effects (Bodner *et al.*, 2006). Nevertheless, mechanisms that promote correct protein folding conformation or enhance the clearance of htt-containing oligomers or aggregates may provide therapeutic approaches if oligomers or aggregates are indeed toxic agents.

Striatal medium spiny neurons receive both glutamate signals from the cortex and dopamine signals from the substantia nigra, and both signaling pathways act synergistically to enhance the sensitivity of striatal neurons to mHtt toxicity. Mutant htt decreases the expression of astroglial glutamate transporter (GLT1) (Huang *et al.*, 2010) and enhances extrasynaptic NR2B-containing NMDAR signaling at ages that precede motor dysfunction and neuronal loss (Milnerwood *et al.*, 2010), which is suggested to cause neuronal death and striatal degeneration. Dopamine signaling pathways play an important role in HD pathogenesis, and antagonists of dopamine pathway such as tetrabenazine or dopamine receptor blockers may have a therapeutic potential for the treatment of HD (Tang *et al.*, 2007). In neuronal cultures derived from *HTT* mutant mice that are selectively killed by dopamine, autophagic vacuoles are formed in response to high dopamine exposure (Petersén *et al.*, 2001), which may be a response to oxyradical stress associated with oxidation of the dopamine molecule itself to a quinone product.

PROTEIN DEGRADATION IN HUNTINGTON'S DISEASE

All cells rely on quality control mechanisms to maintain cellular homeostasis. Malfunction of the systems that contribute to cellular quality control leads to intracellular accumulation of abnormal and damaged components, with subsequent functional decline and loss of cellular viability. In HD, when mHtt is not efficiently removed, it accumulates

inside neurons in the form of toxic oligomeric species and aggregates, which may trigger neuronal death. While preventing the formation and accumulation of misfolded and toxic forms of mHtt may provide protection, htt appears to exert myriad biological functions that maintain a variety of intracellular processes, and so means to suppress htt translation, such as siRNAs, may not be beneficial. Alternatively, activation of pathways that eliminate the improperly folded proteins may represent an encouraging alternative for treatment.

Misfolded and damaged proteins are removed within cells mainly by the ubiquitin–proteasome system and macroautophagy–lysosome pathways. The ubiquitin–proteasome system (UPS) is ubiquitously present in the cytoplasm, nucleus, and various subcellular regions, and is predominantly responsible for the degradation of short-lived proteins tagged with polyubiquitin chains. Macroautophagy is a degradative pathway for long-lived cytoplasmic proteins, protein complexes, or damaged organelles. The activity of the UPS often remains at a high level, whereas basal macroautophagy constitutively occurs at low levels in cells for the performance of homeostatic functions (Levine and Kroemer, 2008). The UPS and macroautophagy have different capacities to remove soluble mHtt in the cytoplasm: the clearance of soluble N-terminal mHtt is more dependent on the function of the UPS (Iwata *et al.*, 2009). Macroautophagy could function as a backup system to clear aggregated mHtt.

Proteasome Dysfunction in HD

Protein clearance by the UPS requires ubiquitination and subsequent degradation of the tagged proteins by the proteasome. Ubiquitin is a small (76 residues), heat-stable, and highly conserved protein that is ubiquitously distributed in eukaryotic cells. Three steps are involved in the ubiquitination reaction. First, a ubiquitin monomer is activated by forming an intermolecular thiol ester with the ubiquitin-activating enzyme (E1) in an ATP-dependent manner. Next, activated ubiquitin is transferred to a Cys residue in the active site of a ubiquitin-conjugating enzyme (E2). Finally, ubiquitin is linked by its C-terminus through an amide isopeptide linkage to the epsilon-amino group of a Lys residue in the target protein, catalyzed by a ubiquitin–protein ligase (E3). Activated ubiquitin molecules are sequentially added to the first ubiquitin proteins, forming a polyubiquitin chain. Proteins tagged with chains of four or more ubiquitins are recognized by the 26S proteasome for degradation.

The 26S proteasome is a multicatalytic protease localized both in the nucleus and the cytoplasm. It is composed of three major subunits: one 20S catalytic core and two 19S regulatory caps. The 19S regulatory caps recognize the substrates for the 20S proteasome and facilitate access of the target proteins to the 20S proteasome by unfolding the substrates and opening the catalytic channel. Upon recognition of the polyubiquitinated substrates, 19S complexes release polyubiquitin chains. De-ubiquitinating enzymes (DUBs) then disassemble polyubiquitin chains into ubiquitin monomers that can be reused. Inside the 20S complex are three types of catalytic subunits that execute the corresponding catalytic activities of the proteasome: trypsin-like, chymotrypsin-like, and peptidylglutamyl-peptide hydrolyzing (PGPH) proteases. After proteasome proteolytic degradation, ubiquitin monomers are released or actively removed by the ubiquitin carboxyl-terminal hydrolases.

In various cellular and animal models of HD, as well as brains of HD patients, nuclear polyQ inclusions are labeled by antibodies against ubiquitin (DiFiglia *et al.*, 1997; Gutekunst *et al.*, 1999), suggesting an attempt at sequestration by UPS components of polyQ inclusions.

This is confirmed by an indirect assay of UPS function based on the expression of degron-reporter proteins in which a UPS degradation signal is fused to a green fluorescent protein (GFPu, [Bence et al., 2001](#)). GFPu has an extremely short half-life and accumulates only if the UPS is inefficient, providing a sensitive tool to measure the UPS in living cells. Using this assay, [Bence et al. \(2001\)](#) found more polyQ aggregates cellular models following pharmacological inhibition of proteasome activity. When GFPu was co-expressed with mHtt in cells, it accumulated more than twice as much as cells expressing wild-type htt. Using a fluorogenic substrate specific for the chymotrypsin activity of the proteasome, [Jana et al. \(2001\)](#) found a reduction in chymotrypsin activity in the cytosolic fraction derived from lysates from both a stable HD cell model with a 150 htt polyQ repeat and in brain lysates from R6/1 HD mice. Consistent with these data, a reduction of chymotrypsin and peptidyl-glutamyl activities was demonstrated in lysates from human HD post-mortem brains and HD patient skin fibroblasts ([Seo et al., 2004](#)).

Several other studies, however, have failed to detect reduced UPS activity in whole-cell homogenates of brain tissues from HD mice, although Lys 48-linked polyubiquitin chains, an endogenous biomarker of UPS function, did accumulate ([Bennett et al., 2007](#)). [Wang et al. \(2008\)](#) generated fluorescent reporters and targeted these reporters to synapses in mouse brain to measure synaptic UPS activity, and found a particular decrease in UPS activity in the synapses of HD mice. It may be that mHtt does not reduce global UPS function in the brain, but affects UPS activity in a subset of neurons or neuronal compartments.

Macroautophagy Defect in HD

Three types of protein degradation via the lysosome have been the most studied: macroautophagy, microautophagy, and chaperone-mediated autophagy. These differ in their physiological functions and by the cargo they deliver to the lysosome ([Massey et al., 2006](#)). Macroautophagy (hereafter referred to as autophagy) is the primary pathway for eukaryotic cells to degrade damaged organelles. Basal autophagy appears to be particularly important for protecting neurons, as deletion of key autophagy genes in the central nervous system results in neurodegeneration with protein aggregate accumulations ([Wong and Cuervo, 2010](#)).

The process of macroautophagy involves the formation of a double-membrane structure (autophagosome) that then encloses a portion of cytosol and delivers its cargo content to the lysosomes for digestion ([Figure 10.1](#)). This nonspecific bulk degradation process can be divided into multiple steps.

1. *Induction of Autophagy.* Formation of the pre-autophagosome structure (PAS), also called the isolation membrane. Discrete regions in the endoplasmic reticulum, mitochondria, and lysosomes may serve as the nucleation site where components required for the formation of the isolation membrane are recruited. The induction of autophagy is regulated by the activation of mammalian Vps34, a class III phosphatidylinositol-3-kinase (PI3K), which generates phosphatidylinositol-3-phosphate required for the nucleation of PAS. Vps34, Beclin 1 (the mammalian orthologue of Atg6) and the myristylated kinase Vps150 (p150 in humans) form a protein complex that regulates the nucleation process. The interaction between Beclin 1 and hVps34 is essential for initiating autophagy, which is reinforced by UV-irradiation resistance-associated gene (UVRAG) or Ambra1, which upregulates

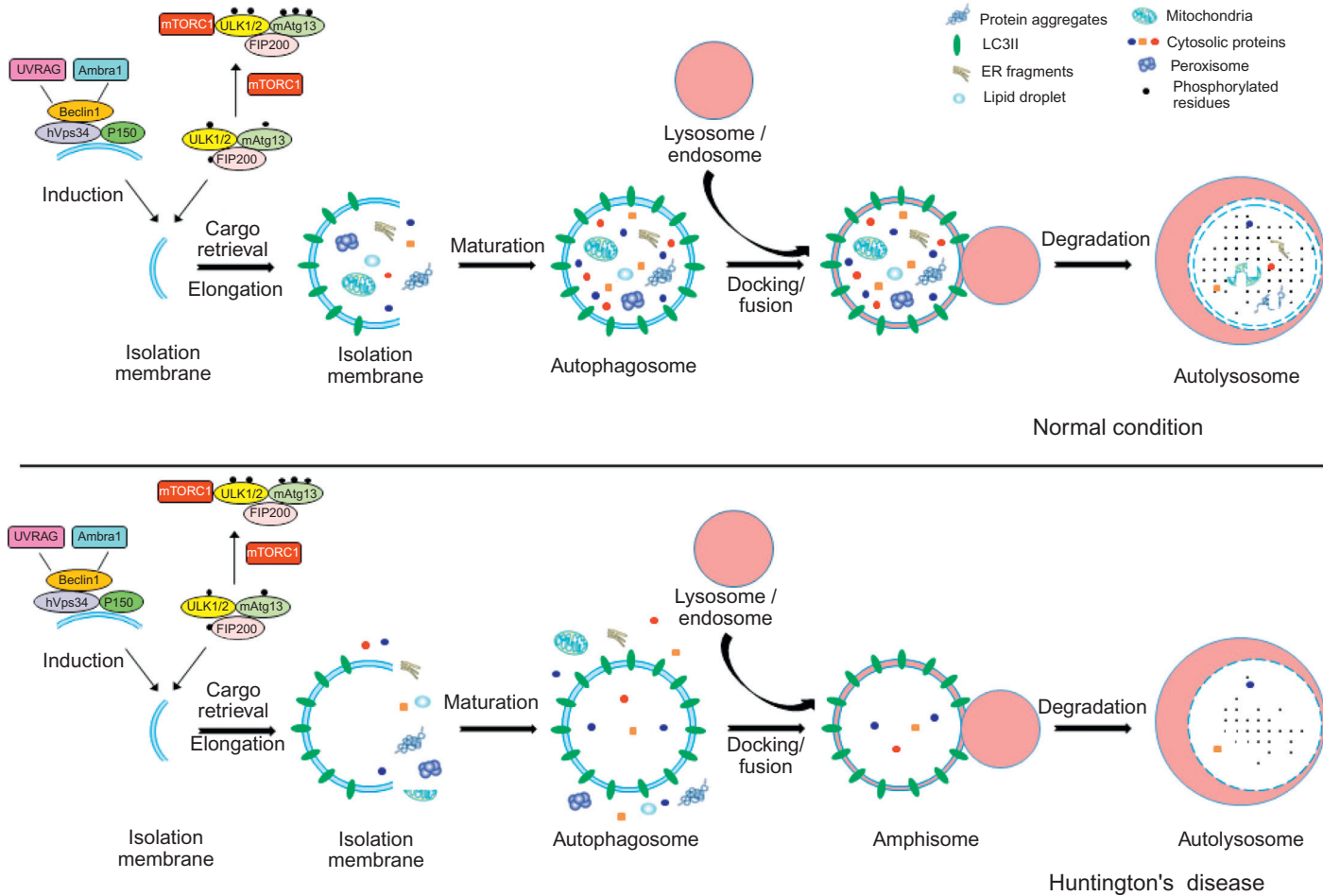


FIGURE 10.1 The mammalian autophagy pathway in normal condition (above) and Huntington's disease (below). In normal condition, mammalian autophagy proceeds through a series of steps, including initiation at the PAS (a.k.a. isolation membrane), cargo recognition and retrieval, membrane elongation, autophagosome formation, docking and fusion with an endosome and/or lysosome, breakdown and degradation of the autophagosome inner membrane and cargo. Regulatory components for autophagy induction include the ULK1/2 (Atg1) complexes that contain ULK1/2-mAtg13-FIP200 proteins that are required for autophagy, and Beclin 1 complexes associated with UVRAG or Ambra1. In HD, autophagosomes fail to efficiently sequester the cargo. Autophagosomes appear to form at normal or even increased rates, and can be degraded by lysosomes as usual. The autophagosome arrives empty at the lysosome, allowing the cargo to accumulate to toxic levels inside the cell.

autophagy, but is inhibited by Bcl-2, an inhibitor of autophagy. Autophagy is also negatively regulated by a major kinase complex, the serine/threonine protein kinase mTOR (mammalian target of rapamycin). mTOR integrates nutrients, energy, growth factors, and calcium and amino acid signaling. Once activated, mTOR inhibits autophagy by acting on ULK1/2 (the mammalian autophagy protein-1 (Atg1) kinase) complex via phosphorylation of mAtg13 protein, which dissociates mAtg13 from ULK1/2 complexes and attenuates ULK1/2 kinase activity. When mTOR is inhibited, reassociation of dephosphorylated mAtg13 with ULK1/2 stimulates its catalytic activity and induces autophagy. Chemical inhibition of mTOR provides an autophagic manipulation to slow the progress of neurodegeneration, and sequestration of mTOR in protein aggregates has been proposed to mediate upregulation of autophagy in HD mouse models (Ravikumar *et al.*, 2004). mTOR-independent pathways involved in autophagy also exist, including calpain and inositol (Ravikumar *et al.*, 2009), although their mechanisms are yet to be fully elucidated.

- Cargo Sequestration.* Autophagy was previously considered a “bulk” process, but evidence now strongly supports selectivity in the sequestration of autophagic cargo. Recognition of post-translational modifications, often polyubiquitination, by molecules that bind both cargo and components of the autophagic machinery mediates this selectivity. p62, the first cargo-recognizing molecule identified, binds preferentially to a particular ubiquitin linkage (Lys63) on the surface of protein aggregates and elicits autophagosome sequestration of these aggregates through its interaction with LC3. Increased p62 expression is commonly interpreted as evidence of reduced autophagic flux, which involves the complete flow of autophagosomes from their formation to fusion with the lysosomes. Cargo recognition by p62 is not limited to protein aggregates, but also includes organelles and even pathogens. Ubiquitin is also the recognition signal for NBR1 and NDP52, recently identified p62-like molecules. The targeted cargo in the case of NBR1 is limited to proteins, whereas NDP52 recognizes ubiquitin-coated bacteria inside human cells.
- Autophagosome Clearance.* Degradation of the sequestered cargo only occurs when autophagosomes fuse to lytic compartments (lysosomes or endosomes). The components that participate in fusion of mammalian autophagosomes to lysosomes or endosomes are poorly characterized, although the participation of additional Rab proteins and several vacuolar-associated SNARE proteins has been proposed. In addition to these components in the membrane of autophagosomes and lysosomes, autophagosome clearance also involves the participation of the cellular cytoskeleton and cytosolic modulators.

The characteristic features of altered autophagy in the postmortem brains of HD patients were first observed by Tellez-Nagel *et al.* (1974), and confirmed by Sapp *et al.* (1997) and Rudnicki *et al.* (2008). mHtt accumulates in neuronal perinuclear and cytoplasmic structures that resemble endosomal-lysosomal organelles in the HD brain. These autophagosome-lysosome-like structures were also observed in lymphoblasts of HD subjects (Nagata *et al.*, 2004). In mouse models with mHtt expressed in striatal neurons, mHtt-labeled vacuoles were identified as autophagosomes (Kegel *et al.*, 2000). The involvement of autophagy in HD was also demonstrated by selective expression of autophagic vacuoles (AVs) in neuronal cultures derived from HD model mice (Petersén *et al.*, 2001) and the induction of autophagy due to impaired mTOR activity, which is mediated by the sequestration of

mTOR in polyglutamine aggregates in cell models, transgenic mice, and human brains (Ravikumar *et al.*, 2004). This induction of autophagy protects against polyglutamine toxicity, as rapamycin attenuates htt accumulation and cell death in cell models of HD, while autophagy inhibition has the opposite effects.

Increased autophagy may represent an important response to expanded repeat polyQ proteins. Autophagy has been shown to play a critical role in the degradation of N-terminal htt (Qin *et al.*, 2003). Blocking autophagy raised levels of exogenously expressed htt, reduced cell viability, and increased the number of cells bearing mHtt aggregates, while stimulating autophagy promoted htt degradation, including breakdown of caspase cleaved N-terminal htt fragments. In addition, post-translational modifications of mHtt, including acetylation and phosphorylation, target mHtt into the autophagic pathway for degradation (Jeong *et al.*, 2009; Thompson *et al.*, 2009).

Recent studies indicate that wild-type htt itself is also implicated in the regulation of autophagy. The dynamic interaction of htt with the endoplasmic reticulum (Atwal and Truant, 2008), an organelle genetically linked to the formation of autophagosomes, the association of htt with late endosomes and autophagic vesicles, and the interaction of htt with Rab5, which is involved in autophagosome formation, support a relationship between htt and the autophagic system. Although these functions are uncharacterized, a failure in the ability of mHtt to perform such functions could underlie altered clearance of the toxic protein in HD.

INEFFICIENT AUTOPHAGIC ELIMINATION OF CYTOSOLIC COMPONENTS ASSOCIATED WITH HD

Defective autophagy exerts different effects depending on the autophagic step affected. Failure to induce autophagosome formation results in cytosolic accumulation of unsequestered cargo, including damaged organelles that may become a source of toxic products. Indeed, accumulation of protein aggregates, higher content of abnormal, nonfunctional mitochondria, deformities of the endoplasmic reticulum, and an increase in the number and size of lipid droplets are each described in conditional autophagy gene knockout mice (Wong and Cuervo, 2010). If autophagic failure originates from a failure in the clearance of autophagosomes, accumulation of cytoplasmic autophagosomes can be detrimental. Although autophagosome formation would prevent the undesirable effects of unsequestered cytosolic cargo, this expansion of autophagic compartments may interfere with intracellular trafficking. Excessive autophagosomes can further provide a source of cytotoxic products, such as the pathogenic amyloid β 42 peptide in cellular and animal models of Alzheimer's disease. Autophagic compartments that persist in the cytosol can become leaky, and if leakage occurs after lysosomal fusion, the release of lysosomal enzymes will cause cell death (Kaasik *et al.*, 2005).

Inefficient Elimination of Cytosolic Components by Autophagosomes in HD

An abnormal number of autophagosomes in the affected neurons is the first sign of altered autophagy in HD, but could result from the impairment in any of the steps of autophagy.

Martinez-Vicente *et al.* (2010) characterized the status of the autophagic system in cell types, including primary neurons, striatal cell lines, fibroblasts, and hepatocytes, from two HD mouse models and lymphoblasts derived from human patients with HD. The results indicate that the massive expansion of autophagic compartments in HD cells previously identified (Kegel *et al.*, 2000; Petersén *et al.*, 2001) does not result in the predicted increase in proteolysis, but instead that the turnover of cytosolic components is impaired in these cells. The formation of autophagosomes was unaffected by mHtt: autophagosomes formed at normal or even increased rates, and could be normally degraded by lysosomes. They found, however, that autophagosomes in HD cells were “empty,” and that these empty vesicles accumulated in the cytosol and failed to correctly recognize and trap cytosolic cargo, including cytosolic proteins, lipid droplets, mitochondria, peroxisomes, and endoplasmic reticulum (ER) fractions (Figure 10.1). This impaired cargo recognition leads to increased numbers of lipid droplets and altered mitochondrial turnover in the cytosol of HD cells, which could contribute to cellular toxicity and damage. Thus, failure to degrade cytosolic cargo through autophagy is not due to a defect in fusion of autophagosomes with lysosomes or reduced proteolytic activity after this fusion, but rather originates for the most part from inefficient cargo loading.

In order to determine the effect of expression of full-length mutant htt on the activity of the autophagic system, total rates of intracellular protein degradation were compared in mouse embryonic fibroblasts (MEFs) from knockin mice in which different CAG repeats (18 in the control or 111 in the HD model) were inserted in exon-1 of the mouse *HTT* homolog, providing ¹⁸Q-htt control and ¹¹¹Q-htt transgenic HD mice. The basal rate of degradation of long-lived proteins was comparable in the two groups of cells. However, upon activation of autophagy by serum removal, rapamycin, or ER stress induced by thapsigargin, the rate of protein degradation was significantly impaired in ¹¹¹Q-htt MEFs. The percentage of protein degradation mediated by lysosomes, determined as that sensitive to inhibition by ammonium chloride that collapses acidic pH in lysosomes, was comparable in the two groups of cells under basal conditions, but there was a significant decrease in lysosomal protein degradation in the ¹¹¹Q-htt MEFs upon autophagy activation.

Rates of protein degradation were comparable in control and HD cells once macroautophagic activity was eliminated through knockdown of the essential macroautophagy gene, *Atg7*, confirming that differences in protein degradation between ¹⁸Q-htt and ¹¹¹Q-htt cells were mainly due to failure of macroautophagy. The decreased autophagic proteolysis was also observed in postnatally derived primary striatal cultures from a second HD mouse line, HD94 mouse expressing mutant human exon-1 htt with 94 CAG repeats. Differences between control and HD in neuronal cultures were noticeable under basal conditions, indicating that the compromise of the autophagic function may be more severe in neurons. A trend was observed for the decreased autophagic proteolysis in lymphoblasts from humans with HD to that in age-matched healthy individuals. These findings suggest that an inhibition of intracellular degradation due to compromised autophagy may be a common feature of many cell types in HD patients and HD models.

Autophagy Flux is not Altered in HD

To elucidate step(s) in autophagy that may be disrupted by mHtt, Martinez-Vicente and colleagues (2010) examined whether the inability of HD cells to engage in productive

autophagy was due to altered signaling in response to autophagic stimuli. Analysis of mTOR, which as above is a negative regulator of autophagy, showed no significant differences between control and HD cells in the global phosphorylation state. In response to stimulation of autophagy, both MEFs and striatal cells showed a comparable decrease in phosphorylation of p70S6K, an mTOR substrate, suggesting that mTOR is inhibited normally in response to nutrient deprivation in HD. Consistently, HD cells reacted to starvation by decreasing their proliferation and phosphorylated mTOR levels. Moreover, levels of Beclin 1, another key component of the autophagy activation complex, were higher in HD cells. Overall, these results support increased or normal autophagy induction and autophagosome formation in htt mutant cells.

The decrease in autophagic degradation in HD cells could thus result from a primary defect in the lysosomal compartment. Levels of principal lysosomal cathepsins, however, showed no differences between ¹⁸Q-htt control and ¹¹¹Q-htt HD MEFs. The degradative activity of these enzymes was moreover well preserved, indicating that lysosomal pH is not markedly altered in HD cells. Indeed, isolated lysosomes from HD cells showed higher proteolytic efficiency than those from control cells, arguing against a primary defect in mature lysosomes due to the mHtt.

To further test whether autophagosome accumulation is due to autophagy induction or a block in downstream steps, autophagic flux was measured by the analysis of the cellular distribution and lysosomal turnover of the light-chain type 3 protein (LC3), a component of autophagosomes. Both ¹⁸Q-htt control and ¹¹¹Q-htt HD cells exhibited few autophagic vacuoles under basal conditions, but markedly increased autophagosomes when lysosomal degradation was inhibited, indicating a constitutively basal autophagic activity in HD cells. The removal of serum, treatment with rapamycin, or ER stress further induced autophagy, indicated by a higher number of LC3 puncta following the inhibition of lysosomal degradation. In contrast to the reduction in total rates of protein degradation observed in ¹¹¹Q-htt HD cells, the increase in LC3 puncta following inhibition of lysosomal proteolysis was comparable to, or even higher than, that in control cells. Thus, autophagic flux was preserved in mutant HD cells.

The lack of inhibition of autophagic flux by mHtt was confirmed by analysis of degradation of LC3-II: the increase in amount of LC3-II after inhibition of lysosomal proteolysis was significantly higher in ¹¹¹Q-htt HD MEFs under basal conditions and upon induction of autophagy, while the amount of p62 was comparable in both cell lines. These results suggest that clearance of autophagosomes by lysosomes was normal or even moderately increased in ¹¹¹Q-htt HD MEFs. The inhibition of autophagy-dependent degradation is not attributed to abnormal levels of the autophagy effectors Atg7, conjugated Atg5 and 12, and Atg4 in ¹¹¹Q-htt HD cells. Overall, these results support normal autophagy induction and the formation of autophagic vacuoles in htt mutant HD cells. There was thus an apparent paradox: How could the reduced autophagic proteolysis in mutant cells be consistent with normal or even increased autophagic flux?

Reduced Ability of Autophagosomes to Recognize Cargo in HD

To explore the apparent conflict between autophagic proteolysis and autophagic flux, [Martinez-Vicente and colleagues \(2010\)](#) further analyzed autophagic vacuole formation

by electron microscopy in neurons from HD94 mice, striatal cells from ¹¹¹Q-htt mice, and lymphoblasts from four humans with HD. These mutant cells all demonstrated a markedly higher presence of autophagic vacuoles than did their controls, consistent with previous studies discussed above. A previously unnoted feature in each of the HD cells, however, was a lower electron density of the autophagic vacuoles: rather than the typical double-membrane vesicles enclosing identifiable cytosolic contents, the most abundant vesicle occupying the cytosol of HD cells was double-membraned but appeared empty. The size of most of these “empty” structures (0.1–0.5 μm diameter) was comparable to that of classic autophagic vacuoles, although in some cells much larger structures were present. Most of these vesicles were positive for LC3, confirming that they are autophagosomes.

These “empty” vesicles were also found in the livers of ¹¹¹Q-htt HD mice, and isolated liver fractions enriched in autophagosomes, immature and mature autophagic vacuoles, autophagolysosomes, and secondary lysosomes. Electron microscopy of the isolated vesicles demonstrated that, in contrast to the presence of recognizable cytosolic structures inside the double-membraned vesicles in the autophagic vacuoles-enriched fraction from the ¹⁸Q-htt control mice, the vesicles from the ¹¹¹Q-htt HD mice were still double-membraned but displayed amorphous electrotranslucent contents in their lumen.

Consistent with the ultrastructural analysis from whole cells and isolated organelles, the analysis of the total protein bidimensional electrophoretic patterns of autophagic vacuoles in HD cells confirmed a deficiency in normal luminal cargo in comparison with controls. Upon separation of membrane and cargo by hypotonic shock, the content of autophagic vacuoles isolated from the ¹¹¹Q-htt mice was markedly less enriched in proteins, with many luminal proteins detected in the vesicles from ¹⁸Q-htt mice low or absent in fractions from ¹¹¹Q-htt mice. While autophagic vacuole membrane-associated proteins such as LAMP-1 (lysosome-associated membrane protein type 1; also detected in autophagic vacuoles), LC3, and histone deacetylase-6 (HDAC6) and dynein (both required for autophagic vacuole trafficking), were significantly higher in autophagic vacuoles isolated from ¹¹¹Q-htt HD mice, common autophagic vacuole cargo such as polyubiquitinated proteins, mitochondrial markers (cytochrome C), lipid droplet structural proteins (ADRP), and soluble cytosolic proteins (GAPDH) were significantly reduced in autophagic vacuoles isolated from ¹¹¹Q-htt HD mice. Both autophagy-related compartments also contained htt, but amounts of htt in autophagic vacuoles from ¹¹¹Q-htt HD mice were markedly higher. Whereas ¹⁸Q htt partitioned almost evenly between the membrane and cargo fractions, mutant htt was more abundant at the autophagosome membrane.

In the *in vitro* fusion assay with isolated organelles, the number of fusion events between LC3-labeled autophagic vacuoles and LAMP-2B-labeled lysosomes was similar in fractions from ¹⁸Q-htt and ¹¹¹Q-htt mice, consistent with comparable LC3 flux in control and HD cells. However, homotypic fusion of autophagosomes (autophagosome to autophagosome) from ¹¹¹Q-htt mice was 33% higher than that of APHs from ¹⁸Q-htt mice, which may explain the abnormally large autophagic vacuoles observed in some HD cells. Unexpectedly, lysosomes from ¹¹¹Q-htt mice fused with autophagosomes from ¹⁸Q-htt mice at rates nearly twice those seen when the two compartments were from the same genotype, probably reflecting increased homotypic fusion of the autophagosomes in these cells. Levels of the endocytic markers mannose-6-phosphate receptor and Rab5 were elevated in autophagic vacuoles from ¹¹¹Q-htt mice, suggesting an enhanced interaction between autophagosomes from

¹¹¹Q-htt mice and endocytic compartments to form amphisomes. Enhanced amphisome formation could explain recent reports that functional multivesicular endosomes are necessary for mHtt clearance.

Inefficient autophagy will slow the normal turnover of intracellular components. Both electron microscopy and fluorescent labeling in ¹¹¹Q-htt MEFs and striatal neurons and HD lymphoblasts showed a marked increase in the content of lipid droplets and mitochondria. Moreover, both HD striatal neurons and MEFs displayed a higher percentage of dysfunctional mitochondria than their corresponding control cells. The observation that lipid droplets and mitochondria are excluded from autophagic vacuoles in HD cells suggests that the defect is in cargo sequestration and engulfment rather than autophagosome formation or degradation in the lysosomal compartment.

Martinez-Vincente *et al.* (2010) also provided evidence in HD cells that (1) there is no accumulation of undegraded protein species inside lysosomes or autophagolysosomes, but rather an accumulation of "empty" vacuoles; (2) there is no difference in the abundance of principal lysosomal proteases; (3) the lysosomal pH is not significantly altered; and (4) there is comparable proteolytic activity of isolated lysosomes in controls and HD. HD cells therefore exhibit the lower rates of inducible autophagy-dependent degradation in HD cells despite similar or higher rates of autophagosome formation and clearance. Thus, although autophagosomes form and fuse with lysosomes normally in HD cells, the low amount of cytosolic cargo in their lumens may lead to low net protein degradation.

The mechanism for the impaired ability to recognize cargo of autophagosomes in HD and HD models remain elusive. It is possible that the mHtt protein, possibly through its abnormal association with p62, is responsible for this failure to engulf cargo. In Martinez-Vincente *et al.*, p62 and htt proteins co-immunoprecipitated directly from autophagic vacuoles isolated from ¹¹¹Q-htt mouse brain. As with htt, amounts of p62 were comparable in ¹⁸Q-htt and ¹¹¹Q-htt mouse autophagic vacuoles, but more p62 bound to autophagic vacuole membranes from ¹¹¹Q-htt mice. When normalized to htt, the amount of p62 associated with ¹¹¹Q-htt was markedly higher than that associated with ¹⁸Q-htt. As p62 participates in the recognition by autophagic vacuoles of polyubiquitinated protein aggregates and organelles, it is possible that this abnormal association between ¹¹¹Q-htt and p62 causes the impairment of cargo recognition in HD cells. Htt associates with various organelle membranes, and the presence of mHtt on these membranes could prevent their recognition by autophagosomes. This could also explain the failure to recognize polyubiquitinated proteins, as mHtt also binds to polyubiquitinated aggregates. Although the cargo-recognition hypothesis may not explain the reduction in cytosolic soluble proteins in autophagosomes, it is possible that some of these proteins could be part of large oligomeric protein complexes or that they associate to the surface of the organelles that fail to be recognized in HD. Alternatively, given the extensive network of proteins interacting with htt, it is plausible that their interaction with mHtt instead prevents those proteins from being engulfed. For example, mHtt can form a complex with GAPDH, one of the cytosolic proteins less abundant in the lumen of autophagic vacuoles from HD cells.

The consequences of impaired organelle degradation arise both from a loss of function and toxic effects due to their accumulation. Abnormal intracellular lipid stores and the persistence of altered mitochondria in HD cells could contribute to increased production of reactive oxygen species and elevated oxidative stress extensively reported in HD.

Indeed, the total content of carbonyl groups, indicative of protein oxidation, in the brain of ^{111}Q -htt HD mice was markedly higher than in ^{18}Q -htt mice. The impaired autophagy of lipid droplets leads to their intracellular accumulation, and may perpetuate a vicious cycle.

THERAPEUTIC STRATEGIES FOR HD TARGETING AUTOPHAGY

As HD is inherited in an autosomal dominant manner and most cases have a family history, one can identify people at risk who carry the mutation. It may be possible to treat asymptomatic mutation-carriers to delay the onset of HD symptoms. Work by Rubinsztein and colleagues suggests that autophagy induction represents an approach for HD treatment. Both mTOR-dependent (Ravikumar *et al.*, 2004) and mTOR-independent (Sarkar *et al.*, 2007) interventions can increase autophagic flux and promote expanded repeat polyQ protein clearance. The apparent success of interventions aimed at increasing autophagic flux in a variety of model systems of HD supports further exploration of such treatments.

Prolonged activation of autophagy might, however, become detrimental in the context of massive accumulation of undegraded autophagic vacuoles. Indeed, treatments that inhibit autophagosome formation have been shown to improve neuronal viability in models of frontotemporal dementia and Alzheimer's disease, where most of the autophagosome accumulation originates from a failure in lysosomal degradation (Boland *et al.*, 2008).

The initial increase in autophagosomes and autophagic activity observed in HD models may represent an attempt by HD cells to remove mHtt, but the autophagy machinery may become dysfunctional over time. Rubinsztein and colleagues (Ravikumar *et al.*, 2004) report that rapamycin did not efficiently reduce htt aggregate levels when ~30% of cells contained aggregates, but did reduce aggregates in populations in which 10% of cells contained aggregates. Thus, the therapeutic induction of autophagy may provide protection at early stages of protein aggregation. Such a therapeutic window needs to be identified, as excessive mutant htt may exert a negative effect on the sequestration of the autophagic cargo (Martinez-Vincente *et al.*, 2010). Indeed, if HD arises from defective cargo recognition, activation of autophagosome formation may increase the amount of cargo randomly sequestered and degraded through autophagy, but the loss of selectivity in recognizing the cargo is likely to decrease the efficiency of the process.

Developing the means to improve recognition of cargo in HD cells may yield a therapy. Such treatment can be combined with enhanced autophagosome clearance by the lysosomal compartment, as proposed by promoting lysosomal biogenesis via overexpression of the transcription factor EB (Wong and Cuervo, 2010). In this case, new and healthy lysosomes may mediate removal of the accumulated autophagosomes. Another strategy is to promote htt acetylation. Indeed, histone deacetylase inhibitors have been shown to block mHtt-dependent neuronal degeneration in *Drosophila* by recruitment of the protein to the autophagosome (Jeong *et al.*, 2009).

Alternative autophagy-related mechanisms may also be explored for HD therapy. For example, accumulated mHtt recruits Beclin 1 and impairs the Beclin 1 mediated long-lived protein turnover. The sequestration of Beclin 1 in the vulnerable neuronal population of HD patients may further reduce Beclin 1 function and autophagic degradation of mHtt (Shibata *et al.*, 2006). In human brains, Beclin 1 expression decreases with age. Because the

heterozygous deletion of the Beclin 1 gene is insufficient for the regulation of autophagosomes, this age-dependent decrease of Beclin 1 expression may lead to a reduction of autophagic activity, which in turn promotes the accumulation of mHtt and the progression of the disease.

Additional therapeutic options could be attained through a better understanding of the compensatory mechanisms and autophagic alternatives that are induced when autophagy fails (Wong and Cuervo, 2010). Autophagy acts in a coordinated manner with other cellular proteolytic mechanisms, including chaperone-mediated autophagy (CMA) and other non-autophagic lysosomal pathways such as endocytosis. Indeed, CMA has been found to be constitutively upregulated in neurons and other cells with HD mutation, probably in response to the inhibition of autophagy (Koga *et al.*, 2011). It is possible that increased CMA activity can help compensate for the inhibition of autophagic turnover of some components. In support of this possibility, cleaved htt fragments have been shown to be targeted to CMA for degradation. However, the htt-dependent upregulation of CMA was observed in mice up to 7 months of age, and not in older mice. The loss of this elevated CMA in older mice may be due to a toxic action of the htt mutation on CMA itself, or due to a lack of normal lipid turnover caused by a defect in autophagic lipid droplet degradation. In either case, means to increase the targeting of htt to CMA degradation, perhaps by post-translational modification of htt, including peptide cleavage to a CMA substrate or enhanced CMA activity by LAMP2A overexpression, could provide means for autophagic/lysosomal therapy by an alternate method.

Cross-talk is also present between autophagy and the UPS: cells respond to acute proteasome blockage by upregulating autophagy, and chemical upregulation of autophagy in mice protects them from the neurodegeneration induced upon inhibition of proteasomes. The microtubule-associated deacetylase HDAC6 links polyubiquitinated proteins and autophagy, and may be essential for rescue of the degeneration associated with proteasome failure in an autophagy-dependent manner (Pandey *et al.*, 2007). Another possible modulator of the autophagy and UPS is p53 (Du *et al.*, 2009): failure to degrade p53 by the UPS will increase its cytosolic levels, leading to activation of autophagy. Connections between autophagy and the UPS are not limited to the removal of cytosolic ubiquitinated proteins, but also involve removal of organelles. For example, ubiquitination of constituent proteins in the membranes of peroxisomes mediates their autophagic degradation. Therefore, multiple means to harness degradative pathways for HD treatment should be considered for exploration.

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