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Does Huntingtin play a role in selective macroautophagy?

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The accumulation of protein aggregates in neurons appears to be a basic feature of neurodegenerative disease. In Huntington disease (HD), a progressive and ultimately fatal neurodegenerative disorder caused by an expansion of the polyglutamine repeat within the protein Huntingtin (Htt), the immediate proximal cause of disease is well understood. However, the cellular mechanisms which modulate the rate at which fragments of Htt containing polyglutamine accumulate in neurons is a central issue in the development of approaches to modulate the rate and extent of neuronal loss in this disease. We have recently found that Htt is phosphorylated by the kinase IKK on serine (S) 13, activating its phosphorylation on S16 and its acetylation and poly-SUMOylation, modifications that modulate its clearance by the proteasome and lysosome in cells.[1] In the discussion here I suggest that Htt may have a normal function in the lysosomal mechanism of selective macroautophagy involved in its own degradation which may share some similarity with the yeast cytoplasm to vacuole targeting (Cvt) pathway. Pharmacologic activation of this pathway may be useful early in disease progression to treat HD and other neurodegenerative diseases characterized by the accumulation of disease proteins.

Key words: Huntington disease, Huntingtin, polyglutamine, autophagy, IKK

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An age-related reduction in protein clearance mechanisms has been implicated in the pathogenesis of neurodegenerative diseases including the polyglutamine (polyQ) repeat diseases, Alzheimer disease (AD), Parkinson disease (PD) and Amyotrophic Lateral Sclerosis (ALS). These diseases are each associated with the accumulation of insoluble protein aggregates in diseased neurons. Huntington Disease (HD), caused by an expansion of the polyQ repeat in the protein Huntingtin (Htt), is one such disease of aging in which mutant Htt inclusions form in striatal and cortical neurons as disease progresses. Clarification of the mechanisms of Htt clearance is paramount to finding therapeutic targets to treat HD that may be broadly useful in the treatment of these currently incurable neurodegenerative diseases.

The Kinase IKK Regulates Htt Degradation

In an effort to better understand the factors which influence mutant Htt degradation, an important focus has been the changes in pathways of mutant Htt clearance which occur as a consequence of cellular stress. Cellular stress can be generated as the direct consequence of mutant Htt expression or by inflammatory stimuli, oxidative stress, DNA damage or any combination of these factors. No matter what the combination of stimuli, activation of the downstream signaling IKK kinase complex appears to be a key regulator of Htt degradation. When IKK is activated, the pattern and control of mutant Htt degradation is altered. We have focused on the details of the cellular response to IKK activation and the consequences on the rate and extent of degradation of mutant Htt.[1]

In cell models of HD, we find that expanded polyQ mutant Htt is less efficiently phosphorylated and cleared upon activation of IKK than unexpanded Htt, resulting in mutant Htt accumulation and reduced degradation by the proteasome and lysosome.[1] IKK is activated in cell culture and mouse models of HD with

expression of mutant Htt.[2] HD patients exhibit innate immune activation over a decade before overt neurological manifestation of disease[3] consistent with a compensatory activation of IKK in vivo to help remove mutant Htt from the cell.

Taking a biochemical approach to study the effects of IKK activation in HD, we have found that IKK directly modifies Htt when activated, phosphorylating Htt on serine (S) 13, inducing its subsequent phosphorylation on S16 and acetylation on lysine (K) 9. These modifications occur on a transient species of Htt that is degraded by both the proteasome and the lysosome in the presence of activated IKK.

A central element to this process now appears to be the recently demonstrated activation of the lysosomal clearance mechanism associated with autophagy by IKK.[4] Consistent with this mechanism, we find that degradation of phosphorylated Htt involves lysosomal-associated membrane protein LAMP-2A and the heat shock protein Hsc70.[1] Phosphorylation of Htt on S16 creates a consensus Hsc70 interaction motif. Further, mimicking Htt phosphorylation reduces its abundance in cell culture but increases its interaction with Hsc70 in vitro, suggesting that phosphorylation may activate Htt recognition by a chaperone complex required for selective protein degradation.

In addition, we have shown that the autophagy protein Atg7, essential for autophagosome formation, regulates clearance of phosphorylated Htt which implies that vesicles may mediate phosphorylated Htt autophagic degradation. S13/S16 phosphorylated and K9 acetylated wild-type (wt) Htt is present in mouse brain and appears to be relatively insoluble, consistent with oligomerization of the species of Htt being cleared by the cell. This species is also phosphorylated on threonine (T) 3; phosphorylation of T3 appears to increase Htt aggregation.[1,5]

IKK Regulates Htt Post-Translational Modification and Cellular Localization

Ubiquitination, SUMOylation and acetylation are post-translational modifications that have been previously demonstrated to regulate protein degradation, and these modifications can be modulated upstream by phosphorylation (reviewed in ref. 1). We find that IKK-activated phosphorylation of Htt S13/S16 regulates multiple post-translational modifications of Htt including ubiquitination, SUMOylation and acetylation and induces its nuclear localization, all events that may be involved in its clearance.[1] The order in which these modifications occur and their specific functions in Htt degradation have yet to be determined. We previously reported that Htt amino acids 1–17 can function as a cytoplasmic retention sequence.[6] I suggest that phosphorylation of S13S16 modifies the structure of this putative cytoplasmic retention sequence to increase nuclear localization and activate Htt poly-SUMOylation.

SUMOylation has been reported to target proteins to subnuclear structures called PML bodies, which contain the acetyltransferase CBP/p300.[7] CBP/p300 interacts directly with and is phosphorylated by IKK in the nucleus, activating its acetyltransferase activity.[8] We previously found that Htt also interacts directly with this acetyltransferase,[9,10] which has been implicated in the acetylation of Htt on K444, a modification that increases mutant Htt clearance by macroautophagy.[11] Clearance of K444-acetylated Htt has been suggested to be mediated by p62/SQSTM1, a protein that shuttles between the cytoplasm and nucleus and recruits polyubiquitinated proteins to PML bodies.[11,12]

PML bodies may also be the site of Htt caspase cleavage initiated by IKK,[13] as caspase cleavage of proteins has been demonstrated to occur within this subnuclear structure.[14] The PML body is a site of proteasomal degradation of poly-SUMOylated proteins, a process dependent on the SUMO-binding ubiquitin E3 ligase RNF4,[15,16] and polyQ aggregates colocalize with PML bodies.[17] I propose that Htt is phosphorylated and forms a complex with IKK in the cytoplasm, which then increases the extent of its nuclear localization. In this view, Htt is then poly-SUMOylated, targeting it to PML bodies, acetylated on lysines 9 and 444, cleaved by caspases, and either degraded by the proteasome in an SUMO/RNF4-dependent fashion or targeted to the cytoplasm where it is cleared by macroautophagy.

Mutant Htt Post-Translational Modification Modulates Its Toxicity

The post-translational modifications described above appear to influence degradation of the Htt protein. With aging, both proteasomal and lysosomal function are reduced.[18-21] We find that inhibition of the proteasome or the lysosome results in an accumulation of phosphorylated and acetylated Htt and Htt fragments. It has been previously shown that a build-up of toxic amino-terminal mutant Htt fragments in the nucleus is a marker of HD pathogenesis, and that IKK may facilitate formation of these fragments.[13,22]

We find that mimicking phosphorylation of mutant Htt exon 1 protein (Httex1p) in an acute striatal slice culture model or full-length mutant Htt in BACHD transgenic mice reduces its toxicity,[1,23] implying that mutant Htt clearance is activated in these phosphomimetic models. In contrast, mimicking phosphorylation of S13 and S16 in a Drosophila model of HD increases mutant Httex1p toxicity and its accumulation into aggregates (Marsh JL, personal communication). This difference could reflect the absence of lysosomal degradation of phosphorylated human mutant Httex1p since lysosomal clearance mechanisms are not closely conserved in Drosophila. For instance, there is no structural homolog of LAMP-2A and the amino-terminal domain of Drosophila Htt does not contain S13 or S16. Therefore, this Drosophila model could mimic patients already having impaired lysosomal clearance upon aging, at which point induction of the IKK kinase by innate immune activation no longer is protective due to nuclear accumulation of a modified form of Htt that is toxic. Consistent with the idea that IKK could have both beneficial and toxic effects depending upon the cell's ability to clear post-translationally modified Htt, inhibition of IKK activity can reduce mutant Htt toxicity and the protein RIP-2, an activator of IKK, has been implicated in enhancement of HD pathogenesis.[2,24]

In keeping with the idea that modifications may have different consequences depending on the cellular milieu, SUMOylation can be both protective and pathogenic in expanded polyQ-repeat diseases, which may reflect that poly-SUMOylation of Htt increases its clearance while undegraded SUMOylated Htt is toxic.[6,25-27] Acetylation may also control pathogenicity of Htt as both inhibition and activation of specific histone deacetylases can reduce Htt-mediated toxicity.[9,28-30] Therapies to treat HD may thus be challenging to develop if activation of Htt phosphorylation, SUMOylation and acetylation early on is protective, but later in the course of disease may result in the formation of an accumulating, toxic species. Therefore, significant care must be taken to match potential therapeutics for HD to the stage of disease progression reached by the patient.

Selective Autophagy, Conserved Across Phylogeny, Has Been Well Defined in Yeast

Selective lysosomal protein degradation mechanisms are essential to the viability of the cell to maximize efficiency of cellular function. These mechanisms have been particularly well defined in yeast. The yeast vacuole is the equivalent of the mammalian lysosome. Genetic screens in *Saccharomyces cerevisiae* have led to the identification of several mechanisms in which select proteins or organelles are directed to be degraded by the vacuole. For example, under glucose and nutrient-rich conditions, yeast gluconeogenic enzymes such as fructose-1,6-bisphosphatase (FBPase) and cytoplasmic malate dehydrogenase (MDH2) become unnecessary. Using biochemical and genetic approaches, the vacuole import and degradation (Vid) pathway has been defined, which selectively targets these enzymes for degradation by the proteasome, or by the vacuole via Vid vesicles.[31] The yeast heat shock protein Ssa2 is required for import of FBPase into Vid vesicles, which cluster at actin patches and merge with endocytic vesicles as they are forming on the plasma membrane. FBPase is degraded following vacuolar fusion with the endosome.[32-34]

Another selective pathway of vacuolar targeting exists in yeast under glucose-rich conditions: the cytoplasm to vacuole targeting (Cvt) pathway. In this pathway, the target protein can be recognized by yeast heat shock proteins Ssa1 and Ssa2,[35] oligomerizes and interacts with the Cvt receptor protein Atg19, which binds Atg11, a scaffolding protein that interacts with actin. Actin and the actin-binding complex Arp2/3 are required for the Cvt pathway and are suggested to act as a track to guide cargos to the site of vesicle formation through interaction with Atg11.[36] The Atg19/Atg11-coated oligomers are surrounded by the Cvt vesicle which then fuses with the vacuolar membrane, dependent on Vam3, an integral vacuolar membrane t-SNARE protein. Atg19 binds to Atg8-phosphatidylethanolamine localized to the forming Cvt vesicle, an event that may trigger completion of the vesicle.[37] While this targeting pathway has been considered biosynthetic, and was defined for resident vacuolar proteins aminopeptidase I (Ape1) and α-mannosidase (Ams1), it may also target proteins for vacuolar degradation.[38]

By both the Vid and Cvt pathways, select proteins are targeted to vesicles that ultimately fuse with the endosome/vacuole when yeast are grown in glucose-rich media. Perixosomes, unnecessary for energy generation under nutrient-rich conditions in yeast can become selectively degraded by another autophagic mechanism requiring Atg11, pexophagy.[39] Pexophagy and the Vid and Cvt pathways allow selective targeting of proteins and organelles to the vacuole under nutrient-rich conditions, regulating vacuolar degradation to maximize cellular efficiency.

In contrast, under starvation conditions, alternate vacuolar targeting pathways are activated in yeast. In glucose-poor media, macroautophagy is induced to generate energy through vacuolar digestion of cytoplasmic proteins and organelles, freeing necessary amino acids that are then transported out of the vacuole to the cytoplasm for use by the cell. Starvation-induced macroautophagy is generally a nonspecific mechanism of vacuolar protein clearance where autophagosomes form and engulf organelles and cytoplasmic contents, but in some physiological conditions, unnecessary proteins or dysfunctional organelles are selectively degraded in the vacuole by macroautophagy.[37,40] Also under starvation conditions, nuclear proteins can be targeted to the vacuole by piecemeal microautophagy of the nucleus (PMN), during which small nuclear envelope blebs pinch off and fuse with the vacuole, requiring Atg11 and Vam3.[41]

Autophagic Pathways in Mammalian Cells

Autophagic pathways used in mammalian cells appear to show significant similarities to those used in yeast. Many of the key proteins in yeast autophagic pathways have orthologs in mammals judged by both sequence and functional similarity. For example, Atg5, Atg7, Atg8 (LC3) and Atg12, required for vesicle formation in starvation-induced macroautophagy, PMN and the Cvt pathway, have clear orthologs in mammals.[37] Macroautophagy, stimulated by starvation conditions and by inhibition of mTOR complex 1 (mTORC1) with rapamycin, has been extensively defined in mammalian cells, while PMN has not yet been directly demonstrated, although a process similar to PMN has been defined in Bloom's syndrome where nuclear microvesicles are released into the cytoplasm.[42]

Signals such as nutrient availability in mammalian cells appear to be involved in the induction of autophagy and activity of autophagy components.[43] Under nutrient-rich conditions, activation of insulin-signaling pathways, Akt, mTORC1 and S6 kinase increase mutant Htt fragment clearance by autophagy in mammalian cells despite the fact that these pathways generally inhibit starvation-induced macroautophagy.[44] Similarly, Akt, IKK and Cdk5, kinases active in nutrient-rich conditions, directly phosphorylate mutant Htt and reduce its toxicity.[1,45-47] mTORC1, downstream of Akt, can activate the IKK complex while rapamycin, an inhibitor of mTORC1, can inhibit IKK activation[48] and activate starvation-induced macroautophagy.[37] We find that the phosphorylated/acetylated species of wt Htt has reduced solubility, suggesting this clearance intermediate may

oligomerize like substrates for the Cvt pathway.[1] Under starvation conditions, the transcription factor FOXO3a activates macroautophagy[49] but under nutrient-rich conditions, both Akt and IKK directly phosphorylate FOXO3a and target it for degradation[50] thereby inhibiting nonspecific macroautophagy.[49]

It is possible, therefore, that the mechanism of IKK-mediated selective autophagic clearance of Htt[1] may be similar to the vegetative yeast Cvt or Vid pathways rather than to starvation-induced macroautophagy. Since in yeast, oligomerized proteins normally selectively targeted to Cvt vesicles in glucose-rich conditions may be delivered to the vacuole by macroautophagy under starvation conditions,[51] nonspecific macroautophagosomal targeting of Htt to the lysosome induced by mTORC1 inhibition[52] may be compensatory for a reduction in function of a mammalian Cvt-like macroautophagic pathway.

Since starvation conditions increase lysosome numbers and levels of LAMP-2A,[53] periodic nutrient limitation may later bolster lysosomal activity under nutrient-rich conditions, as has been demonstrated for the yeast FBPase protein. Yeast cells that are glucose-starved for short periods largely degrade FBPase by the proteasome upon introduction of glucose to the media, while cells starved for longer periods primarily utilize the vacuole upon transfer to glucose.[31] Periodic fasting, thought for centuries to be healthful, may generally boost lysosome numbers and function and later improve lysosomal clearance of proteins under nutrient-rich conditions.

Atg proteins required for non-selective macroautophagy are conserved from yeast to man, while those involved in selective autophagy like the Cvt pathway or pexophagy show poor conservation, suggesting that specialized selective autophagy genes have evolved.[39] In particular, close structural homologs for the proteins essential for the yeast Cvt pathway, Vac8, Atg11, Atg20, Atg23 and Atg27 have not been identified in mammals.

Atg19, the substrate receptor protein for the Cvt pathway, is functionally similar to mammalian proteins p62/SQSTM1 and NBR1 because they all interact with oligomerized cargo, directly bind Atg8/LC3 and are degraded by the mechanism of selective autophagy that they regulate.[54,55] These considerations suggest that a version of the Cvt pathway may exist in mammals.

Atg20 is a sorting nexin family member required for the Cvt pathway and endosomal sorting which interacts directly with Atg11.[56,57] I find that yeast Atg20 shares some structural similarity with human Optineurin (**Suppl. Fig. 1**), a protein that can regulate inflammatory pathways and membrane trafficking,[58,59] interact directly with Htt and is mutated in the neurodegenerative diseases ALS and glaucoma.[60,61] Another sorting nexin that interacts with Atg20, Atg24/SNX4, shares some similarity with Huntingtin-associated protein-1 (HAP-1, **Suppl. Fig. 2**), proteins that both interact with the largest subunit of the yeast/human molecular motor dynactin complex, NIP100/p150^{Glued} respectively.[62,63]

Three proteins in the Atg1 kinase complex, involved in the energetic toggle between starvation-induced macroautophagy and the Cvt pathway, yeast Atg1, Atg13 and Atg17, have been proposed to be Ulk1/Ulk2, mAtg13 and FIP200 in mammalian cells.[39,64,65] While Ulk1/Ulk2 are strongly homologous to the *C. elegans* Atg1-like protein Unc-51, the putative mammalian Atg13 and Atg17 functional homologs share little similarity to the yeast proteins by sequence. In fact, FIP200, a protein that promotes mTORC1 activation,[66] is a member of the NCBI Pfam: *Family*: *ATG11* (PF10377) and is structurally similar to *S. Pombe* (Taz1-interacting factor 1) and *C. elegans* (WBGene00020334) Atg11s. Yeast Atg11 and Atg17 both act as scaffolds for PAS (phagophore assembly site or preautophagosomal structure) organization as part of the Atg1 kinase complex, Atg11 under nutrient-rich conditions and Atg17 under starvation conditions.[67] I find that yeast Atg17 may show similarity to the recently reported mAtg13-binding protein, Atg101,[67] (**Suppl. Fig. 3**), and suggest that FIP200/Atg101 may be the mammalian counterparts of Atg11/Atg17 respectively (**Table 1**). Yeast Vam3 is a vacuolar integral membrane t-SNARE protein involved in fusion of Cvt, PMN and starvation-induced macroautophagy vesicles with the vacuole.[41,68] Vam3 plays a role in vacuolar assembly and trafficking, and interacts genetically with NIP100, part of the dynactin complex.[69] Vam3 may have some functional similarity to mammalian LAMP-2, an integral lysosomal membrane protein involved in autophagy, vesicular trafficking and lysosome biogenesis.[70]

Htt Has Similarities to Several Proteins Required for Selective Autophagy in Yeast

The Huntingtin protein may play a role in the mechanism of protein degradation involved in its own clearance. Conditional knockout of Htt in the central nervous system results in an accumulation of neuropil protein aggregates containing ubiquitin and p62/SQSTM1 (Zeitlin S, personal communication), demonstrating a loss of function in autophagy similar to that observed with neuronal knockdown of mammalian autophagy proteins Atg5, Atg7 and FIP200.[71-73] Conversely, deletion of the polyQ stretch within wt mouse Htt stimulates autophagy via an mTOR-independent pathway in vivo,[74] suggesting that wt Htt has a function in cellular autophagy. Htt may play a role in autophagic cargo recognition as this process has been recently demonstrated to be impaired in HD, where autophagosomes form normally and are eliminated by lysosomes, but fail to efficiently trap cytosolic cargo in their lumen.[75] Based on these considerations I propose the hypothesis that Htt is a protein integral to the function of a Cvt-like mammalian macroautophagic pathway and may have evolved through fusion of proteins essential for the yeast Cvt pathway (**Fig. 1 and Table 1**).

Yeast Atg23 is a peripheral-membrane protein that co-localizes with Atg9 and Atg27, integral membrane proteins essential for autophagic vesicle formation at the PAS.[76] These three proteins may be required for bringing membrane to forming vesicles. Atg9 is needed for both nutrient-rich and starvation-induced vesicle formation, while Atg23 and Atg27 largely function in the Cvt pathway but may aid in the formation of starvation-induced macroautophagic vesicles. Atg23 interacts directly with Atg9 and Atg27 at

Table 1. Yeast Cvt pathway proteins and their hypothetical human functional homologs

Yeast protein	Function	Interactors	Human protein	Function	Interactors
Atg1	Kinase required for vesicle and PAS formation	Atg1, Atg8, Atg11, Atg13, Atg14, Atg17, Atg18, Atg21, Atg29, Slx5	**ULK1/2**	Kinase that regulates autophagy[156]	mAtg13
Atg8	Vesicle component, phagophore expansion, vesicle fusion	Atg1, Atg3, Atg4, Atg7, Atg19, Atg32, Vam3	**LC3**	Recruited to the phagophore membrane and remains associated with completed vesicle[102]	p62/SQSTM1
Atg11	Directs receptor-bound cargo to the PAS for packaging into vesicles; required for recruiting other proteins to the PAS	Atg1, Atg9, Atg11, Atg12, Atg17, Atg19, Atg20, Atg29, Atg32	**Huntingtin aa 1743–3144**	Regulation of early endosome motility[89]	HAP40/Rab5 complex
			FIP200	Regulation of cell growth, proliferation, cell migration, apoptosis, microtubule dynamics, stress response[156,157]	mAtg13, ULK1/2, PIASy, p53, FAK, TSC1, Pyk2, ASK1 stathmin, TRAF2
Atg13	Regulatory subunit of Atg1 complex, required for vesicle formation, involved in Atg9/23/27 cycling	Vac8, Atg1, Atg17	**mAtg13**	Plays an essential role in autophagosome formation, phosphorylated by ULK and mTOR[156,158]	ULK1/2, FIP200, Atg101
Atg17	Scaffold protein responsible for PAS site organization, stimulates Atg1 kinase activity	Atg1, Atg9, Atg11, Atg12, Atg13, Atg17, Atg24, Atg29, NIP100	**Atg101**	Important for stability and phosphorylation of ULK and mAtg13[158]	mAtg13
Atg19	Cvt pathway receptor protein delivers cargo to PAS	Atg8, Atg11, Ubi4	**p62/ SQSTM1 NBR1**	Shuttle proteins transport ubiquitinated proteins to lysosome[54,55,93,102,159]	LC3, Huntingtin, ubiquitin
Atg20	Sorting nexin for Cvt pathway and endosomal sorting	Atg11, Atg24	**Optineurin**	Membrane trafficking, cellular morphogenesis[59]	Huntingtin, Rab8
Atg23	Peripheral membrane protein required for Atg9/Atg27 shuttling/membrane trafficking	Atg9, Atg27	**Huntingtin aa 1–586**	Contains polyQ repeat, vesicle/membrane trafficking, localizes to microtubules[59,77,85,160]	HAP-1, Optineurin, p53, Sp1, Akt, CBP, SH3GL3, RasGAP, PSD-95, HIP-1, HYP-J, PACSIN, actin
Atg24	Sorting nexin involved in Cvt pathway and in retrieval of late-Golgi SNAREs from post-Golgi endosomes to the trans-Golgi network	Atg17, Atg20, Cog6, NIP100	**HAP-1**	Adaptor protein, mediates vesicle trafficking[62,161]	Huntingtin (171-230) PCM-1, kinesin-like protein, neurofilament M, p150^{glued}
Vac8	Vacuole inheritance, the Cvt pathway, mitophagy, PMN, vacuole/vacuole fusion	Atg13, Vam3, Act1	**Huntingtin aa 807–1653**	DNA-damage response signal protein[45]	Cdk5
Vam3	Integral vacuolar membrane protein required for vacuolar assembly and protein trafficking, Golgi to vacuole transport, vesicular fusion, PMN	Atg8, Vac8, Vam3, Vam7	**LAMP-2A**	Integral lysosomal membrane protein involved in vesicular trafficking and lysosome biogenesis[70,150,162]	UCH-L1/Hsc70/Hsp90 complex
NIP100	Large subunit of dynactin complex	Atg24	**p150^{glued}**	Large subunit of dynactin complex[62]	HAP-1

Select direct interactions listed. Yeast protein data obtained from the Saccharomyces Genome Database: www.yeastgenome.org.

the perivacuolar PAS and punctate non-PAS peripheral cytoplasmic locations and cycles between the periphery and the PAS. Atg23 and Atg11 together are required for movement of Atg9 towards the PAS, while Atg1 and Atg13 aid in retrograde movement of Atg23 away from the PAS.

I find that the amino-terminal 586 amino acid caspase-6 fragment of Htt has some structural similarity to yeast Atg23 (**Suppl. Fig. 4**). Like Atg23, the Htt protein also plays a role in vesicular trafficking.[77,78] Htt localizes to microtubules and is associated with proteins of the molecular motor machinery including dynein and HAP-1, that interact with p150^{Glued} and kinesin-1. The first 480 amino acids of wt Htt can activate vesicular movement in cells in which full-length Htt expression is knocked down; this ability becomes impaired with expansion of the polyQ

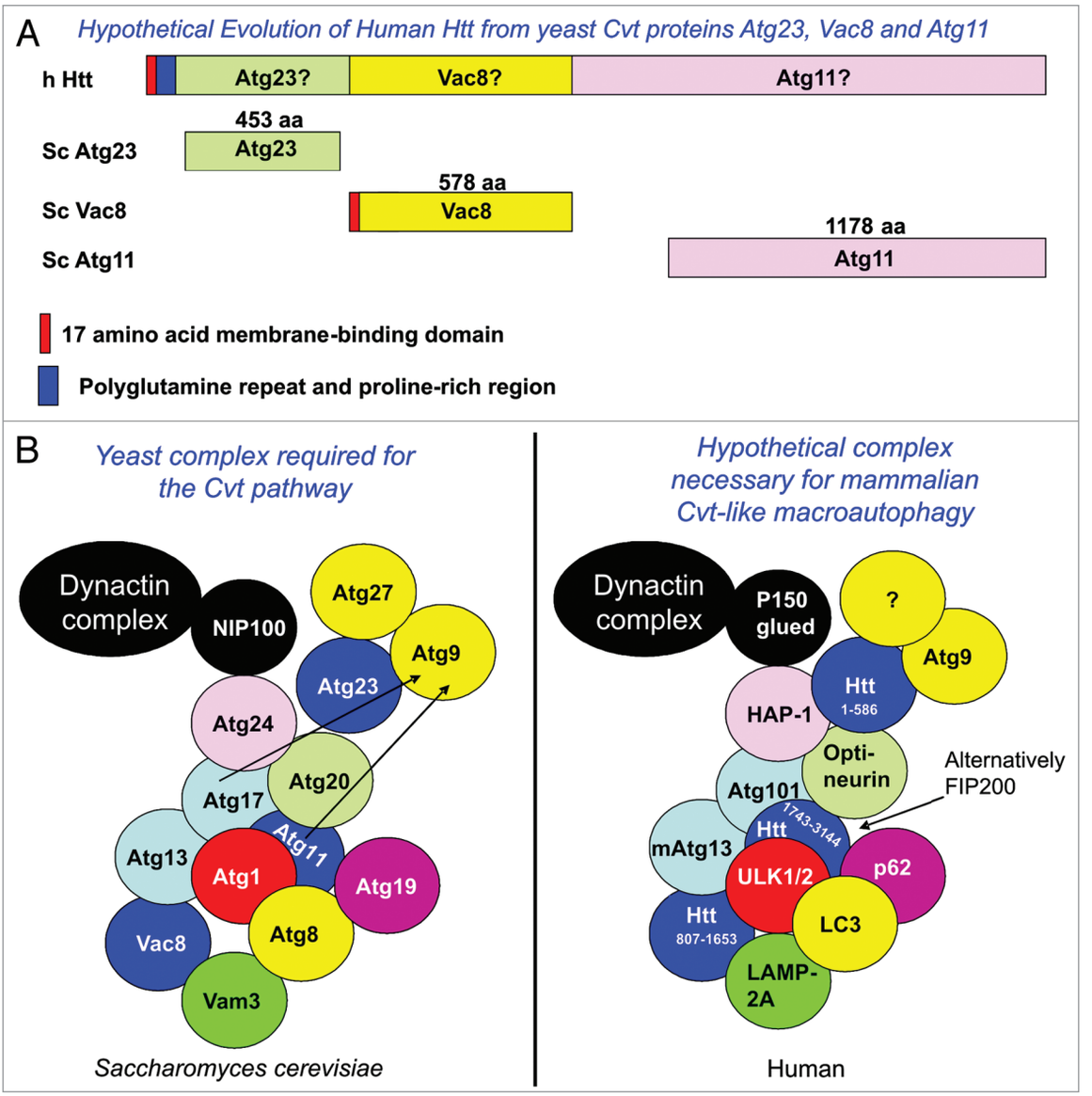

Figure 1. Htt may have evolved from Cvt proteins Atg23, Vac8 and Atg11. (A) The yeast Vac8 protein contains 11 armadillo repeats which can be described as ancestral HEAT repeats. Throughout evolution, Vac8 may have fused with yeast protein Atg23 on the amino-terminus and Atg11 on the C-terminus. These yeast proteins are essential for the cytoplasm to vacuole targeting (Cvt) selective autophagic pathway, which is activated by TORC1 in yeast grown under nutrient-rich conditions. The cartoon is not drawn to scale. (B) A protein complex essential for the yeast Cvt pathway may be similar to a hypothetical human complex required for selective macroautophagy under nutrient-rich conditions.

repeat. This suggests that Htt's role in vesicular trafficking is contained within its amino-terminal 480 amino acids that are similar to yeast Atg23.

The yeast Vac8 phosphoprotein is essential for multiple vacuolar processes including vacuole inheritance, the Cvt pathway, pexophagy, PMN and vacuole/vacuole fusion and co-sediments with actin filaments.[79-81] Vac8 contains 11 armadillo repeats that can be described as ancestral HEAT repeats, spanning its entire sequence.[82] Vac8 is both myristoylated and palmitoylated, modifications required for its targeting to membranes via its first 18 amino acids which contain a Src kinase SH4 domain.[81,83] Similar to the yeast Vac8 protein, the Htt protein contains 12 HEAT repeats between amino acids 745 and 1710,[22] can be palmitoylated,[84] associates with actin,[85] and interacts with membranes via its first 17 amino acids.[86,87] Both Vac8 and Htt regulate longevity.[74,88] I find that the Vac8 protein shares some structural similarity with Htt amino acids 807–1,652 (**Suppl. Fig. 5**).

Within the C-terminal end of Htt, between amino acids 1,815 and 3,144, there exists a domain required for interaction with Hap40/Rab5 and regulation of early endosome motility.[89] This domain contains weak similarity to the yeast Atg11 protein (**Suppl. Fig. 6**), essential for the Cvt pathway, PMN, pexophagy and selective mitochondrial degradation by autophagy: mitophagy.[90] Starting at amino acid 3,037, Htt may also contain a highly conserved consensus WXXL LC3-binding domain preceded by an aspartic

acid residue.[91] Atg11 is an adapter protein necessary for cargo loading that oligomerizes and interacts directly with Cvt receptor protein Atg19.[57] Like Atg11, Htt has been described as a protein scaffold[92] and we have found that the phosphorylated/acetylated wt Htt species may have a tendency to aggregate.[1] Htt aggregates interact with p62/SQSTM1,[93] a protein with similarity to Atg19, which has also been found to be required for targeting of a high molecular weight species of K444-acetylated Htt for lysosomal degradation.[11] p62/SQSTM1 can regulate activation of the IKK complex through multiple mechanisms including oxidative stress[94,95] and may therefore induce Htt post-translational modification and caspase cleavage, activating Atg23 and Atg11 for functioning in a mammalian Cvt-like pathway in which the amino-terminal Atg23-like fragment ultimately oligomerizes and is cleared.[1]

Like the C-terminal domain of Htt, FIP200 may also be a mammalian Atg11 protein by structural comparison and is similar to Htt amino acids 1,743–3,144 (**Suppl. Fig. 7**), but may specifically affect the cerebellum, as neuronal knockdown of FIP200 leads to cerebellar degeneration.[73] A loss of Htt function may particularly affect the striatum which degenerates in HD. The differential function of Atg11-like proteins in brain regions may cause the neuronal specificity of degeneration found in many neurodegenerative diseases. The recent observation of a lack of cargo recognition in HD cells[75] may reflect a loss of Atg11-like function.

Some functional and structural similarities may exist between Htt and yeast Atg23, Vac8 and Atg11, all proteins required for the yeast Cvt pathway. Vac8 and Atg11 are also essential for PMN and pexophagy and Atg11 for mitophagy.[41,90] These proteins may have fused throughout evolution to create Htt (**Fig. 1A**), which may be a protein whose function is integral for mammalian versions of the Cvt and PMN pathways, pexophagy and mitophagy essential for neuronal homeostasis in the striatum. Evolutionary fusion of yeast protein interactors (Slx5/Slx8) to form one functional mammalian protein (RNF4) has been previously described for proteins involved in a poly-SUMOylation/ubiquitination clearance pathway,[96] which may be tied to this mechanism of selective protein degradation. Fusion has also been proposed for *Saccharomyces cerevisiae* Atg29 and Atg31 proteins to form Atg28 in *Pichia pastoris*.[97]

Similar to the function of heat shock proteins Ssa1 and Ssa2 in recognition of proteins targeted to the vacuole in the yeast Cvt pathway, human Hsc70 may regulate lysosomal targeting of proteins selectively bound for degradation under nutrient-rich conditions. The Hsc70 consensus binding sequence is a pentapeptide with a glutamine on either end, a large hydrophobic residue (isoleucine, leucine, valine or phenylalanine), a positively charged residue (lysine or arginine), a negatively charged residue (aspartate or glutamate) and a repeated basic or hydrophobic amino acid.[98] This sequence shares some overlap with the consensus SUMOylation four amino acid motif containing a large hydrophobic residue followed by the lysine to which SUMO is conjugated, any amino acid and ending in a glutamate.[99] We find that phosphorylation of Htt S16 may create a consensus Hsc70 binding sequence with phosphorylated serine mimicking aspartate/glutamate.[1] Similarly, acetylation of lysine can mimic glutamine to potentially create an Hsc70-binding pentapeptide. SUMOylation and acetylation, activated by protein phosphorylation, may be linked to target protein interaction with Hsc70 in the induction of selective protein degradation by the proteasome and by the lysosome.

Mutant Htt Activates the IKK Kinase Complex Impacting Transcription

Short-lived nuclear proteins, in addition to long-lived cytoplasmic proteins, may be selectively targeted for lysosomal as well as proteasomal degradation, as the MEF2D transcription factor was demonstrated to be exported from the nucleus and cleared by the lysosome, dependent on LAMP-2A function.[100,101] p62/SQSTM1 has been shown to activate both proteasomal and lysosomal protein clearance and these two mechanisms may be intimately related.[102-104] We previously found that truncated mutant Htt (exon 1 protein, Httex1p) expression represses p53-activated transcription.[10] If the Htt protein physically plays a role in transcription factor clearance by p62/SQSTM1-mediated selective autophagy and/or proteasomal degradation, then activation of a transcription factor's degradation may effectively result in a repression of transcription mediated by the factor. Expression of mutant expanded polyQ Htt results in activation of the IKK complex in cell culture and in vivo.[2] I hypothesize that the repression of p53-mediated transcription we previously reported may reflect expanded polyQ Httex1p activating IKK and wt Htt to in turn induce clearance of p53, which itself is a target of IKK.[105]

As this protein clearance mechanism becomes compromised in HD, it may result in accumulation of active p53, effectively enhancing neurodegeneration as previously described in HD transgenic mice.[106] Similarly, there is an early reduction in Sp1 and CREB-mediated transcription in cells expressing mutant Htt, while these transcription factors accumulate in HD transgenic mice in parallel with pathogenesis.[107-114] In addition, the transcriptional repressor REST accumulates in the nucleus with mutant Htt expression and represses transcription.[115] I propose that expression of mutant Htt activates IKK, which induces wt Htt to increase clearance of active, acetylated transcription factors including p53, Sp1, CREB and REST, but that these factors all accumulate when protein clearance mechanisms fail, dysregulating transcription, which may precipitate HD pathogenesis.

Activation of IKK by mutant Htt expression may also have direct effects on Htt post-translational modification and the efficacy of drugs and proteins to induce Htt clearance. Mutant expanded polyQ "specific" Htt SUMOylation[27] or acetylation[11] may be driven by endogenous IKK activation in the presence of mutant Htt; these modifications may occur even more efficiently on the wt protein with independent activation of the kinase. Similarly, drugs found only to increase mutant Htt clearance by "mTOR independent autophagy"[116] may actually work to enhance wt Htt clearance in the presence of IKK activation. Recently, the Alfy protein was found to be a scaffold

between p62/SQSTM1-bound proteins and the molecular machinery that builds autophagosomes.[117] Alfy was shown to enhance aggregated mutant Httex1p clearance while having no effect on wt soluble Httex1p. IKK increases levels of insoluble phosphorylated wt Htt;[1] wt Htt oligomerization, activated by IKK, may normally regulate its Alfy-mediated lysosomal degradation, which is not induced without activation of the kinase.

Induction of Selective Protein Clearance Pathways Early in Disease Progression May Slow Neurodegeneration

I propose that activating selective protein clearance pathways in presymptomatic or early phases of disease progression may be generally useful to treat neurodegenerative disease. In later stages, when LAMP-2A levels and lysosome and proteasome function are reduced, activation of these pathways may create acetylated, SUMOylated and caspase-cleaved protein clearance intermediates that accumulate and are toxic to the cell. Evidence for both protective and pathologic outcomes involving activation of the signaling cascades that may induce selective clearance pathways in models of neurodegenerative disease provide support for my thesis. Signal transduction pathways that may induce selective clearance mechanisms include insulin signaling, DNA damage, oxidative stress and inflammation. These stimuli activate Akt, Cdk5 and IKK,[48,118-122] kinases that have been found to phosphorylate neurodegenerative disease-causing proteins and influence their toxicity and clearance.

The pro-survival kinase Akt, activated by insulin signaling,[122] can modulate neurodegeneration in multiple disease models and can function to activate IKK.[48] Akt phosphorylates S776 of Ataxin-1, the disease protein in the polyQ disorder Spinocerebellar Ataxia type 1 and enhances its toxicity in cell culture, transgenic Drosophila and mice.[123,124] Similarly, an inactivation of insulin signaling/Akt in mouse models of AD rescues behavioral deficits.[125-127] Conversely, Akt phosphorylation of the androgen receptor, mutated in Spinal and Bulbar Muscular Atrophy, also a polyQ disorder, increases its clearance and reduces its toxicity in vivo and in vitro.[128] Akt phosphorylates Htt at S421 which has been shown to be neuroprotective and to reduce inclusion formation in cell culture.[129] Activation of Akt is also associated with increased Htt clearance by macroautophagy.[44]

IKK activation has been implicated in neurodestructive outcomes in HD, AD and PD[2,13,24,130,131] while immuno-activities and inflammatory processes may also be neuroprotective.[132] We find that Htt phosphorylation by IKK can both reduce and increase its toxicity, which may depend on the ability of the cell to clear the modified species.[1]

Cdk5, which may activate Akt in neurons, is a protein kinase involved in the maturation and maintenance of the central nervous system with roles in the regulation of the cell cycle, migration, survival and neuronal integration.[121,133] Cdk5 phosphorylates the tau protein, which intraneuronally accumulates in a temporal pattern that parallels pathogenesis of AD and Frontotemporal Dementias; upregulation of Cdk5 activity has been generally observed to enhance AD pathogenesis.[134] However, we have found that increases in the levels of the Cdk5 coactivator p25, previously shown to correlate with improved learning and memory,[135,136] are associated with reduced abundance of phosphorylated tau in brain and improved phenotype of a mouse model of AD[137] implicating a role for Cdk5 in a phosphorylation-driven mechanism of tau clearance. Cdk5 phosphorylates Htt serines 434, 1181 and 1201 and reduces mutant Htt-mediated toxicity and aggregation in cell culture.[45,46] Phosphorylation of both Htt and tau by Cdk5 may similarly regulate their clearance, and accumulation of the toxic phosphorylated protein may reflect a loss of age-related protein degradation mechanisms. Cdk5 may therefore be a third protein kinase, similar to Akt and IKK, which may regulate selective protein clearance that becomes impaired during aging.

Recently proteins have been found to be acetylated by acetyltransferases under nutrient-rich conditions and deacetylated by Class III histone deacetylases with starvation; the level of protein acetylation may reflect energy status and regulate unneeded protein degradation.[138,139] p62/SQSTM1 levels are increased by acetyltransferase p300/CBP, while starvation-induced macroautophagy requires Sirt1, a Class III histone deacetylase.[43,140] I hypothesize that IKK may induce Htt acetylation at K9 and K444[1,11] within its Atg23-like domain, activating its cleavage, function in vesicle trafficking and degradation as well as inducing the activity of the carboxy-terminal Htt Atg11-like fragment in selective autophagy. p62/SQSTM1-mediated selective autophagy may be increased by inhibition of Class III histone deacetylases with nicotinamide, a treatment which we have found reduces levels of phosphorylated tau and slows neurodegeneration in a mouse model of AD.[137] This treatment works early in disease progression but has no effect in older animals, which may reflect a loss in protein clearance mechanisms with age.

I propose that activation of a Cvt-like mechanism of mammalian macroautophagy may be very useful therapeutically to treat neurodegenerative diseases of aging (**Fig. 2**). This mechanism may previously have been defined as "mTOR Independent Autophagy",[141] "Chaperone-Assisted Selective Autophagy (CASA)",[142] or "Quality Control Autophagy."[143] Activation of this protein clearance mechanism may occur with inflammatory stimuli, insulin signaling, DNA damage or oxidative stress activating Cdk5, Akt, mTORC1 and ultimately IKK.[48,121,144] IKK activation to induce this Cvt-like pathway may be enhanced by two proteins mutated in PD, PINK1 and parkin which have recently been demonstrated to activate mitophagy.[145-147] IKK activates CBP/p300 and JNK1 increasing levels of p62/SQSTM1,[4,8,43,148,149] and reducing the interaction of Bcl-x_L/Bcl-2 with Beclin-1[4,13,148] thereby potentially inducing Cvt-like macroautophagy. The complex interacting with the substrate protein may include Htt, BAG-3, p62/SQSTM1, CHIP, Hsc70 and HspB8.[1,142] Function of this complex may be impaired with expansion of the Htt polyQ repeat, as mutant Htt is not as efficiently phosphorylated by IKK,[1] Cdk5,[46] and Akt,[47] all of which may activate the complex's function. Alfy may assist in bridging this complex to the

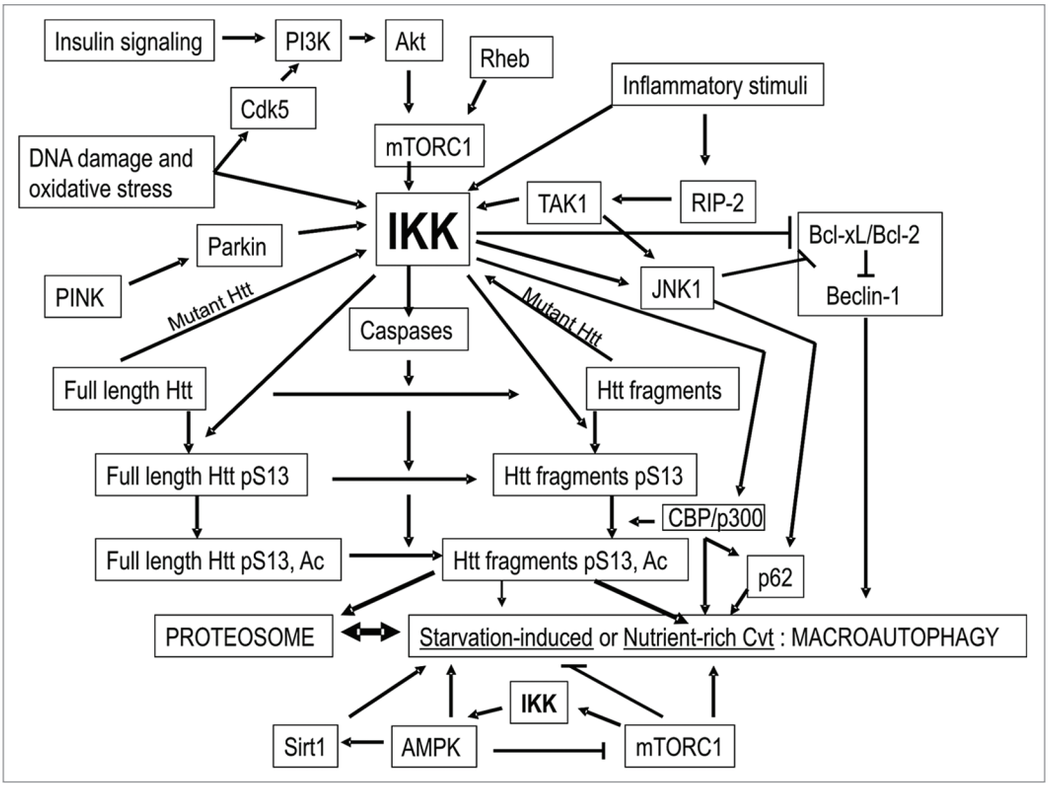

Figure 2. Model: The kinase IKK may activate Htt degradation by a mammalian version of the yeast Cvt pathway. The kinase IKK, activated by inflammatory stimuli, insulin signaling, DNA damage and oxidative stress pathways,[48,118-120,153,154] Rheb[155] and by PINK/parkin,[146,147] phosphorylates Htt S13 and activates Htt acetylation by CBP/p300 and caspase cleavage,[13] targeting the amino-terminal fragments of Htt for degradation by the proteasome and lysosome.[1] IKK reduces interaction of Bcl-x_L/Bcl-2 with Beclin-1,[4,13,148] thereby priming activation of both starvation-induced and the proposed mammalian Cvt macroautophagic pathways. IKK activates JNK1 and CBP/p300 to increase levels of p62/SQSTM1,[4,8,43,148,149] required for the Cvt pathway and AMPK[4] which may then activate starvation-induced macroautophagy as a compensatory feedback mechanism. Mutant Htt expression activates IKK.[2] The proposed mammalian Cvt pathway, activated by CBP/p300 or inhibition of Sirt1 by nicotinamide, may be involved in vesicle-mediated degradation of phosphorylated/acetylated Htt N-terminal fragments by the lysosome. With aging and mutant Htt expression, the proteasome and this Cvt-like mechanism of autophagy may become impaired, and this loss of function may be compensated for by activation of starvation/rapamycin/AMPK/Sirt1-induced macroautophagy.

molecular machinery that builds autophagosomes.[117] F-actin and cortactin regulated by HDAC6 may facilitate fusion of the vesicle to the lysosome[143] and LAMP-2A may be required for optimal association of lysosomes with dynein/dynactin to activate vesicular trafficking which is essential for vesicle/lysosome fusion.[70,150] All of these proteins may be therapeutic targets for the activation of Cvt-like selective macroautophagy. The polyphenol resveratrol, previously shown to induce autophagy, may therapeutically activate this Cvt-like mechanism via induction of JNK1-mediated p62/SQSTM1 expression and caspase activation,[148] consistent with a possible role for resveratrol in the upstream activation of IKK. Proteins involved in vesicle expansion, completion and nucleation are equivalent for both nutrient-rich and starvation-induced autophagic pathways, and therefore may not be useful to specifically increase Cvt-like selective macroautophagy. If this mechanism becomes overwhelmed as in later stages of disease when LAMP-2A levels are reduced,[151] starvation/AMPK/rapamycin/Sirt1-induced macroautophagy may compensate by clearing the oligomerized substrate protein complexes which may have accumulated to form inclusions. At this point, activation of starvation-induced macroautophagy and Sirt1 may also be protective against the further progression of the disease.

Conclusions

Htt may share some functional and structural similarities with three yeast proteins, Atg23, Vac8 and Atg11, required for selective vacuolar targeting in the yeast Cvt pathway. Htt may therefore play a functional and regulatory role in a selective protein clearance mechanism ultimately involved in its own processing. Phosphorylation of Htt by the IKK complex activates its poly-SUMOylation and acetylation; I propose that these modifications may regulate a normal role for Htt in a selective mechanism of protein clearance requiring both the proteasome and the lysosome, involving protein oligomerization. This mechanism may be activated by Akt/Cdk5/mTORC1/IKK kinases, and have some similarities to the yeast Cvt pathway, which functions under glucose/nutrient-rich conditions, when starvation-induced macroautophagy is inhibited, and in which oligomers are selectively targeted

to the vacuole. If the process becomes inefficient, mutant Htt may accumulate in inclusion bodies, which can be cleared by starvation-induced macroautophagy[52] potentially reflecting a compensatory response. This mammalian Cvt-like autophagic clearance pathway may decline with age and become compromised by expansion of the polyQ repeat within Htt, precipitating neurodegeneration. This mechanism may be particularly important in the brain as it depends on glucose as an energy substrate.[152] Therapeutically activating this selective protein clearance mechanism may be a valuable approach to the early treatment of many inclusion-associated diseases like HD and the polyQ-repeat diseases, PD, AD and ALS and to extend lifespan. It will be of great interest to carry out the challenging experimental work that will be required to investigate and potentially validate this approach to therapeutic intervention in these disorders.

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Note

Supplementry materials can be found at: www.landesbioscience.com/supplement/SteffanCC9-17-sup.pdf

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