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Huntingtin proteolysis and toxicity

Marie-Thérèse El-Daher

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UNIVERSITE PARIS-SUD

ÉCOLE DOCTORALE:

SIGNALISATION ET RÉSEAUX INTÉGRATIFS EN BIOLOGIE

LABORATOIRE

De Frederic Saudou "SIGNALISATION CELLULAIRE ET NEUROBIOLOGIE"

DISCIPLINE:

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THÈSE DE DOCTORAT

Soutenue le 17/06/2013

Par

EL-DAHER Marie-Thérèse

Monitoring huntingtin proteolysis reveals toxic events independent of the polyglutamine mutation

Composition du jury:

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Le vivant est une joie à ressources infinies, ressemblant à un tableau dont les couleurs se puisent dans l'immensurable imagination humaine et dont les formes se construisent dans l'observation scientifique.

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Research project

Specific Huntingtin Proteolysis Causes Endoplasmic Reticulum Dilation and Death

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Chapter I: Introduction

Part I: The disease

1. The Huntington disease.

1.1 From a social illness to a genetic disease.

Before the 19th century, patients suffering from Huntington disease (HD) were thought to be possessed by spirits and exiled from society or even persecuted as witches. In deed the primary feature of HD is a compelling need to move, involuntary agitation and irrepressible dance-like shaking. Later on, the disease was described as Huntington's chorea given the fact that it accounts on dancing tendency. Throughout the nineteenth century, doctors (Charles Gorman and Johan Christian Lund) had attracted the attention on clinical forms of the chorea occurring in adults raising the hypothesis of an inherited disease. In 1872, George Huntington published the paper "on chorea" describing precisely HD by combining the study of cases examined by himself or other colleagues (figure 1). Shortly, he described the disease with three marked peculiarities: 1 its hereditary nature, 2 a tendency to insanity and suicide, 3 it's manifesting itself as a grave disease only in adult life. It has a hereditary nature as it is transmitted between generations and confined between families (Huntington, 2003). Among symptoms, there is a predominance of muscular affection of the body coming along with epilepsy and hemiplegia; paralysis of limbs; changes in the sound of the heart and respiratory failure. Also, the propensity of insanity in some cases is marked. Regarding the pathology, Dr. Huntington believed that the disease is due to some dysfunction of the cerebellum as physiologists supposed that it was the region that controlled our movements. As for the treatments, patients were given tonics or electric shock delivered to their spine in order to relax their muscles, also they were pushed to exercise and followed a strict diet. Dr. Huntington concluded by the absence of any recovery or an amelioration of the symptoms. Unfortunately, HD became knotted in the Eugenics movement early on the 20th century, although, Charles Davenport, eugenic himself, discovered that the Huntington chorea was an autosomal dominant disorder.

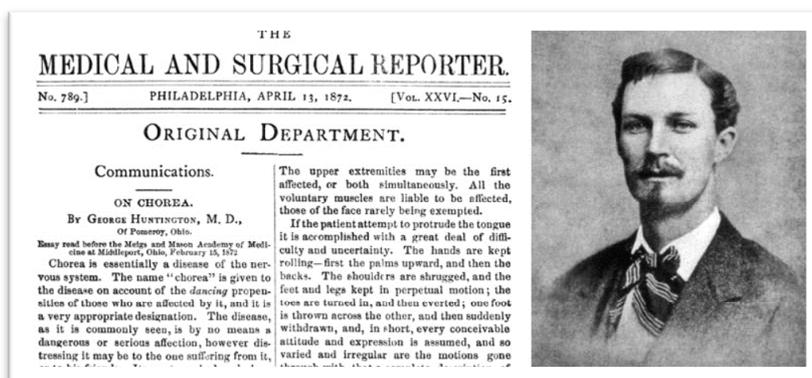


Figure 1 : First page of the article of Georges Huntington « on chorea ».

The interests in the Huntington chorea was steadily gaining importance all along the 20th century. In 1963 Milton Wexler, a famous psychiatrist, created the “Hereditary Disease Foundation” (HDF) after experiencing the disease through his wife. His daughter Nancy Wexler is the current president of the foundation. The HDF was an instrumental in initiating an international research project aiming to search for a cure for HD. This program was established in 1979 and named “The U.S.-Venezuela Collaborative Research Project”. It consisted of a team of doctors and scientists who studied the genome of a population of over 18000 subjects localized in two Venezuelan villages where an exceptionally high prevalence of the disease is occurring. A major breakthrough was the localization of the gene on chromosome 4 in 1983 using newly developed techniques for DNA analysis such as the Restriction Fragment Length Polymorphism (RFLP) and genetic linkage (Wexler, 2012) (The Huntington’s Disease Collaborative Research Group, 1993) (Gusella et al., 1983). Thus, Huntington’s chorea was classified as a genetically inherited disorder due to the mutation of a single gene. The transmission is done in a dominant fashion meaning that 50% of the offspring are carrier of the mutation.

1.2 Clinical diagnosis.

HD patients display specific symptoms correlated with physiological defects.

1.2.1 The triad of symptoms.

The clinical symptoms, in most cases, appear around the midlife of the patients, although there are some juveniles’ form of the disease in 5% of the cases. Patients survive 15 to 20 years after the onset of the first symptoms. In the pre-diagnostic phase, they display subtle changes of (i) cognition, (ii) behavior and (iii) motor control. A real diagnosis takes place when this triad of symptoms becomes sufficiently developed (Walker, 2007).

Cognitive impairment can appear many years before the motor impairment and patients themselves can be unaware of it (Novak and Tabrizi, 2011). Patients face problems in their daily life, at work or within the family. Cognition disorder deteriorates over time and strongly affects the independence of the patient as it affects executive tasks such as life organizing, speech, comprehension and acquisition of new skills.

Regarding behavioral changes, depression is highly prevalent and the suicide rate is higher than the general population (Di Maio et al., 1993). All individuals encounter suicidal thoughts at least occasionally. Anxiety, irritability and obsessive-compulsive behaviors are also relatively common in HD. A recent survey of 2,835 patients with HD found that 40% currently had symptoms of depression and 50% reported having pursued management for depression in the past (Paulsen et al., 2005).

The motor symptoms can be classified in two categories: first, patients are overwhelmed with involuntary movements and second, they lose their capacity to accomplish voluntary movements (Novak and Tabrizi, 2011). The chorea worsen with times and tends to make the patient fall. Rigidity and bradykinesia appear later while the over excitation of muscles disappear.

1.2.2 Neuropathology.

Macroscopic analyses of the disease were performed through the autopsy of post-mortem patients. The main affected region of the body is the brain, with a reduced brain size and several regions atrophied. The major neuro-pathological feature is the minified striatum with a selective atrophy occurring in the putamen and the caudate nucleus and the loss of white matter under the cortical mantle (Bates, 2002) (figure 2).

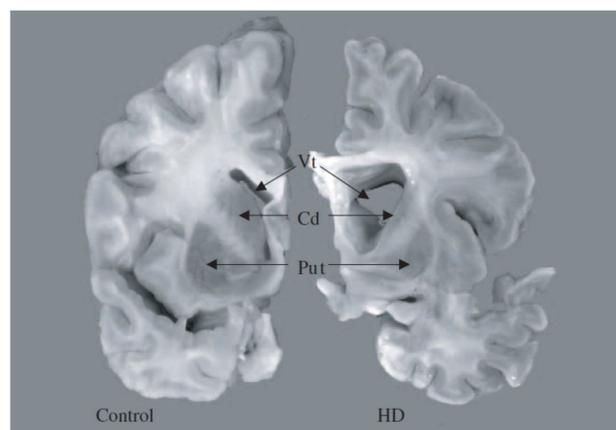


Figure 2: Photographs of coronal sections of fixed cerebral hemispheres from an HD case (right) and an age-matched normal specimen (left). Note the marked atrophy of the caudate nucleus and putamen, along with cortical atrophy and white matter loss in the HD brain. (Vt), Lateral ventricle, (Cd) caudate, (Put), putamen. (Bates, 2002)

At the microscopic level, quantitative studies have shown reductions of the cross-sectional areas of various anatomical compartments: 21-29% in cerebral cortex, 28% in thalamus, 57% in the caudate, and 64% in the putamen in moderately affected cases (Rosas et al., 2005) (Heinsen et al., 1994) (de la Monte et al., 1988). The neostriatum undergoes a massive neuronal loss along with the cerebral cortex. The earliest and most striking neuropathological changes were found in the neostriatum however striatal atrophy correlated with the severity of cortical atrophy, suggesting an associated disease process (Halliday et al., 1998). Along disease progression, the neurodegeneration of the striatum and the cortex contribute to the major symptoms developed early in HD (Rosas et al., 2008). Within the four types of neostriatal neurons, the most vulnerable neurons are the medium-sized spiny neurons (Graveland et al., 1985). Moreover, the striatal neurons that project to the

external globus pallidum are more susceptible to death than neurons projecting to the internal globus pallidum. Interneurons are spared (Walker, 2007).

This is in accordance with the fact that the chorea dominates early in the course of HD. Indeed, the basal ganglia are important to initiate the movement through the direct pathway but are also important at inhibiting the unwanted movements via the indirect pathway. The motor cortex sends glutamate to the striatum which in turn sends an inhibitory message to the external globus pallidum via GABA releasing, eventually leading to the inhibition of the thalamus and thus to a decrease in the excitatory flow toward the motor cortex leading to less muscular excitation (Hauber, 1998). In HD, as the striatal neurons projecting to the external globus pallidum are lost, the thalamus is no longer inhibited and stronger excitatory signals are sent to the motor/cerebral cortex which in turn, sends signal for movement to the spinal cord and ultimately to the muscles. In result this leads to the abnormal rate of movement observed in Huntington’s chorea (figure 3).

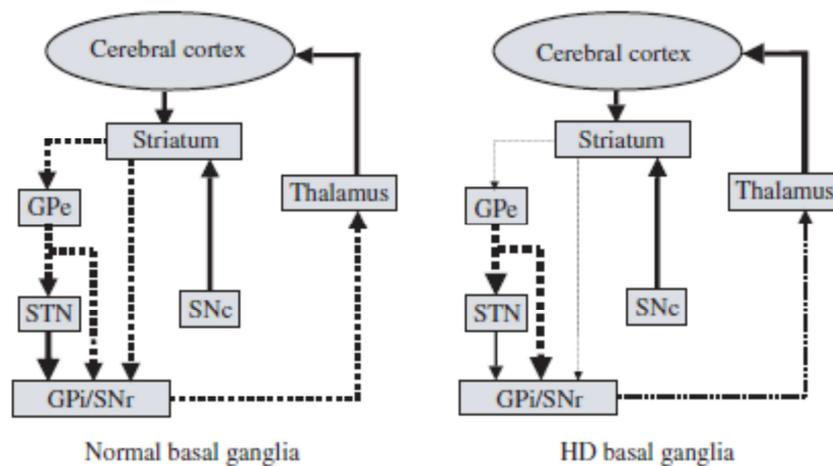


Figure 3: Basal ganglia pathways. Schematic of some of the interactions in the basal ganglia under normal and HD condition. The plain and dots lines indicates excitatory and inhibitory paths respectively. The width of line indicates the relative level of activity. (GPe), Globus pallidus external segment. (Gpi) globus pallidus internal segment. (STN), subthalamic nucleus. (SNr) substantia nigra pars reticulata. (SNc) substantia nigra pars compacta.

Other brain areas are also strongly affected in HD. These include the substantia nigra, cortical layers 3, 5, and 6, the CA1 region of the hippocampus and few Purkinje cells of the cerebellum (Jeste et al., 1984). Cortical neurons show decreased staining of nerve fibers, neurofilaments, tubulin, and microtubule-associated protein 2 and diminished complexin 2 concentrations (DiProspero et al., 2004). These elements that are associated with synaptic function, cytoskeletal integrity, and axonal transport suggest an important role for cortical dysfunction and cortico-striatal connections in the pathogenesis of the disorder.

Imaging the brain by functional magnetic resonance imaging (MRI) studies showed that the atrophy of the striatum becomes apparent long before the onset of the clinical symptoms (Aylward et al., 2004), thus making the volume of the striatum an excellent predictor of HD. Functional neuroimaging techniques such as PET revealed changes in brain activity before the onset of clinical symptoms. However, currently, the disease is declared when a combined physical and psychiatric symptoms are diagnosed (figure 4).

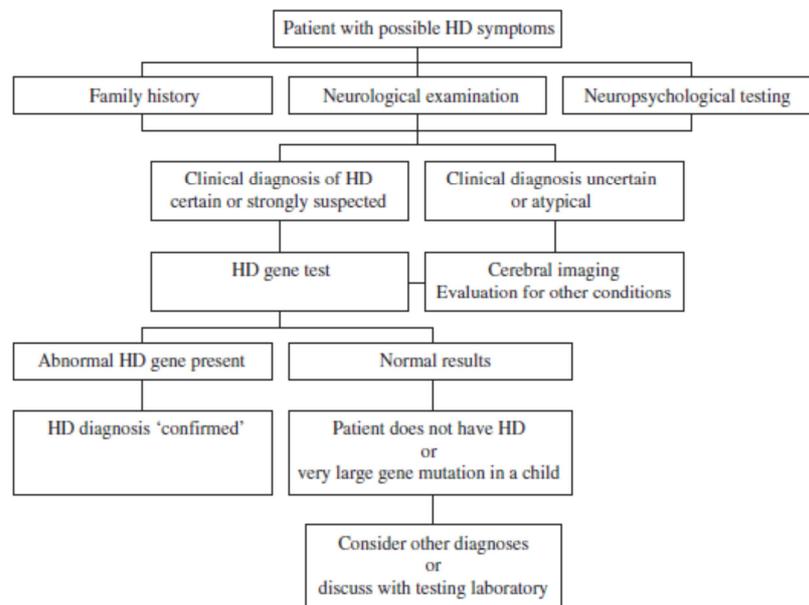


Figure 4: Evaluation of a patient suspected to have HD. (Bates 2002)

1.3 Care and therapeutic options in HD

HD is so far incurable, so the medical treatments are based on improving the quality of patients' life. Both social and medical cares are required for the benefit of the patients and their families. As HD is a long-term devastating disease with motor, cognitive and psychiatric symptoms, it is a very difficult disease to face.

Two types of therapeutics approaches are being pursued in HD: research for symptomatic treatments and research to cure or prevent the disease.

Medications do not help in limiting the involuntary movement at an early stage of the disease, however exercise and warm pool have been reported to be effective in relaxing the patients. When chorea gets severe, dopamine blocking drugs such as haloperidol can be used. Due to their cognitive impairment, speech and language therapy is helpful. Anxiety and additional movement causes insomnia heavily perturb the sleep-wake cycle. In this case, doses of sedatives are given to the patient.

Concerning depression symptoms, treatments are based on the use of different anti-depressant medications. Also, neuroleptics are often used to reduce hallucinations and disordered thoughts. The efficiency of drugs remains modest and choice of those drugs is not easy as many side effects are reported like agitation and insomnia, which are already two serious problems in HD (Bates, 2002).

The second concern is to treat the illness in order to prevent its development. Many trials exist but few are promising. Some drugs have shown efficiency in animal models and are now in clinical trials. The most advanced experimental treatments are the use of the coenzyme Q10 and creatine that have shown some improvement in human and are currently being funded for ulterior test (Walker, 2007; Wexler, 2012).

1.4 Prevalence and neuro-pathological classification.

Huntington's disease shows a steady prevalence of 7/100 000 in populations of European origin. The distribution is not equal between countries: for example the prevalence drops to 0.5/100 000 in Japan (Warby et al., 2011). The rate of HD is expected to constantly increase along with newly appeared mutation and the aging of the population with elderly patients with reduced CAG number developing symptoms. In addition, since the onset of the disease occurs at midlife, most of the patients have children before appearance of clinical symptoms (Pringsheim et al., 2012). Venezuela has the highest concentration of Huntington's disease in the world, particularly focused in the State of Zulia, particularly along the shores of Lake Maracaibo. This Venezuelan community illustrates the rapid expansion of a dominantly inherited genetic disorder that can occur when the gene is introduced into a confined population with a high natural rate of increase (Bates, 2002).

HD is graded on the base of macroscopic and microscopic criteria resulting in five grades (0-4). Indeed, according to Vonsattel (Vonsattel et al., 1985), the severity of the pathology of HD autopsy brain can be rated from 0 to 4. The first stage of the disease (grade 0) is characterized by a major loss of cannabinoid CB1, dopamine D2 and adenosine A2a receptor binding in the caudate nucleus, putamen and globus pallidus externus and an increase in GABAA receptor binding in the globus pallidus externus. The second, intermediate grades (grades 1&2) showed an additional decrease of CB1 receptor binding in the caudate nucleus and putamen; this is associated with a loss of D1 receptors in the caudate nucleus and putamen and a loss of both CB1 and D1 receptors in the substantia nigra. The final stages (grades 3&4) of HD showed an almost total loss of CB1 receptors and the further depletion of D1 receptors in the caudate nucleus, putamen and globus pallidus internus, and an increase in GABAA receptor binding in the globus pallidus internus.

In summary, with the progression of the disease, sequential neurodegeneration occurs leading eventually to a complete loss of GABAergic striatal projecting neurons in the late stages (Glass et al., 2000).

1.5 The genetic mutation of HD.

In 1993, the causal gene of HD, called *HTT* gene, which encodes a protein termed huntingtin (HTT), was localized on the short arm of the chromosome 4. The mutation was identified as a CAG repeat in the exon1 of the gene and is translated into a polyglutamine stretch (The Huntington's disease Collaborative Research group, 1993). When mutated, *HTT* gene contains 37-86 repeats, whereas genes displaying 11-34 repeats do not cause disease (Duyao et al., 1993). However, incomplete penetrance happens with 36-40 repeats due to the instability of CAG repeats that exceed 28 during replication. Indeed in most of the cases, replication of CAG expands the number of repeats although contractions can occur but with much lower frequency (Duyao et al., 1993; Yoon et al., 2003). Homozygosity is rare in HD due to the low prevalence of the disease. It is observed more frequently in the region of the Lake Maracaibo due to the abnormally high prevalence of the disease in this region. Interestingly, in patients carrying two mutated alleles the onset of the disease is similar to the heterozygote patients although more severe clinical course is reported (Finkbeiner, 2011). Importantly there is a strong inverse correlation between the onset of the disease and the number of CAG repeats (Andrew et al., 1993) (figure 5).

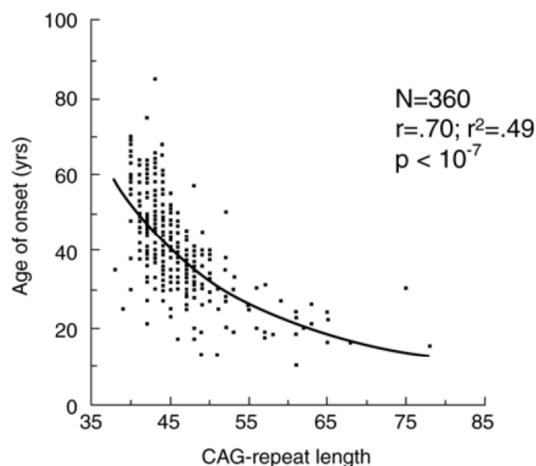


Figure 5: The correlation between CAG repeat length and age of symptom onset (Andrew et al. 1993)

For most of HD patients, age of onset can be explained by the length of the repeats. However other factors have to be considered as 40% of the remaining variance in age of onset is attributable to genes other than the *HTT* and 60% accounts from environmental influence (Wexler et al., 2004).

2. The Huntingtin protein.

2.1 Structure.

The protein HTT does not exhibit any homology with other known proteins. It is composed of the subsequently described domains: An N-terminal region of 17 amino-acids, the polymorphic polyglutamine region, a proline-rich region; the remaining of the protein is composed of HEAT repeats (Imarisio et al., 2008). In intermission between HEAT repeats, PEST domains are found. A PEST sequence is a peptide sequence which is rich in proline, glutamic acid, serine and threonine. This sequence is associated with proteins that have a short intracellular half-life; hence, it is hypothesized that the PEST sequence acts as a signal peptide for protein degradation (Ehrnhoefer et al., 2011; Rogers et al., 1986).

The first 17 amino-acids (N17) form an amphipathic alpha helix (Kim et al., 2009) which acts as a nuclear export sequence (NES). It is unknown if the HTT contains a nuclear localization sequence (NLS), yet stretches between amino acids 90-100 and 1188-1204 are suggestive of NLS. Also, in the C-terminus HTT resides another NES, targeting HTT in and out the nucleus (Bessert et al., 1995; Rockabrand et al., 2007; Xia et al., 2003). The phosphorylation state of amino-acids 13 and 16 regulates HTT localization and toxicity (Atwal et al., 2011; Gu et al., 2009). Also, mutation of methionine residue 8 to proline disrupt the N17 alpha-helical structure and lead to nuclear localization of HTT, dramatically increasing the toxicity of mutated HTT (Atwal et al., 2007). Moreover, N17 has been reported to mediate endoplasmic reticulum and cilia localization (Maiuri et al., 2013; Yan et al., 2011).

The polymorphic proline-rich region is an important mediator of protein-protein interactions and may regulate the aggregation state of HTT (Bhattacharyya et al., 2006). Five clusters of HEAT (huntingtin, elongation factor 3, the PR65/A subunit of protein phosphatase 2A and the lipid kinase Tor) containing 28 to 36 of these motifs are predicted to be distributed along the entire length of HTT (figure 6) (Li et al., 2006) (Imarisio et al., 2008). The sequence of a HEAT repeat is around 50 amino acids disposed in two anti-parallel alpha helices forming a hairpin (figure 6). They normally appear in tandem and are separated with non-helical region (Takano and Gusella, 2002). In addition, the large HEAT domains are interrupted by disorganized regions that could serve as regulatory domains as they often contain post-translational sites (see below). These domains are involved in protein-protein interaction suggesting that HTT is a large scaffolding protein. The structure of these HEAT repeats (stack of alpha helices denoted in the figure legend as alpha rod) could confer a solenoidal conformation to HTT whose function could also be modulated via intramolecular interactions (figure

7) (Palidwor et al., 2009). Dissecting HTT structure gives evidence of domains in HTT that mediate intra- and inter-molecular interactions which determine HTT function as it will be discussed below.

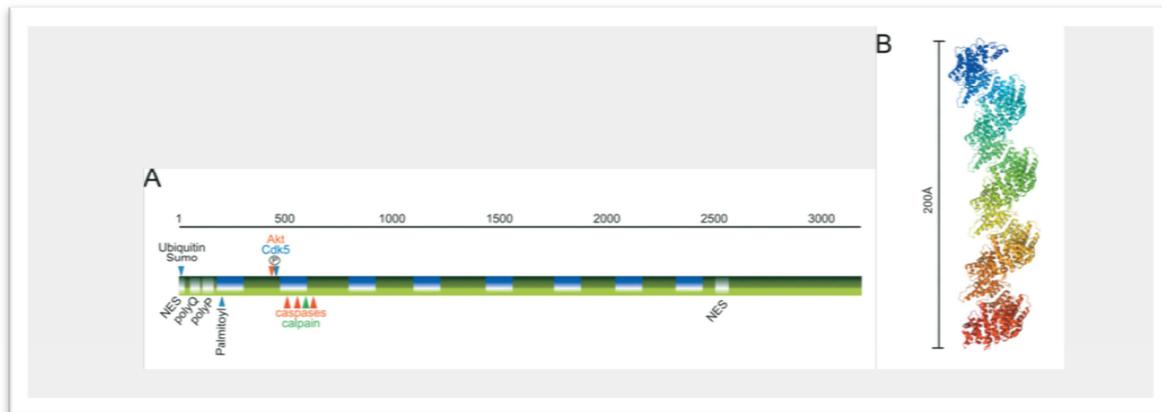


Figure 6: (A) Linear structure of the HTT protein molecule. The locations of the main HTT polypeptide sequence features are shown, including the polyglutamine (polyQ) and polyproline (polyP) sequences, NES and clusters of HEAT motifs (blue bars). Sites of post-translational modifications such as ubiquitination, SUMOylation, palmitoylation, phosphorylation and cleavage by proteases are also shown. (B) Probable three-dimensional structure of HTT as an elongated superhelical solenoid containing multiple HEAT repeats. The structure has been modelled on another HEAT repeat protein that has a molecular mass similar to that of HTT.

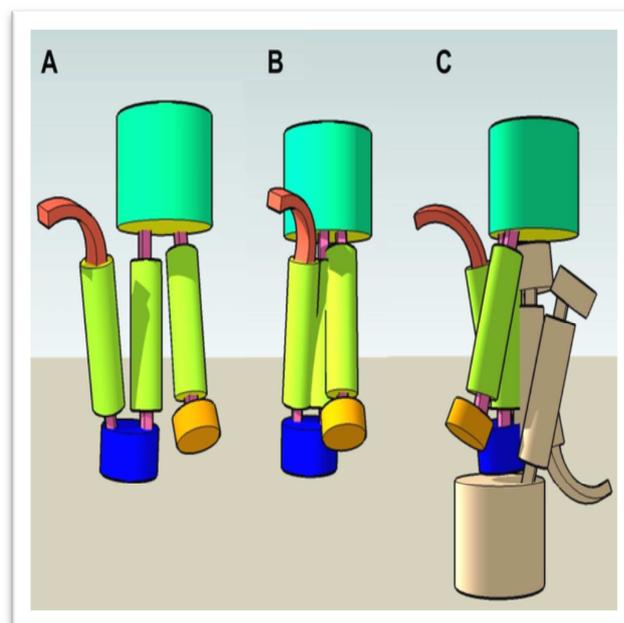


Figure 7: Hypothetical 3D structure of HTT. (A) The N-terminus with the poly-glutamine stretch (red arch) is followed by the H1 alpha-rod domain (residues 114 to 431, yellow cylinder), a small domain (432 to 671, blue), the H2 alpha-rod domain (672 to 969, yellow), a large domain (970 to 2666, green), the H3 alpha-rod domain (2667 to 2938), and a small C-terminal domain (2939–3144). (B) H1/2/3 intra-molecular interaction. (C) Formation of a HTT homodimer with a second molecule of HTT (gray). The N-terminal poly-Q stretch and the H1 domain remain exposed and can interact with other proteins (Palidwor et al., 2009).

2.2 Huntingtin post-translational modifications.

Posttranslational modifications occur on HTT protein and have an important role in regulating its function.

Lysine residues located upstream of the polyglutamine stretch, undergo SUMOylation or ubiquitination, a process that may be involved in the turn-over of the protein and in its cellular localization (Steffan et al., 2004). Also, acetylation of HTT on residue K444 regulates its clearance by macroautophagy as it facilitates its trafficking into autophagosomes (Jeong et al., 2009). The enzyme catalyzing this reaction is the CREB-binding protein (CBP) while the de-acetylation enzyme is the histone de-acetylase 1 (HDAC1).

Ubiquitination and SUMOylation of HTT compete for the same lysine residues 6, 9, 15 and 91. While ubiquitination is linked to a reduction of mutant HTT toxicity through an increase in its clearance, SUMOylation is suggested to stabilize mutant HTT in its soluble form and exacerbate its toxicity. Indeed, the E3 ligase Rhes SUMOylates the mutant HTT that leads to its disaggregation and the increase in its toxicity by augmenting the soluble pool of mutant HTT within the cell. Rhes shows a better affinity to the mutant than the wild type HTT. This prevents its ubiquitination and subsequent degradation (Steffan et al., 2004; Subramaniam et al., 2009).

HTT toxicity can be also modulated through phosphorylation modifications. HTT is a substrate for many kinases phosphorylating HTT at different sites. I previously mentioned the two phosphorylations at serine 13 and 16 that are involved in mutant HTT-induced toxicity (Gu et al., 2009). These residues are phosphorylated by the inflammatory kinase IKK enhancing the normal clearance of HTT by the proteasomes and lysosomes (Thompson et al., 2009), thus modulating mutant HTT neurotoxicity depending on the cell's ability to degrade the toxic HTT. Phosphorylation at serine 421 was shown to be important in regulating HTT function and toxicity as well (Humbert et al., 2002). AKT phosphorylation of HTT on serine 421 inhibits mutant HTT-induced cell death and reveals a neuro-protective effect of the IGF-1/AKT pathway in HD that is mediated by direct phosphorylation of HTT. Consistently, reducing HTT dephosphorylation at S421 by inhibiting the phosphatase calcineurin is protective against mutant HTT toxicity (Pardo et al., 2006). A well-defined function of HTT is its role in vesicular transport (described below). Interestingly the phosphorylation at S421 tunes the association of HTT with the motor complexes that are responsible for the transport of cargoes along microtubules within the cells: when phosphorylated, HTT stimulates the anterograde transport via an enhanced interaction with Kinesin-1, conversely, absence of phosphorylation reduces this interaction thus favoring the retrograde transport (Colin et al., 2008). Two other phosphorylation sites at serine 1181 and 1201, phosphorylated by the cdk5 kinase (Anne et al.,

2007), were recently shown to mediate anxiety/depression-related behaviors in mouse model through a regulation of BDNF transport and hippocampal neurogenesis (Ben M'Barek et al. 2013).

Another modification, I mention here but will develop later in the introduction is the cleavage of wild type and mutated HTT by a series of proteases, which includes caspases, calpain and not yet identified aspartic proteases (Lunkes et al., 2002).

In summary HTT undergoes different post translational modifications including phosphorylation, SUMOylation, ubiquitination, acetylation and proteolytic cleavage (figure 8). Other phosphorylations have been described whose functions are not well defined for some of them (Luo et al., 2005; Schilling et al., 2006). In addition, palmitoylation of HTT has been described and that could play a role in its targeting to membrane (Yanai et al., 2006). In the presence of the polyglutamine mutation, some of these modifications are altered leading to cellular dysfunction participating in mutant HTT toxicity. Targeting the enzymes involved in these modifications could represent promising therapeutic targets for HD.

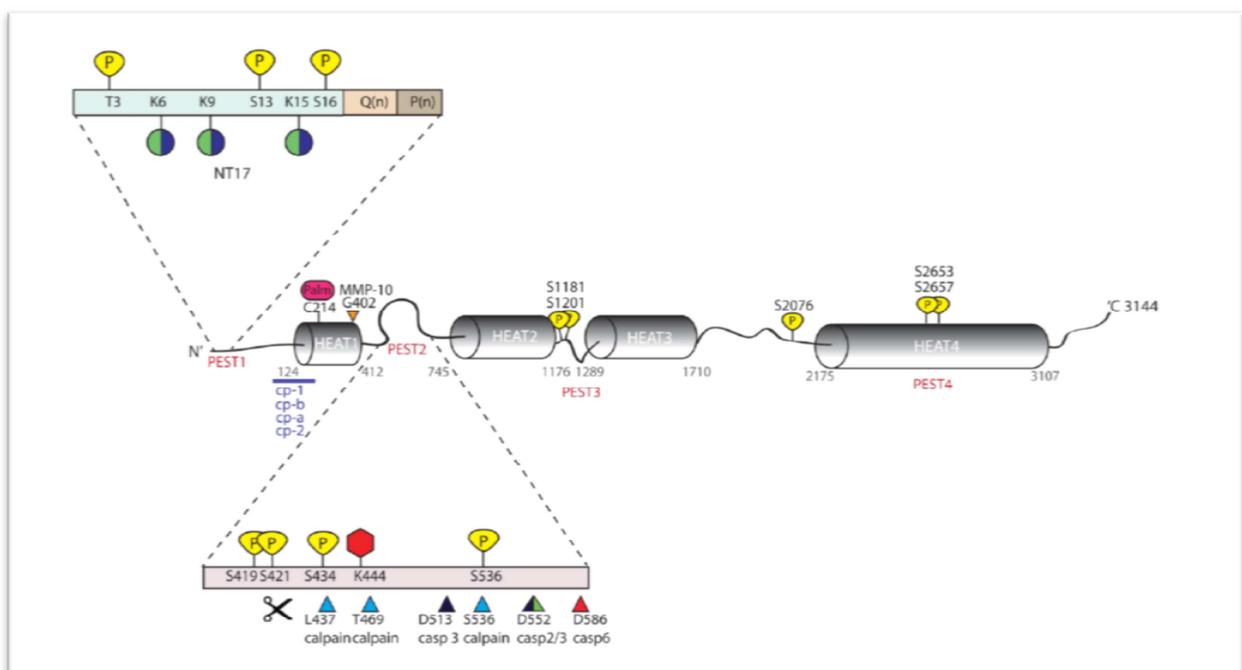


Figure 8: Post translational modifications occurring on HTT protein. Predicted HEAT repeats and PEST domains are marked along the length of the protein. Phosphorylation sites at threonine (T) or serine (S) residues are indicated in yellow. Lysines (K) which are modified either by the addition of ubiquitin or sumo-1 are indicated by green and purple coloured circles. Acetylation at lysine (K) 444 is represented as a red hexagon and palmitoylation at cysteine (C) 214 is represented as a pink oval. Protease cleavage sites are marked by triangles; calpain sites are indicated in blue, caspase -2, -3 and -6 sites are indicated by green, black, and red, respectively and matrix metalloprotease-10 (MMP-10) site at 404 is indicated in orange. Cleavage between amino acid 104-114, 205-214, 81-129 and at R167 generate cp-A, cp-B, cp-1 and cp-2 fragments, respectively (Ehrnhoefer et al., 2011).

2.3 Huntingtin, a player in many games.

The ubiquitous expression of HTT, its widespread localization at the subcellular level, its interaction with a large number of partners as well as the observations that HTT adopts multiple conformations is likely to confer multiple functions to the protein. Wild type HTT is mostly found in the cytoplasm but shuttles in and out from the nucleus (Kegel et al., 2002). HTT is associated with a variety of organelles, including the nucleus, endoplasmic reticulum, Golgi complex, synaptic vesicles and mitochondria (Sharp et al., 1995).

HTT is a protein of 3144 amino acids, with a molecular mass of 348 kDa. It is a ubiquitous protein with a particular enrichment in the brain and testes, significant amounts are found in the liver lungs and heart (Sharp et al., 1995). It is conserved between species, suggesting an essential role of this protein in the development (Gissi et al., 2006). The protein has no sequence homology with other proteins and is highly expressed in neurons and testes in human and rodents (Cattaneo et al., 2005).

HTT presents a large panel of interactor and is believed to be a scaffolding protein in many cellular complexes. Interactors of HTT are proteins that can be classified in diverse categories: transcription, trafficking, endocytosis, survival, signaling and metabolism (Kaltenbach et al., 2007). HTT-interacting proteins have been identified by either yeast two-hybrid or other biochemical approaches, such as affinity chromatography or immunoprecipitation. Interestingly, many HTT-interacting proteins identified to date bind to the N-terminal region of HTT (amino acids 1–588) as most of the bait used to pull down interactors span the N-terminal region of HTT, most probably because of the polyglutamine expansion presence in this region (Li and Li, 2004). Thus, HTT, by interacting with a large panel of membranous and cytosolic protein, is a multifunctional protein in cell. In its mutated form, HTT interactions can be modulated by the expanded polyglutamine presence and therefore altering its functions.

2.3.1 HTT & transcription.

HTT tunes the expression of several genes as HTT interacts with many transcription factors. Mutated HTT impairs the transcription machinery probably through two concomitant ways: loss of normal function and gain of new toxic functions. Abnormal protein-protein interactions with cytosolic transcription factors and nuclear localization of the mutated N-terminal HTT disturb gene expression regulation.

BDNF (brain derived neurotrophic factor) is an important growth factor for the survival of neurons (Strand et al., 2007). HTT enhances its transcription by regulating the transcription factor REST (repressor element-1 silencing transcription factor), which is a repressor of transcription of neuronal genes in non-neuronal tissues. Upon the binding of HTT to REST, REST is sequestered in the cytosol, making it unable to bind to NRSE (neuron-restrictive silencer element), an upstream DNA element, found in almost 2000 genes including BDNF. Hence, HTT regulates positively BDNF expression in neurons (Zuccato et al., 2003). When HTT is mutated, it abnormally binds to REST leading to the translocation of REST to the nucleus and to the repression of BDNF expression. A lack of trophic support to surrounding neurons is generated, contributing largely to neurodegeneration in HD (Zuccato et al., 2001; Zuccato et al., 2003) (figure 9). The abnormal translocation of REST/NRSF promotes histone deacetylation of the chromatin and inhibits the expression of genes implicated in survival signaling such as BDNF (Zuccato et al., 2003). As a consequence, striatal neurons are deprived from this important neurotrophic factor, from which their survival depends on (Strand et al., 2007). Recently, in cultured neurons and sections of the rat cortex, it was demonstrated that, BDNF mRNA associated with HTT and components of neuronal RNA granules, which are centers for regulating RNA transport and local translation. Hence, HTT may play a role in post-transcriptional transport/targeting of mRNA for BDNF, thus contributing to neurotrophic support and neuron survival (Ma et al., 2010) (Gauthier et al., 2004) (figure 9).

HTT interacts with Sp1, a transcription factor that binds to GC-rich elements in certain promoters and activates transcription of the corresponding genes (Sugars and Rubinsztein, 2003). Immunoprecipitation assays showed that polyglutamine expansion enhances the interaction of N-terminal HTT with Sp1 resulting in reduction in the binding of Sp1 to its corresponding promoters, therefore reducing the expression of Sp1-regulated genes. As a consequence, suppression of transcriptional activity of nerve growth factor is detected in cultured cells and HD transgenic mice (R6/2), leading to toxicity (Dunah et al., 2002; Li et al., 2002).

The HTT exon 1 protein directly binds the acetyltransferase domains of two transcriptional co-activators: CREB-binding protein (CBP) and p300/CBP-associated factor (P/CAF) (Chrivia et al., 1993). *In vitro*, HTT exon1 inhibits the acetyltransferase activity of both CBP and P/CAF. *In vivo*, histone deacetylase (HDAC) inhibitors arrest ongoing progressive neuronal degeneration in *Drosophila* models of polyglutamine disease (Steffan et al., 2001). Moreover, in cell culture, HD transgenic mice, and human HD postmortem brain, it was shown that CBP was depleted from its normal nuclear location and was present in polyglutamine aggregates in HD. Expanded polyglutamine repeats specifically interfered with CBP-activated gene transcription, and

overexpression of CBP rescued polyglutamine-induced neuronal toxicity (Steffan et al., 2000) (Nucifora et al., 2001).

While the full length HTT is mostly cytoplasmic, the generation of truncated mutated HTT by proteolysis results in nuclear and perinuclear forms which heavily affect the survival of the cell (Martindale et al., 1998; Saudou et al., 1998). TBP (TATA box binding protein) and CBP, two essential transcription factors for cell survival signaling, contain a stretch of glutamine in their sequence. When HTT is mutated, it abnormally interacts with them through their polyglutamine region. Interestingly it was shown that in the presence of a full length mutated HTT, TBP and CBP are taken out from the nucleus to the cytoplasm and both localize in cytoplasmic HTT polymers. Instead, when mutated HTT was directed into the nucleus (addition of an NLS sequence), TBP and CBP colocalized with the nuclear inclusion formed (Schaffar et al., 2004). The coaggregation of these transcription factors with mutant HTT is a secondary event, as the soluble form of HTT is enough to trigger functional loss of TBP and CBP.

TAFII130 was found to bind HTT *in vivo*. It is an important transcription factor that binds TBP and that is involved in SP1- and CREB-dependent gene transcription. Overexpressing SP1 and TAFII130 in striatal cells obtained from an HD mouse model suppresses the transcriptional inhibition caused by mutated HTT and abrogates the cellular toxicity induced by mutant HTT (Dunah et al., 2002). Mutant HTT was also found to associate with p53 in inclusions generated in cell culture and to interact with p53 *in vitro*. This interaction is restricted to mutated HTT which up-regulate levels of nuclear p53 as well as p53 transcriptional activity in neuronal cultures (Bae et al., 2005). Indeed, expanded HTT represses transcription of the p53-regulated promoters, such as p21 (Steffan et al., 2000). It was also noted that genetic deletion of p53 suppressed neurodegeneration in HD flies and neurobehavioral abnormalities of HD mice. As p53 acts on mitochondria homeostasis (Mihara et al., 2003), this underlines a link between nuclear p53 and mitochondrial pathologies characteristic of HD (Bae et al., 2005).

Finally, the N-terminal HTT 1-171 amino acids interact with the C-terminal region of the nuclear receptor co-repressor (N-CoR). The N-CoR functions as silencing mediator for retinoid and thyroid hormones receptors. This co-repressor is delocalized in HD brain which may induce transcriptional alteration contributing to the pathology of HD (Boutell et al., 1999; Nagy et al., 1997).

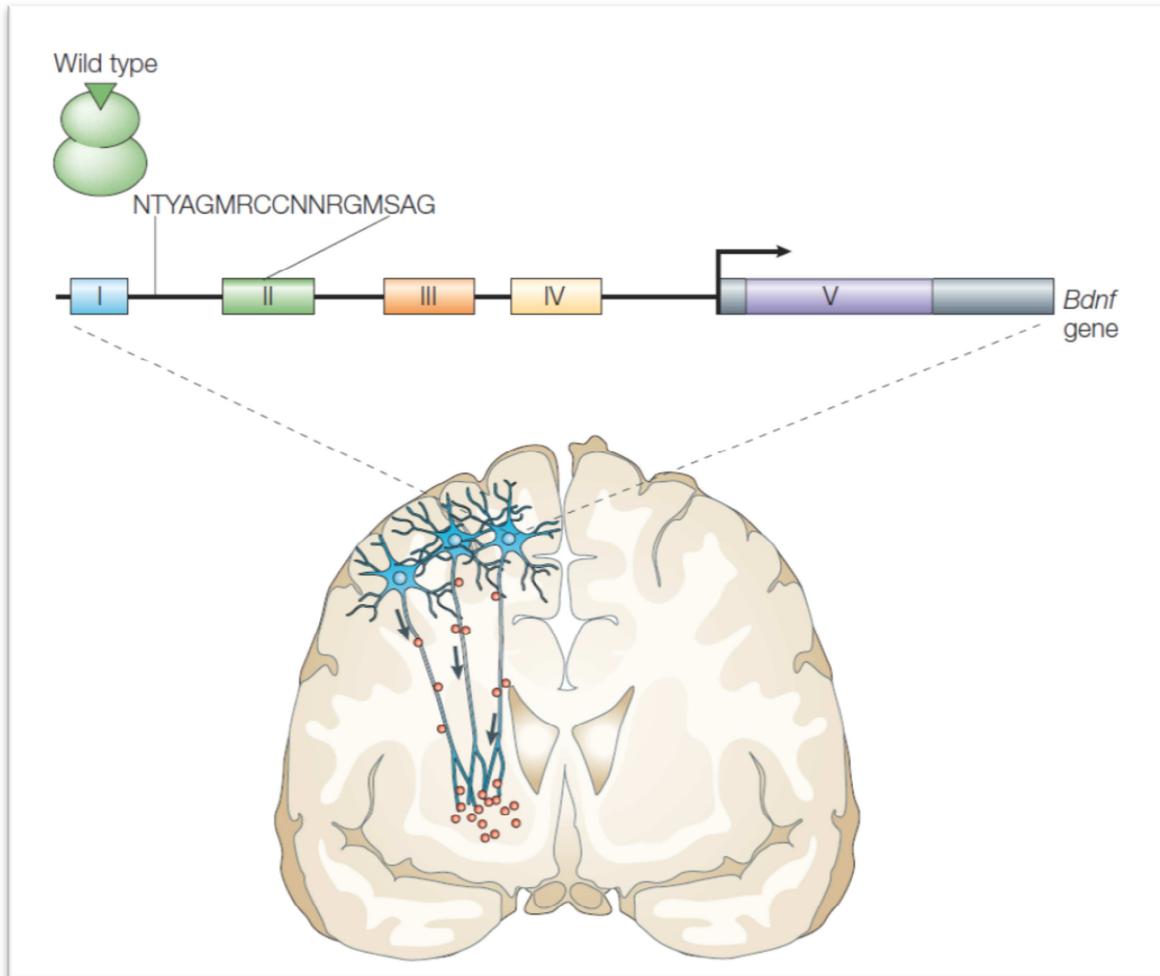


Figure 9: Wild-type but not mutant HTT facilitates cortical BDNF mRNA production. HTT contributes to brain-derived neurotrophic factor (BDNF) transcription in the cortical neurons that project to the striatum by inhibiting the Repressor element 1/neuron-restrictive silencer element (RE1/NRSE) that is located in BDNF promoter exon II. I–IV indicate BDNF promoter exons in BDNF; V indicates the coding region. The RE1/NRSE consensus sequence is shown. Inactivation of the RE1/NRSE in BDNF leads to increased mRNA transcription and protein production in the cortex. BDNF, which is also produced through translation from exons III and IV is then made available to the striatal targets via the cortico-striatal afferents. HTT also facilitates vesicular BDNF transport from the cortex to the striatum (Cattaneo et al., 2005).

2.3.2 HTT & trafficking.

HTT associates with vesicles and microtubules (DiFiglia et al., 1995; Hoffner et al., 2002).

HTT is a scaffold protein for complexes serving the cellular trafficking. HTT binds directly to dynein (Caviston et al., 2007) and indirectly with kinesin light chain and p150Glued/dynactin via Huntingtin-associated protein 1 (HAP1) a neuronal protein that binds to HTT. HAP1 binds more tightly to HTT with an expanded glutamine repeat than to wild type HTT, and the binding is enhanced by lengthening of the glutamine repeat (Li et al., 1998b). HAP1 interacts with kinesin light chain, a subunit of the kinesin motor complex that drives anterograde transport along microtubules in

neuronal processes (McGuire et al., 2006). HAP1 associated with membranous organelles, and binds to p150Glued/dynactin, an accessory protein for cytoplasmic dynein that participates in microtubule-dependent retrograde transport of membranous organelles. Hence, HAP1, p150Glued/dynactin, dynein and HTT form a complex that participates in microtubule-dependent transport (Li et al., 1998a) (Engelender et al., 1997) (Caviston and Holzbaur, 2009; Gauthier et al., 2004). The alteration of the HTT/HAP1/p150 (Glued) complex correlates with reduced association of motor proteins with microtubules which induced transport deficit of BDNF along axons resulting in the loss of neurotrophic support and neuronal toxicity (Gauthier et al., 2004). BDNF transport is attenuated in the disease and reduction of striatal BDNF plays an important role in HD pathogenesis. In agreement, overexpression of BDNF within the forebrain of HD mouse model prevented loss and atrophy of striatal neurons (Xie et al., 2010). The role of HTT in axonal transport was established in mice and *Drosophila* HD model. Indeed, reduction or expression of mutant HTT causes axonal defects in *Drosophila* (Gunawardena et al., 2003). This function of HTT in transport was recently shown to be conserved between flies and mammals as *Drosophila* HTT knock-Out flies show a reduced fast axonal transport of synaptotagmin vesicles in motoneurons *in vivo* (Zala et al., 2013a). Posttranslational modifications of HTT at serine 421 (as previously described), regulate the bi-directionality of vesicular transport further implicating a loss of the wild type HTT function in HD (Colin et al., 2008).

Molecular motors involved in axonal transport require energy for movement. Unexpectedly, mitochondria, the main source of cellular energy, were shown to be dispensable in this process. Energy is provided by vesicular GAPDH and PGK, glycolytic enzymes, which localize on motile vesicles through a HTT-dependent mechanism. Silencing HTT led to the delocalization of GAPDH from BDNF transporting vesicles (Zala et al., 2013b). Thus, HTT is a scaffolding protein for motor complexes maintaining the vesicular cargos to microtubule and is also a scaffolding protein working for the correct position of its partners such GAPDH, being critical for energy support in fast axonal transport.

Acetylation of microtubules favors the recruitment of molecular motors such as dynein and kinesin1, enhancing the transport of vesicle along the cells. It was shown that tubulin acetylation is reduced in HD brains and that the inhibition of the histone deacetylase HDAC6 with trichostatin A, compensates for the BDNF transport- and release-defect phenotypes that are observed in disease. It remains to identify the cause of the change of microtubule acetylation in HD, however inhibiting deacetylation may be a therapeutic target of interest counteracting the harmfulness of the defective intracellular transport in HD (Dompierre et al., 2007).

Wild type HTT is essential for protein trafficking to the centrosome and normal ciliogenesis. Indeed, wild type HTT regulates ciliogenesis by binding to HAP1 and to pericentriolar material 1

protein (PCM1). Polyglutamine expansion led to the accumulation of PCM1 in the centrosome resulting in a cilia defect in mouse model of HD and in HD patients (Keryer et al., 2011). HTT also participates in post-Golgi trafficking of proteins that follow the regulated secretory pathway such BDNF (del Toro et al., 2006). It regulates post-Golgi transport via the optineurin/Rab8 complex. Optineurin, an HTT-interacting protein, colocalizes with HTT in the Golgi apparatus and mutant HTT impairs the presence of optineurin/Rab8 complex in the Golgi apparatus (del Toro et al., 2009; Sahlender et al., 2005) (figure 10).

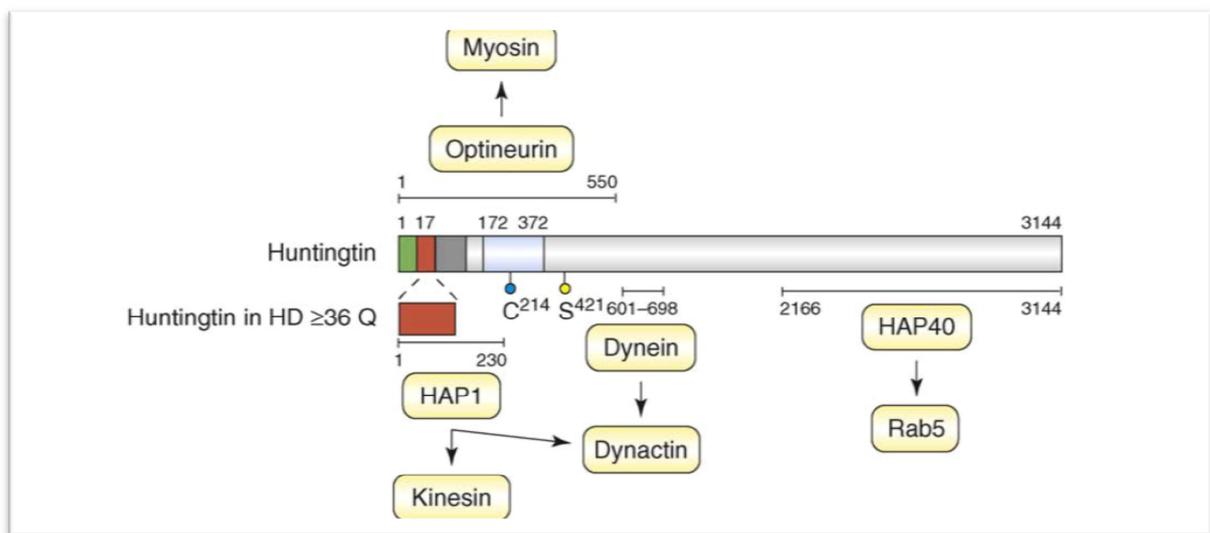


Figure 10: Schematic of HTT binding sites for molecular motors. The N-terminal membrane localization signal (aa1–18) is shown in green and the polyglutamine repeat region (beginning at aa17) is shown in red. The blue region (aa172–372) has been shown to associate with acidic phospholipids at the plasma membrane. The site of palmitoylation of Cys214 by HIP14 is indicated by a blue lollipop and phosphorylation of Ser421 is indicated by a yellow lollipop. The myosin VI linker protein optineurin is known to associate with the N-terminal region of HTT. HAP1 interacts with the N-terminal region of normal HTT and the expanded polyglutamine repeat found in mutant HTT enhance binding. HAP1 also interacts with the plus-end-directed microtubule motor kinesin and dynactin. The minus-end-directed microtubule motor dynein interacts with HTT (aa600–698) and with the dynein activator dynactin. HAP40, an effector of the small GTPase Rab5, binds to the C-terminal region of HTT (Caviston and Holzbaur, 2009).

2.3.3 HTT, endocytosis & vesicular trafficking.

HTT is involved in endocytosis. Endocytosis takes place mainly at two cellular zones: at the plasma membrane where newly generated vesicles are pinched off and send inside the cells (uptake), and at the other membranous organelles of the cell where vesicles are targeted towards other organelles or back to the plasma membrane (recycling).

In proteomic studies, HTT is found in brain membranes fraction suggesting that HTT is targeted and associates to membranes within the cell (Suopanki et al., 2006). HTT is suggested to link to membranes through electrostatic interactions with acidic phospholipids (Kegel et al., 2005) while it is seen to be present at endocytic zones of the plasma membrane (Kegel et al., 2005).

HIP1 (Huntingtin interacting protein 1), was discovered as the one of first interactors of HTT in 1997 by a yeast two hybrid approach (Wanker et al., 1997). It is a multidomain protein containing a central coiled-coil forming region and a C-terminal actin-binding domain. HIP1 is implicated in clathrin assembly via a direct interaction with clathrin light chains (Legendre-Guillemain et al., 2005). Moreover, HIP1, HTT, clathrin and endocytosed transferrin were observed in clathrin coated vesicles indicating that HTT is involved in vesicular trafficking as it interacts with a clathrin-binding protein to mediate endocytosis (Velier et al., 1998; Waelter et al., 2001). In addition, HTT was determined to be important for iron homeostasis. In a zebrafish model, the knock down of HTT lead to a lack of iron uptake by the cells although iron concentration was unchanged within blood cells. This revealed a functional role of HTT in the endocytosis of transferrin receptors (Lumsden et al., 2007).

HTT is important vesicles for recycling as it play a role in the activation of Rab11, a GTPase involved in endosomal recycling. Mutant HTT reduce Rab11 activity leading to a defect in vesicle formation at recycling endosomes. The mechanism by which mutant HTT inhibits nucleotide exchange from Rab11GDP to Rab11GTP is not established (Li et al., 2009).

Interaction between HAP40 and HTT is an effector of Rab5 activity. Rab5 is a key regulator of endocytosis that orchestrates the recruitment of multiple effector proteins on the early endosome membrane to regulate organelle tethering, fusion, and microtubule-dependent motility (Zerial and McBride, 2001). HTT/HAP40 complex regulates the dynamic of early endosomes through a switch from microtubules to actin cytoskeleton (Pal et al., 2006). In mutant conditions, the elevated level of HAP40 impairs endosomes motility (Pal et al., 2006). All these data strongly support the role of HTT in intracellular trafficking of vesicles along microtubules and their targeting to membranous organelles, while mutated or loss of HTT damage the cellular trafficking (figure 10).

The role of the GTPase dynamin in endocytosis is well established where it acts at fission sites for clathrin-mediated endocytosis. *Drosophila melanogaster* shibire mutants show paralysis resulting from the neuronal activity-dependent depletion of synaptic vesicles, which is accompanied by the accumulation of arrested endocytic pits at the presynaptic plasma (Koenig and Ikeda, 1989) (Ferguson and De Camilli, 2012). HTT was shown to interact with dynamin by Yeast two hybrid (Kaltenbach et al., 2007) and recently shown to be involved in the reduction of the endocytosis of the ErbB2/HER2 receptor tyrosine kinase by mutant HTT (Moreira Sousa et al., 2013).

Furthermore, HTT interacting protein 14 (HIP14) is a partner of HTT and is involved in regulating endocytosis protein trafficking. This protein is predominantly expressed in neurons in the brain and localizes to Golgi membranes and cytoplasmic vesicles (Singaraja et al., 2002).

HIP14, is a neuronal palmitoyl transferase (PAT) which shows substrate specificity for neuronal proteins, including SNAP-25, PSD-95, synaptotagmin I, and HTT (Huang et al., 2004). Palmitoylation is important for vesicular trafficking and clustering of a subset of synaptic proteins (Huang et al., 2004; Kang et al., 2004; Rathenberg et al., 2004). For instance, postsynaptic targeting and clustering of the postsynaptic density (PSD) protein PSD-95 requires palmitoylation, and this process regulates glutamate receptor retention at the synapse (Craven et al., 1999). HTT is normally palmitoylated at cysteine 214 by HIP14 (Yanai et al., 2006). Expansion of the polyglutamine tract of HTT, results in reduced interaction between mutant HTT and HIP14 and consequently in a marked reduction in palmitoylation. This leads to abnormal distribution of HTT within the cell and a lack of HTT trafficking to the Golgi (Yanai et al., 2006). How the disturbance of HTT palmitoylation contributes to alterations in synaptic transmission in HD remains to be determined (Zeron et al., 2002). HIP14's interaction with HTT provides another link in the function of HTT in intracellular transport and endocytosis.

2.3.4 HTT & Survival.

HTT has a pro-survival role within the cell. Depletion of HTT causes embryonic lethality at days 8.5 and 10.5 of gestation (Nasir et al., 1995) (Zeitlin et al., 1995) with an increased apoptotic cell death in the embryonic ectoderm suggesting that HTT is involved in processes counterbalancing the operation of an apoptotic pathway during development. Also HTT is required for embryonic development and neurogenesis (White et al., 1997) as well as in extra-neuronal tissue formation (Dragatsis et al., 1998). Moreover HTT's function during embryogenesis is independent of the size of the polyglutamine stretch as it is shown that a mutated HTT can compensate for the loss of the endogenous HTT in mice lacking both copy of the *HTT* gene (Leavitt 2001).

Evidence comes from the demonstration that overexpression of HTT protects the cells from apoptosis stimuli by preventing the activation of caspase-3 possibly by acting upstream, at the level of caspase-9 activation (Rigamonti et al., 2000). Conversely, cells depleted from HTT show increased level of caspase-3, as compared to control cells, and are more sensitive to apoptotic cell death (Zhang et al., 2006b). Importantly, Leavitt and colleagues brought strong evidence for the anti-apoptotic role of HTT. In YAC72 mice depleted from the endogenous HTT, they observed a massive atrophy of the testes explained by an uncontrolled apoptotic cell death (Leavitt 2001). Similarly, in

the zebrafish embryos, the knock-out of HTT showed a massive apoptotic cell death indicated by the increase of caspase-3 activity within the brain of the developing embryo (Diekmann et al., 2009).

One explanation for the anti-apoptotic role of HTT might be given by the sequestration by HTT of two activators of the pro-caspase-8, Hip1 (HTT interactor protein 1) and HIPPI (Hip1 protein interactor). The presence of HTT with an expanded polyglutamine repeat favors the formation of Hippi–Hip-1 oligomers and might be participating in mutant HTT-induced toxicity (Gervais et al., 2002). HIPPI also binds to specific upstream sequences of the caspase-1, caspase-8 and caspase-10 genes and alters their expression suggesting a role in the transcriptional regulation of apoptotic genes (Majumder et al., 2007) (figure 11).

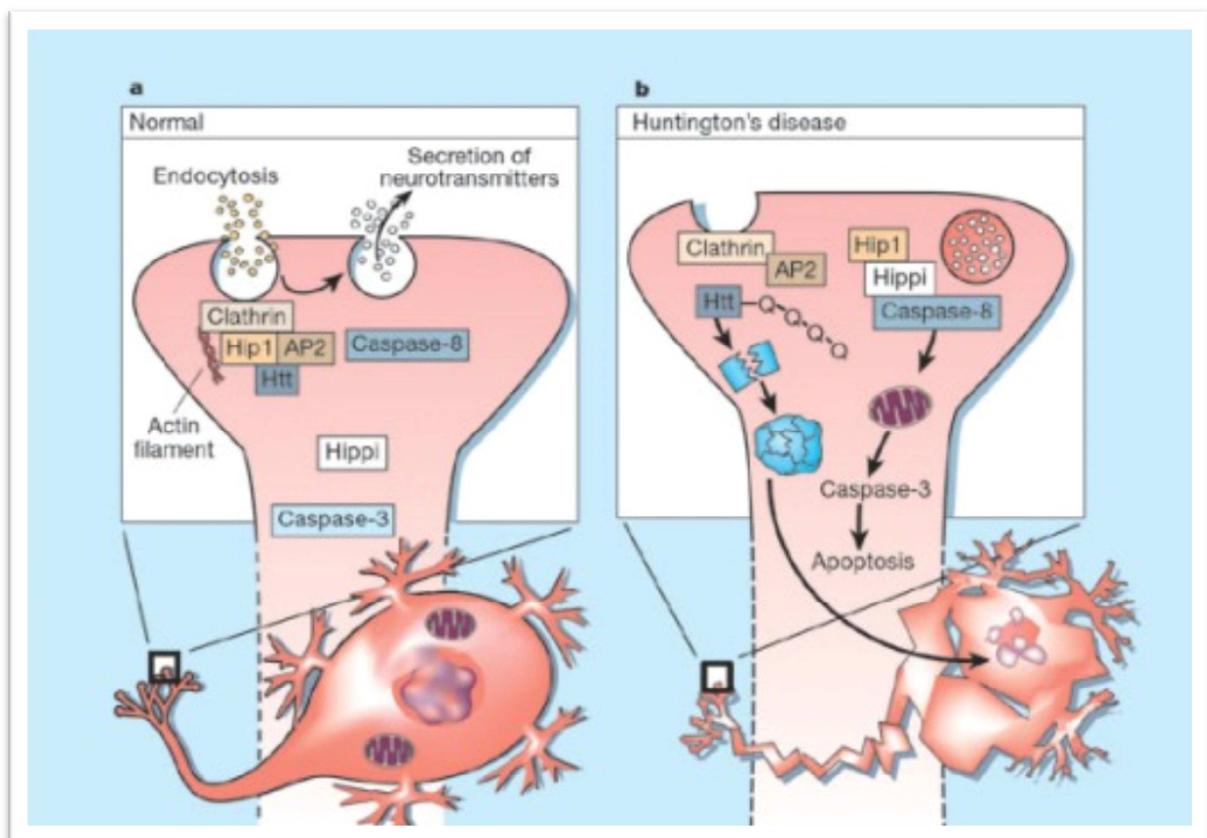


Figure 11: (a) Normal HTT bind Hip1, modulating endocytosis, (b) mutated HTT enhances complex formation of Hip1 and Hippi which eventually promote cell death.

2.3.5 HTT & synaptic transmission.

Normal communication between neurons is regulated by a number of protein in the synapse. By ultrastructure analysis of cortical neurons, HTT was detected in the matrix cytoplasm and around the vesicles membranes (DiFiglia et al., 1997; Trottier et al., 1995). Although HTT is not only localizing at the axonal terminal, it plays an important role at the synapse. Mutated HTT deregulates synaptic transmission by reducing the level of important protein at the synapse and by impairing essential protein-protein interactions. In addition, a direct consequence of a defect in the axonal transport is a local decrease of factors at the synapse (Smith et al., 2005).

HTT binds to the C-terminal-located SH3 domain of PACSIN 1 (Protein kinase C and casein kinase substrate in neurons protein 1). The rat homologue of PACSIN 1, synadapin 1, has been shown to interact with important proteins for endocytosis such as dynamin (Modregger et al., 2000). This interaction is enhanced by mutant HTT and causes the sequestration of PACSIN 1. PACSIN 1 normally located along neurites and within synaptic boutons, but in HD patient neurons, there is a progressive loss of PACSIN 1 in synapse. Mutated HTT alters the distribution of PACSIN1 in pathological tissues, and by doing so, might contribute to the synaptic dysfunction observed in HD (DiProspero et al., 2004; Modregger et al., 2002). Moreover, an association has been defined between PACSIN1 and neuronal Wiskott–Aldrich syndrome protein (N-WASP), a potent activator of the actin-related protein 2 and 3 (Arp2/3), a complex that regulates actin polymerization (Miki et al., 1996). Thus, an interaction between HTT and PACSIN1 might affect vesicle detachment by influencing both actin dynamics and coated-pit formation.

Complexin II is involved in the fusion reaction occurring between the synaptic vesicles and the pre-synaptic plasma membrane. This protein is progressively lost from the brains of HD mouse model suggesting a contribution to the neuronal dysfunction in HD (Morton and Edwardson, 2001).

Synapsin I plays a role in the attachment of synaptic vesicles to the actin and microtubule cytoskeletons (Baines and Bennett, 1986). Phosphorylation state of Synapsin I at site 3-5 is modified in an HD mouse model leading to a decrease affinity to the actin filaments and hence to a decrease in the reverse pool of vesicles (Lievens et al., 2002).

Mutant HTT can also induce synaptic dysfunction by disrupting the activity of pre- and post-synaptic receptors. A higher release of glutamate, an excitatory neurotransmitter is detected in the striatum of a polyglutamine HD mouse model with a defect in its clearance by the glial cells. This is probably due to a feedback control due to a lack of metabotropic glutamate receptors regulating the release of glutamate on the pre-synaptic plasma membranes. As a consequence, a higher excitability

of the NMDA (*N*-methyl-D-aspartate) glutamate receptors is occurring at the post-synaptic plasma membrane (Starling et al., 2005).

In addition, HTT associates with N-methyl-d-aspartate (NMDA) and kainate receptors via postsynaptic density 95 (PSD-95) through its SH3 domain. PSD-95 is a membrane-associated guanylate kinase concentrated at glutamatergic synapses and may participate in synapse development (Cho et al., 1992; El-Husseini et al., 2000; Kistner et al., 1993). Polyglutamine expansion restricts the ability of HTT to interact with PSD-95 and causes sensitization of NMDA receptors. This result in neuronal toxicity and apoptosis induced by glutamate in the pathology. This effect can be counteracted by wild HTT or an antagonist of the NMDA receptor (Sun et al., 2001).

The cysteine string protein (CSP) is a chaperone protein expressed on synaptic vesicles in secretory cells and that interacts with HTT. At the synapse, chaperone proteins are essential for the control of speed and the fidelity of neurotransmitter release (Morgan et al., 2001). It is showed that only the mutated HTT but not the wild type HTT, binds and sequesters CSP, preventing its inhibitory effect on the N type calcium channel, channels involved in neurotransmitter release (Miller et al., 2003).

There are abundant evidence for the role of HTT in synaptic communication and dysfunction induced by mutated HTT (figure 12). The complete picture remains complex as several abnormalities could contribute together to the defect in synaptic vesicles exocytosis and recycling.

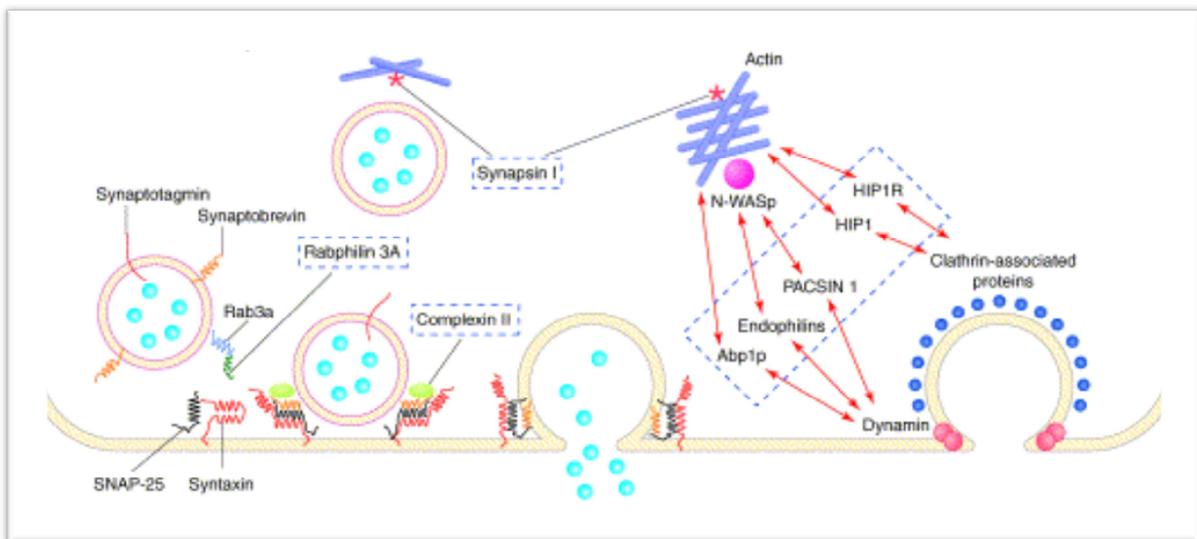


Figure 12: Protein–protein interactions among key factors involved in exocytosis and endocytosis. Red arrows between individual proteins indicate specific binding interactions between those components. Dashed blue boxes indicate protein–protein interactions that potentially could be altered by mutant HTT, thereby disturbing endocytosis and exocytosis (Li et al., 2003).

2.3.6 HTT & mitosis.

The orientation of the mitotic spindle control cell fate choices, tissue architecture, and tissue morphogenesis. The mitotic spindle is an elongated dynamic structure consisting of microtubules. The Dynein/dynactin complex, a microtubule minus end-directed motor, appeared to be a major actor in the regulation of mitotic spindle orientation pathway. Similarly, expressing the p50/dynamitin subunit of dynactin prevents the formation of a functional dynactin complex and subsequently impairs mitotic spindle morphology (Merdes et al., 2000). In support to the observation that HTT mediates transport along microtubules, it was also demonstrated to function during mitosis. HTT localizes at the spindle pole and its absence impairs the proper division axis of the cell (Godin et al., 2010). In this respect, HTT plays a role during cell fate determination, a necessary step during embryonic and adult neurogenesis (unpublished data).

2.3.7 HTT & organelles network maintenance.

HTT is found on Golgi apparatus (Borrell-Pages et al., 2006a; del Toro et al., 2009). HTT shows discrete punctate, perinuclear localization overlapping largely with the trans-Golgi and cytoplasmic clathrin-coated vesicles, implicating HTT in vesicle trafficking (Strehlow et al., 2007). Absence of HTT in cell lead to the disruption of the Golgi network via a mechanism implicating dynein/dynactin-dependent vesicle transport. HTT binds to dynein and acts in a complex along with dynactin and HIP-1 to facilitate vesicular transport and maintain the Golgi apparatus (Caviston et al., 2007; Pardo et al., 2010).

The endoplasmic reticulum (ER) is composed of sheets and tubules spreading all over the cytoplasm. Several proteins are implicated in the well maintenance of the ER shaping as this compartment is constantly under formation with a main mechanism based on fusion/fission reactions of ER forming membranes (see chapter 2). The first 18 amino acids of the HTT forms an amphipathic alpha-helical membrane-binding domain which is actively targeted to ER. In the absence of this domain HTT translocates to the nucleus through an active nuclear import of the region within the 81-588 amino acids. Also, after induction of the ER stress pathway, HTT 1-18 delocalizes to the nucleus (Atwal et al., 2007). This functional domain of HTT ties it to the ER. Recently it was shown that the N17 HTT and its phosphorylation modulate the localization of HTT between the cytoplasm, nucleus and primary cilium (Maiuri et al., 2013). Moreover, depleting HTT using Si-RNA leads to an aberrant configuration of the ER network (Omi et al., 2005) and striatal cell lines deriving from hdhQ111 HD mouse model exhibit aberrant ER network with an enlarged ER compartment (Trettel et al., 2000).

Furthermore, HTT is implied in the maintenance of mitochondria network in the cell (figure 13). Mutant HTT triggered mitochondrial fragmentation in several model of HD before the presence of neurological deficits. It was discovered that mutant HTT abnormally interacts with dynamin-related protein-1 (DRP1), a GTPase, entailed in the mitochondrial fission events (Barsoum et al., 2006), this enhancing its enzymatic activity. Subsequently, mitochondrial fission-fusion balance was lost and thereby could contribute to neuronal injury observed in HD (Song et al., 2003).

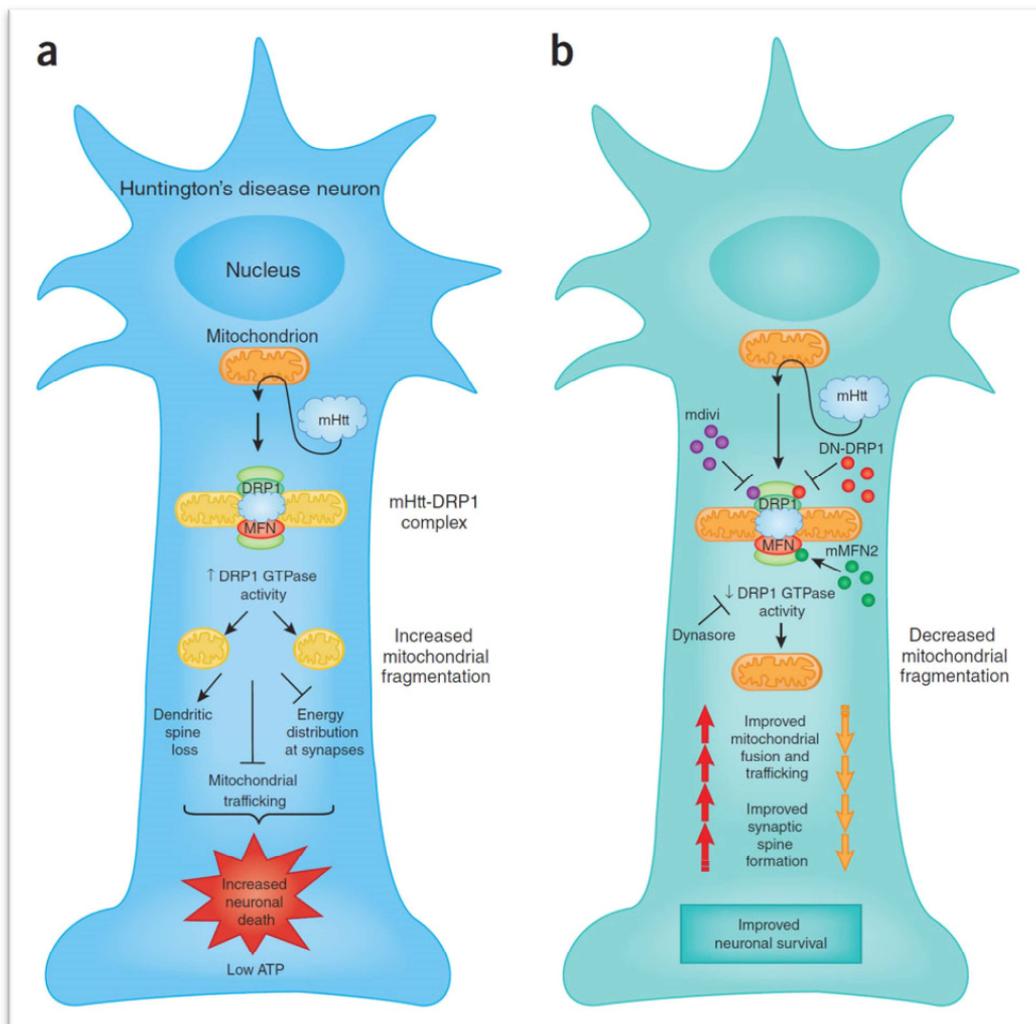


Figure 13: **(a)** Mutant HTT (mHtt) abnormally binds the mitochondrial fission factor DRP1, resulting in increased GTPase enzymatic activity, mitochondrial fragmentation, defective transport of mitochondria to synapses, synaptic spine loss and consequent neuronal cell death. **(b)** Co-expression of dominant-negative DRP1 (DN-DRP1, with defective GTPase activity) to prevent fission and constitutively active mutant MFN2 (mMFN2) to stimulate fusion, increased transport of mitochondria to and from synapses, overall mitochondrial motility and mean velocity, improved synaptic spine formation and decreased neuronal cell death (Johri et al., 2011).

2.3.8 HTT & signaling.

HTT modulates cellular metabolism and interferes with signaling pathways essential for cell survival. As a matter of fact, it was described that HTT interacts with membranes through specific phospholipid associations. Mutant HTT associated more with certain phospholipids not recognized by wild-type HTT such as the phosphatidylethanolamine and phosphatidylinositol (3,4,5)-triphosphate. Therefore, mutant HTT may disrupt membrane trafficking and signaling at membranes (Kegel et al., 2009).

The type 1 inositol (1,4,5)-triphosphate receptor (InsP3R1) is an intracellular calcium release channel that plays an important role in neuronal function. It is showed that InsP3R1-HAP1A-HTT associate into a ternary complex *in vitro* and *in vivo*. Mutated HTT sensitized InsP3R1 activation by InsP3 and induced abnormal calcium release from the endoplasmic reticulum in neurons. This identified a molecular mechanism in which HTT regulates calcium homeostasis and provides an explanation for the imbalance of cytosolic calcium signaling in HD patients and HD mouse models (Tang et al., 2003).

HTT interacts with CIP-4 (Cdc42-interacting protein 4) and PAK1, two proteins involved in the Rac/Cdc42/PAK pathway which regulates many key cellular processes affected during tumor development and metastasis. CIP4 is involved in cytoskeletal organization and was described to interact *in vitro* with HTT and co-localized to ubiquitin-positive aggregates in the striatum of HD patients (Holbert et al., 2003). The overexpression of CIP4 induced the death of striatal neurons suggesting that CIP4 accumulation is toxic and may have a role in HD pathogenesis. PAK proteins, a family of serine/threonine p21-activated kinases, serve as targets for the small GTP binding proteins Cdc42 and Rac and have been implicated in a wide range of biological activities such as cell motility and morphology. PAK1 binds to HTT *in vivo* and *in vitro* and co-localized with mutant HTT-aggregates in cell models and in human HD brains. While PAK1 overexpression enhanced HTT toxicity in neurons, it also stimulated aggregates formation. This advocates a mechanism by which PAK1 might regulate mutant HTT oligomerization (Luo et al., 2008). The mechanism by which mutated HTT disturbs the proper signaling of these pathways is still unknown. Others HTT interacting proteins important in cellular signaling, such as calmodulin and Ras, were determined to interact preferentially with mutated HTT, which consequences still unresolved (Bao et al., 1996; Liu et al., 1997).

HTT might modulate Rac1 signaling and actin dynamics in dendrites via its interactions with HAP1, PSD-95 and CIP-4. Rac1 signaling plays an important role in the remodeling of the actin cytoskeleton by rearranging dendritic spine morphology. A constitutively active Rac1 mutant caused a sharp increase in dendritic protrusions. Protein complexes containing HTT could regulate Rac1

activity supporting the assumption that HTT might participate in adjusting dendritic spine morphology (Colomer et al., 1997; Nakayama et al., 2000).

In conclusion, HTT, through its wide panel of interactors, manages several functions in the cell. It is a scaffold protein important for the proper function of different complexes within the cell. The mutation is dominant and profoundly changes the normal functions of HTT. Polyglutamine expansion can also modify HTT's conformation and structure which can confer to HTT novel toxic properties such as abnormal interactions with nuclear or cytoplasmic protein or exacerbation of the degradation machinery of the cell. This aspect will be discussed hereafter.

3. Mutated Huntingtin: polyglutamine expansion.

Polyglutamine expansion within the N-terminal part of the HTT confers toxic properties for the protein but do not abolishes the entire functionality of HTT. Indeed, the knock-out of HTT is lethal while HD patients develop the disease at late onset during adult hood (see above). Moreover, one mutated allele is enough to develop HD, the rare case of patients carrying both mutated HTT do not show a more severe symptoms. By analyzing others known human genetic disorders with polyglutamine repeats, such as spinocerebellar ataxia or spinobulbar muscular atrophy (SBMA), none of them are haplo-insufficient. Also, the depletion of the androgen receptor, which when contains polyglutamine causes SBMA, does not lead to the neuromuscular defects observed in SBMA patients.

3.1 Formation of aggregates.

A hallmark of HD and others polyglutamine disorders, is the formation of neuronal intranuclear inclusions (Margolis and Ross, 2001).

Aggregate formation is linked to the structure of the polyglutamine stretch and the size of the repeat as well as it depends on its concentration. Aggregation starts by forming dimers rather than oligomers and the higher the polyglutamine repeats are, the faster this process is. This fact that the propensity to aggregate correlated well with the size of the CAG repeats and the age at onset led to the postulate that aggregates are the toxic species in HD, with aggregation accelerating the disease when they are more prone to be formed (Walker, 2007).

The tertiary conformation of HTT is changed by the presence of an abnormal range of polyglutamine. Two mechanisms of aggregation are proposed, through hydrogen bonds and transglutaminase action. Indeed, molecular modelling and X-ray diffraction analysis of synthetic polyglutamine reveals that it is organized in beta-sheets structure which are tightly linked together through hydrogen bonds (Perutz, 1994) (Ross and Poirier, 2004) (figure 14). Another proposed mechanism for aggregates formation was the observation of the transglutaminase, enzymes that catalyze the formation of covalent bond between a free amine groups and the gamma-carboxamide group of glutamines. In HTT protein, the higher the number of polyglutamine, the more it forms polymers and this could be due to the enzymatic cross linking of transglutaminase (Kahlem et al., 1998). In accordance, transglutaminase was shown to be over activated in HD (Karpuj et al., 1999). However, subsequent studies showed that knock-Out mice for transglutaminase-2 when crossed with R6/2 mice prolong their lifespan without affecting aggregates formation suggesting a mechanism independent of polyglutamine cross-linking (Bailey and Johnson, 2005). Similarly, treating mice with cystamine, an inhibitor of transglutaminase, delays the onset of the disease in the R6/2 mice but with no effect on aggregation (Bailey and Johnson, 2006). It was thereafter reported that transglutaminase regulate BDNF vesicles and their release in the striatum. Indeed, cystamine increases BDNF secretion from the Golgi region that is blocked by overexpressing transglutaminase. Another mechanism could involve the anti-oxidant properties of the cysteamine, the FDA-approved reduced form of cystamine, which is also found to be neuroprotective in HD mice by increasing BDNF levels in brain (Bailey and Johnson, 2006; Borrell-Pages et al., 2006a).

Studies have also proposed that the propensity of mutated HTT to aggregate might mediate toxicity in HD as aggregation could sequester other proteins and thus deprive the cell of essential protein activities (Thakur et al., 2004). In support, many proteins have been found to be trapped in the aggregates. For instance, CREB-binding protein (CBP), a transcriptional co-activator that orchestrates nuclear response to a variety of cell signaling cascades, was detected into nuclear inclusions formed by polyglutamine-containing proteins in cultured cells, transgenic mice and tissue from patients with SBMA (McC Campbell et al., 2000). Aggregates formed by the polyglutamine expanded androgen receptor were positively stained for heat shock proteins and mitochondria (Stenoien et al., 1999). However, as it will be discussed after, this theory is no longer supported. In particular, papers reported that inhibition of CBP was aggregate independent (Yu et al., 2002).

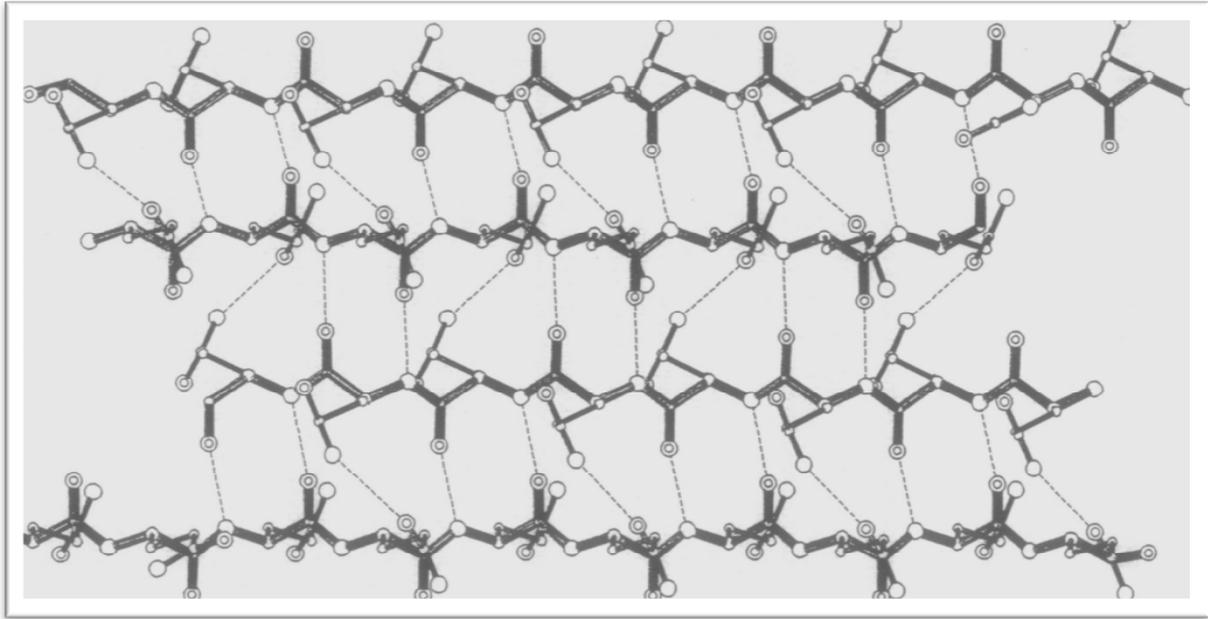


Figure 14: computer generated structure of two paired anti-parallel beta-strands of polyglutamine linked together by hydrogen bonds between the main chain and side chain amides (Bates, 2002).

3.2 Polyglutamine repeats and toxicity.

The formation of intra-nuclear and intra-cytoplasmic inclusions are a hallmark of HD. The early focus on inclusions led to the concept of toxic protein species which are particular forms of aggregated polymeric HTT. The main gain of toxic function of mutated HTT are summarized below. It is to be noted that the gain of toxic function of mutated HTT through its polyglutamine stretch requires flanking region and glutamines themselves are not sufficient (Duennwald et al., 2006).

3.2.1 HTT proteolysis & activation of proteases.

1/ HTT proteolysis: cleavage sites and proteases.

The first evidence for a cleavage of HTT was reported in 1996 through experiments assessing the role of HTT in the apoptotic cell death (Thomas et al., 1995). Apopain, the human homologue of CED-3, a gene required for programmed cell death in *Caenorhabditis elegans* and more commonly termed caspase-3, cleaved efficiently HTT. The cleavage of HTT occurred near a cluster of DXXD sites, the caspase-3 recognition site (Porter et al., 1997) and was mapped to be approximately located between the 1900- 3000 nucleotides. The cleavage was shown to increase with the number of

polyglutamine repeats (Goldberg et al., 1996). The precise location for caspases cleavage sites on HTT were determined in 1998. HTT contains seven predicted caspase cleavage sites. In vitro assays using purified caspases or apoptotic extracts revealed active cleavage of HTT by caspase-3 and caspase-1 within the N-terminal 548 amino acids. These two caspases cleaved less efficiently the C-terminal part of HTT.

Neither caspase-7 nor caspase-8 were able to cleave HTT. Site-directed mutagenesis at putative caspase-3 cleavage sites revealed that caspase-3 cut HTT at position 513, suggesting that caspase-3 is likely to contribute to the generation of N-terminal HTT fragments (Wellington et al., 1998). As two proteolysis products of 67 and 80 kDa were derived from caspase-3 cleavage of the full length HTT with 15 glutamines, screening for other sites were pursued. HTT was found to be cleaved in vitro by caspase-3 at a new cleavage site at position 552. The cleavage site at position 552 is shared between the caspase-3 and caspase-2. Additional experiments confirmed that HTT is processed by other caspases and a caspase-6 cleavage site, at the position 586, was identified (Wellington et al., 2000). The others identified putative caspase sites tested in this study did not appear to be processed by any caspases and are probably not used *in vitro* and *in vivo*.

In support to the toxic fragments hypothesis, inhibiting HTT cleavages by caspases inhibitors decreased death in cells expressing mutant HTT. Also measurement of caspases activity in cells expressing mutant HTT demonstrated that activity of caspases significantly increase with the polyglutamine length. In contrast cell expressing non-cleavable HTT showed a reduced caspases activation and toxicity (Wellington et al., 2000).

Using antibodies developed to detect specifically the N-terminal fragment generated after cleavage, caspase-3-cleaved HTT was detected both in brain lysate from early-grade HD postmortem tissue and young transgenic mice tissue. However this cleavage was occurring similarly in control and in affected tissues raising the possibility of a normal regulation of HTT's catabolism by caspase (Kim et al., 2001; Wellington et al., 2002). The majority of caspase-3 generated-N-terminal fragment was detected in the cytoplasm confirming previous studies that suggests that HTT cleavages occur in the cytoplasm and that the generated N-terminal fragments translocate into the nucleus. Although cleavage occurs in all cases, the distribution of generated N-terminal fragments is not similar. Hodgson and colleagues analyzed the localization of N-terminal fragments of HTT by gross microscopy and immunogold electron microscopy. By light microscopy, N-terminal HTT-containing aggregates were only observed in the nuclei of the medium spiny neurons of mutated YAC72 mice. However, immunogold electron microscopy revealed clustered immunogold particles in the cytoplasm indicative of small aggregates outside the nucleus. In addition it revealed gold labelled-N-terminal HTT traversing the nuclear pore from the cytosol to the nucleus. In contrast, in control YAC18 mice, immunogold particles were also seen in the nucleus and the cytoplasm of medium spiny

neurons but particles were not aggregated (Hodgson et al., 1999). This suggests a shuttling of HTT between nuclear and cytoplasmic compartment of the cell in a normal condition. Another observation indicated that the caspase-generated fragments undergo successive cleavages before accumulating in the nucleus. HTT antibodies raised against the caspase-3-generated fragment of HTT (N-terminal 1-513 amino acids) detected cytoplasmic aggregates in cultured cells expressing mutated HTT but did not detect nuclear inclusions, positive for EM48 staining (an antibody that detects HTT aggregates in HD brain (Li et al., 1999)). This suggests that additional cleavage events may cleave the caspase-3-generated HTT fragment into smaller ones, which afterwards accumulate in the nucleus.

In support, N-terminal caspase-3-generated fragments can be processed by calpain, a calcium activated non-caspase cysteine protease that is enriched in brain and associated mainly with membranes. Biochemical analysis of N-terminal fragments in HD and control brains revealed an immunoreactive band smaller than the caspase-3 HTT fragment. This product of about 65 kDa depends on calpain and was detected in HD brains (Kim et al., 2001). Thus, polyglutamine expansion leads to a differential proteolytic cascade on mutant HTT. This could be partly due to glutamate excitotoxicity occurring in HD and that leads to excessive levels of intracellular calcium (Chen et al., 1999).

Four major cleavage products are generated by calpain cleavage of HTT and predicted cleavage sites were determined based on sequence analysis of HTT and the size of generated fragments. The work of Gafni and Ellerby demonstrated that calpains I and II, the two main isoforms, cleaved HTT (15 polyglutamine) at the position 536-540 amino acid producing an intermediate fragment of 72 kDa, then at positions 465-469 and 437 producing a final cleavage product of 47 kDa (Gafni and Ellerby, 2002). Moreover, calpain cleaves more efficiently the mutated form of HTT than the wild type form. Indeed, *in vitro* cleavage assay, the wild type HTT stayed intact after two hours of incubation of HTT with calpain recombinant proteases whereas a major portion the polyglutamine expanded HTT was processed by calpain (Gafni and Ellerby, 2002). This *in vitro* study correlated well with the observations that calpain expression and HTT processing are increased in the striatum of HD patient's brain when compared to control brains. Thus calpain accessibility to its HTT-cleavage sites is enhanced by the polyglutamine stretch rendering the mutated HTT more susceptible to calpain cleavage (Gafni and Ellerby, 2002). Another site for calpain II was later found in the N-terminal part of HTT between amino acids 63 and 111 that contain a number of hydrophobic amino acids and arginines which tend to be recognized by calpain proteases (Cuerrier et al., 2005). This putative proteolytic site, could generate even smaller fragment that might correspond to the fragment observed in HD patients and animal models (Sun et al., 2002) suggesting a physiological relevance of this putative cleavage site.

Similarly, by dissecting fragments present in cytoplasmic and nuclear aggregates in HD brains and in a cellular model of HD, two distinct mutated fragments of HTT, termed cp-A (cleavage product-A) and cp-B (cleavage product-B) were identified. Antibodies, which epitopes map to amino acids 115-125, reacted to cytoplasmic aggregates but not the nuclear one, suggesting that fragments that form inclusions are different in length. Further biochemical analyses revealed that Cp-A fragment, derived from a cleavage between amino acid 104 and 114, corresponds to the 55 kDa N-terminal HTT fragment prone to form nuclear aggregates.

A second proteolytic event that cleaves between the amino acids 115-125 and 214 generates the cp-B fragment, which is larger than cp-A, and remains in cytoplasmic inclusions. The proteases releasing cp-A and cp-B belong to the family of aspartic endopeptidases as only pepstatin, an inhibitor of aspartic endopeptidases inhibited HTT proteolysis (Lunkes et al., 2002). Pepstatin-sensitive aspartic endopeptidases include the cathepsin D and E. Interestingly, analysis of postmortem HD tissue demonstrated significant increases in the activity cathepsin H and cathepsin D (Mantle et al., 1995). Therefore, the potential role of the cathepsins in the proteolysis of HTT requires further investigation.

Importantly cp-A and cp-B fragments are faintly detectable by western blot under normal condition in cultured cells expressing wild type or mutant HTT. Their accumulation requires the inhibition of proteasome or activation of the upstream proteases. Abnormal proteases activity and proteasomes impairment are conditions reported in HD (Bence et al., 2001; Bennett et al., 2007; Jana et al., 2001), This favors the amplification of cp-A and cp-B fragments with high aggregation potential, leading to the formation of inclusions observed exclusively in affected brain (Lunkes et al., 2002).

Two others cleavages site located close to the cp-A and cp-B cleavages sites have been described. The cp-1 cleavage site is located at position 90-105 and the cp-2 cleavage site localized beyond 115-129 epitope of HTT. These two sites were demonstrated to be distinct from cp-A and cp-B sites as abolishment of amino acids necessary for the production of cp-A and cp-B fragments did not alter the generation of short N-terminal HTT fragments by cp-1 and cp-2 cleavages in PC12 cell model of HD (Ratovitski et al., 2007). Cp-1 and cp-2 cleavage products accumulate in cytoplasmic and nuclear inclusions and their formation is independent of HTT cleavage by caspases. Increased accumulation of these fragments in PC12 cells was observed after inhibition of calpains due to a diminution of their clearance (Ratovitski et al., 2007).The precise site for cp-2 cleavage was determined later by mass spectrometry and located at the arginine 167 of HTT (Ratovitski et al., 2009). Two major candidates, bleomycin hydrolase (BLMH) and cathepsin Z, were found to carry out the cp-2 cleavage. Both enzymes are cysteine proteases of a papain-like structure. Knockdown of

either proteases reduced cp-2 cleavage, whereas their overexpression increased the cp-2 cleavage (Ratovitski et al., 2011).

A recent study revealed HTT as a substrate for metalloproteinase (MMP). Using a high throughput approach, 514 siRNA targeting the repertoire of known human proteases were screened. Eleven proteases were sorted out to be important in HTT proteolysis including proteases some belonging to the MMP family. Both wild type and mutant full length HTT are preferred substrates for MMP-10, which proteolytic sites resides at the amino acid 402 (Miller et al., 2010) (figure 27).

In summary, HTT undergoes series of proteolytic events ranging from caspases cleavages (figure 15), generating the largest N-terminal HTT fragment; calpains cleavages with intermediate generated N-terminal fragments and unknown proteases cleavages generating the smallest N-terminal HTT fragments forming nuclear inclusions. HTT fragments deriving from Cp-A/cp-B and cp-1/cp-A are likely to be unstable in the cells as they go through others potential proteolytic actions facilitating their complete breakdown by the proteasome machinery. Alteration in HD, such as unspecific proteases activation and impairment in the misfolded response system favor the accumulation of those fragments participating in the formation of aggregates in HD (DiFiglia et al., 1997; Wu et al., 2004).

In HD, proteolysis of HTT is accelerated as a consequence of activation of proteases (Gafni and Ellerby, 2002; Mantle et al., 1995; Miller et al., 2010). Most studies on HTT proteolysis focused on the generation of N-terminal fragments as they are the fragments containing the polyglutamine stretch. Only one study has investigated cleavage of HTT in its C-terminal part (Mende-Mueller et al., 2001) suggesting that additional cleavage sites might be found on HTT.

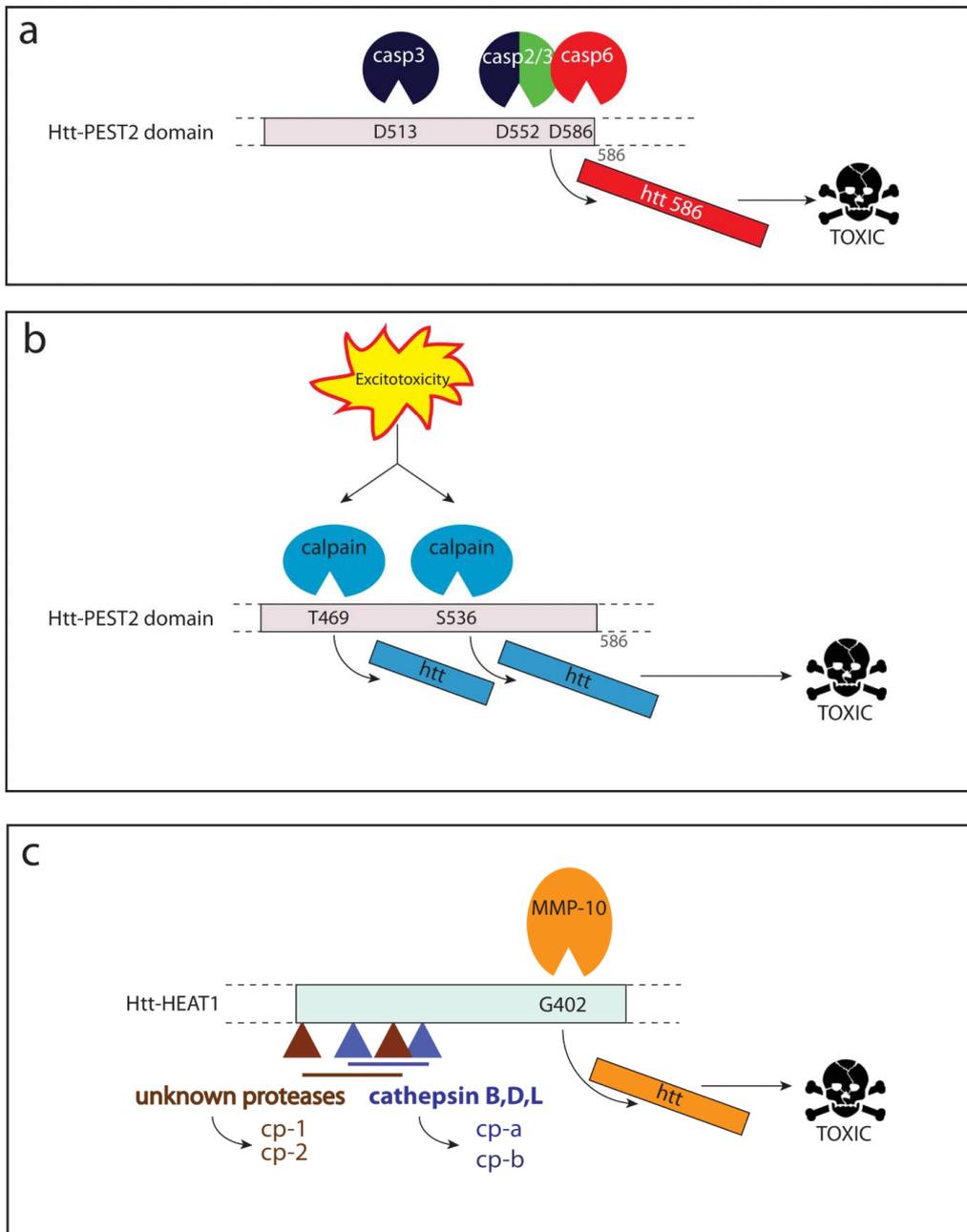


Figure 15: HTT is cleaved by a number of (a) caspases (b) calpains and (c) matrix-metalloprotease-10 (MMP-10), cathepsins and other unidentified proteases generating truncated HTT fragments (Ehrnhoefer et al., 2011).

2/ HTT proteolysis and toxicity.

Expressing different N-terminal fragment in primary culture of rat neurons showed that the smaller the N-terminal fragments are, the more they are toxic. Constructs expressing successively smaller N-terminal fragments of HTT were used to study the influence of HTT size on its localization and toxicity in cells. Smaller N-terminal HTT containing 128 glutamines formed both intranuclear and perinuclear aggregates. In contrast, larger fragments formed exclusively perinuclear aggregates. Furthermore, expression of mutant HTT resulted in increased susceptibility to apoptotic stress which was greater with smaller HTT fragments and increased polyglutamine stretch (Hackam et al., 1998). Similar findings were obtained in primary cultures of striatal neurons although there was no correlation with aggregation (Saudou et al., 1998). Short mutated-N-terminal HTT fragments associate preferentially with synaptic vesicles and may contribute to excitotoxicity occurring in HD (Kim et al., 2001; Li et al., 2000).

In support for a crucial role of proteolysis generating smaller and more toxic N-terminal fragments, several evidence points at a protective effect of the inhibition of HTT proteolysis.

Indeed, cells expressing a cleavable form of full length HTT showed a significantly higher level of caspases activation that was enhanced by the polyglutamine stretch length. In parallel, cleavable HTT induces a higher rate of cell death compared to the non-cleavable form of HTT. Also, inhibition of HTT proteolysis by general inhibitors of caspases reduces overall toxicity, suggesting that caspase contributes to the toxic events occurring in HD (Wellington et al., 2000). Moreover, mutations of two calpain-cleavage sites in HTT rendered the mutated HTT less susceptible to proteolysis and aggregation, ensuing in decreased toxicity in a cell culture model. Calpain family members, calpain-1, -5, -7, and -10 are upregulated in HD tissue culture and transgenic mouse models, suggesting that they may play a key role in HTT proteolysis and disease.

Similarly, the reduction in MMP activities improved the neuronal dysfunction occurring in a *Drosophila* model of HD. Flies genome contains only two MMP family members, Dm1-MMP and Dm2-MMP, where only Dm2-MMP is expressed in adult brain (Llano et al., 2000). A genetic reduction of Dm2-MMP lead to an amelioration of motor performance and retinal integrity of flies expressing an N-terminal HTT with 128 glutamines repeats. This suggests a role for MMP-induced-HTT proteolysis in HD pathogenesis (Miller et al., 2010).

The group of Hayden engineered YAC transgenic mice expressing the caspase-3 and caspase-6-resistant mutant HTT (Graham et al., 2006). The mouse resistant to cleavage at position 586 did not develop striatal neurodegeneration; in contrast to control YAC128 mouse or the mouse resistant to the cleavage at caspase-3 site which both exhibited selective neuronal loss and brain atrophy reproducing HD pathogenesis (Slow et al., 2003). This protection was effective even if mice resistant for caspase-6 cleavage still produce the caspase-3 fragment of mutant HTT (Graham et al., 2006). Hence, the cleavage of the HTT at position 586 appears to be essential in HD pathogenesis. This site, first identified as a caspase-6 site, may not require caspase-6 itself *in vivo* as the proteolysis of mutant HTT at this site still occurred in Hdh150 knock-in mice model of HD crossed with caspase-6 knock-Out mice (Landles et al., 2012). It remains unknown why these two very close cleavage sites of HTT have so distinct effect on mutant HTT toxicity.

In Summary, N-terminal fragments of HTT, indicative of HTT proteolysis, have been found in brain samples from HD patients, early in the onset of the disease; in HD mouse models expressing a mutated full-length HTT as well as in cell culture model.

Away from this view, some studies indicated that the polyglutamine stretch confers to HTT a resistance for proteolysis. Cynthia McMurray's team noted a prevalence of a non-cleaved mutant HTT in human brains, transgenic mice and cells. More surprisingly, they observed that the small N-terminal cleavage products detected in HD samples arose from the wild type and not the mutant HTT and that they are associated with the insoluble full length mutant HTT. The model proposed that proteolysis of wild type HTT is ensuring the normal turnover of the protein leading to survival. When HTT is mutated, the turnover is blocked, thus mutant HTT accumulate in aggregates and sequester essential proteins for the cell. This induces energy deficit and caspases activation contributing to the disease progression (Dyer and McMurray, 2001). One concern in this study is that HTT fragments may be unstable and consequently difficult to detect in certain approaches such as gel filtration and immunoblotting used in this study. Another possibility is that mutant fragments are less detectable either because mutant cells die faster or because these fragments, in contrast to wild type fragment, are processed faster.

Similar biochemical analysis, using native gel techniques, analyzed HTT in human HD samples and HD mice model. The predominant found form in wild type and mutant condition was the full length HTT. In mutant samples, only one N-terminal HTT band of 180 kDa was detected in these conditions, composed of 43-50 kDa fragments (Sapp et al., 2012). This is consistent with previous results showing that the major fragment identified in nuclear inclusions is about 55kDa (Lunkes et al., 2002). Nevertheless, the 180 kDa fragment was detected at a low levels compared to full length HTT.

Interestingly, mutant HTT was found to be less stable and thus more prone to release its N-terminal domain by an unknown process (Sapp et al., 2012).

Identification and characterization of HTT proteolysis is of a major interest for a better understanding of HD pathogenesis. Efforts should be provided in order to identify of new proteases cleaving HTT, to track HTT processing *in vivo* and to assess the fate of each fragments. As HTT plays a role in several cellular functions by interacting with numerous proteins in the cell (Imarisio et al., 2008), this requires an intact HTT, whereas proteolysis of mutant HTT has a major impact on HTT structure, localization and function (Kahlem et al., 1998; Karpuj et al., 1999; Lunkes and Mandel, 1998; Perutz, 1994; Sun et al., 2002). As it will be demonstrated in the next chapter of my thesis, my researches point at proteolysis of HTT as a modulator of a newly established role of HTT on ER-dynamin1 activity.

3.2.2 Polyglutamine expansion & ubiquitin proteasome system.

Many evidence links misfolded proteins and neurodegeneration in HD. Protein homeostasis involves a network of the molecular chaperones system, the ubiquitin proteasome pathway, and the autophagic protein clearance. Through this interconnected network, all the cells sense misfolded protein and either try to repair them or to send them to degradation.

Heat shock proteins are chaperones acting in the refolding of misfolded proteins. In *Caenorhabditis elegans*, it was demonstrated that the presence of polyglutamine stretch impaired the correct folding and functioning of other unrelated proteins in the cells. These results show that protein with a slightly abnormal range of glutamines access in priority to the chaperones system, leaving no place for other misfolded proteins that require correction (Gidalevitz et al., 2006). The working load of the chaperones could be exceeded by polyglutamine presence leading to a global disruption of the cellular protein folding control. In agreement with this idea, it was shown that the two chaperones, HSP40 and HSP70 are sequestered by the polyglutamine inclusions (Wytttenbach et al., 2000).

When misfolded proteins are unable to be properly folded, they are eliminated. One known pathway is the Ubiquitin proteasome system (UPS). Polyubiquitin chains are catalyzed on the proteins, making the signal for the proteasome to come and to target them to degradation (Hershko and Ciechanover, 1998). It is well proven that polyglutamine intranuclear inclusions are ubiquitinated

in HD brain, even prior to the development of neurologic phenotype (Paulson et al., 1997) (Davies et al., 1997). It is proposed that components of the ubiquitin proteasome system are also impaired and trapped by ubiquitinated aggregates (Stenoien et al., 1999). Normal N-terminal HTT did not alter the cellular distribution of the 20S proteasome component, at the opposite, in cells expressing N-terminal HTT with 60 or 150 glutamines, the proteasome were redistributed to the formed aggregates. By doing so, the proteasome was able to degrade polyglutamine-formed-aggregates and thus limits their propagation in the cell. Nevertheless, proteasome degradation capacity is inversely proportional to the length of the polyglutamine stretch.

It was demonstrated that the general activity of the proteasome is diminished by the presence of expanded HTT leading to an activation of apoptotic pathway and triggers cell death (Jana et al., 2001). Consistently, the dysfunction of the ubiquitin proteasome system was suggested to be a feature of HD. Using a mass spectrometry approach on samples issued from HD brain an HD mouse model, the ubiquitin proteasome system function was assessed. Experiments demonstrate that Lys-48 polyubiquitin chains accumulates early in the pathogenesis highlighting a defect in their elimination by the UPS (Bennett et al., 2007).

As a consequence, in the presence of polyglutamine aggregates, neurons may progressively have an altered efficient degradation system.

Other studies proposed that the UPS activity is unchanged or even increased in response to mutant HTT expression. The activation of the system could be a response to the presence of a misfolded polyglutamine protein in the cell. When mutant HTT expression is turned off, aggregates can be cleared by the neurons after inhibiting the expression of the transgene by tetracycline analogues (Yamamoto et al., 2000). This highlights that the repair degradation system of the neurons still operational.

Finally, a very elegant study was conducted in a mouse model for the spinocerebellar ataxia 7, a polyglutamine neurodegenerative disease. Using a fluorescent-tagged ubiquitin reporter, they observed an increase of the ubiquitin level in susceptible neurons which was correlated with an increase of the mRNA level of the reporter and a normal activity of the UPS (quantified by *in vitro* assay) (Bowman et al., 2005).

3.2.3 Cellular degradation machinery cope aggregates.

Although polyglutamine inclusions presence correlated with the onset and severity of symptoms in HD, they were fairly associated as pathogenic species. However this direct causal link to disease broke apart in 1998 when Saudou and colleagues show that overexpressing a dominant negative form of a ubiquitin conjugating enzyme that blocks mutant HTT degradation, reduced aggregates formation but enhanced cell death (Saudou et al., 1998). In support, the same year, another group demonstrated a similar effect in spinocerebellar ataxia1 (SCA1) mouse model. When the SCA1 protein was engineered to dimerize less and is therefore less prone to form polymeric aggregates, the pathology worsened. Both works showed the importance of nuclear translocation of a truncated form of the mutated protein to develop the neuropathology. The presence of intranuclear inclusions was not required and even predicted to be protective (Klement et al., 1998). Indeed, upon these findings, single cell studies have shown that neurons containing the highest numbers of inclusions survive better and the HTT monomers are the toxic form. Therefore, the level of diffused mutated HTT is rather the predictor of cell death (Arrasate et al., 2004).

Why toxicity and inclusions formation correlate in some cases and not in other cases? S. Finkbeiner proposed an interesting model. Incorporating misfolded proteins into aggregates reduces their toxicity. An experiment which increase the load of misfolded protein is going to increase cell death as it exhausts the homeostatic response. A reaction which favors the formation of inclusions reduces the exposure of misfolded proteins and this will reduce cell death (Finkbeiner, 2011).

In summary, molecular chaperones might block toxicity by blocking inappropriate protein interactions and by facilitating disease protein elimination especially through the UPS. Interestingly, the chaperone HSP70 has a rapid kinetics of association/dissociation from mutant HTT (Chai et al., 2002; Kim et al., 2002). They can also block downstream signalings which trigger apoptosis and cell death. In stress condition, cells activate a program known as heat shock protein response (HSPs), crucial for recovery. Mutation in HSPs causes several human autosomal dominant genetic diseases (Hansen et al., 2002) (figure 16).

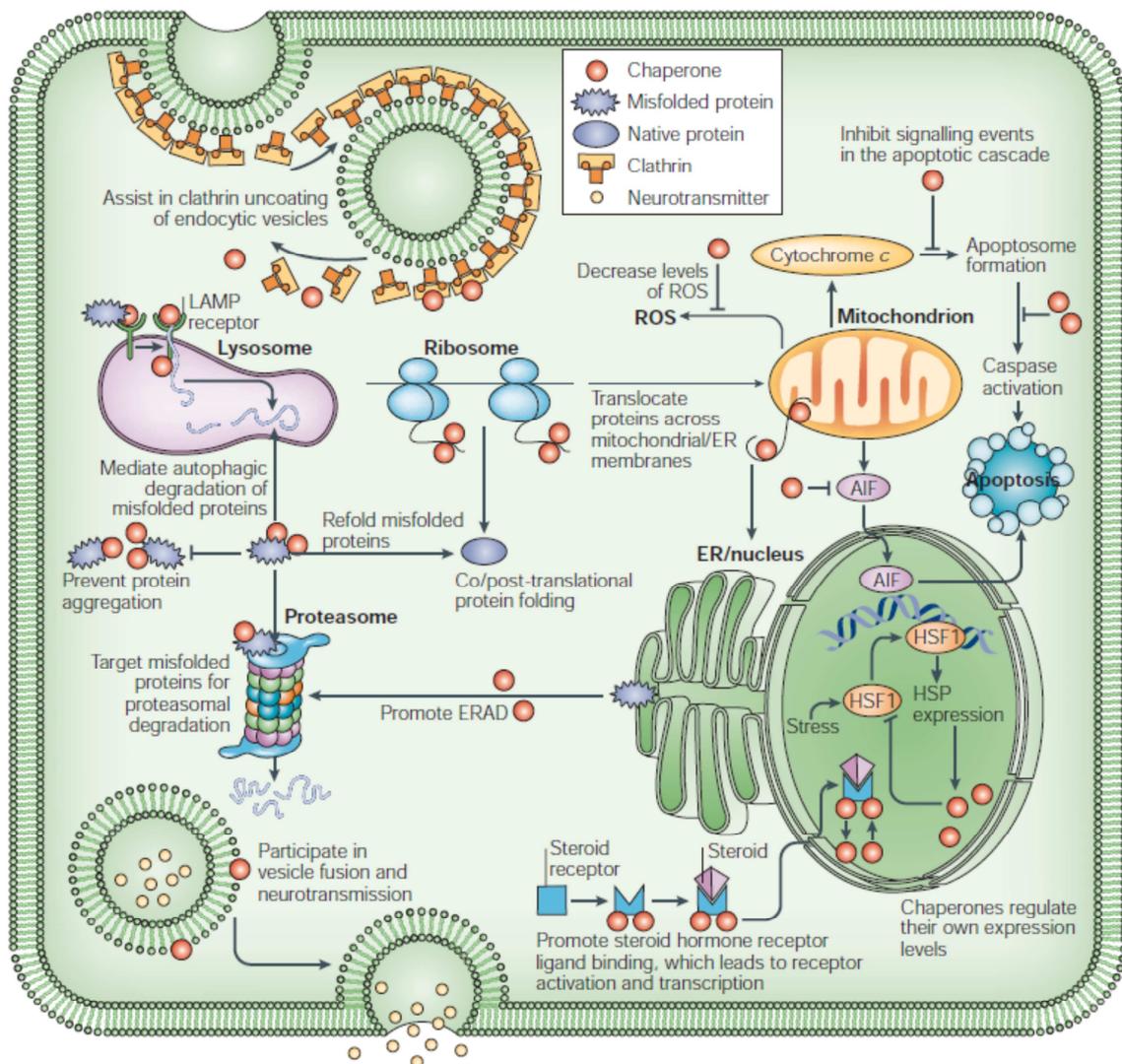


Figure 16: Molecular chaperones regulates several important cellular processes. They facilitate protein folding and prevent protein aggregation. However molecular chaperones regulates other processes such as autophagy, signal transduction, vesicle fusion, apoptosis and proteasomal degradation. AIF: apoptosis inducing factor, ER: endoplasmic reticulum, ERAD: endoplasmic reticulum associated degradation, HSP1; heat shock transcription factor1, LAMP: lysosomal associated membrane protein, ROS: reactive oxygen species. (Muchowski and Wacker, 2005)

3.3 Animal models of HD.

Several in vivo models of HD have been generated. Each created model is unique as they differ by their genetics background, the number of CAG repeats, the size of the mutated HTT protein and the promoter driving the expression of the transgene.

The earliest animal models of HD were developed in 1970 based on the selective vulnerability of striatal neurons to excitotoxic aminoacids. Kainic acid is neuroexcitotoxic and epileptogenic, acting through specific kainate receptors involved in the excitatory neurotransmission.

Kainic acid receptors are non-NMDA ionotropic receptors which respond to glutamate. When administered to mice, Kainic acid induces the appearance of striatal lesions, modelling HD pathology (Coyle and Schwarcz, 1976). Striatal neurons are enriched in glutamate receptors because the cortico-striatal pathway uses this excitatory amino acid as the primary neurotransmitter.

Other chemically-induced models of HD were obtained by intrastriatal injection of malonate, a reversible inhibitor of the succinate dehydrogenase (SDH) or by injection of the mitochondrial toxin 3NP (3-nitropropionate), an irreversible inhibitor of SDH (Beal et al., 1993). The degeneration defect produced by the malonate was slighter than those induced by 3NP. Among defects, analysis showed an increase in lactate concentration in the striatum, indicative of a reduced activity of the respiratory chain. Interestingly, lesions were similar to those observed in HD patients, as 3-NP-induced neuronal death was characterized by the loss of the medium-sized spiny neurons while aspiny neurons are kept intact (Beal et al., 1993).

After the discovery of the gene causing HD, a number of genetically modified mice have been generated in order to model HD pathogenesis. Initially, the first attempt was to produce a yeast artificial chromosome of the mutant gene of human HTT with the goal of investigating in mouse genetic instability of the CAG repeat. Due to its instability, transgenic lines were established using the upstream promoter regions of the human genomic HTT and the exon 1 carrying around 130 CAG repeats coding about 3% of the human HTT. Those transgenic mice were named R6, four lines were established and three of them developed a neuropathology: R6/1, R6/2 and R6/5. In these lines, symptoms appear very early during life hood (3 to 6 month old). Polyglutamine repeats ranged from 115 to 150 CAG, which is a size generally associated with extremely juvenile form of HD (Mangiarini et al., 1996).

The R6 mice have been extensively studied along the last decade. First, the major benefit of this model is the demonstration that the mutated exon 1 fragment was sufficient to produce several main features of HD. These include deficits of motor co-ordination, altered locomotor activity, impaired cognitive performance and seizures (Murphy et al., 2000). R6 mice also exhibit pathological changes in skeletal and cardiac muscles and develop diabetes (Hurlbert et al., 1999) (Bjorkqvist et al., 2005). At this stage, there is significant atrophy of the striatum and neocortex and widespread occurrence of neuronal and non-neuronal intranuclear inclusions of mutant HTT (Davies et al., 1997) (Sathasivam et al., 1999). The limits of such model consist in the fact that only 3% of the HTT is studied and that, surprisingly, little evidence for neuronal death have been demonstrated. The mice die at week 12–14 for reasons which remain to be clarified. Also R6/1 and R6/2 mice are resistant to the NMDA receptor agonist quinolinic acid (QA). This correlates with an increase in the intracellular

calcium levels (Hansson et al., 2001). This could be explained by the adaptation of their neurons to excitotoxic stress such as QA administration. Such features make the R6 model a model of polyglutamine disease rather than a faithful model of HD.

As R6 mice express a large number of CAG, not typical of adult form of HD, Von Horsten and colleagues created a transgenic rat expressing 51 CAG repeats inserted in the rat HTT fragment of complementary DNA (cDNA). Expression of this gene is under control of the endogenous rat promoter. The relatively smaller number of CAG repeats results in an adult-onset progressive phenotype. They exhibit a progressive phenotype with cognitive and motor deteriorations starting at 40 to 50 weeks with no premature death. Rats, compared to mice, are more suitable for repetitive in vivo imaging (such as PET and MRI), which is a technique allowing to compare directly the pathological alterations of the human condition with the corresponding animal model in longitudinal studies (Jacobs and Cherry, 2001; von Horsten et al., 2003). However, this model remains limited as it uses exon1 and many of the pathogenic processes occurring in HD will be lacking.

N171-82Q transgenic mice created by Borchelt and colleagues express the first 171 amino acids of the human HTT protein bearing 82 CAG repeats (Schilling et al., 1999). Expression of the transgene is driven by the mouse prion promoter and is restricted to the central nervous system. Behavioral and anatomical symptoms in this model develop around 15 weeks similarly to R6 mice. At the opposite of the R6 mice, N171-82Q mice exhibit apoptotic neurons highlighting a neurodegeneration occurring in this model (Yu et al., 2003).

Among HD model generated so far, mice expressing N-terminal mutated HTT have the most severe behavioral disturbances and exhibit abbreviated life span. Mice expressing full length version of mutant HTT have less severe phenotype and later onset of symptoms with normal life span, better reproducing the adult hood manifestations of HD in patients. Studies using N-terminal models have provided many important insights into HD, however it is now clear that protein context is important for pathogenesis. This is best illustrated by a transgenic mouse expressing 120 CAG repeats in the context of exons 1 and 2 of HTT, which shows no neuronal dysfunction or degeneration in spite of abundant neuronal inclusions (Slow et al., 2005).

The first full length mouse was created in 1998 with the full length cDNA under the CMV promoter. Mice with 89 repeats display a neuronal loss in the striatum and hippocampus along with DNA fragmentation (TUNEL assay) highlighting apoptotic events. No defects was detected in age-matched HD16 strain. Neuronal inclusions in less than 1% of striatal neurons and in other brain regions (Reddy et al., 1998). However the strain was lost and no other studies have been published.

This may be due to the tendency of CMV promoters to be hyper-methylated in mouse as a protective response to viral infection.

As a strategy to create a model that expressed full length form of HTT under the control of the endogenous HTT promoter and regulatory elements, Hayden and colleagues used the yeast artificial chromosome (YAC) system as it fulfills the requirements of spanning the entire genomic region of the human HD gene, including promoter, intronic, upstream and downstream regulatory elements. Human wild type and mutant HTT under the influence of its native promoter, despite differences to the murine protein, are functional in a murine background and can compensate for loss of the murine protein in the homozygous embryonic lethal mice (Hodgson et al., 1996).

Thus, the development of YAC transgenic mice involved cloning an artificial yeast vector that contains full length HTT into the mouse genome (Hodgson et al., 1999). YAC HD models contain either 72 or 128 CAG repeats. The YAC 72 gave two concerns: the presence of intra-variability and the slow manifestation of the pathology and is no longer used. The YAC 128 lines show a decrease in the number of neurons, preferentially in the lateral striatum (Hodgson et al., 1999) (Slow et al., 2003). They exhibit behavioral defects, loss in the body weight and increase in HTT nuclear staining starting at 12 weeks of ages. No nuclear inclusions were detected in this model thus bringing the demonstration that neurodegeneration can be present in the absence of HTT forming inclusions. Interestingly, as YAC128 mice live much longer than their R6 and N171-82Q transgenic counterparts, this is an attractive model for long- term therapeutic studies.

A bacterial artificial chromosome (BAC) transgenic mouse model (BACHD) was developed expressing full length HTT with 97 glutamine repeats under the control of endogenous HTT regulatory machinery on the BAC. The originality of this model is the engineering of a mutant HTT containing a polyglutamine stretch composed of CAA-CAG repeat from the reiteration of the CAA CAG CAG CAA CAG CAA sequence, which encodes a pure polyglutamine stretch (Kennedy and Shelbourne, 2000). This strategy allowed to test whether somatic repeat instability is necessary for the selective neuropathology in HD. These mice do not exhibit any repeat expansion or retraction but still exhibit a selective brain atrophy similar to that of the HD patients. BACHD mice exhibit progressive motor deficits, neuronal synaptic dysfunction, and late cortical and striatal atrophy (32% reduced volume) with striatal dark neuron degeneration at 12 months of age. BACHD model mice display low abundance of aggregation in the striatum and lack striatal nuclear translocation of mutant HTT. This reveals that the slowly progressive and selective pathogenic process in BACHD brains can occur without early and diffuse nuclear accumulation of aggregated mutant HTT (Gray et al., 2008).

Beyond transgenic models, scientist engineered the genome of the mouse to replace the first part of the endogenous HTT gene with the corresponding wild type or mutated part of Human HTT gene. Those mice are referred as Knock-In model. Initial studies reported mild functional changes and the lack of phenotypic abnormalities suggesting a pre-symptomatic state (Shelbourne et al., 1999). In the HdhQ111 Knock-In mouse model, the earliest nuclear accumulation phenotype involves the full-length mutant HTT, rather than the N-terminal fragment (Wheeler et al., 2000). Indeed, a nuclear localization of the full-length protein is predominant in medium spiny neurons, and subsequent formation of N-terminal inclusions and insoluble aggregate occur. These mice also show an anxiodepressive like phenotype at 13 weeks of age which is relevant to the anxiety and depression manifestations observed at high frequency in HD patients (Duff et al., 2007; Orvoen et al., 2012). Moreover, primary and immortalized striatal cultured neurons from HdhQ111 mice are a valuable tool for the study of mutant HTT in vitro as they reproduced defect in cellular transport and indicate a disruption of striatal cell homeostasis by the mutant protein (Pineda et al., 2009; Trettel et al., 2000).

The CAG140 knock-In mouse, which expresses 140 CAG repeats, displays an increased locomotors activity and rearing at 1 month of age, followed by hypoactivity at 4 months and walk anomalies at 1 year. Behavioral symptoms preceded neuropathological anomalies (Menalled et al., 2003). Neuronal cell loss has not yet been quantified in these mice. Perhaps the most promising of the knock-in mice is the CAG150 mouse model (Lin et al., 2001). These mice show progressive motor defects starting at 4 months. They exhibit loss in weight and mood problem after 25 weeks of age. Brain analysis show a relative preservation of the cell number but gliosis was observed. Importantly, at 14 months, the striatum shows an increase staining of the GFAP (glial fibrillary acidic protein) in mutant mice, which is an early marker of neuronal damage in HD (Hedreen and Folstein, 1995) (Yu et al., 2003). Hence, a more severe pathology is seen with the CAG150 knock-In relative to the trivial pathology seen with the CAG140 knock-In mice. This may be due to the two different strategies and/or the inserted sequences used to generate these knock-in mice.

The fly is an excellent choice for modeling neurodegenerative diseases because it contains a fully functional nervous system where olfaction, vision and learning and memory can be easily assesses. Also, foreign genes are engineered to be expressed in a tissue-specific and temporally regulated patterns. Fly is also a very good system that allows for genetic screens and rapid experimentation using large numbers of animals. Therefore, HD has also been modeled in *Drosophila*.

Flies expressing mutant human genes or polyglutamine peptides alone mimic the human disease in important ways. For example: the pathology is function of the polyglutamine length, it is progressive, it leads to nerves retina degeneration when expressed in the eye, leads to motor dysfunction when expressed in the nervous system and eventually causes early cell death (figure11). Similar to mouse model, transgenic flies expressing the N-terminal part of mutant HTT show photoreceptor degeneration and a shorten life span (Jackson et al., 1998). Transgenic *Drosophila* that express the first 548 amino acids of the human HTT gene with the pathogenic polyglutamine tract of 128 repeats show a defect in axonal transport (Lee et al., 2004). Also the effect of mutant HTT on transcriptional deregulation is studied in HTT exon1 Q94 transgenic flies and Inhibitors of HDAC were identified as chemical compounds that may ameliorate HTT-induced toxicity (Steffan et al., 2001).

A fly model expressing full length HTT with 128 polyglutamine was also characterized. The expanded full-length HTT led to behavioral, neurodegenerative, and electrophysiological phenotypes. It revealed a partial loss of function in synaptic transmission (an increase in neurotransmitter release efficiency), an early mechanism that occurs during the course of the disease, before mutated HTT is imported into the nucleus in detectable amounts. Flies can be powerful tool for high throughput screening of drugs relevant for HD therapy (Marsh et al., 2003). HD *Drosophila* models were also used to identify chemical compounds that may ameliorate HTT-induced toxicity. Fly models are also very useful in the identification and validation of genetic modifiers pertinent to trinucleotide repeat expansion disorders and other neurodegenerative diseases (Kaltenbach et al., 2007; Sang and Jackson, 2005).

In corroboration, a large scale study aimed to test whether genetic modifiers of HD neurodegeneration can be part of HTT protein interactors. By regulating HTT-interacting proteins, toxic effect of polyglutamine HTT was modulated in a *Drosophila* HD model. Thus HTT-interacting proteins could be modifiers of the HD phenotype represented a diverse collection of cellular function counting from synaptic transmission, cytoskeletal organization, signal transducers and transcription (Kaltenbach et al., 2007).

Importantly, HTT function is conserved in flies both for axonal transport and in control of spindle orientation and neurogenesis as theses phenotypes were validated in knock-out flies (Godin et al., 2010; Zala et al., 2013a; Zhang et al., 2009)

Part II: Cell death.

1. Signaling of cell death.

1.1 Apoptosis signaling.

1.1.1 Features.

In multicellular organisms, the timely execution of cell death is critical for numerous physiological processes including embryogenesis, development and adult tissue homeostasis. Apoptosis, a term which comes from Greek meaning “falling away”, is the most well characterized type of death, known as programmed cell death type I. Apoptosis consists of the suicide of the cell in a programmed manner. Cellular proliferation and death are tightly regulated and need to be balanced for the proper development of all organisms.

Apoptosis is characterized by morphological changes: cell shrinks and turns up round and the cytoplasmic content is compacted; pyknosis occurs as a result of chromatin condensation and DNA fragmentation, this is one of the main characteristic of apoptosis. Another important feature is the change in the repartition of the phosphatidylserine. Normally located at the plasma membranes facing the inner site of the cell, this phospholipid faces both inner and outer side of the cell in apoptosis (Fadok et al., 1992). The ultimate step is the budding off of apoptotic bodies after extensive blebbing of plasma membrane. This mechanism is an active process as it requires ATP consumption (Kim et al., 2003).

Cells dying by apoptosis do not release their cellular contents into the surrounding tissues as apoptotic bodies will be engulfed and digested by macrophages. Apoptosis is an irreversible process, when cell is engaged in apoptotic death, it can rarely recover. Nevertheless, a research performed in *C. elegans*, showed that a cell programmed to die by apoptosis can survive upon inhibition of certain engulfment genes. Thus, engulfing cells might act to ensure that cells triggered to undergo programmed cell death die rather than recover after the initial stages of death (Reddien et al., 2001). Activation of apoptosis occurs through two main pathways: (i) the extrinsic pathway through activation of cell surface death receptors and (ii) the intrinsic pathway which originates from the mitochondria and the release of cytochrome C. Both pathways end at the point of the execution pathway the final stages of apoptosis.

1.1.2 Caspases.

Apoptosis involves a set of different genes. The central core of the machinery involves a proteolytic system based on cysteine proteases, the caspases (Thornberry and Lazebnik, 1998). Most of the caspases are synthesized as precursors that have no catalytic activity. The precursor is converted into an active enzyme through a proteolytic event that are tightly regulated. Cleavage and activation of caspases are mediated by other proteases or by autocatalysis triggered by the binding of cofactors.

Two classes of caspases have been described: the initiators and the effectors, all being substrate specific. All these enzymes are expressed as proenzymes of 30 to 50 kDa containing three regions: the N-terminal region with the large and the small domains. After cleavage, these domains join together and form heterodimers. The formation of a tetramer by association of two heterodimers leads to the formation of an active caspase with two functional catalytic domains (Rotonda et al., 1996). Beyond this classical scheme, in some cases, cleavage of initiator caspases are neither required nor sufficient for activation (Fuentes-Prior and Salvesen, 2004) (figure 17).

How caspases kill cells? The cell contains all the needed “reagents” to provoke her suicide, although these are constantly inhibited. When apoptosis is triggered, caspases act to release this inhibition conducting to cell death. As a rule, initiator caspases cleave the inactive form of the effector caspases which in turn inactivate proteins essential for the survival of the cell, leading to cell death. Caspases-3/6 and 7 are the executioner caspases cleaving various substrates.

For instance, in non-apoptotic cells, to prevent DNA fragmentation, the endonuclease CAD (Caspase-activated DNase) is inhibited by ICAD (Inhibitor of Caspase Activated DNase). During apoptosis, the cleavage of ICAD by caspase-3 induces DNA cutting (Enari et al., 1998). Another essential cellular component that is cleaved is Bcl2. Caspase-3 cleaves Bcl-2 (B-cell lymphoma 2) at the aspartic acid 34, releases its C-terminal domain which triggers cell death (Cheng et al., 1997). Bcl2 family comprises several members that are either inhibitors (i.e. Bcl-2, Bcl-xL, mcl-1) or activators (Bad, Bax, Bid) of apoptosis. The propensity of cells to undergo apoptosis depends critically on the balance between anti- and pro-apoptotic members of Bcl-2 family. Another substrate of caspases is the nuclear lamina that is cleavage during apoptosis leading to the disorganization and the condensation of the chromatin (Takahashi et al., 1996).

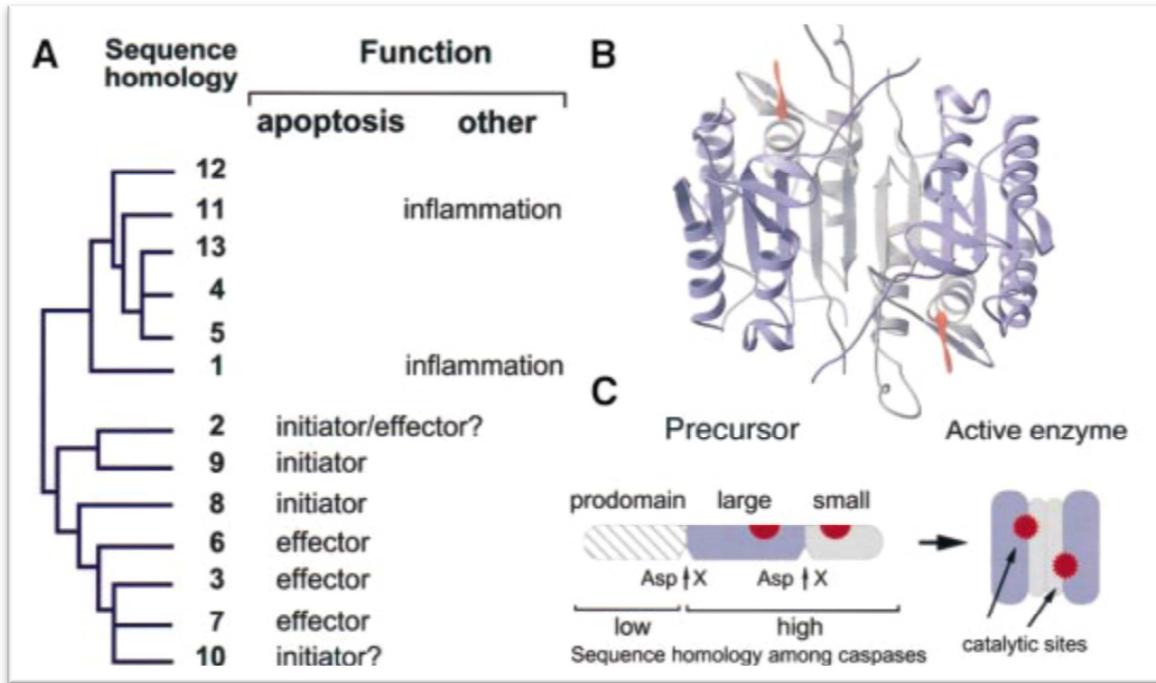


Figure 17: Caspases structure and function. (A) The 13 identified mammalian caspases have different role in apoptosis and inflammation. The family contains two murine homologues with no human counterparts. (B) The crystal structure of caspase-3. The active enzyme is composed of a large (lavender) and small (gray) subunit. Amino acids of each subunit contribute to the active site. In the two crystal structures that are available, two heterodimers associate to form a tetramer. (C) Schematic representation of caspases proteolysis from precursor's state to active enzyme (Thornberry and Lazebnik 1998).

1.1.3 Extrinsic and intrinsic pathway.

The extrinsic pathway that initiates apoptosis involves transmembrane death receptors that are activated by cytokines. Two death factors, Fas ligand and tumor necrosis factor alpha (TNF α) bind to their respective receptors FasR and TNFR1 and initiate the pathway. Upon ligand binding, cytoplasmic adapter proteins are recruited to receptors, respectively FAAD (Fas-Associated protein with Death Domain) and TRADD (TNF receptor-associated death domain). This leads to oligomerization of the death receptors and association with pro caspase-8, forming the death-inducing signaling complex (DISC). This results in the autocatalytic activation of pro caspase-8 (Kischkel et al., 1995). Once caspase-8 (or 10) are activated, the execution phase is launched.

The intrinsic signaling pathway that initiates apoptosis involves non-receptor-mediated stimuli that produce intracellular signals that act directly on targets within the cell and are mitochondrial-initiated events. The stimuli which activate the pathway are: loss of the suppressors of

apoptotic signals or gain in the level of positive triggers such as free radical, toxins, DNA damage or hypoxia. All these stimuli cause changes in the inner mitochondrial membrane that results in an opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential and release into the cytosol of two main groups of pro-apoptotic proteins normally sequestered in the mitochondria (Saelens et al., 2004).

The first group contains the cytochrome *c*, smac and omi (Garrido et al., 2006) and the second one AIF (apoptosis-inducing factor), endonuclease G and CAD (Joza et al., 2001). Cytochrome *c* binds and activates Apaf-1 (Apoptotic peptidase activating factor 1) and procaspase-9 forming the apoptosome, leading to caspase-9 activation and apoptosis (Chinnaiyan, 1999). Smac and omi are reported to promote death through the removal of inhibitors of apoptosis (IAPs) (Schimmer, 2004). AIF and endonuclease G are released from the mitochondria in response to death stimuli, translocate to the nucleus and induce cleavage of the chromatin. These mechanisms are uncoupled to effector caspases (Li et al., 2001).

When mitochondria receive a death signal (free radical, toxins, DNA damage or hypoxia), the outer mitochondrial membrane (OMM) undergoes permeabilization. Members of the Bcl-2 family are key players in OMM opening: The BH3-only protein, such as BIM and tBID, can activate BAX and BAK and participate into the process (Kuwana et al., 2002). A well-known upstream stimulus is the translocation of p53 to the mitochondria. P53 is a transcription factor involved in cell cycle arrest. Targeting p53 to the mitochondria is sufficient to induce apoptosis as it binds to the protective Bcl-XL protein *via* its DNA binding domain and leads to the formation of a complex that induces cytochrome *c* release (Mihara et al., 2003). P53 can also mediate apoptosis through transcriptional activation of several pro-apoptotic genes pushing the balance toward cell death (figure 18).

Caspases substrates are numerous and contribute to cell disassembly in various ways: activation of proteolytic cascade, mitochondrial permeabilization, DNA damage. It remains that a high number of essential proteins contain caspase-cleavage sites making the overall understanding challenging. As we will see in chapter IV, HTT is itself a substrates of several caspases.

Importantly caspases have strict specificity of substrate consistent with the observation that apoptosis is not accompanied by global protein digestion; rather, a select set of proteins are cleaved in a coordinated manner, usually at a single site, resulting in a loss or change in function.

Apoptosis is activated during development and necessary for proper tissue morphogenesis (Martin-Belmonte et al., 2008). However, it can occurs also as a consequence of an uncontrolled stress such as stress induced by the polyglutamine expanded HTT (Saudou et al., 1998).

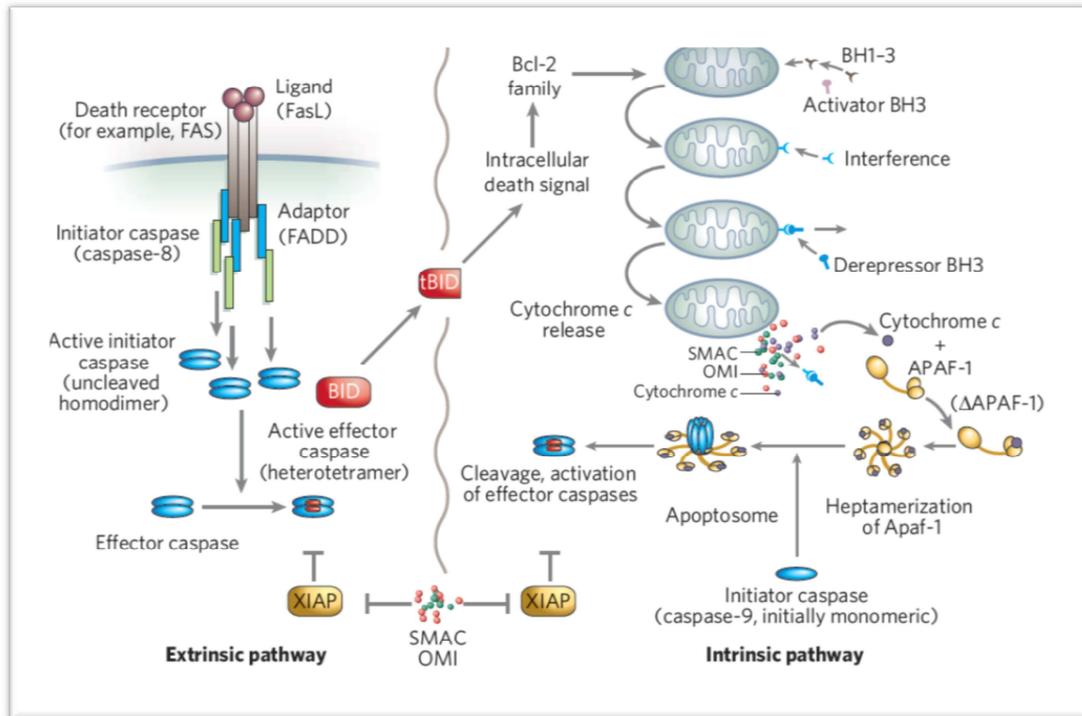


Figure 18: A schematic representation of the apoptotic extrinsic and intrinsic pathways described in the text above.

1.2 Autophagic signaling.

1.2.1 Description of autophagic pathways.

Autophagy (self-eating) is a process which occur under normal condition to recycle long lived molecules and organelles and to maintain homeostasis of the cell (Klionsky and Emr, 2000). However under stress conditions, autophagy may contribute to cell death.

Autophagy is implicated in the removal of damaged organelles or proteins and complements the proteasomal pathway. This final degradation is performed by the lysosomal pathway. There are several forms of autophagy: micro-autophagy, chaperones mediated autophagy (CMA), mitophagy, macro-autophagy and cytoplasm to vacuole targeting (Cvt) pathway (Mizushima, 2007).

- (i) Micro-autophagy is a local uptake of damaged protein by the lysosomes which are cellular organelles that contain acid hydrolase enzymes and break down engulfed material.
- (ii) In CMA, chaperones mediate the delivery of undesired proteins to lysosomes where they undergo enzymatic digestion.
- (iii) In mitophagy, damaged mitochondria release inter-membranes or internal molecules that trigger autophagy. Depolarization of mitochondria, such as by photo-irradiation,

induces their autophagic clearance (Ashrafi and Schwarz, 2013; Kim and Lemasters, 2011).

- (iv) Macroautophagy is a vacuolar mechanism that relies on autophagosome formation. Autophagosomes are double membrane structure that isolates a part of the cytosol. They mature by fusion with endosomes or lysosomes forming respectively amphisomes or auto-lysosomes entities where digestion occurs (Bredesen et al., 2006; Seglen and Bohley, 1992).
- (v) The Cvt is an autophagy-like pathway specific to yeast. It is responsible for the delivery of the hydrolase aminopeptidase I (Ape1) to the vacuoles and its activation. Ape1 is synthesized in the cytosol as a proenzyme that is relatively inactive. Under rich nutrient conditions, the precursor aminopeptidase 1 (prApe1), first oligomerizes, then, is packed into double membrane Cvt vesicles. In the vacuole, it is activated by cleavage of the propeptide (Cueva et al., 1989).

Among these different mechanisms, macro-autophagy is believed to be responsible for the majority of intracellular degradation (hereafter termed autophagy). The molecular mechanism of autophagy depends on the activation of a set of genes termed Atg genes which were first identified in yeast (Klionsky et al., 2003). It can be divided into five steps: initiation of the isolation of membranes (phagophore), elongation, closure of the phagophore and autophagosome formation, autophagosome–lysosome fusion, and lysosomal degradation (Tanida, 2011).

Autophagy is induced by nutrient deprivation via inactivation of the rapamycin-sensitive Tor complex1 (TORC1). Inhibition of TORC1 unphosphorylates Atg13, an essential regulatory component of autophagy, resulting in the activation of the Atg1 kinase complex and induction of autophagy (Kamada et al., 2010).

Nucleation of phagophore requires the ATG1/ULK1 complex activating a class III phosphatidylinositol 3-kinase (PI3K) complex that includes ATG14, Vps34 and Beclin1 (Atg6). Elongation is then mediated by two ubiquitin-like conjugation systems. The first involves the binding of Atg12 to Atg5; then dimers of Atg16 conjugate to Atg12-Atg5 and this newly formed complex associates with the phagophore. The second system requires microtubule-associated protein light chain 3 (LC3) (also called MAP1LC3 or LC3B, the ortholog of yeast ATG8). LC3 is synthesized as a precursor. It is cleaved by Atg4 at its C-terminal part to produce cytosolic LC3-I. Under autophagy activation, Atg7 and Atg3 activate LC3-I that is then conjugated by an ubiquitin-like reaction with phosphatidyl ethanolamine (PE) forming LC3-II. LC3-II localizes to phagophores and is required for elongation and closure of this membrane to form mature autophagosome (Mizushima et al., 2001; Tanida et al., 2004) (figure 19).

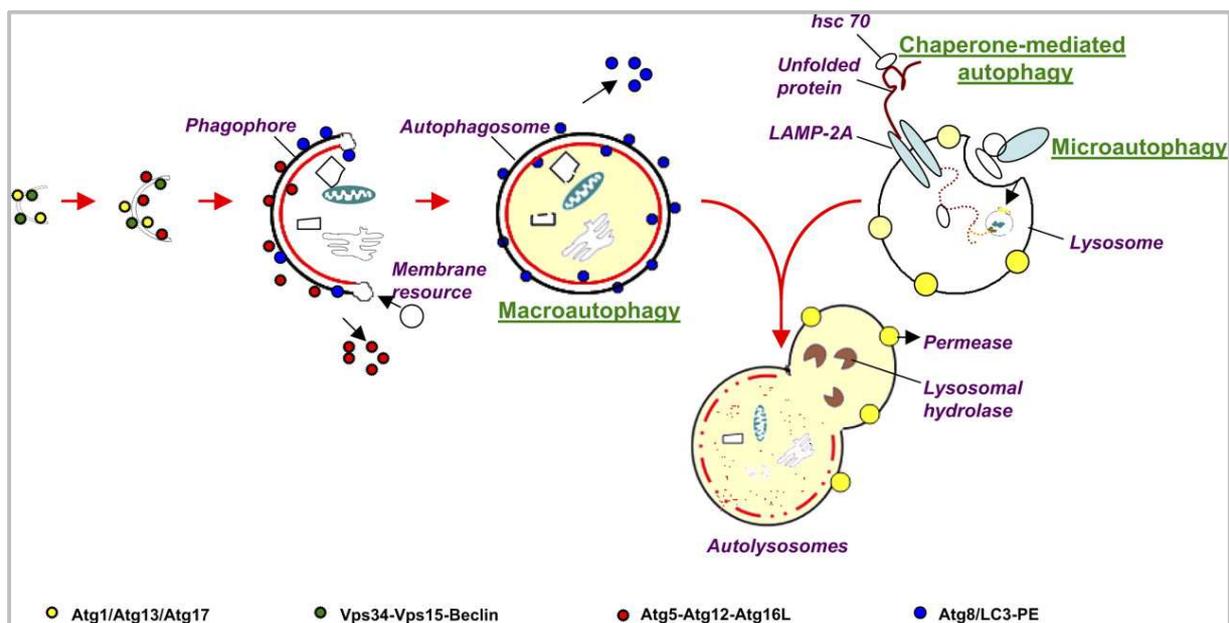


Figure 19: The 3 forms of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy starts with the de novo formation of a cup-shaped isolation double membrane that engulfs a portion of cytoplasm. Microautophagy involves the engulfment of cytoplasm instantly at the lysosomal membrane by invagination, protrusion, and separation. Chaperone-mediated autophagy is a process of direct transport of unfolded proteins via the lysosomal chaperone hsc70 and LAMP-2A. All forms of autophagy subsequently lead to the degradation of intra-autophagosomal components by lysosomal hydrolases. PE, phosphatidylethanolamine (Periyasamy-Thandavan et al., 2009).

1.2.2 Autophagic cell death.

Autophagy has a dual role in cell survival and cell death. It is implicated in many diseases where autophagy dysregulation can be harmful for the cell. In the absence of apoptosis, autophagy can trigger cell death, a form called programmed cell death type II. Autophagy is active at a basal level for the self-cleaning of the cell. It can also bring energy to the cell when nutritive supplies are reduced. This goes through the inhibition of protein synthesis via an increase of the phosphorylation of ribosomal protein S6 (S6) as a result of the inhibition of PI3K and mTOR (mammalian target of rapamycin) signaling (Blommaert et al., 1995).

Whether autophagy is a death mechanism has been controversial mainly from a lack of knowledge in its underlying genetic mechanisms.

The association of autophagic vacuoles with cell death has been made in animal models (Clarke, 1990) but it is not been clear whether it serves to rescue or damage the cell. In 2004, two

groups demonstrated that autophagy could *per se* be a cell death mechanism. The group of Michele Lenardo used *Drosophila*'s cells to monitor death and showed that inhibiting caspases induced an autophagic cell death regulated by caspase-8 inhibition and requiring Atg7 and beclin1 expression. These results defined a molecular pathway in which activation of the RIP (receptor interacting protein), a serine-threonine kinase, and JNK (c Jun N-terminal kinase) induced cell death with the morphology of autophagy (Yu et al., 2004). In support of this work the team of Tsujimoto observed that embryonic fibroblasts from Bax/Bak double knockout mice, and that are resistant to apoptosis, still underwent a non-apoptotic death after death stimulation. This non-apoptotic cell death was modulated by Atg5, beclin1 and Bcl-x. This study put forward a new function of Bcl2 and Atg family members in regulating programmed cell death type II (Shimizu et al., 2004).

Furthermore, research from Avery's team showed that the fine tuning of autophagy influx is key for the cell to decide whether to live or die. Using *C. elegans*, they demonstrated that, autophagy is required for optimal survival of worms during starvation as siRNA of beclin was toxic in worm undergoing starvation. Starvation activates MAPK (mitogen-activated protein kinase) signaling in muscle *via* a muscarinic acetylcholine receptor signaling pathway. When this receptor is over-activated in *gbp-2* mutant worms, it induces excessive autophagy leading to death under starvation condition (You et al., 2006). Interestingly, knock-down of beclin1 rescued starvation-induced cell death in *gbp-2* mutant's worms (Kang et al., 2007). In summary, physiological level of autophagy is beneficial for the body but lack or excess of autophagy is toxic (figure 20).

Although a number of reports on autophagic cell death have been made (Aki et al., 2003; Bursch et al., 1996; Kroemer and Levine, 2008), the mechanism is still unclear. What are the signals that turn autophagy from a protective program to a cell death program? One model involves over-digestion of the cellular content, another proposed that autophagy activates the apoptotic machinery to kill the cell. The third model proposes that autophagy could selectively destroy the survival factors leading to cell death. For example, when autophagy is over-activated, it can degrade catalase, an enzyme that catalyzes the toxic hydrogen peroxide into water and oxygen. As a consequence, oxidative stress-causing reagents (ROS) accumulate in the cell, leading to cell death (Yu et al., 2006).

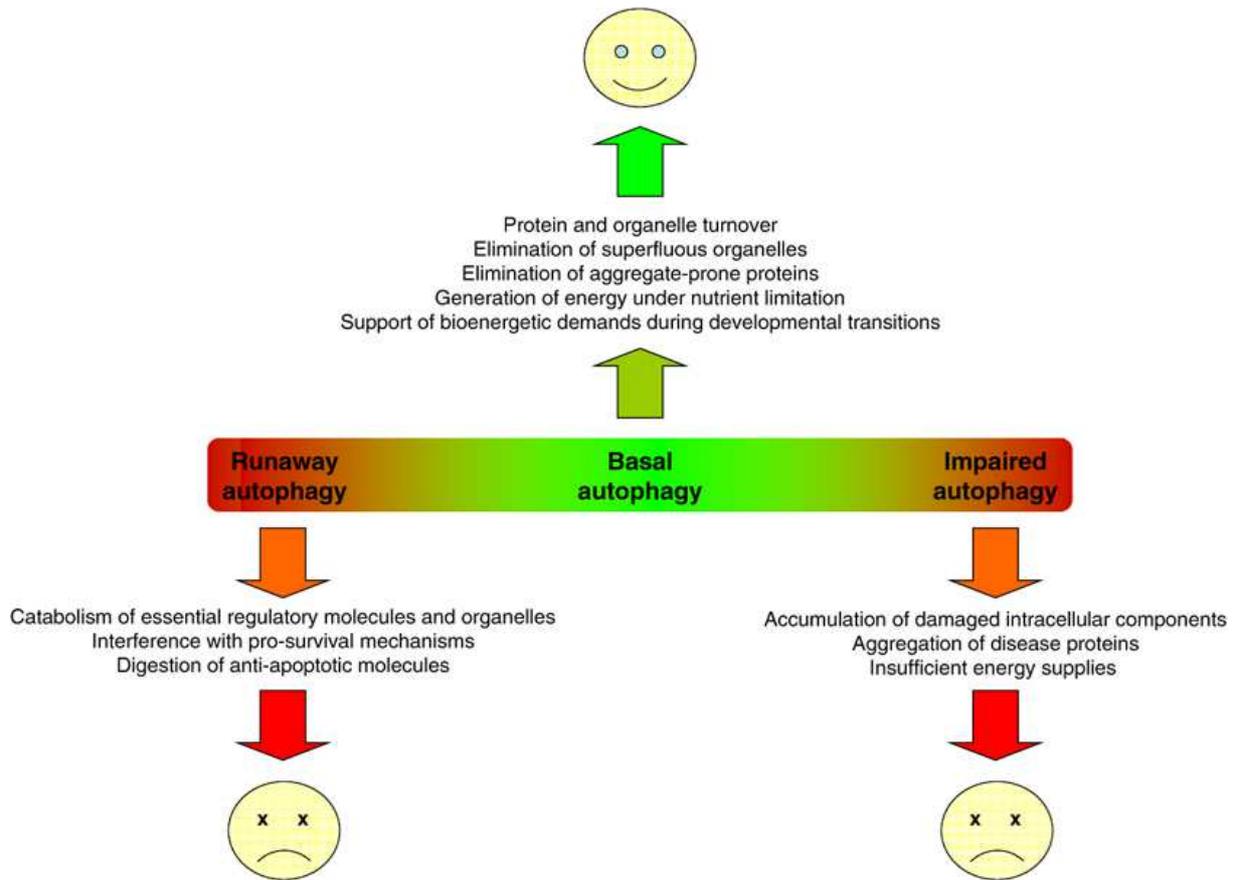


Figure 20: The role of autophagy in cell survival and death. Basal autophagy acts as a cyto-protective mechanism and serves homeostatic functions such as cytoplasmic, protein and organelle turnover. Moreover, autophagy provides metabolic substrates when cell energetic demands are increased, during developmental transitions or nutrient/growth factor deprivation. Uncontrolled, 'runaway' autophagy can digest vital amounts of cell components and survival factors, thus leading to the demise of the cell. Similarly, impaired autophagy can be deleterious by allowing damaged proteins and organelles to accumulate and by failing to provide energy for essential cell functions (Kourtis and Tavernarakis, 2009).

1.3 Apoptosis and autophagy crosstalk.

Apoptosis and autophagy are interconnected as autophagy is activated when apoptosis is inhibited, and conversely, inhibition of autophagy triggers apoptosis.

Interestingly, inhibition of apoptosis could raise up autophagy levels for survival purposes. Indeed, in 2002 Lum et al. showed that Bax^{-/-}Bak^{-/-} cells, generated from the bone marrow of Bax^{-/-}Bak^{-/-} mice, activated autophagy, following the withdrawal of growth factors. As cells were unable to die by apoptosis, they managed to survive through a long autophagic process that led to a significant reduction of the cell size (Lum et al., 2005).

Conversely, there is also convincing studies suggesting that apoptosis is turned on after inhibition of autophagy. For instance, Inhibiting LAMP-2 causes a decrease in autolysosomes formation and an accumulation of autophagic vacuoles, indicative of a blockade in the autophagic signaling. In these conditions, cells exhibit apoptotic features like caspase activation and loss in mitochondria transmembrane potential. Over-expression of Bcl-2 suppress the toxicity of autophagy impairment arguing for a cross-talk apoptosis and autophagic pathways (Gonzalez-Polo et al., 2005). When autophagy is inhibited at early stages, via beclin1 knocking down, no autophagic vacuoles are formed and cell die by apoptosis (Boya et al., 2005).

Common regulators of apoptosis and autophagy such as p53 have been described. P53 is a pro-apoptotic protein by interfering with Bcl-2 family members and by activating the transcription of pro-apoptotic genes. Tazdemir *et al.* found that the cytoplasmic p53 is also an inhibitor of autophagy. Consistently, inducers of autophagy must destroy the pool of cytoplasmic p53 to launch autophagy processing (Tasdemir et al., 2008).

All these data indicates that caspases and members of Bcl-2 family are not only part of the apoptotic pathway but they are also implied in normal processes such as cell differentiation and can interconnect with other pathways such as autophagy. Therefore, it is not surprising that, under certain stresses, cells implement several ways to survive and/or to die. A shift from a mechanism to another reflects the complexity of the cell and highlights the existence of multiple pathways to ensure a proper regulation of cell fate in response to environmental cues (figure 21).

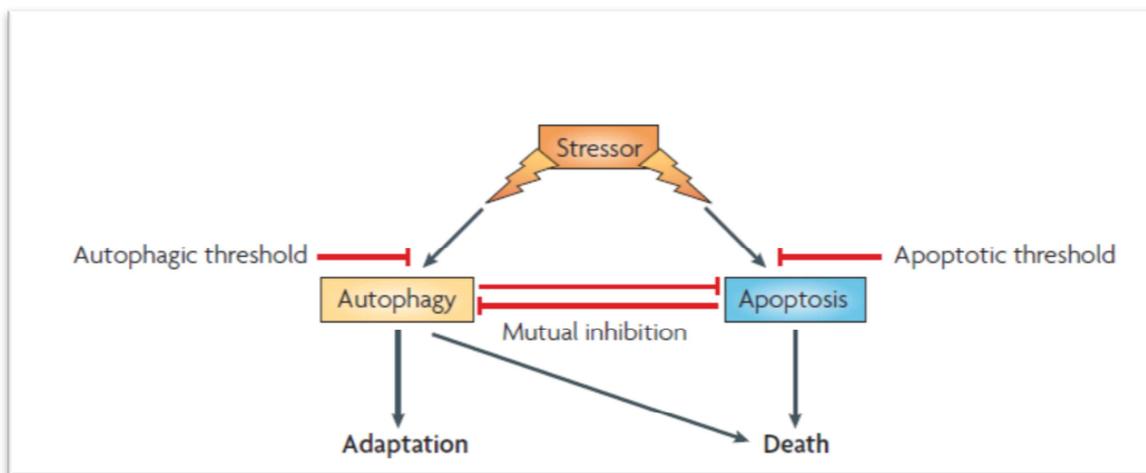


Figure 21: The relationship between apoptosis and autophagy. Similar stressors can induce either apoptosis or autophagy in a context-dependent fashion. Alternatively, the choice between apoptosis and autophagy is influenced by the fact that the two catabolic processes exhibit some degree of mutual inhibition. In some cases, a mixed phenotype of apoptosis and autophagy can be detected at the single-cell level. Although autophagy mostly allows cells to adapt to stress, massive autophagy can also kill cells (Maiuri et al., 2007).

1.4 Alternatives cell death.

Cell death mechanisms that do not respond to the criteria of apoptosis or autophagy have been described. These include: necrosis and oncosis, paraptosis, aponecrosis and caspase independent cell death.

Necrosis is a somewhat primitive path to cell death. In contrast to apoptosis, cleanup of cell debris by phagocytes of the immune system is generally less efficient, as necrosis does not generate cell signals to the nearby phagocytes. Necrosis is a passive process, characterized by cytoplasmic swelling, rupture of plasma membrane, swelling of cytoplasmic organelles and moderate chromatin condensation. It is proposed to be the end result of a bioenergetic catastrophe resulting from ATP depletion to a level incompatible with cell survival and is thought to be initiated mainly by cellular “accidents” such as toxic insults or physical damage (Edinger and Thompson, 2004). A nuclear protein, prothymosin- α 1 (ProT α), was identified as a key protein inhibiting necrosis (Ueda et al., 2007). Slightly different, oncosis (onkos meaning swelling) or ischemic cell death, is a form of accidental, or passive cell death that often is considered as lethal injury. The process is characterized by mitochondrial swelling, cytoplasm vacuolization, and swelling of the nucleus and cytoplasm.

Paraptosis, described by Dale Bredesen, was first observed in a variety of cell types in response to insulin derived growth factor 1 receptor (IGFR1). It has none of the morphological features associated with apoptosis. Instead, paraptosis is characterized by the formation of vacuoles in the cytoplasm, along with mitochondrial swelling, reminiscent of necrosis. However, paraptosis, in contrast to necrosis, requires new RNA and proteins synthesis, suggesting that, like apoptosis, it represents a distinct and programmed biochemical event. Paraptosis involves caspase-9 but the pathway appears to be different from apoptosis as it is not inhibited by the apoptosis inhibitor BAF (Sperandio et al., 2000). Thus caspase-9 activates at least two different death pathways: apoptosis through the cleavage and activation of the effector caspase-3 and paraptosis. Interestingly, IGFR1-induced paraptosis was shown to be mediated by mitogen-activated protein kinases (MAPK) and inhibited by AIP-1/Alix (a protein which participate in a spectrum of activities including pro-apoptotic signaling (Odorizzi, 2006)). This suggests that apoptosis and paraptosis are complementary mechanisms (Sperandio et al., 2004). This form of morphological death has been observed during neural development and degeneration and was referred as cytoplasmic cell death (Clarke, 1990; Dal Canto and Gurney, 1994; Schweichel and Merker, 1973) (figure 22).

Aponecrosis is referred as a mechanism that involves both apoptosis and necrosis. When cells receive apoptotic stimuli at a high concentration, cells die before being able to achieve their

programmed-cell-death. As an example, actinomycin A, a pro apoptotic drug, used at different concentrations, induced death characterized by an incomplete execution of the apoptotic program and necrosis when concentrations were high (Formigli et al., 2000). Also cultured neurons overstimulated with glutamate, die by necrosis or apoptosis probably depending on ATP availability. This depends on mitochondria functionality indicating that mitochondrial functions are critical factors that determine the mode of neuronal death in excitotoxicity (Ankarcrona et al., 1995).

A non-apoptotic, caspase independent, calpain and cathepsin dependent-cell death has also been described. It is triggered by calcium and forms whorls in the cytoplasm. This cell death was first described in *C. elegans* in 1997 (Hall et al., 1997). Two mutations in *mec-4* and *deg-1* genes, genes encoding sodium channels, are responsible of the necrosis-like neuronal cell death observed. Death is characterized by the production of membranes infoldings and whorls. The cytoplasm becomes progressively less dense for electron as whorls and vacuoles accumulate and enlarge. At middle degeneration stage, the ER and Golgi bodies get swollen, but the mitochondria stay intact. Then, vacuoles deform the nucleus by invagination of a large vacuole and dislocate it toward the plasma membrane. The perimeter of the cell is irregular and a complete breakdown of cellular structures occurs (Hall et al., 1997).

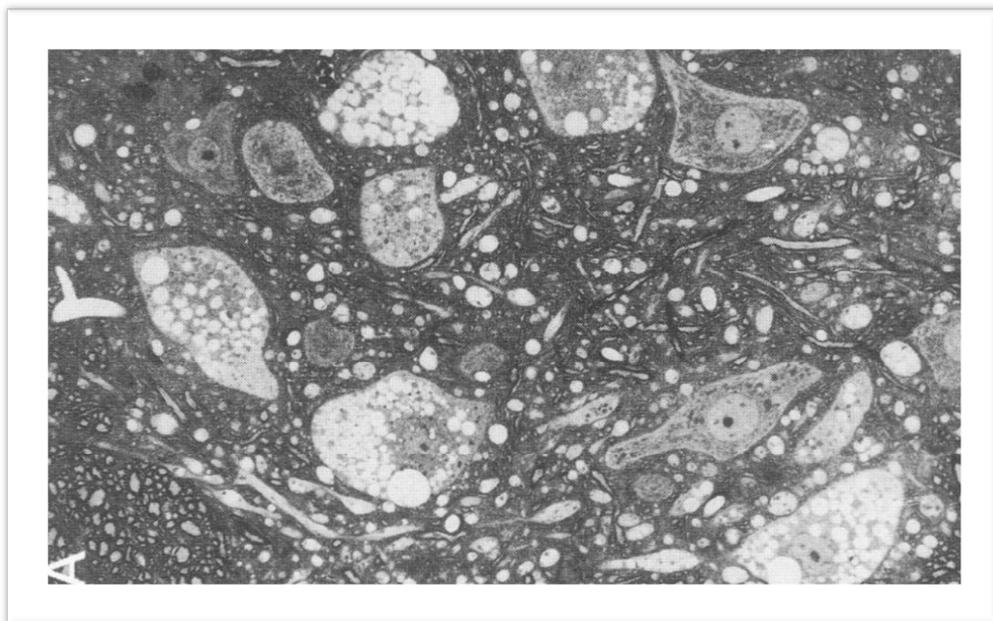


Figure 22: Anterior horn of the spinal cord from a mouse with a motor neuron disease (SOD mutant) sacrificed at 73 days of age, in the preclinical stage. Numerous large motor neurons harbor vacuoles of various dimensions in their cytoplasm. In some cells the cytoplasm appears to be almost completely replaced by vacuoles. A few vacuoles are present in the neuropil, representing vacuolated dendrites (Dal Canto and Gurney, 1994).

In the nematode *C. elegans*, two classes of proteases, calpain and aspartyl proteases are involved in necrotic cell death. Their abnormal activity rely on a high level of intracellular calcium caused by deleterious mutations in the ion channels (Syntichaki et al., 2002).

Another necrosis-like cell death was described by William Maltese's team in 2008. Expression of dominant-active Ras (a small GTPase implicated in cell proliferation), induces accumulation of empty cytoplasmic vacuoles followed by cell death. Death was initiated by the dysregulation of macropinocytosis and it termed methuosis meaning drinking to intoxication (Overmeyer et al., 2008).

Finally, a non-autophagic LC3 dependent cell death was defined in 2009. It is induced by a prostaglandin drug (Thiol reactive cyclopentenone prostaglandin, 15-deoxy-Delta (12,14)-prostaglandin J(2) (15d-PGJ2)) used in cancer research for its anti-proliferative property. It is characterized by a massive cytoplasmic vacuolization. Vacuoles were identified as dilated cisternae of the ER, up-regulation of ER stress signaling occurred and silencing LC3 expression was protective against the induced-cell death (Kar et al., 2009) (for summary see figure 23).

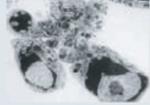
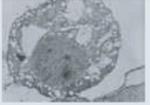
Table 1 Characteristics of dying cells						
Characteristics	Established forms of cell death		Atypical forms of cell death			
	Apoptosis	Autophagic	Paraptosis	Calcium-mediated	AIF/PARP-dependent	Oncosis
Morphology	 Chromatin condensation, nuclear fragmentation, apoptotic bodies	 Autophagic vacuoles	 ER swelling, mitochondrial swelling	 Membrane whorls	 Mild chromatin condensation	 Cellular swelling
Triggers	Include death receptors, trophic-factor withdrawal, DNA damage, viral infections	Serum, amino-acid starvation, protein aggregates	Trophotoxicity	Calcium entry, <i>C. elegans deg</i> mutants	DNA damage, glutamate, nitric oxide	Ischaemia, excitotoxicity
Mediators	Caspases, BH1-3, BH3 proteins	JNK1? MKK7? ATG orthologues	ERK2, NUR77	Calpains, cathepsins	PARP, AIF	JNK
Inhibitors	Caspase inhibitors, BH1-4 proteins	JNK inhibitors?	U0126 (MEK), DN NUR77	Calreticulin, Some calpain inhibitors?	PARP inhibitors	JNK inhibitors
Examples	Type I PCD, nuclear PCD	Type II PCD	Type III PCD, cytoplasmic PCD	<i>C. elegans deg</i> mutants	Some excitotoxic PCD	Ischaemic PCD

Figure 23: Summary of the cell death types reported. This table highlight the differences in morphology present in each form of death, as well as the differences in biochemical mediators, inducers, and inhibitors. At present, only apoptosis and autophagic programmed cell death (PCD) are generally accepted as being legitimate forms of PCD; however, ongoing research should reveal which of the additional candidates represent novel pathways of PCD. DN, dominant-negative; DEG, degeneration; U0126, a mitogen-activated protein kinase inhibitor.

2. Cell death in HD

HD brains show massive neuronal loss. Mutant HTT induces several dysfunction which progressively leads to neuronal degeneration in the striatum, several layers of the cortex and other regions of the brain. Various mechanisms have been proposed to participate in cell death: apoptosis, autophagy, excitotoxicity, dopamine toxicity, metabolic impairment and mitochondrial dysfunction.

2.1 Apoptosis in HD.

Many of the pathways that seem to be altered in HD or models of the disease, and that are discussed below, would be expected to end in apoptosis. The classic hallmark of apoptosis, fragmented DNA as observed by TUNEL staining, has been reported in HD brain (Thomas et al., 1995).

Evidence for the classical apoptotic pathway operating in HD is not clear although much evidence for the involvement of players in apoptosis have been reported. The first evidence comes from studies in postmortem brains.

Early studies have used the TUNEL (Tdt-mediated dUTP-biotin nick end labeling) technique to visualize apoptosis in postmortem brains from patients affected by Alzheimer disease (AD) or HD (Dragunow et al., 1995; Thomas et al., 1995). DNA fragmentation was detected in striatum from HD brains compared to scattered DNA fragmentation positive cells in normal human brains. This suggests that apoptosis may be involved in HD neuronal death. However, the presence of DNA fragmentation in AD brain tissues was controversial as one study reported the observation of TUNEL positive neurons in both AD and HD while the latter study proposed a unique positive staining in HD compared to the other neurodegenerative diseases.

The development and characterization of cellular models in HD gave evidence of apoptotic mechanisms. Indeed, death induced by mutated HTT is blocked by Bcl-XL (B-cell lymphoma-extra-large) a pro-survival protein binding to mitochondria; and by caspase-3 inhibitors (DEVD) (Saudou et al., 1998).

Similarly, Kim et al. 1999 explored the contribution of intranuclear inclusions to cell death. They showed that the formation of inclusions and the neuronal survival by caspases inhibition is separable. Indeed, in clonal striatal cell, the caspase-1 & -3 inhibitor Z-VAD-FMK promoted cell survival but didn't change the rate of inclusion's formation. A debatable observation indicated that inhibitor of caspase-3 (Z-DEVD-FMK) reduced inclusions but did not restore survival of cell. This would imply that inhibiting caspase 1 could prevent cell death due to mutant HTT whereas

inhibiting caspase 3 cannot, although it affects aggregation of HTT. The involvement of caspase 1 within mutant HTT-induced cell death was not confirmed later on and the use of minocycline (inhibitor of caspase-1 & -3) in clinical assays had no beneficial effect on HD patients (Huntington Study Group, 2010).

Another mechanism of apoptosis activation could involve caspase-8. It has been found to be important for cell death in primary rat neurons expressing mutant HTT. Mutant HTT was shown to induce caspase-8 oligomerization and subsequent activation. Concomitantly, Western blot analysis revealed an activation of caspase-8 in affected caudate from HD patients (Sanchez et al., 1999). Thus mutated HTT may have a dominant negative effect on FADD (Fas-Associated protein with Death Domain), abnormally inducing apoptotic signaling and cell death; this in support with dysregulation of the extrinsic pathway.

As previously discussed, wild type HTT protects from apoptosis. HTT binds HIP1 that interacts with Hipp1, a modulator of apoptosis. This complex modulates activation of caspase-8 and therefore apoptosis through components of the extrinsic cell-death pathway. (Gervais et al., 2002; Hackam et al., 2000; Rigamonti et al., 2001). The loss of function of HTT by the polyglutamine expansion may introduce a loss of anti-apoptotic properties of HTT leading to apoptotic cell death.

Eventually, morphological analysis of brain's regions from HD patients or R6/2 mice, by electron microscopy showed no defining characteristic of apoptotic cell death (Turmaine et al., 2000). In parallel, biochemical analysis show TUNEL negative stain indicative of no DNA fragmentation but histological analysis showed that affected neurons underwent dark cell degeneration, contained nuclear inclusions and exhibited invagination of the nuclear membrane (Turmaine et al., 2000).

2.2 Autophagy in HD.

The first indications of a possible involvement of autophagy come from the observations that inhibition of mTOR by rapamycin is beneficial in flies and cellular models of HD (Ravikumar et al., 2004; Sarkar et al., 2007). Indeed, rapamycin administration lead to an increase in the autophagic pathway and to a considerably slower neurodegeneration. This beneficial effect could be linked to the role of autophagy in clearing mutant HTT (Ravikumar et al., 2008). In support, HTT is itself acetylated at lysine 444 and this site is important for targeting HTT to autophagic degradation (Jeong et al., 2009). Also, the survival of *C. elegans* expressing mutated HTT is altered when autophagy

genes are mutated. This suggest that affected worms relied on autophagic clearance to prevent accumulation of polyglutamine toxicity (Jia et al., 2007).

Although, enhancing autophagy is of therapeutic interest, mutant HTT could itself alter autophagosomes formation. A work performed by Cuervo AM's team showed that, in several models of HD, there is a defect in the ability of autophagic vacuoles to recognize cytosolic cargo (Martinez-Vicente et al., 2010). Autophagosomes are properly formed and fused correctly with lysosomes, however they failed to trap cytosolic cargos in their lumen. This reduced the turnover of misfolded proteins and eventually led to cell death. There are evidence of UPS (ubiquitin-proteasome system) alterations in HD since autophagy is another pathway that can use the cells to eliminate misfolded proteins. It could act as a compensatory degradation system when the UPS is impaired (Pandey et al., 2007). These findings suggest that in HD, in addition to possible alteration of the UPS, autophagy could be altered, hence, and this might be increasing the potential of cell death in HD.

In addition to the alteration of autophagosomes formation, trafficking could also impact on autophagy machinery. HDAC6 (histone deacetylase 6) associates to microtubule and to poly-ubiquitinated proteins and was identified to be a crucial player in the cellular management of misfolded protein-induced stress (Kawaguchi et al., 2003). First, HDAC6 was identified to regulate autophagy by acting at the level of autophagosome-lysosome fusion. Thereby, it is a critical component that defines the quality control of autophagy (Lee et al., 2010). When the microtubule network is disrupted by nocodazole treatment, an increased toxicity of mutant HTT is engendered and correlated with the slowdown of misfolded turnover. This was explained by a loss in autophagosme-lysosome fusion (Webb et al., 2004). Also, HDAC6 overexpression could rescue neurodegeneration in a polyglutamine fly model (Pandey *et al.* 2007) and was suggested to be required for autophagic clearance of HTT aggregation (Iwata et al., 2005). All these results taken together suggest that HDAC6 is important for the proper function of autophagy in an HD context.

HDAC6 could act as a double edge sword as in contrast HDAC6 inhibition was proposed as a therapeutic target of interest counteracting the defect in intracellular transport in HD (Dompierre et al., 2007).

In summary, several studies suggest defects in autophagic pathways in cells expressing mutated HTT and that up-regulating autophagy may be beneficial in disease. However the real contribution in HD of autophagic cell death type II is still missing (figure 24).

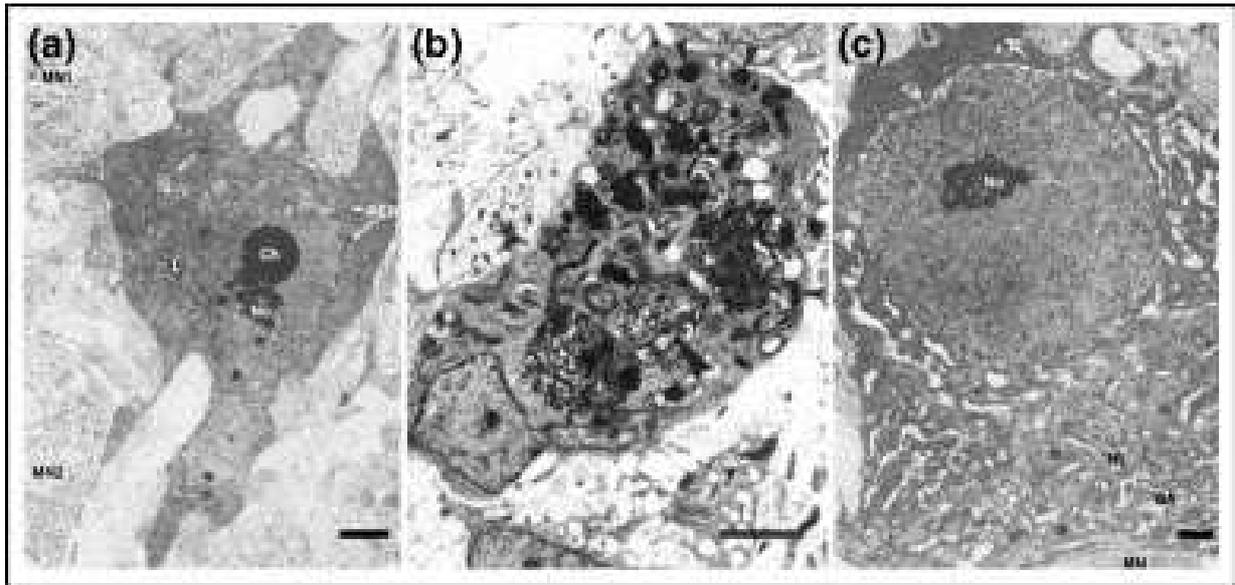


Figure 24: Neuronal cell death: diversity of morphological patterns. Schweichel and Merker (Schweichel and Merker, 1973) and later Clarke (Clarke, 1990), described three general morphological types of dying cells in the developing nervous system: apoptotic (Type 1), cytoplasmic (non-lysosomal, Type 2), and autophagic (Type 3), and they provided these examples. (a) Type 1: a motoneuron exhibiting cell shrinkage and condensation, misshapen nucleus with chromatin masses, and small rough endoplasmic reticulum (RER) cisternae. (b) Type 2: a deafferented isthmo-optic neuron in developing chick brain after uptake of horseradish peroxidase to highlight (electron dense) endocytic and autophagic compartments. (c) Type 3: a motoneuron displaying markedly dilated RER, Golgi and nuclear envelope, late vacuolization and increased chromatin granularity. The cell death pattern features abundant autophagic vacuoles, pyknosis and sometimes dilated endoplasmic reticulum and mitochondria. Scale bars, 2 μ m (Nixon, 2006).

2.3 Excitotoxicity

The medium spiny neurons that are the most susceptible to degenerate are characterized by the presence of N-methyl-D-aspartate (NMDA) receptors, which are sensitive to the excitatory amino acid glutamate. Over-activation of these receptors allows increased calcium influx into the cell and leads to cell dysfunction and death through apoptosis (Zeron et al., 2001). The excess of calcium has been hypothesized to be critical in activating many pathways leading to death (Bezprozvanny and Hayden, 2004).

In support of this mechanism as being critical in HD, quinolinic acid (QA), an agonist of NMDA receptors (NMDAR), induces neuronal loss both in rat and primates (Ferrante et al., 1993). In HD transgenic mice, the consequences of QA treatment differed according to the mouse model. Mice were affected by the excitotoxic insult in a case, or completely resistant in another (Hansson et al., 1999; Zeron et al., 2002). This discrepancy is related to the size of the mutant human HTT expressed

in those murine lines. R6/1 and R6/2 expressed 3% of the total gene and are resistant to QA-induced excitotoxicity; whereas in a transgenic mouse carrying a larger fragment of the human *HTT* cDNA showed a similar susceptibility for QA-induced excitotoxicity than wild type littermates (Petersen et al., 2002). The inhibitory GABA receptors were found to be increased in the medium spiny neuron projections of symptomatic R6/2 mice and this could provide a neuroprotective pathway to counteract excitotoxicity (Cepeda et al., 2004).

Enhanced excitotoxicity could be explained by the binding of HTT to PSD-95 (postsynaptic density protein 95), a protein recruited to NMDA receptors. Polyglutamine expansion of HTT interferes within this interaction resulting in an augmented NMDA sensitivity. Wild type HTT or an NMDA antagonist significantly inhibited neuronal toxicity mediated by NMDA receptors in mutant HTT context (Sun et al., 2001). Moreover, a study showed that mutant HTT elevated the phosphorylation level of NMDA receptors 2B and altered its proper activation, further promoting its sensitization (Song et al., 2003).

Another factor that could contribute to excitotoxicity is the diminished capacity of the glial cells to remove glutamate from the synapse (figure 25). Reduced level of the transcript of GLT1, an astroglial transporter of the glutamate, is observed in R6/1 and R6/2 mice, suggesting an impairment of glutamate re-uptake in HD model mice (Lievens et al., 2001). This could be due to a role of mutant HTT dysregulating the expression of glutamate transporters. In a neuron-glia co-culture, wild-type HTT expressed in unaffected glial cells protected affected neurons against mutant HTT-mediated neurotoxicity, whereas affected glial cells increased neurons sensitivity to glutamate excitotoxicity. These results suggest that decreased glutamate uptake caused by glial expressing mutant HTT may unfavorably contribute to neuronal excitotoxicity in HD (Shin et al., 2005).

Increase in the production of excitotoxins in HD may also participate in excitotoxicity. The kynurenine pathway is the major route of tryptophan degradation in eukaryotes, and leads to synthesis of NAD⁺, an oxidizing agent. The kynurenine pathway (KP) of tryptophan degradation contains three neuroactive metabolites: the neuroinhibitory agent kynurenic acid (KYNA), the free radical generator 3-hydroxykynurenine (3-HK) and the excitotoxin quinolinic acid (QA). The level of 3-HK and QA were depicted to be greater in HD mouse model (Guidetti et al., 2006). Also, Intrastratial injection of QA in rodents reproduces behavioral and pathological features of HD suggesting an activation in the kynurenine pathway in HD along with an increase in NMDA agonist, contributing in the excitotoxicity in HD pathogenesis (Schwarcz, 2004; Schwarcz et al., 1983).

Moreover, the inhibition of KP is beneficial in HD model. A genetic yeast screen, aiming at identifying loss-of-function mutations suppressing mutant HTT toxicity, identified *BNA4* (kynurenine 3-monooxygenase), an enzyme involved in the KP of tryptophan degradation. *Bna4* is activated in HD patients and in animal models of HD (Schwarcz, 2004) and deletion of *BNA4* suppressed mutated HTT-mediated toxicity (Willingham et al., 2003) (Giorgini et al., 2005).

NMDAR activity is modulated by its subcellular location (Kohr, 2006). Synaptic activation of the receptor promotes CREB phosphorylation, BDNF transcription and antioxidant defense, whereas extrasynaptic signaling leads to dendritic blebbing, mitochondrial breakdown, and cell death (Hardingham et al., 2002; Leveille et al., 2008; Papadia et al., 2008). Increase in extrasynaptic NMDARs expression was reported in full length HD mouse model and their pharmacological blockade with memantine reversed signaling and motor learning deficits in mouse (Milnerwood et al., 2010).

Under conditions of chronic activation of NMDA receptors, the intracellular calcium concentration is increased and this could have deleterious effect on mitochondria homeostasis, calcium-dependent proteases activation and ROS production. Indeed, important effectors of neuronal cell death in HD are calcineurin, a calcium/calmodulin-dependent protein phosphatase and calpain, a calcium-dependent cysteine protease. Under excitotoxic stress, calpain is activated by excessive cytosolic calcium and cleaves calcineurin. Calcineurin proteolysis converts the enzyme to a constantly active form that activates caspases and cell death (Wu et al., 2004). One proposed mechanism is that calcineurin dephosphorylates the pro-apoptotic BH3 protein, BAD which in turn translocates from the cytosol to the mitochondria where it binds Bcl-2. Subsequently, the release of cytochrome c is enhanced leading to caspase activation and cell death (Springer et al., 2000). Interestingly, abnormal calcium level and subsequent calcineurin activation could directly impact on HTT itself phosphorylation at the serine 421. Inhibiting calcineurin in vivo and in cells by FK506 prevented dephosphorylation of polyglutamine HTT on S421 and led to neuroprotection (Pardo et al., 2006). However, this mechanism is rather linked to the role of HTT in regulating the fast axonal transport of BDNF vesicles in neurons and may not be directly associated with excitotoxicity.

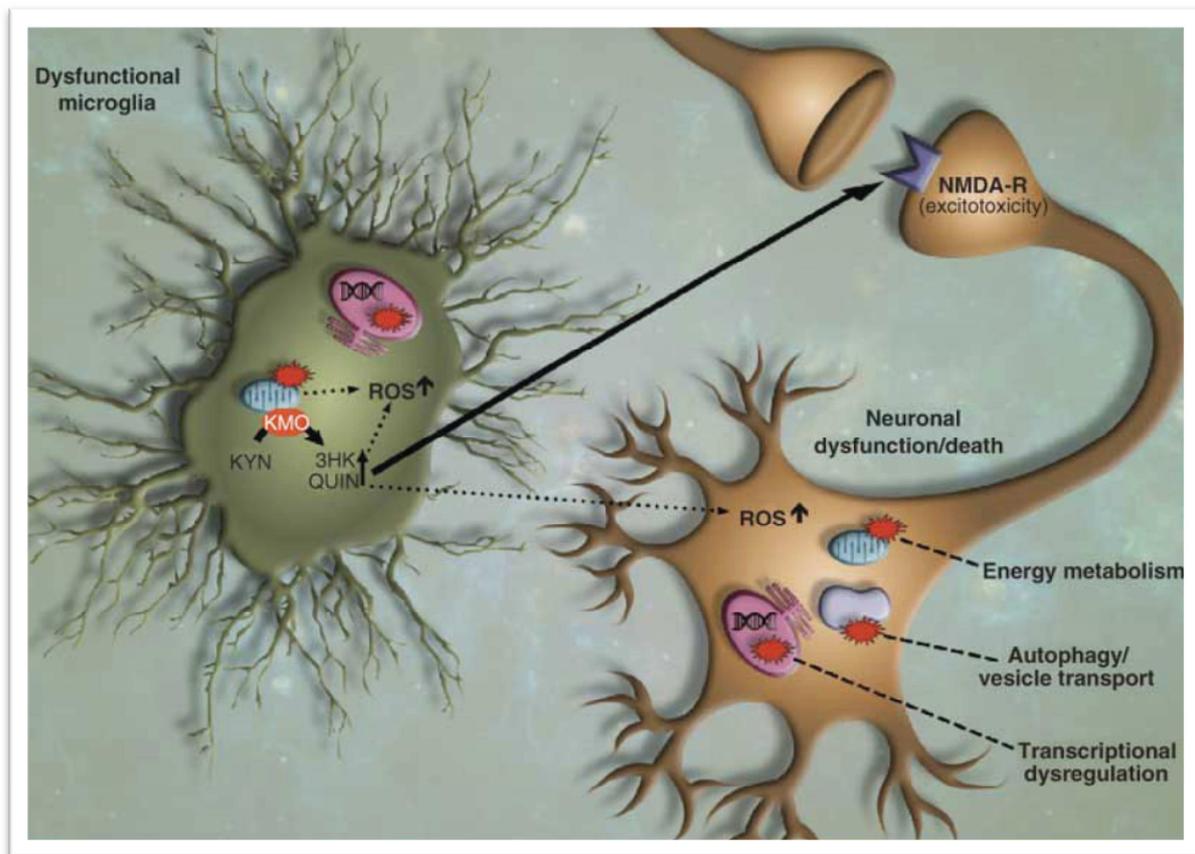


Figure 25: Model depicting the non-cell autonomous contribution by microglia to neuronal dysfunction in HD. In this model, cell-autonomous expression of mutant HTT in microglia causes dysfunction, perhaps by interactions with mitochondria or the mitochondrial membrane protein KMO, leading to upregulation of 3HK and QA (QUIN) synthesis, and thereby increased levels of ROS. The combined effects of ROS and NMDA receptor-mediated excitotoxicity by QA contribute to the dysfunction of neurons expressing mutant HTT. Other functional impairments implicated in HD, are highlighted in the dysfunctional neuron: intracellular events in neurons expressing mutant HTT → impact on organelle functions, nuclear transcription and cell death (Giorgini et al., 2005).

2.4 Dopamine toxicity

The striatum receives dopaminergic input from the substantia nigra. Dopamine is a neurotransmitter that binds to and activates the dopamine receptors. The dopamine receptors are a series of five G protein-coupled receptors, which consist of the D1, D2, D3, D4, and D5 receptors. They signal by modulating the cyclic adenosine monophosphate (cAMP) second messenger system to produce an action potential. The five receptors are divided into two distinctive groups based on their varying effects: the D1-like (containing D1+D5) and D2-like (containing D2+D3+D4) subfamilies. The former increases the cellular concentrations of cAMP by the activation of the enzyme adenylyl cyclase. The latter decreases the cellular concentrations of cAMP by inhibition of adenylyl cyclase. Ultimately, the cAMP second messenger system, through several downstream mechanisms,

modulates the opening of ion channels that allow positively charged ions such as Na⁺ and K⁺ to enter or leave the cell, thereby generating (D1-like receptors) or inhibiting (D2-like receptors) an action potential.

One well-studied molecular target for the actions of dopamine is DARPP-32 (dopamine- and cAMP-regulated neuronal phosphoprotein), which is highly enriched in all medium spiny neurons in the striatum (Walaas et al., 1983). Dopamine, acting on D1-like receptors, causes phosphorylation of DARPP-32. Conversely, dopamine, acting on D2-like receptors, causes the dephosphorylation of DARPP-32 (Nishi et al., 1997). DARPP-32 acts as a switch to potentiate dopaminergic signaling.

In pre-symptomatic R6/2 mouse model, deficiencies in dopamine signaling have been reported in the striatum. These include selective reductions in total levels of dopamine and DARPP-32 protein in medium spiny neurons (Bibb et al., 2000). In addition, D1 and D2 receptors mRNAs expression and protein were shown to be decreased in HD postmortem brain (Augood et al., 1997). The correlation between the progression of illness and loss of D1- and D2-receptors led to their use as *in vivo* markers (as PET markers) for the Huntington's disease degenerative process.

Alterations in dopamine signaling in HD might impact HD neurodegeneration. A study by Charvin et al. in 2005 demonstrated that low doses of dopamine act with mutant HTT and c-Jun N-terminal kinase (JNK), through the oxidative stress pathway, leading to an increase in death of cultured-neurons. In addition, low level of dopamine increased aggregates formation in the presence of mutant HTT, highlighting even more the toxic effect of changes in dopamine homeostasis. In support of these observations, double mice knock-out for dopamine transporter and knock-in for HTT with 92 CAG repeats showed acceleration in nuclear inclusion appearance, compared to simple HD model mice (Cyr et al., 2006).

Another example of dopamine toxicity in HD, could involve the synergistic action of glutamate and dopamine signaling pathways to induce elevated Ca²⁺ signals and to cause apoptosis of YAC128 medium spiny neurons. Elevation in action potential and excitotoxicity were mediated by D1-like receptors and NMDA receptors. The use of the dopamine pathway inhibitor, tetrabenazine, alleviated motor deficits and striatal cell loss in those mice (Tang et al., 2007). Interestingly, this drug is used in HD patients to reduce abnormal movements (Savani and Login, 2007).

Taken together, dopamine signaling is most probably participating in HD pathogenesis and neuronal cell death.

2.5 Metabolic and mitochondrial impairments.

Observations from HD patients and HD mice indicate a metabolic impairment that could contribute to HD pathogenesis. First, using positron emission tomographic (PET) glucose metabolic studies and magnetic resonance imaging (MRI) scans, HD patients showed a significant 3.1% loss of glucose metabolic rate per year in the caudate nucleus very early in the disease, in a pre-symptomatic stage (Grafton et al., 1992) (Kuwert et al., 1993). A decrease ATP synthesis was reported in striatal cells and rodents carrying the mutated HTT (Gines et al., 2003) and can be detected outside the brain such as in lymphoblast of the patients and was inversely correlated to the length of polyglutamine mutation. The ATP/ADP ratio worsens with CAG repeats, highlighting a dependency of the energy status to the dominant CAG mutation (Seong et al., 2005). Consistently, a hyper-metabolic state and weight lost is reported early in patients (Mochel et al., 2007).

At the cellular level, mitochondria is a major producer of ATP. The first focus has been made on the succinate dehydrogenase (SDH) due to the fact that the 3NP (3-nitropropionate), an inhibitor of SDH recapitulates features of HD (Palfi et al., 1996). SDH is an enzyme of the respiratory chain located at the inner membranes of the mitochondria and is part of the complex II. It was determined, by analyzing striatal neurons infected with a truncated N-terminal mutated HTT, that the loss of complex II subunit preceded neuronal death. Also correcting SDH loss suppressed cell death. Thereby, mitochondrial complex II defects is directly link to cell death (Benchoua et al., 2006) and suggests a mitochondrial defect in HD.

Mitochondria is the cradle of the oxidative stress. Oxidative stress is triggered by the generation of toxic species for the cell known as ROS (reactive oxidative species) including superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-). The activation of oxidative stress damages proteins, lipids and nucleic acids degradation. Many studies indicate an activation of oxidative stress in HD: strand break in DNA, accumulation of lipofuscin (a product of the oxidation of unsaturated fatty acids), induction of oxidative stress defense mechanism by overexpression of several anti-oxidant enzymes. (Browne and Beal, 2006; Browne et al., 1999; Sorolla et al., 2008). Interestingly, superoxide can directly inactivate aconitase, a mitochondrial iron sulfur protein in charge of electron carriage. Measurement of aconitase activity in HD lymphocytes and post mortem brain show that its activity drop to 8% in the caudate, while being unchanged in lymphocytes (Tabrizi et al., 1999). Therefore, oxidative stress observed in HD might contribute to cellular damage and death. However, except cysteamine that possess anti-oxidant properties, no anti-oxidant drugs have proved useful in HD.

Mitochondria are also central players in the process of caspases activation and apoptosis. Results showed a robust mitochondrial membranes depolarization in HD fibroblasts (Sawa et al., 1999). This lead to cytochrome c release and to caspases activation which in turn can cleave mutant HTT and promote the translocation of its mutated N-terminal fragments into the nucleus. Moreover, p53 was defined as interacting with mutant HTT in neuronal cells. In mutant HTT expressing neurons, a greater level and activity of p53 was measured (Bae et al., 2005). Genetic depletion of p53 in flies HD model represses neurodegeneration occurrence. As p53 regulates members of the Bcl-2 family and thus regulates the mitochondrial pore opening, it is most probably that mutant HTT, by affecting p53 activity, induces mitochondrial impairment, leading to neuronal death in HD.

New insights connect mitochondrial impairment in HD and transcription via PGC-1 α (peroxisome-proliferator-activated receptor γ coactivator-1 α). PGC-1 α is a transcription co-activator of genes implicated in mitochondrial biogenesis. A reduced level of its transcripts was observed in brain from HD mouse model and HD patients, raising the possibility of a negative effect of mutant HTT on PGC-1 α transcription (Cui et al., 2006) (figure 26).

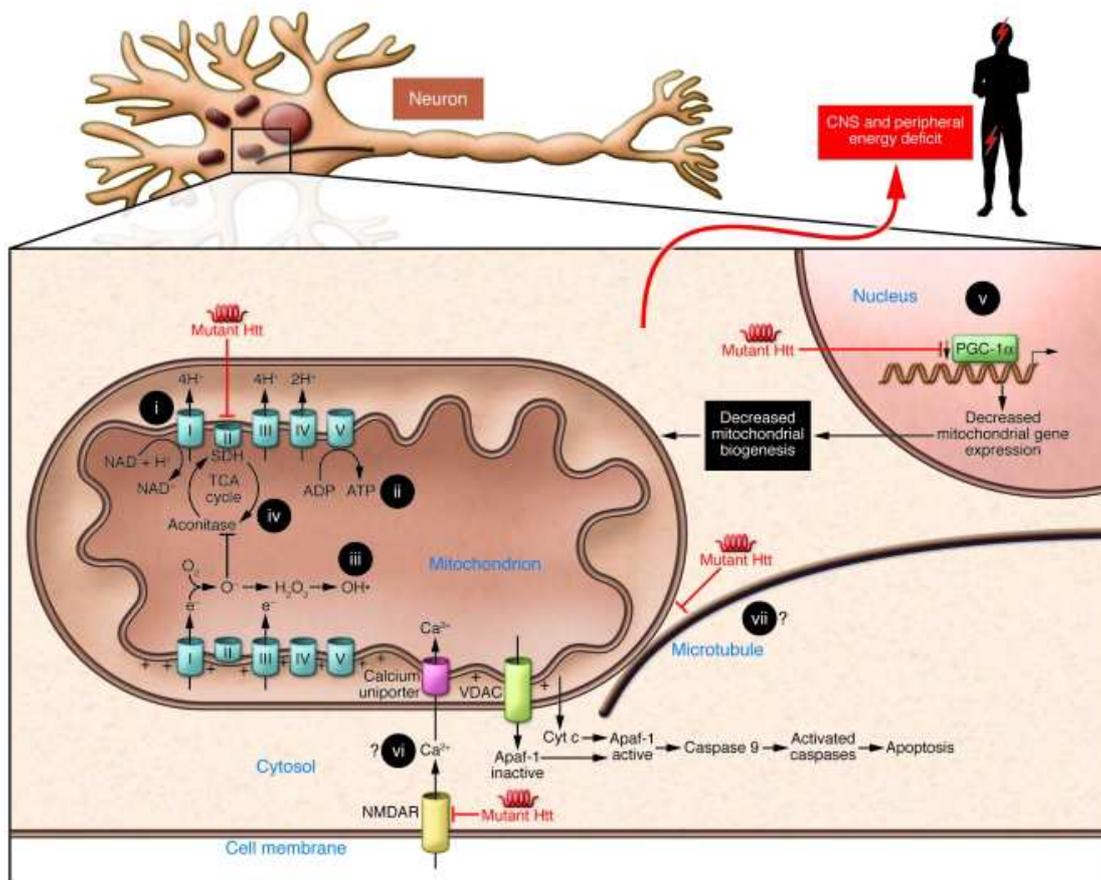


Figure 26: Summary indicating the main putative mechanisms by which mutated HTT causes mitochondrial dysfunction (Mochel and Haller, 2011). Such mechanisms would result in decreased mitochondrial biogenesis, oxidative stress, ATP deficit, increased apoptosis, and, ultimately, a central and peripheral energy deficit. Apaf-1, apoptotic protease activating factor 1; NMDAR, N-Methyl-D-aspartic acid receptor; VDAC, voltage-dependent anion channel.

Part III: Endoplasmic Reticulum in disease.

1. ER shaping.

1.1 Network structure.

The endoplasmic reticulum (ER) is a continuous membrane system made of the nuclear envelope, the ribosome-studded peripheral sheets and an interconnected network of smooth tubules extend throughout the cytoplasm (figure 27). The ER is a single entity with functionally and structurally distinct domains. The ER is related to several functions in the cell and proper maintenance of the network is crucial for cellular homeostasis. ER functions include membrane translocation, folding, post-transcriptional modifications and transport of both membranes and soluble proteins. ER is also consider to be the place for phospholipid synthesis and calcium homeostasis regulation (Baumann and Walz, 2001).

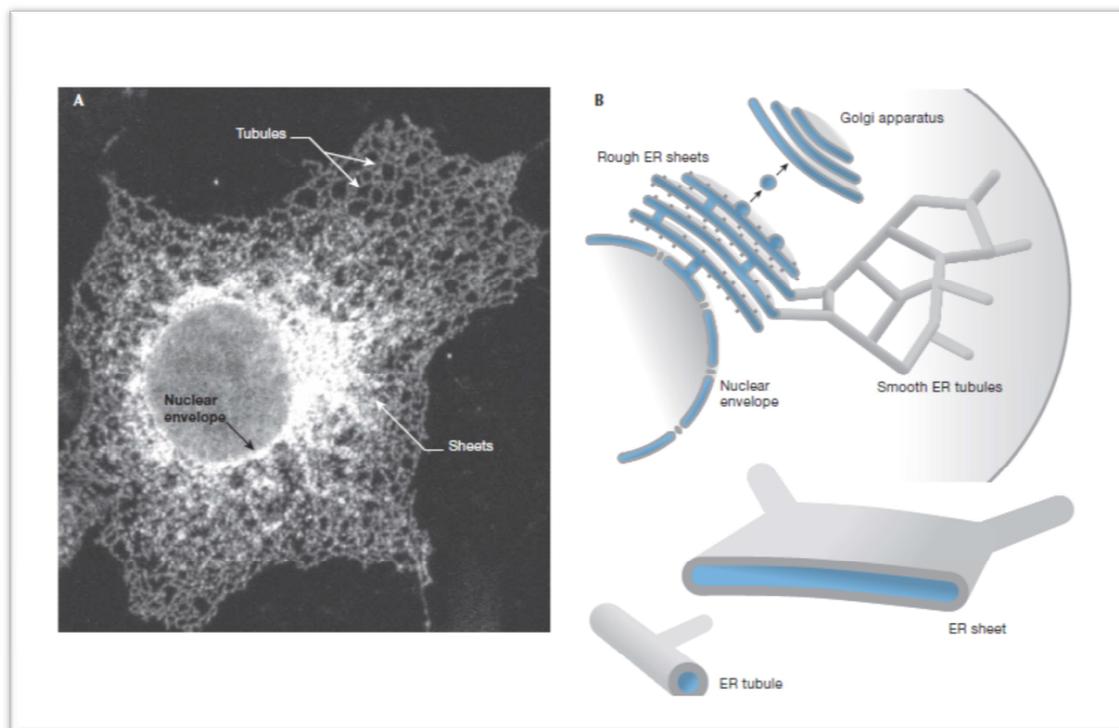


Figure 27: Morphology of the endoplasmic reticulum. (A) COS7 cell immunostained with the ER markers Sec61 β and DP1/REEP5 reveals the different domains, as indicated. (B) Schematic diagram showing the domain organization of the ER, with tubular and sheet morphologies (Park and Blackstone, 2010).

Tubules and sheets are thought to be connected all together so that ER membranes could harbor a single lumen which might fills 10% of the cell (Pendin et al., 2011). Peripheral ER sheets are decorated with ribosomes along their bilayer membranes to form the rough ER (RER) that is associated with the biosynthesis and quality control of secreted protein. According to the cell types,

RER can be different, in secretory such as pancreatic acinar cells, the RER exhibits extensive stacks related to the wide need of production and secretion of proteins. In contrast to ER sheets, ER tubules comprises mostly the smooth ER (SER), which are ribosomes-free and are associated with lipid synthesis and delivery. It is also important in forming contacts with other organelles (Shibata et al., 2006). In muscle cells, the SER is called the sarcoplasmic reticulum and is essential for the sequestration of the calcium and its release during a muscle contraction (Endo, 1977). Finally the nuclear envelope (NE) is perforated with multiples pores that regulates protein transport between the nucleoplasm and the cytoplasm. These morphological and functional differences of each compartment forming the ER is not well defined as ER domains are constantly dynamic. ER continuously undergoes membranes rearrangement of its structure. Vesicles bud from and fuses to the ER, new tubules are formed, some retract, reciprocal transition between sheets and tubules happens. All these events make the ER a highly dynamic organelle. Also during cell division and differentiation ER membranes reorganized (Lu et al., 2009; Puhka et al., 2007). In neurons, ER shape changes in dendritic spines to regulate the synaptic signaling pathway (Park et al., 2008a).

Mechanisms exist to generate and maintain the different domain of the ER. Indeed a machinery that regulate ER shape has been described and will be summarized below.

1.2 ER dynamicity.

1.2.1 The cytoskeleton.

The outgrowth and the retraction of tubules are highly dependent on the close association between ER and microtubules. Alteration of ER dynamics can be achieved by inducing depolymerization of microtubules in mammalian cells (Terasaki et al., 1986).

The commonly accepted model has emerged that microtubules and kinesin (kinesin-1) are essential determinants in establishing and maintaining the structure of the ER by active membrane expansion. Besides, ER membrane expansion is lost when cytoskeleton-linking ER membrane protein of 63 kDa protein of 63 kDa (CLIMP-63) is mutated, revealing stable attachment of ER membranes to the microtubule as a novel requirement for ER maintenance (Vedrenne and Hauri, 2006) (figure 28).

Thus, ER tubules associate with molecular motors along the microtubules (Waterman-Storer and Salmon, 1998). This mechanism is termed ER sliding and is the most common mechanism for controlling ER dynamic. It involves ER tubule extension along acetylated microtubules as experiments demonstrated that ER sliding occurs on nocodazole-resistant microtubules that are post-translationally modified by acetylation. High microtubules curvature was found to be a good

indicator of microtubule acetylation and ER sliding was seen to occur predominantly on these curved, acetylated microtubules. One effect of ER sliding could be to regulate its inter-organelle contact (Friedman et al., 2010). In yeast, ER movement is achieved along actin filaments but ER does not collapse with actin depolymerization (Prinz et al., 2000). The ER sliding mechanism is not fully understood but it may involve residual ER protein such as climp-63, known to have microtubule-binding domains (Shibata et al., 2010).

In addition, in mammalian cells, the tip of ER tubules is attached to the tip of plus end growing microtubules. One possible mechanism involves the ubiquitous ER protein, STIM1 that interacts with EB1, a protein at the plus end of microtubules. Stromal interaction molecule 1 (STIM1) is a transmembrane protein that is essential for calcium entry in response to the depletion of calcium stores in the endoplasmic reticulum. STIM1 is not motile by itself, it attaches and follows EB1 waves, this allowing tubules remodeling (Grigoriev et al., 2008). Hence, the cytoskeleton seems to be required for the maintenance of ER network, spreading over the cytoplasm. Instead, actors that shape the ER membranes are known as curvature-stabilizing proteins.

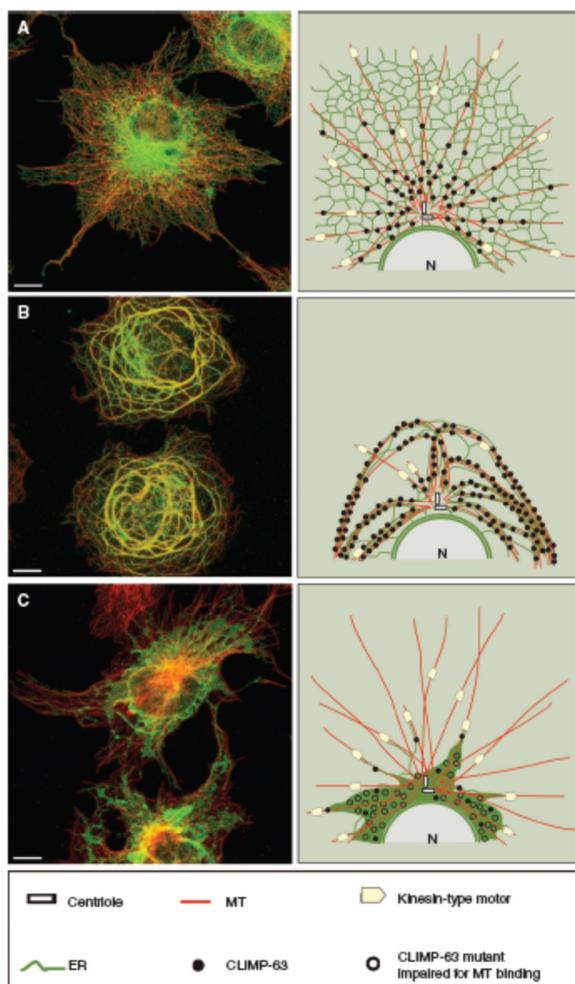


Figure 28: Role of CLIMP-63 in maintaining ER structure. Immunofluorescence microscopy (left panels) for CLIMP-63 (green) and microtubules (MTs) (red) in COS cells. Corresponding schematic interpretations are illustrated in the right panels. (A) Control cell. The ER extended network superimposed to microtubules. The extension of ER membranes is driven by molecular motors (yellow triangles). The ER network is stabilized by CLIMP-63-mediated anchoring of ER membranes to MTs (black circles). (B) Wild-type CLIMP-63-transfected cells. In cells overexpressing CLIMP-63, ER membranes and MTs form rope-like structures. (C) Cells overexpressing a mutant form of CLIMP-63 that do not bind MT. Newly formed ER tubules are not stable enough to elongate, and the ER concentrates in the center of the cell (Vedrenne and Hauri, 2006).

1.2.2 Curvature-shaping proteins.

Sheets are flattened structure with low membranes curvatures whereas, tubules show high membranes curvatures. Difference in membranes curvature seems to be what differentiate the rough and smooth subdomains from the ER. The shape of the membranes involves protein endowed with the ability to deform lipid bilayers. Indeed, peripheral membrane protein with an intrinsic curvature can bend the membranes, also transmembrane proteins with hydrophobic domains can insert a physical curvature into the outer monolayer of membranes (Shibata et al., 2009) (for hypothetical model see figure 29).

To identify protein involved in tubules curvature, Voeltz et al. used an *in vitro* system to demonstrate that network formation requires the integral membrane protein reticulon 4a (Rtn4a), a member of the ubiquitous reticulon family (a group of evolutionary conservative proteins residing predominantly in the ER). This was confirmed by the loss in network formation when inhibitors of Rtn4a (sulfhydryl reagents and antibodies) were used (Voeltz et al., 2006). Rtn4a interacts with another family called DP1 in mammals and Yop1p in yeast. Like Rtn4a, DP1/Yop1p exhibits a conserved domain with two hydrophobic hairpins in the membrane (Hu et al., 2011) (figure 29). Rtn4a and DP1/Yop1p are enriched at ER tubules and at the edges of ER sheets, suggesting a role in the formation of highly curved membranes. Indeed, in mammalian cells, depleting these proteins converted most of the cellular ER to sheets (Voeltz et al., 2006).

Similar results were obtained *in vivo*. Depletion of RET-1 and YOP-1 (homologues of human Rtn4a and DP1) in *C. elegans* leads to ER impairment and enhanced embryonic lethality (Audhya et al., 2007). Audhya and colleagues also showed that, *in vitro*, ER tubules formation required Rab-5 (a small GTPase implicated in endocytosis). The effect of Rab-5 depletion on ER structure phenocopied the depletion of YOP-1/RET-1. However, perturbing endocytosis by other means does not perturb the ER morphology.

Rtn and DP1 are able to reconstitute 15 to 17 nm tubules from liposomes (Hu et al., 2008). This ability comes from their affinity to oligomerize. Using fluorescence recovery after photobleaching, Shibata and co-workers found that yeast Rtn1p and Yop1p are less mobile in the membrane than normal ER proteins. Sucrose gradient centrifugation and cross-linking analyses showed that they form oligomers. Mutants with oligomerization defects did not exclusively localize to tubules and were unable to generate tubules *in vitro* (Shibata et al., 2008).



Figure 29: The Reticulons and DP1/Yop1p Shape ER Tubules (Hu et al., 2011).

“The reticulons and DP1/Yop1p might stabilize the high curvature of ER tubules seen in cross-section by using two cooperating mechanisms, hydrophobic insertion (wedging) and scaffolding. (1) The hydrophobic insertion mechanism is based on the two hydrophobic hairpins, which are proposed to form a wedge-like structure that displaces the lipids preferentially in the outer leaflet of the lipid bilayer, thus causing local curvature. (2) The scaffolding mechanism assumes that the reticulons and DP1/Yop1 form arc-like oligomers that mold the lipid bilayer into tubules. Arc-like scaffolds are consistent with the observation that the reticulons also localize to sheet edges. Because arcs are not entirely encircling a tubule, they would not block the long-distance diffusion of other membrane proteins, explaining the high mobility of these proteins.”

In contrast to tubules formation, we have a poor understanding of the mechanisms underlying sheets formation. The diameter of sheets are similar than tubules, around 30 nm in yeast and 50 nm in mammals (Bernales et al., 2006). They are stacked tightly with regular distances between them and interconnect with tubules. How sheets are form and maintain their curvature?

Only one work recently described a set of membrane proteins to be potentially implicated in this mechanism. These include proteins that modify newly synthesized polypeptides and coiled-coil membrane proteins. These proteins localize to polysomes (clustered ribosomes) and associate to the RER sheet’s membrane. Climp63 could be important in forming sheets. Indeed, it is implicated in the interaction of ER membranes with microtubules (Klopfenstein et al., 1998). Climp63 has a coiled-coil

domain, which was demonstrated to be located on the luminal side of the ER membrane. Although, the depletion of Climp63 did not abolish the existence of ER sheets, it was proposed to serve as a lumen spacer. In addition, the reticulons and DP1/Yop1p universal mechanism appeared to be involved as well in ER sheets formation (Shibata et al., 2010) (figure 30).

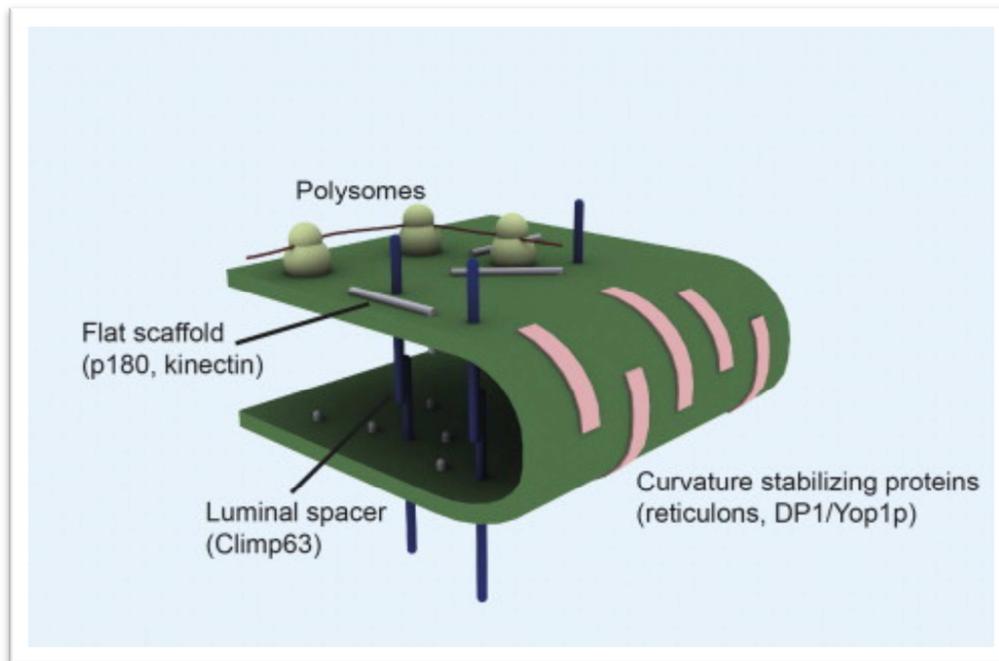


Figure 30: Mechanisms for generating ER sheets. Proteins like Climp63 (blue) form a luminal bridge via the coiled-coil domain. Proteins like p180 and kinectin (gray) are also enriched in sheets and form a flat scaffold with their cytosolic coiled-coil domains. Curvature-stabilizing proteins (pink) localize at the edge and maintain the high curvature. Polysomes may also facilitate sheet formation (Lin et al., 2012).

1.2.3 Fusion and fission.

Beyond tubules and sheets generation, the ER membranes must interconnect in order to form a functional network. This requires a fusion that could involve the p97/p47/VCI135 complex. p97 (an abundant ATPase of the AAA family) with its cofactor p47 was previously implied in the fusion steps of isolated ER membranes *in vitro* (Hetzer et al., 2001). Kano and colleagues developed an assay system to investigate the role of those factors in ER structural changes *in cellulo*. In cells that constitutively expressed heat shock protein-47 fused to the green fluorescent protein as an ER marker, they showed that p47 was essential for the maintenance of the ER network, this process requiring ATP/GTP hydrolysis. This implied that the connection within the ER network requires a membrane fusion process mediated by p97/p47. In addition, they found that phosphorylation of p47

by cdc2 kinase resulted in ER network disruption during mitosis. Thus, the cell cycle-dependent morphological changes of the ER network are regulated through phosphorylation/dephosphorylation of p47 (Kano et al., 2005).

In 2002, a novel essential factor for p97/p47-mediated membrane fusion, named VCIP135 (valosin-containing protein VCP p97/p47 complex-interacting protein, p135) was identified. VCIP135 binds to the p97/p47/syntaxin5 complex and dissociates it via a p97 catalyzed ATP hydrolysis. In living cells, VCIP135 and p47 were shown to function in Golgi and ER assembly (Uchiyama et al., 2002).

In 2009, two groups, the teams of Blackstone and Daga, discovered Atlastin 1 as an important mediator of ER fusion *in vivo*. Atlastin are large GTPases belonging to the family of dynamin-related GTPases that associate with different intracellular membranes compartments (Praefcke and McMahon, 2004). Dynamin-1 is the typical protein belonging to this family and act in vesicular budding from the plasma membranes during endocytosis. Mutations in atlastin-1 causes hereditary spastic paraplegia SPG3A characterized by degeneration of long axons in the ascending and descending tracts of the spinal cord. In mammals, there are 3 isoforms of atlastin, while invertebrates possess only one ortholog.

Atlastin-1 is predominantly expressed in brain and was found to be localized at the cis-Golgi and the ER. Cross-linking studies showed that it forms oligomer *in vivo*. Membrane fractionation and protease protection assays revealed that atlastin-1 is an integral membrane protein with two predicted transmembrane domains; the N-terminal GTP-binding and C-terminal domains are exposed to the cytoplasm (Zhu et al., 2003).

The Knockdown of atlastin-2 and -3 in HeLa cells using siRNA caused disruption of Golgi morphology and expression of a dominant-negative atlastin-1 lacking GTPase activity causes prominent inhibition of ER reticularization, suggesting a role for atlastin GTPases in the formation of three-way junctions in the ER. However, the assessment of the secretory pathway from ER to the Golgi, vesicular trafficking was normal in both knockdown and dominant-negative overexpression conditions for all atlastins. This study demonstrated that the atlastin family of GTPases functions prominently in both ER and Golgi morphogenesis, but they do not appear to be required for ER-to-Golgi trafficking (Rismanchi et al., 2008).

The single *Drosophila* atlastin ortholog has been demonstrated to be the GTPases responsible for homotypic fusion of ER membranes. In contrast with human's atlastin, fly's atlastin is restricted to the ER. In vivo depletion of the *Drosophila* atlastin caused ER fragmentation while its overexpression caused an excessive ER fusion. The suggested mechanism is the following: *Drosophila* atlastin tethers ER membranes together through trans-oligomer formation. GTP hydrolysis brings closely the two adjacent ER membranes, promoting lipid fusion (Orso et al., 2009) (figure 31).

The same mechanism of ER homotypic ER fusion occurs in yeast (Hu et al., 2008). The Sey1p shares the same signature motifs than atlastin and interacts with both ER-shaping protein, Yop1 and reticulons. Blackstone and colleagues observed that deficiency in Sey1p directly affect ER branching and result in the generation of long, unbranched ER tubules. The results raise the possibility that Atlastin/Sey1p might simply regulates the reticulons and Dp1/Yop1 activity. Loss in Sey1p may lead to an excess activity of ER-curving proteins and hence, the formation of unbranched tubule may be an interpretation of an excessive ER tubulation.

Recently, a member of the conserved Lunapark family, was reported to act in ER network formation. Lnp1p binds to the reticulons and Yop1p and resides at ER tubule junctions in both yeast and mammalian cells (figure 32). In yeast, localization of Lnp1p is regulated by Sey1p, the yeast ortholog of atlastin (Chen et al., 2012). Thus, the model proposed is the following: Atlastin and its yeast homologue, Sey1p, mediate homotypic ER membrane fusion, which leads to the formation of new three-way junctions within the polygonal network. At these junctions, the Lunapark protein, Lnp1p, works in conjunction with the reticulons, DP1/Yop1p, and in antagonism to atlastin/Sey1p to maintain the network in a dynamic equilibrium (Chen et al., 2012, 2013).

However some questions remains unanswered concerning the regulatory mechanisms of ER shaping. Does the ER shape maintenance require fission events? No studies have demonstrated that ER fission occurs. However, fusions events are likely to be balanced by fission events. Moreover, during cell division, cells are able to break down their ER network in order to proceed in mitosis. This might be regulated by fission mechanisms. Therefore, ER could be regulated similarly to mitochondria whose shape are conserved by the mean of fusion and fission events which are mediated by the large GTPases of the dynamin family, mitofusins (OPA1 and DRP1) (Chan, 2006).

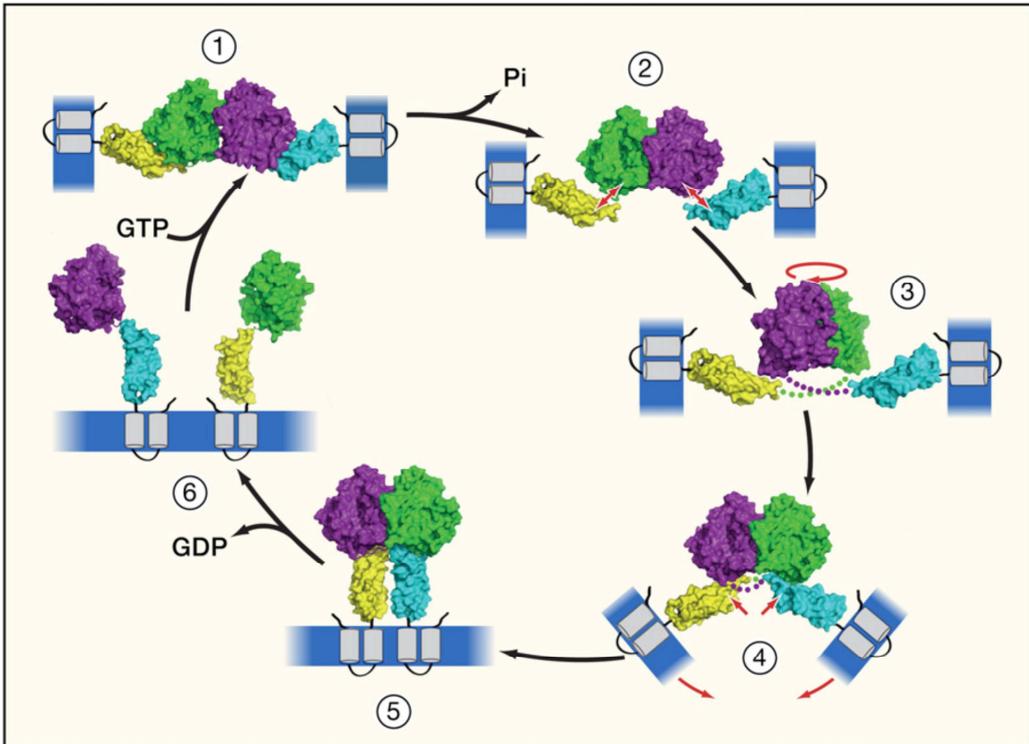


Figure 31: The Fusion Reaction Mediated by the Atlastins. Atlastin in one membrane is colored with its GTPase domain in green and the helix bundle in yellow. In the opposing membrane, it is colored with in purple and cyan respectively. Upon GTP binding, the atlastins dimerize and tether the membranes. GTP hydrolysis then causes conformational changes that pull the membranes together. Finally, GDP is released to reset the fusion machinery (Hu et al., 2011)

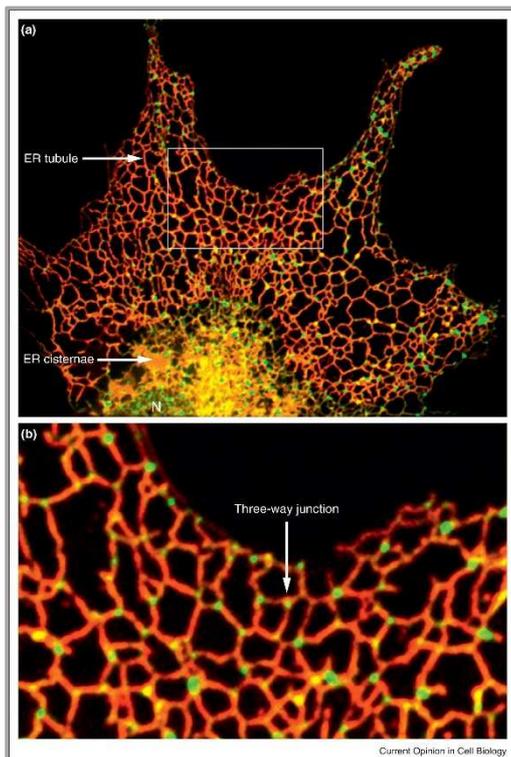


Figure 32: The ER network. (a) The ER network of COS-7 cells is labeled with mCherry-KDEL (red) and the junctions are labeled with Lnp1-GFP (green). Also marked: nucleus (N), ER cisternae and ER tubules. (b) The box area marked in the top panel is magnified below. Interconnected ER tubules form a network with three-way junctions marked by Lnp1-GFP (Chen et al., 2013).

The current picture on the mechanisms involved in the organization of the ER within the cell can be summarized as the following (figure 33):

(A) ER tubules move by attaching to the end tip of microtubules (left) or by a sliding mechanism (right).

(B) The reticulons and DP1/ Yop1 shape flat membranes into curved tubules. Their hydrophobic segments insert like a wedge in the outer lipid layer causing the bilayer to bend and their ability to oligomerize may produce an arc-like scaffolds around the tubules.

(C) ER sheets can be generated by the presence of the reticulons and DP1/ Yop1 at their edges to stabilize locally the high curvature. In addition, climp-63 serves as a luminal spacer to maintain the two membranes flat and at a constant distance.

(D) Two ER tubules in the process of being merged by the fusogenic activity of the atlastin GTPase. (Pendin et al., 2011)

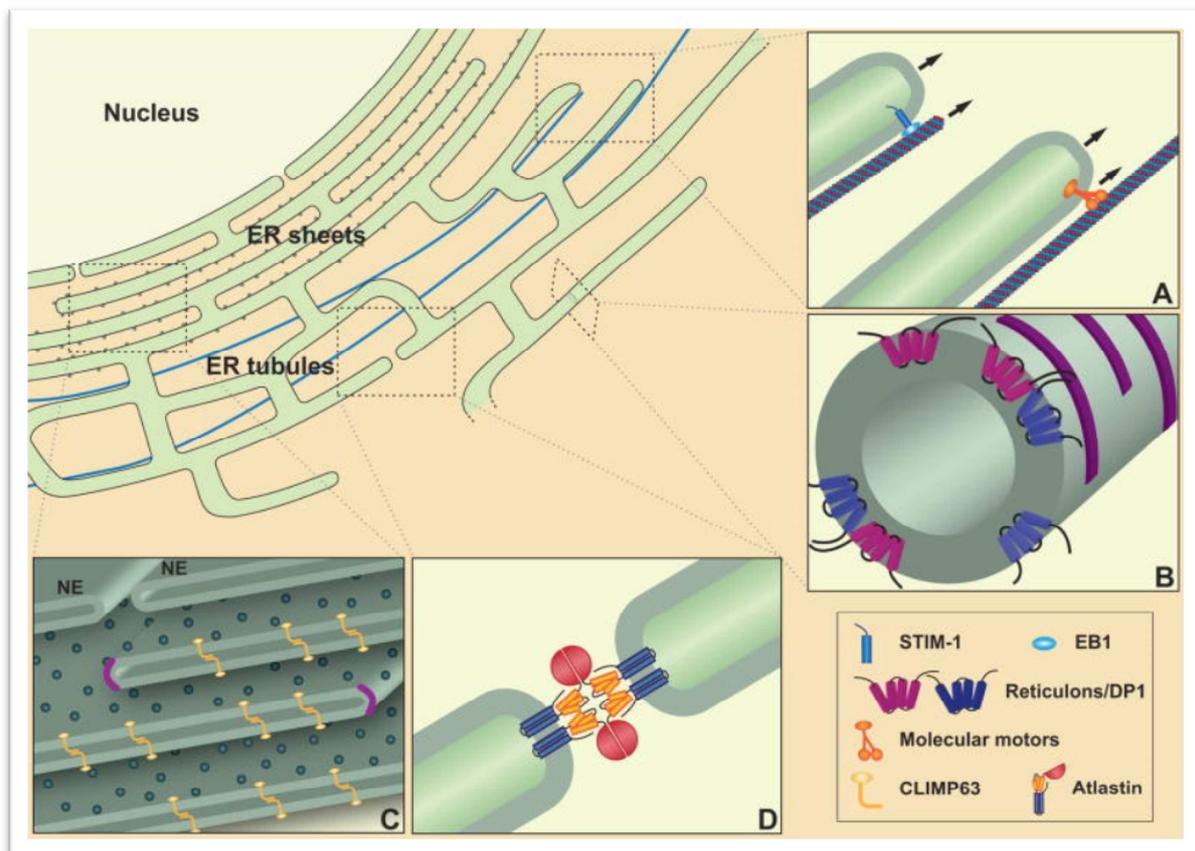


Figure 33: Domain organization of the endoplasmic reticulum network within the cell (Pendin et al., 2011).

2. ER stress pathways.

The ER is the house keeping of secretory and membrane proteins synthesis. Such protein must be properly folded and post-translationally modified before exiting the organelle. It is estimated that around 30% of newly synthesized proteins are degraded (Schubert et al., 2000). Thus cells constantly face the problem of misfolded proteins production. The situation worsen when the cell undergo perturbations such as changes in the calcium level or the redox states. The ER function will be then compromised leading to an overload of misfolded proteins. Under such conditions, the cell activates signaling cascades that attempt to restore the favorable folding environment. This is termed ER stress. The response for ER stress is the unfolded protein response (UPR). UPR works to undo the harms of misfolded proteins accumulation. If not sufficient, cell death pathways are triggered.

The UPR consists of three main signalings initiated by three ER localized stress sensors: IRE1, PERK and ATF6, all of which activates transcription factors that mediate the induction of ER stress response genes.

2.1 The IRE1 pathway.

IRE1 (inositol requirement 1) is a type 1 transmembrane protein with serine/threonine kinase activity. It was identified by a genetic screen in yeast as an ER stress sensor that would mediate communication between the ER lumen and the nucleus. Thus IRE1 is a the transmitter of the unfolded protein signal across the ER lumen to the nucleus (Cox et al., 1993). The luminal domain of IRE1 is involved in the sensing of unfolded proteins, whereas the cytoplasmic domain contains a kinase domain and an RNase domain. Activation of IRE1, like other tyrosine kinases, is done through oligmerization and trans-phosphorylation (Shamu and Walter, 1996). Once, activated, the ensuing signaling cascade occurs: the RNase activity of the C-terminal part of IRE1 catalyzes the splicing of XBP1 (x-box binding protein 1) pre-mRNA into mature mRNA by an unconventional splicing mechanism (Calfon et al., 2002). A protein translated from this mature mRNA translocates into the nucleus, binds the UPR promoter and activates the transcription of various gene required for protein folding and ERAD (endoplasmic reticulum associated protein degradation) (Travers et al., 2000).

In addition to the splicing of XBP1, IRE1 cleaves additional mRNA targeted to the ER and thus play a role in restricting the translation attenuation occurring during ER stress (Iwawaki et al., 2001).

How does IRE1 sense the ER stress? Studies have identified the ER resident chaperones protein Grp78 (glucose regulated protein 78) also termed BiP, as a regulator of IRE1 activity. Experiments showed that the loss of BiP correlated with the formation of high-molecular-mass complexes of activated IRE1, and overexpression of BiP attenuates its activation. This suggests that BiP represses ER stress signaling in normal homeostasis; the accumulation of misfolded proteins relieves this repression by effecting the release of BiP from IRE1 luminal domains (Bertolotti et al., 2000).

2.2 The PERK pathway.

Similar to the IRE1 receptor, PERK is a type I transmembrane protein located in the ER, which senses the accumulation of unfolded proteins in the ER lumen. The luminal portion of PERK is involved in sensing unfolded proteins, whereas the cytoplasmic portion contains a kinase domain. In the absence of ER stress, BiP binds to the luminal domain of PERK and keeps it from being activated through oligomerization and trans-phosphorylation. Once activated, PERK phosphorylates the serine 51 of eukaryotic initiator factor 2 α (eif2 α) (Harding et al., 1999), this prevents eif2 α from promoting translation of proteins in the cell. As a result a drastic decrease in global protein synthesis is observed in the cell (Ron, 2002).

Moreover, PERK signaling initiates the translation of the ATF4 transcription factor which in turn upregulates CHOP and anti-oxidative stress genes. CHOP is a bZip transcription factor that belongs to the C/EBP family known to induce the expression of a set of genes involved in the UPR through binding to a specific DNA element present in their corresponding promoters (Mori et al., 1998; Wang et al., 1996). The phosphorylation of PERK is transient as the protein is dephosphorylated by specific phosphatases such as CReP (constitutive repressor of eIF2 α phosphorylation), protein phosphatase 2C-GADD34, and p58IPK. CReP is constitutively expressed, whereas the expression of GADD34 and p58IPK is induced on ER stress by PERK and activating transcription factor 6 (ATF6) pathways, respectively (Jousse et al., 2003; Novoa et al., 2001; van Huizen et al., 2003; Yan et al., 2002).

ATF4 is only translated when eif2 α is phosphorylated. Indeed, ATF4 encompasses several ORFs (Open Reading Frame). When eif2 α is active (none phosphorylated) only the small ORF are translated by the ribosomes as ribosomes are released before they reach the ATF4 ORF. Upon ER stress, most of the eIF2 α becomes inactive (phosphorylated), and translation rarely starts at the small ORFs, thus ribosomes can reach the ATF4 ORF and induce translation of ATF4 protein.

Translation attenuation is thought to be an adaptive response to the ER stress to relieve the ER protein load. PERK signaling also coordinates with cell cycle arrest, which reduces energy consumption and allows cells to re-establish homeostasis. In support, PERK activation triggers phosphorylation of eif2 α and leads to repression of protein synthesis by inhibiting cyclin D1 translation thereby contributing to cell cycle arrest (Brewer et al., 1999). However the situation is likely to be more complex. It was shown that cells depleted from PERK have an attenuated cell cycle arrest that is not attributable to cyclin D1 loss. Indeed, the UPR could trigger p53 accumulation and activation leading to a p53-dependent cell cycle arrest (Zhang et al., 2006a).

2.3 The ATF6 pathway.

The third ER stress sensor molecule is ATF6. Two isoforms exist in mammalian cells (α and β) with both showing a ubiquitous expression. ATF6 is a type II transmembrane protein, the luminal domain is responsible for the sensing of unfolded proteins. The cytoplasmic portion of ATF6 has a DNA-binding domain containing the basic-leucine zipper motif (bZIP) and a transcriptional activation domain. The activation of the pathway involves ATF6 proteolysis. The mechanism is termed RIP (regulated intra-membrane proteolysis) as cleavage occurs in the transmembrane region of ATF6 on the Golgi compartment. In the absence of ER stress, BiP binds to the luminal domain of ATF6 and impedes the Golgi-localization signal, leading to inhibition of ATF6 translocation (Chen et al., 2002). Under ER stress, Bip is removed revealing two Golgi localization sequence. This allows ATF6 to be targeted to COPII vesicles and to be anterogradely transported to the Golgi. The Full length ATF6 is then subjected to cleavage by two proteases that resides in the Golgi, S1P (site-1 protease) and S2P (site-2 protease). Cleavage releases the cytosolic part of ATF6 and generates a 50 kDa protein which consists of a transcription factor that translocates to the nucleus. This factor, like IRE1 and ATF4, binds to a set of genes involved in ER chaperones expression.

Recently, several bZIP transcription factors located at the ER and regulated by RIP have been reported. CREBH (cAMP response element-binding protein H), enriched in liver, is processed by S1P and S2P in response to ER stress. This stimulates the transcription of genes involved in acute inflammatory responses. OASIS (old astrocyte specifically induced substance) is also cleaved by S1P and S2P in response to ER stress in astrocytes and activates the transcription of BiP. These tissue specific ATF6-like molecules may contribute to the ER stress response (Kondo et al., 2005). (for summary see figure 34)

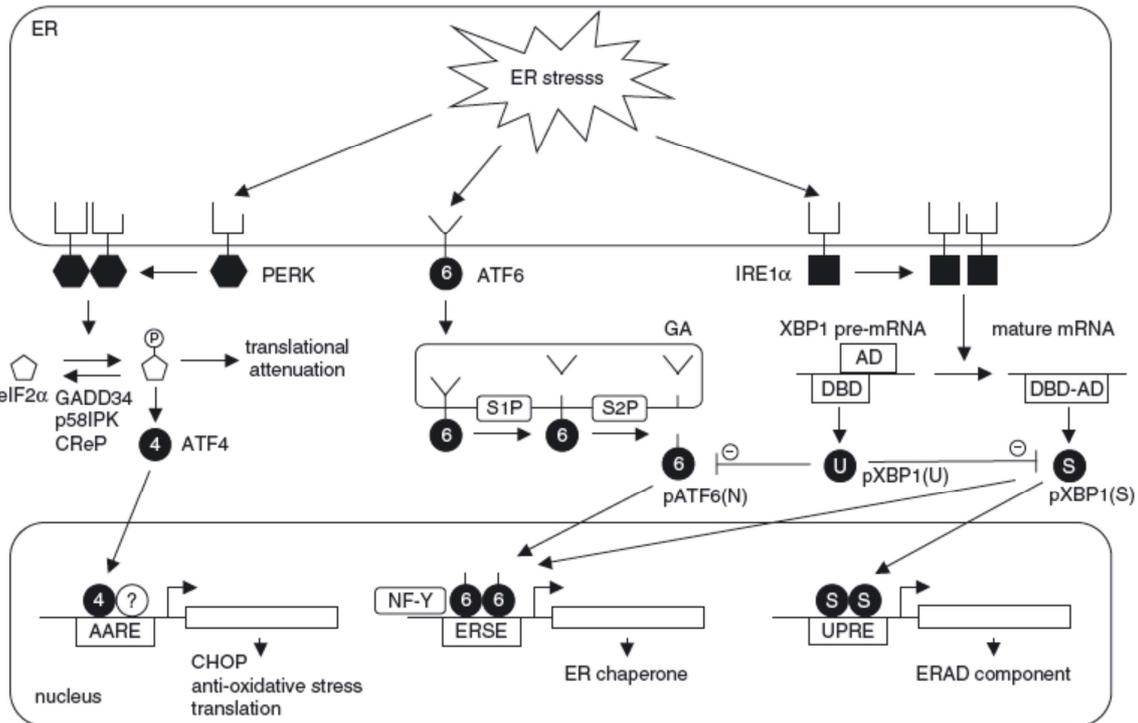


Figure 34: Mammalian response pathways for ER stress. Three response pathways (PERK, ATF6, and IRE1 pathways) regulate the mammalian ER stress response. The sensor PERK, phosphorylates eIF2 α to weaken translation, and to up-regulate expression of ATF4, leading to greater transcription of target genes such as CHOP. ATF6, a transmembrane transcription factor, is translocated to the Golgi apparatus and cleaved by proteases such as S1P and S2P, leading to enhanced transcription of ER chaperone genes. IRE1, a transmembrane RNase, splices XBP1 pre-mRNA, and pXBP1(S) translated from mature XBP1 mRNA activates transcription of ERAD component genes.

2.4 ER stress-associated apoptosis.

When PERK, IRE1 and ATF6 signaling cannot suppress the ER stress, survival is no longer ensured and cell death is triggered mainly by apoptosis. CHOP (C/EBP homologous protein transcription factor), JNK (c-Jun NH2-terminal Kinase) and caspases have been implicated in mediating apoptotic signaling in response to ER stress.

During prolonged ER stress, CHOP is highly upregulated. *CHOP*^{-/-} cells are more resistant to ER stress-induced apoptosis. *CHOP*^{-/-} mice were injected with tunicamycin, a drug that induces ER stress. In *CHOP*^{+/+} and *CHOP*^{+/-} mice, ER stress was associated with the early expression of CHOP in the proximal tubules (tubular cells form the tubules that transports urine to the ureters), followed by the development of an illness characterized by transient renal insufficiency. In the *CHOP*^{-/-} animals,

despite of a severe impairment of renal function, evidence of cellular death (TUNEL positive cells) in the kidney was reduced compared to wild type mice. This study demonstrated a role of CHOP in the induction of cell death under conditions associated with ER stress (Zinszner et al., 1998). It was also shown that CHOP down regulates the expression of the anti-apoptotic protein Bcl-2 and increases cellular level of ROS, two events that could be associated with cell death under ER stress (McCullough et al., 2001).

Inflammatory signals, changes in levels of reactive oxygen species, ultraviolet radiation, protein synthesis inhibitors and a variety of stress stimuli can activate JNK (Vlahopoulos and Zoumpourlis, 2004). In response to ER stress, IRE1 activation lead to the recruitment of TRAF2 (TNF-receptor-associated factor 2) and JIK (c-Jun NH2-terminal inhibitory Kinase), forming a complex that in turn recruits and activates apoptosis signal-regulating kinase 1 (ASK1). Consequently, the JNK pathway is activated and leads to cell death (Nishitoh et al., 2002).

Cleavage of caspase-12 is associated to ER stress-induced cell death. Caspase-12 is located at the ER and is processed by ER stress induction. Stress signals include disruption of ER calcium homeostasis and/or accumulation of excess proteins in ER, but not mitochondrial-targeted apoptotic signals. Mice that are deficient in caspase-12 were resistant to ER stress-induced apoptosis, but their cells undergo apoptosis in response to other death stimuli. However, in these experiments, absence of caspase-12 causes only a reduction of about 12% in ER-stress-induced apoptosis (Nakagawa et al., 2000). Controversial studies showed that melanoma cells die when treated with the ER stress-inducing agent whether they express caspase-12 or not (Kalai et al., 2003). This suggests that other mechanisms may be operating. Caspase-8 deficiency also inhibited the cytochrome c release and DNA fragmentation induced by ER stress suggesting that caspase-8 may be another ER-stress-associated protease. Thus, in parallel with the caspase-12 activation, ER stress triggers caspase-8 activation, resulting in cytochrome c/caspase-9 activation (Jimbo et al., 2003).

Caspase-7 was reported to cleave caspase-12 after ER stress induction leading to its activation (Rao et al., 2001). Also m-calpain may be responsible for cleaving procaspase-12 to generate active caspase-12 (Nakagawa and Yuan, 2000). However, the processing of caspase-12 by other proteases requires further investigation. *In vitro* studies suggest that the proteolytic activity of caspase-12 is confined to its own proenzyme. It is proposed that caspase-12 mediates its autocatalytic maturation and is not able to cleave other substrates. Caspase-12 form a complex with and inhibits caspase-1. During inflammatory response, its auto-cleavage could release its inhibitory effect on caspase-1 (Roy et al., 2008).

Failure in calcium homeostasis can follow ER stress as calcium is released from ER cisternae. Indeed, Bak and Bax undergo conformational changes and oligomerization under ER stress conditions resulting in calcium release from the ER (Zong et al., 2003). Increase in the cytosolic concentration of calcium activates calcium dependent proteases such as calpain and calcineurin. Calpain led to the activation of caspase-12 subsequently activating caspase-9 and thus release the pro-apoptotic activity of caspase-3 (Morishima et al., 2002). Embryonic fibroblasts derived from mice genetically depleted for calpain show resistance to endoplasmic reticulum (ER) stress-induced apoptosis, and this was directly related to a calpain requirement for activation of both caspase-12 and the ASK1-JNK cascades. This revealed a novel role for calpains in ER stress-induced apoptosis and JNK activation (Tan et al., 2006).

In conclusion, three different pathways respond to stress emanating from the ER. In diseases associated with proteins misfolding such as polyglutamine diseases, ER stress signaling is probably an important element that could participate in the pathogenesis (figure 35).

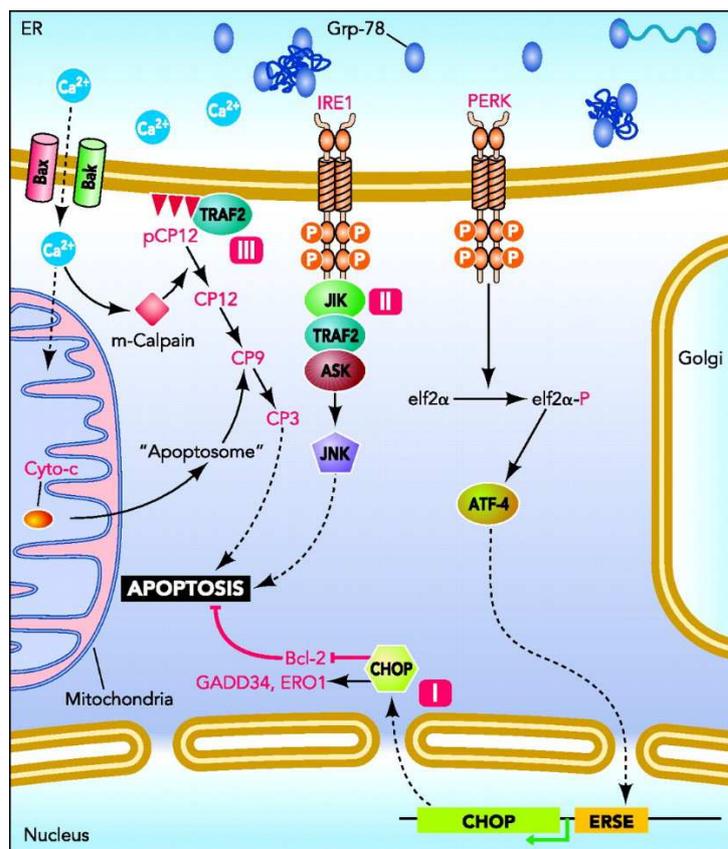


Figure 35: ER stress pathways implicated in mediating cell apoptosis. I: activation of the PERK pathways & induction of CHOP, which downregulates the expression of Bcl-2. II: activated IRE1 binds JIK and recruits TRAF2. III: the recruitment of TRAF2 to IRE1 and dissociation from procaspase-12 (pCP12), allowing pCP12 activation. During ER stress, Bax and Bak in the ER membrane oligomerize and allow the release of Ca²⁺ from the ER to the cytosol, which activates m-Calpain, which subsequently activates pCP12. This activates caspase-9 (CP9), and caspase-3 (CP3). Ca²⁺ released from the ER causes mitochondrial inner membrane depolarization and cytochrome c release into the cytoplasm. This allows the formation of the apoptosome and CP9 activation, leading to cell apoptosis (Lai et al., 2007).

3. ER stress and neurodegenerative diseases.

The ER stress is associated with a wide range of diseases including neurodegenerative diseases (Scheper and Hoozemans, 2009). As described earlier, neurons are sensitive to misfolded proteins and their survival depend on quality control machineries, such as the UPS and ERAD.

Disruption of the co-chaperone BiP results in the accumulation of protein aggregates and neurodegeneration in mice. Mice homozygous for *woozy* (*wz*), a spontaneous recessive mutation in the *SIL1* gene, leads to adult-onset ataxia with loss in Purkinje cell. Affected cells displayed proteins accumulation, evocative of protein inclusions, in both the ER and the nucleus. Upregulation of the unfolded protein response, indicative of ER stress, was observed in those mice. The mechanism involved SIL1, a nucleotide exchange factor which resides in the ER, suggesting the importance of ER machinery in neuronal survival (Zhao et al., 2005).

The ER stress response is activated in patients with Alzheimer's disease, a common neurodegenerative disease, characterized by cerebral neuritic plaques of amyloid β -peptide (A β) (Hoozemans et al., 2005). So far, a set of genes have been identified to be link to the disease appearance, including the amyloid precursor protein (APP) and PS-1. Mutations in the presenilin-1 (PS-1) gene caused early onset familial Alzheimer's disease (FAD) by a mechanism believed to involve perturbed endoplasmic reticulum (ER) function and altered proteolytic processing of the amyloid precursor protein (Mattson et al., 1998). PS1 is an integral membrane protein expressed in neurons throughout the brain where it is localized primarily in the ER (Selkoe, 2001).

In mice expressing FAD-PS1 mutations, it was reported a marked increase of the pro-apoptotic transcription factor CHOP, also known as Gadd153 (growth arrest- and DNA damage-inducible gene 153). An increase in CHOP response induced by ER stress agents was observed in PS1 mutant cells, leading to an enhanced cell death. This effect is skirted by silencing CHOP and suggests a contribution of CHOP in the toxicity observed in FAD (Milhavet et al., 2002). Also, PS-1 mutation sensitized cells to ER stress-induced cell death as it could disrupt IRE1, PERK and ATF6 ER stress pathways (Katayama et al., 2001; Katayama et al., 2004). Upregulation of PERK pathway correlates with abnormally phosphorylated tau in AD (Unterberger et al., 2006).

Parkinson's disease (PD) is another common neurodegenerative disease, which is characterized by a loss of dopaminergic neurons. Three of the genes linked to disease encode proteins that could have an effect on ER homeostasis: Parkin, α synuclein and ubiquitin C-terminal estherase L1.

The α -synuclein forms aggregates termed Lewis bodies typical of PD (Yoshida, 2007). Accumulation of α -synuclein within the ER leads to chronic ER stress and neurodegeneration. Salubrinal, an ER stress inhibitor compound, significantly attenuated neurodegeneration in a mouse model of α -synucleopathies (Colla et al., 2012).

Hereditary mutations in the ER-associated E3 ubiquitin ligase Parkin have also been associated with ER stress-induced cell (Takahashi et al., 2003). Parkin was shown to be involved in the ERAD system and interestingly, expression of Parkin is induced by ER stress, and neuronal cells overexpressing Parkin are resistant to ER stress (Shimura et al., 2000). Also, expression of ER chaperones, PDI, involved in the formation of disulfide bonds in the ER, is up-regulated in the brain of PD patients, and PDI is accumulated in Lewy bodies (Conn et al., 2004).

Recently, the communication of mitochondria with ER compartments has been associated with neurodegeneration in PD. Mutations in *DJ-1* gene, first identified as an oncogene, have been found to be causative for autosomal recessive familial Parkinson disease. DJ-1 has a role in the maintenance of mitochondria structure by favoring the endoplasmic reticulum (ER)-mitochondria tethering. Decrease of DJ-1 levels results in mitochondria fragmentation and loss of mitochondrial-calcium buffering in cells. Thus, the impairment of ER-mitochondria communication, when DJ-1 is mutated, may at the basis of mitochondrial and/or ER dysfunction observed in Parkinson disease (Ottolini et al., 2013).

Prion diseases are neurodegenerative diseases due to an accumulation of infectious agent composed of misfolded proteins (prion proteins). Accumulation of prion proteins caused persistent translational repression of global protein synthesis by P-eIF2 α that is associated with synaptic failure and neuronal loss in prion-diseased mice. Promoting translational recovery is protective in those mice. Given the incidence of protein misfolding, these results suggest that modulating ER stress response level is beneficial for survival of neurons in neurodegenerative diseases (Moreno et al., 2012).

In HD, mutant HTT induces, as mentioned previously, the misfolded protein response and in some models causes a global reduction in proteasome activity, which is an essential component of

ERAD (Thomas et al., 2005). ER stress could activate ASK1 in models of HD, and cells lacking this protein are protected from polyglutamine-HTT-induced cell death (Nishitoh et al., 2002). In support, the levels of ASK1 protein and ER stress markers were increased in the striatum and cortex of R6/2 mice. ASK1 and mutant HTT were found to interact. Inhibition of ASK1 prevented the translocation of HTT fragments to the nucleus, this correlated with improved motor function and reduced neuronal atrophy. Thus active Ask1 may help HTT fragments to enter the nucleus, while inactivated Ask1 hinders this translocation (Cho et al., 2009).

In addition to abnormal ER stress activation that may be due to the accumulation of misfolded HTT, an enlarged ER compartment was detected in immortalized striatal cells derived from HD knock-in mice (Trettel et al., 2000). Some dysregulations of the ER can be also due to the normal function of HTT. Indeed, depletion of HTT in cells led to an aberrant configuration of the ER network suggesting a function of the HTT over ER shaping, nevertheless, further studies are required to determine whether distorted ER network participates to HD pathogenesis (Omi et al., 2005).

Examples of the importance of ER morphology and distribution in highly polarized cells, such as neurons, are provided by the study of the hereditary spastic paraplegias. These diseases are characterized by an axonal degeneration of the cortico-spinal motor neurons, which can reach up to 1 meter in length. In almost 60% of all affected individuals, the disease is due to autosomal dominant mutations in one of three proteins that have a role in ER network formation: spastin (SPG4), atlastin 1 and REEP1 (belong to DP1/Yop1 family); Salinas et al, 2008). These proteins interact together and are localized to the ER in the cortico-spinal neurons, where they mediate ER shaping and interactions of the tubular ER network with the microtubule cytoskeleton (Salinas et al., 2008).

Chapter II: Results

News interactors:

Yeast two hybrid screen on the C-terminal HTT fragment.

If HTT cleavage generates short N-terminal fragments whose amino-acid position range from 1-105 to 1-586, it also generates corresponding C-terminal fragments. Such fragments have been observed in HD post mortem brain samples (Mende-Mueller et al., 2001). Two publications have reported that C-terminal fragments localize in the cytoplasm (Hackam et al., 1998; Takano and Gusella, 2002). However, no studies have investigated whether C-terminal HTT interacts with other proteins and/or could participate to disease. Therefore, we search for potential interactors of the C-terminal HTT fragment were chased using a yeast two hybrid (Y2H) approach.

As discussed in the manuscript, the published Y2H experiments mainly used the N-terminal fragments as a baits and these studies focused on preys that show differential affinity with normal and mutated HTT. Interestingly, in the search for genetics modifiers, Kaltenbach and colleagues used C-terminal fragments ranging from 1100 to 3144 amino acids in their pull down assays and yeast two hybrid screening. Nevertheless, they failed to identify new HTT interactors with these fragments (Kaltenbach et al., 2007). To optimize the strategy for identifying potential C-terminal HTT interactors, we searched the primary sequence of HTT ortholog for highly conserved regions. We analyzed 32 orthologous sequences and generated a residue conservation plot using the EMBOSS package tool 'plocon'. Smoothing was applied to the results revealing regions with higher degree of identity. These regions encompass residues 170-400, 1400-1820, 2000-2350 and 2550-3144. Pair wise comparison of human htt versus *C. elegans*, *A. mellifera*, *C. intestinalis*, *D. rerio*, *F. rubripes* or *G. gallus* also revealed the best non-intersecting alignments (Figure 36). These representations confirm a high level of similarity in discrete regions along the C-terminal HTT, suggesting that these regions could mediate important cellular functions. We therefore used the most conserved part of C-terminal HTT to perform the screen for new yeast two hybrid preys, picked from a mouse brain lysate.

We obtained 13 highly confident interactors (Figure 36). Except dynamin that was found to interact also with the N-terminal part of HTT (Kaltenbach et al., 2007), the proteins identified are new interactors of HTT. Among them, several of these interactors could play a role in HTT-induced toxicity. For example, HUWE1 (also known as Mule/ARF-BP1/LASU1) is a unique BH3-containing E3 ubiquitin ligase targeting the degradation p53 and Mcl-1 (members of Bcl-2 family) and therefore

could participate in HD pathogenesis (Chen et al., 2005; Zhong et al., 2005). We also found NECAB as a potential interactor linking Ca²⁺ signaling to HTT. Interestingly, NECAB1 is particularly enriched in the brain and especially in the striatum (Sugita et al., 2002). Another target of interest is ANKRD25 that potentially links HTT to the actin cytoskeleton. These findings are in agreement with a possible role for HTT in actin remodeling as previously suggested (Colomer et al., 1997; Munsie et al., 2011; Nakayama et al., 2000). Dynamin 1 was already found to interact with the N-terminal part of HTT by Y2H and to full length HTT by immunoprecipitation (Kaltenbach et al., 2007; Moreira Sousa et al., 2013). In addition, our Y2H screen fished dynamin1 as an interactor of the C-terminal part of HTT, suggesting that HTT could bind to dynamin1 via two independent domains. The other interactors found span a variety of functions which are summarized in the table below (table 1) ((Bagni et al., 1993; Castle et al., 2010; Harada et al., 2005; Jakobsson et al., 1999; Park et al., 2008b; Swingle et al., 2004; van der Blik et al., 1993; Zhang et al., 1999).

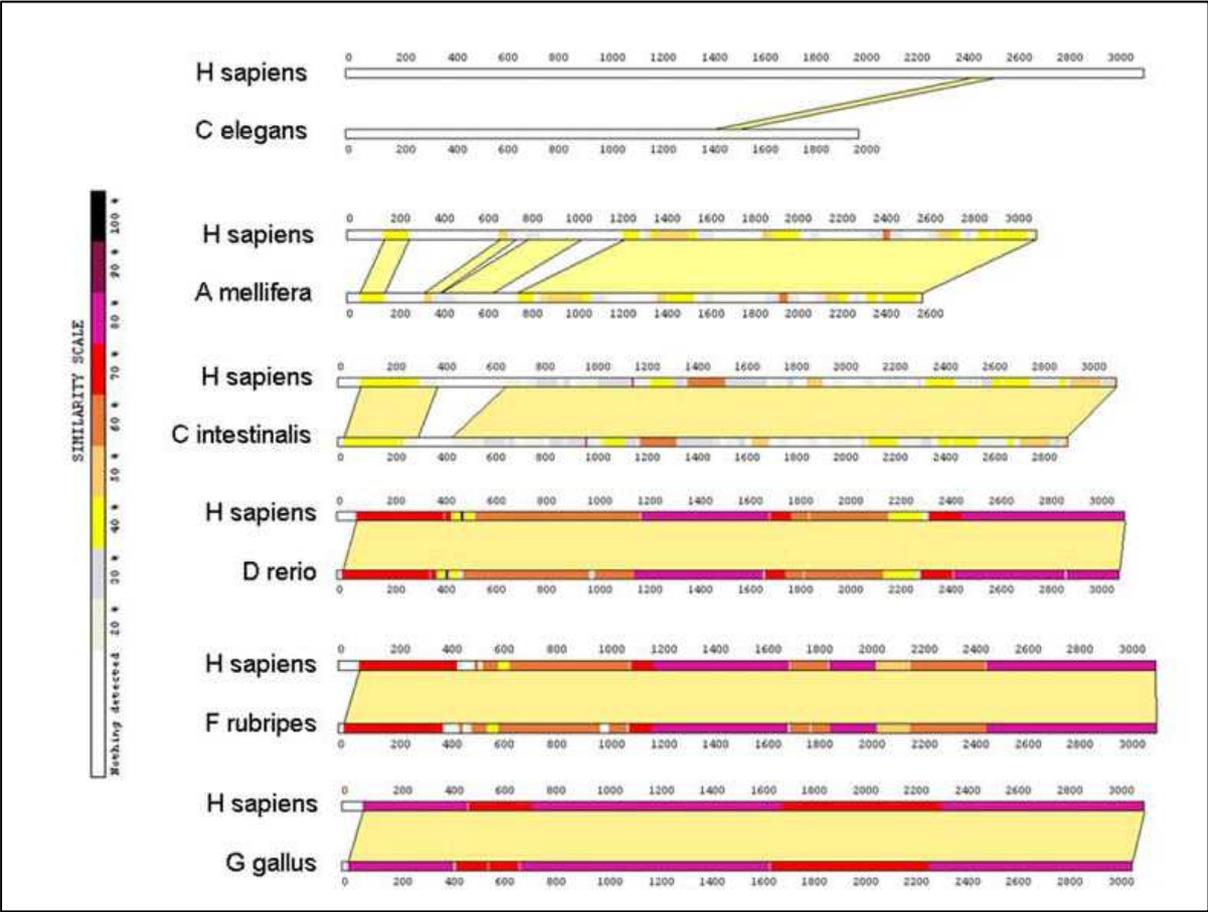
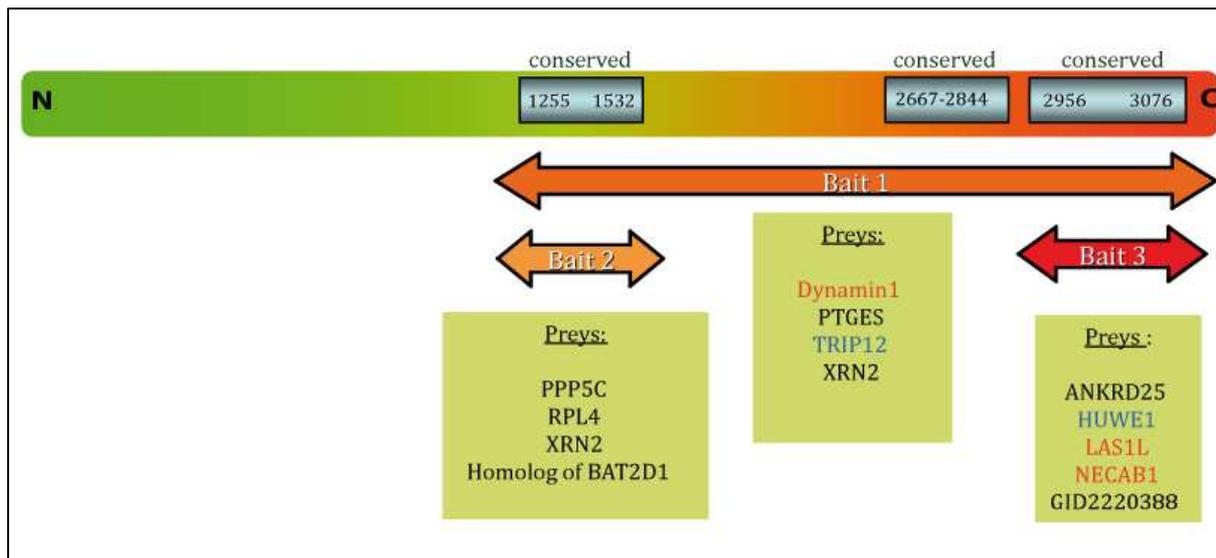


Figure 36: Pair-wise comparison of human HTT with lower species reveals a high level of similarity in C-terminal regions of HTT.



Genes	Functional description	References
PP5C	Serine/threonine phosphatase	Swingle <i>et al.</i> 2005
RPL4	Ribosomal protein L4	Bagni <i>et al.</i> 1993
XRN2	Exoribonuclease 2	Zhang <i>et al.</i> 1999
DNM1	Dynamine1, GTPase	Van Der Blicke <i>et al.</i> 1993
PTGES	Prostaglandine E synthase	Jakobsson <i>et al.</i> 1999
TRIP12	E3 ubiquitin ligase	Park <i>et al.</i> 1994
ANKRD25	Ankyrin repeat domain 25, growth regulatory factor	Carninci <i>et al.</i> 2005
HUWE1	E3 ubiquitin ligase	chen <i>et al.</i> 2005
LAS1L	nucleolar protein implicated in ribosome biogenesis	castle <i>et al.</i> 2010
NECAB1	Neuronal Calcium binding protein 1	Sugita <i>et al.</i> 2002

Table 1: (Above) List and interacting regions of C-terminal HTT partners. (Below) List of the proteins interacting with the C-terminal HTT and their relative functions.

Research context: Relevance and Aims.

The described yeast two hybrid screen suggest roles of HTT in cellular mechanisms yet unexplored. Apart from the N-terminal part of HTT, other domains of HTT are poorly studied. However, it is of a great interest to investigate the contribution in neurotoxicity of both N-terminal and C-terminal fragments generated after HTT cleavage during the course of the disease.

Polyglutamine expansion was shown to severely impact HTT functions through loss and gain of toxic mechanisms (Cheryl L. Wellington et al., 2003). As cleavage of mutated HTT has a major impact on its toxicity, aggregation and subcellular localization, HTT processing was extensively studied.

Both wild type and polyglutamine expanded HTT are subjected to proteolytic processing however abnormal activation of proteases described in HD may confer to mutated HTT a specific proteolysis pattern. HTT proteolysis could be regulated in time and in space and might also represent a mechanism impairing the normal function of HTT in the cell (DiFiglia et al., 1997; Lunke et al., 2002; Mende-Mueller et al., 2001; Wellington et al., 2000).

Modeling HTT proteolysis, using tools allowing to monitor cleavage of HTT, might be a valuable approach to decipher the effects of individual cleavage in a wild type or mutant conditions. Indeed, the main approach so far employed was to engineer HTT constructs resistant for proteases action; this allowed us to understand the importance of HTT cleavage in the neurotoxicity of HD. It remain the following question: How cleavage of HTT induced cellular damage? How some cleavages are toxic and others are not? The formation of aggregates containing mutated N-terminal HTT fragments are not directly link to neurotoxicity (see part I of this chapter) (Saudou et al., 1998). In addition, very few studies addressed the question of which from the full length or the N-terminal HTT is the more toxic. Even though transgenic mouse expressing the mutated exon1 HTT displayed a more acute HD-like phenotype compared to mouse expressing mutated full length HTT (Mangiarini et al., 1996; Shelbourne et al., 1999; Slow et al., 2005; Slow et al., 2003), any conclusion should be made with caution as the expression level of both transgenes is not equal. Indeed, full length HTT generally express in much lower concentration than truncated HTT.

Upon cleavage, what are the consequences of C-terminal HTT generation. Indeed we have no knowledge whether, concomitantly to the generation of mutated N-terminal HTT, the generation of C-terminal HTT fragment impact some cellular functions? Thus, dissecting the mechanisms leaning behind full length HTT proteolysis and toxicity would be of a great interest in the comprehension of neurotoxicity in HD.

Specific Huntingtin Proteolysis Causes Endoplasmic Reticulum

Dilation and Death

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ABSTRACT

Cleavage of mutant huntingtin (HTT) is an essential process in Huntington's disease (HD), a devastating neurodegenerative disorder. Cleavage generates an N-ter fragment that contains the polyglutamine stretch and that translocates into the nucleus. However, when cleaved, HTT will also generate a C-ter fragment whose contribution to pathogenesis remains unknown. Here we report, that upon synthetic cleavage at caspase-6 site, both N- and C-ter fragments remain associated and mutant HTT shows little toxicity. Further cleavage of the N-ter fragment is necessary to release fragments from the N-C interaction and to induce toxicity. Surprisingly, we found that the C-ter fragment remains in the cytoplasm and elicits toxicity through the dilation of the endoplasmic reticulum. The mechanism involves the specific binding of HTT and the dysregulation of dynamin1 activity on the ER. Together with the presence of such ER dilation in HD patients and HD mouse models, our findings suggest that non-polyglutamine fragments of HTT generated by proteolysis contribute to disease.

INTRODUCTION

Huntington's disease (HD) is an autosomal dominant inherited neurodegenerative disorder. 5-10 people are affected for 10.000 individuals in the population of European origin. Patients usually die 15-20 years after the first symptoms appeared, as there is currently no effective treatment to prevent or delay disease progression (Borrell-Pages et al., 2006a; Cattaneo et al., 2005; Imarisio et al., 2008). The most typical symptoms of HD are the involuntary choreiform movements and hypokinesia, cognitive deterioration and personality changes. HD is characterized by the preferential loss of neurons from the striatum and the cortex. HD is caused by an abnormal polyglutamine (polyQ) expansion in the N-terminus of the protein huntingtin (HTT). HTT is a large (350kDa) protein composed of 3144 aas with the polyQ repeat starting at position 18. Although the mechanisms that lead to HD are not fully understood, several studies have revealed a series of events that ultimately lead to the death of neurons in the brain (Borrell-Pages et al., 2006a; Cattaneo et al., 2005; Imarisio et al., 2008). A crucial step in HD pathogenesis is the cleavage of full-length HTT into smaller N-terminal fragments that contain the polyQ stretch and that are toxic to neurons.

Mutant N-terminal (N-ter) fragments have been observed in human HD brains and HD mouse models (Mende-Mueller et al., 2001; Wang et al., 2008; Wellington et al., 2002). Several proteases including caspases, calpains, cathepsins, metalloproteinases cleave HTT at various sites producing toxic mutant N-ter fragments (Gafni et al., 2004; Goldberg et al., 1996; Graham et al., 2006; Hermel et al., 2004; Lunkes et al., 2002; Miller et al., 2010; Ratovitski et al., 2009; Tebbenkamp et al., 2012). In support for a crucial role for proteolysis, inhibiting cleavage reduces toxicity *in vitro* and *in vivo* (Gafni et al., 2004; Graham et al., 2006). Furthermore, short fragments of HTT that contain the polyQ stretch can model some aspects of HD. In particular, exon1 of HTT with an expanded polyQ stretch is sufficient to induce neurological phenotype in mouse (Mangiarini et al., 1996). Over the last years, many studies have investigated the mechanisms by which short N-ter fragments containing the polyQ expansion lead to neurodegeneration. Also, toxicity requires the nuclear translocation of the N-ter fragment (Saudou et al., 1998). When in the nucleus, this fragment leads to transcriptional dysregulation that is detrimental for neuronal survival (Landles et al., 2010; Ratovitski et al., 2011; Sugars and Rubinsztein, 2003). The N-ter fragment containing the polyQ stretch also induces neuronal dysfunctions including defects in signal transduction, autophagy, calcium and mitochondria homeostasis (for reviews, see (Borrell-Pages et al., 2006b; Li et al., 2010; Sarkar et al., 2007)). Together, these studies have established that N-ter fragment(s) containing the polyQ stretch are the crucial pieces responsible for HD pathogenesis.

Therefore, a major consequence of HTT proteolysis is the generation of a variety of small N-ter fragments that contain the polyQ stretch. Importantly, the shorter the fragments containing the polyQ stretch, the more toxic they are (Hackam et al., 1998; Landles et al., 2010). How these various fragments are generated during pathogenesis remain to be understood. Also, the cascade of proteolytic events leading to the generation of more toxic fragments is unclear. This could involve specific proteases that would function only in some cell types or that would be more effective on neo-generated N-ter fragments of HTT (Tebbenkamp et al., 2012). Another crucial question is why some N-ter fragments are more toxic than others. Increased toxic properties might be linked to an increased accessibility or conformation switch of the polyQ stretch in smaller N-ter fragments such as in Exon 1. However, this does not explain why some proteolytic cleavages are more toxic than others independently of a major modification of fragment sizes. As an example, whereas blocking HTT proteolysis at the putative Caspase 6 site at position 586 reduces mutant HTT-induced toxicity *in vivo*, mutations at the Caspase 3 sites 513 and 552 do not (Graham et al., 2006).

Since HTT is subjected to proteolysis by multiple proteases including some yet to be discovered, the consequences of specific cleavages are difficult to achieve. In addition, although N-ter fragments recapitulate features of HD *in cellulo* and *in vivo*, the picture remains incomplete when compared to HD patient situation. Indeed, in human HD, the full-length protein is present from early stages and if HTT cleavage generates short N-ter fragments whose amino-acid position range from 1-105 to 1-586, it will also generate corresponding C-terminal (C-ter) fragments. Such C-ter fragments are also observed in HD post mortem brain samples (Landles et al., 2010; Mende-Mueller et al., 2001) but their participation in the pathogenic process remain to be investigated.

Here we investigated the consequences of full-length mutant HTT proteolysis by developing a time and site-specific controlled system for HTT proteolysis *in vitro* and *in vivo*. We report that successive proteolysis of HTT disrupts full length HTT conformation and generates more toxic N-ter fragments as they are released from an intramolecular interaction with their corresponding C-ter fragments. We observed that full-length HTT proteolysis at specific sites not only generates toxic N-ter fragments as they translocate into the nucleus but also that the generated C-ter fragments also participate to HD pathogenesis via a novel mechanism. These findings highlight the complexity of HD pathogenic mechanism and support the importance of studying HTT as a full-length protein as in HD patients.

RESULTS

Time and Site-Specific Control of Huntingtin Proteolysis

The main caveat of investigating cleavage of HTT by proteases is that proteases cleave many other substrates whose toxicity will mask the toxicity specifically induced by HTT cleavage. To specifically address the relationship between cleavage and toxicity, we took advantage of the recent demonstration that the tobacco etch virus (TEV) protease is able to specifically cleave engineered proteins in mammalian cells (Gray et al., 2010). This system is based on the introduction of a seven amino-acid sequence (ENLYFQ/S) recognized by the TEV protease as a cleavage site and that is not present in the mammalian proteome. As stated previously, HTT is cleaved at many sites. We first investigated the cleavage of full-length HTT at the putative caspase 6 site at position 586 as this site was suggested to play a role in HD pathogenesis (Graham et al., 2006). We also generated HTT constructs whose cleavage can be induced at position 167. Cleavage at amino acid position 167 is induced by Cathepsin Z and Bleomycin Hydrolase (BLMH) and was referred as cp2 cleavage site (Ratovitski et al., 2011) (figure 1A). It also generates an intermediate short N-ter fragment whose toxic role is well established (Schilling et al., 1999). As it is difficult to manipulate HTT protein, we used our synthetic RNA insensitive HTT construct, pARIS-HTT that can be easily mutated using cloning cassettes present throughout the protein (Pardo et al., 2010). As expected, replacing the endogenous caspase 6 site by the TEV Recognition cleavage site (RCS) made the FL-HTT-586TEV constructs insensitive to caspase 6 (Figure S1A). We next expressed the various constructs in HeLa cells and protein extracts were incubated with increasing quantities of recombinant TEV protease (Figure 1B). Whereas the addition of TEV recombinant protease in the lysate of HeLa transfected cells was unable to cleave the WT FL-HTT which does not contain TEV RCS, it cleaved both WT and polyQ FL-HTT-167TEV or FL-HTT-586TEV constructs (Figure 1B). The cleavage pattern of FL-HTT-167TEV and FL-HTT-586TEV was comparable to the migration pattern of N-HTT167 and N-HTT586 constructs, generating N-terminal and C-terminal fragments of expected size (Figure 1B).

FL-HTT-586TEV-Q23 and FL-HTT-586TEV-Q100 were both cleaved efficiently by adding 0,1 µg of recombinant TEV. However, FL-HTT-167TEV-Q23 and FL-HTT-167TEV-Q100 required 100 folds more of protease (10 µg) to generate detectable proteolytic product. This suggests that in contrast to the cleavage site at position 586, the cleavage site at position 167 might be less accessible given its location within the first HEAT domain of HTT.

We next analyzed proteolysis of HTT in cells using the SNIPer-TEV system that consists in two vectors expressing both N and C parts of the TEV fused respectively to FRB and FKBP fragments.

Addition of 10 or 20 nM of rapamycin induces a rapid heterodimerization of the fragments and the TEV protease activity (Gray et al., 2010). We incubated cells expressing the HTT-TEV constructs and the SNIPer-TEV constructs with rapamycin for 30 min, 2 or 6 hrs. At this concentration, rapamycin had no effect on HTT endogenous proteolysis neither on its toxicity (Figure 1C and Figure 2E).

A short treatment of rapamycin led to almost complete cleavage of constructs that contain the TEV site at position 586 confirming the accessibility of this site within FL-HTT. In contrast and as observed in vitro, proteolysis at 167 was less efficient as the N-HTT1-167Q23 and N-HTT1-167Q100 fragments were detected only after 6 hrs of treatment (Figure 1C). Interestingly, we observed an enhanced production of N-HTT1-167Q23 and N-HTT1-167Q100 fragments when HTT contained both 167 and 586 TEV sites. This suggests that a first cleavage at position 586 may lead to HTT conformational changes and facilitate the accessibility of the 167 site. Further quantification revealed a 2.5 fold increase in short fragment production when HTT is first cleaved at position 586 or when produced directly from the N-HTT586 fragment (data not shown). To further analyze the consequences of a first cleavage at 586 position we generated a HTT-TEV construct corresponding to the N-terminal HTT 586 amino-acids fragment arising from the cleavage of FL-HTT by the caspase 6 (N-HTT586) and containing a TEV RCS at position 167 (N-HTT-167TEV) (Figure 1D). Interestingly we observed that the presence of polyQ stretch in HTT favoured the appearance of the N-HTT1-167 fragment suggesting that polyQ expansion -in agreement with the many studies that reported a change in HTT conformation when containing an abnormal polyQ stretch- could render the 167 site more accessible (Figure 1E).

Our findings validate the use of the TEV approach to study intrinsic proteolytic properties of HTT in WT and disease condition and suggest that HTT is subjected to sequential proteolysis that could be more efficient when HTT contains a pathogenic expanded polyQ stretch.

Specific Huntingtin Proteolytic Events Accelerates Toxicity

Having set up the conditions that allow the controlled cleavage of HTT, we analyzed the toxicity induced by HTT proteolysis at specific positions in cells. We choose to express the various HTT constructs into striatal *STHdh^{+/+}* cells. Such cells were previously generated by immortalization of primary mouse striatal neurons (Trettel et al., 2000) and are widely used as they reproduce some features of striatal neurons. Indeed, we could not achieve sufficient expression in primary rat striatal neurons. We first transfected the different constructs in striatal cells in presence of the two SNIPer-TEV plasmids or pcDNA as a control. 20 hrs post-transfection when cells can be identified thanks to the presence of the mCherry tag on HTT constructs, we added 10 nM of rapamycin and, using robotic

automated videomicroscopy, we followed during 24 hrs the fate of the individual cells that express HTT. As a first step we measured, the toxicity of the different constructs containing the TEV RCS and compared it to the toxicity elicited by the FL-HTTQ23 and Q100 constructs. Insertion of TEV RCS had no obvious effect on FL-HTTQ23 and Q100 toxicity (Figure 2E & Figure S1B). As expected, FL-HTTQ100 constructs induced a higher toxicity compared to the FL-HTTQ23 although this was not significantly different (Figure S1B). However, this is expected as in these conditions, we expressed the full-length HTT and assessed toxicity only after 24 hr. Rapamycin treatment had no significant effect at the dose used which is 1000 less than the doses used to inhibit mTOR (figure 2E). We next analyzed the toxicity of the different constructs upon cleavage (Figure 2A). As expected induction of SNIPer-TEV in cells expressing FL-HTT Q100 construct had no effect. It also had no obvious effect in on FL-HTT-167TEV-Q100 construct. This might be expected since SNIPer-TEV was unable to cleave efficiently HTT at this site (Figure 1). Surprisingly, although proteolysis of HTT is very efficient at position 586, we found no difference in FL-HTT-586TEV-Q100-mediated toxicity suggesting that this cleavage has no direct consequences on polyQ-HTT toxicity. However, we found that inducing simultaneous cleavage at positions 586 and 167 led to a marked increase in polyQ-HTT induced toxicity (Figure 2A).

These results indicate that sequential proteolysis induces a marked increase in mutant HTT toxicity.

Both N- and C-Terminal HTT Fragments Induce Toxicity that Depends on the Size of N-Terminal Fragments

To understand better the absence of toxic effect after HTT cleavage at the caspase 6 putative site but its increase upon further proteolysis, we first verified the toxicity induced by N-HTT586Q100 fragment. As expected, we found that the toxicity induced by the N-HTT586Q100 construct in striatal cells is significantly higher when compared to the N-HTT586Q23-induced toxicity (Figure 2B). We next generated the C-HTT587-3144 construct that corresponds to the C-terminal fragment produced by proteolysis at position 586 and expressed it with various N-terminal constructs in striatal cells. As previously reported, when expressed alone, the shorter the N-terminal fragments are, the more they are toxic with N-HTTExon68 being significantly more toxic than N-HTT167Q100 and N-HTT586Q100 (Hackam et al., 1998)(Figure 2C). We next co-expressed the different fragments together (Figure 2D). Strikingly, co-expression of C-HTT587-3144 with N-HTTExon1Q68, N-HTT167Q100 or N-HTT586Q100 induced statistically significant increases in the toxicity elicited by these N-ter fragments. The additional toxicity induced by the presence of the C-ter fragment was particularly evident with the N-HTTExon1Q68, N-HTT167Q100 but was less effective with the N-HTT586Q100 fragment. This

suggests that the C-ter fragment could potentiate the toxicity elicited by mutated N-ter fragments, or alternatively, that the C-HTT587-3144 fragment has intrinsic toxic properties that add to the toxicity of short N-ter fragments. To test this possibility, we measured the toxicity of the C-HTT587-3144 fragment alone in striatal cells. We found that this fragment was highly toxic and to a similar extent to the N-HTTExon1Q68 fragment (Figure 2D). Interestingly, this toxicity was reduced in presence of its complementary N-HTT586Q100 fragment but not with the short ones. Also, and as stated previously the toxicity of the N-HTT586Q100, in contrast to short N-terminal fragments is only weakly increased by the presence of the C-HTT587-3144 fragment suggesting potential functional interactions between the complementary fragments issued from caspase-6 cleavage.

As the toxicity of the C-ter fragment was unexpected, we reasoned that sequential proteolysis of wild-type HTT should also generate the C-HTT587-3144 fragment and be potentially toxic. Therefore, we co-expressed FL-HTT-167/586TEV-Q23 with the SNIPer-TEV or with pcDNA as a control and treated the cells with 10 nM rapamycin as in Figure 1. Strikingly, sequential proteolysis of wild-type HTT led to a marked toxicity compared to the control condition (Figure 2F). These results indicate that HTT proteolysis, in conditions that generate short wild-type N-HTT167Q23 and C-HTT587-3144 fragment, is toxic and is in agreement with the found toxicity of the C-HTT587-3144 fragment.

Taken together, these results suggest that 1) proteolysis of HTT produces both N- and C-terminal fragments that potentially contribute to toxicity and 2) Full length HTT-induced toxicity depends on the proteolytic pattern of the N-terminal fragments.

Huntingtin Intramolecular Interaction is Lost upon Specific Proteolysis

Our findings that cleavage of FL-HTT-586TEVQ100 does not induce significantly more toxicity than mutant FL-HTT and, the observation that co-expression of N-HTT586Q100 fragment -but not shorter N-terminal fragments- with C-HTT587-3144 fragment does not show the expected additive increase due to the toxicity of both N and C-ter fragments (Figure 2D) led us to postulate potential interaction between the N-HTT586 and the C-HTT587-3144 fragments. This hypothesis was supported by recent observations than N-terminal regions of HTT are able to interact with more C-terminal regions (Palidwor et al., 2009).

As a first step, we co-expressed N-HTT586Q100, N-HTT167Q100 and N-HTTExon1Q68 with the C-HTT587-3144 fragment in Hela cells and tested whether the different fragments interact together. Immunoprecipitation of N-HTT586Q100 but not of N-HTT167Q100 using anti-mCherry

antibody led to the co-immunoprecipitation of the C-HTT587-3144 fragment as revealed by GFP antibody (Figure 3A). Similarly, using another set of N-ter constructs that are HA-tagged, we found that immunoprecipitation of N-HTT586Q100 but not of N-HTTExon1Q68 using anti-HA antibody led to the co-immunoprecipitation of the C-HTT587-3144 fragment as revealed by GFP antibody (Figure 3B).

To further confirm the interaction of N-HTT586 fragment but not shorter ones with C-HTT587-3144, we expressed simple or double FL-HTT-TEV constructs in Hela cells and added an excess of recombinant TEV protease on cell lysates to ensure efficient cleavage of the FL-HTT-TEV constructs. We next immunoprecipitated the C-HTT587-3144 fragment that is GFP tagged using anti-GFP antibody. The C-HTT587-3144 fragment produced by cleavage at position 586 was able to co-immunoprecipitate the corresponding wild type or polyQ N-HTT586 fragments. We found no interaction between the C-HTT587-3144 fragment with N-HTT167Q23 or N-HTT167Q100 fragments issued from sequential proteolysis of the N-HTT586Q23 and N-HTT586Q100 fragments. Taken together, we conclude that N-HTT586Q23 and N-HTT586Q100 fragments interact with the corresponding C-HTT587-3144 fragment and that further proteolysis of N-HTT 1-586 at positions 167 induces a loss of intramolecular interaction between the N-ter HTT and C-ter HTT fragments. PolyQ expansion did not modify N-C interaction (Figure 3C). In order to determine the association of the intermediate HTT-168-586 fragment, we similarly performed immunoprecipitation on both N-ter- and C-ter HTT fragment generated after sequential cleavage at site 167 and 586. We found that HTT-167-586 fragment (revealed with 4C8 antibody) immunoprecipitate with the C-HTT 587-3144 and not with the N-HTT 1-167, in wild type and mutant conditions (Figure 3D).

These findings, together with the observation that double cleavage increases toxicity, suggest that HTT intramolecular interaction between N and C fragments of HTT prevents or delays mutant HTT-induced toxicity.

N-ter and C-ter Fragments Differentially Localize after Sequential Proteolysis

Short N-ter fragments are known to translocate into the nucleus and form intranuclear inclusions (Saudou et al., 1998). To investigate within cells the fate of the N- and C-terminal fragments depending on their sizes and their interaction, we co-expressed the various constructs in *STHdh* striatal cells and investigated 20 hr post-transfection the localization of the various constructs as the N-ter and C-ter fragments are fused respectively to mCherry and GFP. Co-localization measurement was achieved by the use of JACOP plug-in of image J software. This co-localization tool is based on distance between center of detected objects from two distinct fluorophores (Bolte and

Cordelieres, 2006). In agreement with the biochemical data (Figure 3A-C) we found that N-HTT586Q100 (mCherry) co-localized in a higher manner with C-HTT587-3144 (GFP) than N-HTT167Q100 and N-HTTExon1Q68 (Figure 3F). Interestingly, concomitant to the loss of interaction between the C-HTT587-3144 fragment and the short N-terminal ones, we observed a progressive increase in nuclear localization of these fragments whereas the C-terminal one remained in the cytoplasm (Figure 3F).

These findings are in agreement with previous reports showing an inverse correlation between nuclear localization and the size of the N-ter fragments (Hackam et al., 1998; Saudou et al., 1998).

C-Terminal Huntingtin Fragment Induces Endoplasmic Reticulum-Derived Vacuolization, Stress and Toxicity

To understand better the consequences of the loss of N-C interaction when N-terminal fragments become shorter, we co-expressed C-HTT587-3144 with N-HTT586Q100, N-HTT167Q100 or N-HTTExon1Q68 and analyzed cells at longer time point (48 hr). As expected and in agreement with Figure 3F we observed a nuclear accumulation of the short N-terminal fragments that is inversely proportional to the size of the N-terminal fragment (Figure 4A). Interestingly, when short N-terminal fragments translocate in the nucleus, we observed a progressive appearance of large cytoplasmic vacuoles (Figure 4A). These vacuoles were observed in cells expressing the C-ter fragment alone and in cells co-expressing the C-terminal fragment with non-interacting N-ter fragments such as N-HTTExon1 and N-HTT1-167. However the number of vacuolated cells was reduced when the C-HTT587-3144 fragment was co-expressed with N-HTT586Q100 (Figure 4A). None of the polyQ N-ter HTT fragments were able to induce such a vacuolar phenotype. These results indicate that the C-HTT 587-3144 fragment produced by caspase-6 cleavage of HTT (Graham et al., 2006; Wellington et al., 2000) and that is observed in post-mortem brain samples, induce cytoplasmic alterations characterized by an abnormal formation of vacuoles.

To start to investigate the nature of such vacuoles, we performed, using various classical markers of cytoplasmic structures and organelles, immunostainings in cells expressing the C-HTT587-3144 fragment. We found that the C-HTT587-3144 fragment-induced vacuoles were immuno-negative for GM130, Cter433 and TGN38 (Figure S2A) and for Rab5 and EEA1 negative (Figure S2C) and therefore are unlikely to derive from the Golgi apparatus or the trans-Golgi network and from early endosomes. In addition, we found the vacuoles not to localize with LC3-GFP and LAMP2 suggesting they are not linked to autophagosomes or lysosomes (Figure S2B). However, we

found that calnexin and ATF6, chaperone proteins localizing at endoplasmic reticulum (ER) membranes (Bergeron et al., 1994), decorated C-terminal HTT-induced vacuoles (Figure 4C). To confirm this finding, we co-expressed C-HTT587-3144 fragment with a vector encoding GFP fused to KDEL motif and this is a luminal marker of the ER (Haugejorden et al., 1991). We found, using videomicroscopy, that vacuoles were progressively being filled by GFP-KDEL (Figure 4B). To further investigate whether ER vacuolization could affect ER stress signaling, we analyzed the levels of calnexin, BIP and nuclear ATF6 that are induced upon ER stress (Walter and Ron, 2011). We found that expression of C-HTT587-3144 fragment led to an upregulation of ATF6 with a marked increase of nuclear ATF6 indicative of ER stress (Figure 4D)(Ye et al., 2000). We also found that expression of both calnexin and BIP were induced by expression of the C-HTT587-3144 fragment to levels comparable as those induced by tunicamycin, a drug described to activate ER stress signaling (Figure 4D) (Samali et al., 2010). To further understand the link between ER vacuolization, induction of ER stress and toxicity upon, we investigated the possible mechanism by which C-HTT587-3144 fragment generated by HTT proteolysis induces toxicity. A pan-caspase inhibitor, Z-VAD-FMK, as well as the caspase-3 inhibitor Z-DEVD-FMK, failed to reduce cellular toxicity induced by the Cter-HTT fragment, (Figure S3B & S3C). We also found that cells expressing the C-HTT587-3144 fragment are TUNEL negative (Figure S3A) suggesting that classical apoptosis is not involved. In addition, we found that autophagy, a mechanism that when dysregulated can induce cell death (Kroemer and Levine, 2008) was unlikely to be involved. Indeed, silencing of Beclin1 had no effect on C-HTT587-3144 fragment-induced toxicity (Figure S3D). We therefore tested the possibility that the ER-stress induced by the formation of these vacuoles could be responsible for the increased toxicity. We expressed the C-HTT587-3144 fragment in striatal cells and treated them with salubrinal, an eif2 α inhibitor. We observed a significant decrease in cell death C-HTT587-3144 fragment-induced toxicity (Figure 4E). Interestingly, we observed an increased percentage of cells containing vacuoles in the salubrinal-treated cells suggesting that toxicity could be secondary to the formation of ER-derived vacuoles and subsequent activation of ER stress.

To further confirm that ER-derived vacuoles originate from the C-HTT587-3144 fragment that is generated by FL-HTT proteolysis and that this fragment is relevant to HD pathogenesis, we aim to induce cleavage of FL-HTT-167/586TEV and evaluate vacuole formation. Since these cells express endogenous HTT that could mask the effect of the cleavage of the transfected constructs we replaced endogenous HTT by WT or mutant FL-HTT-167/586TEV that are insensitive to the siRNA used as they are derived from the pARIS-HTT constructs (Pardo *et al.* 2010). We therefore co-transfected the cells with HTT siRNA and FL-HTT-TEV constructs and the SNIPer-TEV or with pcDNA as a control and treated the cells for 24 hours with 20 nM rapamycin, a condition that generates

significant proteolysis at both 586 and 167 sites (Figure 1) and that induces toxicity (Figure 2). In these conditions, we observed the presence of a disorganized ER network with the presence of dilated lumen, as shown by calnexin staining, before the appearance of marked toxicity (Figure 4F). Importantly, vacuoles and dilated ER was detectable in both FL-HTT 167/586TEV Q23 and Q100 constructs further indicating that this phenotype depends on the generated C-ter fragment issued from FL HTT double cleavage.

Dynamin1 Localization and Interaction with HTT is Altered upon HTT Proteolysis

How does C-HTT587-3144 fragment induces ER-vacuolization? Several studies have reported that expression of polyQ HTT induce ER stress. However, these studies were based on the expression of N-terminal fragments containing the polyQ stretch such as the first 588 amino acids (Yang et al., 2010) or shorter fragments such as N-HTTExon1 (Lajoie and Snapp, 2011). However, we found that such fragments, in contrast to the C-HTT587-3144 fragment, were unable to induce ER dilation and vacuoles.

We obtained 13 highly confident interactors by yeast two hybrid screen as mentioned previously. However none of these interactors gave us a direct link to ER function or localization. However, we decided to further investigate dynamin1 for several reasons. First, dynamin1 has been described to play a role in membrane fission and fusion reactions during several cellular processes (Praefcke and McMahon, 2004). Secondly, dynamin1 was identified as a HTT interactor via its N-terminal region (Kaltenbach et al., 2007) and it was recently reported to interact with full-length HTT and regulate endocytosis of EGF receptor in mammary cells (Moreira Sousa et al., 2013). Therefore, we speculated that dynamin1 could interact with full-length HTT via N and C-terminal domains and that proteolysis could affect dynamin1 interaction with HTT. We performed immunoprecipitation experiments by co-expressing with dynamin1, FL-HTT, C-HTT587-3144, N-HTT586Q100 as well as the shorter N-HTT167Q100 fragment. We found that both FL-HTT, C-HTT587-3144, N-HTT586Q100 but not the shorter N-HTT167Q100 fragment interacted with dynamin1. These results agree with our and other yeast two-hybrid studies. Similarly, we found that N-ter HTT interacted with dynamin1 after a simple cleavage at position 586 but did not after a sequential cleavage at both 586 and 167 sites (Figure 5A). This suggests that dynamin1 interacts with N-HTT586Q100 and C-HTT 587-3144 when expressed alone or together. However dynamin1 did not significantly interact with the N-HTT167Q100 in both conditions.

Since the C-HTT587-3144 fragment generated by FL-HTT proteolysis keep interacting with dynamin1, we tested whether manipulating dynamin1 activity influences C-HTT587-3144 fragment-

induced vacuolization and death. Strikingly, we found that co-expression of dynamin1 WT resulted in a statistically significant decrease in the toxicity induced by the expression of the C-HTT587-3144 fragment (Figure 5B). In contrast, co-expression of dynamin1 K44A (DN) had no protective effect suggesting that dynamin1 activity is required to inhibit C-HTT587-3144 fragment-induced death. Taken together, these results demonstrate that the C-HTT587-3144 fragment interacts with dynamin1 and suggest that the C-HTT587-3144 fragment-induced toxicity is linked to a dysregulation of dynamin1 activity as toxicity can be rescued by WT but not inactive (K44A) dynamin1.

Although HTT has been found associated with the ER (Atwal and Truant, 2008; Atwal et al., 2007), dynamin1 has not been directly associated to ER remodeling processes. Instead, proteins such as atlastin/Sey1p and Lnp1p have been reported to mediate fusion/fission of the ER (Chen et al., 2012). We therefore investigated whether dynamin1 and specific HTT proteolytic fragments could localize at ER membranes through biochemical approaches. ER-derived membranes were purified as shown by the presence of calnexin, an ER specific marker and the absence of a mitochondrial marker (mitochondrial HSP70), cytosolic components (α tubulin and actin) or plasma membrane marker (Annexin V). ER membranes fractionation revealed that a substantial amount of dynamin1 WT and dynamin1 K44R (DN) can be detected on purified ER membranes suggesting that localization of dynamin1 to the ER is independent of its GTPase activity (Figure 5C). We found that the C-HTT587-3144 was enriched at ER membranes as compared to FL-HTT suggesting that upon proteolysis a significant fraction of the C-HTT587-3144 fragment associates to the ER membranes. In contrast, a significant fraction of N-HTT586Q100 and most of N-HTT167Q100 fragments were found in the cytosolic fraction (Figure 5D). Loss of N-ter fragments localization and the enriched Cter fragment localization at the ER were accompanied by a reduction in the level of dynamin1 at the ER (Figure 5D). Expression of N-ter fragments alone did not alter the level of dynamin1 at ER membranes (data not shown). Thus, these results indicate that dynamin1 localization within cellular compartment is altered upon the release of the Cter-HTT. In support, we found by immunofluorescence that dynamin1 localization at the plasma membrane is reduced by the C-HTT587-3144. Indeed, whereas in control cells (non transfected cell), dynamin1 is enriched at the plasma membrane, immunostaining of dynamin1 is decreased at the cellular edges and enriched at a perinuclear region in C-HTT587-3144 expressing cells (Figure 5E). In support of a general inhibition of dynamin1 activity, we observed a decrease in endocytosis as shown by the reduced transferrin uptake in cells expressing the C-HTT587-3144 fragment and the intact endocytosis upon over-expression of WT dynamin1 (Figure 5F).

These findings suggest that FL-HTT is present with dynamin1 at the ER. Upon double proteolysis, the C-HTT587-3144 remains at the ER while dynamin1 as well as short N-ter fragments

mislocalize from the ER. Alteration of dynamin1 localization is accompanied by an inhibition of dynamin activity and death.

Dynamin1 Activity is Disrupted upon HTT Cleavage and Mediates ER Network Disruption

To test whether a defect in the activity of ER-localized-dynamin1 is the cause of ER disruption induced after HTT proteolysis, we engineered a construct expressing WT or K44R dynamin1 tagged with an HA sequence in its N-terminus end and fused to the trans-membrane domain of the atlastin1 in its C-terminus end (Figure 6A). Atlastin 1 belongs to the dynamin family and was shown to regulate the fusion between ER membranes (Orso et al., 2009). We first validated these constructs, termed respectively ER-DNM1-WT and ER-DNM1-DN by immunofluorescence and cellular fractionation experiments. ER isolation showed that both ER-DNM were found exclusively in the ER but not in the cytosolic fractions (Figure S4A). Analysis of immunofluorescence showed that both forms were recognized by dynamin1 antibodies and co-localized with calnexin and with ER-dsRed marker in cells. ER-DNM1-WT and ER-DNM1-DN also surrounded ER tubules when identified by the luminal ER marker KDEL-GFP (Figure S4B & S4C). This indicates that the constructs are correctly located on the cytosolic face of the ER.

We next addressed whether ER-localized-dynamin1 DN is toxic and is able to trigger cytoplasmic vacuolization by itself. We first assessed ER-DNM-WT and ER-DNM-DN toxicity at 48 hr post-transfection in striatal cells and observed a significant increase of cell death in cells expressing inactive ER targeted dynamin1 compared to wild type (Figure 6B). Interestingly enough, we found that inactive ER targeted dynamin1, when co-transfected with an empty mCherry vector to follow the transfected cells by videomicroscopy, induced the formation of vacuoles gradually invading the cytoplasm (Figure 6B and S4C-D). This construct showed higher toxicity when compared to normal DNM-DN (Figure 6C&6D). Moreover, cells expressing normal dynamin1 K44A (DN) did not display such phenomenon, demonstrating that ER-localized-dynamin1 activity is required for the proper maintenance of ER network-

We further tested the interaction of ER targeted dynamin1 with HTT by co-expressing ER-DNM1 and various HTT fragments in striatal cells. We found that a significant fraction of FL-HTT and of proteolytic fragments interacted with ER-DNM. Interestingly, we observed an increased interaction of the C-HTT 587-3144 fragment with the ER-targeted dynamin1 as compared to FL-HTT or N-HTT586Q100. As expected, the shorter N-HTT167Q100 fragment did not interact with ER-targeted dynamin1 (Figure 6E). To make sure that the interaction of the C-HTT 587-3144 occurs on ER membranes, we isolated ER fraction and selectively analyzed whether HTT fragments interact the ER-

targeted dynamin1 by immunoprecipitation. We observed a strong interaction between the ER-DNM and FL-HTT as well as with C-ter HTT. The N-HTT586Q100 interacted with ER-DNM while the short N-HTT167Q100 was not found in ER fraction (Figure 6E). Taken together, these results indicate that both FL-HTT and the proteolytic products generated by cleavage at position 586 interact with dynamin1 at the ER. In contrast upon HTT proteolysis at position 586 and position 167, only the C-ter HTT product remains associated with the ER-localized-dynamin1.

We next assessed the consequences of dynamin1 expression on ER vacuolization induced by Cter-HTT fragment. Importantly, we found that the percentage of both cell death and vacuolated cells were significantly reduced when striatal cells co-expressed ER-DNM-WT. No such effect was observed when cells co-expressed ER-DNM-DN (Figure 6F). In contrast, a slight acceleration of the death and vacuolization process was observed when cells co-expressed the C-HTT587-3144 with ER-DNM-DN.

To further confirm that defective dynamin1 at ER membranes is responsible for the ER vacuolization and death, we co-expressed FL-HTT-167/586TEV-Q23 or Q100 with ER-DNM1-WT in presence of the SNIPer-TEV and treated the cells with 10 nM rapamycin. As shown previously (Figure 2), double cleavage of FL-HTT-167/586TEV-Q23 or Q100 by the SNIPer-TEV induced significant toxicity compared with the pcDNA control condition; However, we found that expressing selectively wild type dynamin1 at the ER completely abolished the toxicity induced by double cleavage of both wild type and mutated HTT at position 167 and 586 (Figure 6G). Thus re-establishing dynamin1 activity at the ER membranes is sufficient to prevent cell death induced by double cleavage of HTT. In contrast ER-DNM1-DN was unable to restore cell survival after HTT cleavage.

Taken together, our results indicate that ER-located HTT generates upon proteolysis a C-terminal fragment that localizes on the ER membranes and inactivates dynamin1 leading to abnormal ER vacuolization and death.

Toxic Events Induced by HTT Proteolysis are Observed In Flies and in a HD Mouse Model.

To investigate this process in vivo, we generated transgenic flies expressing FL-HTT or N- and C-Terminal fragments. As a model organism, the fruit fly *Drosophila melanogaster* has many experimental advantages and is particularly suitable for studying neurodegenerative disease (Bilen and Bonini, 2005; Marsh et al., 2003).

We generated transgenic flies expressing the FL-HTTQ100 containing the various TEV sites and next crossed these flies with UAS-TEV flies and ElaV-Gal4 flies or with flies expressing TEV under a heat-shock promoter (hs-TEV). These flies express the various FL-HTT constructs at similar levels

(Figure S5A) and similar to in cells experiments, cleavage at 586 is efficient compared to cleavage at 167 position but appearance of the 167 fragment is facilitated by a prior cleavage at 586 (Figure S5A, right panels). We next determined fly survival upon crosses to UAS-TEV or hs-TEV (Figure 7A). Whereas cleaving FL-HTT at position 167 slightly enhanced toxicity, cleavage at positions 586 and more evidently at both sites dramatically reduced survival of the flies. Confirming the toxicity of C-ter HTT released upon proteolysis, cleaving FL-HTTQ23 at both sites was sufficient to induce major toxicity in flies indicating that the C-ter fragment generated is also toxic in flies.

Finally, to further confirm that the generated C-HTT 587-3144 fragment is sufficient to induce toxicity we expressed C-ter fragments in flies. We failed to obtain sufficient expression on the C-HTT 587-3144 fragment and therefore used a shorter C-terminal fragment (C-HTT 1722-3144) that also interacts with N-HTT 1-480 fragment but not the N-HTT 1-171 fragment (data not shown). This fragment, as the C-HTT 587-3144 fragment, is also able to induce vacuolization and death (Figure S6A & S6B). As expected, flies expressing, in their neuronal system, mutated FL-HTT Q128 (Romero et al., 2008) or N-HTT 1-548 Q128 (Lee et al., 2004) showed a decreased survival rate compared to flies expressing wild type N-HTT 1-548 Q0. Surprisingly, life span of three independent strains of flies expressing C-HTT 1722-3144 fragment was even more shortened as compared to polyQ N-ter HTT expressing flies (Figure 7A). By western blotting and immunoprecipitation, we found that expression of the C-HTT 1722-3144 fragment in fly brains did not exceed the level of N-ter HTT or FL-HTT transgenic flies excluding the possibility of an over-expression side effect (Figure S5D). In addition, C-HTT 1722-3144 expressing flies displayed a reduction in their climbing ability to the same extent than the N-HTT 1-548 Q128 flies (Figure S6C). We sectioned aged fly brains and found both in C-HTT 1722-3144 and in N-HTT 1-548 Q128 flies the presence of lesions with massive neuronal loss. We also performed electron microscopy on fly brains. As previously reported, N-HTT 1-548 Q128 fly brains showed the presence of dark condensed nuclei in the dying cells that is reminiscent of what is observed in the R6/2 mouse models of HD (Turmaine et al., 2000). In contrast, C-HTT 1722-3144 fly brains showed disperse organelles with an enlargement of the nuclear volume (Figure 7B). Thus, both N and C-terminal products of polyQ FL-HTT proteolysis are both toxic when expressed separately in fly and leads to neurodegeneration.

FL-HTT is found to be cleaved into C-terminal and N-terminal fragments within brains of HD patients and HD knock-In models (Mende-Mueller et al., 2001). Since C-terminal fragment is able to induce toxicity in vivo in flies, we reasoned that features of C-HTT 1722-3144 fragment-induced toxicity such as dilated ER should also be observed in the brain of HD knock-In mice. We therefore assessed ER stress signaling and performed electron microscopy on the striatum and cortex of 20 months old *Hdh*^{Q111Q111} mice. Strikingly, we detected, in neurons containing nuclear aggregates, the

presence of swollen ER tubules that are reminiscent of ER dilation/vacuolization occurring in striatal cells expressing the C-HTT 587-3144 fragment or when double cleavage of FL-HTT is induced through the SNIPer-TEV system (Figure 7D). We next analysed ER stress signaling in the striatum from the same aged mice. We found that the levels of nuclear ATF6 and of eif2 α phosphorylation were significantly increased in mutant Hdh^{Q111Q111} compared with wild type Hdh^{Q707} mice, indicative of ER stress activation (Figure 7C). Together, these results indicate that proteolysis of mutant FL-HTT leads to ER dilation and stress via the generation of C-terminal proteolytic fragments.

EXPERIMENTAL PROCEDURES

Flies:

Crosses:

All flies were reared on standard cornmeal food at 25°C. However, after Gal4 driven-expression, flies were raised at 29°C in order to enhance the transgene expression.

The following transgenic lines were generated by Bestgene using the P-element transformation in a *w¹¹¹⁸* host line: *UAS-FL-HTT-167TEVQ100*, *UAS-FL-HTT-586TEVQ100*, *UAS-FL-HTT-167/586TEVQ100*, *UAS-FL-HTT-167/586TEVQ23*, *UAS-HTT-C1722-3144*, *UAS-HTT-N1-548Q0*, *UAS-HTT-N1-548Q128*. The *UAS-FL-HTT-Q128* flies were a gift from Juan Botas lab (Baylor College of Medicine, Houston). *ELaV-Gal4* flies were a gift from Jean-René Martin's lab (Unit UPR 9040, Gif-sur-Yvette, France).

In all crosses, *ELaV-Gal4* virgin females were crossed with males of each of the UAS-HTT strains.

Lifespan measurement:

Newborn flies were scored daily and males were separated from females and raised at 29°C. Vials were changed three times a week. We registered the age of dead flies and % of survival was then calculated. Three independent survival assays were performed. The survival curves shown are a mean of three independent experiments. The number of individuals is indicated in the figure legend. Statistical analyses were done using Kaplan-Mayer test by Statview.

Climbing assay:

Flies with the desired genotype were analyzed for their locomotors capacity through the climbing test here described. During a month and three times a week, female flies (>4 days of ages) were put in a closed empty vial of 8cm high. There were tapped down and the number of flies failing to climb

to the top within 30 seconds was scored. This procedure was repeated ten times to make a mean. Around 40 flies were subjected to the test for each genotype.

Validation of the transgene expression:

Flies were snapped frozen with liquid nitrogen at 4 days of age. As the expression of all transgenes was driven by an ELaV promoter, total heads were mechanically lysed (25mM tris pH 7.4, 5 mM EDTA, 250 mM NaCl, 0.1% tritonx100) to analyze the protein level of transgenes by western blotting. The lysate was clarified by centrifugation (10.000g, 10min, 4°C). When immunoprecipitation was achieved prior to Western blotting, it was performed as the following: Briefly, 50 heads of adult flies were lysed, and volume was adjusted to 1 ml. Samples were incubated with G-sepharose beads (sigma P3296) coupled to the anti-mCherry antibodies (curie, dilution 1/50) for four hours at 4°C. Beads were then washed three times with lysis buffer, resuspended in 2X loading Buffer, heated and analyzed by SDS-PAGE.

Electron and light microscopy

For ultrastructural studies, cell monolayers and/or small fragments of tissues were fixed for 1h at 4°C with 2% glutaraldehyde (Prolabo, France) in 0.1 M Sörensen phosphate buffer, pH7.3. Cells were scraped during fixation and centrifuged. The fixed pellets and/or the tissues were rinsed for 1h in ice-cold phosphate buffer, post-fixed with 2% aqueous osmium tetroxide (EMS, United-States) and dehydrated in increasing concentrations of ethanol prior to Epon embedding. Polymerization was carried out for 48 hours at 64°C.

For observation, ultrathin sections of 90 nm were done using a Reichert Ultramicrotome III, were collected on 200-mesh grids coated with Formvar and carbon, stained with uranyl acetate and lead citrate and observed with a FEI Technai Spirit transmission electron microscope at 80 Kv. Digital images were taken with a SIS MegaviewIII CCD camera.

For optical observation, semi-thin sections of 1 µm were mounted on a microscope slide, put in sodium hydroxyde saturated ethanol for 20 min, rinsed, stained with 1% thionine blue (PolySciences) for 30 min at 56°C and washed in ethanol absolute then xylene.

DNA constructs.

The ER-DNM-WT and ER-DNM-DN constructs correspond respectively to human dynamin1-WT (DNM-WT) or dominant negative K44A (DNM-DN) fused to the endoplasmic reticulum transmembrane domain of human atlastin-1 at the C-terminal part. DNM-WT and DNM-DN sequence are from peGFP containing vectors provided by addgene. To engineer these proteins we used the following strategy: We first synthesized (Proteogenix) the sequence here described:

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ACCGGTCACC ATG GCC TAC CCC TAT GAT GTG CCA GAC TAC GCC CGG CGC GCC GCG GCC GCA CGA
ATT CCA GCC ACA CTG TTT GTA GTC ATC TTT ATC ACA TAT GTG ATT GCT GGT GTG ACT GGA TTC ATT
GGT TTG GAC ATC ATA GCT AGC CTA TGC AAT ATG ATA ATG GGA CTG ACC CTT ATC ACC CTG TGC ACT
TGG GCA TAT ATC CGG TAC TCT GGA GAA TAC CGA GAG CTG GGA GCT GTA ATA GAC CAG GTG GCT
GCA GCT CTG TGG GAC CAG GGA AGT ACA AAT GAG GCT TTG TAC AAG CTT TAC AGT GCA GCA GCA
ACC CAC AGA CAT CTG TAT CAT CAA GCT TTC CCT ACA CCA AAG TCG GAA TCT ACT GAA CAA TCA GAA
AAG AAA AAA ATG TAA GGC CGG CCA TAC GTA
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It contains a human kozak (CACCATGGCC) followed by a HA tag (TAC CCC TAT GAT GTG CCA GAC TAC GCC); *Ascl*, *NotI* and *EcoRI* sites and finally the Cterminal part of atlastin-1 (from Proline 446 to the end) corresponding to the Altastin1 transmembrane domain (TM). This sequence was cloned in a pUC19 vector surrounded by *AgeI* and *FseI/SnaBI* sites. Human dynamin-1 WT or DN were subcloned in a pENTRY vector. *Ascl* and *NotI* digestion removed out dynamin1 which was subsequently inserted into the pUC-HA-TM-COOH synthesized by Proteogenix. We obtained at this time a pUC-HA-DNM-TM construct. This last construct has been digested by *Ascl* and *EcoRI* and cloned in a pENTRY Gateway vector. A recombination into the pCDNA3.2DEST vector has been finally performed to obtain the ER-DNM-WT and ER-DNM-DN constructs used in this paper.

The FL-HTT-TEV constructs have been specially designed to encode the HTT protein containing a TEV recognition cleavage site (TEVrcs) at specific key positions. For this purpose, the proteolytic site of the endogenous enzyme (R167 for CTSZ/BLMH or D586 for CASP6) has been substitute by the seven amino acids (ENLYFQS) TEV rcs sequence. As it is difficult to manipulate the HTT protein, we used our synthetic RNA insensitive HTT construct, pARIS-FLHTT that can be easily mutated using cloning cassettes present throughout the protein and called pUC19 vectors. Directed mutagenesis by PCR has been used to replace the amino acid R167or D586 by the TEV rcs, respectively in the pUC19-F2 and pUC19-F3 fragments. To this aim, we used the following oligos:

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GACAGCAACCTTCCAGAAAACCTGTACTTCCCAGTCCCTACAGCTCGAACTG (oligo sens, 167TEV
mutagenesis); CAGTTCGAGCTGTAGGGACTGGAAGTACAGGTTTTCTGGAAGGTTGCTGTC (oligo antisens,
167TEV mutagenesis); TCTGAAATTGTGTTAGAAAACCTGTACTTCCAGTCCGGTACCGACAACCAG (oligo
sens, 586TEV mutagenesis); CTGGTTGTCGGTACCGACTGGAAGTACAGGTTTTCTAACACAATTTCAGA
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(oligo antisens, 586TEV mutagenesis). Then, the pUC19-F2-HTT-167TEV and the pUC19-F3-HTT-586TEV vectors have been digested respectively by SacI/SacII and SacII/KpnI and subcloned in a pENTRY Gateway vector containing a mCherry-tag in its Nterminal part. Finally, a recombination into the pcDNA-DEST-47 (Invitrogen) vector has been achieved, using the LR-clonase-II from Invitrogen, to obtain the pcDNA-pARIS-HTT-N(His;mCherry)-167TEV-C(GFP)-Q23 or –Q100 and the pcDNA-pARIS-HTT-N(His;mCherry)-586TEV-C(GFP)-Q23 or –Q100 constructs.

To obtain the HTT-167TEV-586TEV constructs, the pUC19-F2-HTT-167TEV has been digested by SacI/SacII and cloned in the pENTRY-HTT-N(His;mCherry)-586TEV constructs (Q23 or Q100). A recombination into the pcDNA-DEST-47 (Invitrogen) vector has been finally performed to obtain the pcDNA-pARIS-HTT-N(His;mCherry)-167TEV-586TEV-C(GFP)-Q23 or –Q100 constructs.

Cleavage of HTT.

HTT constructs used in the study come from the pARIS synthetic genes engineered by our lab and its sequence details are already published (Pardo et al., 2010). Using this construct, amino acids corresponding to the cleavage site of caspase 6 and/or bleomycin were replaced by the recognition site of the TEV protease “Glu-Asn-Leu-Tyr-Phe-Gln-(Gly/Ser). In vitro cleavage was performed as follow: Hela cells were plated and transfected using lipofectamine LTX (Invitrogen) following manufacturer instructions. The quantity of plasmid was adjusted for each construct in order to obtain a similar level of expression. 24h after transfection, cells were pelleted and washed with PBS. Cells were then lysed in 100 µl of 50 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl solution using a 25G needle and followed by sonication. The lysate was cleared by centrifugation (10 min at 13000rpm). Digestions were performed using 50 µg of lysate with 0.1 µg or 10 µg of recombinant TEV (gift from Carsten Yanke laboratory) at 34°C during 2h. The reaction was stopped by adding protease inhibitor (sigmaP8340) and loading buffer. Samples were boiled for 5 minutes at 95°C and then analyzed on SDS-PAGE (6% gels and 4-14% precasted-gel).

Cleavage within the cells was conducted using the SNIPer constructs coding for the N-terminal and C-terminal domains of the TEV protease (plasmids FKBP-NTEV and FRB-CTEV) (Gray et al., 2010). Two types of cells were used: Hela and the *SThdh*^{+/+} (Trettel et al., 2000). Cells were co-transfected with the HTT constructs and the SNIPer-TEV (or pcDNA empty vector, as a control), using Lipofectamine LTX and following supplier recommendations. 24h after transfection cells were treated with 10 or 20 nM of rapamycin (Tocris cat no 1292) and incubated 30, 120, 360 minutes or 24h, this induced the association and reconstruction of the TEV and induced HTT cleavage. Then cells were lysed for 10 minutes in a buffer consisting of 20 mM Tris pH8; 120 mM NaCl; 1 mM EDTA; 0,5% NP40 supplemented with proteases inhibitors. Cells were centrifuged 10 min at 13 000 rpm. For each

condition, 60 µg of proteins were analyzed by western blotting was performed. Nter-HTT fragments were revealed using an anti-mcherry antibody and Cter-HTT using an anti-GFP antibody.

For HTT-TEV cleavage by the recombinant caspase-6, striatal *SThdh*^{+/+} cells were electroporated with the different HTT-TEV constructs. Cells were lysed 24 hr post transfection in a buffer devoided from protease inhibitors (150mM NaCl, 50mM Tris, pH7.5, 5mM EDTA, 50mM MgCl₂ and 0.5% Triton X-100). Lysates were briefly sonicated and then centrifuged for 10 min at 13 000 rpm. Cleavage by recombinant caspase-6 was conducted as followed: 50 µg of proteins were incubated with 2,5 Units of the recombinant enzyme (Clinisciences) for 1 hr at 37°C. The reactions were stopped by adding SDS containing buffer. Products were analyzed by Western Blotting using an anti-GFP antibody.

Cell lines

Hela, *SThdh*^{+/+} and HEK293T cell lines were grown at 5% CO₂ in a standard media: DMEM (Dulbecco's modified Eagle's medium, Gibco) supplement with 2 mM of L-glutamine and 10% of inactivated bovine calf serum. *SThdh*^{+/+} are cell lines deriving from immortalized striatal neurons of Hdh^{Q7Q7} mice, media also includes 400µg/ml of geneticin (Gibco) and cells are raised at 33°C (Trettel et al., 2000).

Immunoprecipitation

1-HTT and dynamin1 interaction:

SThdh^{+/+} or HEK293T cells were used (see figures legend). For *SThdh*^{+/+} cells, transfection was done using lipofactamine 2000 (invitrogen) according to the guidelines, for HEK293T cells, calcium chloride was used for transfection. HTT plasmids were co-transfected with SNIPer-TEV, pcDNA, dynamin1 WT or ER-DNM-WT (see cloning section). The DNA complexes were added to cells, raised in a full growth media, and left for 24 h. In experiments requiring HTT cleavage, cells were treated with 20 nM of rapamycin during 24 h. When treatment is over, the cells were washed twice with cold PBS on dishes. Cells were scraped within the lysis buffer (20mM Hepes pH7.4/100mM NaCl/2mM CaCl₂/1mM MgCl₂/0.5% tritonX100) supplemented with protease inhibitor cocktail (sigmaP8340) and phosphatase inhibitors (sigma P5726). After 15 min of incubation, collected cells were centrifuged 15 min at 11300g. 0.5-1mg/ml of the supernatant was pre-cleared with 50µl of slurry G-beads (sigma P3296) ½ during one hour at 4°C. 4 µl of anti-mCherry (Curie) antibody with 5mM ADP (sigma A4386) were added to the pre-cleared lysate and left under rotation overnight. G-beads were then added to

samples and left on rotation during 3h30 at 4°C. G-beads were primarily blocked with 5% BSA to reduce unspecific binding. After washing, beads were re-suspended with 2X loading buffer. Immunoprecipitated complexes were analyzed on a SDS-PAGE followed by western blotting.

2- Nter and Cter-HTT interactions:

Similarly to before, HeLa cells were transfected and lysed with a solution of 20 mM Tris pH8, 120 mM NaCl, 1 mM EDTA, 0.5% NP40. Supernatant was taken after 10 min centrifugation at 10000rpm. 0.5-1mg/ml was incubated for 1 h with pre-formed complexes of antibodies/G-beads (2h incubation). Results were then analyzed by immuno-blotting.

Endoplasmic reticulum (ER) isolation.

The protocol for ER isolation was performed as described (Bozidis et al., 2007). HEK293T cells are transfected by calcium chloride for 24 hours. For figure 5D, cells were co-transfected with HTT fragments, wild type dynamin1 and siRNA targeted against endogenous HTT. siRNA target the human HTT at position 4487 and the sequence is: GCAGGUUUUAGAUUUGCUG ; HTT fragments used are insensitive to the siRNA as they derived for the synthetic RNA insensitive HTT construct, pARIS-FLHTT. For ER isolation: cells were first washed with PBS, scraped and collected by centrifugation. Cells were subjected to breakage in an osmotic solution consisting of 270 mM D-Mannitol, 10 mM Tris pH 7.6, 0.1 mM EDTA (MTE buffer) and then sonicated. Non-lysed cells were removed by centrifugation (1400g for 10 min at 4°C). To discard undesired organelles, the supernatant was centrifuged at 15000g for 10 min at 4°C. Cleared lysate was added onto the top of the ER gradient (from bottom to top: 2 ml of 2M sucrose, 3 ml of 1.5 M sucrose and 3 ml of 1.3 M sucrose) which was then ultra-centrifugated during 70 min at 152 000 g (41Ti swing rotor) at 4°C. We collected cytosolic and ER fraction. Finally the ER fraction was pelleted after an ultracentrifugation at 54000 rpm (TLA100.3 rotor) during 45 min.

Transferrin Assay.

SThdh^{+/+} cells were transfected with lipofectamine 2000 with HTT fragments tagged with mCherry with or without dynamin1 WT or dynamin1 K44A tagged with GFP (addgene obtained plasmids). 24 h after transfection, cells were starved by incubation in DMEM media for 30 min at 33°C. Cells were harvested and washed with PBS. The experiment was entirely conducted in a cold room to correctly monitor endocytosis. Surface cell labelling is achieved by incubating the cells in 2ml of DMEM +

1%BSA + 20mM Hepes + 5µg/ml A647 labelled transferrin (Invitrogen T23366) for 1h and mixing gently from time to time to ensure efficient binding of transferrin to its receptors. Cells were washed with DMEM, 1%BSA, 20mM Hepes and then pelleted. To start endocytosis measurement, cells were put in a water bath at 37°C and aliquots are taken at different time points. The endocytosis of transferrin was stopped with cold PBS. When all time points were collected, cells were pelleted and submitted to an acid treatment for 5 min (50mM Glycine, 100mM NaCl, pH3 (stripping)) to detach remaining transferrin at the cell surface. Finally cells from each time point were pelleted and the intensity of A647-transferrin was analyzed on a FACSAriaTMIII (BD Biosciences). A mean intensity of the fluorescence at 647 was calculated for 10000 cells co-transfected with HTT-mCherry and/or dynamin-GFP. The background value was settled as the following: cells were labelled with A647-transferrin and submitted to the stripping buffer with no endocytosis step in between. The mean of intensity was considered the background value and was subtracted from all other time points.

Cell death analysis.

SThdh^{+/+} cells were electroporated with HTT's constructs using the cell line nucleofactor kit L (Lonza) according to the manufacturer's instructions. Cells were fixed 16, 24 or 48 h after transfection with 4% of paraformaldehyde (in PBS) for 20 min at room temperature. For N-HTT Exon1, immunostaining was performed on the HA tag to detect the transfected cells (see immunofluorescence section and microscopy). Cell death was assessed by the condensation of the nucleus stained with DAPI under an inverted fluorescent microscope (Nikon). Statistical analyses were done using the Graphpad software. Salubrinal (21 µM, calbiochem, Cat No 324895), ZDEVD-fmk (10 µM, calbiochem cat no 264155) and Zvad-fmk (50 µM, calbiochem 6276105) were added on cells for 48 hours. For the knock down of beclin1, two different predesigned siRNA were electroporated (sigma SASI_Mm01_00048143 and 00048144) at a final concentration of 100µM. The siRNA control used is a universal negative siRNA (eurogentec OR-0030-neg05) which does not interfere with any known sequence of the murine genome. TUNEL assay was done using the in situ cell death detection kit (Roche ref 12 156 792 910) and following the guidelines for fixed cells.

For assessing toxicity of HTT cleavage, we recorded cells by video microscopy. *SThdh*^{+/+} cells were electroporated with HTT and SNIPer-TEV or empty pcDNA plasmids or ER-DNM plasmids. 24h after transfection cells were treated with 10 nM of rapamycin and 40 cells for each of the conditions were tracked during 24 hours using the mCherry tag. The time of death were registered and survival curves were calculated as a percentage. Dying cells were recognized by shrinkage, detachment from the

plate and explosion of the cells. Survival statistics were done using Kaplan-Mayer test by Statview software.

Immunofluorescence and microscopy.

SThdh^{+/+} cells were electroporated with HTT's constructs and plates on coverslips. Cells were fixed with 4% paraformaldehyde warmed up at 37°C for 20 min at room temperature (RT). Antibodies used are: anti-LAMP-2 (santa cruz cat no sc-18822 (1/250)), anti-calexin (sigma cat no C4731 (1/200)), anti-HA (roche cat no 11867423001 (1/100)), anti-mcherry (curie (1/50)), anti-GFP coupled with AF488 (molecular probes (1/100)), anti-dynamin1 (santa cruz cat no sc-12724 (1/50)), anti-ATF6 (abcam cat no ab 11909 (1/100)), anti-EEA1 (Millipore cat no 07-1820 (1/50)), anti-GM130 (BD transduction laboratory, cat no 610822), anti-TGN38 (BD biosciences cat no 610899 (1/100)). All secondary antibodies used are coupled to alexa flore 488, 555 or 647 and purchased from molecular probes (Invitrogen). For all immunostainings, first antibodies were incubated with the blocking solution over night at 4°C. Cells were counterstained with DAPI (Roche) and mounted with Moviol anti-bleaching solution.

For LAMP-2 staining, blocking step was performed with PBS-0.2% saponin-3% Bovine calf serum (BSA, euromedex ref 04-100-811-C). Antibody was incubated in the blocking solution over night at 4°C. Coverslips were washed and secondary antibody (1/200) were incubated for 2h at RT. For ER markers and dynamin1 staining, cells were blocked with PBS-5% normal Goat serum (Gibco 16210)-0.3% Tritonx100 during 1.30 hours at RT. After incubation with the first antibodies, coverslips were washed and incubated at RT for 2 hours with the desired secondary antibody diluted in PBS-1%BSA-0.3%tritonx100. To better fix the antibodies and avoid their spreading over time, post-fixation was performed with 2% paraformaldehyde during 10 min at RT. When GFP staining was needed, Ab was incubated, after the post-fixation step, during 2.30 h at 37°C.

Images acquisition and analysis.

Images were acquired using a Leica DM RXA microscope with a HCL PL APO CS oil 63 x NA of 1.4-0.6 objective or a HCL PL APO CS oil 100x NA of 1.4 objective coupled to a piezzo and a coolSNAP HQ camera monitored by Metamorph software. Z-stack step was settled to 200 nm. Some experiments were imaged using a Leica SP5 laser scanning confocal microscope equipped with a 63 X oil-immersion objective. For live-cell imaging, cells were recorded by a Leica DM IRB microscope with an air-40 x objective coupled to a moving stage and a photometric CoolSNAP camera controlled by

Metamorph software. Cells were excited using a mCherry filter and snap shots of each point were taken every 4 min during 24h.

For HTT co-localization studies, cells were doubly stained with mCherry (or HA) and GFP to stain both the N-terminal and C-terminal part of HTT. Quantification of co-localization of HTT fragments was achieved using the ImageJ JACoP plugin (Bolte and Cordelieres, 2006), see figure legend.

Nuclear and cytoplasmic intensity of HTT fragments were quantified using the ImageJ software (measurement of integrated intensity). Background signal was subtracted from intensity given for each cellular compartment.

Immuno-blotting.

For all immunoblottings, cell pellets were lysed in the following buffer: 20 mM Tris pH8, 120 mM NaCl, 1 mM EDTA, 0.5% NP40 and protease inhibitor cocktail. Cellular lysates were centrifuged at 10.000 rpm for 10 min at 4°C. Equal amount of proteins was subjected to SDS-PAGE on polyacrylamide gels and transfer to PVDF membranes (Millipore cat .no IPVH00010). Membranes were saturated with 5% BSA or 5% dry milk in TBS-0.1% Tween-20 for 30 min at RT. All primary antibodies were incubated in the blocking solution over night at 4°C. Antibodies used are: anti-calexin (sigma cat no C4731 (1/2000)), anti-HA (roche cat no 11867423001 (1/1000)), anti-mcherry and anti-GFP (curie (1/500)), anti-dynamin1 (novus biological cat no NB 110-56933 (1/1000)), anti-ATF6 (abcam cat no ab11909 (1/250)), anti-HTT (4C8 (1/1000)), anti-calcineurinA (Millipore (1/1000)), antiBIP/GRP78 (santa-cruz sc-1050 (1/1000)), anti-tubulin (sigma T6199 (1/1000)), anti-actin (sigma A5441 (1/5000)), anti-eif2 α (cell signaling cat no 5324, 1/1000), anti-eif2 α pS53 (Invitrogen cat no 447228G (1/1000)), anti-beclin1 (santa cruz sc-11427 (1/1000)), anti-mitochondrial hsp70 (pierce cat no MA3-028, (1/400)), Annexin V (epitomics 2792-S (1/1000)).

ER stress analysis.

For ER stress analysis, C-HTT 587-3144 GFP or peGFP empty vectors were transfected with lipofectamine 2000 on *SThdh*^{+/+} cells during 24h. Expressing cells were sorted on a FACSAriaTMIII (BD Biosciences) cell sorter on the basis of GFP expression with the assistance of BD FACSDiva Software (BD Biosciences). Non-transfected cells were treated with 2 μ g/ml of tunicamycin (sigma T7765) over night.

For cortex and striatum protein extracts, the brains of HD knock-In mouse were lysed. We used wild type (Hdh^{Q7Q7}) or mutant male homozygote mice. Cortex and striatum were dissected and lysed with

a potter homogenizer. Samples were centrifuged twice at 10000 rpm for 10 min and supernatants were analyzed by SDS-PAGE and Western blotting.

FIGURE LEGENDS

Figure 1. Cleavage of wild type and mutant FL-HTT-TEV constructs by the TEV protease.

- A.** Schematic drawing of HTT protein containing the TEV recognition site at the indicated position. Wild type HTT used in this study contains 23 glutamine repeats whereas the mutated HTT contains a stretch of 100 glutamines. TEV at 586 replaces the caspase-6 cleavage site (referred as FL-HTT-586TEV). TEV at position 167 replaces the cathepsinZ/bleomycin Hydrolase cleavage site (referred as FL-HTT-167TEV). HTT constructs containing the two TEV sites at position 167 and 586 were also engineered (referred as FL-HTT-167/586TEV).
- B.** *In vitro* cleavage of FL-HTT-TEV constructs by the recombinant TEV protease. Cells lysates from Hela cells transfected with the different constructs of HTT were subjected to *in vitro* proteolysis by the TEV protease used at increased concentrations. Left and right panels show Western blots assessing the proteolysis profile of the wild type and mutant HTT constructs respectively. The upper panels reveal the presence of the C-terminus end of the HTT as shown by anti-GFP antibody, the lower panels reveal mCherry tag localized at the N-terminus part of the HTT. TEV protease had no effect on HTT with no TEV sites (CT-HTT). TEV protease induces the cleavage of the various HTT constructs containing TEV sites and generated products of expected size as shown by the last two lines that show the migration of N-HTT1-167 and N-HTT1-586 fragments with the C-HTT586-3144. 10 µg of the recombinant TEV is necessary to cleave HTT at position 167 whereas 0.1 µg of TEV was enough to cleave significantly HTT at position 586 and to generate the expected N and C fragments.
- C.** Cleavage of FL-HTT TEV constructs in cells by the SNIPer TEV expressing plasmids. Cells lysates were prepared from Hela cells co-transfected with the FL-HTT-TEV and the SNIPer plasmids and treated with 10 nM of rapamycin to induce TEV activity. Cleavage was assessed as in A. Cleavage at position 167 occurred 6 h after TEV induction by the rapamycin whereas the cleavage at position 586 occurred 30 min after induction. Cleavage of mutant HTT at position 167 is more efficient than WT HTT. ND corresponds to cells transfected with an empty pcDNA vector instead of the SNIPer-TEV and treated with rapamycin for 6 hr.
- D.** Cleavage analysis of the wild type HTT versus mutant HTT. *SThdh*^{+/+} cells were co-transfected with the FL-HTT-TEV or N-HTT-167TEV (N-HTT1-586 with a TEV site at position 167 replacing cathepsinZ site) and the SNIPer-TEV. Cells were treated during 24h with 20 nM of rapamycin and protein extracts were analyzed as in A. In the absence of the SNIPer constructs (pcDNA), none of the HTT-TEV constructs were cleaved. At the right, cleavage is induced and proteolysis pattern show that the amount of the generated N-HTT1-167Q100 is greater than N-HTT1-167Q23 in all

three conditions FL-HTT-TEV167, FL-HTT-167/586TEV and N-HTT-167TEV. However, the amount of N-HTT1-586 generated after cleavage is the same in wild type and polyQ HTT.

- E.** Graph showing the levels of N-HTT1-167 products generated after FL-HTT-TEV or N-HTT-TEV proteolysis obtained in **D**. The calculated percentage (ratio over the non-digested construct) is a mean of three independent experiments. Error bars: SEM, one way Anova with Bonferroni multiple comparisons tests * P<0.05, ** P<0.01, *** P<0.001.

Figure 2. Specific proteolysis of both wild type and mutated HTT revealed toxic events.

- A.** *SThdh*^{+/+} cells were co-transfected with the different FL-HTT-TEV constructs and pcDNA or SNIPer vectors. 20hrs post-transfection cells of all conditions were treated with 10 nM of rapamycin and recorded via multiposition videomicroscopy for 24h. Controls are cells co-transfected with HTT and pcDNA vector. For each condition, the toxicity of non-cleaved (pcDNA) to cleaved HTT (SNIPer) is shown as a percentage of survival over time. Double proteolysis leads to significant increase in toxicity compared to uncleaved polyQ HTT. Cleavage of HTT at 586 although efficient has no marked toxic effect. Cleavage of HTT at position 167 showed little toxicity most probably due to inefficient cleavage at this position. Two to four independent experiments are shown. The total numbers of cells assessed in this assay are the following: FL-HTTQ100+pcDNA=71 or +SNIPer= 70, FL-HTT-167TEVQ100+pcDNA=134 or +SNIPer= 155, FL-HTT-586TEVQ100+pcDNA=88 or +SNIPer= 134, FL-HTT-167/586Q100+pcDNA=217 or +SNIPer= 257. Kaplan-Meier, Log-rank test was used for statistics. *** P<0.001.
- B.** N-terminal HTT fragments referred as N-HTT1-586 Q23 or Q100 were transfected in *SThdh*^{+/+} cells and cell death was scored on fixed cells according to nuclear condensation, 16 hours post transfection. Error bars: SEM, *** P<0.001, unpaired t-test.
- C.** Toxicity of various polyQ N-terminal fragments was analyzed as in B: N-HTTExonQ68, N-HTT1-167Q100 and N-HTT1-586Q100. Error bars: SEM, ** P<0.01, one way anova with Bonferroni multiple comparisons tests.
- D.** Various N-terminal HTT fragments were transfected in *SThdh*^{+/+} cells alone or in combination with the C-HTT587-3144 fragment. The graph summarizes the percentage of cell death quantified by nuclear condensation, on fixed cells, 16h after transfection. Error bars: SEM, ** P<0.01, *** P<0.001, Unpaired t test. For **B**, **C** & **D**, three independent experiments were performed. A range of 100 to 200 cells were counted in each independent experiment.
- E.** Survival curves of cells transfected with FL-HTT-TEV constructs and recorded by multiposition videomicroscopy over 24hours. The replacement of cathepsinZ and caspase-6 sites by TEV recognition sites at position 167 and 586 did not alter the survival of cells expressing the wild type FL-HTT-167/586TEV. The co-transfection of FL-HTTQ23 with the SNIPer constructs also did

not change the survival curve of cells expressing FL-HTTQ23. All cells were treated with 10 nM of rapamycin indicating that at such concentration rapamycin had no toxic effect. Two to three independent experiments are shown. The total number of cells assessed in this assay is: FL-HTTQ23+pcDNA= 97, FL-HTT-167/586TEVQ23+pcDNA=200, FL-HTTQ23+SNIPer=120. Kaplan-Meier, Log-rank test was used for statistics. Ns: non-significant.

- F.** Survival curves of cells transfected with Wild Type (Q23) and mutant (Q100) FL-HTT-167/586TEV constructs. Double cleavage of WT HTT is almost as toxic as the proteolysed polyQ HTT. Three to four independent experiments are shown. The total number of cells assessed is: FL-HTT-167/586 Q23+pcDNA=200 or +SNIPer= 199, FL-HTT-167/586Q100+pcDNA=217 or +SNIPer= 257. Kaplan-Meier, Log-rank test was used for statistics. * P<0.05, ** P<0.01, *** P<0.001.

Figure 3. Intramolecular interaction between HTT fragments defines their toxicity.

- A.** HeLa cells expressing various Nter-HTT and Cter-HTT fragments, as indicated, were used for subsequent immunoprecipitation assays and Western blot (WB) analysis. Immunoprecipitation of N-HTT1-586Q100 and N-HTT1-167Q100 with anti-mCherry antibody reveals an interaction with C-HTT587-3144 GFP only for the mCherry N-HTT1-586Q100 fragment.
- B.** N-HTT1-586Q100 and N-HTTExon1Q100 were immunoprecipitated with HA antibodies. WB using anti-GFP antibody reveals an interaction of C-HTT587-3144 GFP with N-HTT1-586Q100 but not N-HTTExon1Q100.
- C.** HeLa cells were co-transfected with wild type or mutated FL-HTT-TEV constructs. Cleavage of HTT at specific TEV sites was induced by addition of the recombinant TEV on cells lysis for two hours. At the left, WB showed the inputs of non-cleaved HTT and the products obtained after cleavage. At the right, the upper WB displayed the results of the immunoprecipitation performed using an anti-GFP antibody to precipitate the non-cleaved FL-HTT and the Cter-HTT fragments. The lower panel shows that only the N-HTT1-586 are efficiently immunoprecipitated by the C-HTT586-3144 fragment. Stars indicate non-specific bands.
- D.** HeLa cells were co-transfected with wild type or mutated FL-HTT-167/586TEV constructs and extract were cleaved *in vitro* by recombinant TEV. The N-ter and C-ter fragments were respectively immunoprecipitated using mCherry or GFP antibodies (IgG for control). The intermediate fragment of HTT from amino acid 168 to 586 detected using a 4C8 antibody interacts only with the C-ter fragment.
- E.** Cartoon of the different interactions between HTT fragments.
- F.** Immunofluorescences of HTT fragments expressed in *SThdh+/+* cells. Mutated Nter-HTT fragments are stained with mCherry or HA antibodies and the C-HTT587-3114 is labeled with GFP. Co-localization is measured using JACoP, an ImageJ plugin based on the distance between

the mass centers of particles from two different fluorophores. The maps provided by the plugin and the percentage of co-localization are shown at the right. 100% corresponds to the co-localization between mCherry and GFP tags of the FL-HTT (control). Results are the mean of three independent experiments with 25 cells quantified each time in each condition. Error bars: SEM, “ns” non-significant, ** P<0.01, *** P<0.001 (one way anova, Bonferroni multiple comparisons test).

Figure 4. Cter-HTT, released from its Nter-HTT partner, elicits ER swelling and stress.

- A.** Representative immunostainings of *SThdh*^{+/+} cells expressing C-HTT587-3114-GFP and various Nter-HTT constructs fused to mCherry of HA. Cytoplasmic vacuolization appears in cells in which C-HTT587-3114 is released from its interaction with the N-ter fragment. The upper histogram presents the ratio of nuclear HTT over cytoplasmic HTT (integrated intensity). Around 50 cells were quantified per condition (two independent experiments). Lower histogram presents the percentage of cells undergoing vacuolization (three independent experiments in duplicates). Error bars: SEM, *** P<0.001 (one way anova, Bonferroni multiple comparisons test).
- B.** Time lapse imaging for GFP-KDEL localization, a luminal endoplasmic reticulum (ER) marker, in cells expressing C-HTT587-3114 fragment recorded over 18 hours.
- C.** Immunostaining of *SThdh*^{+/+} cells showing the localization of calnexin and ATF6, two membranes ER markers, surrounding the formed-vacuoles in C-HTT587-3114 expressing cells.
- D.** Western blot showing the levels of ER stress-related proteins in *SThdh*^{+/+} cells expressing the C-HTT587-3114 GFP fragment sorted by FACS. Control cells are negative for GFP. Cells treated with tunicamycin (2µg/ml overnight) were used as positive control. The mean intensity level is indicated below (three experiments +/- SEM).
- E.** *SThdh*^{+/+} cells transfected with C-HTT587-3114 GFP were treated with inhibitors along the transfection period (40h) and cell death was analyzed by chromatin condensation after fixation. The upper graph displays the percentage of death of C-HTT587-3114 expressing cells treated or not with 21 µM of salubrinal, an ER stress inhibitor. The lower graph displays the percentage of vacuolated cells with or without salubrinal treatment. For all graphs the condition Cter-HTT is set at 100%. Error bars: SEM, ** P<0.01, *** P<0.001, unpaired t test on three independent experiments in triplicates.
- F.** Huntingtin proteolysis disrupts ER network. Calnexin immunostaining in *SThdh*^{+/+} cells expressing the wild type or mutant FL-HTT-167/586TEV. In control condition (left, +pcDNA), and after induction of the TEV (+SNPer) with 20 nM of rapamycin during 24 hours (right, +SNPer). Cells are co-transfected with a siRNA down-regulating the endogenous HTT and not the HTT-TEV

constructs (data not shown). Analysis of ER network reveals a loss in the proper connection of the tubules that normally form polygonal structures (see enlargements). Scale bar: 10 μ m.

Figure 5. Proteolysis of FL-HTT changes its interacting with dynamin1, impairing its activity.

- A.** Huntingtin interacts with dynamin1. Left: HEK 293T cells were transfected with mCherry mutant FL-HTT, mCherry Cter-HTT or mCherry mutant Nter-HTT fragments and wild type dynamin1. Immunoprecipitation was performed using the mCherry antibody. FL-HTT, C-HTT587-3144 and N-HTT 1-586 but not the short N-HTT 1-167 interact with dynamin1. Right: HEK 293T cells were transfected with dynamin1 and FL-HTT-586TEVQ100 or FL-HTT-167/586TEVQ100 and pcDNA or SN1per constructs and treated during 24h with 20 nM of rapamycin to induce complete SN1per-cleavage of HTT-TEV constructs at specific sites. Immunoprecipitation was performed using the mCherry antibody. Western blot for dynamin1 demonstrate that FL-HTT and the proteolytic product N-HTT1-586 interact with dynamin1. In contrast, upon sequential proteolysis of HTT at position 167 and 586, dynamin1 do not interact with the Nter-HTT products.
- B.** *SThdh*^{+/+} cells were transfected with C-HTT 587-3144 and dynamin1 wild type (DNM1-WT) or a dominant inactive form (K44A) of dynamin1 GFP (DNM1-DN). The graph represents the percentage of cell death quantified by nuclear condensation on fixed cells, 48h after transfection. Means are from four independent experiments in triplicates. Error bars: SEM, ***P<0.001, one way anova, Bonferroni multiple comparisons test.
- C.** ER membranes were isolated from *SThdh*^{+/+} cells transfected with a wild type form of dynamin1 (WT) or an inactive form of dynamin1 (DN) and fractions were analyzed by Western blot. T: total fraction, C: cytosolic fraction, E: ER isolated fraction. Calnexin is a marker for ER membranes; AnnexinV is a marker of plasma membranes; alpha-tubulin and actin are cytosolic proteins, mitochondrial hsp70 is a mitochondria marker. Dynamin1 antibody reveals the presence of dynamin1 WT and DN on ER membranes.
- D.** HEK263T cells were transfected with different HTT fragments and analyzed as in **C**. In all conditions, the endogenous HTT is down-regulated by siRNA and replaced by the desired HTT fragments derived from RNA insensitive HTT construct (pARIS-FLHTT constructs) (data not shown). FL-HTT, C-HTT587-3144 and N-HTT1-586 constructs are associated to ER membranes. In contrast, the short N-HTT 1-167 is cytosolic. The ratio of dynamin1 in ER fraction / cytosolic dynamin1 is reduced by the Cter-HTT alone (as compared to the FL-HTT expressing cells set to 100%) or when co-expressed with Nter-HTT fragments (the percentage below is a mean of three independent experiments +/- SEM).

- E. Representative image of C-HTT587-3144 expressing cell (*SThdh*^{+/+} cells) (purple) immunostained with dynamin1 antibody. Enlargement: dynamin1 shows a decreased level at the plasma membrane (red lines) as compared to an adjacent non-transfected cell (control).
- F. Cter-HTT expressing cells have a reduced dynamin1 activity as shown by reduced endocytosis. Transferrin uptake assay was performed on *SThdh*^{+/+} cells transfected with the C-HTT587-3144 alone or with dynamin1 constructs. Left graph shows that C-HTT587-3144 cells have a reduced transferrin uptake capacity compared to control cells expressing an empty vector (100%). The right graph show that Cter-HTT-induced defect in endocytosis is lost by overexpression of WT dynamin1. The mean intensity of transferrinAF647 uptake is calculated by cell sorting on 10.000 cells. Three independent experiments, error bars: SEM, * P<0.05, ** P<0.01, unpaired t test.

Figure 6. HTT proteolysis impairs the activity of ER-localized dynamin1 inducing ER dilation and death.

- A. Cartoon of wild type (WT) or dominant inactive form (DN) of dynamin1 fused to atlastin1 transmembrane sequence (ER-DNM) and fused with an HA tag in its N-terminal part.
- B. *SThdh*^{+/+} cells were transfected with ER-DNM-WT or DN and fixed 48hr later. Cells were immunostained using an HA antibody and cell death quantified by chromatin condensation. Three experiments in triplicates, error bars: SEM, *** P<0.001, unpaired t test.
- C. Survival curves of *SThdh*^{+/+} cells expressing an empty mCherry vector with DNM-DN or ER-DNM-DN. Cells were recorded during 24 hours by videomicroscopy using the mCherry tag and the time of death was scored as well as the number of cells forming vacuoles (x% vacuolated cell). Total number of cells: DNM-DN=125, ER-DNM-DN=160. Three independent experiments, survival analysis were done by Kaplan-Meier, Log-rank tests, P=0.0026.
- D. Time-lapse images of an ER-DNM-DN and mCherry empty vector expressing cell followed by videomicroscopy and undergoing cytoplasmic vacuolization.
- E. *SThdh*^{+/+} cells were co-transfected with different mCherry-tagged fragments of HTT and ER-DNM-WT. immunoprecipitation (IP) assays were performed using a mCherry antibody: on total cell lysate (left) and on ER isolated membranes (right). Western blot using an HA antibody reveals the interaction between ER targeted dynamin1 and FL-HTT, C-HTT587-3144 and N-HTT1-586 but not the short N-HTT1-167 at ER membranes.
- F. *SThdh*^{+/+} cells were transfected with C-HTT587-3144 and ER-DNM-WT or ER-DNM-DN. The graphs show the percentage of cell death (condensed nuclei) and vacuolated cells (showing cytoplasmic vacuolization) quantified by on fixed cells, 48h after transfection. Means are from three independent experiments in triplicates. Error bars: SEM, ***P<0.001, one way anova, Bonferroni multiple comparisons test.

- G.** Toxicity induced by the double proteolysis of HTT is blocked by ER-targeted dynamin1 WT and not by ER-targeted dynamin1 DN. *SThdh*^{+/+} cells were co-transfected with FL-HTT167/586TEV Q23 (above panel) or Q100 (below panel) with ER-DNM-WT (left) or DN (right). Cells were treated with 20 nM of rapamycin and followed by videomicroscopy as in Figure 2 (pcDNA: uncleaved HTT-TEV, SNIPer: cleaved HTT-TEV). Graphs show the survival curves of cells in each condition. Three independent experiments, the total number of cells assessed in this assay are the following: FL-HTT167/586 Q23 + ER-DNM-WT: pcDNA=270 & SNIPER= 239; FL-HTT167/586 Q23 + ER-DNM-DN: pcDNA=265 & SNIPER= 196; FL-HTT167/586 Q100 + ER-DNM-WT: pcDNA=272 & SNIPER= 245; FL-HTT167/586 Q100 + ER-DNM-DN: pcDNA=204 & SNIPER= 209. Kaplan-Meier, Log-rank test was used for statistics. Ns: non-significant, ** P<0.01.
- H.** ER targeted dominant negative Dynamin1 induces vacuoles formation independently of HTT-TEV cleavage. The percentage of vacuolated cells was quantified using the videomicroscopy experiments performed in **C**. Error bars: SEM, ** P<0.01, *** P<0.001, one way anova, Bonferroni multiple comparisons test.

Figure 7. Toxic events induced by HTT proteolysis are observed in flies and in a HD mouse model.

- A.** Lifespan and half life analysis of transgenic flies expressing different HTT-TEV constructs. All transgenes are under the control of UAS promoter. UAS-HTT-TEV flies were crossed with flies expressing the Gal4 diver under the pan-neuronal ElaV promoter (ElaV-Gal4). Under such condition, the HTT-TEV is expressed in the nervous system of generated flies but is not cleaved and is considered here as the control (ctr). Cleavage of HTT is induced at position 167, 586 or both by using two independent strains expressing the TEV protease under the heat-shock promoter (hs-TEV) or the UAS promoter (UAS-TEV). Homozygote flies containing the hs-TEV or the UAS-TEV transgenes on the chromosome 2 and the UAS-HTT-TEV transgenes on chromosome 3 were crossed with the ELA-V-Gal4 flies to induce the expressing of the transgenes. Offspring were raised at 31°C along the lifespan assay and the number of dead flies was recorded. Total offspring: (i) FL-HTT-167TEVQ100: ctr=135, hs-TEV=129, UAS-TEV=119; (ii) FL-HTT-586TEVQ100: ctr=164, hs-TEV=83, UAS-TEV=105; (iii) FL-HTT-167/586TEVQ100: ctr=144, hs-TEV=119, UAS-TEV=39; (iiii) FL-HTT-167/586TEVQ23: ctr=124, hs-TEV=99, UAS-TEV=106. Kaplan-Meier, Log-rank test of survivorship curves were performed. In each of the four conditions comparisons are made between control flies and flies with cleaved HTT; All differences are significant, P<0.001.
- B.** Lifespan of transgenic flies expressing FL-HTTQ128, FL-HTTQ128, N-HTT1-548Q128 and C-HTT1722-3144 were assessed. All transgenes are under the control of UAS promoter. UAS-HTT flies were crossed with ElaV-Gal4 flies. Three different strains expressing the Cter-HTT are shown (number 140, 143, 145). Three independent crosses with the total number of flies indicated

hereafter: FL-HTTQ128: 287, N-HTT1-548 Q0: 440, N-HTT1-548 Q128: 478, C-HTT1722-3144 #140: 234, C-HTT1722-3144 #143: 223, C-HTT1722-3144 #145: 223. Kaplan-Meier, Log-rank test was used for statistics. N-HTT 1-548 Q0 **vs** N-HTT 1-548 Q128: $P < 0.001$, C-HTT 1722-3144# 143 & #145 **vs** N-HTT 1-548 Q128: $P < 0.01$.

- C.** Brain sections of aged flies expressing wild type (Q0) Nter-HTT, mutant (Q128) Nter-HTT or Cter-HTT fragments. Upper panels: thionin blue counterstaining where optical lobes (OL) and ellipsoid body (EB) are marked with blue dashed lines. Lower panels: ultrastructure images obtained by electronic microscopy (EM). Red arrowheads indicate lesions and dying cell, red dashed lines point at a loss in neuronal tissue indicative a neurodegeneration.
- D.** ER stress signaling was assessed in two brain regions of Hdh^{Q7Q7} and $Hdh^{Q111Q111}$, the cortex and the striatum by Western blotting. The ratio of nuclear ATF6 and the phosphorylation level of eif2 α is shown. 6 to 8 mice of 20 month old and of each genotype were used for the quantification. Error bars: SEM, * $P < 0.05$, unpaired t test.
- E.** Ultrastructure analysis of striatal sections from $Hdh^{Q111Q111}$ mice by electronic microscopy (EM). Left: nuclear aggregate in the nucleus of striatal neurons. Middle: ER dilated lumen observed in those striatal neurons. Right: Dilated ER observed by EM in striatal cells expressing Cter-HTT.

Figure 1

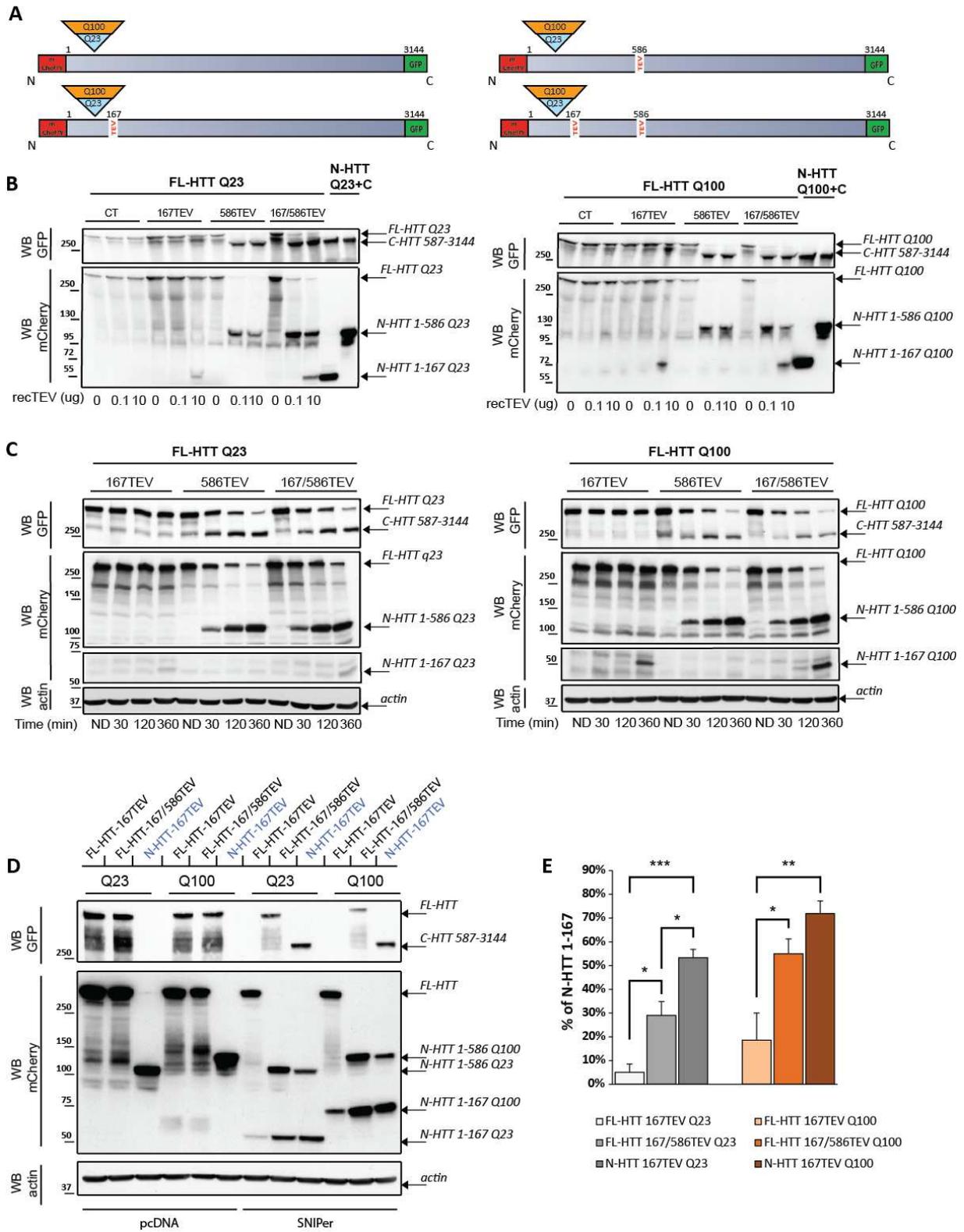


Figure 2

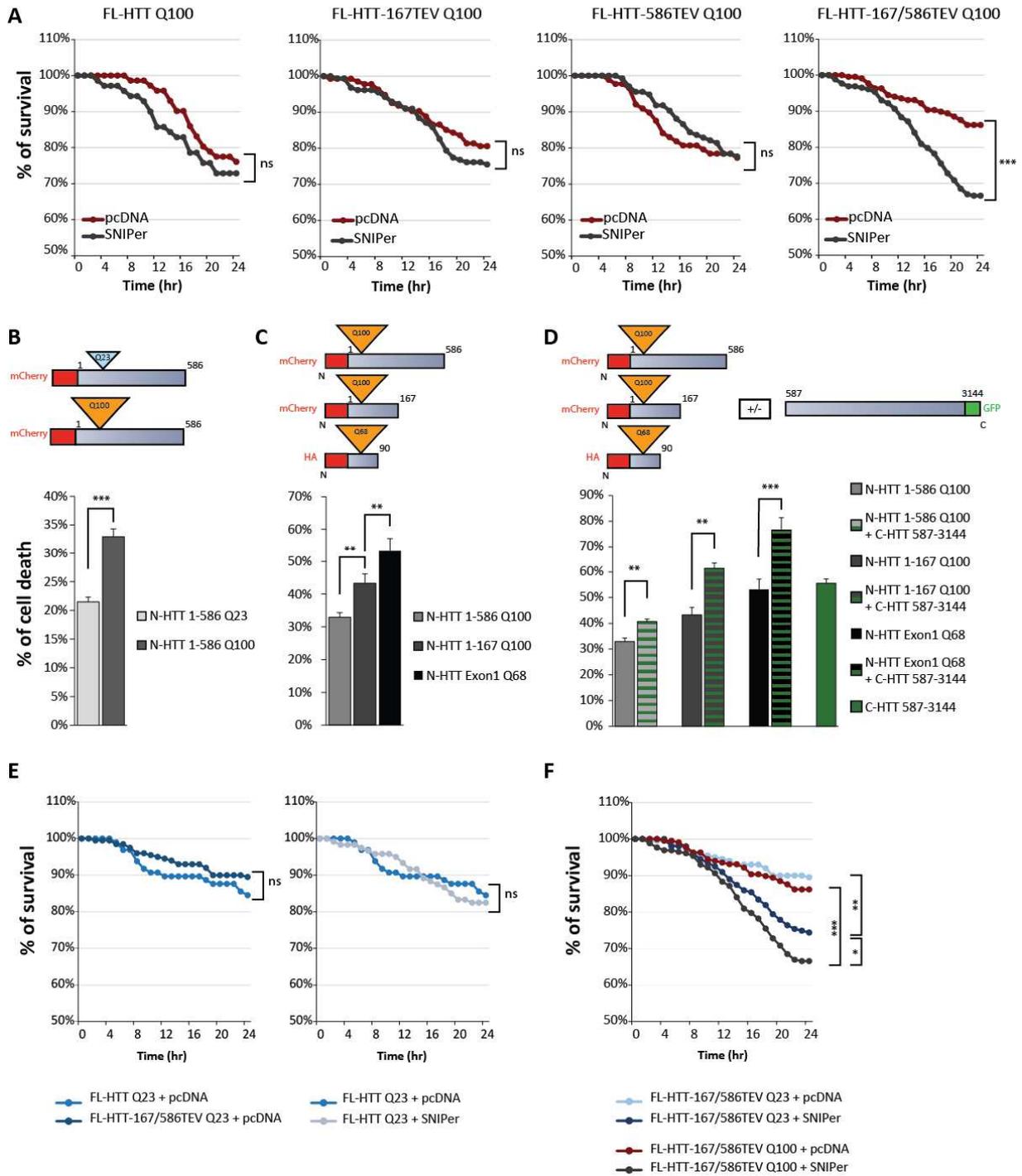


Figure 3

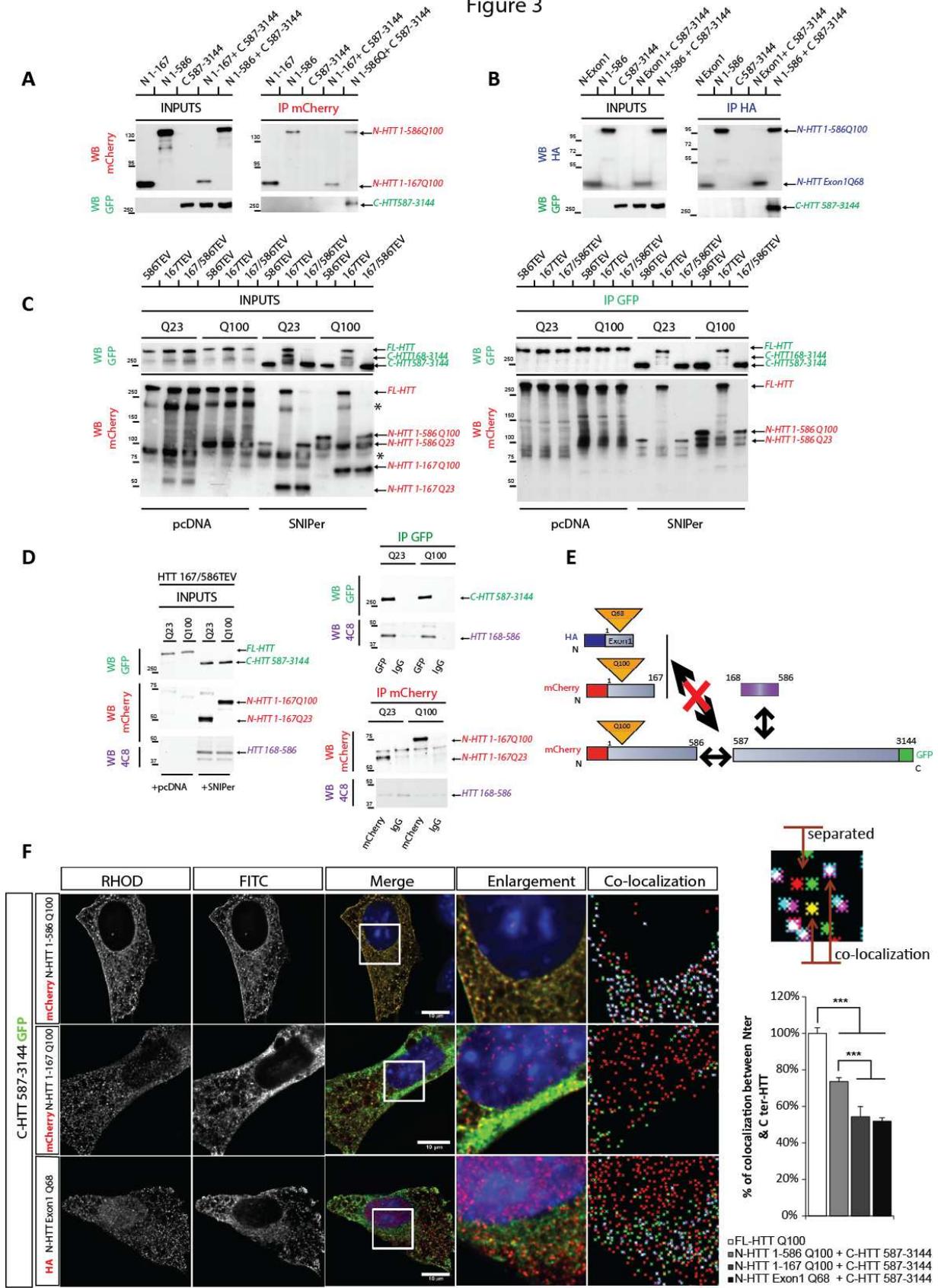


Figure 4

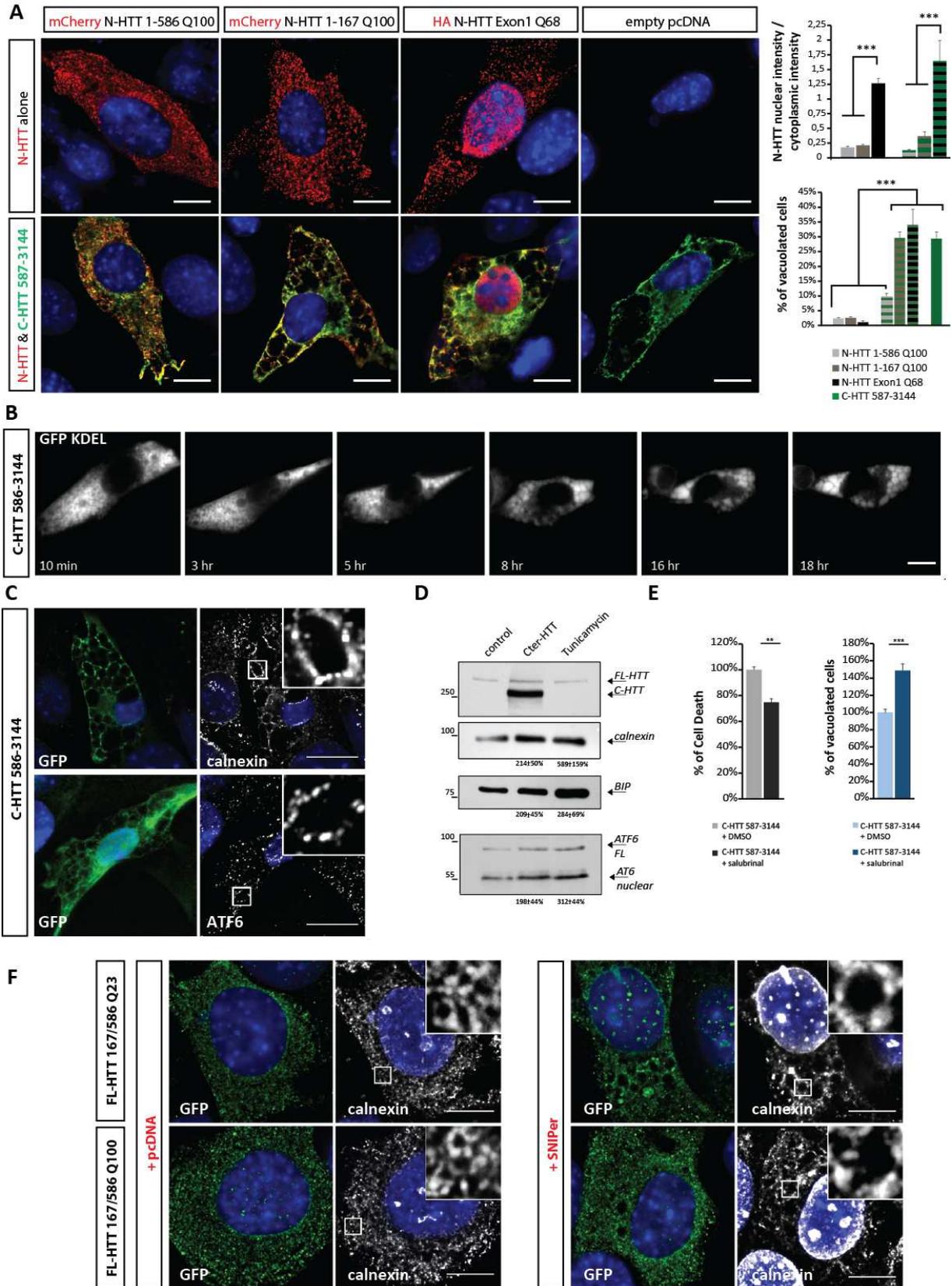


Figure 5

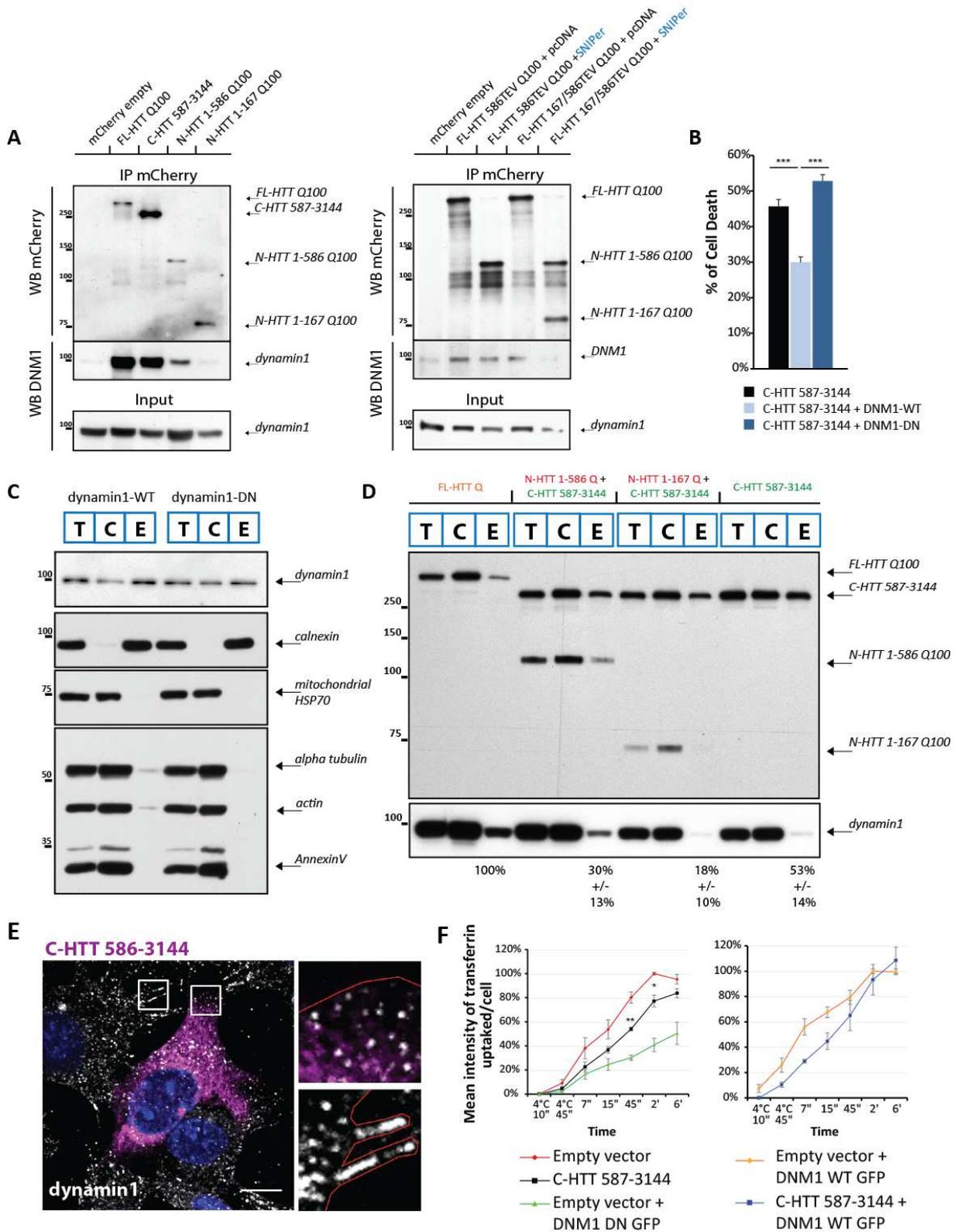


Figure 6

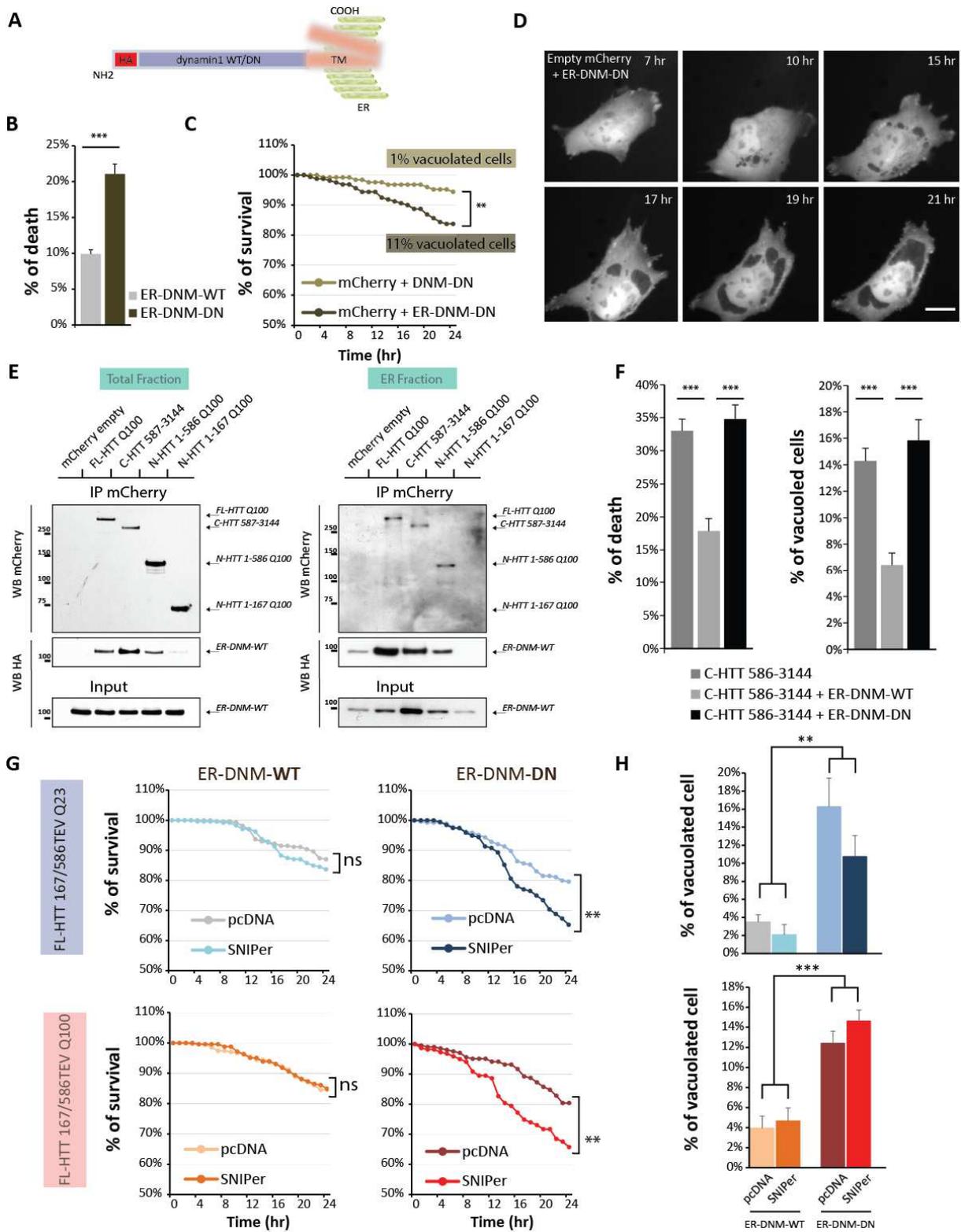
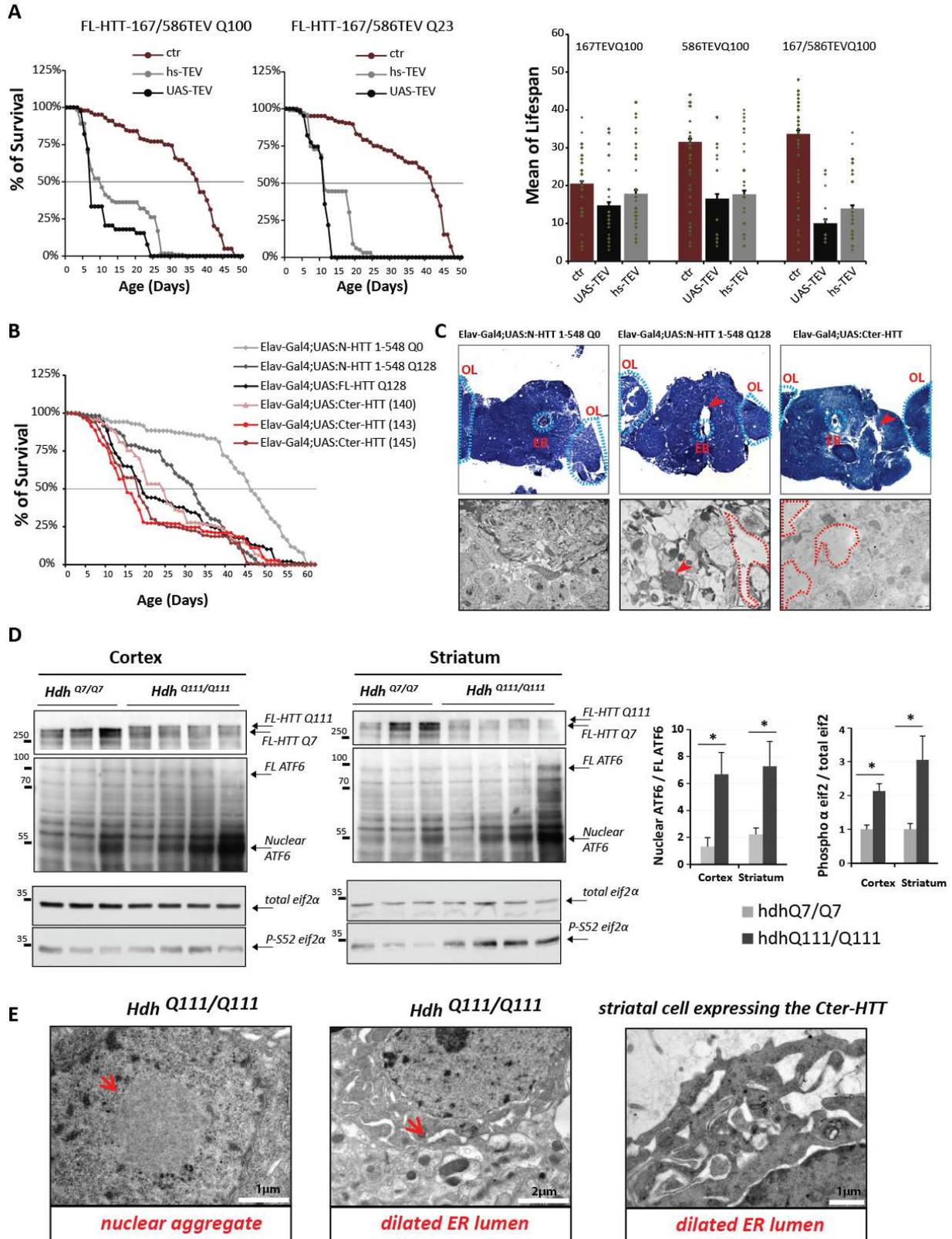


Figure 7



SUPPLEMENTAL PROCEDURES

Figure S1. Replacement of HTT cleavage sites by TEV recognition sites does not alter HTT toxicity.

- A.** Cleavage of FL-HTT-TEV constructs by the recombinant caspase-6. Only the FL-HTT with a TEV site at position 586 were resistant to caspase 6 cleavage.
- B.** Analysis of wild type (Q23) and mutant HTT (Q100) toxicity by videomicroscopy in *SThdh+/+* cells expressing wild type or mutant constructs of HTT. Cells were recorded during 24 hours using the mCherry fluorescence and the time of death was scored. Curves show a decreased survival of cells expressing mutant HTT compared to wild type HTT. Two or three independent experiments; Kaplan-Meier, Log-rank test was used for statistics. Ns: non-significant. Below: histogram represents the overall cell death accumulated during 24 hours. The mean toxicity of mutant FL-HTT constructs is higher than the wild type indicating that the insertions of the TEV sites did not alter significantly HTT toxicity within the time frame of the performed experiments.

Figure S2. Vacuoles formed in Cter-HTT expressing cells do not derive from the Golgi Apparatus, autophagic vesicles or endosomes.

- A.** Assessment of Golgi Apparatus compartments by immunofluorescence (GM130 stained the cis-Golgi; cter433 the medial-Golgi and the TGN38 the trans-Golgi) in *SThdh+/+* cells. At the left, control cells are non-transfected cells, at the right is shown Golgi staining in cells transfected with the mCherry C-HTT 587-3144.
- B.** Assessment of autophagosome and lysosome markers in Cter-HTT expressing cells. Autophagosomes are marked with GFP-LC3 expressing plasmid. Lysosomes are stained with LAMP-2 antibody.
- C.** Assessment of early endosomes in Cter-HTT expressing cells. Up: *SThdh+/+* cell co-expressing the C-HTT587-3144 and a vector coding for a wild type Rab5-GFP. Below: EEA1 staining of a Cter-HTT expressing cell. Scale bars: 10 μ m.

Figure S3. Cter-HTT-induced cell death does not show classical apoptosis or autophagy.

- A.** DNA fragmentation in Cter-HTT expressing cells was assessed using a TUNEL assay. Alive and dead Cter-HTT GFP expressing *SThdh+/+* cells are shown with the corresponding TUNEL staining in red. Cells treated with the enzyme DNase I prior to TUNEL staining represent positive DNA fragmentation in this assay. The nucleus is counterstained with DAPI (blue).
- B.** Percentage of cell death (based on chromatin condensation) counted on cells fixed 48 hours after transfection in cells expressing the C-HTT587-3144 fragment treated with DMSO (1/1000) or 10

μM of Z-DEVD-FMK, a caspase-3 inhibitor. We validated the efficiency of this drug on cell death induced by H_2O_2 treatment (1mM) that potently activates caspase-3, during 2h on non-transfected cells. Data are from 2 independent experiments performed in triplicates. For the quantification of cell death after H_2O_2 treatment, one experiment was performed in duplicate however more than 1000 cells were analyzed Ns: non-significant, * $P < 0.05$, unpaired t test.

- C. Percentage of cell death in C-HTT 587-3144 expressing cells treated or not with 50 μM of Zvad-FMK, a pan caspase inhibitor. Data are from three independent experiments performed in duplicates. Ns: non-significant, unpaired t test.
- D. Two sets of siRNA (n1 & n2) targeted against beclin1 were co-transfected with C-HTT587-3144. Cell death was determined as before and the down-regulation of beclin1 expression was verified by Western blot.

Figure S4. Inactive dynamin1 targeted to ER membranes induced ER dilation and toxicity.

- A. ER membranes were isolated from *SThdh*^{+/+} cells transfected with a wild type form of ER-targeted dynamin1 (ER-DNM-WT) or an inactive form of dynamin1 (ER-DNM-DN). Fractions analyzed by Western blot are: “T” stands for total fraction of extracted protein, “C” for cytosolic fraction and “E” for ER isolated fraction. Calnexin is a marker for ER membranes; AnnexinV is a marker of plasma membranes; tubulin and actin are cytosolic proteins, mitochondrial hsp70 is a mitochondria marker. ER-DNM is revealed using anti-HA antibody.
- B. Immunostainings showing the localization of ER-DNM-WT, using an HA antibody, on the ER network as identified by calnexin antibody and ERdsred vector (a fluorescent marker for ER network). ER-DNM-WT is also recognized by an anti-dybamin1 antibody as showed in the above montage.
- C. The image represents a 3D reconstitution of ER-DNM-WT staining (HA tag) in red and GFP-KDEL, a luminal marker of the ER, in green. It is a magnification of a *SThdh*^{+/+} cell co-transfected with the ER-DNM-WT and GFP-KDEL constructs showing a localization of ER-DNM outside the ER lumen.
- D. Immunostaining of the ER network by calnexin antibody in ER-DNM-DN (HA staining) expressing cells. The ER compartments are enlarged indicating an ER dilation in ER-DNM-DN expressing cells not in ER-DNM-WT expressing cells as shown in C.

Figure S5. HTT expression and cleavage by the TEV protease is effective in flies.

- A. Expression and cleavage of HTT-TEV transgenes within *D. melanogaster*. All transgenes are under the control of UAS promoter and offspring raised at 31°C. Left: UAS-FL-HTT-TEV flies were crossed with flies expressing the Gal4 diver under the pan-neuronal ElaV promoter (ElaV-Gal4).

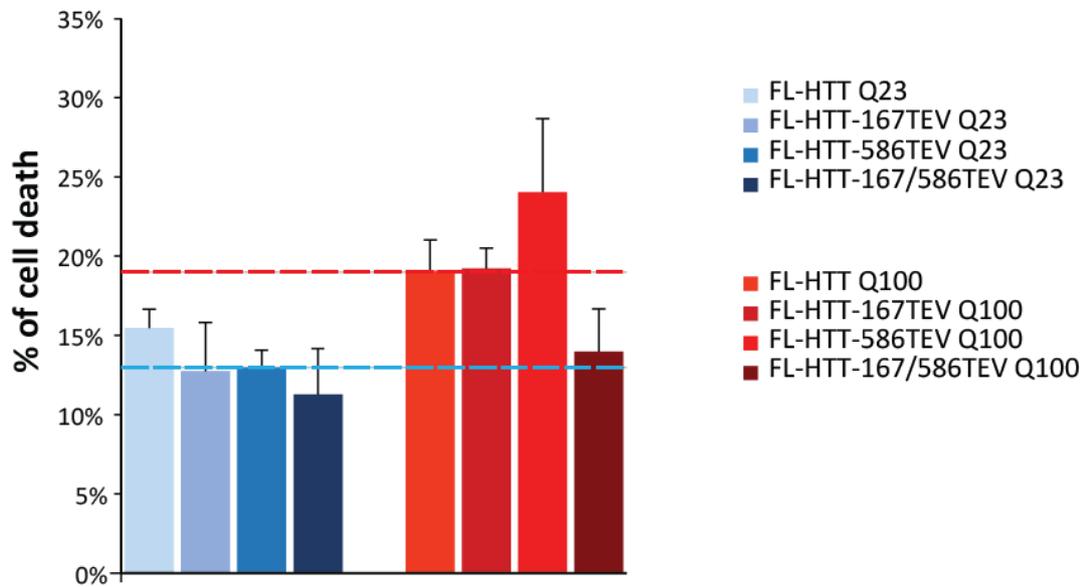
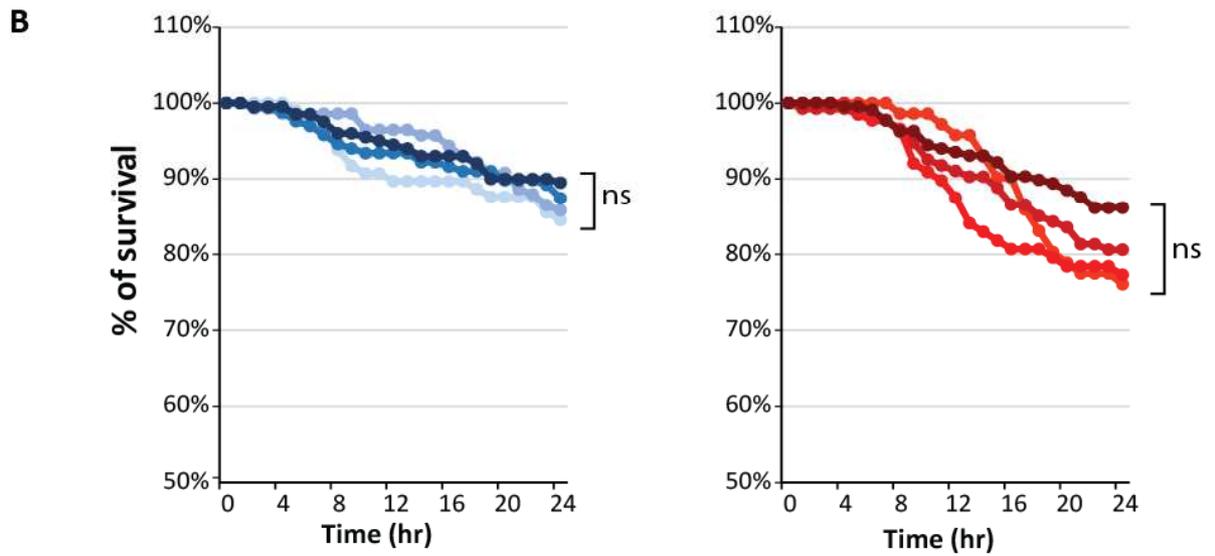
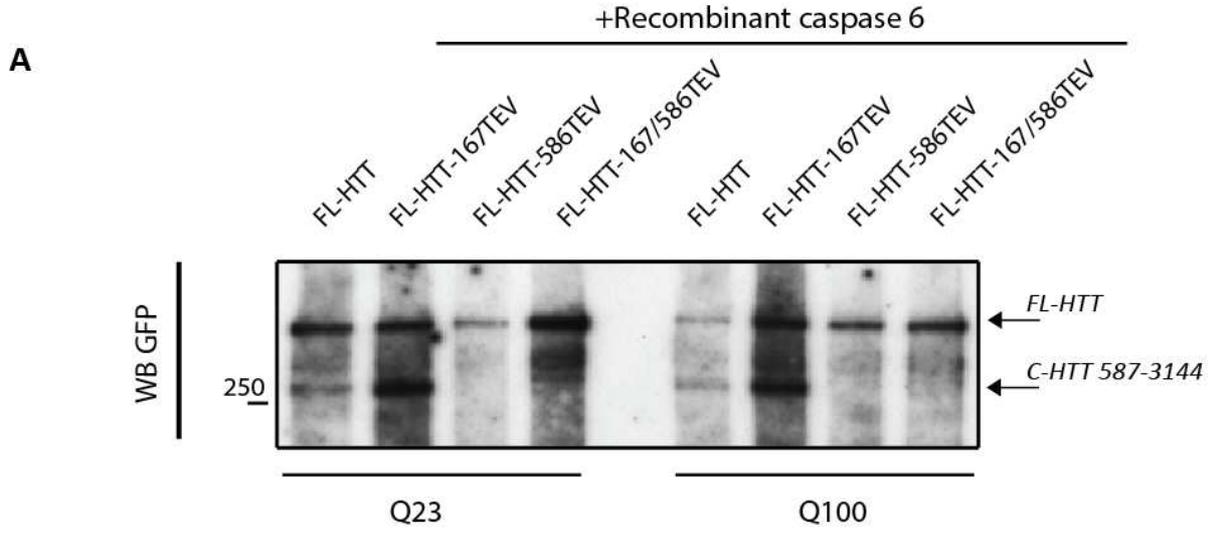
Under such condition, the FL-HTT-TEV is expressed in the nervous system of generated flies but is not cleaved as the TEV protease is lacking. The heads of adult flies (4 days of age) is lysed and submitted to immunoprecipitation (IP) assay using the anti-mCherry antibody (Ab) followed by SDS-PAGE. Right: Homozygote flies containing the TEV transgenes on the chromosome 2 and the FL-HTT-TEV transgenes on chromosome 3 were crossed with the ELaV-Gal4 flies. Cleavage of HTT is induced at position 167, 586 or both by using two independent strains expressing the TEV protease under the heat-shock promoter (hs-TEV) or the UAS promoter (UAS-TEV). Similarly, expression is assessed as followed: C-HTT587-3144 generated fragment is IP and revealed using the HTT 2E8 Ab (aa 2146-2541), Nter-HTT generated fragment are IP using the anti-mCherry Ab and revealed after Western blotting by HTT 2B4 Ab (aa1-82) and polyglutamine 1C2 Ab.

- B.** Expression level of HTT fragments within *D. melanogaster*. All HTT transgenes are under the control of UAS promoter and are crossed with ELaV-Gal4 flies, offspring are raised at 29°C. Left: Total brain lysate are subjected to SDS-PAGE analysis. Nter-HTT fragments are detected using HTT 4C8 Ab (aa around 400). Right: 5 strains of flies expressing the mCherry-C-HTT1722-3144 transgene, negative control (ctr) are W1118 flies. The expression was detected after IP using anti-mCherry Ab as the level of expression was non-detectable in the total inputs.

Figure S6. Both mutated N-ter and C-ter HTT are toxic in vivo.

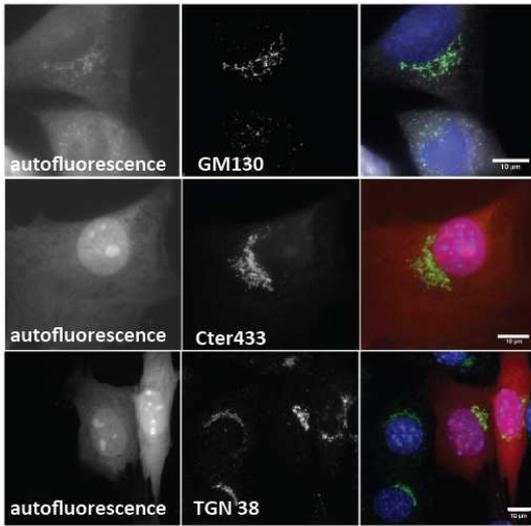
- C.** Percentage of cell death induced by Nter- and Cter-HTT fragments. Two Cter-HTT fragments are analyzed, the 1722-3144 and the 587-3144 fragment. Cell death, quantified by chromatin condensation on fixed cells 48 hours after transfection, show a high toxicity of both Cter-HTT fragment compared to mutant N-HTT1-586Q100 expression.
- D.** The image represents a cell expressing the C-HTT1722-3144 undergoing cytoplasmic vacuolization. A and B suggest that the C-HTT1722-3144 fragment is inducing ER vacuoles formation and toxicity similarly to C-HTT587-3144 fragment.
- E.** Motors capacity of female transgenic flies expressing Nter and Cter-HTT fragments. The expression of N-HTT1-548 Q0, N-HTT1-548 Q128 and C-HTT1722-3144 (#147 and #143) transgenes are under the control of UAS promoters and are crossed with ELaV-Gal4 flies. Female offspring are raised at 29°C and are assessed for their climbing capacity over time.

Supplemental figure 1

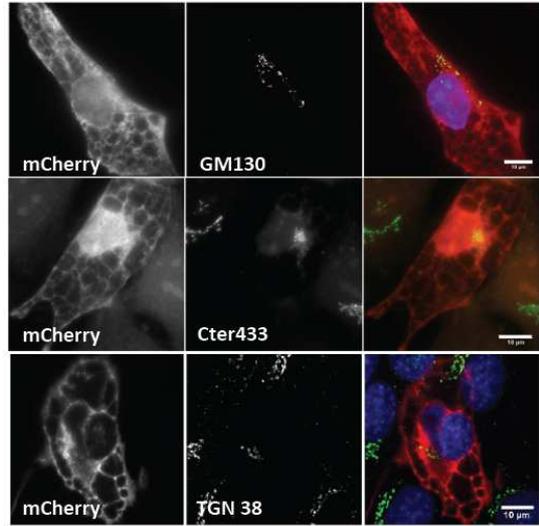


Supplemental figure 2

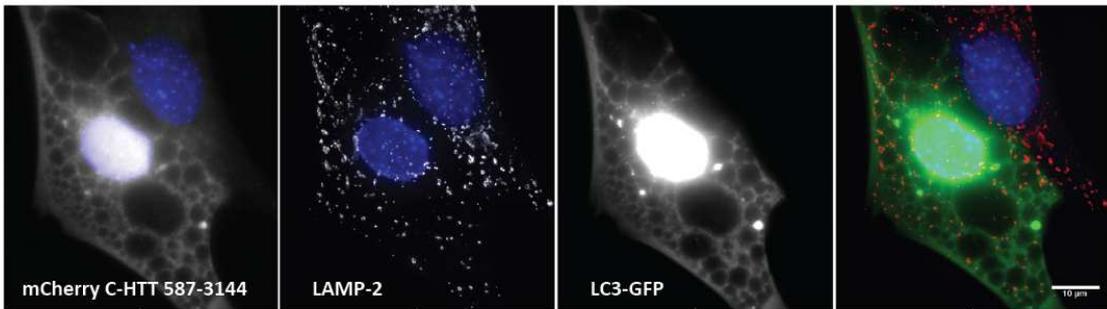
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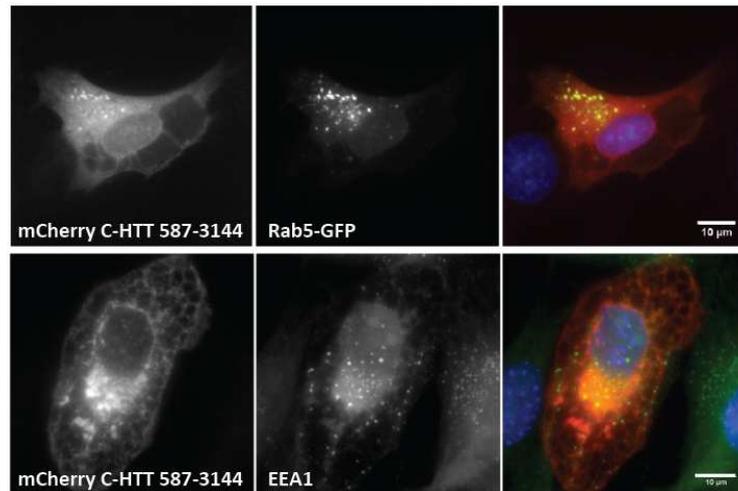
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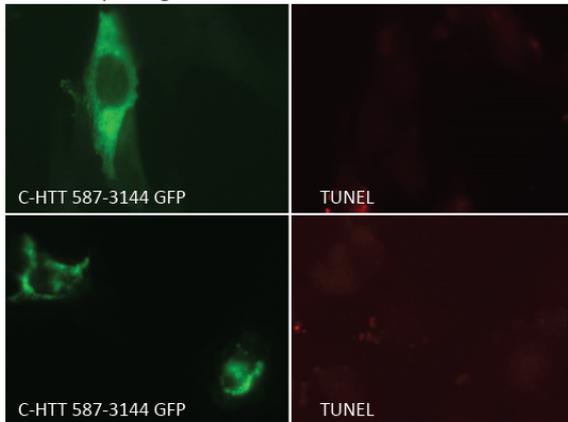
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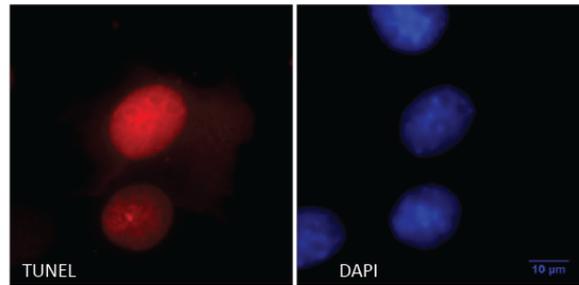
Supplemental figure 3

A

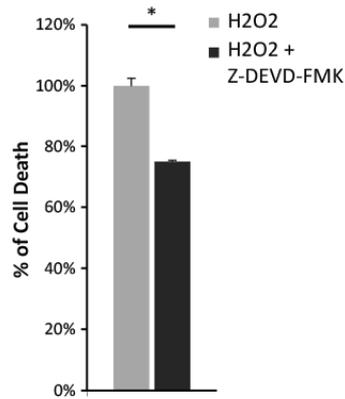
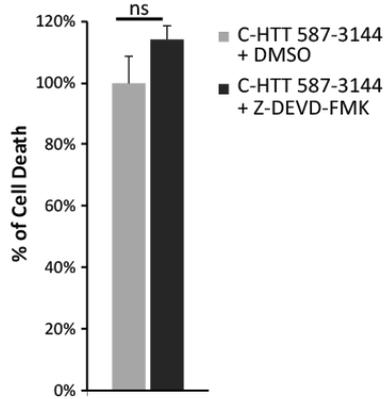
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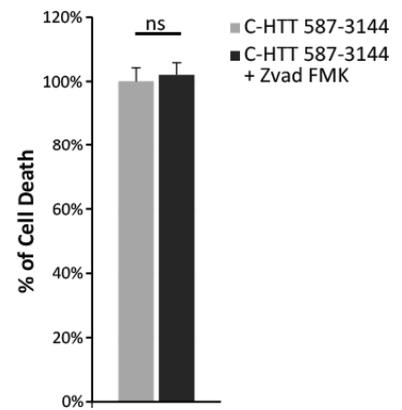
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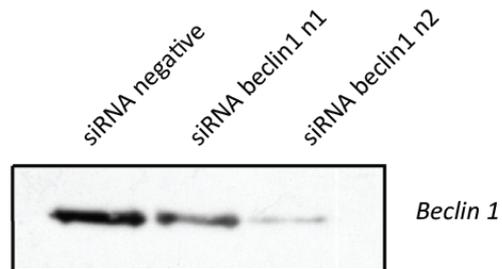
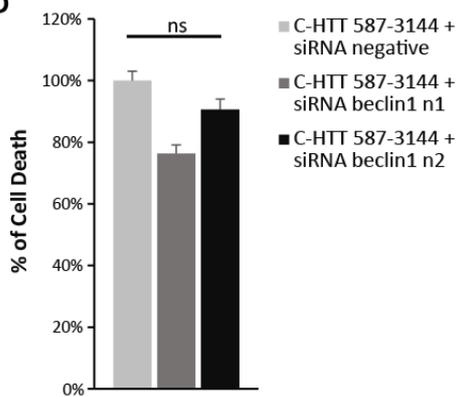
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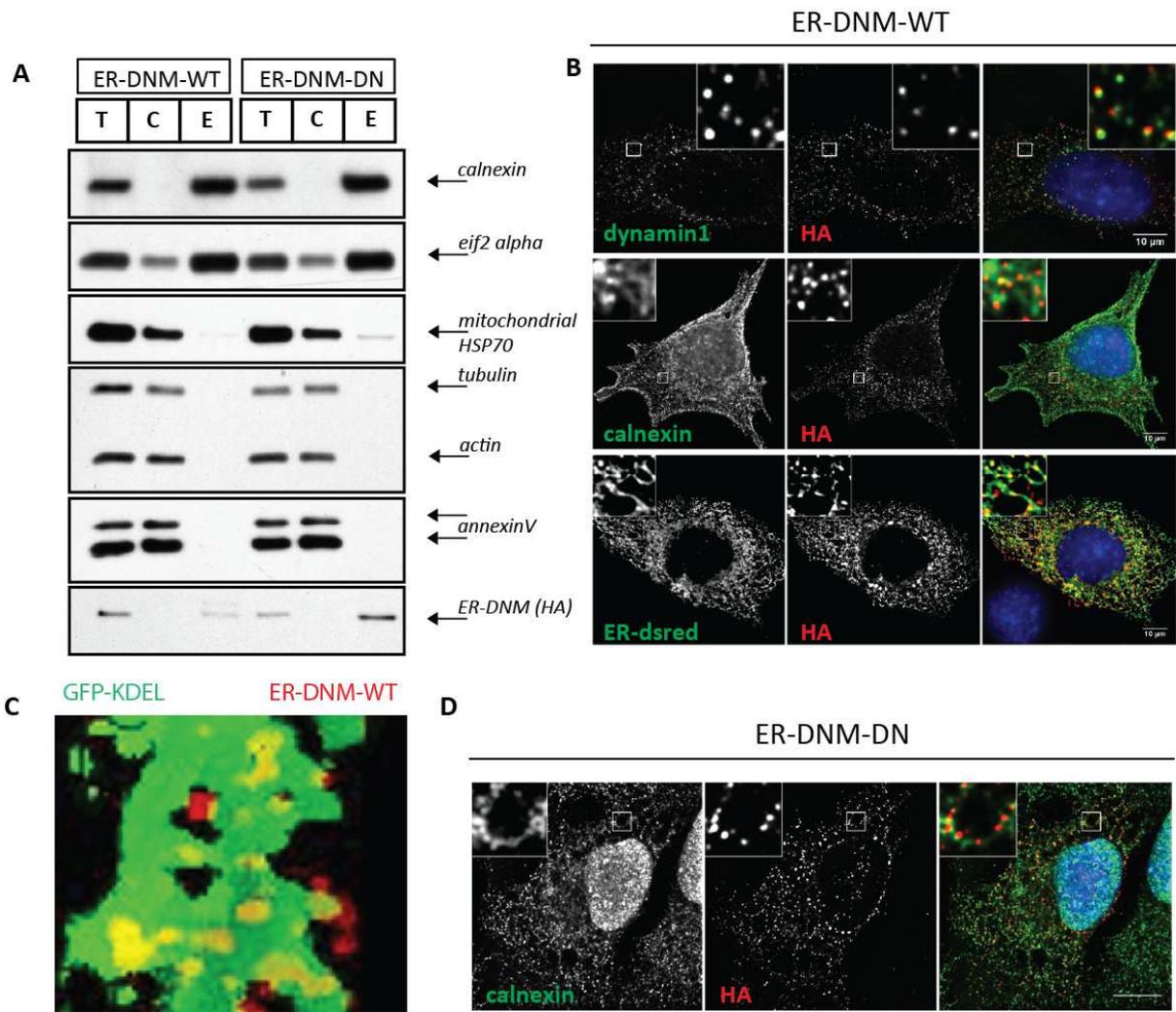
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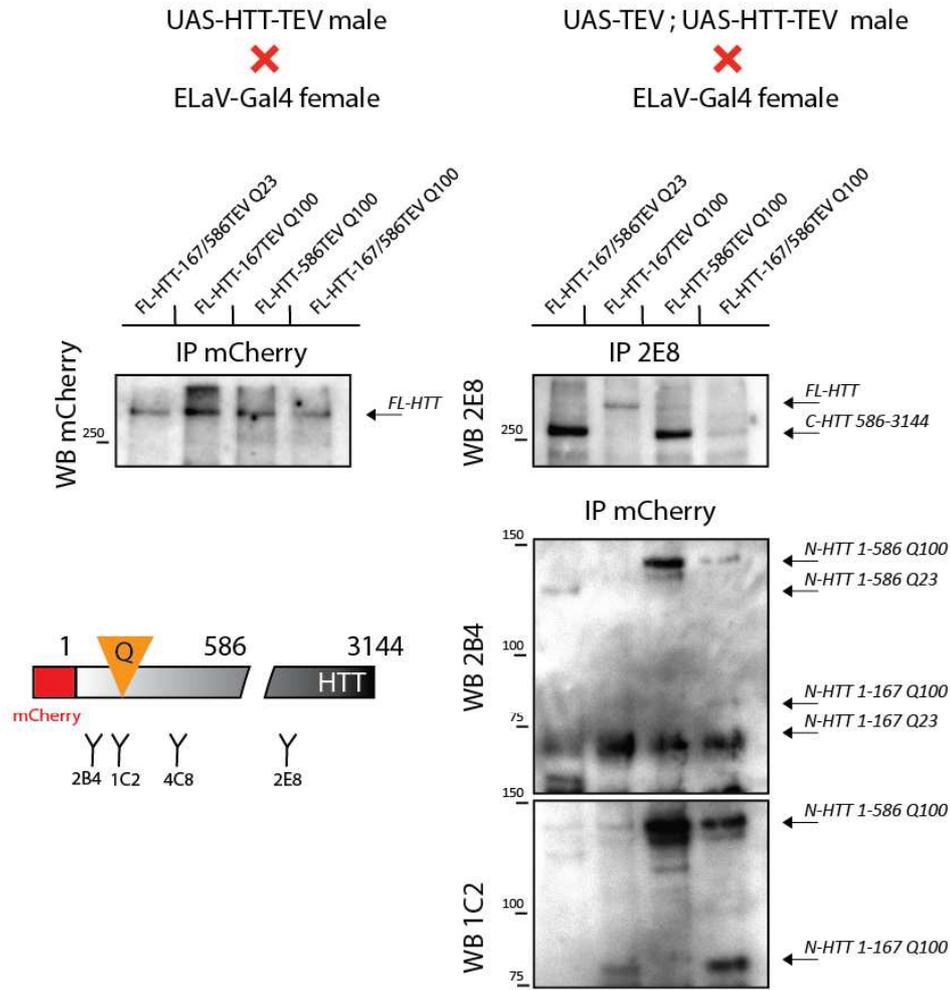


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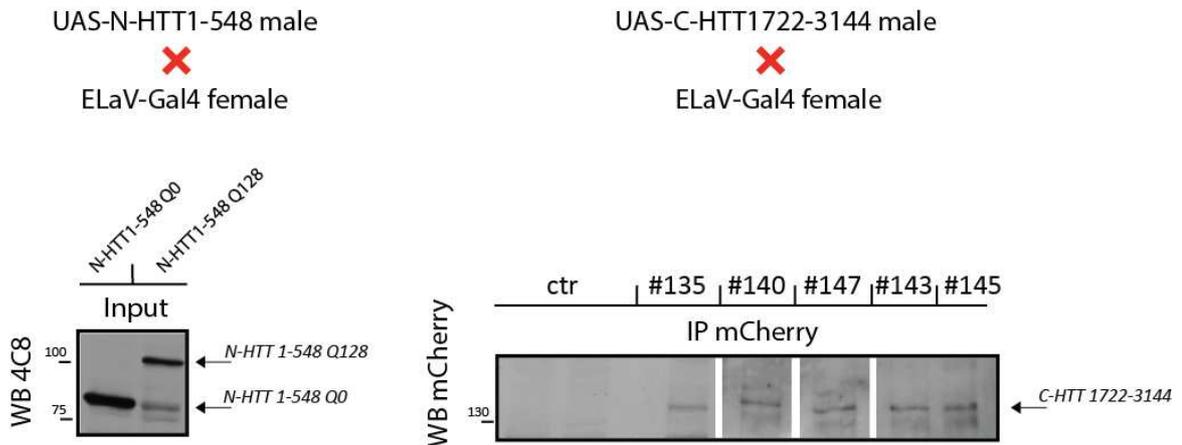


Supplemental figure 5

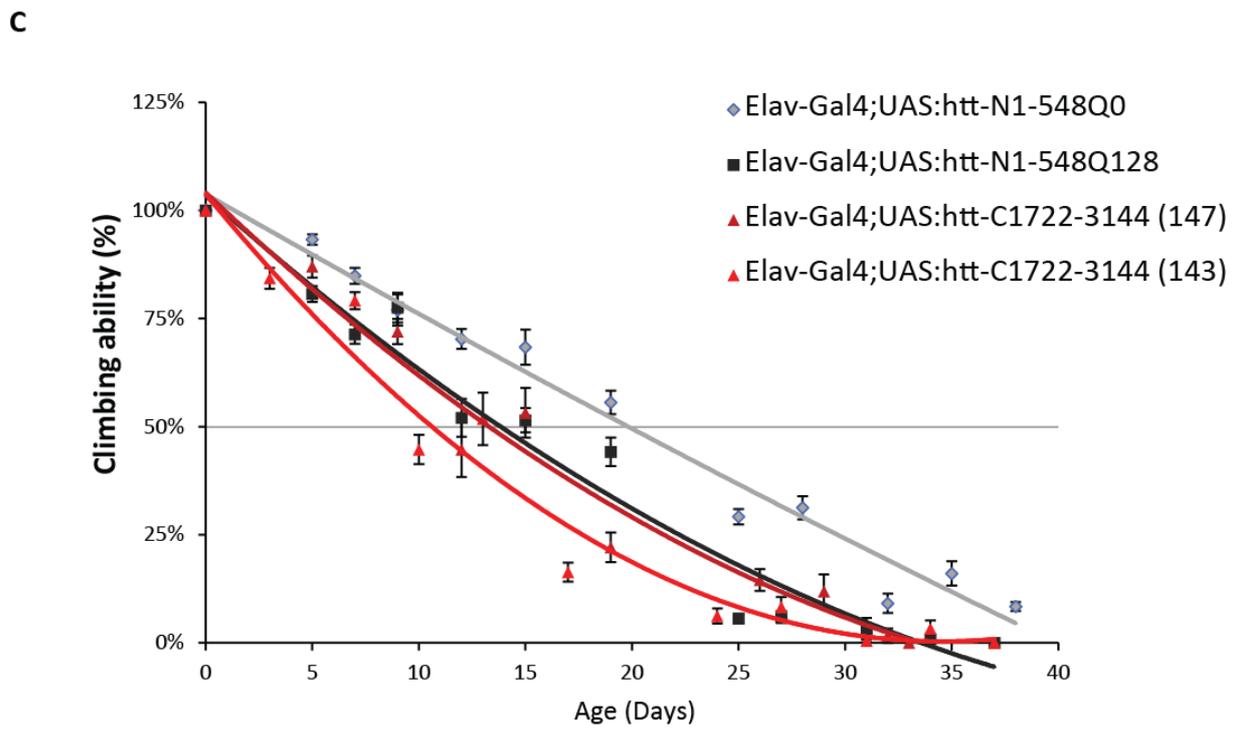
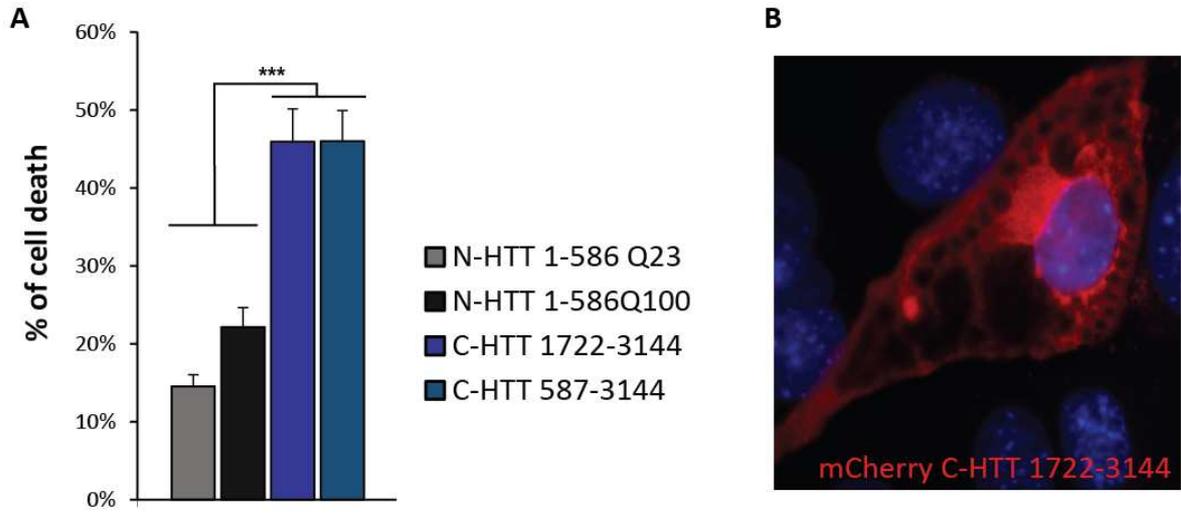
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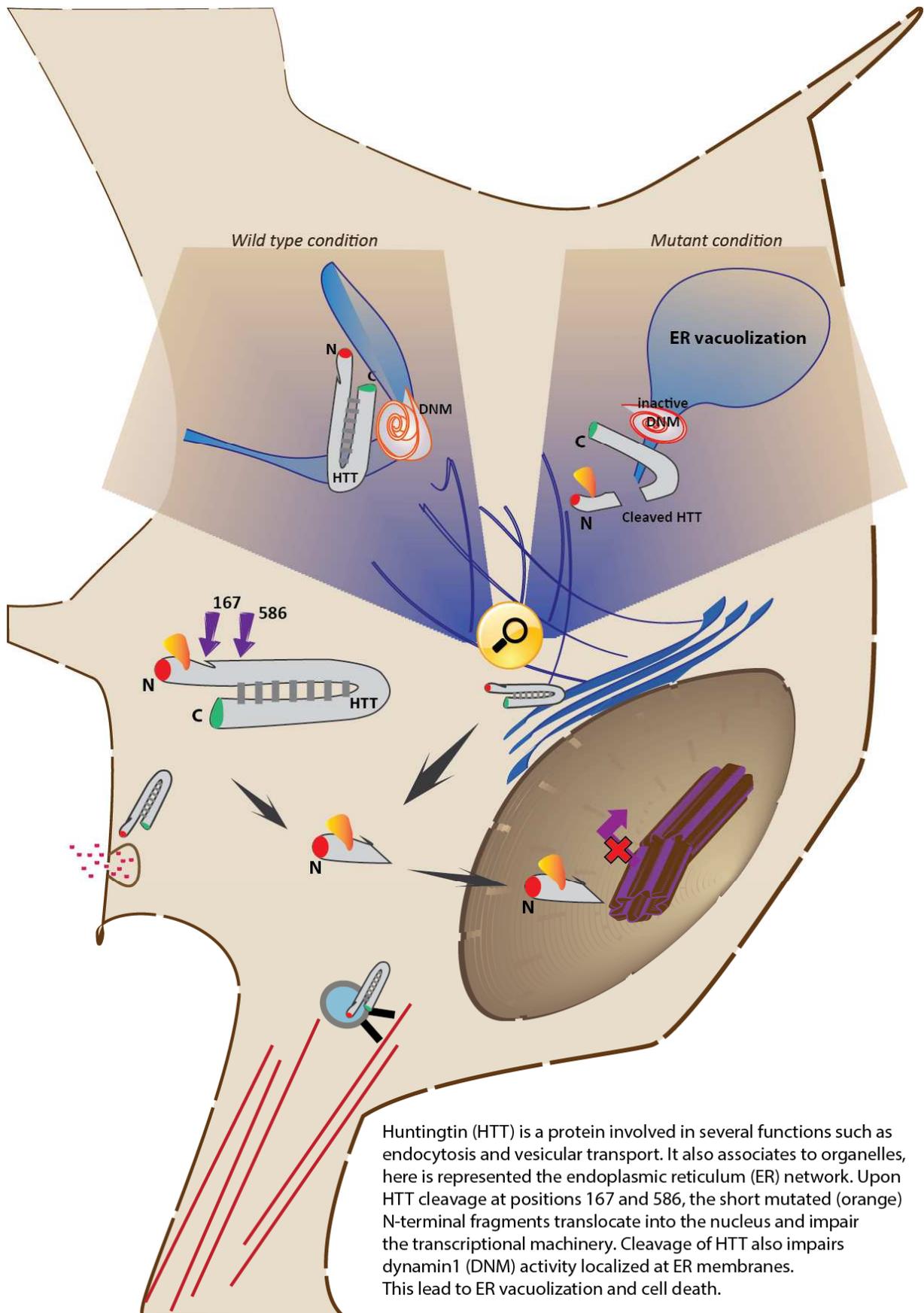


Supplemental figure 6



*Chapter III: General Discussion
& Perspectives*

Working model



1. Cleavage of HTT in disease and normal conditions.

In HD, proteolysis of HTT is deregulated upon abnormal activation of proteases and inhibiting HTT cleavage reduced toxicity. In healthy tissue, wild type HTT proteolysis occurs and is most probably tightly regulated and might participate in cell fate during development or aging processes (Mende-Mueller et al., 2001; Wellington et al., 2002). A particular processing of HTT seems to be a required step in HD pathogenesis (Gafni and Ellerby, 2002; Mantle et al., 1995; Miller et al., 2010). The regulation of proteases cleaving wild type and mutant HTT are poorly understood. However, when comparing normal to disease condition, the proteolysis pattern of mutated HTT appeared to be different with the appearance of small N-ter HTT fragments only in mutant condition (Gafni and Ellerby, 2002; Mende-Mueller et al., 2001). In addition, examining different regions of HD brain, mutated HTT showed tissue-specific proteolysis suggesting different protease-susceptible domains according to the cell type. Hence, HTT-induced toxicity might be close-fitting to a particular cleavage pattern which takes place in disease upon abnormal activation of proteases triggered by the polyglutamine mutation.

We aimed at understanding the consequences of HTT cleavage by analyzing specific cleavage sites of HTT. To do so, we resorted to an artificial cleavage system allowing us to assess HTT proteolysis beyond abnormal proteases activation which can hide important mechanisms involved in cellular toxicity. Thus, we employed the TEV cleavage system to model HTT proteolysis and showed that such tool represent a valuable approach to decipher the effects of individual and combined cleavage of HTT in wild type and mutant conditions. Our results demonstrated that not all cleaves are equal in toxicity. We observed that according to the proteolysis pattern, HTT-induced toxicity is different. We provided an explanation for such correlation based on the findings of intramolecular interactions within the HTT protein. Hence, although the putative C-HTT587-3144 fragment is found in post-mortem control brains, absence of small N-ter fragments might explain the non toxicity of such cleavage as, under such configuration, our results showed that both N- and C-ter HTT remain associated together and exhibit low toxicity. We showed that additional cleavages in the N-ter HTT caused a loss in HTT intramolecular interaction leading to the release of a toxic C-terminal fragment along with the known induced toxicity of mutated N-ter generated fragments.

What does determine the cleavage pattern of HTT?

As the presence of the polyglutamine mutation induces conformation changes in HTT (Ross and Poirier, 2004), this could partly explain the specific proteolytic pattern of mutant HTT when compared to wild type HTT (Gafni and Ellerby, 2002; Kim et al., 2001; Landles et al., 2010). We found that the accessibility of the TEV site at position 167, replacing the cathepsinZ/bleomycin cleavage site, was enhanced by the polyglutamine expansion. The presence of an expanded stretch of

glutamines may modify HTT tridimensional structure and rendered HTT cleavage sites more accessible to proteases as compared to wild type HTT. Moreover, cleavage at 586 position occurred much faster than cleavage at position 167 and facilitated this latter cleavage. This reflects a differential accessibility of HTT cleavage sites according to their positioning along HTT sequence. Thus, altering HTT structure by the polyglutamine stretch or by a first cleavage can induce changes in the accessibility of HTT cleavage sites and therefore can regulate HTT toxicity.

Is HTT subjected to a randomized proteolysis or followed a defined proteolysis cascade? Beyond changes in mutated HTT conformation that can facilitates some cleaves along with an activation of several proteases cleaving HTT during disease, we are lacking direct evidences indicating the cascade of HTT cleavage in HD patients. Analysis of post mortem brains indicates the presence of multiples cleavages products which reflects the end course of the disease. Moreover, HTT proteolysis is probably occurring at specific localization within the cells depending on the cellular localization of specif proteases such as lysosomes for cathepsinZ processing. The use of HD mouse model could be helpful in this respect by following HTT proteolysis by biochemical and immunofluorescence studies along life span. Enzymatic tests or gene reporter can be used in combinations in order to analyse concomitantly proteases activation responsible for HTT cleavage.

2. Both mutated N-terminal and C-terminal proteolytic products of HTT cleavage are toxic.

The mechanisms by which mutant HTT kills neurons is not fully understood, however proteolysis of HTT is a crucial step in HD pathogenesis. A considerable amount of studies have focused on HTT proteolysis in order to better understand the generation of the mutated N-terminal fragment as it is the toxic fragment forming aggregates in HD. My thesis work considered the consequences of the co-existence of mutated Nter-HTT fragments and of their corresponding Cter-HTT fragments that are generated after a proteolytic cascade. Surprisingly, my results show that both fragments contribute to the neurotoxicity.

In HD, proteolysis of HTT at different sites generates toxic small N-terminal fragments that contain the polyQ expansion and that translocate into the nucleus (Graham et al., 2006; Landles et al., 2010; Mende-Mueller et al., 2001; Saudou et al., 1998). We defined here an additional pathogenic mechanism that is occurring upon HTT proteolysis and that is likely to contribute as well to neuronal toxicity. We described a link between ER dilation, ER stress and toxicity that is induced by C-terminal fragments released upon HTT proteolysis. This mechanism points to a novel role for HTT in regulating dynamin1 activity located on ER membranes. Indeed, we showed that concomitantly to the generation of small N-terminal fragments, C-terminal fragments accumulate at ER membranes and dysregulate dynamin1 activity causing ER dilation, ER stress and toxicity.

Dynamin1 GTPase activity requires its proper targeting and self-assembly at membranes. At one hand, our results showed that FL-HTT is required for the localization of dynamin1 at cellular membranes. Thus, HTT mediates dynamin1 targeting to membranes as dynamin1 itself lacks transmembrane domains. At another hand, our preliminary results indicated that cleavage of HTT impaired dynamin1 oligomerization indicative of an impairment in dynamin1 GTPase activity required for membranes fission. Thus, the intact FL-HTT may be required for some of HTT functions in which several domains of HTT could be involved. As N-ter and C-ter HTT bind to dynamin1, we can postulate that both domains might be required for the proper regulation of dynamin1 activity by HTT. Thus, when mutated, cleavage of HTT, releasing the N-ter HTT from its interaction with C-ter HTT, would lead to a loss of HTT function upon dynamin1 regulation.

These findings highlight the importance of assessing the toxicity of HTT in its full length (FL) version. Our laboratory and others have already proven the requirement of the total HTT in cellular mechanisms such as vesicular transports and mitosis (Godin et al., 2010; Zala et al., 2013a; Zala et al., 2013b). Beyond, the toxic mutated N-terminal fragments of HTT, changes triggered by the polyglutamine mutation can result in the loss of HTT integrity such as structural modifications and an enhanced susceptibility for proteolysis.

3. The effect of HTT proteolysis on its intramolecular interaction.

We found that sequential proteolysis, occurring preferentially on mutant HTT, induced a loss in the intramolecular interactions existing between N-ter and C-ter HTT fragments (Palidwor et al., 2009), thus releasing the N-ter and C-ter domains apart from each other.

A first prediction and validation of interactions between HTT domains were provided by Palidwor and colleagues using a computational method based on a neural network to identify alpha-rod which are structural domains formed by a stack of alpha helices (Palidwor et al., 2009). These alpha rod domains form the HEAT repeats in HTT protein (Andrade and Bork, 1995). Interactions between HTT1-506Q23 and Htt507-1230, HTT507-1230 and HTT507-1230, HTT507-1230 and HTT2721-3144 fragments were observed in this assay. Those findings are consistent with our results demonstrating an interaction between the HTT1-586 and HTT586-3144 fragments. In addition we showed that cleaving the HTT1-586 at position 167 disrupts HTT intramolecular interactions. The first HEAT domain of HTT sit between the amino acid 114 and 413 (Palidwor et al., 2009) and interactions between domains composed of HEAT-repeats are known (Li et al., 2006). Thus, cleaving HTT at amino acid 167 may destroy the HEAT repeat structure leading to a loss of interaction with the others HEAT domains of HTT.

Intermolecular interactions may also exist between HTT proteins (Palidwor et al., 2009). I haven't addressed this aspect during my PhD, however studying of HTT dimerization or oligomerization could be of interest especially regarding potential sequestration of wild type HTT by mutant HTT.

4. HTT proteolysis affects its association with ER membranes.

We found that FL-HTT is enriched in ER membranes fraction. Upon HTT proteolysis at 167 and 586 sites, the C-ter HTT fragment remained associated with the ER membranes. At the opposite, the short N-ter HTT showed a cytosolic enrichment in parallel to their nuclear accumulation.

HTT was proposed to play a biological function as an ER-associated protein. Indeed, the N 1-18 HTT is an amphipathic alpha helical membrane-binding domain that can target HTT to vesicles and to ER (Atwal et al., 2007). In response to ER stress, HTT is released from membranes and translocate to the nucleus (Atwal and Truant, 2008). Consistently, we found that HTT cleavage induced ER stress signaling and that mutant N-HTT1-167 proteolytic product is released from ER membranes and showed a nuclear enrichment with increased toxicity. Particularly, the C-HTT 587-3144 showed an enrichment in ER fraction even though it lacks the first eighteen amino acids. Further investigations should be made in order to understand what targets C-ter HTT to ER: intrinsic sequence properties or via defined interacting domains with ER-associated proteins? Also, the presence of palmitate on a protein affects how the protein interacts with lipids and proteins in a membrane compartment (Linder and Deschenes, 2007). A palmitoylation site has been identified at position 214 of HTT (Yanai et al., 2006), a thorough examination of post-translational palmitoylations of HTT on C-ter HTT fragment may provide novel insights for the comprehension of HTT targeting to ER membranes. In the light of these results, the N 1-18 amino acids of HTT itself is not enough to trigger HTT localization at ER membranes, additional elements within HTT sequence seems to be required.

5. Dynamin1 and ER shaping.

So far, the role of dynamin1 in ER membranes remodeling was not explored. Here we bring a set of results pointing at a role of dynamin1 in ER shaping. We propose that the presence of the C-ter HTT domain, released from its intramolecular interaction with the N-ter HTT, exerts a dominant negative effect on dynamin1 activity required for the proper ER network maintenance.

The mechanisms by which the C-ter HTT affect dynamin1 activity are still to be uncovered. Dynamin 1 GTPase activity is regulated by self-assembly and by its association with lipids (Marks et al., 2001; Slepnev et al., 1998). De-phosphorylation of dynamin 1 by calcineurin stimulates its binding to phospholipids and is required for the fission step during synaptic vesicles endocytosis (Cousin and

Robinson, 2001; Lai et al., 1999). We have analyzed the level of phosphorylation of dynamin1 at serine 795 and serine 774 and found an increased level of phosphorylation in C-ter HTT expressing cells (data not shown). As phosphorylation of dynamin1 regulates its protein-lipids interaction, we propose that, upon HTT proteolysis, the localization of dynamin1 on ER membranes is altered causing a disruption in membranes remodeling shapes the network. How does dynamin1 shape the ER? A balance between fusion and fission reactions is required for the proper maintenance of ER network as well as other organelles structure. Members of dynamin family can interplay between membranes fission and fusion events. For instance, a yeast dynamin protein, Vps1p, is involved in both process, indicating that both antagonist reactions might be interconnected and shared similar steps (Peplowska and Ungermann, 2005). Also, disruption of Atlastin1, a member of dynamin's family, led to ER fragmentation as the ER membranes failed to fuse together correctly in order to form a continuous network (Orso et al., 2009; Rismanchi et al., 2008). Even though fission events have not been described yet in ER-forming structure, our results indicates dynamin1 as a candidate for this role. Dynamin1 is a mechanoenzyme that self-associates into rings or spirals playing a key role in membranes fission at region with high curvature (Hinshaw, 2000). It is thus not excluded that dynamin1, as other reticulons proteins, may senses the bending of ER membranes and participates in the proper structuring of ER compartements in order to limit fusion events trigerring ER dilation.

6. C-ter HTT-induced toxicity: several mechanisms involved.

The yeast two hybrid (Y2H) experiment we performed revealed twelve confident interactors of the C-terminal part of HTT. As described previously, these proteins are involved in different cellular functions. Dynamin1 was found to interact with the largest bait of HTT, ranging from amino acid 1100 to 3144. Mule, was found to interact with the most C-terminal bait (from 2927 to 3144 amino acid). Mule (Mcl-1 Ubiquitin Ligase E3) is an E3 HECT-containing-ubiquitin ligase (Chen et al., 2005). It is also known as ARFBP1 or HUWE1 and regulates the degradation of Mcl-1, an anti-apoptotic protein member of Bcl-2 family.

Mule ubiquitinylates Mcl-1 and triggers its degradation by the UPS (Zhong et al., 2005). Interestingly, studies suggest that Mcl-1 might regulate cellular homeostasis between autophagy and cell death. Also, members of the Bcl-2 family such as Bcl-2 and Bcl-xL are dual regulators of apoptosis and autophagy as, in addition to regulate cytochrome C release, they bind to the essential autophagy protein Beclin1 (Levine et al., 2008) (Erlich et al., 2007; Kihara et al., 2001). We validated the interaction of Mule with the Cter-HTT by immunoprecipitation assay and our preliminary results suggest that down regulating Mule expression by shRNA reduced C-HTT587-3117 induced toxicity.

Thus HTT by linking to Mule could regulate members of Bcl-2 family and death mechanisms. However several questions remain to be answered: What are the contributions of the Mule/Mcl-1 pathway to cell death? Does HTT proteolysis affect Mule activity, if so, how?

We generated a construct expressing the C-ter HTT depleted from the Mule-interacting domain (C-HTT 587-2927) termed C-HTT Δ MID. This fragment showed a decreased level of toxicity as compared to the C-HTT 587-3144 fragment. However both were inducing formation of massive vacuoles within the cytoplasm. This indicated that the 587-2927 domain of HTT is sufficient to induce cytoplasmic vacuolization and that the additional presence of the 2927-3144 domain accelerates C-ter HTT-induced toxicity through mechanisms implicating Mule. Thus, toxicity induced by the C-ter HTT required at least two distinct mechanisms acting together to induce cell death.

In support, we have observed that cells expressing an inactive dynamin1 targeted to the ER could recover from ER vacuolization in few cases as vacuoles disappeared and the cells survived. This interesting observation raised the possible existence of cellular defenses which are used by the cell to correct ER disorganization. Such recovery was not observed in C-ter HTT expressing cells. These observations, with the observation that the more C-terminal domain of HTT (2927-3144) accelerated cell death, suggests additional mechanisms by which the C-ter HTT induces toxicity.

A potential mechanism for ER vacuolization recovery may involve the chaperone protein Bip/Grp78. Indeed Bip/Grp78 participates in the cellular defense against ER vacuolization induced by cyclosporine treatment as overexpression of Bip reversed ER vacuolization process in cells (Cheng et al., 2013).

Cyclosporine is an inhibitor of calcineurin used as an immuno-suppressant in organ transplantation. A described side effect of cyclosporine is ER vacuolization of renal cells in immune-suppressed patients (Liptak and Ivanyi, 2006). Thus inhibiting calcineurin induces ER vacuolization probably through its regulation of dynamin1 phosphorylation and consequently dynamin1 activity.

7. HTT: a regulator of calcineurin?

HTT phosphorylation is protective against HTT mediated toxicity (Humbert et al., 2002; Pardo et al., 2006). In particular, phosphorylation of HTT at S421 is crucial to regulate HTT function in axonal transport (Colin et al., 2008; Zala et al., 2008). Increased HTT phosphorylation can be produced either by inhibition of the calcium dependent phosphatase calcineurin or by activation of the Akt and/or SGK kinases (Humbert et al., 2002; Rangone et al., 2004). Calcineurin inhibitors are neuroprotective in HD mouse model by inducing HTT phosphorylation at S421 (Pardo et al., 2006;

Pineda et al., 2009). In addition, *RCAN1* gene, that encodes a regulator of calcineurin, is depressed in HD. *RCAN1-1L* overexpression is protective against mutant HTT as it increases phosphorylation of HTT via calcineurin inhibition.

Thus, calcineurin is an important element in neurotoxicity as its activity is increased in the disease (Xifro et al., 2008). Our result provides novel elements suggesting that calcineurin activity is decreased upon HTT proteolysis and could participate in ER vacuolization. First, the expression of a constitutively active form of calcineurin protected against C-ter HTT-induced cytoplasmic vacuolization and toxicity. In addition, we found that the level of dynamin1 phosphorylation was increased in C-ter HTT expressing cells indicating an inhibition of calcineurin activity. Calcineurin activity, by regulating dynamin1 phosphorylation and thus localization, might play a role in ER dilation caused by HTT proteolysis.

We can hypothesize the following: the activity of calcineurin fluctuates along the course of the disease: at early stage, prior to the release of C-ter HTT fragment, calcineurin activity is increased while, at later stages and upon substantial proteolysis of HTT, calcineurin activity is decreased. As calcineurin is involved in several cellular processes, both perturbations of calcineurin activity can trigger toxic effects and lead to cell death.

HTT is a substrate and an interactor of calcineurin. Calcineurin is known to carry on the dephosphorylation of many substrates and that those substrates display a consensus sequence that acts as a calcineurin docking site. We identified two consensus docking sites for calcineurin within HTT sequence: one corresponding to a SP motif found on NFAT (Nuclear Factor of Activated T-cells) (Dodge and Scott, 2003) within the N-ter part of HTT and a PXIXIS/T sequence commonly found in many other calcineurin substrates, within the C-ter part of HTT (Feske et al., 2003). In agreement, unpublished works from our lab suggest that both N-ter and C-ter HTT interact with calcineurin. Thus HTT binds to calcineurin and might regulate its activity.

How HTT regulates calcineurin still to be determined, however, a possible mechanism would be the following: Calcineurin is a heterodimer composed of two subunits A and B. The subunit A is a catalytic domain, B is a regulatory domain. Calmodulin binds to the regulatory domain causing a conformational change that displaces the auto inhibitory domain from the catalytic active site, resulting in the activation of the phosphatase (Rumi-Masante et al., 2012). We can hypothesize that HTT binds to the regulatory domain of calcineurin, competing with calmodulin binding sites and thus buffering calcineurin activation. Mutation or cleavage of HTT may disrupt calcineurin activity.

8. C-terminal fragment of HTT: relevance to HD.

As mentioned earlier, examination of human HD cortex and striatum indicated that mutant HTT undergoes tissue-specific proteolysis suggesting different protease-susceptible domains according to the cell type. This study described an additional proteases susceptible sites in the C-terminal part (between 2000 and 3000 amino acids) of HTT in the striatum of HD patients (Mende-Mueller et al., 2001). The particular cleavage pattern in HD striatum might explain the more severe neuronal loss compared to the cortex. During my PhD, I investigated the effect on cell survival of different C-ter-HTT fragments: C-HTT 1722-3144, C-HTT 2417-3144 and C-HTT 2768-3144. All were toxic to cell, however the C-HTT 2417-3144 and C-HTT 2968-3144 did not exhibit cytoplasmic vacuolization preceding death. Moreover, while the C-HTT587-3144 exhibits a cytoplasmic localization, the small C-HTT2968-3144 showed an enriched nuclear localization as it loses the Nuclear Export Sequence (NES) around position 2404.

Gathering all our observations we can draw the following picture: The C-HTT1722-2417 fragment triggers ER dilation by acting within the cytoplasm. Specific cleavages generating shorter C-terminal proteolytic fragments (such as C-HTT 2968-3144) in the striatum of HD postmortem brains increases HTT cleavage toxicity through nuclear mechanisms still uncovered. This highlights a complex system implicating different pathways leading to neuronal death in HD. Beyond proteolysis cascade generating mutated N-ter HTT fragments, analysis of C-ter HTT proteolysis, yet unexplored, would be of a great advance in understanding both neuronal death and high vulnerability.

9. Therapeutic perspectives.

Few pharmacological or non-pharmacological treatments have proven efficacy in HD, leaving space to develop pioneer studies with the intention of improving treatments for the patients. Looking at the future, there is a major drive to find disease-modifying and new symptomatic treatments for HD (Kaltenbach et al., 2007; Novak and Tabrizi, 2011). Treatments will, in practice, probably comprise a combination of compounds which will target several key pathogenic pathways to achieve optimal effect. Some potential therapeutic strategies are: enhancing clearance of mutant HTT by cellular mechanisms (Jeong et al., 2009); gene silencing of the mutant gene itself (DiFiglia et al., 2007; Harper et al., 2005); Histone deacetylase inhibitors which target the transcriptional deregulation that occurs early in HD pathogenesis (Ferrante et al., 2003) and inhibitors of proteolytic cleavage of mutant FL-HTT to prevent production of toxic N-ter and C-ter fragments (Graham et al., 2006; Miller et al., 2010). The FL-HTT-TEV system we developed will be a valuable tool to screen modifiers of cellular toxicity upon HTT proteolysis on several protease sites of HTT in cells as well as in animal models.

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Summary

Huntington's disease (HD) is an autosomal dominant inherited neurodegenerative disorder caused by an abnormal polyglutamine (polyQ) expansion in the N-terminus of the protein huntingtin (HTT). A crucial step in HD pathogenesis is the cleavage of full-length HTT into smaller N-terminal (N-ter) fragments that contain the polyQ stretch and that are toxic to neurons. If HTT cleavage generates short N-ter fragments whose amino-acid position range from 1-105 to 1-586, it will also generate corresponding C-terminal (C-ter) fragments. Such C-ter fragments are also observed in HD post mortem brain samples but their participation in the pathogenic process remain to be investigated.

Therefore my PhD research aimed to consider the contribution of such fragments in HD pathogenic process. I investigated the consequences of full-length mutant HTT proteolysis by developing a time and site-specific controlled system for HTT proteolysis. My results show a contribution of both fragments to neurotoxicity *in vitro* as well as *in vivo*.

Briefly, we describe an intramolecular interaction within the N-ter and C-ter domains of HTT which is impaired upon successive proteolysis of HTT. We found that HTT intramolecular interaction buffer mutant N-ter and C-ter HTT-induced toxicity. Moreover, specific cleavages of the mutant HTT not only generated toxic N-ter fragments as they translocate into the nucleus but also generated C-ter HTT fragments which participate to toxicity via a novel mechanism.

We show that the Cter-HTT fragments remain in the cytoplasm and elicit toxicity through the dilation of the endoplasmic reticulum (ER). The mechanism involves the specific binding of Cter-HTT and subsequent dysregulation of dynamin1 activity on ER membranes leading to ER vacuolization and death.

These findings, together with the presence of such ER dilation in HD mouse model, suggest that specific proteolysis of HTT, release non-polyglutamine-containing fragments that contribute to disease. This highlights the complexity of HD pathogenic mechanisms and supports the importance of studying HTT as a full-length protein.

Key words: Huntington disease, proteolysis, huntingtin, endoplasmic reticulum, dynamin1.

Résumé

La maladie de Huntington (MH) est une maladie neurodégénérative héréditaire autosomique dominante. Elle est due à l'expansion anormale de polyglutamine dans la partie N-terminal de la protéine huntingtine (HTT). Une des étapes clés de la pathologie est le clivage de la HTT pleine longueur en fragments N-terminaux plus petits, contenant l'expansion de polyglutamine, et qui sont toxiques pour les neurones. Cependant, si le clivage de la HTT génère des fragments N-terminaux (N-ter) de tailles comprises entre les acides aminés 1-105 et 1-586, celui-ci produit également les fragments C-terminaux (C-ter) correspondants. Bien que ces fragments C-ter soient observés dans des extraits de cerveaux de patients MH post-mortem, leur implication dans les processus pathologiques ne sont pas connus.

Aucune étude n'a examiné la coexistence des fragments N-ter avec les fragments C-ter correspondants également générés. Par conséquent, mes travaux de thèse ont visé à étudier la contribution de ces deux fragments dans la pathogenèse.

Au cours de ma thèse j'ai développé un outil permettant de contrôler le clivage de la HTT dans le temps et à des sites spécifiques. J'ai étudié le clivage de la HTT à deux sites stratégiques : les positions clivées par la caspase-6 et par la bléomycine hydrolase/cathepsine Z. A l'issue de ces clivages, des fragments N-ter ainsi que des fragments C-ter correspondants sont générés. A l'aide de cet outil, j'ai montré que la partie N-ter mutante de la HTT ainsi que la partie C-ter de la HTT sont toxiques *in vitro* et *in vivo* et contribuent ensemble à la mort neuronale induite par la HTT mutante.

Plus précisément, j'ai décrit une interaction intramoléculaire au sein des domaines N-ter et C-ter de la HTT. Mes résultats indiquent que cette interaction protège les cellules de la toxicité induite par le clivage de la HTT mutée. En effet, les clivages successifs de la HTT brisent cette interaction, ce qui induit la libération des fragments N-ter mutants et provoque la mort cellulaire à l'issue de leur translocation nucléaire. En parallèle, ceci libère la partie C-ter de la HTT. J'ai démontré que ce fragment présente une localisation cytoplasmique et induit une mort cellulaire atypique précédée par une hyper vacuolisation du réticulum endoplasmique (RE). Le mécanisme cellulaire mis en jeu implique la dérégulation de l'activité de la dynamine 1 au niveau des membranes du RE. J'ai établi une interaction entre la partie C-ter de la HTT et la dynamine 1 localisée au RE et démontré que l'activité de la dynamine 1 associée aux membranes du RE était nécessaire pour le maintien du réseau. Ainsi j'ai identifié un rôle de la HTT dans l'organisation du RE. Le clivage de la HTT induit une dérégulation de l'activité de la dynamine 1 conduisant à la dilation des compartiments du RE et causant par la suite la mort neuronale.

Pour conclure, au cours de ma thèse, j'ai montré que la protéolyse successive de la HTT induit des processus cytotoxiques impliquant non seulement les fragments N-ter mutés mais également les fragments C-ter de la HTT. L'acquisition des fonctions toxiques du fragment C-ter de la HTT souligne la complexité des mécanismes pathogéniques de la MH et soutient l'importance d'étudier la HTT pleine longueur.

Mots clés: Maladie de Huntington, protéolyse, huntingtine, réticulum endoplasmique, dynamin1.