SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

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By Azzam Aladdin, Biotechnology MSc

Supervisor: Krisztina Tar, PhD

Doctoral School of Molecular Medicine, University of Debrecen

Head of the **Examination Committee**:	János Szöllősi, PhD, DSc, MHAS
Members of the Examination Committee:	Tamás Ivanics, PhD
	Gabriella Czifra, PhD

The examination takes place at the Life Science Building, Faculty of Medicine, University of Debrecen, at 11:00 am, 07th of January, 2019.

Head of the **Defense Committee**:	János Szöllősi, PhD, DSc, MHAS
Reviewers:	László Bodai, PhD
	Gyula Batta, PhD
Members of the Defense Committee:	Attila Tóth, PhD, DSc
	Zoltán Lipinszki, PhD

The PhD defense takes place at the Department of Internal Medicine "A" Building, Lecture Hall, Faculty of Medicine, University of Debrecen, at 01:00 pm, 7th of September, 2021.

Huntington's disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease. HD patients are affected by a combination of symptoms ranging from movement disorders to psychiatric disturbances, and death usually occurs after 15-18 years of symptom onset. The spread of this disease is estimated between 10.6 to 13.7 per 100,000 individuals in Europe. The main hallmark of HD is the expansion of CAG repeats (≥ 40) in exon 1 of the Huntingtin gene (*HTT*) located in chromosome 4. The mutation in the *HTT* gene leads to the generation of mutant huntingtin (Htt) with abnormal polyglutamine repeats (polyQ). The expanded polyQ makes the protein susceptible to be misfolded and forming oligomers and aggregates with different levels of cellular toxicity. Moreover, the length of polyQ correlates severity with disease progression. For example, greater than 60 glutamines (Q)s repeat in mutant Htt produces a more aggressive phenotype even in a juvenile HD patient.

Huntingtin functions

Huntingtin is a large protein coded by the Huntingtin gene. *HTT* consist of 67 exons, where exon 1 contains the CAG repeats the vulnerable part in HD. Htt with a normal polyQ repeat such as 23 glutamines (Q23) includes 3144 amino acids. Wild-type Htt (MW 348 kDa) is a completely soluble protein. This protein is ubiquitously expressed throughout the human body with the highest level in neuronal cells. Most HD reports extensively study the toxic effects of the mutant N terminal Htt fragments, because it contains expandable poly-glutamine (polyQ). This fragment coded in exon 1 of mutant *HTT*. The first 17 amino acids of N terminal Htt is N- region (N17) and end with ~40 amino acids of a proline-rich domain (PRD). The structure of N17 tends to fold as amphipathic α-helix. The function of N17 acts as a nuclear export signal (NES) in the context of huntingtin and is subject to post-translation modifications (PTMs). The polyQ stretch is a polymorphic region that adopts several structures like a random coil, extended loop, and α-helix. The structure of polyQ is also affected by the other protein regions. While the structure of PRD stretch is a polyproline helix, it plays a role in the interaction of Htt with the other proteins. The C terminal of the Htt protein coded by the remaining exons of *HTT* after exon 1 is not well studied. The most known structure in the C-terminal of Htt is HEAT-repeats (HTT, Elongation factor 3, protein phosphatase 2A, and TOR1). The structure of HEAT is antiparallel α-helices linked with loop. These domains have essential roles in protein-protein interaction. However, the structure of Htt is variable. The structure depends on the structures of Htt domains, functions, and interactions with each other

and other proteins. In general, the structure and function of Htt are mainly controlled by PTMs.

Post-translational modifications have essential roles to regulate different functions of Htt. PTMs such as SUMOylation, ubiquitination, phosphorylation, palmitoylation, and acetylation regulate the physiological function of Htt in healthy individuals. For example, SUMOylation helps to increase the capacity of Htt to suppress the transcription in the cell, and ubiquitination affects several aspects of Htt such as subcellular localization and clearance. The full-length Htt has several sites to serve as a substrate for proteolysis. These sites are between ~ 400-600 amino acids of the Htt sequence. Several reports identify several proteases that cleave within Htt such as caspases 2, 3, 6, calpain, and matrix metalloproteinase-10 (MMP10). The cleavage sites for these proteases are present within both wild-type and mutant Htt. These enzymes are expressing in several human tissues with different levels and activities. For example, caspase 6 is more active in the brain of HD patients, which promotes the more generation of N-terminal Htt fragments contain mutant polyQ. Moreover, mutant Htt probably prevents the normal function of Htt, which inhibits the function of caspase 6 in the case of expressing both proteins. While caspase 3 can cleave within wild-type and mutant Htt in neuron cells, which also could form N-huntingtin fragments. The role of protease in wild-type Htt might inactivate some of the protein functions in normal individuals. Thus, the PTMs of Htt play as switchers to activate and deactivate the particular or the whole protein function. The understanding of the different sights of Htt functions leads to a comprehensive knowledge of mutant Htt and suggests potential approaches for HD therapy.

Mutant huntingtin

Mutant Htt contains an expanded polyQ tract in the N terminal region of the Htt protein. As we discussed before, several proteases can cleave in mutant Htt within the range of 400-600 amino acids, which then releases the toxic N-terminal Htt in neuronal cells. The released fragments with mutant polyQ have more cytotoxicity than the mutant Htt protein itself. It was also suggested that the mutant N-terminal Htt fragments may initially be present in the cell as soluble fragments, and later can form oligomers and insoluble aggregates. This occurs when the concentration of mutant polyQ reaches a certain threshold in the cytosol, followed by the formation of aggregates in the cells. The presence of mutant polyQ leads to a change in the structure of mutant Htt and influences the disease effects in the cell. Emerging evidence suggests the importance of studying the effects of mutant Htt in extraneuronal and neuronal

HD tissues. These studies could provide a better understanding of the disease and more HD models for drug testing.

Cellular proteostasis in HD

The dysregulation of proteostasis is another hallmark of Huntington's disease. Proteostasis is a balance between protein expression in the cell with the exact concentration, at the precise location with appropriate folding. Protein concentration in cells is mainly controlled by protein clearance or turnover, which is organized by ubiquitination. The process of ubiquitination is catalyzed by three enzymes cascade, consist of ubiquitin-activating (E1) enzyme, a conjugating (E2) enzyme, and ligase (E3) enzyme**.** It is believed that E3 ligases such as parkin are the key for substrate recognition and contribute to the specificity of the ubiquitin reaction. The polyubiquitin (polyUB) chains are conjugated at different lysine residues (K6, K11, K27, K29, K33, K48, and K63) or N-terminal of methionine (M1) with different ubiquitin (Ubs) chain topologies. Each chain structure defines how the protein will behave or be turned over in the cell. For example, the K48 linked ubiquitin chain will be the signal for protein proteolysis. The attached polyUb can also be removed by the deubiquitinating (DUB) enzymes family. It is well known that the huntingtin protein can be ubiquitinated and degraded by the major proteolytic systems. These systems are the ubiquitin-proteasome system (UPS), and the autophagy/lysosome machinery. Therefore, understanding these pathways in HD will give a great advantage to eliminate the toxicity of mutant Htt in HD.

The ubiquitin-proteasome system in HD

The ubiquitin-proteasome system is the pathway for protein degradation by the proteasome in the cytosol and nucleus. The UPS regulates a large array of substrates and several cellular processes. The degradation of the substrate by UPS is tagged by polyUb that is linked with K48 residue. The unfolded protein can enter the 26S proteasome for degradation. The 26S complex consists of a 20S core particle (CP), which is capped at one or both ends by a 19S regulatory particle (RP) proteasome activator (PA)700.

Proteasome activator PA700 (MW~700 kDa) is a large complex protein of several subunits with diverse activities such as ATPase, reverse chaperon, and deubiquitinating enzyme, etc. For example, PA700 from *S. cerevisiae* contains 17 subunits, such as AAA-ATPase (Rpt1–6) and non-ATPase (Rpn1–12) subunits. These subunits are arranged into two sub-complexes: a base and a lid. The base contains 9 subunits including polyubiquitin-interacting protein S5a,

whereas the lid contains 8 subunits with a lack of ATPase activity. The lid is the upper sub-complex of PA700, which has the deubiquitinating function. The base acts as a reverse chaperone to unfold the substrate and to facilitate the gate opening of 20S-CP. Then the unfolded protein can be "sneaking" into the narrow pore of CP-20S for degradation. The UPS regulates several pathways in the cell, due to the diverse proteins that are degraded by the 26S.

The UPS provides highly effective regulation of mitochondria dynamics. The UPS is involved in the degradation of outer mitochondrial fission-fusion proteins. In human cells, the mitochondria undergo the fission process, when Fis1 and Drp1 accumulate on the mitochondria outer membrane, whilst the Mfn1/2 are ubiquitinated and degraded by the 26S proteasome. On the other hand, the mitochondria are fused (fusion) in case of degradation of Drp1 and Fis1 by UPS, where Mfn1/2 accumulates on the outer mitochondria membrane, which plays a pivotal role in the mitochondria fusion process**.**

It was reported that mutant Htt can be ubiquitinated and be a potential substrate for the 26S proteasome complex for degradation. It was also found that the proteasome is present in the aggregates of mutant Htt, which indicates the direct implication of UPS in HD. It was suggested also that the proteasomes can assembly with the insoluble aggregates. The assembly of proteasomes to aggregates is probably reversible and dynamic. Thus, the proteasomes are still functional and able to reach the substrates even in the presence of mutant Htt species. In an i*n vitro* study, it was reported that mutant Htt aggregates do not inhibit the proteasome activity, whereas the extracted mutant polyQ fibril from *in vivo* studies can inhibit the proteasome activity, which suggests that the ubiquitination of aggregates could play an essential role in the defect of 26S proteasome complex in HD cells. Besides, it was proposed that mutant polyQ stretches could clog the proteasome. The activity of the clogged proteasome is inhibited, and this clog prevents the peptides from passing the proteasome. However, these data are conflicted with other data showing that N terminal fragments are completely degraded by the proteasome. Therefore, it is difficult to conclude the role of the UPS activity in the degradation of mutant Htt. Hence, more studies are needed to confirm the exact mechanism of proteasome function in HD pathogenesis.

The core particle 20S

The core particle (CP) consists of 28 subunits, assembles in 4 heptameric with dyad-symmetric rings, which form a cylinder shape proteasome. The unfolded protein can enter each of the two gates. Each gate is formed from seven α-type subunits. The other two rings in

the middle form the inner chamber. Each ring of the inner chamber is created from seven β-type subunits. The inner chamber is the house for proteolytic active sites for the potential cleaving of peptides. In eukaryotic cells, only three of seven β-type subunits have active sites, therefore the proteasome has six active sites. These sites are caspase-like, trypsin-like, and chymotrypsin-like activities, which are presented by the β1, β2, and β5 subunits, respectively.
The 20S-CP proteasome itself can degrade short peptides in a ubiquitin- and ATP-independent manner. The mechanisms that control the gate opening at the α ring level are not well understood. It is thought that the binding of an unfolded protein to the α ring could induce the gate opening. It was reported that one-fifth of the total proteins in the cell are degraded by the proteasome in a ubiquitin-independent manner. This pathway may provide an alternative mechanism for UPS to degrade unfolded and small proteins. For example, p53 a key regulator of the cell cycle is degraded by both ubiquitin independent and dependent pathways. Moreover, the highly oxidized or misfolded proteins are degraded by 20S-CP proteasome under cellular stress. The degradation by the 20S-CP proteasome is a passive pathway, which needs only an unstructured region in the protein to perform this process.
In general, the degradation by proteasomes is often dependent on ubiquitination and ATP. However, a wide range of proteins is degraded by ubiquitin-independent or by both degradation pathways. The specificity and function of the 20S-CP are well regulated by proteasomal activators PA700, and families Blm10/PA200 and PA28αβγ. PA700 is a proteasomal activator in ubiquitin and ATP-dependent manner as we discussed before. On the other hand, PA28αβγ and Blm10/PA200 families regulate the proteasomal function in a ubiquitin and ATP-independent manner. Moreover, these activator families can form hybrid proteasome complexes with PA700. The exact cellular function of hybrid proteasomes is still not fully understood. These hybrid proteasomes may have more proteolytic specificities than proteasomes capped with a single or double of one type of proteasome activator. Thus, the diversity of protostomes can control vast different substrates that put proteasome complexes as the main target to study several diseases.

The regulation of 20S-CP proteasome in a ubiquitin-independent manner

The capacity of the 20S-CP proteasome in a ubiquitin-independent manner is significantly enhanced by PA28 (αβγ) and Blm10/PA200 activators families. PA28 is known as the 11S regulator. It has three different subunits PA28α, PA28β, and PA28γ, and these subunits are arranged in a heptameric structure. These subunits are assembled in three different structures in mouse PA28α7, PA28β7, and PA28α4β3. While mammalian PA28 is mainly assembling in

asymmetric PA28α4β3 complex. In general, the PA28α and PA28β are found only in the cytosol, but all three PA28 (αβγ) isoforms predominantly present in the nucleus. The diverse locations of PA28 and its function in the degradation of unfolded small proteins make it a target to study its role in HD. The overexpression of PA28γ drives to recover the proteasome function and increases cell viability in different HD cell models. Several studies indicate the importance of enhancing the 20S-CP proteasome function in a ubiquitin-independent manner. This also attracted us to study the Blm10/PA200 family as a proteasome activator and its role in the degradation of peptides such as N terminal Htt fragments.

The Blm10/PA200 family

PA200 is a proteasomal activator with MW∼200 kDa, it is mostly located in the nucleus and found in the cytosol of human cells. The homolog of yeast *S.cerevisiae* is bleomycin resistance 10 (Blm10) with MW ∼245 kDa. Blm10 shows about 20 % homology sequence compared to PA200 protein. The structural studies by cryo-electron microscopy show that PA200 is a monomeric protein, it contains 32 HEAT-repeats-like modules from almost every α-helix. While Blm10 also contains HEAT-repeats, they are formed from all α-helix present in the protein. The Blm10/PA200 family has a dome architecture that binds to the gate of CP-20S, and HEAT-repats associate this binding. PA200 utilizes its C-terminal (Tyr-Tyr-Ala) to trigger an α-ring of CP-20S gate to rearrange the α subunits for gate opening. The structure of PA200 has two holes formed by a lot of positively charged residues, which allow unfolded proteins to go through PA200 into the 20S-CP for potential degradation. Whereas, Blm10 has a small opened slot that also leads unfolded protein to move toward the axial channel of the proteasome. Blm10/PA200 family can assemble with 20S-CP forming several proteasome complexes which were determined in either *in vivo* or *in vitro* studies. The Blm10/PA200-CP proteasomes are found as different complexes. For example, Blm10/PA200-CP can form a hybrid proteasome complex with PA700, which is found *in vivo***.** Moreover, the core particles with PA200 can form singly and doubly capped proteasomes. These proteasomes were recognized *in vitro* and isolated from testes of bovine and mammalian, while Blm10-CP complexes were found *in vitro* and isolated from yeast. The current knowledge about specific substrates for the Blm10/PA200 family is very limited. Blm10/PA200 proteasome activators enhance caspase-like and trypsin-like protease activities of β1 and β2 –subunits, respectively. The Blm10/PA200 family accelerates the turnover of Dnm1 in yeast and unstructured proteins such as Tau. PA200 facilitates the degradation of acetylated histone in a ubiquitin- and ATP-independent manner. Furthermore, PA200 is essential for cell survival in stressed cells.

Collectively, the Blm10/PA200 family plays several roles in the cell starting from its function as a proteasomal activator to its role as the regulator of several cellular functions.

Aims

Protein degradation by proteasome complexes plays the main role in the regulation of a wide range of basic cellular processes and cleaning misfolded proteins. The implication of proteasomal complexes in HD pathogenesis is still not fully understood. Therefore, we investigated the roles of proteasomes in the pathogenesis of HD in two different manners. On one hand, we studied how the activity of the proteasome in a ubiquitin-dependent manner affects mitochondria function in extraneuronal cells. On the other hand, we explored the potential role Blm10/PA200 activator family on the proteasomal degradation of N-Htt in a ubiquitin-independent manner. Thus, our aims in this project are:

1- To investigate the crosstalk between the ubiquitin-proteasome system and mitochondria in juvenile HD fibroblasts.
2- To determine the role of the proteasomal activator Blm10/PA200 family in the degradation of the toxic N terminal Htt fragments.

Objectives

1- Measure intracellular ROS production and mitochondria membrane potential, which reflect mitochondria function in juvenile HD fibroblasts.
2- Evaluate the glycolysis and mitochondria respiration in juvenile HD fibroblast.
3- Identify the cellular viability, which may be affected by mitochondrial alterations.
4- Determine the alteration in mitochondrial dynamics and function.
5- Evaluate the activity of UPS in juvenile HD fibroblast, and the effect of this activity in the turnover of specific UPS substrates.
6- Determine the effects of absence Blm10/PA200 proteasome activator on aggregates formation from the toxic N-Htt, and cellular toxicity of the aggregates in an HD yeast model.
7- Investigate the interaction between the Blm10/PA200 family with soluble fragments and aggregates from toxic N-Htt.
8- Confirm the role of the Blm10/PA200 family regarding aggregate formation in the human neuroblastoma cell line.
9- Evaluate the activity of the proteasome in the presence of Blm10/PA200 family in degradation of soluble N terminal Htt.
10- Determine and specify the contribution of Blm10/PA200 in the degradation of soluble fragments of wild-type and mutant N-Htt fragments by mass spectrometry (MS).

Material and Methods

Cell models

We performed our study using different kinds of cells as experimental HD models to study various aspects of Huntington's disease. The first model is juvenile HD fibroblast. The primary fibroblasts were cultured in Minimum Essential Medium Eagle (MEM) supplemented with 10 % non-heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 100 units/ml penicillin (Pen) and 100 µg/ml streptomycin (Strep) termed as complete MEM (CMEM). The cells grew in a cell culture incubator under standard conditions at 37° C and 5% CO_2. To avoid any effects of cellular senescence on human fibroblast cells, we performed our experiments using the cells within passage numbers 7 and 14.

The second model is the SH-SY5Y neuroblastoma cell line. SH-SY5Y is a human cell line derived from the SK-N-SH cell line. The growth medium for SH-SY5Y consists of Dulbecco's Modified Eagle's Medium-high glucose (DMEM), supplemented with 10 % heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 units/ml Pen - 100 µg/ml Strep antibiotic solution. The cells grew under standard conditions at 37° C and 5% CO_2. We used a previously generated stable PA200 depleted SH SY5Y cell line (shPA200) and corresponding control with an empty pGIPZ-GFP vector. The stable shPA200 and control grew in DMEM, supplemented with 10 % heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate solution, Pen-Strep as an antibiotic, and the cells were maintained by 1.25 µg/ml puromycin selection, which removed before 24 hr of the day of the experiment.

The third model in our study is the yeast model of HD. Yeast strains used in this study are isogenic to BY4741 and BY4742, which derive from *S. cerevisiae* S288C. Complete gene deletion and yeast transformation were accomplished by the Lithium Acetate. Briefly, yeast and plasmid DNA resuspended in polyethylene glycol (PEG) and lithium acetate (LiAc) solution (40 % w/v PEG, 0.1 M LiAc in Tris-EDTA buffer), followed by 40 min incubation at 30 °C, and 7 min at 42 °C. Then, the yeast was plated on a selection medium. The cells grew in standard synthetic complete media (0.67 % w/v yeast nitrogen base, 2 % w/v glucose and necessary amino acids) or yeast extract, peptone, and dextrose (YPD) media (2 % w/v of Difco peptone, 1 % w/v of yeast extract, 2 % w/v of glucose). Then, cells were seeded onto solid selective synthetic complete media with reduced amino acids at 30 °C for selecting the transformed yeast. The yeast strains grew in YPD media at 30 °C and were harvested at the log phase with approximate optical density $(OD)_{660}$ nm ~1 for potential study.

Moreover, we studied the phenotype of yeast strains expressed N huntingtin fragments contain a specific length of polyglutamine (N-Htt polyQ) such as N-Htt25Q as wild type or N-Htt103Q as mutant fragments in the strains. The cells grew overnight in synthetic media with 2 % v/v raffinose as a carbon source and reduced amino acids for plasmid selection. The yeast induced with 2 % v/v galactose for polyglutamine expression, or with 2 % v/v raffinose as uninduced yeast. The cells were incubated for 18 hr to reach the OD_{660} nm ~ 0.4 mid-log phase. The induced and uninduced cells were spotted by drop-out the media onto solid synthetic media. Then the colonies were observed daily for yeast phenotype study.

Filter trap analysis for detecting and quantifying N-Htt aggregates formed in human cells

Control and shPA200 cells were transiently transfected with pHM6-Q23 and pHM6-Q74 plasmids for 48 hr. Cells were raised with 1 X PBS and lysed on the ice with lysis buffer (40 mM HEPES, pH 7.5, 50 mM KCl, 1 % v/v Triton X-100, 2 mM DTT, 5 mM EDTA, supplemented with protease inhibitor cocktail). The lysate was fractionated into supernatant (S) and pellet (P) by centrifugation (16,000 rpm for 20 min, at 4 °C). To avoid DNA and RNA interfering the analysis, the pellet was suspended with 75 µl DNA digestion buffer (40 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 10 mM NaCl, 10 mM $CaCl_2$) supplemented with 100 µg/ml RNase, 30 U DNase I and incubated for 1 hr at 37 °C. The reaction was stopped with 2 X termination buffer (4 % SDS, 40 mM EDTA, 100 mM DTT). Samples with protein amount (10 µg) were suspended with 200 µl filter trap buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl (TBS), 2 % SDS), and loaded onto 0.2 µm nitrocellulose membrane using Minifold I-96 well dot-blot system. The dotes were washed two times with (TBS, 0.1 % SDS). The membrane was incubated with shaking for 1 hr at RT with Odyssey blocking buffer. Specific antibodies for PA200 and HA-tag were diluted by Odyssey blocking buffer contains 0.1 % Tween and shacked overnight in the cooled room. The membrane was washed with (TBS, 0.05 % Tween) and probed with secondary antibody in Odyssey blocking buffer also contains 0.1 % Tween for 1.5 hr at RT. The secondary antibody conjugates with infrared fluorescent dyes (IRDye® 800 CW, IRDye® 680 RD). The dotes blots were visualized by LI-COR ODYSSEY CLx infrared imaging system and the signals were analyzed by Image studio software v 5.2.

Proteasomal activity assay

Proteasome activity assay is based on the hydrolysis of fluorogenic substrate Suc-LLVY-AMC by chymotrypsin-like protease activity of the proteasome. To investigate the

proteasome activity in the cell lysates, fibroblasts were seeded with cell density 2×10^6 in a T150 flask. On the next day, one flask of each cell type was treated with 10 μM of MG132 for 1 hr before the experiment to use as a negative control. Then, the cells were collected and lysed on ice with lysis buffer (20 mM HEPES, 5 mM $MgCl_2$, 320 mM sucrose, 0.2% NP-40, 2 mM EDTA, 1 mM ATP contains protease inhibitor cocktail). Cell lysates were cleared by centrifugation for 10 min at 16,000× g and 4 °C. The protein amount (50 μg) from cleared supernatant was mixed with loading buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 6 % glycerol, and 12 ng/mL xylene cyanol). The samples were loaded into 3-8 % Tris-acetate native gel. Electrophoresis was performed by running buffer (90 mM Tris, 90 mM boric acid, 5 mM $MgCl_2$, 0.5 mM EDTA, pH 8.3-, and 0.5-mM ATP) applying voltage 60 V for 12 hr. The gel with separated proteasomes was incubated with 20 ml of reaction buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM ATP, 100 μM Suc-LLVY-AMC in DMF, and 0.02 % SDS) for 30 min at 30 °C in dark. Fluorescent bands were analyzed using ChemiDoc imager, and band densitometry was quantified by Image Lab software 6.0.1. Proteasome activity was normalized to 1 μg protein in each sample. In this experiment, all buffers were filtered sterilized with a 0.2 μm filter and all steps were performed at 4 °C unless stated otherwise. In parallel, to confirm the equal quantity of core particles loaded for activity assay, equal protein 50 μg amounts were loaded into typical SDS-PAGE and performed Western blot. We determined the β1 subunit as representative of 20S-CP.

Peptidomics analysis of N-Htt degradation *in vitro*

The purified GST-Htt18Q and GST-Htt51Q were degraded into short peptides by 20S-CP or Blm10-CP proteasome complexes *in-vitro*. The peptides were cleaned up by using a stage-tip microcolumn. The cleaned peptides were suspended with 0.1 % formic acid in distilled water (dH_2O). The samples were measured by a Q-Exactive mass spectrometer (MS) system coupled to a Proxeon nano-liquid chromatography (LC) system in data-dependent acquisition mode. It was selected as the top 10 peaks for higher-energy collisional dissociation (HCD) fragmentation. Each sample with 5 μl was injected and applied for 3 hr) gradient solvent A (5 % acetonitrile, 0.1 % formic acid) and solvent B (80 % acetonitrile, 0.1 % formic acid), using an in-house prepared nano-LC column (0.075 mm× 250 mm, 3 μm Reprosil C_{18}) from Maisch GmbH, Germany. The sample was eluted with 3 hr gradients of 4-76% acetonitrile and 0.1% formic acid in dH_2O at flow rates of 0.25 μL/min. The acquisition of MS was carried out at a resolution of 70,000 m/z in the scan range from 300 to 1700 m/z. Dynamic exclusion was set at the 30s and normalized collision energy at 26eV. The mass window for precursor ion

selection was adjusted to 2.0 m/z. Data were analyzed using the MaxQuant software. The internal Andromeda search engine was used to search MS/MS spectra against the *S. cerevisiae* proteome database and the recombinant huntingtin protein sequences of Htt51Q and Htt18Q containing forward and reverse sequences. The search included the variable modification of methionine oxidation and fixed modification of carbamidomethyl cysteine. The smallest peptide length for analysis was adjusted to seven amino acids without any selection for the digestion pattern. The false discovery rate (FDR) was set to 0.01 for peptide identification. The data was further analyzed using the R software and the tidyverse analysis package.

Results

Increased reactive oxygen species production and mitochondria membrane potential indicate changes in mitochondrial function of juvenile HD fibroblasts

Several studies have been demonstrated that HD is associated with increased production of reactive oxygen species (ROS). Increased ROS leads to cell death in the striatum and the cortex of the brain of HD patients. To date, the cellular dysfunction in HD peripheral tissues has not been fully understood. Therefore, we determined ROS production in juvenile HD fibroblasts (68Q and 86Q) using carboxy-H2DCFDA dye. Interestingly, we found a significant increase of cytosolic ROS in HD fibroblast (86Q) compared to healthy control (16Q), which indicates oxidative stress in the juvenile HD model.

The intracellular ROS production is mainly generated from mitochondria dysfunction in HD neuron cells. Consequently, we measured mitochondria membrane potential ($\Delta\Psi m$) by two methods. First, we stained the mitochondria with Mitotracker CXMRos (MTR), and second, we stained the mitochondria with tetramethylrhodamine ethyl ester (TMRE). MTR is a sensitive indicator for $\Delta\Psi m$ changes and oxidative stress, while TMRE is an indicator for the polarization of the mitochondrial membrane. In the case of TMRE, we used a negative control to confirm the specificity of TMRE. Our negative control was generated by treating cells with 20 μM FCCP (Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) to depolarize the mitochondrial membrane. FCCP disrupts ATP synthesis by transporting protons across the mitochondrial inner membrane, which leads to a loss of mitochondrial membrane potential, and eliminates TMRE staining. In both MTR and TMRE staining assays, we detected a significant increase in $\Delta\Psi m$ of 68Q and 86Q compared to 16Q.

Juvenile HD fibroblasts show maintained mitochondrial ATP production, suppressed glycolysis, and disturbed cell cycle

It was previously reported that adult-onset HD fibroblasts tend to show slower cell proliferation compared to healthy counterparts. This observation is caused by disturbances in the oxidative phosphorylation (OXPHOS) machinery in HD cells. We studied the OXPHOS in juvenile HD fibroblasts, and we analyzed mitochondria respiration by Seahorse XF. In our study, we normalized the mitochondrial respiration measurements data to the protein amount in each well. Our data revealed that HD fibroblasts exhibited slightly reduced ATP generation, basal, and maximal mitochondrial respiration.

Glycolysis is another energy source in the cell. To determine glycolysis in HD cells, we measured extracellular acidification rate (ECAR) by Seahorse XF. We detected a significant decrease of glycolysis in 68Q diseased cells compared to healthy cells with 16Q. While this decrease was not significant in 86Q cells. In summary, these data suggest that mitochondrial OXPHOS is maintained while glycolysis is suppressed in juvenile HD fibroblasts.

Increased generation of intracellular ROS suppressed glycolysis led to reducing cell viability in neurodegenerative diseases. Therefore, we analyzed the cell death/viability in HD fibroblasts by flow cytometry analysis (FACS). The analyzed data showed that juvenile HD fibroblasts are viable and both apoptosis and necrosis are relatively low like healthy control**.**

During our daily care of fibroblasts, we observed low cell proliferation in juvenile HD fibroblasts compared to control fibroblasts. Therefore, we analyzed the cell cycle to determine whether the cell cycle disturbance is a possible reason for decreased cell proliferation in juvenile HD fibroblasts. We assessed the percentage of cells in G0/G1, S, and G2/M phases. Data showed that the percentage of S and G2/M phases are significantly lower in both HD fibroblasts, while the percentage of G0/G1 phase is significantly higher in 68Q, and 86Q compared to healthy 16Q. These data indicate that HD cells are accumulated in G0/G1 before proceeding to the S phase, which might lead to reduced mitosis in juvenile HD fibroblasts compared to healthy fibroblasts.

During monitoring our cells also, we observed that 68Q, and 86Q are larger than healthy cells 16Q. Therefore, we analyzed the cell area by high content screening (HCS) confocal microscopy after F-actin staining with Texas Red. Data analysis showed that the cell size of juvenile HD fibroblasts is significantly larger compared to healthy cells**.** We suggest that increased cell area might be related to the cell cycle disturbance in juvenile HD fibroblasts.

Juvenile HD fibroblasts demonstrate alterations in mitochondrial fission-fusion protein levels

Mitochondria function is regulated by the fission-fusion machinery. The increase of ΔΨm might be associated with the disturbed mitochondrial fission-fusion process. Thus, we performed qPCR analysis to investigate the gene expression of several mitochondrial fission and fusion genes. We determined fission genes such as *DNM1L* (Drp1), *FIS1* (Fis1), *MIEF1* (Mid51), *MIEF2* (Mid49), and *MFF* (Mff). The fusion genes were *MFN1* (Mfn1), *MFN2* (Mfn2), and *OPA1* (Opa 1). We found that the gene expression of *DNM1L* for mitochondrial fission protein Drp1 was significantly decreased in 86Q compared to 16Q. The gene

expression of mitochondria fusion proteins did not change significantly in HD cells compared to healthy control cells.

We also evaluated the protein levels for several mitochondrial fission-fusion proteins. Our results revealed that Drp1 protein level is decreased in 68Q, and 86Q with a significant decrease in 86Q compared to healthy control 16Q. The decrease in the Drp1 protein level is by the decrease of *DNM1L* gene expression in HD fibroblasts. Mff did not change significantly in both gene and protein levels in 68Q, and 86Q compared to 16Q. The protein levels of mitochondrial fusion proteins such as Opa1 and Mfn1 were significantly decreased in juvenile HD fibroblasts, but the protein level of Mfn2 did not show a significant decrease in diseased cells compared to healthy control. Our data suggest that the alteration in fission-fusion proteins could lead to changes in mitochondrial morphology.

Therefore, we studied mitochondrial morphology by HCS. The live-cell high content images were analyzed based on a quantitative evaluation of mitochondrial morphology. We found a significant decrease of mitochondrial branching in both juvenile HD fibroblasts compared to the healthy control, which was determined by an increased mitochondria fragmentation score**.** Simultaneously, we also found that mitochondrial length was significantly shorter in HD fibroblasts 68Q and 86Q compared to the control 16Q suggesting mitochondrial fragmentation.

Increased activity of the proteasome accompanied by a normal rate of autophagy in juvenile HD fibroblasts

The deficit of the UPS has been well-studied in neuronal HD cell models, but our knowledge about the activity of the UPS and its role in HD peripheral tissues is still limited. We hypothesized that juvenile HD fibroblasts counterbalance the mitochondrial morphology changes with altered proteasome activity to promote cell survival. We studied the relative distribution of the proteasome complexes using total lysate by in-gel activity assay. We performed this assay in two groups of cells. In the first group, the cells were treated with the proteasome inhibitor MG132 (carbobenzoxy-Leu-Leu-leucinal). MG132 is a peptide aldehyde, which potently blocks the proteolytic activity of the 20S proteasome. In the second group, the cells grew without treatment. Then, we loaded the cell lysates into the native gel to fractionate proteasome complexes based on their molecular weights, followed by an in-gel activity assay. The proteasomes can be fractionated into three main bands based on the molecular weight of proteasomal complexes. In our assay we show the core particle (20S-CP), 20S-CP capped with one proteasome activator (PA700-20S), and the 20S-CP capped with two

proteasome activators (PA700-20S-PA700). To visualize the activity of the core particle of these species, the native gel was incubated with the specific fluorogenic substrate Suc-LLVY-AMC. This substrate is hydrolyzed by the chymotrypsin-like activity of the proteasome. Both doubly PA700-20S-PA700 and singly PA700-20S capped proteasome complexes showed increased activity in HD fibroblasts compared to healthy control. To confirm the exact amount of core particle was loaded for each sample, we determined the protein level of the β1 subunit of 20S-CP by Western blot. We found that the β1 subunit protein levels were similar in all samples.

The singly and doubly capped proteasomes with PA700 mediate protein degradation in an ATP and ubiquitin-dependent manner. We also determined the higher chymotryptic-like activity of the PA700-20S and PA700-20S-PA700 proteasomes in HD fibroblasts. To confirm this activity on ubiquitinated proteins, we investigated the accumulation of ubiquitinated proteins in the total cell lysates. We found that the level of ubiquitinated proteins was significantly higher in juvenile HD fibroblasts compared to healthy control after proteasomal inhibition by MG132. Our results suggested that proteolytic activity of the proteasome complexes was higher in juvenile HD fibroblasts compared to healthy control.

Autophagy is the other protein degradation pathway to remove dysfunctional or long-lived proteins. To determine the efficiency of autophagy in HD fibroblasts, we treated cells with bafilomycin A1 (BMA1) the autophagy inhibitor. BMA1 inhibits vacuolar H+ ATPase in the lysosome, which leads to preventing the fusion between autophagosomes and lysosomes. We found that ubiquitinated proteins were slightly increased upon autophagy inhibition in HD cell lysates compared to control cells. Furthermore, we compared the protein levels of the autophagy markers such as LC3I, II, and p62 in the presence and absence of the autophagy inhibitor BMA1. LC3I converts to LC3II, which requires the formation of the autophagosome. We found that the level of LC3II significantly increased in HD and healthy fibroblasts after treatment with BMA1 compared to its corresponding vehicle-treated cells. We used p62, an autophagy substrate, and marker for autophagy activity. Western blot analysis of p62 showed that autophagy is active in HD fibroblasts. The accumulation of p62 after autophagy inhibition in 68Q-containing HD fibroblasts was similar compared to the healthy control, while the accumulation of p62 in 86Q-containing HD fibroblasts following autophagy inhibition was significantly lower compared to healthy control. Decreased p62 in 86Q could be a preliminary indicator of the impairment of autophagy in the later progression of the disease. Taken together, our observations reveal that juvenile HD fibroblasts show a higher capacity of 26S proteasomes but not autophagy. These findings were confirmed by

increased proteasomal activity and elevated levels of ubiquitinated proteins after proteasomal inhibition.

Accelerated turnover of Mfn1 might be associated with increased Parkin level in juvenile HD fibroblasts

Parkin is an E3 ubiquitin ligase and has several protective functions in the cell. Parkin is also participating in proteasomal-mediated protein degradation, mitochondrial function, and cell survival. We hypothesized that an elevated level of ubiquitinated proteins after proteasome inhibition, and the preserved cell viability in juvenile HD fibroblasts, might be associated with elevated parkin levels. We investigated the *PRKN* gene expression and Parkin protein levels in healthy and HD fibroblasts. Our results showed that *PRKN* mRNA expression increased in juvenile HD fibroblasts with a significant increase in 86Q. Moreover, Parkin protein level increased significantly in 68Q, and 86Q compared to 16Q**.**

We detected a decreased level of mitochondrial fission-fusion proteins including Mfn1 in juvenile HD fibroblasts. Mfn1 is a substrate for Parkin for ubiquitination and degradation by the 26S proteasome. We performed a cycloheximide (CHX) chase assay to determine the Mfn1 half-life in healthy and HD cells. CHX inhibits translation elongation by binding to the ribosomal E-site. We used 300 μg/ml of CHX to block new protein synthesis in the presence and absence of the proteasome inhibitor MG132. The degradation of a protein by the proteasome can be monitored in cells. We determined an increased turnover of Mfn1 over 18 hr in HD fibroblasts. We propose that elevated Parkin promotes ubiquitination and together with increased 26S proteasome activity leading to increased Mfn1 turnover. Then, we tested if these activates could explain the decreased protein levels of Drp1 and Opa1. Therefore, we performed CHX for Drp1 and Opa1. We found that protein levels for both Drp1 and Opa1 did not show a difference in the presence or absence of MG132. These results suggest that increased proteasomal activity does not influence the degradation of Drp1 or Opa1.

Blm10-CP complexes antagonize the toxic effects of mutant N-Htt in the yeast HD model

HD is an age-related disease. Thus, the cells that expressing mutant polyQ show severe cytotoxicity when the patient's age reaches more than 40 years old. In the same manner, the HD yeast model does not show immediate cell death upon expression of toxic polyQ, but the expression of toxic polyQ in yeast causes disability of cell growth. Therefore, yeast is an ideal model to study cytotoxicity in neurodegenerative diseases. A recent study reported that knockdown of mammalian *PSME4* (PA200) or deletion of its yeast ortholog *BLM10* (Blm10)

accelerates cellular aging. Furthermore, the loss of the PA200/Blm10 family leads to a decrease in proteasome activity during aging. Both findings provide a solid idea that the PA200/Blm10 family might have a significant role in age-related diseases. We examined whether the *BLM10* (Blm10) deletion affects the viability of yeast cells expressing the mutant N terminal Htt fragments containing extended polyQ. We induced the expression of the N-Htt103Q as mutant fragments and the N-Htt25Q as wild-type fragments. Then, we determined the viability of the HD yeast model by a serial dilution assay. Hence, we found that deletion of *BLM10* causes impaired cell growth in the presence of toxic fragments compared to wild-type yeast (WT) or *RPN4* deletion strain (*rpn4Δ*). Rpn4 protein is a transcription factor that catalyzes the transcription of several genes of proteasomal subunits. Rpn4 protein is rapidly degraded by the 26S proteasome, where the Rpn4 level provides a negative feedback cycle to control the proteasome abundance in *S. cerevisiae*. We found that inducing the expression of the nontoxic fragments contains 25Q did not affect yeast viability after deletion of *BLM10* (*blm10Δ*) or *RPN4* (*rpn4Δ*).

Next, we examined the effect of the absence of Blm10 on the formation of insoluble aggregates from mutant N-Htt by filter retardation assay. The cells were induced by galactose to express the mutant N-Htt and collected at different time points of induction. We found in both WT and *blm10Δ* strains that the insoluble aggregates accumulate during the time. We performed statistical analysis from the chemiluminescent intensity of 10 μg protein samples and normalized the values to WT samples. The data showed that after 18 hr of galactose induction, the total lysate (T) and aggregates containing pellet (P) slightly increased in *blm10Δ* strain; however, these changes were not significant.

To determine the insoluble and soluble fractions of mutant N-Htt103Q in WT and *blm10Δ* we induced the expression of N-Htt103Q for different time points. The samples (T, S, P) were loaded onto gradient gel under native conditions. The insoluble aggregates are stuck in the upper part of the gel, while the soluble fraction runs into the separating gel. These data were normalized to the values of the corresponding 10 hr induction of each strain. Data analysis indicated that the trend of toxic N-Htt aggregation is increased in both strains of WT and *blm10Δ* in a time-dependent manner. The aggregates formation from toxic N-Htt was more evident but not significant in *blm10Δ* cells. We analyzed these data again using the main effects ANOVA (Statistica V. 13.6). This type of analysis determines the effects of multiple categorical independent variables. In our case, the categorical variables were the fractions of the cells, time, and the strains we used. We found that the formation of the aggregates in WT and *blm10Δ* strains changed significantly over time ($p < 0.001$). Due to methodological

difficulties to determine and quantify the accumulation of aggregates in our model by native gel and immunoblotting, we also wanted to find another approach for more accurate quantification. Therefore, we used live-cell fluorescent microscopy to determine the wild-type or toxic fragments. These fragments are fused with Cyan Fluorescent Protein (CFP). The live-cell imaging of CFP showed that the CFP-Htt25Q is distributed equally in the cytosol in all strains. The absence of Blm10 caused larger aggregate formation from CFP-Htt103Q in *blm10Δ* compared to WT and *rpn4Δ* strains. Data analysis confirmed that the number of *blm10Δ* cells with large aggregates is significantly higher compared to WT and *rpn4Δ* cells.

Endogenous PA200 colocalizes with wild-type and mutant N-Htt fragments in the SH-SY5Y human neuroblastoma cell line

To determine whether PA200, the human orthologue of Blm10 has any physiological relevance in HD we transiently transfected and overexpressed the wild-type and mutant N-Htt fragments. These fragments are tagged with hemagglutinin (HA). We transfected the SH-SY5Y cells for 48 hr, and then we performed immunostaining using an anti-HA tag antibody to detect N-Htt fragments. It was reported that most PA200 localizes in the nucleus of HeLa cells, and approximately 20 % of PA200 present in the cytosol. Our immunostaining with an anti-PA200 antibody in SH-SY5Y cells showed that endogenous PA200 is distributed in both the nucleus and cytosol. Moreover, the wild-type N-Htt with a normal length of polyQ was evenly distributed in the cytosol of the cells, while mutant N-Htt with an expanded length of polyQ formed aggregates in the nucleus and/or cytoplasm of the cells. We then merged the confocal images at superposition. We analyzed the colocalization between N-Htt and PA200 via the JaCoP plugin tool using ImageJ software (imagej.nih.gov). The JaCop plugin combines several colocalization methods, including Pearson's colocalization coefficient (PCC), and Manders' colocalization coefficient (MCC). PCC indicates linear regression between two continuous variables, whereas MCC determines the percentage of overlap between two variables. Colocalization is a co-occurrence such as the simple spatial overlap of two probes. Besides, the correlation means when the two probes not only overlap but also, they co-distribute in proportion within and between their structures. The distribution of two proteins is expected to overlap but not necessarily in proportion. The values we obtained for PCC are $r = 0.659 \pm 0.093$ for PA200 and the N-Htt23Q and $r = 0.72 \pm 0.097$ for PA200 and the N-Htt74Q. The positive value indicates the moderate correlation between two proteins ($r < 0.7$) and the strong correlation between two proteins ($r > 0.7$). MCC was also calculated using JaCoP. MCC is very sensitive to the background. Therefore, we set a threshold by estimating

the background when we calculated M1 and M2. The values of M1 (or M2) show the proportion of the green signal concurrent with the red signal over its total intensities. M1 indicates the fraction of N-Htt overlapping PA200, and M2 indicates the fraction of PA200 overlapping N-Htt. The M1 value = 0.35 for N-HttQ23 and PA200, which means that the two pixels overlap by 35%. The M2 valve =0.34 for PA200 and N-HttQ23, which means that the two pixels overlap by 34%. In the same manner, M1 for N-HttQ74 and PA200 refers to the two pixels overlap by 30%, and M2 refers to overlap 44.8 % for PA200 and N-HttQ74.

PA200 interacts with wild-type and mutant N-huntingtin fragments *in vitro*

Since PA200 overlap with wild-type and mutant N-Htt, we sought to detect whether PA200 might interact with N-Htt fragments. We performed a pull-down assay to show the potential interaction between the recombinant GST-Htt18Q and GST-Htt51Q with endogenous PA200. These two plasmids expressing the GST-N17 and fused with constructs containing either 18Q (wild-type) or 51Q (mutant). These constructs were modified by the addition of a tobacco etch virus (TEV) protease cleavage site between the GST and Httex1 coding regions. The presence of the GST tag maintains mutant fragments in a soluble form. Once the GST is cleaved by TEV protease, the fragments with the 51Q length rapidly form insoluble aggregates. Both proteins GST-Htt18Q and GST-Htt51Q were produced in bacteria and purified by glutathione Sepharose 4B beads. The purified samples were examined by SDS-PAGE and Coomassie blue staining. The GST-Htt18Q band was detected at the approximate size of 45 kDa and the GST-Htt51Q at 55 kDa. The purified GST tagged proteins were incubated with SH-SY5Y cell lysate with the endogenous PA200. The interactions between GST-Htt18Q and GST-Htt51Q with PA200 were determined by immunoblot using an anti-PA200 antibody. In the same experimental setting, we also examined if we could find an interaction between the GST-N-Htt and β1 subunit of the 20S-CP. But we did not detect any interaction between the indicated fragments and the β1 subunit of the 20S-CP.

Depletion of PA200 leads to increased size and number of the mutant N-Htt aggregates in SH-SY5Y cells

To examine the potential role of PA200 (gene: *PMSE4*) on the formation of the aggregates from mutant N-Htt, we used the previously established stable knockdown cell line (shPA200 cells) and its corresponding control, which is stably expressing the pGIPZ-GFP. We validated again the knockdown efficiency of the *PMSE4* gene by RT-qPCR, Western blot, and confocal microscopy. Our results confirm the successful knockdown of *PMSE4* in shPA200 cells.

These findings also indicate the specificity of the anti-PA200 antibody that was used in this study. We also validated the overexpression of wild type (N-Htt25Q-HA-tag) and mutant (N-Htt74Q-HA-tag) fragments in control and shPA200 cell lines by Western blot.

We performed immunocytochemistry for shPA200 and control cells after overexpression of wild-type and mutant N-Htt fragments for 48 hr. The confocal microscopy images showed that wild-type fragments are evenly distributed in the cytosol of both shPA200 and control cells. We also analyzed the overexpression of the mutant fragments in shPA200 and control cells. The data showed that the stable depletion of PA200 leads to a significant increase of aggregates size and the number of mutant N-Htt compared to the control cells.

Next, we performed a filter trap assay to determine the SDS-insoluble aggregates of mutant N-Htt. The cells were transfected by plasmids containing normal or expanded CAG repeats for 48 hr. Cell lysates were fractionated by centrifugation into supernatant (S) and the pellet (P) containing aggregates. Our results demonstrate that the SDS-insoluble aggregates of mutant N-Htt are significantly higher in the pellet fraction of shPA200 compared to control cells. Furthermore, when we applied an anti-PA200 antibody, we found that PA200 is present in the aggregates of the pellet fraction of control cells. Thus, we propose that PA200 is recruited into mutant huntingtin aggregates.

The Blm10/PA200 family promotes the degradation of wild-type and mutant N-terminal Htt fragments *in vitro*

We hypothesized that the Blm10/PA200 family might be involved in the proteasomal degradation of wild-type and mutant N-Htt. To examine this hypothesis, we performed *in vitro* degradation assay for GST-Htt18Q as wild-type and GST-Htt51Q as mutant proteins. The equal molar amounts of a purified core particle (CP) or CP-Blm10 complexes were incubated with GST-Htt18Q and GST-Htt51Q for different time points. Both wild-type and mutant GST-N-Htt were degraded by CP or CP-Blm10 proteasomes. Data analysis showed that both N-Htt with different polyQ is significantly degraded by the proteasome in the presence of Blm10 in a time-dependent manner. The proteins remain stable in the absence of the catalytically active proteasome. Moreover, the GST protein was not degraded by either CP or CP-Blm10. To confirm that Blm10 specifically promotes the degradation of soluble N-Htt in a ubiquitin-independent manner, we also performed *in vitro* degradation assay for GST-H51Q soluble protein using the 26S proteasome. Our results showed that GST-Htt51Q remains stable over time in the presence of the 26S. Taken together, Blm10 the orthologue of

human PA200 enhances the activity of 20S-CP to degrade the soluble non-ubiquitinated N-Htt fragments.

These proteasome complexes were purified by affinity purification and then subjected to size-exclusion chromatography. To confirm the purity of CP and Blm10-CP complexes and detect whether the purified proteasomes are catalytically active, the purified proteasomes were first subjected to Coomassie staining. Then, we performed a native gel analysis followed by an in-gel activity assay and native gel protein staining. We demonstrate that all purified proteasomes CP, CP with singly, and doubly capped by the activator Blm10 are all fully active**,** and we confirmed the presence of proteasomes in the native gel by protein staining.

Blm10 increases the capacity of the proteasome to cut within mutant N terminal Htt fragments

It was previously reported that Blm10 induces the trypsin-like and caspase-like protease activities without changing the chymotrypsin-like activity of the proteasome. Thus, we studied the proteasomal degradation of soluble N-Htt51Q and N-Htt18Q by mass spectrometry (MS) based peptidomics. The purified GST-Htt18Q and GST-Htt51Q proteins were incubated with purified 20S-CP and Blm10-CP proteasomes. The degradation products were subjected to MS. The MS analysis identified 2417 peptides, and 461 peptides were derived from the N-Htt fragments. Peptide analysis showed that without 20S-CP, one peptide was detected, which was derived from the N-Htt51Q. The addition of the 20S-CP to the reaction led to generate a significant number of peptides from the N-Htt18Q and N-Htt51Q as shown in the second column of the heat map. Moreover, adding Blm10 to the CP in the reaction led to generate more peptides such as (SLKSFQQ). Blm10 also triggered the production of other types of peptides generated from 20S-CP alone such as (KASFESLKSFQQ). The addition of Blm10 to the 20S-CP led to an elevated degradation of wild type with 18Q and mutant with 51Q of N-Htt, while the lengths of peptides were unchanged.

To determine the specific cutting patterns by 20S-CP and Blm10-CP, we probed our peptides data and analyzed the differences between the detected peptides from the *in vitro* degradation assays. The peptides generated by the 20S-CP have 71% similarity with peptides produced by Blm10-CP proteasomes. The number of peptides generated from the degradation experiment with Blm10-CP was considerably higher compared to peptides generated by the 20S-CP or without proteasome. To look deeply and investigate the cleavage site of the recognized peptides, we investigated the peptides sequences upstream of the cleavage sites of the

recognized peptides. To explain the cleavage specificity, we applied the Berger and Schechter nomenclature. The proteolytic cleavage sites are distinguished based on protease recognition of a substrate. We found the amino acid residues around the protease-binding pocket are denoted as P4-P3-P2-P1↓P1′-P2′-P3′-P4′. The arrow demonstrates that the cleavage happens between amino acid residues of P1 and P1′. Then, we analyzed the peptides sequence of cleavage site according to amino acids, and by positional enrichment of the amino acids. We found that the cleavage specificity by the 20S-CP proteasome is not altered in the presence of the Blm10 activator for the degradation of N terminal Htt fragments. The proteasome demonstrates a preference for hydrophobic and charged amino acids in positions P1, P2, and P5. The cleavage preference in position P1 is the chymotryptic-like activity of the β5 subunit in the core particle. This specificity is not the same as previously described, showing that PA200 stimulates the trypsin-like and caspase-like protease activities. This might be because we used only N terminal Htt fragments in our study. N terminal Htt fragments have a specific amino acid composition, which lets the CP or Blm10-CP proteasomes be biased to utilize the specific cleavage site for N-Htt degradation.

Summary

Huntington's disease is characterized by ineffective degradation of mutant Htt, which leads to the accumulation of mutant N-Htt fragments, oligomers, and aggregates in neuronal cells. The toxic species of mutant Htt influence several intracellular pathways including proteasomal degradation. Mutant huntingtin widely expresses throughout patient tissues in early life. Several studies have determined the impairment of ATP synthesis and proteolytic function are the hallmarks of the disease in HD neuronal cells. But no hypothesis clearly describes the crosstalk between mitochondria and ubiquitin-proteasome system in HD peripheral tissues. In the first study, we investigated the role of the ubiquitin-proteasome system in mitochondrial function using juvenile HD fibroblasts. We found elevated ROS levels, accompanied by an increase of mitochondrial membrane potential in diseased cells. The analysis of mitochondrial respiration does not show a significant difference in HD cells compared to healthy cells. Several mitochondrial fission-fusion protein levels were significantly decreased in HD fibroblasts. This decrease led to reduced mitochondria branching. On the other hand, the function of the ubiquitin-proteasome system was increased by an elevated proteasome activity and a high level of Parkin. We compared the proteasomal degradation of Mfn1 a specific substrate for Parkin in healthy and diseased cells by chase assay. We found the turnover of Mfn1 was faster in diseased cells compared to control. Moreover, we demonstrated that juvenile HD fibroblasts are viable with a low rate of mitosis. We suggest that juvenile HD fibroblasts were responded to mutant Htt by triggering the activity of the 26S. This activity promotes the turnover of specific substrates by the ubiquitin-proteasome system to protect cells, whereas mitochondria provide sufficient energy for cell survival.

The 26S proteasome degrades ubiquitinated Htt and the 20S-CP cleaves within polyQ in a ubiquitin-independent manner. Moreover, the proteasome activator Blm10/PA200 family controls the gate opening of the 20S core particle in the ubiquitin and ATP independent pathway. Blm10/PA200-CP proteasomes participate in the degradation of Dnm1, acetylated histones, and unstructured proteins such as Tau. In the second study, we demonstrated the role of the Blm10/PA200 family in the degradation of N terminal Htt fragments in a ubiquitin-independent manner. We demonstrated that PA200 interacts with N terminal Htt fragments. The deletion of *BLM10* in yeast and the silencing of *PSME4* (PA200) in human cells leads to the accumulation of mutant N-Htt aggregates. *In vitro* degradation analysis confirms the potent role of Blm10 in the proteasomal degradation of soluble N-Htt fragments. Our finding indicates that the Blm10/PA200 activator family promotes the degradation of N terminal Htt by Blm10/PA200-CP proteasomes.

In conclusion, our results highlight the value of enhancing the activity of the proteasomes by activators. This process would be a promising strategy to restore mitochondrial function and reduce the accumulation of toxic Htt species in HD neuronal cells.

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List of publications related to the dissertation

1. **Aladdin, A.**, Yao, Y., Yang, C., Kahlert, G., Ghani, M., Király, N., Boratkó, A., Uray, K., Dittmar, G., Tar, K.: The Proteasome Activators Blm10/PA200 Enhance the Proteasomal Degradation of N-Terminal Huntingtin.
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