Innovative approaches to monitor mutant huntingtin and to facilitate its degradation in Huntington's disease models

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TABLE OF ABBREVIATIONS

AD	Alzheimer's disease
AGERA	agarose gel electrophoresis for resolving aggregates
ALS	amyotrophic lateral sclerosis
BDNF	brain-derived neurotrophic factor
DMEM	Dublecco's modified eagle medium
DMSO	dimethylsulfoxide
DN	dominant negative
ES	embryonic stem
Ex1	exon 1 of huntingtin protein
FBS	fetal bovine serum
FRET	Förster resonance energy transfer
GABA	gamma-aminobutyric acid
GP	globus pallidus
HD	Huntington's disease
HDAC	histone deacetylase
HDF	hereditary disease foundation
HEAT	Huntingtin Elongation factor 3, the PR63/A subunit of protein phosphatase 2A and the lipide kinase Tor
*Hdh*Q150	KI full length huntingtin mouse model
HRS	heat shock response
Hsf1	heat shock factor 1
Hsp	heat shock protein
Htt	huntingtin protein
Htt573Q25	fragment of 573 aminoacids of huntingtin protein with 25 glutamines
Htt573Q72	fragment of 573 aminoacids of huntingtin protein with 72 glutamines
KI	knock in
mut Htt	mutant huntingtin
MRI	magnetic resonance imaging
MSNs	medium-sized projection spiny neurons

NES	nuclear export factor
NMDA	N-methyl D-aspartate
PBS	phosphate buffered saline
PGC-1α	peroxisome-proliferator-activated receptor γ coactivator-1α
PD	Parkinson's disease
polyQ	polyglutamine
PTM	post translational modifications
R6/2	transgenic exon1 mutant huntingtin mouse model
RSL	rheoswitch ligand
SBMA	spinal bulbar muscular atrophy
SCA	spinocerebellar atrophy
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tb	terbium
TF	transcription factor
TR-FRET	time resolved FRET
Tris	tris(hydroxymethyl)-aminomethan
UPS	ubiquitin-proteasome system

1 SUMMARY

Huntington's disease (HD) is a dominant genetic neurodegenerative disease caused by a mutation in the exon 1 of the huntingtin gene. The clinical symptoms, such as motor disturbances (chorea), cognitive decline and psychiatric impairments are usually developed by the patients in mid-life. Mutant huntingtin protein presents an amplification of a polyglutamine repeat at its N-terminus, which induces conformational changes and leads to neurotoxicity, impairment of cell homeostasis and neuronal cell death. The neuropathology of HD is characterized by a progressive degeneration of the brain starting from the striatum and spreading to other regions such as cortex, hypothalamus and cerebellum. In addition to the diffused brain atrophy, HD patients are also affected by multiple peripheral symptoms which contribute to worsening disease progression and eventually lead to death approximately two decades after onset.

The mechanisms leading to the toxicity induced by mutant huntingtin are not well understood. However the acquisition of a misfolded conformation and the formation of intracellular inclusions constituted by shorter fragments of the mutant protein are considered important in the neurodegenerative process.

In my thesis project I have investigated mechanisms to enhance the cellular degradation of mutant huntingtin. A second focus was on the development of an immunoassay to detect and quantify aggregates in HD models.

I analyzed the data obtained form a high through-put screen aimed to identify small molecular weight compounds decreasing mutant huntingtin levels in cells. Among all compounds screened, only inhibitors of heat shock protein 90 (Hsp90) showed a significant effect on mutant huntingtin clearance. I therefore investigated the mechanisms of Hsp90 chaperone inhibition and the reduction of soluble mutant huntigtin levels. Data from biochemical assays demonstrated that mutant huntingtin degradation is enhanced upon compound treatment and that the protein is cleared through the ubiquitin-proteasome system. This was independent from the heat shock response induced after pharmacological Hsp90 inhibition. Co-immunoprecipitation experiments suggested that mutant huntingtin is a client protein of Hsp90. The results were replicated in different cellular models including full length mutant huntingtin expressed from the endogenous locus, thus highlighting the importance of Hsp90 in stabilizing soluble mutant huntingtin and suggesting the possible application of Hsp90 inhibitors as therapies in HD.

In the second project I developed a sensitive method to detect mutant protein aggregates in HD models. To this purpose I implemented the already established time resolved fluorescence resonance energy transfer (TR-FRET) based immunoassay for the detection of soluble mutant and wild-type huntingtin. A mixture of either donor or acceptor fluorophore labeled single monoclonal antibody directed against an epitope exposed on the huntingtin aggregate surface was used. This strategy allowed for energy transfer and therefore a measurable TR-FRET signal, only in presence of mutant aggregated protein. I could demonstrate the sensitivity of the bioassay on a microtiter set up both as a single assay and in a duplex combination with the previously developed TR-FRET assay for soluble huntingtin.

I applied the TR-FRET for aggregated huntingtin to samples from R6/2 and *Hdh*Q150 mice, expressing exon 1 and full length mutant huntingtin, respectively. In brain homogenates from both models there was an age-dependent, inverse correlation between soluble and aggregated mutant huntingtin. These findings supported the importance of the relation between aggregated and soluble protein in disease progression. Furthermore, I detected the inverse correlation also in peripheral tissues of R6/2 mice where the presence of aggregates was previously demonstrated with other methods. An in-depth analysis of R6/2 samples in a combination of TR-FRET and size exclusion chromatography suggested a differential specificity of the two antibody combinations used for different aggregate populations. The TR-FRET method provides a new means to characterize the aggregation process as well as to test the efficacy of possible disease modifying treatments for HD.

2 INTRODUCTION

2.1 Huntintgton's disease

Huntington's disease (HD) is an autosomal-dominant neurodegenerative disease, with a prevalence of 5-12 people over 100'000 individuals (Spinney, 2010). Usually the symptoms appear around the 40th year of age and consist of motor dysfunction (chorea) as well as psychiatric disturbances and cognitive decline. Progressive neuronal cell death is observed primarily in the striatum and cortex of the patients, but in late-stages of the disorder it is extended to all brain areas (Henley et al., 2006, Vonsattel, 2008, Vonsattel et al., 2008).

HD is caused by the amplification of a polyglutamine (polyQ) repeat in the huntingtin protein (Htt). Above a threshold of 36-39 glutamines the disease develops with 100% penetrance. Mutant huntingtin (mut Htt) acquires a misfolded conformation gaining aberrant functions and its cleavage products assemble into intracellular insoluble aggregates. These phenomena are thought to be the cause of toxicity, homeostasis impairment and consequent neuronal cell death (Ross and Tabrizi, 2011).

The function of wild-type (wt) Htt and the pathomechanism of mut Htt are largely unknown. Current therapies are mainly directed at improving the symptoms, rather than targeting the cause of disease. Further insights in the molecular pathways involved are thus needed in order to develop new therapeutic strategies aimed to slow the progression or to delay disease onset.

2.1.1 Historical background

The term "chorea" derives from the Greek expression *choreia* (dance) and usually it is used to describe quick pathological movements of the limbs which can be compared to dancing or piano playing. The Swiss physician Paracelsus used for the first time this term in the 16th century, while describing the uncoordinated movements of some of his patients. Only later this phenomenon was shown to be one of the hallmarks of the disease. During the Salem Witch trials (17thcentury) women were persecuted because of odd behaviors and unconventional movements. Nowadays it is thought that some of them were indeed affected by HD. At the beginning of the 19th century multiple American and Norwegian physicians described a disease defined as "chronic hereditary chorea", which was inherited by affected parents and characterized by involuntary movements

and psychiatric disturbances (Waters, 1842, Dunglison, 1848, Lund, 1860). Nevertheless, the first official description of the disease was written in 1872 by the physician George Huntington in his report *On Chorea* (Figure 1). He examined several generations of a family with individuals presenting common symptoms, such as chorea, involuntary movements and psychiatric disturbances. Without knowing it, George Huntington described in detail what then will be defined, following the Mendelian theories, a disorder with autosomic dominant inheritance. In his report he highlighted three peculiar aspects of the disease: the hereditary nature, the tendency to insanity and suicide, and the manifestation of severe symptoms only in adult life. As a result of his accurate observations he provided a meticulous description of the motor symptoms characterizing the disease and their progression (Huntington, 1872).

Figure 1: George Huntington. *On Chorea* (1872). Online source from Columbia University libraries blog.

The first time the disease was addressed as "Huntington's Disease" was after the death of the folk singer Woody Guthrie in 1967, an event that brought to the initiation of the Committee to Combat Huntington's Disease (now the Huntington's disease Society of America). One year later, in 1968, after experiencing HD in his wife's family, Dr. Milton Wexler was encouraged to start the Hereditary Disease Foundation (HDF). Both these associations were aimed at increasing the awareness of HD and rising funding to support research to cure genetic illnesses. The HDF together with a group of researchers in 1983 identified the chromosomal localization of the mutation causing the disorder (Gusella et al., 1983) and a few years later characterized the gene encoding for the huntingtin protein, responsible for HD (The Huntington's Disease Collaborative Research Group, 1993). Since then research has achieved relevant progresses towards the understanding of HD pathogenesis. Multiple animal models mimicking the disorder have been developed (Mangiarini et al., 1996, Hodgson et al., 1999, Wheeler et al., 1999, Lin et al., 2001, Gray et al., 2008) and multiple molecular pathways and aggregation dynamics have been investigated. In spite of all these efforts the pathogenic mechanism of action of mut Htt has not yet been unveiled and all the therapies currently available are ineffective in modulate disease progression (Ross and Tabrizi, 2011).

2.1.2 Clinical features of Huntington's disease

Huntington's disease is caused by a single mutation leading to the amplification of a CAG repeat in the gene encoding for the Htt protein. The motor and cognitive symptoms observed in HD can be confused at early stages with other neurodegenerative disorders, like Huntington's Disease-like 2 (Walker et al., 2003, Margolis et al., 2004, Greenstein et al., 2007, Rudnicki et al., 2008) or dentatorubropallidoluysian atrophy (Nakano et al., 1985, Ross et al., 1997).

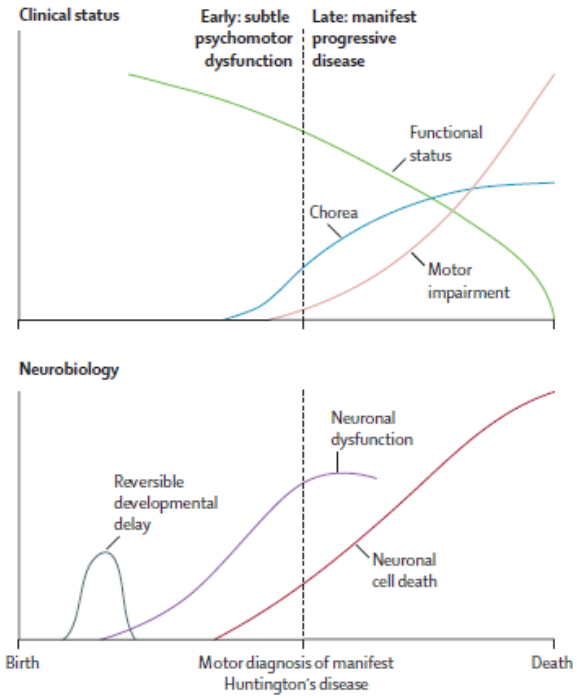

Figure 2. Clinical status and neurobiology of Huntington's Disease progression.
First signs and psychomotor symptoms of Huntington disease begin years before the motor symptoms diagnosis. These consist mainly of behavioral and cognitive changes which correlate with first neurobiological changes. In the early stages of the disease neuronal dysfunction has an important role, however in the progression of the disease neuronal cell death is the predominant feature. Adapted from *Ross and Tabrizi, 2011*

Mut Htt is expressed ubiquitously and throughout the whole life of the patients, however the disease becomes manifest with its first symptoms only in mid-life (Ross and Tabrizi, 2011). The first motor disturbances are mild, as slight uncontrolled involuntary movements normally occurring in the distal extremities. Clumsiness and difficulties in smooth eye movements are also observed (Brandt et al., 1984, Penney et al., 1990, Roos, 2010). The motor symptoms progress slowly, spreading from distal to more proximal and axial, leading to characteristic extra pyramidal disturbances such as walking and movement impairment. Involuntary movements and chorea (rapid, uncontrollable movements) are present all the time the patient is awake. Patients also develop dystonia (sustained muscle contractions causing twisting and repetitive movements or abnormal postures), bradykinesia (slowness of movements) and akinesia (difficulty in starting movements) (Rosenblatt et al., 2003).

Psychiatric, cognitive and behavioral disturbances are present since the early stages of the disease, often before the motor signs become obvious (Figure 2). Psychiatric symptoms include depression, anxiety, low self esteem, increased passive behavior and, in later stages, psychosis and schizophrenia. The patients manifest progressive disturbances in cognitive executive

functions such as planning and organizing simple daily events, as much as language and memory impairment (Marder et al., 2000, Bates, 2002).

Juvenile HD cases express polyQ stretches exceeding 60 repeats and this correlates with an early appearance of the first symptoms, often before the 20th year of age (Perutz and Windle, 2001, Walker, 2007) (see chapter 2.1.4). Young HD patients manifest disturbances which significantly differ from the adult pathology such as learning deficits, bradykinesia, hypokinesia, rigidity and dystonia, without appearance of chorea. Epileptic events are also frequently reported (Quarrell OWJ, 2009).

Beside the neurological features, HD patients develop a variety of peripheral symptoms, such as muscle atrophy, weight loss despite constant caloric intake and metabolic abnormalities (Kirkwood et al., 2001). Interestingly, recent studies showed that weight loss and metabolic dysfunction correlate with CAG repeat length and disease progression (Mochel et al., 2007, Aziz et al., 2008). The general endocrine imbalance is attributed to a dysfunction in the hypothalamic-pituitary-adrenal axis, leading to progressive increase of the levels of corticosteroids (Heuser et al., 1991, Leblhuber et al., 1995, Bjorkqvist et al., 2006, Aziz et al., 2007), reduced levels of testosterone (Markianos et al., 2005) and higher tendency to develop diabetes mellitus (Farrer, 1985). Sleep and circadian rhythm disturbances have been lately described, indicating a possible REM phase reduction occurring before the onset of choreic movements (Arnulf et al., 2008).

HD patients progressively loose the ability to sustain themselves and develop behavioral changes, thus becoming more and more dependent on their families and clinicians for their daily care (Nance, 2007). Death normally occurs 15-20 years after age-of-onset, mainly caused by infections, heart failure, pneumonia and suicide (Chiu and Alexander, 1982, Nance and Sanders, 1996, Roos, 2010).

2.1.3 Neuropathology of Huntington's disease

The pathological hallmark of HD is the progressive atrophy of the brain, leading to a global loss of more than 40% of its mass over disease progression (Gusella, 2001, Gil and Rego, 2008). The shrunken appearance of the brain involves in first place the striatum but is then extended to other regions as cortex (especially layers V and VI), brain stem, spinal cord, hippocampus, cerebellum and thalamus (Marsh et al., 2003, Hedreen et al., 1991, Halliday et al., 1998, Li and Li, 2004, Vonsattel et al., 2008) (Figure 3). Recent studies have shown also a degeneration of the

hypothalamus, consistent with progressive loss of somatostatin-positive neurons and orexin-secreting neurons (Kremer et al., 1990, Kremer et al., 1991, Petersen et al., 2005). Histology studies performed on postmortem brains allowed to establish the first classification of the pathology using a system divided in 5 grades. This classification correlates both with severity of the neuropathology (cerebral atrophy) and clinical progression; with grade 0 identifying a brain with no discernible atrophy and grade 4 identifying the widespread pathology (Vonsattel et al., 1985) (Figure 3). Recent studies have been investigating the development of the disease by using magnetic resonance imaging (MRI), aiming to identify cortical abnormalities also in presymptomatic patients (Rosas et al., 2003, Kassubek et al., 2004, Rosas et al., 2006). Longitudinal studies, such as PREDICT HD and TRACK HD, using in parallel MRI and analysis of behavioral and cognitive parameters, tried to identify early changes and possible early-stage biomarkers (Paulsen et al., 2006, Tabrizi et al., 2009). The selective degeneration of the striatum, presenting massive neuronal loss and astrogliosis sets the base for the uncontrolled and involuntary movements, impulsive behavior and diminished cognitive performances of HD patients (Crossman, 1987, Reiner et al., 1988, Albin et al., 1989, Montoya et al., 2006). Among the different neuronal populations in the striatum the most affected neurons are the medium-sized projection spiny neurons (MSNs), while large and medium-sized aspiny interneurons are relatively spared (Ferrante et al., 1985, Graveland et al., 1985, Vonsattel et al., 1985, Ferrante et al., 1986).

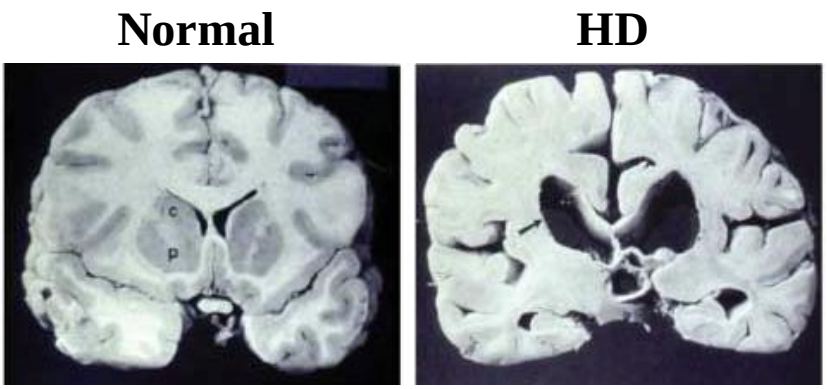

Figure 3: Brain of a Huntington's Disease patient in late stage compared to a healthy individual. A general atrophy of the brain is observed in Huntington's Disease patients. The degeneration affects primarily the striatum but then spreads to cortex and all brain regions. Adapted from *Marsh et al., 2003*

The striatum, which comprises the caudate nucleus and putamen, is one of the nuclei constituting the basal ganglia, together with the substantia nigra (pars compacta and reticulata), the globus pallidus and the subthalamic nucleus. The basal ganglia are deep brain nuclei implicated in multiple crucial functions, but with a major role in the regulation of normal voluntary movements (Gerfen CR, 1996). They do not communicate directly with the spinal cord but receive the

primary input from the cortex, and send their output back to the prefrontal, premotor and motor cortex, via the connection with the thalamus (Andre et al., 2010, Chakravarthy et al., 2010).

The MSNs constitute nearly 95% of the neurons in the striatum. They are inhibitory neurons releasing the neurotransmitter gamma-aminobutyric acid (GABA), and are responsible of carrying the output from the striatum to the globus pallidus (GP). MSNs can be divided into two populations on the basis of their axonal projections, neuropeptide release and expression of dopamine receptors (Gerfen et al., 1990, Gerfen, 1992, Surmeier et al., 2007, Andre et al., 2010), thus constituting two different pathways to their target nuclei.

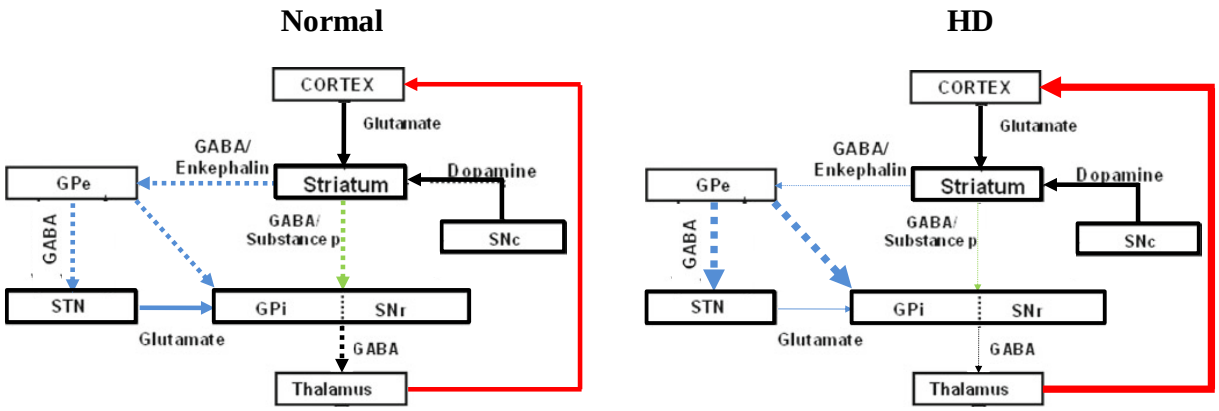

Figure 4. Basal ganglia function in intact brain and during HD progression. Plain and dotted lines represent excitatory and inhibitory pathways respectively. A: Simplified representation showing normal basal ganglia circuitry. In healthy individuals the thalamus is regulated by the direct (green) and indirect (blue) pathways, both acting on the GPi/SNr with opposite effects, thus resulting in a controlled balance of initiations and regulation of voluntary movements. B: HD basal ganglia. The indirect pathway is primarily affected in the disease progression resulting in a stronger inhibition of the GPi and consequent disinhibition of the thalamus. This event causes abnormal movements (chorea) and hyperkinesia. The progressive degeneration of MSNs belonging to both direct and indirect pathways results in both the uncontrolled movements and in the appearance of hypokinesia, probably due to the lack of function of the direct pathway on the GPi. GPe: external segment of the globus pallidus; GPi: internal segment of the globus pallidus; SNc: substantia nigra pars compacta; SNr: substantia nigra pars reticulata; STN: subthalamic nucleus. Modified from *Andre' et al., 2011, Chakravarthy et al., 2010*.

MSNs projecting from the striatum to the external segment of the globus pallidus (GPe) constitute the indirect pathway, express D2 Dopamine receptors and release the neuropeptide Enkephalin (Figure 4, blue lines). MSNs projecting from the striatum to the internal segment of the Globus Pallidus (GPi) and to the substantia nigra pars reticulata, constitute instead the direct pathway, express D1 Dopamine receptors and release Substance P (Figure 4, green line) (Joel, 2001, Gertler et al., 2008). The inhibitory effect generated on the GPi by the direct pathway produces a disinhibition of the thalamus and the consequent initiation of voluntary movements. On the contrary, the indirect pathway projects to the GPe, which connects to the subthalamic

nucleus and the GPi. This pathway results in an inhibition of the thalamic function, thus resulting in a regulation of the voluntary movement (Contreras-Vidal and Stelmach, 1996). Interestingly the two pathways show a differential susceptibility to the neurodegenerative process in HD. The indirect pathway is particularly vulnerable to the cell loss from the very early stages of the disease (Reiner et al., 1988, Albin et al., 1992, Richfield et al., 1995, Deng et al., 2004) leading to the hypothesis that the choreic movements are caused by the loss of inhibitory effect on the thalamus. On the other hand at late stages the neurons of the direct pathway also start to degenerate consequently inducing the appearance of akinesia and dystonia in HD patients (Albin et al., 1990) (Figure 4). The higher susceptibility of MSNs to the HD neurodegenerative process, compared to the relative sparing of medium sized interneurons and cholinergic interneurons, seems to be related also to higher mut Htt expression in MSNs (Ferrante et al., 1997).

2.1.4 The genetics of Huntington's disease and intergenerational anticipation

HD is caused by the amplification of a CAG trinucleotide repeat at the 5' end of the exon 1 (Ex1) of the Huntingtin gene, IT15, which is located on the short arm of the chromosome 4p16.3 (The Huntington's Disease Collaborative Research Group, 1993). The disease is transmitted with autosomal dominant inheritance, following the classical mendelian fashion. Normal individuals have a number of CAG lower than 36, most commonly between 17 and 20 (Imarisio et al., 2008), while patients with over 39 CAG develop the disease with 100% penetrance (Gusella and MacDonald, 2006, Sturrock and Leavitt, 2010, Finkbeiner, 2011). Individuals with a number of glutamines between 36 and 39 show incomplete penetrance and may not develop symptoms or only in late age (Figure 5). Patients homozygous for mut Htt develop symptoms at the same age as heterozygous, although it has been reported that they experience a more severe clinical course (Squitieri et al., 2003).

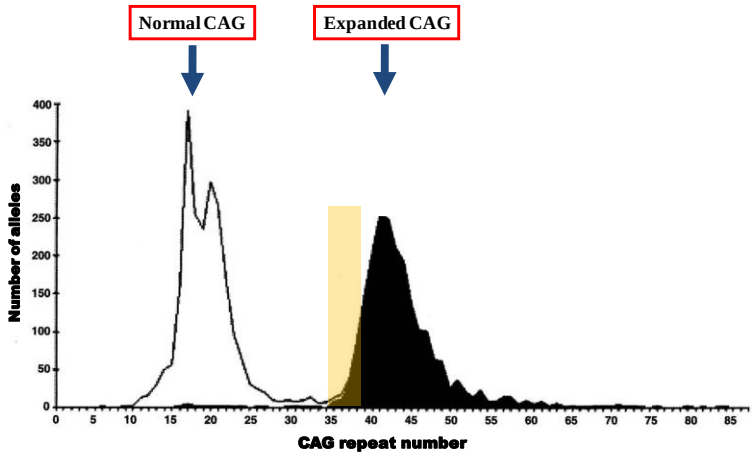

Figure 5. Distribution of normal and expanded CAG allele size in Huntington disease. A clear distinction between the normal individuals and the affected ones can be emphasized by looking at the alleles distribution. Two distinct populations can be identified with normal alleles from controls, unaffected or lower alleles of affected individuals shown in white and expanded CAG alleles depicted in black. The yellow rectangle represents the alleles with 36-39 CAG repeats which lead to disease development with incomplete penetrance. Adapted from *Sturrock and Leavitt, 2010*.

The length of the polyQ stretch correlates with the onset of the disease in 50-70% of the cases (Gusella and MacDonald, 2006, Gusella and MacDonald, 2009, Finkbeiner, 2011), although this correlation is stronger in the presence of longer CAG repeats rather than with shorter polyQ stretches (Myers, 2004) (Figure 6). Besides polyQ length, the onset of the disease seems to be influenced also by modifying genes and environment (Rubinsztein et al., 1996, McNeil et al., 1997, Wexler et al., 2004, Li et al., 2006a, Quarrell et al., 2007). Progression and gravity of the symptoms seem not to be affected by the length of the CAG repeats (Squitieri et al., 2002, Rosenblatt et al., 2006).

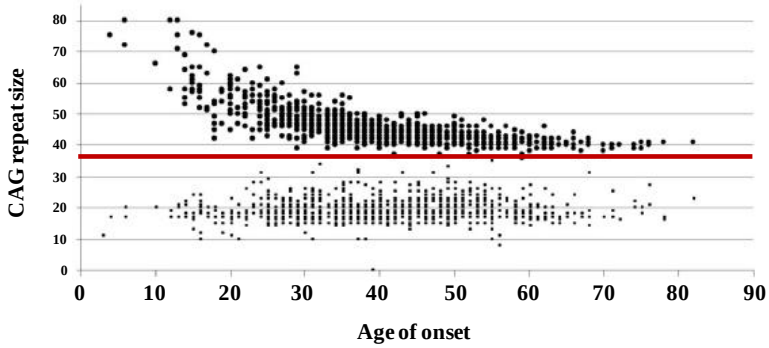

Figure 6. Correlation between age of onset and CAG repeats. There is an inverse correlation between the size of the CAG repeat (y-axis) and the age of onset (x-axis). Among the population with large repeat number the correlation is strong, while it becomes weaker in presence of shorter CAG repeat (55 or lower). The red line represents the threshold between healthy population and HD patients. Adapted from *Myers, 2004*.

Patients with adult onset of the disease show a number of CAG around 40, whereas juvenile cases present with triplet repeats over 60 (Gusella and MacDonald, 2006). CAG triplets between 27 and 35 normally do not lead to HD development however, due to the meiotical instability of the CAG repeats, they can in some cases undergo genetic amplification and reach the pathogenic threshold in the following generation, causing *de novo* cases of HD (Myers, 2004, Semaka et al., 2006). The even higher instability of longer CAG repeats leads to intergenerational amplification and consequent earlier onset of the disease, phenomenon defined as "genetic anticipation" (Ridley et al., 1988, Ridley et al., 1991, Ranen et al., 1995). This event was first studied in myotonic dystrophy, a disorder caused by instable triplet repeats and then extended to other trinucleotide repeat diseases (Howeler et al., 1989). The meiotic instability of polyQ stretches and consequent amplification is more pronounced during male gametogenesis, thus leading to higher paternal transmission of longer CAG repeats (Kremer et al., 1995). Individuals with long CAG stretches present higher rate of amplification, confirming the size-dependent instability of the repeats (Harley et al., 1992, Lavedan et al., 1993, Trottier et al., 1994, Ranen et al., 1995, Bates, 2002). The molecular mechanisms underlying CAG expansion and amplification are not completely clarified. Molecular studies have confirmed a higher instability of the CAG repeats in sperm, which could explain the gender differences in the amplification (Leeflang et al., 1995, Telenius et al., 1995). However, other studies also raised the hypothesis that somatic mosaicism could play a role in modulating the onset of the disease (Swami et al., 2009). Expansions, but to a smaller degree also contractions, are typical events in trinucleotide repeat sequences (Wheeler et al., 2007). One explanation for their occurrence is the formation of single stranded DNA hairpins and unusual structures during DNA replication, recombination events and DNA repair mechanisms (Maurer et al., 1996, Mirkin, 2007). However these events can account mainly for germline and some somatic instability, as they mainly occur in dividing cells, as reported for HD lymphoblasts (Cannella et al., 2009). Interestingly, higher CAG expansions have been observed in striatum and cortex, thus suggesting triplet instability in post mitotic neuronal cells and a correlation with HD neurophatology (Telenius et al., 1994, Shelbourne et al., 1999, Kennedy et al., 2003, Gonitel et al., 2008).

Besides CAG repeat length, environmental factors and other genetic modifiers could also influence HD onset and progression (Wexler et al., 2004, Gayan et al., 2008). One possible modifier has been located in the chromosome 4p16, close to the huntingtin gene itself (Djousse et al., 2004, Norremolle et al., 2009), whereas other genetic modulators of HD development are

TP53, human caspase activated DNase (hCAD) (Chattopadhyay et al., 2005), ASK1 and MAP2K6 (Arning et al., 2008), different subunits of the NMDA receptor, NR2A and NR2B (Arning et al., 2005), PGC-1a polymorphisms and genes downstream of its cascade (Che et al., 2011, Taherzadeh-Fard et al., 2011), as well as HAP-1 (Metzger et al., 2008).
Although these and other modifiers seem to modulate HD onset, no functional studies have yet proved a direct relationship, thus asserting the need for further investigation.

2.1.5 Therapies for Huntington's disease

The treatments currently used in the medical practice are solely aimed to alleviate the motor and psychiatric symptoms occurring in HD, without significantly affecting disease progression (Ross and Tabrizi, 2011). Three strategies have been mainly followed to developed therapeutic approaches: improvement of the motor symptoms, improvement of psychiatric symptoms and enhancement of neuroprotection (Bonelli and Hofmann, 2004, Bonelli and Hofmann, 2007).
Choreic movements, as well as other motor dysfunctions such as dystonia, rigidity and akinesia are targeted. Treatment with Tetrabenazine, a conventional antipsychotic which depletes monoamine uptake, brought improvement of symptom severity but with the appearance of several side effects such as depression, sedation, accelerated functional decline and dystonia (Ondo et al., 2002, Leavitt and Hayden, 2006). Also Haloperidol and several atypical neuroleptics showed promising improvements in the motor symptoms but were accompained by multiple side effects (Barr et al., 1988, Gimenez-Roldan and Mateo, 1989). NMDA antagonists, as Amantadine, Ketamine and Riluzole, have been tested following the theory of neuronal exitotoxicity caused by enhanced release of excitatory neurotransmitters proposed by DiFiglia and colleagues (DiFiglia, 1990). However in spite of improvement on choreic impairment and behavioral symptoms in some trials, several contradictory studies have been reported and side effects have been observed (Mestre et al., 2009, Bonelli and Hofmann, 2007). Important to notice is that not all the trials followed the Unified HD Rating Scale to rate the symptoms (Huntington Study Group, 1996), thus resulting in high inconsistencies and difficulties in their interpretation.
Depression in HD patients correlates with lower metabolic activity in the basal ganglia and in the cingulate cortex (Mayberg et al., 1992). The administration of antidepressant agents such as fluoxetine and clozapine produced potential beneficial effects in some trials (De Marchi et al.,

2001, Bonuccelli et al., 1994, Colosimo et al., 1995), while antidepressants and neuroleptics had only limited beneficial effects (Bonelli and Hofmann, 2007).

Neuroprotection strategies have been also evaluated, aimed to decrease neuronal susceptibility to mut Htt toxicity. Several studies explored the possibility to modulate glutamate transmission, enhancing bioenergetic mechanisms or exerting antioxidant properties. However, the lack of reliable markers to evaluate treatment efficacy results in difficult interpretation (Bonelli and Hofmann, 2007). Trials with Creatine, aimed to potentiate the depleted oxidative mitochondrial functions, showed changes in brain metabolites (Bender et al., 2005) and increase in creatine concentrations, combined with a decrease in 8OH2'dG, a marker of DNA oxidation injury (Hersch et al., 2006). However nor cognitive improvement in HD patients were observed (Verbessem et al., 2003). The administration of coenzyme Q10, a component of the mitochondrial electron transport chain (Crane et al., 1989), showed only a weak trend of improvement (Koroshetz et al., 1997, Huntington Study Group, 2001) without heavy side effects. Promising results of a combinatorial therapy of creatine and coenzyme Q10 in models of PD and HD (Yang et al., 2009, Menalled et al., 2010) supported further studies in this direction.

Other approaches were aimed to decrease the apoptotic rate and cell death. In this context, the enhancement of highly unsaturated fatty acid concentrations in the cell membranes produced contrasting results, showing only weak motor symptoms improvements and atrophy reduction (Rosser, 2002, Puri et al., 2005, Puri et al., 2008, Huntington Study Group, 2008). On the other hand the caspase and neuronal apoptosis inhibitor Minocycline produced improvement in motor and psychiatric symptoms, however rising concerns on its safety (Reynolds, 2007, Bonelli et al., 2003, Bonelli et al., 2004).

Taken together, the inconsistence among the trials and the lack of proofs for drugs efficacy underline the need for further investigation and design of more specific therapies.

2.2 Huntingtin protein

2.2.1 Wild-type huntingtin

Huntingtin is a protein of 3144 amino acids, ubiquitously expressed, and containing at its N-terminus a polyQ repeat followed by a polyproline domain. It has very limited sequence homology with other proteins and its biological functions are only poorly understood. It has an essential role in development, as embryonic lethality has been observed in gene knock out models (Duyao et al., 1995, Nasir et al., 1995, Zeitlin et al., 1995). Interestingly the protein affects adult life as well, since mice with conditional Htt knock down in testis and brain develop neurodegeneration and sterility (Dragatsis et al., 2000). On the contrary, the presence of the mut Htt seems not to abolish essential functions of the wt protein considering that patients undergo normal development and that HD symptoms appear only in late stages of life.

Htt is mainly localized in the cytoplasm with only a small portion in the intranuclear compartment (Hoogeveen et al., 1993, Kegel et al., 2002). Interaction with mitochondria (Rockabrand et al., 2007), endoplasmic reticulum (ER) as well as late endosomes and autophagic vesicles was described (Atwal et al., 2007, Atwal and Truant, 2008). Htt is also thought to play a role in regulating vesicle trafficking to the extracellular matrix via its association to the Golgi apparatus (Strehlow et al., 2007), microtubules and synaptic vesicles (Velier et al., 1998, Gutekunst et al., 1999). Interestingly in patients and cell models of HD the wt Htt localization seems to be influenced by the presence of mut protein (De Rooij et al., 1996, Sapp et al., 1997).

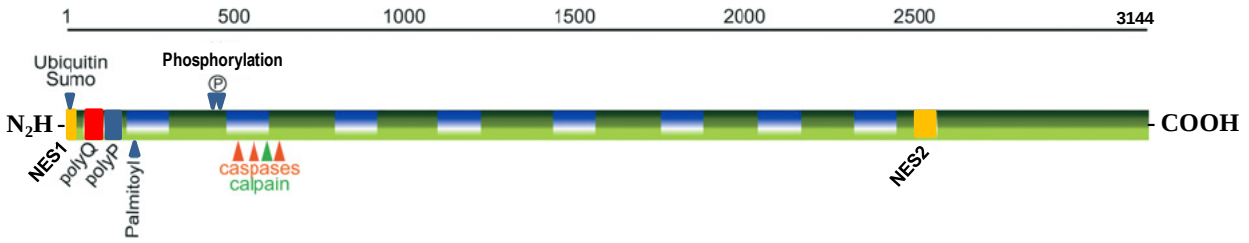

Figure 7. Huntingtin protein. Schematic representation of the structure of the huntingtin protein (3144aa). The localizations of a putative nuclear export sequence (NES1), the polyglutamine region (PolyQ) and the polyproline region (PolyP) are indicated. The blue bars correspond to the HEAT motifs, which are thought to be responsible for protein-protein interactions. Towards the C-terminus there is symbolized a highly conserved Nuclear export signal (NES2). The huntingtin protein undergoes multiple post translational modifications in the aminoterminal region, such as ubiquitination, sumoylation, phosphorylation and palmitoylation (blue arrows) thought to modulate mutant huntingtin toxicity. Multiple proteases cleave the huntingtin protein generating shorter fragments (red and green arrows), which, in the presence of polyglutamine expansions, tend to form intracellular inclusions. Adapted from *Imarisio et al., 2008*.

28-36 HEAT (huntingtin elongation factor 3, the PR63/A subunit of protein phosphatase 2A and the lipide kinase Tor) repeat motifs spread throughout the whole protein length (Andrade and Bork, 1995, Takano and Gusella, 2002). They consist of ~50 amino acids assembled in two anti-parallel α-helices forming a helical hairpin (Li et al., 2006b). They mediate protein-protein interactions important for cytoplasmic-nuclear transport, microtubule dynamics and chromosome segregation (Neuwald and Hirano, 2000) (Figure 7).

Htt contains a highly conserved nuclear export signal (NES) at the C-terminus (Xia et al., 2003) and a putative export sequence in the first 17 amino acids, whose association with the nuclear exporter Trp seems impaired by the presence of mut protein (Cornett et al., 2005). On the contrary Htt does not present a classical importin β-dependent sequence which could explain the nuclear localization of the protein. This suggests a possible interaction with transcription factors mediating its translocation to the nuclear compartment (Truant et al., 2007).

Multiple post translational modifications (PTM) contribute to modulate Htt functions and occur mainly in the first 17 amino acids preceding the polyQ stretch. In this region were identified SUMOylation (Steffan et al., 2004, Truant et al., 2007), ubiquitination (Kalchman et al., 1996) and phosphorylation (Thompson et al., 2009, Warby et al., 2009). Other PTM, which are localized also in more C-terminal regions, are phosphorylation (Humbert et al., 2002, Rangone et al., 2005, Schilling et al., 2006, Colin et al., 2008, Warby et al., 2009), acetylation (Jeong et al., 2009, Cong et al., 2011), and palmitoylation (Yanai et al., 2006, Ohyama et al., 2007, Goytain et al., 2008). The presence of mut Htt could contribute to the modulation of some of the described PTMs, thus influencing the localization, molecular interactions and toxic potential (Luo et al., 2005, Anne et al., 2007, Reijonen et al., 2008, Zala et al., 2008, Jeong et al., 2009).

Both wt and mut Htt are targets for multiple intracellular proteases, including caspase 1,3,6,7 and 8 (Goldberg et al., 1996, Kim et al., 2001, Sun et al., 2002, Graham et al., 2006), calpain (Gafni and Ellerby, 2002, Landles et al., 2010) and some other cellular proteases (Kim et al., 2006, Graham et al., 2011) which cleave the protein and produce fragments of different lengths. The role of shorter fragments is still unknown however, mut Htt fragments assemble in oligomers and aggregates which could play an important role during HD development (Chapter 2.2.4).

The cellular functions of wt Htt are not well understood. Some studies attributed a possible anti-apoptotic role of the protein through the inhibition of caspase 3, caspase 8 and caspase 9 cleavage cascades (Rigamonti et al., 2000, Rigamonti et al., 2001, Gervais et al., 2002, Leavitt et al., 2006, Zhang et al., 2006).

A role of Htt in regulating trafficking and production of brain-derived neurotrophic factor (BDNF), a regulator of striatal cells survival (Nakao et al., 1995, Alcantara et al., 1997), has been proposed. Wt Htt interacts and sequesters in the cytoplasm the transcriptional factor complex repressor-element 1 transcription factor/ neuron-restrictive silencer factor (REST/ NRSF) thus blocking the repressive function of neuron-restrictive silencer element (NRSE) and promoting BDNF transcription (Zuccato et al., 2003). It also regulates BDNF vesicular transport associating with HAP1 (Htt associated protein 1) and the p150 subunit of dynactin (Gauthier et al., 2004, Wu et al., 2010, Yang et al., 2011). Mut Htt impairs these functions thus resulting in lower levels and availability of BDNF in striatal cells (Zuccato et al., 2001, Zuccato et al., 2005) (Chapter 2.2.3).
Htt is involved in the regulation of endocytic and vesicular transport through the association with Huntingtin interacting protein-1 (HIP1) (Kalchman et al., 1997, Legendre-Guillemin et al., 2005) and 14 (HIP14) (Singaraja et al., 2002, Yanai et al., 2006, Huang et al., 2011). Furthermore its interaction with PSD95 (postsynaptic density 95) and the modulation of HIP1 activity influences postsynaptic signaling and suggest a role in the mechanism of NMDA-mediated excitotoxicity in HD (Sun et al., 2001, Fan and Raymond, 2007, Metzler et al., 2007).

2.2.2 Gain or loss of function?

It is still under debate if HD is caused by a loss of function of the wt or a gain of function of mut Htt. Studies on HD lymphoblast showed that mut Htt homozygous condition leads to higher fragments accumulation and more severe mitochondrial impairment than the heterozygous (Maglione et al., 2006, Squitieri et al., 2006), while in Drosophila, depletion of wt Htt accelerates the neurodegeneration caused by mut Htt (Zhang et al., 2009), thus speaking in favor of the loss of function of the wt protein. In contrast, *in vivo* studies reported that the heterozygous inactivation of the wt Htt did not produce any disease phenotype (Duyao et al., 1995), while mouse models expressing mut Htt in addition to normal levels of the wt, develop the disease with obvious neurodegenerative phenotypes (Mangiarini et al., 1996, Reddy et al., 1998, Hodgson et al., 1999, Lin et al., 2001, Shehadeh et al., 2006). The dependence of the manifestation of HD phenotypes on the presence of mut Htt was also confirmed in lentiviral rat models of the disease (de Almeida et al., 2002, Regulier et al., 2003). Mouse embryonic stem (ES) cells expressing Htt with different polyQ length showed HD like gene profiles if compared with the knock out line, thus favoring the hypothesis of a gain of function of the mut Htt (Jacobsen et al., 2011). Furthermore down-modulation of both wt and mutant Htt by RNA interference ameliorated

disease phenotypes *in vivo* (Harper et al., 2005, Rodriguez-Lebron et al., 2005, Machida et al., 2006, Franich et al., 2008). Overall these results seem to support mainly the conclusion that the cell impairment occurring in HD is linked to a gain of function of the mut Htt, although the loss of function hypothesis could not be completely excluded, thus suggesting a possible contribution of both events in HD pathology.

2.2.3 Possible mechanisms of mutant huntingtin toxicity

The amplification of the polyQ tract in the mut Htt protein triggers multiple intracellular events which impair the cell homeostasis, enhance toxicity and lead to neuronal cell death. The cause of mut Htt toxicity is still unknown but several lines of evidence support a contribution of multiple mechanisms rather than a unique toxic event (Figure 8).

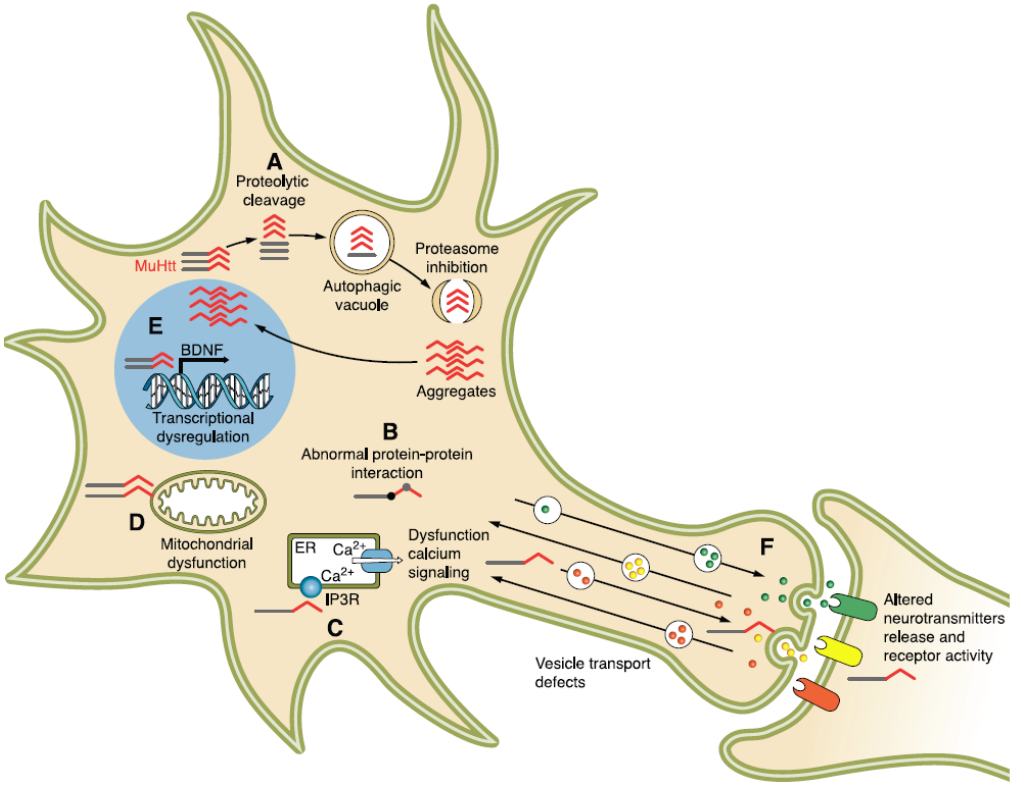

Figure 8. Pathogenic mechanisms in Huntington's disease. Multiple cellular pathways have been implicated in the pathogenesis of HD. A: mut Htt acquires a conformational change that leads to abnormal folding of the protein, inducing molecular chaperones intervention. Full-length mut Htt is cleaved by proteases in the cytoplasm and its fragments are partially ubiquitinated and targeted to the proteasome for degradation, probably impairing the clearance pathways. B/C/D: mut Htt fragments accumulate in the cell cytoplasm and unusually associate with several proteins causing impairment of calcium signaling and homeostasis and mitochondrial dysfunction. E: mut Htt translocates to the nucleus impairing gene transcription and forming intranuclear inclusions. F: the mutation in huntingtin alters vesicular transport and recycling. Adapted from *Zuccato et al., 2010*.

Mutant Htt establishes aberrant associations with several proteins and transcription factors (TF), modulating their activity and leading to transcriptional dysregulation (Cha, 2000, Gomez et al., 2006, Thomas, 2006, Kazantsev and Hersch, 2007, Zuccato et al., 2010) (Figure 8B, E). Aberrant gene expression in HD has been reported in several multiple microarray studies (Borovecki et al., 2005, Hodges et al., 2006, Anderson et al., 2008), mouse models (Luthi-Carter et al., 2002a, Luthi-Carter et al., 2002b) and primary neuronal cultures (Runne et al., 2008), suggesting that transcriptional dysregulation contributes to disease development and proposing potential biomarkers (Borovecki et al., 2005, Runne et al., 2007, Lovrecic et al., 2009).

The association of mut Htt expanded polyQ region with transcriptional regulators containing polyQ-rich sequences can impair gene transcription (Gerber et al., 1994, Perutz et al., 1994). Mut Htt can also associate with CREB-binding protein (CBP) repressing its activity and related transcription (Steffan et al., 2000, Cong et al., 2005). This event alters histone acetylation levels and increases cell toxicity in HD cell and mouse models (Jiang et al., 2006; Klevytska et al., 2010). The connection between mut Htt and histone acetylation suggested histone deacetylase (HDAC) inhibitors as possible therapeutic agents. HDAC inhibition slowed the neurodegenerative process and improved the phenotypes in Drosophila (Steffan et al., 2001, Pallos et al., 2008) and in HD mouse models (Ferrante et al., 2003, Gardian et al., 2005, Thomas et al., 2008). The TF Sp1 has been shown to associate with mut Htt and consequently impair (Li et al., 2002) transcription and activity of members of the transcriptional machinery such as TFIID and TFII130 (Chen-Plotkin et al., 2006, Qiu et al., 2006). The normal interaction with REST/NRSF previously described (Chapter 2.2.1) (Zuccato et al., 2003), is impaired in the presence of mut Htt hence allowing the translocation of the repressor into the nucleus, and decreasing BDNF transcription and transport (Zuccato et al., 2007, Zuccato and Cattaneo, 2007) (Figure 8B).

A role of mut Htt in mitochondrial homeostasis has been proposed (Figure 8D). The CREB-dependent peroxisome-proliferator-activated receptor γ coactivator-1α (PGC-1α), which is important in mitochondrial biogenesis and respiration (Herzig et al., 2001, Puigserver and Spiegelman, 2003), was downregulated in HD mouse models (Cui et al., 2006) and its activity was impaired (Weydt et al., 2006, Cui et al., 2006). Mitochondrial dysfunction seems to play be an important event in neurodegeneration, since neuronal cells require high energy production to support their activities (Beal, 2007, Kann and Kovacs, 2007). During their metabolic functions mitochondria produce reactive oxygen species (ROS) but at the same time are subjected to ROS

damage itself. In neurodegenerative diseases excessive production of ROS and deficits in the antioxidative system have been observed (Facecchia et al., 2011). In HD, reduced glucose uptake in the cortex and striatum (Antonini et al., 1996, Ciarmiello et al., 2006), decreased aconitase activity (Tabrizi et al., 1999, Tabrizi et al., 2000, Kim et al., 2005) and decreased activity of the complexes II, III and IV (Orr et al., 2008, Shirendeb et al., 2011) were described. Additionally, in different models of HD mut Htt has been shown to interact with p53, an important pro-apoptotic factor (Steffan et al., 2000, Ryan et al., 2006), enhancing its activity and leading to mitochondrial dysfunction and apoptosis (Bae et al., 2005, Zhang et al., 2009) (Figure 8D).

The misfolded conformation of mut Htt and its aggregated form induce an impairment of ubiquitin-proteasome system (UPS) mediated protein degradation, thus promoting toxicity and accumulation of non-degraded proteins (Jana et al., 2001) (Figure 8A). The UPS does not efficiently degrade misfolded mut Htt (Holmberg et al., 2004, Venkatraman et al., 2004) probably due to its inability to cleave between polyQ residues, resulting both in a physical blockage for other substrates to reach the 20S catalytic core and in an accumulation of mut Htt fragments (Waelter et al., 2001). UPS impairment has been demonstrated in a HD striatal cell line as well as in a mouse model (Hunter et al., 2007, Wang et al., 2008b). Mut Htt aggregates seem to influence the proteasome activity either by direct interaction (Martin-Aparicio et al., 2001), or by sequestering proteasome subunits, ubiquitin residues and heat shock proteins (Hsp), such as Hsp70 and Hsp40 (Wyttenbach et al., 2000, Bence et al., 2001, Holmberg et al., 2004). In contrast there has been evidence of proteasome impairment and UPS deficit also prior to aggregates formation (Bennett et al., 2005, Bennett et al., 2007). Furthermore, some studies observed enhancement of proteasome activity (Diaz-Hernandez et al., 2003) and enzymatic changes which do not always correlate with an impairment of the UPS (Bett et al., 2006), suggesting that further investigation in the field is required. Mut Htt also impairs calcium signaling and homeostasis, as well as neurotransmitter transport and recycling (Zuccato et al., 2010) (Figure 8C/F).

2.2.4 Mutant huntingtin aggregation

Full length mut Htt is cleaved by caspases, calpains and proteases into shorter fragments which aggregate and potentially contribute to HD neurophathology (Gafni and Ellerby, 2002, Wellington et al., 2002, Ratovitski et al., 2009, Landles et al., 2010).

Mutant protein aggregation is a common feature among neurodegenerative diseases. However the role of inclusions in development and progression of the disorders is still controversial, as they can be considered either a cellular defense mechanism against mutant proteins or toxic species themselves. Intranuclear inclusions were detected in the cortex and striatum of juvenile and adult HD patients (DiFiglia et al., 1997, Hoffner et al., 2007) (Figure 9), in cell culture (Cooper et al., 1998) and *in vitro* (Lunkes et al., 1999).

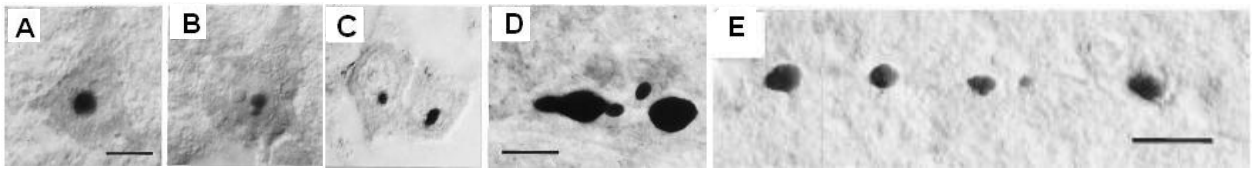

Figure 9. Huntingtin intranuclear inclusions in brain regions from HD patients. A/B. Cortical pyramidal neurons in juvenile HD patients; C. Striatal neurons in juvenile HD patient; D. Cortex of an adult HD patient; E. Cortex of a presymptomatic adult HD patient. Adapted from *DiFiglia et al., 1997*.

The length of mut Htt fragments, as much as the increased number of polyQ residues, influences aggregate formation both *in vitro* and *in vivo* (Li and Li, 1998, Martindale et al., 1998, Legleiter et al., 2010). Mut Htt N-terminal fragments selectively accumulate in the processes and axonal terminals of striatal neurons, inducing neuritic degeneration in cell culture and *in vivo* (Li et al., 2000). The transgenic mouse model, R6/2, expressing the Ex1 form of the protein presents massive aggregation load and HD-like phenotypes develop as early as 3 weeks of age, suggesting a possible toxic role of the inclusions (Mangiarini et al., 1996).

A proposed polymerization process suggests that elongated polyQ stretches acquire a "polar zipper" conformation through hydrogen bonds between the amides (Perutz, 1995, Perutz, 1996) and consequently assemble in water rich β-sheet structures (Figure 10).

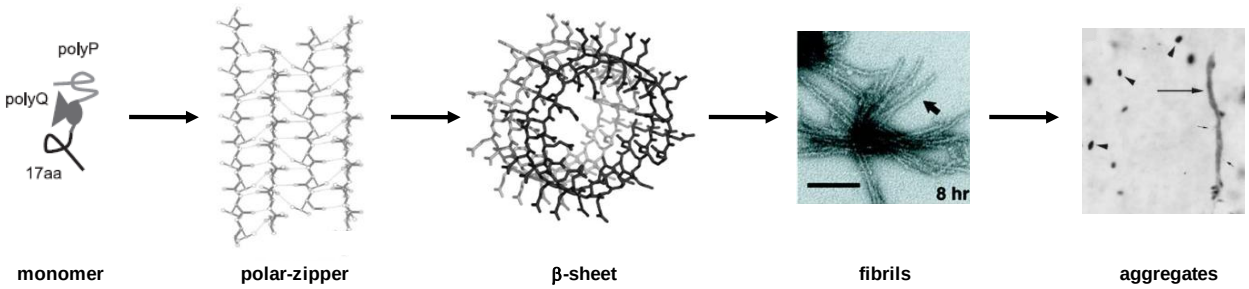

Figure 10. Polymerization process leading to mut Htt aggregates formation. Mut Htt fragments derived from proteolitic cleavage of the full length protein assemble in polar-zipper structures through the polyQ regions, forming intermolecular hydrogen bounds. The association of multiple polar-zippers results in the formation of b-sheet structures which gain stability with higher polyQ repeats. Before forming the high molecular weight aggregates intermediate insoluble fibrils are constituted. Combined figure created respectively from *Zuccato et al., 2010; Perutz et al. 2002; Singer and Dewji, 2006; Poirier et al., 2002; Gutenkust et al., 1999.*

Electron microscopy studies revealed that the β-sheet conformation forms fibrillar structures with a morphology similar to β-amyloid fibrils in Alzheimer's disease, prion rods and yeast prion protein Sup35 (Perutz et al., 2002b). Hence also in HD the phenomenon leading to aggregates formation could be triggered by a nucleation process (Huang et al., 1998, Scherzinger et al., 1999). Since single helical turns with 20 polyQ residues are unstable, the formation of stable β-sheet structures requires more than 40 residues, which assemble in double helical turns, are stabilized by hydrogen bonds and become nuclei for further helical growth (Perutz et al., 2002a).
From the growing nuclei to the large aggregates occurs the formation of protofibrillar intermediates, which are Congo red sensitive, and could represent the toxic species (Poirier et al., 2002).

The sequences flanking the polyQ expansion appear important in the aggregation process. The first 17 amino acids of mut Htt accelerate the polymerization events through hydrophobic interactions within the amphipathic α-helical structure (Rockabrand et al., 2007, Tam et al., 2009, Thakur et al., 2009), while the polyProline region inhibits aggregation in wt conditions by assembling into a helix conformation (Bhattacharyya et al., 2006, Darnell et al., 2007). Both flanking regions acquire aberrant folding in the presence of an elongated polyQ, thus suggesting their possible role in aggregation (Saunders and Bottomley, 2009, Lakhani et al., 2010, Williamson et al., 2010). Interestingly, the formation of oligomeric and fibrillary intermediates during the aggregation process (Olshina et al., 2010, Ramdzan et al., 2010) raises the question if these species rather than the high molecular weight aggregates are the cause of toxicity (Sanchez et al., 2003, Arrasate et al., 2004, Ross and Poirier, 2005).

Several approaches have been pursued aiming to reduce mut Htt aggregation to modulate toxicity. One strategy has been to modulate the cleavage of full length mut Htt acting on the caspase activity with small molecules and thus improving toxicity and cell viability in HD cell and mouse models (Ona et al. 1999, Wellington et al., 2000, Leyva et al. 2010). Modulation of chaperone levels *in vitro* formation of fibrils (Muchowski et al., 2000, Guzhova et al., 2011) as these proteins, such as Hsp70 and Hsp40, are known to interact with mut Htt oligomers (Lotz et al., 2010). Moreover the induction of heat shock response in cell culture reduced mut Htt aggregation and toxicity (Jana et al., 2000, Wacker et al., 2004, Herbst and Wanker, 2007).
Further investigation is required in order to investigate the aggregation dynamics, their role in HD development and possible strategies to modulate these events.

2.3 Heat shock protein 90 (Hsp90)

Heat shock protein 90 (Hsp90) is one of the main abundant proteins in the eukaryotic cell, constituting 1-2% of the total protein. It belongs to the family of molecular chaperones and contributes to maintain cellular homeostasis by regulating protein biogenesis and refolding, as well as cellular proliferation, differentiation and apoptosis. The Hsp90 cycle is a complex and highly regulated mechanism which, in order to be functional, involves several co-chaperons and energy consumption. The destiny of the client proteins depends on this cycle, which can lead either to their stabilization and activation or degradation (Taipale et al., 2010).

2.3.1 Hsp90 isoforms and structure

Hsp90 protein is present in the cell as two major cytoplasmic isoforms, Hsp90α and Hsp90β, inducible and constitutive respectively (Csermely et al., 1998), an endoplasmatic reticulum (ER) associated form, Grp94 (Sorger and Pelham, 1987), and a mitochondrial form, TRAP1/Hsp75 (Song et al., 1995, Chen et al., 1996, Felts et al., 2000). The sequences of Grp94 and TRAP1 have only 50 and 60% homology with the cytosolic forms, due to the presence of targeting motifs and a C-terminal sequence responsible for the retention in the specific compartment (Sorger and Pelham, 1987, Felts et al., 2000). Grp94 plays a role in cell viability and innate immunity (Randow and Seed, 2001) while the function of TRAP1 is not completely understood. It presents an ATP binding domain which can be inhibited by Hsp90 inhibitors (e.g. Geldanamycin) but does not dimerize nor interact with classical Hsp90 co-chaperones, thus suggesting a differential role from the cytoplasmic isoforms (Felts et al., 2000). On the contrary, the functions and role of

Hsp90α and β have been widely studied. Hsp90 exists in a dimeric form, αα or ββ, to be completely functional (Wayne and Bolon, 2007), although αβ monomers or heteromers exist, as well as trimeric and hexameric forms of unknown function (Lee et al., 2011). The C-terminus of the protein is responsible of the dimerization process, since truncated Hsp90 constructs remain in monomeric form (Figure 11A). It also contributes to the ATP/ADP hydrolysis as confirmed by truncated forms with decreased ATP activity (Richter et al., 2001). Crystallographic studies showed that the C-terminus folds in two pairs of helices which pack together in order to constitute a four helices bundle (Pearl and Prodromou, 2006).

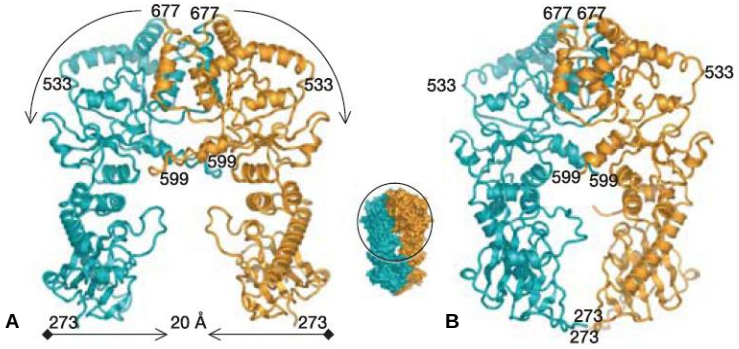

Figure 11. Hsp90 structure and ATP dependent conformational changes. A: Cristal structure of Hsp90 dimeric conformation, through the association of the C-terminal domain. B: ATP dependent shift of the conformation to a closed state. The association of the N-terminal domains due to ATP and the complex with a client brings the monomers approximately 20Å closer. Adapted from *Ali et al, 2006*

The N-terminus of the protein consists of a sequence of 25kDa responsible for the binding with ATP through the formation of a α/β sheet pocket extending from the surface. Classical Hsp90 inhibitors are known to interact with this portion of the protein (Obermann et al., 1998, Panaretou et al., 1998). The N-domains in the dimer associate in an ATP dependent manner, closing the chaperone around the co-factors or client proteins (Ali et al., 2006) (Figure 11B).

The segment connecting the N and the C-terminus consists of two αβα domains and seems responsible for the client protein binding and catalytic activity (Pearl and Prodromou, 2006).

The ATP hydrolysis happening at the N-terminus of the chaperone is associated with the transition from a closed state to an open hydrophobic state (Csermely et al., 1993, Grenert et al., 1997, Sullivan et al., 1997), regulating the association of the Hsp90 dimer with co-chaperones and client proteins.

2.3.2 Hsp90 cycle: Co-chaperones and client proteins

Hsp90 interacts with several co-chaperones (Pearl and Prodromou, 2006). These proteins belong to different classes and associate with Hsp90 through specific domains. The co-chaperones Hop and the E3 ligase carboxyl terminus of Hsc70-interacting protein (CHIP) are characterized by the presence of a so called tetratricopeptide repeat (TRP) domain, a helical coil structure formed by concatenated helical hairpins, which binds at the extreme C-terminus of Hsp90 (Das and Liang, 1998, Scheufler et al., 2000). Another important co-chaperone is p23, a small acidic protein which does not contain a TRP motif and binds directly at the N-terminus of Hsp90. This association is influenced by ATP/ADP hydrolysis, hence the co-chaperone is released from the complex in the presence of Hsp90 inhibitors (Johnson and Toft, 1995, Johnson et al., 1996).

The Hsp90 cycle is divided into an open state, when ADP is bound to the chaperone, and a closed state, when ATP is present in the binding pocket (Csermely et al., 1993, Grenert et al., 1997, Sullivan et al., 1997, Taipale et al., 2010) (Figure 12). The ATP hydrolysis highly influences the kinetics of the cycle by modulating the structure of the complex, as elucidated by fluorescence resonance energy transfer studies (Hessling et al., 2009).

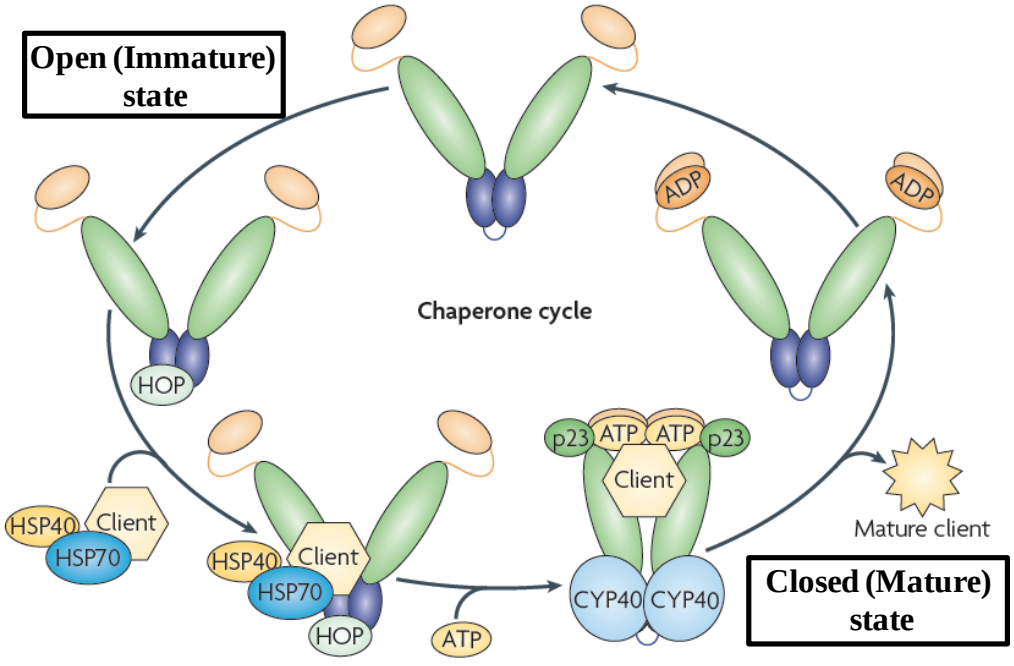

Figure 12. Hsp90 cycle. Hsp90 and other co-chaperones interact in a dynamic, ATP/ADP dependent cycle in order to stabilize and refold client proteins. During the open (immature) state Hsp90 is primarily associated to HOP, and consequently interacts with the complex Hsp70 and Hsp40, which are delivering the client protein. In the later phase, ATP and p23 are binding to the complex, inducing the release of the other co-chaperones and the stabilization of the client protein. When the correct folding is achieved or an activating stimulus is received, the hydrolysis of ATP to ADP leads to the release of p23 and the client, bringing back Hsp90 to the original open state. Adapted from *Taipale et al, 2010.*

The Hsp90 cycle is highly regulated by the interplay of the different co-chaperones, which induce conformational changes in the complex and recruit the client proteins, thus regulating the ATP/ADP hydrolysis and Hsp90 activity (Phillips et al., 2007). Hsp90 activity and interactions can be modulated by several PTM, such as phosphorylation, HDAC6 dependent acetylation and nytrosilation (Taipale et al., 2010).

More than 200 Hsp90 client proteins have been already identified (Picard, 2011). They belong to multiple families, such as steroid hormone receptors (Sanchez et al., 1985, Filipeanu et al., 2011), kinases (Sato et al., 2000, Basso et al., 2002, Lee et al., 2011), transcription factors (Nadeau et al., 1993, Bharadwaj et al., 1999, Muller et al., 2004, Walerych et al., 2004), proteins involved in cell cycle, cellular structure and homeostasis (Basto et al., 2007, Park et al., 2007).

Interestingly, when a client protein after multiple cycles of chaperone binding cannot fold properly, it is released from Hsp90 and degraded through the UPS (Taipale et al., 2010). CHIP is considered a "quality control" E3 ligase which typically associates with Hsp90 and Hsp70 to modulate the balance between protein folding and degradation (Connell et al., 2001, Murata et al., 2001). However other E3 ligases, such as of the RING-cullin family (Petroski and Deshaies, 2005), could be involved in the process, as was recently reported for ErbB2 and Hif-1α degradation (Morishima et al., 2008, Ehrlich et al., 2009).

2.3.3 Heat shock response (HSR)

To respond to acute and chronic proteotoxic damage the cell induces a complex and efficient defense mechanism called heat shock response (HSR). This cascade of events is responsible to ensure stress adaptation, recovery and survival (Gidalevitz et al., 2011). The cellular homeostasis is compromised by several insults, such as temperature and pH fluctuations or exposition to oxidative stress or heavy metals (Lindquist, 1986, Morimoto, 1998). These events influence the protein dynamics, inducing covalent modification and conformational changes, thus favoring the exposure of hydrophobic domains and aggregation. The cell counteracts these unfavorable conditions with activation of trancription factors and chaperones through the HSR (Wu, 1995, Gidalevitz et al., 2011). The first response to the stress is the activation of the transcription factor Hsf1, which in the inactive monomeric form is bound to Hsp90 (Morimoto, 1998, Whitesell and Lindquist, 2009). Four different forms of HSF are expressed in vertebrates but only Hsf1 is

known to actively contribute to the HSR (Morimoto, 1998, Akerfelt et al., 2007). In response to an insult monomeric Hsf1 is released from Hsp90, translocates into the nucleus and forms an active trimer, able to induce the transcription of several Hsp (Lis and Wu, 1993, Vabulas et al., 2010). The Hsp promote protein re-folding and rescuing of cellular homeostasis, also acting as a negative control on the Hsf1 and HSR activation (Shi et al., 1998). The whole mechanism is regulated by fine positive and negative feedbacks loops (Wu, 1995, Gidalevitz et al., 2011) and Hsf1 activation and attenuation is controlled by several PTMs such as phosphorylation, sumoylation and acetylation (Hong et al., 2001, Guettouche et al., 2005, Westerheide et al., 2009).

2.3.4 The role of heat shock proteins in disease: cancer and neurodegeneration

Aggregates and unfolded mutated proteins are a common feature in both oncological and neurodegenerative disorders. Hsp90 and Hsp70 have been studied in oncology in association with mechanisms of resistance to therapy and prevention of apoptosis (Ciocca and Calderwood, 2005, Schmitt et al., 2007), since these chaperones normally prevent cells from undergoing inappropriate autonomous cell death (Khalil et al., 2011). Even though the molecular mechanisms underlying resistance are unknown, the use of Hsp inhibitors as possible therapeutic agents has been explored. This approach would allow a decrease in the levels of Hsp in the cell, thus enhancing cell death and triggering the degradation of mutant oncogenic proteins (Schmitt et al., 2007, Li et al., 2009). Hsp90 inhibitors such as Geldanamycin (Kamal et al., 2004) have shown efficacy as cancer treatments , however further optimizations are needed to improve the potency and to reduce toxicity and side effects (Taldone et al., 2008, Taldone et al., 2009, Sankhala et al., 2011).

Neurodegenerative diseases are often characterized by misfolded proteins which aggregate and induce cell toxicity. Chaperones are known to co-localize with intracellular inclusions (Sherman and Goldberg, 2001, Muchowski and Wacker, 2005) and the HSR is impaired during aging (Meriin and Sherman, 2005). As potential therapeutic strategies Hsp90 inhibition as well as the enhancement of HSR have been discussed (Brown, 2007). The role of chaperones in neurodegeneration has been explored in multiple diseases such as Parkinson's disease (PD) (Auluck et al., 2002, Falsone et al., 2009), Alzeihmer's disease (AD) (Yoo et al., 1999, Evans et al., 2006), Amyotrophic Lateral Sclerosis (ALS) (Takeuchi et al., 2002, Batulan et al., 2006) and polyglutamine diseases (Adachi et al., 2003, Hansson et al., 2003).

A strategy widely explored is the possibility to enhance mutant protein degradation through Hsp90 inhibition. As described previously Hsp90 can bind to client protein and stabilize them (Chapter 2.3.2). The association of a mutant protein to Hsp90 could thus promote disease progression. On the contrary the use of Hsp90 inhibitors, which block the ATP binding site of the chaperone, could facilitate mutant protein release from the complex and UPS mediated degradation (Luo et al., 2010) (Figure 13). This has been demonstrated to be the case for Leucine-rich repeat kinase 2 (LRRK2), a protein kinase involved in PD, which is stabilized by the interaction with Hsp90 (Wang et al., 2008c) and upon Geldanamycin treatment is released from the complex and driven to the UPS by CHIP mediated Ubiquitination (Hurtado-Lorenzo and Anand, 2008, Ding and Goldberg, 2009, Ko et al., 2009).

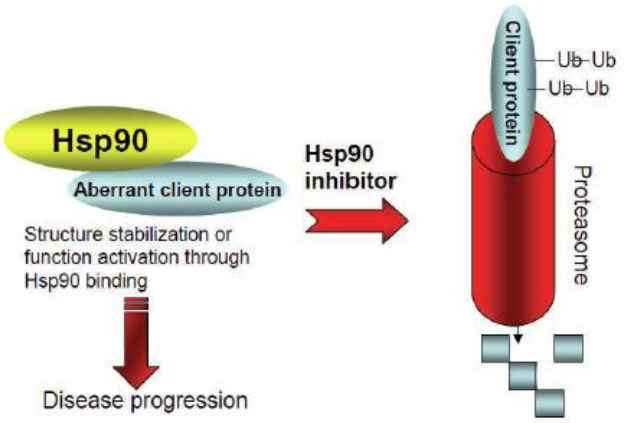

Figure 13. Mechanism of action of Hsp90 inhibitors on client protein degradation. Hsp90 could play a role in stabilizing mutant proteins such as LRRK2 or AR, thus contributing to disease progression. Its inhibition would therefore enhance UPS mediated mutant protein degradation and therefore ameliorate the disease phenotypes. Adapted from *Luo et al., 2010*.

In the context of polyQ diseases it has been demonstrated that the mutant androgen receptor (AR), responsible of spinal bulbar muscular atrophy (SBMA) associates with Hsp90. The use of Hsp90 inhibitors can modulate this interaction inducing AR clearance and improving SBMA phenotypes (Waza et al., 2006a, Waza et al., 2006b, Tokui et al., 2009). Overexpression of CHIP also induces amelioration of phenotypes and UPS mediated degradation of AR (Waza et al., 2005, Adachi et al., 2007).

In HD has been shown that R6/2 mice have reduced levels of Hsp compared to wt (Hay et al., 2004) and the HSR induced by Hsp90 inhibitors is weaker in aging mice (Labbadia et al., 2011). Interestingly, studies on primary neuronal cultures showed that the different levels of expression of Hsp70 could influence susceptibility to mut Htt and toxicity (Tagawa et al., 2007).

Furthermore, Hsp70 and Hsp40 associate with mut Htt oligomers *in vitro* in the classical ATP dependent way (Lotz et al., 2010) and influence aggregates formation (Chai et al., 1999, Jana et al., 2000), also when added exogenously (Novoselova et al., 2005). Interestingly, the Hsp90 inhibitor Geldanamycin improved eye degeneration and body inclusion formation in Drosophila HD models (Fujikake et al., 2008) and reduced the formation of aggregates in cell culture models of HD increasing Hsp70 and Hsp40 levels (Sittler et al., 2001).

Taken together these results suggest a promising role of Hsp90 inhibition in neurodegeneration, to potentiate the impaired HSR, influence aggregation, and modulate mutant protein clearance. Further studies in HD would be needed to characterize the possible use of this approach in modulating mut Htt toxicity.

2.4 Time resolved fluorescence resonance energy transfer (TR-FRET)

2.4.1 Fluorescence resonance energy transfer (FRET)

Energy transfer based on electron transfer or exchanging mechanisms is a phenomenon widely seen in nature (Schaferling and Nagl, 2011). In biological environments, where the distances between molecules are between 2-10nm it is normally referred to as fluorescence resonance energy transfer (FRET). This phenomenon has been described for the first time by the German scientist Theodor Förster in 1948, and it is based on the dipole-dipole energy coupling between two fluorescent molecules in close proximity to each other. In order to consent the energy transfer, the emission and absorption spectra of the two fluorophores involved should be overlapping (Figure 14A). This would allow that the excitation of the donor fluorophore results in the emission of the acceptor (Stryer, 1978, Alvarez-Curto et al., 2010). The efficiency of transfer decays as a function of the inverse sixth power of the distance (r) between the two fluorophores, as depicted in the relation derived by Förster:

$$E=1/[1+(r/R_0)^6]$$

R_0 represents the Förster radius, which is the distance needed between the two fluorophores to obtain an energy transfer of 50% and it can be calculated as:

$$R_0 = [2.8 * 10^{17} * \kappa^2 * Q_D * \varepsilon_A * J(\lambda)]^{1/6} \text{ nm}$$

Where κ^2 is the orientation factor between the dipoles, which usually is different from 90° in order to avoid oscillation abolishing the signal. Q_D is the fluorescence quantum yield of the donor when acceptor is absent, ε_A the maximum acceptor extinction coefficient and J(λ) being the

overlap integral between donor and acceptor spectra. As R_0 is dependent on all these factors the best distance for two fluorophores in aqueous solution is between 2 and 7 nm (Wu and Brand, 1994, Patterson et al., 2000) (Figure 14B).

The FRET technology has been widely applied to investigate intermolecular and intramolecular interatcions. FRET has been used for example in the determination of protein interactions on microarray assays (Schaferling and Nagl, 2011), or in the study of G coupled protein receptors (GPCR) (Alvarez-Curto et al., 2010). In this regard it needs to be said that a FRET signal does not necessarily mean that a direct interaction between two proteins has occured, while on the other hand an absence of signal may not be derived by a missing interaction but may be due to one of the parameters discussed above.

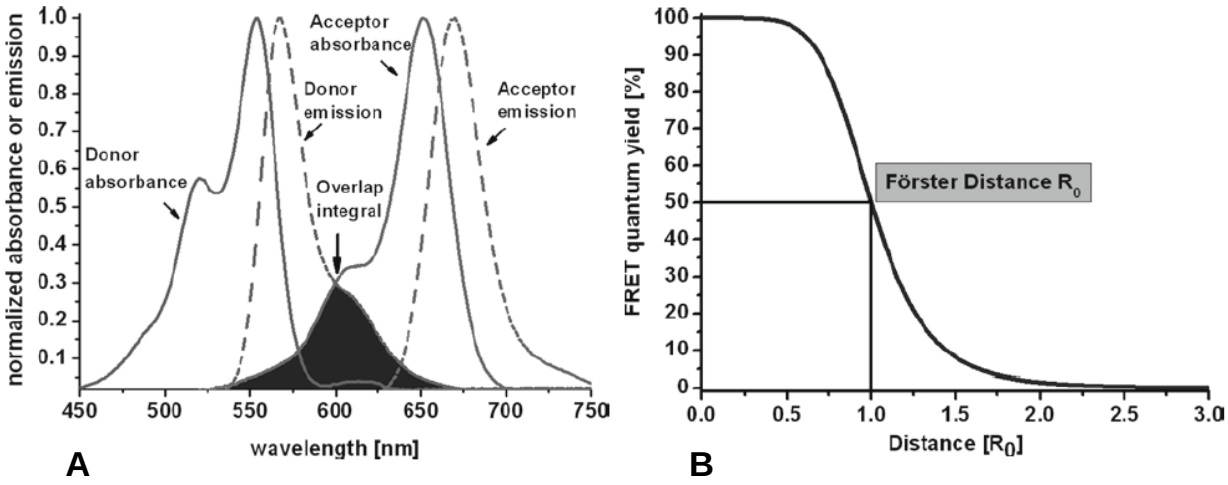

Figure 14. Fluorescence resonance energy transfer. A: spectral representation of the energy transfer. Absorbance and emission are depicted with plain and dotted lines, respectively. In order to obtain the energy transfer the two spectra have to overlap. B: Distance dependence of FRET signal expressed in terms of Förster distance R_0. Adapted from *Schäferling and Nagl, 2011*.

The classical FRET has some limitations, like the overlap between the two spectra which could lead to photobleaching and bleed-through. The excitation of the acceptor in this case would not occurr because of the energy transfer but as a direct effect of the light excitation.

For high-throughput screening purposes the FRET technology shows high susceptibility to artifacts. These events could be caused by autofluorescence of compounds and other components of the solution (buffers, cell lysates, and detergents) which can not be easily distinguished from an authentic signal. The presence of such artifacts in high-throughput screens, where normally the number of replicates and compound concentration are n=1, could heavily affect the robustness, significance and reliability of the results obtained.

2.4.2 Time resolved FRET (TR-FRET)

In order to overcome the limitations of the classical FRET, an improvement of the technology, defined Time Resolved FRET (TR-FRET) has been developed (Degorce et al., 2009). The TR-FRET is also based on the energy transfer between two proximal fluorophores, but takes advantage of the use of rare earth ions as donor fluorohores. These long lived fluorophores coupled with a delay between excitation and emission, prevent the generation of signals due to autofluorescence or bleed-through (Figure 15).

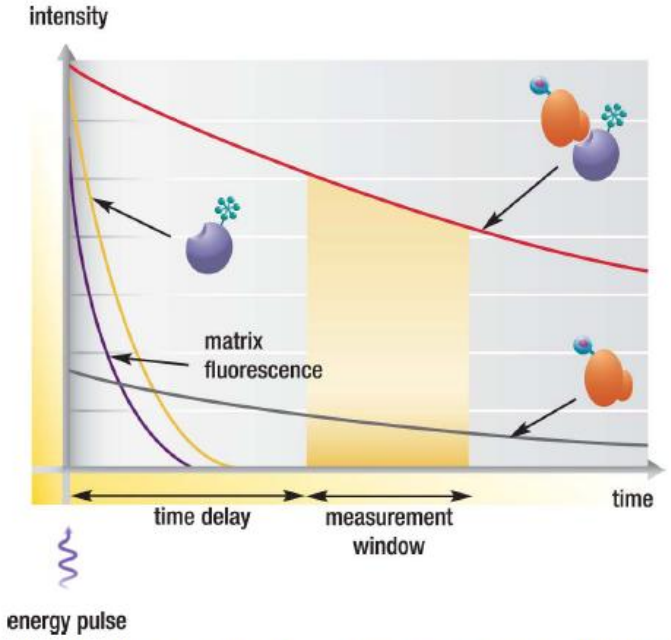

Figure 15. Time resolved FRET. Schematic representation of the fluorescence signals derived from the labeled fluorophores and background in a homogeneous sample system. After excitation of the sample the fluorescence of the background (grey line) and the direct excitation of the acceptor fluorophore (dark blue) decay rapidly. The emission of the donor fluorophore on the other hand has a very long lived emission of up to 1ms (black). The delay of ~100µs before the fluorescence measurement allows the separation between the signal and the background fluorescences described. A measurable signal will be obtained when donor and acceptor fluorophores are in close proximity, thus allowing the energy transfer. Adapted from *Degorce et al, 2009*.

The ions used belong to the family of the lanthanides and the most commonly used are samarium (Sm), europium (Eu), terbium (Tb), and dysprosium (Dy). They show specific photophysic properties, such as a large Stoke's shifts and extremely long emission half-lives (from µsec to msec) when compared to more traditional fluorophores. The cryptate lanthanides are engulfed in a structure constituted of an organic trisbypyridine cryptate (Alpha, 1987), which provides the energy absorption and allows a controlled energy release to the central ion, overcoming the poor absorption capacity of the lanthanides (Mathis, 1993). The ion consequently produces long lived

fluorescence, with excitation and emission separated by hundreds of nanometers. This results in a low background and interference from photobleaching (Dickson et al., 1995). Another advantage of the use of these rare earth ions is the presence of an R_0 of over 9nm, significantly higher than the one normally presented by other fluorophores, and therefore favoring the development of assays to measure the interaction between macromolecules (Mathis, 1993). The possibility to measure independently at the wavelength relative to donor and acceptor allows the generation of a value derived from the ratio between the two signals, thus allowing to correct assay volume errors, as much as signal errors due to quenching or scattering. These properties of TR-FRET confer higher reliability and robustness to the method, if compared to other assays (Imbert et al., 2007). This kind of assay has already been used for several applications such as the detection of enzymatic reactions (Bazin et al., 2001), kinase assays, protease assays and the study of post translational modifications (Dudek and Horton, 2010, Riddle et al., 2006, Horton and Vogel, 2010). In the field of neurodegeneration a TR-FRET based immunoassay has been developed in order to detect and quantify soluble mut Htt, in a high-throughput applicable manner (Paganetti et al., 2009). The use of a combination of two antibodies, directed towards the N-terminus of the protein and the polyQ tract allowed a sensitive measurement of mut Htt in different cell types, HD animal models as well as in human HD patient samples (Weiss et al., 2009a). The use of Tb as a donor fluorophore allows the excitation of two different acceptors simultaneously, d2 and Alexa488, as it has multiple emission spectra (Degorce 2009). This advantage consents the development of multiplex assays which in the case of HD favor the simultaneous detection of wt and mutant protein, using a single donor antibody, Tb labeled, and two acceptors, d2 or Alexa488 labeled, and specific for one or the other Htt form (Weiss et al., 2010).

Overall the TR-FRET technology prevents the appearance of several artifacts, but in high-throughput applications one must be aware of other artifacts, for example driven by the structures of the compounds used (Imbert 2007). The possibility to develop multiplex assay taking advantage of the photophysic properties of the fluorophores suggests the possibility to apply the technology to investigate the modulation of protein-protein interactions and eventually soluble protein and protein aggregates in the same sample.

3 AIMS OF THE THESIS

The mechanisms leading to Huntington's disease pathogenesis and to the huntingtin induced neurotoxicity are currently unknown. The misfolded conformation acquired by mutant huntingtin and the formation of oligomers and aggregates are considered to be potential toxic mechanisms. For this reason, my first goal was to investigate strategies to decrease the cellular levels of mutant misfolded huntingtin in HD cell models and explore the molecular pathways involved.

Starting from Hsp90 inhibitor compounds identified from a high-throughput screen aimed at identifying modulators of mut Htt degradation, I characterized the molecular interaction of mut Htt with the Hsp90 chaperone complex. My data elucidate degradation pathways of mut Htt and point to a role of Hsp90 in maintaining its stability in HD cell models.

Another goal of my studies was the development of a method based on time resolved resonance energy transfer to efficiently detect and quantify mut Htt aggregates in HD models. The first objective was to establish a technology which was sensitive, simple and applicable in a high through-put set up. I used two antibodies binding to different mut Htt epitopes to investigate their specificity for different aggregate species. Together with a previously developed assay for soluble mut Htt, the newly developed assay facilitates the simultanous measurement of soluble and aggregated mutant protein in biological samples. I thus investigated the two forms of mut Htt in different HD models, to better characterize disease progression. The mut Htt aggregate assay is considered a potential means to monitor the effect of therapeutic treatments for HD.

4 RESULTS

4.1 A screen for enhancers of clearance identifies mutant huntingtin as an heat shock protein 90 (Hsp90) client protein.

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(manuscript submitted)

4.1.1 SUMMARY

Mechanisms to reduce the cellular levels of mutant huntingtin (mut Htt) provide promising strategies for treating Huntington's disease (HD). To identify compounds enhancing the degradation of mut Htt we performed a high throughput screen, using a hippocampal HN10 cell line expressing a 573 amino acid mut Htt fragment. Several hit structures were identified as heat shock protein 90 (Hsp90) inhibitors. Cell treatment with these compounds reduced levels of mut Htt as measured by time-resolved Fluorescence resonance energy transferassays and western blots, without overt toxic effects. To characterize the mechanism of mut Htt degradation, we used the potent and selective Hsp90 inhibitor NVP-AUY922. In *Hdh*Q150 embryonic stem (ES) cells and in ES derived neurons, NVP-AUY922 treatment substantially reduced soluble full-length mut Htt levels. In HN10 cells, Hsp90 inhibition by NVP-AUY922 enhanced mut Htt clearance in the absence of any detectable heat shock protein 70 (Hsp70) induction. Furthermore, inhibition of protein synthesis with cycloheximide or overexpression of dominant-negative heat shock factor 1 (Hsf1) in *Hdh*Q150 ES cells attenuated Hsp70 induction, but did not affect NVP-AUY922 mediated mut Htt clearance. Together, these data provided evidence that direct inhibition of Hsp90 chaperone function was crucial for mut Htt degradation, rather than heat shock response induction and Hsp70 upregulation. Co-immunoprecipitation experiments revealed a physical interaction of mut Htt with the Hsp90 chaperone complex. Hsp90 inhibition disrupted the interaction and induced clearance of mut Htt through the ubiquitin proteasome system. Our data suggest that mut Htt is an Hsp90 client protein and that Hsp90 inhibition may provide a means to reduce mut Htt in HD.

4.1.2 INTRODUCTION

Huntington's disease (HD) is a progressive neurodegenerative disease characterized by brain atrophy, motor, cognitive and psychiatric symptoms. Patients also suffer from muscle atrophy, weight loss and metabolic disturbances. HD is caused by the expansion of a trinucleotide repeat resulting in an elongated glutamine stretch close to the N-terminus of the huntingtin protein (The Huntington's Disease Collaborative Research Group, 1993). The length of the polyglutamine expansion correlates with disease onset (Ross and Tabrizi, 2011). The dominant mode of inheritance supports the hypothesis that the extended polyglutamine stretch confers a toxic gain

of function to huntingtin, possibly due to structural changes of the mutant protein, and aberrant interactions with different cellular pathways. Multiple potential pathogenic mechanisms of mutant huntingtin (mut Htt) have been proposed including proteasome impairment, mitochondrial dysfunction, transcriptional dysregulation, impaired intracellular transport, and cell death induced by the formation of toxic aggregates containing N-terminal mut Htt fragments (DiFiglia et al., 1997, Fecke et al., 2009, Ross and Tabrizi, 2011). Reversal of inducible mut Htt overexpression, RNA interference and antisense oligonucleotide studies have all demonstrated amelioration of HD-like symptoms upon reduction of mut Htt expression levels (Yamamoto et al., 2000, DiFiglia et al., 2007, McBride et al., 2008, Sah and Aronin). Therefore, mechanisms to reduce the cellular load of the disease-causing mut Htt protein, such as via enhancement of its clearance and degradation, represent promising therapeutic strategies.

Heat shock proteins play an important role in protein folding and quality control. In the context of polyglutamine diseases, such as HD, heat shock protein 70 (Hsp70, *Hspa1a/b*), Hsp40 (*Dnajb1*) and Hsp90 (*Hsp90aa1, Hsp90ab1*) have been the subject of several studies. Elevation of Hsp70 levels has been found to be neuroprotective in several animal models (Turturici et al., 2011). For instance, Hsp70 overexpression suppressed neuropathology and improved motor function in a spinocerebellar ataxia mouse model (Cummings et al., 2001). Further, Hsp70 and Hsp40 attenuated assembly of polyglutamine proteins into amyloid-like fibrils (Muchowski et al., 2000, Lotz et al., 2010).

Hsp90 comprises about 1-2 percent of total cellular protein (Taipale et al., 2010). It uses ATP hydrolysis to fold and maturate client proteins and interacts with more than 20 co-chaperones. Notably, Hsp90 is described to preferentially bind to partially folded intermediates suggesting a role in the maturation of metastable proteins, late in their folding pathway (Pratt and Toft, 2003, Taipale et al., 2010). Currently more than 200 Hsp90 clients have been identified, including a range of oncogenic proteins (Kamal et al., 2004). Hsp90 inhibitors, such as the geldanamycin derivatives 17-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) as well as other structural classes, including NVP-AUY922, are in clinical development as cancer therapies (Kamal et al., 2003, Kamal et al., 2004). Pharmacological inhibition of Hsp90 induces a heat shock response (HSR) after release of the transcription factor heat shock factor-1 (Hsf1) from the Hsp90 chaperone complex. Hsf1 in turn induces the expression of other heat shock proteins such as Hsp70 (Taipale et al., 2010, Turturici et al., 2011). Upregulation of Hsp70 after Hsp90 inhibition,

using geldanamycin, inhibited mut Htt exon1 protein aggregation (Sittler et al., 2001) whereas loss of Hsp70 exacerbated pathogenesis in a mouse model of HD (Wacker et al., 2009).

We conducted a high throughput screen aimed at identifying molecules enhancing degradation or clearance of soluble mut Htt. Among the non-toxic hits identified reducing mut Htt levels without overt toxic and non-specific effects, a single class of compounds possessed a known mechanism of action, Hsp90 inhibition. Unexpectedly, the HSR induced after Hsp90 inhibition was not required for the degradation of soluble mut Htt. We provide evidence that mut Htt is a client protein of Hsp90 and that the chaperoning function of Hsp90 is critical for maintaining the stability of mut Htt in different cellular systems. Pharmacological inhibition of Hsp90 destabilizes mut Htt and facilitates its clearance through the ubiquitin proteasome system (UPS).

4.1.3 MATERIALS AND METHODS

High throughput screening.

The screen was conducted in a 1536-well format as described previously (Paganetti et al., 2009). Picogreen (Invitrogen, #P11495; 1:1200) and caspase 3/7 (Promega, #G8091) assays to exclude putative toxic compounds were performed according to instructions from the manufacturer.

Cell lines- HN10-Htt cell lines were cultured as described (Weiss et al., 2009b). Htt expression was induced by the addition of 750 nM RSL1 to the growth medium. Mouse embryonic stem (ES) cells expressing 1000 aa or full length mut Htt (Q145) from the ROSA26 locus, and *Hdh*Q150 ES cells (Lin et al., 2001) were cultured in 3i–medium (Ying et al., 2008). Neurons were derived from ES cells as described (Bibel et al., 2007). HEK293T cells were cultured in DMEM supplemented with Glutamax (Invitrogen, #32430) and 10% fetal calf serum.

Plasmids and transient transfections.

HEK293T cells were transfected using Lipofectamine 2000 (Invitrogen, #11668-019). The following plasmids were used in transient transfections: human Htt573Q72/ pER (CMV promoter); hemagglutinin (HA) tagged ubiquitin/ pSG5 (SV40 promoter). Hsf1 dominant negative/ pcDNA5 (Heldens et al., 2010); GFP/ pLL3.7 (CMV promoter).

Western Blot and antibodies.

Cells were harvested and lysed in ice-cooled lysis buffer (phosphate buffered saline, Invitrogen #14190; 1 % Triton X100), containing protease inhibitors (Roche complete, 11836145001). Lysates were kept on ice for 15 min before centrifugation for 10 min at 13000 rpm at 4 °C. The BCA assay (Thermo Scientific, 23227) was used for protein quantification. Samples were diluted in NuPAGE loading buffer (Invitrogen, NP0007; NP0009) and heated 10 min at 95 °C. The samples (ES cells: 15 µg; HN10 cells: 10 µg) were loaded onto 4-12 % NuPAGE Bis-Tris gels or 3-8 % Tris-acetate gels (Invitrogen, NP0335, EA03752). Semi dry protein transfer (Ancos, LV8428062) to PVDF membranes (Millipore, Immobilon-P PVH00010) was performed in NuPAGE transfer buffer (Invitrogen, NP0006-1) for one hour at 15 V and the membrane was then incubated for one hour in 20 mM Tris-Cl, 137 mM NaCl, pH 7.6, 0.1 % Tween 20, 5 % (w/ v) dried milk. Incubation with primary antibodies was done over-night (o/N) at 4 °C; before secondary horseradish peroxidase conjugated goat anti-mouse (Chemicon, AP127P) or goat anti-rabbit antibodies (Jackson ImmunoResearch, 111-035-144) were applied for one hour. The ECL reagent (GE Healthcare, Amersham) was used for protein visualization. Densitometric quantification of Hsp70 was done from X rays using the ImageJ software (signals were normalized to tubulin). The following primary antibodies were used: 2B7 (anti Htt; custom production by NanoTools, Freiburg, Germany); MW1 (anti polyQ; Developmental Studies Hybridoma Bank; (Ko et al., 2001); Hsp90 (Stressgen SPS-771; Stressgen SPA-830); Hsp25 (Stressgen SPA-801); Hsp40 (Stressgen SPA-400); Hsp70 (Stressgen SPA-810); Hsf1 (Stressgen SPA-901); α-tubulin (Abcam ab28037); Akt (Cell Signalling 9272); phospho-Akt (Cell Signalling 9271), ubiquitin (Millipore MAB1510); p23 (Alexis ALX-804-023); HA (Roche 12CA5).

Co-immunoprecipitation.

HN10 and ES cells were lysed (dounce homogenizer) in ice-cold IP lysis buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 20 mM Na-molybdate, 4 mM Na-orthovanadate, 0.02 % Nonidet-P40, Roche complete protease inhibitors). The samples were left on ice for 15 min and centrifuged 10 min, 13000 rpm at 4 °C. 150 µl of lysate (approximately 150 µg protein) were incubated o/N with 2 µg of Hsp90 or p23 antibody. Afterwards the samples were incubated for 30 min with 15 µl of protein G-sepharose 4 fast flow beads (GE Healthcare, 17-0618-01) and subsequently washed three times with lysis buffer and once in lysis buffer

containing 20 mM Hepes pH 8.0. NuPAGE loading buffer was added to the dried beads and the samples heated for 10 min at 95 °C.

Compounds.

NVP-AUY922 was synthesized at Novartis (10 mM stock solution in dimethylsulfoxide (DMSO)). Proteasome inhibitors epoxomicin and MG132 (Calbiochem (#324800; #474790); cycloheximide (Sigma-Aldrich), and RSL1 (New England Biolabs) were used.

Ubiquitination assay.

HEK293T cells were transiently transfected with HA-ubiquitin and Htt573Q72 plasmids. After 24 hours the medium was replaced and the cells treated o/N with Hsp90 and/ or proteasome inhibitors. Two days after transfection the cells were lysed in 50 mM HEPES pH 8.0, 250 mM NaCl, 5 mM EDTA, 0.1 % Nonidet-P40; Roche complete protease inhibitors) and aliquots (approximately 500 µg total protein) were incubated o/N with 5 µg of anti Htt 2B7 antibody and subsequently for 30 min with 15 µl of protein G-sepharose 4 beads. HA-ubiquitin immunoprecipitation was done using an anti-HA magnetic beads kit (µMACS HA, Miltenyi Biotec).

Time resolved fluorescence resonance energy transfer (TR-FRET).

Assays were performed as described previously (Paganetti et al., 2009, Weiss et al., 2009a, Weiss et al., 2010). Briefly, cells were lysed in PBS, 1 % Triton X100 and incubated at room temperature for 30 min with shaking. Then 5 µl of lysate (HN10 cells: 2 µg; ES cells: 5 µg) was transferred to a low-volume plate (Greiner Bio-one, #784080) and 1 µl of antibody mix added. For detection of Htt573Q72 expressed from the HN10 cell line the antibodies 2B7-Terbium (Tb)/ β1-d2 (intact Htt573Q72) or 2B7-Tb/ MW1-d2 (total Htt573Q72) were used. Mut Htt expressed from *Hdh*Q150 cells was measured with 2B7-Tb, MW1-D2. Fluorophore labeling of antibodies was performed by CisBio Bioassays (Parc Marcel Boiteux, France). TR-FRET measurements were done after one hour incubation at room temperature using an EnVision Reader (Perkin Elmer, excitation 320 nm, time delay 100 msec; integration time 400 msec). Values (means from at least 3 experiments ± SD; duplicate or triplicate determinations) are expressed as ratio between the emission at 665 nm and 620 nm; background signals (antibodies in lysis buffer) were subtracted. The *t*-test was used to assess significance.

4.1.4 RESULTS

A high throughput screen identifies Hsp90 inhibitors as enhancers of mutant huntingtin degradation.

Our aim was to identify mechanisms to reduce cellular levels of soluble mutant huntingtin (mut Htt). To this end screening of the Novartis compound library (approximately 2 x 10^6 compounds) was performed, using a mouse hippocampal HN10 cell line expressing an inducible β1 epitope tagged, 573 amino acid N-terminal fragment of human Htt with 72 glutamine residues (Htt573Q72) as described (Weiss et al., 2009b). This cell line does not produce readily detectable mut Htt aggregates. Soluble mut Htt levels were measured using a sensitive, homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET) assay (Paganetti et al., 2009, Weiss et al., 2010). Toxic compounds and structures which interfered with the TR-FRET assay readout were excluded as described (Paganetti et al., 2009). Compounds affecting mut Htt levels by inhibition of the inducible expression system were identified in HN10 cells expressing *Gaussia* luciferase from the expression vector as used for mut Htt (not shown). The remaining hits were then selected for further validation (Figure 16A). Compounds reducing mut Htt protein levels were confirmed in concentration response curves and effects compared to readouts for cytotoxicity, such as caspase 3 activation and DNA fragmentation (Figure 16B). After review of the chemical structures it became apparent that several hits, comprising different structural classes, had previously been characterized to act by a common mechanism of action, ATP competitive inhibition of Hsp90. Hsp90 inhibitory activity of compounds identified from the screen was confirmed in radicicol binding assays as described (not shown) (Schilb et al., 2004). The compounds caused a concentration-dependent reduction of the Htt573Q72 protein expressed in the HN10 cell line as measured by TR-FRET and western blot readouts, without overt cytotoxic effects (Figure 16B).

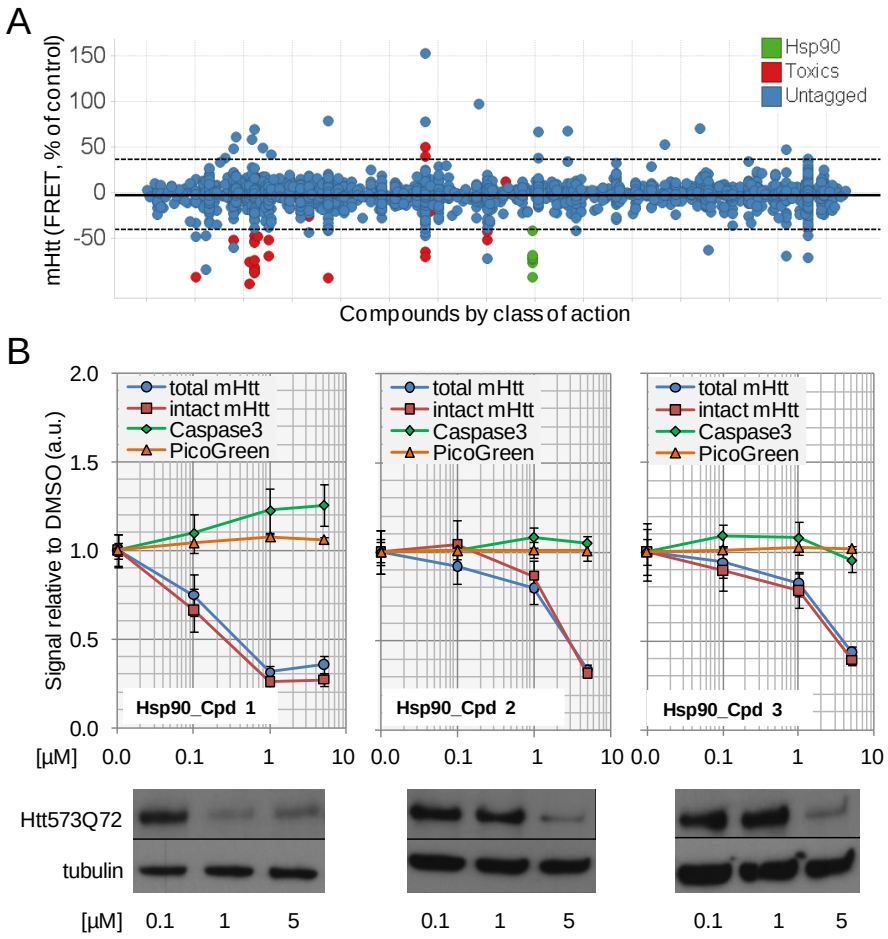

Figure 16. A high throughput screen identifies Hsp90 inhibitors as modulators of mut Htt degradation. A. Visualization of primary screen data for compounds with known mode of action. The Hsp90 inhibitor compounds are labeled. The dotted line denotes the cut-off used for hit selection (3 x SD). B. Examples of profiles of three different Hsp90 inhibitor structures identified from the screen by biochemical (upper panel) and western blot analysis (lower panel). TR-FRET detection of the entire Htt573Q72 fragment was measured with the antibody combination (2B7-Tb and β1-d2) binding to the N-terminal 17 amino acids of Htt and to the C-terminal β1 tag, respectively (intact Htt). MW1-d2 binds to the polyQ region of mut Htt and in combination with 2B7-Tb to detect N-terminal mut Htt fragments (total Htt). DNA fragmentation (picogreen) and caspase 3 activation assays were done to assess cytotoxicity.

For further characterization of the mechanism of mut Htt clearance after Hsp90 inhibition we used a potent and selective Hsp90 inhibitor compound that had previously been described, NVP-AUY922 (Brough et al., 2008, Eccles et al., 2008). In a similar manner to the Hsp90 inhibitors shown under Fig. 16B, NVP-AUY922 concentration-dependently reduced Htt573Q72 protein levels. o/N application of 30 nM or higher concentrations to HN10-Htt573Q72 cells significantly reduced mut Htt protein as evidenced by western blots and quantified using TR-FRET ($p < 0.001$; Figure 17A, C). The expression of endogenous wild-type (wt) Htt in HN10 cells was also reduced, however with lower efficacy (Figure 17A). NVP-AUY922 treatment reduced the levels

of phosphorylated Akt (pAkt) and to a lesser extent also of the non-phosphorylated form, as expected for an Hsp90 client protein (Pratt and Toft, 2003). Interestingly, in HN10 cells NVP-AUY922 did not induce Hsp70 protein expression, an established marker for the HSR after Hsp90 inhibition (Figure 17; Figure 19A) (Taipale et al., 2010, Turturici et al., 2011). The observed potency of NVP-AUY922 correlated well with IC_{50} values from binding experiments of 21 nM and 8 nM at Hsp90β and Hsp90α, respectively (Eccles et al., 2008).

To rule out the possibility that the observed effects of NVP-AUY922 on Htt degradation were mediated by interference with the inducible expression system used, cells were cultured in medium without the inducer ligand RSL1 from the time of Hsp90 inhibitor application onwards (Figure 17D, F). Under these conditions Htt573Q72 protein clearance was also significantly enhanced compared to DMSO, vehicle treatment ($p < 0.001$). To assess the selectivity of the effect over wt Htt, the Hsp90 inhibitor was applied to a HN10 cell line expressing both the mutant (Htt573Q72) and the wt (Htt573Q25) Htt fragments. NVP-AUY922 treatment affected both mutant and wt forms however the decline of mut Htt appeared more pronounced compared to the wt (Htt573Q72) fragment (Figure 17B, E).

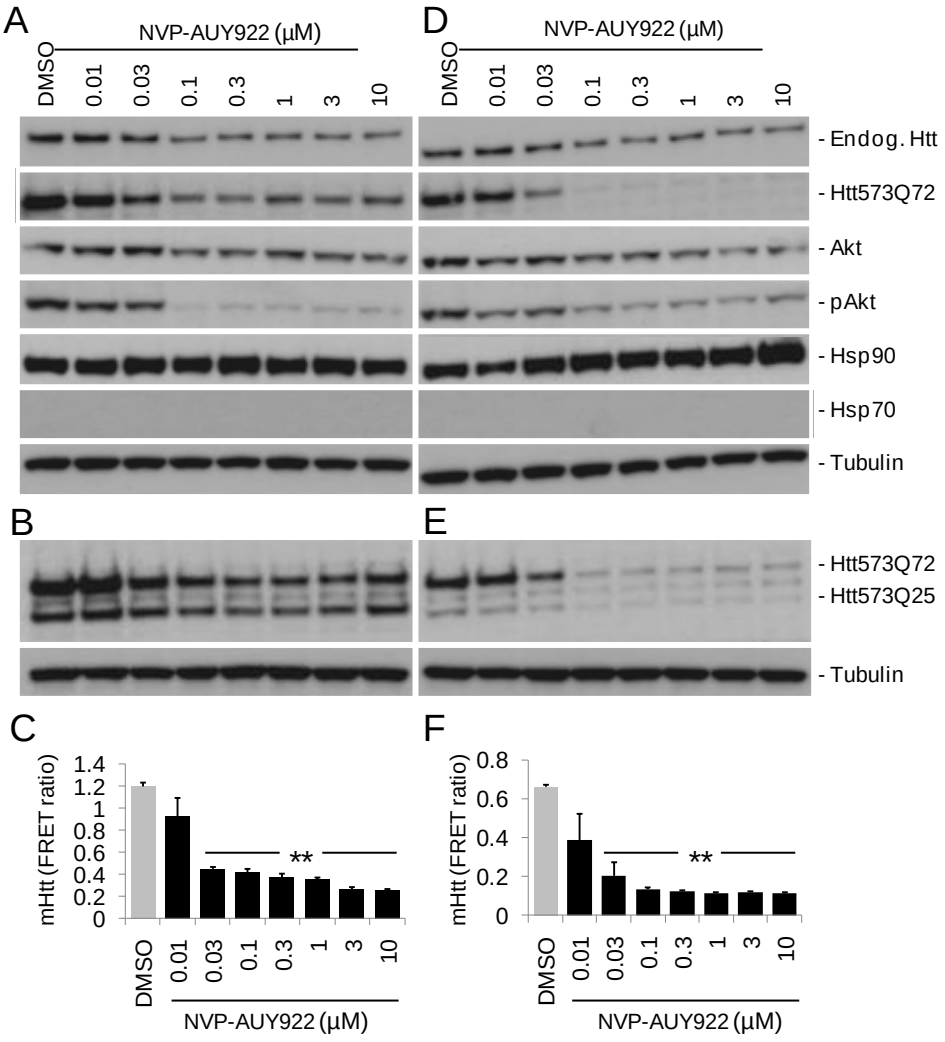

Figure 17. Pharmacological Hsp90 inhibition induces mut Htt degradation. HN10-Htt cell lines were cultured three days in medium containing RSL1 to induce Htt expression. The cells were then treated with NVP-AUY922 as indicated in RSL1 containing medium (A-C, 'steady state', harvest after 24h) or in medium without RSL1 to turn off inducible Htt expression (D-F, 'wash out', harvest after 16h). Western blot analysis (A, D) and TR-FRET quantification (C, F) of NVP-AUY922 effects in HN10-Htt573Q72 cells. B, E. Western blots of HN10 cells co-expressing mutant (Htt573Q72) and wt (Htt572Q25) fragments (2B7 antibody). mut Htt quantification by TR-FRET was done with 2B7-Tb and MW1-d2 antibodies; values are expressed as 665 nm/ 620 nm emission ratios; **p < 0.001, n = 3.

We extended our studies to knock-in *Hdh*Q150 ES cells expressing full-length mut Htt (Figure 18). As observed for the HN10 cell line, the presence of NVP-AUY922 reduced both mutant and wt Htt, as shown by western blots with mut Htt- (MW1) and pan-selective Htt antibodies (2B7; Figure 18A). The MW1 antibody binds to expanded polyQ only and does not detect wt Htt (Weiss et al., 2010). In contrast to HN10 cells NVP-AUY922 induced a strong up-regulation of Hsp70 in ES cells (Figure 17, Figure 18). Lysates were analyzed at different time points after

initiating treatment (Figure 18B). 0.3 µM NVP-AUY922 caused a significant reduction of mut Htt compared to control treated cells after 36 hours ($p < 0.01$; Figure 18B).

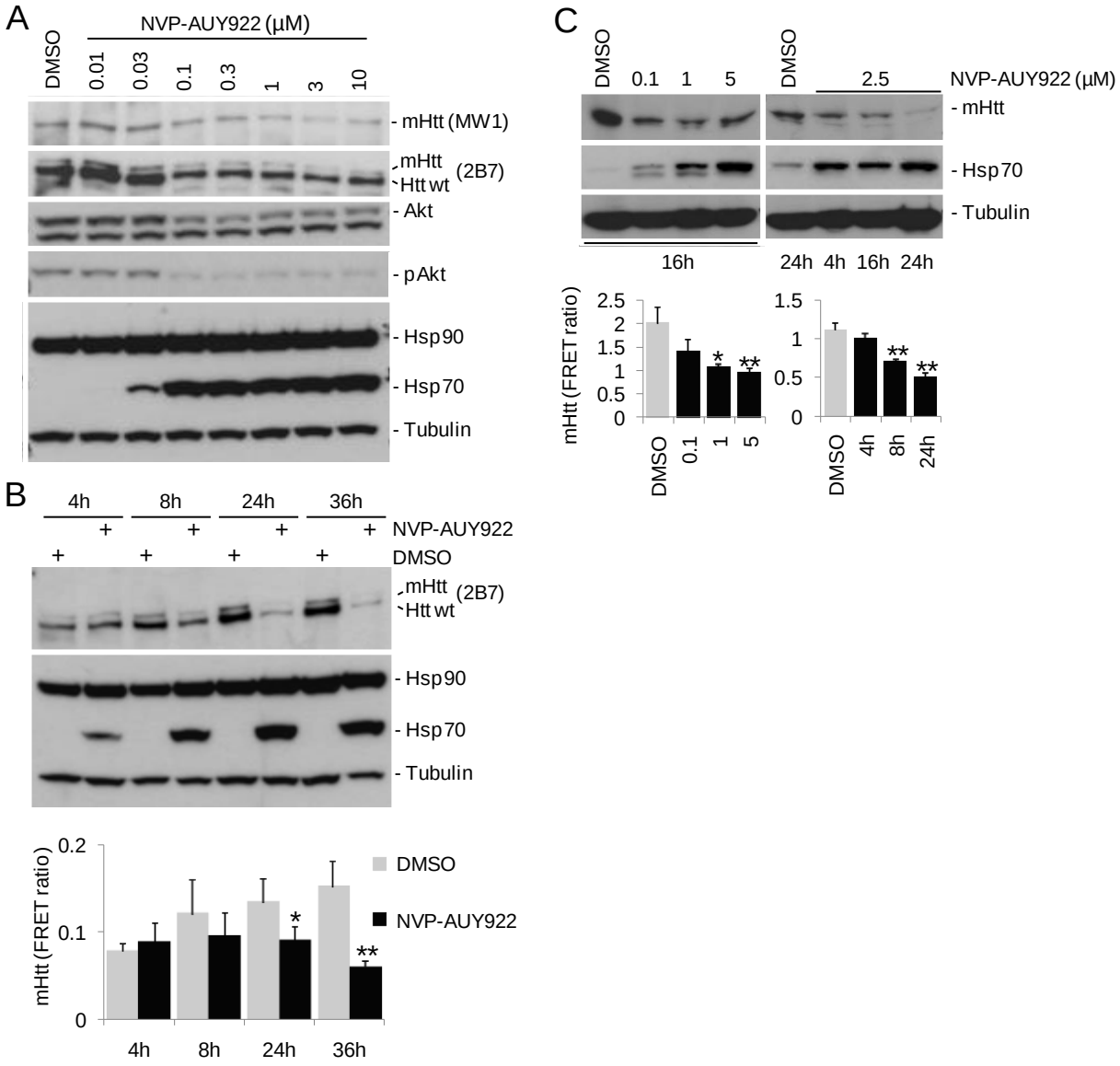

Figure 18. NVP-AUY922 application reduces levels of full-length mut Htt in *Hdh*Q150 ES cells and in ES derived neurons. *Hdh*Q150 ES cell (heterozygous) western blot data for mut Htt and selected marker proteins after o/N application (A) or application of 0.3 µM NVP-AUY922 for different time frames (B) are shown. Lower panel in B: quantification of mut Htt by TR-FRET (2B7-Tb; MW1-d2; *p = 0.012; **p = 0.006 versus DMSO at time points, n = 3). C. ES cells expressing full-length mut Htt with 145 glutamine derived neurons residues were differentiated into neurons and cultured for 14 days. NVP-AUY922 at the different concentrations indicated was applied o/N or for the indicated time points; upper panel: western blots, lower panel: quantification of mut Htt by TR-FRET (2B7-Tb, MW1-d2); *p = 0.002; **p < 0.001 (versus DMSO, n = 3).

To investigate the effect of Hsp90 inhibition on full-length human mut Htt in a neuronal context, mouse ES cells expressing mut Htt were differentiated into neurons as described (Bibel et al.,

2007). NVP-AUY922 reduced concentration- and time-dependently mut Htt protein levels as measured by western blot and TR-FRET (Figure 18C). As observed with *Hdh*Q150 ES cells, NVP-AUY922 caused a concentration-dependent induction of Hsp70 protein expression (Figure 18C).

Inhibition of Hsp90 chaperone function rather than induction of a heat shock response is critical for NVP-AUY922 induced degradation of soluble mut Htt.

Pharmacological Hsp90 inhibition can facilitate protein degradation either through disruption of the Hsp90 chaperone/ client protein complex or indirectly via Hsf1 mediated upregulation of other heat shock proteins, such as Hsp70 and Hsp40. We noted a lack of Hsp70 and Hsp25 (*Hspb1*) induction after NVP-AUY922 treatment in the HN10 cell line used in the primary screen (Figure 19A) suggesting that the HSR is not essential for the degradation of soluble mut Htt after Hsp90 inhibition. Application of 0.3 µM of the protein synthesis inhibitor cycloheximide to HN10 cells for 24 hours did not block NVP-AUY922 induced mut Htt degradation (Figure 19B). Similarly, cycloheximide did not affect mut Htt degradation in *Hdh*Q150 cells, but completely abolished Hsp70 induction (Figure 19C). Furthermore, overexpression of dominant negative Hsf1 (Hsf1-DN) (Heldens et al., 2010) in *Hdh*Q150 cells attenuated Hsp70 induction but did not block mut Htt degradation induced by NVP-AUY922 (Figure 19D). The data suggested that Hsp90 inhibition mediated degradation of soluble mut Htt was independent of Hsp70 induction.

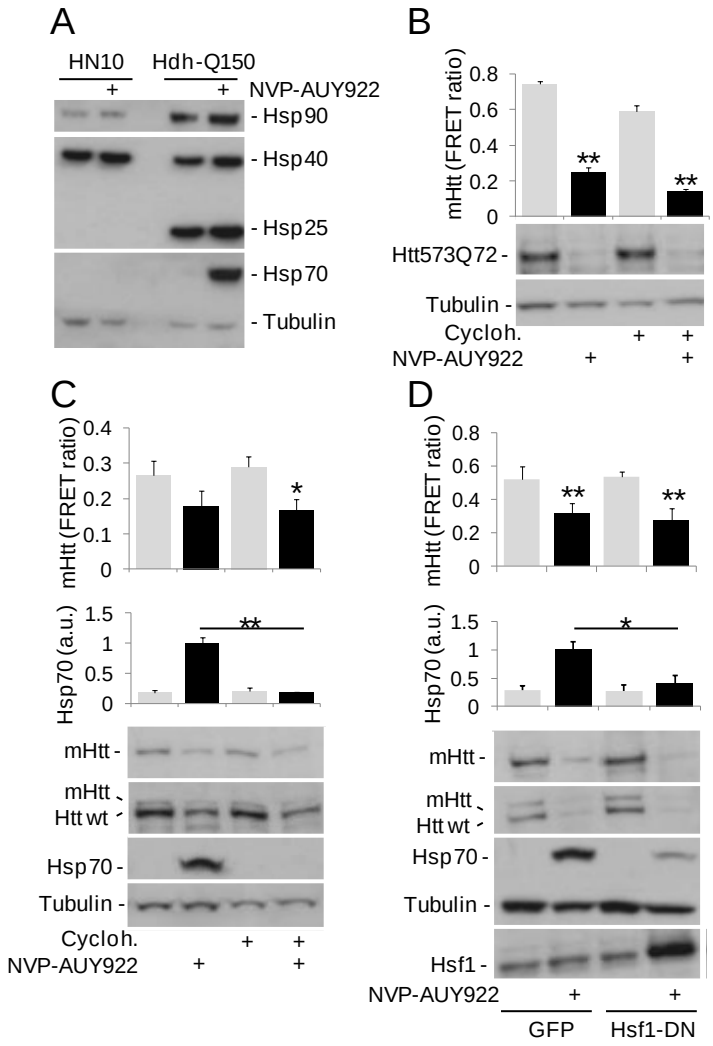

Figure 19. Degradation of mut Htt after Hsp90 inhibition does not require induction of Hsp70. A. Western blots analysis of expression of heat shock proteins in lysates from HN10-Htt573Q72 (HN10) and *Hdh*Q150 cells. In contrast to *Hdh*Q150 cells, 0.3 µM NVP-AUY922 does not induce Hsp70 and Hsp25 in HN10 cells. B. C. Application of 0.3 µM cycloheximide for 24 hours to HN10 (B) or *Hdh*Q150 cells (C) does not abolish mut Htt degradation induced by 0.3 µM NVP-AUY922 (24 h application; control DMSO; n = 4). HN10-Htt573Q72 cells were cultured in medium without expression inducer ligand RSL1 from the time of compound addition on ('wash out'). In *Hdh*Q150 cells 0.3 µM cycloheximide abolishes Hsp70 induction. D. Transient overexpression (36 hours) of Hsf1-DN in *Hdh*Q150 cells attenuates NVP-AUY922 induced Hsp70 induction (*p < 0.01 versus GFP; n = 3) but does not affect mut Htt degradation (**p < 0.001 versus DMSO, n = 6). B-D. upper panels: mut Htt TR-FRET and Hsp70 quantifications, lower panels: western blots.

mut Htt is a client protein of Hsp90.

Co-immunoprecipitation experiments were performed on HN10-Htt573Q72 cells treated for 4 hours with NVP-AUY922 (5 µM) or DMSO (Figure 20A). An antibody directed against Hsp90 co-immunoprecipitated Htt573Q72 as well as the Hsp90 co-chaperone p23 (Johnson and Toft, 1995) from lysates of DMSO, but not from NVP-AUY922 treated cells. Likewise, Htt573Q72

and Hsp90 were co-immunoprecipitated by p23 antibodies from lysates of DMSO but not from NVP-AUY922 treated cells (Figure 20A). Pharmacological Hsp90 inhibition promotes dissociation of p23 from the Hsp90/ client protein complex (Johnson and Toft, 1995) and thus the absence of co-immunoprecipitation after compound treatment, provided a control for Hsp90 inhibition by NVP-AUY922. Hsp90/ mut Htt co-immunoprecipitation was also observed in lysates from ES cells expressing either an N-terminal 1000 amino acid fragment or full-length mut Htt (Figure 20B). Again, the molecular interaction was disrupted after Hsp90 inhibitor treatment. In summary, the co-immunoprecipitation data suggested that mut Htt is a client protein of Hsp90 and that inhibition of Hsp90 chaperoning activity led to destabilization and subsequent degradation of mut Htt.

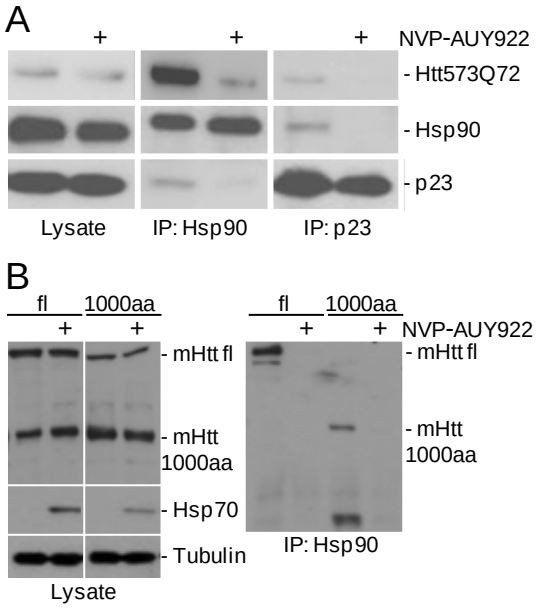

Figure 20. Mut Htt interacts with the Hsp90 chaperone complex. A. Induced HN10-Htt573Q72 cells were incubated for 4 hours with NVP-AUY922 (5 µM) or DMSO in medium without expression inducer ligand RSL1 ('wash out'). Immunoprecipitation was done using the antibodies as indicated. Whole cell lysates (lysate) were loaded as control. B. Anti Hsp90 antibody co-immunoprecipitates mut Htt from ES cell lines expressing either a full length (fl) or a 1000 amino acid N-terminal fragment transgene (1000aa) with 145 glutamine residues. NVP-AUY922 or DMSO application was for 4 hours. Note that full length transgenic mut Htt (fl) migrates close to wt Htt endogenously expressed from the cells, and that there is a non specific band at the size of the 1000aa fragment detected with Htt antibody 2B7.

Hsp90 inhibition facilitates mut Htt degradation through the proteasome.

To investigate further the mechanisms of mut Htt clearance after Hsp90 inhibition HN10-Htt573Q72 cells were treated with NVP-AUY922 at different concentrations in the presence or

absence of the proteasome inhibitor epoxomicin. In the presence of NVP-AUY922 mut Htt levels were again reduced as measured by both western blot and TR-FRET (Figure 21A, B). Epoxomicin co-treatment attenuated mut Htt degradation suggesting that mut Htt clearance after Hsp90 inhibitor treatment was, at least in part, mediated through the proteasome. To further investigate mut Htt degradation kinetics HN10-Htt573Q72 cells were cultured for three days in medium containing 750 nM of the mut Htt expression inducing ligand RSL1. Subsequently the cells were cultured in non-inducing medium (wash out) in the presence or absence of 5 µM NVP-AUY922 and/ or 50 nM epoxomicin. Western blot analysis and mut Htt quantifications by TR-FRET demonstrated that Htt573Q72 degradation was not significantly affected by epoxomicin when applied alone. However, Hsp90 inhibition by NVP-AUY922 facilitated mut Htt degradation, which was partially attenuated by proteasome inhibition with epoxomicin (Figure 21C, D). We concluded that mut Htt became a substrate for the ubiquitin proteasome system (UPS) when released from the Hsp90 complex.

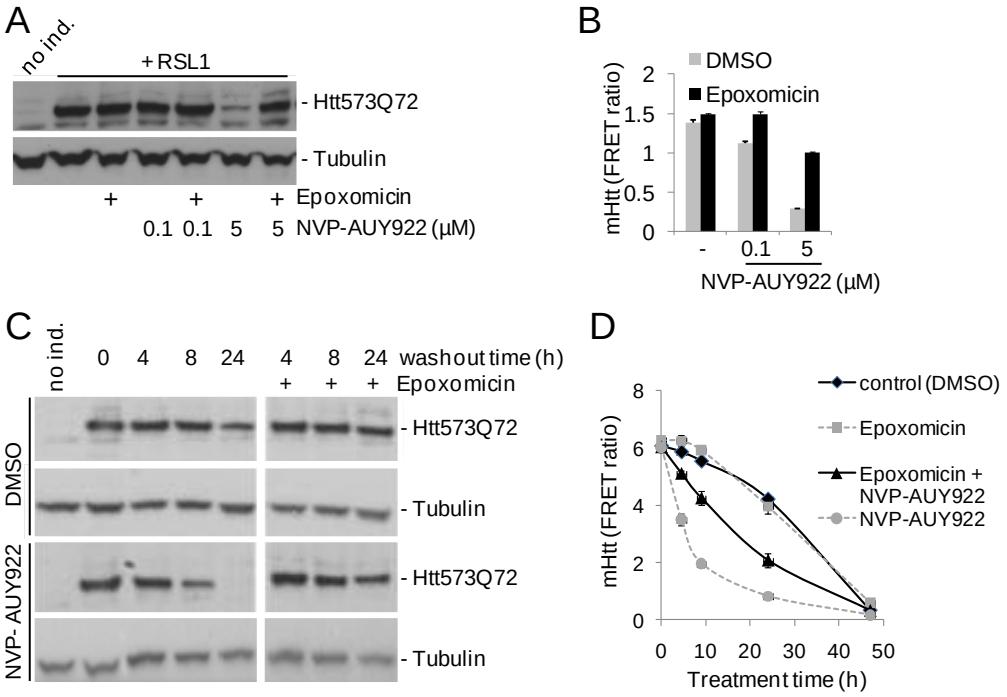

Figure 21. Hsp90 inhibition induces proteasome-dependent Htt degradation. Induced HN10-Htt573Q72 cells were treated o/N with epoxomicin and/ or NVP-AUY922 at different concentrations indicated A, B. Application of NVP-AUY922 reduces mut Htt protein and this effect is partly attenuated by 200 nM epoxomicin. A. mut Htt western blot detection with 2B7; B. mut Htt quantification by TR-FRET; n = 3. C. Hsp90 inhibition accelerates mut Htt degradation kinetics. HN10-Htt573Q72 cells were cultured in medium without expression inducer ligand RSL1 from the time of NVP-AUY922/ epoxomicin treatment on. D. Quantification of NVP-AUY922 induced mut Htt degradation by TR-FRET (n = 3) in the presence or absence of 50 nM epoxomicin. Cells as under (C) were harvested at different time points indicated. NVP-AUY922 induced mut Htt degradation is partly reversed by epoxomicin.

Ubiquitination of mut Htt is increased upon Hsp90 inhibition.

Proteasomal degradation requires protein polyubiquitination. We failed to conclusively demonstrate ubiquitination of the Htt573Q72 fragment expressed from the HN10 cell line, probably due to limited detection sensitivity (not shown). Therefore, we switched to a transient expression system. Plasmid constructs expressing hemagglutinin-tagged (HA) ubiquitin and Htt573Q72 were transiently transfected into HEK293T cells. The cells were then treated over-night with the Hsp90 inhibitor NVP-AUY922 in the presence or absence of the proteasome inhibitor MG132. Immunoprecipitation was conducted with antibodies directed against HA and Htt (Figure 22). Proteasome inhibition revealed basal levels of mut Htt ubiquitination that increased robustly in the presence of Hsp90 inhibition, supporting the conclusion that Hsp90 inhibition triggered Htt ubiquitination (Figure 22).

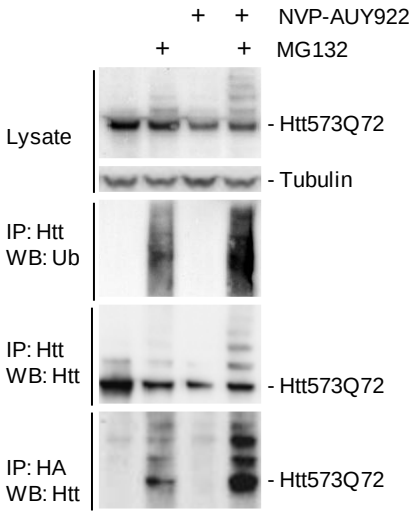

Figure 22. Mut Htt is ubiquitinated upon Hsp90 inhibition. HEK293T cells were transiently transfected with expression constructs for Htt573Q72 and HA-tagged ubiquitin. 24 hours later 1 µM NVP-AUY922, 5 µM proteasome inhibitor MG132 or a combination of the two was applied o/N. Immunoprecipitation was performed using anti-HA (Roche, 12CA5) and anti-Htt antibodies (2B7).

4.1.5 DISCUSSION

We identified Hsp90 inhibitors as enhancers of mut Htt degradation from a high throughput compound screen. The screen was aimed at exploring mechanisms reducing cellular levels of soluble mut Htt as this would be expected to lead to a reduction of the total cellular pool of mut Htt including aggregates and other, potentially toxic intermediate forms of mut Htt. Treatment with the potent and selective Hsp90 inhibitor NVP-AUY922 reduced mut Htt levels in different cell types including ES derived neurons (Figure 17, Figure 18).

To investigate a possible selective effect on mut Htt, a HN10 cell line co-expressing both mutant (Htt573Q72) and wt (Htt573Q25) forms was analyzed. NVP-AUY922 induced reduction of mutant Htt573Q72 appeared to be more pronounced compared to the wt Htt573Q25 fragment (Figure 17 B, E). However, different expression levels of mutant and wt Htt forms in this cell line and potentially different degradation kinetics in the absence of Hsp90 inhibition (see Figure 17E; DMSO) prevented a definite conclusion. In *Hdh*Q150 cells both mutant and wt full-length Htt were influenced by Hsp90 inhibitor treatment. However, substantially lower protein expression levels of mutant compared to wt Htt prohibited a meaningful quantification of NVP-AUY922 effects in this cell line. In summary, although a somewhat preferential effect on mut Htt cannot be excluded it is evident that Hsp90 inhibition by NVP-AUY922 enhanced the clearance of both mutant and wt Htt (Figure 17, Figure 18). Genetic inactivation of both *Htt* alleles is lethal during development, demonstrating essential functions of wt Htt (Duyao et al., 1995). Therefore, the value of mechanisms targeting both mutant and wt Htt remains questionable. However, siRNA and antisense oligonucleotide approaches targeting both mutant and wt Htt ameliorated HD-like symptoms and did not produce overt side effects in animal models (Sah and Aronin, 2011), suggesting that a concomitant decrease of both mutant and wt Htt may be beneficial in HD.

Disruption of the Hsp90-client complex facilitates proteasomal degradation of client proteins (Taipale et al., 2010). Hsp90 inhibitors also induce a stress response via Hsf1 leading to upregulation of other heat shock proteins such as Hsp70 and Hsp40. This can mediate an indirect clearance of protein aggregates especially via Hsp70-dependent E3 ubiquitin ligases (Taipale et al., 2010, Turturici et al., 2011). We identified Hsp90 inhibitors in a screen using an Htt573Q72 overexpressing hippocampal HN10 cell line. In this cell line mut Htt however was reduced after Hsp90 inhibitor treatment in the absence of any detectable Hsp70 induction (Figure 17, Figure

19). In contrast, the Hsp70 antibody used readily detected a strong upregulation of Hsp70 after NVP-AUY922 treatment of mouse *Hdh*Q150 ES cells and in mouse ES derived neurons (Figure 18, 19). In support of the HN10 cell data, a previous study on rat primary hippocampal neurons documented a lack of Hsf1 and Hsp70 induction after heat shock, in contrast to several other neuronal cell types investigated (Kaarniranta et al., 2002). Inhibition of protein synthesis with cycloheximide or overexpression of dominant negative Hsf1 attenuated Hsp70 induction in *Hdh*Q150 cells but did not inhibit NVP-AUY922 induced mut Htt degradation (Figure 19) demonstrating that disruption of the Hsp90-client protein complex, rather than Hsp70 induction, facilitated mut Htt clearance. Studies on the androgen receptor (AR) mutated in spinobulbar muscular atrophy further support the notion that destabilizing the Hsp90/ client protein complex can induce clearance of polyglutamine proteins independently of a HSR. 17-AAG-induced clearance of mutant AR appeared uncoupled from Hsp70 as only limited amounts of Hsp70 and Hsp40 were induced *in vivo* (Waza et al., 2005). Furthermore, the Hsp90 inhibitor 17-DMAG enhanced proteasomal clearance of mutant AR even when Hsp70 induction was blocked by siRNAs (Tokui et al., 2009). Moreover, Hsp90 inhibition blocked the formation of mutant AR aggregates in Hsf1 knock-out mouse embryonic fibroblasts that cannot induce Hsp70 and Hsp40 (Thomas et al., 2006). In summary, the data provide strong evidence that the mechanism of Hsp90 inhibitor mediated degradation of soluble mut Htt is the disruption of the Hsp90/ mut Htt client protein complex. Of note, a recent study has revealed an impairment of the HSR in HD mouse models (Labbadia et al., 2011). Our data suggest that the HSR is not essential for Hsp90 mediated degradation of soluble mut Htt.

Co-immunoprecipitation revealed a physical interaction of mut Htt with the Hsp90 chaperone complex (Figure 20) and pharmacological inhibition of Hsp90 induced mut Htt degradation (Figure 16 - 19). Thus, considering established criteria for Hsp90 clients (Taipale et al., 2010), our data support the conclusion that mut Htt is a client protein of Hsp90. Proteasome inhibition partially attenuated the clearance of mut Htt after NVP-AUY922 treatment (Figure 21), demonstrating that degradation through the ubiquitin proteasome system (UPS) is facilitated after release of mut Htt from the Hsp90 chaperone complex. In support of this degradation pathway mut Htt ubiquitination was increased after Hsp90 inhibition (Figure 22). Possibly, Hsp90-associated mut Htt remains protected from the intervention of ubiquitin-ligases, thereby explaining why mut Htt becomes a substrate for UPS-degradation when dissociated from the Hsp90 complex. Nevertheless, in the presence of NVP-AUY922, mut Htt degradation was only

partially attenuated by proteasome inhibition. This may provide evidence for additional, yet to be elucidated, epoxomicin-independent cellular degradation pathways of mut Htt. The cellular mechanisms of mut Htt degradation and a possible contribution of altered UPS functionality to disease pathology are still under debate. While some studies reported an impairment of the UPS others concluded that mut Htt does not lead to proteasomal dysfunction (Bowman et al., 2005, Bennett et al., 2007, Hunter et al., 2007, Ortega et al., 2010). In the transgenic R6/2 models of HD, which is characterized by rapid disease progression, proteasome activity was not altered compared to wt mice (Bett et al., 2006).

In conclusion, our data show that clearance of soluble Htt in different cellular systems including ES derived neurons can be induced via Hsp90 inhibition. Mut Htt is stabilized by the Hsp90 chaperone complex and pharmacological inhibition facilitates mut Htt release and proteasomal degradation. This effect is independent of a general HSR and Hsp70 induction. Since Hsp90 inhibition is expected to influence a variety of client proteins it is currently uncertain if targeting Hsp90 is selective enough to provide a means for therapeutic intervention in HD. However, further investigation of Hsp90 inhibitors and of mechanisms targeting Hsp90 co-chaperone functions in HD appear warranted.

ACKNOWLEDGEMENTS

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4.2 TR-FRET based duplex immunoassay reveals an inverse correlation of soluble and aggregated mutant huntingtin in mouse models of Huntington's disease

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(manuscript submitted)

4.2.1 SUMMARY

Huntington disease (HD) is an inherited neurodegenerative disorder caused by the amplification of a polyglutamine stretch at the N-terminus of the huntingtin protein. N-terminal fragments of the mutant huntingtin (mut Htt) aggregate and form intracellular inclusions in brain and peripheral tissues. Aggregates are an important hallmark of the disease, translating into a high need to measure and quantify them *in vitro* and *in vivo*. We developed a one-step TR-FRET based immunoassay to quantify soluble and aggregated mut Htt in cell and tissue homogenates.

Strikingly, quantification reveals inverse correlation of soluble and aggregated mut Htt in primary neuronal cell cultures, transgenic R6/2 and *Hdh*Q150 knock-in HD mice. These results emphasize the assay's efficiency for highly sensitive and quantitative detection of soluble and aggregated mut Htt and its application in high throughput screening and characterization of HD models.

4.2.2 INTRODUCTION

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease caused by the amplification of a polyglutamine (polyQ) stretch at the N-terminus of the huntingtin protein (Htt) (The Huntington's Disease Collaborative Research Group, 1993). N-terminal fragments of mutant Htt (mut Htt) are generated by proteolytic cleavage and acquire a misfolded conformation through the polyQ stretch, leading to the formation of nuclear and cytoplasmic aggregates (Hazeki et al., 1999, Lunkes et al., 2002, Wang et al., 2008a, Landles et al., 2010).

HD is characterized by progressive deposition of these insoluble aggregates involving the formation of intermediate states (fibrils and oligomers), whose precise composition and structure is still under investigation (Hoffner et al., 2005, Ratovitski et al., 2009, Legleiter et al., 2010). Whether the aggregates have a toxic role is still controversial (Gutekunst et al., 1999, Kuemmerle et al., 1999, Wanker, 2000) but the correlation between disease status and aggregate load found in post-mortem brains from HD patients highlights the importance to quantify their formation (DiFiglia et al., 1997, Gutekunst et al., 1999, Maat-Schieman et al., 1999).

When expressed in mice, mut Htt is highly pathogenic. For instance, R6/2 mice express an Exon1 mut Htt fragment with ~200 polyQ and develop an aggressive form of the disease with strong aggregation in the brain and peripheral tissues (Mangiarini et al., 1996, Stack et al., 2005).

*Hdh*Q150 knock-in mice, generated by introducing 150 polyQ in the endogenous Htt gene, show slower aggregate formation and disease progression (Mangiarini et al., 1996, Stack et al., 2005, Woodman et al., 2007, Sathasivam et al., 2010).

In order to evaluate a treatment effect on the modulation of aggregate load and to better characterize the role of aggregated and soluble mut Htt in the disease, sensitive and quantitative detection methods for different forms of mut Htt are essential. Currently mut Htt aggregate determination involves labor-intensive biochemical techniques (filter trap or AGERA (Wanker et al., 1999, Weiss et al., 2008)), a Seprion ligand based ELISA (Sathasivam et al., 2010), or immunohistochemistry assays which are often semiquantitative, therefore not sensitive enough to detect small variations (Hazeki et al., 2002, Mitsui et al., 2006).

In this study we combined the advantages of a TR-FRET assay - sensitivity, robustness, speed, usage of small volumes and duplexing potential - with the principle of using a single monoclonal antibody for detection of aggregated mut Htt, as shown for ELISA based detection of multimeric α-synuclein or amyloid-β peptide in human samples (El-Agnaf et al., 2006, Fukumoto et al., 2010). The development of this new technology enables for the simultaneous quantification of small variations in mut Htt aggregate load during disease progression in comparison to changes affecting the soluble pool of the mutant protein. Next to its application for characterizing HD models, due to its sensitivity and simplicity, the method is also applicable to high throughput screenings to evaluate modifiers of disease progression.

4.2.3 MATERIALS AND METHODS

Antibodies.

MW1 and MW8 antibodies were developed by Paul Patterson (Ko et al., 2001) and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Generation and characterization of 2B7 was described previously (Weiss et al., 2009a). 4C9 antibody was raised against the human polyproline region in exon1 of the Huntingtin protein. Generation and characterization of β1 antibody was described previously (Paganetti et al., 1996).

Tissue homogenization and aggregates ultracentrifugation.

Crude tissue homogenates were prepared by homogenization in 10 volumes (w/v) of PBS+1% TritonX100+Complete Protease Inhibitor (Roche, Switzerland) using preCellys tubes (Precellys) and 2 lysis cycles of 10" at 6000rpm. Total homogenate protein concentration was determined with BCA assay (Thermo Scientific).

For the generation of soluble and insoluble, aggregates mutant Huntingtin fractions, 1ml R6/2 and wt brain tissues (~100ug) were homogenized using a dounce homogenizer in PBS+1% TritonX100 and centrifuged at 13.000rpm for 5min. 100ul of the supernatant was kept to be analyzed with AGERA (Start fraction), while 500ul were subsequently ultracentrifuged at 160000g for 1.5h.The resulting supernatant was transferred to a fresh tube (Supernatant fraction). Pellet was resuspended and washed with lysis buffer. After a final ultracentrifugation step at 160000g, the pellet was resuspended in 500ul lysis buffer (Pellet fraction). The three fractions were analyzed in parallel with AGERA and TR-FRET for the detection of soluble and aggregated Htt.

Agarose gel electrophoresis for resolving aggregates.

Biochemical aggregate detection was performed using agarose gel electrophoresis for resolving aggregates (AGERA) assay as previously described (Weiss et al., 2008). Briefly, 1.8g agarose was dissolved in 100ml 375mM Tris HCl pH 8.8 by boiling in a microwave. SDS was added to a final concentration of 0.1% and gels were poured. 50ug of homogenates were loaded per lane. AGERA gel was run at 100V in Tris Glycine SDS Running Buffer (Invitrogen). The gel was then blotted at 15V for 1h on a PVDF membrane (Immobilon-P, Millipore), blocked with 5% milk for 1h at RT and incubated over night with primary antibody, used at a concentration of 1.5ug/ml in 2% milk diluted in TBS+0.1% Tween. After washing, membranes were incubated for 2h with HRP anti mouse secondary antibody 1:10000 in 2% milk diluted in TBS+0.1% Tween and developed using ECL (GE Healtcare).

Time resolved fluorescence resonance energy transfer for soluble and insoluble huntingtin detection.

Antibody labeling with terbium (Tb), d2 and Alexa488 fluorophores was performed by CisBio Bioassays. Time resolved fluorescence resonance energy transfer (TR-FRET) detection of soluble mutant huntingtin protein in a singlex readout was performed as described (Weiss et al., 2009a).

In brief, 5µl tissue homogenate sample was transferred to low-volume wells of white 384-microtiter plates (Greiner, USA). 1µl detection buffer (50mM NaH_2PO_4, 400mM NaF, 0.1% BSA and 0.05% Tween+antibody mix) was added with the final antibody amount per well for soluble mutant huntingtin detection being 1ng 2B7-Tb+10ng MW1-d2. TR-FRET readout was performed with an EnVision Reader (Perkin Elmer, USA). After the excitation of the donor fluorophore terbium at 320nM and a time delay of 100µs, the resulting terbium and d2 emission signals were read at 620nm and 665nm respectively.

Single TR-FRET detection of insoluble, aggregated mut Htt was performed either by using MW8 antibody labeled with Terbium (donor) or d2 fluorophores (acceptor), or by 4C9 antibody labeled with Terbium (donor) or Alexa488 fluorophore (acceptor). TR-FRET protocol was similar to the one used for soluble mutant Huntingtin with following modifications: when using Alexa488 labeled 4C9 antibody as an acceptor antibody, Alexa488 specific emission signal was quantified at 520nm. Duplex TR-FRET assay for simultaneous quantification of soluble and aggregates FR-TRET was performed by addition of 1µl of 1ng 2B7-Tb + 10ng MW1-d2 and 1ng 4C9-Tb +10 ng 4C9-Alexa containing antibody mix per well.

Animal models.

Heterozygous R6/2 mice (Mangiarini et al., 1996) and heterozygous *Hdh*Q150 knock-in mice (Lin et al., 2001) were obtained from the laboratory of G. P. Bates. R6/2 were on a mixed C57BL/6 x CBA/Ca background. The colony was maintained by breeding them with B6xCBA/CaF1 females. *Hdh*Q150 mice were maintained on a C57BL/6J background. The offspring were genotyped by polymerase chain reaction (PCR) using DNA obtained from ear punches. The animals were housed in a temperature-controlled room that was maintained on a 12h light/dark cycle. Food and water were available ad libitum. Animals were sacrificed by decapitation in deep isoflurane narcosis. Tissues were then collected immediately and were snap-frozen on a metal plate placed on dry ice. All experiments were carried out in accordance with local guidelines for the care and use of laboratory animals.

Analysis of soluble and insoluble mut Htt aggregates by Size Exclusion Chromatography.

A) Analysis of mut Htt aggregates in the supernatant by Size Exclusion Chromatography and TR-FRET.

Soluble Fractions: Half brain tissues from R6/2 were homogenized with Precellys according to the manufacturer instructions in 500 µL of sample buffer (1% Triton X-100 in PBS), Complete Protease Inhibitor (Roche) and PhosSTOP phosphatase inhibitor cocktail (Roche) and finally sonicated for 10s.Brain extracts were then clarified by ultra-centrifugation for 30 min at 100,000 g. Supernatant were filtered through 0.45 um membrane and fractionated by SEC on a Superdex 200 10/300 column. All of the SEC experiments were performed at 4 °C with a flow rate of 0.5 ml/min. The elution was one column volume. The total protein loaded on the column were 1.7 mg in a sample volume of 500 µl . Protein standards with 0.5% Triton X-100 in PBS were used to estimate size of Htt aggregatess (standard data not shown). Fractions (250 µl volume/fraction) were collected in 96-well plate format and 10µl from each were applied for TR-FRET measurement with indicated antibody combination.

B) Analysis of mut Htt aggregates in the pellet by TR-FRET.

Insoluble Fractions: Pellet from R6/2 brain homogenate obtained by centrifugation at 100’ 000 g (density separation) were washed once (PBS + 1% Triton X-100) and resuspended in 2% Triton X-100 in PBS. 10µl of the suspension were assayed to TR-FRET measurement with indicated antibody combinations.

Primary neuronal cultures.

Animals were maintained in accordance with Duke University Medical Center Institutional Animal Care and Use Committee guidelines (approval #A248-08-09). Cortico-striatal co-cultures were prepared as described (Kaltenbach et al., 2010). Briefly, striata and cortices were dissected from E18 embryonic rat brains and dissociated separately. $5x10^6$ cells were counted and transfected (Nucleofector, Lonza) with plasmids expressing Htt exon1 fragment carrying 73 CAG expansion or a control plasmid. Neurons were plated onto 96-well plates containing previously isolated astroglia feeder layers (2000 cells/well) and cultured in Neurobasal media (Invitrogen) supplemented with 5% fetal calf serum (Sigma-Aldrich), 2 mM glutamine (Glutamax, Invitrogen), 10 mM potassium chloride, and 5 µg/mL gentamicin at 37°C in 95% 0_2/5%CO_2. BDNF (Sigma) was diluted in Neurobasal media, added to the neurons immediately after plating and replenished every other day until analysis. For TR-FRET analysis, cells were resuspended in

lysis buffer (PBS+1%TritonX100+Complete Protease Inhibitor tablet, Roche), shaken vigorously for 30 minutes at 4°C and then stored at -80°C.

Statistics.

The data in the graphs correspond to the average of TR-FRET signals relative to the wt samples and the bars are representative of the standard deviation (SD) among the replicates. The significance has been calculated with two-tailed, homoscedastic, t-student test.

Error bars = standard deviation; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$

4.2.4 RESULTS

Huntingtin antibodies detect high molecular weight aggregates with different specificity.

We have recently reported a TR-FRET based immunoassay for the detection of soluble mut Htt species in cell lysates and tissue homogenates (Weiss et al., 2009b, Weiss et al., 2010). The first question we addressed is whether we could detect large mut Htt aggregates using the same technology but with aggregate specific antibodies. The combination of both detection systems for soluble and aggregated mut Htt would allow us to track the protein aggregation over time in biological samples.

The assay for soluble mut Htt utilizes two labeled monoclonal antibodies directed towards proximal N-terminal epitopes. One of the antibodies, MW1, is specific for the elongated polyQ stretch through which mut Htt aggregates. The epitope would therefore be masked in presence of mut Htt aggregates, resulting in loss of TR-FRET signal (Figure 23A, upper panel). In contrast, mut Htt aggregates will present multiple binding sites for one single antibody in close proximity. This would allow simultaneous binding of the monoclonal antibody labeled with donor and acceptor fluorophores and thus generation of a TR-FRET signal (Figure 23A, lower panel).

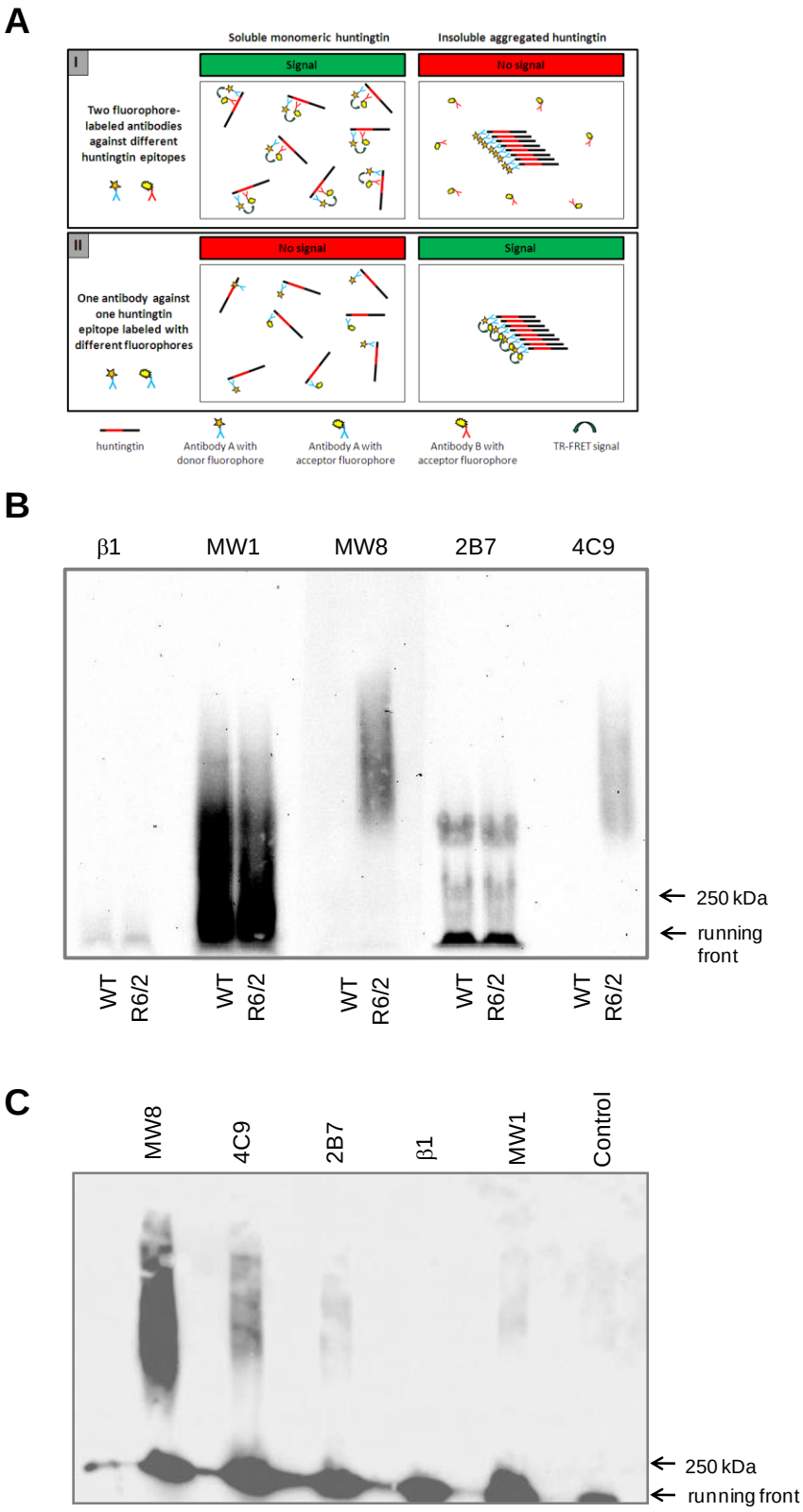


Figure 23. Htt antibodies detect mut Htt aggregates with different affinities. A: Representation of the principle behind Htt-aggregate detection by TR-FRET. Soluble mut Htt protein can be recognized utilizing two antibodies A

and B directed against different, sterically unblocked epitopes. The close proximity of donor (A) and acceptor (B) fluorophores results in a TR-FRET signal in this state (I, left box). When mut Htt protein forms insoluble aggregates only antibody A recognizes it, as the specific epitope is still sterically unblocked. In contrast, antibody B, directed against the expanded polyQ-stretch – red region in Htt-exon1 protein – can no longer bind. Under these conditions, A and B are not in close enough proximity to result in signal (I, right box). On the contrary, a mixture of antibody A labeled with either donor or acceptor fluorophore results in a TR-FRET signal for aggregated (II, right box) but not for the soluble Htt (II, left box).
B: High molecular weight mut Htt aggregates in brain homogenates of 12 week-old wt or R6/2 mice are specifically detected by MW8 and 4C9 antibodies on an AGERA blot. MW1 antibody shows an unspecific signal in both WT and R6/2 brains. Similar to β1, an amyloid-beta specific control antibody, 2B7 antibody does not detect mut Htt aggregates from R6/2 brain.
C: mut Htt aggregates were successfully immunoprecipitated with 4C9 and MW8 antibody, only weakly with 2B7 and MW1, but not with β1 or the control (G-Sepharose beads). Samples were resolved on AGERA blot and detected with MW8.

The first challenge was therefore to find the right antibodies for a TR-FRET based assay specific and selective for mut Htt aggregate detection (antibodies used in this study are presented in Figure 24). We first performed AGERA blotting using brain homogenates of wild-type (wt) and R6/2 transgenic mice. 4C9 and MW8 antibodies showed specific mut Htt aggregate detection in R6/2 when compared to wt homogenates (Figure 23B). In contrast, MW1 and 2B7 antibodies were not aggregates specific and resulted in a diffuse and mut Htt unspecific signal in wt as well as R6/2 samples. Negative control antibody β1 directed against β-amyloid peptide, failed to recognize mut Htt aggregates.

As the TR-FRET assay is based on antibody-antigen interactions in solution, we wanted to further validate the specific binding of 4C9 and MW8 to mut Htt aggregates by immunoprecipitation. Aggregates were efficiently immunoprecipitated by 4C9 and MW8 but not by 2B7, MW1 and β1 antibodies, confirming the specificity of these two antibodies for high molecular weight complexes of mut Htt in solution (Figure 23C). These results indicate that 4C9 as well as MW8 antibodies are mut Htt aggregate specific tools for the development of a TR-FRET based immunoassay for mut Htt aggregates.

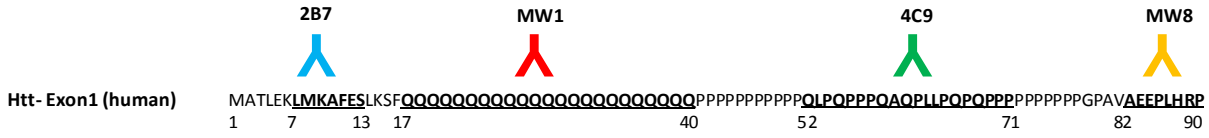


Figure 24. Exon1-protein sequence of human huntingtin containing a 23Q polyglutamine stretch. Epitopes of the anti-huntingtin antibodies used in the study (2B7, MW1, 4C9 and MW8) are underlined.

TR-FRET detection of aggregated mut Htt.

Having demonstrated that 4C9 and MW8 detect mut Htt aggregates by AGERA and immunoprecipitation methods, we proceeded to evaluate whether 4C9 and MW8 could detect mut Htt aggregates via TR-FRET, based on our model in Figure 23A. The antibodies were labeled with donor or acceptor fluorophores, respectively Terbium (Tb) and d2 or Alexa488 (Alexa). Wt and R6/2 brain homogenates from 12 weeks old mice were analyzed with 4C9 Tb - 4C9 Alexa (4C9/4C9), MW8 Tb - MW8 d2 (MW8/MW8) and 2B7 Tb - 2B7 d2 (2B7/2B7) as a negative control pair. As expected, use of the 2B7/2B7 combination did not result in a mut Htt aggregate-specific signal, whereas a significant difference between wt and R6/2 samples was obtained with the two antibody combinations MW8/MW8 and 4C9/4C9 (Figure 25A), indicating the specificity of mut Htt aggregate detection based on TR-FRET.

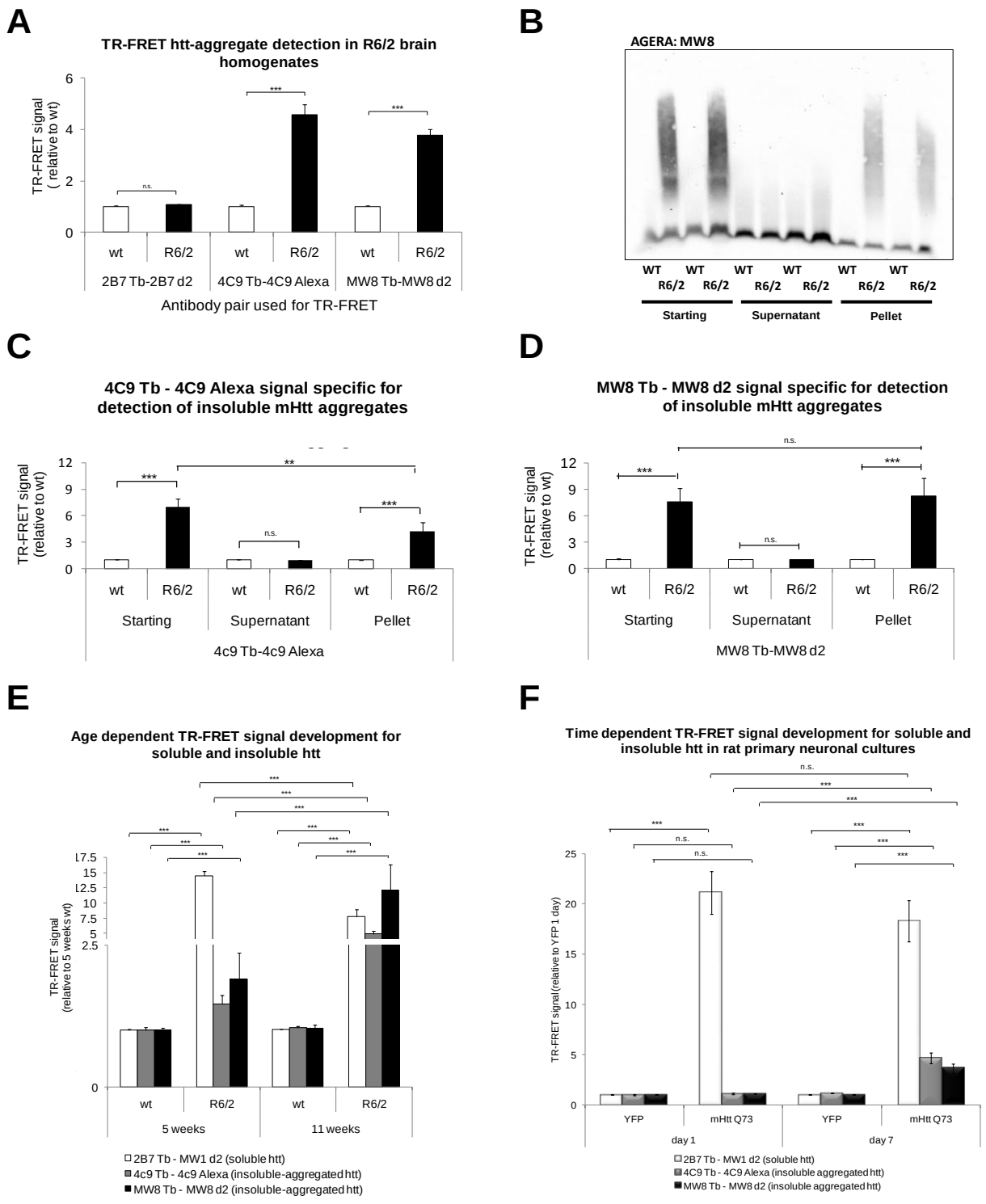


Figure 25. Antibody pairs expected to detect mut Htt aggregates work for TR-FRET. A: TR-FRET assay on brain homogenates of 12 week-old wt or R6/2 with a mixture of one mut Htt antibody labeled with either donor or acceptor fluorophore. Antibodies 4C9 or MW8 detect a mut Htt specific signal in R6/2 brains whereas 2B7 fails to detect TR-FRET signal (n=3, 0.5µg protein loaded per 384-well).

B: Brain homogenates of 12 week-old wt or R6/2 mice (starting fraction) were separated by ultracentrifugation into a soluble (supernatant) and an insoluble (pellet) fraction (n=2). AGERA blot with MW8 antibody shows that all mut Htt aggregates were recovered in the pellet fraction whereas no mut Htt specific aggregates were found in the supernatant fraction.
C/D: TR-FRET assay for ultracentrifugation fractions shows that MW8/MW8 and 4C9/4C9 specifically detect insoluble aggregated mut Htt but not soluble protein recovered in the supernatant fractions.
E: Age dependent increase of insoluble aggregated mut Htt in 4 and 12 week-old R6/2 brain homogenates is detected by TR-FRET using MW8/MW8 and 4C9/4C9. In contrast, TR-FRET analysis with 2B7/MW1 reveals a correlating decrease in soluble mut Htt over time (n=3).
F: Time dependent increase of insoluble aggregated mut Htt is detected by TR-FRET using the MW8/MW8 and 4C9/4C9 in rat primary striatal-cortical co-cultures lysates transiently transfected with YFP or Exon1-Htt with 73Q (n=3 per condition). TR-FRET analysis with 2B7/MW1 shows a decrease in soluble mut Htt over time.
Error bars = standard deviation; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

To further prove the specificity of the new TR-FRET assay for mut Htt aggregates, we separated 12 weeks R6/2 mice brain homogenates into soluble (supernatant) and insoluble (pellet) fractions by ultracentrifugation with a force of 160 000g. Under this condition, all insoluble aggregates sediment. All aggregates present in the starting fraction were recovered in the pellet fraction as shown by AGERA blot (Figure 25B). This result is concordant with previous results where ultracentrifugation was used to identify different populations of purified tagged mut Htt (Olshina et al., 2010). Next we analyzed the three fractions by TR-FRET. Both the 4C9/4C9 and MW8/MW8 combinations specifically recognized the aggregated mut Htt in the starting homogenate and in the pellet fractions, but failed to generate any signal in the supernatant (Figure 25C, D). In contrast, 2B7/MW1 combination generates signal in the starting homogenate and in the supernatant, consistent with the specificity for soluble mut Htt (Weiss et al., 2009a) (Figure 26).

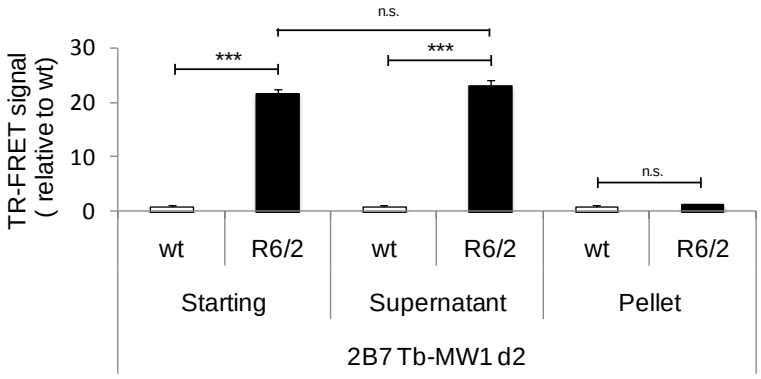


Figure 26. WT and R6/2 brain homogenates were separated into a fraction containing soluble mut Htt (supernatant fraction) or insoluble aggregated mut Htt (pellet fraction). TR-FRET analysis of the fraction reveals that the 2B7/MW1 antibody pair detects only soluble mut Htt present in the supernatant fraction but not aggregated mut Htt.

Taken together, we have shown that 4C9/4C9 as well as MW8/MW8 assays detect specifically insoluble mut Htt aggregates, while 2B7/MW1 antibody combination is specific for soluble mut Htt. These results confirmed the hypothesis we claimed in our aggregation model (Figure 23A). To our knowledge this is the first time that a simple one-step assay detects specifically non-tagged insoluble mut Htt aggregates in solution.

We proceeded to use the new TR-FRET assays to characterize mut Htt aggregation in biological samples over time. Previous works showed an inverse correlation between aggregated and soluble mut Htt as a function of disease progression in R6/2 mice (Woodman et al., 2007, Weiss et al., 2009a, Sathasivam et al., 2010). We therefore asked if we could confirm this inverse correlation with the TR-FRET technology using antibody combinations for soluble (2B7/MW1) and aggregated mut Htt (MW8/MW8 and 4C9/4C9; (Figure 25E)). Indeed, analysis of R6/2 mice at disease onset (5 weeks of age) and late manifest state (11 weeks of age) show progressive formation of aggregated mut Htt with a correlating decrease of soluble mut Htt. This confirmed previous findings and underlined the robustness and specificity of the TR-FRET assay.

Next to its application in tissue samples, we asked whether the mut Htt TR-FRET assay can be used in a screening format to quantify untagged mut Htt aggregates in neuronal cell cultures. To this aim, we used exon1-73polyQ or Yellow Fluorescent Protein (YFP) transiently transfected primary neuronal cortico-striatal co-cultures in 96 well microtiter plate. We monitored mut Htt aggregation over time using the TR-FRET assay. Both MW8/MW8 and 4C9/4C9 combination detected aggregated mut Htt 7 days after transfection, accompanied by a decrease of the soluble mut Htt 2B7/MW1 signal (Figure 25F). These results demonstrate that the TR-FRET assays allow for monitoring changes of aggregated and soluble untagged mut Htt in neuronal co-cultures grown in microtiter plates.

We wanted to further verify the specificity of the 2B7/MW1 combination for soluble and 4C9/4C9 or MW8/MW8 for aggregated mut Htt by an independent method. We therefore proceeded to resolve different mut Htt subpopulations from 4 and 8 week old R6/2 brains by using size exclusion chromatography (SEC) (Lotz et al., 2010). Analysis of the fractions with 2B7/MW1 TR-FRET combination detected low molecular weight mut Htt species of around 300kDa at 4 weeks of ages which decrease in intensity at 8 weeks of age. In contrast, MW8/MW8 and 4C9/4C9 TR-FRET detect high molecular weight mut Htt species around 950kDa, whose intensity increases at 8 weeks of age. Intriguingly, heterogeneity did not change

temporally but the relative abundance of individual mut Htt aggregates did. No mut Htt TR-FRET signals were observed when using wt brain homogenates as a control (data not shown).

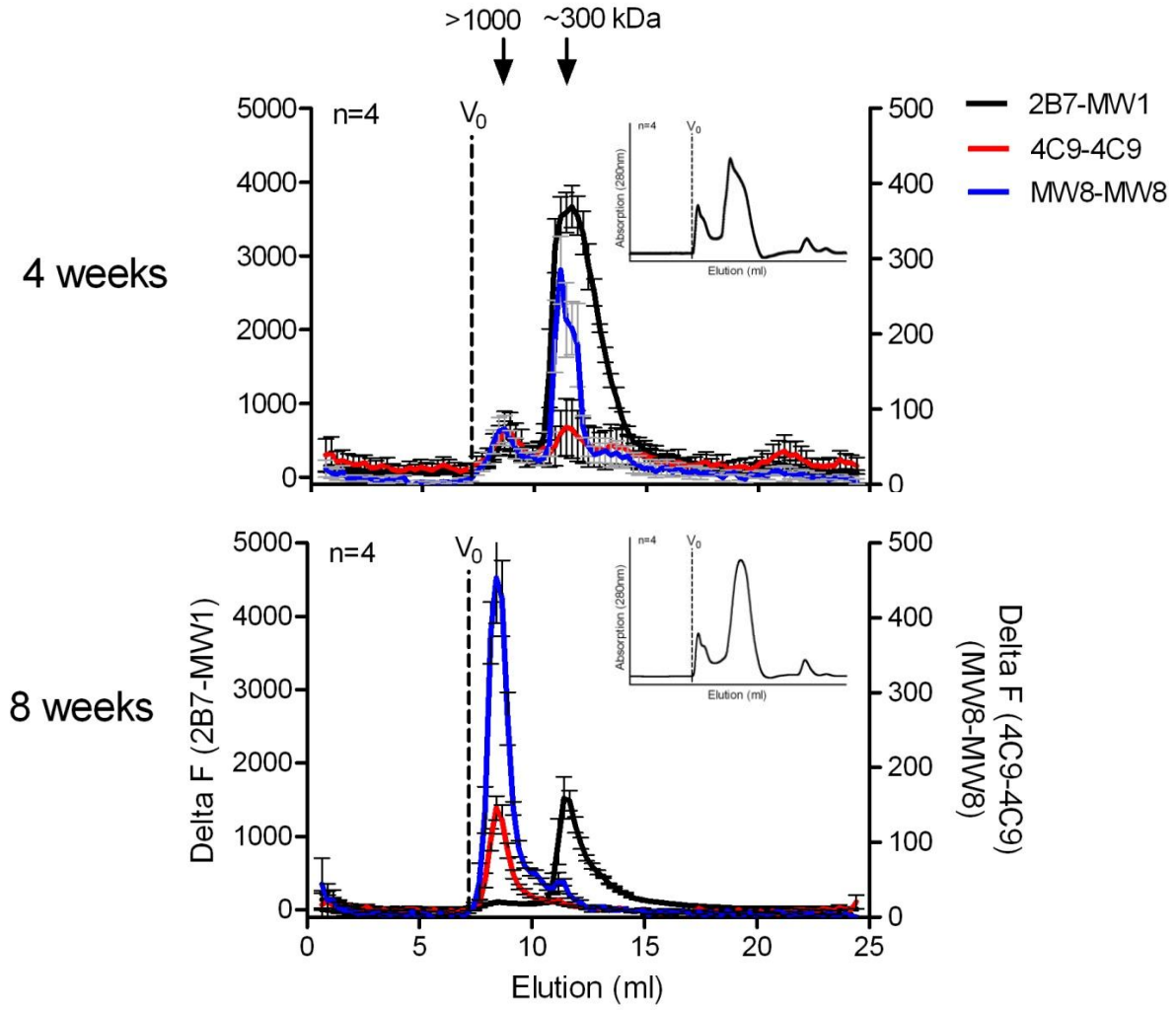


Figure 27. Detection of a subset of soluble mut Htt aggregates in R6/2 brain lysate at different age with a combination of Size Exclusion Chromatography and TR-FRET. Supernatant obtained from centrifuged R6/2 brain homogenate was loaded onto a Superdex200 column and the fractions were analyzed by TR-FRET with indicated antibody combination. Graphs show TR-FRET signal profiles of SEC eluted supernatant of R6/2 brain tissue homogenate at 4 weeks and 8 weeks of age (n=4 per age). Black line corresponds to 2B7/MW1, red to 4C9/4C9 and blue to MW8/MW8 TR-FRET signal profile. Small inner graph indicates total protein elution profile by UV. Arrows indicate estimated size of main peaks in kDa by using protein standards in same running buffer (protein standard data not shown).
Error bars = standard deviation.

Another important observation was that in 4 weeks old mice the MW8/MW8 combination but not the 4C9/4C9 combination detected a subset of low molecular weight mut Htt aggregates, partially overlapping with the 2B7/MW1 signal (Figure 27). This result suggests that all three TR-FRET

assays recognize different Htt species, where 4C9/4C9 and MW8/MW8 preferentially bind to aggregates and 2B7/MW1 to soluble mut Htt. Moreover, these results verify the inverse correlation of soluble and aggregated mut Htt over time in R6/2 mice and identify specific smaller and larger Htt aggregates that are distinguishable with the different TR-FRET antibody combinations.

We continued to investigate whether we could detect an inverse correlation of these mut Htt species during aging in different brain regions. To address this question, we analyzed different brain regions of female and male R6/2 mice at 4, 8, 12 and 15 weeks of age. For both genders and all brain regions we observed a consistent age-dependent decrease in soluble mut Htt associated with a progressive increase in aggregated mut Htt (Figure 28 and Figure 29). No significant differences between genders were found with the exception for soluble mut Htt in the cerebellum, which seemed to decrease more slowly in males than females. In cerebellum and hippocampus, aggregate deposition was less rapid than in the other brain regions when measured with the MW8/MW8 antibody pair but not with the 4C9/4C9 assay. These findings show that the inverse correlation between soluble and aggregated mut Htt found in total brain homogenates also occurs in sub-brain regions.

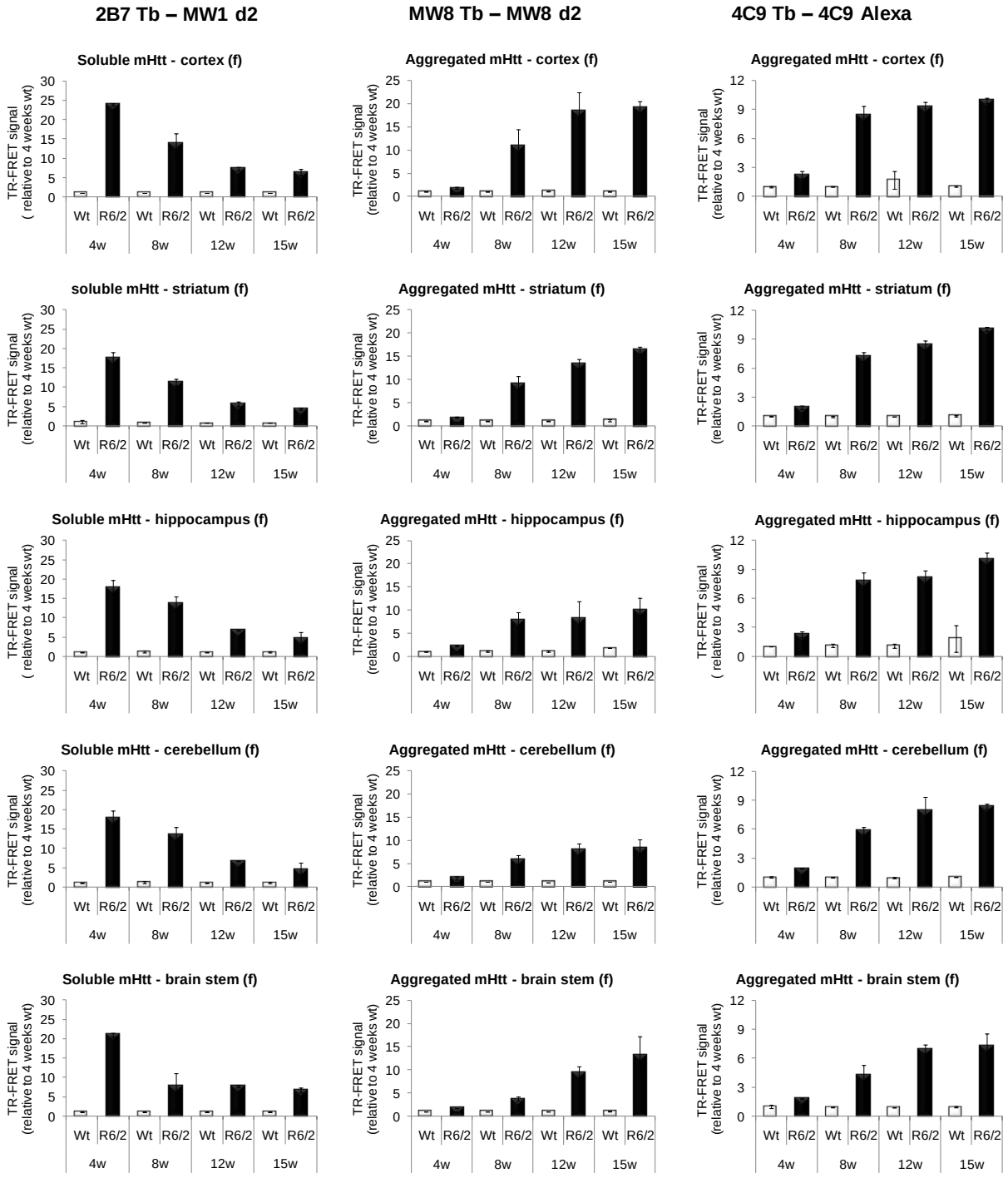


Figure 28. TR-FRET assay detects age- and disease-progression caused changes in soluble and insoluble mut Htt protein levels in female WT and R6/2 brain regions. Homogenates from different brain regions from wt and R6/2 female mice have been analyzed to detect soluble (2B7/MW1) and aggregated mut Htt (4C9/4C9; MW8/MW8) at different age (4, 8, 12 and 15 weeks). All regions analyzed showed a correlation between the decrease over time of soluble mut Htt signal and the progressive increase of the pool of aggregated mut Htt. Cerebellum shows a lower aggregation load, when analyzed with the 4C9 antibody but not with the MW8.

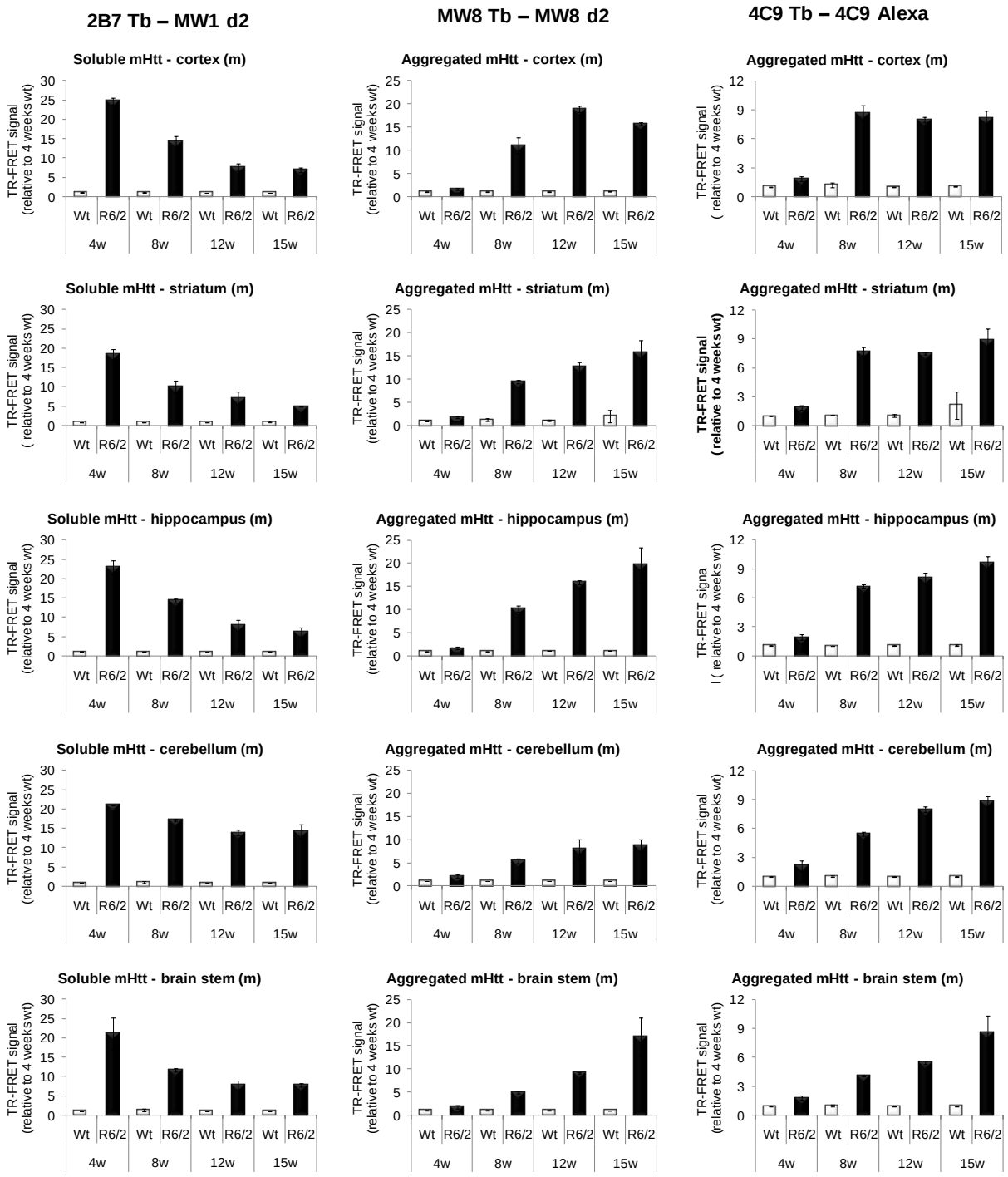


Figure 29. TR-FRET assay detects age- and disease-progression caused changes in soluble and insoluble mut Htt protein levels in male WT and R6/2 brain regions. Homogenates from different brain regions from wt and R6/2 male mice have been analyzed to detect soluble (2B7/MW1) and aggregated mut Htt (4C9/4C9; MW8/MW8) at different age (4, 8, 12 and 15 weeks). All the regions analyzed showed a correlation between the decrease over time of soluble mut Htt signal and the progressive increase of the pool of aggregated mut Htt in male animals (n= 3 per age). Cerebellum shows a lower aggregation load, when analyzed with the 4C9 antibody but not with the MW8.

Duplex TR-FRET assay for simultaneous detection of soluble and aggregated mut Htt.

To further enhance the simplicity of the detection method, we thought to develop a duplex assay which could simultaneously be used to measure both forms of the protein in the same biological sample, following the single pipetting-step procedure depicted in Figure 30 and using two different acceptor fluorophores (d2 and Alexa488) coupled to MW1 and 4C9 respectively which can be simultaneously excited by Terbium donors.

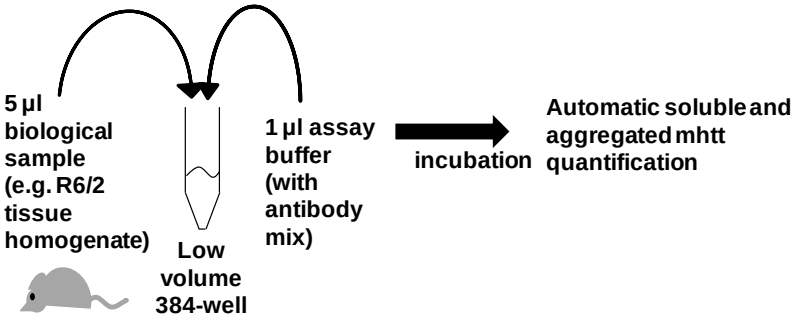


Figure 30. Schematic representation of TR-FRET duplex assay. 5 µl biological sample are pipetted together with 1 µl assay buffer containing 2B7 Tb, MW1 d2, 4C9 Tb and 4C9 Alexa into a low volume 384-well. After an incubation period, soluble and aggregated mut Htt are quantified.

In a first step, to investigate a possible interference between the emission spectra of the two acceptor fluorophores, we measured in both channels the signals generated singularly by the antibody pairs 2B7/MW1 and 4C9/4C9. For this we analyzed brain homogenates of 11 week-old wt and R6/2 mice in presence or absence of the antibodies (Figure 31A, B).

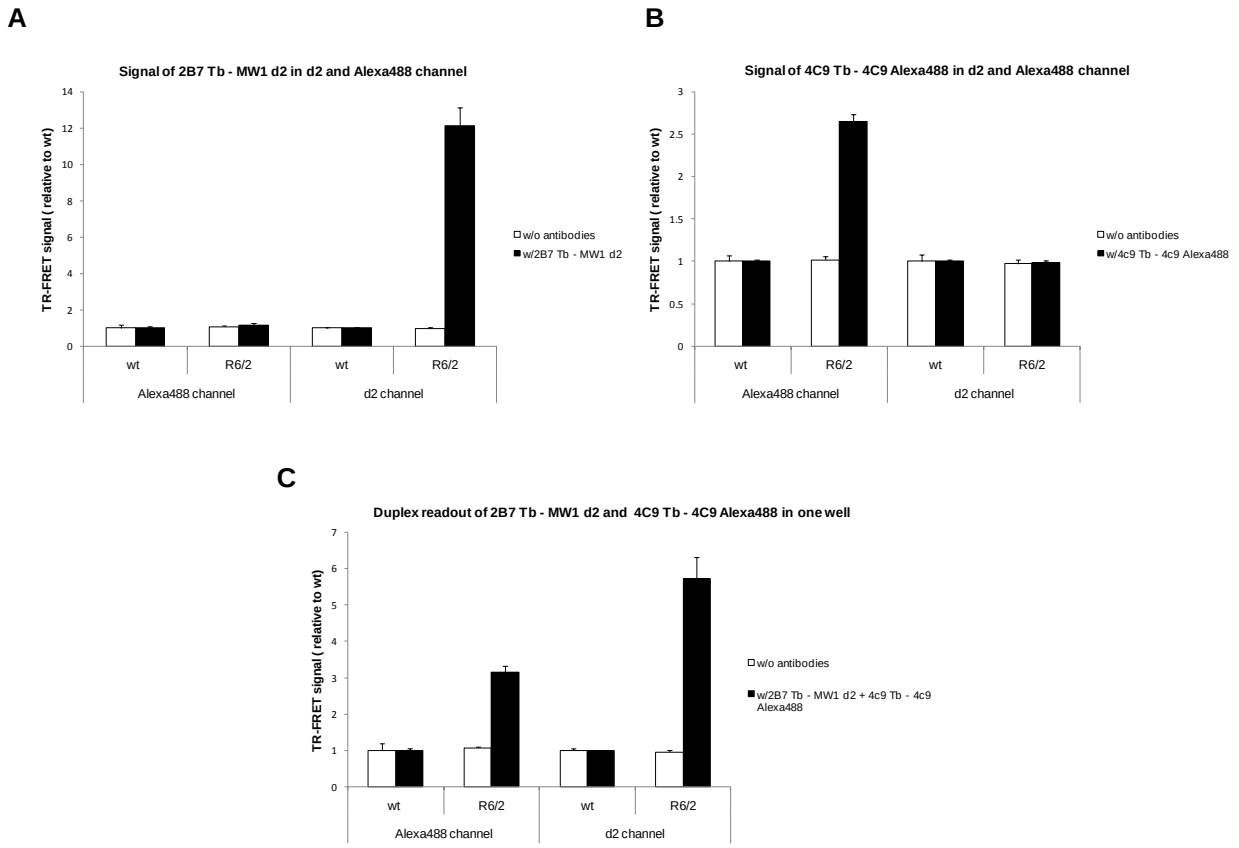


Figure 31. Signal intensity analysis for the two readout channels used to quantify soluble mut Htt (2B7 Tb - MW1 d2 = d2 channel) and aggregated mut Htt (4C9 Tb - 4c9 Alexa = Alexa488 channel). Signal measurement of the two readout channels analyzing 0.5 µg protein of 11 week-old wt or R6/2 brain with or without addition of 2B7 Terbium - MW1 d2 antibody (A) and with or without addition of 4C9 Terbium + 4C9 Alexa antibody (B) reveals no signal bleed-through of one measurement into the other readout channels enabling a homogeneous duplex quantification of soluble and aggregated mut Htt. Duplex quantification of soluble mut Htt and aggregated mut Htt in brain homogenates of 8 week-old wt or R6/2 mice utilizing (C) 1 µl assay buffer containing 1 ng 2B7 Tb, 10 ng MW1 d2, 1 ng 4C9 Tb and 10 ng 4C9-Alexa were added to 0.5 µg brain protein in 5 µl brain homogenization buffer in a 384-low volume well. All graphs: biological triplicates; background signal = 0.5 µg BSA in brain homogenization buffer.

Since no bleed-through of the two channels was detected, we successfully applied the duplex readout for determining soluble and aggregated mut Htt by combining the two pairs of labeled antibodies in the same sample (Figure 31C). Importantly, the readout signals for soluble and aggregated mut Htt displayed an overlapping linear range, allowing for a simultaneous quantitative duplex readout for both forms of the protein (Figure 32). In summary, we have developed and validated a duplex assay using the 2B7 Tb-MW1 d2 combined with the 4C9 Tb-4C9 Alexa488 antibody pair for detection of soluble and aggregated mut Htt in biological samples.

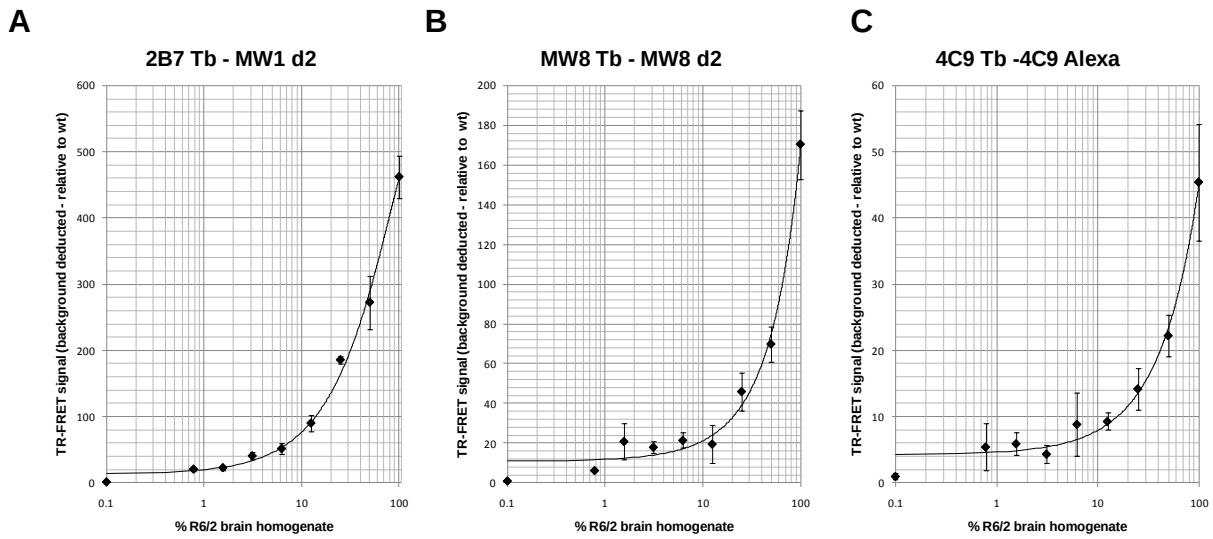
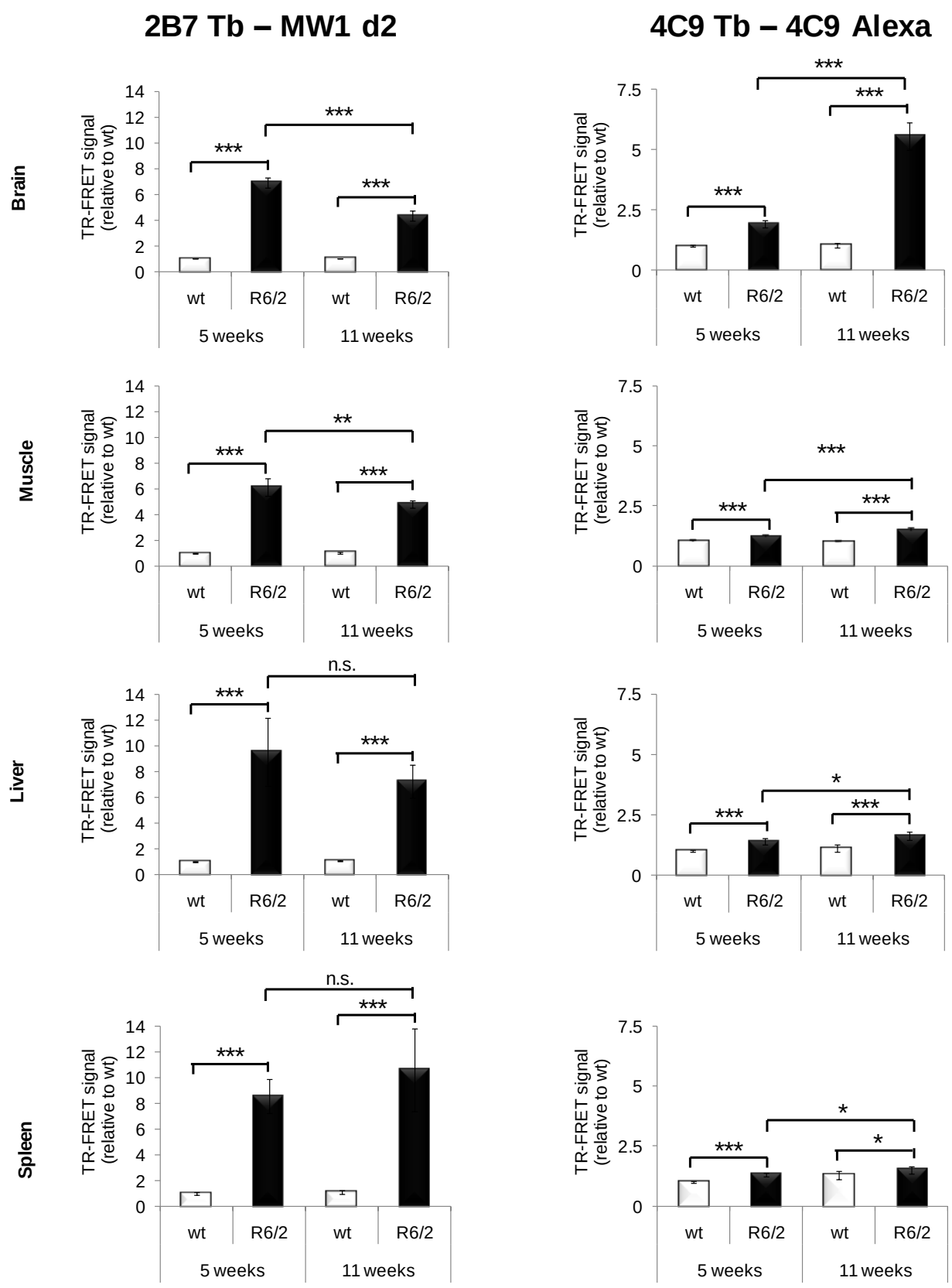


Figure 32. Linear range for readout signals of the TR-FRET duplex assay. R6/2 brain homogenates were spiked into wt brain homogenates in 1:2 step dilutions into 384-wells. The TR-FRET signal obtained produced the linearity curves for soluble mut Htt (A; 2B7/MW1) and insoluble aggregated mut Htt (B-C; MW8/MW8, 4C9/4C9)

Disease-progression related changes in soluble and aggregated mut Htt protein levels in R6/2 and *Hdh*Q150 tissue.

We proceeded to use the validated duplex TR-FRET assay for investigating the progression of soluble and aggregated mut Htt protein in multiple tissues isolated from R6/2 mice at 5 or 11 weeks of age. We observed an age-dependent decrease in soluble mut Htt correlating with an increase in aggregated mut Htt in brain, muscle and liver (Figure 33). Notably, in testis, ear lobe and spleen, while we were able to detect high levels of soluble mut Htt, we did not observe any age-related changes in soluble levels nor any noticeable aggregation.


2B7 Tb – MW1 d2
4C9 Tb – 4C9 Alexa
Brain
Muscle
Liver
Spleen
TR-FRET signal (relative to wt)
wt
R6/2
5 weeks
11 weeks
***
**
*
n.s.

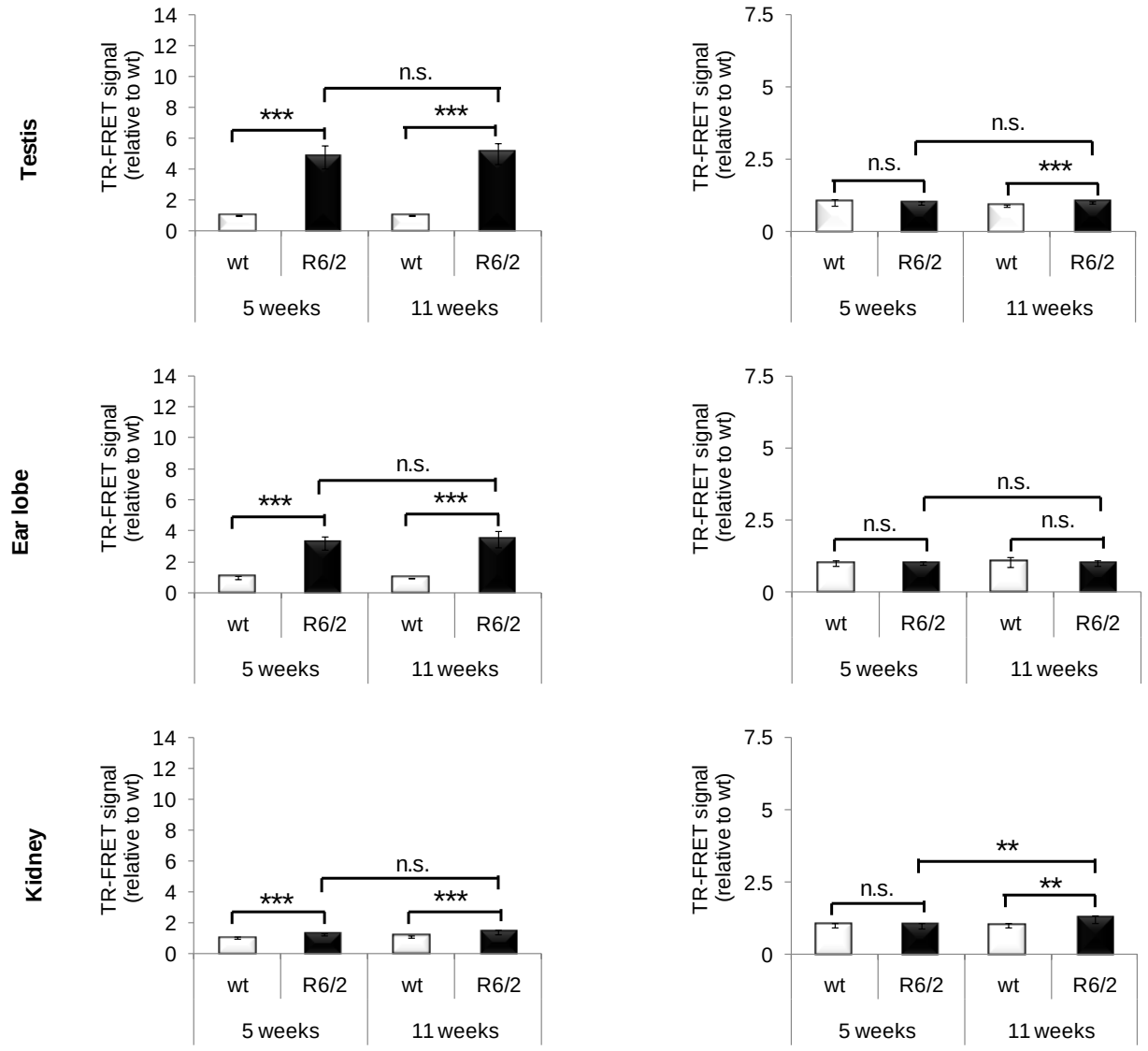


Figure 33. TR-FRET duplex assay detects age- and disease-progression caused changes in soluble and insoluble mut Htt protein levels in WT and R6/2 peripheral tissue homogenates. Tissues homogenates from wt and R6/2 mice of 5 and 11 weeks of age (n=3) were analyzed using the TR-FRET duplex assay. 0.5µg of protein homogenate in 6µl final volume per 384-well were analyzed to detect soluble mut Htt (2B7/MW1) and insoluble aggregated mut Htt (4C9/4C9). An age-dependent decrease in soluble mut Htt correlating with an increase in aggregated mut Htt is detected in R6/2 brain, muscle and liver. No significant changes in levels of aggregated mut Htt or soluble mut Htt levels were detectable in R6/2 testis, ear lobe, spleen and kidney.

Having validated the successful application of the TR-FRET readouts in R6/2 mice, we asked ourselves whether we could also apply our methods to a milder HD mouse model. We therefore assessed the age-dependent soluble and aggregated mut Htt signal development in cortical and striatal homogenates of 2 to 10 months old heterozygote *Hdh*Q150 mice which express one allele of endogenous full-length mut Htt with 150 glutamines. Similar to the data obtained from analysis of R6/2 brain, we observed an inverse correlation of soluble and aggregated mut Htt

signal in both brain regions and genders using 2B7/MW1 and MW8/MW8 (Figure 34 and Figure 35).

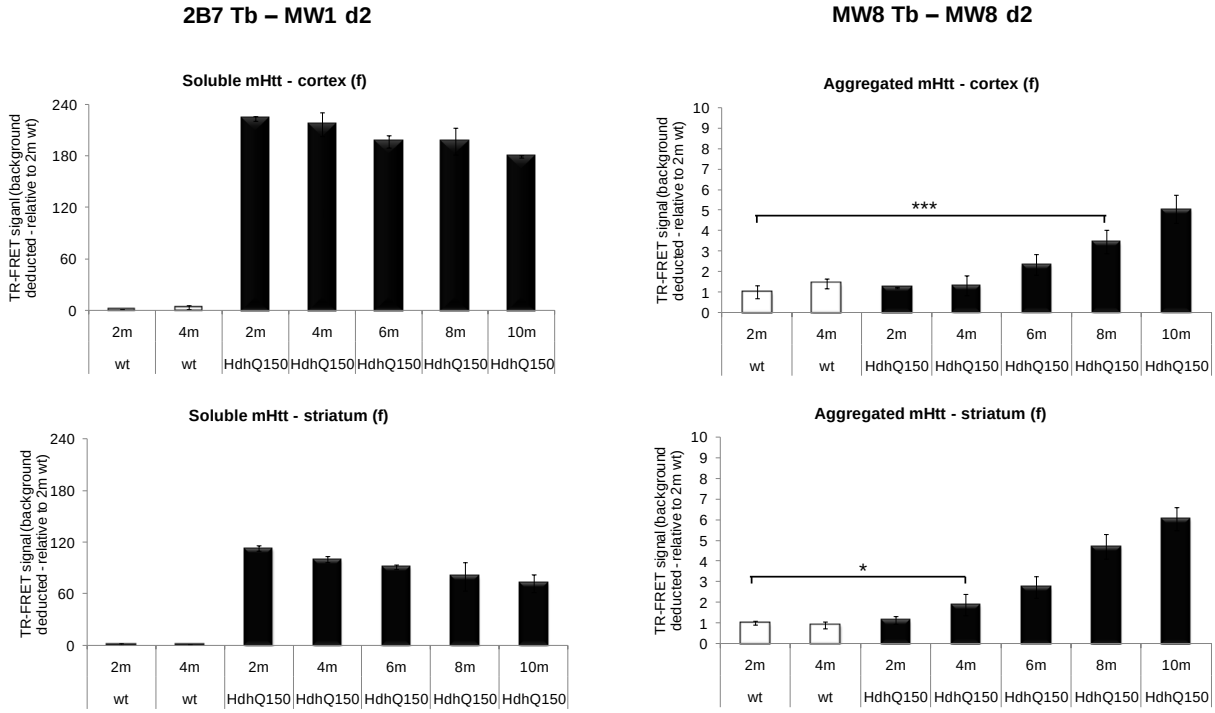


Figure 34. TR-FRET detection of age- and disease-progression caused changes in soluble and insoluble mut Htt protein levels in female WT and heterozygous *Hdh*Q150 brain regions. Cortical and striatal homogenates from wt and heterozygous *Hdh*Q150 female mice at different ages (2, 4, 6, 8, 10 months) were analyzed with 2B7/MW1 (soluble mut Htt) and MW8/MW8 (aggregated mut Htt) TR-FRET (n=3 per age). Both brain regions showed a correlation between a decrease of soluble mut Htt signal and a progressive increase of aggregated mut Htt over time.

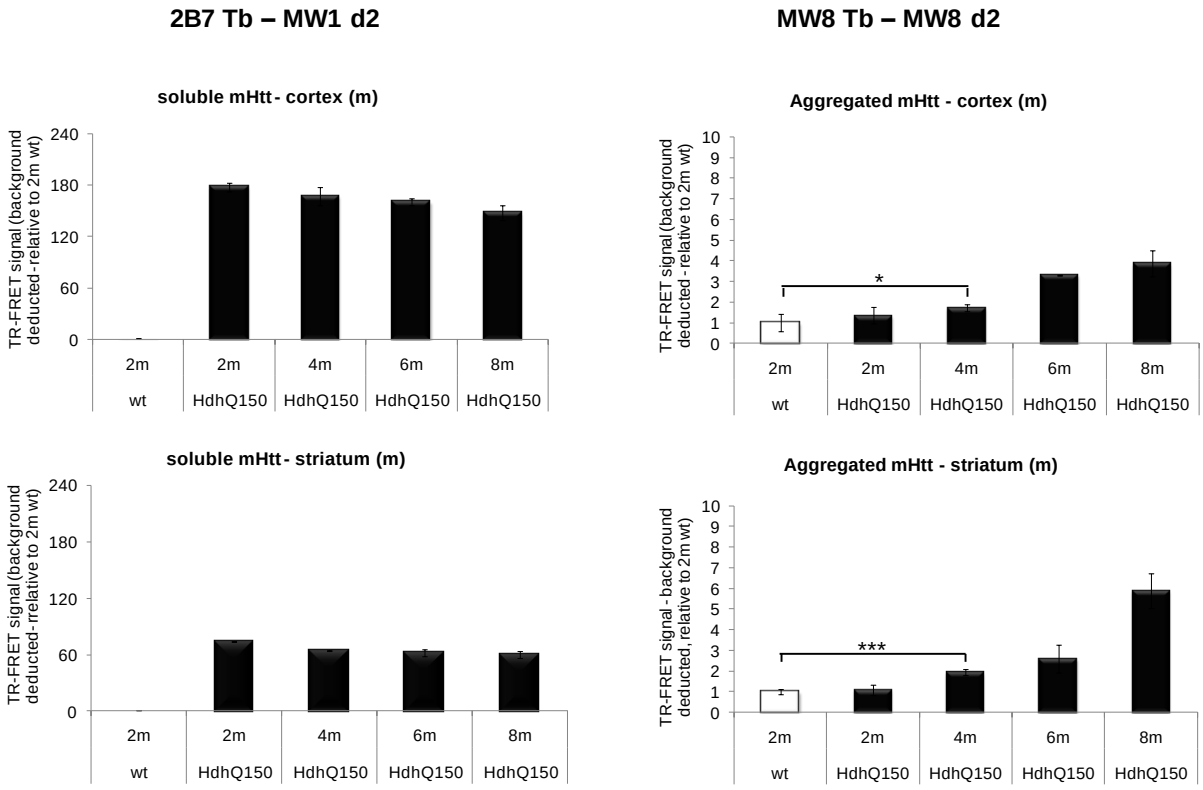


Figure 35. TR-FRET detection of age- and disease-progression caused changes in soluble and insoluble mut Htt protein levels in male WT and heterozygous *Hdh*Q150 brain regions. Homogenates from cortex and striatum from wt and heterozygous *Hdh*Q150 male mice have been analyzed to detect soluble (2B7 Tb – MW1 d2) and aggregated mut Htt (MW8 Tb – MW8 d2) at different age (2, 4, 6, 8 months) (n=3 per age). Both brain regions showed a correlation between the decrease over time of soluble mut Htt signal and the progressive increase of the pool of aggregated mut Htt.

Use of the 4C9/4C9 assay in this model was precluded by the fact that this antibody recognized the human-specific proline-reach region of Htt which is absent in mouse Htt (Figure 36). This result confirmed the value of the assays for quantification of two distinctive conformations of mut Htt and their inverse correlation in a physiologically more relevant animal model of HD.

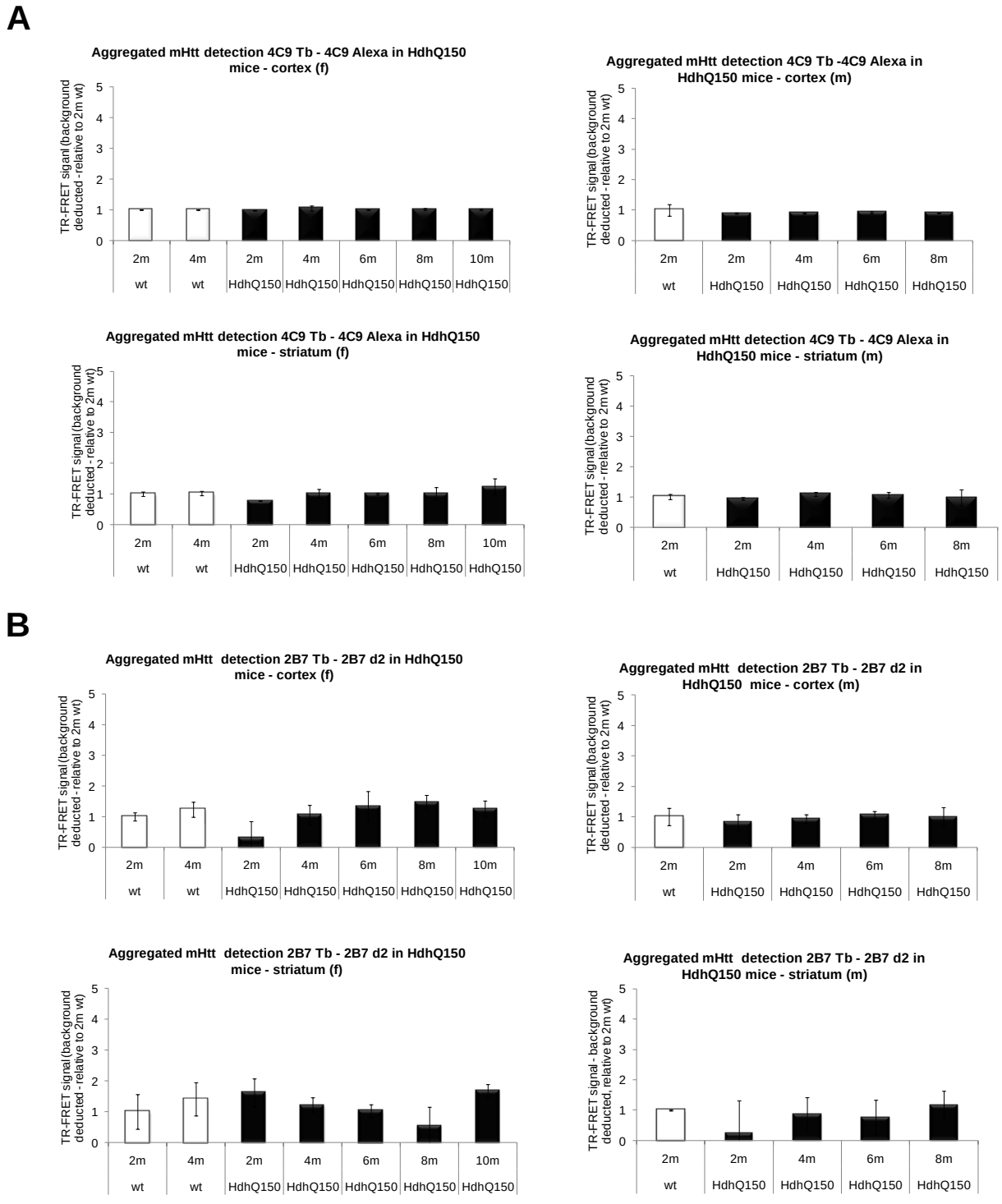


Figure 36. 4C9/4C9 and 2B7/2B7 fail to detect aggregated mut Htt in heterozygous *Hdh*Q150 mice. Samples from cortex and striatum homogenates from aging heterozygous *Hdh*Q150 mice were analyzed with 4C9/4C9 and 2B7/2B7. Both antibody combinations fail to produce a detectable TR-FRET signal. 4C9 is directed towards the polyproline region of the human Htt gene, not included in the knock in construct, therefore can not recognize the correct epitope (A) while 2B7 is specific for soluble mut Htt and it is not able to recognize the aggregated form, as demonstrated also in R6/2 homogenates (B).

4.2.5 DISCUSSION

We have developed a simple and ultrasensitive duplex TR-FRET assay to simultaneously quantify non-tagged soluble and aggregated mut Htt in as little as 5µl biological sample material. By combining the TR-FRET assay with SEC we could show the specificity of our different assay readouts for distinct mut Htt subpopulations in brain homogenates of HD mice. The specificity for a subset of small and large mut Htt aggregates will help to identify and test mut Htt aggregate modifiers and distinguish at what stage (early or late) they could be effective. Interestingly, the combination of SEC and TR-FRET showed that the heterogeneity of mut Htt subpopulations did not change over time, but the relative abundance of individual mut Htt aggregates did. Our analysis of non-tagged mut Htt expressed in *in vivo* HD models leads to similar observations as previous works in which distinct species of GFP-tagged mut Htt in *in vitro* systems were identified (Olshina et al., 2010), even though mut Htt aggregation in cell culture may differ fundamentally from *in vivo* generated aggregates in brain as the neuronal context may lead to pathogenic interactions that still need to be identified.

The ratio of the progressive decrease of soluble mut Htt at around 300kDa with a progressive increase in the aggregates species at around 950 kDa can be monitored and set in direct relation. This is important in order to analyze potential biological activity (e.g. toxicity) of the mut Htt aggregates. Interestingly, both MW8/MW8 and 4C9/4C9 detect high molecular weight aggregates as expected, but MW8/MW8 also recognized a smaller mut Htt aggregate species in young animals. It is likely that MW8 detects aggregate precursors (oligomers) that disappear with age, once large mut Htt aggregates are formed. These oligomers may be prone to be folded into larger aggregates indicating a regulated mechanism of inclusion body formation in cells. With the SEC purification together with the purity characterization by TR-FRET established, it will be interesting to test the biological activity and toxicity of these different mut Htt species in future studies.

It has been previously shown that there is an inverse correlation between aggregated and soluble mut Htt levels in R6/2 brain (Woodman et al., 2007, Weiss et al., 2009a, Sathasivam et al., 2010). Analyzing multiple brain region isolated from R6/2 mice at different age we confirmed the previous observations and validate the specificity and value of the TR-FRET assay to provide a more detailed description of the changes occurring in soluble and aggregated Htt over time. While different regions of the brain contained comparable levels of soluble mut Htt, higher amounts of aggregated protein were detected in striatum and cortex over cerebellum, brain stem

and hippocampus, in line with earlier findings (Sathasivam et al., 2010). Interestingly, these differences were only apparent when using the MW8/MW8 but not the 4C9/4C9 combination, further indicating that MW8 could recognize different aggregates species.

Combining the detection of soluble and aggregated mut Htt in a duplex assay with high specificity for both mut Htt forms, we were able to measure and compare both pools of the protein in brain and peripheral tissues from R6/2 mice. In all samples analyzed where an age dependent decrease in soluble mut Htt was observed, we detected a strong correlating progressive increase in the aggregated pool. These results suggest a mechanistic link between the mut Htt pools most likely through progressive recruitment of soluble mut Htt into aggregates.

In contrast to *in vitro* aggregation studies in which clear concentration dependent aggregation kinetics of mut Htt are observed (Scherzinger et al., 1999, Chen et al., 2002), our findings indicate that the amount of intracellular soluble mut Htt does not always correlate with its propensity to aggregate in a cell. For example, testis contains high amounts of soluble mutant Htt protein but no aggregates were detected. Other proliferating tissues such as the liver displayed similar expression levels of soluble mutant Htt as testis but were characterized by age-dependent aggregate formation. This discrepancy in different tissues suggests that different proliferation rates may influence the propensity to develop aggregates by diluting mut Htt below the threshold needed for aggregation. Of note, previous works have reported that testis is highly degenerative in R6/2 mice and HD patients in absence of aggregates (Sathasivam et al., 1999, Van Raamsdonk et al., 2007, Moffitt et al., 2009). In light of the ongoing debate about mut Htt aggregate toxicity, these observations raise the intriguing possibility that the pathogenic mechanism could be different in the context of different tissues. Importantly, this would also imply that treatment attempts should be aimed not solely at the reduction of aggregates but also at upstream mechanisms to ameliorate the full spectrum of mut Htt pathology.

The detection of mut Htt aggregates in 4 months old heterozygote *Hdh*Q150 mice using the MW8/MW8 assay highlights the sensitivity of the method. Interestingly, it has been recently reported that MW8 is directed against a neo-epitope at the C-terminus of Exon1 (Landles et al., 2010). This could indicate the presence of a pure Exon1 population in this full-length mut Htt model, responsible for the aggregation process and specifically detectable with this antibody.

A limitation in our TR-FRET assays – like in any immunodetection method – is that the antibodies require their respective epitopes to be sufficiently exposed on the aggregate surface. Each assay may thus be specific for a subpopulation of aggregates. In addition, as larger

aggregates have a lower surface to volume ratio than smaller aggregates, the TR-FRET signal intensity for large aggregates may be underrepresented in a heterogeneously sized pool. These limitations could be possibly overcome by further characterization of MW8 and 4C9 affinities to different aggregates populations. In addition, a better understanding of different aggregates populations measured by TR-FRET assays with specific antibodies could allow for a careful comparison to other technologies such as Seprion ELISA and to design experimental conditions to characterize changes occurring during disease progression or in response to treatment.

In conclusion, we developed novel assays that are at the same time sensitive, efficient, simple and highly precise for quantification of aggregated and soluble mut Htt. We made use of our assays to characterize and quantify the inverse correlation between those two conformational states of the protein in different *in vitro* and *in vivo* HD models. The simplicity of the assay protocol coupled with the efficient quantification allows for the use of the method in high throughput screenings to identify modulators of the aggregation process, as well as in the evaluation of translational models of HD.

The research pursued in this study supports the correlation between decrease of soluble and increase of aggregated mut Htt with disease progression in different HD models. Importantly, the ability to measure this tight relationship of the two mut Htt conformational pools over time could yield significant insights for the debate of mut Htt toxicity in HD.

AUTHOR CONTRIBUTIONS

B.B., P.P. and A.W. designed and conceived the experiments. B.B. characterized the method and performed the TR-FRET experiments on R6/2 peripheral tissues. S.G performed the TR-FRET experiments on R6/2 brain regions and *Hdh*Q150 mice. D.M., G.P.L. and A.W. designed and performed the SEC experiments. L.S.K. and D.C.L. designed and performed the experiments with rat primary neuronal cultures. D.A. collected the peripheral tissues from R6/2 mice. D.S. and G.P.B. provided the tissues from R6/2 and *Hdh*Q150 brain regions. B.B. and A.W analyzed the data. B.B. and A.W. wrote the manuscript. P.P., D.C.L., G.P.L., L.S.K. and G.P.B. proofread the manuscript.

5 GENERAL DISCUSSION AND PERSPECTIVES

Huntington's disease (HD) is a progressive neurodegenerative disorder caused by the amplification of a polyQ stretch at the N-terminus of the huntingtin protein (Htt). The events leading to neuronal cell death are not yet well understood but the toxic gain of function of the misfolded mutant Htt (mut Htt) and the formation of intracellular aggregates unequivocally contribute to the pathological cascade (Ross and Tabrizi, 2011). The first goal of my thesis was to explore new approaches to modulate mut Htt levels in the cell, with the aim of unveiling the degradation pathways involved. This could lead to the identification of possible novel therapeutic approaches. Secondly, this work aimed to establish an innovative method to detect and monitor aggregate formation in HD models.

We demonstrated that Hsp90 inhibitors, and in particular NVP-AUY922, are able to significantly reduce mutant huntingtin levels in different HD cell models, at concentrations comparable with previously reported data on the compound activity (Eccles et al., 2008). Interestingly we also showed, using a dominant negative construct of Hsf1 (Heldens et al., 2010), that the decrease of mutant huntingtin occurred independently from the induction of a heat shock response (HSR), thus favoring the hypothesis that huntingtin is a client protein of Hsp90. As was previously demonstrated for mutant androgen receptor, which is the cause of SBMA (Waza et al., 2005, Thomas et al., 2006, Tokui et al., 2009), our work described the interaction between soluble mut Htt and Hsp90, and its modulation by Hsp90 inhibition. Interference with the complex enhances mutant huntingtin degradation through the ubiquitin proteasome system. This involves ubiquitination of mut Htt but degradation probably does not require additional chaperones such as Hsp70 (Chapter 4.1.4). Our findings suggest a possible role for Hsp90 in stabilizing polyglutamine expanded proteins causing neurodegenerative diseases. The effect of Hsp90 inhibitors on wt and mut Htt degradation warrants further studies on this class of compounds as potential therapeutic agents. Moreover, previous studies reported that the induction of the HSR upon Hsp90 inhibition could be beneficial in modulating the toxicity and the by reducing mut Htt aggregate formation (Sittler et al., 2001, Fujikake et al., 2008). The novel findings from my studies suggest that Hsp90 inhibitors have the potential to enhance the degradation of both soluble and aggregated mut Htt protein. It would be interesting to further explore the effect of Hsp90 inhibitors in HD models producing both non-aggregated, soluble as well as aggregated

mut Htt protein, in order to explore whether an Hsp90 inhibition-mediated decrease of soluble mut Htt affects also the aggregation process. It would be also relevant to investigate if other degradation pathways, aside from the UPS, are involved in the clearance of soluble and aggregated protein upon Hsp90 inhibition. Our data suggest that the soluble protein is stabilized by Hsp90 and upon inhibition is degraded for the most part through the UPS. However it cannot be excluded that other pathways, such as chaperone mediated autophagy (CMA, Cuervo, 2010), could be involved in the clearance process. One could speculate that inhibiting Hsp90 triggers greater availability of free soluble mutant protein, hence multiple degradation pathways could be enhanced. A relationship between CMA, huntingtin and chaperones has been already investigated (Koga and Cuervo, 2010, Gamerdinger et al., 2011), thus further investigation could clarify the possible interplay between different clearance mechanisms and provide interesting inputs to design novel therapeutic strategies.

Another interesting aspect of the interaction of Hsp90 and its client proteins is that the modulation of their association can influence the post-translational modification state on the target protein. This has been reported in the past for several Hsp90-client proteins such as Akt (Sato et al., 2000, Yun and Matts, 2005) and the co-factor Hsf1 (Akerfelt et al., 2007). In this work we demonstrated that ubiquitination of mut Htt is enhanced after Hsp90 inhibition and release from the chaperone complex (chapter 4.1.4). Previous studies have shown that mut Htt can undergo multiple post-translational modifications, which can influence the protein degradation, localization and toxicity, as seen for phosphorylation events (Thompson et al., 2009, Atwal et al., 2011, Havel et al., 2011). It would be interesting to investigate the role, if any, of other post-translational mut Htt modifications upon Hsp90 inhibition. This could offer a wider range of possibilities for therapeutic approaches.

Mut Htt fragments assemble into oligomers and aggregates which are thought to contribute to the neurodegenerative process (Ratovitski et al., 2009, Landles et al., 2010). The TR-FRET assay for the detection of aggregates described in this work allows a simple and robust means to monitor aggregation. As it is in a homogeneous format, thus avoiding multi-step protocols, this assay requires minimal handling time prior to signal acquisition, yet does not sacrifice sensitivity or specificity. Moreover, it is readily applicable to high-throughput applications.

Using a TR-FRET based method to detect aggregates in biological samples results in a sound tool to investigate the progression of the pathology and potentially evaluate the effectiveness of HD treatments. The novel method could be used to detect aggregates in different models of the

disease using both *in vitro* and *in vivo* biological samples. These findings suggest the applicability of the TR-FRET for drug discovery purposes and monitoring of therapies aimed to modify the aggregate load. The role of the aggregates in the development of pathology is still controversial, thus a better understanding of different aggregate populations and their assembly kinetics could be an important step to further characterize disease progression. Previous studies showed the existence of different aggregates species (Poirier et al., 2002, Olshina et al., 2010) but their characterization is still only partial and difficult to be translated *in vivo*. The data presented in this thesis suggest that different detection antibodies could have preferential specificity for one aggregated species over another. This indicates that generating multiple assays with aggregate species specific antibodies could allow characterization of various aggregate populations and eventually an investigation of their toxicity and pattern of distribution among different brain regions and tissues. A key finding emerging from my data is the decrease over time of soluble mutant Htt in parallet to a progressive increase of the aggregates in HD models expressing either full length Htt or Ex1 fragment (chapter 4.2.4). This inverse correlation between soluble and aggregated pool suggests a progressive recruitment of the soluble into the inclusions, which would require further investigation. This correlation was detected not only in brain regions but also in peripheral tissues manifesting aggregates. On the other hand, tissues without aggregates, such as testis or ear lobe, did not present any difference in the soluble pool over time. These findings were in agreement with previous studies (Sathasivam et al., 1999, Sathasivam et al., 2010) therefore affirming the significance and sensitivity of the new detection method. However, further experiments would be needed in order to understand the kinetics and dynamics of the aggregation process, as well as to unveil the correlation between aggregate species and degeneration.

The possibility to detect and measure aggregates in peripheral tissue requires further optimization in order to be applied to human biological samples, thus providing a sound tool to monitor the effect of possible therapies modulating aggregation and understand the aggregates formation. The combination of this assay with the one developed previously for soluble mutant huntingtin (Weiss et al., 2009a, Weiss et al., 2010) allows a duplex readout able to explore the changes in both pools of the protein in the same biological sample during disease progression.

The TR-FRET method is based on the use of high affinity antibodies thus suggesting that the assay could be translated to other neurodegenerative or aggregation-prone diseases. Interestingly ELISA assays have been developed for α-synuclein and β-amyloid (El-Agnaf et al., 2006,

Fukumoto et al., 2010) therefore suggesting a possible application of the TR-FRET assay in these diseases.

Overall this thesis provides both new insights into the mechanisms influencing mutant huntingtin stability and degradation, and a robust and sensitive method to monitor aggregate formation and dynamics. Further experiments will be undertaken in order to translate the mechanism involving Hsp90 and Htt degradation *in vivo* or in more relevant models for the disease, and to apply the TR-FRET method to human biological samples and other neurodegenerative diseases.

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APPENDIX

Besides my PhD project, I also collaborated to another study pursued in our laboratory, aimed to explore the role of mTOR and autophagy in modulating mutant huntingtin aggregation. I contributed to this work performing some biochemical analysis and revising the paper, recently accepted in Journal of Neurochemistry.

Induction of Autophagy with Catalytic mTOR Inhibitors Reduces Huntingtin Aggregates in a Neuronal Cell Model.

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Novartis Institutes for BioMedical Research, Neuroscience Discovery, Basel, Switzerland.

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SUMMARY

Huntington's disease is a progressive neurodegenerative disorder caused by a CAG trinucleotide repeat expansion in the huntingtin gene. This expansion produces a mutant form of the huntingtin protein, which contains an elongated polyglutamine stretch at its amino-terminus. Mutant huntingtin may adopt an aberrant, aggregation-prone conformation predicted to start the pathogenic process leading to neuronal dysfunction and cell death. Thus, strategies reducing mutant huntingtin may lead to disease-modifying therapies. We investigated the mechanisms and molecular targets regulating huntingtin degradation in a neuronal cell model. We first found that mutant and wild-type huntingtin displayed strikingly diverse turn-over kinetics and sensitivity to proteasome inhibition. Then, we show that autophagy induction led to accelerate degradation of mutant huntingtin aggregates. In our neuronal cell model, allosteric inhibition of mTORC1 by everolimus, a rapamycin analogue, did not induce autophagy or affect aggregate degradation. In contrast, this occurred in the presence of catalytic inhibitors of both mTOR complexes mTORC1 and mTORC2. Our data demonstrate the existence of an mTOR-dependent but everolimus-independent mechanism regulating autophagy and huntingtin aggregate degradation in cells of neuronal origin.

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CURRICULUM VITAE

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Skills

- Cell culture /cellular assays
- Protein modification analysis
- Nucleic acid prep/analysis
- Imaging/Immunohistochemistry
- Animal handling/dissection
- Assay development (TR-FRET, MSD)
- Strong oral communication
- Scientific writing/publication
- Project design/collaboration

Research

Current

Investigation of the role of molecular chaperons, in particular Hsp90, in the degradation pathways of mutant Hungtintin protein.
Development of a bioassay based on TR-FRET technology, to detect aggregation levels in cellular and animal models of Huntington Disease.

Future

Investigation of cellular pathways and toxicity mechanisms leading to the development of Huntington's disease.
Assay development and biomarker identification in the context of the neurodegenerative disorders.

Education

PhD: Neurobiology **Oct 2008 – present**

NIBR – Novartis Pharma, Basel (CH) and Basel University

Funded by the European Union in the frame of Neuromodel, Initial Training Network (ITN), FP7

- Characterization of the role of Hsp90 in huntingtin protein degradation and stability
- TR-FRET assay development to detect mutant huntingtin aggregates

  Supervisor: Dr. Klemens Kaupmann

  Neuromodel academic mentor: Prof. Gillian Bates, King's College, London

Master's Degree: Medical Biotechnology **Sep 2006 – Apr 2008**

University of Trieste, Italy

- Graduated summa cum laude

  Thesis "The transcription factor GABP in cellular and mouse models of Huntington Disease"

Bachelor Degree: Biotechnology **Sep 2002 – Jul 2006**

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Esearch experience

Doctoral Student: **Oct 2008 - present**

Laboratory of Dr. Paolo Paganetti and Dr Klemens Kaupmann, Neuroscience Discovery, NIBR, Novartis, Basel (CH), under the supervision of Dr. Martin Spiess (University of Basel).

Undergraduate student (Master) **Apr 2007 – Sep 2008**

Neurobiology sector International School of Advanced Studies (SISSA), Trieste (Italy).

Supervisor: Dr. Francesca Persichetti.

Undergraduate student (Bachelor) **Mar 2006 – Jul 2006**

Nucleus of clinical and experimental research in hematology (NRCLE), Oncology Research Centre (CRO) Aviano, PN (Italy).

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Other skills

- **Languages:** Italian (mother tongue)
  Fluent written and spoken English
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- **Other hobbies:** travelling, sports (volleyball, swimming, skiing), reading, cinema, photography

Posters and Publications

Publications:

Baldo B., Paganetti P., Grueninger S., Kaltenbach L.S., Lo D.C., Abramowski D., Smith D, Bates G and Weiss A. (2011) Inverse correlation of aggregated and soluble huntingtin in mouse models of Huntington's disease by duplex quantification of the two protein forms. (submitted)

Baldo B. Parker C, Weiss A, Richter J, Bibel M, Paganetti P and Kaupmann K (2011) A screen for clearance enhancers identifies mutant huntingtin as an Hsp90 client protein. (submitted)

Roscic A., **Baldo B.**, Bibel M., Crochemore C., Mercellin D. and Paganetti P. (2011) Induction of autophagy with catalytic mTOR inhibitors reduces huntingtin aggregate load in neuronal cell models. *J Neurochem* 2011 Aug 19. doi: 10.1111/j.1471-4159.2011.07435.x.
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Posters:

Baldo B. et al (2010) Investigating the role of protein modification in Huntington's disease: the importance of Htt and Hsp90 interaction in protein clearance and stability.
HD2010 "The Milton Wexler celebration of life", Boston (USA), August 2010

Baldo B et al (2011) New insights into the role of Hsp90 in Huntington's disease: influence on protein clearance and stability.
Molecular mechanisms of neurodegeneration, Milan (IT), May 2011