**Scuola Internazionale Superiore di Studi Avanzati**

# Investigating the role of Pin1 and Rrs1 in Huntington's Disease

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"Doctor Philosophiae"

S.I.S.S.A.
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The work described in this dissertation was carried out at the International School for Advanced Studies, Trieste, between October 2004 and August 2008. All work reported here arises from my own experiments and data analysis.

# ABSTRACT

Huntington's disease (HD) is a dominantly inherited neurodegenerative disorder caused by a polyglutamine expansion within the N-terminal region of huntingtin protein. The mutation is likely to confer a novel toxic property to the protein, which initiates a disease cascade that broaden with time and culminates in neuronal loss.

In the present study we have investigated the contribution to the pathogenesis of HD of Pin1 and Rrs1, two proteins already implicated in neurodegeneration.

The identification of cellular factors promoting mutant huntingtin degradation is of great interest in HD pathology as they can lower the level of toxic protein. In this context, we have considered the prolyl-isomerase Pin1 as a good candidate for its role in modulating aggregate formation in other neurodegenerative disorders including Alzheimer's and Parkinson's diseases. In the present work we show that in cell culture Pin1 overexpression reduces the formation of mutant huntingtin intracellular inclusions. In agreement with this observation we demonstrate that Pin1 decreases huntingtin half-life by promoting the activity of the ubiquitin proteasome system. Additional studies are currently underway to unveil the role of Pin1 in modulating huntingtin aggregation *in vivo* using the double $Hdh^{Q111}$:$Pin1^{-/-}$ mouse model we have generated.

The conformational change catalyzed by Pin1 on its substrates regulates a number of cellular processes, which include, among others, the control of gene transcription. We have previously shown that Pin1 is a new huntingtin interactor (unpublished results). In the present work we explore the interplay between Pin1 and mutant huntingtin in deregulating the transcriptional programs in neurons. Gene expression profiling has been carried out on ST*Hdh* striatal cells upon Pin1 silencing and on striatum of $Hdh^{Q111}$/$Pin1^{-/-}$ mice. We report here preliminary lists of genes with change in gene expression in absence of Pin1.

The second study of this thesis work is directed to explore the role in HD of *Rrs1* (Regulator of ribosome synthesis), a gene already implicated in the early phase of the disease process. Rrs1 is an evolutionary conserved protein characterized so far exclusively in *Saccharomyces*

*cerevisiae* where it participates in ribosome biogenesis. Here we present the first description of Rrs1 protein in mammalian cells, where it localizes both in the nucleolus and in the ER. Furthermore, we provide evidence that in striatal cell lines *Rrs1* mRNA is up-regulated upon induction of ER stress. In addition we report that mutant huntingtin induces ER stress in the striatum of $Hdh^{Q111}$ knock-in mice at a very young age, when Rrs1 mRNA levels were found increased.

# CHAPTER 1: INTRODUCTION

## 1.1 Huntington's Disease

### 1.1.1 Brief history of the disease

"*There are three marked peculiarities in this disease: 1. Its hereditary nature; 2. A tendency to insanity and suicide; 3. Its manifesting itself as a grave disease only in adult life"*.

With these words George Huntington in 1872 describes in his paper "*On Chorea*" the distinguishing features and the hereditary nature of this devastating neurodegenerative disorder, named after him Huntington's Disease (HD).

In 1981 the U.S.-Venezuela Huntington's Disease Collaborative Research Project was founded and a team of scientists joined together with the aim to identify the chromosomal region and the gene responsible for HD. Two large families, one from Venezuela, one from the United States were studied and in 1983 the gene linked to the disease was mapped on chromosome 4 (Gusella, 1983).

Ten years later, in 1993, the HD gene was finally isolated (HDCRG 1993). The mutation responsible for the disease was identified as a polymorphic trinucleotide (CAG) repeat in the 5' region of a novel gene that expands beyond the normal range in the disease chromosomes.

Unfortunately more than fifteen years after the isolation of the HD gene, a treatment for this disorder, affecting in Europe 5-10 individuals out of 100,000, is still not available.

### 1.1.2 Clinical manifestation of HD

Although HD is characterized by multiple symptoms, a clinical diagnosis is made following the unequivocal presence of the movement disorder associated with HD in the context of a family history and/or a positive genetic test result (Williams, 2007).

Psychiatric symptoms with changes in cognition and behavior precede the onset of motor disturbances by a decade or more. Early psychiatric disturbances include mood and personality changes, apathy and depression (Paulsen, 2005), alterations in memory (Lemiere,

2004), learning and planning abnormalities (Rosenberg, 1995) and sleep disturbances (Morton, 2005).

As the disease progresses, affected individuals develop overt choreiform movements of head, neck, arms and legs, which increase with time, including facial grimacing and twisting and jerking of the trunk and limbs (Bachoud-Levi, 2001). Weight loss, which also characterizes the disease, may be due to dysphagia as well as degeneration of hypothalamic orexin/positive neurons (Petersen, 2005).

The clinical manifestation of HD typically begin in midlife with a mean age at onset of about 40 years, but its symptoms can also occur as early as 2 years of age and as late as 80 to 90 years. The juvenile-onset individuals have quite a different clinical description compared to the typical adult-onset cases with muscular rigidity, bradykinesia, tremor and the absence of abrupt movements and chorea (Hayden, 1981; Squitieri, 2000). The age at onset is closely correlated with the extent of the mutation as it will be discussed in chapter 1.1.5.

The most common causes of death in HD are cardiovascular disease and pneumonia following general debility from incessant choreic movements, injuries related to serious falls, poor nutrition and infection. Choking, secondary to aspiration of food, and suicide (Di Maio, 1993) are also relatively common causes of death.

### 1.1.3 Neuropathology of the disease

The clinical manifestations are associated with a distinctive neuropathology. A specific and gradual loss of medium spiny projecting neurons (MSN) in the striatum, with some associated degeneration of the cerebral cortex is observed. In the more severe young onset cases the neuronal pathology extends to other brain areas including thalamus and cerebellum. Examination of coronal section reveals atrophy of the striatum in *postmortem* brains of HD patients (Fig. 1). The neurodegeneration of the striatum occurs first in the caudate nucleus, then in the putamen.

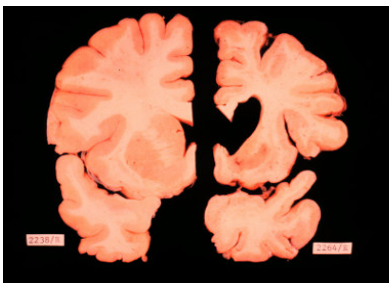

Figure 1: Coronal section passing through the nucleus accumbens of the left cerebral hemisphere of a normal individual, compared to the right cerebral hemisphere of an HD patient *postmortem* brain (Adapted from Vonsattel and DiFiglia, 1998).

At death, about 10 to 20 years after the onset of clinical manifestation, the basal ganglia is ravaged and overall brain weight is reduced by a third (Vonsattel and DiFiglia, 1998). Although the most obvious and striking neuropathology of HD is the dramatic loss of medium spiny neurons, thorough examination shows that other brain regions are affected in HD. Cortical cell loss is often reported even if less severe, mostly affecting the large neurons in layer V and VI, which project to the striatum (Hedreen, 1991).

Hypothalamic atrophy and cell death also occur (Petersen and Bjorkqvist, 2006). Neuropathological studies of HD patients have demonstrated up to 90% neuronal loss in the lateral hypothalamus (Kremer, 1990; Kremer, 1991) and in particular loss of neurons expressing the neuropeptide orexin (Petersen, 2005).

In addition, in *post mortem* studies a significant microglia activation has been described in the areas of neuronal loss, including the striatum, globus pallidus and frontal cortex (Sapp, 2001).

Extra-neural abnormalities reported in literature include increased diabetes (Podolsky, 1972) and peripheral muscle weakness (Homberg and Huttunen, 1994), although most of these data require further epidemiological study (Valenza, 2005). Increased stress-induced apoptotic death in lymphocytes (Sawa, 1999), muscle pathology (Strand, 2005) and endocrine changes have been also described (Petersen and Bjorkqvist, 2006).

### 1.1.4 Motor circuits in Huntington's Disease

The striatum is the main input compartment of a system called basal ganglia (Fig. 2), which are a collection of subcortical nuclei involved in the control of movement.

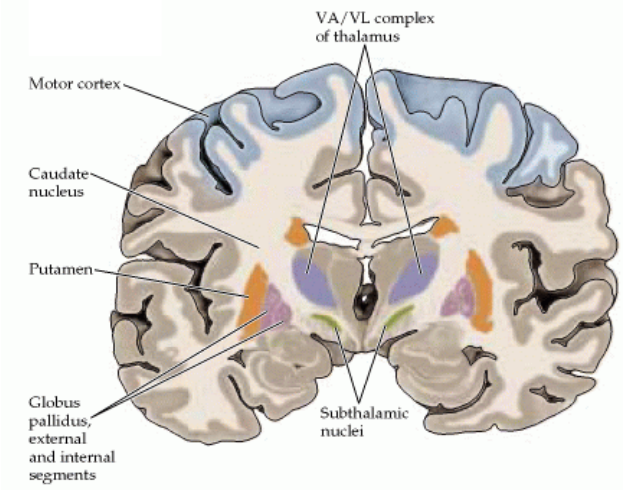


Figure 2: Scheme of a coronal section of the human brain, showing the position of the basal ganglia and their different sub-nuclei. The basal ganglia are composed of four principal nuclei: the striatum, the globus pallidus (GP) consisting of external and internal segments (GPi and GPe), the substantia nigra (SN) consisting of pars compacta and reticulata (SNc and SNr), and the subthalamic nucleus (STN) (Adapted from Neuroscience, 2001 by Sinauer Associates).

The striatum receives massive glutamatergic and dopaminergic innervations from all regions of the cerebral cortex as well as specific thalamic nuclei. The flow of information returns to the cortex through the thalamus, which is the major output structure.

The primary motor dysfunctions in HD are due to the degeneration of the medium-sized spiny neurons (MSNs). The MSNs are projection neurons and represent more than 90% of the striatal cell population. Although all MSNs are GABAergic, they differ in a number of properties including the expression of dopamine and acetylcholine receptor subtypes, peptide content and projection targets.

A classical model describes two major neuronal motor pathways, referred to as direct and indirect pathways, which act in opposition, and correctly predicts the motor impairment in Huntington's disease (Albin, 1989). GABAergic neurons at the origin of the direct pathway mainly express dopamine D1 and muscarinic M4 receptors, substance P, and project to the GPi. On the other hand, GABAergic neurons of the indirect pathway mainly express dopamine D2 receptors and enkephalin, and project to the GPe (Reiner, 1988; Albin, 1991; Richfield, 1995; Sapp, 1995). Early Huntington's disease is characterized by loss of the projection neurons of the indirect pathway which causes an imbalance in favor of the direct pathway. Movements are therefore initiated, but can neither be controlled nor stopped (Albin, 1989). At later stages, the general loss of the MSNs, including those projecting through the direct pathway, induces a drastic motor dysfunction leading to bradykinesia.

### 1.1.5 Etiology of the disease

Huntington's disease was the first autosomal dominant disorder in which the technique of "reverse genetics" was successfully applied. The human HD gene (IT15), located to chromosome 4p16.3, contains 67 exons spanning more than 200 kb and encodes for a ~350 kDa protein named huntingtin (HDRCG, 1993). The 5' end of the gene contains a highly polymorphic stretch of repeated CAG trinucleotides ranging from 6 to 35 repeats. By contrast, HD mutant alleles that produce the full disease phenotype have 36 to more than 250 CAG repeats (Gusella and MacDonald, 2002).

The CAG repeats on HD chromosomes are remarkably unstable through germline transmission. Contractions and expansions are equally likely with maternal transmission, but expansions are more common than contractions with paternal transmission (Wheeler, 2007).
The relationship between the size of the CAG repeat and the age at onset of neurological symptoms has been examined in numerous data sets showing a strong inverse correlation. HD is typically a mid-life pathology associated with CAG allele lengths of 40 to 50 units. Disease alleles with CAG expansion in the 35 to 40 unit range may show very late onset or, in some cases, may be nonpenetrant. By contrast, individuals with more than 60 CAG units typically show onset of HD in their juvenile years.
CAG repeat length however account for about 70% of the variation in age at onset being the remaining variation attributable to environmental factors or to genes other than the HD gene (Li, 2006; Wexler, 2004). Among the potential modifier genes, the serum and glucocorticoid regulated kinase (sgk) and the metabotropic glutamate receptor 1 (mGlur1) are of particular interest (Li, 2006).

### 1.1.6 Triplet repeat disorders

Expanded trinucleotide repeat mutations have been associated with a growing number of inherited human disorders. This class of mutations was first identified in 1991 as the cause of spinal and bulbar muscular atrophy (SBMA) and fragile X syndrome (FRAXA) (La Spada, 1991). Since then, triplet repeat expansions have been found to be the causative mechanisms in 14 other neurodegenerative disorders including HD. Unifying features among these diseases include the unstable behavior of the triplet repeat during germline transmission when the length of the repeat exceeds a critical value (Orr and Zoghbi, 2007).
Trinucleotide repeat diseases can be categorized into two subclasses based on the location of the triplet repeats: diseases involving repeats within coding sequences (exonic) and diseases involving noncoding repeats (untranslated sequences).
Huntington's disease belongs to the first group, which includes nine disorders caused by expansion of the same repeated codon (CAG) into the coding region of different genes (Tab. 1). Since the expansion of the CAG repeats encodes for a polyglutamine tract, these diseases are commonly referred to as polyglutamine (or polyQ) disorders. Interestingly, all polyQ

diseases are characterized by the presence of insoluble ubiquitinated aggregates and by degeneration of specific brain regions, despite the ubiquitous expression of the corresponding proteins.

The second group, non-PolyQ diseases, does not share any specific symptoms and include: Fragile X syndrome, Fragile XE mental retardation, Friedreich's ataxia, Myotonic dystrophy, SCA8 (Spinocerebellar ataxia Type 8), and SCA12.

| Disease | MIM number | Gene product | Normal repeat length | Expanded repeat length | Main clinical features |
|---|---|---|---|---|---|
| HD | 143100 | Huntingtin | 6–34 | 36–121 | Chorea, dystonia, cognitive deficits, psychiatric problems |
| SCA1 | 164400 | Ataxin1 | 6–44[b] | 39–82 | Ataxia, slurred speech, spasticity, cognitive impairments |
| SCA2 | 183090 | Ataxin2 | 15–24 | 32–200 | Ataxia, polyneuropathy, decreased reflexes, infantile variant with retinopathy |
| SCA3 | 109150 | Ataxin3 | 13–36 | 61–84 | Ataxia, parkinsonism, spasticity |
| SCA6 | 183086 | $CACNA1_A$ | 4–19 | 10–33 | Ataxia, dysarthria, nystagmus, tremors |
| SCA7 | 164500 | Ataxin7 | 4–35 | 37–306 | Ataxia, blindness, cardiac failure in infantile form |
| SCA17 | 607136 | TBP | 25–42 | 47–63 | Ataxia, cognitive decline, seizures, and psychiatric problems |
| SBMA | 313200 | Androgen receptor | 9–36 | 38–62 | Motor weakness, swallowing, gynecomastia, decreased fertility |
| DRPLA | 125370 | Atrophin | 7–34 | 49–88 | Ataxia, seizures, choreoathetosis, dementia |

Tab. 1: Polyglutamine disorders (Orr and Zoghbi, 2007).

## 1.2 Huntingtin protein

Human huntingtin (NP_002102) contains 3144 amino acids with a molecular weight of ~350 kDa and very little homology to any other known protein. The polyQ region, encoded by the CAG stretch, is immediately downstream amino acid 17 of huntingtin and upstream to a polyproline (polyP) rich region. Across the entire protein have been identified 36 HEAT-like repeats (Huntingtin, Elongation factor 3, subunit A of protein phosphatase 2A, TOR1) (Takano and Gusella, 2002), which are sequences of about 40 amino acids that form hydrophobic α helices and assemble into an elongated superhelix (Andrade and Bork, 1995). The function of HEAT repeats is still unclear, although they have been found predominantly in proteins involved in intracellular trafficking and chromosomal segregation. Analogous to other HEAT repeat proteins, huntingtin may act as a scaffold molecule facilitating the formation of a variety of complexes (Takano and Gusella, 2002). Huntingtin is expressed ubiquitously in humans with the highest levels in brain and testis. In the brain, high levels are

found in the cerebellar cortex, neocortex, striatum and hippocampus (Schmitt, 1995). In most cells huntingtin is essentially a cytoplasmic protein associated with various organelles including mitochondria, endoplasmic reticulum and Golgi complex (DiFiglia, 1995; Trottier, 1995), however a fraction is also found in the nucleus (Wheeler, 2000; Tao and Tartakoff, 2001; Kegel, 2002).

Huntingtin is an essential protein that is required for normal embryogenesis, as knockout mice die at an early developmental stage (E 7.5) (Duyao, 1995; Nasir, 1995). Conditional knockouts mouse models have demonstrated that huntingtin is also essential at postnatal stages, as the inactivation of the gene in brain and testis leads to degeneration of these two tissues (Dragatsis, 2000). Mice homozygous for reduced levels of huntingtin displayed characteristic aberrant brain development and perinatal lethality, indicating a critical function for *Hdh* in neurogenesis (White, 1997). In Drosophila, the reduction of huntingtin expression causes axonal transport defects in larval nerves and neurodegeneration in adult eyes (Gunawardena, 2003). These studies indicate that huntingtin is required for cell survival and suggest that a loss of function of the protein might induce neurodegeneration (see chapter 1.2.3).

### 1.2.1 Huntingtin, a player of many games

Despite substantial effort has been spent to understanding huntingtin function, a complete picture of its normal activities is not yet available. This is mainly due to the large size of the protein that makes isolation and analysis difficult, to the lack of obvious homology with other proteins and to its ubiquitous localization and promiscuous interactions with more than 200 partners identified to date (Harjes and Wanker, 2003; Li and Li, 2004; Borrell-Pages, 2006; Kaltenback, 2007).

Most of the proteins have been found to interact with the N-terminal region of huntingtin and, in several cases, the strength of the interactions has been shown to be sensitive to the length of the polyQ tract (Tab. 2) (Harjes and Wanker, 2003; Li and Li, 2004).

The different biological roles of the interactors have implicated huntingtin in processes as diverse as transcriptional regulation, RNA splicing, signal transduction, intracellular trafficking, cytoskeletal organization and protein folding and turnover (Li and Li, 2004).

At the same time, other huntingtin partners, involved in post-translational processes, target huntingtin to different subcellular compartments, supporting the hypothesis of a scaffold protein involved in dynamic protein-protein interactions.

| Interactor/modifier | PolyQ length dependence | Region of huntingtin involved | Function |
|---|---|---|---|
| $\alpha$-Adaptin C/HYP-J | yes ↘ | NT (aa 1–550) | endocytosis |
| Akt/PKB | no | S421 | kinase |
| $\beta$-Tubulin | no | unknown | structure, vesicle transport |
| CA150 | no | unknown | transcriptional activator |
| Calcineurin | unknown | S421 | phosphatase |
| Calmodulin | yes ↗ | unknown | calcium-binding regulatory protein |
| Calpain | unknown | aa 430–550 | protease |
| Caspase-3 | no | aa 513, 530 | protease |
| Caspase-6 | no | aa 586 | protease |
| CBP | yes ↗ | NT (aa 1–588) | transcriptional co-activator |
| Cdk5 | no | NT (aa 5–56) | kinase |
| CIP4 | yes ↗ | NT (aa 1–152) | signal transduction |
| CtBP | yes ↘ | PLDLS motif (aa 182–186) | transcriptional co-repressor |
| Cystathionine $\beta$-synthase | no | NT (aa 1–171) | generation of cystein |
| FIP2/HYP-L | unknown | NT (aa 1–150) | cell morphogenesis |
| GAPDH | yes ↗ | PRD | glycolitic enzyme |
| GIT1 | unknown | NT (aa 92–170) | G protein-coupled receptor kinase |
| Grb2 | unknown | PRD | growth factor receptor-binding proteir |
| HAP1 | yes ↗ | NT (aa 171–230) | membrane traffic |
| HAP40 | unknown | CT | endosome motility |
| HIP1 | yes ↘ | NT (aa 1–540) | endocytosis, proapoptotic |
| HIP14/HYP-H | yes ↘ | NT (aa 1–550), C214 | traffic, endocytosis |
| HIP2 | no | NT (aa 1–540) | ubiquitin-conjugated enzyme |
| HYP-A | yes ↗ | PRD | RNA splicing factor |
| HYP-C | yes ↗ | PRD | transcription factor |
| IKK$\gamma$ | yes ↗ | PRD and polyQ | kinase inhibitor |
| $InsP_3R1$ | yes ↗ | NT (aa 1–171) | calcium release channel |
| MLK2 | yes ↘ | PRD | kinase |
| N-CoR | yes ↗ | NT (aa 1–171) | nuclear receptor co-repressor |
| NF$\kappa$B | unknown | HEAT repeats | transcription factor |
| p53 | no | PRD | transcription factor |
| PACSIN1 | yes ↗ | PRD | endocytosis, actin cytoskeleton |
| PQBP-1 | yes ↗ | polyQ | transcription repressor |
| PSD-95 | yes ↘ | PRD | synaptic scaffolding protein |
| RasGAP | unknown | PRD | Ras GTPase activating protein |
| REST/NRSF | yes ↘ | unknown | transcription factor repressor element |
| SGK | no | S421 | kinase |
| SH3GL3 | yes ↗ | PRD | endocytosis |
| Sin3a | yes ↗ | NT (aa 1–171) | transcription repressor |
| SP1 | yes ↗ | NT (aa 1–171) | transcription factor |
| SUMO | unknown | K6, K9, K15 | post-translational modification |
| TAFII-130 | no | NT (aa 1–480) | transcription factor |
| TBP | yes ↗ | unknown | transcription factor |
| Tpr | yes ↘ | NT (aa 1–17) | nuclear export protein |
| tTG | yes ↗ | NT (aa 1–550) | transglutaminase |
| Ubiquitin | yes ↗ | K6, K9, K5 | post-translational modification |

Tab. 2: Proteins that interact with huntingtin. Arrows indicate whether these interactions are increased or decreased by the polyQ expansion. NT, N terminal; CT, C terminal (Borrell-Pages et al., 2006b).

To date, huntingtin protein has been described to undergo post-translational modifications that include palmitoylation, ubiquitination, SUMOylation and phosphorylation.

Palmitoylation at cysteine 214 by huntingtin interacting protein 14 (HIP14) is a critical modification for its normal trafficking to the Golgi (Yanai, 2006). Ubiquitination and SUMOylation occur both at the same N-terminal residues (K6, K9, and K15), but the former targets huntingtin to proteasomal degradation, whereas the latter stabilizes the protein and promotes its capacity to repress transcription (Steffan, 2004).

Phosphorylation of huntingtin at serines 421, 434, 1181 and 1201 has been reported to promote cell survival.

Huntingtin is phosphorylated at serine 421 by the pro-survival signaling kinase Akt (Humbert, 2002) and by the serum and glucocorticoid-induced kinase SGK (Rangone, 2004). Huntingtin is also phosphorylated at serine 434, 1181 and 1201 by Cdk5, a member of the serine/threonine cyclin-dependent kinase (Cdk) family (Luo, 2005).

Both Akt and CdK5 phosphorylation of huntingtin have been shown to be protective against polyQ-expansion-induced toxicity (Humbert, 2002; Luo, 2005; Anne, 2007).

Other huntingtin phosphorylation sites at serine 533-5-6, 2076, 2653 and 2657 have been identified by mass spectrometry (Schilling, 2006) but their role in mediating huntingtin function/dysfunction is still unknown.

It is noteworthy that huntingtin phosphorylation at position 434, 1181, 1201, 2076, 2653 and 2657 occurs at serine (or threonine) residues followed by a proline (Ser-Pro or Thr-Pro). This kind of post-translational modification is indicated as proline-directed phosphorylation. These residues, when phosphorylated, are potential consensus binding sites for the peptidyl-prolyl isomerase Pin1 (see chapter 1.6).

### 1.2.2 Mutant huntingtin and HD pathogenesis

Polyglutamine expansion is likely to confer novel toxic properties to huntingtin. A gain of function mechanism is indeed supported by several data.

Mutant huntingtin has been found to rescue embryonic lethality of knock-out mouse, indicating that the HD mutation does not impair normal embryonic huntingtin functions (Duyao, 1995; Nasir 1995). In humans, a partial deletion of the distal part of the chromosome

4p16.3, which includes the HD gene, results in the Wolf Hirschhorn Syndrome that is characterized by growth and mental retardation and a premature death at 2 years, but lacks of a HD-like phenotype.

On the other hand, the finding of an anti-apoptotic role for wild-type huntingtin has suggested that the loss of the protective functions of the wild-type protein may participate to the disease mechanisms (Rigamonti, 2000; Leavitt, 2001).

In HD target neurons, the trigger event driven by mutant huntingtin is likely to occur many years before the onset of the first signs of neurodegeneration leading to specific neuronal dysfunction. Subsequently, in debilitated neurons, other factors may participate in the disease cascade that leads to neuronal death.

HD pathogenesis can be viewed as a cascade of events that is first triggered by mutant huntingtin through an abnormal interaction with an as yet unknown cellular constituent. Once triggered, this cascade broadens with time, leading eventually to neuronal death (MacDonald, 2003).

Several mechanisms of mutant huntingtin toxicity have been proposed, which partially fit with clinical data obtained from HD patients. Proposed mechanisms of cell dysfunction in HD include, among others, apoptotic insult, defect in protein degradation, aggregation, transcription dysregulation, alteration in intracellular trafficking, glutamate mediated excitotoxicity, mitochondrial dysfunction and oxidative stress (Fig. 3).

These different toxic mechanisms could participate synergistically in the pathology or be subordinate to one of them.

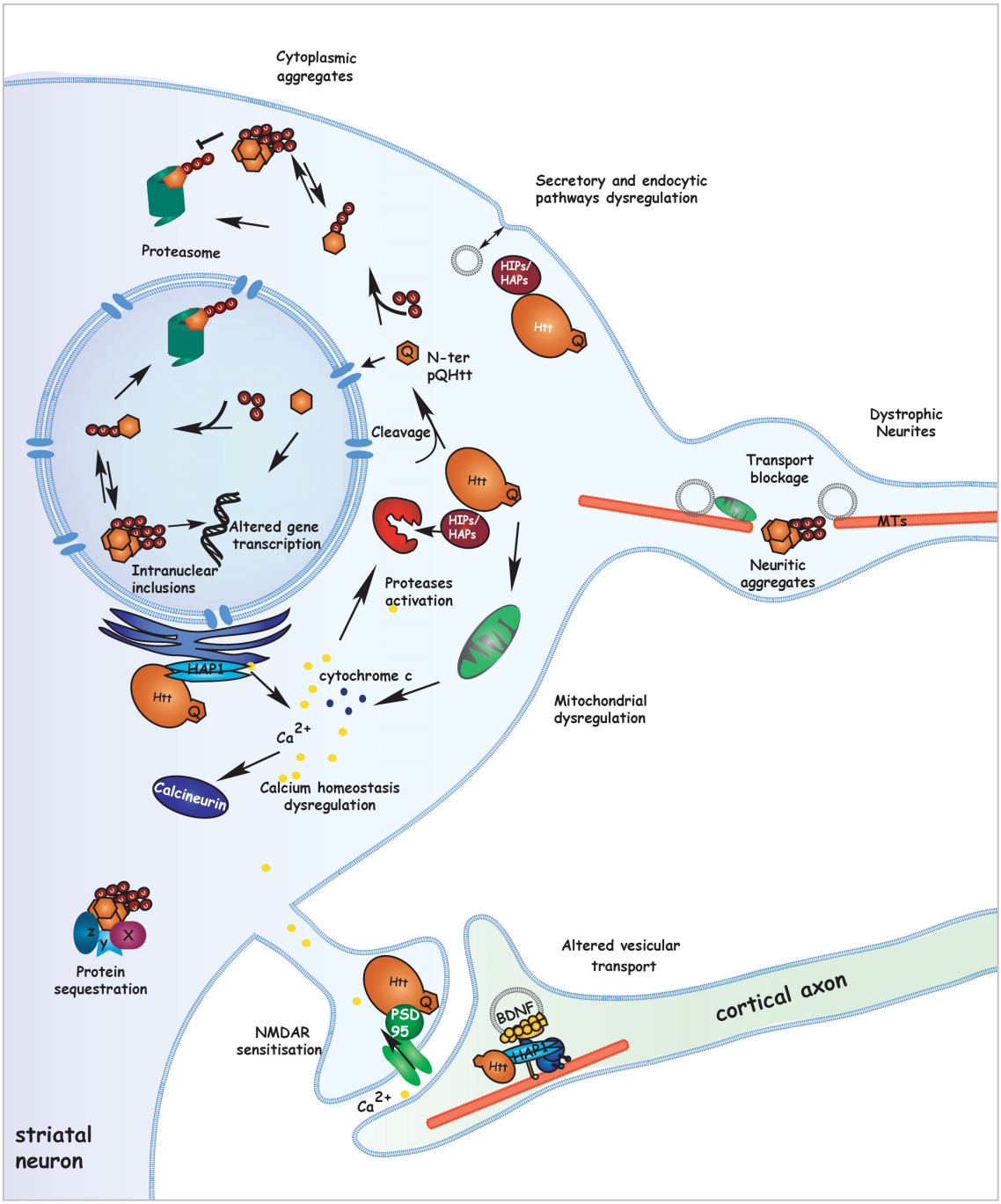


Figure 3: Mutant huntingtin is prone to modifications and induces many intracellular defects. Full-length huntingtin is cleaved by proteases in the cytoplasm, leading to the formation of cytoplasmic and neuritic aggregates. Mutant huntingtin also impairs calcium homeostasis and alters vesicular transport and recycling. Defect in BDNF transport reduces trophic support and increases neuronal death susceptibility. Whereas cytoplasmic and intranuclear aggregates are not directly toxic, neuritic aggregates could physically block transport. N-terminal fragments containing the polyQ stretch translocate to the nucleus where they impair transcription and induce neuronal death. In an attempt to eliminate the toxic huntingtin, fragments are ubiquitinated and targeted to the proteasome for degradation. Intranuclear aggregates could represent temporary storage of soluble and oligomeric forms before degradation or when the proteasome becomes less efficient (Borrell-Pages, 2006b).

**Proteolytic cleavage, protein aggregation and the UPS**

The identification of N-terminal huntingtin fragments in neuronal intranuclear inclusions (NIIs) of HD *postmortem* brains have implicated proteolytic cleavage of the protein in HD toxicity (DiFiglia, 1997). Several proteases cleave huntingtin *in vitro* and *in vivo*: these proteases include caspase 1, 3, 6, 7 and 8, calpain and non-identified aspartyl-proteases (Gafni, 2004; Hermel, 2004) (Goldberg, 1996; Lunkes, 2002; Graham, 2006). Mutant huntingtin cleavage occurs both in transgenic animals and in HD patients (Sieradzan, 1999). The cleavage of huntingtin into fragments containing the polyQ stretch and their subsequent translocation to the nucleus is likely to be a key step in the disease progression (Saudou, 1998). When proteolysis is prevented by inhibition of caspase or calpain activity or by modifying the consensus cleavage site in huntingtin, mutant huntingtin toxicity is reduced and disease progression is slowed (Wellington, 2000; Gafni, 2004; Luo, 2005; Graham, 2006).

How these proteases contribute to the pathological process is not fully understood but recent studies suggest that not all the N-terminal fragments that result from proteolysis are toxic. Mice expressing mutant huntingtin, resistant to cleavage by caspase 6 but not caspase 3, maintain normal neuronal function and do not develop striatal neurodegeneration (Graham, 2006).

Once in the nucleus, huntingtin fragments may aberrantly interact with several transcription factors and form inclusions.

Neuronal inclusions have been found in HD *postmortem* brains (Fig. 4) and in mouse models of the disease. Neuronal Intranuclear Inclusions (NIIs) are a pathological hallmark of HD, and of other polyQ disorders. It is well known that these structures are dynamic and that the pathway from soluble huntingtin to inclusion bodies formation is a multi-step process where many different species are formed before the mature inclusion. The relevance of aggregate formation to the pathogenesis of HD is not totally understood.

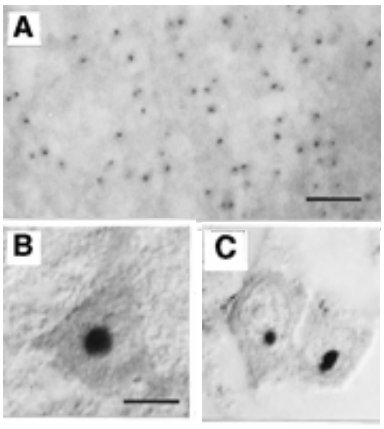


Figure 4: Huntingtin immunoreactivity in HD *postmortem* brain. (A) Cortex of a juvenile patient shows numerous hNIIs prominently stained. (B) Cortical pyramidal neurons contain one hNII. (C) Striatal neurons with hNIIs. A, bar=50µm; B, bar=10µm (Adapted from (DiFiglia, 1997).

Aggregates are not directly correlated with neuronal death, as the highest percentage of NII-containing neurons in HD *postmortem* brain is found in non-degenerating regions (Gutekunst, 1999). Moreover, in mouse models of HD, NIIs are not related to cell death (Kim, 1999; Slow, 2005), and transgenic mice expressing mutant huntingtin exon 1 display only little evidence of cell loss despite a high percentage of intranuclear inclusions (Mangiarini, 1996). Interestingly, in contrast to NIIs, the presence of neuropil aggregates correlates better with HD neuropathology (Gutekunst, 1999). For these considerations, aggregates have been proposed to represent a neuroprotective cellular strategy aimed to reduce the pool of soluble toxic protein.

On the other end, other evidences associate mutant huntingtin aggregates to neuronal dysfunction. Indeed, NIIs have been found to sequester important transcription factors, leading to transcriptional abnormalities. In addition, the accumulation of large aggregates has been proved to physically impair intracellular transport by blocking axonal and dendritic trafficking (Gunawardena, 2003; Lee, 2004).

Aggregation is a characteristic of abnormally folded proteins. Inclusions have been found to be immunopositive for ubiquitin and chaperones, suggesting that the mutant protein is recognized as misfolded and is targeted for degradation to proteasome (Davies, 1997; DiFiglia, 1997; Wyttenback, 2000). Recent data have shown that expanded polyglutamine sequences cannot be digested by proteasome, which therefore release undigested polyQ-containing fragments for further hydrolysis by still unidentified peptidases (Holmberg, 2004; Venkatraman, 2004). The occasional failure of fragments to be rapidly released may compromise proteasome function and accelerate the accumulation of polyQ proteins. These findings hence support the hypothesis that the ubiquitin-proteasome system (UPS) is impaired by polyQ expansions, strongly indicating that the protein turnover is affected in polyglutamine diseases (Bence, 2001). However, controversial results about the impaired proteasome activity in response to mutant huntingtin expression have been reported (Bence, 2001; Jana, 2001; Bowman, 2005; Bett, 2006).

**Autophagy and ER stress in HD**

Proteasome is unable to degrade protein complexes, aggregates or oligomers (Verhoef, 2002). Several evidences have suggested a central role of autophagy in degradation of disease-

associated proteins (Ravikumar, 2002; Rubinsztein, 2006; Settembre, 2008). Autophagy is a bulk degradation process involved in the clearance of long-lived proteins, protein complexes and organelles. Evidences of autophagy in HD brain as well as in cellular and animal models have been demonstrated (Larsen, 2002; Kegel, 2000; Petersen, 2001). Furthermore, induction of autophagy by treatment with the mTOR inhibitor rapamycin has been demonstrated to reduce aggregation and attenuate toxicity in HD cells and mouse models (Ravikumar, 2004). Evidence is beginning to emerge indicating that clearance pathway responsible for elimination of aggregates are closely regulated by the ER through the UPR (Unfolded Protein Response) stress sensors, such as IRE1α and PERK/eIF2α (Kouroku, 2007). Studies aimed to understand the function of wild type huntingtin demonstrated that inhibition of its expression significantly alters the ER morphogenesis (Hilditch-Maguire, 2000; Omi, 2005). In addition, mutant huntingtin perturbs ER calcium homeostasis (Rockabrand, 2007), and the experimental targeting of N-terminal mutant huntingtin fragments to the ER decrease its aggregation propensity (Rousseau, 2004). Thus, increasing evidences suggest that mutant huntingtin may exert its neurotoxic effect by directly causing ER stress. The accumulation of mutant huntingtin inclusions has been shown to trigger activation of the UPR in vitro (Kouroku, 2007; Reijonen, 2007) and subsequently autophagic clearance of such aggregates (Kouroku, 2007; Hoyer-Hansen, 2007, Hoyer-Hansen 2007b). Cytoplasmic aggregates stimulate ER stress signal and induce ER-stress-mediated cell death with caspase-12 activation in mouse cells, presumably by the accumulation of unfolded protein in the ER due to the inhibition of retrotranslocation and ER-associated ubiquitin/proteasome degradation (ERAD) (Kouroku, 2000).

Despite this evidence, the actual involvement of ER-stress related pathways in the disease remains speculative, as no *in vivo* experiments have validated these findings.

**Transcriptional dysregulation**

Transcriptional dysregulation has emerged as a pathogenic mechanism involved in HD. The majority of transcription factors interacting with huntingtin associate with the N-terminal region of the protein and the interactions are in some cases altered by the polyQ expansion (Steffan et al., 2000; Dunah et al., 2002). So far, the transcription factors that have been found

to interact with huntingtin include Sp1, CBP, p53, mSin3A, NCoR, CtBP, CA150, p300/CBP associated factors (P/CAF), TAFII130, NF-Y and REST/NRSF.

The CRE pathway is very interesting for its role in controlling the expression of neuronal genes and neuronal survival. Intriguingly, deletion of CREB causes selective neurodegeneration in the hippocampus and striatum inducing neurological phenotypes resembling those in HD (Mantamadiotis, 2002).

Soluble mutant huntingtin interacts with both the glutamine-rich activation domain and the acetyltransferase domain of the co-activator CREB-binding protein (CBP) altering its function (Steffan, 2001).

Mutant huntingtin binds p53 and induces its stabilization and transcriptional activity toward proapoptotic genes such as Bax and Puma (Bae, 2005).

The Sp1/TAFII130 (TATA-binding protein (TBP)-associated factor) pathway is also altered by soluble mutant huntingtin in a polyglutamine dependent manner, leading to transcriptional downregulation of the nerve growth factor and dopamine D2 receptors (Dunah, 2002; Li, 2002).

Dysregulation of transcription may also result from abnormal interaction between mutant huntingtin and repressors or activators within the nucleus, such as the nuclear receptor corepressor N-CoR (Boutell, 1999), the transcriptional co-repressor C-terminal binding protein (CtBP) (Kegel, 2002), the activator CA150 (Holbert, 2001).

Furthermore, within the nucleus, mutant huntingtin fragments form aggregates which have been shown to sequester transcription factors, as in the case of CBP and NF-Y (Nucifora, 2001; Yamanaka, 2008).

Transcriptional alteration also derives from atypical behavior of mutant huntingtin in the cytoplasm of the cells, as occurs for the repressor element-1 transcription factor/neuron restrictive silencer factor (REST/NRSF) which shows reduced binding to mutant huntingtin with respect to the wild type. Wild-type huntingtin, but not the mutant, promotes BDNF (Brain Derived Neurotrophic Factor) transcription by sequestering REST/NRSF in the cytoplasm thereby preventing it from forming the nuclear co-repressor complex at the NRSE nuclear site and allowing gene transcription (Zuccato, 2003).

Consistent with transcriptional repression playing a role in the pathogenesis of HD, decreased acetylation and increased methylation of histones have been found in HD experimental

models and HD patients (Steffan, 2001; Ferrante, 2003; Ryu, 2006). The HD protein, indeed, binds the acetyl transferase domain of different transcription factors such as CBP and p300/CBP Associated Factor (P/CAF), affecting acetylation of histones (Steffan, 2001), whereas increased level of histone methyl transferase, as ESET, have been reported to affect chromatin structure and, finally, gene transcription in HD patients and mouse models (Ryu, 2006).

**Intracellular trafficking deficiencies**

Altered intracellular dynamics involving defects in axonal transport or alterations of the secretory and endocytic pathways are likely to participate in HD pathogenesis.

The main evidence of a role for huntingtin in cellular trafficking came from the characterization of the first huntingtin partner HAP1. The precise role of this protein in neurons remains to be explored, although some studies have shown that HAP1 is involved in endocytosis, vesicular trafficking and $Ca^{2+}$ regulation (Gauthier, 2004; Kittler, 2004). The binding of HAP1 to huntingtin is enhanced by the expanded polyQ tract (Li, 1995). Mutant huntingtin potentially either disrupt the interaction of HAP1 with other molecules or sequester HAP1-associated protein complexes, thereby affecting HAP1-associated trafficking (Rong, 2007). This idea is supported by the finding that mutant huntingtin attenuates BDNF transport by dissociating htt-HAP1-$p150^{Glued}$ complex from microtubules. Decreased BDNF transport has been linked to loss of neurotrophic support and increased neuronal toxicity (Gauthier, 2004).

Huntingtin is located on plasma and intracellular membranes and associates with vesicles and different organelles (DiFiglia, 1995; Velier, 1998). Sequences in the N-terminal region of huntingtin have been demonstrated to modulate such interactions by being substrate of HIP14, a palmitoyl transferase (Yanai, 2006). Expansion of the polyQ tract results in reduced interaction between mutant huntingtin and HIP14 with consequent reduction of palmitoylation which leads to inclusion formation and increase in neuronal toxicity.

Finally, huntingtin interacts with many proteins involved in secretion and endocytosis. Reduced endosomal motility and endocytic activity in HD fibroblasts and mutant cells was indeed recently reported (Pal, 2006).

**Mitochondrial impairment, oxidative stress and excitotoxicity**

Striatal vulnerability to mitochondrial impairment was discovered after the intoxication with 3-nitropropionic acid (3-NP) (see also 1.3). This toxin leads to a severe neurological disease resembling HD, with a degeneration of basal ganglia and movement dysfunctions characterized by dystonia, chorea and hypokinesia (Ludolph, 1991; Alexi, 1998). A generalized mitochondrial impairment occurs in HD patients and in mouse models of the disease where reduced levels of ATP and membrane potential of mitochondria have been reported (Panov, 2002; Gines, 2003).

Both wild-type and mutant full-length huntingtin are associated with the mitochondrial outer membrane (Choo, 2004). It has been shown that mutant N-terminal huntingtin fragment decreases the calcium threshold required to induce mitochondrial permeability transition (MPT) pore opening. This coincides with cytochrome c release and thus activation of apoptotic death pathways, including activation of caspases (Choo, 2004), calpain (Bizat, 2003), and p53 (Bae, 2005).

Furthermore, the expression of expanded polyQ proteins in PC12 cells and in fibroblasts of HD patients leads to production of reactive oxygen species (ROS) and thus to the activation of DNA damage response (Giuliano, 2003).

The decreased ATP/ADP ratio was linked to enhanced calcium influx through NMDA receptors (Seong, 2005). Impaired energy metabolism probably leads to reduced ATP production, with a concomitant reduced mitochondrial membrane potential and a higher vulnerability to NMDA-mediated calcium influx and excitotoxicity (Panov, 2002; Seong, 2005). Calcium influx could trigger further free radicals production exacerbating cell damage. As cortical glutamatergic processes massively innervate the striatum, it is a structure at risk of glutamate-mediated excitotoxic injury. The overactivation of NMDA glutamate receptors allows high levels of calcium entry, resulting in the death of MSNs (Ferrante, 1985; Lipton and Rosenberg, 1994). Significant increases in the NMDA receptor density associated with an increase of intracellular free calcium levels were found in MSNs from several mouse models for HD (Levine, 1999; Cepeda, 2001; Zeron, 2002).

At a molecular level, huntingtin interacts with postsynaptic density 95 (PSD-95), a scaffold protein that causes clustering and activation of receptors in the postsynaptic membrane, modulating excitatory signaling through the interaction with NMDA receptors (Sun, 2001).

Enhanced activation of NMDA receptors may also reflect an altered interaction of mutant huntingtin with PSD95.

**Loss of normal huntingtin function**

The hypothesis that a loss of normal huntingtin function may play a role in HD pathology came after the finding that huntingtin is an indispensable protein with antiapoptotic function (Rigamonti, 2000). Wild-type huntingtin overexpression was found indeed to be neuroprotective in striatal cells exposed to various apoptotic stimuli such as serum deprivation or 3-nitropropionic acid treatment (3-NP) (Rigamonti, 2001). Overexpression of wild-type huntingtin was also found to protect *in vivo* against ischemic injury or NMDA receptor-mediated excitotoxicity (Zhang, 2003; Leavitt, 2001; Cattaneo, 2001; Leavitt, 2006).

In addition, postnatal inactivation of huntingtin in mouse neurons resulted in a progressive degenerative neuronal phenotype further underlying the role of huntingtin in the surviving of adult neurons (Dragatsis, 2000).

The antiapoptotic effect may occur via the sequestration of proapoptotic molecules such as Hip1 that, once dissociated from huntingtin, forms a complex with Hippi and activates caspase 8 (Gervais, 2002).

The protection of wild-type huntingtin may also occur by activation of BDNF transcription through the interaction with REST and by the stimulation of the vesicular BDNF transport along microtubules (Zuccato, 2001; Zuccato, 2003; Gauthier, 2004).

## 1.3 From chemical to genetic models of HD

A large contribution in elucidating toxic mechanisms involved in HD derives from studies in cellular and animal models of the disease.

Before the emergence of genetic models, different toxins were delivered to rodents and primates to reproduce HD-like phenotypes (Brouillet, 1999).

Glutamate receptors may be over-stimulated with excitatory amino acids such as ibotenic acid, kainic acid, NMDA or quinolinic acid, inducing neuronal death by excitotoxicity (Bruyn and Stoof, 1990). Another strategy to induce striatal degeneration consists in the systemic administration of mitochondrial blocker 3-nitroproprionic acid (3-NP), which inhibits

succinate dehydrogenase, leads to ATP depletion, and reproduces a specific striatal degeneration of GABAergic neurons.

These chemically induced models were, and still are, useful tools to test therapeutic strategies of interest for HD. On the other hand, these models do not reproduce the progressive chronic neuronal degeneration and lack the genetic component of HD. Therefore, their interest has decreased in favor of HD gene-based models.

### 1.3.1 Genetic animal models

A substantial number of animal models of HD is available to conduct studies aimed at understanding and eventually ameliorating the human disease. Differences among these model systems include the length of the huntingtin transprotein, length of polyglutamine repeat, origin of the mutated huntingtin in the species, host organism species and strain and levels of huntingtin expression.

The nematode *Caenorhabditis elegans*, with only 302 neurons, is the simplest genetic animal model of HD (Faber, 1999; Holbert, 2001b; Parker, 2001) that can be used for biochemical, morphological and behavioral studies. In addition, its transparency allows longitudinal live imaging studies *in vivo* with standard microscopy equipment.

*Drosophila melanogaster* is particularly suitable for measuring degeneration of photoreceptors and motor function (Jackson, 1998; Steffan, 2001; Gunawardena, 2003). PolyQ-expressing flies form nuclear inclusions and undergo a progressive neurodegeneration which leads to early cell death.

Mouse models, because of their close genetic and physiological similarities to humans, have been developed as the best mammalian model system for genetic diseases.

The first transgenic HD mouse model was generated in 1996. These mice express the exon 1 of human huntingtin with 115 CAG (strain line R6/1) or 155 CAG (strain line R6/2) repeats and exhibit a severe and rapidly declining molecular and behavioral phenotype, although little evidence for cell loss can be found (Mangiarini, 1996; Murphy, 2000; Turmaine, 2000; van Dellen, 2000).

Various other mouse lines have been developed thereafter, each characterized by different promoter, expression levels, huntingtin length and polyQ expansion (Tab. 3).

Transgenic rat model, which has been also developed, shows phenotypes similar to transgenic mice (von Horsten, 2003).

## CAG *Hdh* knock-in mice

Knock-in mice with the murine protein bearing polyQ expansion are close models of the HD genetic state, as they express mutant huntingtin at endogenous levels.

*Hdh* knock-in mice carrying different expanded CAG repeats have been generated by distinct laboratories. Similarities between these lines have been reported with respect to inclusion formation, brain atrophy, tremors, hyperactivity and clasping phenotype (Tab. 3).

| *Group* | *Promoter gene size polyQ expression* | *Onset behavior* | *Inclusions* | *Cell loss and brain atrophy* | *References* |
|---|---|---|---|---|---|
| Transgenic HD mice | | | | | |
| Bates (R6/1-2) | HD exon1 115Q-155Q < endogenous | 5 weeks Clasping, tremors, abnormal gait, learning deficit, hypokinesis, diabetes | NII and DNI | Fewer dendritic spines, few cells lost, overall brain atrophy | (Mangiarini et al., 1996; Turmaine et al., 2000) |
| Hayden (YAC) | HD full-length 72Q 2x endogenous | 3 months Clasping, hyperactivity and circling | Inclusions in the striatum | Cell loss in the striatum | (Hodgson et al., 1999) |
| | HD full-length 128Q 6x endogenous | Motor abnormalities, hyperactivity, end stage hypoactivity | Inclusions | Striatal neuronal loss Striatal and cortical atrophy | (Slow et al., 2003) |
| Ross/ Borchelt | PrP N171 18,44, 82Q 5x endogenous | 4 months Clasping, tremors, abnormal gait, hypokinesis, weight loss, early death | Inclusions, diffuse nuclear accumulation of htt | dark cells Overall brain atrophy | (Schilling et al., 1999) |
| Tagle | CMV full-length 6, 48, 89Q 1-2x endogenous | 4 months Circling, hyperactivity, end stage hypoactivity, urinary incontinence | Few inclusions | 20% cell loss in striatum in some animals | (Reddy et al., 1998; Reddy et al., 1999) |
| | CMV exons 1-3 89Q 1/5x endogenous | 4 months Prolonged hyperactivity | Few inclusions | not described | Unpublished? |
| Aronin/DiFiglia | rat NSE 3 kb fragment 18,48,100Q > endogenous | 3-4 months Clasping, hyperactivity and endstage hypoactivity | Inclusions | 20% cell loss and brain atrophy in some animals | (DiFiglia et al., 1997) |
| Hen/Yamamoto | tet-off(camKIIα-tTA) HD exon1 97Q < endogenous | 2.5 months Clasping, late onset tremor and gait abnormality | Inclusions and reactive astrocytes | Brain and progressive striatal atrophy | (Yamamoto et al., 2000) |
| Knock-in HD mice | | | | | |
| MacDonald | Hdh 50,92,111Q endogenous | No abnormal behavior | htt nuclear relocalization and inclusions | not described | (White et al., 1997) |
| | neo and HDh 29, 111Q < 1/2 endogenous | 2 months tremor, abnorm gait | htt nuclear relocalization and inclusions | Brain atrophy | (Wheeler et al., 1999; Wheeler et al., 2000) |
| Myers | Hdh prom, 71,82Q 2x endogenous | early onset aggressive behaviour | Late inclusions, LTP impaired, repeat instability in striatum | not described | (Shelbourne et al., 1999) |
| Zeitlin | Hdh 71, 94Q, 150Q ~ 2 endogenous | no phenotype | NMDA sensitivity | No inclusions smaller striatal cell | (Levine et al., 1999) |

Tab. 3: Genetic mouse models of Huntington's disease. The table recapitulates the major characteristics of the available transgenic and knock-in mouse models of Huntington's disease (Adapted from http://www.hdfoundation.org/PDF/hdmicetable.pdf).

In this work we have used *Hdh* knock-in mice generated in the laboratory of Marcy MacDonald (White, 1997), that express endogenous levels of wild-type ($Hdh^{Q7}$) or mutant ($Hdh^{Q111}$) huntingtin. As in humans, *Hdh* knock-in mice with elongated CAG repeat ($Hdh^{Q111}$) presents striatal neuronal degeneration, gliosis, and gait abnormalities at older age (24 months) (Wheeler, 2000). In these mice survival is not compromised, but well before the first signs of neurodegeneration they show early molecular phenotypes evident both in heterozygous ($Hdh^{Q7/111}$) and homozygous ($Hdh^{Q111/111}$) offspring. These changes are first apparent in the striatum, and involve medium spiny projection neurons although at later age other brain regions are involved (MacDonald, 2003).

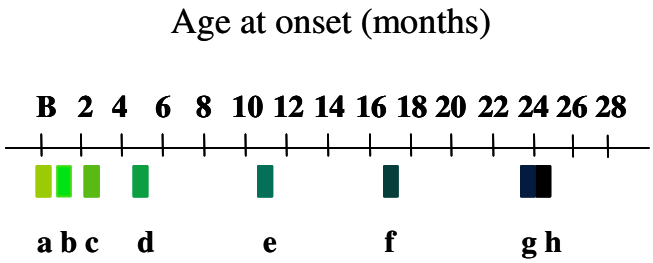


Figure 5: Temporal disease cascade in $Hdh^{Q111}$ mice (Adapted from (MacDonald, 2003).

As depicted in Fig. 5 early phenotypes of $Hdh^{Q111}$ mice include upregulation of *Rrs1* mRNA at 3 weeks of age (a), accumulation of full-length mutant huntingtin in the nucleus of MSNs at 6 weeks of age (b), decreased [ATP/ADP] ratio and cAMP levels (c), reduced cAMP signaling via PKA/CBP/CREB with reduction of BDNF (d). These events precede intranuclear inclusions (e), neuropil aggregates (f) reactive gliosis with neuronal cell dysmorphology (g) and gait deficit (h).

**Cell models**

HD cell models are mainly obtained by expression of mutant huntingtin in different cell types, including yeast, cell lines, or primary cultures.

Several mammalian cell lines stably over-expressing mutant or wild-type huntingtin have been developed (Lunkes and Mandel, 1998; Jana, 2000; Rigamonti, 2000; Wyttenbach, 2001). Neuronal cell lines obtained from *Hdh* knock-in mice are of particular interest because express wild-type and mutant huntingtin at endogenous level and therefore provide genetically precise HD neuronal cell culture models for cell biology and biochemistry.

**ST*Hdh* striatal cells**

In this work we have used immortalized ST$Hdh^{Q7}$ and ST$Hdh^{Q111}$ cell lines, generated from striatal primordia of wild-type $Hdh^{Q7}$ and mutant $Hdh^{Q111}$ knock-in mouse embryos, respectively (Trettel, 2000). ST$Hdh^{Q111}$ striatal cells present an altered nuclear and cytoplasmic distribution of full-length mutant protein. Mutant clones exhibit stress responses that include: elevated levels of p53, an enlarged ER compartment, and an increased basal activity of the iron pathway (Trettel, 2000). Furthermore, reduced concentration of ATP and cAMP in mutant cells suggest impaired energy metabolism. Activation of the Akt pro-survival pathway in mutant striatal cells reflects enhanced NMDA receptor signaling, with excitotoxicity due to aberrant $Ca^{2+}$ influx (Gines, 2003). In addition, as in the human disease, decreased levels of BDNF in ST$Hdh^{Q111}$ cells are evident and associated with altered sub-cellular localization of REST (Zuccato, 2003). These abnormal phenotypes are found both in homozygous and heterozygous mutant clones, suggesting a mechanism that conforms to HD genetic criteria. Thus ST$Hdh^{Q111}$ striatal clones represent a useful cellular model to explore biochemical pathways involving mutant huntingtin and to test hypothesis on the HD-trigger mechanism.

## 1.3.2 Microarray analysis of HD brains and animal models

Characterization of neurotransmitter receptor levels in human HD brains provided the first evidence that transcriptional dysregulation might have a role in HD pathogenesis (Augood, 1997). To elucidate the molecular phenotypes of HD on a genome-wide scale different microarray analyses have been performed both from HD brains and from HD mouse models. The first data set came from a study performed in the striata of R6/2 mice (Luthi-Carter, 2000). This analysis suggested that the dysregulation of the mRNAs encoding neurotransmitter receptors and related second messenger system is an early component of the pathological processes (Luthi-Carter, 2000). More recently, Affymetrix transcription profiles have been generated to compare expression pattern of different brain regions from a large set of HD autopsy brains (Hodges, 2006). Hodges and co-workers found that the transcriptional pathology of HD shows a distinct regional pattern that parallels the known pattern of neurodegeneration: caudate>motor cortex>cerebellum, and a strikingly similar gene

expression profiles of HD caudate and HD motor cortex. This overlap suggested a shared molecular mechanism of HD-related dysfunction in both regions, despite the fact that the HD-sensitive (glutamatergic) corticostriatal pyramidal neurons have a different neurochemistry than that of HD-sensitive (GABAergic) medium spiny neurons of the caudate (Hodges, 2006). Gene ontology analysis of differentially expressed genes suggested increased expression of genes related to central nervous system development in both caudate and motor cortex. Consistent changes in expression were even observed in individual cells and thus the reported decreases in expression in the caudate do not simply reflect cell loss (Hodges, 2006).

In a recent work, seven genetic mouse models of HD and postmortem human HD caudate were compared for changes in mRNA levels (Tab. 4).

A global view suggested that all mutant huntingtin-expressing mice would show relevant mRNA changes if the rodent lifespan allowed sufficient disease progression (Kuhn, 2007). The similarity between the transgenic models and human HD was much stronger for the genes that are down-regulated with the disease. Surprisingly, no differences between models expressing an N-terminal fragment and those expressing a full-length huntingtin protein were observed (Kuhn, 2007; Woodman, 2007). This suggested that these effects are caused by the polyQ-bearing region of the protein. On the other hand, the more protracted timeline of this effect in the full-length models is consistent with the hypothesis that transcriptional dysregulation is dependent on the nuclear accumulation of a proteolytically derived N-terminal huntingtin fragment (Kuhn, 2007).

| R6/1 24w | R6/2 6w | CHL2 15m | Q92 18m | Human | Gene name | Gene symbol |
|---|---|---|---|---|---|---|
| −0.83 | −0.93 | −0.94 | −0.99 | −0.80 | RAS, guanyl releasing protein 2 | RASGRP2 |
| −0.51 | −0.83 | −1.03 | −0.51 | −1.47 | myelin transcription factor 1-like | MYT1L |
| −0.57 | −0.63 | −0.40 | −0.54 | −1.04 | calcium channel, voltage-dependent, alpha2/delta subunit 3 | CACNA2D3 |
| −0.69 | −0.87 | −0.77 | −0.44 | −1.08 | inositol 1,4,5-triphosphate receptor 1 | ITPR1 |
| −0.71 | −0.33 | −0.37 | −0.83 | −1.09 | carbonic anyhydrase 12 | CA12 |
| −0.25 | −0.54 | −0.76 | −0.96 | −1.07 | regulator of G-protein signaling 14 | RGS14 |
| −0.68 | −0.53 | −0.32 | −0.64 | −1.60 | potassium voltage-gated channel, shaker-related subfamily, beta member 1 | KCNAB1 |
| −0.70 | −1.12 | −0.78 | −1.08 | −1.23 | adenosine A2a receptor | ADORA2A |
| −0.56 | −0.79 | −0.44 | −0.49 | −1.34 | hippocalcin | HPCA |
| −0.55 | −0.28 | −0.31 | −0.89 | −1.71 | cannabinoid receptor 1 (brain) | CNR1 |
| −0.71 | −1.42 | −1.08 | −0.84 | −1.68 | cAMP-regulated phosphoprotein 19 | ARPP-19 |
| −0.69 | −0.83 | −0.40 | −0.69 | −0.77 | gamma-aminobutyric acid (GABA-A) receptor, subunit delta | GABRD |
| −0.50 | −0.51 | −0.74 | −0.93 | −0.83 | ST8 alpha-*N*-acetyl-neuraminide alpha-2,8-sialyltransferase 3 | ST8SIA3 |
| −0.47 | −0.41 | −0.24 | −0.54 | −1.03 | Rap 1 GTPase-activating protein | RAP1GAP |
| −0.37 | −0.48 | −0.32 | −0.32 | −0.33 | potassium channel tetramerization domain containing 17 | KCTD 17 |
| −0.48 | −0.33 | −0.46 | −0.63 | −0.60 | UDP-GlcNAc:beta Gal beta-1,3-*N*-acetylglucosaminyltransferase 2 | B3GNT2 |
| −0.83 | −0.79 | −0.63 | −0.80 | −1.68 | coagulation factor C homolog (*Limulus polyphemus*) | COCH |
| −0.33 | −0.23 | −0.38 | −0.57 | −0.29 | POU domain, class 3, transcription factor 1 | POU3F1 |
| −0.54 | −0.74 | −0.55 | −0.48 | −0.72 | homer homolog 1 (*Drosophila*) | HOMER1 |
| −0.40 | −0.78 | −0.35 | −0.59 | −0.42 | D site albumin promoter binding protein | DBP |
| −0.75 | −0.54 | −0.31 | −0.74 | −1.52 | protein tyrosine phosphatase, non-receptor type 5 | PTPN5 |
| −0.57 | −0.42 | −0.47 | −0.35 | −0.79 | ATPase, $Ca^{2+}$ transporting, cardiac muscle, slow twitch 2 | ATP2A2 |
| −0.53 | −0.72 | −0.51 | −0.81 | −0.56 | dopamine receptor 2 | DRD2 |
| −0.45 | −0.32 | −0.67 | −0.36 | −0.33 | zinc finger protein 706 | ZNF706 |
| −0.37 | −0.51 | −0.80 | −0.90 | −0.37 | retinoid X receptor 2 | RXRG |
| −0.81 | −1.41 | −0.24 | −1.26 | −1.38 | preproenkephalin 1 | PENK |
| −0.64 | −0.61 | −1.02 | −0.43 | −1.11 | protein kinase C beta 1 | PRKCB1 |
| −0.34 | −0.33 | −0.40 | −0.22 | −0.43 | spermidine synthase | SRM |
| −0.62 | −1.00 | −0.26 | −0.75 | −0.91 | phosphodiesterase 1B, $Ca^{2+}$-calmodulin dependent | PDE1B |
| −0.64 | −0.55 | −0.61 | −0.40 | −0.58 | ATPase, $Ca^{2+}$ transporting, plasma membrane 2 | ATP2B2 |
| −0.39 | −0.35 | −0.32 | −0.58 | −1.36 | protein phosphatase 3, catalytic subunit, alpha isoform | PPP3CA |
| −0.38 | −0.65 | −0.53 | −0.54 | −0.64 | microtubule-associated serine/threonine kinase 3 | MAST3 |
| −0.47 | −0.35 | −0.39 | −0.51 | −0.47 | sarcolemma-associated protein | SLMAP |
| −0.39 | −0.46 | −0.25 | −0.36 | −0.66 | calcium/calmodulin-dependent protein kinase II, beta | CAMK2B |
| −0.35 | −0.43 | −0.23 | −0.25 | −0.57 | deiodinase, iodothyronine, type II | DIO2 |
| −0.38 | −0.23 | −0.44 | −0.49 | −1.00 | phospholipase C, beta 1 | PLCB1 |
| −0.24 | −0.42 | −0.37 | −0.36 | −1.10 | potassium voltage-gated channel, subfamily Q, member 2 | KCNQ2 |
| −1.17 | −0.25 | −1.35 | −0.81 | −1.56 | regulator of G-protein signaling 4 | RGS4 |
| −0.33 | −0.63 | −0.49 | −0.72 | −0.39 | potassium channel, subfamily K, member 2 | KCNK2 |
| −0.44 | −0.81 | −0.20 | −0.44 | −0.75 | brain-specific angiogenesis inhibitor 1-associated protein 2 | BAIAP2 |
| −0.44 | −0.49 | −0.28 | −0.41 | −0.22 | ubiquitin-specific peptidase 2 | USP2 |
| −0.29 | −0.52 | −0.30 | −0.29 | −0.36 | coronin, actin-binding protein, 2B | CORO2B |
| −0.33 | −0.35 | −0.34 | −0.40 | −0.80 | mannosidase 1, alpha | MANIA1 |
| −0.38 | −0.15 | −0.25 | −0.36 | −0.28 | membrane-bound transcription factor peptidase, site 1 | MBTPS1 |
| −0.61 | −0.73 | −0.55 | −0.40 | −0.75 | chemokine (C-X3-C motif) ligand 1 | CX3CL1 |
| −0.16 | −0.41 | −0.17 | −0.49 | −0.52 | rho guanine nucleotide exchange factor (GEF7) | ARHGEF7 |
| −0.50 | −0.65 | −0.37 | −0.57 | −0.55 | seizure-related gene 6 | SEZ6 |
| −0.32 | −0.55 | −0.33 | −0.42 | −0.51 | myeloid ecotropic viral integration site-related gene 1 | MEIS2 |
| −0.26 | −0.30 | −0.20 | −0.28 | −1.15 | protein phosphatase 1, regulatory (inhibitor) subunit 1A | PPP1R1A |
| −0.34 | −0.55 | −0.49 | −0.32 | −0.62 | cytoplasmic FMR1 interacting protein 2 | CYFIP2 |
| −0.23 | −0.43 | −0.13 | −0.42 | −0.50 | Harvey rat sarcoma virus oncogene 1 | HRAS |
| −0.34 | −0.50 | −0.34 | −0.39 | −0.80 | guanine nucleotide-binding protein, beta 5 | GNB5 |
| −0.18 | −0.24 | −0.13 | −0.26 | −0.30 | selenophosphate synthetase 1 | SEPHS1 |
| −0.25 | −0.39 | −0.19 | −0.27 | −0.70 | adaptor protein complex AP-1, sigma 1 | AP1S1 |
| −0.21 | −0.34 | −0.46 | −0.33 | −0.92 | guanine nucleotide-binding protein, alpha | GNAO1 |
| −0.30 | −0.17 | −0.17 | −0.29 | −0.36 | cDNA sequence BC008155 | C16orf24 |
| −0.38 | −0.69 | −0.26 | −0.70 | −1.13 | retinal-binding protein 4, plasma | RBP4 |
| −0.41 | −0.91 | −0.24 | −0.38 | −1.28 | neuronal guanine nucleotide exchange factor | NGEF |
| −0.69 | −0.55 | −0.72 | −0.35 | −0.44 | calcium/calmodulin-dependent protein kinase II alpha | CAMK2A |
| −0.20 | −0.23 | −0.27 | −0.19 | −0.35 | myeloid leukemia factor 2 | MLF2 |

Tab. 4: Top-ranked early mouse changes concordant with human caudate (Adapted from (Kuhn, 2007)

## 1.4 Toward therapies for HD

Currently, there is no effective therapeutic treatment for preventing or delaying the progression of the disease. Patients are treated with general symptomatic and non-specific HD drugs, such as antidepressants or neuroleptics.

Different drugs, targeting one or more cellular pathway affected by mutant huntingtin, have been proposed and are currently studied (Tab. 5).

As the mutant protein in HD misfolds and aggregates, promoting the degradation of the toxic protein may be of beneficial effect. Many different approaches have been tried to this aim, such as activation of chaperones by *geldanamycin*. The treatment induces expression of heat shock proteins, which promote the heat shock response leading to degradation through the proteasome of misfolded proteins (Sittler, 2001). Another approach is to directly target the proteasome to induce the degradation of toxic soluble proteins (Ciechanover, 2003). Compounds as rapamycin, by activating autophagy, have been found to promote the clearance of aggregates (Ravikumar, 2004).

Chemical compounds that inhibit histone deacetylase might compensate the decrease in the acetylation of histones and the repression of gene transcription caused by the polyQ expansion (Steffan, 2001). They have been found to reduced polyglutamine toxicity in HD models (Ferrante, 2003; Hockly, 2003).

| Candidate drug | Tested in HD | Mechanism of action | FDA approved |
|---|---|---|---|
| Coenzyme Q10 | mouse, rat, human clinical phase I | antioxidant | Yes |
| Creatine | mouse, rat, human clinical phase I | maintains the energy balance in the brain | yes |
| Cystamine | fly, mouse | TGase and caspase-3 inhibitor, antioxidant, increases BDNF | no |
| Cysteamine (Cystagon) | neuronal cells, mouse, monkey, human phase I | TGase and caspase-3 inhibitor, antioxidant, increases BDNF | yes |
| Geldanamycin | mouse neurons and organotypic sclices | activates heat shock response | no |
| Lithium | fly, mouse, rat, human phase I | autophagy inducer | yes |
| Memantine | rat, human clinical phase I | NMDA receptor agonist | yes |
| Minocycline | mouse, rat human | antiapoptotic | yes |
| Mithramycin | mouse, rat | transcription regulator | yes |
| Paroxetine | mouse | serotonin endocytosis inhibitor | yes |
| Rapamycin | fly, mouse | autophagy inducer | yes |
| Remacemide | mouse, human clinical phase I | NMDA receptor agonist | yes |
| Resveratrol | nematode | antioxidant | no |
| Sodium/phenyl butyrate | mouse | transcription regulator | no |
| Suberoylanilide hydroxamic acid | fly, mouse | transcription regulator | yes |
| Tacrolimus (FK506) | neuronal cells, neurons | calcineurin inhibitor | yes |
| Tauroursodeoxycholic acid | mouse, rat | antiapoptotic | yes |
| Trehalose | mouse | protein aggregation inhibitor | yes |

Tab. 5: Possible therapeutic compounds for HD (Borrell-Pages, 2006b).

Evidence of apoptosis in HD *postmortem* brains suggested the use of apoptosis inhibitors. The antibiotic minocycline, which possesses antiapoptotic and anti-inflammatory properties, may be beneficial in HD (Chen, 2000). Although some studies showed opposing results (Diguet, 2003), clinical trials are ongoing and may validate the use of this drug in patients (Bonelli, 2004).

Drugs that improve mitochondrial function, such as coenzyme Q10, a carrier for electron-transfer in the mitochondrial membrane, and creatine, which may stabilize the mitochondrial permeability transition, have been also studied (Verbessem, 2003; Schilling, 2001). These compounds have been proved to be neuroprotective in the transgenic mouse model R6/2 (Ferrante, 2002).

Cystamine is an inhibitor of the transglutaminase (TGase), a calcium-dependent enzyme that is upregulated in the brains of HD patients and HD mice (Karpuj, 1999). Cystamine inhibits also caspase 3 activity (Lesort, 2003), prevents mitochondrial depolarization (Mao, 2006), increases the levels of antioxidants (Fox, 2004), and the secretion and release of BDNF (Borrell-Pages, 2006a).

Neurotrophic factors, such as BDNF and the ciliar neurotrophic factor (CNTF), have been shown to have neuroprotective effects in mouse models of HD (Canals, 2004; Bloch, 2004). The antidepressant paroxetine was identified as a compound that enhances the production or secretion of BDNF (Duan, 2004).

Finally, a promising approach is the reconstruction of neuronal circuits in the brains by intrastriatal transplantation of striatal neuroblasts from human fetuses (Peschanski, 2004). Patients show motor, functional and cognitive improvement, and the transplanted neurons partially rescue the cortico-striatal loop. However, precautions are needed since there are still not enough data to judge the final fate of the transplanted neurons and the clinical improvements might not be permanent (Bachoud-Levi, 2006).

## 1.5 Aims of this work

HD pathogenesis can be viewed as a cascade of events that is first triggered by mutant huntingtin through an abnormal interaction with a yet unknown cellular constituent. Once triggered, this cascade broadens with time, leading first to neuronal dysfunction and subsequently to neuronal death. According to this model, mutant huntingtin might participate either in the trigger mechanism that initiates the disease process or in the myriad subsequent downstream events on the way to cell death (MacDonald, 2003). Several hypotheses have been proposed to elucidate the mechanisms of HD pathogenesis, including excitotoxicity, oxidative stress, impaired energy metabolism, abnormal protein aggregation, transcription dysregulation and abnormal protein interactions. However, because of the incomplete understanding of the interplay among toxic mechanisms in the HD cascade, treatment to delay the onset or slowdown progression remains unavailable at present.
In this context, the description of mutant huntingtin-dependent molecular events is crucial to provide a comprehensive picture of the HD pathogenic mechanisms and to design new therapeutic interventions for this incurable disease.

In this thesis we have taken into account two complementary studies aimed to investigate disease causing molecular mechanisms.

1. The role of Pin1 in Huntington's disease.

   Here we focus our attention on a newly identified huntingtin partner (unpublished data, Michelazzi S., PhD thesis), the peptidyl-prolyl *cis-trans* isomerase Pin1. Pin1 activity has been reported to modulate several cellular processes, including neurodegeneration. In the present work we explore the involvement of Pin1 in HD pathogenic mechanisms.
2. The role of Rrs1 in Huntington's disease.

   In a previous work, the upregulation of *Rrs1* mRNA was identified in a pre-symptomatic mouse model of HD as the earliest detectable event in the HD cascade (Fossale, 2002). Rrs1 protein has been characterized so far only in yeast where it participates in ribosome biogenesis. Here we aim to characterize Rrs1 protein in mammalian cells and to investigate its role in the early phase of the disease process.

# CHAPTER 2: ROLE OF PIN1 IN HD

## 2.1 INTRODUCTION

Proline residues have the unique property of existing in two isomers (Fig. 6) and can provide a potential backbone switch in the polypeptide chain controlled by *cis-trans* isomerization of the peptidyl-prolyl bond. These residues, following a phosphorylated Ser/Thr, are consensus binding site for the peptidyl-prolyl isomerase Pin1 (protein interacting with NIMA (never in mitosis A)-1), which catalyzes the *cis-trans* isomerization of the proline bond. This event couples phosphorylation at these sites with profound changes in protein conformation and has emerged recently as a pivotal signaling mechanism in several processes, such as cell growth regulation, stress responses and neuronal survival.

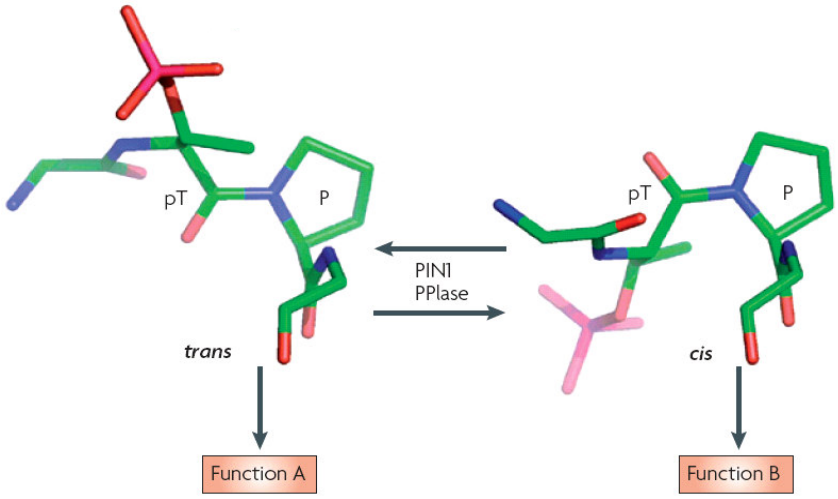


Figure 6: Proline is unique among amino acids in its ability to adopt either the *cis* or *trans* state of the backbone torsion angle, due to its five-membered ring in the peptide backbone. Uncatalyzed isomerization is a rather slow process but can be greatly accelerated by peptidyl-prolyl *cis/trans* isomerases (PPIases). Pin1 belongs to the parvulin subfamily of PPIases, and is the only phosphorylation-specific PPIase. Pin1 specifically recognizes phosphorylated Pro-Ser/Thr (pSer/Thr-Pro) peptide sequences and catalyzes the *cis-trans* conformational changes of the proline bond, thereby modulating the function of its substrates (Adapted from (Lu and Zhou, 2007).

Although Pin1 belongs to the parvulin subfamily of PPIases, it is the only PPIase that specifically recognizes phosphorylated Pro-directed Ser/Thr (pSer/Thr-Pro) motifs (Lu, 1996). This enzyme is a rather small protein (18kDa) present both in nucleus and cytoplasm. Pin1 binds the phosphorylated site of target substrates through its WW domain, while catalyzes the *cis-trans* transition of the proline bond through the isomerase (PPIase) domain (Lu, 1999b).

The change in conformation catalyzed by Pin1 has been shown to perturb protein stability, post-translational modifications and protein interactions of its substrates, that include p53, p73, c-jun, cyclin D1, beta-catenin, bcl-2 and other factors involved in diverse cellular processes (reviewed in (Lu and Zhou, 2007) like cell-cycle control, transcription and splicing regulation, DNA damage response, neuronal survival, and germ cell development (Zacchi, 2002; Atchison, 2003; Wilcox, 2004; Dougherty, 2005). Notably, aberrant Pin1 function has been implicated in several human diseases, including cancer and neurodegenerative disorders. While in normal tissues its expression is associated with cell proliferation, in human cancer Pin1 is prevalently over-expressed working as a critical catalyst for multiple oncogenic pathways (Wulf, 2001; Ryo, 2001; Ayala, 2003). Interestingly, Pin1-deficient mice have been found to be resistant to tumorigenesis induced by specific oncogenes (Liou, 2003), while showing age-dependent neurodegeneration (Wulf, 2004).

### 2.1.1 Pin1 and neurodegeneration

Pin1 dysfunction has a prominent role in age-dependent neurodegeneration and in neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's diseases (PD) and Amyotrophic Lateral Sclerosis (ALS).

In AD brains, Pin1 has been shown to regulate dephosphorylation and function of hyper-phosphorylated forms of tau and of amyloid precursor protein (APP) (Lu, 1999a; Liou, 2003). Since phosphatases such as PP2A can only dephosphorylate pSer/Thr-Pro motifs in the *trans* conformation, Pin1-induced conformational changes may facilitate the dephosphorylation of its substrates (Zhou, 2000). Therefore, a loss of Pin1 function can lead to a build-up of cis-pSer/Thr-Pro motifs. Cis-pAPP is processed by the amyloidogenic pathway, which leads to the accumulation of amyloid-β (Aβ) peptides and the formation of amyloid plaques. Cis-pTau is resistant to protein phosphatases (PPases), which leads to a loss of microtubule (MT) binding, hyperphosphorylated tau and the formation of neurofibrillary tangles. The formation of tangles and plaques might further reduce Pin1 function by sequestering Pin1 and inducing its oxidative modification in a positive feedback loop.

In addition, a lack of proper Pin1 function may lead to further activation of mitotic kinases, which may further increase the phosphorylation of tau, APP and other proteins, eventually

causing neuronal death. Therefore, Pin1 deregulation might act on multiple pathways to contribute to AD pathogenesis and may link both tangle and plaque pathologies.

In PD Pin1 accumulates in Lewy bodies (LB) and its overexpression enhances the protein half-life and insolubility of α-synuclein (Ryo, 2006). By binding to synphilin-1, a α-synuclein partner, Pin1 enhances its interaction with α-synuclein, thus facilitating the formation of α-synuclein inclusions (Fig. 7).

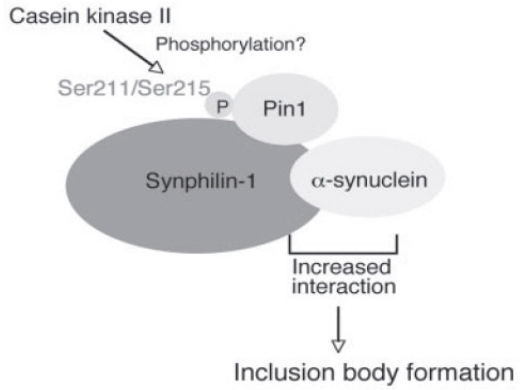


Figure 7: Schematic representation of Pin1-mediated mechanism in PD. Pin1 binds phosphorylated synphilin-1, an α-synuclein partner, via its Ser-211-Pro and Ser-215-Pro motifs and enhances its interaction with α-synuclein, thereby facilitating the formation of α-synuclein inclusions (Ryo, 2006).

Pin1 also colocalized with ALS-affected spinal cord neuronal inclusions and its inhibition reduced glutamate-induced perikaryal accumulation of phosphorylated neurofilament-H in neurons (Kesavapany, 2007).

### 2.1.2 Pin1$^{-/-}$ mice

In a first characterization, Pin1$^{-/-}$ mice were reported to develop normally till the age of six months (Fujimori, 1999).

In a following study, by Liou and co-workers, Pin1$^{-/-}$ mice displayed many severe cell-proliferative abnormalities, including decreased body weight, retinal degeneration, mammary gland retardation, and testicular atrophy (Liou, 2002).

After the implication of Pin1 in Alzeimer's Disease (Lu, 1999), a role for Pin1 in neurodegeneration was postulated.

In normal conditions, Pin1 expression showed a subregional difference in hippocampus and parietal cortex (Liou, 2003). The subregions with low expression of Pin1 are known to be prone to neurofibrillary degeneration in AD, whereas those containing high Pin1 expression

are spared, suggesting the existence of an inverse correlation between Pin1 expression and predicted neuronal vulnerability (Holzer, 2002).

Concomitantly with these findings, $Pin1^{-/-}$ mice showed progressive age-dependent motor and behavioral deficits. Furthermore, the number of NeuN-positive neurons was significantly decreased in the parietal cortex and spinal cord of old, but not young, $Pin1^{-/-}$ mice. However, no obvious neuronal loss was found in other brain regions (Liou, 2003). Degeneration was also observed in some axons and the presence of electron-dense structures in neuronal processes was reported (Liou, 2003).

These results provided genetic evidence for the critical role of Pin1 in protecting against age-dependent neurodegeneration.

The results presented in this section of the thesis are part of a large interlaboratory project aimed to achieve an exhaustive characterization of the role of Pin1 in Huntington's disease. To this purpose, as part of the work performed in our laboratory, Silvia Michelazzi showed for the first time that Pin1 is a novel huntingtin interactor and that mutant huntingtin binding to Pin1 is stronger compared to wild-type (Silvia Michelazzi, SISSA PhD Thesis 2007).

In this thesis we focused our attention on the functional aspects that Pin1 might cover in the context of Huntington's disease.

1. The broad range of cellular pathways in which the prolyl-isomerase Pin1 has been implicated, in particular its characterization as an important player in different neurodegenerative disorders, prompted us to consider a role for Pin1 in mutant huntingtin aggregate formation. Since the exact nature and role of such aggregates in HD is still an open question, whereas the toxicity of the soluble monomeric protein is becoming a well accepted reality, we decided to test the possibility of Pin1 as a regulator of huntingtin stability.
2. Pin1 has been found to alter the activity or the levels of several transcription factors. Since transcriptional dysregulation is an important pathogenic mechanism in HD we decided to asses whether Pin1 may play a role in this process. Microarray analysis is performed on cellular and mouse models of the disease where Pin1 gene has been specifically knocked down.

## 2.2 RESULTS

### 2.2.1 To investigate Pin1 activity in modulating huntingtin half-life and aggregate formation

### Pin1 overexpression reduces mutant huntingtin aggregation

When ectopically expressed in cell cultures, amino-terminal fragments of mutant huntingtin aggregate and generate nuclear and/or cytoplasm inclusions. To investigate whether Pin1 modulates aggregate formation we have used a short huntingtin amino-terminal fragment (residues 1-171) with a pathogenic glutamine tract (Q60) fused at the carboxy-terminus with a GFP-moiety ($htt_{1-171}$Q60GFP) (Persichetti, 1999).

Hek293 cells were co-transfected with mutant huntingtin cDNA ($htt_{1-171}$Q60GFP) and with a construct encoding for HA-tagged Pin1 (HA-Pin1) or with an empty vector (pcDNA3.0-HA) as control. Forty-eight hours after transfection, the proportion of inclusions-containing cells was evaluated by fluorescent microscopy. Interestingly, as shown in figure 8, overexpression of Pin1 remarkably reduces the number of transfected cells with mutant huntingtin inclusions.

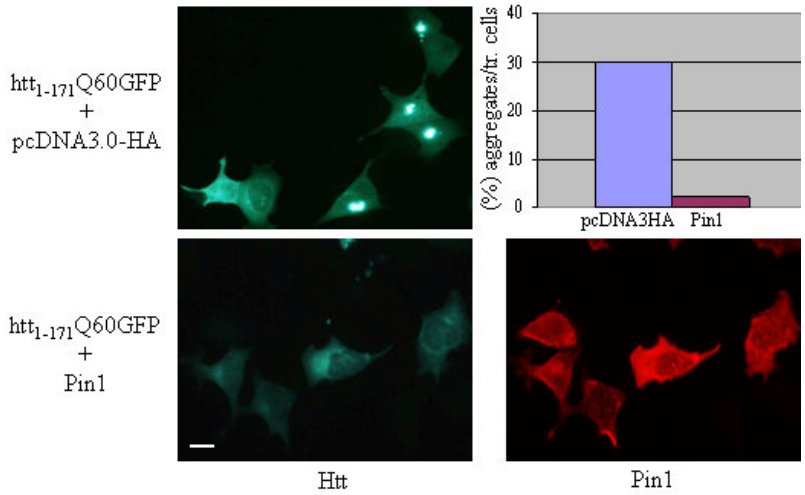


Figure 8: Pin1overexpression reduces mutant huntingtin inclusions. Hek293 cells were co-transfected with $htt_{1-171}$Q60GFP and HA-Pin1 or with empty vector (pcDNA3.0-HA) as control. 48 hours after transfection cells were fixed and stained with anti-HA antibody. Scale bar 10 µm. The percentage of aggregates-containing cells of total transfected cells is shown in the graph.

Pin1 contains an N-terminal WW domain (aa 1-40), which mediates protein interaction and a C-terminal peptidyl-prolyl cis/trans isomerase (PPIase) domain (aa 41-163) that catalyzes isomerization of its substrates (Fig. 9a). Residues Y23 and S67 of the protein have been implicated in regulating the binding and the catalytic activities of Pin1, respectively (Lu, 1999b; Eckerdt, 2005; Behrsin, 2007). By site-directed mutagenesis we have inserted two point-mutations into Pin1 coding sequence to generate an inactive Pin1$^{Y23A;S67E}$ double mutant (HA-Pin1DM) (Fig. 9a). We then used this construct as a more appropriate negative control to repeat overexpression experiments with huntingtin and Pin1.

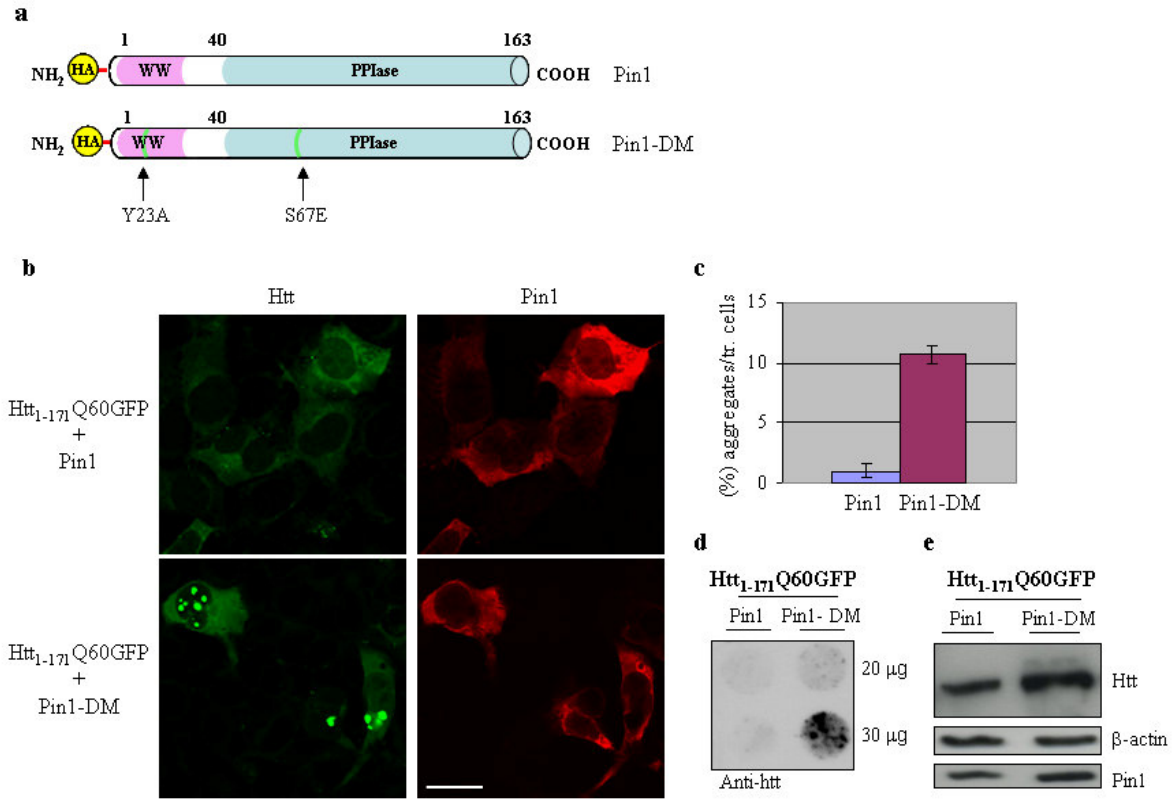


Figure 9: The inactive form of Pin1, Pin1-DM, does not affect htt$_{1-171}$Q60GFP aggregate formation. (a) Schematic representation of Pin1-DM produced by site-directed mutagenesis. (b) Immunofluorescence images of Hek293 cells co-transfected with htt$_{1-171}$Q60GFP and HA-Pin1 or HA-Pin1DM. 48 hours after transfection cells were fixed and stained with anti-HA. Scale bar 10 µm. (c) Percentage of aggregates-containing cells of total co-transfected cells. Error bars represent SDs from 3 independent experiments. (d) Filter trap assay shows absence of detectable htt$_{1-171}$Q60GFP SDS-insoluble material in cells co-expressing HA-Pin1. Hek293 cells were co-transfected with htt$_{1-171}$Q60GFP and HA-Pin1 or HA-Pin1DM. 48 hours after transfection cells were harvested and processed for filter trap. Blot was probed for huntingtin with MAB5490. (e) Western blot analysis of whole-cell lysates probed for huntingtin (MAB5490), β-actin and Pin1 (α-HA). (b), (d) and (e) are representative of at least 4 independent experiments.

Hek293 cells were transfected with $htt_{1-171}$Q60GFP and with HA-Pin1 or HA-Pin1DM. Fluorescent analysis was performed forty-eight hours after transfection. As above, co-expression of Pin1 consistently decreased the number of inclusions in co-transfected cells, which mostly showed diffuse cytoplasmic and nuclear fluorescent distribution of the GFP-huntingtin fragment (Fig. 9b). By contrast, the expression of the inactive Pin1DM did not affect inclusion formation (Fig. 9b and c). Equal transfection efficiency was ensured by counting the number of co-transfected cells in at least 3 independent experiments (data not shown).

We then tested for insoluble complexes using a filter trap assay. SDS-resistant protein extracts of cells co-transfected with $htt_{1-171}$Q60GFP and HA-Pin1 or HA-Pin1DM were applied to cellulose acetate membranes and probed with the anti-huntingtin antibody MAB5490. A strong positive signal for SDS-insoluble material was detected only in protein preparation from cells co-transfected with Pin1DM (Fig. 9d), but not with Pin1, further supporting Pin1 activity in modulating aggregation processes.

Huntingtin aggregation *in vitro* and *in vivo* is dependent on length of the polyglutamine tract, on time and protein concentration (Wanker, 2000; Kaytor, 2004). Since both glutamine length and time are invariable in our experiments, we investigated whether overexpression of Pin1 reduces formation of aggregates/insoluble complexes by affecting huntingtin intracellular concentration. Whole-cell lysates from co-transfected Hek293 cells were analyzed by immunoblot using MAB5490 antibody. As shown in figure 9e, the amount of mutant huntingtin was significantly reduced in cells co-expressing Pin1 compared to negative control. This result suggests that Pin1 activity on aggregation is exerted by lowering the concentration of huntingtin fragment.

We then tested whether Pin1 effect on protein concentration is also exerted on wild-type huntingtin. We used a cDNA construct encoding for the first 171 aminoacids of huntingtin with 21 glutamines fused at the carboxy-terminus with the GFP ($htt_{1-171}$Q21GFP). Typical results, reported in figure 10, indicate that Pin1 reduces wild-type huntingtin concentration compared to control. These data therefore suggest that Pin1 overexpression reduces huntingtin concentration irrespective of polyglutamine length.

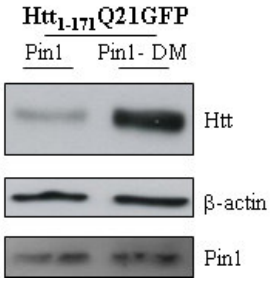


Figure 10: Huntingtin concentration is reduced by Pin1 irrespective of polyQ length. Hek293 cells were co-transfected with $htt_{1-171}$Q21GFP and HA-Pin1 or HA-Pin1DM. 48 hours after transfection cells were harvested and processed for western blot analysis. Blot was probed for huntingtin (MAB5490), β-actin and Pin1 (α-HA). The picture is representative of at least 4 independent experiments.

## Pin1 effect is not exerted through direct interaction with huntingtin amino-terminal fragment

We have previously found that both endogenous full-length huntingtin and ectopically expressed amino-terminal huntingtin fragment, spanning the first 550 aminoacids of the protein ($htt_{1-550}$), interact with Pin1 (unpublished results, PhD Thesis S. Michelazzi). On the contrary, the N-terminal fragment $htt_{1-171}$Q60GFP, which contains a single Pin1 putative binding site (huntingtin $S_{120}$-P), failed to interact with Pin1 in GST-Pin1 pull down experiments. Nonetheless, we decided to test whether Pin1 activity on huntingtin concentration was exerted by direct interaction with the fragment. Using site-directed mutagenesis we generated the mutant construct $htt_{1-171}Q60S_{120}$AGFP, where Serine 120 was replaced with Alanine (Fig. 11a). Hek293 cells were co-transfected with $htt_{1-171}Q60S_{120}$AGFP and HA-Pin1 or HA-Pin1DM as control. The proportion of inclusion-containing cells was evaluated by fluorescent microscopy forty-eight hours after transfection. As reported in figure 11, the overexpression of $htt_{1-171}Q60S_{120}$AGFP and Pin1 did not result in the disappearance of intracellular inclusions (Fig. 11b). However, when we examined by filter trap and western blot analyses the expression of huntingtin we found that, both in SDS-insoluble and in total protein preparations, huntingtin concentration was reduced by Pin1 (Fig. 11c and d).

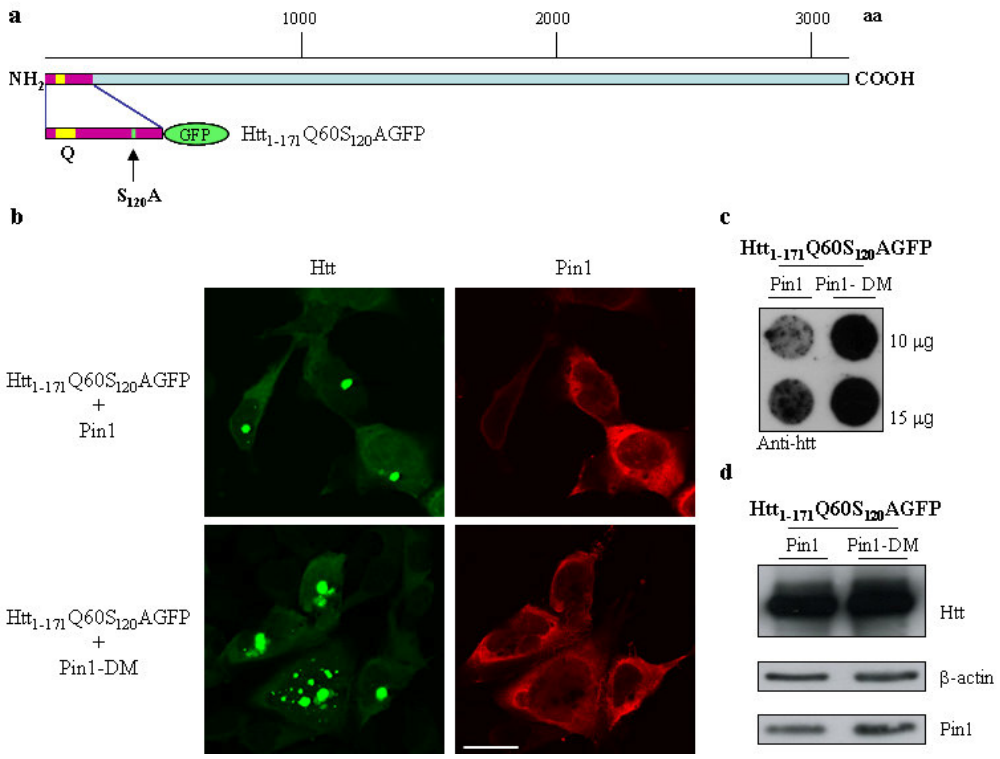


Figure 11: The expression of $htt_{1-171}Q60S_{120}AGFP$ mutant protein is also affected by Pin1. (a) Schematic representation of $htt_{1-171}Q60S_{120}AGFP$ cDNA. The point mutation S120A was inserted by site-directed mutagenesis. (b) Immunofluorescence images of Hek293 cells co-transfected with $htt_{1-171}Q60S_{120}AGFP$ and HA-Pin1 or HA-Pin1DM. Cells were fixed 48 hours after transfection and stained with anti-HA antibody. Scale bar 10 µm. (c) Filter trap assay shows that of $htt_{1-171}Q60S_{120}AGFP$ SDS-insoluble material in cells co-expressing HA-Pin1 is reduced compared to control HA-Pin1DM. Cells were processed 48 hours after transfection. (d) Western blot analysis of whole-cell lysates showing a slight reduction of htt fragment when co-expressed with HA-Pin1 compared to control HA-Pin1DM. Blot was probed for huntingtin (MAB5490), β-actin and Pin1 (α-HA). The pictures are representative of at least 4 independent experiments.

These results are in agreement with those obtained using $htt_{1-171}Q60GFP$ fragment and therefore suggest that Pin1 effects are not exerted via a direct interaction.

To further support this observation we have used a shorter mutant N-terminal huntingtin fragment, namely htt exon 1 (httex1Q60GFP), which does not contain the $S_{120}P$ site. Co-expression experiments in Hek293 cells with httex1Q60GFP and either HA-Pin1 or HA-Pin1DM showed the same reducing effect of Pin1 on the co-expressed shorter mutant huntingtin fusion protein (Fig. 12).

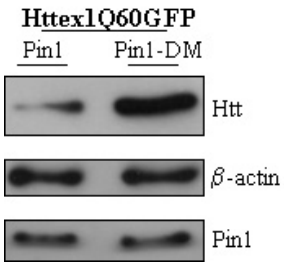


Figure 12: Pin1 decreases httex1Q60GFP protein level. Western blot of Hek293 cells co-transfected with httex1Q60GFP and HA-Pin1 or HA-Pin1DM. Blot was probed for huntingtin (α-GFP), β-actin and Pin1 (α-HA). The picture is representative of 3 independent experiments.

We then addressed whether Pin1 activity is specifically exerted on huntingtin fragments or if is a more general consequence of Pin1 overexpression. We used therefore a cDNA construct encoding for GFP that was co-transfected with Pin1 or with Pin1DM as control. Whole-cell lysates were prepared from transfected cells and analyzed by immunoblot with an anti-GFP antibody. The result shown in figure 13 indicates that GFP protein expression is not even slightly affected by Pin1, suggesting that Pin1 effect is not a general consequence of its overexpression.

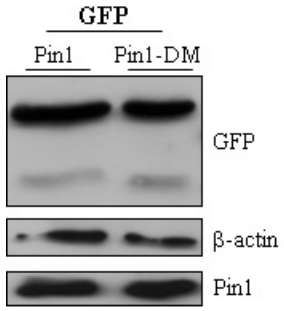


Figure 13: GFP protein concentration is not altered by Pin1. Hek293 cells were co-transfected with GFP and HA-Pin1 or HA-Pin1DM. 48 hours after transfection cells were harvested and processed for western blot. Blot was probed for GFP, β-actin and Pin1 (α-HA). The picture is representative of 3 independent experiments.

## Pin1 affects huntingtin half-life

Reduced expression of huntingtin fragment may be due to enhanced protein degradation mediated by Pin1. To investigate this hypothesis we have evaluated the steady state level of huntingtin in presence of Pin1.

Hek293 cells were co-transfected with $htt_{1\text{-}171}$Q60GFP and HA-Pin1 or HA-Pin1DM as control, according to our standard transfection protocol. After six hours, cells were treated with 40μg/ml of cycloheximide (CHX) (time zero), an antibiotic which inhibits protein

translation by blocking the ribosome activity. Whole cell extracts were then prepared after different times from CHX treatment (2 and 4 hours) and analyzed by immunoblot (Fig. 14). The relative amount of huntingtin at different times was determined by densitometric scanning of western blot signal using β-actin for normalization. As shown in the representative experiment of figure 14 the relative half-life of huntingtin fragment after 2 hrs was reduced of ~ 2.5 fold (ratio htt/β-actin signal = 0.4) in presence of Pin1, compared to a slight reduction of 1.4 fold (ratio htt/β-actin signal = 0.7) with the control. Thus, these results demonstrate that Pin1 overexpression reduces protein half-life of huntingtin fragment.

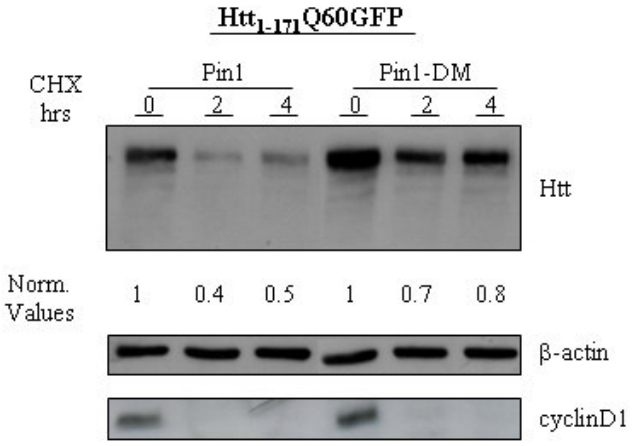


Figure 14: Pin1 reduces huntingtin half-life. Hek293 cells were co-transfected with $htt_{1-171}Q60GFP$ and HA-Pin1 or HA-Pin1DM as control. 6 hours after transfection cells were treated with 40 μg/ml CHX (time zero) for 2 or 4 hours. At the indicated time cells were harvested and analyzed by western blot. Blot was probed for huntingtin (MAB5490), β-actin and cyclinD1. The picture is representative of 4 independent experiments.

Pin1 has been reported to modulate transcriptional activity of RNA polymerase II (RNAP II) by influencing phosphorylation status of the C-terminal domain of the largest subunit. Pin1 overexpression causes formation of a hyperphosphorylated inactive form of RNAP II and specifically inhibits transcription of mRNA precursors in vivo (Xu, 2003; Xu, 2007). We therefore evaluated whether transcription efficiency of the various constructs we had used, may be affected by Pin1. By RT-qPCR we measured mRNA expression of huntingtin ($htt_{1-171}Q60GFP$ and $htt_{1-171}Q60S_{120}AGFP$) and GFP constructs in transfected cells overexpressing Pin1 or Pin1DM. As expected, a reduction in transcription efficiency was observed with Pin1, but not with its negative control. However, the extent of the reduction was identical for huntingtin and for GFP constructs (data not shown).

## Pin1 stimulates activity of the UPS

It is known that GFP is a highly stable protein which is not normally degraded by proteasome (Bence, 2001; Verhoef, 2002). Conversely, it has been reported that N-terminal fragments of huntingtin are proteasome substrates (Jana, 2001; Chandra, 2008). Since GFP protein levels were not altered by Pin1 overexpression (Fig. 14) we have hypothesized that Pin1 might reduce huntingtin half-life by a degradation mechanism that involves the proteasome.

Before testing this hypothesis we assessed whether in our experimental conditions $htt_{1-171}$Q60GFP was a proteasome substrate. Hek293 cells were transfected with the huntingtin fragment and twenty-four hours later treated with the proteasome inhibitor MG132 (10μM), or with DMSO as control, for six hours. Whole-cell extracts were prepared and analyzed by western blot using MAB5490 antibody. As shown in figure 15 the huntingtin fragment accumulates upon proteasome block. We also noted an increase in cyclinD1 on MG-132 treatment, consistent with inhibition of proteasome activity (data not shown).

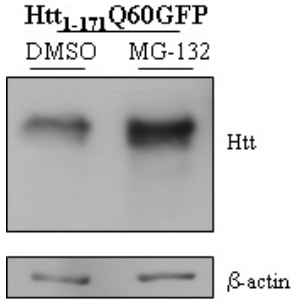


Figure 15: $Htt_{1-171}$Q60GFP is substrate of the proteasome. Hek293 cells were transfected with $htt_{1-171}$Q60GFP. 24 hours after transfection cells were treated with 10μM MG-132 or DMSO for 6 hours, then harvested and analyzed by Western blot. Blot was probed for huntingtin (MAB5490) and β-actin.

To explore the effect of Pin1 overexpression on proteasome activity we used the $YFP^u$ reporter system (Bence, 2001; Bennett, 2005). $YFP^u$ is a protein containing a short degron (CL1), a signal for the ubiquitin-proteasome pathway, fused to the C-terminus of YFP (Bence, 2001). Since $YFP^u$, conversely to YFP, is normally rapidly degraded by the proteasome ($t_{1/2}$ ~30min), it represents an appropriate experimental system to test proteasome activity in the presence of Pin1. In our experimental settings, the $YFP^u$ was transfected at a very low concentration to avoid excessive protein accumulation due to overexpression.

Hek293 cells were co-transfected with $YFP^u$ cDNA and with HA-Pin1 or HA-Pin1DM. Forty-eight hours after transfection the level of $YFP^u$ protein was evaluated by immunofluorescence

and western blot of whole-cell lysates. As shown in figure 16, the YFP$^u$ signal was significantly reduced in cells co-expressing Pin1 compared to negative control both in immunofluorescence and in western blot analysis (Fig. 16a and b).

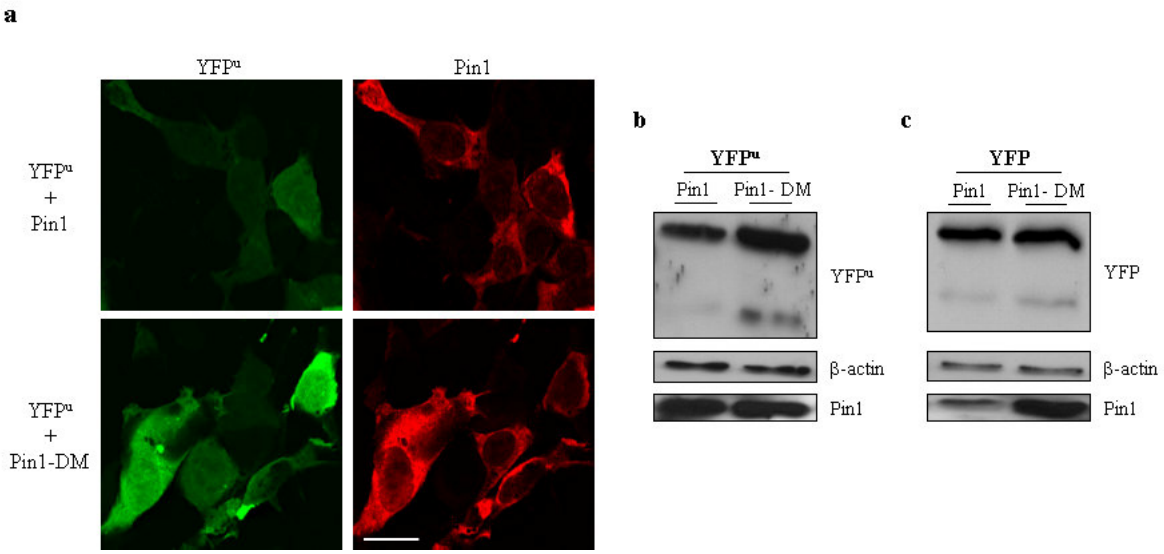


Figure 16: Pin1 promotes proteasome degradation of YFP$^u$. Hek293 cells were transfected with YFP$^u$ or YFP as control and HA-Pin1 or HA-Pin1DM according to the standard protocol. 48 hours after transfection cells were harvested and subject to western blot or fixed in 4% PFA for IF analysis. (a) Immunofluorescence images of Hek293 co-expressing YFP$^u$ and HA-Pin1 or HA-Pin1DM. Cells were fixed 48 hours after transfection and stained with anti-HA antibody. Scale bar 10 μm. (b) Western blot of whole-cell lysates from Hek293 co-transfected with YFP$^u$ and HA-Pin1 or HA-Pin1DM. Blot was probed for YFP$^u$ (α-GFP), β-actin and Pin1 (α-HA). (c) Western blot analysis of whole-cell lysates of Hek293 co-transfected with YFP and HA-Pin1 or HA-Pin1DM. Blot was probed for YFP (α-GFP), β-actin and Pin1 (α-HA). The pictures are representative of at least 4 independent experiments.

The same experiment was repeated overexpressing YFP, which is not normally degraded by proteasome, with either HA-Pin1 or HA-Pin1DM. As shown in figure 16c the presence of Pin1 does not affect YFP concentration. These results, therefore suggest that Pin1-mediated-degradation is occurring by proteasome activity.

To exclude the possibility that transcription efficiency accounted for the differences in the expression level, we performed RT-qPCR using mRNA isolated from cells transfected with YFP$^u$ or YFP and either HA-Pin1 or HA-Pin1DM. In agreement with results obtained with GFP and huntingtin fragments, YFP mRNA level was significantly reduced ($P<0.05$) of ~30% by Pin1 (Fig. 17b). However, interestingly, mRNA level of YFP$^u$ was not reduced in presence of Pin1 (Fig. 17a). Since steady state level of YFP$^u$ protein is determined by a balance between

protein production and protein degradation, RT-qPCR data further support a role for Pin1 in inducing protein degradation by proteasome.

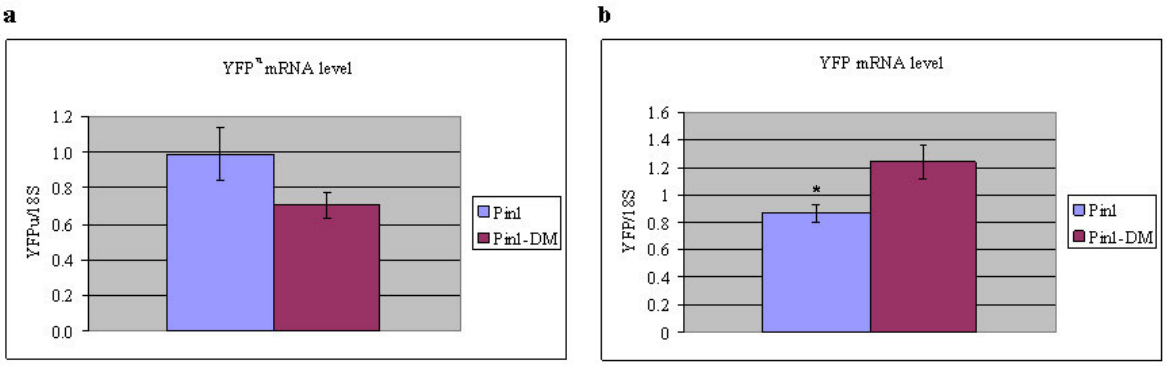


Figure 17: Pin1 reduces mRNA level of YFP but not of $YFP^u$. Graphic representations of normalized mRNA expression values of Hek293 cells co-transfected with $YFP^u$ (a) or YFP (b) and either HA-Pin1 or HA-Pin1DM. 48 hours after transfection cells were harvested for RNA extraction. 1 μg of total RNA was retrotranscribed and analyzed by real time quantitative PCR. mRNA expression values were normalized against 18S RNA and β-actin mRNA values. Error bars represent SDs of 4 independent experiments.

## 2.2.2 Characterization of $htt_{1-171}Q60S_{120}AGFP$

The mutant huntingtin construct we have generated by site-directed mutagenesis ($htt_{1-171}Q60S_{120}AGFP$), has attracted our attention for its ability to generate a large amount of inclusion when transfected in Hek293 cells. We have therefore further analyzed the property of this construct by direct comparison with wild-type $htt_{1-171}Q60GFP$ cDNA.

The expression of the two cDNA constructs was first evaluated in a time course of 12 hours. Hek293 cells were transfected with $htt_{1-171}Q60S_{120}AGFP$ or $htt_{1-171}Q60GFP$ cDNAs and whole-cell protein extracts were prepared at fixed time intervals of 3, 6, 9 and 12-hours after transfection. Western blot analysis revealed that the amount of mutant fragments was increased compared to wild-type, starting from 6 hour after transfection up to 12 hours (Fig. 18). In addition, inspection by fluorescent analysis showed that $htt_{1-171}Q60S_{120}AGFP$ fragment gave rise to inclusion formation already at 12 hours from transfection (data not shown).

We have tested whether unequal transcription efficiency may provide an explanation for the expression level differences between the two constructs. By RT-qPCR transcript levels were found to be similar (data not shown).

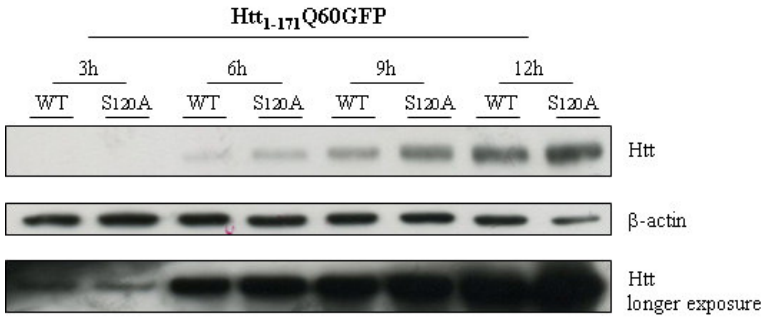


Figure 18: Protein expression of mutant S120A htt1-171 fragment is increased compared to wild-type. Hek293 cells were transfected with $htt_{1-171}Q60GFP$ (WT) or $htt_{1-171}Q60S_{120}AGFP$ (S120A) and collected at the indicated time after transfection for Western blot analysis. Blot was probed for huntingtin (MAB5490) and β-actin.

Higher amount of mutant fragment might be related to its increased stability compared to wild-type. We measured therefore the half-life of the two huntingtin fragments by blocking protein synthesis with CHX. Hek293 cells were transfected with either $htt_{1-171}Q60GFP$ or $htt_{1-171}Q60S_{120}AGFP$ and six hours after transfection were treated with 40 μg/ml of CHX. Whole-cell extracts were prepared after different time from CHX treatment (2 and 4 hours) and analyzed by immunoblot with MAB5490 antibody. The relative amount of both huntingtin fragments was determined by densitometric scanning of western blot signal using β-actin for normalization. As shown in figure 19, $htt_{1-171}Q60GFP$ exhibited a $t_{1/2}$ of ~2hrs, whereas that of $htt_{1-171}Q60S_{120}AGFP$ was ~4hrs.

Thus the substitution of Serine with Alanine at residue 120 of huntingtin leads to a mutant protein with a longer half life.

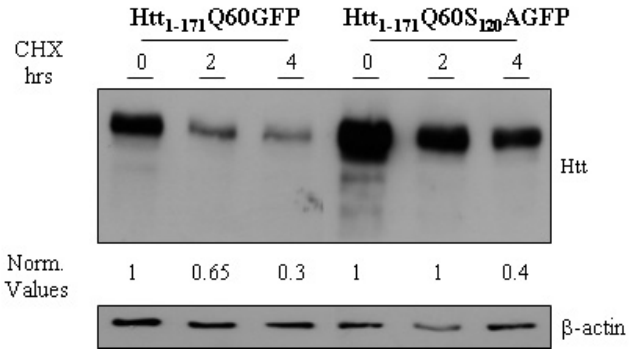


Figure 19: The point mutation S120A stabilizes htt1-171 fragment. Hek293 were transfected with $htt_{1-171}Q60GFP$ or $htt_{1-171}Q60S_{120}AGFP$ and 6 hours after transfection were treated with 40 μg/ml CHX. Cells were collected at the indicated time points and processed for Western blot analysis. Blot was probed for huntingtin ( MAB5490) and β-actin. The picture is representative of 3 independent experiments.

### 2.2.3 To investigate Pin1 activity in regulating gene expression profiling in *in vitro* and *in vivo* model systems of HD

## Pin1 silencing in striatal cells

We took advantage of a well established HD cellular model, represented by immortalized *STHdh* striatal cells derived from wild type and knock-in mouse striata, to set up Pin1 silencing and subsequent gene expression profiling analysis.

For the silencing of Pin1 we used Dharmacon SMARTpool siRNAs specifically designed to block Pin1 expression and Dharmacon RISC-free siRNA as control.

$Hdh^{Q7/Q7}$ and $Hdh^{Q111/Q111}$ striatal cells were transfected with 75 nM of Pin1 SMARTpool (SP) or with RISC-free (RF) for 72 hours, and then collected for RNA extraction. Pin1 silencing was monitored both by western blot with anti-Pin1 antibody, and by real time quantitative PCR, as shown in figure 20.

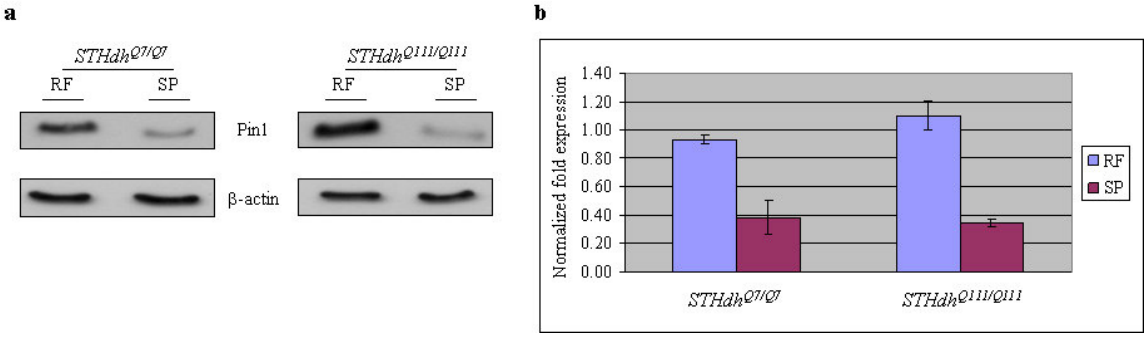


Figure 20: Pin1 silencing in *STHdh* striatal cells. Cells were transfected with 75 nM Pin1 Smart pool (SP) or SiControl Risc Free (RF) and 72 hours later harvested for protein and RNA extraction. (a) Western blot analysis of Pin1 in *STHdh* striatal cells at 72 hour from transfection. Blot was probed for Pin1 and β-actin. (b) Pin1 mRNA level at 72 hours from transfection. The expression value was normalized against β-actin mRNA values (Normalized fold expression). Error bars represent SDs from 3 independent experiments.

## Microarray analysis

Three out of six independent silencing experiments were then chosen for further microarray analysis. Total RNAs were treated with DNAse and purified with the RNeasy Mini Kit from Qiagen. RNA quality was assessed using an Agilent 2001 Bioanalyzer and only samples with an RNA Integrity Number (R.I.N.) higher then 8 were then used. Five µg of total RNA from each sample were templates to generate biotinylated cRNA. They were then hybridized to the

Affymetrix murine 430A 2.0 gene chips that contain approximately 14,000 well-characterized mouse genes. After overnight hybridization, Chips were scanned and image files were processed. Data were then analyzed by using the R Bioconductor package (Gautier L 2004)

The transient nature of Pin1 knock-down led to some variability among the replicates and reduced the number of differentially expressed genes. Nonetheless, statistical analysis using LIMMA on a subset of probes filtered for fold-change threshold of 1.5, returned 8 genes differentially expressed in striatal wild-type cells and 6 genes in mutant cells (Tab.6).

Genes differentially expressed in silenced wild-type striatal cells

| Gene name | Gene symbol | FC |
|---|---|---|
| secreted Ly6/Plaur domain containing 1 | Slurp1 | 1.56 |
| ribonuclease, RNase A family, 1 (pancreatic) | Rnase1 | 0.66 |
| Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 23 | Slc25a23 | 0.65 |
| deiodinase, iodothyronine, type II | DIO2 | 0.64 |
| glycerophosphodiester phosphodiesterase domain containing 2 | Gdpd2 | 0.63 |
| synaptotagmin IX | Syt9 | 0.56 |
| zinc finger protein 91 | Zfp91 | 0.54 |
| protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1 | Pin1 | 0.46 |

Genes differentially expressed in silenced mutant striatal cells

| Gene name | Gene symbol | FC |
|---|---|---|
| DNA-damage-inducible transcript 4-like | Ddit4l | 1.66 |
| scavenger receptor class A, member 5 (putative) | Scara5 | 1.65 |
| HECT, UBA and WWE domain containing 1 | HUWE1 | 1.62 |
| Transformed mouse 3T3 cell double minute 2 | MDM2 | 1.52 |
| Matrix metallopeptidase 9 | Mmp9 | 0.61 |
| protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1 | Pin1 | 0.47 |

Tab. 6: Statistical analysis was performed by means of Linear Models for Microarray Data (LIMMA). All adjusted P-values are $\leq 0.05$. FC= Fold change.

In both experiments Pin1 was at the top of the list of down-regulated genes proving both the efficiency of the siRNA as well as the sensitivity of the array hybridizations.

Interestingly, besides Pin1, no overlap was noted between the two gene lists suggesting specific effects of Pin1 silencing in a wild-type or mutant background.

No Gene Ontology terms enrichment was observed in the list of the wild-type cells while 2 out of the 4 induced genes in mutant cells were E3 ubiquitin ligases: HUWE1 and MDM2.

We then arbitrarily choose these two as well as Ddit4l and Slc25a23 for validation with real-time quantitative PCR.

As shown in figure 22a, differential expression was confirmed for all the genes tested. Interestingly, Ddit4l and Slc25a23 expressions were altered in basal conditions (Fig 21b).

Furthermore, HUWE1 and MDM2 mRNAs were induced only by Pin1 interference on mutant cells with an invariant gene levels in the two cell lines in untreated conditions (Fig.21a and b).

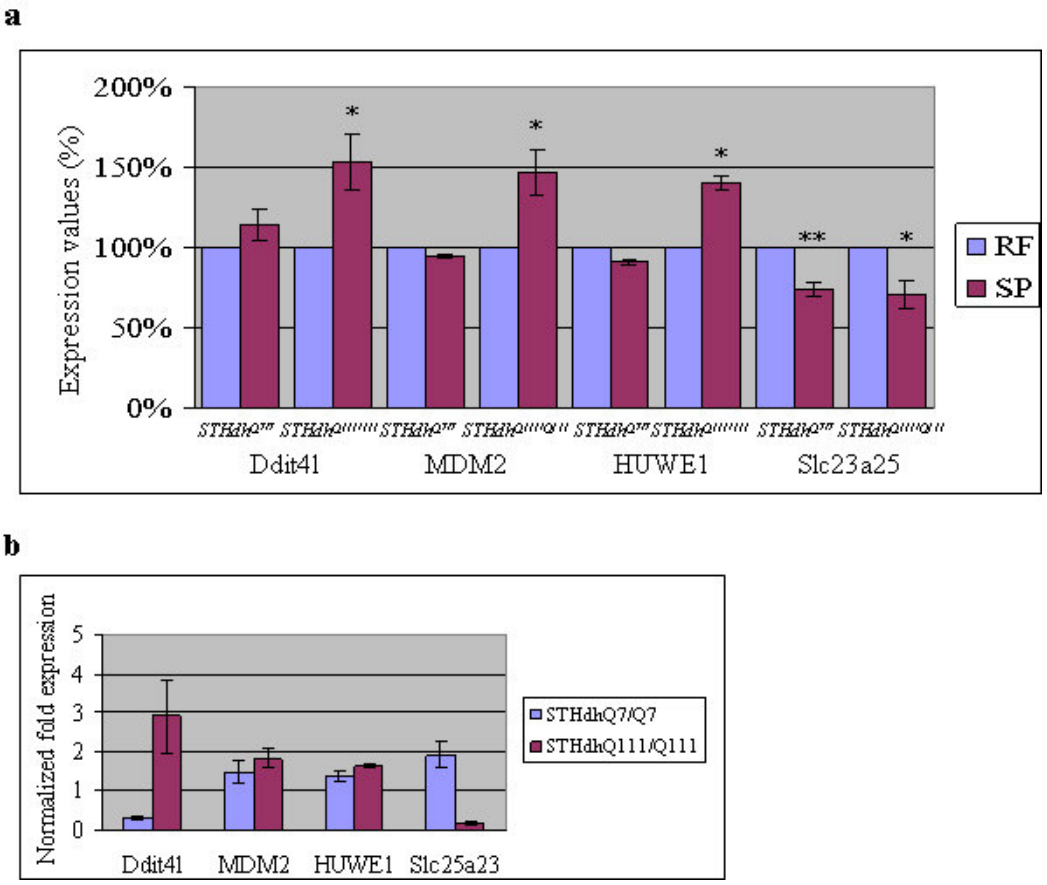


Figure 21: Expression analysis of Ddit4l, MDM-2, HUWE1 and Slc25a23 in *STHdh* striatal cells upon Pin1 silencing (a) and in normal conditions (b). *STHdh* striatal cells were transfected with 75 nM Pin1 Smart pool (SP) or 75nM SiControl Risc Free (RF) and 72 hours later harvested for RNA extraction. 1 µg of total RNA was retrotranscribed and analyzed by real time quantitative PCR. mRNA expression levels were normalized relatively to β-actin mRNA level. Error bars represent SDs from 3 independent experiments.

## Gene expression profile of *Hdh* knock-in mice with a Pin1 knock-out background

### Generation of $Hdh^{Q7/Q111}$ : $Pin1^{-/-}$ mouse line

To dissect the contribution of Pin1 to HD pathogenesis and/or progression, we generated a new mouse line by breeding $Hdh^{Q7/Q111}$mice with $Pin1^{+/-}$ mice. The F1 offspring was intercrossed in order to obtain the following littermates: $Hdh^{Q111/+}$:$Pin1^{+/+}$, $Hdh^{Q111/+}$:$Pin1^{-/-}$, $Hdh^{+/+}$:$Pin1^{+/+}$, $Hdh^{+/+}$:$Pin1^{-/-}$, $Hdh^{Q111/Q111}$:$Pin1^{-/-}$, $Hdh^{Q111/Q111}$:$Pin1^{+/+}$. The exact genotype of the mice was determined by PCR on DNA extracted from tail biopsy.

### Gene expression profile of $Hdh^{Q7/Q111}$:$Pin^{-/-}$ mouse striata

To characterize this new mouse model, we have performed gene expression profiling experiments from the striatum of mice with different genotypes.

Although the first robust gene expression alterations in the striatum of *Hdh* knock-in mouse occur at ~10 months of age, transcriptional dysregulation has been reported as early as 3 weeks of age (Fossale, 2002; Chan, 2002; Woodman, 2007; Kuhn, 2007).

Gene expression profiles of $Pin1^{-/-}$ mice have never been reported so far, nonetheless, evident neurodegeneration of the frontal cortex and spinal cord, with features resembling AD, was described to occur as early as six months of age, suggesting the existence of neuronal dysfunctions preceding the overt neuronal loss (Liou, 2002; Liou, 2003).

Therefore, we decided to analyze mice at 15 weeks of age aiming to avoid the establishment of irreversible neuronal damages due to the loss of Pin1 activity.

RNAs from the striata of three mice for each genotype were purified as described in Methods section. They were then hybridized separately to the very same Affymetrix murine 430A 2.0 gene chips. Normalization, filtration and statistical analysis of the data files were performed as reported in Methods section. A false discovery rate of 10% was applied.

First, we evaluated the effects of mutant huntingtin on a Pin1 wild-type background. The results are summarized in Table 7.

**Genes differentially expressed in *Hdh*$^{Q7/Q111}$: *Pin1*$^{+/+}$ mice**

| Gene name | Gene symbol | FC |
|---|---|---|
| Early growth response 2 | Egr2 | 2.40 |
| FBJ osteosarcoma oncogene | Fos | 1.88 |
| tropomyosin 1, alpha | Tpm1 | 1.68 |
| abhydrolase domain containing 1 | Abhd1 | 1.65 |
| Rho guanine nucleotide exchange factor (GEF) 5 | Arhgef5 | 1.41 |
| poliovirus receptor | Pvr | 1.39 |
| aspartyl aminopeptidase | Dnpep | 1.37 |
| Tumor necrosis factor receptor superfamily, member 25 | Tnfrsf25 | 1.36 |
| zinc finger protein 207 | Zfp207 | 1.36 |
| ankyrin 3, epithelial | Ank3 | 1.35 |
| RIKEN cDNA 8030411F24 gene | 8030411F24Rik | 1.32 |
| class II transactivator | Ciita | 1.28 |
| SNAP-associated protein | Snapap | 0.73 |
| Ras interacting protein 1 | Rasip1 | 0.71 |
| gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 3 | Gabra3 | 0.65 |
| lymphocyte antigen 6 complex, locus H | Ly6h | 0.62 |
| expressed sequence AI467657 | AI467657 | 0.58 |
| Bcl2-associated X protein | Bax | 0.55 |
| expressed sequence AI467657 | AI467657 | 0.48 |
| Protein arginine N-methyltransferase 1 | Prmt1 | 0.44 |

Tab. 7: Statistical analysis was performed by means of the Significance Analysis of Microarrays (SAM). The False Discovery Rate (FDR) was estimated at 10%. FC= Fold Change.

Then, we assessed the effect of Pin1 knock-down on a wild-type huntingtin background. The list of genes differentially expressed is reported in Table 8.

**Genes differentially expressed in *Hdh$^{Q7/Q7}$: Pin1$^{-/-}$* mice.**

| Gene name | Gene symbol | FC |
|---|---|---|
| X-linked lymphocyte-regulated 4B | Xlr4b | 5.09 |
| immunoglobulin kappa chain, constant region | Igk-C | 3.36 |
| neurogenic differentiation 6 | Neurod6 | 3.36 |
| early growth response 2 | Egr2 | 3.12 |
| C1q-like 3 | C1ql3 | 2.66 |
| early growth response 2 | Egr2 | 2.50 |
| C1q-like 3 | C1ql3 | 2.50 |
| RNA-binding region (RNP1, RRM) containing 3 | Rnpc3 | 2.41 |
| Homer homolog 1 (Drosophila) | Homer1 | 2.36 |
| BRCA1/BRCA2-containing complex, subunit 3 | Brcc3 | 2.33 |
| kit ligand | Kitl | 2.30 |
| cerebellin 1 precursor protein | Cbln1 | 2.29 |
| Kruppel-like factor 10 | Klf10 | 2.28 |
| heat shock protein 1B | Hspa1b | 2.27 |
| procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide | P4ha1 | 2.23 |
| FBJ osteosarcoma oncogene | Fos | 2.22 |
| Protein tyrosine phosphatase, receptor type, J | Ptprj | 2.15 |
| microtubule-associated protein 2 | Mtap2 | 2.15 |
| cytoplasmic polyadenylation element binding protein 4 | Cpeb4 | 2.12 |
| Special AT-rich sequence binding protein 2 | Satb2 | 2.11 |
| tetratricopeptide repeat domain 14 | Ttc14 | 2.07 |
| integrin alpha V | Itgav | 2.05 |
| DnaJ (Hsp40) homolog, subfamily B, member 5 | Dnajb5 | 2.04 |
| microtubule-associated protein 2 | Mtap2 | 2.03 |
| gamma-aminobutyric acid (GABA-A) receptor, subunit beta 3 | Gabrb3 | 2.02 |
| brain derived neurotrophic factor | Bdnf | 2.02 |
| ubiquitin protein ligase E3A | Ube3a | 2.02 |
| mitogen activated protein kinase 14 | Mapk14 | 2.00 |
| zinc finger protein 451 | Zfp451 | 2.00 |
| histone cluster 1, H4h | Hist1h4h | 0.48 |
| chemokine (C-C motif) ligand 21a | Ccl21a | 0.47 |
| ribosomal protein S4, Y-linked 2 | Rps4y2 | 0.35 |
| plasma membrane associated protein, S3-12 | S3-12 | 0.24 |
| protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1 | Pin1 | 0.02 |

Tab. 8: Statistical analysis was performed by means of the Significance Analysis of Microarrays (SAM). The False Discovery Rate (FDR) was estimated at 10%. FC= Fold Change.

Finally, we analyzed the consequence of Pin1 knock-down on a mutant huntingtin background (Tab. 9).

**Genes differentially expressed in *Hdh$^{Q7/Q111}$: Pin1$^{-/-}$* mice.**

| Gene name | Gene symbol | FC |
|---|---|---|
| arginine vasopressin | Avp | 2.17 |
| zinc finger protein of the cerebellum 4 | Zic4 | 1.72 |
| peroxisomal biogenesis factor 13 | Pex13 | 1.46 |
| immunoglobulin (CD79A) binding protein 1 | Igbp1 | 1.45 |
| RIKEN cDNA 2700078E11 gene | 2700078E11Rik | 1.45 |
| amylase 1, salivary | Amy1 | 1.44 |
| mitochondrial ribosomal protein 63 | Mrp63 | 1.42 |
| microsomal triglyceride transfer protein | Mttp | 1.42 |
| EGF-like repeats and discoidin I-like domains 3 | Edil3 | 1.41 |
| t-complex-associated testis expressed 2 | Tcte2 | 1.41 |
| DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 | Ddx4 | 1.37 |
| immunoglobulin (CD79A) binding protein 1 | Igbp1 | 1.35 |
| RIKEN cDNA 4921524J17 gene | 4921524J17Rik | 1.34 |
| Tumor necrosis factor alpha induced protein 6 | Tnfaip6 | 1.34 |
| BRCA1/BRCA2-containing complex, subunit 3 | Brcc3 | 1.34 |
| small nuclear ribonucleoprotein D1 | Snrpd1 | 1.33 |
| chemokine (C-C motif) ligand 25 | Ccl25 | 1.31 |
| zinc finger protein 191 | Zfp191 | 1.30 |
| Ly6/neurotoxin 1 | Lynx1 | 1.27 |
| isoleucine-tRNA synthetase 2, mitochondrial | Iars2 | 1.26 |
| Thymopoietin | Tmpo | 1.26 |
| ORM1-like 1 (S. cerevisiae) | Ormdl1 | 1.24 |
| cell cycle associated protein 1 | Caprin1 | 1.24 |
| SRY-box containing gene 7 | Sox7 | 1.24 |
| ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast) | Ube2d3 | 1.22 |
| RIKEN cDNA C330007P06 gene | C330007P06Rik | 1.22 |
| RIKEN cDNA 2700078E11 gene | 2700078E11Rik | 1.21 |
| ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast) | Ube2d3 | 1.18 |
| protocadherin 21 | Pcdh21 | 0.64 |
| Histone cluster 1, H4h | Hist1h4h | 0.57 |
| Protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1 | Pin1 | 0.02 |

Tab. 9: Statistical analysis was performed by means of the Significance Analysis of Microarrays (SAM). The False Discovery Rate (FDR) was estimated at 10%. FC= Fold change.

## 2.3 DISCUSSION

### 2.3.1 To investigate Pin1 activity in modulating huntingtin half-life and aggregate formation

Proteolytic cleavage of huntingtin generates toxic N-terminal fragments that are prone to misfold and aggregate. Although the toxicity of aggregates is controversial, increasing evidences suggest that intermediate oligomeric species, occurring during the aggregation process, are pathogenic (Sanchez, 2004; Schaffar, 2004). The identification of proteins or chemical compounds that control aggregation process, for instance by increasing degradation of toxic fragments, is of large interest in the field of neurodegenerative research.

In this work we have investigated Pin1 as a possible modulator of aggregate formation and protein stability in HD.

Using a cell culture model we found that overexpression of Pin1 remarkably decreases inclusions and SDS insoluble material formed by N-terminal huntingtin fragments with expanded polyglutamine tract. Consistent with a process that links accumulation of misfolded proteins with their rate of degradation, we found that reduced inclusion formation was associated to decreased half-life of wild-type and mutant huntingtin fragments.

There are increasing evidences that Pin1-catalysed prolyl isomerization modulates the half-life of many of its targets by changing protein interaction, subcellular localization and ubiquitination.

For example, Pin1 increases the protein half-life of p53 by inhibiting its binding to the Mdm2 ubiquitin ligase, which regulates the degradation of p53 (Zacchi, 2002). With a different mechanism the interaction of Pin1 to c-Myc facilitates its dephosphorylation at Ser62 by PP2A, triggering a series of events that promote c-Myc turnover by the ubiquitin-proteasome pathway (Yeh, 2004).

We have previously demonstrated that Pin1 interacts with full-length huntingtin (unpublished data, Ph.D. thesis by S. Michelazzi). The N-terminal huntingtin fragment we have used contains a single Ser-Pro motif ($Ser_{120}P$) that if phosphorylated may be target of Pin1. We have therefore hypothesized that Pin1 interaction at $Ser_{120}$-Pro could modulate huntingtin stability.

The mutant protein we have generated by replacing Serine 120 with Alanine ($Ala_{120}$-Pro) exhibited a very high stability compared to wild type ($Ser_{120}$-Pro). However, when tested in co-transfection, we found that its intracellular concentration was also decreased by Pin1 overexpression. Similar results were obtained with a shorter huntingtin fragment (exon 1) that lacks of Pin1 sites. These data, combined to the failure of wild type $Ser_{120}$-Pro to interact with Pin1 in GST pull down assay, suggest that Pin1-mediated activity on huntingtin half-life is not dependent to direct interaction, but it is exerted through the action of a cellular constituent that target huntingtin for degradation.

Misfolded huntingtin fragments have been found to interact with heat-shock proteins (HSPs) such as Hsp40 and Hsp70 (Krobitsch, 2000; Wyttenbach, 2000). The interaction of HSPs with N-terminal huntingtin is likely to represent an attempt of the cell to refold the misfolded protein. In line with this idea several studies have shown that overexpression of HSPs suppresses aggregation of truncated polyQ-containing proteins (Wyttenbach, 2002; Hay, 2004; Kitamura, 2006). The yeast Hsp104 overexpression, for example, was found to rescue aggregate formation in yeast (Krobitsch, 2000), in *Caenorhabditis elegans* (Satyal, 2000), in transgenic mouse and mammalian cell model of HD (Carmichael 2000; Vacher 2005). Of interest, Hsp104 was found to interact with Ess1, the yeast orthologue of Pin1 (Ho, 2002).

When chaperones cannot refold abnormal proteins correctly, then they promote their substrate ubiquitination, which direct them to proteasome for degradation. It is tempting to speculate that Pin1 interaction with yet unknown chaperone may modulate its activity promoting degradation of huntingtin fragments.

The UPP (ubiquitin proteasome pathway) is a major intracellular pathway for degradation of proteins. The substrate is first marked for degradation by covalent linkage to ubiquitin *via* a mechanism that occurs through the action of three classes of enzymes termed E1, E2, and E3. Ubiquitinated proteins, after binding to the 19S regulatory particle of proteasome, are deubiquitinated, unfolded and then translocated into the 20S component for proteolysis. Degradation is mediated by specific proteases that digest proteins to short peptides, which are released by proteasome and then rapidly hydrolyzed to aminoacids by cytosolic or nuclear peptidases.

The ubiquitin ligation step mediated by E3-ubiquitin ligase enzymes is centrally important in determining the selectivity of protein degradation.

So far, four different E2 and E3-ubiquitin ligases have been found to associate with huntingtin fragments and to be responsible for their ubiquitination and degradation by proteasome (Kalchman, 1996; Jana, 2005; Yang, 2006; Mishra, 2008a). Among them, CHIP has been shown to promote also degradation of other disease-proteins as mutant SOD1, Tau, α-synuclein and different polyQ-containing peptides (Cardozo, 2003; Petrucelli, 2004; Shin, 2005; Jana, 2005). Transient overexpression of CHIP increases ubiquitination and rate of degradation of target proteins. E6-AP was recently described to interacts with the soluble misfolded polyQ proteins and associate with their aggregates (Mishra, 2008a). E6-AP, also known as ube3a, promotes degradation of other substrates including p53 (Mishra, 2008b). HIP2 is a member of the E2 family, is highly expressed in brain and it was the first ubiquitin enzyme found to interact with huntingtin (Kalchman, 1996).

We found that overexpression of Pin1 increases the rate of degradation of N-terminus huntingtin fragment. Although we do not know whether ubiquitination of huntingtin is increased in our system, we might hypothesize that Pin1 enhances the rate of degradation by increasing the activity of ubiquitin ligases. Interestingly, by inspection of primary sequence of CHIP, E6-AP and HIP2, several putative Pin1 binding sites are found. Further experiments are obviously required to prove this hypothesis.

Neuronal accumulation of aggregated protein is a feature of many neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD), familial amyotrophic lateral sclerosis (FALS), Huntington's disease (HD) and related polyglutamine (polyQ) expansion diseases.

Pin1 dysfunction has been already linked to neurodegeneration. In AD brains, Pin1 regulate dephosphorylation and function of Tau and APP. Its depletion causes increased formation of neurofibrillary tangles and selectively elevates the production of insoluble Aβ42 (Pastorino, 2006). In PD, Pin1 accumulates in the Lewy bodies and its overexpression enhances the protein half-life and insolubility of α-synuclein (Ryo, 2006). In ALS, Pin1 colocalizes with spinal cord neuronal inclusions and its inhibition reduces glutamate-induced perikaryal accumulation of phosphorylated neurofilament-H in neurons (Kesavapany, 2007).

Our *in vitro* data implicate Pin1 in the clearance of huntingtin fragments. It will be of interest to assess *in vivo* whether Pin1-mediated activity is as well involved in this mechanism. In $Hdh^{Q111}$ knock-in mice (C57BL/6N background), intranuclear inclusions are detected in the

striatum of heterozygous at 6 months of age (Lloret, 2006). We are planning to assess whether timing formation of SDS-insoluble material and/or intranuclear inclusions in $Hdh^{Q7/Q111}$: $Pin1^{-/-}$ mice we have generated is anticipated compared to age-matched $Hdh^{Q7/Q111}$ mice, which express Pin1.

The mutant N-terminal huntingtin fragment we have generated by replacing Serine 120 with Alanine ($Ala_{120}$-Pro) exhibits very high stability and deserves therefore additional comments. In a recent work, Chandra and colleagues have described the pentapeptide motif (FQKLL) at positions 123-127 of huntingtin as a novel proteasomal degradation signal. The motif, if introduced as a 15mer peptide (RNSPE**$F_{123}$QKL$L_{127}$**GIAME) to a heterologous protein (YFP), targets the fusion protein for proteasome degradation. Disruption of the motif by alanine substitution at the hydrophobic residues increases the steady state level of the protein. The role of this motif is not yet known (Chandra, 2008). The 15mer peptide used by Chandra is only three residues apart from the S120P site that we have mutated to generate the fragment ($htt_{1-171}$Q60$S_{120}$AGFP) with increased half-life. We may therefore hypothesize that Serine 120 is also important in regulating the activity of the proteasomal degradation signal but its role require further study.

## 2.3.2 To investigate Pin1 activity in regulating gene expression profiling in *in vitro* and *in vivo* model systems of HD

We have previously shown that huntingtin is interacting with Pin1 and that this interaction is increased by polyQ expansion. Importantly, endogenous Htt/Pin1 protein complex occurs in vivo in a genetically precise mouse model of the disease.

We have also shown that Pin1 may have an effect on degradation of N-terminus huntingtin fragment and, consequently, aggregate formation, without requiring the establishment of a common protein complex. This is important since it suggests that Htt/Pin1 interplay may occur at multiple levels intersecting multiple intracellular pathways.

The intrinsic nature of the biochemical activity of Pin1 on substrate proteins makes this molecule an ideal drug target. Therefore, understanding the functional significance of Htt/Pin1 interplay is of crucial importance.

To this purpose, we have undertaken a genomic approach.

The analysis of gene expression profiles in neurodegenerative diseases has been a powerful tool to characterize metabolic and tissue dysfunctions in cellular and animal models of the diseases as well as in human post mortem brains.

While the studies in humans suffer from the analysis of the final stage of a disease that has lasted many years, arrays data in cell culture and mouse models have provided unexpected insights on the neurodegenerative process. Among others, the analysis of gene expression in a mouse model of HD has identified for the first time an altered cholesterol metabolism that has then been confirmed in human subjects (Sipione, 2002). Furthermore, transcriptome data from the striata of a genetically precise mouse model of the disease has pointed to an altered expression of Rrs1 as the earliest event so far identified in a still asymptomatic animal (Fossale, 2002).

Diverse changes in gene expression may then be correlated in space and time to neuropathological landmarks of disease progression like striatal-specific nuclear accumulation of mutant huntingtin, aggregate formation and, ultimately, to behavioral deficits.

Here we have taken advantage of a widely accepted *in vitro* cellular model system as well as of a genetically precise HD mouse. To eliminate Pin1 expression in these experimental settings we have transiently interfered Pin1 mRNA *in vitro* and bred HD mice with a Pin1 knockout mouse line *in vivo*.

To study gene expression changes we have used the affymetrix platform and analyzed data with the R Bioconductor package.

**Data analysis of *in vitro* Pin1 silencing**

First we discuss the *in vitro* data.

Striatal cells represent a suitable cellular model to explore biochemical pathways involving mutant huntingtin and to test hypothesis on the HD-trigger mechanism.

We performed Pin1 silencing in striatal cells and compared the gene expression profiles of mutant striatal cells to wild-type in order to identify differences that could account for Pin1 knock-down in a mutant huntingtin-dependent way.

As expected, the transient nature of the silencing delivery resulted in a considerable variability in the expression profiles of the replicates, reducing the number of genes differently expressed in a significant manner to a very few probes.

Indeed, we have found 12 genes which expressions were consistently altered in the experiments. We have validated four of them by real time PCR. As shown in Fig. 14a, differential expression was confirmed for all genes. Interestingly, the qPCR revealed that the down-regulation of Slc25a23 mRNA mediated by Pin1 silencing occurs in both cell lines. This effect was not detected in the microarray experiments since Slc25a23 gene was highly down-regulated in mutant striatal cells in basal conditions. Being the level of Slc25a23 mRNA extremely low in mutant cells, a further down-regulation of about ~1.2 fold might be under the threshold of detection.

We were thus confident that our gene expression analysis of transient interference was successful.

Here we discuss some examples of interesting patterns of expression that involve intriguing genes.

1. *DNA-damage inducible transcript 4-like* (Ddit4l) was strongly up-regulated in mutant cells versus wild-type in basal conditions. Pin1 silencing increased its expression in mutant cells only.

This pattern of expression is interesting since it depends on the expanded polyQ tract both in untransfected striatal cells as well as when Pin1 is silenced. Furthermore, the biology of Ddit4l is intriguing. This gene encodes for a protein shown to be a potent negative regulator of mTOR (Corradetti, 2005). It acts downstream to AKT and upstream of TSC2 in coupling with another related protein named Redd1. It has been shown that a variety of stressors causes a rapid and stable increase in Ddit4l (Redd2) to reduce translation through mTOR inhibition (Corradetti, 2004; Proud, 2004). Depending on the cellular context, Ddit4l expression has been considered pro- or anti-apoptosis. Importantly, its biological function may be related to the induction of autophagy, a cellular phenomenon central in HD pathogenesis. If so, Pin1 action may be interpreted as inhibiting autophagic activity. Further studies are needed to unveil Ddit4l potential role in HD and the regulatory function by Pin1.

2. Genes that are not differentially expressed in untreated cells but strongly up-regulated by Pin1 siRNA in mutant cells only.

This class of genes is the only one that seems to contain enriched Gene Ontology terms: *transformed mouse 3T3 cell double minute 2* (MDM2) and *HECT, UBA and WWE containing 1* (HUWE1) are in fact E3 Ubiquitin Ligases.

MDM2 is an E3 ubiquitin ligase that, together with the p300 ‘‘transcriptional co-activator’’ protein (in its capacity as an E4 ligase), mediates the ubiquitination and proteasome-dependent degradation of the p53 tumor suppressor protein and other growth regulatory proteins (reviewed in Meek and Knippschild, 2003). The transcription of the MDM2 oncogene is induced by p53 after DNA damage, and the MDM2 protein then binds to p53 promoting its degradation. These two proteins thus form an autoregulatory feedback loop in which p53 positively regulates MDM2 levels and MDM2 negatively regulates p53 levels and activity (Freedman, 1999).

Pin1 is one of the most important regulators of p53 activity. It specifically binds phosphorylated p53 inducing p53 dissociation from MDM2 and its stabilization, which results in increased transactivation (Zacchi, 2002).

Interestingly, it has been previously shown that mutant striatal cells present a higher level of stabilized p53 (Trettel, 2000). Therefore, the induction of MDM2 mRNA consequent to Pin1 silencing may suggest a potential down-regulation of p53 in mutant cells, which does not occur in wild-type cells with basal levels of p53.

It is extremely provocative that a second E3 ubiquitin ligase induced by siPin1 is HECT, UBA and WWE containing 1 (HUWE1), a central player in p53-dependent effects on cell cycle. In p53 wild-type cells, HUWE1 directly binds and ubiquitinates p53 and inactivation of endogenous HUWE1 is crucial for ARF-mediated p53 stabilization (Chen, 2005).

It seems therefore reasonable that the main effect of Pin1 lack of activity in mutant cells may be related to p53 stabilization.

It will be then important monitoring p53 levels after Pin1 siRNA transfections.

Furthermore, it will be interesting to investigate whether MDM2 and HUWE1 might bind mutant huntingtin and promote its degradation.

### Gene expression profile of *Hdh$^{Q7/Q111}$: Pin1$^{-/-}$* mouse striata

The generation of a new transgenic mouse model harboring the HD mutation in absence of a functional Pin1 gene represented the first step in the dissection of a possible role for Pin1 in HD pathogenesis.

Hdh knock-in mice, designed to recapitulate more precisely the genetic lesion in human HD, were bred with Pin1$^{-/-}$ mice in order to obtain the new strain, double knockin-knockout, *Hdh$^{Q7/Q111}$: Pin1$^{-/-}$*.

Three list of genes resulted by our experiments:

1. Genes induced in the *Hdh$^{Q7/Q111}$* mutant mice include immediate early genes (Egr2 and Fos), while genes down-regulated surprisingly include a pro-apoptotic factor (Bax) and a GABA receptor subunit (Gabra3).

Egr2 has been most widely studied in the context of the nervous system, and its targeting in knock-out mice results in early lethality concurrent to defects in hindbrain patterning, peripheral nerve myelination, and bone formation (Gillian, 2004).

It is noteworthy that the altered expression of Egr2 gene has been widely related to HD pathology even if with opposing results (Chan, 2005; Kuhn, 2007). Egr2 was indeed found down-regulated in transgenic mice expressing short N-terminal mutant huntingtin fragment (R6/2 and N171Q82 mice) (Luthi-Carter, 2000; Chan, 2002; Kuhn, 2007), whereas it is overexpressed in mouse model expressing longer mutant huntingtin fragment (HD46, they express an N-terminal fragment of huntingtin encompassing the first 550 aa) or the full-length protein (YAC72) (Chan, 2002, Kuhn, 2007).

c-Fos is a cellular proto-oncogene that dimerizes with a member of the Jun family to make an AP-1 transcription factor that binds to the AP-1-binding sites of DNA (Karin 1995). Pin1 has been shown playing a key role in regulating c-Fos activity (Monje, 2005). Interestingly, c-Fos has been related to HD as a down-regulated gene in some transgenic mouse models (R6/2 and N171-82Q) (Chan, 2002; Kuhn, 2007).

2. Genes induced in Pin1$^{-/-}$ mice on a wild-type huntingtin background suggest a clear alteration of pathways involved in neuronal networks differentiation and maintenance: among them Homer1, Map2, BDNF and Neurod6.

Homer1 is a constituent of the post-synaptic density structure and is involved in receptor cluster formation (Ango, 2002; Brandstatter, 2004). Its transgenic expression in the striatum has provoked an altered motor function as well a modified response to amphetamine (Tappe and Kuner, 2006). Homer1 up-regulation is concurrent to the induction of Map2, a well known

marker and organizer of dendritic structures. Furthermore, BDNF induction is also observed. This result is of great importance since down-regulation of BDNF has been linked to neurodegeneration in HD (Zuccato, 2003). Among the transcription factors, it is of special relevance the induction of Neurod6, a well know regulator of neuronal development and axonal pathfinding (Uittenbogaard and Chiaramello, 2004).

The E3 ubiquitin ligase ube3a (E6-AP) is responsible for the Angelman syndrome, a genetic disorder with motor impairment and seizure. Importantly, ube3a was recently shown to ubiquitinate and induce degradation of huntingtin (Mishra, 2008a).

3. The list of genes differentially regulated in Pin1$^{-/-}$ mice in a mutant huntingtin genetic background is particularly interesting.

First, no common altered genes between *Hdh*$^{Q7/Q111}$ and *Hdh*$^{Q7/Q111}$*: Pin1*$^{-/-}$ mice are evident at this stage.

This is important since it may suggest that the functional interplay between lack of Pin1 expression and mutant huntingtin may lead to the establishment of an unexpected phenotype.

The most differentially expressed gene was arginine vasopressin (AVP), a neuropeptide that acts as an endocrine and paracrine regulator of important systemic functions, namely, vasoconstriction, gluconeogenesis, corticosteroidogenesis, and excretion of water and urea. Although AVP fibers can be found throughout the CNS, expression of the AVP genes occurs almost exclusively in the hypothalamus (Fields, 2003).

Most importantly, this gene has been found down-regulated in a mouse model of HD (Chan, 2005). In this case, lack of Pin1 expression may reestablish physiological AVP level.

We also noticed the altered expression of the immunoglobulin binding protein 1 (Igbp1) and of the DEAD box polypeptide 4 (ddx4).

Immunoglobulin binding protein 1 (Igbp1) is a regulatory subunit of protein phosphatase 2A (PP2A) sustaining mTOR signaling and acting as a positive feedback mechanism on translation initiation (Kong, 2004).

The Vasa DEAD-box helicases are widespread markers of germ cells across species and are involved in translational control.

Some observations may be discussed on the biological meaning of these gene lists.

We have monitored gene expression in the striatum of mice at 15 weeks of age with the intend to detect early events in HD pathogenesis and to avoid, at the same time, confounding effect

due to neurodegeneration associated with the loss of Pin1. In Pin1$^{-/-}$ mice, indeed, the first evidence of neuronal loss is detected later at six months of age and with features resembling AD (Liou, 2002; Liou, 2003).

Clearly, gene expression analysis must be integrated with the classical phenotype description in HD and AD mouse models of the disease. This analysis has just started since it will require some time: mice will be analyzed by immunofluorescence to monitor some of the hystopathological and biochemical landmark of the neurodegenerative process including mutant huntingtin shuttling into the nucleus, intranuclear inclusion formation, BDNF transcription as well as behavioral tests. Attention will be also devoted to markers of neuronal dysfunction that have been previously described in Pin $^{-/-}$ mice during aging mimicking an AD-like phenotype. This analysis will be complemented with a series of new gene expression arrays at different times up to 2 years of age.

This work is instrumental in answering the fundamental question concerning a potential protective or toxic effect of the lack of Pin1 expression in HD.

No apparent behavioral abnormalities have been so far observed in the double mutant mouse model suggesting that lack of Pin1 does not strongly modify the polyQ-dependent neurodegenerative process.

It is however clear that double mutant mice do not resemble the expression of the HD model or of the Pin1 KO at the same age. This is important since it may suggest that a pharmacological intervention on Pin1 activity may modify disease progression.

Unfortunately there are some considerations we need to make for assessing the value of these data.

First, we decided to consider each hybridization a biological replica consisting of a single animal. Therefore, we avoided multi-animal RNA pooling. This approach is very powerful, although it needs a higher number of replicas to consider an appropriate number of genes as statistically significant. The physiological range of intra-individual variation in the striatum of a 3 months old animal is difficult to judge and has potentially large effects on decreasing the number of genes that can make the threshold.

We are now hybridizing a higher number of mice to increase the statistical significance of some differentially expressed genes.

We also believe that having a homozygous mouse for mutant huntingtin will accelerate neuropathogenesis and will render clearer some differential patterns of expression. We are now collecting three individual striata for each genetic repertory with homozygous mutant huntingtin.

We are thus addressing this important question by increasing the number of mice, by analyzing mutant huntingtin in homozygosity and by following the double mutant mice throughout their lifespan. These in vivo experiments will provide a definitive answer on mutant Htt/Pin1 functional interplay.

In this context, as preliminary information, we looked at those genes that did not pass the significance test, but showed an opposite sign of differential expression with respect to the two background situations. It may be of high biological relevance that some genes are common members of the differentially expressed gene lists but in a discordant direction (Tab. 10). It will be thus worth investigating whether this pattern of expression means that huntingtin and Pin1 act on the same pathway and/or whether lack of Pin1 may induce a partial reversal of the mutant htt-induced phenotype.

| **Gene** | *$Hdh^{Q7/Q111}$: $Pin1^{+/+}$* *Vs* *$Hdh^{Q7/Q7}$: $Pin1^{+/+}$* | *$Hdh^{Q7/Q111}$: $Pin1^{-/-}$* *Vs* *$Hdh^{Q7/Q111}$: $Pin1^{+/+}$* | *$Hdh^{Q7/Q7}$: $Pin1^{-/-}$* *Vs* *$Hdh^{Q7/Q7}$: $Pin1^{+/+}$* |
|---|---|---|---|
| Egr2 | 2.40* | 0.63 | 2.50* |
| c-Fos | 1.88* | 0.61 | 2.22* |
| Avp | 0.19 | 2.17* | 0.73 |
| Cbln1 | 0.76 | 2.56 | 2.29* |

Tab. 10: Fold change of Egr2, c-Fos, Avp and Cbln1 are indicated for the different genotypes. *statistically significant fold change values.

## 2.4 MATERIALS AND METHODS

### Plasmids and mutagenesis

Httex1Q60GFP in pcDNA3.0 (Invitrogen), encoding the first exon (1-85 aa) of human huntingtin with 60 glutamines in frame with the N-terminus of GFP, was constructed by cloning PCR amplified Htt exon1 into EcoRI-XhoI sites of pcDNA3.0GFP vector. The GFP moiety was recovered by XhoI digestion from pGreenLantern-1 (GIBCO-BRL). Htt1-171Q21/Q60GFP, encoding the N-terminal 171 amino acids of human huntingtin, with 21 and 60 glutamines, was constructed by subcloning the NcoI-XhoI fragment of Htt cDNA into pcDNA3.0GFP vector. The point mutant htt1-171Q60S120AGFP was obtained by site directed mutagenesis using the two primer sets: 5'-ACCGGAATTCACCATGGCGACCCT GGAAAAGCTGA-3', 5'-CTGAAATTCTGG**GGC**ATTTCTGACAGA-3', and 5'-TCTGT CAGAAAT**GCC**CCAGAATTTCAG-3', 5'-GTCATTTGCAAAATTGCCAAAAGAAGC CA-3' and subcloned into the same pcDNA3.0GFP vector.

HA-Pin1, encoding the human HA tagged Pin1 in pcDNA3.0 vector, was kindly provided by Prof. G. Del Sal (LNCIB);

HA-Pin1 DM, encoding the human HA tagged Pin1 containing the point mutations Y23A S67E, was constructed by site directed mutagenesis using as template HA-Pin1Y23A, kindly provided by Prof. Del Sal G. (LNCIB) and the two primer sets: 5'-ACGAGGATCC GCGGACGAGGAG-3', 5'-TGGGCCGCCGTTCCTGGCTGTG-3', and 5'-CACAGCCAG GAACGGCGGCCCT-3', 5'-ATCCACTCGAGTCACTCAGTGCGGAGG-3', subsequently cloned into pcDNA3.0-HA.

pEGFP-C2 (Clontech Lab., Palo Alto, CA, USA). pEYFP$^{u}$ was kindly provided by Prof. Poletti (University of Milan, Italy); pEYFP was derived by pEYFP$^{u}$ after elimination of the CL1 degron by XhoI-BamHI digestion.

## Cell cultures

**ST*Hdh*** striatal progenitor cell lines derived from wild-type (ST$Hdh^{Q7/7}$), homozygous (ST$Hdh^{Q111/111}$) and heterozygous (ST$Hdh^{Q7/111}$) knock-in *Hdh* littermate mice embryos (E14 striatal primordial), were cultured at 33ºC in D-MEM (Dulbecco's modified Eagle's medium), 10% FBS (fetal bovine serum), 100 U/ml penicillin, 100 µg/ml streptomycin, 400 µg/ml G418 for selection. The cells are immortalized with a Temperature Sensitive version of the SV40 Large T-Antigen, which allows the cells to proliferate at the permissive temperature of 33ºC (Trettel, 2000).

**HEK293T** (human embryonic kidney) cells were cultured at 37ºC in D-MEM (Dulbecco's modified Eagle's medium), 10% FBS (fetal bovine serum), 100 U/ml penicillin, 100 µg/ml streptomycin.

## Transfection

### Lipofectamine

Hek293T cells were transfected accordingly to the Lipofectamine 2000 (Invitrogen) protocol. Briefly, cells were plated in growth medium without antibiotics in 35mm cell culture dishes the day before transfection. 90% confluent cells were transfected using a total of 4 µg DNA and 10 µl Lipofectamine and medium was changed 7 hours after transfection. Cells were harvested and assayed 48 hours after transfection.

## Treatments

**Cycloheximide (CHX)**: transfected Hek293T cells were treated with 40 µM CHX (Sigma) for 2 to 4 hours.

**MG-132** : transfected Hek293T cells were treated with 10µM MG-132 (MG-132) (Sigma) for 6hours.

## Protein extraction, western blot and filter trap assay

### Western Blot

Cells were washed in PBS, harvested in cold PBS and resuspended in different lyses buffers.
For whole-cell lysates, the entire cell pellet was resuspended in 10% SDS, sonicated for 1 minute and heated for 10 minutes at 95°C. Protein concentration was determined by Bicinconic Acid (BCA) (Sigma) assay using a calibration curve built with standard amounts of BSA (bovine serum albumin).
To test Pin1 silencing, transfected cells were resuspended in Tris-Triton buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2 mM EDTA, 1% Triton X-100, 10% Glycerol, protease inhibitors), incubated 20 minutes in cold room rocking and centrifuged at 13,000 rpm for 15 minutes at 4°C. Protein concentration was determined by Bradford assay (Sigma).
3-10 μg of protein lysates were mixed with 4X SDS sample buffer, boiled for 5 minutes and separated by SDS-PAGE electrophoresis. Proteins were then blotted at 100 V for 1 hour onto nitrocellulose transfer membrane (Schleicher & Schuell 0.2 μm).
Membranes were blocked in 5% dried milk – Tris Buffer Saline (TBS)-0.1% Tween-20 (TBS-T) for 1 hour at RT and then were incubated with the primary antibody, diluted in TBST-milk, with agitation. Blots were then washed three times with TBST and then incubated for 1 hour with secondary HRP conjugated antibody, diluted in TBST-milk, at RT.
Protein bands were detected by HRP/hydrogen peroxide catalyzed oxidation of luminol by an enhanced chemiluminescence system (ECL western blotting detection reagents, Amersham Biosciences).

### Filter Trap Assay

For the filter trap assay soluble/insoluble fractionation, cell pellets were resuspended in buffer 1 (0.15 M sucrose, 15 mM HEPES (pH 7.9), 60 mM KCl, 15 mM NaCl, 5 mM EDTA, 1 mM EGTA) and cleared by centrifugation at 2,000 g for 10 minutes at 4°C. The obtained pellets, containing nuclei, mitochondria, ER and membranes, were resuspended in buffer 2 (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KC1, 5 mM EDTA, 1 mM EGTA and 12.5% Glycerol) and centrifuged at 4,000 g for 15 minutes at 4°C. The remaining pellets were

resuspended in buffer 3 (0.05 M HEPES (pH 7.9), 0.75 mM MgCl2, 0.5 mM EDTA, 0.42 M KC1, 0.1 mM EGTA and 12.5% Glycerol), incubated for 20 minutes in cold room rocking and then spun down at 13,000 rpm for 15 minutes at 4°C.. The final pellet was sonicated and heated at 95°C in a 10% SDS solution to reach maximal resuspension. The obtained insoluble fraction was diluted to 0.2% SDS and 50 mM DTT before filtration. Approximately 10-30 µg of proteins were subjected to filtration through 0.22-µm cellulose acetate membrane (Schleicher & Schuell), the filters were fixed in 0.5% glutaraldehyde solution for 15 min and then treated as western blot.

## Immunofluorescence

Cells were seeded onto 13 mm coverslips and allowed to attach for 24 hours before transfection. 48h after transfection cells were washed in PBS and fixed in 4% paraformaldehyde for 15 minutes at RT. After fixation, cells were rinsed in PBS and incubated 5 minutes with glycine 100 mM to quench autofluorescence. Membrane permeabilization was performed using 0.1% Triton X-100. Cells were then incubated in BSA 1% for 30 minutes to block non specific sites before primary antibody incubation.

Both primary and secondary antibodies were incubated in 1% BSA, 1% NGS.

After primary antibody incubation, cells were washed twice in PBS and subjected to secondary antibody incubation. Nuclei were labeled using DAPI. Cells were washed twice in PBS and mounted on slides using Vectashield mounting medium.

Images were captured with a Leyca confocal microscope by using a 40X oil objective.

## Antibodies

The following primary antibodies were used: Mouse monoclonal anti-huntingtin (MAB5490, Chemicon, WB dilution 1:2.500); Mouse monoclonal anti-Pin1 (Santa Cruz Biotechnology, sc-46660, WB dilution 1:1.000); Chicken monoclonal anti-GFP (Aves Labs, Tigard, OR, GFP-1020, WB dilution 1:5.000); Mouse monoclonal anti-β-actin (Sigma, A1978, Saint Louis, MI, WB dilution 1:5.000); Mouse monoclonal anti-HA tag (kindly provided by Dr. L. Collavin, LNCIB, WB dilution 1:1000, IF dilution 1:100).

The following HRP secondary antibodies were used: HRP conjugated goat anti-mouse antibody (DAKO, WB dilution 1:1.000); HRP conjugated goat anti-chicken antibody (DAKO, WB dilution 1:2.000).
The following secondary antibodies were used for IF: Alexa-594 donkey anti-mouse (1:1000).

## Pin1 silencing by RNAi in striatal cells

Striatal cells were transfected according to Dharmafect 1 protocol (Invitrogen) with 75 nM Pin1siGENOME SMART pool (M-040655-00-0020, Mouse PIN1, NM_023371, Dharmacon), or with non-targeting siRNA with impaired ability for RISC (RNA-induced silencing complex) interaction as negative control, siCONTROL RISC-Free siRNA (D-001220-01-20 Dharmacon). After 72 hours cells were collected by trypsine treatment. 1/10 of total cell suspension volume was used for WB analysis, the rest was centrifuged at 900 rpm for 5 minutes, washed in PBS and centrifuged again. The cell pellet was resuspended in 1 ml of TRIZOL and stored at -80°C for subsequent RNA extraction.

## $Hdh^{Q111/Q111}$ – $Pin1^{-/-}$ mice

To place the $Hdh^{Q111}$ allele on the Pin1-deficient background $Hdh^{Q111/+}$:$Pin1^{+/-}$ mice, generated by crossing $Hdh^{Q111/+}$ and $Pin1^{+/-}$ mice, both on C57Black/6N background, were intercrossed to give $Hdh^{Q111/+}$:$Pin1^{+/+}$, $Hdh^{Q111/+}$:$Pin1^{-/-}$, $Hdh^{+/+}$:$Pin1^{+/+}$, $Hdh^{+/+}$:$Pin1^{-/-}$, $Hdh^{Q111/Q111}$:$Pin1^{-/-}$, $Hdh^{Q111/Q111}$:$Pin1^{+/+}$ littermates.

### Genotyping

DNA was prepared from tail biopsy. CAG repeats were sized by PCR in GB buffer (66 mM Tris-HCl (pH 8.8), 16 mM (NH4)2SO4, 2 mM MgCl2, 0.7% b-Mercaptoethanol), 0.01 mg/ml BSA, 10% DMSO, 200µM dNTPs, 0.5µM primers with 0.4 U/µl RedTaq. Cycling condition were 90”@ 94°C, 35 X (30”@94°C, 30”@ 56/65°C, 90”@ 72°C), 10'@ 72°C. PCR products were resolved in 1% agarose gel. Primer sets were as follow: wild type 5'-CCTGGAAAAGCTGATGAAGG -3', 5'- TGGACAGGGAACAGTGTTGC-3'; knock-in 5'-ATGAAGGCCTTCGAGTCCCTCAAGTCCTTC-3', 5'-GGCGGCTGAGGAAGCTGA GGA-3'.

Pin1 genotype was determined by PCR using RedTaq (Sigma). Cycling conditions were 3'@ 94°C, 35 X (30"@94°C, 30"@ 60/64°C, 90"@ 72°C), 10'@ 72°C. PCR products were resolved in 1% agarose gel. Primer sets were as follow: wild type 5'-AGCACCCGATCCT GTTCTGCAA-3', 5'-AAGGGATTAGAAGCAAGATTCG-3'; knockout 5'-AGCACCCGA TCCTGTTCTGCAA-3', 5'-CAGAGGCCACTTGTGTA-3'.Genotyping of all animals was confirmed after dissection.

### Mouse brain dissections

*Hdh*$^{Q7/7}$-Pin1 wt, *Hdh*$^{Q7/7}$-Pin1 KO, *Hdh*$^{Q7/111}$-Pin1 wt and *Hdh*$^{Q7/111}$-Pin1 KO mice were used for microarray analysis.

Mice at 15 weeks of age were euthanized by $CO_2$ exposure following the CCM regulations and the brains were dissected into striatum, cortex and cerebellum, immediately frozen in liquid nitrogen, and stored at -80°C.

## RNA preparation, analysis and Microarray

### RNA extraction

Cells were washed in RT 1X PBS, then harvested with trypsine and homogenized in cold TRIZOL; mouse striata were homogenized in cold TRIZOL.

Total RNA was isolated with TRIZOL reagent (Invitrogen/Life Technologies, Carlsbad, CA) according to manufacturer's instructions, quantified spectrophotometrically at 260 nm using NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA), and analyzed using the RNA nanochip method on a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).Total RNA was pretreated with DNase I using a DNA-free kit (Ambion Inc., Austin, TX, USA) and additionally purified over RNeasy Columns (Qiagen, Valencia, CA, USA).

### RT-qPCR

Six different silencing experiments were settled, the best three were chosen for successive microarray analysis.

RT reactions were performed in 20 µl with 1 µg of DNase-treated total RNA, 1X reaction mix, and 1 µl of Reverse Transcriptase (Bio-rad, iSript$^{TM}$ cDNA synthesis kit). Reaction conditions

were 5 min at 25°C, 50 min at 42°C and 5 min at 85°C. Quantitative PCR reactions were performed with a BioRad iCycler iQ instrument (Bio-Rad Laboratories, Foster City, CA, USA), using the iQ Custom SYBR- Green Supermix (Bio-Rad, Foster City, CA, USA). PCR reactions were performed in 25 µl reaction volumes containing 1 µl of cDNA, 250 nM of each primer and 1X reaction mix. Each cDNA was run in duplicate for the target and the normalizing gene (β-actin and/or 18S) in the same 96-well plate for all samples. Cycle parameters were 3 min at 95°C (10 s at 95°C, 20 s at 56°C and 30 s at 72°C) for 40 cycles. To quantify the mRNA levels for each sample and primer set, a standard curve was generated with known dilutions of total cDNA. The relative value for each unknown sample was then calculated from its respective standard curve using linear regression analysis. To normalize the differences in the amount of total RNA added to each reaction, we used β-actin as endogenous control. The normalized expression value was then calculated by dividing the relative quantitation value of each sample and primer set by the relative quantitation value of β-actin. Specificity of amplicon was determined by melt curve analysis and gel electrophoresis.

Primers were designed using Beacon Design 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). The following primer pairs were used for pin1 analysis: murine Pin1 (GenBank accession n° NM_023371), 5'-CCAGAAGATTAAGTCAGGAGAGGAAG-3', 5'-CCGTAGAGCAAACGACGCATCCTC-3'; murine β-actin (GenBank accession n° NM_007393), 5'-TGAAATAAGTGGTTACAGGAAGTC-3', 5'-GCAGTACATAATTTACACAGAAGC-3' (TIB MOLBIOL, Genova, Italy).

Primer sequences for significative genes obtained from silenced cells were as follow: MDM2 (GenBank accession n° NM_010786), 5'-GCAAGCACCTCACAGATTCCAG-3', 5'-GCTGCTGCTTCTCGTCATATAACC-3'; HUWE1 (GenBank accession n° NM_021523), 5'-AGTGGTGCTGGCAGTCCTTAAC-3', 5'-GTCAGTAGCGGAGTCCTCTTGTC-3'; Ddit4l (GenBank accession n° NM_030143), 5'-AGAGTTGTTGGACGGTGGCTATC-3', 5'-GGGACCAAGACCTTAGAGCAACC-3'; Slc25a23 (GenBank accession n° NM_025877), 5'-TTGGCAGGAATGGCGAGACC-3', 5'-AACTCATCAGGCACCGTCA GG-3'; murine β-actin as above.

To measure mRNA level of overexpressed constructs, the following primer sets were used: human huntingtin (GenBank accession n° NM_002111), 5'-CTACCAAGAAAGACCGTGTGAATC-3', 5'-CCACCATCCTGACATCTGACTC-3'; htt1-171GFP, 5'-AGTGCTTTTCCAGATACCCAGAC-3', 5'-TCCTTAAAGTCAATGCCCTTCAAC-3'; EGFP (Clontech, GenBank

accession n° U55763), 5'-GCCCGACAACCACTACCTGAG-3', 5'- CGGCGGTCACGAA CTCCAG-3'; human β-actin (GenBank accession n° NM_001101), 5'- CGCCGCCAGCTC ACCATG-3', 5'-CACGATGGAGGGGAAGACGG-3'; 18S RNA, 5'- CGTCTGCCCTATC AACTTTCG-3', 5'-GCCTGCTGCCTTCCTTGG-3'. YFP and $YFP^u$ mRNAs were amplified using EGFP primers.

**Microarray: probe preparation and hybridization**

RNAs for microarray analysis fulfilled the following quality control criteria: R.I.N. > 8.0 and 28S/18S ratio ~2, which were calculated by Bioanalyzer. Approximately 5 µg of total RNA was processed to produce biotinylated cRNA targets. cDNA synthesis was performed with GeneChip® One-Cycle cDNA Synthesis Kit (Affymetrix, Santa Clara, CA, USA; cod. n° 900431). Double-strand cDNA containing the T7 promoter sequence was used as a template for *in vitro* transcription (IVT), amplification and biotin-labeling to prepare cRNA (Affymetrix, cod. n° 900449). Each synthesis step was followed by a purification one with the GeneChip® Cleanup Module (Affymetrix, cod. n° 900371), the same kit was also used for the fragmentation of the cRNA probes. Probes were hybridized on the GeneChip® Mouse Genome 430A 2.0 Array (Affimetrix, cod. n° 900498), a single array representing approximately 14,000 well-characterized mouse genes.

**Microarrays: data analysis**

After hybridization, Affymetrix GeneChip Mouse Genome 430A 2.0 Arrays were scanned and image data was saved as .dat files. Cell intensity data was computed from the image data using the Affymetrix GeneChip Operating Software (GCOS), and saved as .cel files. Further data processing was performed with R and BioConductor, while statistical analysis was performed with the MultiExperiment Viewer, as detailed below.

.cel files were imported in the R environment for statistical computing and graphics (http://www.r-project.org/) using the "affy" package from the BioConductor software project (http://www.bioconductor.org/). Quality assessment of the data was performed with the "affy" and "affyPLM" packages. Data was then processed using the "rma" function from the "affy" package. "rma" (Robust Multi-Array Average) yields probe expression measure by a combination of background adjustment, data normalization and summarization of probe

effects (Irizarry *et al.*, 2003). Normalized data was then filtered according to the Affymetrix detection call, so that only probes that had a "Present" call in at least one of the arrays were retained (McClintick and Edenberg, 2006).

Statistical analysis of silenced striatal cells was performed by means of LIMMA (Linear Models for Microarray Data) (Smyth, 2004) as implemented in the "limma" package in BioConductor. The statistical test was applied to the subsets of probes showing a fold change of at least 1.5. P-values were adjusted for multiple testing using Benjiamini and Hochberg's method (Benjiamini and Hochberg, 1995).

Statistical analysis of mouse striata data was instead performed with the MultiExperiment Viewer (MeV) software (Saeed *et al.*, 2003) using the SAM module (Significance Analysis of Microarrays; Tusher *et al.*, 2001, implemented as in Chu *et al.*, 2002). A False Discovery Rate (FDR) of 10% was applied to detect significant differentially expressed genes.

**Statistical analysis**

Statistical analysis was Student's t-test, choosing $P<0.05$ as significant. Weighted means were calculated for data produced in replica experiments. Calculated means and standard deviations were plotted using the graph tool of Microsoft Excel.

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# CHAPTER 3: RRS1 AND ER STRESS IN HUNTINGTON'S DISEASE

## 3.1 INTRODUCTION

The elongated polyglutamine tract of mutant huntingtin causes the protein to acquire a new property that is particularly deleterious to the medium-sized spiny neurons of the striatum. Onset of clinical symptoms in HD patients precedes death of about 16 years and typically occurs when an estimated ~30% of neurons are lost and the remaining ones are sickened (Vonsattel, 1985). Correlation studies between CAG repeat number and severity of pathology in HD brains suggest that the initial trigger may occur early in life, possibly at birth. The description of these initial molecular events is crucial to design therapeutic intervention for the cure of the disorder.

In $Hdh^{Q111}$ knock-in mice, a presymptomatic genetic model of the disease, the first phenotypic change is observed at 10 weeks of age when mutant huntingtin accumulates in the nucleus of medium-spiny neurons (Wheeler, 2000). Under the hypothesis that this early phenotype is associated with altered gene expression, Fossale and colleagues have screened for genes conserved in evolution, which are likely to encode for essential proteins (Fossale, 2002).

Total RNA extracted from striatum of homozygous HdhQ111 mice was hybridized to Research Genetics Human GENEFILTERS. The microarray analysis revealed a single human cDNA, *Rrs1* (Regulator of Ribosome Synthesis; GenBank accession n°. NM_015169) that was found upregulated in homozygous $Hdh^{Q111}$ mice. By quantitative real time PCR they also demonstrated that *Rrs1* mRNA regulation fulfils the HD criteria of dominance, striatal specificity and polyglutamine-dependence. *Rrs1* mRNA was indeed found increased both in homozygous and heterozygous $Hdh^{Q111}$ striatum at different ages. At 5 months of age, increased *Rrs1* mRNA was also found in homozygous mutant cortex and cerebellum although no significant change was detected in same brain regions from $Hdh^{Q111}$ heterozygous mice. Thus in contrast to striatum, a single copy of mutant huntingtin was not sufficient to yield increased *Rrs1* mRNA in these brain regions at a young age, indicating that the underlying process exhibits striatal specificity.

Moreover, a significant increase of *Rrs1* mRNA was found in striatum of other lines of *Hdh* CAG knock-in mice ($Hdh^{Q50}$ and $Hdh^{Q92}$), consistent with a molecular phenotype that reflects a dominant polyQ-dependent mechanism (Fossale, 2002).
Most importantly, *Rrs1* mRNA expression was also found increased of about 2.4 fold in HD *postmortem* brain compared to age matched control indicating that the expression of this gene is increased as a consequence of mutant huntingtin in human disease.

*Rrs1*, first studied in *Saccharomyces cerevisiae*, encodes for a nucleolar protein essential for ribosome biogenesis (Tsuno, 2000). The biogenesis of ribosomes requires many trans-acting factors to process and modify the primary pre-5S and polycistronic 35S transcripts into mature small subunit (18S) and large subunit (5S, 5.8S and 25/28S) rRNAs. Once assembled the two ribosomal subunits are released from the nucleus and exported to the cytoplasm.
Rrs1 was found to be a member of the yeast Ribosome and rRNA Biosynthesis (RRB) regulon (Wade, 2001) that is a transcriptionally co-regulated set of genes that are required for ribosome biosynthesis. Its depletion in *Saccharomyces cerevisiae* causes defects in the maturation of 25S rRNA and in the assembly and nuclear export of the 60S ribosomal subunit (Tsuno, 2000; Miyoshi, 2004). Recently, Rrs1 was found to be a component of the pre-ribosomal subcomplex which also includes RpL11, RpL5, Rpf2 and 5S rRNA (Miyoshi, 2002; Nariai, 2005; Zhang, 2007). Preribosomes lacking the constituents of the Rrs1 neighborhood are largely intact, but they cannot undergo further maturation, are prematurely released to the nucleoplasm and cannot be exported to the cytoplasm (Zhang, 2007).
It is known that in yeast the alteration of the secretory pathway, from the insertion of the nascent peptide into the endoplasmic reticulum (ER) to the formation of the plasma membrane, prevents the continued synthesis of the components of the ribosomes, causing the repression of both ribosomal protein genes and rRNA genes (Mizuta and Warner, 1994). Of interest, in *Saccharomyce cerevisiae* Rrs1 was reported to be one of the gene essential for the transcriptional repression of rRNA and ribosomal protein genes in response to a secretory defect (Tsuno, 2000).
The impact of polyQ expansions on ribosome synthesis and assembly is currently unknown. Furthermore, the interplay between mutant huntingtin, the integrity of the secretory pathways and ribosome biogenesis remains to be elucidated.

Here we report that mammalian Rrs1 is localized both in the ER and in the nucleolus. This subcellular distribution is shared with its newly discovered binding partner Lyric. Furthermore, Rrs1 and Lyric are components of the ER stress response in neurons. We then show that ER stress is an early presymptomatic event in the striatum of an HD mouse model.

## PREFACE

Rrs1 expression in mammalian cells has been studied in a collaborative effort between our laboratory and the laboratory directed by Marcy MacDonald (MGH, Boston).
Unless otherwise specified, all the data reported here arise from my own experiments and data analysis.

## 3.2 RESULTS

### Detection of Rrs1 in mammalian cells

Rrs1 has been studied so far only in yeast and no literature data are available on Rrs1 protein in mammalian cells. To investigate the role of Rrs1 in HD, Elisa Fossale (MGH, Boston) developed a rabbit polyclonal antiserum, named FUN1, raised against the N-terminal region (1-194 aa) of the mouse Rrs1 protein. The antibody was tested by western blot for its ability to reveal endogenous or ectopically expressed Rrs1 protein in mammalian cells. A band of ~40 kDa, which corresponds to the predicted molecular weight of mouse/human Rrs1, was specifically detected (Fig. 1a).
The intracellular localization of endogenous Rrs1 was studied in *STHdh*$^{Q7/Q7}$ cells by immunoflorescence. Confocal analysis revealed a nucleolar distribution of Rrs1 protein, which was found to co-localize with the nucleolar marker fibrillarin (Fig. 1c). Interestingly, Rrs1 staining was also observed in the cytoplasm, with a distribution pattern reminiscent of a protein associated with the membrane compartment.
Then cellular lysates from *STHdh*$^{Q7/Q7}$ striatal cells were separated into Triton X-100 soluble (supernatant SN) and insoluble (pellet P) fractions to investigate the partition of Rrs1 protein between these two cellular components. As shown in figure 1b Rrs1 was detected both in SN fraction, which contains soluble proteins, and in the P fraction, which contains insoluble and membrane-associated proteins.

These data prove that mammalian Rrs1 is localized in the nucleolus and in the cytoplasm probably associated to the membrane compartment.

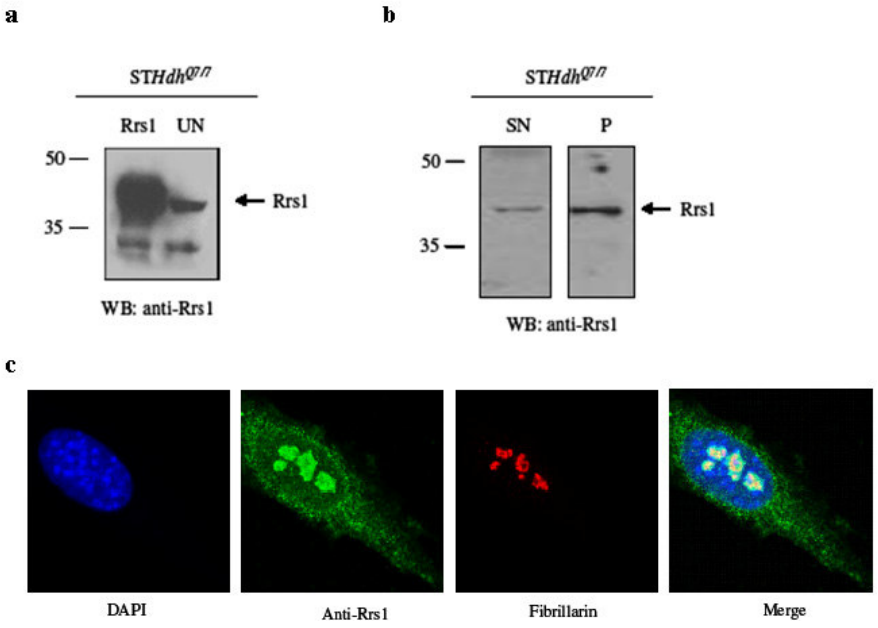


Figure 1: First characterization of Rrs1 in mammalian cells. (a) Wild-type striatal cells (*STHdh*$^{Q7/Q7}$) were transfected with Rrs1 and, after 48 hours, both transfected (Rrs1) and untransfected (UN) cells were lysed and subjected to WB with anti-Rrs1antibody. Anti-Rrs1 antibody specifically recognizes both overexpressed and endogenous Rrs1. (b) WB analysis of Triton-X100 soluble (supernatant SN) and insoluble (pellet P) fractions from *STHdh*$^{Q7/Q7}$ striatal cells. Rrs1 is localized in both fractions, with a higher concentration in the pellet. (c) Immunofluorescence of Rrs1 in wild-type *STHdh*$^{Q7/Q7}$ striatal cells and its colocalization with fibrillarin, a marker for nucleoli. Rrs1 shows also a cytoplasmatic staining. Experiments were performed by E. Fossale.

## A yeast two-hybrid screening to identify Rrs1 interactors

To gain a better understanding of the cellular function of Rrs1, Silvia Michelazzi (SISSA) performed a yeast two-hybrid screening to identify Rrs1 binding proteins. Mouse full-length Rrs1 was used as bait to screen a human fetal brain cDNA library. As expected, some of the interactors are proteins involved in ribosome biogenesis (GRSF1, GNL2, C2f, RPL18A, TSR1). Interestingly, we also found proteins that participate in different biological activities including splicing (C1QBP, SFRS11), transcription (YBX1, zfp690), response to unfolded protein (Hsp90A), and nucleus-cytoplasm transport (KPNA2). Finally, among the stronger Rrs1 interactors, was found the protein Lyric/3D3.

Lyric/3D3, also known as Methaderin and Astrocyte Elevated gene-1 (AEG-1), is a protein highly conserved in mammals with no homologues in nonvertebrate species. Lyric is a novel transmembrane protein of the ER and nuclear envelop and it colocalizes with fibrillarin in the dense fibrillar component (DFC) compartment of the nucleolus. Since the DFC compartment of the nucleolus is the site of rRNA transcription, early posttranscriptional rRNA processing events and ribosomal assembly, Lyric has been suggested to coordinate ER function with protein production at the site of ribosome biosynthesis, in response to ER stress (Sutherland, 2006).

**Rrs1 and Lyric co-immunoprecipitate in mammalian cells**

The interaction between Rrs1 and Lyric was validated by co-immunoprecipitation experiments, using cell line ectopically expressing both Rrs1 and Lyric. Hek293 cells were co-transfected with either Rrs1 and Lyric HA-tagged cDNAs or Rrs1 and an empty HA vector (pcDNA3.0HA) as control. Forty eight hours after transfection protein extracts were immunoprecipitated with the polyclonal anti-HA antibody and analyzed by western blot with anti-Rrs1 antiserum (FUN1). As shown in figure 3, Rrs1 was specifically co-immunoprecipitated with Lyric. We could not perform the reverse co-immunoprecipitation given the inability of FUN1 to immunoprecipitate Rrs1.

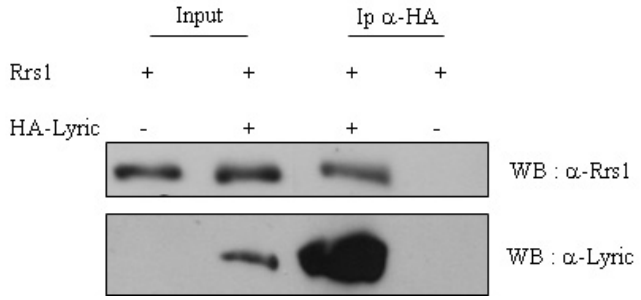


Figure 2: Rrs1 is co-immunoprecipitated by Lyric. HEK293 cells were co-transfected with Rrs1 and HA-lyric or pcDNA3.0-HA, as negative control, and cell lysates were incubated with anti-HA antibody. Immunoblot analysis was performed with FUN1 anti-Rrs1 and anti-lyric antibody.

**Subcellular localization of Rrs1 and Lyric in striatal cells**

The subcellular localization of Lyric in *STHdh*$^{Q7Q/7}$ striatal cells was detected by immunofluorescence with anti-Lyric/3D3 antibody (SS), kindly provided by Dr. Sutherland (Fig. 3a). As expected, Lyric staining was observed both in the nucleoli and linked to ER

(Southerland, 2006). Interestingly, the fluorescent pattern of Lyric strongly resembles Rrs1 (Fig. 3b).

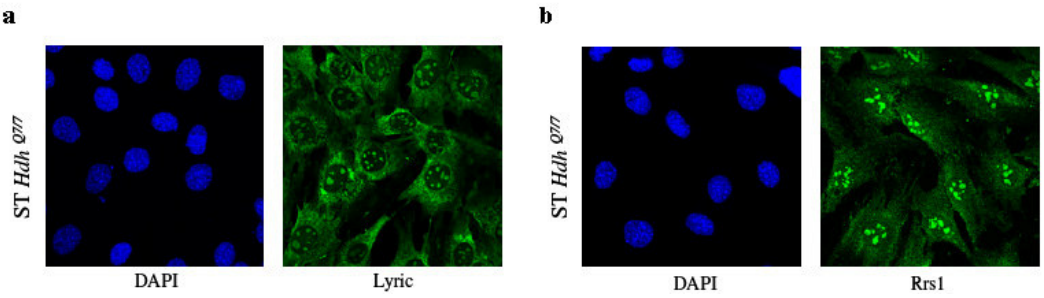


Figure 3: Immunofluorescence of Lyric (a) and Rrs1 (b) in wild-type striatal cells. Experiment performed by E. Fossale.

We have therefore investigated whether the cytoplasmic component of Rrs1, observed by immunofluorescence, is associated to the ER. Confocal analysis was performed in *STHdh*$^{Q7Q7}$ striatal cells by costaining of Rrs1 with the integral ER protein calnexin. As shown in figure 4, Rrs1 was found to co-localize with the marker calnexin to the ER compartment.

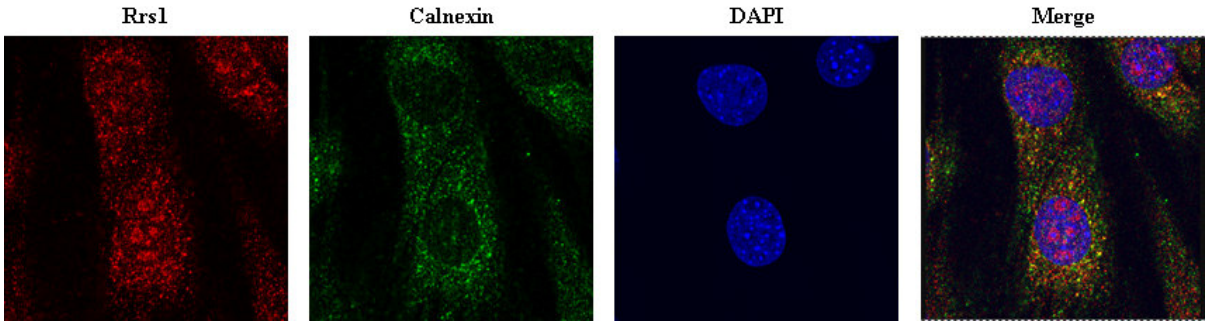


Figure 4: Immunofluorescence of Rrs1 in *STHdh*$^{Q7/Q7}$ striatal cells and its colocalization with the ER marker calnexin.

### Rrs1 mRNA expression is induced by ER stress

The ER fulfils many functions and it is known to be extremely sensitive to alteration of the cellular homeostasis. When stressed the ER sends signals to the cytoplasm and to the nucleus promoting compensative responses that include, among others, the inhibition of protein synthesis exerted through the control of translation and ribosome biogenesis (Sun, 2002).

In yeast Rrs1 expression was found to be essential in repressing ribosome synthesis in response to a defect in the secretory pathway (Tsuno, 2000). In mammals Rrs1 localizes both in the nucleolus and in the ER membranes as its partner Lyric. Therefore we investigated whether these two proteins are involved in the neuronal response to ER stress.

*STHdh*$^{Q7/Q7}$ striatal cells were treated at different times (3, 6 and 12 hours) with Tunicamycin, a well-known inductor of ER stress. Total RNA was extracted and expression of Rrs1 and Lyric were measured by RT-qPCR both in Tunicamycin and in control DMSO-treated cells. As shown in figure 5a, a significant progressive induction of Rrs1 mRNA level was observed starting from 6 hrs up to 12 hrs of treatment. Interestingly, Lyric mRNA was also induced although with a slower kinetics. As shown in figure 5b a significant up-regulation of Lyric mRNA was indeed reached only after 12 hours of tunicamycin treatment suggesting that Lyric might be a component of the later phase of the response.

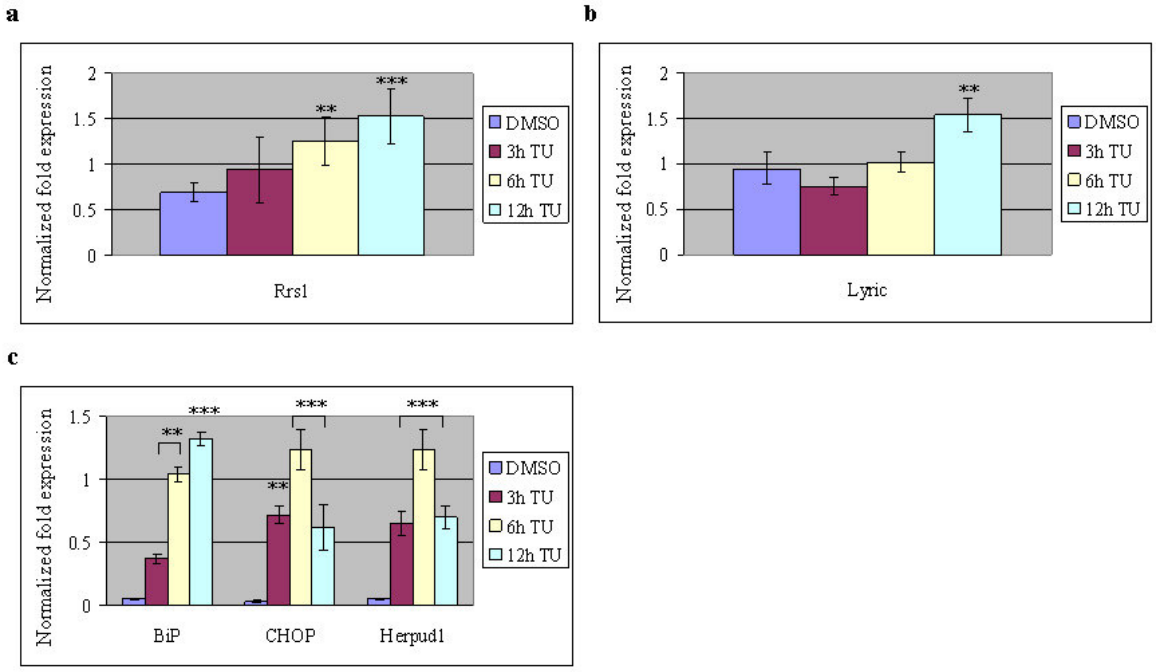


Figure 5: Kinetics of Rrs1, Lyric, BiP, CHOP and Herpud1 mRNAs with persistent ER stress in wild-type striatal cells. ST*Hdh*$^{Q7/Q7}$ striatal cells were treated with 1µg/ml tunicamycin for the indicated hours and harvested for RNA extraction. mRNA expression level was measured by RT-qPCR and normalized relatively to β-actin mRNA level. (a) Rrs1, (b) Lyric, (c) BiP, CHOP and Herpud1 normalized mRNA levels. Error bars represent SDs from five independent experiments. **P<0.005; ***P<0.0005.

We also measured, as control, the expression of BiP, CHOP and Herpud1, which are known markers of the ER-stress response (Li, 1994; Wang, 1996). As expected mRNA level of these genes is specifically induced after treatment in our experimental conditions (Fig. 5c).

These data prove for the first time that Rrs1 and Lyric are components of the ER stress response in mammalian cells.

### Rrs1 and Lyric mRNA expression in ST*Hdh*$^{Q111}$ striatal cells

Fossale and collaborators have shown that mutant huntingtin increases Rrs1 mRNA expression both in HD *postmortem* brains and in *Hdh*$^{Q111}$ knock-in mice.

Here we have extended the analysis to ST *Hdh*$^{Q111/Q111}$ cells.

By quantitative real time PCR we have compared Rrs1 mRNA levels in wild-type (ST*Hdh*$^{Q7/Q7}$) and homozygous mutant (ST*Hdh*$^{Q111/Q111}$) striatal cells. Conversely to data obtained with human *postmortem* brain and with mouse striatum we could not detect differences in Rrs1 expression in the proliferating striatal cells (Fig. 6).

Interestingly, as reported in figure 6 Lyric mRNA showed a significant increase of expression (P<0.0005) in ST*Hdh*$^{Q111/Q111}$ cells.

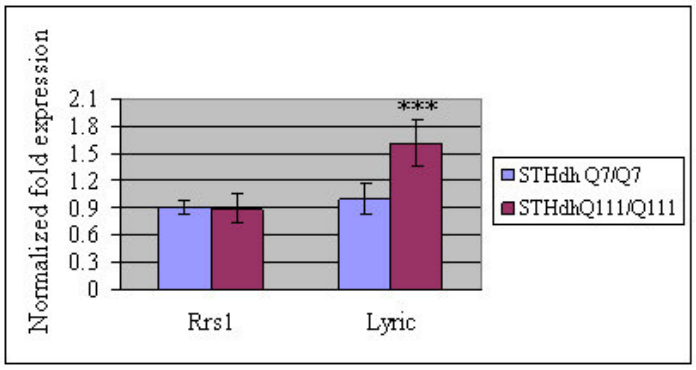


Figure 6: Expression of Rrs1 and Lyric mRNA levels in *STHdh* striatal cells. Equal expression level is found for Rrs1 mRNA, whereas Lyric mRNA is significantly upregulated in *STHdh*$^{Q111/Q111}$ striatal cells with respect to wild-type. Error bars represent SDs from seven independent experiments. ***P<0.0005.

Although *STHdh*$^{Q111/Q111}$ cells present abnormal ER compartment (Trettel, 2000), no literature data are available on presence of an ER stress response in this cell line.

Therefore, by RT-qPCR we compared the endogenous levels of BiP, CHOP and Herpud1 in wild-type and homozygous mutant striatal cells. As shown in figure 7, similarly to Rrs1, no difference in BiP mRNA expression was revealed between wild type and mutant cells. In addition, we found that Herpud1 mRNA was significantly decreased in *STHdh*$^{Q111/Q111}$ cell, whereas the level of CHOP mRNA was significantly increased. Although these observations support an ongoing stress response (induction of CHOP), proliferating *STHdh*$^{Q111/Q111}$ cells do not seem to exhibit the canonical features of a cellular ER stress response. They may present alternative compensative mechanisms for the altered cellular homeostasis triggered by mutant huntingtin.

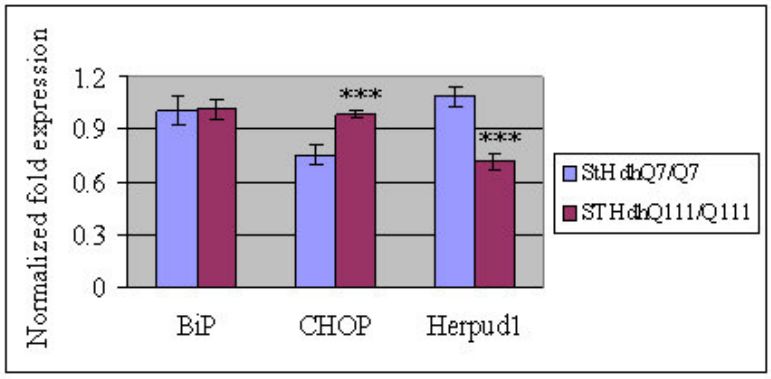


Figure 7: BiP, CHOP and Herpud1 expression in *STHdh* striatal cells. mRNA levels were measured by RT-qPCR and normalized relatively to β-actin mRNA level. *STHdh* striatal cells show no difference in the expression level of BiP mRNA, whereas both CHOP and Herpud1 mRNAs are differentially expressed in *STHdh*$^{Q111/Q111}$ striatal cells with respect to wild-type. Error bars represent SDs from five independent experiments. ***P<0.001.

We then monitored wild-type and mutant striatal cells ability to respond to ER stress inducers. By quantitative real time PCR, mRNA levels of Rrs1, BiP, CHOP and Herpud1 were measured after tunicamycin treatment. Surprisingly, we observed higher induction of Rrs1, BiP, CHOP and Herpud1 in ST*Hdh*$^{Q7/Q7}$ cells compared to mutant (1.6, 26, 41, 25 fold in ST*Hdh*$^{Q7/Q7}$ versus 1.5, 22, 31, 18 fold in ST*Hdh*$^{Q111/Q111}$ respectively).

These data may suggest the existence of compensative mechanisms in mutant cells that counteract ER stress including an altered basal level of expression of CHOP and Herpud mRNAs.

### Increased expression of ER stress-related mRNA markers in *Hdh*$^{Q111}$ striatum

Our findings with tunicamycin treatment in *STHdh*$^{Q7/7}$ cells indicate that Rrs1 expression is induced by ER-stress. We have therefore hypothesized that in *Hdh*$^{Q111}$ knock-in mice the increased expression of Rrs1 mRNA may represent an early response to a perturbation of the ER homeostasis triggered by mutant huntingtin.

To test this hypothesis we have first replicated in our experimental environment the quantitative analysis of Rrs1 mRNA expression in striatum of mutant and wild-type mice. Total RNA was isolated from five mouse striata of each genotype at different ages (3 months and 1 year) and analyzed by RT-qPCR. The results reported in Figure 8a show that Rrs1 was significantly increased of ~2.3-fold (P<0.001) in heterozygous *Hdh*$^{Q111}$ striatum compared to wild-type striatum at 3 months of age. Furthermore, assays of Rrs1 mRNA in homozygous

*Hdh*$^{Q111}$ and wild-type striatum at 1 year revealed a ~2-fold (P<0.02) increase in the mutant striatum, indicating an ongoing impact of mutant huntingtin on Rrs1 mRNA expression (Fig. 8b).

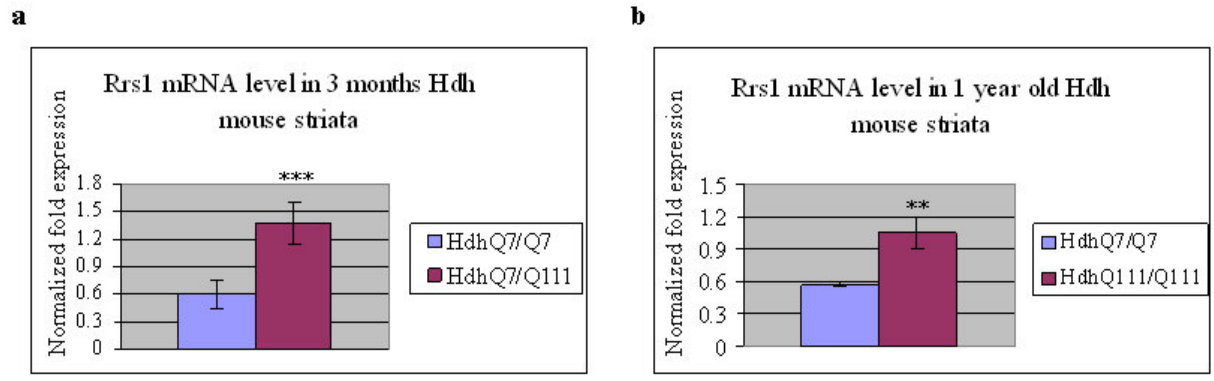


Figure 8: Rrs1 mRNA phenotype in *Hdh*$^{Q111}$ striatum. Quantitative real-time PCR assays were performed with RNA isolated from striata dissected from *Hdh*$^{Q111}$ mice at 3 months (a) and 1 year (b) of age. (a) Rrs1/β-actin mRNA ratios for mutant *Hdh*$^{Q111}$ heterozygous striatum plotted as a histogram, showing a significant 2.3-fold increase, compared with levels of Rrs1 mRNA in wild-type striatum. Bars indicate standard deviation. ***P<0.001. (b) The histogram displays Rrs1/β-actin striatal mRNA ratios determined for wild-type and *Hdh*$^{Q111}$ homozygotes, showing a significant 2.0-fold increase of Rrs1 mRNA. Bars indicate standard deviation. **P<0.01.

To determine whether Lyric mRNA was also increased as a consequence of HD CAG repeat expansions, we performed RT–qPCR assays to assess striata from *Hdh*$^{Q111}$ mice. As shown in Figure 9, four out of five heterozygous *Hdh*$^{Q111}$ mice show a significant increase of Lyric mRNA compared to wild-type mice.

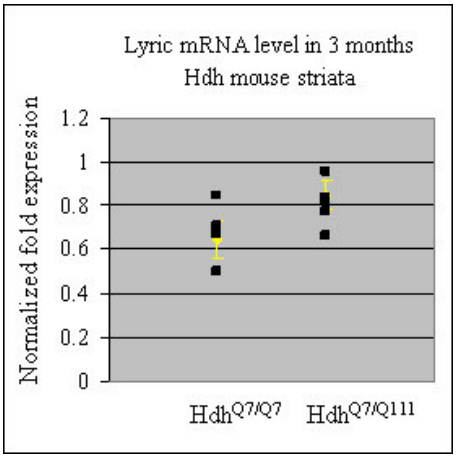


Figure 9: Lyric mRNA expression in *Hdh* mouse striata is affected by mutant huntingtin. Quantitative real-time PCR assays were performed with RNA isolated from four striata dissected from *Hdh*$^{Q111}$ heterozygous mice and four wild-type control littermates at 3 months of age. Lyric/β-actin mRNA ratio show a nearly significant 1.3 fold increase of Lyric mRNA compared with levels in wild-type striatum. Bars indicate SDs.

No significant variation was instead observed at 1 year of age (data not shown).

To evaluate whether ER-stress may account for Rrs1 up-regulation in striatum of knock-in mice, we performed RT-qPCR assays for BiP, CHOP and Herpud1 from single mouse striata. The results reported in figure 10a show a significant up-regulation of the chaperones BiP and Herpud1, as well as of CHOP mRNAs in heterozygous $Hdh^{Q111}$striata compared to wild type at three months of age. Consistent with a progressive dysregulation of many cellular processes at 1 year of age homozygous knock-in mice still show increased expression of Bip and Herpud1 mRNAs, whereas the difference in the level of CHOP mRNA was no longer detectable (Fig 10b).

These data prove for the first time the existence of an ER stress response as an early event in HD pathogenesis.

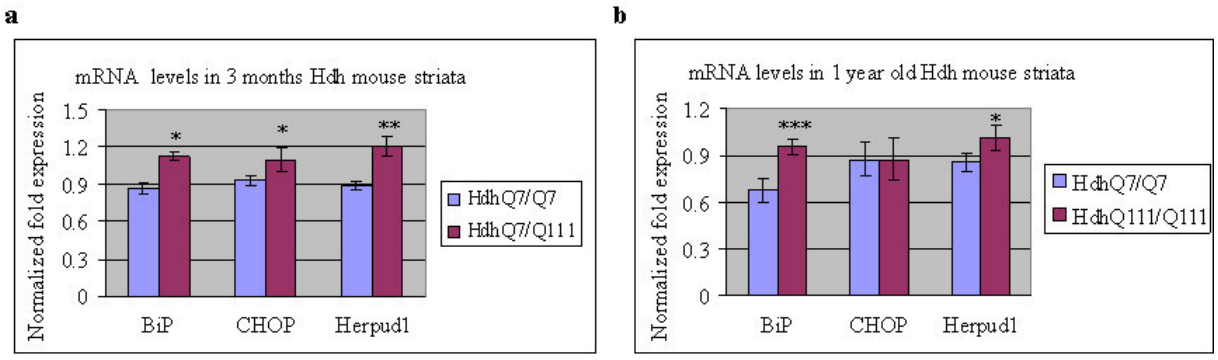


Figure 10: BiP, CHOP and Herpud1 mRNA expression in *Hdh* mouse striata. Normalized mRNA levels were measured by RT-qPCR and are shown relative to β-actin mRNA level. (a) $Hdh^{Q111}$ heterozygous knock-in mice show a significative difference in the expression level of BiP, CHOP and Herpud1 mRNAs at 3 months of age of 1.3, 1.2 and 1.4 fold, respectively. Error bars represent SDs. (b) $Hdh^{Q111}$ homozygous knock-in mice show persistent higher mRNA levels of BiP and Herpud1 also at 1 year of age, whereas the mRNA level of CHOP are unchanged. Error bars represent SDs. Six mice for each genotype were used. *P<0.05; **P<0.01; ***P<0.0005.

## 3.3 DISCUSSION

In this work we have described for the first time Rrs1 expression in mammalian cells.

Using a specific antibody that we have generated, we have found that Rrs1 is a protein of ~ 40 kDa that localizes in the nucleoli, as previously observed in yeast (Tsuno et al., 2000), and in the cytoplasm.

In the nucleolus Rrs1 colocalizes with fibrillarin that is a marker of the dense fibrillar component (DFC) of the nucleoli, which contains actively transcribing rRNA genes along with nascent rRNA transcripts and is the site of maturation of pre-rRNA transcripts. These data are in full agreement with Rrs1 expression and function in yeast, where it was found to be a member of the yeast Ribosome and rRNA Biosynthesis (RRB) regulon (Wade, 2001), a transcriptionally co-regulated set of genes that are required for ribosome biosynthesis.

Here we report for the first time that mammalian Rrs1 is also located in the ER.

By scanning Rrs1 primary sequence, two consensus for ER membrane localization are found both at its N- and C-terminals, XXRR (EGQR) and KKXX (GKRR), providing molecular mechanisms for its subcellular distribution.

In order to gain a better understanding of the cellular function of Rrs1 we performed a yeast two-hybrid screening with a human fetal brain library to identify Rrs1-interacting proteins.

Among the 12 Rrs1-interacting proteins expressed in the nervous system, we isolated proteins located in both ER and nucleolus (Lyric/3D3) as well as involved in ribosome biogenesis (GRSF1, GNL2, C2f, RPL18A, TSR1), splicing (C1QBP, SFRS11), transcription (YBX1, zfp690), response to unfolded protein (Hsp90A) and in the nucleus-cytoplasm transport (KPNA2).

Since Rrs1 and Lyric share their subcellular distribution we focused our attention on this new interactor. Lyric was previously identified as a human immunodeficiency virus-1 inducible and tumor necrosis factor-α-inducible transcript in primary human fetal astrocytes. Its expression is elevated in >95% of human malignant glioma. Furthermore, it downregulates the expression of the glutamate transporter EAAT2, a protein implicated in glutamate-induced excitotoxicity (Kang, 2005).

For its dual localization it has been suggested to play a role in coordinating the activities of the ER and nucleolus (Sutherland, 2006).

In eukaryotic cells, the ER is the primary organelle in the secretory pathway where proteins are synthesized, folded and post-translational modified prior to be delivered to other secretory compartments. In addition, the ER is also the place where intracellular $Ca^{2+}$ is stored and where lipids and sterols are synthesized.

The ER is extremely sensitive to alteration of homeostasis that disrupts its functions. When the load of unfolded proteins stored in the ER reaches a threshold, an imbalance occurs, called "ER stress". Cells respond to ER stress by activation of the unfolded protein response (UPR) pathways where signal are transduced from the ER to the cytoplasm and to the nucleus to induce the expression of genes encoding mediators of host defense. This response changes the expression of specific chaperones, enhances degradation of misfolded protein and inhibits protein synthesis to decrease the load within the ER (Rao, 2004). Protein synthesis is mainly controlled by a translational attenuation mechanism, which entails the phosphorylation of eIF2α by PERK with consequent inhibition of cap-dependent translation (Harding, 1999). A concurrent mechanism of control is mediated by IRE1β protein, which, in response to ER stress, cleaves rRNA to attenuate translation (Iwawaki, 2001). When ER homeostasis cannot be restored, prolonged UPR may induce apoptosis (Breckenridge, 2003).

Increasing evidences connect ER compartment with huntingtin activities. By a variety of methods several investigators have reported huntingtin to target ER membranes and to be essential for normal ER structure (Omi, 2005). The first 18 amino acids of huntingtin have been found to contain a membrane-targeting domain that mediates the association of the protein with the ER. The disruption of this domain or the induction of ER stress causes huntingtin to be actively transported into the nucleus *via* an active process, which involves the residues 81–588 of the protein. Notably, increased nuclear entry of mutant huntingtin due to loss of ER-targeting results in increased toxicity (Atwal, 2007).

Alteration of ER homeostasis is becoming increasingly important in understanding the pathology of several protein-misfolding neurodegenerative diseases, including Parkinson's and Alzheimer's diseases (Lindholm, 2006).

In the present work we found that Rrs1 and its protein interactor Lyric are components of the neuronal stress response. Furthermore, that early dysregulation of Rrs1 and Lyric mRNAs expression in mutant $Hdh^{Q7/Q111}$ heterozygous mice is concomitant to clear evidences of disrupted ER homeostasis. Our results therefore indicate that mutant huntingtin induces ER

stress very early in the disease cascade of Hdh knock-in mice and very likely also in human disorder.

ER stress has been detected as up-regulation of BiP, CHOP and Herpud1 mRNAs at 3 months of age. The rationale for such findings might be represented by the chaperone role of BiP and Herpud1, which are among the first genes to be induced upon ER stress. Furthermore, CHOP is a transcription factor whose expression is associated with an irreversible cellular death through apoptosis due to inability to overcome ER stress (Zinszner, 1998). Interestingly, at 1 year of age the levels of both chaperones, BiP and Herpud1, continue to increase in knock-in mice, whereas the level of CHOP mRNA results down-regulated to the wild type level.

A model for what we observed in knock-in mice may be that at 3 months of age cells try to overwhelm the problem of misfolded mutant huntingtin through the induction of ER chaperones; prolonged stress may then lead to apoptosis through the expression of death effectors as CHOP. Since CHOP transcription requires, among others, NF-Y, it is tempting to speculate that its down-regulation may be due to NF-Y sequestration into huntingtin aggregates, as shown by Yamanaka and colleagues (Yamanaka, 2008). Furthermore, this may provide an original explanation for an increased viability of MSNs-containing aggregates.

Recently, Reijonen and colleagues have reported in PC6.3 cell line overexpressing N-terminal mutant huntingtin fragments clear evidences of ER stress. They observed upregulation of BiP and CHOP mRNAs, phosphorylation of JNK, cleavage of caspase 12 and 3 associated to pronounced cell death. Treatment with salubrinal, that inhibits ER stress, counteracted cell death and reduced protein aggregation caused by the mutant huntingtin fragment (Reijonen, 2008).

Moreover, we found the induction, at the transcriptional level, of BiP, CHOP, and Herpud1 as well as of Rrs1 and Lyric genes in striatal cell lines treated with tunicamycin, a chemical ER stress inducer. In these experimental conditions we observed a lower increase in mutant striatal cells with respect to wild type, possibly underlining a pre-existent stress provoked by the presence of mutant huntingtin. Indeed, mutant striatal cells show altered level of expression of both CHOP (induced) and Herpud1 (reduced), which may explain why these genes fail to reach a higher level of expression in these mutant cell lines upon ER stress.

We believe that Rrs1 and Lyric may be helpful to understand the functional consequence of ER stress in HD

In *Saccharomyces cerevisiae* a defect in the secretory pathway causes the transcriptional repression of both ribosomal protein and rRNA genes. Rrs1 was first identified as an essential protein required for the secretory response (Tsuno, 2000).

Since we have demonstrated that in mammalian cells Rrs1 mRNA expression is increased by ER stress, we hypothesize that Rrs1 might function as an ER stress sensor in HD, shuttling from the ER to the nucleolus to translate stress response into repression of ribosome synthesis.

It will be important to assess whether in mammalian cells, as in yeast, Rrs1 participate in the transcriptional repression of ribosomal components induced by ER stress.

Since Rrs1 depletion in *Saccharomyces cerevisiae* causes defects in the maturation of 25S rRNA and in the assembly and nuclear export of the 60S ribosomal subunit (Tsuno, 2000; Miyoshi, 2004), we also hypothesize that Rrs1 induction in HD may suggest a striatal impairment of ribosome biogenesis.

Interestingly, previous reports are consistent with dysregulation of ribosome synthesis and alteration in the membrane compartments of HD patients (Wyttenbach, 2001; Trettel, 2000; Gauthier, 2004; Atwal, 2007), although further experiments are needed in human and mouse models as well.

We are currently performing experiments to test these hypotheses by monitoring rDNA transcription and rRNA biogenesis in HD mouse models.

These results show that mutant huntingtin activates cellular pathways linked to ER stress with mechanisms that are still unclear. It is likely that association of mutant huntingtin with cell membranes may influence calcium metabolism and activate signaling proteins in the ER (Rockabrand, 2007). This stress response may be more critical to the health of neuronal cell populations such as those in the striatum and the cortex.

The data presented here suggest that compounds targeting ER stress may be considered in designing novel approaches for treatment of HD and possibly other polyQ diseases.

## 3.4 MATERIALS AND METHODS

### Plasmids

Rrs1 in pcDNA3.0 (Invitrogen), encoding full length mouse Rrs1 (Riken clone # 5330427D04 02.7.31) was digested out of the pBlueScript KS (+) plasmid with BamH1 and XhoI and subcloned into pcDNA3.0; HA-Lyric, encoding full length mouse N-terminal HA-tagged Lyric, was digested out of Fantom clone # 4931440A01 with EcoRI and XbaI and subcloned into pcDNA3.0-HA.

### Cell Culture

***STHdh*** striatal progenitor cell lines from wild-type (ST*Hdh*$^{Q7/7}$) and homozygous (ST*Hdh*$^{Q111/111}$) knock-in *Hdh* littermate mice embryos (E14 striatal primordial) were cultured at 33ºC in D-MEM (Dulbecco's modified Eagle's medium), 10% FBS (fetal bovine serum), 100 U/ml penicillin, 100 µg/ml streptomycin, 400 µg/ml G418 for selection.
The cells were immortalized with a Temperature Sensitive version of the SV40 Large T-Antigen, which allows the cells to proliferate at the permissive temperature of 33ºC (Trettel *et al.*, 2000).

**Hek 293T (human embryonic kidney) cells** were cultured at 37ºC in D-MEM (Dulbecco's modified Eagle's medium), 10% FBS (fetal bovine serum), 100 U/ml penicillin, 100 µg/ml streptomycin.

### Transfection: Calcium phosphate method

Hek293T cells were transfected through the calcium phosphate method.
Cells were plated in a 100mm culture dish the day before transfection so to reach about 60-70% confluence at the time of transfection.
A transfection mix composed by 0.25 mM $CaCl_2$, 7 µg plasmid DNA and $H_2O$ to a final volume of 500 µl was added dropwise with bubbling to a tube containing 500 µl HBS 2X (140 mM NaCl, 1.5 mM $Na_2HPO_4 \bullet 2H_2O$, 50 mM HEPES pH 7.1). After 20 minutes incubation at RT the mixture was added to the cells. Cells were incubated overnight with the precipitates

and the medium was changed the day after. Cells were collected and assayed 40-48 hours after transfection.

**Treatment**

**Tunicamycin**: *STHdh*$^{Q7/Q7}$ and *STHdh*$^{Q111/Q111}$ striatal cells were treated with 1 μM tunicamycine for 3-6-12 hrs.

**Protein extraction and western blot**

Cells were washed in RT PBS 1X, then harvested in cold lysis buffers.
Cells were lysed for 20 minutes rocking at 4°C and then centrifuged at 13,000 rpm for 15 minutes at 4°C.
Protein concentration was determined by Bradford assay using a calibration curve built with standard amounts of BSA (bovine serum albumin).
Loading buffer was added and samples were boiled for 5 min. Proteins were separated by SDS-polyacrilamide gel electrophoresis (SDS-PAGE).

**Co-immunoprecipitation assays**

Cells were lysed with a Hepes pH 7.6 lysis buffer (10 mM Hepes pH 7.6, 1 mM EDTA, 150 mM NaCl, 0.2% Triton-X 100), supplemented with protease inhibitor cocktail and phosphatase inhibitors (1mM sodium orthovanadate, 50mM NaF, 10 nM Okadaic Acid). After incubation 20 minutes rocking at 4°C samples were centrifuged 15 minutes at 4°C at 13,000 rpm. Protein concentration was determined by Bradford. The same amount of total proteins (1-2 mg) was used for each sample. The extracts were incubated with the immunoprecipitating antibody (2 μg) for 2 hours at 4°C with rocking, then 25 μl of Protein G Sepharose 50% slurry (Protein G Sepharose 4B, Amersham Biosciences) were added and incubated at 4°C rocking for 1 additional hour. Beads were pelleted at 800 rpm 2 minutes at 4°C and washed 3 times with the same lysis buffer, then dried out with a syringe. Samples were boiled in 20 μl Sample Buffer 2X for 10 minutes before loading on SDS-polyacrylamide gel.

**Immunofluorescence**

Cells were seeded onto 13 mm coverslips and allowed to attach for 24 hours. After medium removal and washing in PBS cells were fixed in 4% paraformaldehyde for 15 minutes at RT.

After fixation, cells were rinsed in PBS and incubated 5 minutes with glycine 100 mM to quench autofluorescence. After rinsing in PBS cells were permeabilized with 0.2% Triton X-100 for 4 minutes, washed again in PBS and incubated in BSA 0.2% for 1hour to block non specific sites before primary antibody incubation.

Both primary and secondary antibodies were incubated in 0.2% BSA, 1% NGS.

After primary antibody incubation, cells were washed 2 times in PBS and subjected to secondary antibody incubation. Nuclei were labeled with DAPI added during secondary antibody incubation. Cells were washed 2 times in PBS and mounted on slides using Vectashield mounting medium.

Images were captured with a Leyca confocal microscope by using a 63X oil objective.

**Antibodies**

The following primary antibodies were used : Rabbit polyclonal anti-Rrs1 (FUN1; E. Fossale, MGH, Boston; WB dilution 1:500, IF dilution 1:30); mouse monoclonal anti-HA tag (kindly provided by Dr. L. Collavin, LNCIB, Ip 2 µg); Rabbit polyclonal anti-Lyric (Zymed Lab.,# 40-6400, WB dilution 1:200, IF dilution 1:50); Anti-Calnexin (Chemicon, MAB3126, IF dilution 1:10), Mouse IgG (Sigma).

The following HRP secondary antibodies were used: HRP conjugated goat anti-rabbit antibody (DAKO, WB dilution 1:2000).

The following secondary antibodies were used for IF: Alexa-594 donkey anti-mouse (1:1000); Alexa-488 donkey anti rabbit (1:1000).

**Genotyping**

DNA was prepared from tail biopsy. CAG repeats were sized by PCR in GB buffer (66 mM Tris-HCl (pH 8.8), 16 mM (NH4)2SO4, 2 mM MgCl2, 0.7% b-Mercaptoethanol), 0.01 mg/ml BSA, 10% DMSO, 200µM dNTPs, 0.5µM primers with 0.4 U/µl RedTaq (Sigma). Cycling conditions were 90”@ 94°C, 35 X (30”@94°C, 30”@ 56/65°C, 90”@ 72°C), 10'@ 72°C. PCR products were resolved in 1% agarose gel. Primer sets were as follow: wild type 5'-CCTGGAAAAGCTGATGAAGG-3', 5'-TGGACAGGGAACAGTGTTGC-3'; knock-in 5'-ATGAAGGCCTTCGAGTCCCTCAAGTCCTTC-3', 5'-GGCGGCTGAGGAAGCTGAG GA-3'. Genotyping of all animals was confirmed after dissection.

**Wild-type and Hdh knock-in mouse brain dissections**

H*dhQ*$^{111/111}$ and H*hd*$^{Q7/7}$ knock-in mice expressing endogenous levels of huntingtin with 111 or 7 glutamines have been described previously (White, 1997).

Mice at 3 months or 1 year of age were euthanized by $CO_2$ exposure following the CCM regulations and the brains were dissected into striatum, cortex and cerebellum, immediately frozen in liquid nitrogen, and stored at -80°C.

**RNA extraction and RT-qPCR**

Cells were washed in RT PBS 1X, then harvested in cold TRIZOL; mouse striata were homogenized in cold TRIZOL. RNA was extracted from six 3 months wild-type mice and six 3 months heterozygous mice, and from six 1 year old wild-type mice and six 1 year old homozygous knock-in mice.

Total RNA was isolated with TRIZOL reagent (Invitrogen/Life Technologies, Carlsbad, CA) according to manufacturer's instructions, quantified spectrophotometrically at 260 nm, and analyzed by agarose gel electrophoresis. Total RNA used for quantitative RT–PCR assays was pretreated with DNase I using a DNA-free kit (Ambion Inc., Austin, TX, USA) and additionally purified over RNeasy Columns (Qiagen, Valencia, CA, USA).

RT reactions were performed in 20 µl with 1 µg of DNase-treated total RNA, 1X reaction mix, and 1 µl of Reverse Transcriptase (Bio-rad, iSript$^{TM}$ cDNA synthesis kit). Reaction conditions were 5 min at 25°C, 50 min at 42°C and 5 min at 85°C. Quantitative PCR reactions were performed with an iCycler iQ instrument (Bio-Rad Laboratories, Foster City, CA, USA), using the iQ Custom Syber Green Supermix (Bio-Rad). PCR reactions were performed in 25 µl reaction volumes containing 1 µl of cDNA, 250 nM of each primer and 1X reaction mix. Each reaction was performed in duplicate for the target and the normalizing gene (β-actin) in the same 96-well plate for all samples. Cycle parameters were 3 min at 95°C (10 s at 95°C, 20 s at 58°C and 30 s at 72°C) for 40 cycles. To quantify the mRNA levels for each sample and primer set, a standard curve was generated with known dilutions of total cDNA. The relative value for each unknown sample was then calculated from its respective standard curve using linear regression analysis. To normalize the differences in the amount of total RNA added to each reaction, we used β-actin as endogenous control. The normalized expression value was then calculated by dividing the relative quantitation value of each sample and primer set by the

relative quantitation value of β-actin. Specificity of amplicon was determined by melt curve analysis and gel electrophoresis.

Primers were designed for each gene using Beacon Design 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). Specific forward and reverse primers (TIB MOLBIOL, Genova, Italy) were as follows: murine Rrs1 (GenBank accession no. NM_021511), 5'-GCAAGTATTGTAAATGGATGCAG-3', 5'-TCTCAAGCACACTCCATATTG-3'; murine Lyric (GenBank accession no. NM_026002), 5'-CTCTCGGGCTGCTCCTGCTCTTC-3', 5'-GGCGGGCTCCTTCGCTTCTTGCG-3'; murine β-actin (GenBank accession no. NM_007393), 5'-TGAAATAAGTGGTTACAGGAAGTC-3', 5'-GCAGTACATAATTTACACAGAAGC-3'; murine CHOP (GenBank accession no. NM_007837), 5'-GAGCTGGAAGCCTGGTATGAG-3', 5'-TGTGCGTGTGACCTCTGTTGG-3'; murine BiP (GenBank accession no. NM_022310), 5'-GAGCGTCTGATTGGCGATGC-3', 5'-TTCCAAGTGCGTCCGATGAGG-3'; murine Herpud1 (GenBank accession no. NM_022331), 5'-GGAGCCAATCAGAACTTGCG-3', 5'- ATAGGTCCAATCCAACCAGTCTCG-3'.

**Statistical analyses**

Statistical analyses were Student's t-test, choosing $P<0.05$ as significant. Weighted means were calculated for data produced in replica experiments. Calculated means and standard deviations were plotted using the graph tool of Microsoft Excel.

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# CONCLUSIONS

The work performed in this thesis was aimed to identify toxic molecular mechanisms in the HD pathogenic cascade using two complementary lines of investigations.

In the first part of this work we have hypothesized that the peptidyl-prolyl isomerase Pin1 may be involved in HD pathogenic mechanisms. This enzyme has been already implicated in other neurodegenerative disorders in modulating fundamental properties of the disease proteins. In this context, Pin 1 could play a pivotal role also in HD and be considered as a potential drug target. In support to our hypothesis we have demonstrated that Pin1, when overexpressed in cell culture, reduces mutant huntingtin aggregate formation. Moreover, we found that Pin1 decreases huntingtin half-life by inducing proteasome pathway activity. The involvement of the proteasome is of particular interest being its dysregulation already linked to HD, as well as to other neurodegenerative disorders.

Several hypotheses may be formulated on the functional consequences of the modulation of huntingtin aggregation by Pin1.

In AD, Pin1 binds the phosphorylated Thr-231-Pro motif of tau and, by enhancing its dephosphorylation, restores tau function and reduces the formation of neurofibrillary tangles (Lu, 1999). Thus, in this context, Pin1 exerts a protective role. Interestingly, analysis of Pin1 activity in AD brain demonstrated that oxidation of Pin1 leads to loss of activity. The *in vivo* oxidative modification of Pin1 as found by proteomics in AD hippocampus suggests that oxidative modification may be related to the loss of Pin1 activity that could be crucial in AD neurofibrillary pathology.

In PD, in contrast to AD, Pin1 inhibits the degradation of α-synuclein and enhances the formation of the Lewy Bodies (LB) cytoplasmic inclusions (Kesavapany, 2007). The question of whether intracellular inclusions protect or are cytotoxic for neurons is still controversial. It is therefore difficult to conclude if Pin1 protects or enhances cell death in the dopaminergic neurons of PD brains.

In HD we do not currently have sufficient data to support either a protective or a toxic role for Pin1. Several scenarios may be played out including a changing role according to the pathogenic stage.

It will be interesting to assess whether Pin1 is a component of HD inclusions in *postmortem* brains and/or it is downregulated.
These data may have profound effects on strategies for therapeutic interventions.
Interestingly, we also found that Pin1 may modulate transcriptional dysregulation induced by mutant huntingtin, further supporting a role for this enzyme in the HD neurodegenerative process. The generation of a double *Hdh*$^{Q111}$KI/*Pin1*KO mouse line and ongoing studies aimed to elucidate the role of Pin1 in modulating HD phenotypes *in vivo* will provide interesting clues.

In the second part of this thesis we focused our attention on Rrs1, a gene whose expression was previously found upregulated in *Hdh*$^{Q111}$ KI mice, a pre-symptomatic model of HD. Up to now, Rrs1 protein has been studied only in yeast where it participates in ribosomes biogenesis. We have considered that knowing Rrs1 function in mammalian cells could provide insight into the early mechanisms of the disease process. In fact, we have described for the first time Rrs1 protein in mammalian cells, where it localizes both in the nucleoli and in the ER. In addition, we have shown that mRNA level of both Rrs1 and its partner Lyric are induced upon ER stress in striatal cell lines. Interestingly, we found that ER stress is a new early phenotype of *Hdh*$^{Q111}$ knock-in mice. In conclusion, studying Rrs1 we have identified a new pathway that is altered in the early phase of the disease cascade and that surely deserve future investigations.

## APPENDIX 2: ISOLATION OF PEPTIDE APTAMERS TO TARGET MUTANT HUNTINGTIN-INDUCED ABNORMAL PHENOTYPES

During the first year of my PhD course, I had been involved in a different project, than those presented in the previous chapters.

In particular, the project I had worked on was focusing on the identification of small molecules, peptides, able to interact with mutant huntingtin.

The strategy of using peptide aptamers that selectively interact with target proteins interfering with the protein functions has been widely validated (Colas, 1996; Guida, 2008). The isolation of peptide aptamers that bind mutant huntingtin may represent therefore a valuable approach to hamper inappropriate interactions and to dissect HD pathogenic pathways. In addition, peptide aptamers provide a platform for the design of pharmacologically active drugs that may specifically slow the disease progression.

We report here the expression of a combinatorial library of constrained 16-residues peptides displayed by the active site loop of *E. coli* thioredoxin and the use of a yeast two-hybrid system to select those that might be able to revert mutant huntingtin-induced abnormal phenotypes.

### Work performed and Results

#### Making and testing the baits

To isolate peptides aptamers that bind huntingtin we first generated bait clones expressing the N-terminal region of huntingtin, with 20 or 62 glutamines, as fusion proteins in pLexA vector (pLexA-HD$_{1\text{-}550}$Q20 and pLexA-HD$_{1\text{-}550}$Q62). The baits were transformed into the EGY48 MAT-alpha yeast strain containing the lacZ reporter plasmid (pSH$_{18\text{-}34}$) to generate the bait strains EGY48Nt-wt (EGY48/pSH$_{18\text{-}34}$/pLexA-HD$_{1\text{-}550}$Q20) and EGY48Nt-mut (EGY48/pSH$_{18\text{-}34}$/pLexA-HD$_{1\text{-}550}$Q60). The accurate expression of the fusion proteins in the bait strains was assessed by western blot (Fig. 1). Potential autoactivation of the baits was tested and excluded by the lack of expression of the reporter genes Leu2 and LacZ.

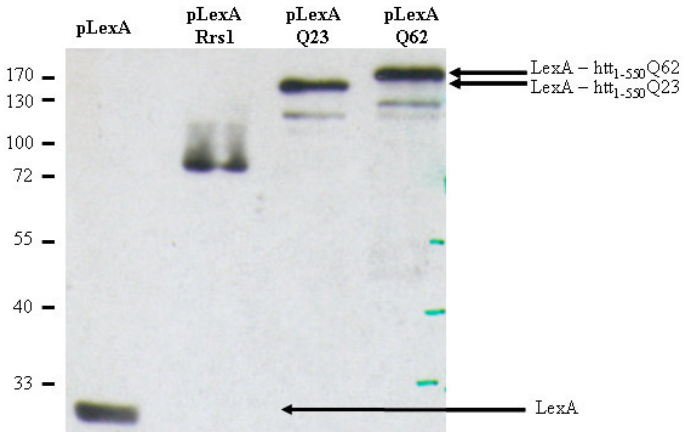


Figure 1: Bait expression test. EGY48/pSH$_{18-34}$ was transformed with pLexA, pLexA-HD$_{1-550}$Q20, pLexA-HD$_{1-550}$Q60 or pLexA-Rrs1 (control). 72 hours after transfection a single colony was cultured ON in liquid medium and the day after cells were lysed for protein extraction. Blot probed for LexA DBD (α-LexA dilution 1:1000).

## Library transformation

The bait strains EGY48Nt-wt/mut were subsequently transformed with a combinatorial peptide library, kindly provided by Prof. G. Del Sal. The library encodes *E. coli* thioredoxin (TrxA) with about $10^9$ random 16-mer peptides in its active site (Guida, 2008). For each bait a total of ~ 7 x $10^6$ colonies were plated into selective media and screened for LacZ and Leu2 expression. As result of this primary screening we have identified 26 positive colonies.

To verify the specificity of the interaction we performed a secondary screening in yeast. Positive prey plasmids were recovered and retransformed into their respective bait strains. Clones positive to the secondary screening were recovered and sequenced; seven peptides were identified.

Bioinformatics analysis of the identified peptide aptamers shows that they have no significant similarity to any known protein, but it is worth noting that they share a positively-charged region (Fig. 2) which could represent a common motif, and so a common way of interaction. The repertoire of binding affinities to mutant and wild-type huntingtin for the various peptides is under investigation. Excitingly preliminary data in yeast suggest that some of the peptides specifically bind mutant huntingtin. Modelling studies of the peptides could reveal interesting insights on this direction.

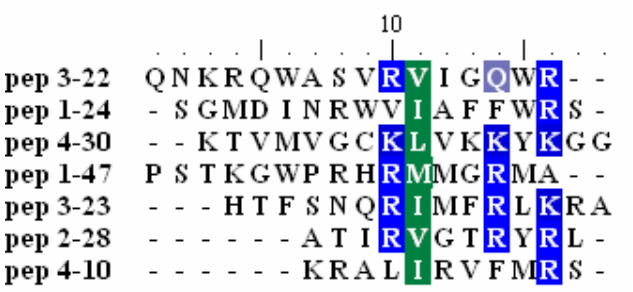


Figure 2: Multiple sequence alignment of the 7 identified peptides. Colours highlight high homology residues.

**Peptide analysis in mammalian cells**

Positive peptides, constrained in the thioredoxin scaffold, were sub-cloned into pcDNA3.0 vector and their expression in mammalian cells was assessed by western analysis.

In order to evaluate the ability of the selected peptides to revert the abnormal phenotypes in *STHdh* mutant striatal cells we first focused our attention on the two peptides which seemed to show specificity in mutant huntingtin binding, and on CBP measurement as single cell assay. No rescue of CBP mutant phenotype was observed for both of them. Unfortunately, our transfection efficiency was not sufficient for assessing cell viability and BDNF levels.

**References**

Colas P et al. (1996), Nature 380:548-550.

Guida E, Bisso A, Fenollar-Ferrer C, Napoli M, Anselmi C, Girardini JE, Carloni P, Del Sal G (2008): *Peptide aptamers targeting mutant p53 induce apoptosis in tumor cells*. Cancer Res. 68 : 6550-6558.

## ABBREVIATIONS

| | |
|---|---|
| 3-NP | 3-nitropropionic acid |
| aa | amino acid |
| AD | Alzheimer's disease |
| ALS | amyotrophic lateral sclerosis |
| APP | amyloid precursor protein |
| AVP | arginine vasopressin |
| BDNF | brain-derived neurotrophic factor |
| CA150 | co-activator 150 |
| CAG | cytosine adenine guanine |
| CBP | (c-AMP-response-element-binding protein) binding protein |
| Cdk5 | cyclin-dependent kinase 5 |
| CHX | cycloheximide |
| CHIP | carboxy terminus of Hsp70p-interacting protein |
| CHOP | C/EBP homologous protein |
| CNS | central nervous system |
| CNTF | ciliary neurotrophic factor |
| CtBP | C-terminal-binding protein |
| DMSO | di-methyl sulfoxide |
| DRPLA | dentatorubralpallidoluysian atrophy |
| ER | endoplasmic reticulum |
| ERAD | ER activated degradation |
| FL | full-length |
| GABA | gamma-aminobutyric acid |
| GFP | green fluorescent protein |
| GPe | external segment of the globus pallidus |
| GPi | internal segment of the globus pallidus |
| GST | glutathione-S-transferase |
| TGase | transglutaminase |
| HAP1 | huntingtin-associated protein 1 |
| HD | Huntington's disease |
| Herpud1 | homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 |
| HIP1 | huntingtin-interacting protein-1 |
| HIP-14 | huntingtin-interacting protein-14 |
| Hippi | Hip-1 protein interactor |
| Hsp40 | heat shock protein 40 kDa |
| Hsp70 | heat shock protein 70 kDa |
| Hsp90 | heat shock protein 90 kDa |
| htt | huntingtin |
| IF | immunofluorescence |
| IT15 | interesting transcript 15 |
| KI | Knock-in |
| KO | Knock-out |
| MSNs | medium sized spiny neurons |

| | |
|---|---|
| N-CoR | nuclear receptor co-repressor |
| NGF | nerve growth factor |
| NII | neuronal intranuclear inclusion |
| NMDA | N-metyl-D-aspartate |
| P/CAF | p300/CBP associated factor |
| PD | Parkinson's disease |
| Pin1 | protein interacting with NIMA (never in mitosis A) 1 |
| PP2A | protein phosphatase 2 A |
| PPases | protein phosphatases |
| PPIases | peptidyl-prolyl isomerases |
| PSD-95 | postsynaptic density-95 |
| Q | glutamine |
| REST | RE1-silencing transcription factor |
| RF | RISC-Free siRNA |
| RISC | RNA-induced silencing complex |
| RRB | ribosome and rRNA biosynthesis regulon |
| Rrs1 | regulator of ribosome synthesis |
| ROS | reactive oxygen specie |
| RT | Room Temperatura |
| SBMA | spinobulbar muscular atrophy |
| SCA | spinocerebellar ataxia |
| SGK | serum- and glucocorticoid-induced kinase |
| siRNA | small interference RNA |
| Sp1 | specificity protein 1 |
| STN | subthalamic nucleus |
| TAFII130 | TATA-binding protein (TBP)-associated factor |
| TBP | TATA-box binding protein |
| UBE3A | ubiquitin protein ligase E3A |
| UPP | ubiquitin-proteasome pathway |
| UPS | ubiquitin-proteasome system |
| Wt | wild-type |
| WB | western blot |
| YAC | yeast artificial chromosome |
| YFP | yellow fluorescent protein |