Role of the MID1/α4 protein complex in Huntington's disease

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Ewa Jastrzebska

aus Wroclaw, Polen

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Ewa Jastrzebska

from Wroclaw, Poland

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1. Gutachter: Prof. Dr. Constance Scharff (Institut für Biologie, Fachbereich Biologie, Chemie, Pharmazie, Freie Universität Berlin)

2. Gutachter: Prof. Dr. Susann Schweiger (Division of Pathology and Neuroscience, University of Dundee; Max-Planck-Institut für Molekulare Genetik)

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1. Introduction

1.1 Huntington's disease (HD)

Huntington's disease is a progressive late-onset neurodegenerative disorder that follows an autosomal dominant mode of inheritance. The disorder was first described in the 19th century by George Huntington, while the underlying genetic defect was discovered in 1993. The defective gene contains an unstable expansion of the CAG repeats in the coding region of the *HD* gene. This mutation leads to an expanded polyglutamine (polyQ) stretch in the N-terminal part of the huntingtin protein (1993).

HD is the most prevalent disorder in a family of several neurodegenerative disorders that are caused by the expansion of CAG trinucleotide repeats (Table 1.1). The prevalence of HD is approximately 3-7 in 100,000 persons worldwide. It is estimated that in Germany about 2,500 persons are affected by the disease. The average age of onset of the disease typically is in the 3th to 5th life decade. (http://www.wrongdiagnosis.com/h/huntingtons_disease/stats-country.htm)

Table 1.1 Disorders with trinucleotide repeats

Disease	Repeat	Normal	Pathological	Region	Inheritance	Protein
Huntington	CAG (5')	<36	>36	ORF	AD	huntingtin
Myotone Dystrophy	CTG	5 to 30	30-80(mild), >2000(sever)	3'UTR	AD	
Friedreich Ataxia	GAA	5 to 30	70-1000	intron1	AR	frataxin
SCA1	CAG			ORF	AD	Ataxin-1
SCA2	CAG (5')	15-24	39-59	ORF	AD	Ataxin-2
SCA3(Machado-Joseph)	CAG	13-36	68-79	ORF	AD	Ataxin-3
SCA4	CAG					CACNA1A
SCA5						
SCA6						
SCA7						
SCA8						
SCA9						
SCA10						
SCA11						
SCA12						
Kennedy-Syndrom	CAG (5')	22+/-3	40-52	ORF	X-chrom.	Androgen receptor
FraX	CGG				X-chrom.	
FragileX tremor/ataxia			50-200	5'UTR	X-chrom.	
Syndaktylie	GCG, GCA, GCT, GCC	Appr. 15	Appr. 24	ORF	AD (haploinsuff.)	HoxD13
Hand-Foot-Uterus-Syndrom				ORF		HoxA13

1.1.1 Clinical features of Huntington's disease

The pathological characteristics of the Huntington's disease is the gradual loss of the striatum (caudate nucleus and putamen) (Vonsattel, Myers et al. 1985). Clinical manifestation involve progressive movement disturbance, cognitive dysfunction and psychiatric symptoms. The initial clinical symptoms usually appear in middle age and vary from person to person. First signs are mild and increase gradually during the progression of the disease (Paulsen, Zhao et al. 2001). The disease starts with involuntary movement of face, finger, feet or thorax. Chorea appears gradually with disease progress and in later stages patients become severely akinetic (Folstein, Leigh et al. 1986).

Psychiatric symptoms however can occur up to 20 years before the onset of choreatic features. They include apathy, anxiety, irritability and depression (Craufurd, Thompson et al. 2001). Cognitive dysfunction including deficits in short-memory, learning and attention also often appear. With the time those symptoms lead to the situation where patients do not recognize people, objects and places (Harper 1996; Paulsen, Zhao et al. 2001).

Weight loss is another characteristic features of HD. It might be due to dysphagia as well as to degeneration of hypothalamic neurons (Li, Yu et al. 2003; Petersen, Gil et al. 2005).

In late stages of the Huntington's disease patients present severe dementia, do not talk and finally are unable to care for themselves. HD patients usually die 10 to 20 years after the first symptoms appeared.

1.1.2 Genetics of Huntington's disease

The genetic defect, which is responsible for HD was discovered in 1993. The gene encodes for the huntingtin (HTT) protein, a ubiquitously expressed protein of 350 kDa (1993). The HD gene was mapped to human chromosome 4p at the position 4p16.3. It consists of 67 exons spanning over 200 kb.

The mutation that causes Huntington's disease is the abnormal expansion of CAG repeats within the coding sequence of the *huntingtin* gene, 17 codons downstream from the ATG start codon. Normal individuals carry between 17-20 CAG repeats. Repeats number between 27-35 are rare (3.2% of all repeats) but do not cause disease. These numbers of repeats however are meiotically unstable in parental

transmission (Myers 2004) and can therefore be the origin of a full HD causing mutation. Repeats number above 36 lead to the disease. The length of the CAG repeat stretch is inversely correlated with the disease onset, which means that patients with more CAG repeats develop first symptoms of the disease earlier than those with shorter repeat numbers (Ross 1995). This is in line with the observation that most patients with a disease onset in adulthood typically carry CAG repeats in the range between 40-55, whereas more than 70 glutamines lead to a juvenile form of HD (Vonsattel and DiFiglia 1998). Nevertheless there is a large variation in given CAG number and the age of onset. Only about 70% cases of age onset could be accounted for the CAG number, therefore the observed variation in the age of onset could be due to the other modifying genes or environmental factors (Rubinsztein, Leggo et al. 1997; Kehoe, Krawczak et al. 1999; Rosenblatt, Brinkman et al. 2001; Djousse, Knowlton et al. 2004; Wexler, Lorimer et al. 2004). Moreover, it was previously believed that HD demonstrate 'complete dominance' meaning that heterozygotes and homozygotes are as badly affected. This was contradicted by recent studies that have shown that homozygous mutation carrier might be associated with a more aggressive progress of the disease (Squitieri, Gellera et al. 2003; Maglione, Cannella et al. 2006).

1.1.3 Huntingtin (HTT)

The human *huntingtin* gene codes for a large 350 kDa, ubiquitously expressed protein called huntingtin (HTT).

Wild type huntingtin is localized to several subcellular compartments and can be found in the cytoplasm as well as in the nucleus (Harjes and Wanker 2003) (Kegel, Meloni et al. 2002). It associates with microtubules, mitochondria, the ER, plasma membrane and also with the Golgi apparatus (Kegel, Sapp et al. 2005; Caviston, Ross et al. 2007; Rockabrand, Slepko et al. 2007; Strehlow, Li et al. 2007). Highest levels of huntingtin are being found in brain and testis (DiFiglia, Sapp et al. 1995; Trottier, Devys et al. 1995).

Huntingtin protein is a multi-domain protein, which has no obvious homology with other known proteins.

The polyglutamine domain starts at amino acid position 18 and is followed by two proline – rich domains, which are required for many protein – protein interactions (Li

and Li 2004). More C-terminal in the huntingtin protein structure are **HEAT** repeat structure (named after the proteins: ***H***untingtin, ***E***longation factor 3, protein phosphatase 2***A***, ***T***OR1). Up to 37 of these motifs are distributed along the entire length of huntingtin (Takano and Gusella 2002; Li, Serpell et al. 2006). HEAT motifs are 50 aa long sequences which form a hairpin of two anti-parallel alpha helices. Those motifs are involved in protein – protein interactions and are usually found in proteins, which play a role in intracellular transport, microtubule dynamics and segregation of chromosomes. Proteins containing these motifs are characterized by a high content of helical and superhelical structures (Neuwald and Hirano 2000; Andrade, Petosa et al. 2001).

Recent biophysical methods used for the characterization of huntingtin showed that the protein is indeed an elongated superhelical solenoid with a diameter of ~200 Å (Li, Serpell et al. 2006) (Fig. 1.1 b.).

It had been shown that the huntingtin protein shuttles between the cytoplasm and the nucleus. However, while a nuclear export signal (NES) has been found within the C-terminus of the huntingtin protein, a nuclear localization signal (NLS) could not be identified. Therefore it is not really clear how the huntingtin can shuttle between nucleus and cytoplasm. N-terminal fragments of huntingtin, although they have neither nuclear import nor nuclear export signal can still accumulate in the nucleus. It has been shown that these fragments interact with the nuclear exporter (Tpr) therefore suggesting that they themselves act as a NES (Cornett, Cao et al. 2005). It is also possible that small N-terminal huntingtin fragments diffuse through the membrane into the nucleus (Hackam, Singaraja et al. 1998; Xia, Lee et al. 2003).

Furthermore, the first 17 aa at the very N-terminus of the huntingtin protein together with a cluster of the first three HEAT repeats has been shown to play an important role in targeting huntingtin to different cellular compartments including plasma membrane, endosomal/autophagic vesicles, mitochondria, the Golgi apparatus and the ER (Kegel, Sapp et al. 2005; Atwal, Xia et al. 2007; Rockabrand, Slepko et al. 2007).

Lysine residues located within the first 17 aa, just before the polyglutamine repeat stretch, undergo post translation modification like SUMOylation or ubiquitination which could regulate the localization, nuclear export and the half life of the wild type

huntingtin in the cells (Kalchman, Graham et al. 1996; Dohmen 2004; Steffan, Agrawal et al. 2004) (Fig. 1.1 a.).

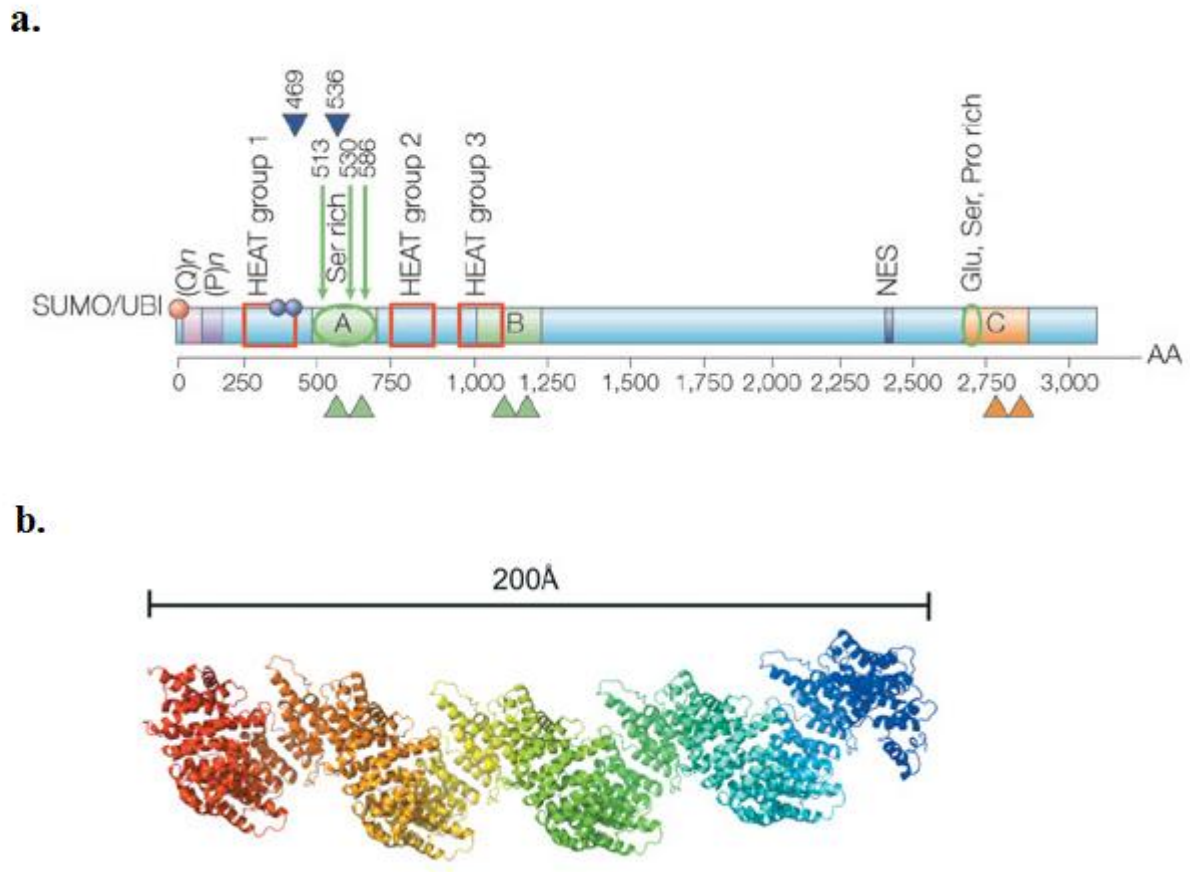

Fig. 1.1 Huntingtin protein.

A. Domain structure of the huntingtin protein.'$(Q)_n$ indicates the polyglutamine tract, which is followed by a polyproline sequence, $(P)_n$, and the red squares indicate the three main clusters of HEAT repeats. The arrows indicate the caspase cleavage sites and their amino acid positions, and the blue arrowheads the calpain cleavage sites and their amino acid position. B identifies the regions cleaved preferentially in the cerebral cortex, C indicates those cleaved mainly in the striatum, and A indicates regions cleaved in both. Green and orange arrowheads point to the approximate amino acid regions for protease cleavage. NES is the nuclear export signal. The red and blue circles indicate post-translational modifications: ubiquitination (UBI) and/or sumoylation (SUMO) (red), and phosphorylation at serine 421 and serine 434 (blue). The glutamic acid (Glu)-, serine (Ser)- and proline (Pro)-rich regions are indicated (serine-rich regions encircled in green)' (Cattaneo, Zuccato et al. 2005).

B. Possible structure of the huntingtin as an elongated solenoid. The structure was modeled on the other HEAT repeat protein that has a similar molecular mass as huntingtin (Takano and Gusella 2002)

1.1.3.1 Proposed function of wild type huntingtin

The cellular functions of the wild type huntingtin are poorly defined. This is mostly due to the large size of huntingtin and the lack of any obvious homology to other known proteins.

A powerful tool to characterize and study gene function is its inactivation in living organisms. Using this strategy a role of huntingtin in embryonic development has been proposed as the loss of protein caused an increased apoptosis rate and led to disruption in nutrients transport to the fetus causing death of mouse embryos on day 8.5 (Duyao, Auerbach et al. 1995; Nasir, Floresco et al. 1995; Zeitlin, Liu et al. 1995). In addition inactivation of the huntingtin orthologue in mouse brain and testes caused degeneration of those organs which may suggest a role for huntingtin in cell survival (Dragatsis, Levine et al. 2000). Recent studies in zebrafish showed that knockdown of huntingtin causes a variety of developmental defects (Lumsden, Henshall et al. 2007). Furthermore, several of the genetic and biochemical experimental evidences suggest, that the wild type huntingtin can act as an anti apoptotic protein. This idea is supported by the observation that overexpression of the wild type huntingtin protects against apoptotic insults including those due to mitochondrial toxins, starvation and also overexpression of mutant huntingtin (Rigamonti, Bauer et al. 2000; Ho, Brown et al. 2001; Rigamonti, Sipione et al. 2001). One explanation for the anti apoptotic role of the huntingtin protein is its inhibitory activity towards several apoptotic proteins including caspase 3, 8 and 9 (Rigamonti, Sipione et al. 2001; Gervais, Singaraja et al. 2002; Zhang, Leavitt et al. 2006). Furthermore, wild type huntingtin can also upregulate the transcription rate of BDNF (brain-derived neurotrophic factor), which is important for the survival of striatal neurons (Zuccato, Ciammola et al. 2001).

Huntingtin has also been shown to be involved in transcriptional regulation. It interacts with transcriptional factors and proteins which are connected to transcription (Harjes and Wanker 2003; Li and Li 2004; Kaltenbach, Romero et al. 2007).
Huntingtin has also been shown to interact with proteins containing multi-tryptophan (WW) domains, which implicates its involvement in pre-mRNA splicing (Faber, Barnes et al. 1998). Based on this and on the analogy to other HEAT proteins huntingtin has been proposed to play a role in nucleo-cytoplasmatic shuttling of

mRNA and transcriptional regulators (Takano and Gusella 2002; Xia, Lee et al. 2003; Truant, Atwal et al. 2007).

A very well known example for huntingtin functioning as a regulator of transcription is a role in controlling BDNF production.

Wild type huntingtin has also been shown to be involved in vesicle trafficking which was proposed based on its localization to the endosomal/endocytic vesicles in axons and synaptic terminals. Furthermore huntingtin interacts with a number of trafficking proteins including: α-adaptin, Hip1, Hap1, PACSIN1 and SH3GL3 (Sittler, Walter et al. 1998; Rong, Li et al. 2007). Recently these interactions of huntingtin has been extended also to endocytic proteins like dynamin or clathrin (Kaltenbach, Romero et al. 2007).

Wild type huntingtin has a well established role in long- and short-distance transport along microtubules. This role is well documented in *Drosophila* and mouse models as well as in mammalian cells (Gunawardena, Her et al. 2003; Gauthier, Charrin et al. 2004; Caviston, Ross et al. 2007). In *Drosophila* reduction of huntingtin levels cause axonal transport defects and neurodegeneration in the eye (Gunawardena, Her et al. 2003). In addition huntingtin through binding with Hap1 interacts with the dynein/dynactin motor complex, which is responsible for the retrograde transport in cells. Moreover Hap1 is also able to bind to the anterograde axonal transporter kinesin. Huntingtin has been shown to directly promote BDNF microtubule-based transport through the interaction with Hap1. Any changes in huntingtin levels show respective changes in intracellular transport of BDNF (Gauthier, Charrin et al. 2004).

Effective strategy for deciphering the function of not well characterized protein like huntingtin include the identification of interacting proteins. Recently, together with the development of new technologies, high throughput approaches have been used to identify huntingtin interacting partners. Studies of Kaltenbach and colleagues led to identification of 234 interacting partners of the huntingtin protein. They could be grouped into distinct functional categories: signal transduction, synaptic transmission, cytoskeletal organization, proteolysis as well as transcription and translation regulation (Kaltenbach, Romero et al. 2007; Li, Friedman et al. 2007).

It seems that the huntingtin protein is involved in many subcellular processes and therefore functions as a scaffold protein. HD pathology could therefore be a result of

an alteration of a variety of network proteins and signalling pathways (Harjes and Wanker 2003).

1.1.4 Pathogenesis of HD

1.1.4.1 Pathomechanisms of CAG-repeat disorder

Based on current evidence, it is believed that mutant huntingtin with an expanded polyglutamine tractus undergoes conformational changes and then lead to a toxic gain of function. The toxic gain of function could be due either to the over-activity of mutant huntingtin or to functions of mutated huntingtin, which are different from the normal biological activity of huntingtin. Thus, it had been shown that mutant huntingtin containing expanded glutamine tracts interacted with other proteins or sequester them into aggregates influencing the activity of some key proteins and therefore several intracellular pathways (Fig.1.2) (Kahlem, Terre et al. 1996).

Gain of function on the RNA level is the one has also been proposed for CAG expansion disorders. Expanded CAG repeats can form a double-stranded RNA structure, stability of which increases with the number of repeats (Sobczak and Krzyzosiak 2005). Several proteins have been shown to bind to such structures. Such complexes are thought to be pathological as they influence RNA metabolism in the cell. In the nucleus they interfere with the RNA splicing machinery and transcription and in the cytoplasm they may interfere with RNA processing and translation (Ranum and Cooper 2006). Furthermore, RNA-binding proteins might interact with the huntingtin RNA and influence the amount of protein produced as well as affecting the localization of the RNA in the cell (McLaughlin, Spencer et al. 1996).
Several transcription factors have been showed to interact with mutant huntingtin due to the presence of the CAG repeats e.g. TBP (Schaffar, Breuer et al. 2004), CREB (Steffan, Kazantsev et al. 2000; Schaffar, Breuer et al. 2004) and Sp1 (Li, Cheng et al. 2002). In the presence of mutant huntingtin, CBP (a co- activator of the CRE-mediated transcription) is recruited into the aggregates, which decreases its active amount in the cell and leads to reduction of CBP mediated transcription. Likewise, mutant huntingtin might also not be able anymore to bind some of the transcription factors, which normally interact with wild type huntingtin thereby also leading to a loss of function. This holds true for NRSE-binding transcription factors. BDNF (brain-derived neurotrophic factor) expression is regulated by REST/NRSF which is able to

recognize and bind to the NRSE. It has been shown that wild type huntingtin bind and sequester cytosolic REST/NRSF keeping it away from the nucleus and therefore allowing BDNF transcription, whereas mutant huntingtin fails to bind and interact with REST/NRSF in the cytosol, thereby increasing its levels in the nucleus and resulting in the suppression of NRSE-sensitive genes like e.g. *bdnf* gene (Schoenherr and Anderson 1995; Zuccato, Tartari et al. 2003).

The extended polyglutamine stretch can also result in huntingtin misfolding. It has been previously shown that heat-shock proteins such as HSP40 and HSP70 that are involved in refolding of misfolded proteins are being found in huntingtin aggregates (Wyttenbach, Sauvageot et al. 2002). Presence of such a proteins in the aggregates may suggest the attempts of the cell to refold the mutant huntingtin. By being sequestered into the aggregates, chaperones with the time will lose their normal protective function in the cell, which finally enhances the accumulation of misfolded protein (Ho, Carmichael et al. 2001). Furthermore, several components of the proteasome are also being caught in huntingtin aggregates (Jana, Zemskov et al. 2001; Wyttenbach, Sauvageot et al. 2002), which results in the impairment of the proteasome system (Bence, Sampat et al. 2001). Mutant huntingtin protein with extended polyglutamine tracts may also physically block the proteasome and hinder the entrance of substrates into the proteasome complex. As a consequence, with the time cells are depleted of functional proteasomes, which also leads to the progressive accumulation of abnormal and misfolded proteins (Jana, Zemskov et al. 2001).

Disruption of axonal transport is another important feature connected with CAG-repeat disorders. As mentioned above, normal huntingtin plays a role in axonal transport through the interaction with HAP1 (McGuire, Rong et al. 2006) therefore promoting retro- and anterograde axonal transport. It had been shown that mutant huntingtin may inhibit this interaction therefore blocking bidirectional transport within the axon. Mutant huntingtin seems to alter the wild type huntingtin/HAP1 complex by stronger interaction with HAP1 leading to weakness of the association between microtubules and the motor proteins and result in attenuated transport (Gauthier, Charrin et al. 2004). Several studies have confirmed that the N-terminal huntingtin with expanded glutamine stretches cause axonal transport defects in *Drosophila* and

cellular models of HD (Gunawardena, Her et al. 2003; Szebenyi, Morfini et al. 2003; Lee, Yoshihara et al. 2004).

Taken all together (Fig. 1.2), the intracellular dysfunction caused by mutant huntingtin containing expanded polyglutamine tracts unavoidably leads to neuronal dysfunction and cell loss in HD.

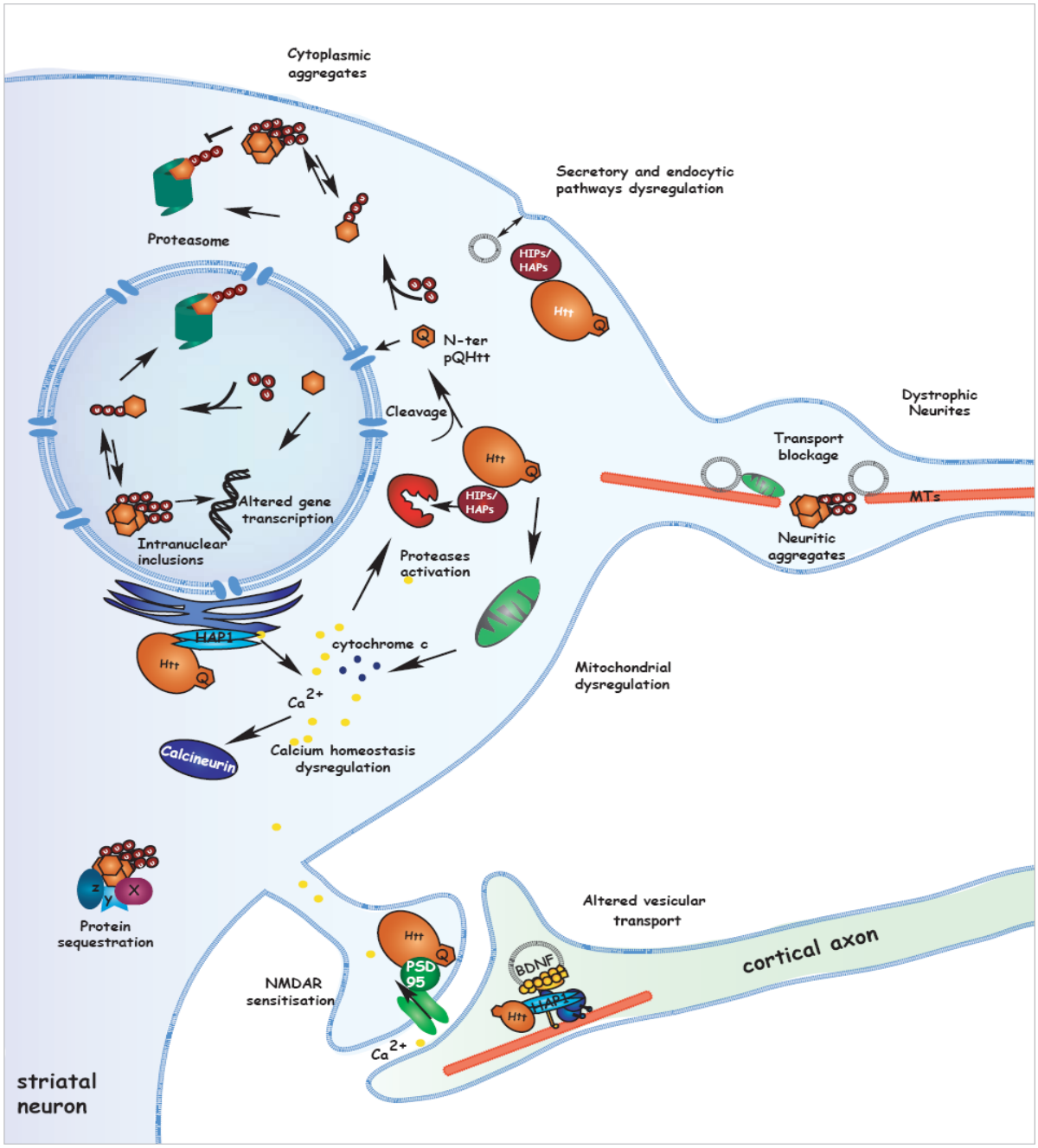

Fig. 1.2 Involvement of mutant huntingtin in different cellular functions.

Mutant huntingtin causes intracellular dysfunction and leads to different defects within the cell (Borrell-Pages, Zala et al. 2006).

1.1.4.2 Huntingtin cleavage

It had been shown that full length huntingtin is being cleaved into smaller N-terminal fragments of different length. Furthermore, there is strong evidence existing that these N-terminal fragments containing expanded polyglutamine tracts are responsible for the pathogenesis of the HD. It is important to notice that N-terminal fragments of mutant huntingtin are sufficient to obtain HD-like phenotype in animal models (Davies, Turmaine et al. 1997; Schilling, Becher et al. 1999; Palfi, Brouillet et al. 2007).

Several proteases have been reported to cleave mutant huntingtin both *in vitro* and *in vivo* producing fragments of corresponding sizes, which could be found in patient's brains as well as in HD animal models (Mende-Mueller, Toneff et al. 2001).

The known proteases found to cleave huntingtin include: caspases -1, -2, -3, -6, -7 and -8, calpains and not yet identified aspartyl proteases (Fig. 1.1 a.) (Goldberg, Nicholson et al. 1996; Lunkes, Lindenberg et al. 2002; Gafni, Hermel et al. 2004; Hermel, Gafni et al. 2004).

Two cleavage sites for caspase 3 were identified at aa positions 513 and 552. This cleavage produces N-terminal fragments of 70 and 75 kDa (Wellington, Ellerby et al. 1998). Position 552 can also be recognized by caspase 2 (Hermel, Gafni et al. 2004), a caspase 6 cleavage site was found at position 586 producing a product of 80 kDa.

As mentioned above, apart from caspases, calpains also take part in mutant huntingtin cleavage. One of the calpain cleavage sites has been identified at position 536 producing an intermediate product, which can be further cleaved to generate a 47 kDa fragment (Gafni and Ellerby 2002). Furthermore mutant huntingtin can also be cleaved by not yet identified aspartic endopeptidases, which target fragments produced by by caspases and calpains thereby generating even smaller fragments (Lunkes, Lindenberg et al. 2002).

Cleavage events of the mutant huntingtin protein, however into small N-terminal fragments might be a rate-limiting steps in the pathogenesis of the HD.

1.1.4.3 Huntingtin aggregation and its role in neurodegeneration

Although it is very well established that mutant huntingtin forms intranuclear and cytoplasmic aggregates (DiFiglia, Sapp et al. 1997), there is still a debate whether aggregates are toxic or rather play a protective role.

In several mammalian models a strong correlation between aggregation and cell death has been observed (Hackam, Singaraja et al. 1998; Lunkes and Mandel 1998; Wyttenbach, Carmichael et al. 2000).

Expression of mutant huntingtin exon1 in mouse models revealed that aggregates are strongly correlated with the onset of the behavioral changes (Davies, Turmaine et al. 1997; Morton, Lagan et al. 2000). In human brain, the density of huntingtin aggregates correlates with the polyglutamine repeat length (DiFiglia, Sapp et al. 1997).

Several mechanisms of aggregate pathology have been developed: (i) As described previously (see section 1.1.4.1) aggregates were found to be not only ubiquitinated but also associated with proteasome components, which supports the hypothesis of Ubiquitin-Proteasome System (UPS) impairment leading to neuronal degradation (Ciechanover and Brundin 2003). (ii) Chaperone proteins are sequestered in aggregates (Wyttenbach, Carmichael et al. 2000) leading to the reduction of the amount of active soluble chaperones in the cell, thereby increasing protein misfolding and mutant huntingtin mediated toxicity. This is confirmed by the observation that overexpression of some HSP proteins has a protective effect against cell death in HD cellular and/or mouse models (Muchowski, Schaffar et al. 2000; Miller, Zhou et al. 2005). (iii) Another potential mechanism of aggregate toxicity might be sequestration of different transcription factors, leading to the perturbation of several vital functions (Landles and Bates 2004).

On the other hand many observations exist describing a neuroprotective role for huntingtin aggregates. Huntingtin inclusions might represent the side-effect of the continuous cell dysfunction or even may play a protective role against polyglutamine-induced neurotoxicity (Kuemmerle, Gutekunst et al. 1999). It has been shown that the R6/2 mouse model displays prominent intranuclear inclusions, but show only little evidence of neuronal cell death (Davies, Turmaine et al. 1997). Moreover, removal of transglutaminase (TG2) reduces neuronal cell death but increases the number of intranuclear aggregates (Mastroberardino, Iannicola et al. 2002). Primary rat striatal

neurons transfected with mutant huntingtin and treated with neurotrophic factors like BDNF (brain-derived neurotrophic factor) exhibit a significant increase of mutant huntingtin aggregates formation and neuroprotection against apoptosis (Saudou, Finkbeiner et al. 1998). Furthermore, a striking dissociation between aggregation and toxicity was observed in cell model systems. Cells containing aggregates showed improved rate of survival in comparison to cells which did not form aggregates (Arrasate, Mitra et al. 2004).

1.1.4.4 Degradation of mutant huntingtin

Cellular metabolism is regulated by the control of the concentrations of specific proteins within the cell. Such regulation might occur on different levels e.g. control of protein synthesis but also by the control of protein degradation (Forster and Hill 2003). Within the cell, proteins are mostly degraded by targeting them to lysosomes or by the Ubiquitin –Proteasome System (UPS) (Hershko and Ciechanover 1998) (Fig.1.3).

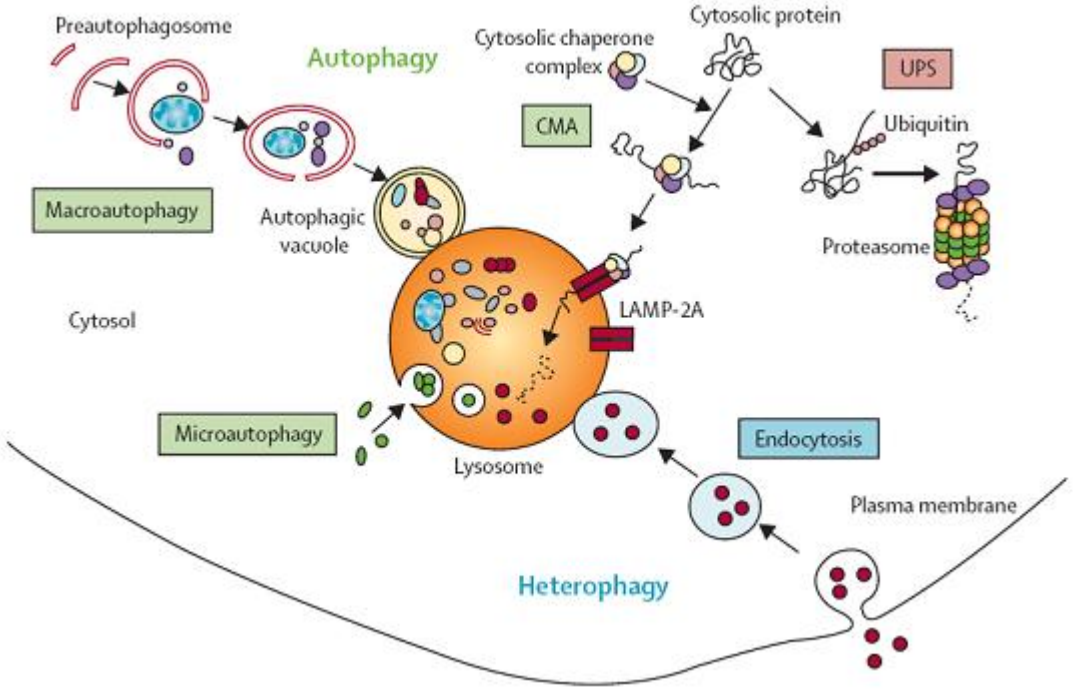

Fig. 1.3 Autophagy and UPS – protein degradation

http://www.mdc-berlin.de/en/research/research_teams/proteomics_and_molecular_mechanisms_of_neurodegenerative_diseases/research/research2/index.html

1.1.4.4.1 Ubiquitin – Proteasome System (UPS)

The ubiquitin-proteasome system (UPS) consists of many components and targets numerous cellular proteins for degradation. Additionally, modifications by ubiquitin-like proteins as well as proteins containing ubiquitin-interacting motifs are able to modulate many others. This highly controlled process involves multiple specific and general enzymes of the system as well as many modifying proteins. Thus, it is not surprising that ubiquitin mediated degradation regulates a broad spectrum of basic cellular processes. Proteasome degradation is of particular importance in protecting cells against harsh conditions (heat shock or oxidative stress) as well as in a variety of diseases (the major neurodegenerative diseases) (Ciechanover and Brundin 2003; Goldberg 2003).

It has been suggested that the UPS is impaired in HD and that it could contribute to the development of the disease. This statement is however very controversial. Many data have been obtained in many different model systems using many different approaches. For example, there are many reports suggesting that in the presence of mutant huntingtin, UPS activity decreases (Bence, Sampat et al. 2001; Jana, Zemskov et al. 2001), while some show no activity change (Ding, Lewis et al. 2002; Bowman, Yoo et al. 2005) or even increase of UPS activity (Diaz-Hernandez, Hernandez et al. 2003; Bett, Goellner et al. 2006).

The hypothesis that the UPS is impaired in HD came from a series of experiments showing that polyglutamine aggregates can be labelled with antibodies against ubiquitin or/and proteasome components, not only in cell models (Cummings, Mancini et al. 1998; Wyttenbach, Carmichael et al. 2000) but also in mouse models and human post-mortem samples (Davies, Turmaine et al. 1997; DiFiglia, Sapp et al. 1997). These experiments suggested that UPS components might be sequestered in huntingtin aggregates and therefore lead to the alteration of proteasome activity.

Other studies suggesting the impairment of the UPS have shown, both in cell models and *in vitro*, that proteins containing expanded polyglutamine stretch can be difficult to be degraded for the eukaryotic proteasome because of its unfolded nature (Holmberg, Staniszewski et al. 2004; Venkatraman, Wetzel et al. 2004).

It has been also reported that the proteasome cannot cleave between glutamines in the polyglutamine stretch. Binding to such a stretch would therefore lead to the blockage of the proteasomal binding domains for other substrates. Moreover,

investigation of purified fibrils from the HD post-mortem brains and from HD mouse models have revealed that those species can decrease proteasome activity *in vitro* (Diaz-Hernandez, Valera et al. 2006).

Other studies however suggest that the UPS is not impaired in HD, like studies in SH-SY5Y cells, where no difference in degradation between mutant and wild type huntingtin has been found (Ding, Lewis et al. 2002). It therefore remains unclear whether the UPS system is impaired in HD.

1.1.4.4.2 Autophagy

Autophagy is a degradation pathway used for degradation of cell components as well as various aggregate-prone proteins e.g. mutant huntingtin.

Autophagy is a highly regulated process that plays an important role in maintaining a balance within the cell between protein synthesis, degradation and cell product recycling. In contrast to the UPS, which is responsible for degradation of the short-lived proteins, autophagy degradation involves long-lived proteins, protein complexes and organelles (Rubinsztein 2006).

As mentioned above the possibility of degradation of protein oligomers as well as organelles made autophagy to be considered as a clearing process for aggregated proteins like e.g. mutant huntingtin protein (Sarkar, Perlstein et al. 2007; Sarkar and Rubinsztein 2008).

It has been recently shown that autophagy plays a protective role in neurodegeneration based on the results showing that loss of autophagy leads to neurodegeneration of neuronal cells in mouse model (Hara, Nakamura et al. 2006; Komatsu, Waguri et al. 2006). Therefore induction of autophagy might be a possible therapeutic strategy in neurodegenerative disorders.

In HD, a positive correlation has been found between the length of the polyglutamine stretch and the number of autophagic vacuoles in a cell (Nagata, Sawa et al. 2004). Furthermore, some data suggest that mutant huntingtin expression induces endosomal/lysosomal activity (Kegel, Kim et al. 2000) and that mTOR (mammalian target of rapamycin), a kinase also involved in autophagy regulation, is being sequestered into mutant huntingtin aggregates. This would result in inhibition of the kinase activity, therefore promoting autophagy (Ravikumar, Vacher et al. 2004) to clear huntingtin aggregates (Qin, Wang et al. 2003; Ravikumar, Vacher et al. 2004).

During the past few years various substances have been used to induce autophagy in mammalian systems. It has been recently shown that rapamycin (Berger, Ravikumar et al. 2006) and few other small-molecule enhancers of rapamycin (Floto, Sarkar et al. 2007; Sarkar, Perlstein et al. 2007), which could act downstream or independently of mTOR, induce clearance of mutant huntingtin fragments in different HD models.

1.2 mTOR

The mammalian target of rapamycin (mTOR) is a central regulator of protein synthesis, ribosome biogenesis and cell growth (Schmelzle and Hall 2000).

The mTOR protein, also known as FKBP12-rapamycin associated protein (FRAP) or rapamycin target (RAPT) has been identified as a 289 kDa serine/threonine kinase (Sabatini, Erdjument-Bromage et al. 1994; Sabers, Martin et al. 1995). It is a highly evolutionarily conserved protein which belongs to the phosphatidylinositol kinase-related protein kinase (PIKK) family (Schmelzle and Hall 2000). The C-terminal region contains a catalytic kinase domain which is located between the FRB (FKBP12-rapamycin binding) domain and the repressor domain (Schmelzle and Hall 2000; Sekulic, Hudson et al. 2000). The N-terminal part of mTOR consists of up to 20 HEAT motifs which are important for the establishment of protein-protein interactions (Andrade and Bork 1995). Other motifs, which have been found within the mTOR protein, are called FAT (FRAP-ATM-TRAPP) and FATC (FAT C-terminus). They also probably play a role in the formation of multiprotein complexes (Keith and Schreiber 1995; Bosotti, Isacchi et al. 2000).

So far mTOR has been found in two different multiprotein complexes (Fig. 1.4) from which only one can bind to FKB12-rapamycin. This complex (mTORC1), activity of which can be inhibited by rapamycin, is composed of mTOR, GβL and raptor protein (Hara, Maruki et al. 2002; Loewith, Jacinto et al. 2002; Kim, Sarbassov et al. 2003). While being similar in its protein composition, the rapamycin-insensitive mTOR complex (mTORC2) contains rictor instead of raptor (Jacinto, Loewith et al. 2004; Sarbassov, Ali et al. 2004).

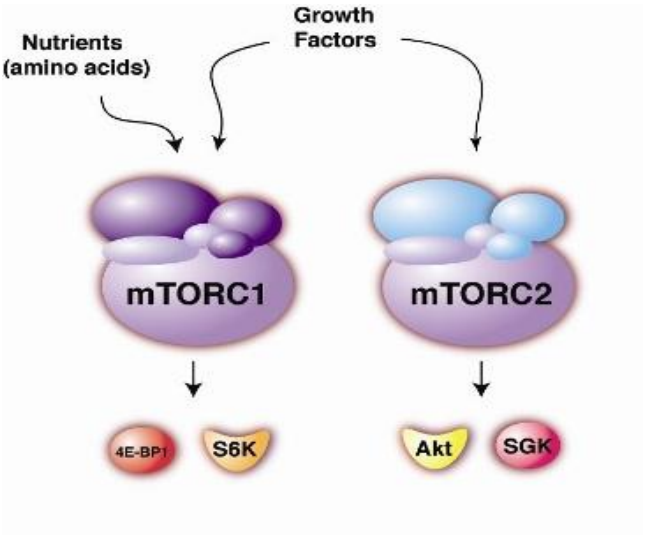

Fig. 1.4 mTOR complexes.
Schematic representation of two mTOR complexes and their downstream targets. www.umassmed.edu/faculty/show.cfm?faculty=1342

1.2.1 mTOR signalling and PP2A

mTOR kinase is a member of the PI3K pathway, activated by growth hormones, nutrients or insulin. In response to those substances it induces the translation machinery through the inhibition of the translation inhibitory eIF-4E binding protein (4E-BP1) (Schmelzle and Hall 2000) and activation of the p70 ribosomal S6 kinase (p70S6K) (Gingras, Raught et al. 2001) (Fig. 1.5).

4E-BP1, one of the main mTOR substrates is a translation inhibitor which in the dephosphorylated status binds and inhibits the translation initiation factor eIF4E. In response to nutrients 4E-BP1 is being phosphorylated by the mTOR kinase and therefore inactivated. Phosphorylation of 4E-BP1 leads to the release of eIF4E, which can then bind the capped mRNAs and promote protein translation (Schmelzle and Hall 2000).

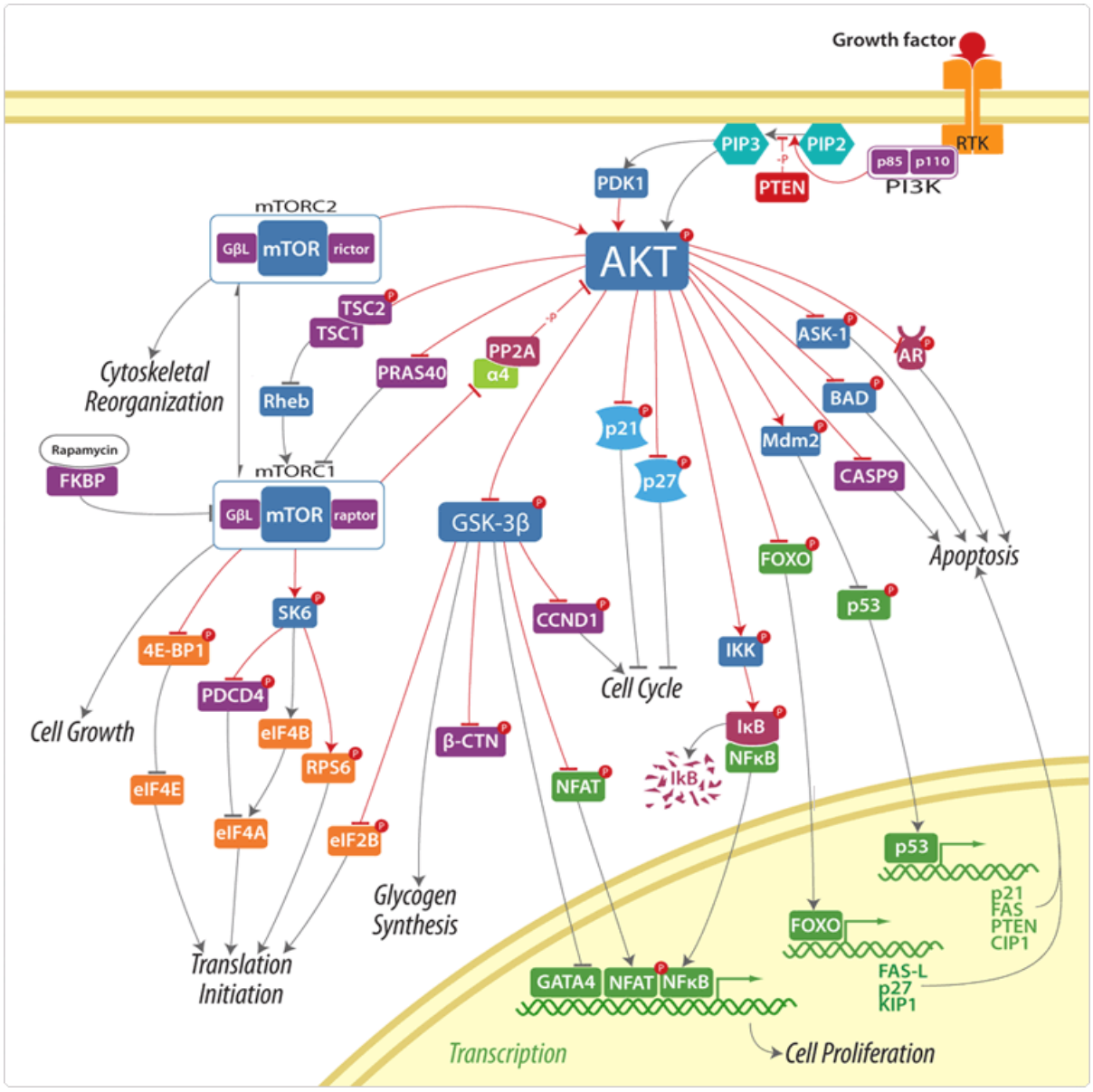

Fig. 1.5 PI3K/AKT/mTOR signalling pathways.

Schematic summary of upstream and downstream proteins of the mTOR pathway and other pathways in which mTOR is involved (www. invitrogen.com).

Another very important target of mTOR is the p70S6 Kinase. Upon phosphorylation by mTOR, this kinase is activated and phosphorylates the ribosomal S6 protein, which results in the activation of the translation machinery (Meyuhas 2000; Ruvinsky, Sharon et al. 2005). Furthermore, p70S6K is able to stimulate protein translation by the phosphorylation of other substrates involved in protein synthesis including the translation initiation factor eIF4B (Ruvinsky and Meyuhas 2006).

The counteracting phosphatase of mTOR on all the mentioned targets is protein phosphatase 2A (PP2A). PP2A in mammals is composed of a core dimer consisting of a 39 kDa catalytic subunit ($PP2A_c$) and a 65 kDa regulatory A-subunit. This core can further associate with a third regulatory B-subunit (Cohen 1990; Mayer-Jaekel and Hemmings 1994).

PP2A has been reported to be phosphorylated *in vitro* by the mTOR kinase, which leads to its inhibition, while rapamycin treatment has been shown to increase PP2A activity *in vivo* (Peterson, Desai et al. 1999). Interestingly *in vitro* studies have revealed that association between a regulatory protein complex including the mammalian α4 protein or its yeast orthologue Tap42 and $PP2A_c$ affects the activity of the $PP2A_c$. While $PP2A_c$ alone is able to dephosphorylate the mTOR substrate 4E-BP1, in complex with α4 or Tap42 respectively its activity towards this substrate is inhibited. It has been shown that association of α4 with $PP2A_c$ is blocked by rapamycin (Murata, Wu et al. 1997). However controversial data exist, showing that treatment with rapamycin not only does not restore $PP2A_c$ activity but also fails to dissociate the complex (Nanahoshi, Nishiuma et al. 1998). This data has been supported by results from another group, which confirmed a rapamycin-insensitive association between α4 and $PP2A_c$ as well as other two PP2A-related phosphatases: PP4 and PP6 in mammalian cells (Chen, Peterson et al. 1998).

1.2.2 mTOR and neurodegeneration – role of rapamycin

Several studies in the past few years provided a better understanding of the translation machinery and the upstream signalling modulating translation, as well as new insight into mechanisms by which nutrients and growth factors control the protein synthesis in the cells.

Strikingly, defects in the proper control of mRNAs translation result in many diseases including neurodegenerative disorders (reviewed in (Proud 2007)).

As described above the mTOR protein combines many different signals that at the end lead to the regulation of various growth related processes e.g. ribosome biogenesis, transcription, translation as well as autophagy (Wullschleger, Loewith et al. 2006).

Pathways that control autophagy play important roles in the pathogenesis of neurodegenerative diseases including Alzheimer's, Parkinson's or HD, which are very often connected with protein misfolding and protein accumulation - aggregation.
It has been reported previously that mTOR is being sequestered in polyglutamine aggregates in cell systems, mouse models as well as in human brains. Sequestration of mTOR seems to lead to inhibition of its kinase activity, and thereby directly to the activation of one of the key clearance pathways – autophagy and the reduction of polyglutamine toxicity (Ravikumar, Vacher et al. 2004) (section 1.1.4.4.2). In support of that, it has been shown, that rapamycin has a similar effect as the mTOR sequestration on polyglutamine toxicity. Thus, specific inhibition of mTOR kinase activity by rapamycin has been reported to decrease accumulation of mutant huntingtin in cell and fly models of HD (Ravikumar, Vacher et al. 2004; Berger, Ravikumar et al. 2006).

Rapamycin is a well characterized macrolytic lactone with antibiotic and antifungal properties, which was first isolated in the 70's from *Streptomyces hygroscopicus*. Intensive studies on rapamycin led to the identification of the mTOR kinase and revealed its growth and proliferation inhibitory function (Pallet, Beaune et al. 2006). As mentioned above, rapamycin is also a well characterized drug activating autophagy (Rubinsztein 2006).
Interestingly, recent data by Wyttenbach and colleagues suggest that rapamycin effects on the aggregation process of proteins with expanded polyglutamine stretches might be independent of autophagy and could be modulated via other pathways (King, Hands et al. 2008; Wyttenbach, Hands et al. 2008). It has also been discussed that the inhibition of mTOR and the subsequent decrease of overall protein synthesis could contribute to some of the HD symptoms like memory loss, due to a reduction of the synthesis of essential proteins at the synapses (Tang, Reis et al. 2002).

1.3 MID1

1.3.1 MID1 protein and its function

The *MID1* gene, located on the X chromosome at the position Xp22 encodes the 667 amino acid phosphoprotein MID1 with a size of approximately 72 kDa. MID1 belongs to the tripartite motif (TRIM) family, also known as the RBCC (RING-B-box-

Coiled-coil) family of zinc binding RING finger proteins. The N-terminal part of MID1 consists of four domains including the RING finger domain, two B-boxes and a coiled-coil domain. The C-terminal part of MID1 contains a COS (C-terminal subgroup one signature) domain, a fibronectin III (FNIII) domain and a B30.2-like domain (Fig. 1.6) (Quaderi, Schweiger et al. 1997; Schweiger and Schneider 2003).

Proteins which belong to the RBCC or TRIM protein family are known to be involved in many processes including scaffolding, protein-protein interactions, formation of macromolecular complexes or they play a role as ubiquitin ligases (Quaderi, Schweiger et al. 1997; Jensen, Shiels et al. 2001).

The RING finger structure in MID1 protein is joined to two B-boxes via a linker region of about 45 aa. The two B-boxes are histidine- and cysteine-rich motifs (Torok and Etkin 2001). Similar to RING finger domains, it is believed that B-boxes are involved in protein-protein interactions and serve as molecular scaffolds in large protein complexes. This is also true for the B-box1 in MID1, which has been shown to be essential and sufficient for the binding of α4 protein to MID1. It was the first specific function of this domain to be reported (Trockenbacher, Suckow et al. 2001; Short, Hopwood et al. 2002). B-box2 domain has been suggested to regulate the binding of B-box1 to α4 (Schweiger, Foerster et al. 1999; Short, Hopwood et al. 2002).

C-terminally located to the B-box2 is a coiled-coil region. Coiled-coil domains are formed by bundles of α-helices which create super coil or super helix. The α- helical coiled-coil domain is one of the principal subunit oligomerizing protein-protein interaction motifs and is, therefore, involved in diverse processes like: transcription, membrane fusion and chromosome segregation (Burkhard, Stetefeld et al. 2001). In the case of MID1, the coiled-coil region was found to be responsible for the formation of homo- and heteromeric complexes (Cainarca, Messali et al. 1999; Trockenbacher, Suckow et al. 2001), which are formed with the close homologue MID2 (Short, Hopwood et al. 2002). Moreover, the coiled-coil domain has been shown to be responsible for homo- and heterodimerization of MID1, which allows the binding of MID1 to microtubules (Cainarca, Messali et al. 1999; Short, Hopwood et al. 2002). MID1 homo- and heterodimerization has been confirmed by finding MID1 in a large macromolecular complexes (Cainarca, Messali et al. 1999).

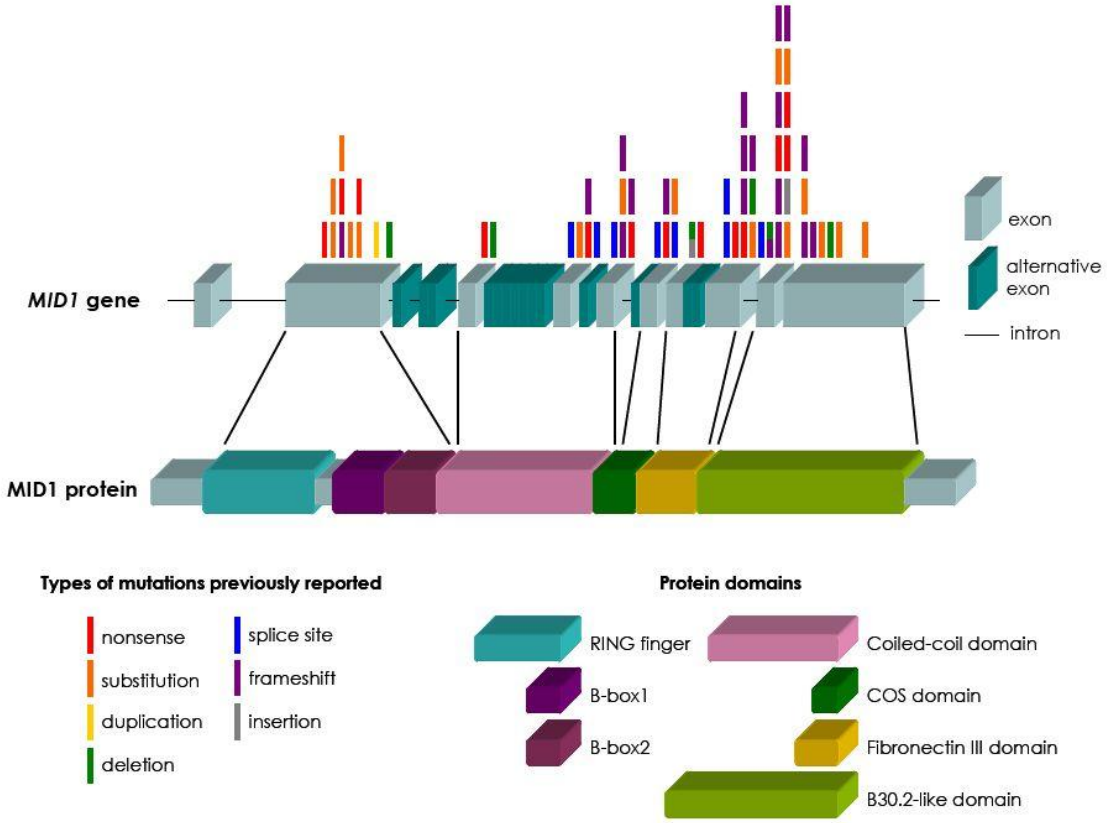

Fig. 1.6 MID1 gene and protein.

Schematic representation of the MID1 gene and protein. Exons coding for each domain are indicated as well as all identified mutations in the gene. Mutations reported in: (Quaderi, Schweiger et al. 1997; Gaudenz, Roessler et al. 1998; Schweiger, Foerster et al. 1999; Cox, Allen et al. 2000; De Falco, Cainarca et al. 2003; So, Suckow et al. 2005). Figure adopted from Schweiger and Schneider 2003, taken from (So 2008)

The function of the FNIII and B30.2-like domains in the C-terminus of MID1 have still to be further elucidated. However, it has been shown that the C-terminus of MID1 plays an important role in the localization of the protein to the cytoskeleton (Quaderi, Schweiger et al. 1997; Henry, Mather et al. 1998; Schweiger, Foerster et al. 1999). FNIII domains have been found in various proteins and are thought to be responsible for the protein-protein interactions (Perry, Short et al. 1999).

Apart from a microtubule stabilizing role (Schweiger, Foerster et al. 1999), it was previously shown that MID1 interacts with the α4 protein through its Bbox1 domain (Trockenbacher, Suckow et al. 2001). α4 binds and negatively regulates the catalytic subunit of phosphatase 2A ($PP2A_c$) (Figure 1.7) (Murata, Wu et al. 1997; Chen, Peterson et al. 1998). This mechanism was first observed in yeast, where

Tap42 (yeast homologue of α4) regulates PP2Ac upon Target of rapamycin (TOR) signalling (see section 1.2.1) (Nanahoshi, Tsujishita et al. 1999; Raught, Gingras et al. 2001). α4 is a cytosolic protein, which in association with MID1 is recruited to microtubules. An excess of α4 can detach MID1 from microtubules and bring it to the cytosol, indicating that both proteins interact and collaborate in determining their subcellular localization.

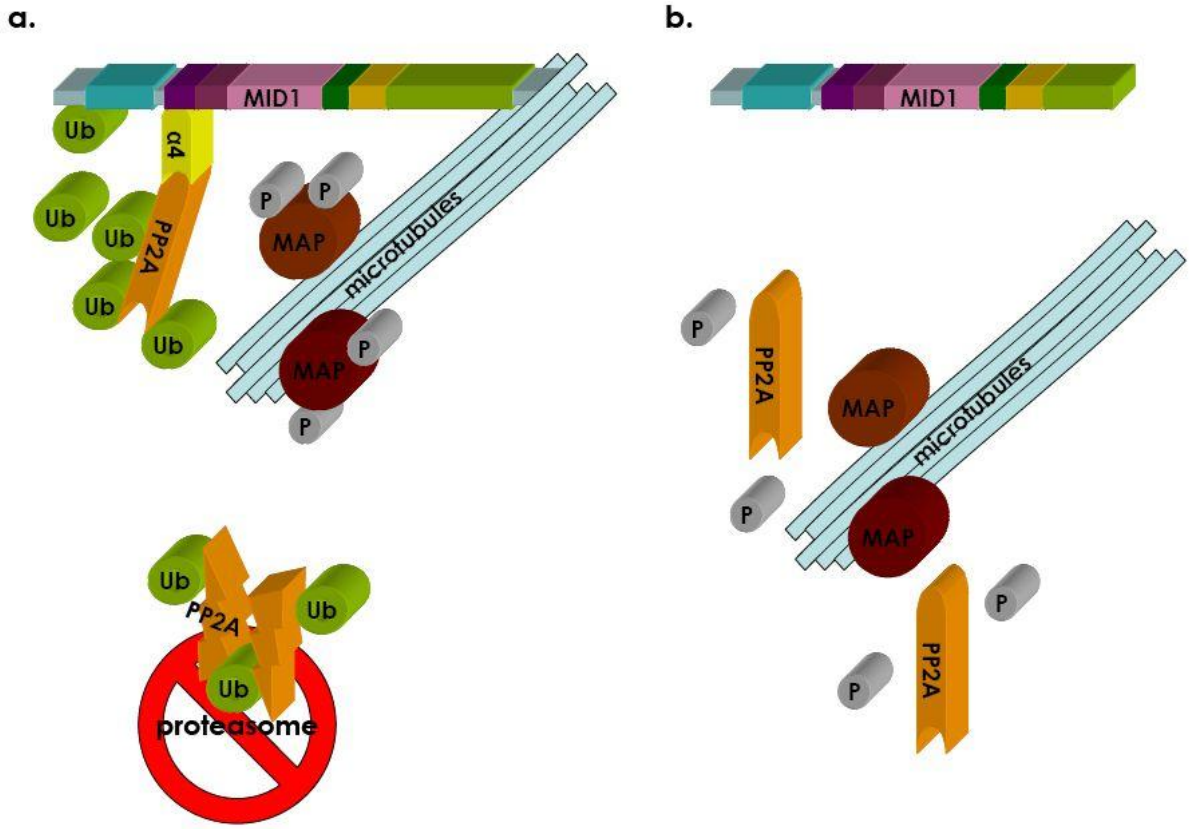

Fig. 1.7 MID1 protein and its function.

Schematic representation of the MID1 protein as a ubiquitin ligase at the microtubules. **a.** The C-terminus of MID1 binds to microtubules and via its B-box1 domain - α4, the regulatory subunit of PP2A. The RING finger domain acts as an E3 ubiquitin ligase, ubiquitinating $PP2A_c$, which results in its degradation. **b.** Mutation in the C-terminus lead to the dissociation of MID1 from the microtubules. $PP2A_c$ cannot be degraded and therefore remains active on the microtubules resulting in hypophosphorylation of MAPs.

Figure adopted from Schweiger and Schneider 2003, taken from (So 2008).

After binding to the microtubules via its C-terminus, interaction between MID1 and α4, brings the RING finger domain and microtubule-associated $PP2A_c$ into close proximity, which leads to the ubiquitin-specific modification and finally degradation of

$PP2A_c$ (Fig. 1.7 a.). As shown in Fig. 1.7 b. mutations in the C-terminal part of MID1 abolish its binding to the microtubules. Therefore, interaction of the MID1-α4 complex with the pool of the microtubule-associated $PP2A_c$ cannot take place. As a consequence, $PP2A_c$ remains at the microtubules resulting in hypophosphorylation of microtubule-associated proteins (MAPs) (Trockenbacher, Suckow et al. 2001; Schweiger and Schneider 2003).

1.3.1.1 MID1 regulates PP2A activity

As described previously (section 1.3.1) MID1 through interaction with α4 protein via its B-box1 domain, and its RING finger E3 ubiquitin ligase activity is involved in the regulation of the PP2A activity and therefore directly implicated in the regulation of the phosphorylation of PP2A downstream targets. PP2A, as described in section 1.2.1, is a central phosphatase in the mTOR signalling pathway, where it targets 4E-BP1 and S6K. Recently it has been shown that PP2A is also involved in the Sonic hedgehog pathway, which regulates the subcellular localization of GLI3 (Krauss, Foerster et al. 2008).

It is worth to notice, that the phosphorylation status of MAPs dependent on PP2A activity has been shown to be implicated in various disorders including Alzheimer's disease (Trojanowski and Lee 1995).

1.3.1.2 MID1 mutations have been found in patients with Opitz BBB/G syndrome

Opitz BBB/G syndrome (OS, MIM 300000) is a congenital disorder characterized by malformations of the ventral midline.

The two main phenotypic manifestations of OS are hypertelorism/telecanthus (abnormally widely spaced/increased innercanthal distance) and hypospadias (urethral cleft in male patients). However, these features are often found together with other typical features such as cleft lip and/or palate, laryngotracheoesophageal (LTE) anomalies, congenital heart defects, imperforate anus, agenesis of the corpus callosum, ear abnormalities, and mental retardation (Cox, Allen et al. 2000; De Falco, Cainarca et al. 2003; So, Suckow et al. 2005). OS is genetically heterogeneous, with both, an X-linked (Xp22.3) and an autosomal locus (22q11.2) (Robin, Feldman et al. 1995). While the X-linked locus was identified in 1997 as the *MID1* gene (Quaderi,

Schweiger et al. 1997), the gene at the autosomal locus at the chromosome 22 still remains unknown.

The *MID1* gene covers a genomic region of approximately 385 kb and consists of 10 exons, from which 9 are constitutively coding exons. Furthermore, *MID1* contains multiple alternative exons (Winter, Lehmann et al. 2004) (Fig. 1.6).

Most of the mutations identified in OS patients are located at the 3′ end of the ORF mostly affecting the C-terminus of MID1. Such mutations may lead to C-terminal dysfunction of the protein (Gaudenz, Roessler et al. 1998) and therefore the dissociation of MID1 from the microtubules (Schweiger, Foerster et al. 1999). The majority of the mutations that have been found are nonsense and frame shift mutations. However, missense mutations, in-frame deletions and in-frame insertions have also been identified (Schweiger and Schneider 2003) (Fig. 1.6).

1.3.1.3 The MID1 protein complex and its function

As described previously (section 1.3.1), the N-terminal part of MID1 is involved in mediating protein-protein interactions, which is essential for the formation of multiprotein complexes (Lupas 1996; Wu, Wang et al. 1996). In confirmation of this, it has been reported that MID1 is being found in large, macromolecular complexes of a size between 232 and 450 kDa (Cainarca, Messali et al. 1999).

Recent work in our group has revealed that MID1 is able to form a microtubule-associated ribonucleoprotein (RNP) complex. By using affinity chromatography and subsequent mass spectrometry analysis it has been found that the MID1/α4 complex associates with several translation regulation factors including elongation factor 1α (EF-1α), several proteins involved in mRNA transport and translation like RACK1, Annexin A2, Nucleophosmin and also active polyribosomes. Interestingly, MID1 complex is also able to associate with mRNAs via G-rich binding motifs. This suggests a role for the MID1 protein in the translation control at the microtubules. It is very likely that the MID1 complex is involved in the compartmentalized translation control of proteins at defined cellular localization thereby providing asymmetric protein production (Orgillés 2006; Aranda-Orgilles, Trockenbacher et al. 2008).

1.4 Aim of the study

Huntington's disease belongs to a group of CAG-repeat expansion disorders (Table.1.1). Most important pathological hallmark is the formation of protein aggregates. Expanded CAG repeats are being translated into abnormally long polyglutamine stretches, therefore leading to protein misfolding and aggregation.
Gain of function on the mRNA level has been proposed to be one of the mechanisms behind HD pathology. It seems that formation of double stranded RNA structures, stability of which increase with the number of repeats, is a critical step in mRNA pathogenesis. Several proteins have been found to bind to such structures. It has been suggested that formation of such RNA-protein complexes might interfere with important cellular processes like i.e. mRNA processing and translation.

The MID1 protein together with α4, a regulatory subunit of $PP2A_c$, forms a microtubule-associated RNP complex, that contains active polyribosomes and binds mRNAs via G-rich RNA motifs with stable secondary structures. This links the translation regulatory mTOR pathway to a microtubule-associated translation unit. Moreover, MID1 via its ubiquitination function, regulates the activity of $PP2A_{c,}$ a central phosphatase in the mTOR signalling pathway and has therefore important influence on the mTOR targets 4EBP and p70S6Kinase. Because CAG repeats are per se G-rich and form stable secondary structure, they are interesting candidates to bind to the MID1 protein complex.

The aim of this study was to (i) investigate the ability of the MID1 complex to bind huntingtin mRNA as an example of an mRNA containing CAG repeats, to (ii) evaluate the influence of the MID1 protein complex on the translation efficiency of the huntingtin mRNA and (iii) to establish the role of the MID-α4 complex in aggregate formation and HD pathogenesis.

2. Material and methods

2.1 Materials

2.1.1 General reagents

Reagent	Manufacturer
ß-mercaptoethanol	Merck
Agarose	Invitrogen
Ammonium persulfate	BioRad
Ampicillin	Sigma
Aqua ad iniectabilia	Baxter
Benzonase	Merck
Biotin-16-UTP	Roche
Bovine Serum Albumin	Serva/Sigma
Bromphenolblue	Serva
Chloramphenicol	Fluka
Chloroform	Merck
Complete mini protease inhibitors	Roche
DAPI (4,6-diamino-2-phenylindole-2HCL $\cdot H_2O$)	Serva
Diethylpyrocarbonate	Sigma
DMSO	Sigma
dNTPs	Fermentas
DTT	Promega
EDTA	Merck
Ethanol	Merck
Ethidium bromide	Serva
Fixing solution	Agfa
Formaldehyde	Merck
Glycerol	Merck
Glycin	Merck
Glycogen	Roche

Reagent	Manufacturer
HCl	Merck
Isopropanol	Merck
Kanamycin	Sigma
KCl	Sigma
LiCl	Sigma
M280 streptavidine coated magnetic beads	DYNAL
3MA	Sigma
Methanol	Merck
$MgCl_2$	Merck/PerkinElmer
Milk powder	Nutricia-Zoetermeer
NaCl	Merck
NaOH	Merck
NBT/BCIP	Roche
NP40	Fluka
Optimem	Gibco
Paraformaldehyde	Sigma
pdN_6	Pharmacia
Penicillin/streptomycin	Cambrex
Phenol	Roth
Prime Rnase inhibitor	Eppendorf
Rapamycin	Sigma
RNA guard	Amersham Pharmacia biotech
RNA loading dye	Fermentas
Rothiphorese gel 30	Roth
Roty-Nylon Plus membrane	Roth
SDS (sodium dodecylsulfate)	BioRad/Serva
Streptavidine-AP	Roche
TEMED	GIBCO BRL
Triton X-100	Serva
Tween20	Sigma

Reagent	Manufacturer
Wortmannin	Stressgen
Zeocin	Invitrogen
Urea	Merck/BioRad

Table 2.1 General reagents

2.1.2 Kits

Kit	Manufacturer
Advantage 2 PCR Kit	Clonetech
Dual-Luciferase®Reporter Assay System	Promega
Endofree Plasmid Maxi Kit	Qiagen
Flexi® Rabbit Reticulocyte Lysate System	Promega
mMESSAGE mMACHINE® Kit	Ambion
Quant-IT protein assay Kit	Invitrogen
Qiaprep Spin Miniprep Kit	Qiagen
Qiaquick Gel Extraction Kit	Qiagen
RiboMAX Large Scale RNA Production System -T7	Promega
Rneasy Mini Kit	Qiagen
SYBRGreen PCR master mix	Applied Biosystems
TaqMan reverse transcription reagents kit	Applied Biosystems
Terminator ready reaction mix	Perkin Elmer
TOPO® XL PCR Cloning Kit	Invitrogen

Table 2.2 Kits

2.1.3 Enzymes

Enzyme	Concentration	Manufacturer
Dnase I	1 U/µl	Promega
GoTaq	5 U/µl	Promega
Proteinase K	10 mg/ml	Roche
Restriction endonucleases	10-20 U/µl	New England biolabs
RNase A	10 mg/ml	Roche
Superscript II reverse transcriptase	200 U/µl	Invitrogen
T4 DNA ligase	400 U/µl	Promega

Table 2.3 Enzymes

2.1.4 Antibodies

Antibodies	WB	Animal	Purchased from
Anti-α4	1:250	Rabbit	Trockenbacher et al., 2001
Anti-MID1	1:500	Rabbit	Sigma
Anti-Actin	1:500	Rabbit	Sigma
Anti-Tubulin MCA 77s	1:1000	Rat	Serotec
Anti-Tubulin MCA 78s	1:1000	Rat	Serotec
Anti-Flag monoclonal	1:1000	Mouse	Stratagene
Anti-Nucleophosmin	1:1000	Mouse	Zymed
Anti-EF-1α	1:1000	Mouse	Upstate
Anti-Annexin II	1:1000	Mouse	BD-transduction laboratories
Anti-Hsp90	1:2000	Rat	Stressgene
Anti-Hsc70	1:10000	Rat	Stressgene
Anti-RACK1	1:2500	Mouse-IgM	BD-transduction laboratories
Anti-GFP	1:500	Mouse	Roche
Anti- GFP	1:1000	Rabbit	abcam

Antibodies	WB	Animal	Purchased from
Anti-GAPDH	1:1000	Rabbit	abcam
Anti-Huntingtin MAB5490	1:1000	Mouse	CHEMICON
Anti-Polyglutamine (CAG)	1:1000	Mouse	CHEMICON
Anti-EP064200 (HTT exon1-N, aa 7-21)	1:500	Rabbit	Kindly provided by E. Wanker
Anti-CAG53b	1:2500	Rabbit	Davies et al., 1997
Anti-Huntingtin HDC8A4 Anti-mouse IgM-POD	1:1000 1:1000	Mouse Donkey	Abcam dianova
Anti-mouse-HRP	1:5000	Goat	dianova
Anti-rabbit HRP	1:5000	Donkey	Amersham
Anti-rabbit HRP non-reduced	1:25000	Mouse	Sigma
Anti-rat	1:1000	Rabbit	Serotec
Anti-rat	1:1000	Goat	Santa Cruz

Table 2.4 Antibodies

2.1.5 Vectors

Clone	Vector backbone	cDNA coding for Insert	Restriction enzymes	Manufacturer
Flag-MID1	pCMVTag2c	MID1 complete cDNA	EcoRI / HinDIII	B. Aranda-Orgilles et al., 2008
HTT-exon1-CAG20	pTetCMV - F° (S)	HTT cDNA coding for amino acid 1-89 with 20 CAG-repeats	NdeI / BamHI	Waelter et al., 2001
HTT-exon1-CAG51	pTetCMV - F° (S)	HTT cDNA coding for amino acid 1- 89 with 51 CAG-repeats	NdeI / BamHI	Waelter et al., 2001
HTT-exon1-CAG83	pTetCMV - F° (S)	HTT cDNA coding for amino acid 1-89 with 83 CAG-repeats	NdeI / BamHI	Waelter et al., 2001

Clone	Vector backbone	cDNA coding for Insert	Restriction enzymes	Manufacturer
HTT-510AA-CAG17	pcDNA3.1	HTT cDNA coding for amino acid 1-510 with 14 CAG-repeats		Kindly provided by E. Wanker
HTT-510AA-CAG49	pcDNA3.1	HTT cDNA coding for amino acid 1-510 with 47 CAG-repeats		Kindly provided by E. Wanker
pGL3m-CAG14	pGL3	HTT cDNA coding for amino acid 1-89 with 14 CAG-repeats (in the 5'UTR of the luciferase)	XbaI	Self made(see section 2.2.1.1)
pGL3m-CAG47	pGL3	HTT cDNA coding for amino acid 1-89 with 47 CAG-repeats (in the 5'UTR of the luciferase)	XbaI	Self made(see section2.2.1.1)
pEGFP-huntingtin exon1 CAG17	pEGFP-C1	HTT cDNA coding for amino acid 1-89 with 17 CAG-repeats	XhoI/PstI	Kindly provided by E. Wanker
pEGFP-huntingtin exon1 CAG49	pEGFP-C1	HTT cDNA coding for amino acid 1-89 with 17 CAG-repeats	XhoI/ PstI	Kindly provided by E. Wanker
pEGFP-huntingtin exon1 CAG72	pEGFP-C1	HTT cDNA coding for amino acid 1-89 with 17 CAG-repeats	XhoI/ PstI	Kindly provided by E. Wanker
pEGFP-huntingtin exon1 CAG83	pEGFP-C1	HTT cDNA coding for amino acid 1-89 with 17 CAG-repeats	XhoI/ PstI	Kindly provided by E. Wanker
pEGFP-C1	pEGFP-C1	Empty vector		Clontech
pCR-XL-TOPO	pCR-XL-TOPO	Empty vector		Invitrogen
pGL3	pGL3	Empty vector		Promega
pRL	pRL	Empty vector		Promega

Table 2.5 Vectors

2.1.6 Buffers and media

Buffer/ Media	Composition
APS 10%	10% w/v APS in water, aliquoted and stored at -20°C
Annealing buffer	100 mM KAc, 30mM Hepes-KOH pH 7,4, 2 mM $MgAc_2$
Blocking buffer	5% milk powder in PBST
Blotting buffer 1 x	5x blotting buffer: MeOH: bidest H_2O 1:1:3
Blotting buffer 5 x	29,11g Tris; 14,65g Glycin; 18,75ml SDS in 1l bidest water
Bradford reagent	Sigma
DMEM (Dulbecco's modified Eagle's medium)	Amino acids, salts ($CaCl_2$, KCl, $MgSO_4$, NaCl, NaH_2PO_4), glucose, vitamins
DPEC H_2O	0,1% DEPC was overnight stirred in water and afterwards autoclaved
EMEM (Eagle's minimal essential medium)	Amino acids, salts ($CaCl_2$, KCl, $MgSO_4$, NaCl, NaH_2PO_4), glucose, vitamins
Ethidium bromide	10 mg/ml EtBr in bidest H_2O
Laemmli buffer	25 mM TRIS p.a, 190 mM Glycin, 0,1% SDS in bidest H_2O
DNA-Loading buffer (LX, LB)	15% Ficoll, 0,25% Bromphenolblue or Xylenecyanol in bidest H_2O
LB (**L**uria **B**ertani) medium	15 g Agar; 10 g Tryptone; 5 g yeast extract in 1 l bidest water; autoclaved
FA lysis buffer	50mM Tris (pH 8,8), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA (pH 8,0), 1% NP40, Bezonase (Merck, Purity Grade II, 250 U/µl, total 100.000 U/vial)
Magic mix 2x	48% Urea (BioRad), 15mM Tris-HCl pH 7,5, 8,7% Glycerin, 1%SDS, 0,004% Bromphenolblue, 143 mM β-mercaptoethanol (add fresh)
Mounting medium	90% glycerol; 0,1 M Tris-HCl pH 8,0; 2,3 % DABCO
Paraformaldehyde/ Paraformaldehyde 3,7%	3,7% formaldehyde in 1,2 PEM Buffer in 1,2 x PEM. Dissolve at 60°C. Store at -20°C

Buffer/ Media	Composition
PBS 1 x	137 mM NaCl; 2,7 mM KCl; 10,1 mM Na_2HPO_4; 1,8 mM KH_2PO_4
PBST	1 x PBS; 1:1000 Tween 20
PEM 10X	1 M PIPES, 0,5 M EGTA, 0,02 M $MgCl_2$ in bidest H_2O, adjust pH 7 with 10N NaOH and autoclave
RNA loading buffer	1 µl MOPS, 5 µl loading dye, 1,5 µl 37% formaldehyde, 10 µl DEPC.water
SDS-PAGE buffer 5x	15% ß-Mercaptoethanol, 15% SDS, 1,5% Bromphenolblue, 50%glycerol
Separating gel buffer	1,5 M Tris-HCl, 0,4 % SDS pH 8,8
SSC 10x	3M NaCl, 0,3M Na-citrate in bidest H_2O, adjust pH 7 with 1M HCl, filter
Stacking gel buffer	0,5 M Tris-HCl, 0,4 % SDS pH 8,8
TAE buffer 50 x	50 mM EDTA, 5,71% v/v acetic acid, 2M Tris-HCl
TBS	10 mM Tris-HCl (pH 8,0); 150 mM NaCl
TBST buffer	10 mM Tris-HCl (pH 8,0); 150 mM NaCl; 0,05% Tween 20
TE	10 mM Tris-HCl (pH 7,5); 1 mM EDTA
TKM buffer	20 mM Tris, 100 mM KCl, 5 mM $MgCl_2$ in DEPC-H_2O

Table 2.6 Buffers and media

2.1.7 Instruments and disposables

Instruments/disposables	Manufacturer
0.025 µm filters	Millipore
0.02 µm acetate membrane	Schleicher and Schuell
BRL dot-blot filtration unit	Life Technologies
CASY1 system	Schärffe system
Centrifuge Rotanta 46R/Rotina 4R	Hettich zentrifugen
Cover slips	Menzel Glaser
Electrophoresis power supply 2	Produced and serviced in MPI for human molecular genetics
Extra thick blot paper	BioRad

Instruments/disposables	Manufacturer
E.A.S.Y Win32 gel documentation system	Herolab
Filter paper	Schleicher and Schuell
Fixogum	Marabu
Gene Pulser cuvettes/ Gene pulser I-Pulser chamber	BioRad
Horizontal gel apparatus Horizon® 11.14 and 20.25	Life technologies
Hypercassete	Amersham biosciences
Inverted microscope Elipse TS100	Nikon
Microscope slides	Roth
Microtitre falcon plates	BD Falcon
Mini-PROTEAN Multi-Casting Chamber	BioRad
Mini- PROTEAN Gel Electrophoresis System	BioRad
Phase lock gel light	Eppendorf
pH-meter	Knick
Pipett boy	Integra biosciences
Pipettes	Gilson
Power Pac 300 electrophoresis power supply	BioRad
PTC200 Peltier Thermal cycler	Biozym
PVDF membrane	Roche
Anthos 2020 (Version1.5) Reader	Anthos
QIAshredder	Qiagen
Qubit fluorometer	Invitrogen
RNA tips	Biozym
Rnase ZapWipes	Ambion
Rotors TLA120.1, TLS-55, SW40	Beckmann
Sonicator, SONOPLUS Homogenisator HD2070	Bandolin electronics
Steril plastic disposables for cell culture	TRP
Table centrifuge 5415C	Eppendorf
UV stratalinker 1800	Stratalinker

Table 2.7 Instruments and disposables

2.1.8 siRNA

Name	Gene	Target sequence	Manufacturer
Non silencing – ns	Non specific	AATTCTCCGAACGTGTCACGT	Qiagen
Alpha4-4	alpha4	CTCGTTGCTATGGCATCTCAA	Qiagen
Alpha4-5	alpha4	AAGTACCTTTTGGTGCCAGCG	Qiagen
MID1-8	MID1	TTGAGTGAGCGCTATGACAAA	Qiagen
MID1-9	MID1	AAGGTGATGAGGCTTCGCAAA	Qiagen

Table 2.8 siRNA

2.1.9 Primers

Name	5'->3' sequence	Manufacturer
httexon1XbaIFor	GCTCTAGAGCGACCCTGGAAAAGCTGATGAAGG	Metabion
httexon1XbaIRev	GCTCTAGACGTCGGTGCAGCGGCTCCTCAG	Metabion
httexon1T7For	CCAAGCTTCTAATACGACTCACTATAGGGAGAATGG CGGACCCTGGAAAAG	Metabion
httexon1Rev	GGTCGGTGCAGCGGCTCCTCAGC	Metabion
ForEndLucipGL3pGL2	AAAGGCCAAGAAGGGCGGAAAG	Metabion
T7pGL3-280-fwd	CCAAGCTTCTAATACGACTCACTATAGGGAGACATG GAAGACGCCAAA	Metabion
pGL3-1989-rev	GTATCTTATCATGTCTGCTCG	Metabion
friefrly-real-f1	TGCACATATCGAGGTGGACATC	Metabion
friefrly-real-r1	TGCCAACCGAACGGACAT	Metabion
friefrly-real-f2	GCGACCAACGCCTTGATT	Metabion
friefrly-real-r2	TCCCAGTAAGCTATGTCTCCAGAA	Metabion
renilla-real-f1	ACGCGGCCTCTTCTTATTTATG	Metabion
renilla-real-r1	CCGCGCTACTGGCTCAA	Metabion
renilla-real-f2	GGTGCTTGTTTGGCATTTCAT	Metabion
renilla-real-r2	TTCAGCGTGAACTATTGCTTTGA	Metabion
GAPDH-real-f1	CCACCCATGGCAAATTCC	Metabion
GAPDH-real-r1	TGGGATTTCCATTGATGACAAG	Metabion
GAPDH-real-f2	CCACATCGCTCAGACACCAT	Metabion
GAPDH-real-r2	AAATCCGTTGACTCCGACCTT	Metabion

Name	5'->3' sequence	Manufacturer
GAPDH-real-f3	ATGGAAATCCCATCACCATCTT	Metabion
GAPDH-real-r3	CGCCCCACTTGATTTTGG	Metabion
GAPDH-real-f4	CAAGGCTGTGGGCAAGGT	Metabion
GAPDH-real-r4	GGAAGGCCATGCCAGTGA	Metabion

Table 2.9 Primers

2.2 Methods

2.2.1 Nucleic acid based methods

2.2.1.1 Cloning of luciferase - pGl3m constructs

httexon1XbaIFor and **httexon1XbaIRev** primers containing XbaI restriction sites (Table 2.9) were used to amplify cDNA of huntingtin exon1 from pTetCMV -F° (S) plasmid containing huntingtin exon1 with 20, 51 or 83 CAG repeats.

2.2.1.1.1 PCR - Polymerase Chain Reaction

PCR was used for the amplification of respective DNA fragments of known sequence using plasmid DNA as a template. GoTaq (Promega) polymerase and PCR touchdown with 72°C annealing temperature were used.

Each PCR reaction was prepared as follows:

Table 2.10 PCR components

Component	Amount
cDNA	1 µl
PCR GoTaq buffer	5 µl
dNTP mix (each dNTP 2.5 mM)	2 µl
Forward and reverse primers (each 10pM/µl)	2 µl
DMSO	4 µl
GoTaq Polymerase	0.6 µl
Aqua ad iniectabilia.	up to 50 µl

Touchdown PCR was performed according to the settings shown in table below:

Table 2.11 Touchdown PCR

Step	Temperature	Time
Initial denaturation	95°C	4 min
Denaturation	95°C	30 sec
Annealing	(TA+10°C)-1°C cycle	30 sec
Elongation	68°C/72°C	1 min/kb amplified DNA
Denaturation	95°C	30 sec
Annealing	Primer specific	30 sec
Elongation	68°C/72°C	1 min/kb amplified DNA
Final elongation	68°C/72°C	10 min
Storage	4°C	Infinite

PCR reactions were carried out in a PTC200 Peltier Thermal cycler (Biozym). When required, PCR products were purified directly with the QIAquick PCR purification kit. DNA was eluted with 30-50 µl EB buffer (Qiagen) diluted 1:10 in bidest water.

2.2.1.1.2 Agarose gel electrophoresis

All PCR products were evaluated by DNA electrophoresis.

1% - 2% w/v ultrapure agarose gels in TAE buffer (Table 2.6) were used to separate RNA or DNA samples. 0,5 µg/µl EtBr was added to the gels to visualize DNA or RNA via UV light. 1 µl of loading buffer was added per each 9 µl of DNA solution before loading the samples on the gel. Samples were run at 80-150 V in an electrophoretic chamber for 30-60 min depending on the fragment size. Different DNA/RNA molecular weight markers were used according to the size of the analyzed products (Table 2.11). Fragments were visualized, and pictures were taken, with the E.A.S.Y Win32 gel documentation system (Herolab).

For DNA fragments bigger than 1 kb LX was used and for shorter fragments LB (Table 2.6). RNA samples were run in RNA loading buffer (Table 2.6) and heated for 10 min at 70°C prior to loading. 1 µl of RNA ladder (Table 2.12) was treated in the same way.

Table 2.12 Nucleic acid markers

DNA/RNA ladders	Fragment size	Manufacturer
Hyperladder I	300-7000 bp	Bioline
Hyperladder V	50-500 bp	Bioline
0.24-9 kb RNA Ladder	all	Invitrogen

When required, PCR products were subsequently extracted with the Gel Extraction Kit (Qiagen, Table 2.2). DNA was eluted with 30-50 µl EB buffer (Qiagen) diluted 1:10 in bidest water.

As an intermediate step in cloning procedure PCR product was then ligated into the pCR-XL-TOPO vector (Table 2.5) according to the manufacturer's protocol and transformed into the bacterial cells as described later in section 2.2.1.1.6.

2.2.1.1.3 DNA digestion

Isolated pCR-XL-TOPO plasmid was digested with XbaI restriction enzyme for 1h in 37°C. The same restriction with XbaI was performed on pGL3m vector (Table 2.5). Both samples were then run on agarose gel and huntingtin exon1 insert containing XbaI restriction sites, as well as linearized pGL3m vector, were excised from the gel and extracted with Gel Extraction Kit (Qiagen, Table 2.2).

2.2.1.1.4 Ligation

Huntingtin exon1 – insert, and pGL3m - linearized vector both containing XbaI sites, were ligated overnight at 16°C with T4 DNA ligase (Table 2.3). For the ligation, insert-vector ratio (3:1) was used and calculated as shown in Fig. 2.1:

$$\text{ng insert} = \frac{\text{ng vector x Kb fragment}}{\text{Kb vector}} \text{ x } 3$$

Fig. 2.1 Insert-vector ratio calculation

2.2.1.1.5 Transformation

Ligation reaction was then transformed into the bacterial cells for its production.

2.2.1.1.5.1 Chemical transformation

Plasmid DNA (10 ng of supercoiled DNA or the whole ligation reaction) was incubated with 30 µl of chemically competent cells (DH 5α) for 30 min on ice, heat shocked at 42°C for 1 min 30 sec and chilled on ice for 2 min. Cells were incubated for 1h in 1 ml LB with vigorous shaking at 37°C. Afterwards, cells were plated onto selective agar plates containing the appropriate antibiotic and incubated overnight at 37°C.

2.2.1.1.5.2 Electroporation

40 µl of competent cells were added to plasmid DNA and the mixture was placed in a 1 mm electroporation cuvette.

Electroporation took place at 25 µF capacity, 1,7 V voltage and 200 Ω resistance with a time constant of about 4,5 msec. Immediately, 1 ml LB was added to the transformed cells. The solution was incubated at 37°C with vigorous shaking for 1h and afterwards, cells were plated onto selective agar plates containing the appropriate antibiotic and incubated overnight at 37°C.

2.2.1.1.6 Plasmid DNA isolation

Single bacterial colonies carrying the plasmid of interest were grown overnight at 37°C with vigorous shaking in 5 ml LB medium with the appropriate antibiotic (Table 2.6). Next day, plasmids were isolated using the Miniprep Kit (Qiagen) according to the manufacturer instructions.

When larger amounts of DNA were required, 5 ml of LB/antibiotic were inoculated with a single colony, and incubated for 8 hours. 0,5 ml of this culture were used to inoculate 100 ml of LB/specific antibiotic, which was incubated overnight at 37°C with vigorous shaking. Next day, plasmid DNA was isolated using either the Maxiprep Kit or the Endofree Maxiprep Kit (for plasmids required for transfection of eukaryotic cells) according to the manufacturer´s instructions.

To verify whether the obtained plasmids contained the expected huntingtin exon1 fragments, the constructs were digested with corresponding restriction enzymes and checked on agarose gels (see section 2.2.1.1.2).

2.2.1.1.7 Sequencing

Clones carrying insert of the correct molecular weight were then sequenced using **ForEndLucipGL3pGL2** and **httexon1XbalRev** primers (Table 2.9).

2 ng of DNA per 100 bp length for PCR products, or 100 ng of plasmid DNA, were mixed with 6 µl H_2O, 1 µl sequencing primer (10 pmol/µl) and 3 µl "Terminator ready reaction mix" (Table 2.2).

Sequencing reaction was precipitated with 100% EtOH. To a 10 µl reaction, 25 µl of absolute EtOH were added and mixed thoroughly. After 10 min incubation, samples were centrifuged for 45 min at 4000 rpm at RT.

Supernatants were discarded and 100 µl of 70% EtOH were added to the pellets. After inverting the tubes, samples were centrifuged for 15 min at 4000 rpm at RT. EtOH was removed and the samples were air-dried. The samples were then analyzed in an ABI377 DNA sequencer by service center in MPI for human molecular genetics.

2.2.1.2 RNA isolation and cDNA synthesis

RNA was prepared with the RNeasy Mini Kit (Table 2.2) according to manufacturer´s instructions. For subsequent removal of DNA in the sample, 1 µg of RNA was digested with 1 µl DNAse I (Table 2.3). The reaction took place for 30 min at 37°C. For purification, samples were subjected to phenol-chloroform extraction and EtOH precipitation (see chapter 2.2.1.3 and 2.2.1.4 respectively). RNA samples were stored at –20°C.

For cDNA synthesis, 2 µg of RNA were mixed with 2 µl dNTPs (2,5 mM each), 6 µl pd(N)6 (100 ng/µl) (Table 2.1) and DEPC-H_20 (Table 2.6) up to 31µl of total volume. The reaction was incubated for 5 min at 70°C and quickly chilled on ice. 10 µl of 5x 1st strand buffer, 5 µl of 0.1 M DTT (Table 2.1) and 1 µl of RNA guard (Table 2.1) were added, and the mixture was incubated for 2 min at 42°C. The samples were divided into two aliquots of 23,5 µl. To one of the aliquots 2 µl of Superscript II reverse transcriptase (Table 2.3) were added. The second aliquot was kept as

negative control for the reaction. RNA was reverse-transcribed by incubation for 1h at 42°C and the enzyme was subsequently inactivated at 70°C for 15 min. cDNAs were stored at –20°C.

2.2.1.3 Phenol-Chloroform extraction

To DNA or RNA containing solutions one volume of phenol-chloroform (1:1) (TE pH:7.5 saturated phenol for DNA, H_2O saturated phenol pH:4,5 for RNA) in a Phase Lock Gel (PLG) light (Table 2.7) was added. The mixture was rotated for 5 min and centrifuged at 16000 *x g* for 5 min. To the aqueous upper phase, one volume of chloroform was added, and sample was again rotated for 5 min and centrifuged for 5 min at 16000 *x g*. The upper phase was transferred to a new tube and DNA or RNA was precipitated with EtOH.

2.2.1.4 EtOH precipitation

For EtOH precipitation of nucleic acids, 2,5 volumes of 95% EtOH, 1:10 LiCl for RNA or NaAc for DNA, and 1:100 glycogen were added to the samples. The mixture was placed at – 20°C for > 30min and subsequently centrifuged for 20 min at 16000 x *g* at 4°C. Afterwards, the pellet was washed with 200 µl 70% EtOH and centrifuged for 10 min at 16000 x *g* at 4°C. The pellet was air dried and resuspended in TE buffer pH 7.5.

2.2.1.5 Real Time PCR

$2x10^4$ HeLa cells were seeded per well of a 12 well plate one day prior to transfection with pGL3m, pGL3m-CAG14/47 as described in section 2.2.3.5.
After 24h total RNA was isolated using an RNeasy Plus Mini kit (Qiagen) following the manufacturer's protocol. cDNA was synthesized using the TaqMan reverse transcription reagents kit (Applied Biosystems), and real-time PCR was carried out using the SYBRGreen PCR master mix (Applied Biosystems) according to the manufacturer's instructions with an ABI7900HT cycler under the following conditions: 50°C for 2 min; 95°C for 10 min; 95°C for 15 s, 60°C for 1 min for 40 cycles; and 95°C for 15 min, 60°C for 15 min, 95°C for 15 min for the dissociation stage. Primers used are listed in Table 2.9

2.2.2 Protein based methods

2.2.2.1 Measurement of protein concentration

2.2.2.1.1 Bradford assay

For the Bradford assay, BSA was used as a protein standard to spectrophotometrically measure protein concentrations. BSA solutions of 1, 2, 4, 5, 7.5 and 10 µg/ml (diluted in a 1:1000 solution of the buffer in which the sample proteins were dissolved) were used to form a standard curve.

For protein measurement samples were diluted 1:1000 in water. 2 x 80 µl of standards and protein samples were loaded on a microtitre falcon plates and 20 µl of Bradford reagent (Sigma) was added. The reaction was incubated for 5 min and then measured in an Anthos 2020 (Version1.5) Reader.

2.2.2.1.2 Quant-IT protein assay

Quant-IT technology from Invitrogen, uses fluorescent dyes to measure protein concentration.

The Quant-IT protein assay was performed according to manufacturer's protocol. Protein samples were diluted 1:200 in working solution and concentrations were measured in Qubit fluorometer (Invitrogen, Table 2.7).

2.2.2.2 SDS – PAGE Gel

To separate proteins according to their molecular weight, samples were first mixed 1:1 with 2x Magic Mix buffer (Table 2.6) and boiled at 95°C for 5 min for denaturation.

5 ml of separating and 1 ml of stacking gel were prepared for one SDS-PAGE gel.

Solutions for Trisglycine SDS-polyacrylamide resolving gels (6-12%) with 5% stacking gels were prepared using Rotiphorese gel 30 (Roth), and the Protean III system according to Molecular Cloning: A Laboratory Manual (Sambrook, Fritsch et al. 1989).

Samples were then loaded onto the SDS – PAGE gels in a Mini-PROTEAN Multi-Casting Chamber (Bio-Rad).

Protein separation was performed on a Mini- PROTEAN Gel Electrophoresis System (Bio-Rad) in 1x Laemmli buffer at 100-200 V.

2.2.2.3 Western blots

The blotting PVDF membrane was equilibrated for 3 sec in MeOH, 2 min in bidest H_2O and then placed together with extra – thick blot paper (Bio-Rad) into 1 x Blotting buffer (Table 2.6).

After electrophoresis gel was first equilibrated in 1 x Blotting buffer (Table 2.6). After 15 min incubation a blotting 'sandwich' was formed on the platinum anode of the transfer cell (Trans Blot SD, Bio Rad) with a layer of extra-thick filter paper at the bottom, then the PVDF membrane, the gel and a second sheet of extra-thick filter paper. Air bubbles were removed out of the 'sandwich' using a glass stirrer. The stainless steel cathode and safety cover were then placed on top of the assembly, and the gel transfer allowed to proceed at 15 V for 30-45 min.

The PVDF membrane with transferred proteins was then blocked in PBST + 5% milk for 30 min prior incubating with primary antibody in PBST + 5% milk for 1h up to overnight. The membrane was then washed 3 times for 5 min with PBST before incubation with secondary antibody conjugated to horse radish peroxidase (HRP) in PBST for 30 min up to overnight. After 3 times washing for 5 min with PBST, HRP-conjugated antibody detection was performed using the Western Lightning Western Blot Chemiluminescence Reagent (PerkinElmer), according to manufacturer's protocol. The membrane was then exposed to Super RX X-ray film (Fuji) and developed in a Curix 60 developing machine (AGFA).

All washings and antibodies incubations shorter then overnight period were performed at RT. Overnight incubations were performed at 4°C.

2.2.3 Cell culturing and cell-based assays

Table 2.13 Cell lines

Cell line	Description	Medium
HeLa (**He**nrietta **La**cks)	Human epithelial cells from cervical carcinoma	DMEM, 10% FCS, 2mM L-glutamine, 100 µg/ml streptomycin, 100 units/l penicillin
293T	Human embryonal kidney cells expressing a temperature sensitive SV40 T-antigene	EMEM, 10% FCS, 2mM L-glutamine, 100 µg/ml streptomycin, 100 units/l penicillin
U-373 MG	Human epithelial cells from malignant gliomas	EMEM, 10% FCS, 2mM L-glutamine, 100 µg/ml streptomycin, 100 units/l penicillin, 0.1 mM non essential aa, 1 mM sodium puryvate
293Q20/Q51/Q83	Human embryonal kidney cells stably expressing the pTetCMV -F° (S) plasmid containing htt-exon1-CAG20/51/83	EMEM, 10% FCS, 2mM L-glutamine, 100 µg/ml streptomycin, 100 units/l penicillin, 100mg/ml G418, 150 mg/ml hygromycin B, and 10 ng/ml doxycycline were added fresh to the medium

All cell lines were grown in the above described media (Table 2.13).
For seeding cells were first trypsinised and counted with the cell counter and analysis system CASY1 (Schärffe system).

2.2.3.1 Transfection

2.2.3.1.1 PolyFect transfection of DNA plasmid

$8x10^5$ ($1x10^5$) of 293T cells in a 75 cm^2 flask (6 well plates) were seeded the day previous to transfection.

Briefly, transfection mix containing all together 9 µg (1.5 µg) of DNA, 240 µl (100 µl) of OptiMEM and 24 µl (10 µl) of PolyFect (Table 2.1) was prepared and incubated 10 min at RT. In the meanwhile, cells were washed with PBS and 10 ml or (1.5 ml) of fresh medium was added to the cells. After 10 min, 2 ml or (0.6 ml) of fresh medium was added to the transfection mixture and after mixing, the solution was placed on the cells. Cells were incubated at 37°C in a humidified incubator with 5% CO_2 v/v for 24h.

2.2.3.1.2 Lipofectamine transfection of DNA plasmid

$3x10^6$ ($2x\ 10^4$) of 293T,HeLa or U-373 MG cells in a 150 cm^2 flask (12 well plates) were seeded the day previous to transfection. A solution '1' containing 15 µg (1 µg) of plasmid DNA and 2,5 ml (100 µl) of OptiMEM, and a solution '2' with 50 µl (1 µl) lipofectamine (Table 2.1) and 2,5 ml (100 µl) of OptiMEM were prepared and kept for 5 min at RT. Subsequently, solutions were mixed and incubated at RT for 20 min. In the meanwhile, cells were washed with PBS and 18 ml (200 µl) of fresh medium without antibiotics was added. After 20 min transfection mixture was slowly added to the cells, which then were incubated at 37°C in a humidified incubator with 5% CO_2 v/v for 24h.

2.2.3.1.3 siRNA transfection

$5x10^4$ of 293Q20, Q51 or Q83 (Table 2.13) cells were seeded, per well in a 6 well plates the day previous to transfection. A solution '1' containing 5 µl of 20 nM siRNA oligo (Table 2.8) and 175 µl OptiMEM, and a solution '2' with 5 µl oligofectamine (Table 2.1) and 45 µl OptiMEM were prepared and kept for 10 min at RT. Solutions were then mixed and incubated at RT for 20 min. In the meanwhile, cells were washed with PBS and 1 ml of fresh medium was added. Transfection mixture was slowly added to the cells, which then were incubated at 37°C in a humidified incubator with 5% CO2 v/v for 24h.

2.2.3.2 Drug treatments

$1x10^5$ 293Q cells (Table 2.13) were seeded per well in 6 well plates. 24h later cells were treated with different drugs. All given concentrations are final concentrations in the medium. For each treatment mock control was prepared.

Table 2.14 Drug treatments

Drug	Final concentration	Time of treatment
Rapamycin	1 µM	48 h
LiCl	5 nM	48 h
3MA	1 mM	72 h
Wortmaninn	50 nM	72 h
MG132	5 nM	24 h

2.2.3.3 Fluorescent imaging

$1x10^5$ 293T cells per well were seeded onto cover slips in 6 well plates. Next day, they were transfected with the corresponding plasmid DNA using polyfect (section 2.2.3.1.1) and grown for 24h. Afterwards, the medium was removed and cells were washed in 1.2 x PEM, fixed in 3,7% paraformaldehyde for 10 min at RT and shortly washed in 1x PBS.

Afterwards cover slips were placed in mounting medium containing 0,5 µg/ml DAPI, dried and fixed to the slide with Fixogum (Table 2.7).

Cell visualization took place using a fluorescence microscope (Zeiss Axioskop). Image acquisition was performed using a cooled CCD camera (Hamamatsu hotonics) and RGB image construction was achieved using the ISIS Image Analysis System (MetaSystems).

2.2.3.4 Filter Retardation assay (FA)

1x 10^5 of 293Q83 cells (Table 2.13) were seeded per well in 6 well plates one day prior transfection or treatments. Doxycycline was washed out 28h after transfection.

The Filer Retardation assay (FA) was performed as described by Scherzinger et al. (Scherzinger, Lurz et al. 1997), with following changes:

Cell pellets were lysed in 400 µl of FA lysis buffer (Table 2.6) for 20 min, after that, protein concentration was measured. Lysates were diluted to 1 mg/ml and mixed 1:1 with denaturing solution (4% SDS, 1mM DTT). After denaturation at 97°C for 7 min, aliquots (80 µl and 40 µl) were diluted into 100 µl of 0.2% SDS and filtered through a cellulose acetate membrane using a BRL dot blot filtration unit (Life Technologies, Table 2.7). Filters were washed with 0.2% SDS, and the SDS-insoluble aggregates retained on the filter were detected by incubation with the anti-CAG53b antibody, followed by an HRP-anti-rabbit secondary antibody detection (see section 2.2.2.3 and Table 2.4).

2.2.3.5 Dual – Luciferase reporter assay

2x 10^4 HeLa or U-373 MG cells were seeded per well in 12 well plates one day prior to transfection.

Cells were transfected with the luciferase vectors pGL3m – CAG14/CAG47 (Table 2.5) together with pRL vector using lipofectamin. Transfection protocol was described in section 2.2.3.1.2.

Cells were kept 24h after transfection. Cell lysates were prepared for luciferase activity measurement using a Dual-Luciferase®Reporter Assay System (Table 2.2), according to manufacturer's protocol (Promega).

2.2.4 RNA-protein binding experiment

2.2.4.1 *In vitro* transcription of biotinylated RNA

Huntingtin exon1 sequences containing 20 and 51 CAG repeats were PCR-amplified using **httexon1T7For** and **httexon1Rev** primers (Table 2.9) and the pTetCMV -F° (S) plasmid containing huntingtin exon1 with 20 and 51 CAGs as a template. The forward primer contained the T7 promotor sequence (5'CCAAGCTTCTAATACGACTCACTATAGGGAGA3') to allow subsequent *in vitro* transcription of the PCR product.

After amplification PCRs were checked on agarose gels and purified by gel extraction. PCRs fragments were additionally digested with Proteinase K by incubation with 100 µg/ml proteinase K, 0.5 % SDS in 50 mM TRIS-HCl (pH 7,5) and 5 mM $CaCl_2$ for 30 min at 37°C. Subsequently, they were purified by phenol-chloroform extraction (see section 2.2.1.3).

Amplified transcripts were then *in vitro* transcribed with the RiboMAX Large Scale RNA Production System -T7 (Promega, Table 2.2), following the manufacturer's protocol with some modifications.

2 µg of purified PCR product were transcribed for 4h at 30°C in the following reaction mix.:

Table 2.15 *in vitro* transcription reaction

Component	Amount
T7 transcription buffer	4 µl
rNTPs (25 mM rATP, rGTP, rCTP, 1,6 mM biotin-rUTP, 2,5 mM UTP)	12 µl
PCR	2 µg
RNA polymerase enzyme mix	2 µl
DEPC-H_2O	up to 20 µl

The transcribed, biotinylated RNA was then purified by phenol-chloroform extraction (section 2.2.1.3) and precipitated with ethanol (see section 2.2.1.4). Products were dissolved in nuclease free TE and stored at -20°C.

RNA concentration was measured with a NanoDrop ND-1000 Spectrophotometer (PEQLAB) and molecular weight was checked on agarose gels.

2.2.4.2 Biotinylation efficiency

The efficiency of the biotinylation was tested by Dot Blotting. Serial dilutions of biotinylated RNA (1:10, 1:100, 1:1000) were made using a 6x SSC buffer.

1 µl from each dilution was spotted on a Roty-Nylon Plus membrane. The RNA was cross linked to the membrane with a UV linker. The membrane was blocked in PBS with 5% BSA for 15 min, followed by incubation with 1:1000 streptavidin-AP in PBS with 5% BSA for another 30 min. The blot was then washed and developed by incubation with freshly prepared NBT-BCIP in the dark until signals appeared. Membranes were finally washed with H_2O and kept in a plastic bag.

2.2.4.3 RNA- protein binding assay

$8x10^5$ of 293T cells were either non transfected or transfected with Flag-MID1 vector (Table 2.5, section 2.2.3.1.1). Cells were first lysed for 15 min on ice in 0.5 ml of TKM buffer supplemented with 1 mM DTT, 1 % NP40 and proteinase inhibitors and then homogenized by sonication. Cell debris were pelleted and discarded by centrifugation for 5 min at 12000 *x g* at 4°C.

2 µg of biotinylated RNA (see section 2.2.4.1) was incubated with 200 µg or 500 µg of cytosolic protein extract containing MID1-FLAG protein or endogenous MID1 protein respectively.

Reaction mix was assembled according to the table:

Table 2.16 RNA-protein binding assay reaction set-up

Component	No RNA sample	RNA sample
Protein lysate	200/500 µg	200/500 µg
RNA	-	2 µg
RNase inhibitors	4 µl	4 µl
TKM buffer	up to 500 µl	up to 500 µl

Samples were incubated for 1h at 4°C rotating. Subsequently, the mixture was incubated for 1h at 4°C with 30 µl of 50% slurry of M280 streptavidin coated magnetic beads (Table 2.1). Beads were then washed 3 times with TKM buffer

containing 0.5 mM DTT, 0.5 % NP40 for 10 min at 4°C and bound proteins were eluted by boiling the beads in 30 µl magic mix for 10 min at 95°C. Bound proteins were detected on Western blots using the respective antibodies (see section 2.2.2.3).

2.2.5 *In vitro* translation

2.2.5.1 *In vitro* transcription of capped RNA

Luciferase sequences containing huntingtin exon1 with 20 and 51 CAG repeats were PCR-amplified using **T7pGL3-280-fwd** and **httexon1Rev** primers (Table 2.9) and the BamHI linearized pGL3m/ pGL3m-CAG14/47 plasmids as a template. The forward primer contained the T7 promotor sequence (5'CCAAGCTTCTAATACGACTCACTATAGGGAGA3') to allow subsequent *in vitro* transcription of the PCR product.

After amplification PCRs were checked on agarose gel and concentration was measured with a NanoDrop ND-1000 Spectrophotometer (PEQLAB).

Subsequently those PCR products were then used for RNA production with the mMESSAGE mMACHINE® Kit (ambion). Reaction took place at 37°C for 2h. Reaction was assembled as follows:

Table 2.17 Capped RNA reaction set-up

Component	Amount
H_2O	up to 20 µl
2 x NTP/CAP	10 µl
10 x Reaction Buffer	2 µl
PCR	1 µg
Enzyme mix	2 µl

Transcribed capped RNA was then purified by phenol-chloroform extraction (section 2.2.1.3) and precipitated with ethanol. Products were dissolved in nuclease free TE and stored at -20°C. RNA concentration was measured with a NanoDrop ND-1000 Spectrophotometer (PEQLAB) and molecular weight was checked on agarose gels.

2.2.5.2 *In vitro* translation assay

Capped RNA (prepared as describe previously in section 2.2.5.1) was used for in vitro translation by Flexi® Rabbit Reticulocyte Lysate System (Promega, Table 2.2) following the manufacturer's protocol with some modifications.

Table 2.18 *in vitro* translation reaction set-up

Component	Amount
Flexi lysate	17.5 μl
AA minus Leu	0.25 μl
AA min Met	0.25 μl
KCl	0.7 μl
Rnase inhibitors	1 μl
Capped RNA	1 μg
H_2O	up to 25 μl

Each reaction was prepared in 4 repetitions for each RNA sample. In vitro translation reaction was performed for 60 min or 90 min in 30°C. Subsequently 6 μl of each reaction was mixed with 40 μl of H_2O.

2x 20 μl for each reaction were placed in a white luminometer plate and chemiluminescence measurement was performed according to manufacturer's protocol (Promega) in Centro Luminometer LB 960 (Berthold).

3. Results

3.1 Establishment of important tools to study huntingtin expression and aggregation

3.1.1 Stable expression of N-terminal huntingtin fragment with 20/51/83 polyglutamine repeats in HEKT cells

In order to investigate huntingtin expression and aggregation, human 293 - Tet-Off cells expressing N-terminal huntingtin fragment (N-HTT), with a polyglutamine repeats in the pathological range, 51 or 83 glutamines, and in the normal range, 20 glutamines, were used (Table 2.7). Cells were previously described by Waelter et al. (Waelter, Boeddrich et al. 2001).

3.1.1.1 Detection of N-terminal huntingtin aggregates in the Filter Retardation Assay

Human 293 - Tet-Off cells expressing huntingtin exon1 containing 51 or 83 CAG repeats (293Q51/Q83) are able to produce aggregates. Those aggregates can be detected on acetate membranes using the Filter Retardation Assay (FA) described previously by Scherzinger et al. in 1997 (see also section 2.2.3.4).

3.1.1.1.1 Tet – off system

The Tet-Off expression system is a binary transgenic system in which expression from a target transgene is dependent on the activity of an inducible transcriptional activator. In this system, expression of the transcriptional activator can be regulated reversibly and quantitatively by exposing cells or transgenic animals to varying concentrations of tetracycline (Tc), or tetracycline derivatives such as doxycycline (Doxy).

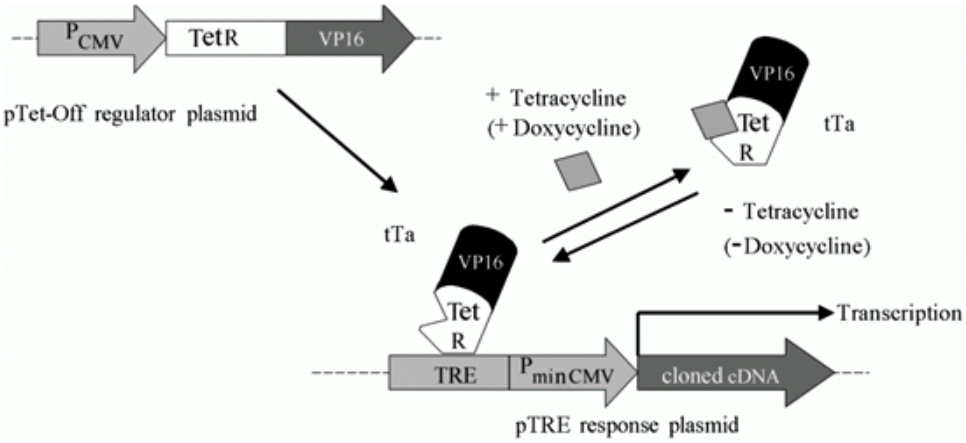

Fig. 3.1 Schematic representation of the Tet – Off expression system (Gossen and Bujard 1992; Baron and Bujard 2000).

In the Tet-Off expression system, a tetracycline-controlled transactivator protein (tTA) regulates the expression of a target gene that is under transcriptional control of a tetracycline-responsive promoter element (TRE).

Transactivator protein (tTA) is a fusion protein of the Tet repressor DNA binding protein (TetR) and the strong transactivating domain of VP16 from Herpes simplex virus. In the absence of Tc or Doxy, tTA binds to the tetracycline-responsive promoter element and activates transcription of the target gene. In the presence of Tc or Doxy, tTA can not bind to the TRE, and expression from the target gene remains inactive (Gossen and Bujard 1992; Baron and Bujard 2000).

3.1.1.1.2 Aggregation in stable cell lines

To establish previously described Tet-Off expression system (Gossen and Bujard 1992; Baron and Bujard 2000) in our lab, the 293Q83 cells were seeded in 6 well plates. 24h later, Doxy was washed out. After 48h without Doxy cells were lysed, and lysates were prepared for Filter retardation Assay (FA) as described in section 2.2.3.4. FA-membranes were then developed using the anti-CAG53b antibody, followed by an anti-rabbit HRP secondary antibody detection (see section 2.2.2.3 and Table 2.4).

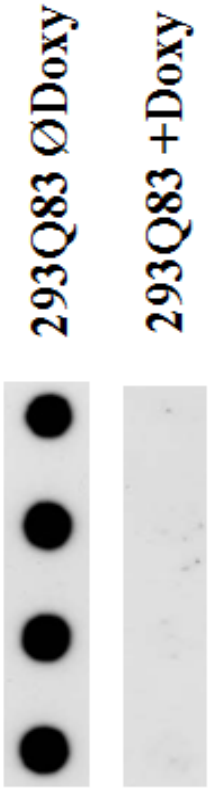

Fig. 3.2 FA of 293Q83 cells with (left panel) and without (right panel) induction of the transgene expression.

As a negative control, cells were grown for 48h in medium containing Doxy, while in control cells no aggregates could be detected. Cells grown in medium without Doxy produced aggregates (Fig. 3.2).

To analyze the differences in aggregation in cell lines containing exon1 of huntingtin with pathological 51 and 83 CAG repeats and non pathological 20 CAG repeats, all 293Q cell lines were analyzed parallel 72h after removing Doxy.

Cells were lysed, FA was performed and FA-membranes were then developed using the anti-CAG53b antibody, followed by an anti-rabbit HRP secondary antibody detection (Fig. 3.3).

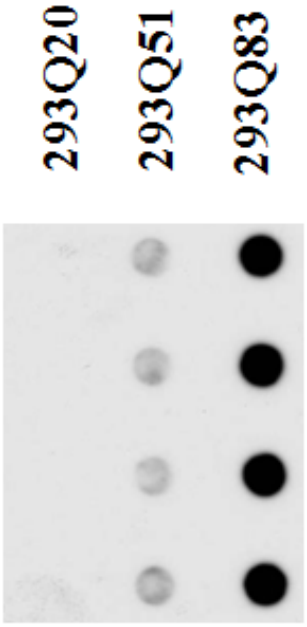

Fig. 3.3 FA of 293Q20/51/83 cells (left, middle and right panel respectively), showing the aggregation pattern after transgene expression.

As shown in Fig 3.3, cells expressing N-terminal huntingtin fragment with 20 glutamines did not produce any aggregates, in contrast to cells expressing N-terminal huntingtin fragment with pathological amount of polyglutamine repeats (51 or 83).
After 72h expression of N-terminal huntingtin fragment with pathological amount of glutamines, there was a clear difference between cells containing transgene with 51 or 83 CAG repeats: the aggregate amount in 293Q51 cells was much lower than in 293Q83 cells.

3.1.1.2 N-terminal huntingtin fragment detection on Western Blots

Two forms of mutant huntingtin exist in cells: aggregates and the fraction of soluble protein.
In the second set of experiments the SDS-soluble fraction of N-terminal huntingtin fragment containing 20/51/83 glutamines was analyzed on western blots.

3.1.1.2.1 Expression of N-terminal huntingtin fragment in 293Q20/51/83 cells

293Q20/51/83 cells were seeded in 6 well plates. After 24h incubation without Doxy, cell lysates were run on SDS-PAGE gels (section 2.2.2.2) and analyzed on western blots. N-terminal huntingtin fragment (N-HTT), with different amount of glutamines was detected using the anti-EP064200 antibody (Table 2.4), detecting aa 7-21 of huntingtin (Fig. 3.4).

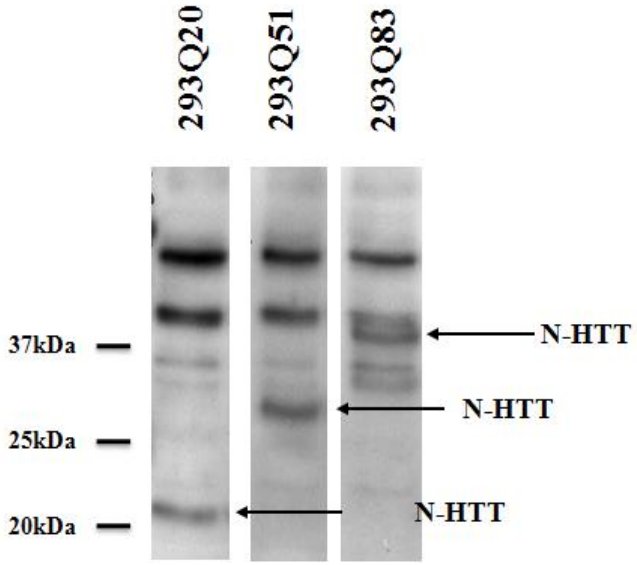

Fig. 3.4 Western blot analysis of 293Q20/51/83 cells (left, middle and right panel respectively), showing products of the transgene expression of respected sizes dependent on the polyglutamine length.

As shown in Fig. 3.4, after 24h expression, SDS-soluble fractions of N-terminal huntingtin fragment of different sizes correlated with polyglutamine stretch length were detected.

3.1.1.2.2 Time course

In the next set of experiments the SDS-soluble fractions of N-terminal huntingtin fragment containing 51 and 83 glutamines were analyzed after different time intervals of transgene expression. Cells were harvested after either 24h, 48h, 72h or 144h of incubation without Doxy. Lysates were then run on SDS-PAGE gels and analyzed on western blots. N-terminal huntingtin fragment, with different amount of glutamines was detected on the resulting western blot with the anti-EP064200 antibody (Table 2.4).

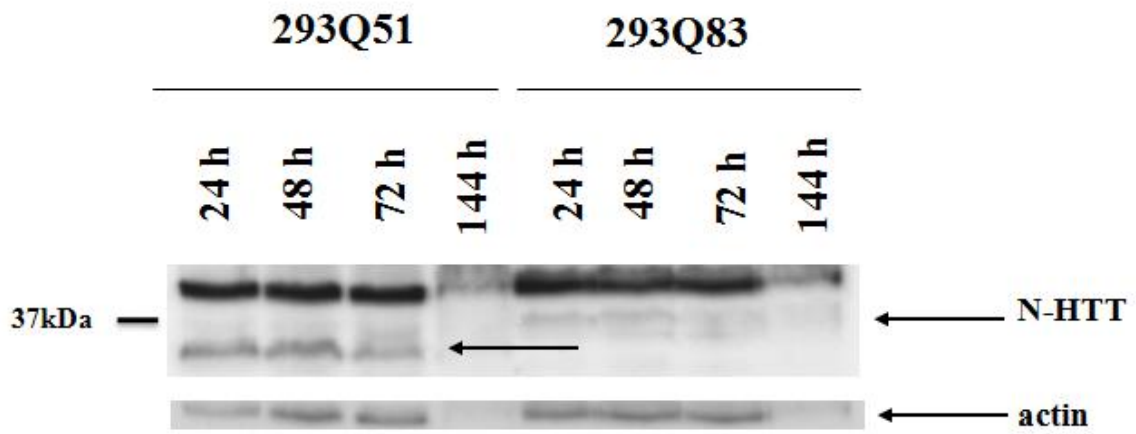

Fig. 3.5 Western blot analysis of the transgene expression in 293Q51/83 cell lines within the time. Size of the product corresponds to the length of polyglutamine stretch.

The amount of N-terminal huntingtin fragment containing 51 glutamines decreased over the time. The overall amount of the N-terminal huntingtin fragment expressed in 293Q83 cell line was lower when compared to the protein amount produced in 293Q51 cells. This difference between two transgenes, might be due to the faster formation of SDS-insoluble aggregates by the fragment containing 83 glutamine stretch. After 144h both N-terminal huntingtin fragments were no longer detected, as well as actin, which would indicate formation of SDS insoluble aggregates.

3.1.2 Transient expression of EGFP-tagged huntingtin exon1 fragment in HEKT cells

3.1.2.1 Fluorescent imaging

EGFP - Enhanced Green Fluorescent Protein is a 29 kDa recombinant protein, which is broadly use as a fusion protein that allows to visualise proteins by fluorescence microscopy.

To visualize aggregates of N-terminal huntingtin fragment in cells, EGFP-tagged huntingtin exon1 constructs carrying 17, 49, 72 and 83 CAG repeats were analyzed. Vectors were kindly provided by E. Wanker (Table 2.5). 293T cells were seeded on cover slips in 6 well plates and transfected with respective EGFP-tagged constructs (see section 2.2.3.1.1 and Table 2.5). After 48h cells were analyzed by fluorescent imaging (section 2.2.3.3).

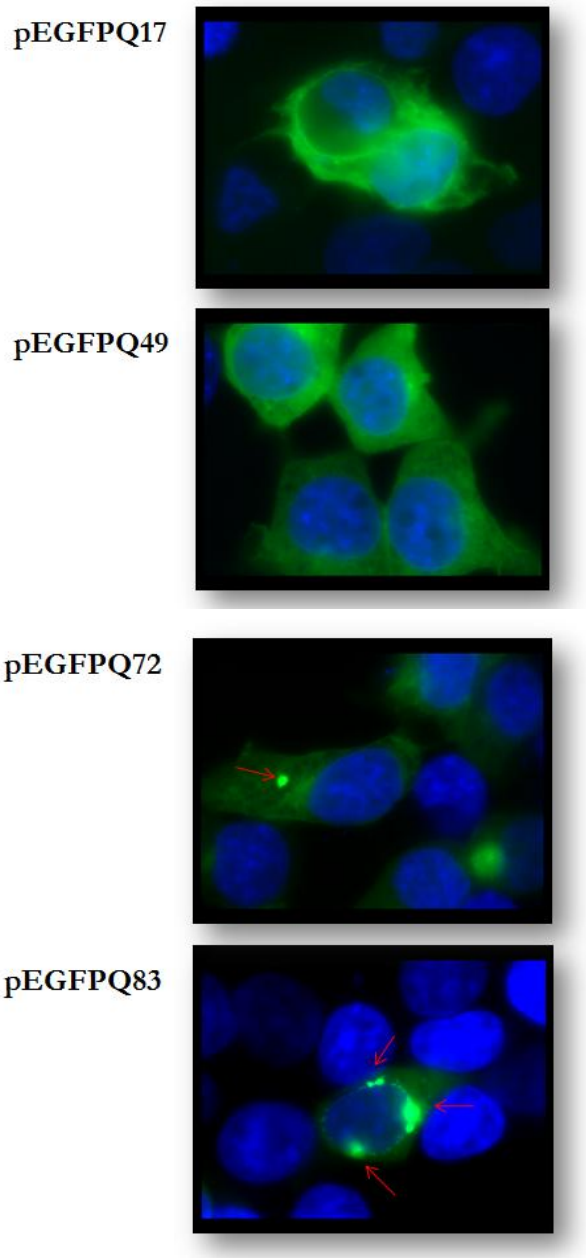

Fig. 3.6 Fluorescent imaging of the 293T cells transiently transfected with EGFP-tagged huntingtin exon1 carrying 17, 42, 72 and 83 CAG repeats. DAPI staining was used to visualize nuclei.
Upper panel: Diffused cytosolic localization of N-terminal huntingtin with 17 glutamines.
Upper middle panel: Diffused cytosolic localization of N-terminal huntingtin with 49 glutamines
Lower middle panel: N-terminal huntingtin with 72 glutamines, small aggregates, are indicated with red arrow.
Lower panel: N-terminal huntingtin with 83 glutamines, aggregates indicated with red arrows.

EGFP-tagged huntingtin exon1 constructs carrying 17 and 49 CAG repeats showed a diffused staining in the cytoplasm and no sign of aggregates (Fig. 3.6 upper and upper middle panel). In contrast, cells expressing fragment with 72 or 83 glutamines, showed a clear aggregation of N-terminal huntingtin (Fig. 3.6 lower and lower middle panel). Here, the aggregate number was much lower for the construct expressing fragment with 72 glutamines compared to the fragment with 83 glutamines, which showed more distinct aggregation pattern and less protein diffused in the cytoplasm (Fig. 3.6 lower and lower middle panel).

Previous experiments, with aggregation for a comparable construct expressing N-terminal huntingtin fragment with 51 glutamines, showed aggregation (see section 3.1.1.1.2), while in fluorescent imaging of N-terminal huntingtin fragment with 49 glutamines no aggregates were detected. Since the incubation time of the cells expressing the huntingtin constructs were different in both assays – 72h for the FA and 48h for the fluorescent imaging – this difference might be explained by a later onset of aggregation in cells (after 72h).

3.1.2.2 Western blots

In next set of experiments a correlation between amount of N-terminal huntingtin fragment diffused in the cytoplasm (Fig. 3.6), and amount of it, which could be detected as an SDS-soluble fraction, was analyzed.

Cells, as described in section 3.1.2.1, expressed EGFP-tagged N-terminal huntingtin containing 17, 49, 72 and 83 glutamines for 48h. Lysates from those cells were then analyzed on western blots.

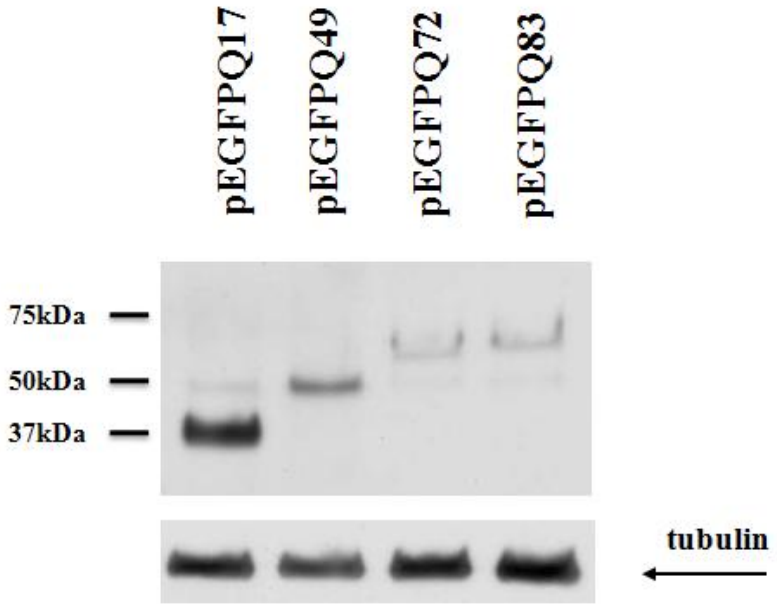

Fig. 3.7 Western blot analysis of lysates from 293T cells transiently transfected with EGFP-tagged N-terminal huntingtin fragment containing different amount of glutamines. Number of glutamines from left to right: 17, 49, 72, 83.

As shown in Fig. 3.7, the SDS soluble fractions of N-terminal huntingtin fragment could be detected in a pattern where increasing amount of CAG repeats resulted in decreased amount of the SDS-soluble protein on the western blot. Together with the observation from section 3.1.2.1, these results show a correlation between the aggregation process in the cells and a decreased SDS-solubility of N-terminal huntingtin fragment as detected by western blotting.

3.2 The MID1 protein complex binds huntingtin mRNA

3.2.1 Binding of the MID1 complex to the huntingtin RNA in a repeat - length dependent manner

It was shown previously in our group that the MID1 protein forms a multi protein complex. It associates with proteins which are involved in mRNA transport and translation. It was also shown that MID1 complex specifically associates with G-rich RNAs, thereby forming a microtubule-associated ribonucleoprotein (RNP) complex (Aranda-Orgilles, Trockenbacher et al. 2008). Therefore, we hypothesized, that the MID1 protein complex could also bind the huntingtin exon1 RNA through the CAG repeat region, since CAG repeats are per se G-rich sequences.

3.2.1.1 *In vitro* transcription and biotinylation of huntingtin exon1 RNA – optimization

A putative binding of the MID1 protein complex to the CAG repeats of the huntingtin mRNA was addressed by RNA-protein pull downs. Therefore, in a first step *in vitro* transcription and biotinylation were optimized (see section 2.2.4.1 and 2.2.4.2).

For the *in vitro* transcription, huntingtin exon1 (Htt exon1) inserts with either 20 or 51 CAG repeats were amplified from the pTetCMV –F (S°) plasmids using **httexon1T7For** and **httexon1Rev** primers (Table 2.9, Fig. 3.8 a.).

The resulting PCR-products - httexon1CAG20/51, which contained T7-promoter sequence were used for *in vitro* transcription with biotinylated UTPs.

After *in vitro* transcription, RNA was purified by phenol-chloroform extraction and precipitated with EtOH (see sections 2.2.1.3 and 2.2.1.4).

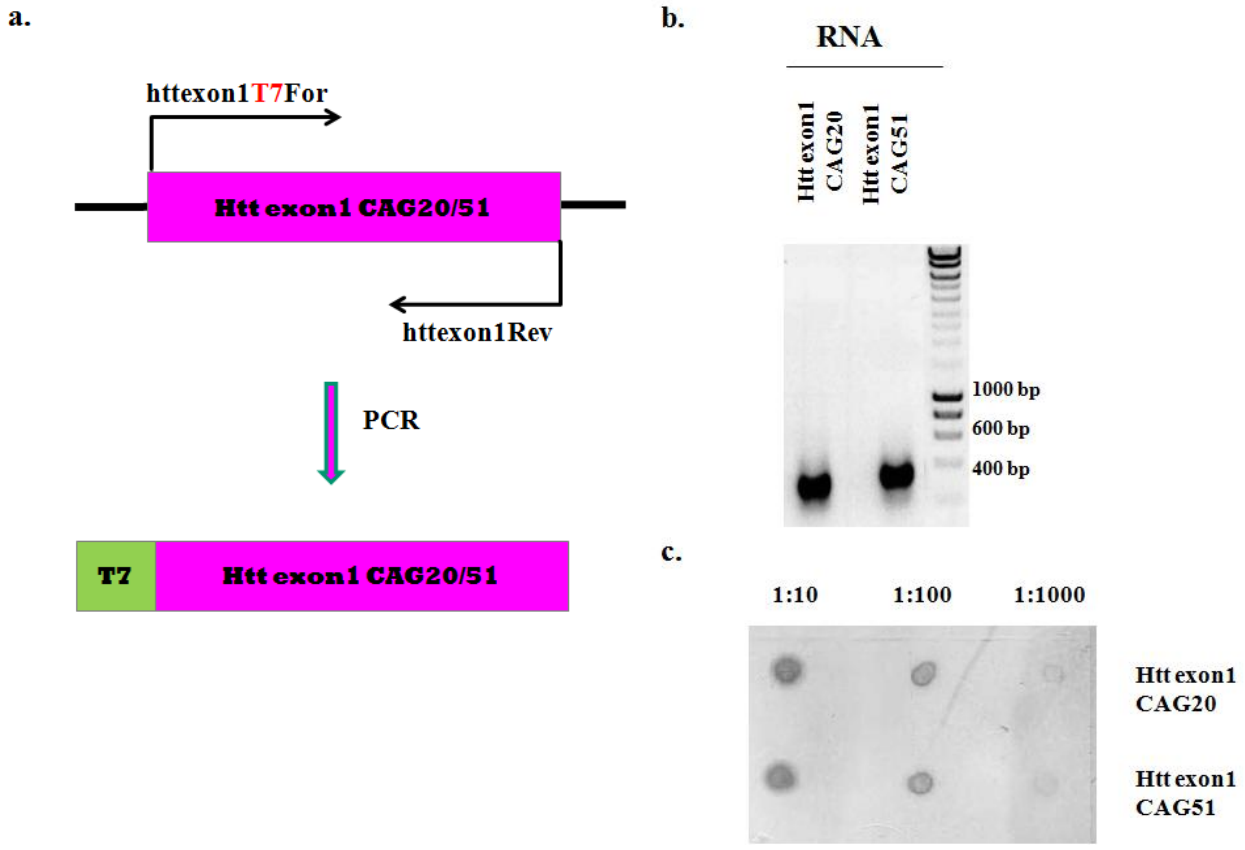

Fig. 3.8 *In vitro* transcription assay. **a**. Schematic representation of huntingtin exon1 insert and primers used for PCR. The PCR product was used for *in vitro* transcription **b.** Agarose gel showing huntingtin exon1 RNAs with either 20 or 51 CAG repeats of a size 280 bp and 370 bp respectively **c.** Dot blot showing the biotinylation efficiency of RNA containing 20 CAGs (upper panel) and 51 CAGs (lower panel). Serial dilutions shown from left to right: 1:10, 1:100, 1:1000.

As shown in Fig. 3.8 **b**, purified RNA was analyzed on agarose gels to check the sizes of *in vitro* transcribed fragments. The size of the amplified RNA fragments was in expected range for both huntingtin fragments: 280 bp - huntingtin exon1 with 20 CAG repeats and 370 bp - huntingtin exon1 with 51 CAG repeats.

During *in vitro* transcription procedure, biotinylated UTPs were incorporated into the huntingtin exon1 RNA. Equal biotinylation of all transcripts was verified by the Dot Blot (see section 2.2.4.2). Fig. 3.8 **c** showed equal biotinylation of both huntingtin exon1 transcripts with 20 and 51 CAG repeats.

3.2.1.2 RNA pull down of overexpressed MID1-Flag protein

To analyze a putative binding of the MID1 protein complex to the huntingtin exon1 transcripts containing either 20 or 51 CAG repeats, protein binding assays were performed as described in section 2.2.4.3. Cytosolic extracts of 293T cells over expressing MID1-Flag protein were incubated with the huntingtin transcripts. Biotinylated transcripts were then purified with streptavidin coated magnetic beads and extensively washed. Finally, proteins bound to the huntingtin exon1 transcripts were analyzed on western blots, using antibodies detecting MID1-Flag and other MID1 complex proteins (Table 2.4 and Fig. 3.9).

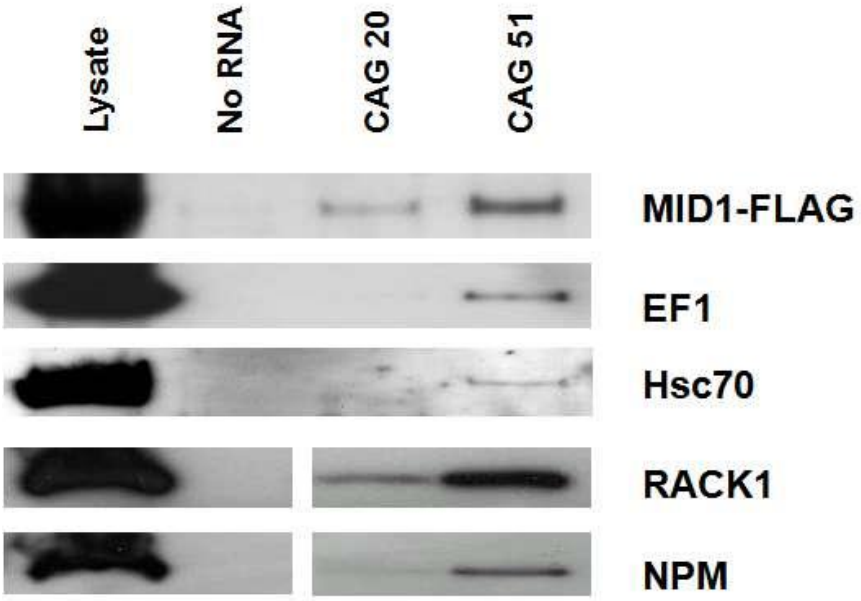

Fig. 3.9 Western blot analysis of RNA pull down assay of cytosolic extracts from cells over expressing MID1-Flag protein, showing the binding of the MID1 protein complex to the biotinylated huntingtin exon1 RNA. From left to right: first line shows the protein lysate, second line - sample where no RNA was added, third and fourth line - samples to which httexon1CAG20 or httexon1CAG51 transcripts were added respectively. Each panel represents detection with antibodies recognizing either MID1-Flag or proteins which belong to the MID1 protein complex.

As shown in Fig. 3.9, MID1-Flag, EF-1α (elongation factor 1α), NPM (nucleophosmin/B2 3.2), RACK1 (receptor for activated kinase 1) and Hsc70 bound to the huntingtin exon1 RNA. A clear correlation between the amount of CAG repeats in huntingtin exon1 RNA and the amount of protein which bound to it could be seen.

3.2.1.3 RNA pull down of endogenous MID1 protein

To analyze the binding of the endogenous MID1 in a similar assay, cell lysates of non-transfected cells were subjected to the RNA pull down procedure.

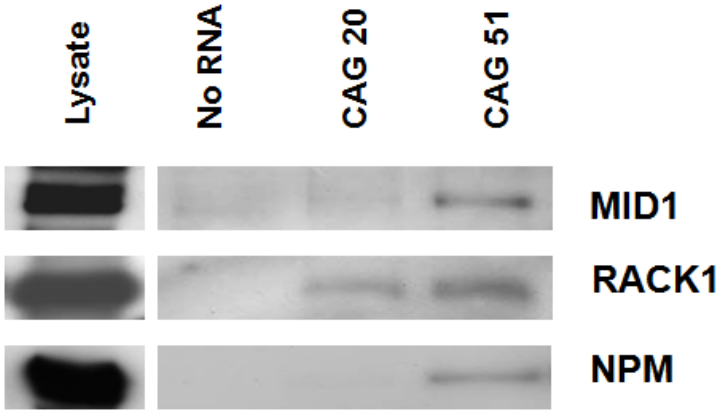

Fig. 3.10 Western blot analysis of RNA pull down of cytosolic extracts from non-transfected cells. From left to right: first line shows the protein lysate, second line - sample where no RNA was added, third and fourth line - samples to which httexon1CAG20 or httexon1CAG51 transcripts were added respectively. Each panel represents detection with antibodies recognizing either MID1 or proteins which belong to the MID1 protein complex. Increased amount of CAG repeats results in increased binding of MID1 protein and some of the interacting partners.

As shown in Fig. 3.10, MID1 and the MID1 complex proteins like NPM and RACK1 could be detected on the resulting western blot. As for the experiment with overexpressed protein, increased amounts of CAG repeats resulted in increased binding of the MID1 protein complex.

3.2.1.4 Salts interfering with RNA-protein binding

It has been previously shown that the Fragile X mental retardation protein (FMRP), in the most common mental retardation, trinucleotide expansion disorder, Fragile-X-syndrome, is able to bind G-rich RNA sequences, through G-quartet motifs. It has been shown, that binding of the FMRP to the RNA depends on ions, namely potassium and sodium cations, which stabilize the RNA structure, whereas cations like lithium destabilize it (Darnell, Jensen et al. 2001; Schaeffer, Bardoni et al. 2001; Ramos, Hollingworth et al. 2003). Furthermore, it has been reported that lithium in addition to its mood-stabilizing effects, shows also neuroprotective effects (Wei, Qin et al. 2001; Wada, Yokoo et al. 2005) and reduces neurodegeneration in Huntington's disease (Senatorov, Ren et al. 2004). The therapeutic mechanisms of lithium are not well understood, although it has been reported that lithium up-regulates cell survival molecules like Hsp70, Bcl-2 and others, whereas down-regulating pro-apoptotic activities (p53, caspase, β-amyloid peptide production), which prevents neuronal cell death. In addition in cell models of HD, pretreatment with lithium reduced polyglutamine toxicity via inhibiting GSK-3β activity (Wada, Yokoo et al. 2005).

Expanded CAG repeats seem to form a double-stranded RNA structure, the stability of which increases with number of repeats (Sobczak and Krzyzosiak 2005). Therefore, it was interesting to investigate if different cations might influence the structure of this CAG repeats and thereby influence its binding to the MID1 protein complex.

3.2.1.4.1 KCl influences the binding of huntingtin exon1 RNA to the MID1 protein complex

To analyze the influence of KCl on the RNA-protein binding of MID1 to huntingtin exon1 CAG repeats, increasing amounts of KCl were added to RNA-protein pull downs as described in section 3.2.1.2.

The RNA-bound proteins were analyzed on western blots with antibodies against MID1-Flag and the MID1complex proteins : NPM and RACK1 (Fig. 3.11).

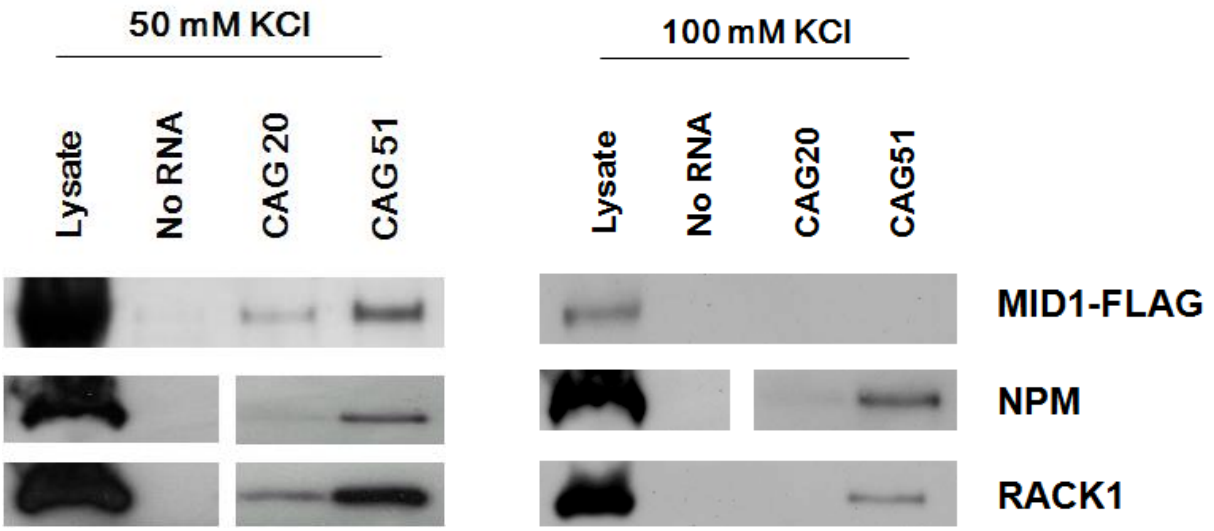

Fig. 3.11 Western blot analysis of influence of different KCl concentrations on the binding abilities of the MID1 protein complex to huntingtin exon1 RNA. From left to right: first line shows the protein lysate, second line - sample where no RNA was added, third and fourth line - samples to which httexon1CAG20 or httexon1CAG51 transcripts were added respectively. Each panel represents detection with antibodies recognizing either MID1-Flag or proteins which belong to the MID1 protein complex: NPM and RACK1.
a. 50 mM KCl concentration in the reaction mix. **b.** 100 mM KCl concentration in the reaction mix.

As shown in Fig. 3.11, different KCl concentrations could indeed influence the binding of MID1-Flag and its interacting partners to huntingtin exon1 RNA.
While 50 mM KCl allowed more efficient binding of proteins to RNA as shown in Fig. 3.11 **a.**, higher concentration of KCl (100 mM), led to decreased binding of proteins from the MID1 complex (NPM, RACK1) and no binding of MID1-Flag to the huntingtin exon1 RNA Fig. 3.11 **b.**
The correlation between amount of protein bound to RNA in relation to amount of CAG repeats present in RNA of huntingtin exon1 was not influenced by salt concentration.

3.2.1.4.2 Na, Mg or Li influence the binding of the exon1 huntingtin RNA to the MID1 protein complex

To investigate the influence of other ions on the interaction between huntingtin exon1 RNA and MID1 protein complex NaCl, $MgCl_2$ or LiCl were added to the previously described assays (see section 2.2.4.3).

Following procedure described in section 3.2.1.2, cytosolic extracts of cells over expressing MID1-Flag protein were incubated with biotinylated huntingtin exon1 transcripts. In addition, different salts were added to the reaction mix for the incubation time.

RNA-bound proteins were then analyzed on western blots with specific antibodies detecting MID1-Flag and other interacting proteins: Hsc70, NPM and RACK1 (Fig 3.12).

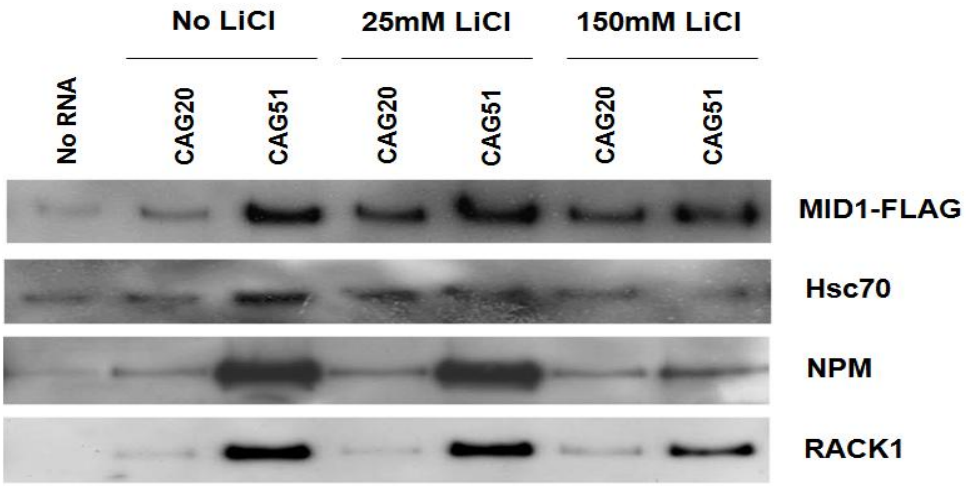

Fig. 3.12 Western blot analysis showing the influence of LiCl on the binding abilities of the MID1 protein complex to the huntingtin exon1 RNA. From left to right: first line shows the sample where no RNA was added, second and third line: samples to which httexon1CAG20 or httexon1CAG51 transcripts were added respectively – control non treated with lithium. Next 4 lines show samples to which httexon1 CAG20 or httexon1CAG51 transcripts were added and which were treated with 25 mM and 150 mM LiCl respectively. Each panel represents detection with antibodies recognizing either MID1-Flag or proteins which belong to the MID1 protein complex: Hsc70, NPM and RACK1.

As shown in Fig. 3.12, the control assay performed without LiCl showed a binding pattern similar to results observed in section 3.2.1.2, where more CAG repeats led to binding of higher levels of protein.

Addition of 25 mM LiCl showed a slight increase of RNA - protein binding, especially for MID1-Flag, to the transcript containing 20 CAGs, but not to the longer transcript containing 51 CAGs. 150 mM LiCl decreased at most the binding of the MID1 complex to the transcript with 51 CAG repeats (Fig. 3.12).

Following the same procedure as described for lithium , influence of NaCl and $MgCl_2$ were tested (Fig. 3.13).

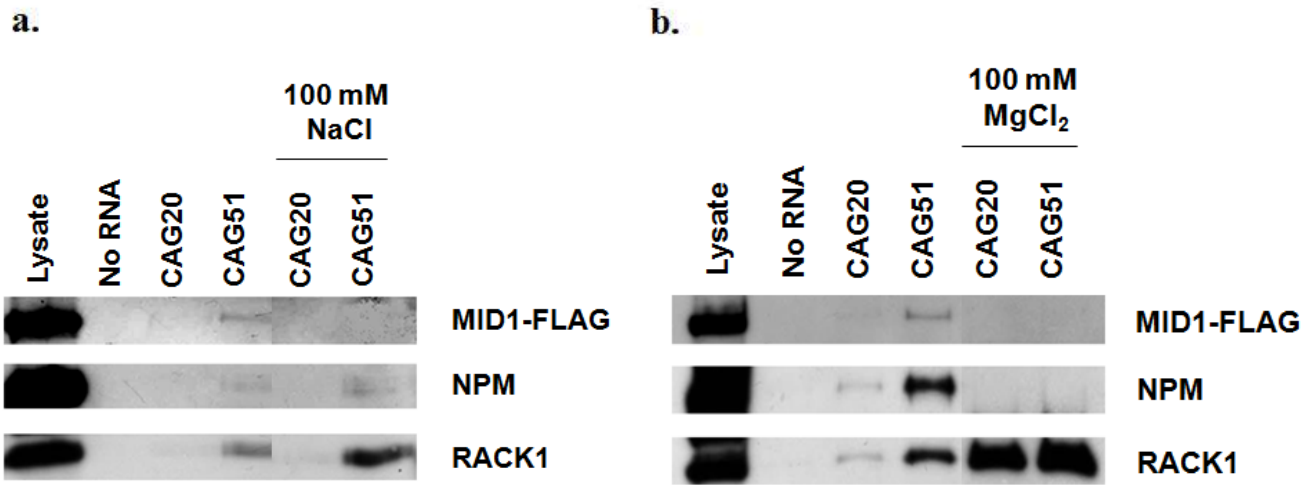

Fig. 3.13 Western blot analysis showing influence of NaCl and $MgCl_2$ ions on the binding abilities of the MID1 protein complex to the huntingtin exon1 RNA. From left to right: protein lysate, second line - sample where no RNA was added, third and fourth line - samples to which httexon1CAG20 or httexon1CAG51 transcripts were added respectively, fifth and sixth line - samples with httexon1CAG20 or httexon1CAG51 transcripts to which $MgCl_2$ (**a.**) or NaCl (**b.**) was added. Each panel represents detection with antibodies recognizing either MID1-Flag or proteins which belong to the MID1 protein complex: NPM and RACK1.

As shown in Fig. 3.13 **a.**, 100 mM NaCl reduced the binding abilities of the MID1-Flag protein. In contrast, NaCl did not seem to have any influence on the other MID1 complex proteins like NPM or RACK1, binding of which was not changed after NaCl

treatment. $MgCl_2$ reduced the binding abilities of Mid1-Flag as well as NPM to the huntingtin exon1 RNA, while the binding of RACK1 was not changed (Fig. 3.13 **b.**).
Taken together those data show, that cations influence the binding abilities of the MID1 complex to the huntingtin exon1 RNA. Potassium cations in low concentration were able to stabilize interaction between MID1 complex and huntingtin exon1 RNA, whereas cations like lithium, sodium or magnesium destabilized it, with the exception for the binding of the RACK1.

3.3 Increased translation of huntingtin exon1 mRNA with elongated CAG repeats

The MID1 protein complex binds proteins, which are involved in mRNA transport and translation i.e. EF-1α, NPM, RACK1. Furthermore, it was shown that MID1 complex associates with active polyribosomes which indicates its involvement in mRNA translation (Aranda-Orgilles, Trockenbacher et al. 2008). Therefore we hypothesized a putative influence of the MID1 complex on the translation of the huntingtin mRNA.
To analyze this, three different assays were performed: (i) transient transfection of a 510 aa N-terminal huntingtin fragment in cell lines, (ii) luciferase reporter assay and (iii) *in vitro* translation assay.

3.3.1 Increased protein amounts produced from constructs containing 510 aa huntingtin with elongated CAG repeats

To investigate translation efficiency of huntingtin mRNA carrying different CAG repeat length, constructs expressing 500 aa huntingtin fragment with either non pathological or pathological amount of glutamines (17 or 47 respectively) were used.
293T cells were transfected with HTT-510AA-CAG17/CAG49 and pEGFP-C1 as an internal transfection control (Table 2.5, section 2.2.3.3.1). After 48h of protein expression, cell lysates were prepared and 50 µg of each sample was then analyzed on western blots using antibodies specifically detecting GFP (anti-GFP) and huntingtin exon1 (Anti-EP064200) (Table 2.4 and Fig. 3.14).

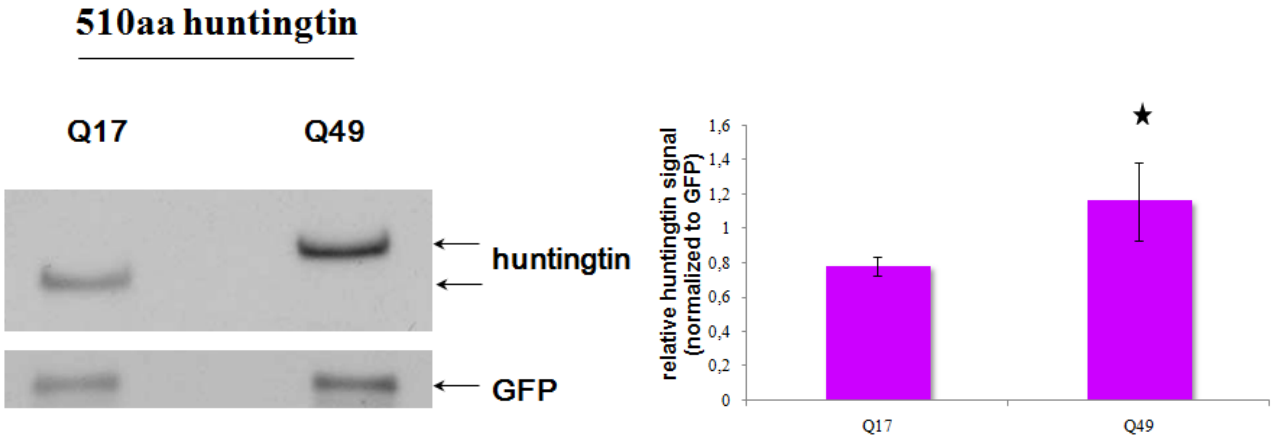

Fig. 3.14 Western blot analysis of 293T cells co-transfected with pEGFP-C1 and HTT-510AA-CAG17/HTT-510AA-CAG49. Quantifications of HTT-500AA normalized to GFP are shown (*p < 0,05; T-test).

The expression of 510 aa huntingtin containing 49 glutamines showed a clear increase in protein amount when normalized to the GFP transfection control, compared to the construct containing 17 CAG repeats (Fig. 3.14).
This could be the first indication, that translation of 510 aa huntingtin fragment with 47 glutamines is being influenced.

3.3.2 Luciferase reporter assay

To investigate further the translation of huntingtin exon1 mRNAs carrying different amount of CAG repeats, a series of reporter assay experiments were performed.

3.3.2.1 Cloning strategy

To investigate the influence of CAG repeat motifs of different length on the translation efficiency of mRNAs, luciferase-reporter constructs containing either 14 or 47 CAG repeats in the 3'UTR of the firefly mRNA were designed (pGL3m-CAG14/CAG47 Table 2.5 and Fig. 3.15). By this strategy, putative effects of protein

aggregation would be avoided. Subsequently those constructs were used for the dual luciferase reporter assays (see section 2.2.3.5).

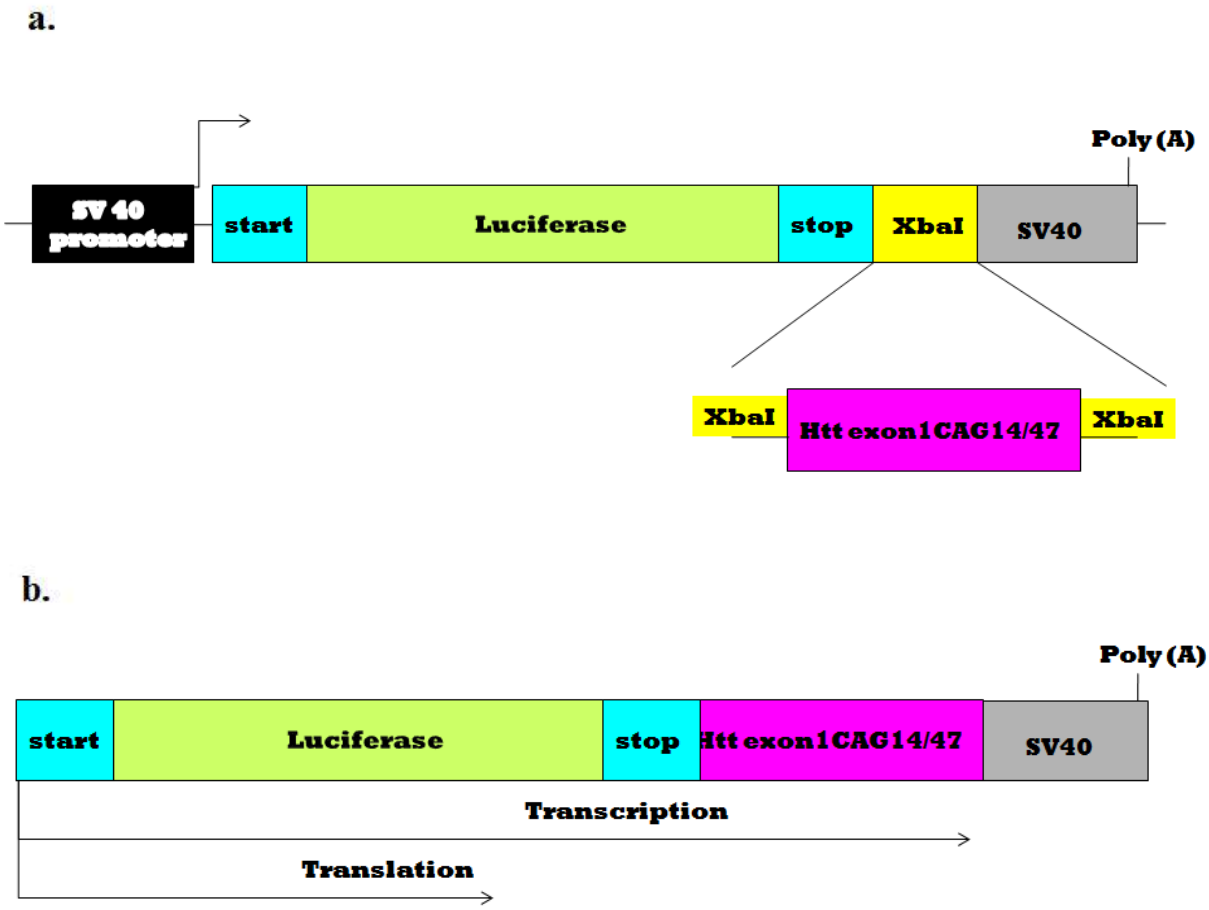

Fig. 3.15 a. Schematic representation of luciferase - pGL3m construct with huntingtin exon1 containing either 14 or 47 CAG repeats cloned behind the stop codon. **b.** Schematic representation of how the construct was transcribed and translated.

3.3.2.2 Dual luciferase assay in HeLa and U373 cells

3.3.2.2.1 Luciferase – reporter signal depends on the amount of CAGs in exon1 of huntingtin

To investigate the influence of different amounts of CAG repeats on luciferase translation, HeLa and U373 cells (Table 2.13) were transfected with previously described pGL3m-CAG14/47 luciferase constructs (see section 2.2.3.1.2).

After 24h of transient expression of pGL3m constructs, cells were lysed and analyzed by the Dual Luciferase reporter assay (Promega) (Fig. 3.16).

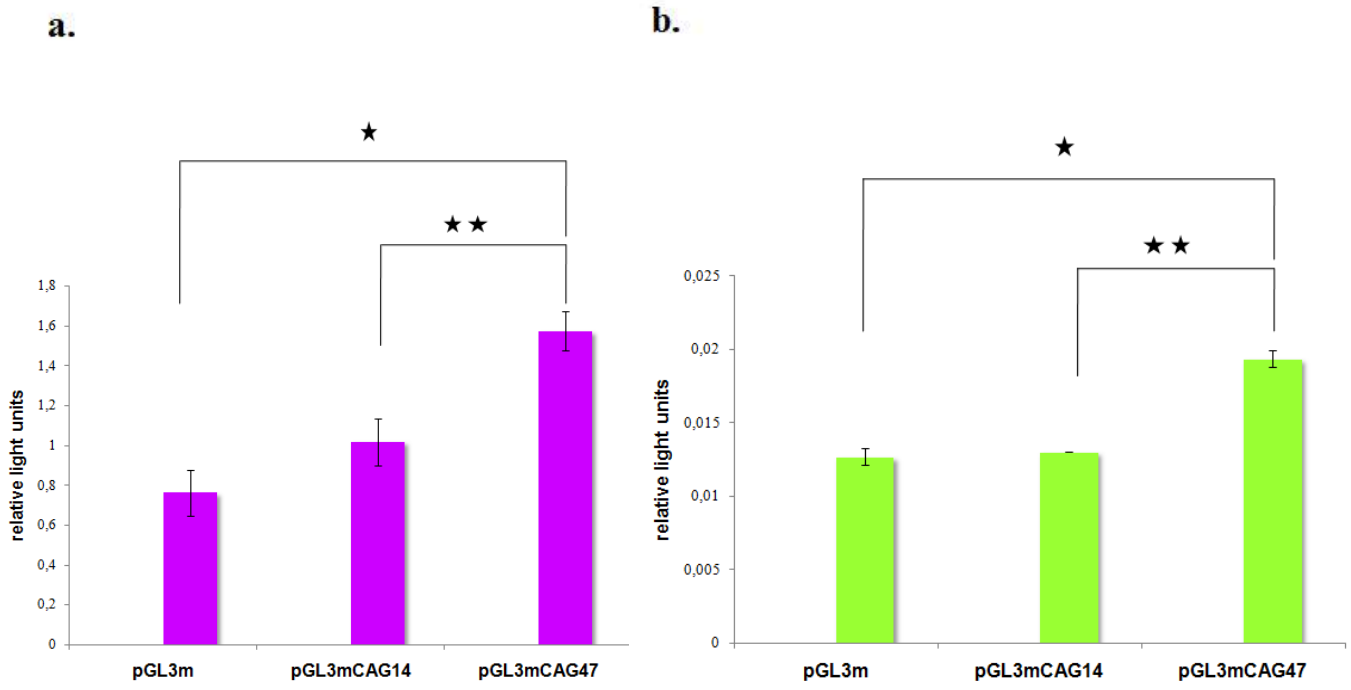

Fig. 3.16 Dual luciferase assay of HeLa (**a.**) and U373MG (**b.**) cells transfected with firefly luciferase carrying 14 or 47 CAG repeats. Columns represent light units of firefly luciferase normalized to renilla ($p<0.001$; T-test).

In both, HeLa and U373 cells there was a significant increase of firefly luciferase signal observed for 47 CAG repeats, while 14 CAG repeats did not induce a significant increase compared to the empty vector control (Fig. 3.16).

3.3.2.2.2 CAG repeats do not influence mRNA levels of luciferase

To check whether the increase of firefly luciferase activity shown in Fig. 3.16 was indeed due to increased translation, and was not caused by an increased transcription, the mRNA levels were analyzed by RealTime PCR (see section 2.2.1.8).

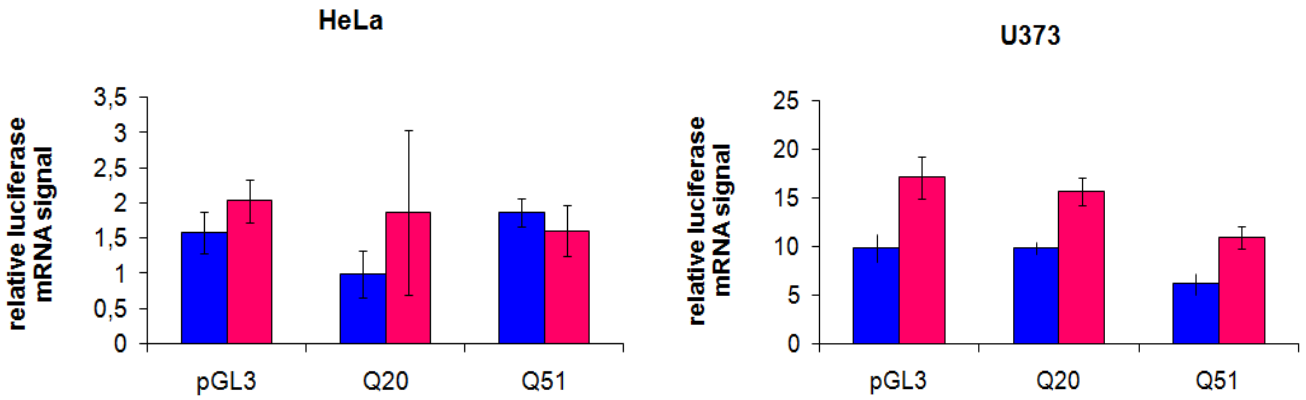

Fig. 3.17 RealTime PCR analysis of HeLa and U373 cells showing firefly luciferase expression normalized to renilla. Blue columns represent GAPDH control signal normalized to pRL, whereas pink columns represent firefly luciferase signal normalized to pRL.

RealTime PCR performed on cDNA from the cells transfected with pGL3m-CAG14/47 plasmids showed no significant difference on mRNA level (Fig. 3.17). These results indicate that the increase in firefly luciferase activity was due to an increased translation regulation of constructs with elongated CAG stretch, while having no influence on transcription of mRNA.

3.3.3 *In vitro* translation assay

To further prove that the translation of mRNAs with elongated CAG repeats is increased, *in vitro* translation assays were performed as described in section 2.2.5.
A firefly luciferase construct containing huntingtin exon1 with either 14 or 47 CAG repeats in the 3'UTR was first *in vitro* transcribed (see section 2.2.5.1), and subsequently equal amounts of those transcripts were used for *in vitro* translation assays (see section 2.2.5.2).

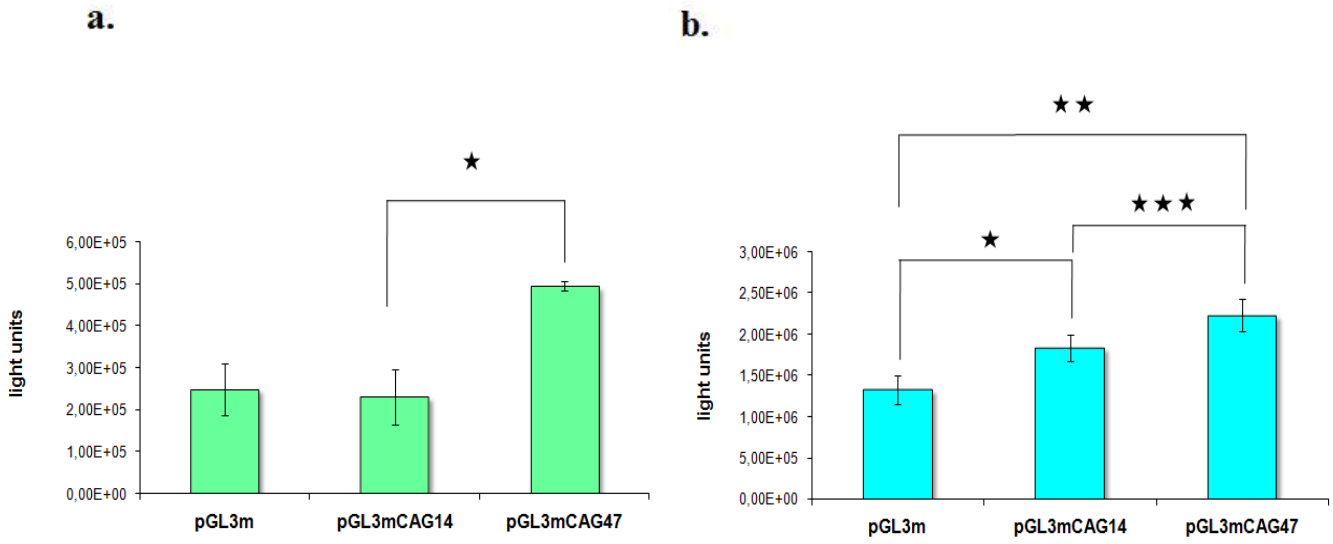

Fig. 3.18 *In vitro* translation assay of transcripts containing either 14 or 47 CAG repeats (constructs from section 3.3.2.2.1) ($p<0.003$; T-test). Columns represent the signal intensity of the firefly luciferase measured after 60 min (**a.**) and 90 min (**b.**)

In vitro translation assay was performed for 60 and 90 min (Fig. 3.18 **a., b.**). After 60 min a significant increase in firefly translation was observed for transcripts containing 47 CAG repeats. Extended experimental time, up to 90 min, showed also a significant increase in translation of transcript containing 47 CAG repeats, and additionally a slight but significant increase in translation of transcript containing 14 CAG repeats.

These data, together with the observation from section 3.3.1 and 3.3.2 suggest, that elongated CAG stretch in huntingtin exon1 increased the translation efficiency of huntingtin mRNA.

3.4 Manipulation of the MID1 complex affects N-terminal huntingtin levels

Prompted by the observation that the MID1 multi protein complex binds to the mRNA of huntingtin exon1 in a CAG length dependent manner (section 3.2) and that the amount of CAG repeats in huntingtin mRNA has an influence on its translation (section 3.3), in a next step I wanted to investigate if the MID1 protein has an influence on the translation of the huntingtin mRNA.

3.4.1 MID1 knockdown effects on expression of huntingtin exon1 containing 20 or 51 CAG repeats

To analyze if the MID1 protein complex could influence the translation of huntingtin exon1 mRNA containing different amount of CAG repeats, the MID1 protein was depleted by siRNA mediated knockdown. Therefore 293Q20 and Q51 cells were transfected with MID1 specific siRNAs (see section 2.2.3.1.3 and Table 2.8). 24h after transfection the expression of N-terminal huntingtin fragment was induced and after additional 48h cell lysates were analysed on western blots using antibodies specific to N-terminal huntingtin fragment, MID1 and GAPDH (Table 2.4, Fig. 3.19).

Knockdown of MID1 caused a slight decrease in protein levels of N-terminal huntingtin fragment containing 51 glutamines, while the production of N-terminal huntingtin fragment with 20 glutamines was not affected. The efficiency of the MID1 knockdown procedure was evaluated by the detection with antibodies recognizing MID1 protein (Table 2.4 and Fig. 3.19).

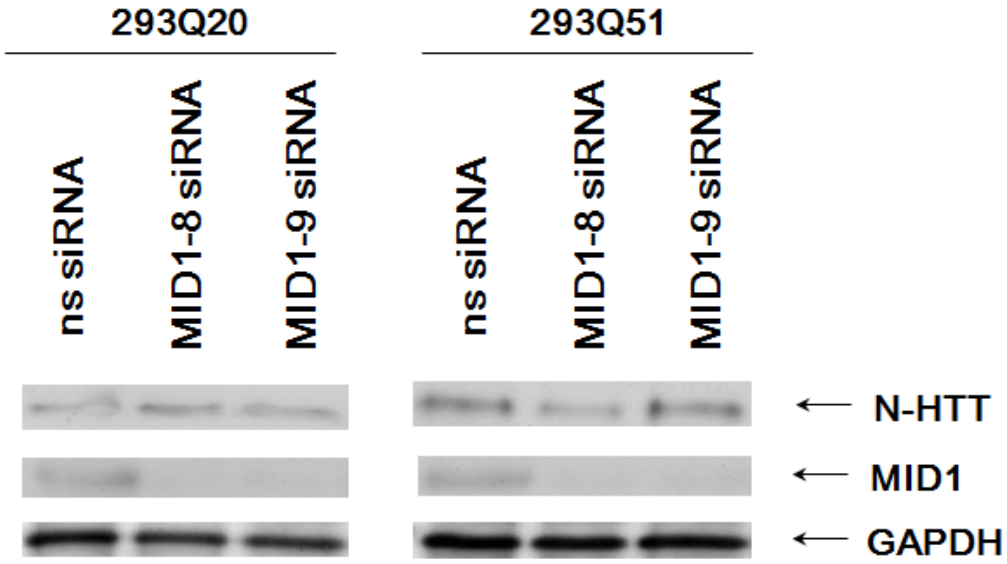

Fig. 3.19 Western blot analysis of cells expressing N-terminal huntingtin fragment (N-HTT) with 20 glutamines (293Q20, left panel) or 51 glutamines (293Q51, right panel) under MID1 knockdown. From left to right: lines with samples after transfection with ns siRNA, MID1-8 siRNA and MID1-9 siRNA respectively. Each panel represents detection with antibodies recognizing either N-terminal fragment of huntingtin, MID1 or GAPDH as a loading control.

3.4.1 α4 knockdown effects on expression of huntingtin exon1 containing 20 or 51 CAG repeats

In a next series of experiments, another important component of MID1 protein complex, the α4 protein was targeted by knockdown.

As described in the previous section, 293Q20 and Q51 cells were transfected with α4 specific siRNA (see section 2.2.3.1.3 and Table 2.8). 24h after transfection, expression of N-terminal huntingtin fragment was induced and after additional 48h cell lysates were analyzed on western blots using antibodies recognizing the N-terminal huntingtin fragment, α4 and GAPDH (Table 2.4 and Fig. 3.20).

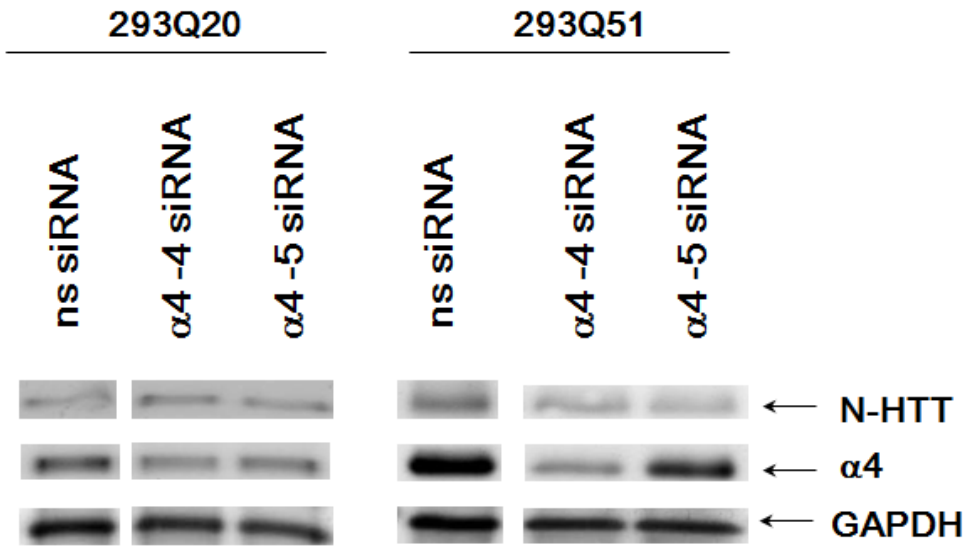

Fig. 3.20 Western blot analysis of cells expressing N-terminal huntingtin fragment (N-HTT) with 20 CAG repeats (293Q20, left panel) or 51 CAG-repeats (293Q51, right panel) under α4-knockdown. From left to right: lines with samples after transfection with ns siRNA, α4-4 siRNA and α4-5 siRNA respectively. Each panel represents detection with antibodies recognizing either N-terminal fragment of huntingtin, α4 or GAPDH as a loading control.

Knockdown of α4 caused a decrease in production of N-terminal huntingtin fragment containing 51 glutamines, while the production of N-terminal huntingtin fragment with 20 glutamines was not affected. The efficiency of the α4 knockdown procedure was evaluated by the detection with antibodies recognizing α4 protein (Table 2.4 and Fig. 3.20).

Taken together, these results suggest that manipulation of the MID1 protein complex, influences the production of N-terminal huntingtin fragment containing elongated polyglutamine stretch.

3.5 The MID1 protein complex influences aggregate formation

One of the pathological hallmarks in Huntington‘s disease is the formation of intracellular, insoluble inclusions of polyglutamine-containing proteins in the brain. Insoluble aggregates have been shown to disrupt several intracellular pathways by sequestering some pathway components into aggregates (Gil and Rego 2008).

To investigate huntingtin protein aggregates, the 293Q83 cell line was used (see section 3.1.1.1.1 and Table 2.13).

Prompted by the observation that: (i) MID1 protein complex binds huntingtin mRNA in a repeat length dependent manner, that (ii) the translation of huntingtin mRNA increases with increasing amount of CAG repeats and that (iii) disruption of the MID1 protein complex reduces production of N-terminal huntingtin fragment, I wanted to analyze, if the MID1 protein complex influences the aggregate formation in cell system.

3.5.1 Optimization of knockdown procedures for the Filter retardation assay

To analyze the putative effects of the MID1 protein complex on the aggregate formation, α4 knockdowns were performed. Different transfection/incubation procedures were tested and the experimental conditions for the α4 knockdown were set. The following experimental procedures were tested: α4 knockdown was performed, either 24h prior to induction of N-terminal huntingtin fragment expression, or 24h after expression induction (Fig. 3.21).

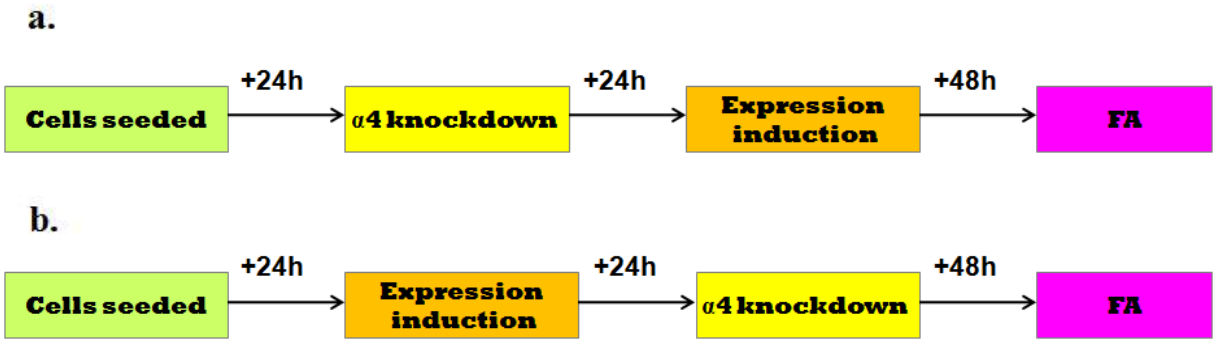

Fig. 3.21 Schematic representation of experimental time line for the knockdown and protein expression. **a.** Knockdown of α4 protein prior to expression induction. **b.** Knockdown of α4 protein after expression induction.

At first, procedure shown in Fig. 3.21 **a**. was performed. Therefore, 293Q83 cells were transfected with α4 specific siRNA, protein expression was induced 24h after transfection, and after additional 48h, cell lysates were prepared and analyzed on FA and on western blots. Membranes FA were analyzed with the specific antibodies recognizing polyglutamine stretch (Anti-CAG53b, Table 2.4). The efficiency of the α4 knockdown procedure was evaluated by the detection with antibodies recognizing α4 protein (Table 2.4 and Fig. 3.22).

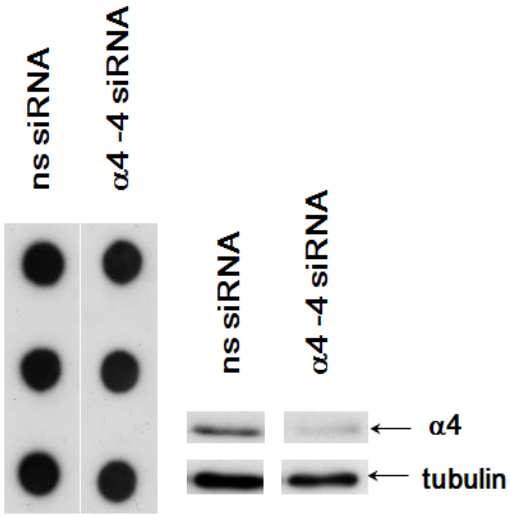

Fig. 3.22 FA of cells under α4 knockdown, followed the procedure shown in Fig. 3.21 (**a.**). Detection of aggregates (left) and Western blots (right) for knockdown controls are shown.

In this set of experiments α4 specific knockdown was successful. Amount of α4 protein was decreased in compare to the control sample. However, there was no change in aggregate amount observed in compare to the control sample (Fig. 3.22).
An efficient α4 knockdown performed prior to the expression induction of N-terminal huntingtin fragment (Fig. 3.22) did not result in decrease in the aggregate amount. Therefore, the second procedure shown in Fig. 3.21 **b.** was performed.
293Q83 cells were transfected with α4 specific siRNA around 24h after expression induction, and after additional 48h, cell lysates were prepared and analyzed on FA and on western bolts. Membranes from the FA were analyzed with the specific antibodies recognizing polyglutamine stretch (Anti-CAG53b, Table 2.4). The efficiency of the α4 knockdown procedure was evaluated by the detection with antibodies recognizing α4 protein (Table 2.4 and Fig. 3.23).

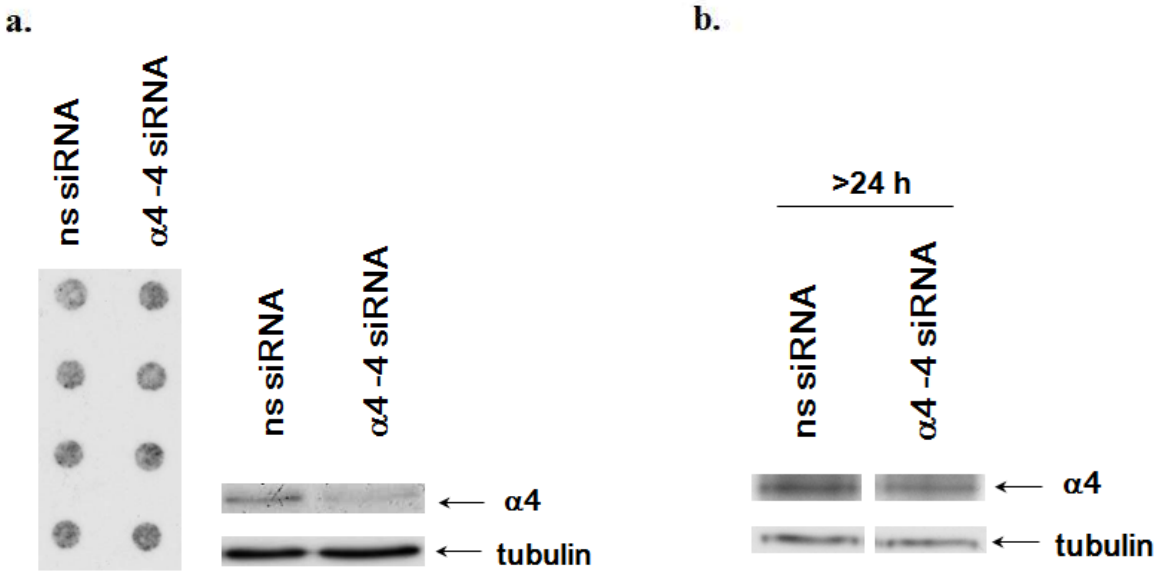

Fig. 3.23 **a.** FA of cells transfected under α4 knockdown, followed the procedure shown in Fig. 3.21 (**b.**). Detection of aggregates (left) and Western blots (right) for knockdown controls are shown. **b.** Western blot for knockdown control analyzed around the time point of induction of protein expression.

As shown in Fig. 3.23 **a.**, no decrease in aggregate formation was observed, although at the end of experiment, the level of the α4 was decreased. Therefore the efficiency of the knockdown was also analyzed around the time point when the

protein production was induced (Fig. 3.23 **b.**). At this time point no obvious decrease in α4 level was detected, in compare to the control sample.

Taken together, all these experiments indicated, that the depletion of the protein which belongs to the MID1 protein complex (α4), before the production of the huntingtin is induced, might have a crucial meaning.

3.5.2 α4 knockdown decreases aggregate formation

Based on the observation from the previously shown experiments, new experimental conditions were set. The knockdown of α4 protein was allowed to establish for a longer time (28h) before the N-terminal huntingtin fragment expression was induced (Fig. 3.24).

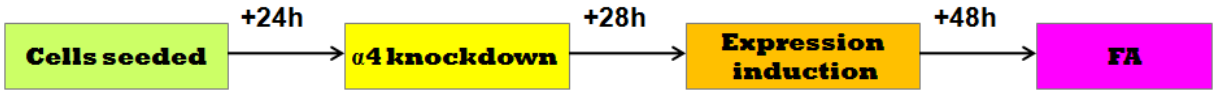

Fig. 3.24 Schematic representation of final experimental time line for the knockdown and protein expression.

Following this procedure, after siRNA transfection and protein production, cell lysates were prepared and analyzed on FA and on western blots. Membranes from the FA were analyzed with the specific antibodies recognizing polyglutamine stretch (Anti-CAG53b, Table 2.4). The efficiency of the α4 knockdown procedure was evaluated by the detection with antibodies recognizing α4 protein (Table 2.4 and Fig. 3.25).

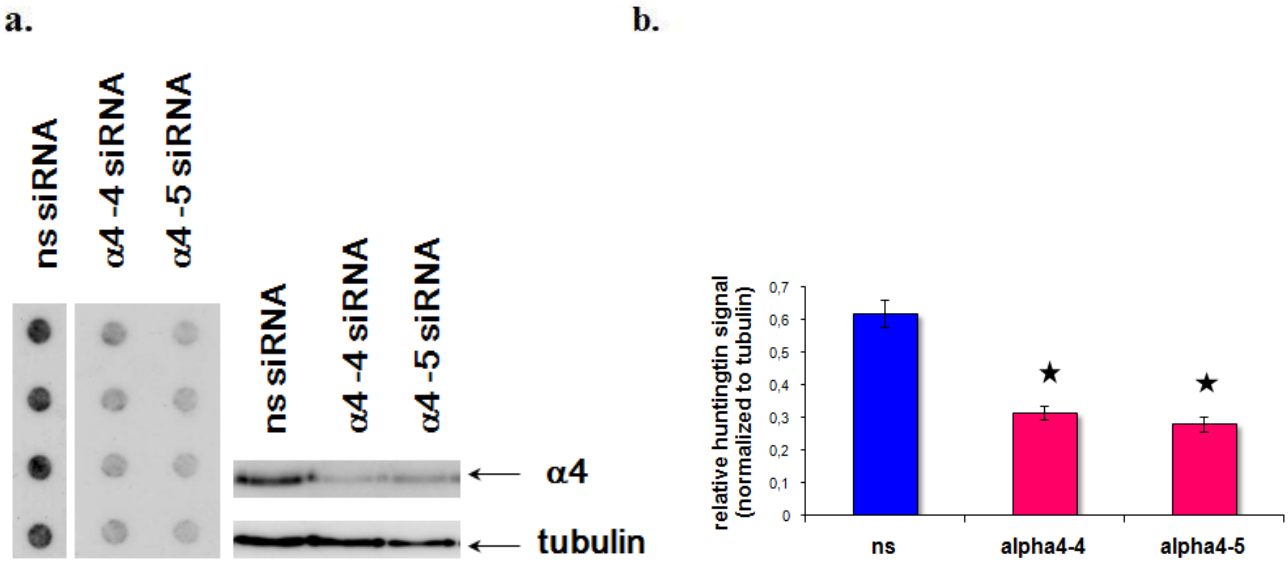

Fig. 3.25 FA of cells under α4 knockdown. **a.** Huntingtin aggregate detection (left), Western blots for knockdown control (right). **b.** Aggregate quantification is shown ($p<0.00001$; T-test).

As shown in Fig. 3.25 **a.**, a decrease in aggregate formation was observed after efficient knockdown of α4 protein with two different specific siRNA (Table 2.8).
Aggregation decrease was quantified using ImageQuant v.5.2. and presented as a graph in Fig. 3.25 **b**.

3.5.3 MID1 knockdown decreases aggregate formation

In a next series of experiments, I wanted to investigate the influence of the MID1 depletion on the aggregate formation.
MID1 knockdown experiments were performed according to the schema shown in Fig. 3.24. After siRNA transfection and protein production, lysates were prepared and analyzed on FA and on western blots. Membranes from the FA were analyzed with the specific antibodies recognizing polyglutamine stretch (Anti-CAG53b, Table 2.4). The efficiency of the MID1 knockdown was evaluated by the detection with antibodies recognizing MID1 protein (Table 2.4 and Fig. 3.26).

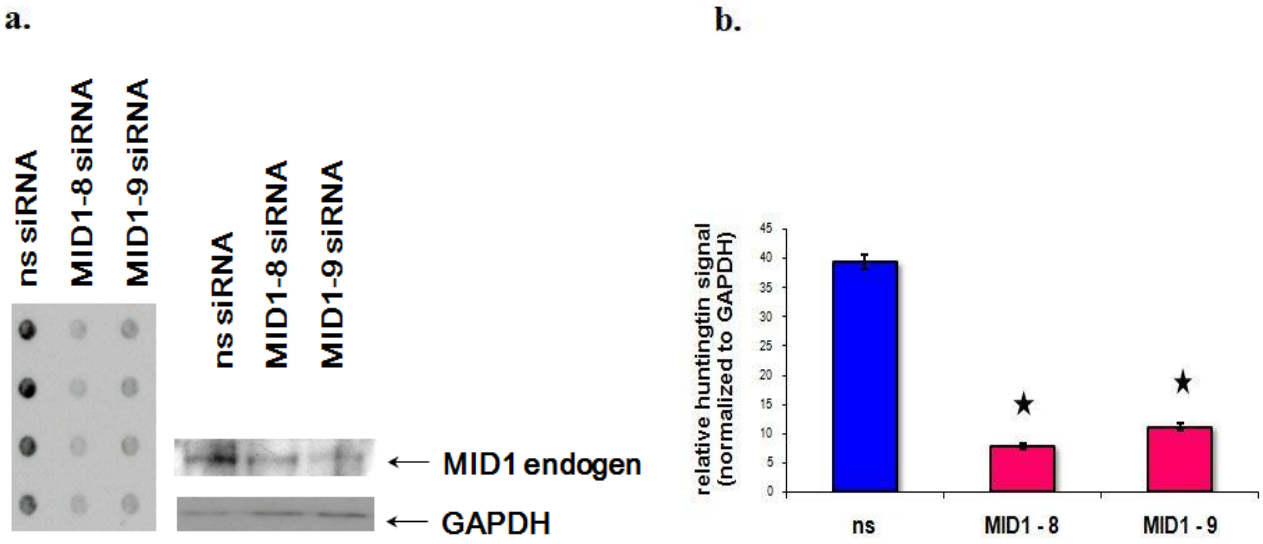

Fig. 3.26 FA of cells under MID1 knockdown. **a.** Huntingtin aggregate detection (left), Western blots for knockdown control (right). **b.** Aggregate quantification is shown ($p<0.00001$; T-test).

A clear decrease of aggregates was observed in the Filter retardation assay after the efficient MID1 depletion (Fig. 3.26 **a.**). Aggregation decrease was quantified using ImageQuant v.5.2. and presented as a graph in Fig. 3.26 **b.**

3.6 MID1 complex does not influence aggregate clearance

There are two main degradation pathways in the cells: autophagy and proteasome degradation. To test whether the MID1 protein complex could also influence clearance of aggregates via autophagy or the proteasome, I investigated if an interruption of the MID1 protein complex could trigger degradation pathways in the cells, thereby leading to degradation of the N-terminal huntingtin fragment and a decrease in aggregate formation.

3.6.1 Autophagy

To investigate if disruption of the MID1 protein complex, for example by α4 knockdown, could decrease aggregate formation by inducing autophagy, experiments in which treatments with specific, well known autophagy inhibitors (3MA, wortmannin) were performed.

293Q83 cells were transfected with specific α4 siRNAs, to knockdown α4. Afterwards the N-terminal huntingtin fragment expression was induced, and autophagy inhibitors were added to the cells at the same time (see section 2.2.3.2). After 48h cell lysates were prepared and analyzed on FA and on western blots. FA membranes were analyzed with the specific antibodies recognizing polyglutamine stretch (Anti-CAG53b, Table 2.4). Western blots were analyzed with specific antibodies recognizing α4 protein to prove the knockdown efficiency, and tubulin antibodies as a loading control (Fig.3.27 **a.** and Table 2.4).

In the control experiment without autophagy inhibitors a decrease in aggregate formation after α4 knockdown was observed (Fig. 3.27 **a.**). 3 MA and wortmannin treatment induced an increase in aggregate amount in all samples (α4 knockdowns and non-silencing) however, the differences in aggregation between the knockdowns and non-silencing control samples did not change by the addition of autophagy inhibitors. The same pattern in aggregation decrease after knockdown as in non treated - control cells was observed (Fig. 3.27 **a, b**). These data suggest, that the MID1 protein complex does not affect autophagy.

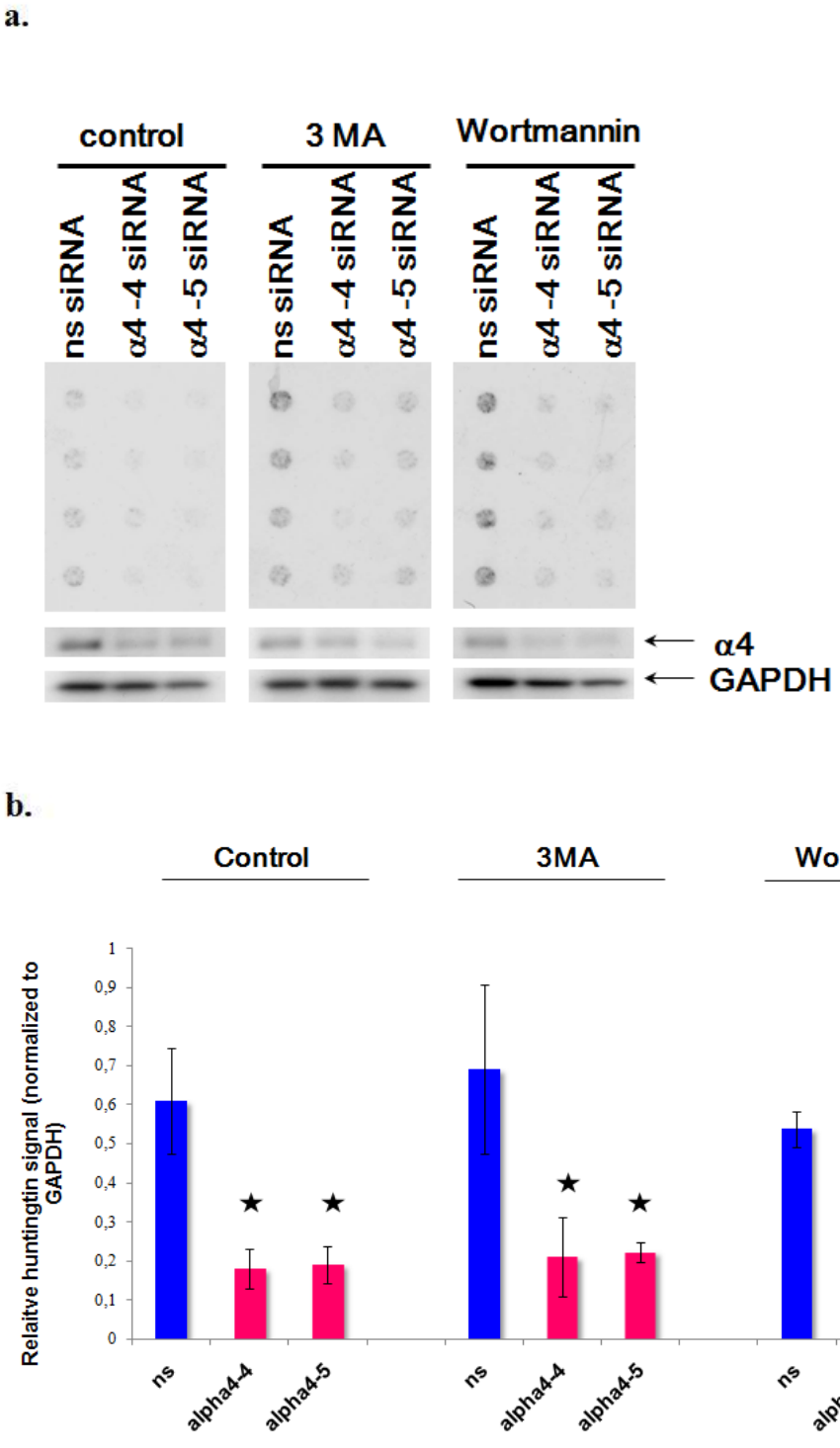

Fig. 3.27 FA of cells under the α4 knockdown and treatment with the autophagy inhibitors 3MA or Wortmannin. **a.** Huntingtin aggregate detection (upper panel) and Western blots for knockdown control (lower panel). **b.** Aggregate quantification is shown (*p<0.01; T-test).

3.6.2 Proteasome degradation

To investigate whether the MID1 protein complex could have an effect on the proteasomal degradation and thereby contribute to aggregate clearance, knockdown experiments together with a specific proteasome inhibitor (MG132) were performed.
293Q83 cells were first transfected with specific siRNA to knockdown α4. The expression of N-terminal huntingtin fragment was induced and for the last 24h of experiment proteasome inhibitor (MG-132) was added to the cells (see section 2.2.3.2). Cell lysates were prepared and analyzed on FA and on western blots. FA membranes were analyzed with the specific antibodies recognizing polyglutamine stretch (Anti-CAG53b, Table 2.4). Western blots were detected with specific antibodies against α4 protein to prove the knockdown efficiency, and tubulin antibodies as a loading control (Fig.3.28 **a.** and Table 2.4).
A decrease in aggregate formation after α4 knockdown with two specific siRNAs was observed in the control experiment without proteasome inhibitors.
A general decrease of protein amount was observed after MG132 treatment, which was probably due to the increased cell death after proteasome inhibition.
The difference in aggregation between the knockdowns and non-silencing control samples did not change by the addition of proteasome inhibitor, however it was observed that α4 knockdown with one of the siRNA used, did not show a significant decrease in aggregate number. Nevertheless, cells showed the similar pattern in aggregation decrease as non treated - control cells (Fig. 3.28 **a, b**).

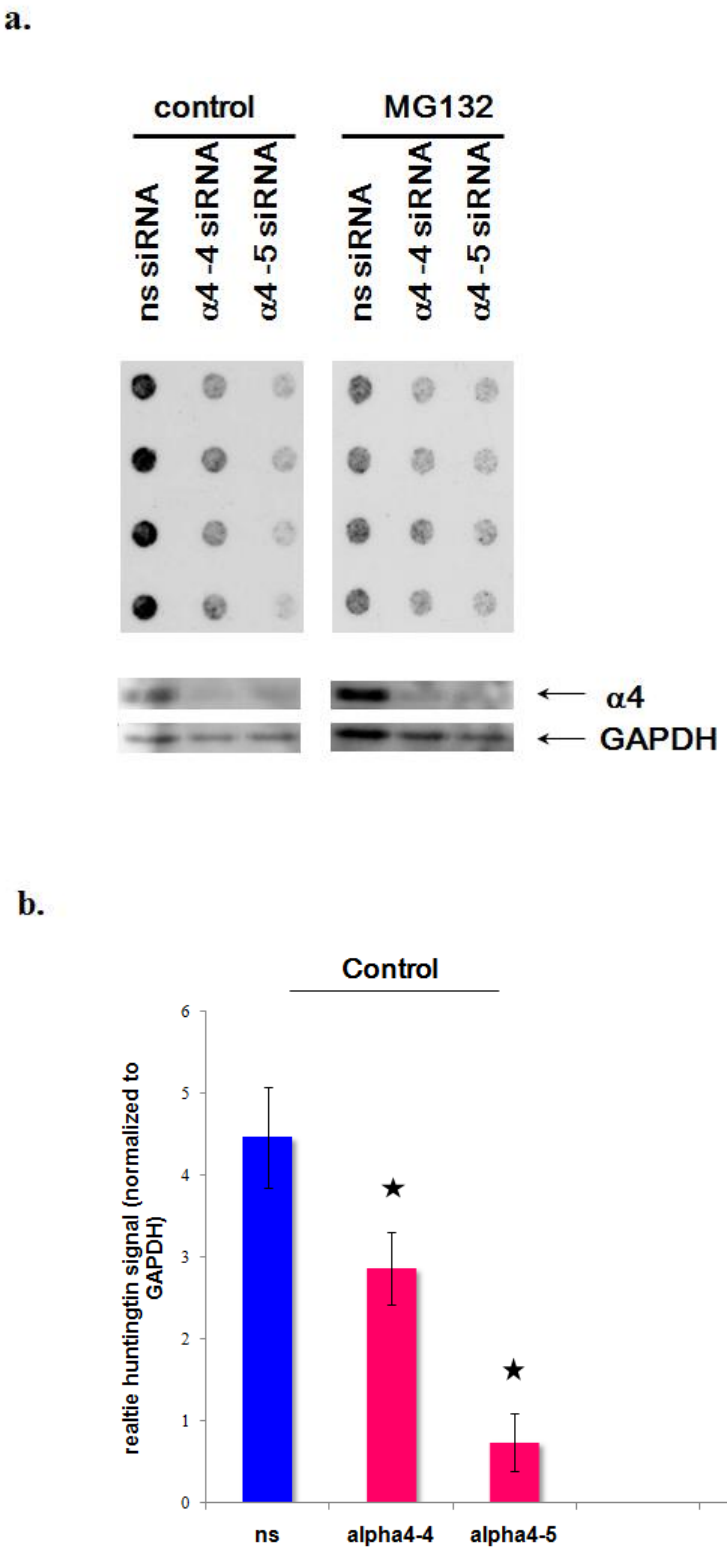

Fig. 3.28 FA of cells under the α4 knockdown and treatment with the proteasome inhibitor MG-132. **a.** Huntingtin aggregate detection (upper panel) and Western blots for knockdown control (lower panel). **b.** Aggregate quantification is shown (*p<0.05; T-test).

Taken together, the experiments with autophagy and proteasome inhibitors suggest that the MID1 protein complex does not affect degradation of the N-terminal huntingtin fragment, but rather reduces translation, finally leading to a decrease in aggregate number.

3.7 Drugs can influence aggregate formation

There are two known drugs, which were suggested to have therapeutic properties in Huntington's disease - rapamycin and lithium.

Rapamycin, a macrolide drug has been already shown to have a neuroprotective effect in a mouse model of Huntington's disease (Ravikumar, Vacher et al. 2004). Rapamycin inhibits mTOR kinase and thereby induces autophagy. Increased autophagy promotes degradation of huntingtin aggregates and reduces polyglutamine expansion toxicity (Ravikumar, Vacher et al. 2004).

Lithium is broadly used as a mood-stabilizing drug in treatment of bipolar diseases. It was previously shown that lithium has neuroprotective effects (Wei, Qin et al. 2001; Wada, Yokoo et al. 2005) and reduces neurodegeneration in Huntington's disease (Senatorov, Ren et al. 2004) by induction of autophagy, thereby leading to clearance of huntingtin aggregates (Sarkar, Floto et al. 2005).
In a next series of experiments I wanted to study the effects of both of this drugs on aggregation in 293Q83 cells.

3.7.1 Rapamycin treatment

To investigate the effects of rapamycin on the aggregation of N-terminal huntingtin fragment containing 83 glutamines, cells were grown and submitted to rapamycin treatment for the entire time period of expression of N-terminal huntingtin fragment (for drug treatment see section 2.2.3.2).
After 72h cell lysates were prepared and analyzed on FA and on western blots. FA membranes were analyzed with the specific antibodies recognizing polyglutamine stretch (Anti-CAG53b, Table 2.4). Western blots were detected with specific antibodies against tubulin as a loading control (Fig.3.29 **a.** and Table 2.4). Aggregation was quantified using ImageQuant v.5.2. and presented as a graph in Fig. 3.29 **b.**

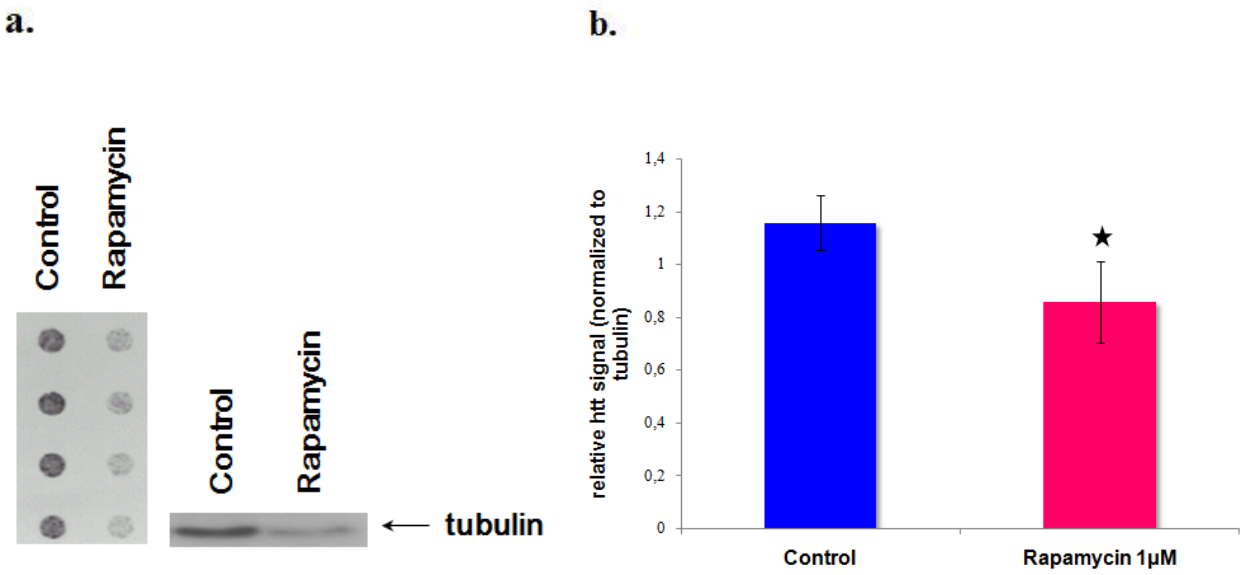

Fig. 3.29 FA of cells under the rapamycin treatment. **a.** Huntingtin aggregate detection (left panel) and Western blots for loading control (right panel).
b. Aggregate quantification is shown (*p<0.05; T-test).

As shown in Fig. 3.29 **a.**, a significant decrease of aggregate formation was detected after 48h treatment with 1 µM rapamycin.
Rapamycin influences both, autophagy and translation. It was not established if decrease in aggregate amount was due to autophagy induction or rather due to influence of protein production through the MID1 protein complex.

3.7.2 Lithium treatment

Prompted by previously published data about neuroprotective effects (Wei, Qin et al. 2001; Wada, Yokoo et al. 2005) and reduced neurodegeneration in Huntington's disease (Senatorov, Ren et al. 2004) through autophagy induction, and by the observation that LiCl in increasing concentration can influence binding of the MID1 multi protein complex to the mRNA of huntingtin exon1 (section 3.2.1.4.2), influence of LiCl on aggregate formation in 293Q83 cells was investigated.
To monitor aggregation of N-terminal huntingtin fragment with 83 glutamines after LiCl treatment, cells were grown and treated with LiCl for the entire time period of expression of huntingtin exon1 protein (for drug treatment see section 2.2.3.2).

After 72h cell lysates were prepared and analyzed on FA and on western blots. FA membranes were analyzed with the specific antibodies recognizing polyglutamine stretch (Anti-CAG53b, Table 2.4). Western blots were detected with specific antibodies against tubulin as a loading control (Fig.3.30 **a.** and Table 2.4). Aggregation was quantified using ImageQuant v.5.2. and presented as a graph in Fig. 3.30 **b.**

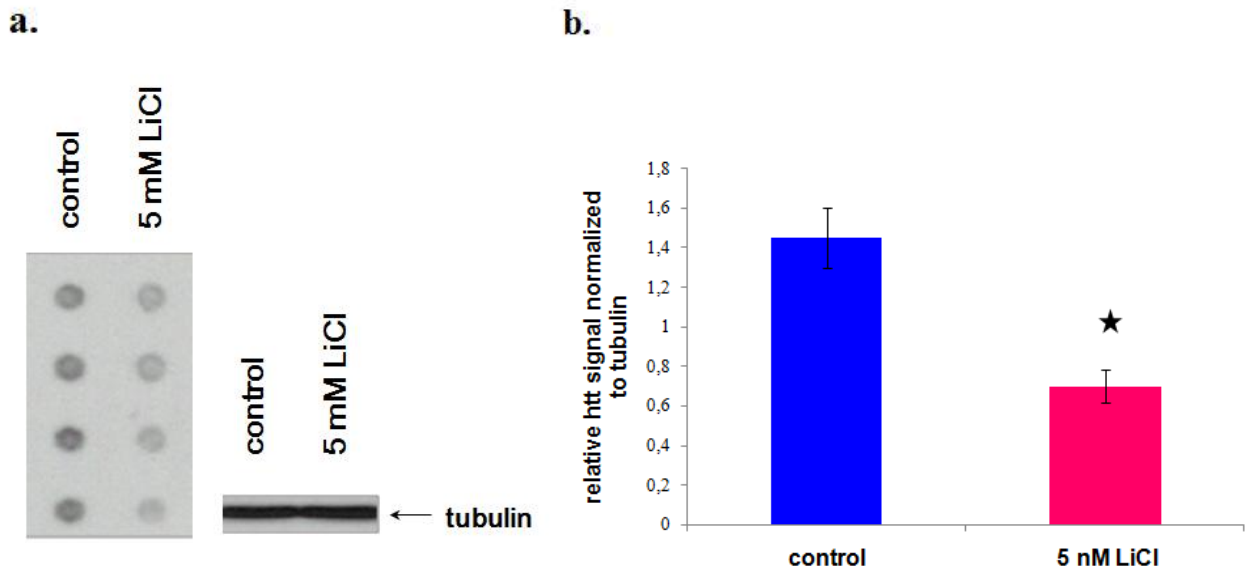

Fig. 3.30 FA of cells under LiCl treatment. **a.** Huntingtin aggregate detection (left panel) and Western blots for loading control (right panel).
b. Aggregate quantification is shown (*p<0.002; T-test).

A significant decrease in amount of aggregates after LiCl treatment was observed (Fig. 3.30). Together with the data showing that LiCl can inhibit binding of the MID1 protein complex to the huntingtin exon1 mRNA with longer CAG stretch, these data suggest that the decrease in aggregate formation might be due to the insufficient binding of the MID1 protein complex to the huntingtin mRNA and a thereby decreased translation.

4. Discussion

CAG repeat mRNA motifs are found in a variety of mRNAs. Expansion of these repeats is a common pathogenic mechanism in diseases like i.e. Huntington's disease (HD). During this thesis I was able to show that the MID1 protein, which is dysfunctional in patients with a midline malformation disorder called Opitz BBB/G syndrome (OS), binds to RNA structures containing such CAG repeats. I have demonstrated, that mRNAs with CAG repeats bind to the MID1 ribonucleoprotein (RNP) complex in a repeat length dependent manner, which can be influenced by different ions, and that the translation of these mRNAs is influenced by the MID1 complex. I found that knockdown of essential components of the MID1 complex result in a significant reduction of protein produced from CAG repeat containing mRNAs, as well as a reduction of aggregate amount. Moreover, I was able to show that the MID1 protein complex does not influence aggregate clearance by autophagy or proteasome.

4.1 MID1 multi protein complex binds CAG rich RNAs

4.1.1 Physiological role of CAG repeat expansion

Trinucleotide repeats are the most common type of simple sequence repeats found in the coding sequence of eukaryotic genomes (Toth, Gaspari et al. 2000). In human genes repeats may undergo expansion, which can cause neurodegenerative and neuromuscular disorders – so-called TREDs (Triplet Repeat Expansion Disorders). Several different types of repeats have been found in 16 genes associated with TREDs: (CUG)n, (CGG)n, (CCG)n, (GAA)n and (CAG)n (Richards and Sutherland 1997; Cummings and Zoghbi 2000; Ranum and Day 2002). The expansion of CAG repeats, which codes for polyglutamine, is a common gene mutation in a disease family called hereditary neurodegenerative disorders. Although CAG repeat stretches are present in many genes, their physiological function is yet not well understood. They might play a regulatory role which is mediated by their interactions with proteins specifically binding to them (McLaughlin, Spencer et al. 1996; Peel, Rao et al. 2001). In the nucleus they might be involved in the regulation of the RNA splicing, maturation and transport (Philips, Timchenko et al. 1998;

Timchenko 1999), whereas in the cytoplasm they could act on mRNA stability and translation regulation (Timchenko, Welm et al. 1999).

4.1.2 Amplification of CAG repeats leads to increased binding of the MID1 protein complex

It has been shown previously that expanded CAG repeats form double-stranded RNA structures, stability of which increases with the number of repeats (Jasinska, Michlewski et al. 2003; Sobczak and Krzyzosiak 2005). Computer modeling of the secondary structure for the huntingtin mRNA predicted the formation of a stable stem-loop sequence encoded by the CAG repeats (Zuker 1989). Our *In silico* RNA structure predictions of CAG repeat stretches with different repeat sizes, showed that, indeed, a hairpin structure is formed, and that the CAG - containing loop is elongated with increasing CAG repeat number (Fig. 4.1) providing space for the binding of proteins. Several proteins have previously been shown to bind to such structures. For example, expanded CUG repeats are able to bind proteins of the muscleblind family in a length dependent manner (Miller, Urbinati et al. 2000), therefore by analogy, one might expect that repeats of CNG family have their specific ssRNA and dsRNA binding proteins (Sobczak, de Mezer et al. 2003). Also, stem-loops of the ferritin and transferin mRNAs, interact with proteins and therefore may regulate gene expression. It has been suggested that complexes like that might stabilize mRNAs, but may also transport RNA from the nucleus to the cytoplasm regulating its subcellular localization (Steward and Banker 1992; Torre and Steward 1992). During this work another role of the CAG repeats was indentified, namely the binding of RNA binding proteins to the CAG repeats that regulate protein translation. CAG repeats in the huntingtin mRNA have been shown previously to bind to proteins in a length dependent manner, with longer repeats binding substantially more protein (McLaughlin, Spencer et al. 1996; Peel, Rao et al. 2001). However, the functional relevance of these protein-RNA interactions has not been defined. However, it has been assumed that these protein-RNA structures are pathological as they influence RNA metabolism in the cell.

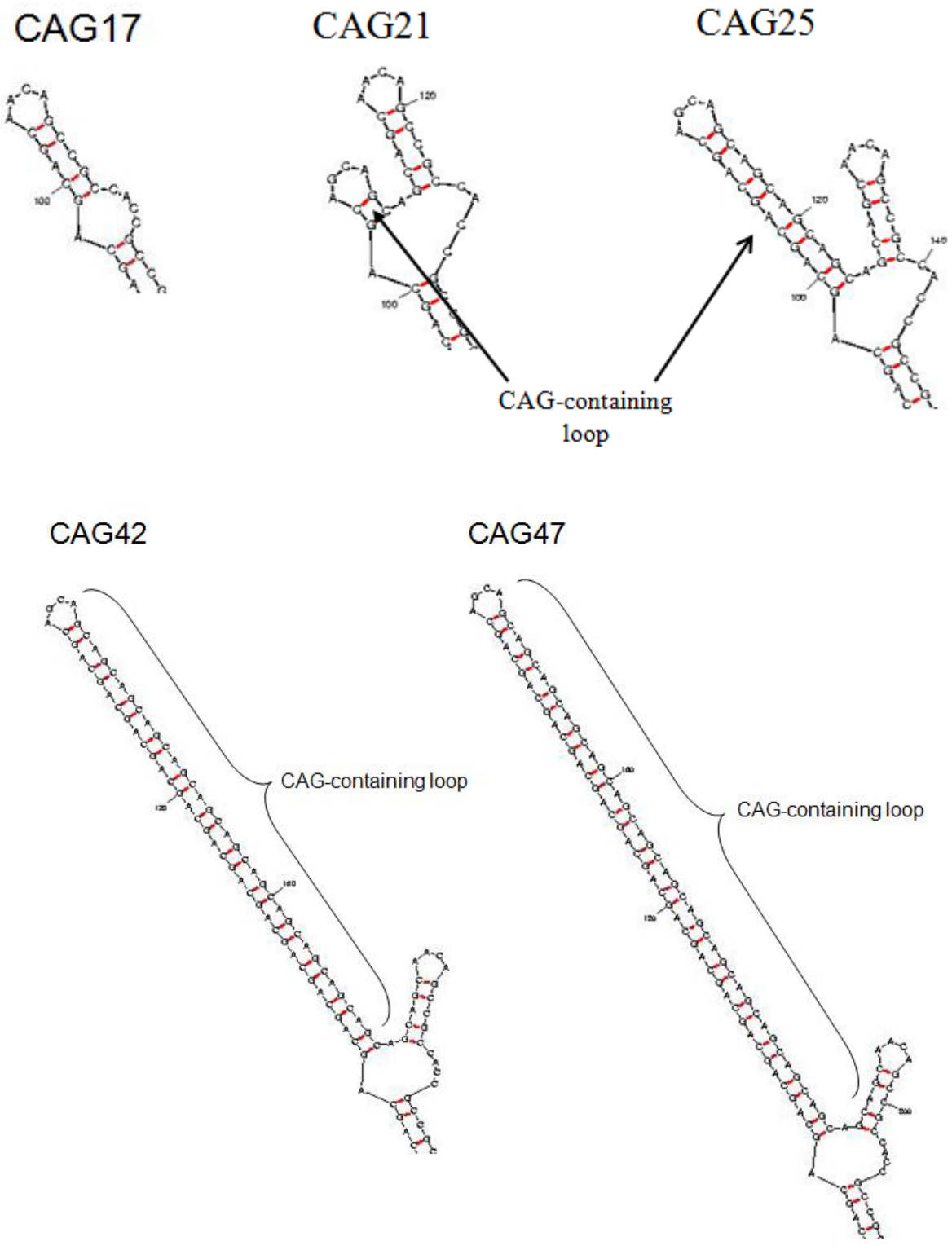

Fig. 4.1 *In silico* prediction of huntingtin exon1 mRNA structures with CAG repeats of different lengths. A CAG repeat - loop, formed by more than 21 repeats, is indicated.

In our group it has been revealed recently that the MID1 ubiquitin ligase forms a microtubule-associated ribonucleoprotein (RNP) complex. Furthermore it had been shown that the MID1/α4 complex associates not only with translation regulation factors e.g. elongation factor 1α (EF-1α), and several proteins involved in mRNA transport and translation like RACK1, Annexin A2 or Nucleophosmin but also with active polyribosomes. Interestingly, the MID1 complex is also able to associate with G- and C- rich sequence motifs within mRNAs (Aranda-Orgilles, Trockenbacher et al. 2008). During this Thesis I could show that the MID1 protein complex binds to the huntingtin exon1 mRNA in a length dependent manner. Previously shown *In silico* predictions (Fig. 4.1), indicate that the increase of MID1 binding with the number of repeats could have sterical reasons. However, both huntingtin exon1 mRNAs, with pathological and non-pathological number of CAG repeats, although with different affinity, are able to bind MID1 protein complex, which goes in line with previous observation made for (CUG)n repeats, showing that proteins do bind not only to the extended but also, to a lesser extent, to CUG repeats of normal sizes (Miller, Urbinati et al. 2000).

Another well known example of an RNP complex, which is responsible for translation control and mRNA transport in neurons (Antar, Afroz et al. 2004; Bagni and Greenough 2005) is the fragile X mental retardation protein (FMRP) complex. FMRP is mutated in patients with FragileX syndrome. It has been established, that the FMRP protein is able to bind G-rich RNA sequences called G-quartet motifs. Moreover, binding of the FMRP to such structures is dependent on ions: potassium and sodium cations are able to stabilize such structures, whereas lithium can destabilize it (Darnell, Jensen et al. 2001; Schaeffer, Bardoni et al. 2001; Ramos, Hollingworth et al. 2003). During this work, experiments showing the influence of different ions on the binding ability of the MID1 protein complex to the huntingtin exon1 mRNA were performed. Interestingly, lithium as well as magnesium and sodium decreased binding of the MID1 complex to the huntingtin exon1 mRNA. Therefore, it is reasonable to assume that ions may destabilize long hairpin structures created by CAG repeats, similar to what had been shown for G-quartet structures and therefore influence binding of proteins, in this case the MID1 protein complex. Assuming that the MID1 protein complex through binding to the CAG

repeats of the huntingtin mRNA is able to regulate its translation, destabilizing its binding to the mRNA would influence translation. However, the underlying mechanism of how ions influence binding of proteins to structures created by CAG repeats still remains unknown .

4.2 MID complex influences huntingtin exon1 gene translation through CAG repeat expansions which could be an important factor in pathogenesis of CAG repeat disorders

4.2.1 "Overexpression" contributes to the HD phenotype

During this thesis it has been shown that the MID1 protein complex – a microtubule-associated translation unit, controls the efficiency of translation from CAG repeat containing mRNAs, thereby suggesting that CAG repeats play an essential role in the regulation of protein translation. This is supported by the observation that complete deletion of the CAG repeat stretch in the androgen receptor (AR) gene results in a significant reduction of AR protein abundance (Harada, Mitani et al. 2009). Thus, in this thesis it has been shown that a pathologically expanded stretch of CAG repeats causes accumulation of the protein translated from a respective mRNA by increased binding of the MID1 complex. Over time, this would result in an accumulation of huntingtin protein in cells of patients with HD, contributing to the formation of protein aggregates and cell damage. This hypothesis is supported by data from Graham et al., that show that in mice expressing varying levels of abnormal huntingtin protein, increased protein levels account for a more severe phenotype, which is characterized by earlier onset, a more rapid progression, and increased striatal volume loss (Graham, Slow et al. 2006). Conversely, it has been demonstrated that the amount of huntingtin aggregates (King, Hands et al. 2008) can be decreased by treating cells with the translation inhibitor rapamycin.

4.2.1 Opitz syndrome phenotype overlaps with WH syndrome phenotype

Regulation of the huntingtin translation by MID1 seems to be relevant in development, as suggested by syndromes caused by loss of either protein respectively. Thus, heterozygous deletion of the distal short arm of chromosome 4

(4p16.3), which includes the huntingtin gene in humans, results in Wolf-Hirschhorn syndrome (WHS), characterized by severe growth retardation, mental defects, closure defects such as cleft lip or palate, and a highly dysmorphic face referred to as 'Greek warrior helmet'. Several patients show also heart defects (Rauch, Schellmoser et al. 2001). This phenotype strikingly overlaps with that in OS patients induced by loss of MID1. Like WHS patients, OS patients may exhibit severe hypertelorism, a broad nasal bridge, cleft lip and palate, and cardiac defects (Cox, Allen et al. 2000; De Falco, Cainarca et al. 2003; So, Suckow et al. 2005). This striking phenotypical overlap between the two syndromes suggest a biochemical link between MID1 and huntingtin. Indeed, in our group it has recently been observed that MID1 regulates the translation efficiency of the huntingtin mRNA, and that huntingtin protein, but not its mRNA, is reduced in cells derived from OS patients. These data offer a molecular mechanism underlying the clinical overlap of two inherited syndromes not previously recognized as related in pathogenesis. Furthermore, these data, together with the established involvement of huntingtin in WHS malformation, suggest that huntingtin may play a role in the formation of facial structures and that its partial loss contributes to the development of ventral midline abnormalities.

4.3 MID1 complex alteration leads to decrease in aggregate formation and translation reduction of CAG rich RNAs

It has been previously discussed that the MID1 protein is at the core of a microtubule-associated ribonucleoprotein (RNP) complex, which interacts with proteins involved in translation and in addition with active polyribosomes. Moreover, PP2A and its regulatory subunit, α4, are also main components of that complex, which integrate the MID1 complex into the mTOR pathway, one of the main translation regulatory pathways (Schmelzle and Hall 2000; Schweiger and Schneider 2003). It has been reported that through interaction with α4/Tap42 (the regulatory component in the rapamycin-sensitive mTOR pathway) PP2A is involved in the rapamycin-sensitive mTOR signalling (Chen, Peterson et al. 1998). PP2A has been shown to be phosphorylated *in vitro* by the mTOR kinase, which leads to its inhibition, while rapamycin treatment has been shown to increase PP2A activity *in vivo* (Peterson, Desai et al. 1999). Moreover, intensive studies on rapamycin revealed the overall growth and proliferation inhibitory function of rapamycin by inhibition of mTOR

kinase activity (Pallet, Beaune et al. 2006). Conversely, selective up- or down-regulation of the PP2A activity by the MID1/α4 complex may participate in the translation control of certain, specific mRNAs, which associated to the MID1 protein complex. Interestingly, it has been reported that defects in the proper control of mRNA translation may result in many diseases including neurodegeneration disorders (reviewed in (Proud 2007)).

4.3.1 Rapamycin affects global protein translation and influences aggregate amount in HD

Rapamycin is a well characterized macrolytic lactone with antibiotic and antifungal properties. As mentioned above, intensive studies on rapamycin revealed that it inhibits cell growth and proliferation (Pallet, Beaune et al. 2006). Moreover, specific inhibition of mTOR kinase activity by rapamycin has been reported to decrease accumulation of the mutant huntingtin in cell and fly models of HD (Ravikumar, Vacher et al. 2004; Berger, Ravikumar et al. 2006). Rapamycin through the inhibition of mTOR kinase activity seems to lead to the activation of autophagy, a process that is responsible for the clearance of aberrant huntingtin protein (Qin, Wang et al. 2003; Ravikumar, Vacher et al. 2004). Interestingly, recent data by Wyttenbach and colleagues suggest that rapamycin effects on the aggregation process of polyglutamine containing proteins might be independent of autophagy and could be modulated via other pathways (King, Hands et al. 2008; Wyttenbach, Hands et al. 2008).

During this thesis it has been shown that, MID1 protein complex binds CAG repeat containing mRNA of huntingtin in a length dependent manner and influences its translation, which would support the idea from Wyttenbach and colleagues, that clearance of aberrant huntingtin by autophagy is not the only process modulating aggregation of polyglutamine containing proteins in cells.

4.3.2 Diverse effects of MID1/α4 on mRNAs containing 20, 51 or 83 CAG repeats

As previously discussed, the possible sterical space to bind MID1 protein complex given by the extended CAG stretch probably leads to the observed increase of MID1 binding with the number of repeats. Within the frame of this thesis, diverse

effects of the MID1/α4 complex on the translation of mRNAs containing different amount of CAG repeats have been analyzed.

In agreement with Wyttenbach and colleagues suggesting, that the decrease in aggregate formation by rapamycin might be due to translation modulation rather than autophagy induction, I show here that incomplete interruption of the MID1 protein complex by siRNA technology particularly influences mRNAs with longer – pathological repeat sizes. MID1 and α4 knockdown experiments performed in cells expressing N-terminal huntingtin fragments with either 20, 51 or 83 glutamines showed that disruption of the MID1 complex influenced their translation with a diverse but very specific efficiency, as the effect of MID1/α4 knockdown on the synthesis of the respective proteins was only significant on the 51 and 83 CAG repeat plasmids. No effect was seen on the production of protein containing 20 glutamines. These data indicate that interference with the MID1 protein complex would influence protein translation from mRNAs carrying pathological sizes of repeats significantly more efficiently than from mRNAs with normal repeat sizes. Effects of the MID1/α4 complex disruption on the translation decrease of specific CAG containing mRNAs are similar to the aggregation decrease effects obtained by rapamycin treatments.

4.3.3 Aggregate toxicity versus toxic soluble protein

As described in the previous section, disruption of the MID1/α4 complex showed most striking effects on the decrease in aggregates amount produced in cells. However, decrease in translation of the N-terminal fragments which contain less CAG repeats in their transcripts was also shown .

Although it is well established that mutant huntingtin forms intranuclear and cytoplasmic aggregates (DiFiglia, Sapp et al. 1997), there is still a debate whether aggregates are toxic or rather play a protective role. In several mammalian models there is a strong correlation between aggregation and cell death (Hackam, Singaraja et al. 1998; Lunkes and Mandel 1998; Wyttenbach, Carmichael et al. 2000). Expression of the mutant huntingtin exon1 in mice models revealed that inclusions are strongly correlated with the onset of the behavioral changes (Davies, Turmaine et al. 1997; Morton, Lagan et al. 2000). On the other hand many observations exists concerning the neuroprotective role of huntingtin aggregates. Huntingtin inclusions might represent the side effect of a continuously growing dysfunction within the cell

(Kuemmerle, Gutekunst et al. 1999). There are currently many attempts to decrease aggregation in cells. One of them would be to promote the degradation of aberrant protein in cells. It had been described previously that aggregates were found to be not only ubiquitinated but also associated with proteasomal components (Ciechanover and Brundin 2003). Activation of chaperons by substances like geldanamycin would promote the unfolding of misfolded proteins and therefore make their degradation through the proteasome easier (Sittler, Lurz et al. 2001). This was confirmed by observations that overexpression of some HSP proteins has a protective effect against cell death in HD cellular and/or mouse models (Muchowski, Schaffar et al. 2000; Miller, Zhou et al. 2005). Other approach suggest direct targeting the proteasome to promote degradation (Tanaka, Machida et al. 2004).

There is a strong evidence that the N-terminal fragments containing expanded polyglutamine tracts are responsible for the pathogenesis of the HD. It is important to notice that the N-terminal fragments of mutant huntingtin are sufficient to obtain HD-like phenotype in animal models (Davies, Turmaine et al. 1997; Schilling, Becher et al. 1999; Palfi, Brouillet et al. 2007). Therefore, another proposed concepts is to rate-limit cleavage of huntingtin to its toxic N-terminal fragments. Inhibitors of proteases that are able to cleave huntingtin may have therapeutic effects, although long term caspase inhibition might increase the cancer risk (Sanchez, Xu et al. 1999; Smith, Woodman et al. 2003; Wang, Zhu et al. 2003).

Other strategies could involve autophagy induction, which lead to clearance of accumulated toxic protein. Rapamycin has been shown to be an interesting drug, which, by inhibition of mTOR, activates autophagy and therefore shows neuroprotective effects in fly and mouse models (Ravikumar, Vacher et al. 2004).
However, rapamycin by inhibition of mTOR kinase activity also influences overall protein translation in cells. However and by contrast it has been discussed that the inhibition of mTOR and a decrease in overall protein synthesis might also contribute to some of the HD symptoms like memory loss, due to lack of certain proteins at synapses (Tang, Reis et al. 2002). In this thesis, it has been shown that, by influencing the MID1/α4 complex, the translation of specific mRNAs containing pathological amounts of CAG repeats was hindered. Moreover, I was able to show that not only aggregate number was decreased but also a specific decrease in protein production from huntingtin exon1 mRNA containing 51 CAG repeats was

observed. By manipulating the MID1 protein complex it therefore is likely to be possible to specifically reduce the production of the aberrant protein in patients with CAG repeat expansion disorders.

4.3.4 Degradation through proteasome or autophagy does not contribute to the MID1 effect

Protein degradation seems to be a very important process and powerful therapeutic strategy in HD. As discussed before aggregates were found to be ubiquitinated and also associated with proteasomal components, supporting the hypothesis of a UPS (Ubiquitin-Proteasome System) impairment in HD (Ciechanover and Brundin 2003). There are many data suggesting that in the presence of mutant huntingtin, UPS activity decreases (Bence, Sampat et al. 2001; Jana, Zemskov et al. 2001) while some show no activity change (Ding, Lewis et al. 2002; Bowman, Yoo et al. 2005) or even increase of the UPS activity (Diaz-Hernandez, Hernandez et al. 2003; Bett, Goellner et al. 2006). Some experiments suggest that UPS components might be sequestered in huntingtin aggregates and therefore lead to the alteration of the proteasome activity (Davies, Turmaine et al. 1997; DiFiglia, Sapp et al. 1997; Cummings, Mancini et al. 1998; Wyttenbach, Carmichael et al. 2000). It is not really clear if proteins like huntingtin, with expanded polyglutamine tracts are good substrates for the UPS. It has been shown that huntingtin interacts with the human ubiquitin-conjugating enzyme E2-25K (Kalchman, Graham et al. 1996) and that it also interacts with the E3 ubiquitin ligase-Parkin, which makes it a possible substrate for the proteasome. Indeed overexpression studies of parkin in cells lead to an induction of clearance of mutant huntingtin (Tsai, Fishman et al. 2003). MID1 fulfills an E3 ubiquitin ligase function through its RING finger domain, binding a microtubule-associated pool of PP2A and leading to its degradation (Trockenbacher, Suckow et al. 2001). It is possible that the MID1 protein is involved in the degradation of huntingtin protein, which has been checked in a knockdown experiment in the presence or absence of proteasomal inhibitors. These experiments did not show a difference in aggregation pattern obtained in cells with and without proteasome inhibitors, therefore confirming that MID1/α4 complex disruption does not influence degradation of huntingtin protein through the proteasome.

Autophagy is another highly regulated process that plays an important role in maintaining a balance between synthesis, degradation and cell product recycling. In contrast to the UPS, which is responsible for the degradation of short-lived proteins, autophageal degradation involves long-lived proteins, protein complexes and organelles (Rubinsztein 2006). The possibility of degradation of protein oligomers as well as organelles made autophagy to be considered as a clearing process for aggregated proteins like mutant huntingtin protein (Sarkar, Perlstein et al. 2007; Sarkar and Rubinsztein 2008). It had been shown recently that autophagy is implicated in neurodegeneration based on the results showing, that loss of autophagy leads to degeneration of mouse neuronal cells (Hara, Nakamura et al. 2006; Komatsu, Waguri et al. 2006). Therefore induction of autophagy might be a possible therapeutic strategy in neurodegenerative disorders. Moreover, it has been shown that the mTOR kinase is involved in autophagy regulation, by being sequestered into mutant huntingtin aggregates which would result in inhibition of kinase activity, therefore promoting autophagy (Ravikumar, Vacher et al. 2004) and possibly clearance of the huntingtin aggregates (Qin, Wang et al. 2003; Ravikumar, Vacher et al. 2004). It has been shown recently that rapamycin (Berger, Ravikumar et al. 2006) and a few other small-molecule enhancers of rapamycin (Floto, Sarkar et al. 2007; Sarkar, Perlstein et al. 2007), which could act downstream or independently of mTOR, induce clearance of the mutant huntingtin fragments in different HD models. However, rapamycin, by inhibition of mTOR kinase activity also influences the overall protein translation in cells. During this work it has been shown that MID1/α4 complex disruption causes decrease in aggregate formation. Using a panel of autophagy inhibitors I have shown here that, although the MID1 protein complex is thought to be a microtubule-associated translation unit linked through PP2A and α4 to the mTOR translation pathway, it does not influence or activate autophagy. Showing that the MID1/α4 complex does not induce aggregate clearance, neither by proteasome nor by autophagy, made it reasonable to assume, that decrease in aggregate formation is due to an influence on the synthesis rate.

4.4 MID1/α4/PP2A complex – drug target in Huntington's disease

4.4.1 Specificity

There is currently no therapy existing for preventing or delaying the progression of HD, which makes therapeutic research a major issue in HD. Many studies in the past few years provided a better understanding of the huntingtin function in cells although the exact molecular mechanisms, by which mutant huntingtin induces cell death are not completely understood. In the ideal case a drug, which targets putative pathological mechanism should be developed. One of the ideas would be to prevent expression of the mutant allele, which would remove the protein responsible for the pathogenesis in HD. It has been shown that humans with only one working copy of *huntingtin* suffer no obvious consequences. Recently it has become possible to use siRNA technology to decrease mutant protein expression in mouse models (Wang, Liu et al. 2005) and thereby to decrease aggregation and extend survival. However, it is clear that the knockdown must specific target the mutant allele while the wild type should be unaffected. However, this seems technically challenging. During this thesis a novel mechanism specifically regulating the translation of CAG repeat containing mRNAs by the MID1 protein complex as a control unit has been shown. Specific binding of the complex to the mRNA of huntingtin in a length dependent manner and influence of the MID1 protein complex particularly on the pathological huntingtin transcripts have been reported. This indicates that, by interfering with the MID1 protein complex, it is possible to reduce the protein produced from CAG repeat containing mRNAs and influence protein translation from mRNAs carrying pathological sizes of repeats significantly more efficiently than from mRNAs with normal repeat sizes.

Based on the data shown in this thesis, I have established evidence that CAG repeats in mRNAs are critical for the regulation of huntingtin protein translation. Furthermore, they suggest that the MID1 protein complex might be a novel and a very promising drug target for the development of therapeutic strategies against CAG repeat expansion disorders.

4.4.2 Expected side effects

It is well established, that dysfunctional MID1 protein causes Opitz BBB/G syndrome (OS), a midline malformation disorder. OS is a congenital disorder, which affects the ventral midline (Trockenbacher, Suckow et al. 2001; Schweiger and Schneider 2003). Due to the anomalies observed in patients, it was suggested that the affected protein is important for human development (Quaderi, Schweiger et al. 1997). Moreover the phenotype of OS suggests, that mutations in *MID1* affect cell migration and apoptosis. The disturbed migration of neural crest cells to the developing ventral midline may play a major role in the pathogenesis of OS (Trockenbacher, Suckow et al. 2001).

Based on these data, it seems obvious that the phenotype the OS patients caused by altered MID1 protein is strictly related to the developmental stage. Therefore, MID1 in the adults should be a good therapeutic target in CAG repeat expansion disorders like HD and its targeting should not cause too many side effects.

Up to date, there are FDA (Food and Drug Administration) approved drugs existing which could be used as a possible therapy for HD like: rapamycin, sodium valproate or lithium showing neuroprotective effects. Rapamycin is a well known immunosuppressant used for organ transplants. The cytotoxic properties of rapamycin could also make it effective in cancer treatment. Moreover, as mentioned before, specific inhibition of the mTOR kinase activity by the rapamycin has been reported to decrease accumulation of the mutant huntingtin in cell and fly models of HD (Ravikumar, Vacher et al. 2004; Berger, Ravikumar et al. 2006). mTOR inhibition directly leads to activation of one of the key clearance pathways – autophagy and reduction of polyglutamine toxicity (Ravikumar, Vacher et al. 2004).

Another drug - lithium has been used for many years as a mood stabilizing agent in manic depressive illness, but recent data from *in vivo* and *in vitro* studies also in humans have revealed, that lithium has a neuroprotective effect in chronic neurodegeneration disorders like: Alzheimer's, Parkinson's and Huntington's disease (Wei, Qin et al. 2001; Wada, Yokoo et al. 2005). During this work both drugs have also shown to decrease aggregate formation in cell model, nevertheless molecular mechanism of their influence has not been established.

4.5 Outlook

In the future, further studies should be performed to elucidate, which protein from the MID1 protein complex is responsible for the binding of mRNA containing CAG repeats. Some *in vitro* studies should be undertaken to evaluate the role of cations on binding efficiency of proteins to hairpins, created by transcripts carrying CAGs. It would also be interesting to investigate if the observed novel mechanism of translation regulation through the MID1 protein complex might be involved in other trinucleotide expansion disorders. Moreover, MID1 protein complex alteration and its influence on translation regulation *in vivo*, in mouse models would be necessary to investigate. Furthermore, as mentioned before MID1 seems to be a novel and promising drug target for the development of therapeutic strategies against CAG repeat expansion disorders. Therefore studies on substances which could be used to influence the MID1 complex should be preformed.

5. Summary

Huntington's disease (HD), is a progressive neurodegenerative disorder in which the defective *HD* gene contains an unstable expansion of the CAG repeats in its coding region. This mutation leads to the translation of expanded polyglutamine (poly Q) stretches in the N-terminal part of the huntingtin protein. CAG repeat motifs are found in a variety of mRNAs. Expansion of these repeats is a common pathogenic mechanism in so called trinucleotide repeat expansion disorders (TREDs).

During this thesis a novel mechanism specifically regulating the translation of CAG repeat containing mRNAs by the MID1 protein complex as a control unit has been identified.

The MID1 protein is a microtubule-associated ubiquitin ligase, which binds to the α4 regulatory subunit of PP2A and thereby controls its activity. Additionally, PP2A and its regulatory subunit, α4, integrate the MID1 complex into the mTOR pathway, one of the main translation regulatory pathways.

Within the frame of this work it has been shown, that the MID1 protein complex, which is dysfunctional in patients with a midline malformation disorder Opitz BBB/G syndrome (OS), binds to RNA structures containing CAG repeats. It has been demonstrated, that amplification of CAG repeats in huntingtin exon1 mRNAs led to increased binding to the MID1 ribonucleoprotein (RNP) complex and that the protein synthesis directed by CAG repeat containing mRNAs increases with the amount of repeats. Moreover, knockdown of essential components of the MID1 protein complex resulted in a significant reduction of protein produced from the CAG repeat containing mRNAs. It has also been established, that the significant reduction in huntingtin protein aggregates amount seen after interference with the MID1 protein complex was not influenced by proteasome or autophagy degradation processes.

Taken together the data presented in this thesis, suggest that CAG repeats in mRNAs are critical for the regulation of huntingtin protein translation. Furthermore, they suggest that the MID1 complex might be a novel and a very promising drug target for the development of therapeutic strategies against CAG repeat expansion disorders.

6. Zusammenfassung

Chorea Huntington ist eine neurodegenerative Erkrankung und wird durch Mutationen im HTT-Gen verursacht, welche zu einer Elongation eines CAG-Trinukleotid-Motivs führen. Die aus dem Triplett CAG resultierende mRNA codiert für die Aminosäure Glutamin, somit führt die erhöhte Zahl der CAG-Tripletts zur Translation eines Polyglutamin-Motivs im N-terminalen Bereich des HTT-Proteins. Derartige Elongationen von Trinukleotid-Motiven spielen neben der Huntington Krankheit auch bei weiteren Erkrankungen eine Rolle.

In der vorliegenden Arbeit wurde eine bislang unbekannte regulatorische Rolle des MID1-Protein-Komplexes auf die Translation von mRNA's mit CAG-Trinukleotid-Motiven beschrieben.

Das MID1-Protein ist eine Mikrotubulus-assoziierte Ubiquitin-Ligase, die eine regulatorische Untereinheit der PP2A – das α4-Protein – binden, und somit die ubiquitin-abhängige Degradation von PP2A steuern kann. Ferner stellen PP2A und α4 eine Verbindung zwischen dem MID1-Protein-Komplex und dem mTOR-Signalweg, welcher bei der Translation-Kontrolle eine wichtige Rolle spielt, her.

In dieser Arbeit wurde gezeigt, dass der MID1-Protein-Komplex, dessen Misregulation mit der Entstehung einer Mittelllinien-Erkrankung – dem Opitz BBB/G Syndrom – verbunden ist, an mRNA's mit CAG-Trinukleotid-Motiven bindet. Eine Vervielfältigung der CAG-Tripletts der HTT mRNA führt zu einer verstärken Bindung des MID1-Ribonukelotid-Komplexes. Nach Verringerung der Expression von MID1-Komplex-Bestandteilen mithilfe von RNA-Interferenz zeigte sich eine deutlich verringerte Translation von mRNA's mit CAG-Trinukleotid-Motiven. Außerdem wurde eine signifikante Verringerung der HTT Aggregate nach Misregulation des MID1-Komplexes beobachtet, welche nicht durch Degradationprozesse wie Autophagie oder proteasomalem Abbau beeinflusst wurde.

Zusammenfassend zeigen die in dieser Arbeit erzielten Ergebnisse, dass CAG-Trinukleotid-Motiven in der HTT-mRNA eine kritische Rolle bei der Translation des HTT-Proteins spielen. Weiterhin machen die hier gezeigten Daten den MID1-Protein-Komplex zu einem vielversprechenden Ziel für die Entwicklung von Substanzen, die bei CAG-Trinukleotid-Motiv-Erkrankungen Anwendung finden könnten.

7. Abbreviations

°C – celcius grad
µg – microgram
µl - microliter
aa – amino acid(s)
Amp - ampicillin
APS – ammonium persulphate
BDNF – brain derived neurotrophic factor
bp – base pair
BSA – bovine serum albumine
CBP – CREB binding protein
cDNA – complementary DNA
CRE – cAMP response element
CREB – co-activator of the CRE-mediated transcription
DAPI – 4,6-diamino-2-phenylindole
DMSO – dimethyl sulfoxide
DNA – deoxyribonucleic acid
dNTP – 2'-deoxynucleotide-5'-triphosphate
Doxy – doxycycline
DTT - dithiothreitol
4EBP1 – eukaryotic translation initiation factor 4E-binding protein 1
EF-1α – elongation factor - 1α
EGFP – enhanced green fluorescent protein
eIF4E – eukaryotic translation initiation factor 4E
ER – endoplasmic reticulum
EtOH - ethanol
FA – filter retardation assay
FDA – food and drug administration
FNIII – fibronectin III
FRAP – FKBP12-rapamycin associated protein
FRB – FKBP12-rapamycin binding domain

Gln (Q) – glutamine

GFP – green fluorescent protein

Hap1 – huntingtin-associated protein 1

HRP – horse radish peroxidase

HD – Huntington's disease

HEAT – huntingtin, elongation factor 3, protein phosphatase 2A, TOR1

Hip1 – huntingtin interacting protein

HSP – heat-shock protein

HTT – huntingtin

kb – kilobase

kDa – kilodalton

LB – Lauria Bertani

LiCl – lithium chloride

LTE – laryngotracheoesophageal

M – molar

MAP – microtubule associated protein

$MgCl_2$ – magnesium chloride

mRNA – messenger RNA

mTOR – mammalian target of rapamycin

NaCl – sodium chloride

NES – nuclear export signal

NLS – nuclear localization signal

NPM – nucleophosmin

NRSE – neuron-restrictive silencer element

NRSF – neuron-restrictive silencer factor

ORF – open reading frame

OS – Opitz BBB/G syndrome

PACSIN1 – Protein kinase C and casein kinase substrate in neurons protein 1

PAGE – polyacrylamide electrophoresis

PBS – phosphatase-buffered saline

PCR – polymerase chain reaction

PP2A – protein phosphatase 2A

$PP2A_c$ – catalytic subunit of PP2A

p70S6K – p70 ribosomal S6 kinase
RACK1 – receptor for activated C-kinase 1
RAPT – rapamycin target
RBCC – Ring-B-boxes-coiled-coil
REST – RE1 silencing transcription factor
RNA – ribonucleic acid
RNP – ribonucleoprotein
SCA – Spinocerebellar ataxia
SDS – sodium dodecyl sulphate
SH3GL3 – SH3-domain GRB2-like 3
siRNA – short interfering RNA
Sp1 – specificity protein 1
TBP – TATA binding protein
Tc – tetracycline
TRE – tetracycline -responsive promoter element
TREDs – triplet repeat expansion disorders
TRIM – tripartite motif
tTA – transactivator protein
UPS – ubiquitin-proteasome system
UTP – uridine-5-triphosphate
UTR – untranslated region
WHS – Wolf-Hirschhorn syndrome

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