Studies of polyglutamine expanded Ataxin-7 toxicity

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Till min familj
List of publications

This thesis is based on the listed publications, referred to in the text as paper I, paper II, paper III and paper IV


Additional publications

I. Abiodun AJAYI, Xin YU, Anna-Lena STRÖM. The role of NADPH oxidase (NOX) enzymes in neurodegenerative disease. Front. Biol. 2013, 8(2): 175–188
Abstract

Spinocerebellar ataxia type 7 (SCA7) is an autosomal dominant inherited neurodegenerative disease for which there is no cure. SCA7 belongs to the group of polyglutamine disorders, which are all caused by the expansion of a polyglutamine tract in different disease proteins. Common toxic mechanisms have been proposed for polyglutamine diseases; however the exact pathological mechanism(s) are still unclear.

The aim of this thesis was to identify and characterize the molecular mechanisms by which polyglutamine expansion in the ATXN7 protein cause SCA7 and how this can be counteracted. We found that mutant ATXN7 can be degraded by Ubiquitin proteasome system (UPS) and autophagy, the two main cellular degradation pathways. However aggregation stabilized the protein against degradation. Moreover, we found that mutant ATXN7 blocked the induction of autophagy by interfering with p53 and the ULK1-ATG13-FIP200 complex. Pharmacological stimulation of autophagy ameliorated aggregation, as well as toxicity.

We also found that oxidative stress plays an important role in mutant ATXN7 toxicity and that the oxidative stress is generated by activation of NADPH oxidase 1 (NOX1) complexes. Furthermore, we showed that the increased NOX1 activity, together with polyQ expanded ATXN7 mediated disruption of the transcription factor p53, results in metabolic alterations in SCA7 cells. The expression of key p53 regulated metabolic proteins like AIF, TIGAR and GLUT1 was altered in SCA7 cells and resulted in reduced mitochondrial respiration, a higher dependence on glycolysis and reduced ATP levels.

In summary, our data indicate that mutant ATXN7 mediated dysregulation of p53, resulting in autophagic and metabolic alterations, could play a key role in SCA7 and possibly other polyglutamine diseases.
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Abbreviations

AD  Alzheimer’s disease
AIF  Apoptosis-inducing factor
ALS  Amyotrophic lateral sclerosis
AMPK  AMP-activated protein kinase
Apaf-1  Apoptotic protease activating factor 1
ATXN  Ataxin
Atg  Autophagy related
Bax  Bcl-2-associated X protein
BBB  Blood Brain Barrier
CoA  Coenzyme A
CPP  Cell penetrating peptide
CREB  cAMP response element-binding
CRX  Cone-rod homeobox
DNA  Deoxyribonucleic acid
DOUX  Dual Oxidase
DRPLA  Dentatorubral-pallidoluysian atrophy
DRAM  Damage-regulated autophagy modulator
FADH2  Flavin adenine dinucleotide (hydroquinone form)
FIP200  Focal adhesion kinase family interacting protein of 200 kD
GCN5  General control nonderepressible-5
GLS2  Glutaminase 2
GPx  Glutathione peroxidase
GST  Glutathione-s transferase
GSTA3  Glutathione S-transferase A3
HD  Huntington’s disease
Htt  Huntingtin
IGF-1  Insulin-like growth factor 1
IP3  Inositol trisphosphate
kDa  Kilodalton
LC3  Microtubule-associated protein 1 light chain 3
LDH  Lactate dehydrogenase
MAPK  Mitogen-activated protein kinases
mTOR  Mammalian target of rapamycin
NAC  N-acetylcysteine
NADH  Nicotinamide adenine dinucleotide (reduced form)
NADPH  Nicotinamide adenine dinucleotide phosphate
NES  Nuclear export signal
NIs  Nuclear inclusions
NLS  Nuclear localization signal
NOX  NADPH-oxidase
NOXO  NADPH oxidase organizer
PDGF  platelet-derived growth factor
PI3K  Phosphatidylinositol 3-kinase
PGAM  Phosphoglyceratemutase
PGC-1 α  Peroxisome proliferator-activated receptor [PPAR]-γ coactivator 1 α
PKA/C  Protein kinase A/C
PolyQ  Polyglutamine
PPP  Pentose phosphate pathway
PUMA  p53 upregulated modulator of apoptosis
Q  Glutamine
QBP1  polyglutamine binding peptide 1
ROS  Reactive oxygen species
SAGA  Spt/Ada/Gcn5/acetyltransferase
SBMA  Spinal and bulbar muscular atrophy
SCA  Spinocerebellar ataxia
SCA7  Spinocerebellar ataxia type 7
SCO2  cytochrome C oxidase 2
Sgf73  SAGA histone acetyltransferase complex 73 kDa subunit
SOD  Superoxide dismutase
STAGA  SPT3-TAF(II)31-GCN5L acetylase
SUMO  Small Ubiquitin-like Modifier
TAFII130  TBP-associated factor 130 kDa
TCA  Tricarboxylic acid cycle
TFTC  TATA-binding protein-free TAF-containing complex
TIGAR  TP53-induced glycolysis and apoptosis regulator
TRRAP  Transformation/transcription domain-associated protein
3-MA  3-Methyladenine
UPS  Ubiquitin proteasome system
ULK  Unc-51 like autophagy activating kinase
Uvrag  UV radiation resistance-associated gene protein
1. Introduction

1.1 Spinocerebellar ataxia type 7 (SCA7)

SCA7 (Spinocerebellar ataxia type 7) is an autosomal dominant inherited neurodegenerative disease associated with progressive cerebellar ataxia (Garden et al. 2008). SCA7 is the most common autosomal dominant spinocerebellar ataxia in Sweden (Jonasson, Juvonen et al. 2000). The average onset age is around 30 and so far there is no cure for SCA7 (David, Durr et al. 1998).

Common symptoms in SCA7 patients are loss of vision, gait ataxia, swallowing and speech problems (David, Abbas et al. 1997; David, Durr et al. 1998; Johansson, Forsgren et al. 1998). The loss of vision distinguishes SCA7 from other cerebellar ataxia diseases (David, Durr et al. 1998). Symptoms are primarily caused by neurodegeneration in the cerebellum, brainstem, and retina (Konigsmark and Weiner 1970; Martin, Van Regemorter et al. 1994; Salas-Vargas, Mancera-Gervacio et al. 2015). The degeneration of Purkinje cells, granule cells and dentate nucleus cells in the cerebellum leads to ataxia, whereas the degeneration of retinal cells (bipolar cells, ganglion cells, rod-cone photoreceptor cells and pigment epithelial cells) results in the loss of vision in SCA7 patients (Martin, Van Regemorter et al. 1994).

Most, but not all, SCA7 patients show only neurological symptoms, however some non-neurological symptoms like muscle hypertonia and patent ductus arteriosus (PDA, an opening between the left lung artery and the aorta that usually closes at birth) have also been observed in infantile SCA7 patients (Anttinen, Nikoskelainen et al. 1986; Benton, de Silva et al. 1998; Johansson, Forsgren et al. 1998).

1.2 The SCA7 gene and mutation

The SCA7 disease causing mutation was identified in 1997 and found to be the expansion of a CAG repeat in a gene named SCA7 (David, Abbas et al. 1997). The SCA7 gene encodes for the Ataxin-7 (ATXN7) protein and the CAG expansion results in an expanded polyglutamine (PolyQ) domain in the N-terminus of ATXN7 (David, Abbas et al. 1997). A healthy person usually has 10-17 CAG/polyQ repeats, while SCA7
patients have more than 37 repeats (David, Abbas et al. 1997; Johansson, Forsgren et al. 1998). The length of the CAG/polyQ expansion is positively correlated with disease severity and negatively correlated with the onset age (David, Durr et al. 1998; Johansson, Forsgren et al. 1998; Martin, Van Regemorter et al. 1999). Furthermore, there is a correlation between the length of the CAG/polyQ repeat and the starting symptom, as ataxia occurs prior to retinal symptoms if the CAG tract is less than 58, while retinal symptoms occur first if the CAG tract is larger than 58 (Johansson, Forsgren et al. 1998).

The expanded CAG repeat is unstable, especially during meiosis and can expand to above 300, which leads to infantile death (David, Durr et al. 1998; Johansson, Forsgren et al. 1998). The expanded allele is often more unstable during paternal than in maternal transmission, however a massive expansion and somatic instability in a maternally transmitted infantile case was recently observed (Trang, Stanley et al. 2014). The mechanism behind the CAG repeat expansion is not fully understood. However, one explanation is that CAG expanded sequences could form abnormal structures called slipped DNAs during processes such as DNA replication, repair, recombination and transcription, as described in reviews (Castel, Cleary et al. 2010; Usdin, House et al. 2015). Studies have shown a correlation between DNA repair system defects and neurodegeneration in polyQ diseases (Jung and Bonini 2007).

1.3 The ATXN7 protein

Wild-type ATXN7 with 10Qs is an 892-amino acid protein and has a predicted molecular weight of 95 kDa (David, Abbas et al. 1997). The ATXN7 protein belongs to a family containing ATXN7, ATXN7-L1 (Ataxin 7 like protein 1), ATXN7-L2 and ATXN7-L3 (Helmlinger, Hardy et al. 2004). The expression of ATXN7 is controlled by a conserved transcription factor CCCTC-binding factor (CTCF) (Sopher, Ladd et al. 2011). So far two isoforms of ATXN7 have been identified, ATXN7a and b (Einum, Clark et al. 2003; Strom A-L. et al., 2005). The only difference between ATXN7a and b is a small sequence in the C-terminus. Both ATXN7a and b are widely expressed in the central nervous system (CNS), whereas cells in the peripheral nervous system (PNS) predominantly express ATXN7a (Lindenberg, Yvert et al. 2000;
Jonasson, Ström et al. 2002; Einum, Clark et al. 2003). ATXN7 isoforms are also ubiquitously expressed in many types of somatic cells, including in heart, muscle, lung, liver, kidney, testis and spleen (Lindenberg, Yvert et al. 2000; Jonasson, Strom et al. 2002). The broad expression of ATXN7 suggests that ATXN7 plays a role in many types of cells, as well as in many different types of neurons (Lindenberg, Yvert et al. 2000; Jonasson, Strom et al. 2002). However, knockout of ATXN7 in Drosophila was recently shown to lead to the degeneration of specifically retinal and cerebellar neurons, suggesting that ATXN7 is especially important for the survival of these neurons (Mohan, Dialynas et al. 2014).

Both ATXN7a and b have 3 nuclear localization signals (NLS) (aa378-393, 704-709, and 834-839) and a nuclear export sequence (NES), indicating that ATXN7 can shuttle in and out of the nucleus (Taylor, Grote et al. 2006). Indeed, ATXN7a was found to localize both to the nucleus and cytoplasm, suggesting that ATXN7 has both cytoplasmic and nuclear functions (David, Abbas et al. 1997; Cancel, Duyckaerts et al. 2000; Jonasson, Juvonen et al. 2000; Jonasson, Strom et al. 2002). In contrast, ATXN7b was observed to predominantly localize to the cytoplasm (Einum, Clark et al. 2003).

In the cytoplasm, ATXN7 is involved in cytoskeleton stabilization and can interact with microtubules (MT) through a large N-terminal region (amino acids 120–400) (Nakamura, Tagawa et al. 2012). However, the polyQ sequence in ATXN7 is not essential for the interaction, and polyQ repeat length does not influence the interaction (Nakamura, Tagawa et al. 2012).

In the nucleus, ATXN7 is a component of the STAGA [SPT3-TAF(II)31-GCN5L acetylase] complex and is involved in gene expression regulation (Helmlinger, Hardy et al. 2004; McMahon, Pray-Grant et al. 2005). STAGA is a highly conserved transcriptional co-activator complex and is the human homologue of the yeast SAGA (Spt/Ada/Gcn5/transferase) complex (Helmlinger, Hardy et al. 2004; McMahon, Pray-Grant et al. 2005). The initiation of gene transcription is regulated by transcription factors and transcriptional coactivators, which can modify histones and thus influence the interaction between transcription factors and promoters (Naar, Lemon et
The SAGA complex has two enzymatic modules, a histone acetyltransferase (HAT) module containing the HAT Gcn5, and a deubiquitinase (DUB) module containing the DUB USP22 (Han Y. et al., 2014). With these two modules, the SAGA/STAGA complex modifies chromatin structure and regulates transcription (Lee, Sardiu et al., 2011; Han Y. et al., 2014). Many stress-induced genes related to neurodegenerative diseases and cancer are regulated by the SAGA/STAGA complex (Mohan, Workman et al. 2014).

Knockdown of ATXN7 results in loss of the DUB module from STAGA and enhances DUB activity in Drosophila (Mohan, Dialynas et al. 2014). ATXN7 has 2 conserved domains, which are important for STAGA DUB function (Helmlinger, Hardy et al. 2004; Bonnet et al. 2010). These two domains are called block I (aa 126-176) and block II (aa 341-400). Block I, at the N-terminus of ATXN7, is required for deubiquitinase module assembly (Lee et al. 2009; Kohler et al 2008). The polyQ tract is located N-terminal of block I in human ATXN7, however absent in Sgf73 in yeast (Helmlinger, Hardy et al. 2004). Block II is believed to be a Zinc finger domain involved in protein-protein interactions within STAGA (Helmlinger, Hardy et al. 2004; Bonnet et al. 2010). Later Bonnet et al at 2010 showed that block II facilitates interaction with nucleosomes (Bonnet et al., 2010).

1.4 SCA7 disease mechanisms

Besides SCA7, there are 8 other neurodegenerative diseases including Huntington’s disease (HD), Dentatorubralpallidoluysian atrophy (DRPLA), Spinal bulbar muscular atrophy (SBMA) and Spinocerebellar ataxias (SCA)-1, 2, 3, 6 and 17 caused by expanded polyQ domains (Fan, Ho et al. 2014). The polyQ diseases are caused by expanded polyQ domains in otherwise unrelated proteins. However these polyQ diseases share several features. In all polyQ diseases, clinical symptom can only be detected when the polyQ expansion is over a threshold of about 30-40 glutamines. Except for SBMA, all polyQ diseases are inherited in an autosomal dominant fashion (Fan, Ho et al. 2014). All expanded polyQ proteins also aggregate and forms inclusions in neurons. Based on these similarities it was early suggested that polyQ diseases are caused by a common gain-of-function mechanism, see review (Pennuto M. and Sambataro F. 2010).
The gain-of-function hypothesis was further supported in 1999, when a transgenic mouse expressing a polyQ domain in a non-polyQ disease related protein HPRT (hypoxanthine phosphoribosyltransferase) was generated (Ordway, Cearley et al. 1999). Expression of polyQ expanded HPRT lead to neuronal degeneration, ataxia and shortened lifespan, similar to symptoms of polyQ diseases, indicating that polyQ tracts are toxic (Ordway, Cearley et al. 1999). Moreover, knockout animal models of polyQ protein encoding genes showed no polyQ disease phenotype (Duyao, Auerbach et al. 1995; Nasir, Floresco et al. 1995). For instance, knockout mice lacking ATXN1 or Htt did not develop HD or SCA1 (Ambrose, Byrd et al. 1994; Matilla, Roberson et al. 1998; Davies, Mirza et al. 1999). Moreover, the expression of mutant Htt rescued the Htt knockout phenotype (White, Auerbach et al. 1997; Leavitt, Guttmman et al. 2001). In contrast, the toxicity of polyQ tracts were shown in several knock-in or transgenic animal models, again indicating the toxic gain-of-function of polyQ tracts (Warrick, Paulson et al. 1998; Yvert, Lindenberg et al. 2000; La Spada, Fu et al. 2001; Lee, Lim et al. 2003; Yoo, Pennesi et al. 2003). In 2000, Yvert et al showed that polyQ expanded ATXN7 (90Q) cause degeneration in cerebellum and retina in transgenic mice, while no toxic effect was detected under wild-type ATXN7 expression (Yvert, Lindenberg et al. 2000). Another transgenic mouse over-expressing polyQ expanded ATXN7 (92Q) constructed by La Spada et al indicated similar toxic effects (La Spada, Fu et al. 2001). Mutant ATXN7 transgenic expression in mice thus leads to similar symptoms as those in SCA7 patients (Yvert, Lindenberg et al. 2000; La Spada, Fu et al. 2001; Yoo, Pennesi et al. 2003). All these evidence suggest that polyQ diseases are gain-of-function diseases.

So far, several possible gain-of-function mechanisms have been suggested, see review (Pennuto M. and Sambataro F. 2010). Since all polyQ proteins, including ATXN7, share the common feature of protein misfolding, accumulation and aggregation, these abnormal features are believed to relate to the toxicity (Weber, Sowa et al. 2014). Misfolded or aggregated polyQ protein species are believed to gain new toxic functions resulting in transcriptional dysfunction, impaired protein degradation pathways, metabolic dysfunction and oxidative stress. Some of these mechanisms will be further discussed in later sections.
However, besides gain-of-function, several recent studies suggest that loss-of-function could also contribute to polyQ diseases (Orr 2012; Clabough 2013; Durcan and Fon 2013; Mohan, Workman et al. 2014). In HD, loss of the normal Htt protein function might actually equally contribute to the pathology, see review (Cattaneo, Rigamonti et al. 2001). Loss-of-function contribution has also been suggested in SCA7. In Zebra fish, wild-type ATXN7 was recently shown to be required for the differentiation of photoreceptor and cerebellar neurons (Yanicostas, Barbieri et al. 2012). Moreover, the loss of ATXN7 led to degeneration of these types of neurons in both Zebra fish and Drosophila (Yanicostas, Barbieri et al. 2012; Mohan, Dialynas et al. 2014). Furthermore, knockout of ATXN7 in Drosophila resulted in reduced mobility and shorter lifespan (Mohan, Workman et al. 2014). All these phenotypes are quite similar to transgenic Drosophila expressing polyQ expanded ATXN7 (Mohan, Workman et al. 2014). As previously described wild-type ATXN7 is involved in regulation of MT cytoskeleton stability, as well as gene expression regulation through STAGA. Mutation/expansion of the polyQ domain in ATXN7 could thus be toxic through loss of these functions. However, so far no reports show that polyQ expansion alters MT regulation. In fact, both wild-type and expanded ATXN7 could stabilize MT to the same degree (Nakamura, Tagawa et al. 2012). However, it was not ruled out that sequestration of wild-type ATXN7 into polyQ expanded ATXN7 inclusions could reduce wild-type ATXN7 levels and thus impair MT stability.

In contrast, evidence showing that polyQ expansion in ATXN7 could alter STAGA function and that this could contribute to SCA7 has been identified (Kohler, Zimmerman et al. 2010; La Spada, Fu et al. 2001). First, transcriptional alterations of many genes have been shown in SCA7 (Chou, Chen et al. 2010). In Chou’s study, altered expression of proteins related to multiple functions including glutamatergic transmission, signal transduction, myelin formation, deubiquitination, axonal transport, neuronal differentiation, glial functions and heat shock proteins were detected (Chou, Chen et al. 2010). In addition, mutant ATXN7 has been shown to incorporate into the STAGA complex, inhibit the HAT activity of STAGA and affect the interaction between the transcription factor CRX (Cone-rod homeobox) and the STAGA complex (Palhan, Chen et al. 2005; Burke, Miller et al. 2013). However, controversial studies indicate
that polyQ expanded ATXN7 do not affect the HAT activity of STAGA (Helmlinger, Hardy et al. 2006). Moreover, the DUB activity has also been shown to be affected by mutant ATXN7. Although no altered DUB enzymatic activity was detected in STAGA containing mutant ATXN7, co-aggregation of polyQ expanded ATXN7 and DUB resulted in reduced DUB activity (Lan, Koutelou et al. 2015). However, nonallele specific silencing of ATXN7 was recently shown to be tolerated in mice, indicating that a decreased level of wild-type ATXN7 is not harmful in mice (Ramachandran, Boudreau et al. 2014). In summary, the contribution of loss of ATXN7 and STAGA function in SCA7 is still unclear.

In this thesis, I have studied different mechanisms involved in mutant ATXN7 toxicity, including mutant ATXN7 aggregation, the role of protein quality control system impairment (Yu, Munoz-Alarcon et al. 2013), the effect of oxidative stress (Ajayi, Yu et al. 2012) and metabolic dysfunction (Ajayi, Yu et al. 2015). Some more background on the role of these mechanisms in polyQ disease will therefore follow below.

1.5. PolyQ protein aggregation

Aggregation of polyQ proteins into inclusion bodies (IBs) is a hallmark in polyQ diseases (Weber, Sowa et al. 2014). The aggregation ability and toxicity of polyQ-expanded proteins is inversely correlated to the length of the polyQ repeat, see review (Li, Li et al. 2008). In SCA7 patients, mutant ATXN7 can be detected and aggregate into inclusion bodies both in affected areas (cerebellar cortex and retina) and “non-affected” areas (hippocampus) (Cancel, Duyckaerts et al. 2000; Einum, Townsend et al. 2001; Jonasson, Strom et al. 2002).

The formation of polyQ protein IBs is initiated from the expression of mutant protein monomers. After a conformational misfolding into a β-sheet structure, the monomers form oligomeric structures, which then further assemble into IBs (Todd and Lim 2013). In order to understand the role of aggregation in polyQ diseases, we need to understand which species are toxic and how polyQ expanded species formed by aggregation induce toxicity. We also need to understand which factors control the aggregation ability of polyQ-expanded proteins.
Both misfolded monomers and oligomers of polyQ proteins are believed to be toxic to neuronal cells (Behrends, Langer et al. 2006; Li, Chevalier-Larsen et al. 2007; Nagai, Inui et al. 2007). However the bidirectional transition between monomers and oligomers makes it difficult to distinguish which form of these polyQ protein species are more toxic and if one of them is sufficient to generate neuronal toxicity (Schaffar, Breuer et al. 2004; Nagai, Inui et al. 2007; Takahashi, Kikuchi et al. 2008). It has been suggested that the formation of higher order aggregates into IBs may be cytoprotective (Taylor, Tanaka et al. 2003). For instance, Muchowski et al demonstrated that Hsp70 and Hsp40 suppress cytotoxicity of mutant polyQ expanded proteins in HD, SCA1 and SBMA models, without affecting IB levels (Muchowski, Schaffar et al. 2000). The formation of IBs also seems protective in striatal neurons in HD models (Takahashi, Kikuchi et al. 2008). However, whether IBs are linked to toxicity or not is still controversial (Weber et al. 2014).

The toxicity caused by misfolded and/or aggregated polyQ species are not fully understood, but several mechanisms, including damage to mitochondria, abnormal axonal transport, the dysregulation of protein degradation mechanisms and the coaggregation of proteins have been suggested (Davies, Turmaine et al. 1997; Nucifora, Sasaki et al. 2001; Morfini, Pigino et al. 2005). Both the ubiquitin proteasome system (UPS) and autophagy, the two main cellular protein degradation mechanisms, have been shown to be affected by aggregating polyQ expanded proteins and this will be discussed further in section 1.6. Co-aggregation of several proteins including chaperons, UPS components and several transcription factors has been identified. Co-aggregation of transcription factors and cofactors like Sp1, p53, CREB-binding protein (CBP) and TAFII have all been detected and suggested to result in transcriptional dysregulation (Yamada, Tsuji et al. 2000). As previously mentioned, ATXN7 is a subunit of the deubiquitinase (DUB) module in the STAGA complex. Recently the DUB module was shown to co-aggregate with mutant ATXN7 and be sequestered away from its substrates in a SCA7 model (Lan, Koutelou et al. 2015).

The misfolding and aggregation of polyQ expanded proteins is influenced by several factors. First, the subcellular localization can affect the accumulation and aggregation of polyQ expanded proteins (Havel, L.S. et
al, 2009). As described before, ATXN7 a and b are two isoforms of ATXN7. The ATXN7a localizes both in the cytoplasm and nucleus, however ATXN7b has been observed predominantly in the cytoplasm (Eینum DD, et al 2003; Strom A-L. et al, 2005). Interestingly, no IBs could be detected in SCA7 patient when using an antibody specific to ATXN7b (Eینum, Clark et al. 2003). The lack of autophagy degradation in the nucleus is one possible reason why enhanced accumulation and aggregation of mutant ATXN7a could occur in the nucleus, see section 1.6.2 for more.

Another factor that can influence aggregation is proteolytic cleavage. Proteolytic cleavage of mutant polyQ proteins resulting in truncated fragments more prone to form toxic structures has been shown to occur in HD (Ratovitski, Gucek et al. 2009), SBMA (Li, Chevalier-Larsen et al. 2007) and SCA7 models (Young, Gouw et al. 2007). The cleavage of full-length ATXN7 at Asp residues 266 and 344 by caspase-7 have been reported and shown to affect the localization and cellular toxicity of mutant ATXN7 (Young, Gouw et al. 2007; Guyenet, Mookerjee et al. 2015). Proteolytically cleaved mutant ATXN7 is predominantly detected in the nucleus, rather than in the cytoplasm (Young, Gouw et al. 2007). The elevated level of cleaved ATXN7 fragments containing expanded polyQ tract in the nucleus could be due to the removal of the NES upon cleavage thus reducing nuclear exit and access to autophagic degradation in the cytoplasm (Young, Gouw et al. 2007).

A third factor that can influence aggregation and toxicity is post-translational modifications. Phosphorylation, acetylation, ubiquitination and sumoylation have been reported to influence polyQ protein aggregation and toxicity (Terashima, Kawai et al. 2002; Warby, Chan et al. 2005; Mookerjee, Papanikolaou et al. 2009; Janer, Werner et al. 2010). For example phosphorylation of mutant Htt at serine 421 might contribute to the selective neurodegeneration in HD (Warby, Chan et al. 2005). Moreover ubiquitination has been shown to target several polyQ proteins to the ubiquitin proteasome system (UPS) for degradation (Terashima, Kawai et al. 2002). In SCA7 models both sumoylation and acetylation has been shown to modify aggregation and toxicity. Preventing the sumoylation of polyQ expanded ATXN7 increased the formation of IBs and enhanced toxicity (Janer, Werner et al. 2010).
acetylation of ATXN7 on lysine 257 stabilized caspase-7 cleaved ATXN7 fragments from autophagic degradation (Mookerjee, Papanikolaou et al. 2009). The acetylation of mutant ATXN7 could therefore promote the accumulation, aggregation and toxicity of mutant ATXN7.

In summary, polyQ expanded proteins misfold and aggregate and this is believed to induce toxicity. The stability and the aggregation of polyQ proteins can be modulated by cellular localization, proteolytic cleavage and posttranslational modifications. Furthermore the activity of protein quality control systems, which can degrade misfolded proteins, can also influence aggregation and toxicity and will be discussed further below.

1.6 Impairment of protein quality control systems

Aggregation-prone polyQ expanded proteins are not efficiently cleared away in cells and enhancing the clearance is believed to be beneficial in polyQ diseases (Weber, Sowa et al. 2014). For example reduction of mutant ATXN7 expression was recently shown to ameliorate symptoms in SCA7 transgenic mice (Furrer, Waldherr et al. 2013). Protein quality control systems can refold or degrade misfolded, potentially damaging, proteins and these systems are crucial for cell survival (Kubota 2009). UPS and macroautophagy (called as autophagy here) are the two main protein degradation systems and both mechanisms are suggested to be affected in polyQ disease (Rubinsztein 2006). In order to develop strategies to improve the clearance of pathogenic polyQ proteins, it is of interest to understand if and how polyQ expanded proteins like ATXN7 is normally degraded and can inhibit UPS and/or autophagy.

1.6.1 UPS dysfunction in polyQ diseases

The UPS system is believed to mainly degrade short-lived or small proteins in the cytoplasm and nucleus of mammalian cells (Ciechanover and Kwon 2015). In UPS, proteins are ubiquitinated and then shuttled to the proteasome where they are unfolded and degraded (Glickman and Ciechanover 2002).

Proteasome components have been reported to associate with polyQ protein inclusions and induce UPS dysfunction resulting in the activation
of apoptosis (Jana, Zemskov et al. 2001). The impairment of UPS activity could be due to the blockage of proteasomes by polyQ-expanded proteins (Bennett, Shaler et al. 2007; Tydlacka, Wang et al. 2008). Interaction between ATXN7 and a portion of the 19S subunit of the proteasome has been observed (Matilla, Gorbea et al. 2001). In addition, the polyQ-expanded form of ATXN7 has been suggested to impair the proteolytic function of proteasomes in mammalian cells (Wang, He et al. 2007). However, in other in vivo studies, global UPS activity was not affected in the brains of SCA7 and HD transgenic mice (Bowman, Yoo et al. 2005; Bett, Cook et al. 2009; Jeong, Then et al. 2009). These controversial results obtained in cell versus animal models was reconciled by Ortega et al, when they showed that UPS-impairment occurred transiently under polyQ expression and the formation of IBs correlated with the restoration of UPS function, indicating that the IB formation results in UPS recovery (Ortega, Diaz-Hernandez et al. 2010).

1.6.2 Autophagy impairment in polyQ diseases

Autophagy is a multistep process in which substrates are degraded through engulfment into autophagosomes as depicted in Figure 1 (Kirkegaard, Taylor et al. 2004). After the induction of autophagy, a phagophore is formed and elongated into an autophagosome. The fusion of the autophagosome with a lysosome then leads to the degradation of the engulfed substrates by lysosomal enzymes. Autophagy is only present in the cytoplasm and it is one of the main pathway by which cells clear away dysfunctional organelles and misfolded proteins (Mizushima 2007). Autophagy is regulated by many mechanisms/signaling pathways (Jing Yang, et al. 2015). The initiation of autophagy can be regulated by both mTOR dependent and / or independent pathways (Vemika C, et al, 2015). In the mTOR dependent pathway, autophagy is activated through the inhibition of mTOR, which results in the release of mTOR inhibition of Unc-51 like autophagy activating kinase (ULK1). The ULK1 kinase is then activated and induces phagophore formation (Mizushima N, 2010).

Autophagic clearance of aggregate-prone proteins is indispensable for post-mitotic cells, especially neurons, which cannot dilute misfolded, potentially toxic protein, by cell division (Carroll, Hewitt et al. 2013).
Many polyQ-expanded proteins can be degraded by autophagy (Ravikumar, Duden et al. 2002; Young, Martinez et al. 2009; Duncan, Papanikolaou et al. 2010). Moreover, enhancing autophagy was shown to ameliorate toxicity in some, but not all polyQ diseases (Menzies, Huebener et al. 2010; Nisoli, Chauvin et al. 2010). However, increasing evidences indicate that autophagic dysfunction occurs in many polyQ diseases (Sarkar 2013; Cortes and La Spada 2015). In SBMA the expression of mutant androgen receptor impairs the maturation of the autophagosome (Cortes, Miranda et al. 2014). In HD models, the expression of polyQ expanded Htt disrupted cargo recognition, autophagic flux and axonal transport of autophagosomes (Heng, Duong et al. 2010; Martinez-Vicente, Talloczy et al. 2010; Wong and Holzbaur 2014). The impairment of autophagic flux has also recently been detected in SCA7 models by us and others, see result sections 4.3 and Alves, Cormier-Dequaire et al. 2014.

1.7 Metabolic alteration in polyQ diseases

Defects in energy production accompanied with dysfunctional mitochondria have been reported in a variety of human neurodegenerative diseases, including Parkinson’s disease (PD), Alzheimer’s disease (AD), amyotrophic lateral sclerosis (ALS) and polyQ diseases (Damiano, Galvan et al. 2010; Oliveira 2010; Esteves, Arduino et al. 2011; Cozzolino and Carri 2012; Wang, Wang et al. 2014).

The main pathway to generate energy in mammalian cells is mitochondrial oxidative phosphorylation (OXPHOX), although glycolysis also generates some energy from glucose (Vander Heiden,
Cantley et al. 2009). Pyruvate generated from glucose during glycolysis is catalyzed by pyruvate dehydrogenase (PDH) into acetyl-CoA which is subsequently used in the tricarboxylic acid cycle (TCA). The NADH produced in the TCA cycle is then used by the mitochondria electron transport complexes (ETC) to generate H₂O and ATP as depicted in Figure 2 (Warburg 1956; Archer, Weir et al. 2010).

Since neurons are highly dependent on ATP and thus proper mitochondria function, neurons are very vulnerable to mitochondria and metabolic dysfunction (Kann and Kovacs 2007). Many studies indicate that energy deficits and alterations in cellular metabolism occur and contribute to neurodegeneration in polyQ diseases (Bauer and Nukina 2009; Chen 2011; Ju, Lin et al. 2012). In HD brains, mitochondrial dysfunction, impaired oxidative phosphorylation and decreased glycolysis have been detected (Chen 2011). Metabolic dysfunction in HD brains could be caused by both ETC dysfunction and other ETC independent mechanisms (Browne, Bowling et al. 1997; Milakovic and Johnson 2005; Powers, Videen et al. 2007; Li, Orr et al. 2010). Mutant Huntingtin has for instance been shown to impair mitochondria via various mechanisms, including the dysregulation of gene transcription, accumulation of polyQ proteins in or on mitochondria and impairment of mitochondria trafficking (Li, Orr et al. 2010). Decreased activity of
mitochondrial respiratory complexes II, III and IV was identified in postmortem samples of HD patients (Gu, Gash et al. 1996; Milakovic and Johnson 2005). In SCA7 patients, changes in mitochondrial morphology, disrupted ETC activity and metabolic acidosis have been detected (Forsgren, Libelius et al. 1996; Johansson, Forsgren et al. 1998). SCA7 patients also generally have difficulties to gain body weight (Johansson, Forsgren et al. 1998). However, the mechanism behind the disrupted metabolism in SCA7 is still far away from clear and clarifying these mechanisms was therefore one of the aim of this thesis, see result section 4.5.

1.8 Oxidative stress and polyQ diseases

Under normal physiological conditions reactive oxygen species (ROS) are formed mainly in mitochondria during oxidative phosphorylation and are at low levels used to regulate various signal transduction pathways (Kirkinezos and Moraes 2001). However, elevated ROS levels and oxidative stress occur in many neurodegenerative diseases, including AD, PD and polyglutamine diseases (Sayre, Perry et al. 2008). Oxidative stress occurs when the ROS level overwhelm the capacity of cellular antioxidant mechanisms and cause oxidative damage to cellular molecules, including DNA, proteins and lipids (Sayre, Perry et al. 2008).

The cause of the elevated ROS levels in neurodegenerative diseases is still not completely understood, but could involve the failure in antioxidant defense systems or activation of mechanism resulting in increased ROS production. Key cellular antioxidant mechanisms include anti-oxidants like glutathione and antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), peroxiredoxins, glutathione peroxidase, and glutathione transferases (GST) (Coyle and Puttfarcken 1993; Fatokun, Stone et al. 2008). Altered levels of antioxidant enzymes, including SOD1, CAT, GSTAs, glutathione peroxidases, have been detected in many polyQ diseases (del Hoyo, Garcia-Redondo et al. 2006; Sorolla, Reverter-Branchat et al. 2008; Reijonen, Kukkonen et al. 2010).

Four main mechanisms; 1) mitochondrial dysfunction, 2) aberrant transcriptional regulation of PGC-1α (peroxisome proliferator-activated receptor-gamma coactivator 1 alpha), 3) ROS generation during the formation of IBs (Firdaus, Wyttenbach et al. 2006), and 4) NADPH
oxidase (NOX) activation (Bertoni, Giuliano et al. 2011; Valencia, A., E. Sapp, et al. 2013) have been suggested to result in more ROS in polyQ diseases. The mitochondrial ROS generation is initiated from the ETC on the inner membrane of mitochondria during the process of oxidative phosphorylation. The leakage of electrons from the ETC results in partial reduction of oxygen to generate \( \text{O}_2^\bullet \) and further produces \( \text{H}_2\text{O}_2 \) (Cadenas and Davies 2000; Li, Fang et al. 2013). Polyglutamine proteins have been suggested to directly affect mitochondria and cause damage to the mitochondria electron transport chain, thus increasing electron escape (Solans, Zambrano et al. 2006; Puranam, Wu et al. 2006). PGC-1\( \alpha \) activates and coordinates gene expression of genes involved in mitochondrial oxidative metabolism (Puigserver and Spiegelman 2003). It has been shown that mutant Htt bind to the promoter of PGC-1\( \alpha \) and interfere with the transcription of PGC-1\( \alpha \) (Cui, Jeong et al. 2006). Mutant Htt was also reported to be associated with reduced expression of PGC-1\( \alpha \)-regulated genes, including proteins involved in the electron-transport chain, which could lead to increased electron escape (Weydt, Pineda et al. 2006). Inclusion bodies have also been suggested to catalyze the generation of ROS via an iron dependent process in a HD model (Firdaus, Wyttenbach et al. 2006). NOX are membrane-bound enzymes that can be activated to generate ROS (Bedard and Krause 2007). These enzymes are most relevant for this thesis and they will therefore be further described below.

### 1.8.1 NOX complexes

NOX enzymes are transmembrane proteins that generate ROS by transferring electrons across biological membranes (Bedard and Krause 2007). So far 7 NOX isoforms have been identified, including NOX 1-5 and Dual Oxidase 1-2 (DOUX 1 and DOUX 2) (Hernandes and Britto 2012). NOX 1–5 are known to produce \( \text{O}_2^\bullet \), whereas DUOX enzymes are able to release \( \text{H}_2\text{O}_2 \) without forming a detectable amount of \( \text{O}_2^\bullet \) (Bedard and Krause 2007).

NOX complexes are ubiquitously expressed in mammals and the ROS generated by NOX enzymes regulate several important cellular functions including differentiation, apoptosis and cell growth (Ajayi A, et al. 2013; Bedard K, et al, 2007). Some NOX enzymes, such as NOX1-3, require additional proteins including p22phox for full activity. In contrast other
NOX enzymes, for instance NOX5, can have activity alone, see review (Lambeth, Kawahara et al. 2007; Ajayi A, et al. 2013). Tissue distributions of different NOX members are markedly different. In neurons, mostly NOX 1, 2 and 4 are expressed. In brain NOX enzymes in astrocytes and microglia also generate ROS, see review (Cooney, Bermudez-Sabogal et al. 2013; Ajayi A, et al. 2013).

In polyQ diseases, several studies indicate that expression of mutant polyQ proteins could activate NOX and lead to oxidative stress and cell degeneration (Bertoni, Giuliano et al. 2011; Valencia, Sapp et al. 2013; Maldonado, Molina-Jijon et al. 2010). For instance, mutant ATXN2 and Htt were detected to interact with NOX2 (also known as gp91) in lipid raft resulting in NOX activation and cellular toxicity (Bertoni, Giuliano et al. 2011). We have also shown that NOX enzymes are activated in SCA7, see results section 4.4. However, how NOX activity is regulated in polyQ diseases and contributing to oxidative stress and toxicity is still not fully understood.

1.9 p53 and polyQ diseases

Several studies indicate that p53 is involved in many neurodegenerative diseases, including AD, PD, prion and polyQ diseases (Feng, Jin et al. 2006; Lane and Levine 2010). The p53 tumor suppressor protein is a key transcription factor involved in anti-oxidant defense, autophagy regulation, genomic stability, proliferation, senescence and cell death (Maiuri, Galluzzi et al. 2010; Jebelli, Hooper et al. 2012; Berkers, Maddocks et al. 2013). In response to cellular stress, p53 can be activated by many factors including genotoxic damage, oncogene activation, loss of normal cell contacts, and nutrient or oxygen deprivation (Horn and Vousden 2007). Mild stress induces a p53 response to attempt to restore homeostasis. However, severe or long-term stress induces a p53 response that irreversibly leads to cell death or senescence (Horn and Vousden 2007). Fluctuations in oxygen or nutrient availability generally lead to more adaptive responses, in which p53 induces metabolic remodeling and promotes catabolism, see review (Berkers, Maddocks et al. 2013). In our studies, we mainly focused on the involvement of p53 in autophagic and metabolic regulation.
It has been shown that p53 depending on the subcellular localization has a dual function in autophagy regulation (Maiuri, Galluzzi et al. 2010). Nuclear p53 predominantly stimulate autophagy, as many proteins involved in autophagy or the regulation of autophagy are transcriptionally regulated by p53, see Figure 3 (Fullgrabe, Klionsky et al. 2014). Proteins such as Sestrins are for instance upregulated by p53 and lead to activation of the AMPK pathway and thereby induction of autophagy (Fullgrabe, Klionsky et al. 2014; Maiuri, Galluzzi et al. 2010). Additionally, many proteins directly involved in autophagy regulation, including ULK1, ATG4, 6, 7, 10 and ultraviolet irradiation resistance-associated gene (UVRAG), have been also identified as potential p53 targets (Maiuri et al. 2010).

In contrast, cytoplasmic p53 inhibits autophagy, although the exact mechanism is still not fully clear (Tasdemir, Maiuri et al. 2008; Livesey, Kang et al. 2012). Recently an interaction between p53 and FIP200, a protein involved in autophagy initiation, was shown to be involved in the inhibitory ability of cytoplasmic p53 on autophagy (Tasdemir, Maiuri et al. 2008; Morselli, Shen et al. 2011). FIP200 is a component of the ULK1·ATG13·FIP200 complex which initiates autophagy (Ganley, Lam et al. 2009). FIP200 knockout mice show abnormal ULK1 stability and disrupted autophagy activity (Hara, Takamura et al. 2008). It is possible that p53 by binding to FIP200 could alter the FIP200-ULK1 interaction and thereby block autophagy induction.

Another important function of p53 is to regulate cellular metabolism and the balance between glycolysis and OXPHOS (Berkers, Maddocks et al. 2013; Liang, Liu et al. 2013). The p53 protein restricts glycolytic flux, but promotes mitochondrial integrity and OXPHOS as described in Figure 4 (Berkers, Maddocks et al. 2013; Liang, Liu et al. 2013).
p53 promotes mitochondria maintenance and OXPHOS by regulating the expression of several metabolic related genes, including AIF, see review (Berkers, Maddocks et al. 2013; Liang, Liu et al. 2013). The apoptosis inducing factor (AIF), which is induced by p53, is involved in keeping the integrity of ETC complex I (Stambolsky, Weisz et al. 2006). Loss of AIF in mice cause neuronal death of primarily cerebellar and retinal neurons, neurons which are highly affected in SCA7 (Polster 2013). Additionally, a key component of ECT complex IV, cytochrome c oxidase 2 (SCO2), is also transcriptionally activated by p53 (Matoba, Kang et al. 2006).

The repressive effect of p53 on glycolysis is mediated for instance via regulation of TIGAR and GLUT1 (Berkers CR. Et al, 2013; Liang Y. et al, 2013). The expression of TP53-induced glycolysis and apoptosis regulator (TIGAR) is transcriptionally activated by p53 and decreases the glycolytic rate and promotes the diversion of glycolytic intermediates into the PPP (Pentose Phosphate Pathway) instead (Bensaad, Tsuruta et al. 2006; Li and Jogl 2009). p53 also reduces the up-take of glucose by both directly (GLUT1 and GLUT4) or indirectly (GLUT3) down-regulating the expression of glucose transporters, (Schwartzenberg-Bar-Yoseph, Armoni et al. 2004; Kawauchi, Araki et al. 2008).

Loss of p53 function, as well as increased p53 activity, has been reported in polyQ diseases. In HD models, p53 interacts with mutant Htt and is sequestrated into the IBs (Steffan, Kazantsev et al. 2000; Ryan, Zeitlin et al. 2006). Moreover, polyQ expanded Htt interrupt CBP mediated transcriptional regulation by disrupting the interaction between p53 and CBP (Steffan, Kazantsev et al. 2000). However, the expression of polyQ expanded Htt has also been reported to elevate the phosphorylation of p53 and induce the expression of apoptotic p53 target genes, thereby causing apoptosis (Grison, Mantovani et al. 2011). Bax and PUMA, transcriptionally regulated by p53, are also elevated and believed to contribute to the neuronal degeneration in both SCA3 and SCA7 models (Wang, Chou et al. 2010; Chou, Lin et al. 2011). Additionally, p53 have been shown to accumulate in the cytoplasm and mitochondria in polyQ models, thereby resulting in induction of apoptosis via the release of cytochrome c from mitochondria (Tsoi, Lau et al. 2012;Illuzzi, Vickers et al. 2011). Deletion of p53 reduced the pathology caused by polyQ
expanded ATXN1 in a mice model (Shahbazian, Orr et al. 2001). The role of p53 in polyQ diseases is thus complicated and not fully clear, further studies on p53 in polyQ disease could contribute to the therapeutic consideration for these disorders.

Figure 4. p53 is involved in glucose metabolism regulation

![Glucose metabolism diagram](image)

G6P: glucose-6-phosphate; F6P: fructose-6-phosphate; F2, 6BP: fructose-1, 6-biphosphate; 3PG: glyceraldehyde-3-phosphate; 2GP: 2-phosphoglycerate; PEP: phosphoenolpyruvate.

1.10 Therapeutic considerations for polyQ diseases

An increasing number of people are now reported to be affected by polyQ diseases worldwide, and currently there is no cure but only symptomatic treatments (Martin 2012). By understanding the pathological mechanisms of SCA7 and other polyQ diseases, many possible therapeutic strategies could hopefully be developed.

Decreasing polyQ expanded protein expression is one potential therapeutic strategy. The expression of mutant polyQ proteins could be reduced by knockdown of mutant gene expression or by enhancing degradation of the mutant protein. Antisense oligonucleotide (AONs) and
RNA interference (RNAi) aiming at silencing the mutant CAG expanded mRNA have showed promising therapeutic effects in polyQ disease models (Gonzalez-Alegre and Paulson 2007; Kim and Rossi 2007; Miller, Smith et al. 2008; Ramachandran, Bhattarai et al. 2014). As most polyQ diseases are autosomal dominant, only 50% of the expressed polyQ protein is expanded. It would be easier for clinical use if nonallele specific silencing of the wild-type and expanded polyQ protein was possible and did not affect the normal function of cells. In SCA7 models, in vivo tests indicate that at least retinal cells tolerate the reduction of both mutant and wild-type ATXN7 expression, suggesting that nonallele specific silencing could work in SCA7 (Ramachandran, Boudreau et al. 2014). However, further careful examinations need to be done to make sure that there are no loss-off-function side effects.

The reduction of mutant polyQ proteins could also be realized by enhancing polyQ protein degradation through stimulation of protein quality control systems. As previously depicted, the stimulation of autophagy can reduce expanded polyQ protein expression and ameliorates toxicity (Waza, Adachi et al. 2005; Wang, Miyata et al. 2013; Cortes and La Spada 2014). Rapamycin, an autophagy activator, has been approved by FDA and is currently used as an immunosuppressor in patients receiving organ transplantations (Lopez, Kohler et al. 2014), and to treat renal cancer (Cloughesy, Yoshimoto et al. 2008). Rapamycin could thus be a potential drug in polyQ disease. However, so far no results from clinical trials using Rapamycin for polyQ diseases have been reported.

Since oxidative stress has been linked to polyQ diseases, stimulation of antioxidant systems or administration of exogenous antioxidants could also have therapeutic effects (Kamat, Gadal et al. 2008). Antioxidants such as coenzyme Q10 (CoQ10), creatine, N-acetyl cysteine (NAC), vitamin C/E, thiols and tauroursodeoxycholic acid (TUDCA), are reported to ameliorate toxicity and prolong the lifespan of HD and SCA1 transgenic mice (Johri and Beal 2012). CoQ10 also showed a trend toward slowing HD disease, on average with 15 percent, in a clinical trial, however due to limited numbers of patients in the study it is hard to recommend HD patients to take CoQ10 (Hyson, Kieburtz et al. 2010). A higher dose CoQ10 phase III clinical trial is ongoing in HD since 2012,
and in addition a phase III clinical trial on creatine has also been approved (Johri and Beal 2012). However, there is a risk that over dosing of anti-oxidants might block basal autophagy and therefore reduce polyQ protein clearance and thus enhance neurodegeneration (Underwood, Imarisio et al. 2010).

Preventing the misfolding of mutant polyQ proteins is another possibility to attenuate polyQ disease. Recognition of various polyQ proteins by the antibody 1C2 indicates that mutant polyQ expanded proteins share a common structure (Trottier Y. et al., 1995; Alexander P. O. et al. 2006). A short peptide named QBP1 (polyglutamine binding peptide 1) composed of 11 amino acids (SNWKWWPGIFD) selectively binds to the polyQ stretch (Nagai, Tucker et al. 2000). Therapeutic effects of QBP1 were shown in tests using both cell and animal models (Nagai, Tucker et al. 2000; Okamoto, Nagai et al. 2009). Pharmacological induction of chaperones that can refold polyQ-expanded proteins and ameliorates the toxicity has also been suggested (Katsuno, Sang et al. 2005).

Stimulation of neuronal survival by neurotropic factors has also been considered as a therapeutic strategy for polyQ diseases (Noma, Ohya-Shimada et al. 2012). Noma et al recently reported that overexpression of hepatocyte growth factor (HGF) could attenuate the degeneration of Purkinje cells and Bergman glia in a knock-in mouse model of SCA7 (Noma, Ohya-Shimada et al. 2012). Implantation of stem cells secreting different neurotropic factor, such as brain derived neurotrophic factor (BDNF), have also been shown to prevent the degeneration of HD mouse striatal neurons (Pineda, Rubio et al. 2007).

Apoptosis is a highly regulated cellular death pathway that has been shown to have a role in polyQ diseases (Wellington and Hayden 2000). The execution of apoptosis normally involves the activation of caspases, and caspase activation has been observed in SCA7 (Dragunow, Faull et al. 1995; Ona, Li et al. 1999; Zander, Takahashi et al. 2001; Young, Gouw et al. 2007; Guyenet, Mookerjee et al. 2015). Caspase inhibitors like zVAD-fmk and minocycline were shown to slow the progression of HD in mice via inhibition of caspase-1 and 3 (Chen, Ona et al. 2000).
SCA7 and other polyQ diseases have also been observed to be associated with transcriptional dysfunction (Orr 2012). Another suggested treatment strategy is therefore to inhibit histone deacetylases (HDAC), which will promote gene expression. HDAC inhibitors like SAHA, phenylbutyrate and sodium butyrate have all been shown to ameliorate the toxicity of polyQ expanded Htt in cells (McCampbell, Taye et al. 2001), Drosophila (Steffan, Bodai et al. 2001) and mouse models of HD (Ferrante, Kubilus et al. 2003). However, the low selectivity of HDAC inhibitors may lead to toxicity when applied in clinical use, limiting the potential of these drugs for neurodegenerative diseases (McCampbell, Taye et al. 2001).
2. Aims of the thesis

The overall aim of this thesis was to investigate the pathological mechanisms by which mutant ATXN7 cause toxicity and how these pathological mechanisms could be counteracted. More specifically, the aim of each paper was as listed below:

**Paper I**

The aim of paper I was to construct a neuronal inducible SCA7 cell model and to investigate whether ATXN7 species are degraded via UPS and/or autophagy, as well as if stimulation of autophagy could ameliorate the mutant ATXN7 toxicity.

**Paper II**

The aim of paper II was to investigate if autophagy is fully functional in SCA7 cells and if not to determine the mechanism behind the autophagy impairment, as well as whether pharmacological reversal of this dysfunction could ameliorate ATXN7 toxicity.

**Paper III**

The aim of paper III was to investigate whether oxidative stress is a factor involved in SCA7 and if so to identify the source of the elevated ROS, as well as whether reduction of ROS is a potential therapeutic strategy in SCA7 disease.

**Paper IV**

The aim of paper IV was to investigate the molecular mechanism behind the metabolic impairment observed in SCA7 patients, and how this potentially toxic mechanism could be counteracted.
3. Methodological considerations

The methods used in this thesis are described in the materials and methods section in each paper; consequently, only general aspects of some key methods used will be discussed below.

3.1 Cell lines

HEK293T, HeLa and stable neuronal PC12 cells expressing ATXN7 under the control of an inducible promoter were used.

3.1.1 HEK 293T cells

Human Embryonic Kidney 293T cells are transformed cancer cells originally derived from human embryonic kidney cells (Graham, Smiley et al. 1977). HEK293T cells were chosen because they are one of the easiest human cells to grow and to transfect. In addition, HEK293T cells endogenously express ATXN7.

3.1.2 HeLa cells

HeLa cells are another type of cells commonly used in scientific research. These cells are derived from human cervical cancer and are remarkably durable and prolific (Scherer, Syverton et al. 1953). NOX1 is highly expressed in HeLa cells, which made this cell line suitable to study how NOX1 is affected by mutant ATXN7 expression (Cheng, Cao et al. 2001).

3.1.3 Stable inducible PC12 cell lines

PC12 is a neuronal like cell line derived from a pheochromocytoma of the rat adrenal medulla (Greene and Tischler 1976). A commercial PC12 Tet-off cell line (Clontech) was transfected with FLQ10-pTRE-tight or FLQ65-pTRE-tight plasmid constructs encoding ATXN7Q10-GFP and ATXN7Q65-GFP respectively. In PC12 Tet-off cells, a regulator plasmid encoding the tetracycline-controlled transactivator (tTA) is present. When doxycycline (Dox) is present in the media, the binding of the tTA protein to the Tet-Response Element (TRE) regulating the ATXN7 expression is inhibited. However upon the removal of Dox from the media the suppression of TRE is released and the ATXN7 expression is induced, see Figure 4. In contrast to the Tet-off system, the target proteins are expressed in the presence of Dox in the Tet-on system. We preferred the
Tet-off system to avoid any potential side effects induced by Dox (Ermak, Cancasci et al. 2003). In the screening of constructed stable PC12 cell lines, cell lines that had similar expression levels of transgenic (wild-type or expanded) ATXN7 as endogenous ATXN7 were selected to avoid over-expression effects. In order to make FLQ65 and FLQ10 cells comparable, cell lines with similar expression levels of ATXN7Q10-GFP and ATXN7Q65-GFP were also chosen. The advantage of this cell model is that the expression of ATXN7 can be controlled at different time point making it possible for us to in a time dependent manner study mutant ATXN7 toxicity, as well as to investigate the clearance of ATXN7.

Fig. 4. ATXN7 expression in FLQ10 and FLQ65 inducible PC12 cells (Tet-off system)
Figure modified from the Tet-Off® Gene Expression Systems User Manual, ClonTech Laboratories, 2012

3.2 Cell treatments
In order to study autophagy in mutant ATXN7 cells, several pharmacological autophagy inhibitors (NH₄Cl, 3-MA and Bafilomycin A1) were used in Paper I and II. NH₄Cl neutralizes lysosomes thereby
interrupting the fusion of autophagosomes and lysosomes. NH4Cl inhibits all types of lysosomal proteolysis (Gordon, Hart et al. 1980; Hart, Young et al. 1983; Wang, Martinez-Vicente et al. 2009). 3-MA inhibits autophagy by suppressing class III PI3K activity and has been widely used as an autophagy inhibitor (Seglen and Gordon 1982; Petiot, Ogier-Denis et al. 2000). Bafilomycin A1 is used as a typical lysosomal inhibitor for autophagy flux studies and inhibits the fusion between autophagosomes and lysosomes (Klionsky, Abdalla et al. 2012; Yamamoto A. et al., 1998.) In paper I, two autophagy activators, Rapamycin and trehalose, were also used. Rapamycin activates autophagy by binding to the mTORC1 complex thus interrupting the inhibitory effect of mTORC1 on autophagy initiation (Guertin and Sabatini 2007). The exact mechanism by which trehalose activate autophagy is still unknown, however the mechanism is known to be mTORC1 independent (Sarkar, Davies et al. 2007).

The degradation of ATXN7 through the UPS pathway was studied by using the UPS inhibitor Epoxomycin. Epoxomycin was used due to the high specificity of this inhibitor compared to latacystin or other UPS inhibitors (Meng, Mohan et al. 1999).

To study the role of oxidative stress, both oxidative stress inducers and ROS scavengers were used. H2O2 and Buthionine sulfoximine (BSO) were used to increase cellular ROS levels. BSO treatment specifically inhibits Glutathione (GSH) biosynthesis and therefore leads to oxidative stress (Drew and Miners 1984). NAC (N-acetyl-L-cysteine) is an essential cell permeable precursor for glutathione and was used as antioxidant (Hoffer, Baum et al. 1996). Vitamin E (α-tocopherol) was also used as an antioxidant in both papers III and IV.

In order to study NOX activity in mutant ATXN7 expressing cells, NOX inhibitors, Apocynin and gp91ds-TAT were applied (Vejrazka M. et al, 2006; Berton, Giuliano et al. 2011). Apocynin inhibits NOX2 by blocking the migration of the p47phox subunit to the membrane, thus blocking NOX activation (Diatchuk, V., O. Lotan, et al. 1997). The interaction between NOX2 and p47phox is inhibited at the presence of gp91-TAT and thereby inhibits NOX assembly and activation. The treatment with apocynin and gp91ds-tat could also affect NOX1 activity.
and assembly due to sequence similarity of NOX1 and NOX2 (Tejada-Simon MV. et al., 2005). Apocynin was also recently reported to function as an antioxidant so the specificity of this NOX inhibitor is questionable (Stolk, Hiltermann et al. 1994; Wind S., et al., 2010). For more specific inhibition of NOX1, ML171 was used (Gianni, Taulet et al. 2010). A specific inhibition of NOX1 was also carried out by siRNA.

In paper IV, inhibitors of ETC complexes were used in order to detect whether mutant ATXN7 leads to mitochondrial respiration chain dysfunction. Rotenone was used as complex I inhibitor, 3-nitropropionic acid (3-NP) as complex II inhibitor, antimycin A as complex III inhibitor, sodium azide (NaN3) as complex IV inhibitor and oligomycin as complex V inhibitor (Forkink M. et al, 2014, Cillero-Pastor B. et al, 2013).

To study the role of p53 in SCA7, a p53 inhibitor and activator were used in paper II and IV. Pifithrin-α is a reversible inhibitor of p53 function and inhibit p53-mediated gene transcription (Komarova and Gudkov 2000). Nutlin-3 was used as a p53 activator and works by interrupting the binding of MDM2 and p53, thereby blocking MDM2-mediated degradation of p53 (Chene 2003).

In order to prevent the aggregation of mutant ATXN7, the polyglutamine binding peptide (QBP1) was used. In order to get the peptide into cells, cell-penetrating peptides (Synb3 or TAT) were conjugated to the core sequence of QBP1. Synb3 was chosen because it is more efficient in terms of delivering different molecules through the blood brain barrier (BBB) and could more likely become a therapeutic candidate (Liu, Zhang et al. 2014). The core sequence of QBP1, which is reported to show the same aggregation prevention ability as the original QBP1 (Ren, Nagai et al. 2001), was used to minimize the length of the conjugated sequence and thereby potentially increase the peptide deliver efficiency and reduce material preparation time.

3.3 Analysis of ATXN7 aggregation

Filter trap assay was used to analyze the level of aggregated ATXN7 species. After cells were lysed, the soluble fraction were applied for western blot analysis, and the pellet fractions containing the aggregated
material was filtered through a nitrocellulose membrane with a pore size of 0.2 µm in diameter using vacuum aspiration. Any aggregated material larger than 0.2 µm in diameter will be trapped on the membrane and can be detected by immunoprobing. Although most other studies have used cellulose acetate membrane for filter trap assay (Zhang, Smith et al. 2005), we found that nitrocellulose is a good option for filter trap assay of mutant ATXN7 (Paper I, Fig. S1). The advantage with the filter trap method is that it allows for an easy and objective quantification of aggregated materials. Another method to analyze aggregated material is immunostaining and microscopy, which is superior to filter trap assay regarding analysis of the localization of inclusion bodies.

3.4 Toxicity measurements

WST-1 assay was used to measure cell viability. In viable cells, the tetrazolium salt WST-1 can be cleaved by mitochondrial succinate-tetrazolium reductase to derive the water soluble formazan, which can be quantified by a spectrophotometer. The signal detected is corresponding to the cell viability. We preferred WST-1 to other assays like MTT, XTT or MTS, since WST-1 is more stable.

Lactate dehydrogenase (LDH) leakage is another indicator for cell viability. In this assay, LDH released into the medium by damaged cells can be measured with a coupled enzymatic reaction that results in the conversion of Resazurin into the fluorescent Resorufin.

Apoptotic cell death markers were also analyzed in mutant ATXN7 expressing cells. Bax level, as a biomarker for apoptosis induction, was analyzed with western blot as reported (Ward, Cummings et al. 2008). Additionally, the number of cells with apoptotic nuclei was analyzed by microscopy.

3.5 Cellular Reactive Oxygen Species Detection

Total ROS levels were analyzed with a DCHF-DA assay. In this assay, a fluorogenic probe 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) is allowed to diffuse into cells and thereafter be deacetylated by cellular esterases to non-fluorescent 2', 7'-dichlorodihydrofluorescein (DCFH). In the presence of ROS, DCFH is rapidly oxidized to 2', 7'-
Dichlorodihydrofluorescein (DCF). The fluorescence intensity from DCF thus corresponds to the ROS level within the cell. The assay is sensitive and the generated signal is stable (Brandt and Keston 1965).

To study ROS produced by mitochondria, the MitoSOX assay was applied. In this assay, the cationic triphenylphosphonium substituent of MitoSOX Red indicator is responsible for the rapid electrophoretically driven uptake of the probe in actively respiring mitochondria. Oxidation of MitoSOX red indicator by superoxide, but not by other ROS or reactive nitrogen species (RNS) generating systems, results in hydroxylation of hydroxyethidium, which binds to mitochondrial nucleic acids, resulting in specific bright red fluorescence in mitochondria.

### 3.6 Autophagy activity analysis

In order to analyze if there is an autophagic dysfunction in SCA7 cells, p62 levels were tested as a direct indicator of autophagy. The level of p62 can be used as a biomarker for autophagy as p62 is degraded by autophagy and dysfunction of autophagy activity leads to the accumulation of p62 (Bjorkoy, Lamark et al. 2009; Moscat and Diaz-Meco 2009). The measurement of LC-3 (a homologue of yeast Atg8) is another typical method to study autophagic flux (Kimura, Fujita et al. 2009). During autophagy, LC3-I is conjugated to phosphatidylethanolamine (PE) and become LC3-II. LC3-II localizes to the inner membrane of autophagosomes and is degraded together with the substrate. The detection of LC3 II level by Western blot is generally used to quantify autophagic flux. However the elevation of LC3-II could be due to either increased conversion of LC3-I or decreased autophagic degradation of LC3-II. The simple measurement of LC3-II level is thus not sufficient to confirm the dysfunction of autophagy. Therefore the ratio of LC3-I/LC3-II should be tested with or without the addition of an autophagy inhibitor like Bafilomycin A1. If autophagic flux is normal, Bafilomycin A1 should result in accumulation of LC3 II. However, if autophagy is already inhibited, Bafilomycin A1 will not result in a further LC3-II increase.
3.7 Analysis of gene/protein expression and stability.

Protein expression was analyzed by western blot in all papers. To distinguish if observed change of protein levels is due to altered transcription or protein stability, we also perform RT-PCR and pulse-chase studies. The semi-quantitative RT-PCR method was applied to analyze mRNA expression and indicate if alterations of expression levels are due to altered transcription. In our studies, p53, NOX1, NOX2, GLUT1, AIF and TIGAR mRNA levels were analyzed. Actin mRNA level was used for normalization.

To investigate if altered expression levels were due to altered protein stability/degradation, we used pulse-chase. The ULK1 stability was analyzed by pulse-chase in paper III. In pulse-chase experiments, cycloheximide was used as an inhibitor of protein synthesis. After the treatment with cycloheximide for different time points, the level of ULK1 was determined with Western blot and the half-life of ULK1 calculated. A similar analysis was used to analyze the stability of ATNX7 in PC12 cells in Paper I. However, for this cycloheximide was not needed as the expression of ATXN7-GFP in this inducible model could simply be blocked by addition of Dox to the media.

3.8 Immunoprecipitation (IP)

Immunoprecipitation (IP) is generally used to analyze protein-protein interactions. In IP an antibody is first bound to beads and then used to pull down the target protein and any interacting proteins. Samples are then denatured and analyzed by regular Western blot. However, in the IP study of p53, it is important to minimize the side effect from antibody heavy chain (HC) that has similar molecular weight as p53 during the Western blot analysis (Muller, Trinidad et al. 2013). Cross-linking of the antibody to the beads is an efficient strategy to avoid this side effect. In the IP analysis, dimethyl pimelimidate (sigma) was used to crosslink the p53 antibody to the beads. The crosslinking of the p53 antibody to the beads prevents the detachment of the p53 antibody from beads during the elution/denaturation and this avoids getting the antibody HC into the Western blot samples.
4. Results and discussion

4.1 Establishment of a novel inducible SCA7 model (Paper I-IV)

In order to study different pathological mechanisms of mutant ATXN7, we constructed a novel inducible SCA7 model as described in the method section. In this model the removal of doxycycline (Dox) from the media induces the expression of either ATXN7Q10-GFP (in FLQ10 cell line) or ATXN7Q65-GFP (in FLQ65 cell line) (Paper I, Fig 1A).

Characterization of the novel SCA7 model showed similar features as those commonly observed in SCA7 patients and other SCA7 models. First microscopic and subcellular fractionation studies showed a predominantly nuclear localization of both ATXN7Q10 and ATXN7Q65 (Paper I, Fig 1E-F). Moreover, accumulation of ATXN7 aggregates could be observed in FLQ65 cells induced for 6 days onwards, whereas no aggregates were detected in FLQ10 control cells expressing ATXN7Q10-GFP (Paper I, Fig. 1C). Furthermore, proteolytically cleaved ATXN7 fragments were present in the new model. Two major fragments (fragment 1: around 45 kDa and fragment 2: around 40 kDa) in FLQ65 cells and one major fragment (between 40 and 50 kDa in size) in FLQ10 cells were identified (Paper I, Fig 1A). Similar proteolytically cleaved mutant ATXN7 products have been found in SCA7 patients, as well as in transgenic models, and suggested to be more toxic than full-length ATXN7 (Garden, Libby et al. 2002; Guyenet, Mookerjee et al. 2015). Moreover, cleavage of ATXN7 at amino acids 266 or 344 by caspase-7 in the nucleus has been shown to underlie the generation of the polyQ-containing fragments (Guyenet, Mookerjee et al. 2015; Young, Gouw et al. 2007). Though we could observe proteolytically cleaved mutant ATXN7 fragments both in the cytoplasm and nucleus, fragments were mostly detected in the nucleus in other studies (Garden, Libby et al. 2002; Young, Gouw et al. 2007). The length of the polyQ tracts could underlie the difference in the subcellular localization of polyQ-expanded ATXN7 fragments in our study. In contrast to our 65Q repeat, ATXN7 containing a larger polyQ repeat was used in other studies. After proteolytic cleavage, ATXN7 fragments lacking the NES are dependent on diffusion through the nuclear pore to leave the nucleus. Since only proteins smaller
than 40-60 kDa are allowed to diffuse through the nuclear pore (Petra P. et al. 2015), a larger repeat could result in larger fragments more unable to leave the nucleus. In addition, the larger the polyQ expansion, the easier and faster mutant ATXN7 fragments could aggregate resulting in large oligomeric species unable to diffuse through the nuclear pore. Another explanation could be that mutant ATXN7 is not cleaved at asp266 or 344 in the new FLQ65 model and the fragments generated in FLQ65 cells still contain the NES.

In the FLQ65 SCA7 model, a progressive toxic effect was detected after the induction of mutant ATXN7 expression using the WST-1 assay (Paper I, Fig. 1D). Decreased cell viability was observed after 6 days of expression onwards, whereas no toxic effects were detected in ATXN7Q10-GFP expressing control cells at any time (Paper I, Fig. 1D). The toxicity in the FLQ65 cell model was associated with plasma membrane leakage (Paper III, Fig. 1D), elevated ROS (Paper III, Fig. 1E) and reduced ATP (Paper IV, Fig. 4H) levels. These three characteristics have been linked to activation of necrotic, rather than apoptotic cell death pathways (Wiem C. et al., 2013). Additionally, we could not observe any markers of apoptosis, like elevated Bax levels or enhanced presence of apoptotic nuclear morphology in FLQ65 cells (Paper II, Fig. S4), further supporting the necrotic rather than apoptotic cell death pathways could be activated in the FLQ65 model. Consistent with this non-apoptotic cell death have been reported in SCA7 mice (Yefimova, Messaddeq et al. 2010). However, apoptotic death has also been reported (Yefimova, Messaddeq et al. 2010; Wang, Yeh et al. 2006; Wang, Chou et al. 2010). For instance have mutant ATXN7 been shown to activate Bax expression and apoptosis (Wang, Yeh et al. 2006; Wang, Chou et al. 2010). Differences in the amount of mutant ATXN7 expression, the length of the polyQ expansion and level of ATXN7 fragments could underlie these differences. In 2015, Guyenet et al observed that proteolytic cleavage of mutant ATXN7 at D266 is an important mediator of neurotoxicity in transgenic mice (Guyenet, S. J., S. S. Mookerjee, et al. 2015). In contrast to our studies, Wang et al who detected apoptosis did not detect any proteolytically cleaved ATXN7 fragments in either cells or mice (Wang, Yeh et al. 2006; Wang, Chou et al. 2010). It is possible that full-length and ATXN7 fragments could induce toxicity and cell death in different
ways, and in our studies, both full-length and cleaved fragment are present.

One large benefit with the FLQ65 SCA7 model is that the expression of ATXN7 can be regulated. This property makes it possible to for instance distinguish early and later pathological events, investigate whether toxic effects can be reversed if mutant ATXN7 is removed and how different mutant ATXN7 species can be cleared away/degraded in cells. Furthermore, it is easy to manipulate the expression of protein of interests in this model with for instance siRNA.

4.2 ATXN7 is degraded by both UPS and autophagy (paper I)

Taking advantage of the ability to switch the expression of ATXN7 on and off in the FLQ65 model, we used this model to study the stability and clearance mechanisms of both wild-type and mutant ATXN7 without having to use protein synthesis inhibitors. This is important since protein synthesis inhibitors, like cycloheximide, disrupt protein degradation mechanisms (Abeliovich, Dunn et al. 2000; Ravikumar, Duden et al. 2002). Following 10 days of induction, mutant ATXN7 expression was turned off and the clearance of ATXN7Q10-GFP and ATXN7Q65-GFP analyzed at different time points. Full-length ATXN7Q10-GFP was cleared away with a half-life of 4.5 hour and a similar clearance half-life of around 4.7 hours was also detected for the ATXN7Q10-GFP cleaved fragment (Paper I, Fig. 2A-B). However, after turn-off of ATXN7Q65-GFP expression, both full-length and proteolytically cleaved mutant ATXN7 fragments showed a dramatic increase during the first 1.5 hours, suggesting that the polyQ expansion makes ATXN7 resistant to degradation (Paper I, Fig. 2A-B). After the first 1.5 hours, a sharp decrease of ATXN7Q65 was observed in the FLQ65 model (Paper I, Fig. 2A-B). This decrease is most likely due to rapid aggregation of ATXN7-Q65, as a corresponding increase of aggregated mutant ATXN7 was observed (Paper I, Fig. 2A-B). Aggregated ATXN7Q65 showed a half-life of 34.3 hours suggesting that aggregation stabilizes mutant ATXN7 from degradation (Paper I, Fig. 2C). Consistent with our findings, a previous study by Yvert et al showed similar stabilization and aggregation of mutant ATXN7 in SCA7 animal models (Yvert, Lindenberg et al. 2001).
In order to study the role of UPS and autophagy in ATXN7 degradation, UPS and autophagy inhibitors (epoxomycin, 3-MA and NH₄Cl) or activators (rapamycin and trehalose) were applied after ATXN7 expression were turned off and ATXN7 levels then analyzed at various time points. For non-expanded ATXN7Q10-GFP, the inhibition of UPS led to dramatic elevated levels of both the full-length ATXN7 Q10 and Q10 fragment (Paper I, Fig. 3A, C). In contrast, inhibition or activation of autophagy showed no statistical effects on full-length or Q10 fragment levels in FLQ10 cells (Paper I, Fig. 3B-C). This indicates that UPS plays the predominant role in clearance of wild-type ATXN7. This conclusion was further supported by data showing that endogenous rat ATXN7 accumulated after UPS, but not autophagy inhibition, in FLQ10 cells (Paper I, Fig. 3A-C). Furthermore, data from transiently transfected HEK293 cells expressing ATXN7Q10-myc again showed the predominant role of UPS in Q10 ATXN7 clearance (Paper I, Fig. 4).

In FLQ65 cells, the inhibition of UPS, but not autophagy, resulted in significantly elevated levels of full-length ATXN7Q65-GFP (Paper I, Fig. 5 and 8). This suggest that UPS is also the main degradation pathway for mutant full-length ATXN7. However, inhibition of autophagy led to a slight accumulation of both Q65 fragment 1 and 2 in continuously ATXN7Q65-GFP expressing cells (Paper I, Fig. 8C). Moreover, both the inhibition of UPS and autophagy resulted in elevation of ATXN7 aggregated material (Paper I, Fig. 5B and 8B, D). In addition, autophagy activation resulted in a significant effect on the clearance of aggregated ATXN7 both in FLQ65 PC12 cells and in HEK293 cells (Paper I, Fig. 5B, Fig. 6B). The results above indicate that although UPS mainly degrades full-length mutant ATXN7, autophagy is involved in the clearance of mutant ATXN7 fragments and especially the clearance of aggregated ATXN7 species. In support of our findings, another study showed that proteolytically cleaved ATXN7 fragments are cleared away by autophagy and this clearance is affected by posttranslational modification of the fragments (Mookerjee, Papanikolaou et al. 2009). In our study, the full-length ATXN7Q65 level is not significantly affected by autophagy inhibition and this might be due to the mainly nuclear localization of full-length ATXN7, as autophagy only exists in the cytoplasm. However, even though autophagy had little effect on full-length mutant ATXN7,
pharmacological stimulation of autophagy by rapamycin reduced the level of mutant ATXN7 toxicity in our study (Paper I Fig. 9). Positive effects of autophagy stimulation have also been observed in other polyQ disease models (Ravikumar, Duden et al. 2002; Menzies, Huebener et al. 2010). Moreover, in some polyQ models the expression of the expanded polyQ protein resulted in elevation of autophagy without external stimulation, see review (Maria J-S, et al, 2012). However, not all studies have indicated positive effects after pharmacological stimulation of autophagy and autophagy dysfunction have been reported in some polyQ models (Tanaka, Machida et al. 2004; Menzies, Huebener et al. 2010; Nisoli, Chauvin et al. 2010). In fact, an analysis of the LC3-II levels in the FLQ65 model in Paper I (Fig. 7) indicated that autophagy did not seem to be up-regulated in response to mutant ATXN7 expression. In a follow-up study we therefore decided to further investigate if there is an autophagy dysfunction under polyQ expanded ATXN7 expression.

4.3 Autophagy dysregulation in SCA7 cells (paper II)

To investigate whether autophagy is affected by mutant ATXN7 expression, we first analyzed the autophagy activity in SCA7 cells using two assays; analysis of the p62/SQSTM1 level and the autophagy flux assay, see material and method. Under the expression of mutant ATXN7, a gradual and significant increase in the p62 level was detected, indicating that the autophagy activity is decreased (Paper II, Fig. 1A). In the autophagy flux assay the LC3-II level in the presence or absence of the lysosomal inhibitor bafilomycin A1 (BafA1) is measured and if autophagy is fully functional the treatment with BafA1 should lead to a significant accumulation of LC3-II compared to the no BafA1 treatment control (Klionsky, Abdalla et al. 2012). However, no significant accumulation in the LC3-II level was detected in FLQ65 or HEK293T cells expressing mutant ATXN7 (Paper II, Fig.1B-C), again suggesting that mutant ATXN7 disrupts autophagy activity. In contrast, no change in p62 or autophagy flux could be detected in control cells expressing wild-type ATXN7 (Paper II, Fig. 1). Consistent with our findings others have also recently shown that autophagy is impaired in both patients and a knock-in mice model of SCA7 (Alves, Cormier-Dequaire et al. 2014). However, enhanced autophagy activity has also been reported in SCA7 transgenic mice (Mookerjee, Papanikolaou et al. 2009). Mookerjee et al,
based their conclusion on an increased level of both LC3-II and LC3-II-positive structures in SCA7 cells. However, although the accumulation of LC3-II could be due to increased LC3-I conversion and autophagy activity, blockage of autophagy and thus reduced LC3-II clearance could also result in the accumulation of LC3-II (Klionsky, Abeliovich et al. 2008). The accumulation of LC3-II and LC3-II positive structures observed by Mookerjee et al could thus in fact also support a dysregulation of autophagic function.

Autophagy is a complicated process regulated by many factors, including p53, see review (Maiuri, M. et al. 2010). Since p53 alterations have been identified in polyQ diseases (Shahbazian, Orr et al. 2001; Bae, Xu et al. 2005; Wang, Chou et al. 2010; Illuzzi, Vickers et al. 2011), we next investigated whether alterations of p53 properties could be involved in the autophagy dysfunction in the FLQ65 model. In fact, under mutant ATXN7 expression, about a 50% decrease in the nuclear p53 level and a 20% down-regulation of the overall p53 transcriptional activity was identified (Paper II, Fig. 2A-B). Nuclear p53 induces autophagy through transcriptional activation of several autophagy related genes, see review (Napoli and Flores 2013). However with RT-PCR only small, not significant, decreases of specific p53 regulated autophagy associated genes, like Sestrin 1 and AMPK β1, was detected (Paper II, Fig 2C). These data indicate that a reduced nuclear p53 level and transcriptional activity might contribute, but is not likely to be fully responsible for the perturbed autophagy in SCA7 cells.

In addition to the decreased nuclear p53 levels, a 50% increase in cytoplasmic p53 was identified in FLQ65 cells (Paper II, Fig. 2a). Cytoplasmic p53 is known to inhibit autophagy via directly interacting with FIP200, a component of the ULK1-FIP200-Atg13-Atg101 autophagy initiation complex (Morselli, Shen et al. 2011; Choi, Ryter et al. 2013). Strikingly an enhanced interaction between FIP200 and p53 was observed in ATXN7Q65 expressing cells (Paper II, Fig. 3A). Furthermore, a dramatic decrease of the soluble FIP200 level was identified (Paper II, Fig. 3A). This decrease was not caused by the decreased nuclear p53 transcriptional activity, since no significant decrease of FIP200 mRNA level was detected (Paper II, Fig. 3C). Instead we found that the reduced FIP200 level correlated with a gradual increase
of p53 and FIP200 co-aggregation with mutant ATXN7 (Paper II, Fig. 6). The decrease of soluble FIP200, as well as the co-aggregation of ATXN7, p53 and FIP200 was also confirmed in HEK293 cells expressing mutant ATXN7 (Paper II, Fig. S3). Consistently with our findings, p53 has also previously been shown to be sequestered into polyQ expanded ATXN7 inclusions (Zander, Takahashi et al. 2001).

The exact effect of the FIP200-p53 interaction on autophagy regulation is not clear, however FIP200 is believed to stabilize the ULK1 protein, an important autophagy initiator (Hara, Takamura et al. 2008). Consistent with this, we detected a decreased ULK1 stability and level in mutant ATXN7 expressing cells (Paper II, Fig. 4A-B). Our results suggest that mutant ATXN7 results in autophagic dysfunction and toxicity by enhancing the p53-FIP200 interaction, sequestration of p53-FIP200 into IBs, and depletion of soluble FIP200 levels causing ULK1 destabilization. In order to further confirm this hypothesis, we tested if 1) overexpression of FIP200, 2) treatment with the p53 inhibitor Pifithrin-α, known to block p53-mediated inhibition of autophagy (Tasdemir, Maiuri et al. 2008), or 3) treatment with the anti-aggregation peptide QBP1, could reverse the decreased autophagy activity and toxicity in SCA7 cells. Indeed, all three treatments not only restored the autophagic flux, but also rescued the cell viability (Paper II, Fig. 4C-D; Fig. 5 and 7).

Together our data suggest that enhanced cytoplasmic p53 activity and FIP200 disruption contribute to mutant ATXN7 toxicity. Consistent with our findings, others have shown that depletion of p53 in SCA1 mice ameliorated cellular pathology and reduce the number of IBs (Shahbazian, Orr et al. 2001). Interestingly, deletion of FIP200 in mice has been reported to result in degeneration of Purkinje and retinal cells, the neurons most vulnerable in SCA7 (Liang, Wang et al. 2010; Yao J et al. 2015). This suggests that mutant ATXN7 disruption of FIP200 and the initiation of autophagy could play a central role in SCA7 pathology. However we must keep in mind that how the expression of mutant ATXN7 alters the subcellular localization of p53 and that p53-FIP200 interaction is still not clear. One mechanism might be that mutant ATXN7 affects the level of post translational modifications which control p53 localization and activity (Ashcroft and Vousden 1999). In order to clarify this hypothesis, further studies are needed.
Additionally, it is worth to point out that the positive effects of the anti-aggregation peptide QBP1 when conjugated to the cell penetrating peptide synb3 in the FLQ65 model, suggest that Synb3-QBP1 could be considered for polyQ disease therapy. Cell penetrating peptides (CPPs), including TAT, have previously been used to deliver QBP1 into cell and animal models. However, although the TAT conjugated QBP1 showed good effects in cells and Drosophila (Nagai, Tucker et al. 2000; Okamoto, Nagai et al. 2009; Popiel, Nagai et al. 2007), only limited effects were detected in mice (Popiel, Nagai et al. 2009), possibly due to degradation, limited diffusion in the brain and/or low passage across the blood brain barrier. Synb3 has higher capability that TAT to pass through the blood-brain barrier in humans (Liu, Zhang et al. 2014). In our SCA7 model, Synb3-QBP1 showed better effect on aggregation prevention and restoration of cell viability than TAT-QBP1 (paper II, Fig. 7A, F). Further studies using Synb3-QBP1 in animal models might therefore be worth to do in the future.

4.4 Oxidative stress induced by NOX1 activation in SCA7 cells (paper III-IV)

Reactive oxygen species (ROS) is thought to play harmful roles in neurodegenerative disorders due to their high reaction capability. ROS induced oxidative stress damage to macromolecules like DNA and proteins have been detected in many neurodegenerative diseases (Kim, Kim et al. 2003; Goswami, Dikshit et al. 2006; Miyata, Hayashi et al. 2008). However, the role of oxidative stress in polyQ diseases, especially in SCA7, is still not fully understood.

To clarify the relationship between oxidative stress and SCA7, we investigated whether mutant ATXN7 leads to oxidative stress and if so by which molecular mechanism. We found that induction of mutant ATXN7 expression, in a time dependent manner, led to elevated ROS levels (Paper III, Fig. 1E) and a reduction of the GSH level (Paper III, Fig. 1F). This shows that mutant ATXN7 expression results in an oxidative environment. Moreover, we found that the increased ROS level promoted ATXN7 aggregation and led to decreased cell viability in ATXN7Q65-GFP expressing cells (Paper III, Fig. 2, 3, 4). Antioxidants, including Vitamin-E and NAC, could ameliorate the ATXN7Q65-GFP toxicity
(Paper III, Fig. 2A, D). This is consistent with other studies linking elevated ROS levels and oxidative stress to toxicity in HD, DRPLA and SCA1 (Goswami A. et al., 2006; Kim S.J., 2003; Miyata R. et al, 2008; Chen 2011)

The elevated ROS level and oxidative stress observed in the mutant ATXN7 cells could be caused by either anti-oxidant defense system dysfunction or increased production of ROS. In order to address this, some antioxidant related enzymes, including glutathione transferase A3 (GSTA3), superoxide dismutase [Cu-Zn] (SOD1) and catalase (CAT) were analyzed in ATXN7Q65-GFP cells. Increased SOD1 and GSTA3, but decreased CAT levels, were observed in FLQ65 cells expressing mutant ATXN7 (Paper III, Fig.5 A-D). Altered levels of antioxidant enzymes, including SOD1, CAT and GSTAs, have also been detected in other polyQ diseases (del Hoyo, Garcia-Redondo et al. 2006; Sorolla, Reijonen-Branchat et al. 2008; Reijonen, Kukkonen et al. 2010). The elevation of SOD1 and GSTA3 in the FLQ65 cells could be a response to the mutant ATXN7 induced elevation of ROS. If so, depletion of ROS by ROS scavengers such as NAC and Vitamin E should restore the level of SOD1 and GSTA3. Indeed, a restoration of GSTA3 and SOD1 protein levels were detected after NAC treatment (Paper III, Fig. 5E-G), suggesting that at least some anti-oxidant defense components are activated under mutant ATXN7 expression. In fact increased levels of several antioxidants including SOD2, peroxiredoxins and glutathione peroxidases have been observed in HD patients (Sorolla, Reijonen-Branchat et al. 2008). However, contradictory to our results, Sorolla et al reported that neither the protein level, nor activity of SOD1, was significantly affected in the striatum and cortex from HD patients (Sorolla, Reijonen-Branchat et al. 2008). These contradictory results between our study and Reijonen et al could reflect differences between mutant Htt and ATXN7.

In contrast to elevated GSTA3 and SOD1 levels, a decrease in CAT level was observed in SCA7 cells (Paper III Fig. 5A, D). Decreased CAT levels have also been shown in HD models and could contribute to the development of oxidative stress in polyQ diseases (Yu, Kuo et al. 2009; del Hoyo, Garcia-Redondo et al. 2006; Reijonen, Kukkonen et al. 2010). However, an increased CAT activity has also been reported in a HD
model (Sorolla, Reverter-Branchat et al. 2008). Interestingly, we could see that the decrease in CAT was not reversed by anti-oxidant treatment, which reduced toxicity and restored GST and SOD1 levels (Paper III, Fig. 5E, H). This suggests that the mechanism by which mutant ATXN7 affects CAT is not easily reversed. It is possible that mutant ATXN7, as a component of the STAGA complex, might selectively affect regulation of the catalase gene expression. According to the study of Reijonen et al, the decreased expression of CAT induced by polyQ-expanded Htt is due to decreased NF-κB mediated gene activation (Sorolla, Reverter-Branchat et al. 2008; Reijonen, Kukkonen et al. 2010). In conclusion, mutant ATXN7 alters the levels of some anti-oxidant defense system components and the reduced CAT level could contribute to the oxidative stress in SCA7 cells. However, further investigations on which components of the anti-oxidant system are altered and how these alterations contribute to reduce or worsen the oxidative stress and toxicity in different polyQ diseases is required.

Next we investigated whether increased ROS production could contribute to the elevated ROS in mutant ATXN7 cells. As previously mentioned, mitochondria and NOX complexes are key ROS producers, see section 1.8. However under the expression of mutant ATXN7, no elevated levels of mitochondrial ROS production was detected (Paper III, Fig. 6A). On the contrary, inhibition of NOX activity by apocynin or gp91-TAT completely blocked the increase in ROS and restored the level of GSH (Paper III, Fig. 6B, D). Furthermore, NOX inhibition reduced ATXN7 aggregation and ameliorated toxicity (Paper III, Fig. 6B-C). These data suggest that ROS generated from NOX play a key role in inducing the oxidative stress in SCA7 cells. Consistent with this, other polyQ proteins, including ATXN2 and Htt, have also been shown to stimulate NOX activity and lead to oxidative stress in cell and animal polyQ models (Bertoni, Giuliano et al. 2011; Valencia, Sapp et al. 2013).

To determine which isoform of NOX that is involved in the mutant ATXN7 induced toxicity, the expression of different NOX isoforms in FLQ65 cells was analyzed (Paper IV, Fig. 1). NOX1 mRNA and protein, but not NOX2, was induced when the expression of mutant ATXN7 was induced (Paper IV, Fig. 1). Two forms of the NOX1 protein (75 kDa and 65 kDa in size) were detected and both of them increased under mutant
ATXN7 expression (Paper IV, Fig. 1 and 2 A-B). The 75 kDa NOX1 form is believed to be a post-translationally modified form of NOX1 (65 kDa) (Pleskova, Beck et al. 2006). Treatment with the NOX1 inhibitor ML171 or NOX1 siRNA not only restored the ROS levels, but also ameliorated the toxicity induced by mutant ATXN7, suggesting that the elevated NOX1 activity is involved in SCA7 toxicity (Paper IV, Fig. 2C-F). Interestingly, NOX2 have been implied in HD (Bertoni, Giuliano et al. 2011; Valencia, Sapp et al. 2013). In fact, using a PC12 model similar to ours Bertoni et al showed that mutant Htt activates NOX2 rather than NOX1 (Bertoni, Giuliano et al. 2011). Furthermore, they suggested that a direct interaction between polyQ expanded Htt and NOX2 in lipid rafts underlies the activation of NOX2 (Bertoni, Giuliano et al. 2011). Though a direct interaction has been questioned by others (Valencia, Sapp et al. 2013), different polyQ expanded proteins might thus activate different NOX isoforms, likely due to the sequence outside the polyQ domain.

4.5 Metabolic defect caused by altered NOX1 and p53 activity (Paper IV)

As our previous studies have shown that the activity of both NOX1 and p53 are affected by mutant ATXN7 expression and both these proteins are involved in metabolic regulation, we next investigated whether the altered NOX1 and p53 activity could contribute to the observed metabolic dysfunction in SCA7 (Forsgren, Libelius et al. 1996; Johansson, Forsgren et al. 1998). Indeed the expression of mutant ATXN7 resulted in metabolic alteration and reduced ATP levels in FLQ65 cells (Paper IV, Fig. 3-4). Whereas mitochondria membrane potential and oxygen consumption was decreased, glucose consumption, hexokinase-2 (HK2) expression and lactate dehydrogenase (LDH) activity were elevated in mutant ATXN7 cells (Paper IV, Fig. 3-4). These data indicate that a shift from oxidative phosphorylation to more glycolysis occurs in SCA7 cells and results in lower ATP production.

Under normal conditions p53 inhibits glycolysis through the induction of TIGAR and inhibition of GLUT expression (Berkers, Maddocks et al. 2013; Liang, Liu et al. 2013). In contrast, p53 promotes mitochondrial respiration by stimulating expression of AIF and SCO2 (Berkers, Maddocks et al. 2013; Liang, Liu et al. 2013). We therefore investigated
whether the reduced nuclear p53 level observed in SCA7 cells contributed to the metabolic dysregulation by disrupting the expression of these genes. Indeed a significant decrease of TIGAR and AIF mRNA levels, but a mild increase of GLUT1 mRNA level was seen in ATXN7 cells (Paper IV, Fig. 6B). Corresponding to the changes in mRNA levels, protein levels of TIGAR, AIF and GLUT1 changed as well (Paper IV, Fig. 6C-F). Furthermore, increasing the p53 level by nutlin-3 restored the protein levels of TIGAR, AIF and GLUT1, as well as rescued the metabolic dysregulation (Paper IV, Fig. 7-8), confirming that p53 is involved in the metabolic dysregulation in SCA7 cells.

The activation of NOX1 was recently shown to be critical for up-regulation of glycolysis in cancer cells with mitochondrial respiration defects (Lu, Hu et al. 2012). To investigate whether the observed activation of NOX1 in SCA7 cells is also involved in the metabolic dysregulation in FLQ65 cells, knockdown of NOX1 or scavenging of NOX1 induced ROS was done. As suspected, the inhibition or scavenging of NOX1 produced ROS resulted in a reversal of the metabolic dysfunction in SCA7 cells (Paper IV, Fig. 5). However, the mechanism by which NOX1 activation is induced and result in metabolic dysregulation in SCA7 cells is still not fully understood. However, elevating the p53 level in mutant ATXN7 expressing cells reversed the increase in NOX1 expression (Paper IV, Fig. 8C) indicating that p53 might negatively regulate the expression of NOX1. Moreover, the metabolic dysregulation in SCA7 cells could be blocked by ROS scavenging, indicating that the NOX1 produced ROS is essential for the metabolic dysregulation. Many transcription factors, including p53, contain cysteine residues with redox sensitive thiol groups and oxidation of p53 was shown to reduce p53 transcriptional activity, see review (Haddad 2002; Turpaev 2002). It is thus possible that NOX1 generated ROS inhibit p53, thereby causing metabolic dysfunction. A feed-forward loop where p53-ATXN7 co-aggregation and NOX1-mediated p53 inactivation could result in reduced p53 transcriptional activity, metabolic dysregulation and further NOX1 activity, as well as oxidative stress could thus be created, see figure 5.
In summary, we have identified a novel mechanism in which mutant ATXN7, by disrupting p53 function and inducing NOX1 activity, leads to metabolic dysregulation, reduced ATP generation and toxicity. However, additional studies are required to further clarify the cross talk between p53 and NOX1 in mutant ATXN7 toxicity.
5 Conclusions

- A novel stable inducible SCA7 model, FLQ65, has been constructed for SCA7 studies.
- UPS is the major degradation pathway for full-length and proteolytically cleaved fragments of wild-type and mutant ATXN7. However, autophagy contributes to the clearance of proteolytically cleaved mutant ATXN7 fragments and especially aggregated ATXN7 species.
- Pharmacological stimulation of autophagy ameliorates mutant ATXN7 toxicity and could have therapeutic potential in SCA7 disease.
- A novel mechanism by which polyQ expanded ATXN7 inhibits autophagy initiation was identified. By enhancing the p53-FIP200 interaction and depleting soluble FIP200 through coaggregation of mutant ATXN7, p53 and FIP200, mutant ATXN7 cause destabilization of ULK1 and dysfunction of autophagy initiation. Preventing the coaggregation of p53, FIP200 and ATXN7, as well as p53 inhibition ameliorated the autophagy defect and toxicity induced by mutant ATXN7 expression.
- Oxidative stress plays an essential role in promoting mutant ATXN7 aggregation and toxicity. Mutant ATXN7 causes oxidative stress through activation of NOX1 complexes. Scavenging ROS with antioxidants or inhibition of NOX1 ameliorated the toxic effects of mutant ATXN7 and could have therapeutic benefits.
- Cellular metabolism and ATP production is altered by mutant ATXN7 expression. Both NOX1 activation and altered p53 transcriptional regulation of important metabolic proteins like AIF, TIGAR and GLUT1 are involved in causing the metabolic dysregulation.

In summary, the results in this thesis show that mutant ATXN7 expression cause toxicity by disrupting autophagy induction, inducing oxidative stress and causing metabolic dysfunction. Interestingly p53 plays important roles both in regulation of autophagy and metabolism, and appears to play a key role in mutant ATXN7 toxicity. Counteracting the toxic mechanisms mentioned above ameliorated cellular toxicity and could be potential therapeutic strategies in the future.
6. Populärvetenskaplig sammanfattning på svenska

Den mänskliga kroppen är sammansatt av miljarder celler som i olika organ utför diverse funktioner. Molekyler som DNA, RNA och proteiner är väsentliga för att cellen ska fungera korrekt och obalans i dessa molekylers placering eller nivåer kan leda till celldöd och sjukdom.

I denna avhandling har jag studerat den neurodegenerativa sjukdomen SCA7 (Spinocerebellär ataxi typ 7). SCA7 tillhör gruppen polyglutaminsjukdomar och idag finns inget botemedel för dessa sjukdomar. SCA7 kännetecknas av att nervceller i ögat och lillhjärnan dör, vilket leder till symptom som blindhet och ataxi. SCA7 orsakas av en mutation, förändring, i proteinet Ataxin-7 (ATXN7).

Syftet med våra studier är att identifiera hur muterat ATXN7 leder till att den normala funktionen hos nervcellen inte fungerar och nervcellen dör, samt hur detta skulle kunna motverkas. I vår första studie visar vi att jämfört med det friska ATXN7 proteinet så är muterat ATXN7 mer stabilt, ackumulerar och klumpar ihop sig inne i cellerna. Vi fann dock att muterat ATXN7 kunde rensas bort från celler genom två mekanismer; autofagi och UPS. Vidare kunde vi visa att behandling med rapamycin och trehalos, som kan öka autofagiaktiviteten, förbättrade överlevnaden av celler som uttryckte muterat ATXN7. I vår nästa studie undersökte vi därför vidare hur muterat ATXN7 påverkar autofagi. Vi kunde visa att muterat ATXN7 blockerar den normala aktiveringen av autofagi och på detta sätt inhiberar sin egen nedbrytning.


Sammanfattningsvis så har vår forskning identifierat flera mekanismer / molekyler, tex inhibering av autofagi, oxidativ stress och NOX1.
aktivering, som påverkas av muterat ATXN7. Förhoppningsvis så kan identifiering av dessa mekanismer/molekyler hjälpa oss att hitta nya möjliga terapeutiska metoder för att bota SCA7 och de andra polyglutaminsjukdomarna i framtiden.
7. Acknowledgements:

It is a pleasure to thank the many people who made this thesis possible.

It is difficult to overstate my gratitude to my Ph.D. supervisor, Dr. Anna-Lena Ström. With her enthusiasm, inspiration, and great efforts to explain things clearly and simply, she helped to make this research fun for me. Throughout my thesis-writing period, she provided encouragement, sound advices, good teaching, good company, and lots of good ideas. I would have been lost without her support. The respect for you in my heart is much more than any verbal description.

I wish to thank my friends in our group. Ajayi, thanks for grateful ideas and supports.

I would like to thank many people who have given me a lot of help since I arrived in Sweden. Marie-Louise, Sylvia and Siv, thank you for always answering my boring questions patiently, and provided me the first place to sleep in Sweden. I wish to thank all the teachers in this department for giving many good suggestions and ideas on my study and private life.

I am especially grateful to Tom and Johan for your experienced help on my lab work and student life here. Linda, Kristin Jacobson and Kristin Webling thank you for academic suggestions, Santosh and Hakim for the technique discussions. Special thanks to Jessica and Andrés for the positive attitude on research and life. Thanks Henrik for giving the computer support. Grateful thanks to Staffan, Rania and Veronica for the happy lab assistant work together. And thanks all the people in this department.

I wish to thank my entire extended family for providing a loving environment for me, my parents and my wife. Special thanks to my sons, Lucas and Edward, who are my supporting angel in soul.

Lastly, and most importantly, I wish to thank my supervisor Anna-Lena again.
8. References


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