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**Inhibition of autophagy via p53-mediated disruption of ULK1 in a SCA7 polyglutamine
disease model**

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Abstract

Spinocerebellar ataxia type 7 (SCA7) is one of nine neurodegenerative disorders caused by expanded polyglutamine domains. These so called polyglutamine (polyQ) diseases are all characterized by aggregation. Reducing the level of aggregating polyQ proteins via pharmacological activation of autophagy has been suggested as a therapeutic approach. However, recently evidence implicating autophagic dysfunction in these disorders has also been reported. In this study we show that the SCA7 polyglutamine protein ataxin-7 (ATXN7) reduces the autophagic activity via a previously unreported mechanism involving p53-mediated disruption of two key proteins involved in autophagy initiation. We show that in mutant ATXN7 cells, an increased p53-FIP200 interaction and co-aggregation of p53-FIP200 into ATXN7 aggregates result in decreased soluble FIP200 levels and subsequent destabilization of ULK1. Together, this leads to a decreased capacity for autophagy induction via the ULK1-FIP200-Atg13-Atg101 complex. We also show that treatment with a p53 inhibitor, or a blocker of ATXN7 aggregation, can restore the soluble levels of FIP200 and ULK1, as well as increase the autophagic activity and reduce ATXN7 toxicity. Understanding the mechanism behind polyQ-mediated inhibition of autophagy is of importance if therapeutic approaches based on autophagy stimulation should be developed for these disorders.

Keywords: polyglutamine, neurodegeneration, autophagy, p53, FIP200, ULK1

Abbreviations:

Atg Autophagy-related gene

ATXN7 Ataxin-7

BafA	Bafilomycin A ₁
GFP	Green fluorescence protein
HD	Hungtinton's disease
kDa	Kilodalton
PolyQ	Polyglutamine
Q	Glutamine
QBP1	Polyglutamine binding peptide 1
SCA7	Spinocerebellar ataxia type 7

Introduction

Spinocerebellar ataxia type 7 (SCA7) is a neurodegenerative disorder characterized by cerebellar ataxia and visual impairment due to a progressive loss of neurons within the retina, cerebellum and brainstem (Konigsmark and Weiner, 1970; Martin et al., 1994). The disease is caused by the expansion of a polyglutamine domain in ataxin-7 (ATXN7), a ubiquitously expressed protein implicated in the regulation of cytoskeleton stability and STAGA (SPT3-TAF(II)31-GCN5L acetylase) mediated gene transcription (Helmlinger et al., 2004; Jonasson et al., 2002; Lindenberg et al., 2000; Nakamura et al., 2012; Palhan et al., 2005). Besides SCA7, eight other neurodegenerative disorders including Huntington's disease (HD), dentatorubral pallidolusian atrophy (DRPLA), spinal bulbar muscular atrophy (SBMA) and SCA1-3, 6 and 17, caused by polyglutamine expansions have been identified (Katsuno et al., 2008). These so called polyglutamine (polyQ) diseases are characterized by misfolding and aggregation of the expanded polyglutamine protein and a negative correlation between repeat length and aggregation, as well as toxicity can be observed (Li et al., 2008).

Macroautophagy (hereafter referred to as autophagy) is an important intracellular pathway by which cytoplasmic proteins and organelles can be degraded (He and Klionsky, 2009). Autophagy is essential in neurons and impairment of this process in mice results in accumulation of neuronal protein inclusions and neurodegeneration (Hara et al., 2006; Komatsu et al., 2007). Several of the expanded polyglutamine proteins, including ATXN7, can be degraded by autophagy (Qin et al., 2006; Ravikumar et al., 2002; Young et al., 2009; Yu et al., 2012). Furthermore, chemical induction of autophagy has been shown to ameliorate the toxicity in several polyQ disease models (Ravikumar et al., 2004; Tanaka et al., 2004; Wang et al., 2009). We recently showed that rapamycin-mediated stimulation of autophagy could ameliorate the toxicity in a SCA7 model (Yu et al., 2012). However, we could not observe any evidence of endogenous up-regulation of autophagy in the presence of mutant

ATXN7. This suggests that the autophagy response to proteotoxic stress could be altered or disrupted in SCA7 cells. In fact, mounting evidence implicating autophagy dysfunction in polyQ diseases have recently arisen, for review see (Cheung and Ip, 2011; Wong and Cuervo, 2010). In HD models aberrant cargo recognition, as well as alterations of autophagic flux, has been reported (Heng et al., 2010; Martinez-Vicente et al., 2010). Furthermore, a polymorphism in the autophagy-related gene 7 (Atg7) was recently shown to influence the age-of-onset in HD patients (Metzger et al., 2010). However, not much is known about how other polyglutamine proteins affect the autophagy process.

During autophagy an isolation membrane (phagophore) is generated and progressively enwraps the cargo forming a double membrane structure called the autophagosome. These autophagosomes then fuse with lysosomes resulting in degradation of the cargo by lysosomal hydrolases (He and Klionsky, 2009). The autophagy process is orchestrated by a number of autophagy-related (Atg) proteins and in response to stress the level of autophagy is usually up-regulated (He and Klionsky, 2009; Mizushima, 2010). In mammalian cells activation of a complex consisting of ULK1 (or ULK2), FIP200, Atg13 and Atg101 is essential for autophagy induction (Mizushima, 2010). The exact mechanism by which this complex induces autophagosome formation is not completely understood. However, the kinase activity of ULK1 appears to be essential and FIP200 can enhance this activity, for review see (Chen and Klionsky, 2011; Mizushima, 2010).

Under non-stress conditions the activity of the ULK1-FIP200-Atg13-Atg101 complex and hence autophagy is inhibited by the serine/threonine protein kinase mTOR (He and Klionsky, 2009; Mizushima, 2010). mTOR interacts with, phosphorylates and inactivates ULK1 and Atg13. However, during various stress conditions, or treatment with the autophagy inducer rapamycin, mTOR activity is inhibited and the ULK1 complex is activated (He and Klionsky, 2009; Mizushima, 2010). p53 is a transcription factor essential for the cellular response to

stress and has been shown to stimulate autophagy by activating the expression of several proteins participating in down-regulation of mTOR activity, for review see (Maiuri et al., 2010). However, cytoplasmic p53 was recently shown to have an opposite effect on autophagy and inhibit this process via direct interaction with FIP200 (Morselli et al., 2011; Morselli et al., 2008; Tasdemir et al., 2008).

In this study we have characterized the autophagy process in SCA7 cells and identified a novel mechanism by which a polyQ expanded protein can inhibit autophagy. We show that mutant ATXN7 expression results in an increased interaction between p53 and FIP200, as well as co-aggregation of p53 and FIP200 with mutant ATXN7. In turn, this results in a loss of soluble FIP200 protein, destabilization of ULK1 and reduced autophagic activity. Treatment with a p53 inhibitor or an ATXN7 aggregation blocker both restored the soluble levels of FIP200 and ULK1, as well as increased the autophagic activity and reduced the mutant ATXN7 toxicity.

Material and Methods

Cell culture and transfections

The generation and characterization of FLQ10 and FLQ65 PC12 cell lines, expressing N-terminal FLAG and C-terminal GFP tagged ATXN7 with 10 (ATXN7Q10-GFP) or 65 (ATXN7Q65-GFP) glutamines respectively, have been described previously (Ajayi et al., 2012; Yu et al., 2012). PC12 cells were grown at 37°C and 5% CO₂ in DMEM (Invitrogen) supplemented with 10% horse serum (Invitrogen), 5% Tet System Approved fetal bovine serum (PAA), 100 µg/ml G418 (Invitrogen), 100 units/ml PEST (penicillin G sodium and streptomycin sulphate), 100 µg/ml hygromycin (Invitrogen) and when desired 1 µg/ml doxycycline (Sigma) to inhibit ATXN7-GFP expression.

Human Embryonic Kidney 293T (HEK 293T) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (PEST, Invitrogen) at 37°C, 5% CO₂.

PC12 or HEK 293T cells were transfected with the indicated plasmids at 50–80% confluency using Lipofectamine 2000 (Invitrogen) and following the manufacturer's recommendations. Plasmids used for transfections included pCMV-Bgal (Strom et al., 2005), p3xFLAG-CMV10-hFIP200, a gift of Noboru Mizushima (Addgene plasmid #24300) and PG13-luc (Addgene plasmid #16442), a gift from Bert Vogelstein. FLQ10-pEGFP-N1 and FLQ65-pEGFP-N1 encoding N-terminal FLAG and C-terminal GFP-tagged ATXN7 with 10 or 65 glutamines were generated as previously described (Yu et al., 2012). To generate GFP-tagged truncated ATXN7 constructs TrQ10-pEGFP-N3 and TrQ65-pEGFP-N3, FLAG-tagged truncated ATXN7 fragments were excised from the previously published TrQ10 and TrQ65 plasmids (Strom et al., 2005) using SacI and SalI. The fragments were then ligated into the pEGFP-N3 vector in frame with the C-terminal GFP tag.

Cell treatments and synthesis of QBP1 peptides

Where indicated, cells were treated with 100 nM bafilomycin A₁ (Sigma) or 100 μM pifithrin-α (Sigma). The QBP1 peptides, Synb3-QBP1 (RRLSYSRRF-WKWWPGIF-NH₂), Synb3-Random (RRLSYSRRF-LSLMEFGCRGA-NH₂), Tat-QBP1 (YGRKKRRQRRR-WKWWPGIF-NH₂) and Tat-Random (YGRKKRRQRRR-LSLMEFGCRGA-NH₂) were synthesized on a SYRO multiple peptide synthesizer (MultiSynTech GmbH) using Chemmatrix Rink amide resin (0.2 mmol/g). Standard Fmoc (9H-fluoren-9-ylmethoxycarbonyl)-AA-OH were coupled using HCTU/ 6-Cl-HOBt as activating reagent and DIPEA as base. The peptides were purified by RP-HPLC, C18 preparative column 5mm, 20% acetonitrile (ACN)-water (0.1% TFA); 20-70% ACN over 55 min for all peptides except

SynB3QBP1 which was purified at 20-100% ACN over 50 min. The products were identified by MALDI-MS (Voyager-DE STR, Applied Biosystems) and the peptides used at a final concentration of 700nM.

Cell lysis, filter trap assay and Western blotting

Total, soluble and insoluble protein fractions were obtained after lysing cells with RIPA buffer (Millipore). See supplementary Figure S1 for a schematic presentation of how fractions were collected. In brief, total fractions containing both soluble and insoluble material were collected directly after lysis. Following centrifugation at 21,000 x g the soluble fractions, i.e. the supernatants, were then collected. To obtain insoluble fractions, pellets were resuspended in DNase I buffer and DNase I treated for 1 hour. SDS and DTT were then added to a final concentration of 2% and 100mM, respectively. The protein concentrations in total, soluble and insoluble fractions (before SDS and DTT addition) were measured using Bradford assay (Bio-Rad). The same amount of total, soluble or insoluble fractions were then analyzed by western blot, dot blot or filter trap analysis as indicated.

Western blot, dot blot and filter trap assay membranes were blocked and then probed with primary antibodies at the following concentrations: ataxin-7 1:700 (Strom et al., 2005), actin 1:500 (SC-1616, Santa Cruz), LC3 1:800 (PM036, MBL international), tubulin 1:1000 (T9026, Sigma-Aldrich), histone H3 1:1000 (ab1791, Abcam), FIP200 1:1000 (SAB4200135, Sigma), ULK1 1:1000 (SAB4200106, Sigma), p62/SQSTM1 1:700 (SC-28359, Santa Cruz) and p53 1:700 (SC-1313, Santa Cruz). After secondary antibody incubation, the protein of interest was visualized using SuperSignal West Pico or SuperSignal West extended duration substrates (Pierce) and the ChemiDoc XRS+ imaging system (BioRad). The signal intensities of protein bands or dots were quantified by Image Lab software (BioRad). For western blotting intensities were normalized against actin or tubulin and the quota for the control

sample set to 100%. For filter trap analyzes of insoluble fractions, the intensities from the insoluble fractions were normalized against the level in total fractions from the same cells detected by dot blot analysis.

Immunoprecipitation

Non-induced or FLQ10 and FLQ65 cells induced to express ATXN7-GFP for 12 days were lysed using Triton X-100 lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA) and supernatants collected after centrifugation at 1000 x g for 10 min. Eight hundred µg of extracts were then added into fresh tubes and pre-cleared with protein G beads for 1 hour. To immunoprecipitate p53 from the extracts, the p53 antibody (SC-1313, Santa Cruz) was cross-linked to Protein G beads (GE Healthcare) using 6.5 mg/ml Dimethyl Pimelimidate (Sigma) and 20 µl of cross-linked p53-protein G bead slurry was added to each extract. After 2 hour incubation the beads were collected, washed and 0.6 M glycine (pH 2.5) was used to elute p53 and p53 interacting proteins. After addition of 6xSDS loading dye and denaturation at 95 °C for 5 min, samples were subjected to SDS-PAGE and Western blot analysis as described above.

Nuclear and cytoplasmic fractionations

Nuclear and cytoplasmic fractions were isolated from parental PC12, non-induced or 12 days induced FLQ10 and FLQ65 cells as previously described (Yu et al., 2012). In brief, cells were lysed in hypertonic lysis buffer and the supernatants, i.e. the cytoplasmic fractions, collected after centrifugation at 11 000 x g. The pellets were resuspended in high salt buffer and the nuclear fractions collected after centrifugation at 20 000 x g. Western blot analysis of the extracts were done as described above, using tubulin as loading control for cytoplasmic and histone H3 for nuclear extracts.

WST-1 cell viability test

The viability of non-induced or FLQ65 cells induced to express ATXN7Q65-GFP for 12 days were measured using the WST-1 assay (Clontech) as previously described (Yu et al., 2012). Where indicated cells were treated with Pifithrin- α for 48 hours prior to the analysis. To determine the effect of QBP1 peptide treatment on viability, cells were treated with the peptides for 9 days prior to the toxicity analysis. The viability was normalized against the protein concentration measured by the Lowry assay (Bio-Rad) and the value obtained from the untreated non-induced sample was set to 100%.

Luciferase reported assay

FLQ10, FLQ65 and PC12 cells were transfected as described above with 0,6 μ g internal control (pCMV-Bgal) and 0,6 μ g PG13-luc reporter containing p53 binding sites. Twenty-four hours after transfection cells were lysed with 1x cell lysis buffer (Promega) and luciferase activity measured using a Glomax 96 microplate luminometer (Promega). The luciferase activity from each sample was normalized against the β -galactosidase internal control to account for variations in transfection efficiency. β -galactosidase activity was measured at 420 nm using ONPG as a substrate. All assays were performed in duplicates or triplicates, and all data presented in figures represent the average of at least three independent experiments.

RNA purification and reverse transcription-polymerase chain reaction (RT-PCR)

Transcriptional expression of p53, Sestrin 1, AMPK β 1, FIP200 and ULK1 were evaluated by the semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) method. RNA from non-induced and 12 days induced FLQ65 cells were purified using the GeneJet

RNA purification kit (#KO731, Thermo Scientific). 2000 ng total RNA was used for reverse transcription-polymerase chain reactions (RT-PCR) using RevertAid H minus First strand cDNA synthesis kit (#K1631, Thermo Scientific). Transcribed mRNAs in each sample were amplified using validated primers for actin, GAPDH, p53, Sestrin 1, AMPK β 1, FIP200 and ULK1 (QuantiTect primer assays, Qiagen) and quantified at individual cycle number for each transcript using a ChemiDoc XRS+ imaging system (BioRad) and the Image Lab software (BioRad). Actin mRNA levels were used for normalizations.

Pulse-chase analysis

For pulse-chase analysis of ULK1 stability, non-induced or FLQ10 and FLQ65 cells induced to express ATXN7-GFP for 12 days were treated with 100 μ g/ml cycloheximide for 0, 2, 4 or 8 hours. Cells were then harvested and the ULK1 protein level analyzed by western blot as described above. The half-lives, i.e. the time required to reduce the ULK1 level by 50% were calculated from three separate experiments using the formula: $M_t = M_0 \times (1/2)^{t/T}$, where T= the half-life, M0 = the amount at 0 hours and Mt = the amount at 8 hours.

Statistical analysis

Statistical analyzes were done by one-way ANOVA followed by Tukey's post-hoc test using Prism graph pad 5.0.

Results

Reduced autophagic activity in mutant ATXN7 cells

The total cellular amount of p62/SQSTM1 can serve as an indicator of autophagic activity as this adaptor protein is degraded by autophagy (Klionsky et al., 2012). To characterize the impact of mutant ATXN7 on autophagy, we therefore analyzed the p62 levels in stable

inducible PC12 cell lines expressing GFP-tagged ATXN7 with 10 (ATXN7Q10-GFP) or 65 (ATXN7Q65-GFP) glutamines, respectively (Ajayi et al., 2012; Yu et al., 2012). A clear and gradual accumulation of p62, suggesting that the autophagy activity is decreased, could be observed in FLQ65 cells upon induction of mutant ATXN7 expression (Fig. 1A). In contrast, no change in p62 levels were detected in FLQ10 control cells induced to express ATXN7Q10-GFP. To confirm the decreased autophagic activity in ATXN7Q65-GFP cells we next measured the autophagic flux, by monitoring the LC3 levels in the presence and absence of the lysosomal inhibitor Bafilomycin A₁ (BafA₁) (Klionsky et al., 2012). Upon autophagy induction the LC3 protein is converted to lipidated LC3 (LC3II). LC3II associates with the autophagosome until fusion with the lysosome at which point intra-autophagosomal LC3II is degraded (He and Klionsky, 2009; Klionsky et al., 2012). If autophagic flux is occurring normally the amount of LC3II should hence be increased in the presence of BafA₁. Indeed, in control FLQ10 and non-induced (+Dox) FLQ65 cells BafA₁ treatment resulted in increased LC3II and decreased non-lipidated LC3 (LC3I) levels (Fig. 1B). However, in induced (-Dox) FLQ65 cells expressing mutant ATXN7 no significant change in LC3II or LC3I levels were observed after BafA₁ treatment (Fig. 1B). These results suggest that mutant ATXN7 reduces the autophagic flux.

To further confirm the inhibitory effect of mutant ATXN7 and make sure that the observed effect is not specific to PC12 cells, we also measured the autophagic flux in transiently transfected HEK 293T cells. Similarly as in PC12 cells, BafA₁ failed to significantly increase LC3II levels in HEK 293T cells expressing either truncated or full-length ATXN7 with 65 glutamines. Whereas increased LC3II levels could be detected in HEK 293T cells expressing truncated or full-length ATXN7 with 10 glutamines (Fig. 1C). Taken together these results show that mutant ATXN7 expression reduces the autophagic activity.

The sub-cellular localization of p53 is altered in mutant ATXN7 cells

As p53 is a known autophagy regulator and altered p53 activity has been implicated in polyQ pathology, we examined the level and sub-cellular localization of p53 in FLQ10 and FLQ65 cells (Bae et al., 2005; Chou et al., 2011; Grison et al., 2011; Illuzzi et al., 2011; Shahbazian et al., 2001; Steffan et al., 2000; Tsoi et al., 2012; Wang et al., 2010). In FLQ10 cells p53 showed a similar sub-cellular pattern regardless of whether ATXN7Q10-GFP expression was induced or not (Fig. 2A). However, in FLQ65 cells induced to express ATXN7Q65-GFP for 12 days, a dramatic shift in the sub-cellular localization of p53 was observed (Fig. 2A). This shift resulted in approximately a halving of the nuclear and a doubling of the cytoplasmic p53 level. As nuclear p53 can stimulate autophagy, whereas cytoplasmic p53 inhibits autophagic activity, both these changes could contribute to the decreased autophagy in mutant ATXN7 cells (Maiuri et al., 2010).

To determine whether the reduced nuclear p53 level has any effect on p53-mediated autophagy regulation, we first analyzed the overall transcriptional activity of p53 using a reporter assay. An approximate 20% decrease in general p53-mediated transcription could be detected in mutant ATXN7 cells (Fig. 2B). We next analyzed the mRNA level of two p53-regulated genes, Sestrin 1 and AMPK β 1, known to activate autophagy. Small (14-20%), non-significant decreases in both Sestrin 1 and AMPK β 1 mRNA levels were detected in mutant ATXN7 cells (Fig. 2C). These data suggest that reduced p53-mediated transcription might contribute, but is unlikely to fully explain the decreased autophagic activity in the SCA7 cells.

Mutant ATXN7 inhibits autophagy by inducing p53-mediated destabilization of ULK1

Cytoplasmic p53 is known to inhibit autophagy via interaction with FIP200 (Morselli et al., 2011). Next we therefore analyzed whether the increased cytoplasmic p53 levels observed in our mutant ATXN7 cells resulted in an increased p53-FIP200 interaction. Indeed, a three-fold

increase in p53-FIP200 interaction could be detected by immunoprecipitation (IP) in ATXN7Q65-GFP expressing FLQ65 cells (Fig. 3A, left panel). Surprisingly, analysis of the cellular extracts used as input for the IP also revealed a dramatic reduction, by more than 50%, of soluble FIP200 levels (Fig. 3A, right panel). Similarly, reduced levels of FIP200 could also be detected in HEK 293T cells transfected with truncated mutant ATXN7 (Fig. 3B). Analysis of FIP200 mRNA levels revealed no significant difference between induced and non-induced FLQ65 cells, suggesting that the reduced FIP200 protein level is not due to reduced gene expression (Fig. 3C).

The mechanism by which the p53-FIP200 interaction interferes with autophagy is unclear, however, FIP200 is known to bind and stabilize the ULK1 protein (Hara et al., 2008; Liang et al., 2010). We therefore hypothesized that the increased p53-FIP200 interaction and reduced soluble FIP200 levels could result in destabilization of the ULK1 protein. Consistent with this hypothesis we could observe no change in ULK1 mRNA level, but a decrease in ULK1 protein level in FLQ65 cells induced to express mutant ATXN7 (Fig. 3C and Fig. 4A). While no change in ULK1 protein level was observed in induced FLQ10 control cells (Fig. 4A). Furthermore, pulse-chase experiments showed that the half-life of ULK1 was reduced approximately 3-fold in mutant ATXN7 cells (Fig. 4B). Taken together, these results suggest that mutant ATXN7 expression results in destabilization of ULK1 and reduced capacity for autophagy induction by the ULK1-FIP200-Atg13-Atg101 complex.

To further confirm this hypothesis, we tested whether increasing the FIP200 level could restore the ULK1 level and the autophagic flux in FLQ65 cells. Due to the low transfection efficiency of PC12 cells, FIP200 levels were only slightly increased after transfection with a FIP200 expression construct (Hara et al., 2008), (Fig. 4C). However, despite this ULK1 levels were increased (Fig. 4C, right panel) and more importantly the mutant ATXN7 induced inhibition of autophagic flux was improved (Fig. 4D and supplementary Fig. S2A). Taken

together these results suggest that mutant ATXN7 reduces the autophagic activity by altering p53 function, thereby resulting in reduced FIP200-mediated stabilization of ULK1.

The p53 inhibitor alpha-pifithrin increases the autophagic flux and viability of mutant ATXN7 cells

To further validate that mutant ATXN7 decreases the autophagic activity via the identified cytoplasmic p53-mediated mechanism we treated mutant ATXN7 cells with the p53 inhibitor pifithrin- α . This inhibitor has previously been shown to block p53-mediated inhibition of autophagy (Tasdemir et al., 2008). In agreement with this pifithrin- α decreased the p53-FIP200 interaction (Fig. 5A), and restored the levels of soluble FIP200 and ULK1 in mutant ATXN7 cells (Fig. 5B-C). Furthermore, with pifithrin- α treatment the mutant ATXN7 inhibition of the autophagic flux was reversed (Fig. 5D and supplementary Fig. S2B). In addition, pifithrin- α treatment also ameliorated the mutant ATXN7 toxicity (Fig. 5E). These results support the finding that altered cytoplasmic p53 activity resulting in an increased p53-FIP200 interaction and destabilization of ULK1, is responsible for the decreased autophagy activity in mutant ATXN7 cells. Moreover, this also indicates that the reduced autophagic activity contributes to the polyQ toxicity.

Sequestration of p53 and FIP200 into mutant ATXN7 aggregates

The p53 protein has been reported to be sequestered into inclusions in several polyQ diseases, including SCA7 (Zander et al., 2001). We therefore considered that sequestration of the p53-FIP200 complex into ATXN7 aggregates could be involved in the destabilization of ULK1 and the autophagy inhibition. To test this hypothesis we used the filter trap assay to quantitatively analyze the insoluble/aggregated material in FLQ10 and FLQ65 cells induced to express ATXN7 for 0-12 days (Fig. 6A-C). As expected, aggregated ATXN7 material was

detected in insoluble fractions from induced FLQ65 cells, whereas no ATXN7 aggregation was detected in FLQ10 cells (Fig. 6A). More importantly, a gradual increase in p53 (Fig. 6B), as well as FIP200 (Fig. 6C), was detected in the insoluble fractions/aggregated material in induced FLQ65 cells. A similar co-aggregation of ATXN7, p53 and FIP200 could also be detected in HEK 293T cells expressing truncated ATXN7 with 65 glutamines, see supplementary Fig. S3. These results suggest that the increased p53-FIP200 interaction and sequestration of the p53-FIP200 complex into ATXN7 aggregates could be responsible for the decreased levels of soluble FIP200 in SCA7 cells. To evaluate if the sequestration also affected the level of soluble p53, we performed western blots and p53 mRNA analyzes. Western blot analysis showed reduced soluble p53 levels in mutant ATXN7 cells (Fig. 6D and supplementary Fig. S3), while no changes in p53 mRNA levels could be detected (Fig. 2C). Together, this suggests that co-aggregation of p53 with mutant ATXN7 indeed causes a slight decrease in soluble p53 levels.

Treatment with Synb3-QBP1 restores the ULK1 level and the autophagic flux

We then reasoned that if sequestration of the p53-FIP200 complex is contributing to the decreased soluble FIP200 level and reduced autophagic activity, blocking ATXN7 aggregation should ameliorate the autophagic dysfunction. In order to test this we treated the mutant ATXN7 cells with the polyglutamine binding peptide 1 (QBP1), previously shown to block polyQ aggregation (Nagai et al., 2003; Nagai et al., 2000). To facilitate the cellular delivery of QBP1 we conjugated the peptide to two cell penetrating peptides (CPP), Tat and Synb3. Consistent with previous reports both Tat-QBP1 and Synb3-QBP1 ameliorated mutant ATXN7 aggregation. Synb3-QBP1 showed the highest efficiency and reduced the aggregation with almost 60% (Fig. 7A). Moreover, QBP1 treatment also reduced the amount of p53 detected in the insoluble fractions/aggregated material (Fig. 7A) and restored the levels of

both soluble p53 (Fig. 7B), FIP200 (Fig. 7C) and ULK1 (Fig. 7D). Most importantly, the QBP1 treatment increased the autophagic flux in mutant ATXN7 expressing cells (Fig. 7E and supplementary Fig. S2C) and also ameliorated the mutant ATXN7 induced toxicity (Fig. 7F). Taken together the results presented above suggest that mutant ATXN7 by altering p53 function result in an increased p53-FIP200 interaction and sequestration of p53-FIP200 into ATXN7 aggregates. In turn this causes decreased FIP200-mediated stabilization of ULK1 and reduced autophagic capacity.

Discussion

Reducing the level of toxic polyglutamine (polyQ) proteins by pharmacological activation of autophagy has been suggested as a therapeutic approach for polyQ diseases. However, autophagic dysfunction has also been reported in these disorders (Cheung and Ip, 2011; Wong and Cuervo, 2010). If therapies based on autophagy activation should be developed it is hence important to understand the mechanism by which mutant polyQ proteins affect the autophagy process. In this study we identify a novel mechanism, involving p53, by which the SCA7 polyglutamine protein ATXN7 reduces the autophagic activity.

The p53 protein is a stress-induced regulator of cell function and can respond to stress by trans-activating genes that either permit cells to re-establish homeostasis or, if the damage is too severe to be repaired, activate apoptosis (Maiuri et al., 2010). Several studies have implicated altered p53-mediated transcriptional activity and apoptosis in polyQ diseases (Bae et al., 2005; Chou et al., 2011; Grison et al., 2011; Illuzzi et al., 2011; Shahbazian et al., 2001; Steffan et al., 2000; Tsoi et al., 2012; Wang et al., 2010). However, to our knowledge no studies investigating the effects of mutant polyQ proteins on p53-mediated regulation of autophagy have so far been published. Autophagy has recently been recognized as one of the pivotal responses to cellular stress and whereas nuclear p53 acts as an autophagy-promoting

transcription factor, cytoplasmic p53 inhibits autophagy via interaction with FIP200 (Maiuri et al., 2010; Morselli et al., 2011). In this study we show that mutant ATXN7 decreases the nuclear, while elevating the cytoplasmic p53 levels. In the nucleus this resulted in a small, non-significant decrease in p53-mediated trans-activation of two autophagy stimulating genes, suggesting that this mechanism is unlikely to fully explain the reduced autophagy in mutant ATXN7 cells.

The cytoplasmic regulatory role of p53 on autophagy has been suggested to dominate over the role of nuclear p53 (Morselli et al., 2011). Consistent with this, we could observe clear statistical alterations of cytoplasmic p53-mediated autophagy regulation in mutant ATXN7 cells. The p53-FIP200 interaction was increased at least 3 times in mutant ATXN7 cells (Fig. 3A and Fig. 5A). Furthermore, both p53 and FIP200 co-aggregated with mutant ATXN7 and together this lead to a more than 50% reduction of soluble FIP200 levels. In turn, this resulted in decreased FIP200-mediated stabilization of the ULK1 protein. Elevating the soluble FIP200 expression increased the ULK1 protein level and ameliorated both the ATXN7-induced autophagy inhibition and toxicity in the SCA7 cells. These findings are consistent with previous studies showing that FIP200 is required for ULK1 stability and autophagy induction (Hara et al., 2008; Mizushima, 2010). Taken together our results suggest that mutant ATXN7 causes reduced autophagy activity via p53-mediated disruption of the autophagy inducing ULK1-FIP200-Atg13-Atg101 complex.

Interestingly, neuronal specific deletion of FIP200 in mice has been shown to result in progressive loss of cerebellar granule and Purkinje cells, as well as ataxia (Liang et al., 2010). This is similar to what can be observed in SCA7 patients (Konigsmark and Weiner, 1970; Martin et al., 1994). It is hence tempting to speculate that p53-mediated disruption of autophagy activity, resulting in decreased clearance of expanded polyQ protein, might contribute to the high vulnerability of cerebellar neurons in SCA7 and other spinocerebellar

ataxias. Consistent with this hypothesis, we could also observe an increased autophagic activity and reduced toxicity, when treating the SCA7 cells with the p53 inhibitor pifithrin- α . Although, pifithrin- α -mediated inhibition of p53's pro-apoptotic functions could also contribute to the reduced toxicity (Wang et al., 2010). We could, however, not detect any signs of apoptosis in our SCA7 model at the time when autophagy inhibition and toxicity occur (supplementary Fig. S4). These results suggest that the positive effect of pifithrin- α is indeed due to blockage of the p53-mediated autophagy inhibition. Further supporting this finding, deletion of p53 in SCA1 mice has previously been shown to reduce cerebellar pathology and the number of polyQ inclusions, through a mechanism independent of p53-mediated apoptosis (Shahbazian et al., 2001).

Proteolytic processing of ATXN7, believed to occur in the nucleus, has been observed in SCA7 and is thought to generate smaller more toxic polyQ fragments (Garden et al., 2002; Young et al., 2007; Yu et al., 2012; Yvert et al., 2001). We have previously shown that in the stable PC12 cell model used in this study, full-length ATXN7 is mainly found in the nucleus, whereas ATXN7 fragments localize and aggregate both in the nucleus and the cytoplasm (Yu et al., 2012). ATXN7 could thus alter the p53 activity by both nuclear and cytoplasmic mechanisms. The huntingtin protein, which is expanded in HD, has been shown to directly interact with p53 in a polyQ length dependent manner (Bae et al., 2005; Steffan et al., 2000). It is hence possible that expanded ATXN7 could also interact with p53 and through this interaction shift the p53 localization towards the cytoplasm and increase the p53-FIP200 binding. This hypothesis is supported by the observation of p53 in SCA7 inclusions (Zander et al., 2001), and co-aggregation of p53 with ATXN7 and FIP200 in our model. However, ATXN7 could also indirectly alter p53 function by activating signaling pathways resulting in post-translational modification of p53. Acetylation of lysine 382 in p53 is known to regulate both the sub-cellular localization and the interaction with FIP200 (Morselli et al., 2011;

Nakamura et al., 2000). During starvation-induced autophagy, K382 acetylation is increased, suggesting that this modification could disrupt the p53-FIP200 interaction and thereby induce autophagic activity (Morselli et al., 2011). Interestingly, decreased acetylation of this particular residue has been reported in a HD model (Illuzzi et al., 2011). Clearly, additional studies to clarify the exact molecular mechanism(s) by which mutant ATXN7 alters the p53 function is needed.

We have previously shown that treatment with the mTOR inhibitor rapamycin can stimulate autophagy and reduce toxicity in the SCA7 model used in this study (Yu et al., 2012). Rapamycin can also ameliorate the toxicity in several other polyQ disease models, suggesting that any p53-mediated autophagy inhibition could be over-come by this chemical autophagy inducer (Ravikumar et al., 2004; Tanaka et al., 2004; Wang et al., 2009). Consistent with these findings autophagy inducers, like rapamycin, were recently shown to deplete p53 in the cytoplasm. In fact, rapamycin treatment was shown to completely eliminate the difference in autophagy inhibition between p53^{+/+} and p53^{-/-} cells (Tasdemir et al., 2008).

In conclusion, we have shown that mutant ATXN7 inhibits autophagy activity via p53-mediated disruption of FIP200 and ULK1, two key proteins involved in initiation of autophagosome formation. Moreover, treatment with a p53 inhibitor or an anti-aggregating peptide could reverse the autophagy inhibition and also reduce the mutant ATXN7 toxicity.

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References

- Ajayi, A., Yu, X., Lindberg, S., Langel, U. and Strom, A.L. (2012) Expanded ataxin-7 cause toxicity by inducing ROS production from NADPH oxidase complexes in a stable inducible Spinocerebellar ataxia type 7 (SCA7) model. *BMC Neurosci* 13, 86.
- Bae, B.I., Xu, H., Igarashi, S., Fujimuro, M., Agrawal, N., Taya, Y., Hayward, S.D., Moran, T.H., Montell, C., Ross, C.A., Snyder, S.H. and Sawa, A. (2005) p53 mediates cellular dysfunction and behavioral abnormalities in Huntington's disease. *Neuron* 47, 29-41.
- Chen, Y. and Klionsky, D.J. (2011) The regulation of autophagy - unanswered questions. *J Cell Sci* 124, 161-70.
- Cheung, Z.H. and Ip, N.Y. (2011) Autophagy deregulation in neurodegenerative diseases - recent advances and future perspectives. *J Neurochem* 118, 317-25.
- Chou, A.H., Lin, A.C., Hong, K.Y., Hu, S.H., Chen, Y.L., Chen, J.Y. and Wang, H.L. (2011) p53 activation mediates polyglutamine-expanded ataxin-3 upregulation of Bax expression in cerebellar and pontine nuclei neurons. *Neurochem Int* 58, 145-52.
- Garden, G.A., Libby, R.T., Fu, Y.H., Kinoshita, Y., Huang, J., Possin, D.E., Smith, A.C., Martinez, R.A., Fine, G.C., Grote, S.K., Ware, C.B., Einum, D.D., Morrison, R.S., Ptacek, L.J., Sopher, B.L. and La Spada, A.R. (2002) Polyglutamine-expanded ataxin-7 promotes non-cell-autonomous purkinje cell degeneration and displays proteolytic cleavage in ataxic transgenic mice. *J Neurosci* 22, 4897-905.
- Grisson, A., Mantovani, F., Comel, A., Agostoni, E., Gustincich, S., Persichetti, F. and Del Sal, G. (2011) Ser46 phosphorylation and prolyl-isomerase Pin1-mediated isomerization of p53 are key events in p53-dependent apoptosis induced by mutant huntingtin. *Proc Natl Acad Sci U S A* 108, 17979-84.
- Hara, T., Nakamura, K., Matsui, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., Yokoyama, M., Mishima, K., Saito, I., Okano, H. and Mizushima, N. (2006) Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* 441, 885-9.
- Hara, T., Takamura, A., Kishi, C., Iemura, S., Natsume, T., Guan, J.L. and Mizushima, N. (2008) FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells. *J Cell Biol* 181, 497-510.
- He, C. and Klionsky, D.J. (2009) Regulation mechanisms and signaling pathways of autophagy. *Annu Rev Genet* 43, 67-93.
- Helmlinger, D., Hardy, S., Sasorith, S., Klein, F., Robert, F., Weber, C., Miguët, L., Potier, N., Van-Dorselaer, A., Wurtz, J.M., Mandel, J.L., Tora, L. and Devys, D. (2004) Ataxin-7 is a subunit of GCN5 histone acetyltransferase-containing complexes. *Hum Mol Genet* 13, 1257-65.
- Heng, M.Y., Duong, D.K., Albin, R.L., Tallaksen-Greene, S.J., Hunter, J.M., Lesort, M.J., Osmand, A., Paulson, H.L. and Detloff, P.J. (2010) Early autophagic response in a novel knock-in model of Huntington disease. *Hum Mol Genet* 19, 3702-20.
- Illuzzi, J.L., Vickers, C.A. and Kmiec, E.B. (2011) Modifications of p53 and the DNA damage response in cells expressing mutant form of the protein huntingtin. *J Mol Neurosci* 45, 256-68.
- Jonasson, J., Strom, A.L., Hart, P., Brannstrom, T., Forsgren, L. and Holmberg, M. (2002) Expression of ataxin-7 in CNS and non-CNS tissue of normal and SCA7 individuals. *Acta Neuropathol* 104, 29-37.
- Katsuno, M., Banno, H., Suzuki, K., Takeuchi, Y., Kawashima, M., Tanaka, F., Adachi, H. and Sobue, G. (2008) Molecular genetics and biomarkers of polyglutamine diseases. *Curr Mol Med* 8, 221-34.

- Klionsky, D.J., Abdalla, F.C., Abeliovich, H., Abraham, R.T., Acevedo-Arozena, A., Adeli, K., Agholme, L., Agnello, M., Agostinis, P., Aguirre-Ghiso, J.A., Ahn, H.J., Ait-Mohamed, O., Ait-Si-Ali, S., Akematsu, T., Akira, S., Al-Younes, H.M., Al-Zeer, M.A., Albert, M.L., Albin, R.L., Alegre-Abarrategui, J., Aleo, M.F., Alirezaei, M., Almasan, A., Almonte-Becerril, M., Amano, A., Amaravadi, R., Amarnath, S., Amer, A.O., Andrieu-Abadie, N., Anantharam, V., Ann, D.K., Anoopkumar-Dukie, S., Aoki, H., Apostolova, N., Arancia, G., Aris, J.P., Asanuma, K., Asare, N.Y., Ashida, H., Askanas, V., Askew, D.S., Auberger, P., Baba, M., Backues, S.K., Baehrecke, E.H., Bahr, B.A., Bai, X.Y., Bailly, Y., Baiocchi, R., Baldini, G., Balduini, W., Ballabio, A., Bamber, B.A., Bampton, E.T., Banhegyi, G., Bartholomew, C.R., Bassham, D.C., Bast, R.C., Jr., Batoko, H., Bay, B.H., Beau, I., Bechet, D.M., Begley, T.J., Behl, C., Behrends, C., Bekri, S., Bellaire, B., Bendall, L.J., Benetti, L., Berliocchi, L., Bernardi, H., Bernassola, F., Besteiro, S., Bhatia-Kissova, I., Bi, X., Biard-Piechaczyk, M., Blum, J.S., Boise, L.H., Bonaldo, P., Boone, D.L., Bornhauser, B.C., Bortoluci, K.R., Bossis, I., Bost, F., Bourquin, J.P., Boya, P., Boyer-Guittaut, M., Bozhkov, P.V., Brady, N.R., Brancolini, C., Brech, A., Brenman, J.E., Brennand, A., Bresnick, E.H., Brest, P., Bridges, D., Bristol, M.L., Brookes, P.S., Brown, E.J., Brumell, J.H. et al. (2012) Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* 8, 445-544.
- Komatsu, M., Wang, Q.J., Holstein, G.R., Friedrich, V.L., Jr., Iwata, J., Kominami, E., Chait, B.T., Tanaka, K. and Yue, Z. (2007) Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration. *Proc Natl Acad Sci U S A* 104, 14489-94.
- Konigsmark, B.W. and Weiner, L.P. (1970) The olivopontocerebellar atrophies: a review. *Medicine (Baltimore)* 49, 227-41.
- Li, X., Li, H. and Li, X.J. (2008) Intracellular degradation of misfolded proteins in polyglutamine neurodegenerative diseases. *Brain Res Rev* 59, 245-52.
- Liang, C.C., Wang, C., Peng, X., Gan, B. and Guan, J.L. (2010) Neural-specific deletion of FIP200 leads to cerebellar degeneration caused by increased neuronal death and axon degeneration. *J Biol Chem* 285, 3499-509.
- Lindenberg, K.S., Yvert, G., Muller, K. and Landwehrmeyer, G.B. (2000) Expression analysis of ataxin-7 mRNA and protein in human brain: evidence for a widespread distribution and focal protein accumulation. *Brain Pathol* 10, 385-94.
- Maiuri, M.C., Galluzzi, L., Morselli, E., Kepp, O., Malik, S.A. and Kroemer, G. (2010) Autophagy regulation by p53. *Curr Opin Cell Biol* 22, 181-5.
- Martin, J.J., Van Regemorter, N., Krols, L., Brucher, J.M., de Barsy, T., Szliwowski, H., Evrard, P., Ceuterick, C., Tassignon, M.J., Smet-Dieleman, H. and et al. (1994) On an autosomal dominant form of retinal-cerebellar degeneration: an autopsy study of five patients in one family. *Acta Neuropathol* 88, 277-86.
- Martinez-Vicente, M., Tallozy, Z., Wong, E., Tang, G., Koga, H., Kaushik, S., de Vries, R., Arias, E., Harris, S., Sulzer, D. and Cuervo, A.M. (2010) Cargo recognition failure is responsible for inefficient autophagy in Huntington's disease. *Nat Neurosci* 13, 567-76.
- Metzger, S., Saukko, M., Van Che, H., Tong, L., Puder, Y., Riess, O. and Nguyen, H.P. (2010) Age at onset in Huntington's disease is modified by the autophagy pathway: implication of the V471A polymorphism in Atg7. *Hum Genet* 128, 453-9.
- Mizushima, N. (2010) The role of the Atg1/ULK1 complex in autophagy regulation. *Curr Opin Cell Biol* 22, 132-9.
- Morselli, E., Shen, S., Ruckenstein, C., Bauer, M.A., Marino, G., Galluzzi, L., Criollo, A., Michaud, M., Maiuri, M.C., Chano, T., Madeo, F. and Kroemer, G. (2011) p53 inhibits autophagy by interacting with the human ortholog of yeast Atg17, RB1CC1/FIP200. *Cell Cycle* 10, 2763-9.
- Morselli, E., Tasdemir, E., Maiuri, M.C., Galluzzi, L., Kepp, O., Criollo, A., Vicencio, J.M., Soussi, T. and Kroemer, G. (2008) Mutant p53 protein localized in the cytoplasm inhibits autophagy. *Cell Cycle* 7, 3056-61.

- Nagai, Y., Fujikake, N., Ohno, K., Higashiyama, H., Popiel, H.A., Rahadian, J., Yamaguchi, M., Strittmatter, W.J., Burke, J.R. and Toda, T. (2003) Prevention of polyglutamine oligomerization and neurodegeneration by the peptide inhibitor QBP1 in *Drosophila*. *Hum Mol Genet* 12, 1253-9.
- Nagai, Y., Tucker, T., Ren, H., Kenan, D.J., Henderson, B.S., Keene, J.D., Strittmatter, W.J. and Burke, J.R. (2000) Inhibition of polyglutamine protein aggregation and cell death by novel peptides identified by phage display screening. *J Biol Chem* 275, 10437-42.
- Nakamura, S., Roth, J.A. and Mukhopadhyay, T. (2000) Multiple lysine mutations in the C-terminal domain of p53 interfere with MDM2-dependent protein degradation and ubiquitination. *Mol Cell Biol* 20, 9391-8.
- Nakamura, Y., Tagawa, K., Oka, T., Sasabe, T., Ito, H., Shiwaku, H., La Spada, A.R. and Okazawa, H. (2012) Ataxin-7 associates with microtubules and stabilizes the cytoskeletal network. *Hum Mol Genet* 21, 1099-110.
- Palhan, V.B., Chen, S., Peng, G.H., Tjernberg, A., Gamper, A.M., Fan, Y., Chait, B.T., La Spada, A.R. and Roeder, R.G. (2005) Polyglutamine-expanded ataxin-7 inhibits STAGA histone acetyltransferase activity to produce retinal degeneration. *Proc Natl Acad Sci U S A* 102, 8472-7.
- Qin, Q., Inatome, R., Hotta, A., Kojima, M., Yamamura, H., Hirai, H., Yoshizawa, T., Tanaka, H., Fukami, K. and Yanagi, S. (2006) A novel GTPase, CRAG, mediates promyelocytic leukemia protein-associated nuclear body formation and degradation of expanded polyglutamine protein. *J Cell Biol* 172, 497-504.
- Ravikumar, B., Duden, R. and Rubinsztein, D.C. (2002) Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. *Hum Mol Genet* 11, 1107-17.
- Ravikumar, B., Vacher, C., Berger, Z., Davies, J.E., Luo, S., Oroz, L.G., Scaravilli, F., Easton, D.F., Duden, R., O'Kane, C.J. and Rubinsztein, D.C. (2004) Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat Genet* 36, 585-95.
- Shahbazian, M.D., Orr, H.T. and Zoghbi, H.Y. (2001) Reduction of Purkinje cell pathology in SCA1 transgenic mice by p53 deletion. *Neurobiol Dis* 8, 974-81.
- Steffan, J.S., Kazantsev, A., Spasic-Boskovic, O., Greenwald, M., Zhu, Y.Z., Gohler, H., Wanker, E.E., Bates, G.P., Housman, D.E. and Thompson, L.M. (2000) The Huntington's disease protein interacts with p53 and CREB-binding protein and represses transcription. *Proc Natl Acad Sci U S A* 97, 6763-8.
- Strom, A.L., Forsgren, L. and Holmberg, M. (2005) A role for both wild-type and expanded ataxin-7 in transcriptional regulation. *Neurobiol Dis* 20, 646-55.
- Tanaka, M., Machida, Y., Niu, S., Ikeda, T., Jana, N.R., Doi, H., Kurosawa, M., Nekooki, M. and Nukina, N. (2004) Trehalose alleviates polyglutamine-mediated pathology in a mouse model of Huntington disease. *Nat Med* 10, 148-54.
- Tasdemir, E., Maiuri, M.C., Galluzzi, L., Vitale, I., Djavaheri-Mergny, M., D'Amelio, M., Criollo, A., Morselli, E., Zhu, C., Harper, F., Nannmark, U., Samara, C., Pinton, P., Vicencio, J.M., Carnuccio, R., Moll, U.M., Madeo, F., Paterlini-Brechot, P., Rizzuto, R., Szabadkai, G., Pierron, G., Blomgren, K., Tavernarakis, N., Codogno, P., Cecconi, F. and Kroemer, G. (2008) Regulation of autophagy by cytoplasmic p53. *Nat Cell Biol* 10, 676-87.
- Tsoi, H., Lau, T.C., Tsang, S.Y., Lau, K.F. and Chan, H.Y. (2012) CAG expansion induces nucleolar stress in polyglutamine diseases. *Proc Natl Acad Sci U S A* 109, 13428-33.
- Wang, H.L., Chou, A.H., Lin, A.C., Chen, S.Y., Weng, Y.H. and Yeh, T.H. (2010) Polyglutamine-expanded ataxin-7 upregulates Bax expression by activating p53 in cerebellar and inferior olivary neurons. *Exp Neurol* 224, 486-94.
- Wang, T., Lao, U. and Edgar, B.A. (2009) TOR-mediated autophagy regulates cell death in *Drosophila* neurodegenerative disease. *J Cell Biol* 186, 703-11.
- Wong, E. and Cuervo, A.M. (2010) Autophagy gone awry in neurodegenerative diseases. *Nat Neurosci* 13, 805-11.

- Young, J.E., Gouw, L., Propp, S., Sopher, B.L., Taylor, J., Lin, A., Hermel, E., Logvinova, A., Chen, S.F., Chen, S., Bredesen, D.E., Truant, R., Ptacek, L.J., La Spada, A.R. and Ellerby, L.M. (2007) Proteolytic cleavage of ataxin-7 by caspase-7 modulates cellular toxicity and transcriptional dysregulation. *J Biol Chem* 282, 30150-60.
- Young, J.E., Martinez, R.A. and La Spada, A.R. (2009) Nutrient deprivation induces neuronal autophagy and implicates reduced insulin signaling in neuroprotective autophagy activation. *J Biol Chem* 284, 2363-73.
- Yu, X., Ajayi, A., Boga, N.R. and Strom, A.L. (2012) Differential Degradation of Full-length and Cleaved Ataxin-7 Fragments in a Novel Stable Inducible SCA7 Model. *J Mol Neurosci* 47, 219-233.
- Yvert, G., Lindenberg, K.S., Devys, D., Helmlinger, D., Landwehrmeyer, G.B. and Mandel, J.L. (2001) SCA7 mouse models show selective stabilization of mutant ataxin-7 and similar cellular responses in different neuronal cell types. *Hum Mol Genet* 10, 1679-92.
- Zander, C., Takahashi, J., El Hachimi, K.H., Fujigasaki, H., Albanese, V., Lebre, A.S., Stevanin, G., Duyckaerts, C. and Brice, A. (2001) Similarities between spinocerebellar ataxia type 7 (SCA7) cell models and human brain: proteins recruited in inclusions and activation of caspase-3. *Hum Mol Genet* 10, 2569-79.

Figure legends

Fig. 1. Reduced autophagy activity in mutant ATXN7 cells.

A) Western blot analysis of ATXN7 and p62 in FLQ10 and FLQ65 cells induced to express GFP-tagged ATXN7 with 10 (ATXN7Q10-GFP) or 65 (ATXN7Q65-GFP) glutamines for the indicated number of days. Actin was used as loading control for quantifications. B) Examination of the autophagic flux in non-induced (+Dox), or FLQ10 and FLQ65 cells induced (-Dox) to express GFP-tagged ATXN7 for 12 days. Flux analysis was done by detecting the increase in LC3II levels and the decrease in LC3I levels, between non-treated and cells treatment with 100 nM of the lysosomal inhibitor Bafilomycin A₁ (BafA₁). LC3 was detected by western blot and the LC3II and LC3I levels were normalized against total LC3. The normalized LC3 levels in non-treated cells were set to 100%. C) Analysis of the autophagic flux in HEK293T cells transfected to express truncated ATXN7 with 10 (Tr10), truncated ATXN7 with 65 (Tr65), full-length ATXN7 with 10 (Fl10) or full-length ATXN7 with 65 (Fl65) glutamines, respectively. All data are shown as means $\pm$ SEM from 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ns = not significant.

Fig. 2. Mutant ATXN7 expression results in increased cytoplasmic p53 levels.

A) The sub-cellular localization of p53 in parental PC12, non-induced (+Dox) or FLQ10 and FLQ65 cells induced (-Dox) to express ATXN7Q10-GFP and ATXN7Q65-GFP for 12 days, respectively. Cytoplasmic (Cyt) and nuclear (Nuc) proteins were separated by fractionation and p53 detected by western blot. Tubulin and Histone H3 (H3) were used as loading control for the cytoplasmic and nuclear fraction, respectively. B) Analysis of p53 transcriptional activity using a luciferase reporter assay. PC12, non-induced (+Dox), or FLQ10 and FLQ65 cells induced (-Dox) to express ATXN7Q10-GFP or ATXN7Q65-GFP for 11 days were transfected with the PG13-luc reporter construct and 24 hours later the luciferase activity was

measured and normalized against the internal control. C) Analysis of p53, Sestrin 1 and AMPK β 1 mRNA levels in non-induced (+Dox) or FLQ65 cells induced (-Dox) to express mutant ATXN7 for 12 days. The mRNA levels of actin were used for normalization. All data are shown as means $\pm$ SEM from 3 independent experiments. ** $p < 0.01$, *** $p < 0.001$ and ns= not significant.

Fig. 3. Mutant ATXN7 increases the p53-FIP200 interaction and results in loss of soluble FIP200.

A) The level of p53-FIP200 interaction in non-induced (+Dox), or FLQ10 and FLQ65 cells induced (-Dox) to express GFP-tagged ATXN7 for 12 days analyzed by p53 immunoprecipitation (IP) and FIP200 western blot analysis (Top left panel). For quantifications (Bottom left panel), the amount of pulled-down p53 was used for normalizations. FIP200 and p53 levels in the extracts used as input for the IPs were also analyzed (Top right panel) and FLQ65 samples quantified using actin as loading control (Bottom right panel). B) Analysis of soluble FIP200 levels in HEK293T cells transfected with truncated ATXN7 with 10 (Tr10), truncated ATXN7 with 65 (Tr65), full-length ATXN7 with 10 (Fl10) or full-length ATXN7 with 65 (Fl65) glutamines, respectively. C) Analysis of FIP200 and ULK1 mRNA levels in non-induced (+Dox) or induced (-Dox) FLQ65 cells expressing ATXN7Q65-GFP for 12 days. The mRNA levels of actin were used for normalization. For all quantifications data are shown as means $\pm$ SEM from 3 independent experiments. * $p < 0.05$ and ** $p < 0.01$.

Fig. 4. Mutant ATXN7 perturbs the autophagy activity by destabilizing ULK1.

A) Detection of ULK1 protein levels in FLQ10 and FLQ65 cells induced to express ATXN7Q10-GFP or ATXN7Q65-GFP for the indicated number of days. Actin was used for

normalization. B) Pulse-chase analysis of ULK1 protein stability in non-induced (+Dox) or FLQ10 and FLQ65 cells induced (-Dox) to express ATXN7Q10-GFP and ATXN7Q65-GFP for 12 days, respectively. Cells were treated with 100 µg/ml cycloheximide for 0, 2, 4 or 8 hours before harvest and ULK1 level then analyzed by Western blot. Tubulin was used as loading control for quantifications. C) Non-induced (+Dox) and FLQ65 cells induced (-Dox) to express mutant ATXN7 for 10 days were transfected with a FIP200 construct and the FIP200 level (left panel) and ULK1 level (right panel) analyzed 48 hours later by western blot. For quantifications, FIP200 and ULK1 levels were normalized against tubulin. D) Analysis of the autophagic flux, by monitoring the increase in LC3II after treatment with the lysosomal inhibitor Bafilomycin A₁ (Baf A₁), in FLQ65 cells transfected and treated as in C. For all quantifications data are shown as means ± SEM from 3 independent experiments. * p<0.05, **p<0.01 and ***p<0.001.

Fig. 5. The p53 inhibitor pifithrin-α restores FIP200 and ULK1 levels as well as the autophagic flux in mutant ATXN7 cells.

A) Analysis of the p53-FIP200 interaction in non-induced (+Dox) or FLQ65 cells induced (-Dox) to express ATXN7Q65-GFP for 12 days while growing in media with or without the p53 inhibitor pifithrin-α. The p53 protein was immunoprecipitated and FIP200 detected by western blot. For quantifications (Bottom left panel), the amount of pulled-down p53 was used for normalizations. B) Analysis and quantification of soluble FIP200 levels in extracts from FLQ65 cells grown and treated as in A. C) Analysis and quantification of ULK1 levels in extracts from FLQ65 cells grown and treated as in A. D) The autophagic flux, analyzed by monitoring the increase in LC3II after treatment with the lysosomal inhibitor Bafilomycin A₁ (BafA₁), in FLQ65 cells grown and treated as in A. E) Viability measured by the WST-1

assay in FLQ65 cells grown and treated as in A. All data are shown as means $\pm$ SEM from 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ and ns = not significant.

Fig. 6. p53 and FIP200 co-aggregates with mutant ATXN7

A) Analysis of ATXN7 aggregation in FLQ10 and FLQ65 cells induced to express ATXN7Q10-GFP or ATXN7Q65-GFP for the indicated number of days. The level of aggregated ATXN7 in insoluble fractions were detected by filter trap assay and normalized against the total ATXN7 level determined by dot blot analysis of total cell fractions from the same cells. B) Analysis of p53 co-aggregation in FLQ10 and FLQ65 cells grown and treated as in A. The level of aggregated p53 material in insoluble fractions were detected by filter trap assay and normalized against the total p53 level determined by dot blot of total cell fractions from the same cells. C) Analysis of FIP200 co-aggregation in FLQ10 and FLQ65 cells grown and treated as in A. The levels of aggregated FIP200 material in insoluble fractions were detected by filter trap assay and normalized against the total FIP200 level determined by dot blot of total cell fractions from the same cells. D) Detection and quantification of the soluble p53 level in FLQ10 and FLQ65 cells grown and treated as in A. Actin was used as loading control for western blots. For all quantifications data are shown as means $\pm$ SEM from 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Fig. 7. QBP1 inhibits mutant ATXN7 aggregation, restores FIP200 and ULK1 levels, as well as ameliorates the autophagy inhibition in SCA7 cells.

A) Analysis of ATXN7 and p53 co-aggregation in FLQ65 cells induced to express ATXN7Q65-GFP for 12 days while growing in media supplemented with 700 nM QBP1 peptides (synb3-QBP1 and Tat-QBP1) or control peptides (synb3-random and Tat-random). Induced FLQ65 cells not subjected to any peptide treatment (ctrl) was also analyzed and for

quantifications the level of aggregation in this sample was set to 100%. B) Western blot analysis of the soluble p53 and ATXN7 level in FLQ65 cells grown and treated as in A. Actin was used as loading control for quantifications. C) Analysis and quantification of soluble FIP200 levels in FLQ65 cells grown and treated as in A. Tubulin was used as loading control. D) Analysis and quantification of soluble ULK1 levels in FLQ65 cells grown and treated as in A. Actin was used as loading control. E) Autophagic flux, measured by LC3II accumulation in the presence of BafA₁, in FLQ65 cells grown and treated as in A. F) Viability measured by WST-1 assay in FLQ65 cells grown and treated as in A. All data are shown as means $\pm$ SEM from 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ns = not significant.

Fig 1

Fig 2

Fig 3

Fig 4

A)

C)

B)

D)

Fig 5

Fig 6

Fig 7

Supplementary Figures S1-S4

Supplementary Figure S1. Aggregation analysis of ATXN7 in FLQ10 and FLQ65 cells.

A) Schematic drawing of how total, soluble and insoluble protein fractions, used for different analyzes in the study, were obtained. B) Filter trap and dot blot analysis of total, soluble and insoluble fractions using 0.2 μm pore size nitrocellulose membrane. In the filter trap assay, where vacuum suction is applied (left panel), no mutant ATXN7 from soluble fractions are trapped on the nitrocellulose membrane. Furthermore, no wild-type ATXN7 can be detected in any fraction. However, aggregated ATXN7 material present in total and insoluble fractions from mutant ATXN7 cells is retained on the membrane. In contrast with dot blot analysis without vacuum suction (right panel), mutant ATXN7 is detectable in all fractions. Whereas the non-aggregating wild-type ATXN7 is only detected in total and soluble fractions.

Supplementary Figure S2. Quantification of LC3 I levels for autophagic flux analyzes.

A) Non-induced (+Dox) and FLQ65 cells induced (-Dox) to express mutant ATXN7 for 10 days were transfected with a FIP200 construct. LC3 was detected by western blot and the LC3I levels compared between non-treated and cells treatment with 100 nM of the lysosomal inhibitor Bafilomycin A₁ (BafA₁). The total LC3 levels were used for normalizations. B) Autophagic flux, measured by LC3I decrease in the presence of BafA₁ in non-induced (+Dox) or FLQ65 cells induced (-Dox) to express ATXN7Q65-GFP for 12 days while growing in media with or without the p53 inhibitor pifithrin-α. C) FLQ65 cells were induced to express ATXN7Q65-GFP for 12 days while growing in media supplemented with 700 nM QBP1

peptides (synb3-QBP1 and Tat-QBP1) or control peptides (synb3-random and Tat-random). LC3 was detected by western blot and the LC3I levels compared between non-treated and cells treatment with the lysosomal inhibitor Bafilomycin A₁ (BafA₁). The total LC3 levels were used for normalizations. All data are shown as means $\pm$ SEM from 3 independent experiments. * $p < 0.05$, and ns = not significant.

Supplementary Figure S3. Co-aggregation of p53 and FIP200 with mutant ATXN7 in HEK293T cells.

A) HEK293T cells were transiently transfected to express truncated ATXN7 with 10 (Tr10), truncated ATXN7 with 65 (Tr65), full-length ATXN7 with 10 (F110) or full-length ATXN7 with 65 (F165) glutamines, respectively. Forty-eight hours later the level of FIP200, p53 and ATXN7 in insoluble fractions were analyzed by filter trap analysis. The total ATXN7, p53 and FIP200 levels determined by dot blots of total cell fractions were used for normalizations.

B) The level of soluble p53 and ATXN7 determined by western blot in HEK293T cells transfected and treated as in A. For quantifications data are shown as means $\pm$ SEM from 3 independent experiments. * $p < 0.05$, *** $p < 0.01$, **** $p < 0.001$.

Supplementary Figure S4. Mutant ATXN7 expression does not result in activation of apoptosis. A) The level of the pro-apoptotic protein Bax was analyzed by western blot in parental PC12, as well as FLQ10 and FLQ65 PC12 cells induced to express ATXN7Q10-GFP and ATXN7Q65-GFP for 0-12 days, respectively. Actin was used as loading control for quantifications. B) The number of apoptotic nuclei, visualized by 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) staining, was determined in non-induced or FLQ10 and FLQ65 cells induced to express ATXN7Q10-GFP and ATXN7Q65-GFP for 12 days, respectively. For quantifications the total number of cells and the number of cells with apoptotic nuclei were counted in 10 randomly chosen view fields (approximately 200-300 cells) for each condition in three independent experiments.