Polyglutamine expanded Ataxin-7 induces DNA damage and alters FUS localization and function

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1. Introduction

Polyglutamine (polyQ) diseases are neurodegenerative disorders caused by expanded CAG trinucleotide repeats, which are translated into abnormally elongated glutamine tracts in the respective mutant proteins (Lieberman et al., 2018; Paulson et al., 2017). Common pathological mechanisms, including aggregation of the expanded polyQ protein, have been suggested in these disorders. However, the specific sequence surrounding the expanded polyQ repeat is believed to modify the toxicity and likely contribute to the selective vulnerability of different subsets of neurons in each polyQ disease (Lieberman et al., 2018; Paulson et al., 2017).

In spinocerebellar ataxia type 7 (SCA7) the CAG/polyQ repeat is situated in the ataxin-7 (ATXN7) gene/protein and results in ataxia, blindness and dysphagia, due to degeneration of neurons in the cerebellum, retina and brainstem (David et al., 1997). ATXN7 is widely expressed in the brain (Jonasson et al., 2002), and is a subunit in the transcription regulation complexes STAGA and TFTC (Helmlinger et al., 2004; Palhan et al., 2005), which carry both histone acetyl transferase (HAT) and de-ubiquitination (DUb) activity. Transcriptional dysregulation has been suggested to play a central role in SCA7. However, studies investigating whether or not the STAGA HAT activity is altered by expansion in ATXN7 have provided conflicting results (Helmlinger et al., 2006; Palhan et al., 2005). In contrast, consistent evidence has shown that STAGA DUb activity is reduced due to sequestration of the whole DUb module into ATXN7 aggregates (vanagt et al., 2015). Moreover, this was shown to result in a global increase in H2B ubiquitination in several SCA7 models (vanagt et al., 2015; Mohan et al., 2014). This suggests that disruption of STAGA/TFTC-mediated gene regulation could contribute to SCA7 pathology. However, disrupted gene regulation due to sequestration of various transcription factors and RNA-binding proteins has also been suggested in SCA7 and other polyQ diseases (Lieberman et al., 2018; Toyoshima and Takahashi, 2014; Woulfe et al., 2010; Yu et al., 2013; Zander et al., 2001).

The DNA/RNA-binding protein Fused in sarcoma (FUS, also known as TLS) has been reported to be a component of mutant ATXN7, as well as other polyQ, aggregates (Alves et al., 2016; Doi et al., 2016; Mori et al., 2019). However, if the polyQ-FUS interaction and co-aggregation

**ARTICLE INFO**

**Keywords:**
Neurodegeneration
Polyglutamine disease
RNA-binding protein
FUS

**ABSTRACT**

Polyglutamine (polyQ) diseases, such as Spinocerebellar ataxia type 7 (SCA7), are caused by expansions of polyQ repeats in disease specific proteins. The sequestration of vital proteins into aggregates formed by polyQ proteins is believed to be a common pathological mechanism in these disorders. The RNA-binding protein FUS has been observed in polyQ aggregates, though if disruption of this protein plays a role in the neuronal dysfunction in SCA7 or other polyQ diseases remains unclear. We therefore analysed FUS localisation and function in a stable inducible PC12 cell model expressing the SCA7 polyQ protein ATXN7. We found that there was a high degree of FUS sequestration, which was associated with a more cytoplasmic FUS localisation, as well as a decreased expression of FUS regulated mRNAs. In contrast, the role of FUS in the formation of γH2AX positive DNA damage foci was unaffected. In fact, a statistical increase in the number of γH2AX foci, as well as an increased trend of single and double strand DNA breaks, detected by comet assay, could be observed in mutant ATXN7 cells. These results were further corroborated by a clear trend towards increased DNA damage in SCA7 patient fibroblasts. Our findings suggest that both alterations in the RNA regulatory functions of FUS, and increased DNA damage, may contribute to the pathology of SCA7.
disrupts FUS’ functions, and if this contributes to pathology still remains to be determined. FUS is a multifunctional protein and plays a role in DNA repair and several RNA regulatory functions, such as transcription, splicing, mRNA nuclear export and translation, reviewed in (Ratti and Buratti, 2016). During normal physiological conditions FUS predominantly localises to the nucleus. However mutations that either mislocalise FUS to the cytoplasm or elevate the total FUS level can cause two neurodegenerative diseases, amyotrophic lateral sclerosis (ALS) and frontotemporal degeneration (FTD) (Butti and Patten, 2018; Kwiatkowski et al., 2009; Neumann et al., 2009; Sabatelli et al., 2013). Depletion of nuclear FUS resulting in partial loss of nuclear functions, as well as cytoplasmic FUS accumulation and aggregation, have been suggested as pathological mechanisms in ALS, reviewed in (Ratti and Buratti, 2016; Xue et al., 2020).

In this study, we show that FUS is an abundant component in ATXN7 aggregates in an inducible SCA7 cell model. Furthermore, we found that induction of polyQ expanded ATXN7 resulted in decreased nuclear and increased cytoplasmic distribution of FUS. Moreover, this was associated with a decreased expression of FUS regulated mRNAs, suggesting that at least some of the transcriptional changes observed in SCA7 could be due to disruption of FUS. However, the ability of FUS in establishing γH2AX foci, required for DNA repair, appeared not to be hindered. In fact, the number of γH2AX foci in both PC12 cells induced to express mutant ATXN7, as well as in SCA7 patient fibroblasts, was increased, indicating an overall increase in DNA damage. Consistent with this both SCA7 PC12 cells and SCA7 fibroblasts showed an increased trend of DNA damage when subjected to comet assays. Hence, although the function of FUS in γH2AX foci formation was not obstructed, expression of expanded ATXN7 appears to cause increased DNA damage.

2. Results

2.1. FUS is abundant in ATXN7 aggregates

To increase the understanding of FUS in SCA7 pathology we first confirmed that FUS is sequestered into ATXN7 aggregates using a previously established stable inducible PC12 cell line (FLQ65) (Ajayi et al., 2012; Yu et al., 2012). Upon removal of doxycycline from the cell medium the FLQ65 cells express full-length GFP-tagged ATXN7 with 65 glutamines (ATXN7Q65-GFP) and show decreased viability from day 9 onwards (Fig. 1 and (Ajayi et al., 2012; Yu et al., 2012)). As a result of ATXN7 processing, polyQ containing N-terminal fragments can also be observed in this model (Fig. 1). Moreover, nuclear and cytoplasmic aggregates were clearly visible in immunostained cells expressing ATXN7Q65-GFP (Fig. 2A, fourth panel). In contrast, wild-type GFP-tagged ATXN7 with 10 glutamines (ATXN7Q10-GFP) induced in the control PC12 cell line (FLQ10) showed diffuse nuclear localisation, with much of ATXN7 localising to the nucleolus (Figs. 1 and 2A, second panel). Staining for FUS revealed a mainly nuclear localisation in both non-induced and induced FLQ10 and FLQ65 cells (Fig. 2A). However, in ATXN7Q65-GFP expressing cells a clear co-localisation of FUS with ATXN7 aggregates, as well as enhanced diffuse cytoplasmic FUS staining could be observed (Fig. 2A). When drawing profile lines across randomly selected aggregates (exemplified in Fig. 2A, white line) in five separate replicates, we found that around 13% of nuclear and around 86% of cytoplasmic ATXN7 aggregates contained an increased staining for FUS, defined as 2-fold higher intensity than the surroundings along a straight intensity profile line through an aggregate (Fig. 2B–C).

To further validate the sequestration of FUS into ATXN7 aggregates, we also performed filter trap analyses on extracted insoluble material from FLQ10 and FLQ65 cells, as previously described (Yu et al., 2013). As expected, a clear ATXN7 signal could be detected following filtration of samples from induced FLQ65 cells, which contain aggregated ATXN7 species that are trapped on the membrane. In contrast, no ATXN7 was detected in the insoluble material from FLQ10 cells or non-induced FLQ65 cells (Fig. 3A). More importantly, FUS could clearly be detected on the membrane following filtration of extracts from induced, but not non-induced FLQ65 cells (Fig. 3A). Thus, the sequestration of FUS into ATXN7 aggregates is strong enough to withstand treatment with detergents, as well as reducing conditions, during extract preparation and filtration. Taken together, these results indicate that FUS is an abundant component in ATXN7 aggregates.

FUS expression and distribution is altered in cells expressing mutant ATXN7.

To investigate if the sequestration of FUS into ATXN7 aggregates had any impact on FUS expression, we next analysed the total soluble FUS expression level using western blot. Interestingly, even though FUS was sequestered into ATXN7 aggregates, the total soluble expression of FUS was markedly increased in cells induced to express ATXN7Q65-GFP (Fig. 3B–C). Surprisingly, FUS expression was also increased by the induction of ATXN7Q10-GFP (Fig. 3B–C). This suggests that the increased FUS level might be a response to increased ATXN7 expression, irrespective of polyQ length.

Next, we analysed the cellular distribution of FUS using cell fractionation and western blot. Analysis of nuclear and cytoplasmic fractions revealed that although induction of ATXN7Q10-GFP did not result in any change in the cytoplasmic to nuclear FUS ratio (Fig. 4A and C), a shift from 30%:70% to 50%:50% could be observed upon induction of ATXN7Q65-GFP (Fig. 4B and D). Hence, mutant but not wild-type ATXN7 appears to promote a more cytoplasmic localisation of FUS.

2.2. The level of FUS regulated mRNAs is altered in cells expressing ATXN7Q65-GFP

Altered expression and cellular distribution of FUS have been shown to affect the level of multiple FUS-regulated mRNAs, and is suggested to contribute to the neuronal death in ALS disease (Lagier-Tourenne et al., 2012; Ling et al., 2019). To investigate if the altered FUS distribution in FLQ65 cells results in disruption of FUS regulated mRNAs we compared the expression of four FUS regulated mRNAs; SOD2 (Ohar et al., 2014; Sánchez-Ramos et al., 2011), HDAC6 (Kim et al., 2010), SMYD3 (Lagier-Tourenne et al., 2012) and CDK6 (Brooke et al., 2011), in non-induced and induced FLQ65 and FLQ10 cells using semi-quantitative RT-PCR. Of these four transcripts, three (SOD2, HDAC6 and CDK6) showed reduced levels upon induction of mutant ATXN7Q65-GFP expression, while one mRNA (SMYD3) remained unaffected (Fig. 5). In contrast, induction of wild-type ATXN7Q10-GFP did not result in alteration of any of the four mRNAs. Hence, although the total level of soluble FUS was
increased upon both wild-type and mutant ATXN7 expression, only the altered cellular distribution of FUS specifically observed upon expression of the mutant form of ATXN7 was accompanied by altered levels of FUS regulated mRNAs.

2.3. The level of DNA damage is increased in SCA7 cells

Phosphorylation of serine 139 in histone H2AX (γH2AX) occurs in response to a broad range of DNA lesions and is important for proper DNA damage response (DDR) signalling (Kopp et al., 2019). FUS is recruited to sites of DNA damage at an early stage and knock-down of this protein results in diminished formation of γH2AX positive foci and downstream DNA damage repair signalling (Mastrocola et al., 2013; Rulten et al., 2014; Wang et al., 2013). Therefore, we next investigated whether or not the observed FUS alterations in mutant ATXN7 cells affected the cells’ ability to form γH2AX foci in response to DNA damage. For this, non-induced and induced FLQ65 cells were treated for 24 h with 300 nM Camptothecin (CPT), a DNA topoisomerase inhibitor that blocks DNA repair, or for 15 min with 100 μM H2O2, a DNA damage inducer. Following treatment the PC12 cells were immunostained and a CellProfiler pipeline for detecting γH2AX foci was employed. As expected, CPT and H2O2 treatments resulted in increased

Fig. 2. FUS is sequestered into ATXN7Q65 aggregates. A) Representative images of immunostained PC12 cells with or without induction of ATXN7Q10/65. ATXN7 labelled green, FUS labelled red, and DRAQ5 (blue) was used as a DNA stain. Thick arrows indicate co-stained cytoplasmic aggregates, thin arrows indicate co-stained nuclear aggregates, and the white line is explained in B). B) Representative plotted intensity profiles of ATXN7, FUS and DRAQ5 staining along the white line displayed in the merged image in the fourth panel seen in A. C) Proportion of ATXN7 cytoplasmic versus nuclear aggregates that also contain FUS staining in the induced FLQ65 cells. FUS staining is defined as having a 2-fold intensity increase compared to background along the plotted intensity profile seen in B. 125 cytoplasmic and 125 nuclear aggregates were sampled across 5 replicates. Data in C shown as mean ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 3. FUS is re-localized into the insoluble fraction containing mutant ATXN7 aggregates. A) Insoluble fractions from non-induced and induced FLQ10 and FLQ65 cells were prepared and subjected to filter trap analysis followed by ATXN7 and FUS immunoblotting. B) Western blot analysis of soluble FUS levels in non-induced and induced FLQ10 and FLQ65 cells. C) Quantification of soluble FUS protein expression, where tubulin was used for normalization ($n = 8$, $p = 0.0300$ for Q10, $p = 0.0045$ for Q65). Data are shown as mean ± SEM, ns = non-significant, $p^* < 0.05$, $p^{**} < 0.01$.

Fig. 4. Induction of mutant ATXN7 disturbs the cellular localization of FUS. A-B) Representative western blots showing the level of FUS in nuclear and cytoplasmic fractions from induced and non-induced FLQ10 (C) or FLQ65 (B) cells. C) Quantified ratios of FUS in the nuclear vs cytoplasmic cellular compartment in induced and non-induced FLQ10 cells ($n = 3$). D) Quantified ratios of FUS in the nuclear vs cytoplasmic cellular compartment in induced and non-induced FLQ65 cells. ($n = 4$, $p = 0.0485$). Data are shown as mean ± SEM, ns = non-significant, $p^* < 0.05$. 

4
DNA damage; both when analysing the total number of γH2AX foci per nucleus, as well as the number of nuclei with ten or more foci (Fig. 6A–C). A similar level of baseline γH2AX foci in untreated cells, as well as increase in γH2AX foci upon CPT or H2O2 treatment could be observed between all FLQ10 cells and the non-induced FLQ65 cells. However, a trend towards an enhanced number of foci could be observed in the induced FLQ65 cells, both with and without treatment. A similar trend could also be observed using a higher concentration of H2O2 (300 μM, see Supplementary Fig. S1). Interestingly, the enhanced level of γH2AX foci in induced FLQ65 cells was most notable and indeed significant in the vehicle treated cells (Fig. 6B–C). Hence, the mutant ATXN7 induced FUS alterations do not diminish the ability of the cells to form γH2AX foci in response to endogenous nor exogenous induced DNA damage. Rather it appears that the presence of mutant ATXN7 increases the number of γH2AX foci. As the number of γH2AX foci is proportional to the production of DNA damage sites (Fillingham et al., 2006), this suggests that expression of mutant ATXN7 induces DNA damage per se.

To further investigate this we also performed γH2AX foci staining in SCA7 patient and control fibroblasts, in the absence or presence of CPT treatment. No increase in the basal level of γH2AX foci could be observed in SCA7 patient fibroblasts (7A-B). This is possibly due to the lack of aggregates and apparently mild pathology in these cells, even though the patient expresses an allele with 62 glutamines. However, upon challenge with CPT, a clear trend towards more nuclei with 20 or more γH2AX foci could be observed in SCA7 fibroblasts (7A-B), supporting the hypothesis that the presence of mutant ATXN7 promotes DNA damage.

Next, we therefore analysed the level of DNA damage in both the SCA7 PC12 cell model and patient fibroblasts using an alternative method, the comet assay. Again we could detect a higher proportion of DNA damage, i.e. amount of DNA in the comet tails, in induced versus non-induced FLQ65 cells, though no statistical significance was obtained (Fig. 6D–E). In contrast, no difference in the comet assay could be detected upon induction of wild-type ATXN7 in FLQ10 cells. Consistent with the findings in the SCA7 PC12 cells, we could also detect a marked shift towards more nuclei with a higher DNA concentration in the tails in SCA7 fibroblasts, compared to control fibroblasts, upon challenging with a low concentration of H2O2 (100 μM) (Fig. 7C). As the fibroblast experiments were only performed on one patient cell line versus one control cell line, the results must be considered with caution. However, the consistent results for both γH2AX and comets between SCA7 PC12 cells and patient fibroblasts point towards mutant ATXN7 either causing DNA damage, or obstructing the DNA damage response.

3. Discussion

Sequestration of FUS into polyQ inclusions has been observed in the brains of patients with several polyQ diseases (Doi et al., 2008). However, if this interferes with FUS’s functions or contributes to polyQ pathology remains to be determined. In this study, we show that FUS is...
Fig. 6. The formation of γ-H2AX positive DNA damage foci is not reduced in cells induced to express mutant ATXN7. A) Representative images of immunostained PC12 cells with or without induction of ATXN7Q10/Q65 and with or without CPT or H2O2 treatment. ATXN7 is labelled green, γ-H2AX is labelled red, and DRAQ5 (blue) was used as a DNA stain. B) Number of γ-H2AX foci per nucleus in FLQ10 and FLQ65 cells treated with vehicle, CPT or H2O2 ($p = 0.0003$) C) Percentage of nuclei with $\geq 10$ γ-H2AX foci in FLQ10 and FLQ65 cells treated with vehicle, CPT or H2O2 ($p = 0.0011$) D) Representative images showing DAPI-stained FLQ10 and FLQ65 nuclei in a comet assay. Arrow indicates comet tails observed in induced FLQ65 cells E) Normalised quantification of the percentage of DNA observed in the comet tail of FLQ10 or FLQ65 cells in a comet assay. Data are shown as mean ± SEM, $n = 5$ (CPT and vehicle), $n = 4$ (H2O2), $n = 4$ (comet assay), $p^{**} < 0.01, p^{***} < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 7. SCA7 fibroblasts show increased DNA damage: A) Representative images of vehicle or CPT treated SCA7 and control fibroblasts. ATXN7 is labelled green, γ-H2AX is labelled red, and DRAQ5 (blue) was used as a DNA stain. B) Quantification of the percentage of nuclei in the respective cell lines and treatments that contained 20 or more γ-H2AX foci. C) SCA7 fibroblasts as well as control fibroblasts were subjected to lysis and single cell electrophoresis. Individual values for each nucleus’ DNA damage, as measured by the percentage of DNA in the comet tail, are shown. n = 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
observe that after both CPT and H2O2 treatment there was in fact a trend towards an increased number of γH2AX foci in mutant ATXN7 cells, compared to control cells. Even more interestingly, a statistically significantly increase in the number of γH2AX foci could be observed in mutant ATXN7 cells even in the absence of treatment. Consistent with this, CPT treatment also resulted in a trend towards more γH2AX foci in SCA7 patient, versus control, fibroblasts. Moreover, using the comet assay, an increased level (although not significant) of DNA single and double strand breaks could also be detected upon mutant ATXN7 expression in FLQ65 cells, as well as in CPT treated SCA7 fibroblasts. Together this suggests that polyQ expanded ATXN7 expression per se could result in DNA damage. In accordance with this, increased levels of DNA damage and γH2AX foci have been reported in models and patients with other polyQ diseases (Castaldo et al., 2019; Giuliano et al., 2003; Illuzzi et al., 2009; Madabhushi et al., 2014).

Oxidative stress is the principle source of DNA lesions (Madabhushi et al., 2014). Interestingly, FUS regulates the expression of several antioxidant genes, including SOD2, catalase and peroxiredoxins (Sanchez-Ramos et al., 2011). In this study we found that the SOD2 mRNA level was decreased in mutant ATXN7 cells. Previously we have also shown that the expression of catalase is decreased and that ROS producing NADPH oxidase (NOX) enzymes are activated in the same SCA7 PC12 cell model (Ajayi et al., 2012; Ajayi et al., 2015). Mutant ATXN7 could hence induce a high level of oxidative stress, DNA damage and toxicity, not only by disrupting the anti-oxidant defence, but also by increasing the ROS production. In fact, we have previously shown that ROS levels increase before any toxicity can be detected by WST-1 or LDH leakage assays in the SCA7 PC12 model (Ajayi et al., 2012; Yu et al., 2012). It should be pointed out that these previous results, as well as most results in this study, have been acquired using a cell model and should be interpreted with some caution. However, our findings suggesting a key role of oxidative stress and DNA damage in SCA7 is supported by a recent study showing a direct association between oxidative stress and disease severity in SCA7 patients (Torres-Ramos et al., 2018). Moreover, genetic variants in DNA repair pathways was recently shown to modulate age of onset in several polyQ diseases including SCA7 (Bettencourt et al., 2016).

In conclusion, our work indicates that increased DNA damage, cytoplasmic redistribution and sequestration of FUS into ATXN7 aggregates, as well as disruption of FUS regulated transcripts may play a role in SCA7 pathology.

4. Materials and methods

4.1. Cell culture

The previously described stable inducible FLQ10 and FLQ65 rat pre-neuronal PC12 cell lines (Ajayi et al., 2012; Yu et al., 2012), were cultured at 37 °C and 5% CO2 in Dulbecco’s Modified Eagle Medium (Gibco #41966-029) supplemented with 10% Horse serum (Gibco #16050-122), 5% Tetracycline-screened FBS (Thermofisher Scientific #SH30070.03), 1% Penicillin-Streptomycin (Gibco #15140-122), 100 μg/ml Hygromycin (Life technologies #10667010), 0.1% G418 (100 mg/ml Gibco #11811031) and when desired 1 μg/ml doxycycline (Merck #D9891-5G) to inhibit ATXN7-GFP expression. Medium was exchanged every 2–3 days. SCA7 patient (GM03561) and control fibroblasts (GM0749) were purchased from Coriell institute and were cultured at 37 °C and 5% CO2 in Minimum Essential Medium (Gibco #21090-022), 20% FBS (Gibco #10270-106), 1% Penicillin-Streptomycin (Gibco #15140-122), 1% l-glutamine (Gibco #25030024), and 1% Non-essential amino acids (Gibco, #11140035). Medium was exchanged every 3–4 days and prior to trypsinization the fibroblasts were pre-incubated with 0.53 mM EDTA in Hank’s Buffered Saline Solution (Sigma).

4.2. Western blots

For western blots, FLQ10 and FLQ65 cells were seeded into 10 cm petri dishes and induced as well as non-induced cells were harvested on day 12 of induction. The cells were rinsed with PBS on ice and lysed with RIPA buffer containing protease inhibitor cocktail for 15 min before they were centrifuged at 4 °C and 20,000 × g for 10 min. The supernatant was collected and the protein concentration was determined using Bradford assay. 6 × SDS-containing loading dye was then added and the samples were boiled for 5 min before equal amounts of proteins were loaded onto 8% polyacrylamide gels for SDS-PAGE using Bio-Rad apparatus. The separated sample was transferred to a 0.2 μm nitrocellulose membrane, which was blocked using 10% dry milk in TBST (100 mM Tris-buffered saline pH 7.4, 0.1% tween-20) for 1 h. The membrane was then incubated overnight at 4 °C with the primary antibody prepared in 2% dry milk in TBST. Primary antibodies used were rabbit α-Ataxin-7 (Jonasson et al., 2002; Strom et al., 2002) (1:700), and rabbit α-FUS (Abcam #124923, 1:1000). After incubation with the primary antibodies the membrane was washed 3 times in TBST and then incubated with the secondary antibody (goat α-mouse and goat α-rabbit conjugated to HRP purchased from invitrogen) prepared in 2% dry milk in TBST for 1 h. Secondary antibody was diluted 1:50,000 and detected using the SuperSignal™ West Dura Extended Duration Substrate (Thermofisher #34075). The blots were imaged on a Chemidoc XRS+ (Bio-Rad), and analysis was performed using ImageLab software (Bio-Rad).

4.3. Filter trap assay

For filter trap assays induced and non-induced FLQ10 and FLQ65 cells were seeded into 10 cm petri dishes and harvested on day 12 of induction. After rinsing the plates with PBS the cells were lysed using RIPA buffer supplemented with protease inhibitor cocktail and centrifuged at 4 °C and 21,000 × g for 10 min. The pellet was washed twice in RIPA buffer and re-centrifuged to obtain a clean pellet. The pellet, i.e. the insoluble cell fraction, was resuspended in DNP in 1 μl and treated with DNase I for 1 h. After determination of the protein concentration, using Bradford assay, equal amounts of proteins (10–20 μg for analysis of ATXN7 and 175–400 μg for analysis FUS) were transferred to fresh tubes and SDS and DTT were added to the final concentrations of 2% and 100 mM, respectively. Following boiling for 5 min, the samples were loaded and vacuum-filtered through a 0.2 μm nitrocellulose membrane in a Bio-Rad dot blot manifold. 0.1% SDS solution was added twice to wash and the nitrocellulose membrane was then removed and immunoblotted as described above for western blot.

4.4. Nuclear and cytoplasmic fractionation

Non-induced and 12-days induced FLQ10 and FLQ65 cells seeded in 10 cm petri dishes were collected and washed with PBS. The cell pellets were then resuspended in 5 times the pellet volume of hypertonic lysis buffer (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 1 mM EDTA, 0.5 mM DTT, protease inhibitor cocktail, and 0.2 mM phenylmethylsulfonyl fluoride) and allowed to swell on ice for 15 min. The cells were pelleted again using centrifugation at 1850 g × 4 °C for 5 min and resuspended in high salt-containing buffer (20 mM HEPES, 1.5 mM MgCl2, 400 mM NaCl, 20% glycerol, 2 mM EDTA, 0.5 mM DTT, protease inhibitor cocktail, and 0.2 mM phenylmethylsulfonyl fluoride), incubated at RT for 30 min and centrifuged at 20,000 × g and 4 °C for 15 min. The supernatant was collected as the nuclear fraction. The samples were analysed using western blot as described above.
4.5. Immunofluorescence

Seven days after induction, induced and non-induced FLQ10 and FLQ65 cells were seeded into 6-well plates containing 0.5% gelatin coated #1.5 coverslips at a density of 400,000 cells per well. On day 12 they were rinsed with PBS and fixed with 4% PFA in PBS for 40 min before they were permeabilised using 0.1% Triton-x in PBS (PBST) 3 × 10 min. The fibroblasts were seeded into glass bottom petri dishes (Thermofisher Scientific #150682) at a density of 100,000 cells per dish. Four days later they were fixed and permeabilised as previously described for the PC12 cells. After permeabilisation the coverslips and dishes were blocked for 30 min in 10% PBS in PBST, after which they were incubated overnight with primary antibodies. Rabbit α-ATXN7 (Jonasson et al., 2002; Strom et al., 2002) (1:500), mouse α-FUS (Santa Cruz #4H11, 1:250-500), and mouse γ-H2AX (Abcam #ab26350, 1:100) were prepared in 1% PBS in PBST. On day two the coverslips were washed by dipping into PBST 20 times, while the dishes were washed 3 × 10 minutes in PBST, before they were incubated with the secondary antibodies for 1 h. AlexaFlour 488 α-rabbit and 568 α-mouse secondary antibodies (Thermofisher) in a 1:5000 dilution in 10% PBS in PBST were used for ATXN7 and FUS/γ-H2AX respectively. DRAQ5 was added to the secondary antibody solution in a 1:1000 ratio as a DNA stain (5 mM, Thermofisher Scientific #62251). After the secondary antibody incubation the coverslips were again washed by dipping into PBST 20 times, before they were mounted with Fluoromount-G (Southern Biotech #0100-01) on slides and sealed with nail polish. The fibroblasts were washed 3 × 10 minutes in PBST after which they were imaged in PBS. For DNA damage experiments, the cells were treated with 300 μM H2O2 for 15 min, 100 μM H2O2 (Sigma) for 15 min, or 300 nM Camptothecin (Sigma #C9911) for 24 h, prior to fixation.

4.6. Image acquisition and handling

3-channel images of immunostained FLQ10 and FLQ65 cells, as well as fibroblasts, were captured using a Zeiss axiovert 200 multipoint confocal microscope, with a 63×/1.4 oil immersion objective and Micro-Manager open source microscopy software (Edelestein et al., 2010). Brightness and contrast were automatically adjusted for all three channels using Fiji open source software (Schindelin et al., 2012). Staining of ATXN7 and FUS in FLQ65 cells induced to express ATXN7Q65-GFP was set to the same brightness and contrast settings as the corresponding channel in non-induced FLQ65 cells. This was repeated for FLQ10 cells, resulting in FLQ10 and FLQ65 cells having separate brightness and contrast settings. This was done due to the large difference in ATXN7 intensity, to be able to visualise the difference in expression between induced and non-induced cells. For co-aggregation the images were scored as follows. Five replicates of FLQ65 cells were performed, staining FUS together with ATXN7 and DRAQ5. From each replicate image set five images were chosen at random, and five aggregates were analysed from each image. Through each aggregate an intensity profile plot line was drawn using the Fiji function ‘plot profile’. If there was an intensity peak of FUS coinciding with the ATXN7 in the images were scored as follows. Five replicates of FLQ65 cells were used to create an image-handling pipeline to measure the number of γH2AX foci (Supplementary Files S1–S2). The thresholds for what was considered a high number of foci per nucleus in the PC12 cells and fibroblasts were set to reflect 2-fold or higher number than baseline.

4.7. Comet assay

PC12 FLQ10/65 cells were induced as previously described. After 12 days of induction the cells were trypsinised, rinsed and resuspended in PBS at a 2 × 10⁶ cells/mL concentration. The cell suspensions were mixed 1:10 with 37 °C 0.75% low melting point agarose and 75 μl were deposited onto a slide precoated with normal melting point agarose. The solution was spread thinly using a coverslip, and after a second coat of cell free low melting point agarose, the slides were submerged in lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM TRIS, 200 mM NaOH and 1% Triton X-100 in milli-Q water (MQ)) for 1 h. After lysing, the slides were equilibrated in alkaline electrophoresis buffer (300 mM NaOH and 1 mM EDTA in MQ) 2 × 30 min, before being subjected to 25 V and 300 mA electrophoresis for 20 min. After electrophoresis the slides were rinsed twice for 5 min in neutralisation buffer (0.4 M TRIS pH 7.5) and once in MQ water. The slides were subsequently dried in 95% ethanol for 10 min, then stored until imaging. For imaging, the slides were rehydrated in MQ, incubated with 10 μg/ml Hoechst for 15 min, rinsed quickly with MQ, then covered with a coverslip, 50–150 nuclei per slide were imaged on a Zeiss Axio Observer 7 widefield microscope using a 10× air objective, and the PC12 images were analysed using the open source software Casplab (casplab.com), while the fibroblast images were analysed using CometScore 2.0 (TriTek Corp). Cells treated with 300 μM H2O2 or 100 μM H2O2 for 15 min were used as a positive controls in the assay.

4.8. Semi quantitative RT-PCR

Non-induced and 12-day induced FLQ10 and FLQ65 cells were collected and washed with PBS. The pellets were then immediately used for RNA extraction using the RNasy Plus Mini kit (Qiagen #74134) essentially according to the manufacturer’s instructions. 2 μg of the extracted RNA was used to synthesize cDNA libraries using the ReverTaid H Minus First Strand cDNA Synthesis kit (Thermo Scientific #K1631) according to the supplier’s protocol and addition of both random hexamers and oligo (dT)₁₈ primers. The cDNA samples of induced or non-induced FLQ10 and FLQ65 were PCR amplified using QuantiTect primers (Qiagen #249900) targeting Actin, CDK6, HDAC6, SOD2 or SMYD3. PCR was prepared using Pfu or Phusion polymerase and run for 50 cycles of cycling between 94 °C, 55 °C and 72 °C. Samples were collected every 5 cycles starting from cycle 15 and mixed with 6 × loading dye (Thermofisher Scientific #R0611), after which the samples were analysed on a 2.5% agarose gel stained with Ethidium bromide using a Chemidoc XRS+ (Bio-Rad). Intensity from CDK6, HDAC6, SOD2 and SMYD3 was normalised to Actin, and induced cells were normalised to non-induced cells.

4.9. Statistical analysis

Induced cells were either normalised within each replicate to the non-induced cells, or for the comet assay in PC12 cells, all measurements were normalised against the mean of the non-induced replicates. The normalised difference between induced and non-induced FLQ10 and induced and non-induced FLQ65 cells was analysed using an unpaired parametric Student’s t-test assuming unequal variance. Non-normalised data was analysed by a one-way ANOVA followed by Tukey’s post hoc test. Graphs are presented as means with standard error of the mean as error bars and all statistical tests were performed in GraphPad Prism 8. P-values >0.05 were considered non-significant (ns), while P < 0.05 was considered *, P < 0.01 was considered **, P < 0.001 was considered ***, and P < 0.0001 was considered ****.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mcn.2020.103584.
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Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by Mag Bergvalls stiftelse (grant 2017-02212), Åhlén stiftelsen (grant mÄ6h17, mÄ2h18 and 193051) and Ljungmanska kulturfonden.

References

Ajayi, A., Xu, Y., Lindberg, S., Langel, U., 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 doi: 10.1186/1471-2202-13-86 [pii]. https://doi.org/10.1186/1471-2202-13-86

Ajayi, A., Xu, Y., Waboba-Swetlin, C., Tsurigotai, G., Karslstrom, V., Strom, A.L., 2015. Altered p53 and NOX1 activity cause bioenergetic defects in a SCA7 polyglutamine disease model. Biochim. Biophys. Acta 1847 (4–5), 418–428. https://doi.org/10.1016/j.bbapap.2014.11.003

Alves, S., Marais, T., Biefert, M.G., Furling, D., Marinello, M., El Hachimi, K., Sittler, A., Brooke, G.N., Culley, R.L., Dart, D.A., Mann, D.J., Gaughan, L., McCracken, S.R., Bettencourt, C., Hensman-Moss, D., Flower, M., Wiethoff, S., Brice, A., Goizet, C., Dhar, S.K., Zhang, J., Gal, J., Xu, Y., Miao, L., Lynn, B.C., St Clair, D.K., 2014. Fused in sarcoma is a subunit of GCN5 histone acetyltransferase-containing complexes. Hum Mol Genet 13 (12), 1257–1265.

Hel Helming, D., Hardy, S., Abou-S Seymour, G., Eberlin, A., Bowman, A.B., Carversoner, A., Devas, D., 2009. Mutations in the 3 untranslated region of FUS causing FUS overexpression are neurodegenerative disease. Annu. Rev. Pathol. https://doi.org/10.1146/annurev-pathol-050809-145938.

Lagier-Trounque, C., Polymenidou, M., Kutt, K.R., Yu, A.Q., Baughn, M., Huelga, S.C., Yeo, G.W., 2012. Divergent roles of ALS-linked proteins FUS/TLS and TDP-43 intersect in processing long pre-mRNAs. Nat Neurosci 15 (11), 1487–1497. https://doi.org/10.1038/nn.3230

Lan, X., Koutelou, E., Schibler, A.C., Chen, Y.C., Grant, P.A., Dant, S.Y., 2015. Poly(Q) expansions in ATXN7 affect solubility but not activity of the SAGA deubiquitinating module. Mol. Cell. Biol. 35 (10), 1777–1787. https://doi.org/10.1128/MCB.01545-14

Lieberman, A.P., Shakkottai, V.G., Albin, R.L., 2018. Polyglutamine repeats in neurodegenerative diseases. Annu. Rev. Pathol. https://doi.org/10.1146/annurev-pathol-051214-014849

Ling, S.C., Dastidar, S.G., Tokunaga, S., Ho, W.Y., Lim, K., Ilieva, H., Cleveland, D.W., 2013. Mutations in the 3 untranslated region of FUS causing FUS overexpression are neurodegenerative disease. Neuron 83 (2), 266–282. https://doi.org/10.1016/j.neuron.2014.06.034

Mastrocola, A.S., Kim, S.H., Trinh, A.T., Rodenkirsch, L.A., Tabbetts, R.S., 2013. The RNA-binding protein fused in sarcoma (FUS) functions downstream of poly(ADP-ribose) polymerase (PARP) in response to DNA damage. J. Biol. Chem. 288 (34), 24751–24741. https://doi.org/10.1074/jbc.M112.409974.

Mohapatra, S., Dyal纳斯, B., Weake, V.M., Liu, J., Martin-Brown, S., Flores, A., Abmayr, S. M., 2014. Loss of Drosophila Ataxin-7, a SAGA subunit, reduces H2B ubiquitination and leads to neural and retinal degeneration. Genes Dev 28 (3), 259–272. https://doi.org/10.1101/gad.214515.113

Morin, M., Visso, E., Jochira, T., Sano, S., Szabo, A., Kondo, H., Ichiki, H., Nishimoto, K., Sasaki, S., Suzuki, S.O., Iwaki, T., 2019. Expanded polyglutamine impairs normal nuclear distribution of fused in sarcoma and poly (rC)-binding protein 1 in Huntington’s disease. Neuropharmacology 39 (5), 358–367. https://doi.org/10.1016/j.neuropharm.2019.08.005

Niu et al. Cellular and Molecular Neuroscience 110 (2021) 103584
Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nature Methods 9 (7), 676–682. https://doi.org/10.1038/nmeth.2015.

Strom, A.L., Jonasson, J., Hart, P., Brannstrom, T., Forsgren, L., Holmberg, M., 2002. Cloning and expression analysis of the murine homolog of the spinocerebellar ataxia type 7 (SCA7) gene. Gene 285 (1–2), 91–99.

Torres-Ramos, Y., Montoya-Estrada, A., Cisneros, B., Tercero-Perez, K., Leon-Reyes, G., Leyva-Garcia, N., Magana, J.J., 2018. Oxidative Stress in Spinocerebellar Ataxia Type 7 Is Associated with Disease Severity. Cerebellum 17 (5), 601–609. https://doi.org/10.1007/s12311-018-0947-0.

Toyoshima, Y., Takahashi, H., 2014. TDP-43 pathology in polyglutamine diseases: with reference to amyotrophic lateral sclerosis. Neuropathology 34 (1), 77–82. https://doi.org/10.1111/neup.12053.

Wang, W.Y., Pan, L., Su, S.C., Quinn, E.J., Sasaki, M., Jimenez, J.C., Tsai, L.H., 2013. Interaction of FUS and HDAC1 regulates DNA damage response and repair in neurons. Nat Neurosci 16 (10), 1383–1391. https://doi.org/10.1038/nn.3514.

Yang, L., Gal, J., Chen, J., Zhu, H., 2014. Self-assembled FUS binds active chromatin and regulates gene transcription. Proc. Natl. Acad. Sci. U. S. A. 111 (50), 17809–17814. https://doi.org/10.1073/pnas.141604111.

Yang, H., Liu, S., He, W.T., Zhao, J., Jiang, L.L., Hu, H.Y., 2015. Aggregation of polyglutamine-expanded Ataxin 7 protein specifically sequesters ubiquitin-specific protease 22 and deteriorates its deubiquitinating function in the Spt-Ada-Gcn5-acetyltransferase (SAGA) complex. J. Biol. Chem. 290 (36), 21996–22004. https://doi.org/10.1074/jbc.M114.631663.

Yu, X., Yu, X., Ajayi, A., Boga, N.R., 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 (2), 219–233. https://doi.org/10.1007/s12031-012-9722-8.

Yu, X., Munoz-Alarcon, A., Ajayi, A., Webling, K.E., Steinhof, A., Langel, U., Strom, A.L., 2013. Inhibition of autophagy via p53-mediated disruption of ULK1 in a SCA7 polyglutamine disease model. J. Mol. Neurosci. 50 (3), 586–599. https://doi.org/10.1007/s12031-013-0012-x.

Zander, C., Takahashi, J., El Hachimi, K.H., Fujigasaki, H., Albanese, V., Lebre, A.S., 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 (22), 2569–2579.