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NSUN5 as a modifier of the stress response in the neurodegenerative disorder SCA3/MJD

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Abstract

Spinocerbellar ataxia 3 (SCA3) is a neurodegenerative disease caused by an expanded CAG repeat in the ATXN3 gene located on 14q32.1. The CAG repeat codes for a long polyglutamine sequence resulting in a unstable misfolded protein ATXN3. This misfold results in ER stress that causes eventually cell death. ER stress factors CHOP, ATF4 and XBP1 play a role in this. A Dutch family cohort of SCA3 patients revealed a missense variant of the gene NSUN5 that resulted in a later AO of SCA3 pathogenesis. NSUN5 has been revealed as potential modifier of stress that may be engaged with the AO of SCA3. This research has shown that NSUN5 both wild-type and variant decrease ER stress factor XBP1 in HEK293T cells. Co-transfected cells with overexpressed NSUN5 and ATXN3 conditions did not show change in expressed ER stress factors, however expression under treatment with tunicamycin revealed increased ER stress factor expression. Both protein and mRNA expressions are limited, which is explained by the tough cell line HEK293T.

Introduction

Spinocerebellar ataxia 3 (SCA3), also known as Machado-Joseph Disease (MJD), is a neurodegenerative disorder that results in impaired movement and coordination due to accruing atrophy in the brainstem and cerebellum (Matos et al., 2019). Although there are more types of ataxias, SCA3 is the most prevalent form known (Klockgether et al., 2019). The cause of pathogenesis is explained by an expanded cytosine-adenine-guanine (CAG) repeat within exon 10 of the ATXN3 gene, located at 14q32.1. Wild-type alleles display a repeat between 12-44 within the same exon region. SCA3's pathogenic phenotype presents in patients when the CAG repeat exceeds the length of 52, up to 87 (Kawaguchi et al., 1994; Costa and Paulson, 2012). The length of the repeat inversely correlates with the age of onset (AO) and only explains up to 50-70% of the variation in this (Leotti et al., 2021).

A Dutch family cohort of SCA3 patients was studied to identify more modifiers of SCA3. Via whole-exome sequencing Huang et al., (n.d.) discovered a missense variant in the gene NSUN5 within this cohort. Patients with this variant, NSUN5-p.G158R, showed a delayed AO of approximately 7.9 years. NSUN5 is certainly a factor that has role in the progression of SCA3. Moreover, NSUN5 has been studied that a knockdown variant shows an increased longevity and stress regulation properties. NSUN5 is a ribosomal RNA methyltransferase involved in translation of proteins. Studies in model organisms show that a reduced expression of NSUN5 increases the lifespan and stress resistance in these models. This can be explained be the absence of methylation that increases the recruitment of stress-specific mRNAs

(Schosser et al., 2015). This all combined, NSUN5 may function as a stress modifier and may cause a later AO in SCA3 patients.

The CAG repeat in ATXN3 encodes for a long polyglutamine (polyQ) sequence in ATXN3, resulting in misfolding of the protein. Wild-type ATXN3 is a deubiquitinating enzyme (DUB) and is also regulated by ubiquitination. The protein binds Ub and polyUb chains to induce the ubiquitination process that can causes proteasomal degradation or other subcellular processes. ATXN3 variant retains its DUB function, however the big polyQ in the protein is instable, which causes a misfolding of the protein. This misfold results in protein aggregates and production of toxic fragments. The misfolded proteins in particular play a significant role in the generation of ER stress in the cell.

Misfolded proteins activate the unfolded protein response (UPR), where processes aim to maintain the balance within the ER and alleviate the stress. However, when these processes cannot withhold the stress, cellular inflammation is triggered, and apoptosis can be induced. The pathways involved in the UPR are protein kinase-like endoplasmic reticulum kinase (PERK) pathway, inositol requiring enzyme (IRE)-1α pathway and the activating transcription factor (ATF)-6 pathway. Respectively, they produce ATF-4, X-box binding protein-1 (XBP1/XPB1s) and ATF-6, which lead to expression of apoptotic genes as C/EBP Homologous Protein (CHOP) and other chaperones (Maamoun et al., 2019; Hu et al., 2018).

For this research, conditions of NSUN5 and ATXN3 plasmids are transfected into HEK293T cells to investigate the affect of the conditions on the expression of ER stress factors XBP1, CHOP and ATF-4 on protein and mRNA level. Further ER stress was induced with Tunicamycin (TM) to activate UPR more.

Materials and Methods

Cell Culture and transfection

HEK293T cells were provided by Dr. Dineke Verbeek (ERIBA) in two flasks and were each propagated in DMEM with 10 % Foetal Bovine Serum and 1% Penicillin-Streptomycin solution. Confluency of cells were checked every three to five days and split in 1:10 dilution when confluency exceeded the confluency of 90%. Six well plates were prepared with approximately 100.000 cells per well for single and co-transfection of ATXN3 and NSUN5. For each plate three different conditions were used to discover the effect of overexpression of different forms of ATXN3 or NSUN5. Table 1 highlights which plasmids were used. ATXN3 plasmids were accompanied with green fluorescence protein (GFP), which enabled to confirm the presence of ATXN3 via fluorescence microscopy. NSUN5 plasmids all contained the FLAG

gene. Transfection was performed 24h post seeding. All plasmids were introduced in the host cells via polyethyleneimine (PEI) with a concentration of 1µg DNA plasmid per well. Co-transfection was performed in a similar manner, with 500ng per plasmid. After approximately 48h of incubation the cells were harvested and analysed.

Table 1: Plasmids

Experiment	Plasmid	Abbreviation
ATXN3	Short polyQ ataxin - GFP	AS
	Long polyQ ataxin (148Q) - GFP	AL
	Empty-GFP	AE
NSUN5	Wildtype NSUN5 - FLAG	NWT
	NSUN5-G158R - FLAG	NMT
	Empty-FLAG	NE

Tunicamycin treatment

Tunicamycin treatment was used to further induce ER stress. Only co-transfection cells received the tunicamycin, where they were treated with a concentration of 0.5 µM, solved in DMSO, approximately 24 hours post-transfection. The cells were then incubated overnight and harvested the following day for further analysis.

Cell lysis

Cell harvesting was performed for Western Blot analysis and qPCR analysis, where three different harvesting methods were used. To obtain protein fractions of cytoplasmic and nuclear proteins, the cells were harvested with the RIPA lysis buffer, following the Thermo Scientific™ RIPA buffer protocol. A 1% protease inhibitor was added to prevent further degradation of proteins. The protein fraction was quantified using the Pierce Detergent Compatible Bradford Assay Kit. Too diluted samples were reconcentrated by using the SpeedVac on max speed for 30 minutes to 1h until the desired concentration was reached. Protein quantification data was used to calculate the amount of protein sample to add to the Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The cells harvested for quantitative Polymerase Chain Reaction (qPCR) followed the Trizol harvesting protocol. In combination with chloroform the samples were prepared for RNA isolation. RNA isolation from the plates treated with Tunicamycin were harvested in the same manner, except that the dead cells were included and not

discarded. Cell collection was performed by centrifuging the cells in a tube containing PBS and followed by aspirating the supernatant, leaving the cells for further analysis.

Western blot

Protein expression of ATXN3-GFP, NSUN5-FLAG, CHOP and ATF-4 was detected via Western Blot. PAGE-gels were prepared for 10%, 12.5% and 15% concerning the size of the proteins of interest. The protein fraction samples obtained from ATXN3, NSUN5 and Co-transfected cells were used to prepare loading samples in 2x, 4x or 5x loading buffer and filled up with RIPA lysis buffer to the maximum volume per well. The gels each were loaded with a marker and the samples of interest. SDS-PAGE had run for approximately 1.5 hours at 100V. Each gel was transferred on PVDF membrane in transfer buffer for 1 hour at 100V.

After the transfer process, the membranes were cut in two for time optimization; both membranes could then be stained with different antibodies. Before staining the membranes were blocked in 5% milk powder solved in TBST for 1 hour at room temperature. The membranes were stained overnight in the primary antibodies against the proteins of interest. The next day, the membranes were stained for 1h with the corresponding secondary antibody. Finally, the membranes were revealed using the ECL-kit.

Images were generated by ChemiDoc Touch Imaging System (Bio-Rad) using the auto-detect chemiluminescence and colorimetric settings. When no detection occurred with auto-detection, the membrane was manually exposed for 60 seconds. Subsequently, the first membranes were stripped with the use of a mild stripping buffer, consisting of 15g Glycine, 1g SDS, 10mL Tween20 diluted in 800 mL demineralized water. Stripping was realized after two times, 10 minutes wash with mild stripping buffer. The membranes were washed and prepared with PBS and TBST respectively to be blocked again for further staining processes.

Table 2: Antibodies utilized for membrane staining

	Antibody	Concentration
Primary antibodies	Mouse Anti-Actin	1:5.000
	Rabbit Anti-CHOP	1:1.000
	Rabbit Anti-ATF4	1:1.000
	Mouse Anti-living-colours	1:5.000
	Rabbit Anti-FLAG	1:1000

Secondary antibodies	Anti-Mouse	1:1.000
	Anti-Rabbit	1:10.000

RNA isolation

Isolation of RNA was performed by the chloroform protocol. Centrifuging the samples, after shaking it vigorously the samples resulted in three layers, where the upper layer was extracted and mixed with isopropanol. After centrifuging again, the supernatant was removed, and 75% ethanol was added. When the pellet was set dry, Nuclease Free Water was used to solve the isolated RNA. Quality and quantity of the RNA was determined by using the NanoDrop in the sense of A260/A280, A260/A230 and the concentration in ng/µL. Furthermore, 1% agarose gel was run to check the stability of the RNA.

First Strand cDNA Synthesis

1 µg of RNA per sample was used, based on the concentrations determined by the Nanodrop together with 1 µL primer and completed with nuclease free water till a total volume of 12 µL was reached. Subsequently, 5X Reaction Buffer, RiboLock RNase Inhibitor (20 U/µL), 10 mM dNTP mix, and RevertAid H Minus M-MuLV Reverse Transcriptase (200 U/µL) were added to reach to a total volume of 20 µL. After 5 minutes at 25 °C followed by 60 minutes at 42 °C. The cDNA was ready for further usage or stored at 4 °C for future purposes.

qPCR

For each gene of interest, a separate Mastermix was prepared including: 2x SYBR green, cDNA and both the forward and reversed primers of the gene of interest, which in total concluded in 10 µL per well. In this experiment GAPDH and Actin were used as the housekeeping genes and the genes of interest were ATXN3, NSUN5, CHOP, and XBP1. The following qPCR run protocol was used: Hold stage (50°C for 2 min and 95°C for 10 min), PCR stage ran for 40 cycles (95°C for 15s, 60°C for 60s), melting curve stage (95°C for 15s, 60°C for 60s, 95°C for 15s). Ct values were collected, and calculations of mRNA expression was performed with the ΔΔ method where they were corrected with the housekeeping Ct values of GAPDH and Actin. Baseline comparison was done with samples with empty transfection.

Statistical analysis

Statistical analysis could not be performed due to lack of replicates per condition. This can be explained by experimental error and limited amount of time to acquire the needed data for statistical analysis. Qualitative analysis will be performed on the results instead.

Results

Fluorescent Microscopy

24h and 48h post transfection ATXN3-GFP transfected cells were checked under the fluorescence microscope, to see if green fluorescence was present. The aim of this procedure was to see if the transfection went successful.

On Figure 1, you can appreciate that ATXN3 148polyQ has the largest coverage of green, fluorescent cells compared to the empty plasmid condition and short ATXN3. Empty plasmid transfected cells show a brighter fluorescent light compared to the 148polyQ ATXN3 and short ATXN3. It is difficult to distinguish protein aggregation and location of the green. In general, the fluorescence is present throughout the whole cell, including the cytoplasm and the nucleus.

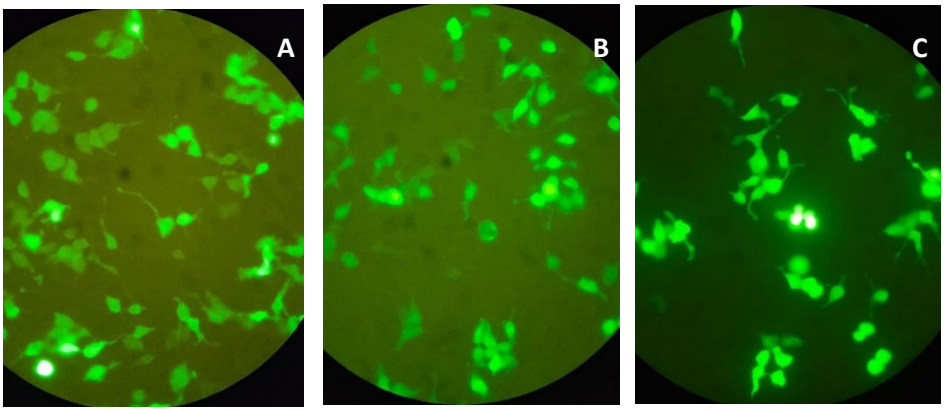

Figure 1: Fluorescence microscopy pictures of single ATXN3 transfection in HEK293T cells. A: 148polyQ ATXN3 transfected cells; B: short ATXN3 transfected cells; C: empty-GFP transfected cells.

Single Transfections

ATXN3

Western Blot

Western Blot was performed on the first set of samples on single transfected ATXN3 cells. This analysis shows three different conditions with two replicates per condition: the empty plasmid which works as the control, ATXN3 148polyQ, and short ATXN3. The goal of this experiment was to visualize CHOP protein expression, and to confirm if all ATXN3 plasmid were successfully transfected. The antibodies used for this

Western Blot are Mouse Anti-Actin, Anti-Mouse, Rabbit Anti-CHOP, Anti-Rabbit, and Mouse Anti-Living-Colours.

The housekeeping protein was equally expressed as depicted in Figure 2. ATXN3-GFP was visualized with Anti-Living-Colours to reveal the ATXN3 bands. The two empty plasmids do not contain bands, as this condition does not contain ATXN3, only GFP, which should result in a lower band on the membrane. Yet, staining for Anti-living-colours was not performed on the lower membrane. Moreover, ATXN3 148polyQ shows bands that end up higher in the membrane compared to short ATXN3, which is explained by the size of the protein. No bands for CHOP appeared in all conditions.

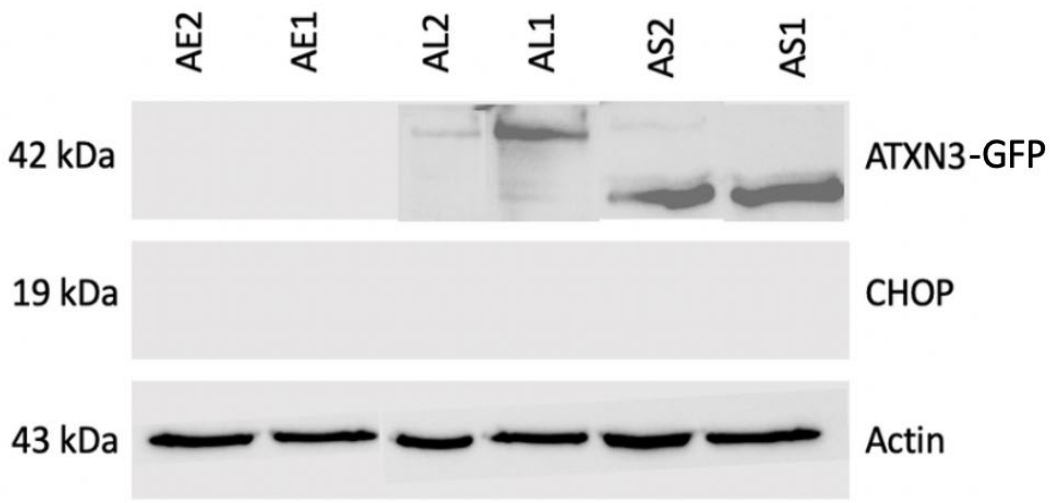

Figure 2: Western Blot results of single transfection of ATXN3 conditions. ATXN3-GFP, CHOP and housekeeping protein Actin were stained for. Conditions: AE: empty-GFP, AL: 148polyQ ATXN3-GFP, AS: short ATXN3-GFP.

qPCR

The qPCR was executed to analyse the mRNA expression of ATXN, CHOP and XBP1 was measured via qPCR for all three conditions: empty plasmid, 148polyQ ATXN3, short ATXN3. Data showed a fifteen-fold overexpression of 148polyQ ATXN3 and a twelve-fold of short ATXN3 mRNA as seen in Figure 3. The transfection with the empty plasmid yielded no extra mRNA expression, as it is not transfected with a plasmid containing ATXN3. Values of this qPCR are obtained from one set of samples, originating from one qPCR plate. The results are therefore not significant.

Subsequently, qPCR data on CHOP and XBP1 resulted in equal expression of mRNA as shown in Figure 4. Both expressions are low, which of CHOP complies with Western Blot data. There are no significant differences between these conditions as the samples here again were obtained from one set.

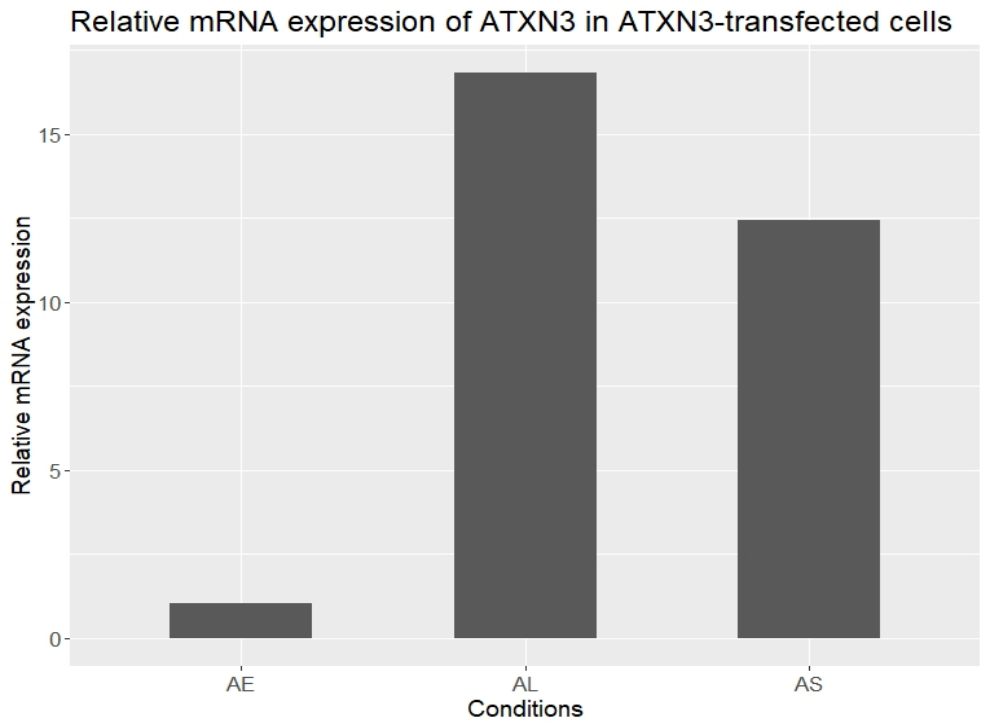

Figure 3: Results of qPCR in relative mRNA expression of single transfection of ATXN3. Gene of interest: ATXN3; Conditions: AE: empty-GFP, AL: 148polyQ ATXN3-GFP, AS: short ATXN3.

Figure 4: Results of qPCR in relative mRNA expression of CHOP and XBP1 in single transfected cells with ATXN3-GFP. Genes of interest: CHOP and XBP1; Conditions: AE: empty-GFP, AL: 148polyQ ATXN3-GFP, AS: short ATXN3.

NSUN5

Western blot

The goal of the second experiment is to visualize if the ER stress factor CHOP is present in cells that are transfected with different NSUN5 plasmids. Besides, staining on anti-FLAG was done to confirm a successful transfection of the NSUN5 plasmids. This experiment contained three different conditions with two replicates per condition: the empty plasmid which works as the control, NSUN5-p.G158R, and wildtype NSUN5.The antibodies used for this Western Blot are Mouse Anti-Actin, Anti-Mouse, Rabbit Anti-CHOP, Anti-Rabbit, Rabbit Anti-FLAG.

Figure 5 shows the expressed housekeeping protein Actin. The expression of Actin for the NMT conditions shows undulate bands. These samples were reconcentrated before loading. Still, the presence of Actin in all conditions make the results viable for further analysis. However, CHOP and NSUN5-Flag could both not be observed in all conditions.

To conclude this experiment, it cannot be said with certainty that the transfection went successful, as NSUN5-Flag has not been detected. On top of that, during this experiment the membranes were stripped, so it is possible that the proteins have been stripped as well.

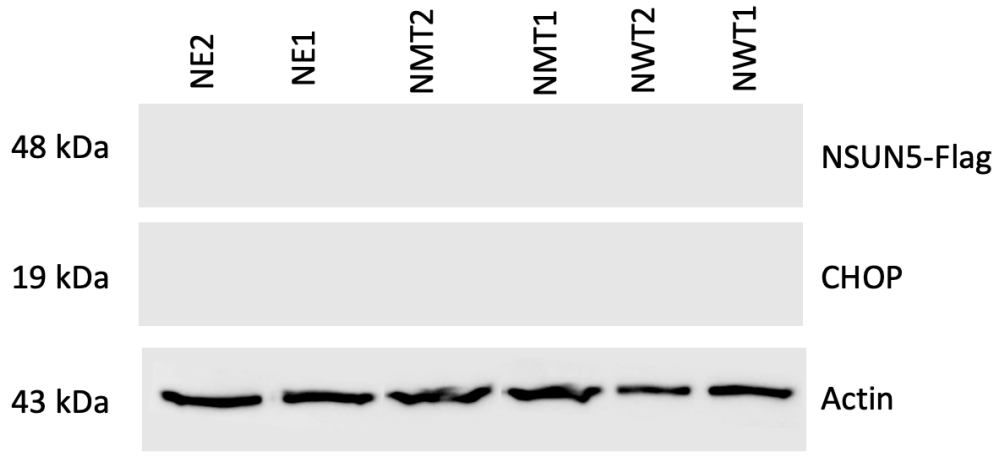

Figure 5: Western Blot results of single transfection of NSUN5. Housekeeping protein Actin together with CHOP and NSUN5-FLAG were stained for. NE: empty-FLAG, NMT: NSUN5-p.G158R-FLAG, NWT: wildtype NSUN5-FLAG

qPCR

For NSUN5 singly transfected cells also a qPCR was performed, where three conditions were used: empty plasmid, NSUN5 G158R, NSUN5 wildtype. qPCR data showed a more than ten-fold mRNA expression for the variant condition and more than twenty-fold for the wildtype condition of NSUN5. Data obtained from this qPCR originated from one sample set, which makes this data not significant.

CHOP mRNA expression was not successfully measured by the qPCR; hence no data can be shown. Subsequently, XBP1 mRNA expression was measured and yielded 0.5-fold less expression compared to the expression of empty plasmid transfected cells. For both the variant and wildtype no significant difference can be observed. Data from this experiment are not significant as it was obtained from one sample set performed from one qPCR.

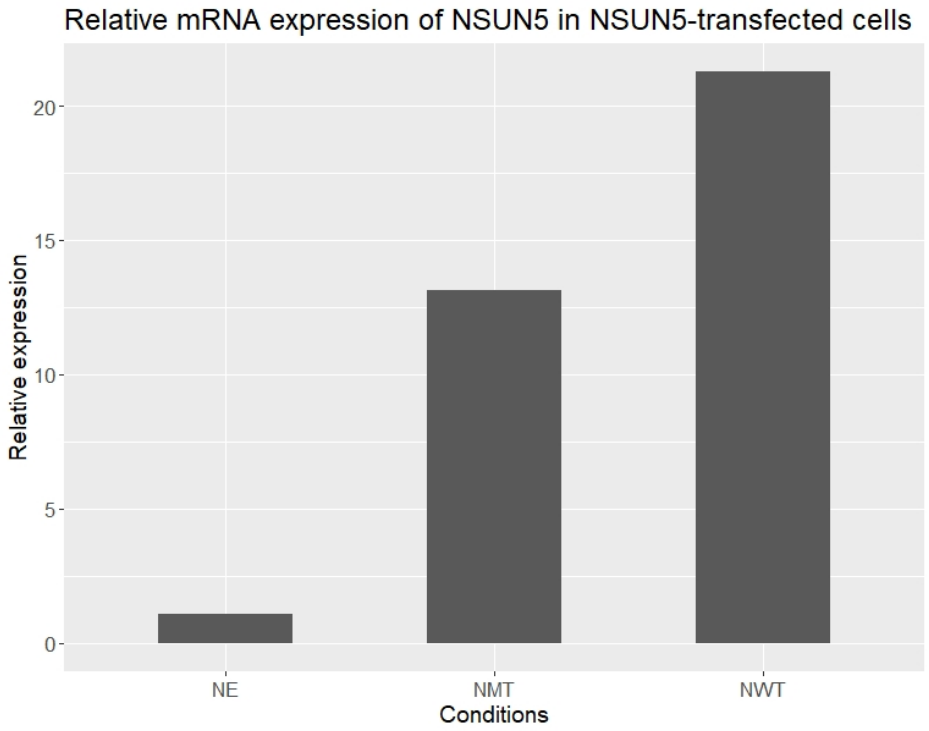

Figure 6: Results of qPCR in mRNA expression of NSUN5 in NSUN5-FLAG transfected cells. Gene of interest: NSUN5; Conditions: NE: empty-FLAG, NMT: NSUN5-p.G158R-FLAG, NWT: wildtype NSUN5-FLAG

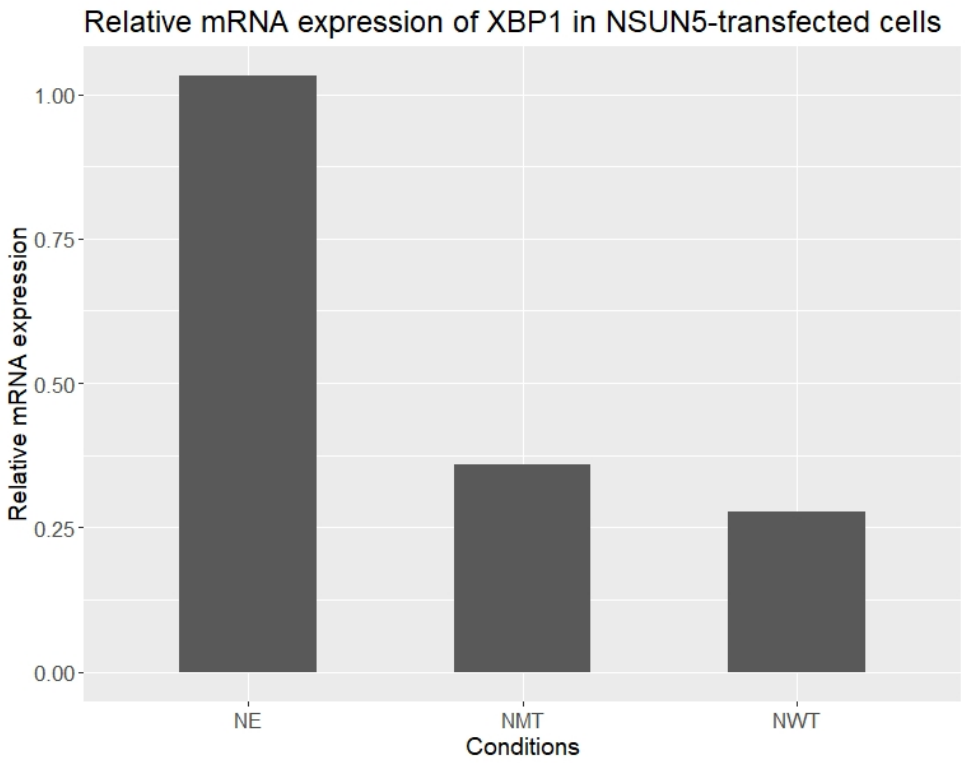

Figure 7: Results of qPCR in mRNA expression of XBP1 in NSUN5-FLAG transfected cells. Gene of interest: XBP1; Conditions: NE: empty-FLAG, NMT: NSUN5-p.G158R-FLAG, NWT: wildtype NSUN5-FLAG

Co-transfection

Western Blot analysis

The aim of this experiment was to confirm the expression of ER stress factors CHOP and ATF4 in a co-transfected cell with NSUN5 and ATXN3. The expression of CHOP has been compared with single transfected cells. This experiment contains three different conditions with two replicates per condition: an empty plasmid with wildtype NSUN5 which works as the control, ATXN3 148polyQ plus NSUN5 wildtype, and short ATXN3 with wildtype NSUN5.The antibodies used during the co-transfection are Mouse Anti-Actin, Anti-Mouse, Rabbit Anti-CHOP, Anti-Rabbit, Rabbit Anti-ATF4, Mouse Anti-Living-Colours. Furthermore, tunicamycin treatment is applied in this experiment on half of the samples (AE1, AL1, AS1).

Figure 8 shows that the housekeeping protein Actin is equally expressed. For CHOP no expression could be observed. While for ATF4 there are bands in every condition visible. The bands of ATF4 show a different intensity of expression between tunicamycin-treated samples and non-treated samples. Where the

samples treated with tunicamycin are a lot thicker and more intense compared to their replicate counterparts.

When looking at ATXN3-GFP, no expression is seen in the empty plasmid which is expected as these cells do not contain ATXN3, so at this height on the membrane, GFP was not visualized. Moreover, double and dragged bands can be observed for the AL1-NWT sample.

Summarizing, it can be said that multiple proteins were visualized from co-transfected samples. Furthermore, it can also be concluded that tunicamycin treatment did affect protein expression, in terms of increasing ATF4 expression.

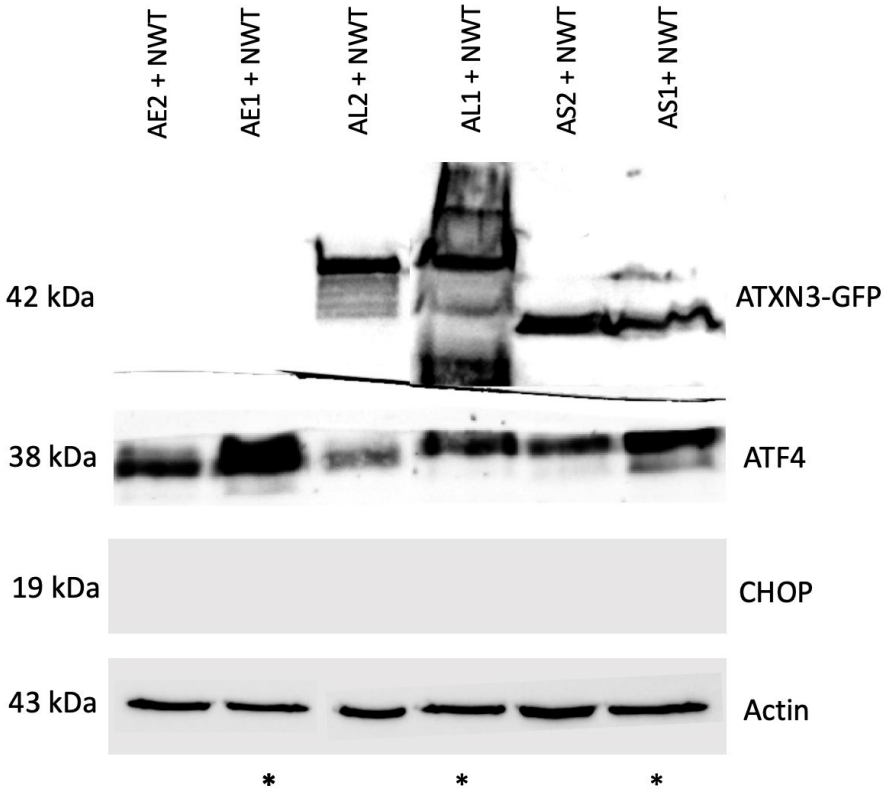

Figure 8: Western Blot results of co-transfection of ATXN3-GFP and NSUN5-FLAG. Proteins of interest are Actin (housekeeping), AFT4, CHOP and ATXN3-GFP.

qPCR

After synthesizing the isolated RNA into cDNA, qPCR was performed on cDNA of the Co-transfection samples to investigate the intensity of mRNA expression of ATXN3, NSUN5, CHOP and XBP1 and what the effect of tunicamycin on their expression is. At first, ATXN3 and NSUN5 mRNA expression were analysed

as seen in Figure 9. Conditions included in the analysis are the empty plasmid and short ATXN3 plasmid transfected samples due to experimental error occurred in the 148polyQ ATXN3.

ATXN3 mRNA expression is more prevalent in AS-NWT compared to the AE-NWT sample as shown in pink. When tunicamycin was added, it showed a tenfold increase in mRNA expression of ATXN3. Subsequently, mRNA expression in AS-NWT has doubled when treated with tunicamycin.

NSUN5 mRNA expression was expected to be equally expressed, as it is the same plasmid for each condition in the co-transfection. Figure X shows that mRNA expression of NSUN5 is not equal compared to the different conditions and compared to different treatment, regarding tunicamycin. When short ATXN3 is present and no treatment is given, it results in a slightly higher expression of NSUN5. Looking at short ATXN3 presence including the treatment, less NSUN5 is expressed compared to the empty plasmid.

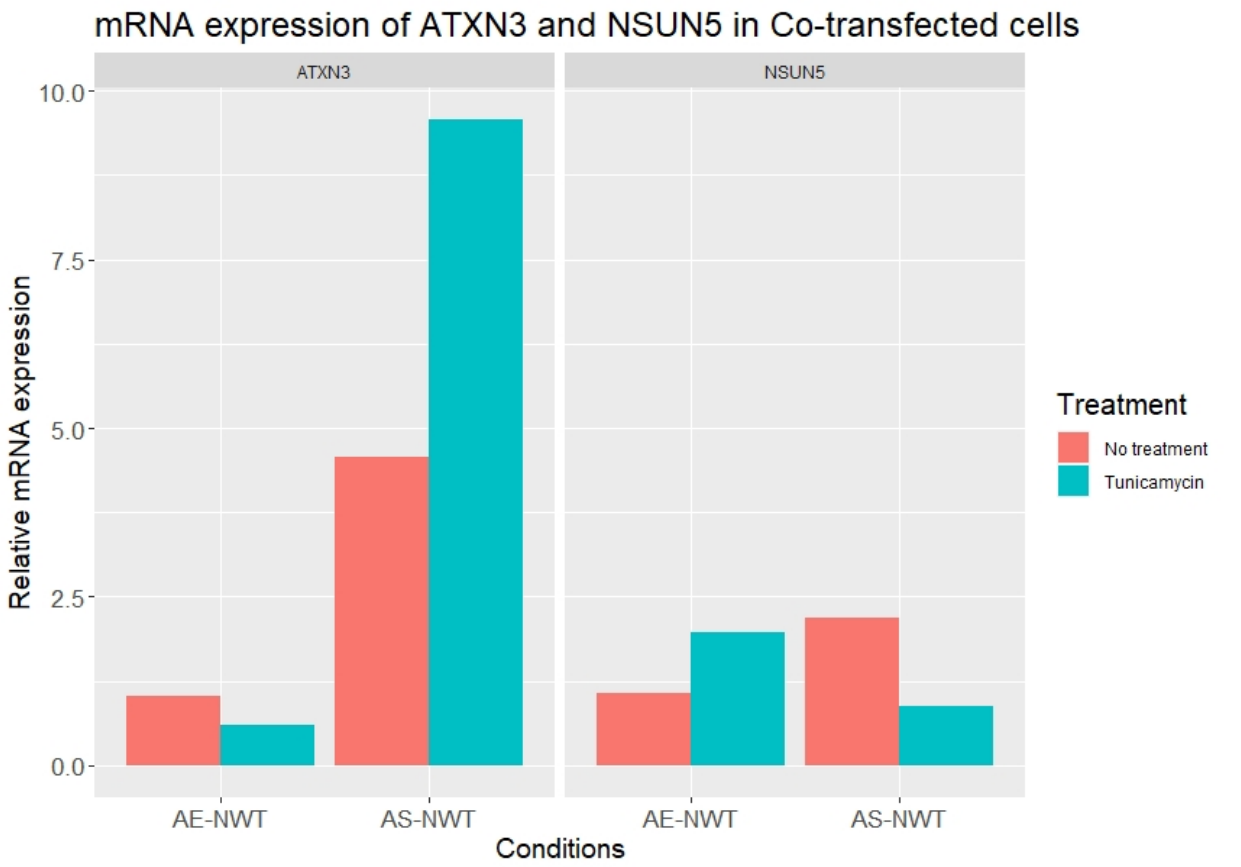

Figure 9: Results of qPCR in relative mRNA expression of NSUN5 and ATXN3 in co-transfected cells. Genes of interest: NSUN5 and ATXN3; Conditions: AE-NWT: Empty-GFP+NSUN5 wildtype-FLAG, AS-NWT: short ATXN3-GFP+NSUN5 wildtype-FLAG.

Lastly, the ER stress factors CHOP and XBP1 were investigated in the co-transfected samples to check the involvement of NSUN5 and ATXN3 in the expression of these stress factors. Basal ER stress factors are relatively low and do not differ between conditions. This accounts for both CHOP and XBP1. However, the treated samples show a fivefold increase in CHOP mRNA expression in both conditions. Looking at the expression of XBP1 a slight increase can be appreciated from the graph for the AS-NWT condition compared to the non-treated sample. AE-NWT condition sample shows an over fifteenfold increase of mRNA expression of XBP1 compared to the non-treated sample. The data is obtained from a qPCR performed on one set of samples, and therefore does not have significant results.

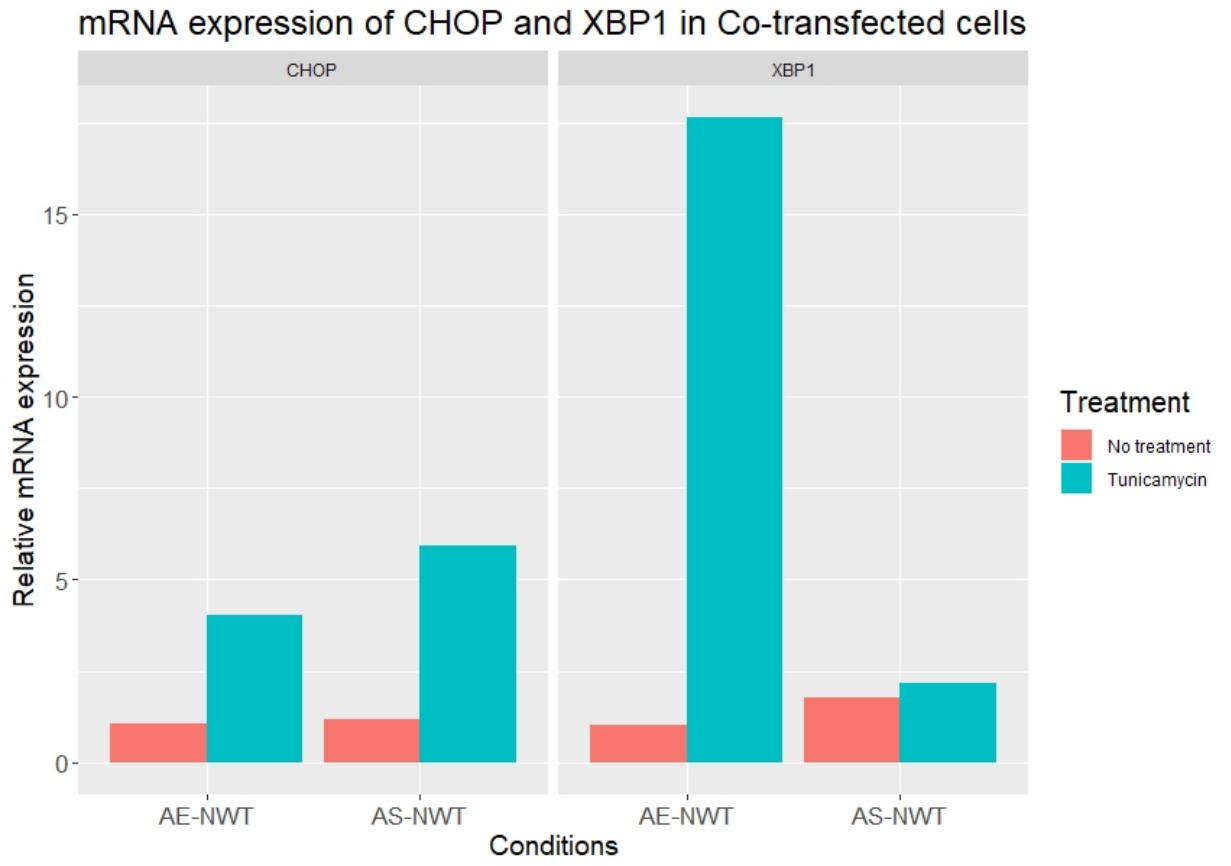

Figure 10: Results of qPCR in relative mRNA expression of CHOP and XBP1 in co-transfected cells. Genes of interest: CHOP and XBP1; Conditions: AE-NWT: Empty-GFP+NSUN5 wildtype-FLAG, AS-NWT: short ATXN3-GFP+NSUN5 wildtype-FLAG.

To sum up the results of both the Western Blot and the qPCR experiments, all ER stress factors of interest are detected. From here further conclusions can be drawn. However, results lack the number of replicates per condition for both Western Blot and qPCR. Therefore, all results shown are not significant.

Discussion

The aim of this research was to discover the effect of NSUN5 and ATXN3 on the expression of ER stress factor on both protein and mRNA levels. The hypothesis stated that NSUN5 has an inhibited function on the expression of stress factors. This research has indeed shown that the presence of overexpressed NSUN5 yielded a decrease in mRNA expression of XBP1 and protein expression of CHOP. No differences of expression in ATF4 were seen within the conditions of NSUN5 as this was only investigated within the co-transfection experiment.

For the single transfected cells, it can be concluded that ATXN3 and NSUN5 were separately transfected correctly in the cells. Green cells appeared under the fluorescence microscope confirming that GFP was present. Besides, ATXN3-GFP was clearly visualized via Western Blot in separate bands per condition and the qPCR data show an increased relative expression of mRNA of ATXN3. For NSUN5 this confirmation was only appreciated from the qPCR data, with a difference between NSUN5 variant and NSUN5 wild-type. Although, no bands on the Western Blot were visualized for NSUN5-FLAG. This can be explained by the stripping process of the membrane, which could have stripped the protein from the membrane.

Regarding the ER stress factors for the single transfections, only CHOP and XBP1 were visualized. CHOP expression was visualized via qPCR only, where the mRNA expression was poorly present. In ATXN3 transfected cells no difference of expression occurred of CHOP and XBP1. It was expected that 148polyQ ATXN3 transfected cell would display more stress, as more protein aggregates should have formed (Bichelmeier et al., 2007). NSUN5 transfected cells did show a lower mRNA expression of XBP1. This indicates that NSUN5 has indeed a role within the regulation of at least XBP1.

Co-transfection results show that ATXN3 and NSUN5 were successfully transfected into the HEK293T cells. ATXN3 expression differs logically between conditions of short and empty transfected samples but shows a higher expression of both mRNA and protein when treated with TM. expression of NSUN5 was expected to be equal in all cells, as it is the constant factor over all conditions. However, the expression of NSUN5 is different on the level of sample and condition, indicating that both TM and ATXN3 engage in the expression of NSUN5, as it decreased the expression of NSUN5. Evert et al. (2006) discovered that ATXN3 represses transcription by recruitments of histones, which can explain why there is a difference in NSUN5 expression. Using TM to induce more stress to investigate the role of NSUN5 might not be of beneficial use. These results can also indicate that the transfection of NSUN5 was not succeeded, which makes all data in this experiment not viable for any conclusions.

When looking at the ER stress factors in co-transfected samples on basal level, no big differences occurred. After treatment with TM increased mRNA levels CHOP and XBP1 were visualized, which comply with other studies (Zamarbide et al., 2013; Lei et al., 2017). XBP1 expression levels were only increased in empty-GFP-NWT samples. Since the other conditions had a transfection of ATXN3, it indicates that ATXN3 inhibits the expression of XBP1, although no studies can confirm this.

The findings from this research require more investigation. The single transfection cells were not treated with TM, making it difficult to compare the ER stress factors per condition with the co-transfection samples. Especially since NSUN5 has shown decreases in ER stress factor expression, treating with TM can show this effect in bigger perspective. Furthermore, this baseline is important, because then it can be ruled out that co-transfection may cause the equal amount of stress to the cells.

Unfortunately, no insights were revealed on 148polyQ ATXN3 transfected samples in the co-transfection experiment. As this condition is quite important for the link with pathogenesis of SCA3, more research with this transfection should be performed. Subsequently, the co-transfection experiment only showed how wild-type NSUN5 engaged in ER stress, whereas the NSUN5 variant is of more interest. Transfection with the variant might shed light upon the function to mediate stress within the human cell.

As earlier mentioned, the expression of CHOP was limited, also when treated with TM. This low concentration of CHOP can be explained by the cell origin, as HEK293T cells are quite tough. Apart from that, NSUN5 expression decreases when treated with TM, which makes TM not a proper tool to induce more ER stress factors for measurement if NSUN5 is the gene and protein of interest. Fluorizoline has found to induce the identical ER stress factors in HEK293T cells (Saura-Esteller et al., 2021), which can be used for inducing more ER stress when necessary. Instead of applying different stress inducers, this experimental setup can be used but on different cells that are less resilient against stress, resulting in more visible stress markers.

In conclusion, NSUN5 and ATXN3 have an influence on the expression on ER stress factors and TM can help with investigating this further. Still, this research was performed in a timespan of four weeks and no significant results were obtained. The conclusions present are only based in qualitative analysis, so more research must be done to fully understand the function of NSUN5 within ER stress.

References

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