The Effect of NSUN5 and ATXN3 on ER Stress Factors in Transfected HEK293T Cells

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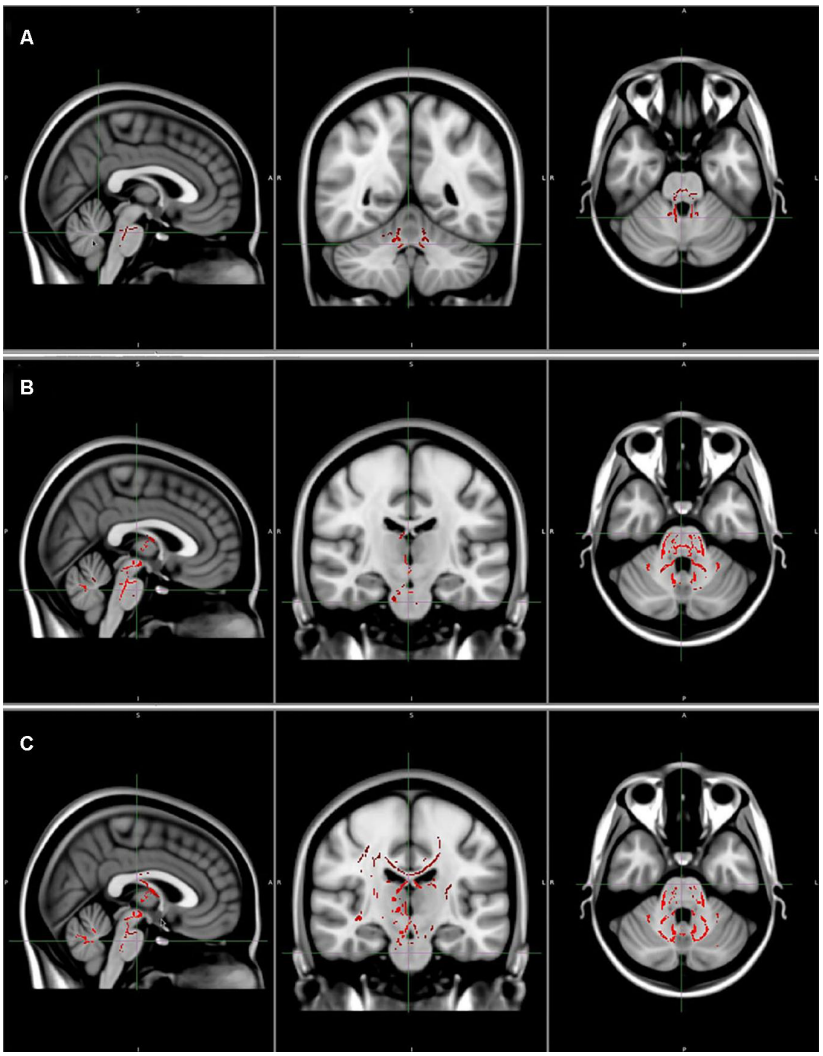

Wan et al. 2020

Abstract

Misfolded proteins can cause a lot of cell stress, by which eventually cells can go into apoptosis. This is also what happens in SCA3 patients, there is still no viable cure found for this disease. Recently, it is discovered that a variant of the NSUN5 gene might be beneficial in SCA3 patients. Therefore, the following research question has been formulated: how does overexpressed NSUN5 affect ER stress factors in ATXN 148Q transfected cells? In this research HEK293T cells are used to study the following ER factors: ATF4, CHOP, and XBP1. These experiments have been done to study these factors are mainly Western Blotting and qPCR analysis. Additionally, tunicamycin treatment is used to further induce ER stress. In the results it is seen that tunicamycin successfully increases the expression of ATF4, CHOP, and slightly that of XBP1. The results also show that NSUN5 decreases XBP1 expression, as also being one of the most important conclusions from this research. In the present study, interesting results about ER factors have come to light. Especially, suggestions for further research can certainly contribute in eventually developing a cure for SCA3 patients.

Introduction

Machado-Joseph disease or SCA3 is the most common subtype of SCA, which stands for Spinocerebellar Ataxia. SCA3 is a rather rare disease, only 3:100.000 people have the disease. SCA3 causes atrophy in the cerebellum and in the brainstem, due to an expended CAG repeat in the Ataxin-3 (ATXN3) gene. This expansion causes protein misfolding which eventually generates toxic fragments and protein aggregation. This interferes with important cellular processes, through which cell stress is induced and eventually neuronal cell death will occur[1].

Misfolded proteins cause the unfolded protein response (UPR) to become active. The UPR activates several signalling pathways, like PERK, ATF6, and IRE1-α. For example, the PERK pathway leads to the production of ATF4, ATF4 enters the nucleus and activates CHOP which subsequently induces apoptosis. On the other hand, spliced XBP-1 (sXBP-1) produces chaperones and endoplasmic reticulum associated protein degradation (ERAD) genes in order to maintain cell homeostasis[2].

Healthy individuals have 12-44 ~CAG~ repeats, compared to 52-87 repeats in people with SCA3[3]. Furthermore, SCA3 is a progressive disease, which means that it gets worse overtime,

[1] Riess, O., R©ob, U., Pastore, A., Bauer, P., & Sch©œls, L. (2008). Sca3: neurological features, pathogenesis and animal models. Cerebellum -Bruxelles-, 7(2), 125–137.

[2] Maamoun, H., Abdelsalam, S. S., Zeidan, A., Korashy, H. M., & Agouni, A. (2019). Endoplasmic Reticulum Stress: A Critical Molecular Driver of Endothelial Dysfunction and Cardiovascular Disturbances Associated with Diabetes. *International journal of molecular sciences*, *20*(7), 1658. https://doi.org/10.3390/ijms20071658

[3] Gardiner, S. L., van Belzen, M. J., Boogaard, M. W., van Roon-Mom, W., Rozing, M. P., van Hemert, A. M., Smit, J. H., Beekman, A., van Grootheest, G., Schoevers, R. A., Oude Voshaar, R. C., Comijs, H. C., Penninx, B., van der Mast, R. C., Roos, R., & Aziz, N. A. (2017). Large normal-range TBP and ATXN7 CAG repeat lengths are associated with increased lifetime risk of depression. *Translational psychiatry*, *7*(6), e1143. https://doi.org/10.1038/tp.2017.116

the age of onset is around 50 years. People with SCA3 struggle with coordination and balance as it affects the cerebellum, other difficulties could be speech, eye movement, and uncontrolled muscle contractions[1].

Recently, research has shown that a mutated form of the NSUN5 gene, that codes for a RNA methyltransferase, might be beneficial in patients with SCA3. NSUN5 is involved in stress regulation of the cell, and has shown to participate in the longevity of other organisms[4]. It is thought that NSUN5 can act as a modifier against protein aggregation and thereby delay the age of onset in SCA3 patients.

The goal of this research is to test the hypothesis that NSUN5's modifying role may act via changing mutant ATXN3's induced cellular stress response. This research is of great importance as the disease is progressive and causes very severe complaints, on top of that there is still no viable cure found. Accordingly, the research question is: can the NSUN5 variant be beneficial in reducing cell stress in patients with SCA3, and thereby delay the age of onset?

For this research HEK293T cells are used, as they grow fast and are easy to transfect. Different conditions are established using two different genes, namely ATXN3 and NSUN5. ATXN3 is used because this is the gene that is involved in SCA3. NSUN5 is used as there is this paper that shows the beneficial effects of a variant of NSUN5 to cell stress and longevity. During this research multiple factors that are produced during ER stress are studied, in order to see what happens to these factors in various conditions. Furthermore, the natural antibiotic tunicamycin is used to induce even more ER stress.

To summarize the main results of this research, it is seen that tunicamycin is very effective in producing more ER factors. On top of that, all factors of interest are detected, either via Western Blot or via qPCR analysis. It is also seen that NSUN5 decreases XBP1 expression. However, due to a limited amount of time and limited amount of replicates, no firm conclusions can be drawn.

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 % Fetal 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 ataxin3 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

[4] Schosserer, M., Minois, N., Angerer, T. B., Amring, M., Dellago, H., Harreither, E., Calle-Perez, A., Pircher, A., Gerstl, M. P., Pfeifenberger, S., Brandl, C., Sonntagbauer, M., Kriegner, A., Linder, A., Weinhäusel, A., Mohr, T., Steiger, M., Mattanovich, D., Rinnerthaler, M., … Grillari, J. (2015). Methylation of ribosomal rna by nsun5 is a conserved mechanism modulating organismal lifespan. Nature Communications, 6(1). https://doi.org/10.1038/ncomms7158

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 polyethylenimine (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-colors	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.

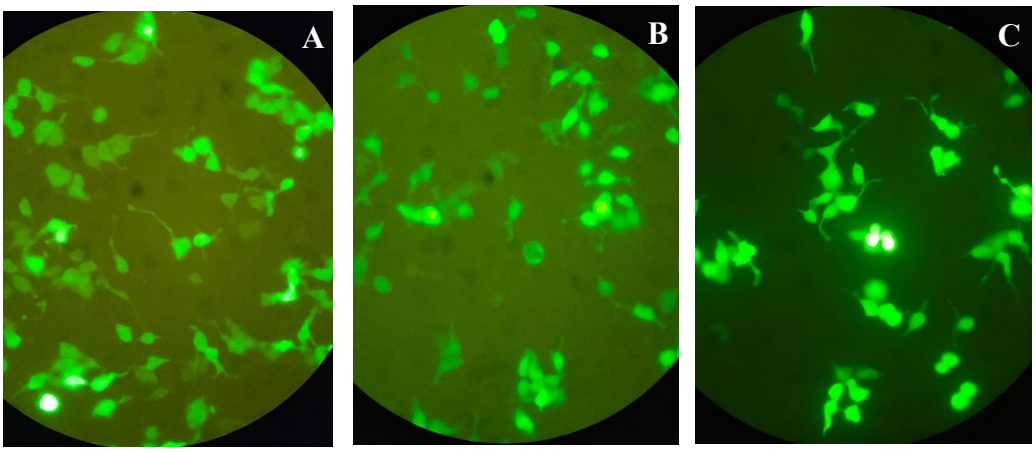

Figure 1: Fluorescence microscopy pictures of single ATXN3 transfection in HEK293T cells. A: 148polyQ ATXN3 transfected cells; B: short ATXN3 transfected cells; C: empty-GFP transfected cells.

Single Transfections

Western Blot Analysis ATXN3

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

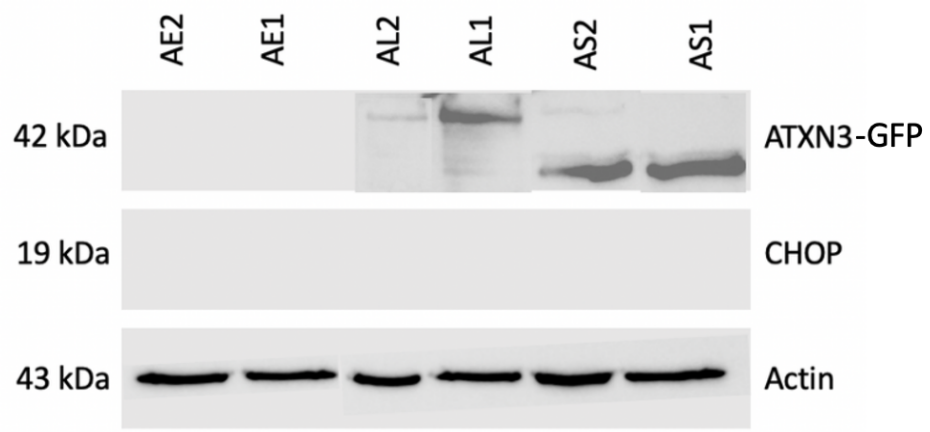

Figure 2: Western Blot results of single transfection of ATXN3 conditions. ATXN3-GFP, CHOP and housekeeping protein Actin were stained for. Conditions: AE: empty-GFP, AL: 148polyQ ATXN3-GFP, AS: short ATXN3-GFP.

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.

qPCR Analysis ATXN3

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 X. 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 X. 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.

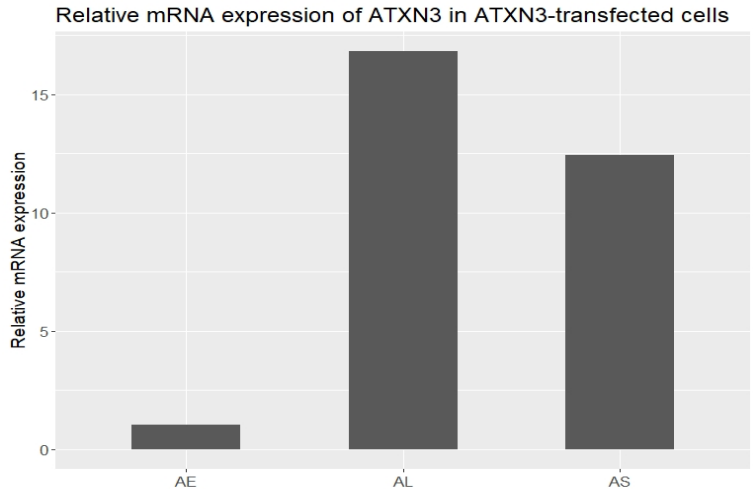

Figure 3: Results of qPCR in relative mRNA expression of single transfection of ATXN3. Gene of interest: ATXN3; Conditions: AE: empty-GFP, AL: 148polyQ ATXN3-GFP, AS: short ATXN3.

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.

Western Blot Analysis NSUN5

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 X 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.

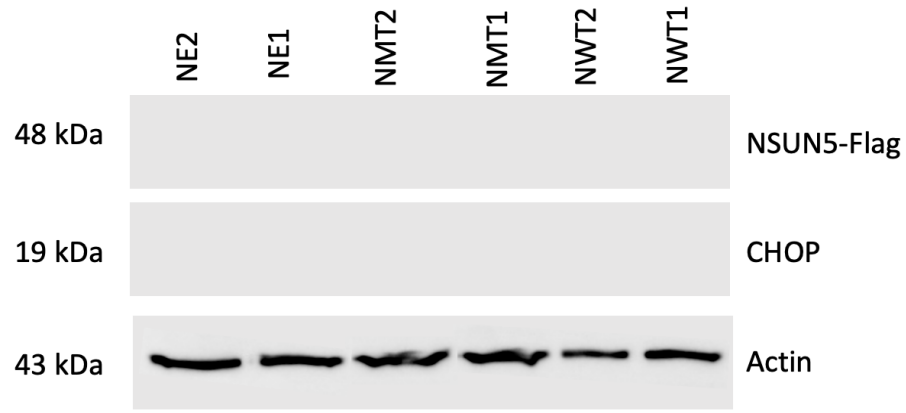

Figure 5: Western Blot results of single transfection of NSUN5. Housekeeping protein Actin together with CHOP and NSUN5-FLAG were stained for. NE: empty-FLAG, NMT: NSUN5-p.G158R-FLAG, NWT: wildtype NSUN5-FLAG

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.

qPCR Analysis NSUN5

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 tenfold 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.

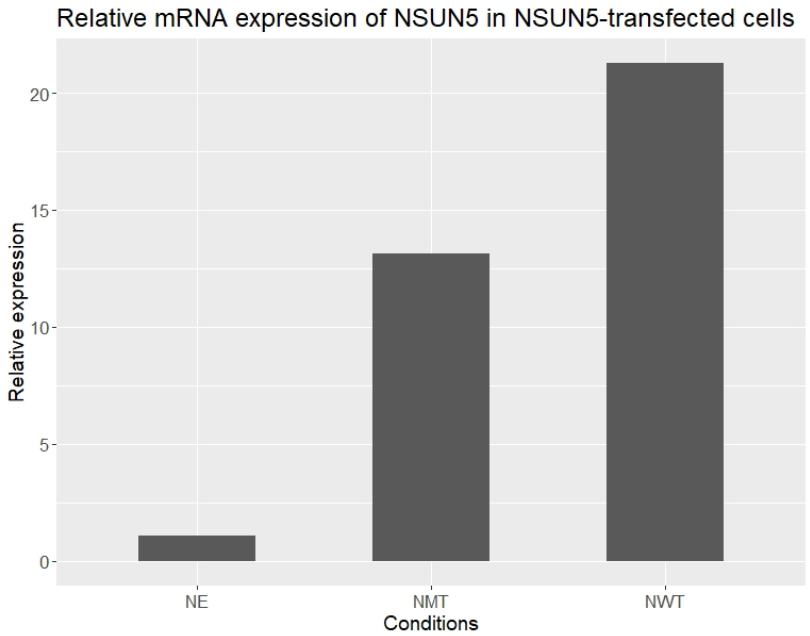

Figure 7: Results of qPCR in mRNA expression of NSUN5 in NSUN5-FLAG transfected cells. Gene of interest: NSUN5; Conditions: NE: empty-FLAG, NMT: NSUN5-p.G158R-FLAG, NWT: wildtype NSUN5-FLAG

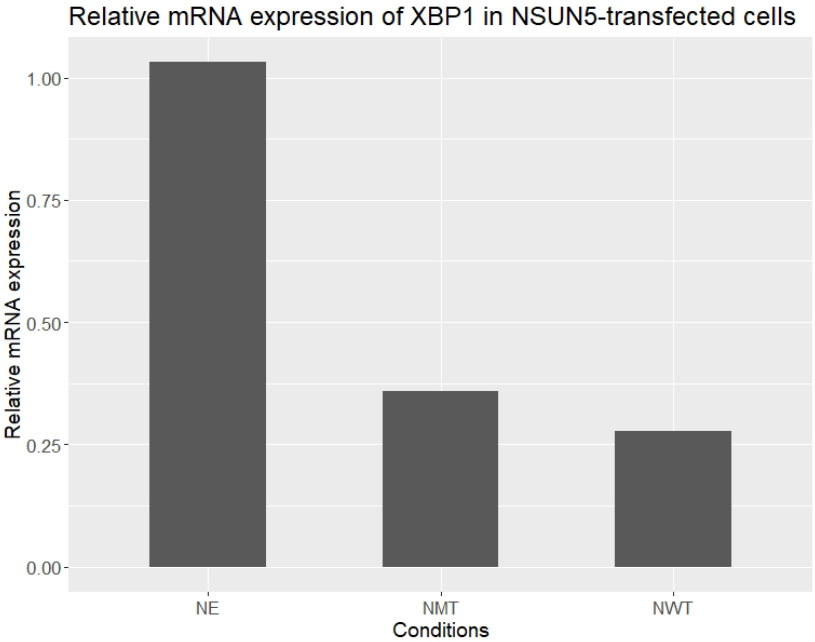

Figure 6: Results of qPCR in mRNA expression of XBP1 in NSUN5-FLAG transfected cells. Gene of interest: XBP1; Conditions: NE: empty-FLAG, NMT: NSUN5-p.G158R-FLAG, NWT: wildtype NSUN5-FLAG

Western Blot Analysis Co-Transfection

The aim of this experiment was to confirm the expression of ER stress factors CHOP and ATF4in 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-Colors. Furthermore, tunicamycin treatment is applied in this experiment on half of the samples (AE1, AL1, AS1).

Figure X 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.

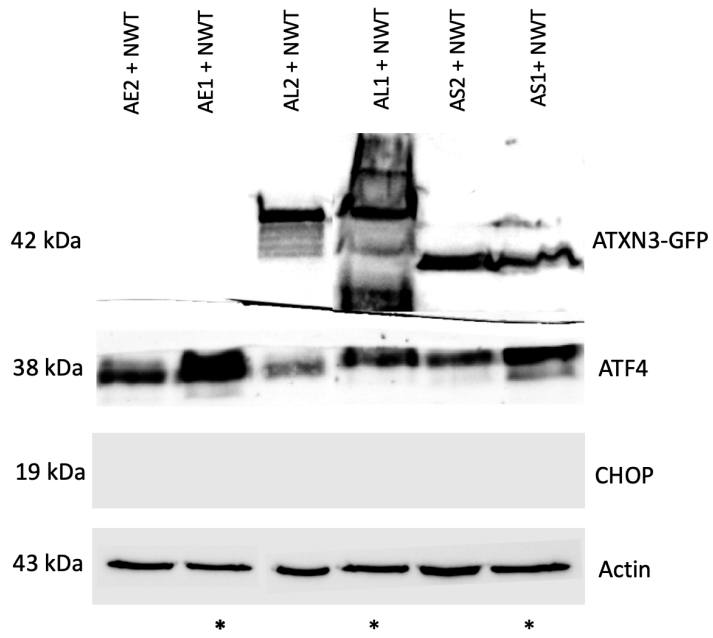

Figure 8: Western Blot results of co-transfection of ATXN3-GFP and NSUN5-FLAG. Proteins of interest are Actin (housekeeping), AFT4, CHOP and ATXN3-GFP.

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.

qPCR Analysis Co-Transfection

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 X. 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 almost 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.

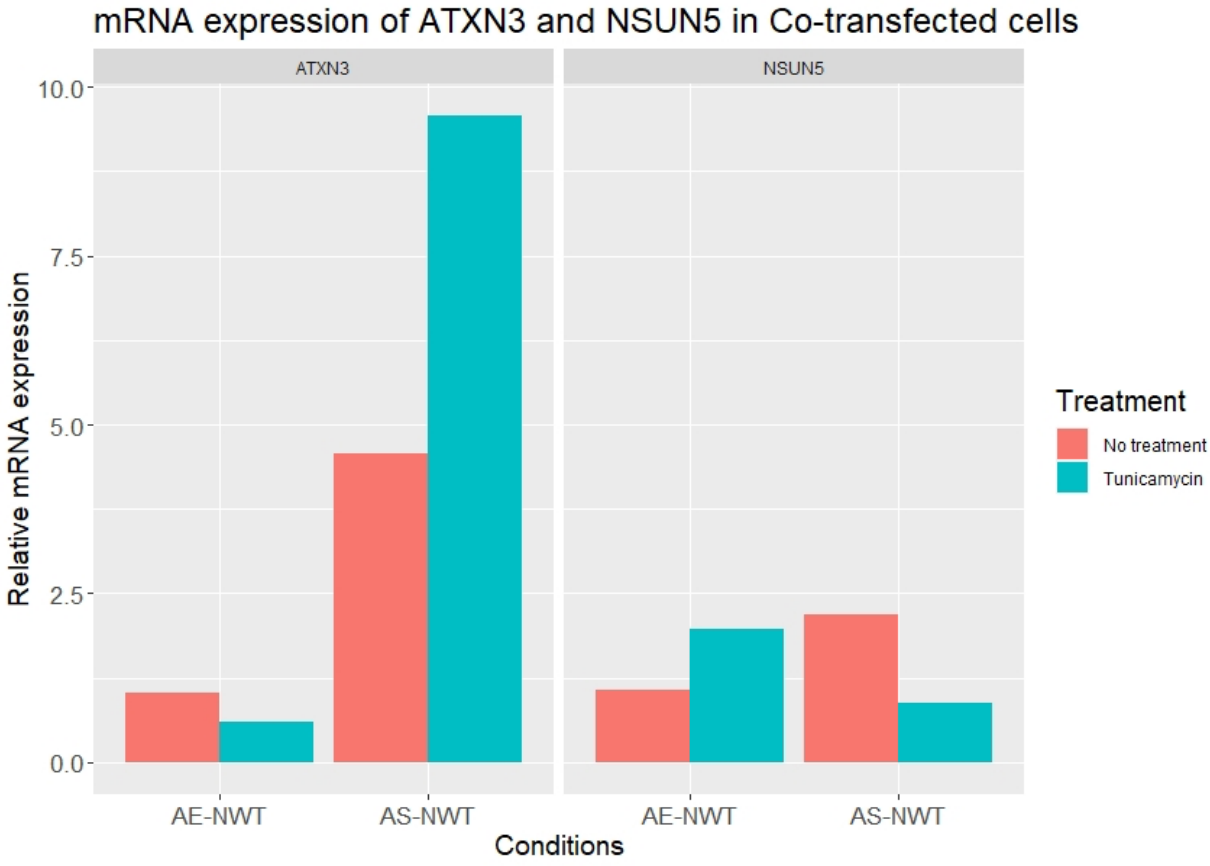

Figure 9: Results of qPCR in relative mRNA expression of NSUN5 and ATXN3 in co-transfected cells. Genes of interest: NSUN5 and ATXN3; Conditions: AE-NWT: Empty-GFP+NSUN5 wildtype-FLAG, AS-NWT: short ATXN3-GFP+NSUN5 wildtype-FLAG.

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.

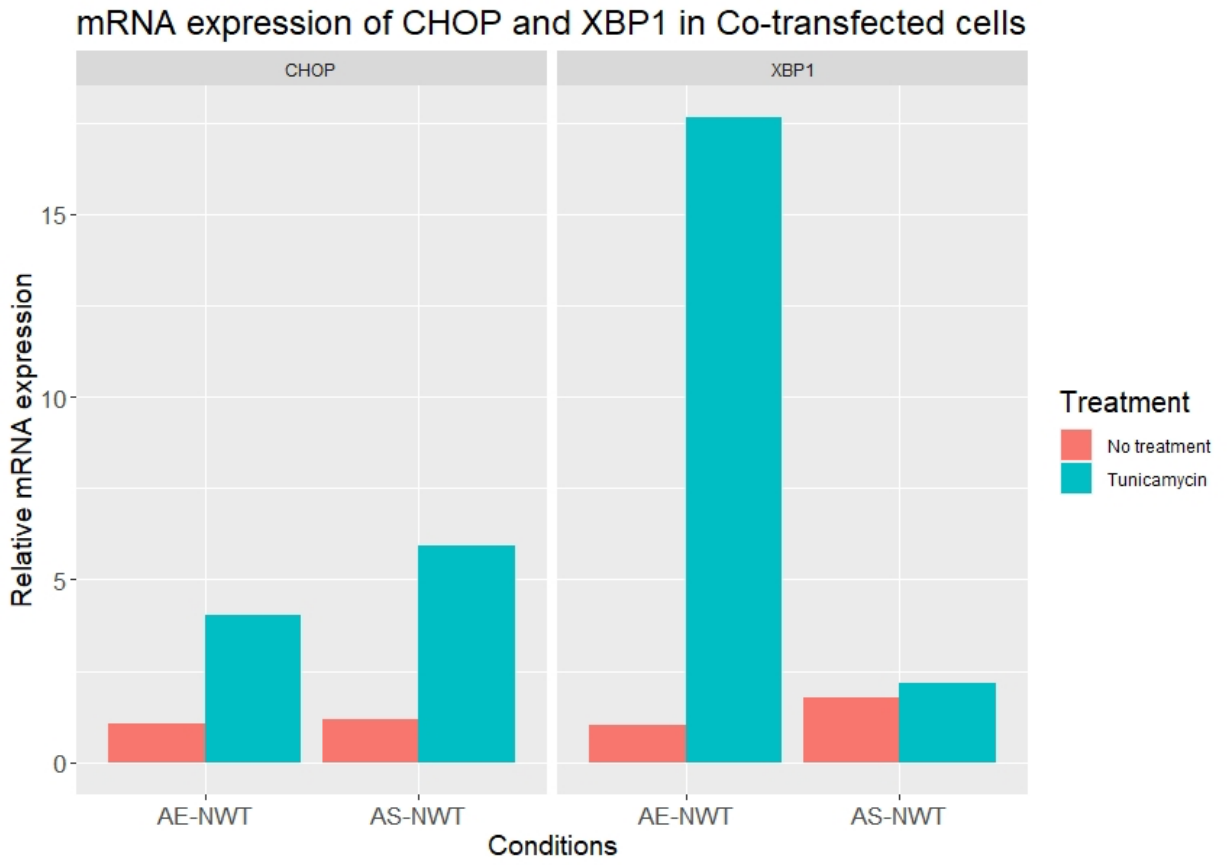

Figure 10: Results of qPCR in relative mRNA expression of CHOP and XBP1 in co-transfected cells. Genes of interest: CHOP and XBP1; Conditions: AE-NWT: Empty-GFP+NSUN5 wildtype-FLAG, AS-NWT: short ATXN3-GFP+NSUN5 wildtype-FLAG.

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.

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 amount of replicates per condition for both Western Blot and qPCR. Therefore all results shown are not significant.

Discussion

The goal of this research was to determine if NSUN5-p.G158R might be beneficial in delaying the age of onset in SCA3 patients, by reducing ER stress factors. Therefore, the main question raised: how does overexpressed NSUN5 affect ER stress factors in ATXN 148Q transfected cells. The approach of this research was mainly done by performing multiple Western Blots and qPCRs. To discover how ER stress factors in ATXN3 and NSUN5 samples differ. Our results indicate that NSUN5 might lower XBP1 expression and also that tunicamycin treatment is an effective tool in inducing more ER stress.

A key finding is seen when looking at the qPCR data of the single transfections. This analysis shows that NSUN5 reduces XBP1 concentrations. This somewhat contradict the claims of Schosserer (2015)[5], in this particular article it is stated that reduced NSUN5 expression can increase the lifespan and stress resistance in various organisms. This is somewhat contrary with results found in this research, as both NSUN5 wildtype and the NSUN5 variant decrease XBP1 expression. Whilst XBP1 can be very beneficial for the cell, as it induces chaperons, foldases, and endoplasmic reticulum-associated protein degradation (ERAD) genes[6]. Though, it has to be taken into account that the results of this research are not significant, for this reason no firm conclusions can be drawn. Another key finding is that in the co-transfected cells CHOP increases after overnight tunicamycin treatment and also XBP1 increases slightly after treatment. This data shows that tunicamycin treatment is effective in increasing the expression of ER factors.

Looking at unexpected findings, CHOP is very hard to detect both during Western Blot and qPCR. This might be due to a too short tunicamycin treatment, the treatment was done for approximately 18 h, so it could be that CHOP in HEK293T cells needs a longer treatment. In various studies researchers have tried different treatment durations[7], to discover what is optimal for CHOP. Moreover, it could also be that there are too low tunicamycin concentration used. Since data shows a difference between CHOP expression before and after treatment, it can be concluded that tunicamycin also affects CHOP. In the future, it can be tested what happens to CHOP expression when a longer incubation of tunicamycin is used or higher tunicamycin concentrations. It could also be that HEK293T cells are very resistant against stress, since they are cancer cells. Therefore it would be interesting to test CHOP expression on another cell line.

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[5] Schosserer, M., Minois, N., Angerer, T. B., Amring, M., Dellago, H., Harreither, E., Calle-Perez, A., Pircher, A., Gerstl, M. P., Pfeifenberger, S., Brandl, C., Sonntagbauer, M., Kriegner, A., Linder, A., Weinhäusel, A., Mohr, T., Steiger, M., Mattanovich, D., Rinnerthaler, M., … Grillari, J. (2015). Methylation of ribosomal rna by nsun5 is a conserved mechanism modulating organismal lifespan. Nature Communications, 6(1). https://doi.org/10.1038/ncomms7158

[6] Maamoun, H., Abdelsalam, S. S., Zeidan, A., Korashy, H. M., & Agouni, A. (2019). Endoplasmic Reticulum Stress: A Critical Molecular Driver of Endothelial Dysfunction and Cardiovascular Disturbances Associated with Diabetes. *International journal of molecular sciences*, *20*(7), 1658. https://doi.org/10.3390/ijms20071658

[7] Zinszner, H., Kuroda, M., Wang, X., Batchvarova, N., Lightfoot, R. T., Remotti, H., Stevens, J. L., & Ron, D. (1998). CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes & development*, *12*(7), 982–995. https://doi.org/10.1101/gad.12.7.982

Another unexpected finding is that tunicamycin treatment lowers NSUN5 expression. This could mean that NSUN5 might not be a very good stress modulator. When NSUN5 gets downregulated after inducing a lot of stress, it might be not that effective to fight cell stress.

The last unexpected finding regards co-transfected cells, there is less XBP1 seen in short ATXN3 compared to the empty plasmid. This could be explained by the splicing process of XBP1. XBP-1 gets spliced in the cytosol, sXBP1 can enter the nucleus to activate chaperones, foldases, and ERAD genes. Hence, it might be that ATXN3 short polyQ interferes with this splicing process or with XBP1 production.

The reliability of this data is among other things impacted by, the amount of replicates per sample. For example, the qPCR data is mostly collected from only one qPCR and from three samples. Therefore, no firm conclusions can be drawn from this. The generalizability of the results is also limited by some experimental errors that occurred and due to the lack of time. However, a major strength of this study is that all stress factors that were aimed to look at, are found. Despite various obstacles and troubleshooting, interesting results came too light. For example the fact that in this study NSUN5 reduces XBP-1 expression.

Further research is needed to establish a more thorough understanding about the functioning of NSUN5-p.G158R, in order to obtain significant results whereafter quantitative research can be done. Furthermore, tunicamycin treatment remains very interesting in the scope of this field. In this research tunicamycin is only used on co-transfected cells, in the future it would also be very interesting to see what the effect of tunicamycin is on cells that are only transfected with ATXN3 or NSUN5. So that a better comparison can be made of what tunicamycin does in different conditions of ATXN3 and NSUN5.

Another aspect to look further into is tunicamycin induced CHOP expression. In this study CHOP has not been detected on the Western Blot, so in the future it would be interesting to see if CHOP expression increases after a longer tunicamycin treatment and/or a higher tunicamycin concentration. Also, due to a lack of data in the 148polyQ ATXN3 samples, more experiments needs to be done on this condition. This condition is very interesting as it mimics the disease of SCA3.

Further, when looking at the co-transfected cells it could be that NSUN5 transfection was not successful. Since the relative mRNA expression of short ATXN3 is lower than its control. Additionally, in this research only wildtype NSUN5 is used during co-transfection, whilst the variant of NSUN5 is especially very interesting to look into. As this NSUN5 variant is said to be beneficial by Schosserer (2015§)[8] in reducing cell stress, and therefore be beneficial for SCA3 patients.

[8] Schosserer, M., Minois, N., Angerer, T. B., Amring, M., Dellago, H., Harreither, E., Calle-Perez, A., Pircher, A., Gerstl, M. P., Pfeifenberger, S., Brandl, C., Sonntagbauer, M., Kriegner, A., Linder, A., Weinhäusel, A., Mohr, T., Steiger, M., Mattanovich, D., Rinnerthaler, M., … Grillari, J. (2015). Methylation of ribosomal rna by nsun5 is a conserved mechanism modulating organismal lifespan. Nature Communications, 6(1). https://doi.org/10.1038/ncomms7158

Something that laid out of the scope of this research, but still might be very interesting would be making a plasmid which contains both ATXN3 and NSUN5. With the intention to induce less stress to the cell during transfection, aiming on having less interfering factors.

To conclude, when in the future these recommendations are brought to practice research will come a lot closer to finding a viable cure for SCA3 patients. Especially by looking into 148polyQ ATXN3 and co-transfecting with NSUN5-G158R.

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