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Introduction of C-terminus Fused in Sarcoma mutation into the Promega Regulated Mammalian Expression System

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disease primarily affecting motor neurons. There is no cure, with death typically occurring within three years of symptom onset. Dozens of genes have been implicated in ALS, including Fused in Sarcoma (FUS), a little-studied RNA/DNA binding protein. FUS mutations have been documented in both late-onset and juvenile-onset ALS, with mutations found in the C-terminus being associated with aggressive juvenile-onset. Specifically, the 1554_1557del mutation in the C-terminus of FUS has been observed in cases of unusually early onset of ALS, which is characterized by an extremely aggressive progression. The 1554_1557del mutation was introduced into a bacterial plasmid via site-directed mutagenesis and transformed into *E. coli*. Bacterial colonies were cultured and lysed whereupon the mutated plasmids were extracted, purified, and submitted for sequencing. Confirmed mutations were subcloned into the Promega Regulated Mammalian Expression System. However, sanger sequencing was inconclusive. Additionally, HEK-293 cells were cultured, and method development for transfection with the Promega System was begun. Future research should focus on analyzing changes in cellular structure, function, and viability due to the introduction of the 1554_57 mutation to the cell line. The collection of cytoplasmic and nuclear RNA and protein fractions from the cells will allow for analysis of changes in RNA and protein expression on a compartmental level. Once analyzed, the data will allow for greater understanding of how the cellular functions of transcription and translation are affected by the 1554_1557del mutation, possibly providing insight into the mechanism of neuronal death and loss of function. Furthermore, this understanding could potentially provide greater insight into how other C-terminus mutations in FUS can drive neurodegeneration in ALS.

1. Introduction

Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disease targeting both upper and lower motor neurons. This neurodegeneration results in cell death eventually leads to respiratory failure due to the death of neurons innervating the lungs. Typically, juvenile onset ALS shows symptom onset between an individual's 20s and 30s and results in a slower progression of the disease and longer life expectancy post-diagnosis. However, late onset ALS shows symptom onset between 50s and 60s and results in aggressive disease progression. The average lifespan of an individual with ALS is between 2-3 years post symptom onset. Males are at a higher risk than females for developing ALS, and at the age of 85, individuals have a 1 in 300 chance of developing ALS.[1] Both genetic and environmental factors have been indicated in ALS incidence.[1-2] Those serving in the US military, athletes with low BMI, and those who have suffered trauma and head injuries are all at a higher risk of developing symptoms.[1] The risk factors correlated with military service and athletics could correspond to the physical trauma incurred from both lifestyles, and this could suggest that such traumas could trigger the onset of ALS. Other environmental factors implicated in ALS-onset include smoking, electromagnetic radiation, as well as heavy metal and pesticide exposure.[1-2]

The genes indicated in ALS have also been indicated in other neurodegenerative diseases, specifically Parkinson's, Multiple Sclerosis (MS), Frontotemporal Dementia (FTD), and Schizophrenia. Families with higher prevalence of

ALS also show higher cases of MS and Schizophrenia, suggesting there may be a heritable link between the diseases.[1] 30-50% of ALS sufferers also show neurocognitive symptoms in the frontotemporal circuits bringing into question whether FTD and ALS are two separate diseases or rather a spectrum of the same disease.[1-2] Additionally, harboring a gene that is an ALS disease variant does not guarantee that an individual will develop ALS.[1] This could provide evidence that there are environmental factors triggering disease variant genes.

There are two main categories of ALS: familial ALS (fALS), which is responsible for 10-20% of cases, and sporadic ALS (sALS), which has associated genetic factors but no indication of heritability.[3] There are over twenty-five different genes associated with ALS, including *TARDP* and *FUS*, which are linked to both fALS and sALS.[1] Transactive response DNA Binding Protein 43 (TDP-43, the protein transcribed from *TARDP*) is responsible for regulating RNA expression and has been indicated in both ALS and FTD, with ubiquitinated inclusions found in 90% of ALS cases.[1,4] Mutated TDP-43 becomes phosphorylated and therefore deactivated and insoluble in aqueous solution.[2,4] This allows for the protein to form aggregates in the cytoplasm.[4] Aggregate formation (though not specifically phosphorylation) has also been shown with Fused in Sarcoma (FUS) mutations.[2-3]

Aggregate formation appears across neurodegenerative diseases.[5] However, the composition of the aggregates and the symptoms that develop from cellular dysfunction vary between diseases. Aggregates consist of prion-like proteins (such as TDP-43, and FUS) that congregate in the cytoplasm and can infiltrate neighboring cells and tissues, spreading the disease.[4-5] The proteins indicated in ALS take on a pathogenic nature when a mutation-driven or chance misfolding causes the alpha-helical secondary structure of the protein to undergo a conformational shift into beta-sheets. These beta-sheets have been shown to be both heat and protease resistant. Normally, TDP-43, FUS, and other prion-like proteins form membraneless organelles or "neighborhoods" held together by liquid-liquid phase separation. These neighborhoods can be disassembled, and their proper function is heavily dependent on the proper equilibrium of temperature, pH, salt concentration, and protein concentration. If these equilibriums are disturbed, aggregate formation can occur.[4] Additionally, mutations causing misfolding or changes in post-translational modifications like methylation or phosphorylation can change the liquid-liquid transition into a liquid-solid transition, leading to aggregate formation.[4,6]

In 2009, researchers linked the RNA binding protein FUS to ALS. FUS-ALS exhibits two distinct phenotypes: aggressive juvenile onset and a less progressive late onset phenotype.[2] FUS-ALS seems to cause primarily distal axon degeneration. Distal axons in affected neurons are shown to contain fewer and smaller mitochondria as well as have less lysosomes and a loss of membrane potential. This leads to loss of skeletal muscle innervation.[7] FUS-ALS is observed in 5% of fALS cases and 1% of sALS cases. Mutations in the C-terminal end of FUS are found in fALS; whereas, mutations in the N-terminal of FUS are found in sALS.[8] Acting to regulate transcription as well as transport and maintain RNA, FUS binds to RNA and is involved in alternative splicing.[8-9] FUS functions within the nucleus of the cell, and mutations in the C-terminal end of the protein appear to block the nuclear transport signal of the protein.[4,6,7,9] The mislocalization of FUS to the cytoplasm correlates with increased aggregation and gain of toxicity, with aggregation appearing to increase with neuron age.[4,6] Additionally, it has been found that DNA-dependent protein kinase (DNA-PK) facilitates the nuclear export of FUS.[7] Thus, it is possible that mutations in FUS could increase the probability of a binding event between DNA-PK and FUS and thus increase nuclear export.

Though attention has been focused on aggregate formation, some researchers wonder if the absence of FUS in the nucleus is responsible for disease pathology. FUS self regulates its own transcription. When there is sufficient protein in the cell, FUS signals for exon 7 to be skipped during alternative splicing. This leads to an early stop codon, and the protein is degraded by nonsense-mediated decay (NMD). However, delocalization of FUS to the cytoplasm renders the protein unable to auto-regulate transcription, which results in excess transcription of FUS.[9] If nuclear-cytoplasmic transport can be rescued within a diseased cell, it could lead to treatment options for ALS.

FUS has been found to colocalize with a number of proteins including TDP-43, Poly [ADP-ribose] polymerase 1(PARP-1), and Ataxin-2. TDP-43 and FUS create colocalized neighborhoods; however, TDP-43 pathology is not observed in FUS-ALS.[2,8] Ataxin-2 interacts with both FUS and TDP-43 and is involved in the polyadenylation of mRNA, miRNA synthesis, and formation of stress granules. Ataxin-2 is found with FUS in FUS-ALS; however, the interactions are not yet clear.[2] PARP-1 allows FUS to undergo liquid-liquid transition in order to form functional neighborhoods.[7] Understanding how these protein-protein interactions shift upon FUS mutation and/or misfolding may shed light on the mechanisms underlying cell toxicity in FUS-ALS.

Furthermore, FUS aids PARP-1 in the recruitment of the XRCC1/DNAligIII to perform nick ligation at DNA sites damaged by oxidation, with FUS directly activating the complex.[7,10] When in the presence of mutated FUS, DNA shows increased single strand breaks due to reactive oxygen species.[10] This could be due to a failure of FUS to properly interact with PARP-1. Additionally, FUS has been found to be recruited by RNA stress granules when a cell is under oxidative stress.[4] Stress granules are membraneless compartments, and mutated FUS has been found to interact with

these granules and change their dynamics.[6] This could further indicate that oxidative stressors (heavy metals, electromagnetic radiation, and pesticides) could be environmental "triggers" that lead to symptom onset in ALS.

The cellular and molecular mechanisms of neuron death in ALS have been investigated through various models, including both wild-type and mutant mouse models as well as the overexpression of gene mutations in motor neuron-like cell lines.[11]

\ The goal of the Kaur Laboratory is to study the effects of TDP-43 and FUS mutations on the structure, function, and viability of motor neurons. Using site directed mutagenesis, individual mutations will be generated and studied by transfecting mutated DNA into mammalian cells. The data collected from cell assays will then be shared with Dr. Brian Drawert in the University of North Carolina Asheville Computer Science Department who is working on generating computational models of ALS progression. Using the data collected, Dr. Drawert will refine his models at different disease scales. The predictions made by these refined models can then be verified (or proven incorrect) via further laboratory work. These cells will be assayed for morphological changes, shifts in calcium signaling, and cell death induction. This work will provide insight into the molecular mechanisms behind ALS and other prion-like diseases.

This work will focus on mutations within the C-terminal of FUS, which contains a non-classical nuclear localization signal.[12] These mutations could affect the ability of nuclear import proteins to recognize FUS's nuclear localization signal and properly localize the protein in the nucleus. This would lead to increased FUS concentration in the cytoplasm, which could lead to aggregation events. The first mutation of interest is the 1554_1557 deletion mutation, which has only been documented twice. Both cases appeared to be *de novo* and presented with bulbar symptom onset and an aggressive progression of disease. Both patients also suffered from slight learning difficulties and congenital joint defects.[12-13] The researchers who discovered this mutation posited that the mutation could lead to changes in pre-mRNA splicing.[12] However, the actual mechanism of disease associated with this mutation is yet to be determined. Like other C-terminal mutations in FUS, this mutation is associated with juvenile onset ALS, with the two patients presenting at the ages of 13 and 18. Understanding the mechanism of cell death and toxicity caused by this severe deletion mutation could potentially lead to answers behind the mechanism of other C-terminal mutations in FUS and could lead to possible therapeutics for juvenile onset ALS.

2. Experimental Methods

2.1. Synthesis of 1554_1557del Mutation

2.1.1. site-directed mutagenesis

The mutation was introduced into a bacterial plasmid (FUS (Homo sapiens) in pENTR223 purchased from DNASU Plasmid Repository (#HsCD00513161)) via PCR site-directed mutagenesis using a NEB Q5 Site-Directed Mutagenesis Kit (#E0554S) and mutation specific primers developed using the software NEBasechanger.

2.1.2. biotransformation

PCR product underwent a Kinase, Ligase & DpnI (KLD) Treatment and was subsequently incubated for five minutes at room temperature before being transformed into the NEB-85 *E.coli* cell line (#C2523I). Transformed cells were allowed to proliferate in 950 µL of SOC medium while incubating for one hour at 37 °C with shaking at 250 rpm. Bacteria were spread on LB-agar plates spiked with the antibiotic spectinomycin and subsequently allowed to grow overnight at 37 °C. The bacterial plasmid transformed into the bacteria contained a gene coding for antibiotic resistance to spectinomycin, and surviving colonies present on the plate indicated a successful biotransformation reaction.

2.1.3. colony PCR:

To confirm the presence of the 1554_57 mutation in the bacterial plasmid, ten colonies from the biotransformation were randomly selected for analysis. The colonies were added to 25 μL PCR reactions containing primers specific to the FUS sequence. The thermocycler program was as follows: 1 cycle at 98 °C for 3min; 34 cycles of 98 °C for 10s, 69 °C for 30s, and 72 °C for 50s; 1 cycle at 72 °C for 10 min; and then held at 4 °C. PCR product was visualized using a 1.2 % agarose gel, and colonies with a bright band at ~1600 bp were selected to move forward.

2.1.4. DNA amplification and purification

Colonies from the successful biotransformation were picked and placed in 2 mL of LB broth laced with spectinomycin and allowed to incubate for 14 hours at 37 °C. The DNA was then extracted and purified using a PureLink MiniPrep Kit (#K210011). The purified DNA product was tested using a NanoDrop Spectrometer for purity (260/280) and product concentration (ng/μL), before being submitted for Sanger Sequencing to confirm presence of mutation.

2.2. Subcloning into Promega Regulated Mammalian Expression System

2.2.1. addition of restriction sites

In order to subclone the desired FUS sequences into the Flexi vector, the bacterial vector underwent the addition of SgfI and PmeI restriction sites via PCR. Two 50 μL reactions were made for each sequence using the NEB Q5 Site-Directed Mutagenesis Kit (#E0554S). The Thermocycler program was as follows: 1 cycle 98°C for 3min; 34 cycles of 98°C for 10s, 61 °C for 30s, and 72°C for 50s; 1 cycle at 72°C for 10min; and then held at 4°C. Both 50 μL reactions were combined and purified using the Wizard® SV Gel and PCR Clean-Up System (#A9281).

2.2.2. co-digestion and ligation

The purified PCR product containing 1554_57del DNA fragment with added restriction sites (Reaction 1) and the Flexi vector (Reaction 2) were co-digested using the Flexi® Enzyme Blend (SgfI & PmeI) enzyme cocktail (#R1851) specific for the expression system. Both R1 and R2 were incubated at 37 °C in a water bath for 30min. R1 was inactivated by undergoing PCR purification using the Wizard® SV Gel and PCR Clean-Up System. R2 was inactivated by heating at 65 °C for 20 min. Both reactions were stored on ice until undergoing ligation using T4 DNA ligase and T4 DNA ligase buffer. The ligation reactions were incubated at room temperature for 10 min and then heat shocked at 65 °C for 10 min. 1 μL of the ligation was biotransformed into the NEB-85 *E.coli* cell line as previously described in 2.1.2.

2.2.3. colony PCR

To confirm the presence of the 1554_57 mutation in the Flexi vector, ten colonies from the biotransformation were randomly selected for analysis. The colonies were added to 25 μL PCR reactions containing a primer specific to the midFUS sequence and the reverse primer used in addition of the restriction sites. The thermocycler program was as follows: 1 cycle at 98 °C for 3min; 34 cycles of 98 °C for 10s, 69 °C for 30s, and 72 °C for 50s; 1 cycle at 72 °C for 10 min; and then held at 4 °C. PCR product was visualized using a 1.2 % agarose gel, and colonies with a bright band at ~800 bp were selected to move forward through DNA amplification, purification, and sequencing confirmation as previously described.

2.3 Preparation of Wild-Type FUS as Control

Wild-Type FUS was subcloned into the Promega Regulated Mammalian Expression System (#C9470) as described previously for the 1554_57del mutation in 2.2.

2.4. Cell Culture & Transfection Overview

2.4.1 cell-culture

HEK293 (human embryonic kidney cells) were donated by Dr. Thomas Meigs and subsequently used for culture and transfection. Cells were plated in vented tissue culture flasks and grown in 1X DMEM modified growth medium with 10% heat-inactivated fetal bovine serum added. Cells were housed in an incubator at 37 °C under 5% CO_2 atmosphere. The cells were monitored and split when the flask reached 95-100% confluency, roughly every 3-4 days. Splitting of the cells was performed under sterile conditions. The cells were rinsed with 1x phosphate-buffered saline (PBS)

solution before being trypsinized and incubating at 37 ℃ for 3 minutes. The cells then underwent a 1:10 split into new growth media.

2.4.2 transfection

A preliminary 6-well plate experiment using .5 μg of eGFP-C1 (donated by Dr. Thomas Meigs) for transfection using the following method resulted in sufficient transfection results for further experimentation with the same method. Cells were transfected when at 85-95% confluence in a 6-well plate. For each well, a solution of 250 μL Opti-MEM (#31985088) and 10 μL of Lipofectamine 2000 (#11668-019) was incubated at room temperature for 5 minutes before being added to a 250 μL solution of Opti-MEM containing .5 μg of eGFP-C1 plasmid. The resulting solution was incubated at room temperature for 20 minutes before being added dropwise to the plate. Cells were incubated overnight at 37 ℃ and 5% CO_2 whereupon the growth media was changed in the morning. Structural analysis via fluorescence microscopy occurred 48 hours post transfection. This would also be the timeframe for cellular collection for post-culture work-up.

2.5 Transfection of e-GFP, Neo, FUS WT and P525L Mutation

In order to determine the concentration of coumermycin required for biologically relevant expression of the Flexi vector, the following experiment was conducted. The transfection media was prepared as previously described. However, only .25 μg of each plasmid was used, and the media was scaled up so that there was one tube of transfection media per control/experimental group (Table 1). Each group was plated across two 12-well plates in triplicate with an additional column of control cells that had zero transfection media added (Table 2). Varying dilutions at the same volume of the antibiotic coumermycin was added to each well immediately after the addition of transfection media (Table 2). Transfection was completed at intervals so that each group had roughly the same incubation times to minimize variables from differences in incubation times if all groups were transfected sequentially. Structural analysis was conducted at ~48 hours using blue wavelength fluorescence microscopy. The P525L mutation was used as the mutant experimental group due to its availability while the 1554_57del mutation underwent trouble-shooting.

Table 1. Experimental groups with plasmid concentrations in transfection media.

	e-GFP-C1	Neo Plasmid	Flexi Plasmid
Control 1	X	X	X
Control 2	.25 μg	X	X
Control 3	.25 μg	.25 μg	X
Experimental Group 1	.25 μg	.25 μg	.25 μg FUS WT
Experimental Group 2	.25 μg	.25 μg	.25 μg P525L

Table 2. Plate map for concentrations of coumermycin added to each column. The control column cells received no transfection media or coumermycin. Each row was plated in triplicate.

[Coumermycin]	0	.5	1	2	5	7.5	10	Control

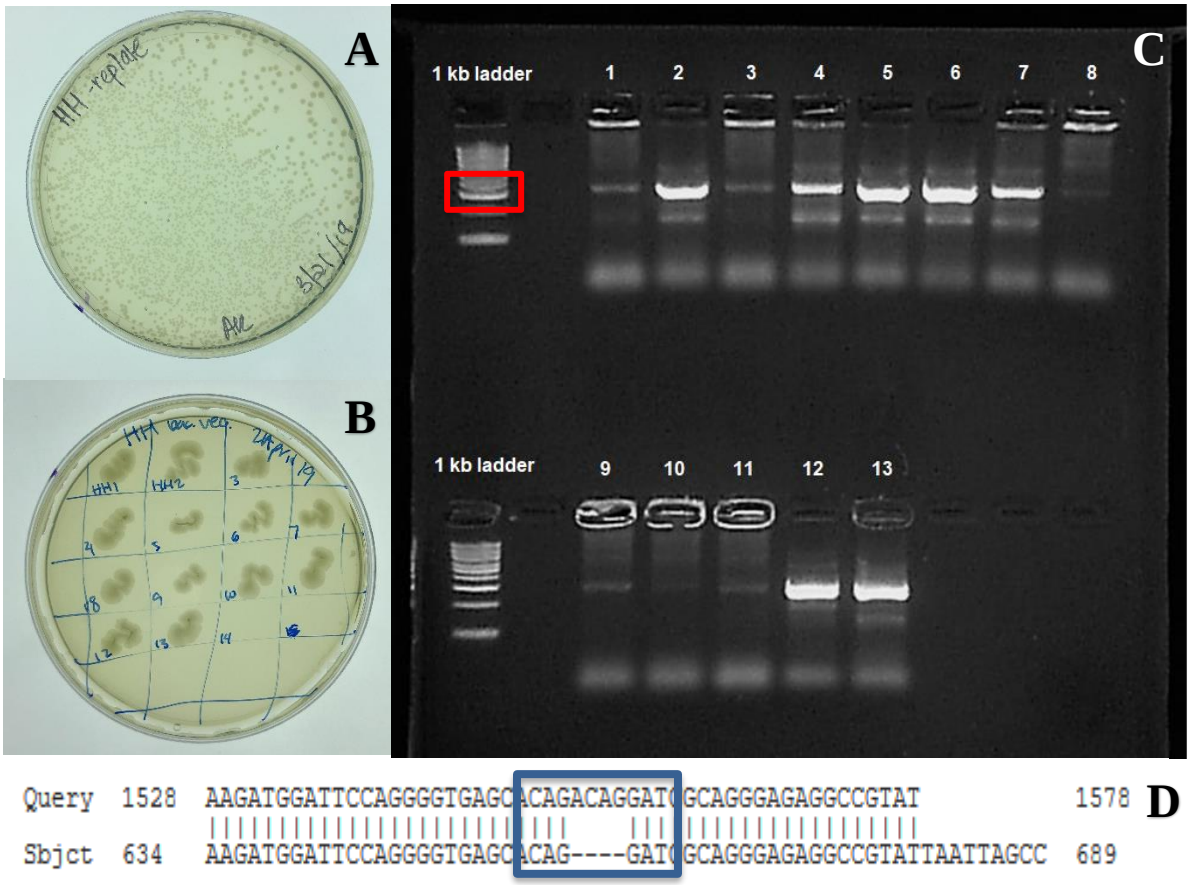
3. Results

Primers were successfully created for inserting the 1554_1557 mutation into a wild-type *FUS* sequence. Bacterial colonies were produced from the biotransformation of the 1554_1557del plasmid into competent *E. coli* cells as indicated by the presence of colonies on the spectinomycin laced LB-agar plate (Figure 1A). Colony PCR was

conducted on 13 randomly selected colonies from the plate in Figure 1A. These colonies were cross-plated for future use (Figure 1B) Colonies showing bright bands at ~1600 bp after gel electrophoresis were selected for DNA purification and submitted for Sanger Sequencing (Figure 1C). Sequencing confirmed presence of 1554_1557del in colonies 2, 5-7, and 12, as shown in the returned sequencing data (Figure 1D). The mutated FUS sequence was introduced into the Flexi vector and underwent successful Biotransformation, producing around thirty usable colonies (Figure 2A). Colonies 1-10 (Figure 2A) were cross-plated before undergoing Colony PCR (Figure 2C). Colonies 1-8 and colony 10 contained a FUS sequence within the vector, as shown by the bright bands at ~800 bp (Figure 2C). These colonies were subsequently worked up from the colony cross-plate (Figure 2B) and submitted for sequencing. The sequencing reaction failed to prime. Thus, an in-house diagnostic PCR and agarose gel was run using the restriction site forward and reverse primers to detect the presence of FUS. All but Colony 5 showed a bright band at ~1600bp (Figure 2D), and the colonies were subsequently processed for sequencing following the aforementioned protocol.

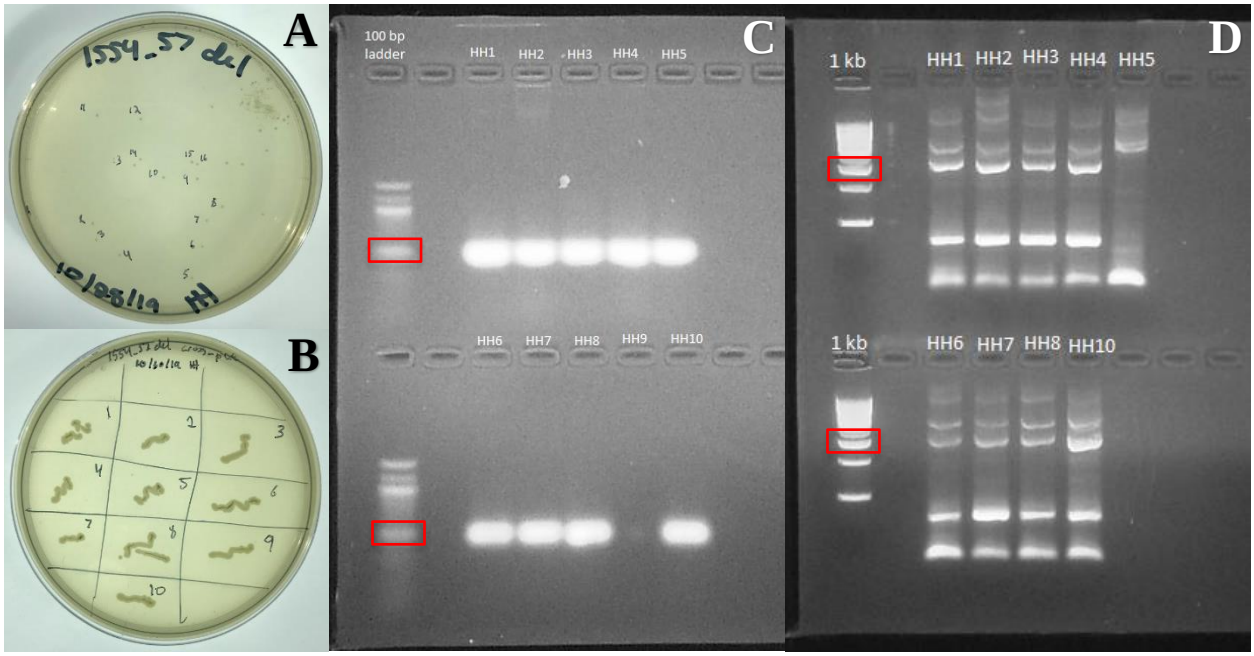
The 6-well transfection with e-GFP-C1 showed what appeared to be successful transfection, with bright fluorescing cells (Figure 3).

Upon evaluation pre-transfection, the HEK-293 cells showed discrepancy in cell density and confluency, leading to the elimination of two plates that exhibited reduced confluency (~30% confluency at time of transfection). At 48 hours post-transfection, the HEK-293 cells had lost adherence to the plate surface, resulting in the cell-layer clumping and overlapping, exposing large patches of bare plate with zero cell density (Figure 4). Loss of adherence and cell density varied across all plates, experimental groups, antibiotic concentrations, and triplicates with no apparent pattern (Figure 4). Due to such a large discrepancy at this stage in the experiment, post-culture work-up past structural analysis by fluorescence microscopy was not completed. Visual analysis indicated that there was partial success of transfection



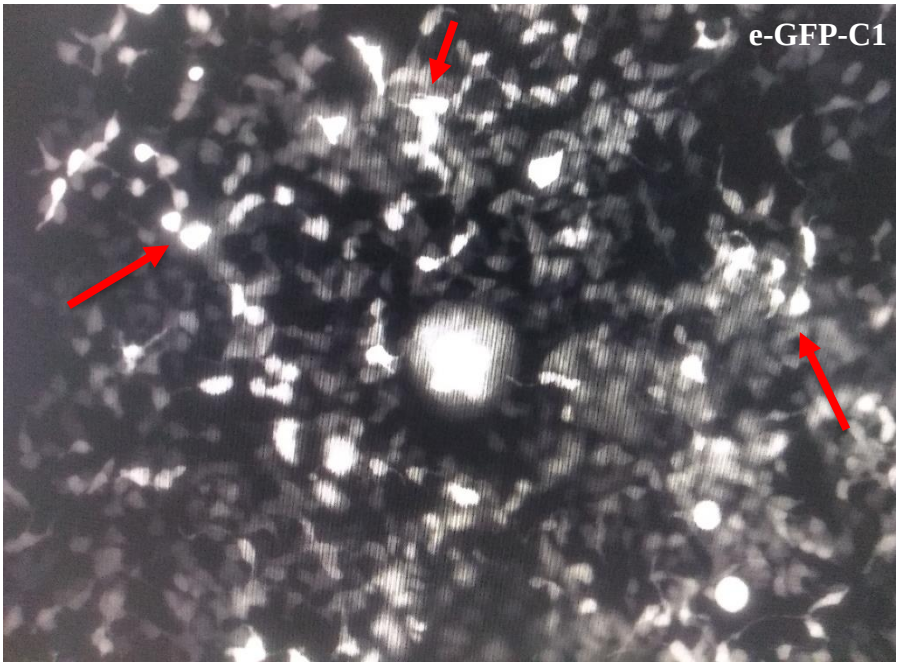
upon comparison of fluorescence of the P525L at the highest antibiotic concentration and the control lane that underwent zero transfection (Figure 5). Additionally, the overlapping cellular layer can be observed along the edges between the cells and the bare plate bottom (Figure 5).

Figure 1) A) Successful biotransformation of mutated bacterial vector into *E. coli.* B) Cross-plate from Colony PCR.



C) Results from Colony PCR showing bright bands at ~ 1600 bp (red box) for colonies 2, 4-7 and 12-13, indicating presence of bacterial vector containing *FUS* sequence. D) Sanger Sequencing results confirming presence of 1554_1557del mutation as noted by the blue box.

Figure 2. A) Subcloning results from biotransformation of Flexi vector with 1554_57del mutation. B) Cross-plate



produced during Colony PCR. C) Results of Colony PCR, with target band of ~800 bp highlighted by red box in 100 bp ladder lane. D) In-house diagnostic gel run on Purified DNA when Sanger Sequencing reaction failed. Red box highlights the targeted ~1600 bp on 1 kb ladder.

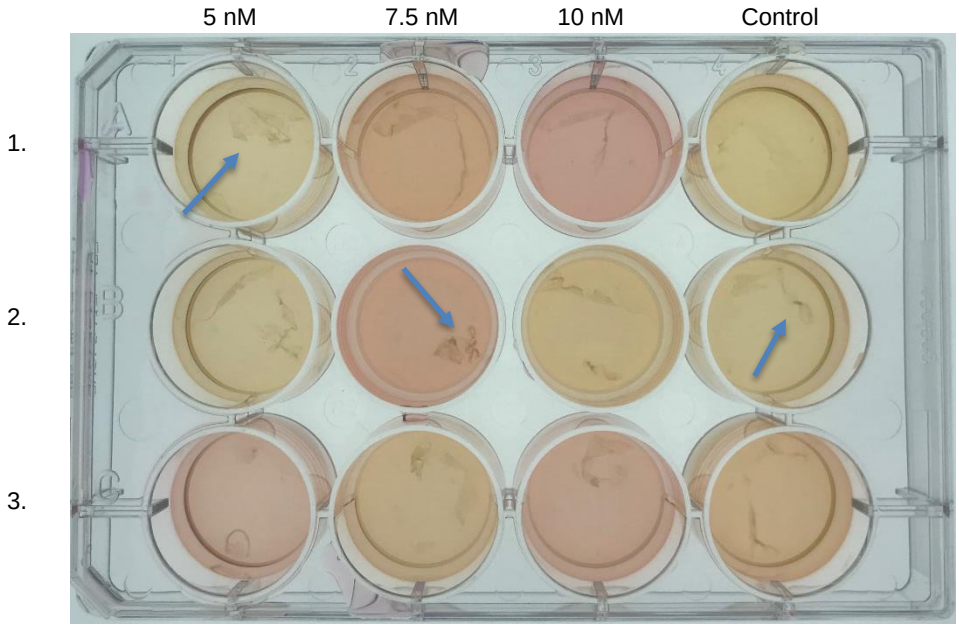


Figure 3. Image of cells at 40X magnification 48 hours after transfection with e-GFP. Bright white cells appear to show fluorescence, indicating successful transfection as indicated by red arrows.

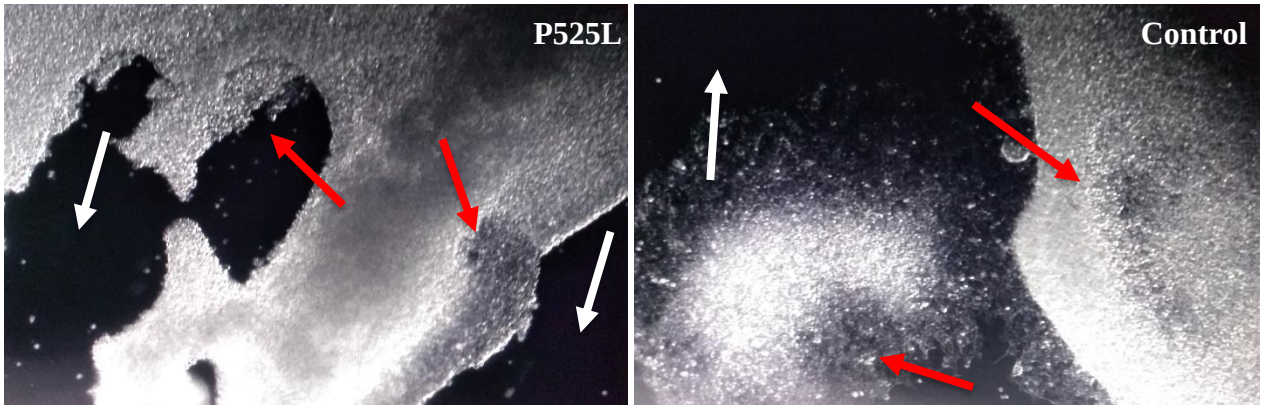


Figure 4. Image of Experiment Group 2 (P525L) ~48hrs after transfection. Coumermycin concentrations shown ranged from 5 nM to 10 nM. Differences in color show variance in cell viability/original cell density with yellower wells indicating more cellular waste than pinker wells. Folded over cell layer is visible on bottom of wells as indicated by blue arrows.

Figure 5. Images of P525L Experimental Group #2 at 10 nM coumermycin concentration (P525L) and the control well from the same row at 10X magnification. There appears to be increased fluorescence in the P525L image compared to the Control image, however, due to the overlapping of the cellular layer (as pointed out by the red arrows), it was difficult to verify. Furthermore, images show areas of plate that are completely clear of cells as marked with white arrows.

4. Discussion

1554_1557del Mutation: Mutation was successfully transformed into a bacterial vector and has potentially been transformed into the Flexi vector. However, this is yet to have been confirmed as the Sanger Sequencing reaction failed to prime. This is probably due to the fact that the DNA purification final elution step used TE buffer, which is Tris-EDTA buffer. EDTA chelates Mg^{2+}, which is essential for proper function of Taq polymerase. Since the concentrations collected from the sub-cloned colonies ~ 50 ng/μL, which is the required concentration for Sanger Sequencing, the samples submitted contained mostly TE buffer, most likely causing the reaction to fail. After the in-house diagnostic gel, colonies 1-4, 6-8, and 10 were chosen to mini-prep again. During the second round of work-up the DNA was eluted in 10mM Tris buffer. Yields were still overall low, ranging from ~80 ng/μL to ~20 ng/μL, along with high A260/280 numbers at >1.90, which indicates RNA contamination. Previous use of this protocol yielded A260/280 values at around 1.80, which indicates a higher concentration of DNA compared to RNA. Due to the high A260/280 values, the samples were discarded and the previously synthesized ligase was used to biotransform into a fresh colony of viable *E.coli*. Nuclease free H_2O at 60 ℃ was used for elution during DNA purification. Concentrations were between 60 and 80 ng/μL with A260/280 numbers between 1.80 and 1.84, indicating less RNA contamination than the previous round. However, sequencing results showed incomplete sequencing with low specificity. An in lab PCR analysis was carried out according to Experimental Methods 2.2.1, and gel electrophoresis showed that the FUS sequence was not present in two of the 1554_57del samples and only showed a small concentration of sequence in the other two samples. This result helps explain the faulty sequencing data. One possible explanation is that the bacterial colonies could have ejected the plasmid due to an elongated time between plating and DNA amplification and purification as well as an additional round of cross-plating

HEK-293 Cell Assay: Due to the variance in cellular confluency at time of transfection and due to separation of the cellular layer from the plate surface, it was impossible to complete post-cell culture analysis that would provide meaningful results as there was variance across all concentrations, all experimental group, and every triplicate. Cell count appeared to decrease along plates as the volume of original growth media and cells from the splitting flask decreased. In the future, frequent swirling of the cell and growth media solution while splitting could help reduce this discrepancy. Furthermore, the HEK-293 cell-line that the lab was working with had been exhibiting longer and longer time increments required for a flask to reach confluency based off of the same ratio split. This indicates that the cell-line itself could have been in poor health or mutated, causing a reduction in proliferation or ability to adhere to the plate as HEK-293 cells are known to lose adherence after multiple splits (Unpublished).[14] Post transfection, the cells could have possibly overgrown leading to additional difficulty adhering to the plate. Due to the described issues, the cells were not viable for post-culture work-up and results could not be verified. In the future, using a fresh cell-line would allow for verification of experimental methods and execution of required techniques. It is important to note, that a previous trial run for transfection of e-GFP-C1 did not show loss of adherence, and cells appeared healthy when checked ~48 hours later. Additionally, the control lanes for each experimental group that underwent zero transfection also exhibited the same loss of cellular adherence and loss of cell density. This indicates that the method and technique were sufficient and that the age of the cell-line could be the most likely culprit.

5. Conclusions

The preceding methodology has been used to transform wild-type FUS, wild-type TDP-43, and two mutations for each protein into the Promega Regulated Mammalian Expression System, a two plasmid system whereupon sequence transcription can be modulated based upon how much coumermycin is added to the cells. The coumermycin interacts with the promoter protein transcribed by the Neo vector. The promoter then dimerizes with the coumermycin amplifying both the Neo vector and promoting transcription of the target gene in the Flexi vector, in this case, *FUS* 1554_57del. This system is superior to the traditional single plasmid system that relies on the overexpression of the protein of interest. Overexpression is a poor experimental model for the study of FUS, as studies have shown that even the overexpression of wild-type FUS will result in aggregation.[15]

Future directions of this research would be to complete the entire process of biotransformation through DNA amplification and purification within a condensed time frame of three days to avoid colony rejection of the plasmid. Furthermore, use of a fresh HEK-293 cell-line would be suggested in order to complete the experiment to determine the correct concentration of coumermycin. Furthermore, fractionation of collected cell lysate into nuclear and cytoplasmic fractions for analysis of changes in protein and RNA expression could elucidate changes in cellular

transcription and translation. Long-term, use of a more biologically relevant cell-line would also be suggested. With the addition of structure, function, and viability data, and the addition of a more biologically relevant cell-line, the puzzling pathology of ALS may become more clear.

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