Analyzing dysfunction of profilin1 in ALS

A Major Qualifying Project

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease which affects the motor neurons in the brain and spinal cord. Profilin1, is a protein that has been associated with the onset of ALS, as mutant profilin1 was discovered in the exome sequencing of those affected with familial ALS. Profilin1 (PFN1) is crucial in the formation of the actin cytoskeleton and maintaining the proper amount of actin assembly in cells. In order to analyze what occurs during profilin dysfunction, multiple experiments were completed. DNA was cloned containing both GFP, BFP, or Cherry DNA and Lifeact which is a marker that allows the visualization of actin in cells. Additionally, lentiviruses were created in order to create stable cells lines with profilin knockdown, allowing future experiments to be completed to analyze the effects of profilin knockdown compared to a non-silencing cell line. Next, three silent mutations were added to the plasmids in order to express endogenous PFN1 resistance to shRNA in stable cell lines. The mutations added did not restore the plasmid back to endogenous PFN1 expression, as the shRNA was still able to recognize the plasmid. Additionally, two different buffers were used to test the solubility of the ALS-mutant-PFN1-C71G protein, along with two different DNA expression vectors. The buffers had no effect on solubility of C71G, but expression of PFN1-C71G was lower in the PFN1-WT in the pDest vector than the pLenti vector. Lastly, the localization of exogenously expressed V5-PFN1 was analyzed in wildtype profilin, V5 vector, and mutant profilin transfected cells by staining with profilin and V5 antibodies and observing by immunofluorescence. Aggregates were observed in the C71G and M114T profilin ALS mutant expressing cells, suggesting further research focus on using these described experimental tools to further analyze profilin dysfunction.

Introduction

The project works under the research of Professor Daryl Bosco and Dr. Jeanne McKeon at Bosco Lab at the University of Massachusetts Medical School, analyzing amyotrophic lateral sclerosis associated proteins. Amyotrophic lateral sclerosis (ALS) is a fatal disease which effects the motor neurons in the brain and spinal cord. About 30,000 Americans are affected by ALS, with a short projected lifespan of 2-5 years after the onset of the disease. There is no cure for ALS and the only FDA approved treatment extends life expectancy by a couple of months. The project focuses on the protein, profilin1 (PFN1). Profilin is crucial for the formation of the actin cytoskeleton in cells. Recently, it has been discovered that the mutations in the PFN1 gene profilin are present in patients with familial ALS via exome sequencing (5).

Through recent findings in Bosco Lab, it has been discovered that mutations cause PFN1 to become destabilized (2). It has been shown that mutant PFN1 aggregates in cells (5) but the protein stability was first analyzed during this study conducted by Bosco Lab. It was discovered that the mutants C71G and M114T destabilized the profilin protein by creating a cavity into the core of the protein which was visualized by X-ray crystallography or determined with predictive modeling studies (2). Since then, these mutants have become of interest in understanding the proteins affected in those with ALS.

This project focuses on gathering tools which can be used further analyze what occurs when profilin is not functioning normally in cells. The study uses various lab techniques which allow for actin to be visualized in cells along with profilin localization using immunofluorescence. In addition, lentiviruses were successfully created in order to generate stable cell lines with profilin knockdown. The study also attempts to rescue the knockdown lines to the wildtype phenotype and observes solubility of the C71G mutant.

The profilin 1 protein & its ALS associated mutants

Profilin 1 is a protein found in many organisms and binds to actin to play a role in the formation of the cytoskeleton. When found at high concentrations, profilin will prevent the polymerization of actin from occurring, while at low concentrations profilin will enhance the formation of the cytoskeleton. Recently discovered, mutations within profilin have been associated with ALS. It was discovered that PFN1 mutants bound lower amounts of actin in addition to altering the F to G actin ratio in cells (see actin dynamics). Such dysfunction which occurs in mutant profilin, have been linked to the pathogenesis of ALS. Through exome sequencing mutant C71G was identified in two families with familial ALS (5). The mutant M114T was identified in 1 family, in addition to mutants G118V, and E117G which were newly identified mutations discovered through exome sequencing (5).

In addition to identifying profilin mutants through exome sequencing, the Bosco Lab collaborated in a study with the Landers Lab in analyzing the destabilization mechanisms of ALS-linked mutant PFN1. It was discovered that the mutants C71G, M114T, and G118V are destabilized when compared to the PFN1 wildtype after undergoing chemical and thermal denaturation (2). Additionally, it was found that the mutants cause the PFN1 protein to misfold. Through gel electrophoresis, multiple slower moving bands were observed for the C71G, M114T, and G118V mutants when compared to the faster moving single bands produced by the PFN1 wildtype and E117V mutant (2). Both these studies provide insight in that identified mutants play a role in the pathogenicity of ALS, driving more studies to be conducted in order to understand the role of profilin further.

Actin Dynamics

Actin is a crucial component in cell motility. The network of actin proteins polymerize into filaments which can take on different types of structures. These structures are vital in allowing the cell to change shape and mobilize (1). There are two forms of actin found in the cell. Filamentous actin (F-actin) is primarily involved with cell motility and muscle contraction (3). Globular actin (G-actin) is the actin which produces F-actin. The profilin gene is responsible for converting G-actin to F-actin (5). G-actin has to successfully polymerize into F-actin in order for the cell to be mobile and signal controlled muscle contractions, an essential component in cellular function.

Using lentivirus to make stable cell lines

Lentivirus was created in order to make stable cell lines allowing the knockdown of profilin. This was done by co-expressing the lenti vector along with the packaging plasmids in HEK293T cells where the virus then gets packaged into particles. The purpose of this is to introduce exogenous DNA into cells enabling expression of the protein in which the introduced DNA encodes. There are many benefits to making lentivirus versus using transient transfection. Although transient transfection is faster, creating stable cell lines allows for more consistent expression (4). Figure 1 shows a lentiviral backbone in which DNA can be easily inserted and packaged. Making stable cells lines from lentiviral packaging plasmids are more efficient than transient transfection. It is proven that using lentivirus to package the DNA is more effective in getting the DNA into the cells. Using virus creates a more uniform gene expression throughout the cells which provides more consistency when analyzing results from experiments (4). Transient transfection commonly does not efficiently deliver DNA into all of the cells which can create skewed experimental results. Thus, it is crucial that DNA be homogenously delivered into the cells, allowing for more accurate results.

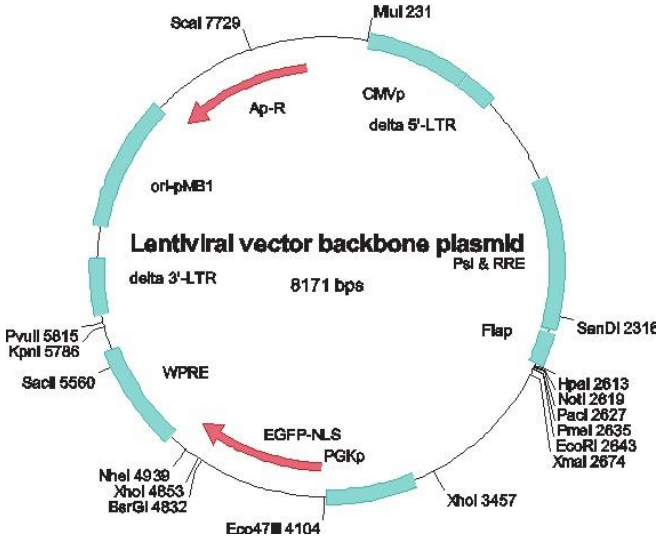


Figure 1: Lentiviral vector backbone plasmid (4).

Methodology

The methodology is organized into six sections where each major experiment was completed.

1. Cloning Lifeact into a pLenti plasmid to make lentivirus and visualize F-actin

DNA constructs, EGFP-Lifact, BFP-Lifeact and mCherry-Lifeact underwent a polymerase chain reaction (PCR) in order to amplify the Lifeact sequence (Table 1). The product was subjected to gel electrophoresis to confirm that Lifeact was amplified from the reaction. Lifeact was then ligated into the pENTR vector cut with SalI and NotI enzymes. The bands were then confirmed by size using gel electrophoresis. Next, the ligated DNA product was bacterially transformed using competent DH5 alpha cells. They were incubated on LB+ kanamycin plates overnight. Five colonies were then picked and cultured for the BFP and Cherry DNA. Six colonies were picked and cultured for the GFP DNA. The DNA was mini prepped and 400 nanograms were digested with 1 microliter of SalI and NotI enzymes and 1 microliter of 10X Cutsmart Buffer. The digestions were analyzed using gel electrophoresis. The clones selected that showed the correct insert and backbone size were then sent out for sequencing to confirm identities. HeLa cells were then transfected using the GFP and Cherry DNA and imaged using the fluorescent microscope.

Table 1: PCR Reaction for Lifeact Cloning

Construct	Primer F	Primer R	Template	Annealing temp	Extension (72 °C)	Product size
EGFP-Lifeact	Lifeact-BGFPSalI_f	LifeactEGFPcherry_r	EGFP-Lifeact	63	30 sec	~790
BFP-Lifeact	Lifeact-BGFPSalI_f	LifeactBFPNotI_r	BFP-Lifeact	62	30 sec	~770
mCherry-Lifeact	LifeactCherrySalI_f	LifeactEGFPcherry_r	mCherry-Lifeact	64	30 sec	~730

Table 2: Ligation of Lifeact and DNA vector

Tube	pEntr	BFP	GFP	Cherry
Insert (Lifeact) (µl)	**0**	**2**	**2**	**2**
Vector (100 ng) (µl)	**1**	**1**	**1**	**1**
10X T4 Ligase Buffer (µl)	**2**	**2**	**2**	**2**
Water (µl)	**16**	**14**	**14**	**14**
Ligase (µl)	**1**	**1**	**1**	**1**

Table 3: Ligation digestion

DNA	EGFP	BFP	Cherry
Enzymes	**SalI/NotI**	**SalI/NotI**	**SalI/NotI**
DNA (500 ng) (µl)	**9.2**	**2.2**	**6**
10X Buffer	**2**	**2**	**2**
Water	**6.8**	**13.8**	**10**
Enzyme (1 µl each)	**2**	**2**	**2**

2. Making lentiviral constructs to make stable cell lines & knockdown PFN1

A lentiviral transfection protocol was followed to make three different strains of lentivirus (Tables 4-5). Two of the viruses (named 74 and 352 after the transfer vector used) were then used to make stable cell lines in HEK293 cells with the help from Dr. Jeanne McKeon. Three stable cell lines were made including a non-silencing lines, and knockdown profilin lines shRNA #1 and shRNA #2. A BCA assay was used to determine protein concentrations. The knockdown lines were then analyzed on a Western Blot to compare knockdown lines to the non-silencing line where 5 micrograms were loaded (Table 7). A GAPDH loading control was used for normalization and the knockdown was quantified by the intensity of the bands and graphed. The knockdown was statistically analyzed using a one-way analysis of variance test and Tukey's multiple comparison test.

Table 4: Lentivirus packaging for transfer vector NS

	Concentration (µg/µl)	Volume (µl)
Transfer vector (NS)	0.9651	18.65
CMV	0.5744	31.34
VSV-G	0.2622	45.77
2.5 mm HEPES		587.35
2M CaCl2		96.90
2x HeBS		780

Table 5: Lentivirus packaging for transfer vector 74

	Concentration (µg/µl)	Volume (µl)
Transfer vector (74)	0.436	41.28
CMV	0.5744	31.34
VSV-G	0.2622	45.77
2.5 mm HEPES		564.66
2M CaCl2		96.90
2x HeBS		780

Table 6: Lentivirus packaging for transfer vector 352

	Concentration (µg/µl)	Volume (µl)
Transfer vector (352)	0.3865	46.57
CMV	0.5744	31.34
VSV-G	0.622	45.77
2.5 mm HEPES		559.42
2M CaCl2		96.90
2x HeBS		780

Table 7: Wildtype and knockdown profilin Western gel volumes

Sample	Concentration (µg/µl)	Volume for 5 µg (µl)	Volume PBS (µl)	Volume 6X Loading Dye (µl)
NS 1	2.36	2.11	12.8	3
74 1	1.52	3.28	11.7	3
352 1	1.77	2.8	12.18	3
NS 2	2.16	2.31	12.68	3
74 2	2.13	2.34	12.65	3
353 2	3.0	1.65	13.34	3
NS 3	2.53	1.97	13.02	3
74 3	2.74	1.8	13.17	3
352 3	3.47	1.43	13.56	3

3. Adding silent mutations to the plasmid in order to express endogenous PFN1

In order to rescue the knockdown lines to the wildtype profilin, three silent mutations were introduced in order to change the gene sequence but not the amino acid sequence (Figure 2). The cell lines were then either induced with doxycycline or non-induced. Next, a Western blot was run in order to compare profilin expression in the rescue constructs and wildtype profilin (Table 8).

shRNA #1

GGT GGT TTG ATC AAC AAG AAA

GGT GG**C** TTG AT**A** AAC AA**A** AAA

Gly Gly Leu Ile Asn Lys Lys

shRNA #2

GAC CGG TCA AGT TTT TAC GTG

GAC CG**C** TCA AG**C** TTT TA**T** GTG

Asp Arg Ser Ser Phe Tyr Val

Figure 2: Silent mutations added to shRNA #1 and shRNA# 2 to rescue knockdown to wildtype phenotype. The silent mutations added are bolded and underlined. The first silent mutation is yellow, the second is blue, and the third is red.

Table 8: Rescue constructs and wildtype profilin volumes run on western gel

Construct	Volume for 5 µg (µl)	Volume PBS	Volume 6X Loading Dye (µl)
1- NS+ pLenti V5-WT	2	13	3
2- NS + pLenti V5-WT-74	2.1	12.9	3
3- NS + pLenti V5-WT-352	2.2	12.8	3
4- NS+ pLenti V5-WT	2.2	12.8	3
5- NS + pLenti V5-WT-74	2.2	12.8	3
6- NS + pLenti V5-WT-352	2.1	12.9	3
7- 74 +pLenti V5-WT	2.5	12.5	3

8- 74+ pLenti V5-WT-74	2.7	12.3	3
9- 74 +pLenti V5-WT	2.4	12.6	3
10-74+ pLenti V5-WT-74	2.5	12.5	3
11-352 +pLenti V5-WT	2.2	12.8	3
12-352+ pLenti V5-WT-352	2.2	12.8	2
13-352 +pLenti V5-WT	2.2	12.8	3
14-352+ pLenti V5-WT-352	2.2	12.8	3

4. Comparing solubility of C71G PFN1 mutant and PFN1 wildtype

In order to analyze the solubility of C71G compared to WT PFN1, two different buffers were used (Table 9). Buffer A was made up of 1% Triton X-100, 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, and 10% glycerol and Buffer B included 1% Igepal, 0.1% Triton X-100, 50 mM Tris-HCl (pH 7.6) and 150 mM NaCl. The PFN1 mutant, wildtype PFN1, and V5 vector control were analyzed in the pLenti vector or the pDest vector. A western blot was run where profilin, V5 and GAPDH antibodies were used to analyze the profilin, V5 and GAPDH expression in the different buffers and vectors.

Table 9: Solubility of C71G, WT, and V5 using two buffers and two DNA vectors

Construct	Concentration (µg/µl)	Volume for 5 µg (µl)	Volume PBS (µl)	Volume 6X Loading dye (µl)
pLenti v5 A	1.15	4.3	10.6	3

pLenti v5 B	1.39	3.57	11.4	3
pLenti WT A	1.11	4.48	10.5	3
pLenti WT B	1.31	3.79	11.2	3
pLenti C71G A	1.49	3.33	11.66	3
pLenti C71G B	1.44	3.45	11.5	3
pDest v5 A	1.42	3.49	11.5	3
pDest v5 B	1.34	3.7	11.3	3
pDest WT A	1.5	3.3	11.6	3
pDest WT B	1.45	3.4	11.57	3
pDest C71G A	1.54	3.22	11.77	3
pDest C71G B	1.36	3.65	11.34	3

5. Determining optimal plating conditions for cells

In order to determine the optimal plating density for HEK cells, cells were plated at different densities to observe which density produced the healthiest confluence. Cells were plated in a 24 well and 12 well plate and incubated overnight. The plating densities are shown in Figure 3. Three densities of cells were determined to produce the healthiest confluence of cells. The cells were then stained with Phalloidin which allows F-actin to be observed in cells. The cells were then imaged under fluorescence microscopy.

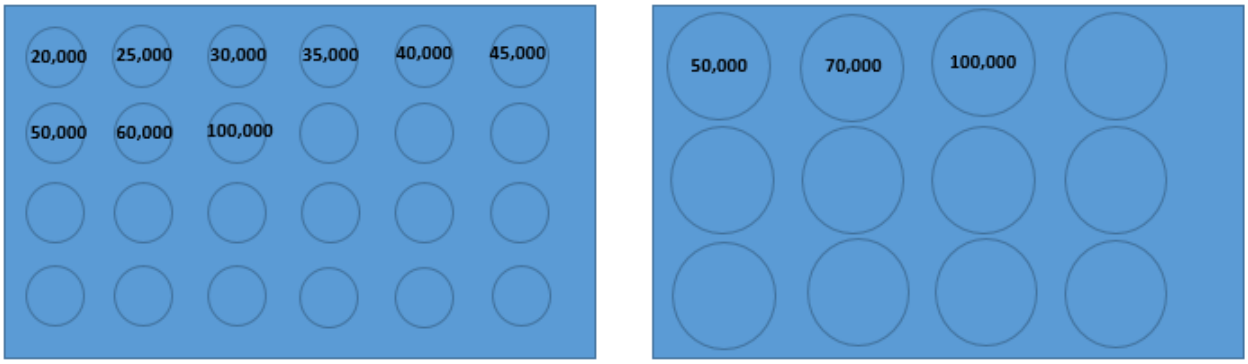


Figure 3: Plating densities (cells/well) for HEK cells in 24 well and 12 well plates.

6. Observing localization for PFN1 mutants, wildtype, and a V5 vector control.

In order to observe profilin and V5 localization in HeLa cells, 4 mutant profilin constructs, wildtype profilin, and a V5 vector were midi prepped. Next, HeLa cells were transfected with the DNA. The cells were stained with profilin and V5 antibody, where the cells were imaged using fluorescence microscopy.

Results

The results are organized into sections of major experiments which provide tools that allowed PFN1 dysfunction to be analyzed.

1. Cloning Lifeact into DNA plasmids to visualize F-actin in Hela cells.

First, DNA constructs, BFP-Lifeact, Cherry-Lifeact, and GFP-Lifeact were amplified using PCR. A no DNA control was used. A DNA gel was run in order to ensure Lifeact was amplified from the constructs. The results are shown below in Figure 4.

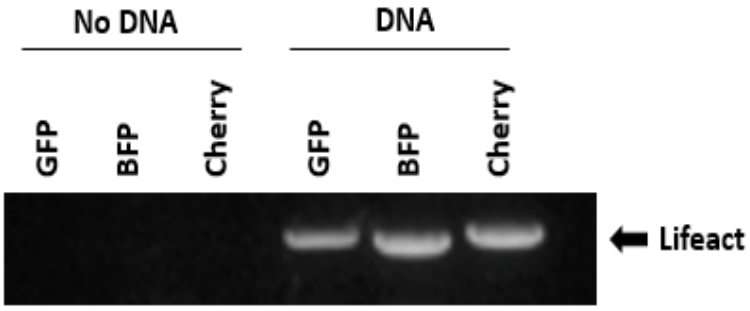


Figure 4: Gel electrophoresis of PCR for the GFP, BFP, Cherry constructs and no DNA controls

The DNA gel shown in Figure 4 shows that the Lifeact sequence was amplified from the constructs according to its expected size. The PCR product was purified from the gel and used for the subsequent ligation. A ligation was then completed with the amplified Lifeact and DNA vectors, BFP, GFP, and Cherry. Gel electrophoresis was used to confirm that the recombinant DNA construction worked shown below in Figure 5.

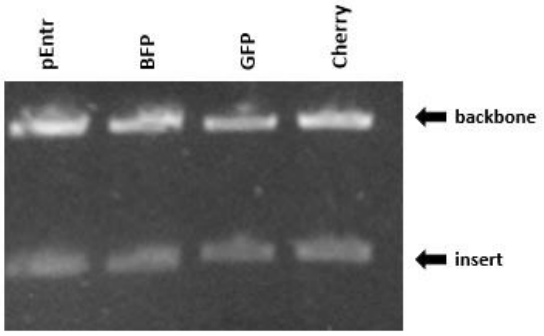


Figure 5: Gel electrophoresis of ligation of Lifeact with the pEntr, BFP, GFP, and Cherry DNA vectors.
Digested with SalI and NotI enzymes.

The ligation was successful for all DNA vectors as the backbone and insert were of expected sizes when cut with SalI and NotI enzymes.

A bacterial transformation was then completed and 5 colonies were picked and cultured for the BFP and Cherry constructs. The cultures were then mini prepped to isolate the DNA and digested using enzymes SalI and NotI. Gel electrophoresis was used to confirm that the cloning was successful from each particular colony (Figure 6).

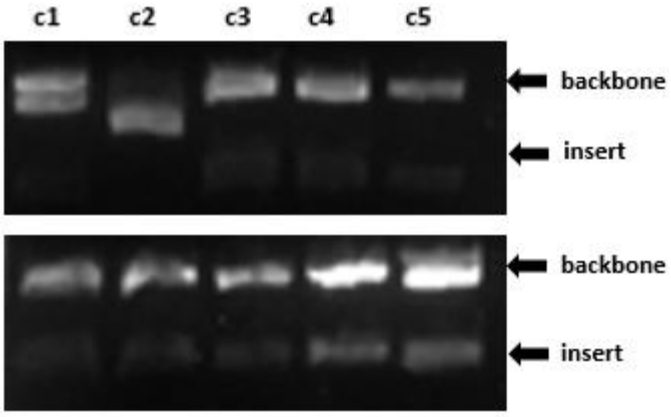


Figure 6: Gel electrophoresis for BFP-Lifeact (top) and Cherry-Lifeact (bottom) recombinant DNA. DNA digested with SalI and NotI. C1-C5 denote clones 1-5 which were picked from each colony.

Clones 3, 4 and 5, showed that the ligation was successful, as the bands were the expected size after digestion for BFP. For the cherry construct, clones 1, 2, 3, 4, and 5 were successfully ligated, as all of the clones were of expected size after digestion.

A bacterial transformation was then completed where 6 colonies were picked and cultured for the GFP constructs. The cultures were mini prepped and digested using SalI and NotI. Gel electrophoresis was used to confirm that the cloning was successful from each particular colony (Figure 7).

Clones 2, 4, and 5 show successful ligation as the digested DNA was of expected size for the insert and backbone.

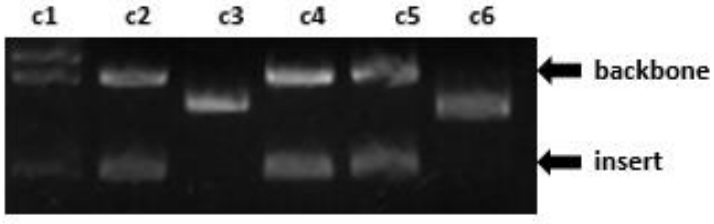


Figure 7: Gel electrophoresis for GFP recombinant DNA. DNA digested with SalI and NotI. C1-C6 denote the clones which were picked from each colony.

Lastly, HeLa cells were transfected with the recombinant DNA. Filamentous actin was observed due to the inserted Lifeact marker. The cells were stained and observed

under a fluorescent microscope (Figure 8). The BFP-Lifeact construct was not analyzed during this time.

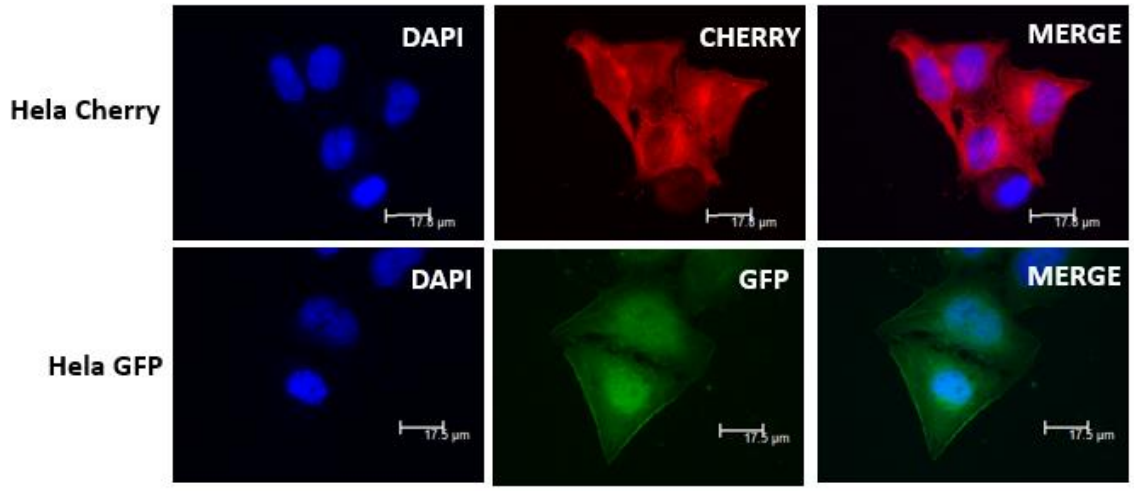


Figure 8: Filamentous actin observed through IF imaging

The nuclei of the cells can be observed through the DAPI channel, while F-actin can be observed on the cherry and GFP channels for the Hela cherry and Hela GFP respectively.

2. Making lentiviral constructs to make stable cell lines & knockdown PFN1

Three types of lentivirus were harvested and named NS, 349, 352, and 74. Types 352 and 74 and HEK293 cells were used to make stable cell lines. Two cell lines contained PFN1 knockdown, while the third was a non-silencing line. In order to quantify knockdown, a Western Blot was run to compare PFN1 knockdown to a GAPDH loading control. The Western Blot is shown below in Figure 9.

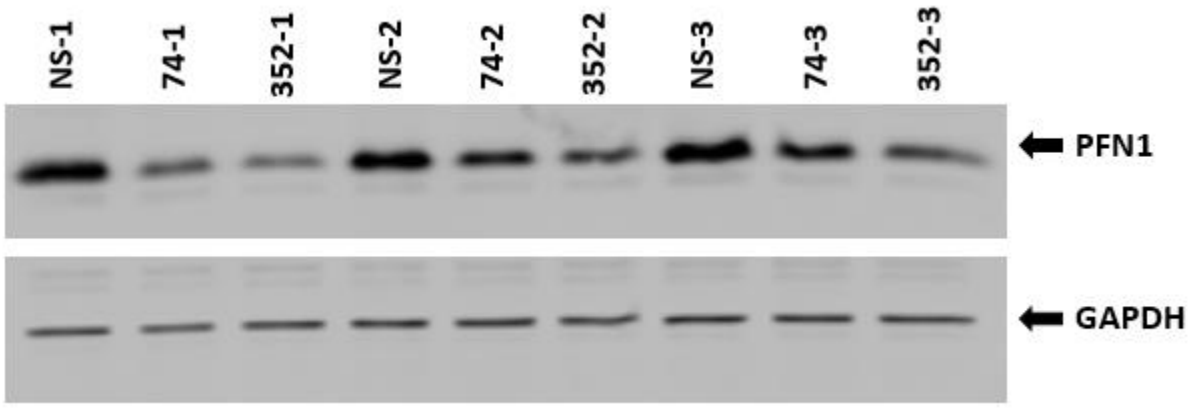


Figure 9: Western blot of PFN1 knockdown lines and non-silencing line (3 replicates for each line from 3 independent experiments).

As shown in Figure 9 all of the non-silencing line samples show a higher expression of PFN1 than the knockdown lines (74 and 352). The knockdown was then quantified by measuring the band intensity and shown in Figure 10. ShRNA #1 is the 74 line and shRNA #2 is the 352 line.

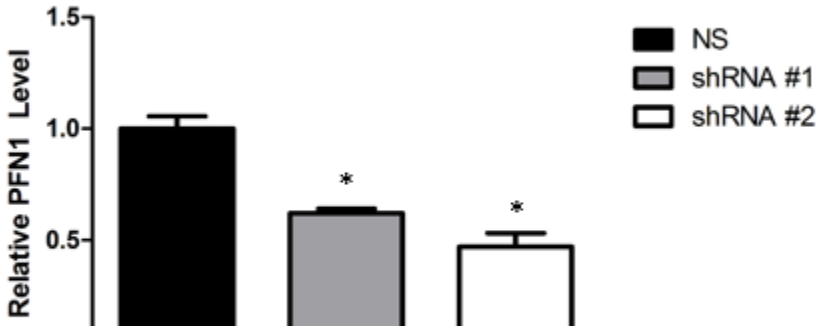


Figure 10: Quantification of PFN1 knockdown compared to non-silencing cell line. The p value was determined to be 0.0007 using ANOVA. When comparing NS to shRNA #1 and NS to shRNA #2 p value was significant (under 0.05). When comparing shRNA #1 to shRNA #2 p value was not significant.

When comparing the PFN1 expression in the non-silencing line and the shRNA 1 and 2 lines, it can be determined that the expression is almost half the expression of NS. A statistical analysis was completed in order to determine if the knockdown data was significant.

The p value was calculated to be 0.0007 after an ANOVA was completed. Using Tukey's multiple comparison test, it was determined that NS vs. shRNA #1 and NS vs. shRNA #2 were both significant. ShRNA #1 vs. shRNA #2 was not significant.

3. Adding silent mutations to the plasmid in order to express endogenous PFN1

Three silent mutations were added to the coding sequence of WT-PFN1 plasmid to disrupt recognition by the shRNAs encoded by the shRNA #1 (74) and shRNA #2 (352) when expressed in those cell lines. This was done so that the exogenously expressed PFN1 would not be targeted by the shRNA and thus restore a WT expression level in the knockdown cells. The cells were then either induced with doxycycline to turn on expression of the shRNA or non-induced. A western blot was run in order to observe expression of the PFN1 protein shown in Figure 11.

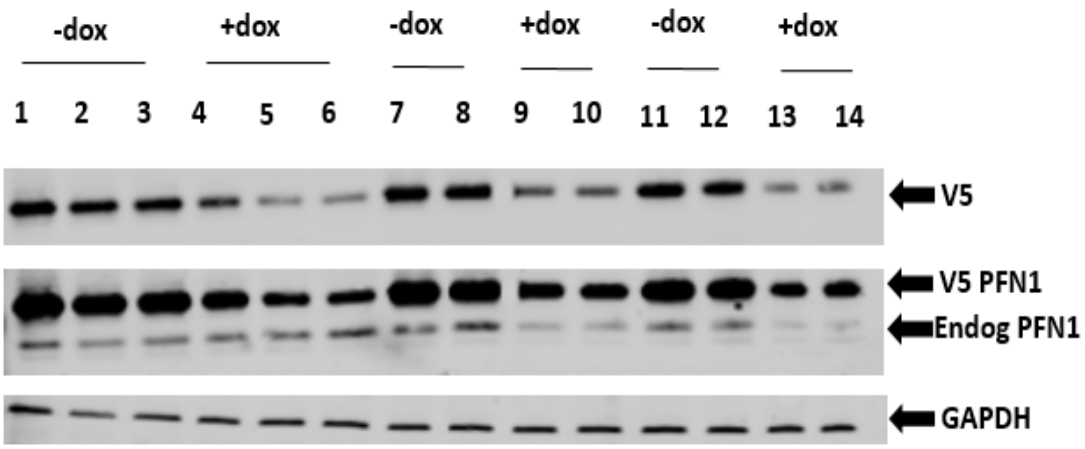
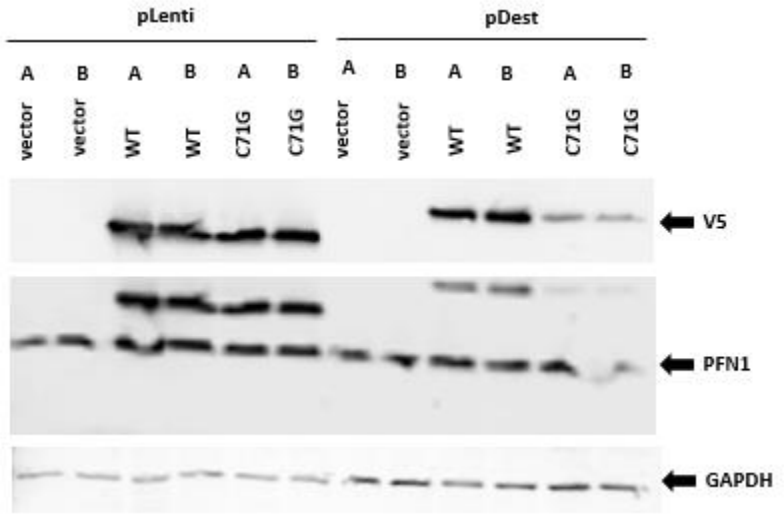


Figure 11: Western blot of rescue constructs induced with doxycycline or non-induced. 1- NS+ pLenti V5-WT; 2- NS + pLenti V5-WT-74 ; 3- NS + pLenti V5-WT-352 4- NS+ pLenti V5-WT;5- NS + pLenti V5-WT-74; 6- NS + pLenti V5-WT-352; 7- 74 +pLenti V5-WT; 8- 74+ pLenti V5-WT-74 9- 74 +pLenti V5-WT; 10-74+ pLenti V5-WT-74; 11-352 +pLenti V5-WT; 12-352+ pLenti V5-WT-352 13-352 +pLenti V5-WT; 14-352+ pLenti V5-WT-352RNA

Following induction, the shRNA#1 (pLenti V5-WT-74) and shRNA #2 (pLenti V5-WT-352) rescued cells showed similar expression as the WT-PFN1 (no silent mutation) rescued cells indicating the exogenously expressed PFN1 is targeted by the shRNA despite the silent mutations. Without doxycycline added, the expression is the same for the wildtype profilin and knockdown profilin. In addition, knockdown was confirmed by blotting with the PFN1 antibody.

4. Comparing solubility of C71G PFN1 mutant and PFN1 wildtype

In order to compare the solubility of the C71G mutant and PFN1 wildtype, two different buffers were used. The experiment was completed with both pLenti and pDest expression plasmids to compare the vectors. The results are shown below in Figure 12.



No difference was observed between the two buffers used, although pLenti showed a higher expression of C71G than the pDest vector.

Figure 12: Solubility of C71G, V5 vector and PFN1 wildtype. Buffer A= 1% Triton; Buffer B= 1% Igepal

5. Determining optimal plating conditions for cells

HEK cells were plated at different cell densities and their confluence was observed under the microscope. Three cell densities which were determined to look the healthiest under the microscope (30,000; 40,000 and 50,000) were Phalloidin stained. This allowed for F-actin to be observed via IF imaging. The results are shown below in Figure 13.

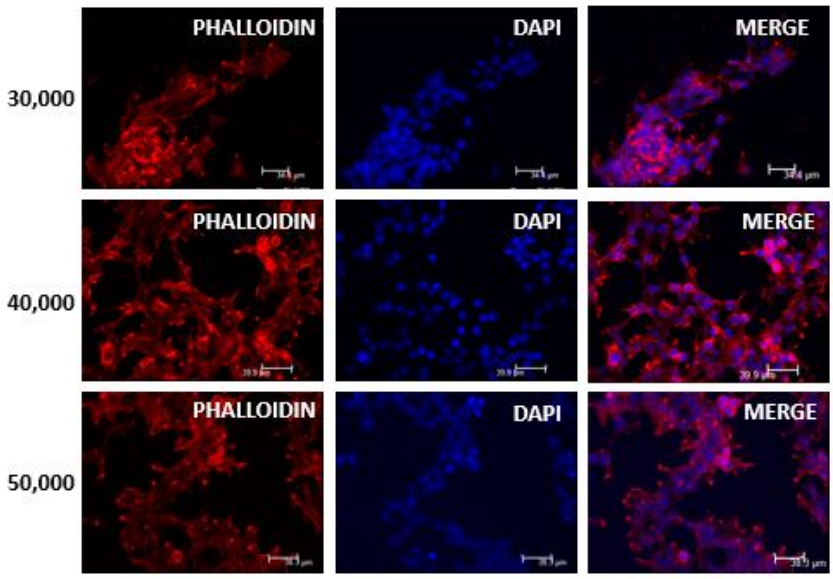


Figure 13: Phalloidin stain of HEK cells using three different cell densities

It was determined from Figure 13 that 30,000 cells showed a sparse confluency, while 40,000 showed a denser confluency. It was determined that 35,000 cells would be the optimal plating density for cells.

6. Observing localization for PFN1 mutants, wildtype, and a V5 vector control.

In order to observe PFN1 and V5 localization, HeLa cells were transfected with a plasmid encoding a wildtype PFN1, a V5 vector, or ALS mutant PFN1. The HeLa cells were then stained with V5 and PFN1 antibodies to observe localization. The results are shown below in Figure 14.

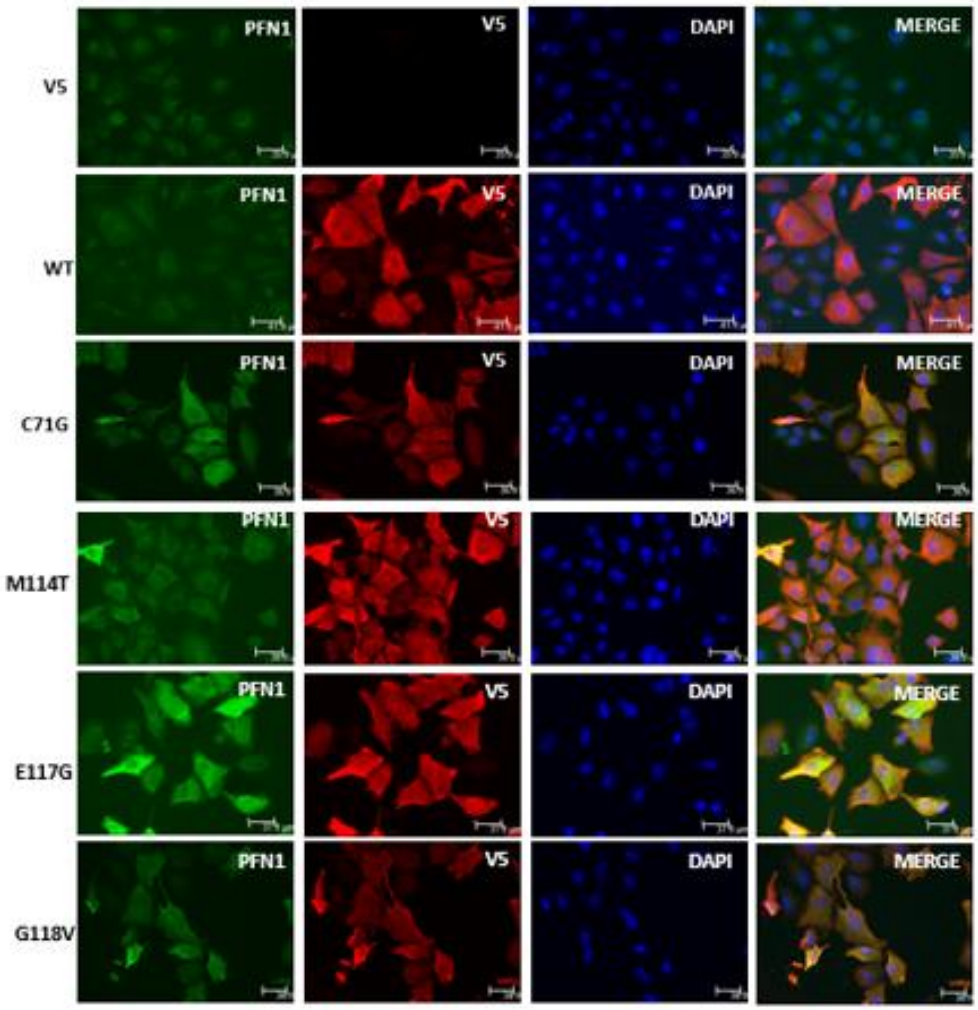


Figure 14: PFN1 and V5 localization in Hela cells transfected with WT PFN1, V5 vector, or ALS PFN1 mutants

Aggregates are observed in the C71G and M114T mutants in the green PFN1 channel. The WT PFN1 shows diffused aggregates in cells that are not transfected. No aggregates are observed in the red V5 channel.

Discussion

Profilin (PFN1) is a protein which is associated with ALS and understanding more about its function can provide more information in discovering potential therapies for the fatal disease. Multiple experiments were completed in order to provide tools which allow for profilin dysfunction to be analyzed.

First, recombinant DNA was created in order to introduce the marker Lifeact into the plasmid so F-actin could be visualized in transfected cells. This tool proved successful, as Lifeact was successfully ligated with the DNA constructs GFP, BFP, and Cherry (Figure 5). Next, F-actin could be visualized in the GFP and Cherry immunofluorescent channels when HeLa cells were transfected (Figure 8) and Lifeact could be put into a pLenti vector to make lentivirus. As profilin is crucial in actin formation, this technique provides a useful tool in observing actin filaments in PFN1 mutant infected cells and in creating lentivirus.

Next, lentivirus was successfully made. The lentiviral constructs created worked, as stable cells lines were able to be created using HEK 293 cells. Additionally, it can be concluded from Figures 9 and 10 that profilin knockdown was successful. Profilin showed knockdown to nearly 50 percent for shRNA #1 and shRNA #2 when compared to the non-silencing line. The profilin knockdown was significant for both shRNA #1 and shRNA #2 compared to the non-silencing line indicating efficient PFN1 knockdown. This provides another experimental tool which can be used to analyze the effects of profilin in ALS. When PFN1 undergoes knockdown, it can be used to simulate the effects of ALS affected cells, allowing more experiments to be completed to understand the role of profilin in ALS more explicitly.

PFN1 knockdown cell lines, shRNA #1 and shRNA #2 were attempted to be rescued to the wildtype PFN1. Three silent mutants were added to a PFN1 plasmid preventing the shRNA from recognizing the sequence. Shown in Figure 11, the knockdown lines were not completely rescued to the wildtype. Expression was lower in the PFN1 knockdown lines than in the wildtype in the induced plasmids. Thus, the shRNA was still recognizing the shRNA targeting sequence from the exogenously rescue plasmids and continuing to knockdown profilin. Resulting from this, more silent mutations should be added to the

knockdown lines. Adding more silent mutations will alter the plasmid sequence more so that the shRNA will not recognize the sequence since the exogenously expressed PFN1 is still targeted by the shRNA when three silent mutations are added.

Next, the solubility of the PFN1 mutant, C71G was analyzed in two different buffers and two different DNA vectors. The solubility of C71G was compared to the wildtype profilin and a V5 vector. When comparing the solubility of C71G in two different buffers, there is no observed difference (Figure 12). Thus, it can be concluded that the two different buffers have no effect on the solubility of C71G as C71G solubility is the same in both buffers (Figure 12). Although, it can be concluded that the vectors have an effect on the expression of C71G. Shown in Figure 12, C71G expression is higher in the pLenti vector than the pDest vector. This could be due to the linker which attached to pDest which could have an effect on the C71G solubility. In order to completely understand why this occurs, further research and experimentation is recommended.

Next, HEK cells were plated in varying densities in order to determine the optimal plating density which would enable the cells to be the best visualized under immunofluorescence. It was determined from Figure 13 that the best plating density would be between 30,000 and 40,000 cells per 12 well plate as the cells showed a healthy confluence and can easily be stained and observed under IF.

HeLa cells were then plated using the optimal plating density of 35,000 cells. Hela cells were then transfected with wildtype profilin, a V5 vector, or mutant profilin. Aggregates were observed for the C71G mutant and M114T mutant in the profilin green channel shown in Figure 14. The aggregates showed a dense population in the middle of the transfected cells, implying the mutants have an effect on profilin localization. Additionally, aggregates were not observed in the V5 and observed in the wildtype green profilin channel. These aggregates appeared more diffuse than the mutant profilin, and appeared only in the non-transfected cells. It is unclear why no aggregates were observed in the red V5 channel for mutant profilin C71G and M114T, as it is expected that the aggregates that were observed in the green channel would also be observed in the red channel. Repeating the experiment could provide more information as to why this occurred, along with further experimentation with the C71G and M114T mutants.

Cloning, transfection, immunofluorescent imaging, and creating stable cells lines of non-silencing and knockdown profilin, are all among experimental tools which were used to analyze profilin dysfunction. These tools can be used in further research to understand the role of profilin in ALS affected cells. As profilin is crucial in actin formation, enabling actin to be visualized by cloning DNA containing the F-actin marker Lifeact is an effective tool to have. Additionally, the stable cell lines created which showed sufficient profilin knockdown can be used to further understand what occurs in cells when the function of profilin is lost. Future directions can involve further experimentation with the knockdown cell lines and comparing what occurs in the knockdown cell lines compared to the non-silencing cell lines. Additionally, more mutations to rescue the wildtype phenotype should be added in order to avoid shRNA recognition to the gene sequence. Rescuing the wildtype phenotype will provide information about how many mutations need to be added in order for knockdown cell line to show the same profilin expression as the wildtype. Additionally, further research can focus on analyzing the C71G an M114T profilin mutants as aggregates were localized in the profilin green channel. Research can also focus on why these aggregates are not present in the red V5 channel and why aggregates are observed in the wildtype and V5 vector controls in the non transfected cells. Such techniques and further directions can broaden understanding of the role of profilin and the actin cytoskeleton in those affected with ALS.

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