BAŞKENT UNIVERSITY
INSTITUTE OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS
MASTER OF SCIENCE IN
MOLECULAR BIOLOGY AND GENETICS

INVESTIGATION OF THE INTERPLAY OF SUMOYLATION AND PHOSPHORYLATION ON PEA3 STABILITY IN NEURONAL CELLS

BY

MEHMET ALP GÜNER

MASTER OF SCIENCE THESIS

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ABSTRACT

Mehmet Alp GÜNER

INVESTIGATION OF THE INTERPLAY OF SUMOYLATION AND PHOSPHORYLATION ON PEA3 STABILITY IN NEURONAL CELLS

Başkent University Graduate School of Natural and Applied Sciences

Department of Molecular Biology and Genetics

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The Pea3 family belongs to the superfamily of ETS domain transcription factors, which includes the Pea3/ETV4, Erm/ETV5, and Er81/ETV1 proteins. The MAPK/ERK signaling pathway regulates Pea3 family members. It has also been shown that Pea3 has some post-translational modifications such as phosphorylation and SUMOylation. However, the specific regions required for these modifications have been defined for Erm and Er81, but these modifications and their effects are not fully known for Pea3. In previous studies on neural cell lines, it was shown that Pea3's Serine 90 and Serine 458 motifs are important in axon elongation. At the same time, according to another study, it is found that Serine 101, 192 and 285 regions may also be effective in neurite elongation. Some findings in other studies in the literature show that regulation of the stability of Pea3 proteins can be achieved by phosphorylation and SUMOylation. Within the scope of this thesis, plasmids encoding Pea3 and phospho-mutant Pea3 proteins, which are thought to have an effect on neurite elongation, were transfected into the NSC-34 cell line. It has been observed that there is a significant connection between the loss of phosphorylation ability of phospho-mutant Pea3 proteins and the loss of SUMOylation ability of these proteins. The effect of these two important post-translational processes on the stability of Pea3 proteins was investigated and it was found that the proteins lost their stability after 3 hours. Determining the regulation of different phosphorylation sites, which are thought to be effective on neurite elongation and phosphorylation and SUMOylation processes, which are directly linked to the functionality of proteins, will contribute to the elucidation of an important molecular mechanism for axon elongation and neuron regeneration.

KEYWORDS: Pea3, SUMO, MAPK, axon elongation, neuronal regeneration

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ÖZET

Mehmet Alp GÜNER

NÖRONAL HÜCRELERDE PEA3 STABİLİTESİ ÜZERİNDE SUMOLASYONUN VE FOSFORİLASYONUN KARŞILIKLI ETKİLEŞİMİNİN İNCELENMESİ

Başkent Üniversitesi Fen Bilimleri Enstitüsü

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Pea3 ailesi, Pea3/ETV4, Erm/ETV5 ve Er81/ETV1 proteinlerini içeren ETS alanı transkripsiyon faktörleri süper ailesine aittir. Pea3 ailesi üyeleri, MAPK/ERK sinyal yolu tarafından düzenlenir. Ayrıca Pea3'nin fosforilasyon ve SUMOlasyon gibi bazı transkripsiyon sonrası modifikasyonlara sahip olduğu da gösterilmiştir. Ancak bu modifikasyonlar için gereken spesifik bölgeler Erm ve Er81 için tanımlanmış olup Pea3 için bu modifikasyonlar ve etkileri tam olarak bilinmemektedir. Nöral hücre hatları üzerinde daha önce yaptığımız çalışmalarda Pea3'ün akson uzamasında Serin 90 ve Serin 458 motiflerinin önemli olduğu gösterilmiştir. Aynı zamanda yapılan diğer bir çalışmaya göre Serin 101, 192 ve 285 bölgelerinin de nörit uzamasında etkili olabileceği düşünülmektedir. Literatürdeki diğer çalışmalarda Pea3 proteinlerinin stabilitesinin düzenlenmesinin fosforilasyon ve SUMOlasyon ile sağlanabileceğini gösteren bazı bulgular bulunmaktadır. Bu tez kapsamında, Pea3 ile nörit uzaması üzerine etkisi olduğu düşünülen fosfo-mutant Pea3 proteinlerini kodlayan plazmitler NSC-34 hücre hattına transfekte edilmiştir. Fosfo-mutant Pea3 proteinlerinin fosforillenme yeteneklerini kaybetmesiyle birlikte bu proteinlerin SUMOillenme yeteneklerini kaybetmesi arasında anlamlı bir bağlantı olduğu gözlemlenmiştir. Bu iki önemli post-translasyonel sürecin Pea3 proteinlerinin stabiliteleri üzerindeki etkisi araştırılmış ve 3. saat itibariyle proteinlerin stabilitelerinin kaybolduğu bulunmuştur. Proteinlerin fonksiyonelliği ile doğrudan bağlantılı olan fosforilasyon ve SUMOlasyon süreçlerinin nörit uzaması üzerinde etkili olduğu düşünülen farklı fosforlanma bölgelerinin regülasyonlarının belirlenmesi akson uzaması ve nöron rejenerasyonu için önemli bir moleküler mekanizmanın da aydınlatılmasına katkı sağlayacaktır.

ANAHTAR KELİMELER: Pea3, SUMO, MAPK, akson uzaması, nöronal rejenerasyon

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FOREWORD

The inability to elucidate the post-translational modifications of the newly identified phosphorylation regions directly associated with axon elongation is a major deficiency in understanding the neuroregeneration mechanism. With this study, the importance of phosphorylation sites on SUMOylation and protein stability will be determined, and an important regulation mechanism will be shed light on.

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LIST OF SYMBOLS AND ABBREVIATIONS

APS	Ammonium Persulfate
DMEM	Dulbecco's Modified Eagle Medium
ETS	E-twenty Six / E-26 Transformation Specific
FGF	Fibroblast Growth Factor
GDNF	Glial Cell-Derived Neurotrophic Factor
HRP	Horseradish peroxidase
MAPK	Mitogen Activated Protein Kinases
NGF	Nerve Growth Factor
NSC-34	Mouse Motor Neuron Cell Line
PBS	Phosphate Buffered Saline
PEA3	Polyomavirus Enhancer Activator 3
PNT	Pointed Domain
PVDF	Polyvinylidene difluoride
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SUMO	Small Ubiquitin-like Modifier
TBST	Tris-Buffered Saline with Tween-20
TEMED	Tetramethylethylenediamine
mM	millimolar
ng	nanogram
µl	microliter

1. INTRODUCTION

The Pea3 (E1AF/ETV4) transcription factor is a member of the ETS family that plays a crucial role in neurite outgrowth [1]. During brain development, various growth factors such as fibroblast growth factor, nerve growth factor, and glial cell line-derived neurotrophic factor regulate it by phosphorylating Serine/Threonine amino acids through the MAPK/ERK pathway [2]. While researchers have identified some Pea3 phosphorylation sites, most of them remain unknown. It has also been shown to have some posttranslational modifications such as SUMOylation and acetylation [3].

Previous studies in our laboratory on neural cell lines have shown that the Serin 90 motif is important on the neurite extension of Pea3 [1]. Other studies in the literature, there are some findings that the regulation of Pea3 stability can be achieved by phosphorylation and SUMOylation [4].

Although we believe that this thesis will illuminate an important molecular mechanism for axon elongation and axonal regeneration, it is thought that Pea3 protein will be important for new treatment approaches and designs in repair and regeneration of the nervous system.

In this thesis study, the importance of Serine 90, 101, 192, 285 and 458 motifs of the Pea3 protein, which we determined to be important for neurite extension, will be investigated in terms of phosphorylation and SUMOylation, and the relationship of Pea3 stability with phosphorylation and SUMOylation will be examined.

2. LITERATURE

2.1. ETS Transcription Factor Family

The ETS-domain transcription factor family also known as E26 (E Twenty-Six) transformation-specific family is unique to metazoans [5]. The avian erythroblastosis virus (E26), which contains the v-ets ("E twenty-six") oncogene, served as the source for the initial name of this protein and its subsequent family. Chickens infected with AEV showed induction of erythroblastosis and sarcoma development [6]. Different animals have different members from the ETS family members. Homo sapiens have 27 [7] members while Mus musculus has 27 [7], Drosophila Melanogaster has 8 [8], and Caenorhabditis elegans has 10 [9]. Although a conserved sequence is observed especially in ETS domain regions and other regulatory domains, they are involved in many different biological pathways [10] including hematopoiesis and immune response [11], epithelial-mesenchymal transition [12], angiogenesis and vasculogenesis [13], cancer and oncogenesis [14], neural development [15], and wound healing and regeneration [16].

2.1.1. ETS Domain Structure

ETS family proteins are transcription factors that recognize certain DNA sequences containing GGAA/T (Ets binding sites, EBS), which have a conserved winged helix-turn-helix DNA binding domain (ETS domain) [17].

The main common feature of ETS family proteins is that they contain 85 amino acid sequences in the C-terminal and the sequence of the ETS domain is highly conserved [18]. This motif is rich in purines [6]. The structure of ETS is a winged helix-turn-helix motif, which consists of 3 α-helices and 4 β-sheets during its transcriptional activity [17]. Third α-helix binds to this purine-rich region. In this region, which is the major groove, it forms hydrogen bonds with the conserved arginine and tyrosine amino acids [12]. Not only are the amino acid arginine and tyrosine located in the recognition site, but also other amino acids located at both the 3' and 5' ends are critical for binding. ETS proteins can recognize up to approximately 9 bases [19].

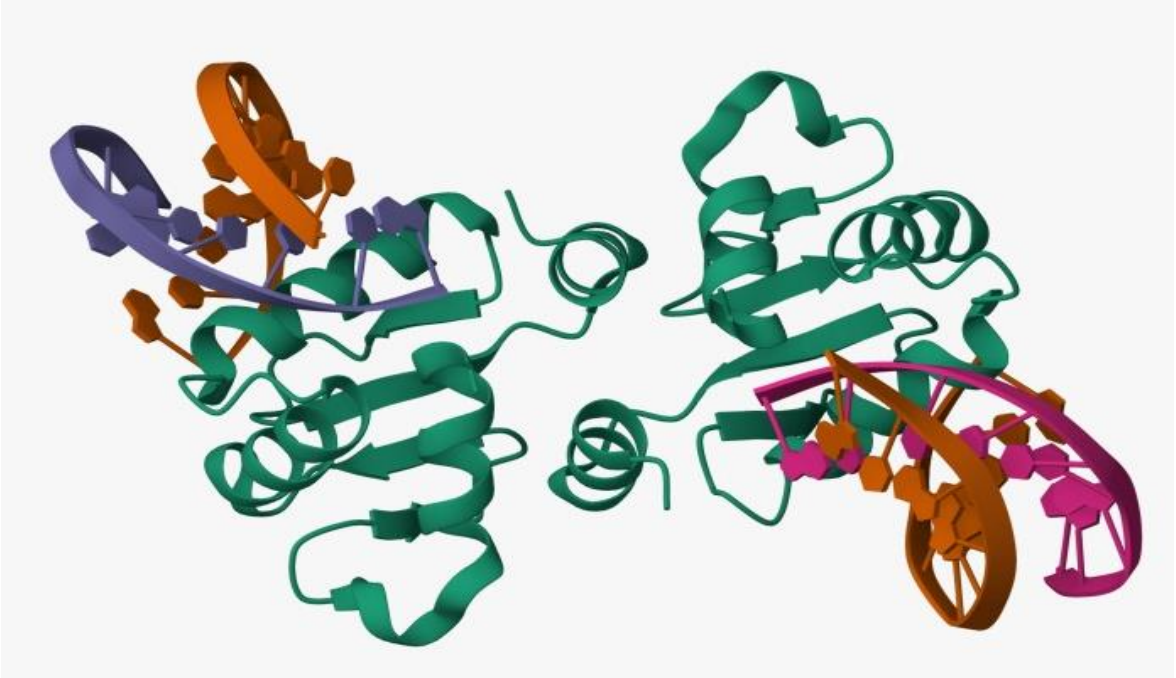

Figure 2.1. 3D structure of Pea3 protein. Pea3 demonstrated in yellow (two molecules), and DNA molecules demonstrated in orange, pink and purple (PDB code 4UUV).

Some members of the ETS transcription factor family have other conserved domains like the pointed, transactivation, and repressor domain. Pointed (PNT) domain is critical for protein-protein interaction proteins [17]. The domain is characterized by a compact, globular structure consisting of several alpha-helices and beta-sheets [20]. The most important role of the domain is the mediating the protein-protein interactions. This allows ETS family members to form heterodimers with other transcription factors and cofactors, enabling them to regulate gene expression in a collaborative manner. And also dimerize with themselves or with other transcription factors. This dimerization can significantly influence the DNA-binding specificity and target gene selection of ETS proteins [21].

2.1.2. ETS Proteins Signaling Pathways

ETS family members can play a role in expression regulation positively and/or negatively. With its signal pathways, the ETS family is involved in many pathways such as embryo development, control of cell division, apoptosis, migration, and cell differentiation, with its activation and/or inactivation [17]. Therefore, they play role in pathogenesis of many diseases, especially in cancer [22]. Overexpression, overactivation, or loss of suppression of ETS protein family members can cause a loss of control of the cell cycle, and an increase in angiogenesis which will accelerate the metastasis can be seen in many types of tumors. ETS1, ETS2, PEA3, ER81, and ELF-1 overexpression can be seen in particularly breast cancer [23, 24, 25, 26, 27].

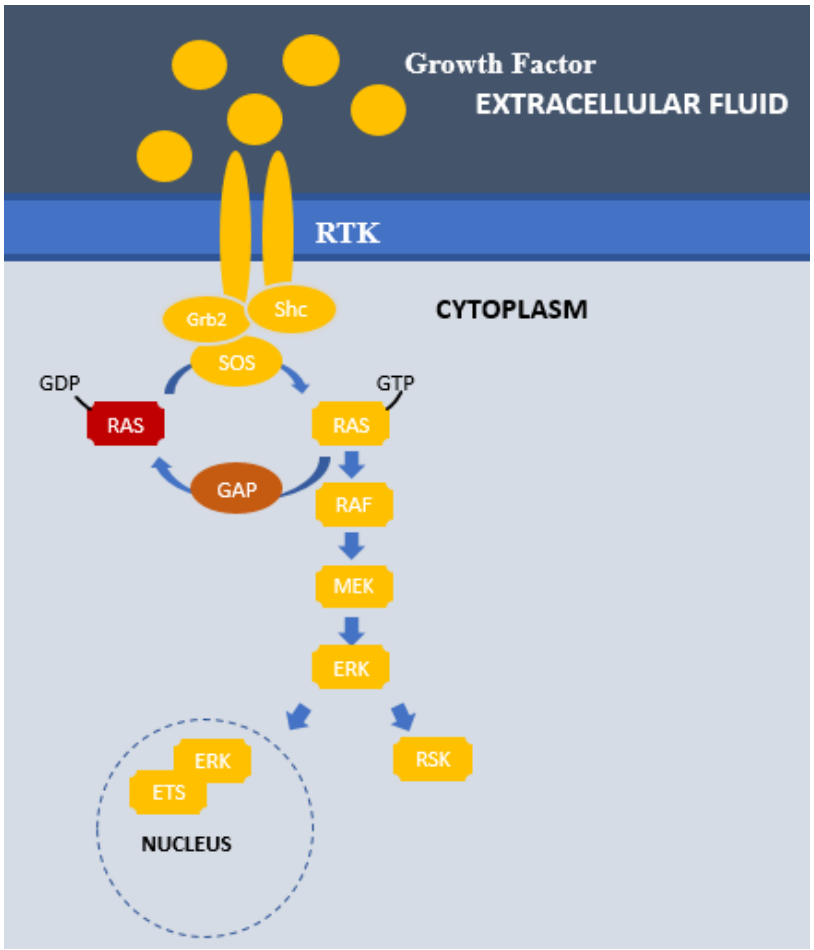

Figure 2.2. Regulation mechanism of ETS factors through MAPK signal cascade

Notably, some ETS family members are phosphorylated by the RAS/ERK/MAPK pathways [28]. This phosphorylation can change the DNA affinity [29], recruitment of other activator proteins [30], and chancing subcellular location [31]. Posttranslational

modifications, including such as phosphorylation and SUMOylation, are mediated via signal cascades [2].

2.2. PEA3 Subfamily

Pea3 subfamily has 3 members: Etv1/Er81 (Ets translocation variant 1), Etv4/E1AF (PEA3), and Etv5 (ERM) [33]. All Pea3 subfamily members have a highly similar sequence for both the C-terminal domain and the N-terminal domain [28]. The PEA3 subfamily is correlated with the coordination of motor movements, hormonal regulation, the developing nervous system, axonal guidance, metabolism, branching morphogenesis, and tumor metastasis [34, 35]. Also, PEA3 subfamily proteins are essential for neuronal differentiation [36].

The Pea3 subfamily is activated by MAPK and Erk signaling pathways. Also regulated by other posttranslational modifications, including SUMOylation, ubiquitination, and acetylation [28].

Figure 2.3. Schematic representation of the members of PEA3 subfamily. The left side is N-terminal, and the right side is C-terminal. The acidic domains are represented in light blue.

As shown in Figure 2.3. Members of the PEA3 subfamily contain a DNA binding domain at the C-terminus and two acidic activation domains at the C- and N-terminus. DNA binding and transactivation of PEA3 proteins. Transcriptional activity is provided by the homologous N- and C-terminal acidic domains, while DNA binding is provided by the ETS domain. The most well-known posttranslational modification mechanisms on Pea3 are SUMOylation and acetylation. According to evidence from the ERK/MAPK pathway, activation encourages acetylation and SUMOylation [37].

DNA binding and transactivation of PEA3 proteins are also regulated by other posttranslational modifications, including SUMOylation, ubiquitination, and acetylation. Acetylation of Lysine 116 of ER81 has been shown to increase DNA binding (a corresponding portion is also present in ERM but not PEA3). Both an activator and a repressor of transcription, Elk-1's activities are triggered by phosphorylation and SUMOylation, respectively. The conserved residues K249 and E251, which were discovered using alanine amino acid scanning mutagenesis of the R motif, are crucial for repressive function [38, 39, 40]. COP1 has been shown to induce polyubiquitination and degradation of all three PEA3 proteins, with modification of Pea3 specifically by ubiquitination, specifically Lysine 222, SUMO over 256 and to a lesser extent 96 [41, 42, 43]. The same Lysine residues on PEA3 can be mutually separately acetylated by SUMOylation, Lysine 96 is likely to be affected by the phosphorylation of SUMOylation on S101 [41]. The SUMO modification of the ERM on Lysines 89 has been shown to inhibit the transactivation capacity of 263, 293 and 350 [43].

Five locations in PEA3 match the ΨKXE (ψ is a hydrophobic amino acid) core SUMO consensus sequence. From humans to zebrafish, PEA3 family proteins share these locations. K96, which complies with the PDSM consensus sequence, has a possible proline-directed serine phosphorylation site three residues after the core motif's conclusion [38]. When the ERK pathway was not activated, increased SUMOylation of K96 by the addition of this phospho-mutant residue was not seen. The half-life of SUMOylated PEA3 was much less than that of bulk PEA3 levels [41].

Acetylation of Pea3 is p300-dependent. This acetylation increases PEA3's ability to transactivate by potentiating ERK MAPK pathway signaling. This acetylation increases PEA3's ability to transactivate by potentiating ERK MAPK pathway signaling. K96 appears to be one of the most crucial residues for both acetylation and in particular for p300 binding, which raises the possibility that it might serve as the initial event in the increasing acetylation of PEA3 [3].

2.2.1. The Biological Roles of Pea3 Proteins

Pea3 proteins are known to be important in nerve differentiation in the neural crown region and retina in the zebrafish model [44], whereas in mouse and chick models, they are

expressed in high amounts in many regions such as the spinal cord and hindbrain, midbrain, ventral forebrain (the highest expression is Etv4/Pea3) are reported [45, 46, 47, 48, 49]. Pea3 has been shown to mediate Fibroblast Growth Factor (FGF) signaling in adult mice [50]. It has also been reported that the regulation of Pea3 protein by FGF18 signaling determines the laminar position in the developing neocortex [51], and it has been shown that Xenopus XER81 is partially stimulated by FGF in the embryo and is primarily expressed in the neural crown, eyes, and kidney [52, 53]. In zebrafish embryos, it has been demonstrated that the expression of fgf3 and fgf8 controls the expression of Erm and Pea3 in the midbrain-hindbrain structure [54].

More recently, it has been shown that Pea3 is upregulated in Dorsal Root Ganglion cells (DRGs) upon NGF (Nerve Growth Factor) signaling during target innervation and is regulated by GDNF (Glial-Derived Neurotrophic Factor) during motor neuron cell body positioning [55]. In contrast, FGF3-mediated ERK1/2 (Extracellular Signal Regulated Kinase) signals have been found to regulate Pea3 in hindbrain development. It has been observed that Etv1/Er81, which is regulated by FGF2 signal, controls the folding of the brain [56].

Despite many reports of regulation of PEA3 family members by growth factor signaling, detailed mechanistic studies of phosphorylation are only available for ERM and ER81. For example, phosphorylation of the ERM by protein kinase A (PKA) at S367 at the border of the ETS domain increases its transactivation capacity while decreasing the DNA binding ability of the ERM [34, 57]. PKA has also been shown to phosphorylate ER81 on residues S191, S216, S334; S334 phosphorylation severely reduces DNA binding but increases transactivation [58].

DeSUMOylation is produced as a result of MAP kinase pathway activation, which also helps to activate transcription. The ERK pathway is activated, which enhances PEA3 SUMOylation, which is a crucial event in enhancing PEA3's transactivation capabilities. Therefore, many functional interactions between the ERK and SUMO pathways ultimately result in transcriptional activation, with various mechanisms [58].

SUMOylation of PEA3 also makes it less stable. Compared to the typical antagonistic relationship between SUMOylation and ubiquitin-mediated proteolysis. ERK pathway

activation results in increased PEA3 ubiquitination. As another member ERM has recently been revealed to be ubiquitinated and exhibit both poly- and monoubiquitination. For PEA3, ubiquitination is a conserved property of the related PEA3 subfamily. PEA3, SUMOylation is crucial for PEA3's polyubiquitination effectiveness. This points to a method whereby ubiquitin ligase is recruited in a SUMO-dependent manner.

3. MATERIALS AND METHODS

3.1. Plasmids

Plasmids were used in this thesis, cloned by Merve Üstün from AxanLab. Mutant mouse Pea3 (mPea3) plasmids were created by site-directed mutagenesis of wild-type mPea3 plasmids expressing pCMV-3 Tag-6 (Stratagene). With this method, S90A, S101A, S192A, S285A, and S458A plasmids were created and used as Pea3 phospho-mutants. Also, we used empty pCMV-Flag plasmid as a control and pCMV-Flag-mPea3 plasmid as a wild type Pea3. To check the transfection efficiency, pEGFP-N2 (Addgene) was used.

3.2. Competent Cell Preparation

To obtain competent cells, the DH5α strain of *Escherichia coli* was used. *E.coli* were inoculated into 30 mL of sterile LB broth for 18 hours incubation at 37^{o}C and shook at 200 rpm. After incubation 0,5 mL of bacteria was re-inoculated into a new 50 ml of LB Broth (w/o antibiotics) in a 250 ml Erlenmeyer flask for more oxygenation of the broth. The new culture was incubated at 37^{o}C with shaking at 200 rpm. OD600 value measurement is taken every 30 minutes until the culture's OD600 value reaches 0,4. The culture was incubated on ice for 15 minutes after the incubation culture was transferred into cold 50 mL falcon tubes. Then the falcon tubes were centrifuged at 4100 rpm for 12 minutes at 4^{o}C. Supernatants were removed and the cell pellet re-suspended gently with pipetting in 1 ml ice-cold sterile 50 mM $CaCl_2$. After resuspension 2 mL 50 mM $CaCl_2$. Tubes were incubated at 4^{o}C for 30 minutes. Then, tubes were centrifuged at 4100 rpm for 12 minutes at 4^{o}C. Supernatants were removed and then cell pellets were re-suspended gently with pipetting with 510 µL ice-cold sterile 50 mM $CaCl_2$. 90 µL of 87% Glycerol was added to tubes and mixed very well. For long term storages cells were stored -80^{o}C freezer.

To obtain fresh competent cells, the DH5α strain of *E. coli* was used. *E. coli* were inoculated into 5 mL of sterile LB broth (w/o antibiotics) for 18 hours incubation at 37^{o}C and shook at 200 rpm. After incubation 0,5 mL of bacteria was re-inoculated into a new 50 mL LB Broth (w/o antibiotics) in a 250 mL Erlenmeyer flask for more oxygenation of the broth. The new culture was incubated at 37^{o}C with shaking at 200 rpm. OD600 value

measurement is taken every 30 min until the culture's OD600 value reaches 0,4. The culture was incubated at 4°C for 15 minutes. Then culture was transferred into cold 50 mL falcon tubes. They were centrifuged at 4100 rpm for 12 minutes at 4°C. Supernatants were discarded and the cell pellet resuspended gently with pipetting in 1 mL ice-cold sterile 50 mM $CaCl_2$. After resuspension 4 mL 50 mM $CaCl_2$. Tubes were incubated on ice for 30 min. Then, tubes were centrifuged at 4100 rpm for 12 minutes at 4°C, again. Supernatants were removed and then cell pellets were re-suspended gently with pipetting with 100 µL ice-cold sterile 50 mM $CaCl_2$.

3.3. Transformation

Stock competent (*E.coli* DH5α) cells were thawed on ice before use, but fresh competent cells were used directly. 300 ng/µL plasmid was added into 50 µL of the competent cell for transformation. Although the amount of plasmid added varied in volume, the added volume of plasmid was not to exceed one-tenth of the total volume to increase the yield. After the incubation, tubes were transferred to 37°C for 90 sec. Then tubes were put in ice for 2 minutes. 950 µL of SOC Medium added to tubes. Cells were incubated for 1 hour while shaking at 200 rpm at 37°C. After incubation cells 200 µL of medium were inoculated to LB broth agar with antibiotic. 700 µL of remaining medium centrifuged at 4100 rpm at room temperature for 12 minutes. Pellet was re-suspended in 200 µL of LB medium and re-inoculated to LB broth agar with the antibiotic (for mPea3 plasmids Ampicillin and for GFP plasmid Kanamycin was used). Then, plates were incubated at 37°C overnight. Two different negative control groups were used to control the contamination of plasmid DNA. For negative control groups, the same transformation protocol was used but instead of the plasmids sterile ddH_2O was used.

3.4. Plasmid Isolation

To isolate the plasmid DNA from transformant bacteria alkaline lysis protocol was used. Single colonies were picked with a sterile loop to inoculate in 100 mL sterile LB broth in a 250 mL Erlenmeyer flask with appropriate antibiotics depending on the plasmid. The flask was incubated at 37°C with shaking at 200 rpm overnight. Then bacterial culture was divided into two 50 mL falcons. Then falcon tubes were centrifuged at 4500 rpm for 15 min at 4°C. 800 µL Solution I (25mM Tris, 10mM EDTA, 50mM glucose, pH 8.0) added into

each falcon and mixed well with pipetting and vortexing and then, incubated at room temperature for 5 minutes. After the incubation, the mixture was divided into two. 800 µL Solution II (0,2 N NaOH, 1% SDS) was added to each falcon. Falcons were mixed by turning them upside down and incubating them for 5 minutes on ice. After 3 minutes falcons are remixed by turning them upside down. For each falcon, 600 µL of Solution III (3M potassium acetate, 11,5% acetic acid) was added. Falcons were mixed by turning them upside down and incubating them for 10 minutes on ice. After every 3 minutes falcons were remixed by turning them upside down. When the incubation was completed, falcons were centrifuged at 9500 rpm at 4°C for 1 hour. Each 800 µL supernatant was transferred into a sterile 1,5 mL microcentrifuge tube. For each tube, 500 µL of isopropanol was added for precipitation. Falcon tubes were incubated at room temperature for 1-hour for maximum yield. Then, tubes were centrifuged at 11000 rpm for 30 minutes at room temperature. After, the supernatant was discarded, and pellets were air-dried until the pellet was transparent. Later, the pellet was dissolved with pre-heated (60°C) sterile distilled water. For every 100 µL plasmid 10 µL RNase A (10mg/ml) was added. Plasmid DNA concentrations were calculated with a spectrophotometer.

3.5. Cell Culture

For the experiments, mouse motor neuron cell line, NSC-34, was used. DMEM medium with 4,5 g/L glucose, L-glutamine and without pyruvate was used. To get a complete medium, 10% fetal bovine serum and 1% penicillin/streptomycin were added. To start the cell culture, cells were thawed and then mixed with complete growth medium pre-heated to 37°C. Then cell-medium mixture started to incubate at 37°C and 5% in a 35 mm tissue culture dish. After 2 hours the medium was changed due to the toxic effects of DMSO. Cells were subcultured when the flask or dish plate had 80% confluency. First the medium was discarded, and the surface was washed to remove all the FBS residue and dead cells and their debris. 0.25% 1X trypsin-EDTA solution was used to harvest cells from the flask or plate. After the addition of trypsin cells were incubated in CO_2 incubator. To inhibit trypsin activity, complete growth media was added. The cell mixture was centrifuged at 2000 rpm at room temperature for 5 minutes. The supernatant was removed, and the pellet was resuspended with fresh complete media. For the seeding of the cells, cells were counted to the desired amount with a hemocytometer and then seeded into T25 or T75 flasks.

To store the cells, a freezing medium was used which includes 20% FBS and 10% DMSO. Respectively, cells were kept at -20ºC for 2 hours then -80ºC overnight and then transferred to liquid nitrogen tank.

3.6. Transfection of NSC-34 Cell Line

Cell transfections were performed with Lipofectamine 2000. Neither the method nor plasmids used for the transfection insert genetic material into the chromosomal DNA. Before the experiment, to control the transfection efficiency, 1500 ng of GFP plasmids were transfected to the NSC-34 cells and protocol supported by manufacturer was performed. Afterward, cells were checked with a fluorescent microscope after 48 hours.

To express the plasmids in NSC-34 cells, firstly, cells were seeded into tissue culture dishes. For global SUMOylation assay and immunoprecipitation experiments, a 100 mm tissue culture dish, 1,5 x 10^6 cells were seeded. Incubated overnight at 37ºC and 5% CO_2. Then two tubes were prepared. For the first tube, 150 µL of plasmid DNA (100 ng/µL) for a total of 15 ng plasmid DNA and 750 µL Opti-MEM medium. For the other tube, 36 µL Lipofectamine 2000 and 864 µL OptiMEM medium. Both tubes were mixed with a 1:1 ratio. And mixed well with gentle pipetting. The mixture was incubated at room temperature for 5 minutes. Later, the mixture was given slowly to the cells in the form of droplets. The dishes were mixed gently. Incubated for 48 hours at 37ºC and 5% CO_2. Later, protein isolation was done.

3.7. Cycloheximide Chase Assay

Cycloheximide was used to inhibit protein synthesis in the transfected cells to investigate protein stability. The transfection was completed as mentioned above but cells were incubated after the transfection for 40 hours. Then, cells were treated with 100 ug/mL cycloheximide and collected at various time points as 0-, 3-, and 7-hour.

3.8. Total Protein Isolation

After the transfection, for cycloheximide assay for 40 hours and both global SUMOylation assay and immunoprecipitation for 48 hours, the medium was removed from tissue culture dishes and washed with ice-cold PBS to dispose of any residual medium and cell debris. Then 0.25% 1X trypsin-EDTA solution was used to remove cells from the plate. After the addition of trypsin were incubated in CO_2 incubator. To inhibit trypsin activity, complete growth media was added. The cell mixture was centrifuged at 2000 rpm for 5 minutes. Supernatant was discarded and the pellet was re-suspended with PBS and centrifuged again at 2000 rpm for 5 minutes at 4^{o}C. Supernatant was discarded and for $1x10^{6}$ cells, 100 µL 1X RIPA buffer was used which includes 1X protease and 1X phosphatase. To increase the cell lysis, the sample was pipetted while on ice and vortexed harshly. Then, the tubes were frozen and thawed 3 times with liquid nitrogen. Tubes were centrifuged at 13000 rpm for 10 minutes at 4^{o}C. After the centrifugation, the supernatant which contains proteins was transferred into a new, sterile pre-chilled micro centrifugation tube. Proteins are stored at -80^{o}C for the long term.

3.9. Nuclear Protein Isolation

To check the SUMOylation levels of mPea3 protein, cell nuclear proteins were isolated. A nuclear extraction kit was used for the isolation. The transfection procedure was done as stated above. After 48 hours, cells were collected into a microcentrifuge tube. The pellet was resuspended with 100 µL 1X Pre-Extraction Buffer per 1x106 cells. Incubated on ice for 10 minutes and vortex vigorously for 10 seconds to burst out the cell membrane. Then tubes were centrifuged at 12000 rpm for 1 minute at 4^{o}C. With the centrifugation, nuclear proteins were formed in a pellet on the bottom of the tube. The supernatant which includes cytoplasmic proteins was discarded. 2 volumes of 1X extraction buffer were used to resuspend the pellet. The extract was incubated on ice for 15 minutes. To increase the protein concentration, tubes were vortexed for 10 seconds every 3 minutes. Then tubes were centrifuged at 14000 rpm for 10 minutes at 4^{o}C.

3.10. Bicinchoninic Acid Protein Assay

To determine the protein concentration, a bicinchoninic acid protein assay was used. With this method, protein levels are determined with colorimetric changes. For the calculation of protein concentration, a bovine serum albumin standard curve was used. 8 different concentrations of bovine serum albumin were used to create a standard curve as in Figure 3.10.1. 2000 µg/mL, 1500 µg/mL, 1000 µg/mL, 500 µg/ml, 250 µg/mL, 125 µg/mL, 25 µg/mL, and 0 µg/mL of BSA concentration was used. 25 µL of each standard and sample protein was mixed with 200 µL of Working Solution, which is Solution A and B in a ratio of 50:1 and mixed well in a 96-well plate. Then the plate was incubated at 37°C for 30 minutes. After the incubation, the plate cooled down to room temperature and was measured with a microplate reader at 562 nm absorbance.

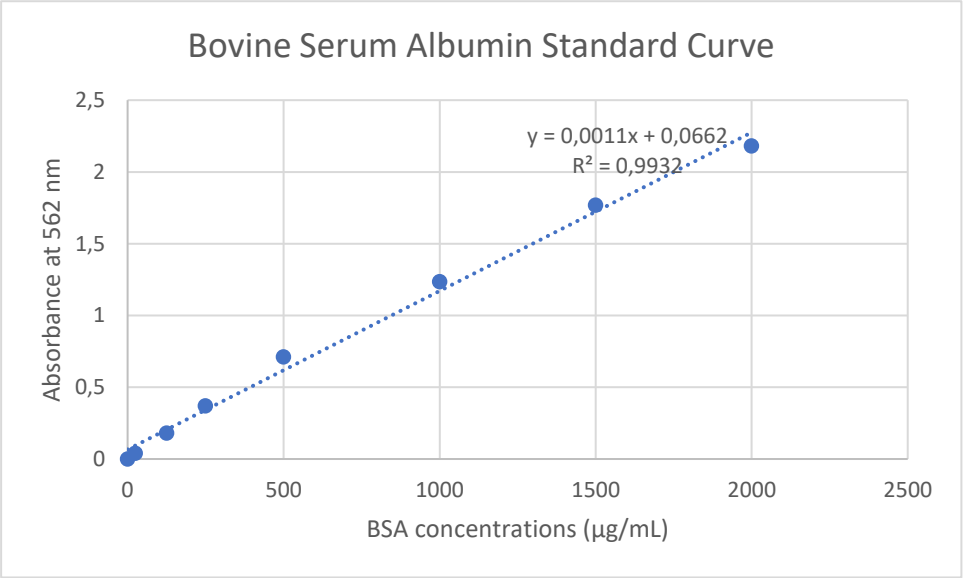

Figure 3.1. Bovine Serum Albumin Standard Curve

3.11. Global SUMOylation Assay

For the global SUMOylation assay, nuclear extraction proteins were used. This assay is used for the detection of the SUMOylation percentages of the mPea3. First of all, mPea3 antibodies and negative control protein were diluted to 2ug/mL with binding buffer. For both mPea3 and negative control, 100 µL of protein was added and covered with

a foil. Incubated for 2 hours at 37°C. Then solutions were removed from each well and 150 µL blocking solution was added. Incubated for 45 minutes at room temperature. Each well was aspirated and washed 3 times with 1X wash buffer. 28 µL of SUMO assay buffer was added to each well and 10 ug of nuclear extract was added along with the negative control. Wells were covered with foil and incubated for 60 minutes at room temperature. Each well was aspirated and washed 3 times with 1X wash buffer.

For the detection of the SUMO proteins, detection solution was prepared. 1 µL SUMO antibody, 0,5 µL signal report solution, and 10 µL 1X were buffer mixed and incubated for 10 minutes at room temperature. Then 20 µL negative control was added and at room temperature incubated for 15 minutes. Lastly 970 µL 1X wash buffer added. 50 µL of detection solution was used for each well and incubated at room temperature for 60 minutes while shaking on an orbital shaker at 100 rpm. Each well was aspirated and washed 6 times with 1X wash buffer. 100 µL of color development solution was added into wells and incubated at room temperature for 10 minutes in a dark environment. 50 µL stop solution was added and measured at 450 nm. Global SUMOylation percentage calculated according to Equation 3.1.

$$\frac{Mutant\ mPea3\ sample\ OD - negative\ control\ OD}{(pCMV\ sample\ OD - negative\ control\ OD)}\ x\ 100 \quad (3.1.)$$

3.12. Immunoprecipitation

To detect the phosphorylation levels of mPea3, mPea3 proteins should be isolated. All mPea3 mutants have a flag tag (DYKDDDDK) of their N-terminus due to plasmid construction. To isolate the mPea3, anti-flag magnetic agarose beads were used. First, the magnetic agarose beads were heated to room temperature and mixed with repeated inversions and gentle vortexing. 50 µL of magnetic agarose beads were used. with 450 µL of binding buffer mixed with agarose bead with vortexing. After, micro centrifuge tubes were put into a magnetic stand. Then beads were collected against the side of the tube. Supernatant were discarded and another 500 µL of binding buffer was added to Eppendorf tubes, mixed well, and with magnetic stand beads were collected to side of the tube and supernatant were discarded. This step was repeated one more time. Total protein samples

which were isolated from transfected cells diluted with binding buffer to 300 μL and then mixed well with gentle pipetting and vortexing with washed magnetic beads. Tubes were incubated at room temperature for 20 minutes. Beads were collected with the magnetic stand. The supernatant was removed. 500 μL wash buffer was added into tubes, mixed well, and with magnetic stand beads were collected to the side of the tube and the supernatant was discarded. This step was repeated one more time.

For the elution, acid elution protocol was used. 100 μL of elution buffer (pH 2.8) was used. Mixed well and incubated for 5 minutes at room temperature with frequent vortexing. With a magnetic stand, beads were collected to the side of the tube, and the supernatant was collected into microcentrifuge tube. 45 μL of neutralization solution was added immediately.

3.13. SDS- PAGE and Western Blotting

Proteins were isolated mixed with 6X Laemmli loading buffer and boiled for 5 minutes at 95^{o}C. Then 15-30 µg protein used for SDS-PAGE gel. Gels were loaded into the western blot tank and the tank filled with 1X running buffer (1 g of SDS, 3,02 g of Tris-base and 14,4 g of Glycine were dissolved in 10 liters of ddH_2O.). The voltage was adjusted at 90V. When protein samples passed to the resolving gel, voltage increased to 120V. At 120V samples were run for 90 minutes. SDS-PAGE gels prepared according to Table 3.1. and 3.2.

Table 3.1. Resolving Gel

Resolving Gel	**Volume (for 1 gel)**
ddH_2O	4,825 mL
40% Acrylamide(Sigma, A9099)	2,475 mL
1.5 M Tris pH: 8,8	2,5 mL
%10 SDS(Merck, 817034)	100 µL
%10 APS(Sigma, 09913)	100 µL
TEMED(Thermo Scientific, 17919)	4 µL

Table 3.2. Stacking Gel

Stacking Gel	**Volume (for 1 gel)**
ddH_2O	2,25 mL
40% Acrylamide(Sigma, A9099)	375 mL
1 M Tris pH: 6,8	380 µL
%10 SDS(Merck, 817034)	30 µL
%10 APS(Sigma, 09913)	30 µL
TEMED(Thermo Scientific, 17919)	4 µL

Proteins were transferred into PVDF membrane after the SDS-PAGE electrophoresis. First, PVDF membranes were prepared to align with gels and wetted with distilled water and absolute methanol for a minute each. Before use, PVDF membranes are maintained in 1X transfer buffer that contains 15% methanol. Additionally, 1X transfer buffer was used to wet fiber pads, filter papers, and cassettes.

Fiber pad located on the anode side of the sandwich apparatus of the transfer system, 2 filter paper were placed. Then, a PVDF membrane or nitrocellulose, two pieces of filter paper, and an additional fiber pad were placed (on the cathode side) on the gels that had been placed on the filter papers.

The system and 1X transfer buffer (14,4g glycine and 3,02g Tris in 1 liter of ddH_2O) with methanol were placed in the blotter system. The system was then powered by a power source for 45 minutes at 100V.

Membranes were twice rinsed with TBS-T for 5 minutes each after transfer. PVDF membranes were treated with blocking solution (%5 non-fat dry milk) with shaking for 1 hour at room temperature to prevent nonspecific protein binding with antibodies. The membranes were then rinsed three times for ten minutes with TBS-T. The primary antibody was made with either 3% BSA or 5% nonfat dry milk. The membranes were then incubated with the primary antibody at 4°C on a shaker for the following day. The primary antibody dilution range and the solution those antibodies were diluted in are displayed in Table 3.12.3. and 3.12.4.

Table 3.3. Primary Antibodies and their dilution

Primary Antibody	**Solution**	**Ratio**
Anti-PEA3 (Santa Cru, sc-113)	5% Non-fat Dry Milk	1:1000
Anti-Tubulin (Abcam, ab4074)	3% Bovine Serum Albumin	1:1000
Anti-Phospho-(Ser/Thr) Phe (Cell Signaling Technology, 9631)	3% Bovine Serum Albumin	1:1000

Membranes were rinsed with TBS-T solution three times for a total of 10 minutes the following day. TBS-T solution was used to dilute the secondary antibodies. Membranes were incubated for one hour at room temperature with the secondary antibody solution while being shaken. Table 3.12.4 displays the secondary antibody dilution range and the solution that the antibodies were diluted in.

Table 3.4. Secondary Antibodies and their dilution

Secondary Antibody	Solution	Dilution
Anti-mouse IgG HRP (Abcam, ab6789)	5% Non-fat Dry Milk	1:5000
Anti-rabbit IgG HRP (Abcam, ab6721)	5% Non-fat Dry Milk	1:5000

After incubating with the secondary antibody, PVDF membranes were rinsed 3 times with TBS-T for 10 minutes.

To visualize protein bands, western HRP substrate was applied. They were incubated for one minute following the application of substrate to the membrane. Blots underwent ChemiDocTM XRS+ System scanning.

3.14. Bioinformatic Analysis of SUMOylation Sites of Pea3

Protein sequences obtained from UniProt and sequenes was analyzed in GPS-SUMO tool [59] to identify SUMO sites and matched with phosphorylation sites which were identified in previous studies [1] with both bioinformatic tools.

4. RESULTS

4.1. Expression Analysis of Mutant mPea3 Proteins in NSC-34 Motor Neuron-Like Cell Lines

In NSC-34 mouse motor neuron cell line, the expression investigation of mutant mPea3 proteins was carried out. Mutant mPea3 plasmids were transfected into cells, and the cells were then cultured with complete DMEM medium at 37°C, and 5% CO2. After 2 days, the proteins were extracted. Only mPea3 and their mutants have the flag-tag so magnetic flag beads were used to isolate the mPea3.

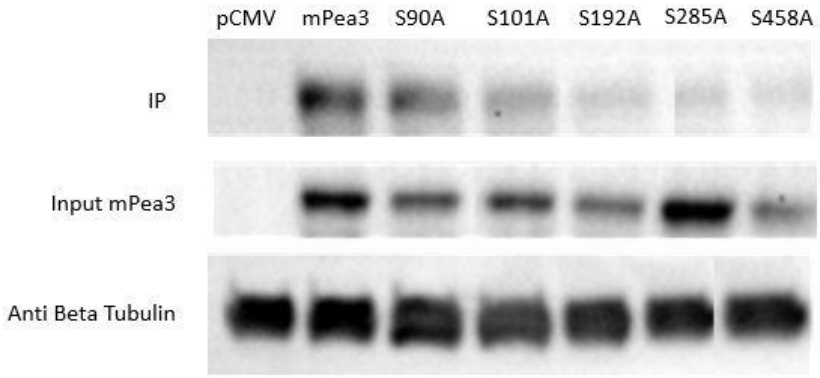

Figure 4.1. Western Blot of mutant mPea3 proteins from transfected NSC-34 cells.

According to the western blot results, endogenous mPea3 can't be seen on the western blot analysis due to its low-level expression. Mutant proteins showed lower levels of expression compared to wild type. S90A and S285A mutants show higher expression level compared to other mutans.

4.2. Phosphorylation Analysis of Mutant mPea3 Proteins in NSC-34 Motor Neuron-Like Cell Lines

To detect the phosphorylation levels of mPea3 and their phospho-mutants, serine/threonine antibody was used for the western blot. After the isolation of mPea3 with flat-tagged magnetic beads, proteins were run in a SDS-PAGE gel and the membrane incubated with serine/threonine antibody.

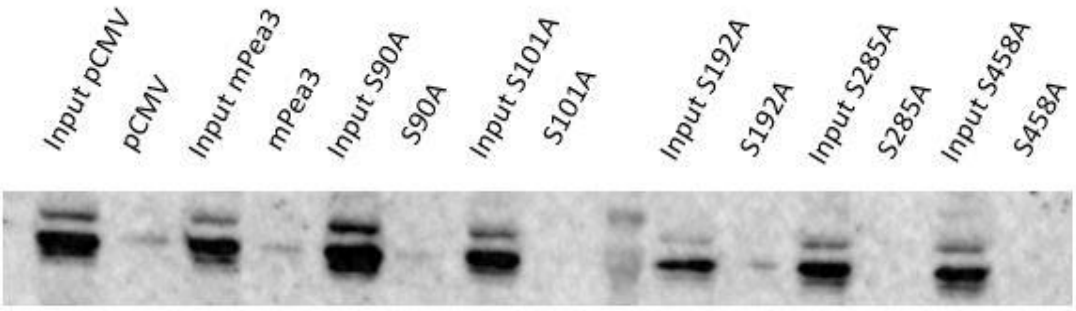

Figure 4.*2.* Western blot of input and phosphorylated mPea3. Input proteins (total proteins) and phosphorylated mPea3 proteins can be seen in figure.

For the pCMV and mPea3 groups, stronger phosphorylation levels can be seen according to mutant proteins. In S101A, S285A and S458A groups there is no phosphorylation. S192A mutant shows the most phosphorylation level among the other mutants.

4.3. Bioinformatic Analysis of Pea3 Subfamily

4.3.1. SUMOylation and Phosphorylation Site Analysis of ETV4/PEA3/E1AF

mPea3 protein sequence obtained from UniProt (P28322) was analyzed in GPS-SUMO tool [59] to identify SUMO sites and matched with phosphorylation sites which were identified in previous studies with bioinformatic tools. With this tool, ΨKXE (ψ is a hydrophobic amino acid) cores were identified.

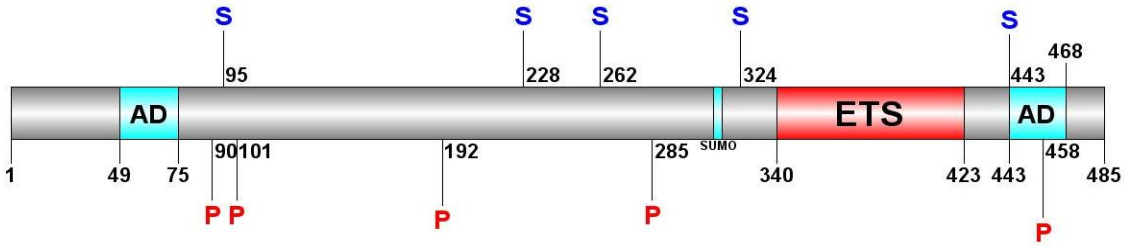

Figure 4.3. Schematic representation of the PEA3. S sites represents predicted SUMOylation sites and P represents phosphorylation sites which were used in experiments. Red box represents ETS (DNA-binding domain), and light blue box represents acidic domains. Light blue line represents SUMO-interaction site.

Table 4.1. PEA3's predicted SUMOylation sites and sequences. Red letters represent the possible amino acids for the SUMOylation.

Amino Acid Number	Sequence	Post-tranlational Modifications
95	HSPTTRIKKEPQSPR	SUMOylation
228	PYPQQNFKQEYHDPL	SUMOylation
262	PGAGVVIKQERTDFA	SUMOylation
312 - 316	LRPFPDD VCIVP EKFEGDI	SUMO Interaction
324	EKFEGDIKQEGIGAF	SUMOylation
443	DNQRPALKAEFDRPV	SUMOylation

According to Table 4.3., K95 site is between the S90 and S101 phosphorylation sites. Also, K228 and K262 sites are between S192 and S285 sites. K324 and K443 sites are very

close to ETS domain. In addition, SUMO site K443 is also the starting point of the N-terminal acidic domain.

Due to the need for further research on the sumo regions of ETV1 and ETV5, other subfamilies were also subjected to the same analysis in order to both test the accuracy of the bioinformatics results and evaluate whether there are common SUMO regions among members of the Pea3 subfamily.

4.3.2. SUMOylation and Phosphorylation Site Analysis of ETV1/Er81

ETV1 protein sequence obtained from UniProt (P41164) was analyzed in GPS-SUMO tool [59] to identify SUMO sites and matched with phosphorylation sites which were identified in previous studies with bioinformatic tools. With this tool, ΨKXE (ψ is a hydrophobic amino acid) cores were identified.

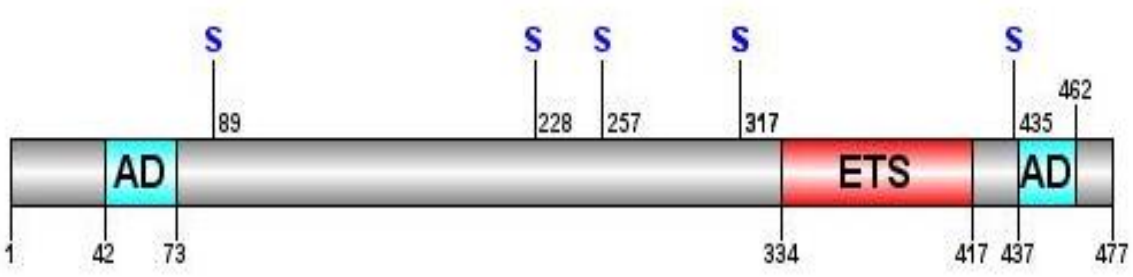

Figure 4.4. Schematic representation of the ETV1. S sites represents predicted SUMOylation sites. Red box represents ETS (DNA-binding domain), and light blue box represents acidic domains.

Table 4.2. ETV1's predicted SUMOylation sites and sequences. Red letters represent the possible amino acids for the SUMOylation.

Amino Acid Number	Sequence	Post-translational Modifications
89	HGLPLKIKKEPHSPC	SUMOylation
228	PFPPQGFKQEYHDPV	SUMOylation
257	FPPPLMIKQEPRDFA	SUMOylation
317	EKFDGDIKQEPGMYR	SUMOylation
435	DNQRPLLKTDMERHI	SUMOylation

K89 SUMOylation site is close to the C-terminal acidic domain and K435 site is very close to the N-terminal acidic domain. K317 SUMOylation site is relatively close to the ETS domain.

4.3.3. SUMOylation and Phosphorylation Site Analysis of ETV5/Erm

ETV5 protein sequence obtained from UniProt (Q9CXC9) was analyzed in GPS-SUMO tool [59] to identify SUMO sites and matched with phosphorylation sites which were identified in previous studies with bioinformatic tools. With this tool, ΨKXE (ψ is a hydrophobic amino acid) cores were identified.

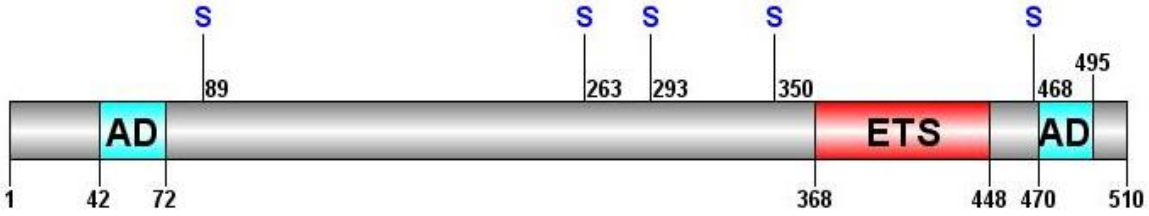

Figure 4.5. Schematic representation of the ETV5. S sites represents predicted SUMOylation sites. Red box represents ETS (DNA-binding domain), and light blue box represents acidic domains.

Table 4.3. ETV1's SUMOylation sites and sequences. Red letters represent the possible amino acids for the SUMOylation.

Amino Acid Number	Sequence	Post-translational Modifications
89	APPPTKIKRELHSPS	SUMOylation
263	PPPLQGFKQEYHDPL	SUMOylation
293	FQSPMGIKQEPRDYC	SUMOylation
350	ERLEGKVKQEPTMYR	SUMOylation
468	DNQRPFLKAESECPL	SUMOylation

K89 SUMOylation site is close to the C-terminal acidic domain and K468 site is very close to the N-terminal acidic domain. K350 SUMOylation site is relatively close to the ETS domain.

According to bioinformatic analysis, we found that our predictions are consistent with the results in the literature [58].

4.4. SUMOylation Analysis of Mutant mPea3 Proteins in NSC-34 Motor Neuron-Like Cell Lines

In NSC-34 mouse motor neuron cell line, the mutant mPea3 proteins SUMOylation levels investigated with global SUMOylation assay kit. Mutant mPea3 plasmids were transfected, and then transfected cells were then cultured with complete DMEM medium at 37°C, and 5% CO_2. After 2 days, the nuclear proteins were extracted. For the detection of SUMO levels in mPea3 proteins, wells covered with anti-Pea3 antibody.

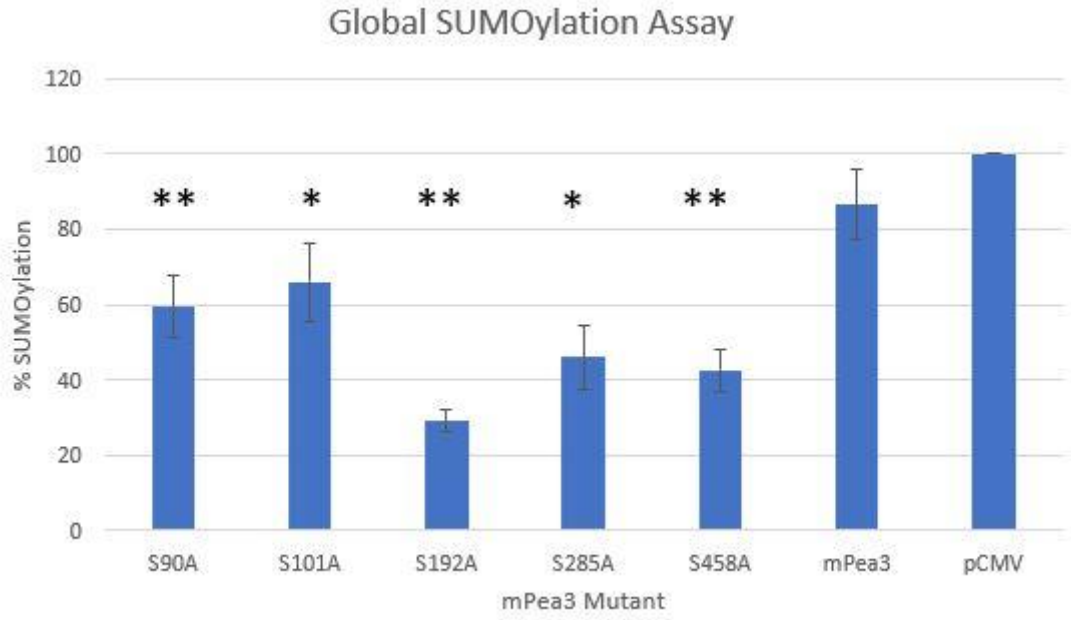

Figure 4.6. Results of the global SUMOylation assay of mutant mPea3 proteins. For statistical of the results, Student-T-Test was used (*p<0,05, **p<0,01). Results were compared against control pCMV group.

According to Figure 4.6. wild type mPea3 didn't show any difference to the control group (pCMV). Transfection processes didn't have any impact on wild type mPea3 SUMOylation level. All the mutant proteins showed a significant decrease in their SUMOylation levels. Especially in S192A, S458A and S90A mutants show a high significant change. S192A mutation causes it to lose its SUMOylation more than 70%.

4.5. Cycloheximide Chase Assay for Analysis of mPea3 Mutants Protein Stability

The stability of mPea3 mutant proteins was analyzed by cycloheximide administration followed by western blot analysis. Firstly, cells were transfected with mutant mPea3 plasmids. Then incubated for 40-hour incubation period instead of the 48-hour incubation period which was in transfection protocol. At the end of the incubation, cycloheximide was applied to the cells. This change aimed to deliver the drug to the cell before the production of mutant proteins produced from the plasmids. Cells were collected after 3- and 7-hour incubation. Control group collected before the drug administration.

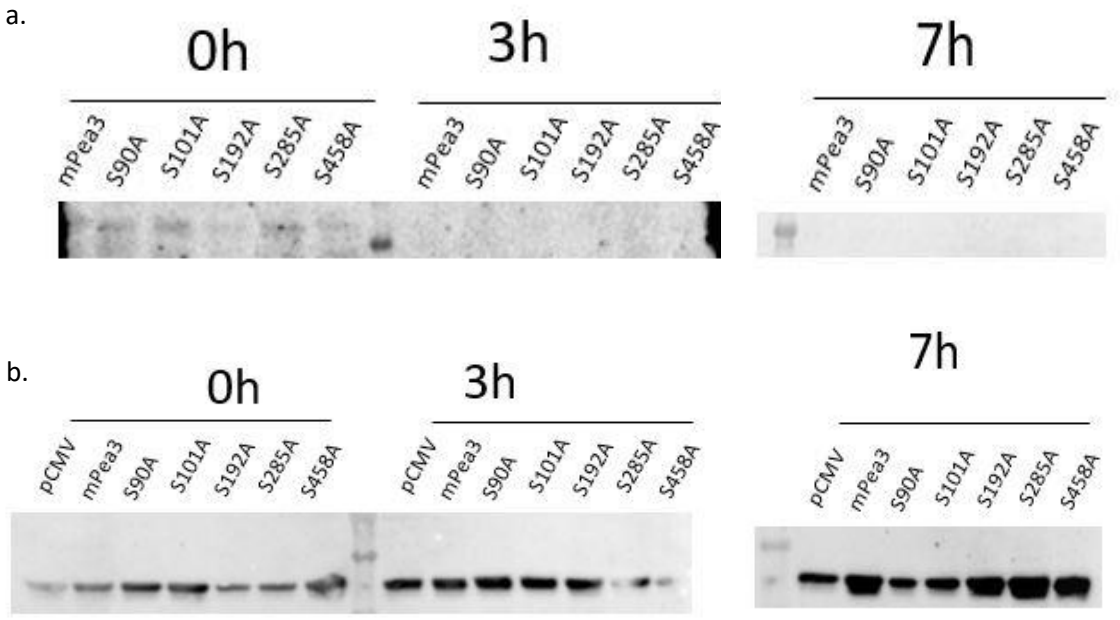

Figure 4.7. Western blot results of the 0-, 3- and 7-hours cycloheximide administration. a. mPea3 levels of cycloheximide administrated NSC-34 cells. b. ß-tubulin levels of cycloheximide administrated NSC-34.

According to cycloheximide chase assay results, in the control group mutant mPea3 proteins production was observed. However, mutant mPea3 protein production was not

observed in either the 3- or 7-hour groups after drug administration. On the other hand, loading control protein, ß-tubulin, was observed in these groups. In this case, it was observed that the cells degraded all mPea3 proteins 3-hours after exposure to the drug.

5. DISCUSSION

The Pea3 family belongs to the ETS domain transcription factor superfamily, which includes Pea3/ETV4, Erm/ETV5 and Er81/ETV1 proteins. The PEA3 subfamily is correlated with coordination of motor movements, hormonal regulation, the developing nervous system, axonal guidance, metabolism, branching morphogenesis, and tumor metastasis [2].

PEA3 subfamily proteins are essential for neuronal differentiation. Pea3 family members are regulated by the MAPK/ERK signaling pathway; it has also been shown to have some posttranslational modifications such as phosphorylation and SUMOylation [3]. The inability to elucidate the posttranslational modifications of the newly identified phosphorylation regions directly associated with axon elongation is a major deficiency in understanding the neuroregeneration mechanism. With this thesis, the importance of phosphorylation sites on SUMOylation and protein stability will be determined, and an important regulation mechanism will be shed light on.

In a previous study, researchers determined that the S90 and S458 phosphorylation sites in NSC-34 cells are extremely important in terms of neurite elongation [1]. When the axon elongation of more extensive phospho-mutants was examined, they determined that S90, S192 and S458 phosphorylation sites significantly increased axon elongation (Üstün, Kandemir et al. prepared for publication).

In this thesis we focused on the interplay of SUMOylation and phosphorylation on Pea3 Stability. Therefore, we used phospho-mutant versions of the mPea3 protein as mentioned above. We transfected motor-neuron cell line NSC-34 with expression vectors which contain these phospho-mutant mPea3 proteins.

According to Figure 4.1. the phosphorylation levels of the mutant proteins differed from both each other and wild type of mPea3. For example, S285A had a higher protein level than all other mutants, while S192A and S458A had a less protein level than all other mutants.

Also, in Figure 4.2. phosphorylation levels differ from each other. Mutant mPea3 proteins, which are already phosphorylated in low amounts due to their mutations, may have appeared to be even lower due to the amount of protein lost during IP isolation. This experiment requires technical replicates to demonstrate better that the loss of protein bands in the western blot is due to mutations. In Figure 4.2. S192A and S90A phosphorylation mutants still show some phosphorylation but S458A, S285A and S101A lost their phosphorylation completely.

To better understand SUMOylation and phosphorylation effect on protein stability, first we determined the SUMO sites with GPS-SUMO prediction tool [59]. ΨKXE (ψ is a hydrophobic amino acid) cores were identified for PEA3 subfamily. For mPea3, 5 SUMO sites were identified, and these SUMO sites are relatively close to the phosphorylation sites. These predicted SUMO sites can vary from organism to organism.

ERK MAPK pathway is responsible for SUMOylation. Activation of ERK MAPK increases the SUMO levels of Pea3. Mutated K96 and K222 amino acids inhibits the SUMOylation and trans-activation of Pea3 increases [58]. SUMOylation levels lowers transcriptional activation of Pea3 and its stability [40].

To point out that, we investigated SUMOylation levels of the mutant mPea3 proteins. According to Figure 4.6, loss of phosphorylation will significantly decrease the SUMOylation levels of the mPea3 proteins. So, there is a strong connection between SUMOylation levels and phosphorylation levels. But to detect the SUMO levels, we used global SUMOylation assay kit. Therefore, there is no way to understand which SUMO site's SUMOylation level decreased due to loss of phosphorylation. To better understand this connection, combinations of SUMO-site mutated mPea3 and phospho-mutant mPea3 can be used.

According to a research paper published, loss of phosphorylation in S101 causes the loss of SUMOylation in K96. Also, adjacent proline mutation (P102A) caused the same result. These loss of SUMOylations can occurred due to conformational changes [58]. According to Figure 4.6. S101A mutant loss of its SUMOylation level around 35% which is compatible with our data. S90 phosphorylation site is also close to the K96 SUMOylation

site. There is 40% loss of SUMOylation in S90 mutation. This finding may have resulted from the same reason.

According to Figure 4.6. the most drastic change is S192 mutation which is around 70%. Then followed by S458 and S90 which is 58% and 40%, respectively. It is found that these 3 phospho-mutants are important for axon elongation (Üstün, Kandemir et al. prepared for publication). The decrease in SUMOylation in these mutants may have increased protein stability. Due to this increase in protein stability, axon elongation can be seen more drastically.

Then we continued with cycloheximide chase assay to understand the stability changes. Unfortunately, due to the dynamic degradation and transcription of mPea3, we couldn't see incubation time difference in cycloheximide chase assay (Figure. 4.7., 4.8). Shorter incubation time with cycloheximide should be used to better understand the differences between different phospho-mutants of Pea3.

6. CONCLUSION

We used 5 different phospho-mutants to detect the interplay between SUMOylation and phosphorylation on Pea3 stability. These 5 different phospho-mutants showed less phosphorylation compared to wild-type Pea3. Then we measured the global SUMOylation levels of both wild type Pea3 and phospho-mutants. We found that all the phospho-mutants lose their SUMOylation levels significantly. S192A, S458A, and S90A mutants lost their SUMOylation more significantly compared to other mutants. These mutants have a direct relationship with increased axon elongation. Cycloheximide chase assay was applied to investigate the effect of loss of SUMOylation levels on protein stability. But both cycloheximide incubation time and instable structure of Pea3 caused the total loss of Pea3 protein. Therefore, no adequate interpretation can be made for the effect of phospho-mutants and their SUMOylation levels on protein stability. Relationship between S192A, S458A, and S90A mutants and increased axon elongation may suggest that stability of Pea3 increases due to loss of phosphorylation and SUMOylation.

This study is anticipated to shed light on a crucial biochemical mechanism underlying axon extension and neuronal regeneration.

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