

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**IDENTIFYING PEA3 PHOSPHORYLATION SITES SPECIFICALLY
REGULATED BY DIFFERENT GROWTH FACTORS**

MERVE USTUN
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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THESIS SUPERVISOR
PROF. DR. ISIL KURNAZ

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2017

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

ÇEŞİTLİ BÜYÜME FAKTÖRLERİ TARAFINDAN
DÜZENLENEN PEA3 FOSFORİLASYON
BÖLGELERİNİN BELİRLENMESİ

MERVE ÜSTÜN
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM
DALI

DANIŞMANI
PROF. DR. IŞIL KURNAZ

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2017

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SUMMARY

The ETS (E26-transformation specific) domain transcription factor family that have regulatory roles in numbers of cellular genes as activators or repressors effects fundamental circumstances such as cellular differentiation, proliferation or development etc. These transcription factors categorized into several members as their sequence homologies contain common purine-rich DNA binding sequence in all members. Pea3 (ETV4/E1AF) transcription factor which is one of the ETS family member known to be regulated by specific growth factors through MAPK/ERK pathway by phosphorylation of Serine/Threonine residues during neural development.

In the thesis, we fundamentally focused the question of which growth factor regulates which phosphorylation sites on Pea3. Substitutional mutation on putative phosphorylation sites was performed to understand whether these motifs are indeed important in growth factor induced activity of Pea3. Firstly, putative phosphorylation sites of Pea3 were mutated with replacement of amino acids from Serine to Alanine or Glutamic acid. After the transfection of mutant Pea3 plasmids into SH-SY5Y human neuroblastoma cells, various growth factors were applied. The protein expression levels were observed and compared with wild type Pea3 and among themselves by cell culture techniques, immunoprecipitation technique, Western Blot Analysis and SDS-PAGE electrophoresis. Regulation between mutant Pea3 and different promoters was also investigated with luciferase reporter assay as well as Pea3 protein expression studies. This thesis could be provided to help understanding of what kind of effects of growth factors have on Pea3 and to identify Pea3 phosphorylation sites.

Pea3 is known to have a role in neurite outgrowth, and the elucidation of Pea3 activation would contribute to understanding of process of neurite outgrowth. Thereby, it would illuminate mechanisms of neuroregeneration.

Key Words: Neurite Outgrowth, Axonal Branching, Pea3, Phosphorylation, MAPK/ERK Pathway.

ÖZET

Çeşitli genlerde aktivatör veya susturucu gibi düzenleyici rolü olan ETS (E26 transformasyon spesifik) domain transkripsiyon faktörü ailesi; hücrel farklılaşma, proliferasyon ya da gelişim gibi temel durumları etkilemektedir. Dizi homologilerine göre çeşitli alt gruplara kategorize edilen bu transkripsiyon faktörleri her bir üyesinde ortak pürince zengin DNA bağlanma dizisi çermektedir. ETS transkripsiyon faktörünün bir üyesi olan Pea3 (ETV4/E1AF) transkripsiyon faktörünün sinirsel gelişim esnasında özel büyüme faktörleri tarafından MAPK/ERK yolağı aracılığıyla Serin/Treonin rezidülerinin fosforillenmesi ile düzenlendiğı bilinmektedir.

Bu tezde temelde, hangi büyüme faktörünün Pea3 üzerindeki hangi fosforlanma bölgelerini düzenlediğı sorusuna odaklanılmaktadır. Büyüme faktörü olduğunda veya olmadan Pea3 protein ifadesinde herhangi bir değışim olup olmadığını anlamak için Pea3'nin öngörülen fosforilasyon bölgelerinde yer değıştirme mutasyonu yapıldı. Öncelikle Pea3'nin öngörülen fosforilasyon bölgelerinde serin amino asitleri ya alanin ya da glutamik asite çevrilerek mutasyonlar yapıldı. Mutant Pea3 plasmitleri SH-SY5Y insan nöroblastom hücrelerine transfekte edildikten sonra çeşitli büyüme faktörleri ile indüklemeye yapıldı. Protein ifade düzeyi yabancı tip Pea3 ve diğeri mutant Pea3'ler arasında karşılaştırılarak hücre kültürü, immunopresipitasyon, Western Blot Analizi, SDS-PAGE elektroforezi teknikleri yardımı ile gözlemlendi. Pea3 protein ifade düzeyi çalışmalarının yanı sıra, Pea3 ve çeşitli promotörler arasındaki regülasyon da luciferase reporter assay tekniğı ile araştırıldı. Bu tez çalışması büyüme faktörlerinin Pea3 fosforilasyon bölgeleri üzerinde ne gibi etkisini anlamada ve Pea3'nin fosforilasyon bölgelerini belirlemede yardımcı olacaktır.

Pea3'nin nörit uzamasında rolünün olduğu bilinmektedir ve Pea3 aktivasyonun aydınlatılması nörit uzama prosesinin anlaşılmasında katkıda bulunacaktır. Dolayısıyla da nörorejenerasyon mekanizmasının anlaşılmasına katkı sağlayacaktır.

Anahtar Kelimeler: Nörit Uzaması, Aksonal Dallenma, Pea3, Fosforilasyon, MAPK/ERK Yolağı.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations</u>	<u>Explanations</u>
<u>and Acronyms</u>	
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
MAPK	: Mitogen Activated Protein Kinases
NF-L	: Human Neurofilament Light Chain
NGF	: Nerve Growth Factor
PEA3	: Polyomavirus Enhancer Activator 3
PLB	: Passive Lysis Buffer
PNT	: Pointed Domain
SDM	: Site Directed Mutagenesis
SDS-PAGE	: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SH-SY5Y	: Human Neuroblastoma Cell Line
TEMED	: Tetramethylethylenediamine

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1. INTRODUCTION

Pea3 (ETV4/E1AF) transcription factor is a member of ETS family and is known to have a role in neurite outgrowth [1]. It is regulated by several growth factors including fibroblast growth factor, nerve growth factor, glial cell line-derived neurotrophic factor etc through MAPK/ERK pathway by phosphorylation of Serine/Threonine amino acids during neural development [2-4]. Although some phosphorylation sites of Pea3 are known, most of them are not elucidated. Bioinformatical studies by alumni graduate student Berrak Caglayan specify the Pea3 MAPK-phosphorylation sites. In the thesis, we focused these putative phosphorylation sites of Pea3 to understand whether they are indeed functional in Pea3 activity, and whether there is any difference in the phosphosites affected upon stimulation with different growth factors. We believe the findings can also help elucidate critical phosphorylation sites of Pea3 for its role in neurite extension.

1.1. ETS Family of Transcription Factors

The E26 transformation specific (ETS) family of transcription factors which involved in metazoans is identified as a tripartite oncogene in E26 avian erythroblastosis virus for the first time [5-8]. After the discovering of this oncogene ‘v-ets’ (Ets-1), which leads to leukemias in chickens, its homologs that are also related to many malignancies have been found in diverse organisms [7], [8]. While *Homo sapiens* have 28 members of ETS factors, mice, *Caenorhabditis elegans* and *Drosophila* have 27 members, 10 members and 9 members of ETS factors, respectively [5], [6], [8], [9]. These genes with structural conservation at the specific domain level of genes in all members encode one of the biggest families of transcription factors that have role as activator or repressor [7], [8], [10].

- **Structure and Members of ETS Transcription Factor Family**

ETS family members are classified into 12 subfamilies based on the presence or absence of subdomains such as activation domain, helix-loop-helix domain etc, and especially on the sequence homology in their DNA-binding domains [7], [8],

[11]. Our laboratory focuses on two of these subfamilies, Pea3 and Ternary Complex Factor (TCF) (Figure 1.1).

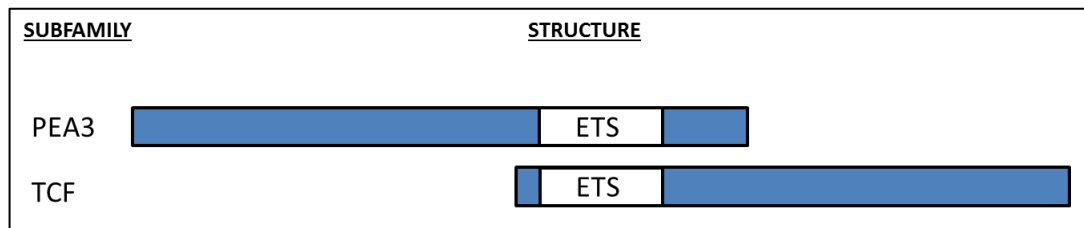


Figure 1.1: Members of ETS factors studied in our laboratory. ETS transcription factor family includes 12 subfamilies and 28 members. PEA3 and TCF subfamilies that are studied in our laboratory are demonstrated in this figure. They contain common conserved DNA binding domain (ETS; express in white) in all members.

The principal common property of ETS proteins is that they contain conserved 85 amino acid sequence in the C-terminus region with the exception that ternary complex factor (TCF) contain it in the N-terminus region [5], [7], [8]. ETS domain provides the structural recognition of specific purine rich 5'-GGAA/T-3' DNA motif and its special structural model has winged helix-turn-helix motif formed from three α -helices and four β -sheets during transcriptional activity [5], [8], [12]. The interaction between ETS domains and the target DNA is provided by third α -helix of the ETS domain (Figure 1.2), and the ETS domain and its near amino acids critical for the affinity of this interaction [7], [10], [2]. ETS-binding site recognizes about 15 bp target sequence on the double strand DNA [9].

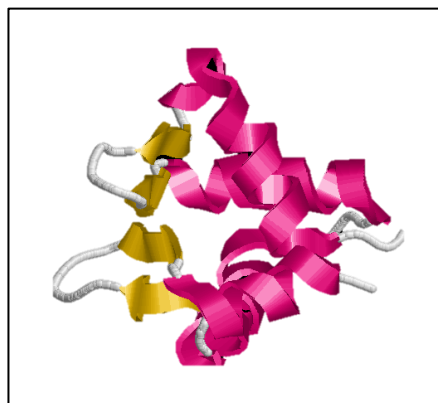


Figure 1.2: Schematic structure of ETS domain. α -helices and β -sheets are demonstrated in pink and yellow, respectively (PDB code 4CO8).

Certain members of ETS factors also include other conserved domains such as pointed domain, repressor domain, transcription activation domain [5], [8]. The most important one is pointed domain (PNT) that contains a basic helix-loop-helix motif within its structure [8]. It is important for transcriptional repression as well as heterodimerization and homooligomerization [2], [8], [9]. Other factors that have effects on DNA-binding and protein-protein interaction are post-translational modifications such as phosphorylation, sumoylation, ubiquitylation etc [8].

While some of ETS transcription factor members are expressed ubiquitously, others are expressed in certain organs or tissues. For an instance, whereas Pea3 proteins are specifically expressed in breast, epidermis and brain, ternary complex factors (TCF) are expressed in almost all tissue types [7].

- **ETS Proteins Signaling Pathways and Biological Functions**

ETS proteins have significant roles in cellular proliferation, cell cycle control, embryonic development, tissue remodeling, migration and in differentiation [5], [6], [8]. Deregulation of these proteins by overexpression or loss of suppression can lead to tumorigenesis and metastasis, for example in breast, prostate and many other tumors [8], [9].

ETS domain transcription factors are downstream targets of mitogen activated protein kinase (MAPK) pathway, which is activated by several growth factors or cytokines [2]. The signal cascades lead the posttranslational modification (such as phosphorylation) of ETS factors and therefore, they cause activation or repression of transcriptional activity (Figure 1.3) [2], [13].

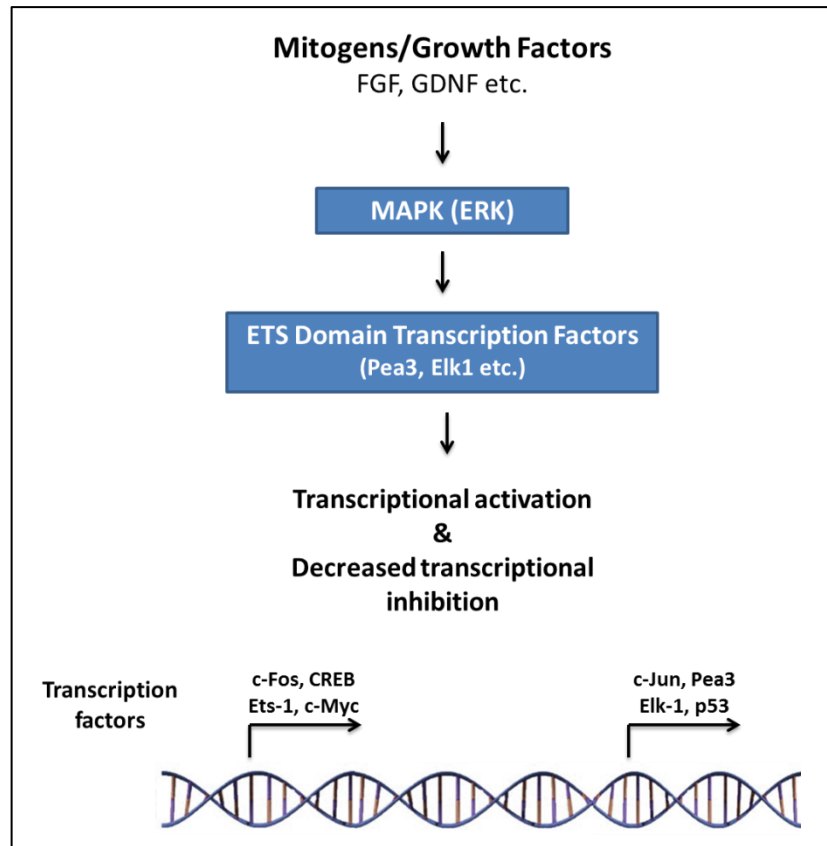


Figure 1.3: Regulation mechanism of ETS factors through MAPK signal cascade.

1.2. PEA3 Subfamily

Pea3 transcription factor subfamily includes three homolog members namely Pea3/E1AF/Etv4, Erm/Etv5 and ER81/Etv1, and ETS-domain have over %95 similarities as well as homology of acidic domain resided N-terminal region and the carboxy-terminal domain [14]. Many post-translational modification sites were also identified on PEA3 subfamilies in addition to negative regulatory regions and transactivation domain (Figure 1.4) [12], [15-17]. The Pea3 (Polyomavirus enhancer activator 3) factor was first identified in mouse 3T6 cells [18].

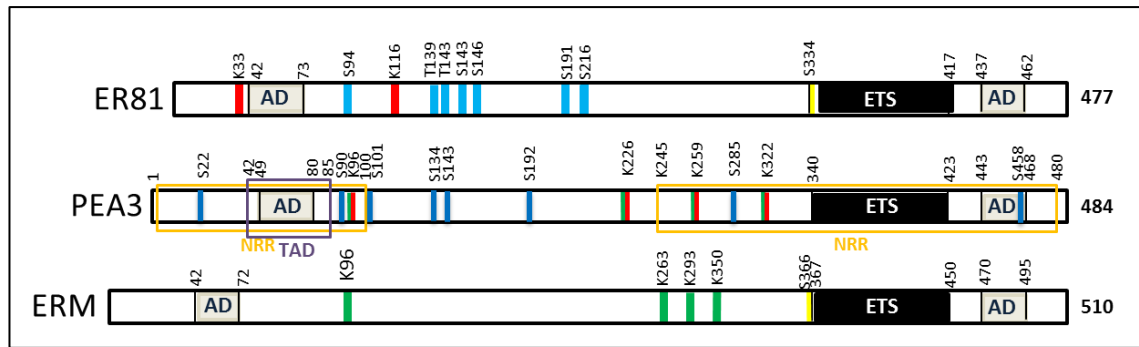


Figure 1.4: Schematic representation of PEA3 subfamily proteins. All members contain acidic domains at the N-terminal and C-terminal regions (represented in grey) and ETS-domain (represented in black). The sites of acetylation and sumoylation are represented in red and green, respectively. Yellow and blue lines represent known phosphorylation sites. While blue lines show the MAPK-directed phosphorylation, yellow lines illustrate PKA-mediated phosphorylation sites. Predicted phosphorylation sites on Pea3 are illustrated by dark blue lines. Pea3 have also two negative regulatory regions (NRR) (represented in yellow boxes) and transactivation domain (TAD) (represented in purple box).

The homologous N and C terminal acidic domains provide transcriptional activity and ETS domain provides DNA binding [19]. Sumoylation and acetylation are the most known post-translational modifications types on Pea3 [20]. Activation of ERK/MAPK pathway is indicated that it promotes the sumoylation and acetylation [20].

Activity of the Pea3 proteins is therefore regulated by posttranslational modifications such as sumoylation, phosphorylation, ubiquitination etc, which in turn results in either activation or repression of Pea3 function, thus leading to various outcomes ranging from carcinogenesis to metastasis [12].

This subgroup have many roles in transcriptional activation and was shown to be responsible for several types of cancers as breast and prostate [21]. The Pea3 transcription factor members were demonstrated to form and regulate specific branching morphogenesis in epithelial organs such as lung and mammary gland [22]. Our laboratory is particularly interested in Pea3 subfamily members with regard to their role in motor and sensory neuron connectivity and specificity [23].

- **The Roles of Pea3 Transcription Factors in Biological Systems**

The Pea3 members also known to express many organs during development [24]. They are expressed in epithelial cells and are involved in epithelial differentiation [22]. They are also involved in branching morphogenesis and organogenesis (particularly during limb development) [14], [22].

With respect to nervous system development, it has been shown that expression of Pea3 family members increase at a time when sensory neurons recognize their target cells and thus innervate their targets, thus they have often been associated with later stages of development [25], [26]. The Pea3 transcription factor was shown to be involved in branching or arborization of motor neurons or sensory neurons during innervation of target motor muscles in periphery, i.e. axonal elongation and synaptogenesis [27]. Glia-derived neurotrophic factor, GDNF, and Met signals are known to be one of the most important factors in positioning and spatial expansion of neurons, exerting their functions through different Pea3 members [3], [28].

Pea3 is also known to mediate retinal development in the chick embryo in response to FGF signaling [4]. In addition to retinal development, Pea3 and Erm were also shown to have role in developing limb bud, regulated by FGF signaling and suppressing sonic hedgehog signals [29].

Pea3 was also shown to be expressed in the hindbrain and activated through MAPK signaling cascade (Figure 1.5) [30]. For instance, BDNF (brain-derived neurotrophic factor) was demonstrated to enhance the Pea3 and Erm factor via the ERK1/2 MAPK signaling cascade [31]. These findings also reveal neurite outgrowth is also mediated by BDNF-regulated Pea3 and Erm proteins [31].

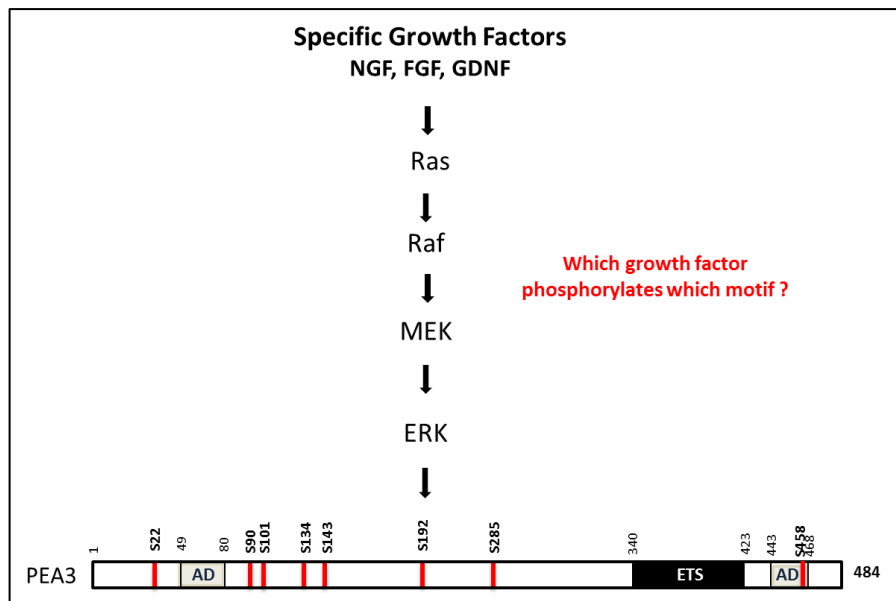


Figure 1.5: Schematic representation of the MAPK pathway. Pea3 is regulated by MAPK signaling cascades by several growth factors. Predicted phosphorylation motifs are represented by red lines. Acidic domains at the N-terminal and C-terminal regions are represented in grey and ETS-domain is represented in black.

Pea3 subfamily members are known to be related to various types of cancers either in response to upregulation or chromosomal rearrangement rendering Pea3 overactive [5], [12]. Epithelial cancers such as prostate, breast and lung cancers result from overexpression of Pea3 subfamily [5], [32]. Upregulation of Pea3 gene causes and controls overexpression of the other genes such as matrix metalloproteinases (MMP) or adhesion molecule of ICAM-1 etc and it leads to tumorigenesis and metastasis in cancerous cells [33]. Yet, these metastasis-related genes are also thought to be responsible for the branching morphogenesis and neurite extension through degradation of the extracellular matrix [33].

2. MATERIALS and METHODS

2.1. Materials

2.1.1. Plasmids

The original Pea3-VP16 expression plasmid is a gift of Prof. A.D. Sharrocks, and mouse Pea3 expression vector is a gift of Prof. Y. De Launoit. Mutant mouse Pea3 (mPea3) plasmids have been prepared through site-directed mutagenesis of wild type mPea3 expressing pCMV-3 Tag-6 plasmids. *Ephrin receptor-1* promoter (EPHA1) and *Ephrin receptor-2* promoters (EPHA2) have been cloned into pGL3 plasmids by undergraduate project student Simge Duman. The SOX2, NANOG and the POU5F1 plasmids were cloned into pGL3 basic vector by graduate student Melis Savasan Sogut. Neurofilament-L promoter was cloned into pGL2-Basic vector by Ozlem Demir.

- pCMV-3 Tag-6 (Stratagene) [34].

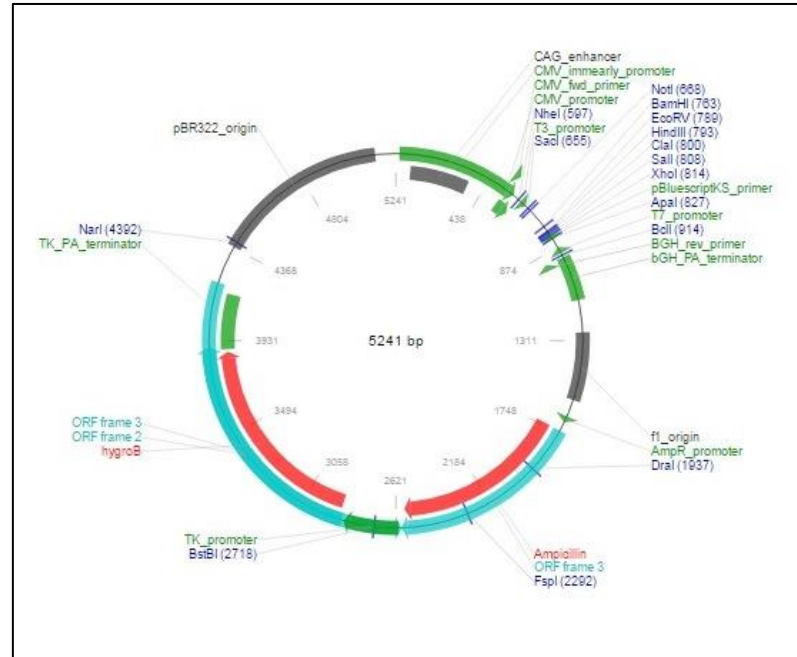


Figure 2.1: pCMV-3 Tag-6 plasmid map.

2.1.2. Cell Lines and Bacterial Strains

SH-SY5Y human neuroblastoma cell lines (ATCC number: CRL-2266) or U87-MG glioblastoma cells (ATCC number: HTB-14) were used in cell culture experiments.

Competent cells were prepared from DH5 α *Escherichia coli* bacterial strains.

2.1.3. Solutions and Kits

2.1.3.1. Enzymes and Buffers

The restriction endonucleases of Hind III and Mlu I were chose for screening of mutant plasmids. Xho I and Hind III were chose for cloning. Their restriction recognition sites are illustrated in Table 2.1.

Table 2.1: Recognition and cutting sites of Hind III and Mlu I restriction enzymes.

Restriction Enzymes	Restriction Sites
Hind III	5'...-A A G C T T-...3' 3'...-T T C G A A-...5'
Mlu I	5'...-A C G C G T-...3' 3'...-T G C G C A-...5'
Xho I	5'...-C T C G A G-...3' 3'...-G A G C T C-...5'

Fast Digest Hind III (Thermo Scientific, #FD0504), Fast Digest Mlu I (Thermo Scientific, #FD0564), Fast Digest XhoI (Thermo Scientific, #FD069), 10X Fast Digest Green Buffer (Thermo Scientific), 10X Fast Digest Buffer (Fermentas), Pfu DNA Polymerase (recombinant) (Thermo Scientific, #EP0502), 10X Pfu Buffer with MgSO₄ (Fermentas), i-Taq DNA Polymerase (iNTRON Biotechnology, 25022), i-Taq dNTP Mixture (iNTRON Biotechnology, 25022), i-Taq 10X PCR Buffer (iNTRON Biotechnology, 25022), 10X Buffer for T4 DNA Ligase with 10 mM ATP (New England Biolabs, #B0202S), T4 DNA Ligase (New England Biolabs, #M0202L).

2.1.3.2. Kits

Q5 Site-Directed Mutagenesis Kit Protocol (New England Biolabs, E0554), Dual-Luciferase Reporter Assay System (Promega, TM040), DNA-Midi, Plasmid DNA Purification Kit (Intron Biotechnology, 17252), DNA-Spin, Plasmid DNA Purification Kit (Intron Biotechnology, 17097), MEGA-quick Spin Total, Fragment DNA Purification Kit (Intron Biotechnology, 17287).

2.1.3.3. Growth Factors, Antibodies and Protein Beads

- FGF-Basic (AA 1-155) Recombinant Human Protein (Thermo Fisher, PHG0266)
- Glial Cell Line-derived Neurotrophic Factor human (G1777, Sigma)
- Recombinant human NGF (Thermo Fisher, 11050HNAC25)
- Anti-Flag monoclonal antibody in mouse (Sigma)
- Pea3 mouse monoclonal antibody (Santa Cruz, sc-113)
- β -Tubulin mouse monoclonal antibody (Santa Cruz, sc-58884)
- Goat anti-mouse IgG-HRP (Santa Cruz, sc-2005)
- Goat anti-rabbit IgG-HRP (Santa Cruz, sc-2004)
- Protein G PLUS-Agarose Immunoprecipitation Reagent (Santa Cruz, sc-2002)
- Anti-flag M2 Affinity Gel (Sigma)

2.1.3.4. Western Blot Solutions

40% Acrylamide:bisacrylamide (Fisher Scientific), Trizma-Base (Sigma), Tris-HCl (Fisher Scientific), Sodium dodecyl sulphate (SDS) (Fisher Scientific), Ammoniumpersulphate (APS) (Fisher Scientific), TEMED (Sigma, T7024-100ML), 3X Blue Loading Buffer (Cell Signaling, #7722S), Page Ruler Plus Prestained Protein Ladder (Thermo Scientific), Tween-20 (Merck), Glycine (Sigma), NaCl (Sigma), HCl (Multicell), NaOH, PhosStop EASYpack - Phosphatase Inhibitor Coctail Tablets (Roche, 04906845001), Protease Inhibitor Coctail (Sigma, P8465-25ML), Smart BCA Protein Assay Kit (Intron Biotechnology, 21071), Dithiothreitol (DTT) (AppliChem), Luminata Crescendo Western HRP Substrate (Millipore,

WBLUR0500), Nonfat Dry Milk (Cell Signaling Technology, #9999S), Albumin-Bovine Serum (Calbiochem, 1265S), Restore Plus Western Blot Stripping Buffer (Thermo Scientific, #46430), Sodium Deoxycholate, Ethanol (Sigma), Methanol (Sigma).

2.1.3.5. Cell Culture Solutions

PEI transfection reagent (Polysciences), Dulbecco's Modified Eagle Medium (DMEM) (1X) with 4,5 g/L Glucose, L-glutamine and pyruvate (Gibco, 41966-029), Penicilin/Streptomycin (10000U-ml Penicillin / 10mg/ml Streptomycin) (PAN Biotech, P06-07100) 0.05% Trypsin-EDTA Solution (1X) (Gibco, 25300-054), Dulbecco's Phosphate Buffered Saline (1X) (DPBS) (Gibco, 14190-94), Fetal Bovine Serum (FBS) (Gibco, 10270), Dimethylsulfoxide (DMSO).

2.1.3.6. Bacterial Media

LB Broth (Caisson Labs, LBP01-500GM), LB Agar (Caisson Labs, LBP02-500GM), Ampicillin (Biomatik), X-Gal (Fisher Scientific), IPTG (Fisher Scientific), 87% Glycerol (Sigma, 15524), CaCl₂ (Acros Organics).

2.1.3.7. Agarose Gel Electrophoresis Solutions

GeneRuler 1kb Plus DNA Ladder (Thermo Scientific, #SM1331), 6X DNA Loading Dye (Fermentas, R0611), RedSafe Nucleic Acid Staining Solution (Intron Biotechnology, 21141), Agarose (Sigma), Tris-Acetate-EDTA (TAE).

2.1.4. Material and Equipments

T25, T75, T150 tissue culture flasks (TRP), 24-well culture plate (TRP), 96-well plate (NUNC), 100 mm, 150 mm tissue culture plate (TRP), petri dish (100 mm) (Lamtek), 5 ml, 10 ml, 25 ml, 50 ml serological pipettes (CAPP), micro pipettes (Eppendorf), 15 ml and 50 ml Falcon (CAPP), microcentrifuge tubes (0,2 ml, 0,5 ml, 1,5 ml, 2ml), cryotubes (1ml, 2ml), hemocytometer (Marienfeld), 0.22 µm syringe

filter (Merck), syringe (1,5 ml, 10 ml, 20 ml, 50 ml), PVDF transfer membrane (Merck Millipore, IPVH00010).

MJ Mini Personal Thermal Cycler (Bio-Rad), Agarose Gel Electrophoresis System (Bio-Rad), Varioskan Flash (Thermo Scientific), Criterion Blotter (Bio-Rad), Western Blot System (Bio-Rad), Power Supply – Power Pac Basic (Bio-Rad), Spin (Sprout), Vortex (JeioTech), Water Bath (JeioTech), Rotator (JeioTech), Laminar Flow Cabinet – Safe 2020 (Thermo Scientific), CO₂ incubator - Stericycle i160 (Thermo Scientific), Microfuge 22R Centrifuge (Beckman Coulter), pH meter (Mettler Toledo), Fluorescence microscope (Leica-DMi1), ChemiDoc™ XRS+ System (Bio-Rad), Heat Block (JeioTech), autoclave (Hirayama HV-110L), Shaking Incubator (FINEPCR), Spectrophotometer – NanoDrop (Schimadzu), -80°C freezer (VWR), UV illuminator (Vilber Lourmat), Centrifuge (Labogene, 1580R), drying-oven (VWR), liquid nitrogen tank (Taylor-Wharton, LS4800).

2.2. Methods

2.2.1. Site-Directed Mutagenesis

Pea3 protein sequence was analyzed in silico by Berrak Caglayan, a former MSc student, for putative MAPK/ERK phosphorylation sites. Substitution mutations of predicted phosphorylation motifs on Pea3 were performed to confirm the predictions. With this aim two kinds of mutations were carried out; first, the mimicking of phosphomotifs (phosphomimetic) by glutamic acid substitution and the second is silencing phosphomotifs by alanine substitution. As for mimicking the serine or threonine amino acid, glutamate is used as it is a negatively charged amino acid and has the second lowest isoelectrical point (pI) value, ensuring the simulation of the phosphorylation by the effect of charge or pI of amino acid. The aspartic acid could also be used for the mimicking mutation. The other mutation is the silencing effect on serine or threonine by preventing the phosphorylation by alanine, a non-polar amino acid devoid of hydroxyl group (Figure 2.2).

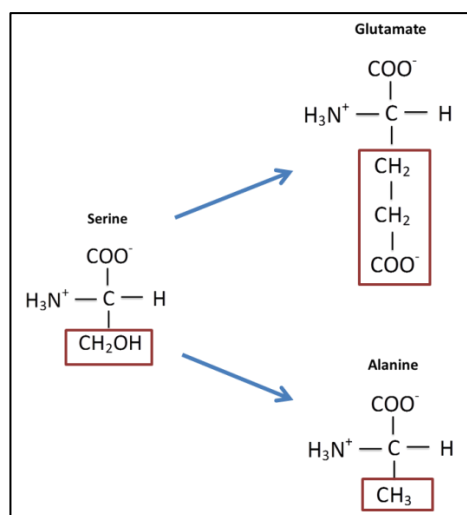


Figure 2.2: Structures of serine, glutamate and alanine amino acids. While glutamate substitution ensure phospho-mimicking, alanine substitution provide silence of phosphorylation because of their radical group (represented by box) in their amino acid structure.

To get these effects, we chose site-directed mutation (SDM) technique. SDM provides site-specific mutation on the DNA. For substitution of amino acid, mutagenic primers are designed and the DNA is amplified with these primers. Then, PCR product is phosphorylated on 5' prime and 3' prime of DNA and re-circularized and also template was discarded with Kinase, Ligase, Dpn I (KLD) enzyme reaction (Figure 2.3) [35].

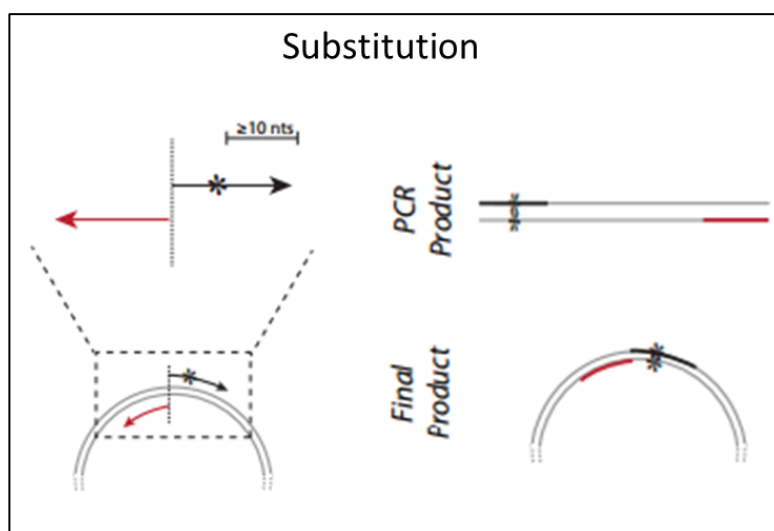


Figure 2.3: Stages of substitution mutation. Mutagenic primers are designed according to desired mutation. Amplification of DNA with these primers ensure to mutant DNA. The final circular product is obtained after kinase, ligase, Dpn I (KLD) reaction. Asterisk expresses the point mutation site on the specific amino acid.

2.2.2. Screening Mutant Plasmids

Some of the existing mutant mPea3 expression plasmids, previously cloned into pCDNA3 vector or pBS-KS as well as pCMV-Flag (pCMV-3 Tag-6) vector in our laboratory, were re-screened to confirm mutations. For the validation, two restriction endonucleases (Hind III, Mlu I) that provide specific restriction sites were used. While Hind III enzyme restricts to pCMV-Flag vector from just one site, Mlu I enzyme restricts from two sites on the pCMV-Flag vector. On the other hand, Hind III and Mlu I enzymes do not restrict within mPea3 coding region. The reaction mixture that is demonstrated Table 2.2 were incubated at 37°C for 1 hour to digest plasmids.

Table 2.2: Reaction mixture for digestion of mutant plasmids with Hind III and Mlu I.

Reaction Component	Volume
Template DNA (200 ng/μl or 500 ng/μl)	Proper volume
Restriction Enzyme (Hind III or Mlu I) (1 U/μl)	0,5 μl or 1 μl
10 X Green Buffer (1 X)	2 μl
dH ₂ O	Up to 20 μl

After incubation, Agarose Gel Electrophoresis was performed; reaction mix was loaded 1% agarose gel, and run at 90-120 Volt. Gel was scanned with Image Lab scanning program (BioRad).

Blue white screening was performed to discriminate between pCMV-Flag and pBS-KS vectors as well as digestion. X-Gal/IPTG plates were prepared to check this circumstance. 40 μl of 100 mM IPTG and 120 μl of 20mg/ml X-Gal were added to agar media plates (with ampicillin) and spread over surface of plates. After drying the plates, transformed competent cells were spread.

2.2.3. Molecular Cloning For Mutants

Mutant mPea3s (S101A-mPea3-pBSKS, S143A-mPea3-pcDNA3, S458A-mPea3-pCDNA3) were used as insert to cloning pCMV-Flag vector. They amplified

with *Pfu* DNA polymerase because of its low error rate, and the cloning primer (demonstrated in primers section) for mPea3 was designed by former MSc student Berrak Caglayan. The reaction mix was prepared in 0,2 ml PCR tubes as shown in Table 2.3.

Table 2.3: Polymerase chain reaction (PCR) components for amplifying inserts with *Pfu* polymerase.

Reaction Component	Volume
Template DNA (150 ng/μl)	Proper Volume
10X <i>Pfu</i> Buffer with MgSO ₄ (1X)	5 μl
2 mM dNTP (0.2 mM)	5 μl
Forward Primer	1,3 μl
Reverse Primer	1,3 μl
<i>Pfu</i> DNA Polymerase	0,8 μl
dH ₂ O	Up to 50 μl

The thermal cycling conditions for amplification are performed as shown in Table 2.4.

Table 2.4: PCR cycling conditions and temperatures to amplify inserts.

Stage	Temperature	Duration
Initial Denaturation	95°C	2 min
Denaturation	95°C	30 sec
Annealing	56°C	30 sec
Extension	72°C	3 min (2 min/kb)
Final Extension	72°C	10 min
	4°C	∞

Agarose gel electrophoresis was performed to check the amplification of insert in 1% agarose gel and it was visualized with UV illuminator. PCR products were purified from proper bands in agarose gel to make ready for digestion.

pCMV-Flag plasmid (pCMV-3 Tag-6) was used as vector, since its flag tag would be used in the protein expression control steps of experiment. Therefore, vector and inserts were double-digested with appropriate restriction enzymes (Hind

III and Xho I) to make suitable integration. While the vector was digested amount of 1 µg/µl, inserts were digested amount of 500 ng/ µl. Digestion reaction mix was prepared as shown in table below, and the mixture was incubated at 37°C for 1 hour.

Table 2.5: Double digestion was occurred with Hind III and Xho I restriction endonucleases.

Reaction Component	Volume
Template DNA (500 ng/µl or 1 µg/µl)	Proper volume
Restriction Enzyme (Hind III) (1 U/µl)	1 µl
Restriction Enzyme (Xho I) (1 U/µl)	1 µl
10 X Green Buffer (1 X)	5 µl
dH ₂ O	Up to 50 µl

Agarose gel electrophoresis was performed to control the digestion in 1% agarose gel and the bands were visualized with UV illuminator. Vector and inserts were purified from proper bands in agarose gels to make ready for ligation.

The molar ratio of 1:3 and 1:5 of vector to insert was used to be joined the DNA fragments of vector and insert from digestion step. For the ligation, amount of vector was used 50 ng, T4 DNA Ligase enzyme and insert was used proper amount as shown in the table below. The reaction mix was incubated at room temperature for 15 min to activation of ligase enzyme. For inactivation of reaction, mixture was incubated at 65°C for 10 min.

Table 2.6: Reaction components for ligation with T4 DNA Ligase.

Reaction Component	Volume
Insert	Proper amount
Vector (50ng)	Proper amount
T4 DNA Ligase	1 µl
10X Ligase Buffer	2 µl
dH ₂ O	Up to 20 µl

After ligation step, plasmid DNA was transferred to competent DH5α *E.coli* bacterial cells. Transformed cells were growth in agar plates with ampicillin.

2.2.4. Sequencing

Mutagenic mPea3 plasmids obtained from site-directed mutagenesis were sent to sequencing for confirmation of the mutations. Restriction-screened plasmids were also sequenced for confirmation of their mutation sites. Sequencing service was provided by Humanizing Genomics MacroGen [36].

Results of sequencing were compared by utilizing ClustalW Tool in Workbench (San Diego Supercomputer Center) [37].

2.2.5. Preparation of Competent Cells

To obtain ultra competent cells, DH5 α strain of bacteria were inoculated in 5 ml of LB Broth (w/o antibiotics) overnight at 37°C with shaking at 185 rpm. 2 ml of inoculated bacteria were grown in new 200 ml of LB Broth (w/o antibiotics) with shaking at 37°C until the value of OD₆₀₀ achieved 0.4. The culture was transferred into pre-chilled 50 ml falcons and they were centrifuged at 2700 g for 10 min at 4°C. Supernatants were removed and the pellets on each falcon were resuspended with 1.6 ml of ice-cold 100 mM CaCl₂. Falcons were incubated on ice for 30 min. Then, they were centrifuged at 2700 g for 10 min at 4°C, again. Supernatants on each falcon were discarded and pellets were resuspended with 1.6 ml of ice-cold 100 mM CaCl₂. All falcons were incubated on ice for 20 min. After, cells were combined to one tube and 1 ml of ice-cold 87% Glycerol was added onto cells and mixed together. Cells were frozen as aliquots in liquid nitrogen.

To obtain fresh competent cells, bacteria cells (DH5 α strain) were inoculated overnight in 10 ml of LB Broth w/o ampicillin at 37°C with shaking at 185 rpm. 1 ml of inoculum was added into fresh 50 ml of LB Broth and incubated for 3.5 hours at 37°C with shaking. Then the culture was centrifuged at 5500 rpm for 5 min at 4°C. Pellets were resuspended with 5 ml of ice-cold 100 mM CaCl₂. Falcons were incubated on ice for 1 hour. They were centrifuged at 2000 rpm for 5 min at 4°C and pellets were resuspended with 1 ml of ice-cold 100 mM CaCl₂. They were stored at 4°C.

2.2.6. Primer Design

The mutagenic primers were designed by using NEBaseChanger Tool for mutation of putative mPea3 phosphorylation sites [38]. Mutagenic primers were designed as two main substitutional mutations which are converting from Serine (Ser) to Alanine (Ala) (to provide silencing in certain phosphorylation sites) and from Serine (Ser) to Glutamic acid (Glu) (to provide mimicking in certain phosphorylation sites). Annealing temperatures were recommended by this tool and there were no needed gradient PCR for optimization of the annealing temperatures.

Table 2.7: Mutagenic primers and the annealing temperatures. Converted sites of amino acids were highlighted with yellow.

Mutation	Forward Primer (5' -> 3')	Reverse Primer (5' -> 3')	Ta
S22A mPea3	CTGCAGCAAAgctCCCG GAAATG	AAGGTGTAGGGCACT CGC	69°C
S90A mPea3	AGCTTTCCATgccCCCAC CACCAG	AAGTTTTCTGAATGGA AATCAGG	61°C
S101A mPea3	GGAACCCCAGgctCCCC GCACAG	TTCTTGATCCTGGTGG TG	62°C
S134A mPea3	CGCCATCAAGgccCCCG CTCCCG	ATTTGTCTGGAGTAAA GGCACTGCTCTCCATG GTG	72°C
S143A mPea3	CCCTGGACAGgcgCCCC TGCAGC	GCACCGGGAGCGGGG GAC	72°C
S192A mPea3	CTCCTTCACAgctCCTCA GGGAG	TGGCACATGTCCACG GGC	69°C
S285A mPea3	CTCTGGACCCgctCCAGG TGATG	AAGCCCTCTGGGTGG AGG	70°C
S458A mPea3	CTTGGATGAGgctCCTGC CTACCTCC	TGGGACAAAGGGACT GTG	60°C
S22E mPea3	CTGCAGCAAAgaaCCCG GAAATGGG	AAGGTGTAGGGCACT CGC	64°C
S101E mPea3	GGAACCCCAGgaaCCCC GCACAG	TTCTTGATCCTGGTGG TGG	65°C
S134E mPea3	CGCCATCAAGgaaCCCG CTCCCG	ATTTGTCTGGAGTAAA GGCAC	63°C
S143E mPea3	CCCTGGACAGgagCCCC TGCAGCCC	GCACCGGGAGCGGGG GAC	72°C
S192E mPea3	CTCCTTCACAgaacCCTCA GGGAGGGGG	TGGCACATGTCCACG GGC	68°C
S285E mPea3	CTCTGGACCCgaaccAG GTGATGGAGTG	AAGCCCTCTGGGTGG AGG	67°C

Universal primers (T3, T7 and BGH) were chosen for sequencing of mutant plasmids (Table 2.8).

Table 2.8: Sequences of universal primers (T3, T7 and BGH).

Name of the primer	Primer (5' -> 3')
T3 Promoter	ATTAACCCTCACTAAAG
T7 Promoter	TAATACGACTCACTATAGGG
BGH Reverse	TAGAAGGCACAGTCGAGG

Primers for amplifying the mPea3 in molecular cloning part of experiments are shown in Table 2.9.

Table 2.9: Sequences of mPea3 cloning primers.

Name	Forward Primer (5' -> 3')	Reverse Primer (5' -> 3')	Tm
mPea3	AGACAAGCTTCCATGGAG CGGAGGA	AGACCTCGAGCTAGTAAG AAT	56°C

2.2.7. Mutagenesis of Phosphorylation Sites of mPea3

Site-directed mutagenesis was performed to ensure mutagenesis on putative phosphorylation sites of mPea3. Serine (S) or Threonine (T) amino acids of putative phosphorylation sites on mPea3 were replaced with alanine (A) or glutamic acid (E). Substitution from serine to alanine ensures to silence certain phosphorylation sites, and substitution from serine to glutamic acid ensures to mimic this phosphorylation sites. Eight phosphorylation sites were mutated with two substitution mutation, 22th serine to alanine (S22A) and to glutamic acid (S22E), 90th serine to alanine (S90A) and to glutamic acid (S90E), 101th serine to alanine (S101A) and glutamic acid (S101E), 134th serine to alanine (S134A) and glutamic acid (S134E), 143th serine to alanine (S143A) and glutamic acid (S143E), 192th serine to alanine (S192A) and glutamic acid (S192E), 285th serine to alanine (S285A) and glutamic acid (S285E), 458th serine to alanine (S458A) and glutamic acid (S458E).

At first, mPea3-pCMV wild type plasmids were amplified with mutagenic primers and High-Fidelity Master Mix supplied from SDM Kit. Amount of components is shown in Table 2.10.

Table 2.10: PCR amplification with mutagenic primers.

Reaction Component	Volume
Q5 Hot Start High-Fidelity 2X Master Mix (1X)	10 μ l
Forward Primer (0.5 μ M)	1 μ l
Reverse Primer (0.5 μ M)	1 μ l
Template DNA (25 ng/ μ l)	Proper Volume
dH ₂ O	Up to 25 μ l

The reaction reagents were added in 0,2 ml PCR tubes and PCR cycling was initialized according to PCR cycling conditions as shown in table below. Annealing temperatures of mutagenic primers were used according to recommended temperatures of NEBaseChanger Tool that were mentioned in Primers Section (Table 2.11).

Table 2.11: PCR cycling condition to prepare mutagenic plasmids.

Stage	Temperature	Duration
Initial Denaturation	98°C	30 sec
Denaturation	98°C	10 sec
Annealing	50-72°C (depend on mutagenic primers)	20 sec
Extension	72°C	3,3 min (20-30 sec/kb)
Final Extension	72°C	2 min
	4°C	∞

In the second step, Kinase, Ligase and DpnI (KLD) treatment was occurred after amplification of the mutant plasmids. The enzymes mix catalyzes rapid circularization for plasmids and provide to remove template DNA. The reaction component was prepared as shown in table below. The reaction components was mixed well and incubated at room temperatures for 5 minutes.

Table 2.12: Kinase, Ligase and DpnI (KLD) Reaction.

Reaction Component	Volume
PCR Product from first step	1 μ l
10X KLD Enzyme Mix (1X)	1 μ l
2X KLD Reaction Buffer (1X)	5 μ l
Water	3 μ l

In the third step, 10 μ l of KLD reaction mix was transformed into NEB 5-alpha Competent *E. coli* cells. KLD mix was added into thawed cells and mixed well without vortex. Then, the mixture incubated for 30 minutes on ice and it was blocked with high temperature at 42°C for 30 seconds. The mixture incubated for 5 minutes on ice, again. 950 μ l of SOC medium supplied from SDM Kit was added into the mixture and they were incubated at room temperature for 1 hour with shaking. After 1 hour, mixture of 100 μ l was spread onto plates containing ampicillin and incubated at 37°C, overnight.

2.2.8. Transformation of Plasmids

The routine transformation protocol in our lab is followed. DH5 α *Escherichia coli* bacterial competent cells are thawed on ice. 2 μ l of (50-100 ng/ μ l) plasmids of interested added into 50 μ l of thawed competent cells and they were incubated for 15 minutes on ice. To control the contamination, negative control was performed without any plasmid as well as plasmid transformation. Then, all tubes were incubated for heat shock at 42°C for 60 seconds on heat block. The tubes were put on ice for 2 minutes, again. 200 μ l of LB Broth (without antibiotic) was added into tubes and the cells were incubated at 37°C for 1 hour with shaking at 180-250 rpm/min. After incubation, bacterial cells were spread onto 10 cm petri dish with LB Agar containing with ampicillin. They were grown overnight at 37°C.

2.2.9. Plasmid Isolation

For MiniPrep, single colonies were picked from LB Agar plate, which was grown overnight after transformation and inoculated in 5 ml of LB Broth with

ampicillin at 37°C with overnight shaking. Then, bacterial cells were precipitated with centrifugation at 4500 rpm for 5 min at room temperature. Pellets were re-suspended with 250 µl of resuspension buffer containing RNase A with vortex. 250 µl of lysis buffer was added onto cells, and tubes were inverted ten times and incubated for 3 min at room temperature. 350 µl of neutralization buffer was added and tubes were inverted ten times and incubated on ice for 5 min. Lysate was centrifuged at 13000 rpm for 10 min at 4°C. Supernatant was transferred into columns and they were centrifuged at 13000 rpm for 1 min. Flow-through that was precipitated in collection tube was discarded and 500 µl of first washing buffer was added into filter columns. Tubes were centrifuged at 13000 rpm for 1 min. Flow-through was discarded, again. 700 µl second washing buffer was added into filter columns and they were centrifuged at 13000 rpm for 1 min. Flow-through was discarded from collection tubes. Collection tubes with filter column were dried with centrifugation at 13000 rpm for 1 min. For elution of plasmid DNA from filter column new clean microfuge tubes was changed with collection tubes and 30 µl of dH₂O was added into columns. Lastly, they were centrifuged at 13000 rpm for 1 min.

For MidiPrep, single colonies were picked from bacterial plate and inoculated overnight at 100 µl of LB Broth at 37°C with shaking. Then they were precipitated with centrifugation at 4000 rpm for 10 min at room temperature. Pellets were suspended with 2 ml of suspension buffer including RNase. 2 ml of lysis buffer was added onto suspended cells and tubes were inverted ten times to mix well. Cells were incubated at room temperature for 3 min. Then, 2 ml of neutralization buffer was added into mixture; they were inverted ten times and incubated on ice for 5 min. All lysate was transferred into pre-column and they were centrifuged at 4000 rpm for 5 min. Flow-through was transferred into binding column and tubes were centrifuged at 4000 rpm for 5 min, again. Flow-through was discarded and 10 ml first washing buffer was added into binding column and they were centrifuged at 4000 rpm for 5 min. Then the binding columns were washed with 10 ml of second washing buffer with centrifugation at 4000 rpm for 5 min. The binding columns were dried with centrifugation of tubes at 4000 rpm for 15 min. Then the filters were dried at 56°C to evaporate the remaining liquids such as ethanol from washing buffers. New collection tube was used for elution of plasmids from binding column. 600 µl of dH₂O was added onto filter of column and incubated at room temperature. Finally, they were centrifuged at 4000 rpm for 5 min.

DNA concentration of plasmid DNA was measured using spectrophotometric measurement (NanoDrop, Shimadzu).

2.2.10. Purification of DNA from Agarose Gel

PCR product from cloning step or DNA fragment from digestion step was extracted from agarose gel. Proper bands from agarose gel electrophoresis were excised from agarose gel and weight of gel slices was measured. Three volumes more agarose gel lysis buffer was added into tubes containing gel slices. The tubes were incubated at 55 °C until gel slices were dissolved. To make easy for dissolving, tubes were mixed with vortex. Gel and buffer mixture was added to filter column and they were centrifuged for 1 min. Flow-through was discarded and 700 µl of washing buffer was added to filter column and centrifuged at 13000 rpm for 1 min. To dry the filters, tubes were centrifuged at 13000 rpm for 1 min. the collection tubes were replaced with clean microcentrifuge tubes. To elute the product, 30 µl of dH₂O was added to column and incubated at room temperature for 1 min, and they centrifuged at 13000 rpm for 1 min. Flow-through product was stored at -20°C.

2.2.11. Cell Culture

As per routine procedure in our laboratory, subculturing procedure was performed when flask or plate reached about 80% cell confluency. For the SH-SY5Y human neuroblastoma cell line, standard culture medium, DMEM with 4,5 g/L glucose, L-glutamine and pyruvate, was used after the addition of 10% FBS and 1% penicillin/streptomycin antibiotics. To culture, firstly medium was discarded from flask/plate and cells were washed with PBS to remove cell debris that are not bound. 0.05% 1X Trypsin-EDTA solution was added onto cells to remove from flask/plate, and cells were incubated 2-3 min at 37°C. To inhibit trypsin, complete medium was then added to the trypsin cell mix. They were centrifuged at 1500 rpm for 5 min and supernatant was discarded. Pellet was suspended with particular amount of complete medium. Cells were transferred into T75 or T150 flasks. If cell seeding was needed, cells were counted with hemocytometer and required amount of cells were plated into tissue culture plates. They were incubated at 37°C and 5% CO₂.

Cryogenic preservation method was used for storage of cells. Cells were frozen with 10% DMSO in liquid nitrogen or -80°C freezer (for short term storage). Before this process, cells were counted with hemocytometer, and 1×10^6 or 2×10^6 cells were used to freeze in each cryotubes. Before transferring to liquid nitrogen, they were kept waiting in -20°C and then -80°C freezer.

Frozen cells were thawed immediately and they were slowly transferred into complete medium. After centrifugation to provide removing of DMSO toxic effect, they were transferred into T25 tissue culture flask and growth in 5% CO₂ incubator at 37°C.

2.2.12. Protein Expression Analysis of Mutant mPea3 with or without Growth Factor Induction

2.2.12.1. Transfection and Growth Factor Treatment

Transient transfection was performed with polyethylenimine (PEI) transfection reagent. The reagent is used to transfect plasmid DNA of interest into cells without integration of chromosome. Before transfection process, 1.5×10^6 (for 60 cm plate) or 2×10^6 (for 150 cm plate) cells were seeded into tissue culture plates. The day after, plasmids were diluted to provide 10 µg/µl or 12 µg/µl concentration for transfection according to seeded cell confluency (1.5×10^6 or 2×10^6 cells). Optimized DNA:PEI ratio (generally 1:4) was preferred to obtain successful transfection efficiency. The protein expression control of mutant mPea3 plasmids was performed by undergraduate project student Busra Nur Cicek.

In the control of mutant protein expression experiment, 100 µl of 100 ng/µl or 120 ng/µl plasmid DNA was added into 1 ml of serum free DMEM. Then, 40 µl or 48 µl of PEI reagent was gently added into plasmid-medium tube. The tube was mixed with vortex and incubated at room temperature for 15 min. During the incubation, medium was discarded from seeded cell plate. The plasmid PEI and medium mixture was added into seeded cell plates. 4 µl (for 60 cm plate) or 9 µl (for 150 cm plate) of complete medium (including 10% FBS and antibiotics) was also added into plates. Plates were incubated at 37°C for 2 hours. After incubation, 5 ml of complete medium was added into all plates. To control transfection efficiency,

GFP plasmids were co-transfected into cells. Cells were incubated about 48 h in 5% CO₂ incubator at 37°C. Then, protein isolation was performed.

In the growth factor induction experiment, 100 µl of 100 ng/µl or 120 ng/µl plasmid DNA was added into 1 ml serum free DMEM. Then, 40 µl or 48 µl of PEI reagent was gently added into plasmid-medium tube. The tube was mixed with vortex and incubated at room temperature for 15 min. During the incubation, medium was discarded from seeded cell plate. The plasmid-PEI-medium mixture was added into seeded cells. 4 µl of complete medium (including 1% FBS and antibiotics) was also added into plates. Plates were incubated at 37°C for 2 hours. After incubation, 5 ml or 10 ml of complete medium (including 1% FBS and antibiotics) was added into all plates. For controlling transfection efficiency, GFP plasmids were also transfected into cells under the same conditions. Cells were incubated about 48 h in 5% CO₂ incubator at 37°C.

For this set of experiments, prior to protein isolation cells were induced with growth factors (bFGF, GDNF and NGF) for 15 min, 30 min, 45 min, 1h or 2h. Duration of induction is important for understanding of most active time of growth factors during treatment. After determination of most active time of growth factors, experiments were continued with specific growth factors.

The concentration of bFGF was 20 ng/ml in all FGF treatment. The ideal concentration of GDNF was checked in the literature and 50 ng/ml and 100 ng/ml of GDNF concentration was performed in growth factor treatment experiments to achieve the optimum concentration. The NGF was treated at the concentration of 100 ng/ml. Growth factors were added into 1% FBS-included DMEM, and medium was replaced with old one. Then, incubation was started through determined duration at 37°C, 5% CO₂ condition.

Preparation of required solution in transfection;

- To prepare 1 mg/ ml PEI transfection reagent; PEI powder was dissolved in 80°C dH₂O and the pH was adjusted to 7.0 with 5M HCl. Then, solution was sterilized with 0.22 µm filter. They were stored at -20°C.
- Cell culture medium (DMEM) was used with 1% penicillin/streptomycin and 10% FBS. However, in the growth factor experiments, medium was used with 1% penicillin/streptomycin and 1% FBS.

- Lyophilized growth factors were dissolved in dH₂O with desired concentration and stored with aliquoted in -80°C.

2.2.12.2. Protein Isolation

About 48 hours after transfection, plates were put into ice and their medium was discarded. Then, all plates were washed with PBS to clear from remaining medium and cell debris. 200-500 µl of lysis buffer – RIPA (including 10X phosphatase inhibitor and 100X protease inhibitor) was added into cells and incubated with shaking at 4°C for 30 min. Attached cells were scraped with RIPA buffer on the ice. During cell lysis, inhibitors contained in RIPA buffer inhibit the phospholytic and proteolytic enzymes from the cells. All cell lysates were transferred into pre-chilled 1,5 ml of microcentrifuge tubes. Cell lysates were precipitated with 30 min centrifugation at 4°C, 13000 rpm. Supernatants (proteins) were collected in clean pre-chilled tubes. Proteins were stored at -80°C for long-term storage. Concentration of proteins was measured with Smart BCA Assay Kit at 562 nm absorbance with Varioskan micro-plate (96-well plate) reader.

Preparation of protein isolation solutions;

- To prepare 100 ml RIPA (lysis buffer); 150 mM NaCl, 50 mM Tris (pH 8.0), 0,1% SDS, 0,5% Sodium Deoxycholate, 1% NP-40 were mixed together. During the protein isolation 10X phosphatase inhibitor and 100X protease inhibitor were added into RIPA buffer.

2.2.12.3. Immunoprecipitation

In order to carry out immunoprecipitation (IP) using Flag tag, for all samples 50 µl of Flag agarose beads were transferred into microcentrifuge tubes. Washing protocol of beads were performed. Firstly, they were centrifuged at 4°C, 600 xg for 1 min. They were washed two times with PBS. Third wash was performed with TBS and finally they were washed with lysis buffer (RIPA). After 1 min centrifugation at 4°C, 600 xg, beads were suspended with 50 µl of RIPA buffer (including inhibitors). Then, 250 µg total proteins were added into the flag-beads. They were incubated

overnight with rotator at 4°C. The day after, beads were washed three times with RIPA buffer and two times with washing buffer. After precipitation, pellets (beads) were suspended with 20 µl of 3X Blue Loading Dye. They were stored at 4°C (about 1-2 hours) or -20°C (for long-term storage) until it was loaded onto SDS-PAGE gel.

- Preparation of washing buffer in flag immunoprecipitation; 50 mM Tris-HCl (pH: 7.4) and 150 mM NaCl were mixed.

In order to immunoprecipitate with Pea3 antibody, 250 µg total proteins were incubated with 2 µg total Pea3 Antibody and they were incubated overnight on rotator at 4°C. The next day, 30 µl of Protein G was added into tubes and incubated with them for 1 hour on rotator at 4°C. Tubes were centrifuged at 4°C, 2500 xg for 1 min. Supernatant was removed and beads were washed with 100 µl of lysis buffer (RIPA) three times and with ice-cold PBS two times. They were centrifuged for 1 min at 4°C, 5000 xg. Supernatant was discarded and pellets were suspended with 50 µl of loading buffer. Protein-Protein G beads were separated from each other with heating at 95°C for 5 min. After 5 min centrifugation at 10000 xg, supernatant was collected and run in SDS-PAGE gel. Normal mouse IgG was used for control.

2.2.12.4. Protein Analysis

2.2.12.4.1. Sodium Dodecyl Sulfate Polyacrylamide (SDS-PAGE) Electrophoresis

Proteins were mixed with 1M DTT (0,2M final) and 3X Blue Loading Dye. Then, they were boiled for 5 min at 95°C. After removing combs from SDS-PAGE gels, 20-30 µg protein and protein ladder were loaded onto SDS-PAGE gel. Gels were put onto western tank containing 1X electrophoresis (running) buffer and the voltage was adjusted to 80 V. When samples were achieved resolving gel, voltage was made high (100-120 V). Samples were run about 3-3,5 h.

In order to prepare 10% SDS-PAGE gel, resolving gel was prepared as shown in Table 2.13.

Table 2.13: Resolving gel components for 2 gels (for 1 mm glass plates).

Resolving Gel	Volume
dH ₂ O	4,825 ml
40% Acrylamide	2,475 ml
1.5 M Tris pH: 8.8	2,5 ml
10% SDS	100 µl
10% APS	100 µl
TEMED	4 µl

In order to prepare 10% SDS-PAGE gel, stacking gel was prepared as shown in Table 2.14.

Table 2.14: Stacking gel components for 2 gels (for 1 mm glass plates).

Stacking Gel	Volume
dH ₂ O	2,25 ml
40% Acrylamide	375 µl
1 M Tris pH: 6.8	380 µl
10% SDS	30 µl
10% APS	30 µl
TEMED	3 µl

- To prepare 1 M DTT; 1.54 g of DTT powder was dissolved in 10 ml dH₂O. The solution was sterilized with filtration. 0.22 µm syringe filter was used.
- To prepare 10X Electrophoresis (Running) Buffer; 10 g of SDS, 30,2 g of Tris-base and 144 g of Glycine were dissolved in 10 liter of dH₂O. It was diluted to 1X concentration before using.
- To prepare 10% Ammonium persulfate (APS); 0,1g of APS was dissolved in 1 ml of dH₂O.

2.2.12.4.2. Western Blotting

Criterion Blotter Wet Transfer System was used to transfer of proteins.

After SDS-PAGE electrophoresis, proteins were transferred to PVDF membrane from gel. Firstly, PVDF membranes were cut as same size with gels and were wetted with absolute methanol (for 1 min) and distilled water, respectively. PVDF membranes and gels were kept waiting into 1X transfer buffer which also include 15% methanol, before using. Also fiber pads, filter papers and cassette were wetted with 1X transfer buffer.

According to Criterion Blotter (wet transfer) system, 2 pieces of filter papers were put on fiber pad that is on the anode side of sandwich apparatus of transfer system. Then, gels were put on filter papers, and PVDF membrane, 2 pieces of filter papers and other fiber pad (on the cathode side) was put on gels, respectively. The system was put into Criterion Blotter tank including 1X transfer buffer without methanol. Then, the system was run at 100V for 1 hour utilizing power supplier.

- To prepare 10X Transfer Buffer; 144 g of Glycine and 30,2 g Tris were dissolved in 1 liter of dH₂O. Before using, it was diluted to 1X concentration.

2.2.12.4.3. Immunostaining and Imaging

After transfer, membranes were washed with TBS-T for 5 minutes, two times. To avoid nonspecific binding of proteins with antibodies, membranes were incubated with blocking solution with shaking for 1 -1,5 hours, at room temperature. Next, membranes were washed three times with TBS-T for 10 minutes. Primary antibody was prepared with 5% nonfat dry milk or 3% BSA. Then, the membranes were incubated overnight with primary antibody at 4°C on shaker. Dilution range of primary antibodies and solution where antibodies were diluted in are showed in Table 2.15.

Table 2.15: Dilution ranges and solution types for primary antibodies used in experiments.

Primary Antibody	Solution	Ratio
Flag	3% BSA	1:20000
Pea3	5% Nonfat Dry Milk	1:1000
Total ERK	3% BSA	1:5000
B-Tubulin	3% BSA	1:1000

The day after, membranes were washed with TBS-T solution for 10 minutes three times. Secondary antibodies were diluted in TBS-T solution. Membranes and secondary antibody solution were incubated together with shaking at room temperature for 1 h. Dilution range of secondary antibodies and solution where antibodies were diluted in are showed in Table 2.16.

Table 2.16: Dilution ranges and solution types for seconder antibodies used in experiments.

Primary Antibody	Solution	Ratio
Anti-mouse IgG HRP	3% BSA or 5% Nonfat Dry Milk	1:2000 or 1:5000
Anti-rabbit IgG HRP	5% Nonfat Dry Milk	1:5000

Then, membranes were washed three times in TBS-T for 10 minutes, after secondary antibody incubation.

- To prepare 10X TBS; 24,2 g of Tris and 80 g of NaCl were dissolved in 1 liter of dH₂O and it was used at 1X concentration. pH value was adjusted to 7.6 with HCl.
- To prepare TBS-T; 0,1% Tween20 was added into 1X TBS.
- To prepare nonfat dry milk; 5% nonfat dry milk powder was dissolved in TBS-T.
- To prepare BSA solution; 3% BSA powder was dissolveed in TBS-T.

Luminata Crescendo Western HRP Substrate was used to image protein bands. After substrate was poured on membrane, they were incubated for 1 minute. Blots were scanned with ChemiDoc™ XRS+ System.

2.2.12.4.4. Stripping

To strip the membranes, Restore Plus Western Blot Stripping Buffer was used. Membranes were washed in TBS-T for 10 minutes at room temperature before stripping. Next, stripping buffer was added into membranes and incubated at 37°C for 15 minutes on shaker. Then, membranes were washed in TBS-T for two times. Protocol was continued as blocking, immunostaining and imaging steps that are mentioned before.

2.2.13. Luciferase Assay

2.2.13.1. Transfection for Luciferase Assay

Transient transfection was performed with polyethylenimine (PEI) transfection reagent essentially as described before. To analyze regulation of *EPHA1* and *EPHA2* promoters with wildtype vs mutant mPea3, 6.10⁴ of U87-MG cell lines were seeded into 24-well tissue culture plates. The day after, DNA concentrations were adjusted according to Table 2.17.

Table 2.17: DNA concentrations for EPHA promoters and mPea3 luciferase assay.

Name	mPea3 (mutant or wild type)	Empty Vector (pCDNA3 or pCMV)	EPHA (Luciferase)	Renilla
Amount	-	1000 ng/μl	300 ng/μl	50 ng/μl
	300 ng/μl	600 ng/μl	300 ng/μl	50 ng/μl
	600 ng/μl	300 ng/μl	300 ng/μl	50 ng/μl
	1000 ng/μl	-	300 ng/μl	50 ng/μl

All repetition mix (usually two) was prepared in one tube. After addition of DNAs, 500 µl of serum free DMEM was add into tubes. 2,75 µl of PEI was also added into tubes. All samples were mixed and incubated at room temperature for 15 min. 300 µl of complete medium was added into tubes and all samples were distributed on cells after discarding old mediums from seeded cells. Cells were incubated at 37°C for 1,5 h in the incubator. Next, 300µl of complete medium was added on cells after 2 hours. Cells were incubated about 48 hours at 37°C in 5% CO₂ incubator. GFP was also used for checking transfection efficiency.

For regulation of *SOX2*, *POU5F1*, and *NANOG* promoters by mPea3, 6.10⁴ of SH-SY5Y human neuroblastoma cells were seeded into 24-well tissue culture plates, before transfection process.

The next day, DNAs were diluted appropriate concentrations for transfection. Optimized DNA:PEI ratio (generally 1:4) was preferred to obtain successful transfection rate. Amount of DNA is showed in Table 2.18 that is used in *SOX2*, *NANOG* and *POU5F1* promoters and mutant vs wildtype mPea3 interaction assays. All samples were repeated for three times in the assay. When Pea3VP16 plasmids provide continuous expression of mPea3, mPea3-pCMV provides overexpression of exogenous mPea3. pCMV also provides endogenous expression of mPea3.

Table 2.18: DNA concentration for *SOX2*, *POU5F1* and *NANOG* promoters and mPea3 luciferase assay.

Name	SOX2,POU5F1 or NANOG	Renilla	pCMV	Pea3VP16	mPea3- pCMV (mutant or wild type)
Amount	550 ng/µl	100 ng/µl	550 ng/µl	-	-
	550 ng/µl	100 ng/µl	-	550 ng/µl	-
	550 ng/µl	100 ng/µl	-	-	550 ng/µl

DNA amount was 1,2 µg per well. All repetition mix (in this case three repeats) was prepared in one tube. After addition of DNAs, 750 µl of serum free DMEM was add into tubes. 14,4 µl of PEI was also added into tubes. All samples were mixed with vortex and incubated at room temperature for 15 min. During

incubation, old mediums from seeded cells were discarded. 300 μ l of complete medium was added into tubes and all samples were distributed on cells. Cells were incubated at 37°C for 2 hours in the incubator. Next, 500 μ l of complete medium was added on cells after 2 hours. Cells were incubated about 48 hours at 37°C in 5% CO₂ incubator. GFP was also used for checking transfection efficiency.

In *Neurofilament-L (NFL)* and mPea3 regulation assay, 6.10⁴ cells of SH-SY5Y were seeded in 24-well plates. The day after, concentration of samples was adjusted according to Table 2.19. All samples were repeated for two times in the assay. DNA: PEI ratio was adjusted as 1:4.

Table 2.19: DNA concentrations for *NEUROFILAMENT-L* and mPea3 luciferase assay.

Name	mPea3-pCMV (mutants or wild type)	pCMV	NFL Luciferase	Renilla
Amount	-	1200 ng/ μ l	400 ng/ μ l	200 ng/ μ l
	400 ng/ μ l	800 ng/ μ l	400 ng/ μ l	200 ng/ μ l
	800 ng/ μ l	400 ng/ μ l	400 ng/ μ l	200 ng/ μ l
	1200 ng/ μ l	-	400 ng/ μ l	200 ng/ μ l

All repeated samples were prepared in one tube. After addition of DNAs, 400 μ l of serum free DMEM was added into tubes. Then, 9,6 μ l of PEI was added and tubes were mixed with vortex. They were incubated at room temperature for 15 minutes. Next, 400 μ l of complete medium was added into tubes. After discarding of old media from seeded cells, transfection mix were transferred on cells. After incubation of 2 hours at 37°C, 400 μ l of complete medium was added on cells. Cells were incubated about 48 hours at 37°C in 5% CO₂ incubator. GFP was also used for checking transfection efficiency.

2.2.13.2. Firefly-Renilla Luciferase Assay

Dual Glo Luciferase Assay System (Promega) was used for all luciferase assay.

At first, all wells (24-well plates) contained transfected cells were washed with 200 μ l of PBS and then the PBS was discarded. 5X PLB (Passive Lysis Buffer) was diluted 1:5 with distilled water before use. 100 μ l of PLB was added in all wells and plates were shaken with vortex for 20-30 seconds. Then, 24-well plates incubated at room temperature for 15 minutes with shaking. Attached cells were scraped from wells and 75 μ l of PLB-cell mix were transferred to 96-well plate one by one.

75 μ l of Dual Glo Firefly Reagent (luciferase detection reagent) that is was stored on dark at -80°C with aliquoted was added into all well and incubated at room temperature for 15 minutes in the dark. Firefly Luciferase activity was measured with microplate luminometer (Varioskan). After measurement of firefly luciferase activity, second reagent was prepared as 1:100 ratio of Stop & Glo Substrate and Dual-Glo Stop & Glo Buffer. 75 μ l of second reagent was added in wells. 96-well plates were incubated at room temperature for 15 minutes in the dark. Measurement of *Renilla* Luciferase activity was measured with microplate luminometer (Varioskan Flash, Thermo Scientific).

3. RESULTS

3.1. Screening Mutant mPea3 Plasmids

Mutant mPea3 plasmids, some of which were cloned previously in different base plasmids such as pCMV, pBlueScript or pCDNA3 in Molecular Neurobiology Laboratory before their relocation to Gebze Technical University, were screened to confirm and validate base plasmids and mutations before starting experiments. The screening was done by digestion with Mlu I and Hind III restriction enzymes that result in unique band sizes depending on whether the base plasmid is pCDNA3.1, pBS-KS or pCMV-3 Tag-6. After the screening, S90E mutant mPea3 and S458E mutant mPea3 was found to be in pCMV-3 Tag-6 vector (data not shown) and they were used in our ongoing experiments. Other mutant mPea3 plasmids were found to be in pCDNA3.1 or pBS-KS vectors.

The positive mutant Pea3 plasmids were subcloned into pCMV-3 Tag-6 vector. However, not all cloning attempts were positive (data not shown), and the strategy of obtaining full set of mPea3 mutants was changed. All mutant mPea3 plasmids, except for S90E and S458E mutants, were obtained by myself with site-directed mutagenesis using the wild-type mPea3 in pCMV-3 Tag-6 vector present in the lab.

3.2. Confirmation of Mutant mPea3 Plasmids by Sequencing

Mutations of putative phosphorylation sites of Pea3 (shown in Figure 3.1) occurred by site-directed mutagenesis technique and mutant mPea3 plasmids obtained before were checked to validate mutations with sequencing technique. Results of sequences are demonstrated in 'Appendix A' section of the thesis.

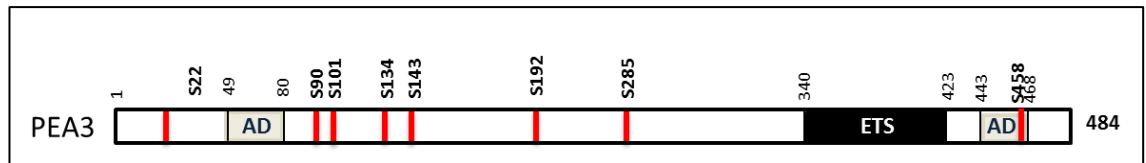


Figure 3.1: Putative phosphorylation sites of Pea3 transcription factor for MAPK-activated pathway. Totally, eight phosphorylation sites were chosen to mutagenesis illustrated with red lines. All chosen sites were mimicked or silenced with changing specific amino acids from Serine to Glutamic Acid or Alanine, respectively. Acidic domains of Pea3 are highlighted with grey and black box illustrates ETS domain (DNA-binding domain).

3.3. Expression Analysis of Wildtype and Mutant mPea3 Proteins in SH-SY5Y Human Neuroblastoma Cell Lines

The expression analysis of mutant mPea3 proteins was performed in SH-SY5Y human neuroblastoma cells. After transfection of mutant mPea3 plasmids into cells, the cells were incubated with 10% FBS, high glucose DMEM media at 37°C, 5% CO₂. After 48 hours, proteins were isolated and their expression was firstly controlled with Flag antibody, because of the plasmid constructs containing flag tag. Pea3 antibody was also used to validate specific Pea3 bands and β -tubulin was utilized for normalization. The expression analysis experiments were performed by Busra Nur Cicek, an undergraduate project student under my supervision.

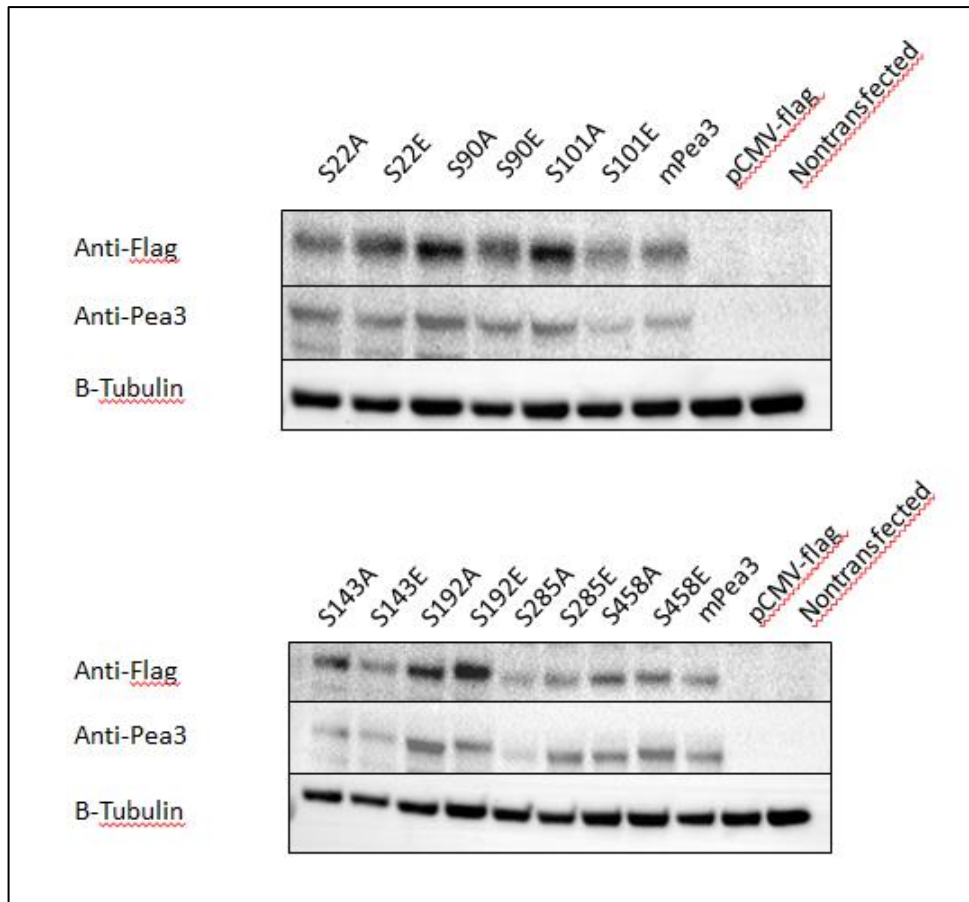


Figure 3.2: Results of immunoblot analysis of mutant mPea3 proteins that were transfected into SH-SY5Y cells. The normalization analyses are also shown in Figure 3.3.

According to analysis results (Figure 3.3) especially mutant types of S22E, S90A, S101A, S192A and S192E shows high expression level when we normalized to tubulin.

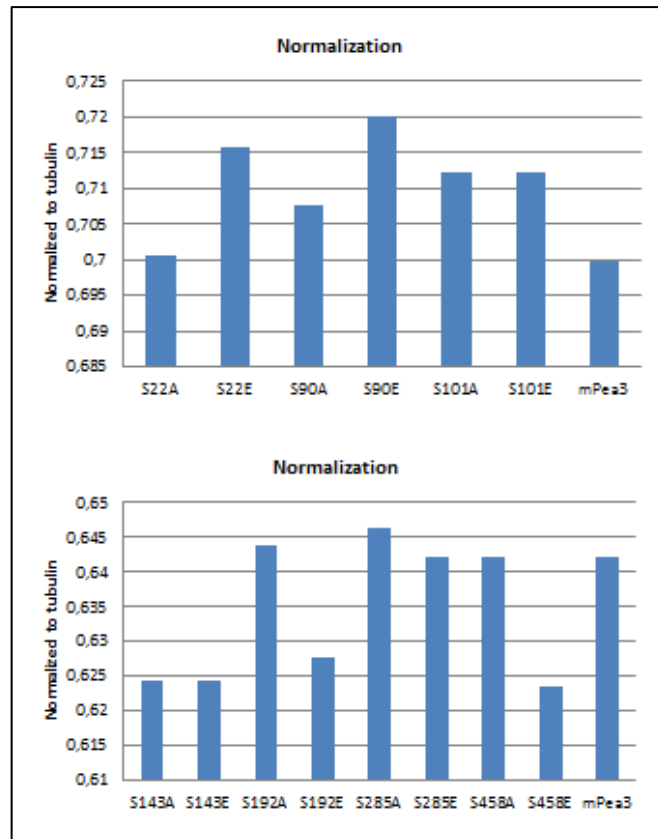


Figure 3.3: Normalization of mPea3 proteins which are demonstrated the protein expression levels in Figure 3.2 according to tubulin.

3.4. Analysis of Protein Expression of Mutant mPea3 Protein upon Growth Factor Induction

In previous studies of our laboratory as well as other researchers have revealed that different types of growth factors regulate the Pea3 proteins via MAPK/ERK pathway with phosphorylation [1]. However, the specific target for this phosphorylation was not identified in literature. Thus, to understand how growth factors affect the Pea3 protein and on which amino acids, we performed the assays that are described in this study.

Before starting the analyses, we have investigated which growth factor receptors were present in SH-SY5Y. We have determined that among many growth factors, bFGF, NGF and GDNF that are known to be important for Pea3 activity in the nervous system and whose receptors are present in SH-SY5Y cells would be the focus of our work.

3.4.1. Induction with Basic Fibroblast Growth Factor

First of all, wild type mPea3 plasmids were transfected into SH-SY5Y cells and 20 ng/ml of bFGF induction was performed 2 hours before protein isolation. Growth factor concentration (20 ng/ml) was determined based on previous studies in the literature. Western blot was performed with lysates of cells transfected with empty pCMV plasmid, mPea3 expression plasmid, or mPea3 expression plasmid followed by FGF induction. Expression level of mPea3 in the input (lower panel, Figure 3.4) as well as IP sample (upper panel, Figure 3.4) with or without bFGF induction was quite similar. However, any phosphorylation, if at all present, could not be observed as a retarded band in this assay.

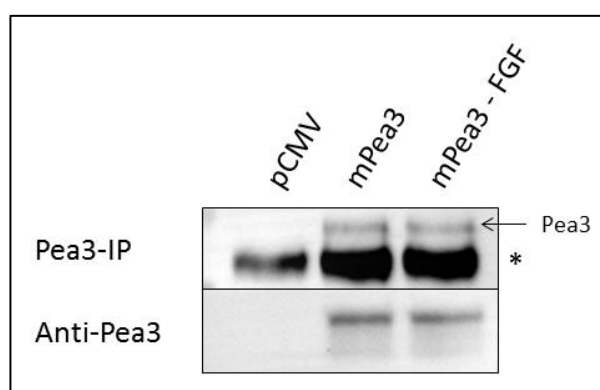


Figure 3.4: Western blot of Pea3-IP (upper panel) and whole cell lysate (lower panel) samples with or without basic fibroblast growth factor induction in SH-SY5Y cells.

Bands shown with arrow indicate the immunoprecipitated mPea3 (~62 kDa), and bands marked with asterisk shows IgG in the upper panel. Lower panel is the input control.

We then performed a duration analysis of the bFGF, since MAPK/ERK activation upon bFGF stimulation is known to be transient. About 48 hours after from transfection of wild type mPea3 plasmids, bFGF was added at the time of 15 minutes, 30 minutes, 45 minutes and 1 hour before protein isolation. Duration analysis was demonstrated in Figure 3.5 and normalization was done according to tubulin expression. Induction with bFGF through 30 minutes was decided as the ideal duration for our analysis. The following experiments were carried out with this time point.

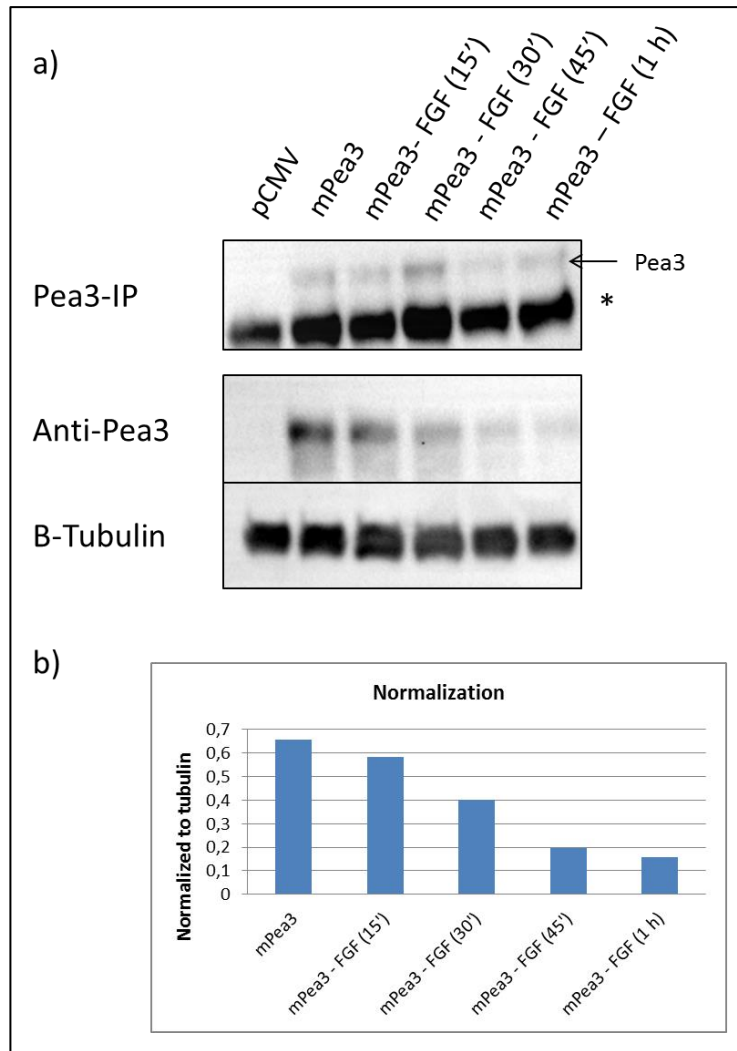


Figure 3.5: Duration analysis of bFGF activity. a) Immunoprecipitation and Western Blot analysis of mPea3 transformation protein induced with bFGF in certain times (15 min, 30 min, 45 min and 1 hour). Asterisk demonstrates IgG bands, b) Normalization of mPea3 proteins according to tubulin.

Since we could not directly investigate whether site-directed mutagenesis constructs had any effect on phosphorylation of Pea3 by growth factors, due to lack of phosphor-specific antibodies for Pea3, we first started to analyze expression levels of phosphomutant Pea3, by transfecting SH-SY5Y cells with these mutants, followed by bFGF induction for 30 minutes (Figure 3.6). In order to compare first level of expression and then phosphorylation (if at all possible as a retarded band), the mPea3 band in each lane was normalized to tubulin level in corresponding blot. Interestingly, not every mutant was expressed to the same level as wildtype mPea3, with or without bFGF stimulation. Upon normalization, it was observed that while S90A mutant was expressed to a similar level as the wildtype protein and increased

upon bFGF induction, S90E mutant had significantly higher level of expression as compared to mPea3 wildtype, while this expression was decreased upon stimulation (Figures 3.6, 3.7). A similar profile was observed with S143A and S143E mutants (Figures 3.6, 3.7). On the other hand, expression level of S192A mutant was increased around twofold before bFGF induction, and about six fold after bFGF induction when compared to wildtype mPea3 without bFGF stimulation; whereas S192E mutant expression level was increased around fourfold without induction, and decreased to the same level as wildtype mPea3 (Figures 3.6, 3.7). It is, unfortunately, impossible to directly deduce phosphorylation from these analyses, however certain mutations seem to effect level of expression of Pea3 (since anti-Pea3 antibody is used, these blots show a combination of both exogenous and endogenous Pea3 expression), thereby indicating to a possible feedback loop in the presence of bFGF signaling. To that end, future studies should use anti-Flag antibody to complement anti-Pea3 antibody blots and to distinguish exogenous Pea3-Flags from endogenous Pea3.

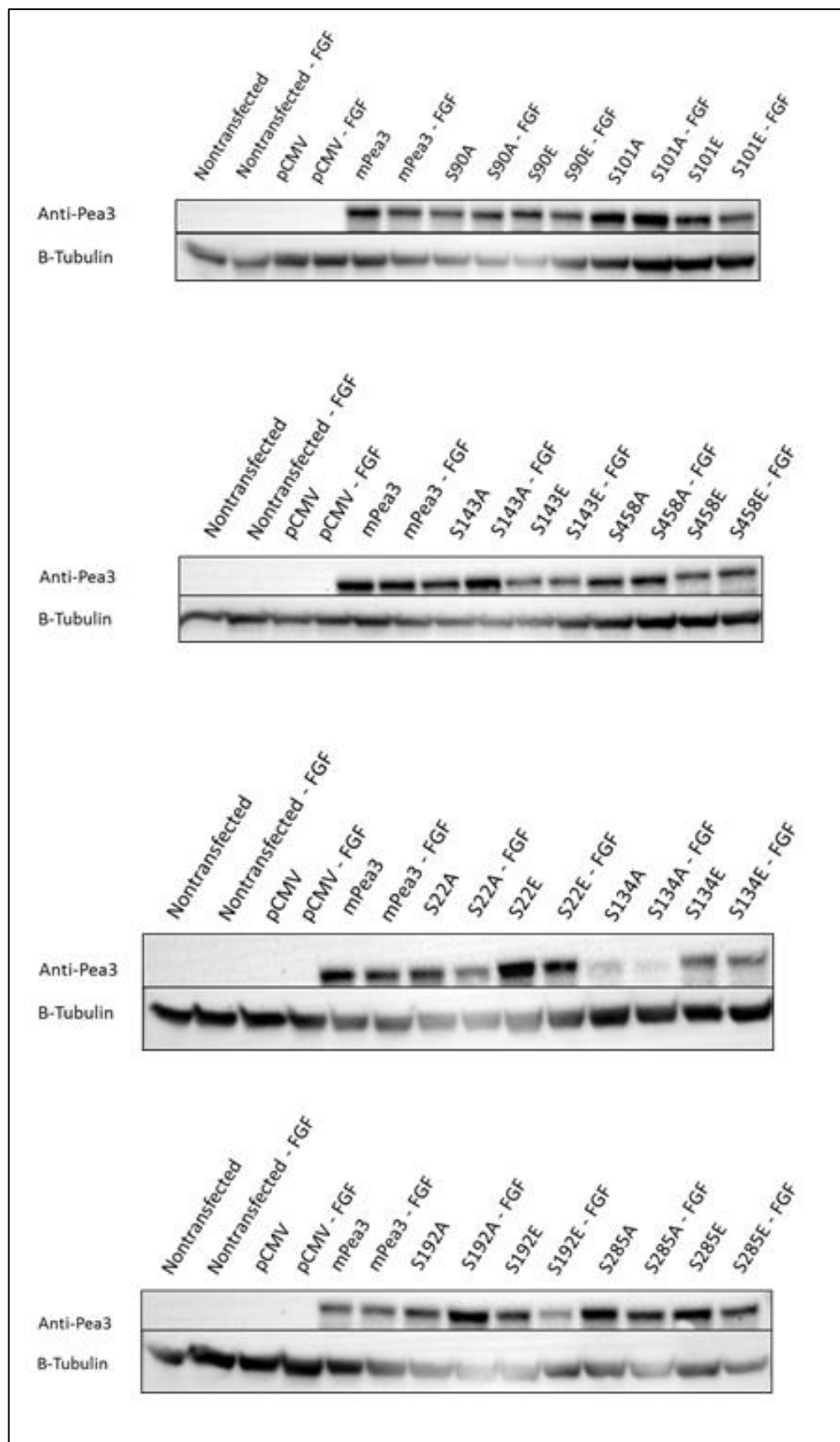


Figure 3.6: Immunoblot analysis of mutant mPea3 expression transfected into SH-SY5Y cells. The normalization results were shown in Figure 3.7.

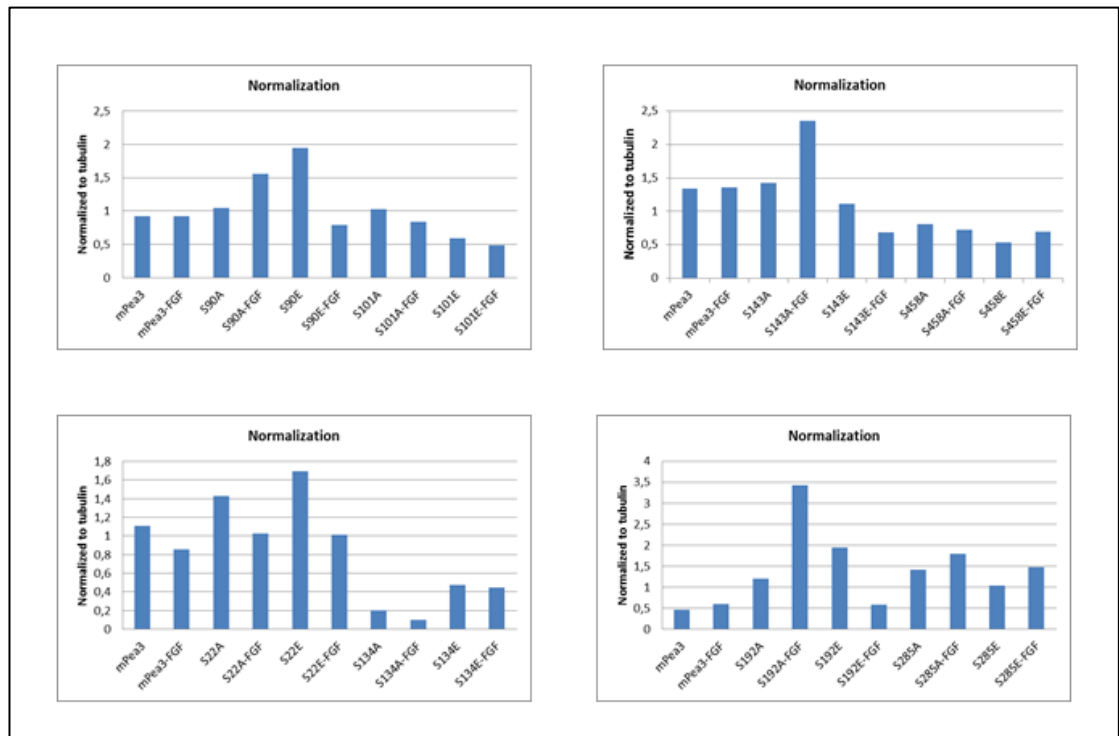


Figure 3.7: Normalization of protein levels of mutant mPea3 that were demonstrated their immunoblot analysis in Figure 3.6 according to tubulin.

In Figure 3.6, faded bands in the middle of the membranes were concerning, and could have resulted in mis-normalization of images. To clarify whether it was a transfer problem or not, all cell lysates were re-run in a different order of lanes (Figure 3.8).

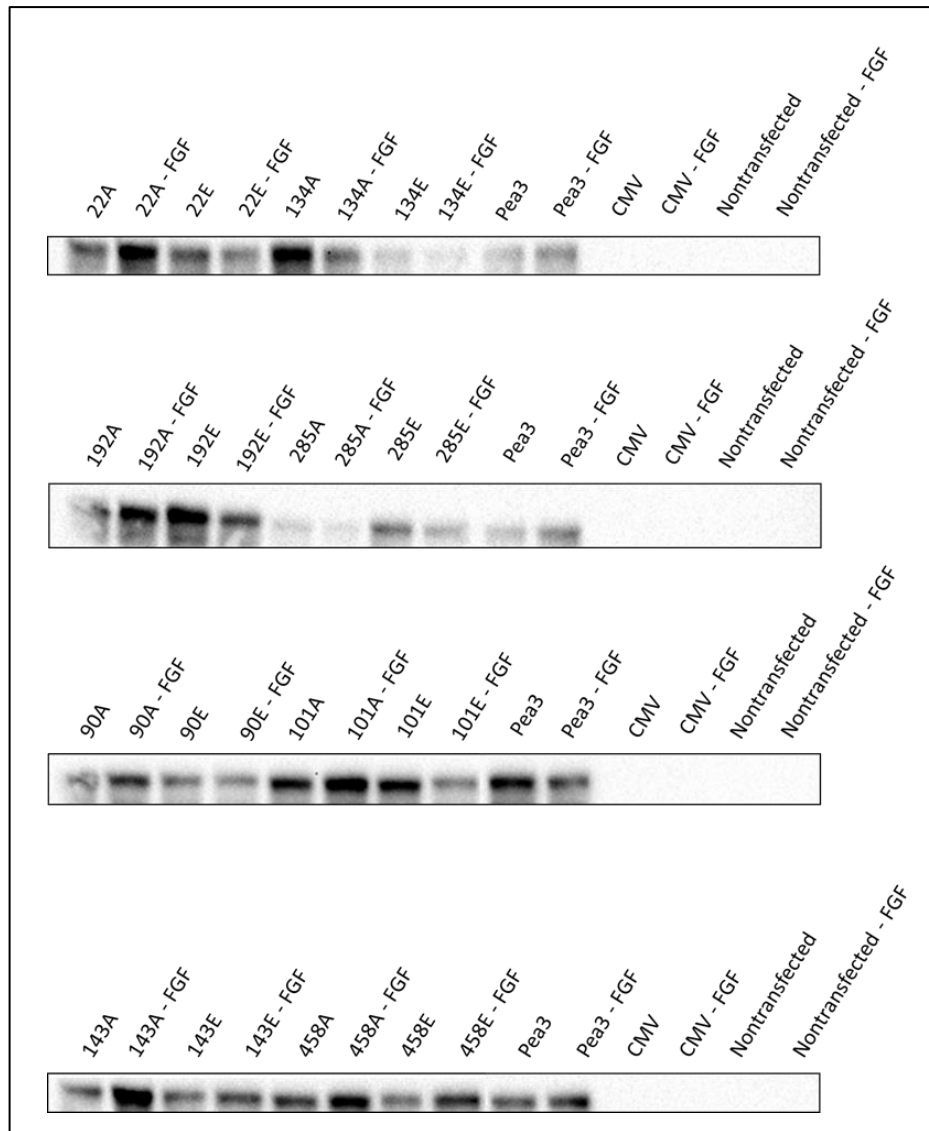


Figure 3.8: Protein analysis of mutant mPea3 expression transfected into SH-SY5Y cells with or without FGF induction.

Second protein analysis is shown that there was indeed a transfer problem in the first analysis (Figure 3.6). Tubulin control and normalization will be performed to compare and reproduce normalizations in Figure 3.7, as well as confirm the conclusions from the original analyses.

3.4.2. Induction with Nerve Growth Factor

The other growth factor analyzed is nerve growth factor (NGF). Similar to before, wild type mPea3 plasmids were initially transfected into SH-SY5Y cells and 100 ng/ml of NGF induction was performed in certain durations (30 minutes and 1

hour) before collecting cell lysate to understand the most active time of phosphorylation of NGF. Growth factor concentration (100 ng/ml) was determined from previous studies in the literature. Expression level of NGF-induced wild type mPea3 was checked using Pea3 antibody and the ideal induction duration is determined as 1 hour (Figure 3.9). Pea3 bands were at the size of 62 kDa. The following experiments used 1 hour induction of NGF.

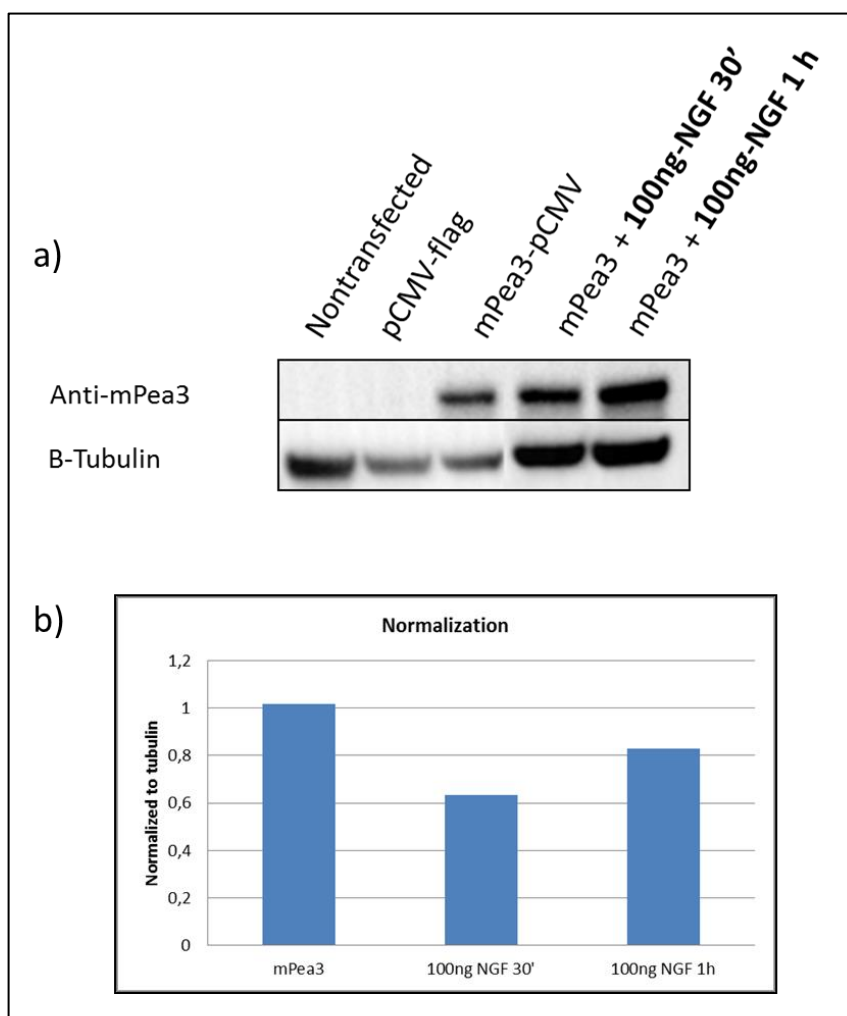


Figure 3.9: a) The immunoblot analysis of NGF-induced SH-SY5Y transfected cells, b) Normalization of mPea3 expression levels over the tubulin levels.

3.4.3. Induction Glial Cell Line-Derived Neurotrophic Factor

The last growth factor used in our experiments is glial cell line-derived neurotrophic factor (GDNF).

The growth factor concentration was investigated in literature and the most common practice of 50 ng/ml and 100 ng/ml concentrations were used in our study. Essentially, wild type mPea3 plasmids were transfected into SH-SY5Y cells, and 50 ng/ml and 100 ng/ml of GDNF inductions were performed for 30 minutes and 1 hour before collecting cell lysate. Expression level of GDNF-induced wild type mPea3 was controlled with Pea3 antibody and the ideal induction duration is decided to 50 ng/ml of GDNF induction through 30 minutes according to tubulin normalization which is demonstrated in Figure 3.10. Pea3 bands were at the size of 62 kDa. The following experiments were performed under these selected conditions. Nonetheless, it should be noted that both for NGF and GDNF, upon normalization mPea3 expression levels are significantly decreased in our assay system, as opposed to our expectations based on previous reports in the literature.

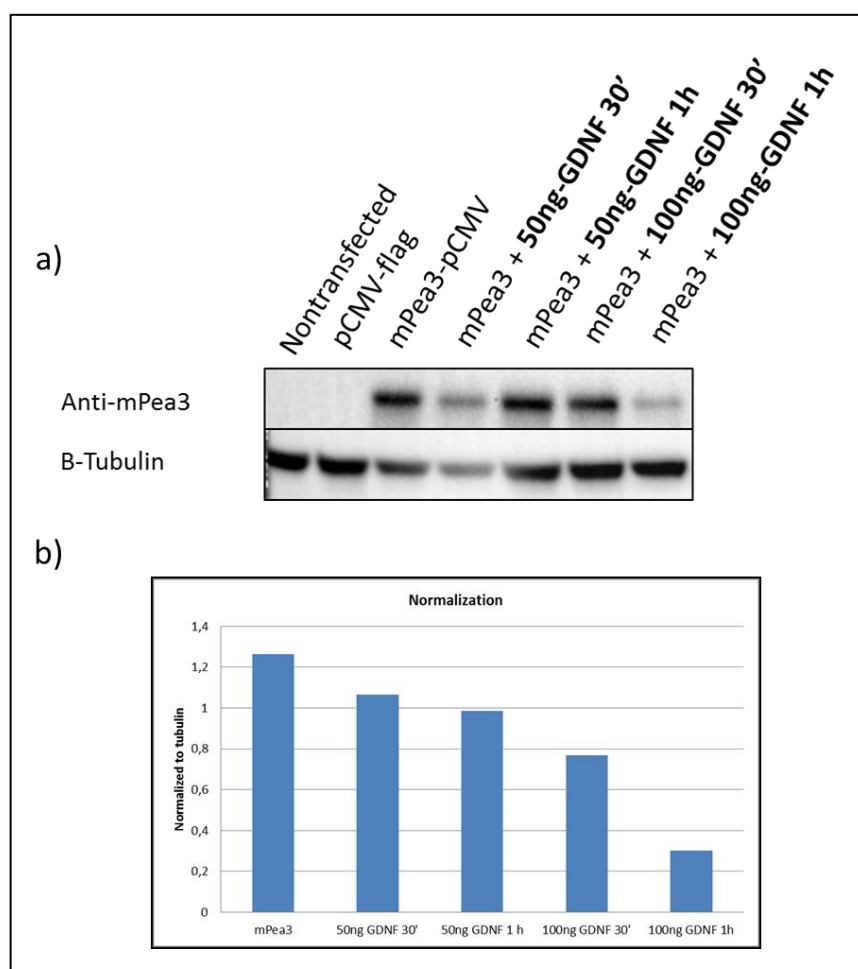


Figure 3.10: a) The immunoblot analysis of GDNF-induced SH-SY5Y transfected cells, b) Normalization of mPea3 expression levels over the tubulin levels.

3.5. Regulation Analysis between Specific Promoters and Wild Type or Mutant mPea3 Proteins

Parallel to previous assays studying the expression and phosphorylation of Pea3, we also wanted to analyze what effect the mutations would have on transcriptional activity of this transcription factor.

To that end, we examined the regulation of various known target promoters by wildtype and mutant mPea3 using luciferase reporter assay. Co-transfection of phospho-mutant or wild type mPea3 with different promoter-luciferase constructs to SH-SY5Y cells was used to determine transcriptional activity of these different mutants. The Firefly luciferase activities are normalized to *Renilla* luciferase activity, used as an internal control.

3.5.1. The Effects of Wild Type mPea3 on *hSOX2*, *hPOU5F1*, *hNANOG* Promoters

According to a recent study, *PEA3* and *ERM* are expressed in embryonic stem cells and this expression is correlated with self-renewal capacity and pluripotent properties of these cells [39]. In this study, it is shown that Oct3/4 regulates *PEA3* and *ERM* expression but not the other way around, and they have role in embryonic stem cell proliferation and differentiation [39]. In our laboratory, we had been studying the role of Elk-1, another ETS domain factor, in relation to maintenance of stemness, therefore we wanted to check in our assay system whether Pea3 indeed regulated from stem cell-specific promoters such as embryonic stem cell markers *OCT3/4* (*POU5F1*), as *SOX2*, *NANOG* and whether mutagenesis of Pea3 had any effect on this regulation.

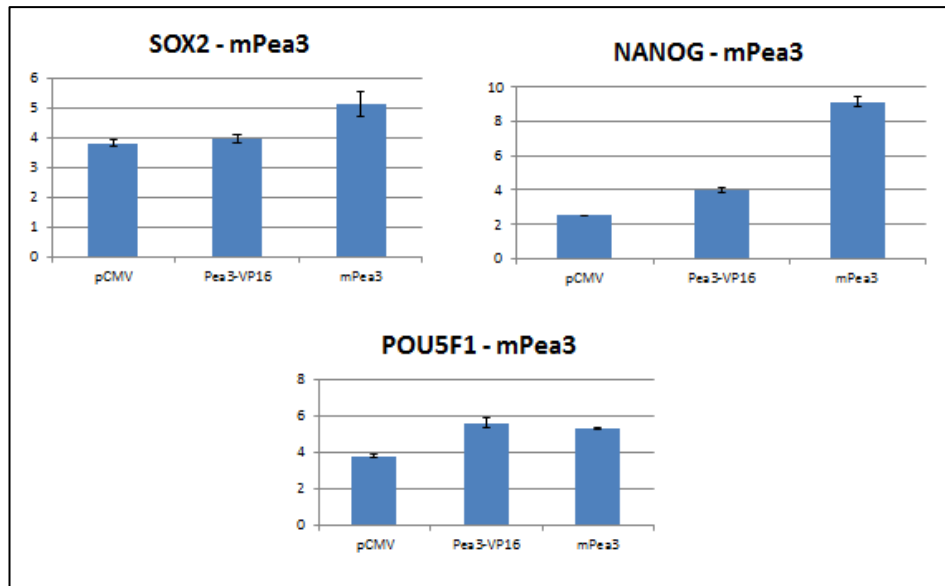


Figure 3.11: Results of luciferase assays for the regulation of hSOX2, hNANOG and hPOU5F1 promoter for wild type mPea3 protein.

In this study, we observed that POU5F1 promoter was hardly responsive to exogenous Pea3 overexpression in SH-SY5Y cells. The regulation of SOX2 promoter by mPea3 or its constitutively active version, Pea3-VP16, was minimal, as shown in Figure 3.11. However, interestingly NANOG promoter regulation was significantly activated, nearly fourfold by mPea3 (Figure 3.11).

3.5.2. The Effects of Mutant mPea3 on *hSOX2* Promoter

Although there was minimal increase in SOX promoter activity in response to Pea3 expression, we nonetheless wanted to check the effect of phosphomutants of Pea3. As shown in Figure 3.12, S101A and S192A mutant mPea3 showed greater activity on *hSOX2* promoter activity as compared to other mutants, while S101E and S192E showed similar activities. One thing to note here is that serine 101 is near the negative regulatory region of Pea3, indicating there could be an inhibitory phosphorylation on this residue, which is relieved when this Serine is mutated. Another interesting point to note is that *hSOX2*-luciferase activity of wild type mPea3, as well as phosphomutants, were much higher than that of constitutively active Pea3-VP16.

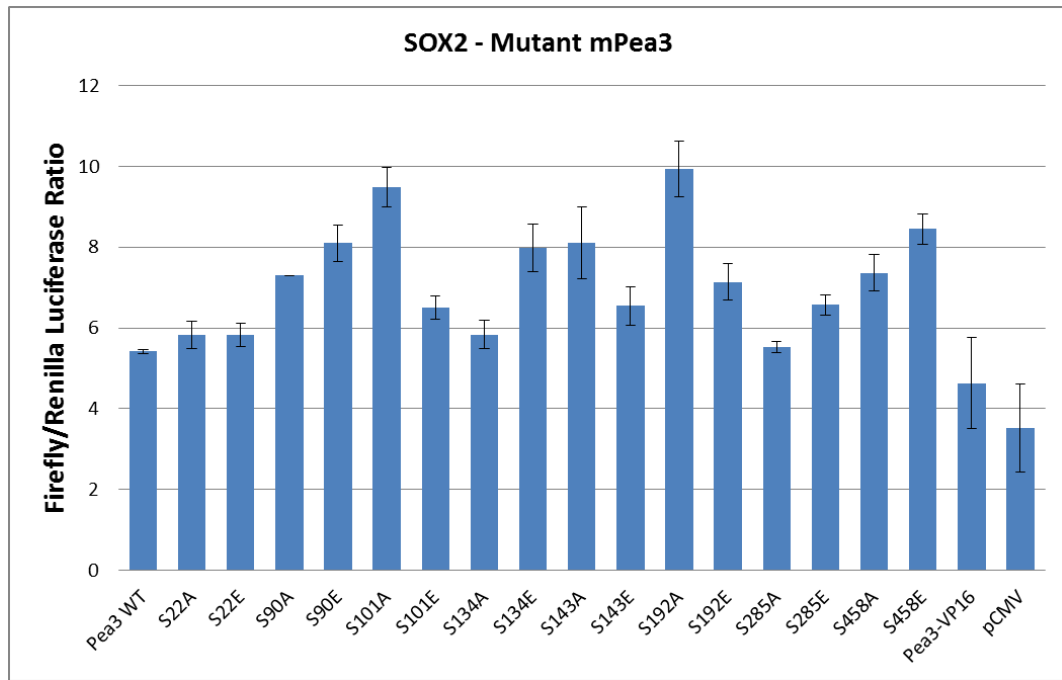


Figure 3.12: Results of luciferase assays for the regulation of hSOX2 promoter for mutant mPea3.

3.5.3. The Effects of Mutant mPea3 on *hNANOG* Promoter

Pea3 had shown the highest transcriptional activity on the *hNANOG* promoter, hence we have also analyzed all phosphomutants for their effect on this promoter. As can be seen in Figure 3.13, transcriptional activity from *hNANOG* promoter was nearly 1.6-fold higher with S143A mutant mPea3 than any other. S285A mutation interfered with this activity, while S285E restored the transcriptional activity of Pea3 on this promoter, indicating that phosphorylation of Pea3 on this residue may indeed be important for the reported effect of Pea3 on stem cell renewal. On this promoter, both S90A and S90E had a slightly negative effect on transactivation function of Pea3.

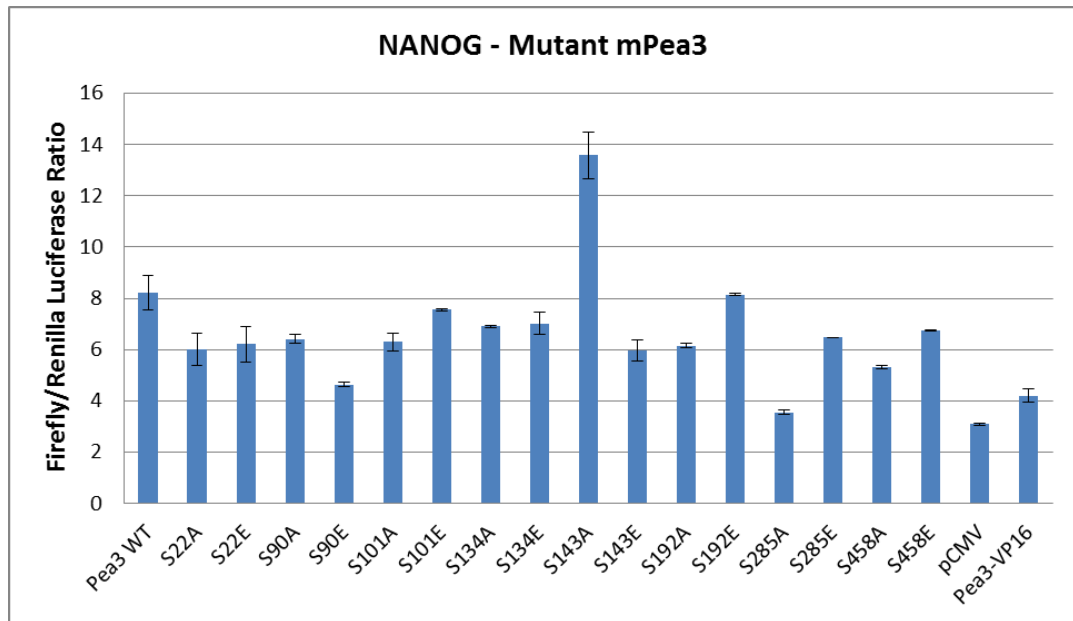


Figure 3.13: Results of luciferase assays for the regulation of *hNANOG* promoter for mutant mPea3.

3.5.4. The Effects of Mutant mPea3 on *EPHA1* and *EPHA2* Promoters

Unlike previous report stated above, our laboratory is mainly interested in axonal outgrowth-related function of Pea3, and other graduate students in the laboratory have identified several novel targets of Pea3 [40]. Among them, ephrins and their receptors are particularly interesting. Ephrins and ephrin receptors are known to be related to axonal development and glioblastoma progression. We have therefore set out to analyze Pea3 phosphomutants on *EPHA1* and *EPHA2* promoters with luciferase analysis.

The activity of *EPHA1* and *EPHA2* promoters are initially analyzed for S458A and S458E mutants in U87-MG cells. As can be seen in Figure 3.14, *EPHA1* promoter activity is slightly affected by S458A mutation when compared to wildtype mPea3, and its S458E counterpart, particularly at low doses (Figure 3.14). This effect is lost at higher doses of expression plasmid.

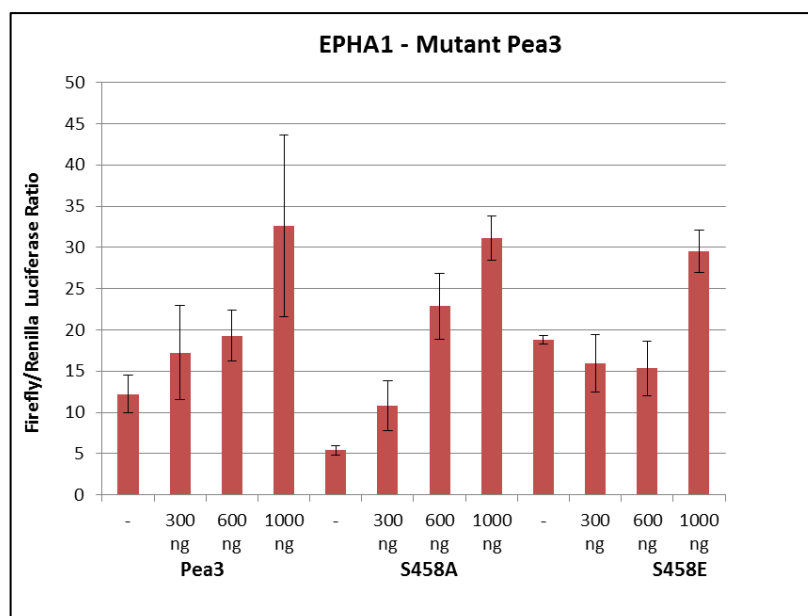


Figure 3.14: Results of luciferase analyses for the activity of EPHA1 promoter.

As can be seen in Figure 3.15, *EPHA2* promoter activity Pea3 mutants could not be studied in U87 MG cells, due to high variability and low transfection efficiency of these cells as compared to SH-SY5Y cells, therefore these experiments need to be repeated with SH-SY5Y cells and with full range of Pea3 phosphomutants in the future.

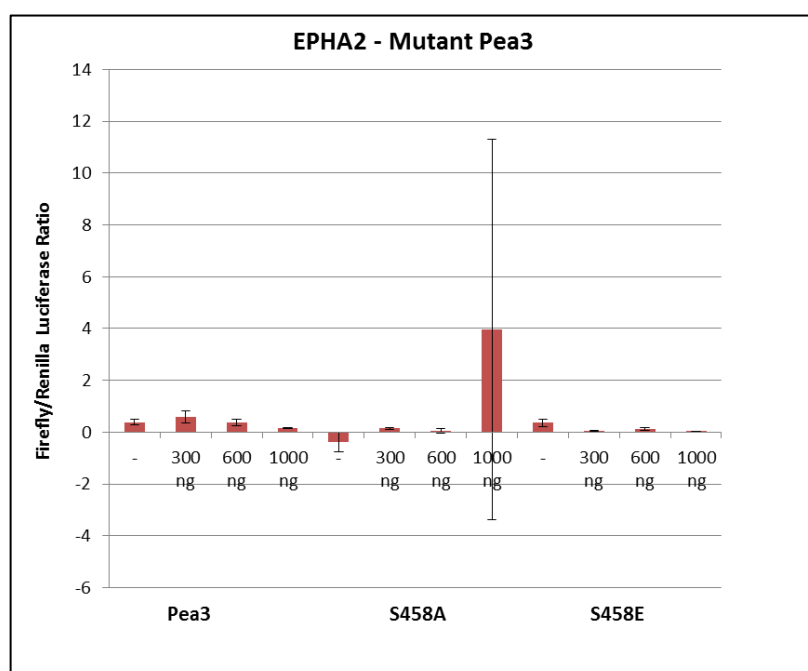


Figure 3.15: Results of luciferase analyses for the activity of EPHA2 promoter.

3.5.5. The Effects of Mutant mPea3 on *NEUROFILAMENT-L* Promoters

According to previous studies in our lab, *Neurofilament-L* and *Neurofilament-M* are demonstrated as putative target for Pea3 proteins and the microarray data shows about 2 fold upregulation in mPea3 transfected cells [1]. To analyze the regulation of this promoter with phosphomutant mPea3, luciferase analysis was performed.

As can be seen in Figure 3.16 and Figure 3.17, *NF-L* promoter activity was particularly lower with S101A, S101E, S134A and S285E phosphomutants when compared to wildtype. Since phosphomimic and phosphomutants do not exhibit opposing transcriptional activity in this context, one can assume that the effect observed is not due to phosphorylation affecting transactivation, but could be related to interaction with other proteins, or intra-molecular structural side effect of these mutations.

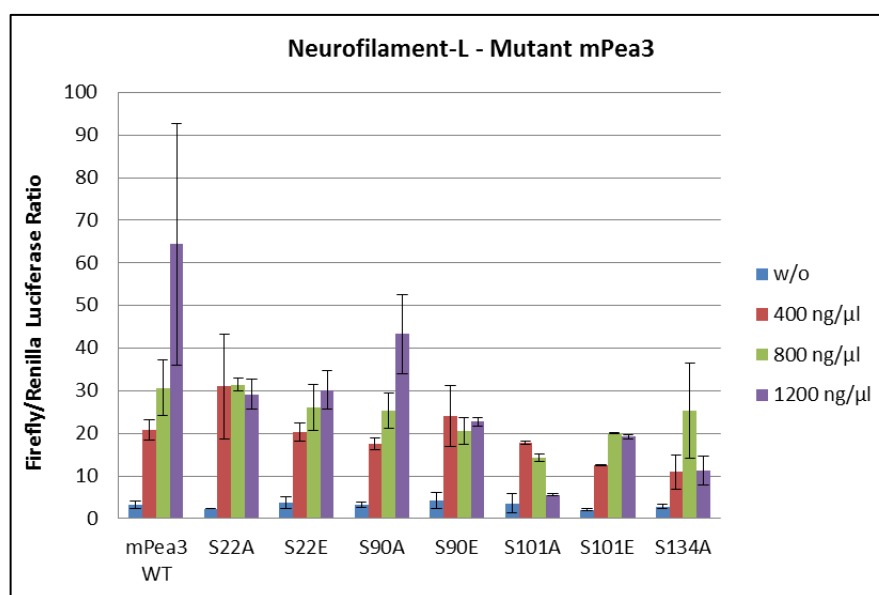


Figure 3.16: Results of luciferase analyses for the activity of NF-L promoter.

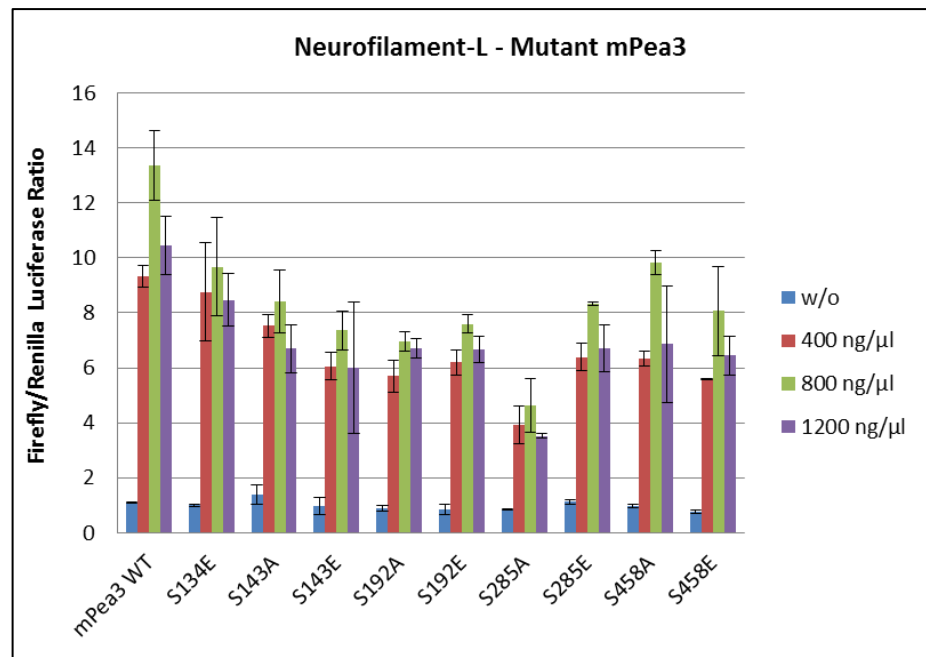


Figure 3.17: Results of luciferase analyses for the activity of NF-L promoter.

4. DISCUSSION

Pea3/E1AF/ETV4 is a member of the ETS domain superfamily of transcription factors downstream target of MAPK/ERK signaling pathway, which phosphorylates Serine or Threonine residues within an S/T-P motif upon growth factor stimulation.

Pea3 subfamily of proteins has been implicated in many processes in the developing nervous system, among others, and was shown to be regulated by various growth factors. Fibroblast growth factor, for example, is known to regulate Pea3 expression in developing chick retina and limb bud [4]. GDNF and Met signaling were both shown to be involved in motor neuron selectivity and motor neuron body positioning through Pea3 subfamily of proteins. Yet, to this date, the exact phosphorylation site for Pea3 had not been studied.

Our laboratory had been studying for quite some time several predicted putative phosphorylation motifs, albeit incomplete. Previous research from our laboratory had indicated Study of Serine 90 mutant Pea3 also revealed that Pea3 factors have a crucial role in axon guidance [1]. In this study, I have focused on completing the full set of putative phosphorylation sites, with the aim to understand of which phosphorylation sites on Pea3 gene is affected by which growth factor, and how that relates to the function of Pea3 in neurons.

As can also be seen in Table 4.1; we have shed some light on the questions stated above:

The phosphorylation mutations appear not to be expressed at the same level; for instance 134A mutant mPea3 expression is lower than wildtype mPea3, and this decrease is not depend on bFGF induction. 458A mutant mPea3 is expressed at significantly low levels irrespective of bFGF. Serine 458 is located within the activation domain of mPea3. S90E phosphomimic mutant of mPea3 was much higher, however this expression was decreased in response to bFGF stimulation. We believe the mutations created do not only knockdown or mimic phosphorylation, but may also have an effect on other posttranslational modifications on adjacent domains of the mutation site, and/or have an effect on intramolecular interactions that are seen with other ETS proteins.

Pea3 protein does not regulate POU5F1 promoter, and only has a minimal effect on hSOX2 promoter in our cell system.

Pea3 regulates *hNANOG* promoter, and S143A mutant has an even higher transactivation activity as compared to wildtype mPea3.

Table 4.1: Comparison of mutant mPea3 types according to FGF induction or luciferase assay. All mutants are compared in order to wildtype mPea3. Fold change $0 < + \leq 1$, $1 < ++ \leq 2$, $2 < +++ \leq 3$, $3 < ++++ \leq 4$ and so on.

	bFGF		NFL				NANOG	SOX2
	-	+	-	400ng/ μ l	800ng/ μ l	1200ng/ μ l		
WT Pea3	1,00	1,02	+	+++++	+++++	+++++	+	+
S22A	1,29	0,93	+	+++++	+++++	+++++	+	+
S22E	1,53	0,92	++	+++++	+++++	+++++	+	+
S90A	1,13	1,68	+	+++++	+++++	+++++	+	+
S90E	2,10	0,85	++	+++++	+++++	+++++	+	+
S101A	1,11	0,90	++	+++++	+++++	++	+	+
S101E	0,64	0,53	+	++++	+++++	+++++	+	+
S134A	0,18	0,09	+	++++	+++++	++++	+	+
S134E	0,43	0,40	+	+++++	+++++	+++++	+	+
S143A	1,06	1,76	++	+++++	+++++	+++++	++	+
S143E	0,83	0,51	+	+++++	+++++	+++++	+	+
S192A	2,63	7,48	+	+++++	+++++	+++++	+	+
S192E	4,23	1,26	+	+++++	+++++	+++++	+	+
S285A	3,09	3,92	+	++++	++++	++++	+	+
S285E	2,27	3,23	+	+++++	+++++	+++++	+	+
S458A	0,61	0,54	+	+++++	+++++	+++++	+	+
S458E	0,40	0,52	+	+++++	+++++	+++++	+	+

Unfortunately, our analyses could not directly shed light to the phosphorylation of this protein, due to lack of phosphospecific Pea3 antibodies. Phosphoserine/threonine antibody in combination with Pea3-IP as well as kinase assays should be performed in the future now that the entire set of mutants are available.

In bFGF induction studies, some of the mutant mPea3 plasmid were shown to have different levels of expression, indicating the mutations somehow interfered with stability of the protein, as discussed above. Therefore, parallel to their effect on phosphorylation, these mutants should also be analyzed with respect to their effect on other posttranslational modifications such as SUMOylation or ubiquitylation, as well as for their targeting for degradation.

The induction of NGF and GDNF experiments should be continued with all the phosphomutants as in bFGF analyses.

Regulation between the mutant mPea3s and *NEUROFILAMENT-L*, *EPHA1* and *EPHA2*, that is known to be a target of Pea3 [1], [40] was assessed luciferase

assay. According to analysis, there are no significant effects of mutant mPea3 on these promoters. All mutant set must be completed for EPHA promoters.

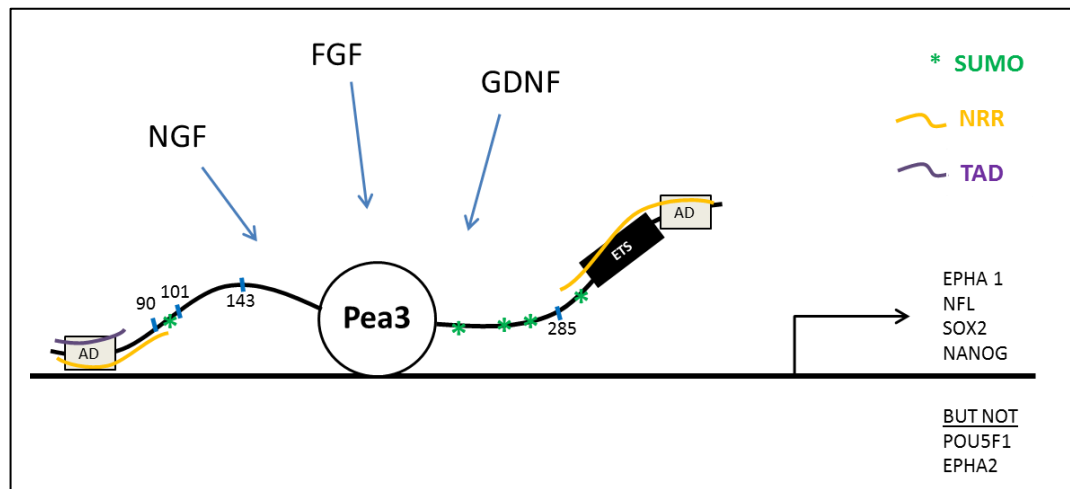


Figure 4.1: The brief summary of the Pea3 activation.

In the future, once the biochemical analyses are completed, these mutants should also be tested for their effect on neurite outgrowth and thus neuroregeneration.

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BIOGRAPHY

Merve Ustun was born in Istanbul, 1992. She achieved her bachelor's degree from Department of Molecular Biology and Genetics, Gebze Institute of Technology in 2014. She has continued master degree program in the Department of Molecular Biology and Genetics at Molecular Neurobiology Laboratory, Graduate School of Natural and Applied Sciences, Gebze Technical University, since 2014 fall. She presented posters in 10th FENS Forum of Neuroscience namely 'Identifying Pea3 Phosphorylation Sites Specifically Regulated by Different Growth Factors', in 15th National Neuroscience Congress namely 'Analysis of Pea3 Phosphorylation Sites' and in GTU Graduate Studies Symposium and Introductory Days 2017 namely 'Analysis of Pea3 Phosphorylation Sites Depending on Specific Growth Factors' during master degree program. She has also participated Brain Awareness Week activities, which is a funded-project by FENS, for three years. She would like to study on neuroscience especially on connectome and synaptic plasticity.

APPENDICES

Appendix A: Sequence Analysis of Mutant mPea3 Plasmids

To confirm the mutations of putative phosphorylation sites on mPea3, they were sequenced and the sequences are compared with wild type forms of mPea3.

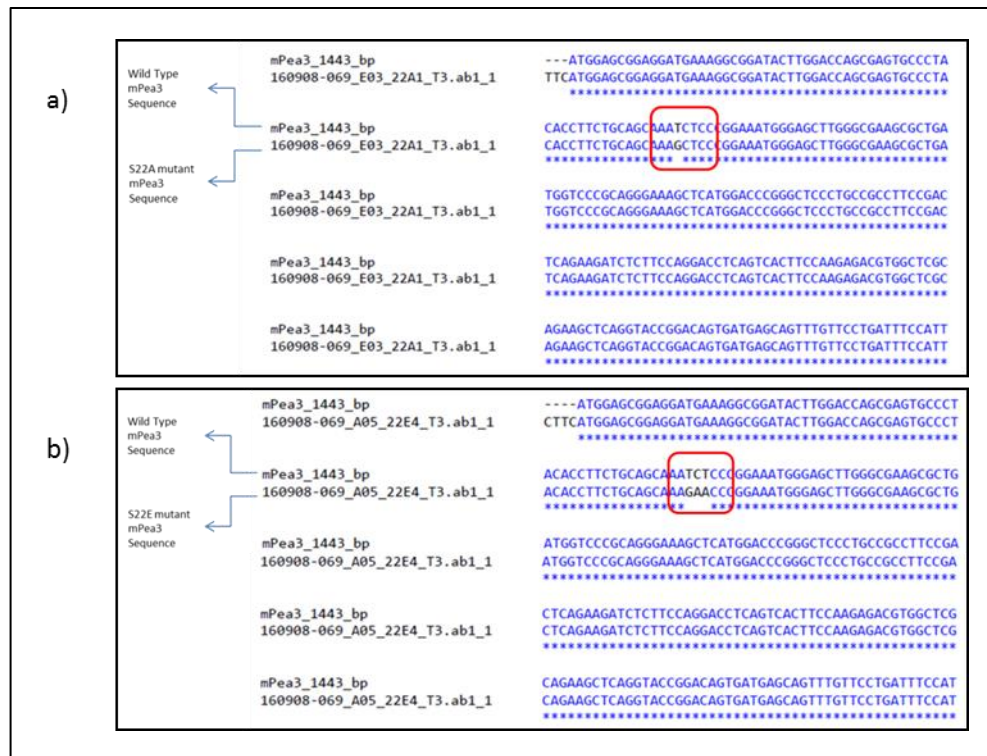


Figure A1. 1: Sequence analysis for a) S22A mutation, b) S22E mutation.

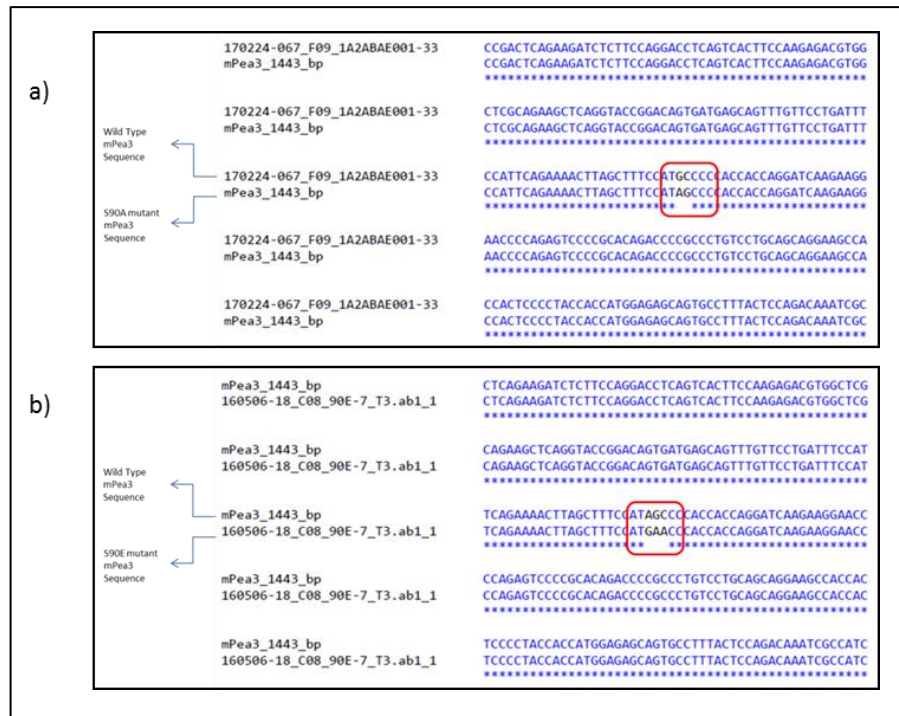


Figure A1. 2: Sequence analysis for a) S90A mutation, b) S90E mutation.

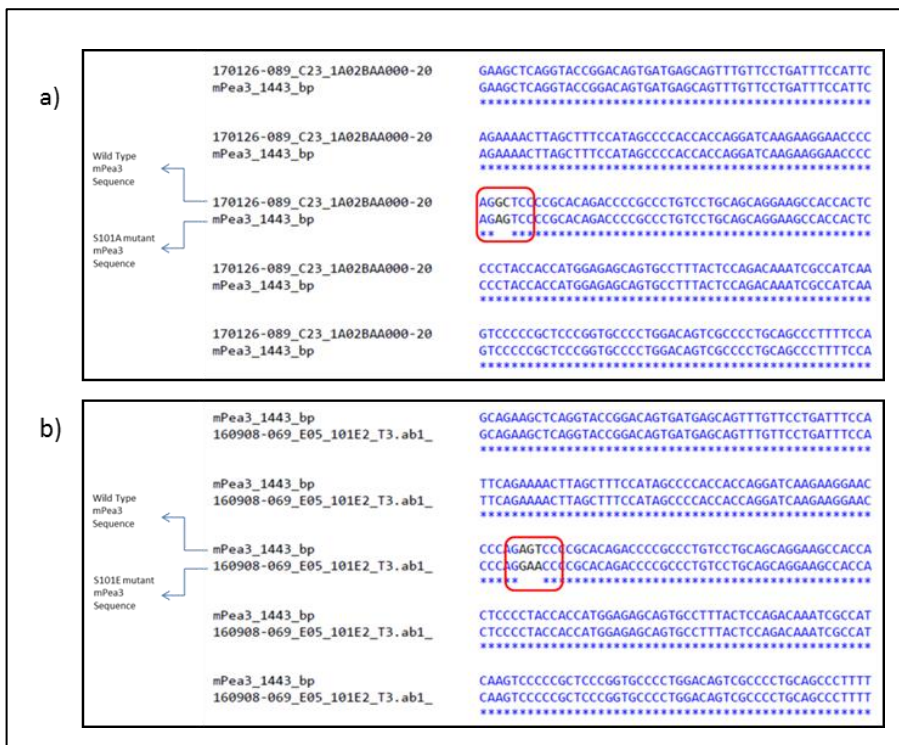


Figure A1. 3: Sequence analysis for a) S101A mutation, b) S101E mutation.

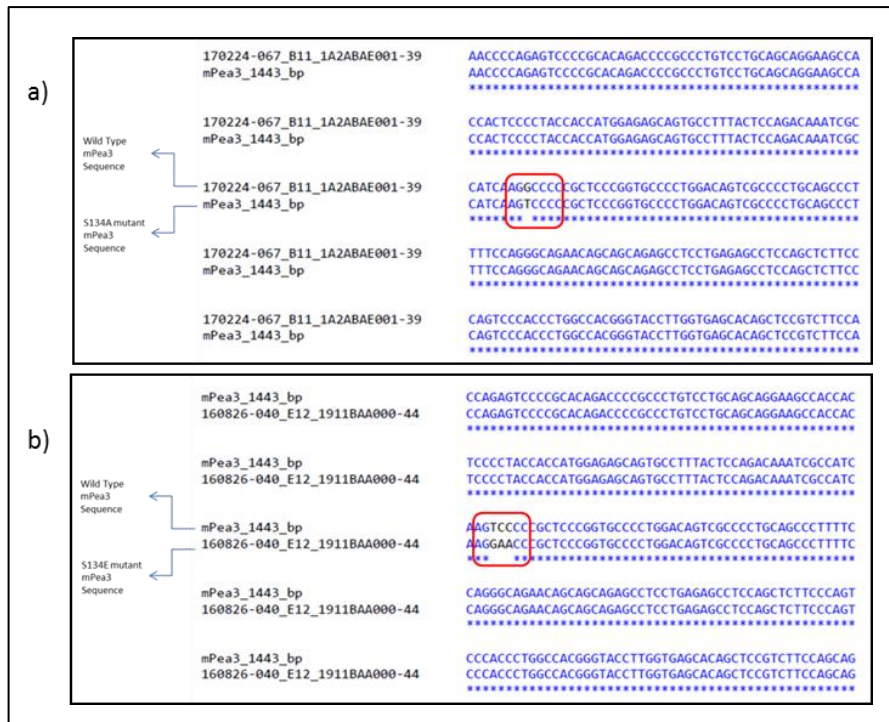


Figure A1. 4: Sequence analysis for a) S134A mutation, b) S134E mutation.

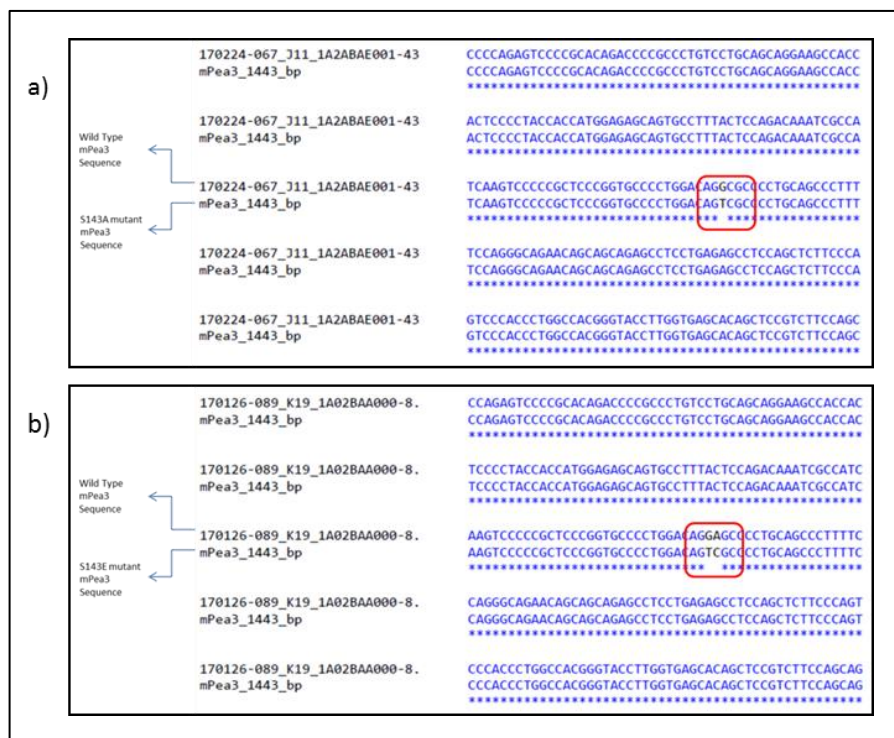


Figure A1. 5: Sequence analysis for a) S143A mutation, b) S143E mutation.

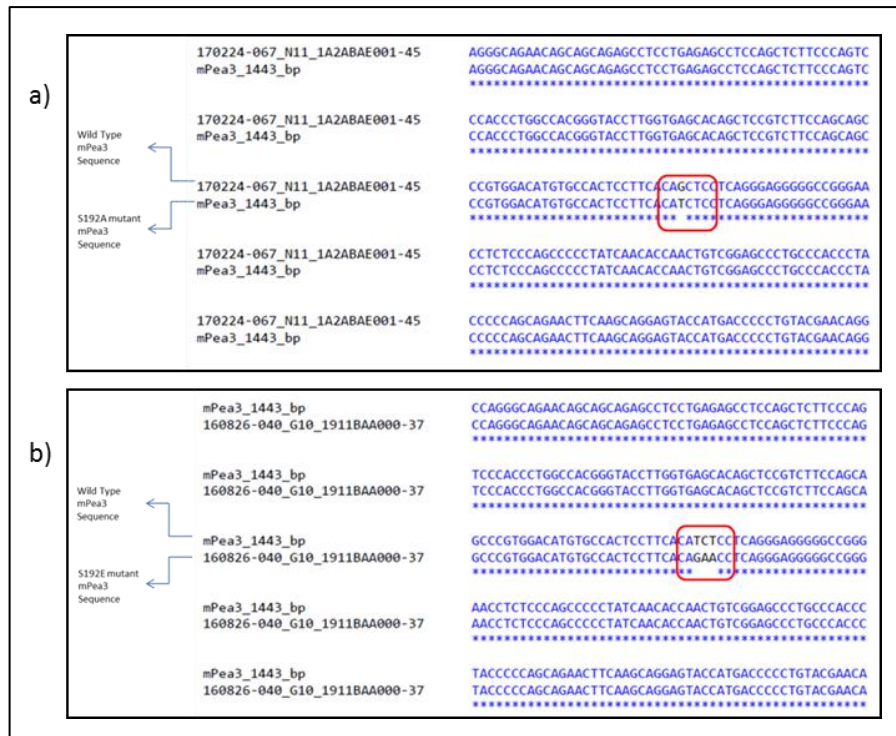


Figure A1. 6: Sequence analysis for a) S192A mutation, b) S192E mutation.

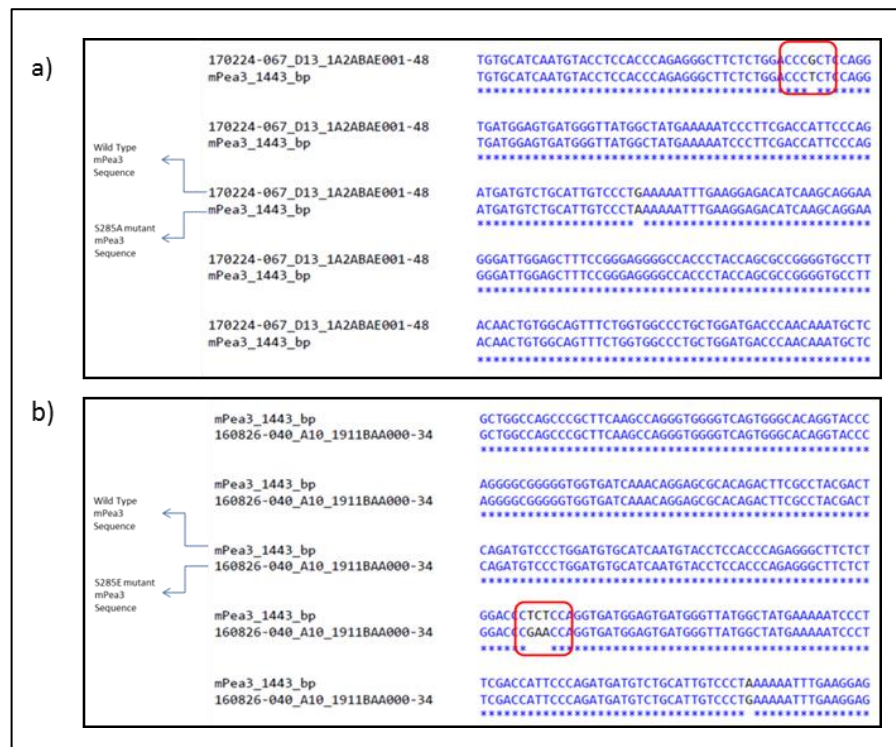


Figure A1. 7: Sequence analysis for a) S285A mutation, b) S285E mutation.

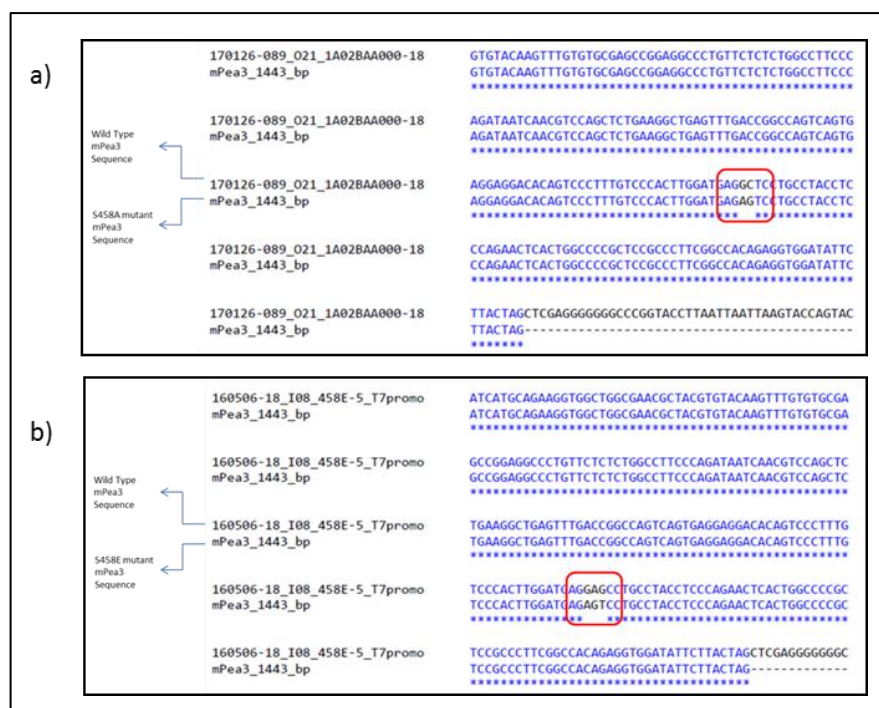


Figure A1. 8: Sequence analysis for a) S458A mutation, b) S458E mutation.

Appendix B: Luciferase Assay Datas for *EPHA1*, *EPHA2*, *hSOX2*, *hPOU5F1*, *hNANOG*, *NEUROFILAMENT-L*

- Activity of *hSOX2*, *hPOU5F1*, *hNANOG* Promoters for mPea3 Protein

Table B1.1: Standard deviation for luciferase analysis for *hSOX2*, *hNANOG*, *hPOU5F1* promoters and mPea3.

	SOX2	NANOG	POU5F1
pCMV	0,11851	0,0033081	0,1115495
Pea3-VP16	0,143656	0,1481619	0,2512641
mPea3	0,422165	0,2967433	0,0482609

Table B1.1: Firefly/Renilla luciferase values for analysis of *hSOX2*, *hNANOG*, *hPOU5F1* promoters activity for mPea3.

	SOX2	NANOG	POU5F1
pCMV	3,827597	2,5456726	3,7873486
Pea3-VP16	3,978018	3,9766333	5,6203874
mPea3	5,120054	9,1184831	5,3417961

- **Activity of *hSOX2* Promoter for Mutant mPea3 Proteins**

Table B1.3: Standard deviation for luciferase analysis for *hSOX2* promoter and mPea3.

	SOX 2
Pea3 WT	0,046576643
S22A	0,333631078
S22E	0,289006507
S90A	0,005723279
S90E	0,443426691
S101A	0,49230307
S101E	0,285887458
S134A	0,342514313
S134E	0,586956688
S143A	0,897034835
S143E	0,475907491
S192A	0,690702717
S192E	0,456732431
S285A	0,14327288
S285E	0,255006807
S458A	0,455085257
S458E	0,376862787
Pea3-VP16	1,128760015
pCMV	1,084916997

Table B1.4: Firefly/Renilla luciferase values for analysis of *hSOX2* promoter activity for mPea3.

	SOX 2
Pea3 WT	5,412147535
S22A	5,826484416
S22E	5,834102802
S90A	7,29170701
S90E	8,094481542
S101A	9,481519756
S101E	6,506870544
S134A	5,836835772
S134E	7,983553957
S143A	8,100640827
S143E	6,548794834
S192A	9,93574037
S192E	7,139033795
S285A	5,521554655
S285E	6,565229445
S458A	7,357957461
S458E	8,446516707
Pea3-VP16	4,635141506
pCMV	3,528316535

- **Activity of *hNANOG* Promoter for Mutant mPea3 Proteins**

Table B1.5: Standard deviation for luciferase analysis for *hNANOG* promoter and mPea3.

	NANOG
Pea3 WT	0,663841469
S22A	0,632676039
S22E	0,694467405
S90A	0,163030734
S90E	0,093559839
S101A	0,342299939
S101E	0,036807595
S134A	0,060589878
S134E	0,454529873
S143A	0,900352347
S143E	0,413635548
S192A	0,084276187
S192E	0,037204613
S285A	0,08321084
S285E	0,004123604
S458A	0,064062441
S458E	0,020113235
pCMV	0,052935192
Pea3-VP16	0,26677166

Table B1.6: Firefly/Renilla luciferase values for analysis of *hNANOG* promoter activity for mPea3.

	NANOG
Pea3 WT	8,227359957
S22A	5,991648753
S22E	6,217158495
S90A	6,419251251
S90E	4,637827438
S101A	6,304728353
S101E	7,561611198
S134A	6,897225832
S134E	7,027114232
S143A	13,5737797
S143E	5,951561348
S192A	6,157742158
S192E	8,150767686
S285A	3,55130415
S285E	6,476411807
S458A	5,327488599
S458E	6,734964335
pCMV	3,094623427
Pea3-VP16	4,203097297

- **Activity of *EPHA1* and *EPHA2* Promoters for Mutant mPea3 Proteins**

Table B1.7: Standard deviation for luciferase analysis for *EPHA1* and *EPHA2* promoters and mutant mPea3.

	Pea3			
Concentration	-	300 ng	600 ng	1000 ng
EPHA1	2,321379	5,717804	3,106593	10,97346
EPHA2	0,117616	0,228652	0,119983	0,015072
	S458A			
Concentration	-	300 ng	600 ng	1000 ng
EPHA1	0,574355	3,005948	4,042973	2,696279
EPHA2	0,387287	0,047551	0,086278	7,345932
	S458E			
Concentration	-	300 ng	600 ng	1000 ng
EPHA1	0,505024	3,490995	3,30673	2,585128
EPHA2	0,151105	0,023957	0,052597	0,007081

Table B1.8: Firefly/Renilla luciferase values for analysis of *EPHA1* and *EPHA2* promoters activity for mutant mPea3.

	Pea3			
Concentration	-	300 ng	600 ng	1000 ng
EPHA1	12,23878	17,26331	19,30021	32,62918
EPHA2	0,382516	0,596453	0,364423	0,147592
	S458A			
Concentration	-	300 ng	600 ng	1000 ng
EPHA1	5,408486	10,83983	22,86191	31,09507
EPHA2	-0,38805	0,140839	0,038981	3,955413
	S458E			
Concentration	-	300 ng	600 ng	1000 ng
EPHA1	18,76142	15,92657	15,34354	29,56726
EPHA2	0,35362	0,05134	0,128233	0,035671

- **Activity of *NEUROFILAMENT-L* Promoters for Mutant mPea3 Proteins**

Table B1.9: Standard deviation for luciferase analysis for *NEUROFILAMENT-L* promoter and mutant mPea3.

	w/o	400 ng/μl	800 ng/μl	1200 ng/μl
mPea3 WT	0,933324947	2,440359745	6,486140528	28,42199551
S22A	0,0158492	12,36215949	1,526118979	3,427382433
S22E	1,409005645	2,041101545	5,309132238	4,535201861
S90A	0,58631692	1,342796543	4,121224038	9,281740104
S90E	1,855174172	7,069051471	3,193725392	1,037215743
S101A	2,262960655	0,267107433	0,877215188	0,232660884
S101E	0,291808002	0,177107963	0,140297035	0,523815689
S134A	0,407123529	4,031618893	11,15690404	3,298926273

Table B1.10: Firefly/Renilla luciferase values for analysis of *NEUROFILAMENT-L* promoter activity for mutant mPea3.

	w/o	400 ng/μl	800 ng/μl	1200 ng/μl
mPea3 WT	3,197324368	20,74920972	30,69351926	64,36524324
S22A	2,310326172	30,98830604	31,42312035	29,17013013
S22E	3,71680417	20,27659668	26,09861906	30,10905186
S90A	3,278157867	17,56257434	25,33251373	43,34224603
S90E	4,154099065	24,07420531	20,5849066	22,69847171
S101A	3,551763773	17,80058206	14,36777255	5,575044335
S101E	2,032561458	12,55723577	20,07466376	19,26654164
S134A	2,844744667	10,96319483	25,38236364	11,27714

Table B1.11: Standard deviation for luciferase analysis for *NEUROFILAMENT-L* promoter and mutant mPea3.

	w/o	400 ng/μl	800 ng/μl	1200 ng/μl
mPea3 WT	0,019927306	0,390302517	1,260840436	1,063433573
S134E	0,047995769	1,791528166	1,794613071	0,953050938
S143A	0,346688415	0,411980607	1,147211667	0,874053253
S143E	0,322919838	0,480006863	0,709552094	2,374420092
S192A	0,106243003	0,599066448	0,347316103	0,345014208
S192E	0,170860377	0,455916593	0,336814655	0,48018738
S285A	0,019164451	0,66790927	0,981054424	0,073030015
S285E	0,081587962	0,511527646	0,044875261	0,852046838
S458A	0,056158084	0,268854849	0,428944878	2,120626509
S458E	0,061238184	0,005863671	1,609855635	0,711242196

Table B1.12: Firefly/Renilla luciferase values for analysis of *NEUROFILAMENT-L* promoter activity for mutant mPea3.

	w/o	400 ng/μl	800 ng/μl	1200 ng/μl
mPea3 WT	1,079923002	9,320150515	13,34474708	10,4467559
S134E	1,000808836	8,752203314	9,671919505	8,465658057
S143A	1,38734274	7,521574076	8,406751359	6,68866679
S143E	0,981275771	6,061684915	7,356778879	5,999696111
S192A	0,893590216	5,692390184	6,942844958	6,705675832
S192E	0,84710651	6,204988784	7,597696924	6,674900458
S285A	0,843152793	3,92150536	4,616317632	3,524161517
S285E	1,120040015	6,391692154	8,32863	6,702049969
S458A	0,97630873	6,320402978	9,828053065	6,861367094
S458E	0,750087545	5,581744631	8,059554057	6,437606616