EXPRESSION ANALYSIS OF TRANSCRIPTION FACTORS ELK-1 AND PEA3

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ABSTRACT

EXPRESSION ANALYSIS OF TRANSCRIPTION FACTORS ELK-1 AND PEA3

Transcription factors are the proteins that regulate the gene expression via binding to the promoter or enhancer regions of DNA sequences. They perform this function alone or with other proteins in a complex, by promoting or suppressing expression. These proteins have a key role in regulation of cell growth, cell proliferation and apoptosis. Elk-1 is a member of ETS- domain transcription factor, which is activated by MAPK pathway and regulates immediately early genes such as c-fos gene, which provides neuronal survival and proliferation. Pea3 transcription factor family belongs to ETS domain transcription factor superfamily and acts as nuclear targets of signal-transduction pathways, including cell proliferation, differentiation, development and apoptosis like, the other members of ETS super-family. A wide variety of the regulatory events that involve ETS domain proteins require MAPK pathways in order to mediate the phosphorylation processes. In this study, we tried to analyze expression and function of two different transcription factors, namely Elk-1 and Pea3 from different aspects. In the first part of the study, we aimed to identify the alternative splicing forms in open reading frame of transcription factor Elk-1 in different cell lines. In the second part of the study, the purpose was to optimize expression of wildtype and mutant GST-Pea3 fusion proteins for further phosphorylation analyses.

ÖZET

ELK-1 VE PEA3 TRANSKRİPSİYON FAKTÖRLERİNİN EKSPRESYON ANALİZLERİ

Transkripsiyon faktörleri, DNA dizilerinin promotör ya da artırıcı bölgelerine bağlanarak gen anlatımını düzenleyen proteinlerdir. Bu düzenlemeyi tek başlarına ya da diğer proteinlerle kompleks oluşturarak gen anlatımını artırır ya da baskılarlar. Bu proteinler hücre büyümesinde, çoğalmasında ve apoptozda anahtar rol oynarlar. Elk-1, ETS ailesine üye olan bir transkripsiyon faktörü olup MAPK yolağı tarafından aktive edilen ve nöronal sağ kalım ve farklılaşmayı sağlayan c-fos gibi genleri düzenler. Pea3 transkripsiyon faktörü ise ETS domain ailesinin üyelerinden biri olan ve diğer üyeler gibi hücre çoğalması, farklılaşması, gelişmesi ve ölümünü içeren sinyal transdüksiyon yolağının nükleer hedefi olarak görev yapar. Düzenleyici olayların birçoğu ETS domain proteinlerinin MAPK yolağındaki fosforlanma olaylarını kapsar. Bu çalışmada, Elk-1 ve Pea3 transkripsiyon faktörlerinin fonksiyon ve gen anlatımlarını farklı yönlerden analiz etmeye çalıştık. Çalışmanın ilk aşamasında, farklı hücre hatlarında Elk-1 transkripsiyon faktörünün açık okuma penceresinde alternatif kırpılma formlarını tanımlamayı amaçlamış olup, ikinci kısmında ise, ileriki fosforilasyon analizlerinde kullanılmak üzere doğal ve mutant GST-Pea3 füzyon proteinlerinin anlatımlarını optimize ettik.

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LIST OF SYMBOLS/ABBREVIATIONS

A	Alanine
AD	Activation domain
AD	Alzeihmer's Disease
AMP	Ampicillin
ATCC	American Type Culture Collection
$CaCl_2$	Calcium Chloride
CIDD	Central Inhibitory DNA Binding Domain
CT	Carboxy Terminal
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
E1AF	E1A enhancer-binding protein
E. coli	*Escherichia coli*
E26	E-Twenty Six
EDTA	Ethylenediaminetetraacetic Acid
ERK 1/ 2	Extracellular Regulated Kinase 1/ 2
ETS	E-Twenty Six
ETV 1/ 4/ 5	ETS Variant Gene 1/ 4/ 5
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
GDNF	Glial cell-line Derived Neurotrophic Factor
Glu	Glutamic acid
HCL	Hydrochloric Acid
HD	Hungtington's Disease
HEK293	Human Embryonic Kidney Cell Line
IGF	Insulin-like Growth Factor
JNK	c-Jun N Terminal Kinase

l	Liter
LB	Luria-Bertani
MAPK	Mitogen Activated Protein Kinase
ml	Milliliters
mM	Milimolar
MMP	Matrix Metalloproteinases
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
ng	Nanogram
NGF	Nerve Growth Factor
nM	Nanomolar
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEA3	Polyomavirus Enhancer Activator 3
pH	Negative log of hydrogen ion concentration
PNT	Pointed Domain
Rpm	Rotation per minute
RT	Room Temperature
S	Serine
SDS	Sodium Dodecyl Sulphate
SH-SY5Y	Human Neuroblastoma Cell Line
SMA	Spinal Muscular Atrophy
SRE	Serum Response Element
SRF	Serum Response Factor
SUMO	Small Ubiquitin-Like Modifier
TAD	Transactivation Domains
TBS	Tris-buffered saline
TCF	Ternary Complex Factor
TF	TransFast
UV	UltraViolet
µl	Microliters

1. INTRODUCTION

1.1. ETS Transcription Factors

Transcription factors are the proteins that regulate gene expression via binding to the promoter or enhancer regions of DNA sequences [1]. They perform this function alone or with other proteins in a complex, by promoting or suppressing expression. These proteins play roles in the regulation of cell growth, cell proliferation [2] and apoptosis [3]. Moreover, co-activators, co-repressors and different factors can control the activity of the transcription factors. Stabilizing or blocking the RNA polymerase binding to DNA, catalyzing the acetylation or deacetylation of the histone proteins and recruiting the co-activator or co-repressor proteins to the transcription factor – DNA complex are the regulation mechanism of transcription factors [4]. The rate of the transcription factor synthesis, nuclear localization [5], activation by ligand binding, phosphorylation [6] or interaction with other proteins, accessibility of the DNA-binding site [7], availability of other cofactors or transcription factors are key steps for the regulation processes.

Most of the transcription factors can bind to specific DNA sequences, and these trans-regulatory proteins can be classified according to the similarities in DNA binding sequences and their structures. Within such a transcription factor family, proteins possess a common core structure in their DNA-binding sites and as a general rule, slight differences in the amino acids at the binding site can change the transcription factor's ability to bind to a specific DNA sequence. In addition to having this sequence-specific DNA-binding domain, transcription factors contain a domain involved in activating the transcription of the gene whose promoter or enhancer that it is bound to. Generally, the trans-activating domain allows that transcription factor to interact with proteins involved in binding RNA polymerase. This interaction enhances the efficiency with which the basal transcriptional complex can be built and bind RNA polymerase II [1]. Regulation of gene expression is controlled by the action of multiple transcription factors that activate or repress transcription through binding to cis-regulatory elements present in the target genes. Identification of functional target gene promoters that are regulated by specific transcription factors and identification of specific upstream regulators of a molecular

mechanism are critical for understanding the molecular mechanisms that control signaling events [1,8].

ETS (E Twenty Six) proteins, which are encoded by highly conserved 85 amino acids, form to ETS domain [9]. This domain has 30 members that defined in mammals and it also has several subfamilies. Member of the ETS –domain superfamily have common region with avian erythroblastosis virus (Figure 1.1). ETS-domain transcription factors have crucial roles in development. They can be activators or repressors of signal transduction cascades and these signaling pathways can regulate cellular processes such as proliferation, differentiation in embryonic development and in adults, immune response, apoptosis, migration, metastasis, tissue remodeling, transformation or angiogenesis [10].

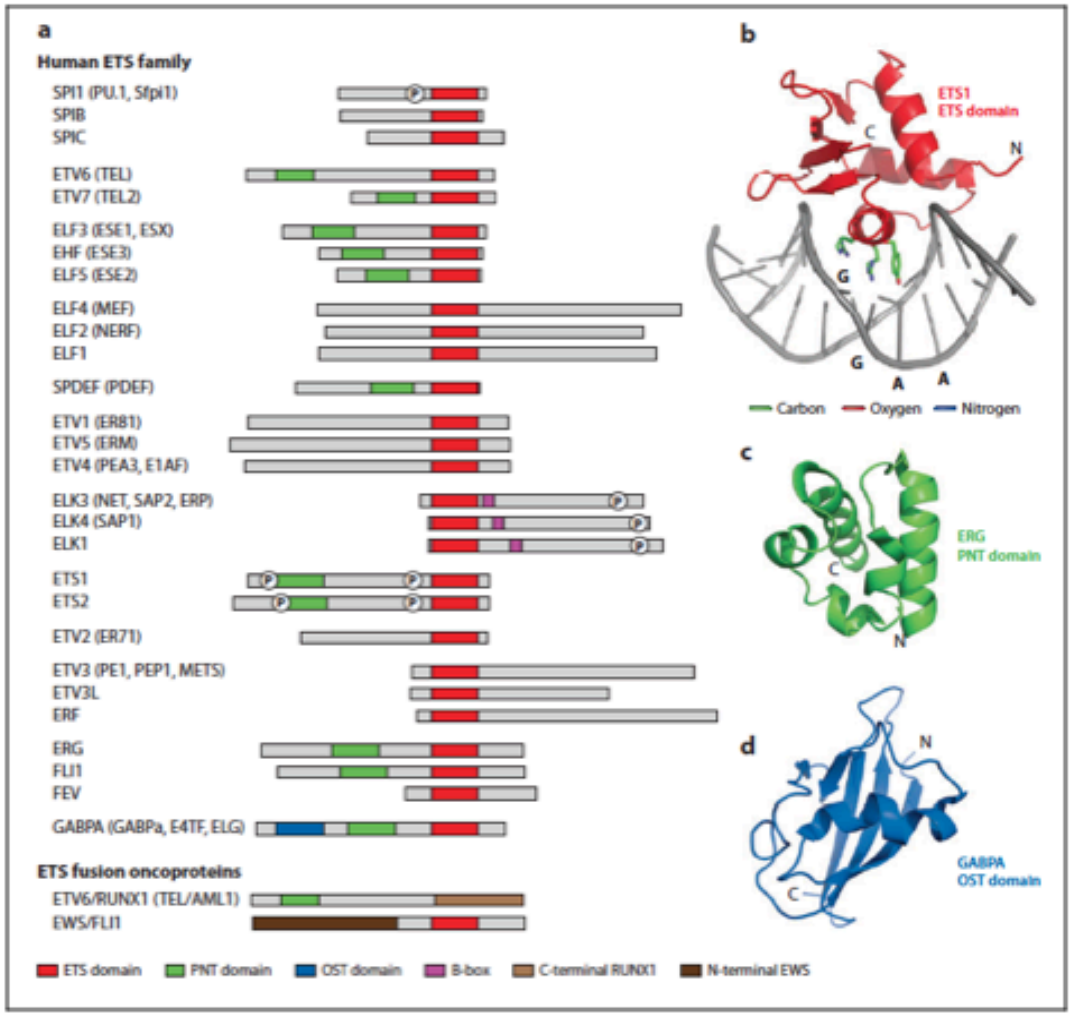

Figure 1.1. ETS transcription factor family. (a) Human ETS family and their domain localizations. (b) ETS domain (c) PNT domain (d) GABPA domain [11]

The expression profile of the ETS protein and the presence of the specific domains are the standard features of DNA-binding specificity of the ETS domain [12]. The alterations of the DNA–binding site of ETS proteins result in the different ETS domains. Furthermore, even small changes in the DNA sequences flanking the core consensus, which is coded as GGAA/T, changes the binding affinity of the ETS proteins to DNA [9]. Moreover, NMR analysis showed that these proteins are in helix-turn-helix super family [13]. The conserved ETS domain consists of three α-helix on a small four-stranded, antiparallel β-sheet scaffold (Figure 1.2) [14].

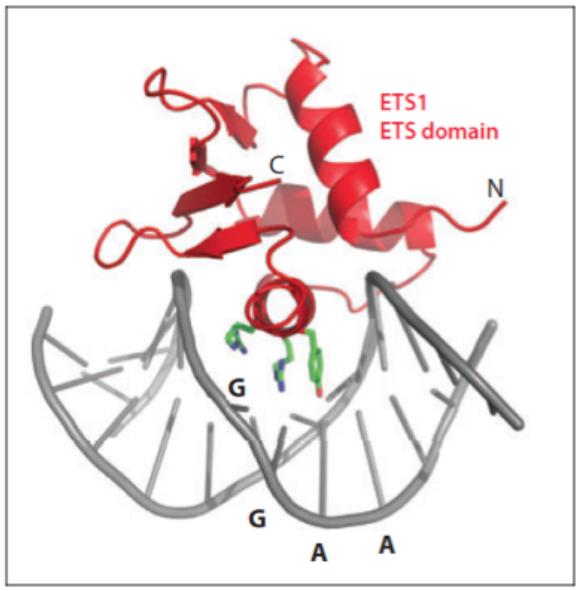

Figure 1.2. Ribbon structure of ETS domain while it is bound to DNA duplex which posses core GGAA. Green color represents carbon and red color represents oxygen and blue color represents nitrogen [11].

ETS-domain transcription factor family involves signal transduction pathways such as Mitogen Activated Protein Kinase (MAPK) mediated phosphorylation. Ternary Complex Factor (TCF) is a subfamily of ETS domain proteins, which show this activity and commonly found to bind promoters in a complex with SRF. Elk-1, SAP-1 and SAP-2/Net, comprise the TCF subfamily of ETS-domain transcription factors. The DNA-binding activity of TCF subfamily of ETS-domain is stimulated by MAP kinase phosphorylation [15]. Mitogenic stimuli are transduced through pathways that terminate in the activation of ERK-1 and ERK-2 MAPKs, whereas cellular stress such as UV irradiation lead to the

activation of the stress-activated kinases SAPK/JNK and p38. A combination of biochemical, molecular biological and genetic approaches have recently demonstrated that the activation of MAPK cascades leads to changes in the activity of many ETS-domain transcription factors; several of which have been demonstrated to be direct MAPK targets. Some of the ETS family, which are nuclear proteins, have nuclear export signals (NES) and also nuclear localization signals (NLS). The phosphorylation of these proteins affects their sub localizations [16].

1.2. PEA3 SUPERFAMILY

PEA3 (Polyoma enhancer activator) subfamily belongs to ETS-domain transcription factor family. This family consists of Pea3, Erm and Er81, which are highly conserved and well characterized proteins. Pea3, Erm and Er81 have human homologous, which are called E1AF, ETV5 and ETV1. ETV5 (Erm) is located on the long arm of chromosome 3 at 3q27-29 [17,18]. E1AF (Pea3) is located on the long arm of chromosome 17 at 17q21 [19] and ETV1 (Er81) is located on the long arm of chromosome 7 at 7q21 [20]. However they localize on different chromosomes, both of them share similar encoding sequences. [16] They possess 95% identical ETS-domain, 85% identical 32 residue acidic domain (AD) and 50% identical carboxyl–terminal tail domain (Ct) [21] (Figure 1.3).

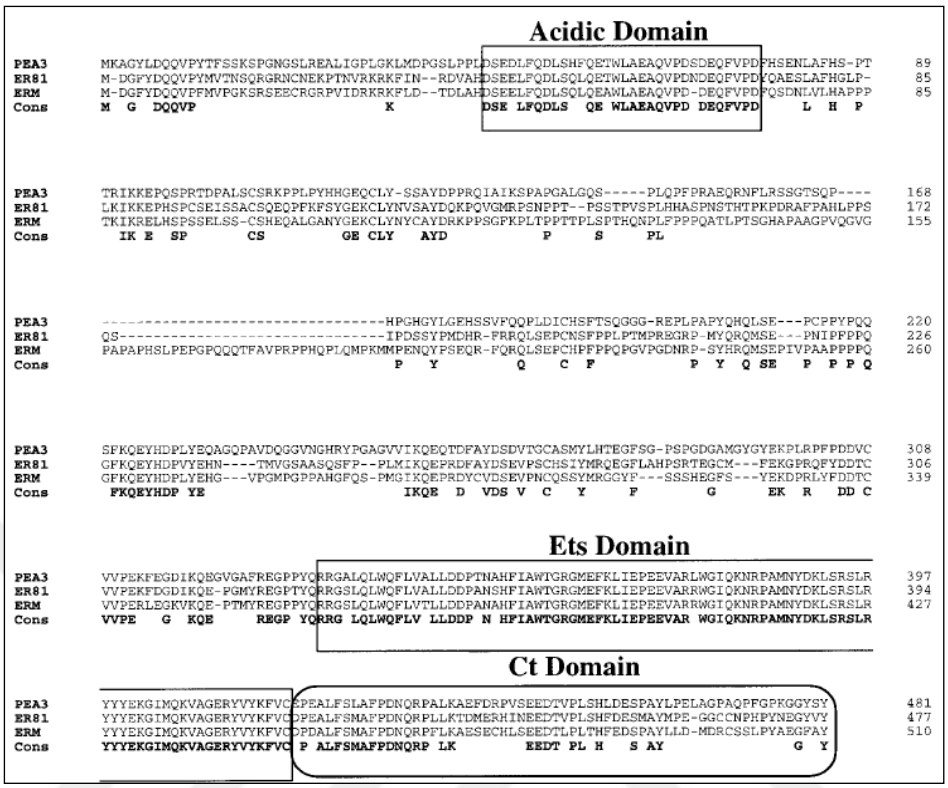

Figure 1.3. Sequence alignment of human Pea3 subfamily [21].

Studies had shown that this highly conserved domains, one in the amino terminal and one in the carboxy terminal, serve as activation domain and are responsible for the transactivation of gene expression. There is also an inhibitory domain resides in the middle, which negatively regulates DNA binding [21] (Figure 1.4).

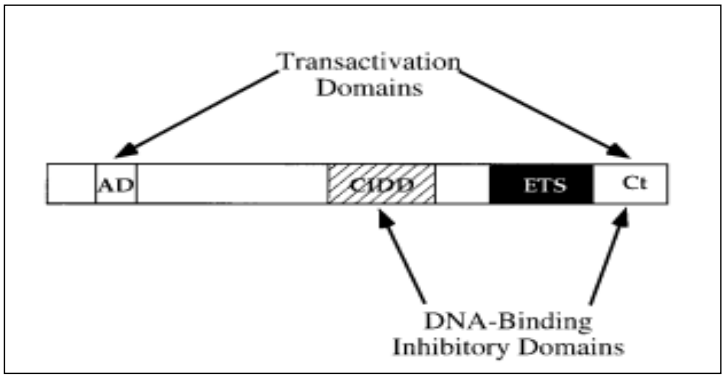

Figure 1.4**.** Regulatory domains of Pea3 group ETS transcription factor. AD represents acidic domain, CIDD represents central inhibitory DNA binding domain, ETS represents DNA binding domain, and CT represents carboxy-terminal [21]

The members of PEA3 subfamily are activated by MAP kinases. Both ERK and JNK MAPK cascades have a role in transcriptional activation of PEA3 subfamily [22] (Figure 1.5). It is shown that ERK signaling pathways also significantly enhances ER81 and ERM-mediated transactivation [23]. This finding indicates that all members of this subfamily are regulated by MAP kinases.

Ras is the upstream target of MAPK pathway and the activation of Ras by extracellular signals cause the activation of MAPK. The findings indicate that activated Ras cause an increment on transactivation capacity of Pea3, Erm, Er81. [23,24] Not only Ras but also SEK, which stimulates JNK/SAPK, was shown to increase the transactivation of Pea3 [25]. Although it is known that their N-terminal transcriptional activation domains of these transcription factors control transactivation, it is currently unknown how the activity of members of PEA3 subfamily are enhanced. In spite of having potential MAPK sites, it is not known whether they are direct targets of these pathways or not [23].

Er81 acetylation by Ras/MAPK phosphorylated p300 acetyltransferase increase the transactivation capacity. Sumo modification of Erm negatively regulates the transactivation capacity. On the other hand, sumoylation on lysine residues increase the Er81 transactivation. [26] Sumolyation and acetylation enhance not only Erm, but also the transactivation of Pea3 [27].

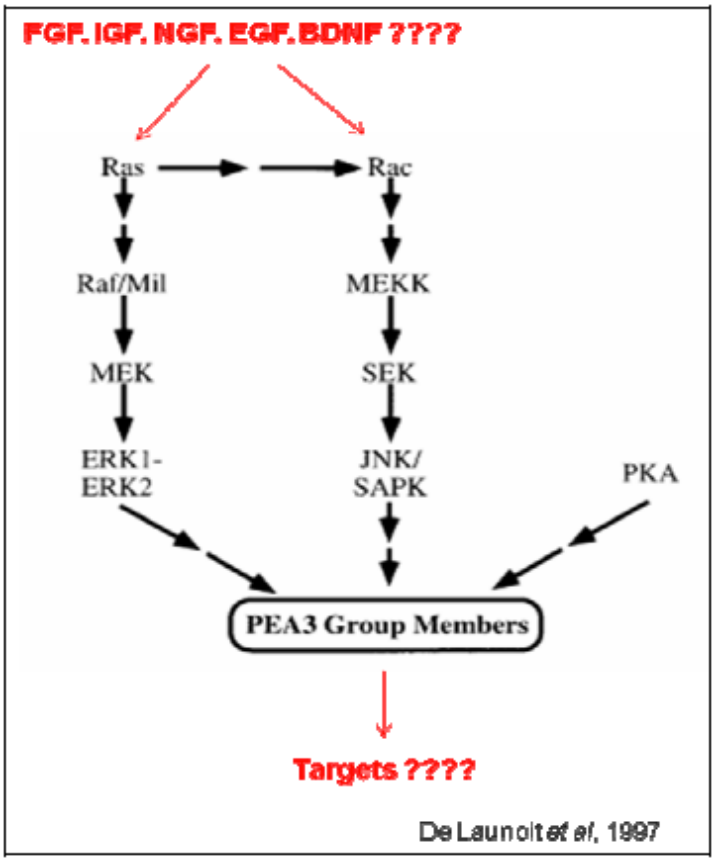

Figure1.5. A model demonstration of transactivation of PEA3 group members via different signal transduction pathways [28].

Pea3 involves in metastatic transformation. In other words, it has long been known as a metastatic marker gene. Phosphorylated Pea3 cause transactivation of metastatic genes. Especially in Her/Neu expressing breast cancer cells and tissues, the expression level of Pea3 is high [29]. Matrix metalloprotease enzymes such as MMP2, MMP9 are the main targets of Pea3. MMP genes are necessary for initiation of cell migration. Overexpression of Pea3 also increases the expression level of vascular endothelial growth factor (VEGF) [30], osteopontin [31], the intercellular adhesion molecule ICAM-I, [32] and vimentin. Another cancer type that Pea3 is highly expressed is prostate cancer. The overexpression of Pea3 due to the high level activation of TMPRSS2 promoter results in prostate cancer, [33]. Colorectal tumors [34], human gastric tumors [35], lung adenocarcinomas [36], ovarian [37] and endometrial carcinomas [38] are the cancer types, where Pea3 is overexpressed and involves in the cancer progression.

Pea3 has role in motor neuron innervation on muscles, studies with ER81 and Pea3 were showed that deletion of ER81 interfere with the growth of sensory neurons in the spinal cord but does not affect its projection to the spinal cord. Therefore sensory neurons can reach to the spinal cord but unable to form connections with the motor neurons [39].

Pea3 is not necessary for the normal development of sensory neurons, but Pea3 is crucial for determination of motor pool neuron identity. In the absence of Pea3, motor neurons lost their branching axons through the target muscles. Moreover, they may die because of the lack of functional synapse and hence activation [40].

Pea3 is regulated by neurotropic factor GDNF. Peripheral neurotropic factors are required to induction of Pea3 expression in the motor neurons and this way these motor neurons are able to branch on target muscle [39].

In zebrafish model, Pea3 is important for early development process. FGF signaling pathway regulates Pea3 expression and disruption of this signal transducer system cause to disruption on cardiac and isthmic organizer. In addition, FGF-activate Pea3 has a role in retinal development and ganglion cell differentiation [41].

Pea3 has a role in mammary gland. During early pregnancy, Pea3 promotes the proliferation and differentiation. As a result of this, arborization in mammary gland occurs [42].

1.3. TRANSCRIPTION FACTOR ELK-1

Elk-1 is the member of ETS oncogene family of transcription factors. These phosphoproteins have roles in cell growth, differentiation, survival, hematopoiesis, angiogenesis, wound healing, cancer and inflammation [43]. Elk-1 is the member of TCF family whereas, Ets motif is located in the N terminus of the proteins. TCF family forms a ternary complex with two molecules of Serum Response Factor (SRF) on Serum Response Element (SRE) at c-fos promoter [44].

Elk-1, Sap-1 and Elk-4 is the members of TCF family and they all contain four conserved

domains with high frequency. First conserved domain is the ETS domain. It is located on N- terminal region and it contains motifs of nuclear localization signals (NLS) and nuclear export signal (NES). (Figure1.6) Another conserved domain is the B domain, which is involved in the binding of Elk-1 to a dimer of its cofactor, the SRF and promotes the TCF formation [45]. Another conserved domain is D domain and it contains the docking site for ERK and JNK. These docking sites are critical for binding to MAP Kinases of ERK, JNK and p38. Last conserved domain on Elk-1 is C-terminal domain and it is transcriptional activation domain. There are multiple S/TP motifs targeted by activated MAP Kinases [46,47].

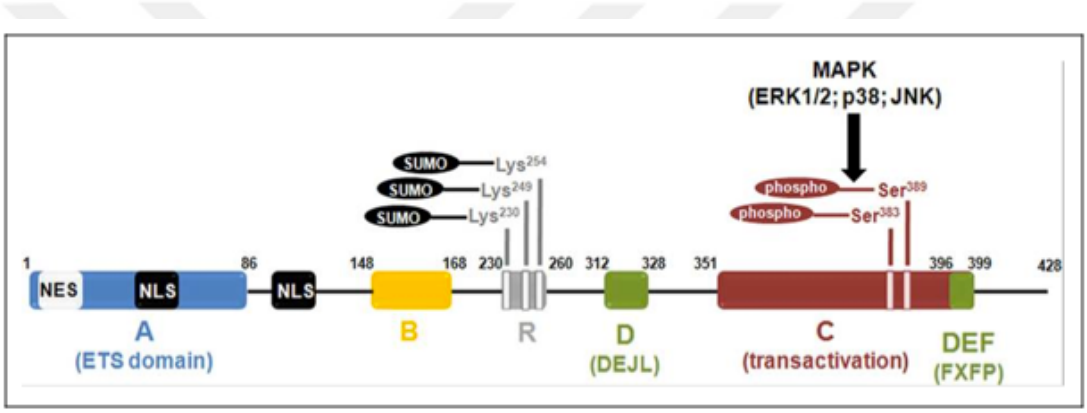

Figure 1.6. Functional domains and major post-translational modifications of the Elk-1 protein [46].

The transcriptional activity of Elk-1 depends on its phosphorylation and it is regulated by MAP Kinase signaling pathway. The three subclasses of MAP kinases, which are ERK, JNK and p38, lead to phosphorylation of Elk-1 [48,49,50,51,52]. Growth factors and mitogens alert ERK, whereas JNK and p38 cascades response to cytokines and stress. The phosphorylation of Elk-1 can occur on nine residues on C-terminal [52] .Two of them are serine 383 and serine 389. They are very critical for activation of Elk-1. Silencing mutation of these residues lead to blockage of Elk-1 by SRE-dependent gene induction [53]. In Elk-1 DEF motifs are phosphorylated by ERK and this phosphorylation causes conformational changes [54].

Elk-1 has role in neurodegenerative disease such as Alzheimer's Disease (AD), Hungtington Disease (HD), Parkinson's Disease (PD). Elk-1 is potent repressor of

transcription factor presenilin 1 gene (PS1), and PS1 gene involves in the final step of the sequential proteolytic processing of amyloid precursor protein. Moreover, PS1 is genetically linked to majority cases of early onset of hereditory AD [55]. ERK, Elk-1 and CREB are hyperphosphorylated in the striatum of a transgenic model model of HD [56]. In this mouse model, defiency of MSK-1 expression is only in striatum region of the brain not in the cortex. Restoring MSK-1 expression reversed neuronal dysregulations induced by expanded Htt [56]. Elk-1 ad MSK-1 has neuronal protection in HD. In Parkinson's disease (PD), phosphorylated Elk-1 was detected but the role of Elk-1 is unclear [58].

Elk-1 is also involved in Down syndrome [59], depression [60], learning and memory [61].

1.3.1. Alternative Splicing and Elk-1

Almost all protein-coding genes undergo alternative splicing process. Alternative splicing is a key regulator for eukaryotic gene expression because it increases the coding capacity of human genome. In other words, it increases the usage of genetic information [61]. More than 88% of the encoding genes undergo alternative splicing process [62]. Studies indicate that more than 90% of pre-mRNA is removed as introns and only 10% of pre-mRNA join as exons via RNA splicing and it happens in the nucleus. The cell decides removing of introns or including the alternative exons [63]. Alternative splicing leads to protein isoforms and it gives different biological properties. This difference causes different protein-protein interactions, subcellular localizations, or catalytic abilities [64]. Mapping studies suggest that most alternative exons are in coiled or loop regions that are located on the surface [65].

Alternative splicing leads to some disease such as Spinal Muscular Atrophy (SMA) [66], Hutchinson-Gilford Progeria [67], and cancer [68]. The expression level of alternative or tumor –specific variants may affect cellular responses such as motility, proliferation, and drug response [69]. These responses are also critical for cancer. Changes in the concentration, localization or composition may cause alteration of alternative splicing [70]. Missplicing, point mutations on the splicing site [7] or loss of splicing factors [71] lead to the disease.

The human elk-1 gene has seven exons. The protein is encoded from exon 3 to exon 7. 5'UTR of elk-1 consists of exon 1, exon2 and the first 34 nucleotides of exon3. Elk-1 is found in both the nucleus and the cytoplasm. In one study, a short isoform of elk-1 was detected in the rat brain. Long isoform is a 55kDa protein, but short form is only 45kDa. Moreover, short isoform was firstly identified in the brain, but later short isoform was detected in kidney as well. Short isoform is only found in the nucleus. Furthermore, short form does not have first the 54 amino acids at the N-terminus and many DNA binding domains are located there. It is compromised in its ability to activate SRE genes. In NGF-treated PC12 cells, short isoform of Elk-1 is expressing and the distribution of Elk-1 has similarities between mature brain and NGF-treated PC12 cells. Furthermore, overexpression of sElk-1, but not Elk-1 increased neurite extension in the PC12 model system. [72].

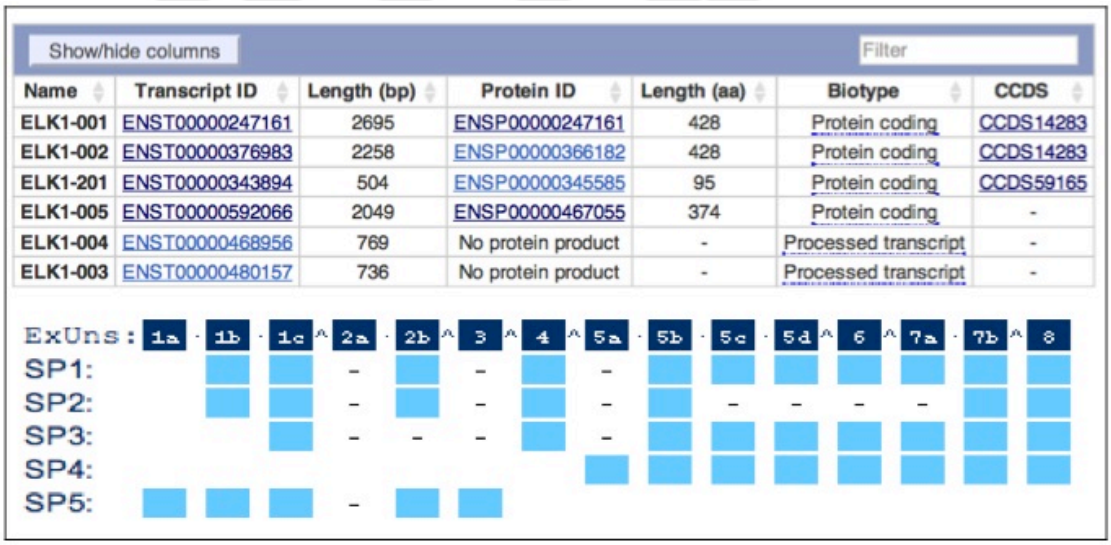

Figure 1.7. Scheme of Alternative Splicing of Elk-1. [It is a screenshot from Ensemble and Genecard databases.]

Transcription factor Elk-1 has 6 alternative splicing forms and only four of them encode protein. First 3 exons forms the UTR of Elk-1 and they do not involve the open reading frame. Two of alternatively spliced forms (1 and 3) encode 428 amino acid, but they have different 5'UTR length. In spliced form 3, absence of exon 2 and partially exon 1 lead to shorter UTR length. Exon 4,5,6,7 and 8 are involved in protein coding for both spliced forms 1 and 3. In spliced form 2, lacking of exon 6, partially exon 5 and exon7 cause

shorter production. This form encodes 95 amino-acids. Alternative spliced form 4 encodes 374 amino acids and it does not have only exon 4 in its ORF and also there is no UTR sequence. (Figure 1.7)

2. MATERIALS

2.1. BIOINFORMATIC TOOLS

Table 2.1 Bioinformatics tools that were utilized in the thesis

Bioinformatic Tools	Purpose	Website
Pubmed	Database where sequences of genes can be retrieved	http://www.ncbi.nlm.nih.gov/pubmed
IDT Oligo Analyzer	Where primers can be design and ordered	http://eu.idtdna.com/analyzer/applications/oligoanalyzer/
Primer BLAST	Database where primers can be align	http://www.ncbi.nlm.nih.gov/tools/primer-blast/
SDSC Biology	Give serves with several tools	http://workbench.sdsc.edu
Finch TV	To rewiev sequencing results	http://www.geospiza.com/Products/finchtv.shtml

2.2. CELL CULTURE

2.2.1. Cell Lines

- A172 Human Glioblastoma Cell Line
- HEK-293 Human Embryonic Kidney Cell Line
- SH-SY5Y Human Neuroblastoma Cell Line
- U87 Human Glioblastoma Cell Line

2.2.2. Cell Culture Maintaining Medium and Supplements

- Dulbecco's Modified Eagle Medium (DMEM) 1g/L Glucose (Gibco)

- Dulbecco's Modified Eagle Medium (DMEM) 4,5g/L Glucose (Gibco)
- Fetal Bovine Serum (Gibco)
- Penicillin/Streptomycin/Amphotericin B (100X) (Kibbytz Beit Haemek)
- 0.5% Tyrpsin-EDTA (Sigma)
- Phosphate Buffered Saline (PBS) (Lonza)

2.2.3. Cell Culture Maintaining Plates

- T25 Tissue Culture Flasks (Corning)
- T75 Tissue Culture Flasks (Corning)
- 100mm x 20mm Tissue Culture Dish (Corning)

2.3. BACTERIAL ASSAYS

2.3.1. Bacterial Asaay Reagents and Equipments

- Luria-Bertani(LB) Broth (Sigma)
- Luria Bertani (LB) Agar (Sigma)
- Ampicillin (Biomatik)
- Petri Plates (Isolab)
- 100 mM$CaCl_2$ solution

2.3.2. Bacterial Strains

- JM109 (Promega)
- BL21 (Promega)

2.4. PLASMIDS

A commercially available empty plasmid vector and several cloned plasmid construct were used in this study.

- PGEX-4T-2 empty plasmid (GE Health Care) (Figure 2.1)
- pCMV-3Tag-6 mPea3 wild type construct
- pCMV-3Tag-6 S90A mutant construct
- pCMV-3Tag-6 S90E mutant construct
- pCMV-3Tag-6 S101A mutant construct
- pCMV-3Tag-6 S101E mutant construct
- pCMV-3Tag-6 S143A mutant construct
- pCMV-3Tag-6 S143E mutant construct
- pCMV-3Tag-6 S458A mutant construct
- pCMV-3Tag-6 S458E mutant construct

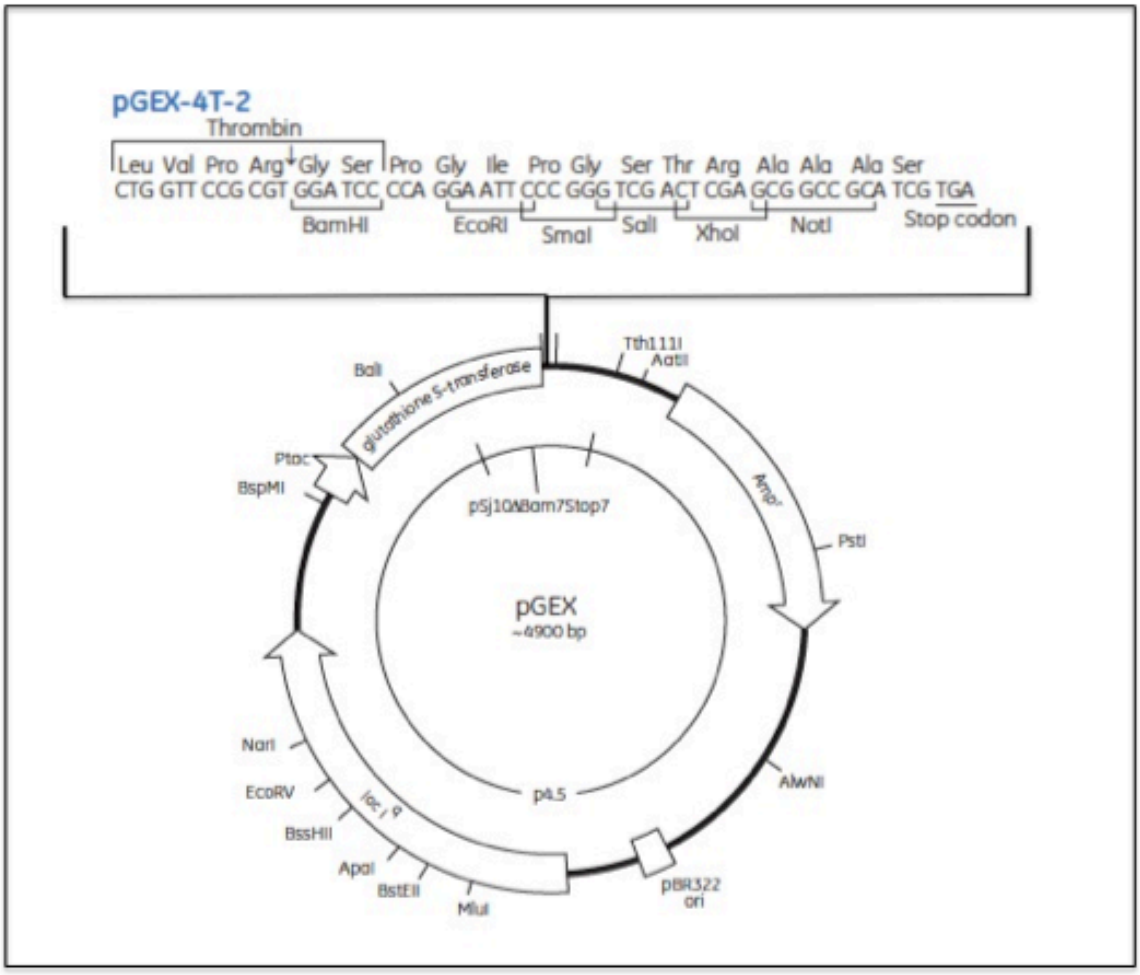

Figure 2.1. Map of pGEX-4T-2 Plasmid

2.5. GST PULL DOWN ASSAY

2.5.1. PCR Amplification of mPea3 Mutants

- pCMV-3Tag-6 mPea3 wild type construct (100ng)
- pCMV-3Tag-6 S90A mutant construct (100ng)
- pCMV-3Tag-6 S90E mutant construct (100ng)
- pCMV-3Tag-6 S101A mutant construct (100ng)
- pCMV-3Tag-6 S101E mutant construct (100ng)
- pCMV-3Tag-6 S143A mutant construct (100ng)
- pCMV-3Tag-6 S143E mutant construct (100ng)
- pCMV-3Tag-6 S458A mutant construct (100ng)
- pCMV-3Tag-6 S458E mutant construct (100ng)
- Long PCR Enzyme (Thermo)
- 10mM dNTP mix (Sigma)
- 10X Long PCR Buffer with 15mM $MgCI_2$ (Thermo)
- DMSO (Thermo)
- Nuclease Free Water
- Forward and Reverse Primers (Table 2.2)
- Bio-radThermocycler
- Agarose (Sigma)
- Ethidium Bromide (Promega)
- 6X Loading Dye (Intron)
- ChemiDoc Imaging System (Bio-RAD)
- PCR Purification Kit (Invitrogen)

Table 2.2. Primer couples designed to clone mPea3 mutants into PGEX-4T-2 vector. Red sequence indicates the BamHI recognition site, whereas blue sequence indicate EcoRI recognition site here.

pGEX-4T-2 Forward Primer	5' - AGAC GGATCC TGG AGC GGA GGA TGA AAG GC - 3'
pGEX-4T-2 Reverse Primer	5' - AGAC GAATTC CTA GTA AGA ATA TCC ACC TC - 3'

2.5.2. Digestion and Ligation of PCR Amplicons and pGEX-4T-2 Empty Vector

- PCR products purified from the previous step
- pGEX-4T-2 empty vector
- EcoR I and BamH I restriction enzyme and 10 X Buffer (Fermentas)
- RNase-free water
- PCR purification Kit (invitrogen)
- T4 ligase and 10X Buffer (NEB)

2.6. PROTEIN ASSAY

2.6.1 SDS Page and Western Blot

- Whole protein lysates from HEK293, A172, SH-SY5Y and U87 cell lines
- RIPA Buffer
- Phosphatase Inhibitor Cocktail
- Protease Inhibitor Cocktail
- 100mM DTT
- Bradford Reagent (Sigma)
- 40%/0.8% Acrylamide/Bis Acrylamide Solution
- 1M Tris-Cl Buffer at pH 6.8
- 1.5M Tris-Cl pH 8.9
- 10% SDS
- 10% APS

- Temed
- 4X Protein Loading Dye (Bio-Rad)
- Broad Range Ladder (Santa Cruz)
- Bio-Rad Semi-Dry Blotting Device
- Nitrocelluose membrane (Roche)
- 1X TBS and 1X TBS-T
- Commasie Blue (Sigma)
- Ponceu S (Chem Cruz)
- Blocking Buffer (5% skimmed milk in TBS-T)
- Primary Antibodies: rabbit anti-Elk-1 polyclonal (Santa Cruz),
- Secondary antibody: goat anti-rabbit (Cell Signaling)
- ECL Western Blotting Substrate Kit (Intron)
- Bio-Rad ChemiDoc Imaging System

2.6.2. GST-Pull Down Assay

- IPTG
- NET Buffer (10mM Tris.Cl [pH:8], 1 mM EDTA, 100mM NaCl , 1x protease inhibitor cocktail)
- Photospectrometer Device

2.7. EXPRESSION LEVEL ASSAYS

- RNA isolation kit (Invitrogen)
- cDNA synthesis kit (Applied Biosystems)
- I-taq Enzyme mix (Intron)

Table 2.3. Primer couples designed to indicate alternative splicing form of Elk-1.

Alternative Splicing Forward 1	5'ATGGACCCATCTGTGACGCTG 3'
Alternative Splicing Forward 1I	5'CATGAATTACGACAAGCTCAGCC3'
Alternative Splicing Reverse I	5'ACATTTGGCATGGTGGAGGTAAC 3'
Alternative Splicing Reverse II	5' AAGCTCTTCCGATTTCAGGTTT 3'
Alternative Splicing Reverse III	5'GCGCTGCCACTGGATGGAAA 3'

Table 2.4**.** GAPDH Primer couple

GAPDH Forward	5' GCATTGCTGATGATCTTGAGG 3'
GAPDH Reverse	5' TCGGAGTCAACGGATTTGG 3'

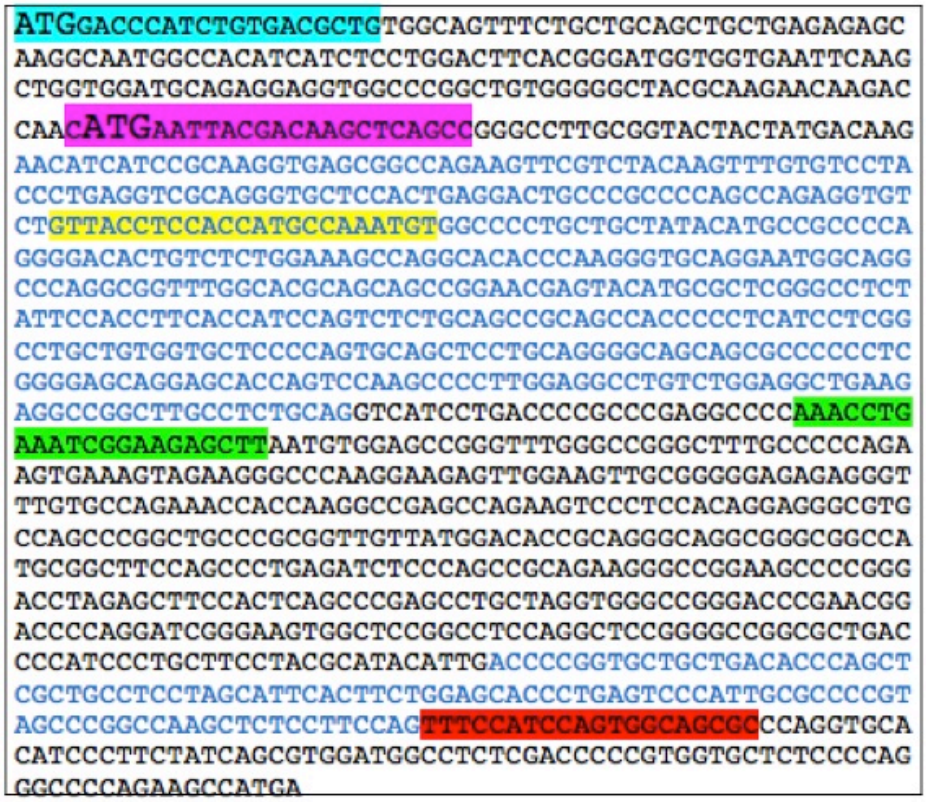

Figure 2.2. Primer alignment for alternative splicing of Elk-1.

Blue letters represent alternate sequences. Primer couples were designed according to these sequences. Forward 1 primer is highlighted with blue and forward 2 primer is highlighted with purple. Reverse primer 1 is indicated by yellow, reverse primer 2 is highlighted with green and lastly, reverse primer 3 is indicated by red.

3. METHOD

3.1. PREPARATION OF MUTANT PLASMID CONSTRUCTS

3.1.1. Insert Preparation

First step of cloning is amplification of insert by PCR. Each insert was prepared according to Table 3.1.

Table 3.1. Ingredients of the PCR

Component	Volume
Template	1 µl
10X Buffer	5 µl
dNTPs (10 mM each)	1 µl
pGEX Forward Primer	0,6 µl
pGEX Reverse Primer	0.6 µl
DMSO	2 µl
Long enzyme	0.3 µl
Water	µl
Total	50 µl

PCR reaction was carried out at an annealing temperature of 56 °C (Table 3.2). PCR amplicons were run in 1% Et-Br stained agarose gel and visualized with Bio-rad Chemidoc Imaging system. Amplicons were purified with PCR purification kit (Invitrogen).

Table 3.2. PCR steps for pGEX forward and pGEX reverse primers

	Temperature	**Duration**	**# of Cycle**
Initial Denaturation	94°C	2 min.	1 cycle
Denaturation	94°C	20 sec.	40 cycles
Annealing	56°C	30 sec.	
Extension	68°C	1.5 min.	
Final Extension	68°C	10 min.	1 cycle

3.1.2. Double Digestion with BamH I and EcoR I Restriction Enzymes

For double digestion, each sample was prepared according to Table 3.2.

Table 3.3. Double digestion reaction of wild type and mutant Pea3 and pGEX-4T-2 vector

Ingredients	**Volume**
Insert / Vector	Varies
BamH I Restriction Enzyme	1 µl
EcoR I Restriction Enzyme	1 µl
10X FastDigest Buffer	2 µl
dH_2O	up to 20 µl

The mixture was incubated for 1 hour at 37 °C. DNA sequences were purified with PCR purification kit. In order to understand digestion was successful, digested plasmid sample was run on the 1% agarose gel, because plasmids are in circular form and after digestion process they become linear. Because of this property, the success of digestion can be understood depending on the band number.

3.1.3. Ligation of PCR Product and pGEX-4T-2 Vector

Only 1:3 (vector: insert) reaction was carried out. The ligation reaction was incubated at room temperature for 15minutes. (Table 3.4)

Table 3.4. Ligation reaction components and volumes

Components	Volumes
10X Buffer	2 µl
Insert	Varies
Vector	Varies
T4	1 µl
dH_2O	Up to 20 µl

For calculation of insert and vector amounts, the equation below was used:

Insert (ng)=[lenght of insert/ lenght of vector x amount of vector (ng)]x(insert:vector ratio)

3.1.4. Preparation of Competent Cells

JM109 cells were inoculated in 10mL LB broth and incubated at 37 ° C for overnight. On the following day 49 mL fresh LB broth and 1 mL inoculum were mixed and incubated on 37 ° C shaker incubator for 3 hours. At the end of the 3 hours, cells were centrifuged at 5500 rpm for 10 minutes. Supernatant was removed and pellet was resuspended with 5 mL ice-cold $CaCI_2$ .It was incubated for 1 hour on ice and then it was centrifuged at 3500 rpm for 5 minutes. Pellet was resuspended with 1 mL $CaCI_2$. Competent cells were kept on +4 ° C.

3.1.5. Transformation

LB agar and LB broth medium were prepared according to manufacturer's orders. LB agar plates were poured with 25 µl ampicillin addition. 50 µl competent cells and 3 µl ligation product were mixed and incubated for 15 minutes on ice. Then tubes were incubated 1.5

minutes at 37 ° C and 2 minutes on ice. 200 µl LB broth was immediately added to tubes and mixtures were incubated in 37 ° C shaker incubator for 1 hour. After incubation, all mixture was spread on agar plates. Plates were placed in 37 ° C incubator for overnight incubation. Following day, positive colonies were collected. Each positive colony was inoculated in fresh LB broth with ampicillin for overnight. The following day, plasmid isolation was carried out in order to obtain plasmids.

3.1.6. Screening with Restriction Enzyme

Double digestion was carried out for screening procedure. (Table 3.5)

Table 3.5 Ingredients of restriction enzyme screening

Components	**Volumes**
10X Buffer	2 µl
Template	Varies
BamH I	1 µl
EcoR I	1 µl
dH_2O	Up to 20 µl

Screening process is necessary for controlling of positive colonies. With this method, whether the insert is placed into multiple cloning sites of the vector or not can be figure out. After digestion with restriction enzymes, products were run on 1% agarose gel and depending on the band size, products were sent to sequencing analysis. The sequencing analysis was carried out by GATC company.

3.1.7. Evaluation of the Cloned Gene Sequence

After promoters were amplified by PCR with the appropriate target primers and cloned into vector plasmids, they were sent to commercial sequencing in order to prove that they had the correct sequence as noted in the bioinformatic resources. The results of the analyses were converted to nucleotide sequences using FinchTV DNA sequencing chromatogram trace viewer and comparison of sequences were done by utilizing the SDSC

Biology WorkBench (http://workbench.sdsc.edu/) bioinformatics tool, the wild type and the phosphor-mutant sequences were aligned.

3.2. GST-PULL DOWN ASSAY

3.2.1. Bacterial Expression of GST-Fused Proteins

To produce GST-fusion proteins, BL21-pGEX transformants were inoculated in 50 ml ampicillin containing LB broth and it was incubated at at 37^{o}C rotary shaker overnight. 450 ml fresh LB broth containing ampicillin was added and incubated at 37 C rotary shaker until the OD $_{600}$ reached 0.2-0.4. The expression of GST-fusion protein was induced by the addition of IPTG. Induced cells were cultured for a further 0-6 hours (t=0,1,2,4,6 hours) and centrifuged at 5500rpm for 10 minutes at 4 ^{o}C. The pellet was resuspended in 1 ml ice-cold NET-Buffer. It was incubated on dry ice for 10 minutes, thawed on ice and then they were treated with DNase I. The mixture was centrifuged at maximum speed for 15 minutes. Proteins were loaded on SDS-PAGE.

3.2.2. Preparation of SDS-PAGE

Resolving gel (10%) was poured and waited until all gel solidify (Table 3.6).

Table 3.6 Ingredients of resolving gel

Ingredients	Volumes
40% acrylamide/bis-acrylamide	3,2 ml
1,5M Tris (pH:8.8)	2,5ml
SDS (10%)	100µl
APS (10%)	100 µl
TEMED	4 µl
dH_2O	4 ml

Then resolving gel, stacking gel(5%) was poured to obtain good protein separation (Table 3.7).

Table 3.7 Ingredients of stacking gel

Ingredients	Volumes
40% acrylamide/bis-acrylamide	300 µl
1M Tris (pH:6.8)	380 µl
SDS (10%)	30µl
APS (10%)	30 µl
TEMED	3 µl
dH_2O	2,1 ml

The comb was placed and waited until all gel solidifies. The gel was placed into running tank. Protein samples were loaded in SDS-PAGE gel and run at constant voltage around 2 hours. (First 30 minutes 100V, later 150V). At the end of 2 hours, gel cast was splitted and gel was removed from the glass holders and washed with distilled water for two times. Gel was stained with Coomassie Blue. Gel was washed twice with distilled water for 1 hour to remove all extra stain. The gel was visualized with Bio-RAD chemi-doc device.

3.3. ALTERNATIVE SPLING ASSAYS

3.3.1. RNA Isolation and cDNA Synthesis

A172, HEK-293,SH-SY5Y and U87 cell lines were used for this experiment. From each cell line, 2 million cells were harvested and washed with PBS twice. Later total RNA isolation was carried out with RNA isolation kit (Invtrogen) according to manufacturer's order. Later, cDNA was synthesized according to protocol below Table 3.8 , Table 3.9 and Table 3.10).

Table 3.8 Ingredients of RT mix

Ingredient	volumes
RNA	varies
dH_2O	Up to 10 µl

Table 3.9 Ingredients for cDNA synthesis

Ingredient	Volume
10 X Buffer	2 µl
25X dNTP	0.8 µl
10X RT Random Primers	2 µl
Reverse Transcriptase	1 µl
dH_2O	4.2 µl
RT mix	10 µl

Table 3.10. cDNA synthesis conditions

Temperature	Time
25 °C	10 minutes
37 °C	120 minutes
85 °C	5 minutes

3.3.2. PCR Analysis of Alternative Splicing

cDNAs were used for expression analysis. Firstly, they were used as a template with GAPDH primers for optimization. Until all of them have equal expression level, this process was repeated. After optimization, PCR with different alternative primers were carried out.

Table 3.11. PCR ingredients for expression analysis

Components	**Volume**
Template	Varies
10X Buffer	2 µl
dNTP (2,5mM each)	2 µl
Forward Primer	0,6 µl
Reverse Primer	0,6 µl
i-taq	0,2 µl
dH_2O	Up to 20µl

Table 3.12. Cycling parameters for PCR

	Temperature	**Product Size**			**# of Cycle**
		100-500bp	**500-1000bp**	**1bk-5kb**	
Initial Denaturation	94°C	2 min	2 min	2 min	1 cycle
Denaturation	94 °C	20 sec	20 sec	20 sec	25 cycles
Annealing	Varies	10sec	10 sec	20 sec	
Extension	72 °C	30 sec	50 sec	1min/kb	
Final Extension	72 °C	5 minutes			1 cycle

Different annealing temperature and extension temperature were used depends on the product sizes (Table 3.12)

Table 3.13. Annealing temperatures for GAPDH and alternative splicing primers

Primer Couples	**Annealing Temperature**	**Product Size**
GAPDH F+R	50 °C	400 bp
ASF1+ASR1	53 °C	341 bp
ASF1+ ASR3	55 °C	1047 bp
ASF2+ASR2	55 °C	1208 bp
ASF2+ASR2	65 °C	541 bp

In order to analyze the results obtained from gel electrophoresis imaging, firstly, normalization of each band belonging to different primer pairs were carried by taking the GAPDH bands as basement value. For that purpose, average density values of each band were measured using the QuantityOne software program. Each band density values of primer pairs were divided by the values of GAPDH band densities, and this calculation was repeated for all cell lines separately. In order to represent the relative folding amounts of PCR products, these calculated ratios of the ones, produced in different cell lines by using same primer pairs, were compared with respect to the least value.

3.3.3. Western Blotting Analysis

3.3.3.1. Preparation of Cell Lysate

2 million cells were harvested with trypsin-EDTA and then washed with PBS twice. Pellet was resuspended with 200 μl lysis buffer (1X RIPA Buffer, 1X Protease Inhibitor Cocktail, 1X Phosphatase Inhibitor Cocktail, 100mM DTT). It was incubated for 30 minutes on ice and centrifuged for 10 minutes at 15000 rpm. Supernatant was collected. Protein concentrations of each whole protein lysates were calculated with Bradford assay. 30 μg protein was loaded to SDS-PAGE.

3.3.3.2. SDS-PAGE and Western Blotting

Each protein sample was mixed with loading dye and incubated at 100 °C for 5 minutes and cooled on ice. Later, protein samples were loaded in SDS-PAGE gel and run at constant voltage around 2 hours. (First 30 minutes 100V later 150V). At the end of 2 hours, gel cast was splitted and gel was removed from the glass holders and washed with distilled water for two times. Transfer of proteins from the gel to nitrocellulose membrane was done by Bio-Rad Semi-Dry Blotting device at 25V for 10 minutes. Successful transfers were visualized with Ponceau S solution and remaining protein in the gel with Commesie Blue solution. Nitrocellulose membrane was washed with distilled water until all Ponceau S stain was removed. Membrane was blocked with 5% skimmed milk powder in TBS-T for 1 hour. Primary antibody was diluted in 1X TBS-T skimmed milk solution and incubated overnight at 4°C with gentle agitation. Membrane was washed with TBS-T

twice and once TBS. Later secondary antibody, which is diluted in 1X TBS-T skimmed milk, was incubated for 1 hour at room temperature and membrane was washed with TBS-T twice and then once with TBS. Membrane was developed with ECL reagent and visualized with Bio-Rad Chemi-doc Chemiluminenscent Imaging System.

4. RESULTS

4.1. Alternative Splicing of Elk-1

4.1.1. Primer Optimization for Alternative Splicing Primer

According to Ensemble database, for the Open Reading Frame (ORF) of Elk-1, there are two alternative sequences. Primer couples were designed to cover these alternative sequences. For this reason 2 forward and 3 reverse primer were designed. Primers were analyzed in IDT Oligo-analyzer program and then blasted with NCBI.

The first step is determining the annealing temperature of primer couples. In order to determine annealing temperature of primers, gradient PCR was carried out. There is an additional step, gradient PCR were also carried out with repeated two different cell lines, which were SH-SY5Y (neurablastoma) and U87 (gliablastoma). For each primer couple, this procedure was repeated. (Figure 4.1)

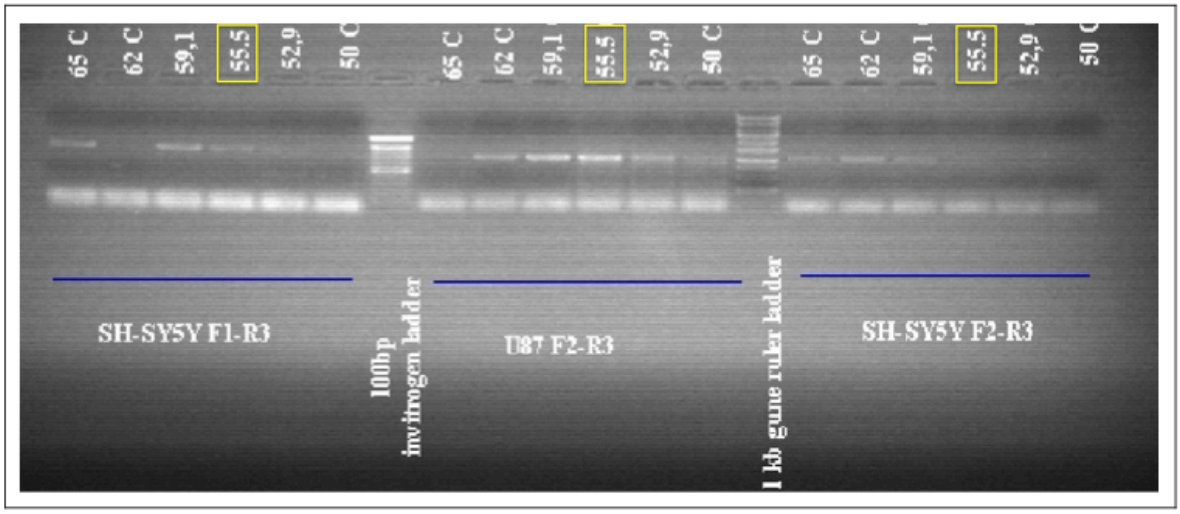

Figure 4.1. An example of Gradient PCR result. Yellow rectangle shows the optimum annealing temperature. Only one representative primer optimization result is shown here.

4.1.2. PCR Analysis of Alternative Splicing Elk-1

Glyceraldehyde 3- phosphate dehydrogenase (GAPDH) is one of the commonly used housekeeping genes, and it is generally used for normalization of expression. In this experiment, GAPDH was used as as our internal control and all normalizations were carried out using GAPDH primers. In all cell lines, expression levels of GAPDH was equalized and then further analysis was carried out. The same amount of template was used for other PCR as used with GAPDH primers.

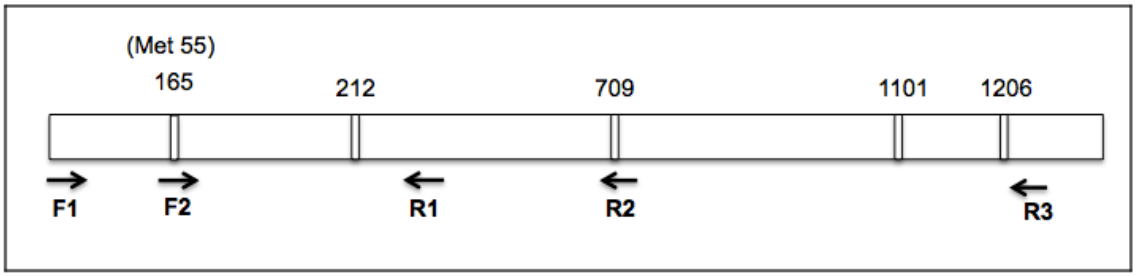

Figure 4.2. Scheme of different primer pairs used to determine alternative splicing isoforms of Elk-1 reported in Ensemble database.

In figure 4.2 and table 4.1 shows the primer pairs and PCR products of these primer couples.

Table 4.1. Alternative splicing products of primer couples

	SP1	SP2	SP3	SP4
ASF1+ASR1	341 bp	341 bp	341 bp	No product
ASF2+ASR2	541bp	No product	541bp	No product
ASF1+ASR3	1208 bp	No product	1208 bp	No product
ASF2+ASR3	1047 bp	No product	1047 bp	No product

Alternative splicing form 1 and 3 have the same coding sequences, but they have different UTR length so all primer couples give the same results for both alternative splicing forms in PCR amplifications. For alternative splicing form 2, F1 & R1 primers gives 341 base pair product, but other primer couples give no product as sequences exist between F1 &

R1, and for the rest there are no sequences being covered by all primers. Alternative splicing form 4 starts in exon 4 and F1 and F2 are staying in exon 3. Therefore primers cannot give PCR products for alternative splicing form 4.

When A172 human glioblastoma, HEK293 human embryonic kidney, SH-SY5Y human neuroblastoma and U87 human glioblastoma cells were analyzed for expression of Elk-1 message, all cells were shown to express only the long form of Elk-1, albeit to different levels. Any alternative form was not detected in our analyses. PCR analysis was repeated two times and the same result was obtained.

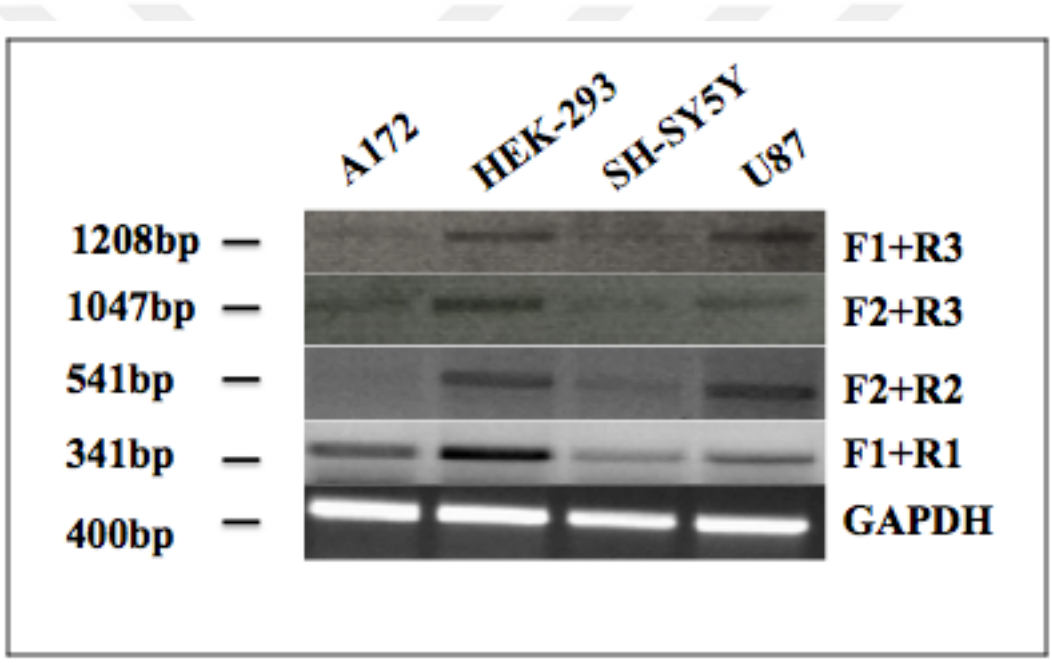

Figure 4.3. PCR analysis of alternative splicing Elk-1

As described in 3.3.2, the calculation of the folding amounts of PCR products amplified by using same primer pairs in different cell lines gave the values shown in Figure 4.4.

a.

	F1&R1
A172	1.4 fold
HEK-293	1.9 fold
SH-SY5Y	1
U87	1.3 fold

b.

	F2&R2
A172	1.1 fold
HEK-293	1.2 fold
SH-SY5Y	1
U87	1.2 fold

c.

	F1&R3
A172	1.1 fold
HEK-293	1.2 fold
SH-SY5Y	1
U87	1.2 fold

d.

	F2&R3
A172	1.1 fold
HEK-293	1.2 fold
SH-SY5Y	1
U87	1.1 fold

Figure 4.4. Calculated folding values for PCR products obtained by using a. F1&R1, b. F2&R2, c. F1&R3 and d. F2&R3 primer pairs, independently, from A172, HEK293, SH-SY5Y and U87 cell lines

In each primer couple, SH-SY5Y cells possess lowest Elk-1 expression in mRNA level and also HEK-293 cells have highest Elk-1 expression respectively.

4.1.3. Western Blot Analysis

PCR analysis results were then confirmed with Western blot analysis. In a parallel set of experiments, protein lysates of each cell line were obtained and used in Western blot analysis. First, anti-βtubulin antibody was used as an internal control and normalization. Later, anti-Elk-1 antibody was used for detecting levels of Elk-1 protein in different cell lysates. Surprisingly, in Western blot analysis, short isoform was also detected in SH-SY5Y and HEK 293 cell lines in addition to the long Elk-1. Western Blot analysis was repeated 3 times and same results were obtained. It should be noted that this short Elk-1 species is not an alternative splicing isoform, but an alternative translation product, hence it can only be detected by protein analysis. (Figure 4.5)

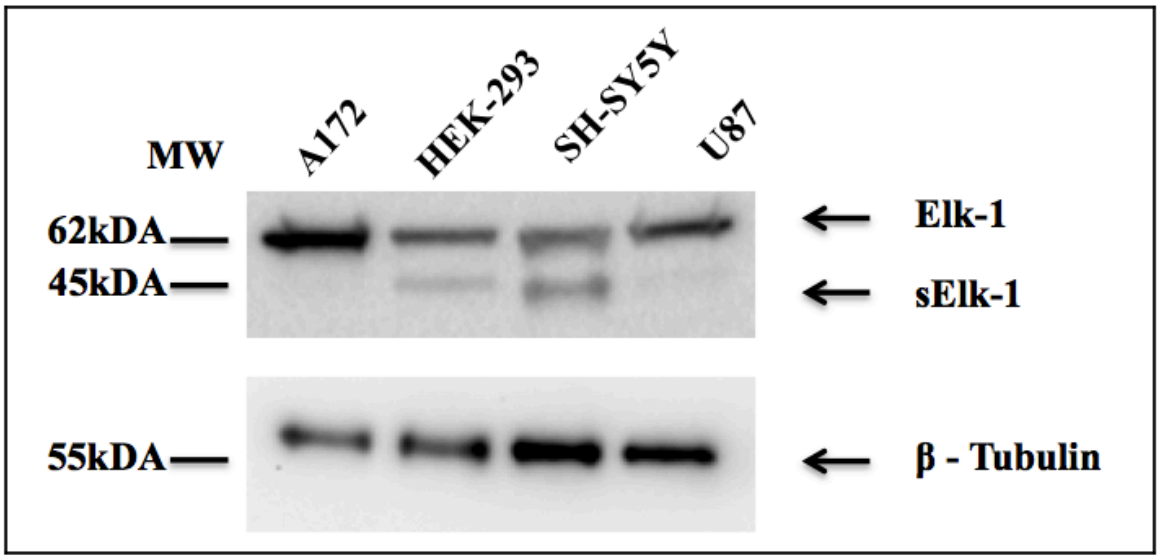

Figure 4.5. Western blotting results

In Western Blot analysis, both short and long forms of Elk-1 were detected. In HEK-293 and SH-SY5Y cell lines, besides long form, the short form exists. In PCR analysis, only long form of Elk-1 was observed at all cell lines. Any absence of alternate sequence was detected.

4.2. Wildtype and Phosphor-mutant GST-PEA3 Expression

4.2.1. Preparation of Wildtype and Phosphor-mutant GST-PEA3 Fusion Proteins

Previously in our laboratory, potential phosphorylation sites (Figure 4.6) were identified bioinformatically, and phosphor-mutants and phosphor-mimics for all possible MAPK phosphorylation motifs on mouse Pea3 were generated for mammalian expression systems. In phosphorylation defective mutants, all potential MAPK target Serines were converted to Alanine through site-directed mutagenesis, and similarly for phosphor-mimic mutagenesis Serines were converted to Glutamic acid. In this study, all these phosphor-mutants and phospho-mimics were sub-cloned into pGEX plasmid for bacterial expression, intended for future protein-protein interaction and kinase assays. Our ultimate purpose is to identify which of the possible upstream signals regulate Pea3 through phosphorylation of which specific motif, with respect to transcriptional activity and neuronal differentiation.

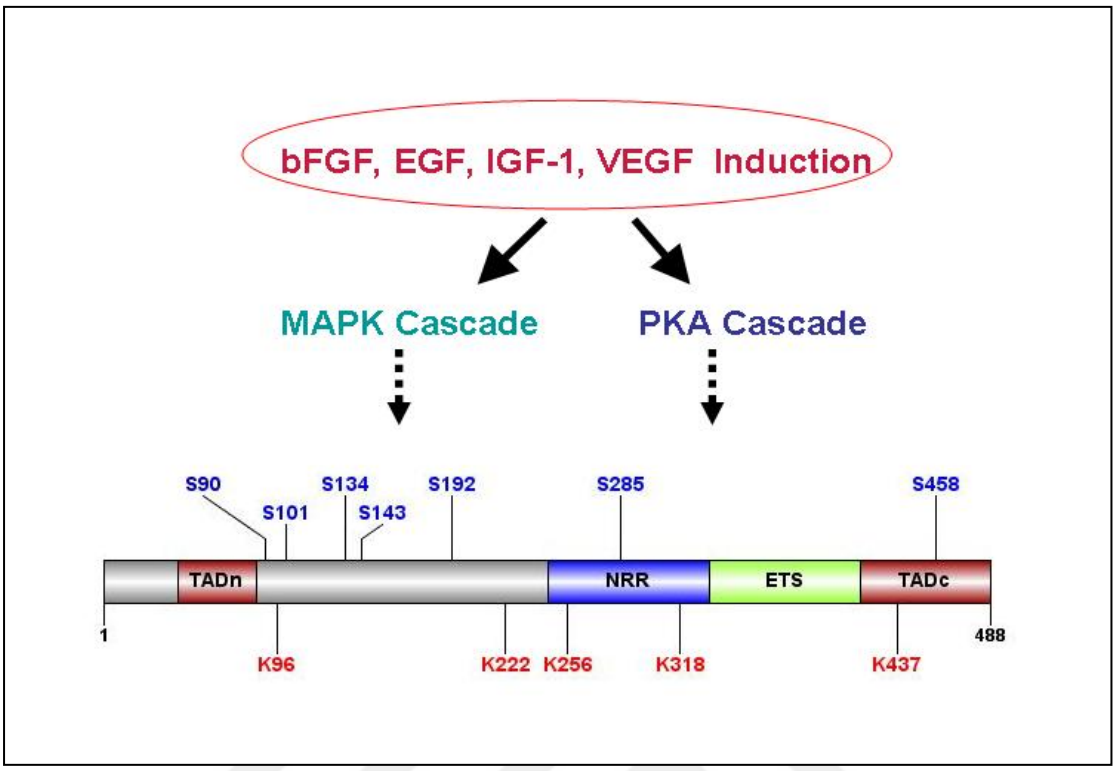

Figure 4.6. Predicted phosphorylation sites of mouse Pea3. Transactivation domain at the N terminal (TADn) and at the C terminal (TADc), negative regulation region (NRR) and ETS domain are shown in this figure.

4.2.1.1. Preperation of Inserts

Mutated Pea3 products were PCR-amplified, processed and then cloned from pCMV-Flag into pGEX-4T-2 plasmid. Same primer couple (forward and reverse) was used for all mutants during cloning.

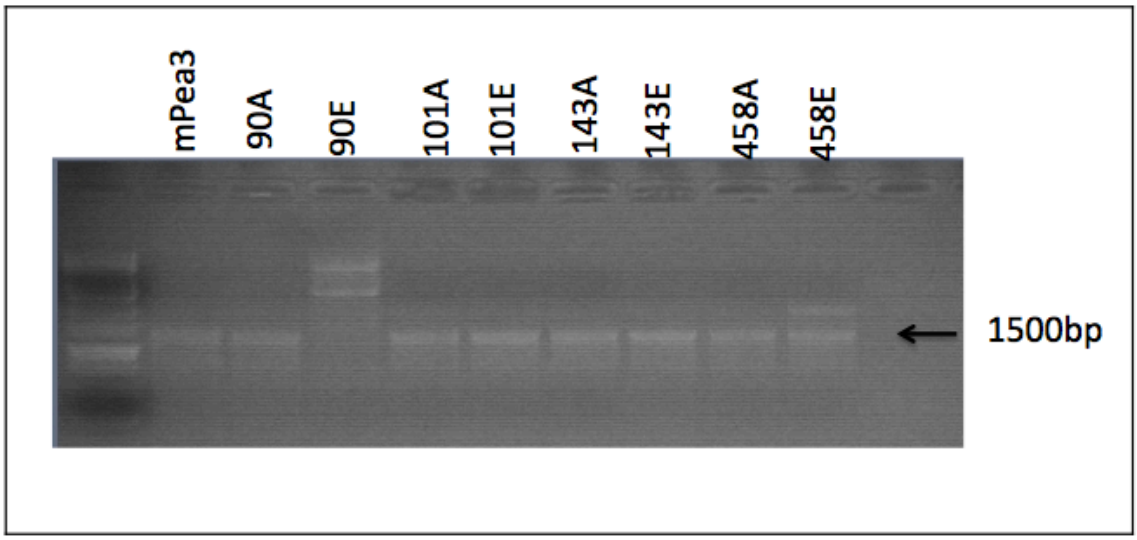

Figure 4.7. PCR amplification of Pea3 mutants from pCMV-Flag backbone templates.

PCR products (Figure 4.7 and Figure 4.8) were run in the 1% agarose gel and then mPea3, S90A, S101A, S101E, S143A, S143E and S458E products were purified. After purification step, concentrations of elutes were calculated respectively (Table 4.2). Only mPea3, S101A and S101E products were found to have high enough concentration for further restriction digestion step for cloning (data not shown).

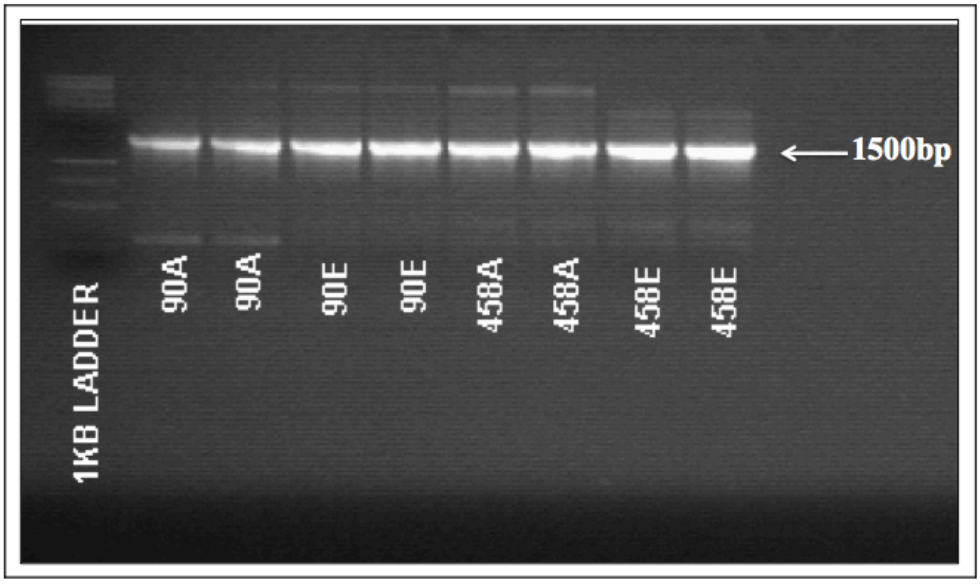

Figure 4.8. PCR amplification mPea3 wild-type phosphor mutants of mPea3 wild-type phosphor mutants

PCR procedure was repeated for S90A, S90E, S458A and S458E mutants. PCR products were run in the 1% agarose gel and then all of them were purified with PCR purification kit. The concentrations of PCR products were measured with Nanospectrophotometer.

Purified PCR products (insert) and pGEX-4T-2 (plasmid) were double digested with BamH I and EcoR I restriction enzyme. And then all of them were purified with PCR purification kit and the concentrations were measured with Nanospectrophotometer.

Table 4.2. Concentration values of PCR Amplification Product and Double Digestion Products

Samples	**PCR Amplification Product**	**Double digestion Products**
mPea3	58,5ng/µl	19,5 ng/µl
S90A	52 ng/µl	15,5 ng/µl
S90E	36 ng/µl	30,5 ng/µl
S101A	48,5 ng/µl	15,5 ng/µl
S101E	44 ng/µl	14 ng/µl
S458E	46,5 ng/µl	13,5 ng/µl
pGEX-4T-2	-	7,5 ng/µl

According to their base number and concentration ratio, the amount of Pea3 mutants products and vector were calculated (ideally, a 3:1 insert:vector ratio was aimed for), and ligation was done to ligate mutate products with pGEX-4T-2 plasmid. After ligation process, transformation was done with ligation products. Colonies were collected and plasmid isolation was carried out.

4.2.1.2 Screening of GST-Pea3 Plasmids

After plasmid isolation, restriction enzyme screening was carried out in order to understand whether insert is localized in the multiple cloning site of the plasmid or not.

There was no need for orientation confirmation because of utilizing two different restriction enzyme digestion.

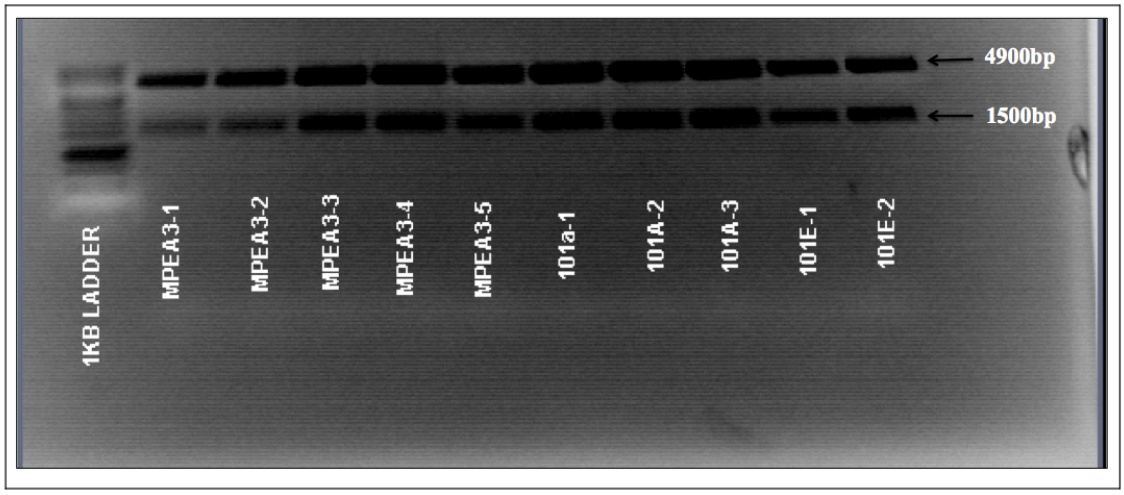

Figure 4.9. Screening results of wild-type Pea3, S101A, S101E

Upper band (4900bp) represent the vector and lower band (1500bp) is the insert. All constructs were found to have wildtype and mutant Pea3 inserts. (If the construct were not to contain any insert, only a single band would be seen).

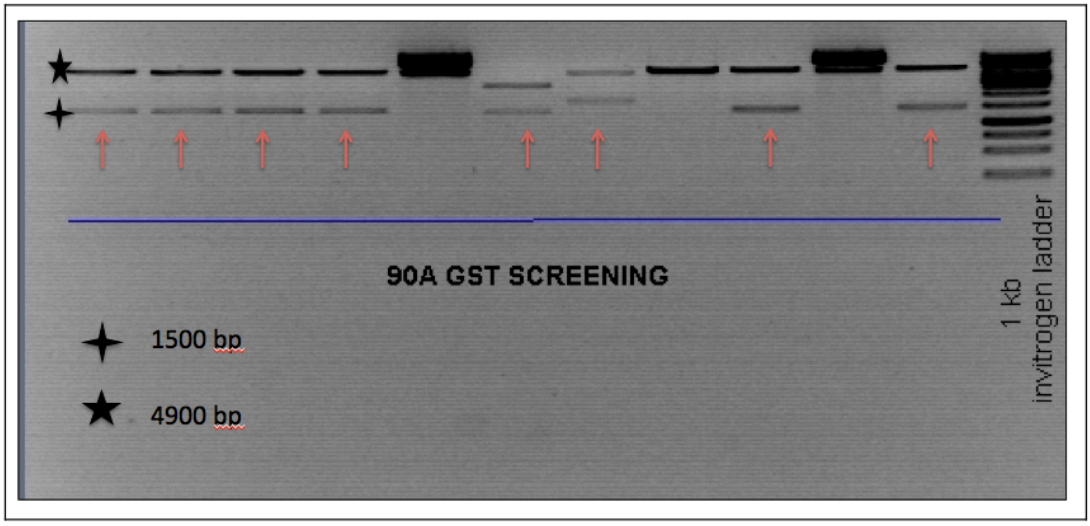

Figure 4.10. Screening results of GST-mPea3-S90A mutant.

In Figure 4.10, pink arrows represent insert containing plasmids. They were selected for sequencing.

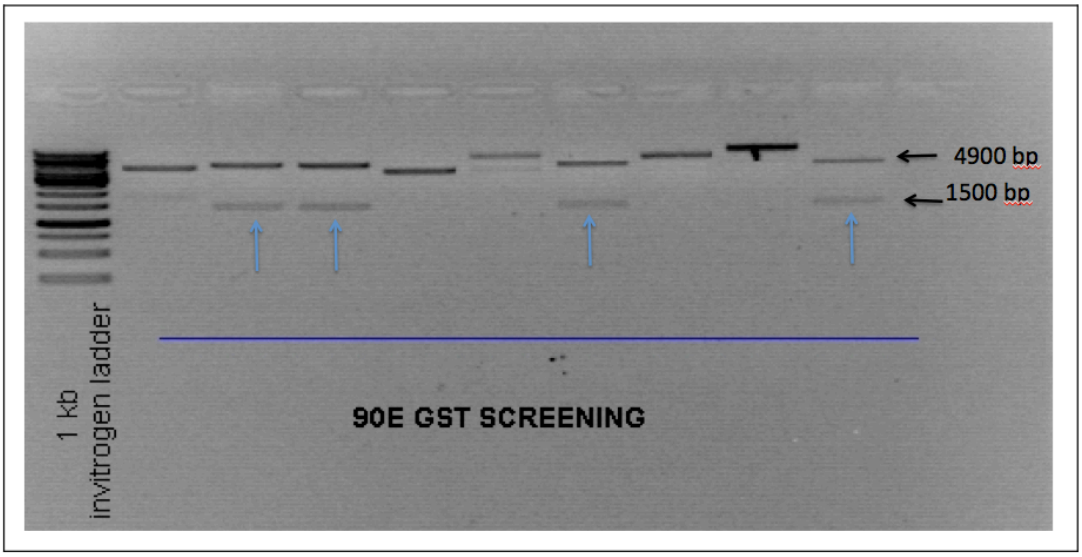

Figure 4.11. Screening results of GST-mPea3-S90E mutant.

In Figure 4.11, blue arrows represent true recombinants. They were selected for sequencing.

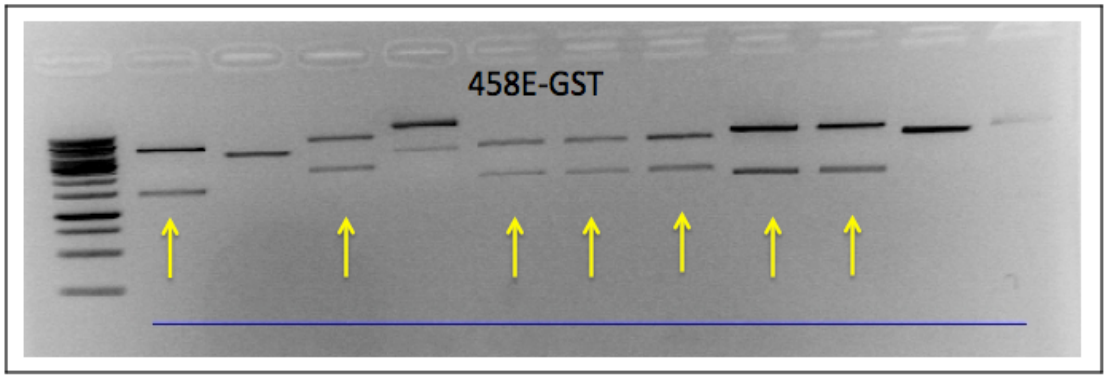

Figure 4.12. Screening results of GST-mPea3-S458E mutant

In Figure 4.12, yellow arrows represent insert containing plasmids. They were selected for sequencing.

Insert containing vectors were sequenced. Wild–type and mutant sequences were compared with sequences in NCBI.

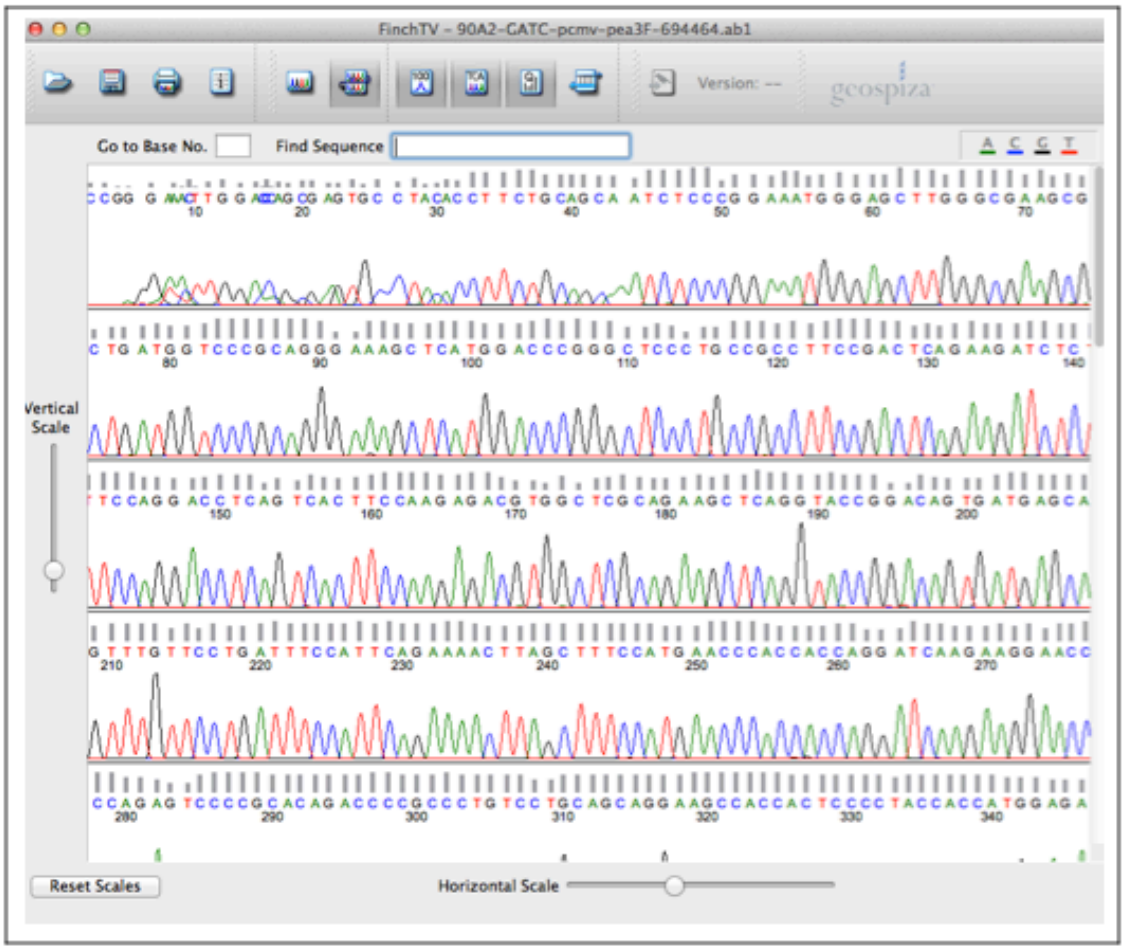

Figure 4.13. A representative example of sequencing data

DNA sequencing was done by GATC company as a service provider. The results were provided in *ab1* format and was analyzed through its specific software, Finch TV (http://www.geospiza.com/finchtv/help/pages/A-Introduction/04-QuickStart.shtml). (Figure 4.13)

The sequences from Finch TV were then analyzed in SDSC Biology Workbench data-based tool (http://workbench.sdsc.edu). Using the alignment tool of Biology Workbench, wildtype and mutant Pea3 sequences were aligned, mutagenesis was confirmed, and clones were amplified and stored for further analyses.

4.2.2. Expression Optimization of Wild-type and Mutant Pea3 GST Plasmids

GST-Pea3 mutants were transformed into BL21 competent cell and 50 ml of overnight culture of pGEX plasmid was started with LB broth containing 200 µg ampicillin. 450 ml

fresh medium was added and incubated on 37°C rotary shaker, and when OD_{600} reached 0.3 GST expression was induced with 200 µM IPTG addition. 1 ml samples were collected at t=0, t=1 hr, t=2 hrs, t=4 hrs and t=6 hrs. Samples were then lysed and DNase I treated as explained in Methods, and lysates were loaded onto 10 % SDS-PAGE gel for analysis (Figure 4.14, Figure 4.15, Figure 4.16, Figure 4.17).

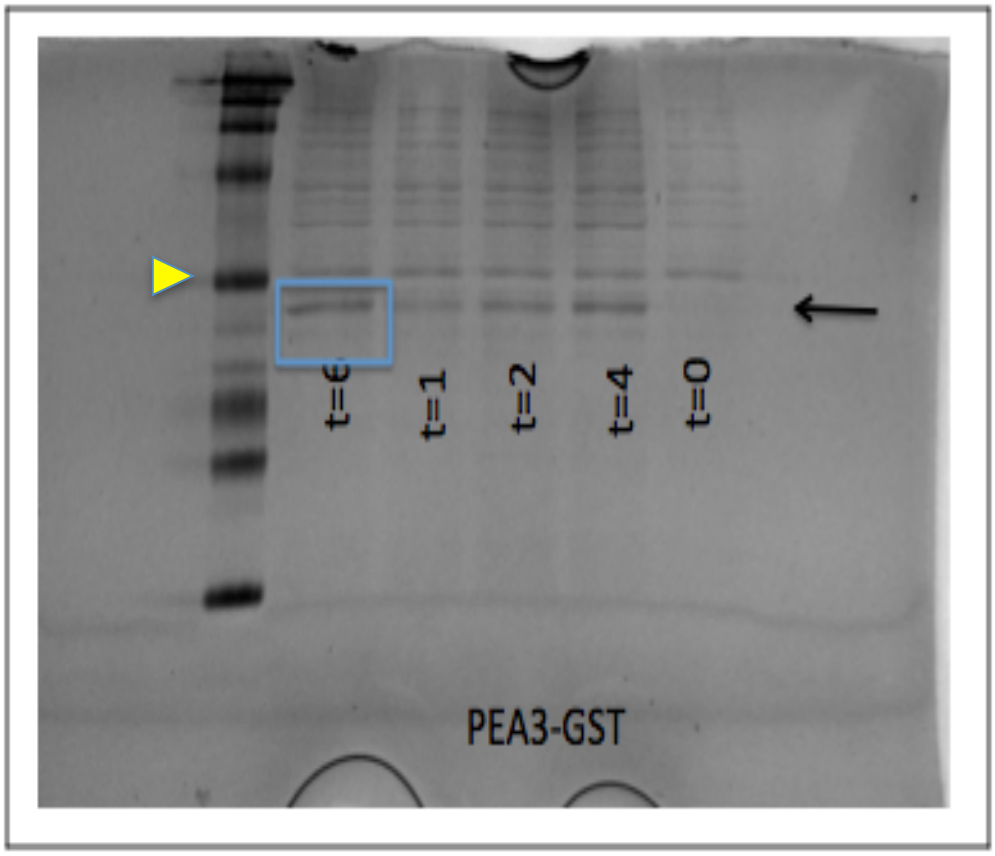

Figure 4.14. Bacterial expression optimization of IPTG induced GST-mPea3 (wildtype). Blue rectangle represents maximum expression time-point. Yellow arrowhead shows 97kDa size marker.

30 µl sample of GST-mPea3 (wild-type) was loaded in each well and the best expression was observed at 6hours. (Figure 4.14)

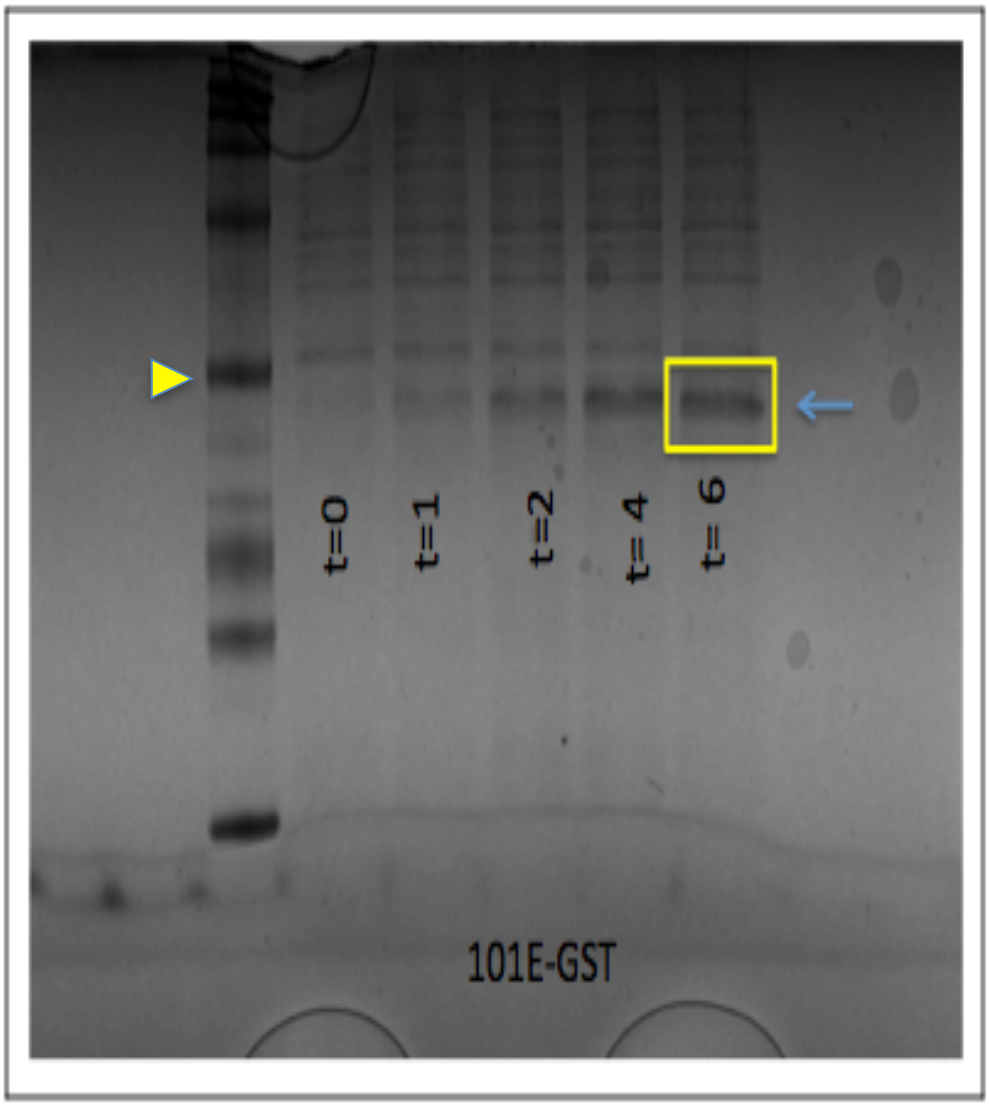

Figure 4.15. Bacterial expression optimization of IPTG induced GST-mPea3-S101E. Yellow rectangle represents maximum expression time-point. Yellow arrowhead shows 97kDa size marker.

30 µl sample of GST-mPea3-S101E was loaded in each well and the best expression was observed at 6hours. (Figure 4.15)

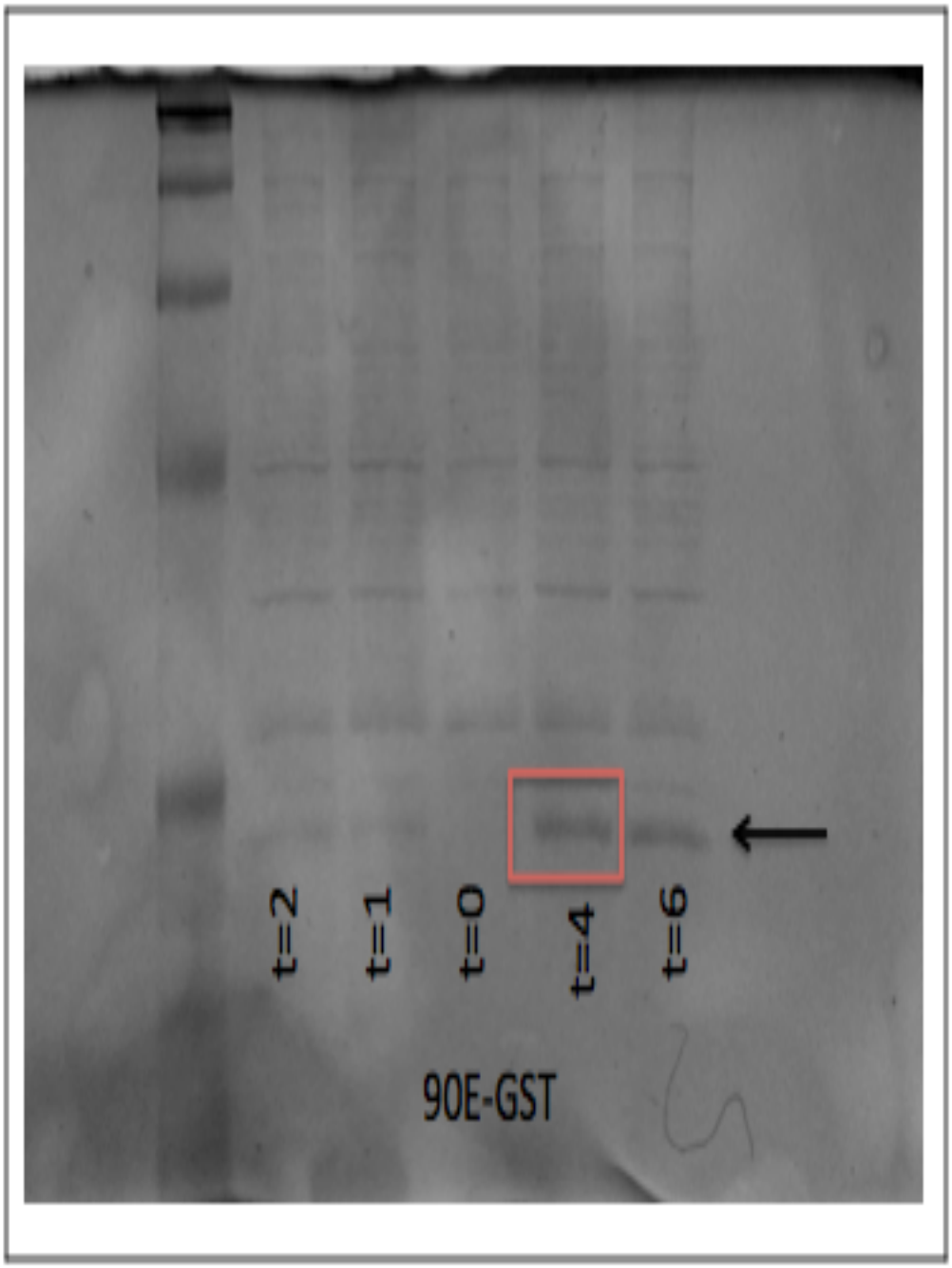

Figure 4.16. Bacterial expression optimization of IPTG induced GST-mPea3-S90E. Pink rectangle represent maximum expression time-point.

30 µl sample of GST-mPea3-S90E was loaded in each well and the best expression was observed at 4 hours. (Figure 4.16)

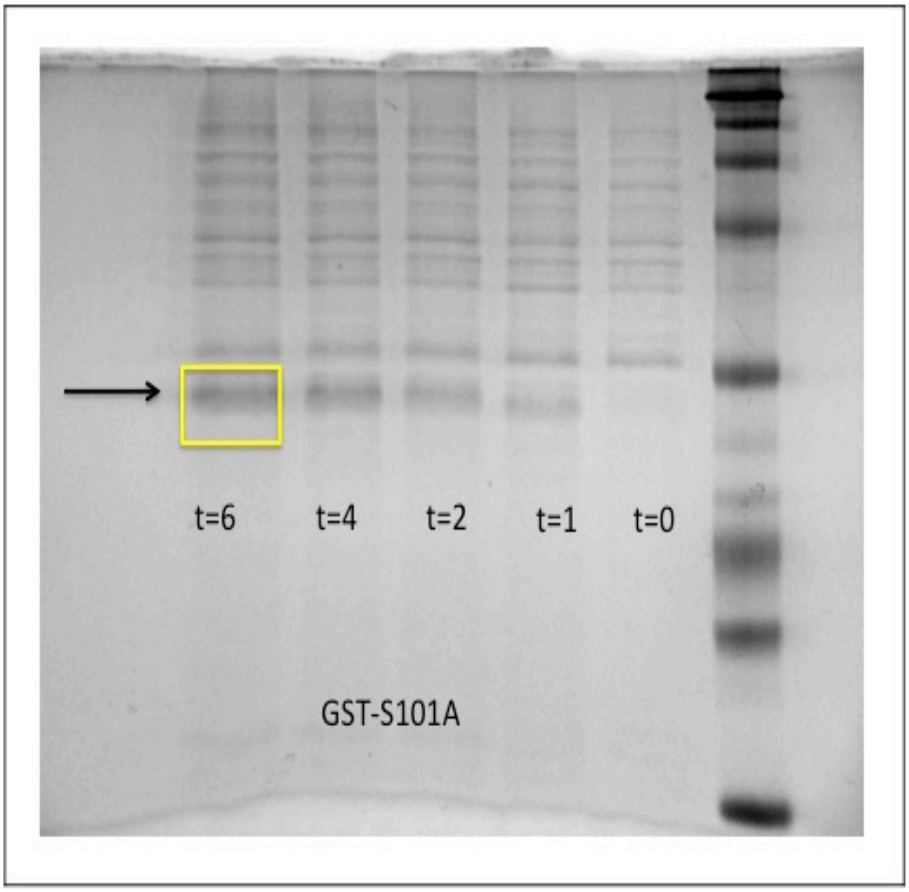

Figure 4.17. Bacterial expression optimization of IPTG induced GST-mPea3-S101A. Yellow rectangle represents maximum expression time-point.

30 µl sample of GST-mPea3-S101A was loaded in each well and the best expression was observed at 6hours. (Figure 4.17)

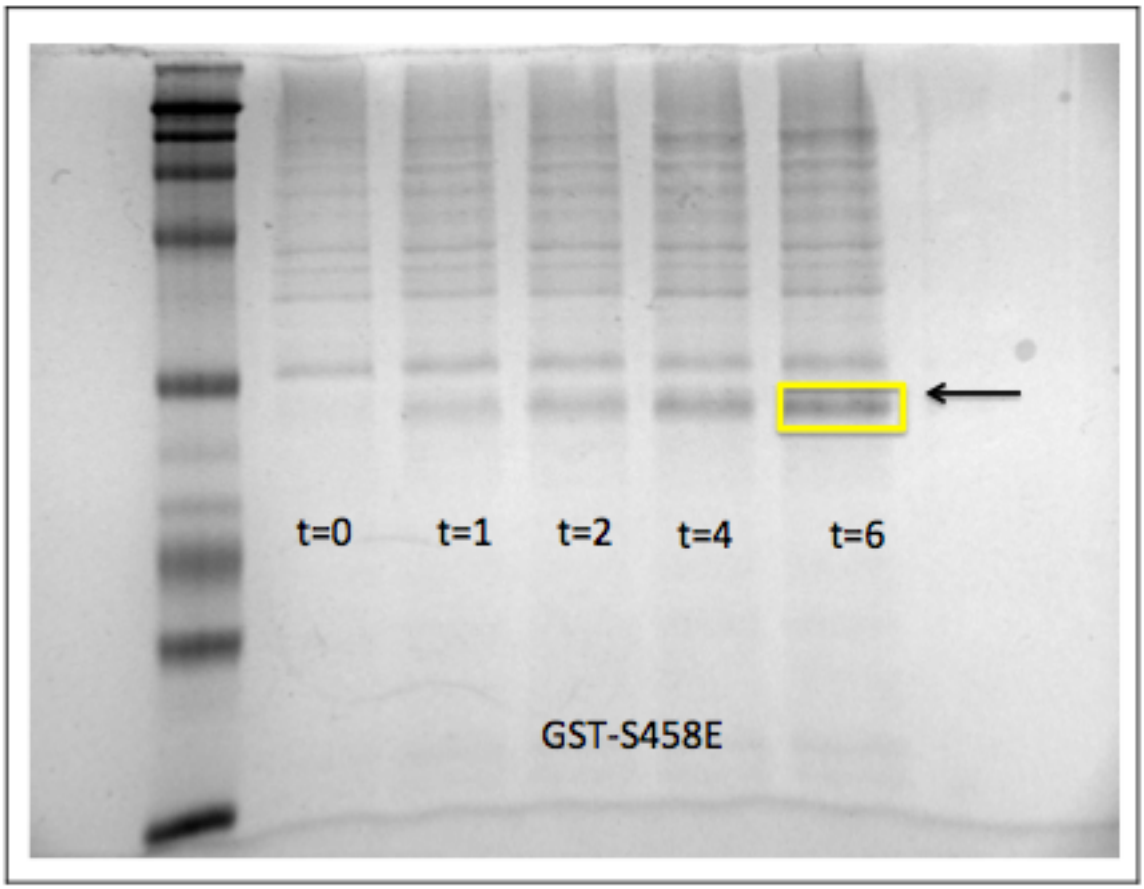

Figure 4.18. Bacterial expression optimization of IPTG induced GST-mPEA3-S458E. Yellow rectangle represents maximum expression time-point.

30 µl sample of GST-mPea3-S458E was loaded in each well and the best expression was observed at 6hours. (Figure 4.18)

These GST-mPEA3 fusions will be used in protein-protein interaction as well as kinase assays in the future.

5. DISCUSSION

Elk-1 belongs to TCF subfamily, whose members are part of the ETS transcription factors, which involve in differentiation, development, transformation and cellular proliferation. When they are activated they form a ternary complex with SRF on SRE of the c-fos promoter In postmitotic neurons, Elk-1 is detected in both the nucleus and cytoplasm, including neuritic extensions. While studying the localization of Elk-1 in the rat brain, an isoform sELK-1 (*s*hort Elk-1) was identified. This 45 kDa protein was expressed uniquely in brain tissue and was exclusively nuclear. The 54 amino-acid N-terminal deletion removes most of the DNA binding domain, explaining why it is compromised in its ability to activate SRE genes. Moreover, with deletion of the first 54-amino acid, short isoform of Elk-1 loses its Nuclear Export Signal (NES). Curiously, in nerve growth factor (NGF)-treated PC12 cells, sElk-1 protein expression was turned on, and this correlated with the redistribution of Elk-1into the cytoplasm. This distribution was similar with mature brain. In addition, the morphology resembles synaptic neurons. Furthermore, overexpression of sElk-1 but not Elk-1 increased neurite extension in the PC12 model system.

In this study, we aimed to identify the alternative splicing forms of the transcription factor Elk-1 in different cell lines. In some studies, different alternatively splicing forms were indicated but all of them based on 5'UTR of Elk-1. We want to show alternative splicing form in Elk-1 open reading frame (ORF). According to Ensemble database Elk-1 has six alternative spliced forms, but only four of them encodes of protein. In addition, Elk-1 has two alternate sequences as well. The primer pairs were design based on these alternate sequences. Spliced form 1 and spliced form 3 have the same amino acid number but they have different UTR length. The primer which was cover the UTR were not designed, so spliced form 1 and spliced form 3 were not separate each other. In spliced form 3 has 95 amino acid and only one primer couple, which were F1 and R1, give PCR product. One primer couple is insufficient to detect an alternative splicing form. One additional primer couple should be designed. In alternative spliced form 4 starts in different start codon. Any primer couple can amplify the spliced form 4 so new primer couple should be designed. I
In PCR analysis, it cannot be analyzed clearly. Any alternative spliced form cannot be detected. Only long isoform of Elk-1 was observed. It could be spliced form 1 or spliced

form 3. On the other hand, short isoform of Elk-1 was detected clearly in Western Blotting analysis. In SH-SY5Y and HEK293 cell lines, short isoform of Elk-1 was detected. In literature, short isoform was indicated in the rat brain but in this study, short isoform was indicated in HEK-293 (embryonic kidney). However mRNA level of Elk-1 is highly low, Elk-1 has the highest protein expression. In further study, primary brain tumor cells will be used and it will give more clear results rather than cell line study.

Elk-1 has role in many diseases such as Alzheimer's disease, Huntington's disease and Parkinson's disease. Identification of spliced form of Elk-1 will help us focus on new strategies for neurodegenerative disease.

Pea3 belongs to PEA3 subfamily with Erm and Er81. This ETS–domain transcription factor is known to play a critical role in axonal branching, axonal migration and cell migration. Most of the ETS-domain transcription factors have post–translational modifications to regulate their transactivations as Pea3 subfamily members have. The most common modification is the phosphorylation and it was induced by MAPK pathway. Until now, the specific phosphorylation sites of Er81 and Erm were done but there is no such phosphorylation mapping for Pea3.

Previously in our lab, the phosphorylation sites, where MAPK kinase phosphorylates Pea3, were determined and two different mutation series were constructed. In one series, serine amino acid was converted to alanine amino acid at the predicted serine/pro sites. With this mutation, the phosphorylation of that docking site is blocked. In the other series, serine amino acid is converted to glutamic acid at the same residues and this mutation type mimics the phosphorylation. Disruption of phosphorylation sites is used as a method to identify their effect in the DNA binding and transactivation of Pea3, and to obtain specific phosphorylation mapping of Pea3.

In this study, we tried to establish a system to study phosphorylation of Pea3 based on docking site specificity in different MAPKs such as NGF, IGF and BDNF. For this purpose, Pea3 wild-type and phopsho-mutants were sub-cloned into pGEX-4T-2 vector.

Firstly, all phospho-mutants and will type Pea3 were tried to be cloned into pGEX-4T-2

vector. Only wild-type, S90A, S90E, S101A, S101E and S458E mutants were subcloned successfully. The study S143A, S143E and S458A construct will be subcloned. With successfully cloned plasmids, optimization of bacterial expression was done. The plasmids were transformed into BL21 competent cell and then they were incubated until their OD_{600} reached 0.3. Then IPTG induction was done because vector has a lac promoter. After induction process, samples were collected in time interval (t=0-6 hours). Expression levels in different time points were compared by SDS-PAGE analysis. The time point, which resulted in thicker protein band on gel, is chosen as optimum hour for expression of that construct. In my samples, I observed increase expression but it is not enough for my further analysis. I will be increased my time intervals and also I will use less lysis buffer. The possible reason behind the failure in thicker band observation might arised from the dilution in higher amounts. In the future, GST-Pull Down assay will be used to study the relationship between Pea3 phosphorylation and upstream signals stimulating MAPKs.

Regeneration of damaged axons became an important topic in the treatment of several neurodegenerative disorders, such as Alzheimer's or Parkinson's disease. A new strategy, which involves application of molecules, is responsible for neurite outgrowth and neuronal differentiation is being widely investigated worldwide. Development of a novel therapy by using proteins promoting differentiation and axonal elongation appears to represent a promising treatment in such disorders. In this context, we focused our research on identifying the regulation mechanisms of Pea3. We focused on phosphorylation mechanism of Pea3 due to the fact that elucidating roles of each phosphorylation motif and the responsible growth factors will eventually be used in designing treatments in animal disease models in future studies.

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