IDENTIFICATION OF UPSTREAM REGULATORS OF MAPK-STIMULATED PEA3 MEDIATED NEURAL DIFFERENTIATION OF PC12 CELLS

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IDENTIFICATION OF UPSTREAM REGULATORS OF MAPK-STIMULATED PEA3 MEDIATED NEURAL DIFFERENTIATION OF PC12 CELLS

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ABSTRACT

IDENTIFICATION OF UPSTREAM REGULATORS OF MAPK-STIMULATED PEA3 MEDIATED NEURAL DIFFERENTIATION OF PC12 CELLS

Pea3 transcription factor family belongs to ETS domain transcription factor super-family and act 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 a previous study performed in our laboratory, it was observed that over-expression of Pea3 protein induces neuronal differentiation in PC12 cells via MAPK cascade when induced with growth factors which alone induce proliferation in the absence of Pea3. Although the phosphorylation sites for other members of Pea3 transcription family were identified and there were a few studies that investigate the post-translational modification of Pea3 protein, phosphorylation is still unknown. In this thesis project, first approach to study the upstream regulators of Pea3 mediated neuronal differentiation, is blocking suggested MAPK phosphorylation sites on Pea3, by converting suspected Serine residues into Alanine residues. MAP kinases are proline-directed Serine/Threonine kinases and phosphorylate the serine or threonine in the dipeptide S/T-P motif. Therefore, conversion of Serine to Alanine will silence phosphorylation, whereas converting Serine to Glutamate will mimic it. Comparison of the signals obtained from a mutated phosphorylation site with the wild-type will be used to identify the phosphorylation sites on Pea3. Second approach involves deletion of suggested MAPK docking sites on Pea3. MAPKs are known to interact with their targets and regulators via high affinity docking sites in order to obtain specific and efficient selectivity. To be able to phosphorylate their targets efficiently, MAPKs require the integrity of the docking site. Therefore, deletion of docking sites on Pea3 will result in decreased activity as a result of inefficient interaction with the kinases. Docking site specificity in different MAPKs will help us identify the upstream regulators of Pea3-mediated neuronal differentiation mechanism in PC12 cells. This study will permit

us to better understand the regulation of ETS domain transcription factor Pea3, in neuronal differentiation. The findings from this work might be considered as a first step in the treatment of neurodegenerative disorders in the future.

ÖZET

PC12 HÜCRELERİNDE MAPK UYARILI PEA3 ARACILI NÖRAL FARKLILAŞMANIN ÜST AKIŞ DÜZENLEYİCİLERİNİN TANIMLANMASI

Pea3 transkripsiyon faktörü ailesi ETS domain transkripsiyon faktörü süper-ailesine aittir ve diğer ETS süper-ailesi üyeleri gibi hücre proliferasyonu, farklılaşması, gelişim ve apoptoz gibi sinyal-dağıtım yollarının çekirdek hedefleri olarak görev yapmaktadır. ETS domain proteinlerini içeren düzenleyici olayların büyük çoğunluğu, fosforilasyon işlemlerine aracılık etmek üzere MAPK yollarına ihtiyaç duymaktadır. Laboratuvarımızda gerçekleştirilen önceki bir çalışmada, Pea3 proteininin yokluğunda tek başına proliferasyonu sağlayan büyüme faktörleriyle birlikte indüklendiğinde, Pea3 proteininin aşırı ekspresyonunun MAPK kaskadı yoluyla PC12 hücrelerinde nöronal farklılaşmayı tetiklediği gözlenmiştir. Pea3 transkripsiyon faktörü ailesinin diğer üyeleri için fosforilasyon bölgeleri tanımlanmış olmasına karşın ve Pea3 proteininin post-translasyonel modifikasyonlarını inceleyen birkaç çalışma yapılmasına karşın, fosforilasyon motifleri hala bilinmemektedir. Bu tez projesinde, Pea3 aracılı nöronal farklılaşmanın yukarı akış düzenleyicilerinin çalışılmasına dair ilk yaklaşım, şüphelenilen Serin bölgelerinin Alanin bölgelerine dönüştürülmesi yoluyla, Pea3 üzerinde tahmin edilen MAPK fosforilasyon bölgelerinin bloklanmasıdır. MAP kinazlar prolin hedefli Serin/Treonin kinazlardır ve dipeptid S/T-P motifi üzerinde serin veya treonini fosforlamaktadır. Bu nedenle, Serinin Alanine dönüştürülmesi fosforilasyonu sustururken, Serinin Glutamata dönüştürülmesi fosforilasyonu taklit edecektir. Bir mutant fosforilasyon bölgesinden elde edilen sinyalin doğal tür ile karşılaştırılması, Pea3 üzerindeki fosforilasyon bölgelerinin tanımlanmasında kullanılacaktır. İkinci yaklaşım, Pea3 üzerinde tahmin edilen MAPK kenetlenme bölgelerinin delesyonunu içermektedir. Spesifik ve etkili seçicilik elde etmek amacıyla MAPK'lerin hedefleriyle ve düzenleyicileriyle yüksek afiniteye sahip kenetlenme bölgeleri yoluyla etkileşime girdiği bilinmektedir. Hedeflerini etkin şekilde fosforlayabilmek için, MAPK'ler kenetlenme bölgesinin sağlam kalmasını gerektirmektedir. Bu nedenle, Pea3 üzerinde kenetlenme bölgelerinin delesyonu kinazlar ile yetersiz etkileşimin bir sonucu

olarak aktivitede azalmayla sonuçlanacaktır. Farklı MAPK'lerinde kenetlenme bölgesi özgünlüğü, PC12 hücrelerinde Pea3 aracılı nöronal farklılaşma mekanizmalarının yukarı akış düzenleyicilerinin tanımlanmasına yardımcı olacaktır. Bu çalışma, nöronal farklılaşmada ETS domain transkripsiyon faktörü Pea3'ün regülasyonunu daha iyi anlamamızı sağlayacaktır. Bu çalışmadan elde edilen bulgular, gelecekte nörodejeneratif hastalıkların tedavisinde bir ilk basamak olarak kabul edilebilir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

EGF	Epidermal Growth Factor
ETS	E26 Transformation Specific
IGF-1	Insulin-like Growth Factor - 1
MAPK	Mitogen Activated Protein Kinase
NFL	Neurofilament Light Chain
PCR	Polymerase Chain Reaction
Pea3	Polyoma Enhancer Activator 3
SAPK/JNK	Stress-activated protein kinase/c-Jun NH2-terminal kinase

1. INTRODUCTION

1.1. ETS DOMAIN TRANSCRIPTION FACTORS

Transcription factors are proteins that bind to the enhancer or promoter regions in order to control that transcription occurs from only a small group of promoters in any cell. 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, 2].

ETS (E26 Transformation Specific) - domain transcription factors family is composed of more than 30 eukaryotic transcription factors [3]. Members of ETS - domain super family have a highly conserved primary sequence that has a homology region with the *v-ets* oncogene encoded by the E26 (E twenty six) avian erythroblastosis virus [4]. ETS - domain transcription factors are known to be conserved in metazoans and also known to be important regulators of development, by serving as downstream repressor or activators of signal transduction cascades that regulate a broad range of cellular processes such as

proliferation, differentiation during both embryonic development and in adults, immune response, apoptosis, migration, metastasis, tissue remodeling, transformation or angiogenesis [5]. ETS - domain transcription factors share a highly conserved 85 amino acid long DNA-binding region [6]. These transcription factors bind to the GGAA/T core recognition sequence, which is also called the ETS-binding site (EBS). The regions outside the EBS vary within this transcription factor family [6].

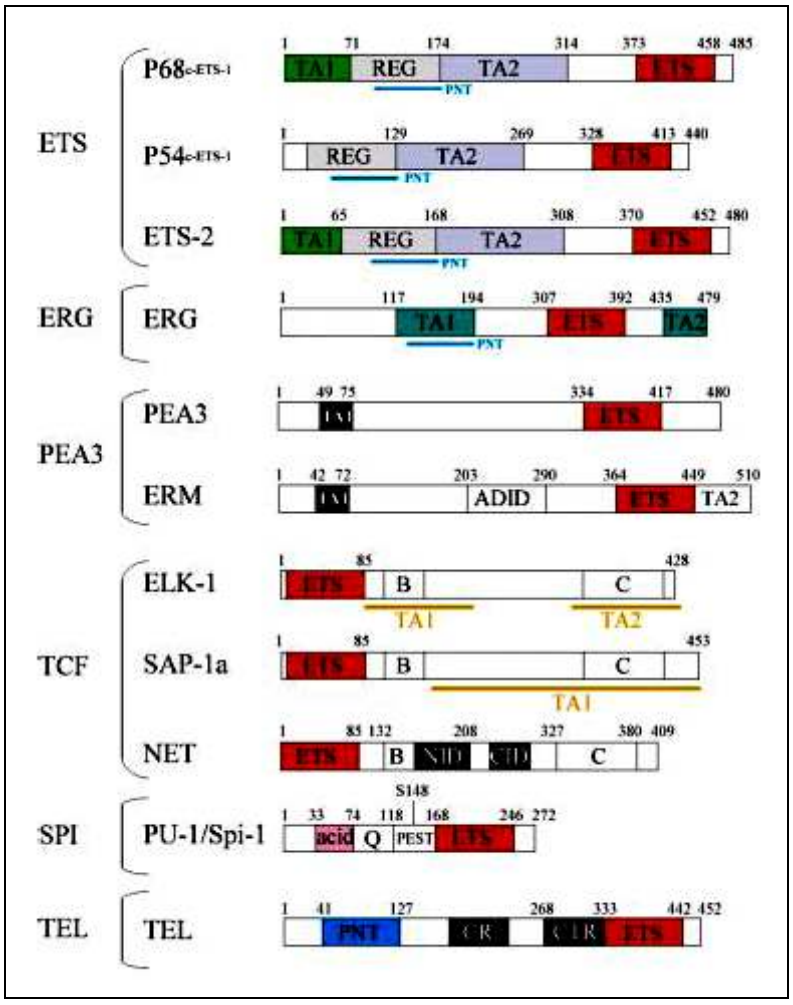

Figure 1.1. ETS Domain Transcription Factor Family [13]. ETS domain is conserved in all members of ETS domain super family of transcription factors, although the location varies within each sub family. Additional domains are located on some sub families, however it is not common in all members

The recognition of DNA target sites is one of the major mechanisms responsible for maintaining the specificity of transcription factor function and is mediated by structural

modules referred to as DNA-binding domains. The EBS domain is characterized by the presence of three conserved tryptophans separated by 17-21 amino acids [8]. Since ETS-domain does not resemble a similarity with other known DNA binding domains, it was thought to represent a novel structural DNA-binding motif. However, after the elucidation of the three-dimensional structure of some of the super family members, (such as Fli-1 [8], Ets-1 [9] and PU.l/Spi-1 [10], it was identified that these transcription factors contain a winged helix-turn-helix (wHTH) which is formed by a three α-helices and four stranded anti parallel β-sheet. The turn in the HTH motif is found to have a 6-7 amino acid loop.

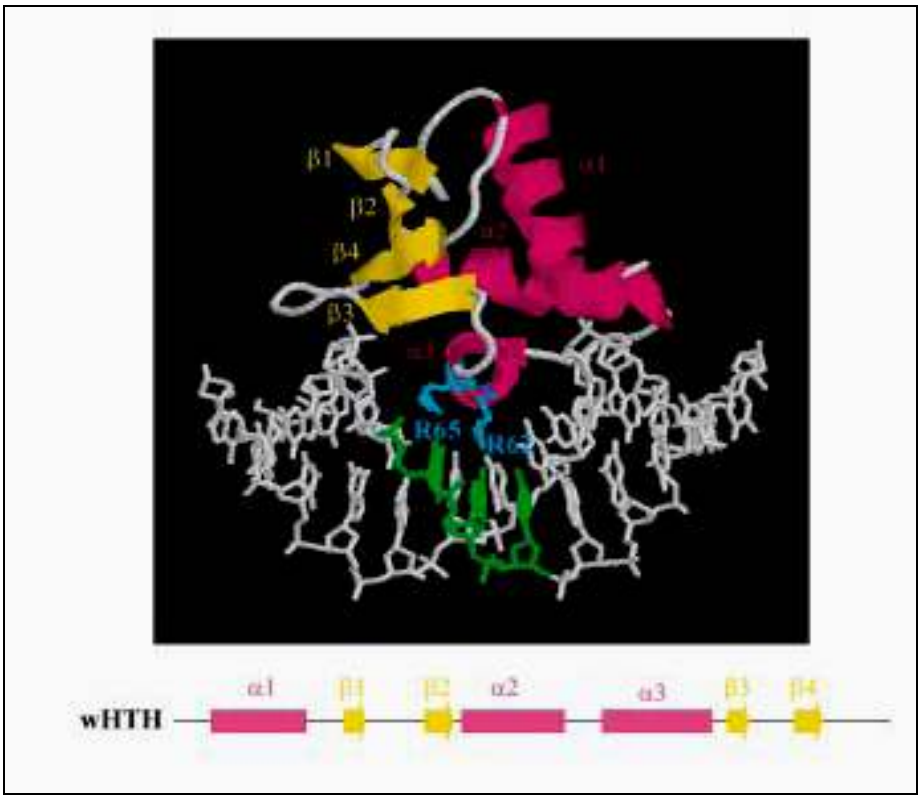

Figure 1.2. ETS domain transcriptional factors contain a conserved winged helix-turn-helix motif which is formed by a three α-helices and four stranded anti parallel β-sheet [13]

Most of the ETS-domain members are regulated by their auto-regulatory mechanisms and this mechanism inhibits their DNA-binding activity. This function is thought to be important in preventing promiscuous DNA-binding of these transcription factors due to their relatively non-stringent DNA-binding specificities. For instance, the DNA-binding activity of Ets-1 transcription factor is inhibited by the DNA sequences

outside its ETS-domain [9]. Recently it was identified that the molecular mechanism underlying this auto-inhibition process is an inhibitory module, composed of two α-helices located N-terminally to the ETS-domain and a single C-terminal α-helix. These motifs work in conjuction to regulate the DNA-binding properties of this transcription factor. [11]. A conformational change involving unfolding of one of the N-terminal α-helices is associated with relief of this intramolecular inhibition [12]. DNA recognition helix α3 recognizes the DNA core with the help of base specific contacts, made by two conserved arginine residues, on the other hand a β-hairpin wing and a loop together make multiple DNA phosphate backbone contacts to nucleic acids outside the core EBS. Even though the mechanism underlying this process is not fully known, when all these information are taken together, it is safe to suggest that DNA contacts from conserved and non-conserved protein residues along with sequence specific DNA structural properties play a role in the individual "innate" ETS protein specificity. Moreover, it is assumed that the other interactions with key transcriptional factor partners (for instance bZIP, Runt or Pax family members [6,14,4]) play a crucial role in this process [10].

Another potential DNA-binding activation mechanism of ETS-domain transcription factor family involves signal transduction pathway such as Mitogen Activated Protein (MAP) kinase mediated phosphorylation. 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 stresses such as UV irradiation lead to the activation of the stress-activated kinases SAPK/JNK and p38 [16]. 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.

Ets family proteins are nuclear proteins and some of them have nuclear export signals (NES) as well as nuclear localization signals (NLS). Phosphorylation of ETS family proteins changes their subcellular localization under different stimulation. Stress caused by UV irradiation or heat shock to cells induces active nuclear exclusion of Net (a member of the TCF subfamily), through phosphorylation of NES which involves JNK/SAPK [16]. Similar type of regulation of a protein by changing subcellular

localization has also been reported in Ets-2 repressor factor (ERF). ERF was initially isolated from the c-ets-2 promoter and it is thought to be an important controller of cell proliferation during G0/G1 phase of the cell cycle. However ERK2 MAP Kinase is involved in this case, instead of JNK pathway [17]. Therefore it can be concluded that, post-translational modification of ETS - domain proteins modulates DNA-binding activities, association with co-regulatory partners, transcriptional activation capacities, and subcellular localization.

Several ETS - domain transcription factors are expressed in the central nervous system. In Drosophila, two ETS - domain proteins, (PntP2 and Yan) were identified such that they work antagonistically in R7 photoreceptor neuron induction and differentiation in the eye [17]. Yan represents a repression activity, after being phosphorylated through a Ras-MAP kinase signaling pathway [18]. In mammals, it is known that Ets-1 and Ets-2 expression exists in specific regions of the brain. Furthermore, Ets-1 is expressed in astrocytes and its expression is shown to be up-regulated during retinoic acid-induced neural differentiation of multipotent mouse embryonic carcinoma P19 cells [17]. Moreover, Ets-1 and GHF-1/Pit-1 (a pituitary-specific POU homeo-domain transcription factor), act synergistically with Ras-MAP kinase signaling in stimulating prolactin promoter activity in pituitary cells. However, Ets-2 has no synergistic effect on Ras activation of the promoter [19]. This difference is thought to be based on the lack of functional interactions between Ets-2 and GHF-1/Pit-1. Neural differentiation of rat pheochromocytoma PC12 cells is known to be induced by nerve growth factor (NGF) through the induction of the brain-specific isoform of Elk-1. Data suggest that the novel short isoform of Elk-1 (sElk-1) has a contrary role compared to the wild-type of Elk-1 in neuronal cell differentiation and proliferation [20].

ETS domain proteins such as Ets-1, Ets-2, Pea3, Er81, Pet-1, Elk-1 and GABPa are shown to interact with the control regions of the neural genes including the synapsin II [21], peripherin [22] and d-opioid receptor genes [23]. It was also shown that expression of the human presenilin gene, which is related to early onset of Alzheimer's disease, is regulated by Ets-1 and Ets-2 [24].

1.2. PEA3 SUBFAMILY

Members of the PEA3 subfamily appear to be involved in development of the central and peripheral nervous system. PEA3 is expressed in specific bundles of motor neurons that innervate limb muscles and appear in afferent sensory neurons of these same muscles [25].

PEA3-deficient male mice have been reported to be infertile probably due to mechanisms proximal to cavernosal smooth muscle or an ejaculatory dysfunction based on functional disorder of neuron connections [26]. Er81, is also expressed in distinct neuronal subsets of the developing spinal cord to control a late step in the establishment of functional sensory-motor neuron connections [27]. Erm (Ets related molecule), is expressed in satellite glia [28].

PEA3 subfamily of transcription factors also belong to ETS-domain transcription factor family. This subfamily has three well characterized, highly conserved proteins; Pea3, Erm and Er81. Although the genes of these proteins are located on different chromosomes (16), they all share a common architecture composed of 14 equivalently sized exons that encode similar sequences of the respective proteins. They have 95% identical DNA – binding (ETS - domain), 85% identical 32 – residue acidic domain (AD), at amino terminal part of, and 50% identical in the final 61 residues carboxyl – terminal tail domain (Ct) [25]. PEA3 subfamily members also appear to be activated by MAP kinases. In the case of PEA3, both ERK and JNK MAPK cascades stimulate PEA3-mediated transcriptional activation [29]. It is shown that stimulation of the ERK signaling pathway also significantly enhances ER81 and ERM-mediated transactivation [30]. As a whole, these findings indicate that all members of this subfamily are regulated by MAP kinases.

Ras is the upstream target of MAPK pathway; the activation of Ras by extracellular signals leads to the activation of MAPK. It is shown that the transactivation capacity of Pea3, ER81 and ERM was increased with activated Ras [30, 31]. It is demonstrated that the activation of SEK which stimulate JNK/SAPK, increases the Pea3 transactivation [32]. Although it is known that transactivation enhancement is controlled by their N-terminal

transcriptional activation domains of these transcription factors, it is currently unknown how the activity of members of the PEA3 subfamily is enhanced. Moreover, despite having potential MAPK sites, it is unknown whether they are direct targets of these pathways or not. ERM is shown to be activated by PKA, however this pathway does not appear to synergize with the ERK pathway [30].

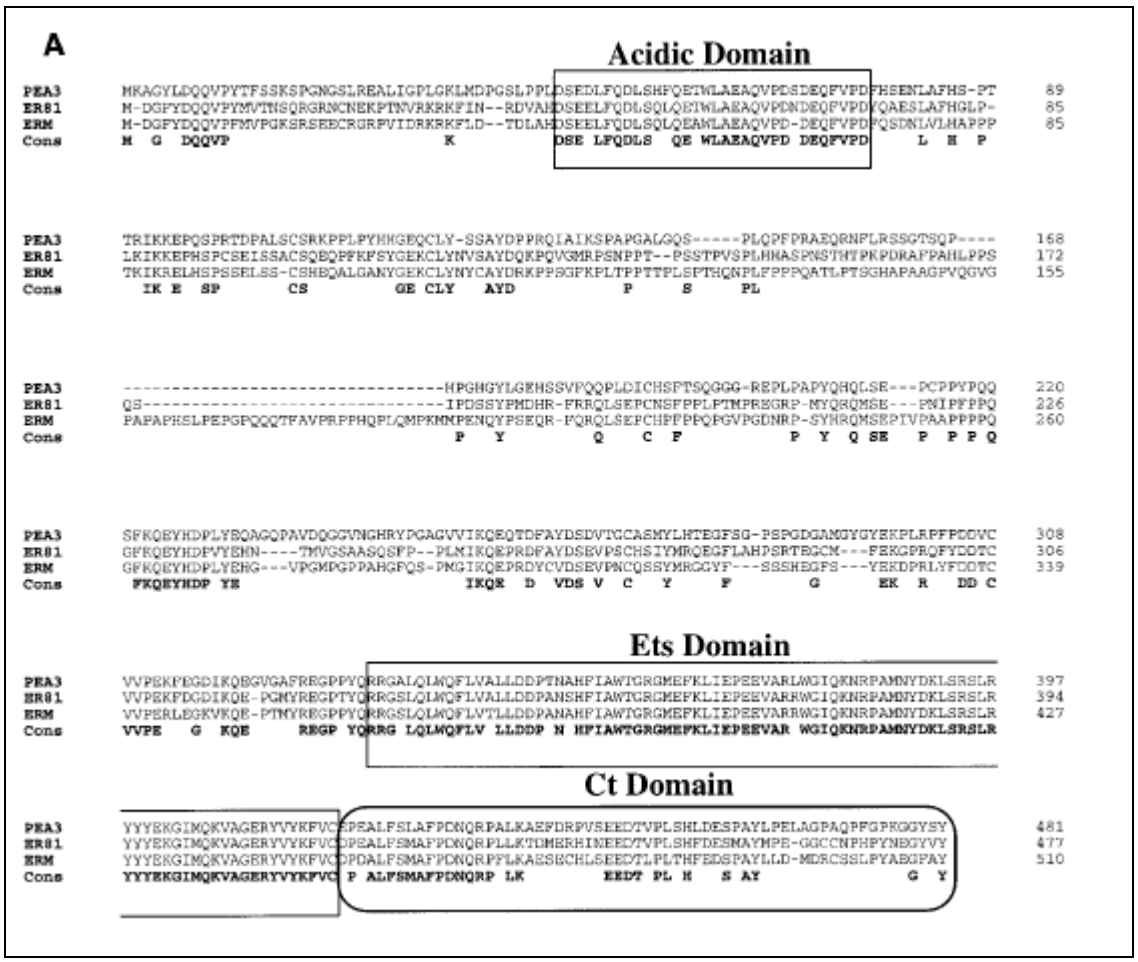

Figure 1.3. Sequence alignment of human Pea3 subfamily [5]

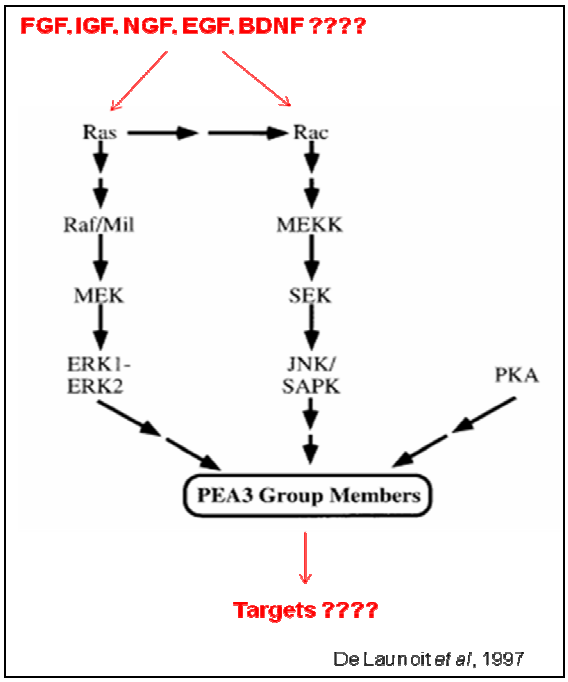

Figure 1.4. Model demonstrating the transactivation of Pea3 group members by different signal transduction pathways. The activation of Ras and SEK promote the increase in the expression of Pea3 group members. PKA stimulate the activation of ERM and ER81. This figure was adapted from Ref. 25

In zebrafish model Pae3 expression was observed during the early development; this suggest that Pea3 can play role in gaining of specific cell fates during the development of somites and the neuronal tube. It was also observed that Pea3 is expressed in the entire mesoderm during the gastrulation and also expressed in the paradaxial mesoderm [33]. The overexpression of Pea3 in metastatic mammary tumors [34], the expression of Pea3 in the neuronal crest cells which play a role in the formation of pharyngeal arches and also the regulatory role of Pea3 on metalloproteinase gene promoters are considered as proofs for the role of Pea3 in cell motility [35].

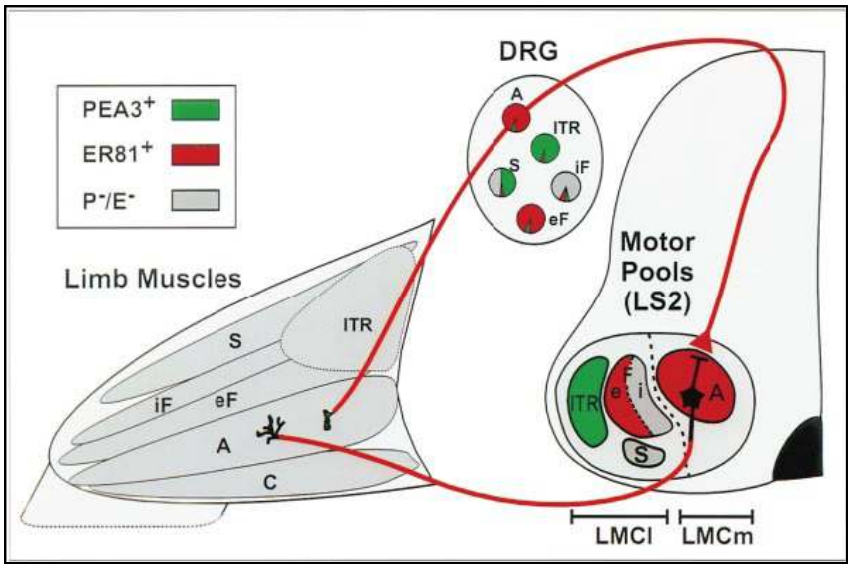

Figure 1.5. Motor neurons are functionally related, but represent a diverse collection of cells that show strict preferences for specific axon pathways during embryonic development. During development, motor neurons subtypes develop axons and extend them together to their specific targets. Although these motor neuron sets send axons together in the same columnar organization, each of these motor neuron pools find its own target muscle sensory afferents. This condition was shown to be related with Pea3 and Er81 expression in these neurons, in the sense that once Pea3 or Er81 expressing neuron subsets find their targets, it was shown that these target sensory affernt neurons do not overlap and there is always a precise match [37]

Pea3 expression was observed in the spinal motor neurons during the development of chick [36, 38]. In other study, it was demonstrated that Pea3 is responsible for the axonal branching and axonal migration [37]. Taken these data from the literature, it was aimed to mutate predicted MAPK - phosphorylation sites on Pea3, so that regulation mechanisms of Pea3 during neural differentiation of PC12 cells could be elucidated.

2. MATERIALS AND METHODS

2.1. PLASMIDS

Several commercially available empty plasmid vectors or cloned plasmid constructs were used in this study.

- pBlueScript KS (pBS-KS) II (+) empty plasmid
- pcDNA3.1 empty plasmid
- pCMV-3Tag-6 empty plasmid
- NFL-Luciferase reporter plasmid
- SRE-Luciferase reporter plasmid
- SMN-Luciferase reporter plasmid
- Pea3-Luciferase reporter plasmid
- Renilla Luciferase plasmid

2.2. PREPARATION OF MUTANT PLASMIDS

2.2.1. Primers and Optimization of Primer Annealing Temperatures

- Wild-type mPea3-pCDNA3 plasmid (100ng)
- 5X GoTaq buffer (Promega)
- GoTaq DNA polymerase (Promega)
- 25mM $MgCl_2$ (Promega)
- 10mM dNTP mix
- Reverse and forward mutant primers used are shown in Table 2.1
- dH_2O (autoclaved and aliquoted for use in PCR)
- Bio-Rad Thermocycler
- Agarose (Sigma)
- Ethidium Bromide (Promega)
- Bio-Rad ChemiDoc Imaging System

2.2.2. PCR Based Site - Directed Mutagenesis

Step 1:

- Wild-type mPea3-pCDNA3 plasmid (100ng)
- 10X Pfu buffer (Fermentas)
- Pfu DNA polymerase with Mg_2SO_4 (Fermentas)
- 10mM dNTP mix
- Reverse and forward mutant primers used are shown in Table 2.1 and 2.2
- Pea3 reverse and forward primers (Table 2.3)
- dH_2O (autoclaved and aliquoted for use in PCR)
- Bio-Rad Thermocycler
- Agarose (Sigma)
- Ethidium Bromide
- 6X loading dye
- Bio-Rad ChemiDoc Imaging System
- Promega Wizard SV Gel and PCR Clean Up Kit (Cat No. A9280)

Step2:

- 5X PrimeStar buffer (Takara)
- PrimeStar DNA polymerase (Takara)
- PrimeStar dNTP Mix (Takara)
- Short product from first step PCR reaction
- Long product from first step PCR reaction
- dH_2O (autoclaved and aliquoted for use in PCR)
- Bio-Rad Thermocycler
- Agarose (Sigma)
- Ethidium Bromide
- 6X loading dye
- Bio-Rad ChemiDoc Imaging System
- Promega Wizard SV Gel and PCR Clean Up Kit
- Nanospectrophotometer

Table 2.1. Primer couples designed to introduce Ser to Ala mutations in mPea3 amino acid sequence

mPea3 S22A Forward Primer	5’ - GCA GCA AAG CTC CCG GAA ATG - 3’
mPea3 S22A Reverse Primer	5’ - CAT TTC CGG GAG CTT TGC TGC -3’
mPea3 S90A Forward Primer	5’ - GCT TTC CAT GCC CCC ACC - 3’
mPea3 S90A Reverse Primer	5’ - GGT GGG GGC ATG GAA AGC - 3’
mPea3 S101A Forward Primer	5’ - CCC AGG CTC CCC GCA CAG -3’
mPea3 S101A Reverse Primer	5’ - CTG TGC GGG GAG CCT GGG -3’
mPea3 S134A Forward Primer	5’ - CCA TCA AGG CCC CCG CTC -3’
mPea3 S134A Reverse Primer	5’ - GAG CGG GGG CCT TGA TGG - 3’
mPea3 S143A Forward Primer	5’ - GGA CAG GCG CCC CTGC - 3’
mPea3 S143A Reverse Primer	5’ - GCA GGG GCG CCT GTCC - 3’
mPea3 S192A Forward Primer	5’ - CCT TCA CAG CTCCTC AGG - 3’
mPea3 S192A Reverse Primer	5’ - CCT GAG GAG CTG TGA AGG - 3’
mPea3 S285A Forward Primer	5’ - GGA CCC GCT CCA GGTG - 3’
mPea3 S285A Reverse Primer	5’ - CACC TGG AGC GGG TCC - 3’
mPea3 S458A Forward Primer	5’ - GAT GAG GCT CCT GCC TACC - 3’
mPea3 S458A Reverse Primer	5’ - GGTA GGC AGG AGC CTC ATC - 3’

Table 2.2. Primer couples designed to introduce Ser to Glu mutations in mPea3 amino acid sequence

mPea3 S22E Forward Primer	5’ - GCA GCA AAG AGC CCG GAA ATG - 3’
mPea3 S22E Reverse Primer	5’ - CAT TTC CGG GCT CTT TGC TGC -3’
mPea3 S90E Forward Primer	5’ - GCT TTC CAT GAA CCC ACC - 3’
mPea3 S90E Reverse Primer	5’ - GGT GGG TTC ATG GAA AGC - 3’
mPea3 S101E Forward Primer	5’ - CCC AGG AGC CCC GCA CAG -3’
mPea3 S101E Reverse Primer	5’ - CTG TGC GGG GTC CCT GGG -3’
mPea3 S134E Forward Primer	5’ - CCA TCA AGG GAA CCG CTC -3’
mPea3 S134E Reverse Primer	5’ - GAG CGG TTC CCT TGA TGG - 3’
mPea3 S143E Forward Primer	5’ - GGA CAG GAG CCC CTGC - 3’
mPea3 S143E Reverse Primer	5’ - GCA GGG GCT CCT GTCC - 3’
mPea3 S192E Forward Primer	5’ - CCT TCA CAG AGC CTC AGG - 3’
mPea3 S192E Reverse Primer	5’ - CCT GAG GCT CTG TGA AGG - 3’
mPea3 S285E Forward Primer	5’ - GGA CCC GAG CCA GGTG - 3’
mPea3 S285E Reverse Primer	5’ - CACC TGG CTC GGG TCC - 3’

Table 2.3. Primer couples designed to amplify mPea3 DNA sequence.

mPea3 Forward Primer	5' - AGACAAGCTTCCATGGAGCGGAGGATGAAAGGC - 3'
mPea3 Reverse Primer	5' - AGACCTCGAGCTAGTAAGAATATCCACCTCTGT - 3'
mPea3-pCMV-3Tag-6 Fw Primer	5' - AGACAAGCTTCATGGAGCGGAGGATGAAAGGC - 3'
mPea3-pCMV-3Tag-6 Rv Primer	5' - AGACCTCGAGCTAGTAAGAATATCCACCTCTGT - 3'

2.2.3. Mega Primer Based Site - Directed Mutagenesis

Step 1:

- Wild-type mPea3-pCDNA3 plasmid (100ng)
- 10X Pfu buffer (Fermentas)
- Pfu DNA polymerase with Mg_2SO_4 (Fermentas)
- 10mM dNTP mix (Fermentas)
- Reverse and forward mutant primers used are shown in Table 2.1 and 2.2
- mPea3 reverse and forward primers (Table 2.3)
- dH_2O (autoclaved and aliquoted for use in PCR)
- Bio-Rad Thermocycler
- Agarose (Sigma)
- Ethidium Bromide (Promega)
- 6X loading dye (Fermentas)
- Bio-Rad ChemiDoc
- Wizard SV Gel and PCR Clean Up Kit (Promega)

Step2:

- 5X PrimeStar buffer (Takara)
- PrimeStar DNA polymerase (Takara)
- PrimeStar dNTP Mix (Takara)
- Short product from first step PCR reaction
- Long product from first step PCR recation
- dH_2O (autoclaved and aliquoted for use in PCR)
- Bio-Rad Thermocycler

- Agarose (Sigma)
- Ethidium Bromide (Promega)
- 6X loading dye (Fermentas)
- Bio-Rad ChemiDoc Imaging System
- Wizard SV Gel and PCR Clean Up Kit (Promega)

2.2.4. Plasmid Based Site - Directed Mutagenesis

- Wild-type mPea3-pCDNA3 plasmid (100ng)
- Long PCR polymerase mix and 10X polymerase buffer (Roche)
- 10mM dNTP mix
- Reverse and forward mutant primers for this method are shown in Table 2.4
- dH_2O (autoclaved and aliquoted for use in PCR)
- Bio-Rad Thermocycler
- Agarose (Sigma)
- Ethidium Bromide (Promega)
- Bio-Rad ChemiDoc Imaging System
- Wizard SV Gel and PCR Clean Up Kit (Promega)

Table 2.4. Primer couples designed to introduce point mutations in mPea3 DNA sequence through plasmid based Site Directed Mutagenesis reaction. In this method, only forward primer has to have the mutation site. Red sequences indicate Ser to Ala mutation whereas blue sequences indicate Ser to Glu mutation.

mPea3 S22A Forward Primer	5' - CTACACCTTCTGCAGCAAA**GCT**CCCGGAAATG - 3'
mPea3 S22A Reverse Primer	5' - TTTGCTGCAGAAGGTGTAGGGCACTCGCTG - 3'
mPea3 S22E Forward Primer	5' - CTACACCTTCTGCAGCAAA**GAG**CCCGGAAATG - 3'
mPea3 S192A Forward Primer	5' - GATGTGCCACTCCTTCACA**GCT**CCTCAGGGAG - 3'
mPea3 S192A Reverse Primer	5' - TGTGAAGGAGTGGCACATCATGTCCACGG - 3'
mPea3 S192E Forward Primer	5' - GATGTGCCACTCCTTCACA**GAG**CCTCAGGGAG - 3'
mPea3 S285A Forward Primer	5' - AGAGGGCTTCTCTGG ACCC**GCT**CCAGGTGATG - 3'
mPea3 S285A Reverse Primer	5' - GGGTCCAGAGAAGCCCTCTGGTGGAGGTAC - 3'
mPea3 S285E Forward Primer	5' - AGAGGGCTTCTCTGGACCC**GAG**CCAGGTGA TG - 3'

2.2.5. Digestion of Second Step PCR Products and pBSKS Empty Vector

- Second step PCR products purified from previous steps
- pBSKS II (+) empty vector (Clontech)
- XhoI and HindIII Fast Digest restriction enzymes and 10X buffers (Fermentas)
- RNase free water
- Promega Wizard SV Gel and PCR Clean Up Kit

2.2.6. Ligation of Second Step PCR Pproducts and pBSKS Empty Vector

- Second step PCR products purified from previous steps
- pBS-KS II (+) empty vector (Clontech)
- T4 DNA Ligase and 10X Ligase buffers (Invitrogen)
- RNase Free Water
- Wizard SV Gel and PCR Clean Up Kit (Promega)

2.2.7. Blue - White Screening

- *E.coli* JM109 strain (Promega)
- LB broth and agar medium (Applichem)
- $CaCl_2$ solution (Applichem)
- Amphicillin (Applichem)
- X-Gal (Applichem)
- IPTG (Applichem)

2.2.8. Colony PCR

- GoTaq DNA polymerase and 5X GoTaq buffer (Promega)
- 25mM $MgCl_2$ (Promega)
- 10mM dNTP mix (Fermentas)
- Pea3 reverse and forward primers
- dH_2O (autoclaved and aliquoted for use in PCR)

- Bio-Rad Thermocycler
- Agarose (Sigma)
- Ethidium Bromide (Promega)
- Bio-Rad ChemiDoc Imaging System
- High Pure Plasmid Isolation Kit (Roche)

2.2.9. Cloning Mutant mPea3 Sequences of Positive Colonies into Empty Plasmid Vectors

- Plasmids from positive colonies
- pcDNA3 (+) (Invitrogen) or pCMV-3Tag-6 (Stratagene) empty vectors
- XhoI and HindIII Fast Digest restriction enzymes and 10X buffers (Fermentas)
- RNase Free Water
- Wizard SV Gel and PCR Clean Up Kit (Promega)
- T4 DNA Ligase and 10X Ligation Buffer (Invitrogen)

2.3. CELL CULTURE

2.3.1. Cell Lines and Transfection

- Pheochromacytoma (PC12),
- Neuroblastoma (SH-SY5Y),
- Human Embryonic Kidney (HEK293) Cell lines
- Dulbecco's Modified Eagle Medium(DMEM) supplemented with 1g or 4.5g/l glucose (Gibco)
- L-Glutamine (Gibco)
- Fetal Bovine Serum (Gibco)
- Penicillin/Streptomycin solution (1X) (Gibco)
- D-PBS (Gibco)
- 0.5% Trypsin/EDTA solution (Gibco)
- Tissue culture grade flasks (Nunc)
- 24-well culture plates (Nunc)

- TransFast transfection reagent (Promega)
- mPea3 wild type and mutants cloned in pCDNA3 plasmid vector

2.4. PROTEIN ASSAYS AND LOCALIZATION

2.4.1. SDS Page and Western Blot

- Whole protein lysates from HEK293 cells transfected with mPea3 wild type or mutants or non-transfected cell lysates to serve as negative controls
- Bradford Reagent (Sigma)
- 30%/0.8% Acrylamide/Bis Acrylamid Solution
- 1M Tris-Cl Buffer at pH 6.8 and 8.9
- 10% SDS
- 10% APS
- Temed
- 6X Protein Loading Dye
- 5X SDS Running Buffer (15g Tris, 72g Glycine, 5g SDS)
- PVDF membrane (Roche)
- 1X TBS and 1X TBS-T
- Blocking Buffer (5% skimmed milk in TBS)
- Antibodies used are: mouse anti-Pea3 monoclonal (Santa Cruz), goat anti-mouse secondary (Pierce)
- ECL Western Blotting Substrate Kit (Pierce)
- Bio-Rad ChemiDoc Imaging System

2.4.2. Immunofluorescence

- PC12, SH-SY5Y and HEK293 cells transfected with mPea3 wild type or mutants
- 1X PBS
- 4% Paraformaldehyde solution
- Blocking solution (10% FBS in PBS)
- 0.1% Triton-X in 1% FBS / PBS

- Antibodies used are: mouse anti-Pea3 monoclonal (Santa Cruz), Alexa Fluor 488 goat anti-mouse IgG (Invitrogen)
- Propidium Iodide
- Mowiol mounting reagent
- Leica laser confocal microscope

2.4.3. Luciferase Assay

- PC12, SH-SY5Y, HEK293 cell lines
- Neurofilament Light Chain (NF-L), Pea3, SRF Promoter luciferase plasmids
- Renilla luciferase plasmid (Promega)
- TransFast transfection reagent (Promega)
- pcDNA3 empty plasmid and mPea3 wild type and mutant plasmids
- Dual-Lucifease Reporter Assay System (Promega)

2.4.4. GST Pull Down Assay

2.4.4.1. PCR Amplification of mPea3 Mutants

- Wild-type mPea3-pCDNA3 plasmid (100ng)
- Pfu DNA polymerase and 10X Pfu polymerase buffer (Fermentas)
- 10mM dNTP mix (Fermentas)
- Reverse and forward mutant primers used are shown in Table 2.5
- dH_2O (autoclaved and aliquoted for use in PCR)
- Bio-Rad Thermocycler
- Agarose (Sigma)
- Ethidium Bromide (Promega)
- 6X loading dye (Fermentas)
- Bio-Rad ChemiDoc Imaging System
- Wizard SV Gel and PCR Clean Up Kit (Promega)

Table 2.5. Primer couples designed to clone mPea3 into pGEX-3X plasmid vector. Red sequence indicates the BamHI recognition site, whereas blue sequence indicate EcoRI recognition site here.

mPea3-pGEX-3X Fw Primer	5' - AGAC **GGATCC** TGG AGC GGA GGA TGA AAG GC - 3'
mPea3-pGEX-3X Rv Primer	5' - AGAC **GAATTC** CTA GTA AGA ATA TCC ACC TC - 3'

2.4.4.2. Digestion and Ligation of PCR Amplicons and pGEX-3X Empty Vector

- PCR products purified from the previous steps
- pGEX-3X empty vector (GE Life Sciences)
- EcoRI and BamHI Fast Digest restriction enzymes and 10X buffers (Fermentas)
- RNase free water
- Wizard SV Gel and PCR Clean Up Kit (Promega)
- PCR products purified from the previous steps
- T4 DNA Ligase and 10X Ligase buffers (Invitrogen)
- RNase Free Water
- Wizard SV Gel and PCR Clean Up Kit (Promega)

3. METHODS

3.1. PREPARATION OF MUTANT PLASMIDS

3.1.1. Optimization of Annealing Temperatures of Primer Couples

PCR reaction:

- mPea3-pCDNA3 wild type (100ng) 0.5µL
- 5X Taq buffer 10µL
- GoTaq polymerase 0.5µL
- $MgCl_2$ 8µL
- dNTP 1µL
- Mutant forward primer 0.5µL
- Mutant reverse primer 0.5µL
- dH_2O 24.5µL
- Final Volume 50µL

Gradient PCR was performed from 50C to 60C, 15µL from the PCR mixtures were run in 1.5% agarose gels and visualized with Bio-Rad ChemiDoc system in order to determine the optimum annealing temperature.

3.1.2. PCR Based Site-Directed Mutagenesis (SDM)

3.1.2.1. First Step SDM PCR

First step PCR Program was prepared as follows;

- mPea3 plasmid (100ng) 0.5µL
- 10X Pfu buffer (Promega) 5µL
- Pfu DNA polymerase (Promega) 0.5µL
- 10 mM dNTP mix 1µL
- Mutant reverse/ forward primers 0.6µL
- Pea3 forward/reverse primers 0.6µL

- dH_2O 41.8µL
- Final Volume 50µL

First Step PCR was performed at annealing temperature 56^0C; PCR products were run in 1.5% agarose gel and gel picture was taken with Bio – Rad ChemiDoc. PCR products were cut from the gel under the UV. Site – Directed SDM first step products were purified with Promega Wizard SV Gel and PCR Clean up. In this step, two products were obtained carrying the mutation; one small and one long product. The concentration of small and long products was calculated with Nanospectrophotometer.

3.1.2.2. Second Step SDM PCR

Second step PCR reaction contained two reactions. First mixture was prepared as follows;

- 10X PrimeStar buffer (Takara) 5µL
- PrimeStar DNA polymerase (Takara) 0.5µL
- PrimeStar (Takara) dNTP 4µL
- Short product from first step PCR reaction 1µL
- Long product from first step PCR reaction 6µL
- dH_2O 32.5µL
- Final Volume 50µL

Second mixture was prepared as follows;

- 10X PrimeStar buffer (Takara) 4µL
- PrimeStar DNA polymerase (Takara) 0.2µL
- PrimeStar (Takara) dNTP 1.6µL
- Pea3 forward primer 0.5µL
- Pea3 reverse primer 0.5µL
- dH_2O 13. 2µL
- Final Volume 20µL

Second Step PCR was performed with first PCR mixture at annealing temperature 46°C for 6 cycles. PCR was stopped and then, second mixture was added and PCR program was continued at annealing temperature 56°C.

Steps two - four were repeated for 4 times and steps five - seven were repeated for 30 times. Firstly, PCR program was run with the first PCR reaction to permit the annealing of the two products obtained from the first step PCR reaction. After the 4 step of second step PCR program, the second mixture was added to tubes and the rest of the program was done.

PCR products were run in 1.5% agarose gel and gel picture was taken with Bio – Rad ChemiDoc and PCR products was cut from the gel under the UV. Site – Directed Mutagenesis (SDM) first step products were purified with Promega Wizard SV Gel and PCR Clean up.

3.1.3. Digestion of Second Step Pproducts and pBS-KS Vector

Second step products and pBS-KS vector was digested with restriction enzymes to obtain sticky ends on both sides.

3.1.3.1. Double Digestion with XhoI and HindIII Restriction Enzymes

1. Second step PCR products (insert)/Empty pBSKS plasmid vector 10µL
2. XhoI restriction enzyme 0.5µL
3. HindIII restriction enzyme 0.5µL
4. 10X restriction enzyme buffer 2µL
5. RNase Free Water 7µL

The mixture was incubated for 1 hour at 37°C and digested DNA sequences were purified with Promega Wizard SV Gel and PCR Clean up.

3.1.4. Ligation of second step PCR products and pBSKS plasmid vector

Concentrations of double digested SDM products and pBSKS empty vectors were measured with Nanospectrophotometer. According to these measurements, the amounts of insert (SDM double digested products) and vector (pBSKS here) to be added into the ligation reaction were calculated. Ligation mixture was prepared with 0.5U T4 DNA ligase and 10X buffer in a 10µL reaction.

3.1.5. Blue – White Screening

Blue - white screening was important in choosing the positive colonies containing the plasmid we desire.

3.1.5.1. Competent Cell Preparation

1. JM109 cells were inoculated in 10mL LB broth and were incubated for overnight at 37°C shaker incubator.
2. On the following day, 500µL of the inoculum was transferred into 10mL fresh LB broth and was incubated for another 3hours at the 37°C shaker incubator.
3. Upon the end of incubation period, cells were centrifuged and resuspended in 10mL ice-cold $CaCl_2$ solution and incubated for 1 hour on ice.
4. After 1 hour incubation on ice, cells were pelleted and resuspended in 10mL of ice cold $CaCl_2$.
5. LB agar medium was prepared according to the manufacturer's orders.
6. 40µL of Amphicillin, 40µL of X-Gal and 100µL IPTG solutions were spreaded on LB agar poured plates beforehand.

3.1.5.2. Transformation

1. 100µL competent cells and all ligation mixtures prepared for each mutation were mixed in 1mL eppendorf tubes separately.
2. The tubes were incubated on ice for 10 minutes, then at 37°C for 1 minute and again on ice for 1 minute.
3. 600µL LB broth was immediately added to tubes and mixtures were incubated at 37°C for 1 hour.

4. After 1 hour incubation, tubes were centrifuged and 500µL from the supernatant were aspirated.
5. The pellets were resuspended in the remaining supernatant solution.
6. The obtained mixtures were separately spread on agar plates containing amphicillin, X- gal and IPTG.
7. Plates were incubated overnight at 37°C.
8. On the following day, agar plates were checked for positive white colonies.

3.1.6. Colony PCR

PCR reaction was prepared as follows;

- White colonies that are carefully picked from LB agar plates.
- 5X GoTaq buffer 10µL
- GoTaq DNA polymerase 0.5µL
- 25mM $MgCl_2$ 8µL
- 10mM dNTP mix 1µL
- Mutant forward primer 0.8µL
- Mutant reverse primer 0.8µL
- dH_2O 23,9µL
- Final Volume 50µL

PCR reaction was carried out at an annealing temperature of 56°C. PCR amplicons were run in 1.5% agarose gel and visualized with Bio-Rad ChemiDoc Imaging System. Bacterial colonies that gave positive results were picked to further use in cloning experiments. Plasmid isolation was carried out for these colonies with the help of Roche's High Pure Plasmid Isolation Kit and obtained plasmids were further confirmed with digestion and DNA sequencing.

3.1.7. Cloning Mutant mPea3 Sequences of Positive Colonies into Empty Plasmid Vectors

Cloning mutant mPea3 sequences from positive colonies into empty pcDNA3.1 vector was performed after digesting both the insert DNA sequence and empty vector with same enzymes and thorough purification of both.

3.1.7.1. Double Digestion of Plasmids from Positive Colonies and Empty Plasmid Vectors

1. Plasmids isolated from positive colonies (insert) / empty plasmid vectors 10µL
2. XhoI restriction enzyme 0.5µL
3. HindIII restriction enzyme 0.5µL
4. 10X restriction buffer 2µL
5. RNase Free Water 7µL

The mixture was incubated for 1 hour at 37°C and digested DNA sequences were purified with Promega's Wizard SV Gel and PCR Clean Up Kit.

3.1.7.2. Ligation of Plasmids from Positive Colonies and Empty Plasmid Vectors

Concentrations of double digested plasmid from positive colonies and pCDNA3 vector were calculated with Nanospectrophotometer. According to these measurements, the amounts of insert (SDM double digested products) and vector (pCDNA3 here) to be added into the ligation reaction were calculated. Ligation mixture was prepared with 0.5U T4 DNA ligase and 10X buffer in a 10µL reaction.

3.1.7.3. Transformation to Screen Ligation of Plasmid from Positive Colonies and pCDNA3

Preparation of competent cells and transformation protocols were the same as 3.1.5 blue-white screening protocols. The only difference is, transformation mixtures were spread on LB agar plates containing amphicillin but not containing X-Gal or IPTG. Plates were incubated overnight at 37°C. On the following day, plates were visually inspected for the presence of bacterial colonies. Colony PCR amplification was the first confirmation

method of choice and it was followed by plasmid isolation and confirmations with restriction enzyme reactions.

3.2. CELL CULTURE

3.2.1. Transfection

1. $5x10^4$ cells/well PC12 cells were split on 24-well plates.
2. Transfection mediums were prepared in 400µL serum free DMEM medium by adding 200ng pcDNA3, 400ng Pea3 Luciferase or 400ng NFL Luciferase or 400ng SRE Luciferase or 400ng SMN Luciferase, 400ng Renilla Luciferase, 200ng mPea3 or one of the mutants.
3. 3µL of TransFast was added to each transfection medium and immediately vortexed. Transfection mixtures were incubated for 15 minutes at room temperature.
4. On the other hand, growth medium of cells on 24-well plates were aspirated and transfection mixtures were added gently.
5. The transfection medium was discarded after 1 hour incubation and 1mL of fresh growth medium was added to each well.
6. Cells were incubated for 48 hours.

3.3. PROTEIN AND LOCALIZATION

3.3.1. SDS Page and Western Blot

3.3.1.1. Preparation of Cell Lysates

1. Cells were transfected according to the same procedure in 3.2.1. Transfection and incubated for 48 hours.
2. All cells were collected at the end of this period and washed with PBS.
3. Then they were lysed with 100 µl lysis buffer on ice for 10 min and centrifuged at the highest speed for 10 min at 4°C; pellet was discarded and supernatant was collected.
4. Protein concentrations of each whole protein lysate were calculated with Bradford assay.
5. 40µg whole protein lysate per well was calculated and loaded to SDS-PAGE.

3.3.1.2. Preparation of SDS-PAGE

1. Resolving gel: 24 ml of a 9% gel
   5.4 ml 30% acrylamide/bis-acrylamide
   3 ml Tris-Cl, pH 6.8
   15.6 ml water
   12 µl Temed
   60 µl 10% APS
2. Stacking gel: 8 ml of a 5% gel
   1 ml 30% acrylamide/bis-acrylamide
   2 ml Tris-Cl, pH 8.9
   5 ml water
   8 µl Temed
   21.6 µl 10% APS

After the polyacrylamide gel was solidified, protein mixtures were loaded and the gel was run around 3 hours with constant current (at a voltage of 300V). At the end of 3 hours, gel cast was splitted and gel was removed from its glass holders and washed with distilled water for two times. Transfer of proteins in the gel to PVDF membrane was carried out with Bio-Rad Semi Dry Blotting Device at 20V for 2 hours. Successful transfers were visualized with Ponceau S (Applichem) solution and remaining proteins on the gel were stained with Comassie Blue solution. PVDF membrane then washed 2 times with 1X TBS solution and blocked with 5% skimmed milk in TBS. Primary antibody was diluted in 1X TBS and incubated overnight at 4°C with gentle agitation. Secondary antibody was incubated 1 hour at room temperature. Membrane was developed using Pierce's ECL Western Blotting Substrate Kit and visualized with Bio-Rad Chemidoc Chemiluminescent Imaging System.

3.3.2. Immunofluorescence

1. Cells were splitted onto cover slips the day before transfection and transfection was carried out as stated in Section 3.2.1 Transfection.

2. After 48 hours, growth medium was aspirated from the wells and cells were washed once with 1X PBS and fixed with 4% paraformaldehyde in PBS for 25 min at room temperature.
3. Cells were rinsed with 1X PBS, then permeabilized with 0.1% Triton X-100 in 1% FBS in PBS solution for 10 min at room temperature.
4. Cells were then blocked with blocking solution (10% FBS in PBS) for 1 hour at room temperature.
5. After blocking, cells were incubated with mouse anti-Pea3 monoclonal antibody in blocking solution for 1 hour at room temperature.
6. Cells were washed with 1X PBS 3 times for 10 min, then incubated for 1 hour with secondary antibody (Alexa Fluor 488 goat anti-mouse antibody) in blocking solution.
7. Cells were washed with 1X PBS 3 times for 10 min, then incubated with PI in PBS for 5 min.
8. Cover slips were mounted by using Mowiol and the borders were sealed with clear nail polish.
9. Cover slips were left to dry overnight at room temperature and studied with Leica laser confocal microscope.

3.3.3. Luciferase Assay

1. Complete growth medium was discarded from each well.
2. Cells were lysed with 100µL Passive Lysis Buffer (1X) per well.
3. 50µL from the lysed cells were transferred into a white 96-well plate.
4. 50µL of Luciferase Assay Reagent II substrate was added and luminescence was measured immediately with Luminoscan Ascent.
5. After the measurements were recorded, 50µL of Stop-Glo reagent was added and luminescence was measured immediately with Luminoscan Ascent.
6. All of the luciferase assays were carried out in triplicates.
7. The averages of measurements, ratios of Firefly / Renilla measurements, and standard deviations were calculated with Microsoft EXCEL program and the data were graphed.

3.3.4. GST Pull Down Assay

For GST Pull Down Assays, the following PCR mixture was prepared:

- mPea3-pCDNA3 wild type (100ng) 0.5µL
- 10X Long PCR polymerase buffer 5µL
- Long PCR polymerase mixture 0.5µL
- 10mM dNTP mix 1µL
- Pea3-pGEX-3X forward primer 0.8µL
- Pea3-pGEX-3X reverse primer 0.8µL
- dH_2O 41.4µL
- Final Volume 50µL

PCR reaction was carried out at an annealing temperature of 56°C. PCR amplicons were run in 1.5% agarose gel and visualized with Bio-Rad ChemiDoc Imaging System. Successful amplicons were purified from agarose gel and double digested with BamHI and EcoRI restriction enzymes. The resulting DNA sequences were further purified to be able to use in ligation reaction. On the other hand, empty pGEX-3X plasmids were also digested with BamHI and EcoRI enzymes and purified. Ligation and transformation reactions were carried out as stated in Section 3.1.5. for Blue-white screening.

4. RESULTS

4.1. PEA3 SUBFAMILY ACTIVITY IN PC12 CELLS

4.1.1. Effects of Growth Factor Induction of Pea3

In a previous study, overexpression of wild-type mouse Pea3 in PC12 cells, together with an induction with Epidermal Growth Factor (EGF) was shown to induce axon-like neurite extensions in these cells (*Kurnaz A I, Sharrocks A; unpublished data*). To be able to determine the downstream pathway(s) of this effect, one should inhibit several signaling cascades by inhibiting different protein kinases. In this context, we used a Mitogen Activated Protein Kinase 1 and 2 (MEK 1 and 2) inhibitor, UO126; an inactive MEK1 and MEK2 inhibitor, PD098059; a specific p38-MAPK pathway inhibitor, SB203580 and a PI3K inhibitor, LY294002.

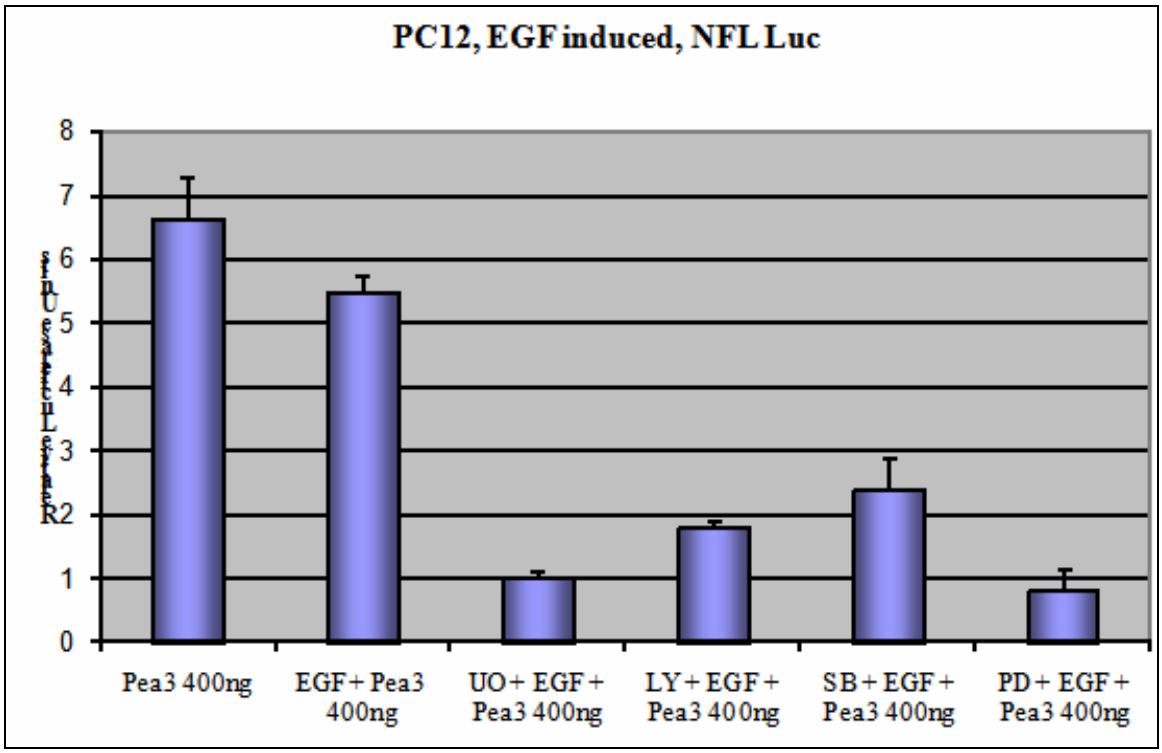

Figure 4.1. PC12 cells were transfected with wild-type mouse Pea3, 24 hours later, specific inhibitors were introduced to culture media and only then, PC12 cells were treated with EGF for 2 days. NFL-Luc results indicate a decrease in the reporter activity of NFL-Luc in the presence of either inhibitor. This result suggested a more complex regulation of Pea3 than it was predicted earlier and urged us to elucidate the phosphorylation motifs of Pea3 and also the upstream signaling cascades responsible for the regulation of each motif

In addition to EGF induction studies, we observed neurite-like extensions of PC12 cells when induced with Insulin-like Growth Factor I (IGF-1).

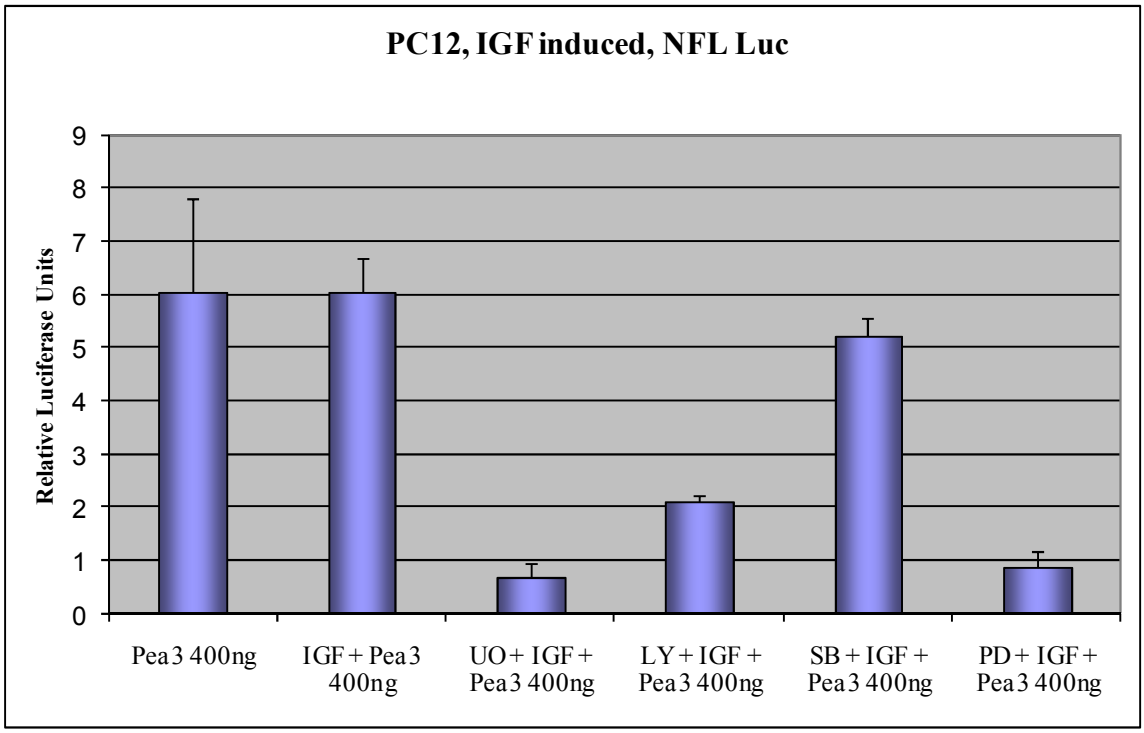

Figure 4.2. PC12 cells were transfected with wild-type mouse Pea3, 24 hours later, specific inhibitors were introduced to culture media and only then, PC12 cells were treated with IGF-1 for 2 days. NFL-Luc results indicate a decrease in the reporter activity of NFL-Luc in the presence of UO126, LY294002 and PD098059 inhibitors. A decrease obtained with SB203580 is negligible. This result suggested us to primarily focus on MAPK phosphorylation motifs on Pea3, however PI3K phosphorylation motifs were also predicted, for further studies

4.1.2. Pea3 Subfamily Activity in PC12 Cells

Pea3 subfamily of transcription factors represent relatively high homology, to be able to conclude Pea3-specific activity in PC12 cells, we also performed studies with mouse Erm and Er81 proteins. In order to address the roles of Erm and Er81, we used a conditional chimera proteins Erm.ER and Er81.ER (from de Launoit). These proteins contain a widely-used ligand binding domain of Oestrogen Receptor (ER) which was fused to C-terminals of Erm or Er81. These chimera proteins were used to determine whether Erm and/or Er81 transactivate NFL promoter by binding to the Pea3 binding sites. Chimera proteins that were fused to ER, display a ligand (β-oestradiol) regulated activity.

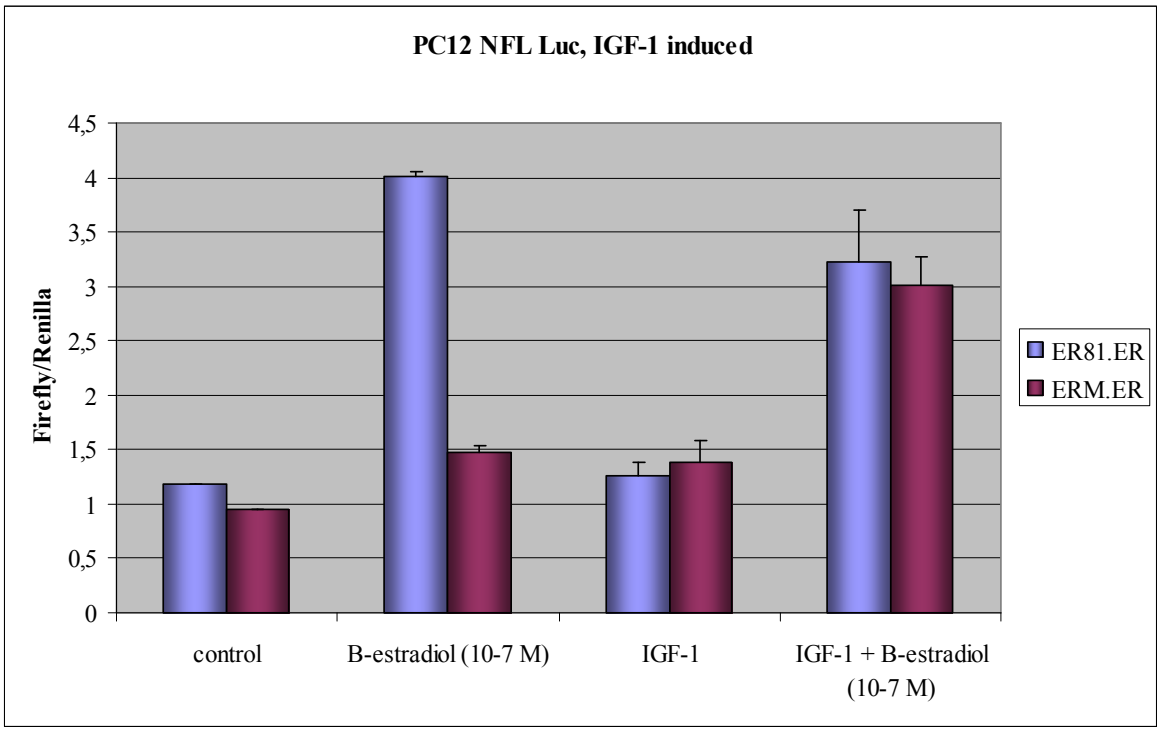

Figure 4.3. PC12 cells were transfected with either Er81.ER or Erm.ER as a control and next, treated with β-oestradiol. Hormonal induction of PC12 cells represented an increase in the Er81.ER mediated transactivation of NFL promoter. It was also shown that IGF-1 induction solely does not have an effect on the transcriptional activity, however when given with β-oestradiol, IGF-1 could boosted up the activity of Erm.ER on NFL promoter.

4.2. PREPARATION OF MUTANT PLASMIDS

In order to investigate which growth factor phosphorylates Pea3 on which residue, one first needs to identify the functional phosphorylation sites, and then carry out the kinase assays and protein-kinase interaction assays. To that end, we have started out with generating phosphor-mutants and phosphor-mimics for all possible MAPK phosphorylation motifs for mouse Pea3.

4.2.1. Plasmid Based Site-Directed Mutagenesis of Pea3 Protein

MAP kinase phosphorylation motif is known to be Ser/Tyr-Pro, therefore, firstly we have identified all possible phosphorylation sites on the amino acid sequence of mPea3,

and in order to investigate which of these potential sites are indeed phosphorylated by MAPK, silencing and mimicking mutations were performed.

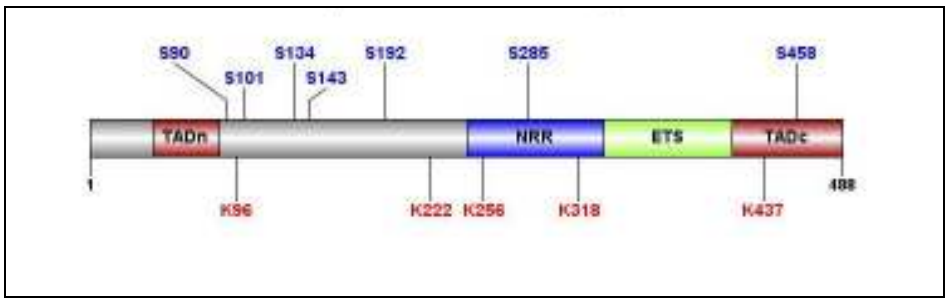

Figure 4.4. Predicted phosphorylation sites of mouse Pea3. Transactivation domain at the N terminal (TADn) and at the C terminal (TADc), negative regulation region (NRR) and ETS domain are presented in this figure

Primers designed to convert Serine to Alanine and Serine to Glutamic Acid residue to introduce mutations to the Ser/Pro motifs of Pea3 were shown in Table 2.1 and Table 2.2, respectively. Primers were designed such that the mutation points are always located in the middle of the primer.

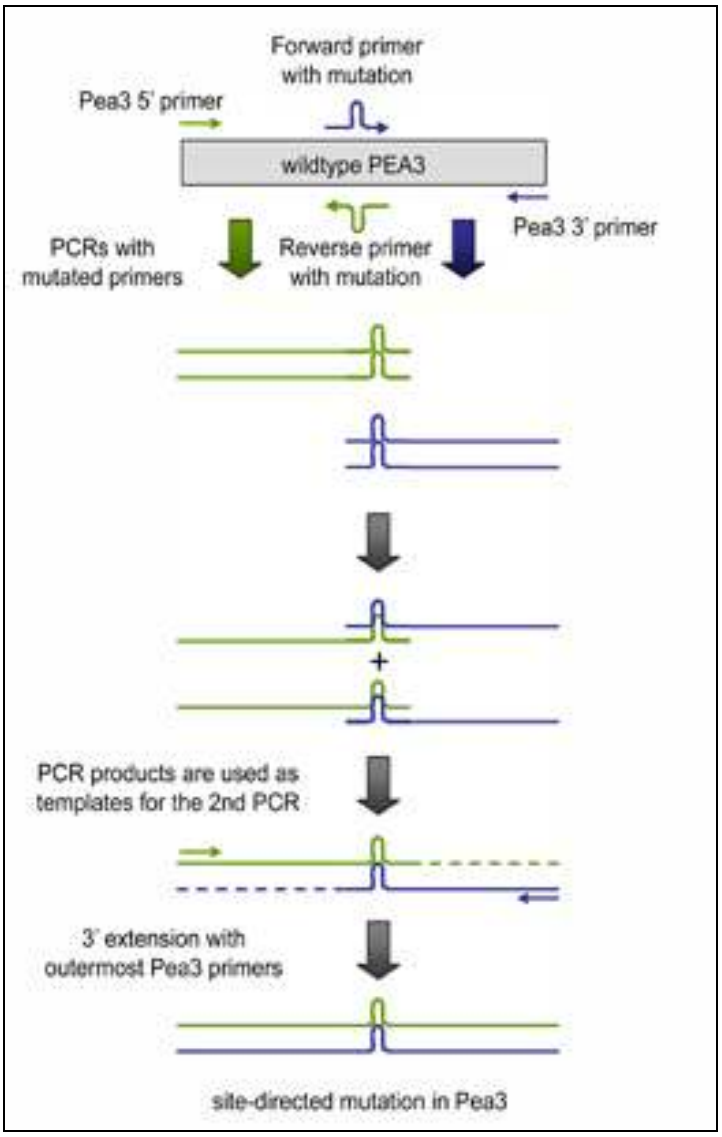

Figure 4.5. PCR based Site – Directed Methodology is shown in this figure. The method has two steps. In the first step, two PCR products are obtained by both Pea3 5' and mutant 3' primer couple and mutant 5' and Pea3 3' primer couple. In the second step, these two products were united in a separate tube and PCR reaction was carried out at a low annealing temperature so that the products from the first step PCR can act as primers over each other to produce full size Pea3. Last of all, Pea3 3' and 5' primers are added to the reaction to amplify the full size Pea3

The PCR based Site-Directed Methodology was chosen as the main method and a brief summary about this method is given in the Figure 4.2 above. This method has two separate PCR steps. At the first step PCR, wild-type mouse Pea3 sequence was amplified

with mPea3 forward primer plus mutant reverse primer and with Pea3 reverse primer and mutant forward primer in two separate PCR tubes. At the end of this PCR step, for each single mutation, there are two PCR amplicons obtained; one is from the 5’ end to mutation site and the other is from 3’ end to mutation site.

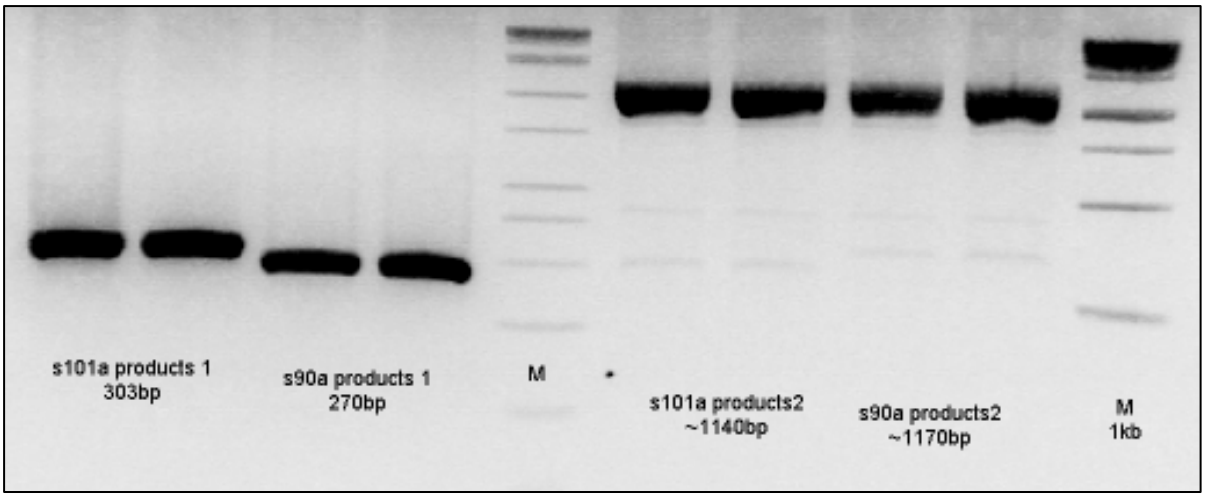

Figure 4.6. The PCR products of the first step PCR for Ser101Ala and Ser90Ala mutations. First two lines of the picture shows the small product obtained for Ser101Ala with Pea3 5’ primer and Ser101Ala 3’ primer. The third and forth lanes shows the single band at around 270 bp for Ser90Ala short product. Sixth and seventh lines are for long product obtained for Ser101Ala with Pea3 3’ primer and Ser101Ala 5’ primer. The remaining two lines are for long product obtained for Ser90Ala with Pea3 3’ primer and Ser90Ala 5’ primer. These bands were removed from the gel, purified and used in the second step PCR

The first step PCR products were purified from the agarose gel and combined in a new PCR tube to use in the second step PCR. The annealing temperature was adjusted as 42°C to allow them to denature and re-anneal over each other. After 4 cycles with this annealing temperature, the program was stopped, another mixture of second step PCR was added and the program was continued. The second mixture contained Pea3 primers in order to fully amplify the Pea3 sequence plus the desired mutation.

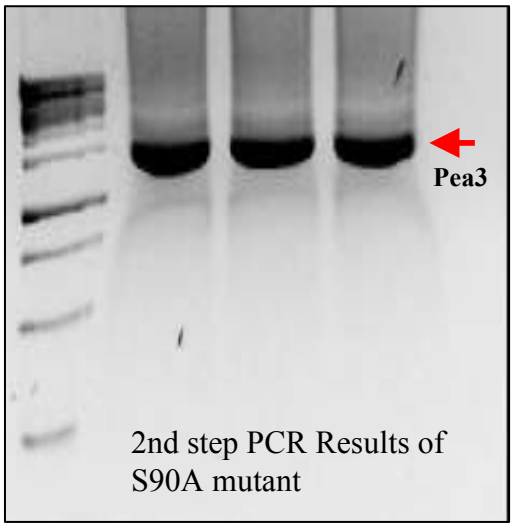

Figure 4.7. Second step of Site – Directed Mutagenesis PCR results for Ser90Ala mutant is shown (Red arrow indicate 1500bp). PCR products from the first step PCR were isolated, purified and used in the second step PCR, using the methodology shown in Figure 4.2

4.2.2. Cloning of the Mutants

Mutated Pea3 products were cloned in p**B**lue**S**cript-**KS** (pBS-KS) II (+) vector for DNA sequencing. For this, both mutated products and pBS-KS vector were double digested with Hind III and XhoI.

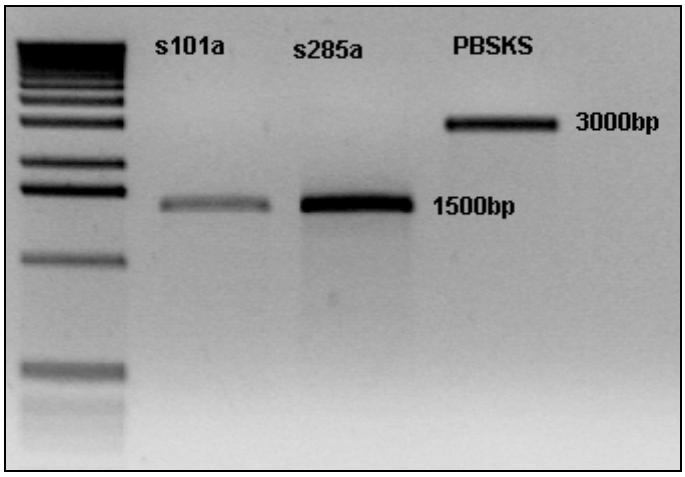

Figure 4.8. Double digestion products are shown. The primers designed as outmost primers contain restriction enzyme cut sites so that they can be cloned into the vector of choice, hence pBS-KS in this case. As a result, products from the second step PCR required a digestion reaction with two separate enzymes. Products from the digestion reaction were purified and run on an agarose gel before proceeding with the ligation reactions. Empty pBS-KS vector was also cut with these same enzymes and run on the same gel to confirm that the reaction was completed

The concentration of double digested pBS-KS plasmid vector and Pea3 mutants were calculated with Nanospectrophotometer. Then, according to their base and concentration ratio, the amount needed from pBS-KS plasmid vector and mutant Pea3 products were calculated and the ligation reaction was done to ligate mutated products with pBS-KS. After the ligation process Blue – White screening was done to select the colonies which have pBS-KS with its insert in it. pBS-KS plasmid vector encodes the alpha subunit of LacZ protein and its multiple cloning site is located in this gene sequence. A suitable *E.coli* strain which contains the remaining subunit to be able to form the functional Beta-galactosidase enzyme was chosen. In the case when the digestion products are successfully inserted in the multiple cloning site of the vector, production of Beta-galactosidase enzyme is disrupted and bacteria are unable to digest X-gal substrate that was spread on the agar plates and white colonies are obtained. However, in the case of no insertion, there would be no disruption of LacZ and bacteria could easily digest X-gal causing bacteria to colored blue. In this context, only white colonies are chosen to use in colony PCR method.

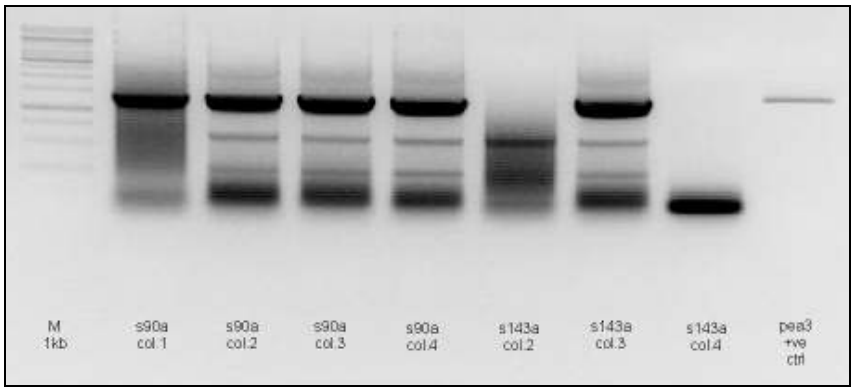

Figure 4.9. Colony PCR results for Ser90Ala mutant. Colony PCR method involves a PCR reaction carried out with Pea3 5' and 3' primers with bacterial colonies taken directly from the agar plates. This method uses a relatively long initial denaturation step in order to burst the bacteria and use the plasmids as a template. In this gel picture, 1st, 2nd, 3rd, 4th and 6th wells indicate positive results for colony PCR and the remaining 5th and 7th wells indicate false positive white colonies. These false positives might result from satellite colonies that are in close proximity to real positive colonies

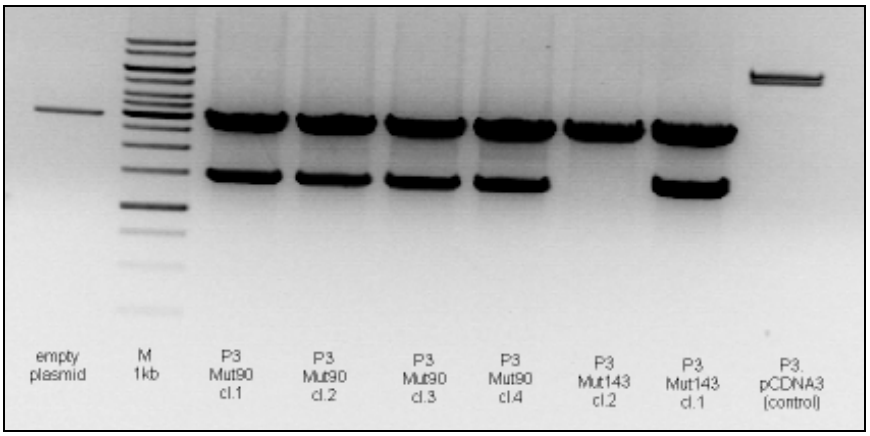

Figure 4.10. Restriction enzyme digestion confirmation of results is shown. Positive colonies are further confirmed with digestion reactions with two separate enzymes. Plasmids containing Pea3 sequence gives two bands; one is for Pea3 and the other is for plasmid. Empty plasmids are seen as single band in the gel as they do not contain Pea3. In this gel picture, 5th well gives one single band that is equal to the size of the empty plasmid vector. This colony is depicted as Pea3 Mutant 143 colony 2. This colony was negative in the colony PCR method and used as an internal control in this method. The negative result was confirmed in both steps

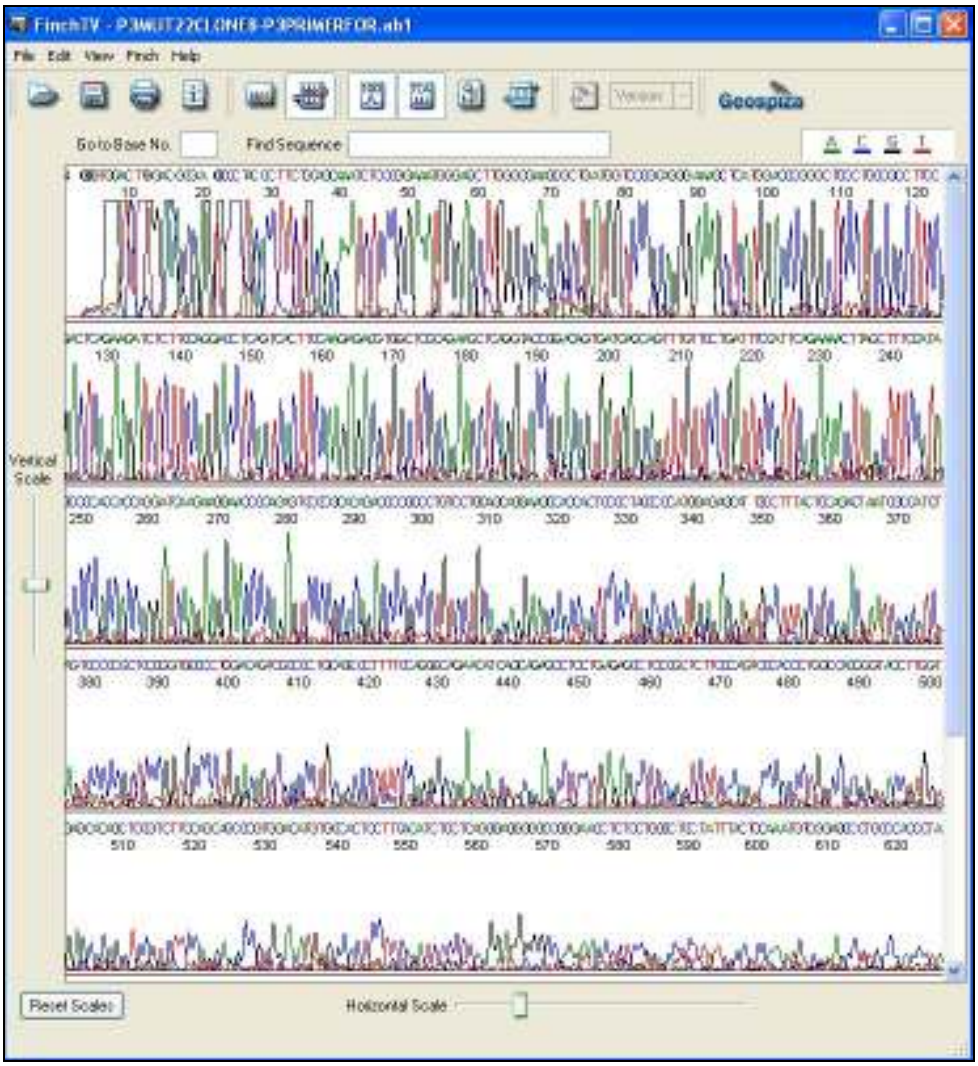

Figure 4.11. An example of sequencing data of mutation S22A, colony number.8. DNA sequencing is done by Refgen Gen Araştırmaları ve Biyoteknoloji Ltd Şti. Results of DNA sequencing is provided electronically as ab1 files and FinchTV free software from Geospiza Inc was used to process these files. Sequences from FinchTV were analyzed using the SDSC Biology Workbench web-based tool. Mutant and wild type sequences were compared, sequences that does not contain a mutation or that contain additional mutations were discarded. Only the sequences with correct mutations were chosen

4.2.3. Confirmation that the Mutant Proteins are Expressed as well as Wild-Type Pea3

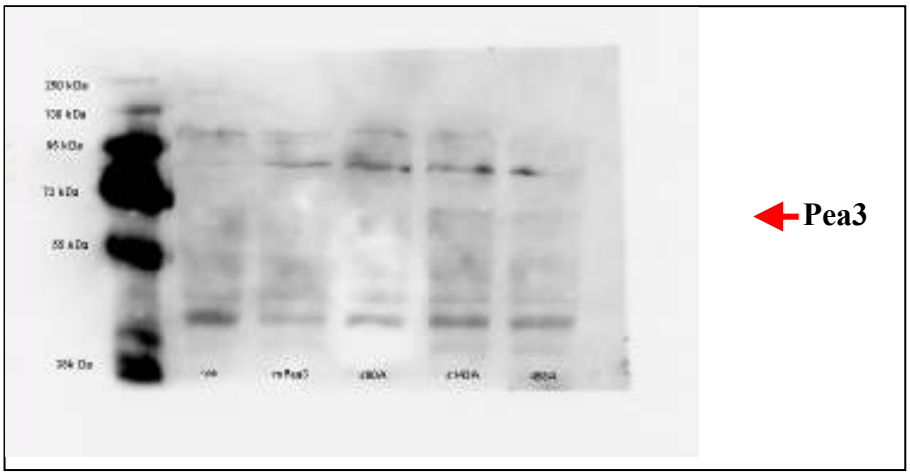

Figure 4.12. Western Blot result of mPea3 wild type and mutants. Western Blot is carried out to confirm that mutant Pea3 proteins are the same size as wild type protein. In order to obtain protein lysates, plasmids were transfected to HEK293 cells. Protein lysates were prepared and run on an SDS-PAGE and transferred to a PVDF membrane. When the membrane was treated with anti-Pea3 antibody, Pea3 band could be seen for all mutants and wild-type. Negative control is untransfected HEK293 cells. Since there is no endogenous Pea3 expression in these cells, there was not a Pea3 band for this control

Figure 4.13. Western Blot results of mPea3 wild type and S90A, S90E, S101A, S101E, S143A, S143E, S458A, S458E single mutants and S90-143A double mutant are shown. This assay was carried out by Melis Savaşan to confirm that mutant Pea3 proteins are the same size as wild type protein. In order to obtain protein lysates, plasmids containing a DNA sequence coding for Flag-tagged Pea3 or mutant proteins were transfected to HEK293 cells. Protein lysates for each mutation were prepared and run on an SDS-PAGE and transferred to a PVDF membrane. When the membrane was treated with anti-Flag antibody, Pea3 band could be seen for all mutants and wild-type proteins

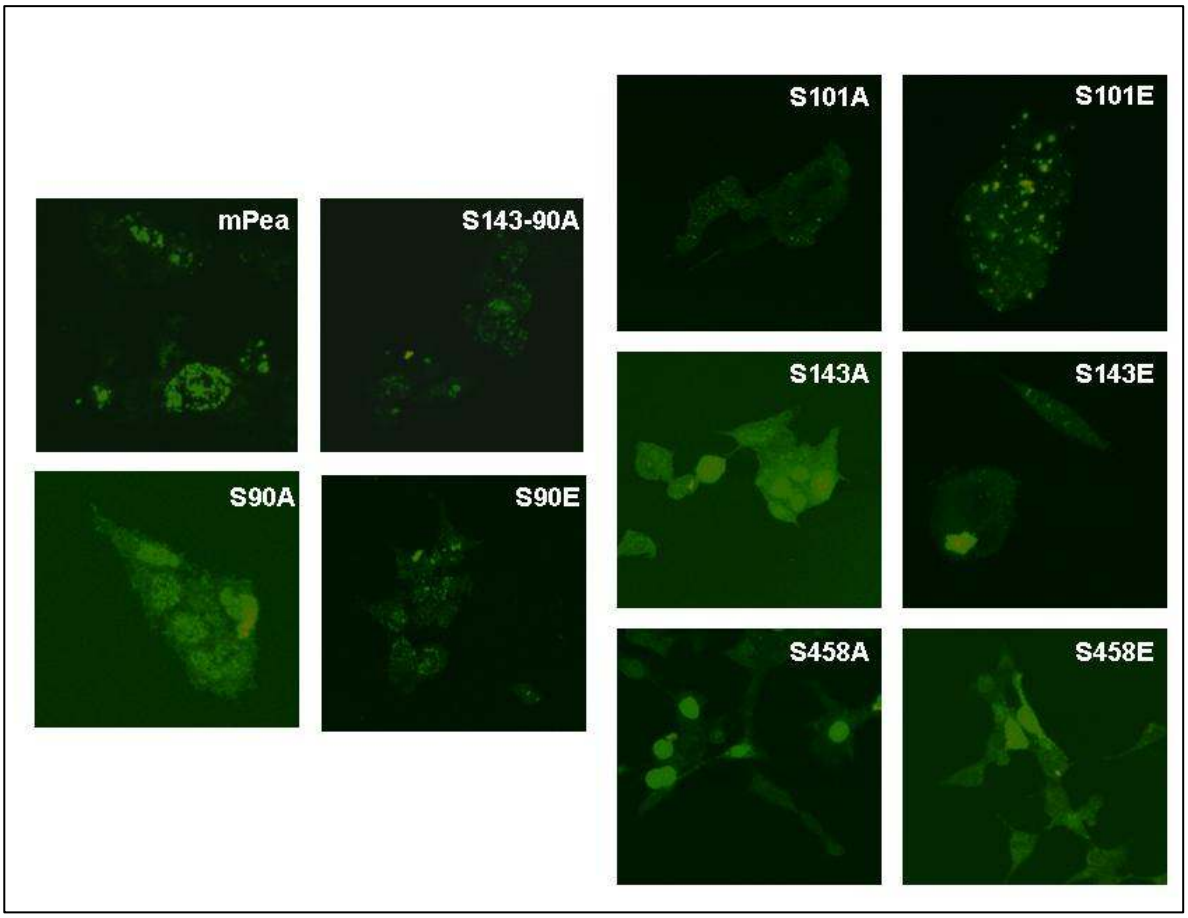

Figure 4.14. Immunofluorescence (IF) results of mPea3 wild type and mutants are shown. IF was carried out by to determine whether the mutants are able to enter the cell nuclei or not. Mutations in amino acid sequence of a transcription factor like Pea3 might lead to lowering of its ability to locate into the nuclei by disruption of its nuclear localization signals. Pea3 mutants were first stained with anti-Pea3 primary antibody in PC12 cells (data not shown). After cloning Pea3 wild type and mutants into flag tag vector, IF assay was repeated for all the mutants constructed to date as well as for wild type. This assay was carried out by Melis Savaşan by using anti-Flag antibody. IF results suggest that even though the cytoplasmic and nucleic distribution of mutant proteins are dependent on the mutant, all the mutants were shown to be able to locate into the nuclei and an increase or decrease observed in transcriptional activity of Pea3 cannot be attributed their not being present in the nuclei. Green, anti-Flag antibody (pseudo-colored)

4.2.4. The Effect of Phosphorylation Mutations on Transactivation Activity of Pea3 on Different Promoters

In order to investigate which phosphorylation motif(s) is(are) important for the transcriptional activity of Pea3, we have performed luciferase assays with some Pea3 target promoters that were identified in our laboratory (such as Neurofilament-L, NFL, or survival of motor neuron, SMN) and artificial response reporters Pea3-Luc (containing 5 repeats of a generic Pea3 binding motif upstream of a basal promoter) and SRE-Luc (containing Serum Response Element upstream of a basal promoter). We have studied reporter activities in a neural differentiation model system, PC12 cells (pheochromocytoma cells).

In PC12 cells, activity of Pea3 mutants were investigated with promoters that Pea3 was shown to bind; such as NF-L, Pea3, Sre and Smn promoters (*Onder Z, Demir O, Kurnaz A I, 2008; unpublished data*). Therefore, Pea3-Luc, NFL-Luc, SRE-Luc and SMN-Luc constructs was used to see whether there are any changes in the level of Pea3 transactivation function due to mutations.

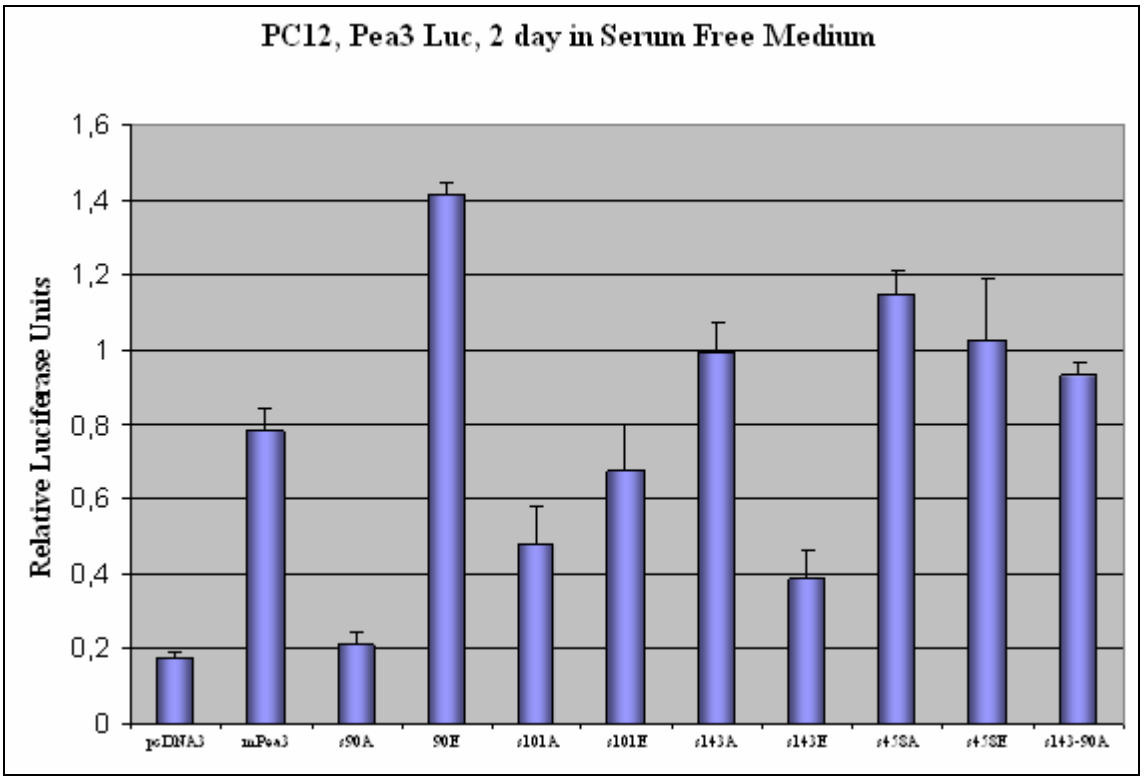

Figure 4.15. Pea3 Luciferase result of mutant and wild type Pea3 proteins is shown. Ser90Ala mutation caused decrease in the reporter activity of Pea3, while the observed increased in the reporter activity of Pea3 with the Ser143Ala and Ser458Ala mutations

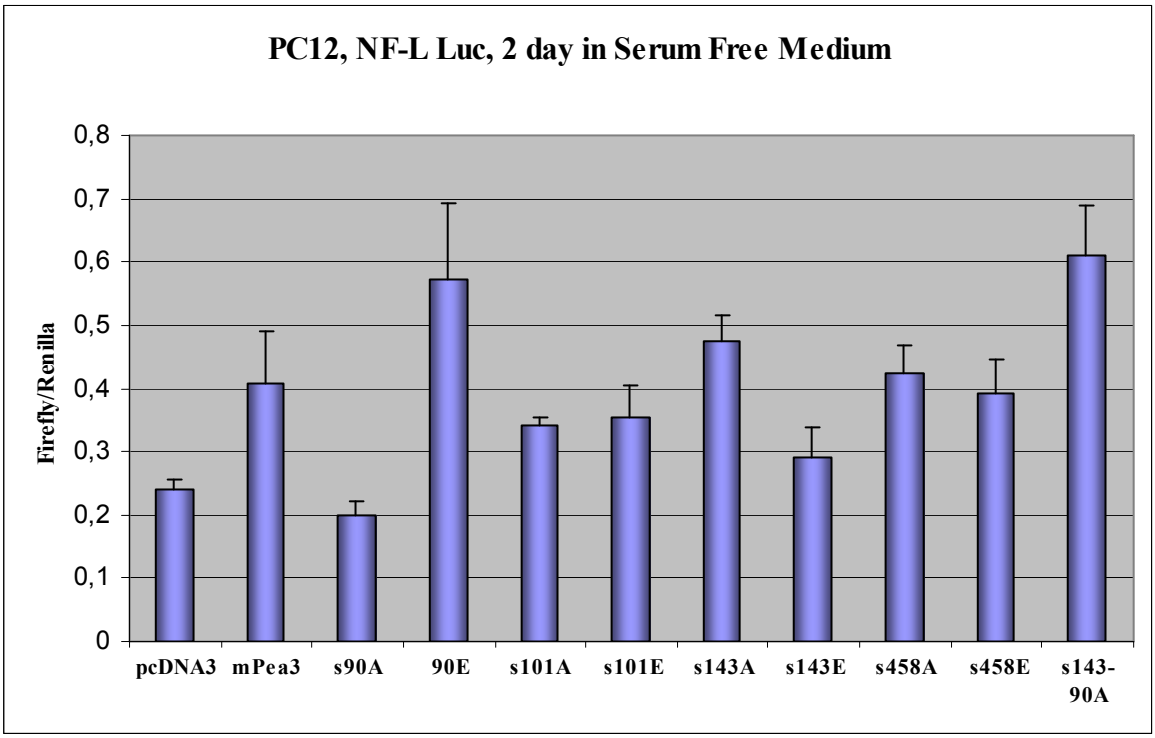

Figure 4.16. NFL Luciferase result of mutant and wild type Pea3 proteins is shown. Ser90Ala mutation caused a decrease in the reporter activity of NFL-Luc, while the observed increased in the reporter activity with the Ser143Ala and Ser458Ala mutations

4.2.5. siPea3 Analysis

In order to further confirm whether an upregulation of Neurofilament Light chain subunit promoter is caused by overexpressing Pea3 in PC12 and SHSY-5Y cells, one should be able to knock down activation on these promoters by introducing Pea3 siRNA.

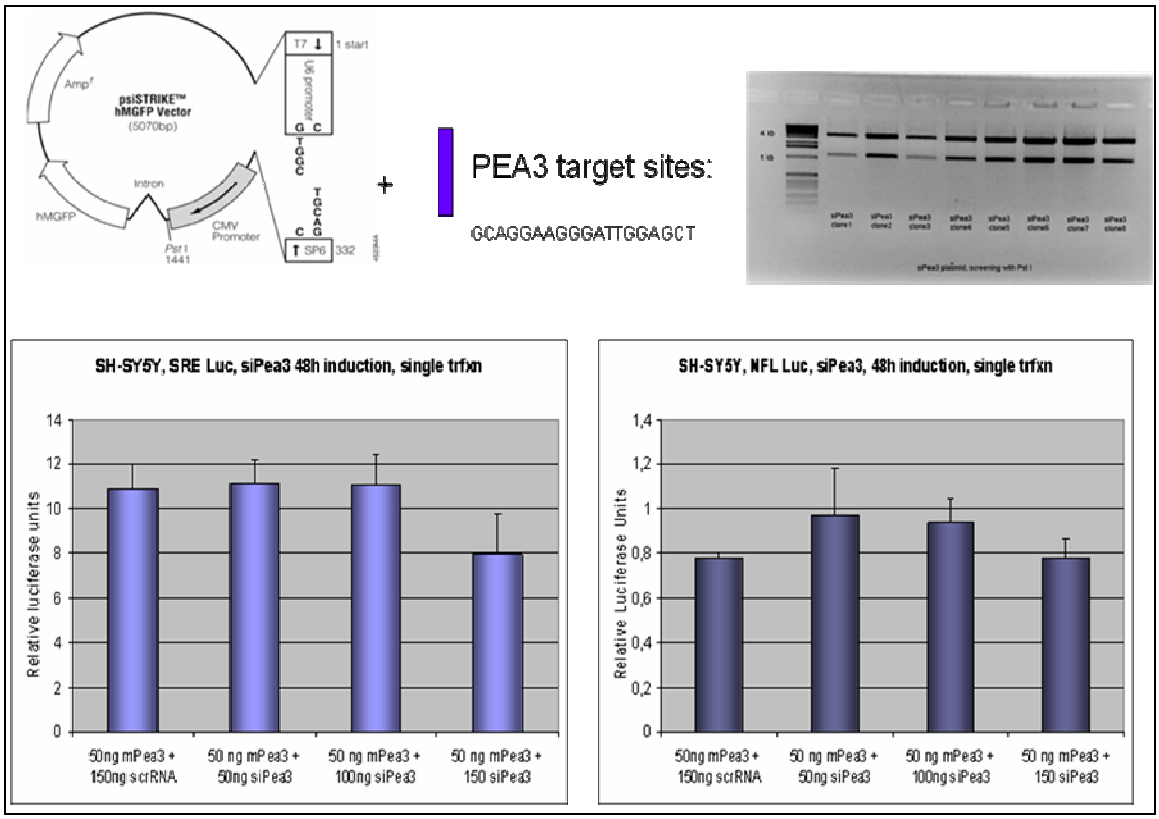

Figure 4.17. In siPea3 analyses, we inserted a 19 bp Pea3 target site (from De Launoit, 2008) into the psiSTRIKE hGMP plasmid vector (Promega) and confirmed the insertion of this fragment as recommended by the manufacturer. SH-SY5Y cells do not express Pea3 endogenously, therefore we co-transfected Pea3 with siPea3 in these cells. SRE-Luc and NFL-Luc reporter assays with single transfection by siPea3 were unsuccessful to knock down the effect of Pea3 on these promoters. These experiments will be further performed with double transfection with siPea3

5. DISCUSSION

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

In our study, we tried to find the phosphorylation sites where MAPK kinase phosphorylate Pea3. For this purpose, in order to prevent phosphorylation, Serine amino acids (polar) were mutated to Alanine (nonpolar) at the predicted Ser/Pro sites, then the obtained mutants were compared to the wild-type Pea3 in their transcriptional activity.

Until now, we had three mutated Pea3 products cloned in pCDNA3. We made Luciferase assay with these mutations. We chose as reporter genes Pea3, NFL (neurofilament light chain) and SMN (survival of motor neurons). It was previously shown that Pea3 binds to NFL promoter in PC12 cells. However, it was newly found by Zeynep Önder and Özlem Demir that Pea3 binds also to SMN promoter to regulate its activation (manuscript in submission).

Luciferase results demonstrate that Serine90 mutants inhibit the transactivation on four of the promoters used in this study, when compared to the wild type. After obtaining the results from the luciferase assays and the lowered luciferase activity with Serine90Alanine mutant was observed in all of these promoters. There might be several reasons for this result; the protein might be expressed at low amounts, compared to wild type or the protein might not be expressed full size. So, we wanted to check whether this mutant is expressed at a relatively low portion in the cells or not, if expressed at all. Therefore, the Western Blot study was performed. The results from Western Blot suggest that the Ser90Ala mutant protein is expressed at a similar degree to the wild type. Thus, the lowered transactivation cannot be explained by the presence of decreased amount of

Ser90Ala mutant protein. Western Blot also suggested this mutant is at the same size with the wild type, so, the decreased activity cannot be a reason of not obtaining full size protein with this mutant. These results suggest that phosphorylation of Serine90 amino acid position is important in the sense that, once this site is phosphorylated, it increases the transcriptional activity of the protein. We observed significant decrease in the luciferase activity of all Pea3, NF-L, SRE and SMN luciferase with Ser90Ala mutated Pea3. Although all these promoters have different functions in the cell and they have different positive and negative regulators, silencing mutation on Serine90 consistently represented a decrease in their luciferase activity. From the schematic structure of mouse Pea3 (Figure 4.1), Serine90 is known to be at a close proximity to the Transactivation Domain present in through the N-terminal. In the light of these data, it can be suggested that this phosphorylation site is an important upregulation site of Pea3 protein.

Luciferase assays show that, Ser143Ala mutant activated the Pea3 and NFL-luc reporters more than the wild type molecule in PC12 cells (Figure 4.9, 4.10 and 4.11). It is confirmed with Western Blot analysis that this activation is not due to higher amount of the Ser143Ala mutant present in the cells. In fact, it can be suggested that because silencing enhances the relative luciferase activity, phosphorylation of Serine143 site inhibits the transcriptional activity of the protein in the promoters mentioned above.

Ser143Ala mutation along with Ser90Ala and Ser458Ala caused a decrease in the luciferase activity with SMN promoter. One possible explanation for this result could be that SMN gene has a tightly controlled regulation mechanism due to its importance in transcriptional regulation, telomerase regeneration and cellular trafficking. SMN protein is found in all cells throughout the body and especially in high degrees in spinal cord. It plays an important role in maintenance of motor neurons and defects in SMN gene causes spinal muscular atrophy. Since SMN is an important protein in neurons and neurons are vulnerable to changes in the maintenance genes more than the other cells, it is not surprising that this gene is tightly controlled than the others (results not shown).

Ser458Ala mutation does not show a specific trend in the transactivation of Pea3. It is suggested that phosphorylation of Serine458 site does not have a primary importance when compared to Serine90 or Serine143. Although this site is located inside the

Transactivation Domain through the C-terminal of Pea3 (shown in Figure 4.1) and although phosphorylation of Serine458 is expected to activate Pea3, the results suggest that this site is more likely to be dependent on the gene to be regulated.

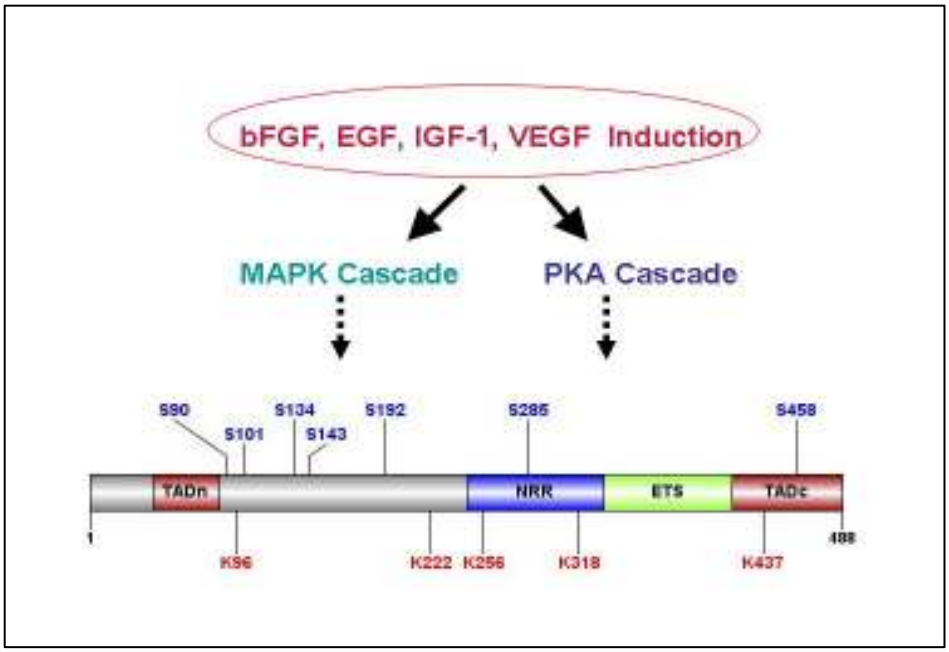

Figure 5.1. Schematic representation of post-translational modification sites on Pea3. Pea3 transcriotion factor has five identified sumoylation motifs on 5 Lysine amino acids (represented in red, below the bar). In this thesis, we predicted 8 SP phosphorylation motifs (represented in blue, above the bar). Upstream modulators of these motifs, including specific cascades is yet to be identified

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. Silencing mutation of Serine90 demonstrates a decrease in the transaction of Pea3 on Neurofilament Light Chain-Luciferase reporter (NFL-Luc) and silencing mutation of Serine143 demonstrates an increase in the transaction of Pea3 on NFL-Luc. Neurofilament proteins are intermediate filaments found in neurons. Neurofilament Light Chain is a subunit of Neurofilament protein and runs at 68 kDa in SDS-PAGE. Neurofilaments play an important role in axonal growth by incorporating new proteins along the axons and in intracellular transport guidance to axons. NFL promoter contains two predicted Pea3 binding sequences (confirmed with online biochemical tools, such as TFSearch) and

experiments show that Pea3 protein regulates NFL promoter (*Demir O, Aksan Kurnaz I, data not shown*). Our purpose was to determine whether phosphorylation of these sites is important in regulation of NFL, hence important in regulation of axonal growth in PC12 cells or not. In this context, we used NFL-Luc reporter with Pea3 mutants in Luciferase assays. It is suggested that phosphorylation of Serine90 might be an important positive regulator for axonal growth in PC12 cells that were maintained in normal growth medium. Upon stimulation of axonal growth in PC12 cells by known stimulants, such as growth factors, roles of phosphorylation sites will be further analyzed and will be identified in a more realistic manner.

The ETS-domain proteins are clearly an important class of transcription factors involved in the regulation of numerous genes with a variety of functions. Significant progress has been made in the understanding of molecular and biological functions of these transcriptional factors. However, there are still areas that require further investigation. First of all, it is not known how DNA-binding specificity play a role in binding of an individual ETS domain transcription factor to its promoter and how this specificity can be altered for an individual protein with the help of post-translational modifications. For example, it is shown that PEA3 group proteins undergo SUMO modification by SUMOylating enzymes and they are regulated by SUMOylation. It is also shown that SUMOylation inhibits transcriptional activity of PEA3 group proteins, however SUMO modification can be reversed by signaling via the ERK-MAPK pathway, which induces a switch from the repressed phase to the transcriptionally active phase. Moreover, for many ETS-domain transcription factors, it is not clear which partner proteins play a role in DNA-binding to a promoter region or which partner proteins alter their DNA-binding specificity in response to upstream regulators.

Altogether, all of these possibilities remain to be studied in our project to identify the role of phosphorylation in the regulation of neurite outgrowth in PC12 cells. In this context, the results of this thesis suggest important phosphorylation sites on Pea3 and these sites will be further determined with the help of different upstream regulators in order to find out whether they are directly regulated by MAPK.

Present work is considered as a first step in understanding the mechanism of regulation of Pea3 protein via phosphorylation by MAPK. Having the role of Pea3 in axonal outgrowth and motor neuron circuit selectivity during development in mind, understanding the growth factors that control and activate Pea3 is going to help us identify new treatments in various neurodegenerative diseases, including Alzheimer. Pea3 is also known as an important modulator of proteins involved in cell-cell adhesion. Overexpression of Pea3 protein in mammary cancers is found to increase metastatic and invasive potential of the cancer tissue. Elucidating the phosphorylation mechanism of Pea3 in different cell types in different contexts and upon different stimulators will help us focus on new treatment strategies in various disease models.

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 that are responsible for neurite outgrowth and neuronal differentiation is being widely investigated world wide. 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. In this first step research, we focused on phosphorylation mechanism of Pea3 due to the fact that elucidating the roles of each phosphorylation motif and the responsible growth factors will eventually be used in designing treatments in animal disease models in future studies. In a side project in our laboratory, we are also focusing on the downstream targets of Pea3 during neural differentiation. Altogether, we believe that this research is essential for understanding the whole image of neurite outgrowth machinery.

APPENDIX A: PEA3 IN NEURITE OUTGROWTH IN ADULT RAT DRG AFTER GROWTH FACTOR INDUCTION

Basic fibroblast growth factor (bFGF) has an important role in neuronal development, in which it promotes axonal growth and maintains the survival of certain cell types. It was also shown that bFGF is upregulated after nerve injury in order to support regeneration at the injury lesion [40].

FGF signal transduction is initiated by the binding of FGF with the help of heparan sulfate to one of the four different cell type specific tyrosine kinase receptors, called FGF receptors (FGFR). This leads to FGFR dimerization and autophosphorylation and subsequent activation of the cytoplasmic part of the receptor. This event in turn, stimulates downstream signaling molecules, including Ras. Through Ras, FGF receptors can stimulate ERK/MAP kinase pathways as a common response. Active ERK phosphorylates cytoplasmic targets or translocates to nucleus and stimulates expression of several genes by phosphorylating different transcription factors [40-42].

bFGF is known to promote the survival, differentiation or branching of various types of neuronal cells in vitro. Of these cell types, being one of the most widely studied cell types, pheochromacytoma (PC12) and DRG were shown to differentiate upon over-expression of FGFR-1 by Klimaschewski's Group [39]. Their work proved that upon activation of FGFR in PC12 cells, sustained ERK levels lead to differentiation; in contrast activation of EGFR induces transient ERK levels that lead to proliferation. However the cellular mechanism of FGFR mediated axonal growth remains unclear.

In the scope of a COST - STSM Project No. B30-4425, a collaboration with Prof Dr Lars Klimaschewski's laboratory in Neuroanatomy Division, University of Innsbruck, Austria was carried out in 2009 in order to investigate whether Pea3 or its mutant forms play a role in axonal growth upon bFGF and/or other growth factor stimulation of primary neurons. At the time when the project was carried out, Ser143Ala silencing mutant was found to be more active and Ser90Ala mutant was found to be less active, compared to

wild type Pea3; therefore wild type Pea3 as well as these two mutants was used in this project.

In order to focus on the role of Pea3 in neurite outgrowth in adult sensory neurons, DRGs were collected from adult rats according the routine protocol used in Klimaschewski's laboratory. [39] Dissociated DRGs were co-transfected with Pea3 wild type and RFP plasmids by using Amaxa Nucleofector Neural cell transfection system. Successfully transfected DRGs were photographed after 48 hours incubation in 37°C 5% CO_2 incubator. Cells were then induced with either bFGF, EGF or IGF-1 and the same cells were photographed exactly 24 hours after induction and compared with pictures taken in the previous day.

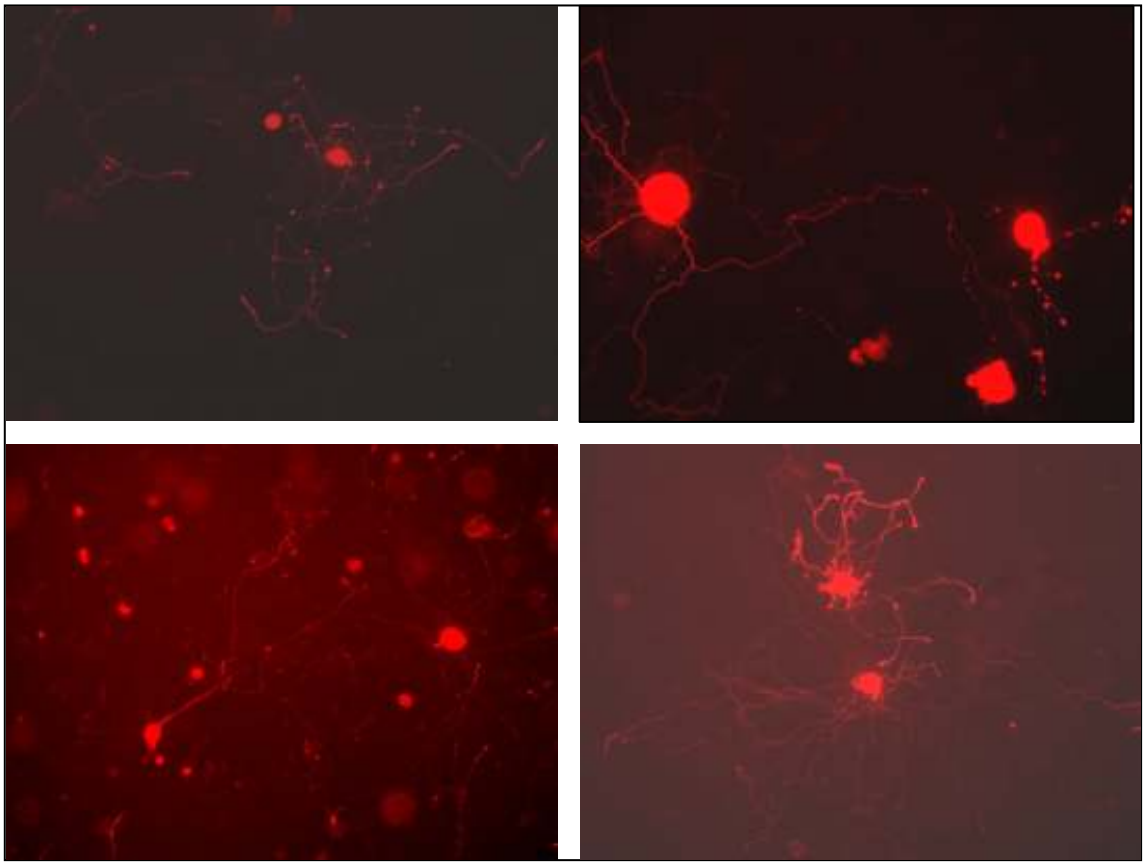

Figure A.1. IGF-1 induced DRGs overexpressing Pea3. DRGs were co-transfected with Pea3 and RFP and 48 hours after transfection, they were induced with IGF-1 and examined under fluorescent microscopy. Total axon lengths, maximal axon distances and branch points and statistical significance were calculated (pseudocolored with red)

After 24 hour incubation with IGF-1, the total axonal length and maximal distance increased significantly in Pea3 wild type overexpressing neurons. When compared to the untreated neurons, an increase with bFGF treatment could be observed. However, this increase was not as much as the increase obtained with the IGF-1 induction. EGF treatment did not result in any significantly important positive or negative effect on the total axonal length or maximal distance of the neurons when compared to the control group.

IGF-1 induction of Pea3 wild type overexpressing primary neurons also caused an increase in the branch point at day 3. In branch point numbers after 72 hours, when compared to control group, there was a slight but significantly not important increase with bFGF treatment and there was a negligible increase in EGF group.

To our surprise, bFGF seemed to induce total axonal length in primary neurons transfected with Pea3 Ser143Ala mutant, when compared to IGF-1 group (Figure A.2). bFGF also induced a slight increase in the maximal distance and branch point counts; however, both of those increases were not significant (Figure A.3 and A.4).

In order to find out whether IGF or Pea3 wild type overexpression was responsible from the significant increase in axonal lengths in DRGs, we transfected DRGs with dsRed plasmid in the absence of Pea3 and treated these neurons with growth factors.

Even though this transfection is not repeated to conclude a reliable result, the number of transfected neurons in each plate was still higher than expected. The data of this experiment clearly indicates the effect of IGF-1 on primary neurons alone. The total axonal growth in 24 hour IGF-1 treatment significantly increases, whereas bFGF treatment itself does not create an important increase in total axonal lengths. Upon treatment with IGF-1, obtained maximal distance length difference was also found to be significant, according to one-way ANOVA analysis and Tukey's post test on the data.

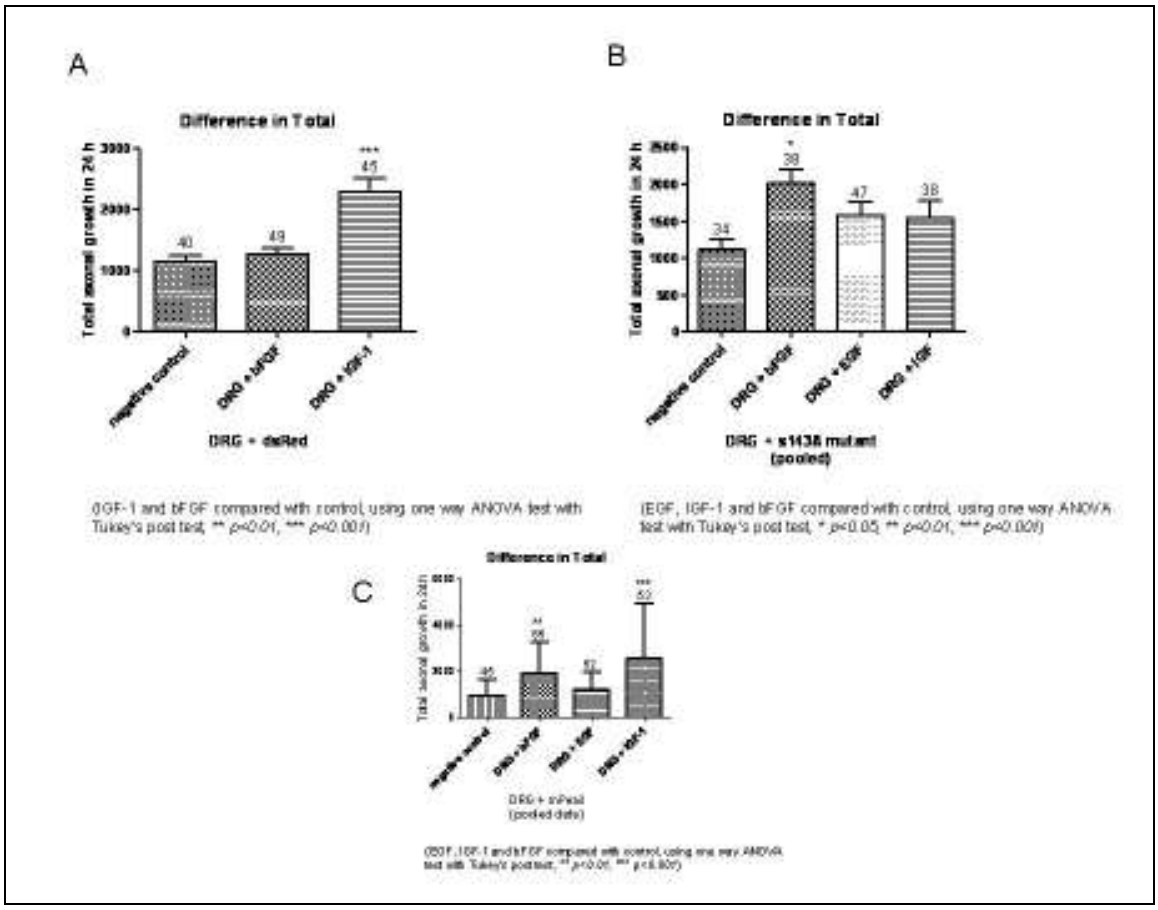

Figure A.2. Differences in total axon lengths. Difference in total axon lengths upon growth factor induction in DRGs were calculated for RFP only transfected control group, mPea3 wild type + RFP co-transfected group and Ser143Ala + RFP co-transfected group. IGF-1 induction has a role in increasing the amount of axonal elongation on DRGs (A). When DRGs that overexpress Ser143Ala mutant Pea3 protein were induced with bFGF, an increase in total axon lengths boosted up even more than IGF-1 induction (B). Wild type Pea3 overexpression is also shown to have a role in increasing the total axon length in DRGs after 24 hours of bFGF induction (C). Statistical significance was compared to control, using one-way ANOVA test with Tukey's post test

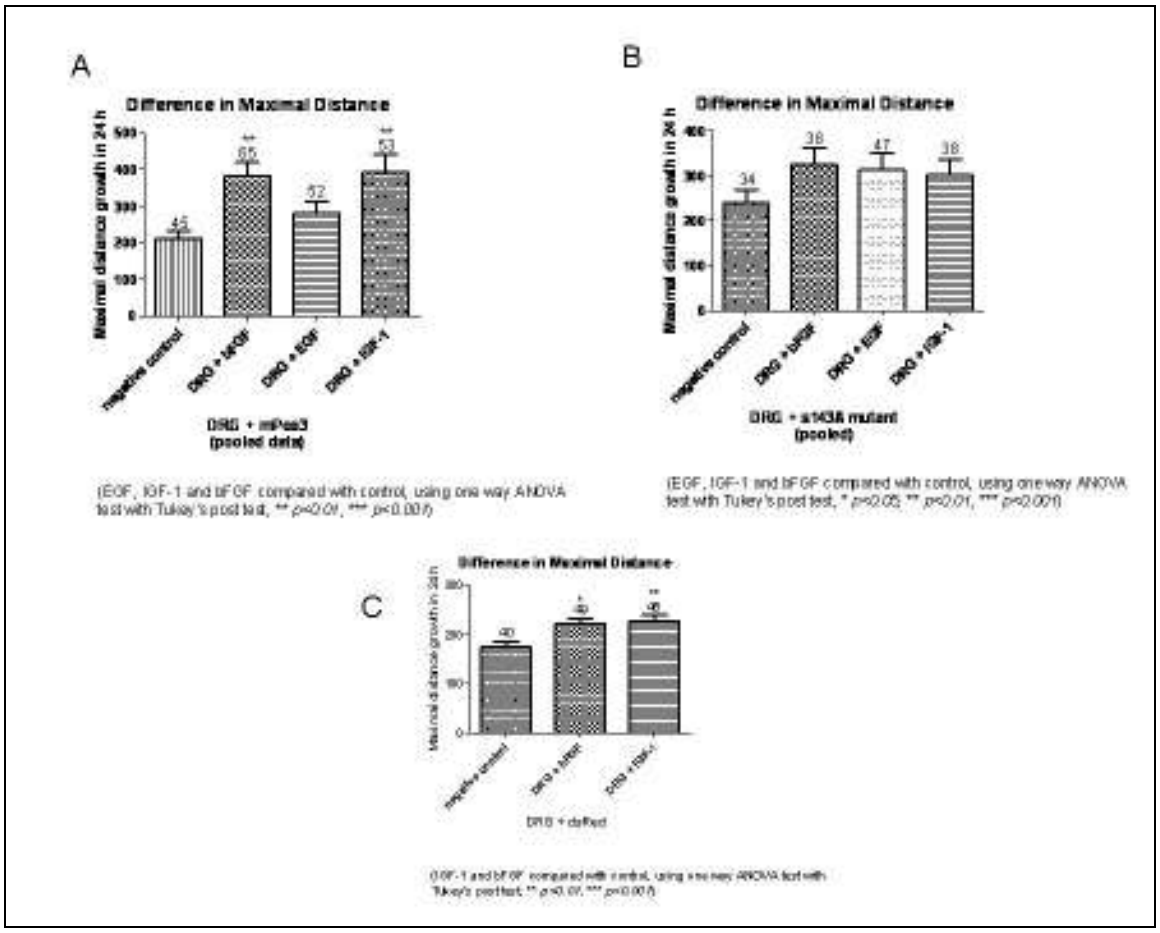

Figure A.3. Differences in maximal distances. Difference in maximal axon distances upon growth factor induction in DRGs were calculated for RFP only transfected control group, mPea3 wild type + RFP co-transfected group and Ser143Ala + RFP co-transfected group. IGF-1 and bFGF induction have a role in increasing the amount of maximal distance of axons on DRGs (A). When DRGs that overexpress Ser143Ala mutant Pea3 protein were induced with bFGF, there is no statistically significant increase in maximal distances (B). On the other hand, wild type Pea3 overexpression is also shown to be statistically significant in maximal distance increase when induced with IGF-1 (C). Statistical significance was compared to control, using one-way ANOVA test with Tukey's post test

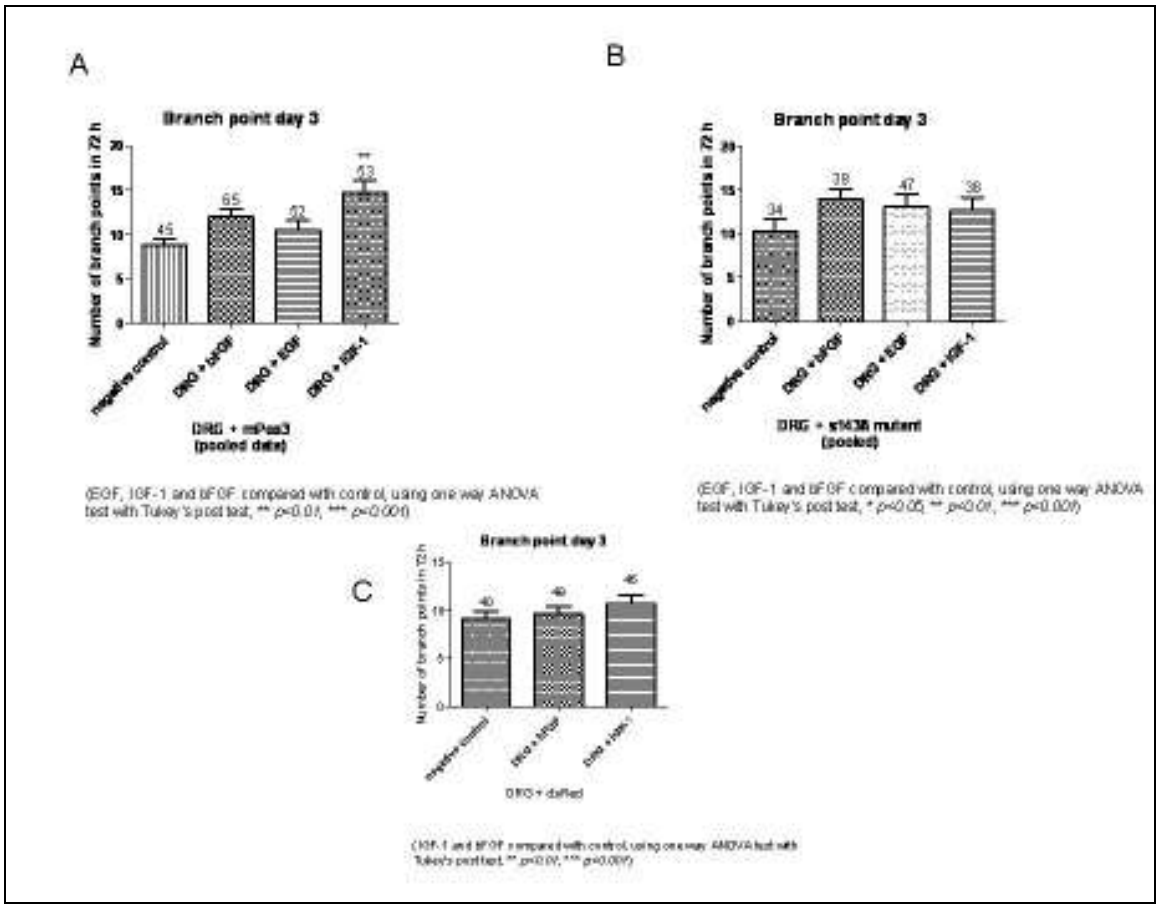

Figure A.4. Differences in branching points. Difference in branch points on axons upon growth factor induction in DRGs was calculated for RFP only transfected control group, mPea3 wild type + RFP co-transfected group and Ser143Ala + RFP co-transfected group. IGF-1 induction has a role in increasing the amount of branch points of axons on DRGs (A). When DRGs that overexpress Ser143Ala mutant Pea3 protein were induced with bFGF, there is no statistically significant increase in branch points on axons (B). Likewise, wild type Pea3 overexpression is also shown to be statistically insignificant in branch points on axons when induced with IGF-1 (C). Statistical significance was compared to control, using one-way ANOVA test with Tukey's post test

In conclusion, the preliminary results obtained in COST - STSM project provided us an insight about a possible role of Pea3 in enhancing neurite formation in DRGs even when transiently overexpressed without a growth factor induction. Results also suggest a possible relationship between Pea3 transcription factor and bFGF signaling pathway as an upstream regulator. Overexpression of Pea3 wild type and especially Ser143Ala mutant

dramatically increased neurite formation and neurite length when given with bFGF induction. Although these results point out a possible relationship between Pea3 and bFGF in primary neuron cultures, obtained data should be further confirmed with additional experiments. Regulation of Pea3 by bFGF is known to occur in motor neuron development in the embryo, but these studies were not carried out in adult rat sensory neurons.

Moreover, concerning the data obtained in our experiments, it is not clear whether Pea3 is a downstream transcription factor in the IGF-1 signaling pathway in primary neurons. Therefore, in order to obtain a successful conclusion about the role of Pea3 in axonal growth in the presence of IGF-1 induction should also be studied in the future experiments.

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