# TRANSACTIVATION CAPACITY OF PEA3 TRANSCRIPTION FACTOR ON PROMOTER OF *NEUROD1*

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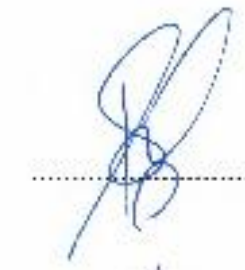

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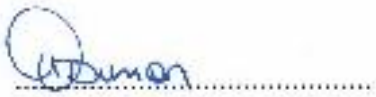

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# ABSTRACT


## TRANSACTIVATION CAPACITY OF PEA3 TRANSCRIPTION FACTOR ON PROMOTER OF *NEUROD1*

Pea3, a member of the ETS (E26 transcription-specific) family of transcription factor is a nuclear phosphoprotein and has been shown to be involved in developmental processes with its expression during embryonic stages, in cancer metastatis by regulating the transcription of matrix metalloproteases or in branching morphogenesis of several organs like lung or mammary glands. In addition to this organ branching, Pea3 is involved in the axonal arborization. It has been shown that Pea3 expression is important for connection of the motor axons to the target muscles. Pea3 presence in branching and axonal projection processes led us to study the genes which can be regulated by Pea3. Among many of its potential target genes we are particularly interested in the novel target NeuroD for its role in differentiation of newborn neurons where they might be involved in the differentiation and allowing axon growth. In this study we identify the regions on promoter of NeuroD where Pea3 binds and regulates its expression in addition to this we also show how Pea3 phosphorylation on specific Serine /Proline residues by MAP kinase pathways affects its transactivating capacity for the expression of NeuroD. The data from this study may give us insight about how and with which target genes Pea3 is involved in the development of a neuron.

# ÖZET

## TRANSACTIVATION CAPACITY OF PEA3 TRANSCRIPTION FACTOR ON PROMOTER OF *NEUROD1*

ETS (E26 transcription-specific) transkripsiyon ailesinin bir üyesi olan Pea3 transkripsiyon faktörü, embriyonik gelişimde, kanser metastazında, branşlaşmanın görüldüğü akciğer ve süt bezlerinde çeşitli genlerin anlatımını sağlayarak rol olan bir nukleer fosfo-proteindir. Bunun yanında Pea3 transkripsiyon faktörünün, akson dallanmasında da önemli rol oynadığı görülmüştür. Çalışmalar, hedef kas dokusuna ulaşmış motor nöronlarının bu doku üzerindeki dallanmasında Pea3 transkripsiyon faktörünün sorumlu olduğunu ve Pea3 eksikliğinde bu dallanmanın ve bozulduğunu göstermiştir.Pea3 transkripsiyon faktörünün yukarıda belirtilen özelliklerini gözeterek bu çalışmada, akson uzamasında ve dallanmasında önemli olan genlerin anlatımında, bu proteinin olası rolü araştırılması hedeflenmiştir. Birçok hedef gen arasından NeuroD (Neurogenic Differentiation) geni seçilmiş ve bu genin regülasyonunda Pea3 proteinin yeri araştırılmıştır. Bu amaçla bir transkripsiyon faktörü olarak Pea3 proteinin, NeuroD geni promotör bölgesideki olası bağlanma motifleri…

Bununla birlikte bir fosfo protein olan Pea3 transkripsiyon faktörünün olası Serin /Prolin bölgelerinden MAPK yolağı sayesinde fosforlanmasının, bu proteinin hedef gen NeuroD'nin aktivasyonuna etkisi araştırılmıştır. Bu çalışmalardan çıkacak sonuçlar ile Pea3 transkripsiyon faktörünün sinir gelişiminde hangi genleri nasıl aktive ettiğine dair, bir görüş kazanılması amaçlanmaktadır.

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# LIST OF SYMBOLS / ABBREVIATIONS

| | |
|---|---|
| AD | Activation domain |
| Ala | Alanine |
| AMP | Ampicillin |
| ATCC | American Type Culture Collection |
| ATP | Adenosine Triphosphate |
| b-HLH | Basic Helix-Loop-Helix |
| $CaCl_2$ | Calcium Chloride |
| ChIP | Chromatin Immunoprecipitation |
| CIDD | Central Inhibitory DNA Binding Domain |
| CT | Carboxy Terminal |
| del | Deletion |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic Acid |
| dNTP | Deoxyribonucleotide triphosphate |
| DRG | Dorsal Root Ganglion |
| 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 |
| HEK293 | Human Embryonic Kidney Cell Line |

| | |
|---|---|
| HEPES | Hydroxyethyl piperazineethanesulfonic acid |
| HLH | Helix-Loop-Helix Domain |
| ID | Inhibitory domain |
| JNK | c-Jun N Terminal Kinase |
| KCl | Potassium Chloride |
| l | Liter |
| LB | Luria-Bertani |
| MAPK | Mitogen Activated Protein Kinase |
| MgCl2 | Magnesium Cloride |
| ml | Milliliters |
| mM | Milimolar |
| MMP | Matrix Metalloproteinases |
| NaCl | Sodium Chloride |
| NaOH | Sodium Hydroxide |
| NeuroD | Neurogenic Differentiation |
| ng | Nanogram |
| NGF | Nerve Growth Factor |
| nM | Nanomolar |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PEA3 | Polyomavirus Enhancer Activator 3 |
| PEI | Polyethylenimine |
| pH | Negative log of hydrogen ion concentration |
| PKA | cAMP-dependent Protein Kinase |
| PLB | Passive Lysis Buffer |
| PNT | Pointed Domain |
| PTM | Post-Translational Modification |
| RCF | Relative centrifugal force |
| RIPA | Radio-Immunoprecipitation Assay |
| Rpm | Rotation per minute |
| RT | Room Temperature |
| Ser | Serine |
| SDM | Site Directed Mutagenesis |

| | |
|---|---|
| SDS | Sodium Dodecyl Sulphate |
| SH-SY5Y | Human Neuroblastoma Cell Line |
| 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 |
| TLB | Tissue Lysis Buffer |
| TRED | Transcriptional Regulatory Element Database |
| UV | UltraViolet |
| µl | Microliters |

# 1. INTRODUCTION

Transcription factors are important molecules that play role in the transcription of their target genes. The regulation of gene expression by transcription factors arise due their transactivation effect. Transcription factors bind gene regulatory regions of DNA by their binding domains and as a result regulate the gene expression. These binding sites are specific to a transciption factor. Sharing same binding sites gather transcription factors and make them called as family.

The ETS (E26 transformation specific) genes are a family of transcription factors and they share a common conserved DNA - binding domain. Pea3 is a member of these ETS family transcription factors and involved in the regulation of gene expression which are important for cell growth, development, differentiation, oncogenic transformation and apoptosis as well.

On the other hand the target NeuroD is expressed in both mitotic and post-mitotic neurons and important for both differentiationand the survival of the alreday differentiated neurons.

This project mainly focused to the Pea3 mediated NeuroD promoter regulation by revealing exact Pea3 binding motifs suggested by the web based virtual labarotories, such as ALGGEN PROMO. Results, gathered from the studies showed the presence of an interaction between Pea3 and NeuroD promoter in such way that increases the expression. Results with our phosphorylation mutants further showed that upstream signals, in particular those that result in MAPK (mitogen activated protein kinase) pathway, have great impact on the transactivation capacity of Pea3 as a transcription factor.

# 2. THEORETICAL BACKGROUND

## 2.1. THE ETS FAMILY OF TRANSCRIPTION FACTORS

ETS (E26 transcription-specific) family of transcription factors are classified in the winged helix-turn-helix superfamiliy (wHTH) which consist of three α-helices and four antiparallel β-sheet structure. They have a functional domain which involves evolutionarily preserved 85 amino acid residues enables binding to a purine-rich DNA sequence with central 5'-GGAA/T-3' core sequence (Figure 2.1.). This functional domain enables DNA binding and called 'ETS Binding Site' (EBS) [1]. ETS family of transcription factors are subdivided into gropus according to the sequence and position of ETS domain, flanking sequences around it. The presence of conserved domains rather than ETS domain attributes their classification within family [2].

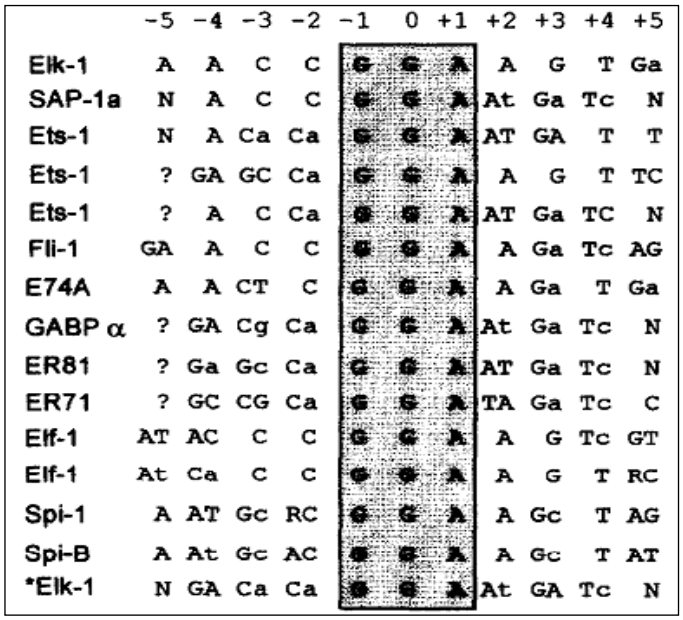


Figure 2.1. DNA binding sites of ETS family of transcription factors. The core DNA binding motif is 5'- GGA- 3' which is followed by A or T [1]

ETS proteins direct gene expression upon binding to the enhancer or promoter of a gene regulatory machinery. However the activation of this machinery by ETS proteins are

influenced by the involvement of other conserved domains or motifs as well. The presence of different domains may enhance DNA binding or may lead to the interaction with partner proteins on particular binding motif. Beside having these structural and functional regulatory elements and motifs, function of ETS proteins can be altered by the post-translational modifications. ETS proteins are downstream effector of signal transduction pathways and phopshorylation has its particular importance on regulation of ETS proteins.

### 2.1.1. Structural Features of ETS Family of Transcription Factors

ETS family of transcription factors are so called since they share evolutionarily conserved purine rich DNA binding domain the ETS domain. Interaction of ETS domain with a particular DNA motif happens due to having winged helix-turn-helix (wHTH) structured ETS domain. It is composed of three α-helices and four β-sheets arranged in the order α1- β1- β2- α2- α3- β3- β4 [3, 4] . The third α-helix is responsible for binding to groove in the GGA(A/T) DNA motif. Additional DNA interactions are carried by the loop between third and fourth β-sheets (the "wing") and the loop between the second and third α-helices (Figure 2.2.) [1].

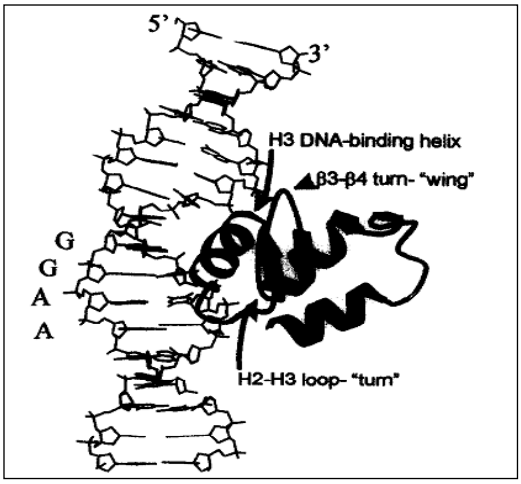


Figure 2.2. The ETS domain binding to GGAA DNA motif [1]

However the member of family has not merely the ETS domain but has other functional domains and hence family is subdivided into groups. Pointed (PNT) domain is one of those conserved domains and serves for protein-protein interactions by forming helix-loop-helix

structure. B-box which is only found in the TCF (ternary complex factor) family members is important for providing interaction with SRF (serum response factor) on SRE (serum response element) DNA binding region of target genes [6]. The location of ETS domain also contributes that classification. ETS domain generally resides on the C-terminal whereas in the TCF family members for instance ETS had located in the N-terminal region (Figure 2.3.).

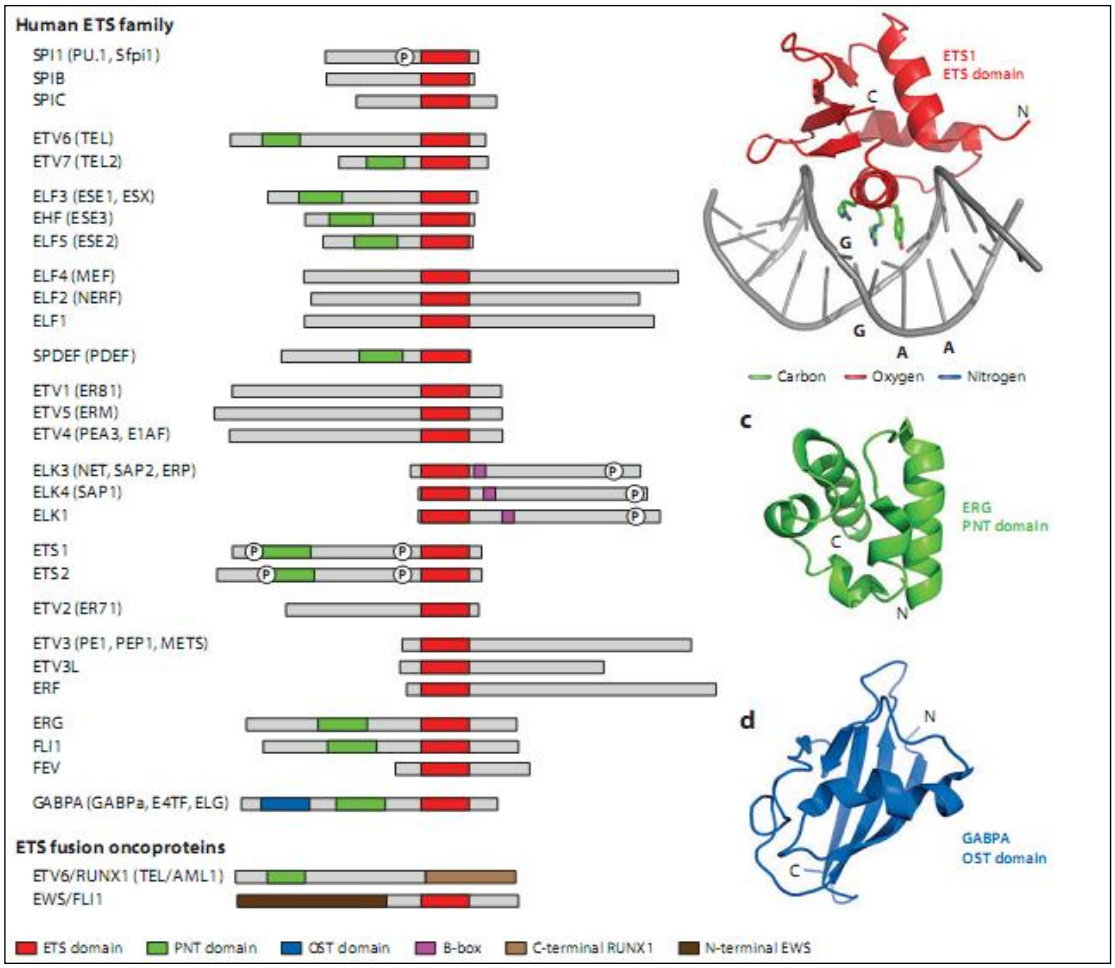


Figure 2.3. ETS family of transcription factors and their specific domains; ETS domain, PNT domain (pointed domain), B-box [7]

### 2.1.2. Post Translational Modifications (PTMs) and Their Implications in Activation

Gene expression is regulated by transcription factors upon binding of these factors to the specific sequences on promoter or enhancer, or so called gene regulatory regions.

However, the regulation of transcription factor itself is necessary in order to gain a function. Regulation of transcription factor happens through their modifications mediated by enzymes either addition of chemical groups or cleavage of some parts from the peptide. PTMs can alter the cellular localization, DNA binding, interaction with other protein or degredation of transcription factor, thereby regulates its activity over target genes.
PTMs occur as a result of signals coming from intra- or intercellularly. Therefore, if we put the events in order, a transcriptipn factor lies in the middle as a transmitter which receives message from its upstream effectors and delays it to the donwstream targets.

### *2.1.2.1. Post Translational Modifications (PTMs): Phosphorylation*

Phosphorylation is the most common and well-studied PTM. Phosphorylation is mediated by the protein kinases, by addition of a phosphate group to the serine, threonine, tyrosine, histidine and asparagine residues. Phosphorylation can be reversed as phosphate group is removed by the phosphatases.

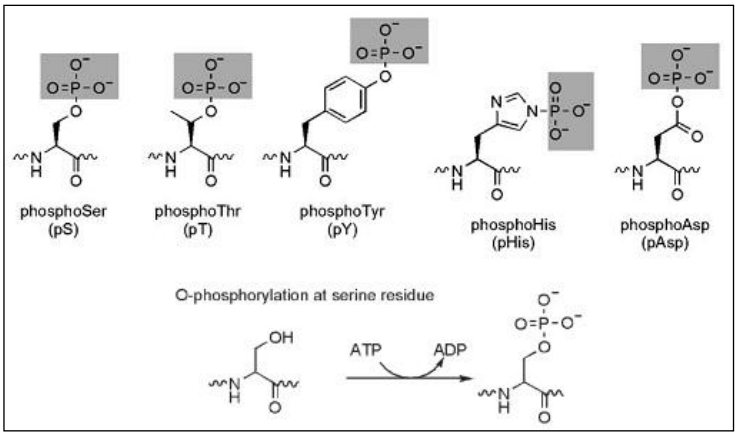


Figure 2.4. Phosphorylation of aminoacids; Serine (Ser), Threonine (Thr), Tyrosine (Tyr), Histidine (His), Aspartic acid (Asp) [8]

Phosphorylation of transcription factors are mediated by the mitogen activated protein (MAP) kinases. MAPKs relay signals received by the cell-surface receptors to the downstream regulatory targets in the pathway. The signals received by the receptors could be chemical or physical stresses or it could be a mitogenic signal which are at the end control the growth, differentiation, development or survival of the cell. Depending on the type of the signal pathway terminates with different kind of MAPKs. Mitogenic stimuli

transfer is end up with the activation of ERK-1 and ERK-2 MAPKs, while cellular stresses transfer is end up with the activation of the stress- activated kinases SAPK/JNK and p38 (Figure 2.5) [9].

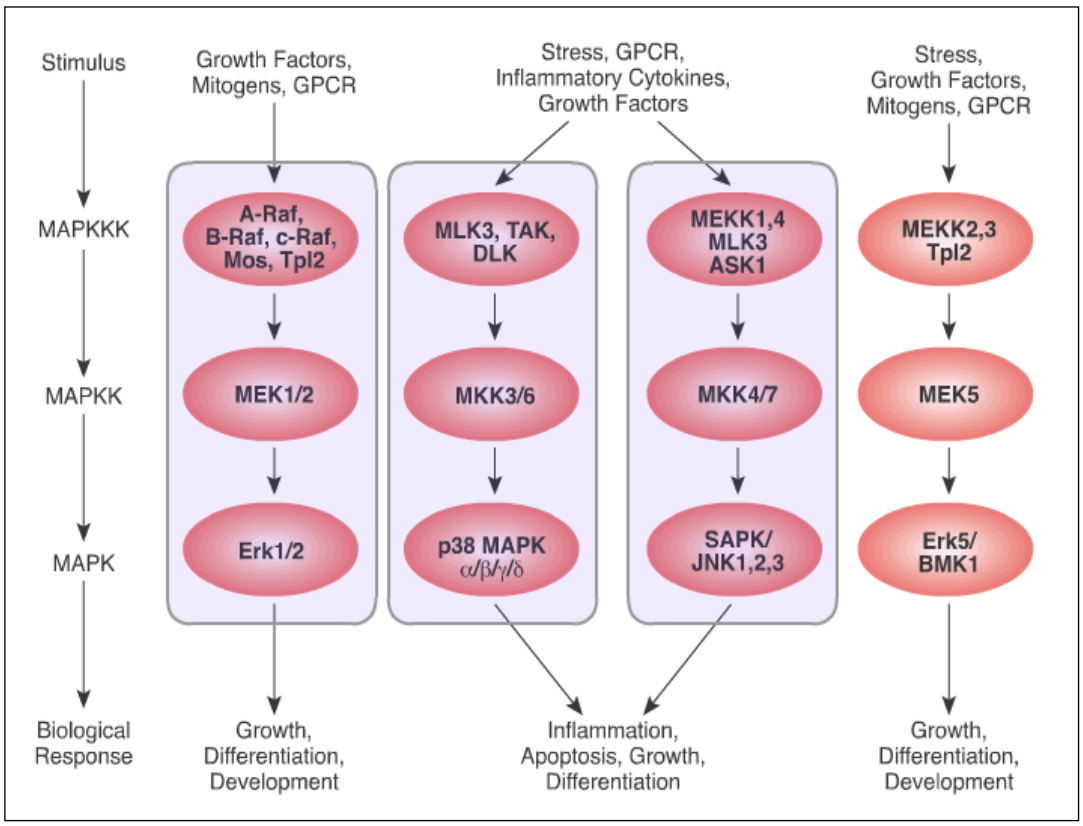


Figure 2.5. Mitogen Activated Protein Kinase (MAPK) signaling pathway [10]

Phosphorylation of transcription factors upon this signaling pathways may alter the regulatory action of proteins in several ways. Phosphorylation of auto-regulatory domains which can inhibit DNA binding or transactivation of capacity of protein may switch protein on or off hence end up with activated or inactivated protein [11] .Phosphorylation can also recruit partner proteins and promote protein-protein interaction which may have an important effect on the action of phosphorylated protein.

An example for this kind of, phosphorylation enhanced, activation can be seen in the Elk-1 transcription factor of TCFs family. Phosphorylation of Elk-1 at Ser383 promotes the formation of ternary complex with Serum Response Factor (SRF) and as a result of this interaction the DNA binding capacity of Elk-1 to the regulatory elements of immediate

early genes is enhanced [12]. The subcellular localization of transcription factor is also altered by their phophorylation status.

## 2.2. THE PEA3 GROUP

### 2.2.1. The PEA3 Group of Ets Transcription Factors

Pea3 (Polyoma enhancer activator) is a group of proteins under the ETS transcription factor family and comprises three related transcription factors, PEA3, ERM and ER81 which are the mouse homologues of human ETS translocation variant 4 (ETV4 or E1AF) , 5 (ETV5), and 1 (ETV1) respectively. Genes expressing these transcription factors are located in different chromosomes. Human ERM (ETV5) is located on the long arm of chromosome 3 at 3q27–29 [13, 14], Pea3 (E1AF) is located on the long arm of chromosome 17 at 17q21 [15] and ER81 (ETV1) is located on the long arm of chromosome 7 at 7q21 [16]. Although they are located on different chromosomes, they share highly related architecture with sequence similarity in their ETS domain and activation domains [17] which suggests that they are formed as chromosome duplication from the same ancestor [1].

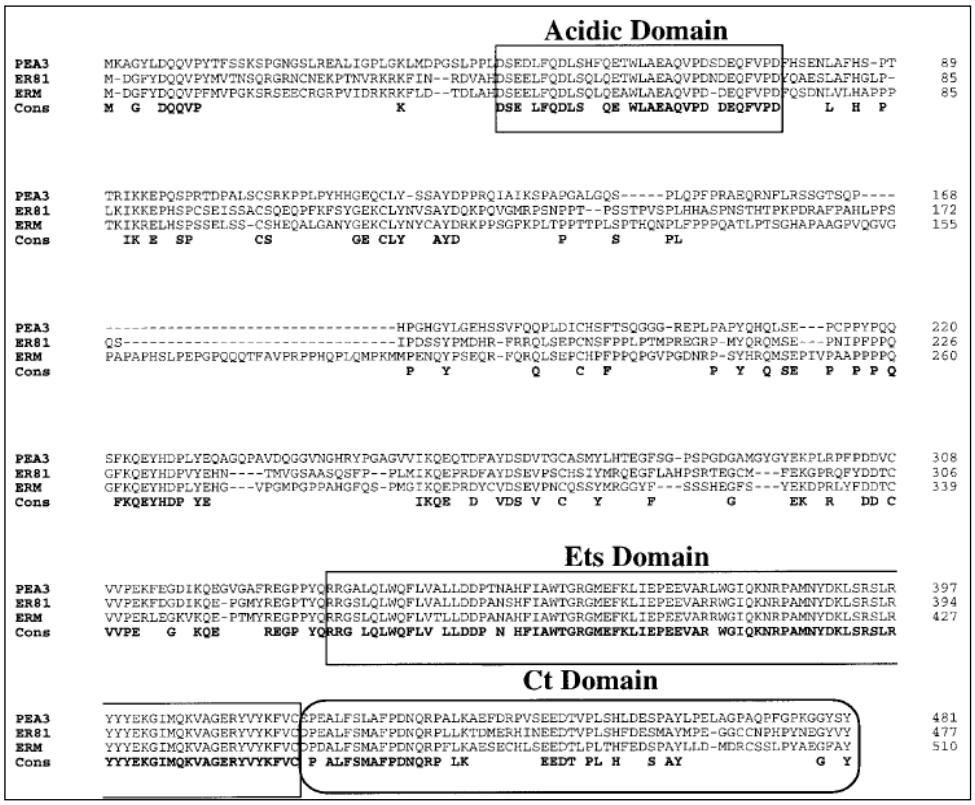


Figure 2.6. Sequence similarity of Pea3 family members; PEA3, ER81 and ERM. 95% identical in ETS domain, 85% identical in acidic domain and 50% identical in carboxyl domain. According to the sequence similarities, ER81 and ERM are more related than the Pea3 [17]

Studies had shown that this highly conserved domains; one in the amino terminal an 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. [17].

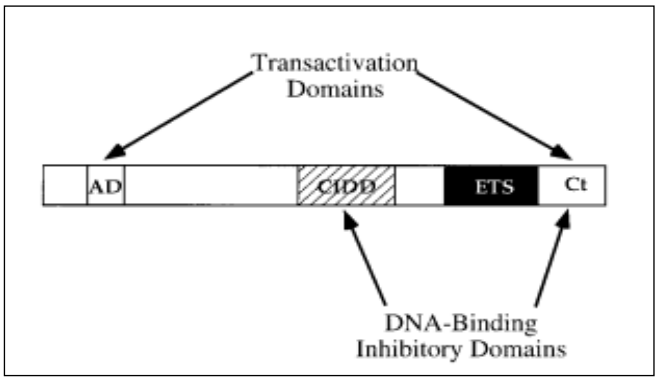


Figure 2.7. Regulatory domains of Pea3 group ETS transcription factor. AD; acidic domain, CIDD: central inhibitory DNA binding domain, ETS: DNA binding domain, CT: carboxy terminal [17]

### 2.2.2. Activation of Pea3 Group by Post-translational Modifications

As noted earlier regulation of gene expression involves the binding of transcription factors to their regulatory regions, promoter or enhancer. This regulation is highly effected by modifications happens to those transcription factors that are involved in this regulation. Sumoylation, acetylation and phosphorylation are one of those modifications anda re called as the post-transational modifications.

The transactivating capacity of Pea3 group is highly effected due to those modifications. It has been shown that Pea3 group members are target for MAPKs and hence get phosphorylated. Pea3 has shown to be phosphorylated upon mitogenic signals by extracellular regulated kinases ERK-1 and ERK-2 and also gets phosphorylated upon stress activated protein kinase (SAPK) or c-Jun N-terminal kinase (JNK) independently [18].

ERM and ER81 also had shown that phosphorylation by both ERK-1 and ERK-2 and stress activated protein kinase (SAPK) or c-Jun N-terminal kinase (JNK) increases their transactivation capacity. In addition to the MAPKs both ERM and ER81 are also phosphorylated by protein kinase A (PKA) [5, 19]. Although the positive regulatory effect of phosphorylation on Pea3 group proteins is known, the exact residues on Pea3 that get phosphorylated in response to particular growth factors are stil unidentified.

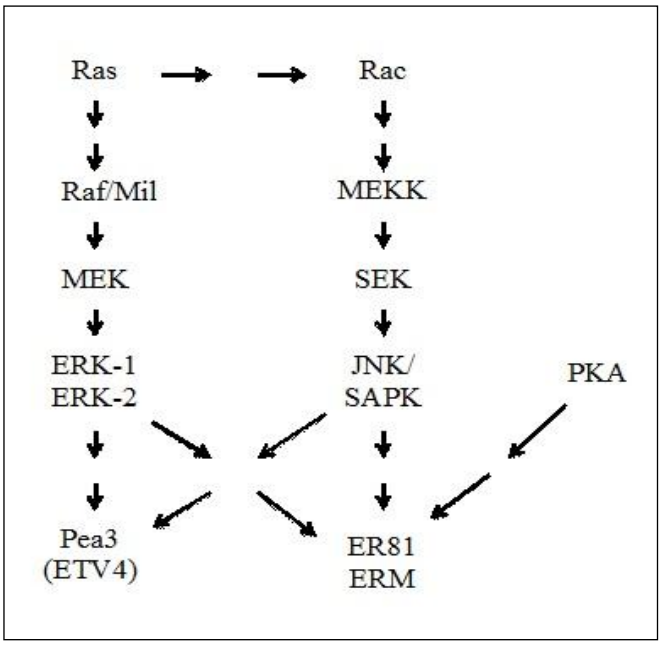


Figure 2.8. Phosphorylation of Pea3 group members by MAPKs.(modified from Yvan de Launoit, 1997) [17]

Sumoylation (covalent conjugation of small ubuquitin like modifier to lysine) and acetylation (addition of a acetyl moeity to lysine) are also one of the post-translational modifications and effects the transactivation capacity of transcription factors.

ER81 acetylation by the Ras/ MAPK phosphorylated p300 acetyltransferase had been shown to increase its transactivation capacity [20]. In addition to that sumoylation also happens at the lysine residues increases ER81 activation. However, SUMO modification of ERM negatively regulates it transactivation capacity [21].

Sumoylation and acetylation of Pea3 also contributes and enhance the transactivation capacity of Pea3 transcription factor [22].

It is important to know such modifications since the presence of one modification may enhance or inhibit the other one to occur.

### 2.2.3. Pea3 Transcription Factor

So far Pea3 transcription factor has been tried to be explained under the Pea3 group, In this section the role of Pea3 in neurons is emphasized mostly.

Pea3 has role in motoneuron innervation on muscles, studies with ER81 and Pea3 were showed that deletion of ER81 interfere with the grow of sensory neurons in the spinal cord but does not effect of its projection to the spinal cord. Therefore sensory neurons can reach to the spinal cord but unable to form connections with the motor neurons [24].

Pea3, whereas is not necessary for the normal development of sensory neurons and this neurons still can make connections in the spinal cord. However it was showed that Pea3 is important in the determination of motor pool neuron identity. The lack of Pea3 motor neurons become unable to branch their axons through the target muscles and may die due to the lack of functional synapse and hence activation [25].

This motor neuron innervation of Pea3 is mediated by the presence of the neurotrophic factor, GDNF. The axons of motor neurons leaving the spinal cord requires neurotrophic factors to approach tov the target muscles. This dependency also regulates the gene expression in these motor neurons and Pea3 is oe of them. Studies show that peripheral neurotrophc signals are required to induce Pea3 expression in the motor neurons which enable them to branch on target muscle [26].

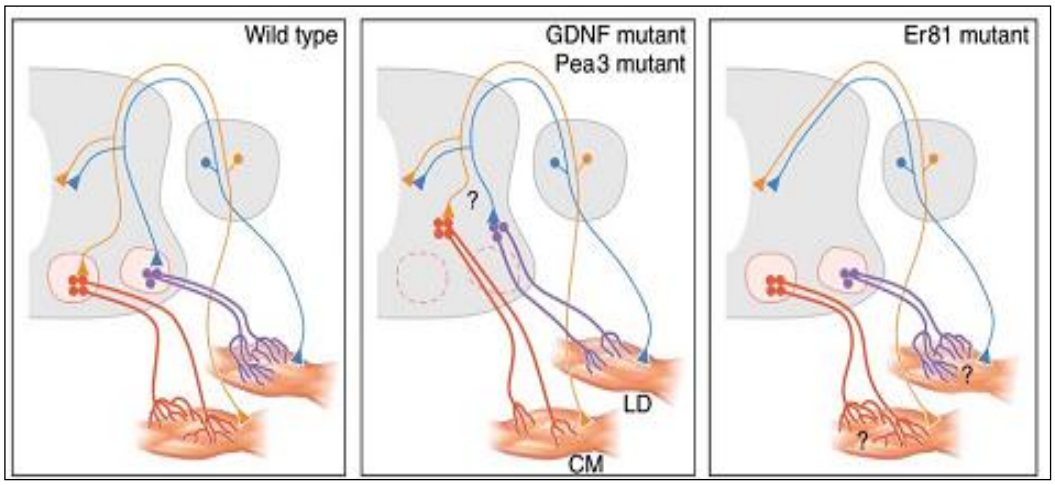


Figure 2.9. Schematic representation of motor neuron innervation on muscles and sensory neuron projection to the spinal cord. Yellow and blue colored neurons are sensory neurons that projects to the motor neuron pools in the spinal cord. Red and prurple colored neurons are the motor neurons that are targeting the muscles. GDNF and Pea3 mutant phenotype is showing that motor neurons are mislocated in the spinal cord and target innervation is disrupted, while sensory neurons are not effected. ER81 mutant shows that sensory neurons have trouble to connect to the motor neurons [27]

Pea3 can also turn non-metastatic tumour to a metastatic one by involving in the regulation of metastatic genes like matrix metalloprotease enzymes, which degrade extracellular matrix proteins and free the cell. MMPs not only involve in metastasis but also cell proliferation, migration and differentiation.

## 2.3. NEUROD1 (NEUROGENIC DIFFERENTIATION) GENE

NeuroD, also defined as BETA2, is a transcription factor with the basic-helix-loop-helix (bHLH) structural feature. These bHLH transcription factors are involved in the fate determination of cells and differentiation. They mediate the expression of cell-type specific genes by binding to consensus sequence, E-box on the promoters of those genes. Such as MyoD drives the differentiation of embryonic cells into the skeletal muscle whereas NeuroD is responsible for the differentiation of embryonic cells into neurons [28].

NeuroD expresison can be seen in stomach, gut, adult lung, pancreas and the nervous sytem. Studies showed that NeuroD can be thought as neuronal differentiation factor since it is able to transform non-neuronal ectodermal cells into neurons when ectopically expressed in the frog. In addition to this NeuroD is also importat for the already diffeferentiated neurons in cerebellum, olfactory bulbs and hippocampus [29].

To understand the importance of NeuroD in the central nervous sytem NeuroD null mice were intended to create, however lack of NeuroD cause to death of these mice and studies were carried out with the mice in which, they are rescued with the insulin promoter mediated NeuroD expression. This study showed that granule cells in the cerebellum are depleted and the dentate gyrus of the hippocampus completely abolished [30].

Liu M. and colleagues also showed that lack of NeuroD shows that proliferation of precursor cells in hippocampus are defected and differentiation is impaired as well, leading to the cell death at granule cell layer of dentate gyrus in the hippocampus [31].

NeuroD expression in cerebellum and in hippocampus starts at early embryogenesis, continue at post-natal development and reach a stable level in the adult brain, however the NeuroD expression in cerebral cortex, spinal cord and pancreas decreases as embryogenesis proceeds [32].

# 3. MATERIALS

## 3.1. BIOINFORMATICS TOOLS

Targets which are going to be cloned into appropriate vectors are determined and analysed through following *in silico* applications.

Table 3.1. Bioinformatics tools utilized during this project

| **Bioinformatics tools** | **Purpose** | **Web site** |
|---|---|---|
| Transcriptional Regulatory Element Database, Cold Spring Harbor Laboratory | Promoter database where promoter sequences can be retrieved | http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home |
| ALGGEN PROMO | A Virtual lab. for t he identification of putative transcription factor binding sites in DNA sequences from species | http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8. 3 |
| IDT DNA Technologies, Oligo Analyzer | Where primers can be designed and ordered | http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/ |
| SDSC Biology WorkBench | Give services with several tools like protein tool, nucleic tools (sequence aligning, restriction enzyme mapping etc.) and also it enables to store nucleic acid and protein sequences. | http://workbench.sdsc.edu/ |
| FinchTV (Geospiza) | To view the raw sequencing data | http://www.geospiza.com/Products/finchtv.shtml/ |

## 3.2. CELL CULTURE

### 3.2.1. Cell Lines

- SH-SY5Y – Human Neuroblastoma Cell Line (ATCC numer: CRL-2266)
- HEK293 – Human Embryonic Kidney Cell Line (ATCC number: CRL-1573)

### 3.2.2. Cell Culture maintaining medium and supplements

- Dulbecco‟s Modified Eagle Medium (DMEM) 1 g/liter Glucose (Gibco)
- Dulbecco‟s Modified Eagle Medium (DMEM) 4,5 g/liter Glucose (Gibco)
- Fetal Bovine Serum
- Penicilin/Streptomycin Solution (100X) (Biochrom)
- 0.5% Trypsin-EDTA Solution (Sigma)
- Phosphate Buffered Saline (PBS) (Gibco)

### 3.2.3. Cell Culture maintaining plates

- T75 Tissue Culture Flasks (Nunc)
- 100mm x 20mm Tissue Culture Dish
- 24-well Culture Plate (Nunc) 25

### 3.2.3. Transfection Reagents

- TransFast
- Polyethylenimine (PEI)

## 3.3. BACTERIAL ASSAYS

### 3.3.1. Bacterial Strain

- *E.coli* JM109 strain

### 3.3.2. Bacterial Assay Reagents and Equipments

- Luria-Bertani (LB) Broth (AppliChem)
- Luria-Bertani (LB) Agar (AppliChem)
- Ampicillin (AppliChem)
- Petri Plates (IsoLab)

## 3.4. PLASMID CONSTRUCTS

- pCMV- 3Tag-6 flag empty vector
- pCMV- 3Tag-6 flag- mPea3 wild type*
- pCMV- 3Tag-6 flag- mPea3 S90A mutant **
- pCMV- 3Tag-6 flag- mPea3 S90E mutant **
- pCMV- 3Tag-6 flag- mPea3 S101A mutant **
- pCMV- 3Tag-6 flag- mPea3 S101E mutant **
- pCMV- 3Tag-6 flag- mPea3 S143A mutant **
- pCMV- 3Tag-6 flag- mPea3 S143E mutant **
- pCMV- 3Tag-6 flag- mPea3 S458A mutant **
- pCMV- 3Tag-6 flag- mPea3 S458E mutant **
- pGL3- Basic Luciferase Reporter Vcetor
- pGL3 – *NeuroD1* promoter wild type***
- pGL3 – *NeuroD1* ets1 mutant promoter****
- pGL3 – *NeuroD1* ets3 mutant promoter****
- pGL3 – *NeuroD1* ets4 mutant promoter****
- pGL3 – *NeuroD1* ets4 deletion mutant promoter****
- pGL3 – *NeuroD1* del1 promoter****
- pGL3 – *NeuroD1* del2 promoter****
- pGL3 – *NeuroD1* del3 promoter****
- pGL3 – *NeuroD1* del4 promoter****

(*, plasmid is kind gift from A.D Sharrocks; **, mutations were created by Berrak Çağlayan; ***, plasmid was constructed by Özlem Demir; ****, mutations were created by Burcu Erdoğan)

## 3.5. COMMERCIAL KITS AND REAGENTS

- High Pure Plasmid Isolation Kit (Roche)
- High Pure PCR Product Purification Kit (Roche)
- Dual-Luciferase Reporter Assay System (Promega)
- T4 DNA Ligation Kit (Takara)
- EZ-ChIPTM (Upstate, Millipore)
- RIPA Buffer (Sigma)
- Protease Inhibitor Coctail (Sigma)
- Phosphatase Inhibitor Coctail (Sigma)
- DNA Ladder (1 Kb and 100 Bp) (Invitrogen)
- 5X Loading Dye (Fermentas)
- FastDigest Restriction Enzymes (Fermentas)

  KpnI 5'...G G T A C^C...3' / 3'...C^C A T G G...5'

  HindIII 5'...A^A G C T T...3' / 3'...T T C G A^A...5'

# 4. METHODS

## 4.1. PREPARATION OF PLASMID CONSTRUCTS

Hereby, the procedure of how to reveal a possible binding sites on a given promoter sequence and how to design primers were shown. Since this study already had chosen the *NeuroD1* as a potential target, all evaluations were done according to that gene.

### 4.1.1. Retrieving Pea3 TF Target Gene Promoter Sequences

Transcriptional Regulatory Element Database Cold Spring Harbor Laboratory (TRED) was utilized to find the promoter sequence of target genes'.

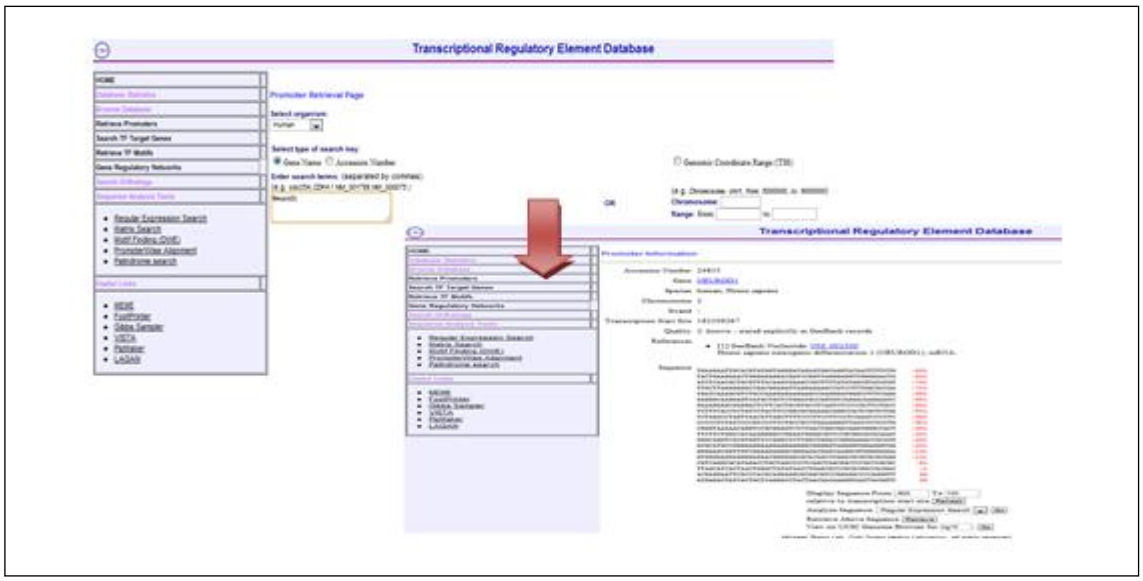


Figure 4.1. The work flow of *Transcriptional Regulatory Element Database*. (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=searchPromForm)

### 4.1.2. Analyzing target sequence for possible Pea3 TF binding sites

ALGGEN PROMO, a virtual laboratory for the study of transcription factor binding sites in DNA sequences was utilized to find, from which sites Pea3 TF binds to the given promoter sequence.

(http://alggen.lsi.upc.es/cgibin/promo_v3/promo/promoinit.cgi?dirDB= TF_8. 3)

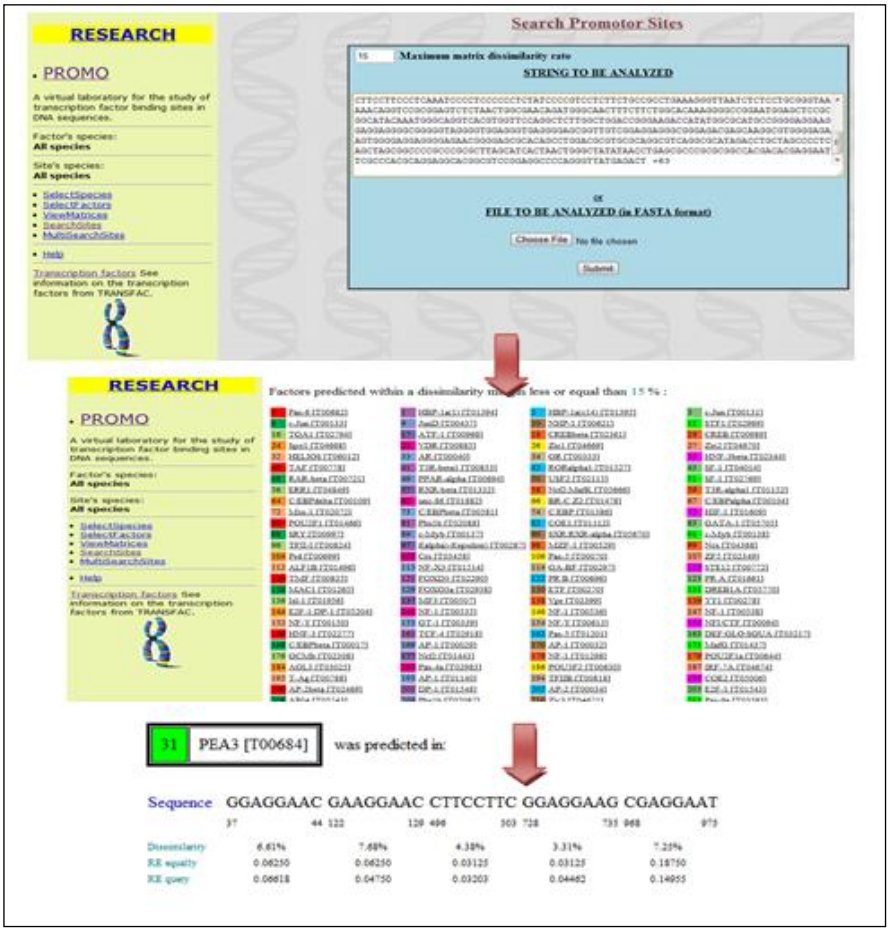


Figure 4. 2. The work flow of *ALGGEN PROMO*

### 4.1.3. Primer Design

1000bp long *NeuroD1* promoter which encomapass 5 possible Pea3 TF binding sites had previously cloned (by Özlem Demir) into the pGL3 Basic Luciferase Reporter Vector (Promega). In order to study whether these ets motifs are indeed responsible for Pea3

binding, mutations were introduced with site ditected mutageneis technique (SDM) to block this possible interaction. Later on deletions were introduced to NeuroD1 promoter to delete ets motifs sequentially. The online tool *Integrated DNA Technologies* was utilized to check the quality of designed primer pairs concerning their GC contents, melting temperatures (Tm), hairpin structure, and BLAST control through out the genome to avoid non-specific binding.

Table 4.1. Sequences of forward and reverse primers used to create mutant NeuroD1 promoter

| | |
|---|---|
| Ultimate Forward (*hNeuroD1*-Luc) | 5'- ACG AGA CGG TAC CAC TGA CGT AGT GAG AGG GTC TGG -3' |
| Ultimate Reverse (*hNeuroD1*-Luc) | 5'- ACG AGA CAA GCT TGT GAT AGT CTC ATA ACC CTG GGC -3' |
| ets motif-1 mutation Forward (*hNeuroD1*-ets1mut-Luc) | 5'- ACG AGA CGG TAC CCG TAG TGA GAG GGT CTA AAC ACA C- 3' |
| ets motif-1 mutation Reverse (*hNeuroD1*-ets1mut-Luc) | (ultimate reverse primer vas used to have complete NeuroD1 sequence since ets-1 motif resides at the very beginning of the sequence) |
| ets motif-3 mutation Forward (*hNeuroD1*-ets3 mut-Luc) | 5'- AAC ATT AGC TTT TAC ACA CAC ACC CTC AAA TCC C – 3' |
| ets motif-3 mutation Reverse (*hNeuroD1*-ets3mut-Luc) | 5'- GGA GGG GAT TTG AGG GTG TGT GTG TAA AAG CTA A -3' |
| ets motif-4 mutation Forward (*hNeuroD1*-ets4mut-Luc) | 5'- ATG GCG CAT GCC GGA AAA AAA AGA GGA GGG-3' |
| ets motif-4 mutation Reverse (*hNeuroD1*-ets4mut-Luc) | 5'- GGC CCC TCC TTC TTT TTT TTC GGC ATG C-3' |
| ets motif-4 deletion mutation Forward (*hNeuroD1*-ets4 del mut-Luc) | 5'- CAT GCC GGG GAA GGA GGA-3' |
| ets motif-1 truncation Forward (*hNeuroD1*-del1-Luc) | 5'- AGA CGG TAC CAG TCG TAG CTG AAG GTC AGG-3' |
| ets motif-2 truncation Forward (*hNeuroD1*-del2-Luc) | 5'- AGA CGG TAC CTG GGA GAG GAC GAT CCG GTT AG-3' |
| ets motif-3 truncation Forward (*hNeuroD1*-del3-Luc) | 5'- AGA CGG TAC CCC TCA AAT CCC CTC CCC CCT C-3' |
| ets motif-4 truncation Forward (*hNeuroD1*-del4-Luc) | 5'- AGA CGG TAC CGA GGA GGG GCG GGG GTA G-3' |

### 4.1.4. Site Directed Mutagenesis

Site Directed Mutagenesis (SDM) is a technique where we can introduce targeted mutations into the site of interest with the PCR method by amplifying desired string with the mutagenic primers (Table 4.1.). Two steps PCR method was used in order to generate mutant sequence. In the first step of the two steps PCR method mutagenic primers were coupled with the primers that constrains the NeuroD1 promoter.

In the second step of SDM, PCR products from 1st step PCR and ultimate forward and reverse primers were put into reaction to have complete NeuroD1 promoter sequence.

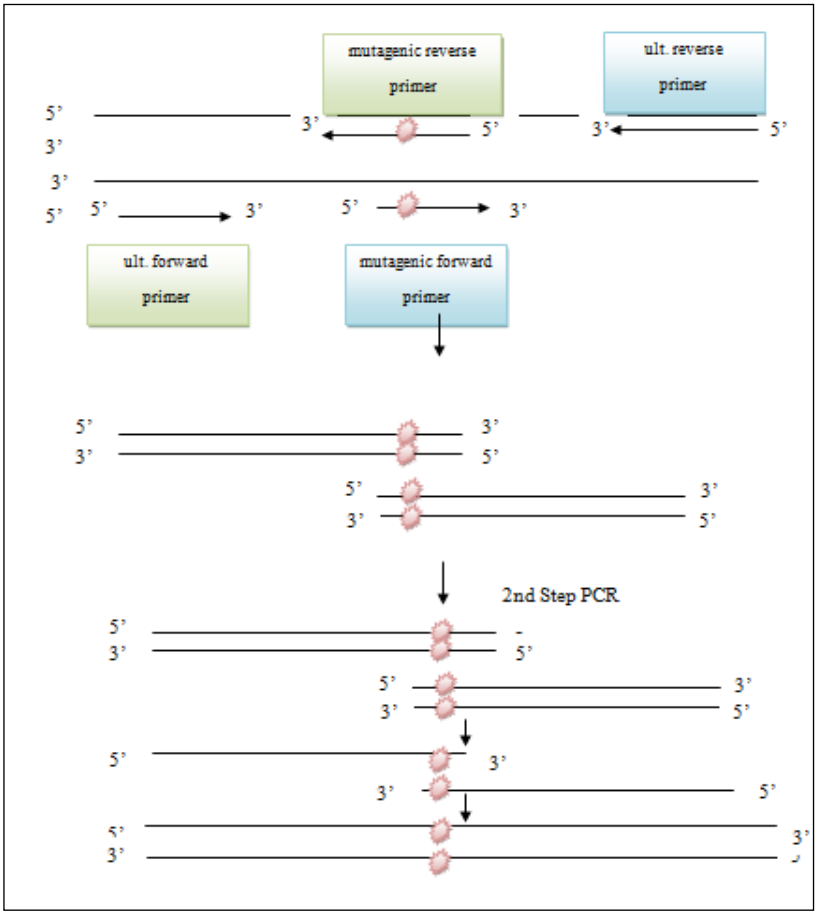


Figure 4.3. Schematic representation of 1st step PCR of Site Directed Mutagenesis (SDM). Mutagenic forward primer is coupled with the ultimate reverse primer and mutagenic reverse primer is coupled with the ultimate forward primer in the first step of the SDM. 2nd step of SDM. PCR products from 1st step PCR were annealed each other and create a

template for ultimate forward and reverse primers in the 2nd step PCR. Since 1000bp long NeuroD1 had already cloned, pGL3-NeuroD1 was used as a template in the SDM reactions

Reaction protocol is as follows:

Table 4.2. Ingredients of the 1st step PCR (common for all mutation reactions)

| **Ingredient** | **Volume** | **Final concentration** |
|---|---|---|
| Template (100ng) | 0,5 µl | 1 ng |
| Pfu DNA polymerase 10X Buffer with $MgSO_4$ | 5 µl | 1X |
| Primer (ult.F / ult R.) | 1 µl | 1µM |
| Primer (mut. R. / mut. F ) | 1 µl | 1µM |
| dNTP (2mM for each) | 5 µl | 0,2 mM |
| Pfu DNA polymerase | 0,5 µl | 1.25 u |
| $dH_2O$ | up to 50 µl | |

Table 4.3. 1st step PCR. for ets-1 mutation with mutant forward & ultimate reverse primers

| | **Temperature** | **Duration** | **# of Cycle** |
|---|---|---|---|
| **Initial Denaturation** | 95°C | 3 min. | 1 cycle |
| **Denaturation** | 95°C | 30 sec. | 30 cycles |
| **Annealing** | 52 °C | 30 sec. | |
| **Extension** | 72°C | 1.5 min. | |
| **Final Extension** | 72°C | 7.5 min. | 1 cycle |

Table 4.4. 1st step PCR. for ets-3 mutation with mutant forward & ultimate reverse primers

| | Temperature | Duration | # of Cycle |
|---|---|---|---|
| **Initial Denaturation** | 95°C | 3 min. | 1 cycle |
| **Denaturation** | 95°C | 30 sec. | 30 cycles |
| **Annealing** | 60 °C | 30 sec. | |
| **Extension** | 72°C | 1.5 min. | |
| **Final Extension** | 72°C | 7.5 min. | 1 cycle |

Table 4.5. 1st step PCR. for ets-3 mutation with mutant reverse and ultimate forward primers

| | Temperature | Duration | # of Cycle |
|---|---|---|---|
| **Initial Denaturation** | 95°C | 3 min. | 1 cycle |
| **Denaturation** | 95°C | 30 sec. | 30 cycles |
| **Annealing** | 58.8 °C | 30 sec. | |
| **Extension** | 72°C | 1.5 min. | |
| **Final Extension** | 72°C | 7.5 min. | 1 cycle |

Table 4.6. 1st step PCR. for ets-4 mutation with mutant forward and ultimate reverse primers

| | Temperature | Duration | # of Cycle |
|---|---|---|---|
| **Initial Denaturation** | 95°C | 3 min. | 1 cycle |
| **Denaturation** | 95°C | 30 sec. | 30 cycles |
| **Annealing** | 53.1 °C | 30 sec. | |
| **Extension** | 72°C | 1.5 min. | |
| **Final Extension** | 72°C | 7.5 min. | 1 cycle |

Table 4.7. 1st step PCR. for ets-4 mutation with mutant forward and ultimate reverse primers

| | **Temperature** | **Duration** | **# of Cycle** |
|---|---|---|---|
| **Initial Denaturation** | 95°C | 3 min. | 1 cycle |
| **Denaturation** | 95°C | 30 sec. | 30 cycles |
| **Annealing** | 52 °C | 30 sec. | |
| **Extension** | 72°C | 1.5 min. | |
| **Final Extension** | 72°C | 7.5 min. | 1 cycle |

The outcome the 1st PCR products were run on the 1.5% agarose gel and the gel was visualized under ultra-violet light (UV) with BioRad® Gel Imager. The desired bands were cut out of the gel and purified with PureLink PCR Purification Kit (Invitrogen) according to protocol that the kit suggests.

Isolated PCR products namely template down and template up were used as templates of the 2nd step PCR with appropriate ingredients and reaction protocol as given below.

Table 4.8**.** Ingredients of the initial mix of 2nd step PCR. PCR products from 1st step PCR

| **1st mix** | | |
|---|---|---|
| **Ingredients** | **Volume** | **Final Concentration** |
| Template Up | varies | 1ng |
| Template Down | varies | 1ng |
| Pfu Polymerase<br>10X Buffer with $MgSO_4$ | 3 µl | 1X |
| dNTP (2mM) | 3 µl | 0,2 mM |
| Pfu DNA Polymerase | 0,3 µl | 1.25 u |
| $dH_2O$ | up to 30 µl | |
| After first reaction was run for 10 cycles 2nd mix was added to the reaction tube | | |
| **2nd Mix** | | |
| **Ingredients** | **Volume** | **Final Concentration** |
| Pfu Polymerase<br>10X Buffer with $MgSO_4$ | 2 µl | 1X |
| Ultimate forward primer | 0,5 µl | 1µM |
| Ultimate forward primer | 0,5 µl | 1µM |
| dNTP (2mM) | 2 µl | 0,2 mM |
| Pfu DNA Polymerase | 0,2 µl | 1.25 u |
| $dH_2O$ | up to 20 µl | |

Table 4. 9. Reaction for the 2nd step PCR. for ets-3 mutation with PCR products from 1st step

| | **Temperature** | **Duration** | **# of Cycle** |
|---|---|---|---|
| Initial Denaturation | 95°C | 3 min. | 1 cycle |
| Denaturation | 95°C | 30 sec. | 10 cycles |
| Annealing | 56 °C | 30sec | |
| Extension | 72 °C | 2min | |
| Final Extension | 72 °C | 10 min | 1 cycle |
| 2nd reaction after addition of 2nd mix (containing ultimate forward and reverse primers) | | | |
| Initial Denaturation | 95°C | 3 min. | 1 cycle |
| Denaturation | 95°C | 30 sec. | 30 cycles |
| Annealing | 52 °C | 30sec | |
| Extension | 72 °C | 2min | |
| Final Extension | 72 °C | 10 min | 1 cycle |

Table 4.10. Reaction for the 2nd step PCR. for ets-4 mutation with PCR products from 1st step PCR

| | **Temperature** | **Duration** | **# of Cycle** |
|---|---|---|---|
| Initial Denaturation | 95°C | 3 min. | 1 cycle |
| Denaturation | 95°C | 30 sec. | 10 cycles |
| Annealing | 56 °C | 30sec | |
| Extension | 72 °C | 2min | |
| Final Extension | 72 °C | 10 min | 1 cycle |
| 2nd reaction after addition of 2nd mix (containing ultimate forward and reverse primers) | | | |
| Initial Denaturation | 95°C | 3 min. | 1 cycle |
| Denaturation | 95°C | 30 sec. | 30 cycles |
| Annealing | 52 °C | 30sec | |
| Extension | 72 °C | 2min | |
| Final Extension | 72 °C | 10 min | 1 cycle |

Initial mix and reaction provides the template up and down to anneal each other and serve as template for the ultimate forward and reverse primers in the final reaction.

The resultant PCR product after this final step was the whole NeuroD1 promoter clone in which desired mutations were introduced to. As noted before pGL3-NeuroD1 construct had been used as a template, however, with the polymerase chain reactions mutant NeuroD1 promoter was amplified seperately from the rest of the plasmid. So it has to be cloned into pGL3 Basic Luciferase Reporter Vector again. Following protocols explaines the clonning procedures.

### 4.1.5. Promoter Truncation

Truncation as the name refers is a technique where a regions of DNA are sequentially removed from the entire string. Truncation is carried out with PCR. Primers are designed so as to border the entire sequence to be truncated. The Figure below shows the NeuroD promoter sequence. The highlighted strings are the sequences where forward primers will bind.Primers bind immediately after the putative Pea3 binding motif in order to discard them. A common reverse primer was paired to each of these forward primers so as toamplify the confined region. Sequences of primers and reaction protocol are given below (Table 4.11 - 4.13.)


Figure 4.4. Truncation primers for *hNeuroD1*promoter ( ). Primers exclude the ets motifs for Pea3 binding. Truncation starts from the first nucleotide of the NeuroD1 promoter and ends at the last nucleotide of the binding motifs (shown in red). The first truncation is 29 bp (del1), second truncation is 114 bp (del2), third truncation is 488 bp (del3) and the last truncation is 720 (del4). The last Pea3 binding, ets5 motif, was excluded in the truncation studies since the remaining promoter will not work efficiently

Table 4.11. Sequence of *NeuroD1* truncation primers

| | | **Flanking sequence** | **Kpn*I*** | | |
|---|---|---|---|---|---|
| Del1 | 5'- | AGAC | GGTACC | AGTCGTAGCTGAAGGTCAGG | -3' |
| Del2 | 5'- | AGAC | GGTACC | TGGGAGAGGACGATCCGGTTAG | -3' |
| Del3 | 5'- | AGAC | GGTACC | CCTCAAATCCCCTCCCCCCTC | -3' |
| Del4 | 5'- | AGAC | GGTACC | GAGGAGGGGCGGGGGTAG | -3' |
| A common Reverse Primer was paired to all forward primers | | | | | |
| | | **Flanking sequence** | **Hind*III*** | | |
| Reverse | 5'- | ACGAGA | CAAGCTT | GTGATAGTCTCATAACCCTGGGC | -3' |

Table 4.12. Ingredients for the reaction mix

| **Ingredient** | **Volume** | **Final concentration** |
|---|---|---|
| Template (100ng) | 0,5 µl | 1 ng |
| Pfu DNA polymerase 10X Buffer with $MgSO_4$ | 5 µl | 1X |
| Primer (ult.F / ult R.) | 1 µl | 1µM |
| Primer (mut. R. / mut. F ) | 1 µl | 1µM |
| dNTP (2mM for each) | 5 µl | 0,2 mM |
| Pfu DNA polymerase | 0,5 µl | 1.25 u |
| $dH_2O$ | up to 50 µl | |

Table 4.13. Reaction protocol for PCR. Annealing temperatures were given for all truncation primers

| | **Temperature** | **Duration** | **# of Cycle** |
|---|---|---|---|
| **Initial Denaturation** | 95$^{o}$C | 3 min. | 1 cycle |
| **Denaturation** | 95$^{o}$C | 30 sec. | 30 cycles |
| **Annealing (del1/ del2/ del3/ del4)** | 56.1 $^{o}$C / 60 $^{o}$C /61. 7 $^{o}$C/ 62. 7 $^{o}$C | 30 sec. | |
| **Extension** | 72$^{o}$C | 1.5 min. | |
| **Final Extension** | 72$^{o}$C | 7.5 min. | 1 cycle |

### 4.1.6. Restriction Digestion

Before restriction digestion the resultant PCR product from the 2nd step of the SDM PCR was purified with PureLink PCR Purification Kit (Invitrogen). Concentration of the purified PCR product was measured with the IMPLEN Nanodrop.

To be able to clone the insert to the vector, both the insert and the vector had to be digested with restriction enzymes. Restriction enzymes' were selected from the multiple clonning site of the pGL3 Basic vector namely Kpn*I* and Hind*III* and their recognition sequences had been added to the 5' end of the forward and reverse primers respectively. Hence digestion reaction with restriction enzymes Kpn*I* and Hind*III* (FastDigest Fermentas) were used to create complementary overhangs both in vector and insert. The restriction digestion reactions for inserts and vector were prepared according to Table 4.7.

Table 4.14. Restriction digestion reaction for NeuroD1 mutant inserts and pGL3 Basic Luciferase Reporter vector (Promega)

| Ingredients | Volume | Final Concentration |
|---|---|---|
| Insert / Vector (NeuroD1 mutant promoter) | Varies | 200 ng / 1000 ng |
| Kpn*I* Restriction Enzyme | 1 µl | 1: 20 |
| Hind*III* Restriction Enzyme | 1 µl | 1: 20 |
| 10X FastDigest Buffer | 2 µl | 1X |
| $dH_2O$ | up to 20 µl | |

Digestion reaction was carried out at 37$^{o}$C for 2 hours. In order to check whether digestion with vector was successful or not, digested plasmid vector was run on the 1,5 % agarose gel. Since plasmid vector is in circular form it should be linearized when digested from the multiple clonning site and give single band on the gel which corresponds to the length of the vector. There was no need to check the insert since it was alredy linear. After digesting

the inserts and plasmid vector, samples were purified with PureLink PCR Purification Kit (Invitrogen). Concentration of the purified digested products were measured with the IMPLEN Nanodrop.

### 4.1.7. Ligation Reaction

Ligation reaction mixture was prepared with the different vector: insert ratios given in Table 4. 8.in order to get the best ligation reaction. Ligation mixtures were kept in +4$^{o}$C for 16 hours.

Table 4.15. Ligation reaction ingredients with different vector: insert ratios

| | **Vector: Insert Ratio** | | **Final Concentration** |
|---|---|---|---|
| **Ingredients** | **1: 3** | **1: 5** | |
| Digested Plasmid Vector | Varies | varies | 100 ng / 150 ng |
| Digested Insert | Varies | varies | varies |
| 10X FastDigest Buffer | 6 µl | 6 µl | 1X |
| T4 DNA Ligase (Invitrogen) | 1 µl | 1 µl | 1u |
| ATP (100mM) | 1,5 µl | 1,5 µl | 5mM |
| $dH_2O$ | up to 30 µl | up to 30 µl | |

The equation to calculate how much of vector should be added to the insert for given vctor: insert ratios is as follows;

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

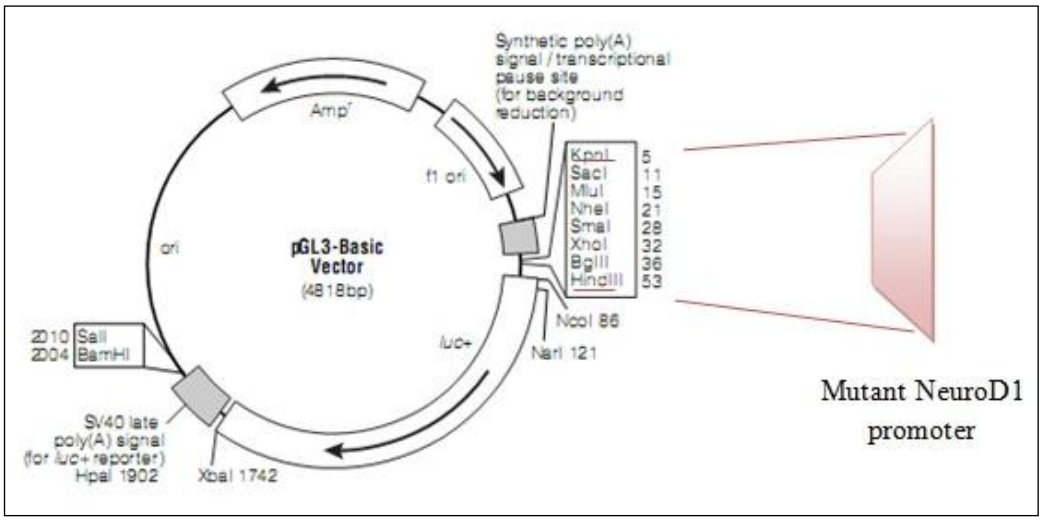


Figure 4.5. Schematic representation of NeuroD1 promoter ligation into pGL3 Basic Luciferase Reporter Plasmid ( Promega)

### 4.1.8. Competent Bacterial Cell Preparation

After ligation reaction was completed, pGL3 vector, containing mutant NeuroD1 promoter had to be amplified. Amplification was carried out by bacterial cells, since they enables the replication of plasmid DNA while they are replicating their own genome. JM109 strain of E.coli were used as bacterial strain in order to incorporate plasmid DNA into them. Bacteria were passed through several applicaions in order to make them competent for plasmid DNA. Previously frozen bacterial stock was thawed, 2 µl of bacteria was transferred to 10 ml LB broth medium without ampicillin (Amp) and inoculated a starter culture in shaker at 37$^{o}$C overnight (about 16 hours). Next day, 1 ml inoculum was added to 50 ml Amp (-) LB Broth. The new culture was again placed in 37$^{o}$C shaker for about 3 hours which enables bacteria to reach their growth in log phase. Then, the culture was centrifuged at 5500 rpm for 10 minutes at 4$^{o}$C. The supernatant was discarded and the pelleted bacteria was gently resuspended in 5 ml ice-cold CaCl2 (100 mM) and incubated 1 hour on ice. Then, the mixture was centrifuged at 5500 rpm for 5 minutes at 4$^{o}$C. The supernatant was discarded and the pellet was resuspended in fresh 1 ml ice-cold $CaCl_2$.

### 4.1.9. Transformation

Bacterial cells were amplified and become competent for the plasmid DNA but still needs modification for DNA uptake. Calcium treatment together with heat shock enable bacterial membrane to hold and take the plasmid DNA. Bacterial cell membrane is permeable to chloride ions, yet calcium ions cannot pass through the membrane. Water molecules accompany the chloride ions when they are entering the cell. This influx of water causes the cells to swell. CaCl2 treatment is necessary for the heat shock proteins to be expressed. Heat shock is another fundamental part of the transformation processes that enables foreign DNA intake by shrinkage and swelling of cells due to the changing temperatures. If the foreign DNA has an antibiotic resistance gene, after transformation, that gene is transcribed and the bacteria can be selected for that incorporation.

50 µl of competent cell was put into a 1,5 ml eppendorf tube together with 5 µl of each ligation mixture, then eppedorf tubes were placed into ice and kept in there for 15 minutes. Then, they were placed into a heater block which was set previously to the 37$^{o}$C for 90 seconds and again to ice for 2 minutes in order to carry out the heat shock. On bench, 500 µl of Amp free LB-Broth was added to the tubes and they were placed in 37$^{o}$C shaker for 1 hour to enable bacterial cell to express ampicillin from the plasmid DNA.

### 4.1.10. Selective growth of the transformed bacterial cells on Amp (+) plates

After an hour, mixture was centrifuged at 4000 rpm for 4min. at RT to settle down the bacteria. 400 µl of the LB broth supernatant was discarded and pelleted bacteria were resuspended in l00 µl of the LB broth. Resuspended bacteria were transferred onto the Amp positive LB agar plates and with a help of inocolumn loop they were spreaded onto the plate. The plates were inverted and placed in an 37$^{o}$C incubator overnight.

The aim of this selection is to seperate bacterial colonies that do not have desired plasmid DNA, because only the ones that posses the plasmid DNA will survive and grow on the Amp (+) plates, since plasmid carries Amp$^{r}$ gene and will provide resistance to that antibiotic.

### 4.1.11. Colony PCR for plasmid with insert verification

Before inoculating and isolating plasmids, colonies had grown on the amp (+) should be checked whether they carry the plasmid with insert, instead plasmid only. Colony PCR was done for that purpose with the given protocol in Table 4. 9. In the colony PCR reaction, with a help of tip very small amount of colony is chosen from the plate and suspended in a 5µl of distilled water in a PCR tube. Then the rest of the ingredients of a PCR with the primer pairs specific to the insert were added into the reaction tube. If there is a plasmid with insert DNA then there is going to be amplification of insert DNA which then can be verified on agarose gel.

Table 4.16. Ingredients of Colony PCR

| **Ingredients** | **Volume** | **Final Concentration** |
|---|---|---|
| Template (Bacterial Colony suspended in 5µl of $dH_2O$) | 5µl | varies |
| 10X DFS-Taq Polymerase Reaction Buffer with $MgCl_2$ | 3 µl | 1 X |
| Forward primer | 0,3 µl | 75 nM |
| Reverse primer | 0,3 µl | 75 nM |
| dNTP (2mM) | 2,4 µl | 160 µM |
| DFS Taq DNA polymerase (BIORON) | 0,25 µl | 1,25 u |
| $dH_2O$ | up to 30 µl | |

Table 4.17. Reaction of the colony PCR

| | **Temperature** | **Duration** | **# of Cycle** |
|---|---|---|---|
| Initial Denaturation | 94$^{o}$C | 5 min. | 1 cycle |
| Denaturation | 94$^{o}$C | 10 sec. | 30 cycles |
| Annealing | varies | 20 sec. | |
| Extension | 72 $^{o}$C | 1 min. | |
| Final Extension | 72 $^{o}$C | 10 min. | 1 cycle |

### 4.1.12. Plasmid Isolation

The following day after transformation, sample plates and control plates were checked if there were any bacterial colonies. If no colonies observed with the negative controls than that means no contamination was involved during transformation and any colonies on sample plates can be used for plasmid isolation.

For the plasmid isolation step, bacteria had grown on the agar plates should be amplified in an amp (+) LB Broth. The amount of LB Broth in which bacteria are going to be grown dependes on the type of the kit that is going to be used and the origin of replication (ORI) of the plasmid ( which determines the level of replication of the plasmid). In our case pGL3 posses ORI with a high copy numeber so inocolumn volume varies with the kit acoording to the protocol kit suggests.

### 4.1.13. Evaluation of the cloned gene sequence

After promoters were amplified by PCR with the appropriate target primers and cloned into vector plasmids, they are sent to commercial sequencing in order to proove they had the correct sequence as noted in the bioinformatic resources. The results of the analysis are 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 promoter and the expected mutant promoter sequences were aligned.

## 4.2. LUCIFERASE REPORTER ASSAY

### 4.2.1. Transient Transfection of Cells

Cells (HEK293, SH-SY5Y) were seeded in triplicate into 24 - well plates at a density of 5 x $10^4$ cells / well, and 24h later cells in each well were transfected with a total of 500 ng of DNA using g Polyethylenimine (PEI) (CellnTech).

The ingredients of transfection mix were given in Table 4.11. The mix was prepared in a 600 µl of serum free medium and vortexed for 10sec. briefly spinned down and then left to wait at RT for 15 min. By the end of waiting of mix medium of cells were aspirated and transfection mix was distributed as 200 µl mix per well. Cells with transfection mix were incubated in 37 $^oC$ in a humidified 5% $CO_2$ atmosphere for 2 hours. After 2h incubation medium of the cells were completed to 500 µl with the DMEM (1g / L glucose, for SH-SY5Y and 4,5 g / L glucose for HEK293 cell lines) supplemented with 10% new-born calf serum 1% antibiotic andplaced into 37 $^oC$ in a humidified 5% $CO_2$ atmosphere for 48 hours.

### 4.2.2. Luciferase Assays

Luciferase assay is a commonly used technique to monitor the regulation (either up or down) of gene elements. Dual-Glo® Luciferase Assay System (Promega) provides powerful technique when considering the presence of an internal control, Renilla Luciferase. While the expression of experimental construct (promoter of interest mediated Luciferase expression) happens with the experimental conditions, the activity of internal control Renilla Luciferase serve as baseline response. Hence, the activity of expermental construct can be normalized to the activity of internal control to eliminate negative effects which may be caused by cell death, transfection efficiency or cell lysis efficiency and increase experimental accuracy.

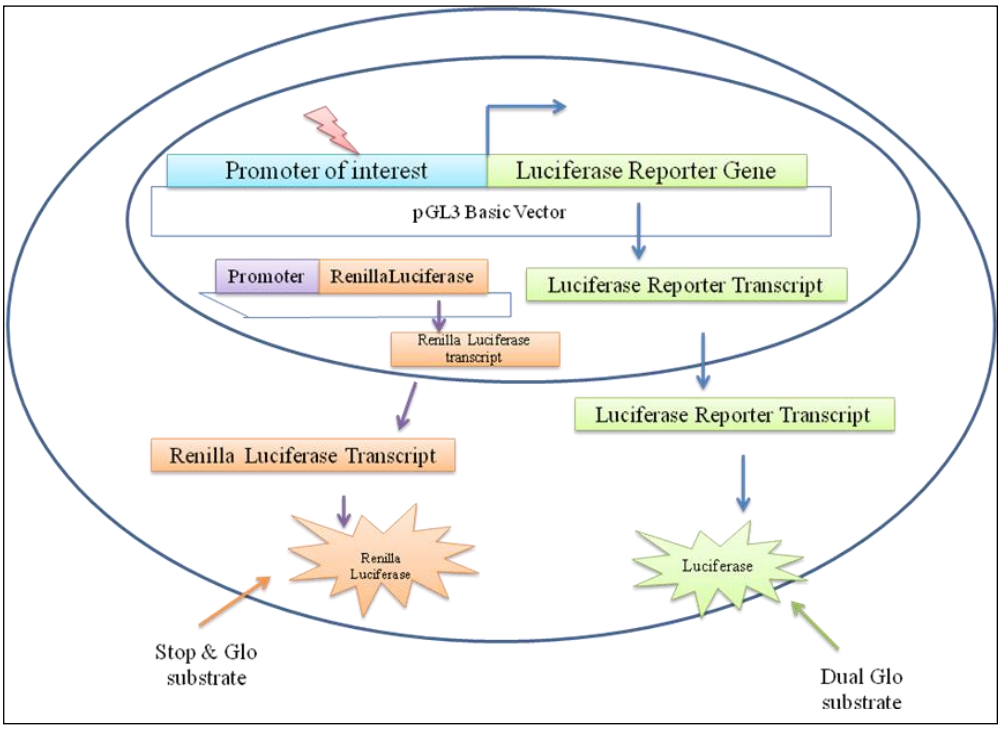


Figure 4.6. Schematic representation of Dual-Glo® Luciferase Assay System within the cell

#### *4.2.2.1. Preparation of Luciferase Substrates*

Lyophilized Assay Substrate was resuspended in 10 ml Luciferase Assay buffer II. The solution was aliquoted and kept at -80$^{o}$C. Lyophilized Stop and Glo substrate was resuspended in 200 ml Stop and Glo substrate solvent to make 50X substrate. 1 volume of50X Stop and Glo substrate was added to 50 volumes of Stop and Glo buffer. The solution was aliquoted and kept at -80$^{o}$C.

#### *4.2.2.2. Cell Lysis*

5X Passive Lysis Buffer (PLB) which is provided with Dual-Glo® Luciferase Assay System (Promega) was used in order to lyse the cells. Firstly 5X PLB was diluted to the 1X with the $dH_2O$. The medium on the cells were aspirated and cells were washed with 1X PBS in order to remove any residual medium. After then, 150 µl of PLB was added onto cells and plate containing the cells was vigorously shaked on the vortex for 30sec. Additional 15 min incubation on a shaker platform was done at RT.

#### *4.2.2.3. Luciferase Assay*

Lysed cells were then measured for their luciferase activity. 30µl of cell lysate triplacate for each sample was transferred to the white opaque luminometer microtiter 96-well plate. Then equal volume of Dual-Glo was added onto each well and firefly luciferase luminescent signal was measured for 10sec. The Renilla Luciferase luminescent signal was measured for 10sec. as Stop&Glo was added into each well. Results were exported to Microsoft Excell and relative Luciferase activity was calculated by taking the ratio of firefly luciferase luminescent signal: Renilla Luciferase luminescent signal for each well, and then avarage of all ratios were plotted and error bars were put according to the standard deviations.

Table 4.18. Ingredients for Transfection of Wild Type Pea3 Titration

| pCMV -3Tag-6 flag- (ng) | pCMV-3Tag-6 flag- mPea3 wild type (ng) | pGL3-NeuroD1 wild type or mutant (Firefly Luciferase) (ng) | Renilla Luciferase (ng) | PEI (µl) |
|---|---|---|---|---|
| 200 | - | 200 | 100 | 2 |
| 195 | 5 | 200 | 100 | 2 |
| 175 | 25 | 200 | 100 | 2 |
| 150 | 50 | 200 | 100 | 2 |
| 100 | 100 | 200 | 100 | 2 |
| 50 | 150 | 200 | 100 | 2 |
| - | 200 | 200 | 100 | 2 |

Table 4.19. Ingredients for transfection of Pea3 Phosphorylation Mutants

| | (ng) | pCMV -3Tag-6 flag- (ng) | NeuroD-Luc (FireflyLuciferase) (ng) | Renilla Luciferase (ng) | PEI (μl) |
|---|---|---|---|---|---|
| pCMV | - | 200 | 200 | 100 | 2 |
| Pea3 (wt) | 100 | 100 | 200 | 100 | 2 |
| S90A | 100 | 100 | 200 | 100 | 2 |
| S90E | 100 | 100 | 200 | 100 | 2 |
| S101A | 100 | 100 | 200 | 100 | 2 |
| S101E | 100 | 100 | 200 | 100 | 2 |
| S143A | 100 | 100 | 200 | 100 | 2 |
| S143E | 100 | 100 | 200 | 100 | 2 |
| S458A | 100 | 100 | 200 | 100 | 2 |
| S458E | 100 | 100 | 200 | 100 | 2 |

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## 4.3. CHROMATIN IMMUNOPRECIPITATION (CHIP) ASSAY

Chromatin Immunoprecipitation (ChIP) is a techniques that is used to reveal the protein-DNA interactions. Different than other protein- DNA interaction studies, with ChIP exact binding sites can be revealed. Assay relays on fixing or crosslinking, interacting proteins on DNA pieces with the chemical action of formaldehyde. Then crosslinked DNA is sheared with nuclease enzyme reaction. DNA pieces carrying interacting proteins were precipitated with a antibody specific to the interacting protein. Immunoprecipitated DNA-protein complex then treated with high salt solution and heat in order to seperate protein from DNA piece which is called as reverse-crosslinking. Reverse-crosslinked DNA then eluted and purified and subjected to the PCR with the primers that constrains binding motif for the interacting protein.

### 4.3.1. Primer Design for Chromatin Immunoprecipitation (ChIP)

The resultant sample obtained from the ChIP is the eluted and purified DNA from immunoprecipitant. The evaluation of DNA in hand was done with primers that were designed to constraint the possible binding sites for Pea3 TF on NeuroD1 promoter. The following DNA sequence belongs to the 1000bp (-947 to +63) NeuroD1 and showing the possible Pea3 TF binding sites in red. The 20bp long sequences underlined with arrows are showing the primer pairs confining the binding motifs highligted with yellow color.

Table 4.20. Sequence of ChIP primers

| | Putative Pea3 binding sequence | Forward Primer | Reverse Primer |
|---|---|---|---|
| Ets 1 | 5'-GGAGGAAC-3' | 5'-ACTGACGTAGTGAGAGGGTCT- 3' | 5'-CGCAGCGTTGAGATTAGTTCC-3' |
| Ets 3 | 5'–CTTCCTTC–3' | 5'-TTCACTGCGTGCCTCAGTCTCC-3' | 5'-TTACCCGCAGGAGAGATTAACCC-3' |
| Ets 4 | 5'–GGAGGAAG–3' | 5'-AACAGATGGGCAACTTTCTTCTGGC-3' | 5'-ACAACCGCTCCCCTCACC-3' |


Figure 4.7. Human NeuroD Promoter Sequence with putative Pea3 binding sites (highlighted as nn; ets1, ets3 and ets4 sequentially) and primers (→) encompassing these sites for ChIP analysis

Table 4.21. Estimated PCR product lengths of the regions confining binding sites

| | |
|---|---|
| PCR product for ets1 motif on Human NeuroD after ChIP | 166 bp |
| PCR product for ets3 motif on Human NeuroD after ChIP | 191 bp |
| PCR product for ets4 motif on Human NeuroD after ChIP | 175 bp |

### 4.3.2. Transient Transfection for ChIP Assay

HEK293 cells were seeded in 100mm x 20mm tissue culture dishes (Corning) at a density of 1,5 x $10^6$ cell / plate. 24h later cells in each well were transfected with a total of 6 µg of DNA using 45 µl of 1µg/ µl Polyethylenimine (PEI) transfection reagent (CellnTech).

The ingredients of transfection mix was given in Table 4.14. The mix was prepared in a 200 µl of serum free medium and vortexed for 10sec. briefly spinned down and then left to wait at RT for 15 min. By the end of waiting of mix medium of cells were aspirated and 3ml of serum free medium was added onto cells gently and transfection mix was added. Cells with transfection mix was incubated in 37 $^oC$ in a humidified 5% $CO_2$ atmosphere for

2 hours. After 2h incubation medium of the cells were completed to 10 ml with the DMEM (containing 4,5 g/ l glucose for HEK293 cell lines) supplemented with 10% new-born calf serum 1% antibiotic and placed into 37 $^{o}$C in a humidified 5% $CO_2$ atmosphere for 48 hours.

Table 4.22. Ingredients for Transient Transfection for ChIP Assay

| | pCMV -3Tag-6 flag- | pCMV -3Tag-6 flag-mPea3 wild type | pGL3-NeuroD1 (Firefly Luciferase) | PEI Transfection Reagent (µl) |
|---|---|---|---|---|
| wt ets-2 | 3 µg | - | 3 µg | 45 |
| | - | 3 µg | 3 µg | 45 |
| mut ets-2 | 3 µg | - | 3 µg | 45 |
| | - | 3 µg | 3 µg | 45 |
| wt ets-3 | 3 µg | - | 3 µg | 45 |
| | - | 3 µg | 3 µg | 45 |
| mut ets-3 | 3 µg | - | 3 µg | 45 |
| | - | 3 µg | 3 µg | 45 |

### 4.3.3. Chromatin Immunoprecipitation Assay

#### *4.3.3.1. In vivo Crosslinking and Lysis*

48h incubation following transfection cells were removed from the incubator and 270 µl of 37% formaldehyde (final concentration 1%) was added onto the cells in 10 ml of medium. The dishes were placed on the shaker platform to be gently swirled for 10 min at RT. After incubation 2 ml of 10X Glycine (1.25M) was added to the each dish to quench unreacted formaldehyde. The dishes were placed on the shaker platform to be gently swirled for 5 min at RT. After 5 min incubation dishes were placed on ice and the medium containing formaldehyde and glycine was aspirated. Cells were rinsed with the 10 ml of ice cold 1X PBS for three times. By the time in a falcon tube 2ml of ice cold 1X PBS was aliquoted together with 10 µl of Protease Inhibitor Cocktail II ( Roche). The mixture was added onto

each dish 1ml at a time and with a help of syringe rubber cells were scraped from the dishes. Cell lysate was transferred into the eppendorf tubes and Centrifuge at 700 RCF for 5min. at 4$^{o}$C. Supernetant was removed and pellet was saved fort he lysis. After removal of supernatant, cell pellet can be stored at -80$^{o}$C for further analysis.

#### *4.3.3.2. Cell Lysis*

For the lysis of the cells Nuclei Isolation Buffer was prepared with the final concentration given in Table 4.23.

Table 4.23. Ingredients of Nuclei Isolation Buffer required final concentrations

| **Chemical** | **Final Concentration** |
|---|---|
| HEPES | 100mM |
| MgCl2 | 1.5mM |
| KCl | 10mM |
| Just before use followings were added | |
| DTT | 1mM |
| Protease Inhib. Cocktail II | 1: 100 |
| $dH_2O$ | up to desired final volume (depends on the number of samples) |

Each cell pellet was resuspended in the 1ml of ice cold Nuclei Isolation Buffer. Resuspended cells were incubated on ice for 10 min. After incubation on ice 100 µl of 10% Igepal (final concentration to 1% ) was added and vortexed for 10 seconds followed with 2min. incubation on ice. Cells were resuspended by centrifugation at 5000 rpm for 3min at 4$^{o}$C. Supernatant was removed and nuclei pellet was resuspended in 90 µl $dH_2O$.

#### *4.3.3.3. DNA Shearing*

Isolated nuclei was digested with Microccal Nuclease with the digestion mixture given in Table 4.16.

Table 4.24. Ingredients for the Micrococcal Nuclease Digestion Reaction

| Chemicals | Volume | Final Concentration |
|---|---|---|
| Micrococcal Nuclease Rxn Buffer (10X) | 10 µl | 1X |
| Micrococcal Nuclease (NEB) | 1 µl | 2000 gel units |
| Nuclei | 88 µl | varies |
| BSA (100X) | 1 µl | 1X |

Micrococcal Nuclease Digestion mix added nuclei were put in a heater block which was set to 37$^{o}$C previously and incubated in there for 10 min. with frequent mixing. The digestion reaction was then stopped with the addition of 100µl of 0.5M EDTA and then samples were centrifuged at 15.000 RCF at 4$^{o}$C for 10 min. Resultant supernatant containing sheared DNA was saved as 100 µl aliquotes in the eppendorf tubes. Sheared crosslinked DNA can be stored at -80 $^{o}$C for further studies.

Some sheared crosslinked DNA supernatant was saved as INPUT and continued with the reverse crosslinking. Reverse Crosslinking of the spared INPUT samples would give us information about the succes of the DNA shearing before passing to the immunoprecipitation.

#### *4.3.3.4. Immunoprecipitation*

Pea3 TF is the protein to be checked for its binding motif on NeuroD1 promoter which is the intreacting DNA partner. Immunoprecipitation relies on the precipitation of desired DNA segments by aid of a specific antibody that recognizes the protein sit on the DNA. Here is the Pea3 TF is expressed together with Flag protein which creates a fused Pea3-Flag couple. Since Pea3 is flag tagged antibody that is used for immunoprecipitation is the flag antibody conjugated ANTI-FLAG M2 Affinity Gel.

The ANTI-FLAG M2 affinity resin is stored in 50% glycerol with buffer and resin should be cleared with TBS buffer prior to use.

20-40 µl gel suspension equals to the 10 to 20 µl packed volume respectively and 10 µl of packed volume is enable to bind 1 µg Flag fused protein.

Since resin is kept in a glycerol containing buffer, to transfer it succesfully the end of the tip was cut off to ease the transfer of resin. 20 µl of resin suspension for each sample was transferred to an eppendorf tube. Resins were briefly centrifuged at 5000 rcf for 30 sec.. Supernatant was removed with a narrow tip in order not to remove any beads. Beads were washed twice with 500 µl of TBS buffer (20 times the packed bead volume) and 1X TBS was discarded at the end. Resins were then resuspended in 30 µl of TLB (Ingredients for 1X TBS and TLB was given in table 4. 17).

Table 4.25. Ingredients for TBS and TLB

| **10X TBS** | |
|---|---|
| **Ingredients** | **Final Concentration** |
| Trizma Base | 25Mm |
| NaCl | 150mM |
| KCl | 2mM |
| $dH_2O$ | 800 ml |
| pH was adjusted to 7. 4 using HCl | |
| $dH_2O$ | up to 1000ml |
| **TLB** | |
| **Ingredients** | **Final Concentration** |
| Trizma Base | 20 mM |
| Triton X-100 | 1% |
| Glycerol | 1% |
| NaCl | 137 mM |
| EDTA | 2 mM |
| Protease Inhib. Cocktail II | 1: 100 |
| Phosphatase Inhib. Cocktail II | 1: 100 |
| $dH_2O$ | up to 10ml |

100 µl of sheared DNA was mixed with the 900 µl of dilution buffer (ingredients were given in Table 4.18) which was containing 4,5 µl of Protease Inhibitor Cocktail II (Roche) and 30 µl of ANTI-FLAG M2 affinity resin previously resupended in TLB. Precipitation mix was incubated overnight at 4$^{o}$C on a shaker platform.

Table 4.26. Ingredients for Dilution Buffer

| Ingredients | Final Concentration |
|---|---|
| SDS | 0,01% |
| Triron X-100 | 1,1% |
| EDTA | 1,2mM |
| Tris-HCl (pH: 8.1) | 16,7mM |
| NaCl | 167mM |

#### *4.3.3.5. Elution*

Immuoprecipitated sample was centrifuged at 3000-5000 RCF for 1min. and supernatant was removed with a narrow end tip. 500 µl of TBS were added to the pelleted beads and beads were gently suspended in order to be rinsed and removed by centrifugation at 3000-5000 RCF for 2min. Beads were washed and supernatant was measured for protein concentration with at 280nm till obtain 0.05 absorbance.

100µl of elution buffer, 0. 1 M Glycine-HCl Ph: 3.5, was added to the cleared bead pellet and incubated with gentle shaking for 5min.at RT. Resin was pelleted with centrifugation at 5000-8200 RCF for 1min. and supernatant was transferred to a tube containing 10 µl of TBS. Samples were store at +4$^{o}$C for immediated use and at -20$^{o}$C for long term storage.

#### *4.3.3.6. Reverse Crosslinking*

After DNA-protein complex was seperated from resins, DNA will be seperated from protein by reverse crosslinking. Reverse crosslinking happens in the presence of high salt containing environment and enhanced with heat treatment. To each 50 µl sample and INPUT tubes 100 µl of nuclease-free water 6 µl 5M NaCl and 2 µl of RNase A were added. Mix was vortexed briefly and then incubated at 37°C for 30 minutes To each RNAse A-digested sample, 2 µl Proteinase K was added, vortexed to mix and incubated at

65°C for 2 hours. After then, DNA in the samples were purified by using PureLink PCR Purification Kit (Invitrogen).

#### *4.3.3.7. Analysis of ChIP Samples and INPUTs*

After reverse crosslinking and following purification of ChIP samples and INPUTs, DNA will be the resultant material which is needed to be analyzed with the primers specifically designed that confines the putative binding region.

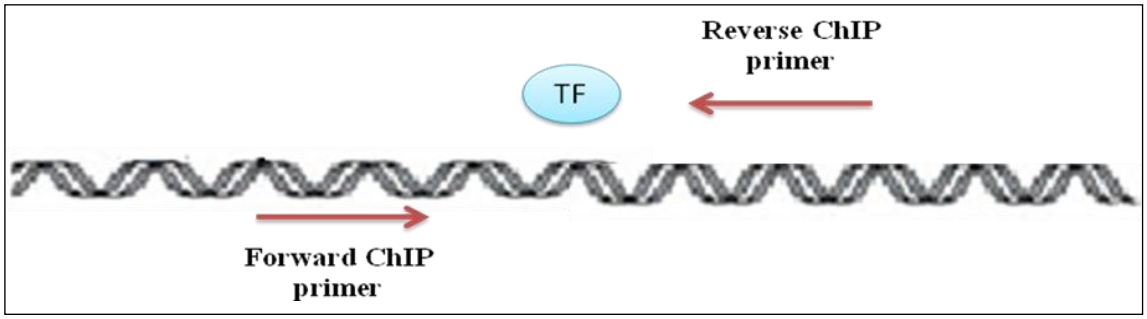


Figure 4.8. Schematic representation of ChIP primers encompassing transcription factor binding site.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) was the method of choice to quantify the precipitated DNA material. qRT-PCR is superior to the conventional PCR since it enables user to monitor amplification. FastStart SYBR Green Master (Roche ) was used in the qRT-PCR analysis.

SYBR Green is a fluuorescent dye that binds selectively to the groove of a double stranded DNA molecule, therefore with each amplicon produced at each cycle will fluorence since SYBR gren will bind to the newly produced double stranded DNA. A qRT-PCR mix contains the following ingredients given in Table 4.27.

Table 4.27. Ingredients for qRT-PCR

| | **Volume** | **Final Concentration** |
|---|---|---|
| SYBR Green | 6,25 µl | 1X |
| Primer Forward | 0,3 µl | 75 nM |
| Primer Reverse | 0,3 µl | 75 nM |
| Template DNA | 1 µl | 100 ng |
| $dH_2O$ | Up to 12,5 µl | |

Reaction mix was prepared in triplicates for each of the ChIP samples. Reaction mix was distributed as 12,5 µl into the 96 PCR-well plate. Mineral oil was then added in equal amounts ontoeach sample well. Plate was then covered with the sealing film and placed into the IcycleriQ Multicolor Real Time PCR Detection Sytem (BioRad). Reaction was done according to the protocol given in Table 4.28.

Table 4.28. Reaction Protocol for qRT-PCR

| **Cycle 1** (40X) | Temperature (ºC) | Time |
|---|---|---|
| Step 1 | 95 | 15 sec |
| Step 2 | 55 | 15 sec |
| Step 3 | 72 | 30 sec |
| **Data collection enabled.** | | |
| **Cycle 2** (1X) | | |
| Step 1 | 72 | 5 min |
| **Cycle 3** (110X) | | |
| Step 1 | 45 | 12 sec |
| Increase setpoint temperature after cycle 2 by 0.5ºC<br>**Melt curve data collection and analysis enabled.** | | |
| **Cycle 4** (1X) | | |
| Step 1 | 4 | HOLD |

Quantification of the amplified PCR product was monitored by measuring how much fluorescence is produced correlatively to the amplified product. Primary detection is done after each extension is completed and secondary detection is done after the final extension is completed and melt curve analysis is started. The these data are used to plot PCR amplification /cycle and melt curve graphs.

Reaction in qRT-PCR goes as in the conventioanl PCR, however, at the end of final extension temperature increases starting from 45$^{o}$C with 0,5$^{o}$C increments within each 2 cycles for 110 cycles. This procedure is called as Melt Curve Analysis which enables to monitor if there is any primer dimer or any other contaminant PCR product interfering with fluorescence emission of sample amplicon.

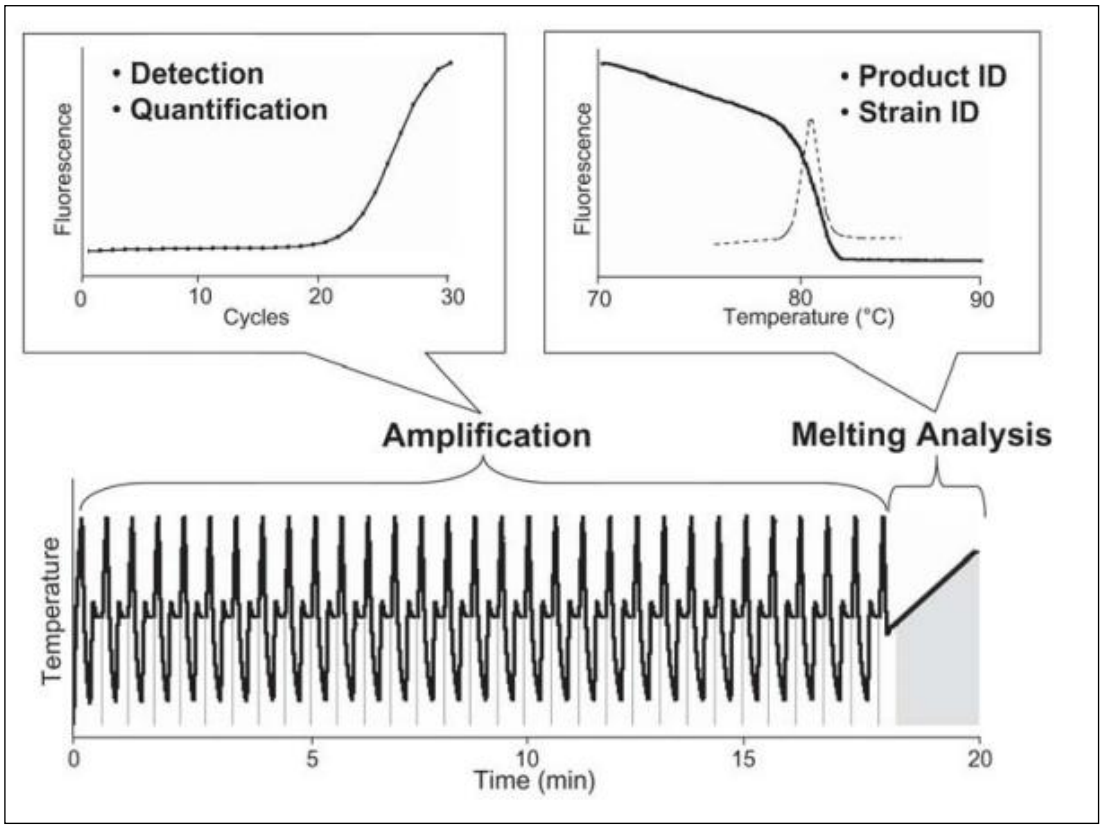


Figure 4.9. Schematic representation of the qRT-PCR. First graph is demonstrating the how quantification, obtained with the fluorescence detection at each cycle, and how melting analysis is graphed [33]

# 5. RESULTS

## 5.1. ASSESSMENT OF PEA3 MEDIATED PUTATIVE REGULATION OF HNEUROD1 PROMOTER BY LUCIFERASE REPORTER ACTIVITY

Luciferase Reporter Assay is a technique which is used to monitor the activity of a promoter by quantifying the expression of a reporter gene which is regulated by the promoter of interest. The reporter gene in this case luciferase, from the firefly *Photinus pyralis*, encodes an enzyme which oxidizes its substrate D-luciferin and releases light which is then detected by the luminometer. Dual-Glo® Luciferase Assay System (Promega) was the assay system that was used. As the name refers, this sytem enables the expression of experimental reporter Firefly Luciferase and the control reporter Renilla Luciferase which is important to normalize experimental result to the control result in order to avoid any difference caused by non-experimental conditions.

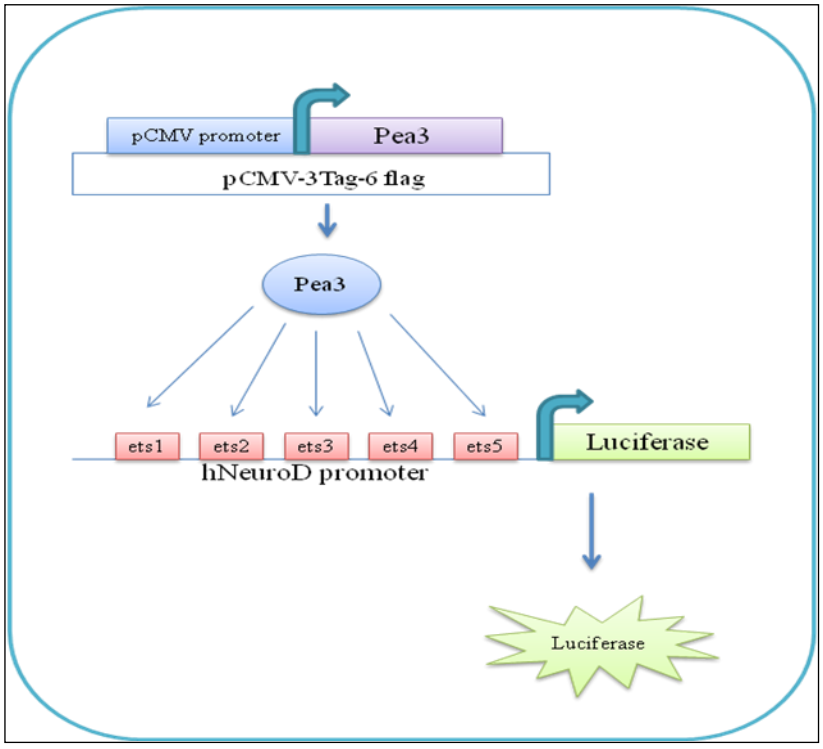


Figure 5.1. Schematic representation of the putative mPea3 TF mediated hNeuroD1 promoter activation

According to this experimental set up SH-SY5Y neuroblastoma cell line and HEK293T cell lines were transfected with the plasmid constructs; pCMV-3Tag-6 flag empty vector pCMV- 3Tag- 6 flag- m*Pea3* wild type (pCMV-3Tag-6 flag- *ERM* wild type, pCMV-3Tag-6 flag- *ER81* wild type) and wildtype *hNeuroD1*-Luc.

Luciferase expression in cells (SH-SY5Y neuroblastoma cell line and HEK293T cell) at a density of 5 x $10^4$ per well transfected with plasmid constructs; pCMV-3Tag-6 flag empty vector, wildtype pCMV- 3Tag- 6 flag- m*Pea3* and wildtype *hNeuroD1*-Luc. 48hr after the transfection cells were lysed. Substrates for firefly luciferase and renilla luciferase were added to the lysates sequentially and relative luciferase activities were measured and plotted.

According to the plotted relative luciferase activities, as Pea3 concentration is increasing NeuroD promoter driven luciferase expression showed an increase in the relative luciferase activities. This interpretation was made in comparison to the luciferase reporter gene expression level of negative control, which only bears pCMV-3Tag-6 flag empty without Pea3. Therefore in SH-SY5Y cells, in comparison to the negative control relative luciferase activity at all Pea3 concentrations were increased with only a small decrease with 200ng Pea3.

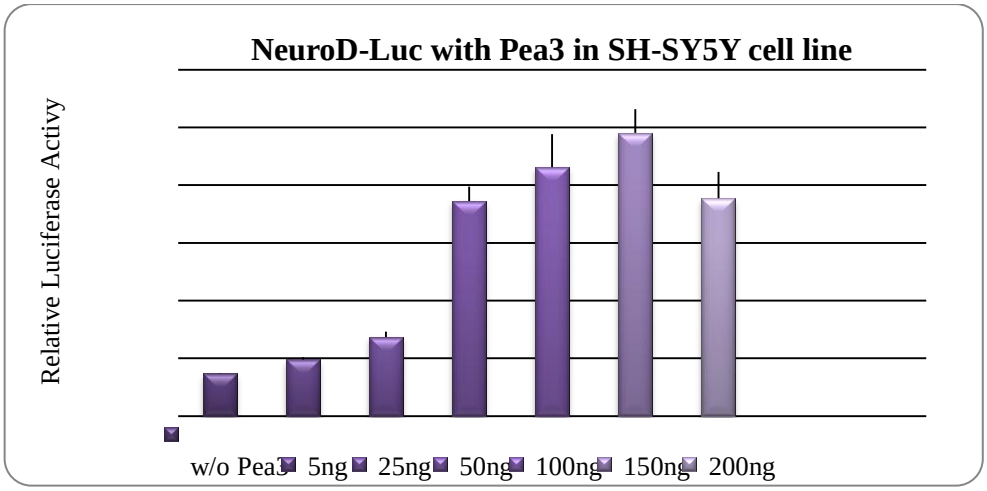


Figure 5.2. Activation of *NeuroD1* promoter by Pea3 in SH-SY5Y neuroblastoma cell line. Cells seeded at a density of 5 x $10^4$ per well and in triplicates for each Pea3 concentration, 24 prior to transfection. Transfection mix was prepared with the 5ng, 25ng, 50ng, 100ng,

150ng and 200 ng (amounts are given under each column) pCMV-3Tag-6 flag-mPea3 wild type vector construct and each of this Pea3 vector concentration is completed to total 200ng DNA with pCMV-3Tag-6 flag- empty vector where needed. In addition 200 ng of *hNeuroD1*-Luc was added together with the 100 ng of Renilla Luciferase and PEI transfection reagent. 48h later cells were lysed and luciferase activity was measured. Data were presented relative to the internal control Renilla Luciferase. Standard deviation calculated for the each triplicate is indicated with the error bars

The same reporter experiment was carried out in HEK293 cell line as well for evaluation of results in non-neuronal environment. Relative luciferase activities at all Pea3 concentrations were inreased as with SH-SY5Y cells. However, relative luciferase activity values are so much lower than SH-SY5Y cells. The luciferase activity obtained with endogenous Pea3 is 10 fold lower than the activity obtained in SH-SY5Y cells. Nevertheless, Pea3 transactivation capacity on NeuroD promoter has increased.

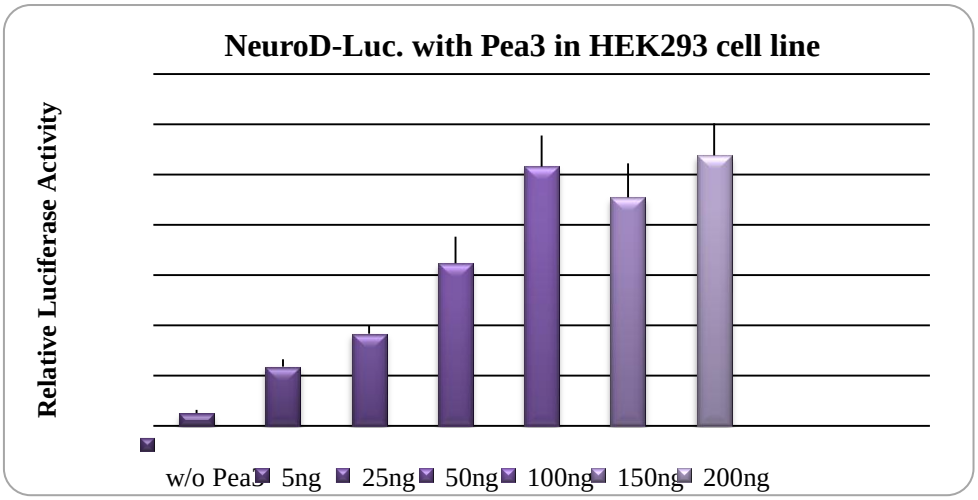


Figure 5.3. Activation of *NeuroD1* promoter by Pea3 in HEK293 (Human Embryonic Kidney) cell line. Cells seeded at a density of 5 x $10^4$ per well and in triplicates for each Pea3 concentration, 24 prior to transfection. Transfection mix was prepared with the 5ng, 25ng, 50ng, 100ng, 150ng and 200 ng (amounts are given under each column) pCMV-3Tag-6 flag-mPea3 wild type vector construct and each of this Pea3 vector concentration is completed to total 200ng DNA with pCMV- 3Tag-6 flag-empty vector where needed. In addition 200 ng of *hNeuroD1*-Luc was added together with the 100 ng of Renilla Luciferase and PEI transfection reagent. 48h later cells were lysed and luciferase activity

was measured. Data were presented relative to the internal control Renilla Luciferase. Standard deviation calculated for the each triplicate is indicated with the error bars.

## 5.2. ASSESSMENT OF ERM AND ER81 REGULATED *NEUROD1* PROMOTER ACTIVITY BY LUCIFERASE REPORTER ASSAYS

ERM and ER81 are members of the Pea3 subfamily and all three members of the family, Pea3, ER81 and ERM exhibits high sequence similarity both in conserved ETS-domain and in N-terminal activation domain [6]. This high similarity among the group members and the fact that they are expressed in the same organ even though they show differential expression sites [23] led us think whether they may exert similar effects on the same target.

Luciferase reporter assay results for ERM is given in Figure 5. 4 below. In comparison to the control (lacks exogenous Pea3) the activity of *NeuroD1* promoter was not changed significantly at any concentration. Also the relative luciferase activity values are too low to talk about a sigificant change that ERM can mediate.

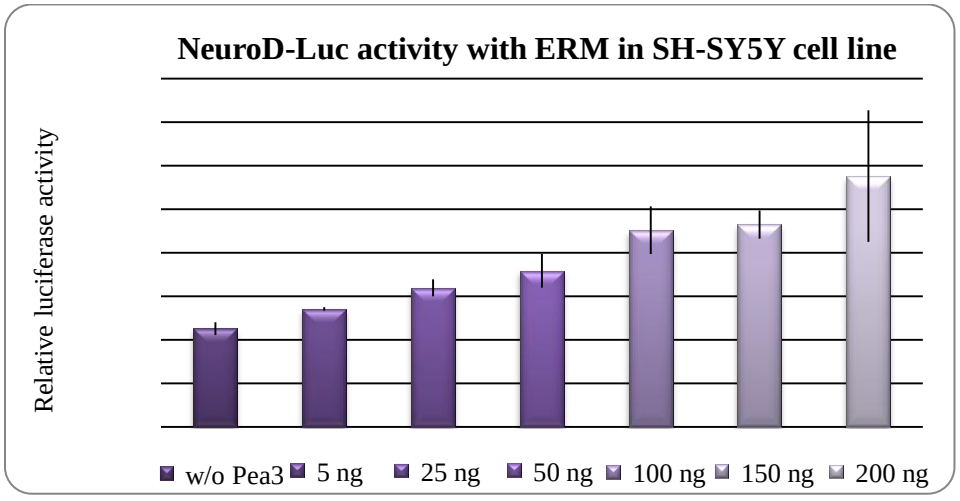


Figure 5.4. Activation of *NeuroD1* promoter by ERM in SH-SY5Y cell line. Cells seeded at a density of 5 x $10^4$ per well and in triplicates for each ERM concentration, 24 prior to transfection. Transfection mix was prepared with the 5ng, 25ng, 50ng, 100ng, 150ng and 200 ng (amounts are given under each column) pCMV- 3Tag-6 flag- ERM wild type vector construct and each of this ERM vector concentration is completed to total 200ng DNA with pCMV- 3Tag-6 flag- empty vector where needed. In addition 200 ng of

*hNeuroD1-Luc* was added together with the 100 ng of Renilla Luciferase and PEI transfection reagent. 48h later cells were lysed and luciferase activity was measured. Data were presented relative to the internal control Renilla Luciferase. Standard deviation calculated for the each triplicate and indicated with the error bars

Transctivation capacity of ER81 on *NeuroD1* promoter in SH-SY5Y cell line and HEK293 cell line are given in Figure 5. 5 and Figure 5. 6. respectively below. Unlike ERM, ER81 mediated activity of NeuroD1 promoter in SH-SY5Y cell line was increased almost 4fold with 50ng ER81, 3-fold with 100ng ER81and nearly 8-fold with the 200ng ER81 in comparison to the pCMV control which lacks exogenous Pea3 (Fig.5.5). However, in HEK293 cells relative luciferase activity values were low and no significant change was observed at any Pea3 concentration (Fig.5.6).

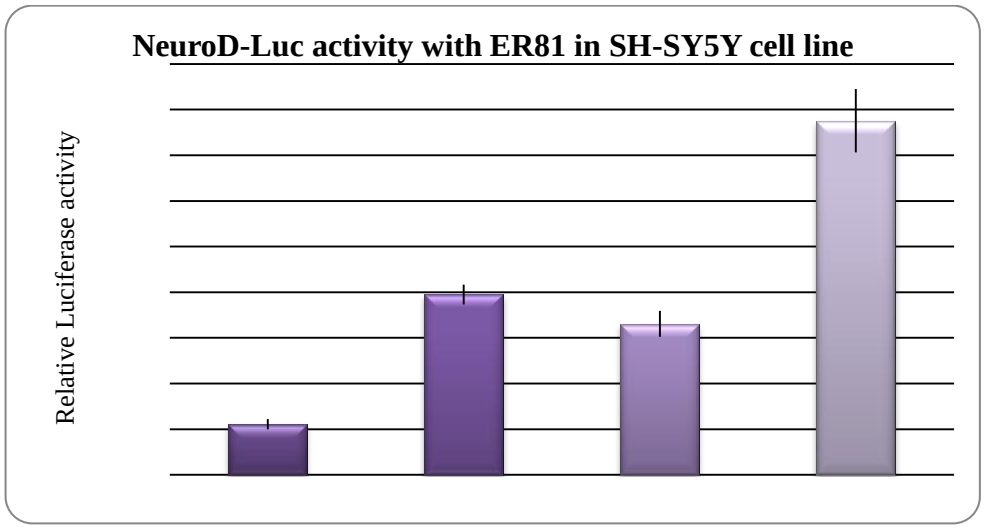


Figure 5.5. Activation of *NeuroD1* promoter by ER81 in SH-SY5Y cell line. Cells seeded at a density of 5 x $10^4$ per well and in triplicates for each ERM concentration, 24 prior to transfection. Transfection mix was prepared with the 50ng, 100ng, and 200 ng (amounts are given under each column) pCMV-3Tag-6 flag-ER81wild type vector construct and each of this ER81 vector concentration is completed to total 200ng DNA with pCMV-3Tag-6 flag-empty vector where needed. In addition 200 ng of *hNeuroD1-Luc* was added together with the 100 ng of Renilla Luciferase and PEI transfection reagent. 48h later cells were lysed and luciferase activity was measured. Data were presented relative to the

internal control Renilla Luciferase. Standard deviation calculated for the each triplicate and indicated with the error bars.

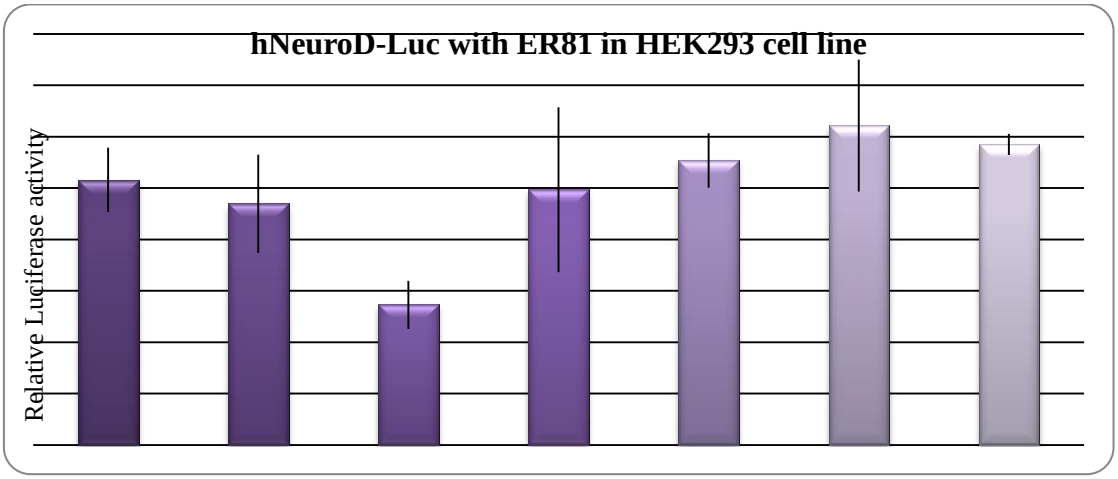


Figure 5.6. Activation of *NeuroD1* promoter by ER81 in SH-SY5Y cell line. Cells seeded at a density of 5 x $10^4$ per well and in triplicates for each ER81 concentration, 24 prior to transfection. Transfection mix was prepared with the 5ng, 25ng, 50ng, 100ng, 150ng and 200 ng (amounts are given under each column) pCMV-3Tag-6 flag-ER81wild type vector construct and each of this ER81 vector concentration is completed to total 200ng DNA with pCMV- 3Tag-6 flag-empty vector where needed. In addition 200 ng of *HNeuroD1-Luc* was added together with the 100 ng of Renilla Luciferase and PEI transfection reagent. 48h later cells were lysed and luciferase activity was measured. Data were presented relative to the internal control Renilla Luciferase. Standard deviation calculated for the each triplicate is indicated with the error bars

## 5.3. EFFECTS OF PEA3 PHOSPHORYLATION STATUS IN REGULATION OF *HNEUROD1* PROMOTER BY LUCIFERASE REPORTER ASSAYS

Luciferase reporter assays with wild type Pea3 showed significant increase in the *hNeuroD1* promoted *luciferase* expression and activity, next we would like to determine how Pea3 phosphorylation status effects its transactivation capacity over the same target, *hNeuroD1*. In order to monitor the effects of Pea3 phosphorylation status, phosphorylation mutations were created in proline followed serine residues (Pea3 phosphhomutants were created by Berrak Çağlayan). Mutations either silenced the phosphorylation by mutating

serine into alanine (Ser→Ala) or mimicked by mutating serine into glutamic acid (Ser→Glu).

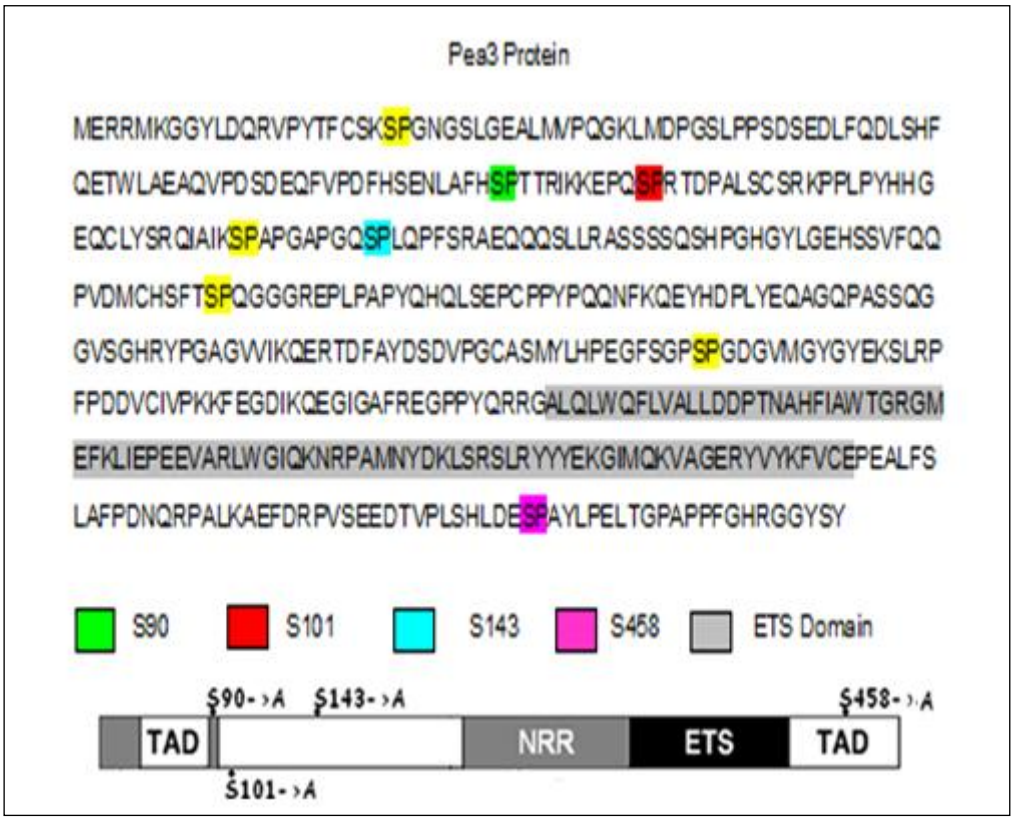


Figure 5.7. Mouse Pea3 aminoacid sequence (480 aminoacids) and a schematic of Pea3structure illustrating the Serine followed Proline (S/P) motifs which are phosphorylation targets for proline-directed protein kinases (Potential phosphorylation sites are proposed by Axan Lab)

*NeuroD1* promoter driven Relative Luciferase activities with Pea3 phospho mutants are given in Figure 5.8 and Figure 5.9. Any change in relative luciferase activities was evaluated comparison to the control group which has wildtype pCMV- 3Tag-6 flag- Pea3. Depending on luciferase activities, mimicking the phosphorylation in Ser90 amino acid residue by a site-directed mutation that replaces serine with glutamic acid (S90E) leads an increse in the expression while non-phosphorylatable alanine (S90A) mutant shows less activity. Results are consistent in both cell lines.

S101A non-phosphorylatable mutant has almost no effect on the relative luciferase activity in SH-SY5Y cells while increase the activity slightly in HEK293 cells. The phospho-

mimicked mutation in the same residue however decreased the relative luciferase activity both in SH-SY5Y and HEK293 cell lines.

S143A non-phosphorylatable mutant, on the other hand, slightly incresed the expression in SH-SY5Y cells while did almost no effect in HEK293 cells. However phospho-mimicked mutant, S143E, cause reductions in the Pea3 transactivation capacity consistently in both cell line.

More interestingly either mutants, S458A and S458E, increased Pea3 transactivation capacity in SH-SY5Y cells, independent of the phophorylation status. However no detectable change was observed in relative luciferase activity of S458 mutants in HEK293 cells.

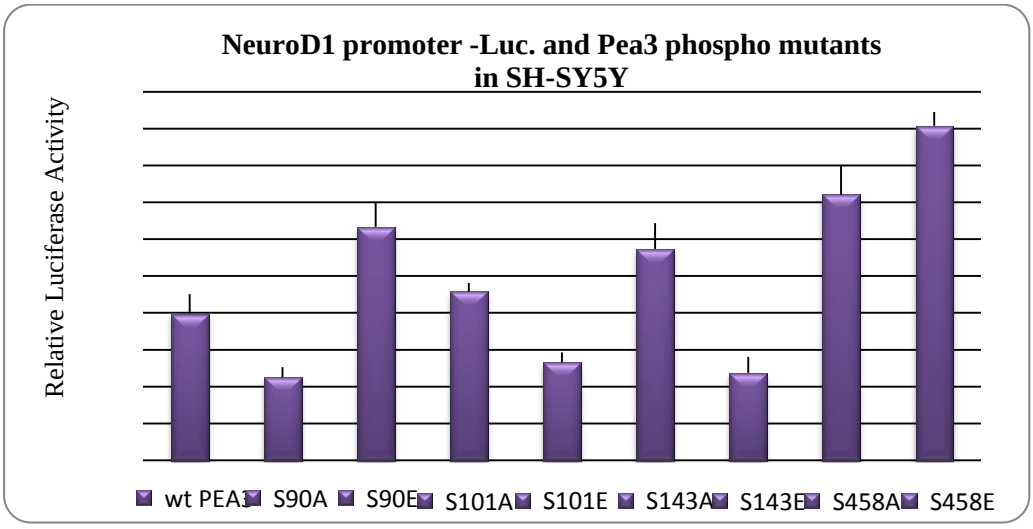


Figure 5.8. Activation of NeuroD promoter by Pea3 phosphorylation mutants in SH-SY5Y cell line. Cells seeded at a density of 5 x $10^4$ per well and in triplicates for each Pea3 concentration, 24 prior to transfection. Transfection mix was prepared seperately for each of the phospho mutants (mutants are indicated under each column) with the, 100ng pCMV-3Tag-6 flag- Pea3wild type/mutant vector construct, 100 ng pCMV- 3Tag-6 flag- empty, 200 ng of *hNeuroD1-Luc* and together with the 100 ng of Renilla Luciferase and PEI transfection reagent were added. 48h later cells were lysed and luciferase activity was measured. Data were presented relative to the internal control Renilla Luciferase. Standard deviation calculated for the each triplicate is indicated with the error bars.

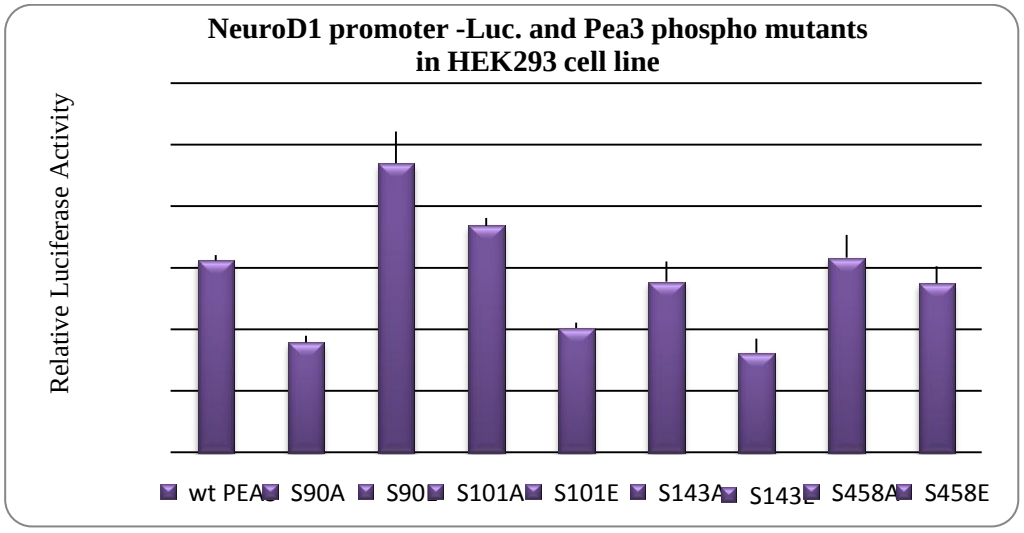


Figure 5.9. Activation of NeuroD promoter by Pea3 phosphorylation mutants in HEK293 cell line. Cells seeded at a density of 5 x $10^4$ per well and in triplicates for each Pea3 concentration, 24 prior to transfection. Transfection mix was prepared seperately for each of the phospho mutants (mutants are indicated under each column) with the, 100ng pCMV-3Tag-6 flag-Pea3wild type/mutant vector construct, 100 ng pCMV-3Tag-6flag-empty, 200 ng of *HNeuroD1-Luc* and together with the 100 ng of Renilla Luciferase and PEI transfection reagent were added. 48h later cells were lysed and luciferase activity was measured. Data were presented relative to the internal control Renilla Luciferase. Standard deviation calculated for the each triplicate is indicated with the error bars

## 5.4. INVESTIGATING THE EFFECTS OF MUTATION IN PEA3 BINDING MOTIFS IN THE REGULATION OF *HNEUROD1* PROMOTER BY LUCIFERASE REPORTER ASSAYS

Bioinformatics tools (ALGGEN PROMO) suggests that *hNeuroD1* promoter posses five Pea3 binding motif which are thought be involved in the promoter activity (Figure 5. 10). Putative binding motifs were named as ets1, ets2, ets3, ets4 and ets5. Three out of those five putative binding motifs with lowest dissimilarity rate were chosen to be used in the following luciferase and ChIP assays. Dissimilarity rates indicate the difference between Pea3 binding motif and the promoter seqeunce and give rates in percentage. The lowest the dissimilarity rate is the highest the possibility of Pea3 binding. Therefore motifs with lowest dissimilarity rates 6.61%, 4.38% and 3.31% corresponds to ets1, ets3 and ets4 were

mutated in order to prevent Pea3 binding by changing the binding sequence to some random non-bindable sequence.

Suggested mutant sequences were checked with ALGGEN PROMO to make sure that Pea3 transcription factor binding is prevented (see RESULTS for Figure 6.5., 6.7, 6.8). Then *hNeuroD1* promoter with desired mutant motifs were cloned into pGL3-Basic Luciferase Reporter plasmid (see "Plasmid construct" in Methods).

The presence of desired mutations was confirmed by FinchTV DNA sequencing chromatogram trace viewer, and comparison of sequences were done with the SDSC Biology WorkBench (http://workbench.sdsc.edu/) bioinformatics tool ( sequenceing results for ets1, ets3 and ets4 were given in Appendix).

Among three mutation trials only ets3 and ets4 motifs were succesfully mutated and used in the Luciferase and ChIP assays.

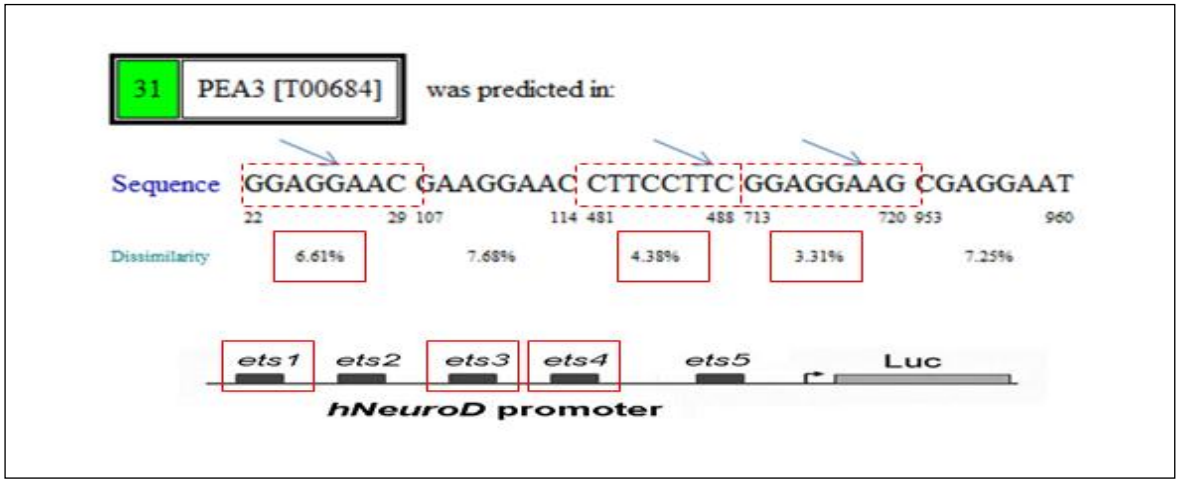


Figure 5.10**.** ALGGEN PROMO results for human NeuroD promoter with putative Pea3 binding sites. The motifs shown in red boxes; ets1, ets3 and ets4 were the ones that gave the lowest dissimilarity rates 6.61%, 4.38% and 3.31% respectively

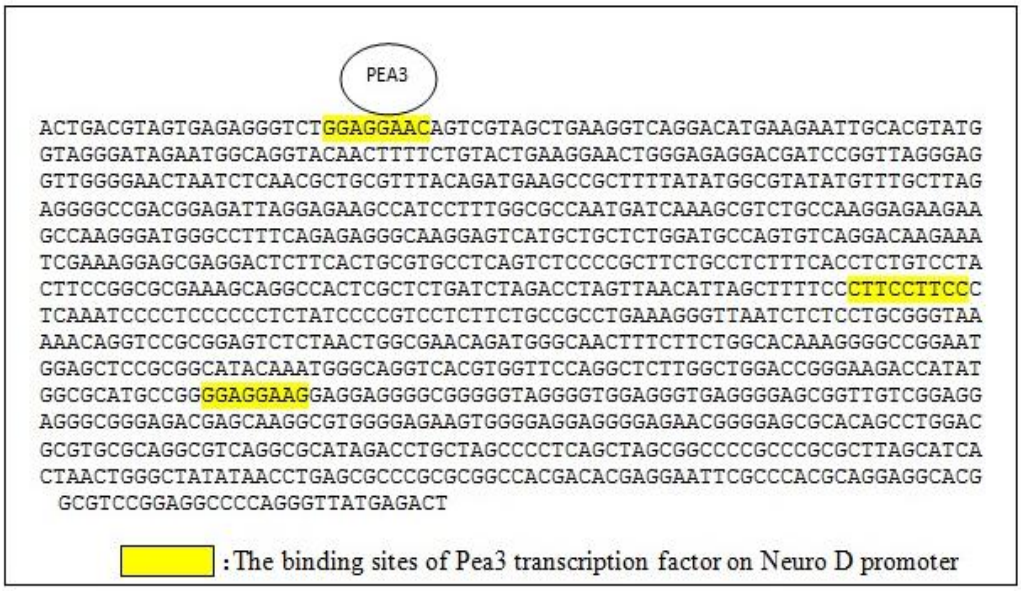


Figure 5.11. Wild-type human *NeuroD1* promoter sequence and putative mPea3 transcription factor binding motifs (ets1, ets3 and ets4, respectively)


Figure 5.12. Representation of mutations introduced to Pea3 binding motifs on human *NeuroD1* promoter (mut ets1, mut ets3 and mut ets4, respectively)

The successful mutant *hNeuroD1*-Luc constructs were then subjected to the luciferase assay in order to investigate whether Pea3 transcription factor binding profile has changed. Results for luciferase assays of the *hNeuroD1*-ets3 mut-Luc and *hNeuroD1*-ets4mut-Luc were given in Figure 5.13. and 5.14.

In comparison to the wildtype *hNeuroD1*-Luc(red columns), relative luciferase activity of *hNeuroD1*-ets3 mut-Luc enhanced by 17 fold in the lack of Pea3 (Pea3(-)). Ascending Pea3 concentrations also increased the relative luciferase activity by only 1 to 2 fold. But

with the addition of 50 ng of Pea3, transctivation capacity of *hNeuroD1* was reduced by 4 fold.

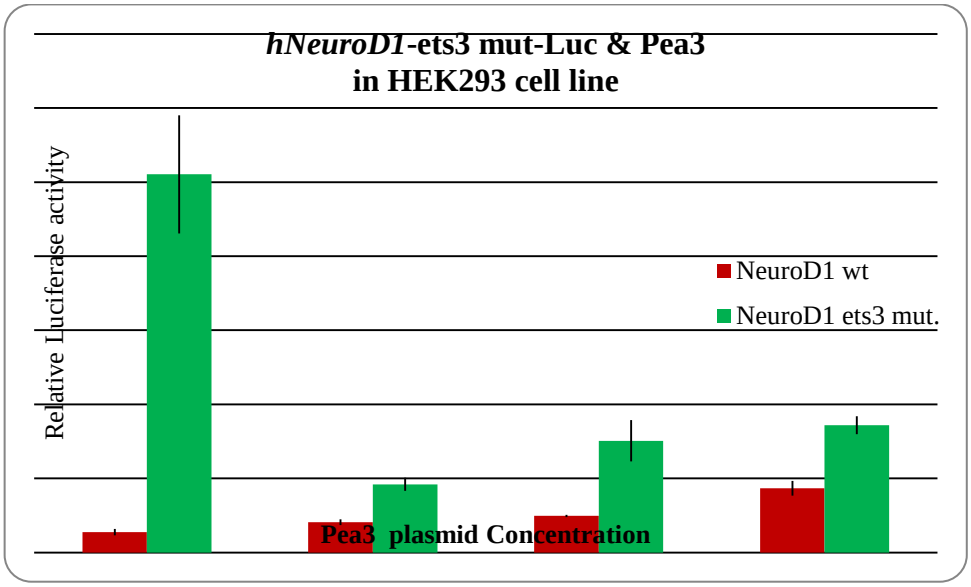


Figure 5.13. Activation of mutant *hNeuroD1* promoter for ets3 motif by mPea3 transcription factor in HEK293 cell line. Green columns indicate the relative luciferase activity of *NeuroD1* ets3 mutant promoter (in which Pea3 binding is blocked at this region) while red columns indicate the relative luciferase activity of wild type *hNeuroD1*-Luc. Cells were seeded at a density of 5 x $10^4$ per well and in triplicates for each Pea3 concentration, 24 hr prior to transfection. Transfection mix was prepared with the 50ng, 100ng and 200 ng (amounts are given under each column) pCMV- 3Tag-6 flag-mPea3 wild type vector. 200 ng of *hNeuroD1*-Luc (either wild type or mutant) together with the 100 ng of Renilla Luciferase and PEI transfection reagent were added as well. 48h later cells were lysed and luciferase activity was measured. Data were presented relative to the internal control Renilla Luciferase. Standard deviation calculated for the each triplicate is indicated with the error bars

The other mutant promoter, *hNeuroD1*-ets4mut-Luc on the other hand showed a different activity pattern. The gradually increased Pea3 concentration increased transactivation capacity of *hNeuroD1* promoter by almost 4-fold , however this activation was increased by 20 fold when Pea3 concentartion was 200ng. One important thing with this result was the relative luciferase activity values. This luciferase assays were carried out in the

HEK293 cells and so far none of the luciferase reporter assays give that high luciferase activity.

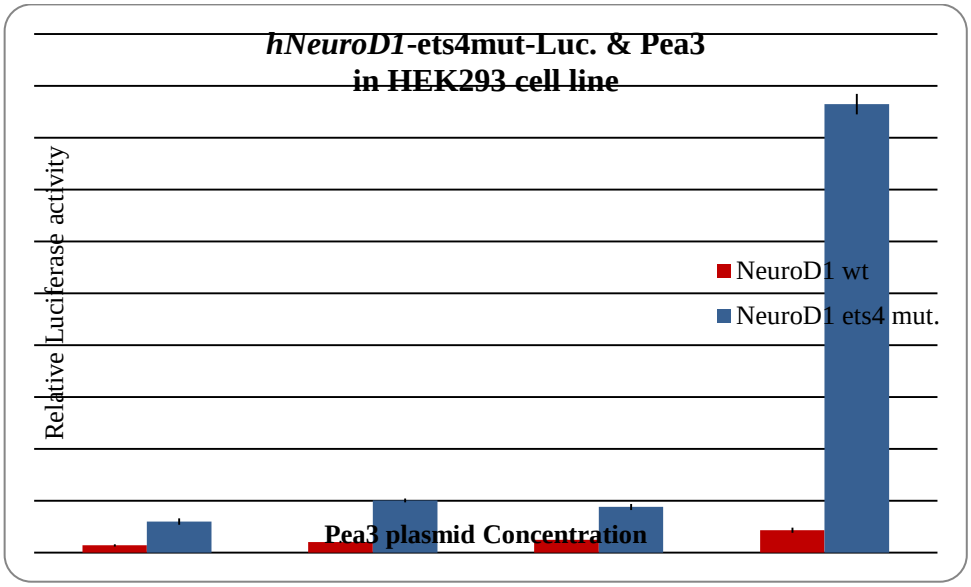


Figure 5.14. Activation of mutant *hNeuroD1* promoter for ets4 motif by mPea3 transcription factor in HEK293 cell line. Blue columns are indicating the relative luciferase activity of *NeuroD1* ets4 mutant promoter (in which Pea3 binding is blocked at this region) while red columns are indicating the relative luciferase activity of wild type *NeuroD1* promoter. Cells were seeded at a density of 5 x $10^4$ per well and in triplicates for each Pea3 concentration, 24 hr prior to transfection. Transfection mix was prepared with the 50ng, 100ng and 200 ng (amounts are given under each column) pCMV-3Tag-6 flag-mPea3 wild type vector. 200 ng of *hNeuroD1*-Luc (either wild type or mutant) together with the 100 ng of Renilla Luciferase and PEI transfection reagent were added as well. 48h later cells were lysed and luciferase activity was measured. Data were presented relative to the internal control Renilla Luciferase. Standard deviation calculated for the each triplicate is indicated with the error bars

Next we would like to see how relative luciferase activity profile will change if the putative motif is deleted. For this purpose ets4 motif deletion mutant was generated and used in the luciferase assays. *h NeuroD1* promoter seqeunce with possible binding motifs ets1,ets3 and ets4 with deleted 5'-GGA-3' core motif is designated in Figure 5.14.


Figure 5.15. Representation of deletion mutations introduced to Pea3 binding motif ets4 on human *NeuroD1* promoter. Highlighted regions ( ) designate putative binding motifs; ets1, ets3 and ets4.5'-GGA-3' core sequence in ets4 motif, highlighted as ( ), is the deleted sequence

Relative luciferase activty with *hNeuroD1*-ets4delmut-Luc was increased as Pea3 concentration increases similar to the activity obtained with *hNeuroD1*-ets4mut-Luc, with only higher fold increase in comparison to the wildtype *hNeuroD1*-Luc. Also Pea3 at 200ng only about 6-fold incresed the luciferase activity in comparison to the wildtype *hNeuroD1*-Luc. In addition to that luciferase activity values are lower than the one with the *hNeuroD1*-ets4mut-Luc.

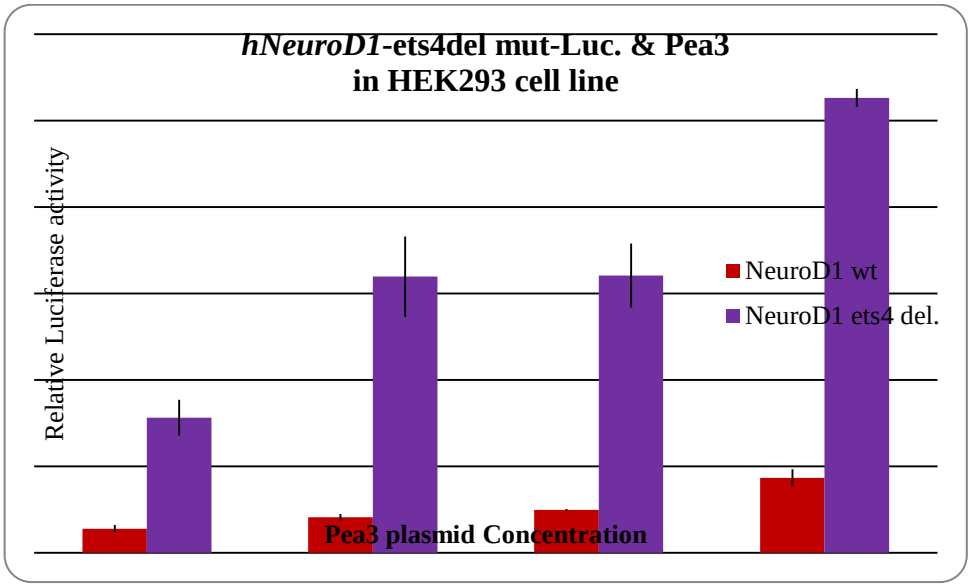


Figure 5.16. Activation of mutant *hNeuroD1* promoter for ets4 motif by mPea3 transcription factor in HEK293 cell line. Purple columns are indicating the relative luciferase activity of *hNeuroD1*-ets4 mut-Luc (in which Pea3 binding is blocked at this region) while red columns are indicating the relative luciferase activity of wild type *NeuroD1* promoter. Cells were seeded at a density of 5 x $10^4$ per well and in triplicates for each Pea3 concentration, 24 hr prior to transfection. Transfection mix was prepared with the 50ng, 100ng and 200 ng (amounts are given under each column) pCMV- 3Tag-6 flag-mPea3 wild type vector. 200 ng of *hNeuroD1*-Luc (either wild type or mutant) together with the 100 ng of Renilla Luciferase and PEI transfection reagent were added as well. 48h later cells were lysed and luciferase activity was measured. Data were presented relative to the internal control Renilla Luciferase. Standard deviation calculated for the each triplicate is indicated with the error bars

We would have expected that mutation of putative Pea3 binding motifs (ets1–ets5) to lead to a decrease in the *NeuroD1* driven luciferase expression. However our results showed that rather than a reduction in the luciferase epression, an increase was observed for all the mutants when compared to the expression from the wildtype *hNeuroD1*-Luc (see Figure 5.15 comparing red columns to the rest).

Generating mutations only on putative binding motifs without disturbing the rest of the sequence was the primary goal of our mutation studies, however as our luciferase results

indicate, the situation may have been more complex than anticipated, and binding sites for other transcription factors may have been created around the *pea3* binding motif mutations (see DISCUSSION section, Figures 6. 9.-6. 11)

To overcome this problem we have decided to generate truncations of *NeuroD1* promoter simply removing the Pea3 binding motifs ets1, ets2, ets3, and ets4 one by one. With these truncations we aimed to directly analyze the removal of the putative ets motifs of *NeuroD1* promoter rather than deal with complexities born due to the addition or variation of the possible protein-DNA or protein-protein interaction which might be important for the activation of promoter. Schematic representation of truncated *NeuroD1* promoter was given in Figure 5.17).

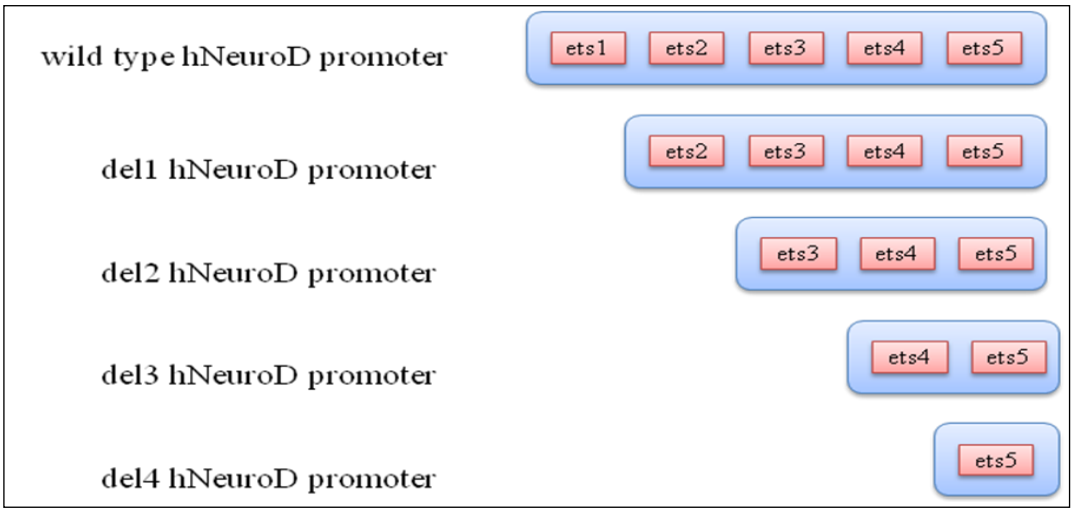


Figure 5.17. Schematic representation of the truncated *hNeuroD1* promoter. With each truncation ets motifs were removed one by one

NeuroD1 promoter truncations were generated by PCR with designed primers that truncates promoter sequence just after the putative Pea3 binding motif (Figure 4.3). Then truncations were named as del1, del2, del3 and del4 and with each truncation an ets binding motif was removed (Figure 5.15, and Materials and Methods).

Truncated NeuroD1 promoter PCR products (Figure 5.17) were then clonned into the pGL3-Basic Luciferase Reporter plasmid for luciferase assays. Cloned plasmids were named as *hNeuroD1-del1-Luc, hNeuroD1-del2-Luc, hNeuroD1-del2-Luc* and *hNeuroD1-*

*del4-Luc.* They were sent to commercial sequencing in order to proove they had the truncation.

The results of the analysis are 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 (see Figure A. 5.–Figure A.7.) for sequencing results.

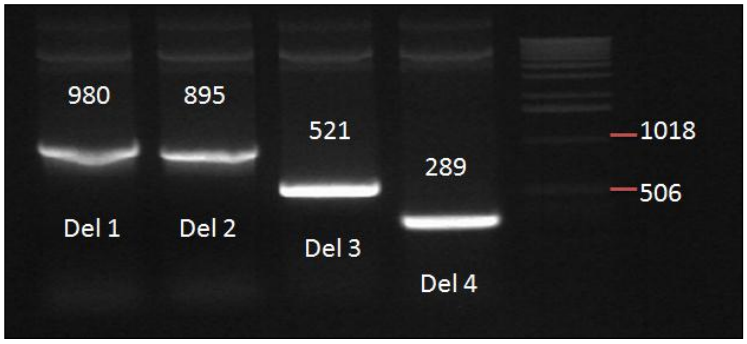


Figure 5.18. Truncated *NeuroD1* promoter PCR products. del1; first ets1 motif was removed, del2; ets1 and ets2 motifs were removed, del3; ets1, ets2 and ets3 motifs were removed, del4; ets1, ets2, ets3 and ets4 motifs were removed. Products were cloned into pGL3 Basic Truncation constructs were used in the luciferase assay and results are given in Figure 5. 19.–5. 22. below.

The first truncation *hNeuroD1*-del1-Luc, where only ets1 motif was removed, was given in Figure 5.19. Blue columns are indicating the activity driven by wildtype *hNeuroD1*-Luc and red columns are indicating the activity driven by *hNeuroD1*-del1-Luc in different Pea3 concentrations.

Activity driven by *hNeuroD1*-Luc was increased gradually with ascending Pea3 doses. There was only a reduction with the 150ng Pea3 but that should be an experimental error, since wildtype *hNeuroD1*-Luc activity consistently increased till 200ng Pea3 8see previous luciferase assay results). On the other hand *hNeuroD1*-del1-Luc demostrated almost 5 fold increase in the absence of Pea3. This activation with the addition of 50ng of Pea3 was draw below in comparison to its wildtype counterpart. However increased doses of Pea3

later reduced the transactivation capacity of *hNeuroD1*-del1-Luc dramatically almost 12 fold in the 100ng of Pea3 and 6 fold in the 150ng Pea3.

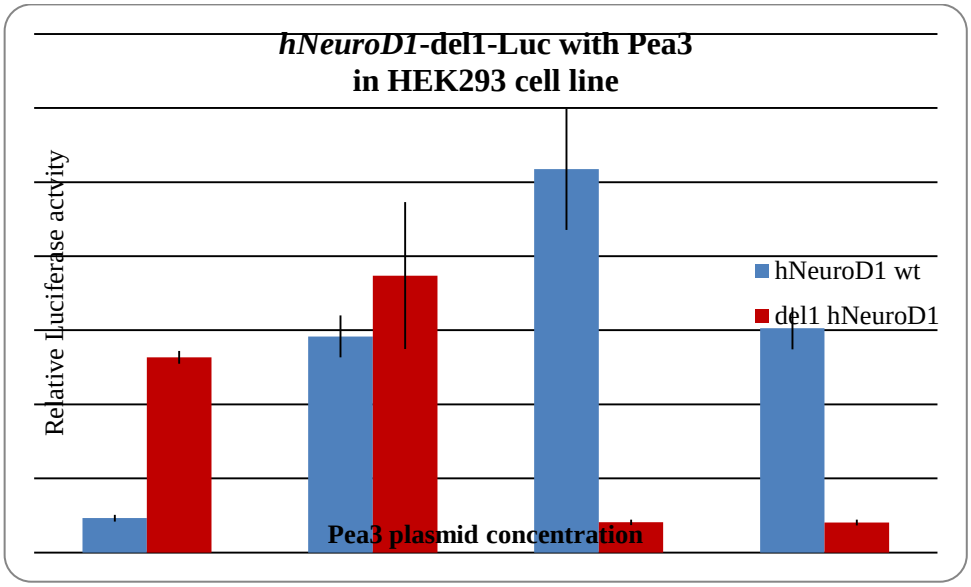


Figure 5.19. Activation of truncated h*NeuroD1* promoter for ets1 motif by mPea3 transcription factor in HEK293 cell line. Blue columns are indicating the relative luciferase activity of wild type *NeuroD1* promoter while red columns are indicating the relative luciferase activity of truncated *NeuroD1* promoter, del1 (in which Pea3 binding ets1 motif was removed ). Cells were seeded at a density of 5 x $10^4$ per well and in triplicates for each Pea3 concentration, 24 prior to transfection. Transfection mix was prepared with the 50ng, 100ng and 150 ng (amounts are given under each column) pCMV-3Tag-6 flag-mPea3 wild type vector. 200 ng of *HNeuroD1-Luc* (either wild type or mutant) together with the 100 ng of Renilla Luciferase and PEI transfection reagent were added as well. 48h later cells were lysed and luciferase activity was measured. Data were presented relative to the internal control Renilla Luciferase. Standard deviation calculated for the each triplicate is indicated with the error bars

The second truncation *hNeuroD1*-del2-Luc, where ets1 and ets2 motifs were removed, was given in Figure 5. 19. Blue columns are indicating the activity driven by wildtype *hNeuroD1*-Luc and green columns are indicating the activity driven by *hNeuroD1*-del2-Luc in different Pea3 concentrations.

Relative luciferase activity driven by *hNeuroD1*-del2-Luc demostrated almost 10 fold increase in the absence of Pea3. Transactivation of *hNeuroD1*-del2-Luc with the addition of 50ng of Pea3 further reduced by 33 fold in comparison to its wildtype counterpart and this reduction even further draw by 63 fold with the presence of 100ng Pea3. However this reduction is rescued when 150 ng of Pea3 is present but still 7 fold reduced in comparison to the the wildtype *hNeuroD1*-Luc with 150ng Pea3. However, it should be noted that the sequence of this construct has not been confirmed, theerfore the results cannot be safely interpreted yet.

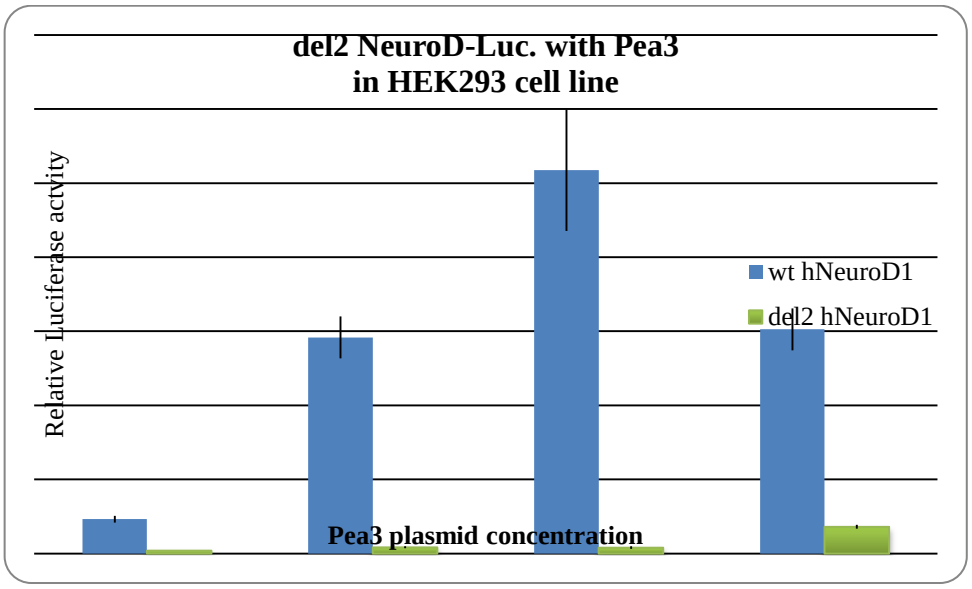


Figure 5.20. Activation of truncated h*NeuroD1* promoter for ets1 and ets2 motif by mPea3 transcription factor in HEK293 cell line. Blue columns are indicating the relative luciferase activity of wild type *NeuroD1* promoter while green columns are indicating the relative luciferase activity of truncated *NeuroD1* promoter, del2 (in which Pea3 binding ets1 and ets2 motif were removed ). Cells were seeded at a density of 5 x $10^4$ per well and in triplicates for each Pea3 concentration, 24 prior to transfection. Transfection mix was prepared with the 50ng, 100ng and 150 ng (amounts are given under each column) pCMV-3Tag-6 flag-mPea3 wild type vector. 200 ng of *HNeuroD1-Luc* (either wild type or mutant) together with the 100 ng of Renilla Luciferase and PEI transfection reagent were added as well. 48h later cells were lysed and luciferase activity was measured. Data were presented relative to the internal control Renilla Luciferase. Standard deviation calculated for the each triplicate is indicated with the error bars

The third truncation *hNeuroD1*-del3-Luc, where ets1, ets2 and ets3 motifs were removed, was given in Figure 5. 21. Blue columns are indicating the activity driven by wildtype *hNeuroD1*-Luc and orange columns are indicating the activity driven by *hNeuroD1*-del3-Luc in different Pea3 concentrations. The Pea3 absence has almost no effect on this truncation *hNeuroD1*-del3-Luc transactivation capacity, no detectable increase or decraese can be observed. A one fold increase can be detected with the addition of 50ng Pea3 in comparison to its wildtype counterpart, but in compasrison to the *hNeuroD1*-del3-Luc transactivation without Pea3 3-fold increase is detectable which was then draw to 2-folds with additional Pea3 doses. A significant reduction by 5-fold observed when 100ng Pea3 is available. 2-fold reduction was observed when Pea3 was increased to 150 ng in comparison to their wildtype counterparts, in other words additional 50 ng Pea3 decrease the transactivation of NeuroD in comparison to activity with 100ng Pea3.

The only certain thing with this particular result is the presence of Pea3 incresed the transactivation capacity of *hNeuroD1*-del3-Luc but without any consistency, since varying doses of Pea3 also varied the activation.

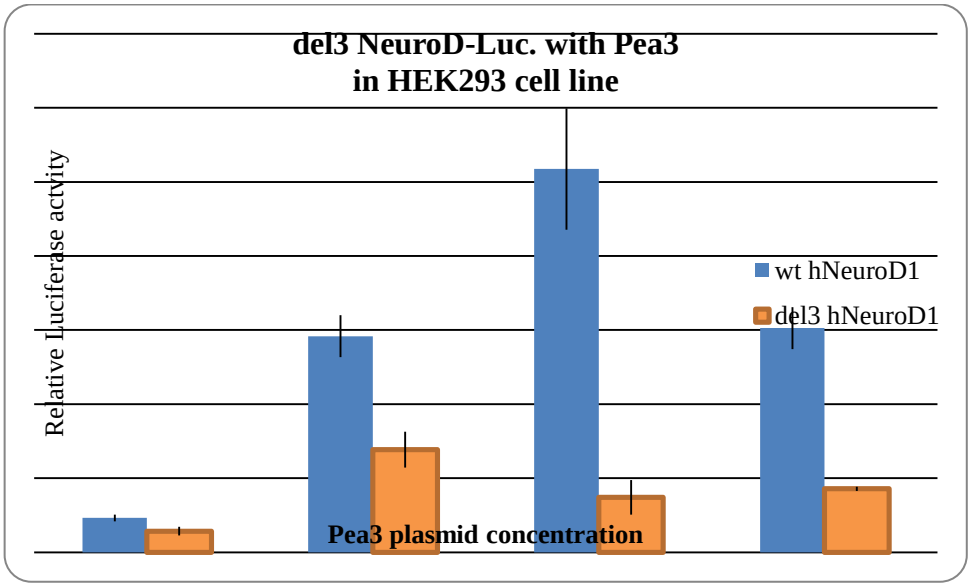


Figure 5.21. Activation of truncated h*NeuroD1* promoter for ets,1ets2 and ets3 motif by mPea3 transcription factor in HEK293 cell line. Blue columns are indicating the relative luciferase activity of wild type *NeuroD1* promoter while orange columns are indicating the relative luciferase activity of truncated *NeuroD1* promoter, del3 (in which Pea3 binding

ets1, ets2 and ets3 motif were removed ). Cells were seeded at a density of 5 x $10^4$ per well and in triplicates for each Pea3 concentration, 24 prior to transfection. Transfection mix was prepared with the 50ng, 100ng and 150 ng (amounts are given under each column) pCMV-3Tag-6 flag-mPea3 wild type vector. 200 ng of *HNeuroD1-Luc* (either wild type or mutant) together with the 100 ng of Renilla Luciferase and PEI transfection reagent were added as well. 48h later cells were lysed and luciferase activity was measured. Data were presented relative to the internal control Renilla Luciferase. Standard deviation calculated for the each triplicate is indicated with the error bars

The forth truncation *hNeuroD1*-del4-Luc, where ets1, ets2, ets3 and also ets4 motifs were removed, was given in Figure 5.22. Blue columns are indicating the activity driven by wildtype *hNeuroD1*-Luc and purple columns are indicating the activity driven by *hNeuroD1*-del4-Luc in different Pea3 concentrations.

The transactivation of this particular construct did not show a sigificant decrease or increase like other truncation constructs. The relative luciferase activity pattern at a glance shows that presence of Pea3 increases the transactivation capacity of *hNeuroD1*-del4-Luc if we compare without Pea3 drived *hNeuroD1*-del4-Luc activity to the Pea3 present drived *hNeuroD1*-del4-Luc. However there is around a 2-fold increase when 50ng Pea3 is available in comparison to its wild type counterpart. Gradual decrease in the *hNeuroD1*-del4-Luc activation was also observed in comparison to both wildtype and truncated constructs.

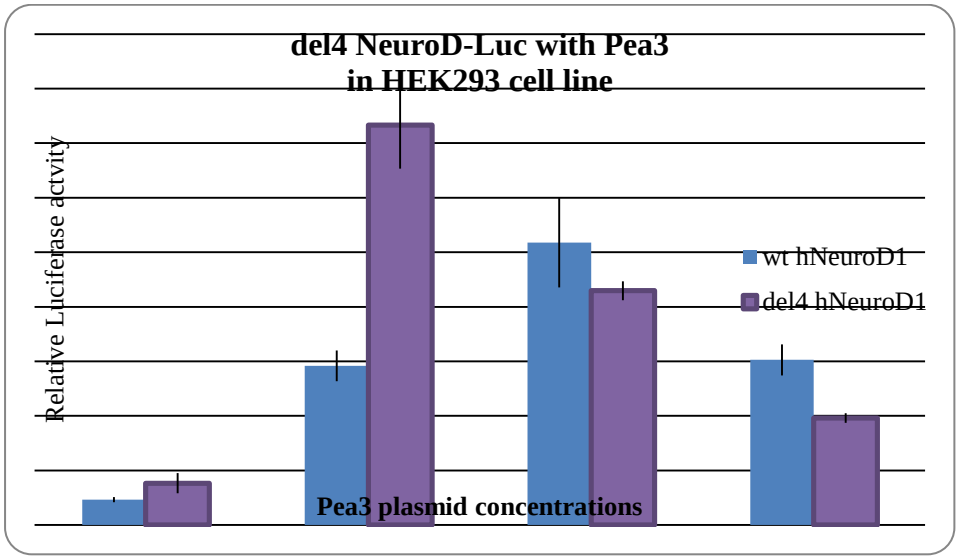


Figure 5.22. Activation of truncated h*NeuroD1* promoter for ets,1ets2, ets3 and ets4 motif by mPea3 transcription factor in HEK293 cell line. Blue columns are indicating the relative luciferase activity of wild type *NeuroD1* promoter while purple columns are indicating the relative luciferase activity of truncated *NeuroD1* promoter, del4 (in which Pea3 binding ets,1ets2,ets3 and ets4 motifs were removed ). Cells were seeded at a density of 5 x $10^4$ per well and in triplicates for each Pea3 concentration, 24 prior to transfection. Transfection mix was prepared with the 50ng, 100ng and 150 ng (amounts are given under each column) pCMV-3Tag-6 flag-mPea3 wild type vector. 200 ng of *HNeuroD1-Luc* (either wild type or mutant) together with the 100 ng of Renilla Luciferase and PEI transfection reagent were added as well. 48h later cells were lysed and luciferase activity was measured. Data were presented relative to the internal control Renilla Luciferase. Standard deviation calculated for the each triplicate is indicated with the error bar

## 5.5. STUDY OF DIRECT BINDING OF PEA3 ON *HNEUROD1* PROMOTER

In our analysis we have shown that Pea3 can activate the NeuroD promoter, although the exact nature and mechanism of this regulation was unclear. In order to study whether Pea3 indeed binds to any one of these *ets* motifs on this promoter, we have next carried out Chromatin Immunoprecipitation (ChIP) assays, hoping this would also help us understand the mutation and deletion results.

Alggen PROMO is an on-line tool enables user to find putative transcriptipon factor binding sites on a given sequence. When 1000bp (-947 to +63) h*NeuroD1* promoter was searched for any possible Pea3 binding sites, 5 predicted sequences were identified for mouse Pea3. Among these five regions three of them with the lowest dissimilarity rate were selected to study. Regions predicted for Pea3 binding were kept in either native form or mutations were introduced to evaluate Pea3 binding. Our initial analyses were done on the motifs with highest probability of binding.

### 5.5.1. Analysing the Chromatin Immunoprecipitation (ChIP) Assay Results

Chromatin IP is a powerful technique to reveal the interaction between protein and DNA. It relies on the precipitation of the interacting protein by aid of antibodies or antibody conjugated beads. In this study ChIP was used in the inveastigation of Pea3 transcription factor bindng to the NeuroD promoter. Pea3 had previously cloned into the pcmv3-tag-6 plasmid vector which posses Flag tag protein aminoacid sequence . Thereby as a result of expression from *cmv* promoter Pea3-Flag fusion protein is produced. If binding regions provided by ALGGEN PROMO is correct, then Pea3-Flag fusion protein will bind those regions (which are previously sheared) and precipitated with the aid of flag antibody conjugated beads (ANTI-FLAG M2 Affinity Gel Sigma) together with the sheared binding regions. Presence of DNA with the binding motif will be proven with polymerase chain reaction carried out with primers confining binding regions which are depicted on h*Neurod1* promoter seqeunce given below. To monitor the binding efficiency more quantitatively qRT-PCR was also applied to the immunoprecipitated samples.

### *5.5.1.1. Quantitative Real Time PCR Analysis of the Chromatin Immunoprecipitation (ChIP) Assay Results*

As noted previously immunoprecipitated DNA samples were analyzed with the qRT-PCR for the presence of interested DNA pieces reserving the putative mPea3 TF binding sites. Primers coupled as forward and reverse which were specially designed to encompass the putative binding motifs were used to amplify any precipitated promter (Figure 5. 23). FastStart SYBR Green Master (Roche) was used to monitor amplified PCR products.

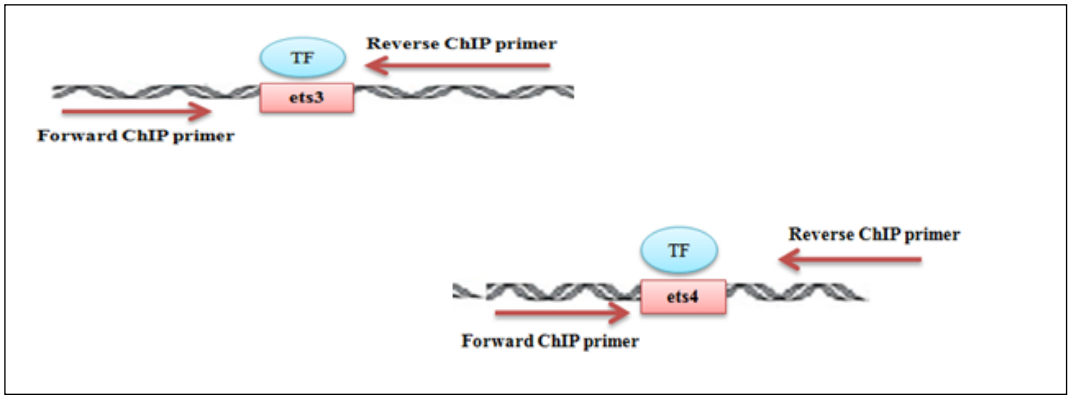


Figure 5.23. Schematic representation of the PCR amplification with forward and reverse primers confining the putative binding ets motifs on the sheared promoter

qRT-PCR was done for ChIP samples subjected to the immunoprecipitation with ANTI-FLAG M2 Affinity Gel and also for the INPUTs which were not subjected to any precipitation. The reason for having INPUT as a control is to assess the amount of starting chromatin material, therefore we can speculate on how much of the chromatin was precipitated due to the DNA-protein interaction. Therefore while we would have all sheared chromatins in the INPUT, the ChIP samples will only have the sheared DNA pieces that posses crosslinked Pea3 transcription factors which has precipitated with the anti-flag affinity resins.

Gel images given in Figure 5. 24 below shows the agorose gel analysis of PCR amplified products of ChIP samples and inputs. Bands, grouped in three with underlying bar belong to the same sample reaction which performed in triplicates. The expected PCR amplicon length is 191 bp for ets3 Pea3 binding motif bearing region. Gel electrophoresis showed that all three INPUT samples of wildtype NeuroD1 without Pea3, wt NeuroD1 with Pea3

and NeuroD1 ets-3 mutant with Pea3 were amplified, indicating the presence of amplicon and success of reaction.

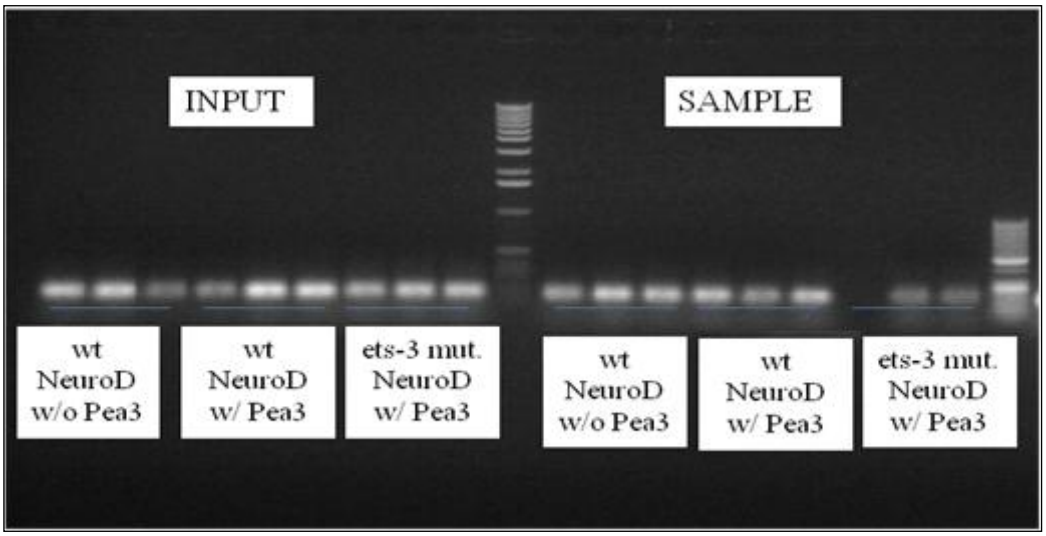


Figure 5.24. Gel electrophoresis of qRT-PCR results of NeuroD ets3 mutataion and wild type INPUT and ChIP samples. qRT-PCR for each sample was done in triplicates and each replica was loaded into gel. Bands are grouped and indicated with a underlying blue bar. Each group belongs to the same sample and goes like, INPUT / SAMPLE; wild type NeuroD1without Pea3, wild type NeuroD wth Pea3, ets-3 mutant of NeuroD with Pea3

Gel images given in Figure 5. 25 below shows the agorose gel analysis of PCR amplified products of ChIP samples and inputs. Bands, grouped in three with underlying bar belong to the same sample reaction which performed in triplicates. The expected PCR amplicon length is 175 bp for ets3 Pea3 binding motif bearing region.

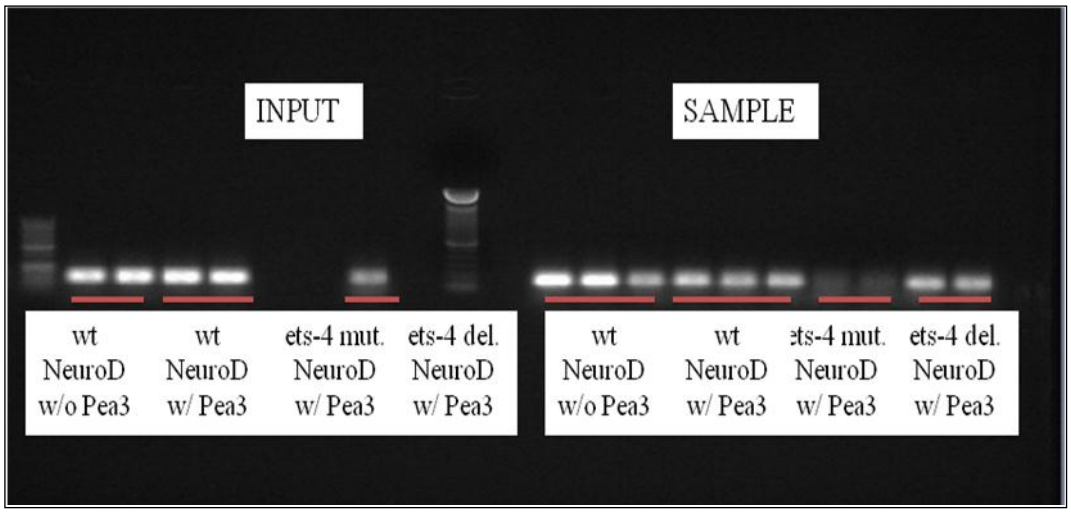


Figure 5.25. Gel electrophoresis of qRT-PCR results of NeuroD ets4 mutataion and wild type INPUT and ChIP samples. qRT-PCR for each sample was done in triplicates and each replica was loaded into gel. Bands are grouped and indicated with underlying red line. Each group belongs to the same sample and goes like, INPUT / SAMPLE; wild type NeuroD1without Pea3, wild type NeuroD wth Pea3, ets-4 mutant of NeuroD with Pea3 and ets-4 deletion mutant of NeuroD with Pea3

# 6. DISCUSSION

Pea3, member of ETS family transcription factor, affect the expression of several genes by directly regulating their promoters. Pea3 is differentially expressed in development. The Pea3 expression starts at embryonic day 9.0-9.5 in the developing mouse embryo in several tissues including brain [23].At E10.5 and E12.5 Pea3 expression is seen at motor neurons and sensory neurons respectively. Pea3 has been highly studied for its role in the branching morphogenesis. Motor neuron branching on muscle target is one of them. Jean Livet and colleagues showed that Pea3 is important for the differentiation of motor neurons so they can localize in the same pool and also it is important for their terminal arborization in the target muscles [34].

NeuroD on the otherhand, as the name implies is known for its role in the differention of neurons, it is also crucial for the survival of already differentiated neurons. Expression of NeuroD starts early as E.10 at dentate gyrus and then seen at regions where neuronal differentiation occurs including dorsal root ganglia, olfactory epithelium,cerebral cortex, and hippocampus. However, its expression gradually decreases through the adulthood [35]. Neuronal differentiation starts with the migration of cell body and the axon initiation and differentiation starts after neuron migrates to its act of location [36]. Therefore by the axon initiation following differentiation we can speculate about the involvement of Pea3 expression since it is involved in axonal branching and following NeuroD expression for terminal differenatiation.

In this study we focused on the Pea3 mediated NeuroD1 regulation. Studies have been done in our lab showed that Pea3indeed transactivates the h*NeuroD1* promoter. Even though Pea increases the NeuroD1 mediated luciferase activity, the levels of activity differentiates in SH-SY5Y and HEK293 cell lines.

Pea3 as noted before, is classified under the Pea3 group of ETS proteins with ERM and ER81. They are generally co-expressed in the same organ but shhows different site of expression (A. C. Lelievre *et al*, 1997) Therefore we would like to check their effect on the same target.

Luciferase reporter assay of ER81 mediated NeuroD promoter in SH-SY5Y cell line showed that increasing concentrations of ER81 enhances the luciferase expression under NeuroD promoter control. However same assay in HEK293 cell line shows no significant increase in the luciferase activity due to the similar reasons stated earlier. Luciferase reporter assay of ERM in SH-SY5Y cell line, on the other hand, has almost no effect on the NeuroD promoter regulation if we consider the error bars (Figure 5. 4.). Therefore effect of Pea3 group members regarding the regulation of gene expression can be both target and cell type dependent.

In the previous studies we had also showed that MAPK signaling pathway plays a role in the transactivation capacity of Pea3 both as inducer and represser depending on both the amino acid residue subjected to the phosphorylation and its location in the protein. In these studies the potential phosphorylation serine residues proposed by our lab were studied.

Pea3 protein bears a transactivation domain in the N-terminus which is flanked by two negative regulatory regions. Another negative regulatory regions also present at either side of the ETS- DNA binding domain [37].

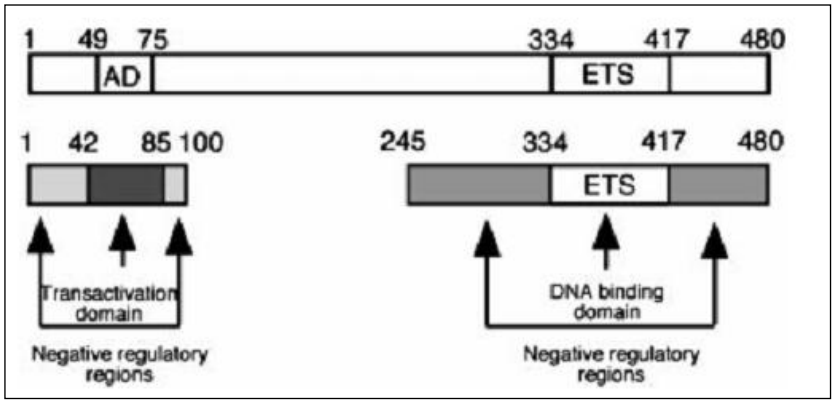


Figure 6.1. Auto-regulatory domains of Pea3 transcription factor. The flanking on either side of the transactivation domain negatively regulates transactivation while regions bearing ETS-DNA binding domain negatively regulates DNA binding [37]

From the regulatory regions point of view, phosphorylation status of Pea3 should be considered in where phosphorylation happens and what other post-translational modifications are happening neigbouring to those putative phosphorylation sites.

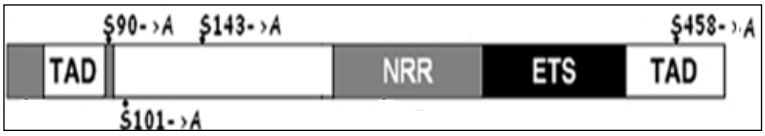


Figure 6.2. Possible MAPK Phosphorylation serine residues on Pea3 (proposed by Axan Lab.). Schema is also representing the non-phosphorylatable serine-alaine conversion but we also studied the serine to glutamic acid conversion to mimick the phosphorylation

Regarding the luciferase assay results phospho-mimicked mutant for S90 positively regulates Pea3 mediated promoter activation and it increases the luciferase expression in comparison to the luciferase expression directed by wildtype *NeuroD1-Luc*. Therefore, regarding the region where S90 resides we can speculate that phosphorylation from this aminoacid residue in the negative regulatory region may reverse its inhibitory function and enhance activation.

However phosphorylation at S101 and S143 residues decreases Pea3 mediated NeuroD activation. S101 phosphorylation is necesseary for the sumoylation of K96 residue [38]. Although sumoylation also enhances the transactivation capacity, sumoylation at K96 residue may enhance protein turnover therefore decrease the Pea3 protein level which also downregulates the NeuroD promoter activation.

The case with Ser143 is less clear since it does not correspond to any revealed regulatory region. But we can speculate that phosphoryation in this residue may alter the folding of the protein and hence decreasing the activity of Pea3.

Like Ser90, Ser458 also coincide with negative regulatory region in the carboxy terminal end, which negatively regulates DNA binding activity of Pea3 [38]. However the effects on Ser 458 mutations are independent of its phosphorylation status. Since in both case either in constitutively phosphorylated form or non-phosphorylatable form, mutant

*NeuroD1* promoter mediated luciferase expression was increased. This can be explained not by phosphorylation status but by the alteration of the serine to any other aminoacid. Serine may be involved in the auto-inhibition of DNA binding and altering it may relax this inhibition and enhance DNA binding hence expression activation. In order to reveal whether serine is responsible for this inhibiton, or this is due its charge or aminoacid character mutations for other aminoacids should be done to see the effect.

Luciferase reporter assays mentioned above were carried out in human neuroblastoma cells (SH-SY5Y) which has neuronal characteristics and Human Embryonic Kidney (HEK293) which is non-neuronal cell line. That is why relative luciferase activities and values were varied for the same sample. Any intrinsic regulation, for instance endogenous expression of Pea3 or ERM and ER81 or the other ETS family members which can have binding affinity for the same motif, might have effected the NeuroD1 promoter driven luciferase expression and hence activity.

Five Pea3TF binding motifs are proposed by the ALGGEN PROMO. Three out of these five motifs with higher binding affinity for Pea3 were chosen to study in luciferase and ChIP assays. For each of these binding motifs non-bindable mutations were created in order to prevent Pea3 binding and mutated sequences were checked wheter Pea3 binding was abolished indeed (Figure 5.16 – 5.19 showing the ALGGEN PROMO search for this mutations). Only two of these mutations were successfuly created for ets3 and ets4. Since the ets1 motif is present at the very beginning of the string, mutation couldn't be achieved.

According to luciferase assay results of *NeuroD1*-ets3mut-Luc, blocking Pea3 binding increased the relative luciferase activity with ascending Pea3 concentration comparing to the wild type promoter. However the absence of Pea3 interestingly enhanced the activation as well. Considering the protein binding profiles and luciferase activities, it could be said that, the mutant motif may create a new binding site for another protein which Pea3 disturbs since NeuroD driven luciferase activity was increased in the absence of Pea3 and decreased with the addition of it. Thus it can be proposed that presence of Pea3 for this mutated motif might inhibit the binding capacity of protein that newly emerged for this binding motif. Figure 5.16 shows the transcription factor binding profile for only the

sequence of wildtype ets3 motif and Figure 6. 3. shows the transcription factor binding profile of the mutant ets3 motif.

Beside Pea3 there are also other ETS family members that can bind to this motif such as, c-ets-1 and Elk-1. According to *in silico* studies, those possible interactions were eliminated with the mutation and all the protein binding profile has changed (Figure 6.3).

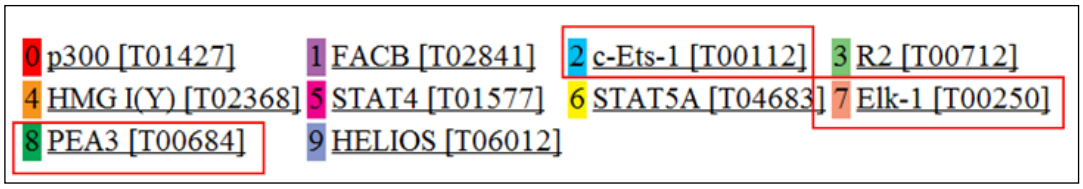


Figure 6.3. Transcription factors, binding to the ets3 motif (putative Pea binding motif) in wild type hNeuroD1 promoter. Only the sequence of ets3 motif was searched for the transcription factor binding profile. Red box is indicating the presence of Pea3, Elk-1 and C-Ets-1 binding

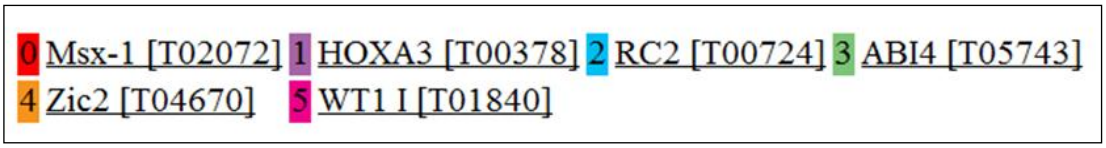


Figure 6.4. Transcription factors, binding to the mutated ets3 motif (putative Pea binding motif) in hNeuroD1 promoter. Only the sequence of mutated ets3 motif was searched for the transcription factor binding profile. Pea3 binding has gone with the mutation

The luciferase assay results for ets-4 however is more consistent. Activation directed by *NeuroD1*-ets4mut-Luc constantly increasing with ascending Pea3 doses. Relative luciferase levels are higher than the wildtype control as well. One possible explanation for this motif might be that its wildtype version can serve as a binding site for an inhibitory protein that newly generated (see Figure 6. 6.) and the mutation in this site may block this inhibitory interaction.On the other hand activity has greatly enhanced when there is high concentration of Pea3 (see Figure 5. 13.) which led us think although Pea3 may not bind to the motif anymore, high doses of it can disturb the inhibitory action of protein and enhance activation to greater extent.

The figure below 6.5. and 6.6 are showing the possible binding sites for wildtype and mutant ets4 motif respectively. Mutation led to removal of any naturally proposed interactions and led to generation of new interactions with two other proteins which may involve in the inhibiton that is mentioned above.

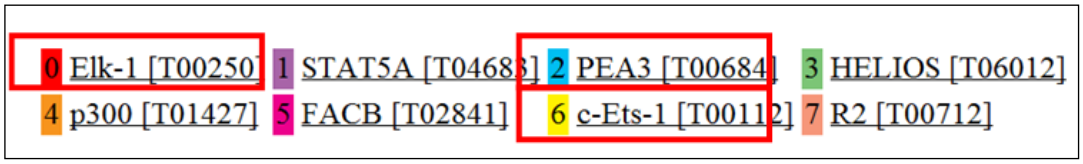


Figure 6.5. Transcription factors, binding to the ets4 motif (putative Pea binding motif) in wild type hNeuroD1 promoter. Only the sequence of ets4 motif was searched for the transcription factor binding profile. Red box is indicating the presence of Pea3 binding, Elk-1 and C-Ets-1 binding

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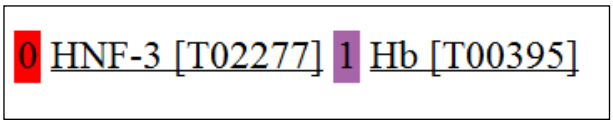


Figure 6.6. Transcription factors, binding to the mutant ets4 motif (putative Pea binding motif) in hNeuroD1 promoter. Only the sequence of ets4 motif was searched for the transcription factor binding profile. Pea3 binding has gone with the mutation

Mutation may have changed the protein binding profile, therefore we delete the 5’ GGA-3’ core motif this time to minimize the alteration in the naturally occurring interactions (Figure 5.18). However, deletion mutation at this ets4 motif gave us similar pattern as mutant ets4 motif. If we compare the the transcription factor binding profile of wildtype and deletion mutant of ets4, we see that deletion of 5’-GGA-3’ only removed some of transcription factors together with the Pea3 and add nothing new.

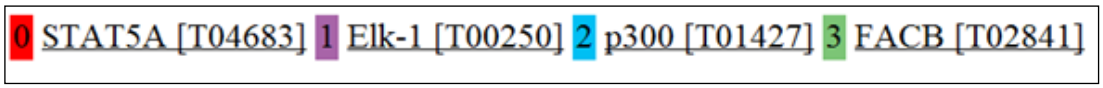


Figure 6.7. Transcription factors, binding to the deletion mutant ets4 motif in hNeuroD1 promoter. Only the sequence of deletion mutant ets4 was searched for the transcription factor binding profile. Pea3 binding has gone with the deletion

Next we truncate the *NeuroD1* promoter to remove Pea3 transcription factor binding motifs sequentially.Since studies with various mutant promoters gave confusing results due to newly generated binding motifs together with binding proteins, we went for the truncation to simply remove the possible binding sites.

First 29 nucleotide truncation eliminated Pea3 binding. *NeuroD1* activation when there is 100ng ad 200 ng of Pea3 was decreasd. This could be linked to the removal of putative Pea3 binding site but *NeuroD1* promoter activation without Pea3 was enhanced.Therefore we cannot make sure that the decrese in the *NeuroD1* activation is merely effected by Pea3 binding blocking. Elimination of other possible interactions (see Figure 6. 8.), beside Pea3, should be considered as well to understand this effect. As seen in the list below, 55 possible interactions were removed with truncation. Among these proteins there are also other ETS TFs like Elk-1, Pu.1, c-Ets1. To clarify enhanced NeuroD activity in the absence of Pea3 we may say that for this putative ets1 motif including its flanking sequences Pea3 may compete to bind there. However when Pea3 binding was eliminated Pea3 can not bind but may still compete with the other protein. So, while the absence of Pea3 mediates the increase in NeuroD activity, its presence reduce activation without bindig to DNA.

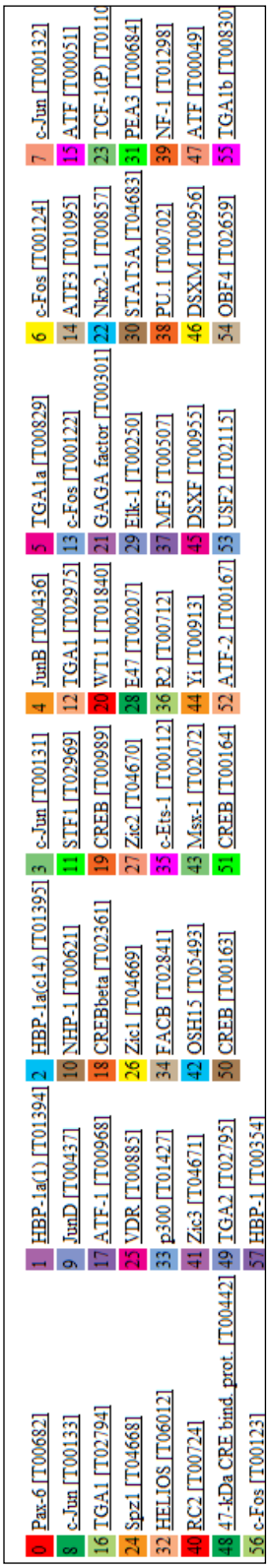


Figure 6.8. Transcription factor binding profile of the first 29bp long truncated sequence of NeuroD1 promoter (see Figure 5.16 for the sequence). These transcription factors are the ones removed with the truncation

Removal of both ets1 and ets2 successfully reduce the activation of *hNeuroD1*-del2-Luc (Figure 6.9.). Considering the previous truncated NeuroD1 activity, removal of ets2 contribute the reduction in NeuroD promoter activity, in which putative ets2 motif can be indeed provide a binding site for Pea3. But together with the truncation complete removal of other putative interaction partners should be considered as well (Figure 6.9.) and more reliable evaluation could be done if this motif is deleted only.

*hNeuroD1*-del3-Luc, truncated NeuroD promoter construct is free of ets1, ets2 and ets3. So far both ets1 and ets2 removal showed enhanced NeuroD1 activation in the absence of Pea3. However with additional removal of ets3 did show no effect on NeuroD1 actvation in the absence of Pea3. In comparison to the wild type counterparts, elimination of ets3 reduced NeuroD1 activation, which makes it to be involved in Pea3 binding. On the other hand when we compare the activity of *hNeuroD1*-del3-Luc with the *hNeuroD1*-del2-Luc, activity of *hNeuroD1*-del3-Luc has increased which suggest a possible positive regulatory region in between this ets2 and ets3.

Unlike the other truncated NeuroD1 activation, elimination of four of the motifs seem to have an effect on *hNeuroD1*-del4-Luc, NeuroD is highly enhanced in the presence of 50ng Pea3, and activity at other concentration are quite high as well even though not exceeds wild type counterparts, in comparison to the first three truncated NeuroD1 promoter activity was enhanced. The seqeuces in between ets3 and ets4 than should bear an inhibitory region that one of these eliminated proteins could bind (Figure 6.11.)

Pea3 mediated activation of NeuroD can be dependent on the interacting partner. Also this interactions might be altered with the changing Pea3 concentrations.

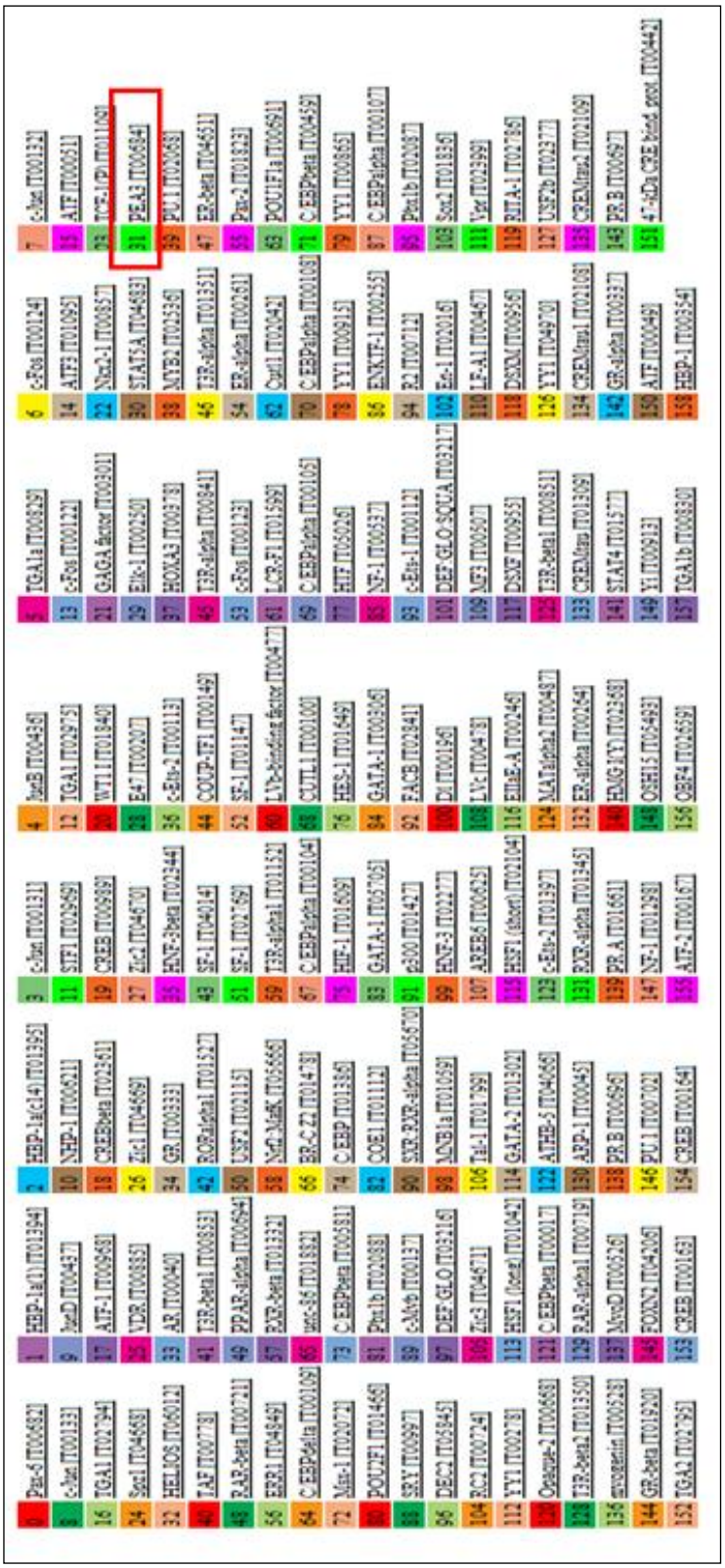

Figure 6.9. Transcription factor binding profile of the first 114bp truncated sequence of NeuroD1 promoter (see Figure 5. 16 for the sequence). These transcription factors are the ones removed with the trunctaion

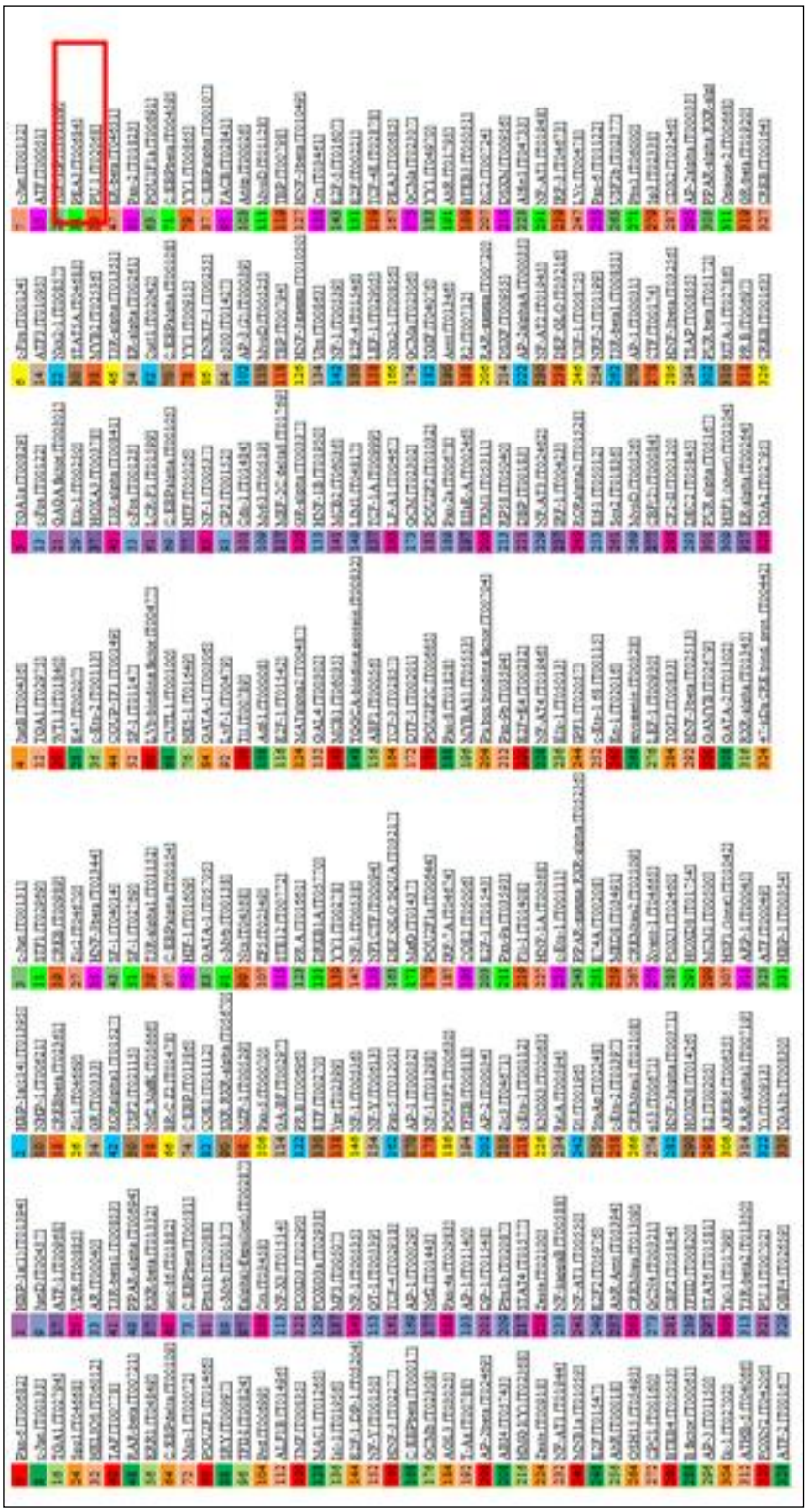

Figure 6.10. Transcription factor binding profile of thefirst 488 bp truncated sequence of NeuroD1 promoter (see Figure 5.16 for the sequence). These transcription factors are the ones removed with the truncation

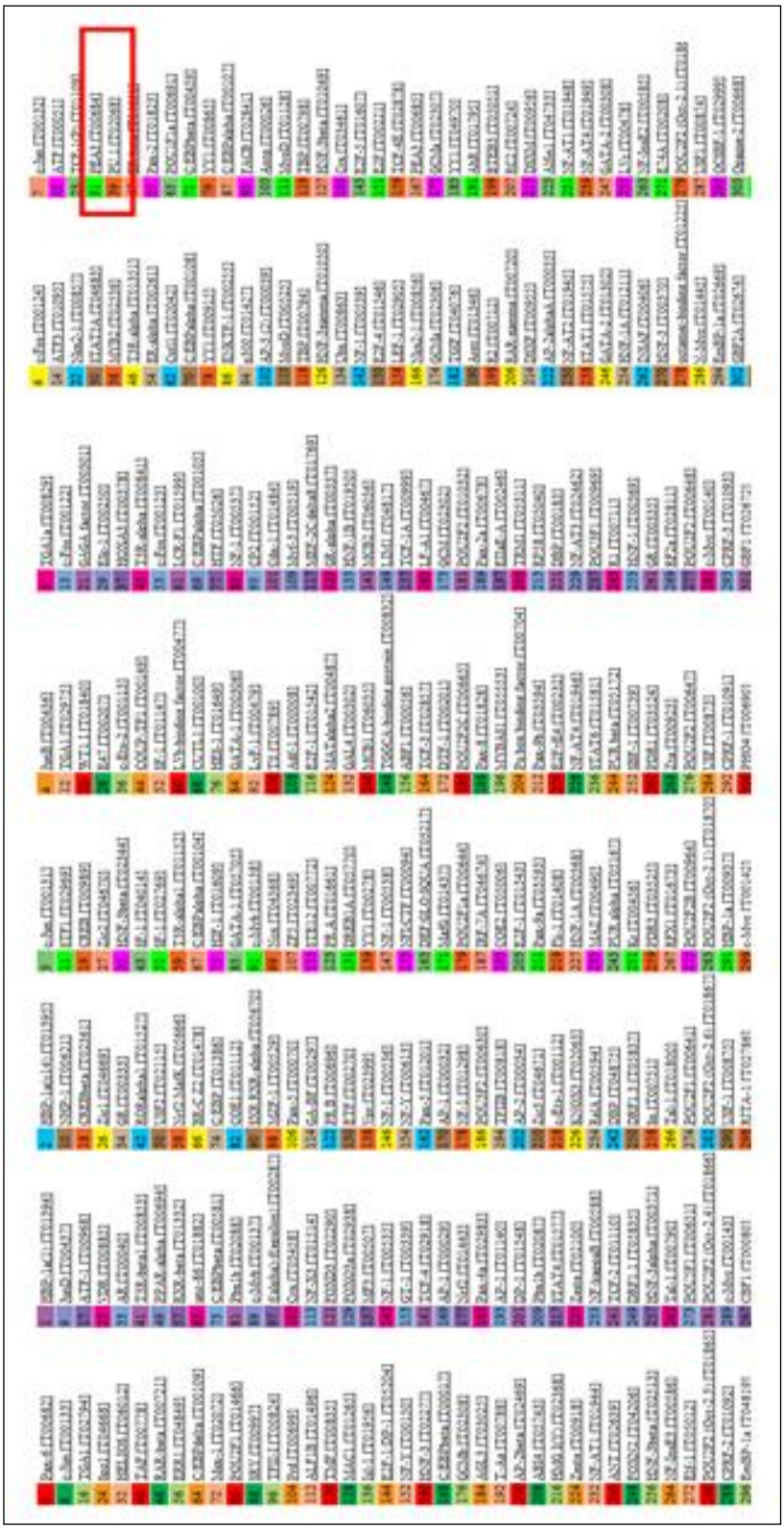

Figure 6.11. Transcription factor binding profile of thefirst 720bp truncated sequence of NeuroD1 promoter (see Figure 5.16 for the sequence). These transcription factors are the ones removed with the trunctaion

Regulatory mechanisms of NeuroD promoter should be considred as well, to see if there are other effects on NeuroD activity independent of Pea3 presence. Takafumi Miyachi and colleagues showed the importance of four E-boxes that human NeuroD bears in 464 nucleotides upstream to transcription start site. E-boxes have shown to increased the activity of NeuroD promoter. E-box 1 and E-box 4 are important for auto-regulation of NeuroD while E-box 3 is important for the transcription of NeuroD. If we consider these E-boxes for the evaluation of truncated *hNeuroD1* promoter activity, the first three truncation did not remove any E-boxes while with truncation del4 all these regulatory elements were gone. However, not a dramatic decrease has observed after removal of these elements.

To be able to state more reliable comment on this constructs studies should be repeated. In addition to this, since those experiments were carried out in HEK293 cell line which has not neuronal characteristic any possible intracellular effects which may involve in Pea3 binding, or activation of Pea3 itself, are avoided.

Also it should be noted that relative luciferase activity values in HEK293 cells were not consistent through the different luciferase assays. They were unusually high in comparison to the luciferase experiments performed with wildtype *hNeuroD*-Luc.earlier. That must be either cells or luciferase substrate batches used in the experiment. In order to eliminate this variation studies should be repeated in SH-SY5Y cell lines with a consistent substrate batches.

Luciferease reporter analysis results prove that Pea3 mediates NeuroD promoted luciferase activation, so Pea3 should bind from any regions that ALGGEN PROMO suggests since those putative regions are proposed referring to the literature.

Pea3 had cloned ino the pCMV-3 tag-6Flag vector and resulted with a Pea3-Flag tag fused protein product.This would enable us to use flag antibody conjugated bead in order to precipitate Pea3 with promoter crosslinked to it. Since only the exogenous Pea3 is fused to flag tag, no endogenous Pea3 would be expected to precipitate.

Detection of DNA precipitate was done with quantitative real time PCR which enables us to evaluate the amount of DNA precipitate and correlate them with the binding affinity of Pea3 on NeuroD promoter. Resultant PCR products were also run on gel to see if amplification indeed occurred. However our results show that even though there is no exogenous Pea3transfected to the cells, ChIP samples obtained from these cells resulted with a DNA precipitate. Two possible reason can be suggested here, either flag antibody conjugated beads did not work sufficiently so endogenous Pea3-DNA crosslink come without any specifity or elution step was not successfully carried out therefore endogenous Pea3-DNA crosslink remain in the sample.

This experiment should be repeated with the new flag beads and elution step should be performed more carefully. In addition to that Pea3-Flag fusion protein expression should be confirmed before continue with the ChIP in order to prove that starting sample material has the protein to be precipitated.

These studies have been carried out *in vitro* with the two different cell line with neuronal and non-neuronal characteristics. However, since NeuroD1 is involved in the differentiation of neuronal precursors into mature neurons with a high expression rate in embryonic development studies should be carried out with more reliable model system like motor neuron progenitors in spinal cord where we can follow the NeuroD expression together with Pea3 during transition from immature neuron to the fully differentiated neuron.

In addition to Pea3 possible interaction of NeuroD1 with ERM and ER81 has been investigated but, Elk-1 could help to understand the regulation of this gene as well, since they are both ETS family proteins and binds to similar DNA motifs on promoter as in silico studies also supported. All putative Pea3 bindnig motifs are also given as potential binding site for Elk-1. In addition to that Elk-1 binding dominancy on this promoter is observed with the in silico analysis. Therefore it will be very valuable and informative if Elk-1 is also studied with NeuroD promoter.

However, in our existing system with neuroblastoma (SH-SY5Y) and embryonic kidney (HEK293) cells. Elk-1 may repress the Pea3 mediated expression for some motifs where

we get high activity in the absence of Pea3, since it is highly expressed in those cell lines, therefore to see the difference experiments should be carried out in embryonic systems as well. Consistency with *in vivo* studies should be also satisfied for more reliable understanding.

Since Pea3 and NeuroD share an important role in neuronal differentiation their incorporation can be carried a step further and used in the researches like neuron replacemet or stem cell therapies where differentiation and functional axon connections are needed.

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# APPENDIX A : MUTATION ANALYSES


Figure A.1. Sequencing result of mutation trial for Pea3 binding site-1 (ets1) on NeuroD promoter. Red window is indicating the region where mutation should have been (Non succesfull trial). *mut_1A_ R1_ rev_comp.*; template where mutation has tried to be done, *neuroD_ prom_ wt*; wild type NeuroD promoter sequence

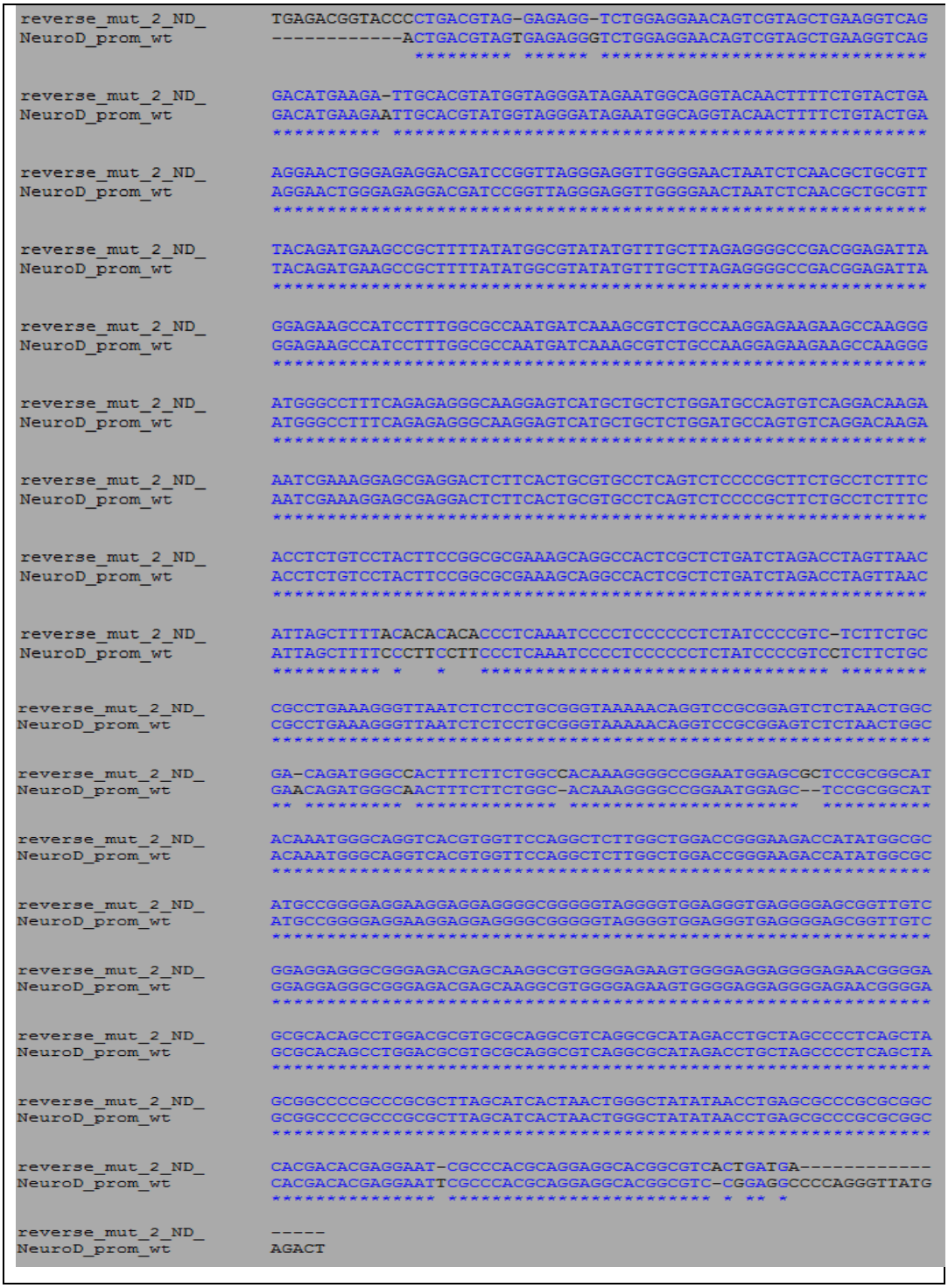


Figure A.2. Sequence analysis of NeuroD promoter with mutation in binding site 2 (ets3). Analyse was done in the direction of reverse primer. Region exhibited with the red window is showing the mutated region. *Reverse mut 2 ND*, is showing the results coming from sequence analysis, and the *neuroD-prom-wt* is showing the sequence of wild-type NeuroD promoter, in order to compare and confirm the existance of the mutation

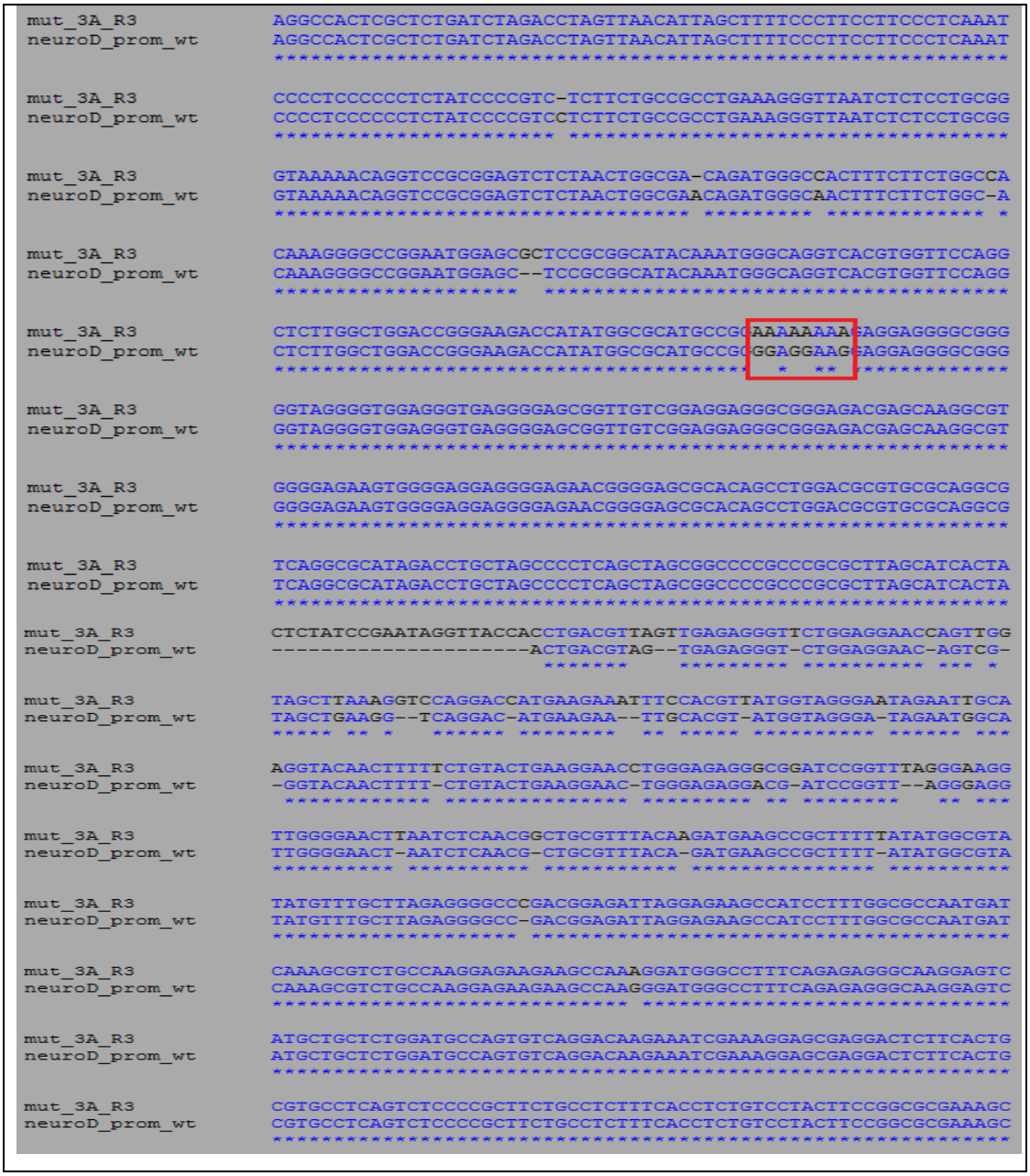


Figure A.3. Sequence analysis of NeuroD promoter with mutation in binding site 3. Analyse was done in the direction of reverse primer. Region exhibited with the red window is showing the mutated region. Mut-3A-R3, is showing the results coming from sequence analysis, and the neuroD-prom-wt is showing the sequence of wild-type NeuroD promoter, in order to compare and confirm the existance of the mutation. Arrows are indicating the non-matching single nucleotides. These non matches are consistent in all three samples and might be a sequenceing problem

Table A.1. Luciferase results of Pea3 and NeuroD interaction in SH-SY5Y cells

| | Firefly Luciferase | | | Renilla Luciferase | | |
|---|---|---|---|---|---|---|
| pCMV | 1,619 | 1,181 | 1,123 | 11,09 | 8,03 | 7,594 |
| 15ng | 1,245 | 1,685 | 1,678 | 6,695 | 8,585 | 8,244 |
| 75ng | 2,286 | 2,292 | 2,24 | 9,032 | 8,307 | 7,715 |
| 150ng | 4,138 | 4,03 | 3,734 | 5,352 | 5,207 | 5,445 |
| 300ng | 4,044 | 3,938 | 3,235 | 5,257 | 4,757 | 3,268 |
| 450ng | 6,846 | 4,798 | 6,625 | 7,65 | 4,867 | 6,246 |
| 600ng | 9,443 | 6,936 | 8,873 | 14,23 | 8,209 | 11,73 |

Table A.2. Relative ratio of luciferase results of Pea3 and NeuroD interaction in SH-SY5Y cells

| | Firefly Luciferase / Renilla Luciferase | | | Average | Standart deviation |
|---|---|---|---|---|---|
| pCMV | 0,145987 | 0,147073 | 0,14788 | 0,146980252 | 0,000949702 |
| 15ng | 0,18596 | 0,196273 | 0,203542 | 0,19525807 | 0,008834943 |
| 75ng | 0,2531 | 0,275912 | 0,290343 | 0,273118486 | 0,018778178 |
| 150ng | 0,773169 | 0,773958 | 0,685767 | 0,744297934 | 0,05069102 |
| 300ng | 0,76926 | 0,827833 | 0,989902 | 0,862331594 | 0,114295057 |
| 450ng | 0,894902 | 0,985823 | 1,060679 | 0,980467895 | 0,08301807 |
| 600ng | 0,663598 | 0,844926 | 0,756436 | 0,75498694 | 0,090672824 |

Table A.3. Luciferase results of Pea3 and NeuroD interaction in HEK293 cells

| | Firefly Luciferase | | | Renilla Luciferase | | |
|---|---|---|---|---|---|---|
| pCMV | 0,0119 | 0,0294 | 0,0086 | 0,8173 | 2,022 | 0,9746 |
| 15ng | 0,043 | 0,0668 | 0,047 | 0,8051 | 1,194 | 0,6991 |
| 75ng | 0,1174 | 0,0969 | 0,1403 | 1,218 | 1,176 | 1,45 |
| 150ng | 0,2566 | 0,2817 | 0,313 | 1,918 | 1,694 | 1,686 |
| 300ng | 0,5023 | 0,5817 | 0,5668 | 2,25 | 2,065 | 2,106 |
| 450ng | 0,3636 | 0,5145 | 0,3792 | 1,905 | 2,203 | 1,472 |
| 600ng | 0,6731 | 0,7241 | 0,7332 | 2,786 | 2,765 | 2,412 |

Table A.4. Relative ratio of luciferase results of Pea3 and NeuroD interaction in HEK293 cells

| | Firefly Luciferase / Renilla Luciferase | | | Average | Standart deviation |
|---|---|---|---|---|---|
| pCMV | 0,01456 | 0,01454 | 0,008824 | 0,012641443 | 0,003305903 |
| 15ng | 0,05341 | 0,055946 | 0,067229 | 0,058861736 | 0,007356696 |
| 75ng | 0,096388 | 0,082398 | 0,096759 | 0,091848033 | 0,008186108 |
| 150ng | 0,133785 | 0,166293 | 0,185647 | 0,161908164 | 0,026207205 |
| 300ng | 0,223244 | 0,281695 | 0,269136 | 0,258025054 | 0,030768506 |
| 450ng | 0,190866 | 0,233545 | 0,257609 | 0,227340001 | 0,033801186 |
| 600ng | 0,241601 | 0,261881 | 0,30398 | 0,269153871 | 0,031819289 |

Table A.5. Luciferase results of Pea3 phosphorylation mutants and NeuroD interaction in SH-SY5Y cells

| | Firefly Luciferase | | | RenillaLuciferase | | |
|---|---|---|---|---|---|---|
| PEA3 | 2,763 | 3,212 | 3,313 | 16,41 | 14,89 | 15,56 |
| S90A | 1,41 | 1,42 | 1,337 | 11,83 | 14,71 | 11,01 |
| S90E | 3,553 | 2,943 | 3,117 | 10,28 | 10,56 | 9,574 |
| S101A | 4,604 | 5,108 | 4,95 | 21,25 | 21,29 | 21,47 |
| S101E | 1,743 | 2,013 | 1,759 | 14,49 | 15,38 | 11,97 |
| S143A | 8,656 | 5,928 | 6,449 | 34,19 | 20,94 | 19,95 |
| S143E | 1,107 | 1,203 | 1,01 | 11,77 | 9,524 | 7,465 |
| S458A | 4,868 | 6,11 | 5,549 | 15,19 | 16,93 | 13,86 |
| S458E | 6,055 | 5,205 | 4,662 | 13,87 | 11,54 | 9,837 |

Table A.6. Relative ratio of Pea3 phosphorylation mutants and NeuroD interaction in SH-SY5Y cells

| | Firefly Luciferase / Renilla Luciferase | | | Average | Standart deviation |
|---|---|---|---|---|---|
| PEA3 | 0,168373 | 0,215715 | 0,212918 | 0,199001975 | 0,026562374 |
| S90A | 0,119189 | 0,096533 | 0,121435 | 0,112385511 | 0,013774579 |
| S90E | 0,345623 | 0,278693 | 0,325569 | 0,316628333 | 0,034348809 |
| S101A | 0,216659 | 0,239925 | 0,230554 | 0,229045978 | 0,011706116 |
| S101E | 0,12029 | 0,130884 | 0,146951 | 0,132708277 | 0,013423694 |
| S143A | 0,253173 | 0,283095 | 0,323258 | 0,286508715 | 0,03516687 |
| S143E | 0,094053 | 0,126312 | 0,135298 | 0,118554403 | 0,021689539 |
| S458A | 0,320474 | 0,360898 | 0,400361 | 0,36057752 | 0,03994434 |
| S458E | 0,436554 | 0,45104 | 0,473925 | 0,453839517 | 0,018842277 |

Table A.7. Luciferase results of Pea3 phosphorylation mutants and NeuroD interaction in HEK293 cells

| | Firefly Luciferase | | | Renilla Luciferase | | |
|---|---|---|---|---|---|---|
| pea3 | 1,182 | 1,795 | 1,058 | 0,8598 | 1,109 | 0,7567 |
| S90A | 0,5471 | 1,448 | 0,5273 | 1,048 | 3,03 | 0,9679 |
| S90E | 0,266 | 0,3511 | 0,4445 | 0,256 | 0,3201 | 0,2903 |
| S101A | 0,8094 | 1,95 | 2,131 | 2,609 | 6,267 | 6,024 |
| S101E | 0,4494 | 1,868 | 1,883 | 1,37 | 4,769 | 5,324 |
| S143A | 1,497 | 1,436 | 0,947 | 0,858 | 0,6473 | 0,7099 |
| S143E | 0,1447 | 0,1326 | 0,1731 | 0,3023 | 0,2614 | 0,3363 |
| S458A | 2,241 | 2,185 | 1,197 | 1,328 | 0,8874 | 0,5521 |
| S458E | 1,39 | 1,345 | 1,016 | 0,5438 | 0,4786 | 0,3402 |

Table A.8. Relative luciferase ratio of Pea3 phosphorylation mutants and NeuroD interaction in HEK293 cells

| | Firefly Luciferase / Renilla Luciferase | | | Average | Standart Deviation |
|---|---|---|---|---|---|
| pea3 | 1,374738 | 1,618575 | 1,398176 | 1,463829965 | 0,134524801 |
| S90A | 0,522042 | 0,477888 | 0,544788 | 0,514905819 | 0,034016065 |
| S90E | 1,039063 | 1,096845 | 1,531175 | 1,222360628 | 0,268996785 |
| S101A | 0,310234 | 0,311154 | 0,353752 | 0,325046376 | 0,024863759 |
| S101E | 0,328029 | 0,391696 | 0,353681 | 0,357802337 | 0,032033009 |
| S143A | 1,744755 | 2,218446 | 1,333991 | 1,7657306 | 0,442600499 |
| S143E | 0,478664 | 0,507269 | 0,514719 | 0,500217045 | 0,019033948 |
| S458A | 1,6875 | 2,462249 | 2,168085 | 2,10594492 | 0,391094867 |
| S458E | 2,556087 | 2,81028 | 2,986479 | 2,784281774 | 0,216370501 |

Table A.9. Luciferase assay measures for Firefly luciferase and Renilla Luciferase of Pea3 motif mutants and NeuroD interaction

| | **Firefly Luciferase** | | | **Renilla Luciferase** | | |
|---|---|---|---|---|---|---|
| wt ND1 +( - ) Pea3 | 0,3676 | 1,373 | 1,454 | 2,865 | 8,406 | 11,85 |
| wt ND1 + 50 ng Pea3 | 4,88 | 4,77 | 3,344 | 22,91 | 21,87 | 18,3 |
| wt ND1 + 100 ng Pea3 | 6,393 | 7,907 | 6,76 | 26,72 | 31,33 | 27,12 |
| wt ND1 + 200 ng Pea3 | 8,223 | 4,578 | 7,97 | 20,06 | 11,47 | 16,28 |
| | | | | | | |
| ets4 mut ND1 +( - ) Pea3 | 9,758 | 8,2 | 5,436 | 14,56 | 14,52 | 9,7 |
| ets4 mut ND1 + 50 ng Pea3 | 24,85 | 20,86 | 20,34 | 24,64 | 21,67 | 19,64 |
| ets4 mut 300 | 28,68 | 27,94 | 26,5 | 33,78 | 33,37 | 27,97 |
| ets4 mut 600 | 56,1 | 55,65 | 52,9 | 6,57 | 6,529 | 5,959 |
| | | | | | | |
| ets3 mut ND1 +( - ) Pea3 | 3,984 | 3,656 | 3,685 | 1,569 | 1,693 | 1,246 |
| ets3 mut ND1 + 50 ng Pea3 | 11,95 | 10,9 | 9,249 | 26,92 | 25,54 | 18,14 |
| ets3 mut ND1 + 100 ng Pea3 | 22,74 | 19,38 | 15,97 | 31,72 | 30,42 | 17,57 |
| ets3 mut ND1 + 200 ng Pea3 | 20,39 | 26,9 | 24,03 | 25,14 | 29,03 | 28,62 |
| | | | | | | |
| del3 ND1 + ( - ) Pea3 | 19,08 | 15,94 | 10,81 | 21,43 | 20,75 | 15,78 |
| del3 ND1 + 50 ng Pea3 | 22,34 | 40,75 | 0,1384 | 15,59 | 23,13 | 88 |
| del3 ND1 + 100 ng Pea3 | 41,27 | 68,12 | 0,076 | 22,74 | 44,72 | 0,0517 |
| del3 ND1 + 200 ng Pea3 | 81,47 | 90,12 | 69,84 | 31,55 | 33,56 | 26,6 |

Table A.10. Relative luciferase ratio of Pea3 motif mutants and NeuroD interaction

| | **Firefly / Renilla** | | | **avarage of f/r** | **standard deviation** |
|---|---|---|---|---|---|
| wt ND1 +( - ) Pea3 | 0,128307 | 0,163336 | 0,1227 | 0,13811443 | 0,022021437 |
| wt ND1 + 50 ng Pea3 | 0,213007 | 0,218107 | 0,182732 | 0,204615552 | 0,019122262 |
| wt ND1 + 100 ng Pea3 | 0,239259 | 0,252378 | 0,249263 | 0,246966477 | 0,006854232 |
| wt ND1 + 200 ng Pea3 | 0,40992 | 0,399128 | 0,489558 | 0,432868713 | 0,049389792 |
| | | | | | |
| ets4 mut ND1 +( - ) Pea3 | 0,670192 | 0,564738 | 0,560412 | 0,598447657 | 0,062170327 |
| ets4 mut ND1 + 50 ng Pea3 | 1,008523 | 0,962621 | 1,035642 | 1,002261803 | 0,036910629 |
| ets4 mut 300 | 0,849023 | 0,837279 | 0,947444 | 0,877915258 | 0,060499034 |
| ets4 mut 600 | 8,538813 | 8,52351 | 8,877328 | 8,646550563 | 0,200005879 |
| | | | | | |
| ets3 mut ND1 +( - ) Pea3 | 2,539197 | 2,15948 | 2,957464 | 2,552047013 | 0,399147001 |
| ets3 mut ND1 + 50 ng Pea3 | 0,443908 | 0,426782 | 0,509868 | 0,460185697 | 0,043869737 |
| ets3 mut ND1 + 100 ng Pea3 | 0,716898 | 0,637081 | 0,908936 | 0,754304803 | 0,139734463 |
| ets3 mut ND1 + 200 ng Pea3 | 0,811058 | 0,926628 | 0,839623 | 0,859102781 | 0,06019707 |
| | | | | | |
| del3 ND1 + ( - ) Pea3 | 0,890341 | 0,768193 | 0,685044 | 0,781192592 | 0,10326368 |
| del3 ND1 + 50 ng Pea3 | 1,43297 | 1,761781 | | 1,597375544 | 0,232504759 |
| del3 ND1 + 100 ng Pea3 | 1,814864 | 1,523256 | 1,470019 | 1,602712944 | 0,185646116 |
| del3 ND1 + 200 ng Pea3 | 2,58225 | 2,68534 | 2,625564 | 2,631051332 | 0,051763254 |

Table A.11. Luciferase assay measures for Firefly luciferase and Renilla Luciferase of Pea3 motif deletions and NeuroD interaction

| | **Firefly Luciferase** | | | **Renilla Luciferase** | | |
|---|---|---|---|---|---|---|
| wt ND1 + (-) Pea3 | 0,7902 | 1,052 | 0,3426 | 1,546 | 2,285 | 0,8122 |
| wt ND1 + 50ng Pea3 | 4,237 | 2,908 | 3,349 | 1,447 | 1,107 | 1,049 |
| wt ND1 + 100ng Pea3 | 4,17 | 3,94 | 3,769 | 0,7053 | 0,741 | 0,8777 |
| wt ND1 + 150ng Pea3 | 3,891 | 4,016 | 2,833 | 1,208 | 1,272 | 1,049 |
| | | | | | | |
| del1 ND1 + (-) Pea3 | 1,761 | 8,089 | 11,83 | 0,6912 | 2,974 | 4,485 |
| del1 ND1 + 50ng Pea3 | 3,096 | 6,509 | 13,08 | 1,105 | 1,791 | 2,738 |
| del1 ND1 + 100ng Pea3 | 8,283 | 8,234 | 6,12 | 22,69 | 19,07 | 14,35 |
| del1 ND1 + 150ng Pea3 | 21,43 | 15,52 | 6,599 | 52,41 | 34,94 | 18,02 |
| | | | | | | |
| del2 ND1 + (-) Pea3 | 4,698 | 2,113 | 1,317 | 117 | 51,53 | 33,09 |
| del2 ND1 + 50ng Pea3 | 2,984 | 4,662 | 4,704 | 42,55 | 49,69 | 52,88 |
| del2 ND1 + 100ng Pea3 | 4,647 | 5,613 | 5,465 | 75,93 | 58,22 | 66,11 |
| del2 ND1 + 150ng Pea3 | 0,318 | 0,7186 | 0,6301 | 0,9127 | 1,83 | 1,838 |
| | | | | | | |
| del3 ND1 + (-) Pea3 | 0,2881 | 0,4672 | 0,6045 | 1,329 | 1,494 | 1,867 |
| del3 ND1 + 50ng Pea3 | 1,225 | 0,8069 | 1,116 | 0,8483 | 0,7206 | 0,7011 |
| del3 ND1 + 100ng Pea3 | 0,375 | 0,2859 | 0,5633 | 0,4725 | 0,5872 | 0,5957 |
| del3 ND1 + 150ng Pea3 | 2,821 | 2,425 | 1,817 | 3,192 | 2,926 | 2,111 |
| | | | | | | |
| del4 ND1 + (-) Pea3 | 6,342 | 5,624 | 2,259 | 7,998 | 6,003 | 3,989 |
| del4 ND1 + 50ng Pea3 | 27,87 | 32,54 | 21,17 | 3,388 | 4,593 | 3,167 |
| del4 ND1 + 100ng Pea3 | 15,65 | 13,91 | 15 | 3,492 | 3,361 | 3,519 |
| del4 ND1 + 150ng Pea3 | 1,16 | 1,255 | 1,279 | 0,6144 | 0,6478 | 0,6224 |

Table A.12. Relative luciferase ratio of Pea3 motif deletions and NeuroD interaction

| | Firefly / Renilla | | | Avarage | Standard deviation |
|---|---|---|---|---|---|
| wt ND1 + (-) Pea3 | 0,511125 | 0,460394 | 0,421817 | 0,464446 | 0,044792 |
| wt ND1 + 50ng Pea3 | 2,928127 | 2,62692 | 3,192564 | 2,91587 | 0,283021 |
| wt ND1 + 100ng Pea3 | 5,912378 | 5,317139 | 4,294178 | 5,174565 | 0,818467 |
| wt ND1 + 150ng Pea3 | 3,221026 | 3,157233 | 2,700667 | 3,026309 | 0,283812 |
| | | | | | |
| del1 ND1 + (-) Pea3 | 2,547743 | 2,719906 | 2,637681 | 2,63511 | 0,08611 |
| del1 ND1 + 50ng Pea3 | 2,80181 | 3,634283 | 4,77721 | 3,737767 | 0,991757 |
| del1 ND1 + 100ng Pea3 | 0,365051 | 0,431778 | 0,426481 | 0,40777 | 0,03709 |
| del1 ND1 + 150ng Pea3 | 0,408891 | 0,44419 | 0,366204 | 0,406429 | 0,039051 |
| | | | | | |
| del2 ND1 + (-) Pea3 | 0,040154 | 0,041005 | 0,039801 | 0,04032 | 0,000619 |
| del2 ND1 + 50ng Pea3 | 0,070129 | 0,093822 | 0,088956 | 0,084302 | 0,012513 |
| del2 ND1 + 100ng Pea3 | 0,061201 | 0,09641 | 0,082665 | 0,080092 | 0,017745 |
| del2 ND1 + 150ng Pea3 | 0,348417 | 0,392678 | 0,342818 | 0,361304 | 0,027314 |
| | | | | | |
| del3 ND1 + (-) Pea3 | 0,21678 | 0,312718 | 0,323781 | 0,284426 | 0,058844 |
| del3 ND1 + 50ng Pea3 | 1,444065 | 1,119761 | 1,591784 | 1,385203 | 0,241454 |
| del3 ND1 + 100ng Pea3 | 0,793651 | 0,486887 | 0,94561 | 0,742049 | 0,233675 |
| del3 ND1 + 150ng Pea3 | 0,883772 | 0,828776 | 0,86073 | 0,857759 | 0,027618 |
| | | | | | |
| del4 ND1 + (-) Pea3 | 0,792948 | 0,936865 | 0,566307 | 0,765373 | 0,186811 |
| del4 ND1 + 50ng Pea3 | 8,226092 | 7,084694 | 6,68456 | 7,331782 | 0,799919 |
| del4 ND1 + 100ng Pea3 | 4,481672 | 4,138649 | 4,262575 | 4,294299 | 0,173698 |
| del4 ND1 + 150ng Pea3 | 1,888021 | 1,937326 | 2,054949 | 1,960099 | 0,085762 |


Figure A.4. Sequencing results for the removal of ets1 motif with *NeuroD1* truncation. Sequenceing was done in one direction with forward primer. NeuroD_del1_new_2012 is the commercially sequenced truncated NeuroD1 seqeunce and the NeuroD_del1_theoretical is the wild type NeuroD1 promoter sequence. The non matching gaps at the beginning of the sequence are the sequence where forward primer binds just after the ets1 motif (see Figure 5.16. for the sequence and primers)

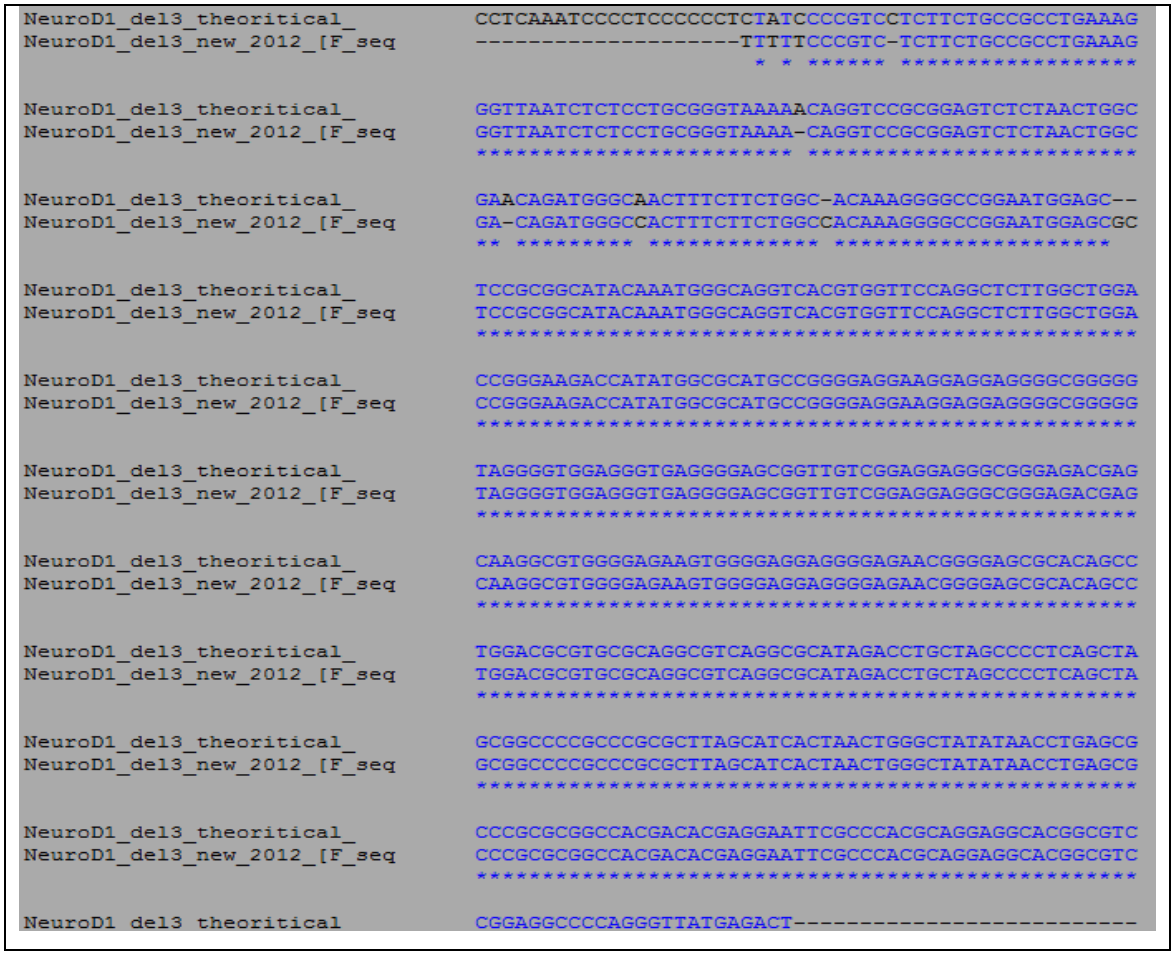


Figure A.5. Sequencing results for the removal of ets3 motif with *NeuroD1* truncation. Sequenceing was done in one direction with forward primer. NeuroD_del3_new_2012 is the commercially sequenced truncated NeuroD1 seqeunce and the NeuroD_del3_theoretical is the wild type NeuroD1 promoter sequence. The non matching gaps at the beginning of the sequence are the sequence where forward primer binds just after the ets3 motif (see Figure 5.16. for the sequence and primers)


Figure A.6**.** Sequencing results for the removal of ets4 motif with *NeuroD1* truncation. Sequenceing was done in one direction with forward primer. NeuroD_del4_new_2012 is the commercially sequenced truncated NeuroD1 seqeunce and the NeuroD_del4_theoretical is the wild type NeuroD1 promoter sequence. The non matching gaps at the beginning of the sequence are the sequence where forward primer binds juct after the ets4 motif (see Figure 5.16. for the sequence and primers)