IDENTIFICATION AND ANALYSIS OF PEA3 TRANSCRIPTION FACTOR TARGET PROMOTERS

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Submitted to the Institute of Graduate Studies in
Science and Engineering in partial fulfillment of
the requirements for the degree of
Master of Science
in
Biotechnology

Yeditepe University
2012

IDENTIFICATION AND ANALYSIS OF PEA3 TRANSCRIPTION FACTOR TARGET PROMOTERS

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DATE OF APPROVAL: .../.../2012

ACKNOWLEDGEMENTS

This study would not have been completed without help and support of many individuals. I would like to thank everyone who has helped me along this way. First and foremost, I would like to gratefully acknowledge to my supervisor, Prof. Dr. Işil Aksan Kurnaz, who has supported me throughout my thesis with her patience and knowledge.

I am very grateful to Prof. Dr. Gürkan Öztürk and Asst. Prof. Dr. Burcu Anıl Kırmızıtaş for being in my evaluation committee, their patience and guidance during this period.

I am heartily thankful to my labmates: Oya Arı, Perihan Unver, Goksu Alpay, Nermin Basak Senturk, Başak Kandemir, Elif Kon, for their support, cooperation, critical feedback, for all the fun and most importantly reminding me I am “a bug between the flowers”.

I would like to specially thank to my roommates and neighbours, Barış Emek, Burhan Karakaş, Neslihan Kızılarslan and Poyraz Yılmaz for their encouragements and endless talks, even though they are still not able to understand what I am doing.

I would like to offer my special thanks to my cats Alexis, Arap, Çirkin and her 5 newborn kittens since they have been sleeping with me and have made me to forget my loneliness at nights.

Burcu Erdoğan deserves a distinct paragraph since she has been my side under any conditions for 7 years and tolerating my imbalanced behavior. This study has not been completed without her presence and her invulnerable supports.

Last but not the least, I would like to express my deepest gratitude to my family for their endless love, confidence, unconditional support and understanding throughout my life.

ABSTRACT

IDENTIFICATION AND ANALYSIS OF PEA3 TRANSCRIPTION FACTOR TARGET PROMOTERS

Transcription factors are the proteins which regulate the expression of the target genes. Improper alterations in the activity of transcription factors may lead to severe diseases. The PEA3 group of transcription factors belong to the ETS family and bind to conserved core DNA sequences 5'-GGAA/T -3' It has been showed that, PEA3/ETV4 transcription factor participates in the motor neuron connectivity and dendritic patterning. In addition to this, it is believed that, PEA3/ETV4 protein is able to regulate the genes involved not only in neuronal differentiation, neuronal development and axonal pathfinding but also in metastatic cases of several cancer types, especially lung and prostate cancer. In this study, we aim to identify the downstream targets of PEA3/ETV4 transcription factor to reveal its signaling pathway. For this purpose, we merged the in silico search and molecular biology techniques. Meanwhile, we developed a novel bioinformatics tool which screens and analyze candidate target promoters for PEA3/ETV4 and other transcription factors.

ÖZET

PEA3 TRANSKRİPSİYON FAKTÖRÜNÜN OLASI HEDEF PROMOTÖRLERİNİN BELİRLENMESİ VE ANALİZİ

Transkripsiyon faktörleri gen anlatımını düzenleyen proteinlerdir. PEA3 grubu transkripsiyon faktörleri, ETS ailesinin bir üyesi olup 5'-GGAA/T-3' DNA dizisine bağlanırlar. PEA3/ETV4 transkripsiyon faktörünün motor nöron bağlantıları ve dendrit şekillenmesinde rol oynadığı gösterilmiştir. Bunun yanında, PEA3/ETV4 proteininin sinirsel başkalaşım, sinirsel gelişim ve aksonal yokbulma ile birlikte akciğer ve prostat kanserleri başta olmak çok sayıda kanser türünün metastazında aktif olarak yer aldığı ispatlanmıştır. Bu çalışmada, PEA3/ETV4 transkripsiyon faktörünün hedef genlerinin belirlenmesi amaçlanmış ve bu kapsamda *in silico* ve moleküler teknikler bir arada kullanılmıştır. Aynı zamanda, PEA3/ETV4 ve diğer transkripsiyon faktörleri için genomu tarayan ve olası hedefleri analiz eden yeni bir çevrimiçi araç tasarlanmıştır.

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LIST OF SYMBOLS / ABBREVIATIONS

ATCC	American Type Culture Collection
$CaCl_2$	Calcium Chloride
cDNA	Complementary Deoxyribonucleic Acid
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
E26	E-Twenty Six
EDTA	Ethylenediaminetetraacetic Acid
Elk-1	Ets Like Transcription Factor-1
ETS	E-Twenty Six
ETV1	ETS Translocation Variant 1
ETV4	ETS Translocation Variant 4
ETV5	ETS Translocation Variant 5
FGF	Fibroblast Growth Factor
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
HEK	Human Embryonic Kidney
ID2	Inhibitor of DNA Binding 2
JNK	C-Jun N Terminal Kinase
KB	Kilobase
L	Liter
LB	Luria-Bertani
MAPK	Mitogen Activated Protein Kinase
mM	Milimolar
MMP	Matrix metalloprotease
NCBI	National Center for Niotechnology Information
Ng	Nanogram
nM	Nanomolar
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

PEA3	Polyomavirus Enhancer Activator 3
PEI	Polyethylenimine
pH	Negative log of hydrogen ion concentration
RNA	Ribonucleic Acid
RT-PCR	Reverse transcription Polymerase Chain Reaction
SH-SY5Y	Human Neuroblastoma Cell Line
TCF	Ternary Complex Factor
U87	Human Glioblastoma Cell Line
µl	Microliters

1. INTRODUCTION

1.1. GENERAL FEATURES OF TRANSCRIPTION FACTORS

Transcription factors are the proteins that regulate gene expression by binding to the specific promoter or enhancer regions of the DNA sequences [1]. The major reflections of the gene regulation are observed during cell growth [2], cell proliferation and apoptosis [3]. Additional proteins such as co-activators and co-repressors may take roles in gene expression processes by promoting or suppressing the rate of the gene expression. In addition to this, the activity of the transcription factors can be controlled by different factors[4].

Transcription factors perform the downregulation or upregulation tasks by stabilizing or blocking the RNA polymerase binding to DNA, catalyzing the acetylation or deacetylation of the histone proteins and recruiting the co-activator or co-repressor proteins to the transcription factor – DNA complex [5]. Transcription factors also regulate themselves in several ways. The rate of the transcription factor synthesis, nuclear localization [6], activation by ligand binding, phosphorylation [7] or interaction with other proteins, accessibility of the DNA-binding site [8], availability of other cofactors or transcription factors are key steps for the regulation processes.

A transcription factor is composed of two main domains named as DNA binding domain and transactivation domain (Figure 1.1). DNA binding domain,recognizes the specific nucleotide sequence on the promoter regions. Different transcription factors may bind to same DNA sequence, which results in these proteins to be classified in the same transcription factor family [1]. Transactivation domain interacts with the components of the basal transcriptional complex, which results in repression or activation of the gene expression.

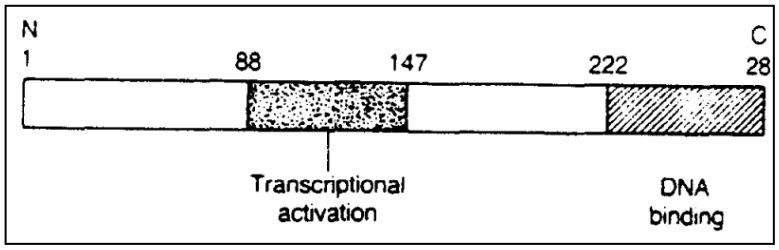


Figure 1.1**.** A prototype image for a transcriptional factor, in particular GCN4 [1]

1.2. ETS FAMILY TRANSCRIPTION FACTORS

ETS proteins are the transcription factors which are characterized by an evolutionarily-conserved ETS domain of about 85 amino acids. This ETS domain facilitates binding to DNA sequences with a central GGAA/T core consensus and flanking nucleotides [9].

28 members of the ETS proteins have been identified in mammals and are categorized within several subfamilies. The basic criteria for characterization are the DNA-binding specificity of the ETS domain, the expression profile of the ETS proteins and the presence of the specific domains which allows to protein-protein interaction [10].

Most of the ETS proteins contain the ETS domains in their C-terminal, although several ETS proteins may have this domain in their N-terminal regions [11]. According to NMR analysis, DNA binding domains of ETS proteins belong the helix-turn-helix superfamily [12, 13]. Amino acid deviations in ETS domains significantly alter the DNA – binding specificities of the ETS proteins [14]. In addition to this, the small changes in the DNA sequences flanking the core consensus affect the binding of the ETS proteins to DNA (Figure 1.1).

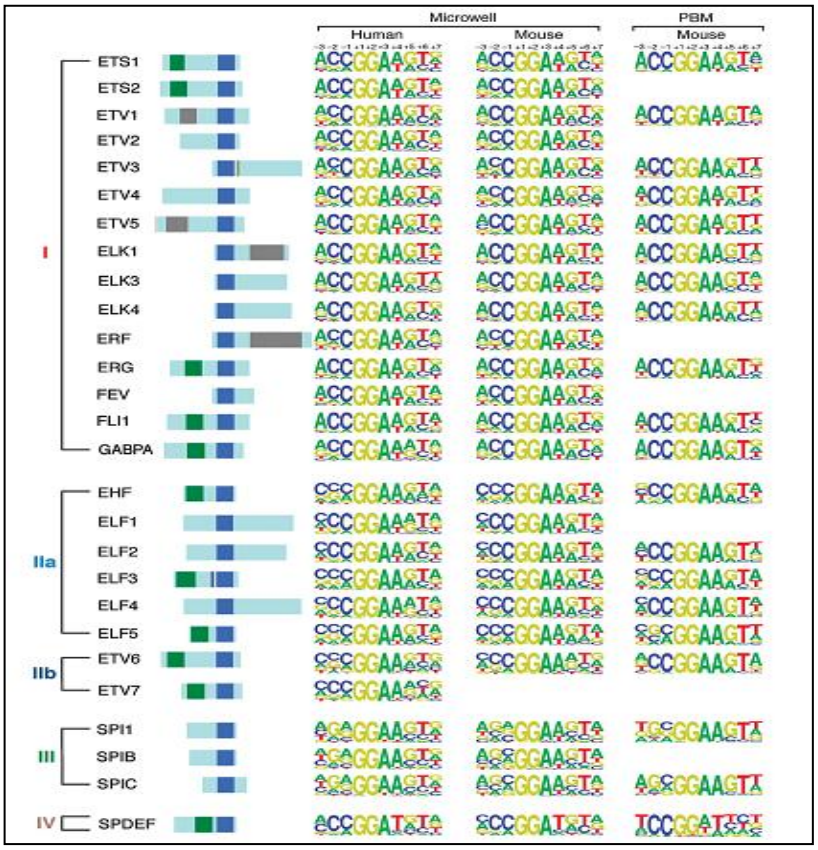


Figure 1.2.The member of ETSfamily of transcription factors [14]

Members of the ETS transcription factor family are targets for several signaling pathways resulting in regulation of diverse cellular functions such as growth, apoptosis, survival, differentiation and oncogenic transformation [15,16].

1.3. PEA3/ETV4 SUBFAMILY OF ETS TRANSCRIPTION FACTORS

1.3.1. General Structures of PEA3 Subfamily Members

The PEA3 group is the subfamily of the ETS transcription factors which is composed of ER81, PEA3/, and ERM. The human homologues of those genes are named as ETV1, ETV4 and ETV5, respectively. The DNA binding ETS domain has 95% identity within PEA3/ETV4 group proteins and these proteins demonstrate 50% amino acid identity overall [10].

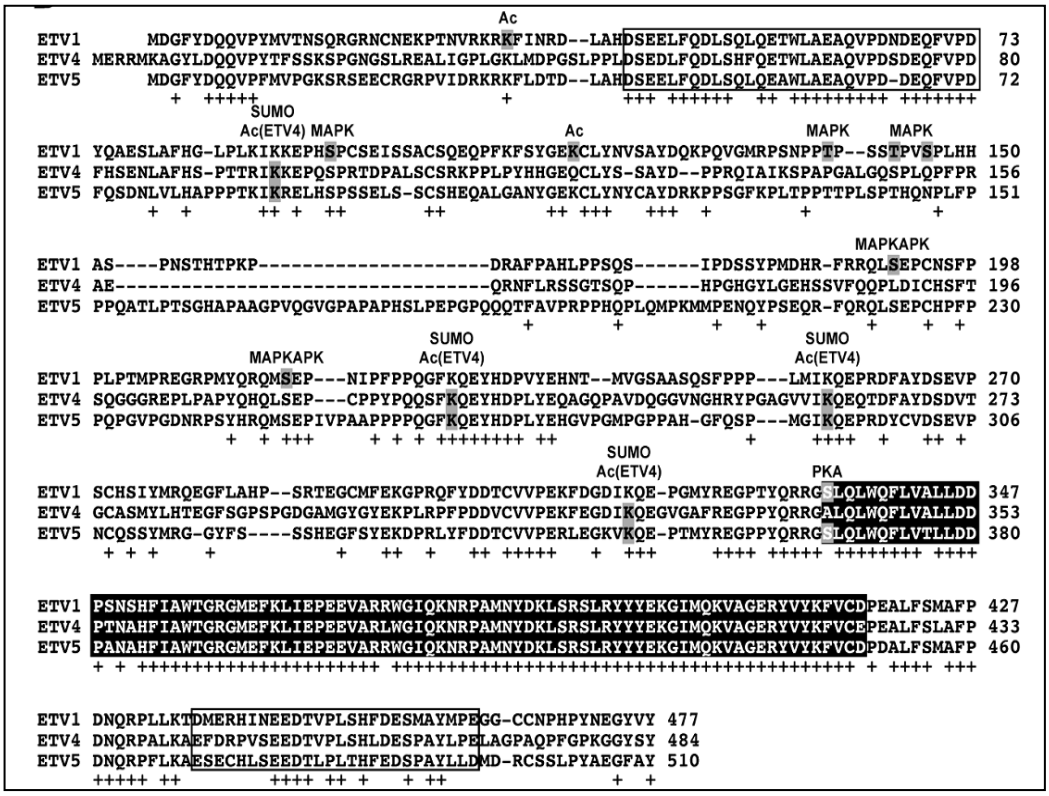


Figure 1.3.The amino acid sequence of PEA3 group of ETStranscription factors [17]

As a characteristic feature of all ETS family transcription factors, PEA3 group proteins bind to the DNA core sequence GGAA/T [18].In addition to this, 32 conserved residues in the acidic domain and 61 conserved residues in the carboxy-terminal domains carry out the transactivation function of PEA3 group proteins [19]. These domains works in a synergetic way to perform the transactivation capacity but several studies show that, N-terminal residues have more potential than the ones on the C-terminal and the flanking sequences around both sides of N-terminal may inhibit the transactivation function as observed in PEA3/ETV4 transcription factor [20]. Similarly, core region of ER81 (ETV1) may exert negative effects on both acidic and carboxylic transactivation sites [21].

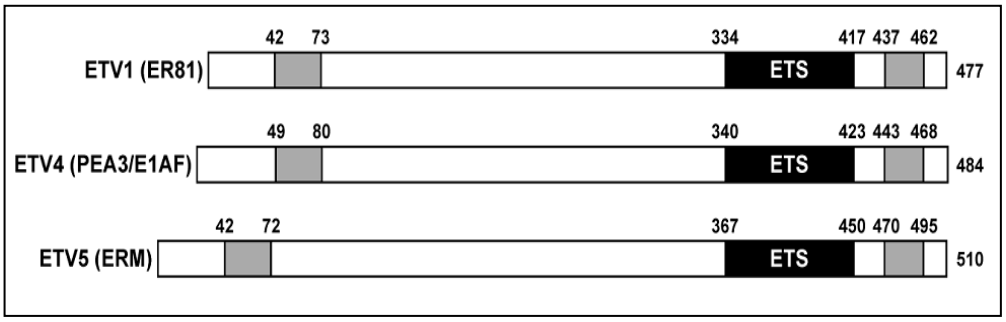


Figure 1.4.Schematic representation of the structures of the PEA3 members [9]

As transcription factors, PEA3 members undergo several mechanisms resulting in alterations for DNA binding. At least two mechanisms may be considered for this phenomenon as interaction with other proteins and posttranslational modifications. To be more specific, upstream stimulating factor (USF-1) interacts with PEA3/ETV4 to increase its capacity to bind to DNA whereas, DNA binding property is suppressed by another ETS domain transcription factor ID2 [22].

An alternative way to affect the transactivation capacity of the PEA3 transcription factors is posttranslational modifications, mainly phosphorylation (Figure 1.5). PEA3 members are mainly considered as targets for MAPK pathway and their activity dramatically changes when they are phosphorylated by this pathway [23,24]. Additionally, it was shown that PKA also stimulates the capacity of PEA3 members by increasing their DNA binding and to promote the gene expression [25, 26].

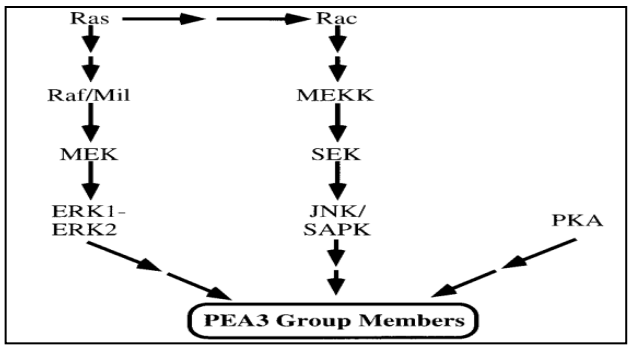


Figure 1.5. General view of phosphorylation cascades for PEA3/ETV4 members [6]

1.3.2. PEA3/ETV4 Transcription Factor

PEA3/ETV4 is one of the three members of PEA3 subfamily. It is located on chromosome 17q21 and composed of 445 amino acids (Figure 1.6).

```
  1 mdpgslppld sedlfqdlsh fqetwlaeaq vpdsdeqfvp dfhsenlafh spttrikkep
 61 qsprtdpals csrkpplpyh hgeqclyssa ydpprqiaik spapgalgqs plqpfpraeq
121 rnflrssgts qphpghgylg ehssvfqqpl dichsftsqg ggreplpapy qhqlsepcpp
181 ypqqsfkqey hdplyeqagq pavdqggvng hrypgagvvi kqeqtdfayd sdvtgcasmy
241 lhtegfsgps pgdgamgygy ekplrpfpdd vcvvpekfeg dikqegvgaf regppyqrrg
301 alqlwqflva llddptnahf iawtgrgmef kliepeevar lwgiqknrpa mnydklsrsl
361 ryyyekgimq kvageryvyk fvcepealfs lafpdnqrpa lkaefdrpvs eedtvplshl
421 despaylpel agpaqpfgpk ggysy
```

Figure 1.6. Amino acid sequence of PEA3/ETV4/ETV4

Upon stimulation, PEA3/ETV4 may activate or suppress the expression of its target genes resulting in different cellular functions.

1.3.2.1. Roles in Breast Cancer

PEA3/ETV4 is considered as participating in metastatic transformation and considered as metastatic marker gene.. As mentioned earlier, PEA3/ETV4/ is target for MAPK pathway and its phosphorylation leads to transactivation of metastatic genes and it was observed that PEA3/ETV4 is highly expressed in Her/Neu expressing breast cancer cells and tissues [6]. The major target for PEA3/ETV4 is matrix metalloprotease enzymes, particularly MMP1, MMP2 and MMP9 which are the genes required for the initiation of the cell migration.Moreover, overexpression of PEA3/ETV4/leads to increased levels of vimentin [27], the intercellular adhesion molecule ICAM-1 [28], osteopontin [29], vascular endothelial growth factor and cyclooxygenase-2 [30] thus providing evidence for the importance of PEA3/ETV4 in tumor formation and metastasis.

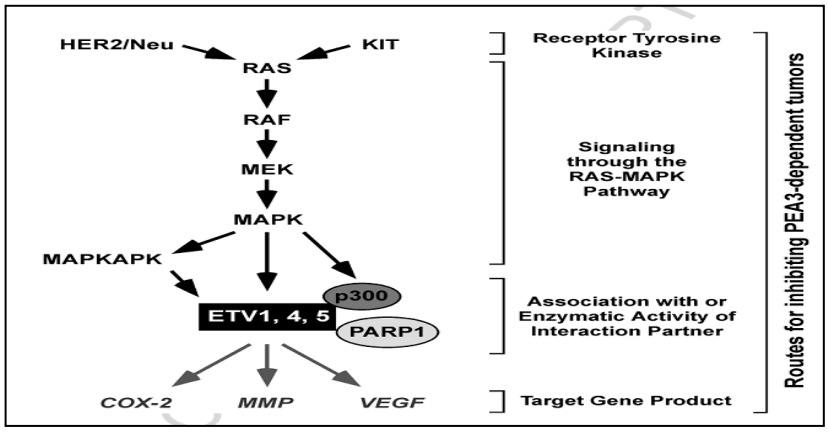


Figure 1.7. The overall picture of PEA3 family transcription factor pathway [31]

1.3.2.2. Roles in Prostate Cancer

Another cancer type wherePEA3 transcription factors are highly expressed is prostate cancer. Chromosomal translocations yielding the fusion of TMPRSS2 promoter, which includes androgen response element, and PEA3/ETV4 protein is observed in prostate cancer cases. The overexpression of PEA3/ETV4 due to the high level activation of TMPRSS2 promoter results in prostate cancer, [32].

1.3.2.3. Roles in Other Cancer Types

Studies indicate that, PEA3/ETV4 is involved a large spectrum of eventsin cancer progression. The overexpression of PEA3/ETV4was observed in colorectal tumors [33], human gastric tumors [34], lung adenocarcinomas [35], ovarian [36]and endometrial carcinomas [37].

1.3.2.4. Roles in Development

The experiments with zebrafish demonstrated the importance of PEA3/ETV4 in developmental processes. It was shown that, FGF signaling pathway regulates PEA3/ETV4 expression and disruption of this signal transducer system lead to both cardiac and left/right patterning defects as well as disruption of the isthmic organizer. Another study showed that FGF -activated PEA3/ETV4 participates in retinal development and ganglion cell differentiation [38].

Another implication of PEA3/ETV4 in development is observed in mammary gland. During early puberty and pregnancy, PEA3/ETV4 promotes the proliferation and differentiation resulting in arborization within the mammary gland [39].

1.3.2.5. Roles in Motor Neuron Connectivity

During development and neurogenesis, newborn neurons migrate to their final destination and once they reach their location they undergo several molecular mechanisms resulting in dendritic arborization and synapse formation. It was shown that PEA3/ETV4 knock-out mice are not able to form synapse and dendritic arborization within the motor neurons of the spinal cord [40]. It was also shown thatPEA3/ETV4 activity was regulated by GDNF [41], but downstream effectors of PEA3/ETV4 have not been revealed yet.

1.4. *IN SILICO* APPROACH FOR TRANSCRIPTION FACTOR ANALYSIS

Online bioinformatics tools play an important role for molecular biologists since they are able to provide an initial clue for their experiments. When the transcription factor studies are considered, databases are utilized for crucial tasks by enabling the user to predict which genes might be regulated by specific transcription factors. For this purpose, several databases are available through the internet.

1.4.1. Promoter Sequence Retrieval

Transcription Regulatory Element Database (TRED) from Cold Spring Harbor Laboratories provides a good insight for promoter sequence access. Once the gene is determined, it is put into the search bar and the tool lists the promoter sequences and ranks them according to reliability as “known curated”,” known”,” predicted” sequences. It gives the user a chance to select start and end sites of the promoter sequence as flanking around the transcription start site (TSS).

1.4.2. Transcription Factor Screening

1.4.2.1. TRANSFAC Tool

TRANSFAC is the product of Gene-Regulation by BioBase and aims to provide data for eukaryotic transcription sites regarding consensus binding sequences by positional weight matrices. It displays the results as similarity between binding motif of the transcription factor and nucleotide sequence on promoter region as percentage. In that manner, higher values indicate the higher possibility for transcription factor binding on that site.

1.4.2.2. TFSearch

This tool is provided by TRC laboratory from Japan and works in same fashion as TRANSFAC. The major difference is that, the search is not restricted to eukaryotic transcription factors and an option is available for arthropods, plants and yeasts. It displays results that cross the similarity threshold, 85%.

1.4.2.3. Promo 3.0

Promo 3.0, which is powered by ALLGEN, might be considered as a more versatile tool found through the internet. It analyzes the promoter regions for a selected transcription factor and displays results as dissimilarity rate. Dissimilarity rate simply implies the variance between the binding motif of the transcription factor and the nucleotide sequence on the promoter as percentage by regarding the binding matrices. From this point of view, the smaller numbersare the indicators of higher possibility for PEA3/ETV4 binding. Unlike the other tools, the user to choose the transcription factor of interest or uses a specific matrix and the search might progress according to this selection. This situation dramatically reduces the processing time of the tool. Regarding these features, Promo 3.0 was utilized in this project.

1.5. AIM OF THE PROJECT

With the lights of this information, the aim of this project is to use bioinformatics and *in silico* approaches to identify possible targets for PEA3/ETV4 transcription factor and, if possible, to confirm a selected subset of genes using molecular techniques.

2. MATERIALS

2.1. BIOINFORMATICS TOOLS

- Gene Database, National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/gene/)
- Transcriptional Regulatory Element Database, Cold Spring Harbor Laboratories (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home)
- Promo 3.0 (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3)
- Oligo Analyzer 3.1, Integrated DNA Technologies (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx)
- Primer Blast, National Center for Biotechnology Information, (http://www.ncbi.nlm.nih.gov/tools/primer-blast/)
- motifTFinder, developed as a collaborative work with Ugur Sezerman, Sabanci University(SeeAppendix B) (http://bioapps.sabanciuniv.edu/sezermanlab/motiftfinder)

2.2. CELL LINES AND BACTERIAL STRAINS

- HEK293 – Human Embryonic Kidney Cell Line (ATCC number: CRL-1573)
- U87–Human Glioblastoma Cell Line (ATCC number: HTB-14)
- *E.coli*, JM109 strain

2.3. COMMERCIAL KITS AND REAGENTS

- EZ10 Spin Column Total RNA Mini-preps Super Kit, BS584 (Bio Basic Inc.)
- Transcriptor High Fidelity cDNA Synthesis Kit (Roche®)
- DNA-Spin Plasmid DNA Purification Kit (Intron)
- Taq DNA Polymerase with 10X buffer (Fermentas)
- Taq DNA Polymerase with 10X buffer containing 25 mM $MgCl_2$ (Sigma-Aldrich)

- KpnI Restriction Enzyme with 10X reaction buffer (Fermentas)
- HindIII Restriction Enzyme with 10X reaction buffer (Fermantas)
- BamHI Restriction Enzyme with 10X reaction buffer (Fermantas)
- T4 Ligase with 5X reaction buffer (Invitrogen)
- pGL3 Basic Expression Vector (Clontech)

2.4. CELL CULTURE

- 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 (Gibco)
- 100X L-Glutamine Solution (Gibco)
- 100X Antibiotic-Antimycotic Solution (Gibco)
- 1X Dulbecco's Phosphate Buffered Saline (Gibco)
- 0,05% Trypsin-0.53 mM EDTA Solution (Multicell)
- T25 Tissue Culture Flasks (Corning)
- T75 Tissue Culture Flasks (Corning)
- T150 Tissue Culture Flasks (Corning)
- 100x20 mm Tissue Culture Petri Dishes (BD Falcon)
- Polyethylenimine, MW 1500 (Polysciences Inc.)

2.5. BACTERIAL ASSAYS

- Luria-Bertani (LB) Agar (Sigma-Aldrich)
- Luria-Bertani (LB) Broth (Sigma-Aldrich)
- Ampicillin (Biomatik)
- Petri Plates (Isolab)
- Inocolumn loops (IsoLab)

2.6. LABORATORY TECHNICAL EQUIPMENT

- 0.5 ml tube (Beckman)
- 1.5 ml tube (Beckman)
- 2 ml tube (Beckman)
- -80 °C freezer (Wisecryo)
- Autoclave (HV-85 – HICLAVE)
- Chemiluminescence Imaging System (Bio-Rad)
- CO2 Incubator (Nuaire)
- Heater (DRI-Block DB.2A – Techne)
- Inverted Phase Contrast Microscope (Nikon)
- Laminar flow cabinet (ESCO Labculture Class II Biohazard Safety Cabinet Type2A)
- Magnetic Stirrer (RH Basic – KIKA Labotechnic)
- Microcentrifuge (Micro 1224–Hettich Zentrifugen)
- Microwave (MD 553–Arcelik)
- Mini-PROTEAN Tetra Cell Electrophoresis System (Bio-Rad)
- Refrigerator (Nu- 6512E- NuAire and Surround Flow- Arçelik)
- Shaker (Innova 4330 – New Brunswick Scientific)
- Ultra-speed Centrifuge (Avanti J-251 - Beckman)
- Water Bath (Memmert)
- Thermal Cycler (My Cycler – Bio-Rad)
- Nanophotometer (IMPLEN)

3. METHODS

3.1. *IN SILICO* ANALYSIS OF PEA3/ETV4 TARGET PROMOTERS

3.1.1. Determination of Possible Targets of PEA3/ETV4

"Neuronal migration" and "Axonal guidance" which are two main functions of PEA3/ETV4 was selected as gene search parameter and the genes related to those functions were obtained by the means of "Gene" tool of NCBI *(http://www.ncbi.nlm.nih.gov/gene/)*.

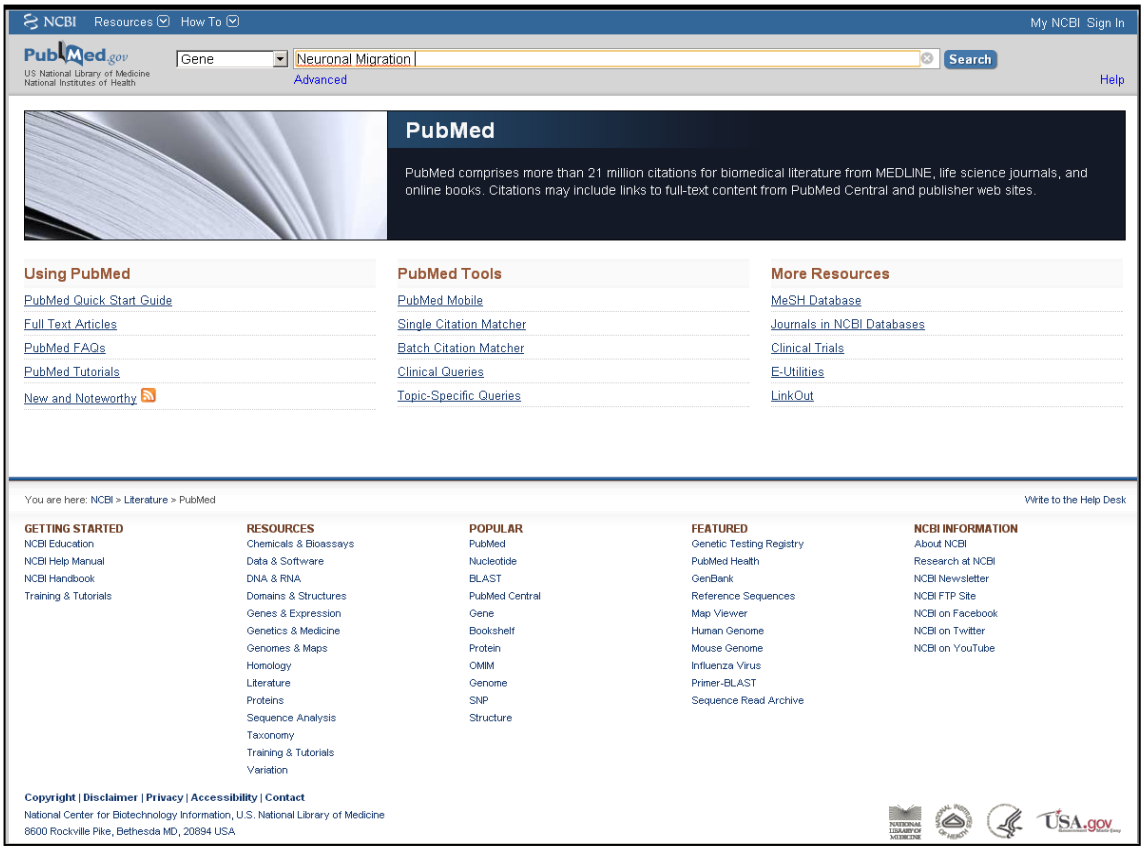


Figure 3.1.The main page of PubMed

3.1.2. Retrieval of Promoter Sequences

The promoter sequences of the genes which had been determined by "Gene" tool of NCBI, were retrieved from the Transcriptional Regulatory Element Database, TRED (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home).This website has a genome-wide database for the promoter sequence. There is an option to choose the start and end points of the promoter region as flanking around the transcription start site (TSS) (Figure 3.2). For convenience, sequences were displayed from -700 to +300 relative to TSS.

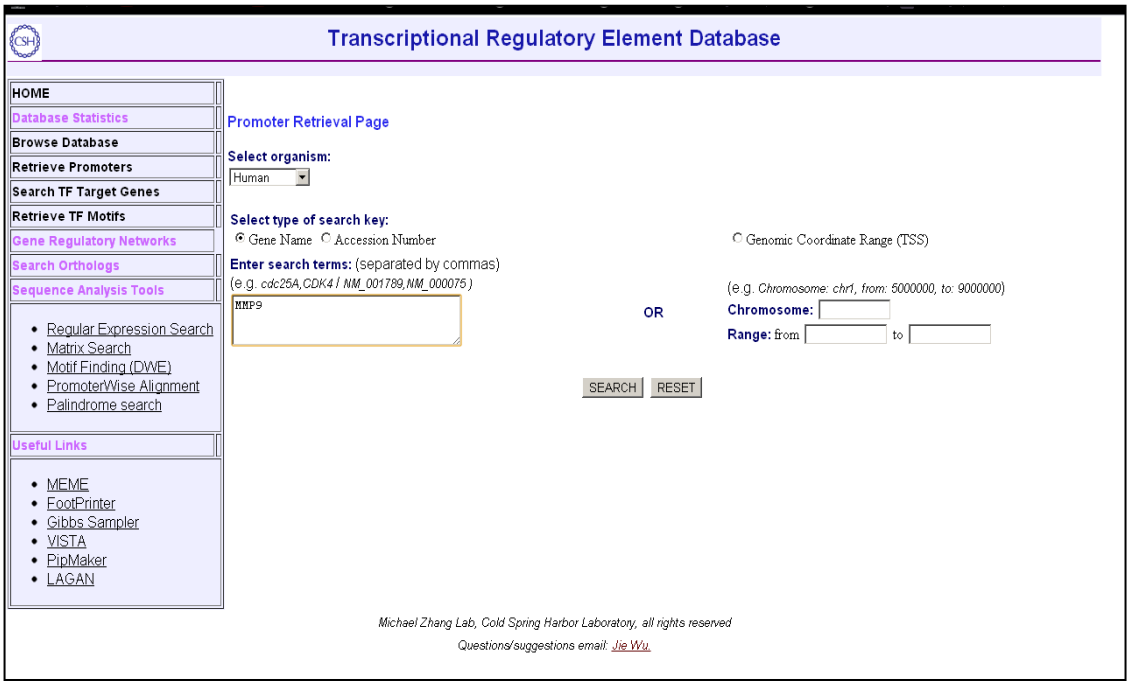


Figure 3.2.Promoter entry site for TRED

3.1.3. Screening of the Promoter Regions with respect to PEA3/ETV4 Binding Affinity

The promoter sequences obtained from TRED were analyzed with Promo 3.0 (*http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3*). The results indicated the dissimilarity rate between the PEA3/ETV4 binding motif and the nucleotides on the promoter regions, where lower dissimilarity rate is the reflection of higher possibility for PEA3/ETV4 binding.

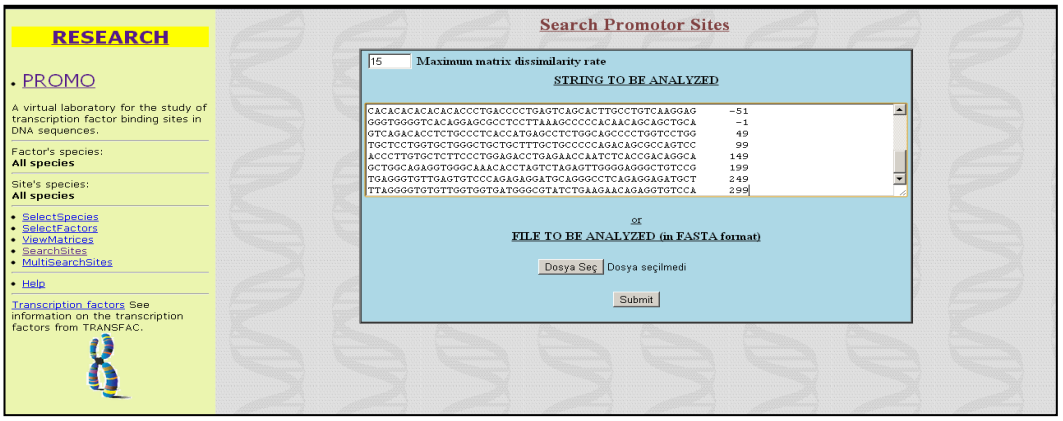


Figure 3.3.Input page of Promo 3.0

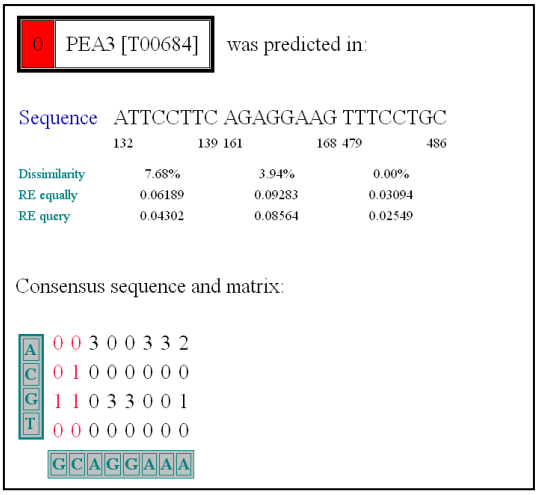


Figure 3.4.An example for Promo 3.0 result page.Screening was done for MMP9 promoter

3.1.4. motifTFinder

At one stage of the project, a collaborative work was carried out with Ugur Sezerman from Sabanci University (http://bioapps.sabanciuniv.edu/sezermanlab/motiftfinder). This tool enables to search all the promoter regions within genome of the selected organism. Moreover, the user is able to select transcription factor of interest and search is done according to this parameter (See Appendix B).

3.2. CLONING OF CANDIDATE PROMOTERS

3.2.1. Primer Design

In order to amplify promoter regions from the genomic DNA, specific primers were designed. Once promoter sequences were retrieved from TRED, sites suitable for primer binding were determined and their parameters in terms of length, melting temperature, GC content and secondary structure possibilities were screened by Oligo Analyzer 3.0 (IDT). Their binding possibilities through the genomic DNA were determined by Primer Blast (NCBI). A flanking region (5' AGAC) and restriction enzyme recognition sites were added to the 5' site of the primers (Table 3.1)

Table 3.1. Primers used for Promoter Amplification

Gene	Forward Primer (5'→3')	Restriction enzyme site	Reverse Primer (5'→3')	Restriction enzyme site	Annealing temperature	Estimated Product length(bp)
AMFR	GACGGTACCCAGAAGAT T GGATAAGACTGG	KpnI	GACCTCGAGTCAGCAGA CTTGGAGAAGC	HindIII	64.1	1000
EPHA4	GACCTCGAGTAGGTCGG AGCGGAGAGTCG	XhoI	GACAAGCTTTCGGGACG CACCATTCACACTG	HindIII	60	1249
EFNB2	GACAGATCTCTACCGTG TTCTTCCTACTGTTGG	BglII	GACAAGCTTTGACTATA GATCCA GTGCAGCG	HindIII	58	1382
DAB1	GACAGATCTTAGCAGGG AGCGGTGCC	BglII	GACAAGCTTATCTCGGA CTCCCTGCCTCTG	HindIII	60	1143

Reverse transcription primers were designed in a similar fashion, but mRNA sequences instead of genomic DNA were used as templates and neither a flanking region nor a restriction enzyme recognition site were added to 5' region of the primers (Table 3.2).

Table 3.2. Primers used for RT-PCR

Name	**Forward Primer** (5'→3')	**Reverse Primer** (5'→3')	Annealing Temperature	Estimated Product Length
AMFR	'TTGACATCTTTTCCACAACTC	AAGTGATTGTGTTGGTTTAAG	52.1	300
EPHA4	TCCAGACCTAACACTGCC	ATCAGGGATGTCTCAGAAGC	50	350
EFNB2	TTGATAAAGACCAAGCCGACA	TTGTCTGGCACACCCCTCCCT	57	300
DAB1	AAGGTCAGGATCGCAGTGAC	TCCAGAATAACAGGTTCAGCCG	50	350
GAPDH	GCATTGCTGATGATCTTGAGG	TCGGAGTCAACGGATTTGG	50	500
PEA3/ETV4	AGACAAGCTTCGCCTACGACTCAGATGTC	AGACTCTAGAAGCTCCAATCCCTTCCTGC	57.5	300

3.2.2. Amplification of the Promoter Regions with PCR

The promoters which had high possibility for PEA3/ETV4 binding were amplified from the human genomic DNA by Polymerase Chain Reaction. The reaction consisted of Template DNA, primers, dNTPs, distilled water, 10X reaction buffer, 25 mM $MgCl_2$ and Taq Polymerase (Fermentas) (Table 3.3).

Table 3.3. Components and their concentrations in PCR

Component	**Volume**	**Final Concentration**
Template	1 µl	100 ng
10x Buffer	3 µl	1X
dNTP (2 mM, each)	3 µl	0.2 mM, each
Primer Reverse	0.4 µl	0.2 µM
Primer Forward	0.4 µl	0.2 µM
25 mM $MgCl_2$	3 µl	2.5 mM
Taq DNA Polymerase	0.3 µl	1.25 unit
Distilled water	Up to 30 µl	

After initial denaturation for 3 min at 95°C, promoter amplification was carried out for 30 cycles and 30 seconds 95°C, 30 secondsannealing temperature (Table 3.1), 90

seconds72°C as cycling conditions. The products were subjected to electrophoresis in a 1% agarose gel which was prepared as dissolving 1.5 grams agarose in 150 ml TAE buffer.

3.2.3. Restriction Digestion of the Amplified Regions

The products from the PCR amplification was considered as insert and underwent the digestion process by the restriction enzymes*KpnI* and *HindIII* (Fermentas) (Table 3.4). The reaction was carried out at 37°C for hours. The pGL3 vector (Figure 3.5) was also digested with the same enzymes and under the same conditions.

Table 3.4. Components and concentrations of the restriction reaction

Component	Volume	Final Concentrations
Insert /pGL3	Variable	1.5 µg
KpnI enzyme	1 µl	1 FDU
HindIII enzyme	1 µ	1 FDU
10X Reaction buffer	3 µL	1X
Distilled Water	Up to 30 µl	

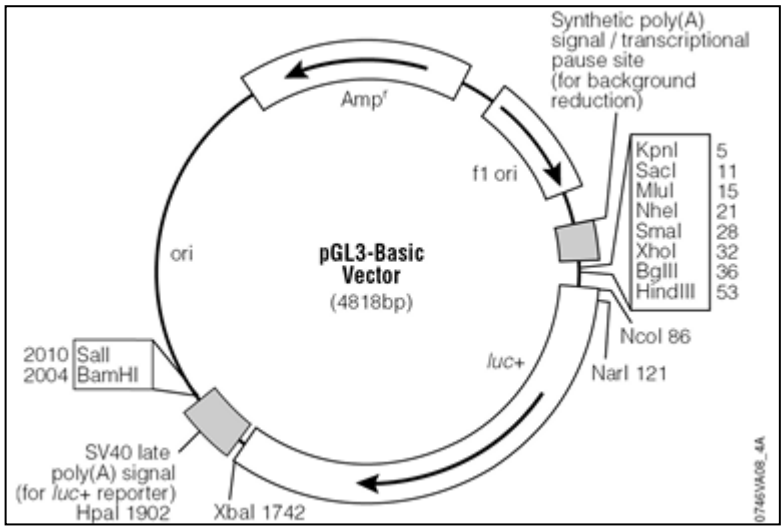


Figure 3.5. Map of pGL3 basic vector (Promega)

3.2.4. Shrimp Alkaline Phosphatase (SAP) Treatment of pGL3 Vector

After the digestion of the pGL3 vector by the restriction enzymes, the digested vector products were treated with Shrimp Alkaline Phosphatase (Roche) (Table 3.5).

Table 3.5.Components and concentrations of SAP Treatment

Component	**Volume**	**Final Concentrations**
10X Reaction Buffer	3.5 µl	1X
SAP Enzyme	1.5 µl	1.5 Unit
pGL3	30 µ	1.5 µg

3.2.5. Ligation Reaction

The ligation process was carried out with different molar ratios of pGL3 vector plasmid and insert by utilizing T4 Ligase enzyme (Invitrogen) (Table 3.6). The reaction was carried out overnight at 16°C.

Table 3.6.Components for Ligation Reaction

	Vector:Insert ratio	
Component	1:3	1:5
Digested pGL3	25 fmol	15 fmol
Digested Insserts	75 fmol	75 fmol
5X Reaction Buffer	4 µl	4µ
T4 Ligase	1 µl	1 µ
Distilled water	Up to 20 µl	Up to 20 µl

3.2.6. Competent Cell Preparation

Cell walls of JM109 bacterial cells were altered in more permeable form for DNA uptake by calcium chloride treatment. For this purpose, bacterial stock of JM109 cells was thawed and 1 µl of the solution was transferred into the 10 µl of LB medium. The mixture was left for overnight incubation at 37°C in shaker incubator. On the following day, 1 ml of the mixture was added into the 49 ml LB broth and this new solution was incubated for 3 hours at 37°C by continuous shaking. After that incubation, the culture was centrifuged at 5500 rpm for 10 minutes at 4°C and supernatant was discarded. The pellet which contained bacterial cells was gently resuspended in 5 ml ice-cold 100 mM $CaCl_2$ and incubated for 1 hour on ice. This mixture was centrifuged at 5500 rpm for 10 minutes at 4°C and supernatant was discarded. The pellet was resuspended in 1 ml ice-cold $CaCl_2$and stored at 4°C for 3 days.

3.2.7. LB Agar Plate Preparation

40 grams of LB Agar powder was dissolved in 1 liter distilled water and the resulting solution was sterilized by autoclaving at 121°C for 15 minutes. After that 1 ml of 200mg/ml ampicillin was added to agar solution. Approximately 15-20 ml LB agar was poured into each petri plate.

3.2.8. Transformation

The transfer of DNA into the host bacterial strain was ensured by heat-shock. 500 ng of DNA was added to 50 µl of competent cells and incubated on ice for 15 minutes. Then they were put into the 37°C heater block for 90 seconds and then put back into the ice immediately for 2 minutes. After heat-shock process 500 µl LB broth without any antibiotics was added and left to incubation for 1 hour at 37°C and finally spread on the plates with LB Agar containing ampicillin. The plates were placed in a 37°C incubator overnight.

3.2.9. Screening by Restriction Enzyme Digestion

Several colonies were picked up from the LB Agar plates after incubation and their plasmids were isolated by DNA-Spin Plasmid DNA Purification Kit (Intron) according to the manufacturer's protocol. The digestion was carried out by BamHI (Fermentas) restriction enzyme for 3 hours at 37°C and samples were run on 1 % agarose gel.

Table 3.7. Components of Restriction Enzyme Screening

Component	Volume	Final Concentrations
Plasmid	Variable	1.5 µg
BamHI	1 µl	1 FDU
10X Reaction buffer	3 µL	1X
Distilled Water	Up to 30 µl	

3.3 EXPRESSION ANALYSIS OF PEA3/ETV4 TARGET GENES

3.3.1. Cell Culture Conditions and Maintenance

Human embryonic kidney (HEK) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) 4.5 g/liter Glucose (Gibco) containing 10% FBS, 1X L Glutamine and 1X antibiotic-antimycotic solution at 37ºC in 5% CO2 incubator. Once the cell confluency reached 80 %, the cells were washed with Phosphate Buffered Saline 2.7 mM KCl, 10 mM sodium phosphate dibasic, 137 mM NaCl and 2 mM potassium phosphate, pH 7.4 andwere deattached from the flask with 0,05% Trypsin-0.53 mM EDTA Solution and transferred into a new flask in 1:4 ratio.

3.3.2. Transient Transfection of the HEK and U87 Cells

For transfection assays, Polyethylenimine (PEI), MW 1500 was used. $1x10^6$ cells were seeded on 100x20 mm Tissue Culture Petri Dishes containing Dulbecco's Modified Eagle

Medium (DMEM) 4.5 g/liter Glucose, one day before the transfection experiment. On the following day, transfection mix was prepared (Table 3.8) and incubated at room temperature for 10 minutes. After that, the medium was aspirated and 3.5 ml Serum-free medium with the transfection mix was applied onto the cells.

Table 3.8.Components of the Transfection Mix

Component	**Amount**
Serum-free medium	150 µl
Plasmid (PCMV-mPEA3/ETV4)	6 µg
PEI (1 µg/µl)	45 µl

The cells were incubated in transfectionmix – serum -free medium mixture for 2 hours in at 37ºC in 5% CO2 incubator. After 2 hours, 6.5 ml complete medium was added and cells were left in the incubator for 48 hours.

3.3.3. Total RNA Isolation and cDNA synthesis

After 2 days incubation, cells were trypsinized and RNA was isolated by EZ10 Spin Column Total RNA Mini-preps Super Kit according to manufacturer's manual. After this step cDNA was synthesized by oligo-dT based method by Roche's High Fidelity cDNA Synthesis Kit (Table 3.9).

Table 3.9. Components for cDNA Synthesis

Component	Amount
Total RNA	1 µg
oligo-dT (50 µM)	1 µl
Distilled water	Up to 11.4 µl
10 minutes at 65 °C and cooling on ice	
5X Reaction Buffer	4 µl
RNAse Inhibitor (40u/µl)	0,5 µl
dNTP mix (10 mM each)	2 µl
DTT (0.1 M)	1 µL
RT Enzyme	1.1 µl
30 minutes at 50 °C and 5 minutes at 85 °C	

3.3.4 Reverse Transcription PCR

cDNA samples were diluted four-fold with distilled water, and their expression was screened by PCR. GAPDH was determined as the housekeeping gene and PEA3/ETV4 primers were used to confirm PEA3/ETV4 overexpression. Once the intensities of the GAPDH bands were roughly equal to each other with a certain amount of starting template, then the expression of the possible target genes were screened according to this amount of template (Table 3.10).

Table 3.10.Components and their concentrations in PCR

Component	Volume	Final Concentration
Template	varies	varies
10x Buffer	3 µl	1X
dNTP (2 mM, each)	3 µl	0.2 mM, each
Primer Reverse	0.4 µl	0.2 µM
Primer Forward	0.4 µl	0.2 µM
Taq DNA Polymerase	0.25 µl	1.25 unit
Distilled water	Up to 30 µl	

After initial denaturation for 3 min at 94°C, PCR was carried out for 25 cycles for housekeeping and 30 cycles for target genes, where the cyclimg conditions were: 60 seconds at 94°C, 120 seconds annealing at temperature (Table 3.2) and 180 seconds 72°C. The products were subjected to electrophoresis in a 1% agarose gel which was prepared by dissolving 1.5 grams agarose in 150 mL TAE buffer.

4. RESULTS AND DISCUSSION

4.1. DETERMINATION OF THE POSSIBLE PEA3/ETV4TARGETS

As a first step of the *in silico* analysis, the genes were retrieved according to their function. Initially, search parameters were neuronal migration and axonal guidance which are two cellular functions of PEA3/ETV4. For this purpose, PubMed Gene tool was utilized.404 human genes were retrieved from the search of "neuronal migration".

Promoter regions of 383 out of 404 genes were accessible on the TRED website. The promoters obtained from that site were screened for containing PEA3/ETV4 binding sites via the PROMO website. PROMO 3.0 tool analyzes the promoter regions for selected transcription factor and displays results as dissimilarity rate. Dissimilarity rate simply implies the variance between the binding motif of the transcription factor and the nucleotide sequence on the promoter as percentage by regarding the binding matrices (Figure 4.3). From this point of view, a smaller number is an indicator of higher possibility for PEA3/ETV4 binding.

As a first step a low-range study was carried out to screen genes that have overlapping functions with PEA3/ETV4 and their results were tabulated (Table 4.1). The genes were selected in a random way by literature search and by considering the current laboratory (AxanLab) studies on *NeuroD*, *NFM*, and*NFL*. Among 50 genes, 16 genes were determined as a good candidate for PEA3/ETV4 binding as they had dissimilarity rates less than 5%.

After the initial screening a wider search followed to retrieve genes for neuronal migration and axonal guidance. The results of the screening against both mouse PEA3/ETV4 and its human homolog with promoter accession number were tabulated (Table A.1). 325 candidates crossed the threshold (5% dissimilarity rate) for mouse PEA3/ETV4 while this number was 105 for the human homolog (Figure 4.1).

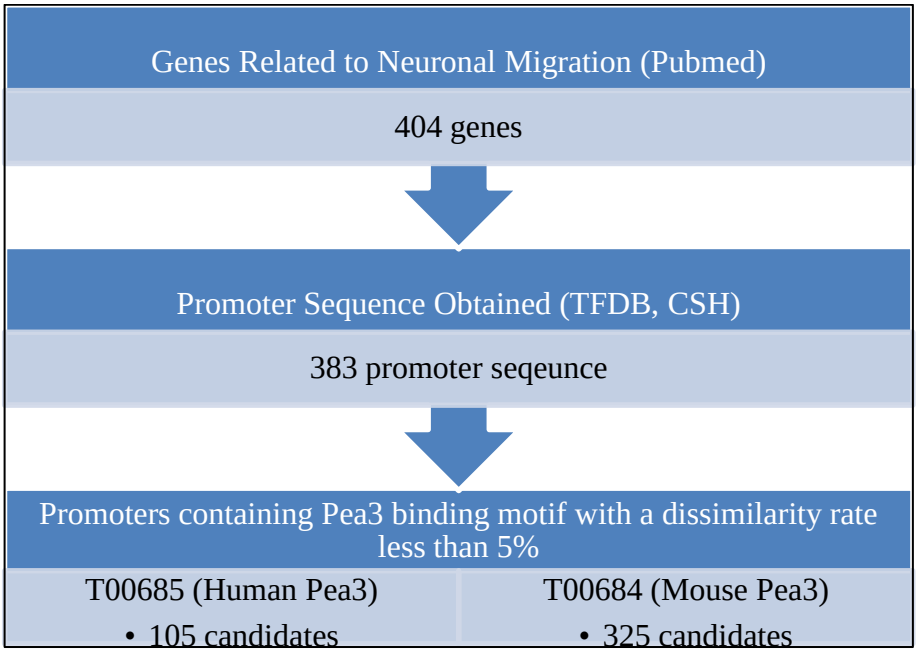


Figure 4.1.The results for neuronal migration genes

The same approach was applied to genes related to axonal guidance. 45 promoters were retrieved from 47 genes and the score for 38 of them was above 5% dissimilarity for mouse PEA3/ETV4 binding site and 18 for ETV4, the human homolog of PEA3/ETV4 (Table A.2, Figure 4.2).

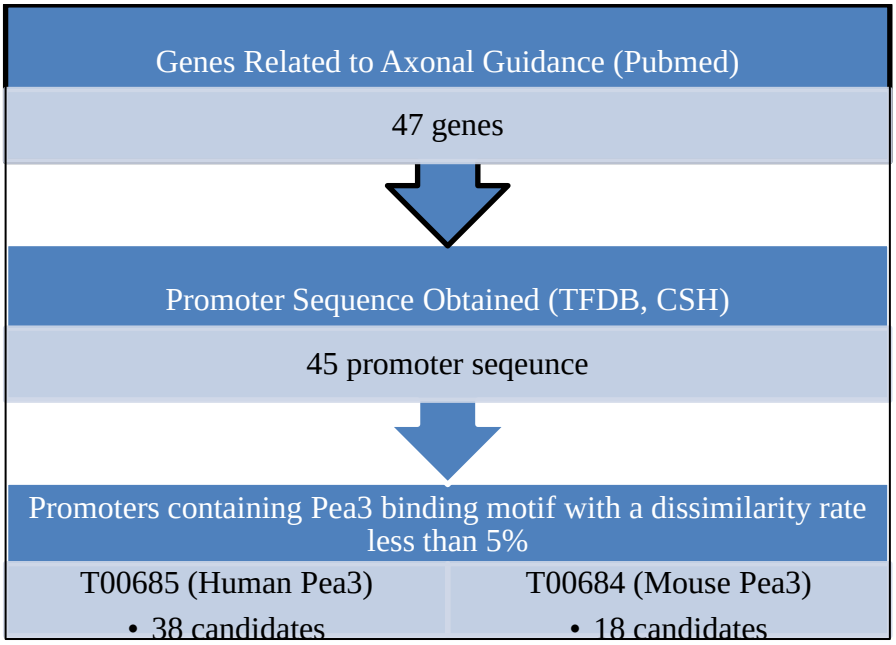


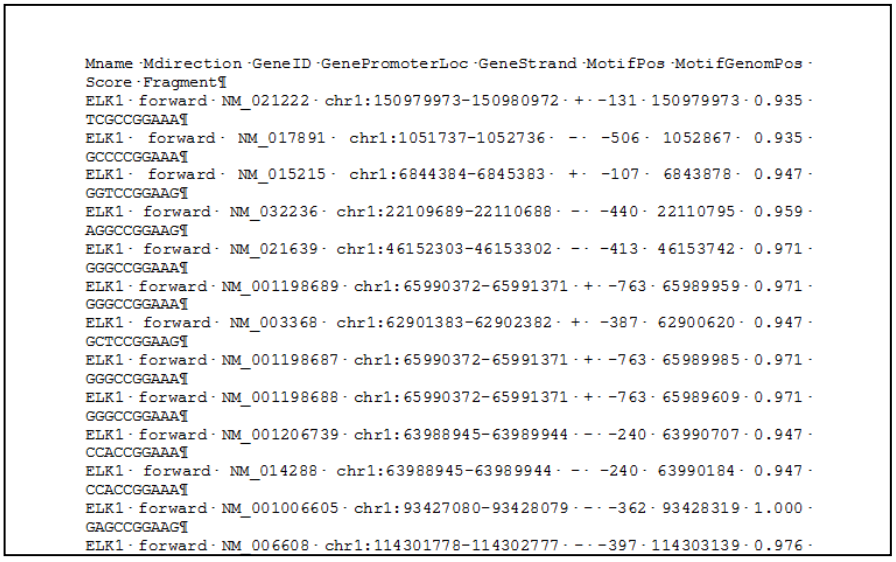
Figure 4.2.The results for axonal guidance genes

Control experiments were performed to control the reliability of the search. MMP3 [42], MMP9 [43] and VEGF [44] were used as positive control since regulation of their expression by PEA3/ETV4 is provenand the dissimilarity rates were 0 for all these inputs.

When the results were compared, it was seen that 19 genes are common and they were tabulated in a distinct table (Table 4.2). They were investigated in a more detailed fashion with respect to their cellular functions and reference articles are included.

This manual curation approach is time-consuming for genome-wide searches. For that purpose a collaborative work was carried out with Ugur Sezerman from Sabanci University. As a first step, we decided to merge 2 different databases which are TRED and Promo. In this fashion, the user simply enters the name of the promoter and the tool automatically screens it for transcription binding sites (Figure B1, B2). Although it reduced time for the search, it was not enough for genome-wide search since the user had to predict a promoter. In the next step, a genome-wide search function was enabled. In this tool, named as *motifTFinder*, user simply selects the type of organism and transcription factor of interest and then the tool lists all the promoters and affinity of the transcription factor on

these all promoters (Figure B3). This is a new approach for transcription factor databases since there is no tool for both predicting and scoring the transcription factor binding possibility in a genome-wide manner. Moreover, this tool basically reduces the time consumed, since it prevents to go back and forth between the sites.



Mname · Mdirection · GeneID · GenePromoterLoc · GeneStrand · MotifPos · MotifGenomPos · Score · Fragment¶
ELK1 · forward · NM_021222 · chr1:150979973-150980972 · + · -131 · 150979973 · 0.935 · TCGCCGGAAA¶
ELK1 · forward · NM_017891 · chr1:1051737-1052736 · - · -506 · 1052867 · 0.935 · GCCCCGGAAA¶
ELK1 · forward · NM_015215 · chr1:6844384-6845383 · + · -107 · 6843878 · 0.947 · GGTCCGGAAG¶
ELK1 · forward · NM_032236 · chr1:22109689-22110688 · - · -440 · 22110795 · 0.959 · AGGCCGGAAG¶
ELK1 · forward · NM_021639 · chr1:46152303-46153302 · - · -413 · 46153742 · 0.971 · GGGCCGGAAA¶
ELK1 · forward · NM_001198689 · chr1:65990372-65991371 · + · -763 · 65989959 · 0.971 · GGGCCGGAAA¶
ELK1 · forward · NM_003368 · chr1:62901383-62902382 · + · -387 · 62900620 · 0.947 · GCTCCGGAAG¶
ELK1 · forward · NM_001198687 · chr1:65990372-65991371 · + · -763 · 65989985 · 0.971 · GGGCCGGAAA¶
ELK1 · forward · NM_001198688 · chr1:65990372-65991371 · + · -763 · 65989609 · 0.971 · GGGCCGGAAA¶
ELK1 · forward · NM_001206739 · chr1:63988945-63989944 · - · -240 · 63990707 · 0.947 · CCACCGGAAA¶
ELK1 · forward · NM_014288 · chr1:63988945-63989944 · - · -240 · 63990184 · 0.947 · CCACCGGAAA¶
ELK1 · forward · NM_001006605 · chr1:93427080-93428079 · - · -362 · 93428319 · 1.000 · GAGCCGGAAG¶
ELK1 · forward · NM_006608 · chr1:114301778-114302777 · - · -397 · 114303139 · 0.976 ·

Figure 4.3. A result page for *motifTFinder* tool. The search was done for ELK1 which has evolutionary conserved site with PEA3/ETV4. The page displays Gene ID, Gene Promoter Location, Strand either minus or plus, position of the motif on the promoter and Promo 3.0 score

Table 4.1. Predictions for PEA3/ETV4 Binding

Gene abb.	Gene Name	Accesion #	mPEA3	hPEA3	Function	Techniques	REF
AMFR	Autocrine Motility Factor Receptor	15993	1,70	0	Signal transduction	Primary Neuron Culture, RT-PCR	[45,46]
EFNB2	Ephrin B2	11196	0	N/A	Ephrin receptor binding	Expression and migration assays	[47,48]
EPHA4	Ephrin Receptor A4	24617	0	N/A	Ephrin ligand binding	Expression and migration assays, electrophysilogical studies	[49,50]
DAB1	Disabled Protein 1	3392	0	9,24	Signal transduction	In vivo RNAi analysis	[51]
NEUROD1	Neuronal Differentiation Factor 1	24855	3,31	9,24	Transcription factor	RNAi, migration assay, immunohistochemistry	[52,53]
NFL	Neurofilament Light Chain	41145	3,94	9,45	constituent of cytoskeleton	Immunohistochemisty, immunoprecipatation	[54]

Table 4.1Predictions for PEA3/ETV4 Binding (continue)

Gene abb.	Gene Name	Accesion #	mPEA3	hPEA3	Function	Techniques	REF
NFM	Neurofilament Medium Cahin	39810	1,07	6,93	Structural constituent of cytoskeleton	Immunoblotting, immunohistochemistry	[55]
PSEN 1	Presenilin 1	11969	3,31	0,21	Calcium ion binding	Yeast-two-hybrid, ChIP	[56]
ALOX15B	Arachidonate 15-lipoxygenase, type B	16817	1,07	7,14	Iron ion binding	RT-PCR, Northern Blotting	[57]
HFE	Hemochromatosis	35056	3,94	7,14	Protein binding	Apoptotic assays, cell culture	[58]
ANGPT-1	Angiopietin	113693	0	0,43	Angiogenesis	Immunoblotting,	[59]
RECK	Reversion-Inducing-Cysteine-Rich	41462	N/A	0,21	endopeptidase inhibitor activity	RT-PCR, hypoxia studies	[60]

Table 4.1Predictions for PEA3/ETV4 Binding (continue)

Gene abb.	Gene Name	Accesion #	mPEA3	hPEA3	Function	Techniques	REF
C4.4A	Metastasis associated GPI-anchored protein	21926	0,63	0	Signal transduction	RT-PCR, Tissue microarray	[61]
CTNNA3	alphaT-catenin	5720	1,70	7,14	Cadherin binding	SNP	[62]
SEMA3A	Semaphorin 3A	117334	1,70	0,21	Chemorepellent activity	RT-PCR, immunocytochemistry	[63]
NTNG1	Netrin G1	959	0,63	7,14	Protein binding	RT-PCR	[64]

Table 4.2.Common genes for neuronal migration and axonal guidance with their PEA3/ETV4 binding scores

Gene abb.	Gene Name	Accesion #	mPEA3	hPEA3	Function	Techniques	REF
CNTN2	Contactin 2	1782	3,94	9,24	Carbonhydrate and glycoprotein binding	Immunohistochemical assays	[65]
SLIT2	Slit Homolog 2	116382	3,94	0,43	GTPase inhibition, Roundabout binding, calcium ion binding	RNAi and expression assays	[66]
Nrp1	Neuropilin 1	5859	3,31	9,24	Growth factor binding, cell-cell signaling	Immunohistochemical and expression assays	[67]
EphB2	Ephrin Receptor B2	323	N/A	9,67	Ephrin receptor activity, nucleotide binding, protein tyrosine kinase activity	Immunohistochemical and expression assays	[68]

Table 4.2 Common genes for neuronal migration and axonal guidance with their PEA3/ETV4 binding scores (continue)

Gene abb.	Gene Name	Accesion #	mPEA3	hPEA3	Function	Techniques	REF
CDK5R1	Cyclin Dependent Kinase 5 regulatory subunit 1	115721	N/A	9,45	Calcium ion binding, protein kinase activity	Survival assays, phosphorylation assays	[69]
NRCAM	Neuronal cell adhesion molecule	38906	8,32	9,67	Ankyrin binding	Immunocytochemistry and Electrophysiological assays	[70]
PTK2	Protein Tyrosine Kinase 2	116986	0,63	0	Nucleotide binding and signal transducer activity	Immunofluorescence staining and in vitro kinase assays	[71]
L1CAM	L1 Cell adhesion molecule	113184	0,63	0	İdentical protein binding for cell adhesion	Metastatic assays	[72]

Table 4.2 Common genes for neuronal migration and axonal guidance with their PEA3/ETV4 binding scores (continue)

Gene abb.	Gene Name	Accesion #	mPEA3	hPEA3	Function	Techniques	REF
EphA8	Ephrin Receptor A8	318	3,94	N/A	ATP binding, nucleotide binding	Expression assays, immunofluorescence	[73]
SEMA4A	Semaphorin 4A	1354	3,94	9,67	Receptor activity	Expression assays, FISH	[74]
NCAM1	Neural Cell Adhesion Molecule 1	7078	0	0,43	İdentical protein binding for cell adhesion	Tissue sample microarray and metastasis assay	[75]
BDNF	Brain Derived Neurotrophic factor	8188	0,63	N/A	Growth factor activity	Phosphorlyation and migration assays	[76]
GNAI2	G protein alpha inhibiting activity polypeptide 2	29399	1,70	9,67	GTPase activity, signal transduces	RNA interference, expression assays	[77]

Table 4.2 Common genes for neuronal migration and axonal guidance with their PEA3/ETV4 binding scores (continue)

Gene abb.	Gene Name	Accesion #	mPEA3	hPEA3	Function	Techniques	REF
NEUROG2	Neurogenin 2	32273	0,63	0,21	Transcription Factor	İmmunohistochemistry, expression assays	[78]
MYH10	myosin, heavy chain 10, non-muscle	19064	6,61	N/A	Actin binding, microfilament motor activity	ATPse assay, in vitro motility assay	[79]
NTF3	Neurotrophin 3	8613	0	7,14	Receptor binding	Molecular cloning	[80]
KAL1	Kallmann syndrome 1 sequence	44617	0,63	9,45	Extracellular matrix structural constituent	Immunocytochemistry and expression assays	[81]
NGFR	Nerve growth factor receptor	17440	1,70	9,24	Receptor and signal transducer activity	Immunoblotting and expression assays	[82]

Table 4.2 Common genes for neuronal migration and axonal guidance with their PEA3/ETV4 binding scores (continue)

Gene abb.	Gene Name	Accesion #	mPEA3	hPEA3	Function	Techniques	REF
MAPK8IP3	mitogen-activated protein kinase 8 interacting protein 3	14609	3,94	7,14	MAP kinase scaffold activity	Immunofluorescence, migration and expression assays	[83]

Regarding the cellular functions, three main targets were selected as AMFR, EphA4 and Dab1.

Autocrine motility factor receptor (AMFR) is a 78 kDa glycoprotein [84]. *AMFR* gene which is located on chromosome 16q21 encodes a 643 amino acid transmembrane protein. AMFR is evenly distributed on the membranes of the normal cells.

Human autocrine motility factor (AMF) and its receptor (AMFR) are responsible for the stimulation of the cell motility in an autocrine manner, meaning that the cell secreting AMF also expresses AMF receptor and AMF binds to this receptor to activate and to regulate the signaling pathways [85].

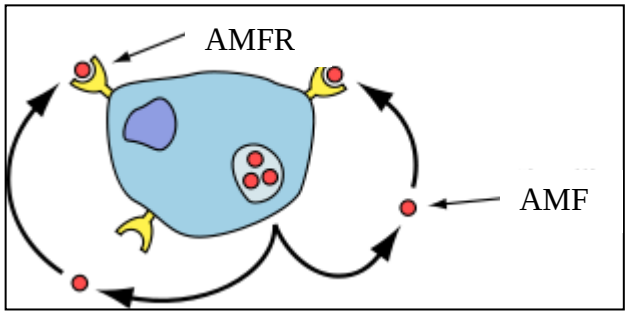


Figure 4.4. Mode of action for AMF and its receptor AMFR

AMF is a multifunctional cytokine. In addition to its motility function, AMF is a glucose-6-phosphate isomerase and neuroleukin meaning that AMF and AMFR have key roles for survival but not the proliferation of the embryonic sensory and spinal neurons [86].

The elevated levels of AMFR in malignant tumors such as gastrointestinal, kidney, colorectal, lung and breast carcinomas, make AMF and its receptor a cancer marker [87]. Recent studies revealed that, AMF and AMFR are highly expressed at protein and mRNA level in human breast cancer cells [88].

AMF and its receptor stimulate the activation of Rho-like GTPases, such as RhoA, RacI and cdc42. These activation patterns lead to actin rearrangement, initiating

metastaticprocesses [85]. In addition to this, AMF and AMFR participate in angiogenesis process in a corporation with VEGF.

It was shown that, mRNA levels of AMF and AMFR are significantly reduced in the hippocampi of the mouse model of Alzheimer Disease and their expression could be upregulated by medical treatments indicating their role in learning and memory [89].

The other target, *EPHA4* gene is located on chromosome 2q36.1 and responsible for synthesis of 986 amino acid transmembrane protein.

Eph receptors are one of the largest subfamily of Receptor Tyrosine kinases and divided into two major groups as A and B family. The members of both A and B family have similar molecular structure but the ligands with which they interact differ. Same as Eph Receptors, Ephrin ligands are divided into A and B group. In most cases, A type receptors interact with A type ligands. The same is true for B types. However, under some circumstances, it is observed that A type receptors may interact with B type ligands, as seen in EphA4 receptors [90]. Eph receptors differ from regular receptor tyrosine kinases as they require a cell surface mediated interaction with their ligands rather than soluble ligands [91]. Once interaction occur, if a downstream effect is observed in the Ephrin expressing cell it is called as reverse signaling and forward signaling in the opposite condition.

Ephrin-Eph interaction results in cell repulsion and plays a critical role in axonal projection. Different combinational effects may be revealed by Eph receptors and ephrins and the outcomes might be projections into different areas [92], synaptic plasticity [93] and even though neuron-glial communication [94].

EphA4 is believed to have a key role for tumor formation and progression. It was shown that, EphA4 is highly expressed in human gastric cancer [95] and pancreatic ductal adenocarcinoma [96]. Beyond this, it was proven that, EphA4 promotes the cell proliferation and migration by creating a complex with FGFR1 and accelerating FGF-based transduction pathway [97].

Studies revealed that *EphA4* expression maintains neural stem cells in an undifferentiated state and EphA4 silencing leads to deficits in neural stem cell proliferation and differentiation [98].

EphA4 participates in optic nervous system development. EphA4 is expressed in astrocyte precursor cells promoting their migration into retina. Also EphA4 has key roles in retinal ganglion cell axon ganglion cell axon growth and projections through the optic chiasm and EphA4 knockdown results in optic nerve pathologies [99].

Finally, *Dab1* gene is located on chromosome 1q31. Its protein product composed of 555 amino acids.

Dab1 plays a vital role in Reelin pathway which is one of the most important cascades regulating the neuronal migration [100]. Dab1 is the target of Reelin and it induces the several signaling cascades including PI3K pathway, within the cell [101]. Regulation of the expression of Dab1 protein still has some unclear points. It is shown that, Dab1 is expressed at the later stages of the neuronal migration, thus it might be considered as a stop signal for migration and initiation for synapse formation.

Dab1 is found as an element for progression of Alzheimer Disease. Upregulation of Dab1 and increased level of interactions with its partners have a consequence as elevated levels of cell proliferation in frontal cortex and results in deregulation of the cellular proteome [102].

In addition to these, increased levels of Dab1 expression is seen in several cancer types. Expression analysis demonstrates that Dab1 is overexpressed in especially brain and endometrial cancer cell lines and tissues and providing an insight for the linkage between nervous system development and tumorigenesis [103].

4.2. PROMOTER CLONING

As mentioned earlier, the promoters of AMFR, EphA4 and Dab1 were selected for further studies. Efnb2 was added to this list as well.

First of all, we attempted to clone the promoter region of AMFR. The annealing temperature was determined by gradient PCR. For this purpose, regular PCR reaction as described in the Section 3.2.2 was followed and annealing temperature was selected within the range of 55°C to 70°C (Figure 4.5).

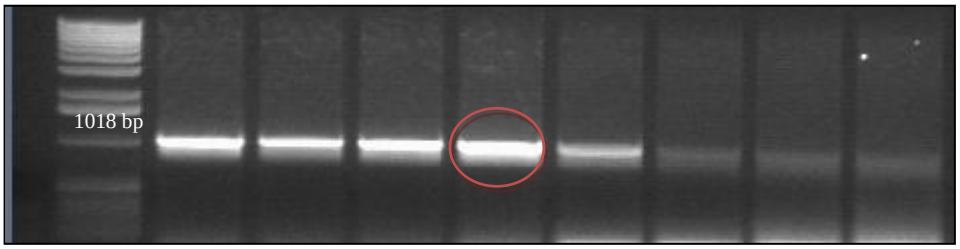


Figure 4.5.Gradient PCR for AMFR Promoter. Invitrogen 1kb DNA ladder was used and annealing temperatures are (from left to right; 70°C, 69.8°C, 67°C, 64.1°C, 60.5°C, 57.9°C, 56°C and 55°C

For later PCR studies, 64.1 °C was selected as annealing temperature and the following reaction was setup according to this value (Figure 4.6).

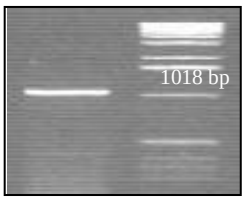


Figure 4.6. Amplification of AMFR promoter

After this step, DNA was purified, both the insert and pGL3 plasmid were digested by the restriction enzymes *KpnI* and *HindIII*. This procedure was followed by ligation with T4 ligase, transformation of bacteria and spreading them on the agar plate containing

ampicillin, since pGL3 has ampicillin resistance gene. Unfortunately, there were no colonies indicating that the restriction or ligation process failed during the experiments.

After that, the same procedure was followed for EphA4 promoter, however products with the desired length could not been obtained during the PCR (Figure 4.7).

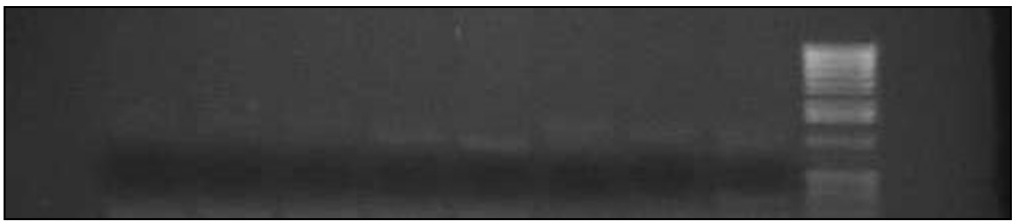


Figure 4.7. Gradient PCR for EphA4 promoter (Invitrogen 1kb DNA ladder was used and annealing temperatures are (from left to right; 70°C, 69.8°C, 67°C, 64.1°C, 60.5°C, 57.9°C, 56°C and 55°C.)

Unfortunately, EphA promoter did not give a significant result. To optimize its reaction, DNA template was titrated from 100 ng and 500 ng. Also it was speculated that unclear results could have been caused by $MgCl_2$ concentration, so various amounts of $MgCl_2$ was used in the range of 1.5 Mm and 4.0 Mm. The quality of the genomic DNA was also susceptible and new genomic DNA isolation was carried out. Although these troubleshooting approaches were followed, the desired results have not been obtained not only for EphA4 promoter but also Dab1 and EFNB2.

4.3 GENE EXPRESSION ANALYSIS

PEA3/ETV4 overexpression was followed by transfection of cell lines with the protein so the effects of PEA3/ETV4 on the gene expression could be observed,. Before starting, the endogeneous PEA3/ETV4 levels were monitored by RT-PCR experiments (Figure 4.8).

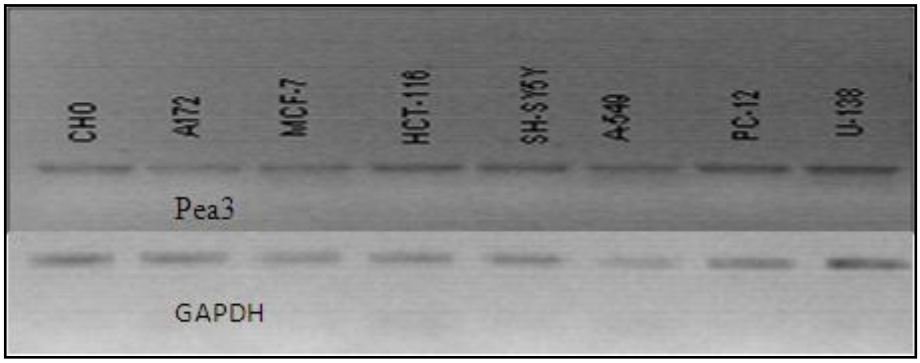


Figure 4.8**.** Endogeneous PEA3/ETV4 levels in different cell lines. GAPDH was used as housekeeping gene

Afterwards, the cells were either transfected or not transfected with mPea3. Two days later total RNA was isolated from both experimental (mPea3-transfected) and the control (untransfected) group and cDNA synthesis was quantified by Oligo-dT based method. Following this step, RT-PCR analysis was carried out where GAPDH was the housekeeping control, and Pea3expression was confirmed, followed by AMFR and EFNB2.

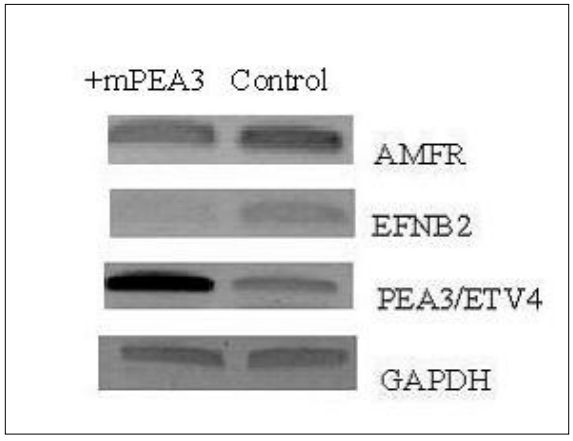


Figure 4.9.Gene expression levels of AMFR, EFNB2, PEA3/ETV4 and GAPDH, in control and PEA3/ETV4 overexpressing cells

The results indicated that, overexpression of PEA3/ETV4 suppresses the EFNB2 expression and it does not significantly changed AMFR expression (Figure 4.10). In fact, real time PCR might be applied for more accurate comments.

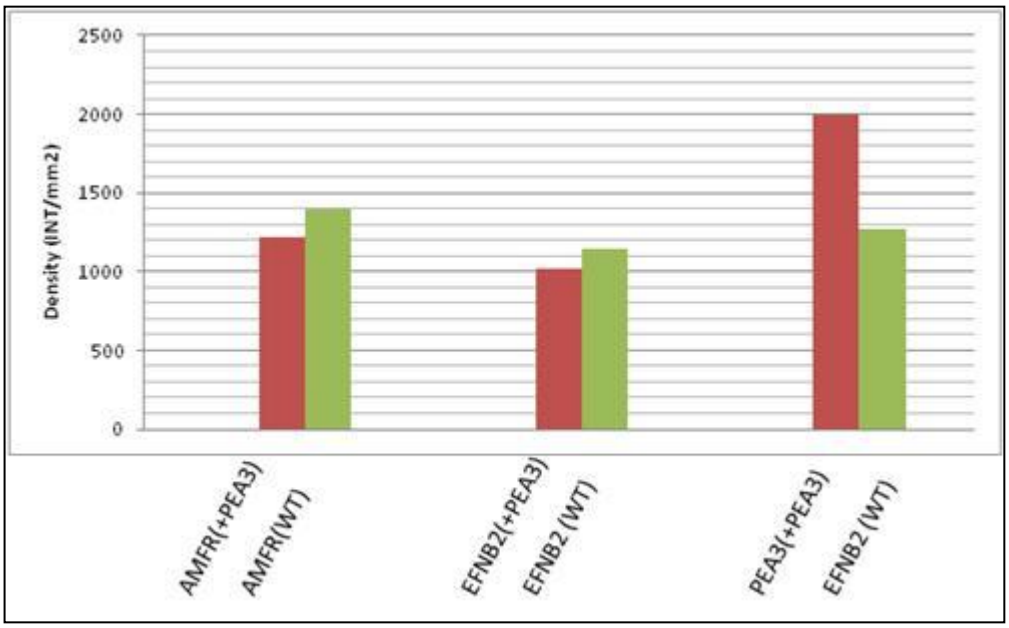


Figure 4.10.Quantification of the AMFR and EFNB2 expression levels, in the presence and the absence of the PEA3/ETV4. The overexpression of PEA3/ETV4 was also confirmed Red bars indicate the expression for PEA3/ETV4 overexpression, while green bars are for wild-type cells

Although AMFR and EFNB2 induce the metastasis [46,47], their expression seems to be repressed by PEA3/ETV4. This might be explained by the pivotal role of PEA3/ETV4. The more accurate comments will be able to be offered after further analysis.

Primers for Dab1 and EphA4 were also designed but the optimal conditions for measuring their expression levels in the presence and the absence of PEA3/ETV4 have not been achieved yet, however studies on this issue are ongoing.

5. CONCLUSION AND RECOMMENDATIONS

The aim of this project was to discover the potential targets for PEA3/ETV4 transcription factor *in silico* and confirmation of these targets with molecular laboratory techniques. For this purpose, the genes related to neuronal migration and axonal guidance were determined and their potential for PEA3/ETV4 binding was compared. The ones which had high scores in that manner and shared similar functions with PEA3/ETV4 were selected for further studies, specifically AMFR, EphA4 and Dab1. Unluckily, the cloning process of those promoters was unsuccessful. In the future, the problems for this failure might be investigated and solved or new promoters could be selected as targets. An alternative way which is thought to give more accurate results is microarray analysis coupled with promoter arrays (ChIP-chips). By doing so, it would be possible to screen PEA3/ETV4targets genome-wide and confirm the *in silico* results.

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APPENDIX A: FULL TABLES OF THE PEA3 AFFINITY ANALYSIS

In this section full list of the genes for neuronal migration and axonal guidance, their accession number for TRED, dissimilarity rates for human and mouse PEA3/ETV4 are available.

Table A.1. Neuronal Migration Genes and PEA3/ETV4 Binding Scores

Gene abb.	Gene Name	Accesion #	mPEA3	hPEA3
DCX	Doublecortin	44150	0,63	0,43
CDH2	Cadherin 2, type 1	19854	N/A	0,21
CDK5R1	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	115721	N/A	9,45
RAC1	Ras-related C3 botulinum toxin substrate	37434	0,63	0,21
NRCAM	Neuronal cell adhesion molecule	38906	8,32	9,67
CHRNA7	Cholinergic receptor, nicotinic, alpha 7	124502	1,07	0,21
NEUROD4	Neuronal differentiation 4	9014	1,70	N/A
GPM6A	Glycoprotein M6A	32061	0	7,36
ACNN1	Acid-sensing (proton-gated) ion channel 2	113662	1,70	0,21
ACNN2	N/A	-	-	-
IL16	Interleukin 16	13565	3,94	0,21
TRIB3	Tribbles homolog 3 (Drosophila)	25974	3,94	9,24
RAE1	RAE1 RNA export 1 homolog (S. Pombe)	26417	3,31	9,24
SCN2B	Sodium channel, voltage-gated, type II, beta subunit	7446	0,63	0,21
BMP2	Bone morphogenetic protein 2	113729	8,32	9,67
FLNA	Filamin A, alpha	43914	0,63	7,14
TMEM18	Transmembrane protein 18	25953	0,63	9,24
SLIT2	Slit Homolog 2	116382	3,94	0,43
VEGFA	Vascular endothelial growth factor A	35486	0	9,67
TGFB1	Transforming growth factor, beta 1	21997	0,63	6,93
ITGB1	Integrin, beta 1	5863	0	9,45
CDK5	Cyclin-dependent kinase 5	38686	3,94	N/A
NRP1	Neuropilin 1	5859	3,31	9,24
Akt1	V-akt murine thymoma viral oncogene	12369	1,70	7,14
Fgf2	Fibroblast growth factor 2 (basic)	44008	6,61	9,24
DRD1	Dopamine receptor D1	113440	8,32	7,36
APOE	Apolipoprotein E	20958	0,63	0
TGFB2	Transforming growth factor, beta 2	1884	3,31	7,36
Psen1	Presenilin 1	11969	3,94	0,21
Pten	Phosphatase and tensin homolog	4722	1,70	7,14
PTK2	Protein Tyrosine Kinase 2	116986	0,63	0
DRD2	Dopamine receptor D2	7493	1,70	7,36
PAFAH1B1	Platelet-activating factor acetylhydrolase 1b, regulatory subt 1	16692	3,31	9,45
Reln	Reelin	38932	0	9,67
Erbb4	V-erb-a erythroblastic leukemia viral oncogene homolog 4	116717	1,70	7,14
ARX	Aristaless related homeobox	44551	1,70	9,45
MYH10	Myosin, heavy chain 10, non-muscle	19064	6,61	N/A
CNTN2	Contactin 2	1782	3,94	9,24
APC	Adenomatous polyposis coli	33308	0,63	9,45
Cx3cr1	Chemokine (C-X3-C motif) receptor 1 [	30958	0,63	0
CCL2	Chemokine (C-C motif) ligand 2	17088	0,63	0,21

Table A.1. Neuronal Migration Genes and PEA3/ETV4 Binding Scores (continue)

Gene abb.	Gene Name	Accesion #	mPEA3	hPEA3
PRKCA	Protein kinase c, alpha	114871	0	9,45
MAPK1	Mitogen-activated protein kinase 1	124425	1,70	7,14
ACVRL1	Activin A receptor type II-like 1	8936	0,63	0,21
TP53	Tumor protein p53	19095	3,94	9,45
CXCR4	Chemokine (C-X-C motif) receptor 4	25069	1,70	0
CCR2	Chemokine (C-C motif) receptor 2	29337	0	N/A
RTN4	Reticulon 4	25612	0,63	N/A
ROBO1	Oundabout, axon guidance receptor, homolog 1 (Drosophila)	30670	1,70	7,14
EGFR	Epidermal growth factor receptor	37723	N/A	N/A
HIF1A	Hypoxia induced factor 1 alpha subunit	11879	1,07	N/A
AGER	Advanced glycosylation end product-specific receptor	36867	3,94	9,45
KDR	Kinase insert domain receptor (a type III receptor tyrosine kinase)	32514	3,31	0,21
FGFR1	Fibroblast growth factor receptor 1	41051	1,07	0,21
PTK2B	PTK2B protein tyrosine kinase 2 beta	39829	0	0
ITGA3	Integrin, alpha 3	17448	0	0,21
IGF1	Insulin-like growth factor 1 (somatomedin C)	9854	7,25	N/A
ILK	Integrin-linked kinase	6243	0,63	0,43
LRP1	Low density lipoprotein receptor-related protein 1	9066	N/A	N/A
NDEL1	61uin nuclear distribution E homolog (A. Nidulans)-like 1	16858	3,31	7,36
MTOR	Mechanistic target of rapamycin (serine/threonine kinase)	4009	0,63	0,21
RET	Ret proto-oncogene	4430	4,38	9,24
CXCL12	Chemokine (C-X-C motif) ligand 12	5815	0,63	N/A
NF1	Neurofibromin 1	17063	0,63	9,45
PRKCE	Protein kinase C, epsilon	23220	0	0,21
AKT2	V-akt murine thymoma viral oncogene homolog 2	22019	1,70	N/A
GNB2L1	G protein, beta polypeptide 2-like 1	33870	N/A	6,93
PTGS2	Prostaglandin-endoperoxide synthase 2	112626	3,94	9,45
BCL2	B-cell CLL/lymphoma 2	19717	0	0,21
SERPINE1	Serpin peptidase inhibitor	38036	0,63	0,43
HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog	6119	3,94	0,21
MMP9	Matrix metallopeptidase 9	26338	0	6,93
SRC	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog	26243	7,25	9,45
RHOA	Ras homolog family member A	30843	4,38	7,36
F2R	Coagulation factor II (thrombin) receptor	33183	3,94	0,21
ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	10119	0,63	N/A
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	112706	3,31	N/A
MAPK14	Mitogen-activated protein kinase 14	35392	1,70	N/A
NKX2-1	NK2 homeobox 1	12851	0	0,21
LRP6	Low density lipoprotein receptor-related protein 6	10493	0	9,24
TAC1	Tachykinin, precursor 1	37967	0,63	0,21
CX3CL1	Chemokine (C-X3-C motif) ligand 1	15262	3,94	0

Table A.1. Neuronal Migration Genes and PEA3/ETV4 Binding Scores (continue)

Gene abb.	Gene Name	Accesion #	mPEA3	hPEA3
TNF	Tumor necrosis factor	35233	0,63	0,21
STAT3	Signal transducer and activator of transcription 3	18595	7,25	0
CASP3	Caspase 3, apoptosis-related cysteine peptidase	32032	0	9,67
BMPR2	Bone morphogenetic protein receptor, type II	24082	1,70	0,21
REST	RE1-silencing transcription factor	31428	3,94	N/A
PROX1	Prospero homeobox 1	1871	0,63	N/A
IGFBP5	Insulin-like growth factor binding protein 5	24681	1,70	N/A
LRRK2	Leucine-rich repeat kinase 2	N/A		
CAV1	Caveolin 1	112864	3,94	6,93
HSPB1	Heat shock 27kda protein 1	37879	1,70	N/A
DISC1	Disrupted in schizophrenia 1	2030	5,01	9,24
NOX1	NADPH oxidase 1	118044	8,32	7,36
APP	Amyloid beta (A4) precursor protein	27897	1,07	0
MAPT	Microtubule-associated protein tau	17352	0,63	7,14
MMP2	Matrix metallopeptidase 2	15206	1,70	7,14
MAPK3	Mitogen-activated protein kinase 3	16165	N/A	N/A
MET	Met proto-oncogene (hepatocyte growth factor receptor)	38183	0	9,45
AGT	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	2219	0	0,43
CALCA	Calcitonin-related polypeptide alpha	8255	0	9,24
RBS6K1	Ribosomal protein S6 kinase, 70kda, polypeptide 1	N/A	-	-
ANG	Angiogenin, ribonuclease, Rnase A family, 5	11590	0,63	N/A
CCL3	Chemokine (C-C motif) ligand 3	18756	3,31	0,43
ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	33039	0	9,45
TSC2	Tuberous sclerosis 2	14637	0	0,43
L1CAM	L1 Cell adhesion molecule	113184	0,63	0
ADAM10	ADAM metallopeptidase domain 10	14166	0	9,45
FSCN1	Fascin homolog 1, actin-bundling protein	37428	8,32	N/A
IL6	Interleukin 6	37500	0	9,67
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	15369	3,94	N/A
CCR5	Chemokine (C-C motif) receptor 5 (gene/pseudogene)	29338	0,63	0,43
HGF	Hepatocyte growth factor	37500	0	9,67
MAPK8	Mitogen-activated protein kinase 8	4485	6,61	6,93
HTT	Huntingtin	31205	3,94	N/a
EGF	Epidermal growth factor	31663	0,63	7,36
PLAUR	Plasminogen activator, urokinase receptor	112794	1,70	7,36
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	10423	1,70	N/A
ASCL1	Achaete-scute complex homolog 1	9256	0,63	N/a
NDN	Necdin homolog (mouse)	1442	0,63	0
WNT11	Wingless-type MMTV integration site family, member 11	7664	7,25	9,45
IL1B	Interleukin 1, beta	25217	5,01	7,14

Table A.1. Neuronal Migration Genes and PEA3/ETV4 Binding Scores (continue)

Gene abb.	Gene Name	Accesion #	mPEA3	hPEA3
MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	N/a	-	-
NCAM1	Neural cell adhesion molecule 1	7078	0	0,43
ALCAM	Activated leukocyte cell adhesion molecule	29583	0	7,36
KIAA0319	Kiaa0319	37133	1,07	7,14
BDNF	Brain Derived Neurotrophic factor	8188	0,63	N/A
GSK3B	Glycogen synthase kinase 3 beta	30585	0.63	N/a
PRKCD	Protein kinase C, delta	29452	3,94	7,14
CXCR3	Chemokine (C-X-C motif) receptor 3	44325	3,94	7,36
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog	3139	3,31	0,21
JAG1	Jagged 1	27116	0,63	9,45
SST	Somatostatin	30203	0	N/A
HES1	Hairy and enhancer of split 1, (Drosophila)	30060	0,63	N/A
EDN3	Endothelin 3	26454	3,97	9,45
NODAL	Nodal homolog (mouse)	5694	0	6,93
DIXDC1	DIX domain containing 1	N/A	-	-
ARC	Activity-regulated cytoskeleton-associated p.	119382	3,31	0,43
SHROOM2	Shroom family member 2	43064	0	0
NOS3	Nitric oxide synthase 3 (endothelial cell)	38471	0,63	9,24
IGF1R	Insulin-like growth factor 1 receptor	13725	N/A	N/A
EGR1	Early growth response 1	33479	3,31	7,14
CD34	CD34 molecule	2391	0	9,24
ID1	Inhibitor of DNA binding 1, dominant negative helix-loop-helix pr.	26136	5,01	N/A
NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	41584	0	N/A
GDNF	Glial cell derived neurotrophic factor	34695	7,25	9,67
ZEB2	Zinc finger E-box binding homeobox 2	11789	3,94	9,45
NOV	Nephroblastoma overexpressed	40195	0	9,24
RRAS	Related RAS viral (r-ras) oncogene homolog	21730	3,94	7,14
LEP	Leptin	38223	3,31	9,24
FAS	Fas (TNF receptor superfamily, member 6)	4737	0,63	9,24
PIN1	Peptidylprolyl cis/trans isomerase, NIMA-interacting 1	32964	0,63	0,21
IQGAP1	IQ motif containing gtpase activating protein 1	13670	0,63	9,24
ADA	Adenosine deaminase	26857	0,63	N/A
DBH	Dopamine beta-hydroxylase (dopamine beta-monooxygenase)	42036	3,94	7,36
TBX21	T-box 21	17380	0,63	9,67
ADORA1	Adenosine A1 receptor	1762	1,07	7,14
DRD5	Dopamine receptor D5	31284	1,07	7,14
APOD	Apolipoprotein D	N/A		
SMO	Smoothened, frizzled family receptor	38242	0	9,45
ANXA3	Annexin A3	31531	0,63	N/A
KATNA1	Katanin p60 (atpase containing) subunit A 1	N/A	-	-

Table A.1. Neuronal Migration Genes and PEA3/ETV4 Binding Scores (continue)

Gene abb.	Gene Name	Accesion #	mPEA3	hPEA3
DNER	Delta/notch-like EGF repeat containing	24585	8,32	7,14
ESR2	Estrogen receptor 2 (ER beta)	12718	0,63	9,45
NOTCH1	Notch1	42193	3,94	0,21
IGFBP3	Insulin-like growth factor binding protein 3	39292	3,31	9,45
CST3	Cystatin C	27045	4,38	9,45
TACR1	Tachykinin receptor 1	25458	7,25	N/A
VCAN	Versican	33234	3,94	9,45
SDC2	Syndecan 2	40122	0,63	N/A
LGALS1	Lectin, galactoside-binding, soluble, 1	28286	6,61	0,21
INS	Insulin	8437	3,94	9,67
FYN	FYN oncogene related to SRC, FGR, YES	36364	3,31	6,93
TRPV1	Transient receptor potential cation channel, subfamily V, member 1	19201	7,25	9,67
AGTR2	Angiotensin II receptor, type 2	43595	0,63	7,14
SGK1	Serum/glucocorticoid regulated kinase 1	36274	6,61	6,93
CCK	Cholecystokinin	N/A		
HDAC6	Histone deacetylase 6	43294	5,01	9,45
PEBP1	Phosphatidylethanolamine binding protein 1	22632	3,94	9,67
PHOX2B	Paired-like homeobox 2b	32560	3,94	7,36
CXCL16	Chemokine (C-X-C motif) ligand 16	19177	0	7,14
FAPB7	Fatty acid binding protein 7, brain	N/A	-	-
RAC3	Ras-related C3 botulinum toxin substrate	17853	N/A	N/A
NRD1	Nardilysin (N-arginine dibasic convertase)	3439	0,63	0,43
ESR1	Estrogen receptor 1	35915	3,94	7,36
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	8728	N/A	9,45
JUN	Jun proto-oncogene	3386	N/A	7,14
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	30284	0	9,24
NRG1	Neuregulin 1	39861	0	6,93
CCR1	Chemokine (C-C motif) receptor 1	30919	3,94	0,45
PFN1	64Uinine64n 1	19165	3,31	7,36
HMOX1	Heme oxygenase (decycling) 1	28253	1,70	9,24
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1	14212	3,94	0,43
DRD4	Dopamine receptor D4	8529	N/A	7,36
CYBA	Cytochrome b-245, alpha polypeptide	15713	0	0,43
NF2	Neurofibromin 2 (merlin)	28183	N/A	N/A
MAP2K1	Mitogen-activated protein kinase kinase 1	13375	3,31	9,67
UCHL1	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	31373	8,32	0,21
IRS2	Insulin receptor substrate 2	11182	N/A	9,45
NR4A2	Nuclear receptor subfamily 4, group A, member 2	25030	0,63	0,43
MYO6	Myosin VI	35601	3,31	9,45
CALD1	Caldesmon 1	38287	5,01	9,24

Table A.1. Neuronal Migration Genes and PEA3/ETV4 Binding Scores (continue)

Gene abb.	Gene Name	Accesion #	mPEA3	hPEA3
MYO5A	Myosin VA (heavy chain 12, myoxin)	14198	3,94	9,45
P2RX4	Purinergic receptor P2X, ligand-gated ion channel, 4	9438	3,94	7,14
SCG2	Secretogranin II	7907	0,63	0
DCDC2	Doublecortin domain containing 2	37135	N/A	0
ARHGEF2	Rho/Rac guanine nucleotide exchange factor (GEF) 2	2844	0,63	0,21
DYX1C1	Dyslexia susceptibility 1 candidate 1	124118	8,32	9,45
DBN1	Drebrin 1	33951	7,25	6,93
POU4F1	POU class 4 homeobox 1	11282	6,61	9,45
ROBO4	Roundabout, axon guidance receptor, homolog 4 (Drosophila)	7370	0,63	6,93
EGR3	Early growth response 3	41169	0,63	9,45
EphA8	Ephrin receptor a8	318	3,94	N/A
NRTN	Neurturin	20211	3,94	0,43
TUBB2B	Tubulin, beta 2B class Iib	N/A		
MDGA1	MAM domain containing glycosyl.	36711	3,31	N/A
ASTN1	Astrotactin 1	2620	1,70	0,21
PPARG	Peroxisome proliferator-activated receptor gamma	29131	0,63	7,14
BRAF	V-raf murine sarcoma viral oncogene homolog B1	38761	1,07	N/A
PAX6	Paired box 6	8176	0,63	0
EPOR	Erythropoietin receptor	22520	1,07	7,36
NGFR	Nerve growth factor receptor	17740	1,70	9,24
ACHE	Acetylcholinesterase	38973	3,31	6,93
ADRB2	Adrenoceptor beta 2, surface	33062	0	0,43
PPARD	Peroxisome proliferator-activated receptor delta	35373	0,63	0,43
CSF1	Colony stimulating factor 1 (macrophage)	112668	1,07	9,45
ATP7A	Atpase, Cu++ transporting, alpha polypeptide	43455	0,63	N/A
TGFB3	Transforming growth factor, beta 3	12596	0,63	7,14
NR4A1	Nuclear receptor subfamily 4, group A, member 1	8941	3,94	0
PITX2	Paired-like homeodomain 2	32277	1,70	0
NTRK3	Neurotrophic tyrosine kinase, receptor, type 3	13873	0,63	N/A
CDKL5	Cyclin-dependent kinase-like 5	43121	3,31	9,45
ATN1	Atrophin 1	8658	0,63	6,93
SMURF1	SMAD specific E3 ubiquitin protein ligase 1	39039	1,70	0,21
MARK2	MAP/microtubule affinity-regulating kinase 2	6648	3,31	9,45
SLC7A11	Solute carrier family 7, member 11	32206	0,63	9,24
ATP1B2	Atpase, Na+/K+ transporting, beta 2 polypeptide	16821	3,31	9,45
POU3F2	POU class 3 homeobox 2	35658	0,63	0,43
EPHA5	EPH receptor A5	32495	0	N/A
DPYSL3	Dihydropyrimidinase-like 3	34133	0,63	0,21
UHMK1	U2AF homology motif (UHM) kinase 1	1502	1,70	N/A
NEUROG2	Neurogenin 2	32273	0,63	0,21

Table A.1. Neuronal Migration Genes and PEA3/ETV4 Binding Scores (continue)

Gene abb.	Gene Name	Accesion #	mPEA3	hPEA3
SIX4	SIX homeobox 4	12732	0	9,45
CLDN19	Claudin 19	3549	4,38	0,21
NAV1	Neuron navigator 1	1734	1,70	7,14
PARK7	66Uinine66n protein 7	119	3,31	0,43
AQP1	Aquaporin 1 (Colton blood group)	37601	1,07	0,43
PRKCZ	Protein kinase C, zeta	N/A	-	-
TNC	Tenascin C	42461	3,94	0,43
CYBB	Cytochrome b-245, beta polypeptide	43188	1,70	0,21
TIMP3	TIMP metallopeptidase inhibitor 3	28241	N/A	9,24
COL18A1	Collagen, type XVIII, alpha 1	27535	3,94	0,21
TSC1	Tuberous sclerosis 1	42275	3,94	N/A
CNR2	Cannabinoid receptor 2 (macrophage)	114186	5,01	0,43
RAP1A	RAP1A, member of RAS oncogene family	1031	0,63	N/A
PRKCG	Protein kinase C, gamma	21242	0	9,67
CXCL1	Chemokine (C-X-C motif) ligand 1	31494	3,31	N/A
COL17A1	Collagen, type XVII, alpha 1	113233	1,70	9,45
EphB2	Ephrin receptor b2	323	N/A	9,67
MAP3K11	Mitogen-activated protein kinase kinase kinase 11	7865	0	9,67
AGAP2	Arfgap with gtpase domain, ankyrin repeat and PH domain 2	10033	3,94	N/A
CD4	CD4 molecule	8642	0,63	0,43
KCNH2	Potassium voltage-gated channel, subfamily H member 2	38691	3,94	6,93
TP73	Tumor protein p73	61	0,63	0,21
E2F1	E2F transcription factor 1	26980	8,32	9,24
GJA1	Gap junction protein, alpha 1, 43kda	35769	0,63	6,93
RAF1	V-raf-1 murine leukemia viral oncogene homolog 1	31064	0,63	0,21
CBL	Cbl proto-oncogene, E3 ubiquitin protein ligase	7161	0,63	9,24
PRKCB	Protein kinase C, beta	14905	0,63	9,45
MIR21	Microrna 21	N/A	-	-
SERPINF1	Serpin peptidase inhibitor, clade F member	16670	0,63	9,24
NGF	Neuronal growth factor	3133	3,94	9,45
GFAP	Glial fibrillary acidic protein	18498	1,07	9,24
AQP4	Aquaporin 4	19861	1,70	7,14
PDPK1	3-phosphoinositide dependent protein kinase-1	14469	0	9,45
MFN2	Mitofusin 2	186	N/A	0
DLG1	Discs, large homolog 1 (Drosophila)	30137	3,94	9,45
KCNN4	Potassium intermediate/small conductance Ca-activated channel N4,	21911	1,70	9,45
PPP1CA	Protein phosphatase 1, catalytic subunit, alpha isozyme	N/A	-	-
SET	SET nuclear oncogene	41931	1,70	7,14
SPRY2	66uinin homolog 2 (Drosophila)	11276	0,63	9,45
MIR34A	Microrna 34a	N/A		

Table A.1. Neuronal Migration Genes and PEA3/ETV4 Binding Scores (continue)

Gene abb.	Gene Name	Accesion #	mPEA3	hPEA3
SORL1	Sortilin-related receptor, L(DLR class) A repeats containing	7188	0	0,21
TUBB3	Tubulin, beta 3 class III	N/A	-	-
RCAN1	Regulator of calcineurin 1	27830	0	N/A
KAL1	Kallmann syndrome 1 sequence	44617	0,63	9,45
GNB1	Guanine nucleotide binding protein	4136	0	9,24
NUMB	Numb homolog (Drosophila)	12642	1,70	9,45
GIT1	G protein-coupled receptor kinase interacting arfgap 1	18831	3,31	0,43
PREX1	Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor1	26795	N/A	N/A
TRIO	Triple functional domain (PTPRF interacting)	32983	0	9,24
LHX5	LIM homeobox 5	9748	0,63	9,45
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	40233	0	0
RELA	V-rel reticuloendotheliosis viral oncogene homolog A (avian)	7862	1,70	9,24
NOS2	Nitric oxide synthase 2, inducible	18883	3,94	7,14
FOXP3	Forkhead box P3	44421	3,94	0
IL2	Interleukin 2	32233	0	0
ABL1	C-abl oncogene 1, non-receptor tyrosine kinase	41981	0	N/A
FASLG	Fas ligand (TNF superfamily, member 6)	1581	0	N/A
CREBBP	CREB binding protein	16406	3,31	9,45
ALDH2	Aldehyde dehydrogenase 2 family (mitochondrial)	N/A	-	-
IL13	Interleukin 13	33399	1,70	N/A
PRKACA	Protein kinase, Camp-dependent, catalytic, alpha	22425	N/A	N/A
TLR3	Toll-like receptor 3	31941	1,07	7,36
PLAT	Plasminogen activator, tissue	41032	0,63	9,24
PROM1	Prominin 1	32633	0,63	7,14
HPSE	Heparanase	32408	0	7,14
TIMP2	TIMP metallopeptidase inhibitor 2	18042	0	0,43
DKK1	Dickkopf 1 homolog (Xenopus laevis)	4522	1,07	9,45
CALR	Calreticulin	20388	0	9,45
YY1	YY1 transcription factor	12193	3,31	0,43
DCN	Decorin	9919	0	N/A
CSK	C-src tyrosine kinase	13468	0	N/A
RASA1	RAS p21 protein activator (gtpase activating protein) 1	33238	0	N/A
MAP3K5	Mitogen-activated protein kinase kinase kinase 5	36258	0,63	0
TJP1	Tight junction protein 1 (zona occludens 1)	14402	1,07	N/A
SCN9A	Sodium channel, voltage-gated, type IX, alpha subunit	115071	1,70	0,43
KCNA5	Potassium voltage-gated channel, shaker-related subfamily, member 5	8612	4,38	9,24
RAB5A	RAB5A, member RAS oncogene family	29177	1,70	0,21
PTDGS	Prostaglandin D2 synthase 21kda (brain)	42110	0	7,14
PRKG1	Protein kinase, Cgmp-dependent, type I	4520	1,70	9,45

Table A.1. Neuronal Migration Genes and PEA3/ETV4 Binding Scores (continue)

Gene abb.	Gene Name	Accesion #	mPEA3	hPEA3
KCNA3	Potassium voltage-gated channel, shaker-related subfamily, member 3	3180	0,63	7,14
VAMP2	Vesicle-associated membrane protein 2 (synaptobrevin 2)	19078	3,94	9,45
GNAI3	G protein, alpha inhibiting activity polypeptide 3	980	N/A	N/A
WASL	Wiskott-Aldrich syndrome-like	38866	6,61	6,93
AKT3	V-akt murine thymoma viral oncogene homolog 3	N/A	-	-
CIB1	Calcium and integrin binding 1 (calmyrin)	13845	4,38	0,21
IKBKAP	Inhibitor of kappa light polypeptide gene enhancer in B-cells	N/A	-	-
AKAP9	A kinase (PRKA) anchor protein (yotiao) 9	37945	1,07	0,43
VAMP7	Vesicle-associated membrane protein 7	43861	0,63	N/A
DAB1	Disabled homolog 1 (Drosophila)	3392	0	9,24
PIP5K1C	Phosphatidylinositol-4-phosphate 5-kinase, type I,	22745	N/A	0,21
PTPRB	Protein tyroine phosphatase, receptor type, B	N/A	-	-
DCLK1	Doublecortin-like kinase	11447	3,94	7,14
NDE1	68uin nuclear distribution E homolog 1 (A. Nidulans)	14815	0,63	N/A
PCDH10	Protocadherin 10	31756	N/A	0
MAPK8IP3	Mitogen-activated protein kinase 8 interacting protein 3	14609	3,94	7,14
GLS2	Glutaminase 2 (liver, mitochondrial)	10070	3,94	6,93
AGTR1	Angiotensin II receptor, type 1	29860	1,70	6,93
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	42533	0,63	N/A
WT1	Wilms tumor 1	8172	0,63	9,45
HSP90AA1	Heat shock protein 90kda alpha (cytosolic), class A member 1	12410	3,31	N/A
NQO1	NAD(P)H dehydrogenase, 68uinine 1	15877	3,94	7,36
C3	Complement component 3	22660	1,70	7,14
CNR1	Cannabinoid receptor 1 (brain)	36466	3,31	N/A
NBN	Nibrin	641	6,61	9,24
EZH2	Enhancer of zeste homolog 2 (Drosophila)	38725	3,94	N/A
HSPA4	Heat shock 70kda protein 4	33414	3,94	0,21
STK11	Serine/threonine kinase 11	20044	5,01	7,14
SKP2	S-phase kinase-associated protein 2, E3 ubiquitin protein ligase	32992	1,70	N/A
FOXO1	Forkhead box O1	11414	3,31	9,67
ADORA2A	Adenosine A2a receptor	28121	3,94	0,21
PTPN1	Protein tyrosine phosphatase, non-receptor type 1	26382	0	0,21
A2M	Alpha-2-macroglobulin	10544	0,63	6,93
NES	Nestin	2813	0,63	7,14
CTSL1	Cathepsin L1	41603	0	9,45
ADRBK1	Adrenergic, beta, receptor kinase 1	6794	3,94	0,21
YWHAB	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation β.	26307	1,07	7,36
HNRNPK	Heterogeneous nuclear ribonucleoprotein K	42681	0,63	7,36
TRPC1	Transient receptor potential cation channel, subfamily C, member 1	29844	3,94	7,14
IL3	Interleukin 3 (colony-stimulating factor, multiple)	33384	3,31	7,14

Table A.1. Neuronal Migration Genes and PEA3/ETV4 Binding Scores (continue)

Gene abb.	Gene Name	Accesion #	mPEA3	hPEA3
GNAI1	G protein, alpha inhibiting activity polypeptide 1	37889	3,31	N/A
ACTN1	Actinin, alpha 1	12678	0,63	9,45
GNAI2	G protein, alpha inhibiting activity polypeptide 2	29399	1,70	9,67
DCC	Deleted in colorectal carcinoma	19497	1,70	0,21
DNM1	Dynamin 1	N/A		
FES	Feline sarcoma oncogene	13679	3,31	9,45
TPM3	Tropomyosin 3	20450	1,70	9,45
TRPM8	Transient receptor potential cation channel, subfamily M, member 8	24334	3,31	0,21
PRDM2	PR domain containing 2, with ZNF domain	209	7,68	0,21
GRM3	Glutamate receptor, metabotropic 3	37909	0	0,21
NTF3	Neurotrophin 3	8613	0	7,14
TUBA1A	Tubulin, alpha 1a	10299	0,63	7,14
CHRNA1	Cholinergic receptor, nicotinic, alpha 1 (muscle)	24932	0,63	N/A
IGF2BP1	Insulin-like growth factor 2 Mrna binding pr.1	17434	0	9,24
AKAP12	A kinase (PRKA) anchor protein 12	35902	0	7,14
ACTN2	Actinin, alpha 2	2058	0	9,45
MST1	Macrophage stimulating 1 (hepatocyte growth factor-like)	30835	5,01	7,14
BAIAP2	BAI1-associated protein 2	17796	3,31	N/A
PDCD10	Programmed cell death 10	30311	3,94	9,67
PLCB2	Phospholipase C, beta 2	116218	0	N/A
PQBP1	Polyglutamine binding protein 1	43299	3,31	7,14
RACGAP1	Rac gtpase activating protein 1	10271	3,94	9,45
DIAPH1	Diaphanous homolog 1 (Drosophila)	34180	1,70	0,21
RB1CC1	RB1-inducible coiled-coil 1	40992	3,31	N/A
P2RY6	Pyrimidinergic receptor P2Y, G-protein 6	6890	0,63	7,14
CHN1	Chimerin (chimaerin) 1	24931	3,94	9,24
ZIC2	Zic family member 2	10974	0	9,45
POMT1	Protein-O-mannosyltransferase 1	41993	N/A	N/A
GNG2	Guanine nucleotide binding protein (G protein), gamma 2	12796	0	0
ARAF	V-raf murine sarcoma 3611 viral oncogene homolog	43257	0,63	9,67
MAPK13	Mitogen-activated protein kinase 13	35397	0	7,14
POMGNT1	Protein O-linked mannose beta1,2-N-acetylglucosaminyltransferase	3494	N/A	9,45

Table A.2**.** Axonal Guidance Genes and PEA3/ETV4 Binding Scores

Gene abb.	Gene Name	Accesion #	mPEA 3	hPEA3
RGMB	RGM Domain Family Member B	33277	3,31	N/A
RGMA	RGM Domain Family Member A	13823	3,94	0
CNTN2	Contactin 2	1782	3,94	9,24
CNTN4	Contactin 4	125841	0	N/A
SLIT2	Slit Homolog 2	116382	3,94	0,43
Sema3a	Semaphorin 3a	117334	1,70	0,21
EphB3	Ephrin Receptor B3	30011	3,94	0
Nrp1	Neuropilin 1	5859	3,31	9,24
EphB2	Ephrin Receptor B2	323	N/A	9,67
Sema3F	Semaphorin 3F	115928	0	0,43
Sema5A	Semaphorin 5A	34733	1,70	0,21
CDK5R1	Cyclin Dependent Kinase 5 regulatory subunit 1	115721	N/A	9,45
NRCAM	Neuronal cell adhesion molecule	38906	8,32	9,67
NRXN1	Neurexin 1	116392	3,31	N/A
GMIP	GEM Interacting protein	120115	3,94	N/A
FEZF2	FEZ Family Zinc Finger 2	N/A		
DPYSL2	dihydropyrimidinase-like 2	39825	0,63	7,14
Sema3B	Semaphorin 3B	29403	0	9,24
UNC5B	UNC5-homolog b	4599	0	N/A
PTK2	Protein Tyrosine Kinase 2	116986	0,63	0
L1CAM	L1 Cell adhesion molecule	113184	0,63	0
Sema4D	Semaphorin 4D	42660	0,63	0,21
Fez1	Zygin1	116617	N/A	9,45
EphA8	Ephrin Receptor A8	318	3,94	N/A
BDNF	Brain Derived Neurotrophic factor	8188	0,63	N/A
PRNP	Prion Protein	112726	3,94	N/A
NCAM1	Neural Cell Adhesion Molecule 1	7078	0	0,43
LIMK1	Lim kinase 1	37847	3,94	0
SCNA8	Sodium channel, voltage gated, type VIII, A	8931	1,07	0,21
SEMA4A	Semaphorin 4A	1354	3,94	9,67
MMP8	Matrix metallopeptidase 8	7558	0,63	0,21
LRRC4C	Leucine rich repeat containing 4C	38852	0	9,24
GNAI2	G protein alpha inhibiting activity polypeptide 2	29399	1,70	9,67
RTN4R	Reticulon 4 receptor	28994	1,70	0,21
FEZ2	Fasciculation and elongation protein zeta 2	25753	1,70	9,24
NEUROG2	Neurogenin 2	32273	0,63	0,21
AGRN	Agrin	N/A		
MYH10	Myosin, heavy chain 10, non-muscle	19064	6,61	N/A
FKBP4	FK506 binding protein 4	8576	3,31	9,45
KCNQ3	Potassium voltage gated channel	40652	3,94	0,21
NTF3	Neurotrophin 3	8613	0	7,14

Table A.2. Axonal Guidance Genes and PEA3/ETV4 Binding Scores (continue)

Gene abb.	Gene Name	Accesion #	mPEA 3	hPEA3
KAL1	Kallmann syndrome 1 sequence	44617	0,63	9,45
NGFR	Nerve growth factor receptor	17440	1,70	9,24
MAPK8IP3	Mitogen-activated protein kinase 8 interacting pro.3	14609	3,94	7,14
NFASC	Neurofascin	1781	0,63	0,21

APPENDIX B: THE INTERFACES OF *motifTFinder*

```
This is a tool where you can search for your TF(transcription factor) on
the gene of interest.
It gives the binding affinities of the TFs.
Please select among these species:  mouse / human / rat
input ->  human

Please enter the gene_names for the sequence search:(ex: cdk4,cdc25a)
input -> AMFR

The search result is in the file 'FastaSeq_Result.txt'

Please type the TRANSFAC access code of the TFs you want to search:
input -> T00684

if you want to search for more TF please select 1,if not please select 0:
input -> 1

for more TF to be searched, please type the TRANSFAC accession number:
input -> T00008

if you want to search for more TF, please select 1, if not please select 0:
input -> 0

Final TF-promoter result is in the file: 'TF_result.txt'
```

Figure B1. The first version of the tool and its entry site

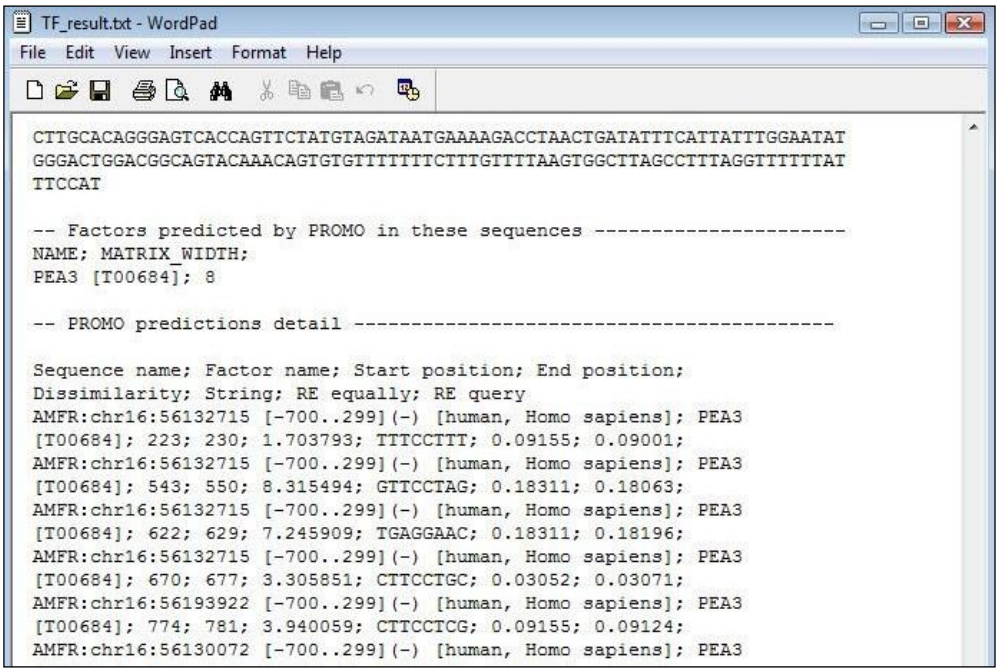

Figure B2. The result page with PEA3/ETV4 binding scores

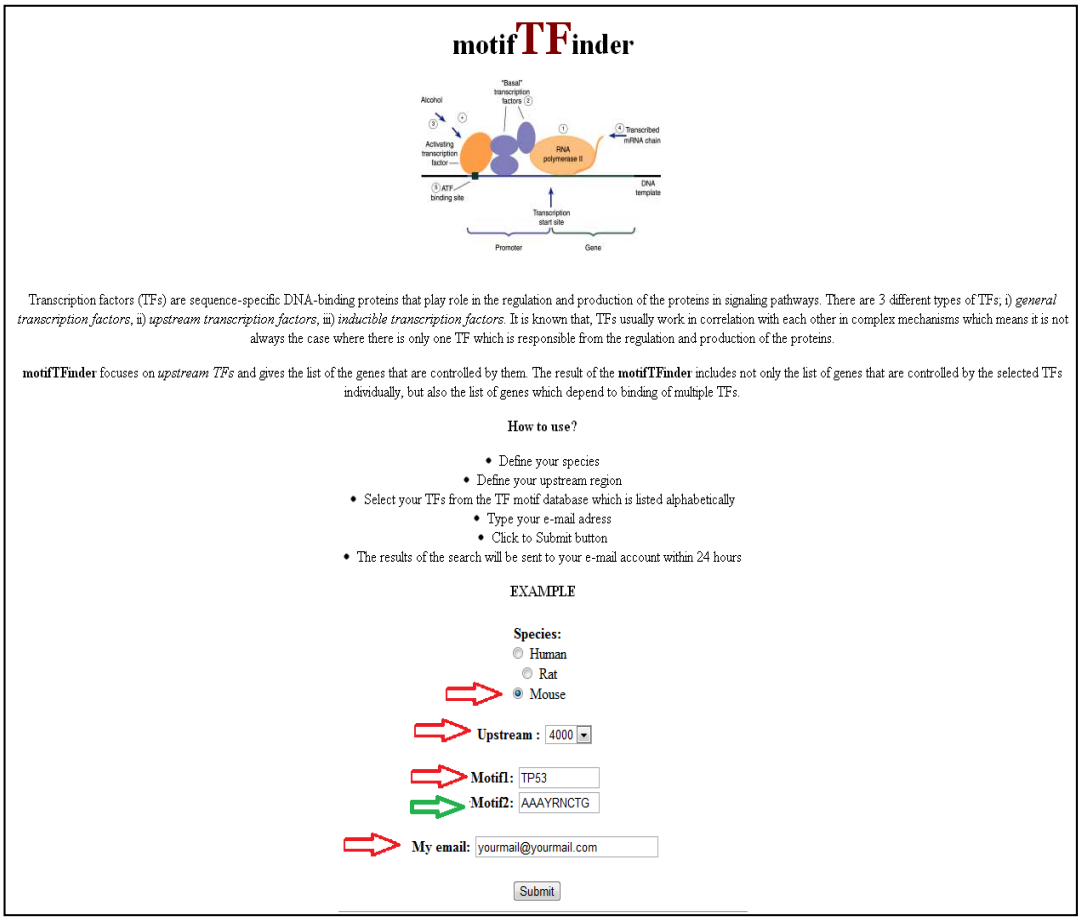

Figure B3. The main page of motifTFinder with search parameters