

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

**MOLECULAR AND COMPUTATIONAL INVESTIGATION OF  
INTRAFAMILIAL TRANSCRIPTIONAL REGULATORY  
NETWORK OF ETS FAMILY TRANSCRIPTION FACTORS**

**YIGIT KORAY BABAL**  
**A THESIS SUBMITTED FOR THE DEGREE OF**  
**MASTER OF SCIENCE**  
**DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS**

**GEBZE**  
**2018**

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

**MOLECULAR AND COMPUTATIONAL  
INVESTIGATION OF INTRAFAMILIAL  
TRANSCRIPTIONAL REGULATORY  
NETWORK OF ETS FAMILY  
TRANSCRIPTION FACTORS**

**YIGIT KORAY BABAL**  
**A THESIS SUBMITTED FOR THE DEGREE OF**  
**MASTER OF SCIENCE**  
**DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS**

THESIS SUPERVISOR  
PROF. DR. ISIL KURNAZ

**GEBZE**  
**2018**

**T.C.**  
**GEBZE TEKNİK ÜNİVERSİTESİ**  
**FEN BİLİMLERİ ENSTİTÜSÜ**

**ETS AİLESİ TRANSKRİPSİYON**  
**FAKTÖRLERİNİN AİLE-İÇİ**  
**TRANSKRİPSİYONEL REGÜLASYON**  
**AĞININ MOLEKÜLER VE HESAPLAMALI**  
**İNCELENMESİ**

**YİĞİT KORAY BABAL**  
**YÜKSEK LİSANS TEZİ**  
**MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI**

**DANIŞMANI**  
**PROF. DR. IŞIL KURNAZ**

**GEBZE**  
**2018**

GTÜ Fen Bilimleri Enstitüsü Yönetim Kurulu'nun 27/06/2018 tarih ve 2018/33 sayılı kararıyla oluşturulan jüri tarafından 12/07/2018 tarihinde tez savunma sınavı yapılan Yiğit Koray Babal'ın tez çalışması Moleküler Biyoloji ve Genetik Anabilim Dalında YÜKSEK LİSANS tezi olarak kabul edilmiştir.

**JÜRİ**

ÜYE

(TEZ DANIŞMANI) : Prof. Dr. İslim Kurnaz



ÜYE

: Doç. Dr. Kozim Yolgın ALGA



ÜYE

: Doç. Dr. Tunahan GAKIR



**ONAY**

Gebze Teknik Üniversitesi Fen Bilimleri Enstitüsü Yönetim Kurulu'nun  
...../...../..... tarih ve ...../..... sayılı kararı.

## SUMMARY

E-twenty-six or E26 Transformation Specific (ETS) Family is one of the largest transcription factor families involved in many diverse tissues and cell types. The family is unique in metazoan lineage and are characterized by an evolutionary conserved DNA binding domain, called ETS domain. ETS transcription factor family has 12 sub-families and 30 members.

Main function of the ETS domain family is transcriptional activation and inhibition. They are not tissue specific, so family members can be seen in many tissues/cell types, regulating diverse molecular and cellular functions including cell proliferation, development or differentiation.

This thesis focused on intra-familial regulation network of ETS family members, based on previous microarray-based studies (Kandemir et al, 2017 and Kandemir et al, unpublished results). In these analyses, it was observed in SH-SY5Y cells overexpressing either Elk-1-VP16 or Pea3-VP16 that expression of various ETS family members were either up- or down-regulated. It was further shown in previous studies in our laboratory that Pea3-VP16 indeed regulated *elk-1* promoter (Kandemir et al, unpublished results). To investigate whether such an auto-regulatory network of ETS family members exist, we have established a molecular and computational methodology, focusing on Pea3 and Elk1, based on the microarray analyses described above. Our main research question was to understand how different ETS members regulate the transcriptional level of other family members, and how this regulation affects their different biological functions. We have successfully recapitulated the computational model simulating results present in literature and have implemented it to provide predictions about the system's behavior.

**Key Words: ETS Family, Transcription Factor, Elk1, Pea3, Kinetic Modeling.**

## ÖZET

E yirmi altı veya E26 Transformasyon Spesifik (ETS) Ailesi çok çeşitli doku ve hücrelerle ilişki en geniş transkripsiyon faktörü ailelerinden birisidir. Aile metazoan hattına özgüdür ve yüksek evrimsel korunmuşluğu olan ETS bölgesi denen bir DNA bağlanma bölgesi ile karakterize edilir. ETS transkripsiyon faktörü ailesinin 12 alt ailesi ve yaklaşık 30 üyesi vardır.

ETS bölgesi ailesinin temel fonksiyonu transkripsiyonel aktivasyon ve inhibisyonudur. Aile üyeleri doku spesifik değildir, bu yüzden bu ailenin üyeleri birçok doku ve hücre tipinde hücre çoğalması, gelişim, farklılaşma veya kanser gelişimi gibi çok çeşitli moleküler ve hücresel fonksiyoları düzenler.

Bu tezde önceki mikro dizin çalışmaları baz alınarak (Kandemir ve ark, 2017 ve Kandemir ve ark, yayınlamış sonuçlar) ETS ailesi üyelerinin aile içi regülasyon ağına odaklanılmıştır. Bu mikro dizin analizlerinde, Elk1-VP16 veya Pea3-VP16 aşırı anlatımı yapılan SH-SY5Y hücrelerinde, çeşitli ETS üyelerinin anlatımında artış veya azalış gözlenmiştir. Laboratuvarımızda yapılan daha önceki çalışmalarda da gösterilmiştir ki, Pea3-VP16, *elk-1* promotorunu regüle etmektedir (Kandemir ve ark, yayınlanmamış sonuçlar). ETS ailesi üyelerinin bu oto-regülasyonunu incelemek için, yukarıda açıklanan mikrodizin analizini baz alan, Pea3 ve Elk1 üzerine odaklandığımız moleküler ve hesaplamalı bir metodoloji kurduk. Bizim temel araştırma sorumuz, ETS üyeleri diğer aile üyelerini transkripsiyonel seviyede nasıl regüle etmektedir ve bu regülasyon farklı biyolojik fonksiyonları nasıl etkilemektediri anlamak içindir. Hesaplamalı model simülasyonlarımız literatürü başarıyla yinelemektedir ve sistemin davranışını tahmin etmek için uygulanmıştır.

**Anahtar Kelimeler: ETS Ailesi, Transkripsiyon Faktörleri, Elk1, Pea3, Kinetik Modelleme.**

## ACKNOWLEDGEMENTS

Firstly, I would like to thank with a great sincerity to Prof. Dr. Isil AKSAN KURNAZ for her supervision, guidance and advice during the period of my research. I feel so lucky to be her student.

I would like to thank all the AxanLab members, my dear lab mates: Basak KANDEMİR, Ekin SONMEZ, Melis SAVASAN SOGUT, Oya ARI UYAR, Merve USTUN, Dogukan METINER, Inanc PASTIRMACI, BÜSRA NUR CICEK, Betül ERTURAL, Esranur YIGIT, Gülbahar CAGIL and Bayram KAS for all their supports, friendship and all lovely memories.

I would like to thank all the OBEDIA lab members and alumni Mehmet Soner TURKUNER, Ozge ONLUTURK, Ilkay Goksu POLAT, Nazlı ERCIYAS ERTURK, Ayşen YAZICI, Gülcan KARTAL for their friendship, moral supports and beautiful memories.

I also thank you Assistant Prof. Dr. Pınar PIR for her advice and positive supports during the period of my research. Additionally, I would like to thank Hamza KARAKURT, Furkan KURTOGLU, Ali KAYNAR, Garip DEMIR and Onur ÖZDEN for their friendship and moral supports.

The most importantly, I specially thank to my lovely mother Pınar BABAL for always being with me, she is most important thing in my life. I also endless thank to my brother Efe BABAL, my grandmother Melek BABAL, my aunt Sukran KASARCI, my uncle Erdal KASARCI, my cousins Taner KASARCI, Erdem BABAL, Didem BABAL and my role model Haluk KASARCI for all their supports, patience and their endless love.

I also very special thanks to my dear Simge SENGUL for always being with me, her support, patience and her precious love.

# TABLE of CONTENTS

|                                                                    |     |
|--------------------------------------------------------------------|-----|
| SUMMARY                                                            | v   |
| ÖZET                                                               | vi  |
| ACKNOWLEDGEMENTS                                                   | vii |
| LIST of ABBREVIATIONS and ACRONYMS                                 | x   |
| LIST of FIGURES                                                    | xi  |
| LIST of TABLES                                                     | xv  |
| <br>                                                               |     |
| 1. INTRODUCTION                                                    | 1   |
| 1.1. ETS Transcription Factor Family                               | 1   |
| 1.2. Molecular and Biological Roles of ETS Family                  | 4   |
| 1.3. Purpose of Thesis                                             | 6   |
| 2. MATERIALS and METHODS                                           | 9   |
| 2.1. Materials                                                     | 9   |
| 2.1.1. Plasmids                                                    | 9   |
| 2.1.2. Cell Lines & Bacterial Strains                              | 9   |
| 2.1.3. Kits                                                        | 9   |
| 2.1.4. Cell Culture Solutions                                      | 10  |
| 2.1.4. Bacterial Media                                             | 11  |
| 2.1.5. Gel Electrophoresis Solutions                               | 11  |
| 2.1.6. Material, Equipments and Devices                            | 11  |
| 2.2. Methods                                                       | 12  |
| 2.2.1. LB Broth and LB Agar preparation                            | 12  |
| 2.2.2. Competent cell preparation                                  | 13  |
| 2.2.3. Transformation of Plasmids                                  | 13  |
| 2.2.4. Plasmid isolation                                           | 14  |
| 2.2.5. Cell Culture                                                | 15  |
| 2.2.6. Transient transfection of Pea3 and Elk1 expression plasmids | 16  |
| 2.2.7. RNA isolation                                               | 16  |
| 2.2.8. Checking RNA integrity                                      | 17  |
| 2.2.9. cDNA synthesis                                              | 17  |

|                                                                                   |    |
|-----------------------------------------------------------------------------------|----|
| 2.2.10. Primer Design                                                             | 18 |
| 2.2.11. Quantitative Real Time PCR                                                | 20 |
| 2.2.12. Molecular Cloning for Promoter of Selected ETS Targets                    | 21 |
| 2.2.13. Promoter Analysis                                                         | 23 |
| 2.2.14. Luciferase Assay                                                          | 23 |
| 2.2.15. Phylogenetic Tree Construction                                            | 24 |
| 2.2.16. Kinetic Model Construction                                                | 25 |
| 3. RESULTS                                                                        | 32 |
| 3.1. Promoter Analysis for Elk-1 and Pea3 on the Promoter of Selected ETS members | 32 |
| 3.2. qPCR Results                                                                 | 34 |
| 3.3. Luciferase Reporter Assays                                                   | 36 |
| 3.4. Kinetic Model Results                                                        | 36 |
| 4. DISCUSSION and CONCLUSION                                                      | 55 |
| REFERENCES                                                                        | 60 |
| BIOGRAPHY                                                                         | 65 |
| APPENDICES                                                                        | 66 |

## LIST of ABBREVIATIONS and ACRONYMS

| <b><u>Abbreviations<br/>and Acronyms</u></b> | <b><u>Explanations</u></b>                      |
|----------------------------------------------|-------------------------------------------------|
| ETS                                          | : E26 Transformation Specific                   |
| PNT                                          | : Pointed                                       |
| FGF                                          | : Fibroblast Growth Factor                      |
| MAPK                                         | : Mitogen Activated Protein Kinase              |
| SRF                                          | : Serum Response Factor                         |
| PEA3                                         | : Polyoma Enhancer Activator 3                  |
| SRE                                          | : Serum Response Element                        |
| SH-SY5Y                                      | : Human Neuroblastoma Cell Line                 |
| DH5 $\alpha$                                 | : <i>Escherichia coli</i> bacterial strains     |
| FBS                                          | : Fetal Bovine Serum                            |
| DMEM                                         | : Dulbecco's Modified Eagle Medium              |
| DMSO                                         | : Dimethyl Sulfoxide                            |
| PEI                                          | : Polyethylenimine                              |
| MOPS                                         | : 3-( <i>N</i> -morpholino)propanesulfonic acid |

## LIST of FIGURES

| <b><u>Figure No:</u></b>                                                                                                                                              | <b><u>Page</u></b> |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------|
| 1.1: Phylogenetic representation of metazoan lineage.                                                                                                                 | 1                  |
| 1.2: ETS domain structure of Ets1 (PDB: 1K79) and PU.1 (PDB: 1PUE).                                                                                                   | 2                  |
| 1.3: Phylogenetic tree of ETS family.                                                                                                                                 | 3                  |
| 1.4: ETS family domain structure.                                                                                                                                     | 4                  |
| 1.5: Molecular and Biological Function of ETS Family.                                                                                                                 | 5                  |
| 1.6: Part of ETS intra-familial interaction network from literature, our published and unpublished data.                                                              | 8                  |
| 2.1: ETS model topology.                                                                                                                                              | 25                 |
| 3.1: Relative expression of Elk-1 in SH-SY5Y cells.                                                                                                                   | 34                 |
| 3.2: Relative expression of Pea3 in SH-SY5Y cells.                                                                                                                    | 35                 |
| 3.3: Relative expression of selected target of ETS members in SH-SY5Y cells.                                                                                          | 35                 |
| 3.4: Elk1-Luc Reporter activity.                                                                                                                                      | 36                 |
| 3.5: mRNA level of basic model simulation with mouse fibroblast parameter values.                                                                                     | 37                 |
| 3.6: mRNA level of basic model simulation with sea urchin parameter values.                                                                                           | 38                 |
| 3.7: Protein level of basic model simulation with mouse fibroblast parameter values.                                                                                  | 38                 |
| 3.8: Protein level of basic model simulation with sea urchin parameter values.                                                                                        | 39                 |
| 3.9: Parameter scan result of basic model showing the effect of different activator level concentration on target mRNA levels with mouse fibroblast parameter values. | 40                 |

|       |                                                                                                                                                                     |    |
|-------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| 3.10: | Parameter scan result of basic model showing the effect of different activator level concentration on target mRNA levels with sea urchin parameter values.          | 40 |
| 3.11: | Parameter scan result of basic model showing the effect of different activator level concentration on target protein levels with mouse fibroblast parameter values. | 41 |
| 3.12: | Parameter scan result of basic model showing the effect of different activator level concentration on target protein levels with sea urchin parameter values.       | 41 |
| 3.13: | Parameter scan result of basic model showing the effect of different inhibitor level concentration on target mRNA levels with mouse fibroblast parameter values.    | 42 |
| 3.14: | Parameter scan result of basic model showing the effect of different inhibitor level concentration on target mRNA levels with sea urchin parameter values.          | 42 |
| 3.15: | Parameter scan result of basic model showing the effect of different inhibitor level concentration on target protein levels with mouse fibroblast parameter values. | 43 |
| 3.16: | Parameter scan result of basic model showing the effect of different inhibitor level concentration on target protein levels with sea urchin parameter values.       | 43 |
| 3.17: | Parameter scan result of basic model showing the effect of different $K_{ba}$ value of activator on target mRNA levels with mouse fibroblast parameter values.      | 44 |
| 3.18: | Parameter scan result of basic model showing the effect of different $K_{ba}$ value of activator on target mRNA levels with sea urchin parameter values.            | 44 |
| 3.19: | Parameter scan result of basic model showing the effect of different $K_{ba}$ value of activator on target protein levels with mouse fibroblast parameter values.   | 45 |
| 3.20: | Parameter scan result of basic showing the effect of different $K_{ba}$ value of activator on target protein levels with sea urchin parameter values.               | 45 |

|       |                                                                                                                                                                   |    |
|-------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| 3.21: | Parameter scan result of basic model showing the effect of different $K_{ba}$ value of inhibitor on target mRNA levels with mouse fibroblast parameter values.    | 46 |
| 3.22: | Parameter scan result of basic mode showing the effect of different $K_{ba}$ value of inhibitor on target mRNA levels with sea urchin parameter values.           | 46 |
| 3.23: | Parameter scan result of basic model showing the effect of different $K_{ba}$ value of inhibitor on target protein levels with mouse fibroblast parameter values. | 47 |
| 3.24: | Parameter scan result of basic model showing the effect of different $K_{ba}$ value of inhibitor on target protein levels with sea urchin parameter values.       | 47 |
| 3.25: | mRNA level of ETS members within model with mouse fibroblast parameter values.                                                                                    | 49 |
| 3.26: | Protein level of ETS members within model with mouse fibroblast parameter values.                                                                                 | 49 |
| 3.27: | mRNA level of ETS members within model with sea urchin parameter values.                                                                                          | 50 |
| 3.28: | Protein level of ETS members within model with sea urchin parameter values.                                                                                       | 50 |
| 3.29: | Parameter scan result showing the effect of ELK1 over-expression at mRNA level of ETS members in the model with mouse fibroblast parameter values.                | 51 |
| 3.30: | Parameter scan result showing the effect of ELK1 over-expression at the mRNA level of ETS members in the model with sea urchin parameter values.                  | 51 |
| 3.31: | Parameter scan result showing the effect of ELK1 over-expression at protein level of ETS members in the model with mouse fibroblast parameter values.             | 52 |
| 3.32: | Parameter scan result showing the effect of ELK1 over-expression at protein level of ETS members in the model with sea urchin parameter values.                   | 52 |

|       |                                                                                                                                                       |    |
|-------|-------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| 3.33: | Parameter scan result showing the effect of PEA3 over-expression at mRNA level of ETS members in the model with mouse fibroblast parameter values.    | 53 |
| 3.34: | Parameter scan result showing the effect of PEA3 over-expression at mRNA level of ETS members in the model with sea urchin parameter values.          | 53 |
| 3.35: | Parameter scan result showing the effect of PEA3 over-expression at protein level of ETS members in the model with mouse fibroblast parameter values. | 54 |
| 3.36: | Parameter scan result showing the effect of PEA3 over-expression at protein level of ETS members in the model with sea urchin parameter values.       | 54 |

## LIST of TABLES

| <b><u>Table No:</u></b>                                                                                                     | <b><u>Page</u></b> |
|-----------------------------------------------------------------------------------------------------------------------------|--------------------|
| 1.1: Up and Down-regulated genes in Pea3 transfected SH-SY5Y cells.                                                         | 7                  |
| 1.2: Up and Down-regulated genes in Elk1 transfected SH-SY5Y cells.                                                         | 7                  |
| 2.1: List of kits.                                                                                                          | 9                  |
| 2.2: List of cell culture solutions.                                                                                        | 10                 |
| 2.3: List of gel electrophoresis solutions.                                                                                 | 11                 |
| 2.4: cDNA synthesis reaction setup.                                                                                         | 17                 |
| 2.5: qPCR primers of selected ETS targets.                                                                                  | 18                 |
| 2.6: Cloning primers of selected ETS targets.                                                                               | 20                 |
| 2.7: qPCR reaction setup.                                                                                                   | 21                 |
| 2.8: Pfu PCR reaction setup for insert amplification.                                                                       | 21                 |
| 2.9: PCR cycling conditions and temperatures to amplify inserts.                                                            | 21                 |
| 2.10: Double digestion reaction setup with XhoI and MluI restriction enzymes.                                               | 22                 |
| 2.11: Reaction setup of ligation procedure with T4 DNA Ligase.                                                              | 22                 |
| 2.12: Luciferase reporter assay transfection setup 1.                                                                       | 23                 |
| 2.13: Luciferase reporter assay transfection setup 2.                                                                       | 24                 |
| 2.14: Biochemical structure of ETS model with references.                                                                   | 26                 |
| 2.15: Different transcriptional regulation under the transcription factor as activator or inhibitor on the target promoter. | 28                 |
| 2.16: Parameter values of mouse fibroblast set of ETS model.                                                                | 29                 |
| 2.17: Parameter values of sea urchin set of ETS model.                                                                      | 30                 |

|      |                                                                                      |    |
|------|--------------------------------------------------------------------------------------|----|
| 3.1: | Promoter analysis of Elk-1 on the promoter of selected targets of ETS members.       | 32 |
| 3.2: | Promoter analysis of Pea-3 on the promoter of selected targets of ETS member.        | 33 |
| 4.1: | Comparison of similarity score of promoter analysis for Pea3 and microarray results. | 56 |
| 4.2: | Comparison of similarity score of promoter analysis for Elk1 and microarray results. | 56 |

# 1. INTRODUCTION

## 1.1. ETS Transcription Factor Family

E-twenty-six (E26 Transformation Specific, ETS) transcription factor superfamily is a one of the biggest transcription factor families that can be seen only metazoan lineage (Figure 1.1 modified from [1]). The characteristic feature of the ETS family is highly evolutionary conserved ~80 amino acid residue DNA-binding domain called ETS-domain which binds 5'-GGAA/T-3' sequence motif to regulate transcriptional processes [2]. The name of this family was firstly derived from avian erythroblastosis virus, E26, the v-ets oncogene [3]. ETS transcription factor family have 12 subfamilies and ~30 members: 28 members in human, 26 in mice, 10 in *Caenorhabditis elegans*, 9 in *Drosophila*. The family subclassified based on their sequence similarities on the ETS-domain and other domain [4]. ETS family plays a critical role in many biological processes such as differentiation, cell proliferation, apoptosis, cancer progression etc. through transcriptional activation and inhibition [5].

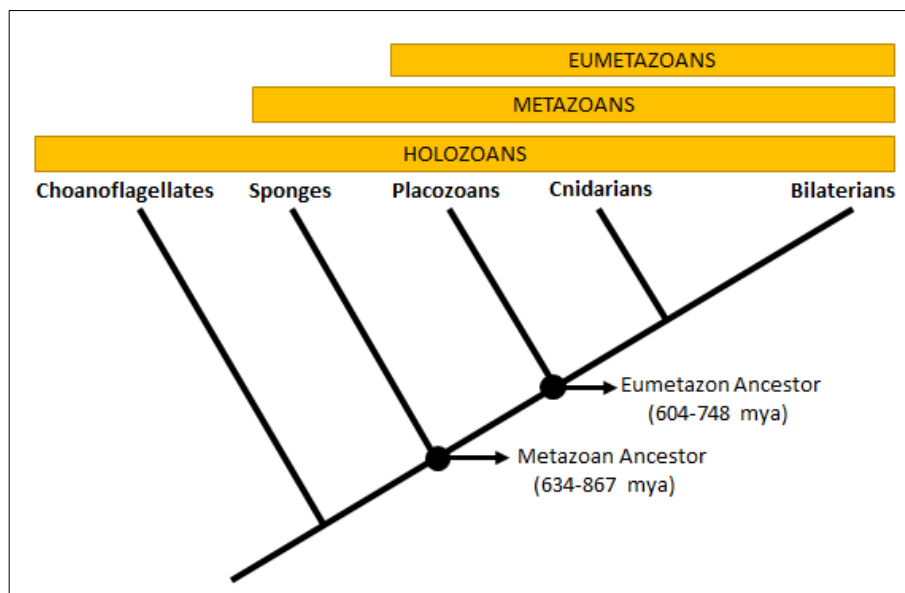


Figure 1.1: Phylogenetic representation of metazoan lineage.

Main characteristic feature of the ETS family is the conserved DNA-binding domain called ETS domain which contains 85 amino acid residues and bind to the consensus 5'-GGAAT/T-3' DNA recognition sequence motif [3]. Structural analysis reveals that, ETS domain have three alpha-helices ( $\alpha 1$ - $\alpha 3$ ) and four beta-sheets ( $\beta 1$ - $\beta 4$ ) indicate the Helix-turn-Helix topology. The third  $\alpha$ -helix, as well as  $\beta$ -sheets 3 and 4 are responsible from the interaction with major groove of DNA (Figure 1.2) [6]. Generally, ETS proteins bind DNA as monomers. ETS activation occurs in three steps: DNA binding, protein-protein interaction and kinase-related phosphorylation [5].

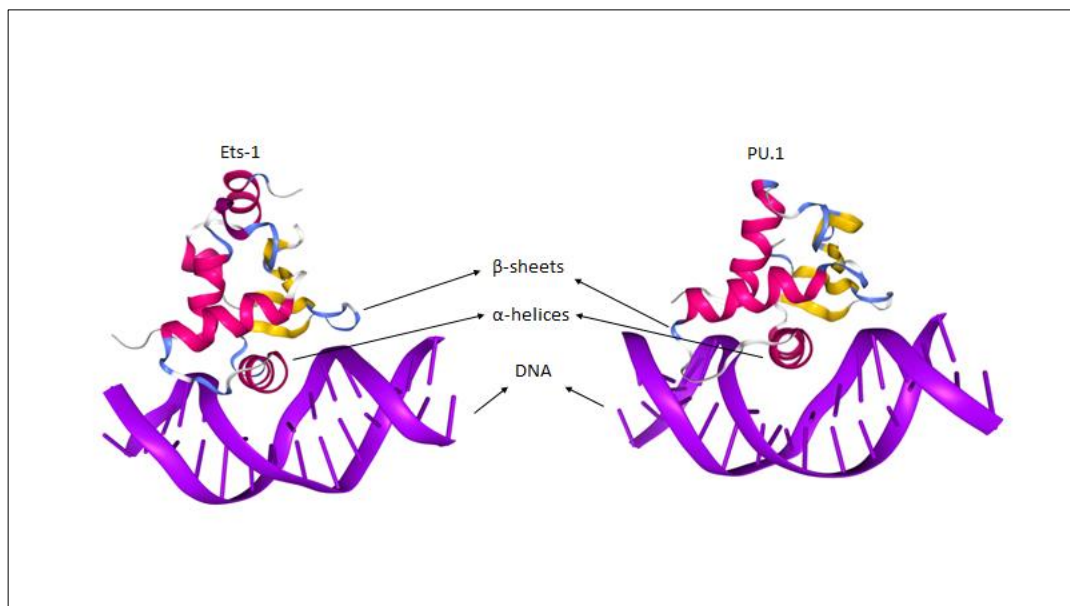


Figure 1.2: ETS domain structure of Ets1 (PDB: 1K79) and PU.1 (PDB: 1PUE).

Another major conserved domain is POINTED-domain (PNT) that firstly identified in *Drosophila melanogaster*. PNT-domain have about 80 amino acid residues that indicate helix-loop-helix structure and generally located in N terminus [6]. PNT-domain plays role in homo-oligomerization, heterodimerization and transcriptional repression [2], [7], [8].

ETS family members have four different DNA-binding specificities clusters (I-IV) that have been revealed through chromatin immunoprecipitation study, and these ETS-binding specificities clusters were found to be correlated with phylogenetic sub-classification (Figure1.3) [9].

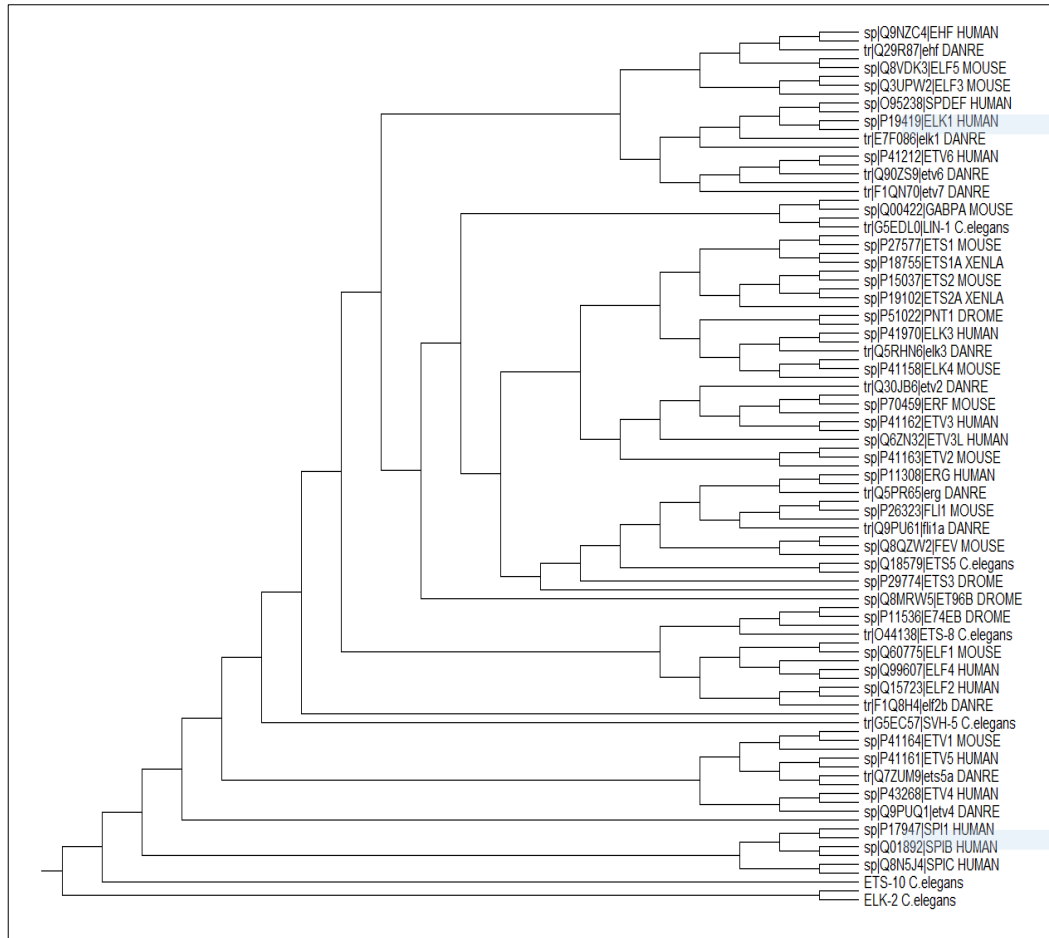


Figure 1.3: Phylogenetic tree of ETS family. sp-codes are Uniprot ID of protein sequences. DANRE indicates zebra fish, XENLA *Xenopus laevis*, DROME *Drosophila melanogaster*. Phylogenetic tree was constructed by using ClustalW (K.Y.B). MEGA X was used for visual arrangement. Blue boxes highlight human ELK-1 and human Pea3/ETV4, focus of this study.

ETS family is a one of the major transcription factor family that has around 30 members. Sub-classification of family is constructed from sequence similarities overall amino acid sequence, ETS domain and another conserved domain, Pointed (PNT). There are 12 subfamilies in this superfamily: ETS, ERG, ELG, ELF, ESE, ERF, TEL, PEA3, SPI, TCF, PDEF, ETV2 (Figure 1.3) [3], [6], [10].

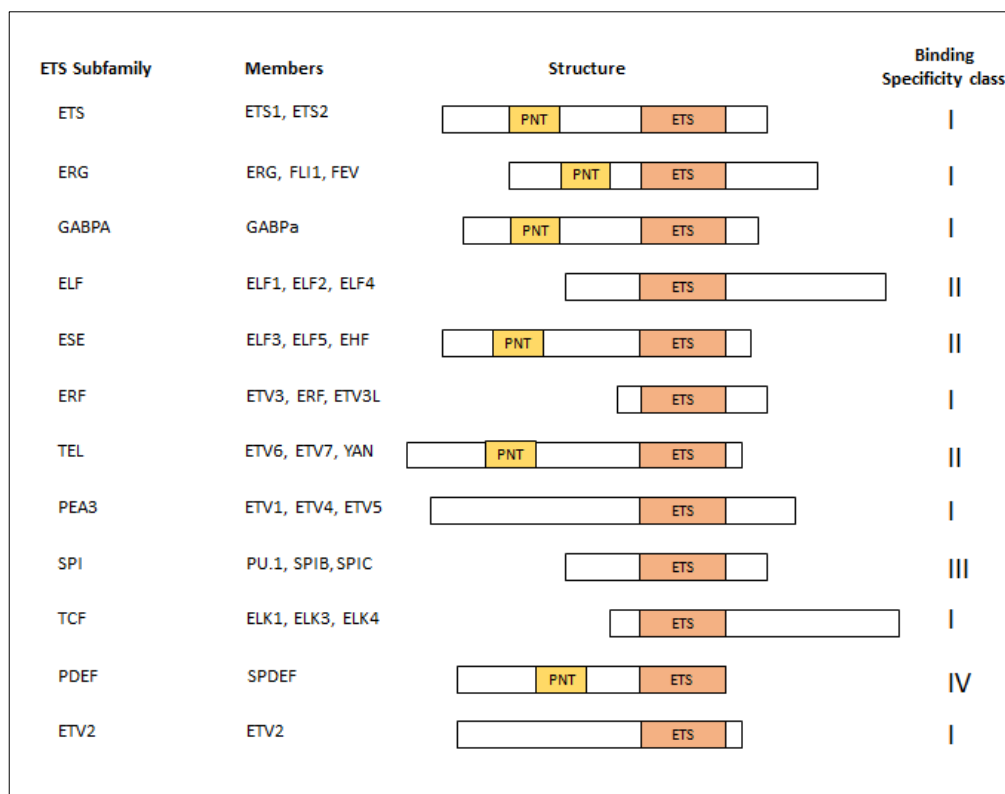


Figure 1.4: ETS family domain structure.

## 1.2. Molecular and Biological Roles of ETS Family

ETS Family transcription factors are not tissue specific proteins, and they play role in many biological processes by participating in different molecular functions. Major molecular functions of family are transcriptional activation and inhibition [11], [12]. In addition, they are downstream targets of different signal transduction cascades such as growth factor-induced MAPK signaling pathway [13], [14]. Protein-protein interaction is also important molecular role of ETS family, hence ETS members have specific protein-protein interaction domains recognizing specific proteins. For instance, Elk-1 interacts with its transcriptional partner, Serum Response Factor (SRF), through its B domain [15].

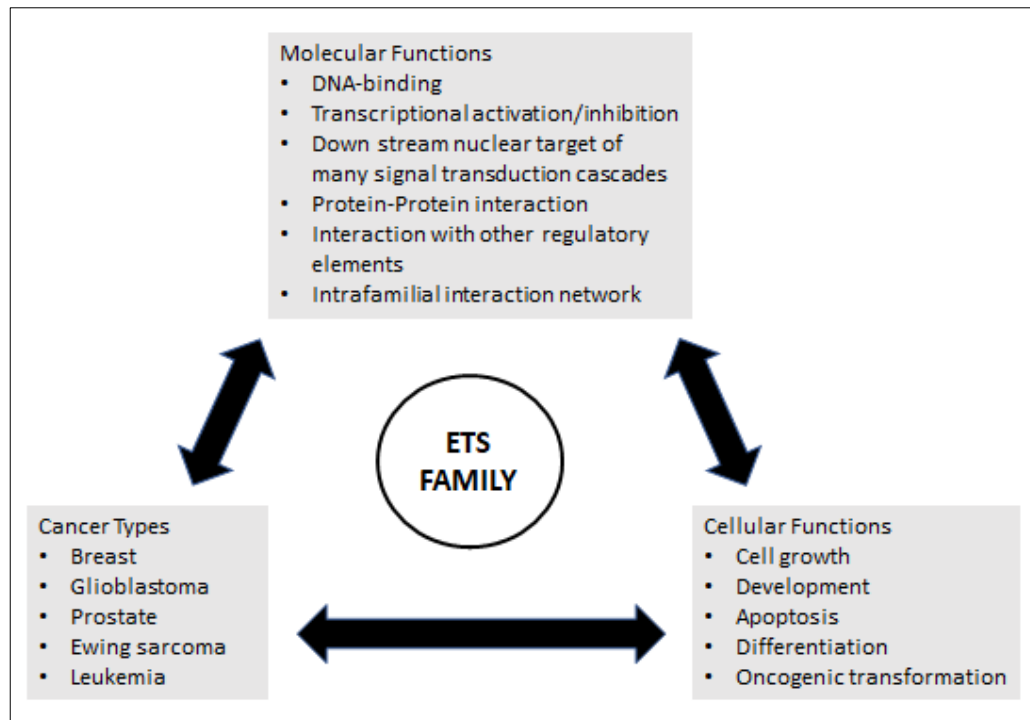


Figure 1.5: Molecular and Biological Function of ETS Family.

Through these various molecular functions, ETS family plays a critical role in many biological processes such as cell differentiation, apoptosis, development and many more by interacting or co-operating with other co-regulatory elements and through the intra-familial network (interaction of ETS members each other) [6]. For instance, Etv4 (Pea3) expression was shown to be important for development of chick embryonic stages, in particular ganglion differentiation by activation of FGF signaling in chicken retina development [16]. In addition, Pea3 induces axonal outgrowth, with significant role of Serine-90 phosphorylation [17]. On the other hand, PEA3 subfamily was also shown to play a role in cancer progression, especially prostate and breast cancer: Etv1 (Er81) and Etv4 (Pea3) regulate prostate cancer progression [18], whereas Pea3/ETV4 was associated with breast cancer progression (reviewed in [19]).

Ternary Complex Factor (TCF) subfamily, of which Elk-1 is the founding member, is characterized by its interaction with SRF on the Serum Response Element (SRE) of immediate-early promoters. Other two members of subfamily are Elk-4 (Sap-1) and Elk-3 (Sap-2), also activated by the MAPK cascade [20]. Human ELK1 was found to downregulate human presenilin-1 gene promoter, while another ETS member Er81 and GABPa was shown to activate the presenilin-1 promoter [21]. Elk-

1 also controls anoikis, a type of apoptosis in anchorage-dependent cells, through regulation of miRNA-200b and affecting Pin1 expression [22].

Elk-1 is also expressed in the nervous system: Ras-Elk-1 signaling regulates CentA1 expression and leads to amyloid beta-induced neuronal dysfunction [23]. In addition, Elk-1 expression mediates neuronal cell survival of neuroblastoma cell line (SH-SY5Y) and pheochromocytoma cells (PC12) by regulation of *egr-1* and *SMN1* promoter respectively [24], [25]. Elk-1 was further shown to regulate other processes in brain such as learning, neuronal plasticity, as well as neuropathological disease progression (reviewed [26]).

Other ETS subfamily members are also seen in regulation of different processes and tissues. For instance, *Erf* and *Etv3l* play key role in the switch between proliferation and differentiation during primary neurogenesis [27]. *Fli-1* is a critical factor for hematopoietic stem cell proliferation, tumor angiogenesis and malignancy, as well as auto-immune disease progression [28].

Chromosomal rearrangement or gene fusion of ETS family members are commonly observed in different cancer types (Figure 1.4) [29], [30]. *Elf-3* is crucial regulatory element of *OCT4* in human embryonic carcinoma cell (NCCIT) differentiation. The study suggests that *OCT4* promoter activity are down regulated by transcription factor of *Elf-3* [31]. *Ets-1*, founder of the ETS family, is expressed highly by activation of Estrogen receptor  $\alpha$  ( $ER\alpha$ ) in neuroblastoma cells (SH-SY5Y) and leads to proliferation of these cells [32].

### **1.3. Purpose of Thesis**

Various ETS members are expressed in many different tissues and regulate different cellular processes through interaction with co-regulatory partners. In previous studies from our laboratory, transcriptomic analyses had shown that in SH-SY5Y human neuroblastoma cells overexpressing *Pea3-VP16* transcription factor, expression of several ETS members were affected, including that of *Elk-1* (Kandemir et al, 2017) [34]. Similarly, in another transcriptomic study using SH-SY5Y human neuroblastoma cells overexpressing *Elk-1-VP16* transcription factor, a different subset of ETS proteins have been up- or down-regulated (Kandemir et al, unpublished data, personal communication) (see in Table 1.1 and 1.3).

Table 1.1: Up and Down-regulated genes in Pea3 transfected SH-SY5Y cells.

| Down regulated |             |             |
|----------------|-------------|-------------|
| Gene           | Fold change | p-value     |
| FEV            | 0.096462    | 0.002515357 |
| ELF2           | 0.406922    | 0.012039108 |
| ERF            | 0.099182    | 0.009349678 |
| ELK1           | 0.340932    | 0.005643042 |
| Up regulated   |             |             |
| Gene           | Fold change | p-value     |
| FLI1           | 1.43        | 0.0146      |

Table 1.2: Up and Down-regulated genes in Elk1 transfected SH-SY5Y cells.

| Down regulated |             |          |
|----------------|-------------|----------|
| Gene           | Fold change | p-value  |
| ETS1           | 0.296353324 | 0.034025 |
| ETS2           | 0.7442764   | 0.154985 |
| FEV            | 0.084799327 | 0.047847 |
| GABPA          | 0.586660936 | 0.309269 |
| ELF1           | 0.499372122 | 0.266235 |
| ELF4           | 0.445593378 | 0.01257  |
| ERF            | 0.202211684 | 0.161429 |
| ETV3L          | 0.733838116 | 0.166175 |
| ETV1           | 0.53745553  | 0.088775 |
| ETV5           | 0.268779377 | 0.165553 |
| ETV6           | 0.482977045 | 0.097468 |
| Up regulated   |             |          |
| Gene           | Fold change | p-value  |
| ERG            | 2.173302452 | 0.017088 |
| ETV3           | 1.09160199  | 0.815843 |
| FLI1           | 1.41858386  | 0.002894 |
| ETV4           | 1.363842155 | 0.636202 |
| SPDEF          | 2.608057261 | 0.007693 |
| GABPA          | 1.344371472 | 0.356193 |
| ETV5           | 2.700583096 | 0.345186 |
| ELK4           | 1.78734808  | 0.014174 |
| ELF2           | 1.373560118 | 0.178179 |
| ETV2           | 2.409405531 | 0.05996  |
| ELF5           | 2.941362008 | 0.03714  |
| ELF3           | 2.37306136  | 0.002324 |
| SPI1           | 1.934392619 | 0.015142 |
| ETV7           | 1.701798057 | 0.060434 |
| EHF            | 2.298199127 | 0.01145  |
| SPIB           | 1.921529357 | 0.091646 |
| SPIC           | 2.501216057 | 0.036708 |

In the light of published reports as well as our own published or unpublished work, we have hypothesized that the ETS protein superfamily not only interact with other proteins but also auto-regulate each other through a transcriptional regulatory network, which we have termed “intra-familial regulatory network”. With data from the literature as well as published and unpublished findings of our laboratory, we have established a schematic for our hypothesized network as figure 1.6. Hence, in this thesis we have tried to address how ETS members regulate the transcription of each other as well as themselves (cross- and auto-regulation) and how that may affect their different biological function. To investigate this question, we used both molecular and computational methodology, and having the previous transcriptomic studies from our laboratory we have initially focused on Elk-1, Pea-3 and their potential ETS member targets.

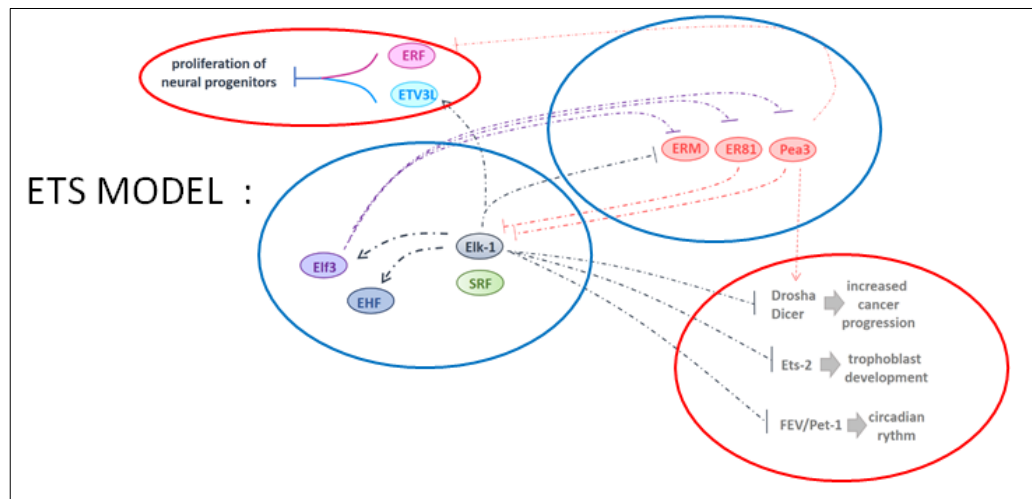


Figure 1.6: Part of ETS intra-familial interaction network from literature, our published and unpublished data. In this figure, arrows indicate activation, straight lines indicate inhibition. Blue circles were implemented in this figure from our data, red circles were implemented from literature data.

## 2. MATERIALS and METHODS

### 2.1. Materials

#### 2.1.1. Plasmids

Expression plasmids of Pea3-VP16 and pCDNA3mPea3 is originally a gift from Prof. Dr. A.D. Sharrocks. Elk1-VP16 and Elk1-pCDNA3 expression plasmids was also used the in vitro experiment. In luciferase reporter assay, pGL3-*elk-1* plasmid vector, *Renilla* plasmid were used. For the normalization with control plasmid, pCDNA3 expression plasmid was used.

#### 2.1.2. Cell Lines & Bacterial Strains

In all cell culture studies, experiments were performed with SH-SY5Y human neuroblastoma cell lines (ATCC number: CRL-2266)

For cloning studies, DH5 $\alpha$  *Escherichia coli* bacterial strains was used for competent cell preparation.

#### 2.1.3. Kits

Table 2.1: List of kits.

| Kit                                             | Company              | Catalog/Lot Number |
|-------------------------------------------------|----------------------|--------------------|
| DNA-midi SV,<br>Plasmid DNA<br>Purification Kit | Intron Biotechnology | 17252              |
| DNA-spin, Plasmid<br>DNA Purification Kit       | Intron Biotechnology | 17097              |
| PureLink RNA Mini<br>Kit                        | Invitrogen           | 12183018A          |

Table 2.1 Continue.

|                                           |         |          |
|-------------------------------------------|---------|----------|
| Dual-Luciferase Reporter Assay System     | Promega | TM040    |
| iScript cDNA Synthesis Kit                | Bio Rad | 1708891  |
| SsoAdvanced Universal SYBR Green Supermix | Bio Rad | 172-5274 |

#### 2.1.4. Cell Culture Solutions

Table 2.2: List of cell culture solutions.

| Solutions                                                                                | Company     | Catalog/Lot Number |
|------------------------------------------------------------------------------------------|-------------|--------------------|
| Dulbecco's Modified Eagle Medium 1X (DMEM) with L-glutamine, pyruvate and 4,5g/L Glucose | Gibco       | 41966-029          |
| Fetal Bovine Serum (FBS)                                                                 | Gibco       | 10270              |
| Penicillin, Streptomycin (10000U-ml Penicillin, 10mg/ml Streptomycin)                    | PAN Biotech | P06-07100          |
| Dulbecco's Phosphate Buffered Saline 1X (DPBS)                                           | Gibco       | 14190-94           |
| 0.05% Trypsin-EDTA Solution 1X                                                           | Gibco       | 25300-054          |
| Dimethyl sulfoxide (DMSO)                                                                |             |                    |

Table 2.2: Continue.

|                                                |              |         |
|------------------------------------------------|--------------|---------|
| Polyethylenimine (PEI)<br>transfection reagent | Polysciences | 24314-2 |
|------------------------------------------------|--------------|---------|

#### 2.1.4. Bacterial Media

LB Agar (Caisson Labs, LBP02-500GM), LB Broth (Caisson Labs, LBP01-500GM), Ampicillin (Biomatik), CaCl<sub>2</sub> (Acros Organics).

#### 2.1.5. Gel Electrophoresis Solutions

Table 2.3: List of gel electrophoresis solutions.

| Solutions                     | Company              | Catalog/Lot/Cas Number |
|-------------------------------|----------------------|------------------------|
| Agarose                       | Sigma                |                        |
| MOPS 10X                      | Fisher Chemical      | 1132-61-2              |
| Tris-Acetate-EDTA (TAE)       | Fisher Scientific    | BP1332-1               |
| Red Safe                      | Intron Biotechnology | 21141                  |
| Sodium acetate                | -                    | 127-09-3               |
| 37% Formaldehyde              | Sigma                | 25249                  |
| GeneRuler 1kb Plus DNA Ladder | Thermo Scientific    | #SM1331                |
| GeneRuler 100bp DNA Ladder    | Thermo Scientific    | #SM0241                |
| 6x DNA Loading Dye            | Fermentas            | R0611                  |

#### 2.1.6. Material, Equipments and Devices

- 0.22 µm syringe filter (Merck)
- 100 mm, 150 mm tissue culture plate (TRP).

- 15 ml and 50 ml Falcon (CAPP)
- 24-well culture plate (TRP)
- 5 ml, 10 ml ,25 ml, 50 ml serological pipettes (CAPP)
- -80°C freezer (VWR)
- 96-well plate (NUNC)
- Agarose Gel Electrophoresis System (Bio-Rad)
- CO<sub>2</sub> incubator - Stericycle i160 (Thermo Scientific)
- Criterion Blotter (Bio-Rad)
- Laminar Flow Cabinet – Safe 2020 (Thermo Scientific)
- Micro pipettes (Eppendorf)
- Microcentrifuge tubes (0,2 ml, 0,5 ml, 1,5 ml, 2ml)
- MJ Mini Personal Thermal Cyclers (Bio-Rad)
- Petri dish (100 mm) (Lamtek)Power Supply – Power Pac Basic (Bio-Rad)
- Rotator (JeioTech), Varioskan Flash (Thermo Scientific)
- Spectrophotometer – NanoDrop (Schimadzu)
- Spin (Sprout)
- Syringe (1,5 ml, 10 ml, 20 ml, 50 ml)
- T25, T75, T150 tissue culture flasks (TRP)
- UV illuminator (Vilber Lourmat)
- Vortex (JeioTech), Water Bath (JeioTech)

## **2.2. Methods**

### **2.2.1. LB Broth and LB Agar preparation**

For LB Broth medium, 25 g powder LB Broth is added into 1L of purified water and it is autoclaved. Then 100µl ampicillin is added for each 100ml LB Broth.

For LB Agar, 40 g powder agar is added into 1L of purified water and it is autoclaved. 100 µl ampicillin is added for each 100 ml LB Agar before the autoclaved agar gelation. Then the agar is poured into 10 cm petri dish and it is stored 4°C.

### **2.2.2. Competent cell preparation**

Firstly, metal loop is flamed until the color turn red and cool down. Small portion of DH5 $\alpha$  cell is scraped by using loop from the glycerol stock. Scraped cells are spread it onto the LB Agar plate and incubated 37°C incubator for over-night. Next day, single colony is picked from agar plate into 5 ml of LB Broth medium and incubated shaker at 250 rpm 37°C for over-night. Next day, 1 ml of over-night culture are picked into 100ml LB Broth medium. Then this medium is shaken 37°C until OD600 is 0.4. This procedure usually takes 2-3 hours. Then cells are placed ice bath for 10 minutes. After this step cells should never touch anything that is warm. Therefore, all solutions, pipette tips, tubes, centrifuge are pre-cooled. After that, the culture is transferred two pre-chilled 50ml falcon and centrifuged at 2700 g for 10 minutes at 4°C. Medium is removed, the cell pellet is resuspended with 1.6 ml ice-cold 100 mM CaCl<sub>2</sub> by swirling on ice gently and it is incubated on ice for 30 minutes. Then the suspension is centrifuged at 2700 g for 10 minutes at 4°C again. Medium is removed, the pellet is resuspended with 16 ml ice-cold 100mM CaCl<sub>2</sub> again and it is incubated on ice for 20 minutes. The cells are combined to one tube. 0.5 ml ice-cold 80 % glycerol is added in to tube and swirled to mix. After that the mix is frozen in liquid nitrogen as 100  $\mu$ l aliquots and stored -80°C.

### **2.2.3. Transformation of Plasmids**

Standard protocol was performed for plasmid transformation in our laboratory. Firstly, DH5 $\alpha$  bacterial competent cells from -80°C storages are thawed in ice. Tubes are labelled for each plasmid samples. 2  $\mu$ l plasmid and 50  $\mu$ l thawed competent cells are mixed in the labelled tube and incubated 15 minutes in ice. Then tubes are taken from ice and tubes are put on 42°C heat-block for 90 seconds and tubes are incubated in ice 2 minutes again. After that 200  $\mu$ l LB Broth without ampicillin is added each tube. Tubes are incubated 37°C in shaker for 1 hour. To check contamination, negative control (50 $\mu$ l competent cell without plasmid) is also prepared same methods. After that mixture within the tubes are spread 10cm ampicillin positive petri agar plate by using loop and incubated 37°C over-night. Next day single bacterial

colony are picked from each sample plates and inoculated to 200 ml LB Broth with ampicillin. Then each sample are incubated 37°C over-night in shaker.

#### **2.2.4. Plasmid isolation**

For midi prep (Intron Biotechnology, 17252), 30-50 ml bacteria cells are collected into 50 ml falcon tubes from over-night incubated LB Broth culture by centrifuged at 4000 rpm for 10 minutes. Supernatants are discarded. For resuspension step, 2 ml resuspension buffer are added into falcon tubes and they are vortexed until no clumps remain. Then 2 ml lysis buffer are added, and they are inverted gently 10 times. Next, 2 ml neutralization buffer are added, and they are inverted 10 times gently. Tubes are incubated on ice for 5 min. After this step, the solution within the tubes are transferred to pre-columns and columns are centrifuged 4000 rpm for 5 minutes. Flow through are transferred into binding columns and columns are centrifuged at 4000rpm for 5 minutes. Flow through are discarded. 10ml first washing buffer are added into binding columns and columns are centrifuged at 4000 rpm for 5 minutes. Flow through are discarded. 10 ml second washing buffer into binding columns and columns are centrifuged at 4000 rpm for 5 minutes. Next, columns are centrifuged at 4000 rpm for 15 minutes without any solution addition (dry centrifuge for the remove ethanol) and columns are incubated 56°C for 5 minutes. Then columns are transferred clean falcon tubes. 600 µl dH<sub>2</sub>O are added into columns for elution and columns are incubated RT for 5 minutes. Then columns are centrifuged at 400 rpm for 5 minutes and extracted plasmids are stored -20°C.

For mini prep (Intron Biotechnology, 17097), bacterial cultures are harvested by at 4000 rpm centrifugation for 2 minutes. Supernatants are discarded. 500µl resuspension buffer are added into tubes and tubes are vortex until no clumps remains. 250 µl lysis buffer are added into tubes and tubes inverted 10 times gently. Tubes are incubated at RT for 3 minutes. Next, 350 µl neutralization buffer are added into tubes and tubes are inverted 10 times gently. Tubes are incubated on ice for 5 minutes and tubes are centrifuged at 13000 rpm for 10 minutes. Supernatants are transferred into columns and columns are centrifuged at 13000 rpm for 1 minute. Flow through are discarded. Then 500 µl first washing buffer are added into columns and columns are centrifuged at 13000 rpm for 1 minute. Flow through are discarded.

Next, 700 µl second washing buffer are added into columns and columns are centrifuged at 13000 rpm for 1 minute. Flow through are discarded. Then columns are centrifuged at 13000 rpm for 1 minute without any solution addition (dry centrifuge). After the dry centrifugation, columns are transferred into clean collection tubes and 50 µl dH<sub>2</sub>O are added into columns. Columns are incubated at RT for 1 minute. Then columns are centrifuged at 13000 rpm for 1 minute and extracted plasmids are stored -20°C.

### **2.2.5. Cell Culture**

Standard cell culture procedure of our laboratory was used: For the culturing of SH-SY5Y human neuroblastoma cell line, standard culture medium DMEM with 4,5g/L glucose, L-glutamine, pyruvate was used with addition of 10 % FBS and 1 % penicillin/streptomycin antibiotics. Firstly, medium from flask or plate is discarded. The cells within flask or plate are washed with PBS to eliminate dead, unbound cells and their debris. Then X Trypsin-EDTA (Gibco, 1721788) is added into flask or plate to remove cells from bounded surface. The complete medium is added into flask to inhibit the trypsin and cells are collected from flask or plate to falcon tube. Then the cells are centrifuged at 1500 rpm for 5 minutes and supernatant is discarded. The pellet is suspended with small amount of complete medium. Suspended cells are counted with hemocytometer and desired amounts of cells transferred into new flask or plate with required amount of complete medium. The cultured cells are incubated 37°C and 5% CO<sub>2</sub>. When cells reach 80 % confluency in flask or plate, the cells are sub-cultured.

For the storage of cells in -80 °C or liquid nitrogen, cryotubes and DMSO are used to freeze the cells. In this procedure, cells are counted with hemocytometer and 1.10<sup>6</sup> or 2.10<sup>6</sup> cells are transferred into cryotube. Then required amounts of DMSO is added into cryotube and cells is frozen firstly -20°C after that -80°C storages.

### **2.2.6. Transient transfection of Pea3 and Elk1 expression plasmids**

Standard polyethyleneimine (PEI) transfection method was used to transient transfection of SH-SY5Y cells with Pea3 and Elk1 expression plasmids. Firstly,  $1.5 \times 10^6$  SH-SY5Y human neuroblastoma cells are seeded into 60 mm cell culture plates. 100  $\mu$ l of 100ng/ $\mu$ l plasmids are mixed into tube with 1 ml of serum-free DMEM. Then optimum amount is determined to be 1:4 (DNA-PEI), and hence 35  $\mu$ l of PEI was added to the DNA mixture. After that tubes are vortexed for 15 seconds, spun and incubated at RT for 15 minutes. On the other hand, medium of cultured cells are discarded and 4 ml of complete DMEM medium are added onto cells. Then DNA/PEI mix is added into plate and plates are incubated at 37°C for 2 hours. After the incubation, 5 ml complete medium are added into plates and plates are incubated at 37°C for 48 hours. To check the transfection efficiency, GFP plasmids are also transfected with same procedure.

### **2.2.7. RNA isolation**

Pure Link RNA Mini Kit (Thermo Fisher Scientific) was commonly used for RNA extraction. Firstly, Medium of cultured cells are discarded and 200  $\mu$ l PBS is added on plate to collect cell with scraper into tube. Tubes are centrifuged at 2000g for 5 minutes and supernatant are discarded. Then 600  $\mu$ l lysis buffer with 6  $\mu$ l 2-mercaptoethanol are added for each sample and tubes are vortexed thoroughly. The mix is then passed from syringe 10-13 times for homogenization. After this step, 600  $\mu$ l ethanol are added into each sample and tubes are vortex. 700  $\mu$ l sample within the tubes are transferred into column and columns are centrifuged at 12000g for 15 seconds. This step is repeated until no sample remain. After that 700  $\mu$ l First Washing Buffer is added into column and columns are centrifuged at 12000g for 15 seconds. Flowthrough is discarded. Then 500  $\mu$ l Second Washing Buffer are added into column and columns are centrifuged at 12000g for 15 seconds. Flow through is discarded and Second Washing Buffer step is repeated. To remove ethanol, columns are centrifuged at 12000g for 1-2 minutes without addition any solution (dry centrifugation) and columns are transferred into clean collection tubes. For elution, 30  $\mu$ l RNase-free water are added into column. Columns are incubated for 1 minute

and columns are centrifuged at maximum speed for 2 minutes and RNA samples are stored -80°C.

### 2.2.8. Checking RNA integrity

To check RNA integrity, 1 % MOPS agarose gel electrophoresis procedure was used. For preparation of 10x MOBS buffer, 200 ml autoclaved water are added into 20.92 g MOPS, 2.05 g sodium acetate, 0.9 g EDTA mixture to dissolve. pH is adjusted to 7 with 2 N NaOH and final volume are filled to 250 ml. Buffer is sterilized with filtration. Buffer can thus be stored at RT. For preparation of 1% MOPS agarose gel, 0.5 g agarose powder are dissolved into 36 ml purified water and agarose is melted. Then 5 ml 10x MOPS buffer are added and solution are cooled to 60°C. When the 60°C, 9 ml fresh 37% formaldehyde and 2.5µl red safe are added into agarose solution. Then agarose gel is poured. For electrophoresis, 1x volume of RNA sample (500 ng/µl) is mixed with 1x volume of RNA loading dye, and in parallel 3 µl RNA ladder are mixed with 3µl loading dye. After that samples and ladder are firstly incubated at 70°C for 10 minutes then on ice for 3 minutes. After the incubation RNA samples and ladder are loaded into gel. Gel are run with 1% MOBS buffer at 50V for 1h and UV imaging is performed.

### 2.2.9. cDNA synthesis

cDNA synthesis was performed by using iScript cDNA synthesis kit (BioRad). For the 20µl total volume 1x reaction, 4µl reaction mix, 1µl reaction enzyme, 500np/µl RNA template and required amounts of nuclease free water were used.

Table 2.4: cDNA synthesis reaction setup.

| Component                     | Volume per reaction, µl |
|-------------------------------|-------------------------|
| 5x iScript Reaction Mix       | 4                       |
| iScript Reverse Transcriptase | 1                       |
| Nuclease Free Water           | Variable                |
| RNA Template                  | Variable (500ng/µl)     |
| Total                         | 20                      |

Reaction protocol:

- 25°C for 5 minutes
- 42°C for 30 minutes
- 85°C for 5 minutes
- 4°C for forever

cDNAs can be stored at -20°C for short time and at -80°C for long-term storage.

## 2.2.10. Primer Design

For designing qPCR, RT-PCR primer, IDT PrimerQuest Tool [35] and NCBI Primer Blast Tool was used [36]. For qPCR, primers are picked with specific parameter settings: primer length 18-24 bp, GC content 40-60%, melting temperature 50-65°C. After the design, primers are analyzed with IDT OligoAnalyzer 3.1 Tool in order to check GC content, melting temperature, hairpin temperature and self-dimer score [37].

For designing promoter cloning primer, same methods and tools was used. Additionally, restriction enzymes cleavage sequence was imported to ends of the forward and reverse primer while designing primers. Compatible overhangs of restriction enzymes were checked.

Table 2.5: qPCR primers of selected ETS targets.

| Target | Primers (5' to 3') |                        | Length (bp) | Product Length (bp) | GC Content (%) | T <sub>m</sub> (°C) |
|--------|--------------------|------------------------|-------------|---------------------|----------------|---------------------|
| ETS2   | Forward            | TTCACAGGAAGTGGACCTA    | 19          | 175                 | 47.40%         | 59.8                |
|        | Reverse            | CGTAGTTCATCTTGGGCTTAT  | 21          |                     | 42.90%         | 59.9                |
| ERG    | Forward            | GAATGGCTCAAGGAAGTCTC   | 20          | 142                 | 50%            | 60.1                |
|        | Reverse            | CTCGTTCGTGGTCATGTTT    | 19          |                     | 47.40%         | 60.2                |
| FLI1   | Forward            | GATGGCAAGGAAGTGTGTA    | 20          | 176                 | 45%            | 60.2                |
|        | Reverse            | GAAGGGTCTTCTTTGACACTC  | 21          |                     | 47.60%         | 60.3                |
| FEV    | Forward            | CGTCCTCTAATCCAGAGTTTAC | 22          | 168                 | 45.50%         | 59.7                |
|        | Reverse            | GACCTGGAGACCTAGAAGAA   | 20          |                     | 50%            | 59.9                |

Table 2.5: Continue

|       |         |                         |    |     |        |      |
|-------|---------|-------------------------|----|-----|--------|------|
| GABPA | Forward | GAATCAGAAGCACATCTTGTTG  | 22 | 172 | 40.90% | 60   |
|       | Reverse | ACTGTATGGGATCATAGGGTAT  | 22 |     | 40.90% | 60.1 |
| ELF1  | Forward | GGAAGAGCACTCAGGTAATA    | 20 | 158 | 50%    | 60.1 |
|       | Reverse | GAAGATAGCGATGGATCTGAAG  | 22 |     | 45.50% | 60.1 |
| ELF2  | Forward | GGATTCAAAGGCTGTCTCTAA   | 21 | 94  | 42.90% | 59.8 |
|       | Reverse | GTAGTAGTATCTCAAAGCTCGTC | 23 |     | 43.50% | 59.8 |
| ELF4  | Forward | GGCGGGCACTAAGATACTA     | 19 | 90  | 52.60% | 60.5 |
|       | Reverse | CCTTGGGCATCTCCTTAAAC    | 20 |     | 50%    | 60.4 |
| ELF5  | Forward | GTACTTCATCCTCCAGAACATC  | 22 | 162 | 45.50% | 60   |
|       | Reverse | CTTTGGAGGCTTGTCTACTAT   | 22 |     | 40.90% | 59.9 |
| EHF   | Forward | CAGCCGAGCTATGAGATATTAC  | 22 | 128 | 45.50% | 59.9 |
|       | Reverse | AGTATTGGCAGCTTCAGTTT    | 20 |     | 40     | 60.1 |
| ERF   | Forward | CTGTCTCTGTGGGTTTCTAAG   | 21 | 131 | 47.60% | 59.8 |
|       | Reverse | AAAGTTCCTGGGTGTTAAGG    | 20 |     | 45%    | 59.9 |
| ETV3  | Forward | GAGGTGGAGGGTATCAGTTT    | 20 | 124 | 50%    | 60.8 |
|       | Reverse | ATGACATGGCGGAACCTCT     | 18 |     | 50%    | 60.9 |
| ETV6  | Forward | ATTCAGGTGATGTGCTCTATG   | 21 | 158 | 42.90% | 59.9 |
|       | Reverse | CCTCTGGACACAGTTATCTTC   | 21 |     | 47.60% | 59.6 |
| ETV7  | Forward | GGAGGAGTCCCTCAACTTAT    | 20 | 139 | 50%    | 60.3 |
|       | Reverse | CTGATACACGTAATCCCACAG   | 21 |     | 47.60% | 59.9 |
| ETV4  | Forward | CTTTACTCCAGTGCCTATGAC   | 21 | 116 | 47.60% | 59.9 |
|       | Reverse | AGGAAATTCGGTTGCTCTG     | 19 |     | 47.40% | 60.1 |
| ETV5  | Forward | GAATACCATGACCCACTCTATG  | 22 | 132 | 45.50% | 59.9 |
|       | Reverse | GCAGTTAGGCACTTCTGAAT    | 20 |     | 45%    | 60.2 |
| ETV1  | Forward | TCTCCAAACTCAACTCATACAC  | 22 | 135 | 40.90% | 60.1 |
|       | Reverse | GGAGTTACAGGGTTCAGAAAG   | 21 |     | 47.60% | 60.1 |
| ETV2  | Forward | GCCAAATTAGGCTTCTGTTTC   | 21 | 92  | 42.90% | 59.9 |
|       | Reverse | GATGAGCTTGTACCTTTCCAG   | 21 |     | 47.60% | 60.4 |
| SPI1  | Forward | GCCAAACGCACGAGTATTA     | 19 | 129 | 47.40% | 60.3 |
|       | Reverse | GCTCCGTGAAGTTGTTCTC     | 19 |     | 52.60% | 60.4 |
| SPIB  | Forward | CCCAGATGGCGTCTTCTAT     | 19 | 136 | 52.60% | 60.8 |
|       | Reverse | CGGGTCGAAGGCTTCATA      | 18 |     | 55.60% | 61   |
| SPIC  | Forward | CCCAGATGGCGTCTTCTAT     | 19 | 149 | 47.40% | 60.1 |
|       | Reverse | CGGGTCGAAGGCTTCATA      | 18 |     | 47.60% | 60.2 |
| ELK4  | Forward | TTTCACAGACACCCATCATAC   | 21 | 145 | 42.90% | 60   |
|       | Reverse | CTGTTCAGTACAGAAGGAAACT  | 22 |     | 40.90% | 60   |
| ELK3  | Forward | TGTGACCAATAAAACCGACA    | 20 | 159 | 40%    | 59.9 |
|       | Reverse | GGTGAGGCGGATGAAATAC     | 19 |     | 52.60% | 60   |
| SPDEF | Forward | GCTTCATTAGGTGGCTCAA     | 19 | 143 | 59.90% | 47.4 |
|       | Reverse | CTTGTAATACTGGCGGATGG    | 20 |     | 50%    | 60.2 |

Table 2.5: Continue

|      |         |                      |    |     |        |      |
|------|---------|----------------------|----|-----|--------|------|
| SRF  | Forward | CCTTTCCCATCACCAACTAC | 20 | 93  | 50%    | 60.1 |
|      | Reverse | CTGTACTCTTCAGCACAGTC | 20 |     | 50%    | 59.6 |
| ELK1 | Forward | CTACGCAAGAACAAGACCAA | 20 | 128 | 45%    | 60.3 |
|      | Reverse | TCAGGGTAGGACACAAACT  | 19 |     | 47.40% | 60.1 |

Table 2.6: Cloning primers of selected ETS targets.

| Target | Primers (5' to 3') |                                    | Length (bp) | Product Length (bp) | GC Content (%) | Tm (°C) |
|--------|--------------------|------------------------------------|-------------|---------------------|----------------|---------|
| ETV6   | Forward            | ATATACGCGTCTCAAGGGTCACCTGACTCATTCC | 34          | 938                 | 54.2           |         |
|        | Reverse            | ACACCTCGAGATACACATAGGGACCCAAAGGC   | 32          |                     | 50             |         |
| ERG    | Forward            | ACACACGCGTATGGAACGAGAGCAGTCCGC     | 30          | 941                 | 60             |         |
|        | Reverse            | ACACCTCGAGCTGTCTTTTACAAGGTCAGTCCA  | 33          |                     | 43.5           |         |

### 2.2.11. Quantitative Real Time PCR

Expression analysis was performed with qPCR by using ssoAdvanced Universal SYBR Green Supermix (Bio Rad). For 10 µl total volume 1x reaction mix, 5 µl SYBR Green supermix, 0.3 or 0.4 µl 10 µM forward and reverse primer, 2.5 ng/µl cDNA template and required amounts of nuclease free water were used. 96 well plate also was used for this procedure. For the normalization process Gapdh and Actin-b qPCR data were used. In addition, pCDNA3 control plasmid was used. Firstly, water and primers are mixed into tubes and tubes are vortexed. Required amounts of SYBR Green Supermix are added into tubes. Then master mixes are divided to 96 well plate and template cDNAs are added into wells. After that, plate is loaded to qPCR machine and reaction protocol is run. When the measurement is finished, threshold cycle ( $C_t$ ) values of samples are normalized with control vector and housekeeping genes.

Table 2.7: qPCR reaction setup.

| Component           | Volume per reaction |
|---------------------|---------------------|
| SYBR Green Supermix | 5µl                 |
| Forward Primer      | 0.3-0.4µl           |
| Reverse Primer      | 0.3-0.4µl           |
| Template            | 2.5ng/µl            |
| Water               | Variable            |
| <b>Total</b>        | <b>10µl</b>         |

### 2.2.12. Molecular Cloning for Promoter of Selected ETS Targets

Promoter of selected ETS targets were used as insert to cloning pGL3 Basic vector. They amplified with *Pfu* DNA polymerase due to its low error rate. Before the *Pfu* PCR amplification, gradient PCR was performed with *Taq* Polymerase to find optimum annealing temperature for each cloning primers. PCR reaction setup for amplification was prepared as shown in Table 2.8.

Table 2.8: Pfu PCR reaction setup for insert amplification.

| Component                                         | Volume per reaction |
|---------------------------------------------------|---------------------|
| 10x <i>Pfu</i> Buffer with MgSO <sub>4</sub> (1x) | 2.5 µl              |
| dNTP                                              | 1 µl                |
| Forward Primer                                    | 1.25 µl             |
| Reverse Primer                                    | 1.25 µl             |
| <i>Pfu</i> DNA polymerase                         | 0.4 µl              |
| Template DNA                                      | 200 ng              |
| dH <sub>2</sub> O                                 | up to 25µl          |

Table 2.9: PCR cycling conditions and temperatures to amplify inserts.

| Stage                | Temperature | Duration   |
|----------------------|-------------|------------|
| Initial Denaturation | 95 °C       | 2 minutes  |
| Denaturation         | 95 °C       | 30 seconds |
| Annealing            | 64 °C       | 30 seconds |
| Extension            | 72 °C       | 2 minutes  |
| Final Extension      | 72 °C       | 10 minutes |
|                      | 4 °C        | Forever    |

Agarose gel electrophoresis was performed to check the amplification of insert in %1,5 agarose gel. PCR product was purified from agarose gel for digestion procedure.

Amplified inserts and pGL3 luciferase vector were double digested with appropriate restriction enzymes (XhoI and MluI) to make integration. Vector was digested amount of 1 µg/µl, inserts were digested amount of 200 ng/µl. Double digestion reaction mix was prepared as shown in table below.

Table 2.10: Double digestion reaction setup with XhoI and MluI restriction enzymes.

| <b>Component</b>          | <b>Volume</b> |
|---------------------------|---------------|
| Template DNA (1µg)        | Variable      |
| Restriction Enzyme (XhoI) | 1 µl          |
| Restriction Enzyme (MluI) | 1 µl          |
| 10x Green Buffer (1x)     | 2 µl          |
| dH <sub>2</sub> O         | Up to 20 µl   |

Agarose gel electrophoresis was performed to check the digestion in %1,5 agarose gel. Vector and inserts was purified from agarose gel for ligation procedure.

The molar ratio of 1:3, 1:5 and 1:10 vector to inserts was used to be joined DNA fragments of vector and inserts from digestion step. For ligation procedure, 50 ng vector was used, and reaction setup was prepared as shown in table below.

Table 2.11: Reaction setup of ligation procedure with T4 DNA Ligase.

| <b>Component</b>  | <b>Volume</b> |
|-------------------|---------------|
| Insert            | Variable      |
| Vector (50ng)     | Variable      |
| T4 DNA Ligase     | 1 µl          |
| 10x Ligase Buffer | 2 µl          |
| dH <sub>2</sub> O | Up to 20 µl   |

After the ligation step, plasmid DNA was transferred to competent DH5α *E.coli* bacterial cells by using transformation method as described.

### 2.2.13. Promoter Analysis

Promoter analysis was used to predict Pea3 and Elk1 binding sites on the promoters of selected ETS genes. Firstly, promoter sequences of a subset of ETS genes studied in this thesis were obtained from Transcription Regulatory Element Database (TRED) as a fasta format [38]. Then promoter sequences were imported into transcription factor binding site identification tool called PROMO [39]. This web-based tool gives us putative binding site sequences of selected transcription factors (in this thesis, Pea3 and Elk1) on the promoter sequences of selected targets (other ETS members) with dissimilarity parameter. The dissimilarity threshold is the parameter that controls how similar a sequence must be to matrix to be reported as a hit.

### 2.2.14. Luciferase Assay

Luciferase assay was performed in order to confirm promoter activation based on predictions above. pGL3-Basic vector was used as the base vector for luciferase reporter assay. Firstly, 15000 human neuroblastoma cells (SH-SY5Y) are cultured in each well of 96-well plate along with 100µl complete DMEM medium.

For the quantification of the effect of Pea3 transcription factor on *elk-1* promoter, pCDNA3, pCDNA3mPea3, pCDNA3-Pea3VP16, pGL3-*elk-1*-Luc, and *Renilla*-Luc plasmids were transfected into cells using standard transfection protocol (see Table 2.7-2.8 below). The cells were incubated for 48 hours and analyzed thereafter.

Table 2.12: Luciferase reporter assay transfection setup 1.

|        | pCDNA3 | pCDNA3mPea3 | <i>elk-1</i> -Luc | <i>Renilla</i> -Luc | PEI   |
|--------|--------|-------------|-------------------|---------------------|-------|
| Tube-1 | 200ng  | -           | 50ng              | 25ng                | 1.1µl |
| Tube-2 | 150ng  | 50ng        | 50ng              | 25ng                | 1.1µl |
| Tube-3 | 100ng  | 100ng       | 50ng              | 25ng                | 1.1µl |
| Tube-4 | 50ng   | 150ng       | 50ng              | 25ng                | 1.1µl |
| Tube-5 | -      | 200ng       | 50ng              | 25ng                | 1.1µl |

Table 2.13: Luciferase reporter assay transfection setup 2.

|        | pCDNA3 | Pea3VP16 | <i>elk-1</i> -Luc | <i>Renilla</i> -Luc | PEI   |
|--------|--------|----------|-------------------|---------------------|-------|
| Tube-1 | 200ng  | -        | 50ng              | 25ng                | 1.1µl |
| Tube-2 | 150ng  | 50ng     | 50ng              | 25ng                | 1.1µl |
| Tube-3 | 100ng  | 100ng    | 50ng              | 25ng                | 1.1µl |
| Tube-4 | 50ng   | 150ng    | 50ng              | 25ng                | 1.1µl |
| Tube-5 | -      | 200ng    | 50ng              | 25ng                | 1.1µl |

For the luciferase reporter assay, Dual-Glo Luciferase Assay System kit (TM040) was used. Firstly, luciferase reagent (stored -80°C) is thawed at RT. Parallely, transfected cells medium is discarded until 75 µl medium remain. Then 75 µl luciferase reagent is added into 75 µl medium within the well. Plate is shaken at RT for 1 minute and incubated at RT for about 20 minutes. After the incubation, plate is imported the VarioScan machine for luminometric measurement (step 1 – firefly luciferase measurement of promoter in question). When the measurement is finished, 75 µl stop-Glo buffer is added into each well. Plate is shaken at RT for 1 minute and it is incubated at RT for same as first incubation time (about 20 minutes) again. After that luminometric measurement is performed again (*Renilla* measurement for internal control). For the luciferase relative expression analysis, firefly luminometric measurement is normalized with *Renilla* luminometric measurement.

### 2.2.15. Phylogenetic Tree Construction

To represent evolutionary relationship of ETS member proteins from different model organisms, a phylogenetic tree was constructed by using Clustal Omega [40] and Mega X [41] software. Firstly, ETS protein sequences of human, mouse, *Drosophila*, *C. elegans*, zebrafish, *Xenopus* organisms were obtained as fasta format by using Universal Protein Resource Database (UniProt) [42]. Then phylogenetic tree was constructed by using Clustal Omega. For visual arrangement, Mega X software was used, and phylogenetic tree was figured.

## 2.2.16. Kinetic Model Construction

Biological processes have highly complex dynamics at the cellular and molecular level. To understanding biochemical reactions under these biological events, mathematical models are useful for the explaining these systems. Mathematical model is a description of any system with mathematical expression methods by using concept and language of mathematics. In biological systems, quantitative kinetic models are widely used to simulate biological system behavior. In quantitative kinetic models, several steps are followed to construct the model. Firstly, mathematical expression is chosen to characterize the relationships between its components. Secondly, parameter values and initial conditions are determined to create reaction of system. Then, numerical simulations are performed to observe system behavior [43].

As described above, quantitative kinetic models are useful method for simulate biological system to understand their behavior. Because of that we implemented this methodology to our transcriptional regulatory network which we wanted to create. To construct our intra familial regulation network of ETS family model that we called as **ETS model**, Complex Pathway Simulator (COPASI) software was used [44]. Firstly, model topology was determined as shown in Figure 2.1. In this topology, four types of reaction were used generally: transcription, translation, mRNA and protein degradation which we assumed possible explanation for our regulation network. This type of topology is also commonly used in modeling of gene regulatory network [45], [46].

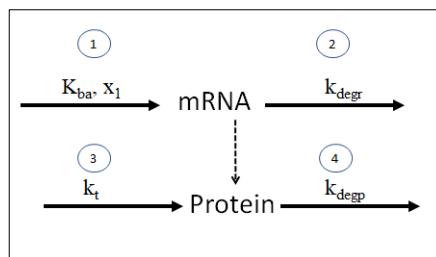


Figure 2.1: ETS model topology. There are four types of reaction in this topology, transcription, translation, mRNA and protein degradation. Arrow of reaction number 1 and 3 indicates synthesis reaction, 2 and 4 are degradation reaction. Parameters of these reactions indicates respectively:  $K_{ba}$  transcription factor binding affinity parameter,  $x_1$  transcription rate,  $k_{degr}$  mRNA degradation rate,  $k_t$  translation rate,  $k_{degp}$  protein degradation rate.

ETS family members are transcription factor, therefore transcription reaction of our ETS model was constructed based on promoter-transcription factor binding affinity and its effect on the mRNA synthesis. Transcription factors regulates mRNA synthesis as an activator or inhibitor. When we tried to find possible mathematical explanation of this transcription reactions, we observed that our description of transcriptional activation or inhibition of transcription factor was not compatible with standard enzyme kinetic equations such as activation, non-competitive inhibition, un-competitive inhibition etc. Therefore, we modified transcriptional activation and inhibition reaction from binding of activator or inhibitor to a promoter by using Michaelis Menten and Hill Equation in [47]. For other reactions, standard mass action reaction was used in the COPASI software. Reaction mathematical explanation and parameters were listed Table 2.14.

Table 2.14: Biochemical structure of ETS model with references.

| Reaction            | Function of rate law                                | Parameter  | Reaction type      |
|---------------------|-----------------------------------------------------|------------|--------------------|
| mRNA synthesis      | $K_{ba} \times [Activator] \times x_1 / 1 + K_{ba}$ | $x_1$      | Modified from [47] |
|                     | $x_1 / (1 + K_{ba}) \times [Inhibitor]$             | $K_{ba}$   |                    |
| mRNA degradation    | $k_{degr} \times [mRNA]$                            | $k_{degr}$ | Mass Action        |
| Protein synthesis   | $k_t \times [mRNA]$                                 | $k_t$      | Mass Action        |
| Protein degradation | $k_{degp} \times [protein]$                         | $k_{degp}$ | Mass Action        |

$K_{ba}$  parameter for activator and inhibitor transcription factor was modified from [47] independently. Firstly, we derived the one activator or inhibitor on the promoter:

For activator:

P: Promoter, A: Activator, PA: Promoter-TF complex,  $P_T$ : Total promoter amount

$$P + A = PA \quad (2.1)$$

$$\frac{d[PA]}{dt} = (k_1 \times [P] \times [A]) - (k_{1r} \times [PA]) \quad (2.2)$$

( $k_1$  : TF association parameter ,  $k_{1r}$  : TF disassociation parameter)

$$[P_T] = [P] + [PA] \rightarrow [P] = [P_T] - [PA] \quad (2.3)$$

The kinetic equation of promoter-TF binding approaches a steady-state in which concentration do not change with time,  $d[PA]/dt = 0$ . Solving this equation at steady state, we find that the balance between P and A and the dissociation of PA leads to the chemical equilibrium equation:

$$k_1 \times [P] \times [A] = k_{1r} \times [PA] \quad (2.4)$$

$$[PA] = \frac{k_1}{k_{1r}} \times [P] \times [A] \quad (2.5)$$

$$\frac{k_1}{k_{1r}} = K_{ba} \quad (2.6)$$

$$\frac{[PA]}{[P_T]} = \frac{K_{ba} \times [A]}{1 + (K_{ba} \times [A])} \quad (\text{promoter activity}) \quad (2.7)$$

Transcription rate =  $x_1$  and promoter activity rate:

$$\frac{x_1 \times (K_{ba} \times [A])}{1 + (K_{ba} \times [A])} \quad (2.8)$$

For inhibition:

P: Promoter, I: Inhibitor, PI: Promoter-TF complex,  $P_T$ : Total promoter amount

$$P + I = PI \quad (2.9)$$

$$\frac{d[PA]}{dt} = (k_1 \times [P] \times [I]) - (k_{1r} \times [PI]) \quad (2.10)$$

( $k_1$  : TF association parameter ,  $k_{1r}$  : TF disassociation parameter)

$$[P_T] = [P] + [PI] \rightarrow [PI] = [P_T] - [P] \quad (2.11)$$

The kinetic equation of promoter-TF binding approaches a steady-state in which concentration do not change with time,  $d[PA]/dt = 0$ . Solving this equation at steady state, we find that the balance between P and A and the dissociation of PA leads to the chemical equilibrium equation:

$$k_1 \times [P] \times [I] = k_{1r} \times [PI] \quad (2.12)$$

$$[PI] = \frac{k_1}{k_{1r}} \times [P] \times [I] \quad (2.13)$$

$$\frac{k_1}{k_{1r}} = K_{ba} \quad (2.6)$$

$$\frac{[P]}{[P_T]} = \frac{1}{1 + K_{ba} \times [I]} \quad (\text{promoter activity}) \quad (2.14)$$

Transcription rate =  $x_1$  and promoter activity rate:

$$\frac{x_1}{1 + K_{ba} \times [I]} \quad (2.15)$$

If there are more than one inter-dependent activator or inhibitor transcription factor on the promoter, different mathematical explanation is required. To overcome this situation, we assumed in our model that, two or more transcription factors as activator or inhibitor on the its target promoter, their activation or inhibition events occur independently, with independent probabilities, and thus their rate law is described as multiplication of independent activation or inhibition rate law described above. Different transcriptional regulation possibilities were shown in Table 2.15.

Table 2.15: Different transcriptional regulation under the transcription factor as activator or inhibitor on the target promoter.

| Reaction       | Rate law                                                                                                                       |
|----------------|--------------------------------------------------------------------------------------------------------------------------------|
| One activator  | $x_1 \times K_{ba} \times [A]/1 + K_{ba} \times [A]$                                                                           |
| One inhibitor  | $x_1/1 + K_{ba} \times [I]$                                                                                                    |
| Two activators | $(x_1 \times K_{ba1} \times [A_1]/1 + K_{ba1} \times [A_1]) \times (x_1 \times K_{ba2} \times [A_2]/1 + K_{ba2} \times [A_2])$ |

Table 2.15: Continue

|                                 |                                                                                              |
|---------------------------------|----------------------------------------------------------------------------------------------|
| Two inhibitors                  | $(x_1/1 + K_{ba1} \times [I_1]) \times (x_1/1 + K_{ba2} \times [I_2])$                       |
| One activator-<br>one inhibitor | $(x_1 \times K_{ba1} \times [A]/1 + K_{ba1} \times [A]) \times (x_1/1 + K_{ba2} \times [I])$ |

After the construction of the reactions, parameter values were determined from literature data. In this step two different parameter sets were found from literature for  $x_1$ ,  $k_{\text{degr}}$ ,  $k_{\text{degp}}$ ,  $k_t$  parameters. First parameter set was obtained from mouse fibroblast cells in [48], and the second parameter set was obtained from sea urchin endomesoderm in [45]. All parameter values were listed in Table 2.16-17 with references.

Table 2.16: Parameter values of mouse fibroblast set of ETS model.

| Parameter         | Organism/cell<br>type | Value                      | Reference                                                                |
|-------------------|-----------------------|----------------------------|--------------------------------------------------------------------------|
| $x_1$             | Mouse fibroblast      | 0.89 molecule/hour         | ETV6<br>transcription,<br>translation and<br>degradation<br>rate in [48] |
| $k_{\text{degr}}$ | Mouse fibroblast      | 0.125117<br>molecule/hour  |                                                                          |
| $k_{\text{degp}}$ | Mouse fibroblast      | 0.0297488<br>molecule/hour |                                                                          |
| $k_t$             | Mouse fibroblast      | 19.04<br>molecule/hour     |                                                                          |
| $K_{ba}$          | -                     | Variable                   | Modified from<br>[47]                                                    |
| $[A]$             | -                     | Variable                   |                                                                          |
| $[I]$             | -                     | Variable                   |                                                                          |

Table 2.17: Parameter values of sea urchin set of ETS model.

| Parameter         | Organism/cell type      | Value               | Reference          |
|-------------------|-------------------------|---------------------|--------------------|
| $x_1$             | Sea urchin endomesoderm | 1 molecule/hour     | [45]               |
| $k_{\text{degr}}$ | Sea urchin endomesoderm | 0.119 molecule/hour |                    |
| $k_t$             | Sea urchin endomesoderm | 2 molecule/hour     |                    |
| $k_{\text{degp}}$ | Sea urchin endomesoderm | 0.3 molecule/hour   |                    |
| $K_{ba}$          | -                       | Variable            | Modified from [47] |
| [A]               | -                       | Variable            |                    |
| [I]               | -                       | Variable            |                    |

As a next step, after the determination of topology, mathematical expression and parameters, we initially constructed basic version of ETS model to confirm our model mathematically. In the basic model, there are two transcription factors: an activator and inhibitor. These TF's are bound their hypothetically promoter of their targets and regulates transcriptional processes. Synthesized mRNAs of targets are leaded to protein synthesis by translation reaction. Additionally, degradation of mRNA and proteins are stabilized all these reactions like in a real cell.

After the mathematical confirmation of basic model, complex ETS model was constructed in Figure 1.6. In this model, our transcription factors are ETS members: ELK1, PEA3, ERF, ETV3L, ER81, ERM, ELF3, EHF. All these ETS members are bound their promoter of ETS member targets and mRNAs are synthesized by transcription reaction. In Figure 1.6, arrows indicate the transcriptional activation reaction, straight arrows indicate the transcriptional repression reaction. For instance, Elk1 is bound to promoter of Pea3 as a transcriptional inhibitor. On the other hands, Pea3 are bound to promoter of Elk1 as a transcriptional inhibitor like feedback. Elk1 also is bound to promoters of EHF, ELF3 and ETV3L as a transcriptional activator. All other arrows in Figure 1.6 indicates these types of reactions and our complex ETS model reactions are happened in our hypothetical cell (called compartment in

COPASI) in parallel. Thereafter we observed their behavior by using numerical simulations in COPASI. All reactions of ETS complex ETS model was listed in Appendix with mouse fibroblast and sea urchin parameter sets. After the set all parameter and initial condition values, numerical simulations (time course task in COPASI) were performed to observe system behavior. Simulation time was set to 48 hours that same as transfection incubation time and deterministic method was used for simulation in COPASI default option. In addition, parameter scan task was also used to observe effect of different activator, inhibitor level and different  $K_{ba}$  level in the system.

### 3. RESULTS

#### 3.1. Promoter Analysis for Elk-1 and Pea3 on the Promoter of Selected ETS members

In order to identify, binding site of Elk-1 and Pea-3 transcription factor on the other promoter of selected target of ETS family, promoter analysis was performed by using PROMO which is a web-based transcription factor binding site (TFBS) tool. In this procedure, PROMO tool gives predictive binding site sequences, their location and dissimilarity score on the targets promoter (Table 3.1). Dissimilarity score indicates binding affinity of transcription factor on the promoter sequence. Before performing the analyses for this particular study, dissimilarity threshold was set to %7.

Table 3.1: Promoter analysis of Elk-1 on the promoter of selected targets of ETS members.

| Promoter | Start position (nt) | End position (nt) | Dissimilarity score (%) | String    |
|----------|---------------------|-------------------|-------------------------|-----------|
| EHF      | 908                 | 916               | 0                       | GGCAGGAAG |
| ELF1     | 169                 | 177               | 5.027151                | CTTCCTAAA |
| ELF2     | 323                 | 331               | 2.299314                | TCCAGGAAG |
|          | 407                 | 415               | 0                       | CTTCCTGCC |
|          | 513                 | 521               | 2.299314                | CTTCCTGTT |
|          | 530                 | 538               | 6.598007                | GGGCGGAAG |
|          | 678                 | 686               | 2.164966                | CTTCCTGTC |
| ELF5     | 451                 | 459               | 4.204473                | TTAAGGAAG |
|          | 503                 | 511               | 3.381796                | TTGAGGAAG |
|          | 662                 | 670               | 3.247448                | GTCAGGAAG |
| ELK3     | 611                 | 619               | 0.134348                | CGCAGGAAG |
|          | 781                 | 789               | 3.121991                | TAAAGGAAG |
|          | 817                 | 825               | 0.134348                | CTTCCTGCT |
| ERF      | 34                  | 42                | 0                       | CTTCCTCCC |
|          | 309                 | 317               | 2.299314                | CTTCCTCGG |
| ERG      | 491                 | 499               | 0                       | CTTCCTCCC |
|          | 157                 | 165               | 2.164966                | CTTCCTCTC |

Table 3.1: Continue.

|       |     |     |          |           |
|-------|-----|-----|----------|-----------|
| ETS1  | 819 | 827 | 0.822677 | GGAAGGAAG |
| ETS2  | 482 | 490 | 2.987643 | CTTCCTTTC |
|       | 143 | 151 | 2.299314 | TACAGGAAG |
|       | 492 | 500 | 6.598007 | GGCCGGAAG |
|       | 737 | 745 | 0.134348 | CTTCCTCCT |
|       | 805 | 813 | 0.134348 | CTTCCTCCA |
| ETV3  | 22  | 30  | 3.121991 | TAAAGGAAG |
|       | 439 | 447 | 2.299314 | CTTCCTGTG |
|       | 736 | 744 | 6.598007 | GGGCGGAAG |
|       | 886 | 894 | 0.134348 | CTTCCTGCT |
| ETV6  | 119 | 127 | 2.299314 | CTTCCTCTG |
|       | 844 | 852 | 3.121991 | ACAAGGAAG |
| ETV7  | 2   | 10  | 2.987643 | CTTCCTTTC |
| FLI1  | 374 | 382 | 0.957025 | CTTCCTTCA |
|       | 439 | 447 | 0.134348 | CTTCCTGCA |
| SPDEF | 429 | 437 | 0.134348 | CTTCCTCCT |
| SPIB  | 417 | 425 | 2.299314 | CTTCCTCGA |
| SRF   | 209 | 217 | 5.027151 | CTTAGGAAG |

Table 3.2: Promoter analysis of Pea-3 on the promoter of selected targets of ETS member.

| Promoter | Start position | End position | Dissimilarity score (%) | String    |
|----------|----------------|--------------|-------------------------|-----------|
| EHF      | 609            | 617          | 0.597316                | TAACATCCT |
| ELF1     | 813            | 821          | 3.710864                | TATCATCCT |
| ELK1     | 976            | 984          | 1.194633                | AGGATGTGG |
| ELK4     | 480            | 488          | 6.824411                | CTACATCCC |
| ERG      | 890            | 898          | 6.824411                | AGGATGCTA |
| ETS1     | 201            | 209          | 6.824411                | CTTCATCCA |
| ETV3     | 852            | 860          | 6.227095                | AGGATGGAA |
| ETV6     | 146            | 154          | 6.227095                | TTTCATCCA |
|          | 278            | 286          | 4.30818                 | AGACATCCA |
|          | 685            | 693          | 3.113548                | TTTCATCCT |

Table 3.2: Continue.

|      |     |     |          |           |
|------|-----|-----|----------|-----------|
| FEV  | 118 | 126 | 6.824411 | AGGATGGGA |
|      | 277 | 285 | 3.710864 | AGGATGAGA |
| FLI1 | 771 | 779 | 6.824411 | TGGCATCCT |
| SRF  | 26  | 34  | 4.30818  | AGGATGATG |

### 3.2. qPCR Results

To verify microarray results, qPCR method was used in pCDNA3-mPea3, Pea3-VP16, pCDNA3-Elk1 and Elk1-VP16 transfected SH-SY5Y cells. Before performing qPCR, RNA of template cDNA's integrity was checked, and thereafter Elk-1 and Pea3 overexpression in transfected cells were confirmed: Elk-1 overexpression could be detected by qPCR (Figure 3.1), however Pea3 overexpression was not observed due to what later on was found to be a primer specific problem (Figure 3.2, note the scale of y axis as compared to Figure 3.1). Therefore, we have continued our analyses with Elk-1VP16-transfected cells to quantify relative expression of selected ETS targets.

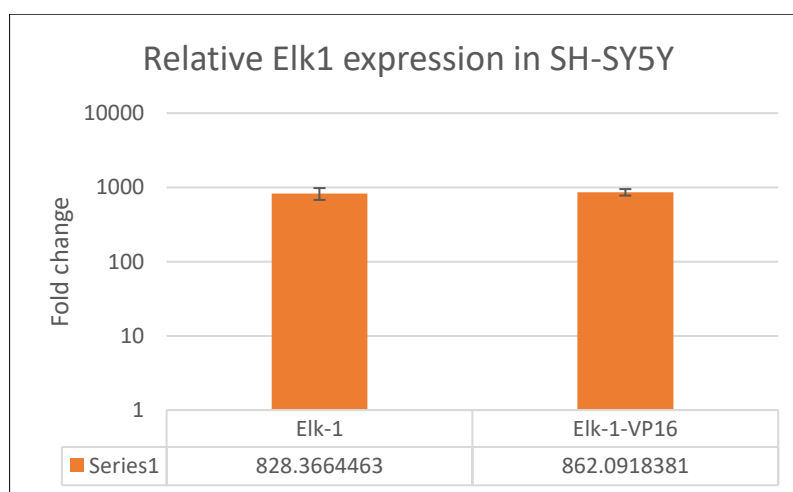


Figure 3.1: Relative expression of Elk-1 in SH-SY5Y cells.

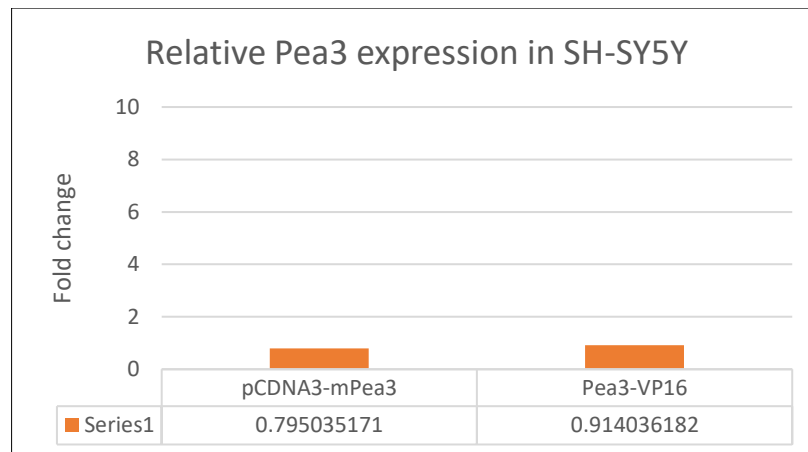


Figure 3.2: Relative expression of Pea3 in SH-SY5Y cells.

Next, we have performed qPCR with Elk1-pCDNA3 and Elk1-VP16 transfected cells to quantify relative expression of ETV3, ETV6, ERF, FEV and ELF2. According to analysis result, relative expression of ETV3, ETV6, ERF and ELF2 are decreased and relative expression of FEV is increased (Figure 3.3.). This study has validated microarray results for ETV3, ETV6 and ERF, but not for FEV and ELF2.

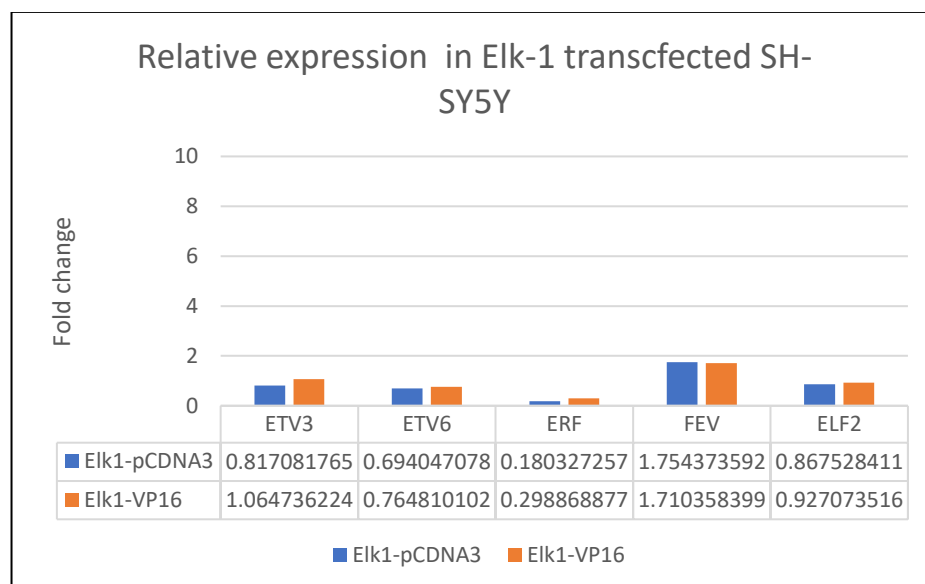


Figure 3.3: Relative expression of selected target of ETS members in SH-SY5Y cells.

### 3.3. Luciferase Reporter Assays

In promoter analysis, only one Pea3 binding site is found on the *Elk1* promoter and dissimilarity value was 1.94%. To confirm and quantify *in silico* promoter analysis results, luciferase reporter assay was performed. *Elk1* promoter activity upon Pea3 over-expression was studied by co-transfection of pGL3 *-elk-1* plasmid and either pCDNA3-mPea3 or Pea3-VP16 expression plasmids in SH-SY5Y cells. Amount of Pea3 plasmids was set from 0 ng to 200 ng (0, 50, 100, 150, 200 respectively). When 50 ng and 100 ng mPea3 expression plasmid were used, *elk-1* promoter activity was increased significantly compared to 0ng mPea3 expression plasmid (Figure 3.3.). However, with 150ng Pea3VP16 expression plasmid transfection, *elk-1* promoter activity was down. In all other plasmid amounts no significant change in luciferase reporter activity was observed.

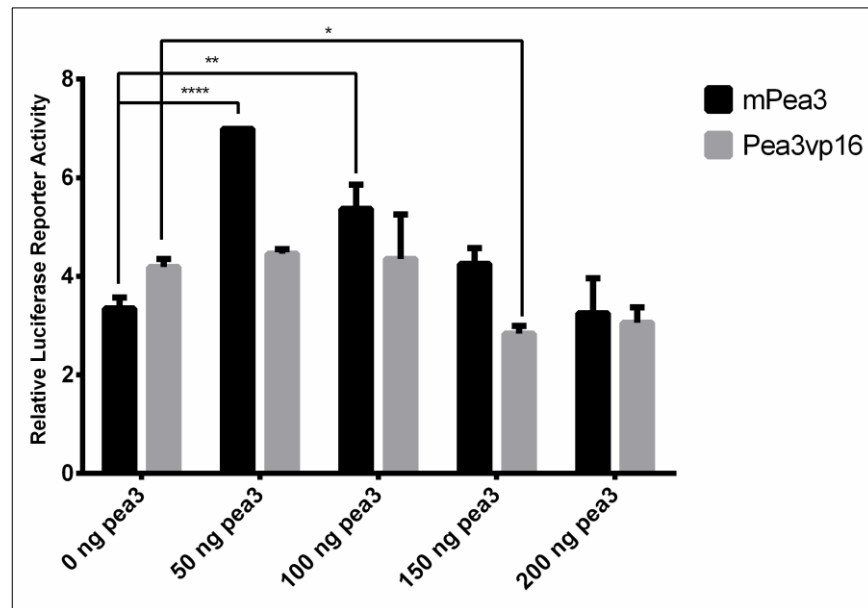


Figure 3.4: *Elk1-Luc* Reporter activity.

### 3.4. Kinetic Model Results

ETS model is constructed as a quantitative kinetic model which describes the values of the variables are determined by numerical analysis of the variables and parameter in the system, based on transcriptional regulation network, especially transcription factor-promoter interaction and its effect on the gene expression.

After the model construction (described in methods), numerical simulation was performed to observe system behavior. First simulations were performed to confirm mathematical expression of our model at the mRNA and protein level with two different parameter sets (mouse and sea urchin) as listed Figure 2.16-17. When the transcriptional activator described in our model binds to its target promoter, mRNA and protein level was increased as expected (Figure 3.5-8), whereas when transcriptional inhibitor binds to the target promoter, mRNA and protein level was briefly increased in the beginning, thereafter quickly degrading as new transcription events were inhibited and existing mRNA or protein was degraded (Figure 3.5-8).

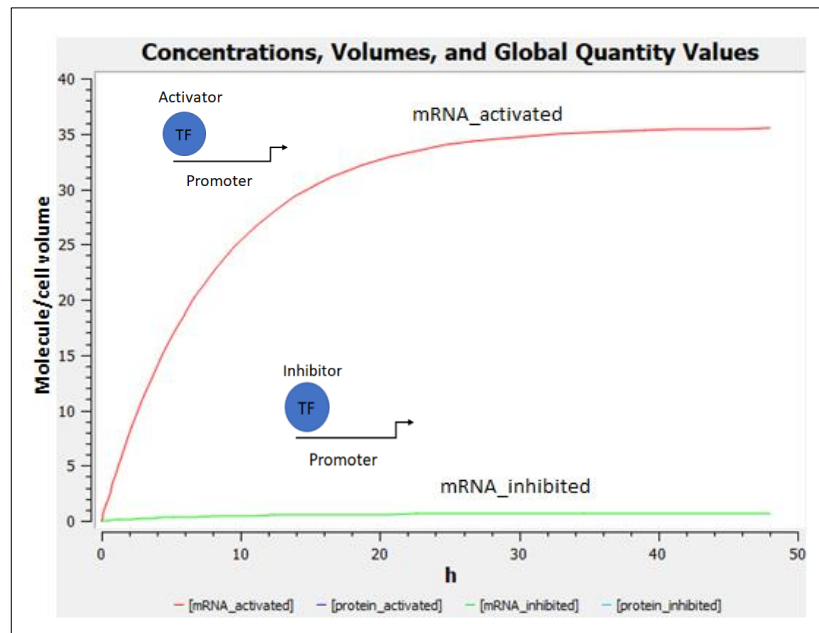


Figure 3.5: mRNA level of basic model simulation with mouse fibroblast parameter values.

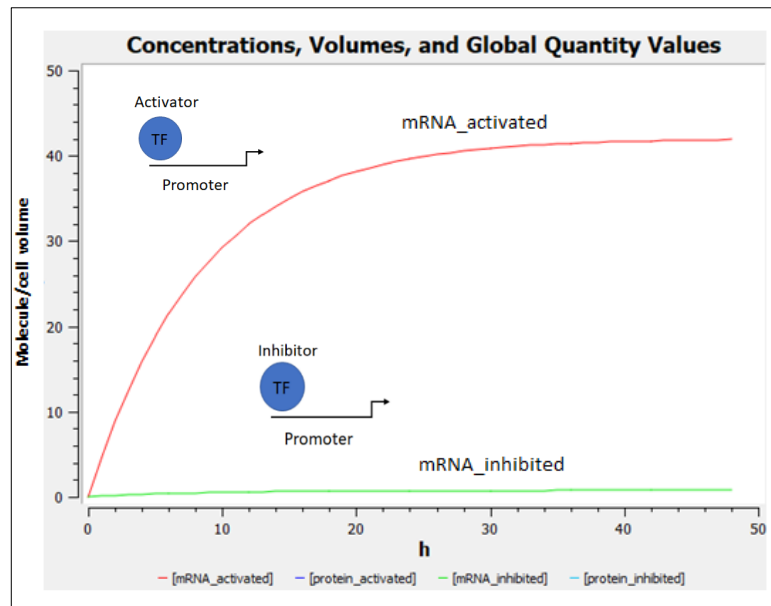


Figure 3.6: mRNA level of basic model simulation with sea urchin parameter values.

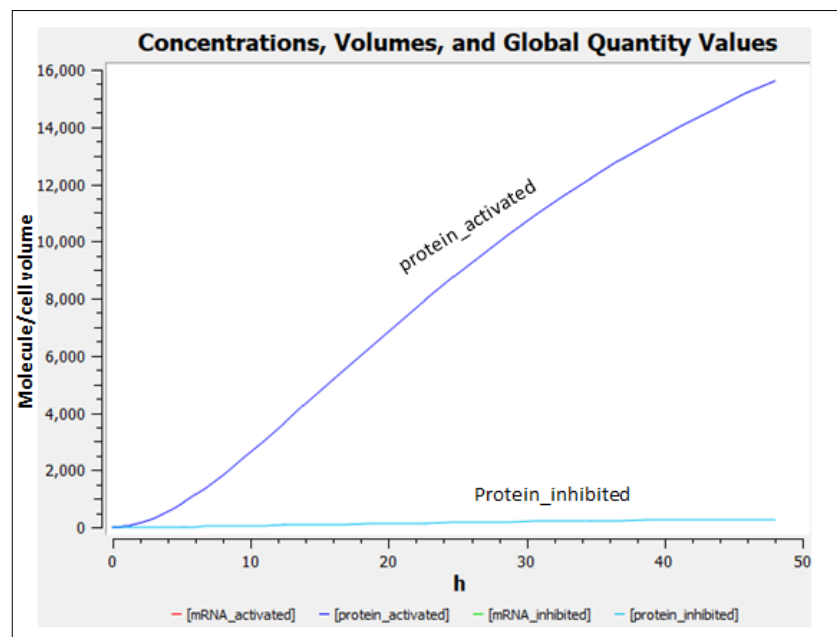


Figure 3.7: Protein level of basic model simulation with mouse fibroblast parameter values.

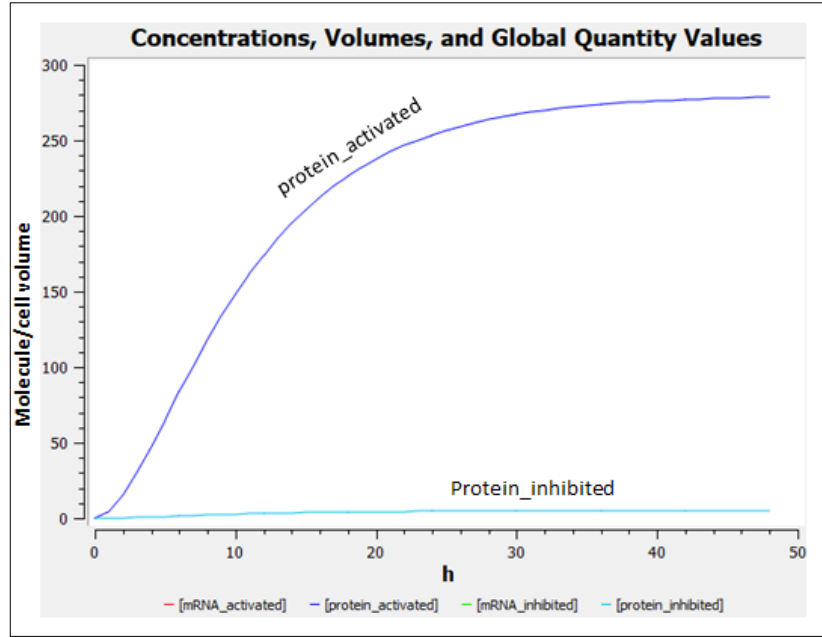


Figure 3.8: Protein level of basic model simulation with sea urchin parameter values.

After the confirmation of the performance of our mathematical expression, we have scanned different activator and inhibitor concentrations using parameter scan task in COPASI. In parameter scan, activator and inhibitor molecule levels were set from 10 to 200 molecule/cell volume and numerical simulation for all parameter was performed in parallel for mRNA and protein levels (Figures 3.9-16). Our results have shown that as the activator and inhibitor molecule levels were increased, mRNA and protein levels were increased or decreased, respectively. One of the values obtained for the activator parameter scan was consistent for data previously reported in literature, indicating that even our basic model was biologically relevant (Figure 3.9-11) [49]. Similarly, for inhibition, parameter scan range included data previously reported in literature for ETS2, validating our model (Figure 3.15-16) [50].

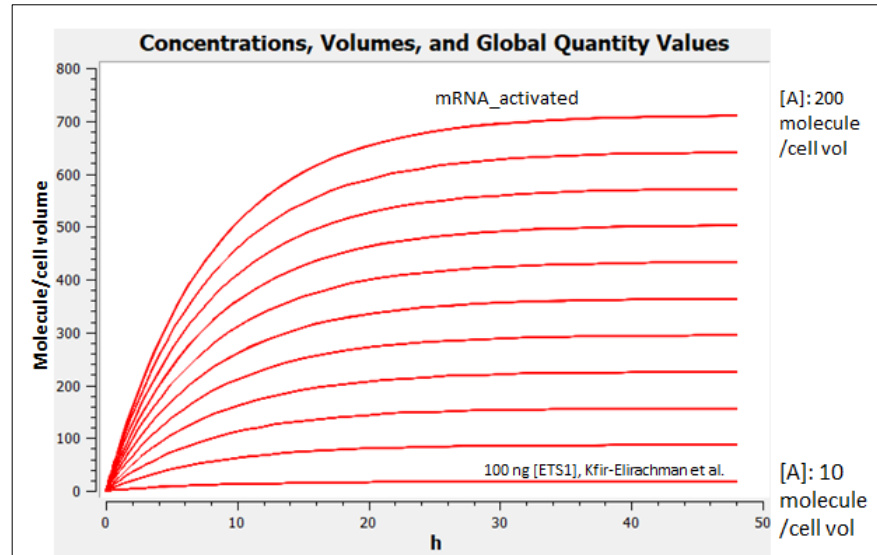


Figure 3.9: Parameter scan result of basic model showing the effect of different activator level concentration on target mRNA levels with mouse fibroblast parameter values.

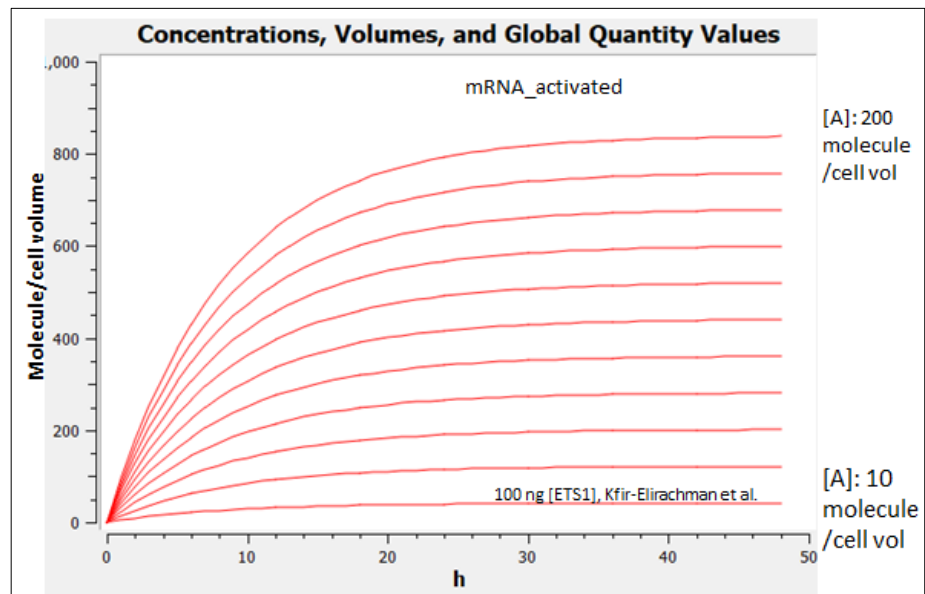


Figure 3.10: Parameter scan result of basic model showing the effect of different activator level concentration on target mRNA levels with sea urchin parameter values.

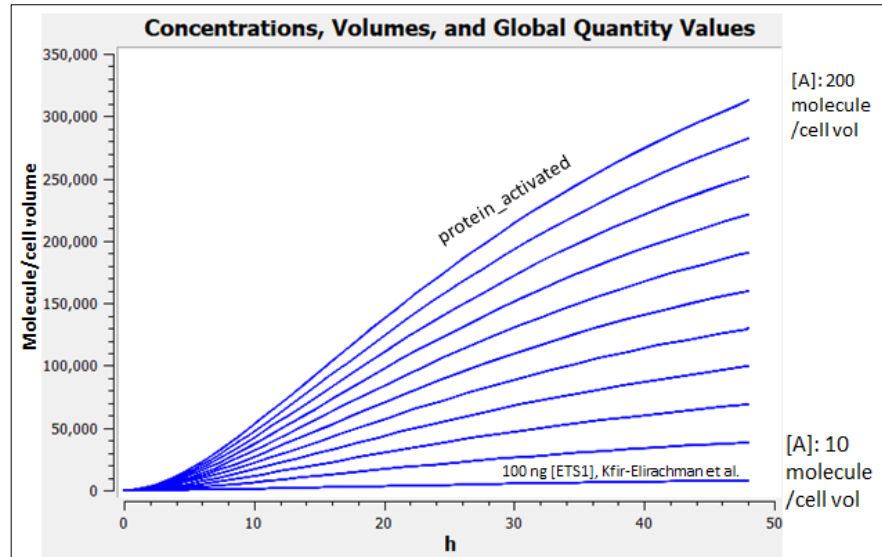


Figure 3.11: Parameter scan result of basic model showing the effect of different activator level concentration on target protein levels with mouse fibroblast parameter values.

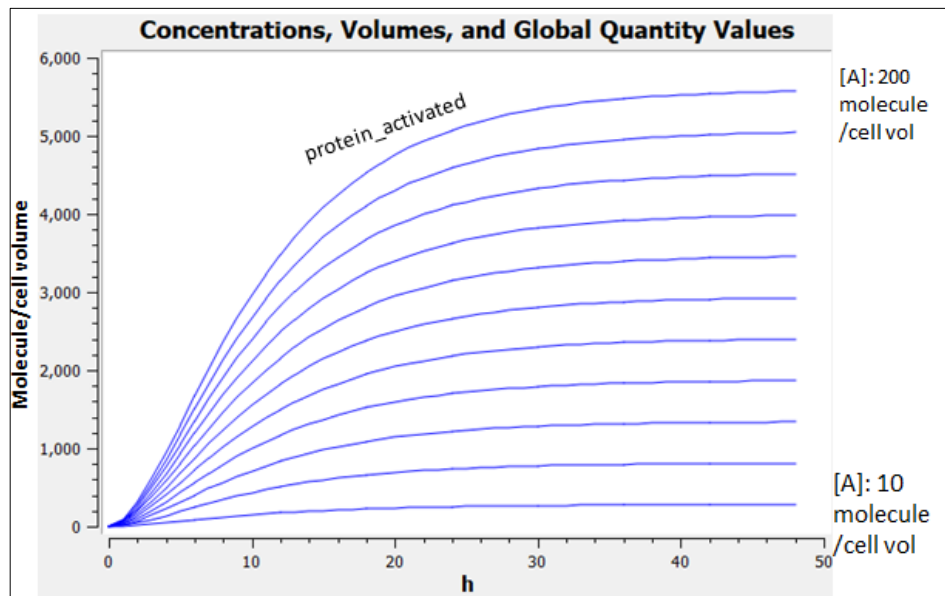


Figure 3.12: Parameter scan result of basic model showing the effect of different activator level concentration on target protein levels with sea urchin parameter values.

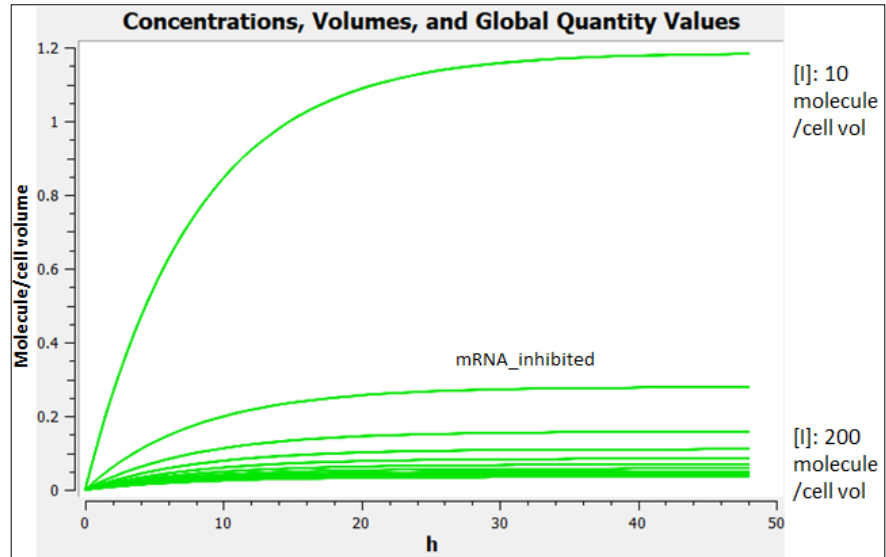


Figure 3.13: Parameter scan result of basic model showing the effect of different inhibitor level concentration on target mRNA levels with mouse fibroblast parameter values.

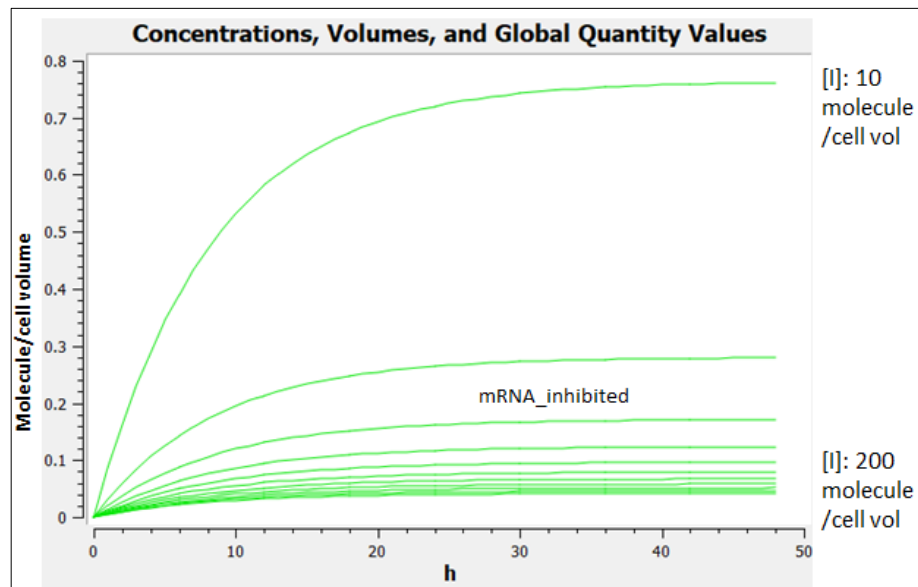


Figure 3.14: Parameter scan result of basic model showing the effect of different inhibitor level concentration on target mRNA levels with sea urchin parameter values.

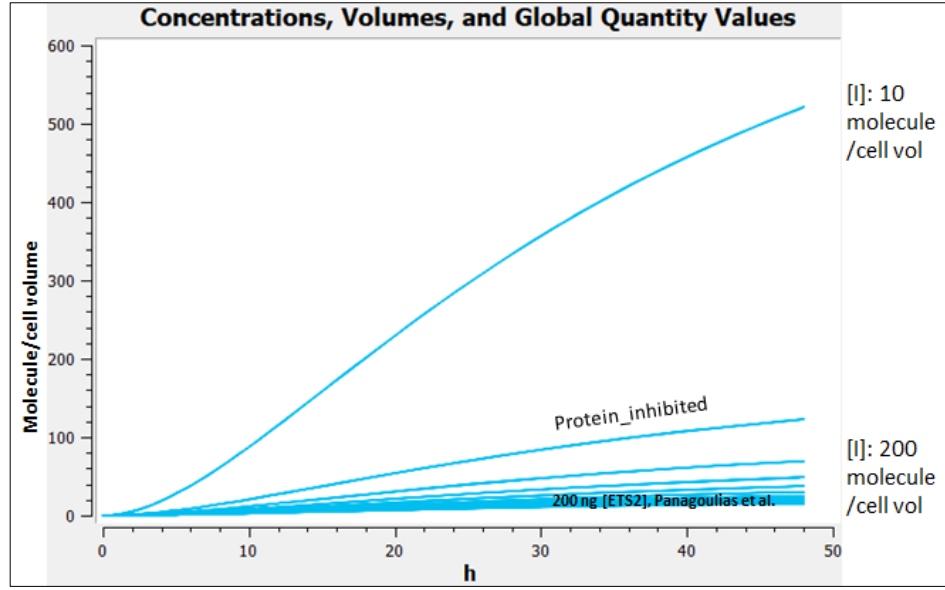


Figure 3.15: Parameter scan result of basic model showing the effect of different inhibitor level concentration on target protein levels with mouse fibroblast parameter values.

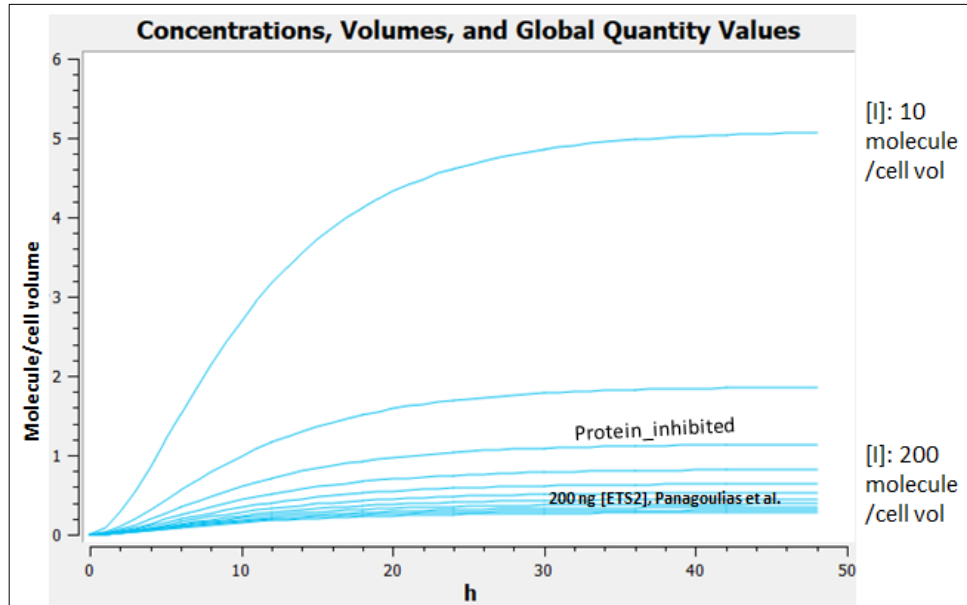


Figure 3.16: Parameter scan result of basic model showing the effect of different inhibitor level concentration on target protein levels with sea urchin parameter values.

Since binding of the transcription factor to its relevant binding motif the promoter is a crucial step in transcriptional regulation, we have next scanned a range of  $K_{ba}$  representing binding in our computational model, using parameter scan task to observe how promoter – transcription factor interaction affects transcription and correspondingly translation outputs.  $K_{ba}$  parameters were set to a range of values from

1 to 100 volume/molecule and simulation was performed for all  $K_{ba}$  parameter values in parallel for both mouse and sea urchin parameter sets (Figure 3.17-24). We have observed that as the  $K_{ba}$  parameter was increased, efficiency of transcription factor as activator or inhibitor was also increased up to a certain value, at which point the transcription or translation reached an equilibrium. Also, we have found different  $K_d$  parameter which indicates disassociation constant of any chemical component, for different ETS members from literature to compare literature  $K_d$  parameter and our derived  $K_{ba}$  parameter [51], [52].

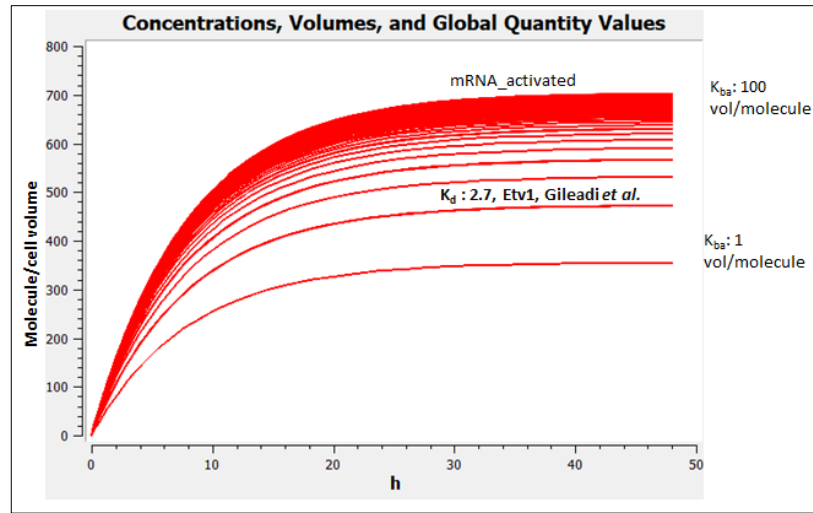


Figure 3.17: Parameter scan result of basic model showing the effect of different  $K_{ba}$  value of activator on target mRNA levels with mouse fibroblast parameter values.

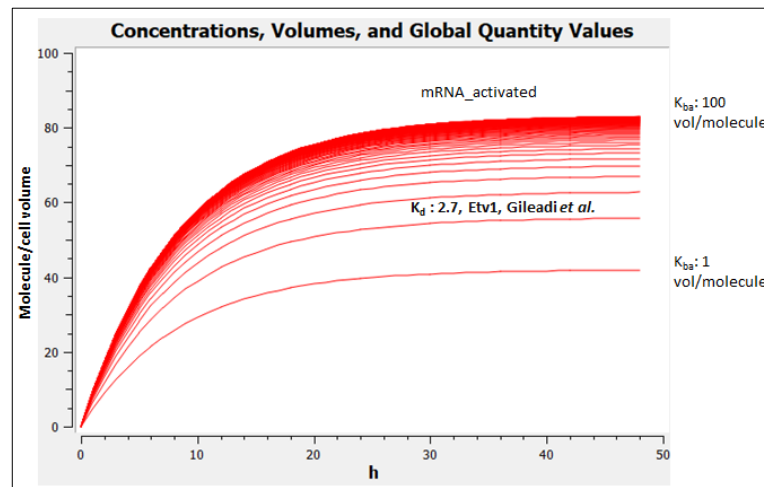


Figure 3.18: Parameter scan result of basic model showing the effect of different  $K_{ba}$  value of activator on target mRNA levels with sea urchin parameter values.

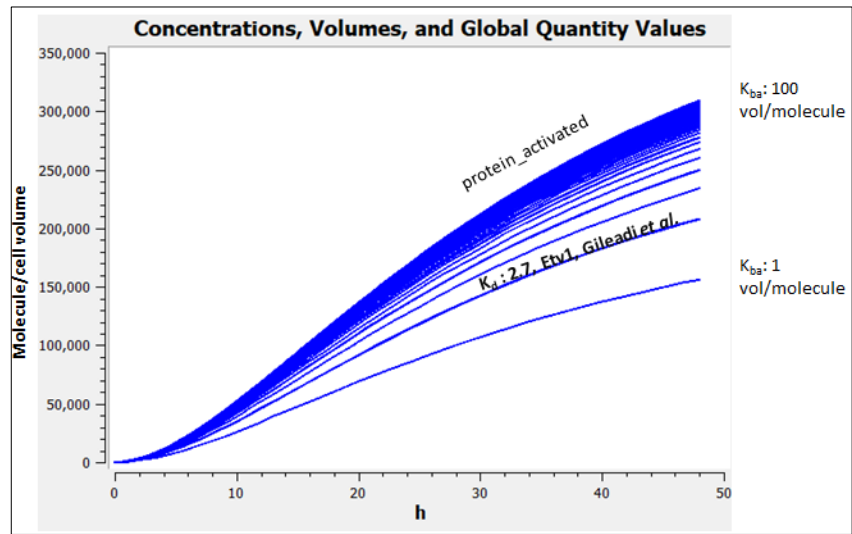


Figure 3.19: Parameter scan result of basic model showing the effect of different  $K_{ba}$  value of activator on target protein levels with mouse fibroblast parameter values.

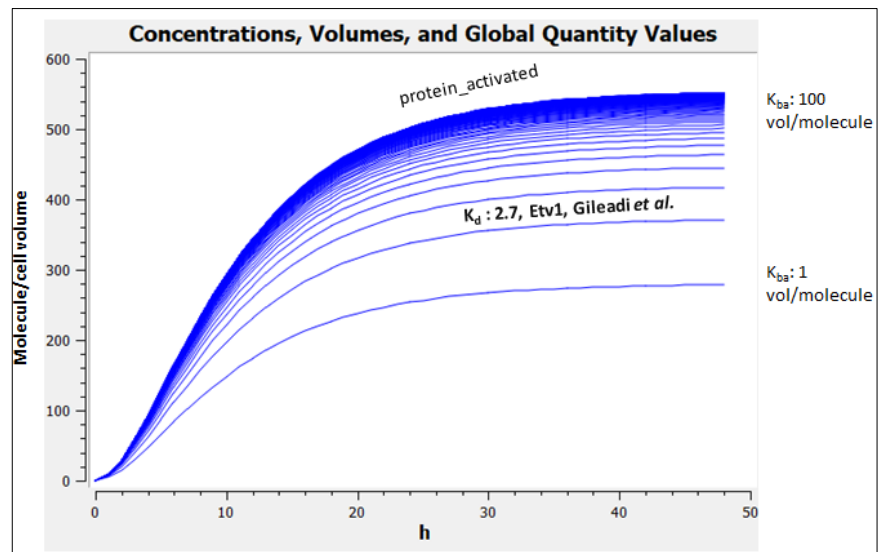


Figure 3.20: Parameter scan result of basic showing the effect of different  $K_{ba}$  value of activator on target protein levels with sea urchin parameter values.

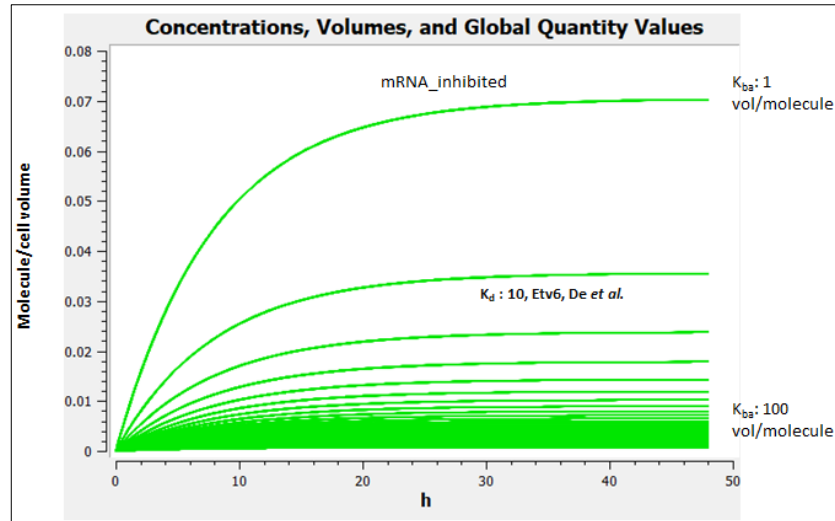


Figure 3.21: Parameter scan result of basic model showing the effect of different  $K_{ba}$  value of inhibitor on target mRNA levels with mouse fibroblast parameter values.

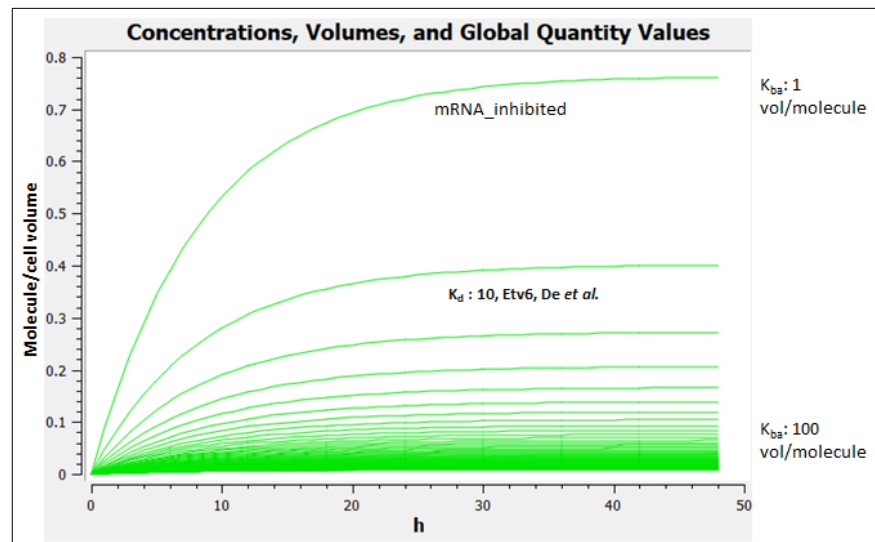


Figure 3.22: Parameter scan result of basic mode showing the effect of different  $K_{ba}$  value of inhibitor on target mRNA levels with sea urchin parameter values.

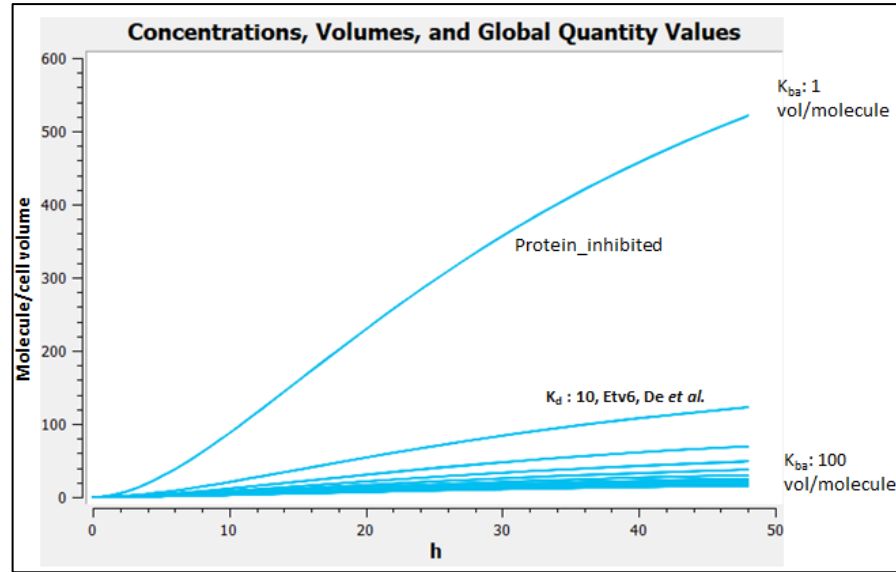


Figure 3.23: Parameter scan result of basic model showing the effect of different  $K_{ba}$  value of inhibitor on target protein levels with mouse fibroblast parameter values.

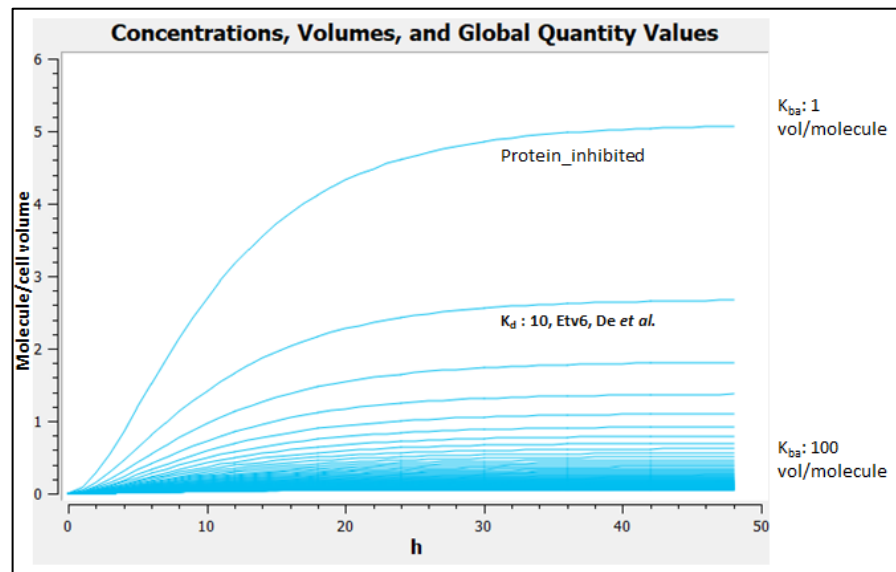


Figure 3.24: Parameter scan result of basic model showing the effect of different  $K_{ba}$  value of inhibitor on target protein levels with sea urchin parameter values.

After the mathematical confirmation of the simple kinetic model for transcriptional regulation, we have then constructed the more complex ETS intrafamilial regulation network presented in Figure 1.6 (see Appendix A, Tables A1.1 and A1.2 for detailed reaction steps of the network). As explained in introduction, we hypothesized that there are intrafamilial regulation network within

ETS family members. Therefore, we tried to simulate this regulation network by using our model topology and mathematical expression. We wanted to understand how all these ETS members behave in the model system. Before the constructing the complex ETS model, we had several assumptions:

- Initial concentration of mRNAs are zero (there is no synthesized mRNA before the simulation).
- Initially  $K_{ba}$  parameter is 1 volume/molecule for all ETS transcription factors (disassociation and association reactions are in equilibrium).
- Initial concentration of all ETS proteins are same.

With these assumptions the ETS model was constructed as described in detail at method section. Transcription factor of complex model are ETS family members. Briefly, in ETS model, mRNA synthesis of ETS members are transcriptionally regulated by other ETS members with promoter-TF interaction and its effect on the transcription processes. Then synthesized mRNAs of ETS members effects protein synthesis of ETS members. Degradation reactions also was determined to stabilize complex ETS model for the simulate transcriptional regulations network at more realistic environment. Thereafter numerical simulations were performed with both mouse fibroblast and sea urchin parameter sets. Model component and reactions was listed in Appendix.

Initially, for ETS model, mRNA and protein level were observed by using numerical simulation 48-hour time scale as same as basic model with mouse fibroblast parameter sets. According this simulation, at the early time points of the simulations, ER81, PEA3, ELK1, ERF and ERM mRNAs level are increased, however at the end of the simulation time their mRNA and protein level decrease and reached the basal level. mRNA and protein level of EHF, ELF3 and ETV3L increased dramatically. Overall, protein level of all ETS members increased in these simulations (Figure 3.25-28).

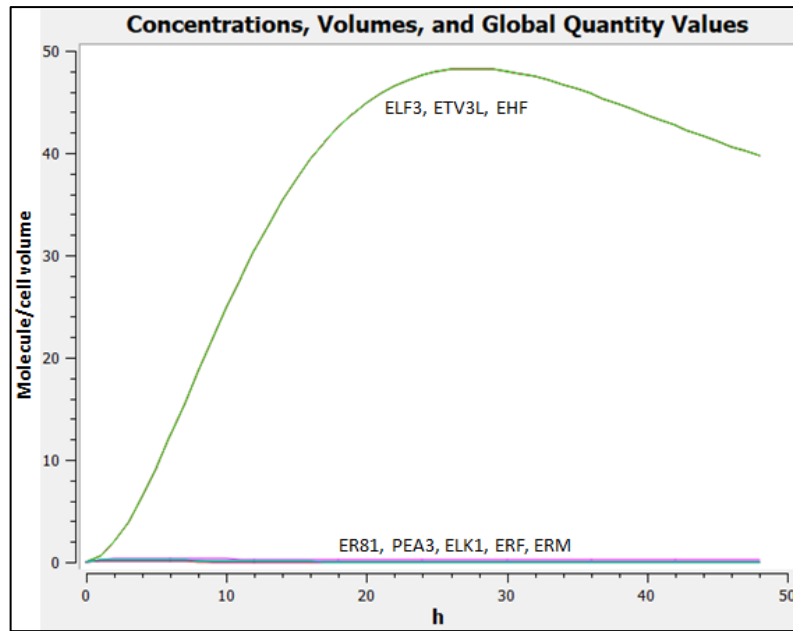


Figure 3.25: mRNA level of ETS members within model with mouse fibroblast parameter values.

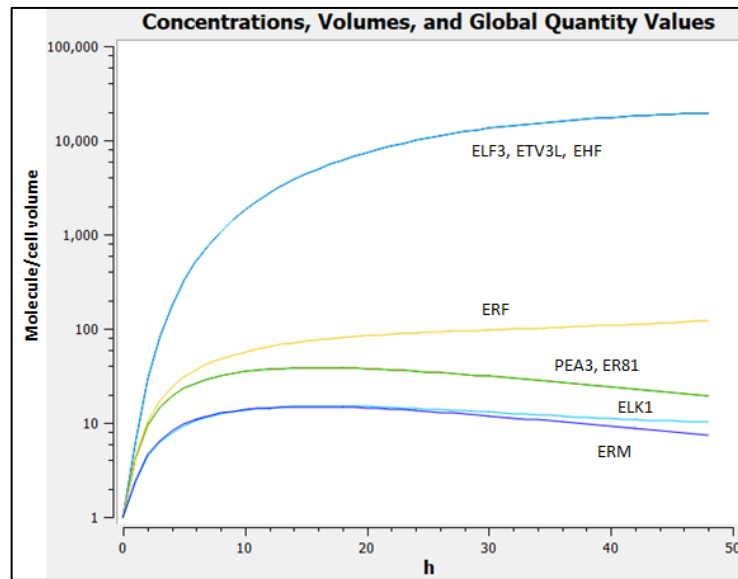


Figure 3.26: Protein level of ETS members within model with mouse fibroblast parameter values.

After that, for ETS model, mRNA and protein levels were analyzed with another numerical simulation set using sea urchin parameter values. In this run, mRNA and protein level of ETV3L, EHF and ELF3 are increased dramatically, although mRNA and protein levels of ERF and ELK1, PEA3, ERM and ER81 are decreased (Figure 3.27-28).

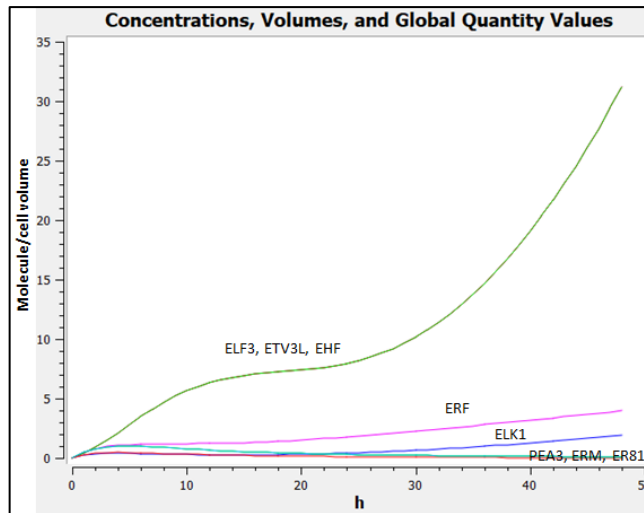


Figure 3.27: mRNA level of ETS members within model with sea urchin parameter values.

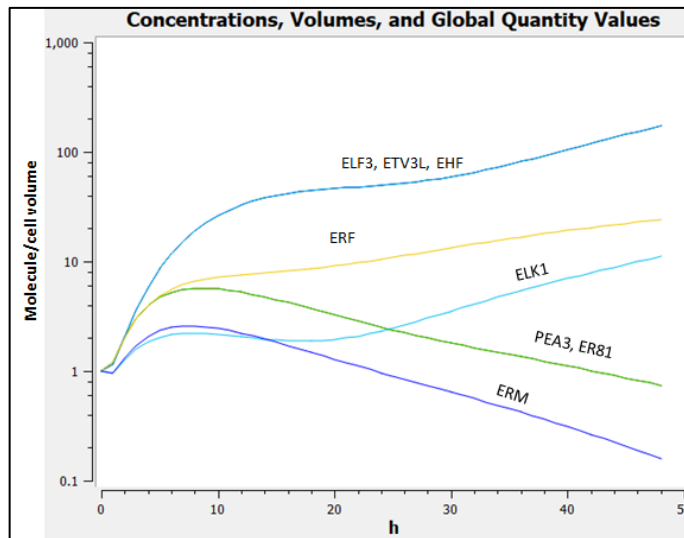


Figure 3.28: Protein level of ETS members within model with sea urchin parameter values.

Next, to model transfection and overexpression of ELK1 and PEA3 in a manner similar to the microarray experiment, we used different initial ELK1 and PEA3 protein concentrations, by changing the concentration by 10-fold from initial concentration with both mouse fibroblast and sea urchin parameter set values using parameter scan task in COPASI.

When ELK1 was thus gradually “over-expressed”, overall behavior of the model was not changed, however EHF, ELF3 and ETV3L concentrations were dramatically increased in early stages of simulation at both mRNA and protein level in both mouse fibroblast and sea urchin parameter set values (Figure 3.29-32).

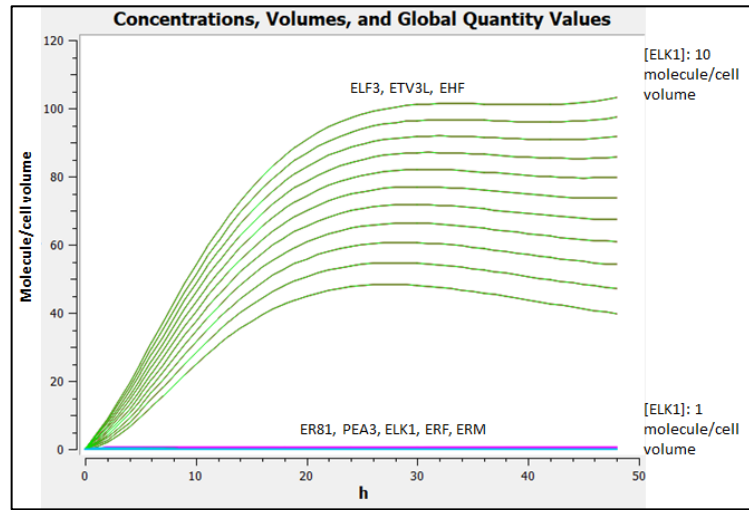


Figure 3.29: Parameter scan result showing the effect of ELK1 over-expression at mRNA level of ETS members in the model with mouse fibroblast parameter values. Each line represents parallel simulation of different ELK1 initial concentration from 1 to 10 molecule/cell volume. Bottom line indicates the ELK1 initial concentration is 1, top line indicates the ELK1 initial concentration is 10.

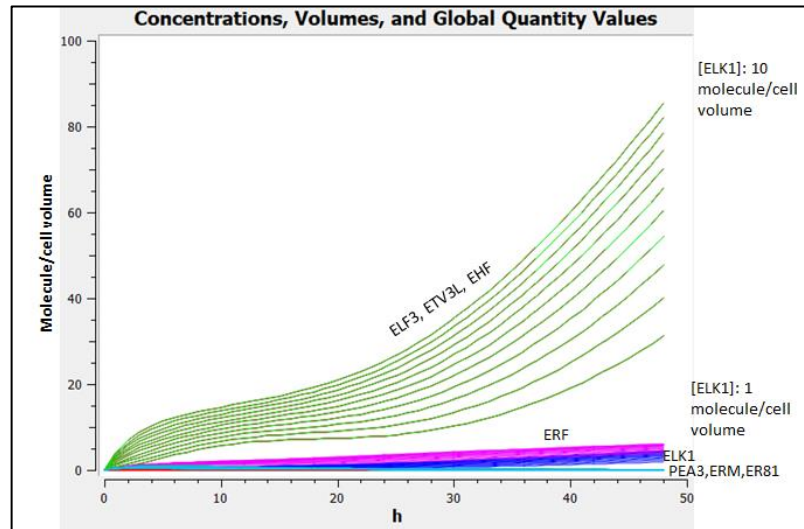


Figure 3.30: Parameter scan result showing the effect of ELK1 over-expression at the mRNA level of ETS members in the model with sea urchin parameter values. Green lines indicate mRNA level of ELF3, ETV3L and EHF, pink line indicate mRNA level of ERF, blue line indicate mRNA level of ELK1, light blue line indicate mRNA level of PEA3, ERM and ER81.

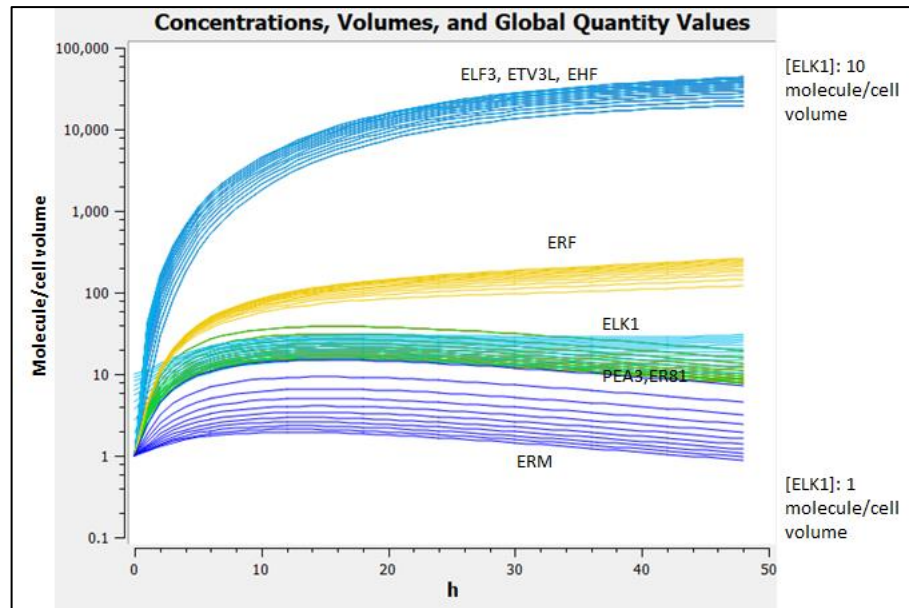


Figure 3.31: Parameter scan result showing the effect of ELK1 over-expression at protein level of ETS members in the model with mouse fibroblast parameter values.

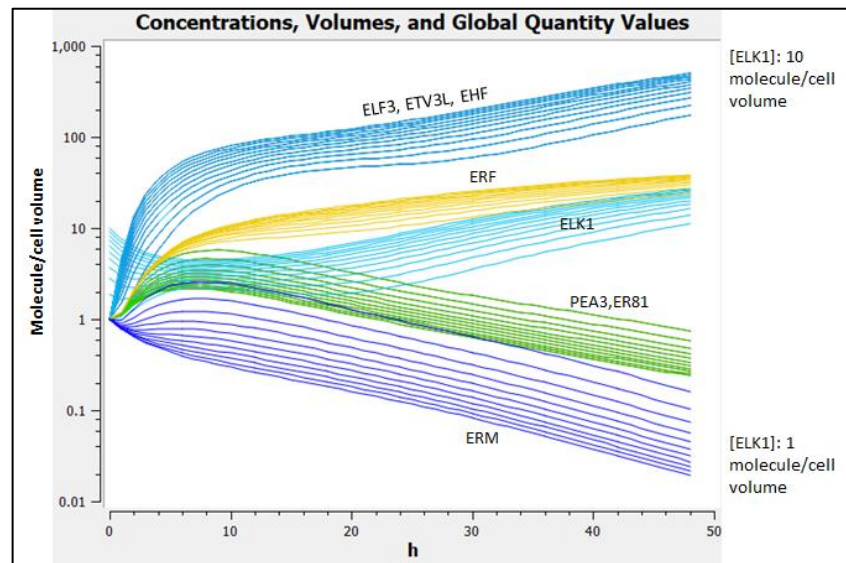


Figure 3.32: Parameter scan result showing the effect of ELK1 over-expression at protein level of ETS members in the model with sea urchin parameter values.

Similarly, when PEA3 was gradually over-expressed, initial behavior of ETS model was again unchanged (Figure 3.33-34).

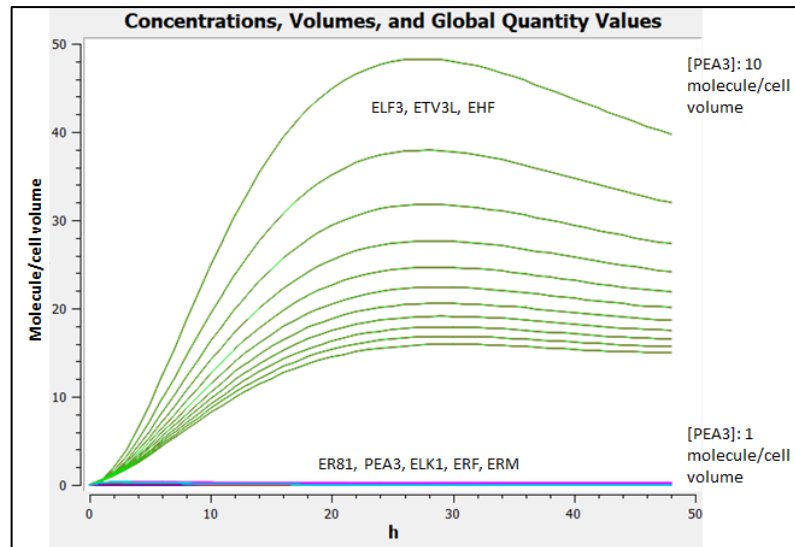


Figure 3.33: Parameter scan result showing the effect of PEA3 over-expression at mRNA level of ETS members in the model with mouse fibroblast parameter values. Each line represents parallel simulation of different PEA3 initial concentration from 1 to 10 molecule/cell volume. Bottom line indicates the PEA3 initial concentration is 1, top line indicates the PEA3 initial concentration is 10.

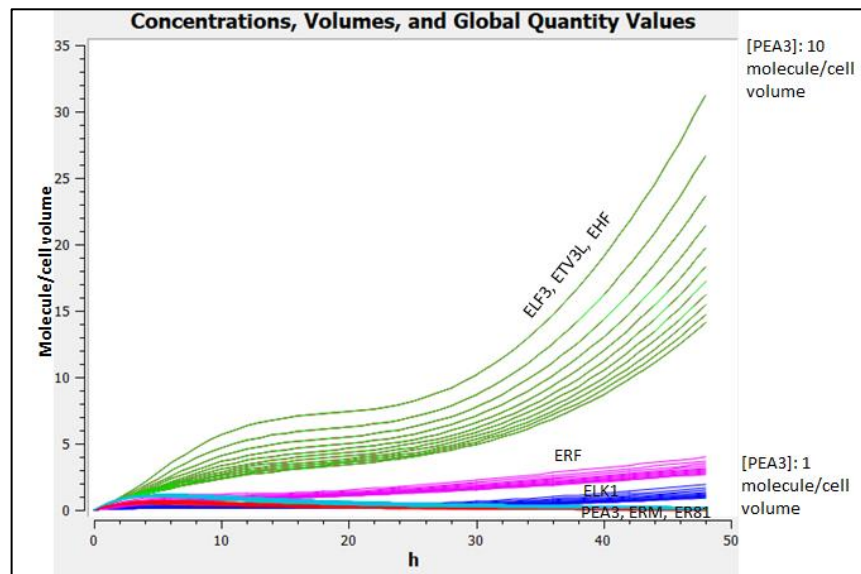


Figure 3.34: Parameter scan result showing the effect of PEA3 over-expression at mRNA level of ETS members in the model with sea urchin parameter values.

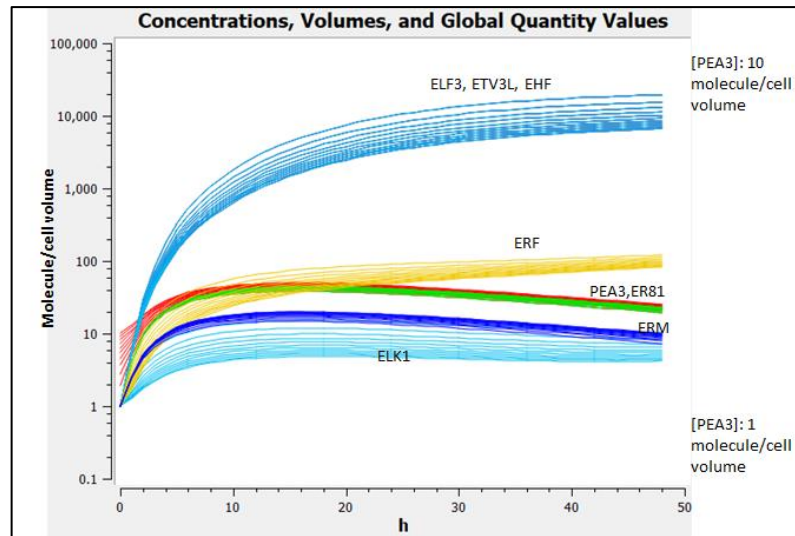


Figure 3.35: Parameter scan result showing the effect of PEA3 over-expression at protein level of ETS members in the model with mouse fibroblast parameter values.

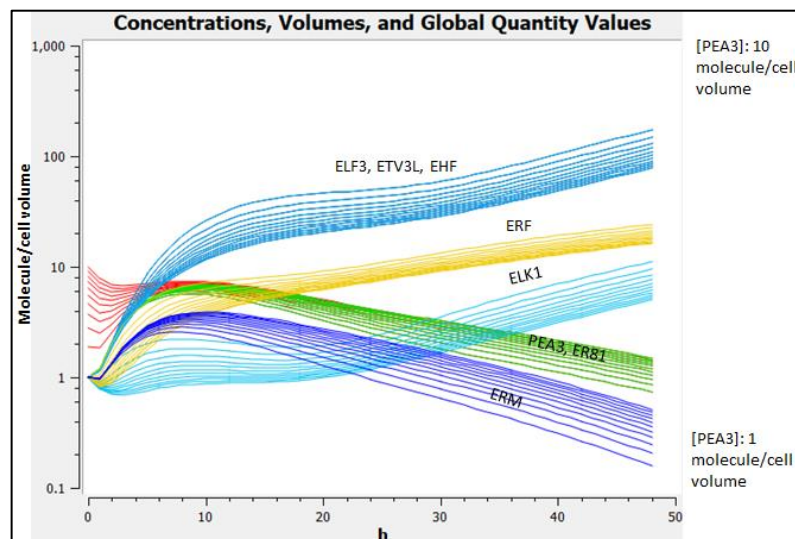


Figure 3.36: Parameter scan result showing the effect of PEA3 over-expression at protein level of ETS members in the model with sea urchin parameter values.

## 4. DISCUSSION and CONCLUSION

ETS protein superfamily has widely different function and regulates so many different biological processes. For example, Pea3 expression is vital chick retina development and directs axonal outgrowth [19], [20]. ETS proteins mediate these processes by interacting with another regulatory element and interacting with each other. ETS family members generally interconnect each other with co-overexpression to regulate biological processes [21], [30].

In this thesis we tried to understand intra-familial regulation network of ETS transcription factor family based on previous work from our laboratory in SH-SY5Y human neuroblastoma cell line, particularly focusing on Pea3 and Elk1. In this thesis, combination of molecular and computational methodology was used. In molecular part, promoter analysis, qPCR and luciferase reporter assay were used to observe ETS regulation network within the family in vitro level. In computational part, quantitative kinetic model was constructed to simulate ETS members regulation network.

In molecular part of thesis, transcriptional measurement methods were used to understand our system. In the confirmation of microarray analysis, qPCR was performed in Elk-1 and Pea3 transfected SH-SY5Y cells. Firstly, we tried to observe overexpression by using qPCR with Elk1 and Pea3 specific primer. Although we could not recapitulate Pea3 overexpression in our assay system due to primer specific problem, Elk1 overexpression was achieved. Thereafter, we continued with Elk-1 transfected cells and quantify relative expression of selected ETS member targets (Figure 3.3) and most of relative expression of these targets are correlated with microarray analysis results within the context of this thesis. In the future, all putative ETS targets from microarray findings should be validated with qPCR and other assays and be incorporated into the computational ETS regulation model.

Promoter analysis was also performed to determine Elk1 and Pea3 transcription factor binding site on the selected ETS targets (Figure 3.1-2), and a preliminary luciferase reporter assay was performed in Pea3-transfected SH-SY5Y cells to quantify luciferase reporter activity on the *elk1* promoter (Figure 3.4). Our luciferase reporter assay results have shown that *elk-1* promoter activity was increased significantly in 50 ng and 100 ng mPea3 expression plasmid transfection compared to 0 ng mPea3 plasmid. However, in previous microarray results from our laboratory,

as well as qPCR validations, had yielded strong downregulation of *elk-1* message. Further analyses are required to elucidate this discrepancy, which might result due to the time required for luciferase assay.

It is interesting to note that when promoter binding predictions, ie dissimilarity scores (ds), were converted to similarity scores (ss, calculated as  $100 - ds$ ), no correlation between predicted binding existed with microarray results (Table 4.1-2).

Table 4.1: Comparison of similarity score of promoter analysis for Pea3 and microarray results.

| Down regulated |             |                      |
|----------------|-------------|----------------------|
| Gene           | Fold change | Similarity score (%) |
| FEV            | 0.10        | 93.18                |
|                |             | 96.29                |
| ELK1           | 0.34        | 98.81                |
| Up regulated   |             |                      |
| Gene           | Fold change | Similarity score (%) |
| Fli1           | 1.43        | 93.18                |

Table 4.2: Comparison of similarity score of promoter analysis for Elk1 and microarray results.

| Down regulated |             |                      |
|----------------|-------------|----------------------|
| Gene           | Fold change | Similarity score (%) |
| ETS1           | 0.30        | 99.18                |
| ETS2           | 0.74        | 97.01                |
|                |             | 97.70                |
|                |             | 93.40                |
|                |             | 99.87                |
|                |             | 99.87                |
| ELF1           | 0.50        | 94.97                |
| ERF            | 0.20        | 100                  |
|                |             | 97.70                |
| ETV6           | 0.48        | 97.70                |
|                |             | 96.88                |

Table 4.2: Continue.

| Up regulated |             |                      |
|--------------|-------------|----------------------|
| Gene         | Fold change | Similarity score (%) |
| ERG          | 2.17        | 100                  |
|              |             | 97.84                |
| ETV3         | 1.09        | 96.88                |
|              |             | 97.70                |
|              |             | 93.40                |
|              |             | 99.87                |
| FLI1         | 1.42        | 99.04                |
|              |             | 99.87                |
| SPDEF        | 2.61        | 99.87                |
| ELF2         | 1.37        | 97.70                |
|              |             | 100                  |
|              |             | 97.70                |
|              |             | 93.40                |
|              |             | 97.84                |
| ELF5         | 2.94        | 95.80                |
|              |             | 96.62                |
|              |             | 96.75                |
| ETV7         | 1.70        | 97.01                |
| EHF          | 2.30        | 100                  |
| SPIB         | 1.92        | 97.70                |

This might be due to the complexity of transcriptional regulation of these ETS genes, and the complex inter-regulatory relationships between various ETS Transcription factors and promoters, as is the main theme of this thesis. Hence, a direct correlation between a single transcription factor binding may not be indicative of the system output.

Thus, in computational part of thesis, initially we tried to address this systems behavior and determine mathematical explanation of transcription factor-promoter interaction as well as their effect on the gene expression. There are different mathematical explanations of TF-promoter interaction in the literature [45], [46]. After having investigated many of these mathematical expressions of TF-promoter interaction, we modified our equations from [47]. In our model topology, four types of reactions were determined to construct our model: transcription which means the mRNA synthesis from genetic code, translation, mRNA and proteins degradations. In the mathematical expression of transcription reaction, we modified the specific parameter we have termed  $K_{ba}$  to be compatible with molecular experimental data. In the literature, binding affinity of chemical component can be deciphered from

dissociation constant  $K_d$ , which essentially is a measure of the disassociation of any molecule to its binding partner, such as in protein-protein, enzyme-substrate, receptor-ligand or in our case DNA-protein binding [51], [52]. If we compare the  $K_d$  parameter with our  $K_{ba}$  parameter for proteins:  $K_d$  parameter is calculate from disassociation constant of proteins under the different conditions, however our  $K_{ba}$  parameter is ratio of association and disassociation constant of proteins (see the derivation of  $K_{ba}$  parameter in methods). Another difference between these two parameters is their units. Unit of  $K_d$  parameter is Molar, but our  $K_{ba}$  parameter unit is volume/molecule. They are thus comparable entities, and our  $K_{ba}$  definition allows us to directly use literature data on our formulas.

After the defining the mathematical expression of our model, we constructed basic version of model to confirm our model mathematically. In this part, when the activator or inhibitor binds to promoter, mRNA and protein synthesis are increased and decreased respectively. Thereafter, we changed activator and inhibitor level to investigate model behavior in different conditions. Results of parameter scan have shown that mRNA and protein level was affected from different concentrations of activator or inhibitor correctly (Figure 3.9-16). For the literature validation, several ETS members concentrations was found as activator or inhibitor [49], [50]. Although their concentrations units were not same as our model quantity units, the results from these studies are in our parameter scan results range, which we claim validates our model's biological relevance.

We also used two parameter sets to construct our ETS model and performed all numerical simulations in parallel with two parameter sets: mouse fibroblast and sea urchin parameter sets. These two parameter values are found from literature listed in method section (Table 2.16-17). All numerical simulations have shown that result of these parameter sets affect our system quantitatively; however, no significant difference in overall system behavior was observed. Furthermore, output from different parameter scans correlated well with previously reported data in the literature, validating the mathematical approach in our model.

Following from this, when complex ETS regulatory network was modeled and Pea3 was overexpressed in this system, ETV3L and ERF levels were found to be dramatically increased. ETV3L and ERF are two ETS proteins required for primary neurogenesis [27], and known to be regulated in response to Pea3, which is quite successfully predicted by our model. All other estimations of different biological

processes related with ETS family regulation network could be implemented by using ETS model predictions.

As a conclusion, in this study we have successfully set up a mathematical model to study intrafamilial transcriptional regulatory network of a selected subset of ETS family transcription factors, complemented in part by experimental validations carried out in this study and in part from previous literature. Because all ETS members could not be implemented in this study, this model could act as a basis of a more complex network of ETS factors, and different conditions, in the future. The model could also be extended to incorporate species-specific or tissue-specific networks to observe more complex transcriptional dynamics in a tissue- and organism-dependent manner.

In the future, it would also provide great mechanistic insight if other proteins or signals involved in the regulation of this network could be incorporated, such as SUMOylation, MAPK signaling or apoptosis. To do that, new kinetic reactions such as protein-protein interactions, protein phosphorylation etc. should be included to the ETS network model presented in this thesis. Our overall aim is construction of multi-dimensional, large scale intra-familial regulatory network of ETS family of transcription factors supported by *in vitro* kinetic data to understand different biological processes under the regulation of ETS members at the different cell types, tissues and different conditions.

## REFERENCES

- [1] Sharrocks A. D., (2001), “The ETS-domain transcription factor family.”, *Nature Reviews Molecular Cell Biology*, 2 (11), 827-37.
- [2] Poon G. M. K., Kim H. M., (2017), “Signatures of DNA target selectivity by ETS transcription factors.”, *Transcription*, 8 (3), 93-203.
- [3] Leprince D., Gegonne A., Coll J., de Taisne C., Schnneeberger A., Lagrou C., Stehelin D., (1983), “A putative second cell-derived oncogene of the avian leukaemia retrovirus E26.”, *Nature*, 306 (5941), 395-7.
- [4] Gutierrez-Hartmann A., Duval D. L., Bradford A. P., (2007), “ETS transcription factors in endocrine systems.”, *Trends in Endocrinology & Metabolism*, 18 (4), 150-158.
- [5] Oikawa T., Yamada T., (2003), “Molecular biology of the Ets family of transcription factors.”, *Gene*, 303, 11-34.
- [6] Kar A., Gutierrez-Hartmann A., (2013), “Molecular mechanisms of ETS transcription factor-mediated tumorigenesis.”, *Critical Reviews in Biochemistry and Molecular Biology*, 48 (6), 522-543.
- [7] Lacronique V., Boureux A., Valle V. D., Poirel H., Quang C. T., Mauchauffe M., Berthou C., Lessard M., Berger R., Ghysdael J., Bernard O. A., (1997), “A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia.”, *Science*, 278 (5341), 1309-12.
- [8] Baker D. A., Mille-Baker B., Wainwright S. M., Ish-Horowicz D. and Dibb N. J., (2001), “Mae mediates MAP kinase phosphorylation of Ets transcription factors in *Drosophila*.”, *Nature*, 411 (6835), 330-4.
- [9] Wei G. H., Badis G., Berger M. F., Kivioja T., Palin K., Enge M., Bonke M., Jolma A., Varjosalo M., Gehrke A. R., Yan J., Talukder S., Turunen M., Taipale M., Stunnenberg H. G., Ukkonen E., Hughes T. R., Bulyk M. L., Taipale J., (2010), “Genome-wide analysis of ETS-family DNA-binding in vitro and in vivo.”, *EMBO Journal*, 29 (13), 2147-60.
- [10] Yordy J. S., Muise-Helmericks R. C., (2000), “Signal transduction and the Ets family of transcription factors.”, *Oncogene*, 19 (55), 6503-13.
- [11] Kfir-Elirachman K., Ortenberg R., Vitzel B., Besser M. J., Barshack I., Schachter J., Nemlich Y., Markel G., (2018), “Regulation of CEACAM1 Protein Expression by the Transcription Factor ETS-1 in BRAF-Mutant Human Metastatic Melanoma Cells.”, *Neoplasia*, 20 (4), 401-409.
- [12] Panagoulas I., Karagiannis F., Aggeletopoulou I., Georgakopoulos T., Argyropoulos C. P., Akinosogou K., Gogos C., Skotelis A., Mouzaki A.,

- (2017), “Ets-2 Acts As a Transcriptional Repressor of the Human Immunodeficiency Virus Type 1 through Binding to a Repressor-Activator Target Sequence of 5'-LTR.”, *Frontiers in Immunology*, 8 (1924).
- [13] Harada H., Omi M., Sato T., Nakamura H., (2015), “Pea3 determines the isthmus region at the downstream of Fgf8-Ras-ERK signaling pathway.”, *Development, Growth & Differentiation*, 57 (9), 657-666.
  - [14] Selvaraj N., Kedage V. and Hollenhorst P. C., (2015), “Comparison of MAPK specificity across the ETS transcription factor family identifies a high-affinity ERK interaction required for ERG function in prostate cells.”, *Cell Communication and Signaling*, 14 (1).
  - [15] Hennenberg M., Strittmatter F., Beckmann C., Rutz B., Füllhase C., Waidelich R., Montorsi F., Hedlund P., Andersson K. E., Stief C. G., Gratzke C., (2012), “Silodosin inhibits noradrenaline-activated transcription factors Elk1 and SRF in human prostate smooth muscle.”, *PLoS One*, 7 (11).
  - [16] McCabe K. L., McGuire C., Reh T. A., (2006), “Pea3 expression is regulated by FGF signaling in developing retina.”, *Developmental Dynamics*, 235 (2), 327-335.
  - [17] Kandemir B., Caglayan B., Housott B., Erdogan B, Dag U., Demir O., Sogut M. S., Klimaschewski L., Kurnaz I. A., (2014), “Pea3 transcription factor promotes neurite outgrowth.”, *Frontiers in Molecular Neuroscience*, 7.
  - [18] Mesquita D., Barros-Silva J. D., Santos J., Skotheim R. I., Lothe R. A., Paulo P., Teixeira M. R., (2015), “Specific and redundant activities of ETV1 and ETV4 in prostate cancer aggressiveness revealed by co-overexpression cellular contexts.”, *Oncotarget*, 6 (7), 5217-36.
  - [19] Kurpios N. A., Sabolic N. A., Shepherd T. G., Fidalgo G. M. and Hassell J. A., (2003), “Function of PEA3 Ets transcription factors in mammary gland development and oncogenesis.”, *Journal of Mammary Gland Biology Neoplasia*, 8 (2), 177-90.
  - [20] Sharrocks A. D., (2002), “Complexities in ETS-domain transcription factor function and regulation: lessons from the TCF (ternary complex factor) subfamily. The Colworth Medal Lecture.”, *Biochemical Society Transactions*, 30 (2), 1-9.
  - [21] Pastorcic M., Das H. K., “Ets transcription factors ER81 and Elk1 regulate the transcription of the human presenilin 1 gene promoter.”, *Molecular Brain Research*, 113 (1-2), 57-66.
  - [22] Zhang X., Zhang B., Gao J., Wang X., Liu Z., “Regulation of the MicroRNA 200b (miRNA-200b) by Transcriptional Regulators PEA3 and ELK-1 Protein Affects Expression of Pin1 Protein to Control Anoikis.”, *Journal of Biological Chemistry*, 288 (45), 32742-32752.

- [23] Szatmari E. M., Oliveira A. F., Sumner E. J., Yasuda R., “Centaurin- 1-Ras-Elk-1 Signaling at Mitochondria Mediates -Amyloid-Induced Synaptic Dysfunction.”, *Journal of Neuroscience*, 33 (12), 5367-5374.
- [24] Demir O., Kurnaz I. A., “Wildtype Elk-1, but not a SUMOylation mutant, represses *egr-1* expression in SH-SY5Y neuroblastomas.”, *Neuroscience Letters*, 437 (1), 20-4.
- [25] Demir O., Aysit N., Onder Z., Turkel N, Ozturk G., Sharrocks A. D., Kurnaz I. A., (2011), “ETS-domain transcription factor Elk-1 mediates neuronal survival: SMN as a potential target.”, *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1812 (6), 652-662.
- [26] Caboche J., Vanhoutte P., Galan-Rodriguez B., Besnard A., (2011), “Elk-1 a Transcription Factor with Multiple Facets in the Brain.”, *Frontiers in Neuroscience*, 5.
- [27] Janesick A., Abbey R., Chung C., Liu S., Taketani M., Blumberg B., (2013), “ERF and ETV3L are retinoic acid-inducible repressors required for primary neurogenesis.”, *Development*, 140 (15), 3095-3106.
- [28] Li Y., Luo H., Liu T., Zacksenhaus E., Ben-David Y., (2014), “The ets transcription factor Fli-1 in development, cancer and disease.”, *Oncogene*, 34 (16), 2022-2031.
- [29] Feng F. Y., Brenner J. C., Hussain M., Chinnaiyan A. M., (2014), “Molecular pathways: targeting ETS gene fusions in cancer.”, *Clinical Cancer Research*, 20 (17), 4442-8.
- [30] Gasi Tandefelt D., Boormans J., Hermans K., Trapman J., (2014), “ETS fusion genes in prostate cancer.”, *Endocrine Related Cancer*, 21 (3), R143-52.
- [31] Park S. W., Do H. J., Ha W. T., Han M. H., Yang H. M., Lee S. H., Song H., Kim N. H., Kim J. H., (2014), “Transcriptional regulation of OCT4 by the ETS transcription factor ESE-1 in NCCIT human embryonic carcinoma cells.”, *Biochemical and Biophysical Research Communications*, 450 (2), 984-990.
- [32] Cao P., Feng F., Dong G., Yu C., Feng S., Song E., Shi G., Liang Y., Liang G., (2015), “Estrogen receptor  $\alpha$  enhances the transcriptional activity of ETS-1 and promotes the proliferation, migration and invasion of neuroblastoma cell in a ligand dependent manner.”, *BMC Cancer*, 15 (1).
- [34] Kandemir B., Dag U., Bakir Gundor B., Durasi İ. M., Erdogan B., Sahin E., Sezerman U., Kurnaz I. A., (2017), “In silico analyses and global transcriptional profiling reveal novel putative targets for Pea3 transcription factor related to its function in neurons.”, *PLoS One*, 12 (2), e0170585.
- [35] Web 1, (2018), <https://eu.idtdna.com/calc/analyzer>, (Date Accessed: 03/01/2018)

- [36] Web 2, (2018), <https://blast.ncbi.nlm.nih.gov/Blast.cgi>, (Date Accessed: 10/02/2018)
- [37] Web 3, (2018), <https://eu.idtdna.com/calc/analyzer>, (Date Accessed: 07/02/2018)
- [38] Web 4, (2018), <https://cb.utdallas.edu/cgi-bin/TRED>, (Date Accessed: 14/02/2018)
- [39] Web 5, (2018), [http://alggen.lsi.upc.es/cgi-bin/promo\\_v3/promo](http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo), (Date Accessed: 16/02/2018)
- [40] Web 6, (2018), <https://www.ebi.ac.uk/Tools/msa/clustalo>, (Date Accessed: 09/03/2018)
- [41] Kumar S., Stecher G., Li M., Knyaz C., Tamura K., (2018), “MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms.”, *Molecular Biology and Evolution*, 35, 1547-1549.
- [42] Web 7, (2018), <https://www.uniprot.org>, (Date Accessed: 15/03/2018)
- [43] Le Novère N., (2015), “Quantitative and logic modelling of molecular and gene networks.”, *Nature Reviews Genetics*, 16 (3), 146-58.
- [44] Hoops S., Sahle S., Gauges R., Lee C., Pahle J., Simus N., Singhal M., Xu L., Mendes P., Kummer U., (2006), “COPASI: a COMplex PATHway Simulator.”, *Bioinformatics*, 22, 3067-74.
- [45] Kühn C., Wierling C., Kühn A., Klipp E., Panopoulou G., Lehrach H., Poustka A. J., (2009), “Monte Carlo analysis of an ODE Model of the Sea Urchin Endomesoderm Network.”, *BMC System Biology*, 3, 83.
- [46] De Caluwe J., Xiao Q., Hermans C., Verbruggen N., Leloup J. C., Gonze D., (2016), “A Compact Model for the Complex Plant Circadian Clock.”, *Frontiers in Plant Science*, 7, 74.
- [47] Aron U., (2007), “An Introduction to Systems Biology: Design Principles of Biological Circuits.”, 1st Edition, Chapman & Hall/CRC.
- [48] Schwanhäusser B., Busse D., Dittmar G., Schuchhardt J., Wolf J., Chen W., Selbach M., (2011), “Global quantification of mammalian gene expression control.”, *Nature*, 473 (7347), 337-342.
- [49] Kfir-Elirachman K., Ortenberg R., Vigel B., Besser M. J., Barshack I., Schachter j., Nemlich Y., Markel G., (2018), “Regulation of CEACAM1 Protein Expression by the Transcription Factor ETS-1 in BRAF-Mutant Human Metastatic Melanoma Cells.”, *Neoplasia*, 4, 401-409.
- [50] Panagoulas I., Georgakopoulos T., Aggeletopoulou I., Agelopoulos M., Thanos D., Mouzaki A., (2016), “Transcription Factor Ets-2 Acts as a

Preinduction Repressor of Interleukin-2 (IL-2) Transcription in Naïve T Helper Lymphocytes.”, *The Journal of Biological Chemistry*, 52, 26707-26721.

- [51] Cooper C. D., Newman J. A., Aitkenhead H., Allerston C. K., Gileadi O., (2015), “Structures of the ETS Protein DNA-binding Domains of Transcription Factors ETV1, ETV4, ETV5, and FEV.”, *The Journal of Biological Chemistry*, 290 (22), 13692-709.
- [52] De S., Chan A. C., Coyne H. J., Bhachech N., Hermsdorf U., Okon M., Murphy M. E., Graves B. J., McIntosh L. P., (2014), “Steric mechanism of auto-inhibitory regulation of specific and non-specific DNA binding by the ETS transcriptional repressor ETV6.”, *Journal of Molecular Biology*, 426 (7), 1390-406.

## **BIOGRAPHY**

Yigit Koray Babal was born in Istanbul, 1994. He achieved his bachelor's degree from Department of Biology, Marmara University in 2016. He has continued master degree program in the Department of Molecular Biology and Genetics at Molecular Neurobiology Laboratory, Graduate School of Natural and Applied Sciences, Gebze Technical University, since 2016 fall. He presented oral presentation in GTU Graduate Studies Symposium and Introductory Days 2017 namely "Molecular and Computational Investigation of Intrafamilial Interaction of ETS Family Transcription Factors". He also participated Brain Awareness Week activities, which is a funded-project by FENS, for two years. He would like to study on neuroscience and system biology.

## APPENDICES

### Appendix A: Reaction list of ETS model with parameter sets

Table A1.1: Reaction list with mouse fibroblast parameter set.

| Number | Reaction name/type                                 | Parameters                                                                                         | Reference |
|--------|----------------------------------------------------|----------------------------------------------------------------------------------------------------|-----------|
| 1      | EHF_mRNA_degradation<br>(Mass Action)              | $k_{\text{degr}}$ : 0.125117<br>molecule/h                                                         | [48]      |
| 2      | EHF_protein_degradation<br>(Mass Action)           | $k_{\text{degr}}$ : 0.0297488<br>molecule/h                                                        |           |
| 3      | EHF_transcription (Derived<br>with one activator)  | $x_1$ : 0.89 molecule/hour<br>$K_{ba}$ : 1 volume/molecule                                         |           |
| 4      | EHF_translation (Mass<br>Action)                   | $k_t$ : 19.04 molecule/hour                                                                        |           |
| 5      | ELF3_mRNA_degradation<br>(Mass Action)             | $k_{\text{degr}}$ : 0.125117<br>molecule/hour                                                      |           |
| 6      | ELF3_protein_degradation<br>(Mass Action)          | $k_{\text{degr}}$ : 0.0297488<br>molecule/hour                                                     |           |
| 7      | ELF3_transcription<br>(Derived with one activator) | $x_1$ : 0.89 molecule/hour<br>$K_{ba}$ : 1 volume/molecule                                         |           |
| 8      | ELF3_translation (Mass<br>Action)                  | $k_t$ : 19.04 molecule/hour                                                                        |           |
| 9      | ELK1_transcription<br>(Derived with two inhibitor) | $x_1$ : 0.89 molecule/hour<br>$k_{ba1}$ : 1<br>volume/molecule<br>$k_{ba2}$ : 1<br>volume/molecule |           |
| 10     | ELK1_mRNA_degradation<br>(Mass Action)             | $k_{\text{degr}}$ : 0.125117<br>molecule/hour                                                      |           |

Table A1.1: Continue.

|    |                                                    |                                                                                              |      |
|----|----------------------------------------------------|----------------------------------------------------------------------------------------------|------|
| 11 | ELK1_protein_degradation<br>(Mass Action)          | $k_{\text{degr}}$ : 0.0297488<br>molecule/hour                                               | [48] |
| 12 | ELK1_translation (Mass<br>Action)                  | $k_t$ : 19.04 molecule/hour                                                                  |      |
| 13 | ER81_mRNA_degradation<br>(Mass Action)             | $k_{\text{degr}}$ : 0.125117<br>molecule/hour                                                |      |
| 14 | ER81_protein_degradation<br>(Mass Action)          | $k_{\text{degr}}$ : 0.0297488<br>molecule/hour                                               |      |
| 15 | ER81_transcription<br>(Derived with one inhibitor) | $x_1$ : 0.89 molecule/hour<br>$K_{ba}$ : 1 volume/molecule                                   |      |
| 16 | ER81_translation (Mass<br>Action)                  | $k_t$ : 19.04 molecule/hour                                                                  |      |
| 17 | ERF_mRNA_degradation<br>(Mass Action)              | $k_{\text{degr}}$ : 0.125117<br>molecule/hour                                                |      |
| 18 | ERF_protein_degradation<br>(Mass Action)           | $k_{\text{degr}}$ : 0.0297488<br>molecule/hour                                               |      |
| 19 | ERF_transcription (Derived<br>with one inhibitor)  | $x_1$ : 0.89 molecule/hour<br>$K_{ba}$ : 1 volume/molecule                                   |      |
| 20 | ERF_translation (Mass<br>Action)                   | $k_t$ : 19.04 molecule/hour                                                                  |      |
| 21 | ERM_mRNA_degradation<br>(Mass Action)              | $k_{\text{degr}}$ : 0.125117<br>molecule/hour                                                |      |
| 22 | ERM_protein_degradation<br>(Mass Action)           | $k_{\text{degr}}$ : 0.0297488<br>molecule/hour                                               |      |
| 23 | ERM_transcription<br>(Derived with two inhibitor)  | $x_1$ : 0.89 molecule/hour<br>$K_{ba1}$ : 1 volume/molecule<br>$K_{ba2}$ : 2 volume/molecule |      |

Table A1.1: Continue.

|    |                                                  |                                                            |      |
|----|--------------------------------------------------|------------------------------------------------------------|------|
| 24 | ERM_translation (Mass Action)                    | $k_t$ : 19.04 molecule/hour                                | [48] |
| 25 | ETV3L_mRNA_degradation (Mass Action)             | $k_{degr}$ : 0.125117 molecule/hour                        |      |
| 26 | ETV3L_protein_degradation (Mass Action)          | $k_{degp}$ : 0.0297488 molecule/hour                       |      |
| 27 | ETV3L_transcription (Derived with one activator) | $x_1$ : 0.89 molecule/hour<br>$K_{ba}$ : 1 volume/molecule |      |
| 28 | ETV3L_translation (Mass Action)                  | $k_t$ : 19.04 molecule/hour                                |      |
| 29 | PEA3_mRNA_degradation (Mass Action)              | $k_{degr}$ : 0.125117 molecule/hour                        |      |
| 30 | PEA3_protein_degradation (Mass Action)           | $k_{degp}$ : 0.0297488 molecule/hour                       |      |
| 31 | PEA3_transcription (Derived with one inhibitor)  | $x_1$ : 0.89 molecule/hour<br>$K_{ba}$ : 1 volume/molecule |      |
| 32 | PEA3_translation (Mass Action)                   | $k_t$ : 19.04 molecule/hour                                |      |

Table A1.2: Reaction list with sea urchin parameter set.

| Number | Reaction name/type                             | Parameters                                              | Reference |
|--------|------------------------------------------------|---------------------------------------------------------|-----------|
| 1      | EHF_mRNA_degradation (Mass Action)             | $k_{degr}$ : 0.119 molecule/hour                        | [45]      |
| 2      | EHF_protein_degradation (Mass Action)          | $k_{degp}$ : 0.3 molecule/hour                          |           |
| 3      | EHF_transcription (Derived with one activator) | $x_1$ : 1 molecule/hour<br>$K_{ba}$ : 1 volume/molecule |           |
| 4      | EHF_translation (Mass Action)                  | $k_t$ : 2 molecule/hour                                 |           |

Table A1.2: Continue.

|    |                                                    |                                                                                           |      |
|----|----------------------------------------------------|-------------------------------------------------------------------------------------------|------|
| 5  | ELF3_mRNA_degradation<br>(Mass Action)             | $k_{\text{degr}}$ : 0.119 molecule/hour                                                   | [45] |
| 6  | ELF3_protein_degradation<br>(Mass Action)          | $k_{\text{degp}}$ : 0.3 molecule/hour                                                     |      |
| 7  | ELF3_transcription<br>(Derived with one activator) | $x_1$ : 1 molecule/hour<br>$K_{ba}$ : 1 volume/molecule                                   |      |
| 8  | ELF3_translation (Mass<br>Action)                  | $k_t$ : 2 molecule/hour                                                                   |      |
| 9  | ELK1_transcription<br>(Derived with two inhibitor) | $x_1$ : 1 molecule/hour<br>$k_{ba1}$ : 1 volume/molecule<br>$k_{ba2}$ : 1 volume/molecule |      |
| 10 | ELK1_mRNA_degradation<br>(Mass Action)             | $k_{\text{degr}}$ : 0.119 molecule/hour                                                   |      |
| 11 | ELK1_protein_degradation<br>(Mass Action)          | $k_{\text{degp}}$ : 0.3 molecule/hour                                                     |      |
| 12 | ELK1_translation (Mass<br>Action)                  | $k_t$ : 2 molecule/hour                                                                   |      |
| 13 | ER81_mRNA_degradation<br>(Mass Action)             | $k_{\text{degr}}$ : 0.119 molecule/hour                                                   |      |
| 14 | ER81_protein_degradation<br>(Mass Action)          | $k_{\text{degp}}$ : 0.3 molecule/hour                                                     |      |
| 15 | ER81_transcription<br>(Derived with one inhibitor) | $x_1$ : 1 molecule/hour<br>$K_{ba}$ : 1 volume/molecule                                   |      |
| 16 | ER81_translation (Mass<br>Action)                  | $k_t$ : 2 molecule/hour                                                                   |      |
| 17 | ERF_mRNA_degradation<br>(Mass Action)              | $k_{\text{degr}}$ : 0.119 molecule/hour                                                   |      |
| 18 | ERF_protein_degradation<br>(Mass Action)           | $k_{\text{degp}}$ : 0.3 molecule/hour                                                     |      |
| 19 | ERF_transcription (Derived<br>with one inhibitor)  | $x_1$ : 1 molecule/hour<br>$K_{ba}$ : 1 volume/molecule                                   |      |
| 20 | ERF_translation (Mass<br>Action)                   | $k_t$ : 2 molecule/hour                                                                   |      |

Table A1.2: Continue

|    |                                                     |                                                                                                         |      |
|----|-----------------------------------------------------|---------------------------------------------------------------------------------------------------------|------|
| 21 | ERM_mRNA_degradation<br>(Mass Action)               | $k_{\text{degr}}$ : 0.119 molecule/hour                                                                 | [45] |
| 22 | ERM_protein_degradation<br>(Mass Action)            | $k_{\text{degp}}$ : 0.3 molecule/hour                                                                   |      |
| 23 | ERM_transcription (Derived<br>with two inhibitor)   | $x_1$ : 1 molecule/hour<br>$K_{\text{ba1}}$ : 1 volume/molecule<br>$K_{\text{ba2}}$ : 1 volume/molecule |      |
| 24 | ERM_translation (Mass<br>Action)                    | $k_t$ : 2 molecule/hour                                                                                 |      |
| 25 | ETV3L_mRNA_degradation<br>(Mass Action)             | $k_{\text{degr}}$ : 0.119 molecule/hour                                                                 |      |
| 26 | ETV3L_protein_degradation<br>(Mass Action)          | $k_{\text{degp}}$ : 0.3 molecule/hour                                                                   |      |
| 27 | ETV3L_transcription<br>(Derived with one activator) | $x_1$ : 1 molecule/hour<br>$K_{\text{ba}}$ : 1 volume/molecule                                          |      |
| 28 | ETV3L_translation (Mass<br>Action)                  | $k_t$ : 2 molecule/hour                                                                                 |      |
| 29 | PEA3_mRNA_degradation<br>(Mass Action)              | $k_{\text{degr}}$ : 0.119 molecule/hour                                                                 |      |
| 30 | PEA3_protein_degradation<br>(Mass Action)           | $k_{\text{degp}}$ : 0.3 molecule/hour                                                                   |      |
| 31 | PEA3_transcription<br>(Derived with one inhibitor)  | $x_1$ : 1 molecule/hour<br>$K_{\text{ba}}$ : 1 volume/molecule                                          |      |
| 32 | PEA3_translation (Mass<br>Action)                   | $k_t$ : 2 molecule/hour                                                                                 |      |

## Appendix B: Ordinary differential equations of ETS model

|                                              |                                                                                                                                                            |
|----------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------|
| $\frac{d([PEA3] \cdot V_{cell})}{dt}$        | $= + V_{cell} \cdot (19.04 \cdot [mRNA\_PEA3])$<br>$- V_{cell} \cdot (0.0297488 \cdot [PEA3])$                                                             |
| $\frac{d([mRNA\_ELK1] \cdot V_{cell})}{dt}$  | $= + V_{cell} \cdot \left( \frac{0.89}{1 + 1 \cdot [ER81]} \cdot \frac{1}{1 + 1 \cdot [PEA3]} \right)$<br>$- V_{cell} \cdot (0.125117 \cdot [mRNA\_ELK1])$ |
| $\frac{d([ER81] \cdot V_{cell})}{dt}$        | $= + V_{cell} \cdot (19.04 \cdot [mRNA\_ER81])$<br>$- V_{cell} \cdot (0.0297488 \cdot [ER81])$                                                             |
| $\frac{d([ELK1] \cdot V_{cell})}{dt}$        | $= + V_{cell} \cdot (19.04 \cdot [mRNA\_ELK1])$<br>$- V_{cell} \cdot (0.0297488 \cdot [ELK1])$                                                             |
| $\frac{d([mRNA\_EHF] \cdot V_{cell})}{dt}$   | $= + V_{cell} \cdot \left( \frac{1 \cdot [ELK1] \cdot 0.89}{1 + 1} \right)$<br>$- V_{cell} \cdot (0.125117 \cdot [mRNA\_EHF])$                             |
| $\frac{d([EHF] \cdot V_{cell})}{dt}$         | $= + V_{cell} \cdot (19.04 \cdot [mRNA\_EHF])$<br>$- V_{cell} \cdot (0.0297488 \cdot [EHF])$                                                               |
| $\frac{d([mRNA\_ELF3] \cdot V_{cell})}{dt}$  | $= + V_{cell} \cdot \left( \frac{1 \cdot [ELK1] \cdot 0.89}{1 + 1} \right)$<br>$- V_{cell} \cdot (0.125117 \cdot [mRNA\_ELF3])$                            |
| $\frac{d([ELF3] \cdot V_{cell})}{dt}$        | $= + V_{cell} \cdot (19.04 \cdot [mRNA\_ELF3])$<br>$- V_{cell} \cdot (0.0297488 \cdot [ELF3])$                                                             |
| $\frac{d([mRNA\_ETV3L] \cdot V_{cell})}{dt}$ | $= + V_{cell} \cdot \left( \frac{1 \cdot [ELK1] \cdot 0.89}{1 + 1} \right)$<br>$- V_{cell} \cdot (0.125117 \cdot [mRNA\_ETV3L])$                           |
| $\frac{d([ETV3L] \cdot V_{cell})}{dt}$       | $= + V_{cell} \cdot (19.04 \cdot [mRNA\_ETV3L])$<br>$- V_{cell} \cdot (0.0297488 \cdot [ETV3L])$                                                           |
| $\frac{d([mRNA\_ERF] \cdot V_{cell})}{dt}$   | $= + V_{cell} \cdot \left( \frac{0.89}{1 + 1 \cdot [PEA3]} \right)$<br>$- V_{cell} \cdot (0.125117 \cdot [mRNA\_ERF])$                                     |
| $\frac{d([ERF] \cdot V_{cell})}{dt}$         | $= + V_{cell} \cdot (19.04 \cdot [mRNA\_ERF])$<br>$- V_{cell} \cdot (0.0297488 \cdot [ERF])$                                                               |
| $\frac{d([mRNA\_ERM] \cdot V_{cell})}{dt}$   | $= + V_{cell} \cdot \left( \frac{89}{1 + 1 \cdot [ELK1]} \cdot \frac{1}{1 + 1 \cdot [ELF3]} \right)$<br>$- V_{cell} \cdot (0.125117 \cdot [mRNA\_ERM])$    |
| $\frac{d([ERM] \cdot V_{cell})}{dt}$         | $= + V_{cell} \cdot (19.04 \cdot [mRNA\_ERM])$<br>$- V_{cell} \cdot (0.0297488 \cdot [ERM])$                                                               |
| $\frac{d([mRNA\_ER81] \cdot V_{cell})}{dt}$  | $= + V_{cell} \cdot \left( \frac{89}{1 + 1 \cdot [ELF3]} \right)$<br>$- V_{cell} \cdot (0.125117 \cdot [mRNA\_ER81])$                                      |
| $\frac{d([mRNA\_PEA3] \cdot V_{cell})}{dt}$  | $= + V_{cell} \cdot \left( \frac{0.89}{1 + 1 \cdot [ELF3]} \right)$<br>$- V_{cell} \cdot (0.125117 \cdot [mRNA\_PEA3])$                                    |

Figure B1.1: ODEs of ETS model with mouse fibroblast parameter set.

|                                              |                                                                                                                                                    |
|----------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------|
| $\frac{d([PEA3] \cdot V_{cell})}{dt}$        | $= +V_{cell} \cdot (2 \cdot [mRNA\_PEA3])$<br>$-V_{cell} \cdot (0.3 \cdot [PEA3])$                                                                 |
| $\frac{d([mRNA\_ELK1] \cdot V_{cell})}{dt}$  | $= +V_{cell} \cdot \left( \frac{1}{1 + 1 \cdot [ER81]} \cdot \frac{1}{1 + 1 \cdot [PEA3]} \right)$<br>$-V_{cell} \cdot (0.119 \cdot [mRNA\_ELK1])$ |
| $\frac{d([ER81] \cdot V_{cell})}{dt}$        | $= +V_{cell} \cdot (2 \cdot [mRNA\_ER81])$<br>$-V_{cell} \cdot (0.3 \cdot [ER81])$                                                                 |
| $\frac{d([ELK1] \cdot V_{cell})}{dt}$        | $= +V_{cell} \cdot (2 \cdot [mRNA\_ELK1])$<br>$-V_{cell} \cdot (0.3 \cdot [ELK1])$                                                                 |
| $\frac{d([mRNA\_EHF] \cdot V_{cell})}{dt}$   | $= +V_{cell} \cdot \left( \frac{1 \cdot [ELK1] \cdot 1}{1 + 1} \right)$<br>$-V_{cell} \cdot (0.119 \cdot [mRNA\_EHF])$                             |
| $\frac{d([EHF] \cdot V_{cell})}{dt}$         | $= +V_{cell} \cdot (2 \cdot [mRNA\_EHF])$<br>$-V_{cell} \cdot (0.3 \cdot [EHF])$                                                                   |
| $\frac{d([mRNA\_ELF3] \cdot V_{cell})}{dt}$  | $= +V_{cell} \cdot \left( \frac{1 \cdot [ELK1] \cdot 1}{1 + 1} \right)$<br>$-V_{cell} \cdot (0.119 \cdot [mRNA\_ELF3])$                            |
| $\frac{d([ELF3] \cdot V_{cell})}{dt}$        | $= +V_{cell} \cdot (2 \cdot [mRNA\_ELF3])$<br>$-V_{cell} \cdot (0.3 \cdot [ELF3])$                                                                 |
| $\frac{d([mRNA\_ETV3L] \cdot V_{cell})}{dt}$ | $= +V_{cell} \cdot \left( \frac{1 \cdot [ELK1] \cdot 1}{1 + 1} \right)$<br>$-V_{cell} \cdot (0.119 \cdot [mRNA\_ETV3L])$                           |
| $\frac{d([ETV3L] \cdot V_{cell})}{dt}$       | $= +V_{cell} \cdot (2 \cdot [mRNA\_ETV3L])$<br>$-V_{cell} \cdot (0.3 \cdot [ETV3L])$                                                               |
| $\frac{d([mRNA\_ERF] \cdot V_{cell})}{dt}$   | $= +V_{cell} \cdot \left( \frac{1}{1 + 1 \cdot [PEA3]} \right)$<br>$-V_{cell} \cdot (0.119 \cdot [mRNA\_ERF])$                                     |
| $\frac{d([ERF] \cdot V_{cell})}{dt}$         | $= +V_{cell} \cdot (2 \cdot [mRNA\_ERF])$<br>$-V_{cell} \cdot (0.3 \cdot [ERF])$                                                                   |
| $\frac{d([mRNA\_ERM] \cdot V_{cell})}{dt}$   | $= +V_{cell} \cdot \left( \frac{1}{1 + 1 \cdot [ELK1]} \cdot \frac{1}{1 + 1 \cdot [ELF3]} \right)$<br>$-V_{cell} \cdot (0.119 \cdot [mRNA\_ERM])$  |
| $\frac{d([ERM] \cdot V_{cell})}{dt}$         | $= +V_{cell} \cdot (2 \cdot [mRNA\_ERM])$<br>$-V_{cell} \cdot (0.3 \cdot [ERM])$                                                                   |
| $\frac{d([mRNA\_ER81] \cdot V_{cell})}{dt}$  | $= +V_{cell} \cdot \left( \frac{1}{1 + 1 \cdot [ELF3]} \right)$<br>$-V_{cell} \cdot (0.119 \cdot [mRNA\_ER81])$                                    |
| $\frac{d([mRNA\_PEA3] \cdot V_{cell})}{dt}$  | $= +V_{cell} \cdot \left( \frac{1}{1 + 1 \cdot [ELF3]} \right)$<br>$-V_{cell} \cdot (0.119 \cdot [mRNA\_PEA3])$                                    |

Figure B1.2: ODEs of ETS model with sea urchin parameter sets.

## Appendix C: Luciferase Assay Data for *Elk-1* promoter

- Activity of *Elk-1* promoter for mPea3 Protein

Table C1.1: Standard deviation for luciferase reporter assay for *Elk-1* promoter and mPea3.

|               | 0 ng pea3       | 50 ng pea3      | 100 ng<br>pea3  | 150 ng<br>pea3  | 200 ng<br>pea3  |
|---------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| mPea3         | 0.3966743<br>09 | 0.8301387<br>5  | 0.9635490<br>24 | 0.2332113<br>93 | 0.3126320<br>52 |
| Pea3-<br>VP16 | 0.4425571<br>93 | 0.7008319<br>16 | 0.3087102<br>99 | 0.2163987<br>31 | 0.4160880<br>27 |

Table C1.2: Firefly/Renilla luciferase values for analysis of *Elk-1* protein activity for mPea3.

|               | 0 ng pea3       | 50 ng pea3      | 100 ng<br>pea3  | 150 ng<br>pea3  | 200 ng<br>pea3  |
|---------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| mPea3         | 3.5032618<br>74 | 6.9909109<br>71 | 5.0254979<br>64 | 4.4771293<br>09 | 2.7462714<br>19 |
| Pea3-<br>VP16 | 4.0752195<br>39 | 4.5259393<br>99 | 4.9931407<br>81 | 2.7223733<br>83 | 3.2798794<br>75 |