ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE, ENGINEERING AND TECHNOLOGY

REGULATION OF *SPG4* AND *KATNB1* GENE EXPRESSIONS BY ELK1 TRANSCRIPTION FACTOR

M.Sc. Thesis by

Ece SELÇUK

Advanced Technologies Department

Molecular Biology-Genetics and Biotechnology Programme

JUNE 2012

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Thesis Advisor: Prof. Dr. Arzu KARABAY KORKMAZ

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

SPG4 VE *KATNB1* GEN ANLATIMLARININ ELK1 TRANSKRİPSİYON FAKTÖRÜ İLE DÜZENLENMESİ

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HAZİRAN 2012

Ece SELÇUK, **a M.Sc.** student of ITU **Graduate School of Science, Engineering and Technology** student ID 521091086, successfully defended the **thesis** entitled "**REGULATION OF *SPG4* AND *KATNB1* GENE EXPRESSIONS BY ELK1 TRANSCRIPTION FACTOR**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission : 04 May 2012
Date of Defense: 07 June 2012

FOREWORD

First, I would like to express my gratitude to my advisors, Prof. Dr. Arzu KARABAY KORKMAZ. She was all the time there for me, guiding,believing and supporting.

I would like to thank Koray Kırımtay for being with me in the lab thorughout this study without complaint and his endless support. I also thank Derya Canbaz for sharing her experience with me. She taught many experimental techniques performed in our lab. I would like to thank other CYTO lab members; Şirin Korulu Koç, Ayşegül Yıldız Ünal and Kutay Deniz Atabay for their help and friendship. They were ready to help in any subject whenever I requested.

I would like to special thank Dr. Deniz Şahin for being with me every time. His encouragement, strong support, companionship have been of great assist to me.

I would like to thank my dear friends; Elif, Nazlı, Salih, Murat, İrem, Ani, Aydın and all my friends. We had great time together in MOBGAM.

Lastly, I would like to thank my family. I am sure they know what I feel about them. Thank you very much for everything.

June, 2012

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ABBREVIATION

µg	**:** Microgram
µM	**:** Micromolar
µm	**:** Micrometer
AAA	**:** ATPases Associated with diverse cellular Activities
AD	: Alzheimer Disease
ADP	**:** Adenosine diphosphate
Amp	**:** Ampicillin
ATP	**:** Adenosine triphosphate
BCA	**:** Bicinchoninic acid
bp	**:** Base pair
BSA	: Bovine Serum Albumin
cDNA	**:** Complementary DNA
cm	**:** Centimeter
cm^2	**:** Centimeter square
Ct	**:** Cycle treshold
DLR	**:** Dual Luciferase Reporter
DMEM	**:** Dulbecco's modified Eagle medium
DMSO	**:** Dimethyl sulfoxide
DNA	**:** Deoxyribonucleic acid
dNTP	**:** Deoxyribonucleotide
DPE	**:** Downstream promoter element
DPM	**:** Downstream Promoter Element
E.coli	**:** *Esherichia coli*
EDTA	**:** Ethylenediaminetetraacetic acid
Elk1	**:** Ets-like 1
ERK	**:** Extracellular Signal Regulated Kinase
EtBr	**:** Ethidium bromide
ETS	**:** E twenty six specific
FBS	**:** Fetal Bovine Serum
g	**:** Gram
GDP	**:** Guanosine diphosphate
GTP	**:** Guanosine triphosphate
h	**:** Hour
HD	**:** Hydrophobic Domain
HDAC	**:** Histone Deacetylase Complex
JNK	**:** c-Jun N-terminal Kinase
kbp	**:** Kilo base pair
kDa	**:** Kilo dalton
L	**:** Liter
LARII	**:** Luciferase Assay Reagent II
LB	**:** Luria-Bertani Broth
***Luc*+**	**:** Luciferase
M	**:** Molar

MAP : Microtubule Associated Protein
MAPK : Mitogen Activating Protein Kinase
min : Minute
mL : Mililiter
mM : Milimolar
mm : Milimeter
mRNA : Messenger Ribonucleic Acid
MTBD : Microtubule Binding Doma**in**
MTOC : Microtubule Organizing Center
NES : Nuclear Export Signal
ng : Nanogram
NLS : Nuclear Localization Signal
nm : Nanometer
nM : Nanomolar
PCR : Polymerase Chain Reaction
pH : Power of Hydrogen
PIPES : Piperazine-N, N'- bis (2-ethanesulfonic acid)
PMA : Phorbol 12-myristate 13-acetate
PTM : Post Translational Modifiction
qRT-PCR : Quantitative Real Time Polymerase Chain Reaction
RNA : Ribonucleic Acid
rpm : Recolutions per minute
sec : Second
sElk1 : Short Elk1
SENP : SUMO1/ sentrin/ SMT3-Specific Peptidase
SOC : Super Optimal Broth with Catabolite Respression
SRE : Serum Response Element
SRF : Serum Response Factor
SUMO : Small Ubiquitin Related Modifier
TAE : Tris-acetate-EDTA
TCF : Ternary Complex Factor
TF : Transcription Factor
Tm : Melting Temperature
UV : Ultraviolet
woR : without R
V : Volt
γ-TuRC : γ-Tubulin Ring Complex

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REGULATION OF *SPG4* AND *KATNB1* GENE EXPRESSIONS BY ELK1 TRANSCRIPTION FACTOR

SUMMARY

Cytoskeleton is a network within the cell responsible for some cellular activities such as determining cell shape, providing cell movement and transport of some molecules. Microtubules are member of this network and have roles in chromosome segregation during mitosis, vesicular transport, axon formation and giving specific shapes of neurons. Microtubule severing is one of the major mechanisms that take role in formation of special neuron morphology. There are three microtubule severing enzymes called spastin, katanin and fidgetin. Spastin is encoded by *SPG4* gene. However katanin is a heterodimer and its subunits p60 and p80 are encoded by two different genes *KATNA1* and *KATNB1*, respectively. p60-katanin has the enzymatic activity for severing microtubules, while p80-katanin regulates role of p60-katanin in terms of stimulation or inhibition of p60-katanin activity.

It is known that these enzymes are related with some neurological diseases. For instance, mutations in *SPG4* gene lead to hereditary spastic paraplegia. Although exact mechanism has not been yet clear, it is thought that mutations in the *SPG4* gene cause lack of short microtubules needed in axon ends, owing to loss of microtubule severing activity of spastin; or failure of vesicular transport due to accumulation of mutant spastin on microtubule in axon ends. Besides of mutations, regulation of *SPG4* gene expression has also an effect on onset of disease. Katanin was found to be related with Alzheimer disease. In its unphosphorylated form, tau protein normally provides stabilization of microtubules and protects microtubules from the activity of severing enzymes. Hyperphosphorylation of tau results in dissociation of tau from microtubules and so microtubules become accessible for severing enzyme katanin. It is thought that this process may result in Alzheimer pathology.

Elk1 is a transcription factor that has repressor and activator domains. The SUMO modification on repressor domain is necessary for inhibition of the target gene expression. However, when Elk1 is phosphorylated by extracellular signal such as a mitotic signal, Elk1 is de-SUMOylated and increases expression of target gene.

In this study, the regulation of *SPG4* and *KATNB1* gene expressions by Elk1was investigated in human neuroblastoma cells.

The repressive effect of Elk1 on spastin promoter was determined by using luciferase reporter assay and the reason for the repression is thought to be SUMO modification. Therefore new construct that did not include the repressor domain was constructed. According to the reporter assay data, Elk1 without repressor domain increased the promoter activity, whereas wt-Elk1 did not. This result was confirmed by analyzing mRNA and protein levels with real time PCR and immunostaning, respectively. Subsequently, PMA treatment was performed in order to de-SUMOylate Elk1 via phosphorylation. Following reporter gene assay, PMA treated and Elk1 transfected

cells showed higher promoter activity compared to untreated and Elk1 transfected cell.

Our observation indicated that, Elk1 acts as an activator on *KATNB1* gene promoter differently than *SPG4* promoter The results for *KATNB1* gene were firstly confirmed in mRNA level by performing real time PCR. Then SUMOylation of Elk1 was triggered by using KCl. According to the reporter assay data, KCl treated and Elk1 transfected cells showed lower promoter activity compared to untreated and Elk1 transfected cells. Increase in p80-katanin protein level by Elk1 and decrease of p80-katanin protein level by KCl treatment were also shown with immunostaining and western blot analysis, respectively.

SPG4 VE *KATNB1* GEN ANLATIMLARININ ELK1 TRANSKRİPSİYON FAKTÖRÜ İLE DÜZENLENMESİ

ÖZET

Hücre iskeleti tüm sitoplazmaya yayılan hücrenin şekil almasında, hareketinde, hücre içi molekül taşınması gibi ana mekanizmalarda görevli bir ağdır. Ökaryotik hücrelerde hücre iskeleti üç farklı yapıdan oluşmaktadır. Bunlar mikrofilamentler (aktin filamentler), ara filamentler ve mikrotübüllerdir.

Mikrofilamentler, hücrede en fazla bulunan proteinlerden biridir ve evrimsel süreçte oldukça korunmuştur. Hücrenin şekil kazanmasında, hücre bölünmesinin sitokinez evresinde önemli roller üstlenen mikrofilamentler, globular aktinlerin ATP hidrolizi yardımıyla polimerizasyonu ile filament aktine dönüşür. Ara filamentler ise genellikle zar yapılarına yakın kısımlara yerleşmişlerdir. Örneğin çekirdek zarına çok yakın kısımlarda lamin adı verilen ara filamentler bulunur. Bunlar zara mekanik destek sağlarlar. Bunların haricinde hücreler arası bağların oluşumunda da görevleri vardır.

Hücre iskeleti üçüncü elemanı olan mikrotübüller hücre bölünmesi sırasında kromozomların kutuplara çekilmesinde, hücrenin hareket edebilmesinde, hücrede vezikül taşınımında, nöronlarda akson oluşumunda ve nöronal şeklin kazanımında önemli rol oynar. Mikrotübüller hücrelerde stabil ve stabil olmayan iki farklı yapıda görülmektedir. Mikrotübüllerin α ve β tübülin adı verilen monomerlerinin polimerizasyonu ve depolimerizasyonu mikrotübüllere "dinamik instabilite" diye adlandırılan özelliği kazandırır. Bu dinamizm, mikrotübüllerin uzayıp kısalmasını sağlar. Stabil mikrotübüller ise nöron gibi özelleşmiş hücrelerde bulunur. Çünkü nöronların, örneğin hücre gövdesinde üretilen proteinlerin akson uçlarına kadar taşınabilmesi için ray görevi görecek sabit bir yapıya ihtiyacı vardır. Bu stabilite mikrotübül ilişkili proteinler (MAP) tarafından kontrol edilir.

Miktotübüller ile ilgili bilinmesi gereken diğer bir nokta da kesim mekanizmasıdır. Nöronların özel morfolojilerinin oluşmasında başı çeken mikrotübül kesimidir. Çünkü polimerizasyon ve depolimerizasyonun neden olduğu sadece uç kısımlarda meydana gelen uzalıp kısalma, nöron morfolojisinin oluşması için gerekli olan dallanma sürecinde zaman ve hız açısından yetersiz kalır. Uzun bir mikrotübülün daha kısa parçalara ayrılması daha avanajlıdır. Ayrıca mikrotübüllerin bir başka yere taşınabilmesi için de kesilmesi gerekir. Yani mikrotübül taşınabilirliği, mikrotübül boyu ile ters orantılıdır. Şu ana kadar spastin, katanin ve fidgetin adı verilen üç kesim enzimi tanımlanmıştır. Her üç enzim de ATP'yi parçalayarak işlev gören ATPaz ailesinin üyeleridir.

Spastin, *SPG4* geni tarafından kodlanırken, bir heterodimer olan katanin *KATNA1* ve *KATNB1* genlerinden kodlanan ve sırasıyla p60 ve de p80 diye isimlendirilen alt birimlerden oluşur. Kataninde enzimatik aktiviteyi gösteren asıl alt birim p60-katanindir. p80-katanin bu enzimatik aktiviteyi düzenleyendir ve bazı tanımlanmış bölgeleri ile p60-kataninin mikrotübülleri kesmesini arttırabilmektedir.

Nöronlarda bu iki enzimin dağılımına bakıldığında, kataninin daha çok akson boyunca yoğunlaştığı, spastinin ise hücrenin kısa uzantılarının olduğu dallanma bölgelerinde fazla bulunduğu gözlemlenmiştir. Böylece spastinin mikrotübülleri daha kısa parçalara ayırdığı, kataninin ise daha uzun parçalar halinde kestiği gösterilmiştir.

Bu iki enzimin çeşitli nörolojik hastalıklarla ilişkili olduğu bilinmektedir. Spastin genindeki delesyon, insersiyon gibi mutasyonların ya da alternatif kırpılmaların herediter spastik paraplejiye sebep olduğu gösterilmiştir. Spastin proteininin bu hastalıkla ilgisi tam olarak aydınlatılamamış olmakla beraber, mutant spastin proteininin mikrotübül kesme fonksiyonunu yitirmesinden dolayı akson uçlarında ihtiyaç duyulan kısa mikrotübüllerin sağlanamaması veya mutant formdaki spastinin akson uçlarındaki mikrotübüller üzerinde birikmesiyle veziküler transportun aksaması olası sebepler olarak bildirilmiştir. Mutasyonların haricinde gen anlatımının düzenlenmesinin de hastalığın ortaya çıkmasında etkili olacağı düşünülmüş ve spastin promotör karakterizasyonu yapılmıştır.

Katanin ise Alzheimer hastalığı ile ilişkilidir. Çünkü mikrotübüllerin stabil kalmasını sağlayan ve normalde mikrotübüllerle etkileşim halinde olan tau proteininin hiperfosforilasyonu, mikrotübüllerin stabilitesini kaybetmesine neden olur. Böylece mikrotübüller katanin ile kesilmeye açık hale gelir. Bu sürecin Alzheimer hastalığı patolojisine neden olabileceği düşünülmektedir. Tüm bunlar göz önüne alındığında spastin ve kataninin hücre içindeki miktarları, dolayısıyla gen anlatım süreçleri ve düzenlenmesi oldukça önem kazanmaktadır.

Bu genlerin anlatımlarının düzenlenmesinde etkili olan Elk1 transkripsiyon faktörünün rol oynadığı bulunmuştur. Elk1 hem gen anlatımını baskılayıcı hem de aktive edici bölgeler içermektedir. Elk1'in hedef gen anlatımını azaltması için aynı zamanda baskılanma bölgesinde SUMO adı verilen bir modifikasyon geçirmesi gerekmektedir. Ancak hücre dışı sinyallere bağlı olarak örneğin mitotik sinyallerle Elk1'in aktivatör bölgesi fosforilasyona uğradığında SUMO modifikasyonu ortadan kalkar ve Elk1 hedef gen anlatımını arttırır.

Bu çalışmada spastin kodlayan *SPG4* geninin ve p80-katanini kodlayan *KATNB1* geninin anlatımlarının insan nöroblastoma hücrelerinde Elk1 transkripsiyon faktörü ile nasıl düzenlendiği araştırılmıştır

Elk1'in spastin promotörü üzerinde baskılayıcı etkisinin olduğu tespit edilmiş ve bu etkinin baskılayıcı bölgede gerçekleşen SUMO modifikasyonundan kaynaklandığı düşünülmüştür. Bu sebeple baskılayıcı bölge içermeyen yeni bir dizi oluşturulmuştur. Elk1 ve baskılayıcı bölge içermeyen Elk1'in spastin geni anlatımına olan etkileri karşılaştırıldığında, baskılayıcı bölge içermeyenin gen anlatımını arttırdığı bildirici gen (lusiferaz) deneyi ile saptanmıştır. Bu sonuç gen anlatım sürecinin kapsadığı transkripsiyon ve translasyon basamaklarında da sırasıyla mRNA ve protein düzeyinde de gerçek zamanlı polimeraz zincir reaksiyonu (PZR) ve immun boyama teknikleri ile doğrulanmıştır. Ardından SUMO modifikasyonunu elimine etmek ve promotör üzerindeki azaltıcı etkiyi tersine çevirebilmek için Elk1'in fosforile olması sağlanmıştır. Bunun için nöroblastoma hücrelerine mitotik bir ajan olan PMA bileşeni uygulanmış ve bildirici gen testi yeniden yapılmıştır. Buna göre PMA ile muamele edilmiş hücrelerde *SPG4* gen anlatımının, PMA ile muamele edilmemiş hücrelere göre arttığı gözlemlenmiştir. Bu deney sonucu da protein düzeyinde doğrulanmıştır.

Elk1'in *KATNB1* promotörü üzerinde *SPG4*'den farklı olarak aktivatör rol oynadığı bildirici gen testi ile belirlenmiştir. Deney sonucu mRNA düzeyinde de gerçek

zamanlı PZR yöntemiyle doğrulanmıştır. Elk1'in KCl kimyasalı ile SUMO modifikasyonuna uğraması tetiklenmiştir. KCl'ye maruz bırakılan hücrelerde diğerlerine göre daha az promotör aktivitesi belirlenmiştir. Elk1'in p80-katanin proteini seviyesini arttırdığı ve KCl uygulanmış hücrelerde p80-katanin protein üretiminin azaldığı immun boyama ve Western blotlama tekniği ile gösterilmiştir.

1. INTRODUCTION

1.1 Cytoskeleton

Cytoskeleton is a network of protein filaments which have role in the formation of cell shape, supporting of the cell membrane, providing self-generated movement and transporting cellular vesicles throughout the cell. Eukaryotic cells contain three main types of cytoskeletal filaments (Figure 1.1). Microfilaments also called actin filament, intermediate filaments, and microtubules can be distinguished from each other on the bases of their diameter, type of subunit, and subunit arrangements (Alberts *et al.*, 2008; Lodish *et al.*, 2003).

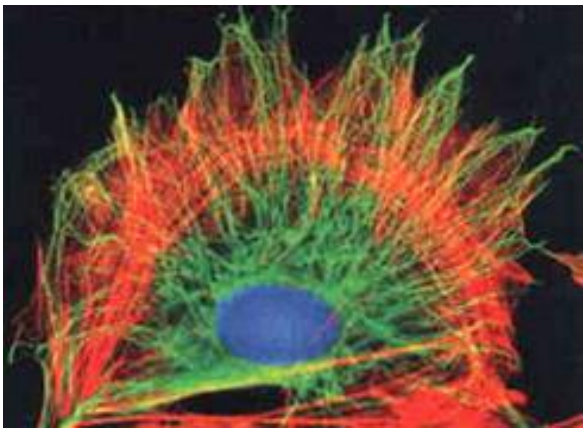

Figure 1.1: A eukaryotic cell stained for visualizing its cytoskeleton. Nucleus (blue), microfilament (red) and microtubules (green) (Alberts *et al.*, 2008).

1.2 Microfilaments (Actin Filaments)

Actin is one of the most abundant intracellular proteins in a eukaryotic cell (Figure 1.2). Actin genes are highly conserved; however the various actin isoforms exhibit minor sequence differences and generally perform different functions. Actin filaments are highly dynamic structures that participate in many cellular functions such as the maintaining cell polarity and morphology, generating force for movements, intracellular trafficking, maintenance of cell junctions and cytokinesis. The organizational flexibility of the actin cytoskeleton permits a cell to gain many different shapes as microvilli on the surface of intestinal epithelial cells

vastly increase the cell surface area and enhance nutrient absorption. On the other hand, the regular network of actin filaments forms stable and differentiated cell morphology such as neurons.

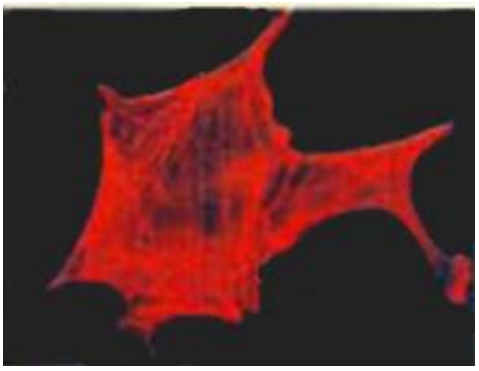

Figure 1.2: Actin filaments stained with red (Campbell and Reece, 2005).

Actin subunit is globular monomer called G-actin and has ATP and Mg^{+2}/ K^{+} binding site at the center of the molecule. ATP is hydrolyzed immediately after the monomer is incorporated into an actin filament. The linear chain of G-actin subunits forms filamentous polymer called F-actin in the presence of ATP, Mg^{+2} and K^{+}. Actin filaments consist of two-stranded helical polymers of the F-actins. The concentration of G-actin is also critical since this process is reversible. Actin molecules polymerize when G-actin is above the critical concentration, and depolymerize when G-actin is below the critical concentration. ATP hydrolysis is not required for polymerization, but it is required to promote depolymerization (when it is converted to ADP). Two of these forms, ATP–G-actin and ADP–F-actin predominate in a cell. They are cross-linked and bundled together via different accessory proteins (Alberts *et al.*, 2008; Lodish *et al.*, 2003).

1.3 Intermediate Filaments

Intermediate filament proteins form parallel homo or heterodimers rope-like structures and typically criss cross the cytosol, forming an internal framework that stretches from the nuclear envelope to the plasma membrane (Figure 1.3). Intermediate filament network is located adjacent to some cellular membranes, where it provides mechanical support. For example lamin proteins are associated with each other and anchored to the inner nuclear membrane. This structure is called nuclear lamina. In the plasma membrane, intermediate filaments are attached by adapter

proteins to form cell junction which mediates cell-cell adhesion and cell-matrix adhesion.

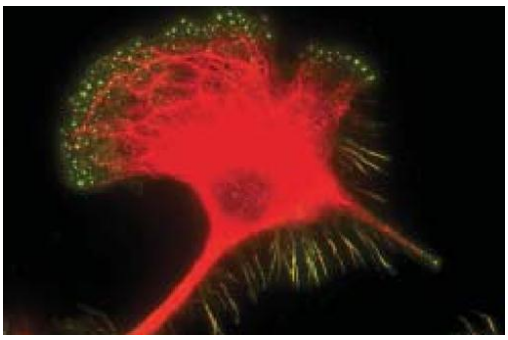

Figure 1.3: Immunofluorescence labeled intermediate filaments (Lodish *et al.*,2003).

Unlike other members of cytoskeleton, intermediate filaments are assembled from a large number of different intermediate filaments and they are more stable. In addition, although microfilaments and microtubules have been strongly conserved in evolution, intermediate filaments family are very diverse. Intermediate filaments, except lamins, are tissue-specific. For example, keratins are found in epithelial cells and also form hair and nails, neurofilaments strengthen the long axons of neurons, vimentins provide mechanical strength to the muscle. The phosphorylation of intermediate filaments early in mitosis leads to their disassembly and break down of nuclear envelope. Reassemble of these intermediate filaments occur in late mitosis after dephosphorylation of the subunits (Alberts *et al.*, 2008; Lodish *et al.*, 2003).

1.4 Microtubules

Microtubules are composed of globular tubulin subunits called α- and β-tubulin (Figure 1.4). They are arranged in a hollow cylinder tubes with 25 nm diameter. Initially, α-tubulin and β-tubulin form heterodimer and subsequently these heterodimers build the protofilament. These protofilaments create the structure of microtubules. Varying in length from a fraction of a micrometer to hundreds of micrometers, microtubules are much stiffer than both microfilaments and intermediate filaments because of their tube-like construction (Alberts *et al.*, 2008; Lodish *et al.*, 2003).

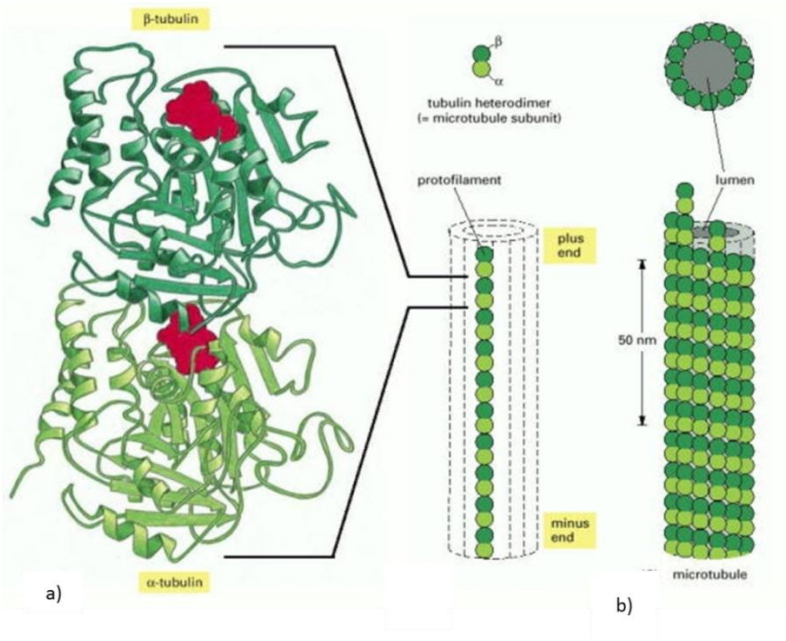

Figure 1.4: (a) Structure of α -tubulin and β –tubulin, (b) assembly of microtubule (Alberts *et al.*, 2008).

Similar to microfilaments, microtubules have roles in certain cell movements, including the beating of cilia and flagella and the molecular trafficking in the cytoplasm. Cells contain microtubules in two different structures; stable, long-lived microtubules and unstable, short-lived microtubules. Stable microtubules are generally found in non-dividing cells. For instance, neurons contain long processes called axons and stable microtubules in axons not only support their structure but also provide transport throughout axons. On the other hand, unstable microtubules are found in cells that need to assemble and disassemble microtubule-based structures quickly. For example, in mitosis the polymerization and depolymerization of microtubules form the mitotic spindle apparatus that provides separation of chromosomes equally to the daughter cells (Lodish *et al.*, 2003).

The structure of microtubule is organized in a polar manner such that the α-tubulin subunit is exposed at the minus end, where rapid depolymerization occurs, while the β-tubulin subunit is exposed at the plus end, where rapid polymerization occurs (Risinger *et al.*, 2009). In a tubulin dimer, non-exchangeable GTP is found at the N terminal site of α-tubulin, whereas the exchangeable nucleotide of β-tubulin is on the surface of the dimer and this determines polymerization or depolymerization state (Alberts *et al.*, 2008). Microtubules comprising GTP-bound tubulin at the plus end are stable since these GTP caps

strengthen the lattice of the microtubule. Hydrolysis an exchangeable GTP disrupts the microtubule stability. GTP cap is lost and microtubules start to shrink if GTP hydrolysis occurs more rapidly than the addition of subunits. This event is called "catastrophe" while a transition from a shortening phase to a growing phase is termed as "rescue". Microtubules are switching between growth and shrinkage phases. This feature is entitled as dynamic instability (Lodish *et al.*, 2003) (Figure 1.5).

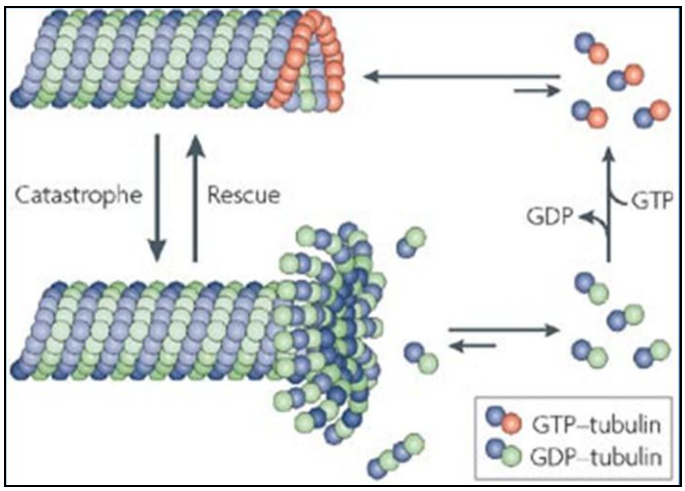

Figure 1.5: Growth and shrinkage of microtubule (Cheeseman and Desai, 2008).

In addition to α-tubulin and β-tubulin, other member of tubulin superfamily is γ-tubulin is localized in **M**icro**t**ubule **O**rganizing **C**enter (MTOC) such as centrosomes. Moreover it is also found in the cytoplasm as a part of a large complex called **γ-Tu**bulin **R**ing **C**omplex (γ-TuRC), which is required to initiate the polymerization of the α/ β- tubulin into a microtubule. Therefore, the minus end of the microtubule is associated with the γ-TuRC complex (Job *et al.*, 2003; Wiese and Zheng, 2006).

Microtubule structure or stability is supported by a class of proteins called **M**icrotubule **A**ssociated **P**roteins (MAPs). They bind to microtubule walls and promote either microtubule polymerization or disassembly of microtubule from microtubules ends (Hartman *et al.*, 1998). Classical MAPs, such as MAP2 and tau, bind to the surface of the microtubules and neutralize the repulsive negative charge on the microtubule surface (Heald and Nogales, 2002). Coordination mistakes of these proteins may lead to diseases such as Alzheimer Disease (AD) (Baas and Qiang, 2005).

Since neurons are specialized cells and they do not divide, microtubules do not take part in the formation of mitotic spindle. Instead, microtubules in neurons are employed in elongation of axons (Karabay *et al.*, 2004). The major events for axonal differentiation such as elongation, branching, navigation, retraction, are accomplished by changes in the configuration and behavior of microtubules (Baas and Buster, 2003). Moreover, microtubules also serve as roadway for transport of organelles or vesicles throughout axons (Baas, 1999). The minus ends of all axonal microtubules are oriented towards the base of the axon, but dendritic microtubules have mixed polarities (Figure 1.6) (Lodish *et al.*, 2003).

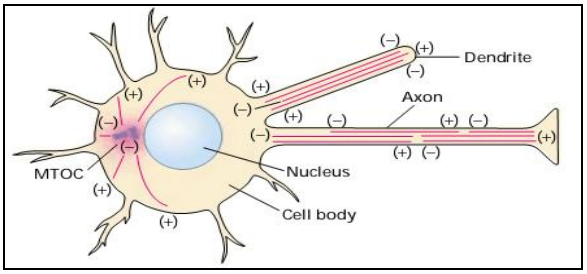

Figure 1.6: Microtubule organization in neurons (Lodish et al., 2003).

1.5 Microtubule Severing

The dynamic instability is not sufficient to explain all behaviors of microtubules observed in the cell. Some specialized cells like epithelial cells and neurons need non-centrosomal microtubules. There are three possible ways to create these non-centrosomal microtubules: (1) the release of microtubules originally anchored at the MTOC, (2) de novo production and growth of microtubules in the cytosol, and (3) severing of microtubules at site remote from the centrosome (Quarmby *et al.*, 2000; Quarmby and Lohret., 1999). Furthermore, the capacity of microtubule to move is inversely proportional to the length of the microtubule; shorter microtubule has more rapid movements (Baas *et al.* 2005). Thus, it is suggested that microtubule movement may be regulated by its length. There are two mechanism in regulation of length of microtubule: (1) addition/loss of tubulin subunits at the end of microtubule, and (2) internal breakage of the microtubule by microtubule severing enzymes (Casanova *et al.*,2009).

According to a recent study related to microtubule movement and called “cut and run”, the motor proteins, which provide transport of vesicle or microtubule, bind to all microtubule regardless of their length, yet it cannot transport longer microtubule. Since of shorter microtubule is mobile, long microtubule can only be transported after being severed into shorter pieces (Baas *et al.,* 2005).

Three microtubule severing enzymes have been identified until now: katanin, spastin and fidgetin. All of these three enzymes belong to the **A**TPases **A**ssociated with diverse cellular **A**ctivities (AAA) family of the ATPases and play critical role in essential cell process such as mitosis, neuronal development, cilia and flagella biogenesis (Casanova *et al.*, 2009).

Microtubule severing also has role in the formation of neuron structure. Especially katanin and spastin are important for the formation of filopodia, neuronal branching and axonal growth (Yu *et al.*,2008). In addition, dendrites and axons contain large numbers of noncentrosomal microtubules that are essential for architectural support and also act as railway for the transport of materials along the axon (Dent, *et al.*, 1999).

1.5.1 Spastin

Spastin is encoded by the *SPG4 (SPAST)* gene composed of 17 exons and 616 amino acids, and approximately 67.2 kDa. It contains two leucine –zipper and coiled– coil dimerization motif (Fonknechten *et al.*, 2000; Hazan *et al.*, 1999). The C-terminal part of the protein contains microtubule severing domain and microtubule binding domain (MTBD), which is necessary to bind to microtubule for severing. The N-terminal part of spastin contain hydrophobic domain (HD), which is important for localization, and MIT domain, which acts role in microtubule interaction and trafficking (Figure 1.7) (Lumb *et al.*, 2012).

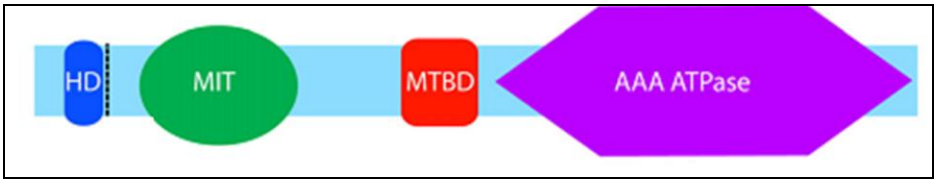

Figure 1.7: Spastin domains organization (Lumb *et al.,* 2012).

The mutations in *SPG4* gene most frequently (~40%) causes genetically inherited disease, **H**ereditary **S**pastic **P**araplegia (HSP) that is a clinically and genetically heterogeneous disorder of which principal feature is progressive lower-limb spasticity, extremity weakness and difficulties in walking (Reid *et al.,* 1999; Morita *et al.,* 2002). The mutations on this gene can be missense, nonsense, splice site mutations, deletion or insertion which result in reduction of mRNA and protein level of spastin (Riano *et al.,* 2009). A previous study shows that in addition to mutations in *SPG4* gene, regulation of spastin gene expression by some transcription factors affects production of spastin protein as well. The reduction of spastin protein level by transcriptional regulation can be associated with HSP (Canbaz *et al*., 2011).

1.5.2 Katanin

Katanin is the most well characterized microtubule severing enzyme whose name is originated from the Japanese word for samurai sword "katana". It is a heterodimer protein consisting of two subunits. Enzymatic subunit p60-katanin, which is named corresponding to its molecular weight (60 kDa), is encoded from *KATNA1* gene and it has ATPase activity and severs microtubules. Other subunit p80-katanin has 80 kDa molecular weight and is encoded from *KATNB1* gene. It localizes katanin to the centrosome and regulates microtubule severing activity of p60 subunit (Hartman and Vale., 1999; Quarmby *et al.,* 2000). In addition, p80 enhances the microtubule severing capacity of p60 subunit (Ahmad *et al*, 1999).

The p60-katanin is 491 amino acid long polypeptide and its N-terminal region binds to microtubules and C-terminal region has ATPase activity, and it severs microtubule. The p80-katanin is 658 amino acids. Its N-terminal contains WD40 repeat domain, a central proline rich domain, and the C-terminal domain is required for dimerization with the catalytic p60-katanin subunit (Karabay *et al.,* 2004; Hartman *et al.,* 1998; Hartman and Vale, 1999). The WD40 domain of p80-katanin can enhance p60 mediated microtubule severing by increasing affinity of p60 to microtubules. However, it is also indicated that the WD40 domain of p80-katanin might inhibit the microtubule-severing activity of p60 (McNally et al., 2000).

According to the experimental data it is suggested that MAPs which provide stability for microtubules, protect the microtubule from severing enzymes. The phosphorylation of these MAPs results in their dissociation with microtubule and

thus enable katanin bind to microtubule to sever it. Not all MAPs, but tau offers strong protection against severing by either katanin or spastin. Therefore, hyperphosphorylation of tau causes it to dissociate from microtubules, so microtubules become more accessible to katanin. Baas and Qiang (2005) suggested that this process can cause AD pathology (Figure 1.8).

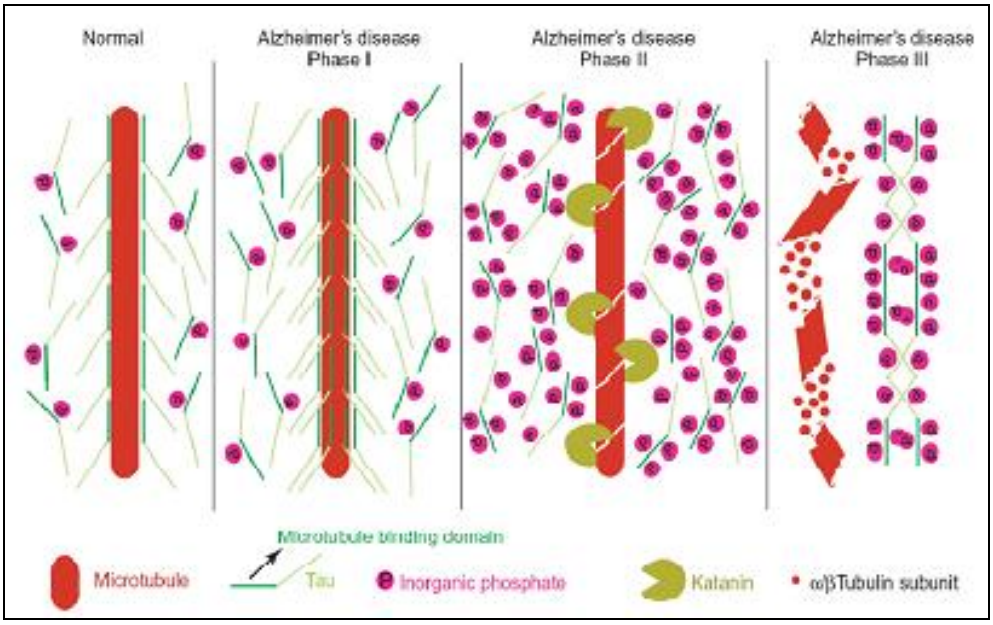

Figure 1.8: Model for microtubule-based axonal degeneration in AD (Baas and Qiang, 2005).

1.6 Transcriptional Regulation of Eukaryotic System

In eukaryotic cells, multiple events such as decondensation of locus, nucleosome remodeling, histone modifications, binding of transcriptional activator or repressor and basal transcription machinery are required for transcription of protein-coding gene (Smale and Kadonaga, 2003). There are thousands of protein-coding genes and each of these proteins has its own specific program for transcriptional control. The control of this process is predominantly mediated by numerous sequence-specific DNA binding factors (Kadonaga, 2004).

Transcription is a complex process that depends on the collective action of the sequence-specific factors along with the core RNA polymerase II transcriptional machinery including chromatin remodeling factors that mobilize nucleosome structures, and variety of enzymes that catalyze the modification of histones and other protein (Kadonaga, 2004).

In eukaryotes, the core promoter providing a control point for regulated gene transcription serves as a platform for assembly of transcription preinitiation complex that includes TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH (transcription factor IIA, IIB, IID, IIE, IIF, IIH respectively) and RNA polymerase II which function collectively to specify the transcription start site (Thomas and Chiang, 2006). This collection results in localization of RNA polymerase II at the correct initiation site, the core promoter (Smale and Kadonaga, 2003). This transcription preinitiation complex could only support basal transcription. This complex need "mediator", a multiprotein complex composed of ~20 different proteins in order to respond to additional regulator proteins such as activator and enhancer (Figure 1.9) (Kornberg, 2005).

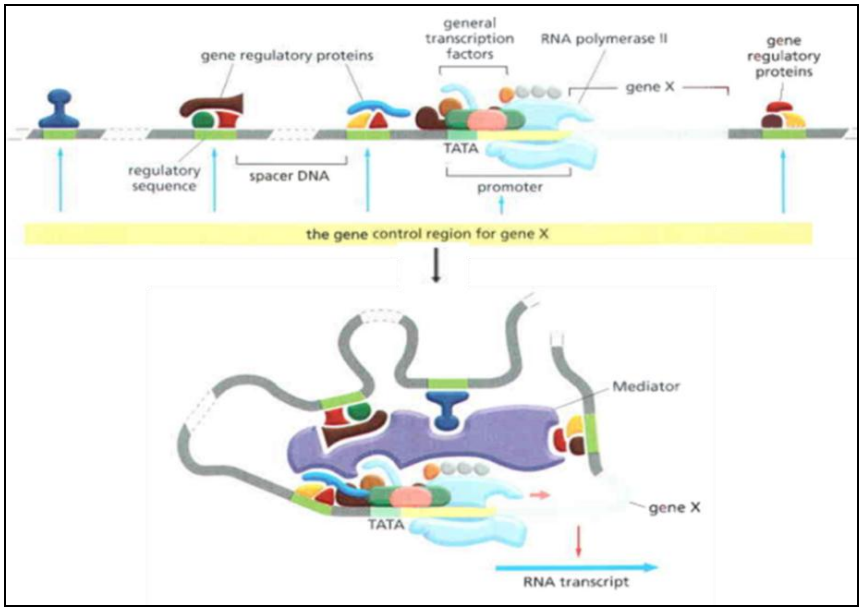

Figure 1.9: The illustration of eukaryotic transcription initiation complex (Alberts *et al.*, 2008).

The TATA box (also named the Goldberg-Hogness box after its discoverers) was the first core promoter element identified in eukaryotic protein-coding genes with TATAAA consensus sequence found 25 to 30 bp upstream of the transcription start site. At first it was speculated that the TATA box might be exactly conserved and essential for transcription initiation. However along with sequencing and characterization of genes, ratio of promoters including TATA box reduced (Smale and Kadonaga, 2003).

1.7 ETS Domain Transcription Factor

The ETS-domain family of eukaryotic transcription factor is composed of more than 30 members and their structure has significant sequence similarity throughout their entire length, especially ETS-domain (Sharrocks, 2001, Oikawa and Yamada, 2003).

Each protein exhibits an evolutionarily-conserved ETS-domain of about 85 amino acids residues (>95 % amino acid identity) that mediate binding to purine-rich DNA sequences with a central GGAA/T core consensus and additional flanking nucleotides (Figure 1.11) (Oikawa and Yamada, 2003).

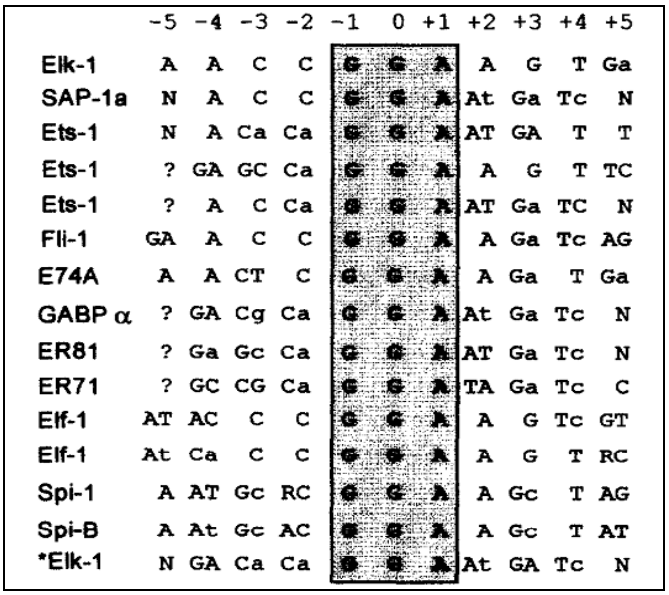

Figure 1.10: DNA-binding site of different ETS-domain transcription factors (Sharrocks *et al.*, 1997).

Many ETS family proteins are downstream target for signal transduction pathways affected by extracellular stimuli. Some post translational modifications occur on the protein and they often change their DNA binding ability, transcriptional activation, association with cellular partners, subcellular localization and/or protein stabilities. They activate or repress transcription of target gene together with other members of transcription factors and co-factors and play crucial role in regulation of numerous cellular function including apoptosis, development, differentiation and oncogenic transformation during both embryonic development and in the adult. In addition, it was shown that some family members have both repressive and simulative abilities, making the strict distinction impossible (Sharrocks, 2001). These signaling pathways

including **M**itogen **A**ctivating **P**rotein kinase (MAPK), **E**xtracellular Signal **R**egulated **K**inase (ERK) 1 and 2, p38 and c-**J**un **N**-terminal **K**inase (JNK) and Ca^{+2}-specific signal activated by growth factors or cellular stress (Yordy and Muise-Helmericks, 2000).

ETS-domain family proteins can be classified into several subfamilies on the basis of their structural composition and their similarities in the DNA-binding ETS domain. The ETS domain localize in C-terminal region of most of them. However several ETS subfamilies like **T**ernary **C**omplex **F**actor (TCF) subfamily have the ETS domain in their N-terminal regions (Figure 1.12) (Oikawa and Yamada, 2003).

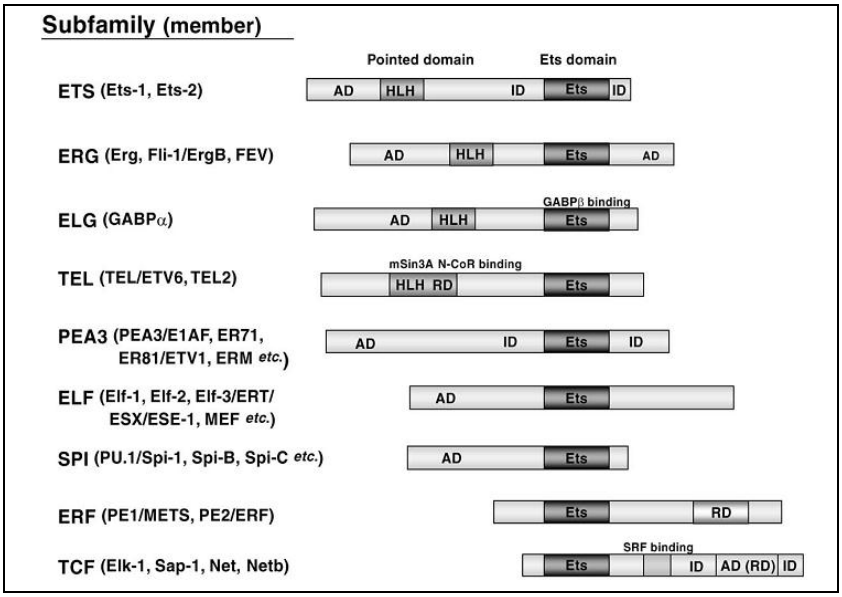

Figure 1.11: ETS-domain transcription factor subfamilies and their structures (Oikawa and Yamada, 2003).

1.7.1 TCF family

Ternary **C**omplex **F**actor (TCF) family is the most studied subfamily of ETS domain factors. The first TCF transcription factor was identified in the nuclear extract of HeLa cells. This novel protein was called p62 due to its 62000 Da molecular weight. Then, it was shown to be homologous to Elk1 (Ets-like 1) protein, which was previously described by Hipskind *et al* (1991). Later, two homologous proteins of Elk1, Sap1 and Net/Sap2 have been identified (Sharrocks, 2002).

Their characteristic property is the ability to form ternary nucleoprotein complex with the Serum Response Factor (SRF) over the Serum Response Element (SRE) of the *c-fos* promoter (Figure 1.13)

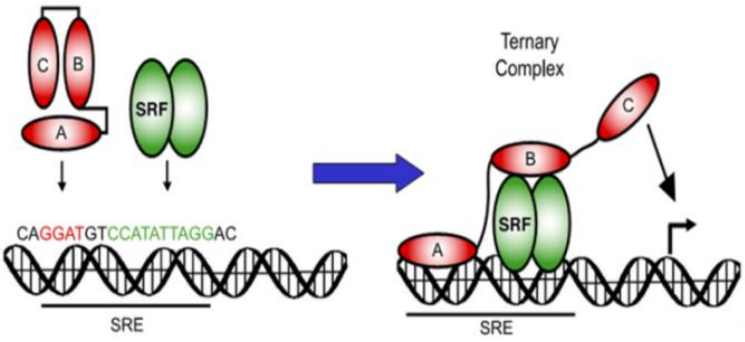

Figure 1.12: Ternary complex formation on SRE site (Buchwalter *et al.*, 2004).

The TCFs have similar four regions known as A, B, C, D domain. The A domain also called ETS domain localizes in the N-terminal is responsible for DNA binding and contains a nuclear localization signal (NLS) and nuclear export signal (NES). It has also been demonstrated to act as a transcriptional inhibitor in a member of TCF subfamily called Elk1. The B domain interacts with the SRF and allows ternary complex formation. The C domain also called transactivation domain localizes in C-terminal and is phosphorylated by MAP kinase pathway. The D domain is a docking site for MAP kinases and lies upstream from C domain. A second docking site FXFP motif within C domain is specific for ERK pathway. (Figure 1.14) (Buchwalter *et al.*, 2004).

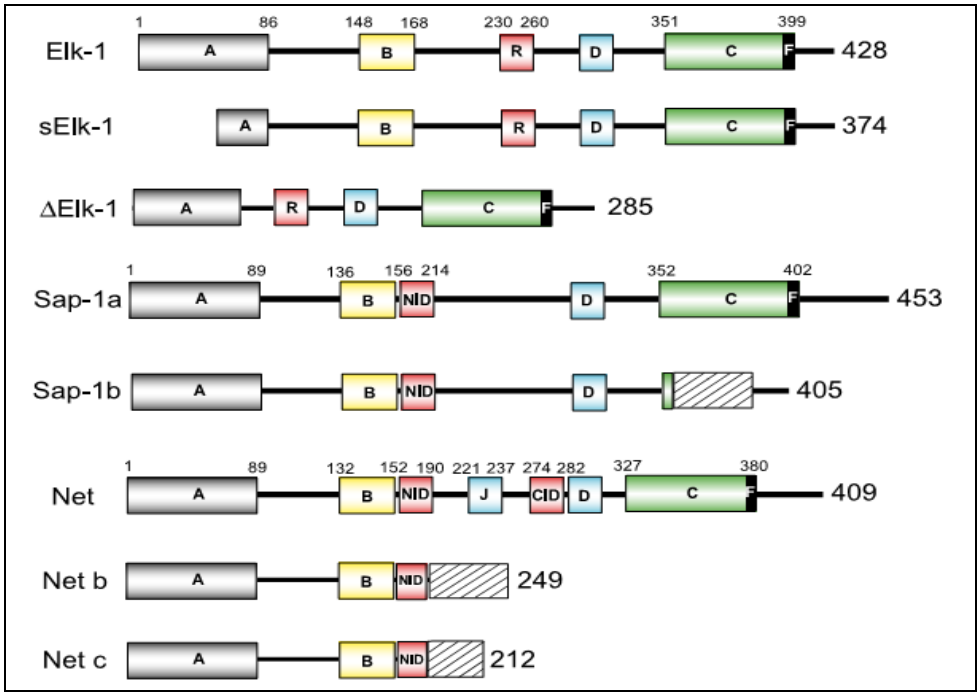

Figure 1.13: Structure of TCF subfamily members (Buchwalter *et al.*, 2004).

1.8 Elk1 Transcription Factor

Elk1 is the best studied member of TCFs family and has been widely studied in the context of cellular signaling (Nilsson *et al*. 2007). In addition to four domains mentioned above, Elk1 has also R motif at the central part of the protein. This motif dampens down the basal transcriptional activity of Elk1 and is sufficient to mediate transcriptional repression (Yang *et al.,* 2002).

In non-neuronal cells, Elk1 is restricted to nucleus as expected for a transcription factor; however in mature neuron it is expressed in both cytoplasmic and neuritic compartments in addition to the nucleus (Sgambato *et al.,* 1998; Vanhoutte *et al*, 2001). Elk1 localization in axon and dendrites of adult rat brain was previously shown (Sgambato *et al.,* 1998). Moreover Elk1 was found to be associated with neuronal microtubules in hippocampal culture and neuroblastoma cells. When Elk1 is phosphorylated, it relocalizes to the nucleus (Demir *et al.,* 2009). Neuronal isoform of Elk1, sElk1(short Elk1), expression of which is restricted to the nucleus, is lacking the first 54 amino acids of the ETS domain due to its translation start site in Elk1 sequence. Therefore truncated protein has different DNA binding property than full length protein. The sElk1 plays an opposite role to Elk1. sElk1 antagonizes Elk1-mediated transactivation of SRE and promotes neuronal differentiation (Vanhoutte *et al.,*2001).

Modifications of Elk1 have crucial roles in alteration of Elk1 activity on the target gene. There are two important modification on Elk1; phosphorylation and SUMOylation. Elk1 is a direct target for MAP kinase pathway and phosphorylation of Elk1 C-terminal transactivation domain stimulates the activator role of Elk1. On the other hand SUMOylation of Elk1 at R motif results in repressive effect of Elk1 on the target gene expression (Yang *et al.,* 2003). The modification of Elk1 is mentioned in details in the section 1.9.1.

1.9 Post-Translational Modifications

Post-translational modifications (PTM) of proteins lead to the functional diversity of the proteome by proteolytic cleavage or by the covalent addition of functional groups on proteins. These modifications include phosphorylation, glycosylation, methylation, ubiquitination, acetylation, SUMOylation. PTMs modulate the activity

of most eukaryote proteins and can affect protein activity, localization, turnover, and interactions with other cellular molecules such as proteins, nucleic acids, lipids, and cofactors (Mann and Jensen, 2003).

Phosphorylation of proteins present in numerous pathways is a major regulatory mechanism in eukaryotic organism and causes conformational change in the structure of protein. Kinases phosphorylate proteins and phosphatases dephosphorylate proteins. Therefore proteins are switched between "active" or "inactive" states. Phosphorylation generally occurs on serine, threonine and tyrosine residues of proteins (Chang and Steward, 1998, Ciesla *et al.*, 2011). The main process in which the major regulator is phosphorylation/dephosphorylation are cell cycle and apoptosis. Phosphorylation induces or inhibits specific cell cycle events. For example, cyclins are phosphorylated by specific cyclin dependent kinase and so cell cycle progress next step, or Rb hypophosphorylation is not sufficient to enter into G1 phase of the cell cycle. Rb should be hyperphosphorylated to begin the cell division mechanism. Furthermore chromosome condensation, nuclear envelope break down durin mitosis and transcription factor nuclear export are also regulated by phosphorylation that increases their affinity for a specific exportin, and many of cell surface receptors are activated by phosphorylation (Lodish *et al.*, 2003).

SUMO (**S**mall **U**biquitin Related **Mo**difier) is ubiquitin-like protein which changes the function of the substrate protein. Although ubiquitin has primary role on proteolytic degradation of target proteins, it can also regulate localization or activity of proteins. Otherwise SUMO conjugates to target protein like ubiquitin, but SUMOylation is not related with protein degradation. SUMO effects protein localization and activation similar to non-proteolytic activity of ubiquitin, and has important role in many cellular process including gene expression, chromatin structure, signal transduction. Although ubiquitin and SUMO have similar non-proteolytic activity, they can cause different events after they modify the target proteins. For instance, modification of histones by ubiquitin usually increases gene expression whereas modification of histone by SUMO usually decreases gene expression. SUMO stimulates interaction with other proteins that have little or no affinity for the unmodified target protein.

Four SUMO homologs have been described in mammals: SUMO-1, SUMO-2/3 and SUMO-4. Human SUMO-2 and SUMO-3 are 95% homologous at the protein level,

whereas SUMO-1 shows 47% homology. SUMO-4 is the least characterized isoform and its expression pattern is generally reported in the kidney (Salinas *et al*., 2004, Gill, 2004). Moreover 18% sequence similarity and similar protein folding are determined between SUMO-1 and ubiquitin by NMR analysis. However the localization of charged amino acid on the surface is very different from that ubiquitin. It is thought that SUMO binds to protein as monomer, however SUMO-1, SUMO-2 and SUMO-3, have been observed to form polymeric chains *in vitro* (Gill, 2004).

SUMOylation is a dynamic process. SUMO is attached to target protein through the action of enzymes including E1 activating enzyme, and the E2 conjugating enzyme Ubc9 that transfers the SUMO to protein. E3 ligase has role as bridge to facilitate the action of Ubc9. On the other hand SUMO is cleaved by SUMO1/ sentrin/ SMT3-specific peptidase (SENPs) (Witty *et al.,* 2010).

A consensus SUMO motif, ψKxE has been identified. Ψ represent a hydrophobic amino acid, x represent any amino acid, and K is the target of SUMO conjugation (Rodriguez *et al.,* 2001). Additionally the SUMO modification site commonly lies within inhibitory domain (Gill, 2004).

In mammalian cells, SUMO modification of some proteins provides localization of these proteins to the nucleus (Gill.,2004). Moreover SUMO modification can negatively regulate gene expression through repressed transcription factor activity or promoted interaction with transcriptional co-repressor. For instance, SUMOylation of p300 stimulates association with histone deacetylase complex 6 HDAC6 and it led to reduction of histone acetylation at the target gene promoter (Girdwood *et al*, 2003).

Another mechanism of SUMO modification is about DNA repair system. Thymine-DNA glycosylase enzyme (TDG) has role in base excision repair in response to DNA damage. SUMO modification on TDG alters its enzymatic activity due to it reduced affinity for DNA (Hardeland *et al.,* 2002).

1.9.1 Modifications of Elk1

Elk1 transcription factor undergoes two post translational modifications; phosphorylation and SUMOylation. These two post translational modifications allow Elk1 to gain both activating or repressing functions. Typically, targets of Elk1 are

immediate-early gene activated by extracellular signals, where Elk1 permits transient and rapid gene activation as response. A tight control mechanism is required for this swift activation, hence dynamic interaction exists between activating MAPK and repressive SUMO pathways (Yang *et al.*, 2003).

Elk1 has two repression sites; one of them is ETS-domain and the other one is repression domain (R motif) that was identified by Yang et al. (2002). This study shows that amino acids between 230 and 260 in Elk1 comprise repression domain and is sufficient to repress transcription. The R motif of Elk1 contains three lysine residues K230, K249, K254, which are SUMO binding sites and are required for the repression of basal transcriptional activation. Moreover, glutamic acid residues are also necessary so that SUMO can recognize the target protein. Especially K249 and E251 are important amino acids to reduce gene expression of target protein. Mutations or deletions in any of these residues result in significant enhancement of gene expression (Yang *et al.*, 2002, Yang *et al.*, 2003). In addition, K249, V250 and E251 residues are also critical for enhancing the activities of histone deacetylase (Figure 1.15). Moreover R motif is thought to maintain Elk1 in an inactive state before activation by MAPK pathway (Yang *et al.*, 2002).

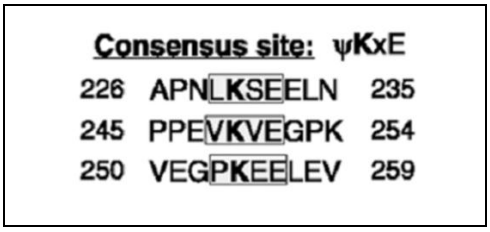

Figure 1.14: The critic SUMO binding residues of WT.Elk1 and consensus SUMO recognition amino acids (Salinas *et al.*, 2004).

Otherwise phosphorylation of Elk1 allows Elk1 to act as an activator and increase gene expression of the target. This phosphorylation occurs on Serine 383 and 389 residues located at the C terminal (Figure 1.16). There are three MAPK pathways identified, whose targets are Elk1. The ERK cascade is stimulated by growth factors and mitogens. The JNK and p38 pathways respond to cytokines signals and stress conditions (Buchwalter *et al.*, 2004). Phosphorylation of Elk1 by these pathways provides loss of SUMO conjugation of Elk1. Therefore Elk1 loses its repressive effect and gains activator effect on the target gene promoter (Yang *et al.*, 2003).

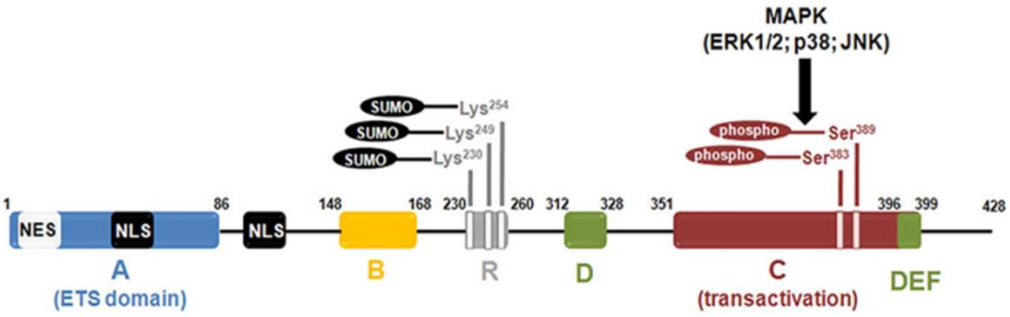

Figure 1.15: SUMOylation and phosphorylation sites of Elk1 (Besnard *et al.,* 2011).

It was shown that phorbol 12-myristate 13-acetate (PMA) treatment induced phosphorylation of MAPK rapidly and increased their ability to phosphorylate Elk1 in a human myeloid leukemia cell line (Hu *et al.,* 2000). In addition, in phosphorylation dependent de-SUMOylation of Elk1by PMA, SENPs have role on cleaving of SUMO from substrate in HEK-293T cells (Witty *et al.,* 2010).

1.10 Aim of the Study

Spastin and katanin sever microtubules by hydrolyzing ATP and this severing activity is required for important processes such as mitosis, neuronal development, axonal branching. Spastin is encoded by *SPG4* gene, whereas katanin subunit p80 and p60 are encoded by *KATNB1* and *KATNA1* genes, respectively. p60-katanin subunit shows enzymatic activity, while p80-katanin has role in regulation and localization of p60-katanin. The activity of p60-katanin can be stimulated or inhibited by p80-katanin.

In our lab, the optimal promoters of *SPG4* and *KATNB1* genes were analyzed to determine the critical region for the regulation of transcription. 700 bp and TATA-less promoter including critical CpG island was identified for *SPG4* as an optimal promoter called S2 construct. 518bp and TATA-less sequence was identified for *KATNB1* as optimal promoter and called F2 construct. In our current study S2 and F2 constructs were used as optimal promoters.

On the other hand, transcription factor Elk, which belongs to ETS domain family and TCF subfamily, regulates expression of *SPG4* and *KATNB1* genes. Elk1 has a repression domain (R motif) in the central part and activation domain at C-terminal region. It can act as either repressor or activator depending on the different promoter sites where these domains bind. When the R motif is SUMOylated, Elk1 has

repressive activity on the target gene promoter; otherwise transactivation domain of Elk1 is phosphorylated and increases expression as an activator.

In this study, our aim is to identify the transcriptional regulation of *SPG4* and *KATNB1* genes by Elk1. Firstly we will intend to construction of Elk1.woR, which is without repression domain at central part of wild type Elk1, in order to understand effect of Elk1 on *SPG4* optimal promoter when Elk1 cannot undergo SUMO modification. On the other hand, we aimed to determine effect of Elk1 on *KATNB1* gene expression. The three experimental methods will be designed to show in expressional, transcriptional and translational levels; dual-luciferase reporter assay, quantitative real time PCR, immunostaining and Western blotting, respectively.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1 Equipment

Equipment used in this study is shown in the table below.

Table 2.1: Laboratory equipment used in the study.

Equipment	Supplier Company
Laminar Air Flow Cabinets	FASTER BH-EN 2003
Pipettes	2.5µL, 10µL, 100µL, 200µL, Eppendorf 1000µL, Finnpipette Thermo
Electronic Pipette	Finnpipette Thermo
Centrifuges	Biolab SIGMA 6K15, Beckman Coultier Microfuge®18, Beckman Coulter Avanti™ J-30 I, IECCL10 Centrifuge, Thermo Electron Corporation, Labnet, Labnet International C1301-230V
Incubator with CO_2	Biolab SHEL LAB
Thermo Cycler	Techne, TC-3000
Vortex	Heidolph, Reaxtop
Quick Spin	Labnet International, C1301-230V
Magnetic stirrer	VELP scientifica
pH Meter	Mettler Toledo MP220
Light Microscope	Olympus CH30 (USA)
Hemacytometer	FisherLab Scientific, 0267110
Nucleofector	Lonza, Amaxa
Luminometre	Thermo, Fluoroskan Ascent FL
High Pressure Steam Sterilizer	TOMY SX-700E
Precision Balance	Precisa 620C SCS
Balance	Precisa BJ 610 C
Ice Machine	Scotsman AF 10

Table 2.1 (cont'd.): Laboratory equipment used in the study.

Electrophoresis Gel System	Thermo EC MiniCell® PrimoTM EC320 Electrophoretic Gel System
Power Supply	EC250-90 Apparatus Corporation BIO-RAD
SDS-PAGE Gel Electrophoresis System	BIO-RAD MiniProtean
Microwave	Arçelik MD582
UVIPhoto MW Version 99.05 for Windows 95 & 98	UVItec Ltd.
Spectrophotometers	Shimadzu, UV-1601 Thermo Scientific Nanodrop™ 2000c BIO-RAD Benchmark Plus
Water Baths	Memmert, Elektro-mag M 96 KP
Thermomixer	Eppendorf Thermomixer Comfort
Shaker	Forma Orbital Shaker, Thermo Electron Corporation
Freezers	UĞUR (+4 °C), UĞUR (-20°C), New Brunswick Scientific (-80 °C)
DNA Sequencer	Applied Biosciences 3100 Avant
Light Cycler® 480	Roche
LightCycler® 480 Multiwell Plate 384	Roche, 04729749001
LightCycler® 480 Sealing Foil 50 foils	Roche,04729757001
Luminomertical 96 well plate	Lumitrac 200
TCS SP2 SE Confocal Microscope	Leica, Microsystems
Trans-Blot® SD Semi-Dry Transfer Cell	BIO-RAD
Kodak Medical X-ray Processor	Kodak

2.1.2 Commercial kits

Commercial kits used in this study are shown in the table below

Table 2.2: Commercial kits used in the study.

Kit	**Supplier Company**
QIAprep Spin Miniprep Plasmid Purification Kit	Qiagen, 27106
QIAGEN EndoFree Plasmid Maxi Kit	Qiagen, 12362
QIAquick PCR Purification Kit	Qiagen, 28104

Table 2.2 cont'd: Commercial kits used in the study.

QIAquick Gel Extraction Kit	Qiagen, 28706
Endofree Plasmid Maxi Kit	Qiagen, 12362
BigDye® Terminator v3.1 Cycle Sequencing Kit	APPLIED Biosystem
Dual-Luciferase Reporter Assay System	Promega, E1910
Transfast Transfection Reagent	Promega, E2431
High Pure RNA Isolation Kit	Roche, 11828665001
Complete Lysis-M	Roche, 04719956001
RevertAid™ H Minus First Strand cDNA Synthesis Kit	Fermentas, K1631
Light Cycler 480 Probes Master	Roche, 04707494001
Amaxa™ Cell Line Nucleofector Kit V	Lonza, VCA-100
20X LumiGLO® Reagent and 20X Peroxide	Cell Signaling, 7003
SuperSignal® West Femto Maximum Sensitivity Substrate	Thermo, 34094
Micro BCA™ ProteinAssay Reagent Kit	Pierce, 23235
Restore Western Blot Stripping Buffer	Thermo Scientific

2.1.3 Molecular cloning assay

- Primers (Alpha DNA)
- 25 mM $MgCl_2$ (Fermentas)
- 2 mM dNTP Mix (Fermentas)
- *Taq* Polymerase 5u/µL (Fermentas)
- 10X *Taq* Buffer with KCl (Fermentas)
- Mass Ruler™ DNA Ladder Mix (Fermentas)
- 6X DNA Loading Dye (Fermentas)
- Agarose Low EEO (Applichem)
- EtBr (Merck)
- KpnI Restriction Enzyme (Fermentas)
- 1X Buffer KpnI (Fermentas)
- SalI Restriction Enzyme (Fermentas)
- 1X Buffer O (Fermentas)
- BamHI Restriction Enzyme (Fermentas)
- 1X Buffer BamHI (Fermentas)

- T4 Ligase (Roche)
- 10X Ligation Buffer (Roche)
- PEG 8000 (Fluka)
- Tris Base (BDH Laboratory)
- EDTA (Merck)
- Boric Acid (Merck)
- Molecular Biology grade Ethyl Alcohol (Fiedel-de Haën)

2.1.4 Bacterial assay

- Tryptone (BDH Laboratory)
- Yeast Extract (Merck)
- NaCl (Fluka)
- Agar (Merck)
- Ampicillin (Sigma-Aldrich)
- Kanamycin (Sigma-Aldrich)
- Glycerol (Fluka)

2.1.5 Cell culture assay

- 25 cm^2, 75 cm^2 Tissue Culture Flask (TPP)
- 6 well, 24 well Culture Plate (TPP)
- 5 mL, 10 mL, 25 mL Serological Pipettes (TPP)
- 250 mL Vacuum Filtration System (TPP)
- Poly-L-Lysine (Sigma-Aldrich)
- 0,22 μm Syringe Filtre (TPP)
- 10 mL, 20 mL Syringe (Set Inject)
- Dulbecco's Modified Eagle Medium (DMEM) 1X (Gibco)
- Fetal Bovine Serum (Gibco)
- L-Glutamine Solution, 200mM (Biochrom)
- Penicilin/Streptomycin Solution 100X (Biochrom)
- Trypsin-EDTA 0,25/0,02 Solution (Biochrom)
- Phosphate Buffered Saline (PBS) 10X pH=7,2 (Gibco)
- DMSO (Fiedel-de Haën)

2.1.6 Protein assay

- SDS (AppliChem)
- TEMED (AppliChem)
- DTT (Merck)
- Acrylamide (Sigma Aldrich)
- Bis-acrylamide (Sigma Aldrich)
- Bromophenol Blue (Merck)
- SeeBlue® Plus2 Protein Standard (Invitrogen)
- Biotinylated Protein Ladder (Cell Signaling)
- Nitrocellulose Paper (BIO-RAD)
- 3MM Whatman Filter Paper (Whatman)
- Goat anti-mouse IgG antibody-HRP conjugate (Promega)
- Goat anti-rabbit IgG antibody-HRP conjugate (Promega)
- Tween-20 (AppliChem)
- Skimmed milk powder (%5) (OXOID)
- BSA (Fluka)
- Rabbit monoclonal β-actin antibody (Cell Signaling)
- Rabbit polyclonal SUMO2-3 antibody (Santa Cruz)
- Rabbit monoclonal SUMO1 antibody (Cell Signaling)
- Rabbit polyclonal Elk1 antibody (Santa Cruz)
- Mouse monoclonal spastin antibody (Sigma)
- Goat polyclonal katanin antibody (Santa Cruz)
- Rabbit polyclonal katanin antibody (Sigma)
- Alexa Flour 488 goat anti-rabbit (Invitrogen)
- Alexa Flour 647 goat anti-mouse (Invitrogen)
- Alexa Flour 647 goat anti-rabbit (Invitrogen)
- Alexa Fluor 488 donkey anti-goat (Invitrogen)
- DAPI (Sigma)
- Mounting Medium (Sigma)

2.1.7 General chemicals

- Ethanol (Riedel- de Haën)
- Methanol (Riedel- de Haën)
- Isopropanol (Fluka)
- KCl (Fluka)
- HCl (Applichem)
- NaOH (Applichem)
- $MgCl_2$ (Merck)
- $MgSO_4$ (Fluka)
- HEPES (Sigma)
- $CaCl_2$ (AppliChem)
- Glucose (Merck)
- Glicial Acetic Acid (Fluka)
- Glycerol (Fluka)
- Glycine (Merck)
- Tricine (Merck)

2.1.8 Buffers and solutions

2.1.8.1 50X TAE buffer

Stock solution 50X TAE buffer was diluted to 1X as a working solution. It was used in DNA gel electrophoresis to prepare the gel and as the tank buffer. The content of this solution is shown in table 2.3.

Table 2.3: Content of 50X TAE buffer.

Content	Concentration	Amount
Tris Base	2 M	242 g
Glacial acetic acid	1 M	57,1 mL
EDTA	50 mM	100 mL (0,5M) (pH=8)
dH_2O		up to 1 L

2.1.8.2 10X TBE buffer

10X TBE buffer was prepared as a stock solution. 1X dilution of this buffer was used for DNA gel electrophoresis to prepare the gel and as the tank buffer. The content of this solution is shown in table 2.4.

Table 2.4: Content of 10X TBE buffer.

Content	Concentration	Amount
Tris Base Boric Acid EDTA dH_2O	890 mM 890 mM 20 mM	108 g 55 g 40 mL (0,5M) (pH=8) up to 1 L

2.1.8.3 Separating acrylamide solution

Separating acrylamide solution was used for preparation separating gel. The amount and content of solution is given in table 2.5.

Table 2.5: Content of separating acrylamide

Content	Amount
Acrylamide Bis-acrylamide dH_2O	46,5 g 1,5 g up to 100 mL

2.1.8.4 Stacking acrylamide solution

Stacking acrylamide solution was used for the preparation of stacking gel. The amount and content of solution is given in table 2.6.

Table 2.6: Content of stacking acrylamide

Content	Amount
Acrylamide Bis-acrylamide dH_2O	48 g 1,5 g up to 100 mL

2.1.8.5 2X sample buffer

2X sample buffer was used to denature protein samples which were loaded on SDS-polyacrylamide gel. Content of 2X sample buffer is shown in table 2.7.

Table 2.7: Content of 2X sample buffer.

Content	Concentration	Amount
Tris-HCl SDS Glycerol Bromophenol Blue DTT dH_2O	0,125 M 4 % 20 % 0,05 % 0,15 M	2,5 mL (0,5M) 4 mL (10%) 2 mL (100%) 5 mg 231 mg Up to 10 mL

2.1.8.6 Running buffer for SDS-PAGE

Both the anode and the cathode buffers were used as SDS-PAGE running buffer, and their contents are given in table 2.8 and table 2.9, respectively.

Table 2.8: Content of 1X anode buffer

Content	Concentration	Amount
Tris-Base dH_2O	0,2 M	24,22 g Up to 1 L (pH=8,9)

Table 2.9: Content of 1X cathode buffer

Content	Concentration	Amount
Tris-Base Tricine SDS dH_2O	0,1 M 0,1 M	182 g 17,92 g 1 gr (1%) Up to 1 L (pH=8,25)

2.1.8.7 Towbin electrotransfer buffer

Transfer buffer was used during Western blot analysis to reduce pH changes of the solution during the transfer of proteins from SDS polyacrylamide gel to nitrocellulose membrane. Content of this buffer is indicated in table 2.10.

Table 2.10: Content of towbin electrotransfer buffer.

Content	Concentration	Amount
Tris-base Glycine Methanol SDS dH_2O	25 mM 192 mM 20 % 0,05 %	3 g 14,4 g 200 mL 0,05 g up to 1 L

2.1.8.8 10X TBS buffer

TBS (with HCl) was prepared and used as blocking and washing buffer during Western blot analysis. 10X stock solution was diluted to 1X concentration with dH_2O to obtain working solution. Content of TBS buffer is shown in table 2.11.

Table 2.11: Content of 10X TBS buffer.

Content	Concentration	Amount
Tris-base Glycine Methanol SDS dH_2O	25 mM 192 mM 20 % 0,05 %	3 g 14,4 g 200 mL 0,05 g up to 1 L

2.1.8.9 TBS-T buffer

TBS-T solution was prepared for washing in Western blot analysis. Preparation of this buffer is shown in table 2.12.

Table 2.12: Content of TBS-T buffer.

Content	Concentration	Amount
TBS Tween-20	1X 0,05 %	1 L 500 µL

2.1.8.10 Blocking and incubation buffer

Blocking and incubation buffer was used for blocking of membrane and incubation with primary antibody. Preparation of this buffer is shown in table 2.13.

Table 2.13: Content of blocking and incubation buffer.

Content	Concentration	Amount
TBS-T Skimmed milk powder	1X	100 mL 5 g

2.1.8.11 PBS solution

PBS solution was prepared by dissolving 1 PBS tablet within 100mL dH_2O.

2.1.8.12 PBST solution

To prepare PBST solution, 2 mL Tween-20 was added into 1 L PBS solution.

2.1.8.13 Elution buffer for immunoprecipitation

50 mM Glycine (pH=2,8) was prepared and used as elution buffer.

2.1.9 Bacterial strains

Escherichia coli DH5α strain [F^-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1]

Escherichia coli dam- dcm- GM2163 strain [F^-, *dam-13::*Tn 9 *dcm-6 hsdR2 leuB6 his-4 thi-1 ara-14 lacY1 galK2 galT22 xyl-5 mtl-1 rpsL136 tonA31 tsx-78 supE44* $McrA^-$ $McrB^-$] (Fermantas, M009).

2.1.10 Bacterial culture media and solution

2.1.10.1 LB medium

Luria Bertani (LB) Medium was prepared by dissolving 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1L dH_2O. The media was sterilized by autoclaving for 15 minutes (min) at 121°C. To prepare a selective media, 50 µg/L ampicillin or kanamycin was added after the media was cooled down approximately to 55°C.

2.1.10.2 LB-agar medium

LB-Agar Medium was prepared by dissolving 10 g tryptone, 5 g yeast extract, 10 g NaCl and 20 g agar in 1L dH2O. The media was sterilized by autoclaving for 15 min at 121°C. To prepare a selective media, 50 µg/L ampicillin was added after the media was cooled down approximately to 55°C. After mixing the medium with ampicillin, the content was poured into 10 mm Petri plates.

2.1.10.3 SOC medium

SOC medium was used to cultivate *E.coli* for 1 hour (h) after heat shock during transformation. 2 g tryptone, 5 g yeast extract, 0,058 g NaCl, 0,0186 g KCl, 0,095 g $MgCl_2$, 0,23 g $MgSO_4$ and 0,36 g glucose was resolved in 100 mL dH_2O and sterilized at 121 ^oC by autoclaving for 15 min.

2.1.10.4 $CaCl_2$ solution

$CaCl_2$ solution was used in the preparation chemically competent *E.coli* cells that were further used in transformation. The solution contains 60 mM $CaCl_2$, 10mM PIPES and 15 % glycerol in dH_2O. pH was adjusted to 6,4 to dissolve PIPES and then was filter sterilized with 0,2 µm filter.

2.1.11 Vectors

2.1.11.1 pGL2-basic and pGL3-basic

The pGL2 and pGL3 luciferase reporter vectors (Promega, E1641 and E1751) provide a basis for the quantitative analysis of transcription factors that potentially regulate mammalian gene expression. These vectors carry the coding region for firefly (*Photinus pyralis*) luciferase that has been optimized for monitoring transcriptional activity in transfected eukaryotic cells. pGL2-basic and pGL3-basic vectors lack eukaryotic promoter and enhancer sequences, allowing maximum flexibility in cloning putative regulatory sequences. Expression of luciferase activity in cells transfected with pGL2-basic or pGL3-basic plasmids depends on insertion and proper orientation of a functional promoter upstream from *luc* or *luc*$^{+}$ respectively. pGL2-basic vector circular map is shown figure 2.1 (Promega Technical Manual, E1641) and pGL3-basic vector circle map is shown figure 2.2 (Promega Technical Manual, E1751).

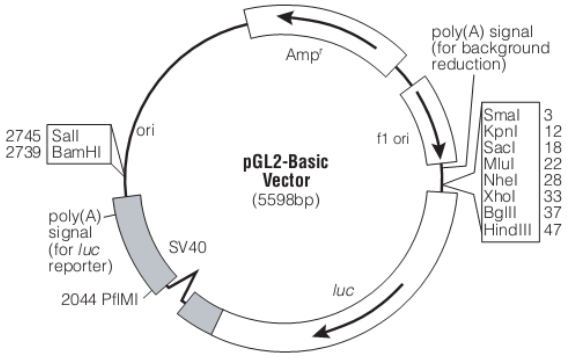

Figure 2.1: pGL2-basic vector circular map (Promega Technical Manual, E1641).

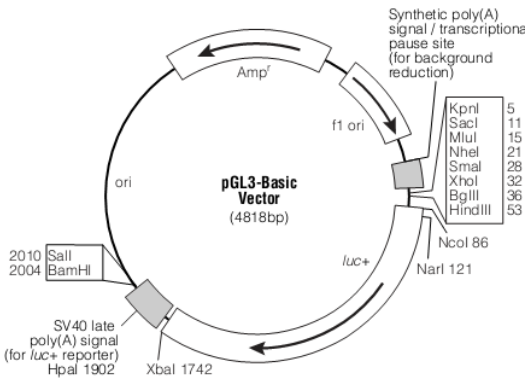

Figure 2.2: pGL3-basic vector circular map (Promega Technical Manual, E1751).

2.1.11.2 pRL-TK

The pRL-TK vectors (Promega, E2241) providing constitutive expression of *Renilla* luciferase *(Rluc)* are control reporter vectors. The pRL-TK vector contains the *Herpes simplex* virus thymidine kinase (HSV-TK) promoter to provide low to moderate levels of *Renilla* luciferase expression in co-transfected mammalian cells. *Renilla* luciferase is a 36 kDa monomeric protein that does not require post-translational modification for the activity. Therefore like firefly luciferase the enzyme may function as a genetic reporter immediately following translation. pRL-TK vector circular map is shown in figure 2.3.

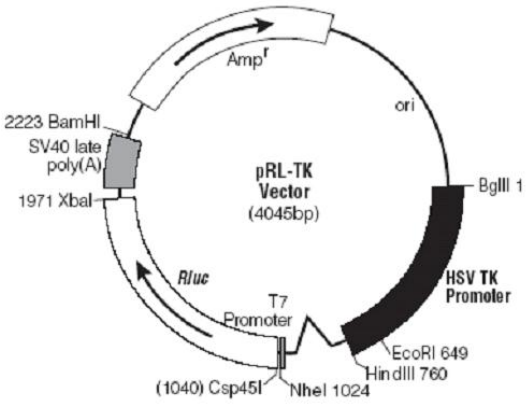

Figure 2.3: pRL-TK vector circular map (Promega Technical Bulletin, E2241).

2.1.11.3 pCMV6 vector

pCMV6 vector is used as an expression vector (Figure 2.4). WT-Elk1, Elk1 without R domain (Elk1-woR) and constitutively active form of Elk1 (Elk1-VP16) sequences were inserted in this vector. *KpnI, SalI, BamHI* restriction endonucleases in multiple cloning site of vector were used for cloning. Wt- Elk1 is cloned into vector by using KpnI and BamHI restriction enzymes. For the cloning of Elk1-woR sequence, firstly the vector was digested with KpnI and SalI restriction enzymes and upstream sequence of repression domain of Elk1 was ligated with the digested vector. Then the vector containing upstream region was again digested with SalI and BamHI restriction enzymes and downstream sequence was ligated to restricted vector.

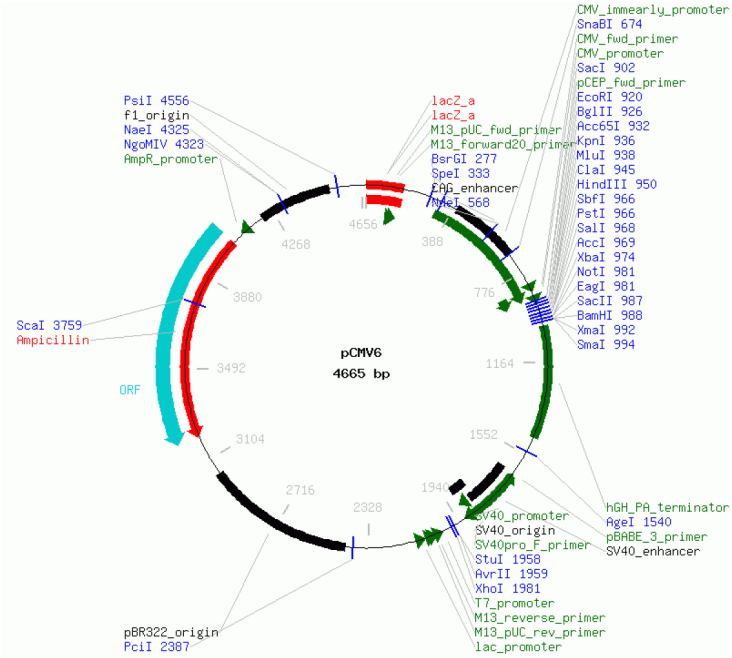

Figure 2.4: pCMV6 vector circle map.

2.1.12 Cell Line

Human metastatic neuroblastoma cell line SH-SY5Y, which is a subline of SK-N-SH neuroepithelioma cell line was used. SK-N-SH cell line had been established from the metastatic neuroblastoma cells including bone marrow biopsy of a 4-year-old girl in 1970.

2.1.13 Cell culture media

2.1.13.1 SH-SY5Y culture medium

SH-SY5Y culture medium was prepared with 10 % FBS, 2 mM L-Glutamine, 1X Penicilin/Streptomycin within DMEM containing 1 g/L glucose and then filter sterilized with 0,2 µm filter.

2.1.13.2 SH-SY5Y freezing medium

To prepare SH-SY5Y freezing medium, 5 % DMSO was supplemented within culture medium and then sterilized with 0,2 µm filter .

2.2 Methods

2.2.1 Primer design

Forward and reverse primers specific for expression vector pCMV6.Elk1.woR construct with appropriate flanking restriction site were designed by considering the general rules of primer design using Integrated DNA Technologies, Oligo Analyser 3.1 tool (Url-1). KpnI, SalI, BamHI enzymes restriction sites were added to 5' end of the primers for the first forward primer, first reverse primer and second forward primer, and second reverse primer respectively. Melting temperatures (Tm) of the primers were selected as close as possible in order to facilitate the optimum annealing temperature selection. GC content, melting temperature, hairpin and self-dimer formations were checked by using IDT Scitools oligo analyzer. All primers are shown in table 2.14.

Table 2.14: Primers used in the study.

Primer	Sequence (5'→ 3')	Length	Tm	GC
1.F	ATAGGTACCATGGACCCATCTGTGACGCT	29	63.8 ^{o}C	51.7 %
1.R	AAGTCGACCGGGGTCAGGATGACCTGCA	28	67,9 ^{o}C	60,7 %
2.F	AAGTCGACCCACAGGAGGGCGTGCCA	26	69,4 ^{o}C	65,4 %
2.R	AAGGATCCTCATGGCTTCTGGGGCCCT	27	66,9 ^{o}C	59,3 %

2.2.2 PCR

Polymerase Chain Reaction (PCR) is used to amplify a specific region of a DNA strand. Therefore unlimited copies of single DNA molecule can be produced by this technique that is based on enzymatic replication *in vitro*. Borders of amplified fragments are defined with oligonucleotides named primers. These primers provide a binding site on template DNA for DNA polymerase. The buffer is used to preserve DNA polymerase in its natural confirmation and dNTP molecules are included to reaction mixture to be integrated into newly forming DNA molecules. After the related components were added, some different PCR programs were performed. For initial denaturation and denaturation steps, where two strands of DNA were separated, 94 ^{o}C was used. Annealing temperatures, where primers bind to the complementary regions of the DNA, were changed according to the primers melting tempetatures. Finally, in elongation and final extension steps where suitable bases are

arranged in an order 72 ^{o}C was used. PCR components are indicated in table 2.15, PCR programs also are indicated in table 2.16 and table 2.17.

Table 2.15: PCR components for amplification.

Component	Volume
Template	200 ng
Forward Primer (25 mM)	1 µL
Reverse Primer (25 mM)	1 µL
10X *Taq* Buffer	2,5 µL
dNTP (2 mM)	2,5 µL
Taq DNA Polymerase	0,5 µL
dH_2O	Up to 25 µL

Table 2.16: PCR program for amplification upstream of R domain.

Step	Temperature	Time	Cycle
Initial Denaturation	94 ^{o}C	3 min	1
Denaturation	94 ^{o}C	1 min	35
Annealing	65 ^{o}C	45 s	
Extension	72 ^{o}C	1 min	
Final extension	72 ^{o}C	5 min	1
Hold	4 ^{o}C	∞	-

Table 2.17: PCR program for amplification of downstream of R domain.

Step	Temperature	Time	Cycle
Initial Denaturation	94 ^{o}C	3 min	1
Denaturation	94 ^{o}C	1 min	35
Annealing	67 ^{o}C	45 s	
Extension	72 ^{o}C	1 min	
Final extension	72 ^{o}C	5 min	1
Hold	4 ^{o}C	∞	-

2.2.3 Agarose gel electrophoresis for detection of PCR product

After PCR amplification of DNA fragments, PCR products were analyzed on agarose gel electrophoresis. For 1,5 % gel, 0,6 g agarose was added in 40 mL 1X TBE buffer and gel solution was boiled in a microwave until the agarose was completely

dissolved. After cooling the solution to about 60 ^{o}C, ethidium bromide was added to the gel solution to a final concentration of 0,5 μg/mL.

Then, the gel solution was poured into a gel casting tray and a proper comb was placed in the gel tray. After the gel had been solidified, the comb was removed and gel was placed in electrophoresis chamber containing the appropriate amount of 1X TBE buffer. An appropriate molecular weight marker (MassRuler^{TM} DNA Ladder, Fermentas) and PCR product samples that were mixed with the 6X loading dye depending on the volume were pipeted into the gel wells. Gel was run at 120 V for 30 min. Ethidum bromide (EtBr) stained DNA bands were visualized with UV trans-illuminator by the help of UV PhotoMW software.

2.2.4 Purification of PCR product

After PCR and visualization by agarose gel electrophoresis, the fragments were purified with Qiagen QIAquick PCR Purification Kit. The kit removes the protein contamination derived from polymerase or restriction enzymes. The principle of this purification is as followed: DNA adsorbs to the silica membrane in the presence of high concentrated choatropic salt while contaminants pass through the column with washing buffer. Finally the DNA molecules are released with low salt concentration elution buffer. The purification protocol is given below:

- 5 volume of Buffer PB to 1 volume of PCR sample was added and mixed.
- After mixing, sample was transferred into collection filter tubes and centrifuged 1 min at 13.000 rpm at table top centrifuge.
- Flow-through was discarded and 750 μL Buffer PE to wash was added. The filter tube was centrifuged at 13.000 rpm for 1 min.
- Flow-through was discarded and filter tube was centrifuged at 13.000 rpm for an additional 1 min.
- The filter was placed into a clean 1,5 mL microcentrifuge tube.
- To elute DNA, 50 μL Buffer EB was added to the center of column, and the column was centrifuged for 1 min at 13.000 rpm.

2.2.5 Determination of DNA concentration

The spectrophotometric analysis was used to determine recovery, purity and concentration of nucleic acid. The ratio of absorption at 260 nm vs 280 nm is commonly used to evaluate the purity of DNA with respect to protein contamination, since protein (in particular, the aromatic amino acids) tends to absorb at 280 nm. According to the literature, the ratio of absorbance (A_{260}/A_{280}) of a pure DNA solution is between 1,8 to 2,0. When protein contamination increases, the ratio decreases. 2 µL sample was placed onto a sensor and its concentration was determined with the help of NanoDrop 2000/2000c Operating Software.

2.2.6 Restriction enzyme digestion of PCR products and the vectors

The restriction endonucleases cut DNA from specific palindromic restriction sites in length of 4 to 8 bases. The restriction endonucleases are commercially available, and are used to generate DNA fragments for cloning experiments. In this study KpnI, SalI and BamHI restriction enzymes were used. Plasmid vector was cut at 37 ^{o}C for 4 h and insert was cut overnight at 37 ^{o}C with 1X appropriate buffer (supplied by the manufacturer). Restriction reaction was completed by thermal inactivation and then DNA was purified from restriction mixture by using QIAquick PCR purification kit. Restriction reaction mixtures are given in table 2.18

Table 2.18: Restriction reaction mixture.

Content	Amount
Plasmid vector	1000 ng
KpnI/ SalI/ BamHI	1 µL
KpnI Buffer/ O Buffer/ BamHI Buffer	2 µL
dH_2O	Up to 20 µL
Insert	1000 ng
KpnI/ SalI/ BamHI	1 µL
KpnI Buffer/ O Buffer/ BamHI Buffer	2 µL

2.2.7 Determination of restricted DNA fragment by agarose gel electrophoresis

After digestion with restriction enzymes, samples were run on 1 % agarose gel prepared with 1X TAE buffer. Each 5 µL sample was mixed with 1 µL of 6X loading dye. MassRuler^{TM} DNA Ladder Mix was used to estimate the sequence size.

Electrophoresis was applied at 120 V for 30 min. Pictures of gels were taken under UV light with a trans-illuminator, with the help of UV PhotoMW software.

2.2.8 Purification of cleaved PCR product and vector

The cleaved PCR product was purified with Qiagen QIAquick PCR Purification Kit as described in section 2.2.4. The purification of cleaved vector from the agarose gel was performed with Qiagen QIAquick Gel Extraction Kit. The purification protocol is given below.

- DNA fragment was excised from the agarose gel with a clean, sharp scalpel.
- After weighing the gel slice in a colorless tube, 3 volume of Buffer QC was added to 1 volume of gel.
- The tube was incubated at 50 ^{o}C for 10 min to dissolve the gel.
- 1 gel volume of isopropanol was added to the sample and mixed.
- A QIAquick spin column was placed in a provided 2 mL collection tube.
- The sample was centrifuged for 1 min at 14.000 rpm at table top centrifuge.
- Flow-through was discarded and the column was placed back in the same collection tube.
- 0,5 mL of Buffer QG was added to the column and centrifuged for 1 min at 14.000 rpm.
- Flow-through was discarded
- To wash, 0,75 mL of Buffer PE was added to wash to the column and centrifuged for 1 min at 14.000 rpm.
- Flow-through was discarded and the column was centrifuged for an additional 1 min at 14.000 rpm.
- The column was placed into clean 1,5 mL microcentrifuge tube.
- To elute DNA, 50 µL Buffer EB was added to the center of column, and the column was centrifuged for 1 min at 14.000 rpm.

2.2.9 Ligation

Purified insert and vectors were ligated with Roche T4 ligase according to 1:3 molecular ratios. Ligation was performed overnight at room temperature. After overnight incubation, T4 DNA ligase was inactivated at 65 ^{o}C for 10 min. Ligation reaction mixture is shown in table 2.19

Table 2.19: Reaction mixture for ligation.

Content	Amount
Plasmid vector	300 ng
Insert DNA	900 ng
10X T4 Buffer	1,5 µL
PEG 8000	1,5 µL
T4 Ligase	1 µL
dH_2O	Up to 15 µL

2.2.10 Competent cell preparation - $CaCl_2$ method

The bacterial competent cells have been chemically treated to allow the foreign DNA or plasmid to be taken in. To prepare competent cells, the protocol is given below.

- *E.coli*-DH5α cells were taken from a glycerol stock culture by scraping with a tip and it was put in 5 mL LB medium and incubated overnight at 37 ^oC in orbital shaker.
- The following day, 100 mL LB medium was inoculated with 5 mL culture solution and was incubated at 37 ^oC in orbital shaker. Cell density was measured by spectrophotometer at OD_{600} and when it was reached to 0.6, the bacteria were transferred to 50 mL prechilled sterile ultracentrifuge tubes and incubated on ice for 10 min.
- The cells were centrifuged at 1600 x g for 7 min at 4 ^oC, and then supernatant was discarded.
- Each bacterial pellet was resuspended in 10 mL ice-cold $CaCl_2$ and centrifuged for 5 min at 1600 x g, the supernatant was discarded.
- Each bacterial pellet was resuspended in 10 mL ice-cold $CaCl_2$ and they were incubated on ice for 30 min.
- Centrifgation was performed again at 1600 x g for 5 min at 4 ^oC
- Each pellet was resuspended completely in 2 mL of $CaCl_2$
- The competent cells were distributed into prechilled sterile microfuge tubes each contains 50 µL and they were stored at -80 ^oC.

For 50 mL $CaCl_2$ solution content is shown in table 2.20.

Table 2.20: $CaCl_2$ solution content.

Content	Concentraiton	Amount
$CaCl_2.2H_2O$	60 mM	0,442 g
PIPES	10 mM	0,15 g
Glycerol	15 %	7,5 mL (from 100 %)
dH_2O	-	Up to 50 mL

2.2.11 Transformation

For the transformation procedure, the competent cells were taken out from -80 ^oC and thawed on ice. The ligation mixture was mixed with competent cells and the tube was incubated on ice for 30 min. Then the tube was placed in 42 ^oC for 45 sec and put back on ice for 2 min. 80 µL SOC medium was added and then culture was incubated at 37 ^oC for 1 h in orbital shaker. Culture was plated on selective medium with appropriate antibiotic (LB-Amp). Plates were incubated overnight at 37 ^oC.

2.2.12 Colony PCR

Colony PCR was performed to ensure selecting right colonies which were carrying inserts. Procedure of the colony PCR is given below:

- 10 µL sterile dH_2O was put into PCR tube and a tip of each colony was resuspended.
- Mixture was incubated in thermal cycler at 85 ^oC for 10 min.
- Then PCR mixture mentioned in table 2.21 was added into tubes.
- The PCR program is shown in table 2.22
- The amplified fragment was controlled with agarose gel alectrophoresis as it was given in section 2.2.3.

Table 2.21: PCR mixture for colony PCR.

Component	Volume
Template+water	10 µL
Forward Primer (25 mM)	1 µL
Reverse Primer (25 mM)	1 µL
10X *Taq* Buffer	2,5 µL
dNTP (2 mM)	2,5 µL
Taq DNA Polymerase	0,5 µL
dH_2O	7,5 µL

Table 2.22: PCR program for colony PCR.

Step	Temperature	Time	Cycle
Initial Denaturation	94 ^{o}C	3 min	1
Denaturation	94 ^{o}C	1 min	30
Annealing	65 ^{o}C	45 s	
Extension	72 ^{o}C	1,5 min	
Final extension	72 ^{o}C	5 min	1
Hold	4 ^{o}C	∞	-

2.2.13 Small scale plasmid DNA preparation (mini-prep)

Plasmid preparation was performed by using QIAGEN, QIAPrep Spin Miniprep Kit for small scale (mini) preparation, following instruction of the manufacturer. In this procedure, plasmid DNA was released from bacteria by alkaline lysis and removed all the RNA in the lysate by RNase. Then in the presence of chaotropic salt (guanidine HCl), DNA has ability to bind selectively to glass fiber fleece in a centrifuge tube and remains bound. Washing steps was performed to remove contaminating bacterial components, and then low salt elution buffer was added in order to remove DNA from the glass fiber fleece.

The protocol of QIAPrep Spin Miniprep Kit, QIAGEN is as follows:

- 5 mL of LB containing selective antibiotic was inoculated with positive colony and the culture was incubated at 37 ^{o}C with 250 rpm shaking overnight.
- Overnight culture was centrifuged 5000 rpm for 5 min. Supernatant was discarded and pellet was resuspended in 250 µL Buffer P1 containing RNase A and transferred to a microcentrifuge tube.
- 250 µL Buffer P2 was added and mixed thoroughly by inverting the tube 4-6 times.
- 350 µL prechilled Buffer N3 was added and mix immediately and thoroughly by inverting the tube 4-6 times.
- Suspension was centrifuged for 10 min at 13.000 rpm in a table top microcentrifuge.
- The supernatant was transferred to the QIAPrep spin column by pipetting.

- Filter tube assembly was centrifuged at 13.000 rpm for 1 min. The flowthrough was discarded.
- 750 µL Buffer PE was added to the wash the column and centrifugation was performed at 13.000 rpm for 1 min.
- Flow through was discarded and tube was centrifuged for an additional 1 min. to remove residual was buffer.
- QIAprep column was placed in a clean 1,5 mL microcentrifuge tube. 50 µL Buffer EB was added to the center of column to elute DNA. Column was let standing for 1 min and centrifuged for 1 min.

2.2.14 Sequencing

DNA sequencing is the process of determining the nucleotide order of a given DNA fragment. For sequencing dye terminator sequencing was performed. This is accomplished by using different fluorescent dye labeled dideoxynucleotide chain-terminators, which fluorescence at different wavelengths.

2.2.14.1 Sequencing PCR

Sequencing PCR is special typeof PCR in which single strand of DNA is amplified. Each dNTP is labeled with a different fluorescent tag; therefore resultant DNA fragment is fluorescently labeled. Single strand of DNA is desired to be exploited since 2 strands apparently interfere with the fluorescence of each other. Thus, only one primer is used in sequencing PCR. The reaction is performed in dark in order to preserve fluorescence. The reaction mixture preparation is shown in table 2.23.

Table 2.23: Sequencing PCR mixture.

Content	Volume
Big dye reaction mix	2 µL
5X sequence mixture	2 µL
Template DNA	200 ng
Forward /Reverse primers (25 mM)	0,5 µL
dH_2O	Up to 10 µL

The sequence reactions were performed using a thermal cycler with the following program mentioned in table 2.24.

Table 2.24: Sequencing PCR program.

Step	Temperature	Time	Cycle
Initial Denaturation	95 ^{o}C	5 min	1
Denaturation	95 ^{o}C	45 sec	40
Annealing	55 ^{o}C	45 sec	
Extension	60 ^{o}C	4 min	
Final Extension	60 ^{o}C	8 min	1
Hold	4 ^{o}C	-	∞

2.2.14.2 PCR purification for Sequencing

Since PCR product was contaminated with polymerase, a subsequent purification was performed. All the purification steps were performed in dark in order to preserve fluorescence. The protocol is as follows:

- 10 µL PCR product was taken into microcentrifuge tube then 2 µL of 3 M NaAC and 50 µL ice-cold 95% ethanol were added to tube and incubated on ice for 30 min.
- Mixture was centrifuged at 14.000 rpm for 30 min. Supernatant was discarded.
- Pellet was resuspended in 200 µL ice-cold 70% ethanol and centrifuged 14.000 rpm for 15 min. Supernatant was discarded.
- Tube was incubated at 95 ^{o}C for 5 min with caps open (in order to evaporate residual ethanol).
- 20 µL of Hi-Di formamide was added to DNA pellet for elution and the mixture was vortexed vigorously and spun down
- Tube was again incubated at 95 ^{o}C for 5 min with caps closed.
- Tube was put on ice.
- Tube was kept at 4 ^{o}C till use.

2.2.14.3 Alignment of sequence results

Nucleotide alignments were made both with BLAST tool (Url-2) and Clustal W program (Url-3) in order to compare the sequences obtained after sequencing with the original sequences.

2.2.15 Large scale plasmid DNA preparation (maxi-prep)

After confirmation of the sequence data and assuring that none of the constructs possess any mutation, each construct was transformed with *E.coli* dam-/dcm- GM2163 strain in order to prevent any methylation that would interfere the transcription factor binding, according to the previously described transformation in section 2.2.11. After the colonies emerged the insert containing plasmids were reproduced in large scale and purified with QIAGEN, Endofree® Plasmid Maxi kit. This kit is chosen because subsequent to large scale production the plasmids are going to be used to transfect SH-SY5Y neuroblastoma cell line which is a sensitive cell line. Endotoxins, which are cell membrane components of Gram-negative bacteria (*E.coli*) strongly influence transfection and increased endotoxin levels lead to sharply reduced transfection efficiencies. Plasmid isolation procedure is as follows:

- 5 mL of LB containing selective antibiotic inoculated with positive colony and the culture was incubated at 37 ^{o}C for 8 h on orbital shaker.
- 100 mL LB medium with appropriate antibiotic was inoculated with 100 µL of starter culture and grown at 37 ^{o}C overnight on orbital shaker.
- Overnight culture was centrifuged at 5000 rpm for 1 h at 4 ^{o}C.
- Supernatant was discarded and pellet was resuspended in 10 mL Buffer P1 and transferred to a 50 mL centrifuge tube.
- 10 mL Buffer P2 was added and mixed thoroughly by inverting the tube 4-6 times and incubated at room temperature for 5 min.
- 10 mL prechilled Buffer N3 was added and mixed immediately and thoroughly by inverting the tube 4-6 times.
- Lysate was poured into barrel of the QIAfilter Cartridge and incubate at room temperature for 10 min. Then the lysate was filtered into a 50 mL tube.
- 2,5 mL Endotoxin removal buffer (ER) was added to the filtered lysate, and mixed by inverting the tube 10 times, and incubated on ice 30 min.
- During the incubation QIAGEN-tip 500 was equilibrated by applying 10 mL Buffer QBT and the column was allowed to empty by gravity flow.
- The filtered lysate was applied to the QIAGEN-tip and allowed to enter the resin by gravity flow.
- QIAGEN-tip was washed with 2 x 30 mL Buffer QC.

- DNA was eluted with 15 mL Buffer QF.
- DNA was precipitated by adding 10,5 mL room temperature isopropanol to the eluted DNA and centrifuged immediately at 5000 rpm for 1 h at 4 ^{o}C.
- After carefully decanting the supernatant DNA pellet was washed with 5 mL of endotoxin-free room temperature 70 % ethanol and centrifuged at 5000 rpm 1 h at 4 ^{o}C.
- The supernatant was carefully decanted without disturbing the pellet.
- The pellet was air-dried for 5 min, and the DNA was redissolved in 300 µL endotoxin-free Buffer TE.

2.2.16 Preparation of cell culture

2.1.16.1 Transferring the stock cell to culture flask

A frozen stock of SH-SY5Y cells were taken -80 ^{o}C and after the cells were thawed in 37 ^{o}C water bath. They were diluted with SH-SY5Y culture media of which content was given in section 2.1.13.1, and centrifuged for 5 min at 900 rpm. Cell pellet was dissolved with medium and transferred to the culture flasks.

2.1.16.2 Cell counting

Growing medium was removed and cells were detached by 1 mL Trypsin-EDTA treatment for 2 min at 37 ^{o}C. Then trypsin-EDTA solution was inhibited by 10 mL medium and cells were suspended Then 10 µL suspension was taken and put onto the hemocytometer, cells were counted as the number of cells per 25 square (1 mm^2). The cell number calculation is given below:

10^4 (constant number) x Amount of counted cell = Cell number/ml

Total cell number = Cell number/ mL x Total volume of cells (10 ml) **(2.1)**

2.1.16.3 Cell passage

After cells were lifted and cell density was determined, cells were put into the centrifuge tubes and centrifuged 900 rpm for 5 min. The supernatant was discarded and pellet was resuspended with culture medium and 1x10^6 cells were transferred to clean culture flask.

2.1.16.4 Cell freezing

After cell passage, cells resuspended in medium were transferred to freezing tube with freezing medium for future experiments. Estimated cell number for per freezing tube was $1x10^6$ cell / mL.

2.2.17 Transfection of SH-SY5Y cells for forced experiment

Forced experiment is co-transfection of cells with vector containing promoter and another vector containing transcription factor. Transfection is the process to introduce a foreign DNA into the cell. In this study DNA was inserted into SH-SY5Y cells by lipofection. The synthetic cationic lipids that can form liposome are incubated with DNA. These liposomes interact with DNA through electrostatic interaction between the negatively charged nucleic acid and positively charged head group of synthetic lipid, and fuse with cultured cells. The liposome complex neutralizes the negatively charge of the nucleic acids, allowing association of the complex with the negatively charged cell membrane. Entry of the liposome complex into the cell may occur by the processes of endocytosis or fusion with plasma membrane via the lipid moieties of the liposome. The optimal amount of control vector to use in cotransfections is the minimum amount that gives significant reporter activity above background (background is measured in samples transfected with only the test vector, in our study pGL2_basic and pGL3_basic). Different amounts were tried for optimization and 7:1 ratio of test vector: control vector were determined as optimum for SH-SY5Y neuroblastoma cell line. Transfection contents are given in table 2.25 and table 2.26, and the transfection procedure is as follow.

- After cells were detached and cell number was calculated (section 2.1.16.2), and 50.000 cells per well were seeded on plated 24 well tissue culture plate 2 day before the transfection so that the cells were approximately 80 % confluent on the day of transfection.
- 1 µg of plasmid DNAs are diluted in 200 µL DMEM (1X) and vortexed and quick spun.
- Promega's transfection reagent Transfast was added to 1:3 ratio to the DNA mixture according to the ratios given in the table below and vortexed and quick spun briefly for the formation of DNA liposome complex, and incubated for 15 min at room temperature.

- The growth medium was removed from the cells and transfection mixture was added gently in order to prevent cell detaching and cells returned to the 37 ^{o}C, 5 % CO_2 incubator for 1 h.
- After incubation, 500 µL medium was added to each well.
- The cells were returned to the 37 ^{o}C, 5 % CO_2 incubator for 48 h before analysis.

Table 2.25: Transfection content for pGL2-S2 forced experiment.

Construct name	*pGL2-S2	wt-Elk1	Elk1-woR	Elk1-VP16	pCMV-Myc.	pRL-TK Control.	Transfast reagent
pGL2-S2	700 ng	-	-	-	200 ng	100 ng	3 µL
pGL2- basic	700 ng	-	-	-	200 ng	100 ng	3 µL
pGL2-S2 Elk1	700 ng	200 ng	-	-	-	100 ng	3 µL
pGL2- basic Elk1	700 ng	200 ng	-	-	-	100 ng	3 µL
pGL2-S2 Elk1-woR	700 ng	-	200 ng	-	-	100 ng	3 µL
pGL2- basic Elk1-woR	700 ng	-	200 ng	-	-	100 ng	3 µL
pGL2-S2 Elk1-VP16	700 ng	-	-	200 ng	-	100 ng	3 µL
pGL2- basic Elk1-VP16	700 ng	-	-	200 ng	-	100 ng	3 µL

*pGL2-S2 construct includes optimal promoter of *SPG4* gene
Each transfection was performed as triplicates.

Table 2.26: Transfection content for pGL3-F2 forced experiment.

Construct name	*pGL3-F2	wt-Elk1	Elk1-woR	Elk1-VP16.	pCMV-Myc	pRL-TK Control.	Transfast reagent
pGL3-F2	700 ng	-	-	-	200 ng	100 ng	3 µL
pGL3- basic	700 ng	-	-	-	200 ng	100 ng	3 µL
pGL3-F2 Elk1	700 ng	200 ng	-	-	-	100 ng	3 µL
pGL3- basic Elk1	700 ng	200 ng	-	-	-	100 ng	3 µL
pGL3-F2 Elk1-woR	700 ng	-	200 ng	-	-	100 ng	3 µL
pGL3- basic Elk1-woR	700 ng	-	200 ng	-	-	100 ng	3 µL
pGL3-F2 Elk1-VP16	700 ng	-	-	200 ng	-	100 ng	3 µL
pGL3- basic Elk1-VP16	700 ng	-	-	200 ng	-	100 ng	3 µL

*pGL3-F2 includes optimal promoter of *KATNB1* gene.
Each transfection was performed as triplicates.

2.2.18 Luminometrical measurement

Genetic reporter system is commonly used in eukaryotic gene expression studies. Dual reporters are generally used to improve experimental accuracy. Normalizing the activity of the experimental reporter to the activity of the internal control minimizes experimental variability caused by differences in cell viability or transfection efficiency. Other source of variability, such as in pipetting volumes, cell lysis efficiency and assay efficiency can be effectively eliminated. In this study, the Dual-Luciferase® Reporter (DLR) Assay System from Promega was used. In the DLR Assay, the activities of firefly *(Photinus pyralis)* and Renilla *(Renilla reniformis)* luciferases are measured sequentially from a single sample. Firstly, cells are harvested by lysing with 1X Passive Lysis Buffer. This buffer is specifically formulated to minimize the low-level autoluminescence emitted by Renilla luciferase substrate, coelenterazine. Firstly, the firefly luciferase reporter is measured by adding Luciferase Assay Reagent II (LAR II) to generate a stabilized luminescent signal. After measurement of firefly, Stop & Glo® Reagent was added into same tube to quench firefly luciferase reaction and initiate Renilla luciferase reaction. Stop & Glo® Reagent also produces a stabilized signal from the Renilla luciferase, which decays slowly over the course the measurement (Promega Technical Manual, E1910).

The luciferase activity depends on the functionally of the promoter upstream from *luc+*; in this study, these promoters are *SPG4* and *KATNB1* genes promoters. For the forced experiments, the luciferase activity depends on functionally of transcription factor on the promoter upstream of *luc+*; in this study these transcription factors are Elk1 and its different forms Elk1 without repression domain (Elk1-woR) and constitutively active form of Elk1 (Elk1-VP16).

The chemiluminescence was measured by Fluoroskan Ascent FL luminometre from Thermo Electron Corporation. The PMT voltage was default setting which is 845 and integration time was chosen to be 10.000. Scaling factor was adjusted to 10 after optimization the measurements. At least 1 h before the assay needed amount of the LAR II and Stop & Glo® Buffer were taken out from -80 ^{o}C and -20 ^{o}C refrigators respectively to be thawed in room temperature. Then Stop & Glo® Reagent was prepared by adding 50X Stop & Glo® Substrate to the Stop & Glo® Buffer to 1X final concentration. All transfections were performed as triplicates and were repeated

at least two times using different DNA preparations. Cells were prepared to assay according to manufacturer instructions,

- 48 h post-transfection, medium was removed from cultured cells, and 60 µL 1X Passive Lysis Buffer was added to each well.
- Homogenous lysates was rapidly prepared by manually scraping the cells.
- The lysate were transferred into microfuge tube for analysis.
- Luminometer was programmed to perform a 2 sec premeasurement delay while shaking the plate, followed by 10 sec measurement period for each reporter assay.
- 50 µL of LAR II was added into one well of luminometer plate and then 50 µL of cell lysate was transferred to the same well.
- Firefly luciferase luminescence was measured and recorded.
- 50 µL Stop & Glo® Reagent was immediately added to the same well.
- Renilla luciferase luminescence was measured and recorded.
- This procedure was repeated for each sample separately.

2.2.19 PMA treatment

50.000 cells were plated in each well of 24 well plate and transfected with pCMV6.Elk1 in indicated amount in table 2.27. After 18 h of growth, cells were serum-starved for 24 h, and then treated with 10 nM PMA for 6 h prior to luciferase assays or immunocytochemistry.

Table 2.27: Transfection content for pGL2-S2 forced experiment under PMA treatment condition.

Construct name	pGL2-S2	wt-Elk1	pCMV-Myc	pRL-TK Control	Transfast reagent
pGL2-S2	700 ng	-	200 ng	100 ng	3 µL
pGL2- basic	700 ng	-	200 ng	100 ng	3 µL
pGL2-S2 Elk1	700 ng	200 ng	-	100 ng	3 µL
pGL2- basic Elk1	700 ng	200 ng	-	100 ng	3 µL
pGL2-S2 Elk1-PMA	700 ng	200 ng	-	100 ng	3 µL
pGL2- basic Elk1-PMA	700 ng	200 ng	-	100 ng	3 µL

Each transfection was performed as duplicates.

2.2.20 KCl treatment

50.000 cells were plated in each well of 24 well plate and transfected with pCMV6.Elk1 indicated amount in table 2.28. After 24 h from transfection the cells were depolarized by KCl in the medium with addition of 31% depolarization buffer (170 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES) at a final concentration of 50 mM KCl for 1 hour. When 48 h post transfection process is completed, cells were prepared for luciferase measurement, immunocytochemistry or Western blotting.

Table 2.28: Transfection content for pGL3-F2 forced experiment under KCl treatment condition.

Construct name	pGL3-F2	wt-Elk1	pCMV-Myc	pRL-TK Control	Transfast reagent
pGL3-F2	700 ng	-	200 ng	100 ng	3 µL
pGL3- basic	700 ng	-	200 ng	100 ng	3 µL
pGL3-F2 Elk1	700 ng	200 ng	-	100 ng	3 µL
pGL3- basic Elk1	700 ng	200 ng	-	100 ng	3 µL
pGL3-F2 Elk1-KCl	700 ng	200 ng	-	100 ng	3 µL
pGL3- basic Elk1- KCl	700 ng	200 ng	-	100 ng	3 µL

*Each transfection was performed as triplicates.

2.2.21 Nucleofection

Nucleofection is a transfection method based on physical method of electroporation which enables transfer of nucleic acid into the cells. It employs a non-viral method based on an optimized combination of electrical parameters, generated by device called Nucleofector, with cell type specific reagents. The substrate is transferred directly into both cell nucleus and cytoplasm. In addition the transfection efficiency is much higher than traditional methods. Nucleofection procedure is as follow:

- Cultured cells were lifted and cell number was calculated (section 2.1.16.2), $1x10^6$ cells for each well of 6 well tissue culture plate were seeded and centrifuged 900 rpm for 5 min.
- Pellet was resuspended carefully with 100 µL room temperature Nuclefector® Solution per sample. (Leaving cell in Nuclefector® Solution for extended periods of time no longer than 15 min.)

- The cell suspension was combined with 8 µg DNA and then cell/DNA suspension was transferred into nucleofector cuvette.
- The appropriate Nucleofector® Program G-004 was selected and cuvette was inserted into Nucleofector® Cuvette Holder and the selected program was applied by pressing the X-button.
- The cuvette was taken out of holder once the program was finished.
- 500 µL culture medium was immediately added to cuvette and the sample was transferred into the prepared 6 well plate (final volume 2 mL media per well). The cells were incubated in 37 ^{o}C, 5% CO_2 incubator until analysis.

2.2.22 RNA isolation

RNA isolation was performed using Roche, High Pure RNA Isolation Kit, following instructions of the manufacturer. This kit is designed for the purification of total RNA from cultured cells. .The protocol of High Pure RNA Isolation Kit, Roche is as follows:

- After 24 h post-nucleofection, cells were detached, collected separately in centrifuge tube and centrifuged 900 rpm for 5 min.
- The pellet was resuspended in 200 µL PBS.
- 400 µL Lysis/Binding Buffer was added into the cell suspension and vortexed for 15 sec.
- The sample was transferred to a High Pure Filter Tube and centrifuged 8.000 x g for 15 sec.
- Flow through was discarded and filter tube was combined again.
- 90 µL DNase Incubation Buffer and 10 µL DNaseI were mixed for each sample. This mixture was added in filter tube containing sample and incubated for 15 min at room temperature.
- 500 µL Wash Buffer I was added into filter tube, centrifuged 8.000 x g for 15 sec and flow-through was discarded
- 500 µL Wash Buffer II was added into filter tube centrifuged 8.000 x g for 15 sec and flow-through was discarded.
- 200 µL Wash Buffer II was added into filter tube and centrifuged maximum speed (approximately 13.000 x g) for 2 min to remove residual wash buffer.
- The filter tube was placed into clean 1,5 mL microcentrifuge tube.

- 50 µL Elution Buffer was added into filter tube to elute RNA and centrifuged at 8.000 x g for 1 min.
- Isolated RNA was stored -80 ^{o}C for future experiments.

2.2.23 cDNA synthesis

cDNA synthesis was performed by using Thermo Scientific RevertAid H Minus First Strand cDNA Synthesis Kit which is a complete system for an efficient synthesis of the first strand cDNA from mRNA or total RNA templates. The procedure for cDNA synthesis is given bellow:

- The components given in table 2.29 were mixed gently and spun down.
- This mixture was incubated at 65 ^{o}C for 5 min and the chilled on ice.

Table 2.29: The first part components of cDNA synthesis reaction.

Component	Amount
Template RNA	1 µg
Random Hexamer Primer	1 µL
Water, nuclease free	Up to 12 µL

- The other components given in table 2.30 were added in mixture, mixed gently and spun down.

Table 2.30: The second part components of cDNA synthesis reaction.

Component	Amount
5 X Reaction Buffer	4 µL
RiboLock RNase Inhibitor (20u/ µL)	1 µL
10 mM dNTP Mix	1 µL
Reverse Transcriptase (200u/ µL)	1 µL
Total Volume	20 mL

- After addition of second part component, mixture was incubated for 5 min at 25°C followed by 60 min at 42°C. To terminate the reaction mixture was heated at 70°C for 5 min. After reaction cDNA's were stored -80 ^{o}C.

2.2.24 Real time PCR

The Real Time PCR (RT-PCR) method is based on PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. The quantity is either an absolute number of copies or a relative amount when normalized to DNA input or

additional normalizing gene.In this study, specific probes and primers for spastin and p80-katanin were purchased from Universal Probe Library (Roche Applied Science Url-4), probe number and primers are given table 2.31.

Table 2.31: Primers and probes for RT-PCR.

Primer	Primer Sequence	Probe Number
Spastin (F)	5'-TTCTGGAGTGAAACAGGGATC-3'	15
Spastin (R)	5'-TTTGTCCTATTTGTTTTCGGAG-3'	
P80-katanin (F)	5'-CGAGCCATCTCAGCTCAATA-3'	1
P80-katanin (R)	5'-AATCAAGGCAGGGGATGG-3'	

Beta-actin (β-actin) (Roche Universal Probe Library Human ACTB Gene Assay) gene was used as reference for RT-PCR studies. RT-PCR reaction was performed with Light Cycler® 480 Probes Master RT-PCR Kit using a Roche Light Cycler 480 according to the following Light Cycler program given in table 2.32.

Table 2.32: Real Time PCR Program.

Step	Temperature	Time	Cycle
Initial Denaturation	95 ^{o}C	10 min	1
Denaturation	95 ^{o}C	10 sec	45
Amplification	60 ^{o}C	30 sec	
Extension	72 ^{o}C	1 sec	

ΔΔCt method was used to analyze RT-PCR results. According to Schmittgen T.D. et al, efficiency rate for each gene should be between 1,8-2,2 and error rate should be below 0,2 to be able to use ΔΔCt method. Therefore, error rate and efficiency values for each gene were determined and all of them were in expected ranges.

2.2.25 Immunostaining

Immunostaining is a biochemical technique used to visualize proteins in the cell. The primary antibodies which are specific for the intended protein, and secondary antibodies which are specific for the IgG heavy and light chains of the primary antibody are used. Detection is usually performed by fluorescence.

- SH-SY5Y cells ($3x10^4$) were seeded on poly-L-lysine coated coverslips and transfected as described in section 2.2.17.
- 48 h post-transfection, culture medium was removed. Cells were washed 2 times with PBS

- Cells were fixed in prechilled methanol and incubated 15 min at -20 ^{o}C.
- Cells were washed 3 times with PBS
- Blocking solution was prepared 10% antibody specific serum, 10 mg/mL bovine serum albumin (BSA) and PBS; cells were blocked with blocking solution 1 h at room temperature.
- Primary antibodies were diluted in PBS (1:500 Rabbit polyclonal anti-Elk1, 1:200 Mouse monoclonal anti-spastin, 1:250 Goat polyclonal anti-katanin antibodies)
- Cells were incubated with 300 µL antibody solution/coverslip overnight 4^{o}C.
- Following day primary antibodies were removed and cells were washed 3 times with PBS.
- Cells were blocked with blocking solution 1 h at room temperature.
- Secondary antibodies were diluted with PBS (1:1000 both Alexa Fluor® 647 goat anti-mouse, 488 goat anti-rabbit Alexa Flour® and donkey 488 anti-goat Alexa Flour®) and cells were treated with secondary antibody solution for 1 h at 37°C in dark.
- Cells were washed 1 time for 5 min with PBS
- Cells were incubated 1:1000 diluted DAPI for 5 min and then washed 3 times with PBS
- 10 µL mounting medium was added into slides.
- Coverslips were put onto object slide and fixed by using nail polisher.
- Samples were visualize and analyzed by using with Leica TCS SP2 SE Confocal Microscope with appropriate laser beams and photos were taken under 63 X magnification.

2.2.26 Total protein isolation

Total protein isolation from cells was performed by using ROCHE, cOmplete Lysis-M Kit. Procedure of protein isolation is as follow:

- 1x10^{6} cells were seeded each well of 6 well plate and transfected as described in section 2.2.17.
- After 48 h transfection, one complete-M Mini tablet was dissolved in 10 mL Lysis-M Reagent to prepare working solution.
- Culture media was removed and cells were washed with PBS.

- 200 µL Lysis- M Reagent solution was added to each well and incubated for 5 min at room temperature.
- The cell lysates were collected and transferred to a microcentrifuge tube.
- The lysate was centrifuged at 14.000 x g for 5-10 min.
- The supernatant containing soluble protein was transferred to clean tube and was stored -80 °C.

2.2.27 BCA Assay

The bicinchoninic acid (BCA) assay was performed by using Pierce Micro BCA™ Protein Assay Reagent Kit to determine the protein concentration. BCA a detection reagent for $Cu^{+1,}$ which is formed when Cu^{+2} is reduced by protein in an alkaline environment, is utilized. A purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{+1}). This complex exhibits a strong absorbance at 562 nm. The procedure for BCA assay is as follows:

- The different diluted Albumin (BSA) standard solutions were prepared between 0-400 µg/mL range (0 µg/mL- 3,125 µg/mL-6,25µg/mL- 12,5 µg/mL- 25 µg/mL- 50 µg/mL- 100 µg/mL- 200 µg/mL- 400 µg/mL).
- fresh working reagent (WR) was prepared by mixing 25 parts of Micro BCA™ Reagent MA and 24 parts Reagent MB with 1 part of Reagent MC (25:24:1, Reagent MA:MB:MC).
- Each sample was diluted 1:20 ratio and 100 µL diluted samples and standard solution duplicate were put into microplate wells.
- 100 µL WR was added into each well and mixed plate thoroughly on a plate shaker for 30 sec.
- Microplate was incubated at 37°C for 2 h.
- After incubation absorbance was measured at 562 nm on plate reader.

2.2.28 SDS-polyacrylamide gel electrophoresis of proteins (SDS-PAGE)

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis is a technique widely used to separate proteins according to electrophoretic mobility. In this method, tertiary structure of proteins is disrupted by SDS and reducing agents such as β-mercaptoethanol. The proteins are separated in gel according to their size.

In this study, Fluka SDS Preparation Kit is used for preparation both 5% stacking gel and 10 % separating gel whose contents were given in table 2.33 and table 2.34 respectively.

Table 2.33: Content of 5% stacking gel.

Contents	Volume
Stacking acrylamide	0,25 ml
Gel buffer	0,75 ml
dH_2O	2 ml
10% APS	20 µl
TEMED	2 µl

Table 2.34: Content of 10% separating gel

Contents	Volume
50% glycerol	2 ml
Separating acrylamide	1,22 ml
Gel buffer	2 ml
dH_2O	0,78 ml
10 % APS	75 µl
TEMED	7,5 µl

- Prepared gels were poured between SDS-glasses and left for polymerization.
- After gel polymerized, samples, that were mixed 5X SDS-loading dye and boiled 95 °C for 5 min to gain primary structure, were loaded in equal concentration into wells.
- The gel was placed into tank and run in running buffer at 25 mA for 2 h.

2.2.29 Western blotting

Western blot is used to detect specific proteins. After separation of denatured proteins according to size by SDS-PAGE, the proteins are transferred to nitrocellulose membrane where they are labeled using antibodies specific to the target protein.

- After SDS-PAGE, gel was taken from the tank and between glasses.
- Nitrocellulose membrane and 2 filter papers were put into towbin electrotransfer buffer for 10 min for equilibration

- After equilibration, the sandwich was prepared between filter papers and nitrocellulose membrane.
- The gel was put on the membrane with care not to disrupt the gel. Air bubbles were removed.
- The protein transfer was carried out in semi-dry blotting apparatus for 20 V for 30 min at 4 °C.
- Then the membrane was blocked with blocking solution to prevent non-specific binding to empty regions on the membrane. In other words, efficient blocking simply reduces the background.
- After blocking, the membrane was incubated with 1:500 diluted with blocking solution primary antibody (Rabbit polyclonal SUMO2-3, rabbit polyclonal Elk1, mouse monoclonal spastin, rabbit polyclonal katanin or mouse monoclonal β-actin antibody) for overnight at 4 °C with gentle shaking.
- Following day membrane was washed with TBS-T buffer for 5 min 6 times with gentle shaking
- 1:5000 diluted secondary antibody was prepared in blocking solution.
- The membrane was incubated with secondary antibody for 1 h at room temperature with gently shaking.
- The membrane was washed for 5 min 6 times with gentle shaking.
- For detection, 20X LumiGLO® Reagent or SuperSignal® West Femto Maximum Sensitivity Substrate was used according to manufacturer instructions. The membrane was exposed to X-ray film for particularly time and then developed in Kodak Medical X-ray Processor according to manufacturer's instruction.

2.2.30 Immunoprecipitation

The cell extracts were treated with specific antibody in order to concentrate target proteins.

- Dynabeads were resuspended in the solution via vortex for 30 sec.
- 50 µl (1,5 mg) beads were transferred into microcentrifuge tube and placed on magnet. The magnet provides separation of beads from solution.
- After beads were separated, supernatant was removed.

- Rabbit polyclonal Elk1 antibody was diluted in 1:50 μg/μl in PBST buffer and diluted antibody solution was added to prepared beads.
- This mixture was incubated with rotation at room temperature for 10 min.
- The beads were separated via magnet, supernatant was removed.
- Bead-antibody complex was treated with PBST by pipetting gently and washed.
- The beads were separated via magnet again.
- ~1 mg cell extracts of samples were added into tubes.
- Mixture was resuspended and incubated with rotation for 10 min at room temperature.
- Bead-antibody-antigen complex was washed 3 times with 200 μl PBS.
- Elk-1 proteins bound with Elk-1 antibodies onto the beads were eluted using 20 μl elution buffer.
- The tubes were incubated for 10 min at 70 °C in order to dissociate the complex.

3. RESULTS AND DISCUSSION

3.1 Production of Elk1 without R domain constructs

Both upstream between (1-669 bases) and downstream sequences (between 831-1287 bases) of WT-Elk1 were amplified with PCR respectively using primers shown in table 2.14. The PCR products were run on agarose gel and expected DNA fragments were observed. The fragments are shown in figure 3.1 in the presence of Fermentas Mass Ruler Mix DNA Marker.

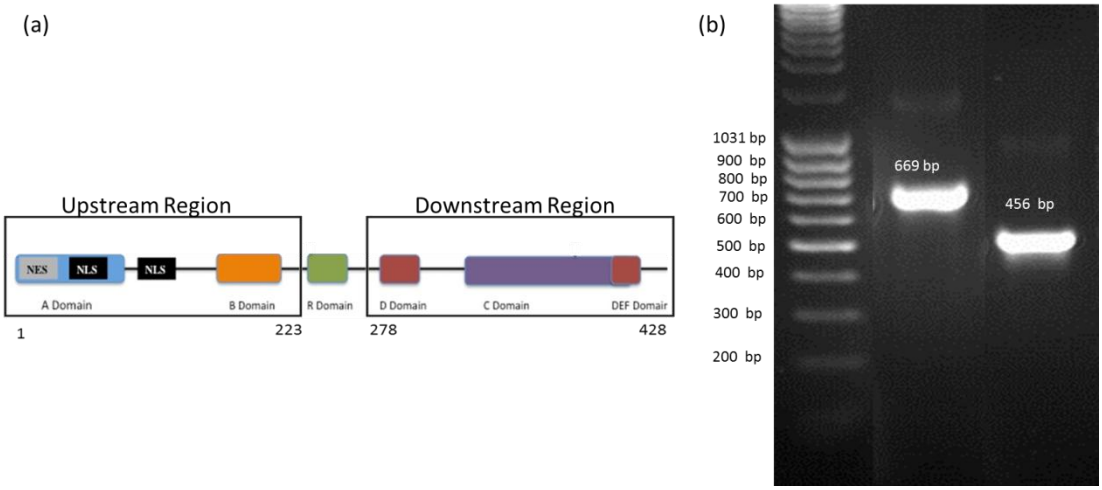

Figure 3.1: (a) Schematic illustration of Elk1. (b)Agarose gel images of PCR products. 2nd lane is the upstream fragment of R domain and 3rd line is the downstream fragment of R domain.

Amplified PCR products of upstream region of R domain and pCVM6 vector were digested by Fermentas's KpnI and SalI restriction enzymes. After digestion and purification by QIAGEN QIAquick PCR Purification Kit, ligation step was performed with Roche T4 DNA Ligase. Subsequently, ligation mixture was transformed into $CaCl_2$ treated *E.coli* DH5α competent cells. When the colonies appeared, colony PCR was performed in order to verify whether the colonies contain the plasmid of interest. PCR fragments are shown in figure 3.2 in the presence Fermentas Mass Ruler Mix DNA Marker.

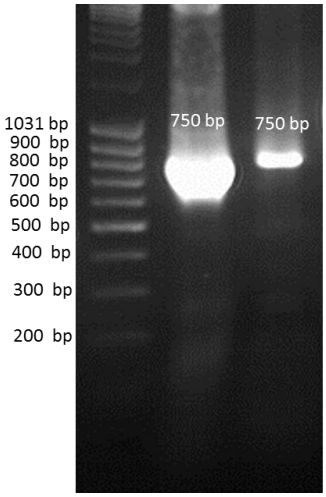

Figure 3.2: Agarose gel image of colony PCR fragments of upstream region of R domain.

Then this construct including upstream of R domain sequence was digested with Fermentas's SalI and BamHI restriction enzymes. Purification and ligation to restricted vector and PCR product were performed with using QIAquick PCR Purification Kit and T4 ligase. The ligation mixture was transformed into *E.coli* DH5α competent cells and colony PCR was performed for verification. PCR fragments are shown in figure 3.3 in the presence of Fermentas Mass Ruler Mix DNA Marker.

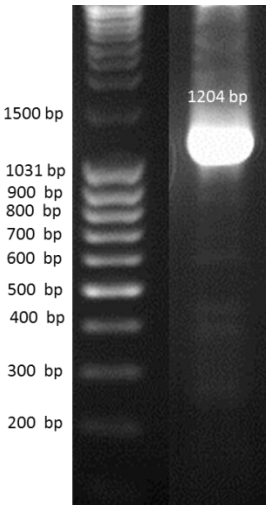

Figure 3.3: Agarose gel image of colony PCR fragments containing upstream and downstream region of R domain.

Plasmid DNA was isolated from colony PCR positive colony with QIAGEN's Qiaprep Spin Miniprep Plasmid Isolation Kit.

3.2 Sequence Alignment Analysis of Constructed Plasmid

Sequence analysis of constructed plasmid was performed via EBI Clustalw sequence analysis tool in order to verify the accuracy of the inserts. It is found that constructs did not contain any mutation. After confirmation, construct was transformed with *E.coli* dam-/dcm-, and produced large scale by QIAGEN EndoFree Plasmid Maxi Kit.

3.3 Results for Spastin Optimal Promoter

3.3.1 Luminometrical measurement data of forced experiments for spastin promoter

In order to determine of Elk1 and Elk1.woR which is not contain repression domain and cannot be SUMOylated, SH-SY5Y neuroblastoma cells were transfected with S2 construct containing spastin optimal promoter, and co-transfected with both S2 and WT-Elk1 (pCMV6.Elk1), or Elk1 without R domain (pCMV6.Elk1.woR), or constitutively active form of Elk1 (pCMV6.Elk1.VP16) expression vectors respectively. Each transfection was performed as triplicate and experiments were repeated 3 times on separate days. Forced experiment, which is co-transfection of cells with promoter and transcription factor, results are indicated table 3.1, table 3.2 and table 3.3. Average of all experiment and graph are shown table 3.4 and figure 3.3 respectively.

Table 3.1: Measured light units of experiment n=1 for spastin promoter.

N=1		S2	S2 Basic	WT.Elk1	WT.Elk1 Basic	Elk.woR	Elk.woR Basic	Elk.VP16	Elk.VP16 Basic
1	Firefly	123,4	1,224	21,96	0,5818	13,48	0,3881	245,8	3,004
	Renilla	219,5	103,1	670,1	386,2	217,7	231,9	352,9	324,5
2	Firefly	141,3	1,236	23,4	0,5957	11,43	0,2936	257,1	4,127
	Renilla	231,4	90,21	739,8	402,6	199,7	185	397,6	393,2
3	Firefly	199,4	1,375	21,33	0,5419	9,506	0,3484	236,4	3,216
	Renilla	335,7	106,3	614,9	380,3	177,4	189,4	356,7	367,4

Table 3.2: Measured light units of experiment n=2 for spastin promoter.

N=2		S2	S2 Basic	WT.Elk1	WT.Elk1 Basic	Elk.woR	Elk.woR Basic	Elk.VP16	Elk.VP16 Basic
1	Firefly	111,6	1,261	22,9	1,058	12,06	0,4093	200,1	3,18
	Renilla	191,8	132,8	660,2	658,4	161,4	158,6	300,5	263,7
2	Firefly	141,5	2,592	29,25	1,202	11,82	0,4065	190,1	2,809
	Renilla	202	189,4	890,6	637,9	163,5	162	279,9	253,4
3	Firefly	158,9	2,271	22,04	1,103	14,91	0,4482	185,4	3,143
	Renilla	203,4	193,3	701,7	623,3	204,9	154,9	267,9	254,8

Table 3.3: Measured light units of experiment n=3 for spastin promoter.

N=3		S2	S2 Basic	WT.Elk1	WT.Elk1 Basic	Elk.woR	Elk.woR Basic	Elk.VP16	Elk.VP16 Basic
1	Firefly	188,1	2,221	21,56	1,192	17,28	0,667	239,4	3,897
	Renilla	215,3	183,5	1162	248,1	267,4	156,1	425,4	491,2
2	Firefly	203,1	2,773	25,4	1,327	18,97	0,6246	276,2	3,652
	Renilla	234,4	245,3	1156	296,1	331,6	164,8	493,3	464,2
3	Firefly	205,2	2,288	20,24	1,17	22,11	0,6326	294,4	3,292
	Renilla	225,8	218,1	1286	303,1	381,8	162,9	496,3	441,7

The following equation is used to determine the normalized fold change in activity between test groups, considered as fold of induction in respect to the activity of the empty vector pGL2-Basic. The calculated fold activity and graph representing the obtained data are given in the table 3.4 and figure 3.4 below.

$$\Delta \text{ Fold Activation} = \frac{\text{Average (Firefly/Renilla) Sample X}}{\text{Average (Firefly/Renilla) pGL2-Basic}} \quad \textbf{(3.1)}$$

Table 3.4: Average of calculated F/R of all experiments and standard deviation for spastin promoter.

Experiment F/R	pGL2-S2	WT.Elk1	Elk1.woR	Elk1.VP16
N=1	45,8808	22,464	33,8702	70,36554
N=2	59,0783	18,8066	27,5297	57,98677
N=3	78,1417	4,2799	15,0456	73,78774
Average F/R	61,0337	15,1836	25,6069	67,4027
Average F/R Standard Deviation	16,2191	9,61826	9,74324	8,5454
Δ Fold Activation	1	0,24877	0,4175	1,10398

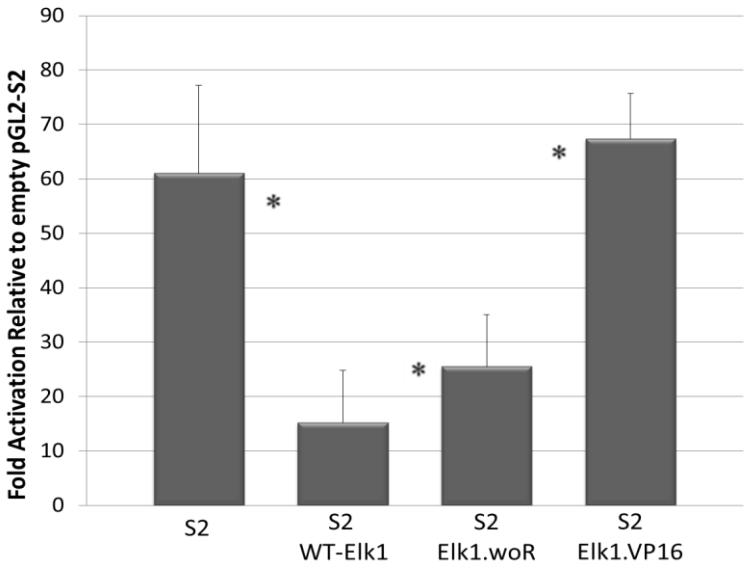

Figure 3.4: Average fold activities of Elk1, Elk1.woR and Elk1.VP16 forced experiment on *SPG4* optimal promoter.

According to forced experiments, WT-Elk1 has repressor activity on *SPG4* optimal promoter, Elk1.woR reversed the repressive activity of WT-Elk1, and Elk1.VP16 increased the promoter activity, as expected as it is the constitutively active form of Elk1. However, the rise in the promoter activity by Elk1.woR was not as high as the unforced promoter activity. Elk1 has also another repression domain coinciding with the ETS DNA binding domain. Transcriptional repression of Elk1 through this domain involves the recruitment of co-repressor complexes such as mSin3A-HDAC complex that possess histone deacetylase activity (Yang *et al.,* 2001).

Another mechanism underlying this phenomenon might be that SENP1 (SUMO1/sentrin-specific protease 1), which impairs SUMO modifications on target proteins, can act as co-activator or recruit co-activators to the target protein while interacting with its target and to do this it might require SUMO modification on target protein. One of the previous study strengthening our speculation demonstrated that SUMO deficient mutant Elk1 could not provide expression of target gene as control cells in the absence of SENP1(Witty *et al.*, 2010).

3.3.2 mRNA expression level of *SPG4* gene

To confirm expressional results of Elk1.woR regulation on spastin mRNA level in SH-SY5Y cells, qRT-PCR analysis was performed. β-actin gene was used as reference for qRT-PCR studies. The values were calculated according to ΔΔCt

method (Schmittgen *et al.*, 2008). qRT-PCR results are shown in table 3.5, table 3.6 and figure 3.5.

Table 3.5: Ct value of experiment n=1 for spastin promoter.

N=1	β-actin	spastin		β-actin	spastin		β-actin	spastin
WT.Elk1	16,23	21,33	Elk1.woR	21,82	27,68	Elk1.VP16	18,12	25,43
	17,41	21,83		22,62	27,51		16,71	23,62
Negative	19,12	26,44	Negative	19,12	26,44	Negative	19,12	26,44
	19,4	26,86		19,4	26,86		19,4	26,86

Table 3.6: Ct value of experiment n=2 for spastin promoter.

N=2	β-actin	spastin		β-actin	spastin		β-actin	spastin
WT.Elk1	28,48	25,89	Elk1.woR	22,58	23,02	Elk1.VP16	23,84	26,71
	28,72	24,51		23,16	23,68		23,23	26,17
Negative	22,17	23,63	Negative	22,17	23,63	Negative	22,17	23,63
	22,39	24,27		22,39	24,27		22,39	24,27

ΔCt= 2^ - (Target gene - Reference gene)

ΔΔCt= 2^- (Sample ΔCt - Control ΔCt) **(3.2)**

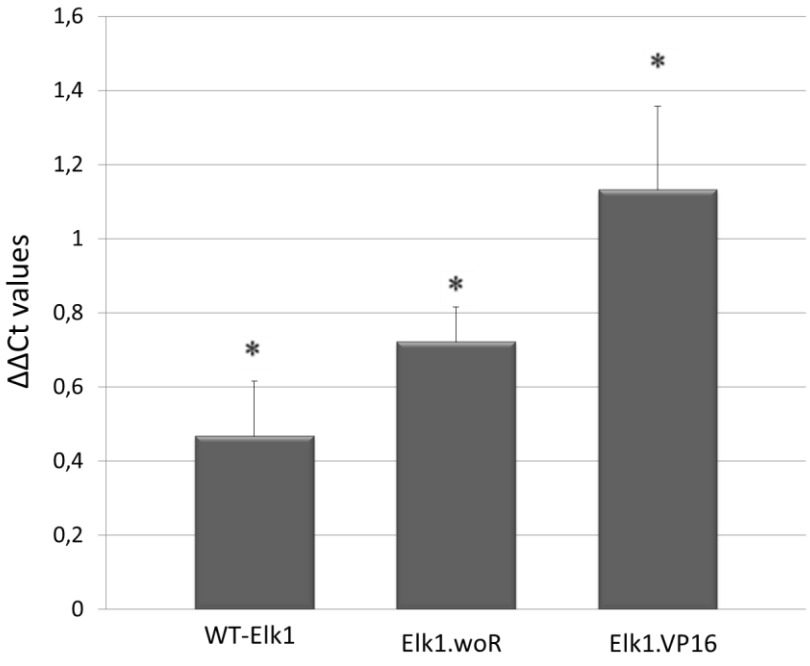

Figure 3.5: $\Delta\Delta C_t$ values of mRNA expression levels of *SPG4* gene of WT.Elk1, Elk1.woR and Elk1.VP16 transfected SH-SY5Y neuroblastoma cells.

It is suggested that SUMOylation of R domain on Elk1 could also decrease mRNA level of spastin by repressing *SPG4* promoter activity that does not allow futher transcription of spastin.

3.3.3 Luminometrical measurement data of forced experiment under PMA treatment condition for spastin promoter

In order to verify that SUMO-modified Elk1 has repressor role on *SGP4* promoter, effect of de-SUMOylated Elk1 on *SPG4* promoter was analyzed by using a reverse perspective. SH-SY5Y neuroblastoma cells were transfected with S2 construct and co-transfected with both S2 and WT-Elk1 (pCMV6.Elk1). After transfection, phorbol 12-myristate 13-acetate (PMA) was added into the medium .The reason of PMA treatment was that PMA is a mitogenic agent and promotes phosphorylation of Elk. The serum starved cells were exposed to PMA for 6 h and luciferase assay was performed. Each transfection was performed as triplicates at the same time and experiments were repeated 2 times on separate days. The data of this experiment is shown below.

Table 3.7: Measured light units of experiment n=1for PMA treatment.

N=1		S2	S2 Basic	WT.Elk1	WT.Elk1 Basic	WT.Elk1 PMA	WT.Elk1 PMA Basic
1	Firefly	123,4	1,224	21,96	0,5818	13,48	0,3881
	Renilla	219,5	103,1	670,1	386,2	217,7	231,9
2	Firefly	141,3	1,236	23,4	0,5957	11,43	0,2936
	Renilla	231,4	90,21	739,8	402,6	199,7	185
3	Firefly	199,4	1,375	21,33	0,5419	9,506	0,3484
	Renilla	335,7	106,3	614,9	380,3	177,4	189,4

Table 3.8: Measured light units of experiment n=2for PMA treatment.

N=2		S2	S2 Basic	WT.Elk1	WT.Elk1 Basic	WT.Elk1 PMA	WT.Elk1 PMA Basic
1	Firefly	0,2336	0,0045	0,0867	0,0019	0,0707	0,0026
	Renilla	1,346	1,162	1,336	1,529	1,023	1,665
2	Firefly	0,1743	0,005	0,079	0,0061	0,0845	0,0055
	Renilla	1,674	0,943	1,12	1,054	1,632	1,224
3	Firefly	0,1962	0,0057	0,0746	0,0041	0,0861	0,0027
	Renilla	1,477	1,058	1,225	1,395	1,362	1,246

The 3.1 equation is used to determine the normalized fold activation between test groups, considered as fold of induction in respect to the activity of the empty vector pGL2-Basic. The calculated fold activity and graph are given below.

Table 3.9: Average of calculated F/R of PMA treatment experiments and standard deviation.

Experiment F/R	pGL2-S2	WT.Elk1	WT.Elk1+PMA
N=1	28,1897	19,6935	32,9182
N=2	29,2753	15,0127	30,7919
Average F/R	28,7325	17,3531	31,8550
Average F/R Standard Deviation	4,8602	3,0350	4,7540
Δ Fold Activation	1	0,6039	1,1086

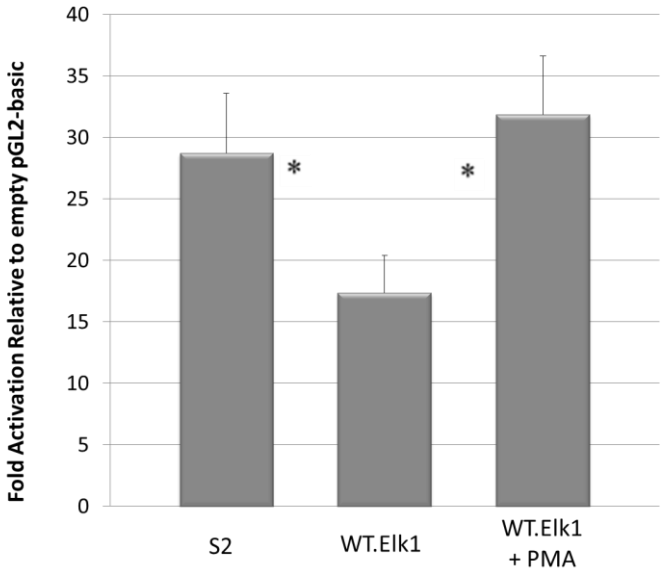

Figure 3.6: Average fold activities of WT.Elk1 forced experiment with PMA treatment on *SPG4* optimal promoter.

According to these results, repressive effect of Elk1 on *SPG4* promoter was reversed in PMA treated cells. It was shown that PMA can activate MAPK pathway by inducing phosphorylation and thus would cause phosphorylation of Elk1 (Hu *et al.*, 2000). PMA also causes deSUMOylation of Elk1 (Witty *et al.*, 2010). These data confirmed that SUMOylated Elk1 represses *SPG4* gene expression and activation of MAPK pathway results in loss of SUMO modification of Elk1 and thus, leads to transcriptional activation of Elk1.

3.3.4 Immunocytochemistry analysis of endogen spastin

After qRT-PCR and forced promoter activity experiment, protein level was obtained by using immunocytochemistry technique. The effects of Elk1-woR overexpression and PMA treatment were analyzed on the level of endogenous spastin protein. Firstly, SH-SY5Y cells were transfected with wt-Elk1, Elk1-woR or Elk1-VP16 expressing vector and after 48 h incubation, to confirm the overexpression of Elk1, Elk1-woR and Elk1-VP16 and spastin protein level, cells were immunostained with rabbit anti-Elk1 polyclonal and mouse anti-spastin monoclonal antibodies as primary, and goat 488 anti-rabbit and goat 647 anti-mouse Alexa Flour® antibody, as secondary antibodies, respectively. Then, Elk1-woR and Elk1-VP16 overexpressed cells were compared to wt-Elk1 transfected cells (Figure 3.7).

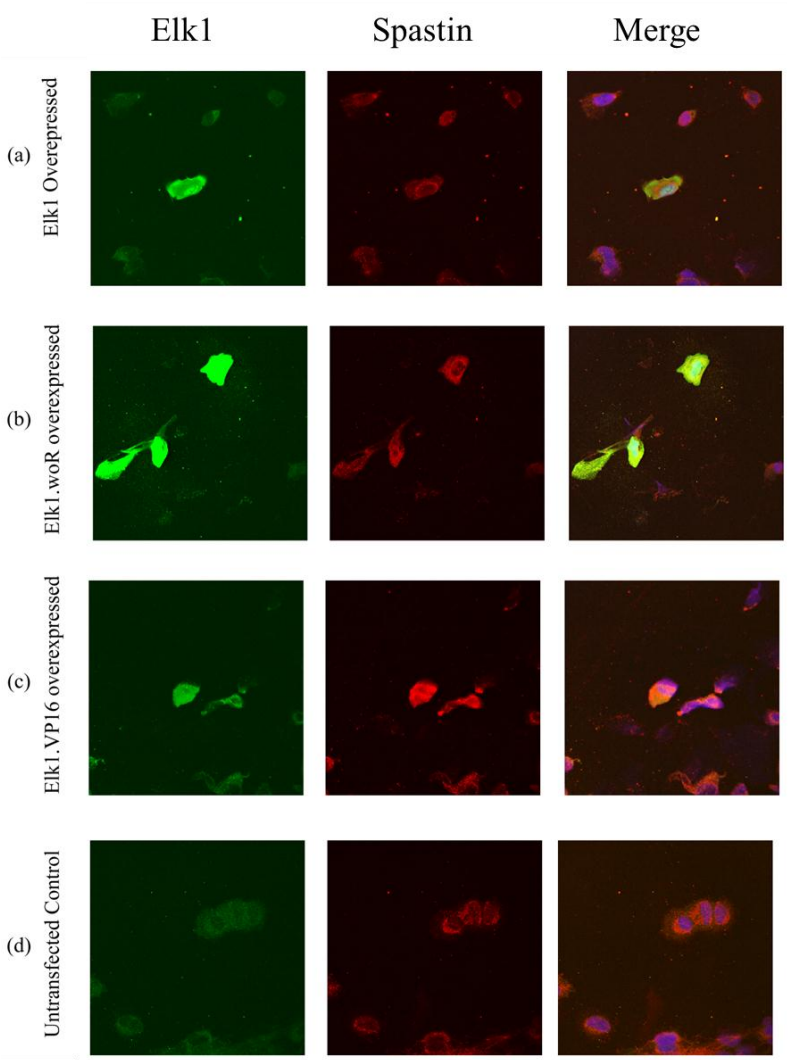

Figure 3.7: The images of immunostained Elk1 constructs (green) and endogen spastin (red).

When Elk1-woR and Elk1.VP16 transfected cells were compared to wt-Elk1 overexpressed cells, spastin level increased in Elk1-woR and Elk1-VP16 overexpressed cells. Moreover wt-Elk1 transfected cells had less spastin protein compared to control cells.

Additionally, SH-SY5Y cells were transfected with wt-Elk1 expression vector. After 18 h growth, cells were serum-starved for 24 h, and then one wt-Elk1 transfected sample was treated with 10 nM PMA. After incubation, cells were immunostained with rabbit anti-Elk1 polyclonal and mouse anti-spastin monoclonal antibodies as primary, and goat 488 anti-rabbit and goat 647 anti-mouse Alexa Flour® antibodies, as secondary antibodies, respectively (Figure 3.8).

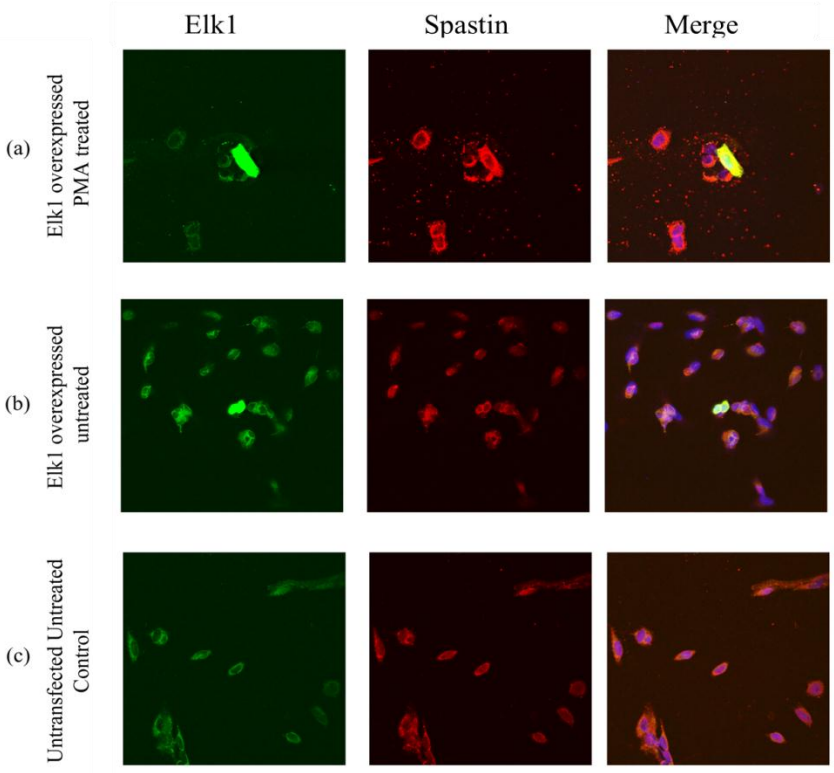

Figure 3.8: The images of immunostained Elk1 (green) and endogen spastin (red) under PMA treatment condition.

When wt-Elk1 overexpressed and PMA treated cells were compared to Elk1 overexpressed and untreated cells, an increase in spastin protein level was observed. On the other hand, spastin protein level in wt-Elk1 transfected cells was less than untransfected control cells, indicating wt-Elk1 transcription factor indeed reduced the spastin level. These immunostaining experiments are consistent with other experiments in expression and transcription level of spastin.

3.4 Results for p80-Katanin Optimal Promoter

3.4.1 Luminometrical measurement data of forced experiments for p80-katanin promoter

In order to determine effect of Elk1 on *KATNB1* optimal promoter, SH-SY5Y neuroblastoma cells were transfected with F2 construct including p80-katanin optimal promoter, and co-transfected with both F2 and WT-Elk1 (pCMV6.Elk1) or constitutively active form of Elk1 (pCMV6.Elk1.VP16) expressing vectors respectively. For statistical analysis each transfection was performed as triplicates at the same time and experiments were repeated 3 times on separate days. Results of forced experiment are indicated table 3.10, table 3.11 and table 3.12.

Table 3.10: Measured light units of experiment n=1for p80-katanin promoter.

N=1		F2	F2 Basic	WT.Elk1	WT.Elk1 Basic	Elk1.VP16	Elk1.VP16 Basic
1	Firefly	20,46	0,5058	7,08	0,1016	37,97	0,2466
	Renilla	2,086	2,997	1,23	1,925	0,7335	1,347
2	Firefly	21,45	0,482	8,715	0,085	45,94	0,2823
	Renilla	2,272	2,573	1,159	1,691	0,9139	1,37
3	Firefly	23,38	0,6229	7,968	0,0648	48,53	0,2316
	Renilla	2,098	2,723	1,576	1,39	1,128	1,38

Table 3.11: Measured light units of experiment n=2 for p80-katanin promoter.

N=2		F2	F2 Basic	WT.Elk1	WT.Elk1 Basic	Elk1.VP16	Elk1.VP16 Basic
1	Firefly	19,18	0,4713	2,893	0,0752	45,49	0,3995
	Renilla	2,75	3,353	0,3843	1,568	0,884	2,218
2	Firefly	20,11	0,5122	3,083	0,0792	44,91	0,3225
	Renilla	3,132	3,485	0,5018	1,563	0,9768	1,653
3	Firefly	18,25	0,5298	3,085	0,0566	50,13	0,3207
	Renilla	2,714	4,053	0,4999	1,272	1,009	1,726

Table 3.12: Measured light units of experiment n=3 for p80-katanin promoter.

N=3		F2	F2 Basic	WT.Elk1	WT.Elk1 Basic	Elk1.VP16	Elk1.VP16 Basic
1	Firefly	6,095	0,2618	5,72	0,0574	25,64	0,1757
	Renilla	1,487	2,392	2,563	2,168	0,8425	0,9982
2	Firefly	7,796	0,2755	5,114	0,0577	23,97	0,1986
	Renilla	1,821	2,841	1,647	1,953	0,9057	0,8273
3	Firefly	6,055	0,2578	5,348	0,0559	19,79	0,1789
	Renilla	1,84	2,421	1,817	2,35	0,6386	1,225

The 3.1 equation is used to determine the normalized fold change in activity between test groups, considered as fold of induction in respect to the activity of the empty vector pGL3-Basic. Average of all experiment and graph are shown table 3.13 and figure 3.9 respectively.

Table 3.13: Average of calculated F/R of all experiments and standard deviation for p80-katanin promoter.

Experiment F/R	pGL3-F2	WT.Elk1	Elk1.VP16
N=1	51,9671	122,4833	260,4444
N=2	48,1044	138,6389	262,2333
N=3	37,2980	103,7510	156,3532
Average F/R	45,7898	121,6244	226,3437
Average F/R Standard Deviation	7,4844	23,1415	54,8993
Δ Fold Activation	1	2,6561	4,9430

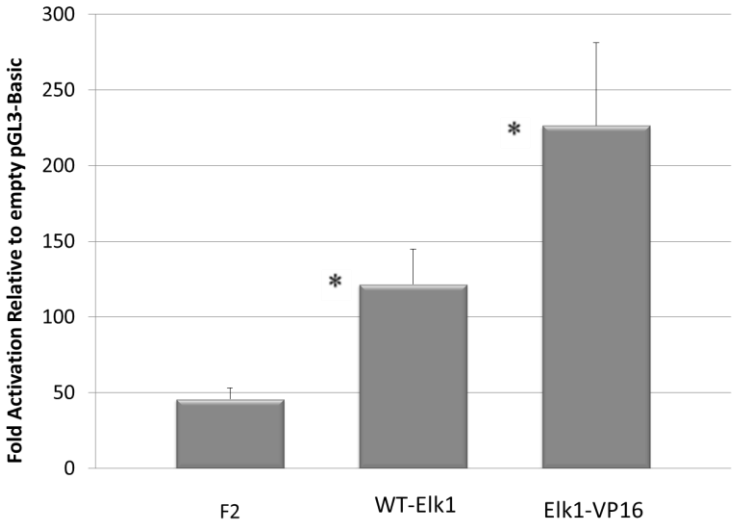

Figure 3.9: Average fold activities of Elk1 and Elk1.VP16 forced experiment on *KATNB1* optimal promoter.

Comparing the luciferase data of F2 construct, Elk1 significantly enhanced the optimal promoter activity and as expected constitutively active form Elk1.VP16 increased gene expression.

These results show that Elk1 has role as an activator role on regulation of *KATNB1* gene expression. Moreover Elk1 might not be SUMOylated, but phosphorylated by MAPK pathway to gain activator capability in by contrast to spastin gene regulation.

3.3.2 mRNA expression level of *KATNB1* gene

qRT-PCR was performed in order to further quantify whether these changes were reflected at mRNA level of p80-katanin. Total mRNA was isolated from SH-SY5Y cells nucleofected with pCMV.Elk1. β-actin gene was used as reference again. Following qRT-PCR experiments, values were calculated according to ΔΔCt method (Schmittgen *et al.*, 2008). qRT-PCR results are shown in table 3.14 and figure 3.10.

Table 3.14: Ct value of experiment n=1.

N=1	β-actin	katanin
WT.Elk1	17,29	27,98
	17,91	27,93
Negative	18,81	27,87
	18,78	28,07

N=2	β-actin	katanin
WT.Elk1	19,42	29,1
	18,65	29,22
Negative	19,86	28,86
	19,28	29,06

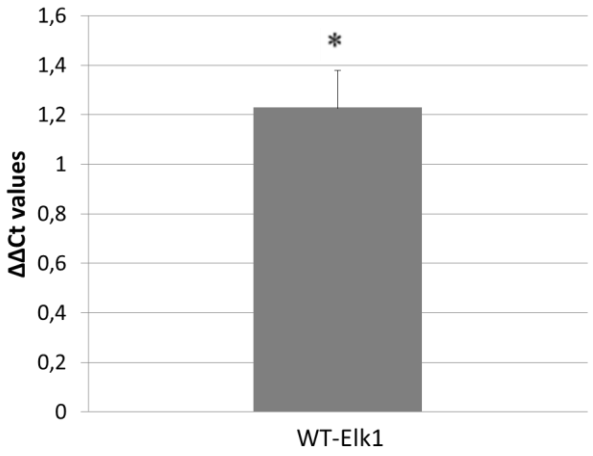

Figure 3.10: $\Delta\Delta C_t$ values of mRNA expression levels of *KATNB1* gene of WT.Elk1, transfected SH-SY5Y neuroblastoma cells.

mRNA levels of human p80-katanin was increased by overexpression of wt.Elk1 in SH-SY5Y cells.

The opposite effect of Elk1 on spastin and p80-katanin promoter might result from interaction with SRF. Elk1 has the ability to form ternary complex with SRF and SRE (Vickers *et al.*, 2004). This complex formation might be responsible for Elk1 deSUMOylation and activation of *KATNB1* promoter because it has a SRE consensus sequence $CC(A/T)_6GG$ at position from -515 to -506 juxtaposition of Elk1 binding site. (Appendix A) On the other hand, *SPG4* optimal promoter does not have this

SRE sequence. Therefore Elk1 might not form ternary complex and be deSUMOylated. Consequently the absence of SRE on *SPG4* promoter may cause the repressive effect of Elk.

3.4.3 Luminometrical measurement data of forced experiment under KCl treatment condition for p80-katanin promoter

A previous study demonstrated that KCl treatment promotes SUMO conjugation (Lu *et al*, 2009). Based on this study, cells were treated with KCl to increase Elk1 SUMOylation in order to understand whether Elk1 increased the promoter activity by MAPK pathway. Cells were transfected with F2 construct and co-transected with F2 and WT.Elk1 constructs. 24 h after transfection, Elk1 transfected cells were incubated in medium containing 50µM KCl for 1 h. Following 48 h post-transfection, luminometrical measurement is evaluated. Each transfection was performed as triplicates at the same time and experiments were repeated 3 times on separate days. The data of this experiment is below.

Table 3.15: Measured light units of experiment n=1for KCl treatment.

N=1		F2	F2 Basic	WT.Elk1	WT.Elk1 Basic	WT.Elk1 KCl	WT.Elk1 KCl Basic
1	Firefly	4,529	0,1483	1,835	0,0544	2,458	0,0521
	Renilla	0,6542	0,7071	0,755	0,7251	0,7523	1,089
2	Firefly	4,107	0,0975	2,264	0,0328	2,337	0,0512
	Renilla	0,7085	0,7521	0,7084	0,6967	0,7284	0,7773
3	Firefly	4,456	0,067	2,411	0,0273	2,513	0,0529
	Renilla	0,9471	0,8388	1,192	0,7582	0,8025	0,5095

Table 3.16: Measured light units of experiment n=2for KCl treatment.

N=2		F2	F2 Basic	WT.Elk1	WT.Elk1 Basic	WT.Elk1 KCl	WT.Elk1 KCl Basic
1	Firefly	4,437	0,064	2,104	0,0401	2,866	0,0542
	Renilla	0,6306	0,602	0,5944	0,5721	0,6007	0,6046
2	Firefly	4,227	0,0563	2,257	0,0386	2,66	0,0265
	Renilla	0,6762	0,6845	0,5899	0,7709	0,9208	0,3239
3	Firefly	4,568	0,0855	2,643	0,0252	2,885	0,0244
	Renilla	0,6397	0,5458	0,7383	0,6877	0,7313	0,3275

Table 3.17: Measured light units of experiment n=3 for KCl treatment.

N=3		F2	F2 Basic	WT.Elk1	WT.Elk1 Basic	WT.Elk1 KCl	WT.Elk1 KCl Basic
1	Firefly	3,807	0,0859	1,803	0,0423	2,213	0,0338
	Renilla	0,5599	0,7723	0,8703	1,065	0,6137	0,609
2	Firefly	3,406	0,1055	1,875	0,0201	2,066	0,0428
	Renilla	0,6294	0,6804	0,6867	0,8021	0,7018	0,4815
3	Firefly	4,075	0,0938	1,979	0,0258	2,421	0,031
	Renilla	0,5376	0,9706	0,5857	0,8698	0,7093	0,4338

The 3.1 equation is used to determine the normalized fold activation between test groups, considered as fold of induction in respect to the activity of the empty vector pGL3-Basic. The calculated fold activity and graph are given in the table 3.18 and figure 3.11.

Table 3.18: Average of calculated F/R of KCl treatment experiments and standard deviation.

Experiment F/R	pGL3-F2	WT.Elk1	WT.Elk1+KCl
N=1	41,5620	61,3749	44,1631
N=2	59,1754	69,8027	47,1811
N=3	54,5319	99,6689	46,1571
Average F/R	51,7564	76,9489	45,8338
Average F/R Standard Deviation	10,1646	22,3592	6,3792
Δ Fold Activation	1	1,4867	0,8855

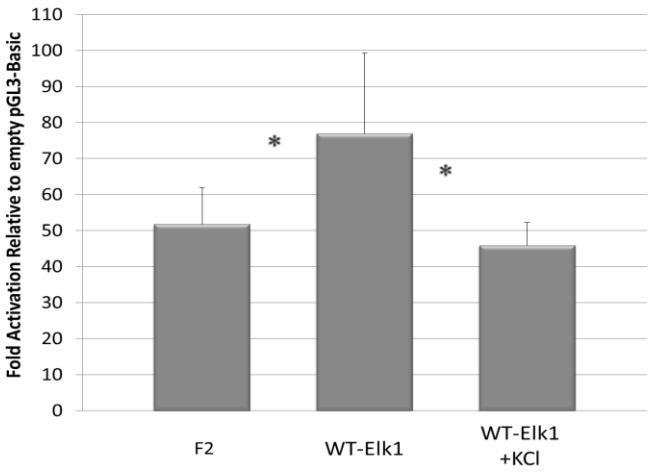

Figure 3.11: Average fold activities of WT.Elk1 forced experiment with KCl treatment on *KATNB1* optimal promoter.

According to these results, KCl treated samples show less promoter activity. Elk1 cannot be phosphorylated by MAPK pathway due to KCl increase of SUMO conjugation. Therefore KCl treated and SUMOylated Elk1 cannot increase expression of p80-katanin gene as much as untreated Elk1.

3.4.4 Immunocytochemistry analysis of endogen p80-katanin

The protein expression level of p80-katanin was analyzed by immunocytochemistry. SH-SY5Y cells were transfected with Elk1 expressing vector and after 48 h incubation cells were immunostained with goat anti-katanin p80 polyclonal antibody as primary antibody and donkey 488 anti-goat Alexa Flour® antibodies as secondary antibodies to visualize the levels of p80-katanin protein. Then Elk1 overexpressed cell, and both KCl treated and Elk1 transfected cells were compared to the control cells (Figure 3.12).

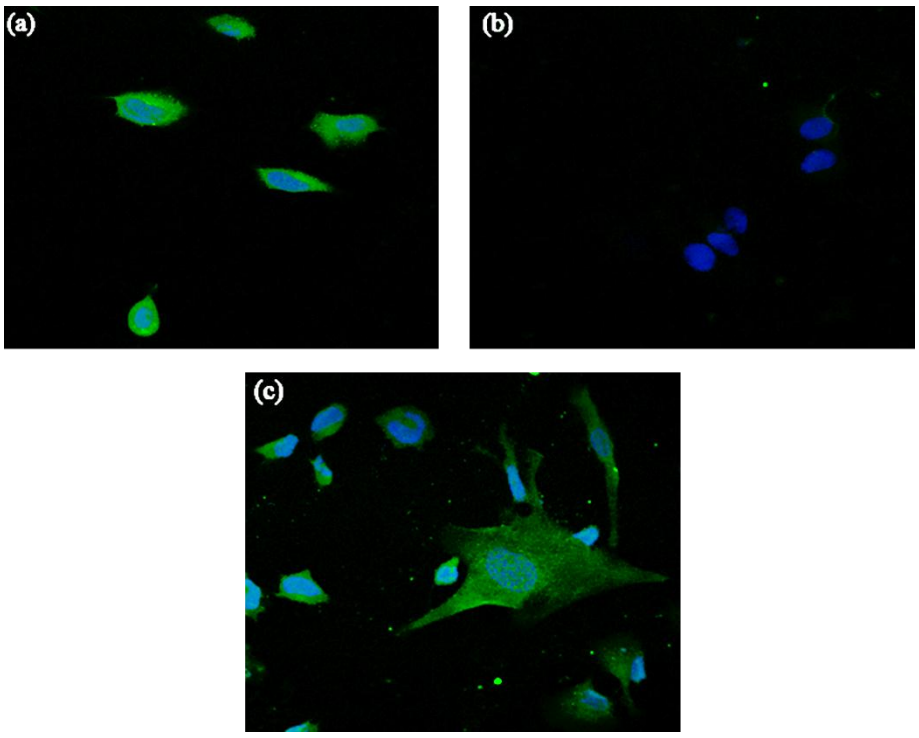

Figure 3.12: The images of endogenous p80-katanin protein. (a) Elk1 overexpressed and untreated cells. (b) Elk1 overexpressed and KCl treated cells. (c) Control cells. All of them p80-katanin were stained in green. All blue staining (DAPI) represented nucleus of the cells.

KCl treated and Elk1 over-expressed cells contained lower p80-katanin levels while untreated Elk1 over-expressed cells had an increased level of p80-katanin compared with the negative control. These results indicated that Elk1 increased the intrinsic p80-katanin protein levels, while KCl treatment reversed the Elk1's activator effects

on p80-katanin protein expression. This data showed that KCl addition trigger SUMO conjugation and lead Elk1 to repress the expression of endogenous p80-katanin protein in SH-SYSY cells.

3.4.5 Western blot analysis of p80-katanin and SUMOylated Elk1

The protein level of p80-katanin also was analyzed by Western blotting. One group of Elk1 transfected cells and one group of untransfected control cells were treated with KCl for 1 h. After total protein was isolated, p80-katanin level was analyzed by Western blotting. p80-katanin was detected with specific antibody. Then stripped membrane was used for the second time for detection of β-actin for normalization of bands density. p80-katanin has 80 kDa molecular weight while, β-actin has 41.7 kDa (Figure 3.13).

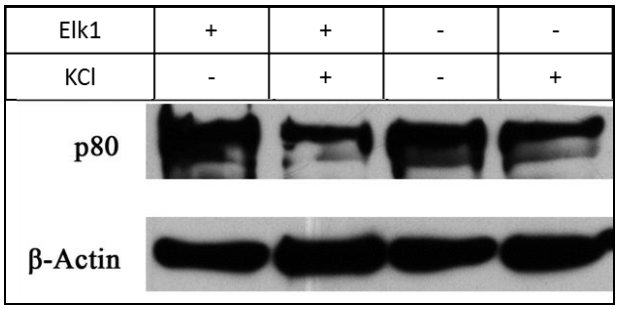

Figure 3.13: The level of p80-katanin and β-actin.

Western blot image was analyzed by Photoshop software in terms of density of bands and results were normalized against β-actin (Figure 3.14).

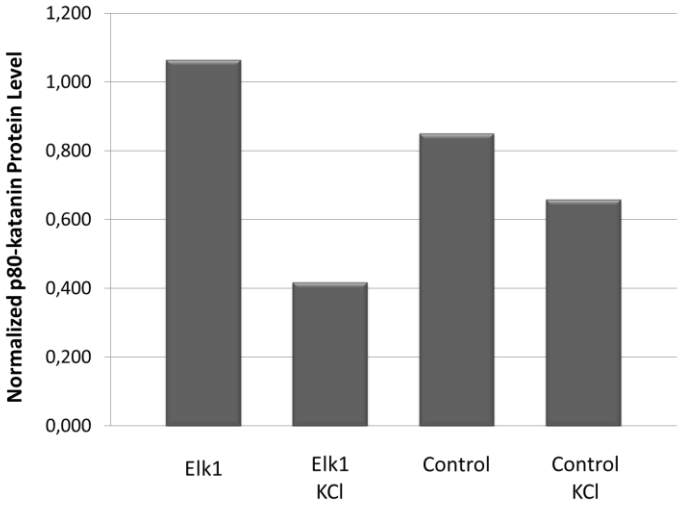

Figure 3.14: Normalized histogram showing p80-katanin level.

Figure 3.14 demonstrates that p80-katanin amount reaches highest level in Elk1 overexpressing cells. On the other hand KCl treated cells decreased p80-katanin expression both in Elk1transfected and untransfected cells.

After Elk1 was eluted from total protein of KCl treated and untreated cells by using immunoprecipitation technique, SUMO proteins were determined with specific SUMO1 and SUMO2/3 antibody, respectively. Figure 3.15 shows SUMOylated Elk1. Elk1 has 44.8 kDa molecular weight without any modifications. SUMO group which is approximately 12 kDa changes the size of Elk1.

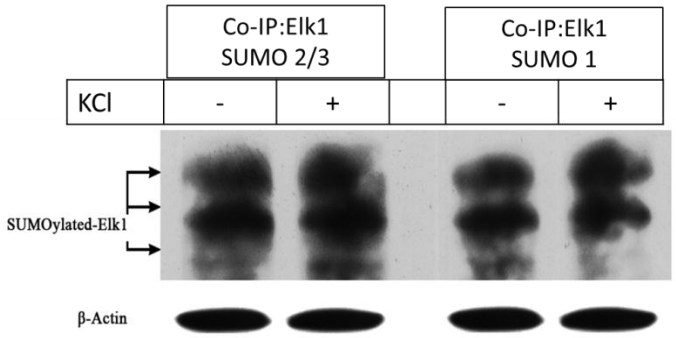

Figure 3.15: Western blot analysis of Elk1 with SUMO modification.

The 3 bands seen each columns belong to SUMOylated Elk1 with different patterns. Results were normalized against β-actin and histograms of total amounts of 3 bands were generated for both SUMO-1 (Figure 3.16) and SUMO-2/3 modifications (Figure 3.17).

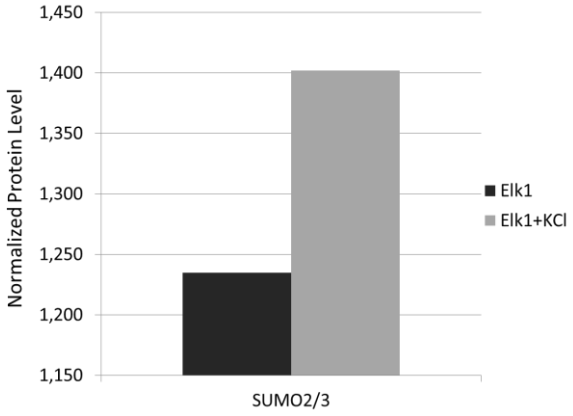

Figure 3.16: Normalized histogram showing Elk1 modified with SUMO 2/3.

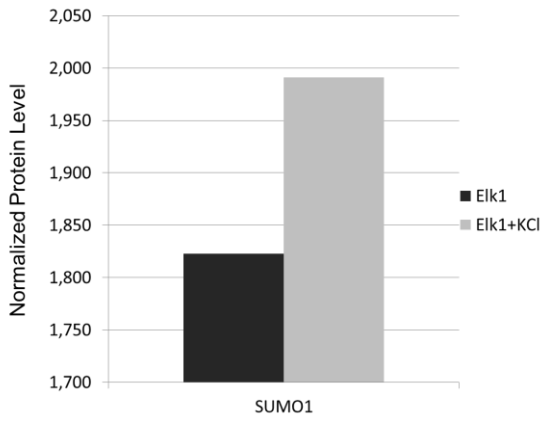

Figure 3.17: Normalized histogram showing Elk1 modified with SUMO 1.

Lu et al. (2009) revealed that when cells were exposed to depolarization buffer including 50 mM KCl, ratio of SUMO conjugation increased. Cells respond to KCl with elevating Ca^{+2} concentration and Ca^{+2} released into cells provides regulation of SUMO modifications (Lu *et al*., 2009).

In the presence of KCl, it was observed that there was a distinct increase in sequential SUMOylation (upper two bands at lane 4) level whereas, single SUMOylation (lower band) level was decreased. Hence, it can be interpreted that, SUMO-1 conjugations are preferentially added to Elk-1 consecutively, rather than singly when cells were promoted with KCl. However, changes in SUMO-2/3 modifications upon KCl treatment were different from SUMO-1 modifications. Multiple and serial SUMOylation was induced for SUMO-2/3 conjugations, furthermore, there was an increase in single SUMOylated Elk-1, as well. In the light of this information it can be concluded that, SUMO-1 may tend to prefer mono conjugated modifications whereas SUMO-2/3 may tend to prefer multi conjugated modifications in normal condition.

4. CONCLUSION AND RECOMMENDATION

In this study, the effects of Elk1on *SPG4* and *KATNB1* gene promoter have been determined. Elk1 has repressive effect on *SPG4* promoter whereas activator effect on *KATNB1* promoter. The results of expression assay have been confirmed both in mRNA and protein levels. Then, the effect of Elk1 on *SPG4* and *KATNB1* promoter has been reversed by PMA and KCl treatment, respectively. PMA treated cells have shown higher spastin promoter activity; while KCl treated cells have shown lower p80-katanin promoter activity than wt-Elk1.

We have shown that regulation of spastin and p80-katanin expression were Elk1 dependent. However, expressional regulations of these proteins could also be investigated with different transcription factors. In addition to this, we know the importance of CpG islands on the promoter regions which would drive the attention to the CpG island methylation as an important mechanism for gene expression. Therefore, we suggest that CpG island methylations, as an epigenetic regulation, might be another regulatory mechanism resulting in different expression levels of spastin and p80-katanin proteins.

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APPENDICES

APPENDIX A. Elk1 Binding Site and SRE on *KATNB1* Optimal Promoter Sequence

ATCTGCAGGCAAAGTGAAGAATGAGGAGCTTCGGCAAAATGCCGACTAAGGCCTCCTTA
GGTTTTGCCCCACTCCAAGATGGAAGGCCTGAGGCTTCACACTGCCCCCGAAGTTCCTTT
CCCATTGGCTATCTGGGAATTGAGTTTTCCAATAATGCGGACGCTGATTGGTCAATCCAG
GACGGTTGCTCAAGCCATTGGCGCAGCCGCCATTGGAGGGCGGCTCTCAAAAGTTTTCAG
ACACAAATTAGGTTCGAGGGAGGAAACGGAGAGGAAAGGGAAAACTTGAGACGGAGGC
GGGACTAAGGAAACGGCAGCTTGCATTGGTTTATTAGAGGCCAAGGGGCGGCTCTTGAA
CGTTCCTTCCTCTTGATTAGTCCTATTTAGGAAAAGAGGGCGGGTACTGAGGAAAAGCGG
CAGAAGCGCCTGCTTCCATTGGTCAGTCCTGGCAGGAGGCGGAGCACCCGCGGCAGCTG
ATTGGTGCGGGAGGCAAGGTGGGCGGGGCTCTGAGCCGGAGGT

* Elk1 binding site and SRE were highlighted yellow and pink respectively.

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