ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

EFFECT OF ELK1 AND YY1 TRANSCRIPTION FACTORS ON *SPG4* AND *KATNB1* PROMOTERS

M.Sc. THESIS

Koray KIRIMTAY

Advanced Technologies

Molecular Biology–Genetics and Biotechnology

Thesis Advisor: Prof. Dr. Arzu KARABAY KORKMAZ

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<u>İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ</u>

ELK1 VE YY1 TRANSKRİPSİYON FAKTÖRLERİNİN *SPG4* VE *KATNB1* PROMOTORLARI ÜZERİNDEKİ ETKİSİ

YÜKSEK LİSANS TEZİ

Koray KIRIMTAY
(521091094)

İleri Teknolojiler Anabilim Dalı

Moleküler Biyoloji-Genetik ve Biyoteknoloji Programı

Tez Danışmanı: Prof. Dr. Arzu KARAB AY KORKMAZ

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Koray Kırımtay, a **M.Sc.** student of ITU **Graduate School of Science, Engineering and Technology** student ID **521091094**, successfully defended the **thesis** entitled "**EFFECT OF ELK1 AND YY1 TRANSCRIPTION FACTORS ON *SPG4* AND *KATNB1* PROMOTERS**", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor : **Prof. Dr. Arzu KARABAY KORKMAZ** ....
İstanbul Technical University

Jury Members: **Assoc. Prof. Dr. Eda TAHİR TURANLI** ....
Istanbul Technical University

Assist. Prof. Dr.Ergül BERBER ....
Istanbul Arel University

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FOREWORD

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ABBREVIATIONS

µg : Microgram
µl : Microliter
µM : Micromolar
µm : Micrometer
aa : Amino acid
AAA : ATPases Associated with diverse cellular Activities
APS : Ammonium persulfate
ATP : Adenosine triphosphate
ATPase : Adenosine triphosphatease
bp : Base pair
CBB : Coomassie Brilliant Blue
cDNA : Complementary DNA
DMEM : Dulbecco's modified Eagle medium
DMSO : Dimethyl sulfoxide
DNA : Deoxyribonucleic acid
dNTP : Deoxyribonucleotide
DTT : Dithiothreitol
E.coli : *Escherichia coli*
EB : Elution Buffer
EDTA : Ethylenediaminetetraacetic acid
Elk1 : E twenty-six (ETS)-like transcription factor 1
Elk1-db : Elk1 DNA binding region
EMSA : Electorphoretic Mobility Shift Assay
ETS : E twenty-six
EtBr : Ethidium bromide
FBS : Fetal bovine berum
g : Gram
GDP : Guanosine tri-phophate
GTP : Guanosine tri-phosphate
His : Histidine
HRP : Horseradish peroxidise
IDT : Integrated DNA Technology
IPTG : Isopropyl β-D-1-thiogalactopyranoside
Kb : Kilo base
kDa : Kilo dalton
L : Liter
LB : Luria-Bertani Broth
M : Molar
mA : Miliampere
MAPs : Microtubule-associated proteins
mg : Miligram
min : Minute
ml : Mililiter
mM : Milimolar

mm : Milimeter
mRNA : Messenger ribonucleic acid
MT : Microtubule
MTs : Microtubules
NCBI : National Center for Biotechnology Information
Ni-NTA : Nickel-nitriloacetic acid
ng : Nanogram
nM : Nanomolar
OD : Optical Density
PBS : Phosphate Buffered Saline
PCR : Polymerase chain reaction
PEG : Polyethylene glycol
pH : Power of hydrogen
RNA : Ribonucleic acid
rpm : Revolutions per minute
SDS : Sodium dodecyl sulphate
SDS-PAGE : Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec : Second
SOC : Super Optimal Broth with catabolite repression
TBE : Tris-borate-EDTA
TBS : Tris-Buffered Saline
TCA : Trichloroacetic acid
TE : Tris-EDTA
TEMED : Tetramethylethylenediamine
T_m : Melting temperature
UV : Ultraviolet
V : Volt
YY1 : Yin Yang 1
YY1-db : YY1 DNA binding region
YY1-woa : YY1 without activator region
YY1-wor : YY1 without repressor region
γ-TuRC : γ -tubulin ring complex

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EFFECT OF ELK1 AND YY1 TRANSCRIPTION FACTORS ON *SPG4* AND *KATNB1* PROMOTERS

SUMMARY

Cytoskeleton is an important network for cellular processes such as mitosis, cytokinesis, cell shape and motility. The cytoskeleton consists of three types of polymeric fibers: intermediate filaments, microfilaments, and microtubules. Intermediate filaments provide cells mechanical strength, such as keratins in epithelial cells and neurofilaments in neurons. Another component of cytoskeleton, actin microfilament, is involved in various cellular functions such as contraction of muscles, maintenance of cell shape. The last member of the cytoskeleton is microtubules. Microtubules are very dynamic structures and they have active roles in cell division, cell morphogenesis and intracellular organelle transport. The dynamic structure of microtubules is provided by two mechanisms. One of the mechanisms is dynamic instability, rapid polymerization and depolymerization; the other mechanism is cleavage of microtubules by microtubule-severing enzymes.

Microtubule-severing enzymes katanin and spastin are members of AAA super family of ATPases. They severe microtubules into shorter polymers which are essential for neurogenesis. Although both of these proteins are microtubule severing enzymes and their mechanism of action is similar, they cut microtubules in different legths. Katanin is composed of two subunits: p60 subunit contains AAA ATPase domain, which severs microtubules and non-ezymatic p80 subunit which enhances severing activity of p60. Katanin is highly expressed in nervous system and its inhibition or overexpression impairs axon formation. Furthermore, mouse model of Alzheimer's disease showed that katanin levels in hippocampus of transgenic mice was less compared to wild-type mice.

The other microtubule-severing enzyme, spastin is encoded by *SPG4* gene and mutation in *SPG4,* causes Hereditary Spastic Paraplegia disease. Studies showed that wild-type spastin overexpressing cells have disrupted microtubule network. It has been shown that downregulation of spastin caused morphologic undergrowth, reduced synaptic area, and increased synaptic microtubules by *in vivo* experiments. Conversely, overexpression of spastin reduced the synaptic strength and amount of synaptic microtubules.

Transcription factors YY1, a GLI-Kruppel class of zinc finger protein, and Elk1 Ets family of transcription factor are able to contribute both in activation and repression of the target gene and are ubiquitously expressed in different tissues as well as in brain.

Our aim in this study was to identify the regulation of *SPG4* and *KATNB1* gene expression by Elk1 and YY1 transcription factors. The optimal promoters of *KATNB1* and *SPG4* have been previously identified by our laboratory. For this purpose, we started with analyzing the binding of these proteins to the corresponding promoter regions. For this purpose, we performed EMSA by using oligonucleotides including related transcription factor binding sites, which are predicted on *SPG4* and

KATNB1 promoter and confirmed the binding of Elk1 and YY1. To further confirm the specificity of the binding, we produced recombinant Elk1 and YY1 proteins that contain only DNA binding domain and performed supershift assay by using these recombinant proteins.
To investigate regulation of *SPG4* and *KATNB1*genes by YY1, different YY1 constructs, which lack of activation or repression domain were obtained. The results demonstrated that YY1 act as a repressor on both *KATNB1* and *SPG4* promoters. It is known that SUMOylation can make a transcription factor to act as a repressor. So that we mutated YY1's SUMO binding site in order to obtain SUMO defficient YY1 transcription factor and to explore the function of SUMO modification on YY1. Luciferase reporter assay results showed that vast majority of repressor function of YY1 is due to its glycine/lysine rich domain rather than SUMOylation.

ELK1 VE YY1 TRANSKRİPSİYON FAKTÖRLERİNİN *SPG4* VE *KATNB1* PROMOTORLARI ÜZERİNDEKİ ETKİSİ

ÖZET

Hücre iskeleti, mitoz, sitokinez, hücreye şeklinin verilmesi, hücrenin hareketi gibi mühim faaliyetlerde görev alan önemli bir ağ yapısıdır. Hücre iskeleti üç temel polimerik yapıdan meydana gelir. Bunlar ara filamentler, mikrofilamentler ve mikrotübüller olarak adlandırılırlar. Sinir hücrelerindeki nörofilamentler ve epitel hücrelerindeki keratin ara filamentlere örnek verilebilir. Bu yapılar hücreye mekanik dayanıklılık kazandırırlar.

Hücre iskeletinin diğer bir üyesi olan aktin filamentler yani mikrofilamentler, bir çok hücresel işlevde görev alırlar. Başlıca fonksiyonları, hücre şeklinin korunması, sitoplazma yoğunluğunun sağlanması, organellere tutunma yüzeyi hazırlanması ve kasların kasılmasını kontrol etmesi olarak sıralanabilir.

Hücre iskeletini oluşturan diğer bir yapı ise mikrotübüllerdir. Mikrotübüller temel olarak hücre morfolojisini, hücre bölünmesini ve hücre içi organel taşımasını sağlarlar. Mikrotübüller oldukça dinamik bir yapıya sahiptirler. Bu dinamizmi sağlayan iki mekanizma vardır. Birincisi intirinsik yol olan "dinamik instabilite" yani mikrotubullerin hızlı bir şekilde polimerizasyonu ve depolimerizasyonudur. İntirinsik mekanizma α-tubulin ve β-tubulin heterodimerlerinin büyüyen ve kısalan mikrotübül zincirine eklenip ya da ayrılımasıyla meydana gelir. α-β heterodimerleri bir ara geldiğinde GTP hirolizi gerçekleşir ve böylelikle mikrotübüllerin gövdesi GDP bağlı tübülin dimerlerini barındırır. Mikrotübüllerin uç kısmında ise GTP bağlı β-tubulinler bulunur. GDP bağlı tubulinler daha stabil bir yapı oluşturlar. GTP bağlı tubulinlerin bulunduğu, hızlı bir şekilde polimerize ve depolimerize olan uç artı (+) diğer uç ise eksi (-) olarak adlandırılır. Eğer GTP ucundaki β-tubulin üzerinde bulunan GTP başka bir GTP-bağlı β-tubulin eklenmeden GDP'ye dönüştürülürse mikrotübüler hızlı bir şekilde depolimerize olurlar. Yalnız GTP-tubulinler sadece artı uçta değil microtubul gövdesinde de bulunabilirler. Böylelikle depolimerize olan microtübüllerin bu noktalara gelindiğindee tekrar polimerize olması sağanır.

Mikrotübüller sinir hücrelerinin akson ve dendritlerinde yoğun demetler halinde bulunarak bu yapıların uzamasını ve korunmasını sağlarlar. Sinir hücrelerinde bulunan mikrotübüller sentrozomlardan çekirdeklenirler ve mikrotübül kesen enzmiler aracılığıyla buralardan ayrılarak kısa parçalar halinde sinir hücresinin uzantılarına taşınırlar. Bu taşınma mikrotübül ne kadar kısa olursa o kadar hızlı gerçekleşir. Diğer bir deyişle taşınma hızı mikrotübül boyu ile ters orantılıdır. Mikrotübüllerin küçük parçalara kesilmesi için AAA ailesi ATPaz'larından olan katanin ve spastin'e ihtiyaç duyulur. Mikrotübül kesen bu iki enzim nöron gelişiminde önemli bir role sahiptirler. Benzer çalışma mekanizmasına sahip olmakla beraber bu proteinler microtübüllerin farklı boyutlarda kesilmesini sağlarlar Spastinden farklı olarak, katanin iki farklı altbirimden oluşur. Bunlar enzimatik faaliyet gösteren ve AAA ATPaz bölgesi içeren p60 ve enzimatik görevi olmayan p80'dir. p80'in görevi ise p60'a bağlandığında p60'ın kesim aktivitesini artırmak ve WD40 bölgesi ile p60'ı sentrozomlara konuşlandırmaktır.

Yapılan araştırmalar sonucu kataninin sinir sisteminde oldukça yoğun olarak ifade edildiği saptanmış ve baskılandığı zaman microtübül uzunluğunda artış gözlemlenmiştir. Bu artışın ise akson gelişimini olumsuz etkilediği ve ayrıca microtubullerin sentrozomlarda birikmesine de sebep olduğu gösterilmiştir. Bu da kataninin mikrotübüllerin sentrozomlardan ayrılması ve akson büyümesi için ne kadar gerekli olduğunu göstermektedir. Fareler üzerinde yapılan bir deneyde ise Alzheimer hastalığı modeli olarak kullanılan transgenik farelerin hipokampüslerinde katanin seviyesinin kontrol farelere göre daha az olduğu gösterilmiştir. Buradan da kataninin nöronların dejenere olup olmamasında belirgin bir rol oynadığı anlaşılmaktadır.
Diğer bir mikrotübül kesen enzim, spastin, otozomal dominant Herediter Spastik Prapleji (HSP) hastalığına sebep olan spastin kodlayan *SPG4* genindeki mutasyonların incelenmesi aşamasında bulunmuştur. Yapılan çalışmalarda spastin proteininin aşırı üretildiği hücrelerde dağılmış bir mikrotübül ağına rastlanmaktadır. Spastin seviyesinin düşürüldüğü diğer çalışmalarda ise, bu azalışın morfolojik olgunluğa erişimi engellediği, sinaptik boşluğu küçülttüğü ve sinaptik mikrotübülleri arttırdığı belirtilmiştir.
Bütün diğer proteinlerde olduğu gibi hücre içindeki katanin ve spastin miktarı da transkripsiyon mekanizması ile düzenlenir. Bu düzenlemenin kontrolü ise gen anlatımını sağlayan promotor bölgelerine bağlanan transkripsiyon faktörleri aracığıyla gerçekleşir. Elk1 ve YY1 bunlardan yalnızca iki tanesidir.
Ets ailesi transkripsiyon faktörlerinden olan Elk1 ve GLI-Kruppel zinc finger proteini YY1 hedef genin hem aktivasyonunu hem de represyonunu sağlayabilirler. Bu iki transkripsiyon faktörüne beyin de dahil olmak üzere çeşitli dokularda sıkça rastlanılmaktadır. İki transkripsiyon faktörünün de fonksiyonunu düzenleyen farklı bölgeleri vardır. Elk1 N-terminal DNA bağlanma bölgesine sahipken, YY1 DNA ile C-terminalde bulunan 4 zinc finger vasıtasıyla bağlanır. Her iki protein de tranlasyon sonrası çeşitli modifikasyonlara uğramaktadırlar. Bu modifikasyonlardan, kinaz yolağı aracılığıyla sağlanan serin (Ser, S) ve treonin (Thr, T) amino asitlerinin fosforilasyonu, transkripsiyon faktörlerinin ilgili gen ifadesinde aktivatör rol üstlenmesine sebep olurken; bir diğer modifikasyon olan SUMOlanma ise gen ifadesinin baskılanmasına yol açmaktadır.
Bütün bu bilgiler bir arada düşünüldüğünde, sırasıyla spastin ve katanin proteinlerinin ifadesini sağlayan *SPG4* ve *KATNB1* promotorlarının YY1 ve Elk1 ile nasıl regüle edildiğinin tespit edilmesi son derece önem teşkil etmektedir. Böylelikle nöronal gelişimde ve nörodejenerasyonda önemli role sahip mikrotübül kesen enzimlerin, YY1 ve Elk1 transkripsiyon faktörlerinin etkisinde miktarlarında ne gibi bir değişikliğe sahip olduğu ve bu etkiyi yaparken ilgili transkripsiyon faktörlerinin hangi yolaklardan etkilendiği saptanabilir.
Bu çalışmada, öncelikle *SPG4* ve *KATNB1* gen promotorlarının YY1 ve Elk1 transkripsiyon faktörlerinin etkisi altında olup olmadığını belirlemedeki ilk adım olan, ilgili gen bölgelerine transkripsiyon faktörlerinin bağlanıp bağlanmadığını belirlemek amaçlanmıştır. Bu etkileşimi saptamak için laboratuarımız tarafından belirlenmiş *KATNB1* ve *SPG4* optimal promotorlarında bu iki transkripsiyon faktörünün olası bağlanma bölgeleri biyoinformatik araçlar kulanılarak belirlenmiştir. Daha sonra EMSA yöntemi ile Elk1 ve YY1'in bu bölgelere bağlandığı doğrulanmıştır. Bu aşama sonrasında bağlanmanın YY1 ve Elk1'e özgünlüğünün tespiti için rekombinant proteinler üretilmiştir. Elde edilen rekombinant proteinler Elk1 ya da YY1'ın yalnızca DNA bağlanma bölgesini içermektedir. Elde edilen sonuçlara göre ilgili bölgelere bağlanan proteinlerin YY1 ve Elk1 olduğu doğrulanmıştır.

YY1 transkripsiyon faktörünün *SPG4* ve *KATNB1* promotorlarına bağlandığı belirlenmesinin ardından YY1'ın katanin ve spastinin gen anlatımındaki rolünün tayin edilmesi gerekmektedir. Bilindiği gibi YY1 hem aktivatör hem de represör olarak görev alabilmektedir. Bundan dolayı YY1'ın aktivatör ya da represör olarak görev almasını sağlayan bölgeler çıkarılarak YY1 fonksiyonunda etkili yolakların da belirli bir oranda etkisi incelenmiştir. Ayrıca transkripsiyon faktörlerinin represör etki göstermesinde önemli olan SUMO modifikasyonu da YY1'in SUMOlanma bölgesi mutasyona uğratılarak incelenmiştir. Sonuçlar göstermiştir ki YY1 transkripsiyon faktörü hem *KATNB1* hem de *SPG4* promotorları üzerinde baskılayıcı rol oynamaktadır ve bu baskılanma SUMO modifikasyonundan ziyade YY1'in glisin/lizin zengin bölgesi ile sağlanmaktadır.

1. INTRODUCTION

1.1 Cytoskeleton

Cytoskeleton, a network of protein filaments extending throughout the cytoplasm, is critical for mitosis, cytokinesis, intracellular transport, regulation of cell shape and cell motility. The cytoskeleton is composed of three principal types of cytoplasmic fibers: microfilaments, intermediate filaments and microtubules. All these fibers are polymers built from small protein subunits held together by noncovalent bonds (Alberts *et al*. 2008).

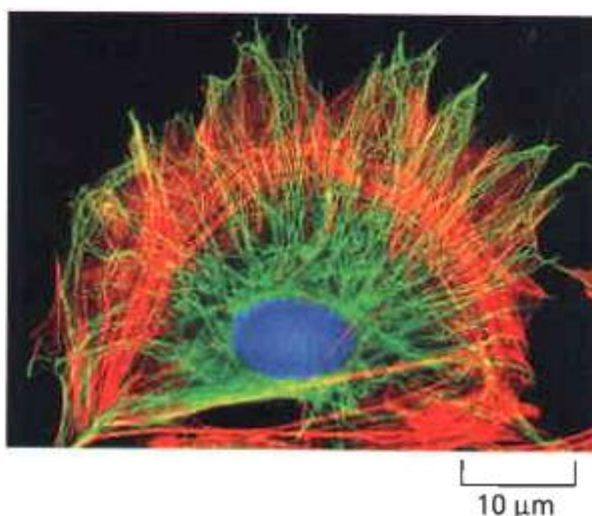

Figure 1.1: The cytoskeleton. A cell in culture has been fixed and labeled to show two of its major cytoskeletal systems, the microtubules (green) and the actin filament (red). The DNA in the nucleus is labeled in blue.(Alberts *et al*. 2008)

Briefly, in contrast to actin filaments and microtubules, the intermediate filaments are not directly involved in cell movements, but they appear to play a structural role by providing mechanical strength to cells. Most types of intermediate filaments are cytoplasmic but one type, lamins, are nucleoplasmic. Intermediate filaments found in cytoplasm can be grouped into three classes: (1) keratin filaments in epithelial cells, (2) vimentin and vimentin-related filaments in connective tissue cells, muscle cells, and supporting cells of nervous system; (3) neurofilaments in nerve cells. All of intermediate filament proteins share a central α-helical rod domain which plays a role

in filament assembly. The variable head and tail domains most likely determine the specific functions (Cooper G.M, Hausman R.E, 2004).
The other component of the cytoskeleton, actin, is one of the most abundant protein and exists in both monomeric (G-actin) and polymeric forms (F-actin). Polymerization of actin monomers results in twisted two-stranded structure known as actin microfilaments. Microfilament ends are biochemically distinct, with different growth rates: the slow growing "minus" end and the fast growing "plus" end. The microfilament network is a highly complex and dynamic structure involved in a variety of cellular functions such as motility, maintenance of cell shape, cell attachment, anchorage of cell organelles and maintenance of cytoplasmic viscosity. To do that actin filaments are associated with a large number of actin-binding proteins, e.g. myosins, cofilins, formins and gelsolins (Alberts B. *et al.* 2003; S.R. da Costa *et al.* 2003).

1.2 Microtubules

Microtubules are dynamic hollow tube like structures present in the cytoskeleton of all eukaryotic cells. They are involved in generation of cell morphogenesis and organization, cell division, cell growth and intracellular organelle transport. A characteristic property of microtubules is their ability to undergo cycles of rapid growth and disassembly known as dynamic instability. For this reason, individual microtubules do not have a stable length, but exist in either polymerization (growth) or depolymerization (shrinkage) state (Poulain F.E, Sobel A, 2010; Conde C, Caceres A, 2009; Antal *et al.*, 2007).
Microtubules are composed of α- tubulin and β- tubulin monomers (450 amino acids each and are about 50% identical at the amino acid level). They are stacked head to tail at 8 nm intervals to form "protofilaments" that run lengthwise along the wall and usually 13 protofilaments associate to form microtubule architecture, a hollow cylinder about 25 nm in diameter (Fig 1.2 a) (Burns, 1991; Meurer-Grob et al., 2001; Watson et al., 2004). They are nucleated at specific structures termed microtubule-organizing centers (MTOC), and γ-tubulin is localized to MTOC and functions as a template for the correct assembly of microtubules (Poulain F.E, Sobel A, 2010). It is also found in the cytoplasm as a part of a large complex called γ-tubulin ring complex (γ-TuRC) (Job et al., 2003). Beside γ-tubulin dependent nucleation mechanism, other mechanisms independent of γ-tubulin complex components exist

(Kuijpers M, Hoogenraad C.C, 2011). The structure of microtubule is organized in a polar manner and polarity is responsible for different polymerization rates at the two microtubule ends, the faster growing "plus" ends being capped by β subunits and the slower "minus" ends by α subunits (Risinger et al., 2009).

By polymerization and depolimerization, constitution of microtubules change between growing and shrinking phases, a process known as dynamic instability (Desai and Mitchison, 1997). In other words, dynamic instability is characterized by the frequencies of catastrophes (transitions from polymerization to depolymerization) and rescues (transitions from depolymerization to polymerization) (Fig 1.2 b) (Conde C, Caceres A, 2009).

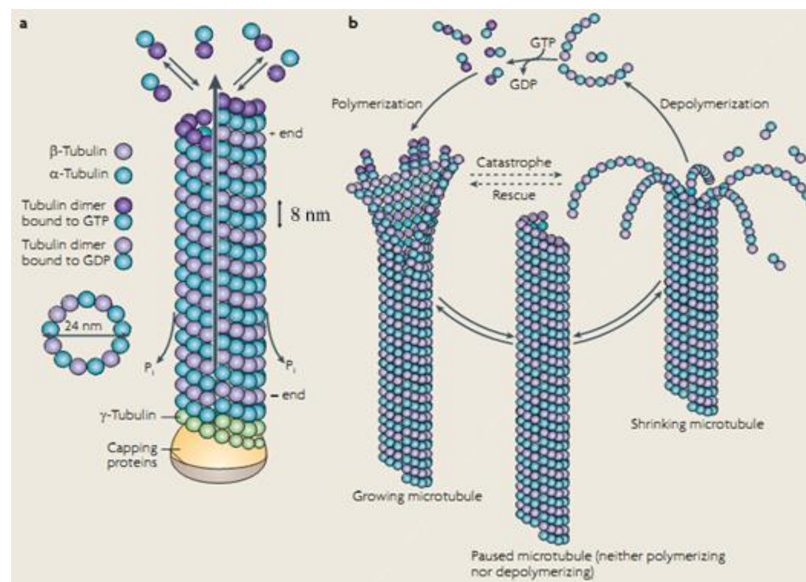

Figure 1.2: Microtubule tructure and dynamic instability (Conde C, Caceres A, 2009).

Microtubule polymerization is a complex process involving a cooperative assembly of α β tubulin heterodimers followed by GTP hydrolysis and there is a hydrolyzable GTP binding site (exchangeable site) on the β- subunit and a non-hydrolyzable site on the α- subunit (Meurer-Grob et al.,2001). The guanoside-triphosphate (GTP)-bound tubulins are added at the plus end of microtubules and form a GTP cap which stabilizes the microtubule structure and facilitates growing of microtubule by addition of new dimers. Addition of new dimer triggers hydrolysis of β-tubulin bound GTP to GDP. Thus, the majority of β-tubulin in the microtubule fiber is in the GDP-bound form and ''capped" with GTP-bound β tubulin at the plus end (Singh et al., 2008). If this GTP cap is lost by means of the GTP molecule on β-tubulin is hydrolyzed to GDP before another GTP-bound β-tubulin is added, the protofilaments

spread out and results in rapid microtubule depolymerization (Risinger et al., 2008; Singh et al., 2008). In addition to this model of action, it has been shown that GTP-tubulin is not only found at plus end, but there are some GTP-tubulins (GTP-islands) exist in the body of microtubules. So that these GTP-tubulins would be exposed upon depolymerization and act as a GTP cap (Dimitrov et al. 2008).

Together with these intrinsic processes, microtubule dynamics are regulated extrinsically by interaction with factors that stabilize or destabilize microtubules. Extrinsic regulation of microtubules is mostly due to the numerous MAPs (microtubule-associated proteins) that bind to microtubules (de Forges H., Bouissou A, Perez F, 2012), microtubule destabilizing factors, microtubule severing proteins and microtubule-based motors of the kinesin and dynein superfamilies (Conde C, Caceres A, 2009).

MAPs such as Tau, MAP2 and MAP4, promote microtubule stability by binding to microtubule surface (Heald and Nogales, 2002). Kinesin and dynein superfamily proteins convey their cargoes along the microtubule (Hirokawa, 1998). Microtubule stabilizing proteins, proteins that promote polymerization or depolymerization and microtubule severing proteins in neurons are schematically shown in Figure 1.3.

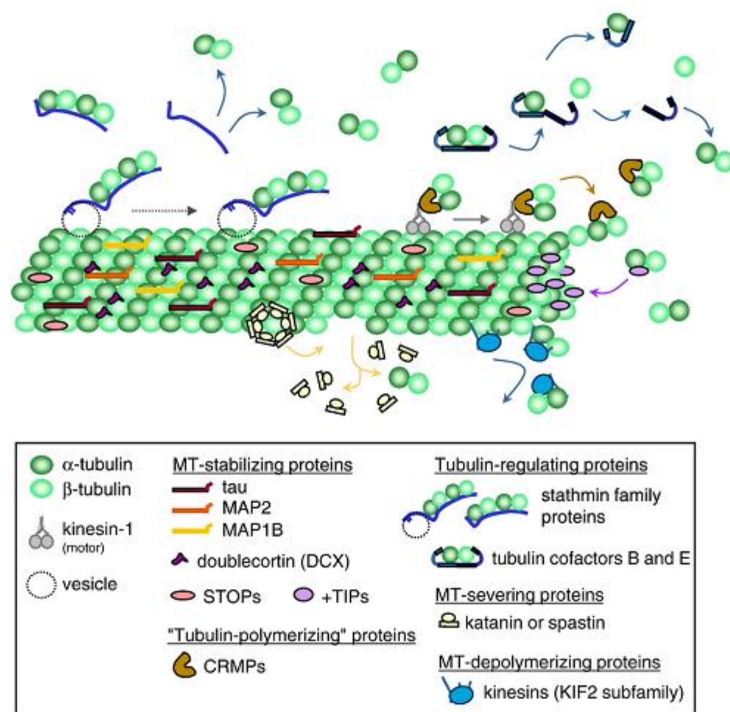

Figure 1.3: Microtubule regulating proteins (Poulain F.E, Sobel A, 2010).

1.3 Microtubule Severing

Beside the dynamic instability, growth and shrinkage of microtubule plus end, or treadmiling, which is described as loss of tubulin subunits from minus end while addition at the plus end, there exists another mechanism, “cut and run” model: in which microtubules can be broken or severed along their length into short pieces in cells such as the reconfiguration of microtubules that have already been stabilized by factors that bind to the surface or the ends of the microtubules, is required for mobility of microtubules (Quarmby L, 2000; Baas P.W, Karabay A. and Qiang L, 2005)

Microtubules are thus essential structures whose nucleation, stabilization and dynamics regulate the formation and restructuring of a complex network within cells (Poulain F.E, Sobel A, 2010). In mitotic cells, microtubule severing on the spindles is thought to contribute to spindle reorganization (Ahmad and Baas, 1995). Different from mitotic cells, in neurons, microtubules are not used for the formation of a mitotic spindles, but rather they form dense parallel arrays (bundles) in axons and dendrites that are required for the elongation and maintenance of these neurites (Conde C, Caceres A, 2009; Karabay et al., 2004). Neuronal microtubules are nucleated at the centrosome, rapidly released by the action of the microtubule severing proteins, and then transported as short polymers into neurites by molecular motors (Yu, W., Centonze, V. E., Ahmad, F. J. & Baas, P. W, 1993; Vale, R. D, 2003; Hirokawa, N. & Noda, Y., 2008).

Elongation, branching, navigation, retraction of axons are accomplished by changes in the configuration of microtubules (Baas and Buster, 2004). It has been shown that motor proteins are essential for transporting microtubules into the axon in neurons (Ahmad et al., 1998) and the length of the microtubule is inversely proportional to its movement speed, the shorter the microtubule is the more rapid is the movement (Dent et al., 1999).

To cut microtubules into smaller pieces microtubule-severing enzymes are required. Up to now three microtubule-severing enzymes, belonging to the AAA (ATPases Associated with diverse cellular Activities) family of ATPases, have been identified: katanin, spastin and fidgetin whose functions are essential for cellular processes such as mitosis, cilia biogenesis, deflagellation, and neurogenesis (Casanova et al., 2009; Roll-Mecak, A. and F. J. McNally, 2010).

1.4 Katanin

The first report of biochemical microtubule severing activity came from observations using Xenopus oocyte extracts (Vale, 1991). After that, katanin was purified from sea urchin eggs and its name derived from katana, which means the samurai sword in Japanese (McNally and Vale, 1993). Katanin was the first microtubule severing protein to be identified, sequenced, and characterized (Baas P.W., Sudo H., 2010).

Katanin is a microtubule-severing protein proposed to be involved in mitosis, neuronal differentiation and flagellar physiology (McNally F., 2000). Katanin is heterodimer composed of two subunits, p60, which is derived from *KATNA1* mRNA, containing the AAA ATPase domain which requires ATP hydrolysis to sever microtubules and the non-enzymatic p80, which is encoded from *KATNB1* gene, containing the N-terminal WD40 repeat domain, a central proline-rich domain and a C-terminal domain (McNally et al., 2000; Hartman *et al.* 1998).

Like the motor proteins kinesin and dynein, katanin's ATPase activity is stimulated by microtubules, and katanin uses ATP hydrolysis to disassemble microtubules at internal sites. Katanin p60 is thought to oligomerize into a hexamer and form a ring that wraps around the microtubule and generates the torque needed to break its lattice (Figure 1.4) (Poulain F.E, Sobel A, 2010).

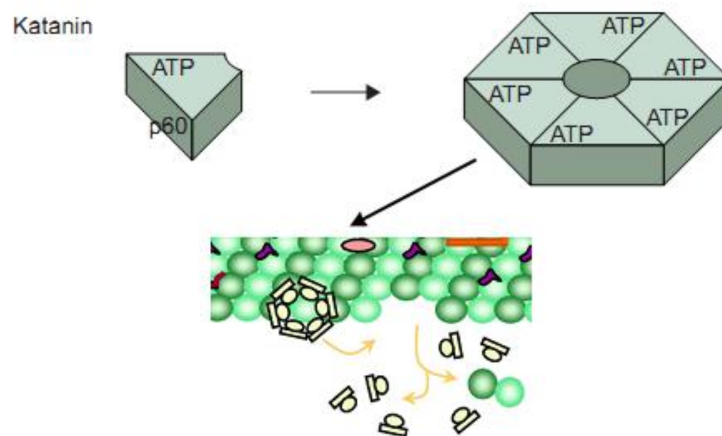

Figure 1.4: Katanin p60 oligomerization (McNally F., 2000; Poulain F.E, Sobel A, 2010).

As seen in Figure 1.5 (red sword handles represent ATP; blue sword handles represent ADP) after ATP binding, katanin oligomerization is triggered and katanin assemble on the wall of the microtubule. When ATP hydrolyzed and phosphate

released, katanin undergoes a conformational change leading to destabilization of tubulin dimers (Quarmby L., 2000).

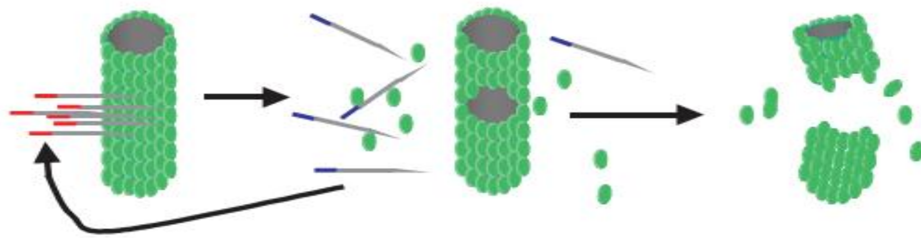

Figure 1.5: Microtubule severing by katanin (Quarmby L., 2000).

Instead of having microtubule-severing properties, p80 is thought to target the p60 subunit to the centrosome and controls the microtubule severing activity of p60 via dimerization with C-terminal domain of P80 (Figure 1.6) (McNally et al., 2000). In addition, p80 enhances the microtubule severing capacity of p60 subunit (Ahmad *et al,* 1999). WD40 domain of p80 localizes p60 to centrosomes instead of having a role in dimerization (Hartman et al., 1998).

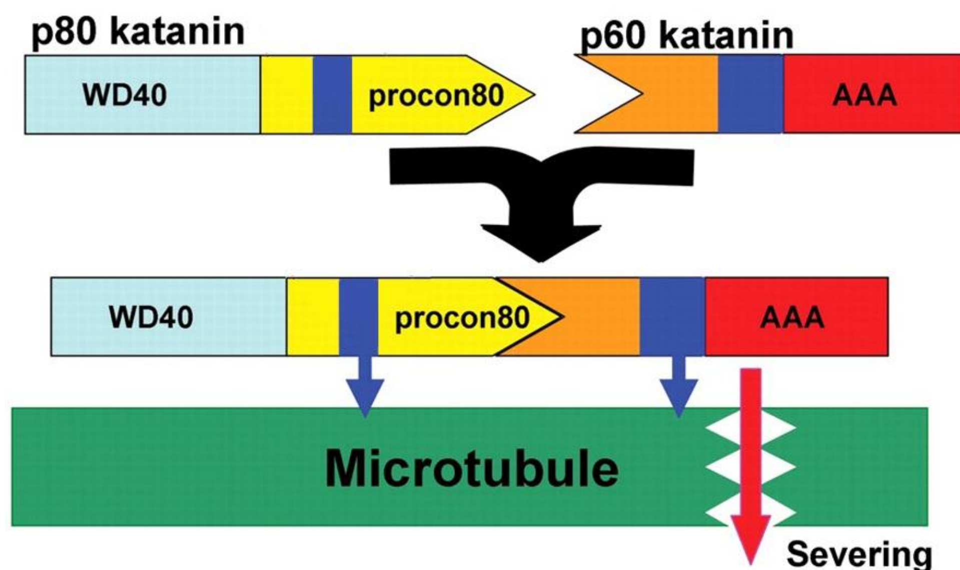

Figure 1.6: The interaction of p60 and p80 katanin (Sudo H. and Maru Y.; 2008).

Katanin is ubiquitously expressed and it is highly detected in the nervous system during periods of axonal growth. It is distributed throughout cytoplasm and centrosomes. In neurons, a large number of non-centrosomal microtubules are required for growth and maintenance of neuronal processes (Ahmad et al., 1999). Inhibition of katanin increases microtubule length and impairs axon growth and also enhances accumulation of microtubules at centrosomes, demonstrating that katanin is essential for releasing microtubules from neuronal centrosome and microtubule

severing and release from the centrosome are essential for axon elongation (Karabay et al., 2004) However, katanin p60 overexpression also impairs axon formation (Yu et al., 2005).

A study conducted on transgenic mice model of Alzheimer's disease (AD) revealed that katanin levels in the cortex and hippocampus of the transgenic mice was decreased compared to non-transgenic normal mice (Nichols et al., 2008). In the model for microtubule-based axonal degeneration in Alzheimer's disease, it has been suggested that hyperphosphorylation of tau which is observed in AD cases causes destabilization of microtubules and thus, microtubules are more accessible to severing proteins such as katanin (Baas and Qiang, 2005).

1.5 Spastin

The 68 kDa microtubule-severing enzyme, spastin was originally identified with mutations in its gene *SPG4,* the defective gene in hereditary spastic paraplegia (HSP), cause the disease characterized by the axonal degeneration of spinal neurons leading to weakness and spasticity of the lower limbs (Hazan et al., 1999). It consists of 616 amino acid (aa) and has two major domains: a microtubule-interacting and endosomal trafficking (MIT) domain at the N terminus and an AAA domain at the C terminus (Figure 1.7) (Salinas et al. 2007).

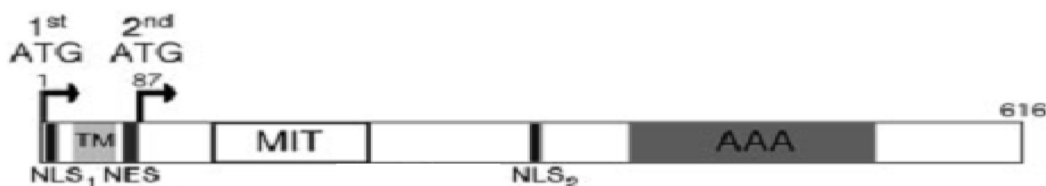

Figure 1.7: Structural organization of spastin.

The MIT domain is responsible for the association of spastin with microtubules (Takasu et al., 2005). The AAA domain consists of approximately 220 amino acids and is responsible for the ATPase activity of the protein (Evans et al., 2005). Moreover, spastin includes two functional nuclear localization signals (NLS), responsible for targeting to the nucleus and nuclear export signals (NES) (Beetz et al., 2004; Claudiani et al., 2005).

By function of its NLS, spastin can localize into nucleus and experimentally in different cell types nuclear localization of spastin was detected. Distribution of spastin whether in cytoplasm or nucleus, is regulated through two mechanisms; the usage of alternative translational start sites and active nuclear export to the

cytoplasm. Thus, *SPG4* mRNA is able to synthesize two spastin isoforms, 68 and 60 kDa, by using two different translational sites (Claudiani et al., 2005).

Like other members of the AAA family, spastin tends to assemble into ring-shaped oligomers. As katanin does, spastin can form hexamers only in the presence of ATP and they can bind their substrate with high affinity only in this oligomeric state (Roll-Mecak A, Vale RD, 2008).

With X-ray crystallography and solution light and X-ray scattering coupled with atomic docking, the active spastin hexamer displaying the ring structure common to AAA ATPases, has been revealed. The hexamer has a prominent central pore and six radiating arms that may be used by the enzyme to dock onto the microtubule. Residues that are important for microtubule severing are shown in red in Figure 1.8 (Roll-Mecak A, Vale RD, 2008).

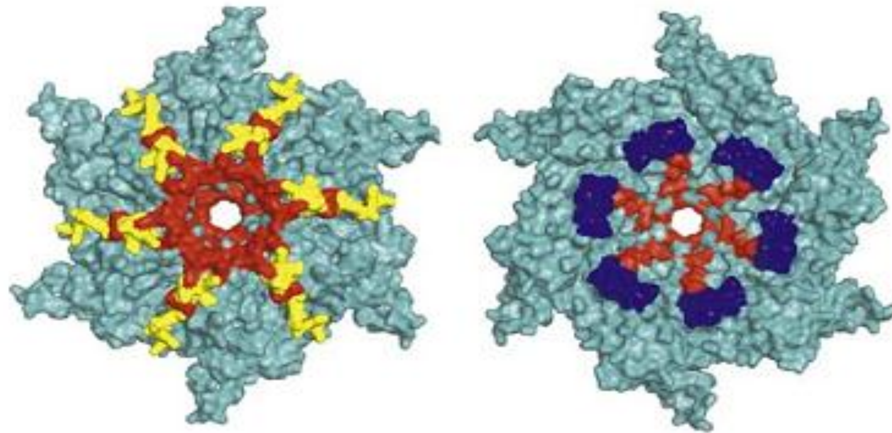

Figure 1.8: Molecular surface of the spastin hexamer.

In cells overexpressing wild-type spastin, disrupted microtubule network was observed. Furthermore, in cells overexpressing spastin with mutations in the AAA domain the protein bound constitutively to MT and altered MT arrays (Errico et al., 2002). By *in vivo* experiments, it has been shown that downregulation of spastin by RNA interference (RNAi) caused morphologic undergrowth, reduced synaptic area, and increased synaptic microtubules. Conversely, overexpression of spastin reduced the synaptic strength and amount of synaptic microtubules (Salinas et al. 2007).

1.6 ELK-1

Elk-1, a member of the Ets (E twenty-six) oncogene family of transcription factors, is able to contribute both activation and repression of target gene expression (Sharrocs, 2001; Buchwalter et al, 2004).

The Ets family transcription factors have a highly conserved ETS DNA-binding domain (DBD) of ~85 amino acids that mediate binding to a DNA target (Mo et al. 2000; Besnard et al. 2011). The ETS domain (A domain) of Elk-1 contains a nuclear localization signal (NLS) as well as a nuclear export signal (NES). A short neuronal isoform of Elk-1, sElk-1, a protein lacking the first 54 amino acids of the Ets DNA binding domain (Vanhoutte et al., 2001; Buchwalter et al. 2003). The B box domain enables TCFs to interact co-operatively with SRF and promotes ternary complex formation (Besnard et al., 2011). The D domain is short MAP kinase docking motif and C domain at the C-terminal of protein is transcriptional activation domain (Yang et al 2002). The R domain contains lysine residues which are target for SUMO (Small Ubiquitin-like Modifier) modification and SUMOylation is required for repressive activity of Elk-1 and can also regulate intracellular localization of Elk-1 (Figure 1.9) (Yang et al 2003).

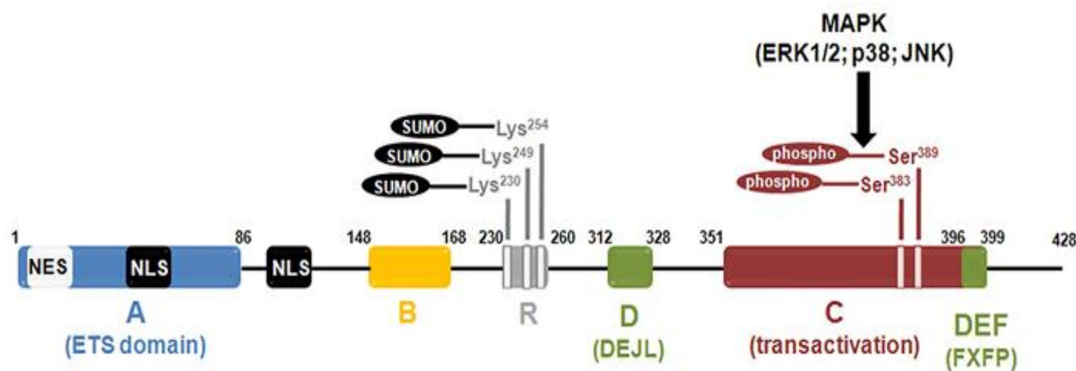

Figure 1.9: Functional domains and major post-translational modifications of the Elk1 protein.

1.7 YY1

Transcription factor YY1 (Yin Yang 1), belongs to the GLI-Kruppel class of zinc finger proteins, is ubiquitously expressed in different tissues including brain, heart, limb and immune system. YY1 is a multifunctional protein that can activate or repress gene expression acting on diverse number of promoters (He Y. and Casaccia-Bonnefil P., 2008; Thomas and Seto, 1999). At its C-terminal, YY1 contains four C_2H_2-type zinc fingers, responsible for the specific DNA-binding activity with the consensus sequence 5'-(C/g/a)(G/t)(C/t/a)CATN(T/a)(T/g/c)-3'. Histidine-rich region flanked by acidic amino acids by the N-terminal region, serves as transcriptional activation domain. The central glycine and lysine rich region interacts with histone deacetylase complex (HDAC), and together with C-terminus it forms

the transcriptional repression domain. Another region within the central part is PHO homology region (amino acids between 205 and 226), which interacts with the homeobox proteins (Figure 1.10) (He Y. and Casaccia-Bonnefil P., 2008).

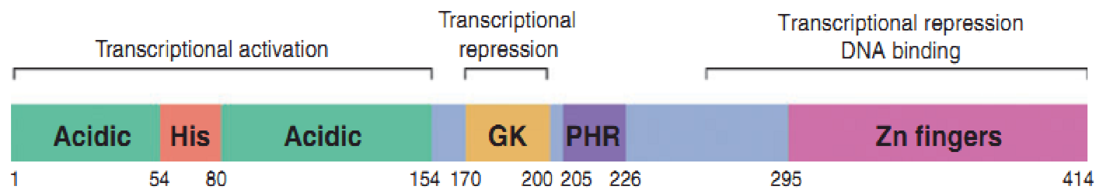

Figure 1.10: Schematic diagram of human Yin Yang 1 (YY1) protein.

YY1 mRNA was identified in numerous tissues in mouse so its expression is thought to be ubiquitous and it is estimated that more than 7% of all vertebrate gene promoters contain at least one YY1 consensus binding site. Therefore it may be involved in expression control of a vast array of genes (Shi et al. 1997; Ohtomo, 2007).

It has been shown in vitro that YY1 directs and initiates transcription when bound to initiator (Inr) elements. YY1 can function like TATA-binding protein (TBP), and it binds to the core promoter and recruits RNA polymerase to the initiation complex. Experimentaly, physical interaction between YY1 and TFIIB and the large subunit of RNA polymerase II was identified and it was sufficient to direct basal transcription of a supercoiled plasmid template in vitro (Shi, 1997).

Three general models for both YY1 mediated activation and repression have proposed (Figure 1.11.a and b respectively). For activation, in the first model, it has been proposed that YY1 stimulates transcription by directly interacting with general transcription factors. In this mechanism, it is most likely that YY1 uses an activation domain. In the second model, it has been proposed that interacting with other proteins YY1 can activate a promoter, which was repressed by YY1 in the absence of those factors. It is likely that this regulation is achieved by anti-repression and/or the unmasking of an activator domain. In the third model, it has been proposed that YY1 recruits a coactivator which modifies or interacts with other transcription factors. In this mechanism, chromatin modification is also likely to achieve an open or accessible chromatin state (Thomas, 1999).

For repressive effect, in the first model, it has been proposed that repression is achieved by displacement of an activator by YY1 through overlapping DNA binding

sites or possibly through some other mechanisms (such as bending of the DNA). In the second model, it has been proposed that YY1 prevents activator function by interfering with the function of other critical transcription factors, which may be activators or general transcription factors. This could also be achieved by squelching of coactivators. In the third model, it has been proposed that YY1 recruits a coactivator, which either negatively interacts with other factors or modifies them. This could also be achieved through chromatin modification to achieve a closed chromatin state (Thomas, 1999).

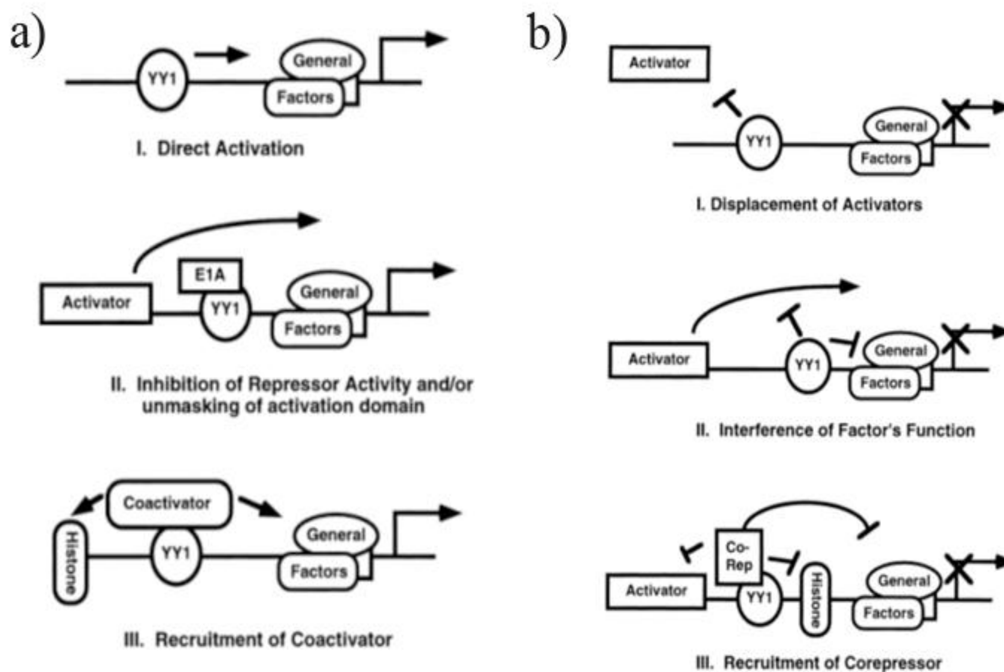

Figure 1.11: Models of YY1 mediated activation and repression (Thomas, 1999).

The potential function of YY1 in the developing nervous system was suggested by the phenotypic analysis of yy1+/− mice, indicating an early embryonic lethality which restained the analysis of later developmental stages caused by complete ablation of yy1 in mice (Donohoe *et al.* 1999). The role of YY1 in late neuronal differentiation has been suggested by the evidence that this molecule positively regulates the basal levels of expression of dopamine beta-hydroxylase (DBH) in noradrenergic neurons (which catalyzes dopamine to noradrenaline), by binding to specific sites in the promoter (He and Casaccia-Bonnefil, 2008). YY1 might also play a role in the neurological diseases such as AD. YY1 acts as an activator of the BACE1 (beta-site amyloid precursor protein-cleaving enzyme 1) promoter (Nowak

et al., 2006) in which its gene product cleaves Aβ proteolytically from amyloid precursor protein (APP) in neurons and astrocytes (Rossner et al., 2006). Moreover, mutations of the YY1 binding site in the BACE1 promoter decrease its activity, while YY1 over-expression increases its transcriptional activity (Nowak et al. 2006).

1.8 Aim of the Study

Optimal promter regions of *KATNB1* and *SPG4* were previously identified by our laboratory. According to deletion constructs, S2 construct containing the 5' upstream region -921/-221 of *SPG4* gene was selected as optimal promoter because of higher promoter activity (Figure 1.12). In the same way, F2 construct containing the region -892/-375 of *KATNB1* gene was chosen as optimal promoter (Figure 1.13). In this study, in order to understand transcriptional regulation of human *KATNB1* and *SPG4*, primarily Elk-1 and YY1 binding sites on *KATNB1* and *SPG4* gene promoters will be identified. To check specificity of their binding, recombinant YY1 and Elk-1 proteins, which contain only DNA binding regions and a 6X Histidine tag (6XHis-tag), will be produced in E. coli and purified via His-tag. Finally, regulation of these promoters by YY1 transcription factor will be investigated using Dual-Luciferase Reporter system.

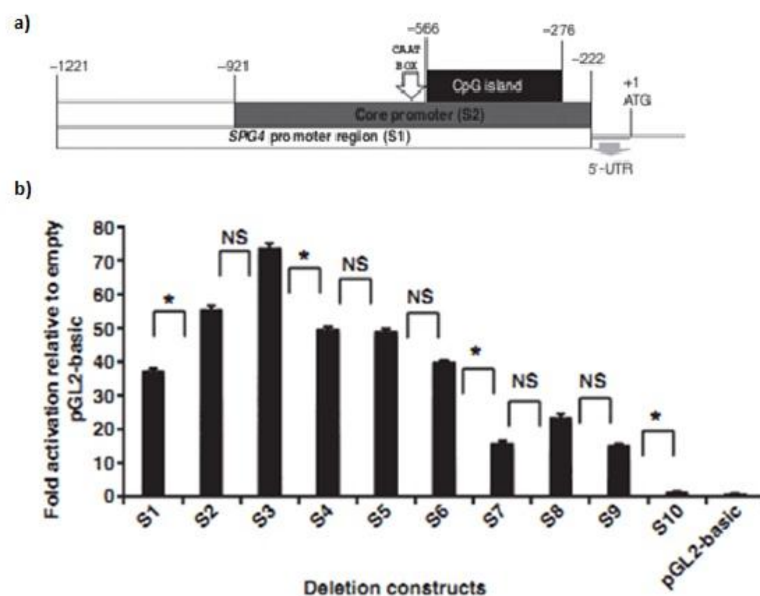

Figure 1.12: Schematic representation of *SPG4* gene promoter (a) and deletion constructs analysis (b).

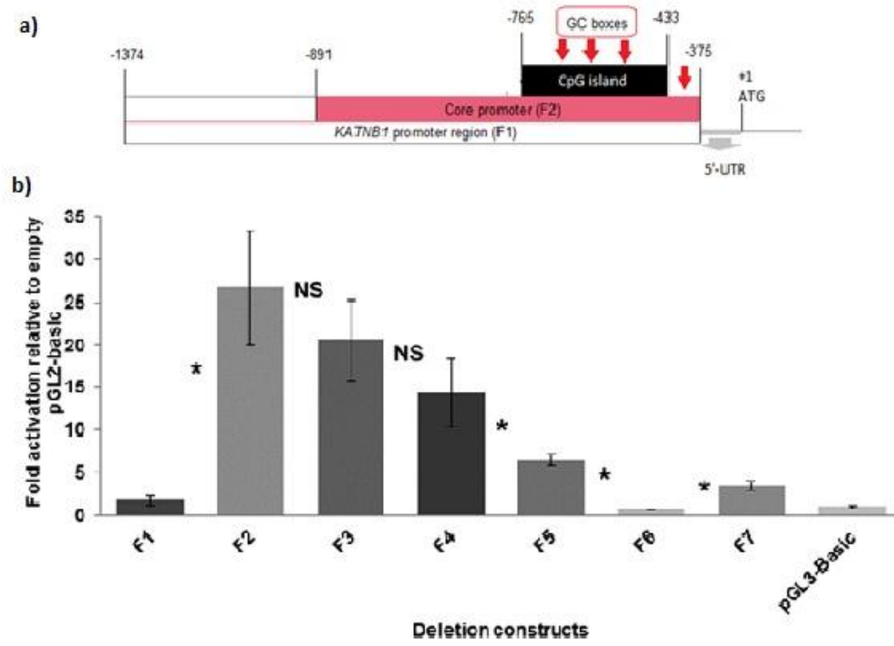

Figure 1.13: Schematic representation of *KATNB1* gene promoter (a) and deletion constructs analysis (b).

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Lab equipment

The equipment used in the study are given in the Table 2.1:

Table 2.1: Laboratory equipment used in the study

Equipment	Supplier Company
Centrifuges	Biolab SIGMA 6K15, Beckman Coulter Microfuge®18, Beckman Coulter Avanti™ J-30 I, IECCL10 Centrifuge, Thermo Electron Corporation, Labnet, Labnet International C1301-230V
DNA sequencer	Applied Biosciences 3100 Avant
High pressure steam sterilizer	TOMY SX-700E
Magnetic stirrer	Labworld Standard Unit
pH Meter	Mettler Toledo MP220
Precision Weigher	Precisa 620C SCS
Weigher	Precisa BJ 610 C
UVIPhoto MW Version 99.05 for Windows 95 & 98	UVItec Ltd.
UV Transilluminator	Biorad UV Transilluminator 2000
Electrophoresis equipments	ThermoEC MiniCell® PrimoTM EC320 Electrophoretic Gel System
Power supply	EC250-90 Apparatus Corporation
Microwave	Arçelik MD582
Ice machine	Scotsman AF 10
Vortex	Heidolph, Reaxtop
Water Baths	Memmert, Elektro-mag M 96 KP
Thermomixer	Eppendorf Thermomixer Comfort
Laminar air flow cabinets	FASTER BH-EN 2003
Incubator with CO_2	Biolab SHEL LAB
UVIPhoto MW Version 99.05 for Windows 95 & 98	UVItec Ltd.
Inverted light microscope	Olympus CK40
Thermal Cycler	Techne TC-3000
SDS-PAGE gel electrophoresis system	BIO-RAD MiniProtean
Power supply	Thermo Electron Corporation EC250-90
Shaker	Forma Orbital Shaker, Thermo Electron Corporation
Freezers	UĞUR (-20°C), UĞUR (+4 ^{o}C), New Brunswick Scientific (-80 ^{o}C)
UV-Visible Spectrophotometers	uv-1700 PharmaSpec Shimadzu, Thermo scientific NanoDrop 2000

Table 2.1 (contd.) : Laboratory equipment used in the study

Microplate Spectrophotometer	BIORAD Benchmark Plus
Pure water system	TKA Wasseraufbereitungssysteme
Mini Blot Module	Thermo Electron Corporation EC140
Syringe filters	0.22µm, TPP
Vacuum filtration- system	150ml, TPP
Pipettes	2.5µl, 10µl, 100µl, 200µl, Eppendorf 1000µl, Finnpipette Thermo
Electronic pipette	Finnpipette Thermo
Microfuge tubes	1.5ml, 2ml, Axygen
Centrifuge tubes	15ml, 50ml Avant Plus
Tissue culture test plates	6 well, 12 well, 96 well, TPP
Tissue culture flasks	25 cm^2, 75cm^2 TPP
Cell Scraper	30cm, TPP
Hemacytometer	Fisher Scientific
Injectors	2ml, inject, 1ml, tuberculin
Serological pipette	TPP
Nitrocellulose membrane	Roche
3MM Whatman Filter Paper	Whatman

2.1.2 Chemicals and enzymes

Chemicals and enzymes used in the study are shown in the Table 2.2:

Table 2.2 Chemicals and enzymes

Material	**Supplier Company**
BamHI restriction enzyme XhoI restriction enzyme KpnI restriction enzyme EcoRI restriction enzyme SalI restriction enzyme MassRuler™ DNA Ladder (Mix, 80bp-10Kb) Mass Ruler Low Range DNA Ladder (80-1031 bp) 10 mM dNTP mix 2 mM dNTP mix 10 X Pfu DNA polymerase Buffer (with MgCl2) 10X Tango Y buffer 25 Mm $MgCl_2$ 10X Y+ Tango buffer 6X Loading dye	Fermentas
T4 DNA Ligase Buffer T4 Ligase	Roche
SeeBlue Plus2 Prestained Protein Ladder	Invitrogen
Primer T7 promoter Primer T7 terminator	Integrated DNA Technologies
IPTG TEMED	AppliChem
$NaH_2PO_4.2H_2O$ KH_2PO_4 K_2HPO_4 NaAc	J.T. Baker
Tryptone	Lab M ™
Isopopanol Glycerol PEG 8000 Coomassie Brilliant Blue	Fluka

Table 2.2 (contd.): Chemicals and enzymes

Ampicillin Kanamycin Albumine, bovine (BSA) N,N'- Dimethyl- bis- Acrylamide 4-Nitrophenyl phosphate di (tris) salt	Sigma
Anti-His penta monoclonal Mouse antibody 50% Ni-NTA agarose suspension	Qiagen
Tris Base Acrylamide	BDH Laboratory
DMSO Absolute methanol Absolute ethanol	Riedel- de Haën
Boric acid APS $CaCl_2.2H_2O$ EDTA SDS Glucose Ethidium bromide Yeast Extract Agar KCl Imidazole PEG 4000 DTT HCl Bromophenol blue	Merck
$MgCl_2.6H_2O$ NaCl	CARLO ERBA
Skimmed milk powder	OXOID
DMEM- High Glucose- Liquid Media (SH30243)	HyClone
FBS	Biochrom

2.1.3 Commercial kits

Commercial kits used in this study are given in the Table 2.3:

Table 2.3: Commercial kits

Kit	**Supplier Company**
QIAquick PCR purification kit	Qiagen, 28104
QiaPrep Spin Miniprep Kit	Qiagen, 27106
Big Dye Terminator v 3.1 Cycle Sequencing Kit	Applied Biosystems
EndoFree Plasmid Maxi Kit	Qiagen 12362
SDS Gel Preparation Kit	Fluka 08091
QIAquick Gel Extraction Kit	Qiagen, 28706
Dual-Luciferase Reporter Assay System	Promega, E1910
Transfast Transfection Reagent	Promega, E2431
LightShift Chemiluminescent EMSA Kit	Thermo Scientific 20148
Chemiluminescent Nucleic Acid Detection Module Kit	Thermo Scientific 89880
Biotin 3' End DNA Labeling Kit	Thermo Scientific 8918
QuikChange II Site-Directed Mutagenesis Kit	Agilent Technologies 200523

2.1.4 Buffers and solutions

2.1.4.1 TBE buffer (10X)

10X TBE buffer was used in agarose gel electrophoresis to prepare the agarose gel that DNA was loaded on and as the tank buffer. 10X TBE buffer was used by diluting to 0.5X TBE buffer with dH_2O. Preparation of 10x TBE is shown in Table 2.4

Table 2.4: Preparation of 10X TBE buffer

Content	Concentration	Amount
Tris Base	890 mM	108 g
Boric Acid	890 mM	55 g
EDTA	20 mM	5.84 g
H_2O		1 L

2.1.4.2 TAE Buffer (50X)

50X TAE buffer was used in agarose gel electrophoresis to prepare the agarose gel that DNA was loaded on and as the tank buffer. 50X TAE buffer was used by diluting to 1X TAE buffer with dH_2O. Preparation of 50x TAE is shown in Table 2.5.

Table 2.5: Preparation of 10X TBE buffer

Content	Concentration	Amount
Tris Base	2 M	242 g
Glacial Acetic Acid		57.1 mL
EDTA	50 mM	14,6 g
H_2O		1 L

2.1.4.3 Protein purification buffers

Protein purification buffers were used for purifying proteins from bacterial pellet by metal affinity chromatography. Buffer types and their preparations are given below:

Lysis buffer: 78 miligram (mg) $NaH_2PO_4.2H_2O$ (50mM), 175.4 mg NaCl (300mM) and 6.8 mg imidazole (10 mM) were dissolved in 10 ml distilled water (dH_2O) and pH was adjusted to 8.0 using NaOH.

Wash buffer: 78 mg NaH2PO4.2H2O (50 mM), 175.4 mg NaCl (300 mM) and 13.6 mg (20mM) were dissolved in 10 ml distilled water (dH_2O) and pH was adjusted to 8.0 using NaOH.

Elution buffer: 39 mg NaH2PO4.2H2O (50 mM), 87.7 mg NaCl (300 mM) and 2.72 gram (g) (4 M) were dissolved in 5 ml distilled water (dH_2O) and pH was adjusted to 8.0 using NaOH.

2.1.4.4 Buffers and solutions for SDS-PAGE analysis

2X sample buffer: 2X sample buffer was used to denature protein samples which were loaded on SDS- polyacrylamide gel. Preparation of 2X sample buffer is shown in Table 2.6.

Table 2.6: Preparation of 2X sample buffer

Content	Concentration	Amount
Tris- HCl pH:6.8	0.125 M	2.5 ml (of 0.5 M)
SDS	4 %	4 ml (of 10%)
Glycerol	20 %	2 ml (of 100 %)
Bromophenol blue	0.05 %	5 mg
DTT	0.15 M	231 mg
dH_2O		up to 10 ml

Tris-Tricine anode buffer (pH: 8.9): Tris-Tricine anode buffer was used to reduce pH of the solution changes. Preparation of Tris-Tricine anode buffer is shown Table 2.7.

Table 2.7: Preparation of Tris-Tricine anode buffer

Content	Concentration	Amount
Tris Base	0.2 M	24,22 g
dH_2O		up to 1 liter

Tris-Tricine cathode buffer (pH: 8.25): Tris-Tricine anode buffer was used to reduce pH of the solution changes. Preparation of Tris-Tricine cathode buffer is shown Table 2.8.

Table 2.8: Preparation of Tris-Tricine cathode buffer

Content	Concentration	Amount
Tris Base	0.1 M	12,11 g
Tricine	0.1 M	17,92 g
SDS	0.1 %	1 g
dH_2O		up to 1 liter

Gel Buffer (pH: 8.45): Preparation of gel buffer is shown Table 2.9.

Table 2.9: Preparation of gel buffer

Content	Concentration	Amount
Tris Base	3 M	182 g
SDS	0.3 %	1,5 g
dH_2O		up to 500 ml

Stacking Acrylamide: Preparation of gel buffer is shown Table 2.10.

Table 2.10 : Preparation of stacking acrylamide

Content	Concentration	Amount
Acrylamide	48 %	48 g
Bis-acrylamide	1.5 %	1.5 g
dH_2O		up to 100 ml

Seperating Acrylamide: Preparation of gel buffer is shown Table 2.11.

Table 2.11 : Preparation of seperating acrylamide

Content	Concentration	Amount
Acrylamide	46.5 %	46.5 g
Bis-acrylamide	1.5 %	1.5 g
dH_2O		up to 100 ml

Coomassie Brilliant Blue (CBB) stain solution: CBB stain solution was used to visualize separated protein bands on SDS polyacrylamide gel. Preparation of CBB stain solution is shown below:

Table 2.12: Preparation of CBB stain solution

Content	Concentration	Volume
CBB R-250	0.1 %	0.5 g
Methanol	45 %	450 ml
Acetic acid	10 %	100 ml
dH_2O	45%	450 ml

Destain solution: Destain solution was used to visualize protein bands clearly by removing the background on SDS polyacrylamide gel. Preparations of CBB destain solution is shown below:

Table 2.13: Preparation of destain solution

Content	Concentration	Volume
Methanol	45 %	450 ml
Acetic acid	10 %	100 ml
dH_2O	45 %	450 ml

2.1.5 Bacterial strains

Escherichia coli (E.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], Invitrogen.

Escherichia coli strain BL21 (DE3)pLysS F^- *dcm ompT hsdS*(rB^- mB^-) *gal* (DE3)[pLysS Cam^r], Novagen.

2.1.6 Bacterial culture media

LB medium was prepared by dissolving 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1 liter distilled water. The media was sterilized by autoclaving at 121°C for 10 minutes (min). After sterilization, antibiotic was added to the LB medium according to the concentration mentioned in Table 2.12 in order to make selection media.

LB- agar plate was prepared by adding 15 g/L of agar to LB medium and sterilized autoclaving as describe above.

SOC medium was used to cultivate *E. coli* cells for 1 hour after heat shock during transformation. It was prepared by dissolving 2 g tryptone, 5 g yeast extract, 0.058 g NaCl, 0.0186 g KCl, 0.095 g $MgCl_2$, 0.23 $MgSO_4$ and 0.36 g glucose in 100 ml distilled water and sterilized at 121 °C with autoclaving for 10 min.

Table 2.14: Stock and working solutions of antibiotics

Antibiotic	Stock Solution Concentration	Working Solution Concentration
Kanamycin	30 mg/ml in distilled water	30 µg/ml
Ampicilin	50mg/ml in distilled water	50 µg/ml

2.1.7 Expression vectors

2.1.7.1 pET-30a vector

The pET-30a (+) vector carries an N-terminal His•Tag®/thrombin/S•Tag™/ enterokinase configuration plus an optional C- terminal His•Tag sequence. The circular map (Fig. 2.1) and the cloning/expression region (Fig. 2.2) are shown below. The vector is 5422 bp long.

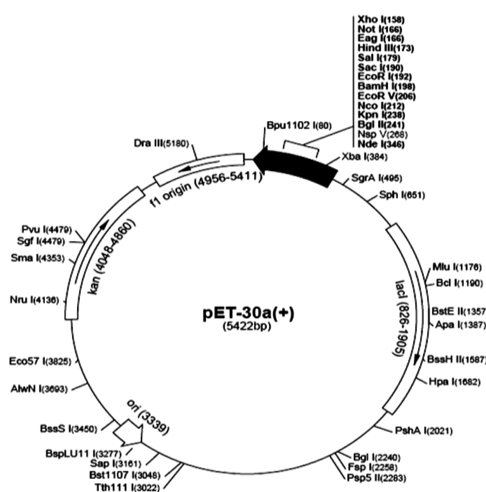

Figure 2.1: Vector map of pET30a (Url-3).

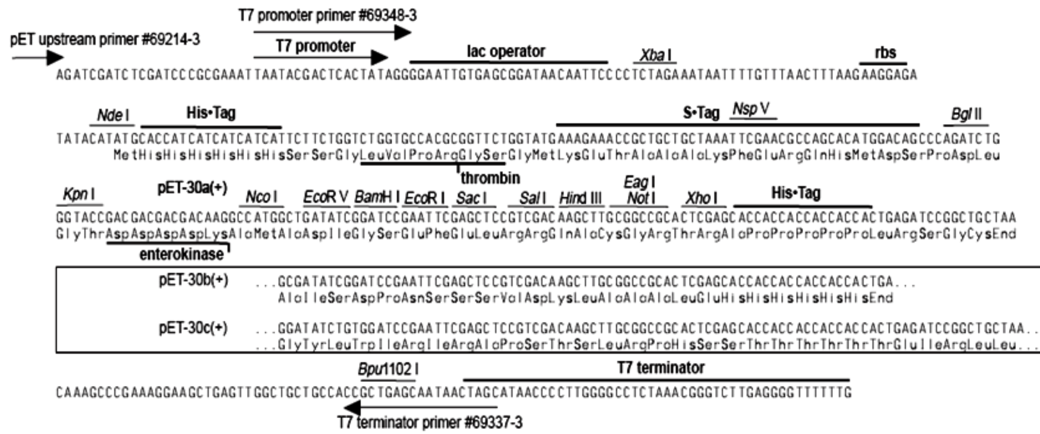

Figure 2.2: Multiple cloning site of pET30a (Url-4).

2.1.7.2 pCMV6 vector

The pCMV6 mammalian expression vector consists of the promoter-enhancer region of the major immediate early gene of the human cytomegalovirus, a synthetic copy of the translational enhancer from the alfalfa mosaic virus 4 RNA, a synthetic polylinker sequence containing unique cleavage sites for 13 restriction enzymes, the transcription termination and polyadenylation region of the human growth hormone gene, and the SV40 virus DNA replication origin and early region enhancer from plasmid pcD-X. After introduction into mammalian cells, the vector is designed such that transcription originating from the strong cytomegalovirus promoter will traverse the translational enhancer and polylinker sequences and terminate in the human growth hormone gene region (Andersson,S. et al, 1989). The circular map (Fig. 2.4) is shown below. The vector is 4665 bp long.

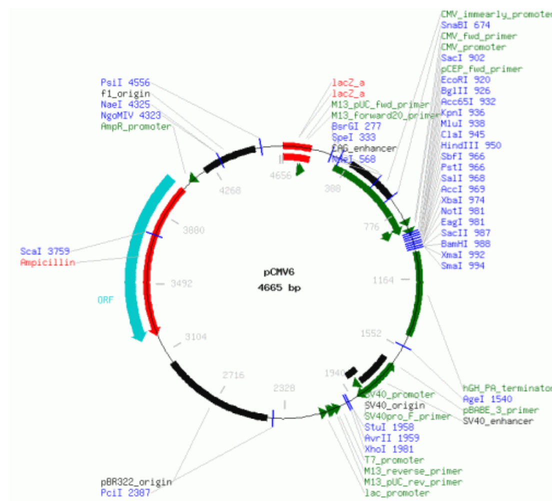

Figure 2.3 Vector map of pCMV6 (Url-2)

2.1.8 Cell culture media

SH-SY5Y Culture Medium

To prepare SH-SY5Y culture medium, DMEM low glucose was supplemented with 10% FBS, 2 mM L-Glutamine and 1X Penicilin/Streptomycin and then filter sterilized with 0,2 µm filter.

SH-SY5Y Freezing Medium

To prepare SH-SY5Y freezing medium, SH-SH5Y culture medium was supplemented with 5% DMSO and then filter sterilized with 0,2 µm filter.

2.1.9 Cell Lines

In this study, SH-SY5Y Human metastatic neuroblastoma cell line was was used. Those cells are clonal subline of the neuroepithelioma cell line SK-N-SH that had been established in 1970 from the bone marrow biopsy of a 4-year-old girl with metastatic neuroblastoma.

2.2 Methods

2.2.1 Cloning studies

2.2.1.1 Primer design

In order to clone desired nucleotide sequence of target DNA, primers with appropriate flanking restriction sites were designed to allow ligation into appropriate vectors. During primer design, melting temperatures of primers were selected as close as possible to each other to apply the optimum annealing temperature. GC content of primers was selected as much as ideal and hairpin and self-dimer formation were checked by IDT Scitools oligo analyzer (Url-1). To introduce mutation to convert YY1 residue lysine 288 to arginine, mutation primers were designed using Quick Change Primer Design tool (Url-2).

Synthesized primers with their properties are given below:

Table 2.15: YY1 and Elk-1 constructs' primers

Primer	Sequence	Length	Tm	GC content
Elk1-db_F	AAGGATCCATGGACCCATCTGTGACGCT	28	64.7°C	53.6 %
Elk1-db_R	AACTCGAGAGTGGAGCACCCTGCGA	25	65.5°C	60.0 %

Table 2.15 (contd): YY1 and Elk-1 constructs' primers

YY1-woa_F	ATAAGGTACCGGCAAGAGCGGCGGCG	26	68.3 °C	65.4 %
YY1-wor1_F	CCGAATTCATGGCCTCGGGCGACACC	26	67.2 °C	65.4 %
YY1-wor1_R	AAAGGTACCGGCCGCCGCCACGGTGA	26	71.2 °C	69.2 %
YY1-wor2_F	AAGGTACCAAGAAGTGGGAGCAGA	24	59.8 °C	50.0 %
YY1-wor2_R	AAGGATCCTCACTGGTTGTTTTTGGC	26	59.7 °C	46.2 %
YY1-db_F	CAGAATTCACAATAGCTTGCCCTCATAAAGG	31	59.3 °C	41.9 %
YY1-db_R	AAGTCGACTCACTGGTTGTTTTTGGCCTTA	30	61.7 °C	43.3 %

Table 2.16: YY1-K288R mutation primers

Primer	**Sequence**	**Length**	**Tm**	**GC content**
YY1-K288R-F	gaatttgctagaatgaagccaagaaaaattagagaagatgatgctcca	48	79.2°C	35.4 %
YY1-K288R-R	tggagcatcatcttctctaatttttcttggcttcattctagcaaattc	48	79.2°C	35.4 %

2.2.1.2 PCR

In order to amplify desired DNA products, WT Elk1 or YY1 containing pCMV6 vectors were used. Following PCR reaction and PCR program were applied.

Table 2.17: PCR reaction set up for cloning YY1 and Elk1 constructs

Content	**Volume**
Template vector	50 – 100 ng
Forward primer (25 µM)	0.5 µl
Reverse primer (25 µM)	0.5 µl
10X DreamTaq Buffer	2.5 µl
dNTP (2mM)	2.5 µl
DreamTaq DNA polymerase (2.5u/µl)	0.5 µl
MQdH2O	Up tp 25 µl
Total reaction:	25 µl

Table 2.18: PCR program for amplification

	Temperature	Time	
Initial denaturation	95 °C	2 min	
Denaturation	95 °C	30 seconds (sec)	35 cycles each
Annealing	Depends on primer temperature	30 sec	35 cycles each
Extension	72 °C	30 sec to 1 min (depends on amplicon length)	35 cycles each
Final extension	72 °C	5 min	

2.2.1.3 PCR for Site Directed Mutagenesis

In order to introduce a mutation to the desired site in YY1 gene, pCMV6 vector containing WT YY1 was used. Following PCR reaction and PCR program were applied.

Table 2.19: PCR reaction set up for cloning YY1 and Elk1 constructs

Content	**Volume**
Template vector	50 ng
Forward primer (25 μM)	0.5 μl
Reverse primer (25 μM)	0.5 μl
10X Reaction Buffer	2.5 μl
dNTP mix	0.5 μl
PfuUltra HF DNA polymerase (2.5u/μl)	0.5 μl
MQdH2O	Up tp 25 μl
Total reaction:	25 μl

Table 2.20 : PCR program for amplification

	Temperature	Time	
Initial denaturation	95 ^{o}C	30 sec	
Denaturation	95 ^{o}C	30 sec	16 cycles each
Annealing	55 ^{o}C	1 min	
Extension	68°C	1 minute/kb of plasmid length	

2.2.1.4 Agarose gel electrophoresis

To separate DNA fragments by their sizes agarose gel electrophoresis is used. In this project, 1 % or 1,5 % agarose gels were used to estimate the size of DNA molecules following PCR reaction and restriction enzyme digestion.

To prepare 1 % agarose gel

- 1g agarose was dissolved in 50 ml (small gel) 0,5X TBE (Tris/Borate/EDTA) or 1X TAE (Tris/Acetic Acid/EDTA) buffer.
- The agarose was solubilized by heating until the agarose was completely dissolved.
- Gel was cooled to ≤ 45ºC and ethidium bromide was added to a final concentration of 0.5 μg/ ml and mixed through gentle swirling.
- The agarose gel was then poured into a horizontal gel tray, and a comb for forming the sample slots was placed into the gel.
- Approximately 15 min later the gel was solidified and then placed into an electrophoresis tank, where the gel was covered with 0,5X TBE buffer.

The DNA was mixed with 6X loading dye in the proportion of 5:1 and the sample was placed into a well on the agarose gel. For the fragment size control, a MassRuler™ DNA Ladder, Mix (80bp-10kb) was used. Electrophoretic separation was achieved by constant current at 120 mV for 30 min.

DNA within agarose gels were visualized under UV light The size of the DNA was determined by comparing their mobility with the fragments of the DNA ladder.

2.2.1.5 DNA cleanup

In order to purify double-stranded PCR products (100 bp – 10 kb) and also to clean up the restriction reaction products for further processes, QIAquick PCR purification kit was used according to instructor's manual.

2.2.1.6 Determination of DNA concentration

Recovery, purity and concentration of nucleic acids were determined spectrophotometrically with NanoDrop 2000. The ratio of absorptions at 260 nm vs 280 nm is commonly used to assess 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 and 2.0. As protein contamination increases, the ratio decreases.

2.2.1.7 DNA cleavage by restriction endonucleases

Restriction enzymes used in this study were BamHI, XhoI, KpnI, EcoRI and SalI of which restriction sites were present in both expression vector and flanking region of PCR product (insert), but not found inside the insert. Plasmid vector was cut at 37°C for 3 - 4 hours and insert was cut overnight at 37°C with 1X Tango Y buffer (supplied by the manufacturer). Restriction reaction was completed by inactivation at 80°C for 20 min and then DNA was purified from restriction mixture by using QIAquick PCR purification kit. Restriction reaction mixtures are given in table 2.21.

Table 2.21: Restriction reaction mixture

Content	Amount	Volume
Plasmid Vector (pET-30a)	~3 µg	Depends on concentration
Enzyme 1	10 - 20 units	1 - 2 µl
Enzyme 2	10 - 20 units	1 - 2 µl
10X Tango/Y Buffer	1X	1.5 µl
Total Reaction Volume		15 µl
Plasmid Vector (pET-30a)	~3 µg	Depends on concentration
Enzyme 1	10 - 20 units	1 - 2 µl
Enzyme 2	10 - 20 units	1 - 2 µl
10X Tango/Y Buffer	1X	1.5 µl
Total Reaction Volume		15 µl

2.2.1.8 Ligation

The insert (YY1, YY1-db, YY1-woR, YY1-woA, YY1K288R or Elk1-db) was ligated to restricted expression vector (pET-30a or pCMV6) by T4 ligase. The ration of vector and DNA fragment 1:3 (vector versus insert DNA) was used in ligation processes.

Ligation was performed overnight at room temperature. After overnight incubation, T4 DNA ligase was inactivated by incubation for 10 min at 65°C. Ligation reaction mixture is shown in Table 2.22 was used.

Table 2.22 Ligation reaction mixtures

Content	Amount	Volume
Plasmid vector	30 ng	Depends on concentration
Insert DNA	90 ng	Depends on concentration
10 X T4 Buffer	1X	1,5 µl
T4 Ligase	1 unit	1 µl
MQdH2O		up to15 µl
Total Reaction Volume		15 µl

2.2.1.9 Competent cell preparation- $CaCl_2$ method

Competent cells were prepared according to the following protocol:

- *E.coli* – DH5α or *E.coli*-BL21 cells were taken from a glycerol stock culture by scraping with a tip and it was directly put in a falcon containing 5 ml LB medium and incubated overnight at 37°C in an orbital shaker.

- The following day, 100 ml LB medium was inoculated with 5 ml of overnight culture solution and was incubated at 37°C in an orbital shaker. Cell density was measured several times by a spectrophotometer at OD_{600}. When the OD_{600} 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 spun down at 1600 x g for 7 min at 4ºC, the supernatant was discarded.
- Each bacterial pellet was resuspended in 10 ml ice-cold $CaCl_2$ and centrifuged for 5 min at 1600 x g at 4ºC, 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.
- Centrifugation was performed again at 1600 x g for 5 min at 4ºC

- 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 – 80ºC.

For 50 ml $CaCl_2$ solution following contents with mentioned concentrations and amounts in Table 2.23 were used.

Table 2.23: $CaCl_2$ solution preparation

Content	Concentration	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	X	up to 50 ml

2.2.1.10 Transformation of competent cells

To introduce the DNA into the host following protocol was applied;

- The competent cells were taken from –80°C and were thawed on ice. 2 µl of purified plasmid DNA or 15 µl of ligation mixture was added to 20 µl of competent cells and the eppendorf tube containing the cells was incubated on ice for 30 min.
- Then, heat shock was done by putting the cells in water bath at 42° C for 45 sec and they were then immediately incubated on ice for 2 min.
- 80 µl of SOC medium was added to competent cells and the eppendorf tube was vigorously shaked at 37° C for 1 hour.
- The cells were then spread onto LB plate containing the appropriate antibiotic (kanamycin). The plates were incubated at 37° C overnight.

2.2.1.11 Colony PCR

After transformation of ligated plasmids, colony PCR was performed to be sure of selecting right colonies which were carrying inserts.

- 10 µl of sterile $MQdH_2O$ was put into PCR tubes for each colony that was taken from the replica plate by sterile tips and incubated at room temperature for 5 min to drop into water.
- Tubes containing water and a piece of colony were incubated at 85°C for 5 min to blow up cells and release plasmids.

- Then, PCR mixture mentioned in table 2.24 was added into tubes. The PCR program was the same as given before in Table 2.18.

Table 2.24: Colony PCR reaction

Content	Volume
Template + water	10 µl
Forward primer (25	0.5 µl
Reverse primer (25	0.5 µl
10X Taq Buffer	2.5 µl
dNTP mix (2 mM)	2.5 µl
Taq polymerase	0.3 µl
MQdH2O	8.7 µl
Total reaction:	25 µl

2.2.1.12 Small scale plasmid DNA preparation

After identification of the right colonies containing the inserts by colony PCR, these colonies were taken into 5 ml LB medium containing 5 µl suitable antibiotic (kanamycin or ampicilin) in order to make selection media. Then, they were incubated overnight with vigorous shaking (200rpm) at 37 ^{o}C.

Plasmid preparation was performed using QIAGEN, QIAPrep Spin Miniprep Kit for small-scale (mini) preparations, following instructions of the manufacturer.

2.2.1.13 DNA sequencing

In order to confirm if correct amplicon was inserted into vector, sequencing was performed.

PCR for sequencing:.

The plasmids with inserts were verified by DNA sequencing using Big Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems). The sequence reaction was prepared according to Table 2.25

Table 2.25 : Sequence PCR set up

Content	Amount	Volume
Big dye reaction mix	X	2 µl
5X sequence mixture	X	2 µl
Template DNA	150 ng	1 µl
T7 Primer (promoter/ terminator)	25 µM	0,5 µl
dH_2O	X	up to10 µl

The sequence reactions were performed using a thermal cycler with the following program mentioned in Table 2.26.

Table 2.26 : Sequence PCR program

	Temperature	Time	
Initial denaturation	95 ^{o}C	5 min	
Denaturation	95 ^{o}C	10 sec	40 cycles each
Annealing	50 ^{o}C/55 ^{o}C	10 sec	
Extension	60 ^{o}C	4 min	
Final extension	60 ^{o}C	8 min	

PCR cleanup for sequencing: The protocol is given below:

- 10 µl PCR product was taken in to microfuge tube then 2µl of 3M NaAc and 50 µl ice-cold 95% ethanol were added to the tube.
- Tubes were incubated on ice for 30 min.
- Mixture was centrifuged at 14000 rpm for 20 min Supernatant was discarded, and pellet was resuspended in ice-cold 70 % ethanol. The mixture was vortexed vigorously for ~30 sec.
- Tubes were centrifuged at 14000 rpm for 20 min. Supernatant was discarded.
- Tubes were incubated at 95°C for 5 min with caps open (in order to evaporate ethanol).
- 20 µl of formamide was added to DNA pellet and the mixture was vortexed vigorously for ~30 sec.
- Tubes were again incubated at 95 °C for 5 min with caps closed.
- Tubes were immediately put on ice and kept at 4 °C until analysis.

2.2.1.14. Alignment of sequence results

Nucleotide alignments were made with EMBOSS Pairwise Alignment Algorithms Tool and NCBI BLAST tool (available online respectively at these web addresses: www.ebi.ac.uk/Tools/emboss/align/index.html and www.ncbi.nlm.gov/BLAST) in order to compare the sequencing results with originally expected one.

2.2.2 Protein expression studies

In this study, cDNA covering only DNA binding domain of Elk-1 and YY1 were cloned separately into pET-30a expression vector that is under the control of T7 promoter and BL21(DE3)pLysS cell strain was used as host in order to express desired protein.

2.2.2.1 Protein expression induction

pET-30a vectors with cloned YY1-db and Elk-1-db were transformed into BL21(DE3)pLysS competent cells, they were spread on LB agar plate containing kanamycin and incubated overnight at 37°C. The next day, a single colony from each plate was taken into 5 ml LB media (with kanamycin) in falcon tube and left for overnight growth at vigorous shaking at 37°C. Next day, 2 ml of overnight culture was diluted with LB media and placed into 50 ml LB media. Culture was incubated with shaking at 37°C until the OD_{600} reaches a value between 0.6 and 0.8. After cells were induced with 0.5 mM IPTG, they were grown for 10 hours with shaking at 37°C. While cells were growing, samples were taken with two hours intervals for further analysis.

2.2.2.2 Total cell protein analysis

- At various times (0-8 hours) following induction, 1 ml cell culture for induction analysis was collected by centrifugation for 5 min at 14000x g in a microfuge.
- Supernatant was discarded. Pellet was mixed with 50 µl 2X SDS sample buffer for SDS-PAGE analysis explained below

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

Tricine/polyacrylamide gel with 15 % acrylamide concentration which is suitable for separating 10-43 kDa proteins was prepared since molecular weight of expressed Elk1-db and YY1-db was expected to be around 20-25 kDa. Preparations of each mini gels were mentioned below:

Table 2.27 : 15 % separating gel solution (6ml)

Contents	Volume
Seperating acrylamide	2 ml
Gel Buffer	2 ml
50 % Glycerol	2 ml
10 % APS	75 µl
TEMED	7.5 µl
dH_20	-

Table 2.28 : 5 % stacking gel solution (3 ml)

Contents	Volume
Stacking acrylamide	0.25 ml
Gel Buffer	0.75 ml
dH_20	2 ml
10 % APS	20 µl
TEMED	2 µl

The separating gel solution was applied into the gel cassette up to ± 6.5 cm, and, the last ± 2.5 cm of the cassette was filled with isopropanol, immediately. After the gel was polymerized for at least 3 min, the isopropanol was carefully removed by filter papers. The stacking gel solution was directly poured into the gel cassette, and the gel comb was placed to form the slots. The stacking gel was polymerized for at least 1 h.

The samples, which were denaturated for 5 min at 95ºC, were loaded on the SDS PAGE gel. As molecular weight marker, 5µl SeeBlue Plus2 Prestained Protein Ladder (Invitrogen) was loaded on the gel.

Electrophoresis was carried out in anode and cathode buffers at constant 25mA.

After electrophoresis, SDS-PAGE was stained in CBB stain solution. To reduce the time gel heated in a microwave oven for ~15 sec then placed on a shaker and then, it was destained in destain solution as staining protocol.

2.2.2.4 Metal affinity purification of 6xHis tagged YY1-db and Elk1-db protein

Purification protocol of recombinant proteins:

- 500 ml of IPTG induced bacterial culture was grown for 8 hours and centrifuged for 5 min at 14000 x g.
- The supernatant was discarded and the pellet was frozen overnight at -80°C.
- Next day, it was thawed on ice, resuspended in 5 ml lysis buffer (see section 2.1.4.2) and centrifuged for 10 min at 14000 x g.
- 15 µl sample of lysate was taken for further SDS-PAGE analysis.
- 500 µl of 50% Ni-NTA agarose was added to lysates and the mixture was gently mixed for an hour in eppendorf tubes at +4°C.
- Then it was centrifuged at 4000 xg for 30 sec to separate resin and supernatant. The supernatant (which is also called flow-through, since it passes through the resin) was transferred to fresh tubes for SDS-PAGE analysis.
- The resin was washed twice with 2.5 ml of wash buffer (see section 2.1.3 “Buffers”). It was centrifuged at 4000 xg for 30 sec after each wash step and the supernatant was taken for SDS-PAGE analysis.
- Finally, the protein was eluted 4 times with 300 µl elution buffer (see section 2.1.3 “Buffers”). Centrifugation at 4000 xg for 30 sec was done after each elution step and the supernatant.

2.2.2.5 Protein concentration determination

Concentration of the purified proteins was determined by Qubit® 2.0 Fluorometer according to manufacturer's instructions.

2.2.3 Cell culture studies

2.2.3.1 Transferring cells from -80°C to culture flasks: Cells which had been stored in -80°C were thawed in 37°C water bath and taken in to 10 ml DMEM medium for centrifugation for 5 min at 900 rpm. Cell pellet was dissolved with DMEM medium and transferred to the culture flasks.

2.2.3.2 Cell counting: Cell pellet was suspended with 10 ml DMEM. Then 10 µl suspension was taken and put onto the hemacytometer, cells were counted as the number of cells per 25 square (1mm^2). The formula of cell number calculation is given below:

10^4 (constant number) X Amount of Counted Cell = Cell Number per ml

Total cell number= Cell Number per ml X Total Volume of Cells (10ml)

2.2.3.3 Cell passage: Cells in the flasks or dishes were maintained in growth medium. Growth medium was removed and 5 ml PBS was added. Cells stuck on the culture dish were lifted by trypsinization. Then cells were resuspended in growing medium and put into the centrifuge tubes and spun at 900 rpm. After the centrifugation, supernatant was discarded and pellet was resuspended with growing medium.

2.2.3.4 Cell freezing: As indicated in cell passage section, after resuspending cells, 1x10^6 cell/ml cells were transferred to freezing tubes with growth medium containing 5-10 % DMSO for furhter applications.

2.2.4 Electrophoretic Mobility Shift Assay (EMSA)

2.2.4.1 Oligo probe Biotinylation and Hybridization

Oligo probes were labeled separately by using Biotin 3' End DNA Labeling Kit that uses terminal deoxynucleotidyl transferase to incorporate 1–3 biotinylated ribonucleotides on to the 3' end of DNA strands. Labeled forward and reverse oligos were then annealed at 1:1 ratio in 10 mM Tris, 1 mM EDTA by heating to 95 ^{o}C for

5 min, slow cooling by 2 °C/min to their annealing temperature, annealing for 30 min and cooling to 4 °C by 2 °C/min (cycle number depends on Tm of the oligos).

Table 2.29: Oligo probes for EMSA

Oligo name	Sequence
Sp-Elk1-WT-1	5'-TACGAAGGCTTCCTGGCAGGAGCTC-3'
Sp-Elk1-Mut-1	5'-TACGAAGGAAAAATGGCAGGAGCTC-3'
Sp-Elk1-WT-2	5'-CGGAGAGGACAGCGACAGGAAGGGAGG-3'
Sp-Elk1-Mut-2	5'-CGGAGAGGACAGCGACATTTTTGGAGG-3'
Sp-YY1-WT	5'-CAGATTGATATCCATTGCTTCTGAG-3'
Sp-YY1-Mut	5'-CAGATTGATATTTGCTGCTTCTGAG-3'
p80-Elk1-WT	5'-GAACGTTCCTTCCTCTTGATTAGTC-3'
p80-Elk1-Mut	5'-GAACGTTCTCCTTCTCCGATTAGTC-3'
p80-YY1-WT-1	5'-AAGCCATTGGCGCAGCCGCCATTGG-3'
p80-YY1-Mut-1	5'-AAGTTGCTGGCGCAGCCGTTGCTGG-3'
p80-YY1-WT-2	5'-GCGCCTGCTTCCATTGGTCAGTCCT-3'
p80-YY1-Mut-2	5'-GCGCCTGCTTTTGCTGGTCAGTCCT-3'

2.2.4.2 EMSA binding reaction, PAGE analysis and detection

For binding reactions, ~2 µg whole extracts of SHSY-5Y cells or Elk1-db or YY1-db protein were incubated with 20 fmol biotinylated oligonucleotides in binding buffer (contents of binding buffer indicated in Table 2.30). In supershift assays, 300 ng of Tetra-His antibody (QIAGEN Inc.) was added prior to the addition of 150 ng Elk1-db protein.

Table 2.30: EMSA reaction buffer contents for Elk-1 and YY1

	Elk-1 binding mix	YY1 binding mix
Contents	**Volume**	**Volume**
MQ-H2O	Up to 20 µl	Up to 20 µl
10X binding buffer	2 µl	-
Poly dIdC	1 µl	1 µl
50 % glycerol	2 µl	4 µl
20 mM EDTA	1 µl	-
6 % BSA (0,5 % for YY1)	1 µl	0.5 µl
300 mM HEPES	-	1 µl
20 mM DTT	-	1 µl
1 mM KCl	-	1.3 µl
100 mM MgCl2	-	0.6 µl
1 % NP-40	-	1 µl
Protease inhibitor	1 µl	1 µl
Unlabeled DNA	2 µl (just for competition)	2 µl (just for competition)
WCE	2 µl	1 µl
Biotin Labeled DNA	2 µl	2 µl
Antibody	5 µl (for supershift)	5 µl (for supershift)

Complexes and free DNAs were separeted on a 6 or 8 % non-denaturating polyacrylamide gel in 0.5X Tris-Borate-EDTA by electrophoresis for 1 h at 120 V at 4 °C. The separated bands on the gel were then transferred to Biodyne A Nylon

Membranes (Pierce) by using Trans-Blot® at 20 V for 30 min at 4 ^{o}C. Cross-link transfer of DNA to membrane was achieved by incubating the membrane with 254 nm UV bulbs for 10 min.

For detection step, Chemiluminescent Nucleic Acid Detection Module Kit (Pierce) was used, and then the membrane was exposed to X-ray film for required amount of time and then developed in Kodak Medical X-ray Processor according to manufacturer's instruction. In supershift assays, 300 ng of Tetra-His antibody (QIAGEN Inc.) was added prior to the addition of 150 ng Elk1-db protein.

2.2.5 Dual-Luciferase Reporter Assay

2.2.5.1 Transfection of SH-SY5Y with promoter construct plasmids and YY1 constructs for forced experiments

Transfection is the process to introduce a foreign DNA into the cell. For eukaryotic cells, lipid-cation based transfection is more typically used, because the cells are more sensitive. In this study, DNA was inserted into SH-SY5Y neuroblastoma cells by lipofection.

In luciferase reporter assay, the amount of vector needed depends on the cell line. 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, pGL2-basic or pGL3-basic). Using optimized transfection conditions for a single reporter, control vector is cotransfected in varying amounts. Typically a ratio of 10:1 test vector:control vector is used; however, 7:1 ratio of test:control vector were defined as optimum previously in our laboratory for SH-SY5Y neuroblastoma cell line.

A frozen stock of SHSY-5Y cells were taken from -80 °C and after the cells were warmed up to room temperature they were diluted with SHSY-5Y culture media. After determining the cell density with hemacytometer, 50.000 cells/well were seeded on 24 well tissue culture plate. Next day, 1 µg of plasmid DNAs are diluted in 200 µl DMEM (1X) media and vortexed and quick spun briefly. Promega's transfection reagent Transfast was added to 1:3 ratio to the DNA mixture according to the ratios given in the table below (Table 2.31 and Table 2.32).

Table 2.31: Transfection content for pGL2-S2 forced experiment.

Construct name	*pGL 2-S2	wt-YY1	YY1-woR	YY1-woA	YY1-db	YY1-K288	pCMV -Myc.	pRL-TK	Transf ast
pGL2-S2	700 ng	-	-	-	-	-	200 ng	100 ng	3 µL
pGL2- basic	700 ng	-	-	-	-	-	200 ng	100 ng	3 µL
pGL2-S2 YY1	700 ng	200 ng	-	-	-	-	-	100 ng	3 µL
pGL2- basic YY1	700 ng	200 ng	-	-	-	-	-	100 ng	3 µL
pGL2-S2 YY1-woR	700 ng	-	200 ng	-	-	-	-	100 ng	3 µL
pGL2- basic YY1-woR	700 ng	-	200 ng	-	-	-	-	100 ng	3 µL
pGL2-S2 YY1-woA	700 ng	-	-	200 ng	-	-	-	100 ng	3 µL
pGL2- basic YY1-woA	700 ng	-	-	200 ng	-	-	-	100 ng	3 µL
pGL2-S2 YY1-db	700 ng	-	-	-	200 ng	-	-	100 ng	3 µL
pGL2- basic YY1-db	700 ng	-	-	-	200 ng	-	-	100 ng	3 µL
pGL2-S2 YY1-K288R	700 ng	-	-	-	-	200 ng	-	100 ng	3 µL
pGL2-basic YY1-K288R	700 ng	-	-	-	-	200 ng	-	100 ng	3 µL

Table 2.32: Transfection content for pGL3-F2 forced experiment.

Construct name	*pGL 2-F2	wt-YY1	YY1-woR	YY1-woA	YY1-db	YY1-K288	pCM V-	pRL-TK	Transf ast
pGL3-F2	700 ng	-	-	-	-	-	200 ng	100 ng	3 µL
pGL3- basic	700 ng	-	-	-	-	-	200 ng	100 ng	3 µL
pGL3-F2 YY1	700 ng	200 ng	-	-	-	-	-	100 ng	3 µL
pGL3- basic YY1	700 ng	200 ng	-	-	-	-	-	100 ng	3 µL
pGL3-F2 YY1-woR	700 ng	-	200 ng	-	-	-	-	100 ng	3 µL
pGL3- basic YY1-woR	700 ng	-	200 ng	-	-	-	-	100 ng	3 µL
pGL3-F2 YY1-woA	700 ng	-	-	200 ng	-	-	-	100 ng	3 µL
pGL3- basic YY1-woA	700 ng	-	-	200 ng	-	-	-	100 ng	3 µL
pGL3-F2 YY1-db	700 ng	-	-	-	200 ng	-	-	100 ng	3 µL
pGL3- basic YY1-db	700 ng	-	-	-	200 ng	-	-	100 ng	3 µL
pGL3-F2 YY1-K288R	700 ng	-	-	-	-	200 ng	-	100 ng	3 µL
pGL3-basic YY1-K288R	700 ng	-	-	-	-	200 ng	-	100 ng	3 µL

Transfection mixture was vortexed and quick spun briefly and incubated for 15 min at room temperature for the proper formation of DNA liposome complex. The growth medium was removed from the cells and the transfection mixture was added gently in order to prevent cell detaching and the cells returned to the 37°C %5 CO_2 incubator. After an incubation period of 90 min, the transfection mixture was removed and 500 µl of complete growth medium was added to each well. The cells were returned to the 37°C %5 CO_2 incubator for 48 hours before analysis.

2.2.5.6 Luminometrical measurement of the transfected cells

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.

To perform the assay, 48 hours after transfection cells were harvested by lysing them with 60 µl 1X Passive Lysis Buffer. The firefly luciferase reporter was measured first by adding 50 µl Luciferase Assay Reagent II (LAR II) to generate a stabilized luminescent signal. After quantifying the firefly luminescence, this reaction was quenched, and the Renilla luciferase reaction is simultaneously initiated by adding 50 µl Stop & Glo® Reagent to the same sample. The procedure was repeated for each sample separetly. The chemiluminescence was measured by Fluoroskan Ascent FL luminometre from Thermo Electron Coorporation. All transfections were performed as triplicates and were repeated at least two times using different DNA preperations.

3. RESULTS & DISCUSSION

3.1 Recombinant YY1-db and Elk-1-db production

3.1.1 Cloning of YY1-db and Elk-1-db

For 363 bp gene region corresponding to YY1 DNA binding domain (YY1-db) and 288 bp region of Elk1 DNA binding domain (Elk-1-db) constructs were amplified with PCR. The resultant PCR products were run on agarose gel and the expected DNA fragments were detected (Figure 3.1.a lane 2 and Figure 3.2.a lane 2, respectively). Following PCR, DNA fragments were purified with QIAquick PCR purification kit and digested with appropriate restriction enzymes (BamHI-XhoI for Elk1-db, EcoRI-SalI for YY1-db).

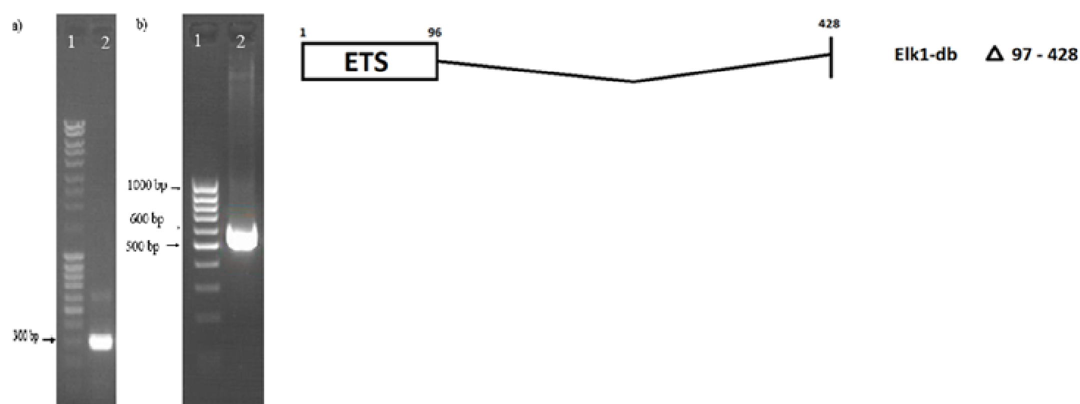

Figure 3.1: PCR amplification of Elk1-db and schematic representation of Elk1 DNA binding domain.

Amplified PCR pruducts were designed to be inserted into pET30a(+) bacterial expression vector. After enzyme cleavage, restricted products were purified by QIAquick PCR purification kit. After that, ligation procedure was performed with Roche T4 DNA ligase overnight at room temperature and the ligation mixture was then transformed into competent *E. coli* BL21 cells. Transformed colonies were selected from selective antibiotic (kanamycin) plates and colonies including Elk1-db and YY1-db were checked with colony PCR using forward and reverse primers of

the vector to verify accuracy. Agarose gel results indicated that amplicons were successfully ligated into vector (Figure 3.1.b and Figure 3.2.b).

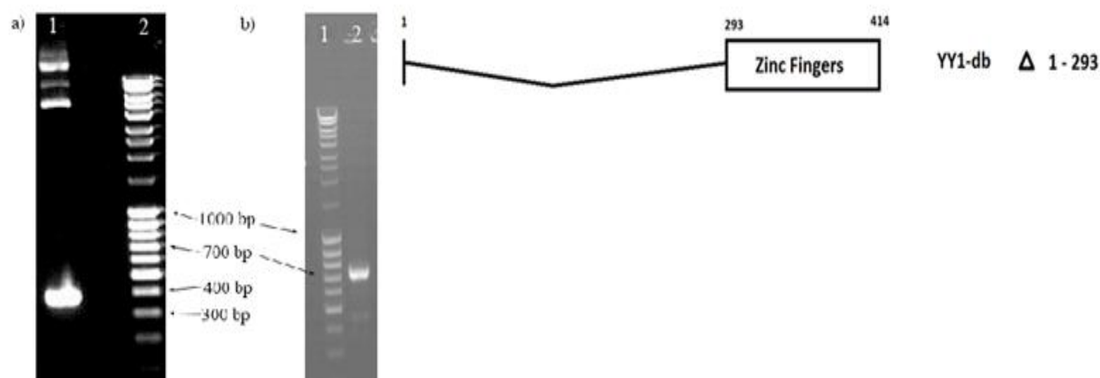

Figure 3.2: PCR amplification of YY1-db and schematic representation of YY1 DNA binding domain.

There is a 250-300 bp shift in bands when comparing primary PCR results and control colony PCR. The reason of the shift is due to using of vector primers, which span 100-150 bp from both forvard and reverse direction. After confirming the success of ligation, the remaining colony that was used in colony PCR, was selected and plasmid purification procedure was performed to collect plasmids. Collected plasmid DNA was subjected to sequence analysis to confirm inframe insertion and correct orientation.

3.1.2 Expression of Recombinant Proteins

In order to express Elk1-db and YY1-db, obtained construct of pET-30a-Elk1-db and pET-30a-YY1-db was transformed into expression strain of *E.coli* BL21 (DE3)pLysS. A colony was picked from LB agar plate containing the selective antibiotic and dropped into LB medium. When the OD_{600} reached 0.6, final concentration of 0.5 Mm IPTG was added to bacterial culture growing in LB at 37 ^{o}C to induce protein expression.

In order to determine the optimum expression time and the expressed recombinant proteins, total cell proteins were analyzed by SDS-PAGE method. For this purpose, bacterial samples were taken before IPTG induction (0 hour) and after IPTG induction at 2 hours intervals for 10 hours. Protein samples were obtained from cell pellets and loaded into 15 % Tricine/polyacrylamide gel (Figure. 3.3 for Elk1-db and 3.4 for YY1-db).

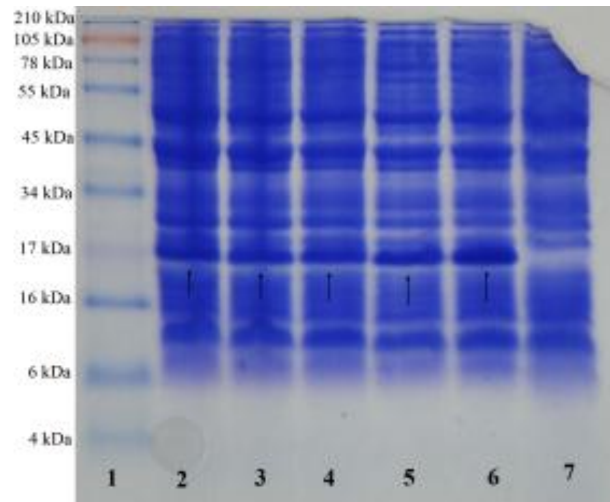

Figure 3.3: SDS- PAGE analysis of total protein samples for Elk1-db expression Lane 1: SeeBlue Plus2 Prestained Protein Ladder (Invitrogen); Lane 2: pET30a-Elk-db induced, 2 hour; Lane 3: pET30a-Elk-db induced, 4 hour; Lane 4: pET30a-Elk-db induced, 6 hour; Lane 5: pET30a-Elk-db induced, 8 hour; Lane 6: pET30a-Elk-db induced, 10 hour Lane 7: pET30a-Elk-db uninduced, 0 hour;

After induction with IPTG, protein of ~17 kDa and ~23 kDa were detected for elk1-db and YY1-db, respectively in protein samples obtained from cell pellet. The expression of recombinant protein increased with time slightly throughout 10 hour time course after IPTG induction. Although, the amount of overexpressed recombinant proteins seemed little at 2 hours comparing the samples taken at further time intervals, it is still very distinguishable. Thus, ~6 hours induction was performed to obtain good quality products. No expressed protein was found in the uninduced samples.

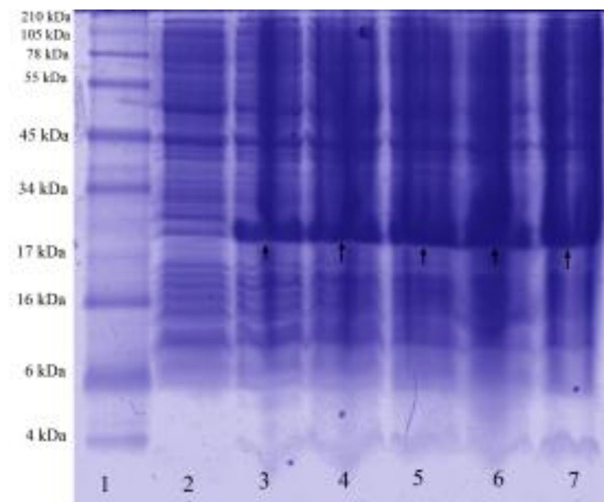

Figure 3.4: SDS- PAGE analysis of total protein samples for YY1-db expression Lane 1: SeeBlue Plus2 Prestained Protein Ladder (Invitrogen); Lane 2: pET30a-YY1-db uninduced, 0 hour Lane 3: pET30a-YY1-db induced, 2 hour; Lane 4: pET30a-YY1-db induced, 4 hour; Lane 5: pET30a-YY1-db induced, 6 hour; Lane 6: pET30a-YY1-db induced, 8 hour; Lane 7: pET30a-YY1-db induced, 10 hour.

3.1.3 Purification of recombinant Elk1-db and YY1-db

All purification steps were given in detail in the Materials and Methods part. At each purification step, the samples were taken and were further analyzed by SDS-PAGE method (see Figure 3.5 for Elk1-db and Figure 3.6 for YY1-db).

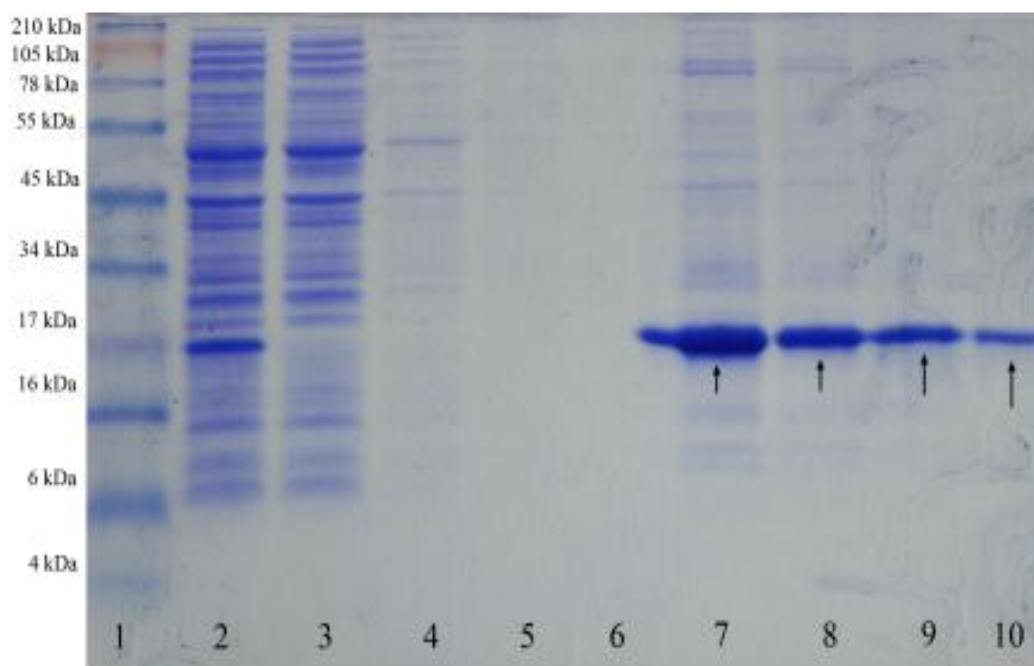

Figure 3.5: SDS-PAGE analysis of purified Elk1-db proteins (Purified protein in elution fractions showed by arrows) Lane 1: SeeBlue Plus2 Prestained Protein Ladder; Lane 2: lysate of induced bacterial culture; Lane 3: lysate flow-through; Lane 4: 1st wash fraction; Lane 5: 2nd wash fraction; Lane 6: 3rd wash fraction; Lane 7: 1st elute; Lane 8: 2nd elute; Lane 9: 3rd elute; Lane 10: 4th elute

Distinct protein bands were seen in elution fractions clearly and the protein amount decreased gradually in elution steps. In order to check whether the purified protein is the same as the overexpressed one, the control sample containing total protein fraction from bacterial cultures was applied to the SDS-PAGE gel as well. In addition, the overexpressed recombinant Elk1-db and YY1-db protein bands were observed in bacterial cell lysate sample and this band was missing in the flow-through sample because overexpressed proteins bound to Ni-NTA agarose with its His-tags during purification process. In wash fractions, desired protein bands were not observed as expected.

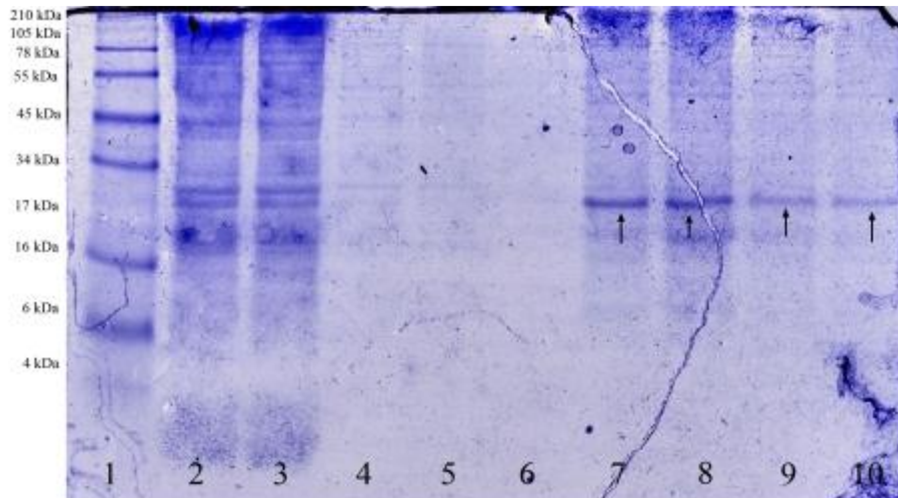

Figure 3.6: SDS-PAGE analysis of purified YY1-db proteins (Purified protein in elution fractions showed by arrows) Lane 1: SeeBlue Plus2 Prestained Protein Ladder; Lane 2: lysate of induced bacterial culture; Lane 3: lysate flow-through; Lane 4: 1st wash fraction; Lane 5: 2nd wash fraction; Lane 6: 3rd wash fraction; Lane 7: 1st elute; Lane 8: 2nd elute; Lane 9: 3rd elute; Lane 10: 4th elute

3.2 Determination of Elk1 and YY1 binding sites on *SPG4* and *KATNB1* promoter regions by EMSA

3.2.1 Identification of Elk1 and YY1 binding sites on the optimal *SPG4* promoter

Based on its functions in neuronal tissues, we theoretically searched the presence of Elk1 and YY1 transcription factors binding sites in the *SPG41* promoter by using PROMO bioinformatics tool (Messeguer *et al.* 2003; Farre *et al.* 2003) restricting the maximum matrix dissimilarity rate 0-3% for *Homo sapiens*. According to these search results, three Elk1 binding sites (positioned at -1074/-1066, -451/-443, and -235/-227) and one YY1 binding site (-614/-610) have been predicted in the promoter (see Appendix A).

Electrophoretic mobility shift assay (EMSA) was performed in order to confirm the binding of Elk1 transcription factor to its predicted sites on the optimal promoter of *SPG4* gene (S2 construct containing the region at -921/-221). For this purpose, we chose two predicted binding site for Elk1 based on their locations in the critical promoter sites that we identified, and these sites are positioned at -451/-443 and -235/-227.

Protein binding to Elk1 and YY1 binding site (WT) was observed using both SH-SY5Y whole cell extract (WCE) (Figure 3.7.a and b -lane 2 and Figure 3.8 for Elk1 and YY1 respectively) and pure Elk1-db and YY1-db protein (Figure 3.9 and Figure 3.10 –lane 1). The signal shifts of the DNA-protein interactions were prevented when

Elk1 oligo mutated in their binding site (Mut) was used with WCE (Figure 3.7.a-b and Figure 3.8 -lane 4) and with Elk1-db (Figure 3.9 and Figure 3.10 -lane 3).

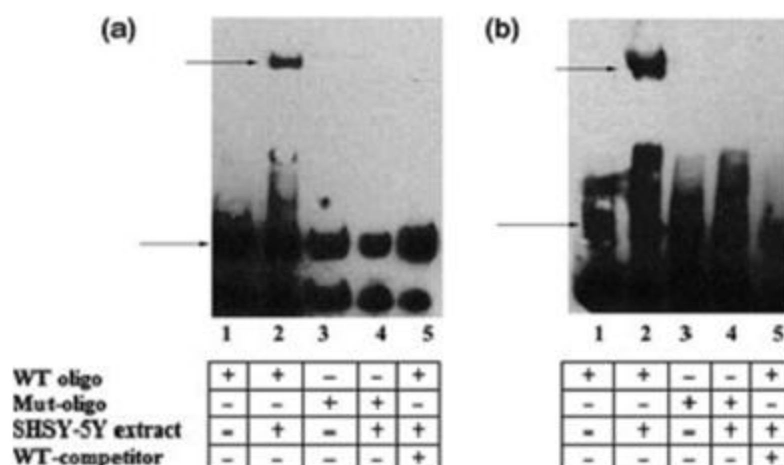

Figure 3.7: Determination of Elk1 binding sites on the *SPG4* promoter by EMSA

We also performed competition analysis using non-labeled WT-Elk1 oligos (1000 fold molar excess) and the band shift was lost (Figure 3.7.a-b and Figure 3.8 -lane 5).

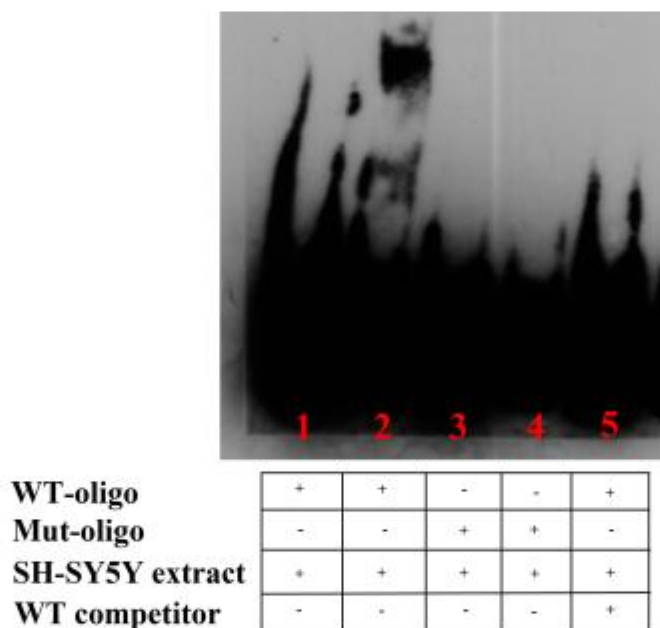

Figure 3.8: Determination of YY1 binding sites on the *SPG4* promoter by EMSA.

Recombinant His-tagged Elk1 protein interaction was supershifted using anti-His antibody (Figure 3.9 -lane 2-4 and Figure 3.10 -lane 2) and no supershift was observed when mutated Elk1 and YY1 oligos used (Figure 3.9.-lane 5-7 and Figure 3.10 -lane 4)

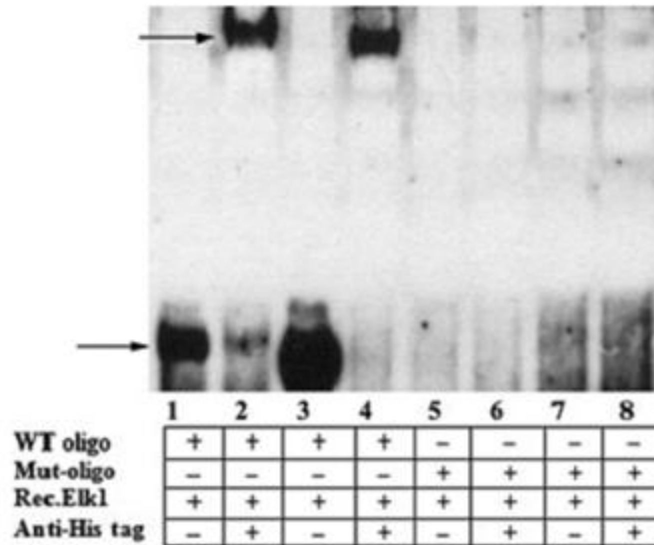

Figure 3.9: Determination of specificity of Elk1 binding to *KATNB1* promoter by EMSA using purified recombinant proteins.

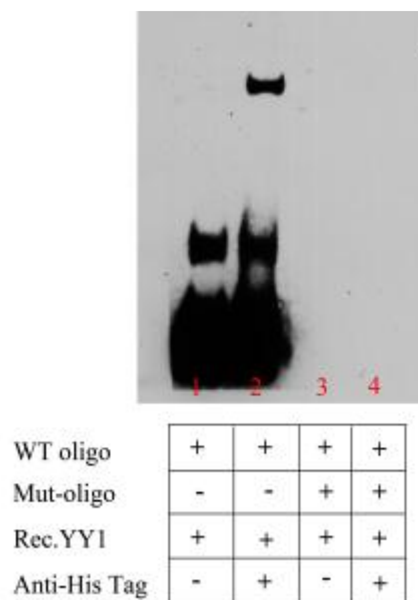

Figure 3.10: Determination of specificity of YY1 binding to *SPG4* promoter by EMSA using purified recombinant proteins.

3.2.2 Identification of Elk1 and YY1 binding sites on the optimal *KATNB1* promoter

The presence of Elk1 and YY1 transcription factors binding sites in the *KATNB1* promoter was predicted by using PROMO bioinformatics tool restricting the maximum matrix dissimilarity rate 0-3% for *Homo sapiens*. According to these search results, only one Elk1 binding site positioned at -531/-523 and two YY1

binding sites with CCAT/ATGG consensus sequence have been predicted in the promoter (see Appendix B).

To confirm the binding of Elk1 and YY1 transcription factors to their predicted sites on the optimal promoter of *KATNB1* gene (F2 construct containing the region at -892/-375) EMSA was performed (Figure 3.11.a and Figure 3.12.a-b for Elk1 and YY1 respectively). Protein binding to Elk1 binding site (WT) was observed using both SH-SY5Y whole cell extract (WCE) (Figure 3.11.a -lane 2) and pure Elk1-db protein (Figure 3.11.b –lane 1). The signal shifts of the DNA-protein interactions were prevented when Elk1 oligo mutated in their binding site (Mut) was used with WCE (Figure 3.11.a -lane 4) and with Elk1-db (Figure 3.11.b -lane 3). We also performed competition analysis using non-labeled WT-Elk1 oligos (1000 fold molar excess) and the band shift was lost (Figure 3.11.a -lane 5). Recombinant His-tagged Elk1 protein interaction was supershifted using anti-His antibody (Figure 3.11.b -lane 2) and no supershift was observed when mutated Elk1 oligo used (Figure 3.11.b -lane 4).

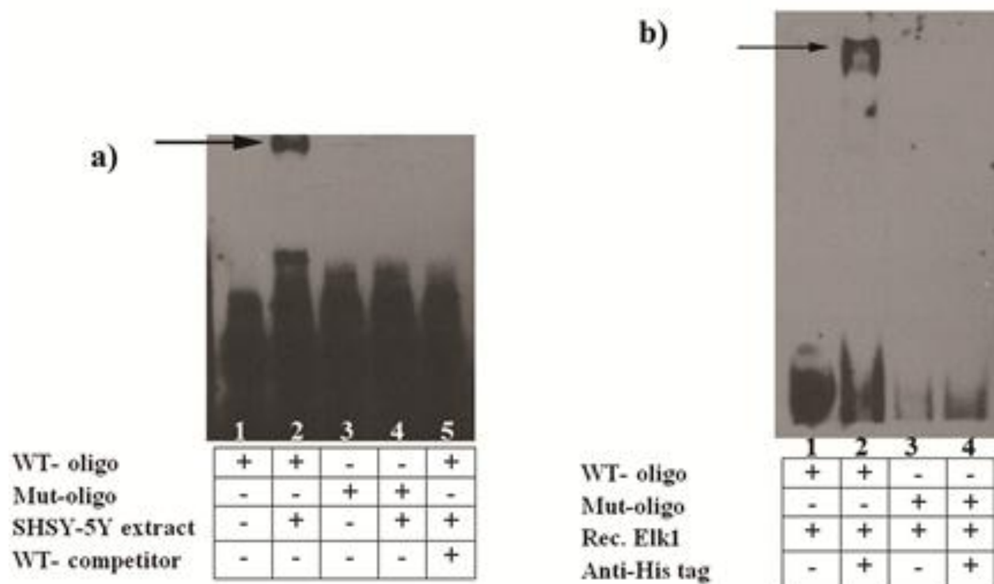

Figure 3.11: Determination of Elk1 binding sites on the *KATNB1* promoter and specifying of binding using recombinant protein by EMSA.

Protein bindings to YY1 binding sites (WT-1 and WT-2) were observed using both SHSY-5Y whole cell extract (WCE) (3.12.a -lane 2 and 3.12.b -lane 2) and pure Elk1-db protein (3.13.a -lane 1 and 3.13.a 1). The signal shifts of the DNA-protein interactions were prevented when YY1 oligos mutated in their binding sites (Mut-1 and Mut-2) were used with WCE (3.12.a -lane 4 and 3.12.b -lane 4) and with Elk1-db (3.13.a -lane 3 and 3.13.b -lane 3). In competition analysis, the band shifts were

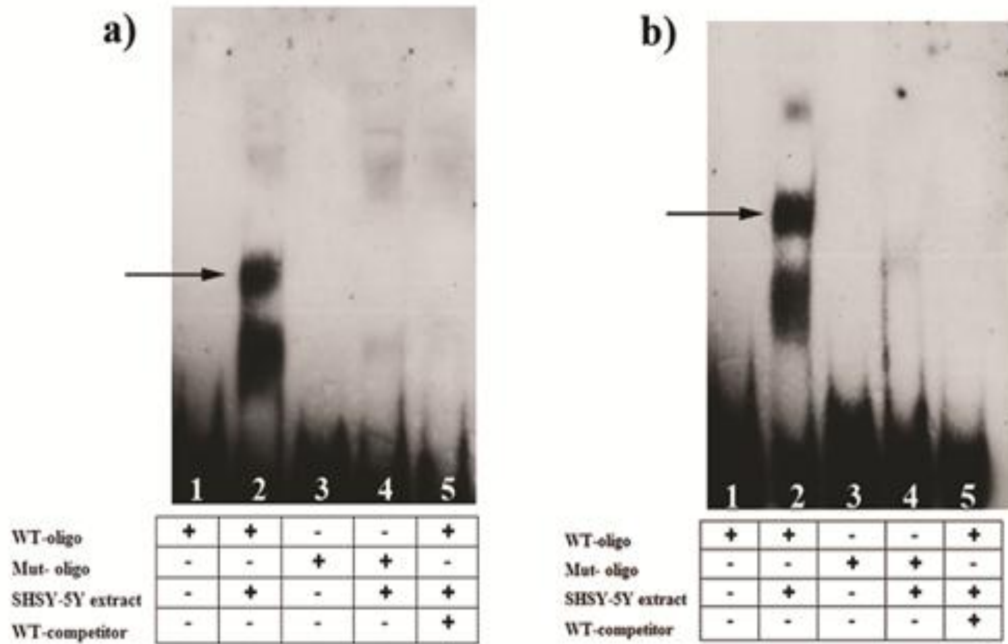

Figure 3.12: Determination of YY1 binding sites on the *KATNB1* promoter by EMSA

lost (3.12.a -lane 5 and 3.12.b -lane 5). The supershifted bands were observed (3.13.a -lane 2 and 3.13.b -lane 2) and the supershift was lost when mutated YY1 oligos used (3.13.a -lane 4 and 3.13.b -lane 4).

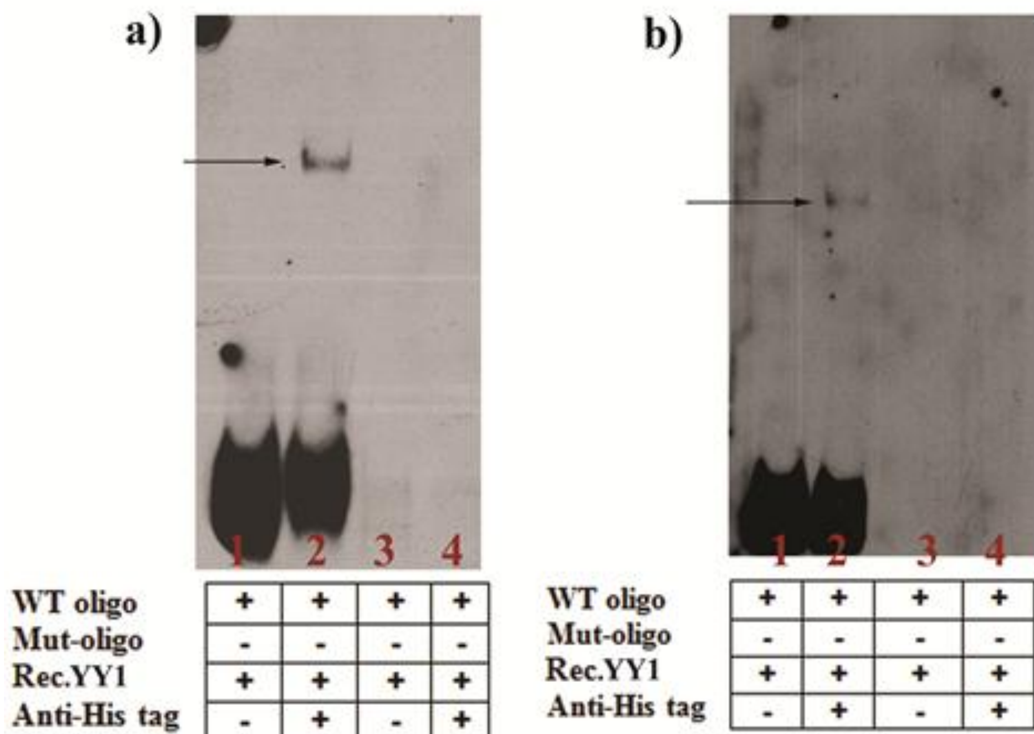

Figure 3.13: Determination of specificity of YY1 binding to *KATNB1* promoter by EMSA using purified recombinant proteins.

3.3 Luminometrical Measurements Data of Promoter Constructs (F2 and S2)

3.3.1 Cloning of YY1 constructs (YY1-woA, YY1-woR)

For 777 bp gene region corresponding to YY1 protein that lacking activator domain (YY1-woA), 468 bp gene region related to upstream of YY1 repressor domain (YY1-woR1) and 639 bp gene region related to downstream of YY1 repressor domain (YY1-woR2) constructs were amplified with PCR. The resultant PCR products were run on agarose gel and the expected DNA fragments was detected (Figure 3.14.a). Following PCR, DNA fragments were purified with QIAquick PCR purification kit and digested with appropriate restriction enzymes (KpnI-BamHI for YY1-woa, EcoRI-KpnI for YY1-woR1 and KpnI-BamHI for YY1-woR2).

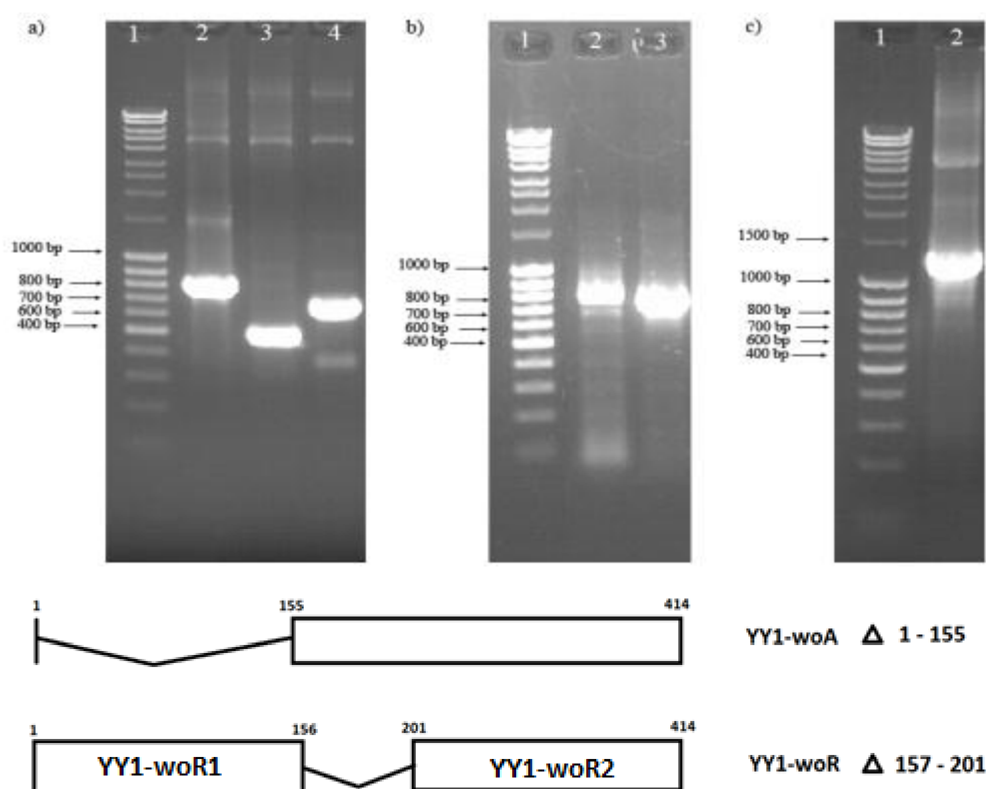

Figure 3.14: PCR amplification of YY1 constructs. a) PCR amplification of YY1-woa (lane2), YY1-woR1 (lane3) and YY1-woR2 (lane4) prior to enzyme digestion. b) colony PCR of YY1-woA and YY1-woR1. c) colony PCR of YY1-woR (includes YY1-woR1 and YY1-woR2)

Amplified PCR pruducts were designed to be inserted into pCMV6 mammalian expression vector. After enzyme cleavage, restricted products were purified by QIAquick PCR purification kit. After that, ligation procedure was performed with Roche T4 DNA ligase overnight at room temperature and the ligation mixture was then transformed into competent *E. coli* DH5α cells. Transformed colonies were

selected from selective antibiotic (ampicillin) plates and colonies including YY1-woa and YY1-woR1 (Figure 3.14.b) were checked with colony PCR using forward and reverse primers of the vector to verify accuracy. To obtain YY1 construct that lacks repression domain, amplicons covering YY1-woR1 and YY1-woR2 ligated into vector sequentially. Ligated two pieces were confirmed by colony PCR (Figure 3.14.c) Agarose gel results indicated that amplicons were successfully ligated into vector.

After confirming the success of ligation, the remaining colony that was used in colony PCR, was selected and plasmid purification procedure was performed to collect plasmids. Collected plasmid DNA was subjected to sequence analysis to confirm inframe insertion and correct orientation.

3.3.2 Production of Mutant YY1 (YY1-K288R)

In order to introduce mutation to YY1 residue 288, mutation primers, which convert lysine 288 to arginine, were used. Following PCR, mutated plasmids were purified and transformed into *E. coli* DH5α strain. Then amplified plasmids were directly used for sequence analysis to confirm the presence of desired mutation. Sequence analysis showed that lysine residue at position 288 was successfully subsituted with arginine (Figure 3.15).

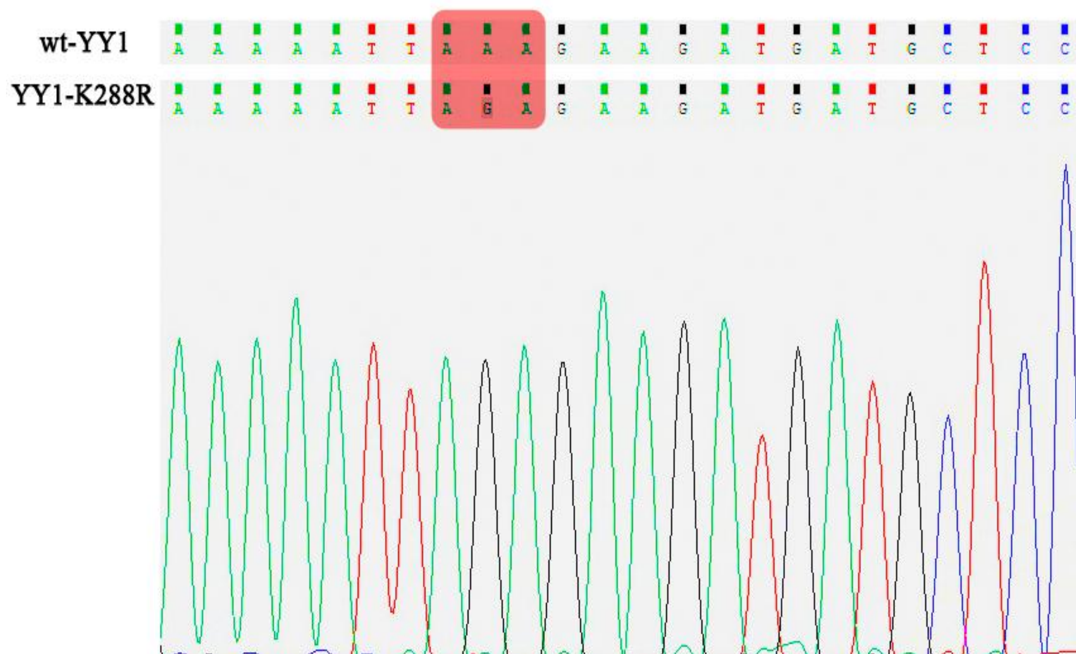

Figure 3.15: Sequence analysis of mutant YY1

3.3.3 Luminometrical Measurement of Data of YY1 constructs for *KATNB1* gene promoter

SH-SY5Y neuroblastoma cells were co-transfected with both F2 construct containing *KATNB1* core promoter and wt-YY1, or F2 and YY1-db or F2 and YY1-woR or F2 and YY1-woA or F2 and YY1-K288R, respectively. As control only F2 construct was transfected. All experiments were repeated 3 times on separate day as triplicates. Light units of independent experiments are given in table 3.1, 3.2 and 3.3.

Table 3.1: Measured light units of experiment n=1for *KATNB1* promoter

N=1		F2	F2 Basic	YY1	YY1 Basic	YY1-db	YY1-db basic	YY1-woR	YY1-woR basic	YY1-woA	YY1-woA basic	YY1-mut	YY1-mut basic
1	Firefly	18,87	0,5467	4,928	0,5767	18,63	0,4175	2,79	0,0599	3,208	0,2309	9,936	0,4937
	Renilla	1,449	2,249	4,934	4,648	2,919	3,731	1,02	0,9841	2,353	3,129	4,572	3,308
2	Firefly	14,25	0,5956	7,698	0,5414	14,97	0,383	2,495	0,0728	3,09	0,2121	11,62	0,5291
	Renilla	1,394	2,669	5,068	4,484	2,306	3,816	1,227	1,097	2,519	2,956	4,694	4,353
3	Firefly	19	0,5361	5,792	0,431	14,37	0,5109	2,686	0,1057	3,302	0,241	11,35	0,4289
	Renilla	1,578	2,063	3,876	3,646	2,935	3,814	1,011	1,384	2,744	3,315	4,152	3,283

Table 3.2: Measured light units of experiment n=2for *KATNB1* promoter

N=2		F2	F2 Basic	YY1	YY1 Basic	YY1-db	YY1-db basic	YY1-woR	YY1-woR basic	YY1-woA	YY1-woA basic	YY1-mut	YY1-mut basic
1	Firefly	12,42	0,3749	6,931	0,2427	12,01	0,1868	3,371	0,0468	3,554	0,1201	11,28	0,4355
	Renilla	0,9124	1,741	4,33	3,32	1,75	2,838	1,017	0,9356	2,645	2,02	4,775	5,448
2	Firefly	16,39	0,3889	6,351	0,2528	16,79	0,2474	2,794	0,0348	2,92	0,1414	11,74	0,4944
	Renilla	1,592	1,694	3,319	3,053	3,682	2,916	0,6478	0,6136	1,622	1,985	4,526	4,691
3	Firefly	16,59	0,3383	4,263	0,292	12,92	0,211	2,281	0,0309	2,86	0,1426	10,51	0,4914
	Renilla	1,506	1,545	1,48	3,272	2,182	2,319	0,7765	0,7121	2,16	2,136	4,086	5,094

Table 3.3: Measured light units of experiment n=3for *KATNB1* promoter

N=1		F2	F2 Basic	YY1	YY1 Basic	YY1-db	YY1-db basic	YY1-woR	YY1-woR basic	YY1-woA	YY1-woA basic	YY1-mut	YY1-mut basic
1	Firefly	8,487	0,1507	4,917	0,2116	13,44	0,1689	4,509	0,1054	4,188	0,1231	5,377	0,2303
	Renilla	0,3262	0,4805	1,438	1,131	0,6079	1,286	0,2963	0,7891	0,6492	0,9627	1,193	1,106
2	Firefly	12,64	0,1152	6,095	0,2209	8,373	0,2482	5,777	0,0832	4,877	0,1128	6,243	0,2411
	Renilla	0,3252	0,4231	1,358	1,028	0,4839	1,513	0,3541	0,4432	0,6593	0,8364	1,255	1,498
3	Firefly	11,57	0,1507	6,742	0,2706	15,57	0,2291	3,643	0,0647	4,597	0,2272	5,668	0,1207
	Renilla	0,3085	0,4649	2	1,143	0,6002	1,553	0,2507	0,567	0,6897	1,335	0,8287	0,8873

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 pGL3-Basic. Fold activity results for all 3 experiments are shown in Figure 3.16.

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

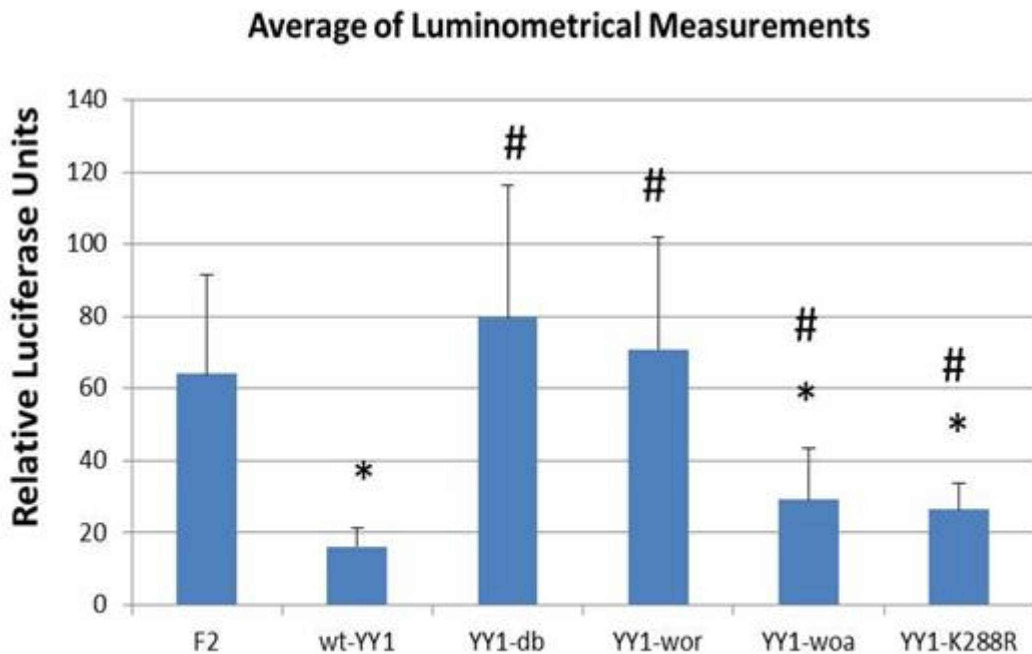

Figure 3.16: Average fold activities of YY1 constructs forced experiment on *KATNB1* core promoter.

According to these data, wt-YY1 acts as a repressor on *KATNB1* promoter. When the repressor domain of YY1 was excluded, repressive properties of YY1 was abolished. The reason of repression could be the recruitment of histone deacetylases to the proximity of the basal transcriptionan apparatus due to the interactions between HDACs and YY1 repressor domain.

Similar to YY1-woR, the YY1-db elevated the expression level. As stated by Austen *et al.* (1997), YY1-db with a spacer sequence, which resides between DNA binding and repression regions, decreased the expression level. However, in this study, YY1-db construct increased the expression level. Lysine 288, which reside in the spacer region, could be SUMOylated so that it might cause repression. However, YY1-db used in this study lacks of SUMOylation site and this might be the reason for increased expression level. Moreover, as stated in Rizkallah and Hurt (2009), the YY1 DNA-binding region includes phosphorylation sites; threonine 348, and 378. Phosphorylation of these residues cause the loss of YY1 DNA-binding activity. YY1 domains excluding DNA binding region, may in charge of recruitment of phosphatases to dephosphorylate residues on DNA binding region in order to recover YY1 DNA-binding activity. In our case, YY1-db which has only DNA-binding domain may not interact with phosphatases and not able to dock on target DNA so that cannot show repressive activity.

Although we were expecting the lowest activity for YY1-woA expressing cells, surprisingly the level of expression was higher than wt-YY1 expressing cells. Thus, activation domain of YY1 may interact with co-repressors. Further identification of YY1 interacting partners remains to be elucidated.

Furthermore, to investigate effect of SUMO modification, lysine 288 was converted to arginine because SUMO-modified proteins accept SUMO modification on lysine residue. According to luminometrical measurement data we can conclude that SUMO defficient YY1 increased expression level slightly but not as much as YY1-woR did. Hence, glycine/lysine rich R domain of YY1 may contribute to major repression activity. Another possibility is that, most SUMO-modified proteins contain the consensus motif ψKxE but, identified YY1SUMOylation site does not contain this motif and far from the gylcine/lysine (G/K) rich domain of YY1. So that, G/K rich region may have additional SUMOylation site that can be further investigated.

3.3.4 Luminometrical Measurement of Data of YY1 constructs for *SPG4* gene promoter

SH-SY5Y neuroblastoma cells were co-transfected with both S2 construct containing *SPG4* core promoter and wt-YY1, or S2 and YY1-db or S2 and YY1-woR or S2 and YY1-woA or S2 and YY1-K288R, respectively. As control only S2 construct were transfected. All experiments were repeated 3 times on separate day as triplicates. Light units of independent experiments are given in table 3.4, 3.5 and 3.6.

Table 3.4: Measured light units of experiment n=1 for *SPG4* promoter

N=1		F2	F2 Basic	YY1	YY1 Basic	YY1-db	YY1-db basic	YY1-woR	YY1-woR basic	YY1-woA	YY1-woA basic	YY1-mut	YY1-mut basic
1	Firefly	0,6279	0,0062	0,9703	0,01	1,6	0,0035	1,197	0,0092	0,7276	0,0067	0,9318	0,0096
	Renilla	0,3172	0,3048	0,8286	0,7994	0,9966	0,5425	0,377	0,2659	1,229	0,3719	0,7564	0,7794
2	Firefly	0,8112	0,003	1,151	0,0147	1	0,0075	0,8785	0,0035	0,5124	0,0028	0,6518	0,0104
	Renilla	0,3009	0,2011	0,8632	0,6368	0,7161	0,4777	0,2133	0,2454	0,8237	0,4002	0,6849	0,5563
3	Firefly	0,992	0,01	1,289	0,0145	1,185	0,001	0,6204	0,0003	0,6411	0,0065	0,9028	0,0079
	Renilla	0,2089	0,2072	0,7505	0,7642	0,5786	0,4029	0,2596	0,1622	0,8934	0,2751	0,6376	0,7038

Table 3.5: Measured light units of experiment n=2 for *SPG4* promoter

N=2		S2	S2 Basic	YY1	YY1 Basic	YY1-db	YY1-db basic	YY1-woR	YY1-woR basic	YY1-woA	YY1-woA basic	YY1-mut	YY1-mut basic
1	Firefly	1,5100	0,0183	0,9156	0,0353	1,6120	0,0180	0,1908	0,0032	0,4113	0,0140	0,8210	0,0273
	Renilla	1,4730	1,8910	3,4610	3,7640	2,9460	2,4640	0,6200	0,9934	1,6920	2,4510	3,1930	3,2600
2	Firefly	1,9890	0,0272	1,2450	0,0310	1,2970	0,0184	0,4297	0,0037	0,5118	0,0104	0,8886	0,0438
	Renilla	1,6590	2,1210	4,9400	3,1710	2,4160	2,7750	1,1540	1,0460	1,7510	2,1540	3,5490	5,8390
3	Firefly	1,6870	0,0220	0,9383	0,0272	1,5170	0,0181	0,3205	0,0074	0,5967	0,0149	1,1070	0,0351
	Renilla	1,4700	2,0230	3,7430	2,6260	2,4480	2,3670	0,9061	1,0900	2,0380	1,8230	4,3240	4,0150

Table 3.6: Measured light units of experiment n=3 for *SPG4* promoter

N=3		S2	S2 Basic	YY1	YY1 Basic	YY1-db	YY1-db basic	YY1-woR	YY1-woR basic	YY1-woA	YY1-woA basic	YY1-mut	YY1-mut basic
1	Firefly	1,8830	0,0077	1,1340	0,0286	1,0510	0,0152	0,8679	0,0080	0,9337	0,0137	1,3020	0,0216
	Renilla	0,8308	0,4994	1,7680	2,3650	0,5347	1,2860	0,5409	0,6894	1,1750	1,3610	1,8670	1,4690
2	Firefly	1,1760	0,0122	1,2860	0,0367	1,4970	0,0183	0,7518	0,0102	0,7603	0,0184	1,2200	0,0216
	Renilla	0,4442	0,7269	1,9590	1,6320	1,0180	1,7130	0,8146	0,7081	1,5640	1,1170	1,3750	1,5780
3	Firefly	0,8130	0,0149	1,6420	0,0330	2,2060	0,0119	0,9531	0,0084	0,9412	0,0160	1,2490	0,0153
	Renilla	0,2476	0,7409	2,2950	2,1190	1,6180	1,3100	0,9109	0,7940	1,4990	1,3750	2,1280	1,6010

The equation (3.1) was 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. Graph of average of the calculated fold activity of all 3 experiments are shown in Figure 3.16.

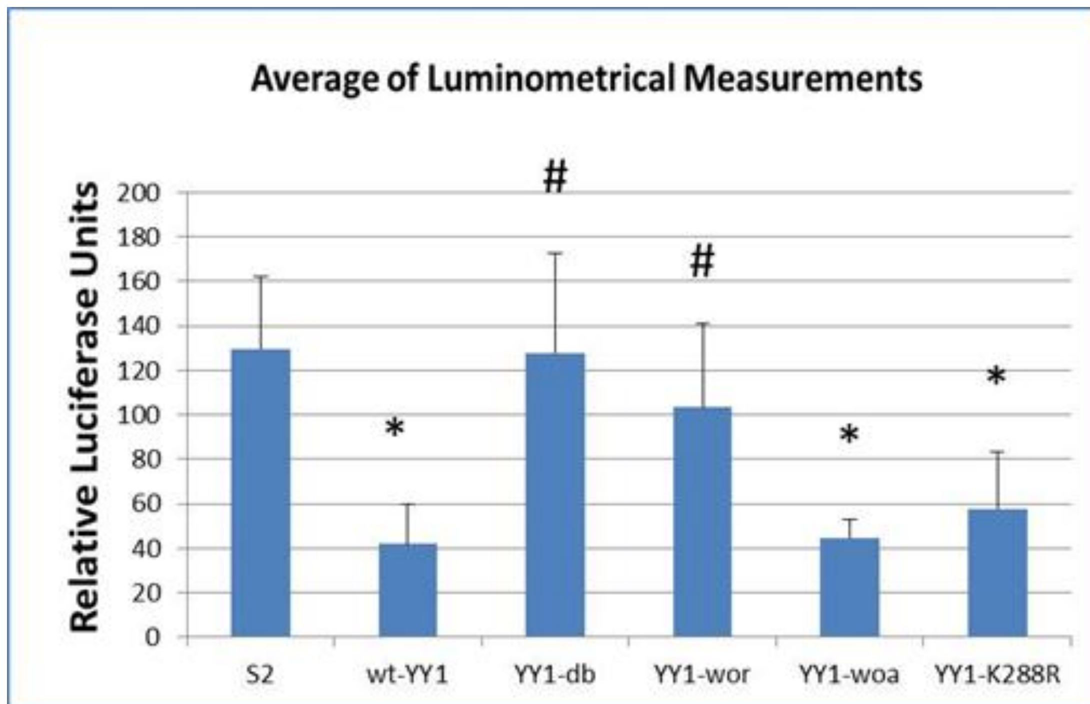

Figure 3.17: Average fold activities of YY1 constructs forced experiment on *SPG4* core promoter.

Results of experiments demonstrated that YY1 constructs had similar effect on *SPG4* promoter as seen in *KATNB1*. wt-YY1 acts as a repressor on *KATNB1* promoter. When the repressor domain of YY1 was excluded, repressive properties of YY1 was

abolished. Again the YY1-db elevated the expression level. Similar to YY1-woA effect on *SPG4* promoter, *KATNB1* promoter activity was elevated compared to YY1-db. According to luminometrical measurement data we can conclude that SUMO defficient YY1 increased expression level slightly but this increment was not significant statistically compared to wt-YY1.

4. CONCLUSION

Both Elk1 and YY1 can act as repressor or activator on gene promoters. Their fuction on *KATNB1* and *SPG4* promoters has not been studied. This study is the first report on regulation of KATNB1 and SPG4 gene promoters by Elk1 and YY1 transcription factors.

After confirming exact binding of YY1 to *KATNB1* and *SPG4* promoters, how YY1 regulates spastin and katanin expression remains to be elucidated. To illuminate this obscurity, we produced different YY1 constructs lacking funtional domains. The results demonstrated that YY1 acts as repressor on both *KATNB1* and *SPG4* promoters. SUMO-modification of YY1 was not effective on YY1 repressor activity. Major repressor function of YY1 was probably due to its glycine/lysine rich domain.

Finally, both *KATNB1* and *SPG4* promoters contain functional CpG islands. It is well known that methylation of these CpG islands affect gene expression. Hence, CpG islands in *KATNB1* and *SPG4* promoters may also alter expression level of katanin-p80 and spastin.

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APPENDICES

APPENDIX A: Elk1 (blue) and YY1 (yellow) binding sites on *KATNB1* promoter

-1374
F1 →
TCATCTGTAAATGGAACAGGAATTCCTACCTCTTATGGTTGTACTGGGCGTTATGTGAGCCCAAGGAGCCCTGACCTGCGGTGAGAGGCTGGAGGCCGAAGGGCA

AGGTTCGAGGGGCTCTGGGTGCCTGGGACAACCTCTGGGCAACAGGGAGAGGCGGCTGCGGGGCCAAGTCCCTCCTAAGCGGCCACGCGAGAGGCCCTGAGTCGAG

AAGGGTTACGGCGCTTCCCAGAGCCCACCCCGCCCAACGTGTCACAAATAAAGCTGGCCTTTCTCCGATTCCCTGATTGCAGCCATTCAGGGAGCAGCCCCTTGCC

CACGCATCCATTAGTCGAGGCGGTGGCTGCGTGTGCAGTGCTACAGGGTTCAGCGCCTGCCTCGGCTCCGGAATCAGGCTGTTTCCTTCTAGACCTCCCATGGGGG

-892
F2 →
ATTCAAGCCGGCGTCCGGGTCAAAGAACAGGGACAAAGTCCTCCTGCCACGGGGACCATCTGCAGGCAAAGTGAAGAATGAGGAGCTTCGGCAAAATGCCGACTA

AGGCCTCCTTAGGTTTTGCCCCACTCCAAGATGGAAGGCCTGAGGCTTCACACTGCCCCCGAAGTTCCTTTCCCATTGGCTATCTGGGAATTGAGTTTTCCAATAA

-699 ↓ -684 ↓ -653 F3 →
TGCGGACGCTGATTGGTCAATCCAGGACGGTTGCTCAAGCCATTGGCGCAGCCGCCATTGGAGGGCGGCTCTCAAAAGTTTTCAGACACAAATTAGGTTCGAGGGA

-600 F4 → -534 F5 →
GGAAACGGAGAGGAAAGGGAAAACTTGAGACGGAGGCGGGACTAAGGAAACGGCAGCTTGCATTGGTTTATTAGAGGCCAAGGGGCGGCTCTTGAACGTTCCTTCC
↑ -531

-461 ↓
TCTTGATTAGTCCTATTTAGGAAAAGAGGGCGGGTACTGAGGAAAAGCGGCAGAAGCGCCTGCTTCCATTGGTCAGTCCTGGCAGGAGGCGGAGCACCCGCGGCAG

-397 F6 → -389 F7 → -375
CTGATTGGTGCGGGAGGCAAGGTGGGCGGGGCTCTGAGCCGGAGGT

APPENDIX B: Elk1 (yellow) and YY1 (blue) binding sites on *SPG4* promoter

−1221
S1 →
GTTACTTTCTATTCGGTGGGTCTCCAGCCAAGATTCCAGGTCCCAGGAGAATCTGACTGACCTAGTGTTTGCTTCCGCCTTTGCGGTCTGGGTTCTG

TGCTTGCAGCTCATTAGAATACAGGGAGCAGAGACAAGCAGGTAGTTTCCCAAAGGAAGGGATGCTGAGTAGATTAAAAAAAAAGTGTAGATTCTTC

AGTAAACTATGGGATGGTAACTATGCAAAACCTAAGATTTCCCTTATTCAAATAAATTATCTTTCATATTAGACATCTAAATATGCACTAATTTAGT

-921
S2 →
TAAACCCCTGGGTTAGTTGATCTCATCACACTGAGCTAACATTTTTGTTGCTGTTGTTTGCAGTGACCTGAAGTTTCTTATCTTCACAATTGCTTTC

CTCTCAAATAATTCCCAGATTTTAAATTTTTATTTTATTTTTTCTGGAGACGGAGTCTCGCTCTGTCGCCCAGGCTGGAGTGCAGTGGCGCGATCTC

AGCTCACTTGCAGCCTCTGCCTCCCGAGTTCAAGCGATTCTCCGGCCTTAGCCTTCCAACCAGCTGGGACTACAGGCGCGCGCCCCCACGCCCGGCT

-614 ↓ −600 S3 →
AATTTAATTCCCAGATTGATATCCATTGCTTCTGAGATGGGCCAATTATCCTTCGGAGAAGACTTAGGTCGCCTGGCAGAAAAAGATGAAAGAAATC

−521 S4 → −471 S5 → − 451 ↓
TAAGAAAACGACGACACTGAGAGAGGAGCCTAGCGAACCAGCAGAGCGACCCCAAGCCGCAATTCCCCCTTCCGTGGATCGATTACGAAGGCTTCCT

−421 S6 → −388 S7 →
GGCAGGAGCTCTCCAGGGCTGCCGACGTGAGCCGAACTGCACATTGGGAACTGTAGTTGAGTGGGAAAGCCGAGAGGCGGGGGCCGCACACGCGTAC

−338 S8 → −310 S9 →
AGGGGCCCCGGTCAACAAAGACGCGCCGTGCGCGCGCGCGCCGGAGAAAAACACGGGAAGACGTGCGCGTGCGCGGCCGCCGCTGGGAGCCACCAGG

−247 S10 → −235 ↓ −222
CGGCGGAGAGGACAGCGACAGGAAGGGAGG

CURRICULUM VITAE

Name Surname: Koray KIRIMTAY

Place and Date of Birth: Eskişehir, 10.06.1985

Address: Yenigelin Zerde Sk. Gül Ap. No:2 D:20 Beşiktaş/İstanbul

E-Mail: koraykirimtay@gmail.com

B.Sc.: Bilkent University, Science Faculty, Department of Molecular Biology and Genetics, 2009

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