

**PROMOTER ELEMENTS ANALYSIS
OF KATANIN P80 GENE**

**M.Sc. Thesis by
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**Programme : Molecular Biology–Genetics and
Biotechnology**

JUNE 2009

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**Date of submission : 04 May 2009
Date of defence examination: 01 June 2009**

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JUNE 2009

**KATANİN P80 GENİNİN PROMOTOR
ELEMENTLERİNİN ANALİZİ**

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Tezin Enstitüye Verildiği Tarih : 04 Mayıs 2009
Tezin Savunulduğu Tarih : 01 Haziran 2009

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FOREWORD

I would like to express my sincere gratitude to my advisor Assoc. Prof. Dr. Arzu Karabay Korkmaz for her guidance, patience, continuous support and for giving me the opportunity to carry out this project and also for helping me increase my laboratory experience throughout this study.

I would like to thank Assoc. Prof. Dr. Işıl Aksan Kurnaz from Yeditepe University Department of Genetics and Bioengineering for providing Elk-1 and PEA3 constructs and critical discussions.

I would like to thank my lab partners and teachers Esra Karaca, Duygu Esen, and Şirin Korulu Koç for sharing all their knowledge, and their support with me at all time. I would like to thank Derya Canbaz for being every time with me in the lab and making the time passes easily till late hours lab work. I would like to thank Ayşegül Ünal, Ayşegül Dilsizoğlu, Meray Akkor and the other CYTO lab members for their help and friendship, and Özlem Demir from Yeditepe University Department of Genetics and Bioengineering for providing me what I needed anytime without any complaint and for sharing her experiences.

I would like to thank Kadir Can Çakıcı for his enduring morale support and encouragement and my dear friend Nazlı İncekara for being always with me, providing me such strong support despite having to put up with my frequent absences.

I would also like to acknowledge the funding agencies. I would like to thank TÜBİTAK for providing me financial support during the study and Istanbul Technical University Molecular Biology Genetics and Biotechnology Research Center for providing me a supportive working environment.

Finally, I gratefully acknowledge my father Zafer Cesur, my mother Nesrin Cesur and my wonderful family for their support, encouragement and love not only during this study but throughout my life.

June 2009

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ABBREVIATIONS

µg	: Microgram
µM	: Micromolar
µm	: Micrometer
AAA	: ATPases Associated with diverse cellular Activities
AD	: Activation domain
ADP	: Adenosine diphosphate
AMP	: Adenosine monophosphate
Amp	: Ampicillin
ATP	: Adenosine triphosphate
ATPase	: Adenosine triphosphatase
bp	: Base pair
BRE	: TFIIB recognition element
BTEB	: Basic transcription element binding protein
CBF	: CCAAT-box± binding factor
cDNA	: Complementary DNA
cm	: Centimeter
cm²	: Centimeter square
Ct	: Carboxy-terminal tail
CTF	: CCAAT-binding transcription factor
DLR	: Dual-Luciferase Reporter
DMEM	: Dulbecco's modified Eagle medium
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
dNTP	: Deoxyribonucleotide
DPE	: Downstream promoter element
<i>E.coli</i>	: <i>Escherichia coli</i>
E26	: E twenty six
EB	: Elution Buffer
EDTA	: Ethylenediaminetetraacetic acid
Elk-1	: Ets-like 1
EtBr	: Ethidium bromide
ETS	: E twenty six-specific
FBS	: Fetal bovine serum
g	: Gram
GFP	: Green fluorescent protein
h	: Hour
HDAC	: Histone deacetylase
Hi-Di	: Highly deionized
ID	: Inhibitory domain
Inr	: Initiator
kbp	: Kilo base pair
kDa	: Kilo dalton
KLF	: Krüppel-like factor

L	: Liter
LARII	: Luciferase Assay Reagent II
LB	: Luria-Bertani Broth
Luc +	: Luciferase
M	: Molar
MAP	: Microtubule-associated protein
MAPK	: Microtubule-associated protein kinase
min	: Minute
ml	: Mililiter
mM	: Milimolar
mm	: Milimeter
mRNA	: Messenger ribonucleic acid
MT	: Microtubule
NF-I	: Nuclear factor I
NF-Y	: Nuclear factor Y
ng	: Nanogram
nm	: Nanometer
nM	: Nanomolar
PCR	: Polymerase chain reaction
PEA3	: Polyomavirus Enhancer Activator 3
pH	: Power of hydrogen
PIC	: Preinitiation complex
PIPES	: Piperazine-N,N'-bis(2-ethanesulfonic acid)
Pi	: Inorganic phosphate
PMT	: Photomultiplier tube
pol II	: Polymerase II
RLU	: Relative Luciferase Unit
Rluc	: <i>Renilla</i> Luciferase
RNA	: Ribonucleic acid
RNAi	: Ribonucleic acid interference
RNase	: Ribonuclease
rpm	: Revolutions per minute
SD	: Standard deviation
SDS	: Sodium dodecyl sulfate
sec	: Second
sElk-1	: Short Elk-1
SOC	: Super Optimal Broth with catabolite repression
Sp1	: Specificity protein 1
SRF	: Serum Response Factor
SUMO	: Small ubiquitin-like modifier
TAE	: Tris-acetate-EDTA
TCF	: Ternary Complex Factor
TE	: Tris-EDTA
TFII	: Transcription factor II
TIEG	: TGF- β inducible early protein gene
TK	: Thymidine kinase
T_m	: Melting temperature
U	: Unit
UV	: Ultraviolet

v	: Volume
V	: Volt
w	: Weight
γ-TuRC	: γ -tubulin ring complex

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PROMOTER ELEMENTS ANALYSIS OF KATANIN P80 GENE

SUMMARY

Katanin is a heterodimeric protein that severs microtubules by hydrolyzing ATP. Katanin consists of 60 kDa and 80 kDa polypeptides. 60 kDa subunit (p60) coded from KATNA1 gene, has the enzymatic activity to break microtubules, whereas 80 kDa subunit (p80) coded from KATNB1 gene, has a role in localization of the protein complex in the cell. Katanin has been shown to have roles for microtubule severing in mitotic cells including release of microtubules from centrosome, depolymerization of microtubule minus ends in the mitotic spindle.

In this study, characterization of promoter site of KATNB1 and its potential effect on katanin gene expression regulation are aimed to be revealed. Identification of transcription factor binding sites in the promoter of the KATNB1 gene is in a great importance to understand temporal gene expression pattern of katanin which slightly differs between different stages of development. Identification of this gene regulation pattern will be a pioneer study for understanding the gene expression regulation of KATNB1 gene.

In this study, we set out sight on the identification of transcription factor binding sites (cis-regulatory elements) of KATNB1 promoter. By using this information, it will be possible to study the genetic and/or epigenetic factors which may be related with the cell division.

For characterization of promoter of KATNB1 gene, polymerase chain reaction (PCR) is used to amplify 1000 bp upstream nucleotides of KATNB1 gene. By using internal primers and nested PCR technique, shorter fragments are obtained. These promoter deletion constructs are cloned into a luciferase reporter plasmid vector which lack eukaryotic promoter and enhancer sequences. We took advantage of luciferase assay system for functional analysis of the promoter by transfecting neuroblastoma cells with these constructs and the effects of promoter parts on katanin expression is examined. 518 bp TATA-less promoter was found to be sufficient for high levels of activity. The luciferase reporter assay in human neuroblastoma cells indicated repression of promoter activity in the presence of overexpressed Elk-1 transcription factor and the assay also showed that overexpression of PEA3 transcription factor had no effect on KATNB1 promoter activity.

KATANİN P80 GENİNİN PROMOTOR ELEMENTLERİNİN ANALİZİ

ÖZET

Katanin 60 kDa (p60) ve 80 kDa (p80) büyüklüğünde iki alt üniteden oluşan ve ATP hidrolizleyerek mikrotubulleri parçalayan bir proteindir. KATNA1 geni tarafından kodlanan p60 alt ünitesi enzim aktivitesi ile mikrotubulleri keserken, KATNB1 geni tarafından kodlanan p80 alt ünitesi enzimin hücre içindeki lokalizasyonunda görevlidir. Mitotik hücrelerde kataninin mikrotubullerin sentrozomdan kesilerek serbest bırakılması, mikrotubul eksi uçlarının depolimerize edilmesiyle mitoz esnasında kromozomların kutuplara çekilmesi gibi hücre içi olaylarda görevli olduğu düşünülmektedir.

Bu çalışma ile KATNB1 geninin promotorundaki nükleotid dizisinin karakterizasyonunu ve bu dizinin katanin gen ifadesinin regülasyonu üzerine olan etkilerini ortaya çıkarmak amaçlanmıştır. Promotor bölgesindeki transkripsiyon faktör bağlanma bölgelerinin aydınlatılması kataninin gelişim süreci boyunca ekspresyonunun regülasyonunu anlamak açısından da büyük öneme sahiptir. Elde edilecek bu bilgi kullanılarak katanin gen ifadenmesi üzerine etkisi olan diğer bazı genetik ve/veya epigenetik etkilerin aydınlatılması mümkün olabilecektir.

Çalışmada promotor bölgesinin karakterizasyonu için polimeraz zincir reaksiyonu (PZR) ile KATNB1 geninin 1000 baz öncesinden başlanarak delesyon konstraktları hazırlanmış ve uygun primerler ile nested PZR tekniği uygulanarak daha kısa parçalar elde edilmiştir. Bu diziler promotor içermeyen fakat raportör (bildirici) bir gen (lusiferaz) içeren bir plazmit vektöre klonlanmıştır. Promotor bölgenin fonksiyonel analizi için bu konstraktların insan nöroblastoma hücrelerine transferiyle gen ekspresyonu üzerindeki etkilerinin incelenmesinde lusiferaz enziminin katalitik aktivitesinden yararlandık. Bu yolla katanin ekspresyonu üzerinde kilit öneme sahip nükleotid dizilerini bulunmuştur. 518 bp'lik TATA-sız promotorun yüksek seviyede promotor aktivitesi için yeterli olduğu gösterilmiştir. Lusiferaz bildirici deneyi ile insan nöroblastoma hücrelerinde, promotor bölgenin Elk-1 transkripsiyon faktörü tarafından baskılandığı, PEA3 transkripsiyon faktörünün ise KATNB1 promotor bölgesi üzerinde etkisinin olmadığı gösterilmiştir.

1. INTRODUCTION

1.1 Microtubules

Microtubules are polar linear polymers that perform major organizational tasks in living cells. Through a unique feature of microtubule assembly, termed dynamic instability, they function as molecular machines that move cellular structures during processes. They are also involved in generation of cell morphogenesis and organization, cell division, cell growth and intracellular organelle transport (Antal et al., 2007).

Microtubules are thought to have the most important roles among cytoskeletal fibers which are separated into 3 groups; intermediate filaments, microfilaments and microtubules. There are three distinct steps in the life cycle of a microtubule, namely, nucleation, assembly and disassembly. A microtubule nucleates, assembles by addition of subunits to its growing ends, and disassembles by endwise loss of grouped subunits (Wade and Hyman, 1997)

Microtubules are composed of a protein called tubulin, which is a heterodimer composed of related α and β chains. Those monomers are proteins of about 450 amino acids each and are about 50% identical at the amino acid level. Each monomer has a molecular mass of about 50 kDa (Burns, 1991). Each tubulin heterodimers form extended asymmetric protofilaments. Each tubulin heterodimer is located in the same orientation within the protofilament. Thirteen different protofilaments associate to form a cylindrical microtubule 25 nm in diameter, and all of the protofilaments are aligned as parallel (Watson et al., 2004). The crystal structure of the tubulin monomer (a) and the tubulin protofilament (b) can be seen in figure 1.1. The α subunit is shown in turquoise and the β subunit is shown in purple.

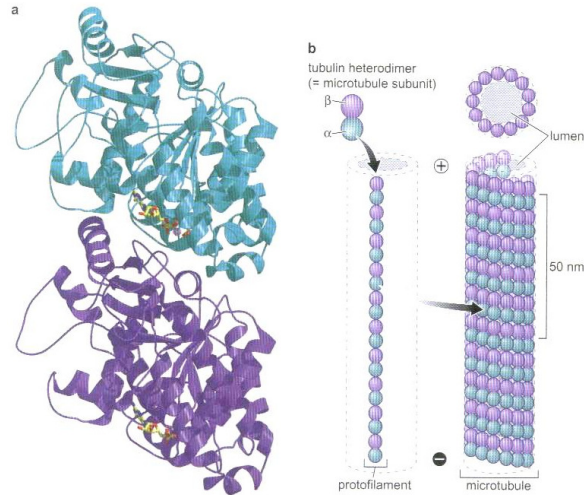


Figure 1.1 : Structures of the tubulin monomer and filament (Watson et al., 2004).

Each monomer binds a GTP molecule, nonexchangeable in α -tubulin and exchangeable in β -tubulin. GTP from β -tubulin is required for microtubule assembly, and its hydrolysis follows addition of a dimer to the microtubule end, upon which it becomes nonexchangeable within the microtubule. Microtubules comprising GTP-bound tubulin at the plus end are stable since these GTP caps strengthening the lattice of microtubule. Therefore, microtubules with tubulin-GTP continue to elongate (Caplow and Shanks, 1996). This phase is known as microtubule growth phase. As soon as the GTP cap of microtubule is detached, the lattice of microtubule becomes unstable and protofilaments are peeled from the lattice (Desai and Mitchison, 1997). This phase is known as shrinkage. Microtubules are switching between growth and shrinkage phases. This feature is entitled as dynamic instability (Mitchison and Kirschner, 1984) illustrated in figure 1.2.

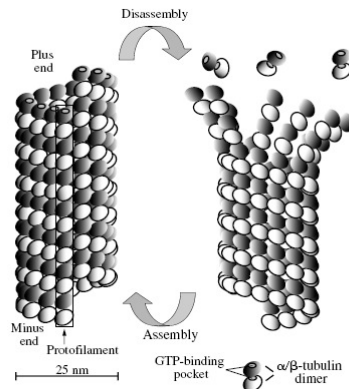


Figure 1.2 : Illustration of dynamic instability (Wiese and Zheng, 2006).

The structures of α - and β -tubulin are basically identical: each monomer is formed by a core of two β -sheets surrounded by α -helices. The monomer structure is very compact, but can be divided into three functional domains: the amino-terminal domain containing the nucleotide-binding region, an intermediate domain containing the taxol-binding site, and the carboxy-terminal domain, which constitutes the binding surface for motor proteins (Nogales et al., 1998). There are two proposed models for microtubule nucleation from tubulin dimers to microtubules shown in figure 1.3. In the first model (a) tubulin dimers associate to form sheets which subsequently close into microtubules. The second model (b) suggests that tubulin molecules associate to form oligomers that subsequently combine to form sheets and microtubules (Valiron et al., 2001).

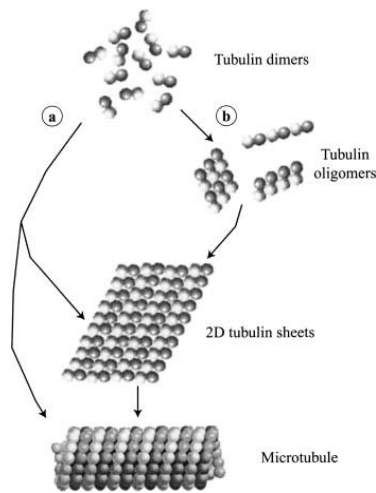


Figure 1.3 : Microtubule nucleation models (Valiron et al., 2001).

There are both lateral and longitudinal interactions between the tubulin heterodimer subunits and these interactions maintain the tubular form of microtubules. Thus, there is an intrinsic polarity in microtubules. One end of the microtubule is called the rapidly growing plus end, while the other end is called the minus end and it is more stable. Although microtubule polarity was originally defined in terms of the preferential addition of tubulin subunits onto the plus end of the polymer, it is now apparent that the polarity of the microtubule is also relevant to its transport properties (Baas, 1999). In addition to α - and β - tubulins, there is a special third type of tubulin named γ -tubulin. It is located in the centrosomal matrix, which is the primary site for microtubule nucleation in animal cells. γ -Tubulin, is related to α/β -tubulin and it is required for initiating the polymerization of microtubules in vivo. γ -

Tubulin has been found in two main protein complexes: the γ -tubulin ring complex and its subunit, the γ -tubulin small complex. Microtubules are thought to be nucleated from γ -tubulin ring complexes (γ -TuRCs) and these microtubules nucleated from γ -TuRCs have minus ends that are physically capped. These caps prevent minus-end polymerization and depolymerization (Moritz and Agard, 2001).

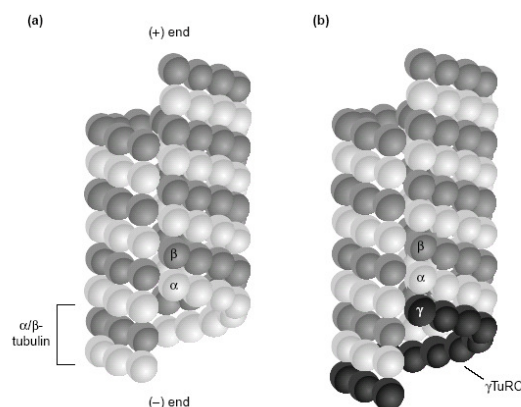


Figure 1.4 : Polymerization of tubulin nucleated by γ -tubulin ring complex (Moritz and Agard, 2001).

The intrinsic microtubule dynamics are further modified in the cell by interaction with cellular factors that stabilize or destabilize microtubules, which operate in both spatially and temporally specific ways to generate different microtubule assemblies during the cell cycle. Microtubule stability is promoted to a large degree by microtubule-associated proteins (MAPs). Classical MAPs, such as MAP2 and Tau, bind to the surface of the microtubule, bridging several tubulin subunits and possibly neutralizing the repulsive negative charge on the microtubule surface (Heald and Nogales, 2002).

Microtubules serve extensively important functions in some specialized cells like neurons. Microtubules in neurons which are post-mitotic cells are not employed for spindle formation but rather they function in elongation of axons (Karabay et al., 2004). Crucial events for axonal differentiation such as elongation, branching, navigation, retraction, are accomplished by changes in the configuration and behavior of microtubules (Baas and Buster, 2004). Microtubules are essential for axonal growth, and yet axons are incapable of locally synthesizing the tubulin subunits that compose microtubules. For this reason, tubulin must be actively transported down the axon from its site of synthesis within the cell body of the neuron (Baas, 1997).

1.2 Microtubule Severing

Although the dynamic behavior of the microtubules results primarily from the regulation of the subunit exchange at the ends of the microtubule polymer other mechanisms are important as well (Karabay et al., 2004). It has been shown that forces generated by motor proteins are essential for transporting microtubules into the axon, and potentially for integrating microtubules with neighboring microtubules and other cytoskeletal elements in neurons. (Ahmad et al., 1998).

Other data strongly suggest that the capacity of a microtubule to move in response to these forces is inversely proportional to the length of the microtubule, shorter the microtubule more rapid is the movement (Dent et al., 1999). Thus, it is obviously clear that microtubule movement may be regulated by regulation of its length. The physical length of these complexes being regulated by two different mechanism: (a) addition/loss of tubulin subunits at the ends of the microtubule polymer; and (b) internal breakage of the polymer by microtubule severing enzymes. This process, which can generate shorter microtubules from longer ones is known as microtubule severing (Casanova et al., 2009).

According to the proposed 'cut and run' model for microtubule transport, the motor proteins bind to all microtubules, regardless of their length, but cannot transport the longer microtubules, due to obstacle imposed on the microtubules as a result of crosslinks with other structures. The long immobile microtubules can only be transported after being severed into shorter pieces (Baas et al., 2005).

The first report of biochemical microtubule severing activity came from observations using *Xenopus* oocyte. A protein component of mitotic oocyte extracts caused the stable microtubules to be fragmented into numerous short segments. It was reported that the severing activity was low in interphase and was activated during mitosis through a posttranslational mechanism and suggested that microtubule severing may complement subunit exchange at the ends of microtubules in disassembling the interphase microtubule network at the onset of mitosis (Vale, 1991).

Three MT-severing enzymes have so far been identified: katanin, spastin and fidgetin, all belonging to the AAA (ATPases Associated with diverse cellular Activities) family of ATPases. These relatively recently discovered proteins play critical roles in essential cell processes such as mitosis, neuronal development and function, axonal branch formation and cilia biogenesis, and, as such, can be the cause of major human diseases (Casanova et al., 2009).

When it comes to possible roles of microtubule severing, there are many different mechanisms involved in a wide range of research with different organisms. These three different microtubule severing enzymes, spastin, fidgetin, and katanin are shown to contribute to the “Pacman-flux” machinery that moves chromosomes in *Drosophila melanogaster* (Zhang et al., 2007). Data from a study about *Chlamydomonas* deflagellation suggest that a katanin-like mechanism may mediate the severing of the outer doublet microtubules during deflagellation (Lohret et al., 1998). In another study, PF15p, which is the *Chlamydomonas* homologue of the katanin p80 subunit is shown to be required for assembly of flagellar central microtubules (Dymek et al., 2004). A very recent work on *Chlamydomonas* affirmed that katanin severs doublet microtubules at the proximal end of the flagellar transition zone, allowing disengagement of the basal body from the flagellum before mitosis (Rasi et al., 2009). A study conducted on *Caenorhabditis elegans* showed that MEI-1/MEI-2 katanin-like microtubule severing activity is required for *C. elegans* meiosis (Srayko et al., 2000) and further studies proved that MEI-1/katanin is required for translocation of the meiosis I spindle to the oocyte cortex (Yang et al., 2003). A recent study about prostate cancer declared that LAPSER1, a putative cytokinetic tumor suppressor showed the same centrosome and midbody subcellular localization pattern as p80 katanin subunit (Sudo and Maru, 2007) and another study showed that LAPSER1/LZTS2 a pluripotent tumor suppressor was linked to the inhibition of katanin-mediated microtubule severing, indicating that microtubule severing at centrosomes is a novel tumor-associated molecular subcircuit in cells, in which LAPSER1 is a regulator (Sudo and Maru, 2008, Maru, 2009). In another work, microtubule-severing proteins are shown to be involved in flagellar length control and mitosis in Trypanosomatids (Casanova et al., 2009). In *Tetrahymena*, katanin activity is shown to be essential and the net effect of katanin on the polymer mass depends on the microtubule type and location, it is proposed that katanin

mediated severing is non-random *in vivo* and that its activity is required to inhibit accumulation of post-translational modifications of microtubules. It is also postulated that katanin preferentially severs older, post-translationally modified segments of microtubules (Sharma et al., 2007).

Microtubule severing also plays a role in specific activities of differentiated cells for instance there are a great number of studies about neurons. It has been shown that microtubule severing by katanin is essential for releasing microtubules from the neuronal centrosome, and also for regulating the length of the microtubules after their release (Ahmad et al., 1999). These data support the idea that microtubule severing by katanin is important for the production of non-centrosomal microtubules in cells such as neurons and epithelial cells (Quarmby, 2000). Axonal growth is found to be sensitive to the levels of p60 katanin, the mechanisms that controls katanin levels and activity could be key to determining the growth properties of the axon (Karabay et al., 2004).

1.3 AAA Family Proteins

AAA ATPases (ATPases Associated with various cellular Activities) play important roles in numerous cellular activities including proteolysis, protein folding, membrane trafficking, cytoskeletal regulation, organelle biogenesis, DNA replication, recombination, restriction, sporulation, chelation, vesicle fusion, and intracellular motility. Sequence analysis indicates that this is an ancient class of proteins and the utility of these proteins is also evident by their abundant genomic representation (Vale, 2000; Neuwald et al., 1999).

The mutual feature of the AAA superfamily is an ATPase domain. It is known that AAA domains assemble into oligomeric structures and this allows proteins to change their shapes during ATPase cycle. ATP binding induces structural rearrangements at the interface region of AAA proteins. This increases interactions between adjacent AAA domains, also increases interactions between AAA protein and its target (McNally, F., 2000). The ATPase domains of these proteins assemble into active ring-shaped hexamers, this allows subunits to switch between tense and relaxed states in a concerted manner. These structures also provide framework for binding target proteins at multiple sites. If ring-binding sites change their positions, this will also cause tension application to bound protein (Vale, 2000).

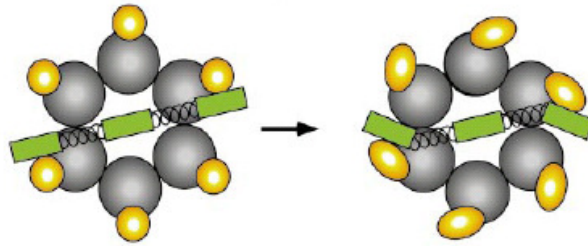


Figure 1.5: Conformational change of AAA protein ring (Vale, 2000).

While AAA proteins are highly homologous within the ATPase domain, usually located at the C-terminus of the protein, other regions of these proteins typically show little sequence similarity. The ATPase domain of AAA proteins is 200–250 amino acids long and is characterized by a number of domains that are important for ATP binding and hydrolysis. These include the Walker A P-loop, Walker B motif, sensor-1 and sensor-2 motifs, arginine finger and a second region of homology that differentiates classically defined AAA proteins such as NSF from the broader AAA+ family that includes members such as dynein. There are two other motifs conserved: the N-linker, which may transduce energy from ATP hydrolysis to the rest of the protein, and the loops that line the pore of a AAA oligomer (White and Lauring, 2007).

Their biological functions can be sub-grouped in three categories. First group is composed of those AAA family members that remodel protein complexes without unfolding or destroying their target proteins and the second group, those that are involved in protein quality control and do unfold their targets. Finally, a third group of microtubule-interacting proteins as their activities are unique in terms of the larger AAA family. This third group can be further divided into two sub-categories, motor proteins including the most well known cytoplasmic dynein and microtubule severing enzymes including katanin, spastin, and fidgetin. Those proteins are associated with microtubules in a different way; they are microtubule severing enzymes that make internal breaks in microtubules (White and Lauring, 2007). Katanin is the most well known microtubule-severing protein which will be discussed in detail in the coming section. Spastin is another microtubule-severing protein that is mutated in around 40% of cases of autosomal dominant hereditary spastic paraplegia (Salinas et al., 2007). Finally, fidgetin is another microtubule-severing protein, whose gene is found to be mutated in fidget mice. The mutant mice have small eyes, associated with cell-cycle delay and insufficient growth of the

retinal neural epithelium, and lower penetrance skeletal abnormalities, including pelvic girdle dysgenesis, skull bone fusions and polydactyly. Fidgetin is the first mutant AAA protein found in a mammalian developmental mutant, thus defining a new role for these proteins in embryonic development (Cox et al., 2000). In a recent study, spastin and fidgetin have been shown to stimulate microtubule depolymerization during anaphase in cultured *Drosophila* cells (Zang et al., 2007). Yet it is unclear how these enzymes use their ATPase activity to sever microtubules.

1.4 Katanin

Katanin is the most well characterized microtubule-severing protein. It was first appeared in scientific field in 1991, a protein component of mitotic *Xenopus* oocyte extracts caused the stable microtubules to be fragmented into numerous short segments (Vale, 1991). After that, a microtubule-severing protein from sea urchin eggs was purified and initially characterized. It was named katanin, a word derived from katana, which means the samurai sword in Japanese (McNally and Vale, 1993). Similar to the severing activity of M-phase *Xenopus* extracts, katanin requires ATP hydrolysis to sever microtubules *in vitro* (Vale, 1991; McNally and Vale, 1993). Video microscopy of the severing reaction showed that katanin rapidly and completely disassembled individual microtubules immobilized on the surface of glass coverslips only in presence of ATP.

This protein is a heterodimeric protein consisting of two subunits (McNally and Vale, 1993). p60 katanin subunit named after its 60 kDa molecular weight is coded from KATNA1 gene located in the long arm of chromosome 6 (6q25.1) in *Homo sapiens* (Url-1). The other subunit p80, named after its 80 kDa molecular weight is coded from KATNB1 gene located in the long arm of chromosome 16 (16q13) in *Homo sapiens* (Url-2).

The subcellular localization of katanin was determined by immunofluorescence in sea urchin, it is highly concentrated at centrosomes throughout the cell cycle, in a region surrounding the γ -tubulin containing pericentriolar region (McNally et al., 1996). The cloning of both katanin subunits from sea urchin and human has revealed that the 60 kDa subunit is a member of the AAA family of ATPases (Hartman et al., 1998; McNally and Thomas, 1998). Its N-terminal domain binds microtubules and C-terminal is the AAA domain (McNally K. et al., 2000).

Recombinant p60 katanin, expressed in Sf9 cells, has microtubule-severing activity and thus, appears to be the catalytic, enzymatic subunit that carries out the ATPase and severing reactions. Baculovirus-expressed other subunit p80 has no ATPase or severing activity in the absence of p60. This evidence suggests that p80 may be responsible for targeting p60 to the centrosome. The fact that p80 is present throughout all compartments of the neuron argues that its function can not be solely to target katanin to the centrosome. Biochemical studies have established that p80 katanin consists of multiple domains with different functions. Through deletion analysis of p80, a p60 dimerization domain, procon80 in its C-terminal domain and a separate centrosome-targeting domain consisting of six WD40 repeats in its N-terminal domain was mapped. The interaction profile of p60 and p80 katanin can be seen in figure 1.6. For p60 katanin, the domain shown in orange is required for association with the C-terminal domain of p80, the domain shown in blue is the microtubule binding domain and the domain shown in red is the ATPase domain. For p80 katanin, the domain shown in bright blue is the N-terminal WD40 domain which is required for centrosome or spindle pole targeting, the domain shown in blue is the microtubule binding domain and the domain shown in yellow is the procon80 domain that includes p60 binding domain.

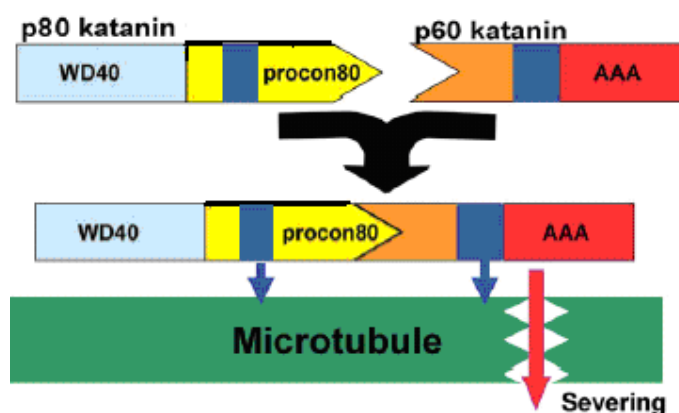


Figure 1.6 : The interaction profile of p60 and p80 katanin, adapted from Sudo and Maru (2008).

It was also shown that deletion of a 130 amino acid domain at the extreme COOH-terminus of p80 resulted in the complete inability of protein to dimerize with p60, whereas deletion of p80's amino-terminal WD40 domain had no effect on p60 dimerization and fusion of the WD40 domain to green fluorescent protein (GFP) revealed targeting of the construct to the centrosome in mammalian cells (Hartman et al, 1998).

The N-terminal WD40 domain of p80 katanin was found to act as a negative regulator of microtubule disassembly activity and also required for spindle pole localization, possibly through interactions with another spindle-pole protein. Both domains of p80 seem to be essential in precisely regulating katanin's activity *in vivo* (McNally K. et al., 2000). Although p60 shows its ATPase and severing activity in the absence of p80 subunit, p80 cannot sever microtubules on its own. Besides targeting katanin to the centrosomes, it also enhances severing capacity of p60. Association of the two subunits increases their affinity for microtubules and also microtubule-severing activity (Hartman et al., 1998).

Katanin is a microtubule stimulated ATPase; thus microtubule concentration affects the enzyme activity. A fluorescence resonance energy transfer assay demonstrated that the p60 subunit of katanin oligomerized in an adenosine triphosphate (ATP) and microtubule dependent manner. ATPase activity increases between 2 μ M-10 μ M microtubule concentrations, but at higher microtubule concentrations (>10 μ M), ATPase activity decreases. This ATPase behavior of katanin is unexpected, not matching the Michaelis–Menten hyperbolic stimulation. There are some explanations for that situation. Katanin binds microtubules at two sites, this increases local microtubule concentration by cross-linking and thereby stimulates katanin's ATPase activity. At higher microtubule concentrations, the ratio of katanin to microtubules is lower, less cross-linking occurs, thus less ATPase stimulation will be observed. A second explanation is about katanin oligomerization into rings. Microtubules promote p60-p60 oligomerization and oligomerization stimulates ATPase activity. While low microtubule concentrations facilitate oligomerization because of p60 monomers being more likely to bind near one another on the microtubule, when the microtubule concentration is high, this will inhibit p60 assembly by sequestering p60 monomers at non-contiguous sites (Hartman and Vale, 1999). Those data suggest a model for microtubule severing by katanin. Katanin-ADP is monomeric, but nucleotide exchange for ATP enhances p60-p60 affinity making oligomerization the most efficient. However, in the presence of its protein substrate, the p60 ring binds to microtubules with high affinity and once katanin oligomers assemble on the microtubule, ATPase activity is stimulated. Katanin ring conformation could change due to nucleotide hydrolysis and subsequent phosphate release that will lead to mechanical strain that destabilizes tubulin-tubulin contacts. This leads to the

dissociation of complex and the recycling of the katanin (Hartman and Vale, 1999; Quarmby, 2000). A model of microtubule severing by katanin is illustrated in figure 1.7.

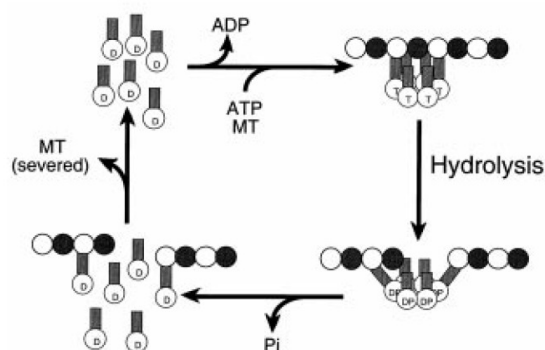


Figure 1.7: Model for microtubule severing by katanin (Hartman and Vale, 1999).

In cells, microtubule severing is controlled by regulatory factors, they can be used to hold the severing complex together after ATP hydrolysis, or might protect the new plus and minus ends from immediate disassembly just like MAPs. At the spindle pole, severing might produce free microtubule ends, allowing for the poleward flux of tubulin and poleward movement of the microtubule. At the centrosome, microtubule severing might release microtubules. In the cytoplasm, microtubule severing might facilitate treadmilling (Quarmby, 2000). Although it is known that katanin forms a transient hexamer in the presence of both ATP and microtubules (Hartman and Vale, 1999), the microtubule binding site to the hexamer remains to be unknown. Possible binding sites include the outside of the microtubule, the MT lumen, or the sides of dimers exposed by holes in the lattice; suggesting that katanin might act specifically at points in the lattice that contain defects. (McNally F., 2000). To interpret the experimental observations, a number of theoretical models were developed and compared quantitatively to the experimental data via Monte Carlo simulation. Models assuming that katanin acts on a uniform microtubule lattice were incompatible with the *in vitro* data, whereas a model that assumed that katanin acts preferentially on spatially infrequent microtubule lattice defects was found to correctly predict the experimentally observed breaking rates, number and spatial frequency of severing events, final levels of severing, and sensitivity to katanin concentration over the range 6–300 nM (Davis et al., 2002). These data are further supported by a study conducted on motile cilia *Tetrahymena*, a cell type with

elaborate microtubule arrays. It has been shown that katanin promotes assembly of ciliary microtubules, and therefore its effects are microtubule-type specific. Katanin-mediated severing is nonrandom *in vivo* and that its activity is required to inhibit accumulation of post-translational modifications on microtubules by regulating the longevity of nonciliary microtubules by preferentially depolymerizing post-translationally modified segments of the polymer (Sharma et al., 2007).

Also, it has been shown that there is an interaction between p60 katanin and P-NDEL1. This protein NDEL1, participates in the regulation of cytoplasmic dynein function during neuronal development. It is found to be mutated in the human neuronal migration defect lissencephaly and along with its partner LIS1. Abnormal accumulation of p60 in nucleus of Ndel1 null mutants supported an essential role of NDEL1 in p60 regulation (Toyo-Oka et al., 2005). It is also known that NDEL1 with its binding partner DISC1 and NDE1 is critical to neurodevelopmental processes aberrant in schizophrenia and NDEL1 significantly influences risk for schizophrenia via an interaction with DISC1 (Burdick et al., 2008). In another genome-wide transcriptome analysis study, using the postmortem brains of bipolar disorder sufferers and schizophrenic subjects confirmed the differential expressions of eight genes in a bipolar-specific manner. Those genes include KATNB1, coding for katanin p80 subunit (Nakatani et al., 2006). Taken together those studies indicate a novel role for katanin in psychiatric diagnosis like bipolar disorder and schizophrenia.

Although katanin's regulation and localization suggest a role in mitosis, its presence in adult brain tissue implies a second function in nondividing cells. Neurons express two different microtubule-severing proteins, namely p60-katanin and spastin. Because katanin is found at centrosomes in a variety of species and cell types, it is likely that katanin is concentrated around centrosomes in neurons as well. It was postulated that katanin concentrated at the centrosomes of neurons could release microtubules from their centrosomal attachment sites in the cell body to allow transport of microtubules down the axon (Baas and Yu, 1996). Axonal growth was found to be influenced of katanin levels, in neuronal cultures, katanin levels are high when axons are allowed to grow avidly but drop when the axons are presented with target cells that cause them to stop growing. Also expression of a dominant-negative p60-katanin construct in cultured neurons inhibits microtubule severing and is found

to be deleterious to axonal growth (Karabay et al., 2004). The ratio of p60 to p80 subunits are shown to vary in different tissues, at different time of development and regionally within the neuron. P80 subunit is found to be more concentrated in the cell body and less variable during development, whereas p60 is found to be often concentrated in the distal tips of processes (Yu et al., 2005). Studies conducted on different cell types show that katanin acts on microtubules according to the proposed 'cut and run' model. This activity is extremely important for microtubule organization and regulation. Motor proteins bind to all microtubules, regardless of their length, but cannot transport the longer microtubules; the long immobile microtubules can only be transported after being severed into shorter pieces by (Baas et al., 2005). The 'cut and run' model is illustrated in figure 1.8.

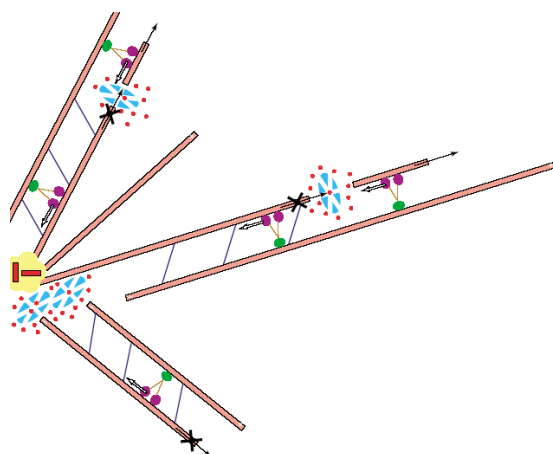


Figure 1.8: The 'cut and run' model for microtubule reconfiguration (Baas et al., 2005).

Another mechanism affecting katanin activity is the presence of microtubule-associated proteins (MAPs). Classical MAPs, such as MAP2 and Tau, bind to the surface of the microtubule and prompts to a large degree katanin activity. Microtubules in the axon are more resistant to severing by katanin than microtubules elsewhere in the neuron. When tau (but not MAP2 or MAP1b) is experimentally depleted from neurons, the microtubules in the axon lose their characteristic resistance to katanin (Qiang et al., 2006). Beside katanin neurons also express another microtubule severing protein known as spastin. However, these two proteins participate differently in axonal branch formation. P60-katanin is more highly expressed in the neuron, but spastin is more concentrated at sites of branch formation. During axonal branch formation, microtubule severing is based on local concentration of spastin at branch sites and on local detachment from microtubules

of molecules such as tau that regulate the severing properties of p60-katanin. It has been shown that tau protection from severing is greater in case of p60 katanin than spastin (Yu et al., 2008). A recent study conducted on transgenic mice model of Alzheimer's disease 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). Microtubule based abnormalities in Alzheimer's disease might consist of multiple phases. The first of which is an increase in the levels of tau, a microtubule associated protein, which blocks anterograde transport. The second phase represents an effort on the part of the neuron to combat the excess tau by hyperphosphorylating it leading to neurofibrillary tangles formation, which is one of the causes of the Alzheimer's disease, thus causing it to dissociate from the microtubules. It is known that hyper-phosphorylated tau cannot bind microtubules. Failure of tau binding to microtubules causes destabilization by increased de-polymerization leaving tau-deprived microtubules more accessible to severing proteins. As a result, axonal degeneration occurs. Model for microtubule-based axonal degeneration in Alzheimer's disease is illustrated in figure 1.9. This observation is completely new and might indicate targeting the microtubule severing protein as a possible therapeutic strategy for Alzheimer's disease (Baas and Qiang, 2005).

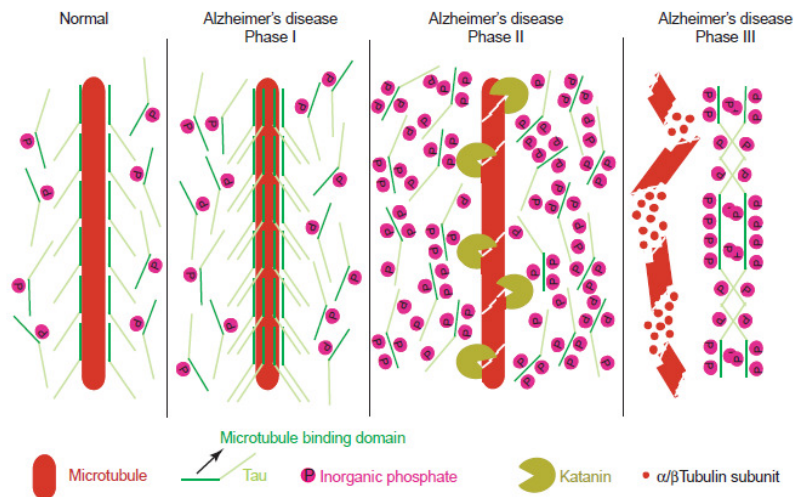


Figure 1.9 : Model for microtubule-based axonal degeneration in Alzheimer's disease (Baas and Qiang, 2005).

1.5 Transcriptional Machinery and Regulation in Eukaryotes

Transcription of a eukaryotic protein-coding gene is preceded by multiple events; these include decondensation of the locus, nucleosome remodeling, histone modifications, binding of transcriptional activators and coactivators to enhancers, promoters, and recruitment of the basal transcription machinery to the core promoter (Smale and Kadonaga, 2003). The many thousands of genes coding for proteins in eukaryotes are transcribed by common multiprotein machinery. The control of this process is predominantly mediated by a network of thousands of sequence-specific DNA binding transcription factors that interpret the genetic regulatory information, such as in transcriptional enhancers and promoters, and transmit the appropriate response to the RNA polymerase II transcriptional machinery.

Transcription is a complex process that relies on the collective action of the sequence-specific factors along with the core RNA polymerase II transcriptional machinery, an assortment of coregulators that bridge the DNA binding factors to the transcriptional machinery, a number of chromatin-remodeling factors that mobilize nucleosomes, and a variety of enzymes that catalyze the covalent modification of histones and other proteins (Kadonaga, 2004). Chromatin assembly is a fundamental biological process by which nuclear DNA is packaged into nucleosomes. The DNA of eukaryotes is ordinarily refractory to transcription because of its organization in nucleosomes (Fyodorov and Kadonaga, 2001). It is wrapped twice around an octamer of histone proteins, which interferes with many DNA transactions. Nucleosomes thus, serve as general gene repressors. They help assure the inactivity of all genes in eukaryotes except those whose transcription is brought about by specific positive regulatory mechanisms (Boeger et al., 2005). The packaging of DNA into chromatin provides the cell with the means to compact and to store its nuclear DNA, but it also creates an impediment to the function of DNA binding factors. To counterbalance the repressive nature of chromatin, a variety of chromatin remodeling factors use the energy of ATP hydrolysis to facilitate the interaction of proteins with nucleosomal DNA (Fyodorov and Kadonaga, 2001).

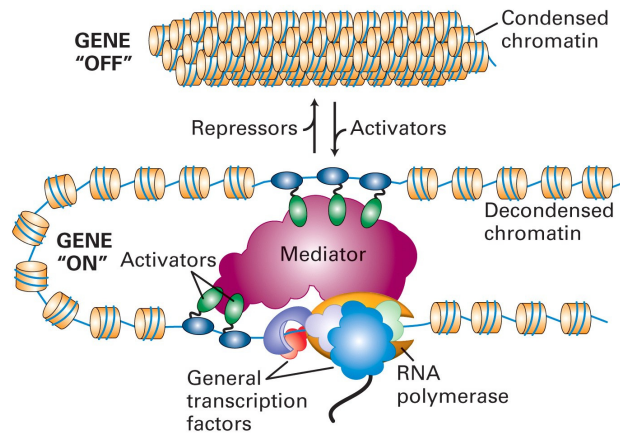


Figure 1.10 : Overview of transcription control in multicellular eukaryotes (Lodish et al. 2004).

A first indication of how repression by the nucleosome is relieved came from nuclease digestion of chromatin, showing an increase in accessibility of promoter DNA upon transcriptional activation (Boeger et al., 2005). Recently, it was found that active promoters are associated with histones modified in various ways, including acetylation, deacetylation, phosphorylation, dephosphorylation, ubiquitylation, deubiquitylation, ADP-ribosylation, methylation and sumoylation. Some of these modifications are correlated with and apparently required for transcription (Wu and Grunstein, 2000, Shiio and Eisenman, 2003). In eukaryotes, the core promoter serves as a platform for the assembly of transcription preinitiation complex (PIC) that includes TFIIA, TFIIB, TFIID, TFIIIE, TFIIF, TFIIF (transcription factor IIA, IIB, IID, IIE, IIF, IIF respectively) and RNA polymerase II (pol II), which function collectively to specify the transcription start site (Thomas and Chiang, 2006). The events leading to transcription of eukaryotic protein-coding genes culminate in the positioning of RNA polymerase II at the correct initiation site, the core promoter (Smale and Kadonaga, 2003). This transcription preinitiation complex could only support basal transcription and does not respond to the addition of gene-specific activator proteins. This observation led to the unexpected discovery and purification of “mediator”, a multiprotein complex composed of ~20 different proteins. The role of mediator in all eukaryotes from yeast to humans is to transfer positive and negative signals from DNA-binding, gene-specific transcription factors to RNA polymerase II and the general transcription factors. With the isolation of mediator, the three essential components of gene regulation and transcription in eukaryotes had been established; namely the general transcription factors, mediator

and RNA polymerase II (Kornberg, 2005). A simple diagram of transcription initiation complex is given in figure 1.11.

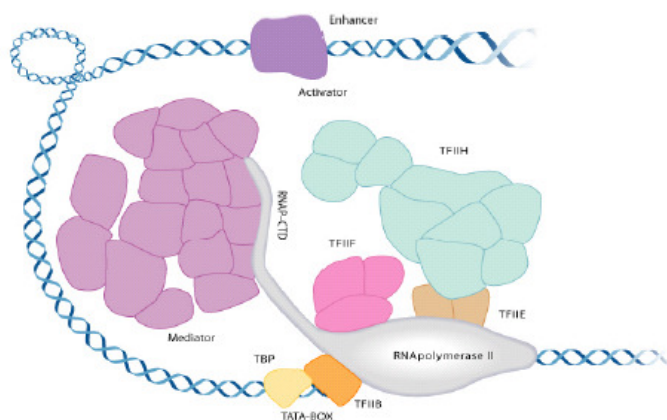


Figure 1.11: A cartoon of a eukaryotic transcription initiation complex (Thelander, 2006).

The TATA box (also named the Goldberg-Hogness box after its discoverers) was the first core promoter element identified in eukaryotic protein-coding genes with TATAAA consensus sequence found 25 to 30 bp upstream of the transcription start site. Following the early studies, it was speculated that the TATA box might be strictly conserved and essential for transcription initiation from all protein-coding genes from yeast to man. However, as the promoters for more and more genes were sequenced and characterized, the prevalence of the TATA box diminished. A database analysis of human genes revealed that TATA boxes were present in 32% of 1031 potential core promoters (Smale and Kadonaga, 2003). Core promoters can be classified into those that contain a functional TATA-box, TATA-box paired with an initiator (Inr), Inr element with downstream promoter elements (DPM), and CpG island-rich promoters which lack all three core elements (Smale, 2001). Figure 1.12 shows some of the sequence elements that can contribute to basal transcription from a core promoter (Smale and Kadonaga, 2003).

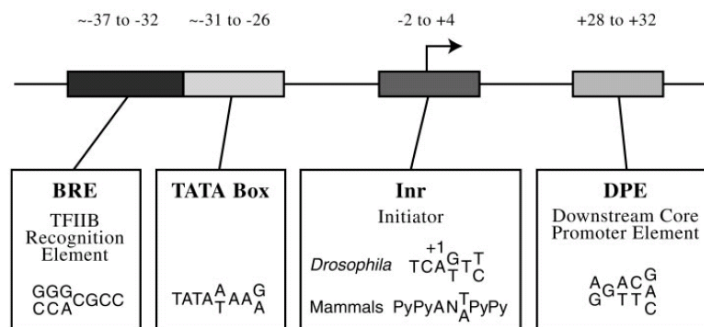


Figure 1.12 : Core promoter motifs (Smale and Kadonaga, 2003).

Numerous housekeeping genes lack both TATAAA and CCAAT sequence motifs but contains multiple GC boxes, determining the functional role of multiple GC boxes in the absence of TATAA and CCAAT motifs is crucial to the understanding of transcriptional regulation of these promoters. Transcriptional initiation is controlled by upstream GC-box interactions in a TATA-less promoter (Blake et al., 1990). The CpG dinucleotide, a DNA methyltransferase substrate, is underrepresented in the genomes of many vertebrates because 5-methylcytosine can undergo deamination to form thymine, which is poorly repaired by DNA repair enzymes. However, 0.5–2 kbp stretches of DNA exist that possess a relatively high density of CpG dinucleotides. The human genome contains ~29,000 of these CpG islands. Most importantly, it has been estimated that, in mammals, CpG islands are associated with approximately half of the promoters for protein coding genes. During early mammalian development, DNA methylation decreases substantially throughout the genome, followed by *de novo* methylation to normal levels prior to implantation. CpG islands are largely excluded from this phase of *de novo* methylation, and most remain unmethylated in all tissues and at all stages of development. Despite the prevalence of promoters associated with CpG islands, the elements that are responsible for their core promoter function remain poorly defined. In general, it has been difficult to identify core promoter elements within CpG islands that are essential for promoter function. One common feature of CpG islands is the presence of multiple binding sites for transcription factor Sp1. Transcription start sites are often located 40–80 bp downstream of the Sp1 sites, this suggests that Sp1 may direct the basal machinery to form a preinitiation complex within a loosely defined window (Smale and Kadonaga, 2003). The dinucleotide CG is underrepresented in vertebrate DNAs, and the presence of a CG-rich region, or CpG island, just upstream

of the start site is a distinctly nonrandom distribution. For this reason, the presence of a CpG island in genomic DNA suggests that it may contain a transcription-initiation region (Lodish et al., 2004).

1.6 Cis-acting regulatory elements

Cis-acting regulatory elements are control regions that regulate transcription of genes. Cis-acting regulatory elements that activate transcription of genes include proximal promoter, core promoters and enhancers and boundary elements.

Core promoters comprise DNA sequence motifs within 240 to 140 nucleotides relative to the RNA start site (such as the TATA box, TFIIB recognition element (BRE), initiator (Inr), and the downstream promoter element (DPE)) that, in the appropriate combinations, are sufficient to direct transcription initiation by the basal RNA polymerase II transcriptional machinery (Blackwood and Kadonaga, 1998).

Most core promoter elements appear to interact directly with components of the basal transcription machinery (Smale and Kadonaga, 2003).

Immediately upstream of the core promoter (from about 250 to 2200 bp relative to the RNA start site), there are typically multiple recognition sites for a subgroup of sequence specific DNA-binding transcription factors, which include Sp1, CTF (CCAAT-binding transcription factor; also called nuclear factor $\pm$ I, or NF-I), and CBF (CCAAT-box $\pm$ binding factor; also called nuclear factor $\pm$ Y, or NF-Y) named proximal promoter (Blackwood and Kadonaga, 1998).

One of the characteristic features of eukaryotic gene expression is the existence of sequence elements located at great distances from the start site of transcription which can influence the level of gene expression. These elements can be located upstream, downstream or within a transcription unit and function in either orientation relative to the start site of transcription. They act by increasing the activity of a promoter, although they lack promoter activity themselves and are hence referred to as enhancers (Latchman, 2004). Transcriptional control regions often contain multiple, autonomous enhancer modules that vary from about 50 bp to 1.5 kbp in size. Each of these modules appears to be designed to perform a specific function, such as the activation of its cognate gene in a specific cell type or at a particular stage in development (Blackwood and Kadonaga, 1998).

Boundary elements are DNA segments (from about 0.5 to 3 kbp) that are thought to function as transcriptionally neutral DNA elements that block, or insulate, the spreading of the influence of either positive DNA elements (such as enhancers) or negative DNA elements (such as silencers, or heterochromatin-like repressive effects) (Blackwood and Kadonaga, 1998).

1.7 Trans-acting Regulatory Elements

Transcription factors are trans-acting regulatory elements that must be stably expressed, translocate to the nucleus, bind DNA or other proteins in order to localize to the target gene, and interact with other factors including the RNA polymerase basal machinery, co-activators or co-repressors, and chromatin-remodeling complexes to regulate transcription (Gill, 2005).

The control of DNA binding by eukaryotic transcription factors represents an important regulatory mechanism. The DNA control elements that bind transcription factors often are located much further from the promoter they regulate. In some cases, transcription factors that regulate expression of protein-coding genes in higher eukaryotes bind at regulatory sites tens of thousands of base pair either upstream or downstream from the promoter. As a result of this arrangement transcription of a single promoter may be regulated by binding of multiple transcription factors to alternative control elements, permitting complex control of gene expression (Lodish et al., 2004).

Although the majority of transcription factors that have so far been described act in a positive manner, a number of cases have now been reported in which a transcription factor exerts an inhibitory effect on transcription initiation. This effect can occur by indirect repression, in which the repressor interferes with the action of an activating factor so preventing it stimulating transcription. Alternatively, it can occur via direct repression in which the factor reduces the activity of the basal transcriptional complex (Latchman, 2003).

Post-translational modification of many transcription factors by SUMO, small ubiquitin-like modifier that is covalently linked to lysine residues, has been correlated with different effects on their activity but mostly inhibition of transcription by modulating their ability to interact with their partners and controlling their

stability (Verger et al., 2003, Hay, 2005). Recent studies suggest that SUMO-dependent interactions with transcriptional co-repressors such as histone deacetylases (HDACs) might be one mechanism that contributes to regulation of transcription by SUMO, suggesting that complex interplay between acetylation and SUMOylation might be important for the regulated expression of many genes (Gill, 2005).

The control elements required for the transcription of a gene can be identified via some experimental approaches. DNA fragments with varying extents of sequence upstream of a start site are cloned in front of a reporter gene. By constructing and analyzing a 5'-deletion series, effects of promoter parts on expression can be examined. In this way, identification of key nucleotide sequences in the promoter which are important in gene expression can be revealed (Lodish et al., 2004). The experimental approach is illustrated in figure 1.13.

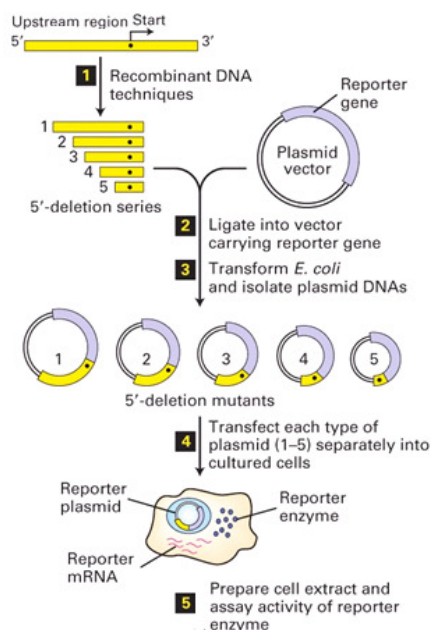


Figure 1.13 : Experimental procedure to identify control elements (Lodish et al., 2004).

1.7 .1 ETS domain transcription factor

Members of this family were originally identified on the basis of a region of primary sequence homology with the protein product of the v-ets oncogene encoded by the E26 avian erythroblastosis virus (Leprince et al., 1983). It was named after the retrovirus E26 (E twenty six-specific) (Dittmer and Nordheim, 1998). After comparing the sequence the sequence-specific DNA binding properties of the proteins encoded by the murine homolog of the ets-1 proto-oncogene and two ets-

related genes, murine PU.1 and Drosophila E74 it has been found that the protein products of these genes share sequence similarity within a region of 85 amino acids. This amino acid sequence similarity, combined with the observation that these proteins bind similar DNA sequences, provided the basis for the ETS-domain a new family of eukaryotic DNA-binding proteins (Karim et.al, 1990).

So far, approximately 30 members of the family have been identified in mammals, which are shown to encode nuclear transcription factors to regulate gene expression. A characteristic feature of this family is that they share an evolutionarily-conserved Ets domain of about 85 amino acid residues that mediate binding to purine-rich DNA sequences with a central GGAA/T core consensus and additional flanking nucleotides (Oikawa and Yamada, 2003).

ETS domain proteins can be subclassified phylogenetically according to the sequence of ETS-domains primarily, the position of this domain in the protein and the presence of other specific conserved domains and motifs. The 13 subfamilies identified are: ETS, TEL, YAN, SPI, ERG, PEA3, ELF, DETS4, ELK, GABP, ER71, ERF, and ESE (Laudet et al., 1999). Some of the members structures are shown in figure 1.14.

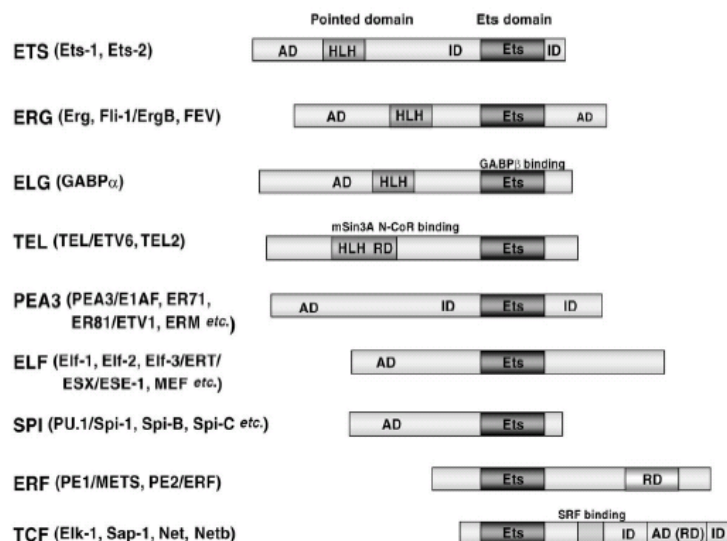


Figure 1.14: ETS-domain transcription factor subfamilies and their structures (Oikawa and Yamada, 2003).

ETS domain proteins can function as either transcriptional activators or repressors and in most cases, their activities are regulated by signal transduction pathways

(Sharrocks et.al., 1997). In addition, it was shown that some family members have both repressive and activating abilities, making the strict distinction impossible (Sharrocks, 2001). These signaling pathways include MAP kinases, Erk1 and 2, p38 and JNK, the PI3 kinases and Ca^{2+} -specific signals activated by growth factors or cellular stresses (Yordy and Helmericks, 2000).

Biologically, ETS-domain transcriptional factors appear to have distinct roles in regulating cellular proliferation and differentiation during both embryonic development and in the adult (Sharrocks et.al., 1997).

Some ETS family proteins are expressed ubiquitously, some in a tissue-specific manner (Oikawa and Yamada, 2003). Expression patterns of representative Ets family proteins are shown in figure 1.15.

Member	Expressing organs and tissues
Ets-1	Lymphoid organs, brain, vascular endothelial cells
Ets-2	Ubiquitous
Erg	Vascular endothelial cells, hematopoietic cells, kidney, etc.
Fli-1	Hematopoietic cells, vascular endothelial cells
GABP α	Ubiquitous
TEL	Ubiquitous
PEA3/E1AF	Epidermis, mammary gland, brain, etc.
Elf-1	Hematopoietic cells, liver, kidney, intestine, etc.
ESE-1/ESX	Epithelial cells
PU.1	B cells, macrophages, neutrophils
TCFs	Ubiquitous

Figure 1.15: Tissue distribution of major ETS family proteins (Oikawa and Yamada, 2003).

All known ETS-domain transcription factors bind to sites containing a central “GGA” trinucleotide motif. The residues flanking this motif decide whether a particular ETS-domain will bind the site. The ETS DNA-binding domain is characterized by the presence of three conserved tryptophans separated by 17-21 amino acids (Karim et al., 1990). Owing to its lack of sequence similarity to other known DNA-binding domains, the ETS-domain was initially thought to represent a novel structural DNA binding motif. However, after revealing of three-dimensional structure of three members of the family; Fli-1, Ets-1 and PU.1/Spi-1 the ETS-domain was placed in the winged helix-turn-helix superfamily of DNA-binding proteins. Due to high amino acid conservation amongst the ETS-domain of individual family members, this structural motif and mode of DNA binding are likely

to be conserved in all ETS domain proteins (Sharrocks et.al., 1997). DNA-binding sites of different ETS-domain transcription factors are shown in figure 1.16.

	-5	-4	-3	-2	-1	0	+1	+2	+3	+4	+5
Elk-1	A	A	C	C		A	A	G	T	Ga	
SAP-1a	N	A	C	C		At	Ga	Tc	N		
Ets-1	N	A	Ca	Ca		AT	Ga	T	T		
Ets-1	?	GA	GC	Ca		A	G	T	TC		
Ets-1	?	A	C	Ca		AT	Ga	TC	N		
Fli-1	GA	A	C	C		A	Ga	Tc	AG		
E74A	A	A	CT	C		A	Ga	T	Ga		
GABP α	?	GA	Cg	Ca		At	Ga	Tc	N		
ER81	?	Ga	Gc	Ca		AT	Ga	Tc	N		
ER71	?	GC	CG	Ca		TA	Ga	Tc	C		
Elf-1	AT	AC	C	C		A	G	Tc	GT		
Elf-1	At	Ca	C	C		A	G	T	RC		
Spi-1	A	AT	Gc	RC		A	Gc	T	AG		
Spi-B	A	At	Gc	AC		A	Gc	T	AT		
*Elk-1	N	GA	Ca	Ca		At	Ga	Tc	N		

Figure 1.16: DNA-binding sites of different ETS-domain transcription factors (Sharrocks et.al., 1997).

Due to high sequence conservation within these DNA-binding domains, members of particular families bind to similar DNA sequences. Thus, mechanisms must be in place to prevent promiscuous binding to inappropriate sites and provide specificity to ensure binding to the correct sites. One such mechanism is autoinhibition, whereby proteins block their own DNA binding by employing cis-acting inhibitory modules. These modules also often act to block other activities of transcription factors such as transcriptional activation or nuclear localization. Many transcription factors are controlled by cis-acting autoinhibitory modules that are thought to act by blocking random DNA binding in the absence of appropriate regulatory clues (Greenall et al., 2001).

1.7.1.1 PEA3 Family

The PEA3 (Polyomavirus Enhancer Activator 3) family of ETS-domain transcription factors contains three different proteins, PEA3 (also called E1AF or ETV4), ERM (also called ETV5), and ER81 (also called ETV1) which are 95% identical within the DNA binding (ETS) domain, more than 85% identical in the 32-residue acidic domain (AD) located in the amino-terminal part of the proteins, and almost 50% identical in the final 61 residues corresponding to the carboxy-terminal tail (Ct) (Launoit et al., 1997). The conserved domains of PEA3 family of ETS-domain transcription factors can be seen in figure 1.17.

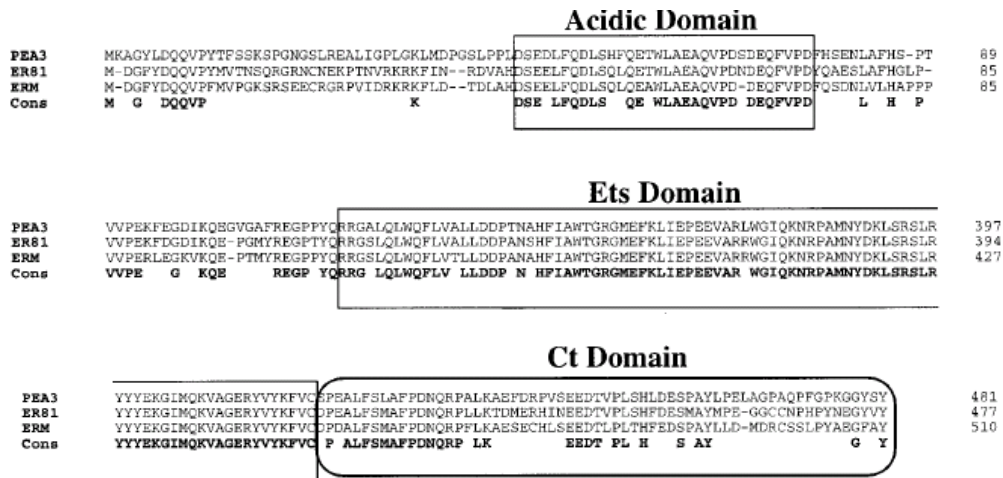


Figure 1.17 : Sequence alignment of human PEA3 group proteins (Launoit et al., 1997).

Members of this group of proteins are conserved at both the sequence and functional levels in vertebrates as diverse as humans and zebrafish. Biological roles for PEA3 family members include promoting muscle cell differentiation and the definition of connecting muscle motor and sensory neurons. Embryonic expression patterns and aberrant expression in metastatic tumors, suggest a role for PEA3 proteins in regulating cell motility during organogenesis/development and during tumor progression (Greenall et al., 2001).

In 1998, zebrafish PEA3 was characterized and a high degree of sequence conservation is observed in the ETS DNA binding domain and acidic transcriptional activation domain. Moreover zebrafish PEA3 also exhibits a virtually indistinguishable DNA binding specificity to mammalian subfamily members and is autoregulated by cisacting inhibitory domains. The developmental pattern of PEA3 expression suggests that it may play a role in the development of the somites and in regionalisation of the nervous system (Brown et al., 1998).

Other roles of PEA3 group transcription factors are shown recently, they are found to be expressed in tissues undergoing branching morphogenesis and promote formation of duct-like structures by mammary epithelial cells *in vitro* (Chotteau-Lelièvre et al., 2003) and also they are thought to play a role in epithelial–mesenchymal interactions during lung organogenesis (Lui et al., 2003).

Recently it was shown that PEA3 group transcription factors have a role in metastatic process, playing a role in many human cancer including oral tract, lung, stomach,

liver, colon, ovary, breast and endometrium. In particular, they regulate the expression of target genes involved in metastasis. Those genes are several matrix metalloproteases, which are enzymes degrading the extracellular matrix during normal remodelling events and cancer metastasis (Davidson et al., 2003, Bieche et al., 2004, Launoit et al., 2006).

The *pea3* ETS transcription factor is overexpressed in the vast majority of human breast tumors, in the epithelial cells of the mammary gland and is found to be required for HER2/Neu-mediated mammary oncogenesis (Sheperd et al., 2001).

The *pea3* subfamily transcription factors also play a key regulatory role in mammary gland development, they are found to be normally expressed in the primitive epithelium of mouse mammary buds during embryogenesis, and these three genes are expressed in epithelial progenitor cells during postnatal mammary gland development (Kurpios et al., 2003).

It has been shown by means of *in situ* hybridization experiments that all three genes are expressed in numerous developing murine organs including brain, kidney, lung, and heart (Chotteau-Lelièvre et al., 1997). At the mRNA level, ERM has been classified in adult humans and mice as a ubiquitously expressed gene with its highest expression in the brain and in the placenta. ER81 displays an expression pattern with high expression levels in human and murine lung, heart, and brain. Interestingly, PEA3, whose expression is very weak in normal human tissues, is expressed in an extensive series of nonhematopoietic cell lines (Launoit et al., 1997).

The human and mouse ER81, ERM, and PEA3 genes are structured in 13–14 exons distributed over more than 15 kbp of genomic DNA. In the human genome, ERM is located on chromosome 3 at position 3q27–29, PEA3 on chromosome 17 at position 17q21, and ER81 on chromosome 7 at position 7q21 (Launoit et al., 2006).

Like many transcription factors, the PEA3 group members also undergo post-translational modification that regulates their transactivation capacity. The most common modification found in the PEA3 group proteins is phosphorylation, as they are targets of the MAPK pathway including Ras, Raf-1, MEK, ERK-1, and ERK-2. Phosphorylation of specific serine and threonine residues generally increases the transactivation capacity of the PEA3 group member. Post-translational modifications

at lysines also play crucial roles in the regulation of transcription, generally on histone proteins but also on transcription factors (Launoit et al., 2006).

1.7 .1.2 TCF Family

Ternary Complex Factor (TCF) family is the most studied subfamily of ETS factors. The first TCF transcription factor was identified in the nuclear extract of HeLa cells which formed a ternary complex with Serum Response Factor (SRF) on the c-fos promoter. This novel protein was called p62 due to its 62000 Da molecular weight. Then, it was shown to be homologous to Elk-1 (Ets-like 1) protein, which had been cloned previously by Hipkind et al. in 1991. Later, two homologous proteins of Elk-1, Sap1 and Net/Sap2 have been identified (Sharrocks, 2002).

Beside the three TCFs Elk-1, Sap-1a and Net identified, there are also their isoforms sElk-1, ΔElk-1, Sap-1b, Net b and Net c.

TCFs share 4 similar regions, known as A, B, C and D domains, which were identified by sequence comparison between Elk-1 and Sap-1. The N-terminal A domain corresponds to the ETS DNA binding domain. It has also been demonstrated to act as a transcriptional inhibitor in Elk-1 by recruiting corepressors and DNA binding inhibitors, and to contain a nuclear export signal in Net. The B domain interacts with the MADS box transcription factor family member SRF and allows ternary complex formation. The C-terminal C domain is an activation domain that is activated by phosphorylation by Mitogen-Activated Protein Kinases (MAPK) containing multiple S/T-P MAP kinase phosphorylation sites. The D domain is a docking site for MAP kinases as well as a nuclear localization signal in Net (Buchwalter et al., 2004). Elk-1 is shown to contain a novel class of repression domain (R motif) in its C-terminal region, which reduce its basal transcriptional activity and dampen its response to mitogenic signals (Yang et al., 2002). Structure of TCF subfamily members is shown in figure 1.18.

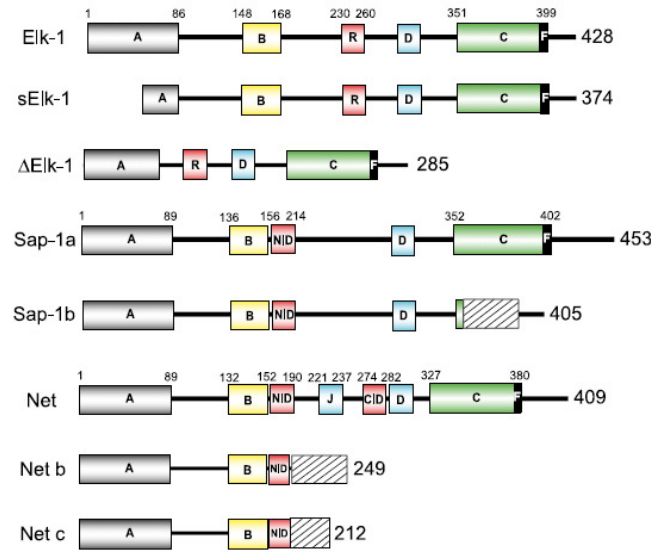


Figure 1.18 : Structure of TCF subfamily members (Buchwalter et al., 2004).

Elk-1 is the best studied of the TCFs, a role for Elk-1 in neuronal function has been proposed based on its expression pattern. Elk-1 mRNA is located in various adult rat brain structures, including the soma, dendrites and axon terminals of neurons (Sgambato et al., 1998). In addition, sElk-1, the shorter isoform, has been shown to play an opposite role to Elk-1 in an *in vitro* model of neuronal cell differentiation. By inhibiting Elk-1 transactivating properties, sElk-1 could promote differentiation, whereas Elk-1 alone drives cell cycle progression through activation of c-fos (Sharrocks, 2002).

Elk-1 is a direct target of the MAP kinase pathways, when phosphorylated by MAP kinases at Serine 383, its activation is triggered via the recruitment of the mediator complex through Sur2 but its R domain causes suppressing of its activity. It has been shown that SUMO modification of the R motif is the cause of this repression. There is a dynamic interplay between the activating MAPK pathway and SUMO pathway. When the MAPK pathway is activated, it stimulates the loss of SUMO conjugation and hence the repressive activity of the R motif is lost (Yang et al., 2003). The dynamic interplay is illustrated in figure 1.19.

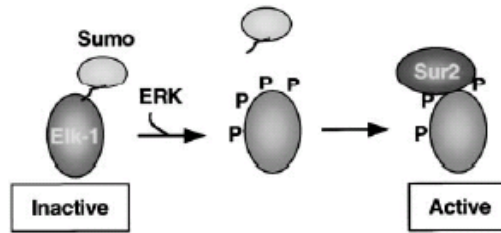


Figure 1.19: Dynamic interplay of SUMO and ERK pathways to regulate the transcriptional activity of Elk-1 (Yang et al., 2003).

The mechanism of SUMO modification leading to Elk-1 repression model is revealed in 2004. It has been shown that histone deacetylase 2 (HDAC2) interacts preferentially with SUMO-modified Elk-1. Inhibition of HDAC2 with chemical inhibitors or by RNAi de-repressed activity of SUMOylated Elk-1, and chromatin immunoprecipitation analysis revealed that increased HDAC2 leads to decreased histone acetylation and hence transcriptional repression at Elk-1 target genes. These studies provided evidence that histone deacetylase HDACs contribute to transcriptional inhibition by SUMO (Yang et al., 2004).

Recently it has been shown that *egr-1* gene is repressed by Elk-1 in normally cycling SH-SY5Y neuroblastoma cell line in a SUMO-dependent manner. Constitutively active Elk-1 construct, Elk-VP16, is found to be capable of activating the *egr-1* gene expression but a SUMOylation mutant of Elk-1, namely ElkK2R, was found to be involved in repression of the *egr-1* promoter. Those findings suggest that neuroblastoma cells regulate the levels of *egr-1* protein through HDAC recruitment to the *egr-1* promoter via Elk-1 and resulting repression of the promoter, thereby escaping apoptosis and surviving (Demir and Aksan-Kurnaz, 2008). This model of action is illustrated in figure 1.20.

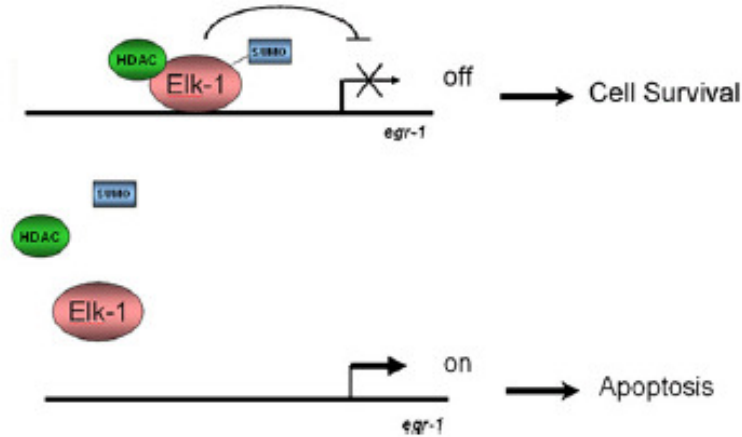


Figure 1.20: This model of action of repression-activation of Elk-1 on *egr-1* promoter (Demir and Aksan Kurnaz, 2008).

The SUMOylation sites for Elk-1 are also identified, SUMO is conjugated on either lysines 230, 249, or 254. Mutation of all three sites is found to be necessary to fully block SUMOylation *in vitro* and *in vivo*. The mutant form Elk-1 (3R) is found to shuttle more rapidly to nuclei of Balb/C cells fused to transfected HeLa cells. When SUMO-1 or -2 are coexpressed, it is shown to strongly reduce shuttling by Elk-1 without affecting that of Elk-1(3R), indicating that SUMOylation regulates nucleocytoplasmic shuttling of Elk-1 (Salinas et al., 2004).

In a recent study, it has been shown that Elk-1 colocalized with microtubules in hippocampal neuron cultures and SH-SY5Y neuroblastoma cell lines, and that Elk-1 protein biochemically interacted with microtubules *in vitro*. It was further demonstrated that upon serum induction, most of the phospho-Elk-1 translocates to the nucleus, which is independent of translation. This leads to the idea that Elk-1 is anchored to neuronal microtubules in resting or unstimulated cells. Upon stimulation, its phosphorylated form, phospho-Elk-1, relocates to the nucleus in neurons where it is proposed to regulate neuronal target genes (Demir et al., 2008).

1.7.2 Sp Family Transcription Factors

The Sp family is a family of transcription factors that bind to cis-elements in the promoter regions of various genes. Regulation of transcription by Sp proteins is based on interactions between a GC-rich binding site (GGGCGG) in DNA and C-terminal zinc finger motifs in the proteins (Kishikawa et al., 2002). The human transcription factor Spl (Specificity protein 1) is one of the very first cellular transcription factors to be identified in the early 1980s as a factor able to bind and

activate the SV40 early promoter (Briggs et al., 1986). Sp1 protein binds to the G-rich sequences present in a variety of cellular and viral promoters and stimulates their transcriptional activity. Sp1 consists of three contiguous Cys₂His₂ zinc-finger domains that bind to the decanucleotide consensus sequence 5'GGGGCGGGGC 3' and similar sequences that are referred to as GC boxes (Park et al., 1998). For some time it has been known that the general transcription factor Sp1 can bind to and act through the GC-boxes and it was generally accepted that this protein is an extremely versatile protein involved in the expression of many different genes. However, it became clear that Sp1 is not the only protein acting through 'Sp1-binding sites' but simply represents the first identified and cloned protein of a small protein family (Suske, 1999). A number of other GC and/or GT box-binding factors homologous to Sp1 have been isolated, namely Sp2, Sp3 and Sp4, and the two more distantly related factors, BTEB1, BTEB2 (basic transcription element binding protein 1 and 2) (Lania et al., 1997), and also TIEG1, TIEB2 (TGF- β inducible early protein genes 1 and 2) (Suske, 1999). In addition, another subgroup of zinc finger, Sp-1 like proteins the so called Krüppel-like factors (KLF) were also identified (Lomberk and Urrutia, 2005). Sp1-like/KLF transcription regulators may take part in virtually all facets of cellular function, including cell proliferation, apoptosis, differentiation, and neoplastic transformation. Individual members of the Sp1-like/KLF family can function as activators or repressors depending on which promoter they bind and the coregulators with which they interact (Kaczynski et al., 2003).

All four human Sp-family members have similar domain structures. They contain three zinc fingers close to the C-terminus and a glutamine-rich domain adjacent to serine/threonine stretches in their N-terminal region. Structural features of Sp-proteins are illustrated in figure 1.21. Colored boxes indicate regions of the proteins that are rich in glutamine (red) and serine/threonine (yellow) residues. The region preceding the first zinc finger (+/-) is rich in charged amino acids. The black boxes represent the zinc fingers, known activation (AD) and inhibitory domains (ID) are also indicated (Suske, 1999).

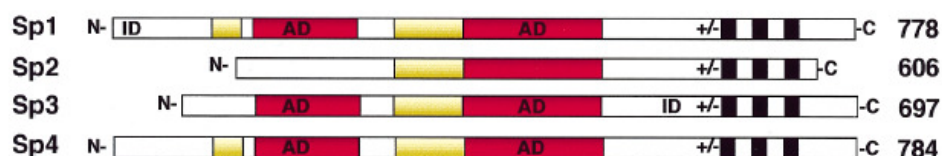


Figure 1.21: Structural features of Sp-proteins (Suske, 1999).

Sp1, Sp3 and Sp4 recognize the classical Sp-1 binding site with identical affinity. Furthermore, Sp1 and Sp3 are structurally and functionally highly related, whereas Sp2 appears to have distinct DNA binding specificity, shown to bind GT-rich elements, and its functional roles are not clearly defined. Sp1 is known to be phosphorylated and glycosylated, and it is capable of forming homotypic interactions leading to multimeric complexes. (Suske, 1999). In contrast to Sp1 and Sp3, which are ubiquitous transcription factors, Sp4 expression is restricted to a few tissues; predominantly in the neuronal tissues and in certain epithelia. Functional analyses have shown that Sp4, like Sp1, is a transcriptional activator, whereas Sp3 has been shown to both repress and activate transcription (Park et. al., 1998). The experimental conditions which are needed for Sp3 to act as a strong activator or a transcriptional inactive molecule, which represses Sp1-mediated activation, are not completely understood. The structure and the arrangement of the recognition sites appear to determine whether Sp3 is transcriptionally inactive and can repress Sp1-mediated activation or whether it acts as a strong activator (Suske, 1999). A study conducted on human dopamine transporter gene promoter demonstrated that both Sp3 and Sp1 serve as strong trans-activators of the promoter, albeit with different efficacy and via different Sp-binding sites (Wang and Bannon, 2005). Sp1 and Sp3 were found to be bound to the same cis-element in DNA methyltransferase gene 1 promoter and in human secretin receptor gene promoter, and the relative ratio of Sp1 to Sp3 is one of the critical factors controlling the tissue-specific and stage-specific expressions of those genes (Kishikawa et al., 2002, Pang et al., 2004).

1.8 Genetic reporter System

Reporter genes or markers provide convenient means to identify and analyze the regulatory elements of genes. Certain genes are chosen as reporters because the characteristics they confer on organisms expressing them are easily identified and measured, or because they are selectable markers. Commonly used reporter genes are chloramphenicol acetyltransferase, β -galactosidase, β -glucuronidase, luciferase,

alkaline phosphatase and green fluorescent protein (Sambrook and Russell, 2001). The purpose of the reporter assay is to measure the regulatory potential of an unknown DNA-sequence. Reporter genes can be used to assay for the activity of a particular promoter in a cell or organism. This can be done by linking a promoter sequence to an easily detectable reporter gene and the reporter gene product's activity is quantitatively measured. The results are normally reported relative to the activity under a consensus promoter known to induce strong gene expression. Standard recombinant methods are used to join the regulatory sequence of interest to a reporter gene in an expression vector. The resulting recombinant is then introduced into an appropriate cell line, where its expression is detected by measurement of the reporter mRNA or the reporter protein, or, in the case of enzyme reporter, by assaying for the relevant catalytic activity. Most of the detection methods are sensitive, specific, quantitative, reproducible, and rapid to perform. Reporter systems are used to measure transcriptional activity in particular to study promoters and enhancers and their interactions with *cis*-acting elements and *trans*-acting proteins (Sambrook and Russell, 2001).

1.8 .1 Dual-Luciferase Reporter Assay System

Dual reporters are commonly used to improve experimental accuracy. The term dual reporter refers to the simultaneous expression and measurement of two individual reporter enzymes within a single system. Typically, the experimental reporter is correlated with the effect of specific experimental conditions, while the activity of the co-transfected control reporter provides an internal control that serves as the baseline response. Normalizing the activity of the experimental reporter to the activity of the internal control minimizes experimental variability caused by differences in cell viability or transfection efficiency. Other sources of variability, such as differences in pipetting volumes, cell lysis efficiency and assay efficiency, can be effectively eliminated.

In the Dual-Luciferase Reporter (DLR) Assay System, the activities of firefly (*Photinus pyralis*) and Renilla (*Renilla reniformis*) luciferases are measured sequentially from a single sample. The firefly luciferase reporter is measured first and after quenching the reaction, the Renilla luciferase reporter is simultaneously measured. Firefly and Renilla luciferases, because of their distinct evolutionary

origins, have dissimilar enzyme structures and substrate requirements. These differences make it possible to selectively discriminate between their respective bioluminescent reactions.

Firefly luciferase, which is a 61 kDa monomeric protein that does not require posttranslational processing for enzymatic activity functions as a genetic reporter immediately upon translation. Photon emission is achieved through oxidation of beetle luciferin in a reaction that requires ATP, Mg^{2+} and O_2 shown in figure 1.18. Under conventional reaction conditions, the oxidation occurs through a luciferyl-AMP intermediate that turns over very slowly. As a result, this assay chemistry generates a flash of light that rapidly decays after the substrate and enzyme are mixed. The reaction equation is given in figure 1.22 (Promega Technical Manual E1910, 2006).

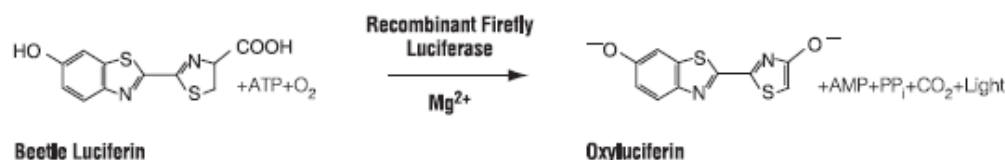


Figure 1.22: Bioluminescent reaction catalyzed by firefly luciferase (Promega Technical Manual E1910, 2006).

Renilla luciferase, a 36 kDa monomeric protein, like firefly luciferase, that does not require post-translational modification may function as a genetic reporter immediately following translation. The luminescent reaction catalyzed by Renilla luciferase utilizes O_2 and coelenterate-luciferin shown in figure 1.23.

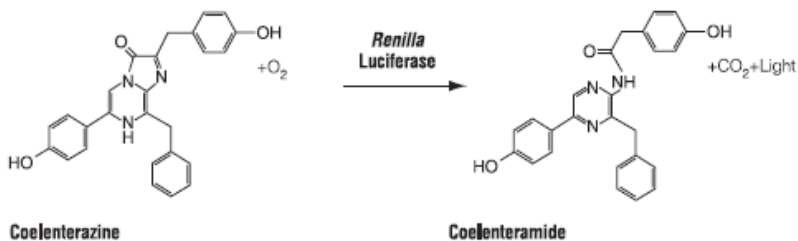


Figure 1.23 : Bioluminescent reaction catalyzed by renilla luciferase (Promega Technical Manual E1910, 2006).

1.9. Aim of the Study

Katanin is a heterodimeric protein that severs microtubules by hydrolyzing ATP. Katanin consists of 60 kDa and 80 kDa polypeptides. 60 kDa subunit (p60) has the enzymatic activity to break microtubules, whereas 80 kDa subunit (p80) has a role in localization of the protein complex in the cell. Katanin has been shown to have roles for microtubule severing in mitotic cells including release of microtubules from centrosome, depolymerization of microtubule minus ends in the mitotic spindle.

Despite the importance of this protein, its transcriptional regulation has not been studied, and the promoter has not yet been characterized.

The promoter site of human KATNB1 gene coding for p80, is high in CG dinucleotides. It is known that the cytosine and guanine rich sequences in the promoter regions of genes are the candidate regions for transcription factors binding.

In this study, in order to understand the transcriptional regulation of human KATNB1, putative promoter region in the upstream of KATNB1 gene will be characterized and its potential effect on katanin expression regulation will be revealed. Identification of transcription factor binding sites in the promoter of the KATNB1 gene is in a great importance to understand temporal gene expression pattern of katanin, which slightly differs between different stages of development. Identification of this gene regulation pattern will be a pioneer study for understanding the gene expression regulation of KATNB1 gene.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Cell lines

SH-SY5Y Human metastatic neuroblastoma cell line was a gift of Assoc. Prof. Dr. Işıl Aksan Kurnaz. 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.

2.1.2. Bacterial Strains

- *Escherichia coli* DH5 α strain [F⁻, ϕ 80dlacZ Δ M15, Δ (lacZYA-argF)U169, *deoR*, *recA1*, *endA1*, *hsdR17*(rk⁻, mk⁺), *phoA*, *supE44*, λ ⁻, *thi-1*, *gyrA96*, *relA1*]
- *Escherichia coli* dam⁻ dcm⁻ GM2163 strain [F⁻, *dam-13::Tn 9 dcm-6 hsdR2 leuB6 his-4 thi-1 ara-14 lacY1 galK2 galT22 xyl-5 mtl-1 rpsL136 tonA31 tsx-78 supE44* McrA⁻ McrB⁻] (Fermentas, M009).

2.1.3. Molecular Cloning Assays

- 25mM MgCl₂ (Fermentas)
- 2mM dNTP Mix (Fermentas)
- MassRuler™ DNA Ladder Mix (Fermentas)
- 6x DNA Loading Dye (Fermentas)
- Agarose Low EEO (Applichem)
- *Taq* polymerase 5u/ μ l (Fermentas)
- 10X *Taq* Buffer with KCl (Fermentas)
- FastDigest® HindIII Enzyme (Fermentas)
- FastDigest® KpnI Enzyme (Fermentas)
- 10X FastDigest® Buffer (Fermentas)
- T4 Ligase (Roche)
- 10X Ligation Buffer (Roche)

- EtBr (Merck)
- Tris Base (BDH Laboratory)
- Glacial Acetic Acid (Fluka)
- EDTA (Merck)
- Hi-Di Formamide (Applied Biosystems)
- Isopropanol (Fluka)
- Molecular Biology grade Ethyl Alcohol (Fiedel-de Haën)
- Primers (Alpha DNA)

2.1.4. Bacterial Assays

- Ampicillin (Sigma-Aldrich)
- $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Merck)
- PIPES (BDH Laboratory)
- Glycerol (Fluka)
- Yeast Extract (Merck)
- Agar (Merck)
- Tryptone (BDH Laboratory)
- NaCl (Fluka)
- MgCl_2 (Merck)
- MgSO_4 (Fluka)
- KCl (Fluka)
- Glucose (Merck)

2.1.5. Cell culture Assays

- Dulbecco's Modified Eagle Medium (DMEM)1X (Gibco)
- L-Glutamine solution, 200mM (Biochrom)
- Fetal Bovine Serum (Gibco)
- Penicilin/Streptomycin solution (100X) (Biochrom)
- Trypsin/EDTA 0,25/0,02 solution (Biochrom)
- Phosphate Buffered Saline (PBS) 10X pH=7,2 (Gibco)
- DMSO (Fiedel-de Haën)
- Transfast Transfection Reagent (Promega)
- 25cm², 75cm² Tissue Culture Flasks (TPP)
- 24-well culture plate (TPP)

- 30cm Cell Scraper (TPP)
- 5ml, 10ml, 25ml Serological pipettes (TPP)
- 150ml Vacuum filtration system (TPP)
- 50ml, 20ml, 10ml Syringes (Set Inject)
- 0.22µm, 0.4µm syringe filters (TPP)

2.1.6. Buffers and Solutions

2.1.6.1. 50X TAE Buffer

50X TAE Buffer used in 1X dilution was used in DNA gel electrophoresis to prepare the gel that DNA is loaded on and also as the tank buffer. The buffer contains 40 mM Tris Base, 20mM Glacial acetic acid and 1 mM EDTA (pH=8) in double distilled water (ddH₂O).

2.1.6.2 CaCl₂ Solution

CaCl₂ solution was used to prepare chemicaly competent *E.coli* cells to use in transformation. The solution contains 60mM CaCl₂, 10mM PIPES and 15% glycerol in ddH₂O. pH was adjusted to 6,4 to dissolve PIPES and then was filter sterilized with 0,4µm filter.

2.1.6.3. LB Medium

Luria Bertani (LB) Medium was prepared by dissolving 10 gram (g) tryptone, 5 g yeast extract, and 10 g NaCl in 1L dH₂O. The media was sterilized by autoclaving for 15 minutes at 121°C. In order to make selection, ampicillin was added after the media was cooled down approximately to 55 °C.

2.1.6.4. LB Agar Medium

Luria Bertani (LB) Agar Medium was prepared by dissolving 10 gram (g) tryptone, 5 g yeast extract, 10 g NaCl and 20 g Agar in 1L dH₂O. The media was sterilized by autoclaving for 15 minutes at 121°C. In order to make selection, ampicillin was added after the media was cooled down approximately to 55°C. After mixing the medium, the content was poured into 10mm Petri plates, approximatly 20ml per plate.

2.1.6.5. SOC Medium

SOC medium was used to cultivate *E. coli* cells after heat shock transformation. 2 g tryptone, 5 g yeast extract, 0,058 g NaCl, 0.0186 g KCl, 0,095 g MgCl₂, 0,23 g MgSO₄ and 0,36 g glucose was dissolved in 100 ml in dH₂O and sterilized at 121°C by autoclaving for 15 minutes.

2.1.6.6. SH-SY5Y Culture Medium

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

2.1.6.7. SH-SY5Y Freezing Medium

To prepare SH-SY5Y freezing medium, DMEM containing 1g/L glucose was supplemented with 10% FBS, 2 mM L-Glutamine and 5% DMSO and then filter sterilized with 0,2 µm filter according to manufacturer instructions.

2.1.7. Vectors

2.1.7.1. pGL3-Basic

The pGL3-Basic luciferase reporter vector (Promega, E1751) provides a basis for the quantitative analysis of factors that potentially regulate mammalian gene expression. The backbone of the pGL3-Basic is designed for increased expression, and contains a modified coding region for firefly (*Photinus pyralis*) luciferase that has been optimized for monitoring transcriptional activity in transfected eukaryotic cells. This vector lacks eukaryotic promoter and enhancer sequences, allowing maximum flexibility in cloning putative regulatory sequences. Expression of luciferase activity in cells transfected with this plasmid depends on insertion and proper orientation of a functional promoter directionally upstream from *luc+*. pGL3-Basic Vector circle map is shown in figure 2.1 (Promega Technical Manual, E1751).

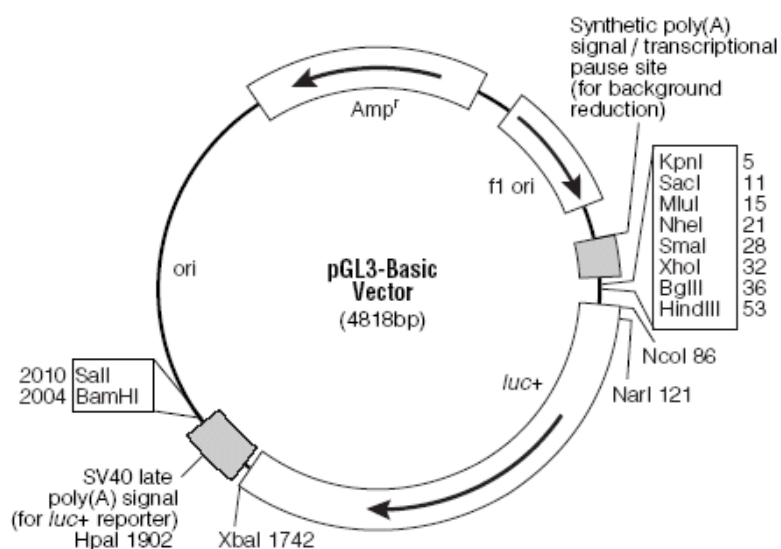


Figure 2.1 : pGL3-Basic Vector circle map (Promega Technical Manual, E1751).

2.1.7.2. pRL-TK

The pRL-TK Vector (Promega, E2241) is intended for use as an internal control reporter and may be used in combination with any experimental reporter vector to co-transfect mammalian cells. The vector contains a cDNA (RLuc) encoding Renilla luciferase, which was originally cloned from the marine organism *Renilla reniformis*. The pRL-TK Vector contains the herpes simplex virus thymidine kinase promoter to provide low to moderate levels of Renilla luciferase expression in co-transfected mammalian cells. Renilla luciferase is a 36 kDa monomeric protein that does not require post-translational modification for activity. Therefore, like firefly luciferase, the enzyme may function as a genetic reporter immediately following translation. pRL-TK Vector circle map is shown in figure 2.2 (Promega Technical Bulletin, E2241).

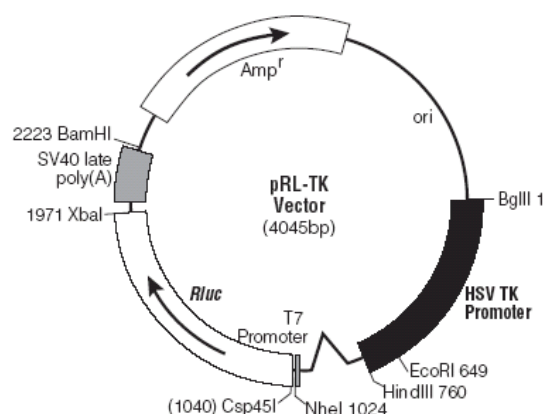


Figure 2.2 : pRL-TK Vector circle map (Promega Technical Bulletin, E2241).

2.1.7.3. Plasmid Constructs

Plasmid constructs used in the forced transcription factor experiments are generous gift of Assoc. Prof. Dr. Işıl Aksan Kurnaz.

- pRSV-Elk-1-VP16, a constitutively active Elk-1 construct is a Rous sarcoma virus promoter-driven vector encoding full-length wild-type Elk-1 fused to residues 410–490 of the VP16 C-terminal sequence (Price et al., 1995).
- pcDNA3-mPEA3, was constructed by Kurnaz, I., in 2001 and encodes full-length mouse PEA3.
- pcDNA3-Elk-1, was constructed by Dolce, in 2000 and encodes full-length human wild-type Elk-1.

2.1.8. Commercial Kits

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

Table 2.1: Commercial kits used in the study

Kit	Supplier Company
8 Lx Magtration Genomic DNA Kit	Precision System Science
QIAprep® Spin Miniprep plasmid purification kit	Qiagen, 27106
QIAquick PCR Purification Kit	Qiagen, 28104
QIAquick Gel Extraction Kit	Qiagen, 28706
Endofree Plasmid Maxi Kit	Qiagen, 12362
Bid Dye Terminator v 3.1 Cycle Sequencing Kit	Applied Biosystems
Dual-Luciferase Reporter Assay System	Promega, E1910
Transfast Transfection Reagent	Promega, E2431

2.1.9. Equipments

Equipments used in the study are shown in the table below.

Table 2.2: Equipments used in the study

Equipment	Supplier Company
DNA sequencer	Applied Biosciences 3100 Avant
Electrophoresis Gel System	E-C Apparatus Corporation, EC250-90 Minicel Primo
Microcentrifuge	Beckman Coulter
pH Meter	Mettler Toledo MP220
Balance	Precisa 620C SCS
UVIPhoto MW Version 99.05 for Windows 95 & 98	UVItec Ltd.
UV Transilluminator	Biorad UV Transilluminator 2000 Vilber Lourmat
Vortex	Heidolph, Reaxtop
Quick Spin	Labnet International, C1301-230V
Water Bath	Memmert Elektro-mag M 96 KP
CO ₂ Incubator	Shel Lab
Hemocytometer	FisherLab Scientific, 0267110
Light Microscope	Olympus CH30 (USA)
Luminometre	Thermo, Fluoroskan Ascent FL
Magnetic stirrer	VELP scientifica
Thermo Cycler	Techne, TC-3000 Corbett Palm Cycler
Spectrophotometer	Shimadzu, UV-1601 Thermo Scientific NanoDrop™ 1000
Ice machine	SCOTSMAN AF10
Centrifuge	Beckman Coulter, Allegra 25R Thermo EC, IECCL10
High Pressure Steam Sterilizer	Tomy 5X-700E
Refrigerator (+4 °C)	UĞUR Derin Dondurucu
Deep Freezer (-20 °C)	UĞUR Derin Dondurucu
Ultra Low Temperature Freezer (-80 °C)	New Brunswick Scientific
Pure Water System	UHQ USF Elga TKA Wasseraufbereitungssysteme
Orbital Shaker	Thermo Electron Corporation, Forma
Thermomixer	Eppendorf Thermomixer comfort
Laminar air flow cabinets	FASTER BH-EN 2003
Automatic pipette	Thermo EC, Finnpiquette
Pipettes	Eppendorff 10µl, 20µl, 100µl, 200µl, 1ml Thermo EC, Finnpiquette 1ml

2.2. METHODS

2.2.1. Promoter sequence analysis of KATNB1 gene

KATNB1 gene is located on chromosome 16 separated from its upstream gene CCDC135 by 4292 base pair (bp) that comprise a putative promoter region for KATNB1 gene. 1000 bp upstream of the transcription start site of KATNB1 gene was determined via University of California Santa Cruz Bioinformatic Genome Browser software using "Get Genomic Sequence Near Gene" tool (Url-3). The "Promoter/Upstream by XXX bases" option represents upstream bases from transcription start site. The promoter region is then considered predicted, by alignment of the mRNA versus genomic. An illustration of the program screen is given in figure 2.3.

Get Genomic Sequence Near Gene

Note: if you would prefer to get DNA for more than one feature of this track at a time, try the [Table Browser](#) using the output format sequence.

Sequence Retrieval Region Options:

☒ Promoter/Upstream by bases

☐ 5' UTR Exons

☐ CDS Exons

☐ 3' UTR Exons

☐ Introns

☐ Downstream by bases

☒ One FASTA record per gene.

☐ One FASTA record per region (exon, intron, etc.) with extra bases upstream (5') and extra downstream (3')

☐ Split UTR and CDS parts of an exon into separate FASTA records

Note: if a feature is close to the beginning or end of a chromosome and upstream/downstream bases are added, they may be truncated in order to avoid extending past the edge of the chromosome.

Sequence Formatting Options:

☒ Exons in upper case, everything else in lower case.

☐ CDS in upper case, UTR in lower case.

☐ All upper case.

☐ All lower case.

☐ Mask repeats: ☒ to lower case ☐ to N

Figure 2.3: Illustration of Genome Browser software.

2.2.2. Bioinformatic analysis of the promoter region

The promoter site of KATNB1 was analysed for the presence of a functional TATA-box, CAAT-box, CG boxes and CpG islands. Presence of TATA box, CAAT box and CG box motif was analysed via The European Bioinformatics Institute (EBI) Clustalw sequence analysis tool (Url-4). CpG island analysis is done through EBI, European Molecular Biology Open Software Suite, EMBOSS CpGPlot/Report tools (Url-5).

2.2.3. Deletion constructs determination

Identifying genomic locations of transcription-factor binding sites, particularly in higher eukaryotic genomes, has been an enormous challenge. Various experimental and computational approaches have been used to detect these sites; methods involving computational comparisons of related genomes have been particularly successful. To predict possible protein-DNA interaction, the promoter region of KATNB1 gene was analysed via TRANSFAC[®] 7.0 Public 2005 (Matys et al., 2006), available online at this web address: <http://www.gene-regulation.com/pub/databases.html>. This database contains data on transcription factors, their experimentally-proven binding sites, and regulated genes. Deletion constructs were determined by reasonably separating the transcription factor binding site in a meaningful way.

2.2.4. DNA Isolation from whole blood

The DNA isolation was performed using Migration System 8Lx Instrument. DNA isolation kits were supplied by Precision System Science. DNA was isolated in an automated system and nearly 4 ml of blood was used. Isolated DNA was kept at -20°C then DNA was diluted 1:100 ratio with sterile dH₂O to a total volume of 500µl and absorbance values at 260nm, 280nm, and 320nm were measured. Concentration and DNA quality calculations from absorbance values are done according to the equations 2.1 and 2.2.

$$\text{DNA Concentration (ng/}\mu\text{l)} = (A_{260} - A_{320}) \times \text{Dilution Factor} \quad (2.1)$$

$$\text{DNA Quality} = (A_{260} - A_{320}) / (A_{280} - A_{320}) \quad (2.2)$$

To obtain the working aliquots, stock DNA was diluted to 50ng/µl with sterile dH₂O.

2.2.5. Primer Design

Forward and reverse primers specific for each deletion construct with appropriate flanking restriction site were designed by considering the general rules of primer design using Integrated DNA Technologies, Oligo Analyser 3.1 tool (Url-6). For forward primers KpnI enzyme restriction site shown in green in table 2.3 were added to the 5' end of the primers and for reverse primer HindIII enzyme restriction site shown in blue in table 2.3 were added to the 5' end of the primer. The upstream stretches of adenine residues shown in red were added to allow proper restriction enzyme cutting. GC content, self-hybridization, hetero-dimer formation between

primer pairs, hairpin formation and presence of secondary priming sites was taken into consideration while choosing the appropriate primer pairs. All the primers used in the study including amplification primers and pGL3-Basic sequencing primers are given in table 2.3.

Table 2.3: Primers used in the study

Primer Name	Primer sequence (5'→ 3')	T _m	% G/C	Length
Katnb1_F1	AAAGGTACCTCATCTGTAAATGGGAACAGGAATT	60.8°C	37.1	35
Katnb1_F2	AAAGGTACCATCTGCAGGCAAAGTGAA	60.6°C	42.9	28
Katnb1_F3	AAAGGTACACACAAATTAGGTTGAGGG	60.3°C	43.3	30
Katnb1_F4	AAAGGTACCGAGGCGGGACT A	60°C	54.5	22
Katnb1_F5	AAAGGTACCTTCCTTCCTCTTGATTAGTCCTATTT	60°C	36.1	36
Katnb1_R	AAAAAGCTTACCTCCGGCTCAGAGC	61.1°C	50	26
Katnb1_F6_F	CGGGCGGGCTCTGAGCCGGAGGTA	71.8°C	-	25
Katnb1_F6_R	AGCTTACCTCCGGCTCAGAGCCCGCCCGGTAC	-	-	33
Katnb1_F7_F	CCTCTGAGCCGGAGGTA	53.4°C	-	17
Katnb1_F7_R	AGCTTACCTCC GGCTCAGAGGGTAC	-	-	25
pGL3_F	TGTCCCCAGTGCAAGTG	55,1°C	58,8	17
pGL3_R	CTTTCCTTATGTTTTGGCGTCTTCCA	56,4°C	37	27

2.2.6. Amplification of KATNB1 constructs

Polymerase Chain Reaction is used to amplify a fragment from template DNA sequence. Borders of amplified fragments are defined with small oligonucleotides named primers. These primers provide a binding site on template DNA for DNA polymerases. The buffers are used to preserve DNA polymerase in its natural confirmation and dNTP molecules are included to the reaction mixture to be integrated into newly forming DNA molecules.

2.2.6.1. PCR of KATNB1 1000bp promoter

For amplification of 1000 bp promoter region of KATNB1 gene following PCR reaction and PCR program was applied. The denaturation time was extended to 20 minutes (min) in order to efficiently denature the template which was genomic DNA.

Table 2.4 : PCR Reaction for cloning KATNB1 1000bp promoter

Stock Concentrations	PCR Ingredients	1X	Final Concentration
10X	<i>Taq</i> Buffer without Mg	5 µl	1X
2 mM	dNTP Mix	4 µl	0,16 µM
10 µM	Forward Primer	2 µl	0,4µM
10 µM	Reverse Primer	2 µl	0,4 µM
50ng/µL	Genomic DNA	2 µl	2 ng/µL
5U/µL	<i>Taq</i> DNA Polymerase	0.5 µl	0.05 U/µL
25 mM	MgCl ₂	2 µl	1 mM
	dH ₂ O	32,5µl	
	TOTAL	50 µl	

Table 2.5 : PCR program for cloning KATNB1 1000bp promoter

Steps	Cycles	Temperature	Time
Initial denaturation	1	95°C	20 min
Denaturation	35	95°C	45 sec
Annealing		60°C	45 sec
Extension		72°C	1 min
Final Extension	1	72°C	5 min
Hold	-	4°C	∞

2.2.6.2. PCR of KATNB1 deletion constructs

For amplification of 4 deletion constructs of 1000 bp promoter region of KATNB1 gene following PCR reaction and PCR program was applied. The template used in those PCR was the first cloned 1000 bp promoter vector. Appropriate primer pairs having each the same melting temperature (60 °C) were used for each PCR.

Table 2.6: PCR Reaction for cloning KATNB1 deletion constructs

Stock Concentration	PCR Ingredients	1X	Final Concentration
10X	<i>Taq</i> Buffer without Mg	5 µl	1X
2 mM	dNTP Mix	4 µl	0,16 µM
10 µM	Forward Primer	2 µl	0,4µM
10 µM	Reverse Primer	2 µl	0,4 µM
-	pGL3-Basic-F1	2 µl	-
5U/µL	<i>Taq</i> DNA Polymerase	0.5 µl	0.05 U/µL
25 mM	MgCl ₂	2 µl	1 mM
	dH ₂ O	32,5µl	
	TOTAL	50 µl	

Table 2.7: PCR program for cloning KATNB1 deletion constructs

Steps	Cycles	Temperature	Time
Initial denaturation	1	95°C	5 min
Denaturation	35	95°C	45 sec
Annealing		60°C	45 sec
Extension		72°C	1 min
Final Extension	1	72°C	5 min
Hold	-	4°C	∞

2.2.6.3. Hybridization of KATNB1 short constructs

Since the 2 last constructs that wanted to be amplified were composed of very short oligonucleotide strands, it was not easily possible to amplify them with PCR. For that reason those sequences were designed as primer pairs and were annealed in thermocycler by decreasing the temperature gradually. The goal here was to denature the complementary strands to remove any secondary structures present and then allow the strands to hybridize. For the hybridization procedure, 10 µl of each primer pair from 100mM stock solutions was mixed together at a 1:1 molar ratio in a PCR tube. The PCR program used in the hybridization of the longer insert is shown in Table 2.8 and the program used in the hybridization of the shorter insert is shown in Table 2.9.

Table 2.8.: Hybridization program for KATNB1 longer construct

Steps	Cycles	Temperature	Time
Step 1	1	95°C	5 min
Step 2	11*	95°C (-2°C /cycle)	1 min
Step 3	1	72°C	30 min
Step 4	34*	72°C (-2°C /cycle)	1 min
Step 5	-	4°C	∞

*The number of cycles in Step 2 and 4 depends on the T_m of the oligonucleotides.

Table 2.9: Hybridization program for KATNB1 shorter construct

Steps	Cycles	Temperature	Time
Step 1	1	95°C	5 min
Step 2	20*	95°C (-2°C /cycle)	1 min
Step 3	1	54°C	30 min
Step 4	25*	54°C (-2°C /cycle)	1 min
Step 5	-	4°C	∞

*The number of cycles in Step 2 and 4 depends on the T_m of the oligonucleotides.

2.2.7. Agarose gel electrophoresis for detection of PCR amplification

The expected DNA fragment length after PCR amplification was about 1000 bp which is estimated to be clearly detectable in 1% agarose gel. To prepare 1% agarose gel in a mini electrophoresis system, 0.5 g agarose was dissolved in 50 µl 1X TAE buffer. 0.5 µg/mL ethidium bromide concentration was used for the gel. For each sample, 5 µl of PCR amplicon was mixed by 1 µl of 6X loading dye. As a standard, MassRuler™ DNA Ladder Mix was used to estimate the PCR amplicon size. Electrophoresis conditions were 40 minutes at 120V (12V/cm of gel). Pictures of the gels were taken under UV light with a transilluminator, by the help of UV PhotoMW software.

2.2.8. Purification of PCR products

After PCR, the fragments were purified with Qiagen QIAquick PCR Purification Kit. This kit removes the protein contamination derived from polymerase enzymes. The principle of the kit is given as followed, DNA is bound to glass fleece with aid of high concentrated chaotropic salt, remains of protein are removed with washes, finally the DNA molecules are released with low salt concentration elution buffer. The purification protocol is given below:

- Total volume of PCR product was brought to 100 µl and 500 µl Binding Buffer (3 M guanidine-thiocyanate, 10 mM Tris-HCl, 5% ethanol (v/v), 2 mg RNase) was added to each PCR tube.
- After mixing the sample well, sample was transferred into collection filter tubes and centrifuged 1 minute at 14000 rpm at table top centrifuge.
- Flow through was discarded and 500 µl Wash buffer (20 mM NaCl, 2 mM Tris-HCl, 80% ethanol) was added. Then again, the filter tube was centrifuged at 14000 rpm for 1 minute.
- Flow through was discarded and the filter tube was centrifuged at 14000 rpm for 1 minute to remove the residual wash buffer.
- The filter was connected to a clean 1.5 ml eppendorf tube. And 50 µl of Elution buffer (10 mM Tris-HCl, pH 8.5) was added then it was centrifuged at 14000 rpm for 1 minute.

2.2.9. Restriction enzyme digestion of PCR products and the vector

DNA fragments are cut with sticky end restriction enzymes forming nicks in the structure of DNA, this phenomenon serves for generating sticky overhangs which have the ability to complement with a fragment containing the same overhang. Purified fragment and target vector pGL3-Basic were double digested with FastDigest Hind III and KpnI restriction enzymes that would generate compatible ends for cloning. Restriction digestion mix was prepared according to the table 2.10.

Table 2.10: Restriction mix for HindIII and KpnI double digestion

Ingredients	Amount	Final Concentration
10X Fast Digest Buffer	5 μ l	1X
FastDigest Hind III	2 μ l	0.5U/ μ l
FastDigest Kpn I	2 μ l	0.5U/ μ l
PCR Amplicon/ pGL3	20 μ l	-
dH ₂ O	21 μ l	-
TOTAL	50 μ l	

Restriction enzyme digestion was performed at 37°C for 1 hour (h) and followed by 10 minute incubation at 80°C for the inactivation of restriction enzymes.

2.2.10. Determination of restriction pattern by agarose gel electrophoresis

Digested samples were run on 1% agarose gel. For each PCR sample, 5 μ l of amplicon was mixed by 1 μ l of 6X loading dye. As a standard, MassRuler™ DNA Ladder Mix was used to estimate the PCR amplicon size. Electrophoresis conditions were 40 minutes at 120V (12V/cm of gel). Pictures of the gels were taken under UV light with a transilluminator, by the help of UV PhotoMW software.

2.2.11. Purification of digested PCR products and vector

Purification of the digested PCR products were performed with Qiagen QIAquick PCR Purification Kit as described in section 2.2.8. The digested vector was purified from the agarose gel via QIAquick Gel Extraction Kit. The purification protocol is given below:

- Gel slice containing the DNA band was excised with a clean, sharp scalpel.
- After weighing the gel slice in a colorless tube, 3 volumes of Buffer QG was added to 1 volume of gel.

- The tube was incubated at 50°C for 10 min. To help dissolve gel, the tube was mixed by vortexing every 2–3 min during the incubation.
- 1 gel volume of isopropanol was added to the sample and mixed.
- A QIAquick spin column was placed in a provided 2 ml collection tube.
- The sample was applied to the QIAquick column and centrifuged for 1 minute at 14000 rpm at table top centrifuge.
- Flow-through was discarded and the column was placed back in the same collection tube.
- 0.5 ml of Buffer QG was added to the column and centrifuged for 1 minute at 14000 rpm.
- To wash, 0.75 ml of Buffer PE was added to the column and centrifuged for 1 minute at 14000 rpm.
- Flow-through was discarded and the column was centrifuged for an additional 1 minute at 14000 rpm.
- The column was placed into a clean 1.5 ml microcentrifuge tube.
- To elute DNA, 50 µl of Buffer EB (10mM Tris-HCl, pH 8.5) was added to the center of the QIAquick membrane and after waiting for 1 minute the column was centrifuged for 1 minute at 14000 rpm.

2.2.12. Ligation of deletion constructs into pGL3-Basic vector

Purified vector and insert gene were ligated with Roche T4 ligase according to 2:15 molecular ratio. The ligation reaction mixture is given table 2.11.

Table 2.11 : Ligation mixture of pGL3-Basic and deletion constructs

Ingredients	Amount
Insert	7 µl
pGL3- Basic	1 µl
10X Roche T4 ligation buffer	1 µl
Roche T4 Ligase	1 µl
Total	10 µl

Ligation mixture was prepared according to table and ligation was performed at room temperature (RT) overnight. Ligation inactivation is performed by heating the samples in thermal cycler to 65 °C for 5 minutes.

2.2.13. Transformation of Constructed pGL3 Basic-insert plasmid

Subsequent to ligation, plasmid constructs were transformed into a bacterium host in order to increase the number of plasmids. Bacterial cells are amenable to modify in order to intake DNA. For this approach; first, the cell wall is disrupted with CaCl_2 and later, DNA binds to disrupted cell wall fractions and finally, DNA is introduced into cell with membrane scaring by heat shock. In our lab, CaCl_2 treatment based chemical competent cells are used. *Escherichia coli* (*E. coli*) DH5 α were modified into competent cells. The chemical competent cell preparation protocol (Sambrook and Russell, 2001) is given below:

- LB plate was streaked with DH5 α frozen stock.
- One good separated colony was chosen for inoculation of 3 ml liquid LB media. Media was incubated at 37 °C with 250 rpm shaking overnight (~16 hours).
- 100 ml LB medium was inoculated with overnight culture, and incubated at 37 °C with 250 rpm shaking for 2.5-3 hours until OD₅₉₀ reaches 0.612.
- Culture was taken into 2 pre-chilled sterile 50 ml falcons and placed on ice for 10 minutes.
- Vials were centrifuged at 2500 rpm for 5 minutes at 4 °C. Supernatant was discarded.
- Each pellet was washed with 10 ml ice cold CaCl_2 solution, and then centrifuged at 2500 rpm for 5 minutes at 4 °C.
- Pellets were resuspended with 2 ml ice cold CaCl_2 solution and 25 μl of resuspension aliquoted into pre-chilled 1.5 ml eppendorf tubes. Aliquots were frozen with liquid nitrogen and stored at -80 °C.

And heat shock transformation was applied accordingly:

- 25 μl aliquots were taken from -80 °C, and thawed on ice.
- 1 μg DNA was added to each vial and left on ice for 30 minutes.
- The vial was placed in 42 °C water bath for 45 seconds and put on ice for 2-3 minutes.
- 80 μl of SOC medium was added, and then culture was incubated at 37 °C for 1 h shaking at 250 rpm.
- Cultures were plated on selective medium with appropriate antibiotic (LB-Amp)
- Plates were incubated upside down at 37 °C overnight.

2.2.14. Colony PCR of transformed colonies

Colony PCR is a specified PCR technique allowing to check if the colonies contain the plasmid of interest. Selected colonies were streaked onto new LB-Amp plates and incubated at 37°C overnight. After the colonies emerged, colony PCR was executed with pGL3 sequencing primers. Procedure of the colony PCR is given below:

- 10 µl of sterile dH₂O was added to adequate number of PCR tubes, and a tip of each colony was resuspended.
- Mixture was incubated in thermal cycler at 85°C for 15 minutes.
- The mixture was vortexed and centrifuged.
- Supernatant of the mixture was employed as sample to PCR reaction given below:

Table 2.12 : Colony PCR reaction

Stock Concentration	PCR Ingredients	1X	Final Concentration
10X	<i>Taq</i> Buffer without Mg	2.5 µl	1X
2 mM	dNTP Mix	2 µl	0.16 mM
10 µM	pGL3 Forward Primer	1 µl	0.4 mM
10 µM	pGL3 Reverse Primer	1 µl	0.4 mM
-	Colony water mix	10 µl	-
5u/µL	<i>Taq</i> DNA Polymerase	0.25 µl	1.25 unit
25 mM	MgCl ₂	1.5 µl	1.5 mM
-	dH ₂ O	6.75 µl	-
	TOTAL	25 µl	

Table 2.13 : Colony PCR program

Steps	Cycles	Temperature	Time
Initial denaturation	1	95°C	5 min
Denaturation	35	95°C	30 sec
Annealing		50°C	30 sec
Extension		72°C	1 min
Final Extension	1	72°C	5 min
Hold	-	4°C	∞

The resultant PCR products were run on 1% TAE Agarose gel and insert containing colonies were selected.

2.2.15 DNA isolation

DNA was isolated from colony PCR positive colonies with Qiaprep Spin Miniprep Plasmid Isolation Kit. The procedure is given below:

- 5 ml of LB was inoculated with positive colony and the culture was incubated at 37 °C with 250 rpm shaking overnight.
- Overnight cultures were centrifuged at 5000 rpm for 5 minutes. Supernatant was discarded and pellet was resuspended in 250 µl Buffer P1 (50 mM Tris-Cl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A) and transferred to a microcentrifuge tube.
- 250 µl Buffer P2 (200 mM NaOH, 1% SDS (w/v)) was added and mixed thoroughly by inverting the tube 4–6 times.
- 350 µl pre-chilled Buffer N3 (3.0 M potassium acetate, pH 5.5) was added and mixed immediately and thoroughly by inverting the tube 4–6 times.
- Suspension was centrifuged for 10 minutes at 14,000 rpm in a table-top microcentrifuge.
- The supernatant from previous step was applied to the QIAprep spin column by pipetting.
- Filter-tube assembly was centrifuged for 30–60 seconds. The flow-through was discarded.
- The QIAprep spin column was washed by adding 0.5 ml Buffer PB (5 M Gu-HCl, 30% ethanol) and centrifuging for 30–60 seconds. Flow-through was discarded.
- The QIAprep spin column was washed by adding 0.75 ml Buffer PE (10 mM Tris-HCl, pH 7.5; 80% ethanol) and centrifuging for 30–60 seconds.
- The flow-through was discarded, and tubes were centrifuged for an additional 1 minute to remove residual wash buffer.
- QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl Buffer EB (10 mM Tris-HCl, pH 8.5) was added to the center of each QIAprep spin column. Column was let standing for 1 minute, and centrifuged for 1 minute.

2.2.16 Sequencing

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

2.2.16.1. Sequencing PCR

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

Table 2.14: Sequencing PCR ingredients

Stock Concentration	PCR Ingredients	1X	Final Concentration
-	Big Dye Terminator	2 μ l	-
5X	Sequencing Buffer	2 μ l	1X
10 mM	pGL3_F/ pGL3_R	1 μ l	1 mM
-	Plasmid	1 μ l	-
-	dH ₂ O	4 μ l	-
	Total	10 μ l	

Table 2.15: Sequence PCR program

Steps	Cycles	Temperature	Time
Initial denaturation	1	95°C	5 min
Denaturation	40	95°C	45 sec
Annealing		50°C	45 sec
Extension		60°C	4 min
Hold	-	4°C	∞

2.2.16.2. PCR Product Purification for Sequencing

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

- 10 µl PCR product was taken in to microfuge tube then 2 µl of 3M NaAc and 50 µl ice-cold 95% ethanol were added to tube.
- Tubes were incubated on ice for 30 minutes.
- Mixture was centrifuged at 14000 rpm for 30 minutes. Supernatant was discarded and pellet was resuspended by vigorously vortexing the sample in 200 µl ice-cold 70% ethanol.
- Tubes were centrifuged at 14000 rpm for 15 minutes. Supernatant was discarded.
- Tubes were incubated with caps open at 95°C for 5 minutes in order to evaporate residual ethanol.
- 20 µl of Hi-Di formamide was added to DNA pellet and the mixture was vortexed vigorously and then spun down.
- Tubes were again incubated at 95 °C for 5 minutes with caps closed.
- Tubes were immediately put on ice and kept at 4 °C until analysis.

2.2.16.3. Analysis of Sequence Results

Analysis of sequence results was performed via EBI Clustalw sequence analysis tool (Url-4). ClustalW2 is a general purpose multiple sequence alignment program for DNA or proteins. It produces biologically meaningful multiple sequence alignments of divergent sequences. It calculates the best match for the selected sequences, and lines them up so that the identities, similarities and differences can be seen.

2.2.17. Large scale plasmid production

After confirming the sequence data and assuring that none of the constructs possess any mutations, each construct was transformed with *E.coli* dam-/dcm- GM2163 strain in order to prevent any methylation that can interfere the transcription factor binding, according to the previously described heat shock transformation in section 2.2.12. After the colonies emerged the insert containing plasmids was reproduced in large scale and purified with QIAGEN, EndoFree® Plasmid Maxi kit. This kit is chosen because subsequent to large scale production the plasmids are going to be used to transfect SHSY-5Y neuroblastoma cell line which is a sensitive cell line. Endotoxins, which are cell membrane components of Gram-negative bacteria (*E.coli*) strongly influence transfection and increased endotoxin levels lead to sharply reduced transfection efficiencies. Plasmid isolation procedure is as follows:

- 5 ml of LB was inoculated with positive colony and the culture was incubated at 37 °C with 250 rpm shaking for 8 h.
- 100 ml LB medium with appropriate antibiotic was inoculated with 100 µl of starter culture and grown at 37°C for 12–16 h with vigorous shaking.
- Overnight cultures were centrifuged at 4100 rpm for 90 min.
- Supernatant was discarded and pellet was resuspended in 10 ml Buffer P1 (50 mM Tris-Cl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A) and transferred to a 50 ml centrifuge tube.
- 10 ml Buffer P2 (200 mM NaOH, 1% SDS (w/v)) was added and mixed thoroughly by inverting the tube 4–6 times and incubated at RT for 5 min.
- 10 ml pre-chilled Buffer N3 (3.0 M potassium acetate, pH 5.5) was added and mixed immediately and thoroughly by inverting the tube 4–6 times.
- Lysate was poured into the barrel of the QIAfilter Cartridge and incubated at room temperature for 10 min then the lysate was filtered into a 50 ml tube.
- 2.5 ml Endotoxin removal buffer (ER) was added to the filtered lysate, and mixed by inverting the tube approximately 10 times, and incubated on ice for 30 min.
- During the incubation QIAGEN-tip 500 was equilibrated by applying 10 ml Buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v); 0.15% Triton® X-100 (v/v)) and the column was allowed to empty by gravity flow.
- The filtered lysate was applied to the QIAGEN-tip and allowed to enter the resin by gravity flow.
- QIAGEN-tip was washed with 2 x 30 ml Buffer QC (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)).
- DNA was eluted with 15 ml Buffer QN (1.6 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)).
- DNA was precipitated by adding 10.5 ml room-temperature isopropanol to the eluted DNA and centrifuged immediately at 4100 rpm for 2 h at 4°C.
- After carefully decanting the supernatant DNA pellet was washed with 5 ml of endotoxin-free room-temperature 70% and centrifuge at a at 4100 rpm for 2 h at 4°C.
- The supernatant was carefully decanted without disturbing the pellet.
- The pellet was air-dried for 5–10 min, and the DNA was redissolved in 300 µl endotoxin-free Buffer TE (10 mM Tris-Cl, pH 8.0; 1 mM EDTA).

2.2.18 Transfection of SH-SY5Y with F2 deletion construct plasmid and ELK-1, ELK-1-VP16 and PEA3 constructs for forced experiments

Transfection is the process to introduce a foreign DNA into the cell. There are various methods of introducing foreign DNA into an eukaryotic cell. Many materials like (cationic) polymers, liposomes, nanoparticles have been used as carriers for transfection and different methods are developed including electroporation, viral infection, lipofection, gene guns and microinjection. 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. Basically synthetic cationic lipids that can form liposomes are incubated with DNA. These liposomes spontaneously interact with DNA and fuse with tissue culture cells. Incubation of cationic lipid-containing liposomes and nucleic acids results in quick association and a compaction of the nucleic acid presumably from electrostatic interactions between the negatively charged nucleic acid and the positively charged head group of the synthetic lipid. The liposome/nucleic acid complex is then presented to the cells that are to be transfected. The liposome complex neutralizes the negative charge of the nucleic acids, allowing closer association of the complex with the negatively charged cell membrane. Entry of the liposome complex into the cell may occur by the processes of endocytosis or fusion with the plasma membrane via the lipid moieties of the liposome. Transfection mixture contents are given in tables 2.16, 2.17, 2.18 and 2.19 and the transfection procedure is as follows:

- A frozen stock of SHSY-5Y cells were taken from -80 °C and after the cells warmed up to room temperature they were diluted with SHSY-5Y culture media which's content was given in section 2.1.6.6.
- After determining the cell density with hemacytometer, 50.000 cells per well were seeded on 24 well tissue culture plate one day before the transfection experiment so that the cells were approximately 80% confluent on the day of the transfection.
- 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 and vortexed and quick spun briefly for the proper formation of DNA liposome complex and incubated for 15 minutes at room temperature.

Table 2.16: Transfection mixture contents for deletion constructs transfections

Deletion Construct name	pGL3 Test Construct	pRL-TK Contol Construct	Total DNA (µg)	Transfast Reagent
pGL3_F1	900 ng	100 ng	1 µg	3 µl
pGL3_F2	900 ng	100 ng	1 µg	3 µl
pGL3_F3	900 ng	100 ng	1 µg	3 µl
pGL3_F4	900 ng	100 ng	1 µg	3 µl
pGL3_F5	900 ng	100 ng	1 µg	3 µl
pGL3_F6	900 ng	100 ng	1 µg	3 µl
pGL3_F7	900 ng	100 ng	1 µg	3 µl
pGL3_Basic	900 ng	100 ng	1 µg	3 µl

*Each transfection was performed as triplicates.

Table 2.17: Transfection mixture contents for ELK-1 forced experiment transfections

Deletion Construct name	pGL3 Test Construct	ELK-1 Construct	pCMV-Myc Construct	pRL-TK Contol Construct	Total DNA (µg)	Transfast Reagent
pGL3_F2_Elk1	700 ng	200ng	-	100 ng	1 µg	3 µl
pGL3_Basic_Elk1	700 ng	200ng	-	100 ng	1 µg	3 µl
pGL3_F2	700 ng	-	200ng	100 ng	1 µg	3 µl
pGL3_Basic	700 ng	-	200ng	100 ng	1 µg	3 µl

*Each transfection was performed as triplicates.

Table 2.18: Transfection mixture contents for ELK-1-VP16 forced experiment transfections

Deletion Construct name	pGL3 Test Construct	ELK-1-VP-16 Construct	pCMV-Myc Construct	pRL-TK Contol Construct	Total DNA (µg)	Transfast Reagent
pGL3_F2_Elk1VP16	700 ng	200ng	-	100 ng	1 µg	3 µl
pGL3_Basic_Elk1VP16	700 ng	200ng	-	100 ng	1 µg	3 µl
pGL3_F2	700 ng	-	200ng	100 ng	1 µg	3 µl
pGL3_Basic	700 ng	-	200ng	100 ng	1 µg	3 µl

*Each transfection was performed as triplicates.

Table 2.19: Transfection mixture contents for PEA3 forced experiment transfections

Deletion Construct name	pGL3 Test Construct	PEA3 Construct	pCMV-Myc Construct	pRL-TK Contol Construct	Total DNA (µg)	Transfast Reagent
pGL3_F2_PEA3	700 ng	200ng	-	100 ng	1 µg	3 µl
pGL3_Basic_PEA3	700 ng	200ng	-	100 ng	1 µg	3 µl
pGL3_F2	700 ng	-	200ng	100 ng	1 µg	3 µl
pGL3_Basic	700 ng	-	200ng	100 ng	1 µg	3 µl

*Each transfection was performed as triplicates.

- 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 CO₂ incubator.
- After an incubation period of 90 minutes, the transfection mixture was removed and 500 µl of complete growth medium of which the content was given in the section 2.1.6.6 was added to each well.
- The cells were returned to the 37°C %5 CO₂ incubator for 48 hours before analysis.

2.2.19. Luminometrical measurement of the transfected cells

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

Expression of luciferase activity in cells transfected with plasmids containing different regions of KATNB1 gene promoter depends on the functionality of the promoter upstream from *luc+*. For the forced experiments, the expression of luciferase activity in cells transfected with plasmids containing different regions of KATNB1 gene and with the transcription factors expressing vectors depends on the functionality of the transcription factor on the promoter upstream from *luc+*.

The chemiluminescence was measured by Fluoroskan Ascent FL luminometre from Thermo Electron Corporation. The PMT voltage was the default setting which is 845 and integration time was chosen to be 10000. Scaling factor was adjusted to 10 after optimization the measurements. At least 1 h before the assay needed amount of the Luciferase assay Reagent and Stop & Glo® Buffer were taken out from -80°C and -20°C refrigerators respectively to be thawed in room temperature water bath. After the reagents have thawed to room temperature, the Stop & Glo® Reagent was prepared by adding 50X Stop & Glo® Substrate to the Stop & Glo® Buffer to 1X

final concentration. All transfections were performed as triplicates and were repeated at least two times using different DNA preparations. Cells were prepared to the assay according to the manufacturer instructions,

- 48 hours post-transfection, growth medium was removed from the cultured cells, and 60 µl 1X passive lysis buffer was added to each well.
- Homogeneous lysates was rapidly prepared by manually scraping the cells.
- The lysates were transferred into microfuge tubes for further analysis.
- Luminometer was programmed to perform a 2-second premeasurement delay while shaking the plate, followed by a 10-second measurement period for each reporter assay.
- 50 µl of LAR II was predisensed into one well of luminometer plate and then 50 µl of cell lysate was transferred to the same well.
- Firefly luciferase luminescence was measured and recorded.
- 50 µl of Stop & Glo® Reagent was added to the same well.
- Renilla luciferase luminescence was measured and recorded.
- The procedure was repeated for each sample separately.

2.2.20. Evaluation of the luminometrical measurement data of promoter deletion constructs

In order to evaluate the luminometrical measurements data, PROMO bioinformatic tool from ALLGEN server, available online at this web address: http://algggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3 was used (Messeguer et al., 2002, Farre et al., 2003). PROMO is a virtual laboratory allowing the study of transcription factor binding sites in DNA sequences from a species or group of species of interest. Transcription factor binding sites defined in the TRANSFAC database (Matys et al., 2006), are used to construct specific binding site weight matrices for transcription factor binding sites prediction. The search for KATNB1 promoter was done by selecting factor's species as *Homo sapiens* and site species as *Homo sapiens* and by restriction the search maximum matrix dissimilarity rate as 5% and also by selecting factor's species as eutheria and site species as eutheria and by restriction the search maximum matrix dissimilarity rate as 5%.

3. RESULTS

3.1. Promoter sequence analysis of KATNB1 gene

Putative KATNB1 gene promoter is located on chromosome 16 between CCDC135 and KATNB1 genes according to NCBI Map Viewer database (Url-7). This 4292 bp region comprise the promoter region of KATNB1 gene. The sequence data of this region is given in Appendix B. Sequence data of 1000 base pair promoter (F1) determined via University of California Santa Cruz Bioinformatic Genome Browser software is given in table 3.1.

Table 3.1 : Sequence data of 1000 base pair promoter (F1)

TCATCTGTAAATGGGAACAGGAATTCCTACCTCTTATGGTTGTACTGGGC
GTTATGTGAGCCCAAGGAGCCCTGACCTGCGGTGAGAGGCTGGAGGCCGA
AGGGCAAGGTTTCGAGGGGCTCTGGGTGCCTGGGACAACCTCTGGGCAACA
GGGAGAGGCGGCTGCGGGGCCAAGTCCCTCCTAAGCGGCCACGCGAGAGG
CCCTGAGTCGAGAAGGGTTACGGCGCTTCCCAGAGCCCACCCGCCCCAAC
GTGTCACAAATAAAGCTGGCCTTCTCCGATTCCCTGATTGCAGCCATTC
AGGGAGCAGCCCCCTTGCCCACGCATCCATTAGTCGAGGCGGTGGCTGCGT
GTGCAGTGCTACAGGGTTCAGCGCCTGCCTCGGCTCCGGAATCAGGCTGT
TTCTTCTAGACCTCCCATGGGGGATTCAAGCCGGCGTCCGGGTCAAAGA
ACAGGGACAAAGTCCTCCTGCCACGGGGGACCATCTGCAGGCAAAGTGAA
GAATGAGGAGCTTCGGCAAAATGCCGACTAAGGCCTCCTAGGTTTTGCC
CCACTCCAAGATGGAAGGCCTGAGGCTTCACTGCCCCGAAGTTCCTT
TCCCATTGGCTATCTGGGAATTGAGTTTTCCAATAATGCGGACGCTGATT
GGTCAATCCAGGACGGTTGCTCAAGCCATTGGCGCAGCCGCCATTGGAGG
GCGGCTCTCAAAAGTTTTTCAGACACAAATTAGGTTTCGAGGGAGGAAACGG
AGAGGAAAGGGAACCTTGAGACGGAGGCGGACTAAGGAAACGGCAGCT
TGCATTGGTTTATTAGAGGCCAAGGGGCGGCTCTTGAACGTTCTTCCTC
TTGATTAGTCCTATTTAGGAAAAGAGGGCGGGTACTGAGGAAAAGCGGCA
GAAGCGCCTGCTTCATTGGTCAGTCCTGGCAGGAGGCGGAGCACCCGCG
GCAGCTGATTGGTGCGGGAGGCAAGGTGGGCGGGGCTCTGAGCCGGAGGT

3.2. Bioinformatic analysis of the promoter region

The search for TATA box and CAAT box within 4292 bp region revealed the presence of several TATA box and a CAAT box located far from the transcription start site. The location of these motifs on the region is given in Appendix B. The 1000 bp selected promoter region is also searched for the presence of TATA box and CAAT box, it is seen that the predicted promoter site of KATNB1 gene does not contain any of them. Furthermore, the analysis for CG boxes showed that this

promoter region contains several CG boxes that are shown in red sequences in the table 3.2. The CpG island analysis revealed the presence of 1 CpG island, 332 nucleotide in length positioned in the 611-942 bp of the 1000 bp promoter region. This CpG island is the only one island that is found within the 4292 bp region. The location of the CpG island is illustrated in the graph given in figure 3.1.

Table 3.2 : CG box analysis of KATNB1 promoter

TCATCTGTAAATGGGAACAGGAATTCCTACCTCTTATGGTTGTACTGGGC
GTTATGTGAGCCCAAGGAGCCCTGACCTGCGGTGAGAGGCTGGAGGCCGA
AGGGCAAGGTTTCAGGGGCTCTGGGTGCCTGGGACAACCTCTGGGCAACA
GGGAGAGGCGGCTGCGGGGCCAAGTCCCTCCTAAGCGGCCACGCGAGAGG
CCCTGAGTCGAGAAGGGTTACGGCGCTTCCCAGAGCCCACCCGCCCAAC
GTGTCACAAATAAAGCTGGCCTTTCTCCGATTCCCTGATTGCAGCCATTC
AGGGAGCAGCCCCCTTGCCACGCATCCATTAGTCGAGGCGGTGGCTGCGT
GTGCAGTGCTACAGGGTTACAGCGCTGCGCTCGGCTCCGGAATCAGGCTGT
TTCCTTCTAGACCTCCCATGGGGGATTCAAGCCGGCGTCCGGGTCAAAGA
ACAGGGACAAAGTCCTCCTGCCACGGGGGACCATCTGCAGGCAAAGTGAA
GAATGAGGAGCTTCGGCAAAATGCCGACTAAGGCCTCCTTAGGTTTGGC
CCACTCCAAGATGGAAGGCCTGAGGCTTCACTGCCCCGAAGTTCCTT
TCCCATTGGCTATCTGGGAATTGAGTTTCCAATAATGCGGACGCTGATT
GGTCAATCCAGGACGGTTGCTCAAGCCATTGGCGCAGCCGCCATTGGA
GGGGCTCTCAAAAGTTTTAGACACAAATTAGGTTTCGAGGGAGGAAACGG
AGAGGAAAGGGAAAACCTTGAGACGGAGGCGGGACTAAGGAAACGGCAGCT
TGCATTGGTTTATTAGAGGCCAAGGGGGCTCTTGAACGTTCTTCCTC
TTGATTAGTCCTATTTAGGAAAAGAGGGGGTACTGAGGAAAAGCGGCA
GAAGCGCTGCTTCCATTGGTCAGTCCTGGCAGGAGGCGGAGCACCCGCG
GCAGCTGATTGGTGCGGGAGGCAAGGTGGGGCTCTGAGCCGGAGGT

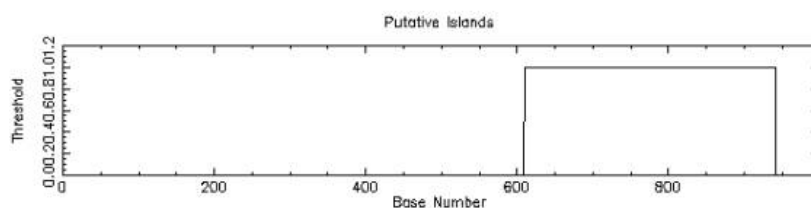


Figure 3.1 : CpG island analysis of KATNB1 promoter.

3.3. Deletion constructs determination

Computer prediction analysis of putative binding sites for different transcriptional elements was accomplished via TRANSFAC[®] 7.0 Public 2005 program (Matys et al., 2006). The database search predicted the presence of some transcription factors including Elk-1 with accession number (AC) T00250, Sp1 with AC T00759 and PEA3 with AC T00685. Sp1 with GCCCGCCCC/**GGGCGG** consensus sequence is represented in red, Elk-1 with **GGAAG** consensus sequence is represented in green and the PEA3 with **AGGAAA** consensus sequence is represented in blue in the alignment given in the table 3.3.

Table 3.3: Putative transcription factor binding site in 1000 base pair KATNB1 promoter

TCATCTGTAAATGGGAACAGGAATTCCTACCTCTTATGGTTGTA
GGCTGGGCTGGGACAACCTCTGGGCAACA
GGGAGAGGCGGCTGCGGGCCAAAGTCCCTCCTAAGCGGCCACGCGAGAGG
CCCTGAGTCGAGAAGGGTTACGGCGCTTCCCAGAGCCCACCCGCCAAC
GTGTCACAAATAAAGCTGGCCTTTCTCCGATTCCCTGATTGCAGCCATTC
AGGGAGCAGCCCCTTGCCACGCATCCATTAGTCGAGGCGGTGGCTGCGT
GTGCAGTGCTACAGGGTTCAGCGCCTGCCTCGGCTCCGGAATCAGGCTGT
TTCTTTCTAGACCTCCCATGGGGGATTCAAGCCGGCGTCCGGGTCAAAGA
ACAGGGACAAAGTCTCCTGCCACGGGGGACCATCTGCAGGCAAAGTGAA
GAATGAGGAGCTTCGGCAAAATGCCGACTAAGGCCTCCTTAGGTTTTGCC
CCACTCCAAGATGGAAAGCGCTGAGGCTTACACTGCCCCGAAGTTCCTT
TCCCATTGGCTATCTGGGAATTGAGTTTTCCAATAATGCGGACGCTGATT
GGTCAATCCAGGACGGTTGCTCAAGCCATTGGCGCAGCCGCCATTGGAGG
CGGCTCTCAAAAGTTTTTCAGACACAAATTAGGTTTCGAGGGAGGAAACGG
AGAGGAAAGGAAACTTGAGACGAGGCGGGACTAAGGAAACGGCAGCT
TGCATTGGTTTATTAGAGGCCAAGGGCGGCTCTTGAACGTTCTTCCTC
TTGATTAGTCCTATTTAGGAAAGAGGGCGGTACTGAGGAAAGCGGCA
GAAGCGCTGCTTCCATTGGTCAGTCTTGGCAGGAGGCGGAGCACCCGCG
GCGAGCTGATTGGTGCGGAGGCAAGGTGGGCGGGCTCTGAGCCGGAGGT

Deletion constructs were generated according to the search results by reasonably separating the transcription factor binding sites in a meaningful way. Deletion constructs sequence data are given in the table 3.4.

Table 3.4: Deletion constructs data

Names	Sequences
F2 (518bp)	ATCTGCAGGCAAAGTGAAGAATGAGGAGCTTCGGCAAAATGCCGACTAAGGCCTCCTTAGGTTTTGCCCCACTCCAAGATGGAAGGCCTGAGGCTTCACACTGCCCCGAAGTTCCTTTCCCATTTGGCTATCTGGGAATTGAGTTTTCCAATAATGCGGACGCTGATTGGTCAATCCAGGACGGTTGCTCAAGCCATTGGCGCAGCCGCCATTGGAGGGCGGCTCTCAAAAGTTTTTCAGACACAAATTAGGTTTCGAGGGAGGAAACGGAGAGGAAAGGGAAACTTGAGACGAGGCGGGACTAAGGAAACGGCAGCTTGCATTGGTTTATTAGAGGCCAAGGGGCGGCTCTTGAACGTTCTTCCTCTTGATTAGTCTATTTAGGAAAAGAGGGCGGGTACTGAGGAAAAGCGGCAGAACGCGCTGCTTCATTGGTCAGTCTTGGCAGGAGGCGGAGCACCCGCGGCAGCTGATTGGTGCGGGAGGCAAGGTGGGCGGGCTCTGAGCCGGAGGT
F3 (279bp)	ACACAAATTAGGTTTCGAGGGAGGAAACGGAGAGGAAAGGGAAA ACTTGAGACGAGGCGGGACTAAGGAAACGGCAGCTTGCATTGGTTTATTAGAGGCCAAGGGGCGGCTCTTGAACGTTCTTCCTCTTGATTAGTCCTATTTAGGAAAAGAGGGCGGGTACTGAGGAAAAGCGGCAGAACGCGCTGCTTCCATTGGTCAGTCTTGGCAGGAGCGGAGCACCCGCGGCAGCTGATTGGTGCGGGAGGCAAGGTGGGCGGGCTCTGAGCCGGAGGT
F4 (226bp)	GAGGCGGGACTAAGGAAACGGCAGCTTGCATTGGTTTATTAGAGGCCAAGGGGCGGCTCTTGAACGTTCTTCCTCTTGATTAGTCCTATTTAGGAAAAGAGGGCGGGTACTGAGGAAAAGCGGCAGAACGCGCTGCTTCCATTG GTCAGTCTTGGCAGGAGGCGGAGCACCCGCGGCAGCTGATTGGTGCGGGAGGCAAGGTGGGCGGGCTCTGAGCCGGAGGT
F5 (160bp)	TTCCTTCCTCTTGATTAGTCCTATTTAGGAAAAGAGGGCGGGTACTGAGGAAAAGCGGCAGAACGCGCTGCTTCCATTGGTCAGTCTTGGCAGGAGGCGGAGCACCCGCGGCAGCTGATTGGTGCGGGAGGCAAGGTGGGCGGGCTCTGAGCCGGAGGT
F6 Long (23bp)	GGGCGGGCTCTGAGCCGGAGGT
F7 Short (15bp)	CTCTGAGCCGGAGGT

3.4. Amplification of KATNB1 constructs

For pGL3-Basic deletion constructs construction, the promoter of 1000 bp (F1) was amplified with PCR. The resultant PCR products were run on agarose gel and the expected DNA fragment of 1000 bp was detected. PCR fragment is shown in figure 3.2 compared to Fermentas Mass Ruler Mix DNA Marker (SM0403).

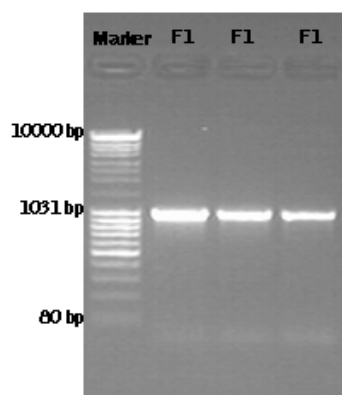


Figure 3.2 : PCR amplified 1000 bp promoter DNA fragment.

Amplified PCR product was designed to be inserted into Promega's pGL3-Basic vector. Therefore, the PCR product and vector were double digested with Fermentas's FastDigest Hind III and KpnI restriction enzymes. After purification step ligation was performed with Roche T4 DNA ligase. Subsequently, ligation mixture was transformed into *CaCl₂* treated *E. coli* DH5α competent cells. After the colonies emerged, in order to verify if the colonies contain the plasmid of interest, colony PCR was executed using pGL3-Basic sequencing primers. Seven colonies were analyzed by colony PCR, and the resultant amplicons were run on agarose gel. The expected DNA fragment of 1000 bp was detected in six colonies. PCR fragments are shown in figure 3.3 compared to Fermentas Mass Ruler Mix DNA Marker (SM0403).

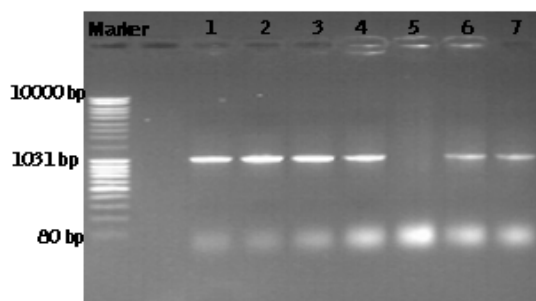


Figure 3.3 : Colony PCR amplified 1000 bp promoter DNA fragment.

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

For the construction of other deletion constructs, pGL3-Basic-1000 bp promoter (F1) containing purified plasmid was used as template for PCR reactions. The resultant PCR products were run on agarose gel and the expected DNA fragments of 518 bp (F2), 279 bp (F3), 226 bp (F4) and 160 bp (F5) were detected. PCR fragments are shown in figure 3.4 compared to Fermentas Mass Ruler Mix DNA Marker (SM0403).

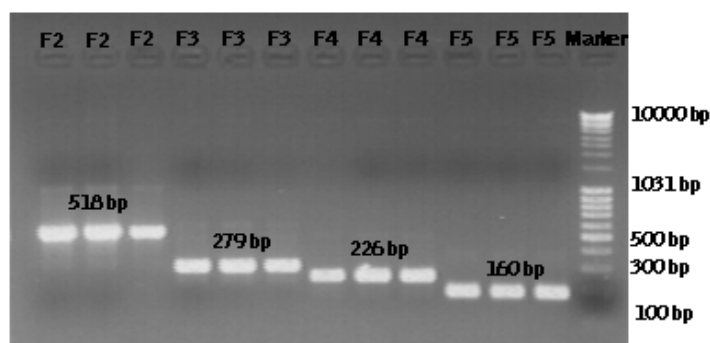


Figure 3.4 : PCR amplified F2, F3, F4, F5 DNA fragments.

Amplified PCR products were inserted into Promega's pGL3-Basic vector the same way as for the 1000 bp promoter. After the colonies emerged following transformation, in order to verify if the colonies contain the plasmid of interest, colony PCR was executed with pGL3 sequencing primers. Three colonies were analyzed by colony PCR for each construct, and the resultant amplicons were run on agarose gel. The expected DNA fragment of 518 bp (F2), 279 bp (F3), 226 bp (F4) and 160 bp (F5) were detected. PCR fragments are shown in figure 3.5 compared to Fermentas Mass Ruler Mix DNA Marker (SM0403).

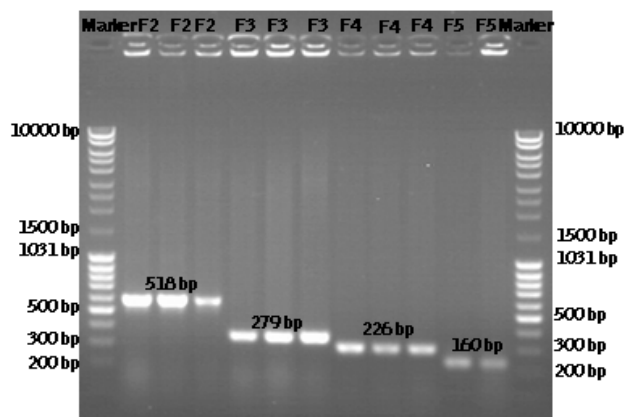


Figure 3.5 : Colony PCR amplified F2, F3, F4, F5 DNA fragments.

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

3.5. Sequence alignment analysis of the constructed plasmids

Sequence analyses of the constructed plasmids were needed to be performed in order to verify the authenticity of the inserts. Even a single base mutation in the promoter region can lead to dramatic decrease of the promoter activity if it takes place in a transcription factor binding site, and that could lead to misinterpretation of the data. Analysis of sequence results was performed via EBI Clustalw sequence analysis tool. It is found that none of the constructed plasmid possesses any mutations. Analysis results are given in Appendix A. After confirming that none of the constructs possess any mutations, each construct was transformed with *E.coli* dam-/dcm- GM2163 strain in order to prevent any methylation that can interfere the transcription factor binding, and after the colonies emerged the insert containing plasmids was reproduced in large scale and purified with QIAGEN, EndoFree® Plasmid Maxi kit.

3.6. Luminometrical Measurements Data of Promoter Deletion Constructs

SH-SY5Y neuroblastoma cells were transfected with each construct on the same day and each transfection was performed as triplicates. Although data are assayed in triplicate, it is not considered $n=3$ for drawing scientific conclusions, for that reason to have a meaningful data for each construct, at least three independent experiments were performed on separate days with independent samples. Recorded light units of independent experiments are given in the following tables.

Table 3.5: Measured light units of experiment $n=1$

n=1	Light Units	F1	F2	F3	F4	F5	F6	F7	pGL3-Basic
1	Firefly	22,84	496	370,8	194,4	131,1	6,239	24,52	3,605
	Renilla	16,65	24,06	25,26	14,52	41,28	23,67	14,04	11,52
2	Firefly	26,67	443,3	286,9	257,5	124,6	8,272	28,02	4,014
	Renilla	20,65	22,89	22,01	25,58	35,55	21,04	14,71	10,90
3	Firefly	26,52	410,2	325,1	221,3	123	9,45	26,84	0,022
	Renilla	18,7	23,32	21,35	25,14	32,3	27,96	16,26	14,33

Table 3.6: Measured light units of experiment n=2

n=2	Light Units	F1	F2	F3	F4	F5	F6	F7	pGL3-Basic
1	Firefly	2,141	137,9	61,72	11,4	11,74	0,1124	0,3179	0,4201
	Renilla	4,103	14,58	3,133	3,133	3,688	0,5655	0,3574	1,361
2	Firefly	3,752	120,8	37,79	26,06	3,276	0,6319	0,885	1,5
	Renilla	6,321	15,28	4,695	4,695	0,9914	2,55	1,025	5,505
3	Firefly	3,691	213,9	49,13	46	0,9217	1,373	0,7318	2,146
	Renilla	3,83	28,56	12,98	12,98	0,7699	4,92	0,7861	7,333

Table 3.7: Measured light units of experiment n=3

n=3	Light Units	F1	F2	F3	F4	F5	F6	F7	pGL3-Basic
1	Firefly	2,813	119,9	165,2	76,05	54,5	4,087	20,69	11,82
	Renilla	17,97	17,43	24,33	21,11	24,5	14,63	11,28	18,17
2	Firefly	3,285	162,7	213,6	83,89	55,27	6,354	19,45	10,09
	Renilla	17,34	21,21	30,67	23,01	24,62	17,6	11,99	17,46
3	Firefly	2,939	166,2	154,1	81,21	79,12	4,9	21,65	11,71
	Renilla	16,01	23,56	27,12	23,25	33,52	18,1	11,5	15,11

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. The calculated fold activity and graphics representing the obtained data are given in the tables and figures below,

$$\Delta \text{ Fold Activity} = \frac{\text{Average (Firefly/Renilla) from Sample X}}{\text{Average (Firefly/Renilla) from pGL3-Basic}} \quad (3.1)$$

Table 3.8: Calculated Δ Fold Activity of experiment n=1

n=1	Ratio	F1	F2	F3	F4	F5	F6	F7	pGL3-Basic
1	F/R	1,371	20,615	14,679	13,388	3,175	1,746	1,746	0.312
2	F/R	1,291	19,366	13,034	10,066	3,504	1,904	1,944	0.368
3	F/R	1,418	17,59	15,227	8,802	3,808	1,650	1,650	-
Average F/R		1.36	19,19	14,313	10,752	10,487	1,766	1,78	0,34
Δ Fold Activity		4,0	56,44	42,097	31,623	30,844	5,194	5,23	1

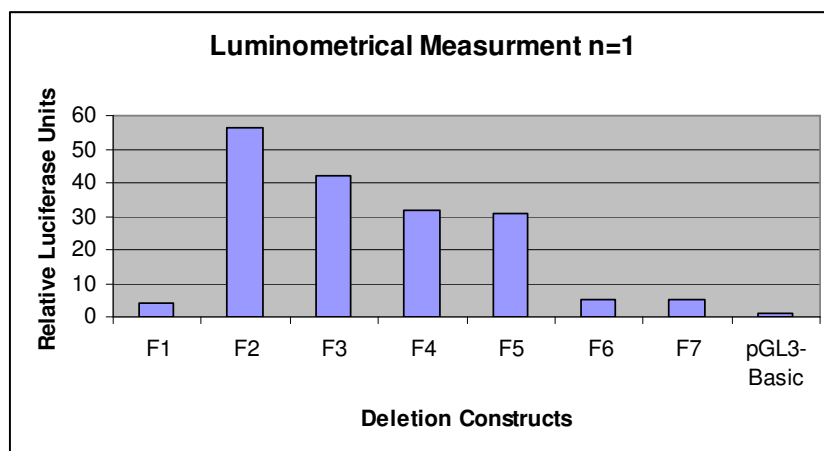


Figure 3.6: Graphic representing the calculated fold activity of experiment n=1.

Table 3.9: Calculated Δ Fold Activity of experiment n=2

n=2	Ratio	F1	F2	F3	F4	F5	F6	F7	pGL3-Basic
1	F/R	0,521	9,4581	6,6508	3,6386	3,1832	0,1987	0,889	0.308
2	F/R	0,5935	7,9057	11,101	5,5505	3,3044	0,252	0,863	0.272
3	F/R	0,9637	7,4894	9,2768	3,5439	1,1971	0,2790	0,9309	0.292
Average F/R		0.6930	8,2844	9,0097	4,2443	2,5616	0,2435	0,8944	0,2912
Δ Fold Activity		2,3793	28,442	30,933	14,572	8,7947	0,8361	3,0709	1

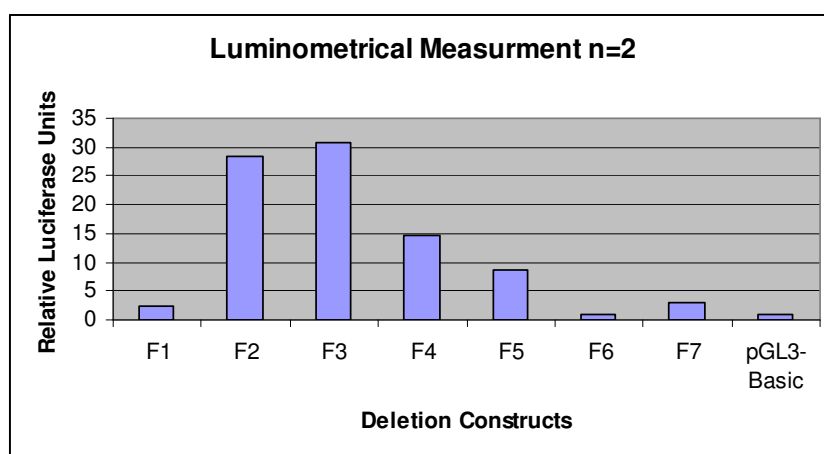


Figure 3.7: Graphic representing the calculated fold activity of experiment n=2.

Table 3.10: Calculated Δ Fold Activity of experiment n=3

n=3	Ratio	F1	F2	F3	F4	F5	F6	F7	pGL3-Basic
1	F/R	0,157	6,879	6,79	3,603	2,224	0,279	1,834	0,651
2	F/R	0,190	7,671	6,964	3,646	2,245	0,361	1,622	0,578
3	F/R	0,184	7,054	5,682	3,493	2,360	0,271	1,883	0,775
Average F/R		0,177	7,201	6,479	3,58	2,277	0,3037	1,78	0,668
Δ Fold Activity		0,265	10,78	9,699	5,359	3,409	0,4555	2,665	1

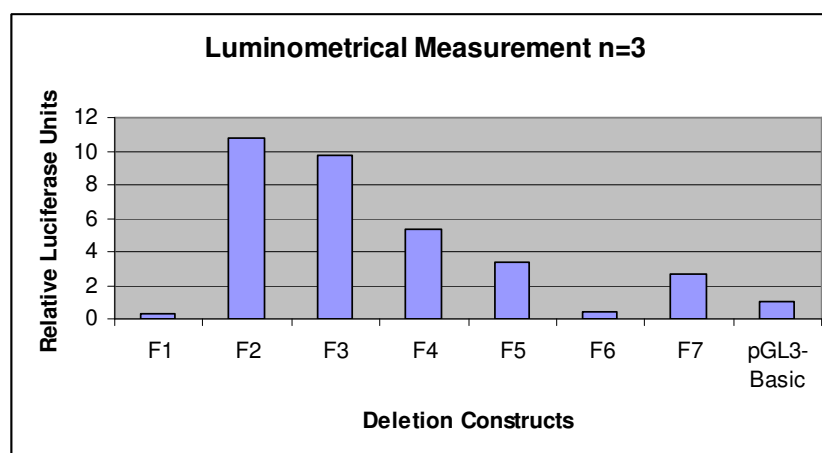


Figure 3.8: Graphic representing the calculated fold activities of experiment n=3.

The normalized fold changes in activity from each experiment are then averaged together, and the standard deviation is calculated in MS Excel. The data is analysed to elucidate whether there is a statistically meaningful difference between the constructs that are following each other via two- tailed unpaired t-test in GraphPad InStat 3 statistic program. Asterisk symbol (*) shown on the average table represents that the p-value is less than 0,005 which is considered a statistically meaningful difference, whereas not significant (NS) symbol represents that the p-value is greater than 0,005 which is not considered a statistically meaningful difference. The data is given in the table 3.11.

Table 3.11: Average of calculated F/R of all experiments and standard deviation

Experiments F/R	F1	F2	F3	F4	F5	F6	F7	pGL3 Basic
n=1	1,36	19,19	14,313	10,75	10,487	1,766	1,78	0,34
n=2	0,693	8,284	9,0097	4,244	2,5616	0,2435	0,89	0,291
n=3	0,177	7,201	6,479	3,58	2,277	0,3037	1,78	0,668
Average F/R	0,743	11,55	9,9339	6,192	5,1085	0,7710	1,48	0,433
Average F/R Standard Deviation	0,593	6,631	3,9979	3,962	4,6600	0,8621	0,51	0,205
Δ Average F/R Fold Activity	1,716	26,69	22,942	14,3	11,798	1,7807	3,42	1

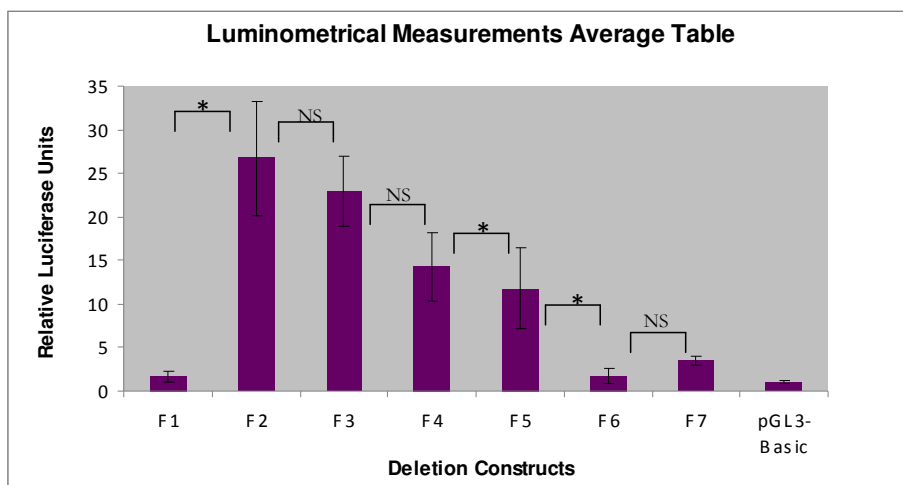


Figure 3.9: Graphic representing the calculated fold activities of average of the experiments and standard deviation.

3.7. Evaluation of the luminometrical measurement data of promoter deletion constructs

For the evaluation of the luminometrical measurements data, PROMO bioinformatics tool was used (Messeguer et al., 2002, Farre et al., 2003). The search for KATNB1 promoter was done by selecting factor's species as *Homo sapiens* and site species as *Homo sapiens* and by restriction the search maximum matrix dissimilarity rate as 5% and the search revealed the presence of 32 transcription factors. Matrix dissimilarity rate stands for the similarity between the given sequence in the promoter and the consensus sequence of the transcription factors. The names and the location of the factors on the KATNB1 1000 bp promoter was given in the following figure 3.10 and figure 3.11, respectively. The research results for Sp1 binding sites shown with number 10 and Elk-1 shown in number 25 can be seen in figure 3.12.

1 GR-beta [T001920]	1 GR-alpha [T00337]	2 STAT4 [T01577]	3 c-Ets-1 [T00112]	4 C/EBPbeta [T00581]	5 ER-alpha [T00261]
8 Pax-5 [T00070]	9 p53 [T00671]	10 Sp1 [T00759]	11 YY1 [T00915]	12 GR [T05076]	13 IRF-2 [T01491]
16 NF-Y [T00150]	17 GATA-1 [T00306]	18 C/EBPalpha [T00105]	19 NF-AT1 [T01948]	20 NF-1 [T00539]	21 ENKTF-1 [T00255]
24 NF-AT1 [T00550]	25 Elk-1 [T00250]	26 E2F-1 [T01542]	27 TFIIID [T00820]	28 NF1/CTF [T00094]	29 RXR-alpha [T01345]
6 AP-2alphaA [T00035]	7 FOXP3 [T04280]				
14 XBP-1 [T00902]	15 TFII-I [T00824]				
22 c-Ets-2 [T00113]	23 IRF-1 [T00423]				
30 RAR-beta [T00721]	31 PPAR-alpha/RXR-alpha [T05221]				

Figure 3.10: *Homo sapiens* factors predicted within a dissimilarity margin less or equal than 5 %.



Figure 3.11: Predicted *Homo sapiens* factors location on the promoter.

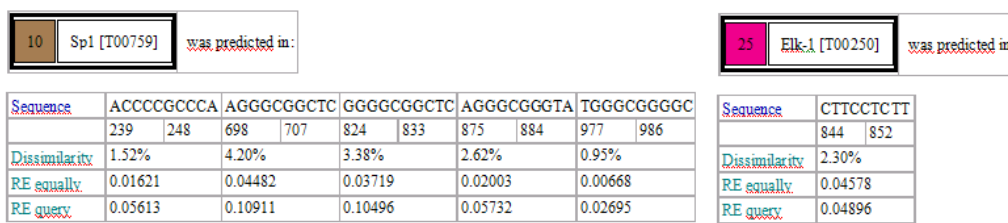


Figure 3.12: Research results for Sp1 and Elk-1 transcription factor binding sites in KATNB1 promoter with matrix dissimilarity rate as 5%.

Another search by selecting factor's species as Eutheria, and site species as Eutheria, and by restriction the search maximum matrix dissimilarity rate as 5% revealed the presence of 124 transcription factors. The names and the location of the factors on the KATNB1 1000 bp promoter was given in the following table 3.13 and figure 3.14 respectively. The research results for PEA3 binding sites shown with number 33 can be seen in figure 3.15.

0	C/EBPbeta [T00017]	1	GR [T00333]	2	HOXA5 [T00377]	3	POU1F1a [T00691]
8	POU2F1 [T00643]	9	YY1 [T00865]	10	GR-alpha [T00337]	11	AR [T00040]
16	C/EBPalpha [T00108]	17	C/EBPbeta [T00459]	18	Nkx2-1 [T00856]	19	JunD [T00437]
24	RAR-beta [T00721]	25	WT1.1 [T01840]	26	Nkx2-1 [T00857]	27	p53 [T01806]
32	HES-1 [T01649]	33	PEA3 [T00684]	34	NF-kappaB [T00590]	35	CP2 [T00152]
40	AP-2alphaA [T00035]	41	p53 [T00671]	42	Sp1 [T00753]	43	Sp1 [T00752]
48	HNF-1C [T01951]	49	NF-1 [T00537]	50	C/EBPalpha [T00105]	51	C/EBPbeta [T00581]
56	DBP [T04875]	57	Crx [T03461]	58	AhR [T01795]	59	fi(alpha)-fi(epsilon) [T00287]
64	c-Jun [T00132]	65	ER-alpha [T00261]	66	AR [T00042]	67	GR [T00335]
72	RXR-alpha [T01345]	73	XBP-1 [T00902]	74	Fli-1 [T01408]	75	NF-Y [T00150]
80	IPF1 [T02057]	81	CBF(2) [T00084]	82	C/EBPgamma [T00216]	83	ENKTF-1 [T00255]
88	PU.1 [T02068]	89	IRF-1 [T00423]	90	NF-AT1 [T00550]	91	VDR [T00885]
96	WT1-KTS [T01839]	97	myogenin [T00528]	98	E2F-1 [T01542]	99	MyoD [T00526]
104	TFIID [T00820]	105	NF-AT3 [T02462]	106	NF-AT2 [T01945]	107	NFI/CTF [T00094]
112	USF-1 [T00875]	113	HNF-3beta [T02344]	114	PPAR-gamma:RXR-alpha [T05236]	115	c-Jun [T00133]
120	XPF-1 [T00906]	121	RXR-beta [T01332]	122	RXR-gamma [T01333]	123	PR B [T00697]
4	C/EBPalpha [T00104]	5	C/EBPdelta [T00109]	6	HNF-3beta [T02513]	7	YY1 [T00915]
12	Elk-1 [T00250]	13	STAT4 [T01577]	14	c-Ets-1 [T00112]	15	c-Ets-2 [T00113]
20	T3R-beta1 [T00853]	21	c-Jun [T00131]	22	JunB [T00436]	23	RAR-alpha1 [T00719]
28	LF-A1 [T00467]	29	COE1 [T01112]	30	NF-1 [T00535]	31	c-Myb [T00138]
36	PTF1-beta [T00701]	37	Pax-5 [T00070]	38	CAC-binding protein [T00076]	39	CACCC-binding factor [T00077]
44	E2F-1 [T01543]	45	Fra-1 [T01208]	46	Cutl1 [T02042]	47	Nkx6-2 [T02050]
52	CREMtau [T01309]	53	CREMtaualpha [T01602]	54	CREMtau1 [T02108]	55	CREMtau2 [T02109]
60	NF-AT1 [T01948]	61	NF-AT1 [T01944]	62	HNF-3alpha [T00371]	63	HNF-3beta [T01049]
68	NF-1/L [T00599]	69	T3R-beta [T00852]	70	IRF-2 [T01491]	71	IRF-2 [T00425]
76	NF-Y [T00613]	77	NF-1 [T00539]	78	GATA-1 [T00306]	79	NF-muNR [T01083]
84	Sp1 [T00754]	85	Sp1 [T00759]	86	HNF-1A [T00368]	87	HNF-1B [T01950]
92	PU.1 [T00702]	93	Crx [T03458]	94	ARP-1 [T00045]	95	WT1.1-KTS [T00900]
100	BTEB3 [T05051]	101	Nr2: MafK [T05666]	102	Elk-1 [T05013]	103	BTEB4 [T05053]
108	C/EBPgamma [T02028]	109	GATA-3 [T00311]	110	HNF-4alpha1 [T00372]	111	AP-2 [T00034]
116	AhR [T00018]	117	PPAR-alpha:RXR-alpha [T05221]	118	NF-1 [T01298]	119	RAR-gamma [T00720]

Figure 3.13: Eutheria factors predicted within a dissimilarity margin less or equal than 5 %.



Figure 3.14: Predicted eutheria factors location on the promoter.

33 PEA3 [T00684]

was predicted in:

Sequence	GCGCTTCC	TCCTTTCC	GGAAACGG	GGAAAGGG	GGAAACGG	GGAAAAGA	GGAAAAGC
	223	230 596	603 743	750 754	761 788	795 808	875 889 896
Dissimilarity	3.80%	0.00%	1.23%	1.23%	1.23%	1.23%	1.23%
RE equally	0.06104	0.06104	0.12207	0.12207	0.12207	0.12207	0.12207
RE query	0.09976	0.07035	0.12394	0.12394	0.12394	0.12394	0.12394

Figure 3.15: Research results for PEA3 transcription factor binding sites in KATNB1 promoter with matrix dissimilarity rate as 5%.

A summary of these computational analysis results is illustrated in the figure 3.16.

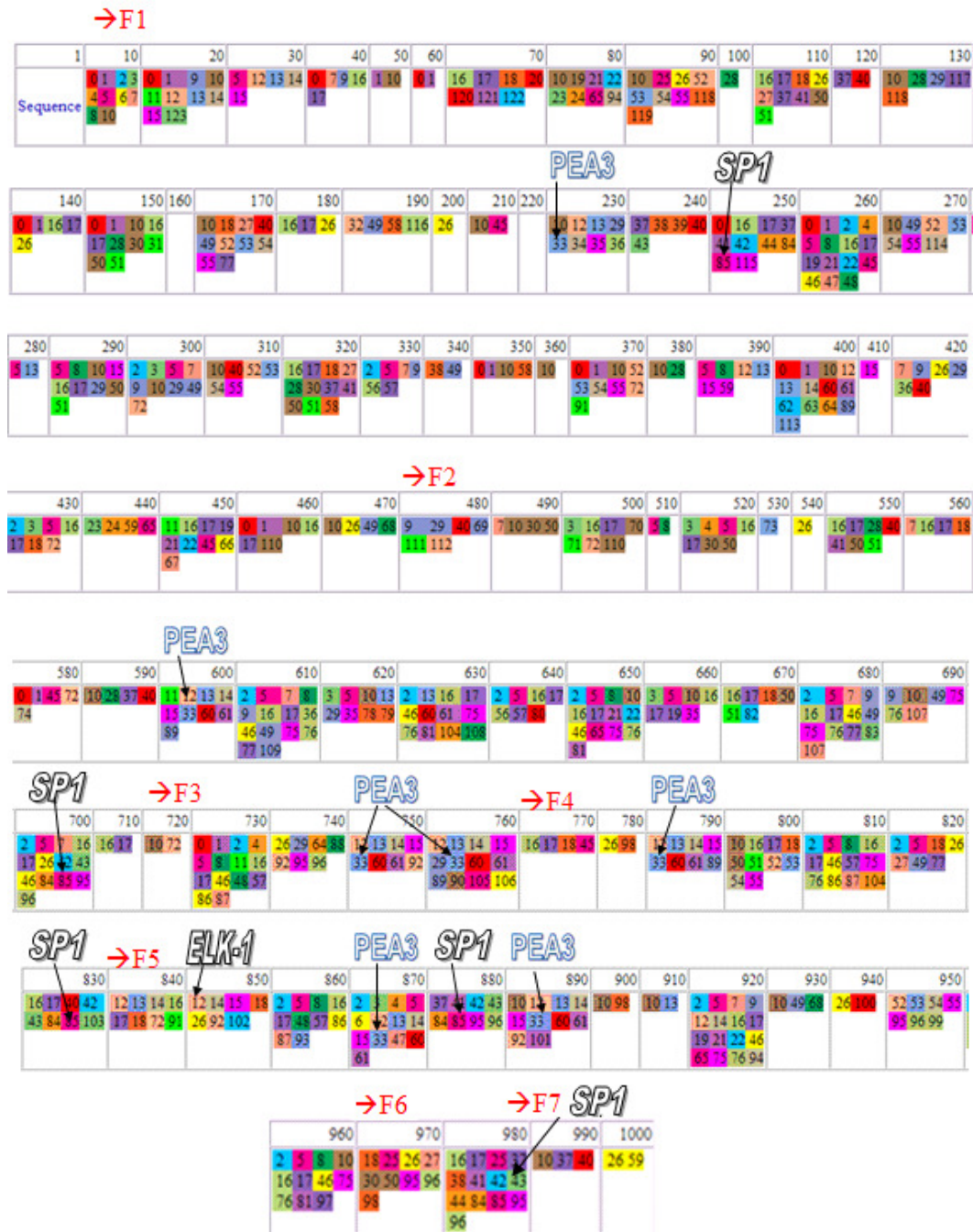


Figure 3.16: Illustration of the KATNB1 promoter binding sites by Elk-1, PEA3 and Sp1 transcription factors.

3.8. Luminometrical Measurements Data of Forced Experiments

SH-SY5Y neuroblastoma cells were transfected with only F2 deletion construct and the same construct were transfected together with Elk-1, Elk-1-VP16 or PEA3 transcription factors expressing constructs. This setup will give the opportunity to evaluate the effects of transcription factors on the promoter. Again the same way as the deletion constructs transfection, the construct was transfected on the same day and each transfection was performed as triplicate and the experiments are replicated at least two times on separate days with independent samples to have a meaningful data. Recorded light units of independent experiments are given in tables below,

- Elk-1 forced experiments,

Table 3.12: Measured light units of Elk-1 forced experiment n=1

n=1	Light Units	F2	pGL3-Basic	F2 Elk-1	pGL3Basic Elk-1
1	Firefly	78,58	3,519	27,48	1,827
	Renilla	10,43	7,1191	55,32	38,92
2	Firefly	85,47	4,238	25,51	1,818
	Renilla	12,23	10,114	58,73	54,54
3	Firefly	60,11	8,17	21,62	2,992
	Renilla	7,513	21,688	47,22	18,74

Table 3.13: Measured light units of Elk-1 forced experiment n=2

n=2	Light Units	F2	pGL3-Basic	F2 Elk-1	pGL3Basic Elk-1
1	Firefly	156,5	3,511	20,08	0,9267
	Renilla	10,39	8,438	64,09	30,24
2	Firefly	159,1	4,238	22,83	1,212
	Renilla	13,76	9,613	62,06	37,61
3	Firefly	0,0052	8,178	16,42	0,8381
	Renilla	0,0083	20,23	57,07	21,42

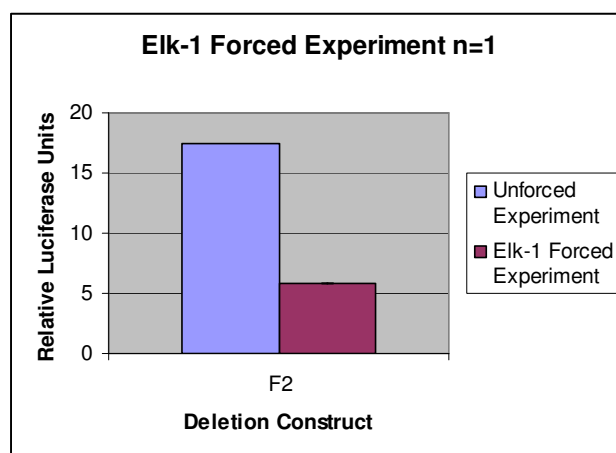
Table 3.14: Measured light units of Elk-1 forced experiment n=3

n=3	Light Units	F2	pGL3-Basic	F2 Elk-1	pGL3Basic Elk-1
1	Firefly	15,823	11,82	42,81	3,51
	Renilla	2,3	18,17	54,03	39,45
2	Firefly	16,344	10,09	21,93	1,346
	Renilla	2,13	17,46	28,42	20,707
3	Firefly	13,102	11,71	31,13	2,57
	Renilla	1,857	15,11	45,43	32,125

The equation used for the analysis of the deletion constructs data to determine the normalized fold change in activity between test groups was used. The calculated fold activity and graphics representing the obtained data are given in the tables and following figures.

Table 3.15: Calculated Δ Fold Activity of Elk-1 forced experiment n=1

n=1	Ratio	F2	pGL3-Basic	F2 Elk-1	pGL3Basic Elk-1
1	F/R	7,5340	0,4943	0,4967	0,0469
2	F/R	6,9885	0,419	0,4343	0,0333
3	F/R	8,0007	0,3767	0,4578	0,1596
Average F/R		7,5078	0,430	0,4629	0,0799
Δ Fold Activity		17,459	1	5,789	1

**Figure 3.17:** Graphic representing the calculated fold activities of Elk-1 forced experiment n=1.**Table 3.16:** Calculated Δ Fold Activity of Elk-1 forced experiment n=2

n=2	Ratio	F2	pGL3-Basic	F2 Elk-1	pGL3-Basic Elk-1
1	F/R	15,0625	0,4160	0,31330	0,030645
2	F/R	11,5625	0,4408	0,36787	0,032225
3	F/R	-	0,4042	0,28771	0,039127
Average F/R		13,3125	0,4204	0,32296	0,03399
Δ Fold Activity		31,6661	1	9,49923	1

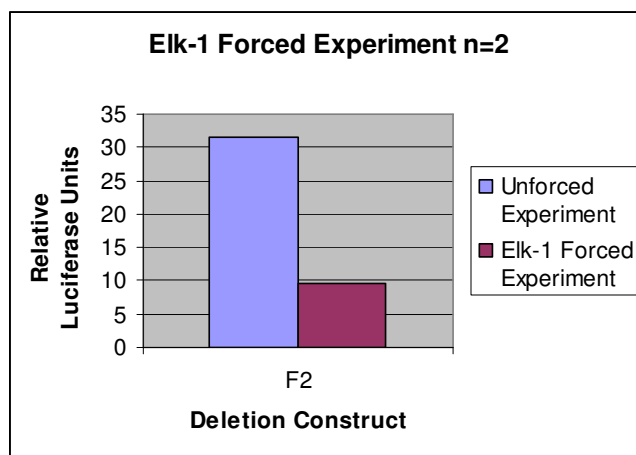
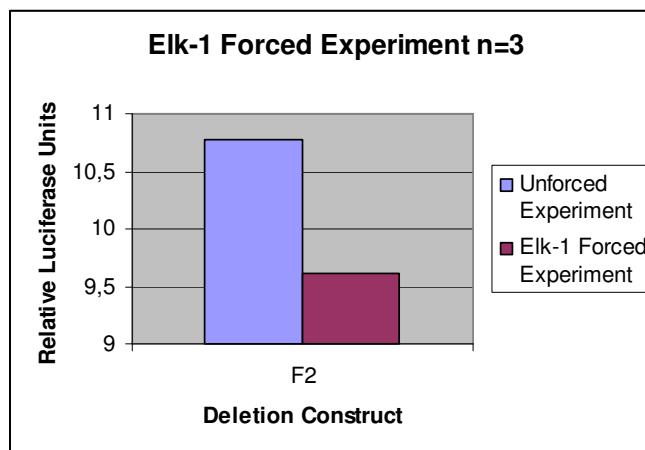
**Figure 3.18:** Graphic representing the calculated fold activities of Elk-1 forced experiment n=2.

Table 3.17: Calculated Δ Fold Activity of Elk-1 forced experiment n=3

n=3	Ratio	F2	pGL3-Basic	F2 Elk-1	pGL3-Basic Elk-1
1	F/R	6,879	0,651	0,792	0,089
2	F/R	7,671	0,578	0,77	0,065
3	F/R	7,054	0,775	0,685	0,08
Average F/R		7,2010	0,668	0,75	0,078
Δ Fold Activity		10,77	1	9,61	1

**Figure 3.19:** Graphic representing the calculated fold activities of Elk-1 forced experiment n=3.

The normalized fold changes in activity from each experiment are then averaged together and the standard deviation is calculated in MS Excel. The data is analysed to elucidate whether there is a statistically meaningful difference between the unforced and the forced experiments via two-tailed unpaired t-test in GraphPad InStat 3 statistic program. Asterisk symbol (*) shown on the average table represents that the p-value is less than 0,005 which is considered a statistically meaningful difference. The data is given in the table 3.18.

Table 3.18: Average of calculated F/R of Elk-1 forced experiments and standard deviation

Experiments F/R	F2	pGL3-Basic	F2 Elk-1	pGL3-Basic Elk-1
n=1	7,5078	0,430	0,4629	0,0799
n=2	13,3125	0,4204	0,32296	0,0339
n=3	7,2010	0,668	0,75	0,078
Average F/R	9,34045	0,50613	0,51198	0,0639
Average F/R Standard Deviation	3,44333	0,14026	0,217693	0,026
Δ Average F/R Fold Activity	18,4545	1	8,008	1

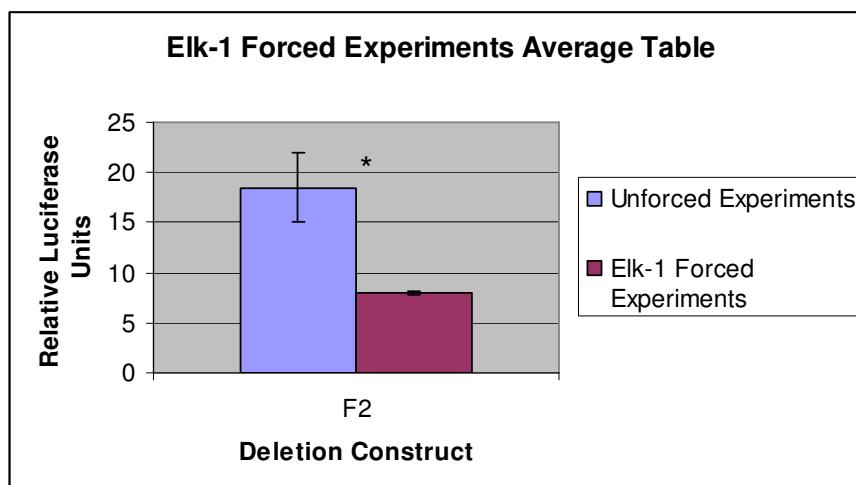


Figure 3.20 : Graphic representing the calculated fold activities of average of Elk-1 forced the experiments and standard deviation.

▪ Elk-1-VP16 forced experiments,

Table 3.19: Measured light units of Elk-1-VP16 forced experiment n=1

n=1	Light Units	F2	pGL3-Basic	F2 Elk-1	pGL3Basic Elk-1-VP16
1	Firefly	78,58	3,519	190,6	5,331
	Renilla	10,43	7,1191	22,25	8,675
2	Firefly	85,47	4,238	245,1	6,666
	Renilla	12,23	10,114	25,59	9,699
3	Firefly	60,11	8,17	257,3	4,849
	Renilla	7,513	21,688	24,12	9,28

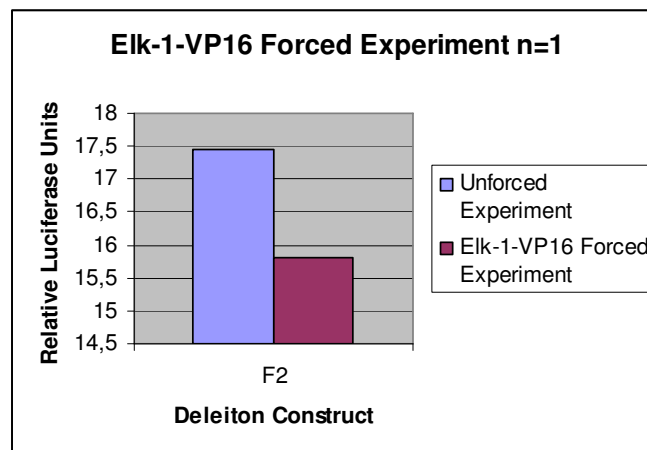
Table 3.20: Measured light units of Elk-1-VP16 forced experiment n=2

n=2	Light Units	F2	pGL3-Basic	F2 Elk-1-VP16	pGL3Basic Elk-1-VP16
1	Firefly	59,75	5,475	104,1	5,331
	Renilla	6,496	6,87	15,73	8,675
2	Firefly	53,44	4,315	96,22	6,666
	Renilla	8,663	7,217	15,92	9,699
3	Firefly	50,06	4,646	93,43	4,849
	Renilla	6,677	8,211	17,08	9,28

The equation used for the analysis of the deletion constructs data to determine the normalized fold change in activity between test groups was used. The calculated fold activity and graphics representing the obtained data are given in the following tables and figures.

Table 3.21: Calculated Δ Fold Activity of Elk-1-VP16 forced experiment n=1

n=1	Ratio	F2	pGL3-Basic	F2 Elk-1-VP16	pGL3-Basic Elk-1-VP16
1	F/R	7,534036	0,4943	8,5662	0,61452
2	F/R	6,988553	0,419	9,577	0,68729
3	F/R	8,000799	0,3767	10,66	0,52252
Average F/R		7,507796	0,43	9,6039	0,6081
Δ Fold Activity		17,45999	1	15,793	1

**Figure 3.21:** Graphic representing the calculated fold activities of Elk-1-VP16 forced experiment n=1.**Table 3.22:** Calculated Δ Fold Activity of Elk-1-VP16 forced experiment n=2

n=2	Ratio	F2	pGL3-Basic	F2 Elk-1-VP16	pGL3Basic Elk-1-VP16
1	F/R	9,19797	0,79694	6,61793	0,61452
2	F/R	6,16876	0,59789	6,04397	0,68729
3	F/R	7,49738	0,56583	5,47014	0,52252
Average F/R		7,62137	0,65355	6,04401	0,6081
Δ Fold Activity		11,6614	1	9,93899	1

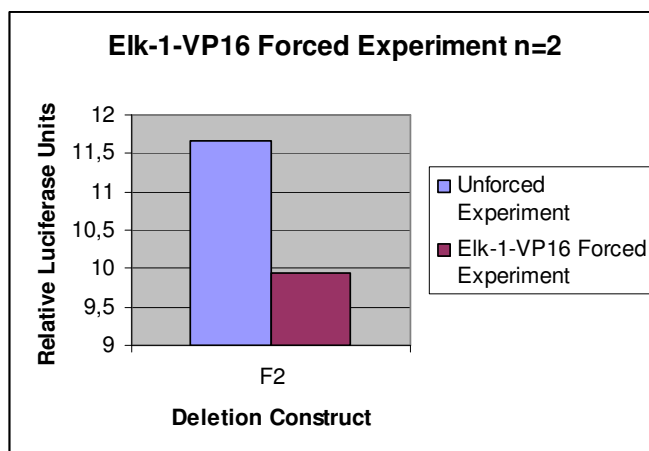


Figure 3.22: Graphic representing the calculated fold activities of Elk-1-VP16 forced experiment n=2.

The normalized fold changes in activity from each experiment are then averaged together, and the standard deviation is calculated in MS Excel. Data is analysed to elucidate whether there is a statistically meaningful difference between the unforced and the forced experiments via two-tailed unpaired t-test in GraphPad InStat 3 statistic program. Asterisk symbol (*) shown on the average table represents that the p-value is less than 0,05 which is considered a statistically meaningful difference. The data is given in the table 3.23.

Table 3.23: Average of calculated F/R of Elk-1-VP16 forced experiments and standard deviation

Experiments F/R	F2	pGL3-Basic	F2 Elk-1	pGL3-Basic Elk-1
n=1	7,50779	0,43	9,6039	0,6081
n=2	7,62137	0,65355	6,04401	0,6081
Average F/R	7,56458	0,54177	7,823955	0,6081
Average F/R Standard Deviation	0,0803	0,1580	2,51722	0
Δ Average F/R Fold Activity	13,9625	1	12,86611	1

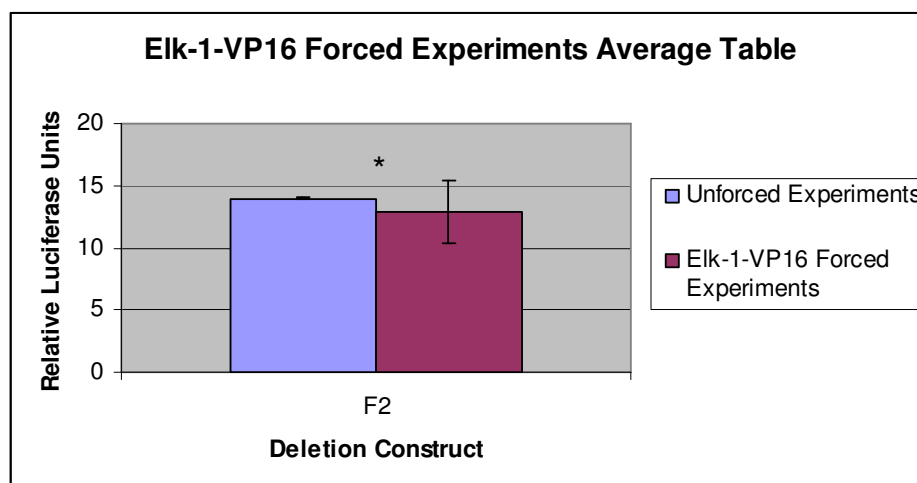


Figure 3.23: Graphic representing the calculated fold activities of average of Elk-1-VP16 forced the experiments and standard deviation.

- PEA3 forced experiments,

Table 3.24: Measured light units of PEA3 forced experiment n=1

n=1	Light Units	F2	pGL3-Basic	F2 PEA3	pGL3-Basic PEA3
1	Firefly	242,6	12,42	148,9	-
	Renilla	27,71	21,26	12,95	-
2	Firefly	266,2	13,02	175,7	7,112
	Renilla	32,02	21,11	13,27	10,26
3	Firefly	200,3	18,31	193,7	8,904
	Renilla	33,6	21,62	14,36	11,14

Table 3.25: Measured light units of PEA3 forced experiment n=2

n=2	Light Units	F2	pGL3-Basic	F2 PEA3	pGL3-Basic PEA3
1	Firefly	156,5	3,511	106,8	4,868
	Renilla	10,39	8,438	16,77	16,86
2	Firefly	159,1	4,238	115,6	4,992
	Renilla	13,76	9,613	21,7	33,37
3	Firefly	0,0052	8,178	96,35	5,311
	Renilla	0,0083	20,23	35,57	24,75

Table 3.26: Measured light units of PEA3 forced experiment n=3

n=3	Light Units	F2	pGL3-Basic	F2 PEA3	pGL3-Basic PEA3
1	Firefly	201,3	11,82	193,2	8,56
	Renilla	29,6	18,17	15,44	11,428
2	Firefly	256,78	10,09	191,6	9,345
	Renilla	34,532	17,46	10,1	11,341
3	Firefly	218,69	11,71	138,6	7,35
	Renilla	29,628	15,11	11,18	11,05

The equation used for the analysis of the deletion constructs data to determine the normalized fold change in activity between test groups was used. The calculated fold

activity and graphics representing the obtained data are given in the tables and figures below,

Table 3.27: Calculated Δ Fold Activity of PEA3 forced experiment n=1

n=1	Ratio	F2	pGL3-Basic	F2 PEA3	pGL3-Basic PEA3
1	F/R	8,75496	0,5842	11,4981	-
2	F/R	8,31355	0,6167	13,2404	0,69318
3	F/R	5,96131	0,8469	13,4889	0,79928
Average F/R		7,67661	0,6826	12,7424	0,746
Δ Fold Activity		11,246	1	17,081	1

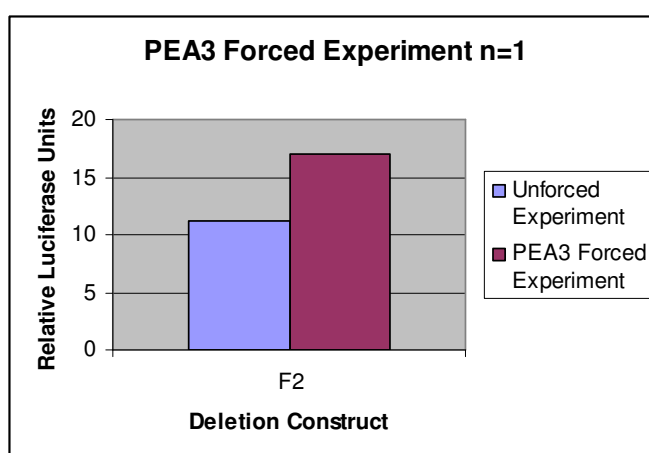


Figure 3.24: Graphic representing the calculated fold activities of PEA3 forced experiment n=1.

Table 3.28: Calculated Δ Fold Activity of PEA3 forced experiment n=2

n=2	Ratio	F2	pGL3-Basic	F2 PEA3	pGL3-Basic PEA3
1	F/R	15,0626	0,41609	6,36852	0,28873
2	F/R	11,5625	0,44086	5,32719	0,1496
3	F/R	0,6265	0,40425	2,70874	0,21459
Average F/R		9,08385	0,4204	4,80148	0,21764
Δ Fold Activity		21,607	1	22,0615	1

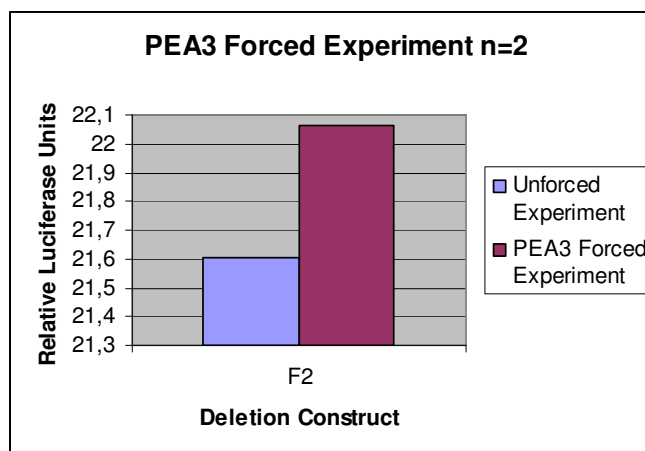


Figure 3.25: Graphic representing the calculated fold activities of PEA3 forced experiment n=2.

Table 3.29: Calculated Δ Fold Activity of PEA3 forced experiment n=3

n=3	Ratio	F2	pGL3-Basic	F2 PEA3	pGL3-Basic PEA3
1	F/R	6,786	0,651	12,512	0,749
2	F/R	7,436	0,578	18,97	0,824
3	F/R	7,381	0,775	12,397	0,665
Average F/R		7,20104	0,668	14,63	0,746
Δ Fold Activity		10,78	1	19,611	1

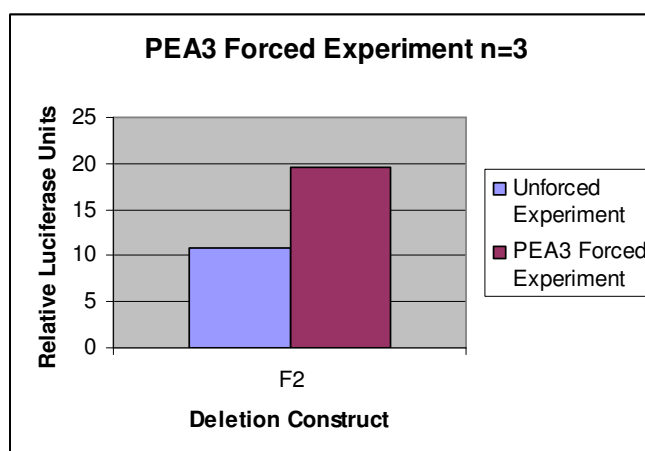


Figure 3.26: Graphic representing the calculated fold activities of PEA3 forced experiment n=3.

The normalized fold changes in activity from each experiment are then averaged together, and the standard deviation is calculated in MS Excel. Data is analysed to elucidate whether there is a statistically meaningful difference between the unforced and the forced experiments via two-tailed unpaired t-test in GraphPad InStat 3 statistic program. Not significant (NS) symbol shown on the average table represents

that the p-value is greater than 0,05 which is not considered a statistically meaningful difference. The data is given in the table 3.30.

Table 3.30: Average of calculated F/R of PEA3 forced experiments and standard deviation

Experiments F/R	F2	pGL3-Basic	F2 PEA3	pGL3-Basic PEA3
n=1	7,67661	0,6826	12,7424	0,746
n=2	9,08385	0,4204	4,80148	0,2176
n=3	7,20104	0,668	14,63	0,746
Average F/R	7,98717	0,59034	10,72463	0,5698
Average F/R Standard Deviation	0,97907	0,14735	5,2157	0,305
Δ Average F/R Fold Activity	13,962	1	18,2059	1

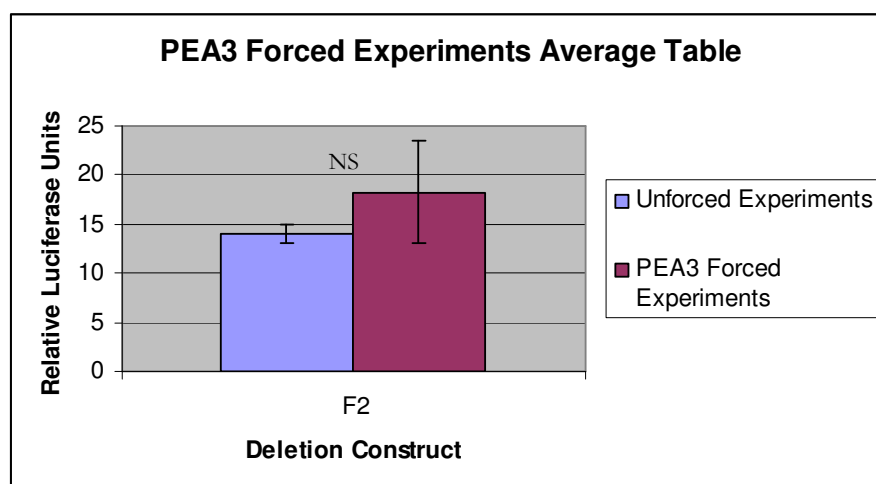


Figure 3.27: Graphic representing the calculated fold activities of average of PEA3 forced the experiments and standard deviation.

4. DISCUSSION

As outlined in the introduction katanin is a heterodimeric protein that severs microtubules by hydrolyzing ATP, consisting of 60 kDa and 80 kDa polypeptides. 60 kDa subunit (p60) has the enzymatic activity to break microtubules, whereas 80 kDa subunit (p80) has a role in localization of the protein complex in the cell. Katanin has been shown to have roles for microtubule severing in mitotic cells including release of microtubules from centrosome, depolymerization of microtubule minus ends in the mitotic spindle. In addition to its roles in mitotic cells, katanin is also important for some specialized cell types such as neurons. It is thought that katanin provides a source of non-centrosomal microtubules that are transported into developing axons and dendrites for structural support.

Despite the importance of this gene, its transcriptional regulation has not been studied, and its promoter has not yet been characterized. We therefore set out to identify the potential cis-regulatory elements including the promoter of the KATNB1 gene coding for katanin p80 subunit.

In order to understand the transcriptional regulation of human KATNB1 gene we have first determined the putative promoter region in the upstream of KATNB1 gene.

Bioinformatic analysis revealed that putative KATNB1 gene promoter is located on chromosome 16 between CCDC135 and KATNB1 genes according to NCBI Map Viewer database. This 4292 bp region comprise the promoter region of KATNB1 gene. The search for TATA box and CAAT box within 4292 bp region revealed the presence of several TATA box and a CAAT box, but those motifs can not be functional because they are located very far from the transcription start site since according to literature data a functional TATA box must be located within 25 to 30 bp upstream of the transcription start site. Furthermore, the analysis for CG boxes showed that promoter region contains several CG boxes and the CpG island analysis revealed the presence of 1 CpG island, 332 nucleotide in length positioned in the 611-942 bp of the last 1000 bp promoter region. This CpG island is the only one

island that is found within the 4292 bp region. The presence of these motifs is important because it is known that the cytosine and guanine rich sequences in the promoter regions of genes are the candidate regions for transcription factor binding. The dinucleotide CG is underrepresented in vertebrate DNAs, and the presence of a CG-rich region, or CpG island, just upstream of the start site is a distinctly nonrandom distribution. For this reason, the presence of a CpG island in genomic DNA suggests that it may contain a transcription-initiation region (Lodish et al., 2004). In this context, we thought that the promoter of KATNB1 gene might be regulated by transcription factor binding to those CG rich regions in the last 1000 bp of the putative promoter region. That is why we have selected the last 1000 bp promoter region for further deletion construct studies. Before creating the 5' truncation of the promoter, computer prediction analysis for putative binding sites for different transcriptional elements was accomplished via TRANSFAC[®] 7.0 Public 2005 program (Matys et al., 2006). The analysis predicted the presence of Elk-1, PEA3, Sp1 binding sites. In order to ascertain which transcription factors are involved in the transcriptional regulation, we designed the deletion constructs by separating the CG boxes and other putative factors. Meaning that, each construct lacks one or more CG box or transcription factor binding site between mentioned transcription factors than its shorter form.

In order to evaluate which part of the promoter has a role in driving transcription, we cloned first the 1000 bp promoter from human genomic DNA isolated from whole blood. Cloning experiment was repeated until we would get the wild-type promoter sequence. We have come across with sequence analysis showing some point mutations. These mutations can either be polymorphism or can be misintegration of nucleotides by the *Taq* polymerase used in the PCR. The deletion constructs were then amplified from the wild type 1000 bp promoter. The deletion constructs are cloned into pGL3-Basic luciferase reporter vector. Those vectors are named as pGL3-Basic-F(1...6), F1 representing the longer 1000 bp construct and F6 the shorter one respectively. Since this vector lacks eukaryotic promoter and enhancer sequences, expression of luciferase activity in cells transfected with this plasmid depends on insertion and proper orientation of a functional promoter directionally upstream from *luc+* providing us the data about which part of the promoter has a role in driving transcription.

The type of cells that will be used in the genetic reporter assay transfections, depends on the anticipated expression pattern of the genes whose regulatory sequences are to be studied. SH-SY5Y human neuroblastoma cell line was chosen to be transfected with pGL3-Basis-F constructs due to its neuronal origin.

We tried to optimize transfection of SH-SY5Y cells first with nucleoporation method. According to literature data, the expression of luciferase activity in cells transfected with the reporter plasmids was measured 48 hours post-transfection. When we measured the full length promoter F1 activity, although the luciferase assay system is highly sensitive and can report even a few femtograms of luciferase we could not detect any luminescence. But, later we found out that optimal analysis time usually is 6-16 hours after nucleofection due to its ability to deliver substrates not only into the cytoplasm but also into the nucleus of the cell and thus reducing time to expression. When we measured the full length promoter F1 activity, 16 hours post-transcription finally, we have detected luciferase activity. Yet, this nucleofection method requires a large number of cells (1×10^6 cells/transfection) and excessive amount of DNA ($10 \mu\text{g}$ /transfection) and this was not very feasible thinking that we were going to repeat each transfection in triplicate for at least two times to have a meaningful data and also would be very hard to handle so many plates, and would consume a lot of culture media and DNA. Hence, we switched our transfection method to cationic lipid mediated transfection. In that method, opposite to nucleofection, a minimum number of cells ($0,05 \times 10^6$ / transfection) and DNA ($1 \mu\text{g}$ /transfection) were needed and it was easy to handle the cells in 24-well cell culture plates with minimal culture media. In case we measured the luciferase activity 48 hour post-transfection. During the transfection, along with the pGL3-Basic-F constructs, pRL-TK vector containing a cDNA encoding renilla luciferase is transfected to cells intended for use as an internal control. The amount of internal control vector needed depends on the control promoter and the cell line. According to manufacturer instruction, we used a ratio of 10:1 test vector:control vector (Schagat et al., 2007). In this study, dual reporters assay system is chosen because dual reporters are commonly used to improve experimental accuracy. Our experimental reporter, pGL3-Basic-F is correlated with the effect of specific experimental conditions, while the activity of our co-transfected control reporter, pRL-TK provides an internal control that serves as the baseline response. Normalizing the activity of the pGL3-Basic-F to the activity of the pRL-

TK minimizes experimental variability caused by differences in cell viability, transfection efficiency, differences in pipetting volumes, cell lysis efficiency and assay efficiency.

The producer of the pGL3-Basic vectors claimed that they removed cryptic regulatory sequences from the *luc+* gene without changing the encoded amino acids and from the vector backbone. Removal process of these cryptic regulatory sequence like a transcription factor binding site and/or a promoter module, sequences resembling splice sites, poly(A) addition sequences, Kozak sequence (translation start for mammalian cells) and *E. coli* promoters or *E. coli* ribosome binding sites said to lead to a greatly reduced number of cryptic regulatory sequences. But the manufacturers also announced that their pGL3-Basic vectors have recently been improved by further cleaning the entire pGL3-Basic vector backbone, removing cryptic regulatory sequences wherever possible, while maintaining its functionality to create unique vector backbone of the pGL4 Luciferase Reporter Vectors (Promega Protocols and Application Guide 8, 2008). The presence of cryptic regulatory sequences in the reporter vector may adversely effect transcription, resulting in anomalous expression of the reporter and to misinterpretation of the data. Taking this into the account, in order to minimize the effects of the possible cryptic regulatory elements in the reporter vector, while interpreting the luciferase measurement data Firefly/Renilla normalized activity is further normalized as fold of induction in respect to the activity of the empty vector pGL3-Basic. Hence, any possible cryptic promoter element within the pGL3-Basic vector that can bind on trancriptrion factors from SH-SY5Y cells is eliminated from the data of the promoter region. The results of luciferase reporter assay for the KATNB1 gene promoter deletion constructs in SH-SY5Y neuroblastoma cells is given in the following graphic.

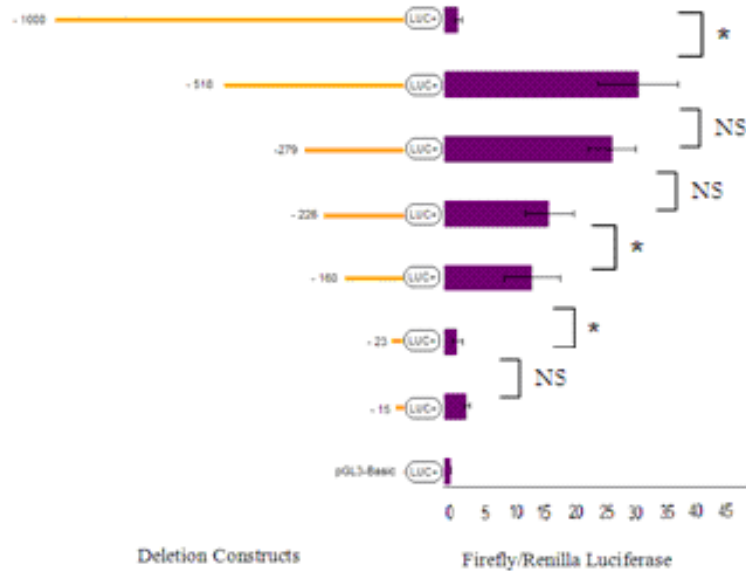


Figure 4.1: Luciferase reporter assay results for the KATNB1 gene promoter in SH-SY5Y neuroblastoma cells.

For the evaluation of the luminometrical measurements data, PROMO bioinformatics tool was used (Messeguer et al., 2002, Farre et al., 2003). Computer prediction analysis was performed many times with different selection of the program. For example, program suggested binding sites for the presence of 14 transcription factors when selecting factor's species as *Homo sapiens* and site species as *Homo sapiens* and by restriction the search maximum matrix dissimilarity rate as 0%. When this dissimilarity rate was increased to 3% it detects 27 factors, when increased to 5%, 32 transcription factors were detected. The importance for us was to see where our predicted factors, namely Sp1, Elk-1 and PEA3 binding sites will be revealed. We could not come across with Sp1 and Elk-1 binding site with 0% dissimilarity rate. When the dissimilarity rate was set to 3%, three putative Sp1 binding sites and one putative Elk-1 binding site appeared. When the rate was set to 5% the number of possible interaction sites were risen to 5 for Sp1 and did not change for Elk-1.

Since we could not come across with the binding site for PEA3 we have changed the selection parameters of the program to factor's species as eutheria and site species as eutheria. Program suggested binding sites for the presence of only one PEA3 transcription factor binding site if chosen the search maximum matrix dissimilarity rate as 0%. This transcription factor was from mouse origin with accession number T00684. When the search maximum matrix dissimilarity rate was increased to 3%,

six binding sites appeared. Finally, with 5% dissimilarity rate, we have come across with seven PEA3 binding sites. Factors binding characteristics of each deletion constructs of the KATNB1 gene promoter are illustrated in figure 4.2. Underlined factors are the ones that differ the construct from the following one.

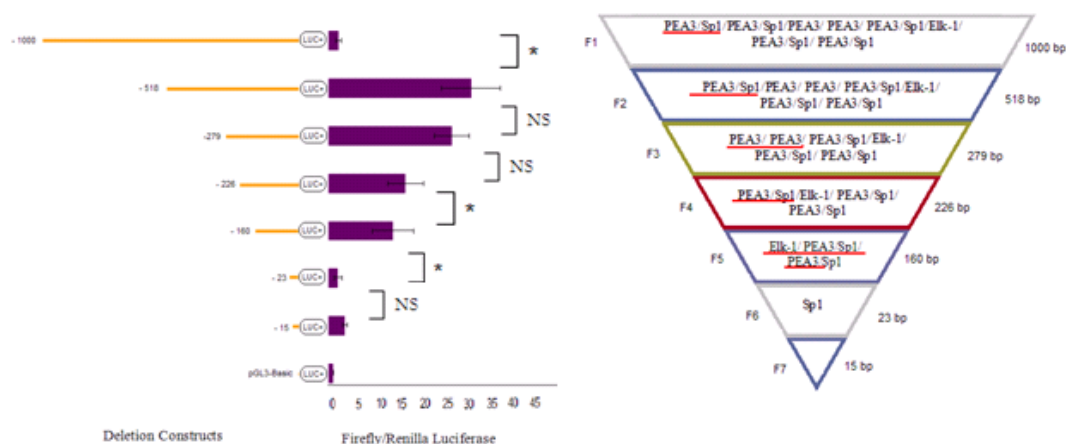


Figure 4.2: Factors binding characteristics of each deletion constructs of the KATNB1 gene promoter.

In the light statistical data of the luciferase reporter assay results for the KATNB1 gene promoter in SH-SY5Y neuroblastoma cells and computational transcription factor binding studies, it can be speculated that the 482 bp portion of the longer construct F1 differing it from its shorter one F2 possesses some inhibitory binding sites for some transcriptional regulators causing its minimal relative luciferase unit (RLU). It is seen that 5'-truncations of F2 construct is leading to a decrease in the promoter activity showing that this part of the promoter is required for the core promoter activity and this 518 bp TATA-less promoter which is statistically different from construct F1 is sufficient for high levels of promoter activity. When the 5'-239 bp were depleted in the F2 construct, it is seen that this deletion does not lead to a statistically different change in the promoter activity. Further 5'-truncation of F3 construct also does not lead to a statistically meaningful decrease in the promoter activity. The 66 bp between the F4-F5 constructs possess one PEA3 and also one Sp1 binding site, and a statistically meaningful decrease in the promoter activity is observed, meaning that PEA3 and/or Sp1 are acting on the promoter activity. The most dramatic statistically meaningful decrease in the promoter activity occurs when 137 bp was truncated from 5'-end of the F5 construct. This region contains two PEA3, one Elk-1 and also one Sp1 site, and we know from executed computational analysis that this region of the promoter contains a CpG island and is found to be rich

for CG nucleotides. This dramatic increase in the promoter activity is concordant with our hypothesis saying that this part of the KATNB1 promoter might be regulated by transcription factor binding to those CG rich regions. This decrease can be due to the truncation of the Sp1 site, because it is known that Sp family transcription factors preferentially binds to CG boxes or maybe due to PEA3 binding site loss. We have designed the last two short construct in order to see if the truncation of the Sp1 binding site from F6 to F7 will lead to a decrease in the promoter activity. Surprisingly, the luminometrical studies results are showing that the depletion of Sp1 binding site does not lead to a statistically different change in the promoter activity, meaning that the Sp1 transcription factor does not bind to this predicted region despite the fact that it contained an intact CG box. We could not further analyze the consequences of Sp1 transcription factor effects on the promoter due to absence of its coding vector.

Regarding to the computational analysis of the promoter and with the light of the luciferase assays data, we further searched the Elk-1 and PEA3 transcription factors activity on KATNB1 promoter. We analyzed their activity with forced luciferase experiments by transfecting plasmids with human Elk-1 coding plasmids and mouse PEA3 coding plasmids along with the identified core promoter region, F2 construct. Since we did not have human PEA3 coding plasmid we used the mouse one, after all those transcription factors consensus sequences show a great homology.

PEA3 transcription factors embryonic expression patterns and aberrant expression in metastatic tumors, suggest a role for PEA3 proteins in regulating cell motility during organogenesis/development and during tumor progression (Greenall et al., 2001). Due to its developmental pattern of expression it has been suggested that it may play a role in the development of the somites and in regionalization of the nervous system (Brown et al., 1998). Other roles of PEA3 group transcription factors are shown recently, they are found to be expressed in tissues undergoing branching morphogenesis and promote formation of duct-like structures by mammary epithelial cells *in vitro* (Chotteau-Lelièvre et al., 2003).

Our protein of interest katanin p80 is widely expressed in the nervous system and in mitotic cells, taking part of severing microtubules with its partner p60 it is mainly responsible of generating free microtubules, participating the regulation of cell cycle progression, regulation of axonal growth, and branch formation.

Both proteins seem to have interacting action areas, while PEA3 is responsible of cell motility during organogenesis/development, tumor progression and expressed in tissues undergoing branching morphogenesis, katanin p80 also has a role in regulation of cell cycle progression, regulation of axonal growth, branch formation and also through its role in mitosis katanin seems to be related with mitogenic tumor cells. To that end, we were expecting that PEA3 factor will lead to an increase in the promoter activity. Forced expression of PEA3 along with F2 construct in transfected SH-SY5Y human neuroblastoma cells did not result with a statistically meaningful change in the promoter activity.

The situation is less clear with Elk-1 transcription factor. Since ETS domain proteins can function as either transcriptional activators or repressors (Sharrocks et.al., 1997), and also some family members including Elk-1 were shown to have both repressive and activating abilities (Sharrocks, 2001). Elk-1 is a direct target of the MAP kinase pathways, when phosphorylated by MAP kinases, its activation is triggered, and it has been shown that SUMO modification of its R motif will lead to its repression. There is a dynamic interplay between the activating MAPK pathway and SUMO pathway (Yang et al., 2003). Elk-1 is important in neuronal systems in particular for processes such as fear conditioning, light stimulus, and glutamate induction. This protein is traditionally known as a mitogenic factor, mostly associated with proliferation thus the molecular mechanism underlying its effects on these learning and synaptic plasticity related events in post-mitotic neurons are not yet understood (Aksan-Kurnaz, 2009). It has been showed that Elk-1 protein biochemically interacted with microtubules *in vitro*. It was further demonstrated that upon serum induction, most of the phospho-Elk-1 translocates to the nucleus, which is independent of translation. This leads to the idea that Elk-1 is anchored to neuronal microtubules in resting or unstimulated cells. Upon stimulation, its phosphorylated form, phospho-Elk-1, relocalizes to the nucleus in neurons where it is proposed to regulate neuronal target genes (Demir et al., 2008). It was also demonstrated that Elk-1 represses pro-apoptotic genes such as *egr-1* in SH-SY5Y neuroblastomas in a SUMO dependent manner (Demir and Aksan Kurnaz, 2008). We were curious as to whether Elk-1 would activate or repress KATNB1 expression. Forced expression analysis of wild type Elk-1 under the control of activating MAPK pathway and repressing SUMO pathway in transfected SH-SY5Y human neuroblastoma cells

along with F2 construct resulted in a statistically meaningful decrease of the promoter activity. In the light of these results, we sought to understand whether its repression domain would be directly involved in the repression and whether Elk-1 would be switched from a repressor to an activator. To test that hypothesis, Elk-1-VP16 a constitutively active Elk-1 construct was transfected along with F2 construct. If the repression was due to the effect of SUMO modification, this transfection would result in an increase of the promoter activity. Strikingly, we have come across with similar results. Since the effect on the promoter activity of these two constructs is statistically different, a comparison between wild type Elk-1 and Elk-1-VP16 transfection results in an increased activity in case of Elk-1-VP16 construct but still much less from the unforced activity of the F2 construct, meaning that the repression occurs in SUMO dependent way, in addition to some other modifications of Elk-1. The comparison between Elk-1 and Elk-1-VP16 on the promoter activity can be seen the figure 4.3. Asterisk symbol (*) shown on the table represents that the p-value is less than 0,05 which is considered a statistically meaningful difference.

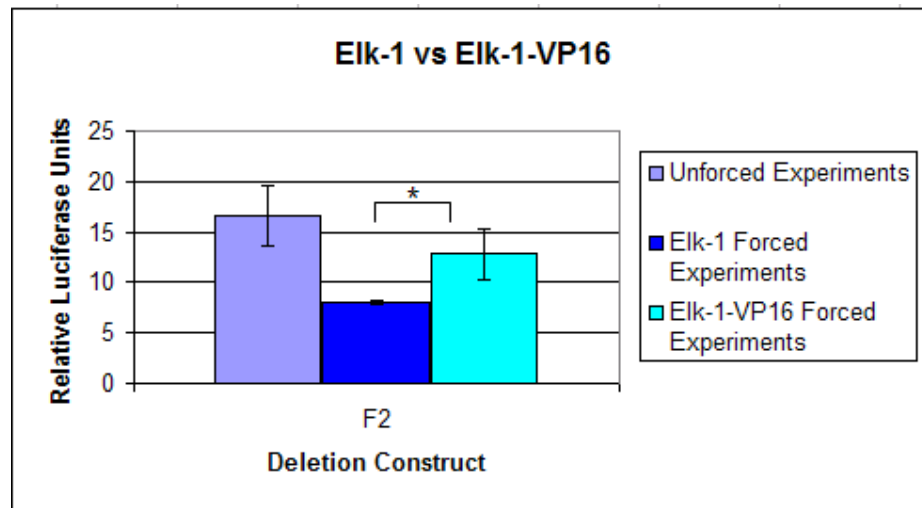


Figure 4.3: The comparison of the effects of Elk-1 and Elk-1-VP16 on the promoter activity.

5. CONCLUSION

This study is an initial report on transcriptional regulation of the KATNB1 gene, representing only the tip of the iceberg. Analysis of deletion constructs showed that a 518 bp TATA-less promoter was sufficient for high level of activity. Effects of ETS-family members transcription factors namely, Elk-1 and PEA3 are studied. While the overexpression of Elk-1 transcription factor acts by repressing the promoter in a SUMO dependent way, in addition to some other modifications of Elk-1, PEA3 was not found to act on the promoter.

This preliminary data help to understand the regulation of this important gene, but further confirmation studies are needed to be done. Site-directed mutagenesis of the mentioned factors binding site may strengthen our data. Currently the chromatin immunoprecipitation technique is the most precise method to identify and analyze transcription factor binding site. Electron mobility shift assay, super-shift assay that have been used extensively to study DNA-protein interactions may reveal the physical interaction of these transcription factors with the KATNB1 promoter.

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APPENDICES

APPENDIX A. Sequence Data of Constructed pGL3-Basic-Deletion Constructs plasmids

▪ Sequence Data of Constructed pGL3-Basic-F1

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F1_For      AGGTACCTCATCTGTAAATGGGAACAGGAATTCCTACCTCTTATGGTTGTACTGGGCGTT 60
pGL3_F1     -----TCATCTGTAAATGGGAACAGGAATTCCTACCTCTTATGGTTGTACTGGGCGTT 53
            *****

F1_For      ATGTGAGCCCAAGGAGCCCTGACCTGCGGTGAGAGGCTGGAGGCCGAGGGCAAGGTTTCG 120
pGL3_F1     ATGTGAGCCCAAGGAGCCCTGACCTGCGGTGAGAGGCTGGAGGCCGAGGGCAAGGTTTCG 113
            *****

F1_For      AGGGGCTCTGGGTGCCTGGGACAACCTCTGGGCAACAGGGAGAGGCGGCTGCGGGGCCAA 180
pGL3_F1     AGGGGCTCTGGGTGCCTGGGACAACCTCTGGGCAACAGGGAGAGGCGGCTGCGGGGCCAA 173
            *****

F1_For      GTCCTCCTAAGCGGCCACGCGAGAGGCCCTGAGTCGAGAAGGTTACGGCGCTTCCCAG 240
pGL3_F1     GTCCTCCTAAGCGGCCACGCGAGAGGCCCTGAGTCGAGAAGGTTACGGCGCTTCCCAG 233
            *****

F1_For      AGCCCAACCCCGCCCAACGTGTGCACAAATAAAGCTGGCCTTTCTCCGATTCCCTGATTGCA 300
pGL3_F1     AGCCCAACCCCGCCCAACGTGTGCACAAATAAAGCTGGCCTTTCTCCGATTCCCTGATTGCA 293
            *****

F1_For      GCCATTACAGGAGCAGCCCTTGCCACGCATCCATTAGTCGAGGCGGTGGCTGCGTGTG 360
pGL3_F1     GCCATTACAGGAGCAGCCCTTGCCACGCATCCATTAGTCGAGGCGGTGGCTGCGTGTG 353
            *****

F1_For      CAGTGCTACAGGGTTCAGCGCCTGCGCTCCGGAATCAGGCTGTTTCCTTCTAGACC 420
pGL3_F1     CAGTGCTACAGGGTTCAGCGCCTGCGCTCCGGAATCAGGCTGTTTCCTTCTAGACC 413
            *****

F1_For      TCCCATGGGGATTCAAGCCGCGCTCCGGGTCAAAGAACAGGGACAAAGTCTCTGCCA 480
pGL3_F1     TCCCATGGGGATTCAAGCCGCGCTCCGGGTCAAAGAACAGGGACAAAGTCTCTGCCA 473
            *****

F1_For      CGGGGGACCATCTGCAGGCAAAGTGAAGAATGAGGAGCTTCGGCAAAATGCCGACTAAGG 540
pGL3_F1     CGGGGGACCATCTGCAGGCAAAGTGAAGAATGAGGAGCTTCGGCAAAATGCCGACTAAGG 533
            *****

F1_For      CCTCCTTAGGTTTTGCCCCACTCCAAGATGGAAGCCTGAGGCTTCACACTGCCCCGAA 600
pGL3_F1     CCTCCTTAGGTTTTGCCCCACTCCAAGATGGAAGCCTGAGGCTTCACACTGCCCCGAA 593
            *****

F1_For      GTTCCTTTCCCATTTGGCTATCTGGGAATTGAGTTTCCAATAATGCGGACGCTGATTGGT 660
pGL3_F1     GTTCCTTTCCCATTTGGCTATCTGGGAATTGAGTTTCCAATAATGCGGACGCTGATTGGT 653
            *****

F1_For      CAATCCAGGACGGTTGCTCAAGCCATTGGCGCAGCCGCCATTGGAGGGCGGCTCTCAAAA 720
pGL3_F1     CAATCCAGGACGGTTGCTCAAGCCATTGGCGCAGCCGCCATTGGAGGGCGGCTCTCAAAA 713
            *****

F1_For      GTTTTCAGACACAAATTAGGTTTCGAGGGAGGAAACGGAGAGGAAAGGGAAGAACTTGAGAC 780
pGL3_F1     GTTTTCAGACACAAATTAGGTTTCGAGGGAGGAAACGGAGAGGAAAGGGAAGAACTTGAGAC 773
            *****

F1_For      GGAGGCGGGACTAAGGAAACGGCAGCTTGCATTGTTTATTAGAGGCCAAGGGCGGCTC 838
pGL3_F1     GGAGGCGGGACTAAGGAAACGGCAGCTTGCATTGTTTATTAGAGGCCAAGGGCGGCTC 833
            *****

F1_For      TTGAACGTTTCCTTCCTCTTGATTAGTCCTATTTAGGAAAGGAGGGCGGGTACTGAGGAAA 878
pGL3_F1     TTGAACGTTTCCTTCCTCTTGATTAGTCCTATTTAGGAAAGGAGGGCGGGTACTGAGGAAA 893
            *****

F1_Rev      TTCCTTAAGACTTGATTAGTCCTATTTAGGAAAAGAGGGCGGGTACTGAGGAAAAGCGGC 146
pGL3_F1     TTCCTTAAGACTTGATTAGTCCTATTTAGGAAAAGAGGGCGGGTACTGAGGAAAAGCGGC 899
            *****

F1_Rev      AGAAGCGCCTGCTTCCATTGGTCAGTCCTGGCAGGAGGCGGAGCACC CGCGCAGCTGAT 206
pGL3_F1     AGAAGCGCCTGCTTCCATTGGTCAGTCCTGGCAGGAGGCGGAGCACC CGCGCAGCTGAT 959
            *****

F1_Rev      TGGTGCGGGAGGCAAGGTGGGCGGGCTCTGAGCCGAGGTAAGCTTGGC 256
pGL3_F1     TGGTGCGGGAGGCAAGGTGGGCGGGCTCTGAGCCGAGGT----- 1000
            *****

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▪ Sequence Data of Constructed pGL3-Basic-F2

```

F2_F      GGTACCATCTGCAGGCAAAGTGAAGAATGAGGAGCTTCGGCAAATGCCGACTAAGGCCT 60
F2      GGTACCATCTGCAGGCAAAGTGAAGAATGAGGAGCTTCGGCAAATGCCGACTAAGGCCT 60
          *****
F2_F      CCTTAGGTTTGGCCCACTCCAAGATGGAAGGCCTGAGGCTTCACACTGCCCCGAAGTT 120
F2      CCTTAGGTTTGGCCCACTCCAAGATGGAAGGCCTGAGGCTTCACACTGCCCCGAAGTT 120
          *****
F2_F      CCTTCCCATTTGGCTATCTGGGAATTGAGTTTCCAATAATGCGGACGCTGATTGGTCAA 180
F2      CCTTCCCATTTGGCTATCTGGGAATTGAGTTTCCAATAATGCGGACGCTGATTGGTCAA 180
          *****
F2_F      TCCAGGACGGTTGCTCAAGCCATTGGCGCAGCCGCCATTGGAGGGCGGCTCTCAAAAGTT 240
F2      TCCAGGACGGTTGCTCAAGCCATTGGCGCAGCCGCCATTGGAGGGCGGCTCTCAAAAGTT 240
          *****
F2_F      TTCAGACACAAATTAGGTTTCGAGGGAGGAAACGGAGAGGAAAGGAAACTTGAGACGGA 300
F2      TTCAGACACAAATTAGGTTTCGAGGGAGGAAACGGAGAGGAAAGGAAACTTGAGACGGA 300
          *****
F2_F      GGCGGGACTAAGGAAACGGCAGCTTGCATTGGTTTATTAGAGGCCAAGGGCGGCTCTTG 360
F2      GGCGGGACTAAGGAAACGGCAGCTTGCATTGGTTTATTAGAGGCCAAGGGCGGCTCTTG 360
          *****
F2_F      AACGTTCCCTTCCTTCTGATTAGTCCTATTTAGGAAAAGAGGCGGGTACTGAGGAAAAGC 420
F2      AACGTTCCCTTCCTTCTGATTAGTCCTATTTAGGAAAAGAGGCGGGTACTGAGGAAAAGC 420
          *****
F2_F      GGCAGAAGCGCTGCTTCCATTGGTCAGTCCTGGCAGGAGGCGGAGCACCCGCGGCAGCT 480
F2      GGCAGAAGCGCTGCTTCCATTGGTCAGTCCTGGCAGGAGGCGGAGCACCCGCGGCAGCT 480
          *****
F2_F      GATTGGTGCGGGAGGCAAGGTGGGCGGGGCTCTGAGCCGGAGGTAAGCTTGGCATTCCGG 540
F2      GATTGGTGCGGGAGGCAAGGTGGGCGGGGCTCTGAGCCGGAGGTAAGCTTGGCATTCCGG 540
          *****

```

▪ Sequence Data of Constructed pGL3-Basic-F3

```

F3_Rev    CACACAAATTAGGTTTCGAGGGAGGAAACGGAGAGGAAAGGGAACCTTGAGACGGAGGCG 540
pGL3_F3   CACACAAATTAGGTTTCGAGGGAGGAAACGGAGAGGAAAGGGAACCTTGAGACGGAGGCG 59
          *****
F3_Rev    GGACTAAGGAAACGGCAGCTTGCATTGGTTTATTAGAGGCCAAGGGCGGCTCTTGAACG 600
pGL3_F3   GGACTAAGGAAACGGCAGCTTGCATTGGTTTATTAGAGGCCAAGGGCGGCTCTTGAACG 119
          *****
F3_Rev    TTCCTTCCTCTTGATTAGTCCTATTTAGGAAAAGAGGCGGGTACTGAGGAAAAGCGGCA 660
pGL3_F3   TTCCTTCCTCTTGATTAGTCCTATTTAGGAAAAGAGGCGGGTACTGAGGAAAAGCGGCA 179
          *****
F3_Rev    GAAGCGCTGCTTCCATTGGTCAGTCCTGGCAGGAGGCGGAGCACCCGCGGCAGCTGATT 720
pGL3_F3   GAAGCGCTGCTTCCATTGGTCAGTCCTGGCAGGAGGCGGAGCACCCGCGGCAGCTGATT 239
          *****
F3_Rev    GGTGCGGGAGGCAAGGTGGGCGGGGCTCTGAGCCGGAGGTAAGCTGGCATCCGGTAC 777
pGL3_F3   GGTGCGGGAGGCAAGGTGGGCGGGGCTCTGAGCCGGAGGT----- 279
          *****

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▪ Sequence Data of Constructed pGL3-Basic-F4

```

F4_F      -----GGAGGCGGGACTAAGGAAACGGCAGCTTGCATTGGTTTATTAGAGGCCAAGGGG 43
F4      GGTACCAGGAGGCGGGACTAAGGAAACGGCAGCTTGCATTGGTTTATTAGAGGCCAAGGGG 60
          *****
F4_F      CGGCTCTTGAACGTTCTTCTCTTGATTAGTCCTATTTAGGAAAAGAGGCGGGTACTG 103
F4      CGGCTCTTGAACGTTCTTCTCTTGATTAGTCCTATTTAGGAAAAGAGGCGGGTACTG 120
          *****
F4_F      AGGAAAAGCGGCAGAACGCGCTGCTTCCATTGGTCAGTCCTGGCAGGAGGCGGAGCACCC 163
F4      AGGAAAAGCGGCAGAACGCGCTGCTTCCATTGGTCAGTCCTGGCAGGAGGCGGAGCACCC 180
          *****
F4_F      GCGGCAGCTGATTGGTGCGGGAGGCAAGGTGGGCGGGGCTCTGAGCCGGAGGTAAGCTTG 223
F4      GCGGCAGCTGATTGGTGCGGGAGGCAAGGTGGGCGGGGCTCTGAGCCGGAGGTAAGCTTG 240
          *****

```

▪ Sequence Data of Constructed pGL3-Basic-F5

```

F5_F      TAGGTACCTTCCTTCTCTTGATTAGTCCTATTTAGGAAAAGAGGCGGGTACTGAGGAA 60
F5      --GTACCTTCCTTCTCTTGATTAGTCCTATTTAGGAAAAGAGGCGGGTACTGAGGAA 58
          *****
F5_F      AAGCGGCAGAACGCGCTGCTTCCATTGGTCAGTCCTGGCAGGAGGCGGAGCACCCGCGGC 120
F5      AAGCGGCAGAACGCGCTGCTTCCATTGGTCAGTCCTGGCAGGAGGCGGAGCACCCGCGGC 118
          *****

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F5_F      AGCTGATTGGTGC GGGAGGCAAGGTGGGCGGGGCTCTGAGCCGAGGTAAGCTTGGCATT 180
F5        AGCTGATTGGTGC GGGAGGCAAGGTGGGCGGGGCTCTGAGCCGAGGTAAGCTTGGCATT 178
          *****

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▪ Sequence Data of Constructed pGL3-Basic-F6

```

L2R      CCCAGTGCAAGTGCAGGTGCCAGAACATTTCTCTATCGATAGGTACCGGGCGGGGCTCT 540
Long     -----GGTACCGGGCGGGGCTCT 18
          *****

L2R      GAGCCGAGGTAAGCTTGGCATCCGGT 567
Long     GAGCCGAGGTAAGCTT----- 35
          *****

```

▪ Sequence Data of Constructed pGL3-Basic-F7

```

L1_Rev   AAATAGGCTGTCCCAGTGCAAGTGCAGGTGCCTGAACATTTCTCTATCGATAGGTACCC 840
Short    AAATAGGCTGTCCCAGTGCAAGTGCAGGTGCCAGAACATTTCTCTATCGATAGGTACCC 695
          *****

L1_Rev   TCTGAGCCGGAGGTAAGC----- 858
Short    TCTGAGCCGGAGGTAAGCTTGGCATTCCGGTA 727
          *****

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APPENDIX B. Sequence Data and analysis of 4292 base pair region between CCDC135 and KATNB1 genes

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TTTGTGCCTTGCGCTTCACAAGCTCCAGCAGCAGCCTTTCTCTTCTTCTGTTATCTATAGCCTGGGAC
CACCCCTTCTCCCTTGGCCTGTCGTTTGTCTTCTGTCCTTCTCTCCGTTGGAATGTCAGGTCTTC
ACAGCACCTCTGCAGATGGTCCCACAGCCATCTGCAAACAACCTGGTTATATCTCAATGTGACTAGGGT
GGCCACCTCACCAAGTGGGATGGGGAAGGCTAAGATGCATGCCCCAGAATTTGAGGACTGAATCCCA
GCTCCTCTCTTACCAGATACCACTTACCCATCTGTGGAATGGGGTAGCATTGTTGCATTAAATAAGA
CTTATGGCCAGGTGTGGTGGCTCACACCTGTAATCCCAACATTTAGGGAGGCTGAGGCAGGAGGATCA
CTTGATCCCGAGGAGTTTGAGACCAGCCTGGGCAACATAATAAGACCCCATCTCTATAAAAAA
ACAAAGAGCCAGGCATGGTGGCATGAGCCTGTAGTCCCAGCTACTCAGGAGGCTGAGGTAGGAGGATC
ACTTTCACCTAGGAAGTCAAGGCTGCAGTGAGCCATGATTGCACCACTGCACCTCAGCCTAAGCAACA
GAGCAAAACCTGTCTCAAAAAAAGAAAAAAGACATAAATTATAAAGCAGGGAGGCTGGGAATGG
CACATAGAAAGCAGTCATTAAATGTTAGCTTACAAAATAGCCAAAAACACATGAAATGGTTGTCAAA
TTCATGAGTCATCTGGCAAATGAAATGTCACAACACCCTCCGGTATGAAAAAATAAAAGTACTGGT
GACGATGAGAAACAACCTGGAACCTCCCAATAGCCTTCTAGTGAAAGTATAAATCAGTACAACCATTT
TGAAAACTGCTTGGCATTATCTATTAAAGCTAAACTAAAGCCCAACAATTCTACTCTTAGGTTCTAC
TCCAATCCATCATTTCATCAAGTAAGAATGATGAACAACCTCAACCGAAATGTAGATAGATAGTTATCTG
CTAAAAGGCATGTGCTAGAACTTTTCATAGCAGCATTATTGATAATAGCACCATACTTGGAAAGTTACCC
AAATGCCCATCAAGAGCAGAATGGATACATAAATTGTGGTCTTCAGATATTTTGGGTTGTCTTTAACT
GCTTAGGGGAGAACTGCTTAGGGAGAATGCTACTGGCATCTAGTGCATACAGGCCAGGGATGCTGCTAA
ACATTCTACAATGCCAGAACAGCCCCCACAGCAATAATTATCTAGGTCTAAATGCCAATAATGTT
GAGGTTATAGAGTATATTAGTCTGTTTTACACTGCTGATTAAGACATACTCAAGACTGGGTAATTTG
TAAAGAAAAAGAGGTTTAAATGGACTCACAGTTCACACGGCTGGGAGGCCCCACAATCATGGCAGAA
GGCCAAAGGCACATCTTACATGGTGGCAGACAAGAAAGAATGAGAACCAAGCAAAAAGGAAAAATCCCT
TATAAAATCATCAGCTCTCGTGAGACTAATTCATACCATGAGAACAGTATGTGGGAAACCCCTCCCA
TGATTCACTTATCTCCCACTGGGTCCCTCCCAATATGTGGGAATTATGGGAGCTACAATTCACAAG
ATGAGATTTGGGTGGGGATACAGCAACCATATCATATAGAGCAATGAAAAATGAATGAACCTGCAACACA
ACTATATGGAATGATATGGTCAAACCTGCCTAAGTATAATGTCAAAAAGATTCAAGACACAAAAGTACAT
ACTCTATTATTCATTTATGGAAGAACAATAAGGAAAATTAATTGGTGATATTAGAAGTCAGGA
CAACCGTCACTCTTGGAGTGTGAGGGTTAGTGAATGGATGTGAGCATGGAGCCCTGGGAGGGGAGGTT
CTGGTTTCTAGTCACGTTCTGCTTTTGGACCTGGGTGTGTTTCGTTTGTAAAAATTCATCAACCTTGT
GCACTTACAATGTGTCTCTGGAGTGTTTTGGACTGCTTGTGTCATGAATCTAATCAAGCCTTTAGC
CCTAACTTAAAGAAATTCAAGGGAAGAAATGCATTTAAGAAGACGTCCATCTAACAAGAACTTCAGGA
AATTCAGGTAATGATACTAAAAGGAAACATTCTAACGTGGGACAATCTAGGTAAATGGCCTGATCT
CTTCAAAAGATCGATGTCATAAAAAATAAAGTTTGGGGACAGAAATGGAAAGAACTTAAAGAGTCAAGT
GTGGTGAATACTGATTGGTTCTTAGTTAGAAAAAAAAGTCCTAAAAGGTTTTTGGGATGACTGGGGA

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CATACGAATATGGACTGGTTGTTACAGAATTAAATTTTCTTAGCAGTGGTGATAACAATGGTTATTGT
 AGAATAAGGGCTTTATTTTATTTTGGAAATGGATCTCACTCTGTCGCCAGGCTGGAGTACAGTGG
 CCCCATCCCAGCTCACCGCAACCTCTGCTTCCCAGTTTCAAGTGATTCTCCTGCCTCAGCCTCCCCAG
 TAGCTAGGATTATAGGCACGTGCGCCACCACGCCCCGGCTAATTTTTGTATTTTTTTGTAGAGACGGGG
 TTTCACCATGTTGGCCAGGTTGGTCTCGGACTCCTGACCTCAGGTGATCCGCCCGCCTTGGCCTCCCA
 AAGTGCTGGGATTGCAGGCGTGAGCCACCGCGCCTGGCCAAGGGCCTTATTTTAAAGATAACATGCT
 CAGTATTTGGTGTGGGGTGAAATGTCATATCTACAATTTTCAAATGGTTCCGAAAAGACACACAGAC
 GAAGCAAATATGGCAAACCTGTTAACAAGTACTGGAATCTACGTGGTATCATTATATTGTCTTTTAAAG
 TTTTCTCTGTTTAGTTTTCTTTTTATAATAACAAAATTAAGGGGAACAGCAGGCTAGACAGTTTTGATC
 CGATGTTATTCTGCTAATCTTTTAAAGGTGTCTTGCGGTGACTCACGCCTGTAATCCCAGCACTTCGG
 GAGGCTGGGGCGGGTGGATCACCTAAGGTCAGGAGTTCAAGACCAGCCTGGCCAAAATGGAGAAACCC
 CGTCTCTACTAAAAATATAAAAACTAGCCTTAGCCCCGTGCTGGTGGCGGGCGCCTGTGGTTCCAGCTA
 CCCAGGAGGCTGAAGCAGGAGAATCTGAAGCAGAAGGCTGAAGCAGAAGGCAGAGTGGGGACTCCCCC
 ACGCTTTGGCTTTTCAACAAGGGTTCGAATTCTGGGGCTATTACTTTCTGACCGTGTGGTTTTCTGC
 AAGCATCTTAACCCGGGCCTCAGTTTCTCATCTGTAAATGGGAACAGGAATTCCTACCTCTTATGGT
 TGTACTGGGCGTTATGTGAGCCCAAGGAGCCCTGACCTGCGGTGAGAGGCTGGAGGCCGAAGGGCAAG
 GTTCGAGGGGCTCTGGGTGCCTGGGACAACCTCTGGGCAACAGGGAGAGGCGGCTGCGGGGCCAAGTC
 CCTCCTAAGCGGCCACGCGAGAGGCCCTGAGTCGAGAAGGGTTACGGCGCTTCCCAGAGCCCACCCCG
 CCCAACGTGTCAAAAATAAAGCTGGCCTTTCTCCGATTCCCTGATTGCAGCCATTTCAGGGAGCAGCCC
 CTTGCCCACGCATCCATTAGTCGAGGCGGTGGCTGCGTGTGCAGTGCTACAGGGTTACAGCGCCTGCCT
 CGGCTCCGGAATCAGGCTGTTTCTTCTAGACCTCCCATGGGGGATTCAAGCCGGCGTCCGGGTCAAA
 GAACAGGGACAAAGTCCTCCTGCCACGGGGGACCATCTGCAGGCAAAGTGAAGAATGAGGAGCTTCGG
 CAAAATGCCGACTAAGGCCTCCTTAGGTTTTGCCCCACTCCAAGATGGAAGGCCTGAGGCTTCACACT
 GCCCCGAAGTTCCTTTCCCATTTGGCTATCTGGGAATTGAGTTTTCCAATAATGCGGACGCTGATTGG
 TCAATCCAGGACGTTGCTCAAGCCATTGGCGCAGCCGCCATTGGAGGGCGGCTCTCAAAAGTTTTCA
 GACACAAATTAGGTTTCGAGGGAGGAAACGGAGAGGAAAGGGAAAACCTTGAGACGGAGGCGGGACTAAG
 GAAACGGCAGCTTGCAATTGGTTTATTAGAGGCCAAGGGGCGGCTCTTGAACGTTCTTCTCTTGATT
 AGTCCTATTTAGGAAAAGAGGGCGGGTACTGAGGAAAAGCGGCAGAAGCGCCTGCTTCCATTGGTCAG
 TCCTGGCAGGAGGCGGAGCACCCGCGGCAGCTGATTGGTGCAGGAGGCAAGGTGGGCGGGCTCTGAG
 CCGGAGGT

TATAAA : TATA Box motif

GGNCAATCT : CAAT Box motif

GGGCGGG : CG Box motif

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