ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

INTERACTION BETWEEN TRANSCRIPTION FACTOR p53 TUMOR SUPPRESSOR and p60-KATANIN (*KATNA1*) PROMOTER

M.Sc. THESIS

Mehtap KAYA

Molecular Biology-Genetics and Biotechnology Department

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

TRANSKRİPSİYON FAKTÖRÜ P53 TÜMÖR SUPRESSÖR PROTEİNİNİN P60-KATANİN (*KATNA1*) PROMOTORU İLE ETKİLEŞİMİ

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Mayıs 2015

Mehtap KAYA, a M.Sc. student of ITU **Graduate School of Science, Engineering and Technology** student ID 52111111, successfully defended the **thesis** entitled **"INTERACTION BETWEEN TRANSCRIPTION FACTOR p53 TUMOR SUPPRESSOR and p60-KATANIN (*KATNA1*) PROMOTER"**, which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission : 04 May 2015
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To my mother,

FOREWORD

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I want commemorate to Gezi people, who defend the tree and resisted for the freedom.

Finally, I would like to thank to all my family for their belief, support and endless love. I love you guys.

May 2015 Mehtap KAYA

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ABBREVIATIONS

µg	**:** Microgram
µM	**:** Micromolar
µm	**:** Micrometer
L	**:** Liter
µL	**:** Microliter
mL	**:** Mililiter
Mm	**:** Milimolar
mm	**:** Milimeter
aa	**:** Amino Acid
AAA	**:** ATPases Associated with diverse cellular Activities
AD	**:** Alzheimer Disease
AD	**:** Transcriptional Activation Domain
ADP	**:** Adenosine Diphosphate
Amp	**:** Ampicillin
APS	**:** Ammonium Persulfate
ATP	**:** Adenosine Triphosphate
Bp	**:** Base Pair
BSA	**:** Bovine Serum Albumin
cDNA	**:** Complementary DNA
cm	**:** Centimeter
cm^2	**:** Centimeter Square
Ct	**:** Cycle Treshold
DLR	**:** Dual Luciferase Reporter
DMEM	**:** Dulbecco's Modified Eagle Medium
DMSO	**:** Dimethyl Sulfoxide
DBD	**:** DNA Binding Domain
DNA	**:** Deoxyribonucleic Acid
dNTP	**:** Deoxyribonucleotide
E.coli	**:** *Esherichia coli*
EB	**:** Elution Buffer
EDTA	**:** Ethylenediaminetetraacetic Acid
EtBr	: Ethidium Bromide
FBS	**:** Fetal Bovine Serum
G	**:** Gram
GDP	**:** Guanosine Diphosphate
GTP	**:** Guanosine Triphosphate
H	**:** Hour
sec	**:** Second
HDAC	**:** Histone Deacetylase Complex
IRES	**:** Internal Ribosome Entry Site
Kbp	**:** Kilo Base Pair
kDa	**:** Kilo Dalton

LARII	**:** Luciferase Assay Reagent II
LB	**:** Luria-Bertani Broth
Luc+	**:** Luciferase
M	**:** Molar
MAP	**:** Microtubule Associated Protein
MAPK	**:** Mitogen Activating Protein Kinase
Min	**:** Minute
mRNA	**:** Messenger Ribonucleic Acid
MTOC	**:** Microtubule Organizing Center
ng	**:** Nanogram
nm	**:** Nanometer
nM	**:** Nanomolar
PCR	**:** Polymerase Chain Reaction
ph	**:** Power of Hydrogen
RNA	**:** Ribonucleic Acid
rpm	**:** Recolutions Per Minute
SOC	**:** Super Optimal Broth with Catabolite Respression
TAE	**:** Tris-acetate-EDTA
TCF	**:** Ternary Complex Factor
TD	**:** Tetramerization Domain
Tm	**:** Melting Temperature
UV	**:** Ultraviolet
V	**:** Volt
γ-TuRC	**:** γ-Tubulin Ring Complex

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SUMMARY

Cytoskeleton is a network of filamentous structures which are spread throughout the cytoplasm. Cytoskeleton play roles in vital functions such as movement of the cells, cell shape, mitosis, cytokinesis, intracellular transport of molecules. Cytoskeleton consists of three distinct polymeric fibers. Microfilaments are required for maintenance of cell shape, cytokinesis, separation of the dividing cell into two and cell surface preparation for adherence. Other component of cytoskeleton, intermediate filaments provide mechanical strenght and mechanical linkage. The final member of cytoskeleton is microtubules that have crucial roles such as drawing the chromosomes apart in mitosis, shaping morphological structure, intracellular traffic to carry vesicules, cell motility, formation of axons and dentrites and therefore, the neuronal structure.

Microtubules are structural polymer proteins and consist of α-tubulin and β-tubulin heterodimers. Microtubules are dynamic structures resulting from rapid polymerization and depolymerization of tubulin monomers by a process known as "dynamic instability". Reconfiguration of the microtubules by these mechanisms is responsible for microtubule growing and shrinking.

When dynamic instability, speed and time for branching for special neuronal morphology are considered, it can be easily understood that cells need another mechanism to explain the movement of intracellular microtubules. "Cut and run" model proposes that microtubules are cut into small pieces by severing enzymes; katanin, spastin and fidgetin. These enzymes are members of AAA family of ATPases. Because microtubules lose their ability to move when they are long, microtubule severing is important in terms of movement capacity.

Katanin and spastin have same working mechanism, but, they provide different severing approach. Spastin encoded by SPG4 gene. Katanin is a heterodimer of p60-katanin encoded by *KATNA1* gene and p80-katanin encoded by *KATNB1* gene. p60-katanin has the enzymatic activity and it has AAA ATPases region but p80-katanin enzyme do not have the enzymatic activiy.

According to studies, katanin is associated with neurologic diseases. Katanin is ubiqutiously expressed in nervous system and its inhibition or overexpression impairs axon formation. Thus, it is clearly understood that katanin has vital role for microtubule reorganisation and axon growing. Hypophosphorylated form of tau protein normally provides stabiliziation of microtubules and protects microtubules from the severing enzymes. Hyperphosphorylation of tau results in dissociation of tau from microtubules and thus, microtubules become accesible for severin enzyme katanin. The enzymatic activity of katanin is proportional to level of enzyme so

regulation of katanin protein level via transcription mechanism comes into prominence.

p53 is important for many cellular mechanisms as a tumor supressor in processes such as cell cycle regulation, metabolism, apoptosis, DNA replication, immunity responses, proliferation and differentiation. In our previous study, we have shown that neuronal processes were retracted upon PKC activation following increases in both p60-katanin and p53 levels in neurons.

This result led us to analyze changes in p60-katanin, which is organizing the neuronal cytoskeleton. We showed that cells which have enhance p60-katanin protein level also had increase in p53 transcription factor, which is related on neuronal differentiation.

In this study, we aimed to identify regulatory DNA sequences of *KATNA1* gene and possible regulation of p60-katanin by p53 trasnscription factor.

For this purpose, we first decided to characterize regulatory regions of *KATNA1* gene. Primarly, the putative transciptional regulatory regions of p60-katanin were identified by bioinformatic methods. p60-katanin regions (336 bp promoter, 448 bp 5'UTR, 784 bp promoter + UTR, 2682 bp intron + UTR and 3000 bp promoter + UTR + intron) were cloned for the identification of their regulatory activities on p60-katanin expression by Luciferase assay. Then, p60-katanin promoter, p60-katanin UTR, p60-katanin promoter + UTR and p60-katanin promoter + p60-katanin UTR + p60-katanin intron regions were amplified by PCR and after required restriction all regions prepared. We identified that 5'-UTR region enhanced transcriptional activity of the reporter gene, but not putative promoter region and the presence of the intron decreased the activity. Thus, expression of katanin-p60 seems to be regulated via 5'-UTR, and intron-1 might have repressor elements. When potential gene regulatory regions were analyzed by bioinformatics software, presence of a CpG island that comprising promoter and 5'-UTR regions (from -615 to -918 bp) was identified.

Next, we identified p53 consensus sequence on *KATNA1* gene bioinformatically. Therefore, it is thought that *KATNA1* gene could possibly be regulated by p53 transcription factor. We started with analyzing the binding of p53 to the corresponding promoter region. To further confirm the specificity of the p53 binding to *KATNA1* promoter was confirmed by Chromosome Immunoprecipitation (ChIP) and also by Electrophoretic Mobility Shift Assay (EMSA) using oligonucleotides including related transcription factor binding sites.

On the other side, our observation indicated that, p53 acts as an activator or repressor *KATNA1* gene promoter. The result for *KATNA1* gene was confirmed in mRNA level by performing real time PCR.

TRANSKRİPSİYON FAKTÖRÜ P53 TÜMÖR SUPRESSÖR PROTEİNİNİN P60-KATANİN (*KATNA1*) PROMOTORU İLE ETKİLEŞİMİ

ÖZET

Hücre iskeleti, hücrenin hareketinde, şekil almasında, mitoz bölünmede, sitokinezde, hücre içi molekül taşınması gibi hayati fonksiyonlarda görev alan tüm sitoplazma boyunca yayılmış bir ağ yapısıdır. Hücre iskeleti üç farklı polimerik yapıdan meydana gelir. Bunlar mikrofilamentler, ara filamentler ve mikrotubuller olarak adlandırılırlar. Mikrofilamentlerin hücrenin şekil kazanması sırasında, sitokenezde , organellere tutunma yüzeyi hazırlanmasında, kas hareketleri gibi fonksiyonel birçok hücre içi görevleri vardır. Ara filamentler ise hücreye mekanik destek sağlarlar ve hücreler arası bağların oluşumunda görevlidirler. Bir diğer hücre iskeleti elemanı da mikrotübüllerdir. Mikrotubuller hücre bölünmesi esnasında kromozomların kutuplara çekilmesinde, hücrenin morfolojik yapısının oluşmasında, hücrede vezikül taşınmasında, hücrenin hareket edebilmesinde, nöronlarda akson oluşumunda ve nöronal şeklin meydana gelmesinde önemli rol oynarlar.

Mikrotubuller alfa (α-tubulin) ve beta (β-tubulin) heterodimerlerinin oluşturduğu polimer yapılı proteinlerdir. α ve β heterodimerlerinin hızlı bir şekilde polimerizasyonu ve depolimerizasyonu ile mikrotubuller dinamik bir yapı kazanmaktadır ve bu dinamizme "dinamik kararsızlık" adı verilir. Hücreler ihtiyaç durumuna göre bu mekanizma ile mikrotubullerini yeniden yapılandırmaktadırlar. Mikrotubullerin uzayıp kısalmasından bu mekanizma sorumludur. Uzama fazı mikrotubullerin uçlarına serbest tubulin hetero dimerlerinin eklenmesiyle oluşurken, kısalma fazıında mikrotubuller uçlarından tubulin dimerlerini kaybederler.

Mikrotubullerin kesilip kısalması bir diğer dinamik mekanizmadır ve nöron morfolojisi açısından çok önemlidir. Çünkü dinamik kararsızlık, nöron morfolojisinin oluşması için gereken dallanma sürecinde hız ve zaman açısından yetersiz kalır. Bu nedenle mikrotubullerin hücre içi hareketini açıklamaya çalışan diğer mekanizmalar devreye girmektedir. Bunlardan birisi de "kes ve koş" mekanizmasıdır.

Mikrotubullerin kesilmesinde görev alan enzimler katanin, spastin ve fidgetindir ve bunlar AAA ailesi ATPaz üyeleridir. Mikrotubullerin kesilmesi hareketi açısından ve taşınabilmesi için önemli bir durumdur. Çünkü mikrotubullerin hücre içerisindeki hareketleri boyları ile ters orantılıdır. Uzun mikrotubuller harekete direnç gösterirken kısa mikrotubuller daha efektif bir şekilde hareket ederler. Ancak, mikrotubuller tekrar uzun hale geldiklerinde hareket yeteneklerini kaybetmektedirler.

Katanin ve spastin benzer çalışma mekanizmaları içerirler. Ancak mikrotubullerin farklı form ve boyutlarda kesilmelerini sağlarlar. Spastin *SPG4* geni tarafından kodlanır. Katanin ise heterodimerdir ve *KATNA1* geni tarafından kodlanan p60-katanin ve *KATNB1* geni tarafından kodlanan p80-katanin alt birimlerinden oluşur.

p60-katanin enzimatik aktivite gösterir ve AAA ATPaz bölgesi içerir. p80-katanin ise enzimatik aktivite içermez ancak p60-kataninin aktivitesini düzenler.

Yapılan araştırmalar sonrasında katanin, nörolojik hastalıklar ile ilişkilendirilmiştir. Kataninin sinir sisteminde oldukça yoğun olarak ifade edilir ve baskılandığı zaman mikrotubul uzunluğunda artışa neden olur. Bu artış ile birlikte akson gelişiminin olumsuz etkilenmesi ve mikrotubullerin sentrozomlarda birikmesi görülür.

Bu nedenle, kataninin mikrotubullerin sentrozomdan ayrılması ve akson büyümesi için hayati önem taşır. Mikrotubullerin sabit kalmasını sağlayan tau proteini hiperfosforilasyon sonucu mikrotubullerden ayrılarak stabilizasyonu engeller. Böylece katanin mikrotubulleri kolayca kesebilir. Bu yüzden kataninin gen anlatım süreci ile düzenlenmesi önem taşımaktadır.

p53, tümör baskılayan protein olarak hücrenin birçok mekanizmasında yer almaktadır. Hücre döngüsünün düzenlenmesinde, metabolizmada, apoptozun tetiklenmesinde, DNA replikasyonunda, bağışıklık cevaplarında, proliferasyonda ve diferansiyasyon kaskadında önemli görevleri vardır.

Daha önce grubumuzun yaptığı çalışmalar sonucunda, nöronlarda PKC'nin yapısında ve aktivitesinde meydana gelen değişimlere bağlı olarak hücre siklusu belirteçlerinden siklin D1 üzerinde oluşan değişiklikler incelendiğinde, PKC aktivasyonu ile birlikte nöronal uzantıların retraksiyonu görülmüştür. Burada yapılan analizlerle de p60-katanin protein seviyesinde artış görülen hücrelerde nöronal farklılaşma ile de ilişkili p53 transkripsiyon faktörünün de arttığı gösterilmiştir.

Buradan yola çıkarak, bu çalışmada ise p60-katanin'in regülatör bölgeleri tespit edilerek p53 transkripsiyon faktörünün *KATNA1* geninin regülasyonu üzerine olan muhtemel etkilerinin belirlenmesi amaçlanmıştır.

Öncelikle p60-katanin promotor bölgesinde olası p53 bağlanma bölgeleri biyoinformatik yöntemler ile saptanmıştır. Bu da, *KATNA1* geninin p53 tarafından regüle edilebileceğine dair önemli bir ipucu olmuştur. Daha sonra p60-katanin bölgeleri (336 bp promoter, 448 bp 5'UTR, 784 bp promoter + UTR, 2682 bp intron + UTR ve 3000 bp promoter + UTR + intron) klonlanmıştır. p60-katanin promoter, p60-katanin UTR, p60-katanin promoter + UTR , p60-katanin promoter + p60-katanin UTR + p60-katanin intron bölgeleri polimeraz zincir reaksiyonu ile çoğaltılmış ve gerekli enzim kesimlerinden sonra hazırlanmıştır. Bundan sonra, p60-katanin ekspresyonunda etkili regülatör bölgeleri lüsiferaz assay ile karakterize edilmiştir. Lusiferaz assay yöntemi ile 5'-UTR bölgesinin transkripsiyon aktivitesini arttırdığı ve tahmin edilen promoter bölgesinin intron varlığında transkripsiyon aktivitesini düşürdüğü bulunmuştur. Bunun sonucunda, intron-1 bölgesinin repressor etkisi görülmüştür. Olası promotor bölgesi biyoinformatik programı ile analiz edildiğinde, guanin ve sitozin (CpG) dinükleotidlerince zengin olduğu bulunmuştur. Bunun yanı sıra yaptığımız analizler sonucunda bu bölgede bir CpG adacığın varlığı (-615 ile -918 bç arası) tespit edilmiştir.

Chromosome Immunoprecipitation (ChIP) ve Elektrophoretic Mobility Shift Assay (EMSA) yöntemleri ile p60-katanin üzerinde p53'ün bağlandığı gözlemlenmiştir. Daha sonra da protein düzeyinde p53'ün ekspresyonunun artışı western blotlama yöntemi ile incelenmiştir. Diğer taraftan bu sonuçlar, gen ekspresyon sürecinin anlatıldığı transkripsiyon basamaklarını kapsayan mRNA düzeyinde gerçek zamanlı polimeraz zincir reaksiyonu (PZR) ile de gösterilmiştir.

p53'ün proliferasyon ve diferansiyon kaskadı arasında kritik bir protein olması nedeniyle p53'ün p60-katanin üzerindeki etkilerinin incelenmesi nörodejenerasyonda nörit retraksiyonu ile karakterize diferansiyon değişikliklerinin p53'e bağımlı moleküler mekanizmasının da aydınlatılmasına olanak sağlayacaktır.

Ayrıca, p60-katanin ve p53 proteini nöronal farklılaşma ve hücre bölünmesinde önemli rollere sahip olduğu için transkripsiyonel ve translasyonel regülasyonun anlaşılması bakımından da büyük önem taşımaktadır.

1. INTRODUCTION

1.1 Microtubules

The cytoskeleton which is a cytoplasmic system of fibers is crucial for cell motility. Cytoskeleton consists of three types of cytoskeletal fibers: intermediate filaments, microfilaments and microtubules. Cytoskeleton fibers are important for meiosis, mitosis, cell morphology, migration, transport of vesicles, support to cell shape and cell movement (Lodish et al., 2003).

In eukaryotic cells, microtubules dynamic property is essential for fundamental processes such as cell cycle, cell migration, cell division and differentiation (McNally, Vale, 1993, Quarmby, 2000) and structural change of microtubule organization occurs quickly in cytoskeleton.

Microtubules are composed of heterodimers of α-tubulin and β-tubulin monomers (Figure 1.1 a). They are arranged in head to tail fashion at 8 nm intervals to constitute protofilaments and 13 protofilaments generate microtubule structure (Figure 1.1 b) (Lodish et al., 2003). In addition, they are nucleated at MTOC and γ-tubulin which is localized at the center of MTOC (Microtubule Organizing Center) functions as a nucleation center for the accurate assembly of microtubules.

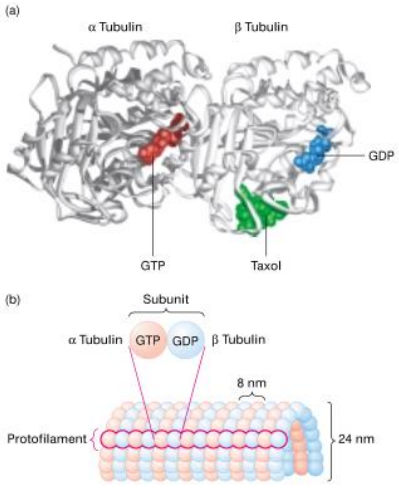

Figure 1.1 : Microtubule structure (Lodish et al., 2003)

Microtubules direct the migration of nerve cell axons and dentrites by guiding the extension of the neuronal growth. In addition to neuronal growth, intrinsic polarity of microtubule is related with neurons dentrites, microtubule array in axons, growth cones and it is associated with assembly. Microtubules as knowledge carriers, play important role in a diversity of ways in neurons. As shown in Figure 1.2, short translocating microtubules are located in axon and microtubules including concentrating +TIP proteins also dynamic microtubules are capable of polymerizing directly into dendritic spines, during polymerization and releasing them upon depolymerization (Lodish et al., 2003, Dent and Baas, 2014).

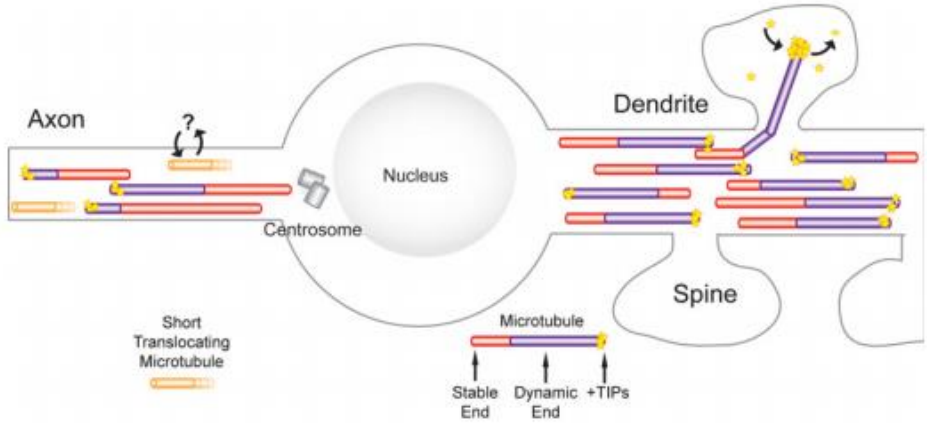

Figure 1.2 : Microtubules are information carriers in axon and dentrite (Dent and Baas, 2014)

Microtubule has two ends that polymerize at different rates: The fast growing end is called plus, end and the slow growing end is called minus end. Addition of the subunits to the structure of polymer in different rates cause the conformational change (Lodish et al., 2003).

Dynamic instability is related with free subunit concentration of inter conversion between growing and shrinking state and it is associated with nucleoside triphosphate hydrolysis, GTP cap on a microtubule. The alteration from growth to rapid shrinkage is called as catastrophe, correspondingly alteration to growth is called as rescue.

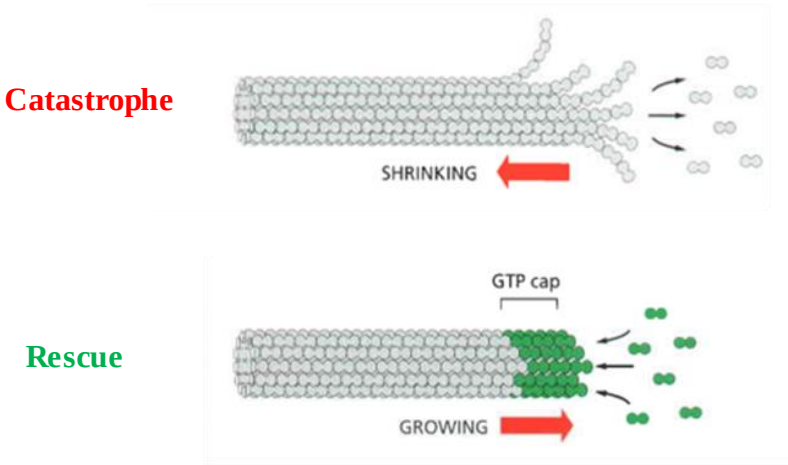

Figure 1.3 : Dynamic instability (Alberts, 2007)

Protofilaments comprise tubulin subunits with GTP bound to the β-monomer generate straight structure that has large amount of energy, that makes strong and regular lateral contacts with another protofilament. However, the hydrolysis of GTP to GDP is associated with a hard conformational change in the protein, which makes the protofilaments curved (Figure 1.3) (Alberts, 2007).

Stabilized or destabilized microtubules originate from the extrinsic factors by microtubule dynamics. Extrinsic regulation of microtubules is mostly derived by numerous MAPs (microtubule-associated proteins) that bind to microtubules, microtubule severing proteins, microtubule destabilizing factors, and microtubule based motor proteins such as kinesin and dynein superfamilies.

There are several protein families discovered to regulate microtubules and their dynamics. While some of them participate directly on microtubule stabilization, promoting their construction, depolymerization or tattering, others control tubulin availability and thus regulate their dynamicity indirectly (Poulain and Sobel, 2010).

1.2 Microtubule Severing

Dynamic instability is a mechanism of assembly and disassembly of microtubules. Nevertheless, dynamic instability is not solely enough to explain the microtubule organization. Many studies suggest a "cut and run" mechanism which is about cell mobility by fulfilling the function of microtubule severing enzymes (Quarmby, 2000; Baas, Karabay and Qiang, 2005).

Microtubule severing in mitotic cells is thought to contribute to spindle reorganization (Ahmad and Baas, 1995). Apart from mitotic cells; in neurons, microtubule form dense parallel arrays (bundles) not spindles, in axons and dendrites that are required for the elongation and maintenance of these neurites (Conde , Caceres , 2009; Karabay et al., 2004).

Neuronal microtubules are nucleated at the centrosome. Therefore, microtubules are rapidly released by the action of the microtubule severing proteins and after that, they are transported as short polymers into neurites by molecular motors. (Yu, Centonze, Ahmad and Baas, 1993; Vale, 2003; Hirokawa and Noda, 2008). However, not all microtubules are nucleated from the centrosome. There are three possible ways to create these noncentrosomal microtubules: (1) severing of microtubules at sites remote from the centrosome (2) de novo production and growth of microtubules in the cytosol, and (3) the release of microtubules originally anchored at the MTOC (Quarmby et al., 2000; Quarmby and Lohret, 1999).

Microtubules can be crosslinked to another close microtubule, and these interactions obstruct movement of motor proteins such as cytoplasmic dynein, on microtubules and eventually inhibits microtubule dynamicity. On the other hand, shortening of microtubules escalate rate of movement as a result of attenuated crosslinking profile (Figure 1.4) (Baas, Karabay and Qiang, 2005).

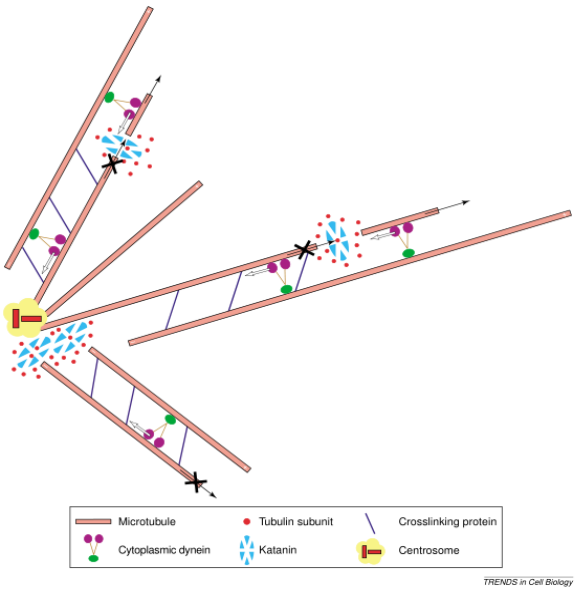

Figure: 1.4 : Microtubule "cut and run" model (Baas, Karabay and Qiang, 2005)

ATPases Associated with diverse cellular Activities (AAA) family has three microtubule severing enzymes: katanin, spastin and fidgetin. These have important roles in cellular processes such as neuronal development, mitosis, cilia and flagella biogenesis (Casanova et al., 2009). Primarily identified severing enzyme katanin, breaks in the lattice of the polymer by hydrolyzing ATP (Figure 1.4). Katanin not only severs microtubule at the centrosome but also severs in all regions (Karabay et al., 2004).

For neurons, microtubule severing is particularly important as it enables axonal growth and neuronal branching (Yu et al., 2008). Yet, these neuronal branches have noncentrosomal microtubules that structurally support the cell and provide cargo transport through the branches (Dent et al., 1999).

1.3 The Role of Katanin

Firstly, biochemical clue for microtubule-severing activity came from observations using Xenopus oocyte extracts (Vale, 1991) and then katanin, an enzyme with an ATP-dependent microtubule severing activity was purified from sea urchin eggs in 1993. Katanin is the best known microtubule severing protein which takes it is name from Japanese samurai sword and its gene is located on the 6th chromosome. (Baas, Karabay and Qiang, 2005).

Another microtubule severing protein that neurons express is spastin. Spastin is concentrated more at the sites of branch formation, whereas p60-katanin is highly expressed in the soma. In the katanin severing mode, as shown in figure 1.5a, tau which is a protective protein maintaining attachment to microtubules in hypophosphorylated state throughout the axon blocks the access of katanin to microtubules.

In the spastin severing mode, as shown in figure 1.5b, microtubule severing is enhanced at sites of branch formation and also spastin mode is not dependent on tau or another molecules for the attachement to the microtubule.

Both modes are quite likely under the regulation of signaling cascades. Besides, these modes arise tandemly during branch formation.

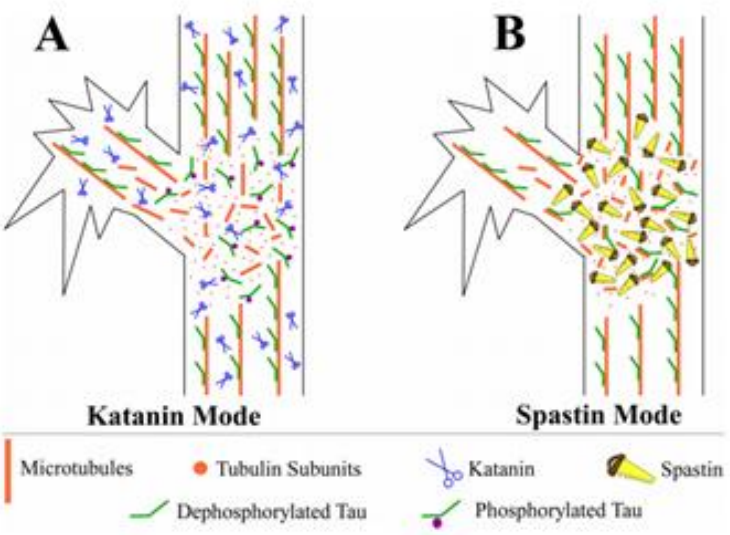

Figure 1.5 : Katanin and spastin severing modes (Baas et.al., 2008)

Katanin is a heterodimeric enzyme that consist of of two subunits; p60 katanin encoded by *KATNA1* gene and p80 katanin encoded by *KATNB1* gene. p60-katanin is composed of an N-terminal domain that binds microtubules (Hartman and Vale, 1999) and a C-terminal domain having a similar structure with AAA family (Hartman et al., 1998) of ATPases. p60 encompasses the microtubules by constituting a hexamer ring and thus produces the torque to crush the lattice (Hartman and Vale, 1999). p60-katanin subunit acts to sever and disassemble microtubules, while p80 subunit targets the enzyme to the centrosome (Baas, Karabay and Qiang, 2005). Microtubule severing is required to obtain small pieces of microtubules. Severing procedure is initiated when ATP dependent katanin oligomerization is triggered and katanin accumulate on the wall of the microtubule. Otherwise, when phosphate is released upon ATP hydrolysis, katanin is conformationally changed and p60-katanin subunits dissociated as shown in Figure 1.6.a. Three type of severing mechanism possible *in vivo* contexts shown in Figure 1.6.b. These are spindle pole, centrosome and treadmilling (Quarmby, 2000).

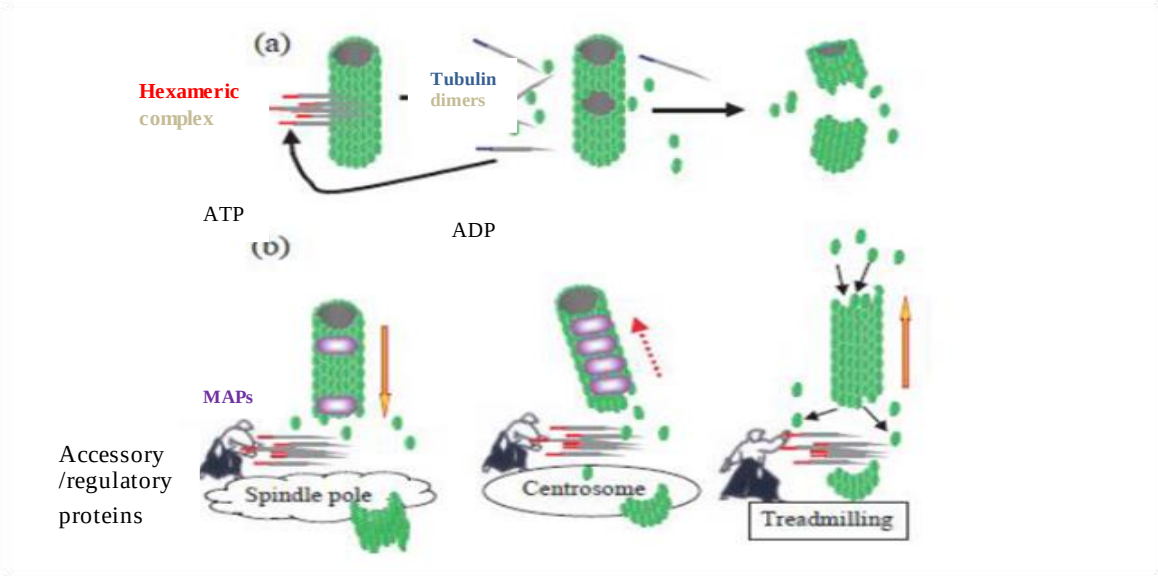

Figure 1.6 : Microtubule severing by Katanin (Quarmby, 2000)

In the nervous system, high amount of katanin is found during axonal growth. Besides its presence in the centrosome where it executes severing activity, it also shows a widespread distribution in the cytoplasm. It has been shown that microtubule severing and relase from the the centrosome are crucial for axon elongation, as katanin inhibition leads to accumulation of microtubules at the centrosome, increases microtubule length and spoils axon growth (Karabay et al., 2004). On the other hand, p60 overexpression is also found to impair axon formation (Yu et al., 2005).

1.4 p53 as a Transcription Factor

The human p53 gene is located on the 17th chromosome and it has 53 kDa molecular mass. Remarking the role of p53, prevention of the genome mutation for conserving stability by p53 it is called as "the guardian of the genome" (Strachan and Read , 1999). Breakthrough discovery of p53 was about its sequence specific DNA binding property (Kern et al., 1991). After that, p53's consensus sequence of response element was identified via in vitro assays (El-Deiry et al., 1992).

In fact, p53 performs as a crucial decision maker not only as a pro-apoptotic factor but also as an inducer of cell cycle arrest and tumor suppressor (Tedeschi and Di Giovanni ,2009). Besides, p53 pathway responds lots of stress signals such as telomere shortening, heat or cold shock, unfolded proteins, improper ribosomal biogenesis, mitotic spindle damage, hypoxia related with DNA damage. (Wang, Xiao and Ren, 2009).

p53 family proteins are comprised of several functional domains. These are: a central sequence-specific DNA-binding domain (DBD), carboxy-terminal region that contains the tetramerization- oligomerization domain (TD), nuclear export signals and also nuclear localization signals domain, proline rich domain and amino terminal the transcriptional activation domain (AD) as shown in figure 1.7. In addition, the DBDs, the ADs, and the TDs are highly conserved (Nature Reviews Cancer, 2004).

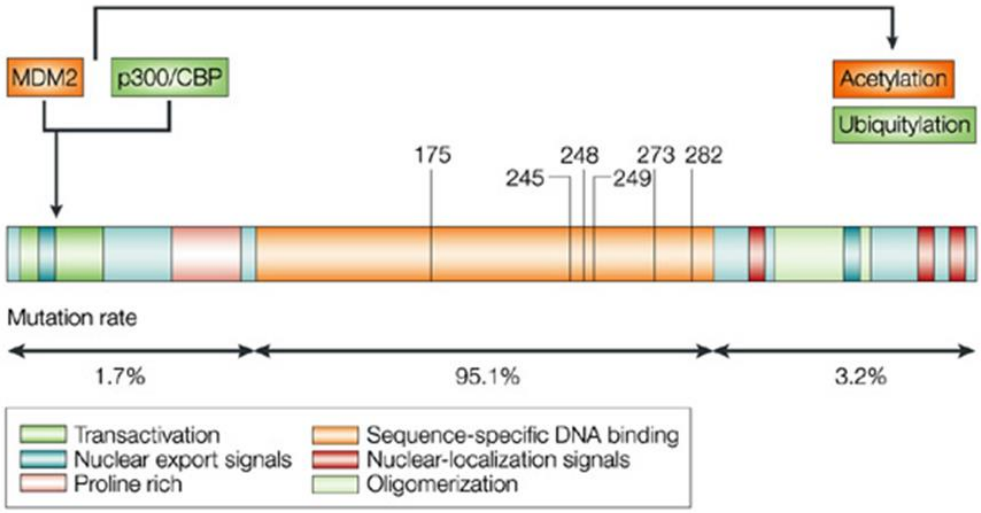

Figure 1.7 : p53 domains (Nature Reviews Cancer, 2004)

The N-terminal domain of p53 protein including transactivation, nuclear export signal, proline rich domains and also phosphorylation sites and MDM2 or p300/CBP are located in the amino terminus. On the other hand, acetylation, ubiquitylation, phosphorylation, sumoylation/ neddylation sites appear in the carboxyl terminus. Central DNA binding core of p53 has broad range of mutation and nearly all of the point mutations are found in cancers (Vousden and Lu, 2002). The DBD is crucial for the function of the p53 family proteins as a central sequence specific transcription factors. Besides, the transcriptional ADs are important for the role of the p53 family proteins. Between the nucleus and the cytoplasm p53 is known to quickly shuttle. Nucleo-cytoplasmic (nuclear import and export) shuttling fulfilment with too large to passively diffuse across the nuclear pore and its important for tetrameric p53.

Importantly, for high affinity transcriptional activation and DNA binding, p53 must be in the tetrameric form (Harms and Chen, 2006).

p53, nuclear transcription factor and the tumor suppressor, is a tetrameric phosphoprotein (Figure 1.8) that can modulate various cellular functions such as DNA

repair, cell cycle regulation, DNA synthesis, senescence and cell death (Shakked et al., 2010).

p53 binds as a tetramer to DNA targets and it has variable spacer for separated two decameric half sites. Besides, four p53 core domains interact with a 20-bp DNA (as shown in Figure 1.8).

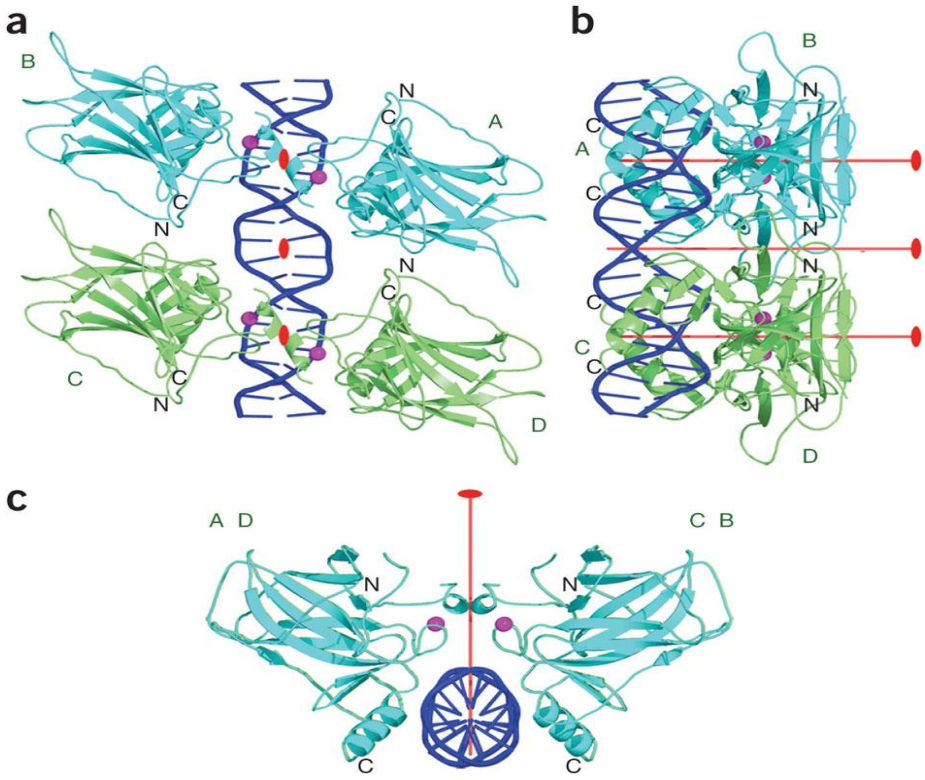

Figure 1.8 : Structure of p53 core domain (Shakked et al., 2010)

p53 repressor and transactivator roles have been described by different mechanisms on different genes. p53 activation can be related with differential target gene activation (Karen, Vousden, Prives, 2009).

Specific core combination within the CWWG motif of the p53RE is important factor that specifies whether p53 transcriptionally activates or represses a target gene. p53 has transcriptional behavior upon CWWG motif which is RRR and YYY triplet sequences comprise. p53 recognizes and binds to DNA containing p53 REs that involve 2 decamer motifs RRRCWWGYYY (in which R is a purine, Y is a pyrimidine and W is an adenine or thymine) separated by a spacer of 0-13 bp (Böhlig and Rother , 2011, Levine et al., 2008). If p53-binding sites match with these consensus sequence are mainly described in p53-dependent transactivation.

According to the recent researches, p53 response elements have diverse sequences which are related to function of p53 as a repressor or an activator. If the central dinucleotide is composed of TT, AA or AT, the binding of p53 intercede strong activation, but the central core dinucleotide TG, CC, CA, CG, GG or GC are found in respressive p53 response elements. Besides, p53 dependent repression may involve other factors such as recruitment of regulatory proteins or epigenetic modifications of chromatin.

There are two mechanism that regulate repressor activity of p53 and these are DNA binding dependent and DNA binding independent mechanisms. Transcriptional repression by p53 occur via binding of p53 to a new p53 response element (Figure 1.9 I). Another mechanism comprise displacement of other transcription factors due to overlapping DNA binding sites (Figure 1.9.II). And also recruitment of another transcription factors along with p53 can lead to repression of the gene (Figure 1.9. III).

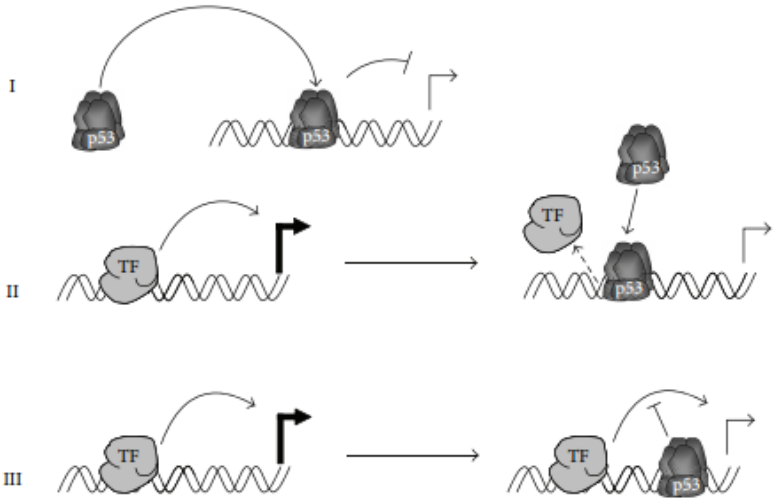

Figure 1.9 : Repression of DNA binding dependent mechanism of p53-binding site (Böhlig and Rother, 2011)

p53 also has DNA binding independent repression mechanisms. One of those includes interaction with other transcription cofactors (Figure 1.10.I) and the other one utilizes interaction of p53 with the basal transcription machinery (Figure 1.10.II).

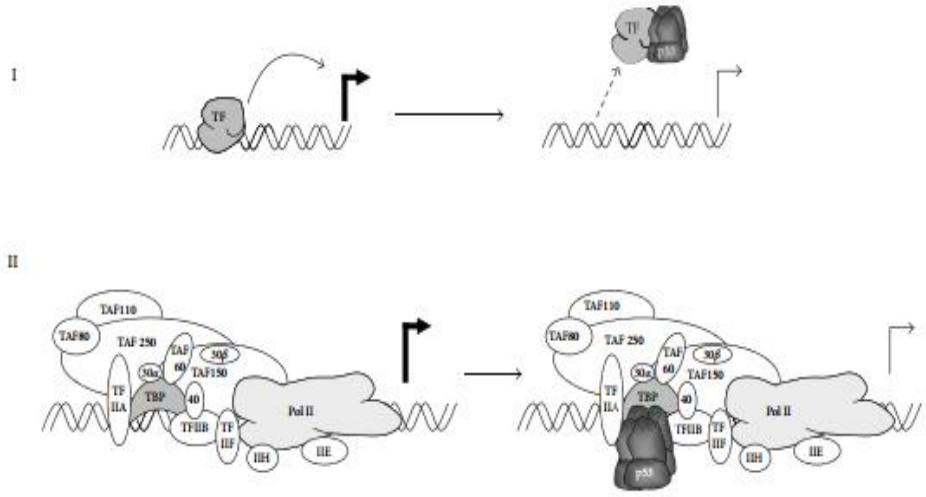

Figure 1.10 : Repression DNA binding independent mechanism of p53 binding site (Böhlig and Rother, 2011)

The diversity of p53-binding sites provides higher flexibility in the sense of cell type and tissue dependent regulation of target genes. p53 transcriptionally down regulates and up regulates diverse target genes in different tissues.

Activation mechanism of p53 consists of three steps as shown in figure 1.11. These are comprised the stress induced stabilization mediated by phosphorilation, DNA binding and interaction with transcriptional machinery. In hemeostasis, p53 is degraded after Mdm2 mediated ubiquitination. When stress signal induces p53 phoshorylation by ATR, ATM and other kinases, it stabilizes p53 and promotes DNA binding. Thus, induction of p53 activates transcriptional machinery (Kruse and Wei, 2009).

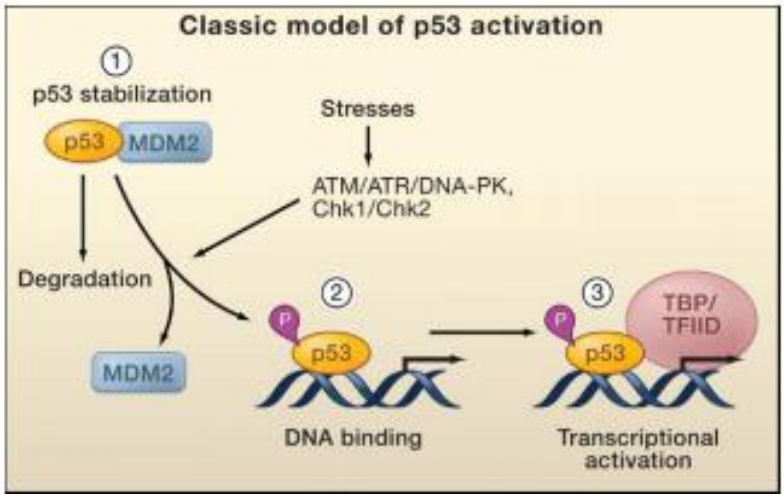

Figure 1.11 : p53 activation model (Kruse and Wei Gu, 2009)

p53's function as a transcription factor is mostly attributed for the ability to regulate numerous target genes at the transcriptional level. The p53 has a pivotal role to activate or repress a gene on the transcriptional level (Moore &Goldberg, 2011, Bohlig and Rother, 2011).

In addition to these mechanisms, many factors can change the mode and level of gene regulation by p53. These factors include spacer lenghts, cofactors, quarter-site orientation, nucleosomes and post-translational modifications of p53 (Böhlig and Rother, 2011).

1.5 Aim of the study

Katanin is encoded by *KATNA1* gene and severs microtubules by hydrolysing ATP. This severing activity is required for cellular functions such as mitosis, axonal branching and neuronal development.

p53 as a transcription factor has important roles for cellular functions and it acts in regulation of many cell cycle related pathways. p53 can act as either repressor or activator depending on different promoter sites where domains bind.

In our lab, the optimal promoters of *SPG4* and *KATNB1* genes were analyzed to determine the critical region for the regulation of transcription. Moreover, we have shown that neuronal processes were retracted upon PKC activation following increases in both p60-katanin and p53 within neurons. After these results, we analyze changes in p60-katanin, which is organizing the neuronal cytoskeleton. We showed that cells which have enhance p60-katanin protein level also had increase in p53 transcription factor, which is related on neuronal differentiation.

In this study, our aim to identify the transcriptional regulation of *KATNA1* gene by p53 as a transcription factor. For this purpose, we performed bioinformatic analysis and specified candidate binding sequences for p53 on p60-katanin promoter. Presence of p53 binding site might also indicate that *KATNA1* gene could possibly be regulated by p53 transcription factor. A putative p53 binding site was identified within -95 to -117 region of the transcription start site. p60-katanin transcriptional regulation regions will be identified by dual-lusiferase reporter assay. The end effect of p53 binding on p60-katanin will be determined by two exprerimental methods; translationally by westem blotting, transcriptionally by quantitative real time PCR.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

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

Table 2.1: The equipment used in this study.

Equipment	Supplier Companies
Centrifuges	Biolab SIGMA 6K15, Beckman Coultier Microfuge®18, Beckman Coulter AvantiTM J-30 I, IECCL10 Centrifuge, Thermo Electron Corporation, Labnet, Labnet International C1301-230V
Centrifuge Tubes	15ml, 50ml Avant Plus
DNA Sequencer	Applied Biosciences 3100 Avant
Electrophoresis Equipment	ThermoEC MiniCell® PrimoTM EC320 Electrophoretic Gel System
Electronic Pipette	Finnpipette Thermo
Freezers	UĞUR (+4 ^{o}C -20^{o}C), New Brunswick Scientific (-80 ^{o}C)
Hemacytometer	FisherLab Scientific, 0267110
Ice Machine	Scotsman AF 10
Incubator with CO_2	Biolab SHEL LAB
Kodak Medical X-ray Processor	Kodak
Luminomertical 96 well Plate	Lumitrac 200
Magnetic Stirrer	VELP Scientifica
Microfuge Tubes 30cm	1.5ml, 2ml, Axygen
Microplate Spectrophotometer	BIORAD Benchmark Plus
Multiwell Plate 384	Roche
Nitrocellulose Membrane	Roche
Nucleofector	Lonza, Amaxa
Quick Spin	Labnet International, C1301-230V
Pipettes	Finnpipette Thermo 2.5µL,10µL,100µL, 200µL,Eppendorf 1000µL
Power Supply	Thermo Electron Corporation EC250-90
Trans-Blot® SD Semi-Dry Transfer Cell	BIO-RAD
Shaker	Forma Orbital Shaker, Thermo Electron Corporation
SDS-PAGE Gel Electrophoresis System	BIO-RAD MiniProtean
Tissue Culture Flasks	25 cm^2, 75cm^2 TPP
Tissue Culture Test Plates	6 well, 12 well, 96 well, TPP
Thermo Cycler	Techne, TC-3000
UV-Visible Spectrophotometers	uv-1700 PharmaSpec Shimadzu, Thermo scientific,NanoDrop 2000
Vortex	Heidolph, Reaxtop

2.1.2 General Chemicals

Chemicals used in this study are shown table below.

Table 2.2 : Chemicals used in this study.

Chemical	**Supplier Company**
Absolute ethanol Absolute methanol	Riedel- de Haën
Albumine, bovine (BSA) Ampicillin Kanamycin	Sigma
DMEM- High Glucose- Liquid Media (SH30243)	HyClone
DMSO	Riedel- de Haën
FBS	Biochrom
Agar APS Boric acid Bromophenol blue $CaCl_2.2H_2O$ DTT EDTA Ethidium bromide Glucose HCl SDS Yeast Extract	Merck
Isopopanol Glycerol	Fluka
MassRuler™ DNA Ladder (Mix, 80bp-10Kb) Mass Ruler Low Range DNA Ladder (80-1031 bp)	Fermantas
SeeBlue Plus2 Prestained Protein Ladder	Invitrogen
Skim milk powder	Oxoid

2.1.3 Enzymes

These enzymes were used in this study :

- BgI restriction enzyme (Fermentas)
- EcoRI restriction enzyme (Fermentas)
- XhoI restriction enzyme (Fermentas)
- HindIII (Fermantas)
- SacI restriction enzyme (Fermentas)
- One Taq Polymerase (New England Biolabs)
- Q5 enzyme (New England Biolabs)

2.1.4 Commercial kits

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

Table 2.3 : Commercial kits used in this study.

Kits	**Supplier Company**
Amaxa ™ Cell Line Nucleofector Kit V	Lonza, VCA-100
Biotin 3' End DNA Labeling Kit	Thermo Scientific 8918
Chemiluminescent Nucleic Acid Detection Module Kit	Thermo Scientific
Complete Lysis-M	Roche, 04719956001
Dual-Luciferase Reporter Assay System	Promega
EndoFree Plasmid Maxi Kit	Qiagen 12362
High Pure RNA Isolation Kit	Roche, 11828665001
QIAquick PCR purification kit	Qiagen, 28104
Light Cycler 480 Probes Master	Roche, 04707494001
RevertAid™ H Minus First Strand cDNA Synthesis Kit	Fermentas, K1631
SDS Gel Preparation Kit	Fluka
SimpleChIP Plus Enzymatic Chromatin IP kit	Cell Signalling technology #9003
SuperSignal® West Femto Maximum Sensitivity Substrate	Thermo, 34094
Transfast Transfection Reagent	Promega

2.1.5 Buffers

2.1.5.1 Running Buffer for Western Blotting

Running buffer was prepared to 1 L with dH_2O and all the components below. Running buffer was used western blot gel running experiment. Components of this solution are shown in table 2.4.

Table 2.4 : Components of Running Buffer

Components	**1L**	**Final Concentration**
Tris base	30.2 g	250 mM Tris
Glycine	144 g	1.92 M glycine
SDS	10 g	1% SDS

2.1.5.2 50X TAE Buffer

50X TAE Buffer was prepared as a stock and 1X TAE was used for DNA gel electrophoresis as tank buffer. Firstly, tris free base and EDTA are dissolved approximately in 700 ml dH_2O then was added the acetic acid and adjusted the volume to 1 L.

The 1X TAE solution is 40mM Tris, 20mM Acetate and 1mM EDTA and typically has a pH around 7.6.

Components of this solution are shown in table 2.5.

Table 2.5 : Components of 50X TAE Buffer

Components	**1L**
Tris free base	242 g
Disodiumn EDTA	18.61 g
Glacial Acetic Acid	57.1 ml
dH_2O	1 L

2.1.5.3 10X TBE Buffer

10 X TBE buffer was used in agarose gel electrophoresis to prepare the agarose gel that DNA was loaded on the as the tank buffer. Tris and Boric acid dissolved with using magnetic stirrer in 800 ml distilled water then added 0.5M Na_2EDTA (pH 8.0) and adjust volume to 1 L. TBE buffer stored at room temperature.

Running buffer was used western blot gel running experiment. Components of this solution are shown in table 2.6.

Table 2.6 : Components of 10X TBE buffer

Compenents	**1L**
Tris	108 g
Boric acid	55 g
0.5 M Na_2EDTA	5.84 g
dH_2O	1 L

2.1.5.4 Towbin Buffer

Towbin buffer was used for SDS polyacrilamide gel for transfer gel to with nitrosellulose membrane. Towbin buffer was stored at 4^oC. Components of this solution are shown in table 2.7.

Table 2.7 : Components of Towbin buffer

Components	1L
Tris base	3.03 g
Glycine	14.4 g
Methanol	200 ml
dH_2O	500 ml

2.1.5.5 10X TBS Buffer

TBS buffer was used 1X TBS buffer for western blot washing and was diluted stock solution 10:1 as a working solution. Tris and NaCl were dissolved in about 800 mL of deionized water. pH to 7.6 with 1 M HCl and completed with deionized water to 1L. It was stored at 4°C. 1X TBS buffer contain 50 mM Tris-Cl, 150 mM NaCl and, pH 7.6.

Components of this solution are shown in table 2.8.

Table 2.8 : Components of 10X TBS Buffer

Component	1L
Tris	0.6 g
NaCl	87.6 g
HCl	1M
dH_2O	1L

2.1.5.6 TBS-T Buffer

TBS-T solution was prepared for washing in Western blot analysis. Preparation of this buffer is shown in table 2.9.

Table 2.9 : Components of TBS-T Buffer

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

2.1.6 Solutions

2.1.6.1 PBS Solution

PBS tablet was used for preparing to PBS solution.

1 PBS tablet was dissolved in 100 mL dH_2O.

2.1.6.2 PBS-Tween Solution

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

2.1.7 Bacterial Strains

Escherichia coli DH5α strain [F-, φ80dlacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk-, mk+), phoA, supE44, λ-, thi-1, gyrA96, relA1]

2.1.8 Bacterial Culture Media and Solutions

2.1.8.1 Luria Bertani (LB) Medium

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

2.1.8.2 LB Agar Medium

LB Agar Medium was prepared by dissolving 10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g agar in 1L dH_2O. The media was sterilized by autoclaving for 15 min at 121°C. To prepare a selective media, 50 µg/L ampicillin was added after the media was cooled down approximately to 55°C near the fire for sterilization. After mixing the medium with ampicillin, the content was poured into 10 mm petri plates.

2.1.8.3 SOC Medium

SOC medium was used to cultivate *E.coli* for 1 h after heat shock during 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 resolved in 100 mL dH_2O and sterilized at 121°C by autoclaving for 15 min.

2.1.8.4 $CaCl_2$ Solution

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

2.1.9 Vectors

2.1.9.1 pJET Vector

The pJET 1.2/blunt is a linearized cloning vector which accepts inserts from 6 bp to 10 kb. It was used for cloning constructs. The vector is 2974 bp long. The circular map is shown below (Figure 2.1).

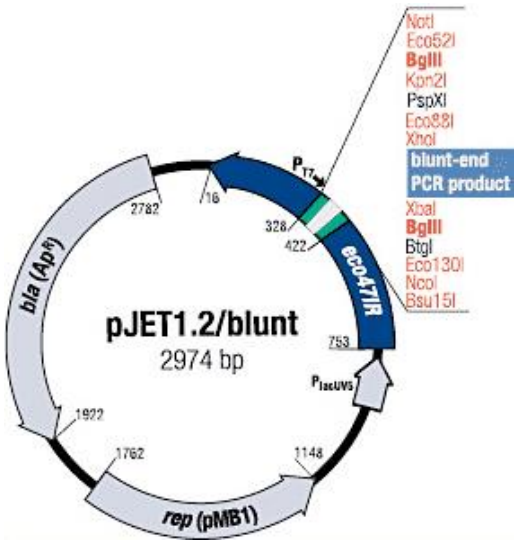

Figure 2.1 : pJET vector map

2.1.9.2 pGL3 Vector

The pGL3 mammalian expression vector is a luciferase reporter vector that produces a fundamental potential regulation. The vector is 4818 bp long. The circular map is shown below (Figure 2.2).

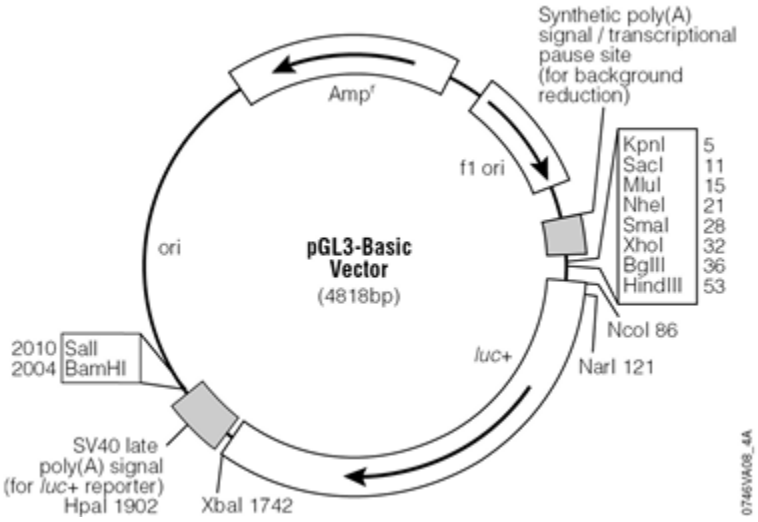

Figure 2.2 : pGL3-basic expression vector map.

2.1.9.3 pCMV6 Vector

The pCMV6 is a mammalian expression vector and it is 4665 bp long. The circular map is shown Figure 2.3.

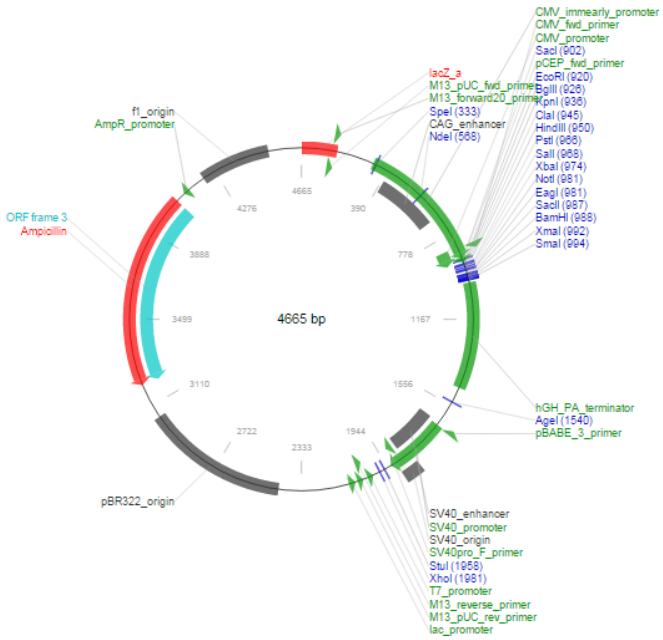

Figure 2.3 : pCMV6 vector map

2.1.10 Cell Culture Media

2.1.10.1 SH-SY5Y Culture Media

To prepare SH-SY5Y medium, DMEM low glucose was supplemented with 10% FBS, 2 mM L-Glutamine and 1X Penicilin/Streptomycin and then filter sterilized with 0,2 µm filter. SH-SY5Y culture media was stored at 4^{o}C.

2.1.10.2 SH-SY5Y Freezing Medium

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

2.1.10.3 Cell Line

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

2.2 Methods

2.2.1 Primer design

Primer design is a part of cloning studies. In order to clone desired nucleotide sequence of target DNA, primers with a favorable flanking restriction sites were designed to allow ligation into matched vectors. During primer design, melting temperatures of primers were selected as close as possible to each other to apply the optimum annealing temperature. Besides, GC content of primers was selected as much as ideal and hairpin and self-dimer formation were checked by IDT Scitools oligo analyzer (Url1).

Synthesized primers and their properties are given below:

Table 2.10 : p60 constructs primers

Primers	Sequence 5'→ 3'	Tm	Length	GC Content
Promoter_F	AAAAGAGCTCGTGGAGATTGAGACTGGAGG	62.6°C	30	50%
Promoter_R	AAAAAAGCTTTCTTGCACCGCCTCCTCC	63.3°C	28	50%
5'UTR_F	AAAAAAGCTTCTGCGGCGGCCCAAGCTC	70.8°C	28	57,14%
5'UTR_R	AAAAGAGCTCATGCGCACGCGCGGCCC	67.1°C	27	66,6%
Promoter+UTR_F	AAAAGAGCTCGTGGAGATTGAGACTGGAGG	62.6°C	30	50%
Promoter+UTR_R	AAAAAAGCTTCTGCGGCGGCCCAAGCTC	67.1°C	28	57,14%
Intron+ UTR_F	AAAAGAGCTCATGCGCACGCGCGGCCC	70.8 °C	27	66.7 %
Intron+ UTR_R	AAAACTCGAGCTAGGTGGGTTTTCTTTGCTTAG	61.6 °C	33	42.4 %
Promoter+UTR+Intron_F	AAAAGAGCTCGTGGAGATTGAGACTGGAGG	70°C	30	50%
Promoter+UTR+Intron_R	AAAACTCGAGCTAGGTGGGTTTTCTTTGCTTAG	70°C	33	42,42%

Table 2.11 : p53 constructs primers

Primers	Sequence 5'→ 3'	Tm	Length	GC Content
p53_**F**	GTCGAATTCATGGAGGAGCCGCAGTCAGAT	64,6 °C	30	53,33%
p53_**R**	TGGGTCGACTCAGTCTGAGTCAGGCCCTTC	66,8 °C	30	60%

2.2.2 Polymerase Chain Reaction (PCR)

PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. DNA polymerase can add a nucleotide only a preexisting 3'-OH group; therefore it needs a primer to which it can add the first nucleotide. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies and these are called amplicons. In the PCR procedure trace amounts of DNA can be quickly and repeatedly copied to produce a quantity sufficient to investigate using conventional laboratory methods.

For the purpose of amplifying requested DNA products , p60 constructs : p60-promoter, p60-UTR, p60-promoter + p60-UTR , p60-intron+ p60-UTR, p60-promoter + p60-UTR + p60-intron containing pGL3 vectors were used.

PCR components are indicated in table 2.12, PCR programs also is indicated in table 2.13.

Table 2.12 : PCR Components

Component	**Volume**	**Final Concentration**
Template	50-100 ng	50-100 ng
Forward Primer (5µM)	2,5 µL	0,5 µM
Reverse Primer (5µM)	2,5µL	0,5 µM
One Taq / Q5 Buffer	5µL	1X
dNTP(2mM)	2,5µL	0,2 mM
One Taq / Q5 DNA Polymerase	0,25µL	2 u/ml
DMSO (Depends on construct)	0,25 µL	1%
dH_20	up to 25µl	-

PCR Program for amplification:

Table 2.13 : PCR program

Step	Temperature	Time	Cycle
Initial Denaturation	94 °C	30 sec	1
Denaturation	94 °C	20 sec	30
Annealing	Depends on primer temperature	45- 68 °C (Depends on primer temperature)	30
Extension	68 °C	1 min/kb (Depends on amplicon length)	30
Final Extension	68 °C	5 min	1
Hold	4 °C	∞	-

2.2.3 Agarose gel electrophoresis for detection of PCR product

The technique of electrophoresis is based on the fact that DNA is negatively charged at neutral pH due to its phosphate backbone. For this reason, when an electrical potential is placed on the DNA it will move toward the positive pole. DNA fragments from PCR amplification, PCR products were analyzed on agarose gel electrophoresis. For 1% gel, 0,3 g agarose was added 30 mL 1X TBE Buffer and gel solution was boiled in a microwave until the agarose was completely dissolved. When gel was cooled, SYBR® Safe DNA Gel Stain added to dissolved agarose to a final concentration of 0.5 µg/mL and mixed through gentle swirling. The agarose gel was then poured into a horizontal gel tray, and a comb for forming the sample slots was placed into the gel. Approximately 15 min later the gel was solidified and then placed into an electrophoresis tank, where the gel was covered with 0,5X TBE buffer.

The DNA was mixed with 6X loading dye (Neb, purple) in the proportion of 5:1 and the sample was placed into a well on the agarose gel. As a fragment size control, a MassRuler™ DNA Ladder, Mix (80bp-10kb) was used. Electrophoretic separation was achieved by constant current at 100 mV for approximatly 30 min. DNA within agarose gels were visualized under UV light. The size of the PCR products was determined by comparing their mobility with the fragments of the DNA ladder.

2.2.4 Purification of PCR product

PCR products were purified with Qiagen QIAquick PCR Purification Kit. Due to discard of protein contamination derived from restriction enzymes or polymerase. Purification kit principle was used according to instructor's manual.

2.2.5 Determination of DNA concentration

The spectrophotometric analysis was used to determine purity, concentration of nucleic acids and recovery. The ratio of absorption at 260 nm vs 280 nm is commonly used to evaluate the purity of DNA with respect to protein contamination because protein (in particular, the aromatic amino acids) tends to absorb at 280 nm. According to literature, the ratio of absorbance (A260 / A280) of a pure DNA solution is between 1.8 and 2.0. Firstly, blank was identified then 2 µL sample was placed onto a sensor and its concentration was determined with the help of NanoDrop 2000/2000c Operating Software.

2.2.6 Restriction enzyme digestion of PCR products and the vector

The restriction endonucleases are commercially available. These endonucleases cut DNA from spesific palindromic restriction sites in length of 4 to 8 bases.

Table 2.14 : Restriction reaction mixture

Content	Amount	Volume per reaction
Plasmid vector(pJET/pGL3)	~3 µg	Depends on concentration
Enzyme 1	10 - 20 units	1 - 2 µl
Enzyme 2	10 - 20 units	1 - 2 µl
10X Tango buffer	1X	1.5 µl
Total Reaction Volume		15 µl

We were cloned pJET vector below conditions:

Table 2.15 : pJET vector enzymes for p60-katanin constructs

p60-katanin	Promoter	UTR	Promoter +UTR	Intron +UTR	Promoter+UTR+ Intron
Enzyme1	SacI	SacI	SacI	SacI	SacI
Enzyme2	HindIII	HindIII	HindIII	XhoI	XhoI

We were cloned pGL3 vector below conditions:

Table 2.16 : pGL3 vector enzymes for p60-katanin constructs

p60-katanin	**Promoter**	**UTR**	**Promoter +UTR**	**Intron+ UTR**	**Promoter+UTR+ Intron**
Enzyme1	SacI	SacI	SacI	SacI	SacI
Enzyme2	HindIII	HindIII	HindIII	XhoI	XhoI

2.2.7 Ligation

Ligation is commonly used in molecular cloning procedure to physically join a DNA vector to a gene of interest. Purified inserts; p60 promoter, p60 UTR, p60 promoter + p60 UTR, p60 Intron + p60 UTR and p60 promoter + p60 Intron+ p60 UTR were ligated to restricted expression vectors.

Purified insert and vector were ligated by T4 ligase according to 1:3 (vector versus insert DNA) molecular ratios. Ligation was performed overnight at room temperature. After overnight incubation, T4 DNA ligase was inactivated by incubation for 10 min at 65^{o}C. Reaction mixture conditions for ligation is shown in table 2.17.

Table 2.17 : Reaction mixture conditions for Ligation

Content	**Amount**	**Volume per reaction**
Plasmid vector	50 ng	Depends on concentration
Insert DNA	150 ng	Depends on concentration
10X T4 Buffer	1X	2 µL
T4 ligase	1 unit	1 µL
dH_2O		up to 20 µL

2.2.8 Competent cell preparation $CaCl_2$ method

The bacterial competent cells were prepared with using chemical treatment for the complete foreign DNA or plasmid. The competent cell preparation protocol is given below.

- Firstly, *E.coli*-DH5α cells were taken from a glycerol stock culture by scraping with a tip and it was put in 5 mL LB medium and incubated overnight at 37 ^{o}C in an orbital shaker.
- Next day, 100 mL LB medium was inoculated with 5 mL culture solution and was incubated at 37 ^{o}C in orbital shaker. Cell density was measured by spectrophotometer at OD_{600} and when it was reached to 0.6, the bacteria were

transferred to 50 mL prechilled sterile ultracentrifuge tubes and incubated on ice for 10 min.

- The cells were centrifuged at 1600 x g for 7 min at 4 ^{o}C, and then supernatant was discarded.
- Each bacterial pellet was resuspended in 10 mL ice-cold CaCl2 and centrifuged for 5 min at 1600 x g, the supernatant was discarded.
- Each bacterial pellet was resuspended in 10 mL ice-cold $CaCl_2$ and they were incubated on ice for 30 min.
- Centrifgation was performed again at 1600 x g for 5 min at 4 ^{o}C.
- Each pellet was resuspended completely in 2 mL of $CaCl_2$.
- The competent cells were distributed into prechilled sterile microfuge tubes each contains 50 µL and they were stored at -80 ^{o}C.

Table 2.18 : The compotent cell preparation content

Content	Final Concentration	Amount
$CaCl_2.2H_2O$	60 mM	0.442 g
PIPES	10 mM	0.15 g
Glycerol	15%	7,5 mL (from 100 %)
dH_2O	-	up to 50 mL

2.2.9 Transformation

The applied transformation protocol is given below;

- Prepared competenet cells were taken from -80 ^{o}C and thawed on ice.
- 2µL purified plasmid was added to 20 µL compotent cells in eppendorf tube after mini vortex then spin, eppendorf tube was incubated on ice for 30 min.
- After incubation, heat shock was done by puttingthe cells in water bath at 42 ^{o}C for 45 second and they were then immediately incubated on ice for 2 min.
- Then, 80 µl of SOC medium was added to competent cells and the eppendorf tube was vigorously shaked at 37 ^{o}C for 1 h.
- The cells were then spread onto LB plate containing the appropriate ampicillin antibiotic. The plates were incubated at 37 ^{o}C overnight.

2.2.10 Colony PCR

Colony PCR is a commonly used method to determine the presence or absence of insert DNA in plasmid constructs. Colony PCR procedure is given below;

- Each colony was resuspend with 10 µL sterile dH_2O was put into PCR tube.
- Mixture was incubated in thermal cycler at 85 ^oC for 10 min.
- Then PCR mixture mentioned in table 2.12 was added into tubes.
- The PCR program is shown in table 2.13.
- The amplified fragment was controlled with agarose gel electrophoresis as given in section 2.2.3.

Table 2.19 : Colony PCR reaction

Component	Volume	Final Concentration
Template + Water	10 µL	variable
Forward Primer (25 mM)	0.5 µL	0.5 µL
Reverse Primer (25 mM)	0.5 µL	0.5 µL
10X Taq Buffer	2.5 µL	1X
dNTP (2 mM)	2.5 µL	0.2 mM
Taq DNA Polymerase	0.3 µL	2 u/ml
dH_2O	8.7 µL	
Total reaction	25 µL	

2.2.11 Small scale plasmid DNA preparation (mini-prep)

After colony PCR, identified the right colonies containing the inserts were taken into 5 mL LB medium containing 5 µL ampicillin antibiotic in order to make selection media. Then, they were incubated overnight with vigorous shaking (200 rpm) at 37 ^oC.

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

2.2.12 DNA Sequencing

For the purpose of finding out the correct amplicon inserted into vector, sequencing was performed via commercial method.

2.2.12.1 Alignment of sequence result

Nucleotide alignments were made using webtool online (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) in order to compare the sequencing results with originally expected one.

2.2.13 Large scale plasmid DNA preparation (maxi-prep)

After correction of the sequence data and assuring that none of constructs involve any mutations, each construct was transformed *E.coli* dam-/dcm- GM2163 strain in order to prevent any methylation that would interfere the transcription factor binding, according to the previously described transformation in section 2.2.9.

Colonies emerged the insert containing plasmids were reproduced in large scale and purified with QIAGEN, Endofree® Plasmid maxi kit. This kit was chosen because subsequent to large scale production, the plasmids were going to be used to transfect SH-SY5Y neuroblastoma cell line which are 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 was performed using QIAGEN, Endofree® Plasmid maxi kit preparations, following instructions of the manufacturer.

2.2.14 Cell culture

2.2.14.1 Transfer to stock cell to the culture flask

Frozen SH-SY5Y cells from were taken -80 ^{o}C and immediately thawed in 37 ^{o}C water bath then taken in to 10 mL SH-SY5Y medium and centrifuged for 5 min at 900 rpm. After that cell pellet was dissolved with medium and transferred to the culture flask.

2.2.14.2 Cell counting

After growing cells, medium was removed and cells were separable with 1 mL of Tyrpsin EDTA treatment for 2 min at 37 ^{o}C. There after tyrpsin EDTA inhibited by 10 mL medium and cells were suspended. From suspended cells were taken 10 µL and put onto the hemocytometer. Cells were counted as the number of cells per 25 square ($1mm^2$).

Cell counting formula is given below:

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

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

2.2.14.3 Cell passage

After cells were lifted with 2 mL tyrpsin EDTA and cell density was determined, cells were put into the centrifuge tubes and centrifuged 900 rpm for 5 min. The supernatant was discarded and pellet was resuspended with culture medium and $1x10^6$ cells were transferred to clean culture flask.

2.2.14.4 Cell freezing

After cell passage with known cell number, cells resuspend in medium were transferred to freezing tube via freezing medium for future experiments. Cell number approximate $1x10^6$ cell / mL per freezing tube.

2.2.14.5 Total protein isolation

From SH-SY5Y cells total protein isolation was performed with mammalian cell & tissue extraction kit by Biovision as the protocol.

2.2.14.6 Determine the protein concentration

After protein isolation, the Bradford assay was performed by using the different diluted albumin (BSA) solutions range between 0-400 µg/mL and BSA reagent. Protein concentration is determined with spektrofotometre device.

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

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis is a method widely used to separate proteins according to electrophoretic mobility. The proteins are separated in gel according to their size by the help of reducing agents.

For this study, Fluka SDS Preparation Kit is used for preparation both 5% stacking gel and 12,5 % separating gel. Preparations of each gels were given below.

Table 2.20 : Content of 12,5 % Separating gel

Content	Volume
PAA stock solution	6,25 mL
Separation gel buffer	5.00 mL
dH_2O	3,50 mL
TEMED	15 µL
10% APS	150 µL

Table 2. 21 : Content of 5% Stacking gel

Content	Volume
PAA stock solution	0,75 mL
Stacking gel buffer	1 mL
dH_2O	3,15 mL
TEMED	5 µL
10% APS	50 µL

Firstly, separation gel solution was applied into the gel cassette and the cassette was filled with isopropanol immediately for fixation. After the gel was polymerized, the isopropanol was carefully washed and removed by filter papers. Then, the stacking gel solution was directly poured into the gel cassette, and the gel comb was placed to form the slots.

After gel polymerized, samples was denaturated for 5 min at 95 ^oC, were loaded in equal concentration into wells. As a molecular weight marker, 5µL SeeBlue Plus2 Prestained Protein Ladder (Invitrogen) was loaded on the gel.

The gel was placed into tank and run in running buffer at 25 mA for 2 h.

2.2.16 Western Blotting

Western blot is often used to identify and separate proteins according to molecular weight. After separation of denatured proteins upon size by SDS-PAGE, transferred to a nitrocellulose membrane producing a band for each protein. Then the nitrocellulose membrane is incubated with labels antibodies specific to the protein of interest.

- After SDS-PAGE, gel was taken from the tank and between glasses carefully.
- Nitrocellulose membrane and 2 pad were put into towbin electrotransfer buffer for 10 min for equilibration at 4 ^oC.
- After equilibration, the sandwich was prepared between pads and nitrocellulose membrane.
- The gel was put on the membrane with care not to disrupt the gel. Air bubbles were removed.
- The protein transfer was carried out in semi-dry blotting apparatus for 20 V for 30 min at 4°C.
- After transfer the membrane was blocked with blocking solution (non fat milk powder and BSA) to prevent non-specific binding to empty regions on the membrane. Moreover, blocking reduced undesirable backround.

- After blocking, the membrane was incubated with 1:500 diluted with blocking solution primary antibody (p53 (1c12) mouse monoclonal antibody, p60-katanin rabbit polyclonal or β-actin mouse monoclonal antibody) for overnight at 4 °C with gentle shaking.
- Following day membrane was washed with TBS-T buffer for 5 min 6 times with gentle shaking.
- Then 1:2000 diluted secondary antibody was prepared in blocking solution.
- The membrane was incubated with secondary antibody for 2 h at room temperature with gently shaking.
- The membrane was washed with TBS-Tbuffer for 5 min 5 times with gentle shaking. The last washing done with TBS buffer for 5 min.
- For detection, 20X LumiGLO® Reagent or SuperSignal® West Femto Maximum Sensitivity Substrate was used according to manufacturer instructions. The membrane was exposed to X-ray film for particularly time and then developed in Kodak Medical X-ray Processor according to manufacturer's instruction.

2.2.17 Chromatin Immunoprecipitation (ChIP assay)

ChIP assay is a powerful, efficient and versatile technique used for probing protein-DNA interactions within the natural chromatin context of the cell. In this method, spesific protein interactions and also histon modifications can be determined. Moreover, ChIP assay useful for analyze binding of transcription factors and co-factors, DNA repair proteins and DNA replication factors. ChIP assay was applied according to manufacturer instructions.

Cells are fixed with formaldehyde for preserving the protein-DNA interactions occuring the cell.

Then appoximately 4 X 10^7 cells were lysed and chromatin harvested. Chromatin was fragmented with using sonication and enzymatic digestion and then DNA fragment size was determined on agarose gel and DNA concentration was measured.

Sonication was applied 20 sec on and 30 sec off for three times.

After sonication, the digested chromatin was immunoprecipitated with p53 and p60-katanin antibodies specific to themselves.

We used SH-SY5Y cells for our experiment;

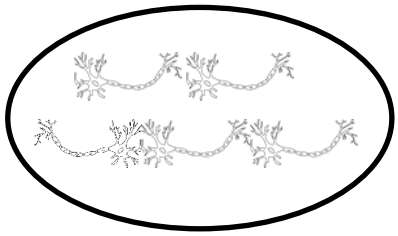

Figure 2.4 : Fixed SH-SY5Y cells via formaldehyde

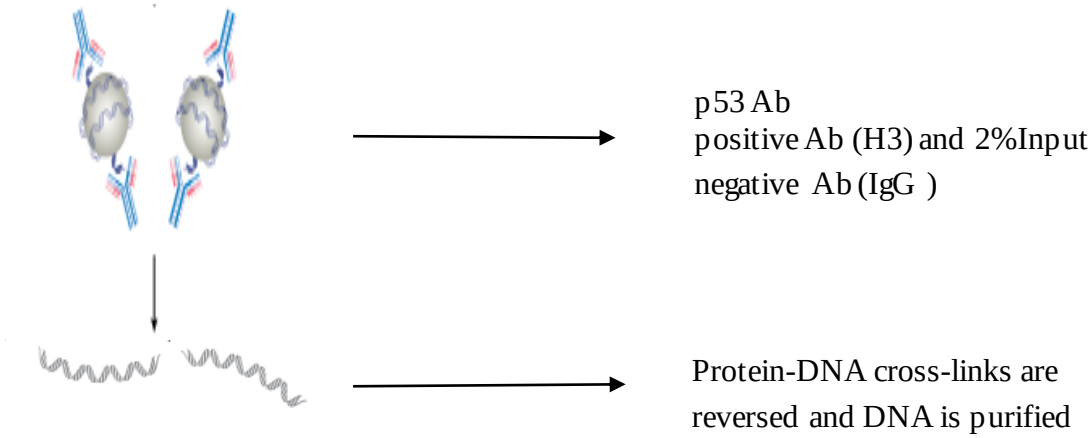

Figure 2.5 : Binding p53 antibody and purify p60-katanin DNA

Standard PCR or Quantitative Real-Time PCR can be used to measure the amount of enrichment of a particular DNA sequence by a protein-specific immunoprecipitation. In this experiment, we used standart PCR according to enzyme and concentration results.

2.2.18 Transfection of SH-SY5Y cells

Transfection is a process by which foreign nucleic acids are inserted into the cell. Forced experiment is co-transfection of cells with vector containing transcription factor and another vector containing promoter. In this study, lipofection was used for DNA insertion into SH-SY5Y cells. In lipofection, the synthetic cationic lipids that can form liposome are incubated with DNA. These liposomes interact with DNA through electrostatic interaction between the negatively charged nucleic acid and positively charged head group of synthetic lipid, and fuse with cultured cells. The liposome complex neutralizes the negative charge of the nucleic acids, allowing association of the complex with the negatively charged cell membrane. Entry of the liposome complex into the cell may occur by the processes of endocytosis or fusion with plasma membrane via the lipid moieties of the liposome.

- The optimal amount of control vector to use in cotransfections is the minimum amount that gives significant reporter activity above background (background is measured in samples transfected with only the test vector, in our study pGL3_basic).
- Eight different amounts were tried for optimization and 7:1 ratio of test vector: control vector was determined as optimum for SH-SY5Y neuroblastoma cell line.
- Transfection contents are given in table 2.22, and the transfection procedure is as follow.
- Cells were detached and cell number was calculated (section 2.2.16.2), and 50.000 cells per well were seeded on plated 24 well tissue culture plate 2 day before the transfection so that the cells were approximately 80 % confluent on the day of transfection.
- 1 µg of plasmid DNA is diluted in 200 µL DMEM (1X) and vortexed and quick spun.
- Promega's transfection reagent TransFast was added to 1:3 ratio to the DNA mixture according to the ratios given in the table below and vortexed and quick spun briefly for the formation of DNA liposome complex, and incubated for 15 min at room temperature.
- The growth medium was removed from the cells and transfection mixture was added gently in order to prevent cell detaching and cells returned to the 37 ^{o}C, 5 % CO_2 incubator for 1 h.
- After incubation, 500 µL medium was added to each well.
- The cells were returned to the 37 ^{o}C, 5 % CO_2 incubator for 48 h before analysis.

Table 2.22 : Optimization of vector: insert ratio

	Construct	pGL3 1X	Renilla 1X	pGL3 2X	Renilla 2X	pGL3 4X	Renilla 4X	TransFast
1	7/1 350:50	0,47 µL	0,17 µL	-	-	-	-	4,8 µl
2	7/1 700:100	-	-	0,94 µL	0,34 µL	-	-	9,4 µL
3	7/1 1400:200	-	-	-	-	1,88 µL	0,68 µL	19,2 µL
4	14/1 700:50	0,94 µL	0,17 µL					9 µL
5	14/1 1400:100	-	-	1,88 µL	0,34 µL	-	-	18 µL
6	14/1 2800:200	-	-	-	-	3,76 µL	0,68 µL	36 µL
7	21/1 1400:50	1,88 µL	0,17 µL	-	-	-	-	17,4 µL
8	21/1 2800:100	-	-	3,76 µL	0,34 µL	-	-	34,8 µL

2.2.19 The Dual Luciferase® Reporter (DLR) Assay

In the Dual Luciferase Reporter Assay, the activities of firefly (Photinus pyralis) and Renilla (Renilla reniformis) luciferases are measured sequentially from a single sample with luminometer. In order to improve experimental accuracy, dual reporters are generally used.

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. The other cause of variability, such as in pipetting volumes, cell lysis efficiency and assay efficiency can be effectively eliminated.

In this study, Promega DLR Assay was used for luminometric measurement. To perform this assay, neuroblastoma cells are harvested by lysing with 1X Passive Lysis Buffer buffer after 48 h. This buffer is specifically formulated to minimize the low-level autoluminescence emitted by Renilla luciferase substrate, coelenterazine. Firstly, the firefly luciferase reporter is measured by adding Luciferase Assay Reagent II (LAR II) to generate a stabilized luminescent signal.

After measurement of firefly, Stop & Glo® Reagent was added into same tube to quench firefly luciferase reaction and initiate Renilla luciferase reaction. Stop & Glo® Reagent also produces a stabilized signal from the Renilla luciferase, which decays slowly over the course the measurement (Promega Technical Manual,E1910).

The luciferase activity depends on the function of the promoter upstream from luc+; in this study, this promoter is *KATNA1* gene promoter. According to forced experiments, the luciferase activity depends on function of transcription factor on the promoter upstream of luc+; for this study this transcription factor is p53.

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

All transfections were performed as triplicates and were repeated at least two times using different DNA preparations from neuroblastoma cells.

Cells were prepared according to manufacturer instructions;

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

2.2.19.1 The Dual Luciferase® Reporter (DLR) Assay for p60-katanin characterization

To determine p60-katanin regions ; p60-katanin promoter, p60-katanin UTR, p60 katanin promoter + p60-katanin UTR, p60-katanin promoter + p60-katanin UTR + p60- katanin Intron characterization, DLR assay was used.

50.000 neuroblastoma cells were plated in each well of 24 well plate and transfected with pGL3_Basic vector and p60-katanin constructs in indicated amount in table 2.23. After 48 h of growth, measured with luciferase assay.

Table 2.23 : p60-katanin characterization with lusiferase assay

Construct name	pGL3+Insert 800 ng x 3	Renilla 30 ng x 3	TransFast reagent
pGL3_Basic	1,5µL	0,18µL	13,8µL
p60 promoter	1,63µL	0,18µL	13,8µL
p60 UTR	1,58µL	0,18µL	13,8µL
Promoter+UTR	0,97µL	0,18µL	13,8µL
Promoter+UTR+Intron	1,48µL	0,18µL	13,8µL

2.2.20 Nucleofection

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

Nucleofection procedure is as follow:

- Cultured cells were lifted and cell number was calculated (section 2.2.14.2), $1x10^6$ cells for each well of 6 well tissue culture plate were seeded and centrifuged 900 rpm for 5 min.
- Pellet was resuspended carefully with 100 µL room temperature Nuclefector® Solution per sample. (Leaving cell in Nuclefector® Solution for extended periods of time no longer than 15 min.)
- Nucleofector solution contains reagent and solution.
- The cell suspension was combined with 10 µg DNA and then cell/DNA suspension was transferred into nucleofector cuvette.

- The appropriate Nucleofector® Program G-004 was selected and cuvette was inserted into Nucleofector® Cuvette Holder and the selected program was applied by pressing the X-button.
- The cuvette was taken out of the holder once the program was finished.
- 500 µL culture medium was immediately added to cuvette and the sample was transferred into the prepared 6 well plate (final volume 2 mL media per well). The cells were incubated in 37 ºC, 5% CO_2 incubator during 24 h.

2.2.21 RNA Isolation

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

2.2.22 cDNA Synthesis

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

Table 2.24 : cDNA synthesis reaction first components

Component	Amount
Template RNA	1 µg
Random hexamer primer	1 µL
Nuclease-free water	up to 13 µL

Components mixed gently and spin down.

This mixture was incubated at 65 ºC for 5 min and the chilled on ice.

Table 2.25 : cDNA syntesis reaction second components

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

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

2.2.23 Real Time PCR

The Real Time PCR (RT-PCR) method is based on PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. The quantity is either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing gene. In this study, specific probes and primers for p60-katanin were purchased from Universal Probe Library (Roche Applied Science Url-4), probe number and primers are given table 2.26.

Table 2.26 : Primers and probes for p60-katanin

Primer	**Primer sequence(5'→ 3')**
p60-katanin L	GCGGACATTACCAACGTGT
p60-katanin R	CATCTGCATTTCTTCTTTGGAA

Beta-actin (β-actin) (Roche Universal Probe Library Human ACTB Gene Assay) gene was used as reference for RT-PCR studies. RT-PCR reaction was performed with Light Cycler® 480 Probes Master RT-PCR Kit using a Roche Light Cycler 480 according to the following Light Cycler program given in table 2.27.

Table 2.27 : Real Time PCR Program

Step	**Temperature**	**Time**	**Cycle**
Initial Denaturation	95 ºC	10 min	1
Denaturation	95 ºC	10 sec	45
Amplification	60 ºC	30 sec	
Extension	72 ºC	1 sec	

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

2.2.24 Electrophoretic Mobility Shift Assay (EMSA)

This assay was used for detection of protein complexes with nucleic acids.

2.2.24.1 Oligo probe Biotinylation and Hybridization

Oligo probes were labeled separately by using Biotin 3' End DNA Labeling Kit that uses terminal deoxynucleotidyl transferase to incorporate 1–3 biotinylated ribonucleotides on to the 3' end of DNA strands.

Labeled forward and reverse oligos were then annealed at 1:1 ratio in 10 mM Tris, 1 mM EDTA by heating to 95 °C for 5 min, slow cooling by 2 °C/min to their annealing temperature, annealing for 30 min and cooling to 4 °C by 2 °C/min (cycle number depends on Tm of the oligos).

Oligo probes for EMSA:

Table 2.28 : EMSA oligo probes for p53

Oligo name	Sequence
p53 prob_WT	5'-GAGGGAAAGTGAGCAAGCACCTGAACAAGGTACAGAGGCGCG -3'
p53_WT_comp	5'-CGCGCCTCTGTACCTTGTTCAGGTGCTTGCTCACTTTCCCTC-3'
p53 prob_mutant	5'-GAGGGAAAGTGAGTGGACACCTGAATGGAGTACAGAGGCGCG -3'
p53mutant_comp	5'-CGCGCCTCTGTACTCCATTCAGGTGTCCACTCACTTTCCCTC-3'

2.2.24.2 Components of EMSA gel

Complexes and free DNAs were separeted on a 6 or 8 % non-denaturating polyacrylamide gel in 0.5X Tris-Borate-EDTA by electrophoresis for 1 h at 120 V at 4 °C. The separated bands on the gel were then transferred to Biodyne A Nylon Membranes (Pierce) by using Trans-Blot® at 20 V for 30 min at 4 °C. Cross-link transfer of DNA to membrane was achieved by incubating the membrane with 254 nm UV bulbs for 10 min. EMSA gel was used given below.

Table 2.29 : EMSA gel components

Component	Amount	Final Concentration
Acrylamide (% 30)	1.4 mL	% 4,2
TBE (10X)	500 µL	0,5 X
APS (% 10)	150 µL	% 0,15
Temed (14.4 M)	10 µL	14,4 mM
H_2O	7.94 mL	-

2.2.24.3 p53 EMSA Optimization

Table 2.30 : EMSA reaction buffer conponents for optimization

	1	2	3	4	5	6	7	8	9
10X binding buffer	2 µL (1X)	2 µL (1X)	2 µL (1X)	2 µL (1X)	2 µL (1X)	2 µL (1X)	2 µL (1X)	2 µL (1X)	2 µL (1X)
Poly dIdC	1 µL (1 µg)	1 µL (1 µg)	1 µL (1 µg)	1 µL (1 µg)	1 µL (1 µg)	1 µL (1 µg)	1 µL (1 µg)	1 µL (1 µg)	1 µL (1 µg)
%50 Glycerol	4 µL (% 20)	4 µL (% 20)	4 µL (% 20)	4 µL (% 20)	4 µL (% 20)	2 µL (% 10)	4 µL (% 20)	4 µL (% 20)	4 µL (% 20)
50mM EDTA	0.2 µL (1 mM)	0.2 µL (1 mM	0.2 µL (1 mM	-	0.2 µL (1 mM	0.2 µL (1 mM	0.2 µL (1 mM	0.2 µL (1 mM	0.2 µL (1 mM
%1 BSA	1 µL (% 0,1)	1 µL (% 0,1)	1 µL (% 0,1)	1 µL (% 0,1)	1 µL (% 0,1)	1 µL (% 0,1)	1 µL (% 0,1)	1 µL (% 0,1)	1 µL (% 0,1)
100mM $MgCl_2$	1 µL (10mM)	1 µL (10mM)	1 µL (10mM)	1 µL (10mM)	1 µL (10mM)	1 µL (10mM)	1 µL (10mM)	1 µL (10mM)	1 µL (10mM)
1M KCL	-	1 µL (100 mM)	2 µL (100 mM)	-	-	-	-	-	1 µL (100 mM)
20mM DTT	-	-	-	-	-	-	4 µL (8 mM)	1.8 µL (3,6 mM)	4 µL (8 mM)
3M NaCl	-	-	-	-	1 µL (300 mM)	-	-	-	1 µL (300 mM)
Protease inhibitor (100X)	1 µL (1X)	1 µL (1X)	1 µL (1X)	1 µL (1X)	1 µL (1X)	1 µL (1X)	1 µL (1X)	1 µL (1X)	1 µL (1X)
Biotin DNA	1 µL (20 fmol)	1 µL (20 fmol)	1 µL (20 fmol)	1 µL (20 fmol)	1 µL (20 fmol)	1 µL (20 fmol)	1 µL (20 fmol)	1 µL (20 fmol)	1 µL (20 fmol)
WCE	2 µL (2 µg)	2 µL (2 µg)	2 µL (2 µg)	2 µL (2 µg)	2 µL (2 µg)	2 µL (2 µg)	2 µL (2 µg)	2 µL (2 µg)	2 µL (2 µg)
H_2O	6.8	4.8	3.8	7	5.8	8.8	2.8	-	0.2

For binding reactions, ~2 µg whole extracts of SH-SY5Y cells or p53 protein were incubated with 20 fmol biotinylated oligonucleotides in binding buffer (contents of binding buffer indicated in Table 2.30). In supershift assays, 300 ng of p53 antibody (QIAGEN Inc.) was added prior to the addition of 150 ng p53 protein.

Table 2.31 : EMSA reaction buffer contents for p53

	Amount				
Contents	**1**	**2**	**3**	**4**	**5**
H_2O	11µL	9 µL	11 µL	9 µL	7 µL
10X binding buffer	2 µL (1X)	2 µL (1X)	2 µL (1X)	2 µL (1X)	2 µL (1X)
Poly dIdC	1 µL (1 µg)	1 µL (1 µg)	1 µL (1 µg)	1 µL (1 µg)	1 µL (1 µg)
%50 Glycerol	2 µL (% 10)	2 µL (% 10)	2 µL (% 10)	2 µL (% 10)	2 µL (% 10)
%1 BSA	1 µL (% 0,1)	1 µL (% 0,1)	1 µL (% 0,1)	1 µL (% 0,1)	1 µL (% 0,1)
100Mm MgCl	1 µL(10mM)	1 µL(10mM)	1 µL(10mM)	1 µL(10mM)	1 µL(10mM)
Protease inhibitor(100X)	1 µL(1X)	1 µL(1X)	1 µL(1X)	1 µL(1X)	1 µL(1X)
WCE	-	2 µL	-	2 µL	2 µL
Biotin DNA	1 µL(20 fmol)	1 µL(20 fmol)	1 µL (20 fmol) (mut)	1 µL(20 fmol) (mut)	1 µL(20 fmol)
Unlabeled DNA	-	-	-	-	2 µL(20 fmol)
Antibody	-	2 µL(1 µg)	-	2 µL(1 µg)	-

For detection step, Chemiluminescent Nucleic Acid Detection Module Kit (Pierce) was used, and then the membrane was exposed to X-ray film for required amount of time and then developed in Kodak Medical X-ray Processor according to manufacturer's instruction. In supershift assays, 300 ng of p53 antibody (QIAGEN Inc.) was added prior to the addition of 150 ng p53 protein.

3. RESULTS AND DISCUSSION

3.1 Production of p60 constructs

Putative transcriptional regulatory regions of p60-katanin promoter were identified and selected by bioinformatic analysis according to H3K27Ac Mark (Often Found Near Active Regulatory Elements) from ENCODE (as shown in appendix c) and these regions (336 bp promoter, 448 bp 5'UTR, 784 bp promoter + UTR, 2234 bp UTR+ Intron and 3000 bp Promoter + UTR + Intron regions) were cloned for the identification of their regulatory activities for p60-katanin promoter by Luciferase assay.

p60-katanin constructs; p60 promoter (336 bp), p60 UTR (448 bp), p60 promoter + p60 UTR (778bp), p60 intron+ p60 UTR (2570 bp) and p60 promoter + p60 UTR + p60 Intron (3000 bp) were amplified with PCR. The PCR products were run on agarose gel and the expected DNA fragments were detected.

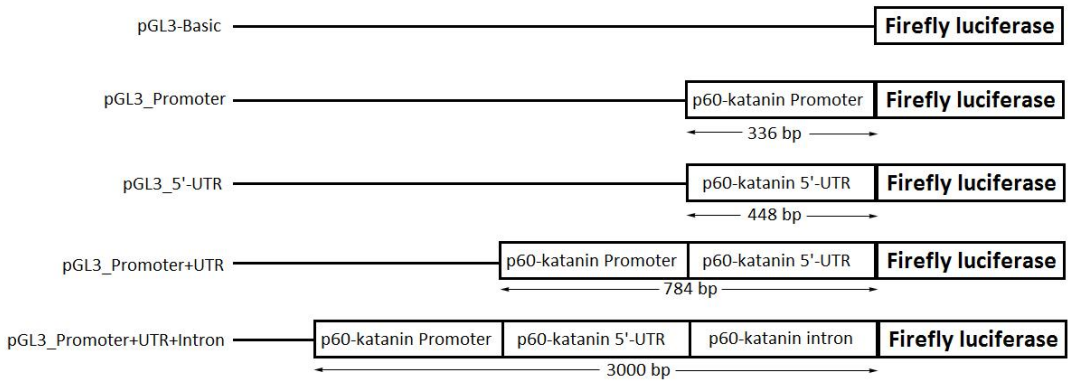

Figure 3.1 : p60 constructs (336 bp promoter, 448 bp 5'UTR, 784 bp promoter + UTR, and 3000 bp Promoter + UTR + Intron) with pGL3_Basic vector

UTR construct of p60-katanin is include CpG island from +219 to -89 region as shown in Figure 3.2. As we know CpG island methylation is critical for gene expression and transcriptional regulation. Therefore UTR region important for p60-katanin regulation.

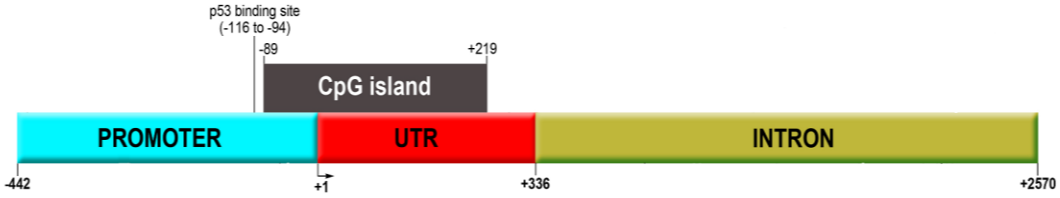

Figure 3.2 CpG island location on UTR region

Amplified PCR products were designed to be inserted into pGL3_Basic expression vector. After enzyme cleavage, restricted products were purified by QIAquick PCR purification kit. After that, ligation procedure was performed with Roche T4 DNA ligase overnight at room temperature and the ligation mixture was then transformed into competent *E. coli* DH5α cells. Transformed colonies were selected from selective antibiotic (ampicilin) plates and colonies including p60 constructs were checked with colony PCR using forward and reverse primers of the vector to verify accuracy. Agarose gel results indicated that amplicons were successfully ligated into vector as shown in Figure 3.3.

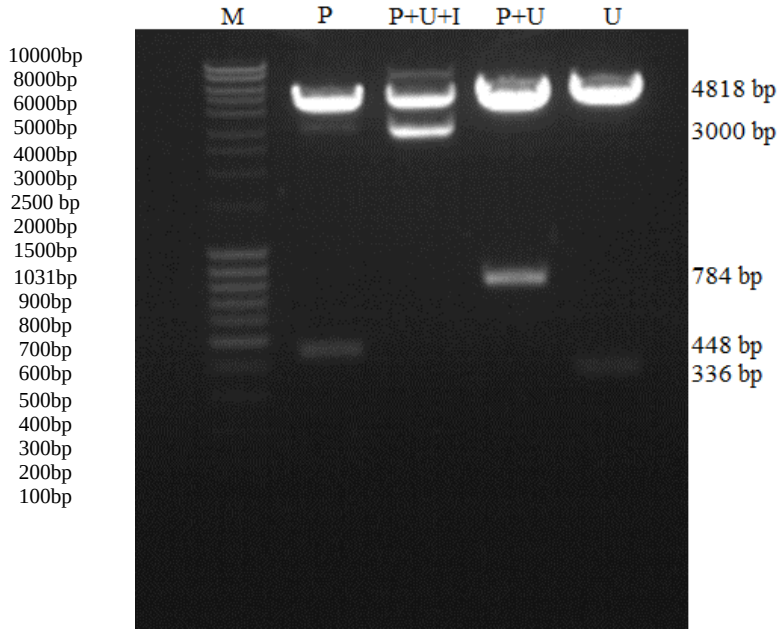

Figure 3.3 : Agarose gel image of of p60-katanin constructs and pGL3_Basic vector

3.2 Luminometrical Measurements Data of p60-katanin Constructs

In order to determine p60-katanin constructs expression, SH-SY5Y neuroblastoma cells were transfected with pGL3_Basic vector and p60-katanin constructs. Each transfection was performed as triplicate and experiments were repeated 4 times on separate days. Transfection with p60-katanin constructs results shown in table 3.1., table 3.3., table 3.5. and table 3.9.

Table 3.1 : Measured light units of experiment n=1 for p60-katanin

		pGL3-Basic	Promoter	UTR	Promoter_UTR	Promoter_UTR_Intron
1	Firefly	0.2203	0.2084	55.4	71.05	47.96
	Renilla	0.0636	0.0519	0.0374	0.0454	0.0426
2	Firefly	0.1591	0.2949	49.3	66.1	42.8
	Renilla	0.0751	0.0978	0.0314	0.0377	0.0488
3	Firefly	0.2247	0.2305	63.39	59.15	47.85
	Renilla	0.0826	0.0766	0.0385	0.036	0.0609

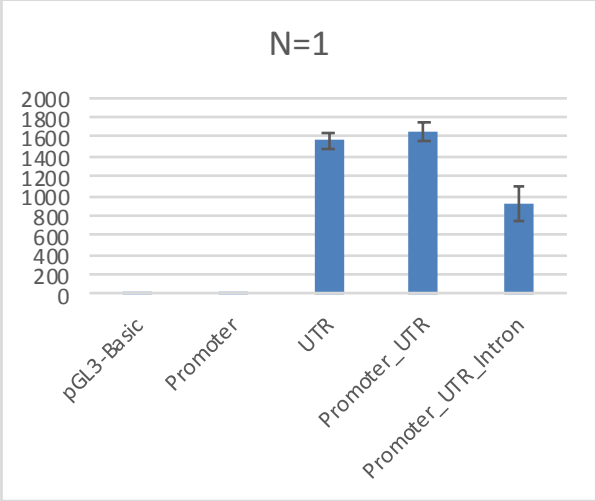

Figure 3.4 : Fold activity of regulatory region N=1 experiment

Table 3.2 : Measured light units of experiment n=2 for p60-katanin

		pGL3-Basic	Promoter	UTR	Promoter_UTR	Promoter_UTR_Intron
1	Firefly	0.5767	0.664	194.2	163.8	147.3
	Renilla	0.1916	0.1997	0.1653	0.1038	0.2478
2	Firefly	0.5189	0.5057	193.9	161.1	154.6
	Renilla	0.1784	0.2138	0.1391	0.1338	0.217
3	Firefly	0.5448	0.5325	179.3	154.9	161.8
	Renilla	0.18	0.1949	0.1327	0.1599	0.2179

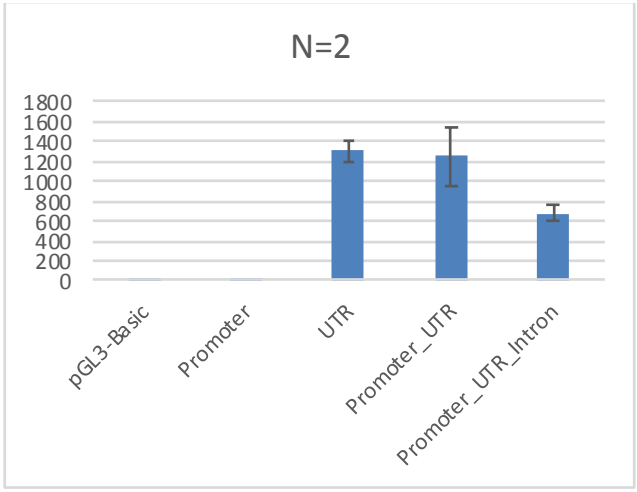

Figure 3.5 : Fold activity of regulatory region N=2 experiment

Table 3.3 : Measured light units of experiment n=3 for p60-katanin

		pGL3-Basic	Promoter	UTR	Promoter_UTR	Promoter_UTR_Intron
1	Firefly	1.063	1.098	362.8	306.9	315.4
	Renilla	0.511	0.507	0.2754	0.3435	0.5553
2	Firefly	1.063	1.12	306.3	276.3	343.2
	Renilla	0.5442	0.4541	0.2544	0.281	0.4818
3	Firefly	1.118	1.107	361.3	319.6	324.9
	Renilla	0.5872	0.4962	0.246	0.3459	0.4497

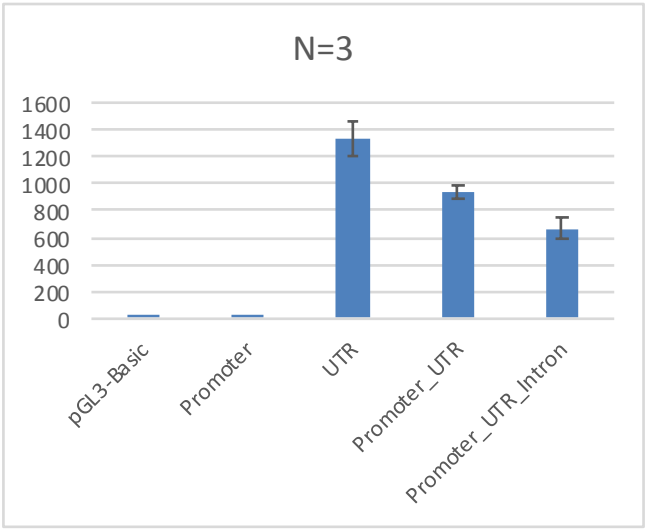

Figure 3.6 : Fold activity of regulatory region N=3 experiment

Table 3.4 : Measured light units of experiment n=4 for p60-katanin

		pGL3-Basic	Promoter	UTR	Promoter_UTR	Promoter_UTR_Intron
1	Firefly	0.5981	0.542	145	161	134.4
	Renilla	0.1286	0.1145	0.0833	0.09	0.1343
2	Firefly	0.506	0.5886	166	145.3	160.2
	Renilla	0.1213	0.1445	0.1032	0.0779	0.1539
3	Firefly	0.5459	0.5611	147	160.4	189.6
	Renilla	0.1146	0.1161	0.0897	0.0673	0.1589

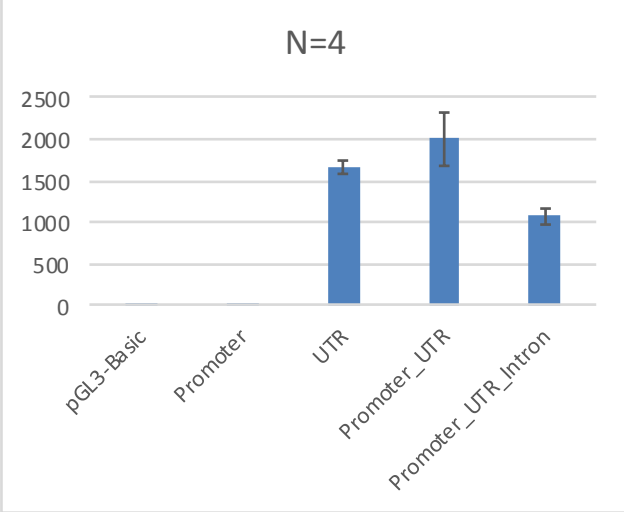

Figure 3.7 : Fold activity of regulatory region N=4 experiment

The normalized fold change activity between four test groups determined with equation calculation and this calculation considered as fold of induction in respect to the activity of the empty pGL3_Basic vector. The calculated fold activity and graph representing the obtained data are given in the table 3.5 and figure 3.8 below.

$$\Delta \text{ Fold activation} = \frac{Average\left({}^{Firefly}/_{Renilla}\right)Sample\ X}{Average\left({}^{Firefly}/_{Renilla}\right)pGL3-Basic}$$

Table 3.5 : Average of calculated F/R of all experiments and standard deviation for p60-katanin

Experiment F/R	pGL3-Basic	Promoter	UTR	Promoter_UTR	Promoter_UTR_Intron
N=1	2.77	3.35	1565.95	1653.78	929.53
N=2	2.98	2.81	1306.65	1250.27	683.14
N=3	1.98	2.29	1330.02	933.56	667.60
N=4	4.52	4.54	1662.67	2012.48	1078.29
Average F/R	2.77	3.35	1465.95	1653.78	839.6396
Average F/R Standard Deviation	0	0.1257	135.3020	79.1595	59.5727
ΔFold Activation	1	1.08	510.80	483.24	285.10

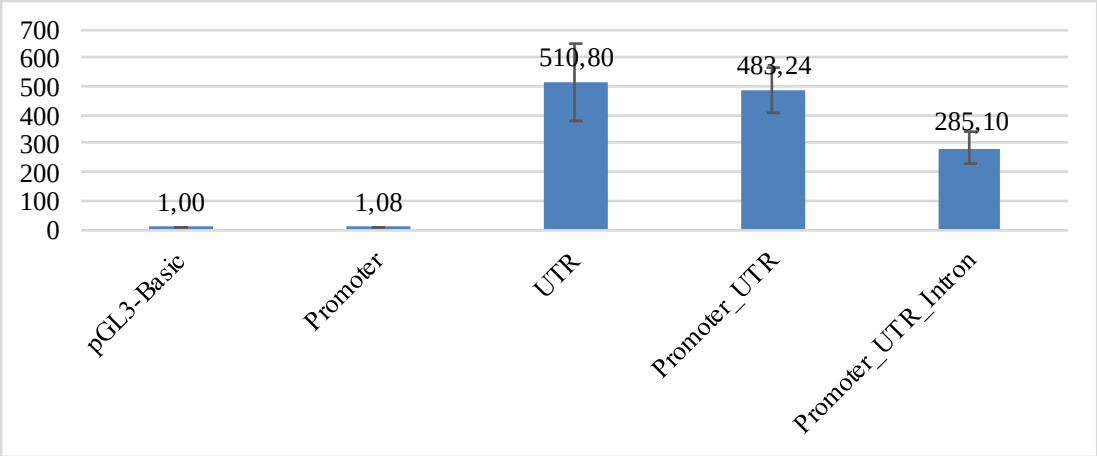

Figure 3.8 : Average fold activities of KATNA1 optimal promoter

The lusiferase reporter activity was measured by using the Dual-lusiferase reporter assay system. For all lusiferase assays, the normalized lusiferase activity of each construct firefly and renilla ratio in the pGL3_Basic vector.

Based on the lusiferase assay, we identified that 5'-UTR region enhanced transcriptional activity of the reporter gene, but not putative promoter region and the presence of intron decreased the activity. Thus, expression of katanin-p60 seems to be regulated via 5'-UTR, and intron-1 might have repressor elements.

The reason for this may be the result of 5' UTR region is including CpG island. CpG islands are important for regulation of transcription. When GC rich region is located gene this cause increases the probability of omnipresent transcription factors will bind (Deaton A.M. and Bird A., 2011). Transcription factors bind inclined to GC-richness

sites because of if gene regions unoccupied by a TF, these regions are occupied by nucleosomes in vivo (Weng Z et. al., 2012).

3.3 Chromatin Immunoprecipitation (ChIP) Method

3.3.1 Identification of p53 binding site on *KATNA1* promoter regions by ChIP

We defined p53 consensus sequence on *KATNA1* gene by bioinformatically. Therefore, it is thought that *KATNA1* gene could possibly be regulated by p53 transcription factor. A previous study demonstrated that neuronal processes were retracted upon PKC activation following increases in both p60-katanin and p53 levels in neurons (Korulu S., Unal A.Y., Yuksel M. and Karabay A., 2013). Based on this study, we started with analyzing the binding of p53 to the corresponding promoter region. Primarily, cells were transfected with p53 in order to understand whether p53 bind to p60-katanin.

3.3.2 In Vivo Crosslinking and Nuclei Preparation Digestion of Chromatin

ChIP assays demonstrate in vivo interaction of p60-katanin and p53 as a transcription factor. ChIPs were performed as described 2.2.20. Formaldehyde fixed protein-gDNA complex was extracted from SH-SY5Y cells and digested with micrococal nuclease as described in SimpleChIP® enzymatic chromatin IP kit protocol. Then, DNA was purified and 10 µl were separated digested DNA product was run on 1 % agarose gel to check success of digestion. Expected DNA fragments (900 – 150 bp) were observed as shown in Figure 3.9.

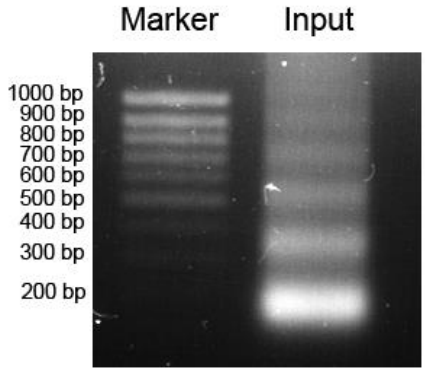

Figure 3.9 : Enzymatic digestion of genomic DNA (gDNA)

3.3.3 Chromatin Immunoprecipitation (ChIP)

For detect of p60-katanin and p53 binding, digested DNA fragments were immunoprecipitated using The SimpleChIP® Enzymatic Chromatin-IP Kit and standard PCR method were used. p53 bound DNA fragments were precipitated with p53 specific antibody. PCR ampification was performed with specific primers complementary to the predicted p53 binding site on p60-katanin promoter region. As enyzme Onetaq was used. Samples were included the 2% input sample, represent equal fractions of extract collected prior to precipitation from which DNA was extracted used as a control, the positive control histone H3 sample, the negative control normal rabbit IgG sample, and a tube with no DNA to control for DNA contamination as shown in Figure 3.10.

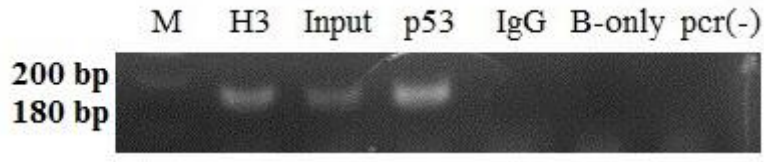

Figure 3.10 : PCR analysis of immunoprecipitated DNA fragments

We found that p53 positive controls H3, input and p53 have a band on 180 bp which p53 sequence length. On the other hand, negative controls IgG, B-only and pcr (-) do not have bands. These results indicate that, p53 transcription factor can actually physically bind to p60-katanin promoter.

3.4 Determination of p53 binding site on *KATNA1* promoter regions by EMSA

Electrophoretic mobility shift assay (EMSA) was performed in order to confirm the binding of p53 transcription factor to its predicted sites on the optimal promoter of *KATNA1* gene.

3.4.1 Identification of p53 binding sites on the optimal *KATNA1* promoter

We theoretically searched the presence of p53 transcription factor binding sites in the *KATNA1* promoter by using PROMO bioinformatics tool (Messeguer et al. 2003; Farre et al. 2003) restricting the maximum matrix dissimilarity rate 0-3% for Homo sapiens. According to these search results, a putative p53 binding site was

identified within -95 to -117 region of the transcription start site by bioinformatic analysis have been predicted in the promoter as shown in Figure 3.11.

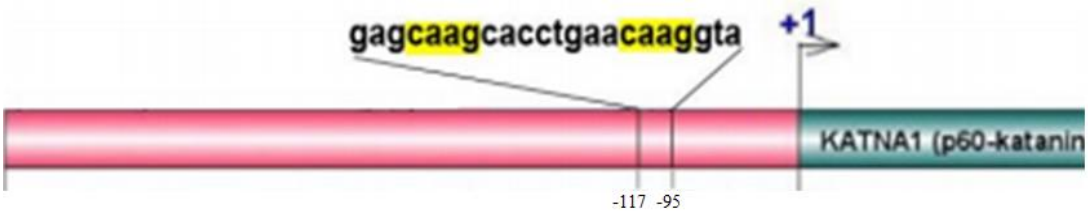

Figure 3.11 : p53 binding sites in p60-katanin promoter region

Protein binding to p53 binding site (WT) was observed using both SH-SY5Y whole cell extract (WCE) and pure p53. The signal shifts of the DNA-protein interactions were prevented when 53 p53 oligo mutated in their binding site (Mut) was used with WCE and with p53.

Extract free biotin-11-UTP labeled p53 oligonucleotides (WT, wild type; Mut, mutated; oligonucleotide sequences were given in Figure 3.12 were shown in lanes 1 and 3, respectively.

p53 overexpressed SH-SY5Y cell extract was added to the reaction mixture and incubated with WT and Mut oligonucleotides to form nucleotide- protein complex (lane 2 and lane 4, respectively). 1000-fold excess of unlabeled WT competitor oligonucleotide was added to the binding reaction mixture (lane 5). p53/WT-oligo complex formation was observed in lane 2. Mutation of p53 binding site drastically decreased p53 binding (lane 4) (Figure 3.12).

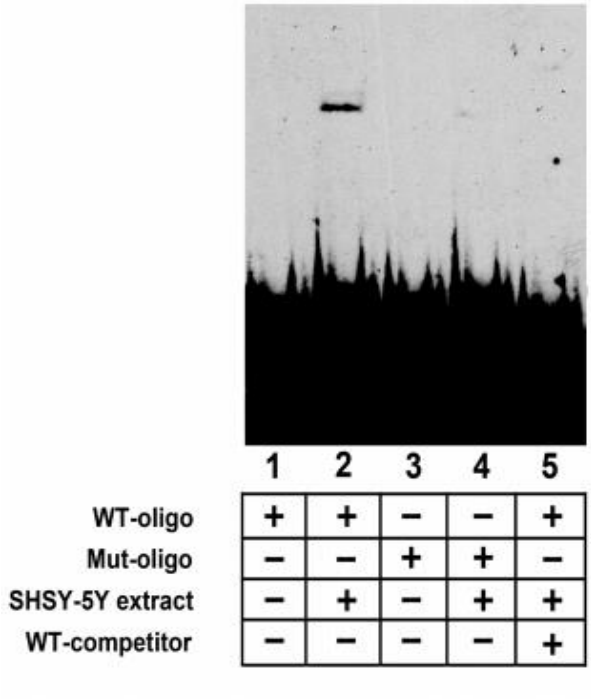

Figure 3.12 : Confirmation of p53 binding to the corresponding site on the *KATNA1* promoter by EMSA

According to these results, we confirmed the p53 binding to *KATNA1* gene by EMSA. WT oligo have a band otherwise, mutation oligo do not have a band. Thus, we ensured the bind p53 on the *KATNA1* gene.

3.5 mRNA expression level of *KATNA1* gene

In order to further quantify whether these changes were reflected at mRNA level of p60-katanin, qRT-PCR was performed. Total mRNA was isolated from SH-SY5Y cells transfected with PCMV and flag p53. β-actin was used as a reference. Following qRT-pcr experiments, values were calculated according to ΔΔCt method (Schmittgen T.D and Livak K.J., 2008). qRT-PCR results shown in Figure 3.13.

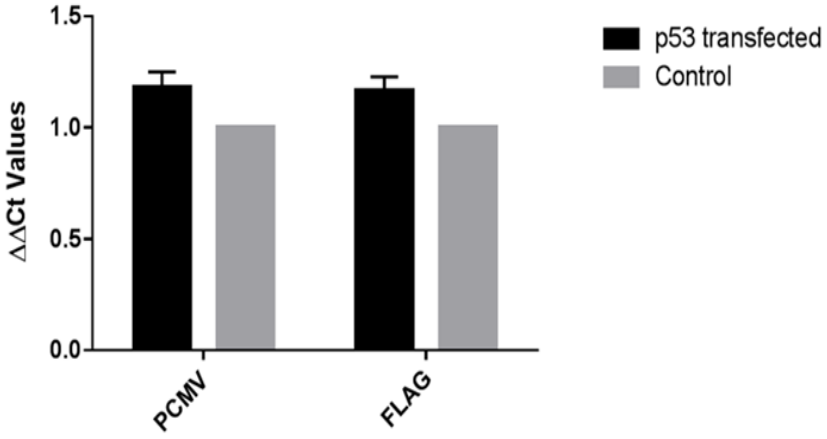

Figure 3.13 : ΔΔCt values of mRNA expression levels of *KATNA1* gene control and p53 transfected SH-SY5Y neuroblastoma cells via flag and PCMV vectors

We transfected p53 in neuroblastoma cells two different vectors these are flag and PCMV. In according to results, two vectors have almost same fold activation. mRNA levels of human p60-katanin was increased by overexpression of p53 in neuroblastoma cells as shown in Figure 3.14.

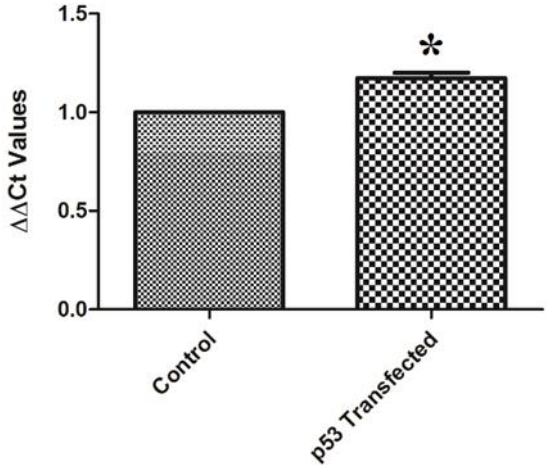

Figure 3.14 : ΔΔCt values of mRNA expression levels of *KATNA1* gene control and all p53 transfected SH-SY5Y neuroblastoma cells.

Consequently, p53 transcriptionally bind to *KATNA1* gene and p53 activation 1.17 fold increased. The experimental results were statictically analyzed by two-tailed paired

student's ttest using Graphpad Prim 5 software. Error bars in the graphs were generated using + SD values. P value under 0.01 was considered very significant.

3.6 Western blot analysis of p60-katanin and p53

The protein level of p60-katanin and p53 were analyzed by Western blotting. p53 transfected neuroblastoma cells and non-transfected neuroblastoma cells were used, one. After total protein was isolated, p53 expression via flag-p53 and p60-katanin level were identified by Western blotting.

Figure 3.15 The level of p53 expression

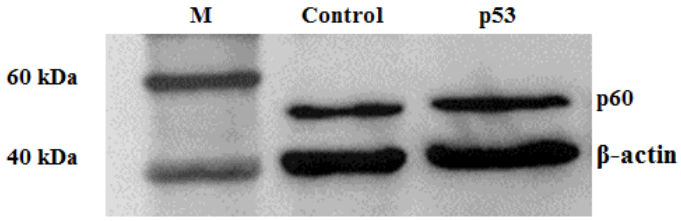

Figure 3.16 The level of p60 expression

p53 expression was detected with specific flag antibody as shown in figure 3.15. Moreover, p60- katanin and p53 expressions were also detected specific antibody as shown in figure 3.16. Besides, β-actin was used for normalization of band density. p60- katanin has 60 kDa molecular weight, p53 has 53 kDa molecular weight and β-actin has 41,7 kDa molecular weight. Experiments were repeated four times on separate days. One of membrane image was shown in figure 3.15.

We repeated four times of western blotting on separate days. After experiments, western blot images were analyzed by Photoshop software in terms of density of bands and results were normalized against β-actin. Then, paired t-test was applied and two-tailed p-value obtained 0.0494. According to western data, we can conclude p53 expression level significant statistically compared to control.

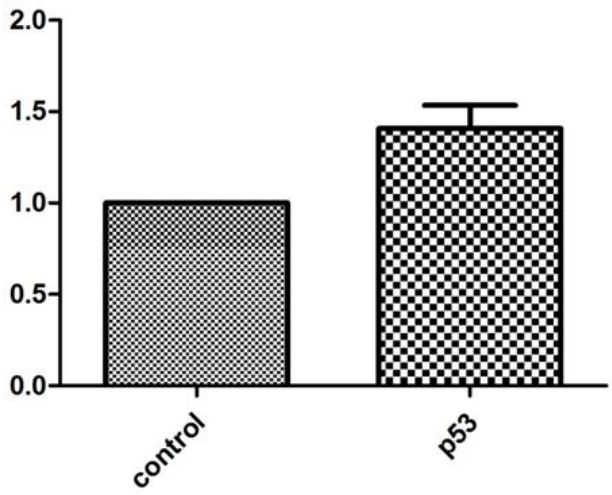

Figure 3.17 : p53 fold activation

Results of experiment demonstrated that p53 expression almost 1.5 fold increase than control cells. p53 can be activator or repressor at the transcriptional level depend on the target gene. This may be related with sequence motif, cell type and binding efficiency **(**Wang B., Xiao Z and Ren E.C., 2009). Our experiment results show that p53 has as an activator role on regulation of *KATNA1* gene expression.

We found that p60-katanin UTR region has strong transcriptional activation potential. The reason of enhanced transcriptonal activity may be presence of CpG islands which regulates expression through DNA methylation. In addition to this, UTR has IRES (Internal ribosome binding site) region. In 5'UTR, IRES complex structure can attenuate translation initiation and it can cause increasing protein synthesis.

4. CONCLUSION

This study is the first report on regulation of *KATNA1* gene promoters by p53 transcription factor. Firstly, we found that 5'-UTR region enhanced transcriptional activity of the reporter gene, but not putative promoter region and the presence of intron decreased the activity. The increased transcriptional activity of 5' UTR region may due to the presence of CpG island and CpG island is crucial for transcriptional regulation. We know the importance of CpG islands on the promoter regions, which would drive the attention to the CpG island methylation as an important mechanism for gene expression. Moreover, 5'UTR region has IRES site and it is crucial for the translation initiation and regulation of protein expression level.

Then, we showed that p53 transcription factor binding to p60-katanin promoter using ChIP and EMSA.

After confirming p53 binding, transcriptional effect of p53 was examined by qRT-PCR in RNA level using SH-SY5Y cells. qRT-PCR results showed that p53 binding to KATNA1 gene promoter activates gene expression and as a result of this upon p53 overexpression 1.17 fold increase was detected in mRNA level.

Finally, we analyzed the protein level of *KATNA1* gene by western blot and results showed that upon p53 overexpression, katanin-p60 protein level was elevated about 1.5 fold comparing to control cells, which were not transfected with p53 expression vector.

In conclusion, we have shown that p53 has an activator role on p60-katanin as transcriptionally and translationally. p53's role on the transcriptional and translational regulation of p60-katanin needs further investigation in order to understand the p53's function on p60-katanin in differentiation and proliferation better. Besides, referring to CpG islands on the 5'UTR regions, methylation studies may be enlightening to understand epigenetic regulation. As p53 and katanin-p60 are both related with cell cycle and differentiation functional studies such as directing SH-SY5Y cells to differentiation and siRNA mediated p53 silencing or nutlin mediated p53 accumulation can be elucidative for co-operation of these proteins.

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APPENDICES

APPENDIX A : Core motif of p60-katanin

gttgagacgttacaagactttaagcccatgaaacattctatttcgtgaccactgtatacccagtgccaacactgtctcacac
atagtagttgctgagtaaacatttgttgactgaatggatgtatttcccctgaattgcttctacatgtctatgttagatgactgggg
tgcttttatttttttagagatagggcctcactttgtctcccagactggagtgcaatggcgccatcatggcccactgcagcctcc
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cgttttaaaggaatgtgtgagaagtgcctattctgcctcaagtgatggcagagggtggagattgagactggaggaagcc
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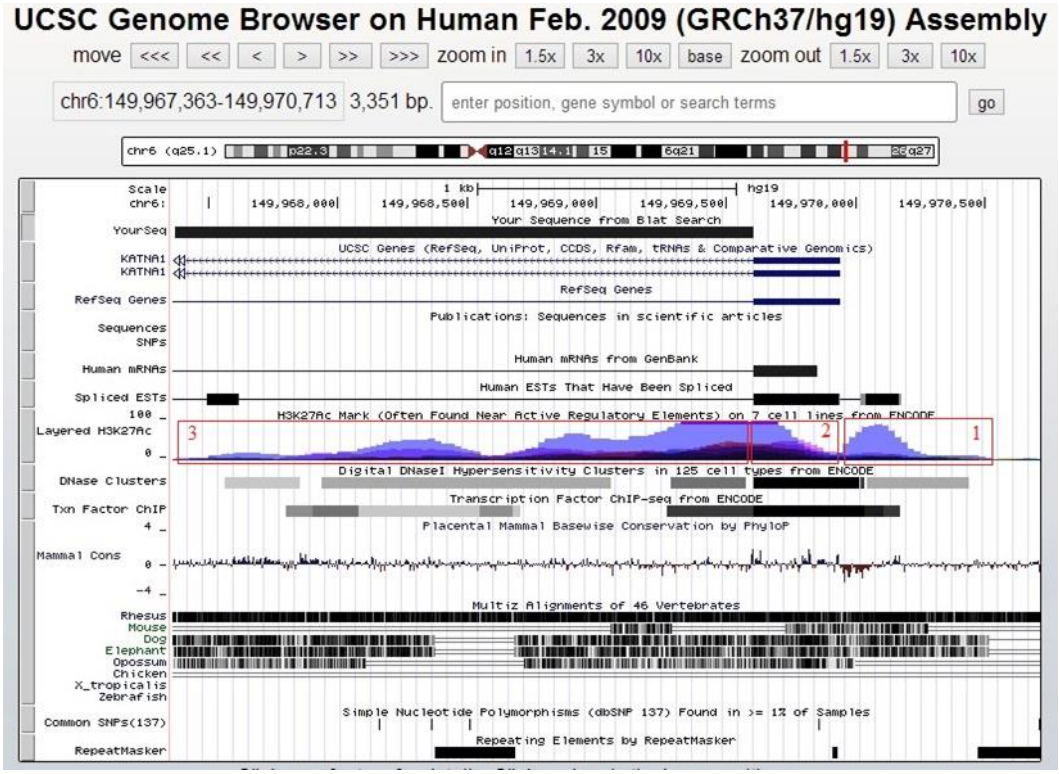
ataaaatcccactagcagtgtgtccgagaatctttgtttacctcctccctattgttatgtcatgacatggagcccagctggg
gaattctgaatactaggttctaaaacaggatttgtcataaacgaattgcttgatcctggacattcacctctaaaatagacacct
gacctcggtgtgtgtgtgagtgtggtgaa**aatcaagtgggagggaaagtgagcaagcac**ctgaacaaggtacagag
gcgcgcgaggtagtaccataataccatctacaccaaggtctatactatccctggtctccgctgcgtccgccgggaggagg
cggtgcaaga

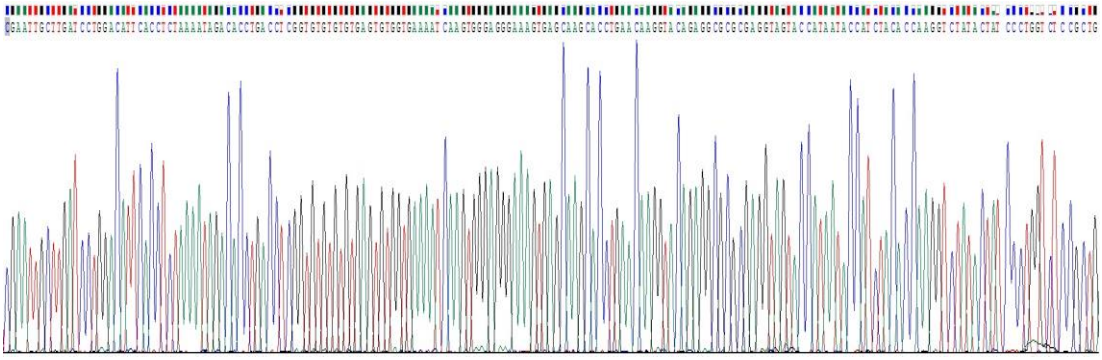
p53 RE in p60-katanin promoter is shown in below.

5'-RRRCWWGYYY-3'	spacer	5'-RRRCWWGYYY-3'
aatcaagtgg	gagggaaagt	gagcaagcac

APPENDIX B : p60-katanin regions from ENCODE



APPENDIX C : Sequence analysis of p53 positive PCR amplification



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