ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

IDENTIFICATION OF INTERACTION BETWEEN SEPTIN3 AND p60-KATANIN (KATNA1) PROTEINS

M. Sc. THESIS

Burcu SUCU

Department of Molecular Biology and Genetics

Molecular Biology - Genetics and Biotechnology Programme

NOVEMBER 2017

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

IDENTIFICATION OF INTERACTION BETWEEN SEPTIN3 AND p60-KATANIN (KATNA1) PROTEINS

M. Sc. THESIS

Burcu SUCU

Department of Molecular Biology and Genetics

Molecular Biology - Genetics and Biotechnology Programme

Thesis Advisor: Prof. Dr. Arzu KARABAY KORKMAZ

NOVEMBER 2017

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

SEPTIN3 VE p60-KATANIN (KATNA1) PROTEİNLERİNİN ETKİLEŞİMİNİN AYDINLATILMASI

YÜKSEK LİSANS TEZİ

Burcu SUCU
(521141103)

Moleküler Biyoloji ve Genetik Anabilim Dalı

Moleküler Biyoloji-Genetik ve Biyoteknoloji Programı

Tez Danışmanı: Prof. Dr. Arzu KARABAY KORKMAZ

KASIM 2017

Burcu SUCU, a M. Sc. student of ITU Graduate School of Science, Engineering and Technology student ID 521141103, successfully defended the thesis entitled "IDENTIFICATION OF INTERACTION BETWEEN SEPTIN3 AND p60-KATANIN (KATNA1) PROTEINS", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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

Jury Members : **Assist. Prof. Dr. Aslı KUMBASAR** .....
İstanbul Technical University

Assoc. Prof. Dr. Nurhan ÖZLÜ .....
Koç University

Date of Submission : 5 May 2017
Date of Defense : 10 November 2017

To my mother,

FOREWORD

This study would not have been completed without help and support of many individuals. I would like to thank everyone who has helped me along this way.

First and foremost, I would like to gratefully acknowledge to my advisor Prof. Dr. Arzu KARABAY KORKMAZ for providing an opportunity to carry out this project and especially for her patience, help and directions that gave me needful guidance to complete the research and write this thesis. It was a great pleasure for me to study under her supervision.

I am especially grateful to all members of CYTO group; especially to Koray KIRIMTAY and Ece SELÇUK ŞAHİN for their technical support and advice during the whole time of this study; Dolunay KELLE, Benan TEMİZCİ, Şeyma AKARSU, Didem BARAN, Ilgın IŞILTAN, Mehtap KAYA and Yağız AKDAŞ for their cooperation, critical feedback, their support and friendliness.

I also would like to thank my dear friends; Sinem SARITAŞ, Nisan CAN, Tuğba KIZILBOĞA, Ezgi BAŞTÜRK, Betül KUTAY, Gökçe CESUR, Aslı KORKMAZ, Tuba BARUT, Özge TATLI and all other MOBGAM members for their support and friendliness.

I acknowledge that this study was supported by The Scientific and Technological Research Council of Turkey (TÜBİTAK) – Project # 114Z244 to Arzu KARABAY KORKMAZ.

Last but not the least, I would like to express my deepest gratitude to my family for their endless love, confidence, unconditional support and understanding throughout my life.

May 2017 Burcu SUCU

TABLE OF CONTENTS

Page

ABBREVIATIONS

AAA : ATPases Associated with diverse cellular Activities
ADP : Adenosine diphosphate
APS : Ammonium persulfate
ATP : Adenosine triphosphate
BCA : Bicinchoninic acid
bp : Base pair
BSA : Bovine Serum Albumin
CC : Coiled-coil domain
cDNA : Complementary DNA
CST : Cell Signaling Technology
co-IP : Co-immunoprecipitation
DAPI : 4',6-Diamidino-2-phenylindole dihydrochloride
dH_2O : Distilled water
DIV : Day *in vitro*
DMEM : Dulbecco's Modified Eagle Medium
DMSO : Dimethyl sulfoxide
DNA : Deoxyribonucleic acid
DNase : Deoxyribonuclease
dNTP : Deoxyribonucleotide
E18 : Embryonic day 18
EDTA : Ethylenediaminetetraacetic acid
FBS : Fetal Bovine Serum
FL : Full length
GBP : Guanosine triphosphate-binding Domain
GC : Guanine and Cytosine
GMPCPP : Guanosine-5'-[(α,β)-methyleno]triphosphate
GTP : Guanosine triphosphate
HBSS : Hank's Balanced Salt Solution
HEPES : 2-[4-(2-hydroxyethyl)piperazine]ethanesulfonic acid
HCl : Hydrochloric acid
HNA : Hereditary neuralgic amyotrophy
ICC : Immunocytochemistry
IgG : Immunoglobulin G
IP : Immunoprecipitation
LB : Luria-Bertani
MAP : Microtubule Associated Protein
MDCK : Madin Darby Canine Kidney
MN : Macherey-Naggel
mRNA : Messenger Ribonucleic Acid
MT : Microtubule
NaCl : Sodium chloride
NEB : New England Biolabs
NEAA : Non-essential amino acid
NP40 : Nonyl phenoxypolyethoxylethanol

NTPase : Nonstandard nucleotide triphosphate pyrophosphatase
P-loop : Phosphate-binding loop
PAGE : Polyacrylamide gel electrophoresis
PB : Polybasic Domain
PBS : Phosphate buffered saline
PCR : Polymerase Chain Reaction
pH : Power of Hydrogen
PKG : Protein Kinase G
PRD : Proline Rich Domain
RNA : Ribonucleic Acid
RNase : Ribonuclease
rpm : Recolutions per minute
SDS : Sodium dodecyl sulfate
SUD : Septin Unique Domain
TAE : Tris-acetate-EDTA
TBS : Tris buffered saline
TBST : Tris buffered saline, 0.1% (v/v) Tween® 20
TEMED : Tetramethylethylenediamine
Tm : Melting Temperature (annealing temperature)
UV : Ultraviolet
WT : Wild-type
Y2H : Yeast two-hybrid

SYMBOLS

A	**:** Ampere
α	**:** Alpha
β	**:** Beta
cm^2	**:** Square centimeter
g	**:** Gram
kDa	**:** Kilo Dalton
L	**:** Liter
M	**:** Molar
mA	**:** Milliampere
mg	**:** Milligram
mL	**:** Milliliter
mM	**:** Millimolar
mm	**:** Millimeter
nm	**:** Nanometer
V	**:** Volt
µL	**:** Microliter
µM	**:** Micromolar
µg	**:** Microgram

LIST OF TABLES

Page

LIST OF FIGURES

Page

IDENTIFICATION OF THE INTERACTION BETWEEN SEPTIN3 AND p60-KATANIN (KATNA1) PROTEINS

SUMMARY

p60-katanin, the small subunit of katanin protein, is an important protein regulating microtubule dynamics of mitotic cells during mitosis and neuronal branching in post-mitotic cells. The C-terminal AAA (ATPases Associated with diverse cellular Activities) domain containing ATPase activity carries microtubule severing function and causes formation of short microtubule pieces from long microtubules. Microtubule severing is an important cellular event which enables rapid re-organization of microtubule network during cell division in the cells. Besides, microtubule severing takes important roles in the formation and branching of neuronal processes such as axons and dendrites.

Although p60-katanin is a protein widely worked on, neither its functioning mechanisms nor its regulatory partners are clearly determined. There are a few proteins identified so far that physically interact with p60-katanin. One of these proteins is the other subunit of katanin: p80-katanin. These two subunits exist as a heterodimer in cells.

In our laboratory, p60-katanin interacting proteins were identified by Yeast Two Hybrid screening system. Human brain cDNA library was screened and twenty-two candidate proteins were identified. Septin3, present in pre-synaptic termini, having roles in synapse formation, has been chosen in this study.

Septin 3 is a novel member of the Septin subfamily of GTPase domain proteins. The septin family consists of multiple genes and protein isoforms; in mammals, 13 septin genes encode for over 30 protein isoforms. Mostly all Septin proteins are expressed in many tissues, but Septin3 appears to be primarily expressed in brain. Septins are abundant in the central nervous system and associated with many neurological diseases such as Parkinson's, Alzheimer's, schizophrenia, and hereditary neuralgic amyotrophy.

In this study, in order to analyze the interaction between p60-katanin and Septin3, co-immunoprecipitation assays were performed using rat brain lysate, and it was found that p60-katanin interacts with Septin3.

Then, immunocytochemistry (ICC) was performed to monitor localization of endogenous Septin3 and p60-katanin proteins. Based on the ICC results, Septin3 mainly localizes along axons and p60-katanin mostly resides in cell bodies. These two proteins have been observed as co-localized along axons and cell body.

In order to observe filament formation of Septin3, wild-type Septin3 construct was overexpressed in RFL6 cells, for better visualization due to its big and flat nature, and they were subjected to ICC. Wild-type Septin3 protein formed form brush-like filaments. Since the GTPase activity of septin proteins is important for filament formation, two critical amino acids, Lys208 and Arg280, for the GTPase activity of Septin3 were mutated both separately and together by site-directed mutagenesis. A mutation on Arg280 showed greater inhibitory effect on the GTPase activity of Septin3 than a mutation on Lys208. The lowest GTPase activity among Septin3

mutants were detected when both amino acids were mutated. Double mutant Septin3 construct was overexpressed in RFL6 cells and subjected to ICC. It was found that double mutant Septin3 protein was observed as being punctiform and accumulating on the cell membranes where they co-localized with microtubules.

Septin3 wild-type and double mutant constructs were overexpressed in RFL6 cells together with p60-katanin and the cells were subjected to ICC. Wild-type Septin3 and wild-type p60-katanin co-transfected cells were observed to be smaller and to have disorganized microtubule structure than the cells expressing only Septin3. In addition, short Septin3 filaments were observed to be localized around p60-katanin proteins, whereas brush-like Septin3 filaments were observed in the cells expressing only Septin3. Double mutant Septin3 and wild-type p60-katanin co-transfected RFL6 cells have been observed to lack the ability to form septin filaments and they were overlaying with microtubules which seemed to have lost their regular meshwork.

In order to identify the interaction domains of p60-katanin and Septin3, deletion constructs of p60-katanin and Septin3 were generated by cloning rat *p60-katanin* fragments into FLAG-tagged mammalian expression vector and by cloning rat *Septin3* fragments into Myc/His-tagged mammalian expression vector. Then the constructs were overexpressed in human colon carcinoma cell line, due to its easily transfectable nature, and the total protein were subjected to co-immunoprecipitation (co-IP) assays. According to co-IP result, p60-katanin strongly binds to Septin3 via when it has ATPase domain at C-terminal end. In addition, Septin3 binds to p60-katanin through its GTPase domain in between 67^{th} and 217^{th} amino acids.

SEPTIN3 VE p60-KATANIN (KATNA1) PROTEİNLERİNİN ETKİLEŞİMİNİN AYDINLATILMASI

ÖZET

p60-katanin, katanin proteininin küçük alt birimi olup üzerinde çok çalışılan, bölünebilen hücrelerde mitoz sırasında mikrotubul dinamiğini etkileyen ve nöronlarda özellikle nöronal dallanma oluşumu açısından önemli bir proteindir. C-ucunda bulunan AAA bölgesi proteine ATPaz özelliği kazandırmakta, dolayısıyla p60-katanin mikrotubulleri kesmek suretiyle büyük parçalardan küçük mikrotubul parçaları oluşumunu sağlamaktadır. Mikrotubullerin bu şekilde kesilmesi hem bölünen hücrelerde hem de bölünmeyen hücrelerde yani nöronlarda akson ve dendrit gibi proseslerin dallanmalarında önemli rol oynamaktadır.

Üzerinde çok çalışılan önemli bir protein olmasına rağmen p60-katanin'in hangi proteinlerce bu görevi düzenlendiği tam olarak aydınlatılamamıştır. p60-katanin'in bilinen regülatörlerinden birisi katanin'in diğer alt birimi olan p80-katanin'dir. Hücre içinde p60-katanin ile p80-katanin heterodimer oluşturmaktadır, böylece p60-katanin ATPaz aktivitesi sayesinde mikrotubulleri kesebilmektedir.

Laboratuvarımızda yüksek lisans tezi olarak gerçekleştirilen p60-katanin ile etkileşen proteinleri aydınlatmak için kullanılan Y2H (maya ikili hibrid) tarama sistemi ile insan beyin cDNA kütüphanesi taranmış ve p60-katanin ile 22 proteinin etkileşimi aydınlatılmıştır. p60-katanin ile etkileştiği tespit edilen bu proteinlerden Septin3, presinaptik terminallerde bulunması, sinaps formasyonundaki rolleri, akson hedeflenmesindeki ve nöronal diferansiyasyondaki etkileri göz önünde bulundurularak bu çalışmada incelenmek üzere seçilmiştir.

Septin3, Septin ailesinin bir üyesi olup GTPaz özelliğine sahip bir proteindir. Memelilerde 13 adet Septin geni 30'dan fazla Septin izoformunu kodlar. Septinler hücre içinde hetero-oligomerler ya da homo-oligomerler halinde bulunur. Oligomer oluşumu sırasında da GTP hidrolizi sonucu açığa çıkan enerjiyi kullanırlar. Bu açıdan GTPaz aktivitesi septinler için önem taşımaktadır. Septinlerin GTPaz aktivitesini etkileyen birçok amino asit bulunmaktadır. Bu amino asitlerin GTPaz aktivitesi üzerine etkileri Septin alt sınıfları arasında farklılık göstermektedir. Septin proteinlerinin çoğu birçok dokuda eksprese edilmektedir. Biyokimyasal saflaştırma çalışmaları göstermiştir ki, septinler hetero-oligomer oluşumu sırasında eksprese edildikleri hücreye göre farklılık göstermektedir. Septinler, gen sekanslarındaki benzerliğe göre SEPT2, SEPT6, SEPT7 ve SEPT3 olmak üzere dört alt sınıfa ayrılmışlardır. Bu açıdan oluşan septin filamentleri; SEPT2/6/7/3 olmak üzere belirli bir kombinasyon doğrultusunda oluşmaktadır.

Septin3 özellikle beyinde eksprese edilmektedir. Diğer septinler gibi hetero-oligomer oluşumuna katılmaktadır ve hetero-oligomerin uçlarında yer almaktadır. Bunun yanı sıra, bazı septinlerden farklı olarak homo-dimer de oluşturmaktadır.

Septinlerin birçok hastalık ile ilişkileri ortaya koyulmuştur. Özellikle merkezi sinir sisteminde yoğun olarak bulunan Septinlerin Parkinson, Alzheimer, şizofreni ve kalıtsal nörolojik amtropy gibi hastalıklarla ilişkili olduğu gösterilmiştir.

Bu çalışmada, Septin3 ile p60-katanin'in etkileşimini aydınlatabilmek için öncelikle sıçan beyin lizatının kullanıldığı ko-immünopresipitasyon (ko-IP) yöntemiyle

Septin3 - p60-katanin etkileşimi incelenmiştir. Ko-immünopresipitasyon sonucunda, Septin3 ve p60-katanin proteinleri birbirlerine bağlanmışlardır. Sadece boncuk ve spesifik olmayan IgG kontrollerinde de bağlanma gözlemlenmemesi, etkileşimin spesifik olarak Septin3 ve p60-katanin arasında gerçekleştiğini ortaya koymaktadır. Sonuç olarak, Septin3 ve p60-katanin sıçan beyninde etkileşmektedir. Ardından immünositokimya deneyleri ile Septin3 ve p60-katanin proteinlerinin lokalizasyonu primer kortikal nöronlarda gözlemlenmiştir. İmmünositokimya deneylerinde endojen Septin3 ve p60-katanin proteinleri spesifik antikorlar kullanılarak görselleştirilmiştir. Nöron görüntülerine göre Septin3 proteinleri literatürde de belirtildiği gibi aksonlarda gözlemlenmiştir. Septin3 ve p60-katanin proteinleri özellikle nöronların gövdesinde ko-lokalize olarak bulunmaktadır. Ardından, Septin3 proteini, büyük hücre yapısına sahip olan ve görüntülenmesi açısından sağlayacağı avantaj düşünülerek RFL6 hücrelerinde fazla-eksprese ettirilmiş ve immünositokimya deneyleri ile Septin3'ün hücre içindeki formasyonu gözlemlenmiştir. Septin3 proteini, literatürde bazı septinler için belirtildiği gibi filament yapı oluşturmuştur.

Septinlerin filament oluşumunda GTPaz aktivitesi önemlidir. Bu açıdan Septin3 proteininin GTPaz aktivitesi için kritik olan iki amino asit, lizin 208 ve arjinin 280, ayrı ayrı ve birlikte mutasyona uğratılarak, mutasyonların Septin3'ün GTPaz aktivitesi üzerine etkisi ölçülmüştür. Arjinin 280'de gerçekleştirilen mutasyon, lizin 208'de gerçekleştirilen mutasyona oranla Septin3'ün GTPaz aktivitesini daha çok düşürmüştür. En düşük GTPaz aktivitesi, her iki amino asidin mutasyona uğratılması sonucu kaydedilmiştir. Bu sonuçlardan yola çıkarak, her iki mutasyonu içeren Septin3 proteini RFL6 hücrelerinde fazla-eksprese ettirilerek immünositokimya deneyleri ile gözlemlenmiştir. Mutant Septin3 proteinleri nokta şeklinde hücre sitoplazmasında ve hücre zarında mikrotubullerle ko-lokalize olarak birikmiş şekilde gözlemlenmiştir.

Ardından, wild-type ve her iki mutasyonu içeren Septin3 konstrakları RFL6 hücresinde p60-katanin ile birlikte fazla-eksprese ettirilmiş ve hücreler immünositokimya analizlerine tabi tutulmuştur. Wild-type Septin3'ün ve p60-katanin'in birlikte eksprese ettirildiği hücreler sadece wild-type Septin3 eksprese eden hücrelere kıyasla daha küçük gözlemlenmiştir. Ayrıca bu hücrelerin düzensiz bir mikrotubul dinamiğine sahip olduğu da görülmüştür. Bunun yanı sıra, Septin3 proteini sadece Septin3 eksprese ettirilen hücrelerde uzun, fırça şeklinde filamentler ya da yoğun Septin3 demetleri şeklinde gözlemlenirken, p60-katanin ile birlikte eksprese ettirildiklerinde hücre içinde kısa Septin3 filamentleri oluştuğu gözlemlenmiştir. Her iki mutasyonu birden içeren Septin3'ün ve p60-katanin'in birlikte fazla-eksprese ettirildiği hücrelerde beklenildiği üzere Septin3'ün filament oluşturamadığı gözlemlenmiştir. Yanı sıra, mikrotubullerin düzensiz ağ yapısına sahip olduğu ve Septin3 proteinleriyle ko-lokalize olduğu gözlemlenmiştir.

Son olarak, Septin3'ün ve p60-katanin'in birbirleriyle etkileşen bölgelerini aydınlatabilmek için p60-katanin'in farklı fonksiyonel bölgelerini içeren gen sekansları FLAG etiketli memeli ekspresyon vektörüne, Septin3'ün farklı fonksiyonel bölgelerini içeren gen sekansları Myc/His etiketli memeli ekspresyon vektörüne aktarılmıştır. Full length Septin3 ile p60-katanin'in konstrakları ve p60-katanin ile Septin3'ün konstrakları olacak şekilde kolay transfeksiyon olabilen HCT116 hücrelerinde fazla-eksprese ettirilmişlerdir. Hücre lizatları toplanmış ve ko-IP analizleri gerçekleştirilmiştir. Ko-IP deneylerinde FLAG etiketli p60-katanin proteinleri FLAG etiketli rezin ile çöktürülmüş, birlikte çöken Myc/his etiketli Septin3 proteinleri ise Myc etiket antikoru ile tespit edilmiştir. Sonuçlara göre, p60-

katanin proteini çoğunlukla Septin3 ile p60-katanin'in ATPaz bölgesi aracılığıyla etkileşmektedir. İlaveten, Septin3 proteini p60-katanin ile GTPaz bölgesi aracılığı ile etkileşime girmektedir.

1. INTRODUCTION

1.1 Components of the Cytoskeleton

A cell maintains its shape through organizing a network of protein filaments. It is necessary for cells to organize themselves by interacting with their environment and also adapting to changing circumstances. Mechanical functions occuring in a cell depends on the system of filaments which are constituted by intermediate filaments, microtubules and actin filaments (microfilaments), all together called the cytoskeleton (Figure 1.1) (Alberts et al., 2014).

All the components of cytoskeleton have dynamic structure and ability of self-assembly. In addition, cytoskeletal filaments are composed of subunits formed as helical assemblies but the differences in the structure of subunits result in variable stability and provide differences in mechanical strength of each type of filament (Lodish et al., 2008). Although they share many properties, they are specialized for different functions in the organization of a cell. Intermediate filaments provide mechanical strength in such a way that forming a strongly twisted cable to hold epithelial cells together or helping neurons to extend their axons, while actin filaments are mainly responsible for determining the shape of a cell's surface by forming cell-surface projections or responsible initiation of cell motility (Mattila and Lappalainen, 2008). On the other hand, microtubules direct intracellular transport by forming tightly aligned bundles which participate in transport of materials (Alberts et al., 2014).

The cytoskeleton network is also supported by a large set of accessory proteins apart from these three main filaments. Those accessory proteins modify dynamic behavior of the filaments as a result of binding either to the filaments or to their free subunits (Cassimeris, 1999).

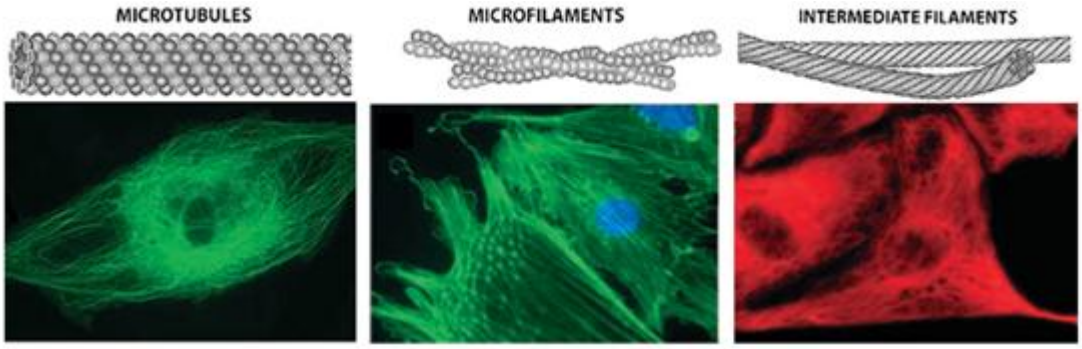


Figure 1.1 : The network of cytoskeletal filaments (Mehrbod and Mofrad, 2011).

1.2 Microtubules

Microtubules are prominent cytoskeletal elements that are formed from αβ-tubulin dimers. The globular α-tubulin and β-tubulin are bound together by non-covalent bonds to constitute a tubulin heterodimer. Although both α and β monomers have the binding sites for one molecule of GTP, the GTP bound to the α subunit is physically trapped at the dimer interphase, so its GTP is never hydrolyzed, while GTP bound to β subunit can be hydrolyzed to GDP (Alberts et al., 2014).

A microtubule is composed of 13 protofilaments which contain αβ-tubulin dimers aligned parallel within a hollow ring structure (Meurer-Grob et al., 2001). The lateral contacts of α-α and β-β together with the longitudinal interaction of α subunit with β subunit in the adjacent heteromer makes the microtubule rigid. However, tubulin conformation can be weakened by inducing with stathmin-like proteins in such a way that stathmin not only binds to free tubulin subunits but also can weaken lateral contacts between tubulin subunits favoring disassembly (Meurer-Grob et al., 2001).

Microtubules have important roles in cell motility, cell growth and cell cycle. During interphase, microtubules constitute mitotic spindle and they are necessary for segregation of chromosomes and specification of the cleavage furrow. In addition, an organization of intracellular compartments depends on the assembly of microtubules, too. In addition to the cell cycle, microtubules are also responsible for cell motility (McNally and Vale, 1993).

Microtubules have dynamic structure such that they polymerize and depolymerize continuously. The state of a microtubule filament can change rapidly from growing to shrinking. The quick transition of a microtubule filament from polymerization to depolymerization process is known as dynamic instability (Figure 1.2) (Desai and

Mitchison, 1997). The rapid growth called as rescue followed by accidental loss called catastrophe of subunits occur at the same end which is GTP-capped plus ends (Conde and Caceres, 2009). Actually, the energy required for catastrophe comes from GTP hydrolysis. The experiments carried out to grow microtubules with the non-hydrolyzable form of GTP, GMPCPP, has shown that microtubules do not undergo dynamic instability upon binding non-hydrolyzable analog of GTP. In other words, the addition of GMPCPP-tubulin prevents depolymerization of microtubules (Mikey and Howard, 1995). Therefore, GTP hydrolysis is required for switching between growth to shrinkage phases (Hyman, Salser, Drechsel, Unwin and Mitchison, 1992).

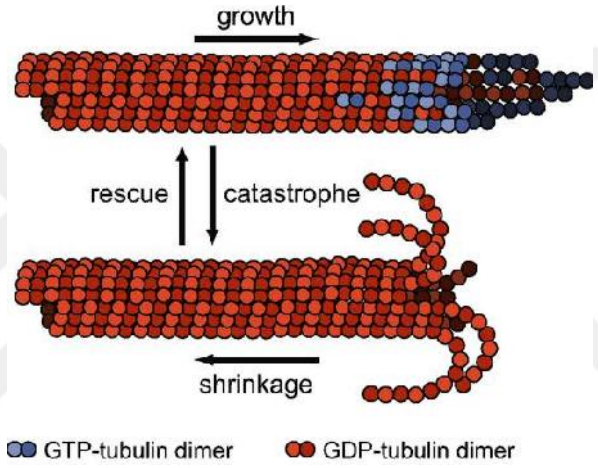


Figure 1.2 : Dynamic instability of microtubules (Bowne-Anderson et al., 2013).

A microtubule filament grows or shrinks in order to meet a specific role in the cytoplasm. At metaphase, the microtubules attached to one side of the kinetochore must grow, whereas those at the other side must shrink at the same time in order to fulfill chromosome alignment at the metaphase plate (Horio and Murata, 2014). Depending on the basis of their function, variable proteins interact with microtubules to control their dynamicity, and they can be classified into two groups as either stabilizing or destabilizing them. Proteins responsible for stabilization of microtubules are called microtubule-associated proteins (MAPs), and they promote the assembly of microtubules by increasing the nucleation rate. Another group of proteins destabilizing microtubules act by promoting catastrophes such as various members of the kinesin family, stathmin, spastin and katanin.

1.3 The Catalytic Subunit of Katanin, 'p60-katanin'

Katanin, an ATP-dependent microtubule-severing protein, was first isolated from *Xenopus laevis* eggs (McNally and Vale, 1993). The protein consists of 60 and 80 kDa polypeptides. p60 has enzymatic activity and it breaks the lattice of the microtubules. Although the function of p80 is not very well understood yet, it has been reported that p80 is not only essential for spindle pole targeting of p60 but also it increases the effectiveness of p60's severing function by binding directly to the N-terminal 29 residues of p60-katanin through its C-terminal con80 domain (conserved among p80 homologs) (McNally K, Bazirgan and McNally F, 2000; O'Donnel et al., 2012). Therefore, p60 is described as the catalytic subunit of katanin, whereas p80 is characterized as the regulatory subunit.

The catalytic subunit of the katanin (p60-katanin) is the best-characterized microtubule-severing protein which is a member of AAA superfamily (ATPase Associated with diverse cellular Activities). The AAA region of p60-katanin found in between 241-382 amino acids has a conserved P-loop that harvests energy released from ATP hydrolysis (Hartman and Vale, 1999; Ghosh, Dasgupta and Guha, 2012). In addition, the two distinct "Walker-A" and "Walker-B" motifs in p60-katanin participate in imparting positional stability of ATP within p60-katanin (Figure 1.3) (Ghosh, Dasgupta and Guha, 2012).

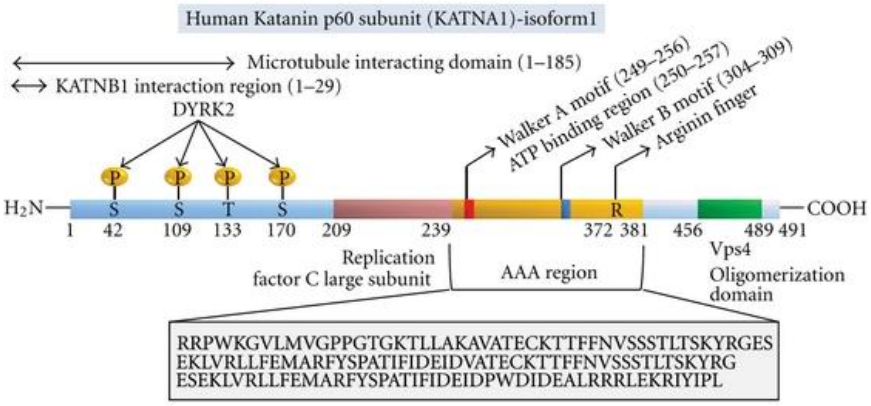


Figure 1.3 : Human katanin p60 subunits: The domains in p60 include replication factor C large subunit, AAA region containing ATP-binding Walker A and Walker B motifs, Vps4 domain (Ghosh, Dasgupta and Guha, 2012).

Katanin is ubiquitously expressed from lower eukaryotes to higher animals and its cell-specific activity arranges the differential formation of microtubule arrays in a

cell. The microtubule fragmentation performed by enzymes such as p60-katanin and spastin has been reported being an important underlying mechanism in cell migration, locomotive organelle formation, cell division and neuronal branching (Baas, Karabay, and Qiang, 2005; Korulu and Karabay, 2011).

1.4 Microtubule Severing Role of p60-katanin

Microtubule severing is primarily induced by p60-katanin that makes breaks in a microtubule array (Quarmby and Lohret, 1999). Inhibition of p60-katanin by function-blocking antibody has shown that new microtubules were nucleated from the centrosome and they were failed to be released upon inhibition of p60-katanin (Ahmad, Yu, McNally and Baas, 1999). As microtubules were not subjected to the severing of p60-katanin due to inhibition, they became longer throughout the cell body (Bass, Karabay, and Qiang, 2005). Microtubules are cut by katanin and run by a mechanism called treadmilling. During treadmilling, tubulin subunits are added to one end and they are also lost from the other end at the same time (Horio and Hotani, 1986).

The driving force for microtubule severing is the ATP hydrolysis by the P-loop of p60-katanin and in turns, it also harvests energy released from ATP hydrolysis (Figure 1.4 and 1.5) (Hartman and Vale, 1999). Physiological status of tubulins affect severing activity of p60-katanin. When the acidic C-terminal segment of either α or β tubulin was removed, the polymerized microtubules became resistant to p60-katanin (Johjima et al., 2015).

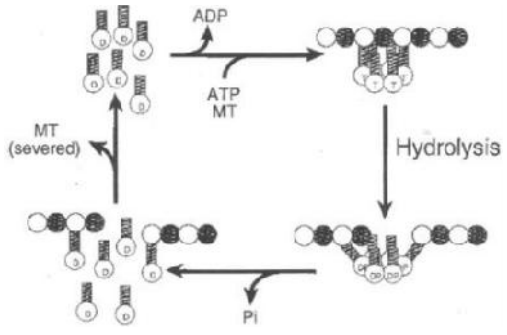


Figure 1.4 : A model for microtubule severing by katanin. A single protofilament shown as T, DP and D represents ATP, ADP+Pi and ADP states, respectively.

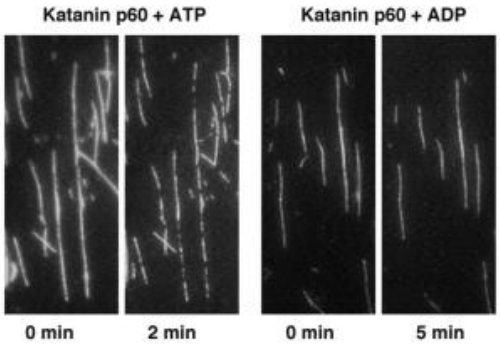


Figure 1.5 : Microtubule-severing activity of p60-katanin in the presence or absence of ATP. Taxol-stabilized rhodamine-labeled bovine microtubules applied onto a microscope slide coated with a mutant kinesin subunit were visualized by fluorescence microscopy (Johjima et al., 2015).

In neurons, microtubules are released from the centrosome after the severing by p60-katanin, then they were relocated into developing processes such as axons and dendrites. The overexpression of p60-katanin results in severe microtubule loss and it is also deleterious to axonal growth in some neurons (Karabay, Yu, Solowska, Baird and Baas, 2004).

1.5 The Discovery of Septins

The discovery of septins has begun with isolation of temperature-sensitive-lethal (ts) mutant *Saccharomyces cerevisiae* which were not able to form colonies due to the defects in cell wall formation (Hartwell, 1967). Therefore, septins have originally been characterized as cell division control proteins of cytokinesis in budding yeast Saccharomyces cerevisiae then, they were named ‘septins’ depending on the basis of localization as rings to the septating bud neck (Longtine et. al., 1996). In 1997, Makoto Kinoshita and his co-workers have presented the first detailed exploration of mammalian septin, SEPT2, which is important for cytokinesis in mammalian cells (Kinoshita M, Tomimoto, Kinoshita A, Kumar and Noda, 1997).

The septin proteins are increasingly recognized as the fourth component of the cytoskeleton because of their filamentous appearance and ability to assemble in bundles. In addition, their association with microtubules and actin filaments, as well as phospholipid membranes, and the effects of these associations on septin filament assembly suggest septins to be a novel component of the cytoskeleton (Mostowy and Cossart, 2012).

1.6 The Septin Family of GTPases

Septins involve an evolutionarily conserved family of GTPases with multiple roles in cytokinesis, vehicle trafficking and cell polarity. The septin family of proteins is broadly conserved among eukaryotes besides plants and protists. They are classified under the GTPase superclass of P-loop NTPases, including Ras GTPases, which contain α-helices, β-sheets and P-loops, as well as GTP-interacting motifs (Figure 1.6) (Weirich, Erzberger and Barral, 2008).

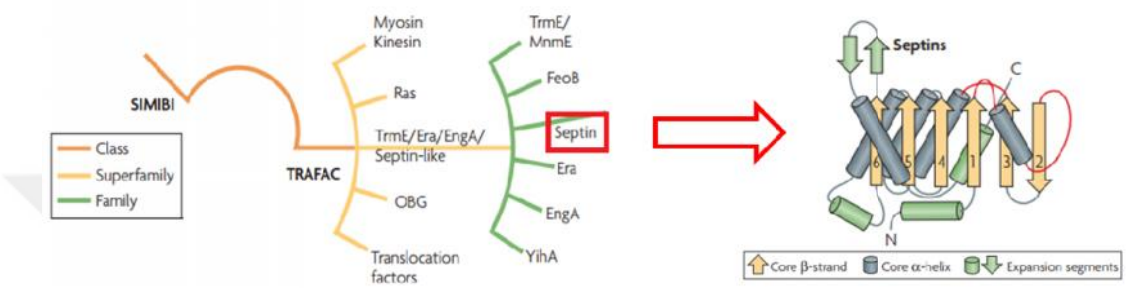


Figure 1.6 : Classification and the secondary structure of septins. Loops are red in the structure (Figure is modified from Weirich, Erzberger and Barral, 2008).

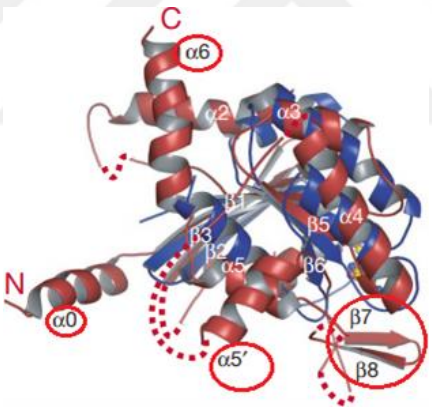


Figure 1.7 : Superimposition of the structures of SEPT2 (in red) and Ras-GppNHp (blue). Four additional elements of SEPT2 are shown in red circles (Sirajuddin et al., 2007).

Monomeric septins contain five α-helices and six β-sheets which is common for GTPase superclass of P-loop NTPases (Sirajuddin et al., 2007). Septins also include four additional elements which are N-terminal α0 and α5'helices, C-terminal α6 helix and antiparallel β7 and β8 strands (Figure 1.7). The GTP-binding pocket of septins (Figure 1.8) includes the G1 motif (Walker A), forming the P-loop that interact with the phosphate residues of GTP, G3 motif (DXXG) which binds Mg^{2+} and coordinates GTP hydrolysis and G4 motif (AKAD) interacts with guanine base of GTP. In addition, G2 motif-Switch I (Thr/Ser) contains threonine residue which is analogous

to the Thr-35 of the G2 sequence of the Ras GTPases (Sirajuddin, Farkasovsky, Zent and Wittinghofer, 2009; Angelis and Spiliotis, 2016).

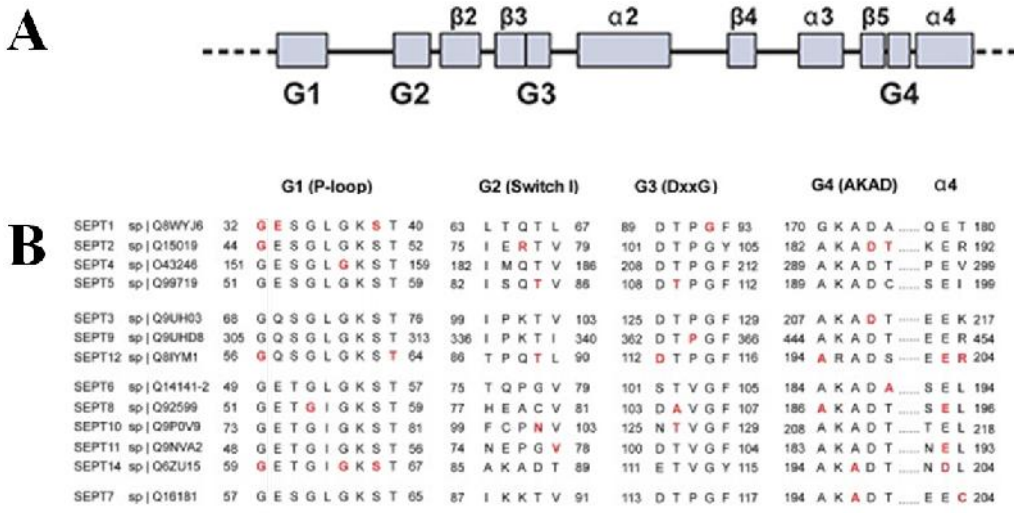


Figure 1.8 : (A) Schematic diagram depicts the secondary structure elements of GTP-binding pocket of septins, (B) Amino acids of septins correspond to the GTP-binding pocket. (Septin residues with missense mutations in cancers are shown in red) (Figure is modified from Angelis and Spiliotis, 2016).

The septins identified in humans consist of thirteen genes which are subdivided into four groups based on sequence similarity; the septin 2 group (SEPT 1,2,4,5), the septin 3 group (SEPT3,9,12), the septin 6 group (SEPT6,8,10,11,14) and the septin7 group (only SEPT7) (Figure 1.9) (Kinoshita, 2003).

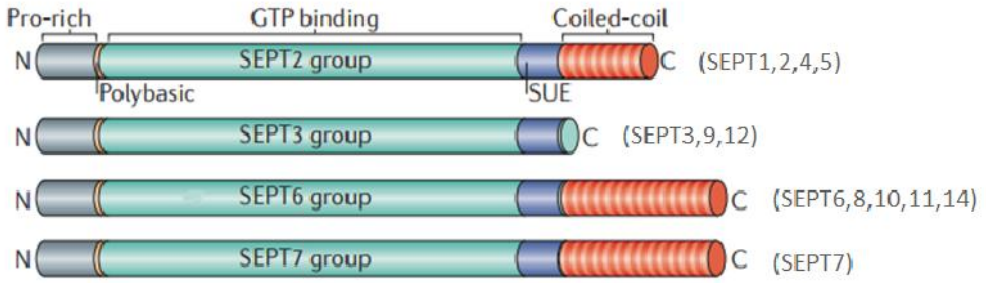


Figure 1.9 : The thirteen human septins are classified into four groups based on sequence similarity. Three conserved domains of human septins are phosphoinositide-binding polybasic domain (Polybasic) in yellow, a GTP binding domain in bluish green and the septin unique element (SUE) in dark blue. The length of the sequence of N-terminal proline rich domain (Pro-rich) in grey and the C-terminal coiled-coiled domain (Coiled-coil) in red. The members of the regarding subgroup are indicated on their left-side in parenthesis (Figure is modified from Mostowy and Cossart, 2012).

The main characteristic of septins, their ability to assemble into filaments, is achieved via the organization of hetero-oligomeric complexes into higher-order

structures. The crystal structures of heterotrimeric human SEPT2/6/7 complex (Figure 1.10) and G dimer of human SEPT2 (Figure 1.12) revealed that filament formation contains conserved interactions between adjacent G (G domain & G domain) interface and NC (N-terminus & C-terminus) interface of the protomer (Sirajuddin et al., 2007). Basically, two copies of heterotrimeric complex form the symmetric heterohexamer, via GTP hydrolysis, which can then form non-polar filaments and high-order structures such as bundles of filaments and rings (Figure 1.10).

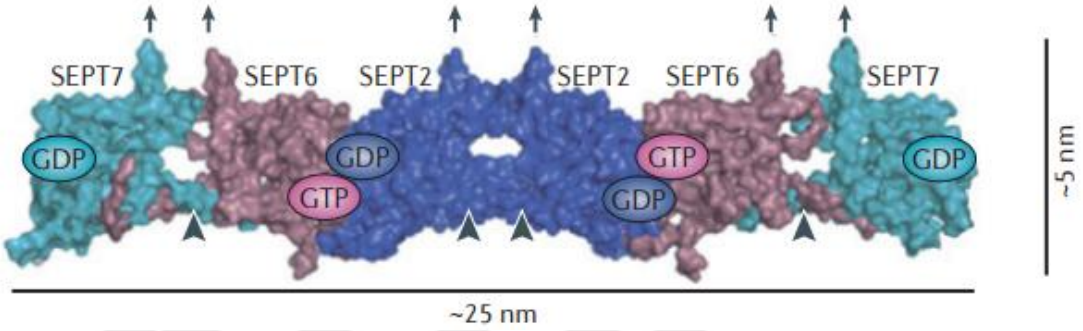


Figure 1.10 : The structure of the SEPT2/6/7 complex; two copies of each septin are symmetrically arranged to generate a hexamer. Septin polybasic regions are indicated by arrowheads. Arrows indicate the position and orientation of the coiled-coil domains (Weirich, Erzberger and Barral, 2008).

Biochemical purification studies of mammalian septin hetero-oligomers have revealed several hexameric complexes which are SEPT2/6/7 (the most studied one), SEPT3/5/7, SEPT7/11/9 and SEPT4/5/8 (Kim, Froese, Estey, and Trimble, 2011). It has also been exhibited that septins are able to form octomeric complexes which are SEPT2/6/7/9, SEPT2/6/7/12, SEPT4/6/7/12 (Sellin, Sandblad, Stenmark and Gullberg, 2011).

Septin complexes have been shown to contain monomers from different septin groups. They form group-specific combinatorial complexes (Sellin, Sandblad, Stenmark and Gullberg, 2011; Kuo et al., 2015). Septins predominantly self-assemble into hetero-hexamers or hetero-octamers in which SEPT3 group of septins, SEPT3,9,12, cap the end of an octamer (Sellin, Stenmark and Gullberg, 2013) (Figure 1.11). Individual septins exist solely in the context of stable six to eight subunit core heteromers, which contain SEPT2, SEPT6, SEPT7 and SEPT3 group septins (Sellin, Sandblad, Stenmark and Gullberg, 2011; Sellin, Stenmark and Gullberg, 2013).

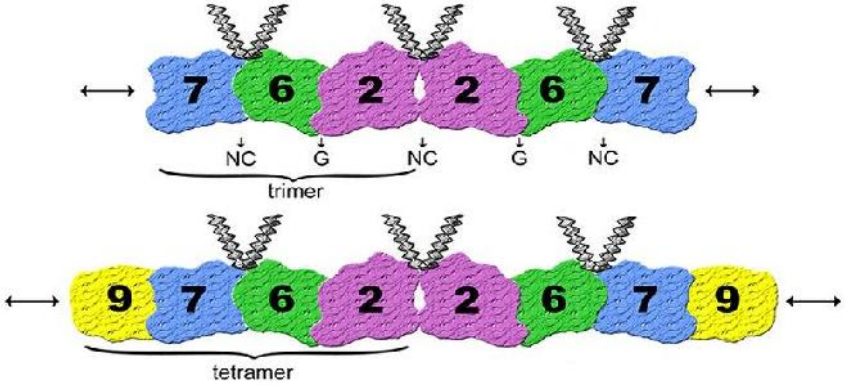


Figure 1.11 : Structure of the SEPT2/6/7 trimer and SEPT2/6/7/9 tetramer. (Two copies of each septin are arranged to generate a hexamer which is at the upper part of the figure and to generate an octamer which is at the bottom part of the figure) (Neubauer and Zieger, 2017).

Septins of the SEPT6 group lack a key threonine residue (T78), which is highly conserved in other septin groups and critical for GTP hydrolysis. In other words, septins belong to SEPT6 group cannot hydrolyze GTP to GDP, so they are constitutively bound to GTP (Sirajuddin, Farkasovsky, Zent and Wittinghofer, 2009). This suggests that GTP hydrolysis plays an important role in the assembly of septin filaments, but it is not essential to form hetero-oligomers (John, Hite and Weirich, 2007; Bertin, McMurray and Grob, 2008; Montagna, Bejerano-Sagie, and Zechmeister, 2014). Since SEPT6 group of septins are unable to hydrolyze GTP, they cannot dimerize. On the other hand, SEPT2 monomers can dimerize via nucleotide-binding domains even they lack C-terminal coiled-coil domain indicating that GTP hydrolysis is important for dimerization, but C-terminal coiled-coil domain is dispensable for dimerization or complex formation (Figure 1.12) (Sirajuddin et. al., 2007). Contrarily, SEPT6 lacking C-terminal coiled-coil domain did not interact with SEPT7 indicating that its coiled coil domain is necessary for the interaction with SEPT7 (Kim, Froese, Estey, and Trimble, 2011). In addition, two independent studies which have performed yeast two-hybrid screening with human septins 1-10 showed that SEPT1,3 and 5 can bind to itself indicating that they are able to form homomeric oligomers (Nakahira et al., 2010; Fujishima, Kiyonari, Kurisu, Hirano and Kengaku, 2007).

The GTPase domain of septins is mainly responsible for polymerization and septin-septin interactions. The crystal structures also showed that not only GTP hydrolysis

influences the stability of hetero-oligomers but also GTP binding to a septin induces change in the loops directly affecting the polymerization state of a complex (Nakahira et al., 2010; Sirajuddin, Farkasovsky, Zent and Wittinghofer, 2009).

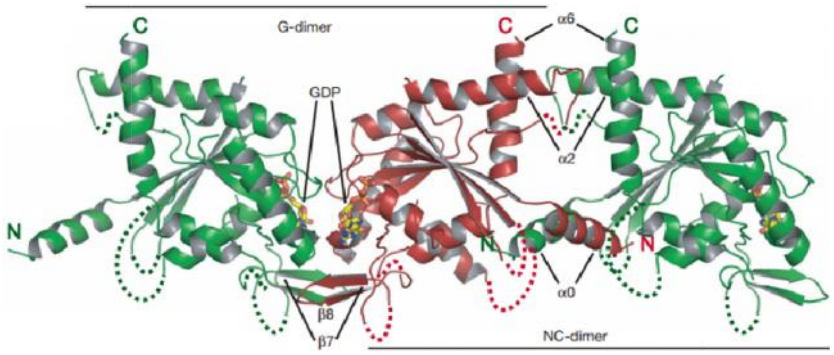


Figure 1.12 : Ribbon representation of two mutant SEPT2 monomers (green) lacking C-terminal coiled-coil domain and one wild-type SEPT2 monomer (red) which dimerize through G and NC interfaces (Sirajuddin et al., 2007).

1.7 Septin Expression and Association with Diseases

Septins have different expression levels in different tissues. Based on the studies providing expression profiles of septins in a variety of tissues, SEPT2,7,9,10 are expressed ubiquitously in different tissues, whereas SEPT3,4,5,8,11 appear to be primarily expressed in cells of central nervous system (Mostowy and Cossart, 2012; Dolat, Hu and Spiliotis, 2014). On the other hand, SEPT1 and 6 were observed at high levels in lymphoid and hematopoietic tissues (Hall, Jung, Hillan, and Russell, 2005). In addition, SEPT12 is specifically expressed in testis, whereas SEPT14 is expressed both in testis and in the nervous system (Peterson et al., 2007). Among all septins, SEPT3 and SEPT5 are expressed primarily in the brain (Beites, Bowser and Trimble, 1999), yet SEPT3 is the only septin classified as neuron-specific. SEPT3 is abundantly expressed in the brain compared to other tissues and its expression increases along with the course of brain development (Xue et al., 2004a; Tsang et al., 2011).

The septin family of proteins is related to a variety of mammalian pathological conditions and diseases as well. SEPT1,2,4 have been shown to be concentrated in the neurofibrillary tangles of Alzheimer's patient brains (Kinoshita A, Kinoshita M, and Akiyama, 1998). SEPT4 has been also implicated in the cell death and formation of Lewy body cytoplasmic inclusions in Parkinson's disease upon co-expression of SEPT4 and α-synuclein (Ihara, Tomimoto, and Kitayama, 2003; Shehadeh, Mitsi,

Adi, Bishopric and Papapetropoulos, 2009). The decreased levels of SEPT7 in the cerebral cortex of fetuses with Down syndrome has suggested the involvement of the septin protein in the cognitive impairment in Down syndrome (Engidawork, Gulesserian, Fountoulakis, and Lubec, 2003). In addition, the mutations in SEPT9 have been identified in hereditary neuralgic amyotrophy, whereas the mutations in SEPT12 have been shown to be related with male infertility (Bai et al., 2013; Cooper, 2005). All the evidence in the literature from tumor formation to neurodegenerative processes clearly suggest the relation of septin mutations in deregulation of variable diseases.

1.8 The Neuronal-Specific Septin, 'Septin3'

SEPT3 is only expressed in neurons and it has only been detected in the brain within twelve examined human tissues. It was enriched in synaptosomes and presynaptic terminals (Xue et al., 2004a; Tsang et al., 2011; 2008). SEPT3 is co-localized with SEPT5, another brain specific septin, and SEPT7 in synapses of hippocampal neurons indicating that SEPT3 may form presynaptic complexes with SEPT5 and SEPT7. Knockdown of SEPT5 or SEPT7 did not change the total number of neurites per cell and double knockout mice (Sept3-/-, Sept5-/-) did not show any significant difference in neurite outgrowth suggesting that septin family of proteins may functionally compensate for loss of the other (Tsang et al., 2011; 2008). Septin3 is phosphorylated on Ser-91 in vitro via cGMP-dependent protein kinase (PKG) in nerve terminals and the phosphorylation of Septin3 might induce translocation from nerve-terminal plasma membrane to the cytoplasm (Xue et al., 2004b). PKG was the only studied binding partner of SEPT3 yet. However, yeast two-hybrid screening of SEPT3 has revealed new binding partners of SEPT3 which are protein inhibitor of activated STAT3 (PIAS3), SMT3 supressor of mif two 3 homolog 1 (SUMO1) and ubiquitin-conjugating enzyme E2I (UBE2I) suggesting that SEPT3 may also be relevant for ubiquitination and sumoylation pathways (Nakahira et al., 2010).

SEPT3 lacks the C-terminal coiled-coil domain (Figure 1.11) and it is able to dimerize in the presence of GTP and Mg^{2+} at low salt concentrations (50 mM NaCl). However, SEPT3 which lacks of the polybasic domain has remained as monomer in the solution suggesting that N-terminal proline-rich domain of SEPT3 may regulate its dimerization (Macedo et al., 2013).

Detailed crystal structure of human SEPT3 has revealed that multiple residues of its nucleotide binding pocket and its septin unique element regulates its oligomerization (Figure 1.12). Ser70 and Ser75 are located in G1 motif and their side chains are oriented to interact with Mg^{2+} ion and α-phosphate respectively. Together with Thr102 which lies in G2-Swith I motif, Ser75 co-ordinates Mg^{2+} ion and make hydogen bonds with β-phosphate. Asp210 and Gly265 bind to the guanine base of GTP, whereas Arg280 resides in SUE domain interacts directly with guanine base and via another Septin3 monomer with ribose moiety of GTP (Macedo et al.,2013).

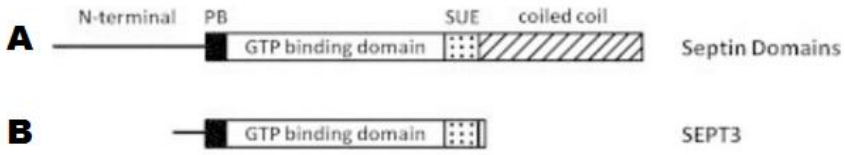


Figure 1.13 : Schematic representations of (A) the typical septin structure and (B) human Septin3, Septin3 contains the highly conserved GTP-binding domain, which is flanked by a variable N-terminal sequence including polybasic domain (PB) interacts with phospholipids. The septin unique element (SUE) is also represented (Macedo et al., 2013).

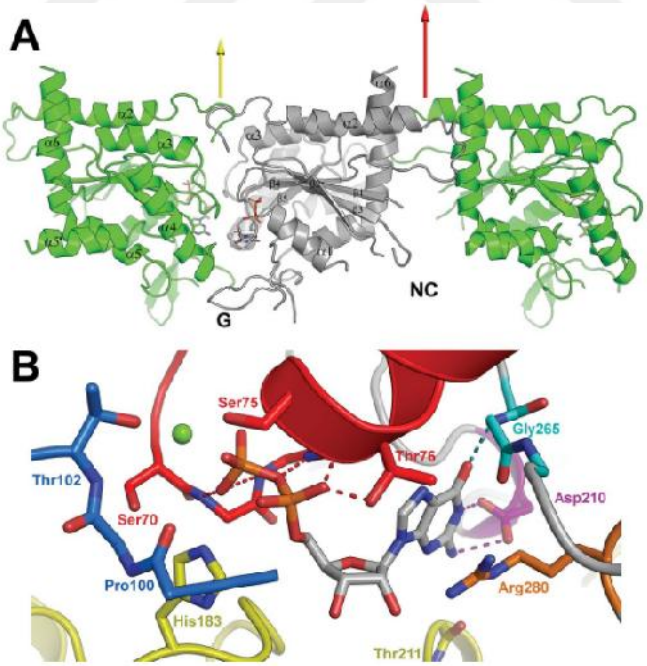


Figure 1.14 : (A) Ribbon representation of SEPT3 monomers, (B) Stick representation of important residues at nucleotide binding pocket of SEPT3. Red arrow in A indicates a crystallographic 2-fold axis and yellow arrow indicates a non-crystallographic 2-fold. Residues from the P-loop and helix α1 are in red, residues from the switch I are in blue, Mg^{2+} ion is in green, Asp210 from the G4 motif (AKAD) is in magenta, Arg280 is in orange, Gly265 is in cyan and His183 and Thr211 from the neighbouring protomer is in yellow (Macedo et al., 2013).

1.9 Relation of Septins and Cytoskeletal Elements

Septins have also important roles in exocytosis, maintenance of cell shape and microtubule-dependent processes. They are generally associated with the sites where the cytoskeleton is rearranged. The co-localization of septins with microtubules has been observed throughout mitosis within a variety of cells (Figure 1.13) (Silverman-Gavrila R, and Silverman-Gavrila L, 2008; Spiliotis 2010). In addition, septins are also involved in dynamic instability of microtubules via an interaction with MAPs. It has been showed that SEPT2 binds and sequesters MAP4, thereby causing inappropriate stabilization of microtubules (Kremer, Haystead, and Macara, 2005).

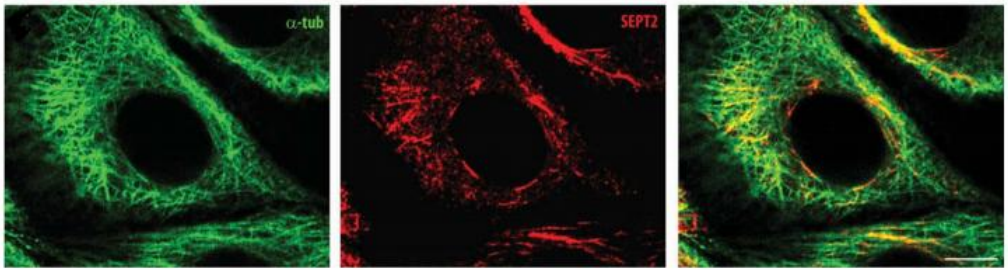


Figure 1.15 : Confocal microscopy image of MDCK cells stained for α-tubulin (green) and SEPT2 (red); SEPT2 on microtubule tracks (Scale bar ≈ 5µm) (Spiliotis, 2010).

Although additional studies are needed to establish the role of septins in the formation of microtubule arrays, septin's deletion and its overexpression have been reported to affect microtubule dynamics in different studies (Kremer, Haystead, and Macara, 2005; Nagata et al., 2003; Amir and Mabjeesh, 2007).

A recent study about the role of septins in microtubule organization gave a different point of view by showing septins spatially guide the organization of microtubules. The septins do not appear to track on microtubule plus ends but they transiently interact with the plus ends of microtubules, as well as, microtubule motor proteins. The interaction possibly mediates the turning of microtubule's plus-end along septin-coated microtubules (Figure 1.14) (Bowen, Hwang, Bai, Roy, and Spiliotis, 2011).

The study group has also found that septins not only affect the stability of microtubules but also the presence of septins influence the direction of microtubule's growth (Figure 1.15) (Bowen, Hwang, Bai, Roy, and Spiliotis, 2011).

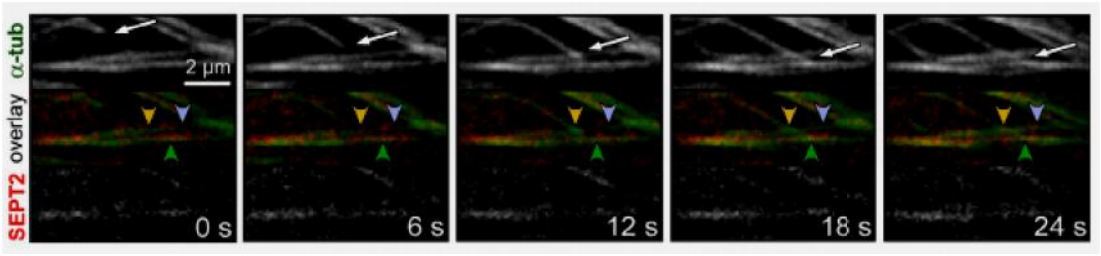


Figure 1.16 : Time-lapse images show docking and coalignment of a microtubule tip with microtubules coated with SEPT2. MT–MT docking, yellow arrowheads; alignment, green arrowheads; a point of no MT overlap, blue arrowheads (Bowen et al., 2011).

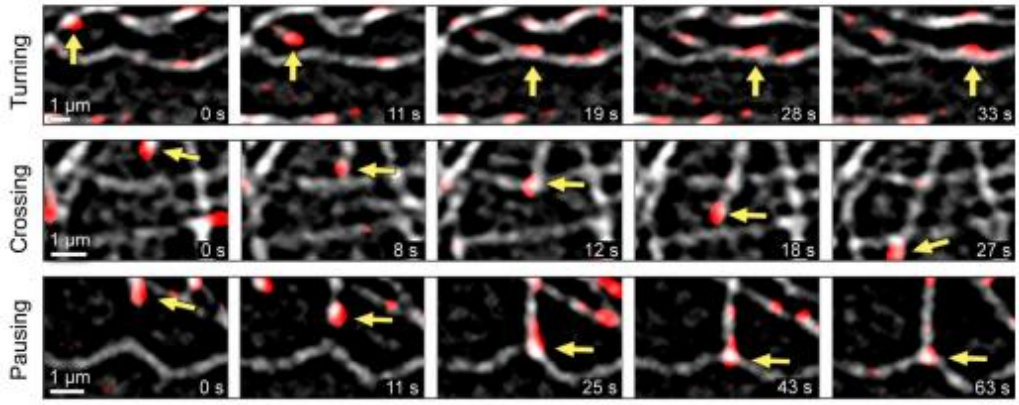


Figure 1.17 : Dual-color time-lapse images of α-tubulin–GFP (grayscale) overlaid with EB1-dsRed (red). Panels depict three types of MT–MT interactions. Yellow arrows point to MT plus-ends moving through the cytoplasm. EB1 is microtubule plus-end binding protein (Bowen et al., 2011).

1.10 Aim of the Study

Septin3 was previously identified as a possible interacting partner of p60-katanin in yeast two hybrid screening performed in our laboratory (Esen, 2008). Since septins assemble into filaments like microtubules which are known to be severed by p60-katanin, possible interaction of Septin3 and p60-katanin raised an intriguing question as to whether Septin3 could be a new substrate of p60-katanin. From this standpoint, aim of this study was to investigate the interaction of Septin3 and p60-katanin in detail. The interaction of Septin3 and p60-katanin was analyzed by co-immunoprecipitation (co-IP) assay and localization of these proteins was visualized by immunocytochemistry (ICC). In order to observe whether Septin3 forms filaments or insoluble aggregates like some other septins, Septin3 was overexpressed in RFL6 cells and visualized by ICC. In order to see if Septin3 and p60-katanin could physically interact, co-IP assay was performed. To find the regions responsible for

the interaction, deletion domains of p60-katanin and Septin3 were tested in co-IP assays.

Since assembly of septins into filaments is based on GTP hydrolysis and critical residues found in GTP binding pocket and septin unique element of Septin3; two critical residues, Lys208 and Arg280, were chosen to be mutated. The effects of these mutations in Septin3 filament formation and p60-katanin interaction were investigated.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bioinformatics tools

During this study, variable bioinformatics tools were used in order to retrieve mRNA sequences of *p60-katanin* and *Septin3*, to control parameters of primers and also to align DNA sequences. The bioinformatics tools utilized during the study are listed in the Table 2.1.

Table 2.1 : Bioinformatics tools.

Bioinformatics Tools	Purpose of Usage	Websites (Url-)
NCBI, National Center for Biotechnology Information	mRNA sequences of *p60-katanin* and *Septin3* were retrieved.	http://www.ncbi.nlm.nih.gov/
IDT DNA Technologies, Oligo Analyzer	The suitability of primers in terms of Tm, GC content and dimers were controlled.	http://eu.idtdna.com/analyzer /Applications/OligoAnalyzer/
NCBI, Primer-BLAST	Any possible non-specific binding of primers to genome was checked.	http://www.ncbi.nlm.nih.gov/tools/primer-blast/
New England Biolabs, Interactive tools, Tm Calculator	Tm (annealing temperature) of primer pairs were determined.	http://www.neb.com/tools-and-resources/interactive-tools
EMBOSS Needle, Pairwise Sequence Alignment, Nucleotide	DNA sequencing results were confirmed by comparing with the sequences in databases.	http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html

2.1.2 Instrument

The instruments used in this study are given in Appendix A.

2.1.3 Equipment

The equipment used in this study is given in Appendix B.

2.1.4 Chemicals

Chemicals used in this study are given in Appendix C.

2.1.5 Buffers and solutions

2.1.5.1 Commercial buffers, solutions and cell culture media

Commercial buffers, solutions and cell culture media purchased from different companies are listed in the Table 2.2.

Table 2.2 : Commercial buffers, solutions and cell culture media.

Buffers and Solutions	Supplier Company
Tris-acetate-EDTA (TAE) Buffer (50X)	Bio-Rad
Acrylamide-bis Solution (40%) (ready to use)	EMD Merck Millipore
Poly-D-Lysine Solution (1.0 mg/mL)	EMD Merck Millipore
Poly-L-Lysine Solution (100 µg/mL)	EMD Merck Millipore
Hank's Balanced Salt Solution (HBSS) (10X)	Gibco Invitrogen
1M HEPES	Gibco Invitrogen
B27 Supplement (50X)	Gibco Invitrogen
GlutaMAX™ (100X)	Gibco Invitrogen
Penicillin-Streptomycin (10K/10K)	Lonza
Trypsin/EDTA	Lonza
Trypsin (2.5%) (w/0 phenol red)	Gibco Invitrogen
Gentamicin® (10 mg/mL)	Gibco Invitrogen
Neurobasal Medium (w/o phenol red) (1X)	Gibco Invitrogen
DMEM High glucose (4.5 g/L) w/L-glutamine (1X)	Lonza
MEM Eagle Non-essential amino acid Solution (100X)	Lonza
Ham's F12 w/L-glutamine (1X)	Lonza

2.1.5.2 Prepared buffers and solutions

Tris buffered saline (TBS) (10X)

10X TBS was prepared by mixing the contents indicated in the Table 2.3. The buffer was diluted to 1X with dH_2O, then its pH was adjusted to 7.6.

Table 2.3 : Contents of TBS (10X).

Content	Concentration	Amount
Tris base	0.4 M	24.3 g
NaCl	1.5 M	88 g
dH_2O	-	up to 1 L

Tris-tricine gel buffer

In order to use during SDS-PAGE, tris-tricine gel buffer was prepared by mixing the contents indicated in the Table 2.4. Then, its pH was adjusted to 8.45.

Table 2.4 : Contents of tris-tricine gel buffer.

Content	Concentration	Amount
Tris base	3 M	182 g
SDS	0.3%	1.5 g
dH_2O	-	up to 500 mL

Tris-tricine anode and cathode buffers

Tris-tricine anode buffer and tris-tricine cathode buffer were prepared as indicated in the Tables 2.5 and 2.6, and their pH values were adjusted to 8.9 and 8.25, respectively.

Table 2.5 : Contents of tris-tricine anode buffer.

Content	Concentration	Amount
Tris base	0.2 M	24.22 g
dH_2O	-	up to 1 L

Table 2.6 : Contents of tris-tricine cathode buffer.

Content	Concentration	Amount
Tris base	0.1 M	12.11 g
Tricine	0.1 M	17.92 g
SDS	0.1 %	1 g
dH_2O	-	up to 1 L

Towbin buffer (1X)

In order to transfer proteins from the SDS gel to a nitrocellulose membrane, towbin buffer (1X) was prepared by mixing the ingredients given in the Table 2.7 and the pH value of the buffer was adjusted to 8.3.

Table 2.7 : Contents of towbin buffer.

Content	Concentration	Amount
Tris base	0.02 M	1.5 g
Glycine	0.01 M	7.2 g
Methanol	20 %	100 mL
dH_2O	-	up to 500 mL

Blocking solutions

Blocking solutions for western blot analysis and immunocytochemistry (ICC) were prepared by mixing the contents given in the Tables 2.8 and 2.9, respectively.

Table 2.8 : Blocking solution for western blot.

Content	Concentration	Amount
Non-fat dry milk	5 %	2.5 g
Tween® 20	0.1 %	50 µL
dH_2O	-	up to 50 mL

Table 2.9 : Blocking solution for ICC.

Content	Concentration	Amount
Bovine serum albumin	3 %	1.5 g
Saponin	0.1 %	50 mg
PBS	1X	up to 50 mL

LB broth and LB agar plates

Luria-Bertani (LB) broth and agar used for the growth of bacteria were prepared by mixing the contents given in the Tables 2.10 and 2.11, respectively. In order to sterilize them, they were autoclaved at 121^0C for 15 minutes prior to use in bacterial cultivation. The LB agar was allowed to cool to 55^0C and ampicillin was added to it at a concentration of either 100 µg/mL or 50 µg/mL prior pouring to petri plates (Isolab).

Table 2.10 : LB broth contents.

Content	Concentration	Amount
Tryptone	1 %	5 g
Yeast extract	0.1 %	2.5 g
NaCl	0.2 M	5.8 g
dH_2O	-	up to 500 mL

Table 2.11 : LB agar contents.

Content	Concentration	Amount
Tryptone	1 %	5 g
Yeast extract	0.5 %	2.5 g
NaCl	0.2 M	5.8 g
Agar	1.5 %	7.5 g
dH_2O	-	up to 500 mL

SDS sample buffer (1.5X)

SDS sample buffer (1.5X) was used for elution of proteins that bound to magnetic beads. The sample buffer was prepared by mixing the contents as indicated in the Table 2.12.

Table 2.12 : Contents of SDS sample buffer (1.5X).

Content	Concentration	Amount
SDS (10% w/v)	3 %	300 µL
Glycerol	15 %	150 µL
Bromophenol blue (10 mg/mL)	0.075 mg/mL	75 µL
0.5 M Tris HCl	93.75 mM	187.5 µL
1.5 M DTT	150 mM	100 µL
dH_2O	-	187.5 µL

NP40 solution

In order to isolate proteins, NP40 solution was prepared by mixing the contents as indicated in the Table 2.13.

Table 2.13 : Contents of NP40 solution.

Content	Concentration	Amount
NP40	1 %	500 µL
Tris base	50 mM	302 mg
NaCl	150 mM	435 mg
1 M EDTA	0.5 M	25 µL
Halt™ Protease Inhibitor Cocktail(100X)	1X	500 µL
dH_2O	-	up to 50 mL

2.1.6 Commercial kits, antibodies and enzymes

The commercial kits and the enzymes used in this study are given in Appendix D.

2.1.7 The mammalian expression vectors

2.1.7.1 p3XFLAG-CMV10 vector

The p3XFLAG-CMV10 (Sigma-Aldrich) is a shuttle vector for transient/stable expression of N-terminal 3XFLAG. The vector is a 6.3 kb derivative of pCMV5 used to establish transient/stable intracellular expression of N-terminal 3XFLAG™ fusion proteins in mammalian cells that encode three adjacent FLAG® epitopes. The vector has kindly been provided by Assist. Prof. Dr. Şirin KORULU KOÇ. The circular map and the polylinker site of p3XFLAG-CMV10 vector is given in Figure 2.1.

2.1.7.2 pcDNA™ 3.1/myc-His A vector

The pcDNA™ 3.1/myc-His A (Invitrogen) which is 5.5kp vector derived from pcDNA™3.1(+) contains a strong promoter for high-level expression in mammalian cells. The vector has C-terminal tag encoding the myc (c-myc) epitope and the following polyhistidine peptide (6X-His) which has kindly been provided by Assist.

Prof. Dr. Aslı KUMBASAR. The circular map and the polylinker site of pcDNA™ 3.1/myc-His A vector is given in Figure 2.2.

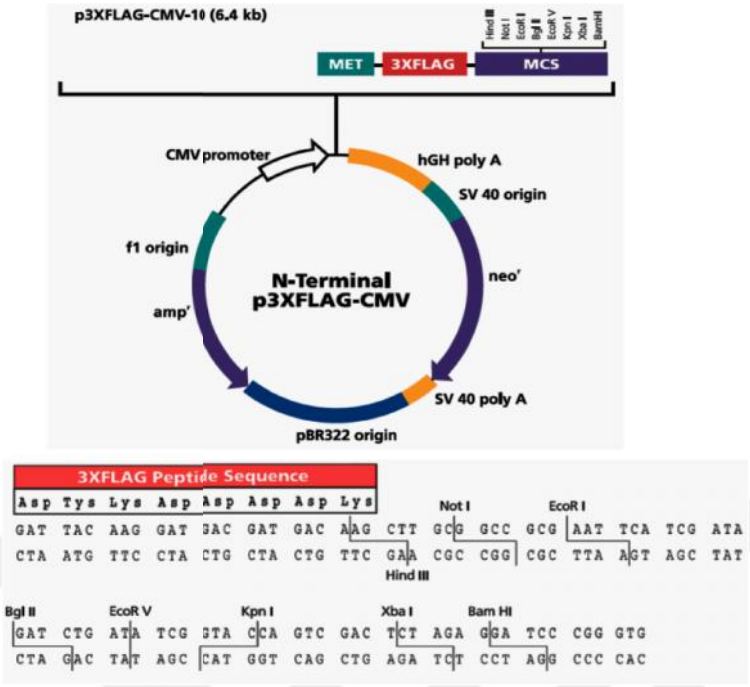
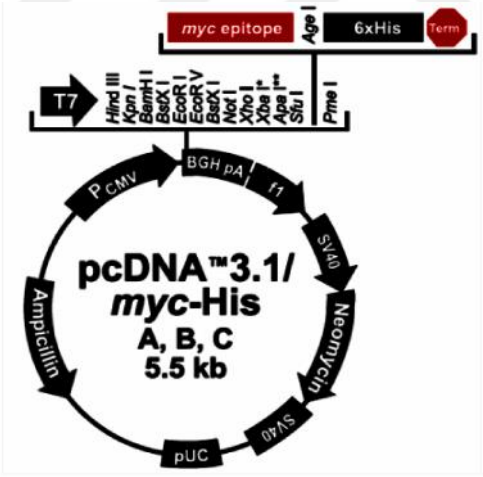


Figure 2.1 : The circular map and the polylinker site of p3XFLAG-CMV10.



pcDNA™3.1/*myc*-His A MCS

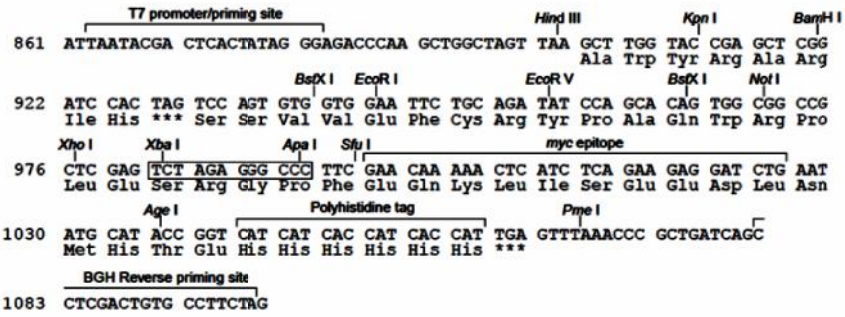


Figure 2.2 : The circular map and the polylinker site of pcDNA™ 3.1/myc-His A.

2.1.8 Mammalian cells and prepared culture media

2.1.8.1 Primary rat cortical neurons

Three different media were used for primary neuron culture in order to meet special purposes. The contents of the media are given in the Tables 2.14, 2.15 and 2.16.

Table 2.14 : Contents of dissection medium.

Contents	Final Concentration	Final Volume
Hank's balanced salt solution (10X)	1X	1 mL
1M HEPES	20 Mm	1 mL
Penicillin-Streptomycin (10K/10K)	-	1 mL
dH_2O	-	up to 100 mL

Table 2.15 : Contents of plating medium (with serum).

Contents	Final Concentration	Final Volume
B-27 supplement (50X)	1X	2 mL
Fetal bovine serum	5% (v/v)	5 mL
45% (w/v) D-glucose solution	-	0.66 mL
GlutaMAX™ (100X)	1X	1 mL
Neurobasal medium (w/o phenol red) (1X)	-	up to 100 mL

Table 2.16 : Contents of serum-free plating medium.

Contents	Final Concentration	Final Volume
B-27 supplement (50X)	1X	2 mL
45% (w/v) D-glucose solution	-	0.66 mL
GlutaMAX™ (100X)	1X	1 mL
Neurobasal medium (w/o phenol red) (1X)	-	up to 100 mL

After the contents of media were mixed well, the media were filtered separately with 0.22 µm pore size filter. All the media were prepared fresh prior to use in culturing neurons.

2.1.8.2 HCT116 cell line

HCT116 cell line (human colon carcinoma cells) has been provided by Prof. Dr. Batu ERMAN. The contents of the culture medium given in the Table 2.17 were mixed and the medium was filtered with 0.22 µm pore size filter.

Table 2.17 : HCT116 complete medium.

Contents	Final Concentration	Final Volume
Penicillin-Streptomycin (10K/10K)	1 % (v/v)	5 mL
Fetal bovine serum	10 % (v/v)	50 mL
DMEM High glucose (4.5g/L) w/L-glutamine (1X)	-	up to 500 mL

2.1.8.3 RFL6 cell line

Rat lung fibroblast (RFL6 cell line) cells derived from the lung tissue of a normal germ-free Sprague-Dawley rat fetus (18 day gestation) were purchased from ATCC (ATCC® CCL-192™). The contents of the culture medium given in the Table 2.18 were mixed and the medium was filtered with 0.22 µm pore size filter.

Table 2.18 : RFL6 complete medium.

Contents	Final Concentration	Final Volume
Penicillin-Streptomycin (10K/10K)	1 % (v/v)	5 mL
Fetal bovine serum	20 % (v/v)	100 mL
Ham's F12 w/L-glutamine (1X)	-	up to 500 mL

2.2 Methods

2.2.1 Co-immunoprecipitation of p60-katanin and Septin3

A previous study done in our laboratory revealed a number of new candidate proteins interacting with p60-katanin. One of these candidates is Septin3 protein which is primarily expressed in brain tissue. Co-immunoprecipitation assays were carried out to confirm the interaction of p60-katanin and Septin3.

2.2.1.1 Isolation of total protein from rat brain

Septin3 is a neuronal-specific septin within Septin family and its expression is only observed in brain tissue (Xue et al., 2004a). Therefore, immunoprecipitation assays were performed with total protein isolated from a rat brain.

100 mg of a rat brain was chopped up with a scalpel and it was placed in an eppendorf tube. The tissue sample was washed twice with 1 mL of PBS (1X) and the tube was centrifuged at 6000 rpm for 5 minutes at 4^0C in order to get rid of the blood. The supernatant was discarded and the pellet was resuspended with 2 mL of NP40 solution prepared as indicated in 2.1.5.2. The volume in the tube was doubled by adding Glass Beads (≤106 µm) (Sigma) and the tube was vortexed vigorously until the tissue sample become dispersed. The tube was incubated on ice for 30 minutes and it was centrifuged at 14000 x g for 10 minutes at 4^0C. The total protein retained in the supernatant was transferred to a new eppendorf tube and its concentration was determined via BCA protein assay using BCA Protein Assay Kit (Pierce™) according to manufacturer's instructions.

2.2.1.2 Immunoprecipitation

In order to perform immunoprecipitation assay, 750 µg total brain lysate was mixed with a 2 µg rabbit polyclonal p60-katanin antibody (KATNA1) (Atlas) (100 ng/µL) in an eppendorf tube. As a negative control of immunoprecipitation assay, 2 µg normal rabbit IgG (CST) (1 µg/µL) was mixed with the same amount of lysate in another eppendorf tube and the tubes were incubated on a rotator at 4^0C overnight.

The next day, 50 µl of protein G magnetic beads (Invitrogen) slurry were incubated with antibody-lysate samples Three different reactions were set as 'IP', 'non-specific IgG' and 'beads only', each containing 50 µL of protein G magnetic beads. The beads were pre-washed with 1 mL of NP40 solution and the supernatants were carefully removed by placing the reaction tubes in a magnetic separation rack. The reaction tubes were separated from the rack and they were mixed with magnetic beads. The tubes were incubated on a rotator for 4 hours at 4^0C. After 4 hours, the tubes were placed in the separation rack and the supernatants were carefully removed. The beads were washed three times each with 1 mL of NP40 solution and the beads were resuspended in 40 µL of 1.5X SDS sample buffer. The beads were boiled for 10 minutes in a heat block set at 95^0C and the tubes were centrifuged at 14000 x g for 3 minutes. The supernatants were used for western blotting. The same protocol was followed for the reciprocal co-immunoprecipitation assay using 2 µg of mouse monoclonal Septin3 antibody (Santa Cruz) (200 ng/µL).

2.2.1.3 Western blotting

In order to perform western blotting, sodium dodecyl sulfate (SDS) polyacrylamide gel was prepared as 4% acrylamide in stacking gel and 10 % acrylamide in separating (resolving) gel. The acrylamide compositions and other contents of SDS gels are given in the Tables 2.19 and 2.20.

Table 2.19 : Contents of separating gel (10% acrylamide).

Content	Concentration	Volume
Acrylamide-bis (40%)	10 %	3.8 mL
Tris-tricine gel Buffer	0.3 %	5 mL
50% (v/v) Glycerol solution	-	1.5 mL
10% (w/v) APS solution	-	150 µL
TEMED	-	15 µL
dH_2O	-	4.7 mL

The separating gel solution was poured into the gel cassette and 1% (w/v) SDS solution was overlayed the separating gel. The gel was left to polymerize for 30 minutes and then the overlay solution was rinsed off. The stacking gel was poured immediately above the separating gel followed by placing a comb (10-well) and then the gel was allowed to polymerize for 45 minutes.

Table 2.20 : Contents of stacking gel (4% acrylamide).

Content	Concentration	Volume
Acrylamide-bis (40%)	4 %	495 µL
Tris-tricine gel Buffer	0.3 %	1 mL
10% (w/v) APS solution	-	50 µL
TEMED	-	5 µL
dH_2O	-	3.4 mL

The polymerized gel was placed in a clamping frame and it was put into the electrophoresis tank. In order to prepare discontinuous buffer system for Tris-Tricine SDS-PAGE, cathode buffer was poured into the clamping frame and anode buffer was placed into the electrophoresis tank.

Immunoprecipitation (IP) samples which were previously mixed with 40 µL of 1.5X SDS sample buffer and boiled were taken from -80^0C and then thawed on ice. The samples were loaded on SDS gel separately and the gel was run at constant amperes (30 mA) until the blue SDS sample buffer totally left the gel.

After about 4 hours, the blue SDS sample buffer was gone off the gel and the gel was separated from plates followed by rinsing with distilled water. As a preparation for protein transfer to a membrane, Trans-Blot® transfer packs and a nitrocellulose membrane (0.22 µm) were soaked into the towbin buffer (1X) for a while. The transfer packs and the gel were aligned horizontally on a transfer cassette as a blotting sandwich. The excess amount of towbin buffer was poured and the transfer cassette was placed on Trans-Blot® Turbo™ (Bio-Rad) instrument running at constant 2.5 amperes (A) for 7 minutes.

The nitrocellulose membrane was separated from blotting sandwich carefully and the success of protein transfer was controlled by staining the nitrocellulose membrane with 0.5 % (w/v) ponceau S solution. Ponceau dye was completely removed from the membrane by washing with TBST (Tris-buffered saline, 0.1% (v/v) Tween® 20). In order to prevent non-specific binding of an antibody, the membrane was incubated in blocking solution on a rocking platform for 1 hour.

After 1 hour, mouse monoclonal Septin3 antibody (Santa Cruz) (200 µg/mL) was prepared at a dilution of 1:500 and p60-katanin (KATNA1) (Atlas) (100 µg/mL) was prepared at a dilution of 1:1000. The membrane of p60-katanin proteins precipitated was incubated with Septin3 primary antibody, whereas the membrane of Septin3 proteins precipitated was incubated with p60-katanin primary antibody on a shaker overnight.

The next day, membranes were washed four times for 7 minutes each with 15 mL TBST (Tris-buffered saline, 0.1 % (v/v) Tween® 20) on the shaker. Anti-mouse IgG 'VeriBlot for IP' (Abcam) solution at a final dilution rate of 1:5000 and anti-rabbit IgG conformation specific (CST) solution at a final dilution rate of 1:3000 were used as secondary antibodies. The membranes were incubated with the secondary antibody solutions on the shaker for 1 hour.

The membranes were washed 4 times for 7 minutes each with 15 mL TBST (Tris-buffered saline, 0.1% (v/v) Tween® 20) on the shaker and the proteins were detected via Gel Doc™ XR+ Imager (Bio-Rad) with Image Lab™ Software (Bio-Rad). In order to visualize proteins on the membrane, Visualizer™ Western Blot Detection Kit (Millipore) was used according to manufacturer's instructions. Then the proteins on membranes were stripped for new primary antibody which was used to precitiate proteins before. The same western protocol was followed to visualize precipitation amounts of proteins.

2.2.2 Immunocytochemistry (ICC) for p60-katanin with Septin3 in rat cortical neurons

2.2.2.1 Primary neuron culture

In this study, rat embryos were used to culture primary cortical neurons at E18 (embryonic day 18) and the protocol was approved by Local Committee on Animal Research Ethics (HADYEK) of İstanbul Technical University and Medipol University. Before culturing neurons, glass coverslips were coated with the poly-D-lysine solution in order to provide a surface for neurons to adhere. From here on in, all the culturing steps were performed in a laboratory hood (Laminar flow hood/Biosafety level Class II).

2.2.2.2 Coating coverslips with poly-D-lysine

The surface of glass coverslips (18 mm diameter) was cleaned up with ethanol and they were soaked into 70% (v/v) ethanol solution for 15 minutes followed by rinsing off the coverslips with distilled water. The glass coverslips were placed in a 12-well tissue culture plate and it was sterilized via UV-lamp for 15 minutes in the hood. Poly-D-lysine solution (1 mg/mL) (Millipore) was diluted to 100 µg/mL with distilled water and the coverslips were coated with the poly-D-lysine solution (100 µg/mL) overnight at room temperature.

The next day, the lysine solution in 12-well plates were removed completely and the coverslips were washed twice for 5 minutes each with 2 mL distilled water. The plate was incubated at 37^0C in a humidified 5% CO_2 atmosphere culture incubator until using.

2.2.2.3 Dissection of cerebral cortices

In order to obtain dissociated primary cortical neuron culture from E18 Sprague – Dawley rat embryos, pregnant rats at embryonic day 18 were used and all operation steps were carried out in Medipol University with the help of veterinarian. The pregnant rat was exposed 2% Forane (Isofluorane) gas through cesarean section and the whole embryos were transferred to ice in a well-cleaned ice bucket to euthanize embryos through anesthesia. At the end, the rat was sacrificed by cervical dislocation with sedation.

The embryos were transferred to our university and they were rinsed with 70% (v/v) ethanol solution prior to dissection. Dissection medium was pre-warmed and all equipment was soaked into 70% (v/v) ethanol solution followed by sterilization via UV-lamp for 15 minutes in the hood. The heads of embryos were transferred onto a culture dish containing dissection medium. The brains were removed from skulls and they were transferred to another culture dish containing dissection medium. The cerebral hemispheres were separated from diencephalon and brain stem with a pair of Dumont-style forceps (no:5) under a dissection microscope. Diencephalon and brain stem were discarded and the lobes were transferred into another culture dish containing dissection medium. Meninges were carefully removed from hemispheres

and each hemisphere was placed lateral side down in order to pull out a block of tissues found in interior part right below the corpus callosum including thalamus, septum and underlying striatum gently. After removing the rest of the limbic lobe containing hippocampus and corpus callosum as well, cerebral cortex was transferred into a culture dish containing dissection medium.

2.2.2.4 Culturing cortical neurons

Dissected cerebral cortices were cut into small pieces and they were put into a falcon tube containing 4.5 mL dissection medium. Then, 0.5 mL trypsin (2.5%) (w/o phenol red) and 0.25 mL DNase (10 mg/ mL) were added into the tube and the tube was incubated in a water bath set 37^0C for 35 minutes. During incubation, the tube was shaken 3 times gently. Cortical pieces were settled down to the bottom of the tube; thus, trypsin and DNase were easily pipetted off without touching any tissue pieces. Tissue pieces were rinsed twice each for 5 minutes with 5 mL plating medium followed by adding 1 mL plating medium pre-warmed to 37^0C. Trituration of cortical pieces was performed 7 times against the side of the tube with fire-polished Pasteur pipettes with different size of narrow mouths. After dissociation has completed, cells were counted via Countess™ automated cell counter (Invitrogen).

$1x10^6$ cells were seeded per each glass coverslip (18 mm diameter) which were precoated with poly-D-lysine described in Section 2.2.2.2 and they were incubated at 37^0C in a humidified 5% CO_2 atmosphere culture incubator for 1 hour.

After 1 hour, Gentamicin® (10 mg/mL) (Gibco) was diluted to 100 µg/mL with serum-free plating medium. Media of coverslips were replaced with serum-free plating medium including gentamicin and they were incubated at 37^0C in a humidified 5% CO_2 atmosphere culture incubator for 2 hours. At the end of the incubation, serum-free plating medium including gentamicin was replaced with serum-free plating medium and the coverslips were incubated at 37^0C in a humidified 5% CO_2 atmosphere culture incubator for 7 days.

2.2.2.5 ICC

In order to perform ICC, the coverslips were washed with PBS (1X) and the cells were fixed with 4 % (w/v) paraformaldehyde solution (dissolved in 1X PBS) by incubating for 10 minutes at room temperature. Then, paraformaldehyde solution

was discarded and the coverslips were washed 4 times for 5 minutes each with 1 mL PBS (1X). The washing step was followed by permeabilization of cell's membrane that the coverslips were incubated with 0.1 % (w/v) saponin solution (dissolved in 1X PBS) at room temperature for 10 minutes. The blocking solution was prepared as indicated in Section 2.1.5.2 and the coverslips were incubated with the solution for 1 hour at room temperature.

After 1 hour, the coverslips were transferred onto parafilm coated petri plates. The primary antibody solution was prepared with rabbit polyclonal p60-katanin antibody (KATNA1) (Atlas) (100 ng/µL) and mouse monoclonal Septin3 antibody (Santa Cruz) (200 ng/µL) at the same dilution of 1:50 with the blocking solution. Before the coverslips get dry, 200 µL primary antibody solution was poured onto the coverslips gently and they were allowed to bind primary antibodies overnight at 4^0C.

The next day, the coverslips were washed 4 times for 5 minutes each with 200 µL PBS (1X) and the secondary antibody solution was prepared with anti-rabbit IgG conjugated to Alexa Fluor® 647 (CST) and anti-mouse IgG conjugated to Alexa Fluor® 488 (CST) at the same dilution of 1:200 with the blocking solution. The coverslips were incubated with 200 µL of secondary antibody solution at room temperature in a dark place for 1 hour.

The coverslips were washed 3 times for 5 minutes each with 200 µL PBS (1X) and they were stained with DAPI (Invitrogen) at a final dilution of 1:1000. After the coverslips were incubated with 200 µL of DAPI for 5 minutes at room temperature in the dark, they were washed additional three times for each 5 minutes with 200 µL PBS (1X) to get rid of unbound antibody residues completely. During washing steps, laboratory slides were cleaned with 70% (v/v ethanol). The coverslips were dried and mounted with Prolong® mounting medium (Invitrogen). They were sealed with a nail polish and the slides were incubated at room temperature in the dark overnight prior to observation via TCS SP2 SE confocal Microscope (Leica).

2.2.3 Cloning of *p60-katanin* and *Septin3*

2.2.3.1 Primer design

Primer is a short strand of nucleotides which binds specifically to template nucleic acid thus, amplify target site during polymerase chain reaction. In order to amplify

rat *p60-katanin* and rat *Septin3*, the primers were designed by considering annealing temperature, GC (guanine and cytosine) content, possibility of hairpin and self-dimer formation of primers IDT Oligo Analyzer.

In-Fusion® cloning system depends on recombinational cloning, as long as vector and DNA fragment share 15 bases of homology at each end, homologous recombination occurs. Therefore, about 15 bases long vector sites were added to 5' ends of each primer just before restriction recognition sites.

Designed primers were ordered from Macrogen Company and they are listed in the Table 2.21.

Table 2.21 : Primers of p60-katanin and Septin3.

Primers	**Primer Sequence**
p60-katanin-FL (1515 bp)	Forward:
	5'AATTCATCGATAGATCAGATGAGCATGAGTCTTCTAATG3'
	Reverse:
	5'CCCGGGATCCTCTAGCTAGCATGATCCAAACTC AAC3'
Septin3 – FL (1107 bp)	Forward:
	5'CTGGCTAGTTAAGCTATGTCCAAAGGGCTCCCAGAG3'
	Reverse:
	5'GCCCTCTAGACTCGATTCAGCGGTGGGGCAGGG3'

2.2.3.2 RNA isolation

RNA isolation was performed from rat brain. 80 mg of rat brain was mixed with 1 mL Trizol reagent (Ambion/Life Technologies) in a falcon tube and the tube was vortexed drastically for 2 minutes. The tissue was pipetted gently until the solution appeared to be homogeneous and it was incubated at room temperature for 5 minutes. After 5 minutes, 200 µL chloroform was added to the sample and it was incubated at room temperature for additional 5 minutes. The tube was centrifuged at 12000 x g, 4^0C for 15 minutes, resulting three layers in solution. The upper layer containing RNA was carefully transferred into a new tube and 500 µL of ice-cold 100% isopropanol was added to the tube. It was pipetted harshly and centrifuged at 4^0C, 12000 x g for 15 minutes. RNA has formed gel-like pellet on the bottom of the tube, therefore supernatant was discarded and the pellet was washed twice with 1 mL

of 75% ethanol by dissolving the pellet in ethanol followed by centrifugation at 4^0C, 12000 x g for 7 minutes.

After washing, remaining ethanol was air-dried in a hood for 10 minutes. Air-dried pellet was resuspended with 20 µL nuclease-free dH_2O by heating in a water bath set 55^0C for 30 minutes. After 30 minutes incubation in the water bath, total RNA was dissolved in 20µL RNase-free water and its concentration was measured with Nanodrop2000 spectrophotometer (Thermo).

2.2.3.3 Complementary DNA (cDNA) synthesis

Complementary DNA (cDNA) is a double-stranded DNA which is synthesized most often from mRNA in a reaction called 'reverse transcription'. In order to amplify target sequences of rat *p60-katanin* and *Septin3* by PCR, cDNA was synthesized from total RNA of rat brain with sequential two steps by using commercial Protoscript II cDNA synthesis kit (NEB). In the first step, components were added to PCR tube with mentioned amounts and the given order in the Table 2.22 and the tube was spun briefly followed by incubating in a T100 thermal cycler (Bio-Rad) at 65^0 C for 5 minutes.

Table 2.22 : Components of cDNA reaction.

Components	Amount
Nuclease-free dH_2O	5 µL
Total RNA (~ 1000 ng)	1 µL
d(T)23 VN (50 µM)	2 µL
Total	8 µL

At the end of the incubation, the tube was placed on ice immediately and 10 µL of ProtoScript II Reaction Mix (2X) was added to the tube. As the last component, 2 µL ProtoScript II Enzyme Mix (10X) was put into the tube and incubated in the T100 thermal cycler (Bio-Rad) at 42^0C for 1 hour. In order to inactivate the enzyme, additional 5 minutes incubation at 80^0C was carried out, then the cDNA was used for PCR.

2.2.3.4 Polymerase chain reaction (PCR)

Rat brain cDNA was used as the template with indicated primers (given in Section 2.2.3.1) in a reaction catalyzed by Q5 DNA polymerase (NEB). Annealing temperatures of polymerase chain reactions were determined via Tm Calculator tool

(NEB). Reaction components and conditions are given in the Tables 2.23 and 2.24, respectively.

Table 2.23 : Components of PCR.

Components	Final Concentration	Amount
Nuclease-free H_2O	(up to 25µL)	14.5 µL
Q5 Reaction Buffer (5X)	1X	5 µL
dNTPs (10mM)	200 µM	0.5 µL
F primer (10µM)	0.5 µM	1.25 µL
R primer (10µM)	0.5 µM	1.25 µL
DMSO	-	0.25 µL
Template (rat brain cDNA)	-	2 µL
Q5 DNA Polymerase	0.02 U/µL	0.25 µL

Table 2.24 : Conditions of PCR.

Step	Temperature	Time	Number
Initial Denaturation	98^0C	30 sec.	1
Denaturation	98^0C	10 sec.	30 cycles
Annealing	63^0C for p60-katanin 70^0C for Septin3	30 sec.	
Extension	72^0C	30 sec.	
Final Extension	72^0C	2 min.	1

Polymerase chain reactions were prepared and the program given in the Table 2.24 was run on the cycler (Bio-Rad).

At the end of the PCR, the samples were mixed with 6X DNA Loading dye (NEB) and were loaded onto 1% (w/v) agarose gel which was prepared with 1X TAE buffer (Bio-Rad) with 1 kb DNA ladder (Promega). The gel was run at 70 volts for 70 minutes followed by visualizing the gel by Bio-Rad Gel/Chemidoc Imaging system.

2.2.3.5 DNA extraction of PCR products from agarose gel

PCR products of p60-katanin and Septin3 which were subjected to agarose gel electrophoresis were cut off from the gel under UV light. 'NucleoSpin Gel and PCR Clean-up Kit (MN)' was used for DNA extraction according to the manufacturer's instructions and DNA concentrations of purified PCR products were measured using Nanodrop2000 spectrophotometer (Thermo).

2.2.3.6 Restriction enzyme digestion of vectors

According to the recognition sites of restriction enzymes found at multiple cloning sites of p3XFLAG-CMV10 (Sigma) (for p60-katanin) and pcDNA™3.1/ myc-His A (Invitrogen) (for Septin3) vectors, p3XFLAG-CMV10 vector was digested with

BglII (NEB) and XbaI (NEB), whereas pcDNA™3.1/ myc-His A was digested with HindIII (NEB) and XhoI (NEB) enzymes. Restriction reaction ingredients for both vectors are given in the Table 2.25.

Table 2.25 : Components of restriction digestion reaction.

Components		**Final Concentration**		**Amount**	
pcDNA™3.1/ myc-His A	p3XFLAG-CMV10	1 µg		1.2 µL	1.5 µL
HindIII	BglII	10 U/ µL	10 U/µL	1 µL	
XhoI	XbaI	10 U/ µL	20 U/ µL	1 µL	
10X NEB Buffer (Cutsmart)	10X NEB Buffer (3.1)	1 X		5 µL	
Nuclease-free dH_2O		-		up to 50 µL	

The restriction reactions were set as indicated in the Table 2.25 and the reaction tubes were incubated in a water bath set at 37^0C for overnight.

After incubation, the reaction mixtures were mixed with 6X DNA Loading dye (NEB) and loaded onto 1% (w/v) agarose gel with 1 kb DNA ladder (Promega). After the gel was run at 70 volts for 70 minutes, DNA bands observed at expected size were quickly cut off from the gel under UV light. 'NucleoSpin Gel and PCR Clean-up Kit (MN)' was used for DNA extraction according to the manufacturer's instructions and DNA concentrations of restricted vectors were measured using Nanodrop2000 spectrophotometer (Thermo).

2.2.3.7 In-Fusion® cloning and following bacterial transformation

In-Fusion® HD Cloning Kit (Clontech) was used to transfer DNA fragments into the vectors that In-Fusion® system depends on recombinational cloning rather than traditional cut-and-paste cloning. Homologous recombination occurs between homologous 15 bases found both in a vector and a DNA fragment. Since the primers were designed considering the working principle of the kit, amplified PCR products contained homologous site. Purified PCR fragments and restricted vectors were diluted at 100 ng/µL concentration and 1 µL PCR fragment was mixed with 1 µL digested vector in an eppendorf tube. The reaction volume was adjusted to 8 µL by adding 6 µL of nuclease-free water to the tube. As a negative control, 1 µL restricted vector was mixed with 7 µL nuclease-free water. After 2 µL In-Fusion® HD Enzyme Premix was added to the tubes, the tubes were incubated in a heat-block set at 50^0C

for 15 minutes. Immediately after the incubation was completed, the tubes were placed on ice.

Stellar competent cells provided by In-Fusion® HD Cloning Kit (Clontech) were taken from -80^{0}C and thawed quickly. After the 50 µL competent cell was mixed with the 2.5 µL reaction mixture in a tube, the tube was incubated on ice for 30 minutes. At the end of the incubation, the tube was placed in a heat-block at 42^{0}C for 45 seconds followed by incubation on ice for additional 2 minutes. 500 µL SOC medium (provided by In-Fusion® HD cloning kit) was poured into the tube and it was incubated for 1 hour at 37^{0}C, 200 rpm on a shaker. After an hour, transformation reaction was centrifuged at 6000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in fresh 100 µL SOC medium followed by spreading the sample on an LB agar plate containing ampicillin. Since the copy number regulation of vectors in this study was different; pcDNA™3.1/ myc-His A (high-copy plasmid) and p3XFLAG-CMV10 (low-copy plasmid), ampicillin at 100 µg/mL concentration was used for pcDNA™3.1/ myc-His A constructs whereas ampicillin at 50 µg/mL concentration was used for p3XFLAG-CMV10 constructs. After spreading, the petri plates were sealed with a piece of Parafilm and they were incubated at 37^{0}C overnight. The next day, two candidate colonies from each agar plate were chosen to be screened for the presence of the insert by colony PCR.

2.2.3.8 Colony PCR

The candidate colonies were resuspended with 15 µL nuclease-free water. 5 µL bacteria-water suspension was stored at 4^{0}C until the colony PCR end up, whereas the rest 10 µL of the bacteria-water suspension was boiled at 80^{0}C for 10 minutes in order to release plasmid by degrading the bacterial walls and membranes. After 10 minutes boiling, the sample was used as template in a polymerase chain reaction carried out with sequencing primers of vectors which are indicated below. Components and conditions of PCR are given in the Tables 2.26 and 2.27.

For p3XFLAG-CMV10 vector (p60-katanin) (Tm 67^{0}C)

- N-CMV30 (F): 5’-AATGTCGTAATAACCCCGCCCCGTTGACGC-3’
- C-CMV24 (R): 5’-TATTAGGACAAGGCTGGTGGGCAC-3′

For pcDNA™3.1/ myc-His A vector (Septin3) (Tm 53^0C)

- T7 promoter (F): 5'-TAATACGACTCACTATAGGG-3'
- BGH (R): 5'-TAGAAGGCACAGTCGAGG-3'

Table 2.26 : Components of colony PCR.

Components	Final Concentration	Amount
Nuclease-free H_2O	(up to 25µL)	8 µL
Bacterial suspension	-	10 µL
dNTPs (10mM)	200 µM	0.5 µL
F primer (10µM) (Sequencing primers)	0.2 µM	0.5 µL
R primer (10µM) (Sequencing primers)	0.2 µM	0.5 µL
OneTaq DNA Polymerase	0.1U/µL	0.5 µL
One Taq Standard Reaction Buffer (5X)	1X	5 µL

Table 2.27 : Conditions of colony PCR.

Step	Temperature	Time	Number of cycle
Initial Denaturation	94^0C	30 sec.	1
Denaturation	94^0C	30 sec.	
Annealing	67^0C or 53^0C	60 sec.	30 cycles
Extension	68^0C	1 min.	
Final Extension	68^0C	2 min.	1

At the end of colony PCR, the samples were mixed with 6X DNA Loading dye (NEB) and they were loaded onto 1% (w/v) agarose gel with 1 kb DNA ladder (Promega). The gel was run at 100 volts for 40 minutes following visualizing under UV light.

2.2.3.9 Isolation of plasmids and DNA sequencing-alignment

According to the colony PCR results, plasmids containing the right insert was needed to be isolated for sequencing. Therefore, resuspended bacterial colonies stored at 4^0C were mixed with 10 mL LB broth containing 100 µg/mL of ampicillin or 50 µg/mL of ampicillin depends on the choise of vector in falcon tubes separately and the tubes were incubated overnight at 37^0C, 200 rpm on a shaker.

The next day, plasmids were isolated by using NucleoSpin Plasmid Kit (MN) according to the instructions of the manufacturer. At the end of the isolation, the concentrations of plasmids were measured with Nanodrop2000 spectrophotometer (Thermo) and they were sent for DNA sequencing to Macrogen Company. After the DNA sequencing results were obtained, the alignments of DNA were accomplished via EMBOSS Pairwise Alignment Algorithms tool with originally expected ones.

2.2.3.10 Expression of FLAG-tagged p60-katanin and Myc/his-tagged Septin3

FLAG-tagged p60-katanin and Myc/his-tagged Septin3 proteins were overexpressed in HCT116 cells separately and the cell lysates were subjected to western blotting in order to control protein expression of constructs.

HCT116 cells which had been stored at -80^{0}C were thawed in a water bath set at 37^{0}C followed by transferring the cells to a falcon tube containing 10 mL DMEM High Glucose medium (Lonza). After the tube was centrifuged for 5 minutes at 1500 rpm, the supernatant was discarded and the pellet was resuspended with 1 mL DMEM high Glucose medium (10% FBS, 1%P/S). The resuspension was seeded to a T75 culture flask and the volume of the flask was completed to 8 mL with DMEM high Glucose medium (10% FBS, 1%P/S). The cells were subcultured for 2 passages prior to transfection. The cells were counted and they were seeded at a density of 550,000 cells per tissue culture dish (60 mm). The cells were incubated at 37^{0}C in a humidified 5% CO_2 atmosphere culture incubator for overnight.

The next day, TransFast™ (Promega) transfection reagent was used for overexpression of constructs according to the manufacturer's instructions. Briefly, 2 mL DMEM high glucose medium, 30 µL TransFast™ reagent, which was dissolved in 400 µL nuclease free water one day prior of transfection, and 5 µg plasmid were mixed and incubated at room temperature for 15 minutes. Just before 15 minutes has ended, the medium in the culture dish was discarded and the mixture was poured into the dish gently. After an hour incubation in the culture incubator, 3 mL DMEM high Glucose medium (10% FBS, 1%P/S) was added to the dish. Co-transfected cells were incubated at 37^{0}C in a humidified 5% CO_2 atmosphere culture incubator overnight.

Medium in culture dish was discarded and the dish was washed with 3 mL of PBS (1X). The cells were scrapped and collected in an eppendorf tube. The tube was centrifuged at 600 x g for 5 minutes at 4^{0}C and the supernatant was discarded. Pellet was resuspended with 1 mL of NP40 solution and the tube was incubated on ice for 30 minutes. After incubation, the tube was centrifuged at 14000 x g for 10 minutes at 4^{0}C and the supernatant was collected as total protein. Protein concentration was determined via BCA protein assay using BCA Protein Assay Kit (Pierce™) according to manufacturer's instructions.

Western blot was performed as previously explained in Section 2.2.1.3 with 10 µg lysate. Rabbit monoclonal FLAG-tag primary antibody (CST) (141 µg/mL) (1:1000 diluted in blocking solution) and mouse monoclonal Myc-tag primary antibody (CST) (318 µg/mL) (1:1000 diluted in blocking solution) were used for FLAG-tagged p60-katanin and Myc/his-tagged Septin3, respectively.

2.2.4 Preparation of mutant Septin3 constructs

2.2.4.1 Site-directed mutagenesis

Site-directed mutagenesis is a method used to create specific, targeted changes in a double stranded plasmid DNA. In this study, the technique was used to make point mutations in wild-type pcDNA3.1/myc-His A-Septin3 construct. Based on the crystal structure of *Septin2*, two critical amino acids which are conserved in *Septin3* as Lys208 and Arg280 were chosen to be mutated with three-step procedure using QuikChange II Site-directed Mutagenesis Kit (Agilent) and In-Fusion® Cloning Kit (Clontech). The mutagenic oligonucleotide primers each containing the desired point mutation for *Septin3* are given in the Table 2.28.

Table 2.28 : Mutagenic oligonucleotide primers for Septin3.

Mutagenic Primers	Primer Sequence
Septin3 K-208-A Forward	5'AACGTCATTCCTGTCATTGCCGCGGCTGACACCATGACC3'
Septin3 K-208-A Reverse	5'GGTCATGGTGTCAGCCGCGGCAATGACAGGAATGACGTT3'
Septin3 R-280-A Forward	5'AAGAGGGTCCTCGGGGCAAAAACACCCTGGGG3'
Septin3 R-280-A Reverse	5'CCCCAGGGTGTTTTTGCCCCGAGGACCCTCTT3'

Three different reactions were set either to mutate one amino acid or both at the same time. The reaction components and conditions are given in the Tables 2.29 and 2.30.

Following temperature cycling, the reaction tubes were placed on ice for 2 minutes to cool the reactions then the product was treated with DpnI endonuclease (5U/l) which targets the sequence of 5´-Gm6ATC-3´ in water bath set at 37^0C for one hour. Since the DpnI endonuclease is specific for methylated and hemimethylated DNA, the enzyme was used to digest the parental vector thereby to select for mutation-containing synthesized vector. After the linearized and digested vector was purified using 'NucleoSpin Gel and PCR Clean-up Kit (MN)' according to the instructions of manufacturer, the linearized vector was re-circularized via the In-Fusion reaction as indicated in the Table 2.31.

Table 2.29 : Components of PCR for Septin3 mutants.

Component	Amount	Final Concentration
dH_2O	17.25 µl	Up to 25µl
PfuUltra HF Reaction Buffer (10X)	5 µl	1X
dNTPs (10mM)	0.5 µl	200 µM
F primer (100 ng)	1.25 µl	125 ng
R primer (100 ng)	1.25 µl	125 ng
DMSO	0.75 µl	3%
Template (Septin3 WT construct)	1 µl	100 ng/µl
PfuUltra HF DNA Polymerase (Agilent)	0.5 µl	2.5 U/µl

Table 2.30 : Conditions of PCR for Septin3 mutants.

Step	Temperature	Time	Cycle Number
Initial Denaturation	95^0C	3 min.	1
Denaturation	98^0C	10 sec.	25
Annealing	55^0C	10 sec.	
Extension	68^0C	6.5 min.	

Table 2.31 : Components of In-Fusion reaction to re-circularize the vector.

Component	Amount
PCR product (treated with DpnI)	3 µl
In-Fusion enzyme mix	2µl
dH_2O	5 µl
TOTAL	10 µl

At the end of the reaction, 50 µl of Stellar competent cell (provided by In-Fusion cloning kit) was mixed with the 2.5 µl reaction mixture in a tube, the tube was incubated on ice for 30 minutes. At the end of the incubation, the tube was placed in a heat-block at 42^0C for 45 seconds followed by incubation on ice for additional 2 minutes. 500 µl SOC medium (provided by In-Fusion cloning kit) was poured into the tube and it was incubated for one hour at 37^0C, 200 rpm on a shaker. After an hour, transformation reaction was centrifuged at 6,000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in fresh 100 µl SOC medium followed by spreading the sample on an LB agar plate containing ampicillin at 100 µg/ml final concentration. After spreading, the petri plates were incubated at 37^0C overnight.

2.2.4.2 Isolation of mutant plasmids and DNA sequencing-alignment

Two candidate colonies were chosen from each agar plate belongs to mutant Septin3 constructs and they were mixed with 10 ml LB broth containing 100 µg/ml

ampicillin in falcon tubes separately followed by incubation overnight at 37^0C, 200 rpm on a shaker.

The next day, plasmids were isolated by using NucleoSpin Plasmid kit (MN) according to the instructions of the manufacturer. At the end of isolation, the concentrations of plasmids were measured with Nanodrop2000 Spectrophotometer (Thermo) and they were sent for DNA sequencing to Macrogen Company.

After the DNA sequencing results were obtained, the alignments of DNA were accomplished via EMBOSS Pairwise Alignment Algorithms tool with expected mutations on the specified amino acids. The schematic representation of three mutant Septin3 constructs is shown in Figure 2.3.

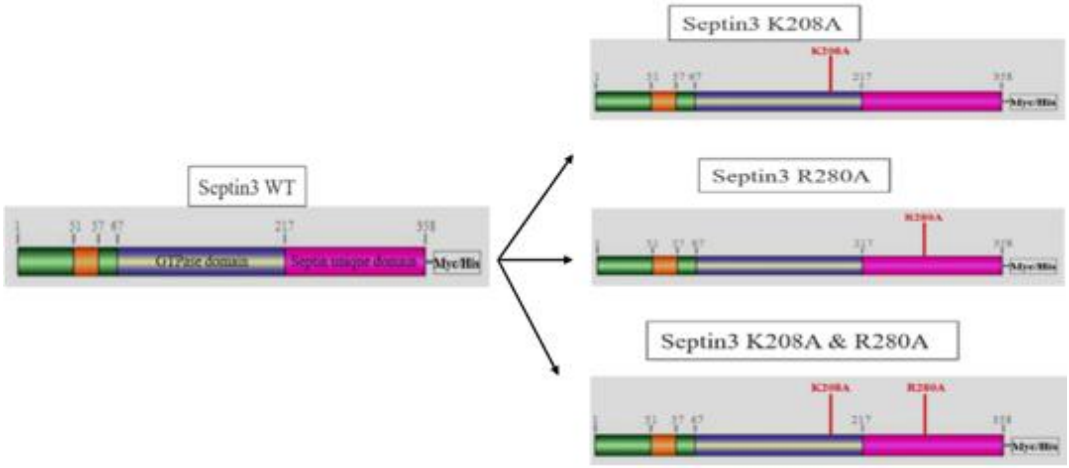


Figure 2.3 : The schematic representations of Septin3 mutants.

2.2.5 GTPase activity analysis of mutant Septin3 constructs

2.2.5.1 Cell culture studies

RFL6 cell line (rat lung fibroblast) which had been stored at -80^0C was thawed in a water bath set at 37^0C followed by transferring the cells in falcon tube containing 10 ml Ham's F12 medium (Lonza). After the tube was centrifuged for 6 minutes at 1500 rpm, the supernatant was discarded and the pellet was resuspended with 1 ml Ham's F12 medium (20% FBS, 1X NEAA, 1%P/S). The cells were seeded into T75 culture flasks and subcultured for 2 passages prior to transfection.

2.2.5.2 Nucleofection of Septin3 mutants to RFL6 cells

RFL6 cells were deattached with 5 ml Trypsin/EDTA (Lonza) from the T75 flask after the incubation in culture incubator (37^0C) for 15 minutes and the suspension

was transferred to a falcon tube containing 7 ml Ham's F12 medium (20% FBS, 1X NEAA, 1%P/S). The tube was shaken gently and the cells were counted via a hemocytometer. The required number of cells ($1x10^6$ cells per nucleofection reaction) were centrifuged at 200 x g for 5 minutes and the supernatants were discarded. Each pellet was resuspended with 100 µl Nucleofector® solution which had prepared by mixing 82 µl of Nucleofector® solution of Kit R with 18 µl of its supplement prior nucleofection. Before RFL6 cells were subjected to nucleofection, 10 µg of plasmids; Septin3 WT (FL), Septin3-K208A, Septin3-R280A and Septin3-K208A&R280A, were combined with the cell suspensions separately. Cell/DNA suspensions were transferred into Nucleofection® cuvette an run the program. Once 1 ml of Ham's F12 medium was added to each cuvette in order to equilibrate cell suspension prior to seeding on a 60 mm culture dish, the dishes were incubated at 37^0C in a humidified 5% CO_2 atmosphere culture incubator for 21 hours.

2.2.5.3 Measuring GTPase activity of Septin3 constructs

Septin3 WT (FL), Septin3-K208A, Septin3-R280A and Septin3-K208A&R280A constructs were transfected to RFL6 cells and then cells were subjected to GTPase activity assay according to the instructions of ATPase/GTPase Activity Assay Kit (Sigma-Aldrich). Briefly, the RFL6 cells on 60 mm culture dishes were scrapped and they were homogenized on ice with 200 µl ice-cold assay buffer (supplied in kit). When the samples were centrifuged at 14000 rpm for 10 minutes, reaction buffer was prepared by mixing assay buffer and 4 mM GTP with a ratio of 2:1. After centrifugation, the supernatants were transferred into new tubes and they were placed in wells of a 96-well plate as 1 µl, 3 µl, 5 µl, 8 µl and 10 µl in duplicate. Then, the volume of wells were completed to 10 µl with assay buffer. 30 µl of reaction buffer was added to each well of the plate and the plate was incubated for 20 minutes at room temperature. After incubation, 200 µl of the malachite green reagent (supplied in kit) was poured into each well and the plate was incubated for 30 minutes at room temperature in the dark. During incubation, 1mM phosphate standard (supplied in kit) was diluted to 0, 12.5, 25, 31.25, 37.5, and 50 µM with ultrapure water and the standards were poured into wells of 96-well plate in duplicate. The absorbance values were measured at 620 nm by spectrophotometric 96-well plate reader (Bio-Rad) and the GTPase activities of Septin3 constructs were calculated via equation of

the manufacturer. Finally, GTPase activities of constructs were plot using Microsoft Excell.

2.2.6 ICC of wild-type p60-katanin with wild-type or double mutant Septin3

2.2.6.1 Coating coverlips

The glass coverslips (18 mm diameter) were cleaned up with ethanol and soaked into 70% (v/v) ethanol solution for 15 minutes followed by rinsing off the coverslips with distilled water. The glass coverslips were placed in a 12-well tissue culture plate and it was sterilized via UV-lamp for 15 minutes in the hood. Poly-L-lysine solution (100 µg/mL) (Millipore) was diluted to 50 µg/mL with distilled water and the coverslips were coated with the poly-L-lysine solution (50 µg/mL) overnight at room temperature.

The next day, the lysine solution in 12-well plates were removed completely and the coverslips were washed twice for 5 minutes each with 2 mL distilled water. The plate was incubated at 37^0C in a humidified 5% CO_2 atmosphere culture incubator until using.

2.2.6.2 Nucleofection to RFL6 cells

RFL6 cells were deattached with 5 ml Trypsin/EDTA (Lonza) from the T75 flask after the incubation in culture incubator (37^0C) for 15 minutes and the suspension was transferred to a falcon tube containing 7 ml Ham's F12 medium (20% FBS, 1X NEAA, 1%P/S). The tube was shaken gently and the cells were counted via a hemocytometer. $2x10^6$ cells per nucleofection reaction were centrifuged at 200 x g for 5 minutes and the supernatants were discarded. Each pellet was resuspended with 100 µl Nucleofector® solution which had been prepared by mixing 82 µl of Nucleofector® solution of Kit R with 18 µl of its supplement prior nucleofection. In term of the optimized protocol, 9 µg wild-type p60-katanin construct and 6 µg of either mutant or wild-type Septin3 constructs were mixed with the nucleofection solution. Cell/DNA suspensions were transferred into Nucleofection® cuvette and run the program. Once 1 ml of Ham's F12 medium was added to each cuvette in order to equilibrate cell suspension and then cells were seeded to coverslips as 90,000 cells per a coverslip. The cells were incubated at 37^0C in a humidified 5% CO_2 atmosphere culture incubator for 21 hours.

2.2.6.3 ICC

The next day, the coverslips were washed with PBS (1X) and the cells were fixed with 4 % (w/v) paraformaldehyde solution (dissolved in 1X PBS) by incubating for 10 minutes at room temperature. Then, paraformaldehyde solution was discarded and the coverslips were washed 4 times for 5 minutes each with 1 mL PBS (1X). The washing step was followed by permeabilization of cell's membrane. The coverslips were incubated with 0.1 % (w/v) saponin solution (dissolved in 1X PBS) at room temperature for 10 minutes. The coverslips were incubated with the blocking solution (as previously explained in Section 2.1.5.2.5) for 1 hour at room temperature. After 1 hour, the coverslips were transferred onto parafilm coated petri plates. The primary antibody solution was prepared with rabbit monoclonal anti-FLAG antibody (CST) (1 µg/µL), mouse monoclonal anti-Myc antibody (CST) (1 µg/µL) and chicken polyclonal anti-α-tubulin (Abcam) (1 µg/µL) antibody at the same dilution of 1:200. Before the coverslips get dry, 200 µL primary antibody solution was poured onto the coverslips gently and they were allowed to bind primary antibodies overnight at 40C. The next day, the coverslips were washed 4 times for 5 minutes each with 200 µL PBS (1X) and the secondary antibody solution was prepared with anti-mouse IgG conjugated to Alexa Fluor® 488 (CST) (1 µg/mL), anti-rabbit IgG conjugated to Alexa Fluor® 594 (CST) (1 µg/mL) and anti-chicken IgG conjugated to Alexa Fluor® 647 (Invitrogen) (1 µg/mL) at the same dilution of 1:200 with the blocking solution. The coverslips were incubated with 200 µL of secondary antibody solution at room temperature in a dark place for 1 hour. After 1 hour, the coverslips were washed 3 times for 5 minutes each with 200 µL PBS (1X) and they were stained with DAPI (Invitrogen) at a final dilution of 1:1000. After the coverslips were incubated with 200 µL of DAPI for 5 minutes at room temperature in the dark, they were washed additional three times for each 5 minutes with 200 µL PBS (1X). During washing steps, laboratory slides were cleaned with 70% (v/v ethanol) and tissue paper. The coverslips were mounted with Prolong® mounting medium (Invitrogen) and sealed with a nail polish and the slides were incubated at room temperature in the dark overnight prior to observation via TCS SP2 SE confocal Microscope (Leica).

2.2.7 Co-immunprecipitation analysis to identify interaction domains

2.2.7.1 Generation of deletion constructs

The schematic representations of rat p60-katanin and rat Septin3 proteins were illustrated based depending on the literature (Ghosh, Dasgupta and Guha, 2012; Mostowy and Cossart, 2012; Macedo et al., 2013). p60-katanin was dissected into four deletion constructs. The schematic representations of domains for full length and truncated p60-katanin are given in Figure 2.4.

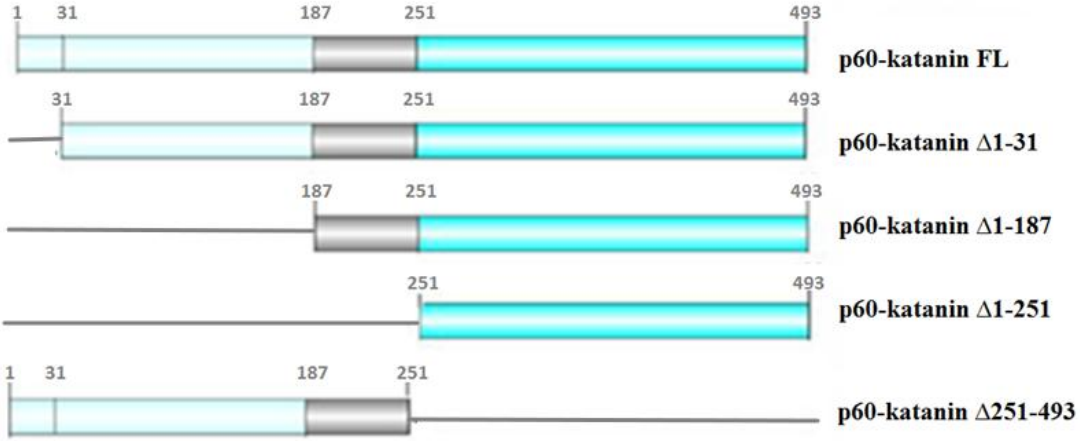


Figure 2.4 : The schematic representations of p60-katanin deletion constructs. (Numbers denote amino acids and lines indicate missing domain. Light blue domain is microtubule binding domain, grey is linker sequence and blue domain indicates ATPase domain)

Septin3 was dissected into two deletion constructs. The schematic representations of domains for full length and truncated Septin3 are given in Figure 2.5.

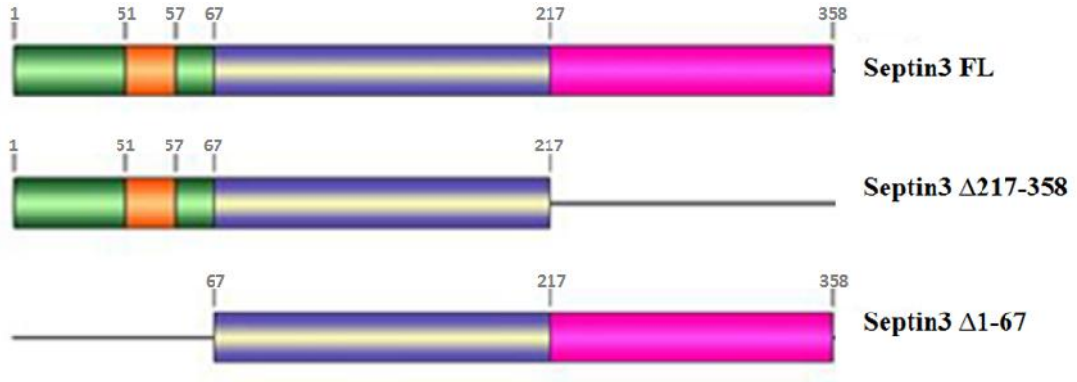


Figure 2.5 : The schematic representations of Septin3 deletion constructs. (Numbers denote amino acids and lines indicate missing domain. Green is proline-rich domain, orange is polybasic domain, purple is GTPase domain and pink is septin unique domain.)

Deletion constructs were cloned into mammalian expression vectors which were p3XFLAG-CMV10 for p60-katanin and pcDNA™ 3.1/myc-His A for Septin3. During cloning, the same steps were followed as previously indicated in Section 2.2.3. Primers used for deletion constructs of p60-katanin and Septin3 are listed in the Tables 2.32 and 2.33, respectively.

Table 2.32 : Primers designed for deletion constructs of p60-katanin.

p60-katanin Primers	Primer Sequence
p60-katanin Δ1-31 (1422 bp)	Forward –5'AAT TCATCGATAGATCAGCAGGGAGTTCTTGACCAAATT3' Reverse –5'CCCGGGATCCTCTAGCTAGCATGATCCAAACTCAAC3'
p60-katanin Δ1-187 (954 bp)	Forward –5'AATTCATCGATAGATCAGGGGTATGACAAGGACTTAGTA3' Reverse - 5'CCCGGGATCCTCTAGCTAGCATGATCCAAACTCAAC3'
p60-katanin Δ1-251 (762 bp)	Forward - 5'AATTCATCGATAGATCAGCCACCTGGCACTGGAAAG3' Reverse - 5'CCCGGGATCCTCTAGCTAGCATGATCCAAACTCAAC3'
p60-katanin Δ251- 493 (783 bp)	Forward - 5'AATTCATCGATAGATCAGATGAGCATGAGTCTTCTAATG3' Reverse - 5'CCCGGGATCCTCTAGTCAGCCAACCATCAGCACACCTTT3'

Table 2.33 : Primers designed for deletion constructs of Septin3.

Septin 3 Primers	Primer Sequence
Septin3 Δ 1-67 (906 bp)	Forward - 5'CTGGCTAGTTAAGCTGGCCAGAGTGGACTGGGC3' Reverse - 5'GCCCTCTAGACTCGATTCAGCGGTGGGGCAGGG3'
Septin3 Δ 217-358 (684 bp)	Forward - 5'CTGGCTAGTTAAGCTATGTCCAAAGGGCTCCCAGAG3' Reverse – 5'CCCTCTAGACTCGAGCTCCTCCAGGGTCATGGT3'

2.2.7.2 Co-transfection and co-immunoprecipitation

The HCT116 cells were seeded at a density of 550,000 cells per 60 mm tissue culture dish. For each set of co-expression, the cells were seeded to 6 different 60 mm culture dishes one day before transfection. Transfection sets are given with amounts of plasmids in the Table 2.34.

Table 2.34 : Co-transfection sets.

Transfection Set	Constructs
Only p60 FL	p60 FL (7 µg) & pcDNA™ 3.1/myc-His A (5 µg)
Only Septin3 FL	p3XFLAG-CMV10 (7 µg) & Septin3 FL (5 µg)
p60 FL & Septin3 FL	p60 FL (7 µg) & Septin3 FL (5 µg)
p60 FL & Septin3 Δ1-67	p60 FL (7 µg) & Septin3 Δ1-67 (5 µg)
p60 FL & Septin3 Δ217-558	p60 FL (7 µg) & Septin3 Δ217-558 (5 µg)
p60 Δ1-31 & Septin3 FL	p60 Δ1-31 (7 µg) & Septin3 Δ217-558 (5 µg)
p60 Δ1-187 & Septin3 FL	p60 Δ1-187 (7 µg) & Septin3 Δ217-558 (5 µg)
p60 Δ1-251 & Septin3 FL	p60 Δ1-251 (7 µg) & Septin3 Δ217-558 (5 µg)
p60 Δ251-493 & Septin3 FL	p60 Δ251-493 (7 µg) & Septin3 Δ217-558 (5 µg)
Empty vectors	p3XFLAG-CMV10 (7 µg) & pcDNA™ 3.1/myc-His A (5 µg)

Co-transfections and protein isolations were performed as previously described in Section 2.2.3.10.

In co-immunoprecipitation assays, ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich) was used according to the manufacturer's instructions. Briefly, 750 µg protein lysates were immunoprecipitated with 20 µl bead slurry FLAG-tag resin overnight and the western blot analysis was carried out with mouse monoclonal Myc-tag (CST) (318 µg/mL) primary antibody at a dilution of 1:1000 in blocking solution. As a secondary antibody, anti-mouse IgG VeriBlot for IP (Abcam) was diluted in blocking solution to 1:3000. The proteins on the membranes were visualized with Visualizer™ Western Blot Detection Kit (Millipore) and they were detected via Bio-Rad Gel Doc™ XR+ Imager.

3. RESULTS

3.1 Co-immunoprecipitation of Septin3 and p60-katanin in Rat Brain Lysate

Septin3 protein was previously identified as one of the interacting partner of p60-katanin in our laboratory (Esen, 2008). In order to reveal the physical interaction of p60-katanin and Septin3 proteins, either endogenous Septin3 or endogenous p60-katanin proteins were immunoprecipitated with specific antibodies from rat brain lysate and co-immunoprecipitations were detected with specific antibodies (Figure 3.1).

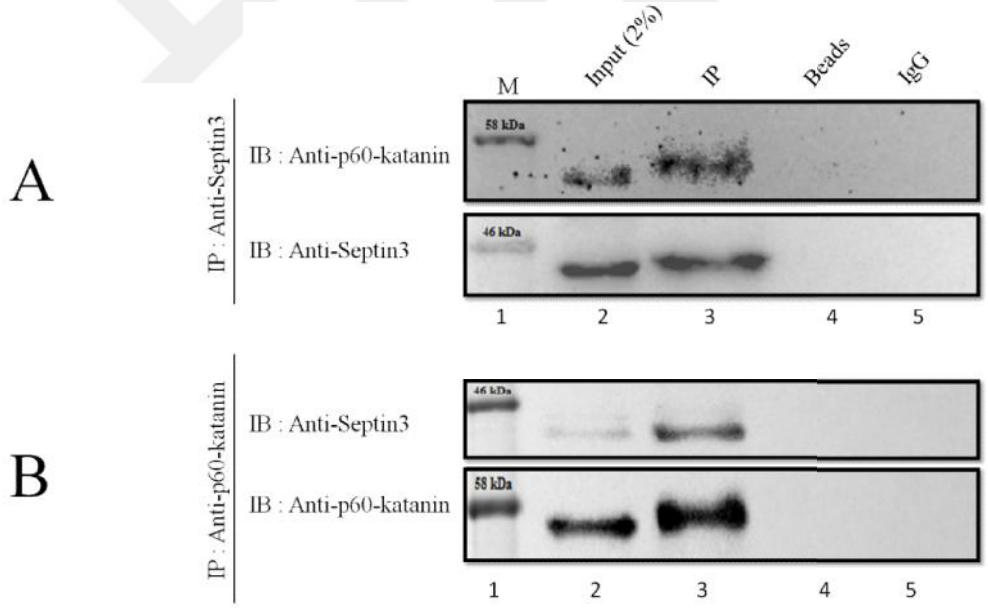


Figure 3.1 : Co-immunoprecipitation results. Part A shows immunoprecipitation with Septin3 specific antibody and part B shows immunoprecipitation with p60-katanin specific antibody. Lane 1: Protein marker, Lane 2: total protein lysate (2%) (15 µg), Lane 3: IP sample, Lane 4: beads only control, Lane 5: Non-specific IgG control (2 µg normal rabbit IgG for upper part, 2 µg normal mouse IgG for lower part of the figure).

Septin3 migrated at ~ 40 kDa as expected (Xue et al., 2004a) and p60-katanin migrated at ~ 55 kDa. The fourth and fifth lanes which were the negative controls of the assay showed that p60-katanin or Septin3 antibodies bound neither to protein G

on the beads nor to normal IgG non-specifically. The result has indicated that p60-katanin interacts with Septin3 in rat brain lysate consistent with the Y2H results from the previous study in our laboratory (Esen, 2008).

3.2 Localization of p60-katanin and Septin3 Proteins in Cortical Neurons

In order to observe in situ localization of p60-katanin and Septin3, primary cortical neurons were subjected to immunocytochemical analysis. p60-katanin and Septin3 proteins stained with Alexa Fluor® 647 (far-red fluorescent dye) and Alexa Fluor® 488 (green fluorescent dye) conjugated secondary antibodies are shown in Figure 3.2 respectively.

In image A and B, Septin3 protein localized in axons and soma of neurons, whereas p60-katanin proteins mostly localized in the cell bodies. Septin3 protein have been were observed as enriched along neurites as indicated previously (Tsang et al., 2011). In image C, p60-katanin protein was observed at the tips of short filaments of Septin3 in a neurite.

3.3 Cloning and Expression of p60-katanin and Septin3 constructs

p60-katanin and Septin3 DNA fragments were amplified from rat brain cDNA by PCR for cloning The PCR products run on agarose gels are shown in part A of Figure 3.3.

DNA fragments of p60-katanin and Septin3 migrated at ~1515 bp and ~1107 bp respectively as expected. Then, they were cloned to mammalian expression vectors. Over-expressed constructs were checked with western blotting to control expressions of FLAG-tagged p60-katanin and Myc/his-tagged Septin3 proteins using epitope tag antibodies (B of Figure 3.3).

FLAG-tagged p60-katanin full length (FL) protein contains 493 amino acids coming from the gene and 31 amino acids coming from p3XFLAG-CMV10 vector. Therefore, full length p60-katanin protein migrated at ~59 kDa as expected. Myc/his-tagged Septin3 full length (FL) protein contains 358 amino acids coming from the gene and 27 amino acids coming from pcDNA™ 3.1/myc-His A vector. Therefore, full length Septin3 protein migrated at ~ 44 kDa as expected.

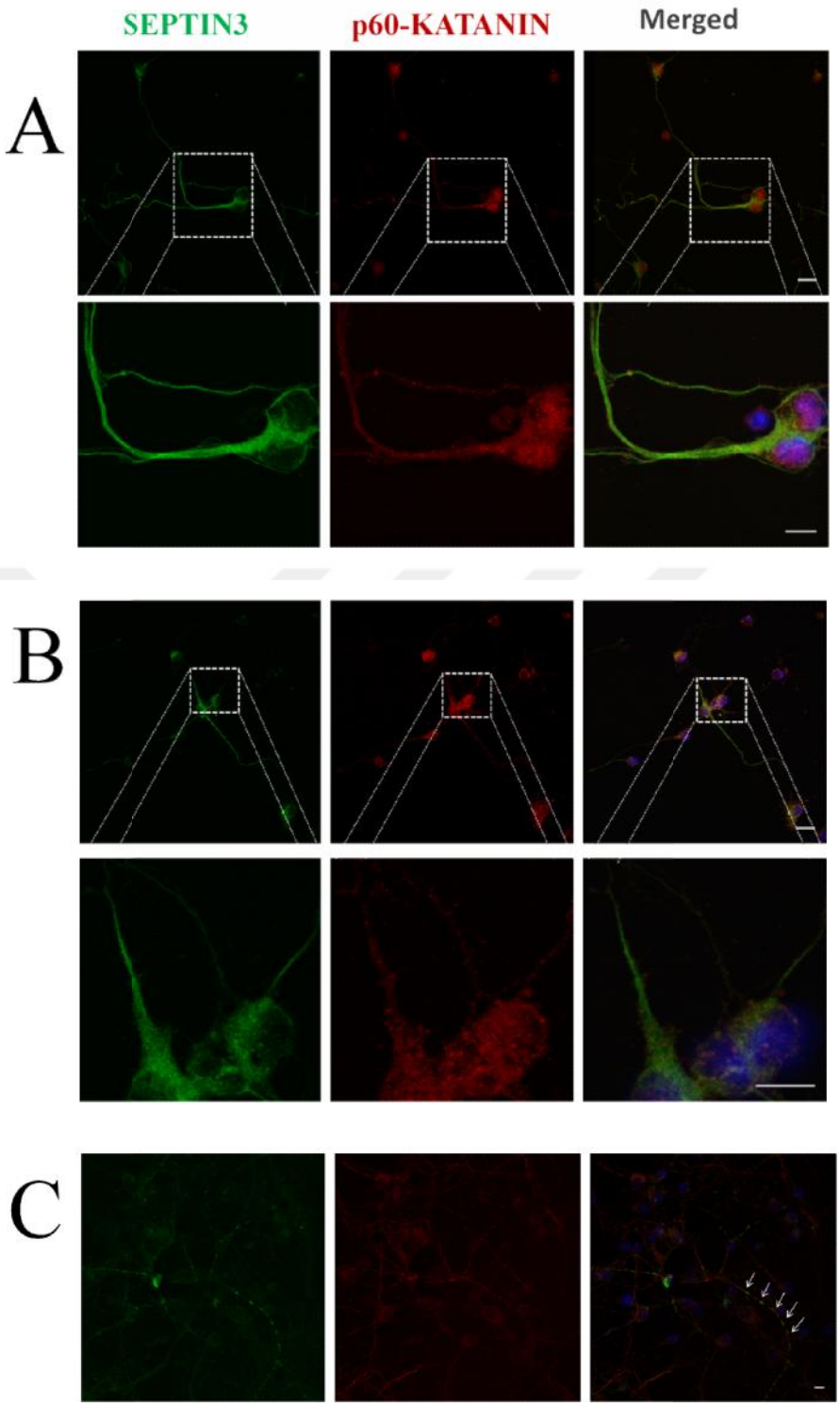


Figure 3.2 : Immunostaining of intrinsic Septin3 and p60-katanin in primary cortical neurons. Septin3 protein appears in green and p60-katanin protein is in red. The nucleus of neurons stained with DAPI is in blue. A and B are the images of two different cells, at DIV3 whereas C is the image of a cell at DIV7. Enlarged images of the framed areas in A and B are given just below them.The arrows in C indicates sequential alignment of Septin3 and p60-katanin proteins one after the other in a neurite. Scale bars, A and B are 40 µm, enlarged images and C are 20 µm.

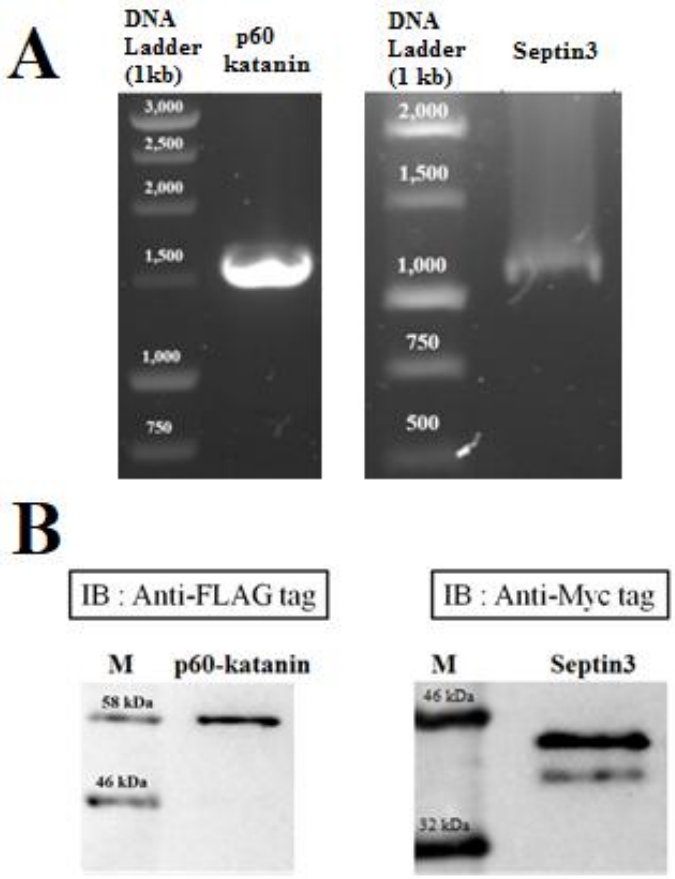


Figure 3.3 : Agarose gel images (A) and western blot (B) of p60-katanin and Septin3.

3.4 GTPase Activity of Septin3 Mutants

The GTPase activities of Septin3 WT (FL), Septin3-K208A, Septin3-R280A and Septin3-K208A&R280A constructs were analyzed measuring amount of free phosphate and the amount of GTP remaining after GTP hydrolysis. The amount of phosphates detected from the standard curve and the calculated GTPase activities according to the manufacturer's equation are listed in the Table 3.1.

Table 3.1 : GTPase activities and amount of phosphates.

Name of construct	GTPase activity (calculated) (U/L)	Amount of phosphate (detected) (µM)
Septin3 WT (FL)	6.83 U/L	51.26 µM
Septin3-K208A	5.59 U/L	41.96 µM
Septin3-R280A	5.09 U/L	38.21 µM
Septin3-K208A&R280A	4.64 U/L	34.83 µM

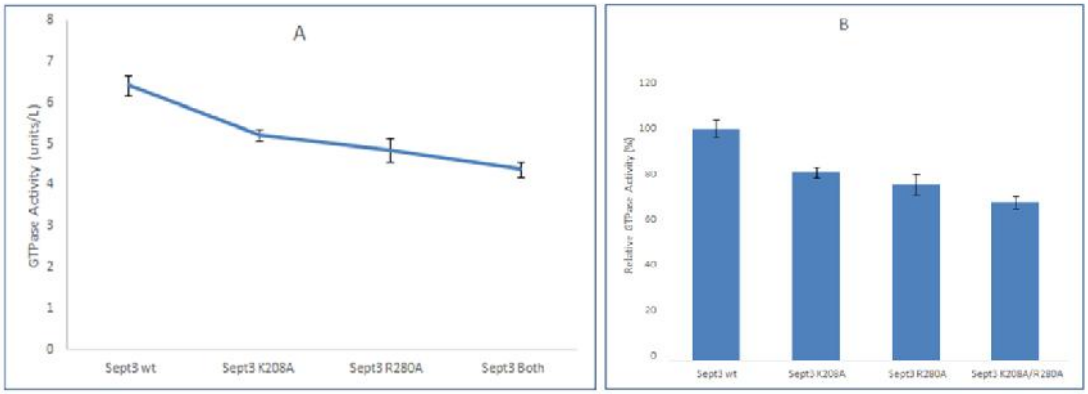


Figure 3.4 : Graphs of GTPase activities of wild-type and mutant Septin3. Panel A shows the calculated GTPase activities of constructs in U/L and panel B shows the GTPase activities of constructs normalized against wild-type Septin3 protein in percentage.

The graph plot according to the GTPase activities of constructs (Figure 3.4, A) showed that mutation of either Lys208 which is found at GTPase domain of Septin3 to Ala or Arg280 which is found at septin unique element of Septin3 to Ala separately decreased the GTPase activity of Septin3 and the lowest GTPase activity was detected when amino acids, Lys208 and Arg280, were both mutated. In addition, the Arg280 showed greater inhibition on GTPase activity of Septin3 than the mutation on Lys208. The GTPase activities normalized against wild-type Septin3 (Figure 3.4, B) showed that K208A mutation inhibited the GTPase activity of Septin3 protein by 18%, whereas R280A mutation inhibited the GTPase activity of Septin3 protein by 25%. Double mutations of K208A and R280A showed greater inhibition on the GTPase activity of Septin3 protein.

3.5 The Effects of Mutations on Filament Formation of Septin3

Septins form hetero-oligomeric complexes via GTP hydrolysis, which can then form bundles of filaments. Crystal structure of Septin3 has revealed multiple critical residues in its nucleotide binding pocket and septin unique element domain. These residues regulate Septin3 oligomerization by binding to variable moieties on GTP (Macedo et. al., 2013). In order to observe the effects of mutations on filament formation of Septin3, two amino acids, Lys208 and Arg280, were chosen based on the crystal structure. RFL6 cells were used for ICC and either wild-type or double mutant Septin3 constructs were over-expressed in RFL6 cells. Septin3 was stained

with Alexa Fluor® 488 (green fluorescent dye) and α-tubulin was stained with Alexa Fluor® 647 (far-red fluorescent dye).

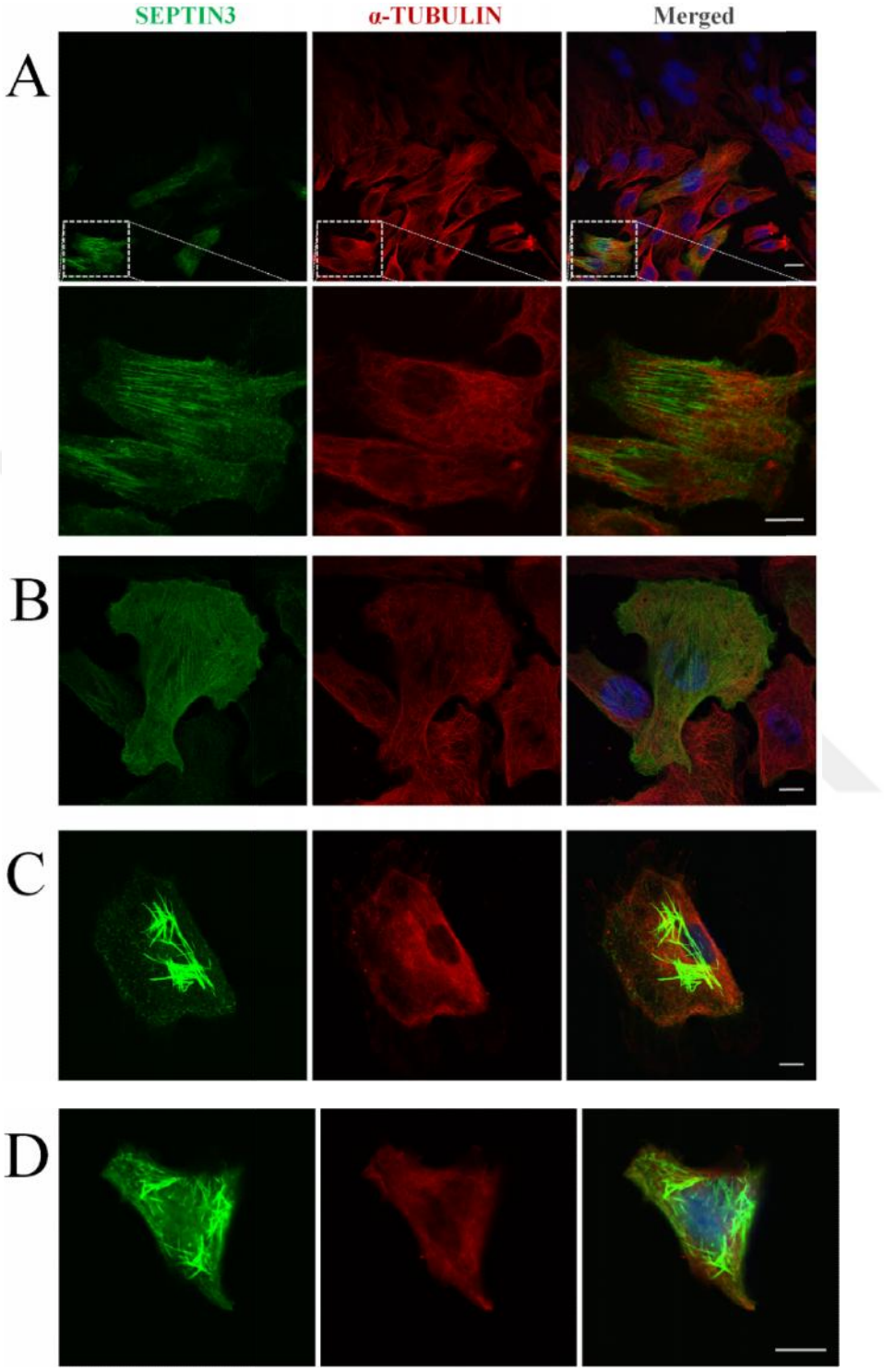


Figure 3.5: Immunostaining of wild-type Septin3 and microtubules in RFL6 cells. Septin3 protein appears in green and α-tubulin is in red. The nucleus of fibroblasts stained with DAPI is in blue. The enlarged images of the framed areas are shown under the images. The merged image is shown at right. Scale bars represent 40 µm for A, B, C and D and 20 µm for the enlarged image of A.

Wild-type Septin3 protein formed brush-like filaments that spread over the cell. Microtubules were observed as meshwork filaments and they were partially overlaying with Septin3 proteins in the cytoplasm. However, they did not exactly show the same manner in filament formation (Figure 3.5).

Double mutant Septin3 protein (Figure 3.6) did not form filamentous structures; rather, they were observed as punctiform all over the cell. Especially, they were seen to accumulate beneath the cell membrane. In addition, microtubules were observed to have distorted organization and they were overlaying with mutant Septin3 proteins at the cell membrane as well.

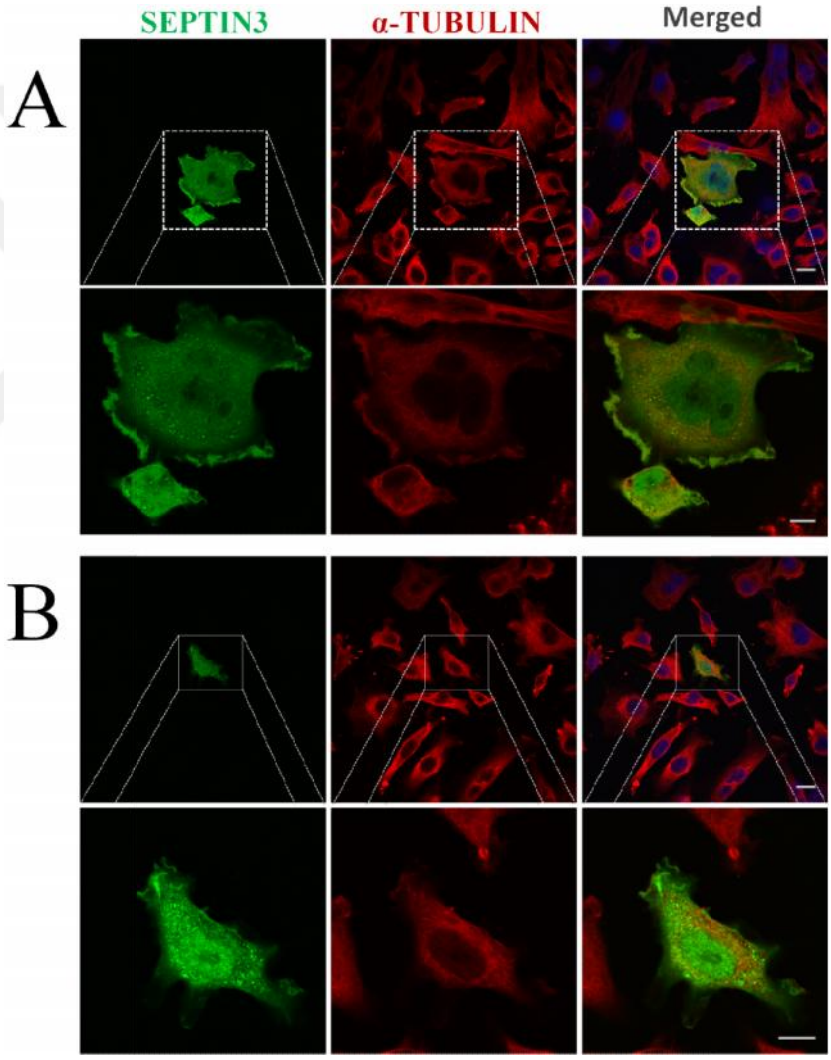


Figure 3.6: Immunostaining of double mutant Septin3 (K208A & R280A) and microtubules in RFL6 cells. Mutant Septin3 protein appears in green and α-tubulin is in red. The nucleus of fibroblasts stained with DAPI is in blue. The enlarged images of the framed areas are shown under the images. The merged images are shown at the right. Scale bars represent 40 µm for A and B, 20 µm for their enlarged images.

3.6 The Effects of Mutations of Septin3 on the Interaction with p60-katanin

In order to observe the effects of mutations of Septin3 on the interaction with p60-katanin, RFL6 cells have transfected with wild-type p60-katanin and wild-type or double mutant Septin3 were subjected to immunocytochemical analysis in which Septin3 was stained with Alexa Fluor® 488 (green fluorescent dye), p60-katanin was stained with Alexa Fluor® 594 (red fluorescent dye) and α- tubulin was stained with Alexa Fluor® 647 (far-red fluorescent dye) conjugated secondary antibodies.

Septin3 bundles present around the nucleus co-localized with p60-katanin (A in Figure 3.7). Septin3 formed brush-like filaments in the cells expressing only Septin3 (B in Figure 3.7), whereas shorter Septin3 filaments spread in cytoplasm were observed in the cells expressing both Septin3 and p60-katanin at the same time (B and C in Figure 3.7). In addition, the cells expressing both Septin3 and p60-katanin were smaller than the cells expressing only Septin3 and the microtubule dynamics of the cells have been observed to be distorted (B and C of Figure 3.7).

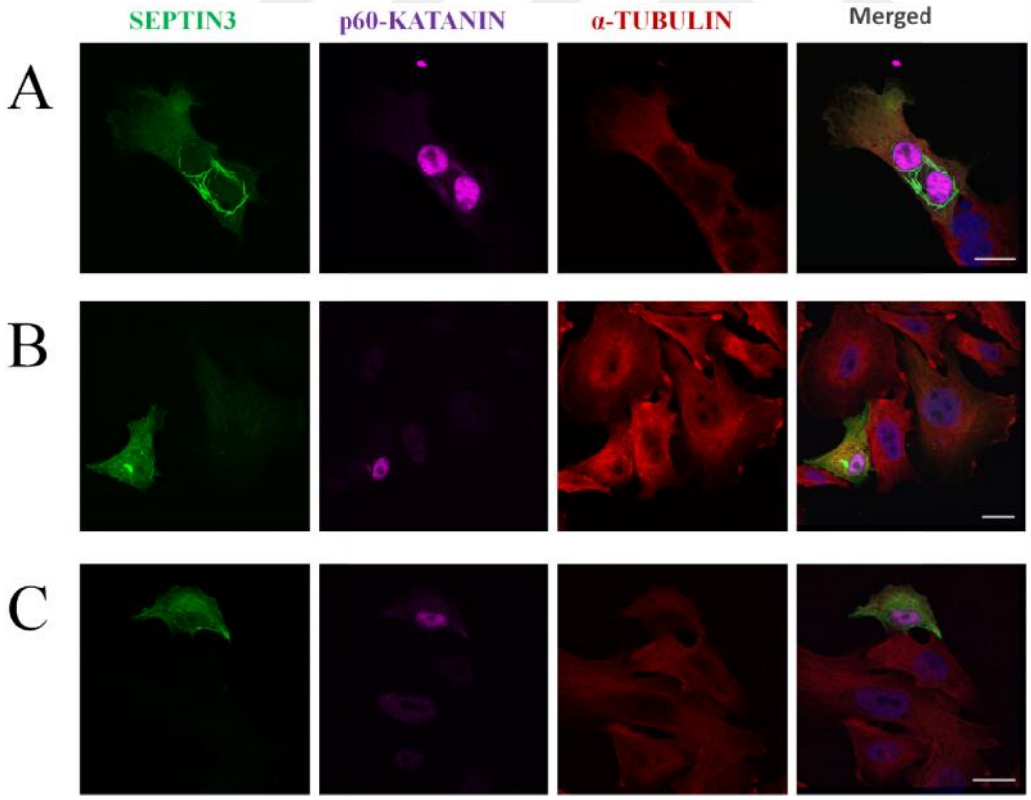


Figure 3.7: Immunostaining of wild-type Septin3, p60-katanin and microtubules in RFL6 cells. Septin3 proteins appears in green, p60-katanin appears in violet and α-tubulin appears in red. The nucleus of fibroblasts stained with DAPI is in blue. The merged images were shown at the right. Scale bars, 40 µm.

Double mutant Septin3 protein did not form filaments and they were observed as punctiform in the cytoplasm in which partially co-localized with p60-katanin. Microtubule structure of the cell expressing double mutant Septin3 and wild-type p60-katanin was seen to be lost their meshwork (Figure 3.8).

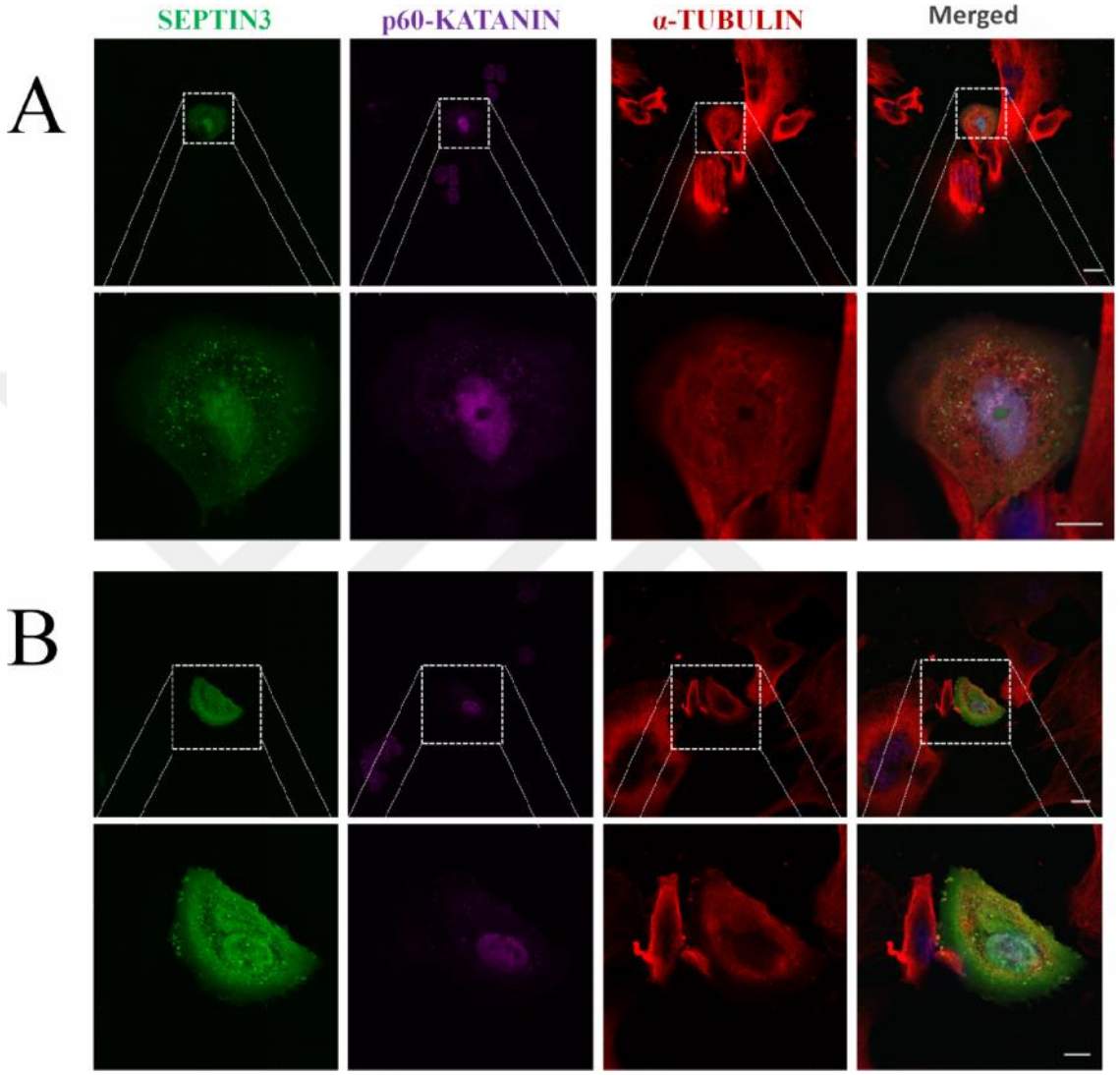


Figure 3.8: Immunostaining of double mutant Septin3 (K208A & R280A), p60-katanin and microtubules in RFL6 cells. Septin3 proteins appears in green, p60-katanin appears in violet and α-tubulin appears in red. The nucleus of fibroblasts stained with DAPI is in blue. The merged image was shown at the right. Scale bars represent 40 µm for A and B, and 20 µm for their enlarged images.

3.7 Identification of Interaction Domains of p60-katanin and Septin3

In order to reveal domains responsible for the interaction of p60-katanin and Septin3 proteins, co-immunoprecipitation assay was performed with the lysates containing

overexpressed Myc/his-tagged Septin3 FL with either FLAG-tagged p60-katanin FL or truncated FLAG-tagged p60-katanin protein (Figure 3.9).

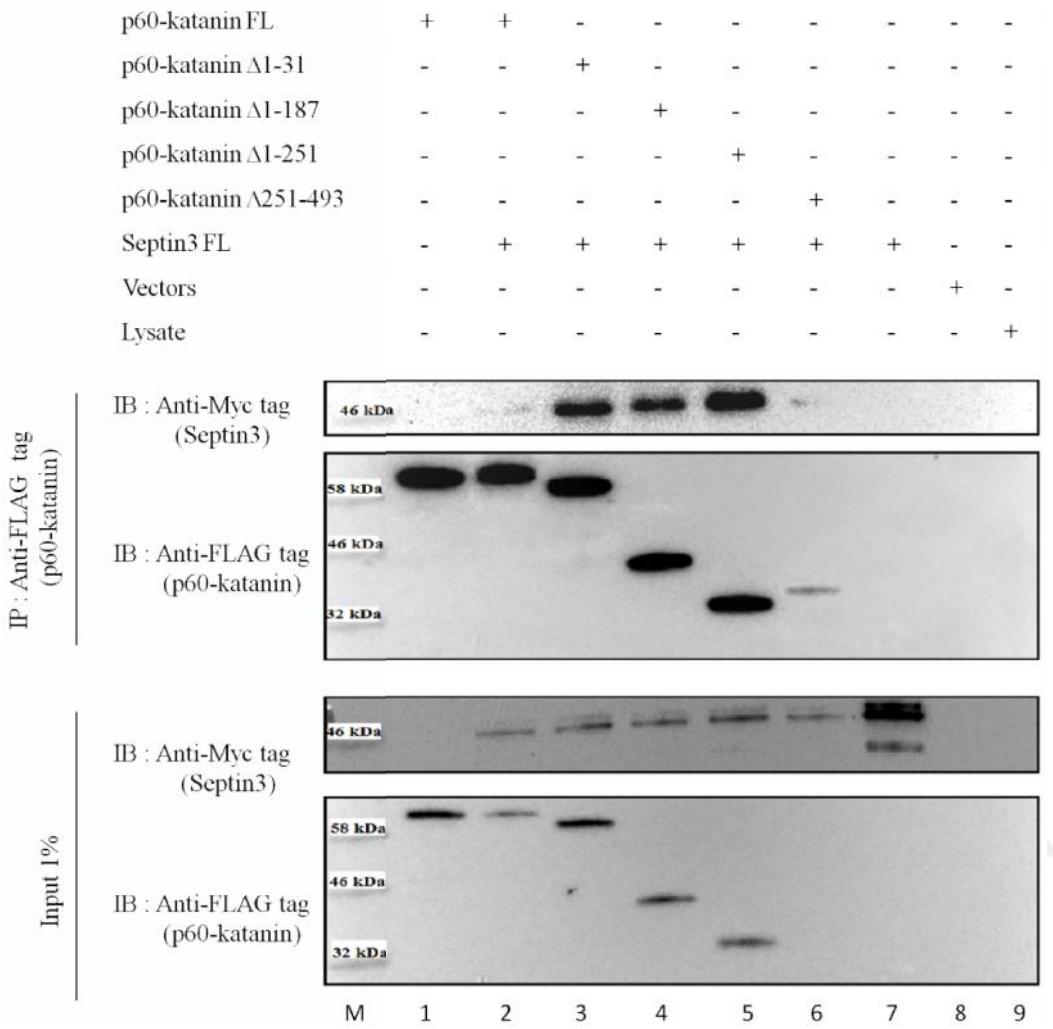


Figure 3.9 : Co-immunoprecipitation analysis to identify interaction domain of FLAG-tagged p60-katanin. (Upper part shows the lysates immunoprecipitated with anti-FLAG tag resin followed by immunoblotting with anti-Myc tag antibody and lower part shows their protein expression levels loaded as 1% of the total lysate (7.5 µg). Letter M denotes protein marker to compare molecular mass of the proteins. From lane 1 to lane 8, co-immunoprecipitation results with cell lysates co-transfected in HCT116 cells with different constructs as indicated in the upper part of the figure. Lane 9 is co-immunoprecipitation results of non-transfected HCT116 cell lysate.

FLAG tag resin precipitated FLAG-tagged p60-katanin proteins well, except FLAG-tagged p60-katanin Δ251-493 proteins. The interaction of full length p60-katanin and Septin3proteins was observed to be weaker than truncated p60-katanin proteins. The results showed that Myc/his-tagged Septin3 strongly bound to FLAG-tagged p60-katanin containing ATPase domain found in 251th and 493rd amino acids.

In order to find domains of Septin3 responsible for the interaction with p60-katanin, co-immunoprecipitation assay was performed with the lysates containing overexpressed FLAG-tagged p60-katanin FL with either Myc/his-tagged Septin3 or Myc/his-tagged truncated Septin3 proteins (Figure 3.10).

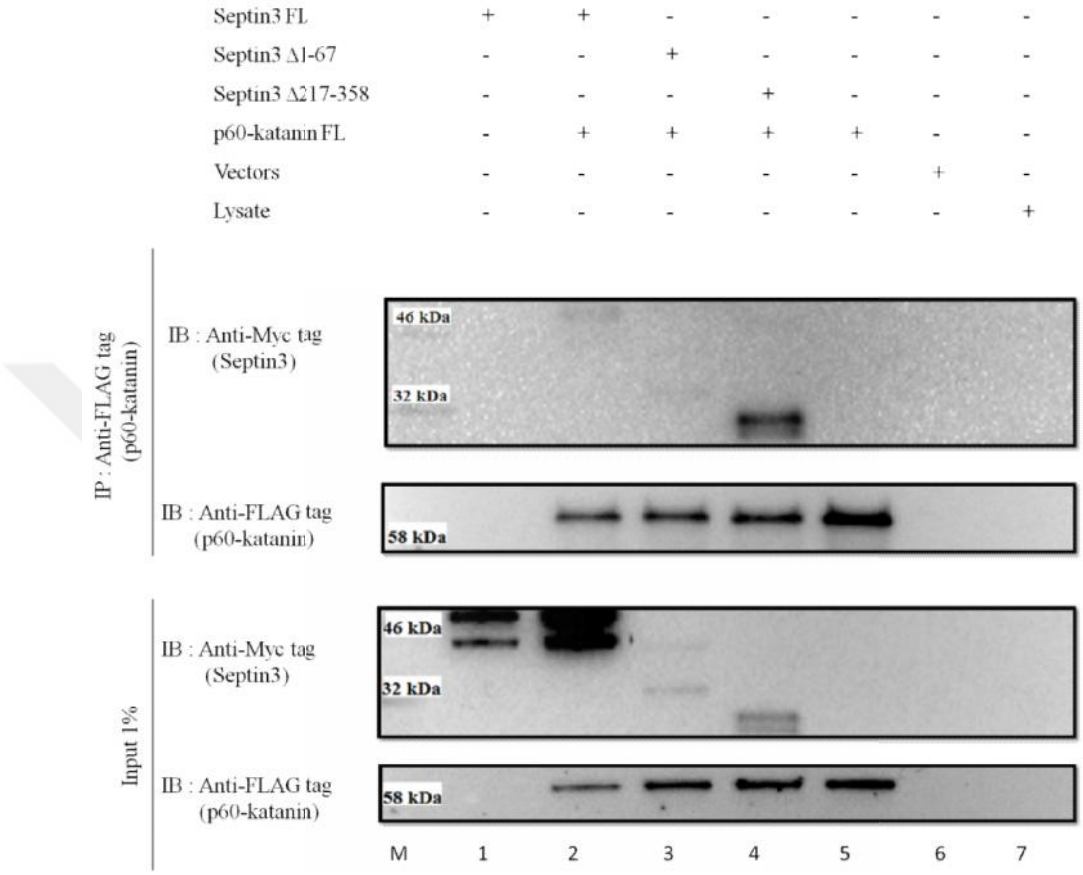


Figure 3.10: Co-immunoprecipitation analysis to identify interaction domain of Myc-tagged Septin3. Upper part shows the lysates immunoprecipitated with anti-FLAG tag resin followed by immunoblotting with anti-Myc tag antibody and lower part shows their protein expression levels loaded as 1% of the total lysate (7.5 µg). Letter M denotes protein marker to compare molecular mass of the proteins. From lane 1 to lane 8, co-immunoprecipitation results with cell lysates co-expressed in HCT116 cells with different constructs as indicated in the upper part of the figure. Lane 9 is co-immunoprecipitation results of non-transfected HCT116 cell lysate.

FLAG tag resin precipitated FLAG-tagged p60-katanin proteins almost at the same amount. Although FLAG-tagged p60-katanin proteins co-immunoprecipitated with all Myc/his-tagged Septin3 proteins regardless of missing domains which are proline-rich domain (Δ1-67) and septin unique element (Δ217-358); p60-katanin protein strongly bound to Septin3 protein when it lacked septin unique element found in between 217th and 358th amino acids.

4. DISCUSSION AND CONCLUSION

In this study, interaction of p60-katanin and Septin3 has been investigated in vitro through co-immunoprecipitation assays. The interaction of endogenous p60-katanin and Septin3 in rat brain lysate was confirmed reciprocally and it was shown that Septin3 binds to p60-katanin in brain lysate.

Different septins other than Septin3, have been shown to form either filamentous structures or insoluble aggregates upon over-expression in variable cells (Kim, Froese, Estey and Trimble, 2011; Abbey et. al., 2016). Therefore, Septin3 was over-expressed in RFL6 cells to observe how it would behave. Septin3 formed filamentous structures like Septin2 and Septin9 and it co-localized with microtubules as shown for other septins previously (Bowen, Hwang, Bai, Roy and Spiliotis, 2011; Sellin et al., 2013).

Septins belong to the superclass of P-loop NTPases, which includes the RAS-like GTP-binding proteins. Unlike RAS-like GTP-binding proteins, septins form hetero-oligomeric complexes in which multiple residues in GTPase domain and septin unique element are involved (Sirajuddin et al., 2007; Sirajuddin, Farkasovsky, Zent and Wittinghofer, 2009; Angelis and Spiliotis, 2016). Two amino acids of Septin3 which are conserved in SEPT2 (Appendix E), a well known septin, Lys208 and Arg280 were chosen to be mutated to Ala. Lys208 lies in GTPase domain and directly interacts with the ribose moity of GTP (Sirajuddin, Farkasovsky, Zent and Wittinghofer, 2009). In addition, Arg280 which lies in septin unique element of Septin3 has been shown that it binds not only to GTP but also to another Septin3 monomer. Thus, Arg280 is responsible for dimerization and septin-septin interaction (Sirajuddin, Farkasovsky, Zent and Wittinghofer, 2009; Macedo et al., 2013). I have shown that Arg280 mutation has decreased the GTPase activity of Septin3 more than the mutation of Lys208. Since, Arg280 binds both second monomer and GTP at the same time, it decreased GTPase activity more than Lys208 as it would be expected. In the GTPase domain of Septin3, there are also many important residues binding to

phosphate moiety and guanine base of GTP such as Ser75, Thr102, Asp210 and Gly265. However, the only two residues appear to interact directly with ribose moiety of GTP are Lys208 and Arg280. Hence, these two residues are specifically important for GTPase activity of Septin3 (Macedo et al., 2013). Another critical amino acid for GTPase activity could have been Thr102 of Septin3. This threonine residue is lack in septins of SEPT6 group which has shown to cause defect in GTP hydrolysis (Sirajuddin, Farkasovsky, Zent and Wittinghofer, 2009). Nevertheless, Lys208 and Arg280 mutations in Septin3 havae blocked filament formation and mutated Septin3 has been observed as punctiform.

The role of septins on microtubule dynamics has been demonstrated and it has been shown that septins not only suppress microtubule plus end catastrophe but also they spatially guide microtubule growth and positioning (Spiliotis, 2010). Septin3 overexpressed RFL6 cells have showed that brush-like Septin3 filaments, yet microtubules have formed meshwork filaments in these cells. Septins have been shown to partially overlay with both microtubules and actins, but they do not totally overlap with either microtubules or actins and therefore considered as the fourth cytoskeletal element (Sellin, Sandblad, Stenmark and Gullberg, 2011; Mostowy and Cossart, 2012). In my results, Septin3 also formed different filament structures than microtubules.

Endogenous Septin3 and p60-katanin proteins were observed in primary cortical neurons and p60-katanin localized at the tips of short Septin3 filaments. In addition, intact Septin3 filaments could not be observed in Septin3 and p60-katanin co-expressed RFL6 cells. This brings up the possibility of Septin3 being a new substrate for p60-katanin. On the other hand, Septins might organize microtubules by regulating their dynamics. In a study, knocking down Septin2 reduced the appearance of septin filaments forming shorter septin filaments which were associated with the ends of microtubules that are failed to properly target to the cell cortex in the absence of Septin2. Indeed, knocking down Septin2 increased the frequency and duration of microtubule “catastrophes” suggesting that septin filaments promote the growth of microtubules by inhibiting their depolymerization. (Bowen, Hwang, Bai, Roy, and Spiliotis, 2011). Since p60-katanin has roles in microtubule dynamics by severing microtubules, septins may be affected by p60-

katanin severing of microtubules. For both possibilities, additional experiments needs to be carried out to enlighten the exact relation.

REFERENCES

Abbey, M., Hakim, C., Anand, R., Lafera, J., Schambach, A., Kispert, A., Taft, M. H., Kaever, V., Kotlyarov, A., Gaestel, M., Menon, M. B., (2016). GTPase domain driven dimerization of SEPT7 is dispensable for the critical role of septins in fibroblast cytokinesis. *Scientific Reports, 6:*20007.

Ahmad, F. J., Yu, W., McNally, F. J., Baas, P. W., (1999). An Essential Role for Katanin in Severing Microtubules in the Neuron. *Journal of Cell Biology*,145(2):305-15.

Alberts, B. Roberts, K. Lewis, J. Raff, M. Walter, P. Johnson, A., (2014). Molecular Biology of the Cell 6e, *Garland Science***.**

Amir, S., Mabjeesh N. J., (2007). SEPT9_V1 protein expression is associated with human cancer cell resistance to microtubule-disrupting agents. *Cancer Biology & Therapy*, 6(12), 1926-31.

Angelis, D., Spiliotis, E. T., (2016). Septin mutations in human cancers. *Frontiers in Cell and Developmental Biology, 4:122.*

Baas, P. W., Karabay, A., Qiang, L., (2005), Microtubules cut and run. *Trends in Cell Biology*, 15, 518-524.

Bai, X., Bowen, J. R., Knox, T. K., Zhou, K., Pendziwiat, M., Kuhlenbäumer, G., Sindelar, C. V., Spiliotis, E. T., (2013). Novel septin 9 repeat motifs altered in neuralgic amyotrophy bind and bundle microtubules. *Journal of Cell Biology*, 203, 895–905.

Beites, C. L., Xie, H., Bowser, R., and Trimble, W. S., (1999). The septin CDCrel-1 binds syntaxin and inhibits exocytosis. *Nature Neuroscience*, 2(5), 434-439.

Bertin A., McMurray M. A., Grob, P., Sang-Shin, P., Garcia, III.G, Patanwala, I., Ng, H., Alber, T., Thorner, J., and Nogales, E., (2008). *Saccharomyces cerevisiae* septins: supramolecular organization of heterooligomers and the mechanism of filament assembly. *Proceeding of the National Academy of Scinces of United States of America*, 105, 8274–8279.

Bowen, J.R., Hwang, D., Bai, X., Roy, D., Spiliotis, E., (2011). Septin GTPases spatially guide microtubule organization and plus end dynamics in polarizing epithelia. *Journal of Cell Biology*, 194(2), 187–197

Bowne-Anderson, H., Zanic, M., Kauer, M., Howard, J., (2013). Microtubule dynamic instability: A new model with coupled GTP hydrolysis and multistep catastrophe. *Bioessays*, 35(5): 452–461.

Cassimeris L., (1999). Accessory protein regulation of microtubule dynamics throughout the cell cycle. *Current Opinion in Cell Biology*, 11, Issue 1, 1 134–141.

Conde, C. and Cáceres, A., (2009). Microtubule assembly, organization and dynamics in axons and dendrites. *Nature reviews, Neuroscience*, 10(5), 319-332.

Cooper T.G., (2005). Cytoplasmic droplets: the good, the bad or just confusing? *Oxford Academic, Human Reproduction*, 20, 9–11.

Desai, A. and Mitchison T. J., (1997). Microtubule polymerization dynamics. *Annu. Rev. Cell Dev. Biol*., 13, 83-117.

Dolat, L., Hu, Q., Spiliotis, E. T., (2014). Septin functions in organ system physiology and pathology. *Biological Chemistry,* 395(2):123-41.

Engidawork, E., Gulesserian, T., Fountoulakis, M., and Lubec, G., (2003). Aberrant protein expression in cerebral cortex of fetus with Down syndrome. *Neuroscience,* 122, 145–154.

Esen, D., (2008). Identification of katanin p60 interacting proteins. Master thesis, İstanbul Technical University, Institute of Science and Technology, İstanbul.

Fujishima, K., Kiyonari, H., Kurisu, J., Hirona, T., Kengaku, M., (2007). Targeted disruption of Sept3, a heteromeric assembly partner of Sept5 and Sept7 in axons, has no effect on developing CNS neurons. *Journal of Neurochemistry,* 102(1):77-92.

Ghosh, D. K., Dasgupta, D., Guha, A., (2012). Models, Regulations, and Functions of Microtubule Severing by Katanin. *ISRN Molecular Biology*, 2012:596289.

Hall, P. A., Jung, K., Hillan, K. J., Russell S. E., (2005). Expression profiling the human septin gene family. *Journal of Pathology*, 206, 269-278.

Hartman, J. J., Vale, R. D., (1999). Microtubule disassembly by ATP-dependent oligomerization of the AAA enzyme katanin. *Science*, 286(5440), 782-5.

Hartwell H. L., (1967). Macromolecule Synthesis in Temperature-sensitive Mutants of Yeast. *Journal of Bacteriology*, 93(5), 1662–1670.

Horio, T., Hotani, H., (1986). Visualization of the dynamic instability of individual microtubules by dark-field microscopy. *Nature*, 321; 605-607.

Horio, T., Murata, T., (2014). The role of dynamic instability in microtubule organization. *Frontiers in plant science*,7;5:511.

Hyman, A. A., Salser, S., Drechsel, D. N., Unwin, N., Mitchison, T. J., (1992). Role of GTP hydrolysis in microtubule dynamics: information from a slowly hydrolyzable analogue, GMPCPP. *Molecular Biology of the Cell*, 3(10), 1155–67.

Ihara, M., Tomimoto, H., Kitayama, H., Morioka, Y., Akiguchi, I., Shibasaki, H., Noda, M., Kinoshita, M., (2003). Association of the cytoskeletal GTP-binding protein Sept4/H5 with cytoplasmic inclusions found in Parkinson's disease and other synucleinopathies. *Journal of Biological Chemistry*, 278, 24095–24102.

Johjima, A., Noi, K., Nishikori, S., Ogi, H., Esaki, M., Ogura, T., (2015). Microtubule severing by katanin p60 AAA+ ATPase requires the C-terminal acidic tails of both α- and β-tubulins and basic amino acid residues in the AAA+ ring pore. *The journal of biological chemistry*, 290(18):11762-70

John, C. M., Hite, R. K., Weirich, C. S., Fitzgerald D. J., Jawhari, H., Faty, M., Schläpfer, D., Kroschewski, R., Winkler, F. K., Walz, T., Barral, Y., Steinmetz, M. O., (2007). The Caenorhabditis elegans septin complex is nonpolar. *The EMBO Journal*, 26, 3296–3307.

Karabay, A., Yu, W., Solowska, J. M., Baird, D. H., Baas, P. W., (2004). Axonal Growth Is Sensitive to The Levels of Katanin, a Protein That Severs Microtubules. *J. Neurosci*., 24, 5778-5788.

Kim, M. S, Froese, C. D., Estey, M. P., Trimble, W. S., (2011). SEPT9 occupies the terminal positions in septin octamers and mediates polymerization-dependent functions in abscission. *Journal of Cell Biology*, 195(5):815-26.

Kinoshita, M., (2003). Assembly of mammalian septins. *Journal of Biochemistry*, 134, 491–496.

Kinoshita, A., Kinoshita, M., Akiyama, H., Tomimoto, H., Akiguchi, I., Kumar, S., Noda, M., Kimura, J., (1998). Identification of septins in neurofibrillary tangles in Alzheimer's disease. *American Journal of Pathology*, 153, 1551–1560.

Kinoshita, M.,Tomimoto, H., Kinoshita, A., Kumar, S., Noda, M., (1997). Up-regulation of the Nedd2 gene encoding an ICE/Ced-3-like cysteine protease in the gerbil brain after transient global ischemia. *Journal of Cerebral Blood Flow & Metabolism*, 17(5), 507-514.

Korulu, S., Karabay, A., (2011). IGF-1 participates differently in regulation of severing activity of katanin and spastin. *Cellular and molecular neurobiology*, 31(4), 497-501.

Kremer, B.E., Haystead, T., Macara, I.G., (2005). Mammalian septins regulate microtubule stability through interaction with the microtubule-binding protein MAP4. *Molecular Biology of the Cell*, 16(10), 4648-4659.

Kuo, Y. C., Shen, Y. R., Chen, H. I., Lin, Y. H., Wang, Y. Y., Chen, Y. R., Wang, C. Y., Kuo, P. L., (2015). SEPT12 orchestrates the formation of mammalian sperm annulus by organizing core octameric complexes with other SEPT proteins. *Journal of Cell Science*, 128(5):923-34.

Lodish, H., Berk, A., Kaiser, C. A., Krieger, M., Scott, M. P., Bretscher, A., Ploegh, H., Matsudaira, P., (2008). Molecular Cell Biology 6e, *Macmillan Learning*, Chp.18.

Longtine, M. S., DeMarini, D. J., Valencik, M L., Al-Awar, O. S., Fares, H., De Virgilio, C., Pringle, J. R., (1996). The septins: roles in cytokinesis and other processes. *Current. Opinion.in Cell Biology.* 8:106-119.

Macedo, J. N., Valadares, N. F., Marques, I. A., Ferreira, F. M., Damalio, J. C., Pereira, H. M., Garratt, R. C., Araujo, A. P., (2013). The structure and properties of septin 3: a possible missing link in septin filament formation. *Biochemical Journal*, 450, 95-105

Mattila, P. K., Lappalainen, P., (2008). Filopodia: molecular architecture and cellular functions. *Nature Reviews, Molecular Cell Biology*, 9, 446-454.

McNally, F. J., Vale, R. D., (1993). Identification of katanin, an ATPase that severs and disassembles stable microtubules. *Cell*, 75, 419-29.

McNally, K. P.,Bazirgan, O. A., McNally, F. J., (2000). Two domains of p80 katanin regulate microtubule severing and spindle pole targeting by p60 katanin. *J. Cell Sci.*, 113, 1623-1633

Mehrbod, M., Mofrad M. R. K., (2011). On the significance of microtubule flexural behavior in cytoskeletal mechanics. *PLoS ONE*, 6(10), e25627.

Meurer-Grob, P., Kasparian, J., Wade, R. H., (2001). Microtubule structure at improved resolution. *Biochem.*, 40, 8000-8008.

Mickey, B., Howard, J., (1995). Rigidity of microtubules is increased by stabilizing agents. *Journal of Cell Biology*, 130, 909–917.

Montagna, C., Bejerano-Sagie, M., Zechmeister, J. R., (2014). Mammalian septins in health and disease. *Research and Reports in Biochemistry*, 5, 59-72.

Mostowy, S., Cossart, P., (2012). Septins: the fourth component of the cytoskeleton. *Nature Reviews Moecular Cell Biology*, 5, 13(3):183-94.

Nagata, K., Kawajiri, A., Matsui, S., Takagishi, M., Shiromizu, T., Saitoh, N., Izawa, I., Kiyono, T., Itoh, T.J., Hotani, H., Inagaki, M., (2003). Filament formation of MSF-A, a mammalian septin, in human mammary epithelial cells depends on interactions with microtubules. *Journal of Biological Chemistry*, 278, 18538-18543.

Nakahira, M., Macedo, J. N., Seraphim, T. V., Cavalcante, N., Souza, T. A., Damalio, J. C., Reyes, L. F., Assmann, E. M., Alborghetti, M. R., Garratt, R. C., Araujo, A. P., Zanchin, N. I., Barbosa, J. A., Kobarg, J., (2010). A draft of the human septin interactome. *PLoS One*, ;5(11):e13799.

O'Donnell, L., Rhodes, D., Smith, S. J., Merriner, D. J., Clark, B. J., Borg, C., Whittle, B., O'Connor, A. E., Smith, L. B., McNally, F. J., de Kretser, D. M., Goodnow, C. C., Ormandy, C. J., Jamsai, D., O'Bryan, M. K., (2012). An essential role for katanin p80 and microtubule severing in male gamete production. *PLoS Genetics*, 8(5).

Peterson, E. A, Kalikin, L. M., Steels, J. D., Estey, M. P., Trimble, W. S., Petty, E. M., (2007). Characterization of a SEPT9 interacting protein, SEPT14, a novel testis-specific septin. *Mamm. Genome*, 18(11):796-807.

Quarmby, L. M., Lohret, T. A., (1999). Microtubule severing. *Cell motility and the cytoskeleton*, 43(1):1-9.

Sellin, M. E., Sandblad, L., Stenmark, S., Gullberg, M., (2011). Deciphering the rules governing assembly order of mammalian septin complexes. *Molecular Biology of the Cell*, 22:3152-3164.

Sellin, M. E., Stenmark, S., Gullberg, M., (2013). Cell type-specific expression of SEPT3-homology subgroup members controls the subunit number of heteromeric septin complexes. *Molecular Biology of the Cell*, 25(10:1594-607.

Shehadeh, L., Mitsi, G., Adi, N., Bishopric, N., Papapetropoulos, S., (2009). Expression of Lewy body protein septin 4 in postmortem brain of Parkinson's disease and control subjects. *Mov. Disord.,* 24, 204–210.

Silverman-Gavrila, R., Silverman-Gavrila, L., (2008). Septins: new microtubule interacting partners. *The Scientific World Journal*, 8, 611-620.

Sirajuddin, M., Farkovsky, M., Hauer, F., Kühlmann, D., Macara, I. G., Weyand, M., Stark, H., Wittinghofer, A., (2007). Structural insight into filament formation by mammalian septins. *Nature,* 449(7160):311-5.

Sirajuddin, M., Farkovsky, M., Zent, E., Wittinghofer, A., (2009). GTP-induced conformational changes in septins and implications for function. *Proc Natl Acad Sci U S A,* 106(39):16592-7.

Spiliotis, E.T., (2010). Regulation of microtubule organization and functions by septin GTPases. *Cytoskeleton (Hoboken),* 67(6), 339-45.

Tsang, C. W., Fedchyshyn, M., Harrison, J., Xie, H., Xue, J., Robinson, P. J., Wang, L. Y., Trimble, W. S., (2008). Superfluous role of mammalian septins 3 and 5 in neuronal development and synaptic transmission. *Mol.Cell Biol.,* 28(23):7012-29.

Tsang, C. W., Estey, M. P., DiCiccio, J. E., Xie, H., Patterson, D., Trimble, W. S., (2011). Characterization of presynaptic septin complexes in mammalian hippocampal neurons. *Biol. Chem.* 392(8-9):739-49.

Weirich, C. S., Erzberger J. P., Barral, Y., (2008). The septin family of GTPases: architecture and Dynamics. *Nature Reviews Molecular Cell Biology*, 9, 478-489.

Xue, J., Tsang, C. W., Gai, W. P., Malladi, C. S., Trimble, W. S., Rostas, J. A., Robinson, P.J., (2004a). Septin 3 (G-septin) is a developmentally regulated phosphoprotein enriched in presynaptic nerve terminals. *Journal of Neurochemistry*, 91(3), 579–590.

Xue, J., Milburn, P.J., Hanna, B. T., Graham, M. E., John, A. P., Rostas, J. A. P., Robinson, P. J., (2004b). Phosphorylation of septin 3 on Ser-91 by cGMP-dependent protein kinase-I. *Biochemical Journal* 1;381,753-60.

APPENDICES

APPENDIX A: The instruments used in this study are listed together with Supplier Companies.

APPENDIX B: The equipment used in this study is listed together with Supplier Companies.

APPENDIX C: The chemicals used in this study are listed together with Supplier Companies.

APPENDIX D: The commercial kits and the enzymes used in this study are listed together with Supplier Companies.

APPENDIX E: Conserved residues of rat Septin3 and Septin2**.**

APPENDIX F: The Chromotogram Images of Septin3 mutants.

APPENDIX A

Table A.1 : The instruments used in this study are listed together with Supplier Companies.

Instrument	Supplier Company
Centrifuges	Biolab SIGMA 6K15, Beckman Coulter Microfuge®18, Beckman Coulter AvantiTM J-30 I, IECCL10 Centrifuge, Thermo Electron Corporation, Labnet, Labnet International C1301-230V
pH Meter	Mettler Toledo
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)
Hemocytometer	FisherLab Scientific, 0267110
Ice Machine	Scotsman AF 10
Tissue Culture Incubator with CO_2	Steri-Cycle, Thermo Scientific
Medical X-ray Processor	Kodak
Magnetic Stirrer	Isolab
Microplate Spectrophotometer	BİO-RAD Benchmark Plus
Magnetic Separation Rack	Promega
Quick Spin	Labnet Spectrafuge C1301 Mini Centrifuge
Micro Pipette	Eppendorf micropipette; 10 µL,100 µL, 200 µL, 1000 µL Mettler Toledo Volumate Liquid System; 0.1-2.5 µL
Power Supply	Bio-Rad Powerpack Basic
Western Blot Transfer System	Bio-Rad Trans-Blot® Turbo™ (Semi-Dry)
SDS-PAGE Gel Electrophoresis System	Mini-Protean® Tetra Cell, Bio-Rad
SDS-PAGE Gel Casting System	Mini-Protean®, Tetra Handcast System, Bio-Rad
Gel Imaging System	Gel Doc™ XR+Imager, Bio-Rad
Thermocycler	Bio-Rad T100™ Thermal Cycler
UV-Visible Spectrophotometers	NanoDrop 2000, Thermo scientific
Vortex	Heidolph Reaxtop
Waterbath	Electro-Mag M96KP
Heat-block	VWR
Rocking Shaker Platform	VWR
Tube Rotator	Rotator SB3, Stuart
Laboratory Hood	Laminar Flow/Biosafety level Class II
Dissection Microscope	Leica
Light Microscope	CK40-F200, Olympus
Confocal Microscope	TCS SP2 SE confocal microscope, Leica
Automated Cell Counter	Countess™, Invitrogen
Nucleofector	Nucleofector™ 2b device, Lonza

APPENDIX B

Table B.1: The equipment used in this study is listed together with Supplier Companies.

Equipment	Supplier Company
Falcon Tubes	Sarstedt
Eppendorf tubes	Axygen
Cell Scraper	TPP
Nitrocellulose Transfer Membrane (0.22 µm)	Bio-Rad
Tissue Culture Flasks	Sarstedt
Tissue Culture Plates	Sarstedt
Tissue Culture Dish	TPP
Inoculation Loop	Isolab
Glass Coverslips (18mm diameter)	Marienfeld-Superior
Petri plate	Isolab
Weighing dish	Isolab
Scalpel	Kruuse
Glass beads (≤106µm)	Sigma-Aldrich
Mini-PROTEAN® SDS gel plates	Bio-Rad
SDS Blot Roller	Bio-Rad
Glass Pasteur Pipette	Isolab
Dumont-style forceps (no:5)	Dumont
Cell Counting Chamber (Countess™ Counting Chamber Slide)	Invitrogen

APPENDIX C

Table C.1: The chemicals used in this study are listed together with Supplier Companies.

Chemicals	Supplier Company
Ethanol (absolute, ≥99.8% (GC))	Sigma-Aldrich
Methanol (for HPLC, ≥99.9%)	Sigma-Aldrich
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Ampicillin sodium Salt	Sigma-Aldrich
Tricine	Sigma-Aldrich
Phosphate buffered saline (PBS) Tablets	VWR Amresco Life Science
Paraformaldehyde	EMD Merck Millipore
D(+) Glucose, Anhydrous	EMD Merck Millipore
SeaKem® LE Agarose	Lonza
Saponin	Sigma-Aldrich
Nonfat Dry Milk	Cell Signaling Technology
Tryptone	LabM, Neogen Company
Sodium chloride (NaCl)	EMD Merck Millipore
Tris Base (Molecular Biology Grade)	EMD Merck Millipore
Yeast Extract	Pronadisa, Conda
Glycine	EMD Merck Millipore
Agar (American Bacteriological Agar)	Pronadisa, Conda
Ammonium persulfate (APS)	Fluka, Sigma-Aldrich
Tetramethylethylenediamine (TEMED)	EMD Merck Millipore
Ponceau S	EMD Merck Millipore
Glycerol	Fischer Scientific
Tween® 20	Fischer Scientific
Nonidet® P40 (NP40)	Applichem
Sodium dodecyl sulfate (SDS)	EMD Merck Millipore
Ethylenediaminetetraacetic acid	EMD Merck Millipore
Saponin	Sigma-Aldrich
Guanosine 5’-triphosphate sodium salt hydrate	Sigma-Aldrich
Hydrochloric acid (HCl) (37%)	EMD Merck Millipore
DMSO	Thermo Fischer Scientific
Fetal Bovine Serum	Biowest
Protein G Magnetic Beads	Cell Signaling Technology
Protein Ladders	GENE’s Protein Ladder PS10 Plus Prestained, GeneOn; Color Prestained Protein Standard, NEB

Table C.1 (continued): Chemicals used in this study are listed together with Supplier Companies

Chemicals	Supplier Company
DNA Loading Dye	6X DNA Loading Dye, New England Biolabs
DNA Ladders	MassRuler DNA Ladder Mix, Thermo Scientific
Protein Sample Buffer	3X SDS Sample Buffer, New England Biolabs
Trizol Reagent	Ambion, Life Technologies
Chloroform	EMD Merck Millipore
Isopropanol (2-propanol)	EMD Merck Millipore
Nuclease-free dH_2O	EMD Merck Millipore
TransFast Transfection Reagent	Promega
Filtration System	Stericup® FilterUnit (0.22µm)
Parafilm	Parafilm M®, Bemis
Mounting Medium	Prolong® Mounting Medium, Invitrogen
DMSO	
dNTP (10mM)	New England Biolabs
Nucleic Acid Stain	Pronasafe, Pronadisa Conda
DAPI(4’,6-Diamidino-2-Phenylindole, Dihydrochloride)	Invitrogen

APPENDIX D

Table D.1 : The commercial kits and the enzymes used in this study are listed together with Supplier Companies.

Kits, Antibodies and Enzymes	Supplier Company
Halt™ Protease Inhibitor Cocktail (100X)	Pierce, Thermo
BCA Protein Assay Kit	Pierce, Thermo
ATPase/GTPase Activity Assay Kit	Sigma-Aldrich
Rabbit pAb p60-katanin (KATNA1)	Atlas
Mouse mAb Septin3 (G-6)	Santa Cruz Biotechnology
Anti-mouse IgG, HRP-linked	Cell Signalling Technology
Anti-rabbit IgG, HRP-linked	Cell Signalling Technology
Rabbit pAb Vinculin	Cell Signalling Technology
Chicken pAb α-tubulin	Abcam
Mouse mAb β-actin	Cell Signalling Technology
Mouse mAb β-tubulin	Cell Signalling Technology
Rabbit pAb β-tubulin	Cell Signalling Technology
Normal rabbit IgG	Cell Signalling Technology
DNase (10 mg/mL)	Sigma-Aldrich
Nuclofector Kit R	Lonza
Anti-chicken IgG conjugated Alexa Fluor 647	Invitrogen
Anti-rabbit IgG conjugated Alexa Fluor 594	Cell Signalling Technology
Anti-rabbit IgG conjugated Alexa Fluor 647	Cell Signalling Technology
Anti-mouse IgG conjugated Alexa Fluor 488	Cell Signalling Technology
Q5 and One Taq DNA Polymerases	New England Biolabs
NucleoSpin Gel and PCR Clean-up Kit	Macherey-Nagel
NucleoSpin Plasmid Kit	Macherey-Nagel
BglII, restriction enzyme	New England Biolabs
XbaI, restriction enzyme	New England Biolabs
XhoI, restriction enzyme	New England Biolabs
HindIII, restriction enzyme	New England Biolabs
In-Fusion® HD Cloning Kit	Clontech, Takara
Mouse mAb Myc-Tag	Cell Signalling Technology
Rabbit mAb His-Tag	Cell Signalling Technology
Mouse mAb FLAG-Tag	Cell Signalling Technology
Rabbit mAb FLAG-Tag	Cell Signalling Technology
Protoscript II cDNA Synthesis Kit	New England Biolabs
Visualizer™ Western Blot Detection Kit	EMD Merck Millipore

APPENDIX E

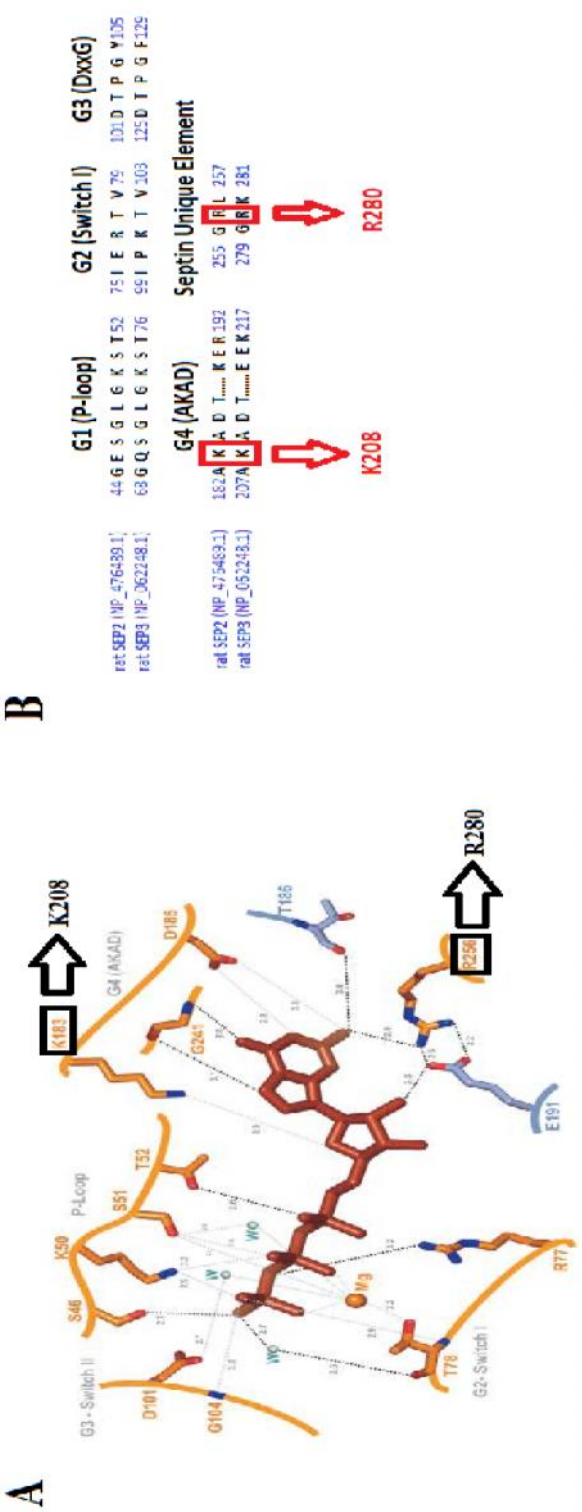


Figure E.1 : A) Details of the nucleotide-binding site of human SEPT2, showing GppNHp and surrounding residues, with the 2 protomers (Sirajuddin, Farkasovsky, Zent and Wittinghofer, 2009), (B) Conserved residues in domains of rat SEP2 and rat SEP3.

APPENDIX F

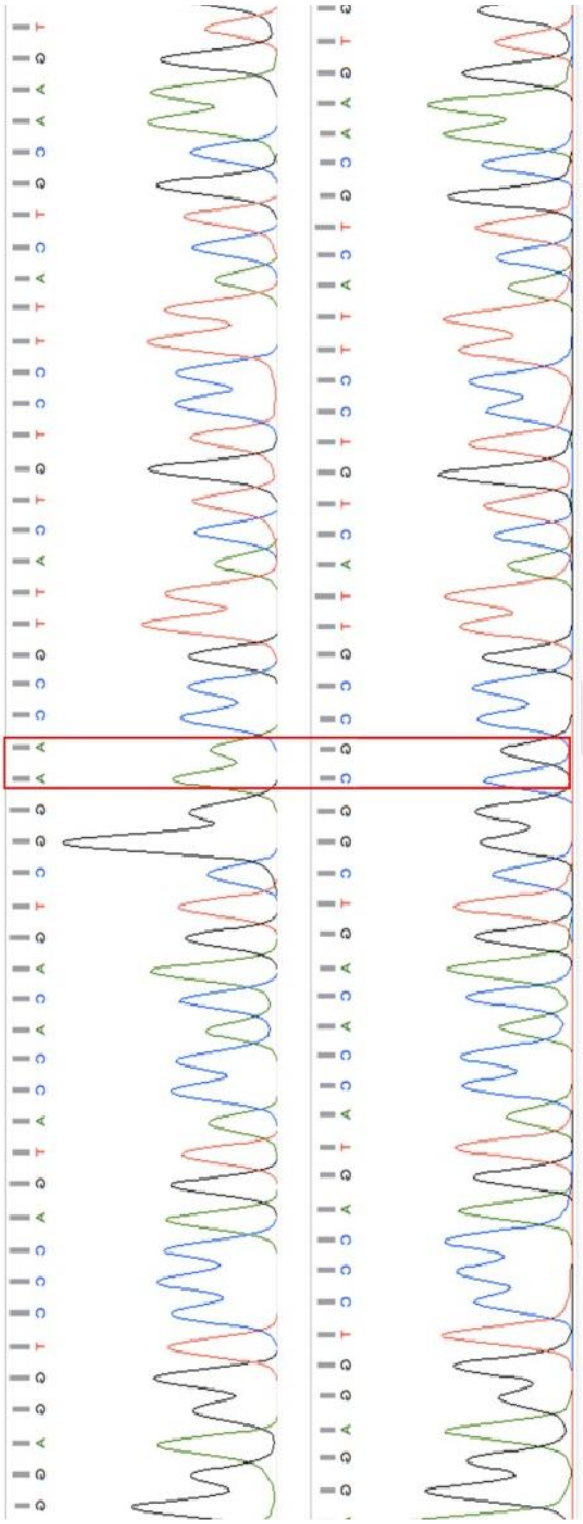
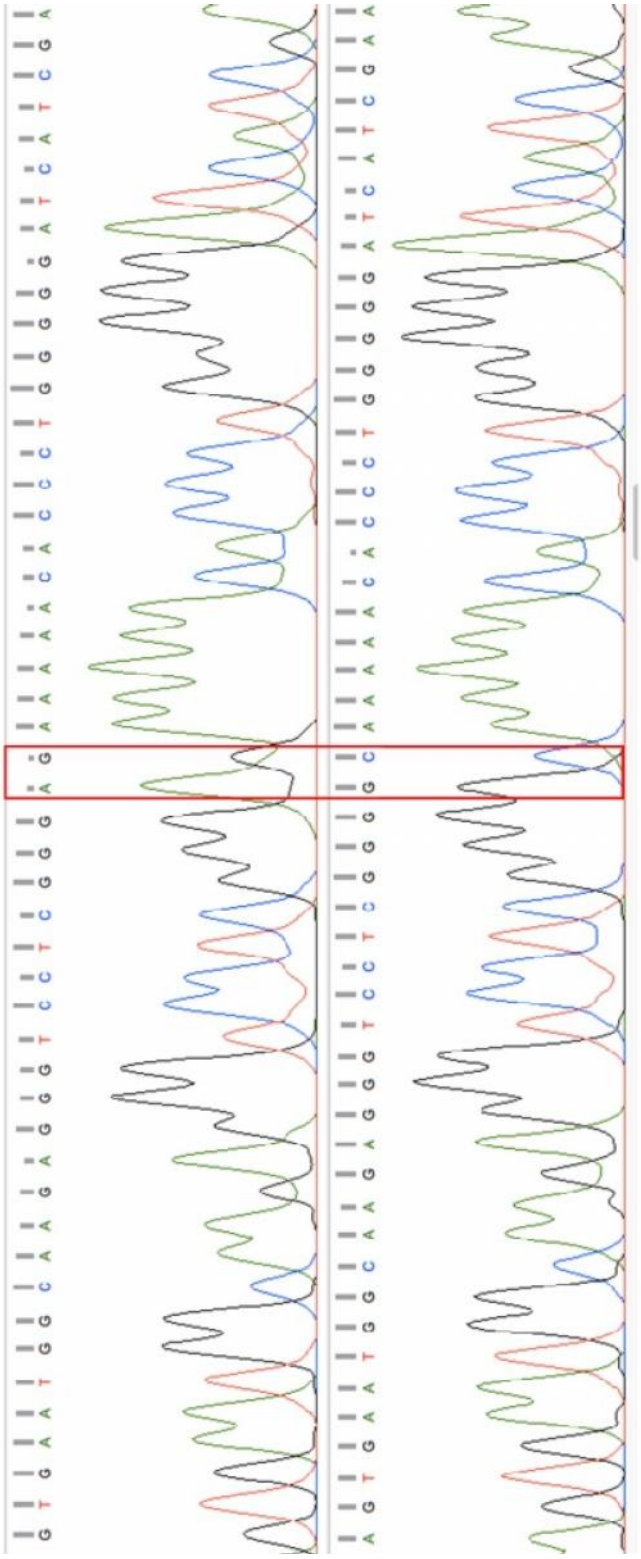


Figure H.1 : The chromatogram image of Lys208Ala mutation.



The Figure H.2 : The chromatogram image of Arg280Ala mutation.

CURRICULUM VITAE

Name Surname **: Burcu SUCU**

Place and Date of Birth **: İstanbul-Turkey / 01.05.1989**

E-Mail **: burcusucu@hotmail.com**

EDUCATION **:**

- **B.Sc.** **:** 2014, Yeditepe University, Faculty of Engineering and Architecture, Department of Genetics and Bioengineering

PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- **Sucu, B., Kırımtay, K., Karabay, A.,** 2015 : Identification of Physical Interaction between Septin3 and p60-katanin. 4th International Congress of Molecular Biology Association of Turkey - Neuroscience, November 27-29, 2015, Middle East Technical University, Ankara, Turkey. (Poster Presentation)
- **Sucu, B., Baran, D., Kırımtay, K., Temizci, B., Akarsu, S., Kelle, D., Karabay, A.,** 2016. Physical Interaction of Septin3 & p60-katanin and Role of Septin3 & Tau Relation in This Interaction. *FEBS J*, 283: P02.05.5-004 (Abstract Instance)
- **Baran, D., Sucu, B., Kırımtay, K., Korulu Koç, Ş., Karabay, A.,** 2017. 'Septin3 Involvement in Microtubul-p60-katanin-Tau Interactions'. 15th International Congress of Histochemistry and Cytochemistry – Structure and function of the cell, May 18-21 2017, Kervansaray Lara Hotel, Antalya, Turkey. (Poster Presentation)