ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

REGULATION OF TAU ON SEPTIN3 IN NEURONAL BRANCHING

M.Sc. THESIS

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Department of Molecular Biology – Genetics and Biotechnology

Molecular Biology - Genetics and Biotechnology Programme

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ISTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

SEPTİN3' ÜN NÖRONAL DALLANMA ÜZERİNDEKİ ETKİSİNİN TAU TARAFINDAN DÜZENLENMESİ

YÜKSEK LİSANS TEZİ

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Didem Baran, a M.Sc. student of İTU Graduate School of Science Engineering and Technology student ID 521151104, successfully defended the thesis entitled "Regulation Of Tau On Septin3 In Neuronal Branching", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my mother,

FOREWORD

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December 2017 Didem Baran

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ABBREVIATIONS

µg : Microgram
µM : Micromolar
µm : Micrometer
α : Alpha
AAA : ATPases Associated with diverse cellular Activities
AD : Alzheimer Disease
Amp : Ampicillin
BCA : Bicinchoninic acid
BSA : Bovine serum albumin
CC : Coiled-coil domain
cm : Centimeter
DAPI : 4',6-diamidino-2-phenylindole
DMSO : Dimethyl sulfoxide
DNA : Deoxyribonucleic acid
DMEM : Dulbecco's modified Eagle medium
DMSO : Dimethyl sulfoxide
DNA : Deoxyribonucleic acid
***E.coli* :** *Escherichia coli*
EDTA : Ethylenediaminetetraacetic acid
FBS : Fetal Bovine Serum
g : Gram
GC : Guanine and Cytosine
GMPCPP : Guanosine-5'-[(α,β)-methyleno]triphosphate
GDP : Guanosine diphosphate
GTP : Guanosine triphosphate
HBSS : Hank's Balanced Salt Solution
HCl : Hydrochloric acid
ICC : Immunocytochemistry
IgG : Immunoglobulin G
IP : Immunoprecipitation
kDa : Kilo Dalton
LB : Luria-Bertani Broth
M : Molar xiv
MAP : Microtubule Associated Protein
MN : Macherey-Naggel
mg : Milligram
mL : Mililiter
mM : Milimolar
mm : Milimeter
MT : Microtubule
MTOC : Microtubule Organizing Center
NaCl : Sodium chloride
NEB : New England Biolabs
NEAA : Non-essential amino acid

ng : Nanogram
nm: Nanometre
NP40 : Nonyl phenoxypolyethoxylethanol
NTPase : Nonstandard nucleotide triphosphate pyrophosphatase
P-loop : Phosphate-binding loop
PAGE : Polyacrylamide gel electrophoresis
PB : Polybasic Domain
PBS : Phosphate buffered saline
pH : Power of Hydrogen
PIPES : Piperazine-N, N'- bis (2-ethanesulfonic acid)
PRD : Proline Rich Domain
RNA : Ribonucleic Acid
rpm : Recolutions per minute
SDS : Sodium dodecyl sulfate
SDS-PAGE : Sodium dodecyl sulphate-polyacrylaimde gel electrophoresis
SUD : Septin Unique Domain
TBS : Tris buffered saline
TBS-T : Tris buffered saline, 0.1% (v/v) Tween® 20
TEMED : Tetramethylethylenediamine
UV : Ultraviolet
V : Volt
WT : Wild-type

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REGULATION OF TAU ON SEPTIN3 IN NEURONAL BRANCHING

SUMMARY

Cytoskeleton is a network which consists of an interconnected filamentous proteins and regulatory motor proteins. In addition, cytoskeleton has important cellular activity roles for surviving and functioning such as determining cell shape and cell movement. Rearrangement of cytoskeleton is responsible for environmental changes and adaptation. Neurons distinguish from other cell types in the nature with their morphology. Their unique morphology allows them to perform their specific tasks. All neurons contain common structural backbone which is microtubule. Neurons have capacity to reconfigure their morphology and it is related to microtubule scaffolding. Neuronal cells undergo major developmental changes. Complex nervous systems require cytoskeleton-based processes that coordinate neuronal activites. Neurons are highly polarized cells and the establishment and maintenance of neuronal polarization is crucial for correct development of nervous system. They develop two types of cytoplasmic extensions; axons and dendrites. In response to electrical signals from the cell body, axons release neurotransmitters at the axon terminals. Dendrites, especially dendritic spines contain receptors for neurotransmitters and important function in signaling. The motility capacities of microtubules are directly related to their length. Mostly, the parent axon is dominated by very long microtubules chopped into short microtubules for direction of axon outgrowth. Microtubule severing is an important mechanism that take role in formation of specific neuron morphology. Neurons are rich in an enzyme called p60-katanin that makes breaks in a microtubule array. p60-katanin severes microtubules from the centrosome of neurons and later develops short microtubules that move more avidly than longer microtubules. There is a model called 'cut and run', in which the longer microtubules are mobilized by enzymes that sever them into shorter polymers. p60-katanin is not phosphorylated itself, yet its severing activity is regulated by phosporylation of microtubule binding proteins. Also, it is indicated that phosphorylation of MAP4 has regulatory effect on p60-katanin microtubule-severing activity in non-neuronal cells. Neuronal cells does not contain MAP4 but in place of MAP4, it has been shown that tau protects microtubules in the axon from severing by p60-katanin. According to this model, microtubule-associated proteins (MAPs) especially the axonal MAP tau bind along the axonal microtubules and has protective activity from severing proteins on microtubules. Tau deattaches from microtubules by phosphorylation and this controls levels of short microtubules in specific axonal regions. Short microtubules can then be transported by motor proteins into collateral branches to promote their growth and stabilization.

Over the last several decades, Septin protein family has been recognized as fourth cytoskeleton compenent because of their filamentous appereance and their association between other cytoskeleton elements such as microtubules. Septins are evolutionarily conserved family classified under the GTPase superclass of P-loop NTPases. Septins have important roles in maintenance of cell shape and microtubule-dependent processes. For instance, it has been demonstrated that Septin2 and MAP4 intract and this interaction results in microtubule destabilization. Septin3 (Sept3) specifically associates with neurons and nerve terminals, which is found as a neuronal spesific

protein after examination of 12 different tissues. Septin3 is found enriched in synaptosomes and presynaptic terminals.

Here we sought to determine Septin3 - Tau interaction from the point of neuronal process formation and branching and how this interaction would effect p60-katanin activity on Septin3 filament formation.

Initially, physical interaction of Tau & Septin3 and Tau & p60-katanin proteins were determined co-immunoprecipitation (co-IP) with rat brain lysate. co-IP results indicated that Septin3 and tau physically interacted, however tau and p60-katanin did not physically interact.

The studies on RFL6 fibroblasts sought seek to test Septin3 – Tau – p60-katanin interaction in non-neuronal cell which do not express Septin3 and Tau endogenously. RFL6 cells were over-expressed both with tau and Septin3 and co-localization of tau then were confirmed. Septin3 over-expression resulted in filament formation, however after over-expression of both Septin3 and p60-katanin, Septin3 filament formation was damaged. This effect of p60-katanin is considered as similar to its effect on microtubules. In addition, RFL6 cells nucleofected with Septin3, tau and p60-katanin and Septin3 filament formation was investigated. According to these results, Septin3 protein formed filamentous appearance, in spite of p60-katanin over-expression.

Several studies showed that septins increase spine morphogenesis and dendritic spine formation. For instance, Septin7 is found to localize at dendritic protrusions and dendritic branch points in cultured hippocampal neurons. Primary cortical neuron studies were performed to determine Septin3 – Tau – p60 - katanin interaction from the point of neuronal processes formation. Primary rat cortical neurons over-expression studies were carried out by nucleofection and gene silencing was carried out by Accell siRNA transfection. Septin3 over-expression increased dendritic branch formation and expedited neuronal development. Also, over-expression of Septin3 resulted in long and thick bundles in microtubules. Upon down regulation of Septin3, dendritic spine morphology and exon elongation were impaired. Following over-expression of both Septin3 and p60-katanin in the same cell, Septin3 was found to be interrupted along the axon in the presence of p60-katanin. Moreover, microtubules remained partially uninterrupted. Septin3 and tau were localized predominantly along the axon and resulted in even thicker bundles of Septin3 and microtubules. Besides Tau' s localization in mainly axons, Septin3 over-expressed and Tau silenced cells still lost their axon elongation.

Observation in this study showed that, in addition to other known septin' s, Septin3 forms filaments in non-neuronal cells. Also, Septin3 has a role on neuronal polarization. Similar to some septins, Septin3 interacts with Tau and this interaction results in long and thicker microtubule bundles on neuronal microtubules. Although Further experiments needs to be carried out. tau may have a protective effect on Septin3 filaments against severing, like it does on the microtubules.

SEPTİN3' ÜN NÖRONAL DALLANMA ÜZERİNDEKİ ETKİSİNİN TAU TARAFINDAN DÜZENLENMESİ

ÖZET

Hücre iskeleti, hücre için son derece gerekli olup hücre içi molekül taşınması, hücre hareketi, hücre bölünmesi gibi önemli mekanizmalarda görevli ağ şeklinde bir yapıdır. Ökaryotik hücrelerde hücre iskeleti aktin filamentler, ara filamentler ve mikrotubullerden oluşur. Mikrotubuller (MT), alfa ve beta tubulin heterodimerlerinden oluşurlar ve hücre bölünmesi sırasında kromozomların kutuplara çekilmesi, hücre hareketi, nöron hücrelerinde yapının şekillenmesi ve akson ile dendritik gibi nöronal yapıların oluşmasında görevlidir. Nöronlar mikrotubul yapısından oluşurlar ve kendilerine özgü bu yapıları oluşturması, özel görevlerini gerçekleştirmesinde etkili olur. Mikrotubuller 'dinamik kararsızlık' adı verilen bir özellikle ile alfa ve beta tubulin monomelerinin polimerizasyonu ve depolimerizasyonu sonucu mikrotubuller yeniden yapılandırılmaktadır. Mikrotubullerin hızlı büyümesine 'kurtarma', GTP-başlıklı artı uçtan alt ünite kaybına uğramasına 'katastrofi' ismi verilmiştir. Katastrofi için gerekli olan enerji GTP hidrolizi ile sağlanmaktadır. GTP'nin hidroliz edilemeyen formu olan GMPCPP ile gerçekleşen deneylerde, mikrotubullerin dinamik instabiliteyi gerçekleştirmediği gözlemlenmiştir. Uzun mikrotubuller hareketsizdir ve hareket edip taşınabilmesi için küçük parçalara kesilmesi gerekmektedir.

Katanin, p60-katanin ve p80-katanin olarak isimlendirilen ve sırasıyla 60 kDa ve 80 kDa büyüklüğündeki iki alt birimden oluşan bir mikrotubul kesim enzimidir. p60-katanin, KATNA1 geni tarafından kodlanırken, p80-katanin KATNB1 geni tarafından kodlanmaktdır. p60-katanin, katanin proteininin küçük alt birimi olup üzerinde çok çalışılan, özellikle nöronlarda dallanma oluşumu açısından önemli bir proteindir. C-terminalinde bulunan AAA bölgesi proteine ATPaz özelliği kazandırmakta, dolayısıyla p60-katanin mikrotubulleri kesmek suretiyle büyük parçalardan küçük mikrotubul parçaları oluşumunu sağlamaktadır. p80-katanin'in enzimatik aktivitesi olmamakla birlikte, p60-katanin aktivitesinin düzenlenmesinde ve p60-katanin'in sentrozoma yönlendirilmesinde görevlidir. Mikrotubullerin kesilmesi hem bölünen hücrelerde hem de bölünmeyen nöronlarda akson ve dendrit gibi proseslerin dallanmalarında önemli rol oynamaktadır. Hareketsiz mikrutubuller nöronlarda bulunmaktadır. Nöronların hücre gövdesinde üretilen proteinlerin akson uçlarına kadar taşınabilmesi için görevli bir sabit yapıya ihtiyacı vardır. Kesilen mikrotubuller taşınmaktadır. Bu mekanizma mikrotubul ile ilişkili proteinler (MAP) tarafından kontrol edilmektedir.

Septinler 1967 yılında *Saccharomyces cerevisiae* ile yapılan bir çalışmada, ısıya-duyarlı-ölümcül mutantların hücre çeperi oluşumundaki sorunlardan dolayı koloni oluşturamamasının sebebi olarak ortaya çıkmıştır. Septinler, evrimsel olarak korunmuş GTPaz ailesidir. Bitkilerde ve protistlerde bulunmazken, mantarlar ve hayvanlarda çok sayıda mevcuttur. Septinlerin temel özelliği, filamentler oluşturabilmeleridir. Filament oluşumu GTP hidrolizine bağlı, hetero-oligomerik komplekslerin daha üst seviye yapılar oluşturmasıyla gerçekleşir. Farklı septin alt gruplarını içeren septin proteinleri 1:1:1 veya 1:1:1:1 sitokiyometrik oranlarla GTP hidrolizinden açığa çıkan enerji sayesinde bir araya gelerek hetero-heksamer veya hetero-oktomer oluşturmaktadır. Bu hetero-oligomerler daha sonra polar olmayan

filament demetlerini ya da sitokinez sırasında meydana gelen dairesel bölümleri oluştururlar. Temel bir septin hetero-trimeri sırası ile Septin2-Septin6-Septin7-Septin3 grubu septin proteinlerinin bir araya gelmesi ile oluşmaktadır. Daha sonra bu trimer uç kısımlarda bulunan Septin2-Septin2 ve Septin3-Septin3 bağlanması ile hetero-oktomer oluşturmaktadır. Bu oluşum, birbirini takip eden septin proteinlerinin GTPaz bölgeleri arasında, korunmuş etkileşimler sayesinde gerçekleşmektedir.Septin ailesinin birçok üyesinin mikrotubul ve aktin ağı ile bir arada ya da etkileşim halinde bulunduğu saptanmıştır. Buna ilaveten, Septinler, MAP'lerle birlikte mikrotubul dinamiğini düzenlemede de önemli rol oynarlar. Septin3 nöron-spesifik bir proteindir ve on iki doku ile yapılan inceleme sonucu yalnızca beyinde saptanmıştır. Sinaptozom ve presinaptik terminallerde yoğun bir biçimde bulunmaktadır. Septin3, başka bir beyin-spesifik Septin olan Septin5 ile ko-lokalize halde bulunmuştur. Hippokampal nöronların sinapslarında yapılan bir araştırmaya göre, Septin3' ün Septin5 ve Septin7 ile beraber presinaptik kompleks oluşturduğu gösterilmiştir.

MT'lerin kesilmesinin ya da stabil kalmasının regülasyonu da tamamiyle MAP'ler aracılığıyla gerçekleşir. MAP'ler üzerinde yapılan fosforilasyon gibi modifikasyonlar p60-katanin gibi kesici enzimlerin MT'lere ulaşabiliriliğini değiştirmektedir. Örneğin, bir mikrotubul bağlanma proteini olan MAP4'ün nöronal olmayan hücreler üzerinde düzenleyici etkisi olduğu görülmüştür. Nöronal hücreler MAP4 içermemektedir; ancak başka bir çalışmada mikrotubule bağlanan bir protein olan nöronal Tau'nun, mitotik MAP4 gibi, aksonların p60-katanin tarafından kesilmesini önlediği gösterilmiştir. Nöronlara spesifik bir MAP olan Tau'nun mikrotubul polimerizasyonu üzerindeki etkisi in vitro olarak incelendiğinde nukleasyonu teşvik ederek MT polimerizasyonunun hızını ve derecesini uyardığı, böylece katastrofi evresine geçiş oranını direkt olarak düşürdüğü gözlemlenmiştir. Çok düşük miktarlarda dahi Tau mikrotubullerin katastrofi sıklığını dramatik olarak azaltmıştır. Aynı zamanda, Tau mikrotubuller ile etkileşim halindedir ve aktin filamentlere bağlanarak bundle (demet) oluşturabilirler. Mikrotubuller, mikrotubul asosiye Tau proteini ile bağlanarak stabilize edilirler. MAP'ler katanin-mikrotubul interaksiyonunun gerçekleşme ihtimalini azaltmaktadır. Bunun sebebi ise MAP varlığında katanin alt ünitelerinin mikrotubul etrafında heksamer oluşturamayarak onu kesme aktivitesi gerçekleştiremiyor olmasıdır. Hiperfosforilasyon ile MT'lerden ayrılan Tau, p60-katanin'in MT'lere erişebilirliğini arttırmıştır. Bu bağlamda p60-katanin'in modifikasyondan bağımsız olarak Tau ile olası etkileşimi de MT dinamiği açısında önem kazanmaktadır. Aynı zamanda, Septinlerin anafazda mitotik iğ ipliğine, telofazda ise orta cisimciğe lokalize olması ve p60-katanin'in benzer şekilde hücre siklusu boyunca yoğun bir şekilde sentrozomlarda ve mitotik mekiği oluşturan mikrotubuller üzerinde lokalize olması; p60-katanin ve septinlerin mikrotubul dinamiğinin düzenlenmesinde birlikte rol alabilecekleri fikrini ortaya çıkarmştır.

Bu çalışmada, p60-katanin ve Septin3 ilişkisi üzerinde bir mikrotubul-bağlanma proteini olan Tau' nun etkisi hem mitotik hem de nöronal diferansiyasyon/dallanma üzerindeki etkisinin araştırılması amacıyla post-mitotik hücrelerde incelenmiştir. Septin3 ve Tau' nun fiziksel etkileşiminin incelenmesi amacıyla yetişkin sıçan beyninden izole edilen protein kullanılmış ve Septin3 ile Tau' nun etkileştiği ancak, p60-katanin ve Tau' nun fiziksel olarak etkileşmediği görülmüştür. Septin3 ve Tau' nun ko-lokalizasyonu RFL6 ve primer kortikal nöron hücrelerinde yapılan immunositokimya (ICC) deneyleriyle gösterilmiştir. Aynı zamanda, primer kortikal nöron hücrelerinde yapılan over (aşırı)-ekspresyon ve gen susturulması deneylerinde, Septin3 over-ekspresyonunun akson uzaması/kalınlığı ile dendiritik-spine (iğne)

oluşumunu arttırdığı görülmüştür. Septin3 ve Tau ağırlıklı olarak aksonda ko-lokalize olmuştur. Septin3 ve/veya Tau yokluğunda ise akson uzamasında hasar meydana gelmiştir.

Bu çalışmadaki bulgularımız, Septin protein ailesinin diğer üyeleri gibi Septin3' ün de filament formasyonu oluşturduğunu göstermiştir. Aynı zamanda, nöronal bir Septin olan Septin3' ün nöronal polarizasyonda görevi olduğu bulunmuştur. Nörotransmitter salınmasında önemli bir göreve sahip olan dendiritik iğne oluşumu Septin3 over (fazla)-ekspresyonu ile artmıştır. Septin3 ve bir mikrotubul ile ilişkili protein olan ve nöronlarda yoğun olarak bulunan Tau' nun etkileşim içinde olduğu ve yoğun olarak aksonda ko-lokalize olduğu ve beraber görev yaptığı gösterilmiştir. Tau' nun p60-kataninin mikrotubuller üzerindeki koruyucu etkisi bilinmekte olup, Septin3 filamentleri üzerinde benzer bir etkisi olup olmadığı araştırılmış, olabileceği düşünülmüş ancak daha ileri deneyler yapılarak kesin sonuca varılabileceği düşünülmüştür.

1. INTRODUCTION

1.1 Cytoskeleton

Cytoskeleton consists of an interconnected network of filamentous proteins and regulatory motor proteins (Fletcher et al., 2010). In order to survive and function, cells need to arrange their internal cytoskeleton components. Rearrangement of cytoskeletal components is required for adapting to environmental changes, growth and division. Also, cytoskeleton is important for cell strength. Eukaryotic cytoskeleton contains three main proteins which are microtubules, microfilaments and intermediate filaments. All cytoskeletal elements distinguish in size (diameter) and subunit composition. Actin filaments are the smallest and as their name implies intermediate filaments are mid-sized. Microtubules also enables to pull daughter chromosomes apart to the opposite poles during cell division (Alberts, 2014).

1.1.1 Microtubules

Microtubules consist of α and β globular tubulin subunits. The microtubule proteins are around 25nm in diameter. They are the largest cytoskeletal elements and appear as filaments. In an eukaryotic cell, there are two types of microtubules which are stable and unstable microtubules. Stable microtubules are mostly found in non-replicating cells. Unstable microtubules are found in the cells in which microtubule-based structures need to assemle and disassemble quickly. Microtubules are hollow tubes composed of a lattice of α-β tubulin heterodimers that stack end-to-end to form protofilaments (Hawkins et al., 2010). The polymerization depends on GTP hydrolysis. α-tubulin monomer is bound to GTP and however, β-tubulin monomer is bound to GDP which is convertible to GTP (Alberts, 2014) (Lodish, 2008). Microtubules continously alternate between periods of elongation and shortening in a process termed dynamic instability which is important for a wide variety of cellular processes (Figure 1) (Janson et al., 2003). During microtubule elongation, if the polymerization is faster than GTP hydrolysis, GTP-cap is generated at the (+) end of

microtubules. If GTP hydrolysis occurs more rapidly than polymerization, GTP-cap is lost and microtubules start to shrink. Transition from growing to shrinking is called "catastrophe" while a transition from a shrinking to growing is called as "rescue" (Figure 1.1) (Lodish, 2008). Whole process of dynamic instability depends on GTP hydrolysis. In order to support this idea, an experiment carried out with GMPCPP, which is a slowly hydrolyzable analogue of GTP. It polymerizes stable tubulin caps onto microtubules originally grown in the presence of GTP.

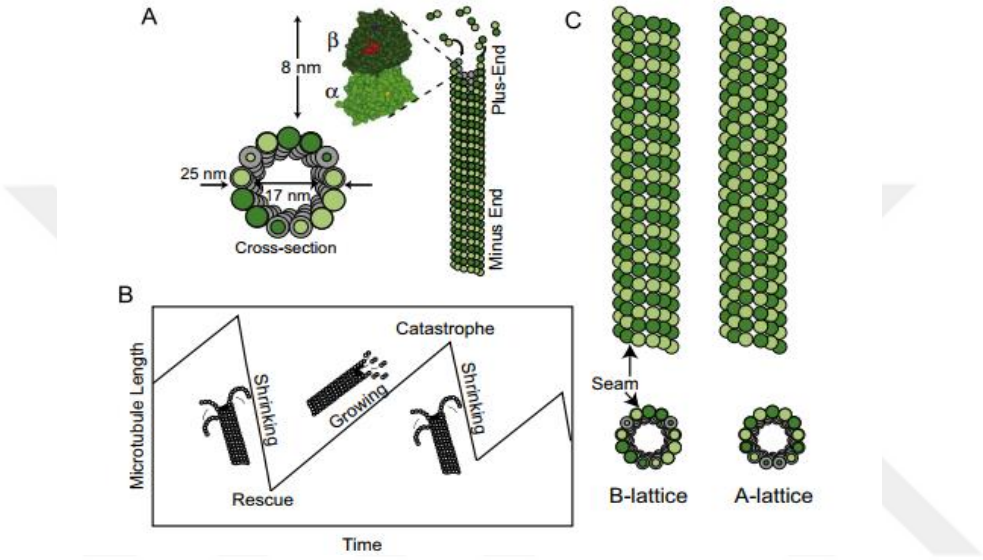

Figure 1.1 : Microtubule structure and dynamic instability (Hawkins et al., 2010).

The dynamic instability of microtubules has valuable roles in eukaryotic cells such as cell cycle, cell activity and differentiation, also organizing intracellular cell compartments (Lodish, 2008). For instance, mitotic spindle is generated from microtubules and the movement of chromosomes to opposite spindle poles depends on the delicate state of balance between assembly and disassembly (Figure 1.2) (Amos and Schlieper , 2005).

The third member of tubulin superfamily is discovered as γ-tubulin which is localized in the centrosomal matrix. γ-tubulin is an essential component of Microtubule Organizing Center (MTOC) and contributes to microtubule nucleation. It is a conserved tubulin that does not contribute into the microtubule wall but, instead binds to the microtubule minus end (Figure 1.2) (Wiese and Zheng, 2006).

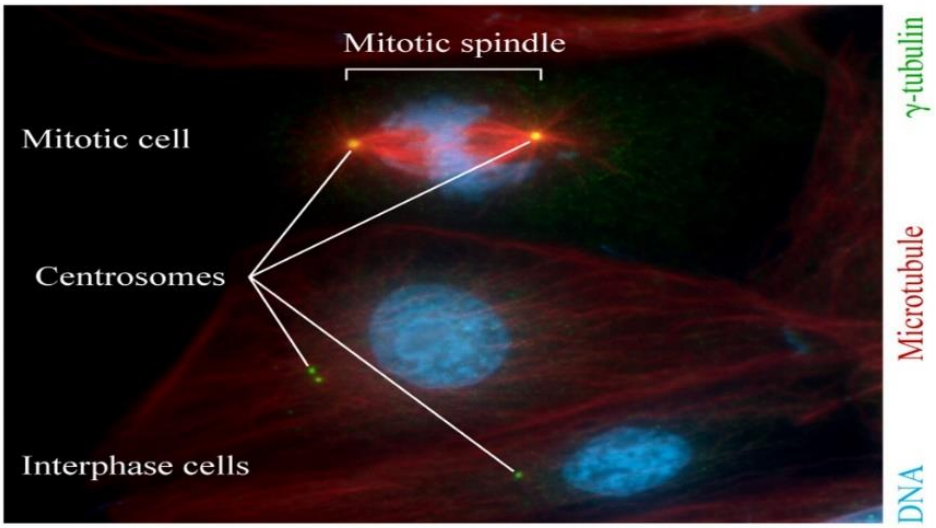

Figure 1.2 : Microtubules are shown during interphase and mitosis, as in these cultured Xenopus laevis kidney epithelial cells (Wiese and Zheng, 2006).

Microtubule behaviours and stability are promoted to a large degree by microtubule-associated proteins (MAPs). For instance, the best known MAPs are the heat-stable proteins MAP2 and tau and they bind to the surface of the microtubule and promote microtubule polymerization (Heald and Nogales, 2002).

1.1.2 Microtubule associated protein: Tau

Most of the major MAPs (MAPlA, lB, 2A, 2B, 2C, tau) are expressed in nerve cells and have their own characteristic distribution. Tau is identified and purified by early studies with mammalian brain microtubules and is thought to be a prominent and potent promoter of tubulin assembly *in vitro* (Cleveland et al., 1977). Tau promotes nucleation and stimulates both the rate and the extent of microtubule polymer assembly; thus decreases the rate of transit into the shrinking phase (catastrophe). The effects of Tau on microtubule assembly is examined *in vitro.* It is suggested that Tau has suppressive activity on microtubule dynamics. Even at low levels (0.1-0.2μM), Tau dramatically reduces the catastrophe frequency of microtubules, Besides, higher concentrations of Tau increases the rate of polymerization of growing microtubules (Figure 1.3) (Dreschel et al., 1992) In addition to that, Tau connects microtubule as well as actin filament networks because tau can also bind to and bundle actin filaments (Morris et al., 2011)

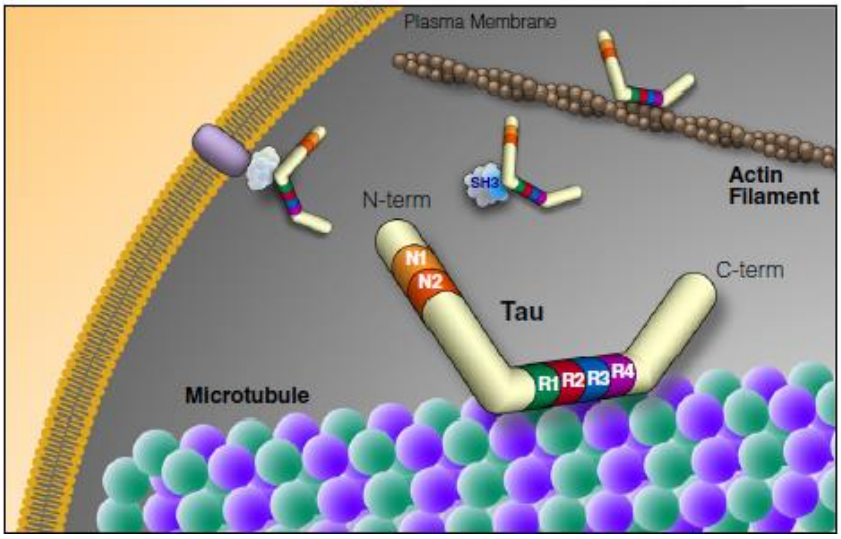

Figure 1.3 : Tau is an intrinsically disordered protein that can be alternatively spliced at N terminal exons (N1, 2) and microtubule repeat domains (R). (Morris et al., 2011)

Tau is localized abundantly in axonal compartments (Figure 1.4) and is thought to stabilize microtubules in axons and provide the basis for axonal transport (Mandelkow and Mandelkow, 1995; Hirokawa, 1994). It has been found that the suppression of Tau, impairs axonal elongation (Caceres et al., 1992) and also, another study showed that tau protein overexpression induces long axon-like processes in non-neuronal Sf9 ovary cells (Knops et al., 1991).

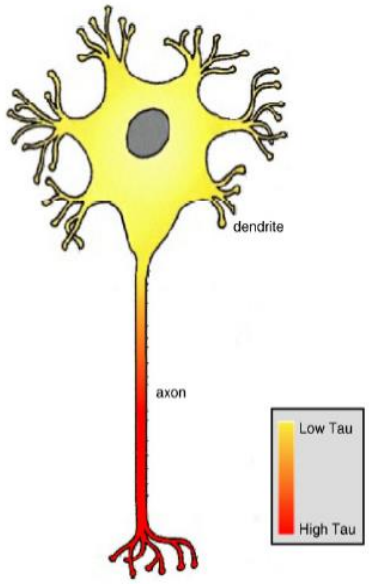

Figure 1.4 : Mature neuron showing a predominant axonal location of Tau protein (Scholz and Mandelkow, 2014)

Tau represents the subunit protein of one of the major hallmarks of disorders known as tauopathies. Neuronal degeneration in tauopathies is associated with the constant accumulation of filamentous tau inclusions (Lee et al., 2001). Hyperphosphorylated, insoluble, filamentous tau has been shown to be the main component of neurofibrillary tangles (NFTs) which is prominent hallmark of Alzheimer disease (AD) (Figure 1.5) (Grundke-Iqbal et al., 1986).

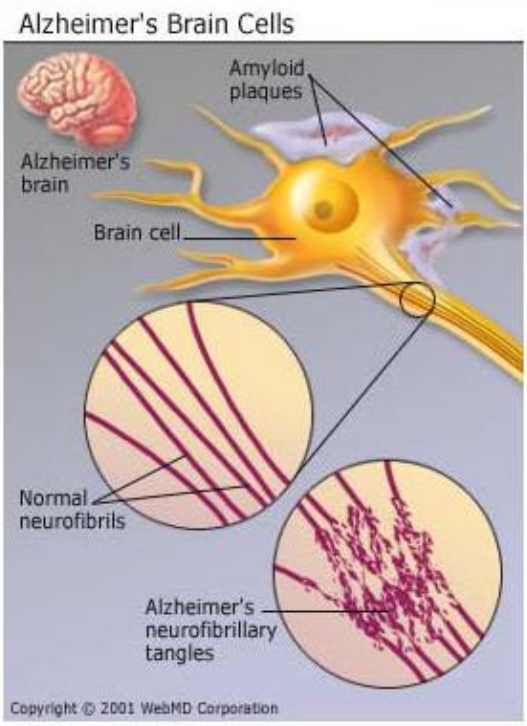

Figure 1.5 : Difference between normal neurofibrils and Alzheimer' s neufibribrillary tangles (Neil Lava, 2017)

1.2 Neuronal Polarization

Neurons are unique cells which develop from ectodermal origin of mitotic cells. Neurons contain all cytoskeletal elements, namely microtubules, actin filaments, and intermediate filaments. Neurons are non-dividing cells, they utilize their efforts of division towards transmitting signals. In order to achieve this activity, they stop dividing in the early developmental stage. At this point, unless neurons become cancerous it never organizes its microtubules into a bipolar spindle. Neurons do not exhibit a radial array of microtubules appearing from centralized centrosome, the centrosome has random place in the cell body (Baas et al., 1999). In addition, microtubules are abundant in the cell body and employed in elongation of axons. Axons contain long microtubules and their minus ends are oriented towards the cell

body; though microtubules in the dendrites are shorter with mixed polarity orientation (Arimura and Kaibuchi, 2007).

Experiments using cultured embryonic hippocampal neurons have shown that neurons initially generate small protrusion veils and a few spikes. Then, due to these protrusions growth cones at their tips, they develop into immature neurons. One neurite then starts to initiate morphological symmetry and establishing the polarity. Eventually, that one neurite elongates as an axon while the remaining neurites become dendrites (Figure 1.6) (Arimura and Kaibuchi, 2007).

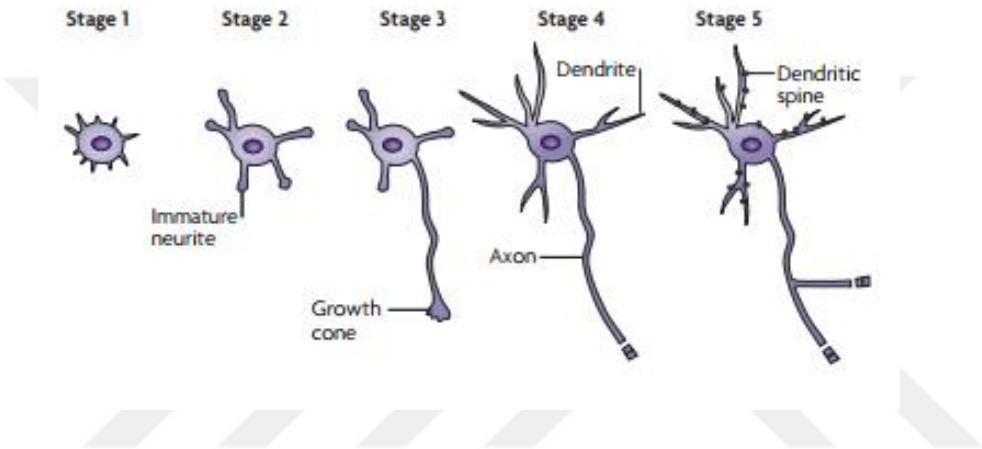

Figure 1.6 : Schematic representation of neuronal polarization in cultured rat embryonic hippocampal neurons (Arimura and Kaibuchi, 2007)

1.3 Microtubule Severing

The dynamic instability is not always enough to explain all behaviors of microtubules, thus an additional pathway is discovered by which microtubule dynamics might be affected: microtubules can be severed along their length. In some specialized cell types, such as epithelial cells and neurons non-centrosomal microtubules are needed, whereas in many cells like fibroblasts, minus-ends of microtubules are localized near the centrosome and the plus-ends are oriented towards the cell periphery. There are three possible way to generate sources of non-centrosomal microtubules: (1) de novo nucleation and growth of the microtubules in the cytosol; (2) release microtubules from the centrosome or (3) severing of microtubules at sites remote from the centrosome (Quarmby, 2000)

There is a model called ‘cut and run’, in which the longer microtubules are immobile and mobilized by enzymes that sever them into shorter polymers (Baas et al., 2006) In

cells, motor proteins ensure transport of vesicles, thus they bind to all microtubule regardless of their length. Neverthless, motor proteins cannot transport longer microtubules, they need to be severed before transportation (Baas et al., 2005)

Axonal growth predominantly depens on microtubule transport and microtubule severing (Gallo and Letourneau, 1999). The motility capacities of microtubules on axons are directly related to their length and studies demonstrated that microtubules are transported down the axon in the form of short polymers. The parent axon is dominated by very long microtubules severed into short microtubules. The growing axon contains a dense array of microtubules; during growth and navigation of the axon, the microtubule array reorganizes and reorients toward the future direction of axon outgrowth (Tanaka and Kirschner , 1991).

The schematic model of how microtubule-severing proteins regulate microtubule transportance axonal branch formation can be found below (Figure 1.7). According to this model, MAPs especially the axonal MAP tau bind along the axonal microtubules and has protective activity from severing proteins on microtubules. Detachment of MAPs from microtubules occurs by phosphorylation. This detachment mechanism controls the levels of short microtubules in specific axonal regions. These short microtubules can then be transported by motor proteins into collateral branches to promote their growth and stabilization (Baas et al., 2006).

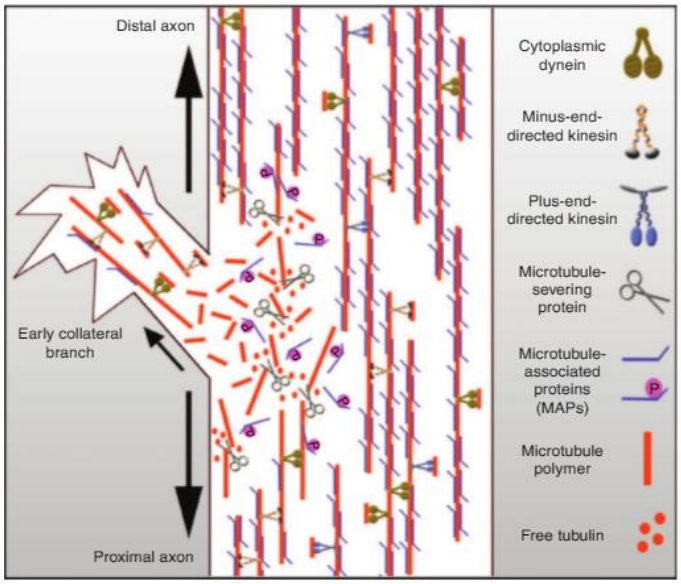

Figure 1.7 : Schematic model of how microtubule-severing proteins regulate microtubule transportand axonal branch formation (Baas et al., 2006)

Katanin, spastin, and fidgetin are all microtubule severing enzymes and belong to the ATPases Associated with diverse cellular Activities (AAA) family and they are closely related microtubule-severing enzymes. They have important roles in neuronal structure, cilia and flagella formation and mitosis (Roll-Mecak and McNally, 2010).

1.3.1 Microtubule-severing enzyme: Katanin

Katanin is first isolated from sea urchin egg as a heterodimeric ATP-dependent microtubule-severing protein. Due to the dramatic nature of the microtubule severing reaction, it is named as katanin, from the Japanese word katana, meaning samurai sword (McNally and Vale, 1993). Katanin is a heterodimer organized into a 60-kDa and 80-kDa subunits. Enzymatic subunit is p60-katanin which carries out the ATPase and microtubule severing activity and it is encoded by KATNA1 gene. p80-katanin is a targeting subunit and localizes katanin to the centrosome and has regulative effect on p60-katanin' s severing activity. p80-katanin is encoded by KATNB1 gene (Hartman et al., 1998).

The catalytic subunit of the katanin, (p60-katanin) is the best-characterized microtubule severing enzyme. It is 491 amino acid long and its N-terminal region binds to microtubules and C-terminal region is ATPase Associated with diverse cellular Activities (AAA) domain. (Quarmby, 2000; Hartman et al., 1998). The AAA region contains a conserved P-loop NTPase and this P-loop harvests energy which is released after ATP hydrolysis in order to unfold and remodel complex tertiary structures of tubulin proteins in microtubules (Ghosh et al., 2012). p80-katanin is 655 amino acid long and its N-terminal contains WD40 repeats which are involved in protein-protein interactions and C-terminal region is involved in dimerization with the catalytic p60-katanin subunit (Hartman et al., 1998). WD40 repeats also function as negative regulator of microtubule disassembly at spindle poles (McNally et al, 2000). This region controls the interaction of p80 with centrosome and any other microtubule associated proteins like dynein (Ghosh et al., 2012).

In various groups of studies, katanin has been shown to be essential factor in neuronal development in axonal elongation by regulating microtubule polymerization dynamics. It is also found that katanin-mediated microtubule severing is required for proper arrangements of microtubules along the axon (Figure 1.8) (Karabay et al., 2004) (Butler et al., 2010). Distribution of p60 and p80 subunits of Katanin varies during

different stages of neuronal development. p60-katanin is more abundant in axonal length and tips to enhance the total number of microtubule processes and on the contrary, p80 is localized and more consantrated in cell body to play limiting role in altering microtubule mass (Yu et al., 2005). In axons, nucleosome assembly protein (NAP) and Tau binding to shorter microtubules prevent further damaging activity of katanin in axonal microtubules (Figure 1.8) (Ghosh et al., 2012).

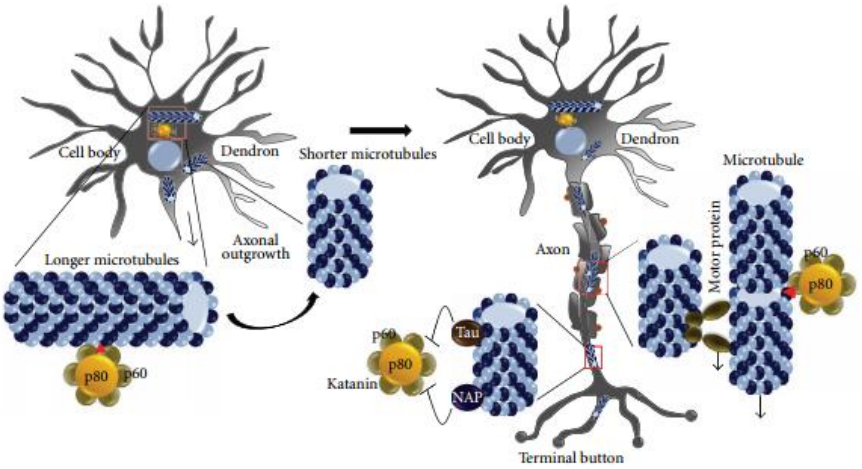

Figure 1.8 : Role of Katanin in neuronal development

1.4 Septin Protein Family

In 1967, Hartwell discovered septins by isolating temperature-sensitive-lethal (ts) mutants of *Saccharomyces cerevisiae (S. cerevisiae).* The mutants had defects in cell wall formation and they were not able to form colonies. In further studies, it has been found that temperature-sensitive mutations in four cell division control (CDC) genes (cdc3, cdc10, cdc11 and cdc12) result in a defect in cytokinesis (Hartwell et al., 1971). Originally, septins have been characterized as CDC proteins in *S.cerevisiae*, eventually they were named as 'septins' due to their localization as rings to the septating bud neck (Mostowy et al., 2012). Although septins were discovered in yeast, they were identified in nearly all eukaryotes, including humans. However, no septins had been found in plants (Nishihama et al., 2011). Septins assemble as bundles and appears as filamentous structure, due to these functions and their association with other cytoskeleton elements, septins are increasingly recognized as a novel component of the cytoskeleton (Mostowy et al., 2012).

Septins are evolutionary conserved small GTP-binding proteins of 30–65 kDa and belong to the superclass of phosphate-binding loop (P-loop) NTPases (Leipe et al., 2002). Septins share a conserved GTP-binding (G) domain and a predicted coiled-coil domain at the C terminus with the exception of Septin3 group which lacks coiled-coil domain (Figure 1.9) (Weirich et al., 2008).

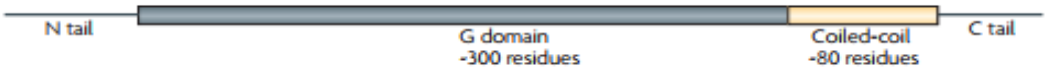

Figure 1.9 : Septin protein structure (Weirich et al., 2008).

The number of septin genes varies per organism. For instance, *Caenorhabditis elegans* has two septin genes, *S. cerevisiae* has seven and humans have thirteen septin genes (Russell et al, 2011). Human septins are classified based on sequence similarity, into four homology groups (Figure 1.10) (Mostowy et al., 2012).

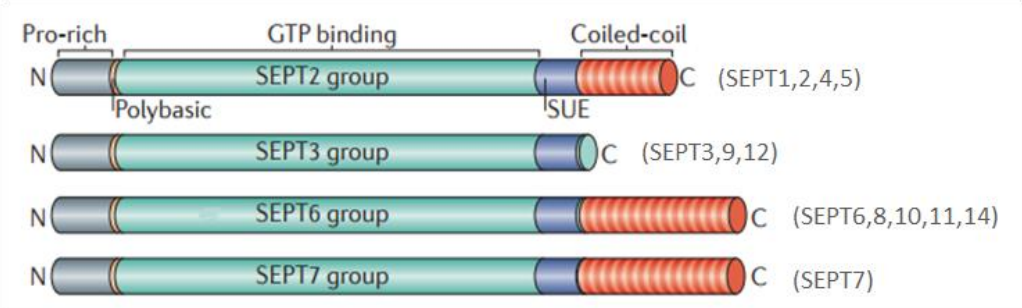

Figure 1.10 : Human septins. Phosphoinositide-binding polybasic domain (Polybasic) in orange, a GTP binding domain in light green and the septin unique element (SUE) in blue, proline rich domain (Pro-rich) in grey and the C-terminal coiled-coiled domain (Coiled-coil) in red.

Septin subunits interact through their GTP-binding domain (called the G interface) and amino-terminal and carboxy-terminal regions (called the NC interface), thus forming rod-shaped heterohexameric complexes (Mostowy et al, 2012). Then, these complexes join end-to-end in order to form non-polar filamentous structures and eventually higher-order structures such as bundles of filaments and rings. Whole process is carried out via GTP hydrolysis. Septin unique structures are involved into control cellular processes that are localized, such as at the division site, the plasma membrane, the bases of cilia, and dendrites (Figure 1.11) (Mostowy et al., 2012; Weirich et al., 2008).

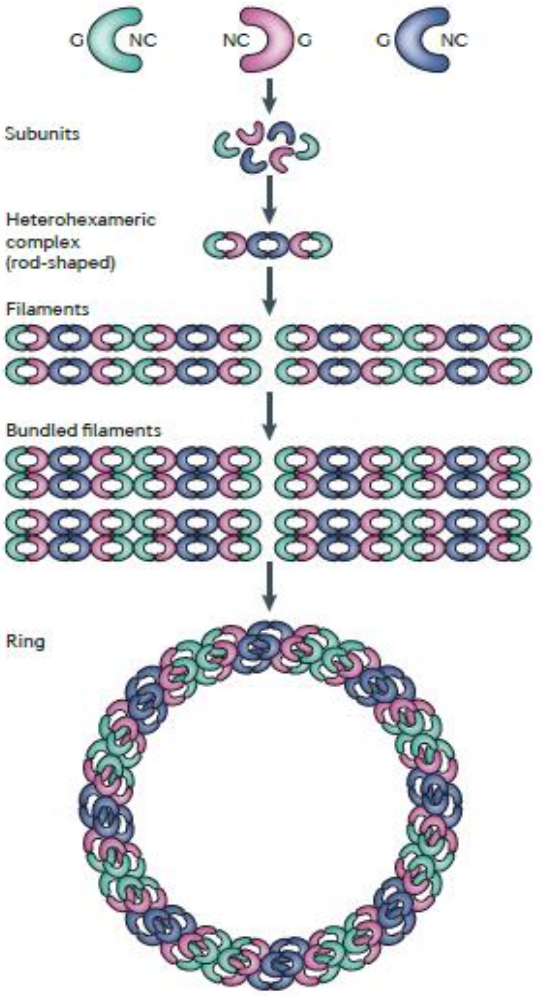

Figure 1.11 : Septin cytoskeleton dynamics (Mostowy et al., 2012).

1.4.1 Septin3, neuronal spesific Septin protein

Septin3 specifically associates with neurons and nerve terminals, which is found as a neuronal spesific protein after the examination of 12 different tissues. Septin3 is found to be enriched in synaptosomes and presynaptic terminals (Xue et al., 2004).

Septins C-terminal domain encodes coiled-coil domain and it has been suggested that the C-terminal domain may have a function on protein–protein interactions and controlling filament formation (Xue et al., 2004). On the other hand, Septin3 group of septins is characterized by having no coiled-coil domain at all (Figure 1.12). Despite Septin3 lacks coiled-coil domain, it is able to dimerize in the presence of GTP and Mg^{2+} at low salt concentrations (50 mM NaCl). However, Septin3 which lacks of the polybasic domain impairs its dimerization in the solution suggesting that N-terminal

polybasic domain of Septin3 has regulatory activity on dimerization (Macedo et a., 2013).

Sept3 is found as a substrate for PKG-I (cGMP-dependent protein kinase-I) in nerve terminals. Sept3 is phosphorylated on Ser-91 in vitro via cGMP-dependent protein kinase (PKG) and the phosphorylation activity might induce Sept3 translocation from nerve-terminal plasma membrane to the cytosol (Xue et al, 2004).

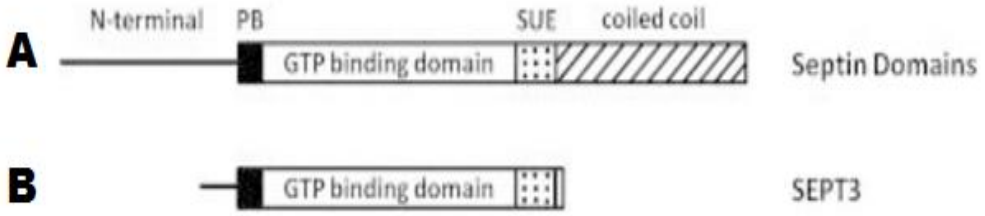

Figure 1.12 : Schematic representation of the typical septin structure (A) with compared to Septin3 structure (B) (Ortore et al., 2015).

1.4.2 Septins and microtubule associated proteins

It has been shown that septins are involved in microtubule dynamic regulation with microtubule-associated proteins (MAPs). For instance, Kremer and colleagues (2005) demonstared that Septin2 and MAP4 intract and this interaction results in microtubule destabilization. Spoliotis (2010) revealed three hypothetical models of how septin GTPases may spatially regulate microtubule-MAP: 1) regulation of microtubule-MAP interactions could be achieved by sequestration of MAPs by cytoplasmic septin complexes and/or competitive exclusion of MAPs by septins on the microtubule lattice (Figure 1.13A) (Kremer et al., 2005) (Spiliotis et al., 2008) 2) regulation of microtubule–motor interactions could be achieved allosterically, through direct binding of septins (Figure 1.13B) or 3) Septins may spatially restrict the electrostatic interactions between microtubules and motors/MAP.

Septins are also thought to be involved in dynamic instability of microtubules via interactions with MAPs. In polarizing epithelia, septins guide the directionality of microtubule plus end movement by suppressing microtubule catastrophe. The interaction possibly mediates the growth and capture of microtubule's plus-end along septin-coated microtubules (Bowen et al., 2011).

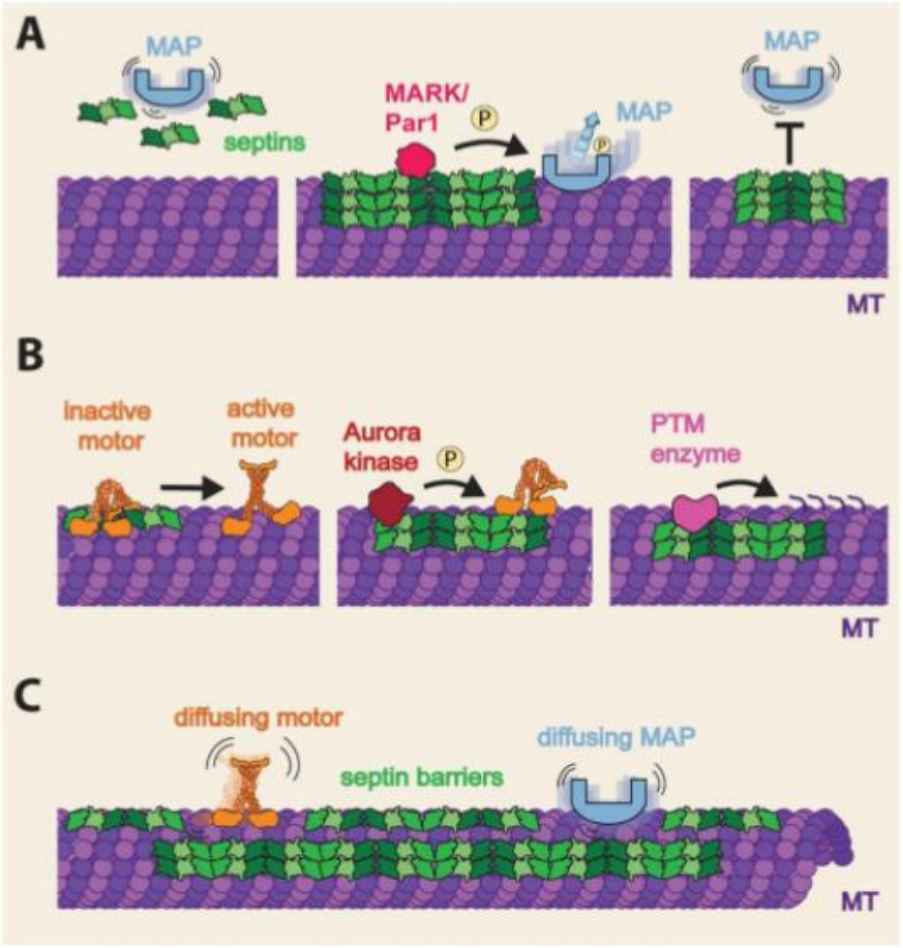

Figure 1.13 : Septin GTPases: three hypothetical models of how septin GTPases may spatially regulate microtubule-MAPs (Spiliotis, 2010)

1.4.3 Septins and neurological disorders

Possible roles for septins in neurological disorders have emerged based on the brain-specific expression of some septins. Septin 1, 2, 4 have been shown to be concentrated in the neurofibrillary tangles of Alzheimer's patient brains, thus three septins are found to be highly interactive and co-localized with extremely basic protein tau (Kinoshita et al, 1998). In another study, it is suggested that polymorphism in exon 11 of Septin3 may have a determinative role in the pathogenesis of Alzheimer' s disease (Takehashi et al., 2004). Eleven proteins that have been deregulated in cerebral cortex of fetal Down syndrome and one of them was revealed as Septin7 (Engidawork et al., 2003).

1.5 Aim of the Study

Septin3 was previously found as a possible interacting partner of p60-katanin in yeast two hybrid screening performed in our laboratory (Esen, 2008). Septins assemble into filaments like microtubules which are known to be severed by p60- katanin, later in our studies Septin3 was identified as a new interacting partner and a substrate of p60-katanin via co-IP and ICC experiments (Sucu, 2017). Since it has been known that microtubule associated protein Tau strongly shield microtubules from severing by p60-katanin, possible interaction of Septin3 and Tau raised an intriguing question as to whether Tau protects Septin3 filaments from severing by p60-katanin as it does on the microtubules. From this standpoint, first aim of this study was to investigate the interaction of Septin3 and Tau and Tau' s regulatory effect on Septin3 filaments in detail. The interaction of Septin3 and Tau was analyzed by co-IP assay and Tau' s possible protective effect on Septin3 filaments was visualized by ICC.

In addition, it is known that Septin3 is exclusively expressed in neurons and localizes in synaptic vesicles (Xue et al., 2004), however knowledge on the role of Septin3 in neuronal branching is limited. Since maintenance of neuronal polarization is critical for correct development of neurons, second aim of this study is to determine the role of Septin3 in neuronal polarization and the regulatory capacity of Tau on Septin3 in neuronal branching. The role of Tau on Septin3 in neuronal branching was analyzed by over-expression and siRNA gene silencing experiments on primary rat cortical neurons.

2. MATERIAL AND METHODS

2.1 Materials

2.1.1 Animals

Embriyonic day 18 (E18) Sprague-Dawley rats were used for cortical neuron culturing. Ethical approval was obtained from Medipol University and Bogazici University. The procedures involving animal experimentation and animal handling were done according to the guidelines by the National Research Council's for the care and use of laboratory animals.

2.1.2 Equipments

Equipment used in this study is shown in the Table 2.1 below.

Table 2.1: Laboratory equipment used in the study.

Equipment	**Supplier Company**
SDS-PAGE Gel Electrophoresis System	Mini-Protean® Tetra Cell, BioRad
SDS-PAGE Gel Casting System	Mini-Protean®, Tetra Handcast System, BioRad
Gel Imaging System	Gel Doc™ XR+Imager, BioRad
Thermocycler	BioRad T100™ Thermal Cycler
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
Micro Pipette	Eppendorf micropipette; 10 µL,100 µL, 200 µL, 1000 µL Mettler Toledo Volumate Liquid System; 0.1-2.5 µL
Power Supply	BioRad Powerpack Basic
Western Blot Transfer System	BioRad Trans-Blot® Turbo™ (Semi-Dry)

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

SDS-PAGE Gel Electrophoresis System	Mini-Protean® Tetra Cell, Biorad
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°C -20°C), New Brunswick Scientific (-80°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	BioRad Benchmark Plus
Magnetic Separation Rack	Promega
Quick Spin	Labnet Spectrafuge C1301 Mini Centrifuge

2.1.3 Chemicals

Chemicals used in this study are given in the Table 2.2.

Table 2.2: Chemicals used in this study

Chemicals	Supplier Company
Trizol Reagent	Ambion, Life Technologies
Chloroform	EMD Merck Millipore
Isopropanol (2-propanol)	EMD Merck Millipore
Nuclease-free dH_2O	EMD Merck Millipore
Filtration System	Stericup® FilterUnit (0.22µm)
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
Ammonium persulfate (APS)	Fluka, Sigma-Aldrich
Tetramethylethylenediamine (TEMED)	EMD Merck Millipore

Table 2.2 (continued): Chemicals used in this study

Ponceau S	EMD Merck Millipore
Glycerol	Fischer Scientific
Tween® 20	Fischer Scientific
Nonidet® P40 (NP40)	Applichem
Hydrochloric acid (HCl) (37%)	EMD Merck Millipore
DMSO	Thermo Fischer Scientific
Fetal Bovine Serum	Biowest
Saponin	Sigma-Aldrich
Protein Ladders	GENE's Protein Ladder PS10 Plus Prestained, GeneOn; Color Prestained Protein Standard, NEB
Protein Sample Buffer	3X SDS Sample Buffer, New England Biolabs
Parafilm	Parafilm M®, Bemis
Mounting Medium	Prolong® Mounting Medium, Invitrogen
DMSO	EMD Merck Millipore
DAPI(4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Invitrogen
Nonfat Dry Milk	Cell Signaling Technology
Tryptone	LabM, Neogen Company
Sodium chloride (NaCl)	EMD Merck Millipore
Tris Base (Molecular Biology Grade)	EMD Merck Millipore
Glycine	EMD Merck Millipore

2.1.4 Commercial kits and antibodies

Commercial kits and antibodies used in this study are given in Table 2.3.

Table 2.3: Commercial kits and antibodies used in this study

Kits and Antibodies	Supplier Company
Mammalian Cell Extraction Kit	BioVision
BCA Protein Assay Kit	Pierce, Thermo
Visualizer™ Western Blot Detection Kit	EMD Merck Millipore
Accell siRNA Green Control Kit	Dharmacon™
Accell siRNA SEPT3 (rat)	Dharmacon™
Accell siRNA KATNA1 (rat)	Dharmacon™
Accell siRNA MAPT (rat)	Dharmacon™
Rabbit pAb p60-katanin (KATNA1)	Atlas
Mouse mAb Septin3 (G-6)	Santa Cruz Biotechnology

Table 2.3 (continued): Commercial kits and antibodies used in this study

Anti-mouse IgG, HRP-linked	Cell Signalling Technology
Anti-rabbit IgG, HRP-linked	Cell Signalling Technology
Chicken pAb α-tubulin	Abcam
Normal rabbit IgG	Cell Signalling Technology
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
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

2.1.5 Buffers and Solutions

2.1.5.1 10X Tris buffer saline (TBS)

TBS was prepared as 10X stock solution by dissolving 24.3 g (0.4M) Tris base and 87.66 g (1.5M) NaCl in 1 lt ddH_2O and then pH was adjusted to 7.6. 1X working solution was prepared by dilution and used through the study.

2.1.5.2 Tris buffer saline – Tween20 (TBS-T)

TBS-T was prepared by dissolving 0.1% (v/v) Tween20 in 1X TBS. TBS-T was used as washing buffer in Western blot experiments throughout the study.

2.1.5.3 Separating buffer for western blotting

Separating buffer was prepared by dissolving 187 g Tris Base (1.5 M) and 4.0 g SDS (0.4%) in 1L ddH_2O and then pH adjusted to 8.8. It is stored in 4^0C.

2.1.5.4 Stacking buffer for western blotting

Stacking buffer was prepared by dissolving 60.5 g Tris Base (0.5 M) and 4.0 g SDS (0.4%) in 1L ddH_2O and then pH adjusted to 6.8. It is stored in 4^0C.

2.1.5.5 SDS Running buffer for western blotting

SDS running buffer was prepared by dissolving 30.3 g (0.025M) Tris base, 144.1 g (0.192M) glycine and 10 g SDS (1%) in 1 L ddH_2O and then pH adjusted to 8.3. The buffer is used in Western blot experiments throughout the study.

2.1.5.6 Towbin buffer

Towbin buffer was prepared by dissolving 1.5 g (25 mM) Tris, 7.5 g (192 mM) Glycine and 20% (v/v) Methanol in 500 mL ddH_2O. Towbin was used as transfer buffer in Western blot experiments throughout the study.

2.1.5.7 PBS solution

PBS solution was prepared by dissolving 1 PBS tablet within 100 mL ddH_2O.

2.1.5.8 Elution buffer for immunoprecipitation

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

2.1.5.9 Blocking solution for western blotting

Blocking solution for western blot analysis was prepared by mixing the 2.5 g (5%) non-fat dry milk and 50 µl (0.1%) Tween-20 in 50ml ddH_2O.

2.1.5.10 Blocking solution for immunocytochemistry

Blocking solution for immunocytochemistry was prepared by mixing 1.5 g (3%) bovine serum albumin and 50 mg (0.1%) saponin in 1X 50 ml PBS.

2.1.5.11 NP40 solution

NP40 solution was used for isolating proteins. It was prepared by mixing 500 µl (1%) NP40, 302 mg (50mM) Tris base, 435 mg (150mM) NaCl, 2,5 µl (0.5M) 1M EDTA, 500 µL 1X Protease Inhibitor Cocktail (100X) in 50 ml ddH_2O

2.1.5.12 Mild stripping solution for western blotting

Mild stripping solution was used for removing proteins from the membrane in order to incubate with new antibody. Mild stripping buffer was prepared by dissolving 15g glycine, 1 g SDS and 10ml Tween20 in 800 ml ddH_2O and pH adjusted to 2.2, later volume bringing up to 1 L with dH_2O.

2.1.5.13 Bacterial strains

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

2.1.5.14 LB medium

Luria Bertani (LB) medium was prepared by dissolving 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1L ddH2O. LB medium was sterilized by autoclaving for ten minutes at 121°C. In order toprepare a selective media, 100 µg/L ampicillin or 50 µg/L kanamycin depending on the plasmid was added after the media was cooled down.

2.1.5.15 LB-agar medium

LB-agar medium was prepared by dissolving 10 g tryptone, 5 g yeast extract, 10 g NaCl and 20 g agar in 1L ddH_2O. LB-agar medium was sterilized by autoclaving for ten minutes at 121°C. In order to a selective media, 100 µg/L ampicillin or 50 µg/L kanamycin depending on the plasmid was added after the media was cooled down. After mixing the medium with the appropriate antibiotic, the LB-agar medium was poured into 100 mm Petri plates.

2.1.5.16 $CaCl_2$ solution

$CaCl_2$ solution was used in order to prepare chemically competent *E.coli* cells that were further used in transformation protocol. The solution was prepared by mixing c 60 mM $CaCl_2$, 10mM PIPES and 15 % glycerol in ddH_2O. pH was adjusted to 6,4, following the sterilization with 0,22 µm filter.

2.1.6 Cell culture mediums preparation

2.1.6.1 Primary rat culture neurons

Three different media were used for primary neuron culturing for different purposes.

Dissection medium:

In order to prepare 100 mL rat cortical dissection medium, 10 mL Hank's balanced salt solution (10X), 1 mL 20mM M HEPES and 1 mL Penicillin-Streptomycin were dissolved in 88 mL ddH2O. Medium was sterilized with 0.22 µm pore size filter.

Platig medium with serum:

Cortical neurons were plated 1 hour with plating medium contains serum. The medium was prepared by mixing 2 ml 1X B-27 supplement (50X), 5 ml (5% v/v) fetal bovine serum, 0.66 ml 45% (w/v) D-glucose solution, 1 ml 1X GlutaMAX™ (100X) up to 100 ml Neurobasal medium (w/o phenol red) (1X). Medium was sterilized with 0.22 μm pore size filter.

Plating medium without serum:

Cortical neurons were plated for further analysis with plating medium without serum. The medium was prepared by mixing 2 ml 1X B-27 supplement (50X), 0.66 ml 45% (w/v) D-glucose solution, 1 ml 1X GlutaMAX™ (100X) up to 100ml Neurobasal medium (w/o phenol red) (1X). Medium was sterilized with 0.22 μm pore size filter.

2.1.6.2 RFL6 plating medium

Rat lung fibroblast cells (RFL6 cell line) derived from the lung tissue of a 18 day gestation, Sprague-Dawley rat fetus from ATCC. The medium was prepared by mixing 5ml 1 % (v/v) Penicillin-Streptomycin (10K/10K), 100 mL 20 % (v/v) fetal bovine serum up to 500ml Ham's F12 w/L-glutamine (1X). Medium was sterilized with 0.22 μm pore size filter.

2.2 Methods

2.2.1 Co-immunoprecipitation of Tau & Septin3 and Tau & p60-katanin

Co-immunoprecipitation assay was carried out to investigate the interaction of Tau & Septin3 and Tau & p60-katanin.

2.2.1.1 Total protein isolation from rat brain

Septin3 is a neuron-specific septin within Septin family. Also, Tau and p60-katanin are known to be abundant in neurons. Therefore, immunoprecipitation assay was performed with total protein isolated from the rat brain.

100 mg of rat brain was chopped and placed in a falcon. The tissue sample was washed twice with 1 mL NP40 solution and the tube was centrifuged at 4300 rpm for 5 minutes at 4^0C for getting rid of the blood. The supernatant was discarded and the pellet was resuspended with 1500 μL of NP40 buffer, which was prepared as mentioned in the

section 2.1.5.11. The volume in the falcon was doubled by adding Glass Beads (≤106 µm) (Sigma) and the falcon was vortexed until the tissue sample become totally homogenized. The falcon was centrifuged at 4300 rpm for 10 minutes at 4^0C and the supernatant was transferred into a new falcon. The protein concentration of total brain lysate was measured via BCA protein assay with using BCA Protein Assay Kit (Pierce™) according to manufacturer's instructions.

2.2.1.2 Co-immunoprecipitation

Immunoprecipitation assay for Tau and Septin3 interaction was performed using 750 µg total brain lysate mixing with 3 µg mouse monoclonal Tau antibody (A-10) (Santa Cruz) (200 ng/µL) in an eppendorf tube. As a negative control of immunoprecipitation assay, 3 µg normal mouse IgG (CST) (1 µg/µL) was mixed with the same amount of protein lysate in another eppendorf tube and the tubes were incubated on a rotator overnight at 7 rpm in 4^0C. Following the over-night incubation, 50 µl of protein G magnetic beads (Invitrogen) were incubated with antibody-lysate samples after the beads were pre-washed with 1 mL NP40 solution. There were three different reactions as 'IP', 'non-specific IgG' and 'beads only', each reaction contains 50 µL of protein G magnetic beads. 750 µg of lysate and beads were mixed as a negative control of immunoprecipitation assay. The tubes were incubation via rocking on a rotator for 1 hour at 7 rpm on room temperature. After an hour, the tubes were placed in the magnetic rack and the supernatant was removed. The magnetic beads were washed four times each with 250µl NP40 solution. After the washing, the supernatants were removed and magnetic beads were resuspended in 35 µL elution buffer (prepared like given in 2.1.5.8). Following the elution step, the beads were mixed with 3X protein sample buffer and boiled for 5 minutes in a heat block set at 95^0C. Then the supernatant were transferred into new eppendorf tubes and used for western blotting. The same protocol was performed for reciprocal co-immunoprecipitation except mixing 750 µg of lysate with a 3 µg mouse monoclonal Septin3 antibody (Santa Cruz) (200 ng/µL).

In order to perform immunoprecipitation assay to analyze Tau and p60-katanin interaction, same protocol was performed for Tau but for reciprocal co-immunoprecipitation 750 µg total brain lysate was mixed with a 3 µg rabbit polyclonal p60-katanin antibody (KATNA1) (ATLAS) (100 ng/µL) and as a negative control 3 µg normal rabbit IgG (CST) (1 µg/µL) was used.

2.2.1.3 Western blotting

Western blotting is performed via sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Initially, SDS-PAGE gel was prepared. Ammonium persulfate (APS) was prepared by mixing 30 mg APS in 300 µl dH_2O. Separating gel was prepared for two gels by mixing 5.30 ml dH_2O, 5 ml separating buffer, 4.68 ml 40% Acrylamide/Bisacrylamide, 150 µL APS and 15 µl TEMED. The separating gel solution was poured into the gel cassette and polymerized for 30 minutes at the room temperature. Then, separating gel solution was removed and washed. Stacking gel was prepared for two gels by mixing 3.275 ml dH_2O, 1ml stacking buffer, 0.625 ml 40% Acrylamide/Bisacrylamide, 50 µl APS, 15 µl TEMED. The stacking gel was poured above the separating gel and 10-well comb was placed, then the gel was polymerized for 45 minutes at the room temperature.

The SDS-PAGE gel was placed in a clamping frame and it was run in the electrophesis tank in a running buffer at 90V for 4 hours. After separation of denatured proteins according to size by SDS-PAGE, the proteins were transferred to nitrocellulose membrane (0.22 µm) via Trans-Blot® transfer packs. Nitrocellulose membrane and transfer packs were incubated into towbin buffer (1X) for 15 minutes before transfer. Transfer pack, nitrocellulose membrane, SDS-PAGE gel and one more transfer pack were placed onto transfer cassette, sequentially. Transfer was performed via Trans-Blot® Turbo™ (Biorad) instrument running at constant 2.5 amperes (A) for 7 minutes.

In order to reduce background staining and prevent non-spesific binding, the membrane was blocked in a blocking solution on a rocking platform for 1 hour at the room temperature. After blocking, the membrane was incubated with 1:500 diluted blocking solution primary antibody mouse monoclonal Tau antibody (A-10) (Santa Cruz), rabbit polyclonal p60-katanin antibody (KATNA1) (ATLAS) and mouse monoclonal Septin3 antibody (Santa Cruz)) for overnight at 4°C on a rotator. Following day, membranes were washed with TBS-T five times for 5 minutes. For secondary antibody, Anti-mouse IgG VeriBlot for IP (Abcam) solution (1:5000 dilution) and anti-rabbit IgG conformation specific (CST) (1:3000 solution) were used and membranes were incubated with secondary antibodies for 1h at the room temperature. Membranes were washed five times for 5 minutes after secondary antibody incubation.

For detection, Visualizer™ Western Blot Detection Kit (Millipore) was used according to manufacturer's instructions and the proteins were visualized via Gel Doc™ XR+ Imager (Biorad) with Image Lab™ Software (Biorad).

2.2.1.4 Mild stripping

The membranes were stripped for precipitation control. Mild stripping was used and incubated on a shaker at room temperature for 7 minutes. Then, buffer was discarded and incubation was repeated with fresh mild stripping buffer with additional 7 minutes. The buffer was removed again an followed by the washing step with 1X PBS for 10 minutes. 1X PBS was removed and the membrane was washed with fresh 1X PBS with 10 more minutes. Whole procedure was included additional washing step with 1X TBS-T for 5 minutes, two times. After that, membrane was stripped and ready for blocking. The same western protocol was used to detect precipitation amounts of proteins after stripping.

2.3 RFL6 Cell Culture Studies

RFL6 cell line which had been stored at -80^{0}C was thawed and mixed with 5 ml Ham's F12 medium (Lonza). The falcon tube was centrifuged for 7 minutes at 1500 rpm, the supernatant was removed and the pellet was resuspended with 1 ml Ham's F12 medium (20% FBS, 1X NEAA, 1%P/S). Cells were seeded to T75 culture flasks and were subcultured for at least 1 passage prior to nucleofection.

2.3.1 Nucleofection of RFL6 cells

RFL6 cells were incubated with 3 ml Trypsin/EDTA (Lonza) for 10 minutes for deattaching. After that, deattached cells were transferred to a falcon tube containing 6 ml Ham's F12 medium (20% FBS, 1X NEAA, 1%P/S) and centrifuged at 1500 rpm for 7 minutes. Following the centifugation; supernatant was removed and pellet was resuspended with Ham' s F12 medium. The resuspended cells were counted via a hemocytometer. 1x10^6 cells per nucleofection reaction and each eppendorf tube was centrifuged at 300 x g for 7 minutes. Nucleofector® solution was prepared by mixing 82 µl of Nucleofector® solution of Kit R with 18 µl of its supplement prior nucleofection. For a single transfection, 10 µg of plasmid was used for each reaction. For co-transfection 6 µg Septin3 with 9 µg p60-katanin and 6 µg Septin3 with 6 µg

Tau were used. All nucleofection reactions were combined with the cell suspensions separately. Cell/DNA suspensions were transferred into Nucleofection® cuvette and the Nucleofector® program O-017 was applied to the cuvettes. After nucleofection, 1 ml of Ham's F12 medium was added to each cuvette in order to equilibrate cell suspension. Cells were seeded on a 60 mm culture dish, the dishes were incubated at 37^0C culture incubator for 21 hours.

2.3.2 Immunocytochemistry of RFL6 cells

For immunocytochemistry experiments, glass coverslips were needed to coat with Poly-L-Lysine. Coverslips (18 mm diameter) were cleaned up with 70% ethanol for 15 minutes at the room temperature. Coverslips then incubated with distilled water in order to remove 70% ethanol. The glass coverslips were put in a 12-well tissue culture plate and it was sterilized under the UV-lamp for 15 minutes in the hood**.** The coverslips were coated with diluted (50 µg/mL) Poly-L-lysine solution (100 µg/mL) (Millipore) and incubated over-night at the room temperature. Following over-night incubation, the lysine solution was discarded completely and the coverslips were washed four times for 5 minutes each with 2 mL distilled and filtered water. Coverslips were incubated at 37^0C in a humidified 5% CO_2 atmosphere culture incubator until using and could be stored at 4^0C for further experiments.

After the cells were seeded on the coverslips and incubated for 21 hours, 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 the room temperature. Then, paraformaldehyde solution was removed and the coverslips were washed 4 times for 5 minutes each with 1 mL PBS (1X) in order to completely discard remaining paraformaldehyde solution. Cells' membranes were permeabilizated with 0.1 % (w/v) saponin solution (dissolved in 1X PBS). The coverslips were incubated with with saponin solution at the room temperature for 10 minutes. Following the permeabilization step, coverslips were incubated with the immunocytochemistry blocking solution for 1 hour at room temperature. After 1 hour, the coverslips were placed onto parafilm layered petri plates. The primary antibodies were used in RFL6 experiments as followed: anti-GFP tag antibody (Invitrogen) (1 µg/µL) 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 all at the same dilution of 1:200. Primary antibodies were incubated over-night at 4^0C. The next day, the coverslips were washed 4 times for 5 minutes each with 500 µL PBS (1X) in order to reduce non-spesific binding of primary antibodies**.** The secondary antibodies which were used in this experimes as followed: 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) all at the same dilution of 1:200 and prepared in the blocking solution. The coverslips were incubated for secondary antibody binding at the room temperature for one hour in a dark place. After 1 hour secondary antibody incubation, the coverslips were washed 3 times for 5 minutes each with 200 µL PBS (1X) to get rid of unbound antibody residues completely. After the coverslips were dried well, they were mounted with Prolong® mounting medium (Invitrogen) on laboratory slides. The coverslips were sticked with a transparent nail polish and the slides were incubated at room temperature in the dark over-night before the imaging via TCS SP2 SE confocal Microscope (Leica).

2.4 Primary Rat Cortical Neuron Culturing

The name of 'primary culture' implies the cultures started from cells, tissues or organs directly taken from organisms. Because neurons are post-mitotic cells, they have completed their division *in situ* and started to extend processes and each different type of neurons expresses its own properties which was an important factor on neuronal studies.

2.4.1 Dissection of cerebral cortex

Primary cortical neuron culture was obtained from E18 Sprague – Dawley rat embryos. Embryos were transferred on ice bucket to obtain anaesthesia and prevent from brain death. Rats were briefly rinsed in 70% ethanol prior to dissection procedure in order to reduce the contamination risk. Also, all equipment for dissection was sterilized via %70 ethanol incubation and UV-lamp sterilization. Heads of the embryos were cut and transferred into a culture dish containing cortical dissection medium pre-warmed to 37°C. The brains were removed from skulls under the dissection microscope with Dumont-style forceps (no:5) and they were transferred to another culture dish containing dissection medium. First step was separating the cerebral hemispheres from

diencephalon and brain stem following the meninges removal. The hemisphere was stabilized with forceps and another forceps were used to remove limbic lobe containing hippocampus and corpus callosum. Then, cerebral cortex was transferred into a cleang culture dish containing cortical dissection medium. Dissected cerebral cortices were cut into smaller pieces and they were put into 15ml falcon tube and the volume was brought to 4.5 mL with dissection medium. Then, 0.5 mL trypsin (2.5%) (w/o phenol red) and 0.25 mL DNase (10 mg/ mL) were mixed into the falcon and incubated in a water bath set 37^0C for 30 minutes with gentle shaking. Following the incubation step, cortical tissue pieces were settled down to the bottom of the falcon tube and trypsin and DNase were pipetted off. Cortical tissues were rinsed 2 times for 5 minutes each with 5 mL pre-warmed cortical plating medium with serum. Trituration of cortical tissues were performed 8 times against the side of the tube using a fire-polished Pasteur pipette with a narrow mouth. After dissociation has completed, the cell density were counted via Countess™ automated cell counter (Invitrogen).

2.4.2 Nucleofection of primary cortical neurons

$1x10^6$ cells per nucleofection reaction and each eppendorf tube was centrifuged at 300 x g for 7 minutes. Nucleofector® solution was prepared by mixing 82 µl of Nucleofector® solution of Rat Neuron with 18 µl of its supplement prior to nucleofection. For a single transfection, 10 µg of plasmid was used for each reaction. For co-transfection 6 µg Septin3 with 9 µg p60-katanin and 6 µg Septin3 with 6 µg Tau were used. All nucleofection reactions were combined with the cell suspensions separately. Cell/DNA suspensions were transferred into Nucleofection® cuvette and the Nucleofector® program G-013 was applied to the cuvettes. After nucleofection, 1 ml of Neurobasal medium was added to each cuvette in order to equilibrate cell suspension. Cells were seeded on either 60 mm culture dish for protein isolation or 18 mm diameter coverslips for immunocytochemistry, the dishes were incubated in a 37^0C culture incubator.

2.4.3 Accell siRNA transfection

siRNA Resuspension protocol was followed at least one day prior to Accell siRNA transfection (Dharmacon™) Dharmacon™. Initially, tubes containing siRNA was briefly spin to ensure that the siRNA pellet was collected at the bottom of the tube. Then siRNA was resuspended in RNase-free 1x siRNA Buffer for the desired final

concentration. In this study 10 nmol of siRNA and 100 µM of stock concentration was used. Thus, 100 µl 1x siRNA Buffer was added onto the siRNA pellet and vortexed. Following the resuspension step, the solution of siRNA stock was placed on an orbital mixer/shaker for 90 minutes at 37°C. Then, tube containing siRNA was briefly spin to collect solution to bottom of the tube. The used final concentration was 1 µM Accell siRNA per well as manufacturer' s recommandation. Due to the toxicity of Accell siRNA delivery medium, siRNA' s for each reaction was mixed with 50% Accell siRNA delivery medium and 50% Neurobasal medium. The growth medium was removed from the cells and delivery medium mix was added to each well. According to optimization results, cells were incubated at 37 ºC with 5% CO_2 for 48 hours.

2.4.4 Western blotting of over-expressing and siRNA-delivered neurons

48 hours later, media were discarded and the culture dishes were washed with 1X PBS Additional 2 mL 1X PBS was poured into each dish and the cells were scrapped from the culture dish. This step was completed two times and each dish was washed out with 2 ml 1X PBS. The cell suspension was transferred into a falcon tube and the tube was centrifuged at 4300 rpm for 7 minutes. Protein isolation was performed using freshly prepared NP40 solution. After the centrifugation, the supernatant was discarded and the pellet was resuspended in 50 µl NP40 solution for siRNA-delivered neurons and 200 µl NP40 solution for over-expressed neurons. Resuspended solutions transferred to the new eppendorf tube, and then it was incubated on ice for 30 minutes. After the incubation, the tube was vortexed briefly and centrifuged at 14,300 x g for 7 minutes. The supernatant was containing proteins and transferred to the new eppendorf and the protein concentration was measured via BCA Protein Assay Kit (Pierce™) according to manufacturer's instructions. Western blotting was carried out as explained in the Section 2.2.1.3. The only difference was for secondary antibody: Anti-mouse IgG (CSR) solution (1:3000 dilution) and anti-rabbit IgG (CST) (1:3000 solution) were used.

2.4.5 Mild stripping

The membranes were stripped for precipitation control. Mild stripping buffer covered nitrocelluose membrane was incubated on a shaker at room temperature for 7 minutes. Then, buffer was discarded and incubation was repeated with fresh mild stripping buffer with additional 7 minutes. The buffer was removed again and followed by the

washing step with 1X PBS for 10 minutes. 1X PBS was removed and the membrane was washed with fresh 1X PBS with 10 more minutes. Whole procedure ncluded additional washing step with 1X TBS-T for 5 minutes, two times. After that, membrane was stripped and got ready for blocking. The same western protocol was used to detect precipitation amounts of proteins after stripping.

2.4.6 Immunocytochemistry of primary cortical neurons

For immunocytochemistry experiments, glass coverslips were needed to coat with Poly-D-Lysine. Coverslips (18 mm diameter) were cleaned up with 70% ethanol for 15 minutes at the room temperature. Coverslips were then incubated with distilled water in order to remove 70% ethanol. The glass coverslips were put in a 12-well tissue culture plate and sterilized under the UV-lamp for 15 minutes in the hood. The coverslips were coated with diluted (50 µg/mL) Poly-L-lysine solution (100 µg/mL) (Millipore) and incubated over-night at the room temperature. Following over-night incubation, the lysine solution were discarded completely and the coverslips were washed four times for 5 minutes each with 2 mL distilled and filtered water. Coverslips were incubated at 37^0C in a humidified 5% CO_2 atmosphere culture incubator until using and could be stored at 4^0C for further experiments.

Immunocytochemistry was carried out as explained on Section 2.3.2. Althoug the protocol was the same, primary antibodies for detecting gene-silencing were as followed: mouse monoclonal Tau antibody (A-10) (Santa Cruz), rabbit polyclonal p60-katanin antibody (KATNA1) (ATLAS) and mouse monoclonal Septin3 antibody (Santa Cruz), all diluted 1:200 concentration. Over-expression analysis was carried out with a tag-spesific antibodies.

2.5 Plasmid Isolation for Experiments

Plasmids were used in this study as followed: Septin3-pcDNA™ 3.1/myc-His A, p60-katanin- p3XFLAG-CMV10 and Tau-EGFP. All plasmids were cloned previously in our research group. In this study, they were not cloned but prior to experiment plasmids were transformed and isolated freshly.

2.5.1 Competent cell preparation

The bacterial competent DH5α cells were chemically treated to allow the foreign plasmid to be passed through the cell membrane. *E.coli*-DH5α cells were taken from a glycerol stock and it was inoculated in 5 mL LB medium and incubated overnight at 37ºC on orbital shaker. After overnight incubation, 100 mL LB medium was inoculated with 5 mL culture solution and was incubated at 37ºC in orbital shaker. When cell density was reached to 0.6, the bacteria were transferred to 50 mL eppendorf tube and incubated on ice for 10 minutes. The cells were centrifuged at 1500 x g for 6 minutes at 4ºC, and then supernatant was removed. Each *E.coli*-DH5α bacterial pellet was resuspended in 10 mL ice-cold $CaCl_2$ solution and centrifuged for 5 minutes at 1500 x g. In addition, each bacterial pellet was dissolved in 10 mL ice-cold $CaCl_2$ and they were incubated on ice for 30 minutes. Centrifugation was performed again at 1500 x g for 5 minutes at 4ºC. Each *E.coli*-DH5α pellet was resuspended in 2 mL of $CaCl_2$. The competent cells were stored at -80ºC.

2.5.2 Transformation

For the transformation procedure, the competent cells were taken out from -80^{0}C and thawed on ice. The plasmids (100μM for each plasmid) were mixed with competent cells and the eppendorf tube was incubated on ice for 30 minutes. Then the tube was placed in 42ºC for 45 seconds for heat-shock and permabilization and put back on ice for additional 2 minutes. 200 μL LB medium was added and then culture was incubated at 37ºC for 1 hour in orbital shaker. Culture was plated on selective medium with appropriate antibiotic (LB-Ampicillin or LB-Kanamycin). Plates were incubated overnight at 37ºC.

2.5.3 Plasmid isolation

Plasmids were isolated by using NucleoSpin Plasmid Kit (MN) according to the manufacturer' s instructions. After the isolation, the concentrations of plasmids were measured with Nanodrop2000 spectrophotometer (Thermo).

3. RESULTS

3.1 Immunoprecipitation of Tau & Septin3 and Tau & p60-katanin in Rat Brain Lysate

Septins are considered as the fourth cytoskeleton elements and some septins have been revealed to be involved in microtubule dynamics via interactions with MAPs. It has been previously identified that Septin2 modulates microtubule dynamics through interaction with MAP4 (Kremer, 2005). Also, it is known that tau provides strong protection against microtubule severing by p60-katanin (Yu, 2008).

Physical interaction of Tau & Septin3 and Tau & p60-katanin proteins were determined by co-immunoprecipitation of endogenous Tau & Septin3 and Tau & p60-katanin with specific antibodies from rat brain lysate and the presence of the proteins were detected with specific antibodies.

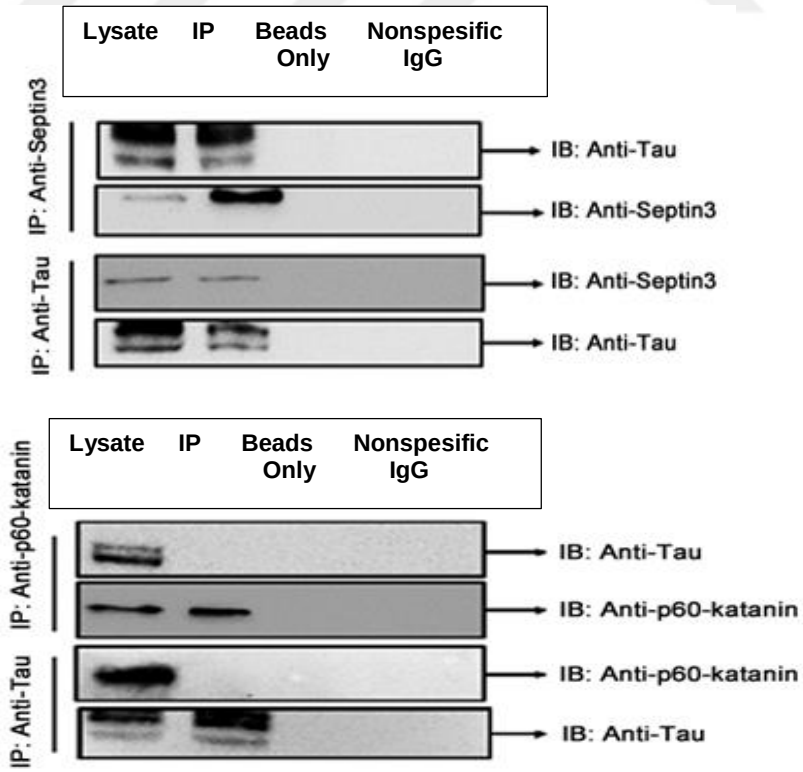

Figure 3.1 : Co-immunoprecipitation results of Tau&Septin3 and Tau&p60-katanin.

3.2 Interaction Between Septin3 – Tau – p60-katanin in Mitotic RFL6 Cells

For these studies RFL6 cells were chosen because they are very flat, and hence excellent for imaging microtubules using immunofluorescence, and because they do not express Tau and Septin3 proteins endogenously.

Initially, Tau over-expression in RFL6 cells were investigated. Tau was over-expressed via nucleofection in RFL6 cells, after 21 hours cells were stained with GFP-tag antibody and microtubules were stained with α-tubulin antibody and subjected to the immunocytochemistry analysis. Microtubules were stained with Alexa Fluor® 647 (far-red fluorescent dye) and Tau is stained with Alexa Fluor® 488 (green fluorescent dye) conjugated secondary antibodies. Consequently, Tau formed filaments and co-localized with microtubules (Figure 3.2).

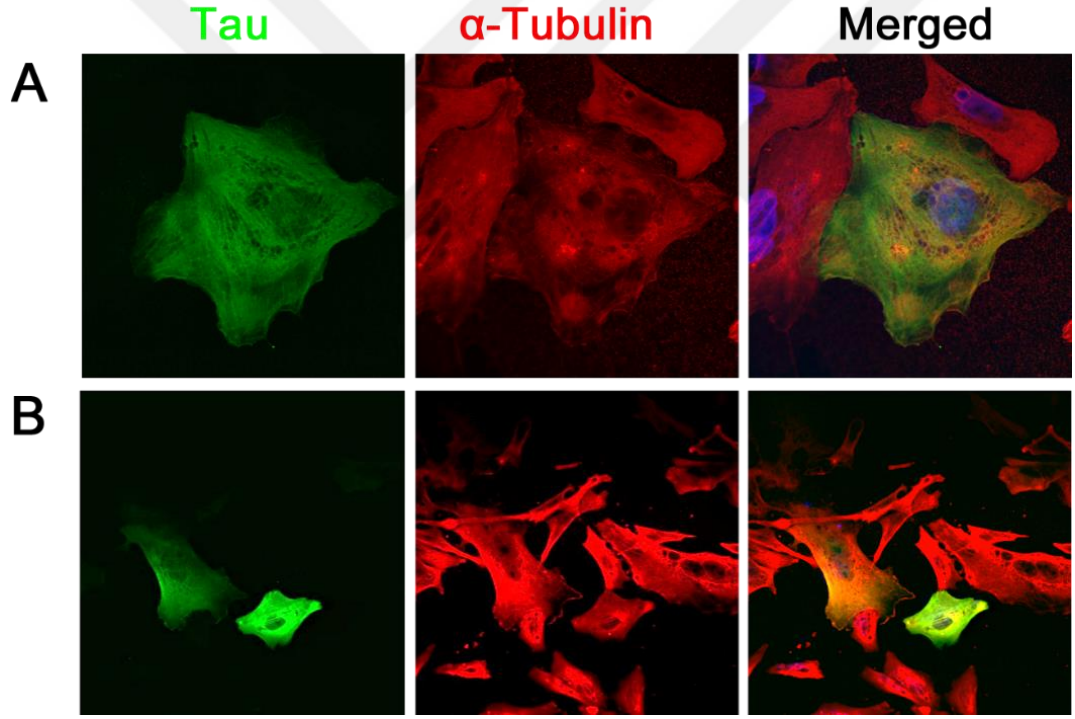

Figure 3.2 : Immunostaining of Tau and microtubules on RFL6 cells. Tau protein is green and α-tubulin is red. The merged image was shown at the right.

The physical interaction between Tau and Septin3 was identified by co-immunoprecipitation. In order to investigate *in situ* interaction and co-localization of Septin3 and Tau, immunocytochemistry (ICC) analysis was performed. Septin3 and Tau constructs were over-expressed via nucleofection in RFL6 cells. After 21 hours, cells were fixed and stained with anti-Myc tag (Septin3 construct' s tag) and GFP-tag (Tau construct' s tag). Microtubules were stained with α-tubulin antibody. As a

secondary antibody, Alexa Fluor® 647 for microtubules, Alexa Fluor® 488 for Tau and , Alexa Fluor® 594 for Septin3 were used. Figure 3.3 showed that Septin3 formed filamentous structure and co-localized with Tau on RFL6 cells.

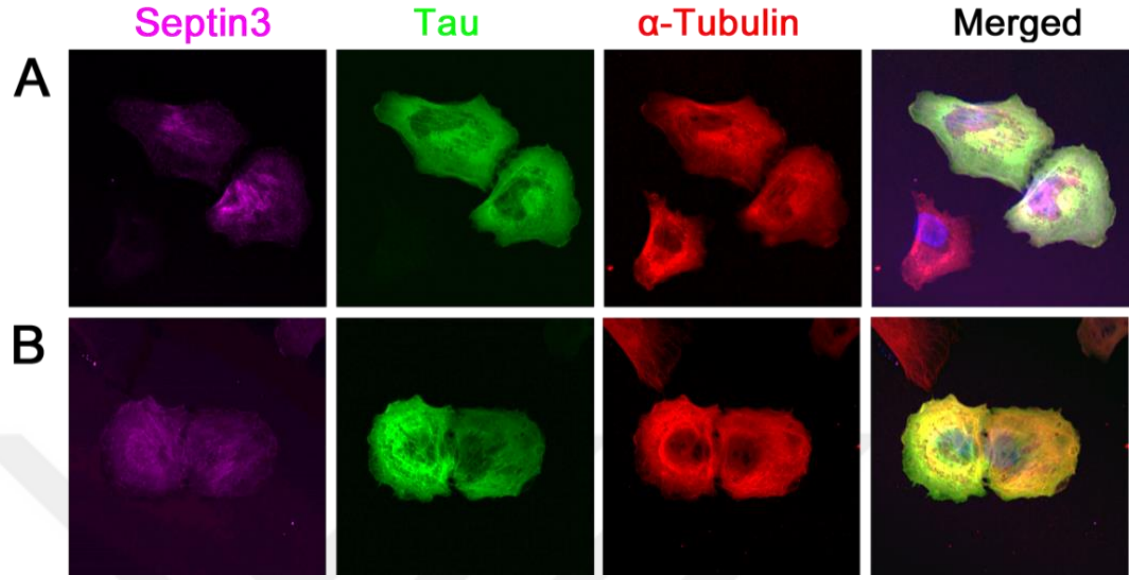

Figure 3.3 : Co-localization of Septin3 and Tau in RFL6 cells. Tau protein is green, Septin3 is violet and α-tubulin is red. The merged image was shown at the right.

Through study, in order to observe the p60-katanin's potential severing effect on Septin3 filaments, RFL6 cells transfected with p60-katanin and Septin3 were exposed to ICC analysis. Septin3 proteins were stained with Alexa Fluor® 488, p60-katanin proteins were stained with Alexa Fluor® 594 and α- tubulin was stained with Alexa Fluor® 647 conjugated secondary antibodies. It is observed that following the p60-katanin over-expression, Septin3 filament formation was impaired. Small Septin3 filaments localized around p60-katanin which was abundant in nucleus (Figure 3.4)

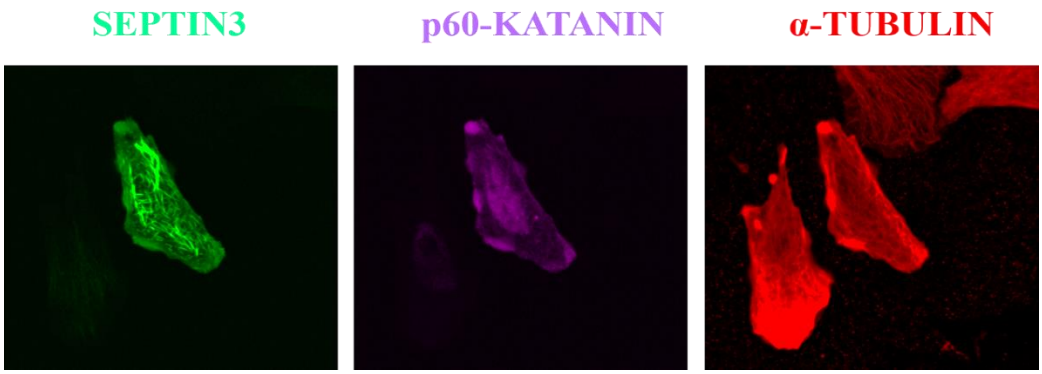

Figure 3.4 : Immunostaining of Septin3, p60-katanin and microtubules in RFL6 cells. Septin3 protein is green, p60-katanin is violet and α-tubulin is red.

To determine how Septin3 and Tau interaction would effect p60-katanin activity on Septin3 filament formation, Septin3, Tau and p60-katanin were co-transfected via nucleofection on RFL6 cells. Septin3 was stained with anti-Myc tag; p60-katanin was stained with anti-FLAG tag, microtubules were stained with α-tubulin. It is shown that despite p60-katanin had severing effect on Septin3 filaments, when it was co-transfected with Tau, it could not change the Septin3 filament formation. Septin3 could still form filaments (Figure 3.5).

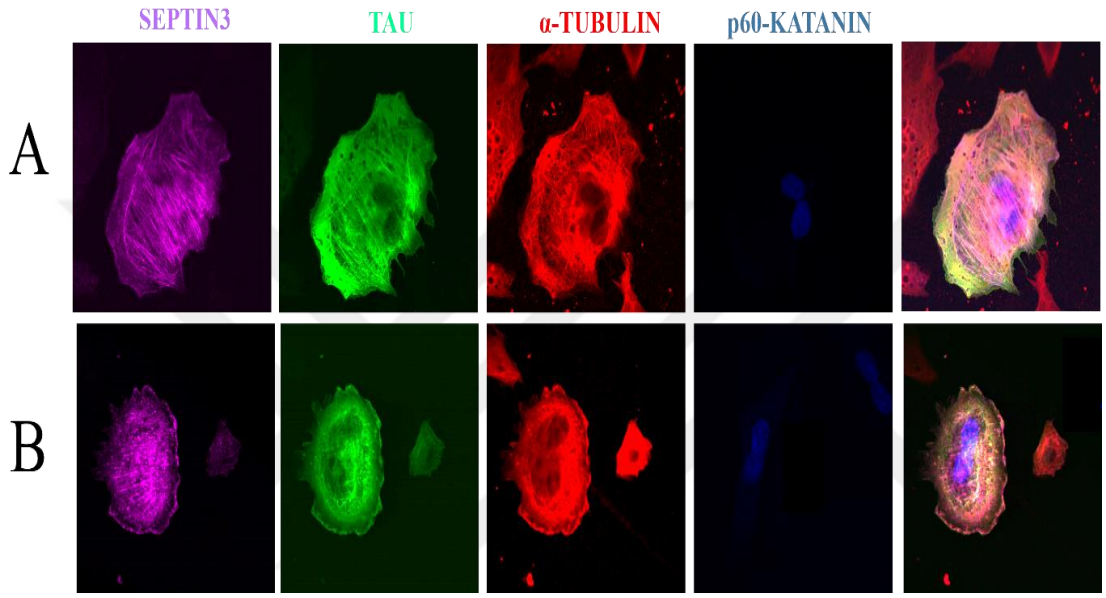

Figure 3.5 : Immunostaining of Septin3, Tau, p60-katanin and microtubules in RFL6 cells. Septin3 is violet, p60-katanin is dark blue, Tau is green α-tubulin is red.

3.3 Effects of Septin3 on Neuronal Branching

In the next part of study, effects of p60-katanin-Tau-Septin3–microtubule interaction network in neuronal branching and microtubule dynamics were analysed by over-expression and gene silencing studies in primary cortical neurons.

In th first set of studies using primary rat cortical neurons, I investigated whether Septin3 has effect on neuronal branching. In order to understand the effects of Septin3 in neuronal branching, primary cortical neuron culture from embriyonic day 18 Sprague – Dawley rat embryos were used. Septin3 protein was over-expressed via nucleofection in neurons and cells were fixed after 48 hours. The samples were stained with Septin3 and β-III-Tubulin antibodies and visualized with fluorescence microscopy (40x). Figure 3.6 showed images of control and over-expressed Septin3.

Axon elongation and thickness were increased by means of Septin3 over-expressing compared to control neurons (Figure 3.6)

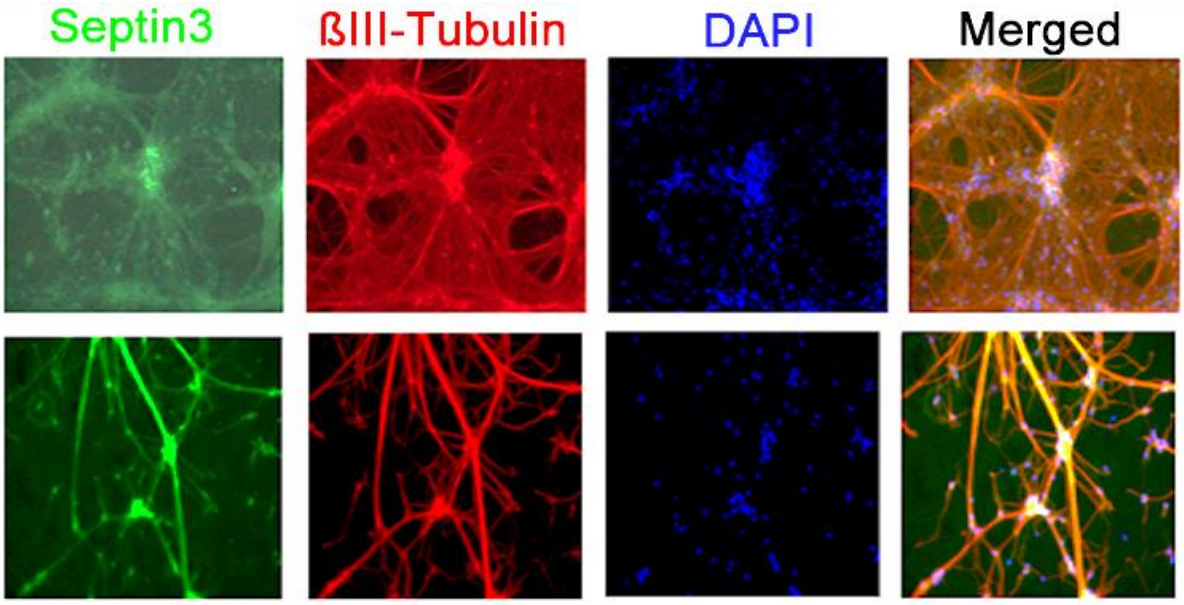

Figure 3.6 : Immunostaining of Septin3 and microtubules in primary rat cortical neurons. Septin3 protein is green, β-III-Tubulin is red. Nucleus is stained with DAPI.

Over the years, a variety of different methods have been used to deplete proteins from neurons. In this study, to better understand the role of Septin3 in neuronal branching, Septin3 was silenced using Accell siRNA transfection. Accell siRNA transfection was performed according to the manufacturer' s protocol. 48 hours after transfection, proteins were isolated and assessed by quantitative western blotting. Normalized protein levels were measured with ImageJ. Figure 3.7 showed that Septin3 expression was silenced 98% via Accell siRNA transfection. In addition, ICC analysis demonstrated that upon down regulation of Septin3, axon extension was impaired (Figure 3.7).

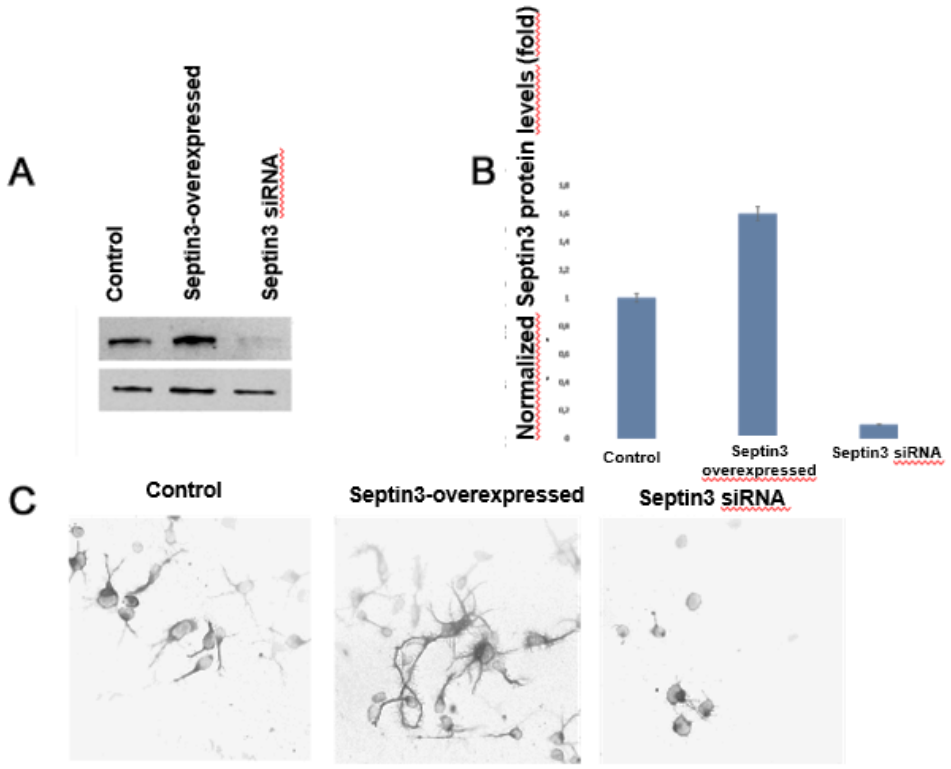

Figure 3.7 : Western blotting and ICC results of Septin3 over-expression and gene silencing ($p<0,01$).

In the literature, very little is known about the functions of septins in mammalian cells, especially in post mitotic neurons. However, Xie and colleagues (2007) found that Septin7 localizes at the bases of filopodia and at branch points in developing hippocampal neurons. As expressed in the aim of the study section, Septin3 is a neuronal spesific protein and it was aimed to test whether it has an effect in neuronal branching. Septin3 was over-expressed in primary cortical neurons and 48 hours after culturing, Septin3 over-expressing neurons reached to stage 5 in neuronal polarization, whereas control neurons reached stage 3. Also, Septin3 over-expression in rat primary cortical neurons increased dendritic-spine branching morphology minor processes and axons became much more developed. Due to the Septin3 over-expression, dendritic spine amount increased 7,5 fold compared to the control neurons. (Figure 3.8).

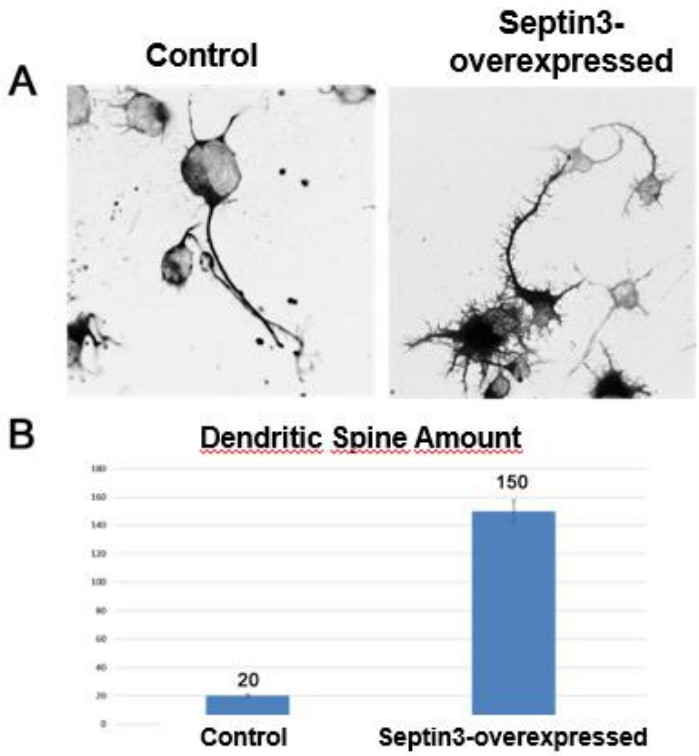

Figure 3.8 : Primary cortical neurons over-expressing Septin3. B showed morphological analysis of Septin3($p<0,05$)

3.4 Effects of Septin3 – Tau – p60-katanin interaction on Neuronal Branching

Since p60-katanin has an important role in neuronal development and Septin3 is a neuronal-specific protein, this interaction has been investigated through neuronal branching. In order to understand the effect of Septin3 and p60-katanin on neuronal branching, primary rat cortical neurons were co-transfected with Septin3 and p60-katanin via nucleofection. 48 hours after nucleofection, cells were fixed and stained with anti-Myc tag and anti-FLAG tag antibodies for Septin3 and p60-katanin, respectively. Septin3 was stained Alexa Fluor® 488, p60-katanin proteins were stained with Alexa Fluor® 594 and α- tubulin was stained with Alexa Fluor® 647 conjugated secondary antibodies. According to ICC results, Septin3 filaments were severed in axons when p60-katanin was over-expressed. Moreover, microtubules were partially remained intact in some areas where Septin3 filaments were interrupted (Figure 3.9).

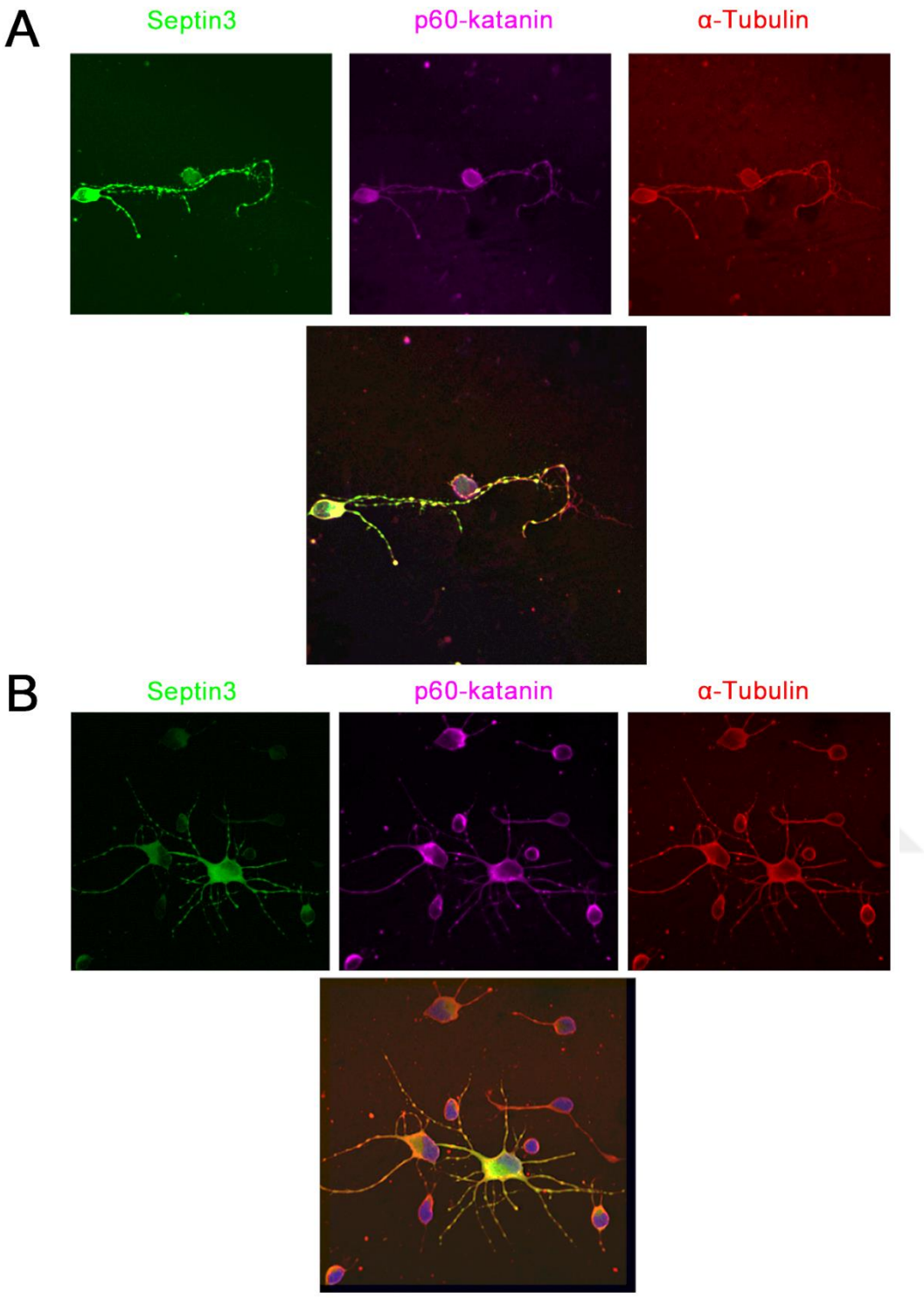

Figure 3.9 : Primary cortical neurons over-expressing both Septin3 and p60-katanin. Neurons were co-nucleofected with Septin3 and p60-katanin.

In this part, studies sought to test whether Tau has any role on Septin3 to protect them from being severed by microtubule severing protein, p60-katanin.

First, as a control Tau was over-expressed in primary rat cortical neurons, fixed and stained with anti-GFP tag antibody. As expected, Tau was observed predominently in axons and did not increase dendrit formation. Neuronal growth was stayed at stage 3 (Figure 3.10).

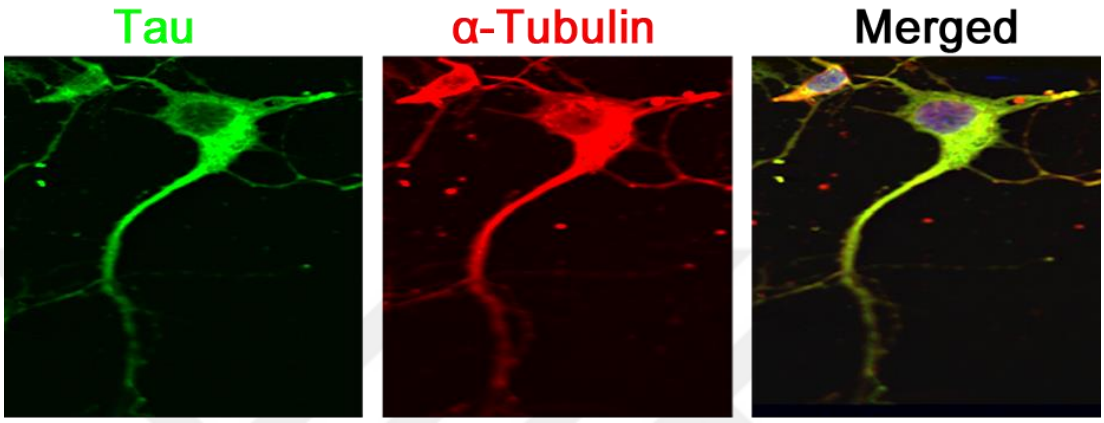

Figure 3.10 : Primary cortical neurons over-expressing Tau

The the question asked was that whether Septin3 could regulate microtubule stability via association with Tau. Tau is a primarily found in neurons and important for neuronal differentiation and growth of neurons. In order to investigate the co-localization of Tau and Septin3, primary rat cortical neurons were co-transfected with Septin3 and Tau via nucleofection. 48 hours after nucleofection, cells were fixed and stained with anti-Myc tag and anti-GFP tag antibodies for Septin3 and Tau, respectively. Tau protein was stained Alexa Fluor® 488, Septin3 proteins were stained with Alexa Fluor® 594 and microtubule was stained with Alexa Fluor® 647 conjugated secondary antibodies. Co-localization of Septin3 with Tau was observed predominantly in axons of neurons, and resulted in thicker bundles (Figure 3.11).

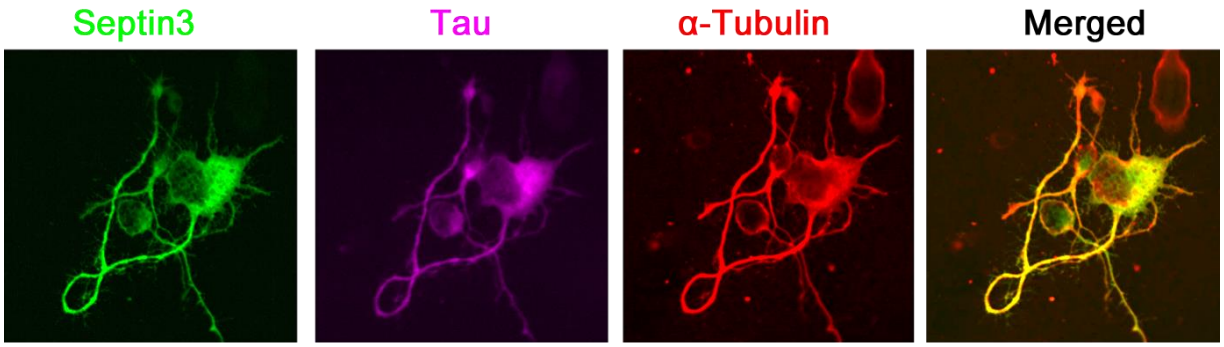

Figure 3.11 : Primary cortical neurons over-expressing both Septin3 and Tau.

It was already mentioned that severing of microtubules increases during mitosis; hence the regulation was thought to be phosphorylation related. There was no evidence from other cell types that p60- katanin itself was phosphorylated (McNally, et al., 2002). This leads to the probability that p60-katanin might be regulated by the phosphorylation of other proteins. In our case, the candidate protein was Tau. Since Septin 3 appears to be a novel cytoskeleton protein and is associated with microtubules, we aim to understand Tau' s activity in Septin3 and p60-katanin interaction. Primary rat cortical neurons were co-nucleofected with Septin3, Tau and p60-katanin in order to achive this purpose. Despite p60-katanin' s effect on Septin3 in axons, Septin3 proteins remained uninterrupted upon co-transfection with Tau. It could be suggested that similar to its function on microtubules, Tau expression could have protective effect on Septin3 filaments against p60-katanin on primary cortical neurons (Figure 3.12)

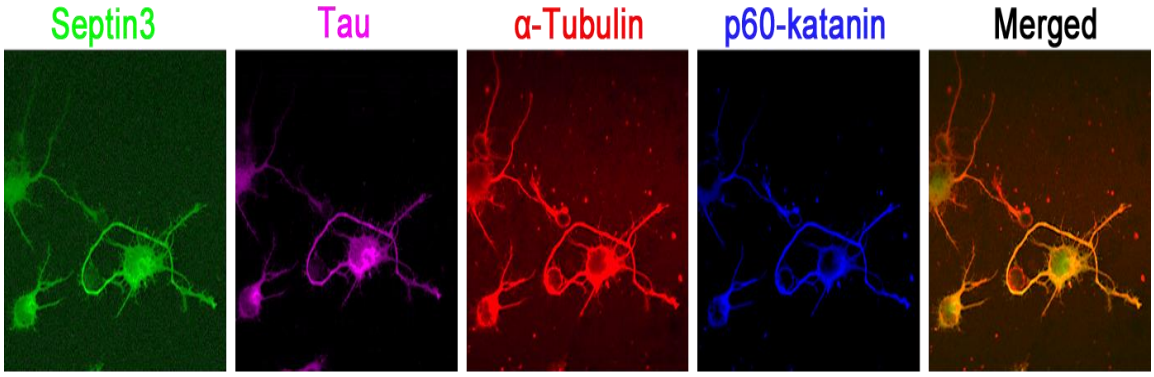

Figure 3.12 : Primary cortical neurons over-expressing Septin3, p60-katanin and Tau

It was previously reported that the ability of p60-katanin to sever microtubules is attenuated by the presence of Tau on the microtubules. Also, depletion of Tau from neurons before over-expressing p60-katanin rendered the microtubules in the axon

equally sensitive to the microtubules in other regions of the neuron (Qiang, 2006). According to this data, we examined the effect of p60-katanin on Septin 3 filaments in the absence of Tau. We performed Tau gene silencing experiment via Accell siRNA transfection. Accell siRNA transfection was performed according to the manufacturoer's protocol. 48 hours after transfection, proteins were isolated and subjected to western blotting. Tau gene silencing occured 90% via Accell siRNA transfection. First, primary rat cortical neurons were co-transfected with p60-katanin and Septin3 and incubated over-night at 37^0C incubator. After over-night incubation, Tau Accell siRNA transfection was performed and ICC experiment was carried out for morphological analysis on primary cortical neurons. Despite the death of most of the cells, remaining cells lacked axon extension and Tau lost its possible protective ability on Septin3 as it did on microtubules (Figure 3.13)

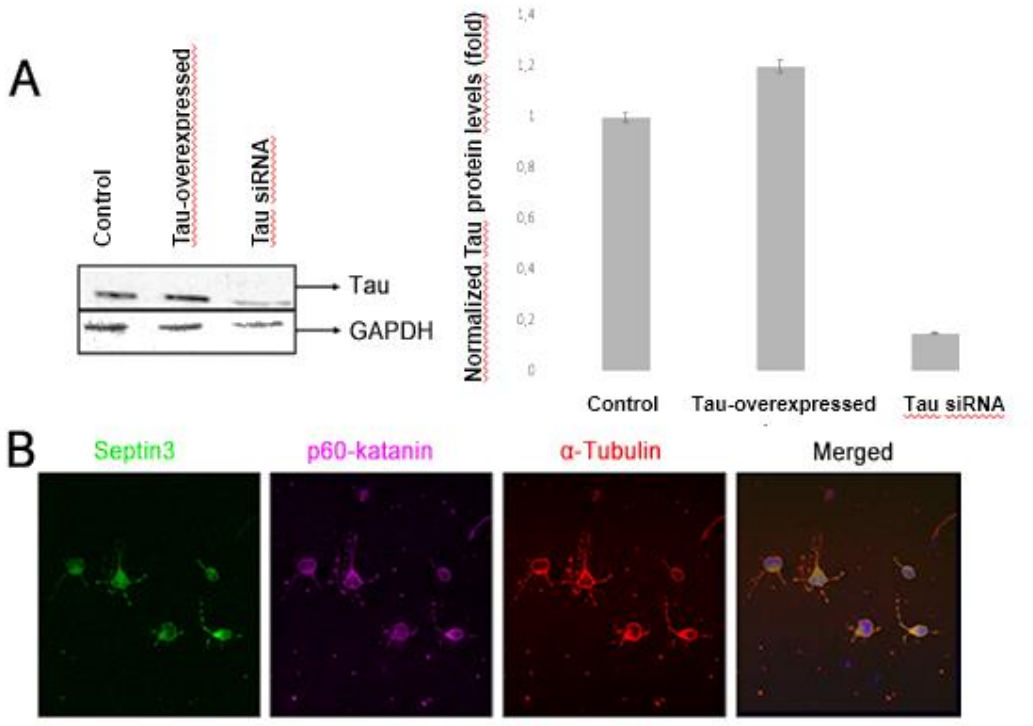

Figure 3.13 : Western blotting results of Tau over-expression and gene silencing.

In previous studies, it was documented inhibition of p60-katanin compromises release of microtubules from the centrosome and results in significantly longer microtubules throughout the neuron (Karabay et al., 2004). Therefore, we tested Septin3 and Tau interaction in the depletion of p60-katanin. We performed p60-katanin gene silencing experiment via Accell siRNA transfection. Accell siRNA transfection was performed

according to the manufacturer' s protocol. 48 hours after transfection, proteins were isolated and subjected to western blotting. p60-katanin were silenced 99% via Accell siRNA transfection. Primary rat cortical neurons were co-transfected with Tau and Septin3 and incubated over-night at 37^0C incubator. After over-night incubation, p60-katanin Accell siRNA transfection was performed and ICC experiment was carried out for morphological analysis on primary cortical neurons. Due to the p60-katanin' s neuronal branching activity, it was shown that upon down-regulation of p60-katanin, primary rat cortical neurons mostly lost their neuronal morphology. Axon-like thick extensions were observed due to Septin3 and Tau over-expression (Figure 3.14)

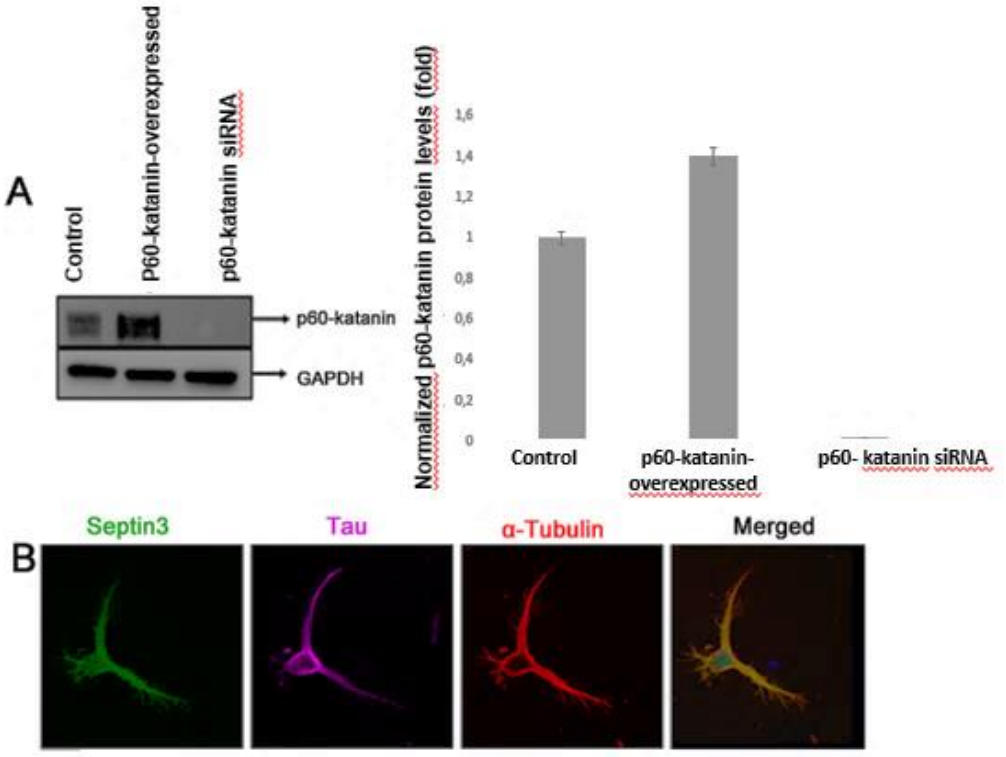

Figure 3.14 : Primary cortical neurons over-expressing both Septin3, Tau and gene-silencing of p60-katanin ($p<0,01$).

Our previous results suggested that Septin3 and Tau co-localized predominantly in the axons. In this study, the effect of Septin 3 on microtubules was examined in the absence of Tau. Similar to Septin3' s siRNA experiments result, upon absence of Tau, primary rat cortical neurons had impaired axon elongation. Some cells still formed dendrites and dendritic-spines due to Septin3 over-expression (Figure 3.15).

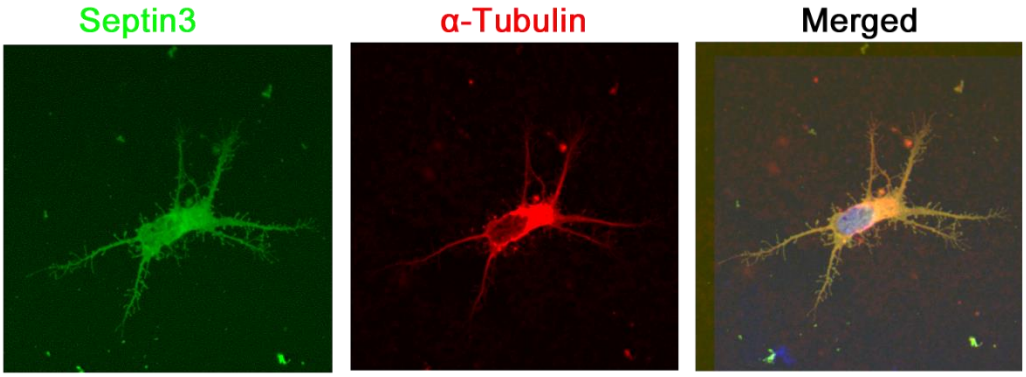

Figure 3.15 : Primary cortical neurons over-expressing Septin3 and lacks Tau.

Neurons express p60-katanin enzyme in high levels because the severing of microtubules is very crucial for axonal growth and branch formation (Yu et al., 2008). To examine the possible role of septins in microtubule dynamics, siRNA was used to deplete the endogenous Septin3. Upon over-expression of p60-katanin, microtubules were stucked in the cell body however, dendritic branches were formed. Septin3 inhibiton and p60-katanin over-expression together, were resulted in impaired axon elongation and mostly, cell death (Figure 3.16).

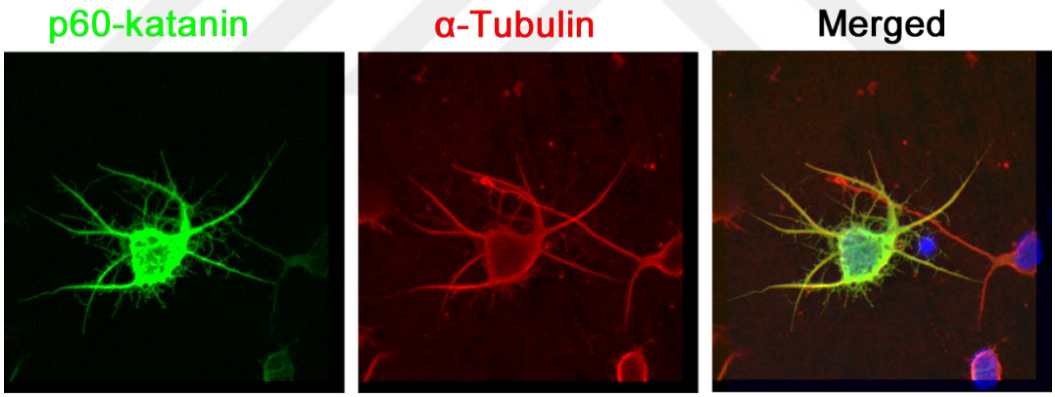

Figure 3.16 : Primary cortical neurons over-expressing p60-katanin and lacks Septin3.

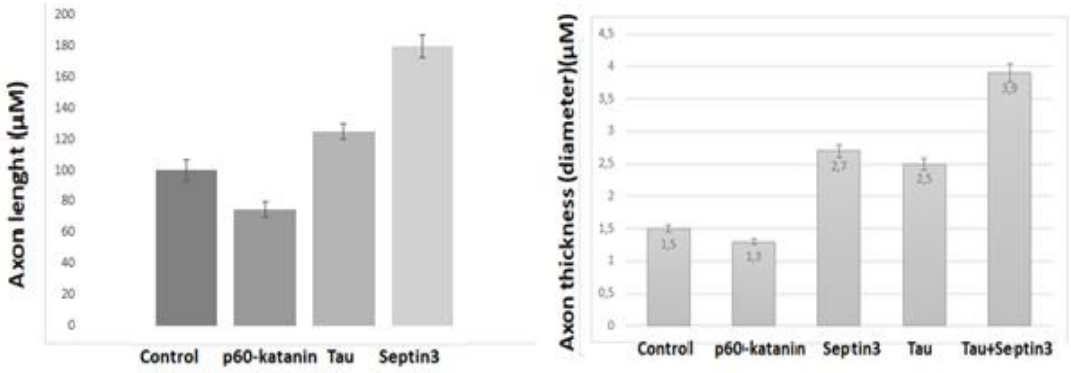

Figure 3.17 : Morphological analysis of primary cortical neurons ($p<0,05$)

Following ICC experiments, axon length and thickness were morphologically analyzed with Aivia 5.1 and Image J programs. It was observed that axon length increased about 2 times in Septin 3 over-expressing neurons compared to control. Tau over-expression did not show a significant increase. Later, the increase in axonal thickness seen in ICC experiments was analyzed. Axon thickness in Septin 3 overexpressing neurons increased 1.8-fold compared to control. At the same time, axon thickness were increased 2,5 fold when Tau and Septin 3 were co-localized. This result suggests that Tau and Septin 3 co-localization has supporting effect on axonal microtubules (Figure 3.18)

4. DISCUSSION AND CONCLUSION

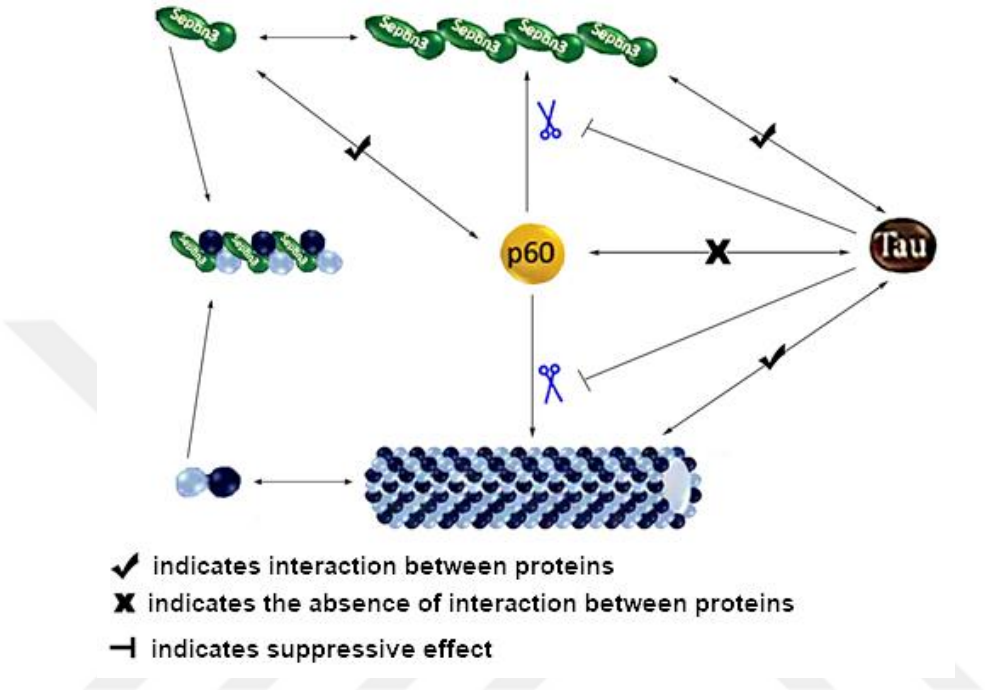

Figure 3.18 : Regulation mechanism of interaction between Septin3 – p60-katanin – Tau proteins

In eukaryotic cells, septins often co-localize with the cytoskeleton elements such as microtubules and actins, which they show some level of interconnectedness for structural integrity. Despite growing interest in these septin proteins, the molecular mechanisms of septin assembly and septin-related mechanisms are much less understood than those of other cytoskeletal elements. It has been demonstrated that septin has a role on microtubule dynamics, also it is thought that septins may spatially guide microtubules in cellular processes such as cytokinesis, during which microtubules determine their positioning (Glotzer, 2004) (Spiliotis, 2010) (Canman et al., 2003). In additon, septins could be important for the Golgi-nucleated microtubule orientation, and proper microtubule targeting to the focal adhesions (Vinogradova, 2009) (Small and Kaverina, 2003). Septins are also revealed to be involved in microtubule dynamics via interactions with MAPs (Spiliotis, 2010). For instance, it is identified that Septin2 modulates microtubule dynamics through interaction with MAP4 (Kremer, 2005).

Since most of the studies were performed with p60-katanin severing effect on microtubules, it may be speculated that p60-katanin has severing effect on septin filaments by reason of septin protein family is recognized as novel cytoskeletal compenents based on their filamentous appearance, as well as their association with microtubules. Due to the same fact about septins, it may also be speculated that septins and MAPs are associated and MAPs have protective effect on septin filaments as they do on microtubules.

Neurons are highly polarized cells and develop two types of cytoplasmic extensions; axons and dendrites. The maintenance of neuronal polarization is critical for correct development of neurons. All neurons contain a common structural backbone which is microtubule, and alterations in neuronal morphology are related to the microtubule scaffolding (Qiang et al., 2006).

In this study, primary cortical neuron experiments were performed to determine Septin3 – Tau – p60 - katanin interaction from the point of neuronal processes formation. Axons release neurotransmitters at the axon terminals in response to electrical signals from the cell body. Dendrites, especially dendritic spines, contain receptors for neurotransmitters (Gallo and Letourneau, 1999) (Tanaka and Kirschner, 1991). Septin3 over-expression increased dendritic branch formation and expedited neuronal development. Cortical neurons reached stage 5 of growth, despite control neurons reached the stage 3 in the same amount of time. Upon down regulation of Septin3, dendritic spine morphology and exon elongation was impaired. Several studies showed that septins have a role in spine morphogenesis and dendritic spine formation. For instance, Septin7 is found to localize at the dendritic protrusions and at dendritic branch points in cultured hippocampal neurons (Xie et al., 2007). This distribution of Septin3 and Septin7 reminisce the septin localization in the bud neck of budding yeast. Therefore, Septin3 might be conserved through budding yeast the mammalian and Septin3 may have a critical role on dendritic spine morphology.

p60-katanin is required for proper arrangements of microtubules along the axon. It is revealed that in p60-katanin inhibited cells, microtubules were not able to be released from the cell body (Karabay et al., 2004). In our study, Septin3 was interrupted along the axon in the presence of p60-katanin. Moreover, microtubules remained partially uninterrupted. Similar to RFL6 experiments, p60-katanin may have a severing activity on Septin3 filaments. Microtubules in the axon are more resistant to severing by p60-katanin than microtubules elsewhere in the

neuron due to the tau' s protective effect. Septin3, p60-katanin and tau were over-expressed on primary cortical neurons and either Septin3 or microtubules were remained uninterrupted. Once again, tau may have a protective activity on Septin3 filaments as it does on microtubules (Figure 3.19) Septin3 and tau' s co-localization was confirmed in primary cortical neurons. They co-localized predominantly along the axon and resulted in even thicker bundles of Septin3 and microtubules. Despite the co-localization, tau has no effect in dendritic-spine morphology. To better understand tau's effect, tau was silenced via Accell siRNA transfection. Despite the death of most of the cells, remaining cells lacked axon extension and Tau lost its possible protective ability on Septin3 filaments. Also, Septin3 over-expressed and Tau silenced cells still lost their axon elongation. This led us that tau and Septin3 co-localize and work together in the axons. In addition, septin3 loss-of-function resulted in impaired microtubule elongation in axons and this data support the guidance role of septins to the microtubules.

Baas et al., (2005) proposed a model for katanin-MAPs interaction, which we think is the similar for septin-MAPs interaction. According to the model, the microtubule is decorated by MAPs such as tau. The MAPs reduce the possibility of katanin-microtubule interaction. Hence, katanin subunits can not form hexamer around the microtubule to break it. Phosphorylation of MARK by MARK kinase causes MARK to phosphorylate the MAP molecule, which dissociates from the microtubule, thereby enhancing the katanin-microtubule interaction. In this situation, katanin can break the microtubule. Specifically in Alzheimer disease, neurons responds to hyperphosphorylation of tau, causing it to dissociate from microtubule lattice. Microtubules become more susceptible to katanin access whereas hyperphosphorylated .tau forms abnormal paired helical filaments. A group of septins has been shown to be concentrated in the neurofibrillary tangles of Alzheimer's patient brains and three septins are found highly interactive and co-localized with extremely basic protein tau (Kinoshita et al, 1998). In another study, it is suggested that that polymorphism in exon 11 of Septin3 may have a determinative role in the pathogenesis of Alzheimer' s disease (Takehashi et al, 2004). These data' s suggest that in order to better understand Alzheimer's pathogenesis, it is very important to understand Septin-Tau relation in neurons.

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6. CURRICULUM VITAE

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PUBLICATIONS

Obakan-Yerlikaya P, Arisan ED, Coker-Gurkan A, Adacan K, Ozbey U, Somuncu B, Baran D, Palavan-Unsal N, Calreticulin is a fine tuning molecule in epibrassinolide-induced apoptosis through activating endoplasmic reticulum stress in colon cancer cells, Mol Carcinog. 2017 Jun;56(6):1603-1619.

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