**İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY**

# REGULATION OF MICROTUBULE SEVERING

**M.Sc. Thesis by**
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**Department: Advanced Technologies in Engineering**

**Programme: Molecular Biology–Genetics and Biotechnology**

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**Date of submission : 8 May 2006**
**Date of defence examination: 8 June 2006**

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**JUNE 2006**

**İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ**

# MİKROTÜBÜL KESİLMESİNİN REGÜLASYONU

**YÜKSEK LİSANS TEZİ**

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**Tezin Enstitüye Verildiği Tarih : 8 Mayıs 2006**
**Tezin Savunulduğu Tarih : 8 Haziran 2006**

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**HAZİRAN 2006**

**ACKNOWLEDGEMENTS**

I would like to thank Associate Professor Arzu Karabay Korkmaz for invaluable guidance, advice, and also for her motivation and morale support at difficult times.

I would like to thank Ayşegüls ☺ (Ayşegül Yıldız and Ayşegül Dilsizoğlu) for being with me in all conditions. They are very enjoyable partners, as well as very good friends. It was easier to work in lab until late hours with such friends.

I would like to thank also Ceren Eke Koyuncu, Eyser Kılıç, Burcu Turanlı, Iraz Toprak Aydın for their friendship.

I would like to thank Prof. Peter W. Baas from Drexel University, Department of Neurobiology and Anatomy for providing me position in his lab. I would also like to thank Dr. Wenqian Yu for sharing all her technical experience during this lab work.

I would also like to acknowledge the funding agencies. This study was supported by TUBİTAK Career Project assigned for Dr. Arzu Karabay Korkmaz.

I would like to thank Ersin Koç for waiting for me ☺ and being the most enjoyable part of my life. He helped me to see that there is another life outside the lab ☺.

Finally, I would like to thank my family for their endless love and support. I always felt their courage.

June, 2006 Şirin KORULU

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## ABBREVIATIONS

| | |
|---|---|
| **AAA** | : **A**TPases **A**ssociated with various cellular **A**ctivities |
| **ATCC** | : The American Type Culture Collection |
| **BSA** | : Bovine Serum Albumine |
| **CNS** | : Central Nervous System |
| **DNA** | : Deoxyribonucleic Acid |
| **GFP** | : Green Fluorescent Protein |
| **HSP** | : Hereditary Spastic Paraplegia |
| **IR** | : Inter Repeats |
| **MAPs** | : Microtubule-associated proteins |
| **MTBR** | : Microtubule Binding Repeats |
| **MTOC** | : Microtubule Organizing Center |
| **MW** | : Molecular Weight |
| **NGF** | : Nerve Growth Factor |
| **PBS** | : Phosphate Buffered Saline |
| **RT** | : Room Temperature |

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**LIST OF SYMBOLS**

| | |
|---|---|
| **$1^0$** | : Primary |
| **$2^0$** | : Secondary |
| **$H_2O$** | : Water |
| **Ab** | : Antibody |
| **dd** | : Double distilled |
| **fig.** | : Figure |
| **GFP** | : Green Fluorescent Protein |
| **g** | : Gram |
| **h** | : Hour |
| **IFs** | : Intermediate Filaments |
| **Lys** | : Lysine |
| **MAPs** | : Microtubule-associated proteins |
| **MFs** | : Microfilaments |
| **mRNA** | : Messenger-ribonucleic acid |
| **µl** | : Microliter |
| **µg** | : Microgram |
| **µm** | : Micrometer |
| **mg** | : Milligram |
| **ml** | : Milliliter |
| **MTs** | : Microtubules |
| **NF** | : Neurofilament |
| **PFA** | : Paraformaldehyde |
| **Tb** | : Tubulin |
| **γ-TuRC** | : γ-tubulin Ring Complex |

## REGULATION OF MICROTUBULE SEVERING


## SUMMARY

Microtubules are cytoskeletal polymers made of alpha and beta tubulin heterodimers. They are essential for the cell and have role in important cellular processes such as cell transport, cell motility and cell division. In neurons, MTs provide support for the growth and maintenance of the axonal and dendritic processes. They also serve as railroads along which organelles are transported within the axon.

Cells reconfigure their MTs by assembly and disassembly phases, known as dynamic instability. However, many cell types, such as neurons, have complex MT organization patterns, and it is difficult to explain their reconfiguration by dynamic instability, especially when the MTs are stabilized by some proteins. All these suggest that there are some other models for MT movement in the cell. According to our model, long MTs are stationary, but short MTs are mobile. Cells mobilize their MTs by severing them into short pieces. Severing activity is performed by enzymes such as katanin and spastin. Once short MTs elongate, they lose their mobility property.

Katanin and spastin are MT severing enzymes. Katanin is one of the best characterized MT severing proteins. It has two subunits, p60 and p80. P60 subunit has high homology with another MT severing protein, spastin. Nowadays, spastin is as popular as katanin because it is known that spastin mutation leads to neurological disorder, hereditary spastic paraplegia.

In this study, our aim was to have some further steps in characterization of spastin and also to identify the regulation mechanisms of MT severing by spastin and katanin. In this study, we specifically concentrated on MAP1b and MAP2c proteins that have protective role over MTs.

In the first part of the study, we have worked with hippocampus cells. In order to identify the role of spastin in neurons, we overexpressed GFP, p60 –katanin and spastin constructs in hippocampal cells. Cells were fixed at the particular time points following transfection, day2 and day4. After fixation, immunostaining was done and then by using primary and secondary antibodies cells were analyzed with fluorescent microscopy. Control cells, p60 –katanin overexpressing and spastin overexpressing cells were compared with each others.

In the second part of the study, MAP's protective functions were analyzed in spastin and p60 –katanin overexpressing cells. P60 –katanin and spastin constructs were overexpressed with and without MAPs such as MAP1b and MAP2c in living fibroblast cells, RFL-6. Cells were fixed on the following day of transfection and stained with primary and secondary antibodies. After immunostaining, cells were analyzed with fluorescent microscopy.

## MİKROTÜBÜL KESİLMESİNİN REGÜLASYONU

## ÖZET

Mikrotübüller, alfa ve beta tubulin heterodimerlerinden oluşan hücre iskeleti polimerleridirler. Hücre için son derece gerekli olup hücre taşınması, hücre hareketi, hücre bölünmesi gibi hücre için hayati önem taşıyan olaylarda görevlidirler. Sinir hücrelerinde, MTler hücrenin büyümesine destek sağlamakla beraber hücresel yapının şekillenmesinde, akson ve dendritlerin oluşumunda da etkilidirler. Ayrıca MTler organel gibi moleküllerin akson boyunca çift yönlü taşınmasında da demir yolu vazifesi görmektedirler.

Hücreler "dinamik kararsızlık" adı verilen mekanizma ile MTlerini yeniden yapılandırmaktadırlar. Ancak sinir hücrelerinde olduğu gibi, birçok hücre tipi karmaşık MT ağına sahiptir. Bu hücrelerin MT yapılanmalarını dinamik kararsızlık ile açıklamak, özellikle de MTler başka proteinler ile de etkileşim halindeyken oldukça güçtür. Bu bulgular MTlerin hücre içi hareketini açıklamaya çalışan başka modellerin de mevcut olduğunu düşündürmektedir. Üzerinde çalıştığımız modele göre ("kes ve koş" modeli), uzun MTler durağan, kısa MTler ise hareketlidirler. Bu modele göre, hücreler MTlerini hareketli hale getirmek için onların katanin, spastin gibi proteinler tarafından kesilip, küçük parçalara ayrılmasını sağlamaktadırlar. Kısa MTler tekrardan uzun hale geçtiklerinde ise hareket yeteneklerini kaybetmektedirler.

Katanin ve spastin MT kesici enzimlerdir. Katanin, karakterizasyonu en güzel yapılan MT kesen proteinlerinden biridir. İki alt üniteden oluşur, p60 ve p80. En az onun kadar popüler olan diğer bir MT bölme proteini de spastindir ve p60–katanin alt ünitesi ile büyük benzerlik göstermektedir. Spastinin en fazla ilgi çeken proteinlerden biri haline gelmesinin nedeni de bu proteinin MT temel hücre biyolojisini nörolojik hastalıklarla birleştirmesidir. Spastinin mutasyonunda kalıtsal spastik parapleji rahatsızlığına neden olduğu bilinmektedir.

Bizim bu çalışmadaki hedefimiz spastinin karakterizasyonunda yeni adımlar atabilmek, MTlerin spastin, katanin tarafından kesilmesinin mikrotübül ilişkili proteinler tarafından düzenlenmesini aydınlatmaya çalışmaktır. Çalışmanın bu kısmında MTler üzerinde koruyucu görevleri olan mikrotübül–ilişkili proteinler, özellikle de MAP1b ve MAP2c üzerinde durulmuştur.

Spastinin görevini aydınlatabilmek için hipokampüs hücrelerinde GFP, spastin, p60–katanin proteinlerinin ekspresyonu gerçekleştirildi. Hücreler transfeksiyonu takip eden 2. ve 4. günlerde sabitlendi. Birincil ve ikincil antikorlar ile boyandıktan sonra hücreler floresan mikroskobu ile incelendi. Kontrol hücreleri, katanin ve spastin proteinlerini aşırı eksprese eden hücreler aralarında karşılaştırıldı.

Çalışmanın ikinci kısmında ise mikrotübül ilişkili proteinlerin, p60–katanin ve spastin eksprese eden hücreler üzerinde herhangi bir düzenleyici rolünün olup olmadığı aydınlatılmaya çalışıldı. P60–katanin ve spastin proteinleri mikrotübül ilişkili proteinlerin varlığında ya da bu proteinler olmadan fibroblast hücrelerinde eksprese edildiler. Transfeksiyonu takip eden günde hücreler sabitlendi. Birincil ve ikincil antikorlarla işaretlendikten sonra hücreler floresan mikroskobunda incelendiler.

# 1. INTRODUCTION

## 1.1. Cytoskeleton

In all eukaryotes, there are fibrous proteins in the cytosol. These proteins are microfilaments, microtubules and intermediate filaments. They are collectively called "cytoskeleton". These cytoskeletal fibers give the cell strength and rigidity. They also have control on movement within the cell, especially microtubules (MTs) have very important role in cell division (Lodish, 1995).

### 1.1.1. Microtubules

Among the cytoskeletal proteins, microtubules are thought to have the most important roles, especially in generation of cell shape and polarity, cell division, cell growth and intracellular organelle transport.

Microtubules are polymers of α- and β- tubulin subunits. These subunits are arranged in a cylindrical tube 24 nm in diameter. There are both lateral and longitudinal interactions between the tubulin heterodimer subunits. These interactions maintain the tubular form of microtubules (Lodish, 1995; Vale et al., 1999).

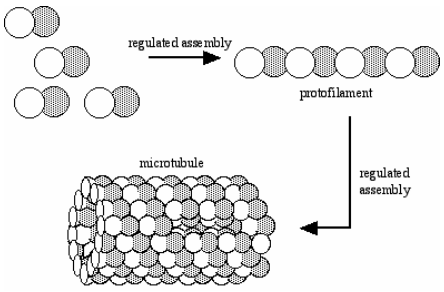


**Figure 1.1:** MT Assembly

Each α-β tubulin heterodimer binds two molecules of GTP. One of the GTP-binding sites is located on α-tubulin; second site for GTP-binding is located on β-tubulin. GTP binding to the α-tubulin site is irreversible but on the β-tubulin site, GTP binding is reversible; thus GTP can be hydrolyzed to GDP.

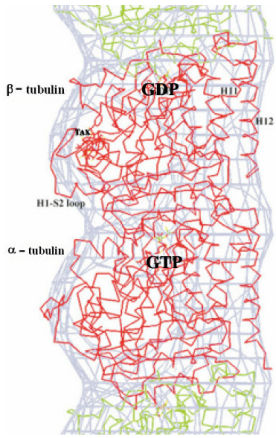


**Figure 1.2:** GTP sites in α- and β- tubulin subunits

In addition to α- and β- tubulins, there is a special third type of tubulin, γ-tubulin. It is located in the centrosomal matrix. In animal cells, centrosomes are primary sites for microtubule nucleation. Microtubules are thought to be nucleated from γ-tubulin ring complexes (γ-TuRCs) and these microtubules nucleated from γ-TuRCs have minus ends that are physically capped. These caps prevent minus-end polymerization and depolymerization (Lodish, 1995; McNally et al., 2002).

#### 1.1.1.1. Dynamic Instability of Microtubules

Microtubules continuously switch between growth and shrinkage phases. Growth phase occurs by polymerization of tubulin at MTs ends while depolymerization occurs by loss of tubulin subunits from their ends. This process is called "dynamic instability". Dynamic instability is characterized by the coexistence of polymerizing and depolymerizing MTs. It is thought to be a function of GTP hydrolysis. GTP-tubulin is incorporated at polymerizing MT ends, the bound GTP is hydrolyzed during or soon after polymerization, and Pi is released. Thus, the MT lattice is predominantly composed of GDP-tubulin (McNally et al., 1998; Hartman et al., 1998).

Rapid loss of GDP-tubulin subunits and oligomers from the MT end is termed depolymerization. Depolymerizing MTs can also transit back to the polymerization phase, “rescue”. If the polymerizing MTs transit to the depolymerization phase, it is called “catastrophe”. With the catastrophe, MTs switch to a rapid shortening phase (Walker et al., 1988, 1991).

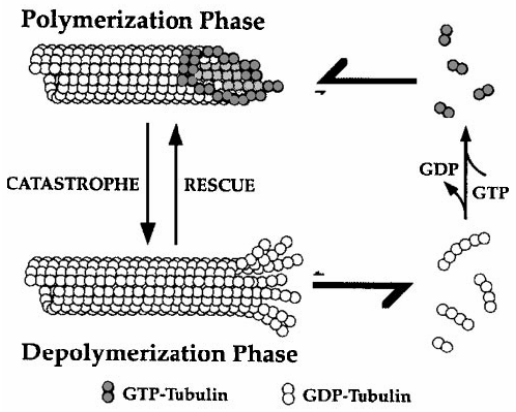


**Figure 1.3:** Dynamic instability

The dynamic behavior of microtubules is essential for fundamental processes in eukaryotic cells such as cell division, cell differentiation, nerve growth, organizing large intracellular compartments such as Golgi apparatus and endoplasmic reticulum as well as for transporting small membrane carrier vesicles in the endocytotic and secretory pathways. Also, during the mitotic cycle, microtubules are primarily needed components of the mitotic spindle for proper segregation of chromosomes and specifying the position of the cleavage furrow (McNally et al., 1993; Quarmby, et al., 2000; David, et al., 1999).

There are also some other proteins that control the microtubule behaviors. They are called microtubule-associated proteins (MAPs). They bind to microtubule walls and promote microtubule polymerization. Other proteins such as OP18, XKCM1 increase the frequency of catastrophes and thus, promote disassembly of microtubules from MT ends (Hartman et al., 1998).

### 1.1.2. Microtubule Associated Proteins (MAPs)

Microtubule Associated Proteins (MAPs) control MT behaviors. It has been proposed that MT – MAP interaction is predominantly electrostatic. MAPs are positively charged and act by screening negative charges (highly acidic sites) on the C – terminal domain of both in α- and β- tubulin. MAPs have N – terminal projection domains, so that they can crosslink and bundle MTs. This promotes rescue and causes stabilization. (Nogales, 2000; Desui et al., 1997).

Neuronal MAPs can be classically divided into two groups: (1) Very high molecular weight polypeptides such as MAP1 and MAP2, which are abundant in adult brain, (2) intermediate – sized proteins, such as tau. There is also MAP4 in non – neuronal cells (Matus, 1988).

MAP2, MAP4 and tau have conserved C – terminal MT binding domain with three or four pseudo repeats. Each repeat represents a MT binding site and is composed of 31 – 32 amino acids including several basic residues. MAP1 has only one N – terminal MT binding domain which is acidic rather than basic (Nogales, 2000).

Most of the identified MAPs are thought to be regulated by phosphorylation. Phosphorylation decreases MAPs ability to bind MTs by weakening the electrostatic interaction between MTs and MAPs. When the phosphorylation increases, MAPs are inhibited in MT stabilization ability.

#### 1.1.2.1. MAP2 and MAP2c

MAP2 is a monomeric protein that has four isoforms 2a, 2b, 2c and 2d. MAP2 shares homology in its MT binding domain with tau and MAP4. It stimulates the growth of MTs in vitro by promoting nucleation and tubulin subunit addition at MT ends. MT – MAP2 binding also leads to stabilization of MTs, in other terms reduction in their dynamic instability and increase in MT rescue (Halpain et al., 2000).

Multiple isoforms of MAP2 are encoded by a single gene as a result of differential alternative splicing mechanisms. MAP2a, MAP2b are high MW isoforms, but MAP2c and MAP2d are low MW isoforms (Halpain et al., 2000). MAP2a and MAP2b are mainly found in dendrites and cell body while MAP2c is particularly

pronounced in developing axons (Mandelkow et al., 1992). MAP2c is also considered as juvenile MAP and it is expressed perinatally in rats, coincident with the period of maximal dendritic outgrowth and synaptogenesis, then it is replaced by MAP2a, MAP2b postnatally. Only some Central Nervous System (CNS) regions that undergo neuritigenesis throughout postnatal life such as olfactory bulb, retina, continue to express MAP2c at high levels into adulthood. This leads to the idea that MAP2c has a specific function associated with dendritic outgrowth and synaptogenesis (Mandelkow et al., 1992; Halpain et al., 2000).

Bloom and Valle suggested that MAP2 may be divided into two structural domains (see fig. 1.4). C terminal domain has MT binding site which is positively charged. It contains three 18 – residue MT binding repeats (MTBR) separated by 13 – 14 residue inter – repeats (IR). This domain promotes MT assembly. N terminal domain is predominantly negatively charged and represents portion of MAP2 observed as a projection of MT surface. Differently, the projection domain of MAP2 does not bind to the MTs and is thought to extend into the solution, away from MT surface. This projection domain contains binding sites for regulatory subunits of protein kinases and these kinases have role in MAP2 regulation via phosphorylation (Vallee et al., 1983; Mandelkow et al., 1992; Milligan et al., 2002).

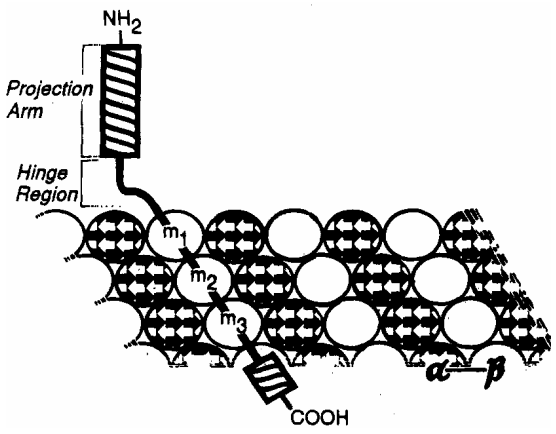


**Figure 1. 4:** Model for MAP2 – MT interaction

### 1.1.2.2. MAP1b

MAP1b is one of the first MAPs to be expressed during embryonic development of the nervous system. It is also known as MAP1x, MAP1.2 or MAP5. Molecular weight of the protein is about 320kDa and its structure is filamentous with a small spherical segment at one end (Kunkel, 1994; Taniquchi et al., 1997; Propst et al., 2000). MAP1b is a multimeric protein complex that contains one heavy chain (regulatory subunit) and at least one light chain (active subunit). There is a MT binding domain in the N – terminal half of the heavy chain. This region is composed of 21 times repeated highly basic KKE(E/I/V) motifs. It has been proposed that this positively charged domain has an α – helical structure that binds to a negatively charged α – helical domain at the C – terminus of β – tubulin which is on the outer surface of the MT (Tögel, et al., 1998; Propst et al., 2000; Franzen et al., 2001).

Beside its MT binding ability, MAP1b can also bind to actin filaments; hence, MAP1b is a link between these two proteins that form the growth cone cytoskeleton.

MAP1b is the earliest MAP expressed in the developing nervous system and is abundant early in development. The level decreases in the adult, but it is still high in adult dorsal root ganglion (DRG) neurons and sciatic nerve axons (Kunkel et al., 1994; Fischer et al., 2000).

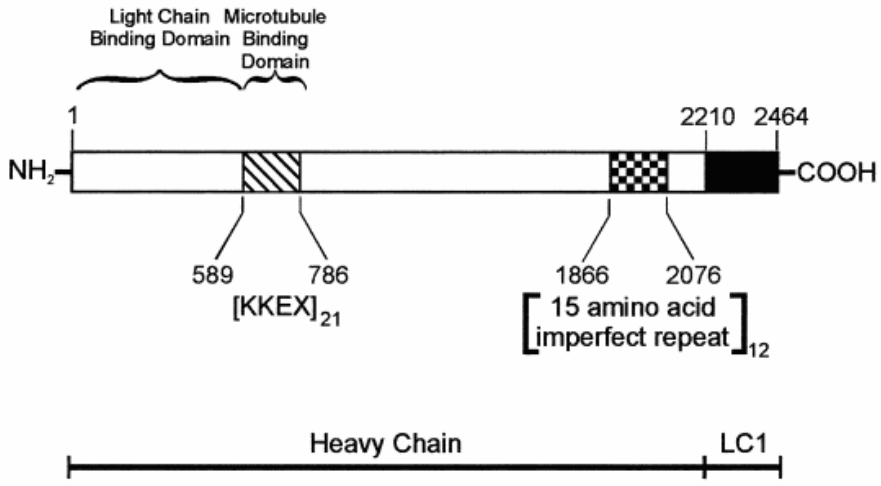


**Figure 1. 5:** Model for MAP1b gene organization

Localization studies have shown that MAP1b expression is much higher in actively growing and developing neurons. It is also present in axons, cell bodies and dendrites of neurons and in glial cells. Because MAP1b is especially expressed in axons during their initial outgrowth, it has been suggested that MAP1b plays important role in neurogenesis. When phosphorylated, it causes growth cone MTs to be in a dynamically unstable form which is necessary for axonogenesis (Noble et al., 1989; Kunkel et al., 1994; Tögel, et al., 1998; Bomont et al, 2003).

MAP1b is regulated by phosphorylation and dephosphorylation. Two modes were identified for MAP1b phosphorylation. Mode I may be catalyzed by proline directed protein kinases (PDPK), whereas the mode II is due to the action of casein kinase II (CKII). MAP1b expression decreases after neuronal maturation; phosphorylation state of MAP1b is also modified. Mode I phosphorylation disappears while the mode II phosphorylation is still present in adult MAP1b (Avila et al., 1994).

### 1.2. Neuronal Cytoskeleton

Neurons develop from mitotic cells of ectodermal origin. After several divisions, these cells begin to express neuron specific proteins. Human nervous system consists of over $10^{11}$ neurons associated with over $10^{12}$ supporting glial cells. Neurons are non – dividing cells, in order to transmit signals they stop dividing early in development (Baas, 1999). Typical neuron has an enlarged cell body that contains a nucleus and most of the cytoplasmic organelles. Cell body is also the place where all the neuronal proteins are synthesized. Neuron has two different extensions from the cell body. Branching ones are called dendrites and long one is called axon. Axons send information over long distances, dendrites act as receptors for incoming information (Baas, 2002). In humans, axons can grow to enormous length. This leads to questions: “How does the neuron support and maintain such a long process?”, “How can materials be transported along the axon?” The general answer lies under the “neuronal cytoskeleton”. Neuronal cytoskeleton is composed three types of filamentous proteins. Microtubules (MTs), microfilaments (MFs) and intermediate filaments (IFs). Each has different types of subunits, tubulin for MTs, actin for MFs, and a family of related proteins for IFs.

### 1.2.1. Neuronal Polarity

Neurons are the most polarized cells in nature. They contain one axon and multiple dendrites. In axons, there are long MTs oriented with their plus ends away from the cell body and MT polarity is uniform; whereas microtubules in the dendrites are short and they have mixed polarity orientation (Baas, 1989; 1999; Vale et al., 1999, Ahmad, et al., 1999). Another difference between axon and dendrites is compartmentalization of some organelles. For example, dendrites have ribosomes and Golgi elements, but axons do not. This explains why each process has specialized roles during neuronal activities.

The most important question in neuronal polarity is that, how the neurons know to extend a single axon and multiple dendrites? Broad agreement is that, this event is due to changes in the cytoskeleton. Laboratory studies showed that when expression of tau, major axonal MAP, was suppressed, transformation of immature neurite into an axon was curtailed. This indicates that MT stability might be the basis of axonal differentiation. However, the mechanism is not clear, since MAPs such as tau are also involved in many activities, not just in MT stabilization (Baas, 2002).

Besides maintaining the cell shape, neuronal cytoskeleton is also important for axonal transport. There is no protein synthesis machinery in axons and dendrites; hence they can not synthesize tubulin subunits locally. Therefore, proteins and tubulins must be transported in some form. There are two types of transport; fast and slow transport.

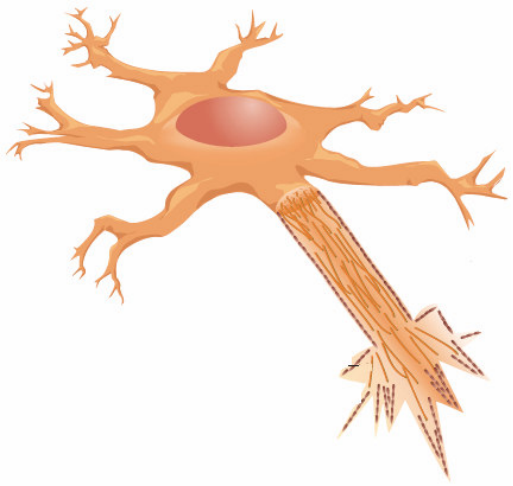

**Figure 1. 6:** Typical vertebrate neuron

Fast transport is responsible for movement of organelles, and the travel rate is 100-400 mm per day. Slow transport moves cytoskeletal proteins and soluble enzymes and travel rate is 0.1 – 3 mm per day. This type of transport is especially important for neuronal growth and process maintenance (Hirokawa, N., 1993; Baas, 1999).

### 1.3. Microtubule Severing

In many cell types, such as fibroblasts, minus-ends of microtubules are anchored near the centrosome, whereas the plus-ends are oriented towards the cell periphery. In other cells, such as epithelial cells and neurons, non-centrosomal microtubules are needed for the activity of differentiated cell. There are three possible ways to produce non-centrosomal microtubules: (1) the release of microtubules originally anchored at the centrosome, (2) de novo nucleation and growth of microtubules in the cytosol, (3) severing of microtubules at sites remote from the centrosome (Quarmby, et al., 2000; Quarmby, et al., 1999). Recent studies support the idea that microtubule severing is an important source of non-centrosomal microtubules. Most of the newly formed minus-ends (as a result of microtubule severing) seem to be stable (Quarmby, et al., 1999).

When it comes to possible roles of microtubule severing, this mechanism may play a role in regulating poleward flux of tubulin in the metaphase spindle during cell division, degradation of sperm axonemal microtubules after fertilization (in sea urchin oocytes), microtubule reorganization during the transition from interphase to mitosis in dividing cells, and the release of centrosome-nucleated microtubules (Lohret et al., 1998).

Microtubule severing also plays a role in specific activities of differentiated cells. For example in neurons, it is important in neuronal branching and axonal growth. The effect of microtubule severing on neuronal cells was investigated by experiments that inhibited the microtubule severing ATPase katanin activity. When anti-katanin antibodies were injected into neurons, centrosomal microtubules accumulated and the neuronal process did not occur (Ahmad, et al., 1999). According to the experimental studies, the following model was developed for the effects of katanin inhibition on process outgrowth. In control cells with active katanin, microtubules

are nucleated from centrosome and they are rapidly released by katanin after their lengths become a few microns. Then, motor proteins transport microtubules outward towards the cell periphery. These severing activities ensure that microtubules remain relatively short. On the other hand, in experimental cells with inactivated katanin, microtubules are not released from centrosomes. The number of individual microtubules cannot be increased by severing the microtubules. As a result of all these events, the process outgrowth is inhibited (Ahmad, et al., 1999). This supports the idea that centrosomal katanin, so microtubule severing is important for the production of non-centrosomal microtubules (Quarmby, et al., 2000).

As already mentioned, dendrites and axons contain large numbers of non-centrosomal microtubules that are essential for architectural support and also act as railway for the transport of materials along the axon (Dent, et al., 1999, Joshi, et al., 1998). There are two possible mechanisms for the formation of non-centrosomal microtubules in neuron cells: (1) these non-centrosomal microtubules are derived from in situ nucleation and assembly, or (2) these non-centrosomal microtubules are transported from the cytosol as polymers. As axons are incapable of locally synthesizing the tubulin subunits, microtubules nucleated at centrosomes must be actively transported from their sites of synthesis within the cell body of the neuron down the axon in the form of assembled microtubule polymer (Baas, et al., 1997). If the second mechanism occurs, these microtubules are probably produced by microtubule severing (Quarmby, et al., 1999, Quarmby, et al., 2000).

### 1.3.1. AAA Family Proteins

Katanin and spastin are members of a large protein family, AAA which stands for **A**TPases **A**ssociated with various cellular **A**ctivities. This family proteins play important role in numbers of cellular activities including proteolysis, protein folding, membrane trafficking, cytoskeletal regulation, organelle biogenesis, DNA replication and intracellular motility (McNally et al., 1993; Vale et al., 2000).

The common feature of the AAA superfamily is an ATPase domain. This domain is composed of about 220 amino acids. It is known that AAA domains assemble into oligomeric structures and this allows proteins to change their shapes during ATPase cycle. ATP binding induces structural rearrangements at the interface region of AAA

proteins. This increases interactions between adjacent AAA domains, also increases interactions between AAA protein and its target (Vale et al., 2000; McNally, F. et al., 2000).

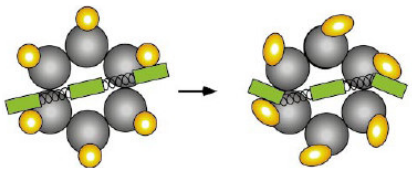

**Figure 1.7:** Conformational change of AAA protein ring

Ring-like structures are useful for AAA enzyme mechanism. This allows subunits to switch between tense and relaxed states in a concerted manner. These structures also provide framework for binding target proteins at multiple sites. If ring-binding sites change their positions, this will also cause tension application to bound protein (Vale et al., 2000).

#### 1.3.1.1. Katanin

Katanin is the most well characterized microtubule-severing protein. It was first purified from sea urchin eggs. It is a heterodimer protein consisting of two subunits. Enzymatic subunit is 60kD (p60) and it carries out the ATPase and severing reactions. Other subunit is 80kD (p80) and it localizes katanin to the centrosome and regulates microtubule-severing activity of p60 subunit (Vale et al., 1999; Quarmby, et al., 2000). N-terminal domain of p60 subunit binds microtubules and C-terminal is AAA domain (Quarmby, et al., 2000; McNally, K. et al., 2000).

AAA domain of p60 affects the binding affinity of the adjacent microtubule-binding domain, and tight binding occurs in nucleotide states (ATP). This stabilizes p60 rings (Vale et al., 1999). N-terminal of p80 subunit is composed of WD40 repeat (proline-rich) domain and a C-terminal domain is required for dimerization with catalytic p60 subunit. Studies showed that WD40 repeat domain of p80 is required for spindle pole localization of katanin. WD40 domain probably binds to another spindle pole protein (McNally, K. et al., 2000). Although p60 shows its ATPase and severing activity in the absence of p80 subunit, p80 cannot sever microtubules on its own.

Besides targeting katanin to the centrosomes it also enhances severing capacity of p60. Association of the two subunits increases their affinity for microtubules and also microtubule-severing activity (Hartman et al., 1998; Quarmby, et al., 2000; Ahmad et al., 1999).

Katanin is a microtubule stimulated ATPase; thus microtubule concentration affects the enzyme activity. Katanin forms ring structures and the ring formation occurs only when katanin subunits bind to adjacent tubulin subunits in the microtubule wall (McNally, F. et al., 2000). If microtubules are not present at the centrosome, katanin is distributed in the cytoplasm. Once microtubules are nucleated at the centrosome tubulin-katanin interactions make ring formation occur (Baas et al., 1997). At low microtubule concentrations (<2μM), ATPase activity increases with increasing microtubule concentration; but at higher microtubule concentrations, ATPase activity decreases until it approaches basal levels.

This ATPase behavior of katanin is unusual, and there are some explanations for the unusual ATPase behavior of katanin. Katanin binds microtubules at two sites; this increases local microtubule concentration by cross-linking and thereby stimulates katanin's ATPase activity. At higher microtubule concentrations, the ratio of katanin to microtubules is lower, less cross-linking occurs, thus less ATPase stimulation will be observed. A second explanation is about katanin oligomerization into rings. Microtubules promote p60-p60 oligomerization and oligomerization stimulates ATPase activity. Low microtubule concentrations facilitate oligomerization because p60 monomers are more likely to bind near one another on the microtubule. On the other hand, when the microtubule concentration is high, this will inhibit p60 assembly by sequestering p60 monomers at non-contiguous sites (Vale et al., 1999; Hartman et al., 1998).

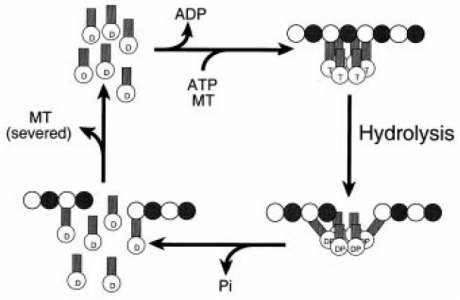


**Figure 1.8:** Model for microtubule severing by katanin

The following question is “how does katanin sever microtubules?” Studies on katanin gave rise to a model of microtubule severing. Katanin-ADP is monomeric molecule. When katanin exchanges its ADP for ATP, p60-p60 affinity is enhanced, and then oligomerization on microtubule is most efficient. Oligomerization allows binding of multiple katanin subunits to multiple adjacent tubulin subunits in the microtubule and 14-16 nanometer katanin ring is formed. Once a complete katanin ring is assembled on the microtubule, the ATPase activity of katanin is stimulated. As a result of ATPase reaction, phosphate group is released. Katanin undergoes a conformational change. This creates a pulling or pushing force on the tubulin subunits, leading to destabilization of tubulin-tubulin contacts. Katanin-ADP monomer has lower affinity for other katanin molecules and for tubulin subunits. This leads to the dissociation of complex and the recycling of the katanin (Vale et al., 1999; Quarmby et al., 2000; McNally, F. et al., 2000).

Because of being hexamer, the katanin ring cannot be docked on the sides of microtubule in such a way as to create identical interactions between katanin subunits and tubulin subunits. There are two possible solutions for this paradox: katanin hexamer can be docked inside the lumen of the microtubule. According to this model, katanin monomers enter microtubule through dynamic lattice defects in the microtubule wall and oligomerization occurs inside the microtubule. Second solution is assembly of higher-order oligomers of hexameric rings on the outside of the microtubules. Unlike a single hexameric ring, these can form multiple homologous contacts with tubulin subunits in the microtubule wall. Coordinated conformational changes in both structures lead to disrupt tubulin- tubulin interactions (McNally, F. et al., 2000).

#### 1.3.1.2. Spastin

Spastin is a member of AAA family and recently became protein of interest because it merges the fundamental cell biology of MTs with a neurological disorder. Spastin mutation leads to a genetically inherited disease spastic paraplegia.

There are also some other genes identified that are responsible for the disease. Most frequent (~40%) form is due to *SPG4* locus mutation which encodes spastin protein. The mutations can be missense, nonsense, splice site mutation, deletion or insertion in spastin gene (Errico et al, 2002).

Hereditary Spastic Paraplegia (HSP) can be in pure or complicated form. In pure form weakness and spasticity of lower limbs are main characteristics. Patience usually experience difficulties in walking. When the disease is in complicated form, it has some additional neurological abnormalities such as retinopathy, deafness and ataxia (Errico et al., 2002; Sherwood et al., 2004).

The degenerative process of the disease is interesting. It selectively affects some of the longest axons in central nervous system. Corticospinal axon is most severely affected. Next one is dorsal column. The degenerative process starts from distal ends of these axons and proceeds toward the cell body. This is called "dying back" axonopathy and the reason of such mechanism is still unclear (McDermott et al., 2003).

There are hypothesis for the situation. Mutant spastin disrupts MT dynamics by causing impairment of organelle transport on MT network. Supporting the hypothesis, studies with rat cortical neurons showed that there was a decrease in kinesin staining in mutant spastin overexpressing cells. Also in the same study, it was observed that spastin do not localize in axonal and dendritic processes while mutant spastin extended into the axons but not into dendrites (McDermott et al., 2003). Another degeneration hypothesis is spastin can cause degeneration by diminishing the supply of short MTs required for process generation. Mutant spastin lacks MT severing activity, so that short MTs can not be formed via severing activity of spastin (Baas et al, 2005).

Spastin is a member of AAA protein family. It belongs to the meiotic subgroup which also contains proteins involved in vesicle trafficking and MT dynamics. P60 – katanin is also a member of this group and it is the most characterized one. Spastin shares great homology with p60 –katanin within AAA domain but they do not have homology in their N – terminal region. Because of this homology, spastin is thought to be MT severing protein as p60 –katanin. To test the hypothesis, several cell

culture experiments were done and overexpression of wild type spastin really caused disassembly of MT cytoskeleton. Drosophila studies also showed that spastin overexpression in muscle erases their MT networks consistent with the idea that spastin is a MT severing protein. The same study also showed that Drosophila spastin (Dspastin) has a positive role in maintaining the synapse by encouraging growth through increasing dynamic instability of MTs. One more hypothesis raised from this study: proteins that destabilize MTs should facilitate synaptic growth (Sherwood et al., 2004; Roll – Mecck, 2005).

Spastin is encoded by *SPG4* (SPAST) locus and it is composed of 17 exons (Fonknechten, 2000). Spastin is 616 amino acid long and approximately 67.2 kDa. It contains two leucine – zipper and coiled – coil dimerization motif (Charvin et. al., 2003; Hazan et al., 1999).

Spastin is composed of three domains. N – terminal region contains putative transmembrane region TM, MIT is a microtubule interacting and trafficking domain. This domain is well conserved in spastin family. Final domain is ATP binding AAA domain (Roll – Mecck, 2005).

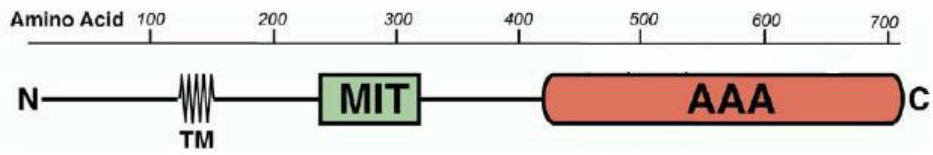


**Figure 1.9:** Domain localization of spastin

Although having similarity with p60 –katanin in function, spastin needs lots of further investigations. Even localization of spastin is controversial. It was reported that there is a putative nuclear localization signal RGKKK at 7 – 11 positions of human aminoacid sequence of spastin, but its sub cellular localization is still not clear (Charvin et al., 2003).

Various studies were done by using different antibodies generated against the protein. Some results pointed spastin to be a cytoplasmic protein however some results reported nuclear localization (Sherwood, 2004).

Charvin et al. found spastin in nuclei, whereas most recent study detected spastin in the cytoplasm and/or in the nucleus depending on neuronal population. All these findings possibly underlies multifaced cellular role for spastin.

It is possible for endogenous spastin to be a nuclear protein but because of conditions in transient transfection and overexpression, factors that localize spastin to the nucleus could be overwhelmed. This may lead spastin to be distributed as aggregates surrounding the nucleus. Alternative idea is that spastin is initially cytoplasmic protein that is transported slowly into nucleus. This process can not occur in transient overexpression conditions (Fink et al., 2004).

In dividing cells, spastin is mainly nuclear in interphase cells, becomes associated with centrosomes, the spindle MTs, the midzone and finally midbody during cell division (Errico, et al., 2004).

When it comes to post-mitotic cells, spastin is localized in discrete nuclear domains, but most interestingly detects a specific signal in the neurites. This signal is characteristically enriched in the distal axon and in the branching regions such as growth cones. According to these results it can be hypothesized that spastin influences MT dynamics in growth cones; thus regulating the stability of axons and axonal transport (Errico, et al., 2004).

### 1.4. Aim of the Study

Microtubules are essential polymers for the cell. Specifically in nerve cells, the neuronal morphology is related to the capacity of the neuron to reconfigure its microtubule scaffold. Recent studies indicate that microtubule movement is directly related to its length; the shorter the microtubule is the faster the movement (Dent et al., 1999). Based on these studies, it can be concluded that reconfiguration and transport of microtubules require that longer microtubule are severed into shorter pieces.

Neurons are rich in protein called katanin, which is a microtubule severing ATPase. It has been shown to severe microtubules from centrosomes and play role in generating short microtubules throughout the neuron (Ahmad et al., 1999). Recently, spastin, another microtubule severing protein expressed in neurons was identified (Errico et al., 2002).

First aim of the study was to analyze the basis of existence for two different proteins with the same functions. In previous studies done with katanin, researchers expected to obtain neurons with increased process formations upon overexpression of katanin (Karabay et al., 2004). Unfortunately, the expectations did not come true. We thought that, although having similar function with katanin, spastin may act in different way in the cell. Our expectation was based on dilemma about the localization of spastin. Besides the studies that support cytoplasmic localization of the protein there are also studies that address nuclear localization. If localization pattern of spastin is different than katanin, it may also lead severing of microtubules in different compartments of the neuron and cause variations in process formations, for instance it may increase dendrite formations. We hypothesized that "spastin, microtubule severing protein, may lead new process formations in neurons by breaking down long microtubules into short pieces throughout the cell"

In order to test the hypothesis, overexpression of spastin, katanin and GFP (as control) constructs were carried out in hippocampal cell cultures.

Neurons contain high levels of katanin and theoretically it can be claimed that katanin continuously severs microtubules into subunits. If not, there should be some regulatory mechanisms for the severing activity of katanin. Observations showed that katanin is more active in mitotic extracts than in interphase extracts, and that the activity is related to phosphorylation in some ways (McNally et al., 2002). However, katanin itself is not phosphorylated so this leads the idea that phosphorylation of other proteins regulates katanin.

It was shown that the frog homolog of MAP4 reduced the microtubule severing *in vitro* (McNally et al., 2002) and this result is the basis of the model where katanin is regulated by microtubule –associated proteins (MAPs).

In 2005, Yu et al., showed that p60 –katanin overexpression in cultured neurons causes widespread severing and loss of microtubules in cell bodies, minor processes, dendrites and not in axons. In axons, microtubules appear to be more resistant to severing. This protection can be due to existence of MAPs like MAP2c, MAP1b, tau that are enriched in the axon. Our hypothesis was "microtubule severing protein, spastin is regulated by microtubule associated proteins and MAP overexpression prevents microtubules to be severed by spastin"

In this part of the study the aim was to test whether individual neuronal MAPs, in this case MAP2c and MAP1b, have the ability to protect microtubules from being severed by overexpressed spastin. Since fibroblasts do not have intrinsic MAP2c, MAP1b expression feature, RFL6 cell line was chosen in the study.

# 2. MATERIALS AND METHODS

## 2.1. Materials

### 2.1.1. Animals

Rat embryos at embryonic day 18 (E18) were used for hippocampal dissection. Embryos were taken from pregnant female-Sprague-Dawley rat with a c-section, which was about to give a birth. Rats usually give birth on the 21$^{st}$ or 22$^{nd}$ day post-conception.

### 2.1.2. ATCC Cell Lines

Fetal, rat lung fibroblasts RFL-6 (ATCC, CCL-192) were obtained from American Type Culture Collection (Rockville, Maryland).

### 2.1.3. Plasmid Constructs

**Table 2.1:** List of plasmid constructs

| Construct | Reference |
|---|---|
| pEGFP-C1- p60 katanin | Karabay, et al, 2004 |
| pcDNA3-spastin-myc-GFP | Errico, et al, 2002 |
| pEGFP-C1 | Clontech, catalog number: 6084-1 |
| pMAP2c – human MAP2c | Gamblin, et al, 1996 |
| pMAP1b – mouse MAP1b | Weeks and Fischer, 2000 |

### 2.1.4. Antibodies

- Goat polyclonal anti-MAP1b antibody (Ab) (Santa Cruz, CA, sc-8971);
- Unconjugated mouse monoclonal anti-beta-tubulin Ab (Sigma, catalog number T4026);

- rabbit anti-GFP polyclonal Ab (Abcam, UK, catalog number ab6556);
- mouse monoclonal anti-MAP2 Ab (Chemicon, catalog number MAB3418X);
- Cy3 (Cyanine)-conjugated mouse monoclonal anti-beta-tubulin Ab (Sigma, catalog number c4585);
- Donkey anti rabbit –alexa 488 (Molecular Probes, catalog number A21206)
- Series of appropriate fluorescent secondary antibodies (Jackson ImmunoResearch, PA).
    - Donkey anti goat – cy3 conjugated (catalog number 705-165-147)
    - Donkey anti mouse –cy5 conjugated (catalog number 715-175-151)
    - Goat anti mouse –cy5 conjugated (catalog number 115-175-003)
    - Goat anti rabbit –FITC conjugated (catalog number 111-095-144)

### 2.1.5. Solutions and Buffers

#### 2.1.5.1. PBS

Stock solution (10X PBS) was prepared by dissolving 81.8 g NaCl, 1.86g KCl, 21.04g Na2HPO4.7$H_2O$, and 2.2g KH2PO4 in 1lt ddH2O. Working solution (1X PBS) was prepared by diluting 10X PBS by ten times. pH was adjusted to 7.2. PBS was stored at room temperature.

#### 2.1.5.2. Borate Buffer

To prepare borate buffer, 190 mg Borax (sigma, 229946), 124 mg Boric acid (sigma, B1934) were put in a 50 ml falcon tube. Final volume was brought to 40 ml with dd$H_2O$. Borate buffer is good for 1 month at room temperature.

#### 2.1.5.3. PHEM

PHEM is a MT stabilization buffer. Stock solution (2X PHEM) was prepared by dissolving 18.14 g Pipes (Sigma, P1851), 5.96 g Hepes (Invitrogen, 11344-041), 3.8 g EGTA (Sigma, E3889) and 0.41 g MgCl2 in 500 ml ddH2O. pH was adjusted to 6.9 with NaOH. PHEM stock solutions aliquots were prepared and stored at -20 $^0$C. Once thawed, it can be kept a few days in refrigerator.

#### 2.1.5.4. Mounting Medium

Mounting medium was prepared by bringing 0.106g N-Propyl gallate (Sigma, P3130), 5 ml 1x PBS and 45 ml glycerol (Sigma, G2025) together. It was vortexed very well and kept at 4 $^{0}$C.

#### 2.1.5.5. Hippocampus Dissection Medium

To prepare Hippocampus Dissection Medium, 10 ml HBSS (Invitrogen, 14185), 1 ml 1M HEPES (Invitrogen, 15630-080), 1 ml Penicillin-Streptomycin (Sigma, P4333) were mixed. Final volume was brought to 100 ml by addition of 88 ml ddH2O. Medium was filter sterilized with 0, 2 µm Nalgene filter. All the filter sterilizations were done with same method. Medium is good for 15 days in 4 $^{0}$C. It has to be kept on ice while working.

#### 2.1.5.6. Hippocampus Plating Medium

To prepare Hippocampus Plating Medium, 45,92 ml Neurobasal Medium (Invitrogen, 12349-015), 1 ml B27 Supplement (Invitrogen, 17504-044), 0, 33 ml 45% Glucose (Sigma, G8769), 0, 25 ml L-Glutamine (Invitrogen, 25030-164) and 2.5 ml FBS (Biological Industries, 04-001-1) were brought up together. Final volume was 50 ml. After filter sterilization, medium was kept in 4 $^{0}$C for 15 days.

#### 2.1.5.7. Hippocampus Serum Free

To prepare Hippocampus Serum Free Medium, 48,42 ml Neurobasal Medium (Invitrogen, 12349-015), 1 ml B27 Supplement (Invitrogen, 17504-044), 0,33 ml 45% Glucose (Sigma, G8769), 0,25 ml L-Glutamine were brought up together. Final volume was 45 ml. After filter sterilization, medium was kept in 4 $^{0}$C for 15 days.

#### 2.1.5.8. Fixative

To prepare 40 ml fixation solution, 160 µl 50% Gluteraldehyde (Sigma, 7651), 400 µl 10% Triton X100, 40µl 10 mM taxol and 20 ml 2X PHEM solution were mixed. Final volume was brought to 40 ml with ddH2O. Fixative was warmed in 37 $^{0}$C water bath before using.

#### 2.1.5.9. Triton X100

Triton X100 is a detergent that is mostly used to extract the membrane from cells so that the cytoskeleton can be accessed. It is usually prepared as 10% solution. To prepare this, 4 ml of pure Triton X100 (Sigma, T-9284) was placed in 50 ml tube. dd$H_2O$ was added into tube up to 40 ml. Tube was capped and covered with parafilm. Tube was rotated overnight in the cold room to fully disperse the tick detergent. Next morning, 4 ml aliquot tubes of 10% Triton X100 were prepared. Tubes were covered with parafilm and stored at -20 $^0$C. When the aliquot is thawed it has to be stored at 4 $^0$C.

#### 2.1.5.10. Blocking Solution

Blocking solution application is important to reduce unspecific bindings. To prepare 1 ml blocking solution 100 µl GOAT (or DONKEY) serum and 10 mg BSA were dissolved in 1X PBS and filtered with 0.8µm Nalgene filter. The final concentrations of ingredients were 10% serum and 10 mg/ ml BSA. Blocking solution is good for 15 days when stored at 4 $^0$C.

#### 2.1.5.11. ATCC Medium

ATCC medium was recommended by the manufacturer of RFL – 6 cell line. To prepare 200 ml of the medium, 40 ml FBS (Biological Industries, 04-001-1) and 160 ml F12K (Sigma, N3520) were mixed. The final concentration of ingredients were 20% FBS and 80% F12K.

#### 2.1.5.12. L – Glutamine

L-glutamine is an essential amino acid that is a crucial component of culture media that serves as a major energy source for cells in culture. L-glutamine is very stable as a dry powder and as a frozen solution; however in liquid media or stock solutions, L-glutamine degrades relatively rapidly. For optimal cell performance, L-glutamine is usually added to the media prior to use. In this study, the L-glutamine was purchased from Sigma, with the catalog number G7513.

### 2.1.5.13. Chemicals

**Table 2.2:** List of chemicals

| | |
|---|---|
| 25% Gluteraldehyde | EMS |
| Taxol | Sigma |
| NaHBo | Sigma |
| BSA | Calbiochem |
| 0.25 % Trypsin/EDTA | Sigma |
| 2.5% Trypsin | Gibco |
| DNase | Sigma |
| Wax | Sigma |
| Nail polisher | |

### 2.1.5.14. Kits

**Table 2.3:** List of kits

| |
|---|
| Rat Neuron Nucleofactor Kit (catalog number: VPG-1003), Amaxa. |
| Plasmid Midi Kit (catalog number: 12143), Qiagen. |
| Endofree Plasmid Maxi Kit (catalog number: 12362), Qiagen. |

### 2.1.5.15. Equipments

**Table 2.4:** List of equipments

| | |
|---|---|
| Water bath | |
| $CO_2$ Incubator | Shel Lab |
| Ultrapure water system | USF-Elga UHQ (USA) |
| Centrifuge | Beckman coulter |
| Hemacytometer | |
| Fluorescent Microscope | Carl Zeiss, Oberkochen,Germany |
| Light Microscope | Olympus CH30 (USA) |
| Micropipettes | Eppendorf (Germany) |
| Filters | Nalgene (USA) |

## 2.2. Methods

### 2.2.1. Coverslips

Much of our work requires microscopic techniques, so that it is difficult to use plastic culture dishes for plating the cells. Because of this, we plate the hippocampal cells onto glass coverslips that have been already treated with poly – L – lysine. These dishes can be prepared by fixing the glass coverslips onto the plate or commercially available cell culture dishes can be used. These dishes have special chamber made of glass, and after coating this chamber with desired substrate, we can plate the hippocampal cells into this hole. Using substrates, like poly-lysine, enhances cell adhesion. Obtaining successful neuronal cultures also depends on finding appropriate substrate. Surfaces treated with basic amino acid polymers such as polylysine and polyornithine are very common.

To prepare the coating substrate, 10 mg poly – L – lysine was dissolved in 10 ml borate buffer (1 mg/ ml) and filter sterilized by using 0,2 µm Nalgene filter.

- Each dish was covered with 0.2 ml of poly – L – lysine solution for 1 hour at room temperature.
- Then, the dishes were washed 6 times for 5 minutes each with sterile double distilled water.
- Water was taken off and 2 ml of hippocampal plating medium was added into the dishes.
- Dishes were kept in incubator with water until the day of culturing or dried.
- On the day of culturing, plating medium was removed and cells were plated immediately
- All the coating procedure was done under the UV sterilized hood.

### 2.2.2. Media

Tissue was dissected and dissociated in Hepes-buffered, calcium- and magnesium-free Hank's balanced salt solution (HBSS), "Hippocampus Dissection Medium". At all stages tissues and cells have to be maintained in an osmotically balanced solution at physiological pH. This medium is intended for short-term use. After obtaining

single cell suspension, neurons were plated into serum- containing medium, “Hippocampus Plating Medium”. The plating medium contains serum that provides the trace nutrients and growth factors needed for long-term growth of the cells, also amino acids and some other ingredients. In this study we used fetal bovine serum (FBS). FBS is rich in mitogenic factors and preferred for proliferating cells.

When the cells were attached to the substrate, medium was changed with serum free medium “Hippocampus Serum-Free Medium” because there are some disadvantages when serum is used to supplement culture medium. Serum represents a major potential route for the introduction of agents including bacteria, fungi and viruses into cell culture. This could be disruptive. The presence of serum also influences downstream processing. At 10% concentration, serum contributes about 4-8 mg of protein per ml while recombinant proteins are frequently expressed at levels of tens of micrograms per ml. In this situation, efficient purification of the required protein may be difficult.

The Neurobasal Medium that was used in plating medium has to be phenol red-free, because phenol red is endocytosed by the cells and can contribute significantly to fluorescence background and to phototoxicity.

### 2.2.3. Primary Culture

In this part of the study primary neuron culture cells were used. When the culture started from cells, tissue or organs taken directly from organisms it is termed “primary culture”. When the tissue is dissociated into single cell suspension and plated into culture dish, go through series of event. If cells are mitotic, they undergo rapid division. When they approach confluence and contact with one another, their division rate slows and they start differentiation. Each type of different cell has different differentiation pattern.

Because they are post-mitotic cells, the situation is different in neuron cells. When the embryonic brain cells are dissociated and plated into culture dish, neurons that have completed their division in situ start to extend processes, form synapses with one another, become electrically active, etc. This makes the study of neurons in culture completely different from other cell types. One of the important things about

neurons is that, in dissociated cultures they also retain their individual identities, and each different type of neurons expresses its own properties.

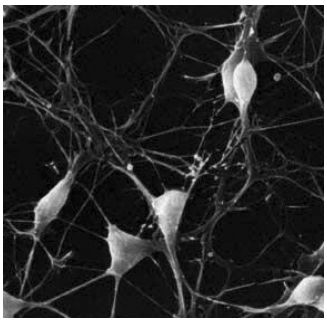

**Figure 2.1:** Primary Cell Culture

By using primary dissociated neuronal culture, it is especially possible to study morphological and physiological techniques, which can be applied on a cell-by-cell basis.

To obtain primary dissociated neuronal culture from hippocampus, we first had to dissect the hippocampus from embryonic rat brains. After serial steps listed below, we obtained single cell suspension of the hippocampal neuron cells.

### 2.2.4. Hippocampal Culture

#### 2.2.4.1. Dissection of the Hippocampus

In our experiment, fetuses were obtained at embryonic day 18 (E18) from pregnant Sprague – Dawley rats.

- The pregnant rat was euthenized with carbon dioxide and its abdomen was wiped with 70% ethanol. To minimize any contamination risk from the fur, first the skin has to be cut and laid back. Then after rinsing the instruments with 70% ethanol again, abdominal wall was cut.
- Uterus was removed and placed in a sterile Petri dish and was carried to a laminar flow hood.
- Fetuses were removed from the uterus, the heads were transferred to a Petri dish containing hippocampus dissection medium pre-warmed to 37 $^{0}$C.

- Brains were removed under a dissection microscope. For this procedure two Dumont-style forceps (no.5) were used.
    - The cerebral hemispheres were separated from diencephalon and brain stem (A, B). Diencephalon and brain stem were discarded.
    - Meninges were carefully stripped away (C).
    - (D) shows one of the hemispheres after removal of meninges. Arrowheads indicate the boundary between the hippocampus and the adjoining cortex; small arrows mark the free edge of the hippocampus.
    - (E) shows the removal of hippocampus. The hemisphere was stabilized with forceps and another forceps was used to cut the hippocampus and to take it out of the hemisphere.
    - (F) shows the hippocampus after it has been removed.

When viewed from the midline, the hippocampus forms a **C** in the hemispheres. When all the required hippocampi have been lift them with the forceps and transfer to a dish containing hippocampus dissection medium. The hippocampi were cut into small pieces and transferred into 15 ml conical centrifuge tube.

- The volume was bringed to 4.5 ml with hippocampus dissection medium.
- 0.5 ml Trypsin 2,5% (Gibco # 25095-19) and 0.25 ml DNase (Sigma#DN-25) (10 mg/ ml) were added into tube, tube was gently mixed and incubated in 37 $^{0}$C water bath for 15 minutes.
- Hippocampi pieces were settled to the bottom of the tube, and after the incubation trypsin and DNase were pipetted off.
- Hippocampi pieces were rinsed 2 times for 5 minutes each with 5 ml hippocampus plating medium.
- 1 ml warm hippocampus plating medium was added into the tube.
- Trituration was done 6-7 times against the side of the tube with a fire-polished Pasteur pipette. The medium was especially pushed out against the side of the tube to prevent frothing because the cells at an air-liquid interface can be lysed.
- Dissociation was completed, and the cell density was determined by using hemacytometer.

Hemacytometer is a specially designed slide with a counting chamber 0.1 mm deep and ruled in a grid pattern. After the density and proportion of the viable cells was determined the required amount of cells were taken and diluted. For transfection, we used $10^6$ cells per dish.

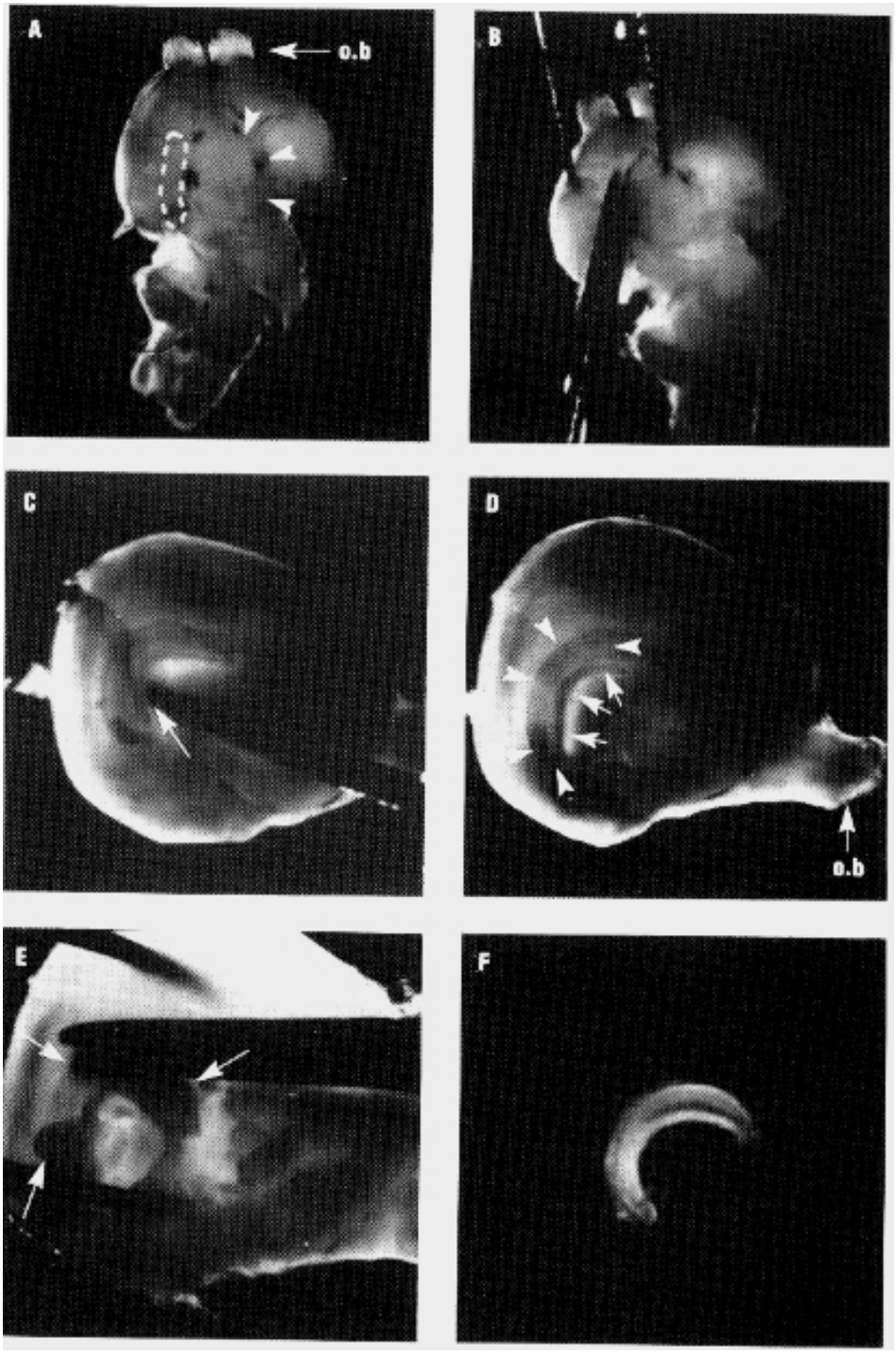


**Figure 2.2:** Hippocampus Dissection

#### 2.2.4.2. Plasmid DNA Preparation (midi scale)

Plasmid mini preparation was performed using Qiagen, Plasmid Midi Kit for large-scale (midi) preparations, following instructions of the manufacturer. The principle of this purification is as follows: alkaline lysis releases plasmid DNA from bacteria and RNase removes all the RNA in the lysate. Then, in the presence of a chaotropic salt (guanidine HCl), plasmid DNA binds selectively to glass fiber fleece in a centrifuge tube. The DNA remains bound while a series of rapid "wash-and-spin" steps remove contaminating bacterial components. Finally, low salt elution removes the DNA from the glass fiber fleece.

The protocol is as follows:

- A single bacterial colony was picked and inoculated into 5 ml selective LB media containing Falcon tube, and grown for 8 hours with shaking (250 rpm) at 37 ºC.
- Starter culture was diluted into 25 ml selective LB media containing flask and grown for 16 hours with shaking (250 rpm) at 37 ºC.
- The following day, culture was distributed into 2 centrifuge tubes (12.5 ml each tube), and the bacteria were harvested by centrifugation for 15 minutes at 6.000 x *g* at 4 ºC. The supernatants were discarded.
- The bacterial pellet was resuspended in 4 ml of suspension buffer, Buffer P1 in each centrifuge tube separately and then collected to one centrifuge tube. Suspension buffer contains RNase which removes bacterial RNA.
- To lyse the cells, 4 ml lysis buffer, Buffer P2 was added (contains NaOH), mixed by inverting the tube 6 times and incubated at room temperature for up to 5 minutes.
- Lysis was stopped by addition of 4 ml ice-cold binding buffer, Buffer P3. Tube was again inverted 6 times and incubated on ice for up to 15 minutes.
- The mixture was centrifuged for 30 minutes at 20.000 x *g* at 4 ºC and supernatant was transferred to a new centrifuge tube. Chromosomal DNA was precipitated with cellular debris during centrifugation and this supernatant contains the plasmid DNA
- Supernatant was centrifuged again for 15 minutes at 20.000 x *g* at 4 ºC.

- Filter tube, Qiagen-tip 100 was equilibrated by adding 4 ml Buffer QBT. Column was allowed to empty by gravity flow.
- When the Qiagen-tip 100 was drained completely, supernatant was applied to Qiagen-tip 100 and allowed to enter the resin by gravity flow. Plasmid DNA is bound to the glass fibers pre-packed in the filter tube. Flow-through was discarded from the collection tube.
- Qiagen-tip 100 was washed 2 times with 10 ml washing buffer, Buffer QC. Buffer QC was allowed to move through the Qiagen-tip 100 by gravity flow. The first wash is sufficient to remove all contaminants in the majority of plasmid DNA preparations. The second wash is especially necessary when large culture volumes or bacterial strains producing large amounts of carbohydrates are used.
- To elute the DNA 5 ml elution buffer, Buffer QF was added. Eluate was collected in 15 ml centrifuge tube. Since polycarbonate tubes are not resistant to alcohol, it is not recommended to use polycarbonate centrifuge tubes.
- Eluted DNA was precipitated by adding 3.5 ml room-temperature isopropanol.
- Tube was mixed and centrifuged immediately at 15.000 x *g* for 30 minutes at 4 ºC.
- DNA pellet was washed with 2 ml of room-temperature 70% ethanol, and centrifuged at 15.000 x *g* for 10 minutes.
- Supernatant was discarded very carefully without disturbing the DNA pellet.
- Pellet was air-dried for 30 minutes.
- When the pellet is dried completely, it was redissolved in 100 µl TE buffer, pH 8.

#### 2.2.4.3. Transfection

Many research techniques in molecular biology require a foreign gene to be inserted into a host cell. Since the phospholipid bilayer of the plasma membrane has a hydrophilic exterior and hydrophobic interior, any polar molecules, such as DNA, are unable to freely pass through the membrane. Many methods have been developed to allow the insertion of DNA and other molecules into the cells to be studied. In dividing cells, if the DNA enters into the cytoplasm, it will enter the nucleus during

break down of the nuclear envelope during mitosis. On the other hand, neurons are post-mitotic cells and they do not have ability to divide so that it is difficult to transfect these cells. If standard methods such as calcium phosphate co-precipitation, lipid mediated transfection, etc… are used to transfect the neurons the efficiency is usually very low, ≤ 1 %. Other methods that have been used for transfecting neurons are microinjection and biolistics. Also some adenoviral transfection methods give very high transfection efficiencies.

The method that we used in this experiment was electroporation, which is also termed electrotransfer or electropermeabilization. In this experimental technique, brief electric pulses, lasting a few microseconds to a millisecond, are applied to the cells. This quick voltage shock disrupts areas of the membrane temporarily, causes the formation of temporary aqueous pores. The electric potential across the membrane of the cell simultaneously rises by about 0.5-1.0 V so that charged molecules (such as DNA) are driven across the membrane through the nucleus. As charged ions and molecules flow through the pores, the cell membrane discharges and the pores quickly close, and the phospholipid bilayer reassembles. The plasmid of interest supposed to be inside the cell for further use or study.

Although the efficiency is high in electroporation, this technique has some disadvantages also; if the pulses are of the wrong length or intensity, some pores may become too large or fail to close after membrane discharge causing cell damage. The transport of material during the electropermeability is relatively nonspecific, this may cause an ion imbalance that could later lead to improper cell function and cell death (Banker & Goslin, 1998)

- Required amount of cells were taken from cell suspension that was prepared by trituration and centrifuged at 3000 rpm for 5 minutes.
- Supernatant was removed and pellet was resuspended in Nucleofactor solution. 100 µl of Nucleofactor solution was used per cuvette.
- 100 µl cell – Nucleofactor solution mix was placed into the tubes that contain interested plasmids:
    - rat p60-GFP → 15 µg/cuvette
    - spastin-myc-GFP → 15 µg/cuvette
    - GFP → 5 µg/cuvette

- Cells, Nucleofactor solution and plasmids were combined.
- Combination was transferred into the electroporation cuvette.
- Electroporation was done by using program G-13 in Amaxa nucleofactor.
- Cells were taken out of the cuvette immediately and transferred into new tube containing 900 µl plating medium by Amaxa certified pipette.
- Cells were diluted with plating medium. The final concentration was 8000 cells/dish.
- 200 µl of cells were plated into 35-mm Petri dishes and placed into 37 $^0$C 5% $CO_2$ incubator.
- 2 – 3 hours later, 2 ml of plating medium was added into the dishes and kept in 37 $^0$C 5% $CO_2$ incubator.
- Cell fixation/co-extraction was done on day 2, day 4.

#### 2.2.4.4. Cell Fixation

To be able to analyze the cells under the microscope they have to be fixed. This also preserves the cells for longer storage periods. There are several methods for fixation. Besides the aldehydes organic solvents can be used in fixation. Aldehydes fix by denaturation and chemical modification of proteins. For example, covalent reactions can occur with free amino groups of lysine residues. When protein cross linking occurs cells become rigid. The cell membrane of intact cells remains relatively impenetrable to larger molecules such as antibodies. Thus, "cell permeabilization" is required before immunostaining.

When it comes to the organic solvents such as methanol, ethanol, acetone, they denature proteins without covalent modifications, they just remove bound $H_2O$ molecules. These solvents also remove membrane and some structural lipids, and cells become permeable to antibodies.

In our study we used gluteraldehyde as fixative. We also used detergent, Triton-100X for cell extraction that led cell permeabilization. Both procedures were done in the same step, termed fixation/co-extraction.

**Table 2.5:** Fixative ingredients for hippocampus cells

| Chemical | Stock concentration | Final concentration |
|---|---|---|
| Gluteraldehyde | 25% | 0,2% |
| Triton100x | 10% | 0,1% |
| Taxol | 10mM | 10µM |
| PHEM | 2X | 1X |
| $ddH_2O$ | - | Up to final volume |

- The fixation/extraction solution consisting chemicals listed above was warmed in 37 $^0$C water bath.
- Petri dishes containing the cultured cells were filled with 2 ml of fixation/extraction solution.
- Cultures were incubated at room temperature for 15 minutes.
- Cultures were rinsed with PBS 3 times for 5 minutes and were kept at 4 $^0$C until the day of immunostaining.

In this study, cell fixation/co-extraction was done in day 2 and 4. At the end of the day 4, fixation/co-extraction and Sodium Borohydrate treatment were done. This treatment is necessary to block free aldehyde groups formed during the fixation process, since they may decrease antigenic site accessibility.

- 10 mg/ ml Sodium Borohydrate solution was prepared in PBS.
- Cells were quenched three times for 10 minutes with Sodium Borohydrate solution.
- Cells then were rinsed with PBS 3 times for 5 minutes.

Cells were blocked with goat-blocking solution at room temperature for 1 hour, this is important to reduce unspecific bindings. To prepare blocking solution 10% GOAT serum and 10 mg/ ml BSA were dissolved in PBS and filtered with 0.8 µm Nalgene filter.

### 2.2.4.5. Immunostaining

Immunostaining is a biochemical technique that applies an antibody to a specific cell protein, which can be used either to detect whether the protein exists in the sample, or to highlight the location of the specific protein. Some immunostaining agents are applied in a single stage - the antibody is directly conjugated to a colored agent. Others are two-stage, first antibody does not have the chemical structure to support a colored agent - in this case, after application of the first (colorless) antibody, a second antibody linked to a colored agent is applied, and the second antibody binds to the first. The colored agent can then be visualized under the microscope. Two – stage method has also some advantages. Variety of coloring agents can be conjugated to any given species of secondary antibody. This gives chance to visualize different types of proteins at the same time.

The constructs used in this study had GFP protein linked to protein of interest. In order to see whether we had expression of the interested protein, "rabbit anti-GFP polyclonal primary antibody" was used. This antibody also amplified GFP signal in the constructs. Secondary antibody was "goat anti rabbit - alexa 488 conjugate" which is green colored. To be able to visualize the microtubule channel, Cy3 conjugated –monoclonal anti-β-tubulin clone 2.1 antibody (produced in mouse) was used.

- Antibodies were diluted in PBS (dilution ratio for rabbit anti GFP polyclonal antibody was 1:500).
- To remove any precipitates formed during storage, diluted antibodies were centrifuged at 10.000 rpm for 10 minutes at $4^0$C.
- 200 µl of $1^o$ Ab solution was put in the hole containing fixed cells.
- $1^o$ Ab containing cells were incubated at $4^0$C for overnight. During this staining period, the dishes were placed in a "humidified chamber," a large Petri dish containing moistened filter paper, which minimizes evaporation of the antibody.
- $1^o$ Ab was removed by vacuum carefully and dishes were washed by using PBS 3 times for 5 minutes each.
- Cells were blocked again for 1 hour at room temperature with goat blocking solution.

- $2^o$ Ab was diluted and centrifuged in the same way as $1^o$ Ab (dilution ratio was 1:200 for goat anti rabbit -alexa 488 antibody and 1:400 for Cy3 conjugated -beta-Tb antibody).
- 200 µl of $2^o$ Ab solution was put in the hole and cells were incubated for 1 hour in 37 $^0$C incubator. Working in dark with $2^o$ Ab is very important.
- Dishes were rinsed with PBS 3 times for 5 minutes each.
- 6-7 drops of mounting medium was added into the dishes.
- Cover slips were placed on the dishes and fixed from the edges by using nail polisher.
- Dishes were visualized and analyzed by using fluorescent microscopy.

#### 2.2.4.6. Imaging / Analyzing

Parameters of the software were kept constant during the imaging process. Images were taken in two different channels.

In spastin / katanin over expression experiments,

- Cy5 (Rhodamine, red) channel was used for MT visualization,
- FITC (green) channel was used to visualize spastin / katanin / GFP expression.

Images were acquired with 40X objective of AxioVert 200M microscope (Carl Zeiss) and images were taken at identical setting of exposure time, brightness, and contrast. After the imaging, cells were analyzed. In spastin / katanin over expression experiments, process numbers, process lengths were determined.

### 2.2.5. RFL – 6 Cultures

#### 2.2.5.1. Passage P0

- Stock tube that contains RFL-6 cells was taken out from the liquid nitrogen tank.
- Tube was shaken very carefully in 37 $^0$C water bath to melt the cells.

- Cells were transferred into 10 ml of 37 $^0$C warmed ATCC medium and centrifuged at 3000rpm for 5 minutes.
- Supernatant was removed, 1 ml fresh 37 $^0$C warmed ATCC medium was added into the tube and the pellet was dissolved.
- Cells were transferred into horizontal flask containing 20 ml of 37 $^0$C warmed ATCC medium and flask was put in 37 $^0$C 5% $CO_2$ incubator.

#### 2.2.5.2. Splitting the Cells

When culture density was a 85-90% monolayer, cells were splitted, resulting cells were termed as P 1 passage:

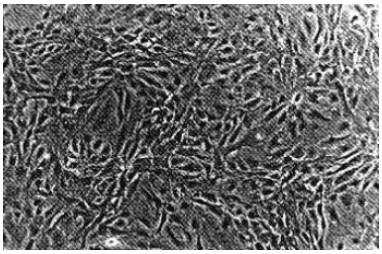

**Figure 2.3:** Monolayer Fibroblast Cells

- ATTC medium was removed from horizontal flask carefully by vacuum.
- Cells were rinsed once with 10 ml sterile 1xPBS to block serum activity, and then PBS was removed by vacuum.
- 1 ml Trypsin/EDTA was added into the flask, and the bottom of the flask was tipped to provide coating.
- Flasks were placed in $CO_2$ free incubator for 3 – 4 minutes in order to detach the cells.
- Flask was smacked couple of times, so that the cells were able to flow freely.
- 10 ml of fresh 37 $^0$C warmed ATCC medium was added into the floating cells and divided into the additional 3 flasks.
- Fresh 37 $^0$C warmed ATCC medium was added into the flasks up to 25 ml.

#### 2.2.5.3. Detaching the Cells:

- Cells in the flasks were rinsed once with 10 ml sterile 1xPBS to block serum activity, and then PBS was removed by vacuum.
- 1 ml Trypsin/EDTA was added into the flask, and the bottom of the flask was tipped to provide coating.
- Flasks were placed in $CO_2$ free incubator for 3 – 4 minutes in order to detach the cells.
- Flask was smacked couple of times, so that the cells were able to flow freely.
- 10 ml of fresh 37 $^0$C warmed ATCC medium was added into each flask.
- All the cells were brought up together and 40 ml of cell suspension was obtained.

#### 2.2.5.4. Endofree Plasmid DNA Purification (maxi scale)

Plasmid maxi preparation was performed using Qiagen, Endofree Plasmid Maxi Kit for large-scale (maxi) preparations, following instructions of the manufacturer. This purification process is specifically important to remove endotoxins.

Endotoxins are cell membrane components of Gram-negative bacteria (e.g., *E. coli*). Bacteria shed small amounts of endotoxins into their surroundings while they are actively growing and large amounts when they die. During lysis of bacterial cells for plasmid preparations, endotoxin molecules are released from the outer membrane into the lysate. Endotoxins strongly influence transfection of DNA into primary cells and sensitive cultured cells, and increased endotoxin levels lead to sharply reduced transfection efficiencies. Furthermore, it is extremely important to use endotoxin-free plasmid DNA for gene therapy applications, since endotoxins cause fever, endotoxic shock syndrome, and activation of the complement cascade in animals and humans. Protocol is as follows:

- A single bacterial colony was picked and inoculated into 5 ml selective LB media containing Falcon tube, and grown for 8 hours with shaking (250 rpm) at 37 ºC.
- Starter culture was diluted into 100 ml selective LB media containing flask and grown for 16 hours with shaking (250 rpm) at 37 ºC.

- The following day, culture was distributed into 2 centrifuge tubes (50 ml each tube), and the bacteria were harvested by centrifugation for 15 minutes at 6.000 x *g* at 4 ºC. The supernatants were discarded.
- The bacterial pellet was resuspended in 10 ml of suspension buffer, Buffer P1 in each centrifuge tube separately and then collected to one centrifuge tube. Suspension buffer contains RNase which removes bacterial RNA.
- To lyse the cells, 10 ml lysis buffer, Buffer P2 was added (contains NaOH), mixed by inverting the tube 6 times and incubated at room temperature for up to 5 minutes.
- During the incubation, QIAfilter Cartridge was prepared and placed in a convenient tube.
- Lysis was stopped by addition of 10 ml ice-cold binding buffer, Buffer P3. Tube was again inverted 6 times and incubated on ice for up to 15 minutes.
- Lysate was poured in the barrel of the QIAfilter Cartridge and it was incubated at room temperature for 10 minutes.
- Plunger was genteelly inserted into the QIAfilter Cartridge and lysate was filtered into 50ml tube.
- 2.5 ml of Buffer ER was added into the filtered lysate and it was mixed by inverting the tube 10 times.
- Tube containing lysate was incubated on ice for 30 minutes.
- Filter tube, Qiagen-tip 500 was equilibrated by adding 10 ml Buffer QBT. Column was allowed to empty by gravity flow.
- When the Qiagen-tip 500 was drained completely, filtered lysate was applied to Qiagen-tip 500 and allowed to enter the resin by gravity flow. Plasmid DNA is bound to the glass fibers pre-packed in the filter tube. Flow-through was discarded from the collection tube.
- Qiagen-tip 500 was washed 2 times with 30 ml washing buffer, Buffer QC. Buffer QC was allowed to move through the Qiagen-tip 500 by gravity flow. The first wash is sufficient to remove all contaminants in the majority of plasmid DNA preparations. The second wash is especially necessary when large culture volumes or bacterial strains producing large amounts of carbohydrates are used.

- To elute the DNA 15 ml elution buffer, Buffer QF was added. Eluate was collected in 50 ml centrifuge tube. Since polycarbonate tubes are not resistant to alcohol, it is not recommended to use polycarbonate centrifuge tubes.
- Eluted DNA was precipitated by adding 10.5 ml room-temperature isopropanol.
- Tube was mixed and centrifuged immediately at 15.000 x *g* for 30 minutes at 4 ºC.
- DNA pellet was washed with 5 ml of endotoxin-free room-temperature 70% ethanol, and centrifuged at 15.000 x *g* for 10 minutes.
- Supernatant was discarded very carefully without disturbing the DNA pellet.
- Pellet was air-dried for 30 minutes.
- When the pellet is dried completely, it was redissolved in 100 µl TE buffer, pH 8.

**2.2.5.5. Transfection of RFL-6 Cells:**

- Plasmids that were used in the experiment were put into tubes.

**Table 2.6:** Constructs used for transfection

| Tube number | Construct | DNA amount |
|---|---|---|
| 1 | GFP | 10 µg |
| 2 | Spastin-myc-GFP | 15 µg |
| 3 | Map1B and MAP2C | 15 µg |
| 4 | Spastin-myc-GFP + Map1B | 15 + 15 µg |
| 5 | Spastin-myc-GFP + Map2C | 15 + 15 µg |

- Required amounts of cells were taken from detached cells.

For this, you have to count the cells in hemacytometer, $7x10^6$ cells that correspond to 25 ml were used for transfection.

- Cells were centrifuged at 3000 rpm for 5 minutes.
- Supernatant was taken out and cells were resuspended with solution R (from Amaxa nucleofactor kit).

- For each set of transformation 100 µl of R solution was used so totally 600 µl of solution R was used for resuspention.
- 100µl (cell + solution R) suspension was put into each tube containing plasmids.
- Cells and plasmids were mixed together and transferred into electroporation cuvette supplied by Amaxa.
- Transfection was done in nucleofactor by using program G-13.
- Electroporated cells were taken out immediately by special pipettes provided in the nucleofactor kit.
- Cells were transferred into new tubes containing 900 µl fresh ATCC medium.
- Cells were diluted with ATCC medium. 8.000cells were plated into 33mm plastic culture dishes.
    - We had ~1x$10^6$ cells per transfection set = $10^6$ cells/ ml.
    - We need 8.000cells x 5 dishes = 40.000cells which is 40 µl.
    - Take 50 µl cells into 750 µl ATCC medium and plate 150 µl per dish.
- 2 – 3 hours later, 2 ml of ATCC medium was added into the dishes and kept in 37 $^0$C 5% $CO_2$ incubator.
- Cells were left in 37 $^0$C 5% $CO_2$ incubator for overnight.

#### 2.2.5.6. Fixation/ Co-extraction of RFL-6 Cells

**Figure 2.7:** Fixative ingredients for RFL-6 cells

| Solution | Stock concentration | Final concentration |
|---|---|---|
| Gluteraldehyde | 50% | 0,2% |
| Triton100x | 10% | 0,1% |
| Taxol | 10 mM | 10 µM |
| PHEM | 2X | 1X |
| dd$H_2O$ | - | Up to final volume |

- The fixation/extraction solution consisting chemicals listed above was warmed in 37$^0$C water bath.
- Petri dishes containing the cultured cells were filled with 2 ml of fixation/extraction solution.
- Cultures were incubated at room temperature for 15 minutes.

- 2 mg/ ml Sodium Borohydrate solution was prepared in PBS.
- Cells were quenched two times for 15 minutes with Sodium Borohydrate solution.
- Cells then were rinsed with PBS 3 times for 5 minutes.
- Dishes were kept in 4 $^{0}$C until the day of immunostaining.

### 2.2.5.7. Immunostaining

- Fixed cells were blocked with blocking solution for 1 hour at room temperature. Blocking solution was different for each set of experiment.
- Blocking solution was removed with vacuum carefully.
- Antibodies were diluted in PBS.

**Table 2.8:** Dilution ratio for primary antibodies

| | |
|---|---|
| Goat Map1B | 1:200 |
| Mouse beta Tb | 1:200 |
| Rabbit GFP polyclonal | 1:500 |
| Mouse Map2c | 1:200 |

- To remove any precipitates formed during storage, diluted antibodies were centrifuged at 10.000rpm for 10 minutes in 4$^{0}$C.
- 200 µl of 1$^{o}$ Ab solution was put in the hole containing fixed cells.

**Table 2.9:** Flowchart for Primary Antibody Application

| Dish | Blocking | $1^o$ Ab |
|---|---|---|
| Map 1B | Donkey blocking serum | Goat Map1B<br>Mouse beta Tb |
| Map1B + Spastin–myc–GFP | Donkey blocking serum | Goat Map1B<br>Mouse beta Tb<br>Rabbit GFP polyclonal |
| Spastin–myc–GFP | Donkey blocking serum | Mouse beta Tb<br>Rabbit GFP polyclonal |
| Map2c | Goat blocking serum | Mouse Map2c |
| Map2C + Spastin–myc–GFP | Goat blocking serum | Mouse Map2c<br>Rabbit poly GFP |
| Spastin–myc–GFP | Goat blocking serum | Rabbit poly GFP |
| GFP control | Goat blocking serum | Rabbit poly GFP |

- $1^o$ Ab containing cells were incubated at 4 $^0$C, overnight. During this staining period, the dishes were placed in a "humidified chamber," a large Petri dish containing moistened filter paper, which minimizes evaporation of the antibody.
- $1^o$ Ab was removed by vacuum carefully and dishes were washed by using PBS 3 times for 5 minutes each.
- Cells were blocked again for 1 hour at room temperature with blocking solution. Blocking solution was different for each set of experiment again.
- $2^o$ Ab was diluted and centrifuged in the same way as $1^o$ Ab.

**Table 2.10:** Dilution ratio for secondary antibodies

| | |
|---|---|
| Donkey anti goat – cy3 | 1:300 |
| Donkey anti mouse– cy5 | 1:100 |
| Donkey anti rabbit-alexa – 488 | 1:200 |
| Goat anti mouse – cy5 | 1:200 |
| mouse IgG | 1:1000 |
| Goat anti rabbit FITC conjugated | 1:100 |

Secondary antibody application was done in different way for the different sets of experiment.

- 200 µl of secondary antibody solution was applied to the Map1b dishes.
- Cells were incubated for 1hour in 37 $^{0}$C incubator.

**Table 2.11:** Flowchart for Secondary Antibody Application for MAP1b Part

| Dishes | Blocking | $2^{o}$ Ab |
|---|---|---|
| Map 1B | Donkey blocking serum | Donkey anti goat-cy3<br>Donkey anti mouse-cy5 |
| Map1B + Spastin–myc–GFP | Donkey blocking serum | Donkey ant goat-cy3<br>Donkey anti mouse-cy5<br>Donkey anti rabbit-alexa 488 |
| Spastin–myc–GFP | Donkey blocking serum | Donkey anti mouse-cy5<br>Donkey anti rabbit-alexa 488 |

**Table 2.12:** Flowchart for Secondary Antibody Application for MAP2c Part

| Dishes | Blocking | $2^{o}$ Ab |
|---|---|---|
| Map2c | Goat blocking serum | Goat anti mouse cy5 ► 1h at 37$^{0}$C<br>▼<br>mouse IgG blocking ► 1h at RT<br>▼<br>Cy3-beta-Tb |
| Map2C + Spastin–myc–GFP | Goat blocking serum | Goat anti rabbit FITC conjugated<br>Goat anti mouse cy5 ► 1h at 37$^{0}$C<br>▼<br>mouse IgG blocking ► 1h at RT<br>▼<br>Cy3-beta-Tb |
| Spastin–myc–GFP | Goat blocking serum | Goat anti rabbit FITC conjugated<br>Cy3-beta-Tb |
| GFP control | Goat blocking serum | Goat anti rabbit FITC conjugated<br>Cy3-beta-Tb |

- 200 µl of secondary antibody solution, except Cy3 – conjugated – beta – tubulin, were applied to the cells in the dishes.
- Dishes were incubated for 1hour in 37 $^{0}$C incubator. Working in dark with 2$^{o}$ Ab is very important.
- 2$^{o}$ Ab was removed by vacuum carefully and dishes were washed with PBS 3 times for 5 minutes each.
- 200 µl of mouse IgG was added into the dishes to block non-specific bindings. Cells were blocked for 1hour at room temperature.
- Blocking solution was removed with vacuum device and 200 µl of Cy3 – conjugated – beta – tubulin antibody solution was applied to the cells.
- Cells were incubated for 1hour in 37 $^{0}$C incubator
- All the dishes were rinsed with PBS 3 times for 5 minutes each.
- 6-7 drops of mounting medium was added into the dishes.
- Cover slips were placed on the dishes and fixed from the edges by using nail polisher.
- Dishes were visualizes and analyzed by using fluorescent microscopy.

**2.2.5.8. Imaging / Analyzing**

Parameters of the software were kept constant during the imaging process. Images were taken in two or three different channels according to the constructs expressed in the cells.

In Map1b experiments,

- Cy5 (Rhodamine, red) channel was used for MT visualization,
- Cy3 (purple) channel was used to visualize Map1b expression,
- FITC (green) channel was used to visualize spastin / GFP expression.

In Map2c experiments,

- Cy5 (Rhodamine, red) channel was used for MT visualization,
- Cy3 (purple) channel was used to visualize Map2c expression,

- FITC (green) channel was used to visualize spastin / GFP expression.

Images were taken by using 40X objective of the microscope. After the imaging, next step was to analyze the cells. The analyses were done by using software of the microscope. In Map experiments, MT fluorescence intensity of the cells was determined.

## 3. RESULTS

There are two sets of studies presented here, one on primary cultured neurons and the other on RFL6 fibroblasts. The studies on cultured neurons, in this case hippocampal neurons, seek to test whether overexpressed spastin causes dramatic increase in the numbers of processes, specifically in the number of dendrite formations. In the second set of studies, MAP experiments on RFL6 fibroblast were performed to investigate whether individual neuronal MAPs have the ability to protect microtubules from being severed by overexpressed spastin. Both primary neurons and RFL6 cells were from rat. We chose RFL6 cells for these studies because they are very flat, and hence excellent for imaging microtubules using immunofluorescence, and because they do not express MAP2c, MAP1b proteins endogenously.

### 3.1. Spastin Overexpression in Hippocampus Cells

In the first set of studies we sought to overexpress spastin (pcDNA3-spastin-myc-GFP; human spastin; Errico et al., 2002) and GFP (pEGFP-C1; BD Bioscience), as control constructs, in cultured hippocampal cells to ascertain how sensitive the neuron is to alterations in expressed protein levels. Based on the great homology with spastin, p60–katanin (pEGFP-C1-p60; rat p60–katanin; Karabay et al., 2004) overexpression was also performed as a control.

To investigate whether expressed constructs were showing the expected effects on neurons, we had to wait for a period of time in which the adequate expression levels were obtained. The most applicable time points were day 4 and day 6 where dramatic changes occurred in the morphology of the cells. Further investigations were performed with the hippocampal cells fixed on the day 4 and day 6.

Figure 3.1 shows images of overexpressed GFP, p60 – katanin and spastin proteins that were immunostained with polyclonal antibodies against the fusion protein, GFP.

Images were acquired with an AxioVert 200M microscope (Carl Zeiss) and images for comparison were taken at identical setting of exposure time, brightness, and contrast. The cells were fixed on the 4th day of expression. The 1st row (1a/1b/1c) shows GFP overexpressing cells, while the 2nd row (2a/2b/2c) represents p60 – katanin overexpressing cells and finally in the 3rd row (3a/3b/3c) spastin overexpressing cells can be seen. Each column represents different channel of visualization. The 1st column (I) shows green channel (GFP), the 2nd column (II) shows merged green and MT channels, where MTs are in red and the 3rd column (III) shows MT channel.

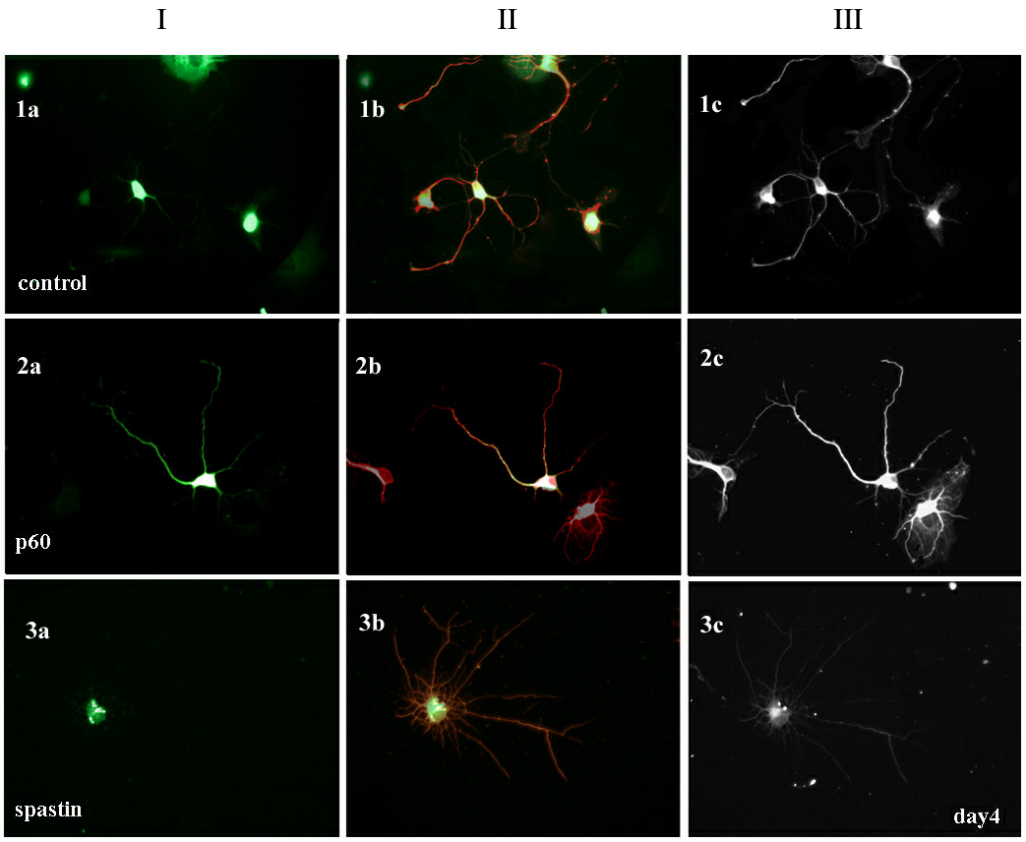


**Figure 3.1** Spastin, p60, GFP overexpression in hippocampus cells (day 4)

As expressed in the aim of study section, in katanin studies, process branching levels were not high as expected. In contrast to these results, immunostaining results and quantitative data indicated that there were significant differences in total process lengths and process numbers in spastin overexpressing neurons compared with p60–katanin and GFP expressing cells.

As illustrated in figure 3.2 and 3.4, the degree of increase was ~60% in process numbers and ~50% in total process lengths (Student's t- Test, $p<0.01$). There were no significant differences statistically between GFP expressing control cells and p60–katanin expressing cells in terms of total process lengths and process numbers (Student's t- Test, $p>0.05$).

The slight distribution of spastin was observed through the dendrites especially in the day 6 fixed cells. Furthermore, spastin localization was substantially obtained as clusters around the cell body.

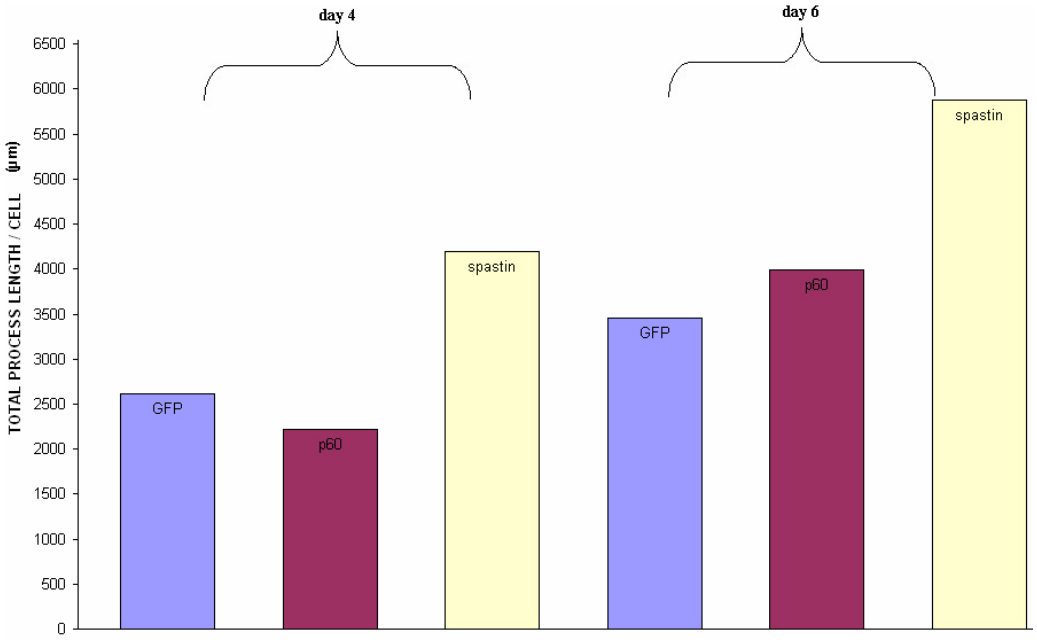


**Figure 3.2:** Total process length per cell

By day 6, minor processes and axons became much more developed. Thus, the process formations increased in all cell types.

Figure 3.3 shows images of overexpressed GFP, p60 – katanin and spastin proteins that were immunostained with polyclonal antibodies against the fusion protein, GFP on the 6$^{th}$ day of expression. Images were acquired with an AxioVert 200M microscope (Carl Zeiss) and images were taken at identical setting of exposure time, brightness, and contrast. The 1$^{st}$ row (1a/1b/1c) shows GFP overexpressing control cells, the 2$^{nd}$ row (2a/2b/2c) represents p60 – katanin overexpressing cells and finally the 3$^{rd}$ row (3a/3b/3c) shows spastin overexpressing cells. Each column represents different channel of visualization. The 1$^{st}$ column (I) shows green channel (GFP), the

2nd column (II) shows merged green and MT channels, where MTs are in red and the 3rd column (III) shows MT channel.

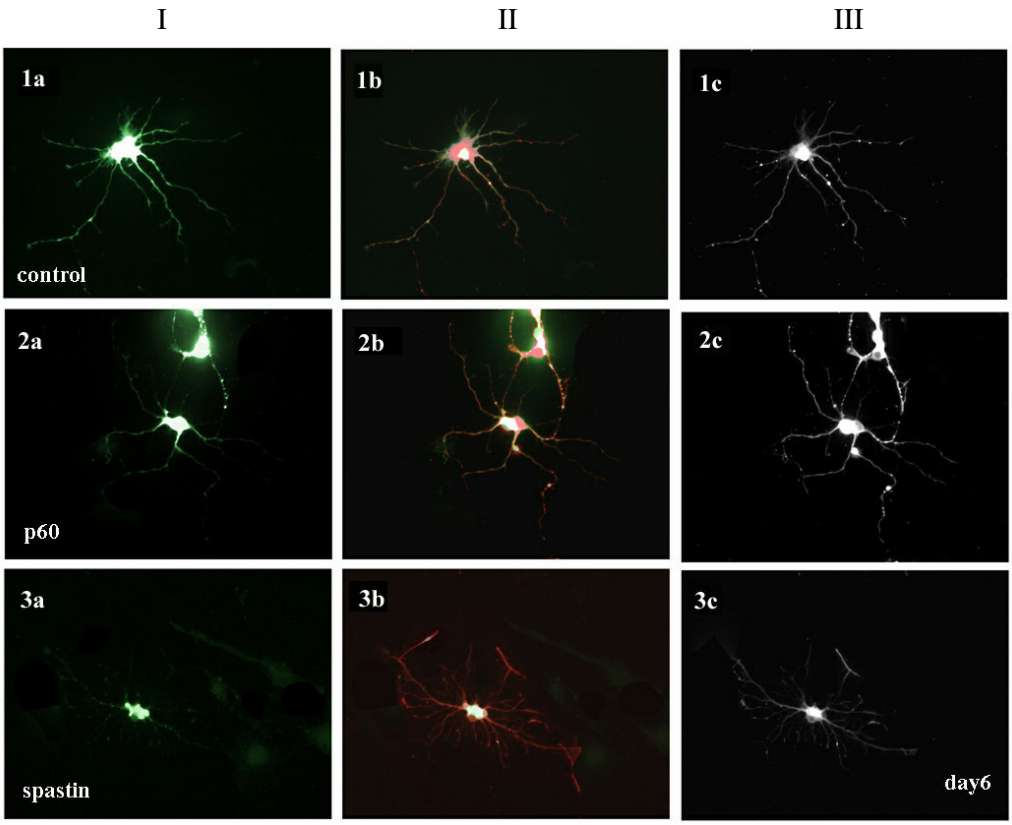


**Figure 3.3:** Spastin, p60, GFP overexpression in hippocampus cells (day 6)

By the day 6, process formations increased relatively in all cells that overexpress different constructs (GFP, p60-katanin or spastin) compared with the day four cells; however, there were still significant differences in total process lengths and process numbers between spastin overexpressing neurons and p60–katanin or GFP expressing cells.

As quantified in figure 3.2 and 3.4, the degree of increase was ~40% in process numbers and ~50% in total process lengths (Student's t- Test, $p<0.01$). correlated with day 4 results, there were no significant differences statistically between GFP expressing control cells and p60–katanin expressing cells in terms of total process lengths and process numbers (Student's t- Test, $p>0.05$).

Spastin expression was substantially obtained as aggregates around the cell body as it was reported in the previous part of the study. However, despite having great homology with spastin, the distribution of katanin was indicated to be rich in axons and dendrites.

If we compare the total process lengths and process numbers of the cells, regardless of overexpressed protein type, gradual increase can be observed in the number of process formations. Quantification results confirmed that the observations on the total length of the processes per cell were also correlating with results obtained in the process numbers analysis.

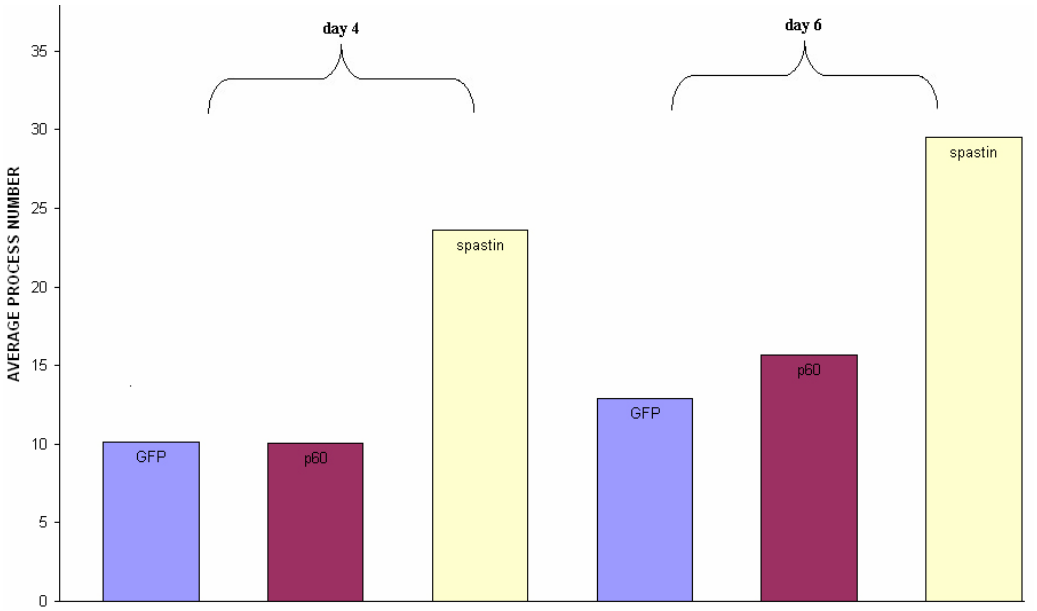


**Figure 3.4:** Average process numbers of cells

### 3.2. Regulation of Spastin, MT Severing Protein, by Microtubule Associated Proteins

In this part, studies sought to test whether MAPs have any role on microtubules to protect them from being severed by microtubule severing protein, spastin. To test the capacity of MAPs to protect the microtubules against severing, MAP1b and MAP2c were overexpressed. The DNA constructs used for these studies were: pEGFP-C1 (Clontech, #6084-1, as a control), pcDNA3 –spastin –myc –GFP (Errico, et al, 2002), pMAP1b (mouse Map1b, Weeks and Fischer, 2000) and pMAP2c (human MAP2c, Gamblin, et al, 1996).

In order to compare the results of the MAPs, we attempted to achieve equivalent levels of expression of the two MAPs. We selected the most highly expressing cells for our analyses.

Figure 3.5 shows the results obtained with MAP1b and spastin overexpression in fibroblasts. To allow the cells to express adequate levels of MAP1b and spastin, they were fixed in the 2nd day of expression. Spastin overexpression was observed after immunostaining with polyclonal antibodies against the fusion protein, GFP, while MAP1b overexpression was detected with polyclonal anti-MAP1b antibody.

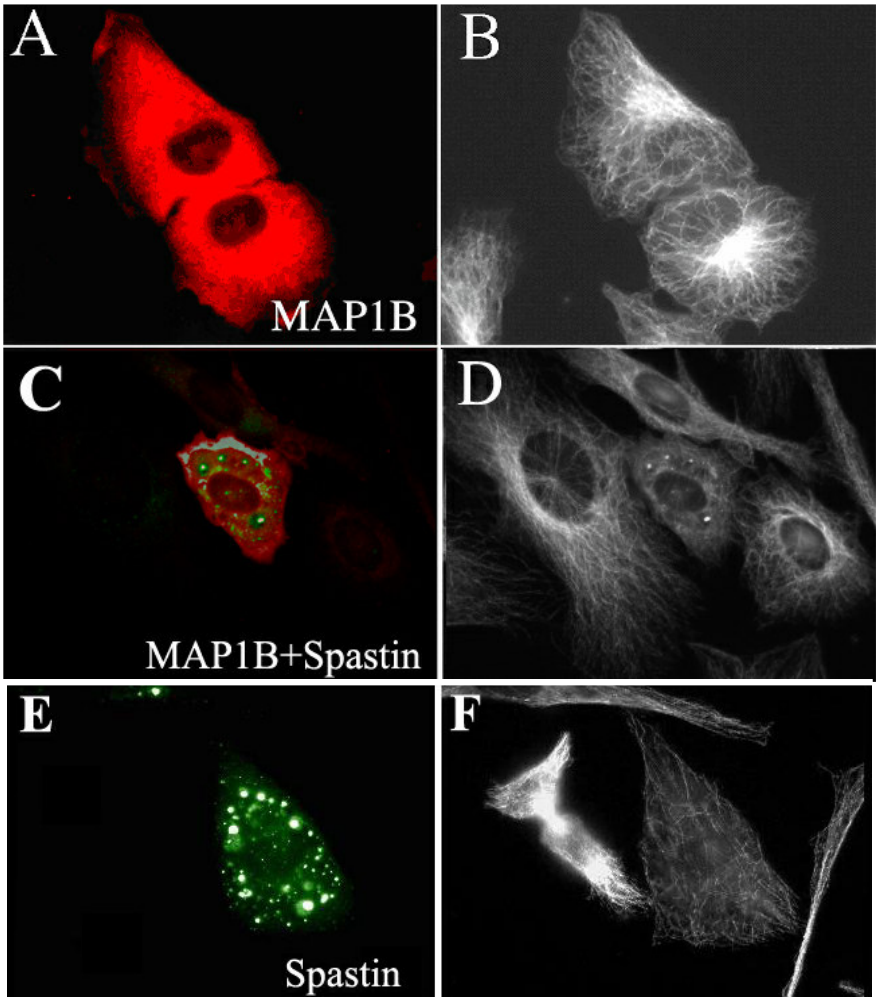


**Figure 3.5:** MAP1b / Spastin co – expression

Images were acquired with an AxioVert 200M microscope (Carl Zeiss) at identical setting of exposure time, brightness, and contrast. The 1st row (A, B) shows MAP1b overexpressing cells, while the 2nd row (C, D) represents MAP1b/spastin co-expressing cells and finally in the 3rd row (E, F) spastin overexpressing cells can be seen. Panel A, C and E are fibroblasts stained for MAP1b (red) and spastin (green). Panel B, D and F show MT staining.

MAP1b overexpression shown in figure 3.5, A and B did not cause cells to form bundles but it is known to stabilize their microtubules (Takemura et al., 1992). No significant changes detected in polymer levels in response to MAP1b. As shown in figure 3.5, C and D, MTs were severed into small pieces in MAP1b and spastin overexpressing cells. The degree of severing was similar to spastin overexpressing cells indicated in figure 3.5, E and F (~66%; $p < 0.05$).

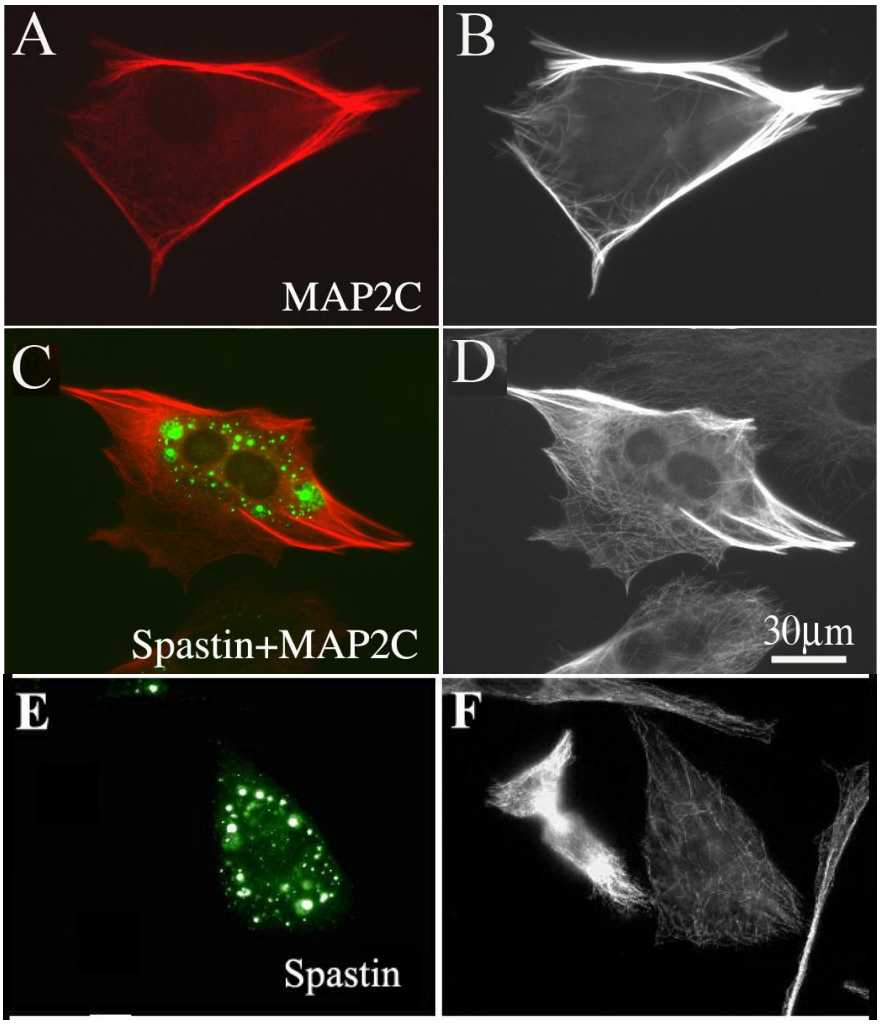


**Figure 3.6:** MAP2c / Spastin co – expression

Figure 3.6 shows the images of overexpressed MAP2c and spastin. As in the MAP1b set, MAP2c expressing cells were fixed in the 2nd day of expression. This short period of time allow cells to express MAP2c ectopically. Cells were immunostained with polyclonal primary antibodies, mouse anti-MAP2 against MAP2c and rabbit anti-GFP against the fusion protein GFP in spastin construct.

Images were acquired with an AxioVert 200M microscope (Carl Zeiss) at identical setting of exposure time, brightness, and contrast. The 1st row (A, B) shows MAP2c overexpressing cells, while the 2nd row (C, D) represents MAP2c/spastin co-expressing cells and finally in the 3rd row (E, F) spastin overexpressing cells can be seen. In panel A, C and E, fibroblasts stained for MAP2c (red) and spastin (green) are shown. Panel B, D and F show MT staining.

Figure 3.6, A and B show noticeable bundling of microtubules by MAP2c, although there were also numerous unbundled microtubules as well. Polymer levels increased in the presence of MAP2c. Unlike in the case with MAP1b, co-expression of spastin and MAP2c did not alter the length of microtubules, their bundling, or the levels of polymer in any detectable way (figure 3.6, C and D). Similar to the observations in bundled microtubules, unbundled microtubules remained long. This result indicated that it was not the bundling itself that prevented severing. There was no significant lose of microtubule mass from either group ($p > 0.05$).

Figure 3.7 shows quantitative analysis of microtubule mass in fibroblasts induced to overexpress spastin together with MAP1b and MAP2c constructs. Digital grey values of image pixels representing arbitrary fluorescence units (AFUs) were obtained. The abbreviations in the figure are: GFP, GFP alone as control; spastin, human spastin alone; MAP2c, MAP2c alone; MAP2c plus spastin, the cells expressing MAP2c and spastin; MAP1b, MAP1b alone and MAP1b plus spastin, the cells expressing MAP1b and spastin. Y axis shows change in MT fluorescence intensity which is related to the microtubule mass and X axis represents different types of expressed constructs. The quantification results indicated that microtubule polymerization increased in MAP2c expressing cells; as expected, in spastin expressing cells microtubule density was decreased. However, spastin failed to show its severing activity in MAP2c/spastin co-expressing cells.

Although MAP1b is also stabilization protein, results with MAP1b did not correlate with the MAP2c results. Microtubule mass of MAP1b expressing cells was very similar to control cells. There was no microtubule protection observed against severing by spastin in MAP1b cells. Hence, in MAP1b/ spastin co-expressing cells there was dramatic diminution in microtubule mass suggesting that spastin performed its severing activity despite the presence of MAP1b.

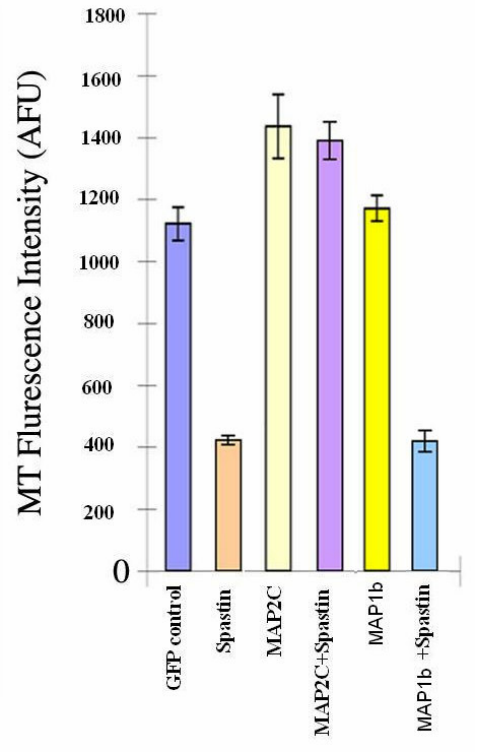


**Figure 3.7:** MT Mass Change Depending on the Expressed Protein

## 4. DISCUSSION

In neurons microtubules are nucleated at the centrosome. However, to be transported from centrosomes down into the axons, microtubules must be severed into short pieces (Ahmad et al., 1994). Specifically, during the formation of collateral axonal brunches, microtubule numbers need to be increased at distal sites of the centrosomes. Hence, it was speculated that long microtubules in the axons might be severed into multiple shorter microtubules at strategic locations such as brunch points (Joshi and Baas, 1993). Since these early studies, the breakage of microtubules at axonal brunch points was visualized by different groups (Dent et al., 1999). Conclusion from all these observations was that microtubule severing is an important phenomenon for regulating microtubule length and number throughout the neuron, as well as for releasing microtubules from centrosome (Dent et al., 1999).

Since most of the studies were performed with p60-katanin, it may be speculated that recently identified severing protein, spastin, based on the great sequence homology with p60–katanin, has same function in the cell as p60-katanin.

Spastin mutation was shown to degenerate corticospinal axons in the neuron. Mutation in spastin might cause degeneration of longer axons by diminishing the supply of short microtubules required for process generations or by the mutant spastin molecules accumulating on the microtubules and impeding their ability to participate in the transport of vesicular cargo (McDermott et al., 2003). This is indicated by the fact that functional spastin does not appear to accumulate on the microtubules, but the mutant spastin does.

There are discrepancies on sub-cellular localization of spastin. Besides the studies reporting cytoplasmic targeting of spastin, studies indicating detection of nuclear localization of spastin are also available (Errico, et al., 2002, 2004; Charvin et al., 2003). Although its function is currently unknown, recently identified spastin variant, short N-terminal truncated isoforms, was shown to have nuclear localization (Claudiani, et al., 2005). In this study, results on hippocampal cells indicated that

spastin overexpression was localized in the cell body of the neuron. There was nearly no spastin observed in the processes of the day 4 fixed cells, however, spastin expression was slightly observed in the processes of day 6 fixed cells. Consistent with these results, independent studies have also found spastin in the cytoplasm and synaptic terminals of pyramidal cells of the cerebral cortex and hippocampus, in Purkinje cells, and in spinal motor neurons (Wharton, et al., 2003). Whereas, our results on another microtubule severing protein, p60-katanin, indicated expression of the protein in the cell body and processes as well. Previous studies also reported p60-katanin to be rich in distal regions of the axon (Karabay et al., 2004). Comparing spastin overexpressing day 4 and day 6 hippocampal cells, there are no distinctive diversities in the expression levels. We can speculate that spastin expression increases during process development stage of the neuron and remain constant after reaching to particular levels in the cell.

Another goal of this study was to ascertain whether spastin increases process branching and if so how the increased levels change comparing with p60-katanin. We found that spastin increased the number of process formation relatively more than p60-katanin. Although the images of results can easily be examined with visual observation, quantification results also confirmed ~50% increase in average process numbers in spastin overexpressing cells compared with control cells and p60-katanin overexpressing cells. Although p60-katanin is also a microtubule severing protein, its overexpression did not yield an increase in the branch formation. There is no clarification indicated for the results on p60-katanin, but one speculation may be different regulation mechanisms of proteins. In neurons, p60-katanin may require sufficient amounts of related proteins such as nerve growth factor, NGF, to be able o show its severing activity *in vitro* or increase in the p60-katanin level may be buffered by certain MAPs. Although there is no observation that p60-katanin co-localizes with another protein, there might be such a protein that acts synergistically with p60-katanin and regulate its activity in neurons.

On the other hand, spastin has microtubule interacting and transmembrane domain both of which lead to the prediction that spastin has a role in membrane trafficking as well. Recent studies have also shown spastin interaction with various proteins that act as spastin binding partners and direct spastin to several membrane sites (Reid et

al., 2005). Although exact localization regions have not been identified yet, it was indicated that spastin co-localizes with atlastin, a protein whose mutation also leads to hereditary spastic paraplegia and with endosomal protein CHMP1B (Reid et al., 2005).

With all of this in mind, it can be concluded that spastin may play a role in local regulation of microtubules, perhaps coupled to the movement of membrane-bound organelles (especially in axons) and/or the remodeling of organelle architecture or even may have a separate role unrelated its microtubule severing function.

Both spastin and p60-katanin are virtually present in the neuron and to be able to prevent microtubules from breaking down into smaller pieces everywhere at all times, severing must be strategically regulated by the cell. 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, so does spastin, might be regulated by the phosphorylation of other proteins. In our case, these proteins were MAPs.

In our study we specifically concentrated on MAP1b and MAP2c. RFL-6 fibroblast cells were chosen in this study because they are very flat and hence excellent for imaging immunostained microtubules and the most importantly MAP4 is the only traditional fibrous MAP endogenously expressed in fibroblasts.

We sought to test whether these MAPs protect microtubules from being severed by spastin. It was indicated that MAP2c provides strong protection of the microtubules against severing by overexpressed spastin. Similar results were shown in the studies done with p60-katanin. Furthermore, the four-repeat form of tau was shown to protect better and have stronger binding affinities to the microtubules than three-repeat form of tau (Qiang et al., 2006). Since MAP2c and tau share similar microtubule binding domains, the level of protection is thought to be related to binding affinity of the MAP to the microtubule.

We think that regulation of microtubule severing by MAPs is based on physically restricted access of the severing protein, in this case spastin, to the microtubule lattice rather than interacting directly with severing protein itself. According to our thought, projection domains in MAP2c and tau provide additional steric hindrance of the access of spastin to the microtubule.

In contrast with MAP2c results, it was shown that MAP1b is not capable of protecting cells against severing by spastin. The difference in protection level of MAP2c and MAP1b is probably based on their different microtubule interacting domains. MAP1b has only one microtubule binding domain whereas MAP2c has three microtubule binding domains plus projection arm as an additional hindrance factor.

In previous studies of our group on katanin, it was tested whether taxol stabilization alters severing response of p60-katanin overexpression. Results indicated that in the presence of taxol, katanin caused microtubules to be broken down into short pieces (Qiang, et. al, 2006). Hence, it can be speculated that protection of microtubules against spastin is MAP2c related rather than stabilization or bundling. Since neuron express excess amounts of MAP2 isoforms in the axon, it can be concluded that MAP2c protects microtubules against severing by spastin in axons.

Baas et al., (2005) proposed a model for katanin-MAPs interaction, which we think is the same for spastin. According to the model in figure 4.1, the microtubule is decorated by MAPs such as tau. The MAPs reduce the possibility of both motor (kinesin)-microtubule and katanin-microtubule interaction. Hence, katanin subunits can not form hexamer around the microtubule to break it and motor cannot move along the microtubule. This would lead long microtubules but insufficient cargo transport.

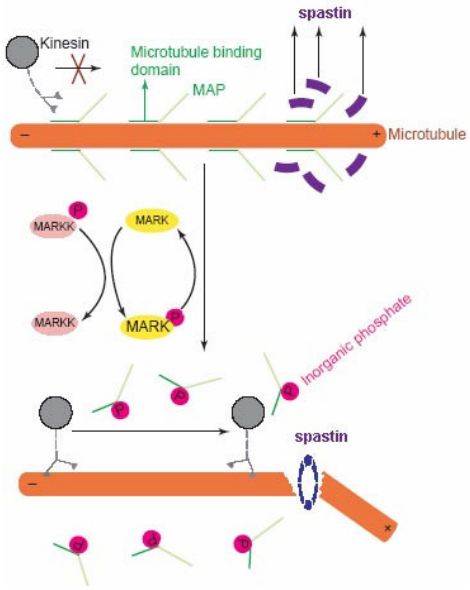


**Figure 4.1** Model for MAP–Microtubule Severing Protein Interaction

Phosphorylation of MARK by MARK kinase causes MARK to phosphorylate the MAP molecule, which dissociates from the microtubule, thereby enhancing the motor-microtubule and katanin-microtubule interaction. In this situation, katanin can break the microtubule, and cargo molecules such as organelles can be transported along the microtubules.

Specifically in Alzheimer disease, tau overexpression impedes motor-based transport, neuron responds to the situation by hyperphosphorylating tau, causing it to dissociate from microtubule lattice. Microtubules become more susceptible to katanin access whereas hyperphosphorylated tau forms abnormal paired helical filaments (Baas et al., 2005).

## 5. CONCLUSION

Microtubules undergo between growth and shrinkage phases continuously. This process is called dynamic instability. This dynamic behavior of microtubules is essential in eukaryotic cells for fundamental processes such as cell division, cell differentiation, and nerve growth. However, the dynamic instability is not enough to explain all microtubule reorganization in the cell, specifically as in the case of process formation in the neurons.

Since microtubule reorganization is thought to be based on microtubule severing via spastin and katanin, in this study we have concentrated on spastin and its possible MAP based regulation mechanism.

Spastin overexpression was performed in hippocampus cells, and to investigate spastin's regulation mechanism, MAP/spastin co-expression studies were performed. Increased branch formation was observed in spastin over-expressing cells, as a result of severing activity of spastin. In MAP based experiments, it was observed that MAP2c protects microtubules from being severed against spastin.

Neurons are terminally differentiated cells, hence degeneration of these cells lead irreversible paralysis of body parts. Regeneration of neurons may be possible with newly branching axons and dendrites. In order to better understand neuro-degenerative disorders like Alzheimer's, it is very important to understand microtubule reorganization mechanism in nerve cells. Since reorganization is thought to be a result of microtubule severing via spastin and katanin, it is important to further analyze both function and regulation of these proteins.

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## APPENDIX

**Table A.1:** Process measurements values of spastin overexpressing cells, day 4 (µm)

| spastin | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DAY 4 | | | | | | | | | | | | |
| cell1 | cell2 | cell3 | cell4 | cell5 | cell6 | cell7 | cell8 | cell9 | cell10 | cell11 | cell12 | |
| 246,61 | 434,18 | 814,47 | 414,08 | 578,15 | 185,93 | 581,58 | 390,97 | 288,67 | 311,37 | 291,78 | 426,29 | |
| 45,8 | 99,45 | 760,76 | 65,97 | 360,59 | 56 | 110,98 | 92,71 | 144,07 | 284,6 | 92,27 | 84,14 | |
| 116,95 | 360,08 | 545,72 | 115,65 | 96,21 | 50 | 246,21 | 1072,65 | 51,31 | 518,22 | 35,65 | 141,91 | |
| 54,14 | 265,85 | 624,23 | 151,15 | 42 | 155,97 | 284,17 | 98,51 | 53,31 | 192,29 | 145 | 390,03 | |
| 50,14 | 159,58 | 178,68 | 470,66 | 105,31 | 125,1 | 202,65 | 255,19 | 97,24 | 134,15 | 24 | 132,89 | |
| 38,63 | 74 | 106,97 | 92 | 47,65 | 38,95 | 295,5 | 432,48 | 66,13 | 129,92 | 127,3 | 241,05 | |
| 64,97 | 124,4 | 69,65 | 74 | 42,97 | 83,65 | 168,91 | 439,12 | 75,8 | 173,73 | 360,84 | 349,71 | |
| 32 | 43,41 | 89,31 | 158,24 | 47,56 | 84,14 | 145,1 | 325,94 | 127,45 | 149,25 | 62,62 | 109,35 | |
| 141,65 | 82,97 | 76,48 | 64 | 28,48 | 196,72 | 142,95 | 238,94 | 136,63 | 160,54 | 429,69 | 133,31 | |
| 68,95 | 277,57 | 53,65 | 83 | 40 | 149,85 | 69,44 | 152,67 | 276,9 | 140,37 | 215,85 | 210,27 | |
| 40,61 | 355,38 | 47,65 | 167,92 | 96,87 | 140,28 | 302,34 | 312,1 | 383,37 | 111,45 | 91,8 | | |
| 65,31 | 572,82 | 34 | 117,78 | 89 | 103,1 | 122,27 | | 65,8 | 199,08 | 252,4 | | |
| 56,14 | 123,93 | | 372,25 | 63,44 | 128,4 | 115,31 | | 209,21 | 44,13 | 300,31 | | |
| 74,97 | 102,25 | | 78 | 76 | 51,57 | | | 75,31 | 48,48 | 299,07 | | |
| 71,9 | 88 | | 72,14 | 44,83 | 149,21 | | | 416,49 | 15,65 | 646,55 | | |
| 67,65 | 121,11 | | 106,48 | 97,1 | 72,61 | | | 437,87 | 44,48 | 193,31 | | |
| 56,47 | 188,28 | | 212,97 | 105,25 | 170,05 | | | 409,5 | 56,14 | 189,08 | | |
| 52,83 | | | 102,27 | 81,78 | 83,8 | | | 55,65 | 42,82 | 66,62 | | |
| 95,31 | | | 591,24 | 26,83 | 347,02 | | | 412,71 | 90,82 | 210,95 | | |
| 85,43 | | | 123,38 | 18 | 164,27 | | | 48,48 | 78,14 | 58,48 | | |
| 113,24 | | | 128,61 | 40,83 | 48,14 | | | 126,83 | 120,97 | 201,94 | | |
| 48,95 | | | 645,07 | | 167,43 | | | 53,65 | | 95,72 | | |
| 78,82 | | | 229,98 | | 95,31 | | | 365,43 | | 214,18 | | |
| 109,31 | | | 130,51 | | 215,1 | | | 254,08 | | 134,78 | | |
| 175,11 | | | 406,48 | | 422,08 | | | 414,86 | | 735,28 | | |
| 97,93 | | | 222,13 | | 129,31 | | | 190,25 | | 133,58 | | |
| 44,97 | | | 115,09 | | 45,65 | | | 866,73 | | 159,09 | | |
| 64,14 | | | 123,44 | | 48,83 | | | 288,61 | | 272,58 | | |
| 78,05 | | | 380,75 | | 60,83 | | | 368,35 | | 250,15 | | |
| 69,31 | | | 96,14 | | 71,8 | | | 112,54 | | 85,65 | | |
| 44 | | | 154,82 | | 26 | | | | | 58,48 | | |
| 26,83 | | | 136,55 | | 95,71 | | | | | 174,88 | | |
| | | | 45,31 | | 76,97 | | | | | 146,66 | | |
| | | | 70,83 | | 71,11 | | | | | 207,55 | | |
| | | | 56,83 | | 124,14 | | | | | 238,39 | | |
| | | | 82,48 | | 107,11 | | | | | 100,13 | | |
| | | | 87,65 | | 88,48 | | | | | 24 | | |
| | | | 143,71 | | 66,62 | | | | | 132 | | |
| | | | 115,44 | | 193,91 | | | | | 206,3 | | |
| | | | 139,6 | | 140,94 | | | | | 98,62 | | |
| | | | 152,07 | | | | | | | | | |
| | | | 113,91 | | | | | | | | | |
| | | | 52,83 | | | | | | | | | |
| | | | 115,14 | | | | | | | | | |
| 2477,12 | 3473,26 | 3401,57 | 7578,55 | 2128,85 | 4832,09 | 2787,41 | 3811,28 | 6873,23 | 3046,6 | 7763,53 | 2218,95 | TOTAL |

**Table A.2:** Process measurements values of p-60 katanin overexpressing cells, day 4 (µm)

| p60 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| DAY 4 | | | | | | | | | | | |
| cell1 | cell2 | cell3 | cell4 | cell5 | cell6 | cell7 | cell8 | cell9 | cell10 | cell11 | |
| 336,81 | 378,67 | 322,28 | 486,21 | 416,18 | 70,83 | 146,77 | 190,64 | 373,35 | 549,28 | 510 | |
| 345 | 333,69 | 219,21 | 428,73 | 85,49 | 83,6 | 197,01 | 76,24 | 262,47 | 259,89 | 169 | |
| 132,4 | 355,63 | 88,62 | 248,65 | 215,17 | 64,25 | 155,03 | 139,65 | 353,28 | 81,1 | 200,7 | |
| 117,8 | 114,97 | 152,27 | 139,57 | 442,28 | 68,97 | 55,31 | 72,4 | 300,68 | 78,48 | 202,3 | |
| 319,7 | 260,17 | 50,65 | 158,8 | | 64,95 | 309,62 | 671,62 | 90,89 | 83,6 | 323,43 | |
| 390 | 463,63 | 162,06 | 113,1 | | 120,05 | 48,83 | 102,74 | 405,87 | 54,8 | 242,01 | |
| 224,48 | 80,47 | 93,91 | 855,12 | | 1003,4 | 43,31 | 99,31 | 261,29 | 67,35 | | |
| 154,75 | 286,44 | 96,83 | 320,34 | | | 42,48 | 78,14 | 181,92 | 63,07 | | |
| 206,33 | 78,97 | 96,72 | | | | 50,14 | 326,87 | 63,44 | 87,78 | | |
| 100,48 | | 607,18 | | | | 722,72 | 104,6 | | 120,6 | | |
| 213,24 | | 132,64 | | | | 90,9 | 183,58 | | | | |
| 113,71 | | 125,81 | | | | 99,45 | 102,97 | | | | |
| 786,63 | | 66,14 | | | | 88,24 | 565,17 | | | | |
| 361,28 | | 126,22 | | | | | | | | | |
| 208,4 | | | | | | | | | | | |
| 122,67 | | | | | | | | | | | |
| | | | | | | | | | | | |
| 4133,68 | 2352,64 | 2340,54 | 2750,52 | 1159,12 | 1476,12 | 2049,81 | 2713,93 | 2293,19 | 1445,95 | 1647,44 | TOTAL |

**Table A.3:** Process measurements values of GFP overexpressing control cells, day 4 (µm)

| GFP | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| DAY 4 | | | | | | | | |
| cell1 | cell2 | cell3 | cell4 | cell5 | cell6 | cell7 | cell8 | |
| 504,27 | 426,21 | 518,85 | 760 | 844,6 | 260,21 | 76,82 | 711,23 | |
| 129,65 | 493,18 | 656,55 | 242,44 | 1166,6 | 368,33 | 194,21 | 679,54 | |
| 98,13 | 760,61 | 196,14 | 195,31 | 518,29 | 137,14 | 127,09 | 742,2 | |
| 49,65 | 516,45 | 206,22 | 428,05 | 319,5 | 463,49 | 140,94 | 366,8 | |
| 104,83 | 150,64 | 182,27 | 125,72 | 177,56 | 61,45 | 247,88 | 84,95 | |
| 155,6 | | 245 | 104,97 | 169,71 | 182,53 | 134,54 | 83,8 | |
| 109,65 | | 119,78 | 107,24 | 126,4 | 90,48 | 174,32 | 169,23 | |
| 117,93 | | 787,45 | 81,44 | | 140,71 | 273,68 | 120,61 | |
| 230,42 | | 452,47 | | | 68,48 | 306,3 | 128,42 | |
| 67,93 | | | | | 242,65 | 145,6 | 49,6 | |
| 56,14 | | | | | 179,37 | 285,6 | 101,81 | |
| 193,4 | | | | | 107,92 | 122,14 | 41,3 | |
| 87,31 | | | | | | | | |
| 38,82 | | | | | | | | |
| 39,31 | | | | | | | | |
| 1983,04 | 2347,09 | 3364,73 | 2045,17 | 3322,6 | 2302,76 | 2229,12 | 3279,49 | TOTAL |

**Table A.4:** Process measurements values of spastin overexpressing cells, day 6 (μm)

| spastin | DAY 6 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| cell1 | cell2 | cell3 | cell4 | cell5 | cell6 | cell7 | cell8 | |
| 785,91 | 425,08 | 958,82 | 199,37 | 374,44 | 243,14 | 865,53 | 132,83 | |
| 108,75 | 238,61 | 373,12 | 203,04 | 371 | 445,3 | 323,88 | 131 | |
| 37,65 | 736,09 | 581,63 | 110,14 | 133,8 | 607,58 | 119,78 | 118,28 | |
| 85,65 | 54,14 | 121,1 | 125,31 | 97,54 | 132,6 | 128,05 | 65,94 | |
| 99,45 | 673,05 | 761,56 | 108,25 | 94,94 | 63,44 | 215,17 | 167,99 | |
| 133,91 | 323,4 | 149,63 | 157,1 | 396,13 | 109,93 | 887,65 | 198,66 | |
| 52,95 | 517,34 | 148,62 | 116,53 | 575,5 | 154,8 | 198,56 | 496,94 | |
| 42,48 | 196,21 | 497,51 | 850,34 | 209 | 152,4 | 105,78 | 869,3 | |
| 221,98 | 169,45 | 107,39 | 136,92 | 494,18 | 34 | 116,4 | 122,97 | |
| 123,8 | 146,21 | 138,33 | 90 | 625,4 | 276,37 | 120,87 | 149,84 | |
| 42,82 | 124,53 | 170,47 | 128,61 | 192,26 | 90,28 | 64,48 | 302,91 | |
| 148,42 | 122,62 | 160,62 | 10 | 236,48 | 139,11 | 86,14 | 106,48 | |
| 456,94 | 150,41 | 150,14 | 94,95 | 213,83 | 281,58 | 84,62 | 54,97 | |
| 162,22 | 116,14 | 88,13 | 88,62 | 249,8 | 346,15 | 97,8 | 130,1 | |
| 290,1 | 288,04 | 130,28 | 51,8 | 191,6 | 110,6 | 210,55 | 110,9 | |
| 273,98 | 114,97 | 73,78 | 53,31 | 775,23 | 67,31 | 38,47 | 60 | |
| 124,48 | 80 | 331,16 | | 158,62 | 59,65 | 569,92 | 71,65 | |
| 104,97 | 123,25 | 122,61 | | 231,31 | 37,31 | 82 | 45,65 | |
| 146,28 | 89,44 | 115,45 | | 89,58 | 88,62 | 150,67 | 261,07 | |
| 117,31 | 101,65 | 57,45 | | 194,5 | 64 | 94,97 | 254,05 | |
| 122,08 | 50,48 | 703,33 | | 72 | 96,97 | 429,57 | 295,25 | |
| 85,7 | 88,48 | 98,05 | | | 26,48 | 157,54 | 130,91 | |
| 305,39 | 106,88 | 94,14 | | | 130,28 | 122,62 | 235,71 | |
| 83,44 | | 100,97 | | | 96,97 | 340,49 | 95,31 | |
| 216,62 | | 170,54 | | | 105,65 | 908,35 | 106,63 | |
| 49,65 | | 94,55 | | | 110,26 | 185,01 | 553,05 | |
| 26 | | 143,38 | | | 64,48 | 297,84 | 146,97 | |
| 46 | | 77,31 | | | 173,1 | 184,24 | 70,61 | |
| | | 64,14 | | | 236,35 | 309,53 | 102,5 | |
| | | | | | 206,61 | 50,83 | 88,84 | |
| | | | | | 124,5 | 223,9 | 155,11 | |
| | | | | | 65,31 | 123,45 | 176,3 | |
| | | | | | 191,7 | 89,11 | 203,44 | |
| | | | | | 223,64 | | 149 | |
| | | | | | 112,58 | | 86,82 | |
| | | | | | 445,81 | | 199,3 | |
| | | | | | 223,6 | | 117,87 | |
| | | | | | 199,58 | | 42,15 | |
| | | | | | 76,62 | | 95,65 | |
| | | | | | 168,62 | | 91,44 | |
| | | | | | 154,04 | | | |
| | | | | | 111,8 | | | |
| | | | | | 73,8 | | | |
| | | | | | 52 | | | |
| | | | | | 105 | | | |
| | | | | | 99,44 | | | |
| **4494,93** | **5036,47** | **6784,21** | **2524,29** | **5977,14** | **7179,36** | **7983,77** | **6994,39** | **TOTAL** |

**Table A.5:** Process measurements values of p-60 katanin overexpressing cells, day 6 (μm)

| **p60** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **DAY 6** | | | | | | | | | |
| **cell1** | **cell2** | **cell3** | **cell4** | **cell5** | **cell6** | **cell7** | **cell8** | **cell9** | |
| 205,31 | 293,11 | 447,46 | 137,45 | 224,22 | 205,05 | 937,04 | 516,37 | 558,9 | |
| 112,2 | 144 | 191,45 | 116,13 | 395,84 | 138,42 | 69,8 | 540,068 | 500 | |
| 169,8 | 117,65 | 46 | 190,55 | 96 | 103,72 | 113,8 | 276,47 | 251 | |
| 108,8 | 46,48 | 92,62 | 56,62 | 928,37 | 577 | 441,56 | 550,38 | 318,02 | |
| 221,8 | 79,8 | 437,1 | 136,48 | 70,03 | 53 | 1017,97 | 163,58 | 70,47 | |
| 74,8 | 177,94 | 62,14 | 608,33 | 73,21 | 168,83 | 718,61 | 517,51 | 484,06 | |
| 58,48 | 144,28 | 69,94 | 28 | 56,13 | 262,41 | 575,42 | 599,96 | 140,95 | |
| 106 | 91,31 | 204,62 | 62,83 | 967,22 | 692,58 | 326,82 | 476,42 | 93,08 | |
| 124,14 | 168,97 | 764,18 | 219,78 | 87,24 | 150,83 | 126,62 | 359,36 | 75,65 | |
| 147,94 | 150,97 | 92,57 | 616,92 | 455,12 | 603,16 | 84,27 | 96,62 | 384,18 | |
| 597,27 | 63,44 | 254,87 | 114,14 | 866,32 | 165,18 | | | 85,79 | |
| 679,11 | 77,65 | 225,54 | 89,43 | | 775,26 | | | 20 | |
| | 77,65 | 156,91 | 208,34 | | 206,04 | | | 544,46 | |
| | 185,11 | 170,27 | | | 228,48 | | | 104,2 | |
| | 37,65 | 81,2 | | | 132,14 | | | 36 | |
| | 49,31 | 161,31 | | | 44,95 | | | 72,62 | |
| | 201,45 | 179,58 | | | 934,77 | | | 56 | |
| | 207,57 | 169,44 | | | 333,98 | | | 26 | |
| | 46,82 | 121,45 | | | 336,55 | | | 45,65 | |
| | 60 | 75,3 | | | 190,89 | | | 39,3 | |
| | | | | | 108,28 | | | 346,35 | |
| | | | | | 125,93 | | | 38 | |
| | | | | | | | | 577,61 | |
| | | | | | | | | 83,22 | |
| | | | | | | | | 63,78 | |
| **2605,65** | **2421,16** | **4003,95** | **2585** | **4219,7** | **6537,45** | **4411,91** | **4096,73** | **5015,29** | **TOTAL** |

**Table A.6:** Process measurements values of GFP overexpressing control cells, day 6 (µm)

| **day 6** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **GFP** | | | | | | | | | |
| **cell1** | **cell2** | **cell3** | **cell4** | **cell5** | **cell6** | **cell7** | **cell8** | **cell9** | |
| 988,41 | 328,14 | 905,038 | 425,56 | 752,52 | 626,73 | 649,71 | 880,51 | 1165,41 | |
| 579,38 | 150,92 | 99,24 | 327,97 | 89,59 | 272,06 | 87,52 | 613,28 | 990,95 | |
| 136,87 | 210,69 | 188,28 | 341,93 | 76,14 | 76,05 | 26 | 153,97 | 217,78 | |
| 141,02 | 270,99 | 132 | 320,03 | 138,63 | 310,79 | 57,92 | 126,39 | 916,76 | |
| 153,5 | 26 | 195,14 | 140,99 | 206,74 | 211,07 | 528,74 | 127,25 | 74,6 | |
| 606,5 | 229,31 | 184,88 | 269,94 | 132,41 | 181,47 | 386,7 | 120,17 | 103,58 | |
| 162,3 | 187,22 | 109,48 | 163,11 | 85,78 | 264,71 | 568,04 | 102,62 | 100,97 | |
| 97,21 | 122,33 | 105,45 | 754,39 | 174,33 | 556,65 | 231,08 | 169,68 | 131,22 | |
| 124,74 | 437,85 | 63,78 | 892,39 | 407,17 | 455,17 | 261,68 | 115,11 | | |
| 181,07 | 274,97 | 87,09 | 515,72 | 67,65 | 538,63 | 100,14 | 109,24 | | |
| 105,6 | 191,81 | 105,45 | | 129,45 | 60,25 | 61,91 | | | |
| | 204,04 | 63,78 | | 110,05 | 268,37 | 237,83 | | | |
| | 434,2 | 87,09 | | 108,04 | 240,16 | 166,8 | | | |
| | 251,017 | | | 87,43 | 58,13 | 71,1 | | | |
| | 229,25 | | | 123,11 | 153,93 | | | | |
| | 170,04 | | | 96,75 | 82 | | | | |
| | 308,34 | | | | 71,14 | | | | |
| | 76,97 | | | | 103,06 | | | | |
| | 71,54 | | | | | | | | |
| | 211,01 | | | | | | | | |
| **3276,6** | **4386,63** | **2326,69** | **4152,03** | **2785,79** | **4530,37** | **3435,17** | **2518,22** | **3701,27** | **TOTAL** |

**Table A.7:** Measurements values of MAP experiments

| MAP2c/spastin | MAP2c | spastin | GFP control | MAP1b | MAP1b/spastin |
|---|---|---|---|---|---|
| 831,38 | 541,08 | 404,41 | 446,82 | 1720,97 | 522,62 |
| 533,89 | 861,1 | 381,39 | 425,67 | 1573,22 | 570,98 |
| 1004,84 | 953,15 | 387,02 | 450,55 | 1130,97 | 755,54 |
| 712,28 | 1134,73 | 433,71 | 509,86 | 1279,6 | 501,01 |
| 1007,58 | 1598,83 | 390,12 | 492,08 | 1934,62 | 566,83 |
| 851,68 | 1019,8 | 376,46 | 710,68 | 1290,62 | 359,56 |
| 916,14 | 1016,32 | 432,92 | 771,2 | 1355,34 | 487 |
| 844,19 | 1362,49 | 432,6 | 883,95 | 1616,11 | 511,74 |
| 1118,81 | 983,85 | 561,77 | 701,62 | | 511,04 |
| 658,12 | 1409,53 | 468,24 | 801,09 | | |
| 642,47 | 1132,54 | 353,03 | | | |
| 947,56 | 847,05 | 427,34 | | | |
| 1129,43 | 1685,71 | 447,2 | | | |
| 1167,97 | 821,93 | | | | |
| 1869,5 | | | | | |
| 1223,62 | | | | | |
| 1471,79 | | | | | |
| 796,54 | | | | | |
| 1156,65 | | | | | |
| 1100,4 | | | | | |
| 1363,89 | | | | | |
| 1201,45 | | | | | |
| 1167,97 | | | | | |
| 1689,73 | | | | | |
| 1038,95 | | | | | |
| 1213,67 | | | | | |

| MAP2c/Hsp | | map2c | | spastin | |
|---|---|---|---|---|---|
| Mean | 1063,865 | Mean | 1097,722 | Mean | 422,7854 |
| Standard Error | 60,90478 | Standard Error | 85,10018 | Standard Error | 14,70377 |
| Median | 1069,675 | Median | 1018,06 | Median | 427,34 |
| Standard Deviation | 310,5546 | Standard Deviation | 318,4157 | Standard Deviation | 53,01519 |
| **GFP control** | | **MAP1b** | | **MAP1b+Hsp** | |
| Mean | 619,352 | Mean | 1487,681 | Mean | 531,8133 |
| Standard Error | 54,27996 | Standard Error | 94,95081 | Standard Error | 34,63322 |
| Median | 605,74 | Median | 1464,28 | Median | 511,74 |
| Standard Deviation | 171,6483 | Standard Deviation | 268,5615 | Standard Deviation | 103,8996 |

**RESUME**

Şirin Korulu was born in Bulgaria in 1980. After getting her high school diploma from Bursa Boys High School in 1999, she has continued her undergraduate degree at Istanbul Technical University, Department of Molecular Biology and Genetics in 1999. She had her Bachelor degree in 2003. She has continued to her graduate studies at Advanced Technologies in Molecular Biology – Genetics and Biotechnology program in the Istanbul Technical University. She has been also working as a research assistant in Department of Molecular Biology and Genetics. She is still pursuing her studies in the same department.