ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

THE INTERACTION BETWEEN ELK-1 AND MICROTUBULES IN NEURONS

M.Sc. Thesis by
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Department : **Advanced Technologies**
Programme: **Molecular Biology, Genetics and Biotechnology**

JANUARY 2008

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

ELK-1 PROTEİNİNİN MİKROTÜBÜL İLE ETKİLEŞİMİNİN SİNİR HÜCRELERİNDE ARAŞTIRILMASI

YÜKSEK LİSANS TEZİ

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PREFACE

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ABBREVIATIONS

MAP	: Microtubule Associated protein
GTP	: Guanosinetriphosphate
NF-L	: Neurofilament-Light Chain
NF-M	: Neurofilament-Medium Chain
NF-H	: Neurofilament-Heavy Chain
ETS	: E-Twenty Six
HTH	: Helix-Turn-Helix
TCF	: Ternary Complex Factor
SRF	: Serum Response Factor
MAPK	: Mitogen Activated Protein Kinase
SRE	: Serum Response Element
NGF	: Nerve Growth Factor
ERK	: Extracellular Regulated Kinase
MLK	: MAP Kinase Kinase Kinase
KIF3	: Kinesin Family
NLS	: Nuclear Localization Sequence
JIP	: JNK Interacting Protein
DMEM	: Dulbecco's Modified Eagle Medium
HBSS	: Hank's Based Salt Solution
FBS	: Fetal Bovine Serum
PBS	: Phosphate Buffered Saline
IGF	: Insulin Lİke Growth Factor
GST	: Glutathione S-Transferase
SDS	: Sodium Dodecyl Sulphate
BSA	: Bovine Serum Albumin
ECL	: Enhanced Chemiluminescence
pNPP	: Phospho Nitro Phenyl Phosphate

THE INTERACTION BETWEEN ELK-1 AND MICROTUBULES IN NEURONS

SUMMARY

Elk-1, which a member of TCF family of ETS domain transcription factors, functions in various cellular mechanisms. Upon different extracellular stimuli, Elk-1 can direct the cell to appropriate fates such as proliferation, differentiation or just survival. Because Elk-1 is a transcription factor, it functions by interacting with DNA in the nucleus on its target promoters. Previous studies have shown that Elk-1 activation is controlled by different signalling pathways, but ERK 1/2 mediated activation of Elk-1 appears to be critical.

Although Elk-1 was known to function mainly in highly proliferative cells by regulating immediate early gene expression followed by cell cycle entry previous studies have shown that Elk-1 was present in post-mitotic neurons, in different regions of brain tissue of adult rat. Due to the fact that neurons are known to be largely post-mitotic, it is very intriguing to observe Elk-1 in neurons. Since Elk-1 is a transcription factor, it would be expected to be in the nucleus and perhaps the cytoplasm. However, both Elk-1 and phospho-Elk-1 was found to be present in the axonal regions, indicating that this protein may have an alternative function or functions in neurons.

Although the effects of microtubules on the spatial organization of signal transduction is studied for years, there are still some mechanisms and components which are not well understood. The discovery of signalling molecules which can interact with microtubules or with other members of cytoskeleton is very intriguing perhaps these molecules transmit signals related to growth, stress or even differentiation to the appropriate parts in the cell mostly by the motor proteins which are the cargo transporters of the cell. To date, the retrograde transport of two important signalling molecules, namely ERK 1/2 and JNK, has been shown.

In this study, we have decided to study Elk-1 protein and its possible interaction with microtubules. As mentioned before, Elk-1 is found abundantly both in the cell body and as well as dendrites and axons of neurons. The interaction of other signalling molecules or transcription factors with cytoskeleton and specifically with microtubules has led us to first consider the presence of such an interaction between Elk-1 and microtubules. In this study, we used both biochemical and cytological techniques to determine initially the co-localization of Elk-1 and microtubules in different neuronal systems. We have then performed biochemical and molecular assays to analyze whether this interaction between Elk-1 and microtubules is direct and specific.

ELK-1 PROTEİNİNİN MİKTOTÜBÜL İLE ETKİLEŞİMİNİN SİNİR HÜCRELERİNDE ARAŞTIRILMASI

ÖZET

ETS transkripsiyon faktörlerinin TCF alt grubu üyesi olan Elk-1 hücre içinde birçok mekanizmada yer almaktadır. Hücre dışından gelen değişik uyarılara karşı, Elk-1 hücreyi farklı akıbetlere yönlendirebilir. Bunların başlıcaları, hücre bölünmesi ve çoğalması, farklılaşma ve hayatta kalmasıdır. Elk-1 transkripsiyon faktörü olduğu için, kendi hedef genlerinin promotör bölgelerinde DNA ile interaksiyona geçer. Son yapılan araştırmalar Elk-1 aktivitesinin ERK1/2 yolağı tarafından kontrol edildiğini göstermektedir.

Elk-1'in çoğunlukla aktif olarak bölünen hücrelerde fonksiyonel olduğu bilinmektedir. Bu hücre tiplerinde, hücrenin mitoza girmesini sağlayan genlerin ekspresyonlarını regüle etmektedir. Ancak son yapılan araştırmlarda Elk1'in bölünmesi durmuş olan sinir hücrelerinde de bulunduğu anlaşılmıştır. Özellikle bu hücrelerde Elk-1 sadece çekirdek ve somada değil, akson ve dendrit boylarında da gözlemlenmiştir.

Diğer taraftan mikrotübüller temel olarak hücredeki molekül ve organel taşınması için yolların yapımı, mitoz esnasında iğ iplikçiklerinin oluşumu ve hücrenin hareti için gerekli olan yapılardır. Ancak mikrotübüllerin bu dinamik görevlerinin dışında hücre içi sinyal iletim mekanizmalarıyla da direkt bağlantısı vardır. Özellikle Elk-1'in aktivatör proteinleri olan ERK 1/2 ve JNK proteinleri mikrotübüllerle etkileşime geçebilmekte ve bu etkileşime bağlı olarak hem kendi lerinde hem de mikrötübüllerde önemli değişiklikler meydana gelmektedir.

Bu çalışmada, Elk-1 proteininin hücrenin iskeletini oluşturan mikrotübül yapısıyla olan etkileşimi sinir hücrelerinde moleküler ve biyokimyasal yöntemler uygulanarak araştırılmıştır. Öncelikle bu iki proteinin, konfokal mikroskobu tekniği kullanılarak hücrenin içinde yerleri belirlenmiş ve ayni bölgelerde lokalize oldukları görülmüştür. Sonrasında yapılan *in vitro* ve *in vivo* bağlanma deneylerinde ise Elk-1 ve mikrotübül arasında direkt interaksiyon olduğu tespit edilmiştir. Daha sonra ise Elk-1 proteininin mikrotübüle bağlanma bölgesinin belirlenmesi amacıyla delesyen mutantları kullanılarak çökertme deneyleri tasarlanmış ve sonuç olarak Elk-1'in değişik bölgelerinin mikrötübüle bağlanma yatkınlığının olduğu bulunmuştur. Elde ettiğimiz bulgular mikrotübüllerin Elk-1 ile interaksiyona geçebildiğini göstermektedir.

1. INTRODUCTION

1.1 Neurons

The human body is made up of trillions of cells. Cells of the nervous system, called nerve cells or neurons, are specialized to carry messages through an electrochemical process (Figure 1.1). Neurons are highly specialized for the processing and transmission of cellular signals. The soma or cell body is the central part of the neuron (Figure 1.1 c). It contains the nucleus of the cell, and therefore is where most protein synthesis occurs. The dendrites of a neuron are cellular extensions with many branches, and metaphorically this overall shape and structure is referred to as a dendritic tree (Figure 1.1. b). This is where the majority of input to the neuron occurs. The axon is a finer, cable-like projection which can extend tens, hundreds, or even tens of thousands of times the diameter of the soma in length (Figure 1.1 a). The axon carries nerve signals away from the soma. Many neurons have only one axon, but this axon may undergo extensive branching, enabling communication with many target cells (Purves et al., 2001).

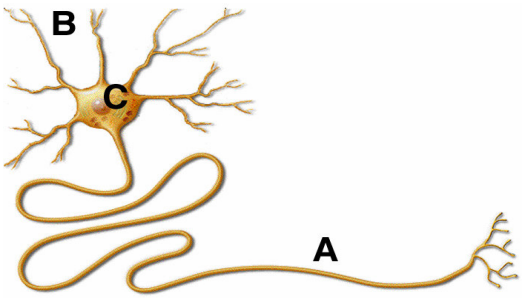

Figure 1.1: Structure of a neuron. **A** axon, **B** dendrites and **C** cell body (URL-1).

1.2 Neuronal Cytoskeleton

The organization of cells in space and their interactions with the environment are fundamental. Cells should have a correct shape and a proper internal structure. This property will allow them to rearrange their internal components

and to adapt to changing environmental conditions. These spatial and mechanical functions depend on a well organized system in eucaryotic cells called cytoskeleton.

Cytoskeleton is composed of three families of protein molecules, which in turn assemble to generate three main types of filaments that have distinct mechanical and dynamic characteristics. 1, microtubules provide roads of intracellular transport and function in the chromosome segragation during mitosis. 2, intermediate filaments give mechanical strength to reduce the environmental stress on the cell. 3, actin filaments or microfilaments detemine the shape of the cell and function in the movement of the cell, if necessary.

Although the filamentous structures are the main components of the cytoskeleton, their functions and assembly-disassembly dynamics are controlled by another set of proteins called "accessory proteins". These proteins include **M**icrotubule **A**ssociated **P**roteins (MAP) and certain signalling proteins of which functions remain to be understood (Alberts et al., 2002).

1.2.1 Microtubules

Microtubules are long and hollow cylinders that have 25 nm diameter. They are composed of tubulin protein. Tubulin has 2 different monomers which are α- and β-tubulin. The tubulin dimers can bound to each other by non-covalent bonds and this generates microtubule structure. The tubulin subunits come together to form protofilaments, and 13 parallel protofilaments form the microtubule filament (Figure 1.2). α- and β-tubulin monomers contain three domains. These include the N-terminal nucleotide-binding domain and intermediate domain connected to it by H7 helix. These two domains are involved in polymerization, however the C-terminal domain containing two anti-parallel helices is exposed on the outside to define the protofilament crest.

Microtubules have a very dynamic character. They switch between growing and shrinking phases, which are actually essential for their function and requires the binding, hydrolysis and exchange of nucleotides (Alberts et al., 2002).

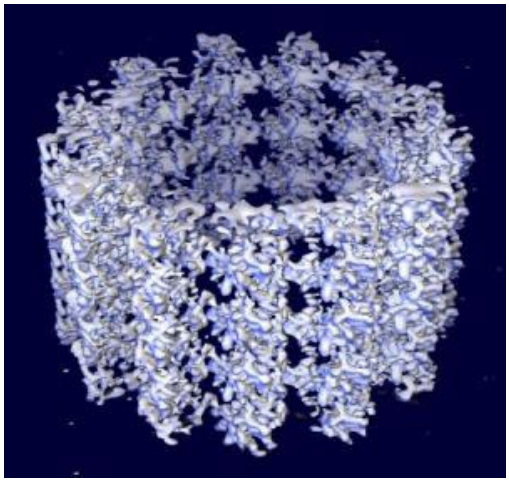

Figure 1.2: The structure of Microtubule. (URL-2)

In tubulin dimer, non-exchangeable GTP is found at the N terminal site of α-tubulin, whereas the exchangeable nucleotide of β-tubulin is on the surface of the dimer which determines polymerization or depolymerization state. Hydrolysis of β-tubulin GTP makes the microtubule energetically unstable, which in turn causes dynamic instability (Figure 1.3). According to GTP cap model, the microtubule structure is stabilized by a layer of GTP-tubulin subunits at the ends and when lost, it causes rapid depolymerization (Alberts et al., 2002). Microtubule structure or stability is supported by a class of proteins called Microtubule Associated Proteins or briefly MAPs. Most abundant MAPs in the mammalian systems are tau, MAP1, MAP2 and MAP4.

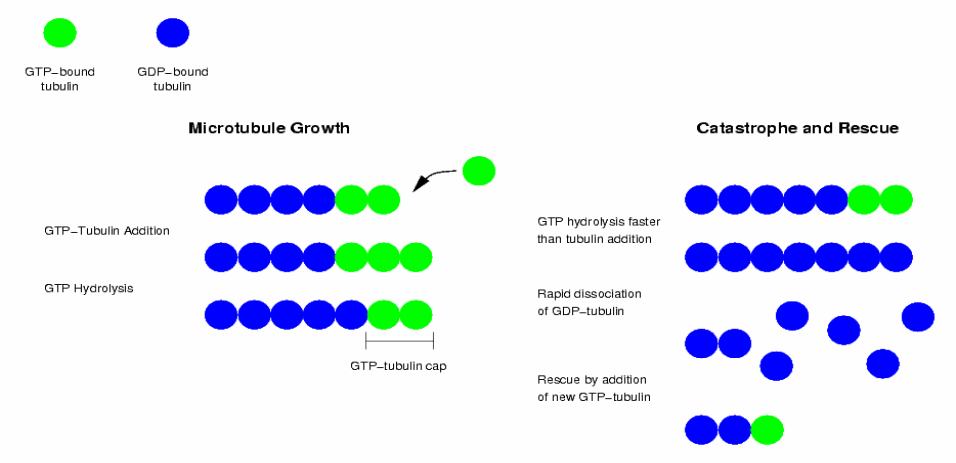

Figure 1.3: Microtubule Polymerization and Depolymerization. (URL-3)

1.2.2 Neurofilament Proteins

Neurofilament proteins constitute a major intermediate filament component of the neuronal cytoskeleton. They are organized as 10 nm thick filaments in axons and dendrites. They are composed of three polypeptide subunits which are identified by their molecular weights as low, NF-L (68 kDa), medium, NF-M (150 kDa), high, NF-H (200 kDa). These polypeptides assemble in a 5:3:1 (NF-L:NF-M:NF-H) ratio and generate the filamentous structure running the length of the axon (Figure 1.4). Side-arms protrude from the filament and interact with neighboring structures and with other filaments. They are also modified by post-translational changes that are regulated in a complex fashion (Al-Chalabi and Miller, 2003).

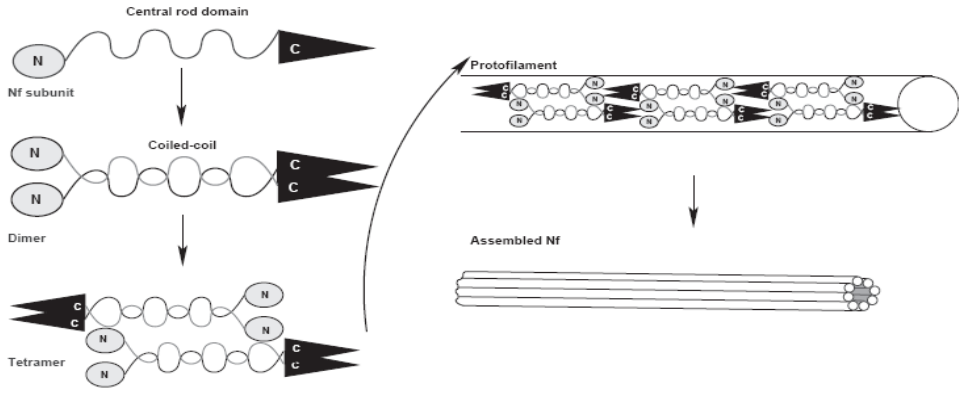

Figure 1.4: The formation of neurofilament assembly (Petzold, 2005)

Neurofilaments generate the neuronal cytoskeleton together with other cytoskeletal components such as microtubules and actin. However, among these cytoskeletal proteins only neurofilaments are unique to neurons. In the previous studies, it has been shown that neurofilaments constitue up to 13% of the total protein but 54% of the total insoluble protein (Morris and Lasek, 1982). Because neurofilaments are highly stable proteins, they have important structural roles in axonal and dendritic branching and promotion of axonal growth and thickening (Leterrier et.al., 1996). Besides these structural functions, neurofilaments can also participate in the dynamic properties of axonal cytoskeleton during neuronal differentiation, axon outgrowth and guidance (Nixon and Shea, 1992).

They are mostly found in phosphorylated form due to their numerous phosphorylation sites that are organized as repeat motifs, and this phosphorylation is dynamic (Grant and Pant, 2000). They are phosphorylated on their amino-terminal head domains and this mechanism regulates the neurofilament assembly (Pant et.al., 2000). Head phosphorylation by protein kinase A and C occurs at many distinct sites in NF-L and NF-M to inhibit neurofilament assembly or leads to disassembly during thinning of axonal diameter (Cleverley et.al., 1998, Hisanaga et.al., 1994). Moreover, there are many KSP (Lysine-Serine-Proline) phosphorylation motifs at the carboxy-terminal side arm domains of NF-M and NF-H (Xu et.al., 1992, Elhanany et.al., 1994) . These phosphorylation sites is believed to be a controlling region for regulating interfilament spacing and axonal calibre (Nixon et.al., 1994), (Figure 1.5). Another function of tail domain phosphorylation is to regulate the conduction velocity in large axons. There is a linear correlation between the rate of myelination and degree of phosphorylation in side arms (Cole et.al., 1994). Although there are many phosphorylation sites on NF proteins, axonal phosphorylation of NFs is a slow process *in vivo* because of the slow transport of NF proteins into the axon, delayed activation of local kinases or inaccessibility of phosphate acceptor sites, because of a closed conformation of assembled polymers (Jung et.al., 2000).

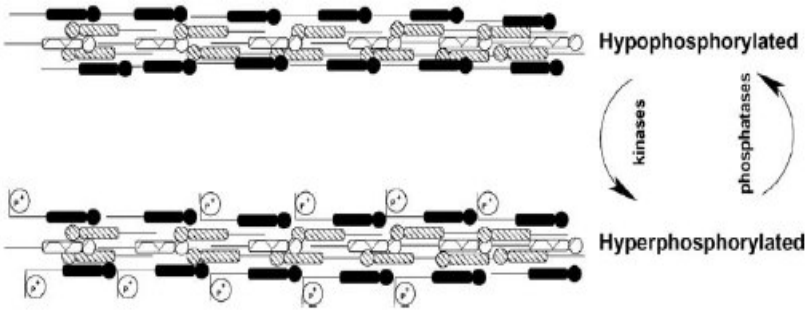

Figure 1.5: Control of phosphorylation on the neurofilament rod (Petzold, 2005)

1.2.3 Actin Filaments

Actin is an abundant protein which polymerizes to form actin filaments or microfilaments, that have approximately 7 nm diameter and several micrometers in length. Actin filaments are organized into higher order structures, forming bundles or three-dimentional networks. The assembly and disassembly of actin filaments, their crosslinking into bundles and networks, and their association with other cell structures are regulated by a variety of actin-binding proteins, which are critical components of the actin cytoskeleton. Actin filaments are particularly abundant beneath the plasma membrane, where they form a network that provides mechanical support, determines cell shape, and allows movement of the cell surface, thereby enabling cells to migrate, engulf particles, and divide (Cooper et al., 2000).

Individual actin molecules are globular proteins of 375 amino acids (43 kd). Each actin monomer has tight binding sites that mediate head-to-tail interactions with two other actin monomers, so actin monomers polymerize to form filaments (Figure 1.6). The first step in actin polymerization is the formation of a small aggregate consisting of three actin monomers which is called nucleation. Actin filaments are then able to grow by the reversible addition of monomers to both ends, but one end elongates five to ten times faster than the minus end. The actin monomers also bind ATP, which is hydrolyzed to ADP following filament assembly. Although ATP is not required for polymerization, actin monomers to which ATP is bound polymerize more readily than those to which ADP is bound.

As discussed below, ATP binding and hydrolysis play a key role in regulating the assembly and dynamic behavior of actin filaments (Cooper et al., 2000).

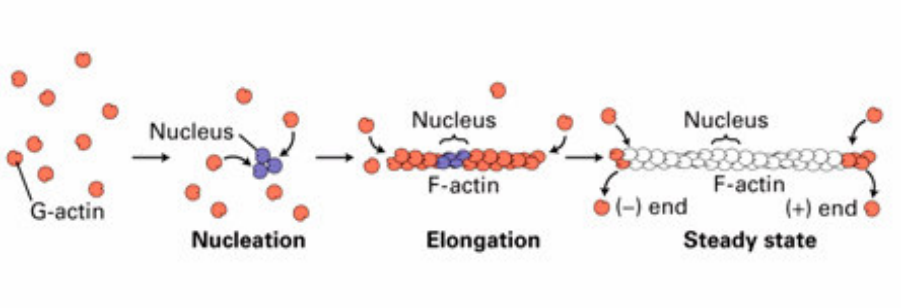

Figure 1.6: Polymerization of actin filaments (URL-4)

Within the cell, both the assembly and disassembly of actin filaments are regulated by actin-binding proteins. The turnover of actin filaments is about 100 times faster within the cell than it is *in vitro*, and this rapid turnover of actin plays a critical role in a variety of cell movements. The key protein responsible for actin filament disassembly within the cell is cofilin, which binds to actin filaments and enhances the rate of dissociation of actin monomers from the minus end. In addition, cofilin can sever actin filaments, generating more ends and further enhancing filament disassembly (Cooper et al., 2000).

Cofilin preferentially binds to ADP-actin, so it remains bound to actin monomers following filament disassembly and sequesters them in the ADP-bound form, preventing their reincorporation into filaments. However, another actin-binding protein, profilin, can reverse this effect of cofilin and stimulate the incorporation of actin monomers into filaments (Figure 1.7). Profilin acts by stimulating the exchange of bound ADP for ATP, resulting in the formation of ATP-actin monomers, which dissociate from cofilin and are then available for assembly into filaments. Arp2/3 proteins, on the other hand, can serve as nucleation sites to initiate the assembly of new filaments, so cofilin, profilin, and the Arp2/3 proteins can act together to promote the rapid turnover of actin filaments and remodeling of the actin cytoskeleton which is required for a variety of cell movements and changes in cell shape (Cooper et al., 2000).

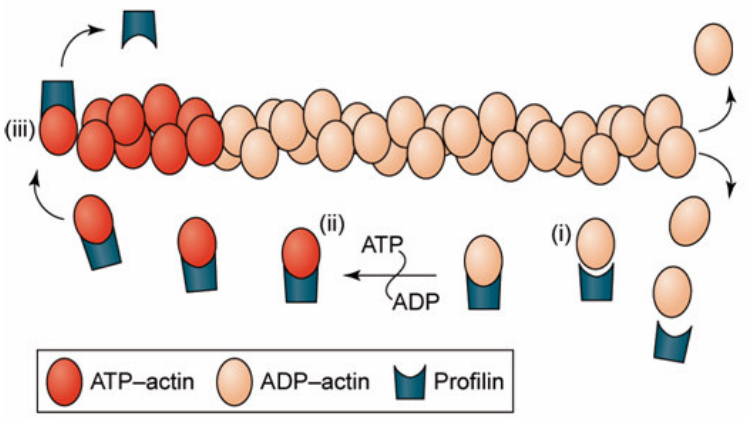

Figure 1.7: Profilin facilitates filament formation (URL-4).

1.3 ETS Domain Transcription Factors

Transcriptional activation of eucaryotic genes during development or in response to extracellular signals involves the regulated assembly of multiprotein complexes on enhancers and promoters. Because of the complex nature of these processes, unlimited possibilities for regulation are found in the cell. Sequence-specific transcription factors are the central players in this process by selecting the genes to be activated and by generating a highly specific response which is actually determined by the arrangement of DNA-binding sites and protein-protein interactions.

ETS (**E**-**T**wenty **S**ix)-domain transcription factor family is involved in different cellular processes such as proliferation, differentiation, immune response and apoptosis. Members of this family were originally identified on the basis of a region of primary sequence homology with the protein product of the v-ets oncogene encoded by the E26 avian erythroblastosis virus (Karim et.al, 1990). The conserved region, termed the ETS-domain, corresponds to the DNA-binding domain of these proteins. ETS-domain transcription factors can be further subclassified primarily because of the high amino acid conservation in their ETS-domains and in addition, the conservation of other domains and motifs (Figure 1.8). These subfamilies include ETS, ERG, PEA-3 and TCF.

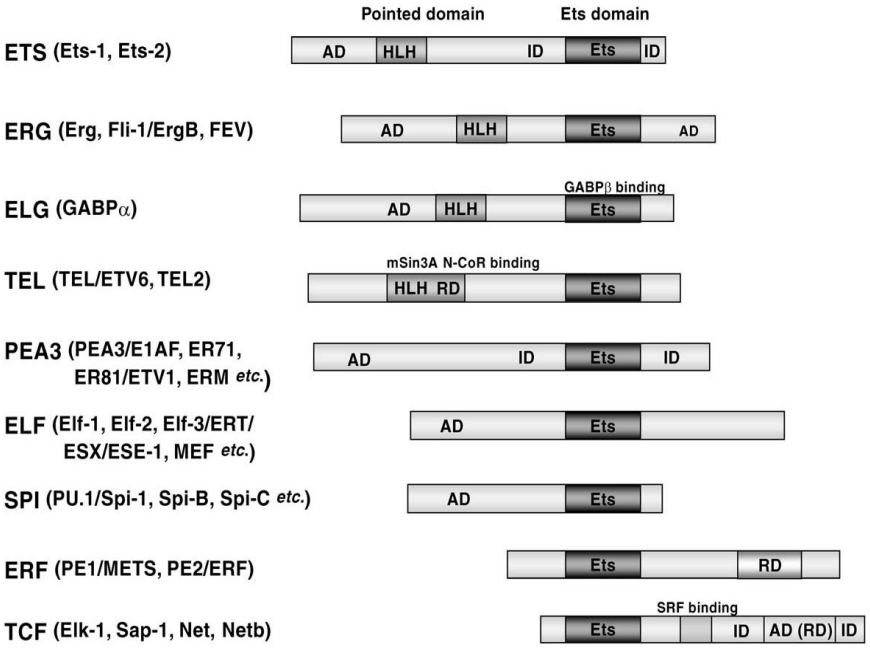

Figure 1.8: Domain similarities of ETS-domain transcription factors (Oikawa and Yamada, 2003).

All known ETS-domain transcription factors bind to sites containing a central "GGA" trinucleotide motif (Figure 1.9). The residues flanking this motif show whether a particular ETS-domain will bind the site. The ETS DNA-binding domain is characterized by the presence of three conserved tryptophans separated by 17-21 amino acids (Karim et al., 1990). Owing to its lack of sequence similarity to other known DNA-binding domains, the ETS-domain was initially thought to represent a novel structural DNA binding motif. However, after revealing of three-dimensional structure of three members of the family, Fli-1 (Liang et al., 1994), Ets-1 (Donaldson et al., 1996) and PU.l/Spi-1 (Kodandapani et al., 1996), the ETS-domain was placed in the winged helix-turn-helix (HTH) superfamily of DNA-binding proteins. Due to high amino acid conservation amongst the ETS-domain of individual family members, this structural motif and mode of DNA binding are likely to be conserved in all ETS domain proteins.

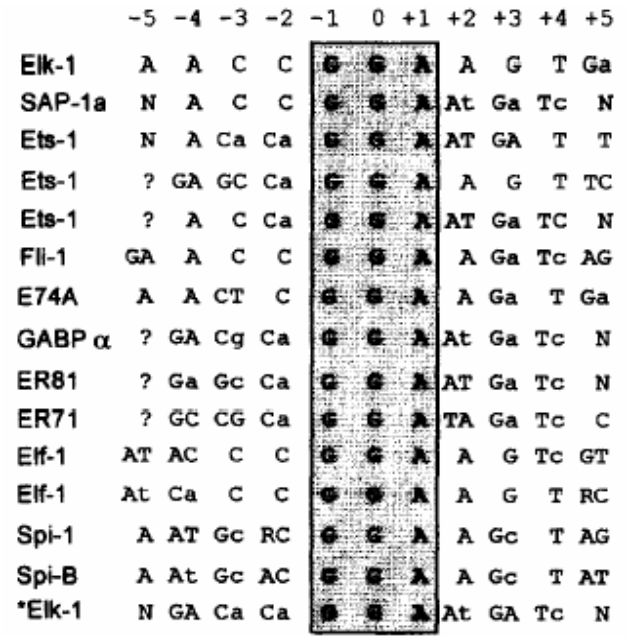

Figure 1.9: DNA-recognition sites of different ETS-domain transcription factors.

A variety of growth modulators affect ETS activity, including growth factors, non-nuclear oncoproteins, activators of protein kinase C, intracellular calcium, and cyclosporin. They intervene at various levels, including post-translational modification, gene transcription and protein stability (Oikawa and Yamada, 2003). ETS domain proteins can be either transcriptional activators or repressors. Their targeting to promoters is promoted by specific DNA-protein and protein-protein interactions. In most cases, the transcription factors are activated by extracellular stimuli and the signal is conducted to transcription factors by signal transduction pathways (Sharrocks et.al., 1997).

1.3.1 TCF Family

TCF (**T**ernary **C**omplex **F**actor) family is the most studied of ETS factors. The first TCF was identified in the nuclear extract of HeLa cells which formed a ternary complex with SRF (**S**erum **R**esponse **F**actor) on the *c-fos* promoter (Figure 1.10). This novel protein was called p62 due to its 62 kDa molecular weight (Shaw et.al., 1989). Then, it has been shown to be homologous to Elk-1 (Ets-like 1) protein which was previously described (Hipskind et al., 1991). Later, two homologous proteins of

Elk-1, Sap1 (Dalton and Treisman, 1992) and Net/Sap2 have been identified (Giovane et al., 1994).

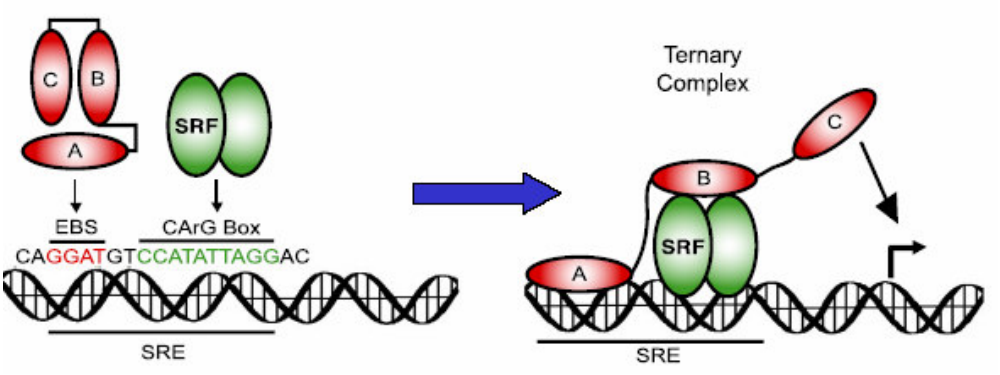

Figure 1.10: Ternary Complex Factor Structure on the SRE site (Buchwalter et al., 2004)

1.3.2 Structure of TCFs

TCFs have 4 different regions that they share. These are known as A,B,C and D domains, each of them having distinct functions (Figure 1.11). The N-terminal A domain corresponds to the ETS DNA binding domain. It has also been demonstrated to act as a transcriptional inhibitor in Elk-1 by recruiting corepressors (Yang et al., 2001) and DNA binding inhibitors (Yates et al., 1999), and to contain a nuclear export signal in Net (Ducret et al., 1999). The B domain interacts with the MADS box transcription factor family member SRF and allows ternary complex formation (Shore and Sharrocks, 1994). The C-terminal C domain is an activation domain that is activated by phosphorylation by **M**itogen-**A**ctivated **P**rotein **K**inases (MAPK) (Janknecht et al., 1993; Marais et al., 1993; Gille et al., 1995). It contains multiple S/T-P MAP kinase phosphorylation sites. The D domain is a docking site for MAP kinases (Sharrocks et al., 2000) as well as a nuclear localization signal in Net. The (FXFP motif) is an additional MAP kinase docking site with different binding properties.

The presence of distinct docking sites in the TCFs generates a modular system, which allows complex integration of the signals from different MAP kinase

pathways. The R motif, specifically found in Elk-1 (Yang et al., 2002), acts as a repressor domain that dampens the activity of the C-terminal activation domain.

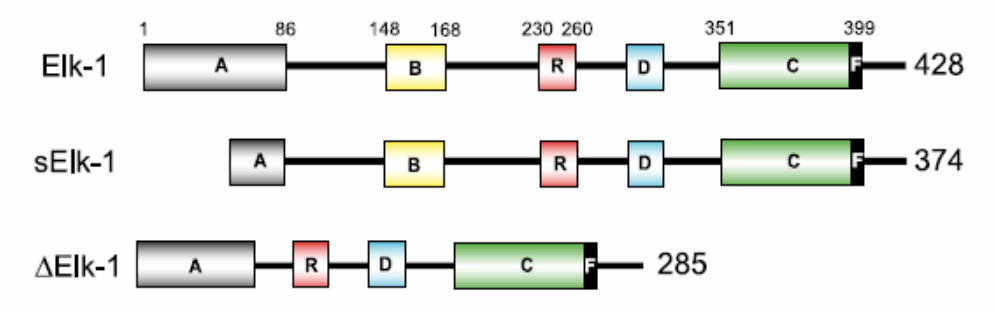

Figure 1.11: Domains of different splice variants of Elk-1 (Buchwalter et al., 2004)

Although TCFs share a high homology among themselves, they also have alternatively spliced forms with different domain organization (Figure 1.11). Several variants have been described, for example for Elk-1, Δ-Elk-1 and sElk-1 are two splice variant form of this protein. sElk-1 arises from an internal translation start site which generates a protein lacking the first 54 amino acids of the ETS DNA binding domain (Vanhoutte et al., 2001). Δ-Elk-1 is an alternatively spliced variant of Elk-1 that has lost the SRF interaction domain and part of the Elk-1 DNA binding domain (Rao and Reddy, 1993). Consequently, the DNA binding ability of these variants is different from that of the full-length protein (Figure 1.8). sElk-1 expression is neuronal-specific and plays an opposite role to Elk-1 in potentiating NGF (**N**erve **G**rowth **F**actor)-driven neuronal differentiation in PC12 cells, whereas ΔElk-1 might function by competing for some of the Elk-1 target sequences (Vanhoutte et al., 2001). It might thereby block transcriptional activation of c-fos by SRF and Elk-1. Generally, the truncated TCFs are considered to antagonize the activity of the corresponding full-length proteins.

1.4 Microtubules and Signal Transduction

Microtubules are very important components of intracellular signal transduction mechanisms. They fill the cytoplasm and usually serve as a rail-road between the plasma membrane and nucleus. This property gives the cell a polarity and motor proteins like kinesin and dynein can carry their cargo on microtubules by utilizing

this polarity. For this reason, microtubules maintain a directional flow of information (Gundersen and Cook, 1999).

Microtubules provide a large surface area in the cell, which is about the same surface area as the plasma membrane. Therefore, microtubule surface has a tendency to interact with other proteins. Because microtubules are dynamic components of the cell, they should immediately respond to any signal transmitted in the cell to re-organize themselves. That is why they are usually found in relation with signal transduction molecules to be able to react in an efficient way. Many different signal transduction pathways have been shown to be related to microtubules. In some pathways, signals are transmitted to microtubules whereas in others microtubules are used to transmit signals from one region to another.

Microtubule stability can effect different cellular mechanisms. Microtubule breakdown or stabilization stimulates or inhibits various signalling pathways. As a result of this action, different sets of genes are activated and cells are directed to proliferation (Crossin and Carney, 1981), apoptosis (Srivastava et al., 1998), movement (Mikhailov and Gundersen, 1998), polarization (Achler et al., 1989), contractility (Danowski, 1989) or neurite extension (Bamburg et.al, 1986, Tanaka et al., 1995) depending on the type of the cell.

Microtubules can be affected by proteins of different signalling pathways. These proteins might be kinases, phosphatases, some upstream adaptor proteins or even transcription factors. Microtubules can either directly interact with these effectors or complexed with them with the help of motor proteins such as kinesin or dynein or by the MAPs (Crepieux et al., 1997, Rezska et al., 1995).

The **E**xtracellular-signal **R**egulated **K**inases ERK1 and ERK2 have been shown to interact with microtubules both *in vitro* and *in vivo* (Rezska et al., 1995, Rezska et al., 1997). Microtubules carry mostly the activated form of these kinases (Morishima-Kawashima and Kosik, 1996) and the amount of these protein does not change after mitogenic stimuli that increases the active form of ERK 1/2 in the cell (Gundersen and Cook, 1999). One suggested hypothesis is that MAPK affects microtubule stability by phosphorylating MAPs and therefore reducing their

microtubule affinity (Hoshi et al., 1992). This hypothesis got stronger after an observation that some mutations on MAPK that do not affect microtubule binding can still disrupt microtubule organization. These data suggest that there are some other players in the microtubule organization relaying the message coming from MAPK to microtubule (Rezska et al., 1997). In addition to the role of MAPs in the regulation of microtubule stability, they can also be significant in the signal transduction. The importance of MAPs in the signal transduction comes from their projection domain extending from the microtubule wall. This property makes MAPs very suitable as a scaffolding protein. In the previous studies, the association between MAP2 and PKA and as well as MAP4 and cyclin B have been found (Yoshida et al., 1996, Berling et al., 1994). MAPs can also convey signalling molecules to actin cytoskeleton and this has been shown to have a functional role (Cunningham et al., 1997). One possibility of targeting these proteins to microtubules is to bring them in proximity to microtubule bound substrates to promote their activity. Other signalling molecules are known to interact with microtubules and this interaction might be supported by a scaffold forced by MAP.

To explain the signalling between microtubules and its associated proteins, 3 different mechanisms have been proposed (Gundersen and Cook, 1999) (Figure 1.12). These are (i) “microtubule sequestering and release”, (ii) “microtubule delivery” and (iii) “microtubule scaffolding of signalling molecules”. In the sequestering and release mechanism (Figure 1.12 a), the target protein directly interacts with microtubules and this association is controlled either by modification of the protein or microtubule or by polymerization and depolymerization dynamics of the microtubule. The interaction between NFκB and microtubule is a good example of this mechanism, in that the activation of NFκB depends on the depolymerization of microtubule (Rosette and Karin, 1995).

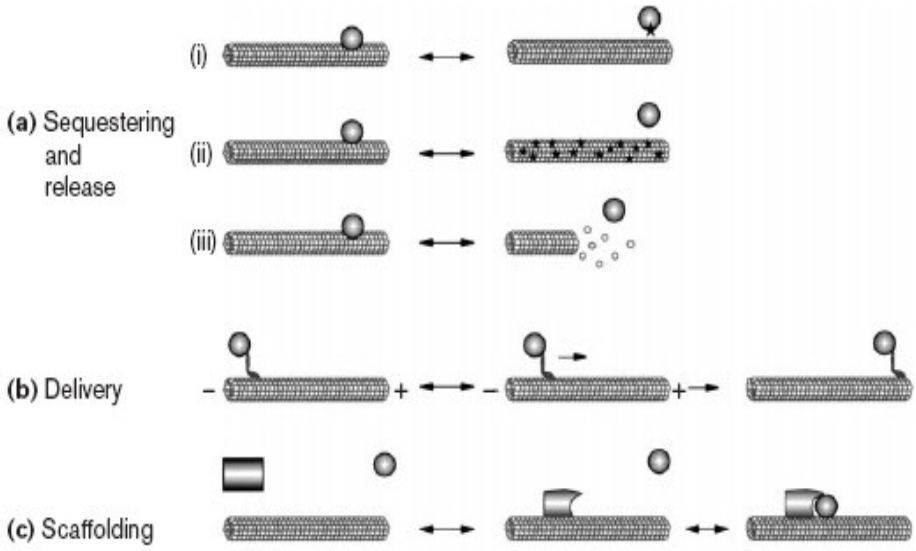

Figure 1.12: Mechanisms proposed for the interaction of microtubules with the signal transduction molecules (Gundersen and Cook, 1999)

In the microtubule mediated delivery mechanism, the signalling factor is delivered on the microtubule surface by motor proteins (Figure 1.12 b). Activation of this mechanism by signal transduction could occur through enhanced motor-cargo interaction, enhanced motor-microtubule interaction or stimulation of the motor itself (Gundersen and Cook, 1999). The interaction between MLK2 (MAP Kinase Kinase Kinase 2) and KIF3 (Kinesin Family Protein 2) is an appropriate example of this mechanism (Nagata et al., 1998). In that respect, kinesin polypeptides are known to be phosphorylated and it is thought that this modification might regulate their interaction with cargo. Moreover, it is known that post-translational modification of tubulin enhances kinesin binding and may contribute to the specific interaction of kinesin with stabilized microtubules (Liao and Gundersen, 1998).

In the microtubule scaffolding mechanism, microtubule surface functions as a template to promote the interaction of two or more factors that would otherwise not interact (Figure 1.12 c). The interaction of one component with the microtubule induces a binding site for a second factor or simply brings low affinity components into proximity so they can interact. Because most of the signal transduction proteins that interact with microtubules are found as large

multimeric complexes on the microtubules, the assembly of them can be promoted by microtubules. Another advantage of the interaction with microtubules is that the diffusion of these proteins is limited to enable their movement by motor proteins to their ultimate targets (Gundersen and Cook, 1999).

The effects of microtubules on the signal transduction have been studied by using drugs that break down or hyperstabilize microtubule structure. These drugs specifically bind to tubulin and studies carried out with these drugs in different cell types indicate that microtubule depolymerizing agents such as colcichine stimulate cell proliferation, whereas for instance microtubule stabilizing agents such as taxol inhibit proliferation (Gundersen and Cook, 1999). This observations suggest that there is a sequestering and release mechanism mediated by microtubules, but whether this effect is direct or indirect is not known.

The interaction of microtubules with the signal transduction proteins is especially important in neurons for the transmission of signals in the anterograde or retrograde direction. Because neurons usually have long and branched axons and dendrites originating from a large cell body, the events occuring in any part of the cell should be somehow relayed to another part which could be far away. Some specific mechanisms were suggested to understand the underlying relay mechanism. In injured neurons for example, the proteins containing NLS (**N**uclear **L**ocalization **S**ignal) sequences are usually directed to retrograde signalling (Hanz and Fainzilber, 2006). Proteins called importins recognize NLS sequences of proteins and direct them to the retrograde transport complex (Weis, 2003). The components of this retrograde transport complex and the mechanism of initiation and transmnission of signal has been studied with the ERK1/2 kinase, the upstream kinase of Elk-1. Concomitant phosphorylation of ERK1/2 in axoplasm generates a signaling moiety that binds directly to vimentin (Figure 1.13). Vimentin can bind to both phospho-Erk and importin-β, thereby linking activated ERKs to importin-mediated retrograde transport (Hanz and Fainzilber, 2006) .

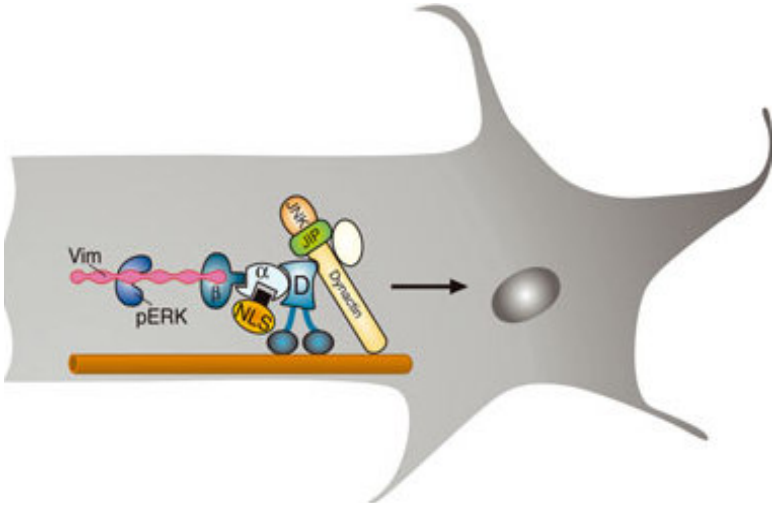

Figure 1.13: Retrograde transportation complex formed on the microtubules after an axonal injury (Hanz and Fainzilber, 2006).

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Animals: Embryos (P18) from pregnant rats

2.1.2 Cell Lines and Bacterial Strains

SH-SY5Y Human metastatic neuroblastoma cell line was a gift of Andrew Sharrocks

E.coli BL21 (DE)pLysS strain was from Promega (Madison)

Bovine Brain from slaughterhouse

2.1.3 Plasmid constructs

Table 2.1: Plasmid constructs used in the experiments

Plasmid Name	Reference
pcDNA3 Elk-1	Yang et al., 1998
pGEX Elk(1-205)	Gille et al., 1995
pGEX Elk(205-329)	Yang et al., 1998
pGEX Elk(349-428)	Yang et al., 1998

2.1.4 Cell Culture Assays

- Dulbecco's Modified Eagle Medium (DMEM) 1g/liter glucose (Gibco)
- Neurobasal Medium (Gibco)
- HBSS (Gibco)
- HEPES (Gibco)
- B27 Supplement (Gibco)

- Fetal Bovine Serum (Gibco)
- L-Glutamine solution (100X) (Gibco)
- Penicilin/Streptomycin solution (100X) (Biochrom)
- 0,5% Trypsin-EDTA solution (Sigma)
- D-PBS (Gibco)
- T25 Tissue Culture Flasks (Nunc)
- T75 Tissue Culture Flasks (Nunc)
- T150 Tissue Culture Flasks (Nunc)
- 24-well culture plate (Nunc)
- Coverslip (Isolab)
- Poly-L lysine (Sigma)
- Laminin (Roche)
- IGF-1 (Promega)
- Transfast (Promega)
- Nucleofector solution (Amaxa)
- Nucleofector Electroporation Device (Amaxa)
- Glutaraldehyde (Sigma)
- Paraformaldehyde (AppliChem)
- Triton X-100 (AppliChem)
- Goat polyclonal anti-rabbit IgG- Alexa 647 conjugate (Molecular Probes)
- Goat polyclonal anti-mouse IgG- Alexa 488 conjugate (Molecular Probes)
- Leica TCS SP2 SE Confocal Microscope

2.1.5 Bacterial Assays

- LB Medium (Applichem)
- LB Agar Medium (Applichem)
- Ampicillin (Applichem)
- Chloramphenicol (Applichem)
- IPTG (Invitrogen)

LB and LB Agar media were prepared according to manufacturer's insructions. 25 g LB was dissolved in 1 liter dH_2O, 32 g LB Agar was dissolved in 1 liter dH_2O. They were autoclaved at 121ºC for 15 minutes and used as sterile.

2.1.6 Protein Assays

- Glutathione agarose beads (Sigma)
- CM Cellulose (Whatmann)
- Transfast Transfection Reagent (Promega)
- RIPA Buffer (Sigma)
- PBS (Gibco)
- Protease Inhibitor Coctail (Sigma)
- Phosphatase Inhibitor Coctail (Sigma)
- Protein G Agarose Beads (Roche)
- Flag Protein G Agarose Beads (Sigma)
- Ultra-speed Centrifuge (Beckman)
- 1,5 ml tube (Beckman)
- Hybridization Chamber
- Polystyrene 96-well microplate (Orange Scientific)
- Donkey anti-rabbit IgG Alkaline Phosphatase conjugate (Promega)
- SIGMAFAST pNPP tablets (Sigma)
- ELISA plate reader (BioTek)
- Goat anti-mouse IgG antibody-HRP conjugate (Pierce)
- Goat anti-rabbit IgG antibody-HRP conjugate (Pierce)

2.1.7 General Chemicals

- 29:1 Acrylamide:bisacrylamide (AppliChem)
- Ammoniumpersulphate (AppliChem)
- SDS (AppliChem)
- TEMED (AppliChem)
- Coomassie Brilliant Blue (Sigma)
- Nitrocellulose Paper (Schleicher and Schuell)
- 3MM Whatman Filter Paper (Whatman)
- Ponceau S Stain (AppliChem)
- Tris (AppliChem)
- NaCl (AppliChem)
- Tween 20 (AppliChem)

- BSA (Fluka)
- Non Fat Dry Milk
- PMSF (AppliChem)
- GTP (Sigma)
- NaOH (AppliChem)
- HCl (AppliChem)
- $CaCl_2$ (AppliChem)
- Bromophenolblue (AppliChem)
- Rabbit polyclonal anti-Elk-1 antibody (Celll Signalling Technologies)
- Rabbit polyclonal anti-beta tubulin antibody (Cell Signalling Technologies)
- Mouse monoclonal anti-beta tubulin antibody (Chemicon)
- Mouse monoclonal anti-GST antibody (Novagen)
- ECL detection substrate (Pierce)

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 SH-SY5Y Culture

SH-SY5Y cell line was used in the experiments. SH-SY5Y cell line was used in immunofluorescence and immunoprecipitation experiments due to its neuronal origin.

SH-SY5Y culture was maintained in DMEM containing 10%FBS, 1X L-Glutamine and 1X Penicilin/Streptomycin. Cells were grown in T75 flasks with 15 ml medium at 37ºC in 5% CO_2 incubator until they reach to confluency. Cells are usually split every 2-3 days by tyripsinization (see below, Splitting of Cells).

2.2.1.2 Splitting of Cells

For immunoflourescence and immunoprecipitation experiments, SH-SY5Y cells were splitted onto poly-L lysine coated coverslips in 6 well plate and onto 10 cm cell culture dish.

- SH-SY5Y cells were plated to 6 well plates at $2X10^5$ cells/well and to 10 cm dishes at 10^6 cell /dish concentrations in their growth medium.

2.2.1.3 Primary Hippocampal Culture

- Isolation and culturing of hippocampal cells were essentially performed as described in Yu et al., 2005.
- Briefly, heads of rat embryos were separated from their bodies.
- Brains were dissected and hippocampi were extracted under the stereomicroscope.
- Hippocampi were pooled in dissection solution.
- Hippocampi were digested in Trypsin-EDTA-HBSS solution at 37C.
- After trypsinization, cell were collected after a brief centrifuge and they were washed with HBSS.
- Then growth medium was added to the cells and after several triturations with a pasteur pipette, cells were counted.
- They were plated at 3000 cells/well to poly-L lysine and laminin coated coverslips in 24-well plate following transfection.

2.2.2 Transfection

Transfection is the insertion of foreign DNA into the cell. In particular, this method is essential for examining the effects of gene products on cellular mechanisms. The experiments requiring transfection are usually done by using cloned DNA containing coding sequences or by control regions such as promoters. Different methods are developed to introduce the foreign DNA into the host cell. They include electroporation, viral infection, lipofection, gene guns and microinjection. In this study, DNA was inserted into cells either through lipofection or electroporation. In electroporation, an external electrical

field is applied to cells to cause a transient membrane permeability in plasma membrane. For this purpose cells are suspended in a special solution and put in a plastic cuvette with aluminium electrodes and voltage is applied. In lipofection, DNA is delivered to cells by means of liposomes which are vesicles that can easily fuse with the cell membrane due to their phospholipid bilayer structure. Basically synthetic cationic lipids that can form liposomes are incubated with DNA. These liposomes spontaneously interact with DNA and fuse with tissue culture cells.This technique is very popular in cell and molecular biology because it is simple, highly reproducible and efficient (Felgner et al., 1987).

- For immunofluorescence experiment, 1 µg of pcDNA3-Elk-1 or 1µg pcDNA3 alone was put in 400 µl serum free medium and vortexed briefly.
- Then 3 µl TransFast transfection reagent (Promega) was mixed with DNA and again vortexed.
- Mixture was incubated for 10-15 minutes at room temperature to allow the formation of DNA-liposome complex.
- Meanwhile, the medium of cells were discarded and cells were washed with D-PBS once.
- Transfection mixture was added to wells and cells were incubated at 37ºC for at least 1 hour.
- At the end of transfection, transfection mixture was removed from cells and 3 ml fresh serum containing growth medium was added to cells.
- The same procedure was repeated for immunoprecipitation experiment with 5 µg plasmid and 15 µl TransFast in 2 ml serum free medium in 10 cm dishes.
- Primary hippocampal cells were transfected by electroporation as described in Karabay et al., 2004. 15 µg Elk-1 expression plasmid was introduced to hippocampal cells using Nucleofector transfection solution and Nucleofector electroporation device (Amaxa).

2.2.3 Immunostaining

Immunostaining is a technique used to visualize proteins in the cell. It is particularly important when studying colocalization of the proteins. Essenatially, after primary antibody incubation with another antibody, called secondary antibody, which is specific for the IgG heavy and light chains of the primary antibody, detection is performed usually by fluorescence.

- Transfection was essentially performed as described previously.
- SH-SY5Y cells were fixed in 3% paraformaldehyde for 15 minutes at room temperature.
- Cells were washed 2 times with PBS.
- Cells were permeabilized in 0,1% Triton X-100 and 1% FBS in PBS for 10 minutes.
- Then cells were washed once with PBS.
- Samples were blocked in 5% FBS in PBS solution.
- Primary antibodies were diluted in PBS-T (0.1% Trition X-100 in PBS) with 1:100 mouse monoclonal anti-β-tubulin and 1:100 rabbit polyclonal anti-Elk-1 dilution factors.
- Cells were incubated with 100 µl antibody mixture/coverslip at room temerature for 1 hour.
- Samples were washed 3 times with PBS-T
- Secondary antibody dilutions were used at 1:400 dilution factor goat anti-mouse IgG-Alexa 488 conjugate and goat anti-rabbit IgG- Alexa 647 conjugate antibodies.
- Cells were incubated with secondary antibodies at room temerature for 1 hour in a closed box to prevent light entry.
- Cells were washed 3 times with PBS-T and they were mounted to glass slides with 10 µl mounting medium.
- Cells were visualized in Leica TCS SP2 SE Confocal Microscope with appropriate laser beams and photos were taken under 63X magnification.

2.2.4 GST-Pulldown Assay

The pull-down assay is an *in vitro* method used to determine physical interaction between two or more proteins. Pull-down assays are useful for both confirming the existence of a protein:protein interaction predicted by other research techniques (e.g., co-immunoprecipitation, yeast two-hybrid and density gradient centrifugation) and as an initial screening assay for identifying previously unknown protein:protein interactions. The minimal requirement for a pull-down assay is the availability of a purified and tagged protein (the bait) which will be used to capture and 'pull-down' a protein-binding partner (the prey).

Confirmation of previously suspected interactions typically utilizes a prey protein source that has been expressed in an artificial protein expression system. This allows the researcher to work with a larger quantity of the protein than is typically available under endogenous expression conditions and eliminates confusing results, which could arise from interaction of the bait with other interacting proteins present in the endogenous system that are not under study. Protein expression system lysates, such as *E. coli* or baculovirus-infected insect cells, *in vitro* transcription/translation reactions, and previously purified proteins are appropriate prey protein sources for confirmatory studies.

2.2.4.1 Competent Cell Preparation and Transformation

For the isolation of GST fusion proteins from the bacteria, the plasmids carrying the desired constructs should be introduced into the cells. For this purpose BL21(DE) pLysS bacteria strain was used. The procedure was essentially performed as described previously (Sambrook et al). Cells were made competent by calcium chloride method as described and transformation was carried out as explained in the following.

- 50 µl of competent cells mixed with 500 ng DNA.
- Bacteria-DNA mixture was kept on ice for 15 minutes.
- Then, the heat-shock was applied at 37 ºC for 60-90 seconds.

- The sample was put again on ice for 5 minutes.
- 500 µl of LB medium was added and bacteria was incubated at 37º C.
- At the last step, bacteria were collected with a quick spin at the bottom of the tube and they were spred on LB Agar plates containing 33 µg/ml chloramphenicol and 50-200 µg/ml ampicillin.

2.2.4.2 Induction of Protein Expression and Protein Isolation

- One colony from the plate was inoculated into 10 ml LB medium with ampicillin and chloramphenicol at the concentrations indicated above.
- Bacteria were grown overnight at 37 ºC with 200 rpm shaking.
- Then, they were diluted at 1:30 ratio in fresh medium and they were grown until they reached OD_{600}=05-07.
- At that time, bacteria were induced for GST-fusion protein expression with 25 µg/ml IPTG.
- After 5 hours of protein production, bacteria were collected by centrifugation at 4 ºC.
- Pellets were resuspended in ice-cold PBS containing protease and phosphatase inhibitors to prevent protein degradation.
- Bacteria were lysed by sonication in the ultrasonic water bath for 2 minutes X5.
- After lysis was completed, the lysate was cleared by centrifugation at 4 ºC, and 15000 rpm for 15 minutes. 100 µl of lysate was stored for further analysis at -80 ºC.
- Meanwhile, 70 mg GST-Agarose beads was weighed and treated as per manufacturer's instructions and 50% bead-PBS slurry was prepared.
- 200 µl of 50% slurry was mixed with 1 ml cell lysate.
- They were incubated at 4 ºC on the end-over-end rotator overnight.
- At the final step, beads were washed twice with ice-cold PBS and once with 0,1% Triton X-100 in PBS.
- Beads were resuspended in 100 µl PBS and they were kept at 4 ºC until GST pulldown assay.

2.2.4.3 Microtubule Isolation

- Bovine brain was obtained from slaughterhouse.
- Cerebellum and meninges were cleaned and weighed as 311,62 gr.
- 235 ml PM buffer with 1 mM PMSF was added and the mixture was blended.
- The mixture was centrifuged at 30.000g for 30 minutes at 4 ºC and supernatant was collected.
- Supernatant was centrifuged again at 50.000g for 40 minutes at 4 ºC and supernatant was again collected.
- 2 mM GTP at final concentration was added.
- The sample was incubated at 37 ºC for 30 minutes in an incubator.
- After incubation, the sample was centrifuged at 50,000 rpm for 30 minutes at 37 ºC.
- The pellet was resuspended in 36,5 ml PM buffer with 1 mM PMSF.
- Sample was left at 4ºC for 30 minutes in cold room and then centrifuged at 60,000 rpm for 30 minutes at 4 ºC.
- Supernatant was collected and resuspended in the same volume of PM Buffer.
- 1 mM PMSF and 2 mM GTP was added in final concentrations.
- Sample was incubated at 37ºC for 30 minutes and then centrifuged at 60,000 rpm for for 30 minutes at 37ºC.
- Pellet was resuspended in 7 ml PM Buffer and homogenized with potters
- Sample was incubated on ice for 30 minutes and centrifuged at 60,000 rpm for 30 minutes at 4ºC.
- Supernatants were collected.
- 9 ml PM buffer with 1mM PMSF was added into the supernatant.
- The samples were kept at 4ºC until column chromatography.

2.2.4.4 Column Chromatography

Ion exchange chromatography relies on charge-charge interactions between the proteins in sample and the charges immobilized on the resin of choice. Ion exchange chromatography can be subdivided into cation exchange

chromatography, in which positively charged ions bind to a negatively charged resin; and anion exchange chromatography, in which the binding ions are negative, and the immobilized functional group is positive. In this experiment, we used cation exchange chromatography to purify negatively charged microtubules from positively charged MAPs.

- 50 g resin was weighed.
- 1250 ml 0,5 M NaOH solution was added to the resin and waited for 5 minutes.
- Supernatant was discarded and resin was washed 7 times with ddH_2O.
- Washing continued until pH<11.
- 1250 ml 0,5 ml HCl was added and waited for 5 minutes.
- Supernatant was discarded and resin was washed 6 times with ddH_2O.
- Washing continued until pH>3.
- Resin was washed in 5X concentrated PM buffer with 600 ml volume and pH was set up to 6,9 with NaOH.
- 5X PM was discarded and 1250 ml 1X PM was added and pH was checked (6,88).
- Resin was poured into 2 liter cylinder and allowed to setle overnight in 1X PM in cold room at 4˚C.
- Next morning, resin volume was measured as 440 ml.
- Resin was transferred to the column and allowed to setle overnight in cold room.
- Next day, 200 ml column buffer (0,1 mM GTP and 1X PM) was passed through the column.
- 50 ml column buffer was added and resin was left in it overnight in cold room.
- 50 ml column buffer was discarded.
- 9 ml semi-purified tubulin was poured from the top.
- If all the sample was in the resin column buffer was added continuously.
- First 43 tubes were collected as 4 ml.
- Other tubes until 139 were collected as 0,6 ml.
- Bradford assay was done from the samples to calculate the amount of tubulin.

2.2.4.5 SDS-PAGE Analysis

SDS-PAGE is a technique used to separate proteins according to their electrophoretic mobility. In this method, tertiary structure of proteins is disrupted by SDS (sodium dodecyl sulfate) and reducing agents such as β-mercaptoethanol. Then proteins are separated in a polyacrylamide gel according to their sizes. During SDS-PAGE, discontinuous gel system is used to increase the resolution of proteins. The recipes of SDS-PAGE for different gel concentrations are given in Table 2.3 and 2.4.

Table 2.2: Components of resolving and stacking gel buffers

Contents	**Resolving Gel Buffer pH 8.9**	**Stacking Gel Buffer pH 6.7**
Tris	18,1 g	5,9 g
SDS	0,4 g	0,4 g
Water	100 ml	100 ml

Table 2.3: Components of the resolving gel

Contents	**9 %**	**12%**
29:1 Polyacrylamide:Bisacrylamide	1,8 ml	2,4 ml
Resolving Buffer	1,5 ml	1,5 ml
10 % APS	37,5 µl	37,5 µl
TEMED	2,5 µl	2,5 µl
Water	2,675 ml	2,1 ml

Table 2.4: Components of the stacking gel

Contents	**3,5 %**
29:1 Polyacrylamide:Bisacrylamide	1 ml
Stacking Buffer	1,5 ml
10% APS	50 µl
TEMED	2,5 µl
Water	3,5 ml

- After gels polymerized, samples were loaded into wells.
- Before loading, protein samples were mixed with 6X SDS-Loading Buffer in 1X final concentration (Table 2.5). Then, they were boiled at 95 ºC for 5 minutes to break non-covalent and ionic bonds.

Table 2.5: Components of the 1X SDS-Loading Buffer

Contents	**Concentration**
Tris-HCl (pH6.8)	62 mM
SDS	2% w/v
DTT	50 mM
Bromophenol blue	0.01% BPB

- When the samples were loaded to the gel, they run in tank buffer (Table 2.6) at 80V for 1 hour, basically until proteins reach to resolving gel. Then, the voltage was increased to 100V for 1 hour and finally 120 V for an additional 1 hour.

Table 2.6: Components of 1X tank buffer

Contents	**Amount**
Tris	63,2 g
Glycine	40 g
SDS	10 g
Water	1 liter

- The gel was either stained in staining solution to visualize protein bands on the gel or it was blotted for western blotting.

2.2.4.6 Pulldown of Purified Microtubules

In order to find out any possible interaction between microtubules and Elk-1, both proteins were isolated and their purities were controlled by SDS-PAGE. The next step was to incubate these protein in a test tube. For this reason;

- 2 µg of purified tubulin was mixed with the same amount of GST-Elk fusion proteins with different deletion constructs.
- To stabilize α- and β-tubulin interaction, 1 µM GTP was added at a final concentration.
- The mixture was incubated at room temperature for 30 minutes on a rotator.

- After incubation, GST-beads were collected at the bottom of the tube by quick spin and the supernatant containing unbound tubulin was discarded.
- The beads were washed twice with 1 ml PBS and once with 1 ml PBS-T to remove all the remaining tubulin. After washing, beads were resuspended in the same volume of PBS and SDS sample buffer added at 1X final concentration.
- GST-pulldown samples were kept at -80 ºC until western blotting analysis.

2.2.4.7 Pulldown from Brain Lysate

- The whole brain was extracted from an adult mouse.
- It was washed twice in ice-cold PBS solution.
- 1 ml lysis buffer (50 mM Tris-HCl, 300 mM NaOH, 1% Triton X-100) was added to the tissue.
- Brain was homogenized with a pestle and mortar in the lysis buffer.
- Homogenate was vortexed vigorously and incubated on ice for 30 minutes for efficient lysis.
- Homogenate was centrifuged at 15,000 rpm for 15 minutes at 4ºC.
- Supernatant was transferred to a new tube.
- The concentration of proteins was measured by Bradford assay and calculated as 5,35 µg/µl.
- Previously isolated GST-protein-beads were incubated with 94 µl (500 µg) brain lysate at room temperature for 30 minutes.
- After 30 minutes, GST-beads were collected at the bottom of the tube after a quick spin.
- Supernatant was discarded and beads were washed once with PBS and then once with PBS-T.
- Beads were resuspendedn in the same volume of PBS and SDS sample buffer at 1X concentration.

2.2.4.8 Western Blotting

- The GST-fusion protein samples which were incubated with microtubules or with brain lysate were run in 9% SDS-PAGE gel. The gel was prepared according to recipie in Table 2.3 and 2.4 and run for 3 hours.
- After the separation of protein bands, proteins were transferred to nitrocellulose membrane.
- Before transfering to the nitrocellulose membrane, filter papers and gel were equilibrated in transfer buffer for at least 15 minutes. The recipe of transfer buffer is given in Table 2.7.

Table 2.7: Components of the transfer buffer

Content	**Concentration**	**Amount in 1 liter buffer**
Tris	48 mM	5,8 g
Glycine	39 mM	2,93 g
SDS	0,0037%	1.875 ml 20% SDS solution
Methanol	20%	200 ml

- After equilibration was finished, the sandwich was prepared as per manufacturer's instructions between fitler papers and nitrocellulose membrane.
- The gel was put on the membrane with care not to disrupt the gel. Air bubbles were removed.
- The protein transfer was carried out in semi-dry blotting apparatus (Bio-Rad).
- Transfer was run for overnight at 15 V in cold room (4 ºC) to prevent protein degradation.
- After transfer was completed, nitrocelluose membrane was stained with Ponceau S stain to see whether proteins are on the membrane or not.
- After transfer the gel was also stained with Coomassie Blue to see the remaining protein amount. If most of the proteins were on the membrane, western blotting was continued with antibody incubations.
- Membrane was washed 2 times with ddH_2O (ultrapure) for 5 minutes to remove Ponceau S from membrane. Because the binding of stain is reversible and the stain itself is water soluble, it is quite easy to eliminate it from the membrane.

- Then the membrane was blocked with blocking solution (Table 2.8) at room temprerature for 1 hour with gentle shaking. This blocking step was done to prevent non-specific of antibodies to empty regions on the membrane. In other words, efficient blocking simply reduces the background.

Table 2.8: Components of blocking solution for Western blotting

Contents	Concentration	Amount
Non-Fat Dry Milk	5%	5 g
Tween-20	0,2%	200 µl
TBS		100 ml

Table 2.9: Formulation of TBS buffer

Contents	Amount
Tris	2,42 g
NaCl	8 g
Water	1 liter

- After blocking, the membrane was washed briefly with TBS-T (0.1% Tween-20 in 1X TBS solution) (Table 2.9) solution to remove excess of protein.
- Then, the membrane was incubated with 1:1000 diluted anti-beta tubulin rabbit polyclonal antibody in blocking solution for overnight at 4ºC.
- The blot was washed with TBS-T.
- 1:1000 dilution of anti rabbit IgG-HRP antibody was prepared in blocking solution.
- The blot was incubated with secondary antibody for 1 hour at room temperature with gentle shaking.
- The blot was washed 3 times with TBS-T.
- For signal development 10 ml of signal development solution was prepared by mixing peroxide and luminol solutions as described (Roche).
- Blot was placed into the solution and after 5 minutes signal was captured with BioRad ChemiDoc EQ Gel Analysis System.

2.2.5 Co-Immunoprecipitation of Microtubules with Elk-1

Immunoprecipitation is a common method used for the analysis of protein-protein interactions in the cell. It is the technique of precipitating an antigen out of solution using an antibody specific to that antigen. This process can be used to enrich a given protein from the lysate to some degree of purity. Co-immunoprecipitation can identify interacting proteins or protein complexes present in cell extracts by precipitating one protein believed to be in a complex, additional members of the complex are captured as well and can be identified. The protein complexes are removed from the bulk solution, once bound to the specific antibody, by capture with an antibody-binding protein attached to a solid support such as an agarose bead. These antibody-binding proteins such as Protein A and Protein G, were initially isolated from bacteria and recognize a wide variety of antibodies. Following the initial capture of a protein or protein complex, the solid support is washed several times to remove any proteins not specifically and tightly bound through the antibody. After washing, the precipitated protein(s) are eluted and analyzed using gel electrophoresis, mass spectrometry, western blotting, or any number of other methods for identifying constituents in the complex. Thus, co-immunoprecipitation is a standard method to assess protein-protein interaction.

- SH-SY5Y cells were splitted to 10 cm tissue culture dishes at 10^6 cells/dish concentration.
- Transfection was performed as described before.
- Transfected cells were incubated at 37ºC in growth medium for 2 days.
- Cells were lysed in 500 µl RIPA buffer was added on each dish and cells were scraped into that and lysed for 30 minutes on ice.
- Lysates were centrifuged at 15.000 rpm for 15 minutes and supernatants were taken to another tube.
- 50 µl was taken in another tube and kept as input for the analysis.
- 50 µl Protein G Agarose was added to each sample and they were incubated at 4ºC on a shaking platform for overnight.
- Next day, samples were briefly centrifuged and supernatants were collected.
- They were again cleared with an additional 50 µl Protein G agarose and incubated for 6 hours at 4ºC on a shaking platform.

- Meanwhile 200 µl Flag-Agarose beads were taken from the stock.
- Beads were briefly centrifuged and supernatant was discarded.
- The remaining beads were washed 4 times with incubation buffer (50 mM Tris-HCl, 150 mM NaCl, pH7.4) and made into a 50%slurry.
- After 6 hours, samples were briefly centrifuged and then supernatant was transferred to a new tube.
- 5% (weight/volume) BSA was added to the samples.
- 100 µl beads were mixed with precleared lysate and incubated at 4ºC for overnight.
- Beads were washed with incubation buffer.
- Then, they were resolved in SDS-loading buffer and boiled at 95ºC for 5 minutes and loaded to 10% SDS-PAGE (as explained before) and run at 120V for 2 hours.
- Proteins were transferred to nitrocellulose membrane and detection of beta tubulin and Elk-1 was carried out with rabbit polyclonal anti-beta tubulin and rabbit polyclonal anti-Elk-1 antibodies by using anti-rabbit IgG-HRP and enhanced chemiluminescence systems.

2.2.6 Microtubule Co-sedimantation Assay

Microtubule co-sedimentation is a frequently used method to identify microtubule interacting proteins. The main principle of this assay is the polymerization of tubulin heterodimers to long microtubule filaments and after incubation of polymerized microtubules with target proteins, to precipitate them by centrifugation. Polymerized microtubules will be collected in the pellet and any microtubule interacting protein will also be with them.

- Tubulin heterodimers isolated from bovine brain were polymerized into microtubules.
- The concentration of tubulin was measured with Bradford Assay.
- Tubulin was polymerized into microtubule in polymerization buffer containing taxol and GTP at 37ºC for 30 minutes.
- Each bacterially expressed GST, GST-Elk (1-205), GST-Elk (205-329) and GST-Elk (349-428) containing lysates were mixed with polymerized tubulin. The

reaction was performed in 100 µl volume with AB buffer (20 mM Pipes, pH 6.9, 1 mM $MgCl_2$, 1mM EGTA) (Karabay and Walker, 1999).

- Mixtures were incubated at room temperature in a rotating platform for 45 minutes.
- After incubation, polymerized microtubules were precipitated at 130,000 rpm at 37ºC for 30 minutes.
- Pellets were dissolved in 1X SDS-loading buffer and in another tube supernatants were also mixed with an equal volume of 2X SDS loading buffer.
- Protein samples including tubulin, bacterial lysates and pellets and supernatants of polymerized microtubules were run in a 10% SDS-PAGE gel prepared as mentioned before.
- Analysis of microtubule bound GST-Elk-1 proteins was done by Western blotting technique with mouse monoclonal anti-GST antibody (Novagen, 1:10,000 dilution) and detection was performed by ECL.

2.2.7 ELISA based *in vitro* Binding Assay

ELISA-based *in vitro* binding assay is another method to find out the interaction between two proteins. In this assay, one of the proteins is immobilized on the wells of a microtiter plate and the binding of the second protein was detected by antibody specific for the added protein, followed by color reaction. The ELISA detection method is more sensitive than Western blotting, in that smaller quantities of protein can be detected.

- 96 well polystyrene microwell plates were coated with the purified tubulin heterodimers.
- To optimize microtubule concentration required to coat the surface of well, microtubule solutions ranging from 10-500 µg/ml concentrations were prepared in PBS solution.
- Wells were incubated with 100 µl microtubule solution at room temperature for 3 hours on a shaker.
- Then solution was discarded and unbound microtubule was washed twice with 200 µl PBS-T solution.

- The remaining empty surfaces on the well were blocked with 1%BSA solution prepared in PBS-T.
- Wells were incubated with 100 µl of this solution for 1 hour on a shaker at room temperature.
- Wells were again washed with PBS-T to remove any unbound protein.
- Wells were incubated with 100 µl of 1:1000 diluted rabbit anti-β-tubulin antibody for 1 hour at room temparature.
- After extensive washes, donkey anti-rabbit IgG-AP conjugate secondary antibody was diluted at 1:1000 in 1%BSA and wells were incubated with 100 µl of this solution for 1 hour at room temperature on a shaker.
- Wells were washed twice with PBS-T after removal of antibody solution.
- Alkaline Phosphatase substrate pNPP (phospho-nitrophenyl phosphate) was prepared by mixing pNPP and buffer tablets from SIGMA (N1891) in 5 ml ddH_2O.
- 200 µl of substrate was added to each well and plate was incubated for 30 minutes at room temperature in the dark.
- After 30 minutes, the color reaction monitored by measuring absorbance at 405 nm in a multiwell plate reader (BioTek).
- As a result of optimization of microtubule concentration for coating 30 µg/ml microtubule concentration was determined as optimum and wells were coated with that concentration of microtubules as described before.
- Tubulin coated wells were incubated with different dilutions of SH-SY5Y lysate containing overexpressed wild-type Elk-1 protein.
- Dilutions of cell lysate ranging from 1:400 to 1:100 were prepared in 1%BSA solution.
- Microtubule coated wells were incubated with these solutions at 4ºC for overnight on a shaker.
- Next day, lysate solutions were discarded and wells were washed twice with 200 µl PBS-T solution.
- Then, rabbit polyclonal anti-Elk-1 antibody was diluted at 1:1000 in 1%BSA solution and well were incubated again with 100 µl of this solution for 1 hour at room temperature on a shaker.
- Solution was discarded and wells were washed with PBS-T twice .

- Donkey anti-rabbit IgG-AP conjugate secondary antibody was diluted at 1:1000 in 1%BSA and wells were incubated with 100 ul of this solution for 1 hour at room temperature on a shaker.
- Wells were washed twice with PBS-T after removal of antibody solution.
- Signals were developed as explained above.

3. RESULTS

3.1 Microtubules Co-localize with Elk-1 in SH-SY5Y Neuroblastoma Cells and Primary Hippocampal Neurons

To explore the possible interaction between two proteins, they should be somehow co-localized in the cell. If a protein is located to the axonal region and the other one is located in the nucleus, the possibility of an interaction will be very low between them and even if it is present it should be under specific conditions. For this reason, we first explored the co-localization between Elk-1 and microtubules in SH-SY5Y human neuroblastoma cell line. Neuroblastoma cells were incubated either in growth medium and serum starved overnight, or in 10 nM IGF-1 containing medium for 3 days for differentiation (Kim et al., 1997). IGF-1 is a well known survival factor of neurons and it has been previously shown that IGF-1 can induce neurite extension in SH-SY5Y cells (Kim et al., 1997). IGF-1 stimulates neurite extension by increasing retinoic acid receptor expression which is critical for the activation of other down-stream target genes. IGF-1 exerts its effects by Ras/Raf/MAPK pathway which is the main upstream activation pathway of Elk-1 (Perez-Juste and Aranda, 1999). To support the attachment of cells to the surface, poly-L lysine coated coverslips were used. Cells were also transfected with an Elk-1 expression plasmid. In immunoflurescence, cells were fixed in paraformaldehyde to preserve the protein structure and then localizations of Elk-1 and microtubules were analysed by protein-specific antibodies. For Elk-1, rabbit polyclonal anti- Elk-1 antibody and for microtubules mouse monoclonal anti-beta tubulin antibody were used. Detections were carried out with Alexa 647 conjugated rabbit IgG specific antibody for Elk-1 and Alexa 488 conjugated mouse IgG specific antibody for β-tubulin. After scanning of samples with appropriate lasers and collecting signals after excitement, we merged two pictures in Adobe Photoshop after pseudo-coloring and examined the localizations of proteins. As a result of this experiment, we have observed that in serum starved cells, Elk-1 was present in the cell body

(Figure 3.1). On the other hand, β-tubulin localized only in cytoplasm not in nucleus as expected. When these these two images overlapped, it was observed that, these two protein co-localized at axonal projections of neurite extensions. (See axon terminals in Figure 3.1).

If we look at the protein localizations in IGF-1 induced cells, we have seen that most of the Elk-1 was localized to the nucleus. Still there was some Elk-1 in the cell body and axonal region but most of the co-localization between Elk-1 and microtubules was in the cell body around the nucleus and axonal regions (Figure 3.2).

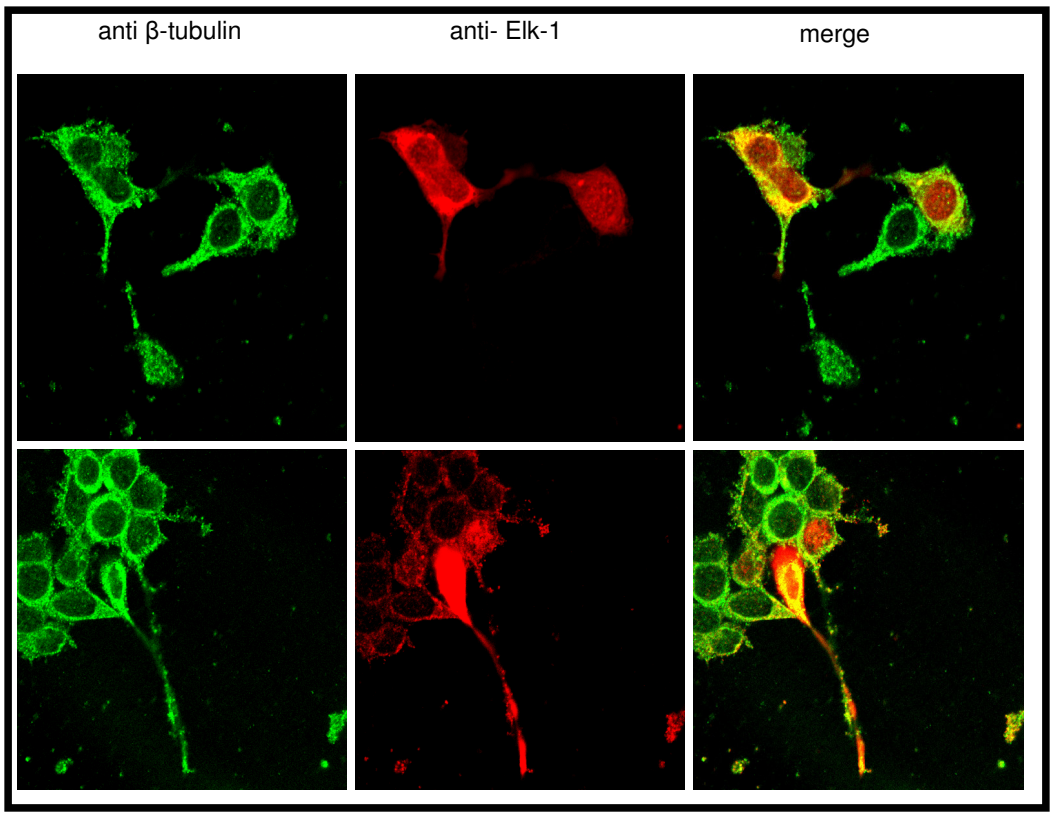

Figure 3.1: Co-localization of Elk-1 and microtubules in serum starved SH-SY5Y neuroblastoma cells.

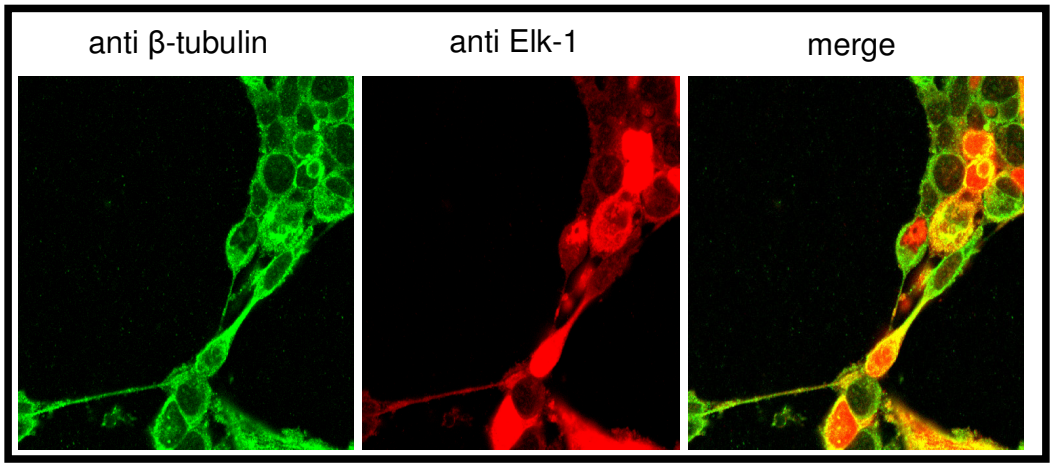

Figure 3.2: Co-localization of Elk-1 and microtubules in IGF-1 induced SH-SY5Y neuroblastoma cells.

Although neuroblastomas are neuronal originated cells, they are transformed cells and hence show tumorigenic characteristics in their morphology, which distinguishes them from healthy neuronal cells. Therefore, we wanted to confirm our results in primary neuronal cells and to that effect we have selected to work with embryonic hippocampal cells.

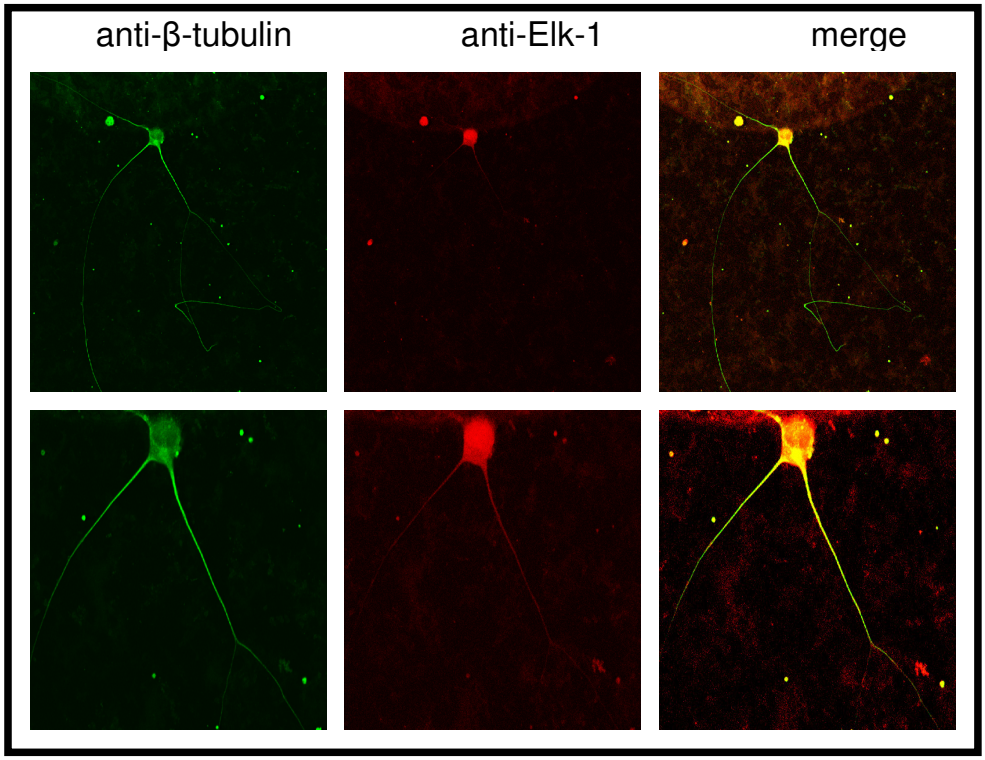

Figure 3.3: Co-localization of Elk-1 and microtubules in P18 rat embryonic hippocampal cells.

When the localizations of the two proteins in primary hippocampal cells were examined a similar pattern to SH-SY5Y was observed. Most of the Elk-1 protein expressed in cells was in the cytoplasm and at the branching sites of axons (Figure 3.3). Microtubules were present along axons beginning at the branching points until the tips of axons and dendrites and as well as in the cell body. In this cell type, most of the co-localization was in the cell body and proximal axonal regions. Although there was a small amount of Elk-1 expression in the axons, it was appeared to disappear towards the tips of extensions.

3.2 Different Regions of Elk-1 Can Interact with Microtubules

After having successfully shown co-localization of Elk-1 and microtubules, the second step was to analyze whether the interaction was direct or not, since there are many intracellular proteins and Elk-1 could be localized to the same region as microtubules but not necessarily interact. We hence used several biochemical and molecular techniques for analysis.

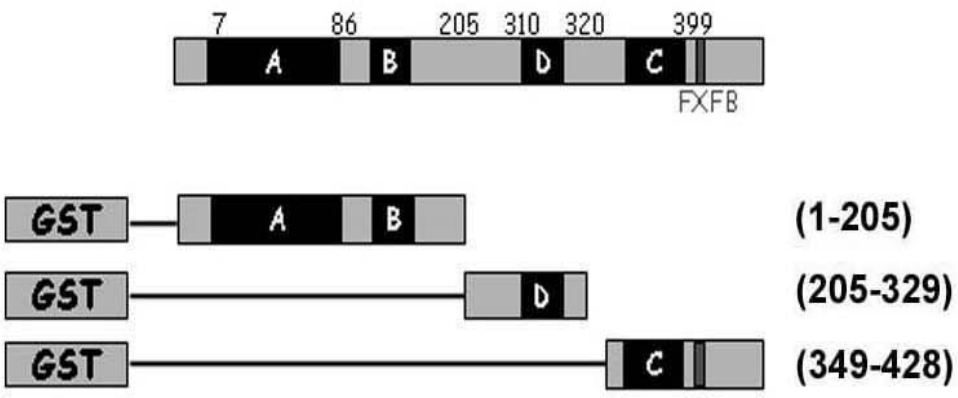

Figure 3.4: Different deletion constructs of GST-Elk-1 fusion proteins used in GST-Pulldown and Co-sedimentation assays.

The first analysis that we have carried out was GST pulldown assay. According to the theory of GST pulldown, proteins fused GST enzyme were produced in *E.coli* immobilized on the agarose beads coated with GST substrate, glutathione. Due to the specificity of substrate-enzyme interaction, desired protein was also immobilized on

the beads. Then these proteins can be incubated either with cell lysate in which another protein is present or with purified protein to study protein:protein interaction. If these two proteins directly interact, second protein would also be immobilized on the beads. We have used 3 different GST-fusion of Elk-1, as depicted in Figure 3.4, and microtubules purified from the bovine brain (See Materials and Methods). We hence intially isolated microtubules in two stages. In the first part, microtubules were isolated from the rest of the brain tissue by multiple incubations at 4ºC and 37ºC depolymerizing and polymerizing, respectively, and then centrifugating. After retainment of microtubules as α- and β-tubulin heterodimers, they were purified from MAPs. Since they have a strong affinity to microtubules, in order to completely remove them from microtubules, cationic resin is used. Because microtubules are highly negatively charged proteins, they can easily bind to positively charged proteins or substances. Due to this fact, we used cationic resin and we made microtubules bind to this resin . After elution with highly ionic elution buffer, purified tubulins were collected in test tubes.

After isolation of microtubules is completed, we continued the experiment by expressing different deletions of Elk-1 as GST fusion (Figure 3.4). These deletions were constructed previously (Yang et al., 1998) and was a generous gift of Andrew D. Sharrocks. In these constructs 1-205, 205-329 and 349-428 amino acids were included.

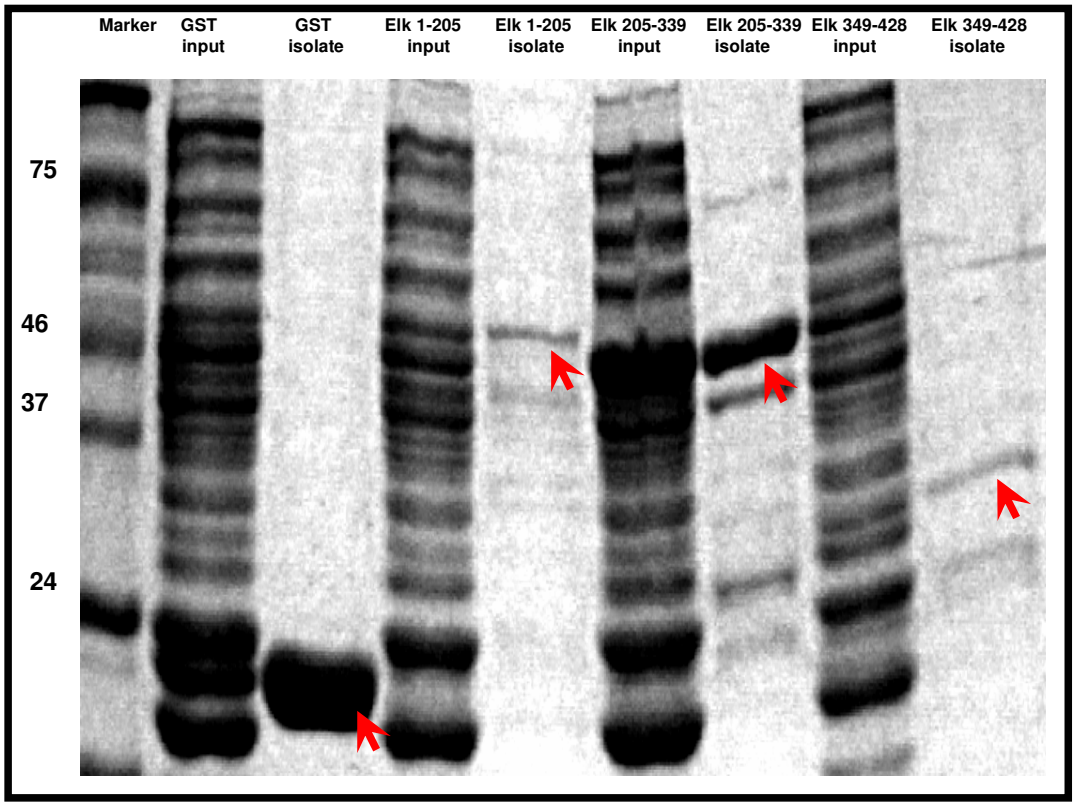

Figure 3.5: SDS-PAGE of isolated GST-Elk-1 fusion proteins.

These proteins were expressed in *E.coli*, as mentioned before. As indicated in the Figure 3.5, proteins were partially purified and protein bands at the expected sizes were confirmed.

After SDS-PAGE of purified GST-Elk proteins, relative protein amounts were estimated by using Quantity One (Bio-Rad) software depending on the pixel number (data not shown). Equivalent amounts of GST and GST-Elk fusion proteins immobilized on glutathione agarose beads were incubated with purified microtubules in the presence of 1 µM GTP. The amount of beads were adjusted by adding empty beads to mixture. After incubation beads were precipitated and detection of bound microtubules was carried out by Western blotting with β-tubulin antibody. As seen in the Figure 3.6, it was observed that microtubules can interact with different regions or domains of Elk-1. Although all the three deletions are shown to associate with microtubules, it appeared that the central region of Elk-1 between 205th and 329th amino acids can interact with microtubules more potently compared to other two regions, because the tubulin band at 55 kDa was more thicker than the other bands.

As input we have included purified microtubules which is in the first lane and as the negative control of experiment, we put only GST. Due to the fact that tubulin was not detected when it was incubated with only GST protein, we can say that the binding of microtubules to GST- Elk-1 and glutathione bead complex occurs by its interaction with Elk-1 protein because in the absence of Elk-1 this association did not occur.

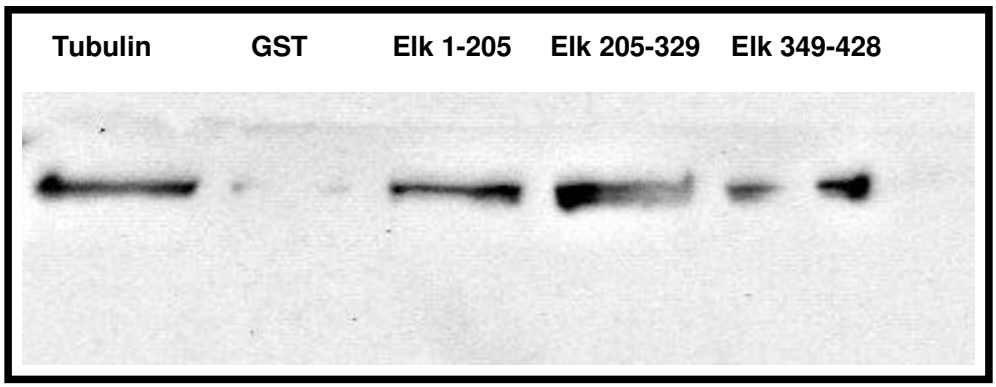

Figure 3.6: Western blotting of GST-Pulldown assay with anti-β-tubulin antibody.

The same GST-pulldown assay was also done with the protein lysate obtained from the whole mouse brain. Because using two highly purified proteins can increase the possibility of a pseudo interaction, it would be more informative to test the same interaction using brain lysates that include many intracellular proteins. GST-Elk-1 fusion proteins were incubated with 500 µg of brain lysate and analysed with anti- β-tubulin antibody in Western blotting. We observed a similar pattern as the previous GST-pulldown assay. β-tubulin was present in the input sample, which is a small aliquot of lysate, and in the three GST-Elk-1 deletion containing pulldown samples. As expected there was not any β-tubulin in GST only negative control (Figure 3.7).

Similar to the GST-Pulldown assay with purified microtubules, microtubules from brain lysates were also present in all three precipitates meaning that they could bind to different regions of Elk-1. However, if we make a relative quantification of microtubules depending on the density of bands, we can say that C-terminal regions of the Elk-1 can bind to microtubules more than the N-terminal part. It should be noted, however, that these assays were not intended to study relative binding affinities, but only to investigate possible binding domains.Altogether, GST-pulldown data suggest that microtubules and Elk-1 can indeed interact *in vitro*.

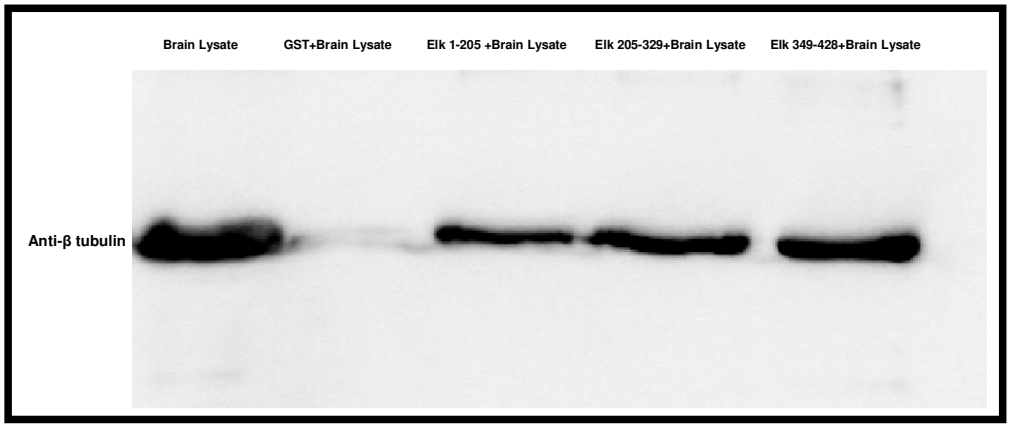

Figure 3.7: GST-Pulldown of microtubule from whole brain lysate with isolated and immobilized GST-Elk fusion proteins.

The second assay that was conducted to analyze the interaction between Elk-1 and microtubules was Microtubule Co-sedimentation Assay. In this assay, we aimed to understand whether Elk-1 could associate with the polymerized microtubules, and if yes which region or regions were involved in this association.

For this purpose, we first polymerized our previously purified tubulin monomers. The polymerization reaction was performed at 37ºC in a buffer containing taxol and GTP as described in Materials and Methods. Taxol is an anti-carcinogenic agent, which is known to stabilize microtubule structure by inhibiting depolymerization of tubulins. After that, polymerized microtubules were incubated with bacterial cell lysates including each GST-fusion Elk-1 protein used in the previous GST-pulldown experiment.

The result of microtubule co-sedimentation assay was monitored by Western blotting technique using anti- β-tubulin and anti-GST antibodies. The results of β-tubulin Western blotting showed that polymerization of microtubules were successful as it is seen that most of the β-tubulin was present in pellet (Figure 3.8).

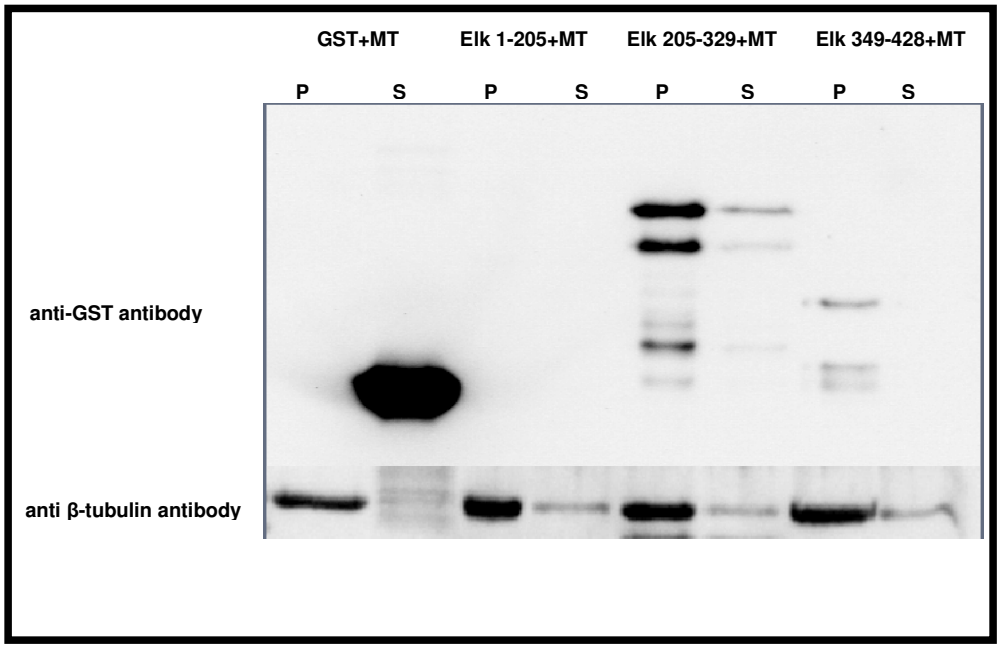

Figure 3.8: Microtubule Sedimentation Assay. Microtubule-bound Elk-1 was detected with anti-GST antibody and microtubule polymerization was detected with anti β-tubulin antibody.

Our microtubule co-sedimentation assay also shows that most of the Elk 205-329 and 349-428 proteins were in the pellet indicating that they were precipitated along with microtubules. Unfortunately, there was not any band either in pellet or supernatant of microtubule and GST-Elk 1-205 incubation. This indicates that either the expression of 1-205 construct or the quality of GST-beads was not good. As a negative control only GST protein was used and it was seen that almost all the GST was in supernatant rather than the pellet indicating that the interaction between GST-Elk and microtubules is specific to the Elk-1 region. These results show that both Elk 205-329 and Elk 349-428 regions can strongly interact with microtubules, although comparison of their binding affinities are yet to be determined.

ELISA-based *in vitro* binding assay was used to confirm the association of Elk-1 to microtubules. For this purpose, 96 well-plate ELISA system was used. To understand which concentration of microtubule is sufficient for coating surface of microtiter plates, we first coated the surfaces with varying amounts (10 µg/ml to 50 µg/ml) of

microtubules. After washing the unbound microtubules, the detection was carried out with anti β-tubulin antibody, as explained in the Materials and Methods.

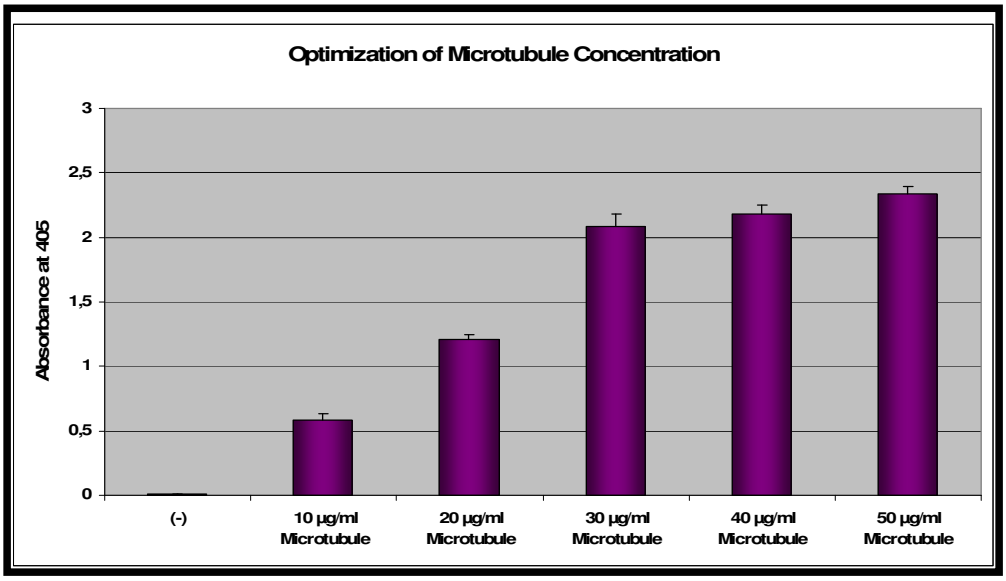

Figure 3.9: Optimization of microtubule concentration for coating.

As a result of the optimization experiment, it was clearly seen that 10 µg/ml and 20 µg/ml microtubule concentrations were not sufficient to completely coat the surfaces (Figure 3.9). However, after 30 µg/ml concentration there was not a significant change in the readings of absorbances meaning that almost all the surface was coated and wells were saturated with microtubules. For this reason 30 µg/ml microtubule concentration was determined as optimum for the rest of the experiment.

After optimization of microtubule concentration for coating, the assay was performed with the total protein lysates of SH-SY-5Y cells overexpressing Elk-1 as described previously. When the results obtained from in vitro binding assay was evaluated, it is very clear that, the binding of Elk-1 increases with a decrease in the dilution ratio or in other words with an increase in the concentration (Figure 3.10). While the absorbance at 405 nm is about zero in negative control, it rises to 0,05 in 1:400 diluted Elk-1 lysate. There was not a significant difference between 1:400, 1:300 and 1:200 dilution ratios. However in 1:100 ratio, a sharp increase from 0,06 to 0,2 was observed. This increase shows that, wild-type Elk-1 protein can interact with microtubules. We did

not observe any signal from negative control which is not coated with microtubules but rather with BSA in the blocking step.

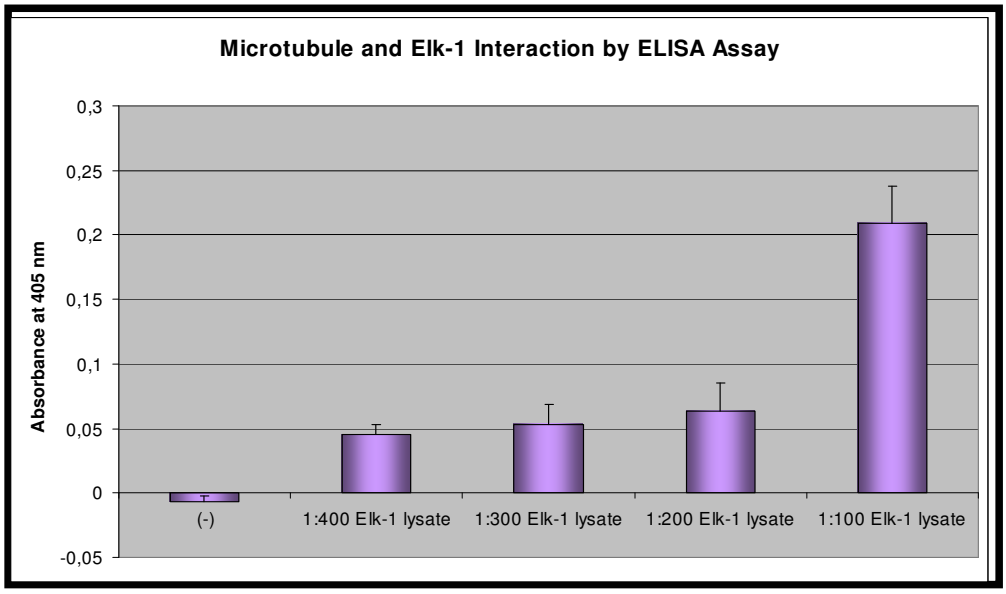

Figure 3.10: *In Vitro* binding of Elk-1 to microtubule-coated microwell plates.

3.3. The Association Between Elk-1 and Microtubules Was Confirmed *In Vivo*

The last experiment that we have conducted was co-immunoprecipitation. In this method, the interaction of expressed exogenous Elk-1 with microtubules were analysed in cell lysates. For this purpose, SH-SY5Y neuroblastoma cell line was transfected with Elk-1 expression plasmid containing the FLAG tag.

As it is seen in Figure 3.11, Elk-1-Flag was successfully immunoprecipitated using Flag agarose beads. Western blotting with anti-Elk-1 antibody showed that Elk-1 was detectable in Elk-1 transfected cells but not to the same level in only pcDNA3 (empty vector) cells. Moreover, it is clear that Elk-1 was successfully immunoprecipitated. On the other hand, β-tubulin was detected in both input samples, but only in the Elk-1-Flag transfected immunoprecipitate. This result once more confirms that, Elk-1 interacts with microtubules *in vivo*. Because microtubules were not detected in pcDNA3 transfected control cells, this indicates that, microtubules directly interact with Elk-1, not with Protein G or Flag tag.

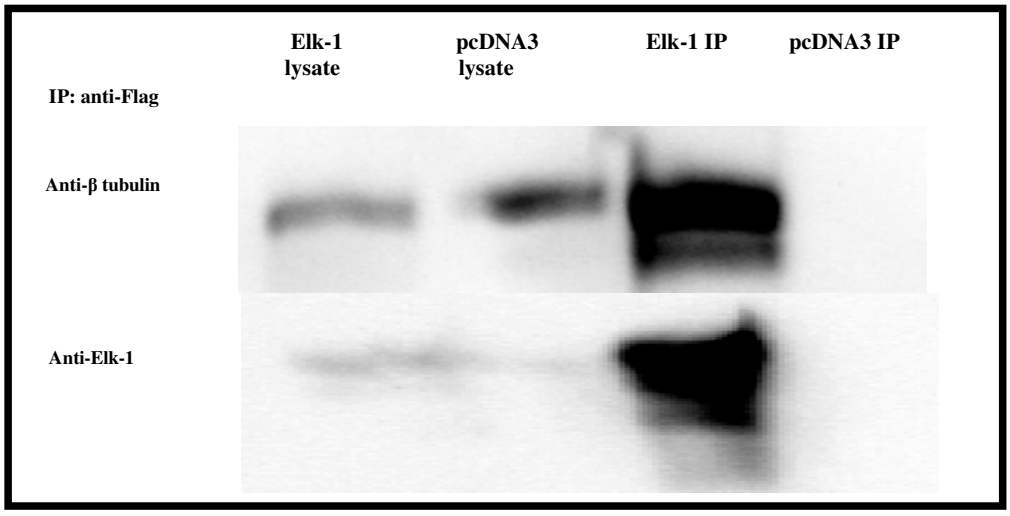

Figure 3.11: Co-immunoprecipitation of microtubule with transfected Elk-1

The interaction between Elk-1 and microtubules in neurons was justified with co-localization and biochemical interaction assays. Co-localization studies in SH-SY5Y neuroblastoma and primary hippocampal cells show that two proteins co-localize in the cell. ELISA-based in vitro binding and immunoprecipitation assays confirm that there is a direct interaction between Elk-1 and microtubules. Finally, our results from GST-pulldown and microtubule co-sedimentation assays indicate that, Elk-1 can bind to microtubules with its different regions, but mostly with C-terminal end.

4. DISCUSSION

In this research, we studied whether the ETS-domain transcription factor Elk-1 could associate with neuronal microtubules. For this purpose, we used both biochemical and molecular techniques such as co-localization of two proteins by immunofluorescence, co-immunoprecipitation of two proteins from the cell lysate and finally to analyze the possible interaction site by using different deletion constructs. At the end of the study, we have demonstrated that Elk-1 co-localizes with microtubules in both SH-SY5Y neuroblastoma and primary hippocampal cells and Elk-1 can bind directly to microtubules both *in vitro* and *in vivo* by pulldown, co-sedimentation and co-immunoprecipitation assays. In this study we also aimed to find the interaction region in Elk-1 structure using three deletion constructs including different domains of the protein but our results indicated that the nature of interaction may not be so simple, different regions of the protein might be involved in this binding. However both microtubule co-sedimentation and GST pull-down assays indicate that there is an enhanced binding between aminoacids 205 and 428 of Elk-1.

Although biochemical assays were all performed in order to identify a domain of Elk-1 that specifically interacts with microtubules, we have unfortunately not been able to pinpoint an exact binding region. However, both GST pull-down assays (Figure 3.6, 3.7) and microtubule co-sedimentation assay (Figure 3.8) indicate an enhanced binding between amino acids 205–428 of Elk-1. Microtubule interaction site of Elk-1 can be predicted by aligning microtubule binding motif to the Elk-1 amino acid sequence (Fig.4.1). Depending on the microtubule binding motifs on various microtubule interacting proteins such as MAPs and motor proteins like kinesins, certain amino acid residues were identified as required for microtubule interaction. These amino acids are mainly lysine (K), arginine (R), proline (P), valine (V) and glycine (G) (Goode et al., 1997). As shown in Figure 4.1 six different microtubule binding sites can be

predicted in the Elk-1 sequence. A consensus binding motif for microtubules has previously constructed based on binding regions of motor proteins, and determined to have the sequence REP**KKVAVVR**TP (Goode et al., 1997). A putative sequence that shows the highest homology among the six predicted motifs on Elk-1 to this consensus sequence appears to be present between amino acids 314 – 325, namely RKPRDLELPLSP in the Elk-1 amino acid sequence. In this sequence, valine residues are replaced by leucines, which are also non-polar amino acids (Figure 4, green box).

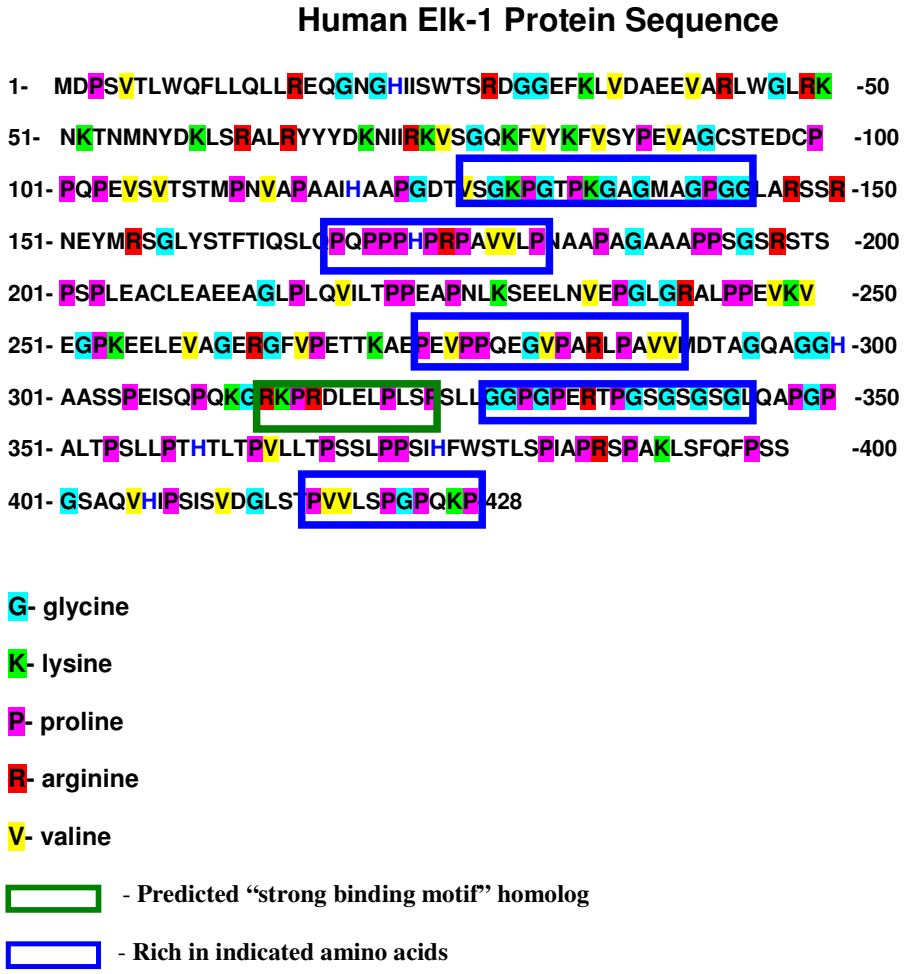

Figure 4.1: Predicted microtubule-binding sites in the Elk-1 protein sequence

In addition to the presence of this “strong binding motif”, there are also other sequences that are rich in K, R, P ,V and G. These sequences are between 126-144, 169-182, 274-290, 329-344, 417-428 amino acids. Since microtubules are acidic proteins, which are highly negatively charged, microtubule interacting proteins can bind to them by their positively charged residues. For this reason, the presence of basic amino acids such as lysine and arginine is important for microtubule interaction. As we look at the strong binding motif homolog in the Elk-1 sequence (green box in Fig.4.1), the first 4 amino acids are “RKPR”, which might be crucial for interaction due to its highly basic nature. To find out which one this predicted motifs is directly related to the microtubule interaction, single or double mutations can be inserted to these sites by site-directed mutagenesis method in the future.

When studying protein:protein interactions, bioinformatics tools are very helpful for analyzing experimental data. Although some predictions can be done by looking at the sequences of proteins, the knowledge of 3-Dimensional structure of protein would say much more about the nature of interaction. At this step, we met with a problem because the whole structure of Elk-1 has not been yet identified. Only the structure of A domain, which is involved in the DNA-binding, interacting with the SRF and DNA was identified (Mo et al., 2000), but the structure of other regions is not yet resolved.

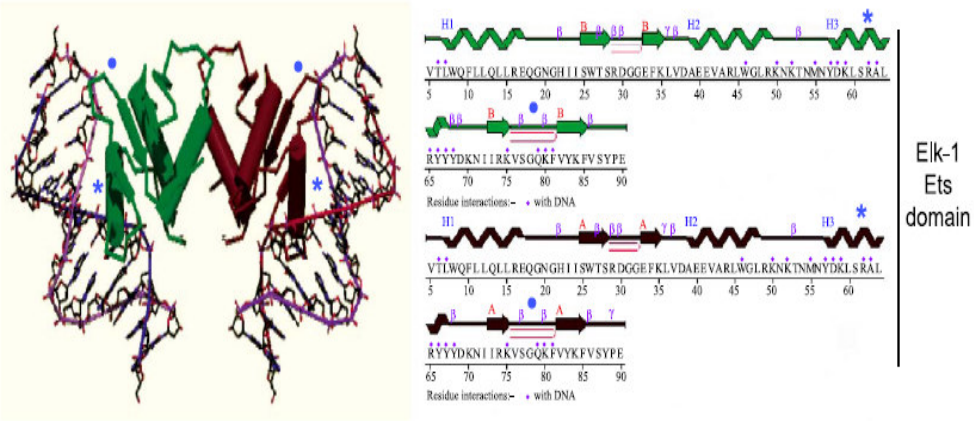

Figure 4.2: The structure of DNA-binding region (A domain) of Elk-1 (Buchwalter et al., 2004).

Having identified a biochemical interaction between Elk-1 and microtubules led us to ask what the physiological function of this interaction could be with respect to neurons. As mentioned earlier, both inactive and active (phosphorylated) forms of Elk-1 is found in cell bodies and axons of neurons (Sgambato et al., 1998). Because of the fact that, Elk-1 is a transcription factor that is mainly involved in the regulation of immediate early gene expression such as *c-fos* and *egr-1,* it is expected to be present mostly in dividing cells. But its presence in a non-dividing cell type shows that, Elk-1 might have other functions than proliferation or survival. On the other hand, microtubules and also other cytoskeletal structures, actin filaments and intermediate filaments, are required not only establish and maintain cell polarity but also provide a scaffold for organizing or translocating signalling molecules by their large surfaces. Therefore, they can serve as a network to bring molecules together to perform specialized functions.

In the previous studies, two transcription factors have been shown to interact with microtubules (Alexandrova et al., 1995, Blackwood et al., 1992, Kato and Dang, 1992). c-myc and NFK-B can associate with microtubules and this interaction important for their proper functioning. In addition to this, c-myc can be stored as bound to microtubules, which may act as a reservoir to sequester the c-myc protein. The same could also be valid for Elk-1, since they have very similar functions and regulation mechanisms - Elk-1 could be held on the microtubules until a relevant signal arrives and then it could be transported to the nucleus in where it performs the transcriptional activation. There is an earlier study that makes this hypothesis stronger. As mentioned in the introduction, the upstream kinase of Elk-1, ERK1/2 is abundantly found in almost all the regions in the cell because it is one of the key signal transduction protein that transmits the signals coming from the extracellular environment. In the previous study conducted by Morishima-Kawashima and Kosik, 1996, ERK has been shown to interact both with microtubules and MAPs and to be able to phosphorylate MAP proteins to reduce their binding to microtubules. As a result of this microtubule stability is disrupted and de-polymerization is favored. Depending on this data, it could be also suggested that, microtubule bound Elk-1 can be activated by ERK upon appropriate signals or de-polymerization of microtubules after phosphorylation of MAPs can make Elk-1 free and accessible to ERK for phosphorylation. This hypothesis would need to be verified in future experiments.

Another possibility of the presence of Elk-1 especially in the axons of neurons might be related to the transmission of messages from the distal regions to the cell body. If the length of an axon is considered, the extracellular environment around the cell body and axon might be different (Figure 1.1). Depending on this, they will recieve different messages that can direct the cell to survive, to become stressed or even to die. Each of these signals have to be transmitted to the nucleus and then integrated, and response at the transcriptional level by up-regulating or down-regulating certain sets of genes. Elk-1 could possibly carry these messages from the axons to the nucleus upon its activation. Moreover, the differences in the extracellular environment may not be the only reason for activation of Elk-1. If we consider the neuronal structure, any injury signal at any region of the neuron should be transmitted to the cell body. There are two significant studies that have been carried out about the retrograde transport of signal transduction protein after an injury signal. It is very well-known that one of the major risks for the survival and functionality of neurons is the neuronal injury, most of the time in the axonal region. Especially, the patients who suffer from stroke are affected from this situation and they cannot move the regions of their bodies controlled by the injured nerves. Neuronal injury induces the local translation of β-importin at injured sites. Synthesized β-importin binds to the activated proteins having NLS sequence. Then β importin form a complex with the α importin and this importin heterodimer-NLS-bearing signalling molecule complex was transported retrogradely with the dynein motor protein on the microtubules (Figure 4.3).

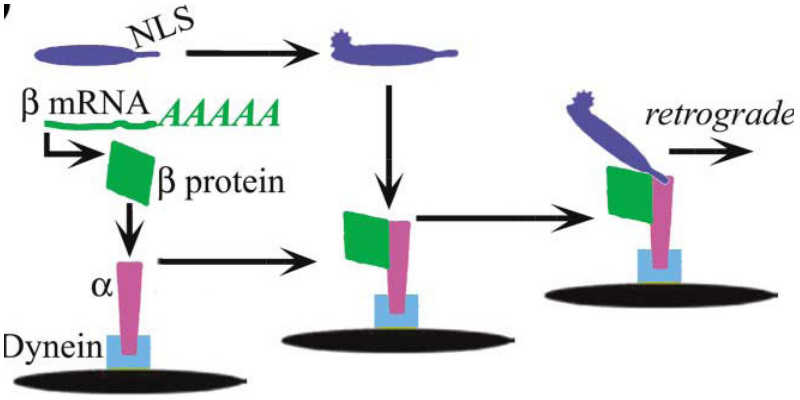

Figure 4.3: Retrograde transport of NLS-containing signalling molecules (Hanz et al., 2003)

Because of the fact that Elk-1 is a nuclear protein in its active form, it has to be transported from cytoplasm to the nucleus after phosphorylation. Elk-1 contains a potential NLS in its A domain, which could also direct the protein to retrograde transport complex. Elk-1 can bind to importin after activation, most of the time phosphorylation, and this binding will recruit Elk-1 to motor proteins and other accessory proteins. The interaction of Elk-1 with the microtubules will make this binding much more efficient. Due to the fact that, both retrograde and anterograde transport complexes assemble and move on the microtubules, microtubule bound Elk-1 will be more close to the importin and motor proteins for the assembly than cytoplasm diffused Elk-1. As related to the transportation of signalling molecules, various scaffolding proteins can interact with motor proteins to facilitate the movement of appropriate signalling molecules, such as JIP 1,2,3, mLin2/7/10, 14-3-3, adaptin and INAD (Verhey and Rapoport, 2001). Among them the interaction between JIP family scaffolding proteins and motor proteins (Verhey et al., 2001) is especially interesting for us, because JIPs are involved in the signal transduction of MAPK pathway. As seen in Figure 4.4, JIP proteins bring the upstream kinases of MAPK, mostly involved in the JNK pathway, to close proximity, so the phosphorylation of kinases happens more rapidly. JIP can also interact with JNK kinase, which can phosphorylate Elk-1. If JIP can bind to motor proteins like kinesin and dynein, it can also potentially bring JNK and as well as Elk-1 to the transportation complex. As hypothesized previously, Elk-1 could thus be transported on the microtubules by participating in transportation complexes after recieving an activation signal. All these hypotheses remain to be addressed in future studies.

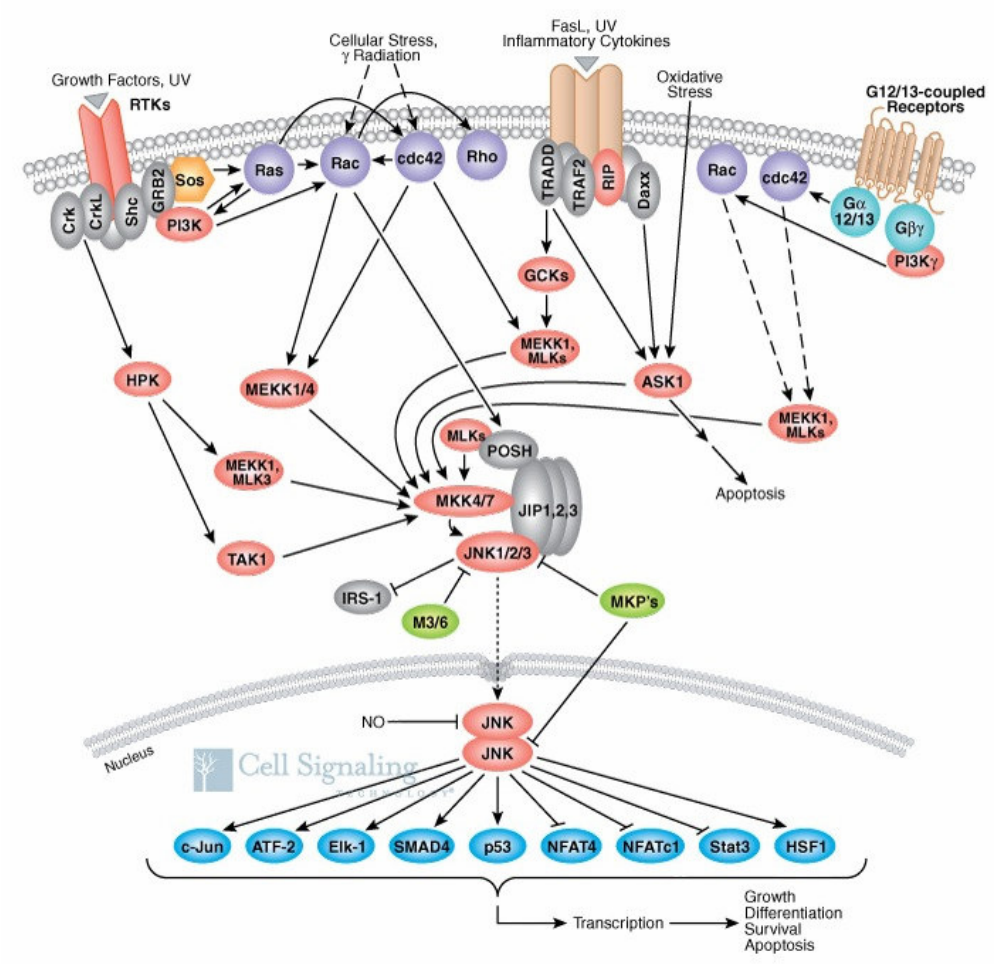

Figure 4.4: Activation of Elk-1 by JNK pathway (URL-5).

SRF, which is the transcriptional partner of Elk-1, has been shown to be activated by cytoskeletal dynamics (Sotiropoulus et al., 1999). Especially, the polymerization of G-actin to F-actin causes the depletion of G-actin in the cytoplasm and this results in the activation of SRF in the nucleus. A similar mechanism can be envisaged for the activation of Elk-1. Depolymerization of microtubules after appropriate signals could change the activity of Elk-1. Because microtubule dynamics is important in the elongation and branching of neurites, signals can thus be transported to and from the growing and branching sites with the help of Elk-1. In addition to this, Elk-1 might even facilitate microtubule stability. In the previous study, the relationship between serum induction and microtubule stability is investigated in 3T3 mouse fibroblast cells (Danowski, 1998) and observed that serum induced factors incerase the stability of microtubules.

5.CONCLUSION

At the end of the study, the interaction between Elk-1 and microtubules has been identified. The co-localization studies showed that, two proteins can localize to the same regions inside the cell in both primary or tumorigenic neurons. Moreover, immunoprecipitation, GST-pulldown and *in vitro* binding assays proved that, Elk-1 can associate with microtubules *in vitro* and *in vivo*. This work has generated even further questions and hypotheses about the function of Elk-1 on the microtubules in relation to various signaling events: for example, the attempt to identify the microtubule interacting region of Elk-1 indicated that six different regions of the protein can serve as microtubule interaction motifs, and it has become apparent that identification of the exact interaction site requires further analyses. Furthermore, the physiological aspect of this interaction can also be studied to find out the effects of microtubules in the Elk-1 activation and signal transmission.

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APPENDIX

Table A.1: Raw data of absorbance measurements at 405 nm of optimum microtubule concentration of coating microtiter plates for *in vitro* binding assay

control	10 µg/ml Microtubule	20 µg/ml Microtubule	30 µg/ml Microtubule	40 µg/ml Microtubule	50 µg/ml Microtubule
0,152	0,82	1,352	2,121	2,345	2,451
0,155	0,763	1,297	2,23	2,369	2,446
0,16	0,672	1,326	2,16	2,312	2,502
0,155	0,741	1,292	2,305	2,405	2,599
0,169	0,745	1,378	2,186	2,285	2,413
0,151	0,723	1,403	2,435	2,399	2,522
0,162	0,725	1,363	2,146	2,32	2,514
0,151	0,658	1,412	2,252	2,184	2,489

Table A.2: Blanked data of absorbance measurements at 405 nm of optimum microtubule concentration of coating microtiter plates for *in vitro* binding assay

control	10 µg/ml Microtubule	20 µg/ml Microtubule	30 µg/ml Microtubule	40 µg/ml Microtubule	50 µg/ml Microtubule
0,002	0,67025	1,20225	1,97125	2,19525	2,30125
0,005	0,61325	1,14725	2,08025	2,21925	2,29625
0,01	0,52225	1,326	2,01025	2,16225	2,35225
0,005	0,59125	1,14225	2,15525	2,25525	2,44925
0,019	0,59525	1,22825	2,03625	2,13525	2,26325
0,001	0,57325	1,25325	2,28525	2,24925	2,37225
0,012	0,57525	1,21325	1,99625	2,17025	2,36425
0,001	0,50825	1,26225	2,10225	2,03425	2,33925

Table A.3: Calculations of average and standard deviations of blanked data

Calculations	control	10 µg/ml Microtubule	20 µg/ml Microtubule	30 µg/ml Microtubule	40 µg/ml Microtubule	50 µg/ml Microtubule
Average	0,007	0,581125	1,22184375	2,079625	2,177625	2,34225
Standard deviation	0,006	0,050921333	0,060787384	0,102750652	0,071556047	0,057296223

Table A.4: Raw data of absorbance measurements at 405 nm of microtubule-bound Elk-1

control	1:400 Elk-1 lysate	1:300 Elk-1 lysate	1:200 Elk-1 lysate	1:100 Elk-1 lysate
0,161	0,209	0,207	0,199	0,369
0,157	0,214	0,214	0,261	0,317
0,154	0,216	0,221	0,219	0,301
0,165	0,219	0,237	0,216	0,394
0,152	0,195	0,246	0,254	0,324
0,158	0,21	0,205	0,235	0,412
0,155	0,202	0,205	0,213	0,423
0,163	0,217	0,21	0,233	0,302

Table A.5: Blanked data of absorbance measurements at 405 nm of microtubule-bound Elk-1

control	1:400 Elk-1 lysate	1:300 Elk-1 lysate	1:200 Elk-1 lysate	1:100 Elk-1 lysate
-0,00462	0,043375	0,041375	0,033375	0,203375
-0,00862	0,048375	0,048375	0,095375	0,151375
-0,01163	0,050375	0,055375	0,053375	0,135375
-0,00062	0,053375	0,071375	0,050375	0,228375
-0,01363	0,029375	0,080375	0,088375	0,158375
-0,00762	0,044375	0,039375	0,069375	0,246375
-0,01063	0,036375	0,039375	0,047375	0,257375
-0,00262	0,051375	0,044375	0,067375	0,136375

Table A.6: Calculations of average and standard deviations of blanked data

Calculations	control	1:400 Elk-1 lysate	1:300 Elk-1 lysate	1:200 Elk-1 lysate	1:100 Elk-1 lysate
Average	-0,0075	0,044625	0,0525	0,063125	0,189625
Std. deviation	0,004549	0,00820714	0,015551183	0,021130547	0,050312594

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