THE ROLE OF ELK-1 IN MITOSIS AND ITS INTERACTION WITH MICROTUBULE BASED MOTOR PROTEINS

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ABSTRACT

THE ROLE OF ELK-1 IN MITOSIS AND ITS INTERACTION WITH MICROTUBULE BASED MOTOR PROTEINS

Elk-1 is a member of the ETS domain superfamily of transcription factors and involved in many biological processes, such as cell growth, differentiation, apoptosis, survival, angiogenesis and cancer. Elk-1 is a protein that is associated with mitogen-induced immediate early gene transcription upon phosphorylation by MAPKs. Recently, the interaction of Elk-1 and microtubules has been shown in neurons in our laboratory. It was further observed that, P-S383-Elk-1 is associated with mitotic spindle poles from metaphase through telophase, and relocates to the spindle midbody during cytokinesis. Microtubules are the dynamic structures that is the main components of the cytoskeleton and important for maintaining cell structure, intracellular transport of organelles, forming the spindle during mitosis and seperation of chromosomes. Many different proteins are identified that bind to the microtubule, including motor proteins, such as kinesin and dynein which are involved in diverse roles during mitosis. In this thesis project, we have analyzed the possible interaction between Elk-1 transcription factor and microtubule motor proteins. Co-immunoprecipitation and GST pull down analyses indicate that Elk-1 interacts with dynein motor protein in response to serum stimulation, independent of Serine 383 phosphorylation. In addition to dynein motor protein, different kinesin family members are analyzed to reveal a potential interaction between Elk-1 and these proteins, but any possible interaction has not yet been observed. Data from our laboratory and this thesis imply that Elk-1 may not only be involved in the transcriptional regulation of anti-apoptotic and proliferative genes and also interfering directly with the cell cycle apparatus.

ÖZET

ELK-1'İN MİTOZDAKİ ROLÜ VE MİKROTÜBÜL TEMELLİ MOTOR PROTEİNLERLE İLİŞKİSİ

ETS transkripsiyon faktörleri ailesinin bir üyesi olan Elk-1, hücre büyümesi, farklılaşma, apoptoz, hayatta kalım, anjiyogenez ve kanser gibi bir çok biyolojik süreçte görev almaktadır. Elk-1, mitojenler tarafından aktive edilen MAPKlerin fosforlamasına bağlı olarak transkripte olan mitozun öncül genleri ile ilişkili olan bir proteindir. Laboratuarımızda yakın zamanda gerçekleştirilen çalışmalar Elk-1'in nöronlarda mikrotübüller ile etkileşim içerisinde olduğunu göstermiştir. Ayrıca S383 bölgesinden fosforlanan Elk-1 in metafazdan telofaza kadar mitotik iğ iplikciğinde bağlı kaldığı ve sitokinez sırasında midbody de bulunduğu gözlemlenmiştir. Dinamik bir yapıpya sahip olan mikrotübüller, hücre iskeletenin temel bileşenleridir ve hücre şeklinin korunması, organellerin hücre içerisinde taşınması, mitozda iğ iplikciklerinin oluşması ve kromozomların birbirinden ayrılması gibi fonksiyonlar için oldukça önemlidir. Bugüne kadar microtübüllere bağlanan birçok protein tanımlanmıştır ve mitoz bölünmede çok çeşitli rolleri olan dynein ve kinezin motor proteinleri bunlara örnek olarak gösterilebilmektedir. Bu tezde, Elk-1 in mikrotübül motor proteinleri ile olan potansiyel etkileşimi incelenmiştir. Immuno çökeltme ve GST çöktürme analizleri Elk-1'in serum uyarısına cevap olarak dynein motor proteini ile birleşiğini ve bu etkileşimin Serin 383 fosforlanmasından bağımsız olduğunu göstermiştir. Dynein proteinine ek olarak, Elk-1 in kinezin ailesinin birkaç üyesi ile potansiyel etkileşimi araştırılmış ancak şu ana kadar böyle bir etkileşim ortaya çıkarılamamıştır. Laboratuarmızda elde edilen sonuçlar ve bu tez çalışması Elk-1'in sadece anti apoptotik ve proliferatif genlerin transkripsiyonel düzenlenmesinde rol oynamadığını ayrıca hücre döngüsü aparatları ile de direkt olarak ilişkili olduğunu göstermiştir.

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LIST OF SYMBOLS / ABBREVIATIONS

AD	Activation domain
AMP	Ampicillin
APS	Ammoniumpersulphate
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
C6	Rat Glioblastoma Cell Line
$CaCl_2$	Sodium Chloride
CENP-E	Centromere Protein E
DC	Cytoplasmic Dynein
DEJL	Docking Site for ERK and JNK LXL
DHC	Dynein Heavy Chain
DIC	Dynein Intermediate Chain
DLC	Dynein Light Chain
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
E26	E-Twenty Six
EDTA	Ethylenediaminetetraacetic Acid
EGR-1	Early Growth Response Gene-1
Elk-1	Ets Like Transcription Factor-1
ERF	Ethylene Response Factor
ERK	Extracellular Regulated Kinase
EtOH	Ethanol
ETS	E-Twenty Six
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
GDP	Guanosine Diphosphate

GST	Glutathione S-Transferase
GTP	Guanosine Triphosphate
HCL	Hydrochloric Acid
HLH	Helix-Loop-Helix Domain
HRP	Horse-Radish Peroxidase
ID	Inhibitory domain
IEG	Immediate Early Genes
JNK	C-Jun N Terminal Kinase
KHC	Kinesin Heavy Chain
KIF	Kinesin Superfamily Proteins
KLC	Kinesin Light Chain
L	Liter
LB	Luria-Bertani
MAP	Microtubule Associated protein
MAPK	Mitogen Activated Protein Kinase
Ml	Milliliters
mM	Milimolar
MT	Microtubule
MTOC	Microtubule Organizing Center
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NES	Nuclear Export Signal
Ng	Nanogram
NGF	Nerve Growth Factor
NLS	Nuclear Localization Signal
nM	Nanomolar
NUMA	Nuclear Mitotic Apparatus Protein
OD	Optical Density
PBS	Phosphate Buffered Saline
PC12	**Rat Adrenal Pheochromocytoma Cell Line**
pH	Negative log of hydrogen ion concentration
PI	Propidium Iodide
PNT	Pointed Domain

PTP	Permeability transition pore
RD	Repression domain
RIPA	Radio Immuno Precipitation Assay
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SH-SY5Y	Human Neuroblastoma Cell Line
SRE	Serum Response Element
SRF	Serum Response Factor
TCF	Ternary Complex Factor
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TF	TransFast™
U87	Human Glioblastoma Cell Line
XKLP-1	Xenopus Laevis Kinesin-Like Protein-1
µl	Microliters

1. INTRODUCTION

ETS family of transcription factors are described in Section 1.1. Detailed information about TCF group and Elk-1 transcription factor is also explained in the same section. Information about the structure and funtions of microtubules are explained in section 1.2. The mitotic spindle and the molecular motors are presented in detail in section 1.3. and 1.4., respectively.

1.1. THE ETS FAMILY TRANSCRIPTION FACTORS

Ets domain transcription factors comprise a large family of gene expression regulators with crucial roles in development and diseases [1]. Most of the members of this family are down-stream nuclear targets of the important signal transduction pathways. Binding to this family of proteins often accompanies with changes in DNA binding, interaction with cellular partners, transcriptional activities, subcellular localization and protein stabilities [2]. The interaction between this group of proteins and other members of transcription factors results in activation or repression on DNA to regulate diverse functions into the cell such as growth, apoptosis, survival, differentiation and oncogenic transformation [3, 4].

Members of this transcription factors family can be characterized by having an evolutionarily-conserved Ets domain of about 85 amino that have a central GGAA/T core consensus and additional flanking nucleotides to mediate binding purine-rich DNA sequences. The conserved region, termed the ETS-domain, corresponds to the DNA-binding domain of these proteins [5].

Ets family proteins can be divided into several subfamilies on the basis of the similarities in amino acid sequences at DNA binding domain and conservation of other domains [6]. Most of the members of this family have the Ets domain in their C-terminal regions, whereas several Ets family proteins such as TCF and ERF subfamilies have their DNA binding domain in their N-terminal regions as shown in Figure 1.1. [7].

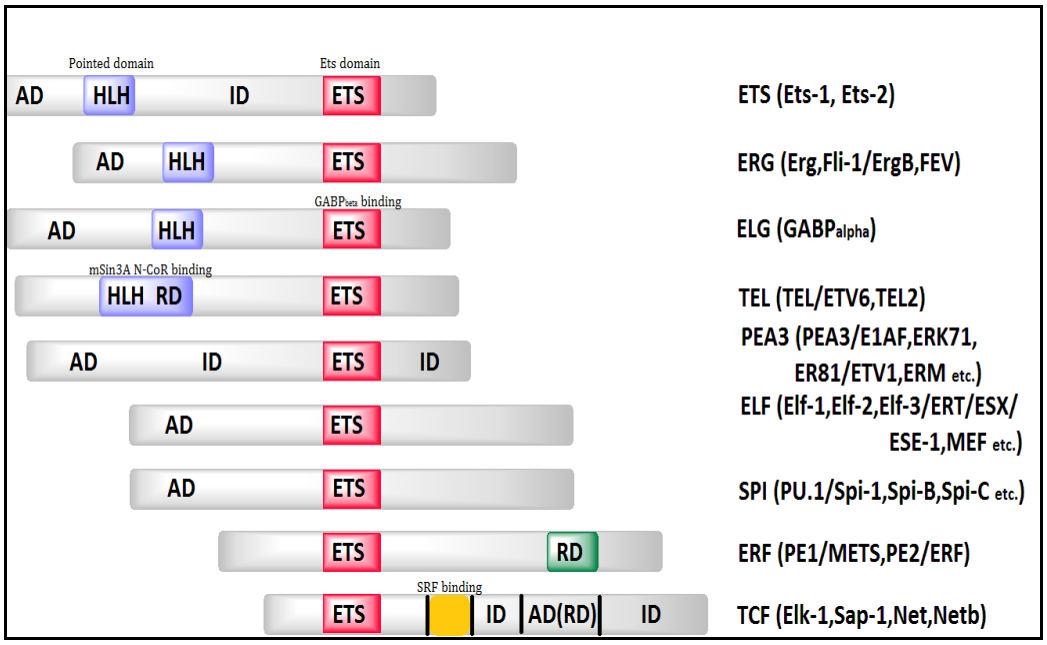


Figure 1.1. Structure of Ets family members. Ets, DNA-binding domain; AD, activation domain; ID, auto-inhibitory domain; RD, repression domain; HLH, helix-loop-helix domain [7]

Ets domain transcription factors family controls crucial biological processes such as cellular proliferation, differentiation, lymphocyte development, haematopoiesis, apoptosis and transformation by either transactivating or repressing the promoter or enhancer of their target genes [8].

Several Ets family transcription factors are expressed in the central nervous system to perform neural cell types specific functions. In Drosophila, two Ets family proteins, PntP2 and Yan have implicated in neuron induction and differentiation in the eye [9]. In mammals, Ets-1 and Ets-2 are expressed in specific regions of the brain to induce neuronal differentiation [10]. It has been reported that induction of the brain specific isoform of Elk-1 by nerve growth factor (NGF) induces neuronal differentiation of PC12 cells. This novel short isoform of Elk (sElk-1) plays an opposite role to the wild-type of Elk-1 in neuronal cell differentiation and proliferation [11].

1.1.1. Ternary Complex Factor Family

TCF (Ternary Complex Factor) family is the most studied group of the ETS domain transcription factors. The first TCF which was called as p62 due to its 62000 Da molecular weight, was identified in the nuclear extract of HeLa cells and it was then shown to be homologous to Elk-1 (Ets-like transcription factor) [12] which forms a ternary complex with SRF (Serum Response Factor) on the *c-fos* promoter [13].

The ternary complex factors family has four conserved regions with high sequence and functional similarities, named A,B,C and D domain. A domain that is located at the N-terminal and corresponds to the DNA binding domain [14]. The B domain which enables TCFs to interact co-operatively with SRF and promotes ternary complex formation, is located downstream of the Ets domain[15]. The C terminal located C domain is an activation domain and activated by phosphorylation by mitogen-activated protein (MAP) kinases [16, 17]. The D domain is a docking site for MAP kinases [18]. The (FXFP motif) is an additional MAP kinase docking site with different binding properties [19].

Phosphorylation of TCFs by MAP kinases stimulates the transcriptional activity and the best known MAP kinase pathways includes ERK (ERK1 and ERK2; extracellular signal regulated kinase), JNK (JNK1, JNK2 and JNK3; c-Jun Nterminal kinase) and p38 (p38a, p38h, p38g and p38y) cascades [20]. Growth factors and mitogens stimulate the ERK cascade whereas cytokines and stress trigger JNK and p38 cascades as depicted in Figure 1.2. [21].

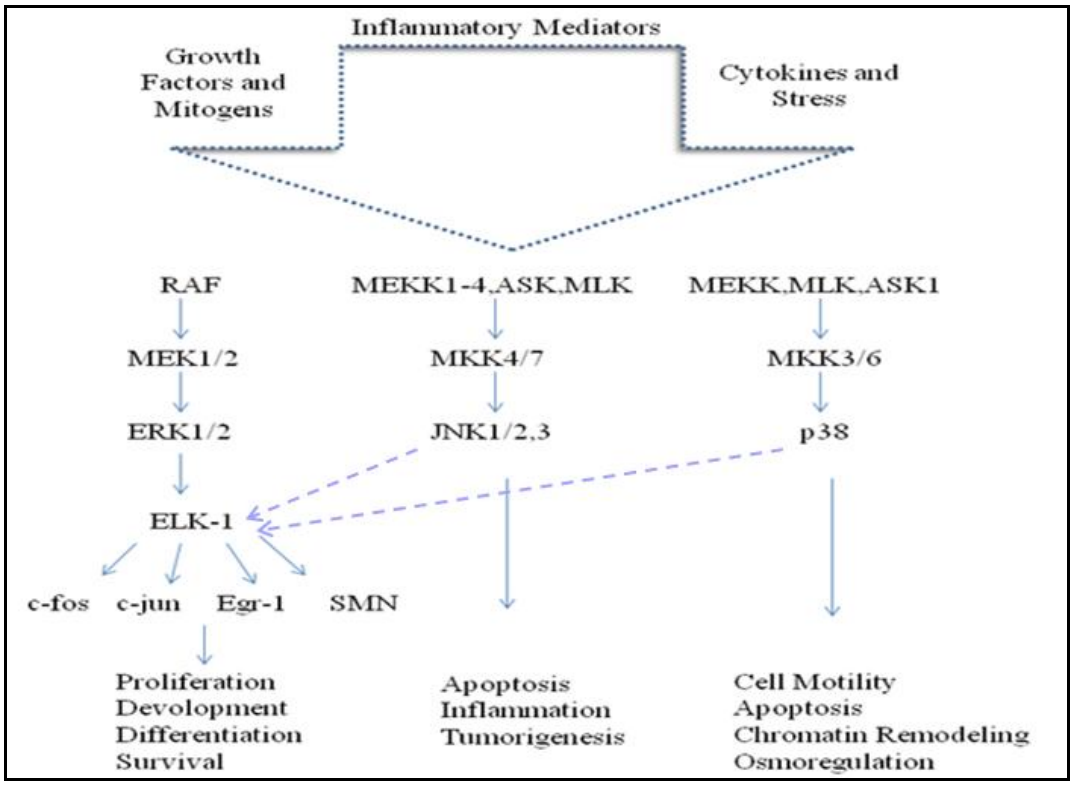


Figure 1.2. Flow chart of the three MAPK modules (ERKs, JNKs, p38) [21,22]

1.1.2. TCF/SRF Interaction

Serum response factor (SRF) is a transcription factor that binds to the serum response element (SRE) in the promoter region of the target genes. This protein regulates the activity of many immediate-early genes, such as *c-fos*, *c-jun*, and *egr-1* and thereby participates in cell cycle regulation, apoptosis, cell growth, and cell differentiation. SRF is the downstream target of many pathways; for example, the mitogen-activated protein kinase pathway (MAPK) that acts through the ternary complex factors (TCFs) [23].

The ternary complex factor proteins form complexes with SRF dimers on SREs found in the *c-fos* and other immediate early genes (IEG) promoters [24]. The TCFs are recruited to the *c-fos* SRE by a combination of protein–protein and protein–DNA interactions. The *c-fos* SRE consists of a weak Ets motif adjacent to an SRF motif, the CArG box, whose consensus sequence is 5V-CC(A/T)6GG-3V as shown in Figure 1.3. [7].

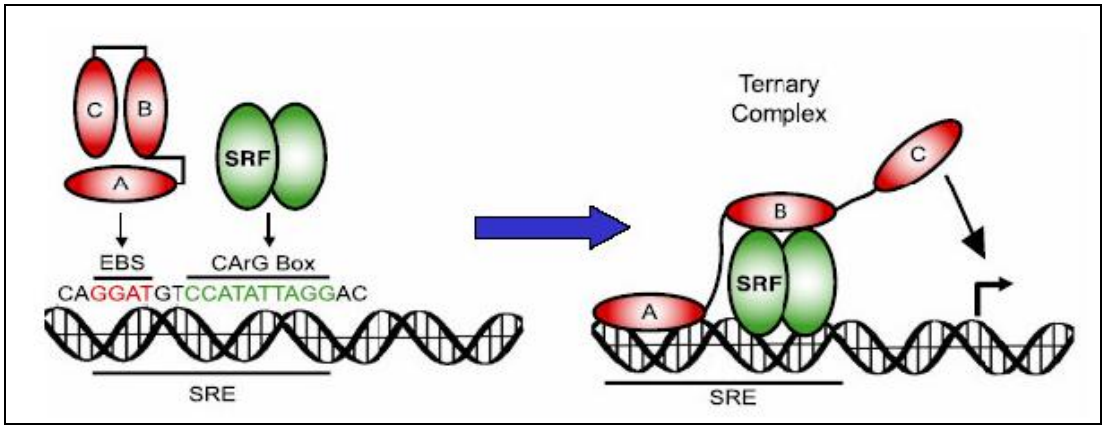


Figure 1.3. Ternary complex factor structure on the SRE site [7]

It has been revealed that SRF has numerous functions in the nerveous system as shown in Figure 1.4. It controls immediate early gene (IEG) activation which is important for learning and memory, synaptic plasticity and differentiation in developing and adult brain [25]. In addition to eliciting immediate transcriptional responses to neuronal activation, SRF also exerts long-lasting cellular effects by modulating actin microfilament dynamics and associated neuronal motility functions [26]. SRF also functions in eary stages of brain developmant such as neuronal induction, neuronal tube formation and neuronal progenitor development [27].

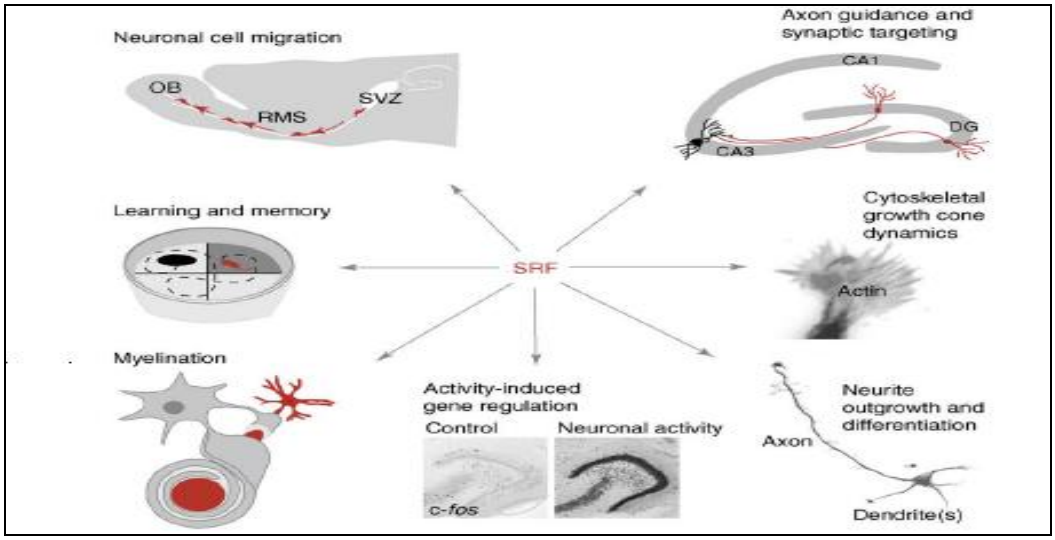


Figure 1.4. Functions of SRF in nervous system [27]

1.1.3. Elk-1 Transcription Factor

Elk-1 is a TCF subfamily member of the Ets transcription factors family and it is involved in many biological processes, such as cell growth, differentiation, survival, hematopoiesis, angiogenesis, wound healing, cancer, and inflammation [28].

As it is mentioned before, each TCFs family members, like Elk-1, contains four conserved domains with high sequence and functional similarities, named A-D as illustrated in Figure 1.5. The A domain which is localized at the N-terminal region, corresponds to the Ets DBD (DNA binding domain). Elk-1 protein also contains a nuclear localization signal (NLS) and a nuclear export signal (NES) within the DNA binding domain that is deleted in an isoform of Elk-1 called short-Elk-1 [11]. The B domain functions in the binding of Elk-1 to a dimer of its cofactor, SRF [29]. The C domain which is a transactivation domain, contains the multiple S/TP motifs targeted by activated MAP kinases [13,30] and within this region there is a docking domain, the FXFP or DEF (docking site for ERK FXFP) motif, which is specifically involved in docking to ERK [18, 31]. The D domain contains the DEJL (docking site for ERK and JNK LXL) sequence that provides binding to MAP kinases of ERK, JNK, and p38 MAPK families [7, 12]. Elk-1 also have R domain is crucial for the repression of Elk-1 transcriptional activity. R domain is rich in lysine residues that are responsible for the inactivation of Elk-1 by SUMOylation, a post-translational event that reinforce the repression of target protein [32, 33].

Elk-1 activation requires the phosphorylation in particular Serine 383 and Serine 389 within the activation domain by the three classes of MAP kinases, ERK, JNK and p38 [27]. When it is activated by phosphorylation, it binds to the target molecules and either activate or repress them. The best-characterized Elk-1 targets are immediate early genes, IEGs, such as *c-fos, junB, egr-1* (early growth response gene-1, also called zif268 or NGF-IA), and *Nur77*, the murine homolog of the human *NR4A1* gene [34]. Its promoter is regulated by mitogens via the SRE which binds the ternary complex formed by two molecules of SRF and one molecule of the Elk-1. The recruitment of Elk-1 to SRE is made by a combination of protein–protein, via its Ets domain, and protein–

DNA, via B box, interactions. The determinant residues in SRF–Elk-1 interaction are Tyrosine 153, Tyrosine 159, and Phenylalanine 162 [35].

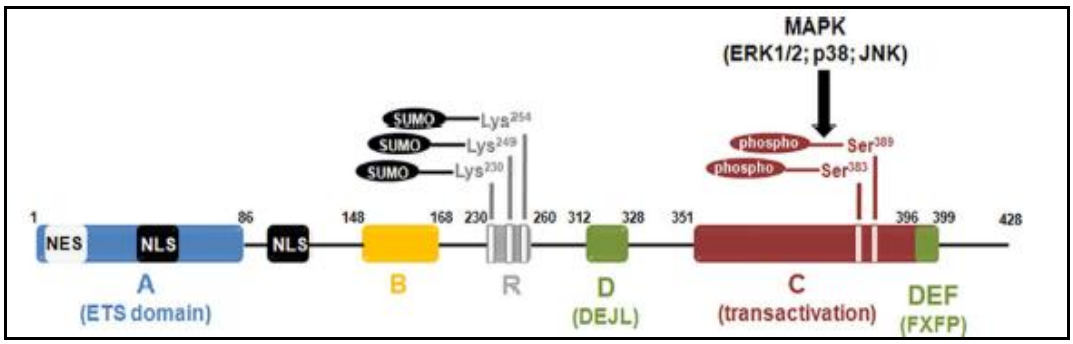


Figure 1.5. Domain structure of Elk-1 [12]

Previous studies performed in rat brain revealed that Elk-1 is expressed in most brain regions, within neuronal and not glial cell types [36]. In non- neuronal cells, Elk-1 is restricted to the nucleus [30, 37], whereas it is expressed in both cytoplasma and nucleus in neuronal cells [38]. As it is mentioned before, the neuron specific isoform of Elk-1, named sElk-1, is restricted to the nucleus and it antagonizes Elk-1 mediated transactivation of SRE and promotes neuronal differentiation [11].

The precise roles of Elk-1 in different cellular compartments are still unclear but it is known that Elk-1 has been implicated in cell survival by protecting cells from apoptosis [39] and downregulating apoptosis-associated genes [40]. In addition to this, Akt-dependent phosphorylation of Elk-1 has been shown to be important for proliferation of glioblastoma cells [41]. However, it has also been shown that Elk-1 binds to mitochondrial permeability transition pore (PTP) and trigger apoptosis in different experimental systems [42].

Recently, it was demonstrated that Elk-1 interacts with neuronal microtubules. It is proposed that Elk-1 is localized to the neuronal microtubules in unstimulated or resting cells and phosphorylation of Elk-1 disrupts its binding to the microtubules, which relocalizes phospho-Elk-1 to the nucleus where it plays an important role in SRE-dependent transcription, promotes neuronal differentiation, and regulates the cytoskeleton dynamics, as depicted in Figure 1.6. [38, 43].

Serum response factor (SRF) behaves as a sensor of cytoskeleton actin dynamics and previous studies which were performed by inhibition of Elk-1 phosphorylation revealed that actin levels are down regulated and growth cone collapse occurred in the absence of Elk-1 [43].

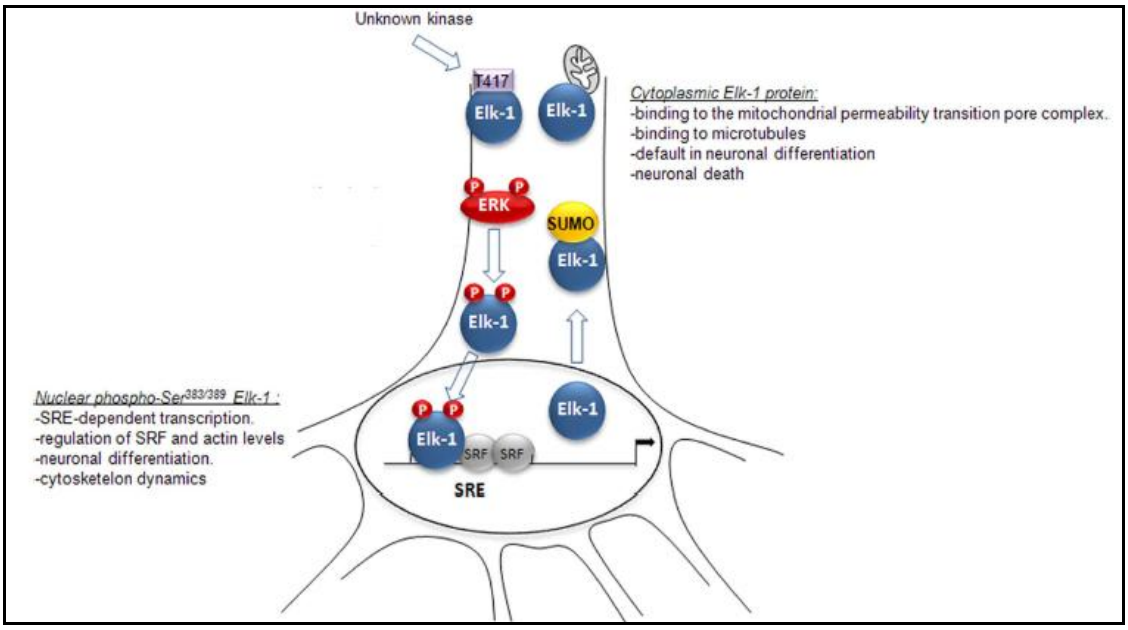


Figure 1.6. Impact of Elk-1 post-translational modifications on its cytoplasm-to-nucleus trafficking and functions in neurons [12]

1.2. MICROTUBULES

Microtubules are hollow cylinders composed of tubulin monomers that have 25 nm diameter and extensively varies in length. Their structure are made of dimers tubulin proteins and these dimers can bound to each other by non-covalent bonds to form microtubule structure. Three classes of protein make up the microtubules which are α-tubulin, β-tubulin and γ-tubulin. While the α-tubulin and β-tubulin proteins come together to form the protofilaments, γ-tubulin plays a role in keeping the stability of the microtubule [44].

In a protofilament, one end of microtubule have α subunit exposed while the other end will have the β subunit exposed. The α subunit exposed ends are known as minus ends

and β subunit exposed ends are known as plus ends. The minus ends of microtubules are capped so coexistence of assembly and disassembly of the tubulin subunits are occured at the plus ends of a microtubule [45].

Each tubulin protein has a GTP (Guanosine Triphosphate) binding site on the plus end surface. This GTP binding site allows for the binding of the next subunit by forming the protofilaments [46]. During polymerization, both the α- and β-subunits of the tubulin dimer are bound to GTP. When β subunit attaches to an α-tubulin, GTP bound β -tubulin is hydrolysed. Only the GTP bound to β -tubulins may be hydrolysed and unhydrolysed GTP provides a site of attachment to the tubulin proteins that will bind in the future. When the β tubulin subunits are hydrolysed, GTP becomes a GDP (Guanosine Diphosphate) which make the β- tubulin subunit prone to depolymerization [47].

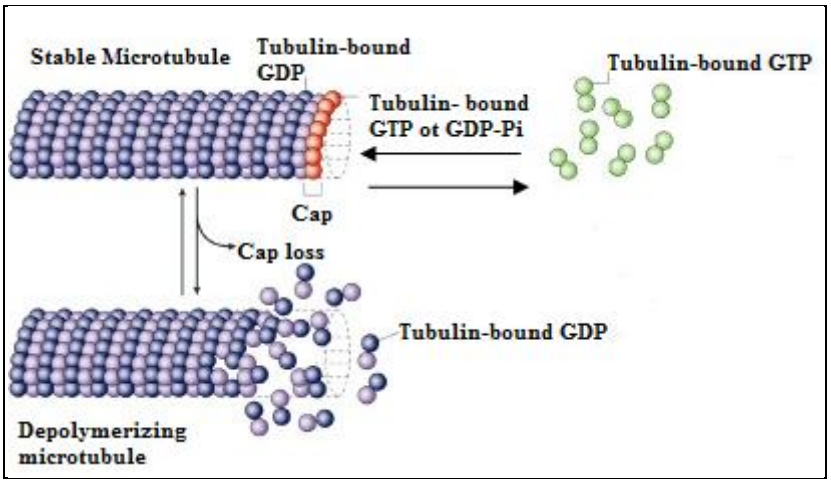


Figure 1.7. The assembly of tubulin proteins into a microtubule structure [45]

Microtubules are involved in different functions including forming an internal transport network for the trafficking vesicles and generating force and movement in motile structures such as cilia and Flagella by microtubule associated motor proteins. Microtubules, with intermediate filaments and microfilaments, are the components of the cell skeleton which determinates the shape and the polarity of a cell by influencing the organization of nucleus, organelles and other cytoskeleton components. Their major

role is organization of chromosomes by initiatiating the assembly of the mitotic spindle [48].

1.3. MITOTIC SPINDLE

After the S phase of the mitosis, the cell contains two sets of chromosomes which will be segregated to the daughter cells. This segregation is carried out by a complex machine called as mitotic spindle that pulls sister chromatids apart and moves a complete set of chromosomes to each pole of the cell. The mitotic spindle is based on a bipolar array of microtubules, the minus end of the microtubule is embedded in a spindle pole and the plus end of the microtubule is pointing outward from the pole. An antiparallel array in the spindle midzone is formed by overlapping plus ends from one pole with plus ends from the other [49].

The spindle contains three classes of microtubules which are kinetochore microtubules, interpolar microtubules and astral microtubules. The function of the kinetochore microtubules is connecting the spindle poles to kinetochores on the sister chromatids and multiple kinetochore microtubules bundle together to form kinetochore fibers. The two spinle poles are linked by interpolar microtubules by interdigitating with each other in the midzone of the spindle. Astral microtubules extend from the poles away from the spindle and are typically involved in anchoring and positioning the spindle in the cell [50].

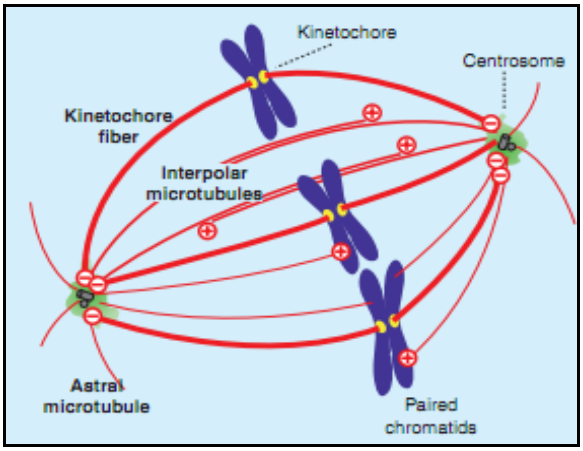


Figure 1.8. Mitotic spindle anatomy [50]

Microtubules are highly dynamic structures that continuously grow and shrink and in the spindle, this behavior is regulated by many different proteins that bind to the microtubules. These are the motor proteins that can travel along microtubules and have crucial roles in the assembly and stability of the microtubule array and the movement of chromosomes on the spindle. The balance of the force generated by motor proteins is the main factor that determines the steady state length of the mitotic spindle [51].

Functional studies in several systems revealed that motor proteins have important functions for microtubule and mitotic spindle self organization. The plus end directed motor proteins, such as Eg5, play a fundamental role in spindle pole seperation and spindle bipolarity due to the cross-linking activity which would bundle microtubules and push antiparallel microtubules apart. The minus-end directed motors proteins counteract the tetrameric kinesins and function to focus microtubule minus-ends at the spindle poles [52, 53]. Figure 1.9. illustrates that plus-end directed kinesins, like Xklp1, localizes to chromosome arms to contribute chromosome attachment and movement toward the metaphase plate, while cytoplasmic dynein can function to orient astral microtubules by focusing microtubule minus ends into the poles [54].

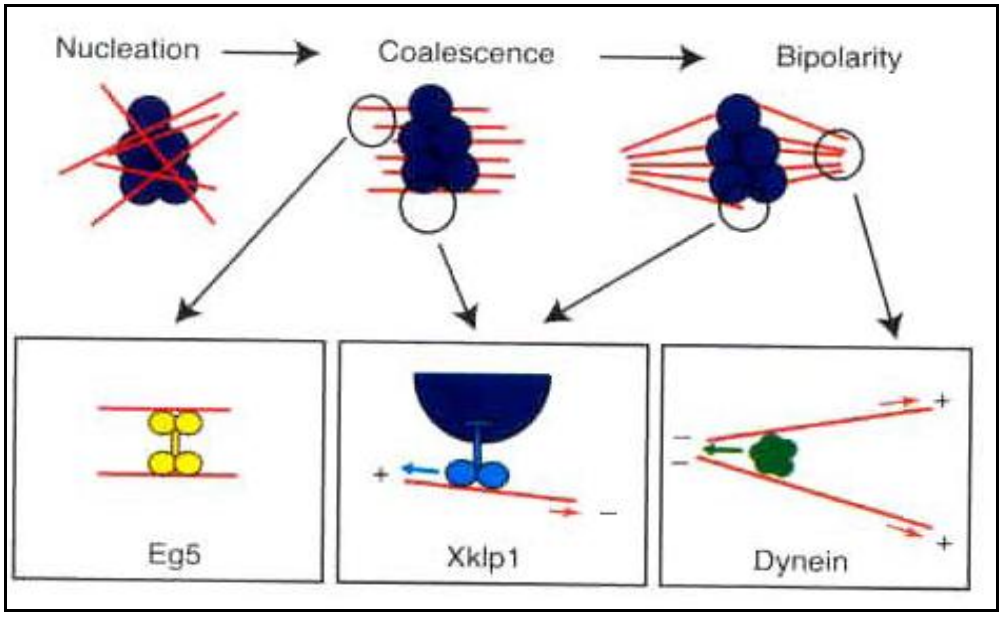


Figure 1.9. Role of motors in spindle self organizations [55]

1.4. MOLECULAR MOTOR PROTEINS

As it is explained above, microtubules function in mitosis, cytokinesis, cell motility as well as maintenance of the golgi apparatus and cellular polarity. Microtubule associated proteins (MAPS) have been thought to play an important role in mediating these functions of microtubules [56]. In neuronal cells, microtubule-associated proteins (MAPs) can be classified into two distinct groups. The first group is involved in microtubule-based movements and they are called as force-producing MAPS or motor molecules. Major components of this group are kinesin and dynein. The second group involves fibrous MAPs that are composed of tau, MAP2, MAP2c, MAP lA, and MAPlB. These proteins promote microtubule assembly and stability and may be involved in the formation and maintenance of neurites [57].

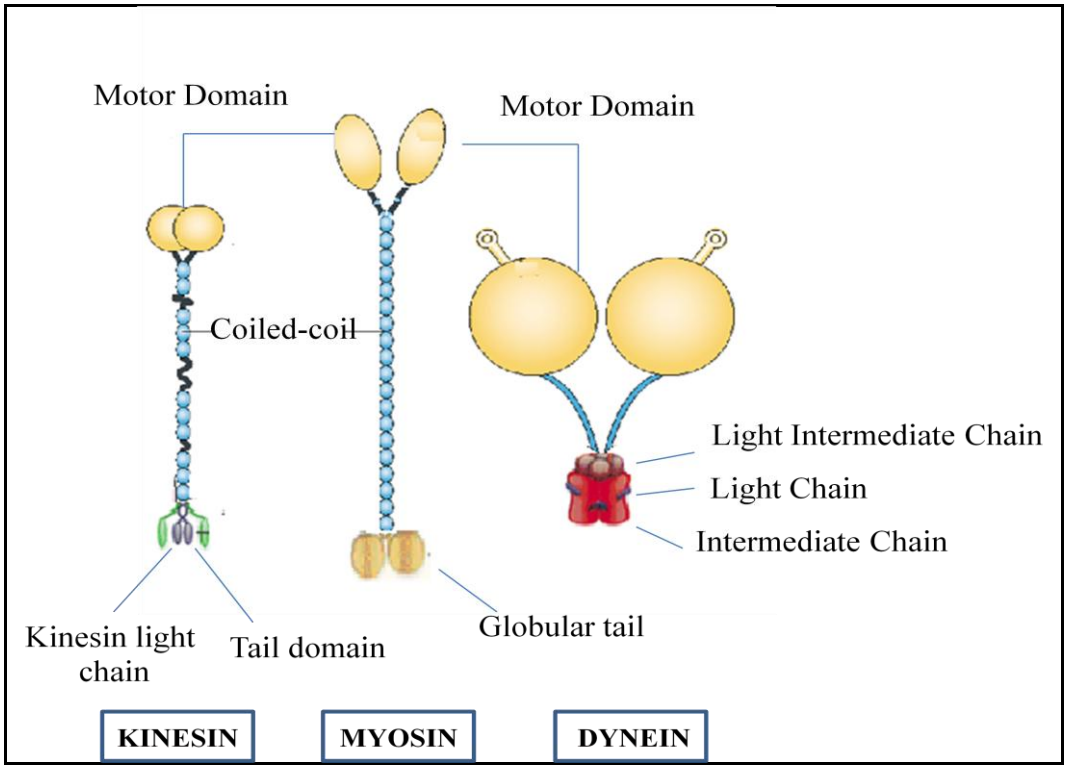


Figure 1.10. Structures of conventional kinesin, cytoplasmic dynein and myosin [58]

Most of the kinesin superfamily proteins (KIFs) move towards the plus end of microtubules, which are known as plus-end-directed motors, and participate in transporting molecules from the cell body to axons and dendrites, known as

anterograde transport [59, 60], whereas cytoplasmic dynein is a minus end-directed motor, transporting molecules from the axonal or dentritic structures to the cell body, known as *retrograde transport* [57, 59].

1.4.1. Dynein Superfamily Proteins

Dynein superfamily member of proteins hydrolyze ATP and produce a force directed to the minus-end of the microtubules [53]. Dyneins can be classified into two groups: axonemal and cytoplasmic dyneins. Axonemal dynein functions in movement of cilia and Flagellae in eukaryotic cells. On the other hand, cytoplasmic dynein is a transport motor and displays diverse roles such as transport of cell organelles, some aspects of chromosome behavior, orientation of mitotic spindle, translocation of nucleus transport and maintenance of golgi [60, 61]. In any case, the cytoplasmic dynein plays the main role in the cell cytoplasm [62].

Cytoplasmic dynein (DC) is a multisubunit complex and its total molecular mass is approximately 1.2 Mda. The complex is composed of two dynein cytoplasmic heavy chains (DCHC or DHC), 530 kDa each, three dynein cytoplasmic intermediate chains (DIC), 74 kDa each, four dynein cytoplasmic light intermediate chains (DLIC), 55 kDA each and several dynein cytoplasmic light chains (DLC), 8 to 20 kDa each [61, 63].

Previous studies indicate that cytoplasmic dynein needs an adaptor protein to perform its function known as dynactin. Dynactin is a multisubunit complex and has been shown to interact directly with cytoplasmic dynein [64]. The main function of dynactin is attachment of cytoplasmic dynein to its cargo [65]. Dynein-dynactin complex plays a key role in the organization of radial microtubule arrays. Dynactin also anchors microtubules at the centrosome without cytoplasmic dynein [66]. Besides, dynactin has been shown to function as an adaptor for at least two motors of the kinesin family: heterotrimeric kinesin-2 [67] and mitotic kinesin Eg-5 [68].

The dynactin complex consists of two morphologically distinct structural domains a rod-shaped domain that binds to the cargo, and an extended projection that mediates an interaction with cytoplasmic dynein and microtubules. The rod-shaped part consists of

an Arp-1 filament which also called centractin. Protein Arp is a homolog of actin and filaments formed by this protein are shorter and more stable. Octamer Arp1 is capped from the one end by dimer of protein CapZ and from the other end, by specific proteins p62, p25, and p27, as well as by one molecule of protein Arp11. However, the projection is formed by a homodimer of a p150*glued* subunit as shown in Figure 1.11. These two parts of the dynactin complex are bridged by the tetramer of a p50 subunit, dynamitin [69]. Microtubule binding by dynactin has been suggested to enhance dynein motor's processivity [70] and also to function at the tips of microtubules by facilitating cargo binding [71].

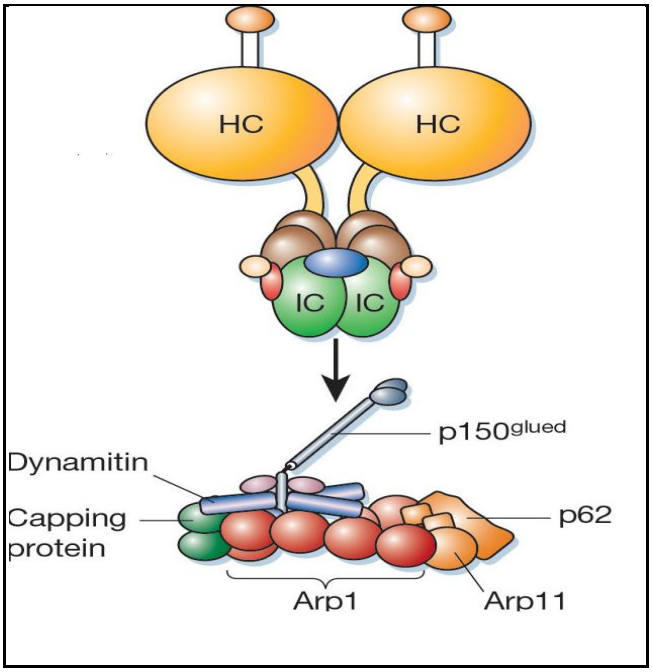


Figure 1.11. Schematic overview of the dynein–dynactin complex [72]

1.4.2. Kinesin Superfamily Proteins

The kinesin sıperfamily is a large family of microtubule dependent-motors which currently consists of 45 members, 10 subfamilies of kinesin-related proteins and rest of them are traditional kinesins. These members are named and classified into four groups according to the positions of their motor domains within the peptide sequence and their functions as KIN N, KIN C, KIN I and orphan kinesins as shown in Figure 1.12. The motor domain of the KIN N kinesins is at the N terminus of the peptide sequence. This

group of kinesins is composed of 39 members comprising several subgroups such as conventional, bipolar, monomeric, heteromeric and chromosome associated kinesins. Many members of this group are associated with the mitotic spindle except conventional KIN N kinesins. The KIN C kinesin motors have their motor domain at the C-terminus of the polypeptide sequence and all of the members of this group have functions in mitosis. The motor domain of the KIN I subfamily is within the peptide sequence and it is known that they modulate microtubule dynamics [73]. The fourth group is the Orphan kinesins whose members have their motor domains at different locations relative to each other [74, 75]

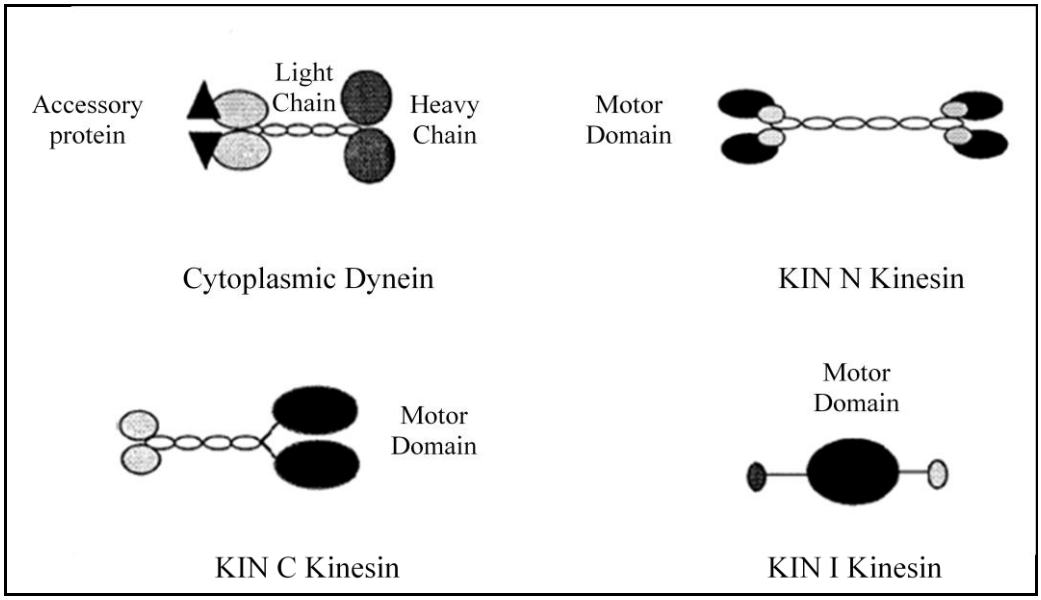


Figure 1.12. Schematic structure of motor proteins [75]

Kinesin family members consist of two heavy chains (120-124 kDa) and two light chains (62-64 kDa). The heavy chains contain binding sites for ATP and microtubules [76]. Previous studies showed that kinesin binds to microtubules by globular domains that contain the heavy chains and attachment sites for organelles are at the opposite, where light chains are located [77, 78]. The role of kinesin was suggested to be that of a motor for anterograde transport because it can produce force toward the plus ends of microtubules [59].

1.4.3. Regulation of Motor Traffic and Attachment

Regulation of the motors must occur in several different contexts and their activity must be coupled in some way to cargo binding. As it is mentioned above, dynein motor activation and attachment to the cargoes are mediated by dynactin complex [64]. For kinesin, there is not enough information about motor attachment and regulation but it is known that motor and enzymatic activities of kinesin-1 are regulated by the tail that is composed of kinesin light and heavy chain [79]. In addition, one potential membrane receptor for kinesin-1 has been identified called kinectin and it may be required for kinesin-1 mediated movements *in vitro* [80, 81].

In neurons, motor proteins must be transported to the synapse in the anterograde pathway before they can perform their retrograde function. Figure 1.13. illustrates three major views that explain the control mechanism of transport direction carried by dynein and kinesin which is described below:

1. Anterograde transport is carried out by kinesins and dynein is inactive and passively carried to the synapse during this process. When they reached to the synapse kinesins are destructed or inactivatied, whereas dynein motor is activated to carry the molecular cargoes back to the cell body [82].
2. Dynein and kinesins are both active during anterograde transport but kinesins which are plus end directed motors are more effective through their higher processivity. When the cargoes reached to the synapse, the plus end directed kinesins are inactivated and this would then free the minus-end motor dyneins to act on their own [82, 83].
3. Regulation and coordination lead to alternating the activation and respression activities of dynein and kinesin motors and the balance controls the direction of the transport [82, 83].

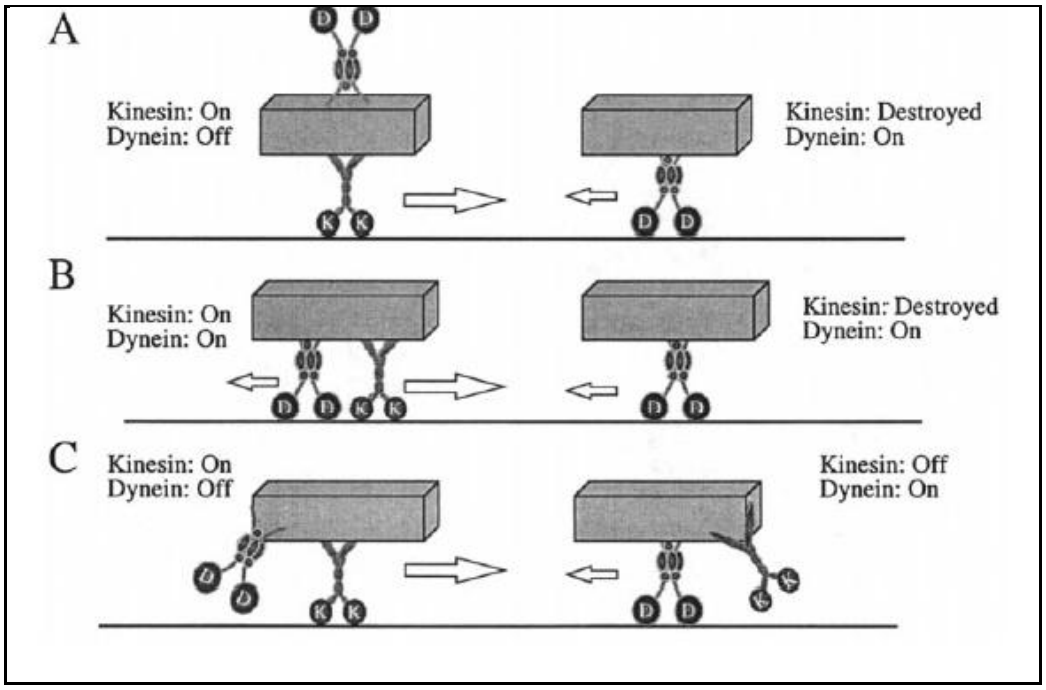


Figure 1.13. Working models for regulation of transport direction (microtubule based transport) [83]

1.4.4. Motor Proteins In Mitosis

Dynein and kinesin superfamily members are essential proteins for the assembly and maintenance of the mitotic spindle. They participate seperating the centrosomes during the early stages of spindle formation, organizing the microtubules within the spindle and chromosome movement as listed in Table 1.1. [84].

1.4.4.1. Centrosome Seperation

During the mitosis, two centrosomes move apart by pushing against one another with the help of the motors sliding towards the microtubules. At least three motors, plus end directed bipolar KIN N kinesin, minus end directed KIN C kinesin and cytoplasmic dynein, are involved in the centrosome seperation which is essential for bipolar spindle formation [85]. Cytoplasmic dynein is essential for the pulling the centrosome apart at the cell cortex which is most important activity for initial seperation of the centrosomes [86]. After the breakdown of the nuclear envelope, KIN N and KIN C kinesins cross

link microtubules derived from the two centrosomes to generate strong microtubule bundles which is necessary for centrosome seperation and spindle bipolarity [85, 86].

1.4.4.2. The Spindle Pole

The mitotic spindle pole is the region in each half of the spindle where the minus ends of the microtubules are collected. One of the essential factor required for spindle pole organization is the minus end directed motor protein dynein. It cross links microtubules to drive the convergence of microtubule minus end at the spindle pole by using its minus end directed motor activity [86].

In addition, dynein delivers the key structural protein NuMA, nuclear mitotic apparatus protein**,** to the spindle poles**.** During interphase, NuMA locates within the nucleus but rapidly redistributes to the separating centrosomes during early mitosis**.** The studies which are performed by using frog egg extract revealed that NuMA associates with cytoplasmic dynein and activating dynactin complex at mitotic centrosomal regions. This NuMA-motor complex binds the free ends of microtubules, converging and tethering spindle microtubule ends to the poles [87].

Besides the cytoplasmic dynein, KIN C kinesin participate in the spindle pole formation and maintanence through the combination of microtubule cross linking and minus end directed motor activity [88].

1.4.4.3. Chromosome Movement

The motor activity of cytoplasmic dynein and kinesin is an essential factor for the chromosome attachment to the spindle and movement during mitosis. The cytoplasmic dynein is a subunit of the outer plate of the kinetochore, the protein structure that links chromosomes to the microtubules from the mitotic spindle, and interacts directly with microtubule plus ends. Cytoplasmic dynein appears at kinetochores in early prometaphase and localizes to the fibrous corona [89]. Kinetochore dynein was initially implicated in the poleward movement of chromatid pairs during early prometaphase. Poleward movement of paired chromatids during prometaphase reflects the initial capture of microtubules to facilitate engagement of chromosomes with the mitotic spindle [90].

Another kinetochore associated motor is the N terminal Orphan subfamily member CENP-E, centromere protein E [91]. CENP-E is located in the corona of the kinetochore and involved in chromosome positioning at the metaphase plate. In addition, it has a role as late prometaphase mitotic arrest associated with unattached or monooriented kinetochores in congression to the metaphase plate [92]. CENP-E has also been implicated in mitotic checkpoint control [93].

1.4.4.4. Microtubule Dynamics

Microtubule dynamics are important for chromosome segregation. Spindle microtubules must be capable of coordinate growth and shortening to promote chromosome attachment and alignment at the metaphase plate and segregation during anaphase. Monomeric KIN I kinesin motors, which are associated with microtubule plus ends at kinetochore, have been implicated in microtubule dynamics. These motors do not move along the microtubules, but directly destabilize the microtubule plus ends. Distruption of KIN I kinesins results in malformed spindles caused by the formation of microtubules that are longer than usual and interfere with spindle [94].

1.4.4.5. Cytokinesis

After the chromosomes have migrated to each spindle poles, the spindle midzone elongates to push two chromosome clusters apart and the cell cytoplasm divides between the chromosomes. The N-Orphan kinesin proteins function in spindle midzone elongation to seperate the chromosome clusters from each other [95]. These kinesins cross link the microtubules and use their motor activities to extend the length of the microtubule bundles by sliding one microtubule relative to the other to make sure that the cleavage furrow is positioned accurately between the two clusters of chromosomes [96].

Table 1.1. Mitotic motor proteins and their functions during mitosis [75]

Class Of Motor Protein		**Individual Class Members**	**Biological Activity During Mitosis**	**Direction Of Motility**
Cytoplasmic Dynein		-	*Centrosome separation and spindle orientation *Microtuble minus end focusing at spindle poles *Microtuble plus end attachment to the kinetochores	Minus
Bipolar KIN N Kinesin		hsEg5-Homo sapiens	*Centrosome and spindle pole body separation *Maintenance of spindle bipolarity *Microtuble minus end focusing at spindle poles *Microtuble crosslinking	Plus
Chromokinesin		Kid-Homo Sapiens	*Microtuble plus end attachment to chromatin *Formation and maintenance of spindle bipolarity *Chromosome segregation	Plus
KIN I Kinesin		MCAK-Homo Sapiens	*Affect microtubule dynamics	Not motile
KIN C Kinesin		HSET-Homo Sapiens	*Microtuble minus end focusing at spindle poles *Microtuble crosslinking *Centrosome separation	Minus
Orphan	N-Orphan-kinetochore	CENP-E Homo Sapiens	*Microtuble attachment at the kinetochores *Chromosome segregation	Minus/Plus?
	N-Orphan-midzone	MKLP-1 Homo Sapiens	*Cytokinesis	Plus

2.MATERIALS

2.1. CELL LINES AND BACTERIAL STRAINS

- C6 – Rat Glioblastoma Cell Line (ATCC number: CCL-107)
- SH-SY5Y – Human Neuroblastoma Cell Line (ATCC numer: CRL-2266)
- U87–Human Glioblastoma Cell Line (ATCC number: HTB-14)
- *E.coli* BL21 (DE) pLysS strain (Promega)
- *E.coli* DH5α strain

2.2. COMMERCIAL KITS AND REAGENTS

- High Pure Plasmid Isolation Kit (Roche)
- Transfast Transfection Reagent (Promega)

2.3. PLASMID CONSTRUCTS

- pcDNA3
- pcDNA3-Elk-1
- pCMV-Elk-1
- pCMV- S383A-Elk-1
- pGEX
- pGEX Elk (1-205)
- pGEX Elk (205-428)

2.4. CELL CULTURE

- Dulbecco’s Modified Eagle Medium (DMEM) 1g/liter Glucose (Gibco)
- Dulbecco’s Modified Eagle Medium (DMEM) 4.5g/liter Glucose (Gibco)
- Fetal Bovine Serum (Gibco)
- L-Glutamine Solution (100X) (Gibco)

- Antibiotic-Antimycotic Solution, 100 X (Gibco)
- 0.5% Trypsin-EDTA Solution (Sigma)
- Phosphate Buffered Saline (PBS) (Gibco)
- Hemocytometer (Bright-Line)
- T25 Tissue Culture Flasks (Nunc)
- T75 Tissue Culture Flasks (Nunc)
- T150 Tissue Culture Flasks (Nunc)
- Coverslip (Isolab)

2.5. BACTERIAL ASSAYS

- Ampicillin (AppliChem)
- Chloramphenicol (Applichem)
- IPTG (Invitrogen)
- Luria-Bertani (LB) Agar (AppliChem)
- Luria-Bertani (LB) Broth (AppliChem)
- Petri Plates (Isolab)
- Phenylmethylsulfonyl fluoride (PMSF) (AppliChem)
- Sodium Deoxycholate (Sigma)

2.6. PROTEIN ASSAYS

- 2-Mercaptoethanol (Gibco)
- 3MM Filter Paper (Whatman)
- 3X Blue SDS Loading Buffer (Cell Signaling Technologies 7722S)
- Anti-Biotin HRP Linked Antibody (Cell Signaling Technologies #7075)
- Anti-Flag Affinity Gel (Sigma)
- Biotinylated Protein Ladder (Cell Signaling Technologies #7727)
- Cell Culture Plates (TPP or Grenier-Bio)
- Filter Paper (Millipore)
- Glutathione Agarose Beads (Bio World)
- Goat Anti-Mouse IgG Antibody-HRP Conjugate (Pierce)

- Goat Anti-Rabbit IgG Antibody-HRP Conjugate (Pierce)
- Mouse Anti-Human Eg5 Antibody (BD Bioscience)
- Mouse Anti-MKLP1 Antibody (BD Bioscience)
- Mouse Monoclonal Anti- Dynein Antibody (Millipore MAB 1618)
- Mouse Monoclonal Anti KLC1 Antibody (Santa Cruz Biotechnology sc-58776)
- Nitrocellulose Paper (Schleicher and Schuell)
- Phosphatase Inhibitor Coctail (Roche)
- Prestained Protein Molecular Weight Marker (Fermentas)
- Protease Inhibitor Coctail (Sigma)
- Protein A Agarose Beads (Roche)
- Rabbit polyclonal anti-Elk-1 antibody (Santa Cruz Biotechnology sc-22804)
- RIPA Buffer (Sigma)

2.7. LABORATORY TECHNICAL EQUIPMENT

- 0.5 ml tube (Beckman)
- 1.5 ml tube (Beckman)
- 2 ml tube (Beckman)
- -80 °C freezer (Wisecryo)
- Autoclave (HV-85 – HICLAVE)
- Blot Transfer (Trans- Blot SD Semi-Dry Transfer Cell- BioRad)
- Chemiluminescence Imaging System (Bio-Rad)
- CO_2 Incubator (Nuaire)
- Heater (DRI-Block DB.2A–Techne)
- Inverted Phase Contrast Microscope (Nikon)
- Laminar flow cabinet (ESCO Labculture Class II Biohazard Safety Cabinet Type 2A)
- Magnetic Stirrer (RH Basic- KIKA Labotechnic)
- Microcentrifuge (Micro 1224–Hettich Zentrifugen)
- Microwave (MD 553–Arçelik)
- Mini-PROTEAN Tetra Cell Electrophoresis System (Bio-Rad)
- Refrigerator (Nu- 6512E- NuAire and Surround Flow- Arçelik)

- Semi-Dry Transfer System (Bio-Rad)
- Shaker (Innova 4330 – New Brunswick Scientific)
- Sonicator (Bandelin Sonorex)
- Spectrofotometer (Multiscan Spectrum- Thermo Labsystems)
- Ultra-speed Centrifuge (Avanti J-251 - Beckman)
- Water Bath (Memmert)

2.8. CHEMICALS

- 29:1 Acrylamide:Bisacrylamide (AppliChem)
- 2-propanol (AppliChem)
- Acetic Acid (AppliChem)
- Ammoniumpersulphate (APS) (AppliChem)
- Bovine Serum Albumin (BSA) (Sigma)
- Bromophenol Blue (Applichem)
- Calcium Chloride ($CaCl_2$) (AppliChem)
- Dithiothreitol (DTT) (AppliChem)
- Glycine (Promega)
- HCl (AppliChem)
- Methanol (Sigma)
- NaCl (AppliChem)
- NaOH (AppliChem)
- Non-Fat Dry Milk (Marvel)
- Ponceau S Stain (AppliChem)
- Propidium Iodide (Sigma)
- Sodium Chloride (NaCl) (Sigma)
- Sodium dodecyl sulphate (SDS) (AppliChem)
- TEMED (AppliChem)
- Tris (AppliChem)
- Triton X-100 (AppliChem)
- Tween-20 (AppliChem)

3. METHODS

3.1. CO-IMMUNOPRECIPITATION (CO-IP) ASSAY

3.1.1. Cell Culture Conditions and Maintanence

Two different cell lines are used for these experiments; SH-SY5Y human neuroblastoma cell line and U-87 human glioblastoma cell line. SH-SY5Y cell line was mainly used in transfection experiments through its high transfection efficiency. SH-SY5Y cell line needs low glucose medium (1g/liter glucose), whereas U-87 cell line needs high glucose medium (4.5g/liter glucose) to grow.

SH-SY5Y culture was maintained in DMEM containing 10% FBS, 1X L Glutamine and 1X antibiotic-antimycotic solution (Anti-Anti). Cells were grown in low glucose DMEM medium at 37ºC in 5% CO2 incubator until they reach to confluency. Cells were usually splitted every 2-3 days by tyripsinization. The same protocol except using high glucose DMEM medium was applied for U-87 cells.

The media that cover the cells was discarded and cells were washed once with PBS solution containing 2.7 mM KCl, 10 mM sodium phosphate dibasic, 137 mM NaCl and 2 mM potassium phosphate, pH 7.4. The cells were detached with the addition of trypsin (0.5 g/l)-EDTA (0.2 g/l) solution (1 ml for T-25 flasks, 2.5 ml for T-75 flasks, and 5 ml for T-150 flasks) and incubated at the flask for 3-5 minutes at 37°C. The detached cells were collected in a centrifuge tube and 2 volumes of media were added. After centrifugation of the mixture at 1500 x g for 5 minutes, the supernatant was discarded and the cell pellet was resuspended in growth media (5 ml for T-25 flasks, 15 ml for T-75 flasks and 25 ml for T150 flasks) to be seeded cells in a new flask.

3.1.2. Cell Counting

After suspenion of adherent cells by trypsinization, the trypsin-cell mixture was transferred to a 15 ml falcon and 5 ml medium added into it. After homogenizing the

solution, 10 µl of solution was pipetted out onto a haemocytometer and the cells were counted under microscope at 10X magnification.

Four big squares located at the corners of the haemocytometer were chosen to count the cells. The resulting number was divided into four to take the mean cell number. The total number of cells per ml was calculated by multiplying the total number of cells found by 10^4.

3.1.3. Cell Freezing

After trypsinization and cell counting, cell suspension was centrifuged at 1500 x g for 5 minutes and supernatant was removed. The cell pellet was resuspended in culturing media. $1x10^6$ cells were dissolved in 900 µl media and after addition of 100 µl DMSO, the mixture placed in cryovials to be transferred to -80°C for short term storage. For long-term storage, the cells were transferred to liquid nitrogen.

3.1.4. Cell Thawing

The frozen cell vial was taken from -80°C freezer or liquid nitrogen and was rapidly thawed at 37°C for 90 seconds. Cell solution was transferred to a T-25 flask which contains 5 ml growth media. The following day, culture media was removed; cells were washed with PBS, and fresh media were added.

3.1.5. Cell Staining With Propidium Iodide

Approximately 1.10^6 cells were suspended in 0.5 ml of PBS and then centrifuged at 600g for 5 minutes. 4.5 ml 70 % ethanol was added onto the cells and kept at 4 Cº for 2 hours. The ethanol suspended cells were centrifuged for 5 minutes at 300g and ethanol was decanted thoroughly. Cell pellet was suspended in 5 ml PBS and waited 30 seconds and centrifuged at 300 g for 5 minutes. Cell pellet was suspended in 1 ml PI staining solution and kept in the dark at room temperature for 30 minutes. Sample was transfered to the flow cytometer and cell fluorescence was measured.

Table 3.1. PI staining solution

Contents	Final Concentration
Triton-X-100	0.1 % (v/v)
Propidium Iodide (PI)	10 µg/ml
DNase-free RNase A in PBS	100 µg/ml

3.1.6. Transient Transfection

A day before the transfection experiment, SH-SY5Y cells were counted and 1.5 $x10^6$ cells were seeded on the 10 cm cell culture grade petri dishes with 10 ml DMEM low glucose complete medium.

Transfection mixtures were prepared in 15 ml falcon tube by mixing 1.5 ml serum-free DMEM low glucose medium, 5 µg plasmids and 15 µl transfection reagent, TransFast™ (TF). The concentration of each plasmid stock was 100 ng/µl. TF amount was adjusted depending on the ratio 1µg DNA: 3 µl TF.

The transfection mixtures were vortexed and incubated for 15 minutes at room temperature. When the incubation time completed, 6 ml serum free medium was added into the tubes. The complete medium in the petri dish were aspirated and the transfection mixture was added directly to the cells. The plates were incubated at 37°C for 1 hour.

After 1 hour, 4ml of DMEM low glucose complete medium was added to each culture dish and they were placed into the 37°C incubator for 2 days.

3.1.7. Protein Lysate Preparation

300 µl of lysis buffer was prepared for each dish by mixing 1X RIPA cell lysis buffer, Phosphatase and Proteinase Inhibitors (1:100) and DTT (final concentration 1mM). Complete medium was aspirated from the dishes and they were washed with 5 ml ice-cold PBS. 300 µl of lysis buffer was dispended to dishes and cells were scraped using

the rubber part of a syringe. Cells in lysis buffer were transferred to 1.5 ml eppendorfs and kept on ice for 15 minutes. They were centrifuged at 4°C for 15 minutes at 15000 rpm. Supernatant was kept and protein concentration was determined with Bradford assay.

3.1.8. Bradford Assay

For determination of the protein concentrations, 10 µl lysate and 990 µl Bradford reagent were mixed, vortexed and incubated 10 minutes at room temperature. The mixtures were transferred to plastic cuvettes and OD values were detected at 595 nm. The concentrations were calculated according to the standard curve equation [y (absorbance) = 0.0177x (protein concentration) + 0.0925].

3.1.9. Immunoprecipitation

U87 glioblastoma cells were spread on 10 cm cell culture dishes at 15.10^5 cells/dish. By the time the cells reach confluency, they were rinsed with ice-cold PBS and treated with 100 ng/ml nocodazole for 16 hours. Then, nocodazole included medium was discarded and cells rinsed with ice-cold PBS. After PBS discarded, complete medium was added into the dishes and cells were collected after 0, 30, 60 and 90 minutes. Cells were centrifuged at 12,000 rpm at 4°C for 5 minutes. The pellet was lysed in lysis buffer that was prepared with RIPA buffer, phospahatase inhibitor coctail and protease inhibitor coctail and incubated 15 min on ice and then centrifuged at 14,000 rpm at 4 °C for 15 min. Protein concentrations are determined as described above and 250 µg total protein was incubated with 2µg mouse monoclonal anti-dynein antibody, mouse monoclonal anti-KLC1 antibody or with rabbit polyclonal anti-Elk-1 antibody for overnight at 4 °C.

Protein A agarose beads were precleared by washing 2X with lysis buffer and 2X with PBS. 50 µl protein A slurry was added into the protein lysate-antibody mixture and incubated for 1 hour at 4°C on a rotator. The tube was centrifuged at 2500g for 30 seconds at 4°C. Supernatant was carefully removed and beads were washed with 1X lysis buffer and 3X with ice-cold PBS. After the last wash, supernatant was removed and beads were dissolved in 20 µl SDS loading buffer. They were heated at 95°C for 5

minutes and then centrifuged at 10.000g for 5 minutes to pellet the beads. The beads were stored at 4°C until the Western blot.

To perform Flag-IP, SH-SY5Y cells were transfected with 5 μg pCMV Elk-1, pCMV-S383A-Elk-1 and empty pcDNA3 plasmids by using transfast reagent as decsribed in the transfection part. After 2 days, cells were lysed in lysis buffer and protein concentrations were determined with Bradford assay. 250 μg total protein was incubated with 30μl Flag-Agarose beads for overnight at 4°C. Beads were collected and washed three times with lysis buffer and two times with PBS. After the last wash, beads were dissolved in 20 μl SDS loading buffer and heated at 95°C for 5 minutes and then centrifuged at 10.000g for 5 minutes to pellet the beads. Beads were stored at 4°C until the Western blot experiment.

3.2. GST PULL-DOWN ASSAY

3.2.1. Competent Cell Preparation

Competent cells have more easily altered cell walls that enable foreign DNA molecules can be passed through the cell membrane easily. By a process which uses calcium chloride and heat shock, *E.coli* bacteria can be made competent. For the isolation of GST fusion proteins from the bacteria, BL21 (DE) pLysS bacteria strain was used.

BL21 (DE) pLysS bacterial stock was thawed and 1 μl of bacteria was transferred to 10 ml steril LB broth that does not include any antibiotics. Cells were allowed to grow in shaker at 37°C for overnight. Next day, 1 ml inoculsum was taken and added into 50 ml LB broth without any antibiotics. This culture was incubated at 37°C for about 3.5 hours. The culture was centrifuged at 5500 rpm for 5 minutes at 4°C and supernatant was discarded. The pelleted bacteria was gently resuspended in 5 ml ice-cold $CaCl_2$ (100 mM) and incubated 1 hour on ice. This mixture was centrifuged at 2000 rpm for 5 minutes at 4°C and supernatant was discarded. The pellet was resuspended in 1 ml ice-cold $CaCl_2$ and stored at 4°C until it was used.

3.2.2. Preparation of LB Agar Plates

40 grams of LB Agar powder was weighed and it was mixed with 1L dH_2O .The solution was autoclaved at 121°C for 15 minutes. After the sterilization process, 1 ml ampicillin (200mg/ml) was added to the LB Agar. 15-20 ml of LB Agar was poured into each petri plate and waited for the cooling.

3.2.3. Transformation

50 µl of competent cells mixed with 500 ng DNA. Bacteria-DNA mixture was kept on ice for 15 minutes.Then, they were placed on a 42°C heater for 60 seconds and again to ice for 2 minutes. 200 µl of Amp (-) LB-Broth was added to the tubes and they were were incubated at 37°C for 1 hour. After an hour, the mixture in each tube was spread on LB Agar plates containing 33 µg/ml chloramphenicol and 50-200 µg/ml ampicillin.The plates were inverted and placed in an incubator at 37°C for overnight.

3.2.4. Induction of Protein Expression and Protein Isolation

One colony from the plate that contains desired plasmids was inoculated into 10 ml LB medium with ampicillin and chloramphenicol at the concentrations indicated above for overnight at 37ºC with 200 rpm shaking. Next day, 250 ml of LB Broth containing ampicillin and chloramphenicol were inoculated with 2 ml of overnight culture and growed until OD_{600} is 0.5-0.7. Then, bacteria were induced for GST-fusion protein expression with 0.5 mM IPTG and grown for 4 hours.

After 4 hours of protein production, bacteria were collected by centrifugation at 5000 rpm for 10 minutes. Pellets were resuspended in ice-cold PBS containing 1:1000 protease inhibitor cocktail, 1 mM sodium orthovanadate and 1 mM PMSF as phosphatase inhibitors to prevent protein degradation. Bacteria were lysed by sonication in the ultrasonic water bath for 2 minutes X5.

After sonication, %1 Triton X 100 and 1mM DTT were added into the tubes and incubated on ice for 30 minutes. Then the lysate was cleared by centrifugation at 4 ºC,

and 15000 rpm for 15 minutes. 100 µl of lysate was stored at -80 ºC for further analysis as an input.

200 µl of GST-Agarose beads were precleared with PBS and mixed with 1 ml of cell lysate. This mixture was incubated at 4 ºC on shaker for 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 the GST pulldown assay.

3.2.5. Pulldown from total protein lysate

Previously isolated GST-protein-beads were incubated with 500 µg protein 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 resuspended in the SDS sample buffer and waited at 4°C until the Western blot experiment.

3.3. WESTERN BLOT

3.3.1. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.3.1.1. Preparing the Glass Setup

Initially, the glass plate was assembled on a clean surface. After all of the components were cleaned with 70% ethanol (EtOH), the longer glass was laid down first, and then 2 spacers were placed along the sides. Next, the shorter glass plate was placed on top of the spacers and glass plates were aligned. The glass plate sandwich was placed onto the casting stand and was tightened with the help of two clips. The setup was checked with the addition of dH_2O. When there was no leakage, the next step was performed.

3.3.1.2. Casting the SDS Polyacrylamide Gel

10% Resolving Gel was prepared according to the Table 3.2. The solution was split between two glass plates. The surface was flattened by pouring isopropanol onto the gel. The gel was incubated at room temperature for 30 minutes to solidify. Upper part of

the gel was washed with dH_2O to remove isopropanol. To continue, 5% Stacking Gel was prepared according to the Table 3.2. and split between two glass plates. The 10-well comb was immediately placed into the stacking gel by avoiding air bubble formation and gel was left to polymerize at room temperature for 1 hour.

Table 3.2. Components of resolving and stacking gel buffers

10% Resolving Gel		**5% Stacking Gel**	
Component	**Volume (µl)**	**Component**	**Volume (µl)**
dH_2O	4000	dH_2O	2100
30 % Acrylamide	3300	30 % Acrylamide	500
1.5 M Tris pH 8.8	2500	1.5 M Tris pH 8.8	380
10 % SDS	100	10 % SDS	30
10 % APS	100	10 % APS	30
TEMED	4	TEMED	3

3.3.1.3. Sample Preparation and SDS-PAGE

Samples for SDS-PAGE were prepared according to the amounts mentioned in Table 3.3.

Table 3.3. Sample preparation for SDS-PAGE

Procedure			
Content	**Western Marker**	**SDS-Marker**	**Sample**
Protein	-	-	40 µg
2X Laemmli Buffer	-	-	Depends on the protein concentration
Boil for 5 min. at 95°C	Boil for 3 min.	Do not boil	Boil for 5 min.
Loading sample into the well	10 µl	5 µl	20 µl

After the stacking gel polymerized, combs were removed from the gels and the glass plates were placed into SDS-PAGE tank filled with 1X Tank Buffer which prepared according to the amounts mentioned in Table 3.4. Laemmli buffer added protein samples and protein markers were loaded to the wells carefully. Lid of the tank was placed on top of the chamber and the connection was checked. Voltage was 100V until the samples run through the stacking gel and it was adjusted to 150V when the samples reached to the resolving gel part until the dye front reached to the bottom of the gel.

Table 3.4. Components of 1X tank buffer

Contents	**Amount**
Tris	63.2 g
Glycine	40 g
SDS	10 g
dH_2O	up to 1 liter

When the dye front reached to the bottom of the gel (after approximately 2 hours) gel was removed from the buffer chamber and clips were taken out to release the glass plates. The spacers were removed gently and by utilizing a scalpel the upper glass plate was separated from the lower glass plate. The stacking gel and the bottom part of the gel were cut out.

After size separation of proteins in SDS-PAGE, the gel was either stained with Coomassie or Silver stain and its picture was taken or it is directly processed for Western blotting, as described below.

3.3.1.4. Coomassie Brillant Blue Protein Staining

Subsequent to the electrophoresis, the apparatus was disassembled and the gel was washed off the glass plates with distilled water. After washing, the gel was covered with staining solution that described in Table 3.5. and incubated on shaker for 30 minutes at room temperature. After thirty minutes incubation, the gel was de-stained by the boiling with distilled water for a few minutes. After enough de-staining, the picture of the gel was taken with Bio- Rad Chemi-Doc. system.

Table 3.5. Gel staining solution

Contents	Final Concentration
Methanol	45 %
dH2O	45 %
Acetic Acid	10 %
Bromophenol Blue	0.2 %

3.3.1.5. Blotting

4 pieces of filter paper and nitrocellulose membrane were cut at the same size of the gel and transferred to a container containing 1X transfer buffer which prepared as described in Table 3.6. SDS gel was also transferred to 1X transfer buffer and kept in that buffer for about 10 minutes.

Table 3.6. Components of 1X Transfer Buffer

Contents	Amount
Tris	14 g
Glycine	3g
dH_2O	up to 1 liter

2 pieces of filter paper was placed on anode plate of Bio-Rad Semi-Dry Transfer Apparatus by avoiding trapping air bubbles. Nitrocellulose membrane was removed from the Transfer Buffer and placed on top of Whatman paper stack by avoiding trapping air bubbles. SDS-PAGE gel was placed on top of the membrane. 2 pieces of filter paper was placed on SDS-PAGE gel as depicted in Figure 3.1. Cathode plate of the blotter was placed on top of the transfer stack.

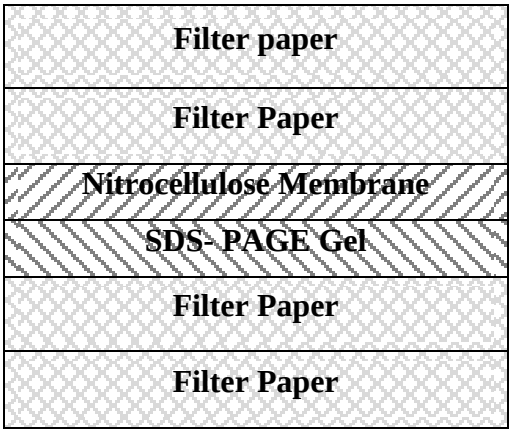


Figure 3.1. Schematic representation of blotting

Bio-Rad Power Supply was adjusted to 10V until the amper decreased and then it was adjusted to 20V, the transfer was carried out for 90 minutes. After the transfer time finished, membrane was submerged in a container containing Ponceau S stain for 1 minute to detect whether blotting was successful or not. Membrane was washed with dH_2O many times in shaker untill getting rid of the stain.

3.3.1.6. Immunostaining

Unoccupied protein binding sites on membrane was blocked by placing the membrane in blocking buffer composed of 5% non-fat dry milk or in 3% BSA prepared in 1X TBS-T. 10 ml of blocking buffer was added into the membrane and incubated at 4°C for 1 hour on a shaker. After one hour, blocking buffer was discarded and primary antibodies, Mouse Monoclonal Anti-Dynein Antibody (1:10000), Mouse Monoclonal Anti KLC1 Antibody (1:1000), Rabbit Polyclonal Anti-Elk-1 Antibody (1:1000), Mouse Anti-MKLP1 Antibody (1:1000) and Mouse Anti-Human Eg5 Antibody (1:1000), that were prepared in blocking buffer were added onto the membranes and the membranes were incubated at 4°C shaker for overnight.

The following day, membranes were washed in 1X TBS-T solution for 15 minutes. This was repeated for 3 times. Secondary antibody solution was prepared by adding HRP Conjugated Antibody (Pierce) (1:5000) to 1X TBS-T solution. Membranes were incubated in secondary antibody solution for 1 hour on shaker. Next, they were washed

in 1X TBS-T for 5 minutes. This was repeated for 5 times and after this step membranes were ready for imaging.

3.3.1.7. Imaging

SuperSignal West Pico Chemiluminescent Substrate (Pierce) was prepared by mixing stable luminal solution and stable peroxide solution together at a one-to-one ration (500 µl each). Membrane was transferred in a plastic membrane protector and the freshly-prepared substrate was poured on it. The membrane was analyzed with Bio- Rad Chemi-Doc equipment using Image Lab software.

4. RESULTS

4.1. CO-IMMUNOPRECIPITATION OF ELK-1 AND DYNEIN

Immunoprecipitation (IP) is a technique that precipitates a protein out of a solution using a specific antibody that binds to desired protein. This technique enables to isolate and concentrate a particular protein from a sample containing thousands of different proteins. Co-Immunoprecipitation (Co-IP) assay, used to analyze protein-protein interactions, involves the precipitation of intact protein complexes. The mechanism of Co-IP assay works by selecting an antibody that targets a known protein that is believed to be a member of a larger complex of proteins. Precipitation of large complexes works when the proteins involved in the complex bind to each other tightly.

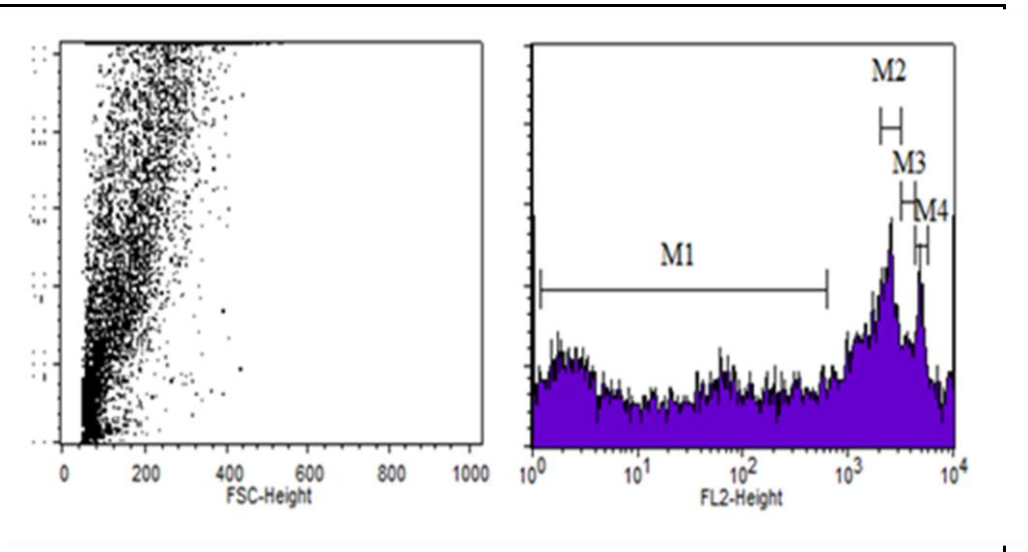
To examine the interaction of Elk-1 with dynein, which was rather elusive in immunofluorescence experiments, we initially used biochemical analyses. U-87 glioblastoma cells were treated with 100 ng/ml nocodazole, an anti-neoplastic agent which exerts its effect in cells by interfering with the polymerization of microtubules, for 16 hours in order to arrest cells at G_2/M transition for synchronizing them. After incubation time completed, nocodazole included medium was discarded and cells rinsed with ice-cold PBS. Complete medium was added into the dishes for inducing the cells to enter mitosis and cells were collected after 0, 30, 60, 90 minutes in an effort to arrest cells in different stages of the cell cycle.

Nocodazole untreated cells were used for the detection of interphase cells. The input, whole proteins, are used as a positive control in order to justify the existence of Elk-1 protein into the different cell lysates such as interphase (normal dividing cells), prometaphase (time=0), metaphase (30th minute), anaphase (60th minute) and telophase (90th minute).

In order to figure out whether the cells are arrested at G_2/M transition or not, DNA of the cells was stained with propidium iodide (PI) which is an intercalating agent and a fluorescent molecule that can be used to stain the nucleus of cells. When excited with

488 nm wavelength light, it fluoresces red and this fluorescence helps to identify cell viability and DNA content in cell cycle analysis by flow cytometry. PI is commonly used for identifying dead cells in a population. After PI stainig of the cells, results were identified by using flow cytometry.

a.



Marker	Left, Right	Events	% Gated	% Tota	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 991(	6615	100.00	100.00	1446.78	147.63	150.59	321.97	1
M1	1, 62(	3151	47.63	47.63	99.25	23.12	150.75	25.03	1
M2	2017, 3191	739	11.17	11.17	2482.65	2462.80	12.87	2414.42	2436
M3	3191, 4294	332	5.02	5.02	3736.81	3722.26	8.84	3718.03	3428
M4	4294, 5777	335	5.06	5.06	4836.81	4821.28	8.15	4740.03	4613

Figure 4.1. Flow cytometry analyses of the cells

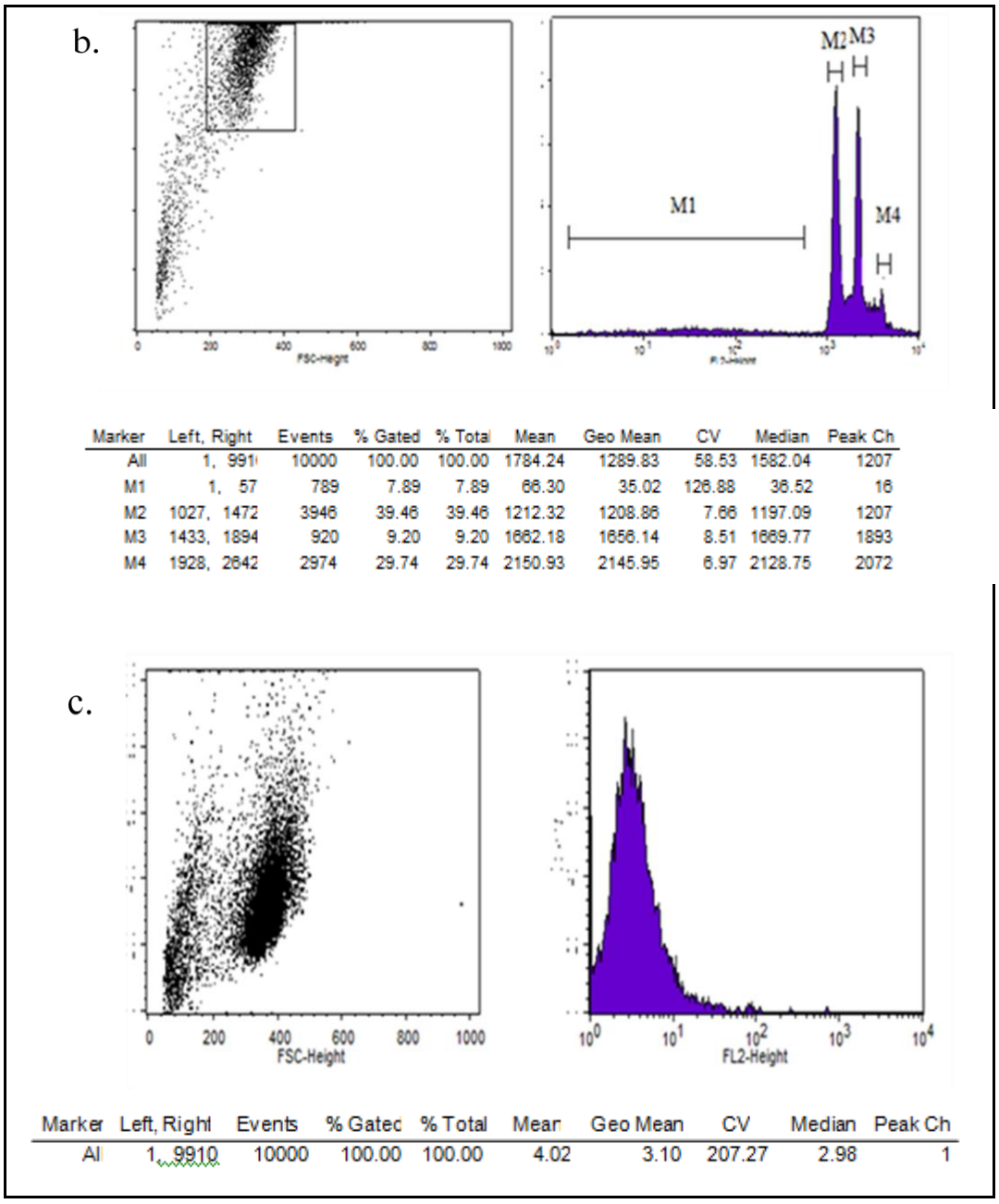


Figure 4.1 Flow cytometry analyses of the cells (continue)

Figure 4.1.a indicates the analysis of the cells that were treated with nocodazole to cease the cell cycle at G_2/M phase. Nocodazole untreated cells were used to compare the results which shown in Figure 4.1.b. Figure 4.1.c is a negative control which includes the cells that were not stained with PI. M values of the histogram statistics indicate the cell cycle phases of the cell given in Table 4.1.1.

Table 4.1. Evaluation of flow cytometry analysis

Region	**Phases**
M1	Apoptosis
M2	G_0/G_1 Phase
M3	S Phase
M4	G_2/M Phase

% Gated values demonstrated that nocodazole treatment resulted in apoptosis in 47.63 % of the cells as shown in Figure 4.1.a. 11.17 % of the cells are at G_0/G_1 phase and 5.02 % of cells are at S phase. The cells that are arrested at G_2/M phase comprise 5.06 of whole cells. These data indicates that after the nocozadole treatment, enter of the most of the cells to the mitosis were halted. When compared to the Figure 4.1.a, apoptotic cell value, 7.89, is markedly decreased in Figure 4.1.b which contains untreated cells. The percentages of M1, M2 and M3 values are 39.46, 9.20 and 29.74, respectively. These results indicate that the cells which are not treated with nocodazole as control, shown in Figure 4.1.a, are at different phases of cell cycle, as expected. Since Figure 4.1.c does not contain any PI stained cell, there are no results showing the different cell cycle phases.

After nocodazole treatment and cell cycle analysis, cell lysates were prepared by using RIPA buffer and they were precipitated by using anti-total Elk-1 antibody and Protein A coupled agarose beads. Subsequently, the possible interaction of Elk-1 protein that was isolated from total cell lysate by immunoprecipitation and dynein was tried to be clarified by using SDS-PAGE and western blot analysis as shown in Figure 4.2.a.

For reverse IP, previously isolated proteins were used to precipitate dynein protein. To that end, protein lysates were incubated with anti-dynein antibody for overnight. After one day, protein A agarose beads were added to the mixture to attach the dynein and dynein-bound proteins to the beads. Proteins attached to the beads were isolated by using SDS loading buffer and then analyzed with western blot experiment by using anti-total Elk-1 antibody.

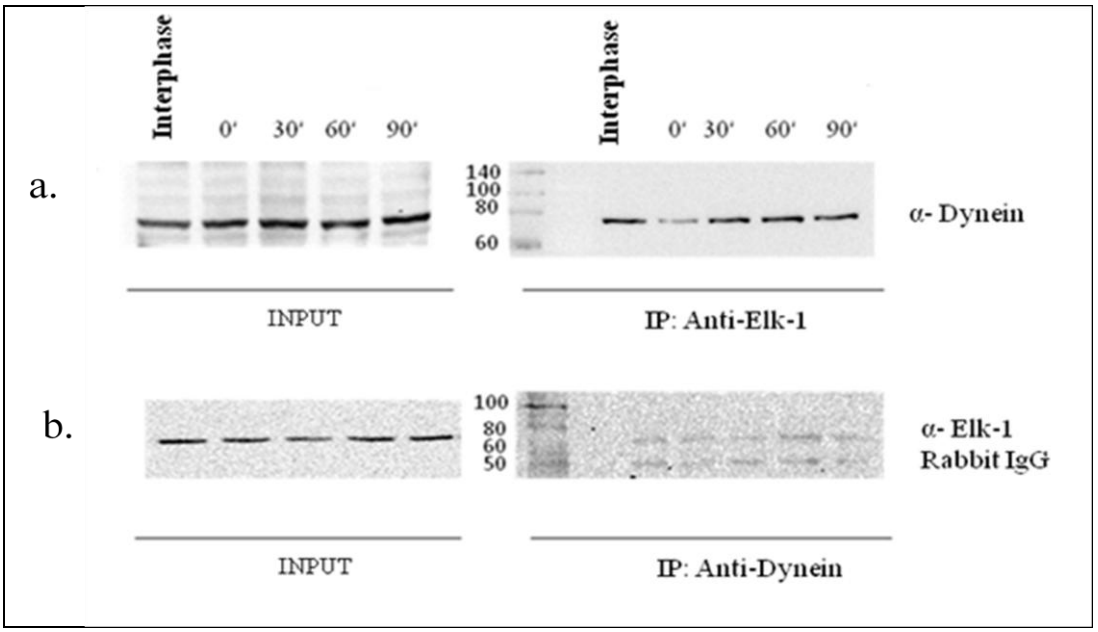


Figure 4.2. Co-immunoprecipitation of Elk-1 and dynein

In Figure 4.2.a, Elk-1 protein is precipitated by using total Elk-1 antibody and the interaction was analyzed with western blot by using anti-dynein antibody. In contrast, in Figure 4.2.b dynein protein was precipitated and presence of Elk-1 was confirmed by performing Western blot. The inputs were used as positive controls. The bands that were observed in Figure 4.2 a correspond to 74 kDa, which is the molecular weight of cytoplasmic dynein intermediate chain, and in Figure 4.2 b they correspond 62 Kda, the molecular mass of Elk-1 protein.

After SH-SY5Y cells have been synchronized with nocodazole and subsequently released into the cell cycle, the samples were collected in 30 minutes intervals between 0 and 90 minutes. According to results of the both two co-IP experiments which have been carried by using these cell lysates, the interaction between dynein and Elk-1 was clearly observed through interphase and all the phases of mitosis.

4.2. ELK-1 INTERACTS WITH DYNEIN UPON SERUM STIMULATION

After revealing a non-specific interaction of dynein and Elk-1 by immunofluorescence and co-IP experiments, we performed a second set of immunoprecipitation experiment,

Flag-IP, that have three different experimental parts as using the cells grown in normal growth medium (10 % FBS), serum-starved and after serum starvation stimulated with 20 % FBS to confirm whether this interaction is specific or not.

The first part of this experiment involves the cells that were grown in normal growth medium containing 10 % FBS (non-synchronized). In the first place, SH-SY5Y neuroblastoma cells were transfected with wild type and S383A mutant (S>A) plasmids that includes Flag tag and with empty pcDNA3 plasmid as a negative control. Then these exogenous Elk-1 protein involved cells were lysed and proteins were isolated. These Flag-tagged proteins were precipitated by using Flag agarose beads which were coated with Flag antibody to recognize the Flag tags. After precipitation, Flag agarose bead bound proteins were isolated by administiration of SDS loading dye that has DTT or β-mercaptoethanol as a reducing agent to break down the disulfide bonds contributing to tertiary structure which SDS was unable to affect and further denaturing the protein by incubation of 95°C for 5 minute. Finally, proteins were analyzed with western blot by the usage of anti-dynein antibody.

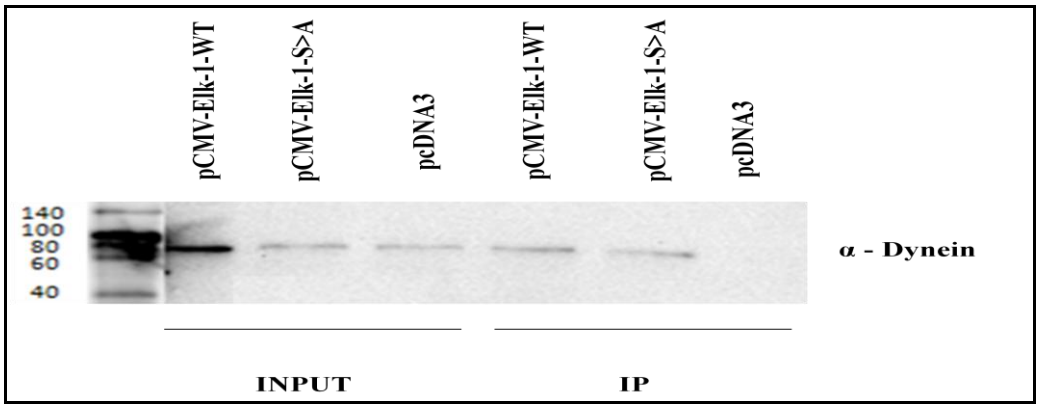


Figure 4.3. Co-immunoprecipitation of dynein and Elk-1 by using the cells that were cultured in normal growth medium

The co-IPs in normal cycling cells showed that both wild type Elk-1 and Elk-1 S>A mutant form could interact with dynein. Inputs, which have total proteins of the cells were used as a positive control. Since the pcDNA3 plasmid has no Elk-1 sequence and Flag tag, any band that corresponds to interaction of dynein and Elk-1 did not observed.

After demonstrating the Elk-1 and dynein interaction in normal cycling cells that were grown in 10 % FBS containing medium, the second part of this experiment were performed by using serum starved cells. Cells were transfected again with pCMV-Elk-1, pCMV-Elk-1-S>A and empty pcDNA3 plasmids and they were grown in medium that contains only 0.5 % FBS to starve cells. Subsequently, proteins were isolated from these cells and Co-IP experiment performed by using Flag agarose beads. Precipitated proteins were analyzed by Western blot.

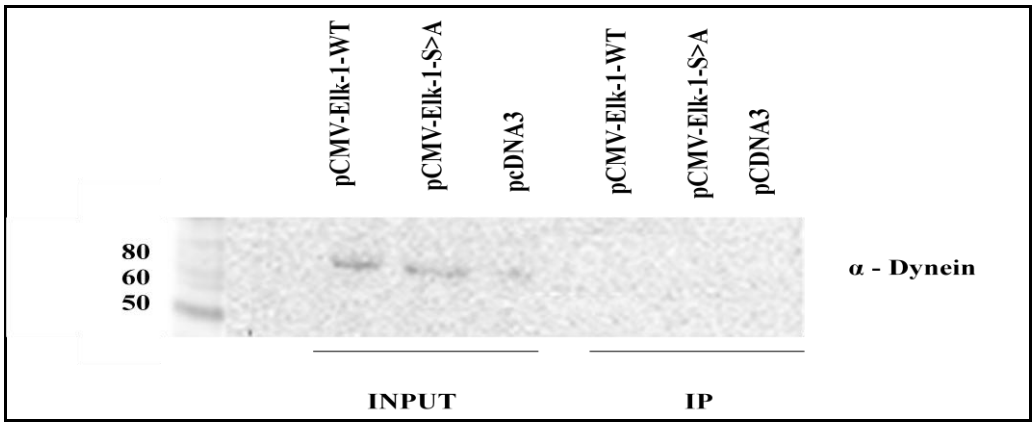


Figure 4.4. Co-immunoprecipitation of Elk-1 and dynein by using the cells that were cutured in 0.5 % FBS containing medium

In contrast to the Figure 4.3. any dynein specific bands, except inputs, could not been observed in immunoprecipitation samples, both in pCMV-Elk-1, pCMV-Elk-1-S>A and empty pcDNA3 plasmids, as shown in Figure 4.4. The presence of Elk-1 and dynein interaction in the IP samples that were prepared by using the cells cultured in 10% FBS and seperation of this interaction in serum starved conditions indicate serum dependence of this interaction.

Final step of this experiment includes the induction of cells with 20 % FBS after they were serum starved. As it is explained before, cells were transfected with previously mentioned plasmids and incubated with 0.5 % FBS for 48 hours. Then, growth of the cells were stimulated by the administration of the culture medium containg 20 % FBS. The proteins of these cells were isolated as explained before and analyed with western blot subsequent to immunoprecipitation.

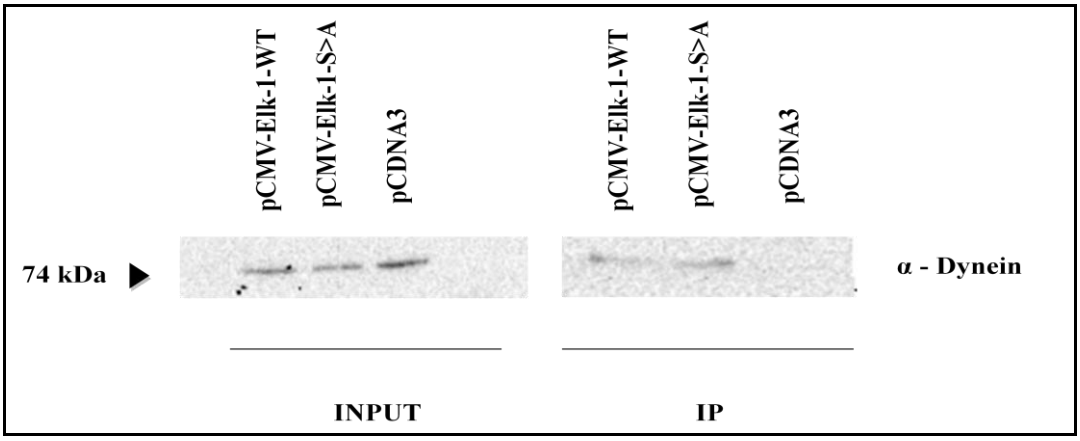


Figure 4.5. Co-immunoprecipitation of Elk-1 and dynein after the serum induction

As it is shown in Figure 4.5., in addition to the inputs, pCMV-Elk-1 and pCMV-Elk-1-S>A samples have the protein bands with the size of 74 kDa that is specific to cytoplasmic dynein. The peresence of the dynein protein in Elk-1 precipated sample, proves the interaction between these proteins, one more time.

All these three westen blot experiment results indicate that Elk-1 and dynein interact to each other in a serum dependent manner and this interaction is free from Serine 383 phosphorylation since S383A mutant (S>A) can be attached to the dynein protein. Destruction of the interaction upon serum starvation which observed in normal cultured cells and after induction with %20 subsequent to serum starvation reveals that Elk-1 and dynein interaction needs serum stimulation.

4.3. THE POSSIBLE INTERACTION BETWEEN ELK-1 AND KINESIN

After the identification of the interaction between Elk-1 and dynein motor protein, our second target was kinesin motors. To reveal the interaction of this motors and Elk-1, we initially used conventional kinesin protein. We used the same experimental set-ups for kinesins that is designed for dynein motor.

Initially, we used the co-immunoprecipitation assay that was performed after cells were synchronized with nocodazole and released into the cell cycle, but we could not observe

any conventional kinesin specific bands after western blot analysis (data not shown). Afterwards we tried to analyze this interaction by using the protein samples prepared by using normal dividing cells, after serum starvation and after serum stimulation.

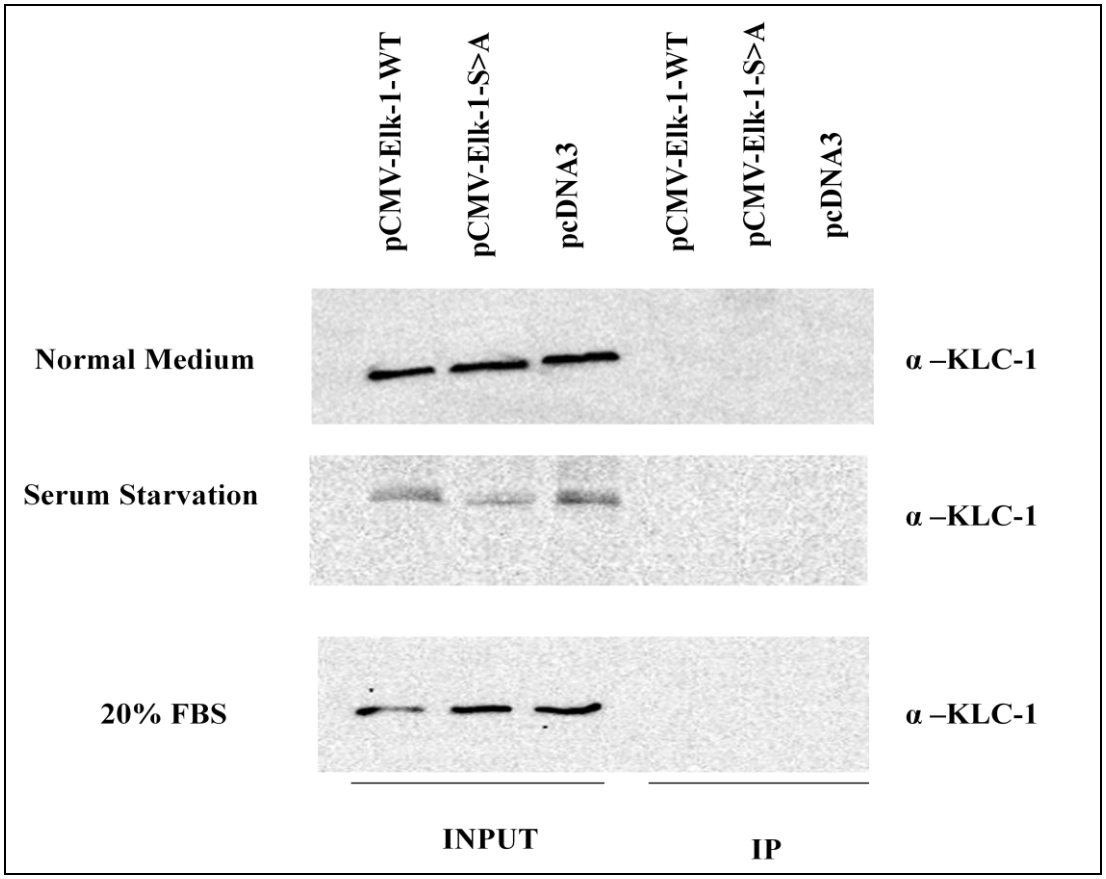


Figure 4.6. Co-immunoprecipitation of Elk-1 and kinesin

As it is obvious in Figure 4.6., only positive controls, inputs, represent conventional kinesin specific bands which have the weight of 61 kDa. Any protein bands specific to kinesin could not been observed in IP samples, so we could not yet identify any interaction between conventional kinesin and Elk-1 by using co-immunoprecipitation assay.

To investigate the interaction of Elk-1 and kinesin motors more specifically, we used different types of kinesin motors, MKLP-1 and Eg5, which had perviously shown co-localization with Elk-1 in immunofluorescence experiments as shown in Figure A.4. [97].

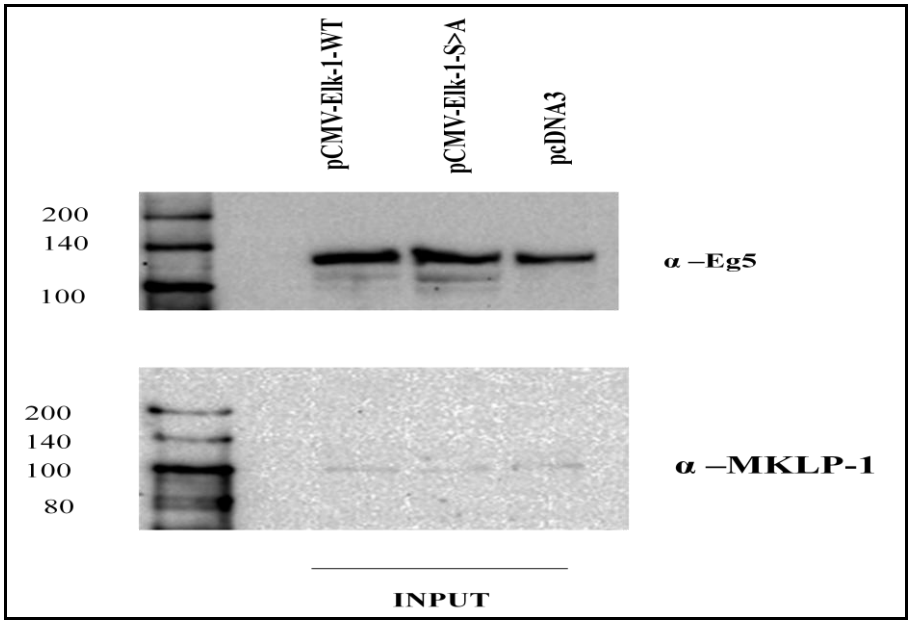


Figure 4.7. Co-immunoprecipitation of Elk-1 with Eg5 and MKLP-1

By using the previoulsy prepared cell lysates, we precipitated Elk-1 by using total Elk-1 antibody and then analyzed the samples with western blot to observe an interaction between Elk-1 with Eg5 and MKLP-1. As well as the conventional kinesin, we could not observe any MKLP-1 and Eg5 specific bands that point out the possible interaction with kinesin and Elk-1.

The lack of a specific interaction between kinesin motors and Elk-1 with this co-immunoprecipitation assay does not mean that these protesins are not associate into the cells. The same experimental set-ups may be repeated by using other antibodies. As it is mentioned in the introduction part, the kinesin superfamily is a large family of microtubule dependent-motors which currently consist of 45 members. To reveal the interaction between Elk-1 and kinesin motors, other isoforms of kinesin family members should be tried; if there is no such interaction, and then a different mechanism for the anterograde transport of Elk-1 should be suggested.

4.4. ELK-1 CAN INTERACT WITH DYNEIN FROM DIFFERENT REGIONS

To analyze the inteaction of Elk-1 and dynein more specifically, we used GST-pull down assay. The pull-down assay is a method used to determine a physical interaction between two or more proteins. Pull down assays are useful tools for both confirming the existence of a protein-protein interaction predicted by other research techniques such as Co-IP and as an initial screening assay for identifying previously unknown protein-protein interactions. This technique relies on the usage of Glutathione-S-transferase (GST) fusion proteins that have a range of applications as tools for synthesis of recombinant proteins in bacteria. In theory, GST pull down experiments are used to identify interactions between a probe protein and unknown targets to confirm suspected interactions between a probe protein which is a GST fusion whose coding sequence is cloned into an isopropyl-beta-D-thiogalactopyranoside (IPTG)-inducible expression vector and a known protein. This fusion protein is expressed in *E.coli* bacterial strains by the induction with IPTG and then can be purified.Target proteins are usually presented with cell lysates and they are incubated with GST fusion protein immobilized on glutathione-agarose beads. Complexes recovered from the beads are resolved by SDS-PAGE and analyzed by Western blotting.

In our experiment, we used 2 different GST-fusion of Elk-1, as depicted in Figure 4.8. These deletions were constructed [98] previously by Andrew D. Sharrocks and were a special gift to us.

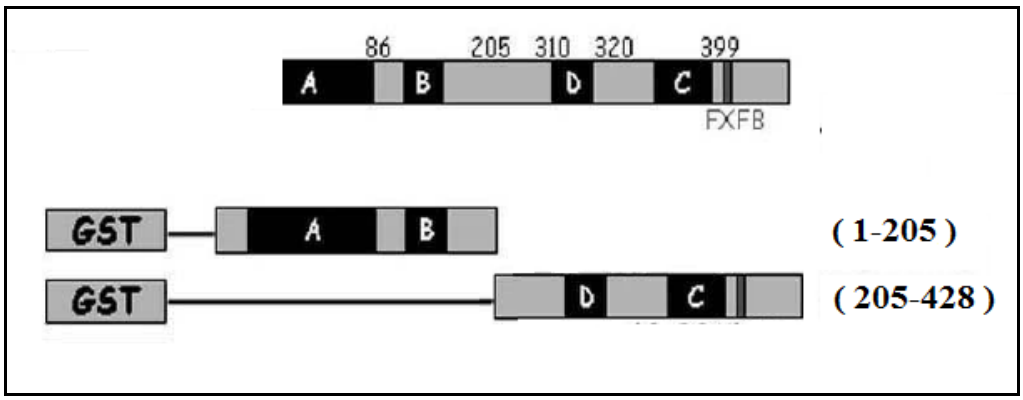


Figure 4.8. Different deletion constructs of GST-Elk-1 fusion proteins used in GST Pull down

E. coli BL21-pLysS strain was initially transformed to 1-205 (N- terminal) and 349-428 (C-terminal) deletion constructs of Elk-1 and the protein expression was induced by the addition of IPTG. With the aim of measuring whether the protein expression in the bacteria is occured or not, bacterial samples were collected before IPTG administration and after 1, 2 and 3 hours IPTG induction and analyzed with SDS-PAGE and subsequently coomasive staining as shown in Figure 4.9.

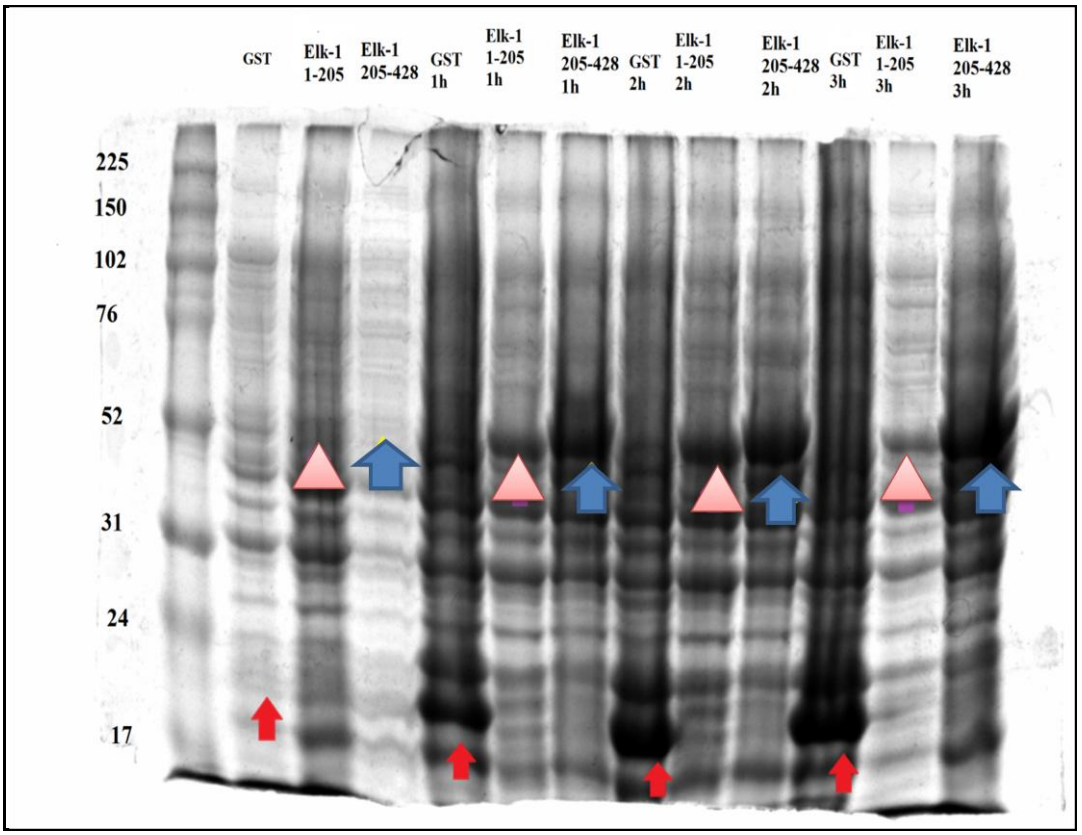


Figure 4.9. SDS-PAGE of GST-Elk-1 fusion proteins after IPTG induction.The bottom and middle arrows indicate GST and Elk-1-1-205 expression, respectively. Whereas, the triangles indicate the expression of Elk-1 205-428 deletion construct

In Figure 4.9., the first 3 lanes indicate the expresion of proteins before IPTG administiration and the rest of the lanes indicate expression after IPTG. As it is seen in the Figure 4.9., proteins could be expressed in *E.coli* bacteria and this expression was gradually increased after IPTG induction.

After the confirmation of protein expression, bacterial lysates containing the expressed protein were cleared by sonication, and incubated with GST sepharose beads as described in material and method section. To perform pull down experiment, partially purifed GST fusion proteins were incubated with previously isolated U-87 glioblastoma cell line proteins. After incubation, beads were precipitated and SDS-PAGE was carried out to measure the desired proteins on the gel as shown in Figure 4.10.

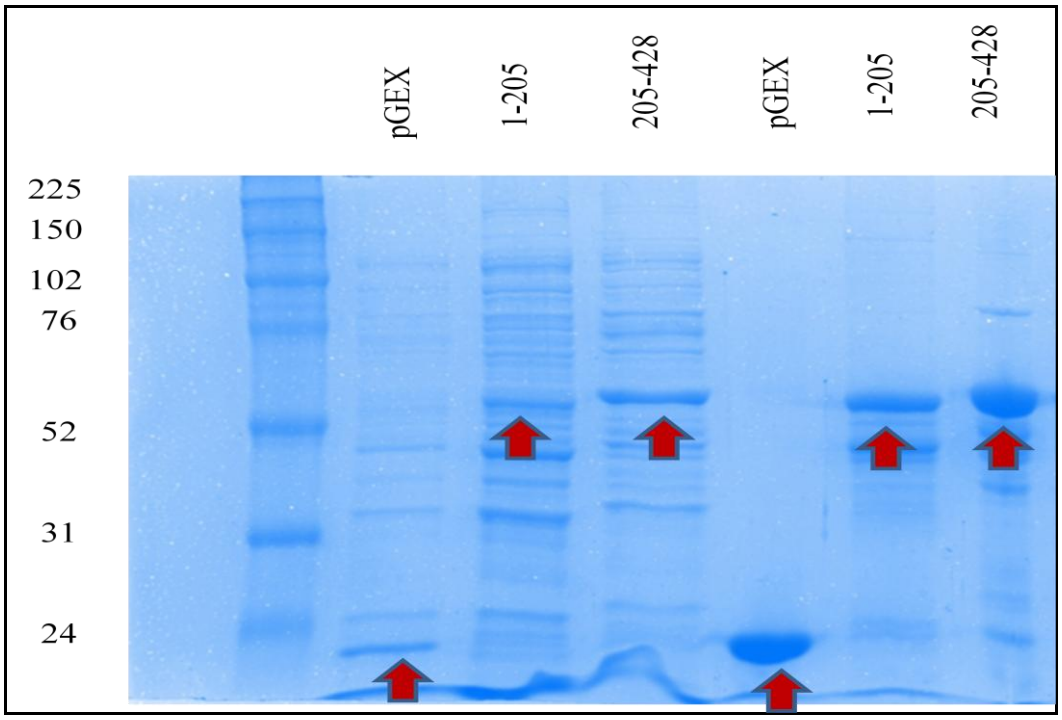


Figure 4.10. SDS-PAGE of purified GST-Elk-1 fusion proteins

Figure 4.10. indicates that GST bound proteins were successfully purified after incubation with cell lysate. After the confirmation of purification of 1-205 and 205-428 Elk-1 deletion constructs, Western blot was carried out to observe any interaction by using anti-dynein antibody.

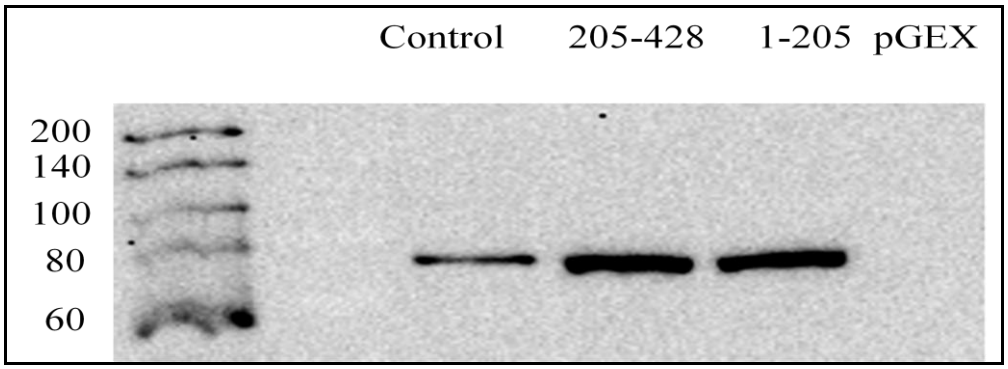


Figure 4.11. Western blotting of GST-Pulldown assay with anti- dynein antibody

In Figure 4.11., the first lane is total protein as an input (positive control) and the last lane includes only GST as a negative control. As seen in the figure, it was observed that the thickneses of the two Elk-1 proteins are almost the same, indicating that Elk-1 can bind dynein from both of these regions with the same intensity. To determine the binding regions of Elk-1 to dynein more specifically, other Elk-1 deletion constructs can be used such as 1-93 and 205-339.

Our initial results indicate that cytoplasmic dynein can bind to Elk-1 protein from different regions or domains. Since the cytoplasmic dynein is a large protein with approximately 1.2 Megadalton (MDa) molecular mass, interaction with Elk-1 from different regions is plausible but more specific analyses are required to reveal this interaction more specifically.

5. DISCUSSION

The microtubule cytoskeleton is the fibrillar structure and functions in virtually all aspects of eukaryotic cell biology including organelle transport, signaling, maintenance of cell shape, mitosis and cytokinesis. During mitosis, nucleation and polymerisation of microtubules is carried out by centrosomes thus it is known as the microtubule organizing center (MTOC). During prophase, the two centrosomes are pushed apart to the opposite ends of the nucleus by the action of moleular motors acting on the microtubules to organize microtubules for the formation of the bipolar mitotic spindle which is required for the seperation of chromosomes [99].

Microtubules are formed by heterodimers of alpha and beta tubulin whose polymerization is modulated by microtubule associated proteins (MAPs). In neuronal cells, microtubule-associated proteins (MAPs) that have motor activity required for the movement of microtubules can be classified into two distinct groups: dynein and kinesin [57].

Dyneins are one of the two known families of force-producing microtubule associated proteins (the other is the kinesin family), which hydrolize ATP for retrograde transport, from cell periphery to the center of the cell, because it can produce force toward the minus ends of microtubules that move load along the microtubules and the other one is kinesin [57]. Dynein is involved in the capture of microtubules by the kinetochore in prometaphase and removal of metaphase checkpoint proteins from the kinetochores, so that in its absence or in the case of its inhibition, mitosis is delayed at metaphase. The main role of dynein for mitosis consists in moving the microtubules extending from the kinetochores along the microtubules extending from the centrosome towards the latter [100].

Kinesins are the second group of force producing microtubule-based motor proteins that function in the movement of microtubules by producing force towards plus ends of the microtubules for anterograde transport, from cell center to the periphery. Kinesin family members have crucial roles during mitosis such as formation and function of the mitotic

spindle, anaphase movement of chromosomes, regulation of microtubule dynamics and cytokinesis [59].

Elk-1 is the member of ternary complex factor (TCF) subfamily of Ets domain transcription factors, the proteins form ternary complexes with serum response factor (SRF) and serum response elements (SRE) found in immediate early genes such as c-fos, egr-1 and c-jun for the activation of these genes [24]. Besides this transcriptional dependent role, the interaction of Elk-1 with microtubules [38], the main component of spindle fibers in mitosis, and mitotic kinases such as Aurora A as shown in Figure A.1., which is revealed by our laboratory, conceive the transcriptional independent role of Elk-1 during mitosis. In addition to these interactions, the 21.000 genes found in the cell were investigated by using RNAi method to reveal the genes which are required for mitosis and found that Elk-1 is one of the 1000 gene that is required for mitosis and in the absence of Elk-1 different types of anomalies occures such as defects in nuclear shape, segregation problems, cell death and mitotic delay [101].

Since dynein and kinesin are involved in the polymerization and regulation of microtubule functions and have diverse roles during mitosis, we initiated our studies to reveal whether Elk-1 can interact with these motors or not. To that end, Demir and Kurnaz performed immnofluresence analysis to determine the positions of Elk-1 during the mitosis and they showed that the presence of total Elk-1 in mitotic spindle is vague, whereas phospho-Elk-1 (Serine 383 phosphorylation) is more prominent and this phosphoform of Elk-1 is mostly at the nucleus in interphase cells and at the spindle poles during metaphase and anaphase and finally it relocalizes to the spindle midbody during cytokinesis in different cell lines as shown in Figure A.2.

When the Serine383-to-Alanine phospho-mutant of Elk-1 transfected were cells stained, the same localization pattern was observed at the spindle poles, but this mutant form failed to relocate on the midzone during cytokinesis and dispersed throughout the mitotic spindle. The other phospho form of Elk-1, P-T417 found to DNA bound during different stages of mitosis and was absent from the spindle midbody during cytokinesis as shown Figure A.3. This data reveal that, mitotic spindle pole localization of Elk-1 is specific to Serine 383 phosphorylation.

The presence of P-S383-Elk-1 at different locations during different stages of the cell cycle, rises the question how Elk-1 gets to these different locations. It is suggested that there could be two main explanations for this case, whether a phosphorylation / dephosphorylation cycle is initiated which changes the phosphorylation status of Elk-1 or P-S383-Elk-1 is translocated from one position to the other via mitotic motor proteins. Due to the vague localization of total Elk-1 at the spindle poles and the S383A mutant was seen to be present at the mitotic spindle during anaphase, it is more plausible that motor proteins carries the P-S383-Elk-1 along the microtubules in different stages of the cell cycle. To that end, we started the analyze the possible interaction between Elk-1 and mitotic motor proteins, dynein and kinesin.

To examine whether Elk-1 can interact with kinesin motors, immofluoresence analysis was performed by Demir and Kurnaz by using kinesin light chain specific antibody and revealed a co-localization between P-S383-Elk-1 and kinesin during all the stages of the cell cycle. Besides the traditional kinesin light chain analysis, spesific mitotic kinesin, such as Eg5 which is involved in spindle pole seperation and spindle bipolarity [68] and MKLP-1, required for the microtubule bundles at the spindle midbody [67], were used to identify possible interaction between kinesin motor and Elk-1 and a co-localization between Elk-1 and these motors also observed as shown in Figure A.4. [97].

In addition, treatment of cells with EHNA-HCl, a dynein inhibitor that interferes with dynein-microtubule binding through inhibition of the ATPase activity of dynein, resulted in interfering with mitosis. Cells that could proceed into mitosis and phospho-Elk-1 species successfully located to the spindle poles but in prophase and metaphase P-S383-Elk-1 appeared in dot-like structures associated with DNA. When compared to the P-S383-Elk-1 staining in untreated cells, it was observed that phospho-Elk-1 staining leaves the DNA and becomes restricted to spindle poles in prophase. In dynein inhibitor treated cells, some P-S383 Elk-1 was stil remained at the metaphase plate, wheras the rest could locate to the spindle. There was no problem with the spindle midbody localization at cytokinesis as shown in Figure A.5 [97]. The dot like structure of Elk-1in the presence of dynein inhibitor suggets that P-S383 Elk-1 is carried by dynein motors from poles to the spindle .

The immunofluorescence analysis need to further confirmation by other techniques to analyze whether this interactions were direct or not, since there are many intracellular proteins and Elk-1 could be localized to the same region with microtubules, dynein and kinesin motors. Hence, we used several biochemical and molecular techniques for the analysis of the interaction between Elk-1 and mitotic motor proteins.

After revealing a potential interaction between Elk-1 and dynein by immunofluorescence analysis, we examined this interaction more specifically with immunoprecipitation experiments. After cell cycle arrest by administration of nocodazole, we collected cells at different time zones to catch the cells at different cell cycle phases. The western blot analysis performed after immunoprecipitation assay revealed that Elk-1 can interact with dynein at interphase and all the phases of the mitosis as prophase, metaphase, anaphase and telophase. In addition to the co-immunoprecipitation, GST pull down result also proved this interaction. This analysis demonstrated that dynein protein can bind to the Elk-1 protein from different regions.

As it mentioned before, cytoplasmic dynein has diverse roles during mitosis. In interphase cells, it is responsible for carrying molecular cargoes such as aggregated proteins, transcription factorsand mRNA containing particles towards the nucleus During mitosis, it has been found at the pole for pole focusing at the cell cortex where it pulls on microtubules attached to the spindle poles [102] and at the spindle pole where it accumulates after transporting factors required at the poles as shown Figure 5.1. [103].

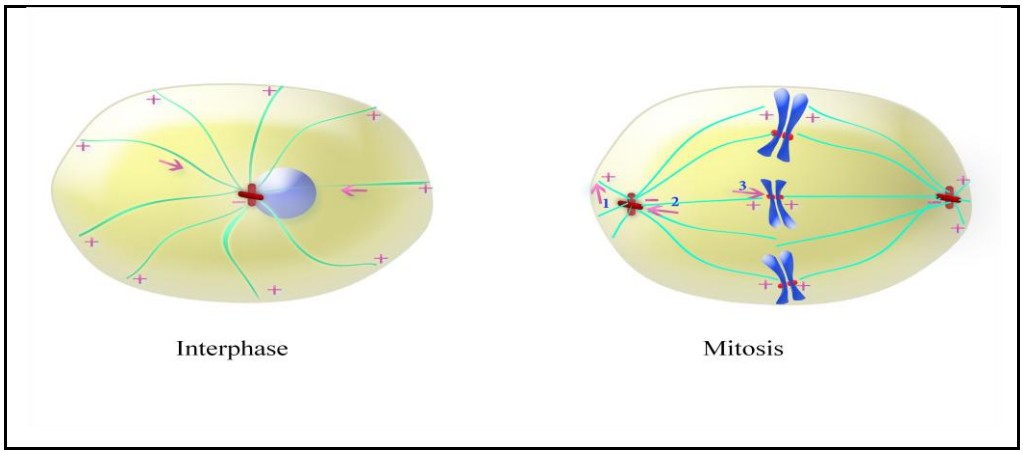


Figure 5.1. Roles of dynein during interphase microtubule transport and mitosis 1 ,2 and 3 indicate cortex, pole and kinetochore, respectively [102,103]

As mentioned before, Phospho-Elk-1 localizes at the nucleus during interphase and it translocates and positions to the spindle pole during prophase and metaphase. These localization of Elk-1 suggests that it is carried from nucleus to the spindle pole by dynein motor throught the minus end of the microtubules.

After metaphase, P-S383-Elk-1 is localized to the midzone and it is finally focused at the spindle midbody during cytokinesis. This localization of Elk-1 requires the tranport of this protein from the minus ends of microtubules , that is embeded in the centrosome, to the plus ends, localized at the cell periphery, with kinesin motors by anterograde transport. At this point, it seems that Elk-1 should be interacting with specific kinesin motors which is required to be identified with addiditonal experiments.

As explained in the introduction part, Elk-1 is a member of the ETS domain superfamily of transcription factors, and has been traditionally associated with mitogen-induced immediate early gene transcription upon phosphorylation by MAPK. Immediate early genes (IEGs) are the genes whose expressions are activated transiently and rapidly by growth factor stimulation. About 40 cellular IEGs have been identified sof far and the best known members include *c-fos, c-myc* and *c-jun* and *egr-1*. Besides functioning many cellular processes, they are particularly important for cell growth and differentiation just because their immediate stimulation in a resting cell by extracellular signals. For instance, *c-fos* is expressed transiently in the G_1 phase of cell cycle after

antigen stimulation. The gene product, *c-fos*, interacts with members of the Jun family to form an activator protein-1 (AP-1) heterodimer that plays an important role in inducing gene expression required for cell proliferation [104].

Activation of immediate early genes such as *c-fos* whose activation is required for cells which have exited the cell cycle to re-enter G_1 phase and subsequently enter mitosis, by serum growth factors, is mediated through a promoter element known as the serum response element (SRE), a sequence comprised of a CArG box [CC(A/T)6GG] and an Ets-like binding site [7,105,106].

The serum response element (SRE) is a regulatory sequence required for the growth factor-induced transient transcriptional activation of many cellular immediate-early genes [107]. Activity of the SRE is dependent on binding of the transcription SRF (serum response factor) [108].

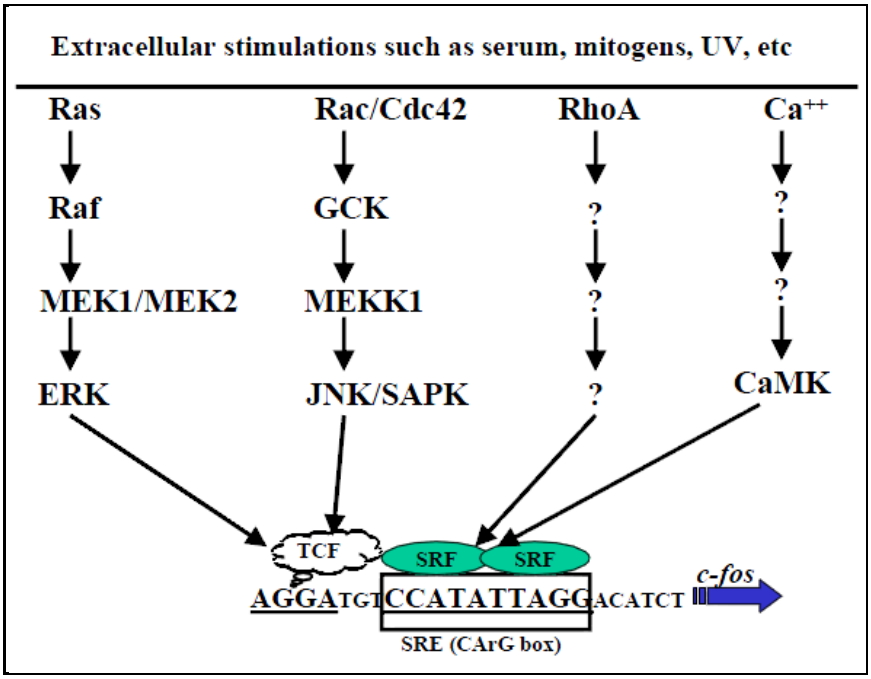


Figure 5.2. Possible pathways involved in *c-fos* gene activation [109]

SRF is an important regulator of numerous genes associated with cell growth and differentiation. SRF can be activated by serum, mitogens, lipopolysaccharides and

cytokines. There are two general classes of signaling mechanisms involving the SRF regulating SRE activity. TCF dependent pathway involves the Ras-Raf-Mapk-Erk cascade which is required for the activation of the SRE. TCF independt pathway involves the Rho family of GTPases. Several MAPK pathways have been found to activate TCF, for instance extracellular signal regulated kinase (ERK) can be activated by extracellular stimulations such as serum induction to activate TCF by phosphorylation as depicted in Figure 5.2. [109].

After confirmation of dynein and Elk-1 interaction by co-immunoprecipitation and GST pull down assay, we used serum starvation and subsequently serum induction experiments to confirm this interaction was specific or not. In the co-IP experiments, we demonstrated that the wild type and S383A (S>A) mutant form of Elk-1 can interact with dynein motor. When the cells were exposed to serum-starvation, interaction of Elk-1 and dynein motor was lost. Whereas, when the serum starved cells were stimulated by 20 % FBS, this interaction is re-gained. This data indicates that serum stimulation enhances the dynein interaction of Elk-1 and this interaction is specific and dependent on serum induction. This serum dependence, probably, based on the interaction of Elk-1 and SRF. Failure of the connection between Elk-1 and dynein in te absence of the serum may be arised from the inactivation of SRF. When the interaction of SRF and Elk-1 protein distrupted, Elk-1 failed to engage to dynein protein.

The TCF family member Elk-1 is not only an activator but also a repressor of SRF dependent *c-fos* regulation. Activation of *c-fos* transcription depends on the ternary complex factor formation by two molecules of SRF and one molecule Elk-1 phosphorlation of Elk-1 by ERK1/2 on SRE region [110].

The phosphorlation of Elk-1 by ERK1/2 is required for its activation upon extracellular stimulations such as serum. In addition to the transcriptional activation by Elk-1, in the presence of mitogenic stimuli, it returns back to the repressor state by recruiting the mSin3A-HDAC-1 corepressor complex to its Ets like DNA binding domain [111].

It is also known that glutamate treatment leads activation of the ERK and JNK/SAPK MAPK cascades which results in a significant increase in Elk-1 phosphorylation and the

appearance of c-*fos* mRNA. In the absence of serum, decreased level of glutamate resulted in the decreased level of Elk-1 phosphorylation that lead to the hindering Elk-1 and dynein interaction [112].

The Rho family GTPases pathway controls diverse cellular processes concerned with the cytoskeleton, including actin polymerization, F-actin bundling, myosin-based contractility, focal adhesion formation, and cytokinesis [113, 114].

The actin cytoskeleton is a static scaffold for the maintenance of cell shape, polarity, and mechanical support. One of the central features of actin filament dynamics and the ability of a cell to migrate is a process known as actin treadmilling plays a key role in RhoA-mediated SRF activation. Nevertheless, it is known that several SRF target genes encode for proteins that play a key role in actin treadmilling [114, 115].

Dividing a eukaryotic cell in two is a complex process that requires tight coupling between the mitotic spindle, the macromolecular machine that drives chromosome segregation, and the actin-based cortex, which underlies the plasma membrane. For the completion of the cytokinesis and sepereation of chromosomes into two daughter cells, the actin and actin based motor myosin are required. Recent studies indicate that, in additon to the role of actin and myosin during the passage from mitosis to cytokinesis, actin and actin polymerisation play a key role in spindle migration and positioning, astral microtubule growth [114-116].

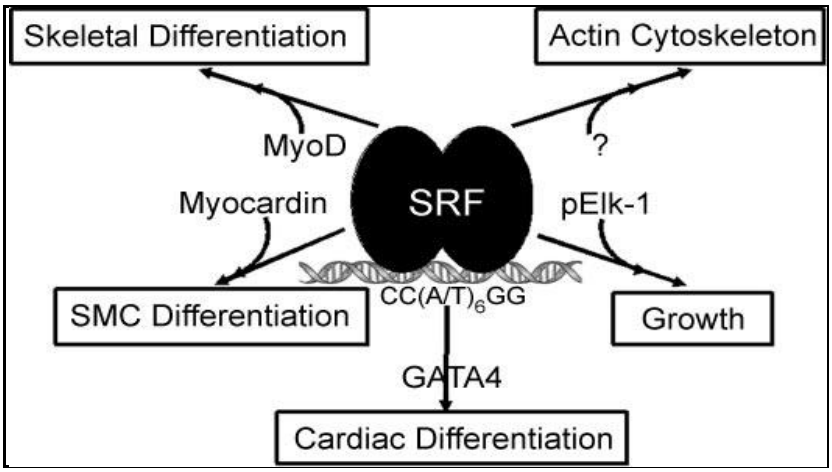


Figure 5.3. Serum response factor (SRF) controls disparate programs of gene expression through cofactor associations [114]

It is known that the pulling and pushing forces required for the proper positioning of mitotic spindle are driven by the concurrent interaction of dynein, astral microtubules and actin that is positioned at the cell cortex [113-115]. Previous studies which were performed by the inhibition of Elk-1 phosphorylation revealed that in the absence of Elk-1 not only actin levels are down regulated and growth cone collapse occurred but also Serum response factor (SRF) that behaves as a sensor of cytoskeleton actin dynamics and expression, depicted in Figure 5.3., decreased [43].

As it is mentioned before, Elk-1 is carried from nucleus to the cell cortex by the retrograde transport with dynein where actin is localized. This co- localization of Elk-1 and actin and decreasing the actin level in the absence of Elk-1 suggest that Elk-1 and actin protein may interact at the cell cortex and this interaction may be required for proper segregation of chromosomes to the daughter cells along with spinde assembly and positioning. This possible interaction and its function sould be identified with biochemical and molecular analysis.

In many cell types, extracellular stimuli, such as serum, growth factors, phorbol esters, neurotransmitters, cytokines, Ca^{2+}, UV light, and redox agents, regulate critical cellular events, such as growth, differentiation and apoptosis through activation of protein kinase cascades. Many of these stimuli trigger mitogen-activated protein kinase

(MAPK) cascades through initial activation of their receptor-associated tyrosine kinases and subsequent phosphorylation of other intracellular substrates. In mammalian cells, three MAPK cascades, which regulate the activity of the extracellular signal-regulated kinase (ERK) subclass, the closely related c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 MAPKs are characterized at present. For instance extracellular signal regulated kinase (ERK) can be activated by extracellular stimulations such as serum induction to activate TCF by phosphorylation [117].

As it is explained above, Elk-1 activation requires the phosphorylation of it by MAPKs upon the external stimulation. The MAPK pathway phosphorylates Elk-1 in particular at S383, S389 and T417 within the activation domain The formation of interaction between Elk-1 and dynein in the case of using S383A mutant form indicates that this interaction is independent of Serine 383 phosphorylation. To reveal the exact mechanism of this interaction, the other potential phosphorylation residues of Elk-1 needs to be analyzed such as S389 and T417.

In this study, we demonstrated that Elk-1 and dynein motors interacts throughout mitosis upon the serum stimulation by using different brain tumor cell lines. The other question that needs to be clarified that whether the interaction observed in normal dividing and tumor cells, between Elk-1 and motor proteins is occured in primary neurons or not.

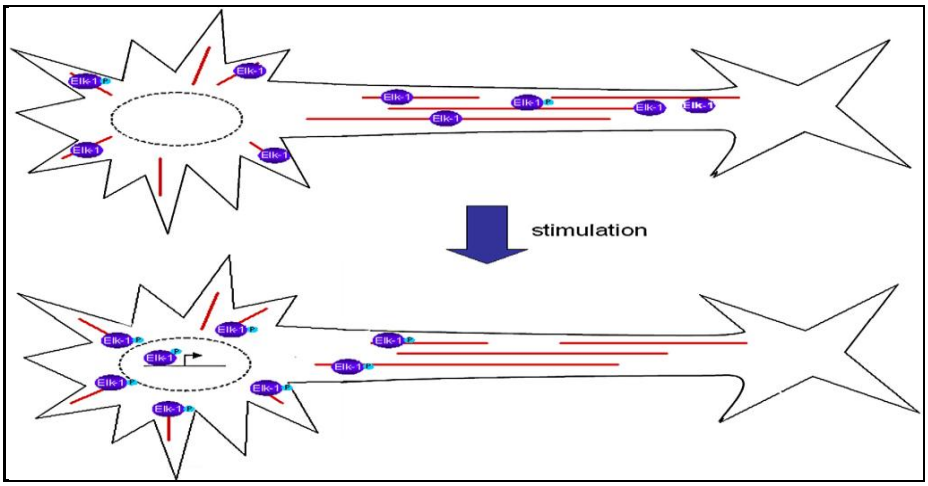


Figure 5.4. A model of the association of Elk-1 with neuronal microtubules [38]

Previous study indicated that Elk-1 interacts with neuronal microrubules and relocalizes to the nucleus upon phosphorylation [38]. In resting cells, the non- phospo and relatively low levels of phospho-Elk-1 are located at the cytoplasm. However, serum or growth factors induction results in the rapid phosphorylation of non-phospho Elk-1 and this phospho form of Elk-1 translocates to the nucleus where it is proposed to regulate neuronal target genes This results indicates the translocation of Elk-1 from cytoplasm to the nucleus upon stimulation but it is stil unclear how Elk-1 is carried from cytoplasm to the nuclues [38].

As it is known that molecular motors dynein and kinesin are able to carry the cargoes such as proteins from long distances, they can also be responsible for the transport of Elk-1 from cytoplasm to nucleus or visa versa. To identify such a mechanism, the interaction between motor proteins, dynein and kinesin, and Elk-1 should be revealed by using primary neurons.

6.CONCLUSION AND RECOMMENDATIONS

In this study, we tried to reveal the possible interaction between dynein and kinesin motors and Elk-1 to identify the role of Elk-1 in mitosis. Immunofluorescence analysis indicates a co-localization of Elk-1 with dynein and kinesin. To investigate the asociation of these proteins, we used co-immunoprecipitation and GST pull down experiments. Our data demonstrated that Elk-1 can interact with dynein motor in interphase and different phases of the mitosis.

We also showed that this inreaction of Elk-1 and dynein is serum dependent. In the absence of serum stimulation, activation of MAPK pathway is decreased which phosphorylates Elk-1. Nevertheless, we demonstrated that the S383 phosphorylation of Elk-1 by MAPK is not essential for this interaction. Inhibion of the activation of Elk-1 hinders its binding to the dynein. Moreover, the interaction of Elk-1 with dynein, the possible interaction of actin which needs to be determined more specifically proves the important role of Elk-1 during mitosis.

We also tried to reveal the interaction between Elk-1 and kinesin motors but unfortunately we could not demonstrate such an interaction with co-immunoprecipitation analyses. Since the kinesin and dynein motors are uni-directional, the cellular transport system could work with the together action of these motors. Kinesin motors move cargoes towards the cell periphery and dynein motors bring them back. So, the interaction with dynein suggests a possible interaction with kinesin that needs to be clarify by using other isoforms of kinesin family.

APPENDIX A: IMMUNOFLUORESCENCE ANALYSES OF ELK-1 AND MOTOR PROTEINS

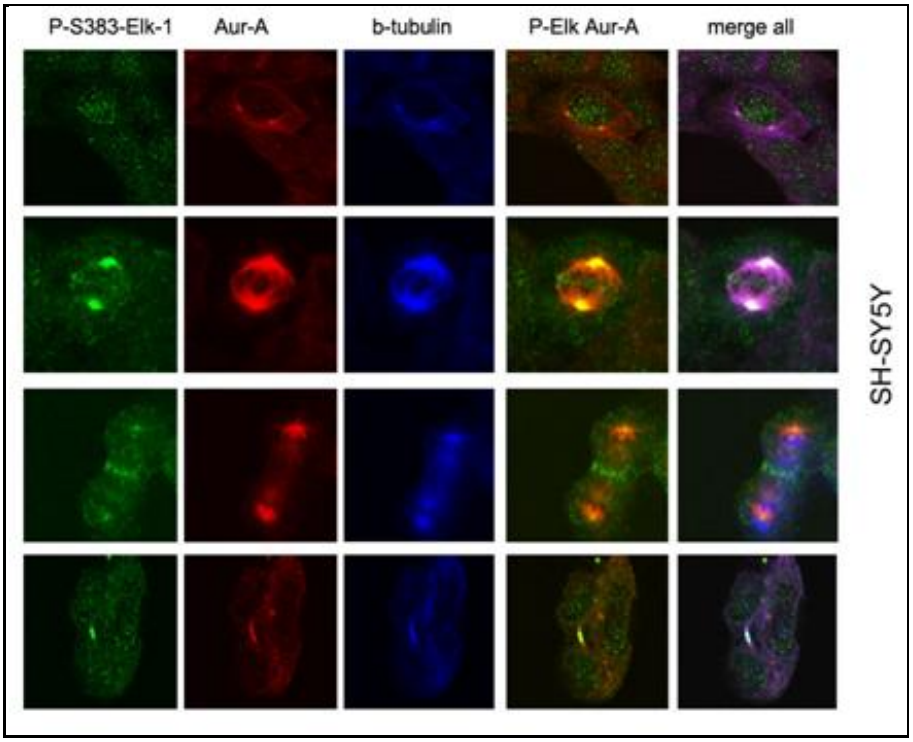


Figure A.1. Co-localization of Phospho-Elk-1 and mitotic kinase Aurora-A in SH-SY5Y human neuroblastoma cells (anti-P-S383-Elk-1, green; anti-Aur-A, red; anti-β-tubulin, blue), (Demir and Kurnaz, submitted)

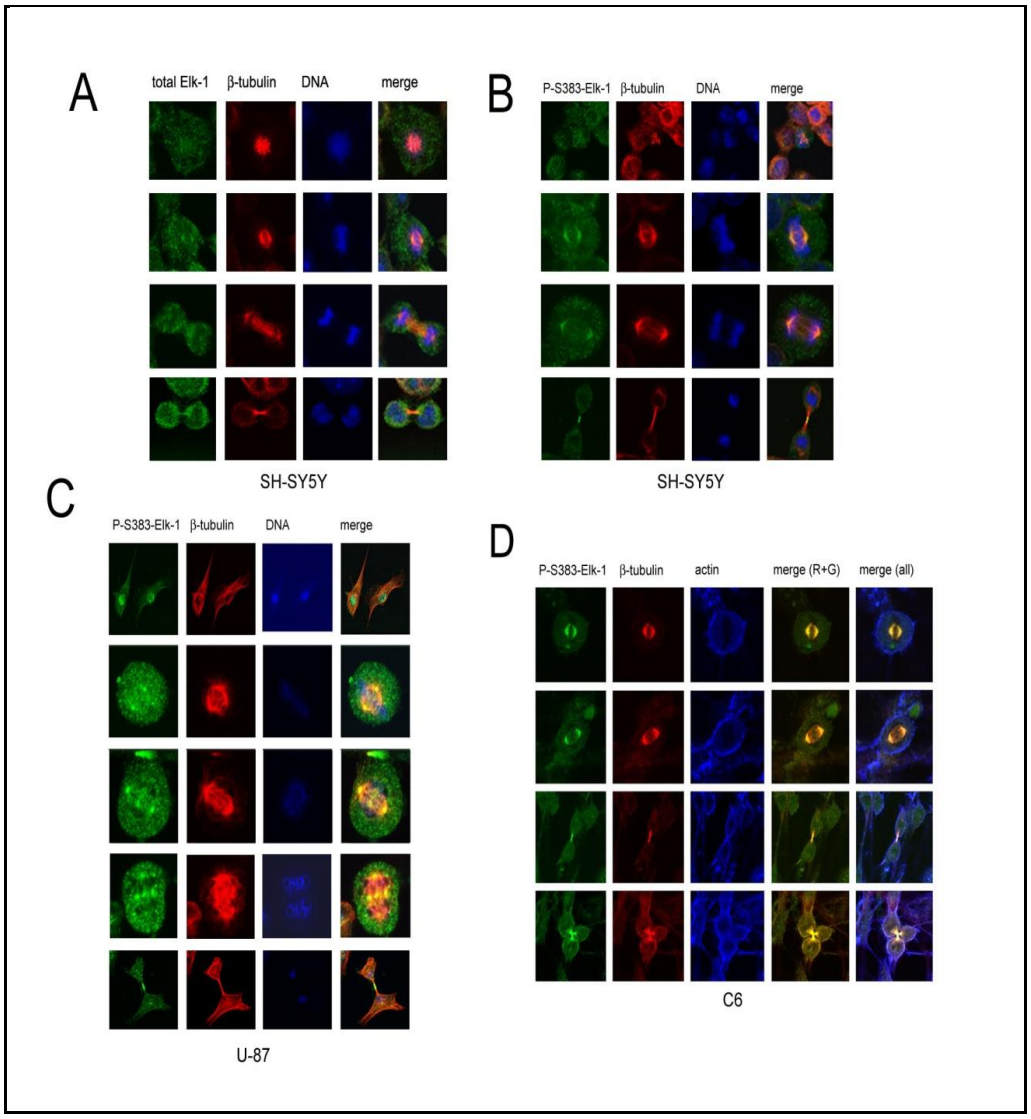


Figure A.2. The localization of total Elk-1 and phospho-Ser383-Elk-1 during different stages of the cell cycle in brain tumor cell lines ;A.Total Elk-1 staining in SH-SY5Y human neuroblastoma cells (anti-Elk-1, green; anti--tubulin, red; DNA is pseudo-colored blue) B. Phospho-Ser383-Elk-1 staining in SH-SY5Y human neuroblastoma cells (anti-P-S383-Elk-1, green; anti-β-tubulin, red; DNA is pseudo-colored blue) C. Phospho-Ser383-Elk-1 staining in U87 human glioblastoma cells (anti- P-S383-Elk-1, green; anti-β-tubulin, red; DNA is pseudo-colored blue), D. Phospho-Ser383-Elk-1 staining in C6 rat glioma cells (anti- P-S383-Elk-1, green; anti-β-tubulin, red; actin is pseudo-colored blue), (Demir and Kurnaz, submitted)

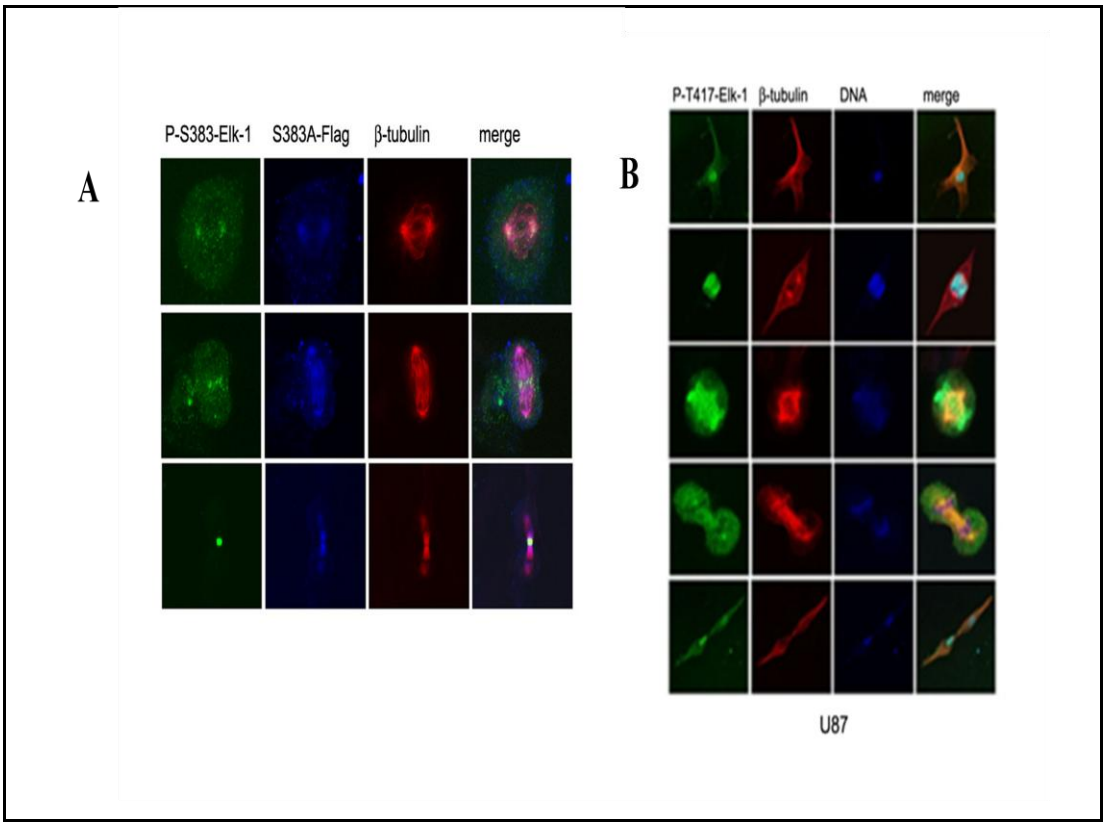


Figure A.3. The localization of phospho-Ser383-Elk-1, S383A-Elk-1 and phospho-Thr-417-Elk-1 throughout mitosis A. Endogenous P-S383-Elk-1 as well as transfected Elk-1-S383A-Flag are located at the spindle pole in U87 cells but S383A mutant fails to relocate to the midzone and instead remains attached to the microtubules all around the mitotic spindle in anaphase, and fails to focus at the midbody as well as endogenous Phospho-Elk-1 during cytokinesis (anti-P-S383-Elk-1, green; anti-Flag, blue; anti-β-tubulin, red) B. Phospho-Thr417-Elk-1 staining in U87 human glioblastoma cells shows nuclear staining in interphase cells, with phospho-Elk-1 remaining DNA-bound throughout the cell cycle (anti- P-S383-Elk-1, green; anti-β-tubulin, red; DNA is pseudo-colored blue (Demir and Kurnaz, submitted)

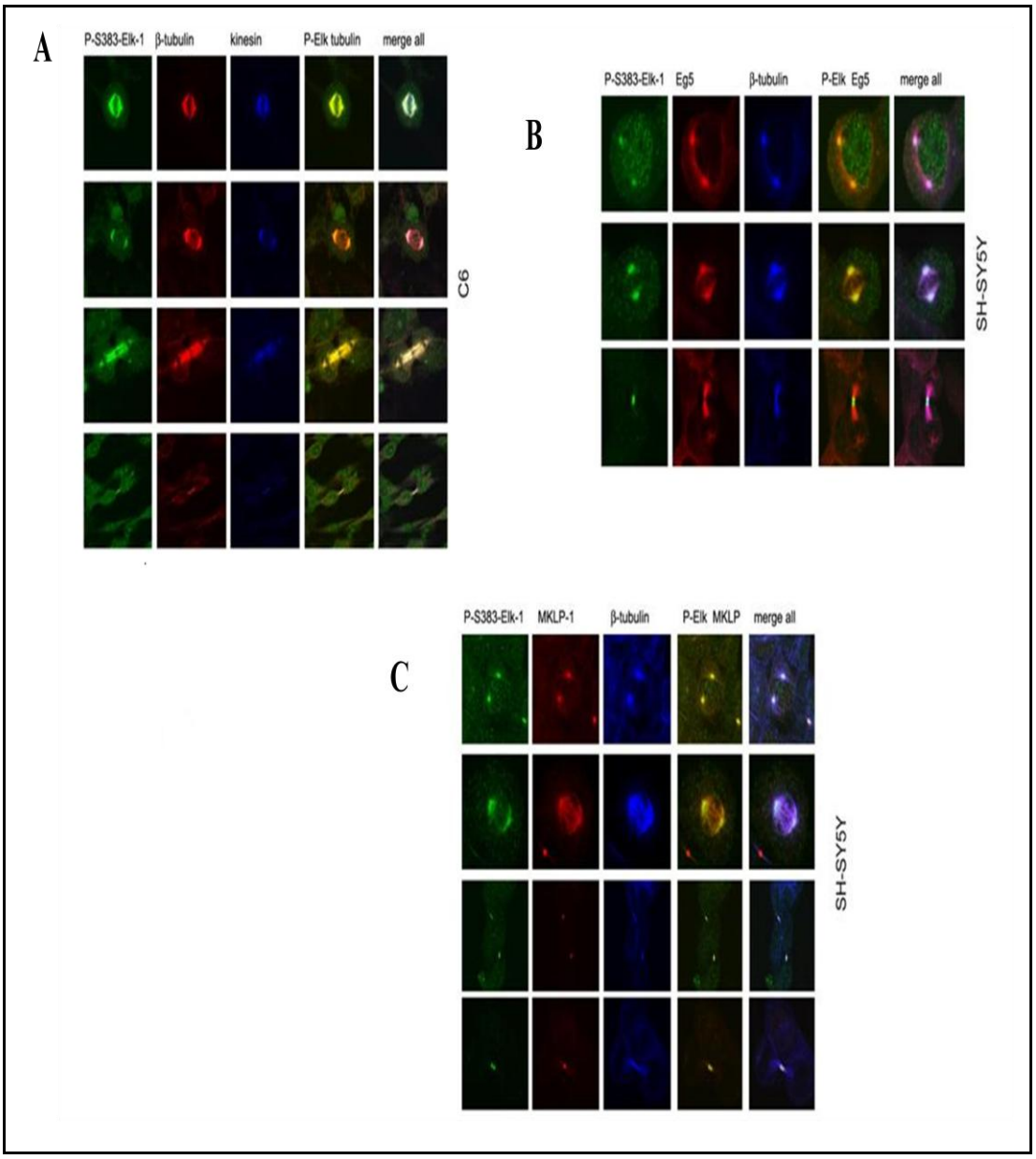


Figure A.4. Phospho-Elk-1 co-localizes with kinesin family of motor proteins throughout the cell cycle. A. Co-localization of Phospho-Elk-1 and kinesin in C6 rat glioma cells (anti-P-S383-Elk-1, green; anti-b-tubulin, red; anti-kinesin, blue), B. Co-localization of Phospho-Elk-1 and mitotic kinesin Eg5 in SH-SY5Y human neuroblastoma cells (anti-P-S383-Elk-1, green; anti-Eg5, red; anti-b-tubulin, blue) C.Co-localization of Phospho-Elk-1 and mitotic MKLP-1 in SH-SY5Y human neuroblastoma cells (anti-P-S383-Elk-1, green; anti-MKLP-1, red; anti-b-tubulin, blue) [97]

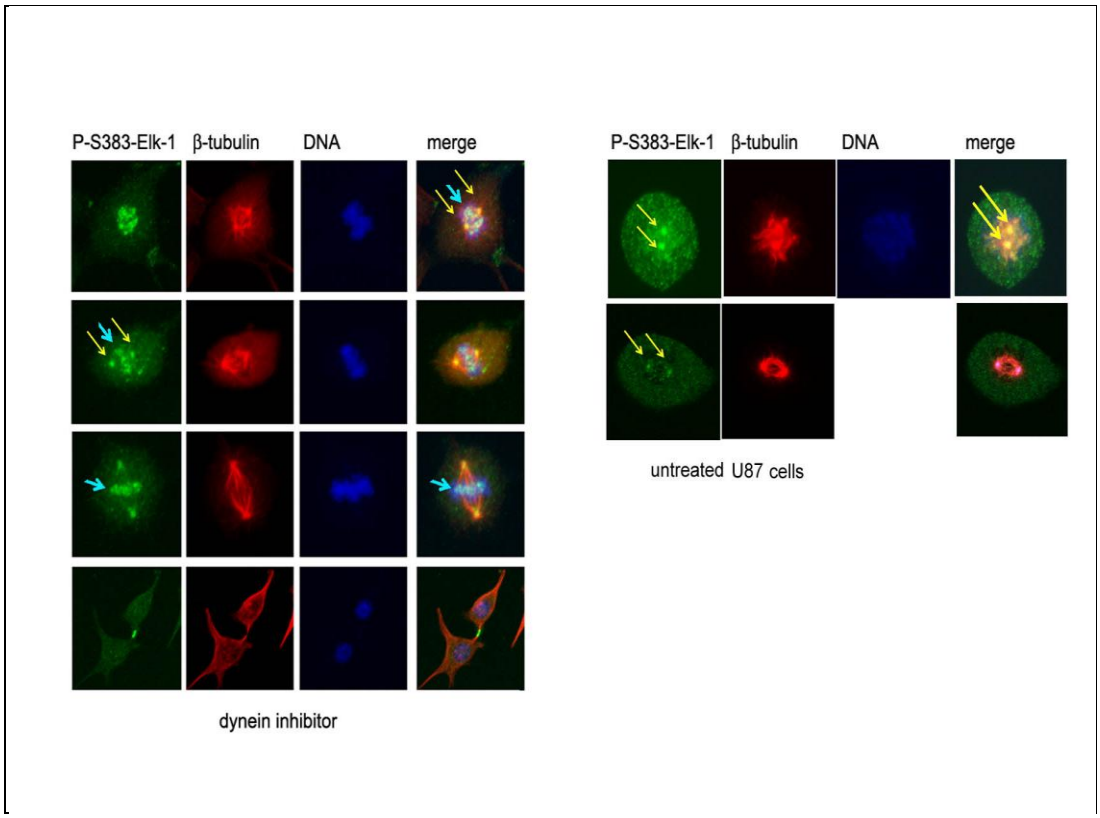


Figure A.5. Co-localization of Phospho-Elk-1, β-tubulin and DNA in the presence of dynein inhibitors in U87 cell line (anti-P-S383-Elk-1, green; anti-b-tubulin, red; DNA, blue). The results shown on the left panels are representative of all mitotic cells from at least two independent experiments from U87 cells treated with dynein inhibitor. The results shown on the right are representative from at least three independent parallel experiments in untreated U87 cells. Yellow arrows indicate P-S383-Elk-1 in spindle poles, whereas short blue arrows show P-S383-Elk-1 on DNA [97]

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