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GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

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GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

ELK-1'İN KİNESİN MOTOR PROTEİNİ İLE NÖRONLARDA ETKİLEŞİMİ

BETÜL ERTURAL
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
PROF. DR. IŞIL KURNAZ

GEBZE
2021

GEBZE TEKNİK ÜNİVERSİTESİ	YÜKSEK LİSANS JÜRİ ONAY FORMU

GTÜ Fen Bilimleri Enstitüsü Yönetim Kurulu'nun 08/01/2021 tarih ve 2021/02 sayılı kararıyla oluşturulan jüri tarafından 21/01/2021 tarihinde tez savunma sınavı yapılan Betül ERTURAL'ın tez çalışması Moleküler Biyoloji ve Genetik Anabilim Dalında YÜKSEK LİSANS tezi olarak kabul edilmiştir.

JÜRİ

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ONAY

Gebze Teknik Üniversitesi Fen Bilimleri Enstitüsü Yönetim Kurulu'nun …..…/…..…/..…… tarih ve ……/…….. sayılı kararı.

İMZA/MÜHÜR

SUMMARY

Elk-1 is a member of Ternary Complex Factor (TCF) subfamily of the ETS superfamily of transcription factors. Elk-1 is activated through phosphorylation upon MAPK pathway and participates in many important cellular processes. In neurons, Elk-1 was found to localize to dendrites and axon terminals, and upon serum stimulation, it relocates to the nucleus. It was shown that Elk-1 interacts with neuronal microtubules after serum stimulation. Phosphorylated Elk-1 from Ser-383 is localized firstly in spindle poles, then midbody during cytokinesis. Elk-1 was found to co-localize with kinesin during mitosis. In this study, we aimed to demonstrate whether Elk-1 could interact with kinesin motor protein in neurons. We firstly identified possible kinesins that could interact with Elk-1 using bioinformatic approaches. To investigate interaction between Elk-1 and these KIFs, immunoprecipitation analysis (IP) would be performed. Before IP, for overexpression of KIFs, we tried to clone them into expression plasmid. As the first step of cloning Gradient PCR analysis was performed for amplification of KIF genes. Since amplification of these genes could not be achieved, we continued with IP analysis of Elk-1 and its phospho-mutant forms with Eg5 that was studied before our laboratory. On the other hand, we have conducted co-localization studies of Elk-1 and Eg5 with immunofluorescence analysis. Due to not accomplish these analyses, cell line and phospho-mutant forms of Elk-1 were changed. Before starting the further analysis, we checked expressions of these mutants, and phosphomimic mutant of Elk-1, S198E was either not expressed or rapidly degraded. Although our studies did not clearly confirm protein-protein interactions aimed in the thesis, we believe that further studies and elucidation of this interaction may be important for mechanisms of tumor formation and neurodegenerative diseases.

Keywords: Elk-1, Kinesin, Protein interaction.

ÖZET

Elk-1, transkripsiyon faktörlerinin ETS süper ailesinin alt ailesi olan Üçlü Kompleks Faktörlerinin bir üyesidir. Elk-1, MAPK yolağı üzerinden fosforillenerek aktive olur ve birçok önemli süreç içerisinde yer almaktadır. Nöronlarda Elk-1'in dendritlere ve akson terminallerine lokalize olduğu ve serum stimülasyonu üzerine çekirdeğe taşındığı bulunmuştur. Serum stimülasyonu sonrasında Elk-1'in nöronal mikrotübüllerle etkileşime girdiği gösterilmiştir. Ser-383'ten fosforile Elk-1, sitokinez boyunca ilk önce kutuplarda daha sonra orta cisimcikte lokalize olmaktadır. Elk-1'in mitoz boyunca kinesin ile kolokalize olduğu bulunmuştur. Bu çalışmada, Elk-1'in kinesin motor protein ile etkileşip etkileşmediğini göstermeyi amaçladık. İlk olarak, Elk-1 ile etkileşebilecek olası kinesinleri biyoinformatik yaklaşımları kullanarak belirledik. Elk-1 ve bu KIF'ler arasındaki etkileşimi araştırmak için immünopresipitasyon (IP) analizi yapılacaktı. IP'den önce, KIF'lerin hücrelerde aşırı ifadesi için, KIF'leri ekspresyon plazmidine klonlamayı denedik. Klonlamanın ilk adımı olarak KIF genlerinin amplifikasyonu için Gradyan PZR analizi gerçekleştirildi. Bu genlerin amplifikasyonu başarılı olamadığından, Elk-1'in ve fosfo-mutant formlarının daha önce laboratuvarımızda çalışılan Eg5 ile IP analizine devam ettik. Bunun yanı sıra, immünofloresan analizi ile Elk-1 ve Eg5'in kolokalizasyon çalışmalarını gerçekleştirdik. Bu analizler tamamlanamadığı için, hücre hattı ve Elk-1'in fosfo-mutant formları değiştirildi. İleriki analizlere başlamadan önce, bu mutantların ekspresyonlarını kontrol ettik. Buna göre Elk-1'in fosfomimik mutantı S198E, eksprese edilmiyor ya da hızla degrade oluyor. Bizim çalışmalarımız amaçlanan protein-protein etkileşimini tam olarak doğrulamasa da ileriki çalışmaların ve bu etkileşimin aydınlatılmasının tümör oluşum ve nörodejeneratif hastalıkların mekanizmaları için önemli olabileceğini düşünüyoruz.

Anahtar Kelimeler: Elk-1, Kinesin, Protein Etkileşimi.

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LIST of ABBREVIATIONS and ACRONYMS

Abbreviations and Acronyms		**Explanations**
µl	:	Microliters
bp	:	Base pair
kb	:	Kilobase
AD	:	Alzheimer's Disease
APS	:	Ammoniumpersulphate
CBP	:	CREB binding protein
CCDS	:	Consensus Coding Sequence
Cdk	:	Cyclin-dependent kinase
DMSO	:	Dimethyl Sulfoxide
egr-1	:	Early growth response-1
Elk-1	:	Ets-like transcription factor
ERK	:	Extracellular signal-regulated kinase
ETS	:	E twenty-six
GFP	:	Green Fluorescent Protein
HD	:	Huntington's Disease
HDAC1	:	Histone deacetylase 1
IDT	:	Integrated DNA Technologies
IP	:	Immunoprecipitation
JNK	:	c-Jun N-terminal Kinase
KAP3	:	Kinesin Associated Protein-3
KIF	:	Kinesin Superfamily Protein
KPM	:	KeyPathwayMiner
LB	:	Luria-Bertani
MAPK	:	Mitogen activated protein kinase
MKLP1	:	Mitotic Kinesin-Like Protein
PCR	:	Polymerase Chain Reaction
PD	:	Parkinson's Disease
PFA	:	Paraformaldehyde

Plk	:	Polo-like kinase
PPI	:	Protein-protein interaction
PSOPIA	:	Prediction Server of Protein-Protein Interactions
Sap-1	:	SRF accessory protein 1
SDS	:	Sodium dodecyl sulphate
SDS-PAGE	:	Sodium Dodecyl Sulfate Polyacrylamide
SRE	:	Serum Response Element
SRF	:	Serum Response Factor
SUMO	:	Small ubiquitin-related modifier
TCF	:	Ternary Complex Factor

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1. INTRODUCTION

Elk-1 is a member of the ETS domain superfamily of transcription factors, and it regulates immediate early gene expression upon phosphorylation by mitogen-activated protein kinase (MAPK). In addition to the stimulation of cell growth, Elk-1 takes part in many biological processes such as survival, differentiation, and cancer. Elk-1 localizes different regions of neurons like axon terminal dendrites. After Elk-1 is stimulated through the MAPK pathway, it is transported to the nucleus for transcriptional activation [Besnard et al., 2011]. In addition, it was shown in our laboratory that Serine 383 phosphorylation of Elk-1 localized first to the spindle poles and then to midbody during mitosis [Demir and Kurnaz, 2013]. Different localization of Elk-1 within the neuron and during mitosis indicates that Elk-1 may interact with motor proteins, and previous studies show that Elk-1 indeed interacts with dynein, as well as co-localize with kinesin [Demir et al., 2012] . In this study, we aimed to show whether previously reported kinesin co-localization with Elk-1 also implies an interaction between these proteins and whether mitotic kinase-mediated phosphorylation of Elk-1 affected this interaction and co-localization.

2. THEORETICAL BACKGROUND

2.1. The ETS Domain Transcription Factor Family

ETS (E twenty-six) is a large family of transcription factors play role in various biological functions such as survival, cell growth, and differentiation [Besnard et al., 2011].

ETS transcription factor family have conserved DNA binding domain called as ETS domain. ETS domain comprises of 85 amino acids and it has winged helix-turn-helix binding motif. ETS domain binds to 11-12 base pair sequence and ETS-binding consensus site, 5'-GGA(A/T)-3' (the *ets* motif), is at the core of this binding sequence [Hollenhorst et al., 2011]. ETS domain provides interaction with proteins as well as binding to DNA. In addition to the ETS domain, ETS proteins have other domains that show also high similarities among family members. Subfamilies are classified according to these similarities of DNA binding and other additional domains. [Sharrocks, 2001].

ETS domain proteins are responsible for similar molecular and biological processes in adults and during embryonic development [Sharrocks, 2001], and are responsible for diverse functions within the cells. This functional diversity is provided by interactions with other proteins, co-regulatory partners, and by specific structural elements that are present in the ETS domain [Hollenhorst et al., 2004]. Transcriptional activation of ETS family members is enhanced by the MAPK pathway [Hollenhorst et al., 2011]. Phosphorylation of ETS proteins by different MAPK pathways alters the transcriptional activity of ETS proteins. This alteration occurs by enhancing the DNA binding affinity of the ETS domain, increasing the activity of co-activators or changing the localization of ETS proteins within the cells [Selvaraj et al., 2015]. More recently, our laboratory has also shown interaction of Elk-1 with mitotic kinase Aurora-A as well as Polo-like kinase 1 (Plk1) and Cyclin-dependent kinase 1 (Cdk1) [Uyar et al., Manuscript in preparation].

Autoinhibition of ETS protein can be regulated by other proteins. For example, a protein that covers the inhibition domain of ETS protein is proteolyzed with alternative splicing and post-translational mechanisms, so the inhibition domain comes off. In addition to regulation with other proteins, the ETS domain has an autoinhibitory

sequence. This inhibitory sequence can be outside of the ETS domain like as Ternary Complex Factors subfamily of ETS superfamily [Hollenhorst et al., 2011].

2.1.1. Ternary Complex Factors (TCFs)

Ternary complex transcription factor family is a subfamily of the ETS domain transcription factors. Net, Sap-1 (SRF accessory protein 1), and Elk-1 (ETS like transcription factor) form TCFs subfamily. TCFs constitute a ternary complex with Serum Response Factor (SRF) on Serum Response Element (SRE) and activate transcription of immediate early genes such as *c-fos* [Buchwalter et al., 2004], [Ducret et al., 1999]. TCFs contain four conserved domains A, B, C and D. A domain is ETS, DNA binding domain, and also contains nuclear localization and export signal. B domain provides interaction with SRF to form a ternary complex. The activation domain is the C domain that is activated by phosphorylation through the MAPK pathway and the docking of MAPKs occurs via D domain [Besnard et al., 2011], [Buchwalter et al., 2004]. Besides these similar domains, other domains are present in different TCFs [Buchwalter et al., 2004].

TCFs can be activated by phosphorylation of three different kinases in the MAPK pathway, ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase), and p38. p38 and JNK cascade respond to cytokines and stress, ERK cascade is stimulated by growth factor and mitogens [Besnard et al., 2011]. According to signalling pathway TCFs act differently, even they have similar sequences. Phosphorylation residue of MAPKs is Ser/Thr-Pro motif at the C domain but the docking site of MAPKs s is at the D domain. This binding is crucial for the transmission of the signal correctly. In other words, TCF is phosphorylated proper kinase with high fidelity and enhanced action. In addition to the D domain, the C domain also has a docking site that is FXFP motif and special for the ERK pathway as well as MAPKs have similar docking site. Phosphorylation TCFs increase DNA binding but also can recruit interaction with co-activators. Specificity and complexity of TCFs can be regulated with interaction with many other proteins such as Pax-5, p53, BRCA1 [Buchwalter et al., 2004].

The absence of MAPK signalling causes transcriptional repression by TCFs. In addition to that, TCFs have inhibitory domains for transcriptional repression, such as

NID and CID domains found in Net and NID domain found in Sap-1. Different from those, Elk-1 additionally has a repression domain called as R domain that can be modified with small ubiquitin-like modifier (SUMO) and this post-translational modification causes Elk-1 repression [Buchwalter et al., 2004].

2.1.1.1. Elk-1 Transcription Factor

Elk-1 transcription factor is a member of TCFs subfamily. Elk-1 is related to lots of biological processes such as differentiation, cell growth and, survival. Elk-1 involves regulation of immediate early gene expression through forming a ternary complex with SRF on the SRE region of DNA. In addition to SRE-dependent transcription regulation, Elk-1 is also important for transcription in general [Besnard et al., 2011].

Since Elk-1 is one of the TCF, it is composed of four conserved domains and a repression domain (Figure 2.1). A domain for DNA binding also contains nuclear import and export signal sequence. B domain interacts with its DNA binding partner, SRF. The docking site called DEJL for the binding of JNK, p38, and ERK is contained in D domain [Besnard et al., 2011]. C domain is a transactivation domain that carries multiple phosphorylation sites that contain S/TP motif. Especially, phosphorylation of Serine 383 and Serine 389 amino acids at the C domain is responsible for Elk-1 mediated transcription [Yang et al., 1999]. At the downstream of the C domain, there is a DEF (FXFP) docking site for the docking of ERK. It is crucial for selective phosphorylation of Elk-1 from ERK. R domain is responsible for repression of Elk-1. Lysine residues presented in R domain are SUMOylated and this post-translational modification enhanced the suppression of Elk-1 transcriptional activity [Besnard et al., 2011].

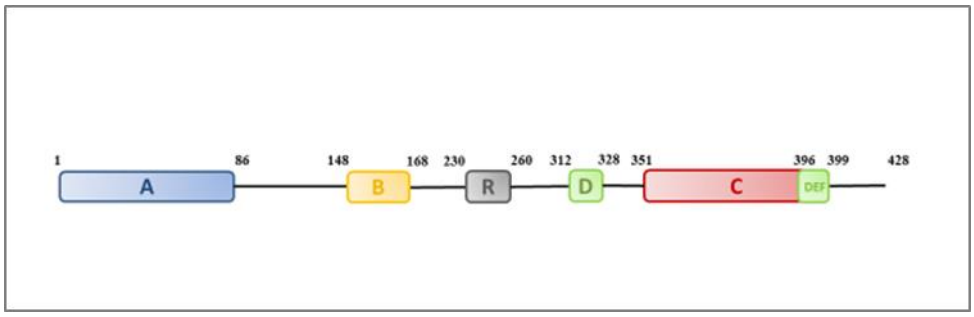

Figure 2.1: Domains of the Elk-1 transcription factor. DNA binding, ETS, domain is the A domain. B domain is required for interacting with SRF. C domain is transactivation domain, MAPKs phosphorylates multiple serine-threonine residues in this domain. DEF is the docking site for ERK. The docking site for all upstream kinases of Elk-1, p38, JNK, and ERK is presented in D domain. R domain is a repression domain, contains several lysine residues for SUMOylation.

Transcriptional activation of Elk-1 happens upon phosphorylation by MAPKs. As aforementioned before, Serine 383 and Serine 389 at the activation domain is phosphorylated for transcriptional activity of Elk-1 [Yang et al., 1999]. If these proteins transform to alanine amino acid, Elk-1 cannot be phosphorylated and so the SRE-dependent transcriptional activation property of Elk-1 is lost. Especially, transforming from Serine 383 to alanine cause total loss of transcriptional activity in general [Besnard et al., 2011]. Binding of Elk-1 with SRF to SRE occurs via protein-DNA interaction and protein-protein interaction. A conformational change in tertiary structure of Elk-1 is stimulated by phosphorylation of Elk-1 so that it can bind to SRF as well as to DNA [Yang et al., 1999]. Elk-1 phosphorylation also recruits coactivators of basal transcriptional machinery like CREB binding protein (CBP) [Ramirez et al., 1997].

The transcriptional activity of Elk-1 can be regulated by kinases and phosphatases. Phosphatases take part in the inhibition mechanism of Elk-1 transcriptional activity in contrast with kinases. Calcineurin (PP2B), the serine-threonine phosphatase, is shown that major phosphatase for dephosphorylation and inactivation of Elk-1 [Sugimoto et al., 1997]. Another repression mechanism of Elk-1 is the SUMOylation of the R domain. At the basal conditions, Elk-1 is inactive and in SUMOylated state. SUMO is conjugated to Lysine 230, 249, and 254. SUMOylated Elk-1 recruits the corepressors complexes to the promoter. Phosphorylation of Elk-1 upon MAPKs cascade especially the ERK pathway leads to loss of SUMO modification, [Yang et al., 2003]. A study has been shown that the phosphorylation

residues of Elk-1 are phosphorylated with different rate by its upstream kinase, ERK. Some residues phosphorylated fast some residues phosphorylated slowly. Different phosphorylation rate of these phosphorylation sites may affect the activation and repression of Elk-1 [Mylona et al., 2016].

Expression analysis showed that Elk-1 is strongly expressed in different regions of adult rat brain such as striatum and hippocampus. Elk-1 is especially expressed in neurons, not glial cells. In addition to the higher expression level of Elk-1 in neurons, Elk-1 is present at the dendrites, soma and, axon terminals of neurons as well as the nucleus, although it is a transcription factor [Sgambato et al., 1998].

In addition to the transcriptional regulatory role of Elk-1, Elk-1 acts variously in neurons like inducing cell death according to its different localization in neurons, and also different phosphorylation patterns [Barrett et al., 2006]. Overexpression of Elk-1 decreases cell viability by causing apoptosis of neurons by interacting with mitochondrial complexes when it is present at the cytoplasmic compartment [Barrett et al., 2006]. In contrast, Elk-1 increases cell viability and displays an anti-apoptotic effect by negatively regulating promoter of *egr-1* (early growth response-1) that is a transcription factor and induces apoptosis in neuroblastoma cells [Demir and Kurnaz, 2008]. In addition to that, it has been reported that Elk-1 has a neuroprotective role by regulating anti-apoptotic gene *MCL-1* and another target *SMN* [Demir et al., 2011]. The study from our laboratory suggests that Elk-1 has an important role in mitosis. P-S383-Elk-1 localizes to the first to spindle poles and co-localizes with Aurora-A and then to midbody during mitosis [Demir and Kurnaz, 2013]. Elk-1 can be phosphorylated by mitotic kinases that are Polo-like kinases (Plks), Aurora kinases and, Cyclin-dependent kinases (Cdks), because of their similar localizations with Elk-1 during mitosis [Uyar et al., Manuscript in preparation].

Elk-1 is also related to neurological diseases such as Alzheimer's Disease (AD) and Parkinson's Disease (PD). A study showed that Ras-Elk-1 signalling is activated by Centaurin-α1 (CentA1) that is overexpressed in AD and accumulates in neuritic plaques. CentA1-Ras-Elk-1 signalling is related to β-Amyloid dysfunction through the impairment of mitochondrial activity [Szatmari et al., 2013]. Phosphosite Threonine 417 of Elk-1 is associated with Elk-1 mediated neuronal death in the context of neurodegeneration. It is shown that phosphorylation of Elk-1 on T417 is colocalized with neuronal inclusions in AD, PD, and Huntington's Disease (HD) [Sharma et al., 2010].

Elk-1 is involved in different neuronal processes such as synaptic plasticity, long-term potentiation and learning [Besnard et al., 2011]. Elk-1 can regulate immediate early genes transcription like *zif268* in neurons via stimulation by ERK signalling cascade for synaptic plasticity, besides cell growth [Sgambato et al., 1998].

Phosphorylated Elk-1 upon serum stimulation, localizes to the nucleus for transcriptional activation. While Elk-1 is relocated to the nucleus, it interacts with microtubules [Demir et al., 2009]. SUMOylation and phosphorylation processes are the important post-translational mechanism for the trafficking of Elk-1 between the nucleus and cytoplasm (Figure 2.2) [Besnard et al., 2011]. Axotomy studies have shown that after nerve injury phosphorylated Erk is transported retrogradely from axon terminal to the cell body via dynein motor protein. Erk is one of the main upstream kinases of Elk-1 [Perlson et al., 2005].

According to these, Elk-1 might be phosphorylated by Erk at the axon terminal and phosphorylated Elk-1 might be transported with motor proteins to the nucleus like Erk. In parallel with the interaction of Elk-1 with motor proteins, it has been shown that after serum stimulation Elk-1 interacts with dynein motor protein during mitosis [Demir et al., 2012]. In the context of axonal trafficking, the presence of Elk-1 in nucleus and axon terminals under different conditions raises the question of whether Elk-1 is transported with motor proteins. Previous studies indeed showed co-localization of Elk-1 with kinesins such as MKLP1 and Eg5 in SH-SY5Y and U87 cells [Demir et al., 2012]. In this study, we tried to investigate whether this co-localization is through direct interaction between Elk-1 and kinesin motor protein, and whether mitotic kinase-dependent phosphorylations can affect this co-localization and interaction.

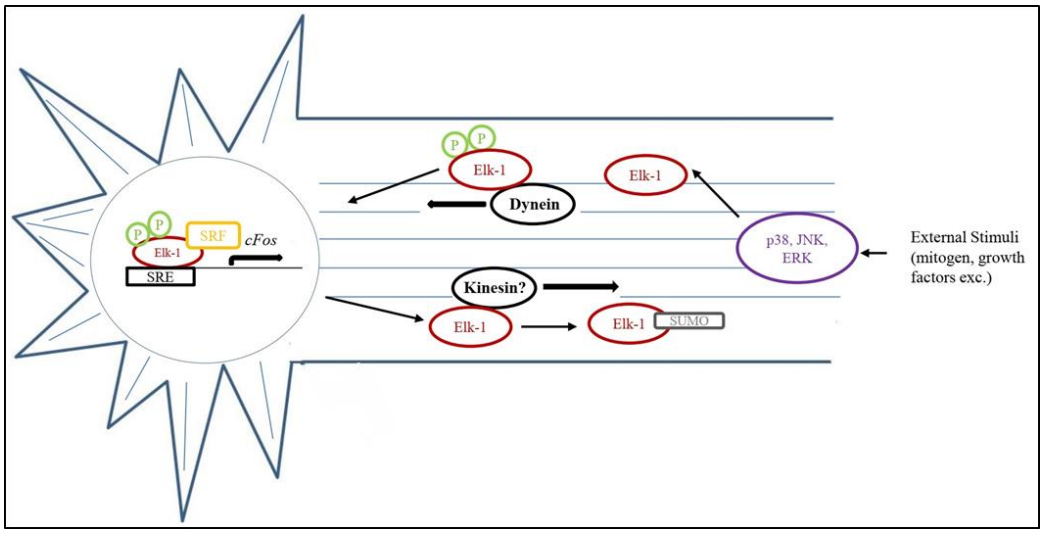

Figure 2.2: Localization and translocation of Elk-1 within the neuron. External stimuli such as growth factors, mitogens cause activation of three MAPKs, p38, JNK, and ERK. Activated kinase phosphorylates Elk-1 from Serine 383 and Serine 389. Normally Elk-1 interacts with microtubules. After its phosphorylation, it is translocated to the nucleus to activates transcription of immediate early genes like *c-fos.* Elk-1 may be translocated to the nucleus with dynein motor protein. After transcriptional activation non-activated form of Elk-1 may be translocated from the nucleus to the axon terminal with kinesin motor protein.

2.2. Kinesin Motor Protein in Neurons

Intracellular transport is critical because of the polarized structure of neurons. Organelles, synaptic vesicles, neurotransmitter proteins are transported from soma to axon terminal. Motor proteins are dynein, kinesin and, myosin that are responsible for the transportation of cargos and moves on microtubules as a track. Most of the kinesins are microtubule plus end-directed motor proteins and mediate anterograde transport which occurs from cell body to cell periphery. On the other hand, most of the dyneins are microtubule minus end-directed motor proteins and mediate retrograde transport which occurs cell periphery to cell body [Hirokawa et al., 2010], [Seog et al., 2004]. Totally 45 kinesin superfamily proteins (KIFs) are identified from mouse and human genomes and these are classified into 14 classes [Hirokawa et al., 2010], [Miki et al., 2001]. KIFs contain the motor domain, a stalk domain, and the tail region (Figure 2.3 taken from [Hirokawa et al., 2010]). All KIFs have conserved motor domain but other parts of the protein exhibit divergence to bind different cargos [Hirokawa et al., 2010]. KIFs are critical for cell division, neural development, and neurological disorders progression [Swarnkar et al., 2018].

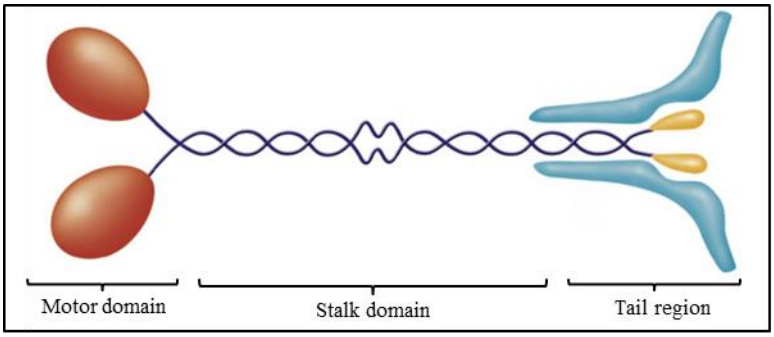

Figure 2.3: Domains of KIFs are indicated through the structure of Kinesin-1 motor protein.

KIFs take part in various processes in neurons since they carry different cargos. KIF1B is responsible for transportation of mitochondria and then it has been shown that its isoform, KIF1B-β, transport synaptic vesicle precursors, and mutation in the motor domain of this protein causes neuropathies [Zhao et al., 2001]. KIF3 is one of the most abundant KIFs and mainly presented in the nervous system [Takeda et al., 2000]. KIF3 motor contains KIF3A/KIF3B/KAP3 (Kinesin Associated Protein-3). This complex associated with the organization of neuroepithelium which is related to the stability between proliferation or cell-cell adhesion of neural progenitor cells in the developing brain [Teng et al., 2005].

Motor proteins, especially KIFs, have an important role not only in intracellular transport but also in mitosis, such as the organization of chromosomes and movement of spindles [Seog et al., 2004]. MKLP1 (Mitotic Kinesin-Like Protein/KIF23/CHO1) is a component of central spindlin complex and it is critical for the formation of the midbody and its phosphorylation by Aurora B is required for implementation of cytokinesis [Guse et al., 2005], [Zhu et al., 2005]. CHO1/MKLP1 is not present only mitotic cells, its expression is important in postmitotic neurons of the developing brain for dendritic differentiation [Lotfi Ferhat, 1998]. Eg5 is required for the separation of centrosomes and bipolar spindle formation [Eibes et al., 2018], [Tanenbaum et al., 2008]. Deacetylation of Eg5 by HDAC1 during prophase of mitosis is important for mitotic progression [Nalawansha et al., 2017]. A previous study from our laboratory showed that, P-383-Elk-1 co-localizes with Eg-5 and also MKLP-1 at spindle poles during mitosis (Figure 2.4 and 2.5) [Demir et al., 2012].

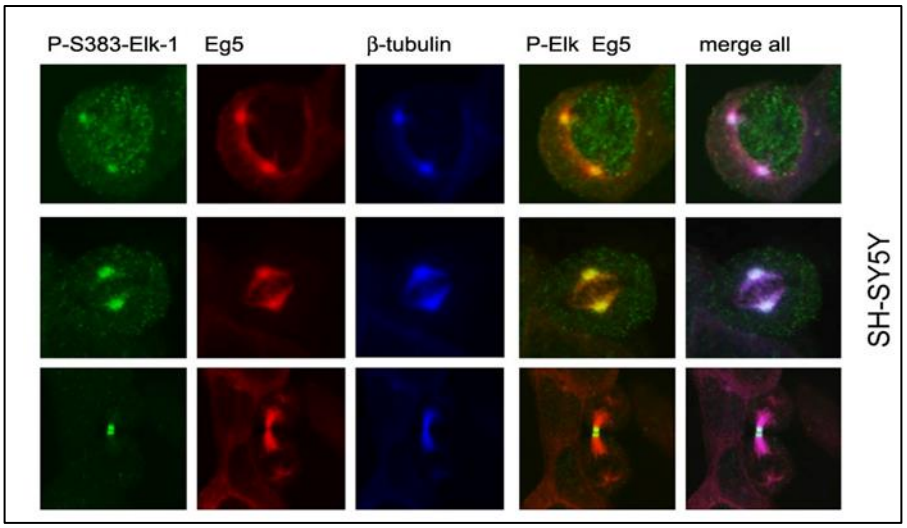

Figure 2.4: Co-localization of Serine 383 phosphorylated Elk-1 with Eg5 during mitotic cycles (anti-P-S383-Elk-1, green; anti-Eg5, red; anti-b-tubulin, blue; DNA).

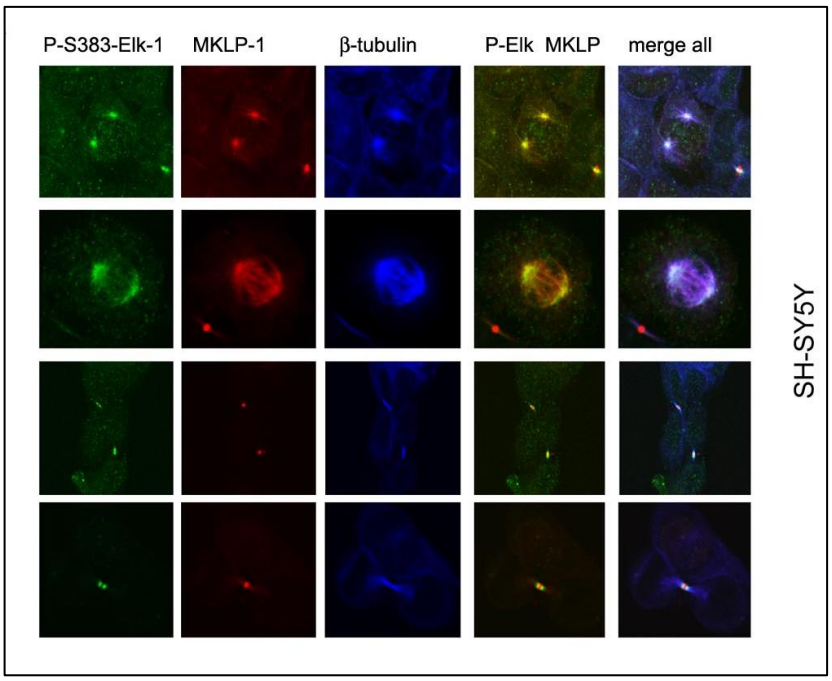

Figure 2.5: Co-localization of Serine 383 phosphorylated Elk-1 with MKLP1 during mitotic cycles (anti-P-S383-Elk-1, green; anti-MKLP1, red; anti-b-tubulin, blue; DNA).

3. MATERIALS and METHODS

3.1. Materials

3.1.1. Plasmids

Original Elk-1 expression plasmid was a gift of by Prof. A.D. Sharrocks (UK); the coding sequence of Elk-1 was sub-cloned to pCMV-5-Flag plasmid in AxanLab (TR). Phospho-mutants of Elk-1, namely S198A, S198E, S303A, S304A, S324A, S326A, S326E, S383A, S389A, T417A, were created by Oya Arı Uyar site-directed mutagenesis on the pCMV-5-Flag-Elk-1 (AxanLab). Serine (S) and Threonine (T) residues of Elk-1 are phosphorylated by kinases. When these residues are converted to Alanine (A) or Glutamic acid (E), they mimic non-phosphorylated and phosphorylated forms of Elk-1, respectively.

3.1.2. Cell Lines and Bacterial Strains

- SH-SY5Y – Human Neuroblastoma Cell Line (ATCC number: CRL-2266)
- SK-N-BE(2)- Human Neuroblastoma Cell Line (ATCC Number: CRL-2271)
- U-87 – Human Glioblastoma Cell Line (ATCC number: HTB-14)
- *E. coli* DH5α bacterial strain

3.1.3. Enzymes and Buffers

- i-*Taq* DNA polymerase (iNTRON Biotechnology, 25022)
- i-*Taq* dNTP Mixture (iNTRON Biotechnology, 25022)
- MyTaq DNA Polymerase (BIOLINE, BIO-21105)
- 10X *Taq* Buffer with $MgCl_2$ (Thermo Scientific)
- 25 mM $MgCl_2$ (Thermo Scientific)

3.1.4. Kits

- DNA-midi SV, Plasmid DNA Purification Kit (iNTRON Biotechnology, 17252)
- DNA-spin, Plasmid DNA Purification Kit (iNTRON Biotechnology, 17097)
- iScript cDNA Synthesis Kit (Bio Rad, 1708891)
- Smart BCA Protein Assay Kit (Intron Biotechnology, 21071)

3.1.5. Cell culture solutions

- Dulbecco's Modified Eagle Medium 1X (DMEM) with 4,5 g/L D-Glucose, L-Glutamine, and Pyruvate (Gibco, 41966-029)
- Fetal Bovine Serum (FBS) (Gibco, 10270)
- Dulbecco's Phosphate Buffered Saline 1X (DPBS) (w/o $CaCl_2$, $MgCl_2$) (Gibco, 14190-94)
- 0.05% Trypsin-EDTA Solution (1X) (Gibco, 25300-054),
- Penicillin/Streptomycin (10000U-ml Penicillin / 10mg/ml Streptomycin) (PAN Biotech, P06-07100)
- Polyethylenimine (PEI) transfection reagent (Polysciences)
- Dimethyl sulfoxide (DMSO)
- 0,1% Poly-L-Lysine solution (Sigma)
- Antibiotic-Antimycotic Solution, 100 X (Gibco)

3.1.6. Antibodies

- Rabbit Polyclonal anti-Elk-1 Antibody (Cell Signaling #9182)
- Rabbit Polyclonal anti-P-Elk1 S383 Antibody (Cell Signaling #9181)
- Purified Mouse Anti-Human Eg5 (BD Transduction Laboratories, 611186)
- Rat Monoclonal Antibody to Tubulin (Abcam, 6160)
- Mouse Monoclonal anti-b -Actin Antibody (Santa Cruz Biotechnology, sc-47778)
- Mouse Monoclonal anti-Flag Antibody (Sigma, F1804)
- Anti-Flag M2 Affinity Gel (Sigma, A2220)

- Goat Anti-Mouse IgG Antibody-HRP Conjugate (Santa Cruz Biotechnology, sc-2005)
- Goat Anti-Rabbit IgG Antibody-HRP Conjugate (Santa Cruz Biotechnology, sc-2004)
- Phospho-specific antibodies of Elk-1 (S303, S304, S324, S326) (GenScript)
- Goat anti-rabbit IgG–Alexa Fluor 488 (Invitrogen)
- Goat anti-rat IgG–Alexa Fluor 546 (Invitrogen)
- Goat anti-mouse IgG–Alexa Fluor 647 (Invitrogen)

3.1.7. Western Blot Solutions

- 40% Acrylamide:bisacrylamide (Fisher Scientific)
- Tris (AppliChem)
- Sodium dodecyl sulphate (SDS) (Fisher Scientific)
- Ammoniumpersulphate (APS) (Fisher Scientific)
- TEMED (Sigma, T7024-100ML)
- Tween-20 (Merck)
- Glycine (Sigma)
- NaCl (Sigma), HCl (AppliChem), NaOH
- 3X Blue Loading Buffer (Cell Signaling, #7722S)
- Page Ruler Plus Prestained Protein Ladder (Thermo Scientific, 26619)
- PhosStop EASYpack - Phosphatase Inhibitor Coctail Tablets (Roche, 04906845001)
- Protease Inhibitor Coctail (Sigma, P8465-25ML)
- Luminata Crescendo Western HRP Substrate (Millipore, WBLUR0500)
- Nonfat Dry Milk (Cell Signaling Technology, #9999S)
- Bovine Serum Albumin (Calbiochem, 1265S)
- Methanol (Sigma)

3.1.8. Agarose Gel Solutions

- Tris-Acetate-EDTA (TAE)

- Agarose (Sigma)
- RedSafe Nucleic Acid Staining Solution (Intron Biotechnology, 21141)
- GeneRuler 1kb Plus DNA Ladder (Thermo Scientific, #SM1331)
- 10X Loading Buffer (TaKaRa Bio)

3.1.9. Bacterial Assays

- Luria-Bertani (LB) Broth (Caisson Labs, LBP01-500GM),
- LB Agar (Caisson Labs, LBP02-500GM)
- Ampicillin (Biomatik)
- 87% Glycerol (Sigma, 15524)
- Calcium chloride ($CaCl_2$)(AppliChem)

3.1.10. Material and Equipment

- Hemocytometer (Marienfeld)
- T25 Tissue Culture Flasks (TRP)
- T75 Tissue Culture Flasks (TRP)
- T150 Tissue Culture Flasks (TRP)
- Coverslip (Isolab)
- µ-Slide 8 Well
- 60 mm, 100 mm, tissue culture plate (TRP)
- Petri dish (100 mm) (Lamtek)
- 5 ml, 10 ml ,25 ml, 50 ml serological pipettes (CAPP)
- Micropipettes (Eppendorf)
- 15 ml and 50 ml Falcon (CAPP)
- Microcentrifuge tubes (0,2 ml, 0,5 ml,1,5 ml, 2ml)
- Cryotubes (1ml, 2ml)
- Mini-PROTEAN Tetra Cell Electrophoresis System (Bio-Rad)
- Varioskan Microplate Reader (Thermo)
- Water Bath (Lab Companion BW-10H)
- Ultra-speed Centrifuge (Avanti J-251 - Beckman)

- Blot Transfer (Trans-Blot SD Semi-Dry Transfer Cell- BioRad)
- ChemiDoc Imaging System (Biorad)
- Confocal Microscope (Zeiss, LSM 880)
- Laminar flow cabinet (Thermo Scientific Class II Safety Cabinet S2020)
- CO2 incubator (Thermo Scientific, Steri-Cycle i160)
- Inverted microscope (LEICA DMi1)

3.2. Methods

3.2.1. Bioinformatic Analyses

3.2.1.1. Protein-Protein Interaction Subnetwork Discovery

Microarray analysis of Elk-1-VP16 transfected SH-SY5Y cells was performed by Dr. Başak Kandemir [Sogut et al, Manuscript in preparation]. Using this microarray data and after determination of differentially expressed genes (DEGs), we aimed to identify subnetworks that DEGs are involved in and focus on pathways related to axonal transport. At first, whole-genome protein-protein interaction network was obtained from BioGRID database [Chatr-Aryamontri et al., 2017]. For the discovery of subnetworks our expression data was mapped to with this PPI network using KeyPathwayMiner (KPM) tool [Alcaraz et al., 2016]. This tool results in several subnetworks that contain mostly significantly changed genes. From these networks we chose the biggest subnetwork for gene enrichment analysis that was performed in g:Profiler web server [Raudvere et al., 2019]. Unfortunately, we could not obtain any result that was related with motor proteins from gene enrichment analysis of this microarray data. We thought that still, this pathway might contain motor proteins especially KIFs, but they were not enriched so that to find out these genes a code manuscript was written by Yiğit Koray Babal. The code checks GO terms of genes in the pathway about motor activity.

3.2.1.2. Identification of Possible Kinesins for Elk-1 Interactions

We have used another bioinformatic tool Prediction Server of Protein-Protein Interaction (PSOPIA) to identify kinesins, possible interaction partner of Elk-1. This server predicts the interaction of two proteins based on the amino acid sequence of proteins. It gives a value between 0 and 1, and the probability of interaction between two protein increases as value approaches to 1. This value is determined according to sequence similarities of interacting proteins, statistical propensities of domain pairs presented in interacting proteins, homologous of these two proteins how much they are in close proximity in known PPI network [Murakami and Mizuguchi, 2018]. Then, we chose the KIFs that their possible interaction value is higher than 0,7 to further cloning studies.

3.2.2. Primer Design

Primers were designed for the amplification of *KIF* genes, coding sequence of KIF proteins which is presented in the Consensus Coding Sequence (CCDS) database and primer pairs are listed in Table 3.1. After the amplification step, since these genes will be used in cloning assay, primers contain restriction enzyme digestion sequence. In addition to restriction enzyme digestion sequence, forward primers have a few bases for the prevention of frameshift. Secondary structure formation, GC content properties were checked by using the Oligo Analyzer tool of Integrated DNA Technologies (IDT).

Table 3.1: Primer pairs of *KIF* genes.

KIF1B	**Forward:** 5'- ATAGGTCGACTTCGGGAGCCTCAGTGAAG-3'
	Reverse: 5'- ACAGGGTACCTTAGTATTTCGACTGGCT-3'
KIF3B	**Forward:** 5'- ATAGGTCGACGTCAAAGTTGAAAAGCTCA-3'
	Reverse: 5'- ATATGGTACCTTACTTTGGAACCAGCCC-3'
KIF3C	**Forward:** 5'- ATATGTCGACTGCCAGTAAGACCAAGGCC-3'
	Reverse: 5'- ATATGCGGCCGCTCACTCATGGTCCGCCACTGT -3'

Table 3.1: Continued.

KIF4	**Forward:** 5'- ATAGGTCGACTAAGGAAGAGGTGAAGGGA -3'
	Reverse: 5'- ATATGCGGCCGCTCAGTGGGCCTCTTCTTC -3'
KIF11	**Forward:** 5'-ATAGGAATTCATGCGTCGCAGCCAAATTCG -3'
	Reverse: 5'- ATAGGTCGACTTAAAGGTTGATCTGGGC -3'
KIF23	**Forward:** 5'- ATATGTCGACTAAGTCAGCGAGAGCTAAG -3'
	Reverse: 5'- ATATGGTACCTCATGGCTTTTTGCGCTT -3'

3.2.3. cDNA Synthesis

cDNA was synthesized with BIO-RAD iScript cDNA Synthesis Kit (Catalog No #17008891). cDNA was synthesized from 1 μg total RNA of SH-SY5Y and U87 cells that are prepared from Ekin Sönmez. The reaction was performed at 5 min at 25℃, 20 min at 46℃, 1 min at 95℃. Reaction components are showed in Table 3.2.

Table 3.2: Reaction mixture for cDNA synthesis.

Components	**Amounts**
iScript Reaction Mix (5X)	4 μl
iScript Reverse Transcriptase	1 μl
RNA template	variable
Nuclease free water	variable
Total volume	**20 $\boldsymbol{\mu l}$**

3.2.4. Gradient PCR

For the amplification of desired genes, PCR conditions must be optimized. Determinations of annealing temperature (Ta) is one of the important steps for PCR optimization and gradient PCR was performed for this. For each *KIF* gene melting temperature was calculated by using the NEB Tm calculator. Five tubes that contain identical mixture was prepared for each primer set. Reaction mixture were prepared according to Table 3.3. PCR was performed to these tubes at gradient annealing temperatures. Gradient temperatures were setting up as lower and higher temperatures

of Tm. While setting up annealing temperatures, determined temperatures were within 55°C-65°C range except for special conditions (These are explained in the result section). Reactions conditions are given at the table 3.4.

Table 3.3: Components of reaction for Gradient PCR.

Components	**Amounts**
10X Taq Buffer with $MgCl_2$	2,5 μl
$MgCl_2$ (25 mM)	0,75 μM
iTaq dNTP mixture (2,5 mM each)	0,5 μl
Forward primer	0,5 μM
Reverse primer	0,5μM
Taq DNA polymerase (5U/μl)	0,1 μl
Template DNA	variable
Nuclease free water	variable
Total Volume	**25 $\boldsymbol{\mu l}$**

Table 3.4: Reaction conditions for gradient PCR.

	Temperature	**Duration**	
Initial Denaturation	94°C	3 min	
Denaturation	94°C	45 sec	34 cycles
Annealing	Gradient	30 sec	
Extension	72°C	1 min for each 1 kb amplicon size	
Final extension	72°C	10 min	

3.2.5. Bacterial Assays

3.2.5.1. Competent Cell Preparation

-80°C stock of DH5α strain of *Escherichia coli* bacteria is streaking on the LB agar plate and incubated overnight at 37°C. Then a single colony was picked up from this bacterial culture and inoculated 5 ml of LB broth medium. Bacterial culture was incubated overnight at 37°C with shaking at 250 rpm. 1 ml of the saturated overnight culture was inoculated to 100 ml of LB broth medium in an Erlenmeyer flask. Erlenmeyer flask was shaken at 37°C at 180 rpm until OD_{600} the value reached 0,4. Then the bacterial culture was placed on an ice bath for 10 min, after this point, all materials used must be pre-cooled. Bacterial culture was transferred to the pre-chilled 50 ml falcon tubes. They were centrifuged at 2700xg for 10 min at 4°C. after centrifugation, the medium was removed, the cell pellet was resuspended with 1,6 ml of ice-cold 100 mM $CaCl_2$ by swirling on ice gently. Tubes were incubated on ice for 30 min and centrifuged at 2700xg for 10 min at 4°C. After centrifugation, the medium was removed, and the cell pellet was resuspended with 1,6 ml of ice-cold 100 mM $CaCl_2$ by swirling on ice gently. Tubes were incubated on ice for 20 min. Then, cells were combined to one tube and 0,5 ml of ice-cold 80% glycerol was added. The mixture was aliquoted as 100 μl and frozen in liquid nitrogen. They were stored at -80°C.

3.2.5.2. Transformation

Plasmids were transferred to the competent bacterial cells for amplification of plasmids. plasmid stocks were diluted as 100 ng/μl. 1 μl of the diluted plasmid is transferred to a 1,5 ml Eppendorf tube. 50 μl of competent cells were added on it. In addition to this, to check contamination in competent cells, just 50 μl of competent cells were put in another Eppendorf tube as a negative control. Tubes were incubated on ice for 15 mins, then at 42°C for 90 sec, and on the ice again for 2 mins. 900 μl of LB broth medium was added to tubes. Tubes were shaken at 37°C for an hour at 180 rpm. 200 μl of the mixture was spread onto antibiotics containing LB agar plates. Plates were incubated overnight at 37°C.

3.2.5.3. Plasmid Isolation

A single colony was picked up the streaked bacterial plate and inoculated to antibiotics containing LB broth medium into Erlenmeyer flask. That was shaken overnight at 37°C at 180 rpm. The bacterial culture was transferred to 50 ml falcon tubes. They were centrifuged for 10 min at 4000 rpm and the supernatant was removed completely. Bacteria pellet was resuspended in 2 ml of M1 (resuspension buffer) buffer by vortexing until no clumps remain. In this step, the important thing is that M1 buffer contains RNase A solution for efficient lysis of the bacterial cell. After that, 2 ml of M2 buffer (lysis buffer) was added. Tubes were inverted 10 times for proper mixing and incubated for 3 mins at RT. This incubation time is optimal for releasing of non-denatured plasmid DNA without releasing chromosomal DNA. 2 ml M3 buffer (neutralization buffer) was added, tubes were inverted 10 times to mix gently and incubated on ice for 5 min. The bacterial lysate was poured to pre-column. Tubes were centrifuged for 5 min at 4000 rpm. After centrifugation cell debris stays on pre-column and flow-through was poured to the binding column. The binding column was centrifuged for 5 min at 4000 rpm and flow-through was removed. 10 ml of washing buffer A was added to the column and centrifuged for 5 min at 4000 rpm. Flow-through was removed and 10 ml of washing buffer B was added. The column was centrifuged for 5 min at 4000 rpm. To distract the washing buffer completely, the column was centrifuged for 15 min at 4000 rpm. Flow-through was removed again and the column was incubated at 56°C for 5 min to remove residual ethanol that is found in washing buffer B. Binding column was re-inserted new 50 ml collection tube. For elution of plasmid DNA from the column, 300 μl nuclease-free water was added. The column was incubated at RT for 5 min and centrifuged for 5 min at 4000 rpm. Then, again 300 μl nuclease-free water was added, and the centrifugation step was repeated. Elution was performed in two steps, as it increases the yield.

3.2.6. Cell Culture

As a general procedure, when cell confluency reaches 80%, cells should be passaged to another flask. Medium on the cells was removed. PBS is added and flask

shaking slowly to distract the rest of the medium. After removing PBS 0,05% Trypsin EDTA is added and incubated at 37°C for 3 min. Medium ten times amount of the trypsin was added for the inhibition of trypsin. Cells were placed in falcon tube and centrifuged at 1500 rpm for 5 min. After centrifugation, the supernatant was removed, and the pellet was dissolved in the medium. After this step, if this is necessary, cells were counted using a hemacytometer. Dissolved cells in the medium were placed in another T75 or T150 tissue culture flask and incubated at 37°C, 5% CO_2.

For cryogenic preservation, cells were frozen with %10 DMSO (Dimethyl Sulfoxide). First, cells were counted with hemacytometer then 100 μl of DMSO is mixed with 1,5 or 2 million cells per ml in a cryotube. Cryotube is placed at -20°C for 2 hrs and then at -80°C for short time storage or liquid nitrogen for long term storage.

While frozen cells are thawing, cryotube is incubated for 1-2 min at 37°C. After cells thaw, dissolved cells were immediately transferred to the medium in the falcon tube to protect cells from the toxic effect of DMSO and centrifuged. After centrifugation, cell pellet was dissolved in medium and transferred into T25 tissue culture flask, incubated at 37°C, 5% CO_2.

3.2.7. Immunoprecipitation

3.2.7.1. Transfection

1,5X 10^6 SH-SY5Y human neuroblastoma cells (American Type Culture Collection ATCC#CRL-2266) were seeded in 100 cm^2 tissue culture dish and the dish was incubated for 24 hours for attachment of cells. Phosphomutants of Elk-1 and GFP plasmid for control of transfection efficiency were transiently transfected by using Polyethylenimine (PEI) reagent (dissolved in dH_2O at 80°C, pH is adjusted 7.0 with using 5M HCl and filtered). To achieve effective transfection, the ratio of the plasmid to PEI in the transfection mixture was 1: 3.5 or 1: 4 that was optimized in our laboratory. For the preparation of transfection mixture 10 μg of plasmid, 1 ml of serum-free medium and 35 μl of PEI (1mg/ml) reagent were mixed by vortexing and after 15 mins 4 ml of complete medium was added to transfection mix. Medium on the cells removed and transfection mix was added to cells slowly, after 2 hours 5 ml of

complete medium was added. Cells were further incubated for 48 hours to complete transfection.

3.2.7.2. Protein Isolation

Medium on the transfected cells in 100 cm^2 tissue culture dish was removed. The dish was placed on ice and further steps performed on ice for protection of protein stability. 3 ml of PBS was added and then removed for washing of residual medium. This step was repeated two times. RIPA buffer was prepared as shown in Table 3.5. 1 ml of RIPA buffer was added. Before the addition of RIPA buffer, phosphatase and proteinase inhibitors (final concentration 1X) was put into RIPA buffer. The dish was shaken at 4°C for 20 min at 15 rpm. After that cells were harvested by using scrapper and collected into 1,5 ml Eppendorf tube. The tube was centrifuged at 14 000 rpm for 30 min at 4°C. The supernatant was transferred to a new tube and stored at -20°C.

Table 3.5: Components of RIPA buffer.

Components	**Amounts**
NaCl (1M)	15 ml
NP-40 (%10)	10 ml
Sodium Deoxycholate	0,5 g
SDS (%10)	1 ml
Tris, pH:8 (1M)	5 ml
dH_2O	Up to 100 ml

3.2.7.3. BCA (Bicinchoninic acid) Assay

For the determination of protein concentration, BCA Assay was performed. Proteins were diluted 1:5 with sterile water. BSA standards were prepared as 25, 125, 250, 500, 1000, 1500 and 2000 $\mu g/ml$. 25 μl of samples and BSA standards were put in 96-well plates as two replicates. 200 μl of Working Solution was added to each well and mixed. Working Solution was prepared by mixing 50 parts of Solution A with 1 part of Solution B (50:1, Solution A:B) (Smart BCA Protein Assay Kit, Intron

Biotechnology, 21071). The plate was incubated for 30 min at 37°C. Absorbance was measured on a microplate reader at 562 nm.

3.2.7.4. Flag-Immunoprecipitation (Flag:IP)

Immunoprecipitation is a method that a specific protein is obtained from a protein mixture using antigen-antibody interaction. Proteins were isolated from cells transfected with flag-tagged expression vectors containing wild type and mutants of Elk1. In order to obtain wild type and mutants of Elk-1 from this protein mixture, immunoprecipitation was performed by using Flag-Agarose beads. The first step of IP is the precleaning of agarose beads. For this, 50 μl of agarose beads were centrifuged at 600 g for 1 min at 4℃. The supernatant was removed, and beads were washed with 1 ml of PBS and centrifuged at 600 g for 1 min at 4℃. This washing step was repeated one more time. Then, beads were washed with 1 ml of TBS and centrifuged after that washed with1 ml of RIPA and centrifuged at 600 g for 1 min at 4℃. At the end of washing steps, beads were resuspended in 50 μl of RIPA buffer supplemented with phosphatase and protease inhibitors. Protein samples were prepared as 250 μg in 500 μl of dH_2O. Proteins were added to the beads and mixture was incubated overnight at 4℃. After incubation, beads were washed 2 times with 150 μl of RIPA buffer and 2 times with washing buffer that contains 50 mM Tris-HCl and 150 mM NaCl at pH 7.4. After washing steps pellet was resuspended in 30 μl of 4X Blue Loading dye and stored at 4℃.

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

Protein samples were mixed with 4X Blue Loading dye according to the desired amount of protein. Samples were boiled at 95℃ for 6 min. Stacking and resolving gels were prepared according to Table 3.6 and Table 3.7. Gels were placed in a gel electrophoresis chamber and fresh electrophoresis buffer(1X) was added until the buffer covered the gels (10x electrophoresis buffer: 30,2 Tris, 144 Glycine and 10 g SDS are dissolved in 1 L dH_2O). Combs were taken out gently from stacking gels. Protein samples were loaded to each well. The power supply was adjusted to 80V.

After proteins migrated from stacking gel to resolving gel, power was increased to 100V. Electrophoresis was continued for 2-2.5 hrs.

Table 3.6: Stacking gel (5%) components for 2 gels (for 1,5 mm glass plates).

Components	**Amounts**
dH_2O	4,5 ml
Acrylamide (40%)	750 μl
1 M Tris (pH 6,8)	760 μl
SDS (10%)	60 μl
APS (10%)	60 μl
TEMED	6 μl

Table 3.7: Resolving gel (10%) components for 2 gels (for 1,5 mm glass plates).

Components	**Amounts**
dH_2O	9,65 ml
Acrylamide (40%)	4,95 ml
1 M Tris (pH 8,8)	5 ml
SDS (10%)	200 μl
APS (10%)	200 μl
TEMED	8 μl

3.2.7.6. Western Blot Analysis

After the separation of proteins in SDS-PAGE analysis, Western Blot Analysis was performed. Wet transfer protocol was applied to transfer proteins from gel to membrane. PVDF membrane is activated in absolute methanol for 1 min after that, it was placed transfer buffer. 2 Whatman paper, gel, activated PVDF membrane and 2 Whatman paper were placed respectively in transfer cassette. Transfer cassette was put on in the transfer system and transfer buffer (1X) (Glycine and Tris) and 20 per cent methanol was added. Proteins were transferred for 1 hr. After the transfer step, the membrane was blocked with 3% BSA (in TBS-T) or 5% skim milk (in TBS-T) for 1 hr to prevent the antibody from binding to non-specific proteins. The membrane was

washed 3 times for 3 min with TBS-T (1X). The membrane was incubated overnight with the primary antibody that was diluted with 5% BSA or 5% skim milk at 4℃. The membrane was washed 3 times for 10 min with TBS-T, then incubated with secondary antibody for 1 hr. After that, the membrane was washed 2 times for 10 min with TBS-T and once with TBS. Before imaging of membrane, it was incubated with 1 ml of ECL for 1 min and imaged with Bio-Rad ChemiDoc Imaging Systems.

After the imaging of membranes, a stripping protocol was performed for checking housekeeping genes. The mild stripping buffer was prepared according to Table 3.8. The membrane was washed twice with stripping buffer for 8 min, then twice with PBS for 10 min, and then twice with TBS-T for 5 min. After stripping of the membrane, protein-bound antibodies were removed. The membrane was blocked and incubated with primary and secondary antibodies as previously described.

Table 3.8: Mild stripping buffer components.

Components	**Amounts**
Glycine	1,5 g
SDS	0,1 g SDS
Tween-20	1 ml
HCl	pH adjusted to 2.2
dH_2O	Up to 100 ml

3.2.8. Immunofluorescence Analysis

3.2.8.1. Cell culture

Chambered coverslip for 8 wells was put on an autoclaved glass slide. This system needs a few numbers of cells and a low volume of reagents. These properties make the system one of the best options for immunofluorescence analysis. The glass slide was coated 0,1% Poly-L-Lysine solution in dH_2O. It was diluted 1:10 with autoclaved dH_2O before use. Diluted Poly-L-lysine was added to cover the surface of well and incubated for 20 min. After incubation, the solution was removed, and wells were washed 3 times with autoclaved dH_2O. 6 X 10^3 U-87-MG glioblastoma cells,

purchased from American Type Culture Collection (ATCC#CRL-2266) were seeded in each well and incubated for 24 hrs at 37℃ and 5% CO_2.

3.2.8.2. Transfection

Cultured cells in 8-well were transfected transiently with phospho-mutants of Elk1, and also GFP plasmid for control of transfection efficiency. PEI was used as a transfection reagent. The transfection mix was prepared from 500 ng plasmid 2 μl of PEI (1mg/ml) reagent and 50 μl of serum-free medium and incubated for 15 min. 25 μl of complete medium was added to transfection mix. The complete medium on the cells was removed and the transfection mix was added slowly. After 2 hrs, 150 μl of complete medium was added gently. Cells were further incubated for 48 hours to complete transfection.

3.2.8.3. Immunostaining of Proteins

After transfection of cells medium on the cells was removed. Cells were washed with DPBS(1X). For the fixation of cells, 100 μl of 4% paraformaldehyde (PFA) (4 gr of PFA is dissolved in 100 ml of dH_2O at 60℃, pH is adjusted to 7.4) was added and incubated for 15 min at RT. 4% PFA was removed and cells were washed 2 times with 100 μl of PBS. 100 μl of blocking solution (3% BSA, 0,3% Sodium azide, 1% serum, 0,1% Triton-X) was added and incubated for 1 hr at RT. Primary antibodies were diluted in 50 μl of blocking solution. The blocking solution on the cells was removed and the primary antibody mixture was added and incubated overnight at 4℃ with shaking slowly. The primary antibody was removed, and cells were washed 2 times with PBS. Secondary antibodies were diluted in 50 μl of blocking solution. The secondary antibody mixture was added to cells and incubated for 3 hrs with slowly shaking. After that, secondary antibodies were removed, and cells were washed 3 times with 0,2% Tween-20 in PBS (PBS-T). 100 μl of 0,1% DAPI in PBS was added and incubated for 3 min. DAPI was removed and cells were washed 5 times with PBS-T. At last, glass slide was mounted with Prolong Gold Antifade Reagent or autoclaved %87 glycerol and analysed under Zeiss Confocal Microscope.

4. RESULTS

4.1. PPI Subnetwork Discovery

To research the effect of Elk-1 on axonal trafficking, we performed some bioinformatics analysis according to differentially expressed genes obtained from microarray analysis results of Elk-1-VP16 transfected cells. We tried to obtained subnetwork from the whole human protein-protein interaction network. We used KPM tool to discover subnetworks and the biggest subnetwork containing 4802 genes was chosen to analyse for the gene enrichment analyses. Gene ontology analysis was performed for this network with g:Profiler web server, but we couldn't obtain any molecular functions or biological processes that motor proteins are involved. To check the presence of genes that are not enriched in the pathway but related to motor activity, the code manuscript was written by Yiğit Koray Babal. The gene names and Entrez IDs were represented in table 4.1.

Table 4.1: Gene names and Entrez IDs after protein-protein interaction subnetwork analysis using KPM.

Gene name	Entrez ID
KIFC3	3801
KIF7	374654
KIF21B	23046
KIF12	113220
KIF19	124602
KIF1C	10749
KIF24	347240
KIF22	3835
KIF2C	11004
KIF2B	84643
KIF5A	3798
KLC4	89953
KLC3	147700
KLC2	64837

In the following step, a different bioinformatic tool called PSOPIA was used for verification of whether these KIFs could be interaction partners of Elk-1.

4.2. Identification of Possible Kinesins for Elk-1 Interactions

There are 45 mammalian *KIF* genes. In this part of the study, we have tried to identify KIFs that are possibly interacting with Elk-1 for the first time in literature. To that end, we have used the bioinformatic web server, Prediction Server of Protein-Protein Interactions (PSOPIA). PSOPIA analysis results in a value between 0 and 1 for interaction probability of two proteins according to amino acid sequences of these two proteins and the probability of interaction between two proteins increases as value approaches to 1. KIF proteins that were identified as possible Elk-1 interaction partners with higher interaction and their interaction values were shown in table 4.2. When this analysis was performed, it was observed that the KIFs identified previously in Table 4.1 do not correspond to KIFs that were identified from PSOPIA. Since PSOPIA gives reliable predictions on protein protein interactions, we have decided to continue our studies with KIFs in Table 4.2.

Table 4.2: Identified KIF proteins written in bold for Elk-1 interaction and their probability value for the interaction.

Gene Names	Value of the interaction with Elk-1
KIF1B	**0,8550**
KIF3B	**0,7150**
KIF3C	**0,7554**
KIF4	**0,7150**
KIF5A	0,6260
KIF6	0,5555
KIF2A	0,4189

KIFs have different roles in various cell types and they are expressed in most tissues but at different levels. KIF1B takes part in axonal transport of synaptic vesicles and especially transportation of mitochondria [Lyons et al., 2009]. KIF3B is found in a cell as a complex that comprises of KIF3A-KIF3B-KAP3 subunits called KIF3 motor [Teng et al., 2005]. KIF3 complex, plus-end directed motor protein, is involved in the transportation of membrane-bound organelles and protein complexes and it is abundantly expressed in neurons [Nonaka et al., 1998]. KIF3 motor complex is important for the left-right asymmetry and neurite building. In addition to important

roles in the nervous system, it is also involved in the accomplishment of mitotic progression [Haraguchi et al., 2006]. KIF3C is strongly expressed in nervous system and takes part in axonal anterograde transport. It is showed that KIF3C is an important element of microtubule dynamics and organization in growth cone after injury [Gumy et al., 2013], [Yang et al., 2001]. KIF4 motor protein is strongly expressed in juvenile tissues like neurons and its expression changes developmentally. KIF4 is related to mitosis and also axonal transport of membranous organelles. During development of brain, KIF4 involved mechanism of activity dependent neuronal survival [Midorikawa et al., 2006]. Eg5 and MKLP1 are especially mitosis related kinesins as mentioned before in detail at section 2.2.

4.3. Cloning of Selected *KIF* Genes

We aimed to study the interaction of KIFs with Elk-1, however specific antibodies for these individual KIF isoforms were not available in the laboratory to study Elk-1/kinesin interactions on endogenous proteins, therefore we have decided to clone selected KIFs that were identified with PSOPIA web server to mammalian expression vector pCMV containing Myc tag for immunoprecipitation experiments. In addition to the identified KIFs by using PSOPIA web server, we also studied the interaction of KIF11 (Eg5) and KIF23 (MKLP1) with Elk-1 due to our previous study that showed co-localization of Elk-1 with KIF11 and KIF23 during mitosis [Demir et al., 2012].

KIF genes were to be cloned into the expression plasmid and overexpressed in cell lines to study protein-protein interaction. In order to overexpress *KIF* genes, they should have been cloned into an expression plasmid. As the first step of molecular cloning experiments, PCR analysis was performed for the amplification of *KIF* genes. Before PCR analysis we should determine the optimal temperatures for amplification of *KIF* genes by performing gradient PCR analysis.

The predicted melting temperature of designed primers for *KIF* genes was calculated using Tm calculator websites. Five different PCR mixtures were prepared for different temperatures that contain higher and lower temperatures of calculated melting temperature. Template cDNA for these PCR mixtures was synthesized from the total RNA of SH-SY5Y neuroblastoma cells. PCR products were analysed with

agarose gel electrophoresis. KIFs are huge proteins so they have very long amino acid sequences and corresponding coding sequences. This makes molecular studies of KIFs more difficult, so we began with gradient PCR of KIF3B that has shorter nucleotide sequences than others. Gradient PCR analysis was performed at first higher and then lower temperatures (Figure 4.1a and 4.1b). Due to the nucleotide sequence length of KIF3B is 2244 bp, we expected to obtain a band at this size. However, we couldn't obtain expected results at any temperature. Bands that are seen at the images might be non-specific and might be primer dimer because of their small sizes.

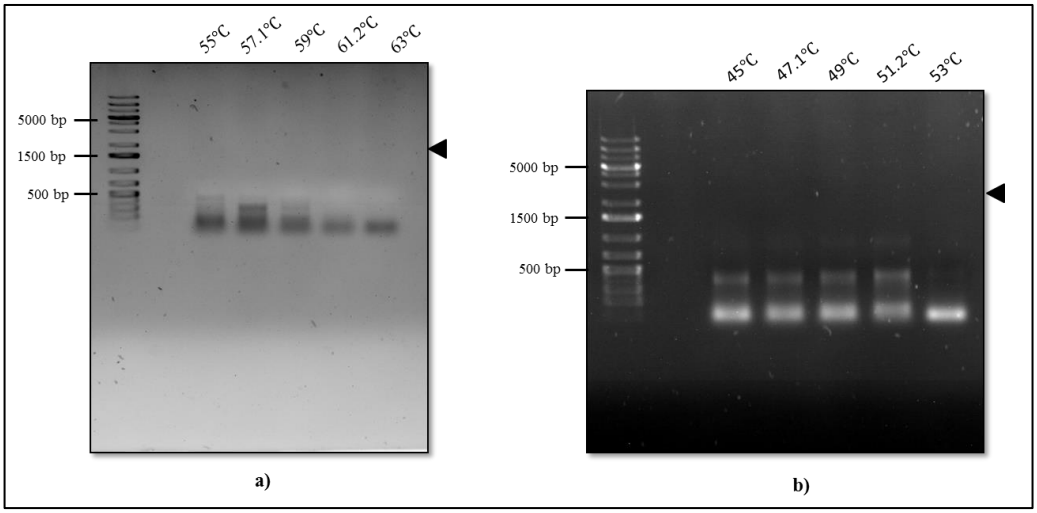

Figure 4.1: Agarose gel images after gradient PCR of KIF3B at a) higher and b) lower temperatures. Arrowheads indicate the expected band size.

Gradient PCR of KIF3B was carried out by adding %5 DMSO, without changing other parameters of the procedure so that if primers cannot bind to template DNA because of secondary structure formation, DMSO would prevent this. (Figure 4.2) However, expected bands were not obtained.

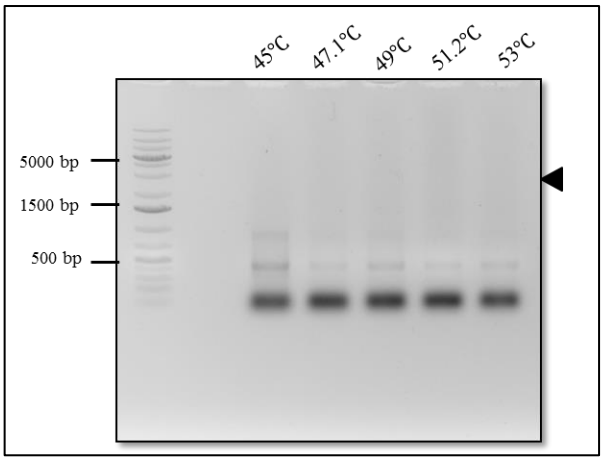

Figure 4.2: Agarose gel image after gradient PCR of KIF3B. %5 DMSO was added to the general protocol. Arrowhead indicates the expected band size.

$MgCl_2$ is an important component of PCR mixture that increases the efficiency of DNA polymerase. 0.75 mM $MgCl_2$ was used for gradient PCR analysis protocol. This amount could not be enough for the efficiency of DNA polymerase since amplicon length is longer. For that reason, the amount of $MgCl_2$ was increased 4 mM (Figure 4.3) but the amplification of KIF3B was not successful.

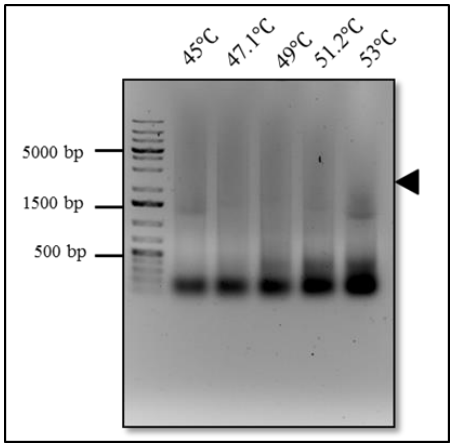

Figure 4.3: Agarose gel image after gradient PCR with 4mM $MgCl_2$ of KIF3B. Arrowhead indicates the expected band size.

Amplification of KIF3B was not accomplished although different components of PCR reactions were changed. KIFs are even one of the most abundant proteins in cells, KIF3B expression could be less in SH-SY5Y cells than other cell types. We compared KIF3B expression levels in SH-SY5Y and U-87 glioblastoma cells and expression level was higher in U-87 cells so that cDNA obtained from total RNA of

U-87 cells was used as the template DNA at next analysis. Gradient temperatures were also reduced a little more, but again desired bands were not seen in the agarose gel image (Figure 4.4).

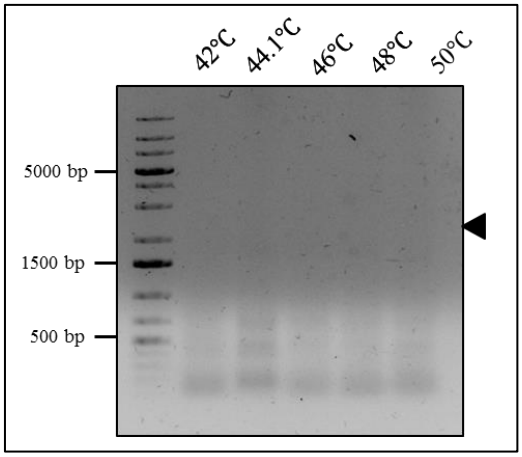

Figure 4.4: Agarose gel image after gradient PCR of KIF3B using cDNA from total RNA of U-87 glioblastoma cells as the template DNA. Arrowhead indicates the expected band size.

The preceding gradient PCR analyses were performed using *Taq* DNA polymerase. Another DNA polymerase that is MyTaq DNA polymerase was used to eliminate the problem that comes from the enzyme. According to enzyme protocol, PCR mixture contains 1 μl of each primer, 1 μl of MyTaq DNA polymerase, 10 μl of 5X MyTaq Reaction Buffer and nuclease-free water up to 50 μl. PCR conditions are 1 min at 95℃ for initial denaturation, 15 s at 95℃ for denaturation, 15 s at gradient temperatures for annealing, and 30s for 1 kb at 72℃ for the extension for 35 cycles. Changing enzyme was not enough for the amplification of KIF3B (Figure 4.5).

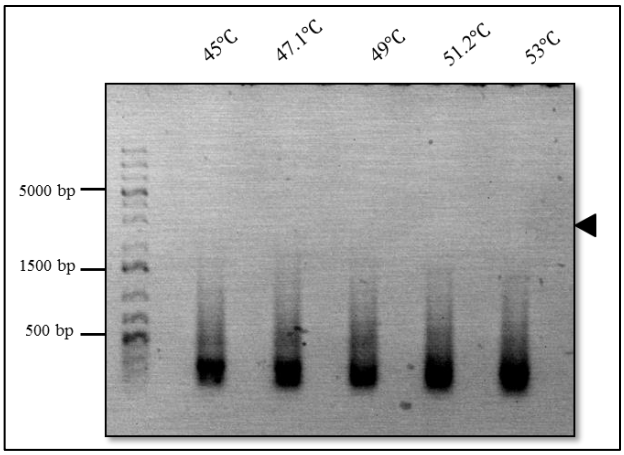

Figure 4.5: Agarose gel image after gradient PCR of KIF3B using different DNA polymerase. Arrowhead indicates the expected band size.

After gradient PCR of KIF3B, we continued with gradient PCR analyses of KIF3C. After prediction of Tm for the KIF3C primers using Tm calculator, gradient PCR analysis was performed for amplification of KIF3C. Similar optimization steps for amplification of KIF3B was also applied to KIF3C. PCR was carried out firstly at higher gradient temperatures, then lower gradient temperatures because amplification of KIF3C was not accomplished at higher temperatures. Reducing temperatures cause amplification of non-specific products but KIF3C that is 2382 bp could not be amplified at higher or lower temperatures (Figure 4.6a and b). Addition of %5 DMSO to general protocol could not enough to obtain correct results (Figure 4.7a). Besides addition of 5% DMSO, concentration of $MgCl_2$ was increased from 0,75 mM to 1.5 mM however KIF3C could not amplified, just non-specific bands have become stronger (Figure 4.7b).

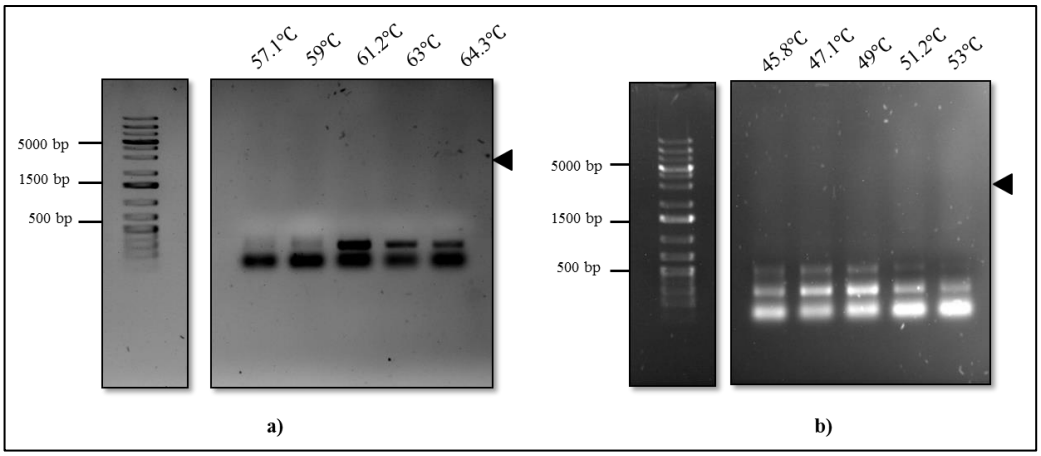

Figure 4.6: Agarose gel images after gradient PCR of KIF3C at a) higher and b) lower temperatures. Ladders are taken from images of KIF3B that was loaded same gel with KIF3C. Arrowheads indicate the expected band size.

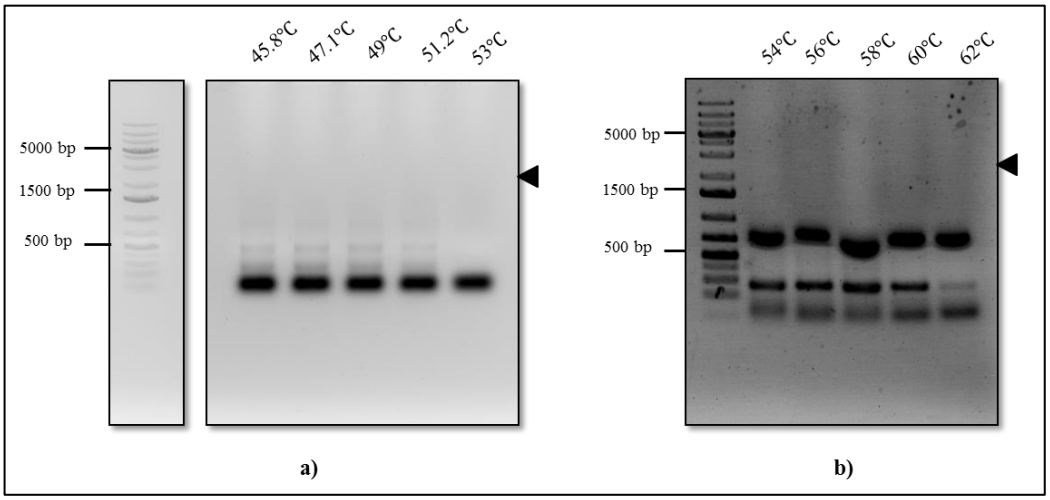

Figure 4.7: Agarose gel images after gradient PCR of KIF3C. a) %5 DMSO was added to the general protocol, ladder is taken from images of KIF3B that was loaded same gel with KIF3C. b) 1.5 mM $MgCl_2$ and %5 DMSO were used additionally. Arrowheads indicate the expected band size.

Since no results can be achieved from gradient PCR analysis of KIF3B and KIF3C despite different optimization steps, we continued with gradient PCR analysis of other identified kinesins, KIF4, KIF1B, and Eg5 (KIF11), MKLP1 (KIF23). We could not any obtain any results from gradient PCR of KIF4, KIF1B, Eg5, and MKLP1 (Figure 4.8).

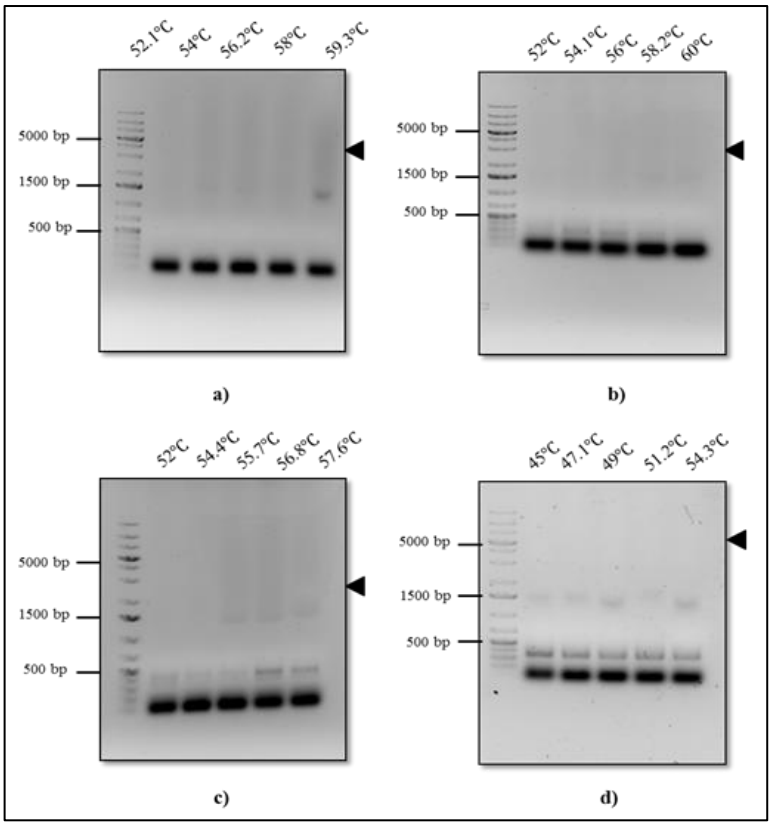

Figure 4.8: Agarose gel images after gradient PCR of a) KIF4 b) Eg5 c) MKLP1 d) KIF1B. Arrowheads indicate the expected band size.

Since these are common motor proteins, cloning of their coding sequences for mammalian expression was expected to be straightforward, however, the amplification of the coding sequences presented significant problems. The primers were designed to have no significant match for other sequences, however in all PCR amplifications either no bands were observed, or amplification products were much shorter than expected. This could be due to the relatively long length of coding sequences of KIF3B, KIF3C, KIF4, Eg5, MKLP1, and KIF1B ranging from 2244 bp to 5313 bp. Since we routinely prepare our cDNA samples using random primers, the full-length sequences may not be present in the cDNA used as template for PCR reactions.

4.4. Analysis of Elk-1 and Kinesin Interaction

Previous studies from our laboratory showed that Eg5 and Elk-1 is co-localized during mitosis [Demir et al., 2012]. We have initially investigated whether co-localization of these proteins also implies interaction between Eg5 and Elk-1. For the

analysis of physical interaction between Eg5 and Elk-1, Flag immunoprecipitation (IP) analysis was performed. If Elk-1 and Eg5 interact strongly with each other, when Elk-1 is precipitated, Eg5 will also co-precipitate with Elk-1. In addition to analysis of Eg5 and Elk-1 interaction, we tried to check whether mutation at specific phosphorylation sites of Elk-1 affects interaction between Eg5 and Elk-1. Elk-1 and its phospho-mutants S383A, S389A, T417A were precipitated with anti-Flag antibody because they were cloned into pCMV-Flag expression plasmid so that they were expressed with Flag tag. 250 μg protein that was obtained from SH-SY5Y cells that overexpress wild type Elk-1 and phospho-mutants of Elk-1 was incubated with anti-Flag antibody. Figure 4.9a shows Western Blot analysis of total protein that was not incubated with anti-Flag antibody. This is control of Flag-IP analysis. In this figure, expression of Elk1-417A could not seem however there was not any problem endogenous expression of Eg5 so that we should have been seen Eg5 after IP analysis if it interacts with Elk-1. In Figure 4.9b, we could not see the expression of Elk1-417A. Eg5 did not precipitate with Elk-1, bands that are seen in anti-Eg5 antibody incubated membrane are non-specific except total protein obtained from non-transfected SH-SY5Y cells because this band was seemed also protein lysate of pCMV-Flag transfected cells that were used for as a negative control of IP analysis. These non-specific bands may arise from Flag:IP assay.

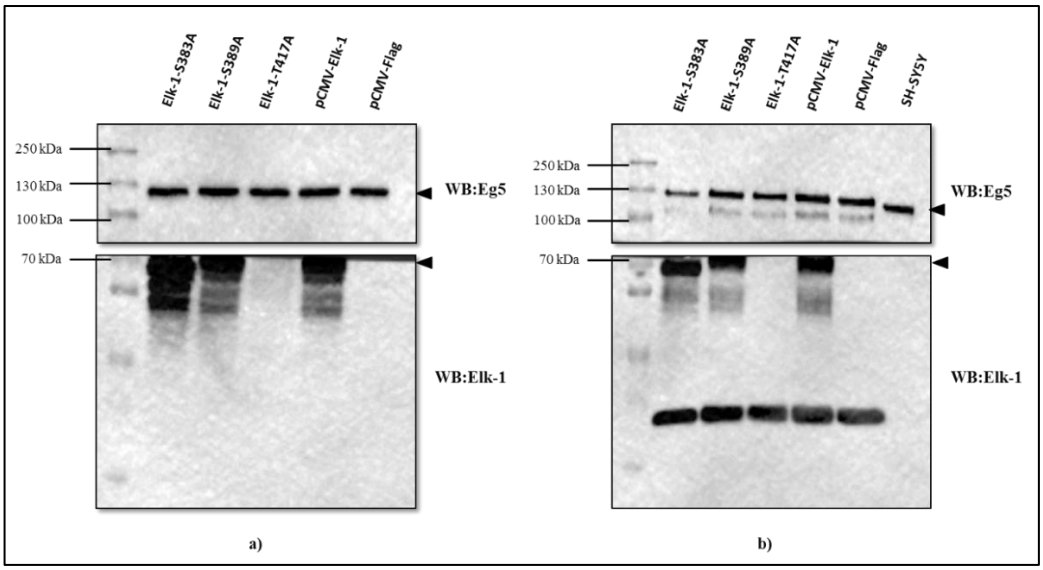

Figure 4.9: The interaction of Elk-1 and its phospho-mutants with Eg5. a) Input and b) Flag-IP analysis. Non-transfected SH-SY5Y cells was used for detection of endogenous Elk-1. pCMV-Flag plasmid is a negative control of Flag-IP:Elk-1. Molecular weight of Eg5 is 120 kDa. Arrowheads indicate the expected band size. WB: Western Blot

Since expression of T417A-Elk-1 could not be observed with previous Western Blot analysis, expression levels of phospho-mutants of Elk-1 was analysed with Western Blot analysis without doing Flag:IP analysis. In this analysis, mouse anti-Elk-1 antibody was changed with rabbit anti-Elk-1 antibody because mouse anti-Elk-1 antibody gave non-specific bands close to Elk-1. Besides this, expression of Elk-1 phospho-mutants was checked with anti-Flag antibody since Elk-1 was expressed with Flag tag. After changes in these parameters, non-specific bands were eliminated but expression of Elk-1-417A mutant could not be observed again in both blots (Figure 4.10a-b). Expression of endogenous Elk-1 could not be observed at protein lysate of non-transfected SH-SY5Y cells (Figure 4.10a) although expression of β-Actin was seen clearly.

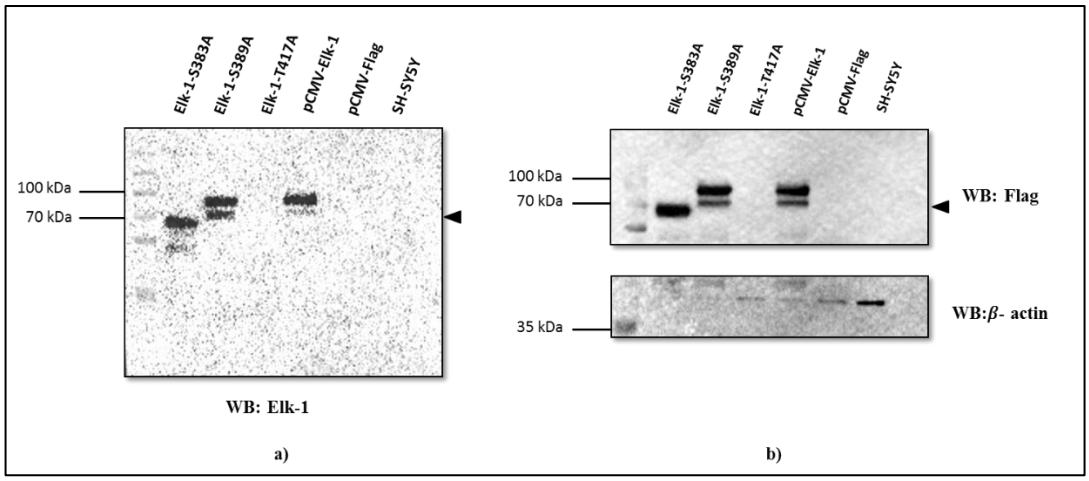

Figure 4.10: Western Blot analysis of a) Elk-1 and b) Flag antibodies on Elk-1 and its phospho-mutants. Phospho-mutants of Elk-1 and wild type Elk-1 are tagged with flag. Non-transfected cells and empty plasmid transfected cells are used for detection of endogenous Elk-1. Arrowheads indicate the expected band size. WB: Western Blot

4.5. Elk-1 and Kinesin Co-localization

Elk-1 and Eg5(KIF11) co-localization during mitosis was first elucidated in the study of Demir, Ari et al. In this study we have addressed whether mitotic-kinase dependent phosphorylation of Elk-1 affected co-localization of Elk-1 with Eg5 during mitosis. For analysis of co-localization of Elk-1 and Eg5, immunofluorescence analysis was performed. Immunofluorescence procedure was tried to optimize at the beginning so that it was performed for U-87 glioblastoma cells that were not transfected with phospho-mutant forms of Elk-1. Tubulin was stained and DNA was counterstained for determination of mitotic stages addition to Elk-1 and Eg5. In the first trial, imaging of tubulin was not achieved for understanding mitotic stages or cell shape (Figure 4.11).

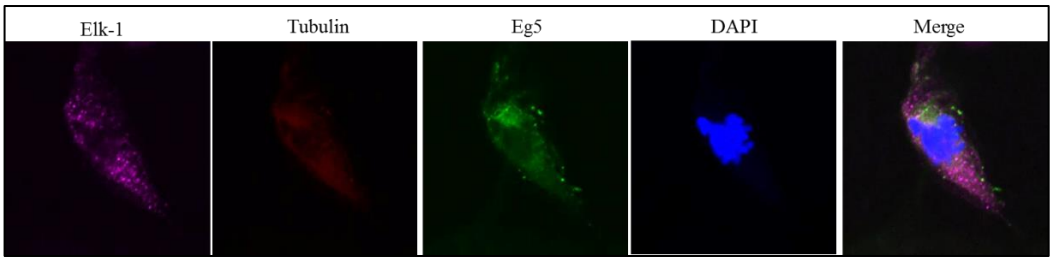

Figure 4.11: Immunofluorescence images of U-87 cells (anti-total Elk-1: magenta, anti-Eg5: green, anti-tubulin: red, DNA: blue). All images were used for generation of merged images.

Co-localization of Eg-5 with endogenous P-S383-Elk-1 that is transcriptionally activated form of Elk-1 was examined beside of endogenous Elk-1 to see whether this phosphorylation specifically associated with colocalization of Elk-1 with Eg5. In addition to that, mutation at different phosphorylation sites of Elk-1 may also affect S383 phosphorylation. In the previous experiment, we used secondary antibodies with 1:1000 dilution. In the following analysis, we checked 1:1500 dilution of secondary antibodies besides this dilution ratio, and also we used different secondary antibody for imaging of P-S383-Elk-1 and total Elk-1 to get better images. 1:1500 dilution was not enough for imaging of proteins, 1:1000 dilution gave better results so that images that visualized with 1:1000 dilution of secondary antibody were presented (Figure 4.12). However, tubulin was not visualized, and P-383-Elk-1 could not be detected in the nucleus. For the attachment of U-87 cells, plate was coated with poly-L-Lysine that caused background staining especially in DAPI staining (Figure 4.12b).

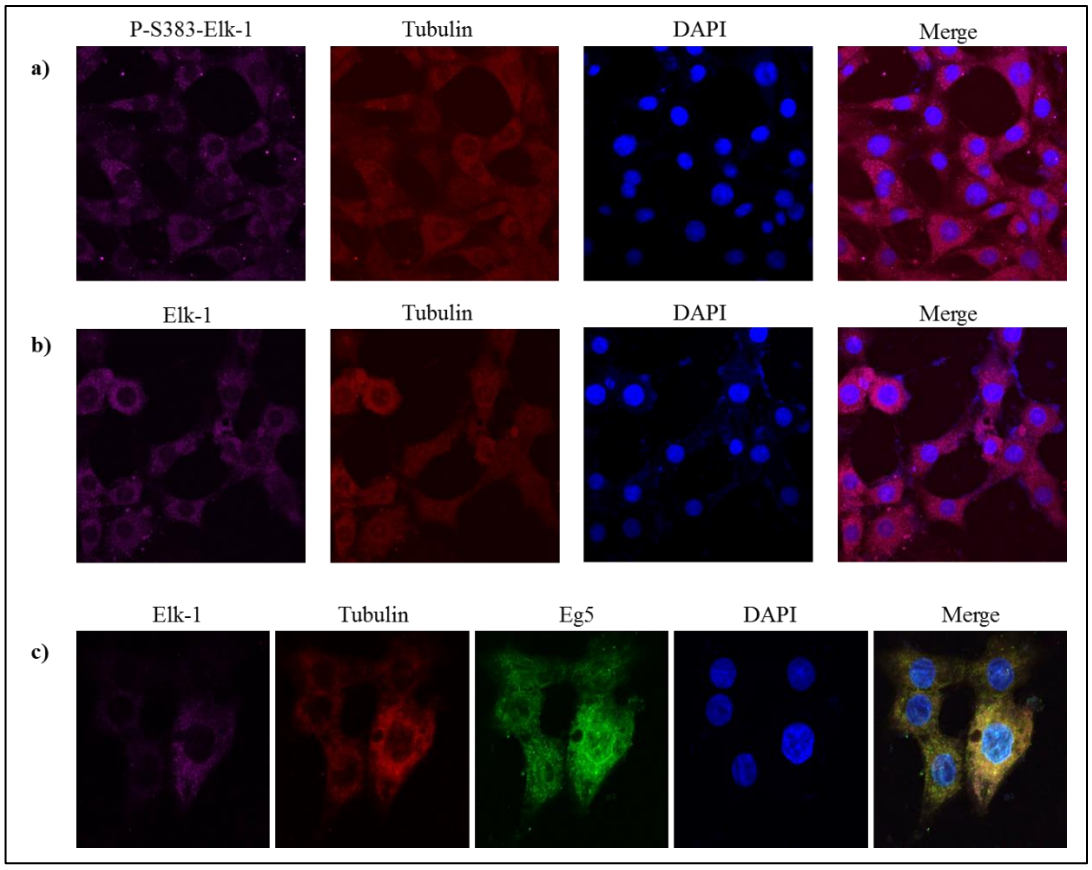

Figure 4.12: Immunofluorescence images of U-87 cells. a) anti-P-S383-Elk-1: magenta b) anti-total Elk-1: magenta c) anti-total Elk-1: magenta and anti-Eg5: green (anti-tubulin: red, DNA: blue in all figures)

Since there were problems of detection of tubulin and Elk-1, we changed primary antibody dilution for tubulin staining and different secondary antibodies were used for the labeling of proteins. To get the best signal, three different primary antibody dilutions 1:50, 1:100, 1:200 were applied for tubulin (Figure 4.13). According to figures 4.13 and 4.14, 1:50 dilution for tubulin detection gave the best result, and change of secondary antibodies did not affect staining outcome. Elk-1 staining was still not observed in the nucleus and also gave low-intensity signal.

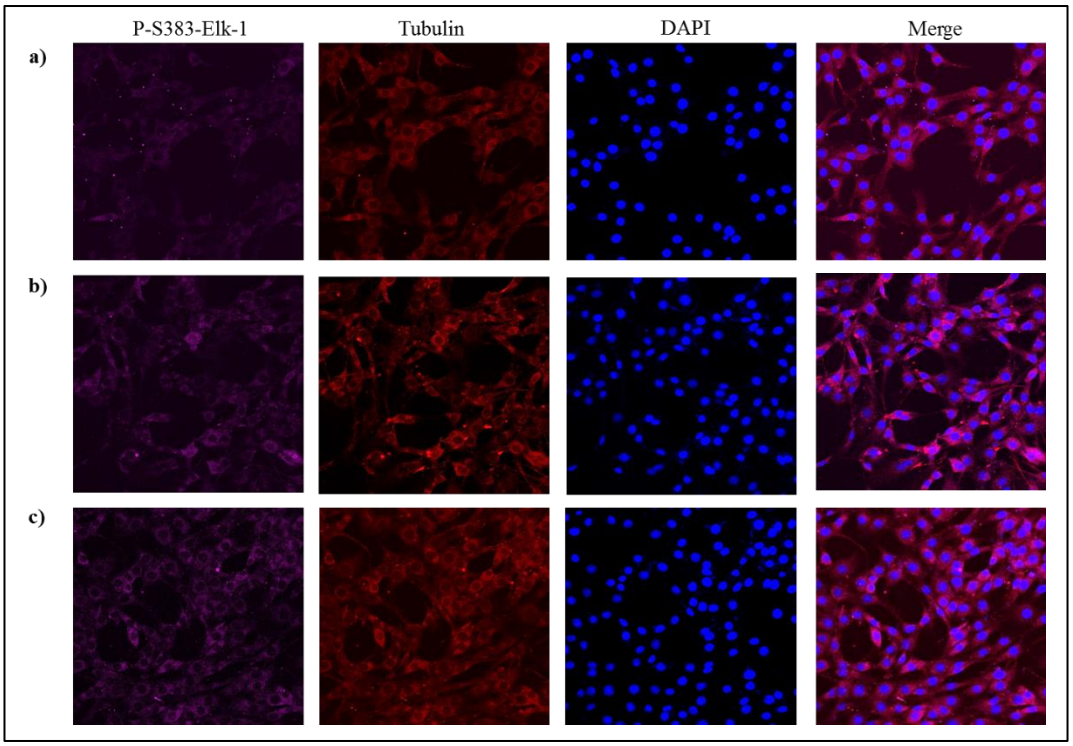

Figure 4.13: Imaging of tubulin with different tubulin antibody dilutions. a) 1:50 dilution b) 1:100 dilution c) 1:200 dilution (anti-P-S383-Elk-1: magenta, anti-tubulin: red, DNA: blue)

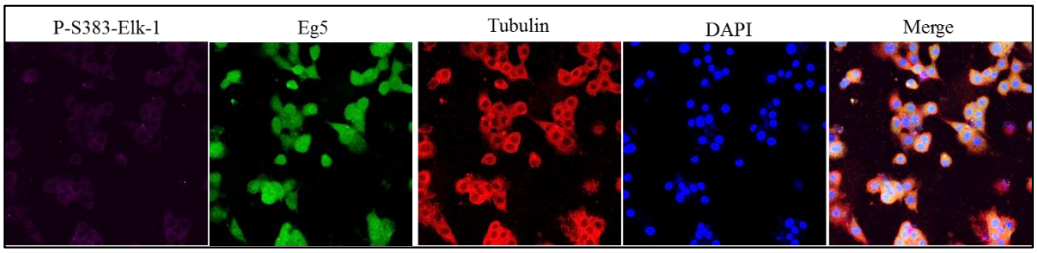

Figure 4.14: Eg-5 localization within U-87 cells (anti-P-S383-Elk-1: magenta, anti-Eg5: green, anti-tubulin: red, DNA: blue).

We next investigated with immunofluorescence analysis whether phospho-null mutants of Elk-1, S303A, S304A, S324A, S326A affected Elk-1 and Eg5 colocalization. U-87 glioblastoma cells were transfected with these phospho-null mutants of Elk-1. Phosphorylated Elk-1 at these phosphorylation sites was also stained to check the absence of signal in the cell that was transfected with phospho-null mutants. Endogenous P-S383 Elk-1 and Elk-1 were positive control for colocalization of Elk-1 with Eg5 in cells transfected with phospho-mutants of Elk-1.

Detection of P-S383-Elk-1 in cells transfected with S303A mutant of Elk-1 is missing because of some problems about eight well system that was used

immunofluorescence analysis to make more simple visualization (In Figure 4.15). Tubulin detection was not proper to understand which mitotic state the cell was in. Labeling of Elk-1 and Eg5 was not as expected in Figure 4.15a and b. P-S303-Elk-1 staining was as expected because cell was transfected with S303A-Elk-1 which is phospho-null mutant (Figure 4.15b).

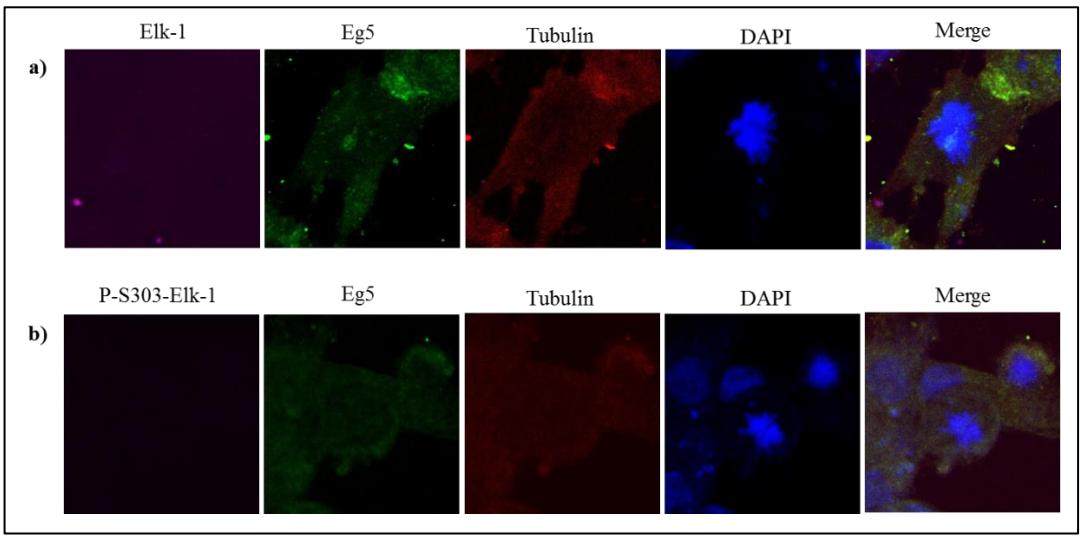

Figure 4.15: Eg-5 localization after transfection of U-87 cells with S303A-Elk-1. a) anti-total Elk-1: magenta b) anti-P-S303-Elk-1: magenta (anti-Eg5: green, anti-tubulin: red, DNA: blue in all figures)

Elk-1 and P-S383-Elk-1 were not detected in U-87 cells transfected with S304A-Elk-1 and also P-S304A-Elk-1 was not detected as expected. Eg5 and tubulin were observed co-localized in anaphase in Figure 4.16b although weak staining of tubulin. Since Elk-1 was not stained properly, we could not analyse effect of phospho-mutant of Elk-1 on Eg5 and Elk-1 co-localization.

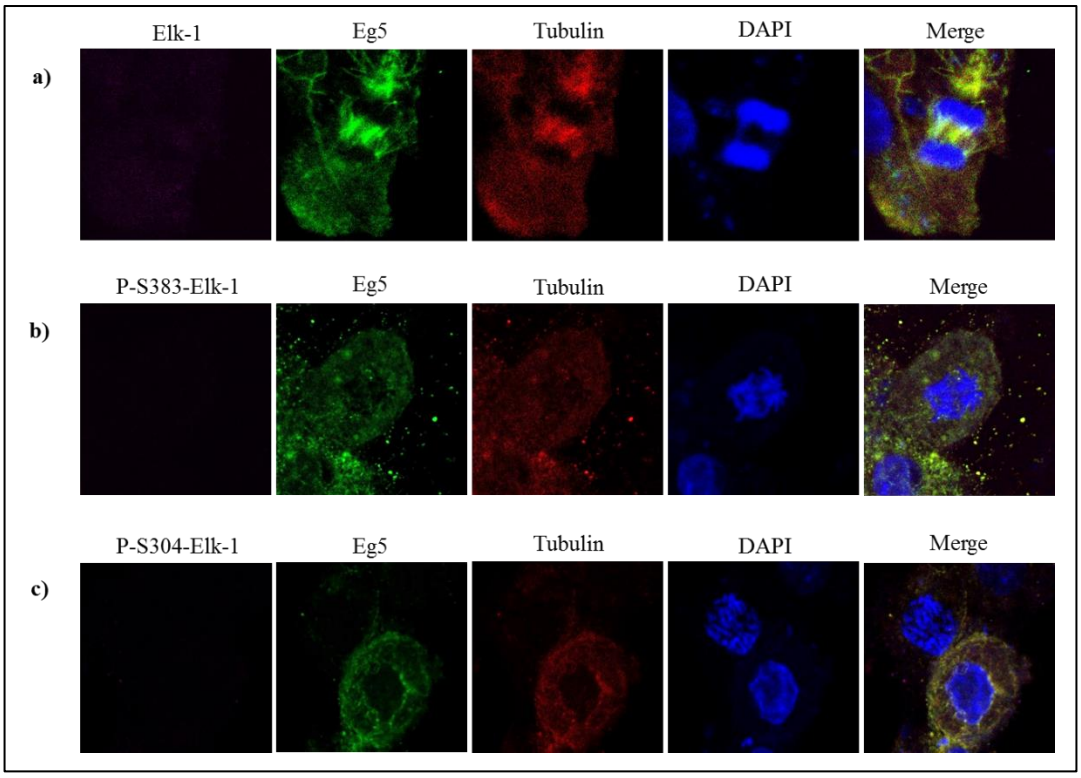

Figure 4.16: Eg-5 localization after transfection of U-87 cells with S304A-Elk-1. a) anti-total Elk-1: magenta b) anti-P-S383-Elk-1: magenta c) anti-P-S304-Elk-1: magenta (anti-Eg5: green, anti-tubulin: red, DNA: blue in all figures)

Cells transfected with S324A-Elk-1 and S326A-Elk-1 were not stained successfully for Elk-1, P-S338-Elk-1, Eg5, and tubulin. In these samples, just DAPI was detected clearly (Figure 4.17 and 4.18). P-S326-Elk-1 was detected despite transfection of cells with S326A-Elk-1 (Figure 4.18c).

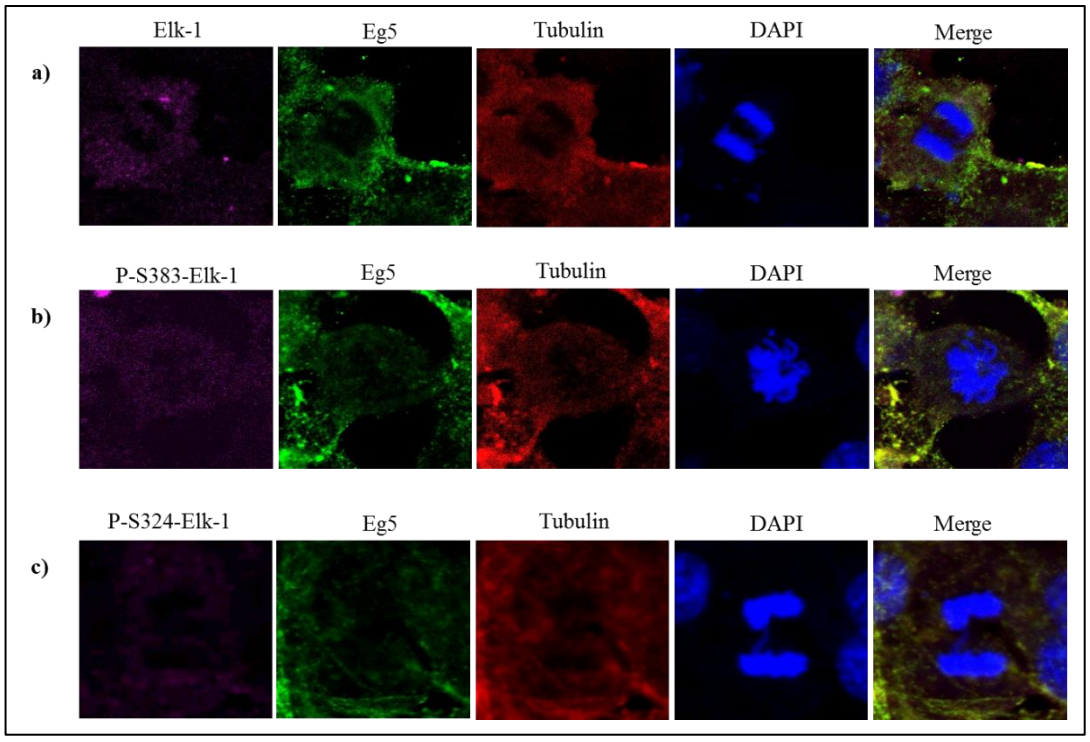

Figure 4.17: Eg-5 localization after transfection of U-87 cells with S324A-Elk-1. a) anti-total Elk-1: magenta b) anti-P-S383-Elk-1: magenta c) anti-P-S324-Elk-1: magenta (anti-Eg5: green, anti-tubulin: red, DNA: blue in all figures)

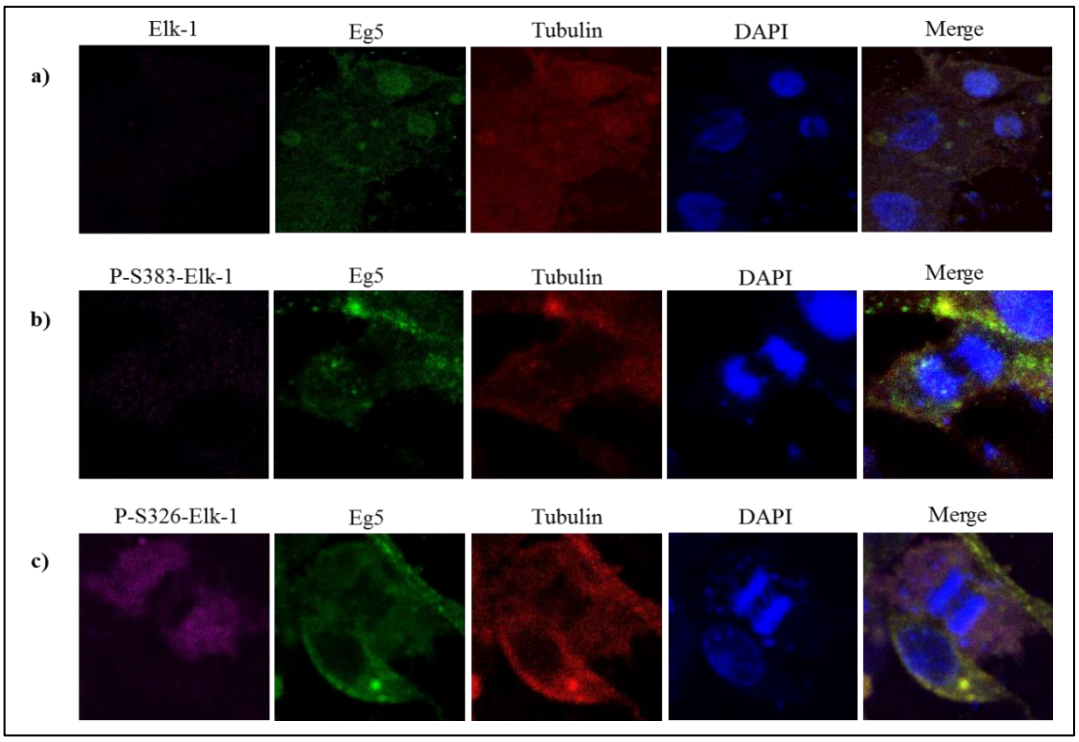

Figure 4.18: Eg-5 localization after transfection of U-87 cells with S326A-Elk-1. a) anti-total Elk-1: magenta b) anti-P-S383-Elk-1: magenta c) anti-P-S326-Elk-1: magenta (anti-Eg5: green, anti-tubulin: red, DNA: blue in all figures)

4.6. Expression Analysis of Elk-1 Phospho-mutants in SK-N-BE(2) Cells

Elk-1 and Eg5 interaction was tried to show in another cell line, SK-N-BE neuroblastoma cells. Before the interaction studies, expression levels of different phospho-mutants of Elk-1 was checked. SK-N-BE(2) cells were transfected with phospho-mutants of Elk1, S198A, S198E, S326A, S326E that were cloned in pCMV-Flag plasmid. After 48 hours of transfection, total protein was isolated from cells. Expression of S198E-Elk-1 could not be observed in Elk-1 Western Blot (Figure 4.19a). Endogenous Elk-1 expression was detected with mouse Elk-1 antibody in neither pCMV-Flag transfected cells nor non-transfected SK-N-BE(2) cells. We saw several bands for Elk-1 for overexpressed wild type Elk-1 and also mutant forms of Elk-1. The membrane which was incubated with anti-Elk-1 antibody was stripped and then incubated with anti-Flag antibody (upper panel) and anti-β-actin antibody (lower panel) (Figure 4.19b). Since phospho-mutant forms of Elk-1 were expressed with Flag tag, we have checked expression of proteins with anti-Flag antibody, but different bands were detected at 130 kDa size that might be because of non-specific binding of Flag antibody. S198E-Elk-1 expression was not detected with anti-Flag antibody, too, and as we expected there was no expression in pCMV-Flag transfected cells and non-transfected cells. β-actin expression was not observed at lanes 1, 4, and 5, it might be due to a problem caused by stripping.

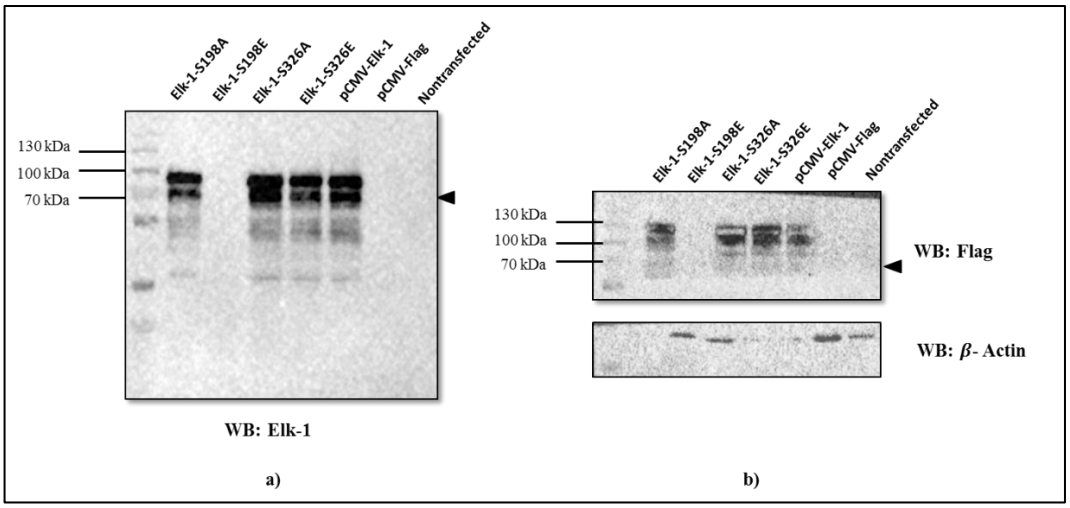

Figure 4.19: Western Blot analysis of a) mouse Elk-1 and b) Flag antibodies on phospho-mutants of Elk-1 and b) anti-β- actin antibody. SK-N-BE(2) cells were transfected with indicated phospho-mutants of Elk-1, pCMV-Elk-1 as a positive control, pCMV-Flag as a negative control. Arrowheads indicate the expected band size.

Another question about previous Western Blot analysis was detection of several bands for Elk-1 in protein lysate obtained from transfected cells with Elk-1 phospho-mutants so that phospho-mutants of Elk-1 expressions were also checked with rabbit anti-Elk-1 antibody (Figure 4.20a). Antibody related non-specific bands were eliminated with rabbit antibody but still S198E-Elk-1 and endogenous Elk-1 expression was not observed. Then, the membrane which was incubated with rabbit anti-Elk-1 antibody was stripped and incubated with anti-Flag antibody again (4.20b). In this result second band that seems slightly in Figure 4.20a became more intense with anti-Flag antibody, one of the reasons that might be host organism of anti-Flag antibody was mouse. The absence of S198E-Elk-1 expression has been demonstrated as well. As a conclusion, mouse antibodies that are anti-Flag antibody and anti-Elk-1 antibody (in Figure 4.18) have shown non-specific binding by comparison with rabbit anti-Elk-1 antibody.

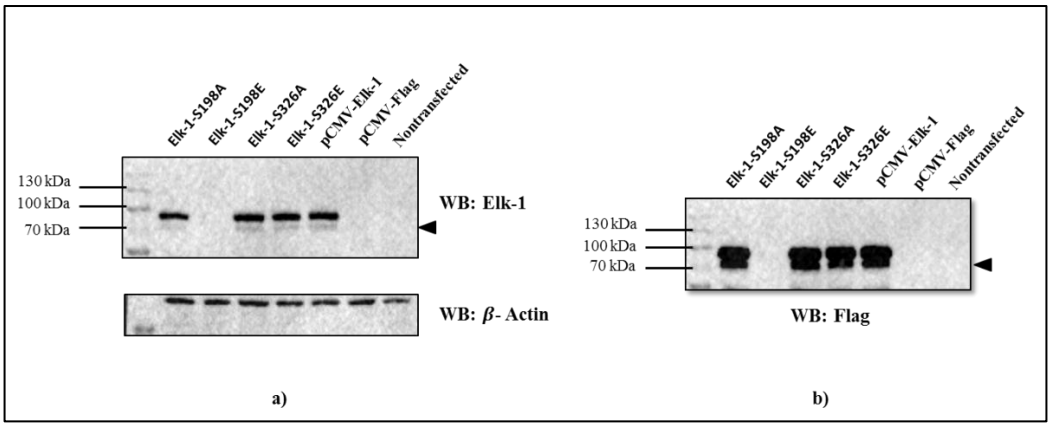

Figure 4.20: Western Blot analysis of a) rabbit Elk-1 antibody and b) Flag antibody on Elk-1 phospho-mutants and a) actin antibody SK-N-BE(2) cells were transfected with indicated phospho-mutants of Elk-1, pCMV-Elk-1 as a positive control, pCMV-Flag as a negative control. Arrowheads indicate the expected band size.

5. DISCUSSION and CONCLUSION

Previous study from our laboratory had shown that Elk-1 co-colocalizes with Eg5 and MKLP1 in the different stages of mitosis [Demir et al., 2012]. Besides this study, different localization of Elk-1 during mitosis and also interaction of Elk-1 with neuronal microtubules were demonstrated [Demir et al., 2009], [Demir and Kurnaz, 2013]. Based on these findings, we have hypothesized that Elk-1 can interact with kinesin motor proteins, including Eg5 and MKLP1, and this interaction and co-localization can be affected by mitotic kinase dependent phosphorylation.

In the beginning, in order to determine specific kinesins that might interact with Elk-1, bioinformatic analyses were performed. Microarray data from Elk-1-VP16 overexpressed cells was analysed and protein-protein interaction subnetworks were obtained. Different KIFs were identified in the biggest subnetwork (Table 4.1). On the other hand, there has been no information about Elk-1 and any KIF protein, we used a bioinformatic tool, PSOPIA that predicts interaction possibility between two proteins. Different KIFs were determined according to this tool (Table 4.2) Then since we wanted to examine protein interaction studies, we continued with KIFs that were identified by PSOPIA bioinformatic tool. KIFs that were determined from microarray data may be studied in further studies.

The interaction between Elk-1 and KIFs would be tried to show with immunoprecipitation and Western Blot analyses. Elk-1 and its mutant forms have been cloned flag tagged plasmid before so that we could precipitate Elk-1 with Flag:IP. For detection of co-immunoprecipitated KIFs with WB analysis, *KIF* genes would be cloned into Myc tagged plasmid. As the first step of cloning, *KIF* genes were tried to amplify with PCR analysis. Amplification of *KIF* genes is not achieved at high annealing temperatures so that annealing temperatures of primers were decreased initially. Although decreasing temperatures would be increased non-specific binding of primers, also our target genes might be produced with them. We could not obtain any result with low temperatures so that we changed different parameters. Since it is considered that problems that may arise in the enzyme, $MgCl_2$ concentration was increased to increase efficiency of *Taq* DNA polymerase. In some PCR reactions, we added DMSO to prevent secondary structure formation of primers like hairpin structure and also prevents primer dimer formation. Expression of KIF proteins varies

in different cell lines so that we used different cDNA synthesized from total RNA of SH-SY5Y or U87 cells and cDNA quality was checked with GAPDH control by Büşra Nur ÇİÇEK. Despite there was not any problem cDNA, we realized that our cDNA synthesis kit is used for production of target mRNAs up to 1 kb however length of KIF genes is much greater than that. It could be one of the reasons coding sequences could not be amplified for cloning.

Since we did not achieve amplification and therefore cloning of identified KIFs, we continued with immunoprecipitation analysis to examine interaction between kinesin and Elk-1 and also phospho-mutants of Elk-1in SH-SY5Y cells using kinesin antibodies present in our laboratory. Eg5(KIF11) was analysed in interaction studies based on previous study from our laboratory. Wild type Elk-1 and mutant forms of Elk-1 were immunoprecipitated with Elk-1 and then Western Blot analysis was performed for Eg5 and Elk-1 (Figure 4.9). Eg5 was not detected after Flag:IP of Elk-1. When we compare with total protein lysate, Eg5 is seemed co-immunoprecipitated with Elk-1 but bands that were at the same location with Eg5 (120 kDa) in membrane were non-specific. These bands might come from Flag:IP because band was also observed pCMV-Flag transfected protein lysate that is negative control of the IP analysis. One of the reasons that we could not observe Eg5 immunoprecipitation with Elk-1 might that Eg5 is a big protein compared with Elk-1. If the interaction between these two proteins is not strong enough Eg5 may not be detected after immunoprecipitation of Elk-1. Besides that, we tried to precipitate Elk-1 with antibody of tag at the end of Elk-1 therefore immunoprecipitation of Elk-1 could already be difficult. To solve this problem Elk-1 might be precipitated with Elk-1 antibody or Eg5 might be precipitate with Eg5 antibody and then Elk-1 was detected with WB analysis. In addition to these, another analysis that is more powerful for revealing molecular interactions may be performed like mass spectrometry.

Another unexpected result was not to detect expression of T417A-Elk-1. There was not any problem with expression of this phospho-mutant western blot analyses that have performed in our laboratory, so we again checked expressions of these mutant forms of Elk-1 in SH-SY5Y cells (Figure 4.10). We did not observe expression of T417A-Elk-1. Then, we decided to check plasmid construct to analyse any problem in expression plasmid. pCMV-Elk-1-T417A plasmid was cut with restriction enzymes that were used for the cloning of Elk-1 before to obtain insert DNA. According to agarose gel analysis, insert Elk-1 was not visualised at the correct size. Same

experiment was performed for pCDNA3-Elk-1, insert Elk-1 in this plasmid was observed correct size (Data not shown). As a result, expression of T417A-Elk-1 could not be observed because of impairment in plasmid construct.

In Western Blot analysis, we observed several bands for Elk-1 when we used Elk-1 antibody which host is mouse. On the other hand, when we used Elk-1 antibody which host is rabbit, number of observed bands decreases. Same results were obtained with flag antibody which host is also mouse. Normally, phosphorylated and SUMOylated Elk-1 can also be detected in Western blot assays, hence some of the upper bands could arise from post-translational modifications of Elk-1, however other bands that were observed with mouse anti-Elk-1 might be non-specific because these were not seen with rabbit antibody. It can be concluded that rabbit antibody binds more specifically than mouse antibodies.

After molecular interaction studies, we continued with immunofluorescence analysis to examine co-localization of Elk-1 and Eg5. In addition to the wild type Elk-1, we analysed co-localization of phospho-mutants of Elk-1. Before transfection of U-87 cells with phospho-mutants of Elk-1, we tried to optimize immunostaining protocol. The main reason that tubulin and Eg5 could not be stained properly might be related to shelf-life of some of the primary and secondary antibodies used. P-S383-Elk-1 and Elk-1 antibodies were used generally in Western Blot analyses therefore they may not work as well in immunofluorescence assays.

It was decided to perform IP and immunofluorescence analyses in SK-N-BE(2) neuroblastoma cell line. In these analyses, we changed phospho-mutants of Elk-1 and these are S198A, S198E, S326A, S326E. In the beginning, we wanted to check expression levels of these mutant forms of Elk-1 in SK-N-BE(2) cells with Western Blot analysis. Interestingly, we did not observe S198E-Elk-1 expression. This is the phosphomimic mutant of Elk-1 so that phosphorylation from this residue might decrease stability of protein and cause degradation of protein immediately in the cell. qPCR analysis might be performed to examine synthesis of S198E-Elk-1 in mRNA level.

As a conclusion, problems that come from materials used in the experiment prevent to deduce about Elk-1 and kinesin interaction. Nevertheless, interaction between Elk-1 transcription factor and kinesin motor proteins in conjunction with mitosis may be crucial for shed light on the underlying mechanism of brain tumor formation. Besides, defect in axonal transport of Elk-1 may play an important role in

progression of neurodegenerative diseases or maybe target for retardation of disease after emergence of neurodegenerative diseases.

REFERENCES

Alcaraz N., List M., Dissing-Hansen M., Rehmsmeier M., Tan Q., Mollenhauer J., Ditzel H. J., Baumbach J., (2016), "Robust de novo pathway enrichment with KeyPathwayMiner 5", F1000Res, 5, 1531.

Barrett L. E., Sul J. Y., Takano H., Van Bockstaele E. J., Haydon P. G., Eberwine J. H., (2006), "Region-directed phototransfection reveals the functional significance of a dendritically synthesized transcription factor", Nat Methods, 3 (6), 455-460.

Barrett L. E., Van Bockstaele E. J., Sul J. Y., Takano H., Haydon P. G., Ebewine J. H., (2006), "Elk-1 associates with the mitochondrial permeability transition pore complex in neurons", Proceedings of the National Academy of Sciences of the United States of America, 103 (13), 5155-5160.

Besnard A., Galan-Rodriguez B., Vanhoutte P., Caboche J., (2011), "Elk-1 a transcription factor with multiple facets in the brain", Front Neurosci, 5, 35.

Buchwalter G., Gross C., Wasylyk B., (2004), "Ets ternary complex transcription factors", Gene, 324, 1-14.

Chatr-Aryamontri A., Oughtred R., Boucher L., Rust J., Chang C., Kolas N. K., O'Donnell L., Oster S., Theesfeld C., Sellam A., Stark C., Breitkreutz B. J., Dolinski K., Tyers M., (2017), "The BioGRID interaction database: 2017 update", Nucleic Acids Res, 45 (D1), D369-D379.

Demir O., Ari O., Kurnaz I. A., (2012), "Elk-1 interacts with dynein upon serum stimulation but independent of serine 383 phosphorylation", Cell Mol Neurobiol, 32 (2), 185-189.

Demir O., Aysit N., Onder Z., Turkel N., Ozturk G., Sharrocks A. D., Kurnaz I. A., (2011), "ETS-domain transcription factor Elk-1 mediates neuronal survival: SMN as a potential target", Biochim Biophys Acta, 1812 (6), 652-662.

Demir O., Korulu S., Yildiz A., Karabay A., Kurnaz I. A., (2009), "Elk-1 interacts with neuronal microtubules and relocalizes to the nucleus upon phosphorylation", Mol Cell Neurosci, 40 (1), 111-119.

Demir O., Kurnaz I. A., (2008), "Wildtype Elk-1, but not a SUMOylation mutant, represses egr-1 expression in SH-SY5Y neuroblastomas", Neurosci Lett, 437 (1), 20-24.

Demir O., Kurnaz I. A., (2013), "Phospho-Ser383-Elk-1 is localized to the mitotic spindles during cell cycle and interacts with mitotic kinase Aurora-A", Cell Biochem Funct, 31 (7), 591-598.

Ducret C., Maira S.-M., Dierich A., Wasylyk B., (1999), "The Net Repressor Is Regulated by Nuclear Export in Response to Anisomycin, UV, and Heat Shock", Molecular and Cellular Biology, 19 (10), 7076-7087.

Eibes S., Gallisa-Sune N., Rosas-Salvans M., Martinez-Delgado P., Vernos I., Roig J., (2018), "Nek9 Phosphorylation Defines a New Role for TPX2 in Eg5-Dependent Centrosome Separation before Nuclear Envelope Breakdown", Curr Biol, 28 (1), 121-129 e124.

Gumy L. F., Chew D. J., Tortosa E., Katrukha E. A., Kapitein L. C., Tolkovsky A. M., Hoogenraad C. C., Fawcett J. W., (2013), "The kinesin-2 family member KIF3C regulates microtubule dynamics and is required for axon growth and regeneration", J Neurosci, 33 (28), 11329-11345.

Guse A., Mishima M., Glotzer M., (2005), "Phosphorylation of ZEN-4/MKLP1 by aurora B regulates completion of cytokinesis", Curr Biol, 15 (8), 778-786.

Haraguchi K., Hayashi T., Jimbo T., Yamamoto T., Akiyama T., (2006), "Role of the kinesin-2 family protein, KIF3, during mitosis", J Biol Chem, 281 (7), 4094-4099.

Hirokawa N., Niwa S., Tanaka Y., (2010), "Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease", Neuron, 68 (4), 610-638.

Hollenhorst P. C., Jones D. A., Graves B. J., (2004), "Expression profiles frame the promoter specificity dilemma of the ETS family of transcription factors", Nucleic Acids Res, 32 (18), 5693-5702.

Hollenhorst P. C., McIntosh L. P., Graves B. J., (2011), "Genomic and biochemical insights into the specificity of ETS transcription factors", Annu Rev Biochem, 80, 437-471.

Lotfi Ferhat R. K., Gary E. Lyons, Bruce Micales and Peter W. Baas, (1998), "Expression of the mitotic motor protein CHO1/MKLP1 in postmitotic neurons", European Journal of Neuroscience, 10, 1383-1393.

Lyons D. A., Naylor S. G., Scholze A., Talbot W. S., (2009), "Kif1b is essential for mRNA localization in oligodendrocytes and development of myelinated axons", Nat Genet, 41 (7), 854-858.

Midorikawa R., Takei Y., Hirokawa N., (2006), "KIF4 motor regulates activity-dependent neuronal survival by suppressing PARP-1 enzymatic activity", Cell, 125 (2), 371-383.

Miki H., Setou M., Kaneshiro K., Hirokawa N., (2001), "All kinesin superfamily protein, KIF, genes in mouse and human", Proceedings of the National Academy of Sciences of the United States of America, 98 (13), 7004-7011.

Murakami Y., Mizuguchi K., (2018), "PSOPIA: Toward more reliable protein-protein interaction prediction from sequence information", ICIIBMS 2017 - 2nd International

Conference on Intelligent Informatics and Biomedical Sciences, 2018-January, 255-261.

Mylona A., Theillet F. X., Foster C., Cheng T. M., Miralles F., Bates P. A., Selenko P., Treisman R., (2016), "Opposing effects of Elk-1 multisite phosphorylation shape its response to ERK activation", Science, 354 (6309), 233-237.

Nalawansha D. A., Gomes I. D., Wambua M. K., Pflum M. K. H., (2017), "HDAC Inhibitor-Induced Mitotic Arrest Is Mediated by Eg5/KIF11 Acetylation", Cell Chem Biol, 24 (4), 481-492 e485.

Nonaka S., Tanaka Y., Okada Y., Takeda S., Harada A., Kanai Y., Kido M., Hirokawa N., (1998), "Randomization of left-right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein", Cell, 95 (6), 829-837.

Perlson E., Hanz S., Ben-Yaakov K., Segal-Ruder Y., Seger R., Fainzilber M., (2005), "Vimentin-dependent spatial translocation of an activated MAP kinase in injured nerve", Neuron, 45 (5), 715-726.

Ramirez S., Ali S. A. S., Robin P., Trouche D., Harel-Bellan A., (1997), "The CREB-binding protein (CBP) cooperates with the serum response factor for transactivation of the c-fos serum response element", Journal of Biological Chemistry, 272 (49), 31016-31021.

Raudvere U., Kolberg L., Kuzmin I., Arak T., Adler P., Peterson H., Vilo J., (2019), "g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update)", Nucleic Acids Res, 47 (W1), W191-W198.

Selvaraj N., Kedage V., Hollenhorst P. C., (2015), "Comparison of MAPK specificity across the ETS transcription factor family identifies a high-affinity ERK interaction required for ERG function in prostate cells", Cell Commun Signal, 13, 12.

Seog D. H., Lee D. H., Lee S. K., (2004), "Molecular Motor Proteins of the Kinesin Superfamily Proteins (KIFs): Structure, Cargo and Disease", Journal of Korean Medical Science, 19 (1), 1-7.

Sgambato V., Vanhoutte P., Pagès C., Rogard M., Hipskind R., Besson M. J., Caboche J., (1998), "In vivo expression and regulation of Elk-1, a target of the extracellular-regulated kinase signaling pathway, in the adult rat brain", Journal of Neuroscience, 18 (1), 214-226.

Sharma A., Callahan L. M., Sul J. Y., Kim T. K., Barrett L., Kim M., Powers J. M., Federoff H., Eberwine J., (2010), "A neurotoxic phosphoform of Elk-1 associates with inclusions from multiple neurodegenerative diseases", PLoS One, 5 (2), e9002.

Sharrocks A. D., (2001), "THE ETS-DOMAIN TRANSCRIPTION FACTOR FAMILY", Nature Reviews Molecular Cell Biology, 2 (11), 827-837.

Sogut M. S., Venugopal C., Kandemir B., Dag U., Singh S., Gulfidan G., Arga K. Y., Yilmaz B., Kurnaz I. A., (Manuscript in preparation) "ETS-domain transcription factor Elk-1 regulates stemness genes in brain tumors and CD133+ brain tumor initiating cells (BTIC)"

Sugimoto T., Stewart S., Guan K. L., (1997), "The calcium/calmodulin-dependent protein phosphatase calcineurin is the major Elk-1 phosphatase", Journal of Biological Chemistry, 272 (47), 29415-29418.

Swarnkar S., Avchalumov Y., Raveendra B. L., Grinman E., Puthanveettil S. V., (2018), "Kinesin Family of Proteins Kif11 and Kif21B Act as Inhibitory Constraints of Excitatory Synaptic Transmission Through Distinct Mechanisms", Sci Rep, 8 (1), 17419.

Szatmari E. M., Oliveira A. F., Sumner E. J., Yasuda R., (2013), "Centaurin-alpha1-Ras-Elk-1 signaling at mitochondria mediates beta-amyloid-induced synaptic dysfunction", J Neurosci, 33 (12), 5367-5374.

Takeda S., Yamazaki H., Seog D. H., Kanai Y., Terada S., Hirokawa N., (2000), "Kinesin superfamily protein 3 (KIF3) motor transports fodrin-associating vesicles important for neurite building", Journal of Cell Biology, 148 (6), 1255-1265.

Tanenbaum M. E., Macurek L., Galjart N., Medema R. H., (2008), "Dynein, Lis1 and CLIP-170 counteract Eg5-dependent centrosome separation during bipolar spindle assembly", EMBO J, 27 (24), 3235-3245.

Teng J., Rai T., Tanaka Y., Takei Y., Nakata T., Hirasawa M., Kulkarni A. B., Hirokawa N., (2005), "The KIF3 motor transports N-cadherin and organizes the developing neuroepithelium", Nat Cell Biol, 7 (5), 474-482.

Uyar O. A., Celik O. D., Yilmaz B., Kurnaz I. A., (Manuscript in preparation)" Interaction of transcription factor Elk-1 with mitotic kinases Aurora-A, Plk-1 and Cdk1 in neuroblastoma and glioblastoma"

Yang S.-H., Jaffray E., Hay R. T., Sharrocks A. D., (2003), "Dynamic Interplay of the SUMO and ERK Pathways in Regulating Elk-1 Transcriptional Activity", Molecular Cell, 12 (1), 63-74.

Yang S. H., Shore P., Willingham N., Lakey J. H., Sharrocks A. D., (1999), "The mechanism of phosphorylation-inducible activation of the ETS-domain transcription factor Elk-1", EMBO Journal, 18 (20), 5666-5674.

Yang Z., Roberts E. A., Goldstein L. S., (2001), "Functional analysis of mouse kinesin motor Kif3C", Mol Cell Biol, 21 (16), 5306-5311.

Zhao C., Takita J., Tanaka Y., Setou M., Nakagawa T., Takeda S., Yang H. W., Terada S., Nakata T., Takei Y., Saito M., Tsuji S., Hayashi Y., Hirokawa N., (2001), "Charcot-Marie-Tooth disease type 2A caused by mutation in a microtubule motor KIF1Bβ", Cell, 105 (5), 587-597.

Zhu C., Bossy-Wetzel E., Jiang W., (2005), "Recruitment of MKLP1 to the spindle midzone/midbody by INCENP is essential for midbody formation and completion of cytokinesis in human cells", Biochemical Journal, 389 (2), 373-381.

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APPENDIX

Appendix A: Primer Design for KIFs

Designed primers for selected KIF proteins and their coding sequence is presented. Green highlighting indicates additional nucleotides for efficient digestion by restriction enzymes that is one of the steps of further cloning assay. Red highlighting indicates restriction enzyme sequences, and some forward primers have additional nucleotides (shown in red) to prevent frameshift. Yellow highlighting indicates sequences that forward and reverse primers were designed according to these sequences.

KIF1B (5313 bp):

F: 5'- ATAG GTCGAC T TCG GGA GCC TCA GTG AAG-3'

R: 5'- ACAG GGTACC TTA GTA TTT CGA CTG GCT-3'

ATGTCGGGAGCCTCAGTGAAGGTGGCTGTCCGGGTAAGGCCC
TTCAATTCTCGAGAGACCAGCAAGGAATCCAAATGCATCATTC
AGATGCAAGGCAACTCGACCAGTATTATTAACCCAAAGAATCC
AAAGGAAGCTCCAAAGTCCTTC……
……AACGACT
GGTTGTATGCCTTCAACCCACTTCTAGCTGGCACAATACGGTC
AAAGCTTTCCCGCAGATGCCCGAGCCAGTCGAAATACTAA

Figure A1.1: Primer pairs of KIF1B and initial and terminal part of its coding sequence.

KIF3B (2244 bp):

F: 5'- ATAG GTCGAC G TCA AAG TTG AAA AGC TCA-3'

R: 5'- ATAT GGTACC TTA CTT TGG AAC CAG CCC-3'

ATGTCAAAGTTGAAAAGCTCAGAGTCAGTCAGGGTGGTGGTT
CGCTGTCGGCCCATGAATGGCAAGGAAAAGGCTGCTTCGTATG
ACAAAGTGGTGGATGTGGATGTTAAGCTGGGGCAGGTGTCTGT
GAAGAACCCCAAAGGGACGGG……
……CT
CCTCTTCCTCAGGAACCCCTGCATCTCAGCTTTATCCACAGTCT
CGGGGGCTGGTTCCAAAGTAA

Figure A1.2: Primer pairs of KIF3B and initial and terminal part of its coding sequence.

KIF3C (2382 bp):

F: 5'- ATAT GTCGAC T GCC AGT AAG ACC AAG GCC-3'

R: 5'- ATAT GCGGCCGC TCA CTC ATG GTC CGC CAC TGT -3'

ATGGCCAGTAAGACCAAGGCCAGCGAGGCCCTCAAGGTGGTG
GCCCGGTGCCGCCCCCTCAGCAGGAAGG……
……
……..AGATCCTGGTGCCAGAGTCCTCAGCG
GCCTCCACCTTCCACCACACATGCCTCCCTGGCCTCTGCTTCTC
TGCGCCCTGCAACAGTGGCGGACCATGAGTGA

Figure A1.3: Primer pairs of KIF3C and initial and terminal part of its coding sequence.

KIF4 (3699 bp):

F: 5'- ATAG GTCGAC T AAG GAA GAG GTG AAG GGA -3'

R: 5'- ATAT GCGGCCGC TCA GTG GGC CTC TTC TTC -3'

ATGAAGGAAGAGGTGAAGGGAATTCCCGTAAGAGTGGCGCTG
CGTTGTCGCCCTCTGGTCCCCAAAGAGATTAGCGAGGGCTGCC
AGATGTGCCTTTCCTTCGTGCCCGGAGAGCCTCAGGTGGTGGT
TGGTACAGATAA……
……GGAAGAAAAAGAA
ACGGGCTCTGGCCAGCAACACCAGCTTCTTCTCTGGCTGCTCC
CCTATCGAAGAAGAGGCCCACTGA

Figure A1.4: Primer pairs of KIF4 and initial and terminal part of its coding sequence.

KIF11 (3171 bp):

F: 5'- ATAG GAATTC AT GCG TCG CAG CCA AAT TCG -3'

R: 5'- ATAG GTCGAC TTA AAG GTT GAT CTG GGC -3'

ATGGCGTCGCAGCCAAATTCGTCTGCGAAGAAGAAAGAGGAG
AAGGGGAAGAACATCCAGGTGGTGGTGAGATGCAGACCATTT
AATTTGGCAGAGCGGAAAGCTAGCGCCCATTCAATAGTAGAAT
GTGATCCTGTACG……
……TAACACACTGGAGAGGTCTAAA
GTGGAAGAAACTACAGAGCACTTGGTTACAAAGAGCAGATTA
CCTCTGCGAGCCCAGATCAACCTTTAA

Figure A1.5: Primer pairs of KIF11 and initial and terminal part of its coding sequence.

KIF23 (2883 bp):

F: 5'- ATAT GTCGAC T AAG TCA GCG AGA GCT AAG -3'

R: 5'- ATAT GGTACC TCA TGG CTT TTT GCG CTT -3'

ATGAAGTCAGCGAGAGCTAAGACACCCCGGAAACCTACCGTG
AAAAAAGGGTCCCAAACGAACCTTAAAGACCCAGTTGGGGTA
TACTGTAGGGTGCGCCCACTGGGCTTTCCTGATCAAGAGTGTT
GCATAGAAGTGAT……
……..CTGTGGCTGTGGAGATGAGAGC
AGGATCCCAGCTGGGACCTGGATATCAGCATCACGCACAACCC
AAGCGCAAAAAGCCATGA

Figure A1.6: Primer pairs of KIF23 and initial and terminal part of its coding sequence.