

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**THE INTERACTION OF ELK-1 WITH MINUS-END DIRECTED
DYNEIN MOTOR PROTEIN**

BÜŞRA NUR ÇİÇEK
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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THESIS SUPERVISOR
PROF. DR. ISIL KURNAZ

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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

ELK-1'İN EKŞİ UÇ YÖNELİMLİ DİNEİN
MOTOR PROTEİNİ İLE ETKİLEŞİMİ

BÜŞRA NUR ÇİÇEK
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
PROF. DR. İŞİL KURNAZ

GEBZE
2021

GEBZE TEKNİK ÜNİVERSİTESİ	YÜKSEK LİSANS JÜRİ ONAY FORMU
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İMZA/MÜHÜR

SUMMARY

Elk-1 is a transcription factor that belongs to the Ternary Complex Factor (TCF) subfamily of ETS transcription factors whose members have a common DNA binding domain called the Ets domain. Elk-1 activity is regulated by post-translational modifications such as phosphorylation, ubiquitination, SUMOylation (small ubiquitin modifier) and protein-protein interaction with Serum Response Factor (SRF). Elk-1 plays role in crucial biological processes such as cell cycle, neuronal survival, and apoptosis. It has been shown that Elk-1 takes part in the regulation of neurodegenerative disease-related genes, and certain phosphorylated form of Elk-1 is colocalized with Lewy bodies and Huntingtin protein. In addition, it was shown that in neuronal model cells, Elk-1 was shown to colocalize and interact with microtubules, and then it was relocalized to the nucleus upon growth factor stimulation of cells. This observation has led to the question of how the microtubule-dependent transport of Elk-1 was achieved. Previous studies in our laboratory have shown that Elk-1 physically interacted with dynein in a serum stimulation-dependent manner, albeit irrespective of Ser383 phosphorylation. In this thesis, we aimed to show the dynamic interaction of Elk-1 with dynein and to study whether other phosphorylations of Elk-1 was responsible for this interaction using a variety of methods such as split-EGFP (enhanced green fluorescent protein) complementation assay, immunoprecipitation and fluorescence microscopy. Gradient Polymerase Chain Reaction (PCR), cloning trials, immunoprecipitation were performed to achieve our aim. Further analysis will illuminate that dynein-mediated transport of Elk-1 is crucial for certain biological processes such as neuroregeneration, cell cycle, and cancer.

Keywords: Elk-1, Split-EGFP Complementation Assay, Motor Protein, Immunoprecipitation.

ÖZET

Elk-1, üyeleri Ets alanı adı verilen ortak bir DNA bağlanma alanına sahip olan ETS transkripsiyon faktörlerinin Üçlü Kompleks Faktör alt ailesine ait bir transkripsiyon faktörüdür. Elk-1 aktivitesi fosforilasyon, sumoylasyon (küçük ubikitin düzenleyici), ubikitinlenme gibi transkripsiyon sonrası modifikasyonlar ve SRF ile olan protein-protein etkileşimi tarafından düzenlenir. Elk-1, hücre döngüsü, nöronal sağkalım ve apoptoz gibi önemli biyolojik süreçlerde rol oynar. Elk-1'in nörodejenaratif hastalıklarla ilişkili genlerin düzenlenmesinde rol aldığı ve belirli fosforillenmiş formunun Lewy cismi ve Huntingtin proteini ile kolokelize olduğu gösterilmiştir. Ek olarak, nöronal model hücrelerde, Elk-1'in mikrotübüllerle kolokelize olduğu ve etkileşime girdiği ve hücrelerin büyüme faktörü uyarılmasının ardından çekirdeğe yeniden lokalize olduğu gösterilmiştir. Bu gözlem, Elk-1'in mikrotübüle bağlı taşınmasının nasıl başarıldığı sorusuna yol açmıştır. Laboratuvarımızdaki önceki çalışmalar, Elk-1'in, Ser383 fosforilasyonundan bağımsız olarak, dinein ile serum uyarımına bağlı olarak fiziksel etkileşime girdiğini göstermiştir. Bu tezde, Elk-1'in dinein ile dinamik etkileşimini ve bu etkileşimden Elk-1'in diğer fosforilasyonlarının sorumlu olup olmadığını, bölünmüş-gYFP (geliştirilmiş yeşil floresan protein) tamamlama deneyi, immün çöktürme ve floresan mikroskopisi gibi çeşitli yöntemler kullanarak göstermeyi amaçladık. Amacımıza ulaşmak için Gradyan Polimeraz Zincir Reaksiyonu (PCR), klonlama denemeleri ve immün çöktürme deneyleri yapıldı. Daha ileri analizler, Elk-1'in dinein aracılı taşınmasının nörorejenerasyon, hücre döngüsü ve kanser gibi belirli biyolojik süreçler için çok önemli olduğunu aydınlatacaktır.

Anahtar Kelimeler: Elk-1, Bölünmüş-Geliştirilmiş Yeşil Floresan Protein Tamamlama Deneyi, Motor Protein, İmmün Çöktürme.

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LIST of ABBREVIATIONS and ACRONYMS

Abbreviations Explanations

and

Acronyms

ml	: Milliliter
mM	: Millimolar
pH	: Negative Log of Hydrogen Ion Concentration
AD	: Alzheimer's Disease
BCA	: Bicinchoninic Acid
BSA	: Bovine Serum Albumin
CaCl ₂	: Calcium Chloride
CBP	: CREB-Binding Protein
CID	: C-terminal Binding Protein Inhibition Domain
DPBS	: Dulbecco's Phosphate Buffered Saline
DYN1H1	: Cytoplasmic Dynein 1 Heavy Chain 1
DYNC1I	: Cytoplasmic Dynein Intermediate Chain
DYNC1LI	: Cytoplasmic Dynein 1 Light Intermediate Chain
DYNCLL	: Dynein Light Chain
DYNLRB	: Roadblock-Related Dynein Light Chain
DYNLT	: Dynein Light Chain Tctex-Type
EDTA	: Ethylenediaminetetraacetic acid
egr-1	: Early Growth Response Gene 1
ELK-1	: ETS Like Transcription Factor 1
ERF	: ETS2 Repressor Factor
ERG	: ETS-Related Gene
ERK	: Extracellular Regulated Kinase
ESE	: Epithelium Specific Ets Like Factor
ETS	: E-Twenty-Six
ETV	: ETS Translocation Variant
FBS	: Fetal Bovine Serum
HAT	: Histone Acetyl Transferase
HD	: Huntington's Disease

HDAC	: Histone Deacetylase
HRP	: Horseradish Peroxidase
Id	: Inhibitor of Differentiation
IEG	: Immediate Early Gene
JNK	: c-Jun-N Terminal Kinase
MAPK	: Mitogen Activated Protein Kinase
NaCl	: Sodium Chloride
NaOH	: Sodium Hydroxide
NES	: Nuclear Export Signal
NID	: Net Inhibition Domain
NLS	: Nuclear Localization Signal
PD	: Parkinson's Disease
PEI	: Polyethylenimine
PTM	: Post Translational Modification
PVDF	: Polyvinylidene Fluoride
SAP	: SRF Accessory Protein
SDS-PAGE	: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SH-SY5Y	: Human Neuroblastoma Cell Line
SMA	: Spinal Muscular Atrophy
SPDEF	: SAM Pointed Domain Containing ETS Transcription Factor
SRE	: Serum Response Element
SRF	: Serum Response Factor
SUMO	: Small Ubiquitin-like Modifier
TCF	: Ternary Complex Family
TEL	: Leukaemia-Related Transcription Factor
TEMED	: N,N,N',N'-Tetramethylethylenediamine
U-87	: Human Glioblastoma Cell Line

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1. INTRODUCTION

The E-Twenty-Six (ETS) Family is a transcription factor superfamily with 12 subfamilies and 28 members. Every member of this family has a common DNA binding domain called Ets domain with a highly-conserved purine-rich core sequence [Kar and Gutierrez-Hartmann, 2013], [Oikawa and Yamada, 2003].

Elk-1 is a member of the Ternary Complex Factor (TCF) Subfamily of the ETS superfamily. It is responsible for the regulation of many genes that take part in several biological processes from cell proliferation to apoptosis [Besnard et al., 2011]. It has different domains for DNA binding (Ets domain), SRF interaction (B domain), repression (R domain), transactivation (C domain), and MAPK docking (D domain) [Buchwalter et al., 2004], [Janknecht and Nordheim, 1993]. Elk-1 activity is regulated through its repression and transactivation domains by post-translational modifications: SUMOylation and phosphorylation, respectively. Major Elk-1 phosphorylation is performed by Mitogen-activated protein kinases (MAPKs), as in other members of the ETS family. Protein-protein interaction is another way of regulating Elk-1 activity. And all these regulations affect the binding of Elk-1 to different kinds of proteins such as Histone-deacetylases (HDAC), Histone-acetyltransferases (HAT), Cre-Binding proteins, which take roles in the regulation of transcription of many immediate early genes (IEGs) such as *c-fos* [Oikawa and Yamada, 2003], [Yamamoto et al., 2002].

In addition to these regulations, it has been shown that Elk-1 is involved in the regulation of certain genes associated with neurodegenerative diseases, and the phosphorylated form of 417th residue of Elk-1 is colocalized with certain bodies related with the pathogenesis of neurodegenerative diseases such as Lewy bodies and ubiquitinated Huntingtin protein [Besnard et al., 2011], implying that the role of Elk-1 goes beyond immediate early gene regulation, at least in neuronal tissues.

Preliminary protein sequence analysis of Elk-1 has shown that it has putative microtubule-binding residues. Based on this, our laboratory had shown that Elk-1 colocalizes to microtubules and interacts with them, and that Elk-1 was relocalized to the cytoplasm upon growth factor stimulation [Demir et al., 2009]. In previous studies of our laboratory, Elk-1 was demonstrated interact with dynein motor protein by the immunoprecipitation assay, indicating this nuclear relocalization could be through retrograde transport of Elk-1 [Demir et al., 2012]. In this study, it was shown that Elk-1 and dynein colocalization was dependent on serum stimulation, however this

interaction was independent of the main MAPK phosphorylation motif, Ser383. The fact that Elk-1 phosphorylation affects its transport from the cytoplasm to the nucleus has raised questions about which alternative phosphorylation sites were responsible for its interaction with dynein.

Dynein is a minus end-directed motor protein with multiple subunits such as heavy chain, light chain, intermediate chain, and light intermediate chain. Dynein has critical roles in the retrograde transport of cargos in the cell. It carries organelles, mitotic checkpoint proteins, and many others through the microtubule along the axon [Dwivedi and Sharma, 2018]. Elk-1, on the other hand, was shown to be regulated by mitotic kinases Aurora-A and -B, Plk-1 and Cdk1 in brain tumor cell lines [Uyar et al., Manuscript in preparation].

Therefore, in this thesis we aimed to study (a) real time interactions of Elk-1 and dynein using Split-EGFP complementation assay, (b) analyze the effects of various mitotic kinase phospho-mutants of Elk-1 on dynein interaction and localization using immunoprecipitation and fluorescence microscopy methods.

2. BACKGROUND

2.1. Elk-1 is a Member of E-Twenty-Six Domain Transcription Factor Superfamily

Elk-1 is a member of the Ternary Complex Factor (TCF) subfamily of ETS Domain superfamily, whose members take roles in many important cellular processes from cell proliferation to cell differentiation [Besnard et al., 2011], [Kar and Gutierrez-Hartmann, 2013], [Oikawa and Yamada, 2003].

ETS family members are characterized by a 85 amino acids long highly conserved DNA binding domain, called the Ets domain, consisting of the specific DNA binding motif that recognizes the purine-rich GGAA/T core sequence, the *ets* motif, on their target promoters [Kar and Gutierrez-Hartmann, 2013], [Oikawa and Yamada, 2003]. ETS transcription factors take roles in the transcriptional activation and/or repression of many genes by interacting with co-activators or co-repressors of transcription such as Cre-binding Protein (CBP/p300) or Histone Deacetylase 1 (HDAC1), respectively [Kar and Gutierrez-Hartmann, 2013], [Oikawa and Yamada, 2003], [Yamamoto et al., 2002], and thus regulate crucial biological processes such as cellular proliferation, survival, apoptosis, cellular differentiation (e.g. hematopoiesis, neuronal differentiation etc), angiogenesis, and cell migration [Findlay et al., 2013], [Oikawa and Yamada, 2003]. Abnormal expression of ETS transcription factors, translocations and fusions etc can cause tumorigenesis and metastasis in many cancer types including prostate cancer, breast cancer, brain tumors and leukemia [Hsing et al., 2020], [Kar and Gutierrez-Hartmann, 2013], [Kasza, 2013], [Watson et al., 2010].

Subclassification of the ETS family is carried out in different ways. Mainly, the ETS family can be divided into subfamilies based upon their Ets domain localizations: subfamilies containing N-term-localized Ets domain, C-term-localized Ets domain, and central Ets domain [Janknecht and Nordheim, 1993]. Also, the ETS family is divided into 12 subgroups according to their Ets domain homology: ELF, ERF, ERG, ESE, ETS, ETV, GABPA, PEA3, PDEF, SPI, TCF, TEL [Findlay et al., 2013]. Elk-1 takes place in the TCF subfamily containing the N-term-localized Ets domain (Figure 2.1) [Janknecht and Nordheim, 1993], [Oikawa and Yamada, 2003].

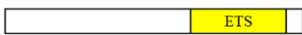
Subfamily	Structure	Members
ETV		ETV2
PDEF		SPDEF
TCF		ELK-1, NET/ELK-3, SAP1/ELK-4
ETS		ETS1, ETS2
ERG		ERG1, FLI1, FEV
GABPA		GABPA
ELF		ELF1, ELF2/NERF, ELF4/MEF
ESE		ESE1/ELF3, ESE2/ELF5, ESE3/EHF
ERF		ERF, ETV3, ETV3L
TEL		ETV6, ETV7
PEA3		ERM, ER81, PEA3
SPI		SPI1, SPIB, SPIC

Figure 2.1: ETS domain transcription factors have a common DNA binding domain called Ets domain and they differ from each other by localization of this domain (either N- or C-terminal, or in some subfamilies central).

Functional activities of ETS proteins are regulated by different mechanisms: i-) expression, ii-) protein-protein interaction, iii-) posttranslational modifications such as phosphorylation, SUMOylation (small ubiquitin-like modifier), ubiquitination, and glycosylation [Besnard et al., 2011], [Oikawa and Yamada, 2003]. Phosphorylation of these proteins is classically controlled by growth factor mediated mitogen-activated kinases, ERK (extracellular-signal-regulated kinase), or stress and cytokine-activated kinases, JNK (c-Jun-N terminal kinase) and p38 [Buchwalter et al., 2004], although recently our laboratory has also shown mitotic kinase-dependent phosphorylation of Elk-1 [Demir and Kurnaz, 2013], [Uyar et al., Manuscript in preparation].

2.2. Ternary Complex Factor (TCF) Subfamily

Ternary complex factor family takes this name due to the construction of a ternary complex with dimers of Serum Response Factor (SRF) on the Serum Response Element (SRE) region of promoters of immediate early genes such as *c-fos*, *egr-1*, and *pip92* [Buchwalter et al., 2004], [Demir et al., 2011], [Demir and Kurnaz, 2008], [Wasylyk et al., 1998]. Elk-1, Net (Elk-3), and Sap1 (Elk-4) take place in this family [Demir et al., 2011], [Wasylyk et al., 1998]. Members of this subfamily have several

conserved domains with small diversities. The N-terminal DNA binding “A” domain, or Ets-domain, is responsible for DNA-binding [Buchwalter et al., 2004], [Janknecht and Nordheim, 1993], [Oikawa and Yamada, 2003], [Wasylyk et al., 1998]. B domain is the SRF binding domain, while C domain contains Mitogen-activated Protein Kinase (MAPK) phosphorylation sites and is responsible for the transcriptional activation. D and F domains are MAPK docking sites [Buchwalter et al., 2004], [Janknecht and Nordheim, 1993], [Wasylyk et al., 1998]. Although these are the classical domains that TCF members have, some members can have additional domains: Elk-1, for example, was shown to possess a repressor (R) domain as an inhibitory domain (Figure 2.2) while Net has 2 different inhibition domains, called Net inhibition domain (NID) and C-terminal binding protein inhibition domain (CID), while J domain as a specific docking site for JNK kinase [Buchwalter et al., 2004], [Janknecht and Nordheim, 1993], [Sharrocks, 2002], [Wasylyk et al., 1998]. Sap1 also has an NID as an inhibitory domain [Buchwalter et al., 2004], [Sharrocks, 2002].

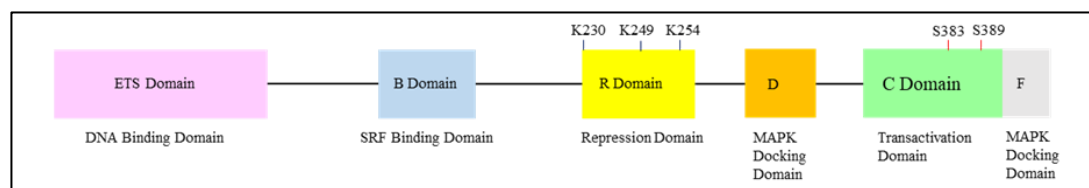


Figure 2.2: Elk-1 domain structure.

2.2.1. Functional Activity Regulation of Elk-1 by Protein-Protein Interaction

To ensure binding of Elk-1 to SRE elements in target gene promoters and regulate its activation, ternary complex formation with SRF dimers is necessary, which is where the subfamily takes its name, TCF, from. Interaction with Elk-1 and SRF through the B domain of Elk-1 affects the recruitment of the complex to the promoter, and Leu158 of Elk-1 was shown to be crucial for this interaction [Demir et al., 2011], [Ling et al., 1997], [Sharrocks, 2002], [Vickers et al., 2004], [Wasylyk et al., 1998].

Another well-known protein-protein interaction that affects the Elk-1 activity is its interaction with Id proteins (inhibitor of differentiation) [Dittmer and Nordheim, 1998]. BRCA1a/1b proteins, alternative splice variants of tumor suppressor gene BRCA1, inhibit the binding of Elk-1 to its target *c-fos* gene in human breast cancer by

binding the Ets domain of Elk-1 [Buchwalter et al., 2004], [Oikawa and Yamada, 2003]. Our laboratory has also shown its interaction with mitotic kinase Aurora-A [Uyar et al., Manuscript in preparation], microtubules [Demir et al., 2009] and dynein [Demir et al., 2012] .

2.2.2. Functional Activity Regulation of Elk-1 by Post-Translational Modifications

Elk-1 activity is also regulated by post-translational modifications such as phosphorylation, SUMOylation, and ubiquitination. Phosphorylation of Elk-1 is carried by ERK, JNK, and p38 kinases [Janknecht and Nordheim, 1993], [Tootle and Rebay, 2005], [Yang et al., 1998]. MAPK-mediated Elk-1 phosphorylation increases its DNA binding affinity, ternary complex formation, its activation, and also inhibits SUMOylation [Janknecht and Nordheim, 1993], [Tootle and Rebay, 2005].

Phosphorylation of Elk-1 by MAP kinases occurs on specific Ser/Thr-Pro amino acid residues on the C domain and particularly Serine-383 and Serine-389 phospho-sites are critical for transactivation function. Serine to Alanine (non-phosphorylatable amino acid) mutations studies showed that non-phosphorylated Elk-1 on these sites causes the blockage of SRE-dependent gene expression [Janknecht and Nordheim, 1993]. Phosphorylation of Elk-1 within its C domain leads to the conformational change of its structure, which causes the alteration of intramolecular interaction between the transactivation domain and the ETS domain, leading to increase in DNA binding and recruitment of coactivators such as p300 and CBP for transcriptional activation [Janknecht and Nordheim, 1993], [Li et al., 2003], [Tootle and Rebay, 2005]. In some cases, phosphorylation may cause transcriptional repression rather than activation. Serine-383 phosphorylation of Elk-1 by growth factor activated ERK-2 was shown to recruit the corepressor complexes such as histone deacetylases (HDACs) that leads to transcriptional repression by chromatin compaction in HEK 293 cells [Besnard et al., 2011], [Oikawa and Yamada, 2003], [Yang and Sharrocks, 2006], [Yang et al., 2001].

The protein sequence analysis of Elk-1 has shown that it is not only phosphorylated by mitogen-activated protein kinases but also contains putative residues to be phosphorylated by some cell cycle-related kinases such as Aurora kinases, Polo-like kinases (PLKs), and cyclin-dependent kinases (CDKs). These

kinases perform serial of events required for cell cycle progression, such as chromosomal biorientation, kinetochore-microtubule binding, transition point regulation. Previous studies from our laboratory has shown that Elk-1 was phosphorylated by these mitotic kinases, and this phosphorylation affected cell cycle progression [Demir and Kurnaz, 2013], [Uyar, 2019], [Uyar et al., Manuscript in preparation].

Elk-1 activity is also regulated by SUMOylation of its R domain. SUMO is an 11 kDa small peptide that is added to specific lysine residues of its targets [Salinas et al., 2004], [Tootle and Rebay, 2005]. Elk-1 has SUMOylation residues in the R domain such as Lysine 230, 249, and 254 [Besnard et al., 2011], [Salinas et al., 2004]. The modification of Elk-1 in these sites causes its inactivation, and SUMOylated Elk-1 leads transcriptional repression by recruiting HDACs to target immediate-early gene promoters such as *egr-1*, *c-fos* [Besnard et al., 2011]. Another post-translational modification of Elk-1 is the adding of 9 kDa ubiquitin peptide causes proteasome-mediated degradation of its targets [Salinas et al., 2004], [Tootle and Rebay, 2005].

Phosphorylation and SUMOylation modifications also affect the localization of Elk-1; it was shown that phosphorylation of Serine-383 and Serine-389 leads to nuclear translocation and regulation of SRE-dependent transcriptional activation [Besnard et al., 2011], [Demir et al., 2009]. SUMOylation within specific lysine residues causes the transfer of Elk-1 from the nucleus to the cytoplasm [Besnard et al., 2011], [Salinas et al., 2004].

2.2.3. Elk-1 Phosphorylations in Neurodegenerative Diseases

Experiments with brain tissues of post-mortem patients have shown that Elk-1 phosphorylations could be linked to neurodegenerative diseases such as Alzheimer's Disease, Parkinson's Disease, and Huntington's Disease [Besnard et al., 2011], [Sharma et al., 2010].

Alzheimer's Disease is characterized by neurofibrillary tangles formation and amyloid-beta (A β) accumulation, called amyloid plaques. It is thought that mutation on presenilin 1 gene (*Psen1*), which encodes a functional proteolytic subunit of gamma-secretase and carries out the final proteolytic process of the amyloid precursor protein (APP), causes the overproduction of toxic amyloid-beta accumulation that leads to Alzheimer's Disease [Pastorcic and Das, 2003], [Sharma et al., 2010]. Elk-1 is

defined as a repressor of this gene [Pastorcic and Das, 2003]. However high concentrations of A β have been shown to interfere with brain derivative neurotrophic factor (BDNF) induced Elk-1 activity [Tong et al., 2004]. Brain tissue samples of post-mortem Alzheimer's disease patients have shown that neurofibrillary tangles are colocalized with Elk-1 phosphorylated on Threonine 417. Likewise, T417 phosphorylation of Elk-1 has been shown colocalized with Lewy body and ubiquitinated Huntingtin protein in Parkinson's Disease and Huntington's Disease, respectively [Besnard et al., 2011], [Pastorcic and Das, 2003], [Sharma et al., 2010].

In addition to these diseases, Elk-1 or phosphorylated Elk-1 may be related to neural processes such as learning, depression, and drug addiction, and also Down syndrome [Davis et al., 2000], [Dwivedi et al., 2001], [Gardiner, 2006].

2.2.4. Elk-1 Targets in Neuronal and Non-neuronal Cells

Elk-1 regulates immediate early gene transcription. *c-fos* takes role in cell proliferation, transformation, apoptotic cell death. Also, its expression is used as a neural activity marker. Another target is the Early growth response 1 (*egr1*) gene. This gene is negatively regulated by Elk-1. SUMOylated Elk-1 recruits the transcription corepressors and represses the *egr-1* expression leading cell survival [Demir and Kurnaz, 2008], [Vickers et al., 2004], while non-SUMOylated Elk-1 derepresses this effect and *egr-1* expression causes apoptotic cell death [Demir and Kurnaz, 2008]. Elk-1 affects neuronal differentiation by regulating *pip92* gene expression [Kelle et al., 2019], [Vickers et al., 2004], [Wasylyk et al., 1998].

Elk-1 has two alternative splice variants, Δ Elk-1, and short Elk-1 (sElk-1). While Δ Elk-1 lacks a part of its SRF interaction domain and negative regulatory domain, sElk-1 lacks the first 54 amino acids that form the nuclear export sequence and a small portion of the DNA binding domain [Buchwalter et al., 2004], [Rao and Reddy, 1993], [Vanhoutte et al., 2001]. The absence of SRF interaction domain in Δ Elk-1 prevents it from regulating genes that have SRE on their promoter, such as *c-fos* [Rao and Reddy, 1993]. Regardless of this, it was shown that expression of sElk-1 causes the differentiation of PC12 cells, and overexpression of sElk-1 but not full-length Elk-1 increased the neurite extension in neurons, and that deletion of the nuclear export signals restricts the localization of sElk-1 to the nucleus [Buchwalter et al., 2004], [Vanhoutte et al., 2001]. In addition to these studies in PC12 cells have shown that

exogenous expression of Elk-1 protects these cells from chemical-dependent apoptosis; this was shown to be mainly through targeting the pro-survival gene *Mcl-1* [Demir et al., 2011], [Townsend et al., 1999], [Vickers et al., 2004].

Another target of Elk-1 was shown to be the survival motor neuron-1 (*Smn-1*) gene which contains two *ets* motifs on its promoter, and reduction of *Smn-1* expression is known to cause the neurological disease called spinal muscular atrophy (SMA). It was shown that the expression of the *Smn-1* gene is increased by overexpression of Elk-1, while RNAi-mediated down-regulation of Elk-1 caused an increase in caspase activity in SH-SY5Y cells [Demir et al., 2011]. Considering all these studies, it can be said that Elk-1 has a neuroprotective effect on neurons.

On the other hand, Elk-1 was shown to bind to mitochondrial permeability transition pore complex and triggers apoptosis in other experiments [Barrett et al., 2006]. Also, overexpression of Elk-1 in primary neurons was shown to cause a decrease in cell survival; interestingly, although overexpression of Elk-1 in distal dendrites triggered neuronal death, its expression in the cell body did not result in neuronal death, indicating that the pro-apoptotic or anti-apoptotic effect of Elk-1 may be determined by its subcellular localization and/or specific protein-protein interactions within the cell [Barrett et al., 2006], [Demir et al., 2011].

2.2.5. Dynein Motor Protein: An Interaction Partner of Elk-1

Dynein is a microtubule-based minus-end directed motor protein that localizes at the plus-end of microtubules and transports its cargos toward the minus-end of microtubules [Rao et al., 2017]. Dyneins are basically divided into two categories as axonemal and cytoplasmic. While axonemal dyneins take role in cilia and flagella which have cytoskeletal structure axoneme and provide their movements, cytoplasmic dyneins are responsible for retrograde transport in all animal cells [Roberts et al., 2013], [Schroeder et al., 2014]. There are two types of cytoplasmic dynein: cytoplasmic dynein 1 and 2 [Schroeder et al., 2014]. Cytoplasmic dynein 1 takes role in the transport of organelles such as lysosomes, mitochondria, endosomes, Golgi Apparatus, viruses, mitotic checkpoint proteins, intermediate filaments, microtubules while cytoplasmic dynein 2 provides transport in cilia [Dwivedi and Sharma, 2018], [Gee and Vallee, 1998], [Gonçalves et al., 2019], [Pfister et al., 2006], [Stuchell-Brereton et al., 2011]. Cytoplasmic dynein 1 has also critical roles in the regulation of

plenty of events in mitotic cells such as positioning of the mitotic spindle, chromosome segregation, centrosome segregation, and nuclear envelope breakdown, also it is responsible for the nuclear positioning in interphase cells [Dwivedi and Sharma, 2018], [Reck-Peterson et al., 2018], [Whyte et al., 2008].

Cytoplasmic dynein 1 consists of 4 different subunits: (i) heavy chain homodimers (DHC, encoded by *DYNC1H1* gene; *DYNC2H1* for cytoplasmic dynein 2), (ii) two intermediate chains (ICs, encoded by *DYNC1I1* and *DYNC1I2* genes), (iii) two light intermediate chains (LICs, encoded by *DYNC1LI1*, *DYNC1LI2* genes; *DYNC2LI1* for cytoplasmic dynein 2) and (iv) additional light chains (LCs, encoded by *DYNLL1*, *DYNLT1-3*, *DYNLRB1* genes) (Figure 2.3) [Asante et al., 2014], [Olenick and Holzbaur, 2019], [Pfister et al., 2006]. The heavy chains have motor activity and perform ATP hydrolysis, providing the force necessary for the movement of dynein through the microtubule, while the intermediate chain acts as a scaffold protein by binding to the other subunits of dynein and its partner dynactin, provides the cargo specificity of dynein [Stuchell-Brereton et al., 2011]. On the other hand, while exact roles of the light intermediate chain and the light chain are not fully known, they are thought to link the dynein to the cargo adaptors, and to provide the stabilization of the dynein complex [Gonçalves et al., 2019], [Olenick and Holzbaur, 2019]. Besides acting as a scaffold, phosphorylation of the dynein intermediate chain was shown to also increase the binding to endosomes and transport them to the nucleus to provide cell survival [Pfister, 2015].

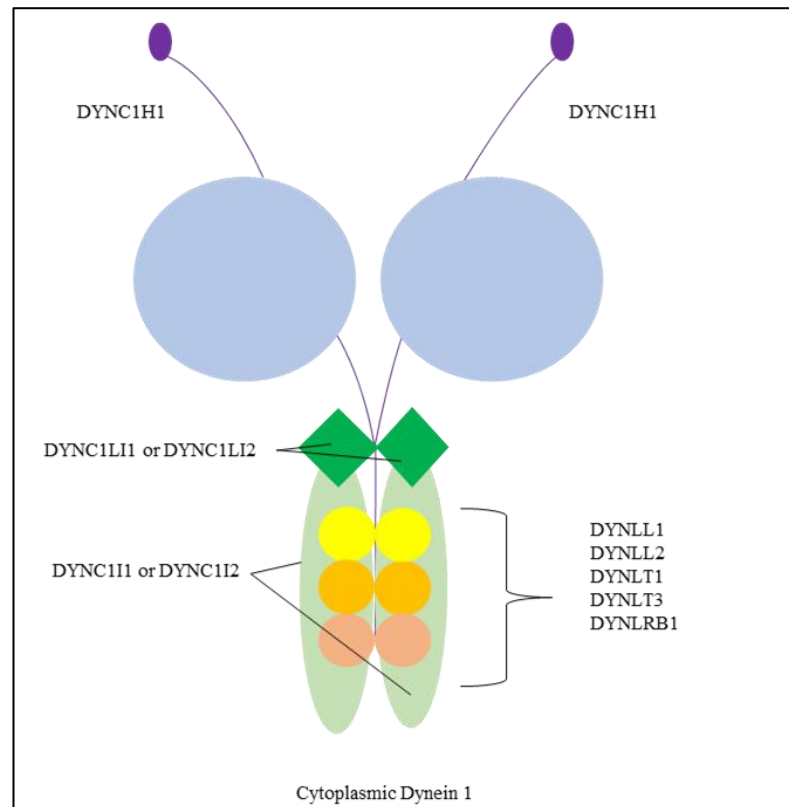


Figure 2.3: Cytoplasmic dynein 1 composed of heavy, light, intermediate, and light intermediate chains.

During cell division, dynein localizes to three different places, which is the basis of its naming. *Spindle dyneins* localize to spindle poles and can be visualized during the cell cycle. *Cortical dyneins* localized to the cortex of the cell mostly on the astral spindle in mammalian cells and can be seen in all stages of the cell cycle. *Kinetochore dyneins* localize to kinetochores of chromosomes until microtubule attachment is completed bioriented way [Hinchcliffe and Vaughan, 2018], [Kasuboski et al., 2011].

Kinetochore dynein localization dynamics depend on microtubule attachments so that this dynein cannot be visualized metaphase. Dynein localization is led by its phosphorylation and dephosphorylation [Hinchcliffe and Vaughan, 2018], [Kasuboski et al., 2011], [Prosser and Pelletier, 2017]. Previous studies in our lab and the movement dynamics of dynein and Elk-1 have brought about the question of whether Elk-1 may be transported by dynein motor protein throughout the cell cycle [Demir et al., 2012], and whether this transport depends on the mitotic kinase phosphorylation of Elk-1, which we aimed to address in this thesis.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Equipments

T25, T75 tissue culture flasks (TRP), μ -Slide 8-wells (Ibidi), 96- well plate (NUNC), tissue culture plates (60 mm, 100 mm) (TRP), 100 mm petri dishes (Lamtek), Coverslip (Isolab), serological pipettes (5ml,10 ml, 25 ml) (CAPP), micro pipettes (Eppendorf), falcons (15 ml and 50) (CAPP), microcentrifuge tubes (0,2 ml, 0,5 ml, 1,5 ml, 2ml)(Beckman), cryotubes (2ml), hemocytometer (Marienfeld), 0.22 μ m syringe filter (Merck), syringes (10 ml, 50 ml), inoculating loops(Isolab).

Polyvinylidene fluoride (PVDF) transfer membrane (Merck Millipore, IPVH00010), 3 MM Whatman Filter Paper (Whatman), MJ Mini Personal Thermal Cycler (Bio-Rad), Agarose Gel Electrophoresis System (Bio-Rad), Varioskan Flash (Thermo Scientific), Criterion Blotter (Bio-Rad), Western Blot System (Bio-Rad), Blot Transfer (Trans-Blot Semi-Dry), Power Supplier Power Pac Basic (Bio-Rad), Spin (Sprout), Vortex (JeioTech), Water Bath (JeioTech), Rotator (JeioTech), Laminar Flow Cabinet – Safe 2020 (Thermo Scientific), CO2 incubator - Stericycle i160 (Thermo Scientific), Microfuge 22R Centrifuge (Beckman Coulter), pH meter (Mettler Toledo), Fluorescence microscope (ZOE) (Leica-DMi1), ChemiDoc™ XRS+ System (Bio-Rad), Heat Block (JeioTech), autoclave (Hirayama HV-110L), Shaking Incubator (FINEPCR), Spectrophotometer (Multiscan Spectrum-Thermo Labsystems), NanoDrop (Schimadzu), -80°C freezer (VWR), UV illuminator (Vilber Lourmat), Centrifuge (Labogene, 1580R), drying-oven (VWR), liquid nitrogen tank (Taylor-Wharton, LS4800), Confocal Microscope (Zeiss, LSM 880).

3.1.2. Cell Culture Solutions

Dulbecco's Modified Eagle Medium (DMEM) (1X) with 4,5 g/L Glucose, L-glutamine and pyruvate (Gibco, 41966-029), Fetal Bovine Serum (FBS) (Gibco, 10270), Penicilin/Streptomycin (100 IU/ml Penicillin / 100 μ g/ml Streptomycin) (PAN Biotech, P06-07100), Dulbecco's Phosphate Buffered Saline (1X) (DPBS) (Gibco, 14190-94), Polyethylenimine (PEI) transfection reagent (Polysciences),

0.05% Trypsin-EDTA Solution (1X) (Gibco, 25300-054), Dimethylsulfoxide (DMSO) (BioFrox, 5BB34CE9), Poly-L-lysine solution 0.1% (w/v) in H₂O (Sigma, SLBG4596V).

3.1.3. Bacterial Media and Chemicals

Luria-Bertani (LB) Broth (Caisson Labs, LBP01-500GM), Luria-Bertani (LB) Agar (Caisson Labs, LBP02-500GM), Ampicillin (Biomatik), 87% Glycerol (Sigma, 15524), CaCl₂ (Acros Organics), Kanamycin Sulfate (Fisher Chemical, 107776).

3.1.4. Antibodies

- Anti-Flag M2 Affinity Gel (Sigma, A2220)
- Goat-anti-rabbit IgG-Alexa Fluor 647 (Invitrogen, #42246A)
- Goat-anti-rat IgG-Alexa Fluor 546 (Invitrogen, 1010045)
- Goat-anti-mouse IgG-Alexa Fluor 488 (Invitrogen, 948490)
- Goat anti-mouse IgG Antibody-HRP Conjugate (Santa Cruz Biotechnology, sc-2005)
- Goat anti-rabbit IgG Antibody-HRP Conjugate (Santa Cruz Biotechnology, sc-2004)
- Mouse Monoclonal anti-β-Actin Antibody (Santa Cruz Biotechnology, sc-47778)
- Rat Monoclonal anti-Tubulin Antibody (Abcam, 6160)
- Mouse Monoclonal anti-Elk-1 Antibody (Santa Cruz Biotechnology, sc-365876)
- Mouse Monoclonal anti-Flag Antibody (Sigma, F1804)
- Mouse Monoclonal anti-Dynein Antibody (Millipore, MAB1618)
- Rabbit Polyclonal anti-Elk-1 Antibody (Cell signaling #9182)
- Rabbit Polyclonal anti-Phospho-Elk-1 Ser383 Antibody (Cell signaling #9181)
- Rabbit anti-Elk-1-Phospho-mutants Antibodies (S303, S304, S324, S326)

3.1.5. Western Blot Solutions

Sodium dodecyl sulphate (SDS) (Fisher Scientific), 40% Acrylamide:bisacrylamide (Fisher Scientific), Tris-HCl (Fisher Scientific), Trizma-

Base (Sigma), TEMED (Sigma, T7024-100ML), Ammonium persulphate (APS) (Fisher Scientific), 4X SDS Sample Loading Dye, Page Ruler Plus Prestained Protein Ladder (Thermo Scientific, 26619), Tween-20 (Merck), Glycine (Sigma), NaCl (Sigma), HCl, NaOH, Protease Inhibitor Cocktail (Sigma, P8465- 25ML), PhosStop EASYpack - Phosphatase Inhibitor Cocktail Tablets (Roche, 04906845001), Smart BCA Protein Assay Kit (Intron Biotechnology, 21071), Nonfat Dry Milk (Cell Signaling Technology, #9999S), Albumin-Bovine Serum (Calbiochem, 1265S), Luminata Crescendo Western HRP Substrate (Millipore, WBLUR0500), Restore Plus Western Blot Stripping Buffer (Thermo Scientific, #46430), Ethanol (Sigma), Methanol (Sigma).

3.1.6. Agarose Gel Electrophoresis Solutions

Agarose powder, 1 X Tris-Acetate-EDTA(TAE) buffer, Red Safe(Intron Biotechnology, 21141), GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific, #00675354), 10X Loading Buffer (Takara, #A2701A).

3.1.7. Plasmids

pCMV-HA-EGFP-P1-N, pCMV-HA-EGFP-P2-C, pCMV-HA-EGFP-P1-Elk-1, pCMV-HA-Elk-1-EGFP-P2 plasmids were produced by graduate student Eray Şahin by using pCMV-HA-N plasmid (Figure 3.1). pCMV-Flag, pCMV-Flag-Elk-1, pCMV-Flag-Elk-1 (T417A), pCMV-Flag-Elk-1 (S383A), pCMV-Flag-Elk-1 (S389A), pCMV-Flag-Elk-1 (S303A), pCMV-Flag-Elk-1 (S303E), pCMV-Flag-Elk-1 (S304A), pCMV-Flag-Elk-1 (S324A), pCMV-Flag-Elk-1 (S326A), pCMV-Flag-Elk-1 (S149A), pCMV-Flag-Elk-1 (S149E) were generated by graduate student Oya Arı Uyar. pRSV-Elk-1 (L158P)-VP16, pRSV-SRF-VP16 plasmids were gifted by A.D. Sharrocks.

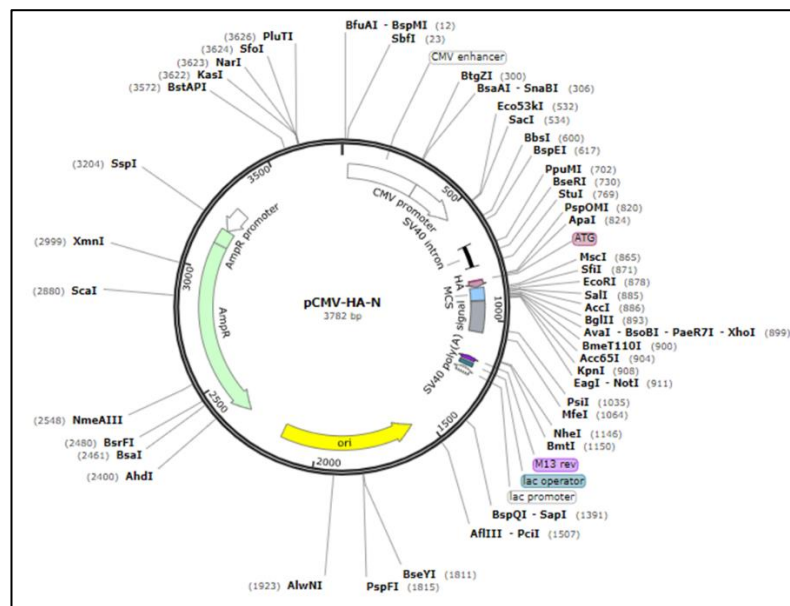


Figure 3.1: pCMV-HA-N plasmid map.

3.2. Methods

3.2.1. Primer Design

To study the real-time interaction of dynein and Elk-1, a Split-EGFP complementation assay was designed. In this method, EGFP encoding gene is split into two fragments as EGFP-N (1-158 aa) encoding and EGFP-C (159-238 aa) encoding. Each of fragments is fused with one of the two interested proteins. If these two proteins interact with each other, EGFP-N and EGFP-C fragments come close proximity and produce fluorescent (Figure 3.2) [Paulmurugan and Gambhir, 2005], [Sahin, 2016]. To decide which subunit of dynein will be cloned, interactions of dynein intermediate and light intermediate chains with heavy chain, each other, and dynactin subunits were checked by STRING database version 10.5 [Szklarczyk et al., 2015] (data available upon request).

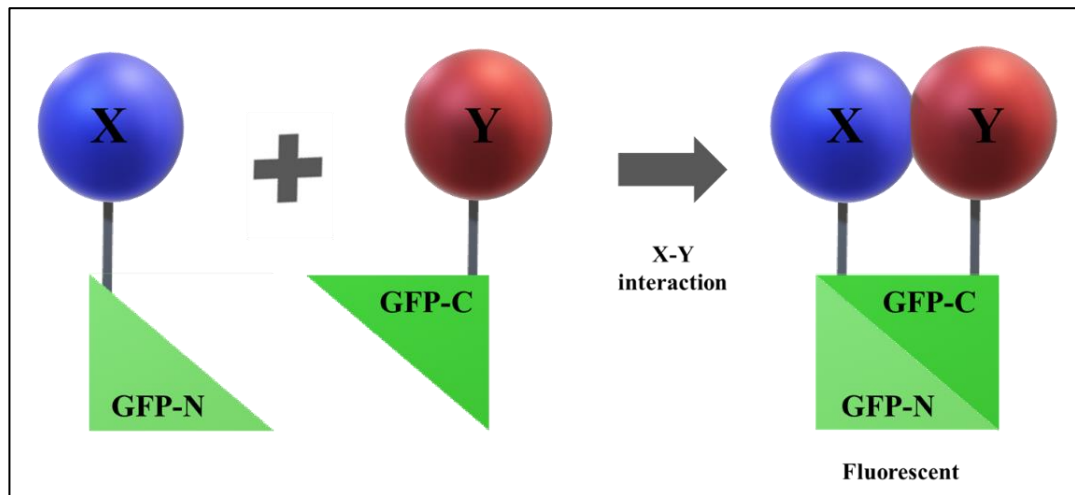


Figure 3.2: Scheme of Split-EGFP complementation assay.

In our study, dynein and Elk-1 interaction was aimed to be shown by using Elk-1-Erk-1 and Srf-Elk-1 positive interactions and Srf-Elk-1 (L158P) negative interaction as controls (Figure 3.3).

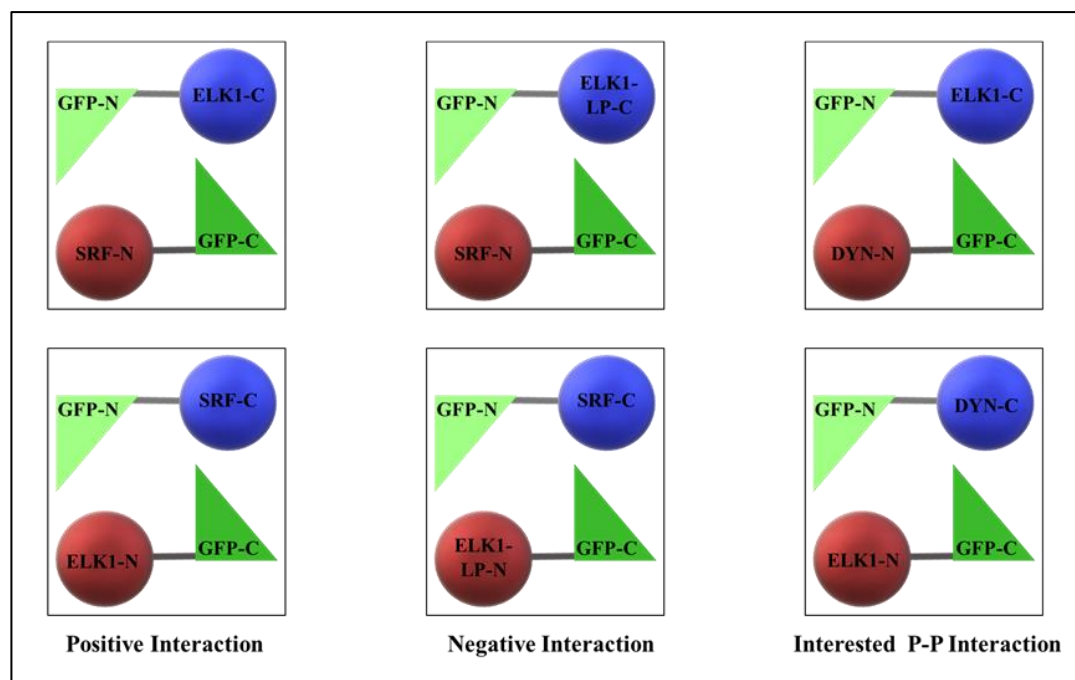


Figure 3.3: Positive interaction and negative interaction controls and interested protein-protein interaction of Split-EGFP complementation assay model.

To clone Dync1li2, Dync2li1, Dync1i2, and Dync1i1 subunits of dynein, as well as Erk-1 as positive control, in split-EGFP expression system either as N-terminal or C-terminal fusion partners, primers were designed for split-EGFP complementation

assay using OligoAnalyzer (Table 3.1). N-term primers and C term primers were designed to have restriction sites for *EcoRI-SalI* and *SalI-BglII* restriction enzymes, respectively. Because *DYNC2LI1*, *DYNC1I2* and *ERK-1* genes did not contain suitable restriction sites for these enzymes, *DYNC2LI1-N* (*MscI-SalI*) *DYNC2LI1-C* (*SalI-XhoI*), *DYNC1I2-N* (*MscI-EcoRI*), *ERK-1-N* (*EcoRI-SalI*) and *ERK-1-C* (*SalI-XhoI*) were designed to have restriction sites for different restriction enzymes.

EGFP-N and *EGFP-C* fragments have been cloned into pCMV-HA-N plasmid by Eray ŞAHİN. Also, *ELK-1-N* and *ELK-1-C* have been cloned into pCMV-HA-EGFP-P2-C and pCMV-HA-EGFP-P1-N plasmids, respectively.

During primer design, following conditions were considered: G/C content should be 40-60%, Gibbs Free Energy (ΔG) values of self-dimer and heterodimer tendencies. Primers were checked by using Primer Blast free software from the National Institute of Health (NIH).

Table 3.1: Primers designed for split-EGFP Complementation Assay. The first four nucleotides were added to increase restriction enzyme interaction efficiency with its recognition site, following nucleotides highlighted with yellow color were recognition sequences of restriction enzyme. The sequences emphasized with gray color represent the linker sequences, and sequences written in blue color were added to prevent frameshift mutation.

*These primers have been designed by graduate student Eray ŞAHİN in the lab.

NAME	PRIMER	SEQ
<i>ERK1-N</i>	Forward	5'-ATAT GAA TTC AA GCG GCG GCG GCG GCT CAG-3'
<i>ERK1-N</i>	Reverse	5'-ATAT GTC GAC CAC CTT CTT GGG GGC CTC CAG CAC TCC-3'
<i>ERK1-C</i>	Forward	5'-ATAT GTC GAC AAG GAA AAG GCG GCG GCG GCG GCT CAG-3'
<i>ERK1-C</i>	Reverse	5'-ATAT CTC GAG CTA GGG GGC CTC CAG CAC TCC-3'
<i>DYNC2LI1-N</i>	Forward	5'-ATAG TGG CCA AG CCC AGT GAA ACT CTC TGG-3'
<i>DYNC2LI1-N</i>	Reverse	5'-ATAG GTC GAC CAC CTT CTT AGA ATC AAG CTC GAT TTG-3'
<i>DYNC2LI1-C</i>	Forward	5'-ATAG GTC GAC AAG GAA AAG CCC AGT GAA ACT CTC TGG-3'
<i>DYNC2LI1-C</i>	Reverse	5'-ATAG CTC GAG TCA AGA ATC AAG CTC GAT TTG TTT CCA AGAC-3'
<i>DYNC1I2-N</i>	Forward	5'-ATAG TGG CCA AG TCA GAC AAA AGT GAA TTA AAG GC-3'
<i>DYNC1I2-N</i>	Reverse	5'-ATAG GAA TTC AG CAC CTT CTT AGC AGG TAT TCG GGT AGC-3'
<i>DYNC1LI2-N</i>	Forward	5'-ATAG GAA TTC AG GCG CCG GTG GGG GTG GAG AA-3'
<i>DYNC1LI2-N</i>	Reverse	5'-ATAT GTC GAC TCC CTT CTT GGC TTC ATT TTC TGT TGA AGA G-3'
<i>DYNC1LI2-C</i>	Forward	5'-ATAT GTC GAC AAG GAA AAG GCG CCG GTG GGG GTG GAG AA-3'
<i>DYNC1LI2-C</i>	Reverse	5'-GCGC AGA TCT TCA GGC TTC ATT TTC TGT TGA AGA GTT TGT-3'
<i>DYNC1I1-N</i>	Forward	5'-AGCC GAA TTC AA TCT GAC AAA AGT GAC TTA AAA-3'
<i>DYNC1I1-N</i>	Reverse	5'-ATAT GTC GAC CAC TTG TTG GGC AGA TAA CTC AAC AGT-3'
<i>DYNC1I1-C</i>	Forward	5'-AGCC GTC GAC AAG GAA AAG TCT GAC AAA AGT GAC TTA-3'
<i>DYNC1I1-C</i>	Reverse	5'-ATTT AGA TCT CTA GGC AGA TAA CTC AAC AGT GC-3'
<i>ELK-1-N*</i>	Forward	5'-AGCC GAA TTC AA GAC CCA TCT GTG ACG CTG TGG-3'

Table 3.1: Continued.

<i>ELK-1-N*</i>	Reverse	5'-TAAT GTC GAC CAC CTT CTT TGG CTT CTG GGG CCC TGG GGA GA-3'
<i>ELK-1-C*</i>	Forward	5'-ATAA GTC GAC AAG GAA AAG GAC CCA TCT GTG ACG CTG TGG-3'
<i>ELK-1-C*</i>	Reverse	5'-TAAT AGA TCT TCA TGG CTT CTG GGG CCC TGG GGA GA-3'
<i>SRF-N*</i>	Forward	5'-ATGA GAA TTC TT TTA CCG ACC CAA GCT GGG-3'
<i>SRF-N*</i>	Reverse	5'-TATT GTC GAC CAC CTT CTT TTC ACT CTT GGT GCT GTG GG-3'
<i>SRF-C*</i>	Forward	5'-ATAA GTC GAC AAG GAA AAG TTA CCG ACC CAA GCT GGG-3'
<i>SRF-C*</i>	Reverse	5'-TATT AGA TCT TCA TTC ACT CTT GGT GCT GTG G-3'

3.2.2. General Cell Culture Procedure

SH-SY5Y and SK-N-BE (2) human neuroblastoma cell lines, U-87 human glioblastoma cell line were used in our studies. Before cell culture studies, Dulbecco's Modified Eagle's Medium (DMEM, Gibco) was supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% Penicillin-Streptomycin (Pen-Strep, Gibco) to prepare complete medium.

Cell passaging was performed before the cell reached confluency (approximately when the cells had 80-90% confluency). The old medium that covers the cell was discarded and the cells were washed with 1X DPBS to remove detached cells. After removal of DPBS, cells were incubated with 0.05% 1X Trypsin-EDTA solution at 37°C for 1-2 min to dissociate the cells from the plate surface. The complete medium was added to the cell mix containing trypsin to inhibit trypsin activity. Then cell mix was centrifuged for 5 min at 1500 rpm and the supernatant was discarded. The cell pellet was suspended with fresh complete medium and cells were transferred into T75 flask. If cell seeding was necessary, cell counting was performed using a hemocytometer and appropriate number of cells were cultivated into 60 mm or 100 mm culture plates. They were incubated at a humidified tissue culture incubator at 37°C and 5% CO₂.

For cell freezing, after centrifugation of cell mix, cell pellet was re-suspended in freezing media having 9:1 ratio of complete medium:dimethylsulphoxide. This mixture was divided into cryotubes at concentration of 2x10⁶ cells/ml. Cryotubes were kept into isopropanol chamber for 1-2 hours. Thereafter tubes were transferred to -80 °C freezer or liquid nitrogen.

Frozen cells were thawed and transferred into complete medium. Mix was centrifuged for 5 min at 1500 rpm, the supernatant containing DMSO was discarded

and cell pellet was re-suspended in fresh complete medium. Cells were cultured into 25 cm² flasks or 10 mm dishes and incubated in tissue culture incubator.

3.2.3. cDNA Synthesis

For cDNA synthesis, I Script cDNA Synthesis Kit was used routinely in the laboratory. Total RNA of SH-SY5Y cell were gifted from Ekin Sönmez. Total RNA diluted as 100ng/µl and 10 µl of this dilution were used as a template for gradient Polymerase Chain Reactions (PCRs). The components and protocol of this reaction were described in Table 3.2, and Table 3.3, respectively.

Table 3.2: cDNA synthesis components.

Component	Amount
Template of 100 ng/ µl	10 µl
5x iScript reaction mix	4 µl
Reverse Transcriptase	1 µl
Nuclease Free Water	5 µl
Total Volume	20 µl

Table 3.3: cDNA synthesis protocol.

Step	Temperature	Time
Priming	25°C	5 min
Reverse Transcription	46°C	20 min
Reverse Transcriptase Inactivation	95°C	1 min
Terminal Step	4°C	Hold

3.2.4. Gradient PCR

125 µL PCR mix was prepared by using components and amounts that are described in Table 3.4. PCR mix was divided into 5 PCR tubes and each tube was put on different annealing temperatures to decide the optimum annealing temperature for primer pairs (Table 3.5). In the case of primers have high hairpin melting temperature, 4-10% Dimethyl sulphoxide (DMSO) was added to PCR mix.

Table 3.4: Gradient PCR components and their amounts.

Component	Amount
Water	to final volume 125 μ L
10X <i>Taq</i> Polymerase Buffer	12.5 μ L
MgCl ₂ (25 mM)	7.5 μ L
iTaq dNTP mixture (2,5mM of each)	2.5 μ L
Forward Primer	as 0.5 μ M final concentration
Reverse Primer	as 0.5 μ M final concentration
Taq DNA Polymerase (5U/ μ L)	0.5 μ L
Template	100-200 ng cDNA or plasmid

After the gradient PCR, optimum annealing temperatures of *DYNC1LI2*, *DYNC2LI1*, *DYNC1I2* primer pairs were decided as 62.8°C, 61.2°C, 60.6°C, respectively. Clear results were not taken for *ERK*, *SRF* and *DYNC1I1* primer pairs despite of different trials.

Table 3.5: For each primer pair 5 different temperatures were chosen as potential annealing temperatures.

Step		Temp.	Duration					
			dync1li2 N-C Term	dyc2li1 N-C Term	dync1i1 N-C Term	dync1i2 N Term	erk N-C Term	srf N-C Term
Initial Denaturation		94°C	3 min	3 min	3 min	3 min	3 min	3 min
Denaturation	x35	94°C	45 sec	45 sec	45 sec	45 sec	45 sec	45 sec
Annealing		Gradient 50-70°C	30 sec	30 sec	30 sec	30 sec	30 sec	30 sec
Extension		72°C	133 sec	95 sec	170 sec	173 sec	103 sec	135 sec
Final Extension		72°C	10 min	10 min	10 min	10 min	10 min	10 min

3.2.5. Transformation

5 μ l of ligation product was added into 50 μ l of competent cell (DH5 α cells). As a negative control and positive control, nuclease-free water and 0.5 ml of intact plasmid were added into 50 μ l of competent cells, respectively. All tubes were incubated on ice for 15 min, 90 seconds at 42°C and 2 minutes on ice, respectively.

900 µl of antibiotic-free LB Broth was added to tubes. Tubes were incubated at 37°C for 1 hour with shaking 180 rpm. 200 µl of mixes were streaked into LB Agar containing ampicillin or kanamycin, plates were incubated overnight at 37°C.

3.2.6. Transient Transfection

SK-N-BE (2) cells were seeded into 60 mm dishes as 7.5×10^5 cells/dish and cultured in complete medium for 24 hours at 37°C incubator supplemented with 5% CO₂. Transfection mixes were prepared by using 5 µg of plasmid, 20 µl of polyethylenimine (PEI: 1 mg/mL), and 500 µL of serum free medium into the 1.5 mL eppendorf tubes. These mixtures were vortexed for 10 seconds and left to incubation at room temperature for 15 minutes to form transfection complex (DNA/PEI complex). The media of seeded cells was discarded, and cells were washed with 1X Dulbecco's phosphate-buffered saline (DPBS). The transfection mix was added dropwise onto the cells. 2 mL of complete medium was added, and cells were cultured for 2 hours at 37°C incubator. After 2 hours, 2.5 mL of complete medium was added onto the cells, and cells were incubated for 48 hours at 37°C.

SH-SY5Y cells were seeded into 10 cm dishes as 1.5×10^6 cells/dish and cultured in complete medium for 24 hours at 37°C incubator supplemented with 5% CO₂. Transfection mixes were prepared by using 10 µg of plasmid, 35 µl of PEI and 1 mL of serum free medium into the 1.5 mL eppendorf tubes. These mixtures were vortexed 10 seconds and left to incubation for 15 minutes at room temperature. The media of seeded cells was discarded, and cells were washed once with 1X Dulbecco's phosphate-buffered saline (DPBS). The transfection mix was added dropwise onto the cells. 4 mL of medium containing FBS at different ratios (1%, 10% and 20%) was added and cells were cultured for 2 hours at 37°C incubator. After 2 hours, 5 mL of complete medium was added onto the cells, and cells were incubated for 48 hours at 37°C.

3.2.7. Protein Isolation

Before protein isolation lysis buffer was prepared by using RIPA, 7X protease inhibitor (Complete ultra-tablets, Mini, EASYpack (Roche) and 10X phosphatase

inhibitory (phospho-Stop, Roche). The buffer was prepared as final concentration of protease and phosphatase inhibitors was to be 1X.

48 hours after transfection, dishes were placed on ice. Fluorescent and brightfield displays of cells transfected with GFP plasmid were taken by using BioRad ZOE Fluorescent Cell Imaging. The media cover the cells were discarded and cells were washed twice with non-sterile 1X DPBS. Then 500 μ L of lysis buffer was added onto the cells seeded into 10 cm culture dishes (the amount of lysis buffer for cells seeded into 60 mm dishes was 300 μ L). Dishes were incubated for 20 min at 4°C by shaking. After 20 min cells were scraped and taken into 1.5 mL eppendorf tubes. Eppendorf tubes were centrifuged for 30 min at 14.000 g, 4°C. Supernatants were placed into clean tubes and stored at -20 °C.

3.2.8. BCA Assay

Firstly, 200 μ L of working solution per samples was prepared by mixing 196 μ L of solution A and 4 μ L of solution B. 25 μ L of each diluted BSA standard was pipetted into microplate as to be two replicates. 10 μ L of unknown protein samples were diluted with 40 μ L of distilled water and pipetted into 2 wells of microplate. Also, RIPA buffer was pipetted into wells as to be two replicates. After that 200 μ L of working solution was added to the samples and the microplate was placed into microplate reader to receive colorimetric absorbance measurement results (Thermo Scientific-Varioskan Flash). The microplate was incubated for 30 min at 37 °C and cooled to room temperature. After that absorbance values of each sample were measured at 562 nm wavelength. By using the absorbance values and concentrations of BSA standards, the standard curve was plotted, and the standard equation was obtained. Concentrations of diluted unknown protein samples were calculated by using the standard equation.

3.2.9. Immunoprecipitation

50 μ L of Flag agarose beads were taken for each sample and centrifuged for 1 min at 1000 g, 4°C. The supernatant was discarded, and the pellet was washed with 1 mL of non-sterile 1X DPBS following centrifuged for 1 min at 1000 g, 4°C. This step was repeated. Then pellet was washed with 1 mL of 1X TBS following centrifuged for 1 min at 1000 g, 4°C. After discarding the supernatant, the pellet was washed with

phosphatase-protease free lysis buffer (RIPA) and centrifuged for 1 min at 1000 g, 4°C. The supernatant was discarded, and Flag beads were resuspended in 50 µL of lysis buffer containing 1X protease and phosphatase inhibitors. At the same time, total protein samples were prepared as 250 µg protein in 500 µL final volume. And these total protein samples were added into the tubes containing beads and tubes were incubated overnight at rotator, 4°C. The next day, washing buffer was prepared by mixing 1 mL of 1M TrisHCl, 1 mL of 3M NaCl, and distilled water to 20 mL final volume, and pH of the solution was adjusted as 7.4 with 10 M NaOH. Tubes were centrifuged for 2 min at 1000 g, 4°C. The supernatant was discarded and 150 µL of RIPA was added to the pellet. Tubes were rotated for 2 min at 15 rpm, and then centrifuged at the same conditions. 150 µL of washing buffer was added, and tubes were rotated for 2 min then centrifuged. The supernatant was discarded and 150 µL of washing buffer was added. Tubes were centrifuged and supernatants were discarded completely. Pellet was dissolved in 30 µL of 4X loading dye and denaturated at 95 °C for 7 min.

In the meantime, protein samples as the input of immunoprecipitation assay were prepared. 30 µg of protein sample was mixed with 7.5 µL of 4X loading dye and distilled water to adjust final volume as 30 µL. Each protein sample was denaturated as previously described.

3.2.10. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

10% resolving gel and stacking gel were prepared by using Mini-PROTEAN Tetra Casting Module (Bio-RAD) at described amounts in Table 3.6. The glass plates that have 1.5 mm thickness were used and mix volume was adjusted by considering this thickness.

Table 3.6: Amount of components for four gels.

Component	Amount for Resolving Gel	Amount for Stacking Gel
Distilled water	19.3 mL	9 mL
40% Bis/Acrylamide	9.9 mL	1.5 mL

Table 3.6: Continued.

1.5 M Tris with pH 8.8	10 mL	-
1 M Tris with pH 6.8	-	1.520 mL
10% SDS	400 μ L	120 μ L
10% Ammonium Persulphate (APS)	400 μ L	120 μ L
Tetramethylethylenediamine (TEMED)	16 μ L	12 μ L

After pouring of resolving gel isopropanol was added onto the gels as it covers the whole gel surface. When gels were solidified, isopropanol was removed by washing with water and filter paper. Then stacking gels were poured on top of the resolving gels and combs (suitable with 1.5 mm thickness of glasses) were inserted between glass plates. Gels were left to polymerization.

After polymerization, gels were placed into Mini-PROTEAN Gel Electrophoresis System, and combs were removed from the gels gently. Fresh 1X Electrophoresis buffer was poured into the tank chamber. Previously prepared protein samples and protein ladder (PAGE-Ruler Plus Prestained Protein Ladder, Thermo Scientific) were loaded into wells. Electrophoresis was run at 100 voltage until loading dyes left from the gel.

For the preparation of 10% Ammonium persulphate (APS): 0.1 g of APS was dissolved in 1 ml of dH₂O.

For the preparation of 10X Electrophoresis Buffer: 30,2 g of Tris-base, 144 g of Glycine, and 10 g of SDS were dissolved in 1 liter of distilled water. Before using, the electrophoresis buffer was diluted to 1X concentration with distilled water.

3.2.11. Western Blot

Polyvinylidene Difluoride (PVDF) membranes were activated with methanol for 1 min and then rinse with distilled water. Then activated membranes, gels, and filter papers were kept in 1 X transfer buffer before blotting stack assembled. The blotting stack was prepared in the order shown in Figure 3.4.

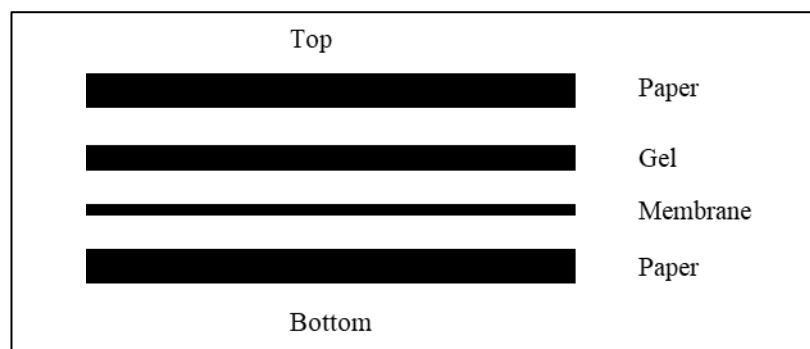


Figure 3.4: Assembly of the blotting stack.

The blotting stack was placed on the transfer cassette, voltage and time were set as 25.0 V and 45 minutes respectively. After transferring of proteins to the membrane, the membrane was blocked for 1 hour at room temperature with 5% or 3% of non-fat dry milk in 1 X TBS-T. Then, membranes were rinsed three times with 1X TBS-T for 10 minutes and were incubated overnight with appropriate primer antibodies in 3% BSA at 4°C by shaking. The day after, primer antibodies were collected, and membranes were washed three times with 1X TBS-T. Then, membranes were incubated with appropriate secondary antibodies in 3% BSA for 1 hour at room temperature on shaker. After incubation with secondary antibodies, membranes were washed two times with 1X TBS-T and one time 1X TBS. Luminata Crescendo Western HRP Substrate was added onto membranes and membranes were incubated for 1 minute by shaking. For imaging of membranes, ChemiDoc™ XRS+ System was used. Image Lab software was used for the analysis of membrane displays.

For the preparation of 10X Transfer Buffer: 30,2 g of Tris-base and 144 g of Glycine were dissolved in 1 liter of dH₂O. Before using, the transfer buffer was diluted 1X. 1X transfer buffer for semi-dry transfer, 1X transfer buffer should contain 20% methanol.

For the preparation of 10X TBS: 24,2 g of Tris-base, 80 g of NaCl were dissolved in 1 liter of dH₂O and pH value of the solution was adjusted to 7.6 with 5 M HCl.

For the preparation of 1X TBS-T: Tween 20 was added to 1X TBS as Tween 20:1X TBS ratio to be 1:1000.

For the preparation of BSA solution: BSA powder was dissolved in 1X TBS-T as ratio of BSA:1X TBS-T to be 3:100 or 5:100.

For the preparation of non-fat dry milk solution: Non-fat dry milk powder was dissolved in 1X TBS-T as ratio of non-fat dry milk:1X TBS-T to be 3:100 or 5:100.

3.2.12. Stripping

For stripping of membranes, two different protocols were used.

At the first stripping method, membranes were washed with TBS-T for 10 minutes at room temperature. Then, membranes were incubated with Restore Plus Western Blot Stripping Buffer at 37° for 15 minutes by shaking. After that, membranes were washed two times with TBS-T. Blocking, washing, and immunostaining steps were repeated as mentioned before.

Mild stripping buffer was prepared for the second stripping method.

Membranes were washed with mild strip buffer two times for 8 minutes with shaking. Then membranes were rinsed with PBS two times for 10 minutes. At the final steps, membranes were washed with TBS-T two times for 5 minutes. Blocking, washing, and immunostaining steps were repeated as mentioned before.

For the preparation of Mild Strip Buffer: 1.5 g of Glycine, 0.1 g of SDS and 1 ml of Tween 20 were dissolved in distilled water as final volume to be 100 ml. pH value of buffer adjusted to 2.2.

3.2.13. Competent Cell Preparation

DH5 α strain of *E. coli* was streaked into the plate containing ampicillin free LB Agar and the plate was incubated in 37 °C incubator for 18 hours. After 18 hours, single colony from this plate was picked and inoculated into 5 ml of ampicillin free LB Broth. LB Broth having colony was incubated overnight at 37°C with shaking at 250 rpm. 100 ml of LB Broth was inoculated with 1 ml of saturated overnight culture and shaken at 37 °C until OD₆₀₀ = 0.4. 100 ml of LB Broth was placed on ice bath for 10 minutes. At the same time centrifuge, pipette tips, falcons, and Eppendorf tubes were pre-cooled. The culture was transferred into two pre-chilled 50 ml falcon and centrifuged at 2700 g for 10 minutes at +4°C. The supernatants were removed, and cell pellets were resuspended with 1.6 ml of ice-cold 100 mM CaCl₂ by pipetting on ice gently. Resuspensions were incubated on ice for 30 minutes and centrifuged at 2700 g for 10 minutes at +4°C. The supernatants were removed, and cell pellets were resuspended with 1.6 ml of ice-cold 100 mM CaCl₂ by pipetting on ice gently. Resuspended cells were incubated on ice for 20 minutes and combined into one tube. 0.5 ml ice-cold 80% glycerol was added into the tube and cell suspension was swirled

gently. Cell suspension was aliquoted in 100 µl tubes and frozen in liquid nitrogen then stored in -80°C for long time usage.

3.2.14. Cloning

In polymerase chain reactions for molecular cloning, *pfu* DNA polymerase was used because of its low error rate that comes from its 3' to 5' proofreading activity. cDNA synthesized from total RNA of SH-SY5Y cells was used as template for amplification of *DYNC1I1-N&C*, *DYNC1LI2-N&C*, *DYNC2LI1-N&C*, *DYNC1I2-N*. pRSV-Elk-1(L158P)-VP16 plasmid was used as template for *ELK-1(L158P)-N&C* amplifications. Reaction mix was prepared as shown in Table 3.7.

Table 3.7: PCR components for amplification of inserts for molecular cloning.

Component	Amount
dH ₂ O	Variable (up to 50 µl)
10 X <i>Pfu</i> Buffer	5 µl
2 mM dNTP	2 µl
Forward primer	1 µM
Reverse primer	1 µM
Template	200 ng
<i>Pfu</i> DNA Polymerase (500 U)	1.25 U

Thermocycler conditions were adjusted as shown in Table 3.8.

Table 3.8: Thermocycler conditions of PCR of inserts.

Step		Temp.	Duration			
			dync1li2 N-C Term	dyc2li1 N-C Term	dync1i2 N Term	elk(lp) N-C Term
Initial Denaturation		95°C	3 min	3 min	3 min	3 min
Denaturation	x35	95°C	45 sec	45 sec	45 sec	45 sec
Annealing		Variable 50-70°C	30 sec	30 sec	30 sec	30 sec
Extension		72°C	180 sec	130 sec	231 sec	160 sec
Final Extension		72°C	10 min	10 min	10 min	10 min

Because of the cloning problem, PCR with *pfu* polymerase for amplification was not performed for all inserts. After amplification, PCR products were loaded into 1% agarose gel, and gels were visualized with a UV transilluminator (Vilber Lourmat). Then PCR products were purified from gel as described below.

Purified PCR products and vector plasmids (pCMV-HA-EGFP-P1-N and pCMV-HA-EGFP-P2-C) were double-digested with appropriate restriction enzyme combinations for 1 hour at 37°C water bath as described in Table 3.9. Restriction enzyme combinations were stated in the text.

Table 3.9: Double-digestion components.

Component	Amount
DNA	1 µg of vector or 200 ng of insert
Restriction enzyme I	1 µl
Restriction enzyme II	1 µl
10 X Green Buffer	2 µl
dH ₂ O	Up to 20 µl

All digested vectors and inserts were loaded into %1 agarose gel and after visualization, they were purified from agarose gel. Then T4 DNA Ligase enzyme was used for ligation of these inserts. 50 ng of vector and the proper amount of insert at the 1:3 and 1:5 molar ratios of vector: insert were incubated in reaction mixture containing T4 DNA Ligase enzyme and T4 DNA Ligase buffer at room temperature for 10 minutes. Then the mixture was incubated at 65°C for 10 minutes for inactivation of the enzyme. Ligation mixtures were prepared as described in Table 3.10.

Table 3.10: Components and their amounts for ligation.

Component	Amount
10 X T4 Buffer	2 µl
T4 DNA Ligase Enzyme	0.2 µl
Vector	50 ng
Insert	Proper amount
dH ₂ O	Up to 20 µl

DH5 α cells were transformed with these ligands as described before and cells were spread to agar plates containing ampicillin to verify the success of cloning.

3.2.15. Purification of PCR products

After PCR or digestion procedure, PCR products and DNA fragments were extracted from agarose gel. Desired bands were excised from agarose gel and weights of these gel slices were measured. Appropriate volume of lysis buffer was added into tubes containing gel slices. Tubes were incubated at 55°C and tubes were vortexed at each 2-3 min until gel slices were melted. The mixture was transferred into tubes containing column, and tubes were centrifuged for 1 min at 11.000 g. The bottom liquid was discarded and 750 μ l washing buffer was added to the column. Afterward, tubes were centrifuged at 11.000 g for 1 min and the bottom liquid was discarded. To dry the filters, tubes were centrifuged at 11.000 g for 1 min. For complete removal of ethanol, columns were incubated at 70 °C for 5 min. Columns were transferred into clean microcentrifuge tubes, 30 μ l of pre-heated autoclaved water was added onto filters, and tubes were incubated at 70 °C for 5 min to increase the product yield. After that, tubes were centrifuged at 11.000 g for 1 min to elute DNA.

3.2.16. Plasmid Isolation

A single colony was picked from bacterial plate and inoculated into 100 ml of LB broth. The liquid culture was incubated overnight at 37°C with shaking. Then, the culture was centrifuged at 4000 rpm for 10 min and the supernatant was discarded. The cell pellet was resuspended in 2 ml of resuspension buffer by vortexing. After that 2 ml of lysis buffer was added to resuspended cells. The mixture in the tube was mixed by inverting the tube 10 times. The tube was incubated at room temperature for 3 min. 2 ml of neutralization buffer was added and the mixture was mixed by inverting the tube 10 times. The tube was incubated on ice for 5 min. Then cell lysate was transferred into the pre-column and centrifuged at 4000 rpm for 5 min. After centrifugation, flow-through was poured into the binding column and centrifuged at 4000 rpm for 5 min. Flow-through was discarded and 10 ml of first washing buffer was added onto the binding column; centrifuged at 4000 rpm for 5 min. Flow-through was discarded and second washing buffer was added; centrifuged at 4000 rpm for 5 min. The binding

column was centrifuged for 15 min at 4000 rpm for the complete removal of ethanol. After that binding column was transferred into a clean 50 ml tube, 600 µl of autoclaved water was added onto the membrane of the binding column and incubated at room temperature for 5 min. Then the binding column was centrifuged at 4000 rpm for 5 min to elute the plasmid. DNA concentration of eluted plasmid was measured by using NanoDrop spectrophotometer.

3.2.17. Immunofluorescence Staining

Firstly, glass slides were autoclaved and coverslip bottom, lid, and glass slides of Ibidi chambered 8-well system were sterilized by UV. For coating of wells, 0.1% (v/v) Poly-L-lysine solution was diluted in a 1:10 ratio by autoclaved distilled water. Sterilized pieces of 8-well system were assembled and well surfaces were coated with 0.01% (v/v) Poly-L-lysine working solution for 15 minutes at room temperature. After that, wells were washed three times with autoclaved distilled water.

U87 cells were seeded into wells at a density of 50,000 cells/well and incubated for 24 hours. Transfection mixes were prepared by using 500 ng of plasmid (pCMV-Flag-Elk-1, pCMV-Flag-Elk-1 (303A), pCMV-Flag-Elk-1 (304A), pCMV-Flag-Elk-1 (324A), pCMV-Flag-Elk-1 (326A), EGFP), 2 µg of PEI (plasmid: PEI ratio was 1:4) and 50 µl of serum free medium. The old medium was discarded, and cells were washed with 1X DPBS. After that, cells were transfected with prepared transfection mixes and incubated for 48 hours.

The medium of cells was discarded, and cells were washed two times with 1X DPBS. Cells were fixed by 4% paraformaldehyde (PFA) for 15 min at room temperature. PFA was discarded and wells were washed two times with 1X DPBS. After that, cells were permeabilized with PBS-T for 10 min by shaking. Wells were washed with 1 X DPBS, then, treated with blocking solution for 1 hour at room temperature. The blocking solution was prepared in 1X DPBS at the ratios shown at Table 3.11. Then, well were washed two times with 1X DPBS.

Table 3.11: Blocking solution ingredients.

Ingredient	Ratio
BSA	3:100
Sodium Azide	3:1000
Goat Serum	1:100
Triton X	1:1000

Primer antibody mixes were prepared in blocking solution at the dilutions shown in Table 3.12. After blocking, primer antibody mixes were added and incubated o/n at 4°C on shaker. At this time, secondary control was incubated with blocking solution.

Table 3.12: Primer antibody ratios.

Antibody	Ratio	Catalog Number of Ab.
Dynein	1:75	Millipore, MAB1618
Total Elk-1	1:300	Cell signaling #9182
Phospho-Elk-1 (303,304,324,326)	1:1000	Synthesized by Genscript
Tubulin	1:50	Abcam, 6160
Phospho-383-Elk-1	1:300	Cell signaling #9181

After primer antibody removal, wells were washed three times with 1 X DPBS and incubated with secondary antibodies; goat anti-rat IgG–Alexa Fluor 546, goat anti-mouse IgG–Alexa Fluor 488, goat anti-rabbit IgG–Alexa Fluor 647 at 1:1000 dilution for two hours at room temperature in dark. Then secondary antibodies were collected, and wells were washed five times with PBS-T. Wells were treated with DAPI for 5 minutes and then, washed two times with PBS-T. 8-well system was disassembled. Surfaces of glass slides were mounted with 80% glycerol and glass slides were covered by coverslips. Glass slides were analyzed by using Zeiss Confocal Microscopy.

Preparation of PBS-T: Triton X was added into PBS in ratio 2:1000 of Triton X: PBS.

3.2.18. Bioinformatical Analysis

Gene expression data of Elk-1-VP16 transfected cells was obtained by microarray analysis performed by Başak Kandemir [Sogut et al., Manuscript in preparation]. Using KeyPathwayMiner (KPM) tool [List et al., 2016], gene expression data of Elk-1-VP16 transfected cells was mapped to the genome-scale protein interaction network taken from BioGrid [Oughtred et al., 2019]. To obtain subnetworks that were rich for significantly changed genes k value was decided as ‘1’. After that largest subnetwork was chosen for further analysis. Gene ontology analysis by using g: Profiler database [Raudvere et al., 2019] was performed for genes placed in this subnetwork to understand whether dynein related pathways are affected or not. Desired results were not obtained, because of the enrichment analysis used by g:Profiler. So that, by using code was written by PhD student Yiğit Koray Babal, genes that are associated with “motor activity” in the gene ontology (GO) database [Ashburner et al., 2000] were compared to our genes found in KPM analysis to find motor activity related genes in the subnetwork.

4. RESULTS

4.1. Split-EGFP Complementation Assay

To show the real-time interaction of dynein and Elk-1 proteins, we have initially decided to use split-EGFP complementation assay. For this purpose, one of the predicted interaction partners were to be cloned into expression vector containing EGFP-N fragment and the other was to be cloned into the vector containing EGFP-C fragment. Because of that dynein is a huge protein complex, we first had to decide which subunit or subunits were likely to interact with Elk-1, and thus candidates for cloning. To achieve this purpose, protein-protein interaction analysis has been done using STRING database (version 10.5). In this analysis, the interaction of dynein intermediate and light intermediate subunits to heavy chain, each other, and dynactin subunits have been checked. Because of the nonconclusive results of these analysis (data available upon request), we next decided to clone dynein subunits that were reported to be expressed in neurons.

4.2. Bioinformatic Analysis of Microarray Data

To understand whether Elk-1 overexpression affects the pathways that are related to neuronal transport or dynein directly, we used the bioinformatic approaches. First of all, we used the gene expression data of the Elk-1-VP16 transfected cells that were previously made in our lab and the whole human protein-protein interaction network obtained from BioGrid. Using KeyPathwayMiner (KPM) tool, we mapped gene expression data to genome-scale protein interaction network and obtained subnetworks that were rich for significantly changed genes (k value = 1). We chose the largest subnetwork that contains 4802 genes for further analysis. To see if the dynein related pathways are affected, we took the node file containing the genes that were placed in these networks and performed gene ontology analysis via the g:Profiler. However, since g:Profiler uses enrichment analysis, we could not get the desired result from here. So we compared genes that are associated with “motor activity” in the gene ontology (GO) database with our genes found in KPM analysis to find motor activity genes in the subnetwork by using code was written by PhD student Yiğit Koray Babal (Table 4.1).

Table 4.1: The genes which are pulled with ‘Motor activity’ GO term as a result of analysis of Elk-1-VP16 microarray data.

Gene Name	Entrez ID
DYNC1H1	1778
DYNC2H1	79659
DYNC2LI1	51626
DNAI2	64446
DNAH3	55567
DCTN2	10540
DCTN1	1639

DYNC1H1, *DCTN1*, *DCTN2* genes have low cell-specificity while *DYNC2H1* is expressed in mostly neuronal cells. *DNAI2*, *DNAH3* are not expressed in neuronal cell line mostly expressed in epithelial and germ cells (Human Protein Atlas, version 20.0). *DYNC2LI1* is the light intermediate chain of cytoplasmic dynein, and it was also obtained from this analysis, so that we decided to clone this subunit of dynein in addition to subunits of dynein mentioned before.

4.3. Gradient PCR Results

To decide the annealing temperatures for PCR, firstly, gradient PCRs were performed. In normal conditions, the annealing temperature is calculated as 5°C below the lower melting temperature (T_m). However, since our primers were very long and PCRs were made by using cDNA template instead of plasmid template, we decided to carry out gradient PCR to determine optimum annealing temperature.

To find the annealing temperature for *ERK-1-N* and *ERK-1-C*, gradient PCR was performed by using the reaction mixture containing *taq* buffer with KCl and 4% DMSO, because of the high temperatures of T_m of primer pairs. As a result of the gradient PCR for *ERK-1-N* and *ERK-1-C*, we expected to see bands at around 1140 base-pair length, but we obtained non-specific bands (Figure 4.1).

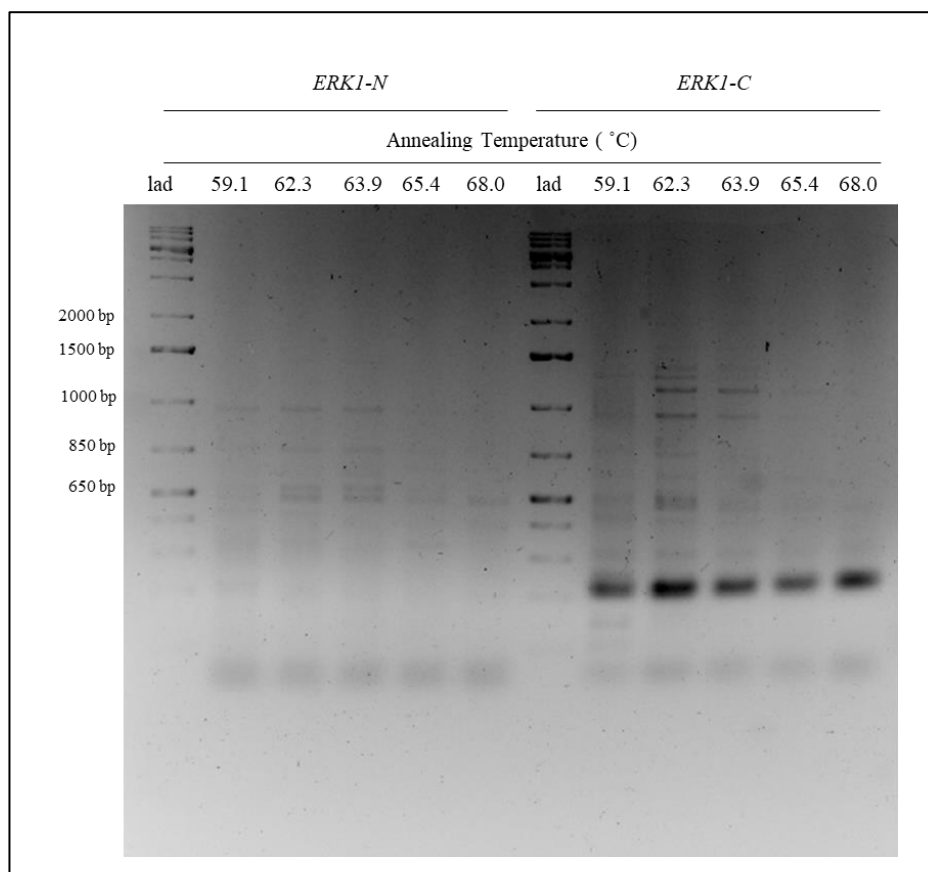


Figure 4.1: Gradient PCR result of *ERK-1-N* and *ERK-1-C*.

Gradient PCR was reperformed at different annealing temperatures for *ERK-1-N*, but we did not get successful results (Figure 4.2.a). As a last resort, we tried to increase the activity and binding capacity of *taq* polymerase by increasing the MgCl_2 concentration and tried to get successful results. In this time *taq* buffer with $(\text{NH}_4)_2\text{SO}_4$ was used. However, we did not get a proper result (Figure 4.2.b).

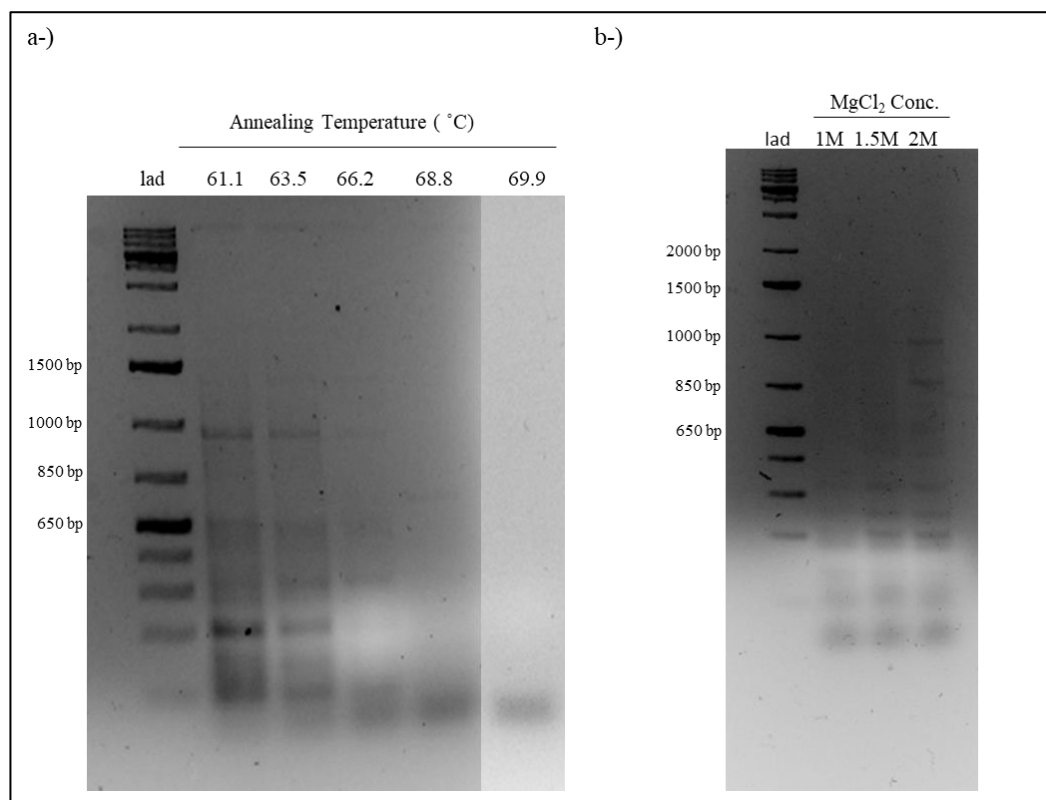


Figure 4.2: Results of Gradient PCR of *ERK-1*. a-) *ERK-1-N* in the presence of 4% DMSO. b-) *ERK-1-C* with increasing $MgCl_2$ concentration in the presence of 4% DMSO and *taq* buffer containing $(NH_4)_2SO_4$.

Similarly, for the *SRF-N* and *SRF-C*, gradient PCR was performed by using the RSV-*SRF*-VP16 plasmid as a template, and gradient PCR was not successful (Figure 4.3). This problem could arise from the formation of primer dimers.

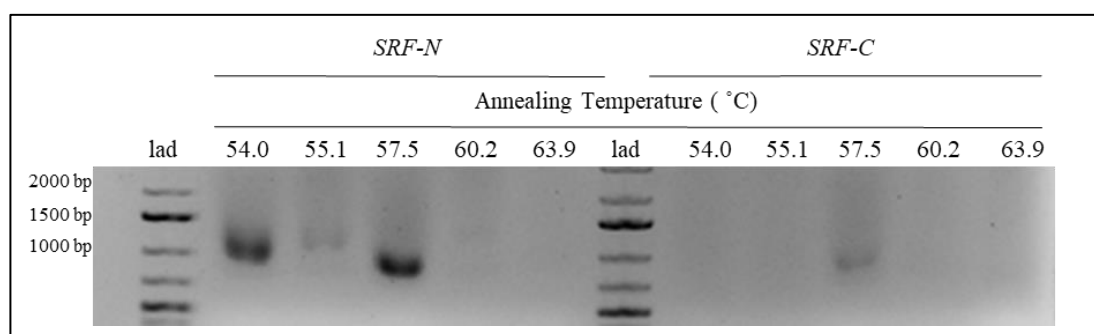


Figure 4.3: Results of Gradient PCR of *SRF-N* and *SRF-C* performed by using RSV-*SRF*-VP16 plasmid as a template.

To prevent the formation of the primer dimer and to dissolve the hairpin structure of the primers, PCR was tried once adding 10% DMSO (Figure 4.4).

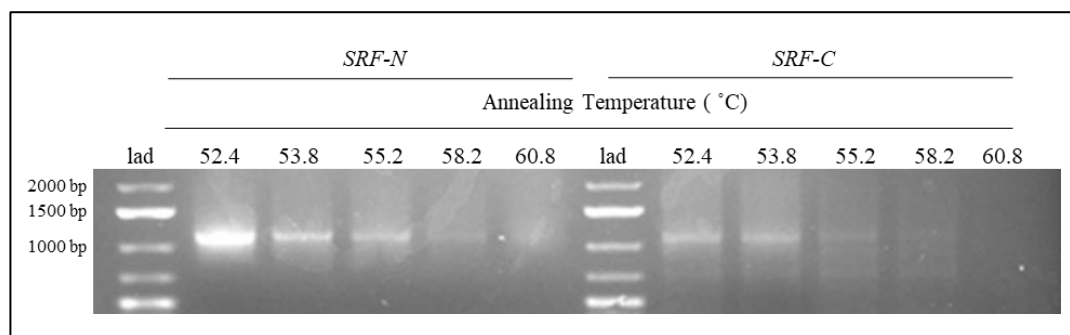


Figure 4.4: Results of Gradient PCR of *SRF-N* and *SRF-C* performed by using 10% DMSO.

Again, since there was no band at the expected location (1527 bp), the expression of *SRF* in SH-SY5Y cells was controlled and this time gradient PCR was performed by using cDNA as a template and reaction mixture containing 10% DMSO (Figure 4.5).

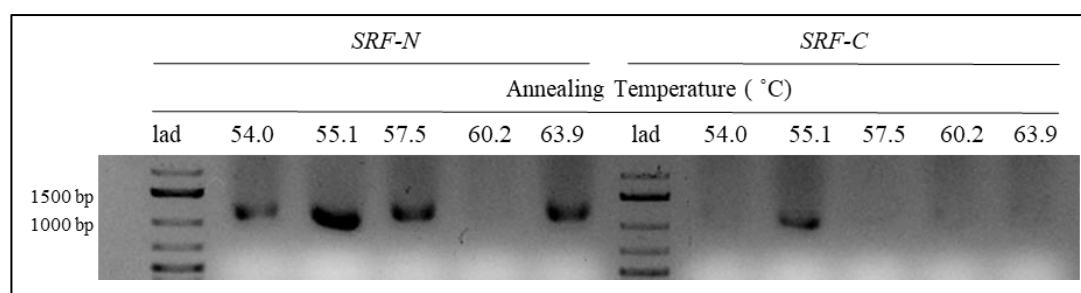


Figure 4.5: Results of Gradient PCR of *SRF-N* and *SRF-C* performed by using 10% DMSO and, cDNA as a template.

Besides all these trials, two steps PCR and PCR with *phusion* polymerase were also tried but these were inconclusive again (data not shown).

Gradient PCR for *DYNC2LI1-N* and *DYNC2LI1-C* were done, PCR products were expected nearly above the 1000 bp bands. After imaging, the bands for both products were visualized at the expected place, but the bands of the *DYNC2LI1-N* were weak (Figure 4.6.a). So that, *DYNC2LI1-N* gradient PCR was tried by adding 4% DMSO into the reaction buffer, and a relatively strong band was visualized at the expected place at 62.5°C (Figure 4.6.b)

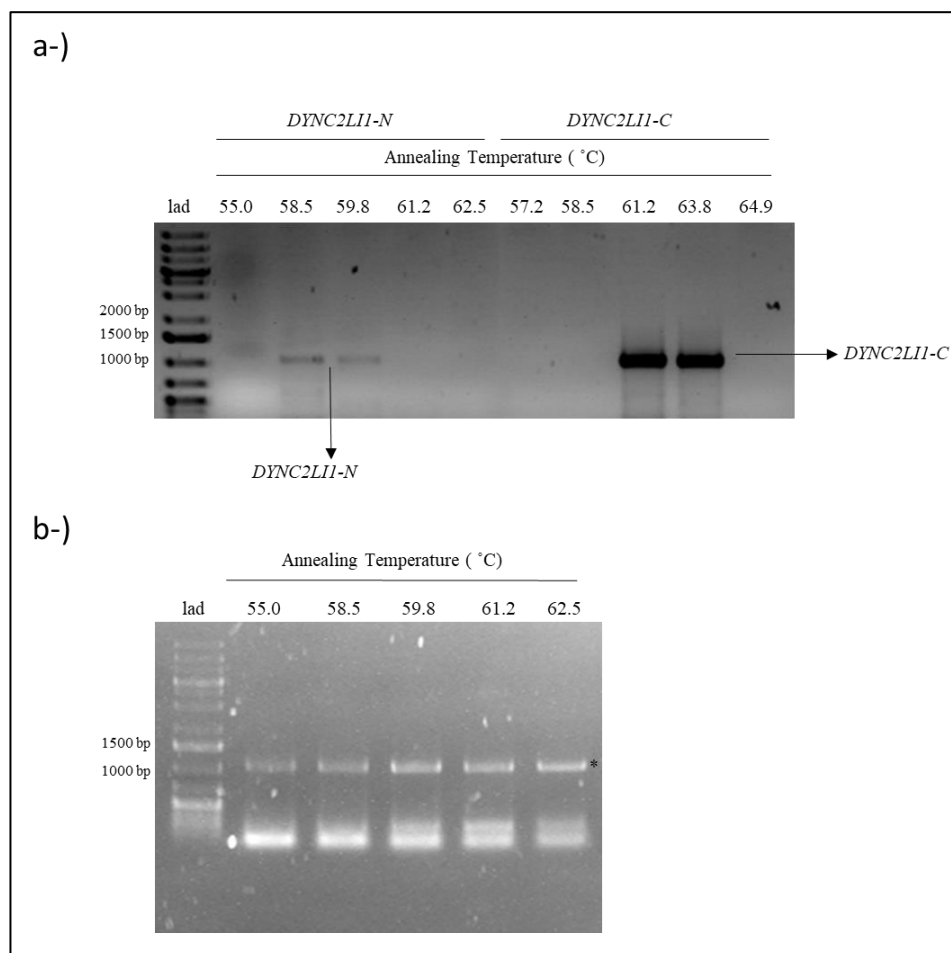


Figure 4.6: Gradient PCR results of *DYNC2LI1*. Results of a-) *DYNC2LI1-N* and *DYNC2LI1-C*, b-) *DYNC2LI1-N* at 4% DMSO condition.

To find the appropriate PCR annealing temperature we performed a gradient PCR for *DYNC2LI1-N*, but we could not get the expected band at any temperatures for it (Figure 4.7.a). After that we reperformed gradient PCR by adding 4% DMSO into the *taq* polymerase buffer, we saw the explicit band at nearly around 2000 bp as we expected (Figure 4.7.b).

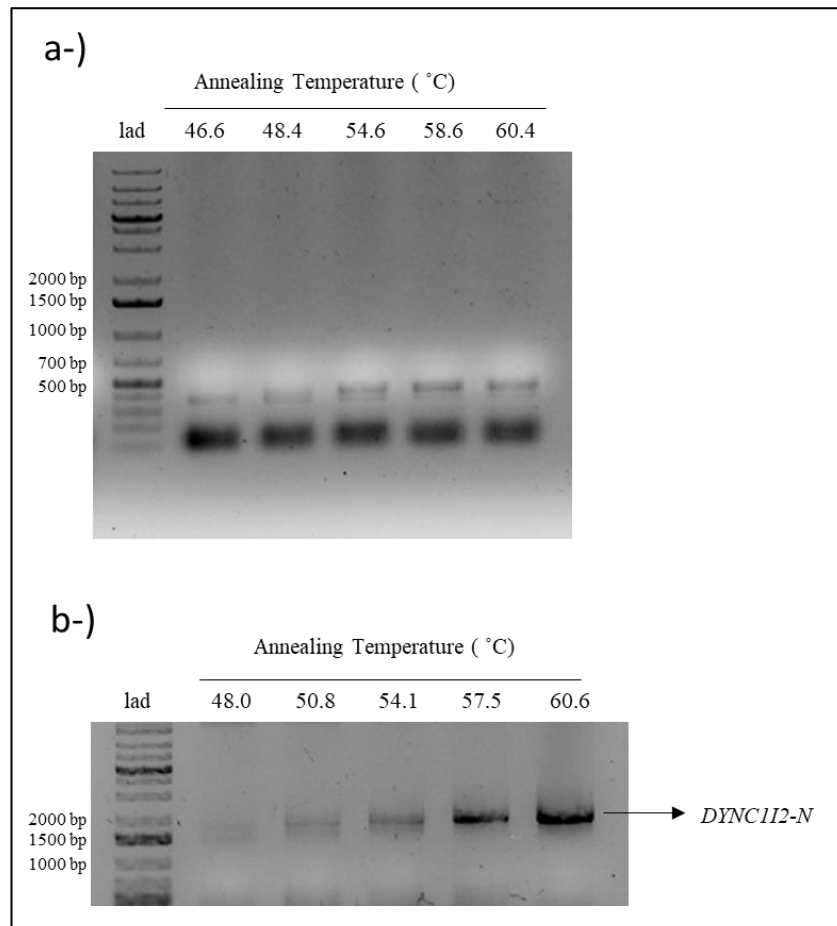


Figure 4.7: *DYNC1I2-N* gradient PCR results using reaction mixture a-) without DMSO and b-) with 4% DMSO.

Gradient PCR was run for the *DYNC1LI2-N* and *DYNC1LI2-C*, after that, we could get the expected band for *DYNC1LI2-C* (Figure 4.8.a right column) but for *DYNC1LI2-N* this process was not successful (Figure 4.8.a left column). For *DYNC1LI2-N*, gradient PCR was reperformed with 4% DMSO in PCR buffer. At this time the expected band was seen at nearly around 1500 bp (Figure 4.8.b).

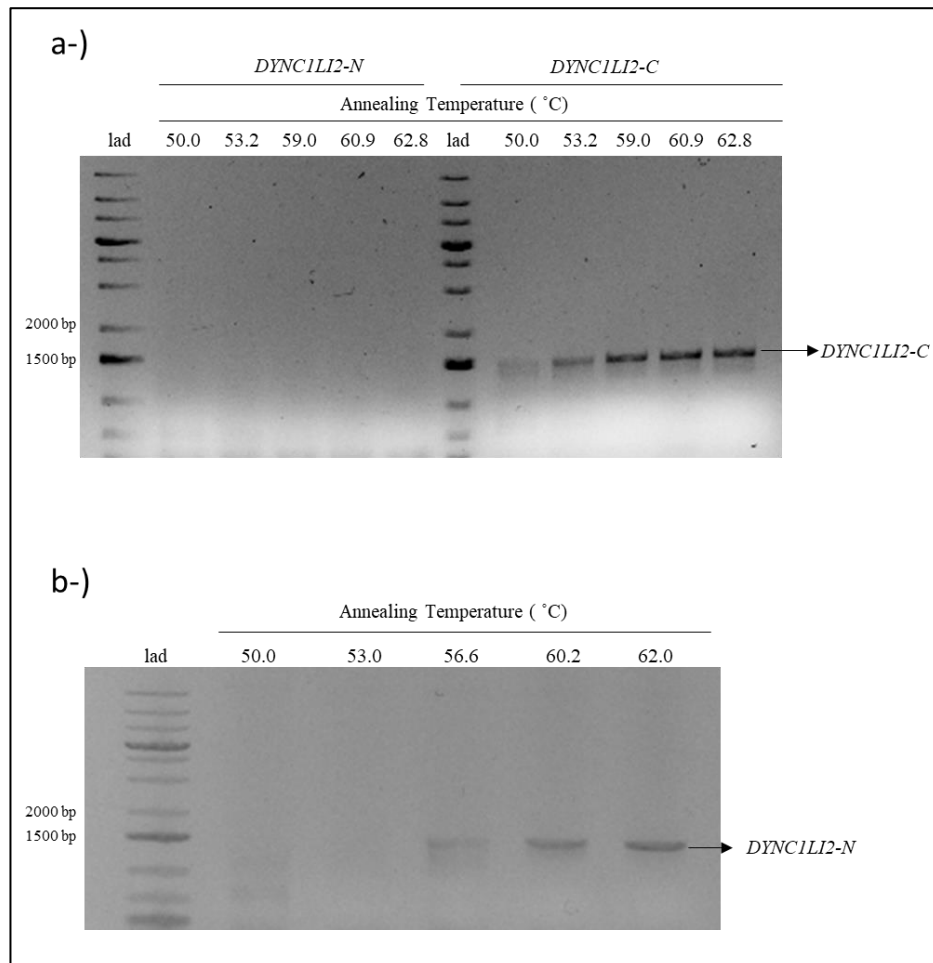


Figure 4.8: Gradient PCR results of *DYNC1LI2*. a-) *DYNC1LI2-N* and *DYNC1LI2-C* gradient PCR results, b-) *DYNC1LI2-N* gradient PCR with 4% DMSO.

Gradient PCR was run for the *DYNC1I1-N* and *DYNC1I1-C*, after that, we could not get the expected bands that should be near below 2000 bp (Figure 4.9.a). Then PCR was repeated with 4% DMSO in buffer without changing annealing temperatures. But again, desired bands were not seen on the gel (Figure 4.9.b).

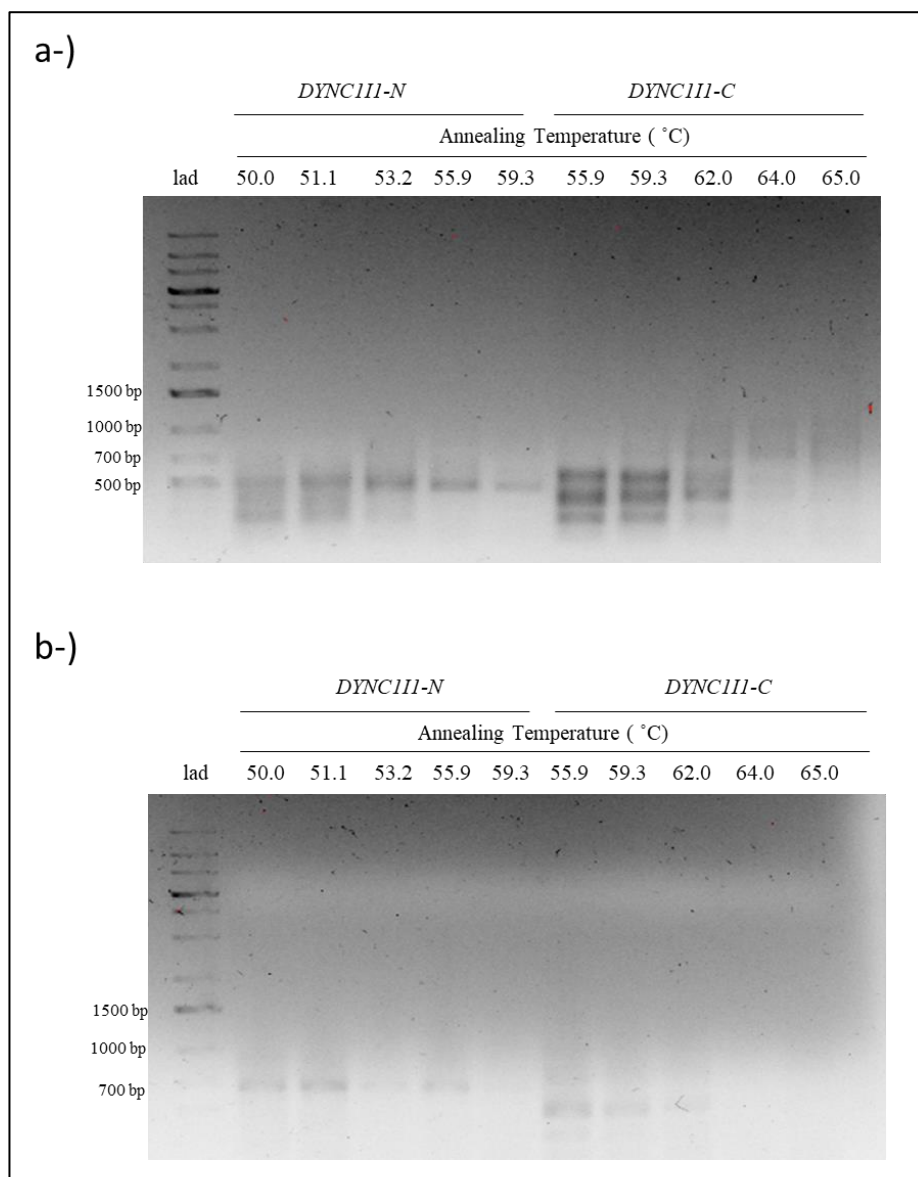


Figure 4.9: *DYNC111-N* and *DYNC111-C* gradient PCR results. Reaction mixture a-) without DMSO, b-) with 4% DMSO.

As a last chance gradient PCR was done with 4% DMSO and at lower MgCl_2 concentration (decreased from 1.5 mM to 1 mM) to decrease non-specific products and at lower annealing temperatures to increase the primer binding to the template. This time, the desired results were not obtained again, however, non-specific bands were seen for *DYNC111-N* and *DYNC111-C* (Figure 4.10).

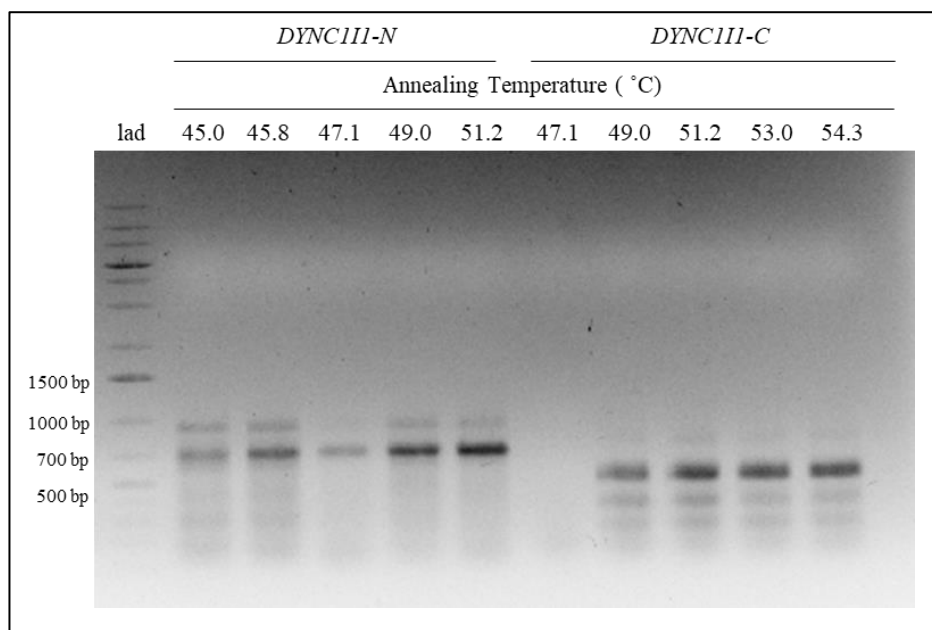


Figure 4.10: *DYNC111-N* and *DYNC111-C* gradient PCR results with 4% DMSO and 1mM MgCl₂ at lower annealing temperatures.

4.4. Cloning

Since PCR has been done previously in our lab with Elk-1 primers, PCR with *pfu* polymerase has been run because of no need to do gradient PCR for *ELK-1-L158P-N* like others (Figure 4.11.a). The PCR product was isolated from the gel, the isolated PCR product and vector (pCMV-HA-EGFP-P2-C) were cut with restriction enzymes and cloning was attempted (Figure 4.11.b). Cloning protocols have been tried at 3:1, 5:1 insert: vector ratios at 22°C overnight, and 3:1 ratio at 16°C overnight, but none of them were successful. After that, despite cloning experiments of single digested vector were tried with a different enzyme and different buffer solution at 16°C and 4°C temperatures overnight, again no success has been recorded.

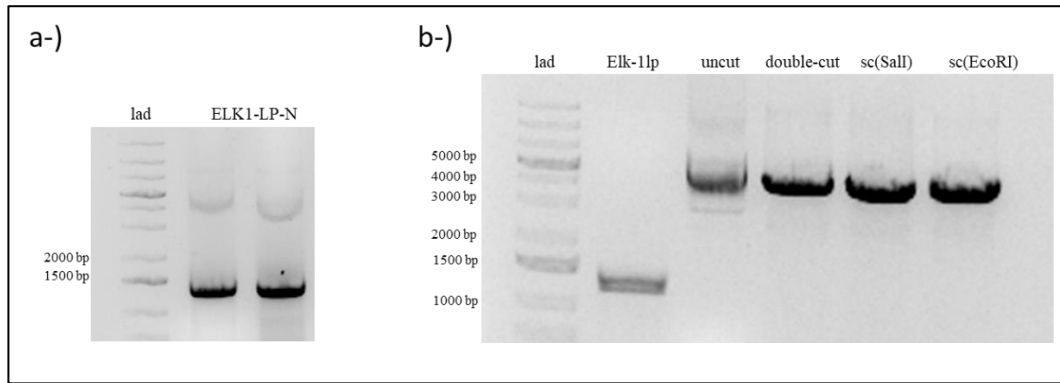


Figure 4.11: PCR for cloning. a-) *ELK1-L158P-N* PCR with *pfu* polymerase at 60°C, b-) Digestion of *ELK1-L158P-N* product and pCMV-HA-EGFP-P2-C vector for cloning.

Unfortunately, for technical reasons the required set of expression vectors could not be cloned to start the split-EGFP complementation assay to study the interaction of Elk-1 with different dynein components, therefore we have continued our studies using immunoprecipitation experiments.

4.5. Flag-IP Analysis to Show the Interaction of Elk-1 and Dynein

Elk-1 and dynein have been shown to interact with each other by the previous Co-IP experiments in our laboratory [Demir et al., 2012]. It was examined whether this interaction was affected by serum stimulation and 383S phosphorylation of Elk-1. In this study, the effects of serum condition and Serine 383, 389, and 417 phosphorylations on this interaction were examined by Flag-IP analysis. For this, SH-SY5Y cells grown in media containing 1%, 10%, and 20% FBS were transfected with Elk-1 mutants and wild type. After concentrations of the obtained proteins were measured, Flag-IP was performed with 250 µg protein from each sample. 30 µg total protein was loaded into the SDS-PAGE as an input. While we could see Elk-1 proteins after Flag-IP we made in cells grown in medium containing 1% FBS (Figure 4.12.a upper panel), dynein bands were not visible after stripping and reblotting (Figure 4.12.a lower panel). Furthermore, several bands were seen in Elk-1 staining. Although we could observe Elk-1 with Western of total protein lysates, we could not observe Elk-1 bands in either IP or input of cells transfected with 417A mutant, wild-type Elk-1, and non-transfected cells (Figure 4.12.a upper panel 4th, 6th, 7th lanes and Figure

4.12.b upper panel 4th, 6th, 7th lanes), which could be a staining problem or reflects failure to immunoprecipitate Elk-1 successfully. Dynein antibody used recognized the intermediate chain epitope, so we should see a single band around 74 kDa, but also an additional band was seen below in the proteins of cells transfected with 383A, 389A, and wild type Elk-1 (Figure 4.12.b mid-panel, 2nd, 3rd, 5th lanes), which could be a degradation product of dynein intermediate chain. β -actin was checked to ensure that proteins were loaded equally (Figure 4.12.b lower panel).

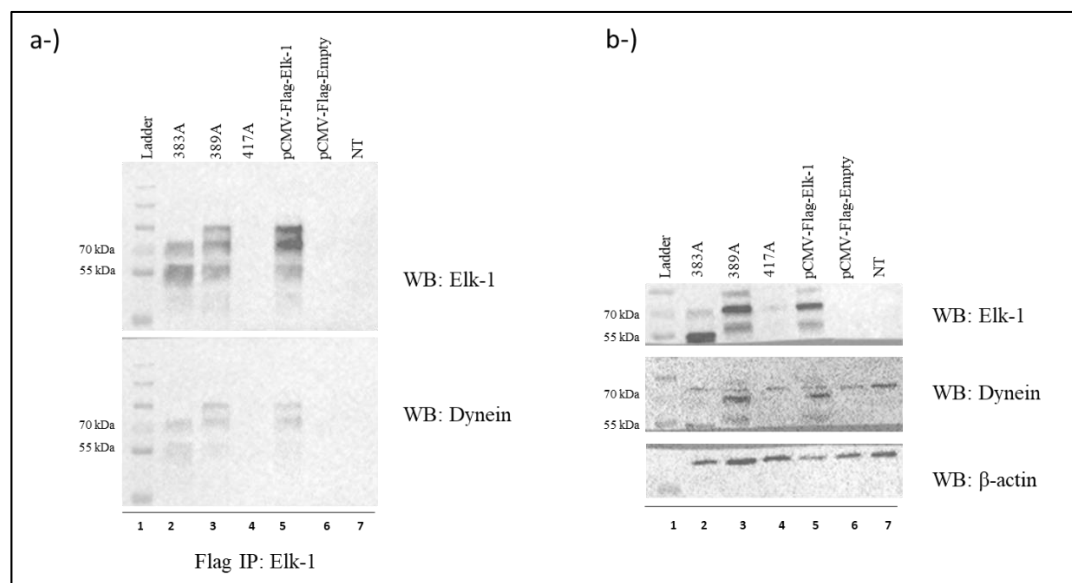


Figure 4.12: The interaction of wild type and phospho-mutants of Elk-1 with dynein at 1% FBS condition. Western blot analysis of a-) Flag-IP and b-) total protein lysate.

Western blot analysis of proteins from cells grown in 10% FBS condition was run according to the same protocol. Results of this analysis were consistent with those in the 1% FBS condition (Figure 4.13). After electrophoresis membrane was treated with Elk-1 primary antibody and images were analyzed (Figure 4.13.a upper panel). Unexpectedly, Elk-1 was not seen in IP of proteins of 417A transfected cells, and also endogenous Elk-1 could not be observed with the antibody used (Figure 4.13.a upper panel 4th and 6th lanes). In addition to this, additional Elk-1 bands were seen at 70 kDa and 100 kDa, which could correspond to SUMOylated isoforms of Elk-1 under these conditions. After that membrane was stripped by using mild strip buffer and then the membrane was incubated with dynein primary antibody, however no dynein bands were observed in the blots (Figure 4.13.a lower panel). This problem could have been

due to the striping protocol, however, since both dynein and Elk-1 primary antibodies were obtained from the same organism.

Western blot of total protein lysates with Elk-1 antibody showed that there were different bands of Elk-1 from the total proteins of 383A, 389A, and wild-type Elk-1 transfected cells, while there was no band from total proteins of 417A and empty-plasmid transfected cells (Figure 4.13.b upper panel 4th, 6th lanes). Western blot with dynein antibody also gave unexpected results. Generally, proteins from 383A, 389A, wild-type Elk-1, and empty-plasmid transfected cells showed a similar pattern to proteins from 1% FBS condition (Figure 4.13.b mid panel). But 417A transfected cells showed a band at 70 kDa (Figure 4.13.b. mid-panel 4th lane). Also, 389A and wild-type Elk-1 transfected cells had a thicker second band at 70 kDa (Figure 4.13.b mid panel).

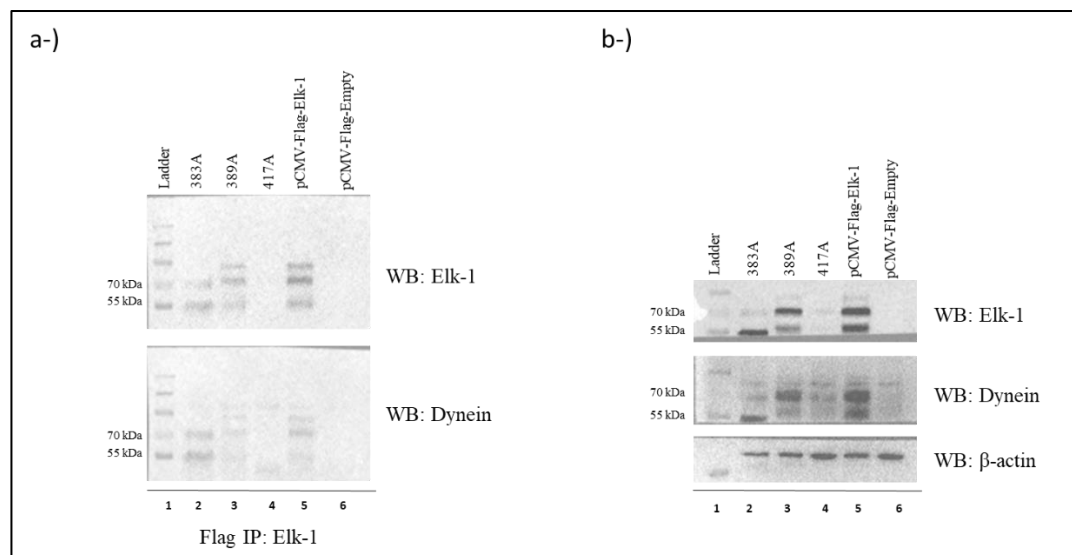


Figure 4.13: The interaction of wild type and phospho-mutants of Elk-1 with dynein at 10% FBS condition. Western blot analysis of a-) Flag-IP and b-) total protein lysate.

Western blot of immunoprecipitated protein samples and total protein samples from cells grown at 20% FBS condition showed similar results to serum-starvation (1% FBS) and normal growth medium condition (10% FBS). As a result of the Flag-IP of protein samples isolated from 383A transfected cells, 2 bands of Elk-1 were seen around 70 kDa and 55 kDa (Figure 4.14.a upper panel 2nd lane). It was also observed that cells transfected with 389A and wild-type Elk-1 gave two thick bands around 100 kDa and 70 kDa and a weak band around 55 kDa (Figure 4.14.a upper panel 3rd, 5th

lanes). However, Elk-1 bands were not seen at 417A and empty-plasmid transfected cells and non-transfected cells (Figure 4.14. upper panel 4th, 6th, 8th lanes). Flag-IP membrane was treated with dynein antibody and a non-specific band around 100 kDa was observed in all lanes (Figure 4.14.a lower panel) except the last lane. It was thought that this band could be arisen from Flag-IP because it was also observed for the protein sample from non-transfected cells incubated with Flag bead (Figure 4.14.a lower panel 7th lane). The total protein sample in the 8th lane was loaded to show the exact place of dynein in the gel and sure that the antibody works correctly.

Interestingly, when the Western blot of total protein samples was examined, neither the band around 70 kDa seen in the IP result of 383A transfected cells nor the band around 100 kDa observed in the IP results of 389A and wild type Elk-1 transfected cells were observed (Figure 4.14.b upper panel 2nd, 3rd, and 5th lanes). When the membrane was incubated with dynein antibody, two bands around 70 kDa and a band around 55 kDa were observed for 389A and wild-type Elk-1 transfected cells (Figure 4.14.b mid-panel 3rd, 5th lanes). One band was observed around 70 kDa for 383A, 417A, and empty-plasmid transfected cells (Figure 4.14.b mid-panel 2nd, 4th, and 6th lanes). In addition to this band, also a band around 55 kDa was observed for 383A transfected cells (Figure 4.14.b mid-panel 2nd lane). Also, one weak band around 70 kDa was observed for the non-transfected cells (Figure 4.14.b mid-panel 7th lane).

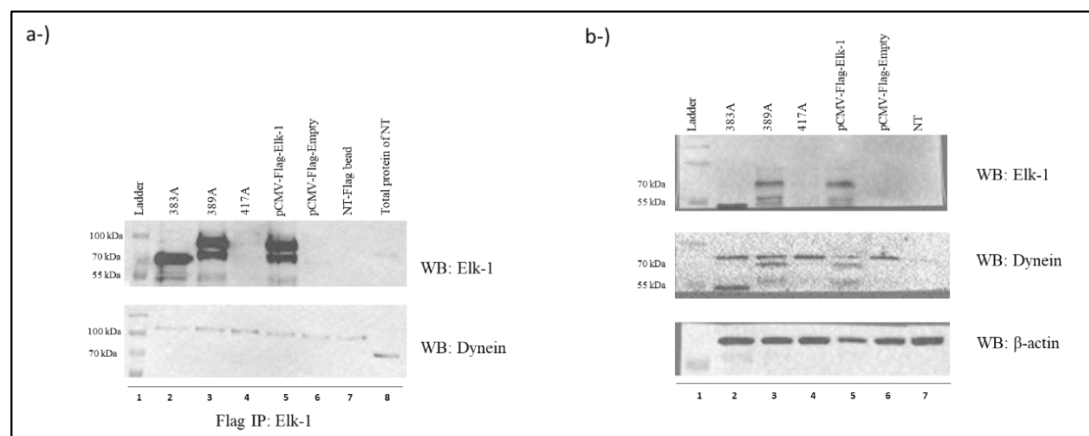


Figure 4.14: The interaction of wild type and phospho-mutants of Elk-1 with dynein at 20% FBS condition. Western blot analysis of a-) Flag-IP and b-) total protein lysate.

4.6. Immunofluorescence (IF) Analysis

In addition to interaction with dynein, Elk-1 wildtype and phosphorylation mutants were also studied with respect to their localizations. To that end, immunofluorescent analysis studies were performed to show the colocalization of Elk-1 and dynein. Firstly, U87 glioblastoma cells were transfected with different Elk-1 phospho-mutants and then treated with the total Elk-1 (or 383S-Elk-1 or phospho-mutant-Elk-1), dynein, tubulin antibodies, and DAPI for the nucleus staining. At the first set, ice-cold 100% methanol was used for the fixation, but at this set staining procedure was not good (data not shown). So, we decided to progress step by step and started with the changing of fixation method and optimization of the concentrations of the primer antibodies. Non-transfected cells were fixed with 4% paraformaldehyde (PFA) and stained with different concentrations of dynein (Figure 4.15) and tubulin (Figure 4.16) antibodies. But in those optimizations, secondary antibodies gifted by Medipol University were used because that was thought that our secondary antibodies could be problematic. According to optimization trials, that was decided that dynein antibody and tubulin antibody will use at 1:75 and 1:50 concentrations, respectively.

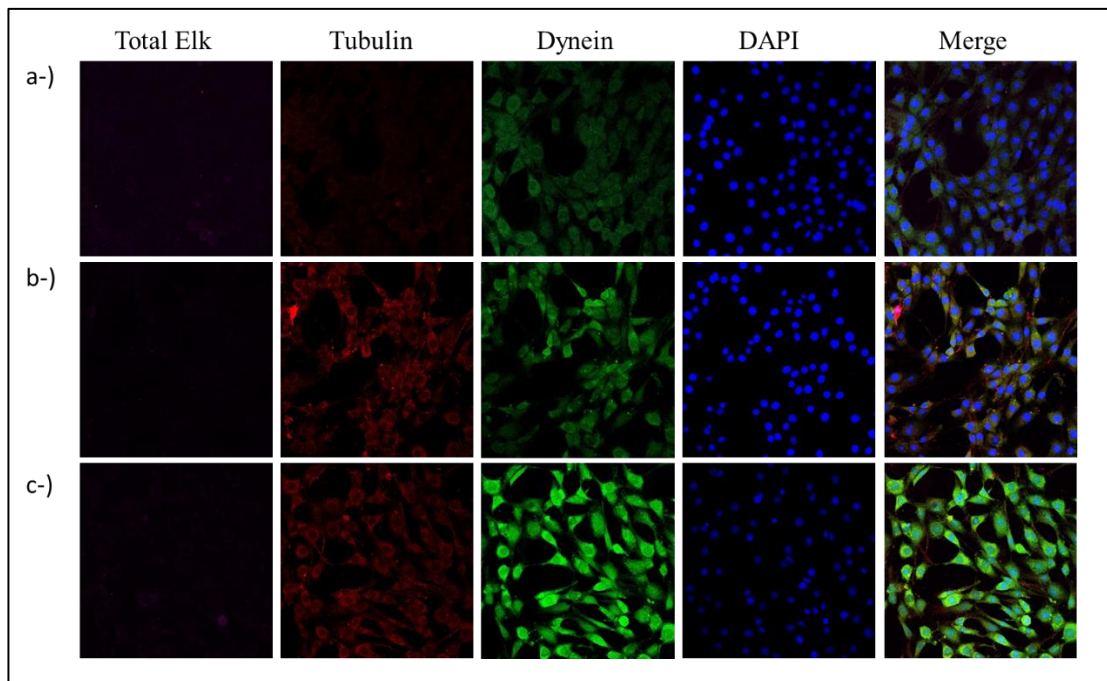


Figure 4.15: Dynein primer antibody concentration trials. Antibody concentrations at a-) 1:50, b-) 1:75 and c-) 1:100 ratios. At all trials total Elk-1 (1:100) and tubulin (1:50) antibody concentrations are constant.

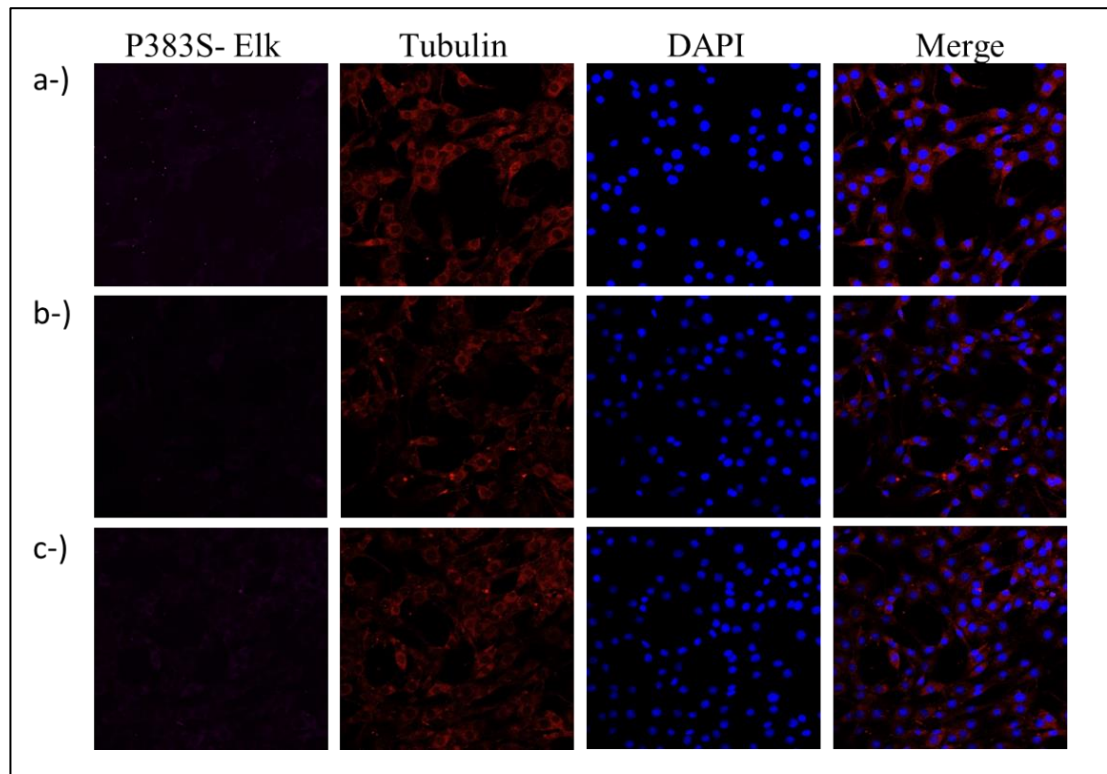


Figure 4.16: Tubulin primer antibody concentration trials. Antibody concentrations at a-) 1:50, b-) 1:100 and c-) 1:200 ratios. At all trials P383S-Elk-1 (1:100) ab concentration is constant.

After these optimizations, the experiments continued with the secondary antibodies of our lab. U87 cells were transfected with 303A, 304A, 324A, and 326A phospho-mutants of Elk-1. And cells were fixed with 4% PFA and treated with primer and then secondary antibodies. Cells were visualized by Confocal Microscopy and images were analyzed by using Zen Blue and Fiji programs. In all figures, green shows dynein, magenta shows Elk-1 (total, 383S or phospho-mutant), red shows tubulin, and blue shows DAPI.

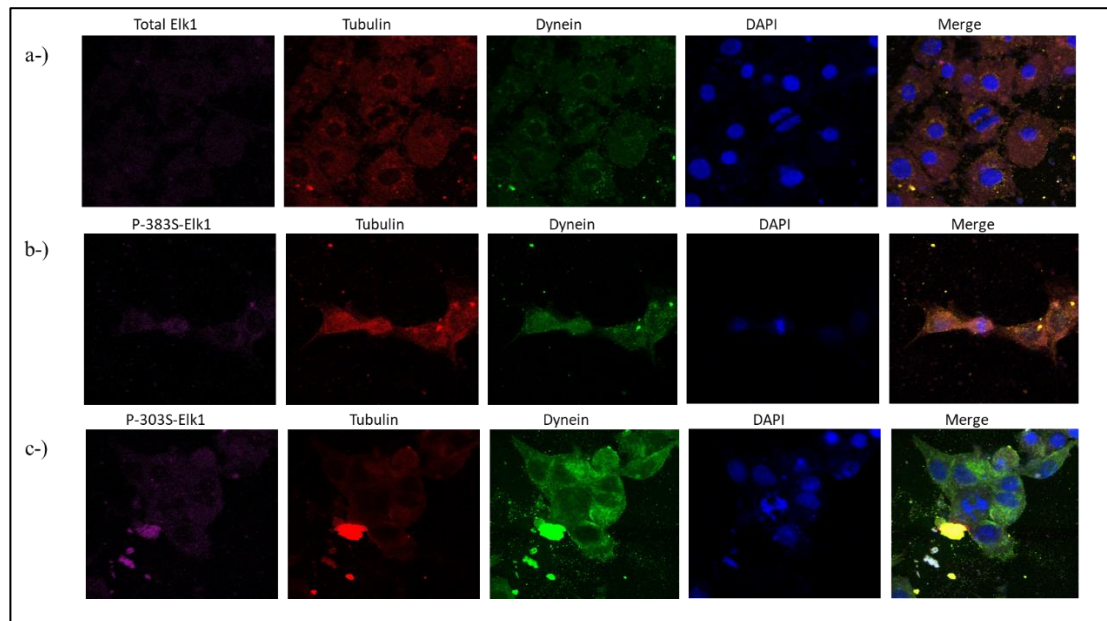


Figure 4.17: Immunofluorescent analysis for colocalization of dynein motor protein with Elk-1 in Elk-1 (303A) transfected U-87 cell lines. Staining with anti-tubulin (red), anti-dynein (green), DAPI (blue) and, a-) anti-total Elk-1 (magenta), b-) P-383S Elk-1 (magenta) or c-) P-303S Elk-1 (magenta) antibodies.

S303A-Elk-1 phospho-mutant transfected cells were visualized under the confocal microscopy. Cells in metaphase (middle cells of the square of Figure 4.17.a and Figure 4.17.c) and prophase (middle cell of the square of Figure 4.17.b) were observed. But neither Elk-1 nor P-383S-Elk-1 nor P-303S-Elk-1 could not be observed clearly (first squares of Figure 4.17). Likewise, tubulin and dynein staining were also not good (red and green of Figure 4.17). So colocalization of Elk-1 and dynein could not be showed.

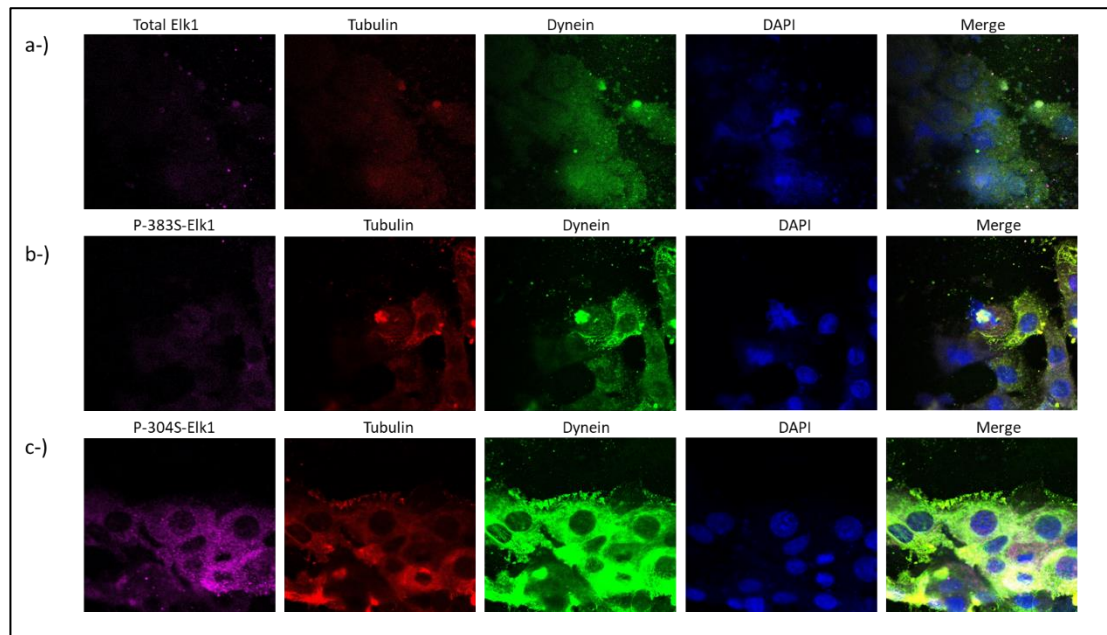


Figure 4.18: Immunofluorescent analysis for colocalization of dynein motor protein with Elk-1 in Elk-1(304A) transfected U-87 cell lines. Staining with anti-tubulin (red), anti-dynein (green), DAPI (blue) and, a-) anti-total Elk-1 (magenta), b-) P-383S Elk-1 (magenta) or c-) P-304S Elk-1 (magenta) antibodies.

S304A-Elk-1 phospho-mutant transfected cells were visualized. Cells were in metaphase (Figure 4.18.a-b) and in prophase (Figure 4.18.c). These cells were treated with total-Elk-1 (Figure 4.18.a), P-383S-Elk-1 (Figure 4.18.b) and P-304S-Elk-1 (Figure 4.18.c) antibodies. Total Elk-1 and P-383S-Elk-1 could not be visualized (First squares of Figure 4.18.a-b, respectively), although cells were transfected with 304A phospho-mutant of Elk-1, phospho-304S-Elk-1 was observed. However, dynein and tubulin could not be shown correctly because staining could not be performed properly. Also, there was a blur in the background in the staining (Figure 4.18.a-b).

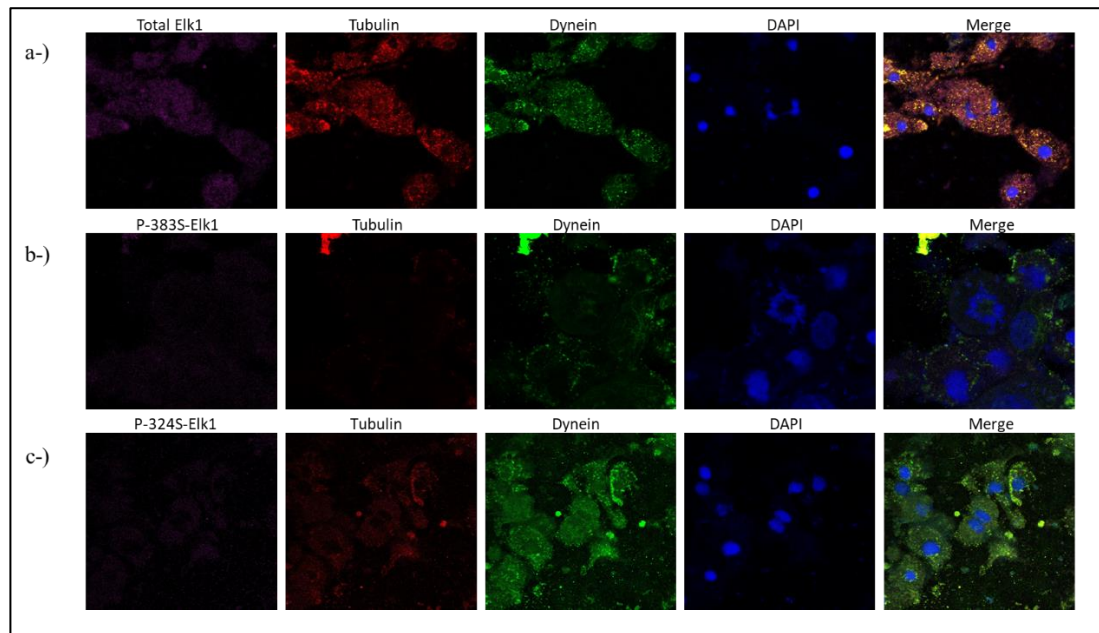


Figure 4.19: Immunofluorescent analysis for colocalization of dynein motor protein with Elk-1 in Elk-1(324A) transfected U-87 cell lines. Staining with anti-tubulin (red), anti-dynein (green), DAPI (blue) and, a-) anti-total Elk-1 (magenta), b-) P-383S Elk-1 (magenta) or c-) P-324S Elk-1 (magenta) antibodies.

In cells transfected with S324A phospho-mutant of Elk-1, while total Elk-1 was visualized very faintly (First square of Figure 4.19.a), phospho-383S and phospho-324S could not be visualized (First squares of Figure 4.19.b-c, respectively). Cells in anaphase (Figure 4.19.a-b) and metaphase (Figure 4.19.c) were monitored. Tubulin staining and dynein staining were also problematic again (Figure 4.19.b-c).

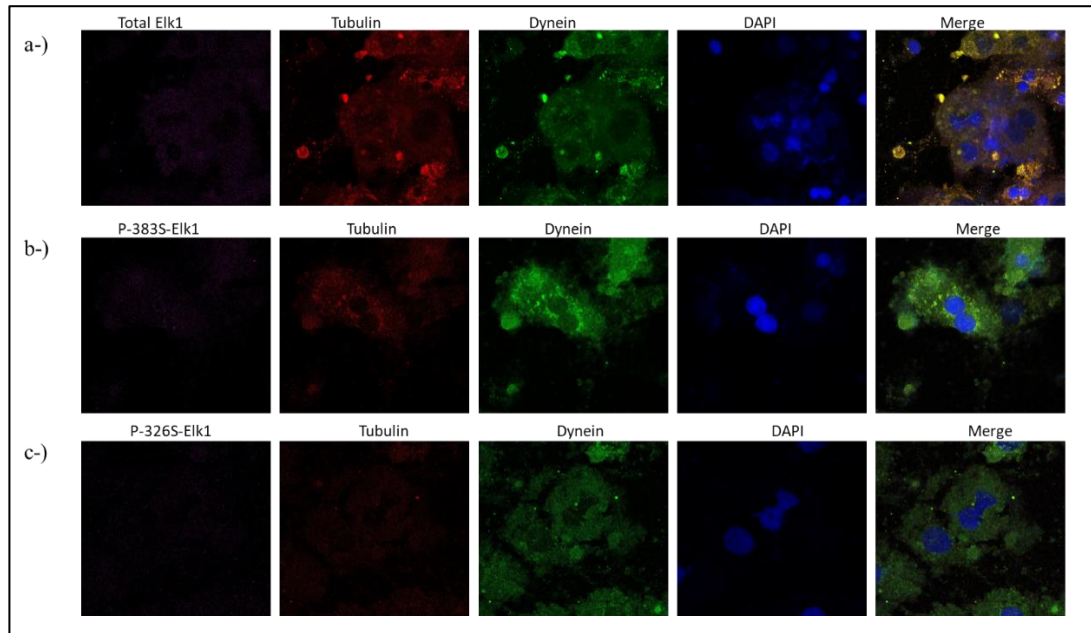


Figure 4.20: Immunofluorescent analysis for colocalization of dynein motor protein with Elk-1 in Elk-1(326A) transfected U-87 cell lines. Staining with anti-tubulin (red), anti-dynein (green), DAPI (blue) and, a-) anti-total Elk-1 (magenta), b-) P-383S Elk-1 (magenta) or c-) P-326S Elk-1 (magenta) antibodies.

In cells transfected with S326A phospho-mutant of Elk-1, neither total Elk-1 nor, phospho-383S-Elk-1 nor phospho-326S-Elk-1 could not be visualized (First squares of Figure 4.20.a, b, c, respectively). Tubulin staining and dynein staining were again unsuccessful as in previous sets (Second and third squares of Figure 4.20.a, b, c). Cells were visualized in anaphase (Figure 4.20.a, c) and prophase (Figure 4.20.b).

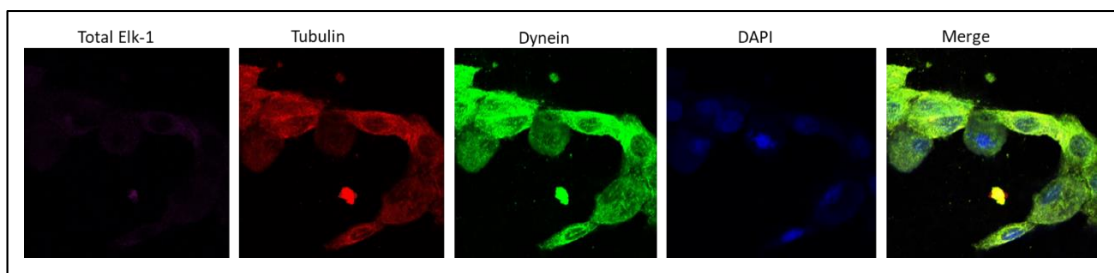


Figure 4.21: Immunofluorescent analysis for colocalization of dynein motor protein with Elk-1 in non-transfected U-87 cell lines. Staining with anti-tubulin (red), anti-dynein (green), DAPI (blue), and anti-total Elk-1 (magenta) antibodies.

Non-transfected U-87 cells were visualized under confocal microscopy as a control of transfection. The cell in interphase was observed (Figure 4.21). In this staining, total-Elk-1 could not be visualized (First square of Figure 4.21). Abnormal

signals were monitored for tubulin and dynein (Second and third squares of Figure 4.21, respectively).

4.7. Protein Expression Analysis of Wild-Type Elk-1 and Elk-1 Phospho-mutants in SK-N-BE (2) Cells

To show the Elk-1 expression of SK-N-BE (2) cells, they were transfected with pCMV-Flag containing wild-type Elk-1, phospho-null and phosphomimic mutants of Elk-1. Proteins were analyzed using western blot. The membrane was treated with mouse anti-Elk-1 antibody (Figure 4.22.a upper panel) and then the membrane was striped for mouse anti-Flag treatment (Figure 4.22.a mid-panel). After that western blot was performed, the membrane was treated with rabbit anti-Elk-1 (Figure 4.22.b upper panel) and no bands below 70 kDa were observed.

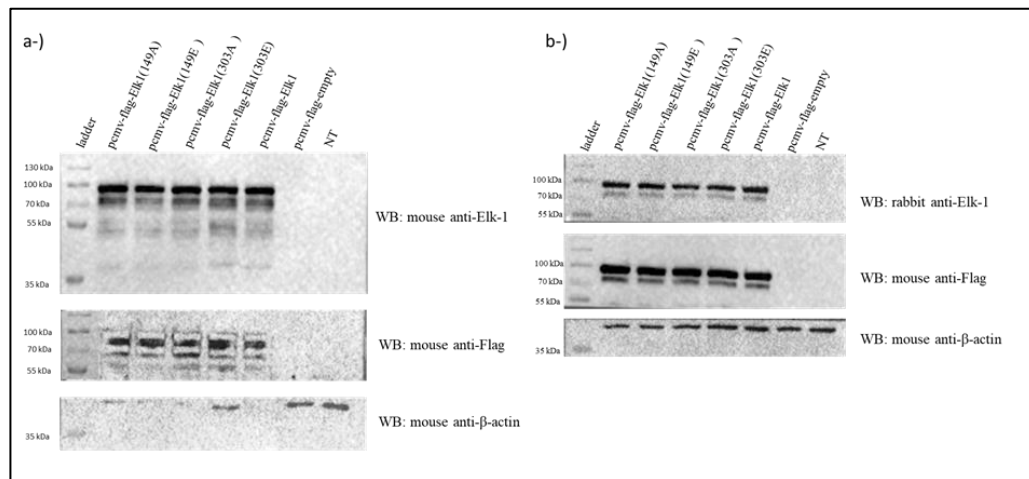


Figure 4.22: Western blot analysis of total protein lysate of non-transfected SK-N-BE (2) cells, and wild type Elk-1, Elk-1 phospho-mutants and empty plasmid transfected SK-N-BE (2). a-) WB analysis with primer antibodies of mouse anti-Elk-1, mouse anti-Flag, and mouse anti-β-actin, b-) WB analysis with primer antibodies of rabbit anti-Elk-1, mouse anti-Flag, and mouse anti-β-actin.

5.DISCUSSION

The main aim of the thesis was to analyze the interaction and co-localization of various phosphorylation mutants of Elk-1 with dynein, in order to provide molecular mechanism of serum-dependent interaction of Elk-1 and dynein [Demir et al., 2012]. Unfortunately, technical problems have proven a challenge in this endeavour. Since the results of the split-EGFP cloning PCRs were not reliable, a few parameters had been checked such as cDNA quality, expression levels of *DYNC1I1*, *DYNC1I2*, *DYNC1LI2*, *DYNC2LI1*, *SRF* and *ERK1* in SH-SY5Y cell line, and Tm's of primers. Additionally, expression profiles of these genes were checked from The Human Protein Atlas database (version 19.3) to confirm whether they were indeed expressed in the cell lines used in our study, which were found to be expressed in the SH-SY5Y cell line (Appendix A). To check the cDNA quality, PCR for *GAPDH* housekeeping gene was done by using this cDNA as a template (*GAPDH* primers were gifted from A. Giangrande). As a result, cDNA was found to be trouble-free (data not shown).

Since the designed primer sizes are longer than normal and their Tm's are very high, PCRs were done by adding DMSO into reaction mixtures. Generally, 4% DMSO was used but because of the hairpin Tm's were very high, 10% DMSO was added into *SRF-N* and *SRF-C* PCR mixes. After the gel electrophoresis, the PCR bands were seen at lower position than they should have been (the expected position was 1527 bp) (Figure 4.3). These bands were seen at similar place when PCR was done by using cDNA or plasmid as a template (Figure 4.4 and Figure 4.5). This band may belong to a new *SRF* alternative variant. To clarify this matter, this band should be isolated and sent for sequence analysis.

The gradient PCRs of *DYNC1I1-N* and *DYNC1I1-C* were also failed, even when they were tried with 4% DMSO and the gradient temperatures were decreased. The length of the gene cannot be a problem because the *taq* DNA polymerase was used for these PCRs and it had previously amplified a gene longer than this gene (*DYNC1I2-N* *DYNC1I2-C*). But the primer blend in the cDNA kit (Bio-Rad iScript™ cDNA Synthesis Kit, #1708891) was used is optimized for targets below 1000 base pairs in length. Probably, it is the reason of PCR problem.

Genes whose PCR results gave bands at the expected place on the gel were amplified with *pfu* DNA polymerase enzyme for cloning. Unfortunately, this time low yield challenge welcomed us. To overcome this challenge, elution water was preheated

to 70 °C and columns were incubated at 70 °C for 5 min after the adding of elution water. After that cloning of *ELK-1-L158P* was tried. Different cloning protocols were tried, different T4 DNA ligase enzymes and buffers were used (data not shown). Considering that the problem may arise from competent cells, the transformation step was also tried with fresh competent cells. Activities of restriction enzymes were controlled by restriction trials and all of them were seen as unproblematic. Single cut ligation was also tried but again it was failed. The cloning problem has not been overcome. Finally, it was thought that there might be problems with cuts of restriction enzymes. They can cause loss of nucleotides at the ends.

Flag-IP was performed to show the interaction of different phospho-mutants and wild-type of Elk-1 with dynein in different serum conditions. In this method, the Elk-1 protein expressed as fused to the Flag protein is precipitated with anti-Flag coated beads. In addition to Elk-1, other proteins that are known to interact with Elk-1 are also expected to precipitate. In this study, Elk-1 proteins were precipitated which were yielded in three different medium conditions and with transfections of different Elk-1 phospho-mutants. However, dynein protein was not observed to precipitate with Elk-1. Since it is thought that the washing and the buffer used may have damaged this interaction, the buffer was refreshed and the washing durations were shortened, but still, dynein could not be shown on IP membranes. The presence of dynein protein in the lysates was proven by direct Western Blot analysis of total protein lysates. In this case, either there was a situation that prevents Elk-1 from binding to dynein, or dynein cannot be precipitated, or a very small amount of dynein was precipitated not enough to visualize with Western Blot analysis. Perhaps the reason dynein was not precipitated as a result of Flag-IP maybe that dynein is a large protein. In future experiments, an IP can be made directly with anti-Elk-1 antibody and a direct examination can be made with dynein antibody, or the lysate precipitated as a result of IP can be sent to mass analysis to obtain more accurate results. Likewise, double-sided verification can be achieved by performing precipitation with anti-dynein and checking the presence of Elk-1 protein in the precipitation lysate.

In addition to these, the Elk-1 protein was neither displayed in IP nor direct Western blot of the protein lysate of the 417A transfected cell. The reason of this was thought to be due to transfection, but since this situation was observed for all three sets, plasmid-related problems were emphasized. As a result of the control digestions with restriction enzymes by Betül Ertural, plasmid accuracy was found to be

problematic. However, to reach a more accurate conclusion, the plasmid should be sent for sequencing.

Besides these, interestingly, the endogenous Elk-1 was not seen in any set. When all the western results were examined, multiple bands at different kDa were seen for different phospho-mutants of Elk-1. It was thought that these bands might be the SUMOylated forms of Elk-1. Because it is known that there are three different SUMOylation sites on Elk-1. To understand whether these bands belong to the SUMOylated forms of Elk-1, membranes were examined with SUMO antibody, but no definite result could be obtained because the preliminary experiments did not yield reliable results (data not shown).

Also, to determine whether these bands were non-specific, proteins of cells transfected at 20% FBS condition, were loaded into SDS-PAGE, and membranes were incubated with rabbit anti-Elk-1 and mouse anti-Flag antibody (Appendix B). As a result of these analyzes, no Elk-1 band was observed below 62 kDa.

In Immunofluorescent analysis, in the first set of staining, ice-cold 100% methanol has been used for fixation of the cells, but clear images were not taken from this set. Therefore, decided to continue with optimization studies. As a result of these optimization studies, optimal concentrations of tubulin and dynein antibodies were defined. The secondary antibodies used for optimization studies have been gifted from Medipol University, and better images were taken but continued with the secondary antibodies of our lab because of financial problems. Unfortunately, clear images could not be taken for further sets of staining, the image was blurred. The reason for this blurring could be the fixation method, the scrolling while treating with mounting medium, or the image acquisition method. Because antibodies used were considerably old, accurate images of interactions could not be observed.

In particular, it was not possible to visualize total-Elk-1 and P389-S-Elk-1 in these studies, which could be due to antibody storage and shelf-life. Also, although cells were transfected with the phospho-mutants of Elk-1, the P304-S-Elk-1 antibody gave signal in the cells were transfected with 304A phospho-mutant of Elk-1. The reason of this could be the failure of transfection or the 304S antibody could take a false signal from tubulin or (merging of the signal taken from) dynein antibodies. Because of so many proteins were investigated, transfection control could not be performed for every transfected cell. Transfection control could be achieved by using anti-Flag antibody but again it could be hard because of the combination of wavelength

of signals of antibodies. Tubulin staining was generally problematic, this could have arisen due to the fixation method, and PFA fixation can cause the damage of tubulin structure [Robinson and Snyder, 2004]. Also, shelf-life of this antibody can be another reason of this problem. Previous studies in our lab have not shown the direct colocalization of dynein at the microtubules, it was shown that dynein was dispersed throughout the cell [Demir et al., 2012]. Unlike previous studies, although the fixation method has been changed, dynein could not be shown as colocalized to microtubules. Since tubulin stainings were not clear in our studies, it could not be determined whether dynein localized to microtubules.

Dynein can be localized in three different positions through the cell division: around the spindle poles, on kinetochore, and on astral spindles. Kinetochore localization is depending on the special phosphorylation of dynein (PT89) so that in future studies, anti-PT89 antibody can be used for the dynein localization on the kinetochores. In addition, Split-EGFP complementation assay could be repeated after cloning problems are sorted out with new primers, new cDNA, or new templates.

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BIOGRAPHY

Büşra Nur Çiçek was born in Bursa, 1995. She achieved her bachelor's degree from Department of Molecular Biology and Genetics, Gebze Technical University in 2017. She has continued Master of Science program in the Department of Molecular Biology and Genetics at Molecular Neurobiology Laboratory, Graduate School of Natural and Applied Sciences, Gebze Technical University, since 2017 fall. She is also employed at Gebze Technical University as Research Assistant. She has presented a poster in Gebze Technical University Graduate Studies Symposium and Introductory Days 2019 named 'The Interaction of Elk-1 with Minus-End Directed Dynein Motor Protein'.

APPENDICES

Appendix A: Expression Levels of Genes

Expression levels of *DYNC1I1*, *DYNC1I2*, *DYNC1LI2*, *DYNC2LI1*, *SRF* and *ERK1* in SH-SY5Y cell line by using Human Protein Atlas Database.

NX annotated the normalized expression of this gene. And this is the maximum expression value of this gene from three different databases.

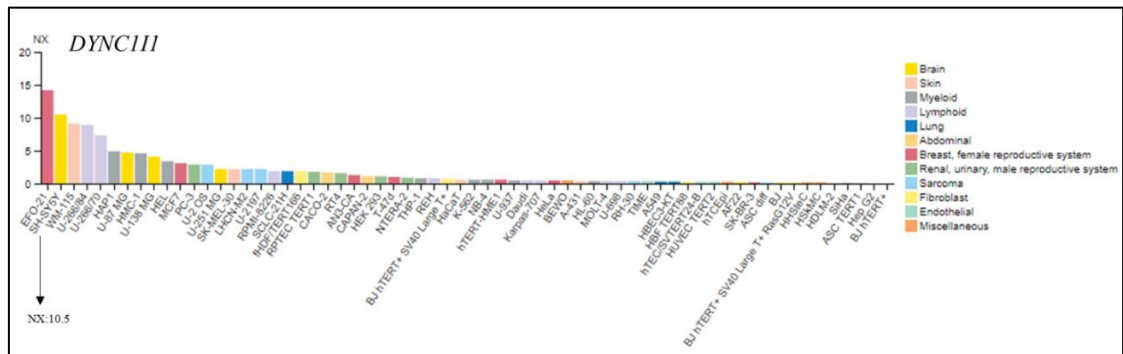


Figure A1.1: Expression level of *DYNC1I1* in SH-SY5Y cell line.

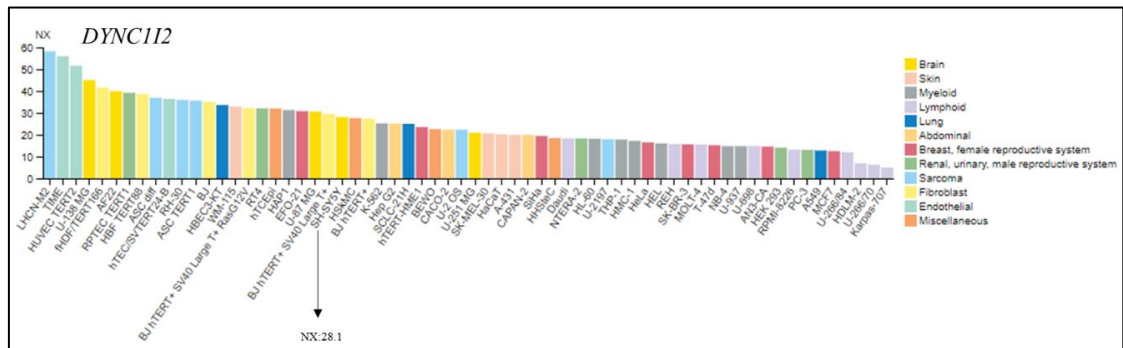


Figure A1.2: Expression level of *DYNC1I2*.

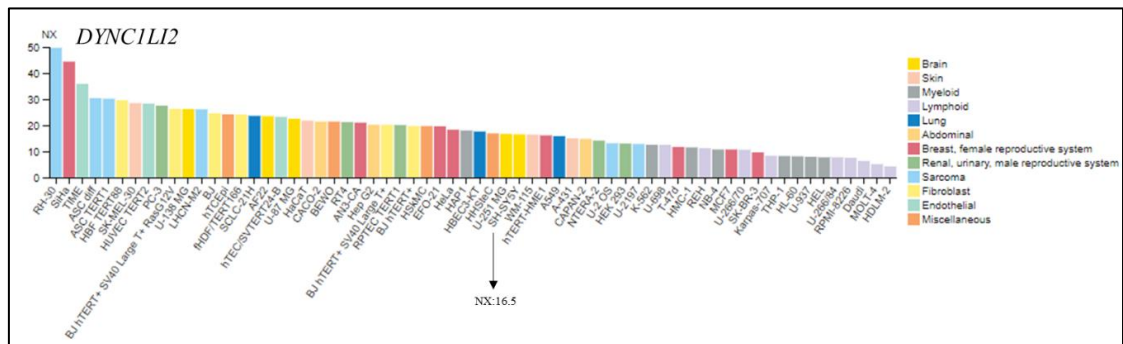


Figure A1.3: Expression level of *DYNC1LI2*.

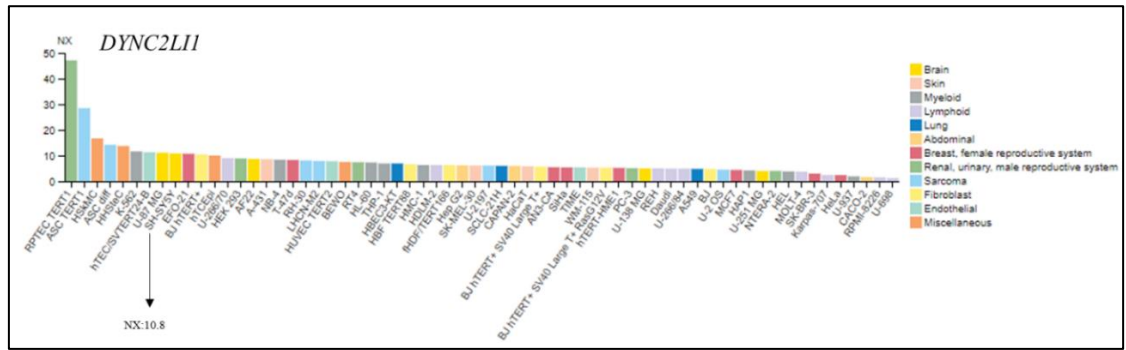


Figure A1.4: Expression level of *DYNC2LI1*.

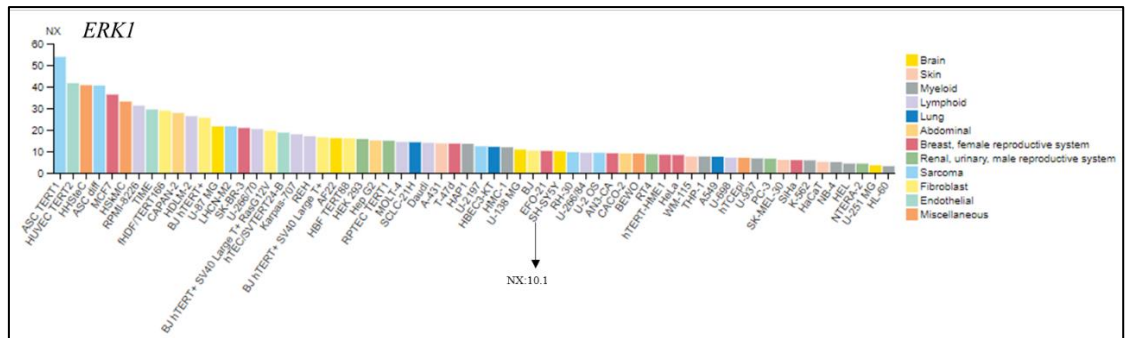


Figure A1.5: Expression level of *ERK1*.

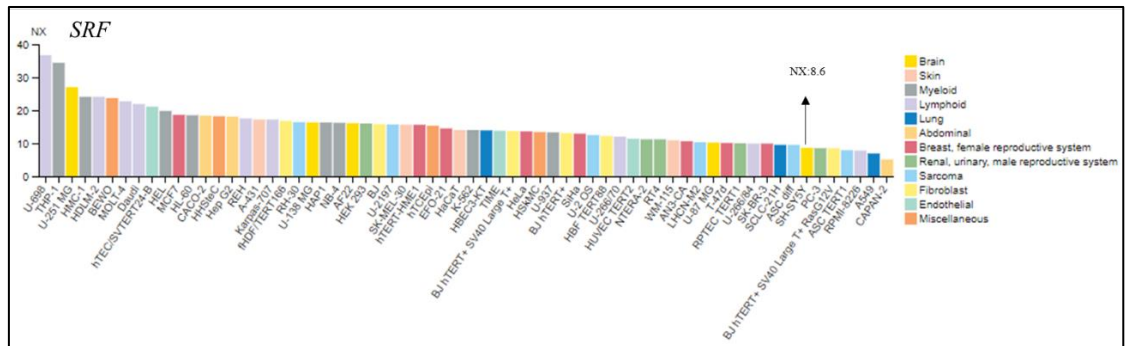


Figure A1.6: Expression level of *SRF*.

Appendix B: Western Blot Analysis of Total Protein of SH-SY5Y cell

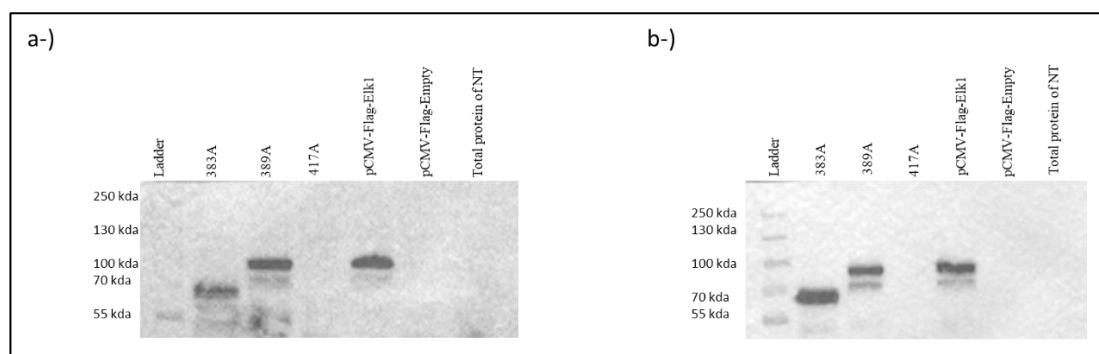


Figure B1.1: Western blot analysis of total protein of non-transfected and transfected with wild type and phospho-mutants of Elk1 and empty vector of SH-SY5Y cells a-) with rabbit anti-Elk1 antibody, b-) with mouse anti-Flag antibody.