THE INTERACTION BETWEEN ELK-1 AND MITOTIC KINASES IN BRAIN TUMORS

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ABSTRACT

THE INTERACTION BETWEEN ELK-1 AND MITOTIC KINASES IN BRAIN TUMORS

Ets-like transcription factor 1 (Elk-1) is a member of E twenty-six (ETS) oncogene superfamily of transcription factors which is composed of nuclear phospho-proteins that have important roles in development and disease. Activation of Elk-1 requires phosphorylation in particular Serine 383, Serine 389 and Threonine 417 residues within the activation domain by mitogen-activated protein kinase (MAPK) pathway in regulation of immediate early genes upon mitogenic stimulation, as well as PI3K/AKT and Protein Kinase C (PKC) pathways. Elk-1 has been implicated in protecting cells from apoptosis and downregulating apoptosis-associated genes, thereby mediating cell survival. Furthermore, Akt-dependent phosphorylation of Elk-1 has been shown to be important for proliferation of glioblastoma cells. Recently Elk-1 was shown to also interact with mitotic kinase Aurora-A in a mitotic phase-dependent manner in brain tumor cell lines, and that Serine 383 phosphorylation was important for its mitotic spindle localization. Furthermore, in a Polo like kinase (Plk) phospho-proteome analysis, novel phosphoforms of Elk-1 were identified. Yet it was not clear whether interaction of Elk-1 with Aurora-A resulted in its phosphorylation, or whether it was involved with other mitotic kinases. In this study we showed for the first time that Elk-1 indeed could interact with not only mitotic kinase Aurora-A but also Aurora-B, Polo-like kinase Plk-1 and cyclin dependent kinase 1 (Cdk1) through the N-terminal amino acids 93-205 of Elk-1. It was also defined for the first time novel phosphorylations of Elk-1 and showed that mutagenesis of these residues interferes with not only its interaction with mitotic kinases, but also mitotic profile of brain tumor cell lines.

ÖZET

BEYİN TÜMÖRLERİNDE ELK-1 VE MİTOTİK KİNAZLAR ARASI ETKİLEŞİM

Ets-benzeri transkripsiyon faktörü 1 (Elk-1), gelişme ve hastalıkta önemli rolleri olan nükleer fosfo proteinlerinden oluşan transkripsiyon faktörlerinin E yirmi-altı (ETS) onkojen süper ailesinin bir üyesidir. Elk-1'in aktivasyonu, aktivasyon bölgesi içerisindeki özellikle Serin 383, Serin 389 ve Treonin 417 bölgelerinin mitojen uyarısının varlığında erken cevap genlerinin düzenlenmesi için mitojen-aktive edilmiş protein kinaz (MAPK) yolağının yanı sıra PI3K / AKT ve Protein kinaz C yolakları tarafından fosforilasyonunu gerektirir. Elk-1, hücrelerin apoptozdan korunmasında ve apoptoza bağlı genlerin aşağı doğru düzenlenmesinde, dolayısıyla hücre sağ kalımına aracılık etmede rol oynamaktadır. Ayrıca, Elk-1'in Akt-bağımlı fosforlanması, gliyoblastoma hücrelerinin çoğalması için önemli olduğu gösterilmiştir. Son zamanlarda Elk-1'in mitotik kinaz Aurora-A ile mitotik faz-bağımlı bir şekilde beyin tümörü hücre hatlarında etkileştiği gösterilmiştir ve Serine 383 fosforlanmasının mitotik iğ lokalizasyonu için önemli olduğu bilinmektedir. Ayrıca, Plk fosfo-proteom analizinde Elk-1'in yeni fosfor formları tanımlanmıştır. Yine de Elk-1'in Aurora-A ile etkileşiminin Elk-1 fosforlanmasıyla mı, yoksa başka mitotik kinazlarla mı ilişkili olduğu bugüne kadar bilinmiyordu. Bu çalışmada ilk kez Elk-1'in sadece mitotik kinaz Aurora-A ile değil, aynı zamanda Aurora-B, Polo-benzeri kinaz Plk-1 ve siklin bağımlı kinaz Cdk1 ile N-terminal amino asitleri üzerindeki 93-205 amino asitler yoluyla etkileşime girebileceğini gösterdik. Aynı zamanda Elk-1'in yeni fosforilasyonlarını tanımlanmış ve bu bölgelerin mutajenezinin sadece mitotik kinazlarla etkileşimini değil, aynı zamanda beyin tümör hücre dizilerinin mitotik profilini de etkilediği gösterilmiştir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

µl	Microliters
AD	Activation domain
ALS	Amyotrophic lateral sclerosis
AMP	Ampicillin
APC	Anaphase promoting complex
APP	Amyloid precursor protein
APS	Ammonium persulfate
ATCC	American type culture collection
ATP	Adenosine triphosphate
A172	Human glioblastoma cell line
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
BUB1	Budding uninhibited by benzimidazoles 1
C6	Rat glioblastoma cell line
$CaCl_2$	Sodium chloride
CAK	CDK activating kinase
CBP	CREB binding protein
CDK1	Cyclin dependent kinase 1
CENP-E	Centromere protein E
CPC	Chromosome passenger complex
CST	Cell signaling technology
DC	Cytoplasmic dynein
DEJL	Docking site for ERK and JNK LXL
DHC	Dynein heavy chain
DIC	Dynein intermediate chain
DLC	Dynein light chain
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DSK	Dual specifity kinase

DTT	Dithiothreitol
E26	E-twenty-six
EDTA	Ethylenediaminetetraacetic acid
EGR-1	Early growth response gene-1
ELG	ETS like gene
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
EtOH	Ethanol
ETS	E26 transformation specific
FACS	Fluorescent activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FSC	Forward scatter
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
HAT	Histone acetyl-transferase
HCL	Hydrochloric acid
HDAC	Histone deacetylase
HLH	Helix-loop-helix domain
HRP	Horseradish peroxidase
ID	Inhibitory domain
IDH	Isocitrate dehydrogenase
INCENP	Inner centromere protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IEG	Immediate early genes
JNK	c-Jun N terminal kinase
KDA	Kilodalton
KLD	Kinase-ligase-DpnI
MAP	Microtubule associated protein

MAPK	Mitogen activated protein kinase
MEK	MAPK/ERK kinase
Mg	Magnesium
MKLP	Mitotic kinase-like protein
Ml	Milliliters
mM	Milimolar
MT	Microtubule
MTOC	Microtubule organizing centre
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromidem
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NES	Nuclear export signal
Ng	Nanogram
NIMA	Never in mitosis gene A-related kinase
NLS	Nuclear localization signal
NP-40	Octyl phenoxypolyethoxylethanol
nM	Nanomolar
NUMA	Nuclear mitotic apparatus protein
OD	Optical density
PAK	P21 activating kinase
PB	Polo box
PBD	Polo box domain
PBS	Phosphate buffered saline
PCM	Pericentriolar material
PDEF	Prostate derived ETS transcription factor
PEI	Polyethylenimine
pH	Negative log of hydrogen ion concentration
PI	Propidium iodide
PI3K	Phosphatidyl inositide–3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLK-1	Polo like kinase 1
PP	Protein phosphatase

PTM	Post translational modification
PTP	Permeability transition pore
RB	Retinoblastoma
RD	Repression domain
RFU	Relative fluorescence unit
RIPA	Radio immuno precipitation assay
RNA	Ribonucleic acid
SAC	Spindle assembly checkpoint
SAP	SRF accessory protein
SCBT	Santa Cruz biotechnology
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGK1	Serum- and glucocorticoid-inducible kinase 1
SH-SY5Y	Human neuroblastoma cell line
SMN	Survival of motor neuron
SOD	Superoxide dismutase
SRE	Serum response element
SRF	Serum response factor
SSC	Side scatter
STK	Serine/Threonine kinase
SUMO	Small ubiquitin-related modifier
TCF	Ternary complex factor
TEL	Leukemia-related transcription factor
TEMED	N, N, N′, N′-tetramethylethylenediamine
TK	Threonine kinase
TPA	12-O-tetradecanoylphorbol-13-acetate
T98G	Human glioblastoma cell line
U-87	Human glioblastoma cell line
WHO	World health organization
WST-1	Water soluble tetrazolium salt-1
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazoliumhydroxide

1. INTRODUCTION

Ets-like transcription factor 1 (Elk-1) is classified as a member of ternary complex factor (TCF) transcription factors, a subclass of the E26 transformation specific (ETS) family, that meditates transcription of many target genes, including immediate early genes, especially *c-fos, egr-1* and *mcl-1*, upon phosphorylation by mitogen-activated protein kinase (MAPK) pathway. Activation of Elk-1 mainly requires phosphorylation of Serine 383, Serine 389 and Threonine 417 residues within the activation domain by ERK, JNK or p38 pathways and this activation induces its DNA binding [1].

Since Elk-1 mediates transcription of numerous target genes, it is thus mainly localized to the nucleus [1]. However, it was also identified that Elk-1 has a neuron-specific localization; in the resting state it localizes at the cytoplasm as microtubule-bound [2] which was unexpected for a transcription factor. Upon further detailed analysis it was shown that stimulation of Elk-1 resulted in its localization from microtubules to the nucleus, and to mediate survival of cells by repressing *egr-1* gene [3] or through *mcl-1* gene transcriptional regulation [4] as well as a novel target identified in our laboratory, survival of motor neuron, SMN [5]. In addition to the association with microtubules, Elk-1 was also shown by our laboratory to colocalize and interact with kinesin and dynein microtubule motors in a serum dependent manner and treatment of cells with specific dynein inhibitors, serine 383 phosphorylated Elk-1 showed altered localization [6].

In this and other studies from our laboratory, it was shown that during interphase, Serine 383 phosphorylated Elk-1 was localized at the nucleus, whereas through prophase to metaphase this phosphor form mainly localized to the spindle poles, which was not the case for other transcription factors studied, such as c-fos. In anaphase, P-S383-Elk-1 transitioned from the spindle poles to the midzone, relocating to the spindle midbody in cytokinesis. Serine to alanine mutation of Serine 383 residue did not affect the spindle pole localization of Elk-1, but during telophase this mutant could not translocate to the midzone, instead it remained in the spindle poles throughout the mitotic spindle [7]. It was further demonstrated by our laboratory that Elk-1 co-localized with Aurora-A kinase during mitosis and when cells were treated with specific Aurora inhibitors, Elk-1 could not localize to the poles and remains associated with DNA [7].

Cell division is tightly regulated to prevent improper segregation of the sister chromatids since any error during this process leads to chromosomal instability and aneuploidy and

thereby tumorigenesis. Therefore, to control segregation of chromosomes properly, cells use spindle checkpoint regulation as a surveillance mechanism. Mitotic kinases bears crucial roles during the regulation of cell division such as progression of cell cycle, maturation of centrosome and mediation of microtubule dynamics that are critical for the accurate segregation of the chromosomes [8].

The best studied kinases involved in modulation of mitosis are Aurora kinases, Polo like kinases and Cyclin-dependent kinases (Cdk). Aurora A kinase is a regulatory kinase of centrosome maturation, bipolar mitotic spindle formation, chromosome segregation, alignment of the chromosomes during prometaphase and completion of cytokinesis [8]. In common with Aurora A, Polo like kinases, especially Plk1 is also implicated in bipolar spindle formation, centrosome maturation as well as regulation of kinetochore function [9]. Many of Cdk family members are related to controlling cell cycle and Cdk1 involved in mitotic entry by mediating G_2/M transition. Moreover, Cdk1 is also involved in chromosome condensation, microtubule nucleation and mitotic exit and dysregulation of Cdk1 expression as well as Aurora and Polo like kinases is related with tumorigenesis [10]. All of these mitotic kinases exhibit similar spindle localization as that of Elk-1 during various stages of mitosis, hence it was intriguing to address whether any interactions exist between Elk-1 and these mitotic kinases.

Indeed, investigation of Elk-1 protein sequence in terms of potential kinase phosphorylation motifs revealed that several residues are putative sites for phosphorylation by cell cycle-related kinases, such as Cyclin dependent kinases (Cdks), Polo-like kinases (Plks) and Aurora kinases. Detailed analysis of these potential mitotic kinase phosphorylation sites to help elucidate the possible mitotic role of Elk-1 in brain tumorigenesis is the main theme of this thesis study.

2. THEORETICAL BACKGROUND

2.1. ETS TRANSCRIPTION FACTOR SUPERFAMILY

The E-twenty-six or E26 transformation-specific (ETS) domain transcription factor superfamily is one of the well-known transcription factor families that are known to play critical roles throughout development. Most of the family members are downstream nuclear targets of various signaling pathways, most notably the MAPK and PI3K/Akt pathways, and regulation of ETS family members by these pathways often bring about changes in DNA binding, interaction with partners and transcriptional activities [11].

The ETS superfamily is defined by having an ETS domain comprising 85 amino acids which binds a GGAA/T core sequence and flanking nucleotides to regulate binding of purine-rich DNA sequences [11]. The vast majority of this family members have the ETS domain in their C-terminal, whereas some of the ETS subfamily members such as TCF and ETS2 repressor factor (ERF) have DNA binding domain in N-terminal as shown in Figure 2.1. [11]. Several ETS subfamilies including ETS, ETS-related gene (ERG), ETS like gene (ELG), Epithelium Specific Ets Like Factor (ESE), Leukemia-related transcription factor (TEL) and Prostate Derived ETS Transcription Factor (PDEF) contain a conserved *pointed* domain that functions as a protein-protein interaction motif, while some subfamilies such as TEL, ERF and TCF comprise a repressor domain and a vast majority of the members have transcriptional activation domain (AD) whereas few have repression domain (RD) or both [12]. Sequence conservation of the domains often reflects similar molecular and biological functions of subfamilies [13].

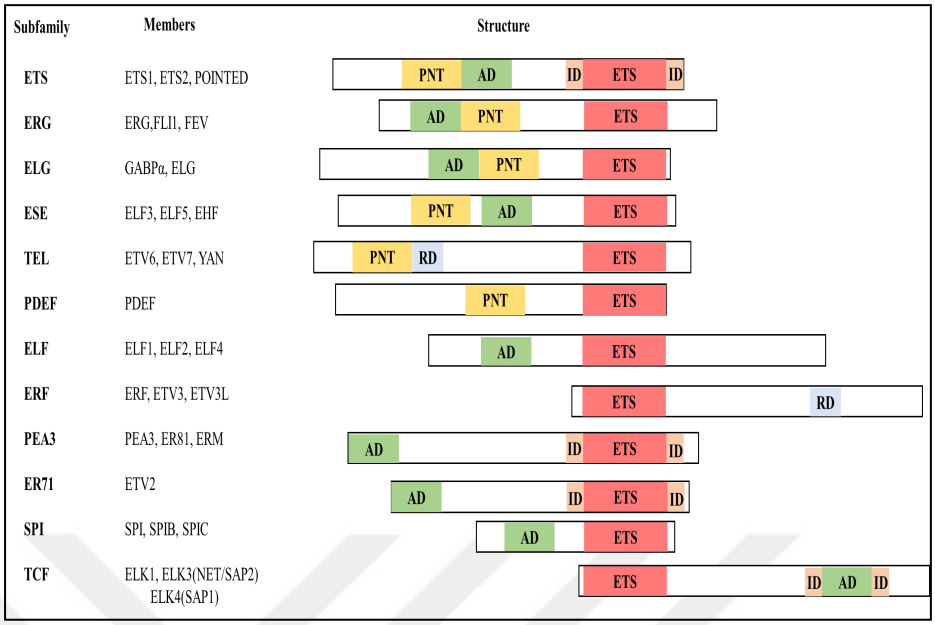


Figure 2.1. Structure of ETS proteins. Ets: Ets domain [12]. ETS: ETS or DNA binding domain, PNT: Pointed domain, AD: Activation domain, ID: Inhibitory domain and RD: repressor domain [13].

The target gene specificity of ETS members is regulated by three mechanisms, DNA binding, protein interactions and post-translational modifications (PTMs). The main PTMs that play vital roles for the functions of ETS family members include phosphorylation, acetylation, SUMOylation, ubiquitinoylation and glycosylation [13]. Phosphorylation is the most important post-translational modification for the modulation of ETS proteins and regulates effects on DNA-binding, transcriptional activity/repression, interaction with other proteins and subcellular localization [14]. Subcellular localization effects biological roles of ETS members, Ternary Complex factor (TCF) subfamily contain a nuclear export sequence which directs them to the nucleus and regulated by mitogen-activated protein kinases (MAPK) [12].

ETS members have a wide range of substrates involved in many biological functions such as proliferation, migration, differentiation, apoptosis, hematopoiesis and oncogenic transformation. Dysregulation of ETS transcription factors by either improper expression or binding with other proteins is believed to be a crucial factor for the generation of numerous types of cancer [11].

2.1.1. Ternary Complex Factor (TCF) Subfamily

Ternary Complex Factor (TCF) transcription factors are the most studied group of ETS superfamily and the first TCF member which was called as p62 was characterized by forming a ternary complex with SRF (Serum Response Factor) [13]. Then, it became clear that this novel molecule was homologues to Elk-1 transcription factor. Two other TCF members were also identified, SRF accessory protein 1(SAP) and SAP-2/ERP/Net which are distinct gene products but they are characterized by forming a ternary complexes on target promoters along with SRF [4].

The TCF members share four structurally and functionally similar conserved regions; A, B, C and D domains. The A or ETS domain is known as the DNA binding domain and it also have a role in transcriptional inhibition of Elk-1 by collecting repressors or DNA-binding inhibitors. The B domain is localized at the downstream of the DNA binding domain, A domain, it is required for the interaction of TCFs with SRF dimer. The C and D domains constitute regulatory part of TCFs; D domain is a docking site for MAPKs and the bound kinases then phosphorylate certain residues in C domain [15].

The TCFs are transcriptionally activated by all three MAPK pathways [15]. Growth factors and mitogens predominantly stimulate the ERK cascade whereas cytokines and stress primarily trigger JNK and p38 cascades as it is shown in Figure 2.2. [16]. Ternary complex factor members, therefore, play vital roles in transducing extracellular stimuli into the nucleus.

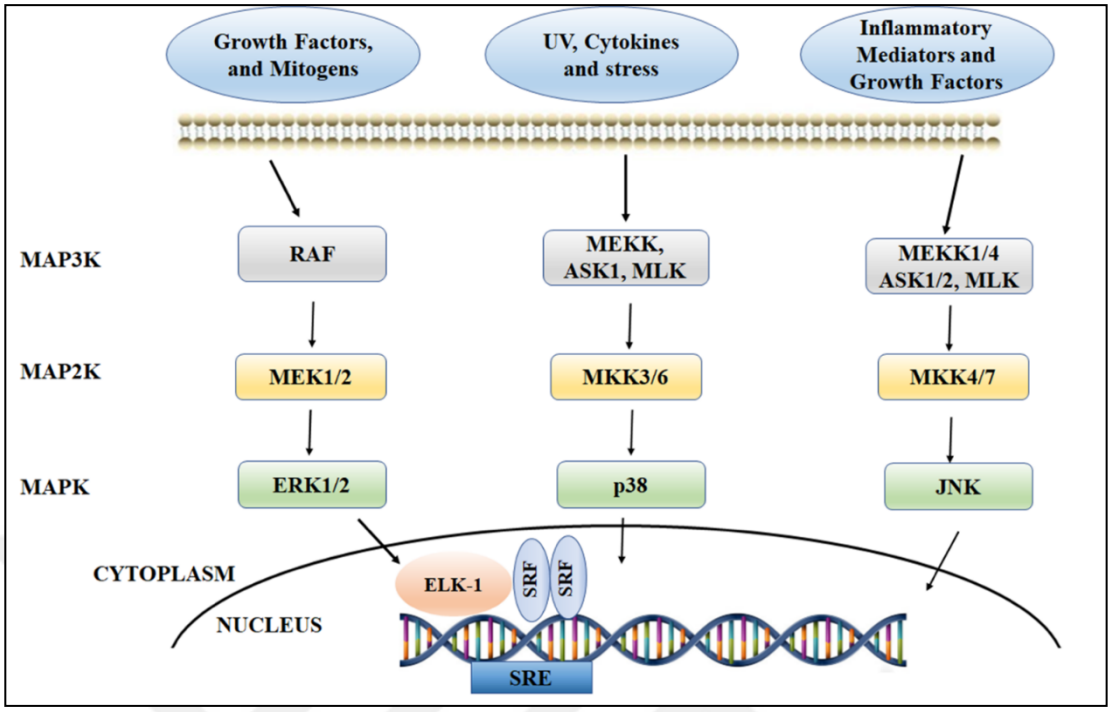


Figure 2.2. Flowchart of MAPK modules [4].

Without MAPK signaling, Net and in some cases Elk1 has been shown to work as a repressor of transcription. In mouse embryonic fibroblast cell line, NIH3T3, all three members of TCF have been shown to act in different roles, Net is a repressor protein, Elk-1is the activator and Sap-1 is in an inactive state [17]. Elk-1 also has repression domains, which recruits mSin3A-HDAC corepressor [18] and the R motif or repression domain suppress the activity both by the recruitment of histone deacetylase complexes and by both by C-terminal activation domain by SUMO modification [19].

2.1.1.1. ELK-1 (Ets-like Transcription Factor)

Elk-1 has different roles in several biological processes including differentiation, cell survival, apoptosis, hematopoiesis, angiogenesis, inflammation, wound healing, cytoskeletal dynamics and cancer [20]. In this section, a detailed picture of this transcription factor will be provided [20].

2.1.1.2. Domain Structure of Elk-1

All TCF family members have four conserved domains: The N-terminal **A domain**, also known as ETS DNA binding domain; Elk-1 also has nuclear import and export signals. **B domain** binds the transcriptional partner SRF [20]. **C domain** is the activation domain which gets phosphorylated by MAPK pathway [21]. Phosphorylation of particularly Serine 383 and Serine 389 residues within this domain has a critical role for the activation of Elk-1. At the end of the C domain, there is a DEF or FXFP motif (docking site for ERK) which is exclusive for ERK and functions as ERK binding site. This motif is responsible for directing particularly serine 383 phosphorylation which plays vital role for Elk-1 function [22].

D domain (DEJL domain) is located upstream of the transactivation domain and functions as a docking site for ERK, JNK and p38 MAPK pathways as shown in Figure 2.3. Recruitment of these kinases leads to the phosphorylation of target residues within the C domain and enhance ternary complex formation with SRF. **R domain** (repression domain) is located between B and D domains and has multiple lysine residues susceptible to be SUMOylated, which was shown to result in transcriptional repression of Elk-1 targets, as will be discussed below [20].

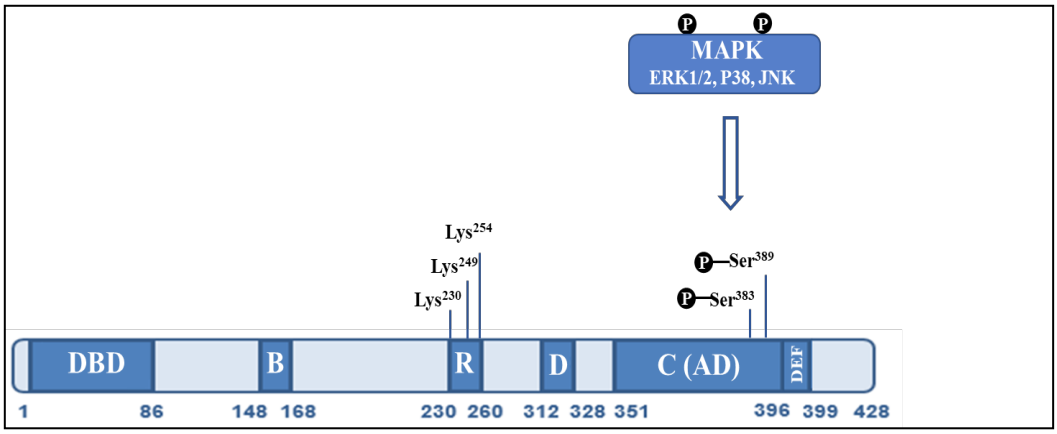


Figure 2.3. Domain structure of Elk1. DBD: DNA binding domain, B: SRF interaction domain, R: repression domain, C (AD): activation domain, D: docking site. DEF: SRF binding site [20].

2.1.1.3. PTMs and Transcriptional Activation of Elk-1

Activation of Elk-1 relies on phosphorylation by three class of MAPKs; ERK, JNK and p38 [23]. In addition to MAPKs, it was shown that Elk-1 can also be activated by Protein Kinase C (PKC) [24] and phosphatidyl inositide–3 kinase (PI3K) pathways. ERK cascade of MAPK signaling is stimulated by growth factors and mitogens, whereas cytokines and stress trigger JNK and p38 cascades. Activated kinases, in turn, phosphorylate Elk-1, in particular from Serine 383 and Serine 389 residues, and initiate its transcriptional activity. [25].

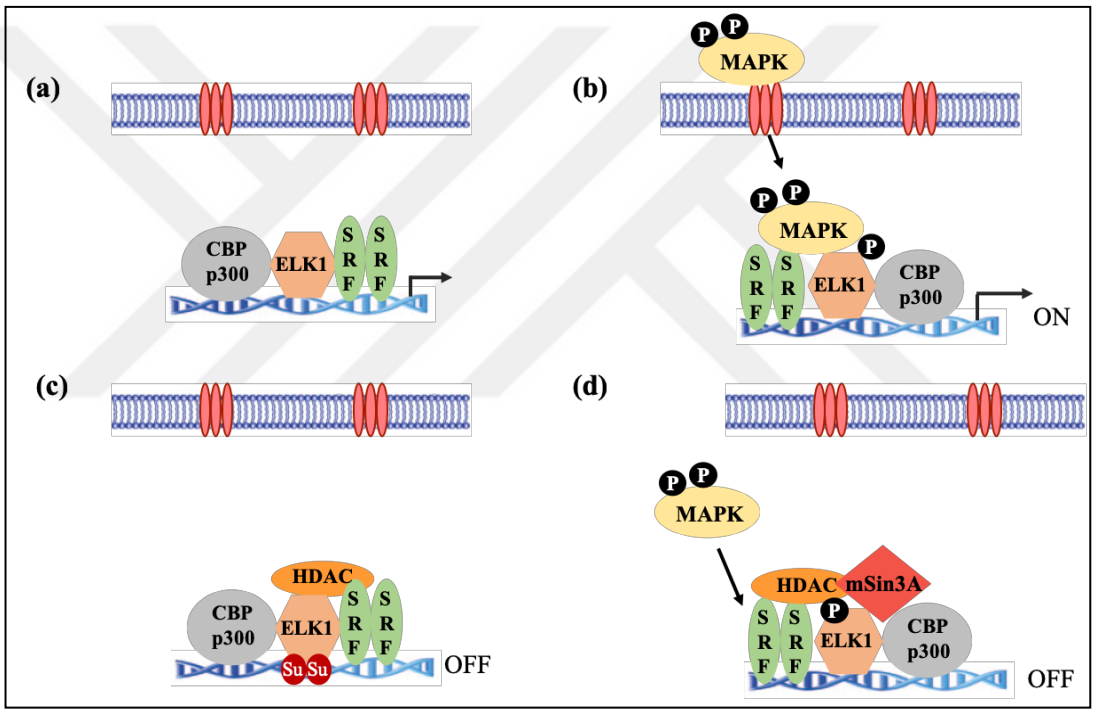


Figure 2.4. Transcriptional regulation of Elk-1. (a) Without MAPK signaling Elk-1 is bound to co-activator CBP. (b) MAPK meditated signaling phosphorylates and activates Elk-1 and its target gene expression. (c) Repression of Elk1 by SUMOylation. (d) MAPK phosphorylation also induces mSin3A binding and repression of Elk-1 [26].

Binding of a dimer of SRF on SRE region potentiates recruitment of Elk-1 to this region and formation of the ternary complex to regulate the functions of the target genes. As it is explained above, B domain is required for the binding of SRF to Elk-1 and in case of the deletion of B box results in blocking of ternary complex formation. Ling et al. identified four hydrophobic residues of Elk-1, Tyrosine 153, Tyrosine 159, Phenylalanine 162 and

Isoleucine 164, as determinant residues in SRF-Elk-1 interaction [27]. Docking of Elk-1 to the serum response element (SRE) of target genes is performed by combined action of protein and DNA interactions and these hydrophobic residues in B box mediates interactions with SRF, intrinsic binding activity and ternary complex formation. Formation of the Elk-1-SRF-SRE complex is a prerequisite for the regulation of immediate early genes which are activated rapidly in response to extracellular signals. The best-known target of Elk-1/SRF complex is the proto-oncogene c-*fos* and it is known that SRF constitutively localizes at the SRE region of the promoter of this gene to recruit TCFs as depicted in Figure 2.4a [27].

Multiple phosphorylations of Elk-1 causes a conformational change that adjust interactions between DNA binding and activation domains resulting elevated DNA binding and transcriptional activity. In addition to this, phosphorylation of Elk1 is also necessary for its interaction with co-activators such as CREB binding protein (CBP) and p300 [28]. Prior to MAPK activation, Elk-1 is found to be in a complex with a co-activator either CBP or p300 as illustrated in Figure 2.4a. Phosphorylation of Elk-1 enhances the stability of this complex results in faster response to extracellular signaling as shown in Figure 2.4b [29].

2.1.1.4. Repression of Elk-1 Activity

The transcirptional activity of Elk-1 relies on the balance between phosphorylation and de-phosphorylation by kinases and phosphatases, respectively and several negative regulatory mechanisms of Elk-1 has been revealed: one major negative regulation is through the action of calcium/calmodulin-dependent protein phosphatase, calcineurin. Specific dephosphorylation site of Elk-1 by calcineurin is Phospho-Serine 383, a pivotal residue targeted by MAPKs for enhanced transcriptional activity of Elk1 [30].

Another repression mechanism of Elk-1 is the small ubiquitin-related modifier (SUMO) family of proteins. Last four amino acids of C terminus region of SUMO are cleaved to link the remained glycine and lysine residues within the target molecule to regulate different biological processes including transcription factor activity [31]. R domain of Elk-1 is rich in lysine amino acids and Lysine-230, -249 and -254 residues within this domain are identified as potential SUMOylation sites [32].

In quiescent cells, Elk-1 is SUMOylated from its R domain and held in an inactive state. SUMOylated Elk-1 is accompanied with the increased histone deacetylase (HDAC) activity, mainly HADC-2, on Elk-1 targeted promoters and transcriptional repression by decreased histone acetylation. SUMOylation of Elk-1 within repression domain holds it in a dormant state, whereas phosphorylation by MAPKs leads to reversing SUMOylation and subsequent segregation of SUMO and Histone deacetylase-2 (HDAC-2) as illustrated in Figure 2.4c. Phosphorylated Elk-1 recruits Mediator, a transcriptional co-activator, and interacts with p300/CBP that has histone acetyltransferase domain. This interaction leads to increased acetylation of histone of target promoters with higher levels of gene transcription [33].

In addition to phosphatases and SUMOylation, Teixera et al., recently demonstrated that Elk-1 activity is also regulated by ubiquitin-proteasome system. Skp, Cullin, F box containing complex (SCF1 complex) is large and well-characterized E3 ubiquitin ligase and F box protein FBXO25 is able to bind to SCF1 complex. FBXO has been shown to bind and mediates proteasomal degradation of Elk-1 via ubiquitination by SCF1 complex [34].

Phosphorylation of Elk-1 by MAPKs at Serine 383, both induces activation of Elk-1 and delays the negative regulatory loop temporally which stimulates collection of the co-repressor mSIN3A-HDAC complex to Elk-1 targeted promoters and thereby restricts the duration of the response by converting Elk-1 to a repressive state, as depicted in Figure 2.4d [18].

2.1.1.5. Biological Roles of Elk-1

Regulation of Elk-1 targets is bi-directional; it can induce activation by phosphorylation and recruitment of co-activators and repress transcription via induction of histone deacetylase complex. More than one thousand of Elk-1 binding targets have been identified and there are two different target selection models among these targets, binding with ETS domain transcription factors via promoter regions or binding with its cofactor, SRF. Elk-1 target network mainly contains genes implicated in transcription, translation and splicing and involved in numerous biological functions as listed in Figure 2.5 [35].

The best-studied target genes of Elk-1 are immediate early genes, *c-fos, junB*, *egr1* and *Nur77,* which are important regulators of cellular proliferation. Elk-1 activation is

stimulated by of the MAPK pathway and subsequently, increased histone acetyl-transferase (HAT) activity which results with the activation of *c-fos* that promotes cell cycle entry [36].

Two different variants of Elk-1 have been identified so far, ΔElk-1 and short Elk-1, sElk-1. ΔElk-1 is an alternative splicing variant of Elk-1 and does not have a part of the DNA binding domain that is required for the interaction with SRF [37]. Short Elk-1 is a neuronal-specific isoform of Elk- and has an intact SRF binding domain but it lacks nuclear export signal site in DNA binding domain, thus it is only expressed in the nucleus of neurons. Both of these isoforms are the truncated forms of full-length Elk-1 and they are able to bind to DNA and thereby inducing multiple signaling processes. Vanhoutte *et al.* demonstrated that sElk1 counteract Elk-1 mediated activation of serum response element and contribute to neuronal differentiation of PC12 cells subsequent to nerve growth factor stimulation. Interestingly, overexpression of sElk-1 but not full-length Elk-1 increases neurite extension in a phosphorylation-dependent manner [38].

Elk-1 phospho-mutation studies also revealed that phosphorylation of Elk-1 regulates dendritic elongation, actin expression levels and cytoskeleton dynamics by regulating the expression of *actin* and *srf* genes [39].

Elk-1 interacts with neuronal microtubules, in dendritic and axonal extensions and Serine 383 phosphorylation of Elk-1 accumulates towards the cell soma including nucleus [2]. In addition to neuronal microtubules, Elk-1 was shown to interact with microtubule-related motor proteins, dynein and kinesin upon serum stimulation, although the detailed dynamics and physiological relevance of these interactions are yet to be elucidated [6].

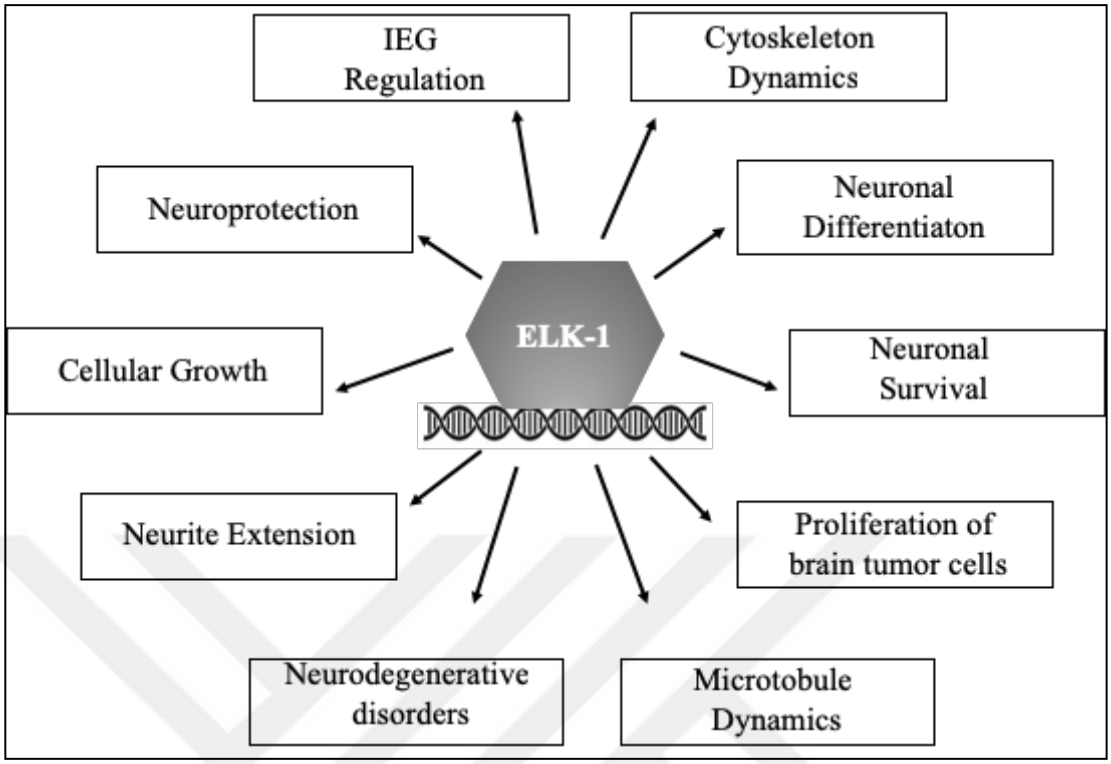


Figure 2.5. Schematic representation of Elk-1 biological functions.

There are contradictory reports in the literature on whether Elk-1 is a pro-apoptotic or apoptotic factor in the nervous system; in one study, Elk-1 was shown to be associated with mitochondrial permeability transition pore (PTP) complex and triggers apoptosis of cortical neurons [40]. Overexpression of Elk-1 decreased cell viability in primary neurons, whereas knockdown of Elk-1 by si-RNA increased cell survival. Region-directed photo-transfection of Elk-1 revealed that overexpression of Elk-1 was shown to be toxic in distal dendrites, but when restricted to the cell body did not give rise to neuronal cell death [41]. On the other hand, Elk-1 was shown to interact with Mcl-1 which is Bcl2-related protein family member and implicated in the regulation of cell survival [4]. Elk-1 also regulates expression of survival of motor neuron (SMN) and thereby induces neuronal survival indicating an anti-apoptotic role of Elk-1 in neurons [5].According to these, it is strongly that specific intracellular localization of Elk-1 along with different upstream signals may determine the fate of the neuron to whether it is going to survive or die.

Epidermal Growth Factor Signaling induces activation of PKC and activated PKC isoforms stimulates ERK and MAPK/ERK kinase-1 (MEK-1). It was observed that, PKC

mediated activation of Elk-1 has been shown to important for the proliferation of glioblastoma cells [24].

Elk-1 phosphorylation has been shown to be involved in the regulation of many neurodegeneration-related genes. α-Synuclein is a pre-synaptic neuronal protein and forms insoluble aggregates which are characteristic profile of Parkinson's disease. It was shown that, α-Synuclein indirectly interacts with Elk-1 via ERK and overexpression of α-synuclein affects Elk-1 phosphorylation and restores decreased cell viability [42]. Accumulation of amyloid-β (Aβ) peptides initiate and/or contributes progression of Alzheimer's disease and it was shown that Elk-1 forms a complex with amyloid precursor protein, APP. Furthermore, *presenilin 1* gene is associated with Alzheimer's disease and ETS motif on the promoter region of this gene was shown to be regulated by Elk-1 [43].

Many lines of evidence designate the conclusion that mutations in copper-zinc superoxide dismutase (SOD1) were found to be causative for amyotrophic lateral sclerosis (ALS) and Elk1 appears to play a positive regulatory role by binding SOD1 promoter [44]. Elk-1 is hyperphosphorylated in the striatum and loss of Elk-1caused apoptosis in mouse Huntington disease models which could contribute to neuroprotection in remaining cells [45].

Schroer *et al*. Identified a co-segregation of Elk-1 T108A Elk-1 with mental retardation. Sequence analysis revealed a transition between A and G nucleotides resulted in T108A amino acid substitution. This nucleotide exchange has been shown in a small three generation family with mental retardation, hypoactivity, low motor and cognitive acquisitions [46]

2.1.1.6. Elk-1 Subcellular Localization

Elk-1 is restricted to the nucleus in non-neuronal cells but expressed in most brain regions within neuronal and non-glial cell types in mature neurons. In the unstimulated or resting state, Elk-1 appears at cytoplasm, where it binds mitochondrial proteins or microtubules. Elk-1 is phosphorylated by MAP kinases and translocates to the cell body and then translocated to the nucleus to regulate chromatin remodelling, SRE-dependent transcription, cytoskeleton dynamics and promotion of neuronal differentiation. This nucleus-cytoplasm shuttling of Elk-1 seems to be determinant for its function in cell

survival or neuronal differentiation. NLS and NES motifs within ETS domain are also critical for the localization of Elk-1 [20].

During cell division, Elk-1 localizes at different positions and this translocation of Elk-1 depends on the interaction of Elk-1 with dynein and kinesin motors [6]. Confocal microscopy studies indicated that Serine 383 phosphorylated Elk-1 is mostly found at the nucleus in interphase cells but then translocates to spindle poles through metaphase and anaphase. When cells proceed to cytokinesis, Elk-1 is mainly re-localizes to the spindle midbody [7].

2.2. EUKARYOTIC CELL DIVISION

Eukaryotic cell division is crucial for growth, development, renewal and repair of the organisms and it consists of successive processes as cell growth, replication of DNA, segregation of the duplicated chromosomes into daughter cells and division of the cell cytoplasm or known as cytokinesis. Interphase has three different and unique stages; G_1 (first gap), S (synthesis) and G_2 (second gap) and proregression through the next stages depends on the proper progression and completion of the previous stage as illustrated in Figure 2.6 [8].

G_1 or first gap is a gap stage between mitosis and the initiation of DNA replication, and during this phase the cell is quite active and grows continuously but DNA does not replicate itself. During G_1, cell accumulates associated proteins and sufficient energy required for the replication of each chromosome in the nucleus. Some cells stop cell cycle at this stage and enter a quiescent state also known as G_0 or resting phase to control external factors such as growth factor and nutrient deprivation. Cells in G_0 phase are neither dividing nor actively preparing to divide and sometimes cells enter G_0 temporarily until the external conditions are optimal for cell division, whereas others permanently cease cell cycle as in nerve cells [47]. If the environmental factors sufficient, G_1 is followed by S phase, in which cell replicates its DNA resulting in the formation of identical pairs of DNA molecules and the centrosome is duplicated in this phase which will further form the mitotic spindle. In the second gap or G_2 phase cell renew its energy stores and synthesize proteins required for the entry of the mitotic phase, so prepare itself for mitosis [48].

Mitosis is highly organized process which culminated by the formation of two identical cells and divided into mainly four different stages as prophase, metaphase, anaphase and telophase which is subsequently followed by cytokinesis. Prophase is the first stage of mitosis and begins immediately after the end of G_2 and composed of centrosome separation, microtubules organization to form mitotic spindle, chromosome condensation, disappear of the nucleolus and disassembly of the nuclear envelope. Prometaphase is a gap between prophase and metaphase and in this phase, chromosomes continue to condense and attach to mitotic spindle via their kinetochore regions. Assembly of chromosomes at the centre of the mitotic spindle, known as metaphase plate, and attachment of each sister chromatids to a spindle fibre originating from opposite poles takes place in metaphase which is followed by moving of sister chromatids to the opposite poles in anaphase. During telophase, chromosomes are located at the opposite poles and began to decondense, nuclear envelope is reformed, and it surrounds each set of chromosomes. Telophase if followed by cytokinesis which is the final step of mitosis and at the end of this stage two daughter cells are formed with identical genetic information [49].

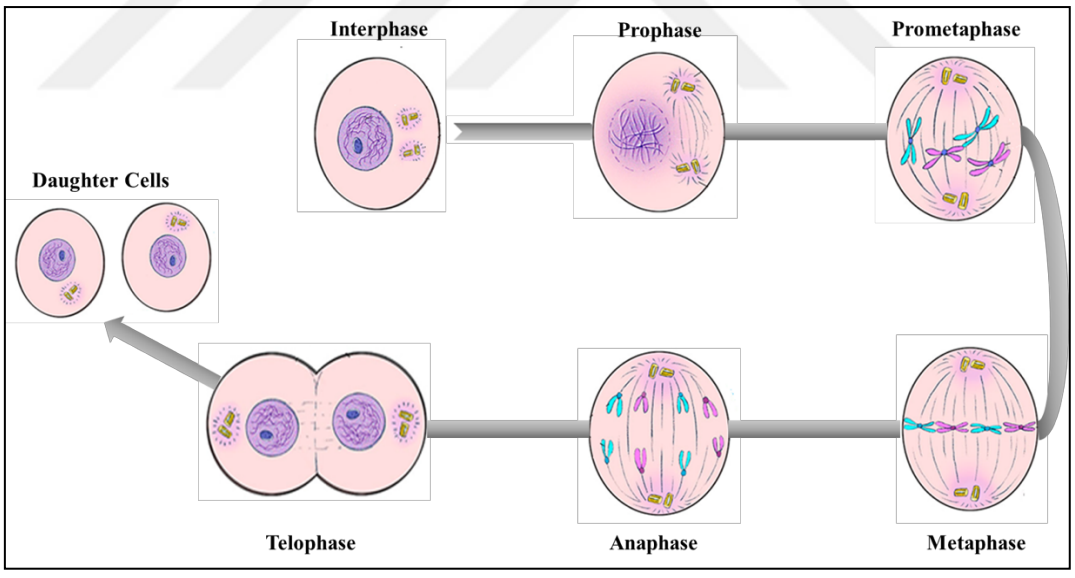


Figure 2.6. Mitotic phases of eukaryotic cell cycle [50].

The progression of the cell division is regulated by both internal and external signals such as growth factors that monitor the fidelity of various events. Cellular processes of cell division must be well-coordinated since any disturbance in the duplication of the chromosomes or distribution of the them give rise to the mutations that can be passed through to the daughter cells. To overcome this, there are several checkpoints but three of

them are crucial for accurate segregation of duplicated chromosomes into daughter cells; G_1, G_2 and spindle assembly checkpoints [48].

The first cell cycle checkpoint occurs at late in G_1 and called as G_1 checkpoint or restriction point, in yeast, which is the main decision point for a cell to divide or not. In this checkpoint, cell controls whether all conditions are favourable for cell division by controlling cell size, amount of nutrients, growth factors, protein reserves and DNA damage. Control of DNA damage is crucial before DNA synthesis since inheritance of damaged DNA contributes to cancer initiation and progression. In G_1 phase, cell double its contents and ensure that each daughter cell will be donated with the appropriate amount of genetic and cytoplasmic materials before division. If the conditions are not appropriate for mitosis, cells does not proceed into the S phase and attempt to normalize the conditions or enter G_0 phase and wait until everything is proper for DNA synthesis [51].

Following DNA replication in S phase, the G_2 checkpoint controls the transition from G_2 to mitosis and in this phase, cell size, nutrient availability, the sufficiency of key proteins and DNA stability before mitotic entry is monitored. In addition to this, the focal point of the G_2 checkpoint is to control of the replication of the chromosomes and DNA damage. If any defects are detected on DNA replication by G_2 checkpoint, the cell cycle will not be proceed into mitosis and attempt to either complete DNA replication or repair the damaged DNA as illustrated in Figure 2.7 [52].

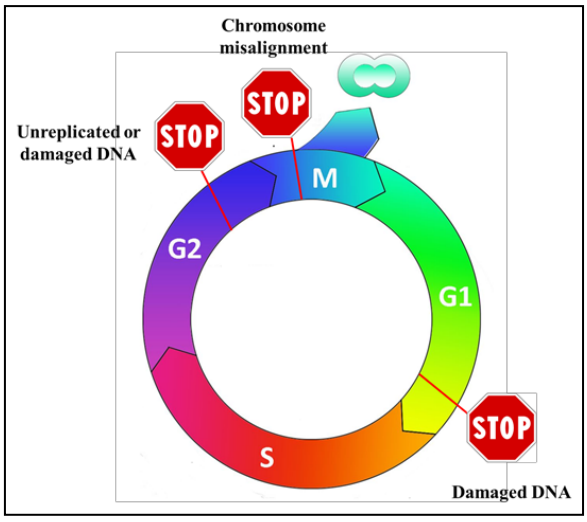


Figure 2.7. Checkpoints of the cell cycle [48].

Attachment of chromosomes to the metaphase plate in a bi-oriented fashion is the focal point for ensuring that a complete set of chromosomes is distributed to the daughter cells accurately. Spindle assembly checkpoint controls whether all sister chromatids are attached at metaphase plate properly or not. Once all kinetochore regions of chromosomes are attached and aligned at the metaphase plate, cells proceed into the next step, anaphase [53].

2.2.1. Regulation of Cell Cycle by Mitotic Kinases

Accurate completion of cell division requires careful coordination of four main processes, DNA replication, entry into mitosis, segregation of chromosomes and cytokinesis. Signaling pathways and molecules coordinate t2hese vital events and several positive and negative feedback loops cooperate with these signaling molecules to ensure chromosomes are accurately separated into the daughter cells and the all events occur in the correct order. These signaling pathways have two key roles, coordinate different events at the same time and arrange checkpoints that halt cell division if the conditions are not proper for cell cycle progression [54].

Cdk1 controls both G_2 phase entry and phosphorylation of substrates during mitosis [45]. Besides Cdk1, Aurora kinase family members also play vital roles in cell cycle regulation. Aurora A is related with G_2-M transition, centrosome assembly and mitotic spindle organization. Aurora B, the other Aurora kinase family member, being a chromosome passenger complex (CPC) protein, regulates chromosome segregation. During mitosis, regulation of Plk1 is critical for mitotic entry, spindle dynamics and completion of mitosis [55].

In response to double-stranded DNA damage, NEK4 arrests cell cycle [56]. During prophase, Budding Uninhibited by Benzimidazoles 1 (BUB1) kinase localizes to the outer kinetochore and involved in the regulation of checkpoint activation in response to DNA damage. It also functions in the spindle assembly checkpoint and alignment of chromosomes on the equatorial plate during metaphase [57].

Extracellular signals are mainly responded by the MAPKs, which are play important roles during cell division. Various cellular stresses, i.e. osmotic shock, ionizing radiation, oxidative stress, cytokine stimulation and growth factors, activate MAPKs and can halt cell cycle at G_2 phase. p38, JNK and ERK1 and ERK2 MAPK pathways are activated by these factors; the p38 pathway is implicated in mitotic entry and cell cycle progression [58],

Erk1/2 are involved G_1/S and G_2/M transition [59] and it is demonstrated that the JNK module of MAPK pathway controls the mitotic entry and mitotic spindle organization [60].

Akt pathway is also implicated mitotic functions; active Phosphoinositide 3-kinase (PI3K) regulates the onset of the mitosis, metaphase progression and spindle organization in association with CDK. Inhibition of the PI3K/AKT pathway delays cell cycle in S phase and promotes mitotic exit and hampers G_2/M transition by decreasing Cdk1 kinase activity [61]. It is also known that inhibition of AKT related with Aurora A kinase expression, which hinders centrosome organization, progression of mitotic phases and mitotic spindle orientation [62].

Key mitotic kinases and their functions are listed in Table 2.1. In this chapter only three kinase family, Aurora Kinases, Cyclin-Dependent kinases and Polo-like kinases, will be explained in detail since their close relationship with Elk-1 protein.

Table 2.1. Key kinases involved in mitotic regulation [55]

Kinase	**Function**
Aurora A	Mitotic spindle formation and centrosome separation
Cyclin-Dependent Kinase 1	Cell cycle regulation, entry into mitosis
Polo-Like Kinase 1	G2/M transition, spindle assembly and cytokinesis
NEK-4	Cell cycle arrest
BUB-1	Spindle assembly checkpoint and chromosome Alignment
P38	Mitotic entry
ERK1/2	G2/M transition
JNK	The onset of mitosis and mitotic spindle regulation
mTORC1	Mitotic progression
AMPK	Mitotic spindle orientation
PI3K/AKT	G2/Mitosis transition and cytokinesis
MAD2	Regulation of spindle assembly checkpoint
ATM and ATR	Response to DNA damage

Chk1 and Chk2	DNA damage checkpoint and mitotic entry
NIMA	Centrosome dynamics and separation and mitotic entry

Since mitotic kinases are crucial for the regulation of cell division, cell cycle progression, spindle assembly checkpoint, microtubule organization, centrosome maturation and separation, they have been implicated in tumor development. Therefore, targeting mitotic kinases in terms of the management of cancer seems to be promising.

2.2.2. Aurora Kinase Family

Mammalian Aurora kinase family is composed of structurally similar but functionally quite different 3 members, Aurora A, Aurora B and Aurora C. These members have an N-terminal domain that determines selectivity during protein-protein interactions, a protein kinase domain and C-terminal domain. N terminal domains of these kinases comprise a KEN motif that is required for the Cdh1 dependent Anaphase Promoting Complex (APC) recognition signal and a D-box activating motif functions for the activation of D-box that is implicated in the proteolysis of these kinases [63].

Aurora B is a member of CPC and involved in numerous functions including chromosomal bi-orientation, sister chromatids cohesion, repair of defective attachments, regulation of the kinetochore-microtubule binding, mitotic spindle assembly and cytokinesis. It is believed that Aurora B also implicated in chromosome condensation and separation since one of the targets of Aurora B is histone H3. The exact function of Aurora C is still unclear but known that it functions in spermatogenesis and since it has similar localization pattern with Aurora B it is believed that also exhibits similar functions with Aurora B in terms of cytokinesis [50].

2.2.2.1. Aurora A kinase

Aurora A, also known as Aurora-2 or IAK , is a main regulator of the mitosis and involved in the proper cell-cycle progression [63]. Besides the roles mentioned above, Aurora A also has important roles in centrosome maturation and microtubule nucleation. This kinase mainly accumulates at the centrosome in the S phase and it is activated between G_2 and M phase [50]. During mitosis, it appears at the centrosome through prophase to telophase and

it also localizes to the spindle pole from prophase to anaphase [64] and finally, it moves to the midbody which is a cytoplasmic bridge between the two daughter cells [65].

Microtubule organizing centre (MTOC), centrosome in animal cells, is made up of a pair of centrioles surrounded by pericentriolar material (PCM) [66]. Aurora A regulates γ-tubulin recruitment to the centrosome through centrosomin of the pericentriolar material [67].

Aurora A mutation or silencing of Aurora A by RNA interference gives rise to density loss of centrosome-associated microtubules, lack of centrosome maturation [68], defects in the maintenance of centrosome separation, formation of multipolar spindle, failure in cytokinesis, abnormal centriole numbers and reduced accumulation of PCM proteins like γ-tubulin and Minispindles (Msps) [69].

2.2.2.2. Regulation of Aurora A Activity

It has been demonstrated that Aurora A activation mainly requires phosphorylation at threonine residue on 288 position. P21 activating kinase (PAK) and Protein Kinase A (PKA) are two kinases that can phosphorylate Aurora A from Threonine 288 [70]. Interestingly, phosphorylation of Serine 342 residue results decreased Aurora A kinase activity [71]. In addition to this, in vitro kinase assays demonstrated that Threonine 287 residue can compensate for T288A mutation [70]. Any signaling pathway that activates Aurora A has not been determined yet, but several Aurora A interacting molecules have been identified, including TPX2 [72]. Ajuba a protein localized to the centrosome [73], Bora, a protein released from the nucleus to the cytoplasm in late G_2 [74], Eg5 which is a kinesin motor, HEF1 (functions in focal adhesion) [75], CDC25B (activates the cyclin-dependent kinase CDC2) [76] and PAK1 (Serine/threonine-protein kinase) [77].

Protein phosphatase type 1 (PP1) binds Aurora A and hinders its activity by dephosphorylation of threonine 288 during mitosis. Interestingly, Aurora A can also phosphorylate PP1 and inhibits its phosphatase activity [78]. In mitosis, Aurora A is activated by autophosphorylation with the help of the binding to its cofactor TPX2. This cofactor binds Aurora A via N-terminal domain and induces moving of the activation segment of Aurora A inside, the catalytic pocket of the kinase [79].

Another inactivation mechanism of Aurora A is the anaphase-promoting complex/cyclosome (APC/C)-ubiquitin proteasome pathway and this degradation is dependent on hCdh1 [70]. D-box activating motif that located at N-terminal domain of Aurora A is required for the activation of D-box (degradation box) that locates at kinase domain and this domain is one of the targets of Fizzy-related proteins [80].

2.2.2.3. Polo-Like Kinases

Polo-like kinases (Plks) belongs to mitotic serine/threonine kinase family whose regulation is crucial for signaling pathways including cellular growth, differentiation, survival and motility. Five polo-like kinases have been identified to date in mammalian cells and all of them comprise a serine/threonine kinase domain regulating ATP binding and enzymatic activities and a polo box domain (PBD) that contains one or two polo boxes (PB) which forms a binding pocket towards phosphorylated motifs on their targets [81]. Polo box domains are not only responsible for binding to phospho-peptides on its targets, but it is also implicated in the regulation of Plk catalytic activity, localization of Plks to different cellular localizations and for mitotic progression [82]. Carboxy-terminal domain of Plks is also a negative regulator of Plk activity since it interacts with kinase domain to prevent substrate binding and kinase activity in the absence of a phosphorylated ligand [9].

In mitotic cells, Polo-like kinases are related to different localizations such as centrosomes, spindle poles, kinetochores, midbody and midzone, indicating a potential role for these kinases in centrosome maturation, kinetochore assembly, bipolar spindle formation, kinetochore-microtubule attachments, regulation of the spindle checkpoint, chromosome segregation and completion of the cytokinesis [83].

Although all Plk family members share a similar domain structure, they have different subcellular localizations and interacting molecules. Expression of three Plk members, Plk2, Plk3 and Plk5, is regulated by numerous stimuli and they function in multiple cells and tissues. It is identified that Plk 2 and Plk3 have important functions in modulating synaptic plasticity in neuronal dendritic spines, DNA damage respond and G_1/S and G_2/M progressions, respectively [9]. Centrosome localization of Plk2 suggests its role in centrosome regulation and S-phase checkpoints. Plk5 is a new member of Plk family and any cell cycle related role of Plk5 has not been identified but it mainly expressed in neurons to modulate neural pathways in response to growth factor stimuli and its tumor

suppressor activity in brain tumor cells has been elucidated. In contrast to Plk2, Plk3 and Plk5, Plk1 and Plk4 are mainly expressed in dividing cells and they are associated with the regulation of mitosis [81].

2.2.2.4. Polo-Like Kinase 1

Among the five mammalian Plks, Plk1 is the most extensively studied family member and it regulates critical roles in the regulation of cell division. It functions substantially during cell division and mediates numerous critical processes. Plk1 expression initiates in late S phase and reaches a peak during G_2 and M phases [83]. It localizes at the centrosomes through metaphase and early anaphase and translocates to the midzone of the spindle through anaphase and then localizes at midbody during cytokinesis. Plk1 contributes several pivotal processes; mitotic entry, bipolar spindle formation, centrosome maturation, kinetochore function, spindle assembly checkpoint (SAC), promotion of mitotic exit and cytokinesis [82].

2.2.2.5. Functions of Plk1 During Cell Division

Plk1 is one of the crucial kinases whose activity is needed for the proper segregation of chromosomes into daughter cells and completion of cytokinesis. Depletion of Plk1 gives rise to mitotic arrest, aberrant spindle formation as well as immature centromeres, on the other hand, overexpression contributes to oncogenic transformation.

Cdk1/CyclinB complex is a crucial regulator of mitotic entry and activity of this complex is organized by the opposed actions of Wee1 and Myt1 kinases and Cdc25 phosphatase. Previous studies indicated that both Myt1 and Cdc25 can be phosphorylated by Plk1 to regulate the activity of Cdk1-Cyclin B complex. It is believed that Plk1 activation of Cdk1 is then promoted mitotic entry [82].

Plk1 is involved in the recruitment of γ-tubulin along with several centromere-associated proteins during centrosome maturation which is required for microtubule nucleation. At spindle poles, Plk1 acts for the formation of bipolar spindle along with several microtubules associated proteins and motor proteins [82]. Moreover, Plk1 phosphorylates NLP, a centrosomal protein that regulates microtubule nucleation and inhibits its dynein-associated transport to centrosome to allow recruitment of other centrosomal protein [84].

Polo-like kinase 1 is targeted to centromere and kinetochore regions by several proteins to check the attachment of spindles through SAK that interferes mitotic progression to be sure that the spindles attached properly and ensure genomic stability. For instance, BuBr1, functions in the spindle attachment checkpoint, phosphorylation of Plk1 is essential for the microtubule-kinetochore attachment. When Plk1 is inhibited, cell cycle arrest is stimulated as it depends on the activity of SAC [85].

The small GTP-binding protein, Rho1/RhoA plays a focal role in cytokinesis. During anaphase Rho-GTP level substantially increases to initiate cytokinesis and its action is seeming to be dependent on Plk1 activation. It is demonstrated that Plk1 triggers recruitment of a Rho guanine nucleotide exchange factor (GEF), ECT2, to the midzone and promotes Rho activation. In brief, Plk1 is necessary to promote accumulation of RhoA and target it to the cell equator to initiate cytokinesis and mitotic exit [86].

2.2.2.6. Regulation of Plk1 Activity

Like many proteins, regulation of Plk1 activity relies on transcriptional activation, phosphorylation, protein interaction and ubiquitin-dependent degradation. Located at the activation loop, Threonine 210 residue is crucial for Plk1 activation. Protein phosphatases 1 (PP1) and its adaptor protein MYPT1 antagonizes Plk1 phosphorylation via the same residue [87]. During late mitosis, Plk1 is also phosphorylated by Serine 137 and this phosphorylation probably alters its substrate specificity [88].It was observed that P21 activating protein kinase 1 (PAK1) phosphorylates Serine 49 residue located at the activation domain of Plk1 and this leads to function Plk1 at metaphase-anaphase transition [89].

Several repressors of Plk1 act after completion of cytokinesis in order to repress Plk1 expression, including p53, p21 and Retinoblastoma (RB) family [90]. Moreover, cell cycle-dependent proteolysis of Plk1 from D-box (degradation box) is achieved by Anaphase-Promoting Complex/Cyclosome (APC/C) which is regulated by the spindle checkpoint. Best known adaptors of APC/C complex are Cdc20 and Cdh1 whose activity responsible for the degradation of Plk1 during late mitosis and G1 or after DNA damage, respectively [91].

As it is mentioned above, Plk1 is asymmetrically localized during mitosis, and its localization at different compartment, centrosomes, spindle poles, midbody and midzone,

relies on the action of target proteins. Numerous proteins have been identified that binds to PBD of Plk1. Until mid-mitosis, Plk1 is associates with INCENP (Inner centromere protein), BUB1 and BUBR1 whose binding is essential for centromere and kinetochore localization of Plk1. Furthermore, through the late mitosis and cytokinesis, Plk1 associates with central spindle proteins such as MKLP2 (mitotic kinase-like protein 2) and PRC1 to regulate their functions [82].

2.2.2.7. Cyclin-Dependent Kinases (CDKs)

Cdks are important for the regulation of cell cycle, initiation of DNA replication and transition both into G_1/S and G_2/M phases. Cdks are serine/threonine kinases and their kinase activity depend on association with their regulatory proteins, cyclins. Each cyclin-dependent kinase associates with one or two cyclins and each cyclin also can bind to one or more Cdks. In the absence of cyclin, Cdk kinase activity is very low. To date, 11 conventional Cdks (Cdk-1-11) and two newly designated family members (Cdk-11 and 12) have been identified. Among these kinases, Cdk 1, 2, 3, 4 and 6 are involved in the cell cycle and functions in numerous pivotal roles [92].

Cyclins are regulatory subunits of cyclin-dependent kinases and classified according to the existence of cyclin box that is responsible for Cdk binding. There are 11 cyclins in human many having sub-family members and they bind to Cdk to regulate the cell cycle. Cyclin and Cdk complexes regulate cell cycle both by promoting actions for respective stages and by arresting actions for forthcoming cell cycle stages. Therefore, cyclins are both formed and degraded for cell cycle progression [93]. Cyclins are generally present at low levels during the cell cycle, but their expression strongly increases at the stage where they are needed. For instance, Cyclin B expression initially starts just after completion of DNA replication in S phase, increases during G_2, peaks at G_2/M transition and it is considerably degraded by the onset of anaphase. Transient accumulation of Cyclin B protein level is important for G_2/M transition and progression of mitosis and degradation of this protein a necessity for mitotic exit. Cyclins, their related kinases and the outcomes of their relations throughout mitosis are listed in Table 2.2. [94].

Table 2.2. Cyclins, cyclin-dependent kinases and their functions during mitosis [95].

Cyclins	**Cyclin-Dependent Kinase (Cdk)**	**Cell Cycle Stage**	**Function**
Cyclin A	Cdk1 and 2	S and G_2	Initiation and progression of DNA synthesis S phase progression S/G_2 transition
Cyclin B	Cdk 1	M phase	G_2/M transition Regulation of M checkpoint Mitotic spindle assembly
Cyclin D	Cdk 4 and 6	G_1	G_0/G_1 transition Cell cycle progression Cellular migration DNA damage response and repair Chromosome stability
Cyclin E	Cdk 2	G_1/S	G_1/S transition

2.2.2.8. Cyclin-Dependent Kinase 1

Cdk1, also known as Cdc2 or Cdc28, is a key player of cell proliferation that decides to enter mitosis or undergo apoptosis in conjugation with its activators and substrates. Cdk1 inactivating phosphorylation, in case of DNA damage, halts cell cycle and replication at G phase to gain time for DNA repair. To date, more than seventy Cdk1 interacting protein have been identified that cooperates to regulate cell division. In addition, Cdk1 is also the

mediator of neuronal cell death which is related to brain development and neurodegeneration [96].

Like other family members, Cdk1 has a catalytic cyclin-dependent kinase (Cdk) subunit and a regulatory cyclin subunit. The kinase activity of cyclin-dependent kinases depends on the cyclin subunit. Cyclin binding to the Cdk subunit provides a minimal kinase activity and full activation of this subunit requires phosphorylation by an activating kinase [97].

During cell proliferation, Cdk1 associates with its activator cyclin A or cyclin B to control activity of its targets, implicated in chromosome condensation (histone H1), DNA replication (MCM2, MCM4, Cdc7, nucleolin, the ribonucleotide reductase R2), regulation of cell cycle progression (P53, BARD1, BRCA),breakdown of nuclear lamina (lamins and nuclear pore complex), microtubule function (β-tubulin, Dynein, Eg5, MAP4, MKLP1, Tau) as well as ubiquitin-dependent proteolysis (Cdc20, Cdc25, Cdh1) [10].

During G phase, Cdk1/cyclin A complex triggers S translation, but cyclin A is degraded at the mid-S phase and instead Cdk1 binds to newly synthesized cyclin B (B1 and B2) to activate G_2/M transition and entry into mitosis [10]. During prophase, Cdk1–Cyclin B complex translocates to the centrosome and phosphorylate a kinesin motor protein (Eg5) to contribute centrosome separation [98]. After chromosomes are condensed and properly aligned at the metaphase plate, Cdk1 activity is turned off. Interestingly, degradation and inactivation of Cdk1–Cyclin B complexes by anaphase-promoting complex/cyclosome (APC/C) is required for mitotic exit [99].

2.2.2.9. Regulation of Cdk1/CyclinB Activity

Cyclin-dependent kinase 1 activity highly depends on several integrated mechanisms: binding to regulatory proteins, cyclins, interaction with activating kinases (CAK), suppression of kinase activity by interaction with inhibitory kinases and binding inhibitory cyclin-dependent kinase inhibitors. Cdk1 activation by CAK stabilizes Cdk1-cyclin B association, enhances its substrate binding and prevents repression by specific inhibitors. Cyclin B binding to Cdk1 triggers relocation of this complex to the nucleus and CAK phosphorylates Cdk1 on Threonine 161 residue to reverse the effect of inhibitory phosphorylations on Threonine 14 and Tyrosine 15 by dual-specific protein kinases Myt1 and Wee1 [100].

The expression of cyclin B is regulated by several transcription factors, e.g. NF-Y, FoxM1, and B-Myc. During S phase cyclin B protein synthesis starts and dramatically increases during late G phase. The mediator mechanism of cyclin B expression is another cyclin/cyclin-dependent kinase, cyclin A–Cdk2 and that regulates the expression of all these transcription factors to restrict cyclin B synthesis at S and G phases [101]. Degradation of cyclin B through metaphase to early S phase controlled by anaphase-promoting complex/cyclosome (APC/C) which is also induced by cyclin A–Cdk2 and an APC inhibitor, Emi1 [102].

When cyclin B reaches a sufficiently high level, binds and forms an inactive complex with Cdk1 and this complex needs to be activated to turn Cdk1 kinase activity on. There two main kinases that repress Cdk1 activity, Wee1 and Myt1 that phosphorylates Cdk1 on Threonine 14 and Tyrosine 15 residues. Cyclin B/Cdk1 is able to Cdc25 dual specificity phosphates which subsequently dephosphorylates Thr14 and Tyr15 residues to antagonize their inhibitory effect [103].

Cdk1 activation is occurred through the cooperation of three the combination of three successive processes. The first step includes phosphorylation of Thr161 residue by Cdk-activating kinase (CAK) at late G2 which results in the opening up the catalytic domain of Cdk1. Secondly, Cdk1 is phosphorylated on Ser147 by Plk-1 to promote nuclear localization of Cdk1/cyclin B1 complex. The final or the activation step of Cdk1 contains dephosphorylation of Thr14 and Tyr15 residues of Cdk1by Cdc25 phosphatase family members, Cdc25A, Cdc25B, and Cdc25C (18). Activated Cdk1/cyclin B Complex regulates its targets in the nucleus to control mitotic entry and later mitotic progression as seen in Figure 2.8. [94].

Cdk1-mediated phosphorylation does not only affect the activity of its targets but also creates a docking site for other kinases including Plk1. It was observed that Plk1 can bind and phosphorylate many targets that already phosphorylated by Cyclin B/Cdk1, e.g. Wee1, Myt1 and Cdc25. Phosphorylation of Wee1 by Plk-l after Cyclin B/Cdk1 phosphorylation renders it more susceptible to degradation [104].

As mentioned above, Bora is an activator of Aurora A kinase and Bora binding to Aurora A induces its phosphorylation of Plk1. Interestingly, Cyclin B/ Cdk1 phosphorylated Bora can bind and activate Plk1. Activated Plk1 then stimulates FoxM1 activation to trigger cyclin B expression that afterwards binds Cdk1 [105].

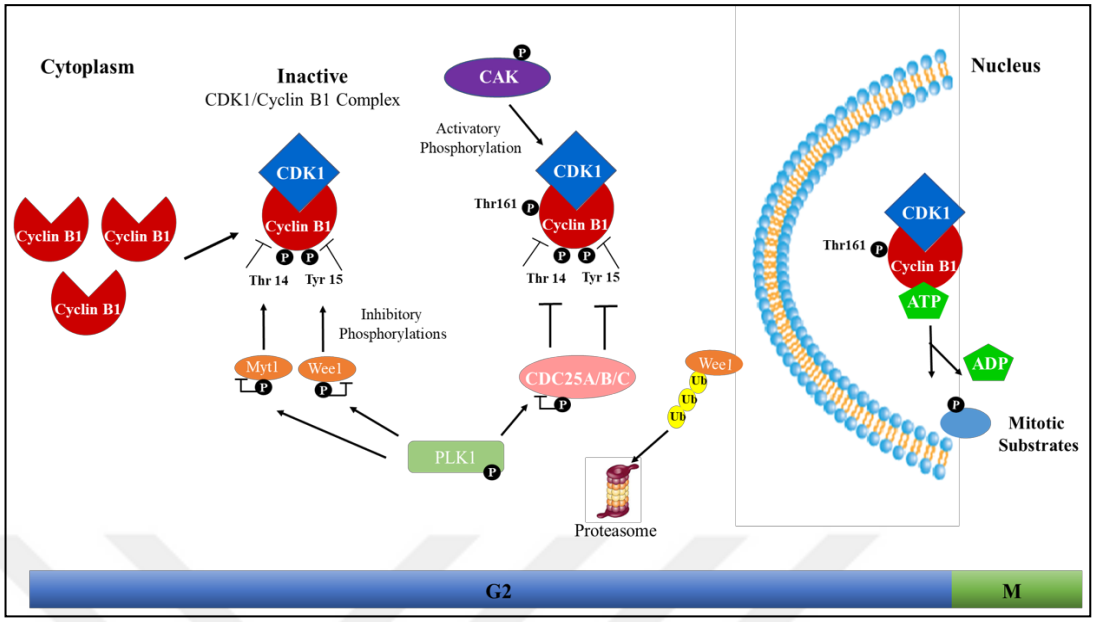


Figure 2.8. Activation of Cdk1/CyclinB1 complex [94].

2.2.3. Key Mitotic Regulators Involved in Cancer

Mitotic kinases play pivotal roles in many aspects of cell division, so deregulation of these key players, including up and/or downregulation, overexpression, mutations and misfolding of proteins, contributes to cancer initiation and progression.

Aurora kinases have numerous important functions during cell division, so aberrant expression of these kinases results in the cell transformation that causes cancer. Previous studies indicated that overexpression of Aurora A in many cell tumors including breast, ovarian, colon, prostate, leukaemia, neural and cervical cells, so Aurora A can be classified as an oncogene. For instance, overexpression of Aurora A has been observed in 60 per cent of gliomas [64]. In addition to the transformation effect of Aurora A overexpression, it also causes loss of contact inhibition that leads to cell growth [106].

Overexpression of Aurora A is not sufficient to start cellular transformation, so additional oncogenic induction is required for transformation, such as activation of Ras-signaling [50]. Aurora A overexpression may be an early stage in tumorigenic transformation [64].

Human malignancies caused by Cdks are most frequently amplifications of regulatory subunits and cyclins. In human malignancies, most observed disturbances are overexpression of regulatory subunits of Cdks and cyclins, inactivation of Cdk inhibitors

and chromosomal translocation as in lymphomas, leukaemias and melanomas [107]. Disruption of Cdk1 activity has also been observed in a number of primary tumors, for instance, lung, colon, prostate, breast oral, lung and oesophagal carcinomas [108].

Overexpression of Plk1 has been observed in various cancer types and Plk1 expression is generally associated with poor prognosis. Plk1 overexpression also plays a part in multinucleation and overrides the cell cycle checkpoints and behave as an oncogene. Furthermore, Plk1 mutations involved in tumorigenesis [109].

2.2.4. Elk-1 and Cancer

Elk-1 is mainly activated by the phosphorylation of upstream MAPK signaling cascades, ERK, p38 MAPK and JNK, by distinct external stimuli and translocates to the nucleus to regulate its downstream targets, including immediate early genes which are also proto-oncogenes, *c-fos*, *junB, c-myc* and *egr1* [35]. Matrix metalloproteinases that contribute to tumor cell invasion, are also targeted by Elk-1 [110]. Activation of Elk-1 regulates various cellular processes such as, cellular proliferation and differentiation, survival and apoptosis. Since Elk-1 is implicated in these crucial functions in terms of tumor formation and progression, dysregulation Elk-1 activation has been related with many cancers, such as brain, breast, colon, lung, ovarian, bladder.

It is known that Serine 383 phosphorylation of Elk-1 by ERK pathway promotes survival and proliferation of many cancer cells. Interestingly, Threonine 417 phosphorylation of Elk-1 was also detected in various normal and cancer tissues, such as colonic adenocarcinomas. [111].

Elk-1 inhibition is shown to enhance the anti-proliferative effects of breast cancer by subsequent inhibition of *c-fos* transcription [112]. Elk-1 is also implicated in EGF mediated cell survival in breast cancer by the regulation of anti-apoptotic protein Mcl-1 [113]. Elk-1 is found to be overexpressed in colon cancer and directly regulates the gene expression of Myc, a proto-oncogene that plays an important role in colon cancer development [114]. Inoue *et al*, have found that up-regulation Serine 383 phosphorylated Elk-1 correlates with the induction of cell proliferation, migration, and invasion of bladder cancer [115]. It was also demonstrated that Elk-1 knockdown resulted in decreased proliferation of the pancreatic cancer cells [116].

Brain tumors in particular glioma and glioblastoma have a high rate of proliferation and expansion. The high mortality rate of glioblastoma, virtually 100 per cent, is based on the rapid proliferation and invasion rate. Recent studies revealed that Elk-1 has an abnormal expression profile in brain tumors. Uht *et al.* demonstrated that a PKC isoform stimulates activation of Elk-1 leading to transcriptional activity and proliferation increase of glioblastoma cells [24].

2.3. BRAIN TUMORS

Cancer is a very complex disease caused by the combined effects of environmental and genetic factors. Genetic alterations lead to dysregulated proliferation, differentiation, growth and development. Certain genes known as oncogenes play pivotal roles in cancer formation and disturbances on the structure and activity of these genes due to genetic or epigenetic factors lead to enhanced cell proliferation and growth and confers increased cell survival and resistance capacity to tumorigenic cells.

Neuroblastoma is a childhood cancer that starts in immature nerve cells of the nervous system and in most cases, *N-myc* protooncogene is overexpressed. Neuroblastoma is the most widespread tumor in children and most children who have this cancer are under the age of five [117].

The origin of gliomas has been an issue for discussion, but it is speculated that they might arise from astrocytes since that is the only cell type that is capable of cell divisions in the adult nervous system. Glial tumors generally exhibit heterogeneous composition with multipotent cells within the tumor with astrocytic, oligodendrocytic and neural properties. These multipotent cells might be arising from progenitor cell dedifferentiation or by oncogenic mutation, particularly in neural stem cells. The subventricular zone is an important area for neural stem cells and it is found to be the origin of most gliomas [118].

Gliomas account for 80 per cent of the brain tumors. Glioblastomas arise from astrocytes whereas gliomas may be originated from any of the supportive tissue cells of the brain, such as astrocytes, oligodendrocytes and ependymal cells or neural stem cells and progenitor cells. Malignant gliomas such as Glioblastoma Multiforme are quite invasive and diffusely infiltrate surrounding brain tissue. However, these tumors are restricted to the central nervous system and do not metastasize [118].

In 2016, The World Health Organization (WHO) re-classified brain tumors and incorporated new entities that are defined not only by histological parameters like increasing degrees of undifferentiation, anaplasia, and aggressiveness but also the genetic basis of tumorigenesis as listed in Figure 2.9. In this new classification system, glioblastomas are incorporated into the diffuse gliomas and divided into three groups, IDH-wildtype (90 per cent of all cases), IDH-mutant and NOS, not otherwise specified, for which full IDH evaluation cannot be performed. IDH wild-type glioblastoma is composed of three groups as giant cell glioblastoma, gliosarcoma and epithelioid glioblastoma and this group corresponds most frequently with the clinically defined primary predominate in patients over the age of 55. IDH-mutant glioblastoma patients are younger and carry mutations in isocitrate dehydrogenase (IDH) 1 and 2 and also have improved prognosis compared to wild-type IDH. According to this new classification system, neuroblastoma is incorporated into embryonal tumors [119].

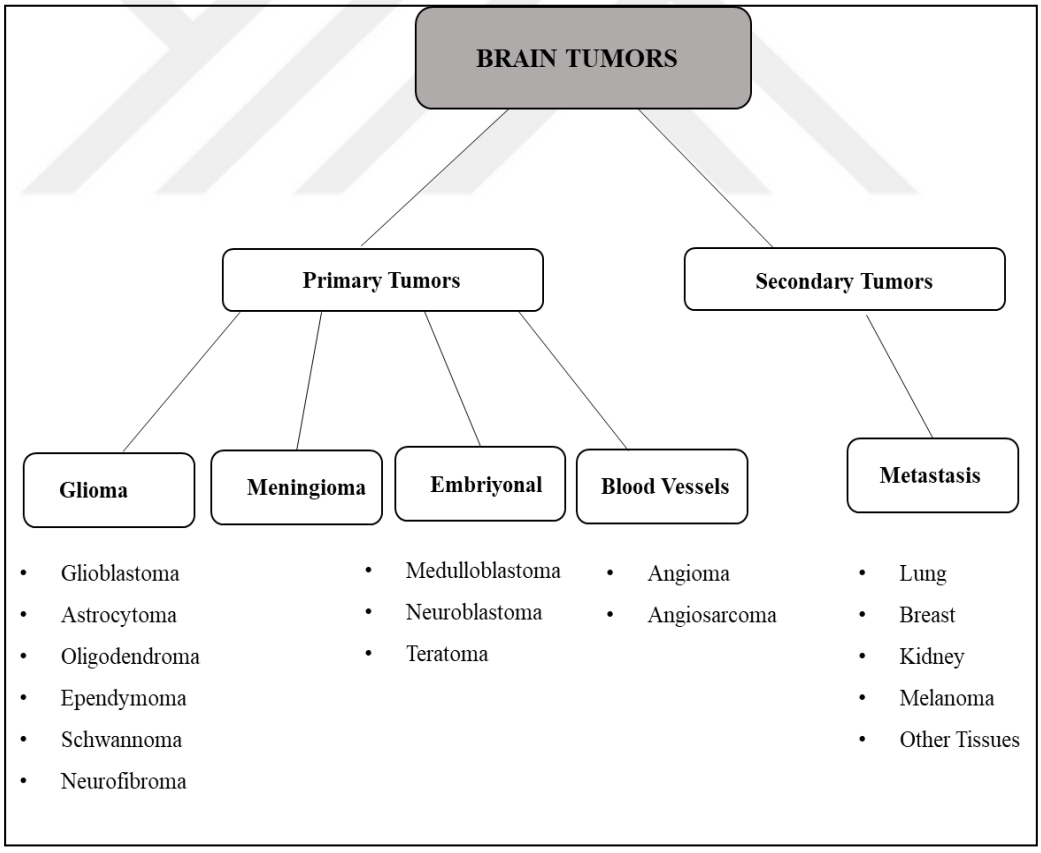


Figure 2.9. WHO classification of brain tumors [120].

2.3.1. Post-Translational Modifications

Post-translational modifications (PTMs) refers to the chemical changes of proteins by covalent and generally enzymatic modification of amino acids of primary protein sequences. Addition or removal of various groups to the side chain or at the protein's C or N terminal of the amino acid backbone of proteins increase the functional diversity of the proteome. [121].

PTMs are able to change the chemical structures of amino acid sequences by modifying an existing functional group or positioning such as phosphate, phosphorylation, or carbohydrate, glycosylation, which then promote protein folding, improve stability and regulatory functions. The covalent binding of a lipid group to a peptide chain referred as lipidation, often targets a protein and can affect the activity of the protein and/or alter its subcellular location. Histone modifications such as methylation (addition of a methyl group), ubiquitylation (covalent linkage of ubiquitin), acetylation (addition of an acetyl group), and SUMOylation (covalent linkage of SUMO) can effect gene expression by altering chromatin structure or through recruiting histone modifiers [122].

In response to specific environmental requirements, post-translational modifications play pivotal roles in many biological processes such as protein localization and function, interactions with other proteins, protein stability, enzymatic activity and regulation of signaling pathways [121]. PTMs can also be used as biomarkers, such as hydroxylation and nitration of aromatic amino acid residues or carbonylation targeting modified proteins for degradation resulting in the formation of protein aggregates, can be used for detection of oxidative stress [123]. Since modifications of histone proteins play important roles in gene expression alterations, they can be used as potential biomarkers of disease progression and prognosis [124]. Therefore, identification and analysis of PTMs are critical in the field of cell biology and the identification and treatment of complex diseases such as cancer.

Several experimental techniques can be used to identify and quantify PTMs, including EDMAN degradation, chromatography conducted with mass spectrometry, Eastern blotting, Western blotting, fluorescence spectrometry, peptide and antibody synthesis and *in silico* tools [125].

2.3.2. Phosphorylation of Proteins

Protein phosphorylation is one of the most widespread post-translational modifications and it regulates protein functions in response to extracellular signals, including growth factors, hormones, neurotransmitters, radiation, neurotrophic factors and all kind of cellular stress It is implicated in almost all aspects of cellular functions including, proliferation, differentiation, growth, survival and apoptosis. Virtually all types of extracellular signals, exert their biological and physiological effects by regulating phosphorylation of specific phosphoproteins in their target cells [126].

The target protein is converted into the phospho form dephosphorylated form by protein kinases, this process is reversed, the phosphorylated protein is converted back to the de-phospho form, by a protein phosphatase. The terminal (γ) phosphate group of ATPs is transferred to the hydroxyl group of target amino acid by protein kinases in the presence of Mg^{2+}, while this phospho-ester bond is cleaved by protein phosphatases through hydrolysis as illustrated in Figure 2.10. [127].

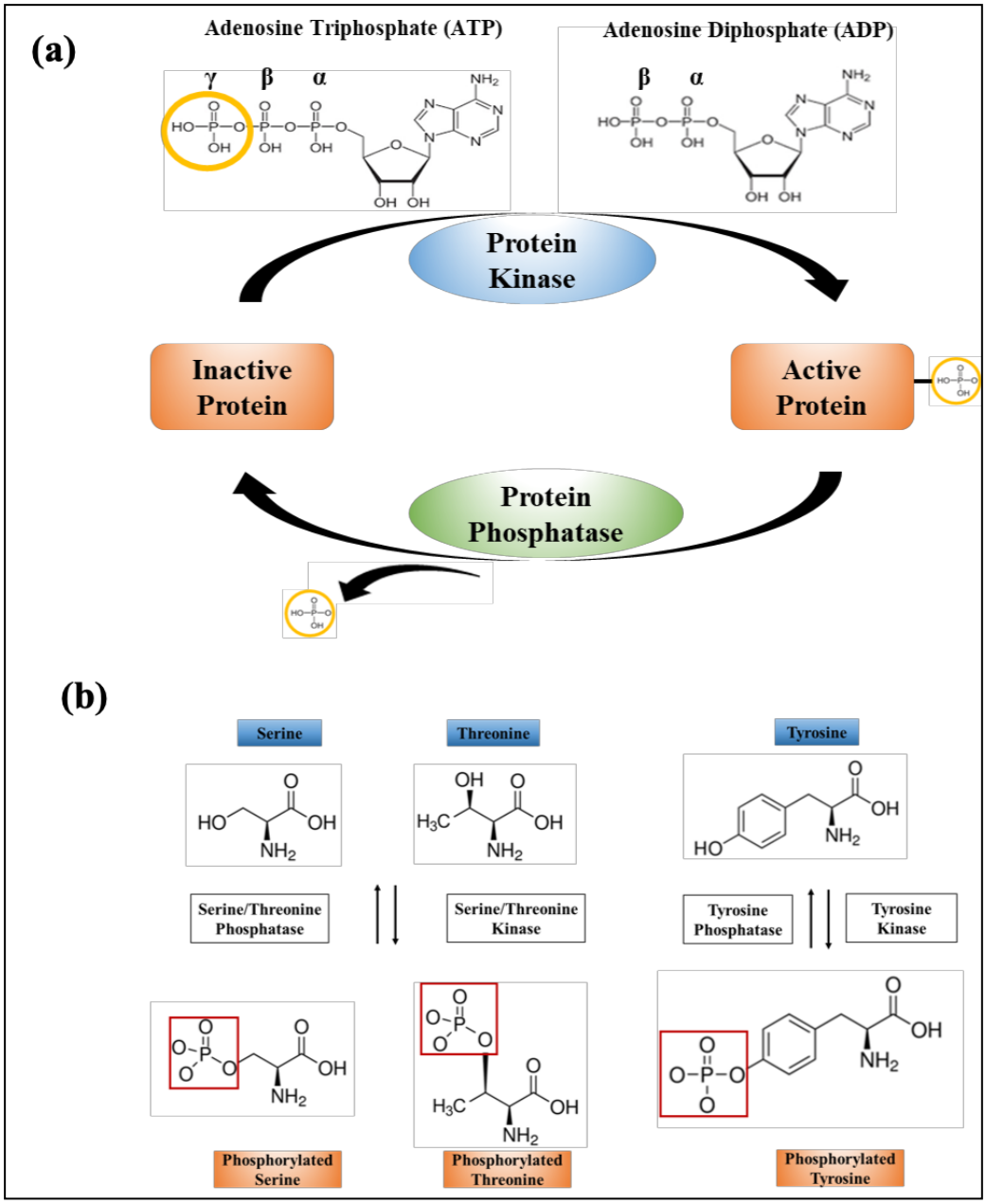


Figure 2.10. Protein phosphorylation and de-phosphorylation. (A) Mechanism of protein phosphorylation and de-phosphorylation by kinases and protein phosphatases, respectively. (B) Phosphorylated serine, threonine and tyrosine residues [29].

Protein kinases are mainly divided into three groups as serine-threonine kinases (STK), tyrosine kinases (TK) and dual specificity kinases (DSKs). STKs phosphorylate substrate proteins on serine or threonine residues (STKs), whereas tyrosine kinases phosphorylate only tyrosine residues. However, dual specificity kinases can phosphorylate substrates from both serine, threonine and tyrosine residues [29], but they predominantly phosphorylates serine residues, over 85 per cent of protein phosphorylation occurs on

serine residues followed by residues of threonine 10 to 15 per cent, whereas tyrosine residues are phosphorylated less 2 per cent. In addition to this residues, histidine and aspartate residues are rarely phosphorylated but both in cases, this phosphorylation is less stable than others [128]. Phosphatases play the opposite role of kinases, they remove the phosphate group from phospho-proteins by hydrolyzing phosphoric acid monoesters. Protein phosphatases are composed of three groups; phosphoprotein phosphatase, metallo-dependent phosphatase and tyrosine phosphatase [129].

Addition of phosphate modifies protein from hydrophobic apolar to hydrophilic polar which can then alter the conformation of the protein and ultimately its functional activity. This reversible process can change protein conformation and stability, protein-protein interactions, enzymatic activation or subcellular localizations [126].

Phosphorylation and de-phosphorylation dynamics are momentous in many cellular processes including protein synthesis, signal transduction, cell division, cellular growth, differentiation, development, aging and cell death. Since protein kinases convert extracellular stimuli into specific cellular responses, overactivity, malfunction or overexpression is implicated in several diseases, in particular, tumorigenesis and cancer progression, so they are ideal targets for cancer therapy lately [29].

2.3.3. Post Translation Modifications of Elk-1

Elk-1 activation is mainly dependent on the balance between different post-translational modifications. It is known that Elk-1 is regulated by three PTMs, phosphorylation, SUMOylation and ubiquitylation. SUMOylation and ubiquitylation are both reversibly modify lysine residues and affect stability, activity and subcellular localization of proteins by the attachment of SUMO and ubiquitin peptides. These modifications have diverse effects on proteins, for instance, ubiquitylation plays crucial roles in the regulation of transcriptional activity, by inducing proteasome-mediated degradation indirectly and by changing transcription factor features, directly [29].

Ubiquitylation is the attachment of ubiquitin to lysine residues via an isopeptide bond and composed of three main steps, activation by E1 enzymes, conjugation by E2 enzymes and attachment to the substrate protein by ubiquitin ligase E3, which signs protein for degradation by the proteasome [130].

In addition to ubiquitylation, SUMOylation also targets lysine residues and R domain of Elk-1 is rich in terms of lysine residues which are susceptible to be SUMOylated an it maintains Elk-1 in an inactive state before phosphorylation and activation by ERK pathway. Lysine 130- 249 and 254 residues potential SUMOylation sites within the R domain and SUMOylated Elk-1 recruits histone deacetylases (HDAC) to the promoter regions of target genes and leads to their repression [131].

Elk-1 is both localized at the nucleus and cytoplasm of the neuronal cells and upon stimulation cytoplasmic Elk-1 translocated to the nucleus to drive SRF mediated transcription of its target genes. Cytoplasmic localization of Elk-1 has been linked to neuronal differentiation and neurite elongation these effects has not been observed when Elk-1 restricted to the nucleus Nucleo-cytoplasmic translocation of Elk-1 is mainly regulated by SUMOylation and co-expression of SUMO-1 or SUMO-2 strongly diminishes shuttling of Elk-1. Overexpression of Lysine 130, 249 and 254 residues of Elk-1 enhances neurite extension inducing cytoplasmic localization [132].

Phosphorylation of two serine residues plays a pivotal role for Elk-1 driven regulation of many cellular processes, Serine 383 and Serine 389. Serine 383 phosphorylation is particularly important since a single mutation on this residue elicits functional inhibition of Elk-1. These residues are mainly phosphorylated by the three classes of MAPKs, ERK, JNK and p38 in response extracellular stimuli such as mitogens, growth factors, cytokines and cellular stress [133]. Blocking S383 or S389 or both T363 and S422 or both T417 and S422 extensively reduced transcriptional activation of Elk-1 [134].

Serine 383 phosphorylation of Elk-1 is important for the nucleo-cytoplasmic shuttling of Elk-1. This phosphorylation seems to play an opposite role as SUMOylation performs and induces nuclear translocation of Elk-1. The balance between SUMOylation and phosphorylation determines the fate of neuronal cells as illustrated in Figure 2.11 [20].

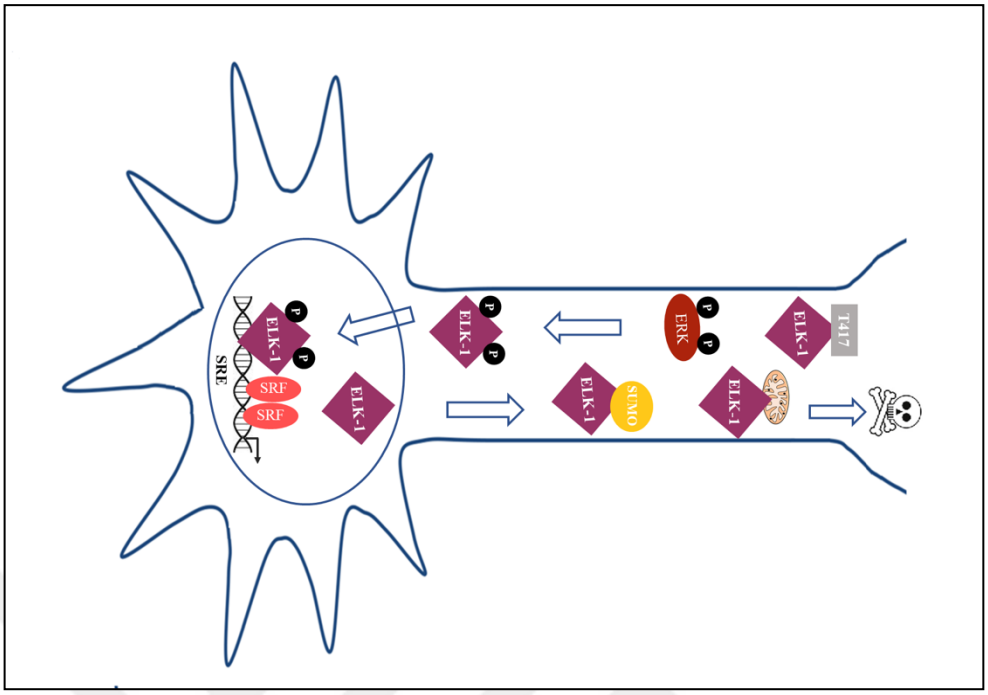


Figure 2.11. Impact of Elk-1 post-translational modifications on its localization and functions in neurons [20].

Threonine T417 is another phosphorylated residue of Elk-1 in neuronal cells but the phosphorylating kinase is still unknown. Elk-1 tends to be toxic in the cytoplasm, but when Threonine 417 phosphorylation of Elk-1 blocked by mutation, Elk-1 is not toxic anymore in hippocampal neurons. Threonine 417 phosphorylation of Elk-1 is involved in neuronal apoptosis induced through dendritic signaling mechanisms and increased phosphorylation of T417 detected in tissue samples from patients with neurodegenerative diseases [135]. SGK1 (serum- and glucocorticoid-inducible kinase 1) signaling pathway has been shown to negatively regulate Elk-1 transcriptional activity. Phosphorylation of Serine 159 and Threonine 160 residues of Elk-1 by SGK1 interrupts its binding to SRF. Phosphorylation of these residues by SGK1 (Serum- and Glucocorticoid-Inducible Kinase) interrupts Elk-1 binding to its cofactor SRF and thereby prevent its transcriptional activity. [136].

2.4. AIM OF THE THESIS

It has been previously shown in our laboratory that Elk-1, a transcription factor commonly studied as a mitogen-regulated activator of immediate-early genes, is present in post-mitotic neurons, can interact with microtubules and binds to microtubule-associated motor

proteins in a phosphorylation-dependent manner. Furthermore, it was shown that Elk-1 localized to mitotic spindle during mitosis which showed similarities to the localization of mitotic kinases at different stages of mitosis Interestingly, an FP7 MitoCheck consortium that had aimed to identify all genes critical for cell division, and to that end examined approximately 20000 genes in *Caenorhabditis elegans* using RNA interference, identified 1000 genes essential for mitosis which included Elk-1 as well as its transcriptional partner, Serum Response Factor, SRF [137]. In the light of these evidence, this thesis aimed to further study the potential mitotic role of Elk-1 by investigating its interaction with and phosphorylation by mitotic kinases Aurora A, Aurora B, Plk1 and Cdk1. We also aimed to understand the nature and implications of these interactions with respect to brain tumorigenesis.

3. MATERIALS

3.1. CELL LINES AND BACTERIAL STRAINS

- A172- Human Glioblastoma Cell Line (ATCC Number: CRL-1620,)
- C6 – Rat Glioblastoma Cell Line (ATCC number: CCL-107)
- E. coli BL21 (DE)pLysS strain was from Promega (Madison)
- E.coli DH5α bacterial strain
- HEK293T- Human Embryonic Kidney Cell Line (ATCC Number: CRL-1573)
- SH-SY5Y – Human Neuroblastoma Cell Line (ATCC number: CRL-2266)
- SK-N-BE (2)- Human Neuroblastoma Cell Line (ATCC Number: CRL-2271)
- T98G- Human Glioblastoma Cell Line (ATCC Number: CRL-1690)
- U87 – Human Glioblastoma Cell Line (ATCC number: HTB-14)

3.2. COMMERCIAL KITS AND REAGENTS

- DNA-Midi Plasmid DNA Purification Kit (Intron)
- DNA-Spin Plasmid Purification Kit (Intron)
- High Pure Plasmid Isolation Kit (Roche)
- i-*Taq* DNA polymerase (Intron 25022)
- *Long* PCR enzyme Mix (Thermo #K0182)
- Mega-Quick-Spin PCR&Agarose Gel Extraction Kit (Intron)
- MTT Cell Proliferation Assay Kit (Intron, 21180)
- PEI Transfection Reagent (BioSciences)
- ProtoScript First Strand cDNA Synthesis Kit (NEB #E6300S)
- Pure-Link RNA Mini Kit (Thermo)
- Q5-Site-Directed Mutagenesis Kit (NEB #E0554S)
- T4 DNA Ligase (NEB #M0202S)
- WST-1 Cell Proliferation Assay Kit (Intron, 15092)
- XTT Cell Proliferation Assay Kit (Cell Signaling #9095)
- Universal Kinase Assay Kit (Abcam, ab138879)

3.3. PLASMID CONSTRUCTS

- pcDNA3
- pcDNA3 Elk-1
- pCMV-Elk-1
- pCMV- S383A-Elk-1
- pCMV- S383E-Elk-1
- pCMV- S389A-Elk-1
- pCMV- S389E-Elk-1
- pCMV- T417A-Elk-1
- pCMV- T417E-Elk-1
- pGEX-4T2
- pGEX-4T2-Elk-1(1-93)
- pGEX-4T2-Elk-1(1-205)
- pGEX-4T2-Elk-1(205-428)
- pGEX-4T2-Elk-1(349-428)
- pcDNA4/TO-Plk1-NT
- pcDNA4/TO-Plk1-T210A-NT
- pcDNA4/TO-Plk1-T210D-NT

3.4. CELL CULTURE EQUIPMENTS

- Dulbecco's Modified Eagle Medium (DMEM) 1g/liter Glucose (Gibco)
- DMSO
- Dulbecco's Modified Eagle Medium (DMEM) 4.5g/liter glucose (Gibco)
- Fetal Bovine Serum (Gibco)
- L-Glutamine Solution (100X) (Gibco)
- Antibiotic-Antimycotic Solution, 100 X (Gibco)
- 0.5 per cent Trypsin-EDTA Solution (Sigma)
- Penicillin/streptomycin (100 IU/ml and 100 μg/ml)
- Phosphate Buffered Saline (PBS) (Gibco)
- Poly-l-lysine (Sigma)

- Hemocytometer (Bright-Line)
- T25 Tissue Culture Flasks (Nunc)
- T75 Tissue Culture Flasks (Nunc)
- T150 Tissue Culture Flasks (Nunc)
- Coverslip (Isolab)

3.5. BACTERIAL ASSAYS

- Ampicillin (AppliChem)
- Bacterial Protease Inhibitor Coctail (Sigma)
- Chloramphenicol (Applichem)
- IPTG (Invitrogen)
- Luria-Bertani (LB) Agar (AppliChem)
- Luria-Bertani (LB) Broth (AppliChem)
- Petri Plates
- Phenylmethylsulfonyl fluoride (PMSF) (AppliChem)
- Sodium Deoxycholate (Sigma)
- Sodium Orthovanadate (NA3VO4) (Sigma)

3.6. PROTEIN ASSAYS

- 2-Mercaptoethanol (Gibco)
- 3MM Whatman Filter Paper (Whatman)
- 3X Blue SDS Loading Buffer (Cell Signaling Technologies 7722S)
- Alisertib (MLN8237) Aurora A Inhibitor (Seleckhem, S1133)
- ATP (10 mM) (Cell Sigmaling #9804)
- Aurora A kinase (Millipore 14-511)
- Aurora B kinase (Cell Signaling #7394)
- Anti-Biotin HRP Linked Antibody (Cell Signaling Technologies #7075)
- Anti-Flag M2 Affinity Gel (Sigma, A2220)
- Biotinylated Protein Ladder (Cell Signaling Technologies #7727)
- BI-2536, Plk1 Inhibitor (Enzo Life Seciences, ENZ-CHM160-0005)

- Cell Culture Plates (TPP or Grenier-Bio)
- Cdk1/Cyclin B Protein (Milliopre 14-450)
- Crescendo Western HRP Substrate (Millipore)
- Filter Paper (Millipore)
- Glutathione Sepharose 4B (Sigma)
- Goat anti-rabbit IgG–Alexa Fluor 488 (Invitrogen)
- Goat anti-rat IgG–Alexa Fluor 546 (Invitrogen)
- Goat anti-mouse IgG–Alexa Fluor 647 (Invitrogen)
- Goat Anti-Mouse IgG Antibody-HRP Conjugate (Santa Cruz Biotechnology, sc-2005)
- Goat Anti-Rabbit IgG Antibody-HRP Conjugate (Santa Cruz Biotechnology, sc-2004)
- Lambda Phosphatase (Santa Cruz, sc-200312A)
- Mouse Monoclonal Anti- Plk1 Antibody (Abcam, ab17056)
- Rabbit Monoclonal Anti Cdk1 Antibody (Abcam)
- Nitrocellulose Membrane (Santa Cruz Biotechnology)
- Phosphatase Inhibitor Cocktail (Roche)
- PageRuler Plus Prestained Protein Marker (Thermo, 26619)
- Prolong Gold Antifade Reagent (Invıtrogen)
- Protease Inhibitor Cocktail (Roche)
- Protein A Agarose Beads (Santa Cruz Biotechnology, sc-2001)
- Protein G Agarose Beads (Santa Cruz Biotechnology, sc-2002)
- Mouse Monoclonal anti-β -Actin Antibody (Santa Cruz Biotechnology, sc-47778)
- Mouse Monoclonal anti-β-Tubulin Antibody (Santa Cruz Biotechnology, sc58884)
- Mouse Monoclonal anti-Aurora A Antibody (BD Biosciences 610938)
- Mouse Monoclonal anti-Aurora B Antibody (BD Biosciences 611082)
- Mouse Monoclonal anti-Elk-1 Antibody (Santa Cruz Biotechnology sc-365876)
- Mouse Monoclonal anti-Plk1 Antibody (Abcam [35-206] ab17056)
- Mouse Monoclonal anti-Flag Antibody (Sigma, F1804
- PhosphoPlus Elk-1 (Ser383) Antibody Kit (Cell Signaling #9180)
- Plk1 Kinase (Cell Signaling #7728)
- Rabbit Monoclonal anti-Cdk1 Antibody (Abcam [E161] ab32384]
- Rabbit Polyclonal anti-Cyclin B1 Antibody (Cell Signaling #4138)
- Rabbit Polyclonal anti-Elk-1 Antibody (Cell Signaling #9182)

- Rabbit Polyclonal anti-GST Antibody (Cell Signaling #2622)
- Rabbit Polyclonal anti-Phospho-Elk1 Ser383 Antibody (Cell Signaling #9181)
- Rabbit Polyclonal Phospho-(Ser/Thr) Antibody (Cell Signaling #9631)
- Rabbit Polyclonal anti SRF Antibody (Santa Cruz Biotechnology, sc-335)
- Rabbit Polyclonal anti-Histone H3 (Ser10)- Alexa Fluor 488 Conjugate Antibody (Cell Signaling #9708)
- Rabbit Polyclonal anti-Phospho-p44/42 MAPK (Erk1/2) (Cell Signaling #9102)
- RO-3306 Cdk1 Inhibitor (Sigma, SML0569)

3.7. LABORATORY TECHNICAL EQUIPMENT

- 0.5, 1.5 and 2 ml tubes (Beckman)
- -80 °C freezer (Wisecryo)
- Autoclave (HV- 85–Hiclave)
- Blot Transfer (Trans-Blot SD Semi-Dry Transfer Cell- BioRad)
- ChemiDoc Imaging System (Biorad)
- Chemiluminescence Imaging System (Bio-Rad)
- CO_2 incubator (Nuaire)
- Confocal Microscope (Zeiss, LSM 880)
- Flow Cytometer, BD Accuri C6
- Heater (DRI-Block DB.2A–echne)
- Hotplate Stirrer (Scilogex)
- Inverted phase contrast microscope (Nikon)
- Laminar flow cabinet (ESCO Labculture Class II Biohazard Safety Cabinet Type 2A)
- Magnetic Stirrer (RH Basic- KIKA Labotechnic)
- Microcentrifuge (Micro 1224 – Hettich Zentrifugen)
- Micro-volume spectrofotometer, Nanodrop (Shimatzu, BioSpec-nano)
- Mini-PROTEAN Tetra Cell Electrophoresis System (Bio-Rad)
- Refrigerator (Nu- 6512E- NuAire and Surround Flow- Arçelik)
- Semi-Dry Transfer System (Bio-Rad)
- Shaker (Innova 4330–New Brunswick Scientific)
- Spectrophotometer (Multiscan Spectrum- Thermo Labsystems)

- Ultra-speed Centrifuge (Avanti J-251 - Beckman)
- Water Bath (Memmert)
- Varioskan Microplate Reader (Thermo)

3.8. CHEMICALS

- 29:1 Acrylamide: bisacrylamide (AppliChem)
- 2-propanol (AppliChem)
- Acetic Acid (AppliChem)
- Ammonium persulphate (APS) (AppliChem)
- Beta-Glycerophosphate (Santa Cruz Biotechnolgy, sc220452)
- Bovine Serum Albumin (BSA) (Sigma)
- Bromophenol Blue (Applichem)
- Calcium Chloride ($CaCl_2$) (AppliChem)
- Dithiothreitol (DTT) (AppliChem)
- Ethanol (Sigma)
- Glycine (Promega)
- HCl (AppliChem)
- Methanol (Sigma)
- NaCl (AppliChem)
- NaOH (AppliChem)
- Nocodazole
- Non-Fat Dry Milk
- NP-40 10 per cent (Abcam)
- Ponceau S Stain (AppliChem)
- Propidium Iodide (Sigma)
- Sodium Chloride (NaCl) (Sigma)
- Sodium dodecyl sulphate (SDS) (AppliChem)
- TEMED (AppliChem)
- Tris (AppliChem)
- Triton X-100 (AppliChem)
- Tween-20 (AppliChem)

4. METHODS

4.1. PREPARATION OF PLASMID CONSTRUCTS

4.1.1. Gradient PCR

To clone Aurora A, Aurora B and Cdk1 coding sequences into mammalian expression vectors, specific primer pairs were designed initially, as listed in Table 3.1. Primers were designed considering guanine-cytosine (GC) content, melting temperature (Tm), nucleotide repetitions and presence of hairpin or dimer and binding specificity.

All these criteria were analyzed by bioinformatic tools including oligoDT analyzer and NCBI Blast. Restriction analysis of primers and plasmid constructs evaluated by Biology WorkBench bioinformatics tool and suitable restriction enzymes were chosen. In order to determine the specificity of the designed primer pairs, Primer Blast software developed by NCBI was used. Designed primers were ordered to Macrogen Europe at a concentration of 0.025 µM and reconstituted in sterile distilled water at a final concentration of 500ng/µl.

Table 4.1. Primer pairs of the cloned genes

Aurora A
Forward: 5’ AGACCTCGAGACGACCGATCTAAAGAAAAC TGC 3’ **Reverse:** 5’ AGACGGTACCCTAAGACTGTTTGCTAGCTG 3’
Aurora B
Forward: 5’ TAAGGAATTCGAGCCCAGAAGGAGAACTCCTAC 3’ **Reverse:** 5’ ATATCTCGAGTCAGGCGACAGATTGAAGGGCAGA 3’
Cdk1
Forward: 5’ GGGCGAATTCCCGAAGATTATACCAAAATAGAG 3’ **Cdk1 Reverse**: 5’ GGGCCTCGAGCTACATCTTCTTAATCTG 3’

Aurora A, Aurora B, Cdk1 and Plk1 coding sequences of were cloned into mammalian expression vectors pCMV-HA or pCMV-Myc purchased from Clontech, illustrated in Figure 3.1. To obtain cDNA from the cells, *Reverse transcription polymerase chain*

reaction (RT-PCR) was performed. Obtained cDNA was used as a template for cloning and melting temperature determination.

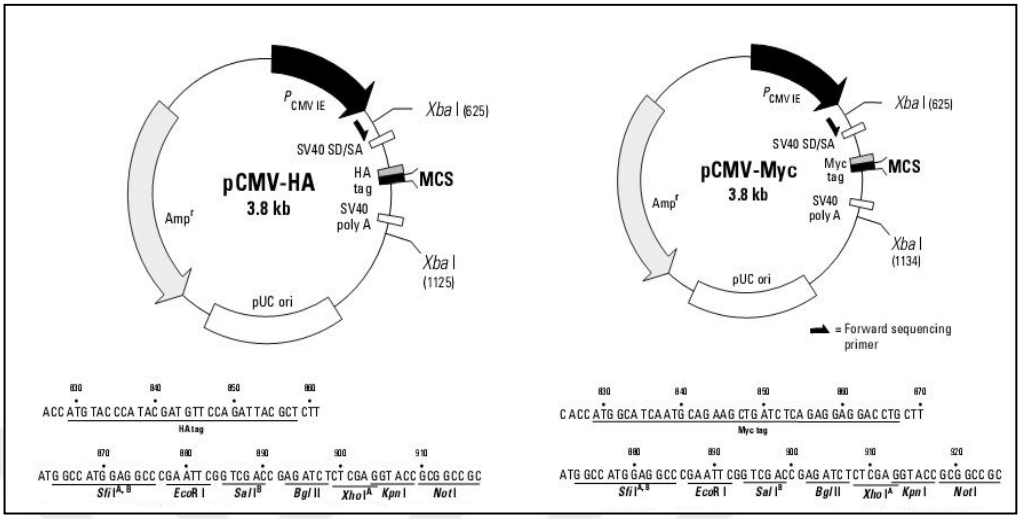


Figure 4.1. pCMV-HA-N and pCMV-Myc-N plasmid maps [Clontech].

To determine optimum annealing temperature (Ta) values of the primer pairs designed which are listed above, gradient PCR was used. For each primer pairs, eight identical reaction mixtures were prepared for each Ta value in the gradient temperature range. The reaction mixtures were prepared according to the Table 3.2. Cycling program and temperatures for each cycle were determined for *i-Taq* DNA polymerase enzyme submitted in Table 3.3.

Table 4.2. A typical reaction mixture for i-Taq DNA polymerase

Component	**Amount**
PCR Buffer (10X)	2 µl
dNTP Mixture (2.5 Mm each)	2 µl
Forward & Reverse Primer (each)	1 µl
Template	Variable
i-Taq DNA Polymerase (5U/µl)	0.2 µl
Nuclease-Free Water	Variable
Total Volume	20 µl

Table 4.3. Thermal cycling program set for gradient PCR

Step		Temperature °C	Duration
Initial Denaturation		95	2 minutes
Denaturation	30 cycles	95	30 seconds
Annealing		Optimized T_a	30 seconds
Extension		72	2 minute/kb
Final Extension		72	10 minutes

4.1.2. PCR for Amplification of Inserts for Clonings

The amplification of the insert sequences was carried out using *Long* PCR enzyme since the *i-Taq* DNA Polymerase lacks proof-reading activity and may cause changes in DNA sequences. PCR reactions were carried out as submitted in Table 3.4 and 3.5.

Table 4.4. PCR reaction mixture for Long PCR enzyme

Component	Amount
10X Long PCR Buffer with 15 mM MgCl2	5 µl
dNTP Mixture (2.5 Mm each)	3 µl
Forward Primer	2 µl
Reverse Primer	2 µl
Template	Variable
Long PCR Enzyme Mix (5 U/µL)	0.5 µl
Nuclease-Free Water	Variable
Total Volume	50 µl

Table 4.5. Thermal cycling program set for Long PCR enzyme

Step		**Temperature °C**		**Duration**
Initial Denaturation		94		2 minutes
Denaturation	35 cycles	94		20 seconds
Annealing		Aurora A	62	30 seconds
		Aurora B	58	
		Cdk1	58.7	
		Plk1	61	
Extension		68		2 minute/kb
Final Extension		68		10 minutes

Amplified PCR products and empty plasmids were cut restriction enzymes for 1 hour at 37°C. Linearized plasmids and cut DNA sequences were ligated by T4 DNA ligase (NEB) as the ingredients were listed in Table 4.6.

Table 4.6. Ligation Protocol with T4 DNA Ligase

Component	**Amount**
T4 DNA Ligase Buffer 10X	2 µl
Vector DNA	50 ng
Insert DNA	Variable
T4 DNA Ligase	1 µl
Nuclease-Free Water	Variable
Total Volume	20 µl

4.1.3. Transformation

In order to transfer newly prepared plasmids into suitable competent bacteria, ligated plasmids were transformed. The transformation reaction was prepared by mixing 5 µl of plasmid and 100 µl of competent bacteria which is prepared by $CaCl_2$ treatment. The positive control was prepared to check transformation efficiency with a well-established plasmid and negative control was prepared with nuclease-free water to check the presence of any contamination. The mixtures were incubated on ice for 15 minutes, at 37°C for 90 seconds and once again on ice for 2 minutes. 200 mL of LB broth solution without any antibiotics was added into each tube. The tubes were incubated in a rotator at 37°C at 180-

200 rpm/min for one hour. This incubation time is critical for the bacteria to have time to express its antibiotic resistant gene present. Then, the mixtures were spread onto antibiotic-containing LB agar plates and incubated at 3°7C overnight. The plates with transformant bacteria colonies were stored at 4°C up to one month.

4.1.4. Site-Directed Mutagenesis (SDM)

The coding sequence of Elk1 was previously cloned into the pCMV-5-Flag plasmid by Prof. A.D. Sharrocks (UK). Target serine (S) or Threonine (T) amino acids were mutated to alanine (A) or glutamic acid (E) residues by specific primers designed according to NEBaseChanger tool as listed in Table 3.7. NEB Q5 site-directed mutagenesis kit was used to mutated target sequences on the pCMV-5-Flag-Elk1 plasmid. The ingredients of the PCR reaction are given in Table 3.8. and cycling conditions are submitted in Table 3.9.

Table 4.7. Mutagenic primer sequences

Primer Name		**Primer Sequence**
S106A	Forward	5’ GCCAGAGGTG**G**CTGTTACCTCC 3’
	Reverse	5’ TGGGGCGGGCAGTCCTCA 3’
S106E	Forward	5’GCCAGAGGTG**GAA**GTTACCTCCACCATGCCAAATG3’
	Reverse	5’ TGGGGCGGGCAGTCCTCA 3’
T108A	Forward	5’ GGTGTCTGTT**G**CCTCCACCATGCCAAATG 3’
	Reverse	5’ TCTGGCTGGGGCGGGCAG 3’
T108E	Forward	5’GGTGTCTGTT**GAA**TCCACCATGCCAAATGTGGCCC 3’
	Reverse	5’ TCTGGCTGGGGCGGGCAG 3’
T133A	Forward	5’ AGCCAGGC**G**CACCCAAGGGT 3’
	Reverse	5’ CCAGAGACAGTGTCCCCT 3’
T133E	Forward	5’ AAAGCCAGGC**GA**ACCCAAGGGTG 3’
	Reverse	5’ CCAGAGACAGTGTCCCCT 3’
S149A	Forward	5’ GGCACGCAGC**GCC**CGGAACGAGTACATG 3’
	Reverse	5’ AAACCGCCTGGGCCTGCC 3’
S149E	Forward	5’ GGCACGCAGC**GAA**CGGAACGAGTACATGCG 3’
	Reverse	5’ AAACCGCCTGGGCCTGCC 3’

S198A	Forward	5’ GCGCCCCCCTCGGGGAGCAGG**GC**CACCAGT 3’
	Reverse	5’CCTGCTCCCCGAGGGGGGCGCTGCTGCCCC 3’
T198E	Forward	5’ GGGGAGCAGGG**AA**ACCAGTCCAAGCCCCTTG 3’
	Reverse	5’ GAGGGGGGCGCTGCTGCC 3’
T199A	Forward	5’ GCGCCCCCCTCGGGGAGCAGGAGCGC**C**AGTCCA 3’
	Reverse	5’ GCTCCTGCTCCCCGAGGGGGGCGCTGCTGC 3’
T199E	Forward	5’ GAGCAGGAGCG**AA**AGTCCAAGCCCCTTGGAGGCC 3’
	Reverse	5’ CCCGAGGGGGGCGCTGCT 3’
S200A	Forward	5’CCCTCGGGGAGCAGGAGCACC**GC**TCCAAGC 3’
	Reverse	5’ GGTGCTCCTGCTCCCCGAGGGGGGCGCTGC 3’
S200E	Forward	5’ CAGGAGCACC**GAA**CCAAGCCCCTTGGAGGCC 3’
	Reverse	5’ CTCCCCGAGGGGGGCGCT 3’
S202A	Forward	5’ CACCAGTCCA**G**CCCCCTTGGAGGCC 3’
	Reverse	5’ CTCCTGCTCCCCGAGGGG 3’
S202E	Forward	5’ CACCAGTCCA**GAA**CCCTTGGAGGCCTGTC 3’
	Reverse	5’ CTCCTGCTCCCCGAGGGG 3’
S303A	Forward	5’ CCATGCGGCT**GCC**AGCCCTGAGATCTC 3’
	Reverse	5’ CCGCCCGCCTGCCCTGCG 3’
S303E	Forward	5’ CCATGCGGCT**GAA**AGCCCTGAGATCTCCCAGCC 3’
	Reverse	5’ CCGCCCGCCTGCCCTGCG 3’
S304A	Forward	5’ TGCGGCTTCC**GCC**CCTGAGATCTCCCAGCCGCAG 3’
	Reverse	5’ TGGCCGCCCGCCTGCCCT 3’
S304E	Forward	5’TGCGGCTTCC**GAA**CCTGAGATCTCCCAGCCGCAGAAG 3’
	Reverse	5’ TGGCCGCCCGCCTGCCCT 3’
S324A	Forward	5’ GCTTCCACTC**GCC**CCGAGCCTGC 3’
	Reverse	5’ TCTAGGTCCCGGGGCTTC 3’
S324E	Forward	5’ GCTTCCACTC**GAA**CCGAGCCTGCTAGG 3’
	Reverse	5’ TCTAGGTCCCGGGGCTTC 3’
S326A	Forward	5’ ACTCAGCCCG**GCC**CTGCTAGGTG 3’
	Reverse	5’ GGAAGCTCTAGGTCCCGG 3’
S326E	Forward	5’ ACTCAGCCCG**GAA**CTGCTAGGTGG 3’
	Reverse	5’ GGAAGCTCTAGGTCCCGG 3’

Table 4.8. Exponential amplification of mutant Elk-1 sequences

Component	25 µl Reaction	Final Concentration
Q5 Hot Start High Fidelity 2X Master Mix	12,5µl	1X
10µM Forward Primer	1,25 µl	0,5 µM
10µM Reverse Primer	1,25 µl	0,5 µM
Template DNA (50 ng/µl)	0,5	50 ng/µl
Nuclease Free Water	9,5	-

Table 4.9. Cycling conditions

Step	Temperature (°C)	Time
Initial denaturation	98	30 seconds
25 cycles	98	10 seconds
	70	20 seconds
	72	120 seconds
Final Extension	72	120 seconds
Hold	4	∞

After PCR reaction, amplified sequences were exposed to Kinase-Ligase-DpnI (KLD) reaction and removal of template DNA by DpnI restriction endonuclease cutting, for 10 minutes at room temperature Plasmids containing mutated Elk-1 sequences were transformed into highly efficient *E. coli* and analyzed by sequencing.

Table 4.10. KLD reaction conditions

Component	Volume	Final Concentration
PCR Product	1	-
KLD Reaction Buffer (2X)	5	1X
KLD Enzyme Mix (10X)	1	1X
Nuclease-Free Water	3	-

4.2. CELL CULTURE

In this study, the experiments were performed on different brain tumor cells SH-SY5Y (CRL-2266, Human Neuroblastoma Cell Line), SK-N-BE (2) (CRL-2271, Human Neuroblastoma Cell Line) U-87 (HTB-14, Human Glioblastoma Cell Line) and C6 (CCL-107, Rat Glioma Cell Line). A172 (CRL-1620, Human Glioblastoma Cell) and T98G (CRL-1690 Human Glioblastoma Cell Line) cells were a gift from Assist. Prof. Tugba Bagci Onder, Koc University. As normal cell line control, HEK293 (CRL-1573, Human Embryonic Kidney Cell Line) cells were used.

For the culture of the cells, 4.5 g/L glucose containing DMEM medium was used as a growth medium. It was supplemented with 10 per cent (v/v) FBS solution and 1 per cent (v/v) penicillin/streptomycin (100 IU/ml and 100 µg/ml, respectively) solution before use. Cells were grown in the humidified tissue culture incubator at 37°C and 5 per cent CO_2 conditions until they reach at 80-90 per cent confluency at cell culture dish. When cells were reached at that confluency, they were sub-cultured. Medium covering the cells was discarded and cells were washed with PBS to remove medium completely. To remove cells, form the surface of the dish, they were treated with 0.25 per cent (v/v) trypsin (0.5 g/L)-EDTA (0.2 g/L) solution for two minutes. Cell counting was performed before transfection experiments by using hemocytometer and the desired number of cells were seeded into a new flask.

Cells were freeze for further studies in freezing media containing 90 per cent (v/v) complete medium and 10 per cent (v/v) dimethyl sulfoxide (DMSO) at 1.10^6 cells/mL. Cells in freezing media were transferred into cryovials and kept at -20^0C freezer for 2 hours. The cryovials were transferred to -80^0C freezer and then put into the liquid nitrogen for long term storage.

4.2.1. Transient Transfection

SH-SY5Y and U87 cells were seeded into 10 cm tissue culture dishes at 1.5 x 10^6 cells or into 6 well plates at $3x10^5$ cells and incubated for 24 hours for the attachment of cells. Transfection mixtures were prepared by combining 1 ml serum-free high without antibiotics, 100 µl of plasmid at a concentration of 100 ng/µl and 35 µl PEI reagent for 1.5 x 10^6 cells. The mixture was vortexed and incubated for 15 minutes at room temperature

for the formation of Polyethylenimine (PEI)/DNA complexes. 4 ml complete medium containing 10 per cent FBS and antibiotics was supplemented onto the transfection mixture and the ultimate mixture was added onto the cells. The cells were incubated at 37°C for two hours and the medium volume was completed to 10 mL by adding 5 ml complete medium. Cells were further placed into the 37^0C incubator and incubated for 48 hours to provide sufficient time for the expression of transferred genes.

4.2.2. Protein Lysate Preparation

Cells seeded on cell culture dish/plate were treated with RIPA Buffer, 50mM Tris-HCI, pH 7.4, 1 per cent NP-40, 0.5 per cent Na-deoxycholate, 0.1 per cent SDS, 2mM EDTA and 150 mM NaCI, to lyse and prepare protein extraction. 1 ml of RIPA buffer supplemented with Phosphatase and Proteinase Inhibitors (final concentration 1X) was dispensed to dishes and cells were incubated at 4°C for 15 minutes Cells were scraped by using a scraper and were transferred to 1.5 ml eppendorf tubes. Tubes were centrifuged at 4^0C for 15 minutes at 15000 rpm and the supernatant was transferred to a new tube and stored at -20°C for several months.

4.2.3. BCA (Bicinchoninic acid) Assay

To determine protein concentrations, proteins were diluted 1:5 in sterile water and 25 µl protein sample mixed with 200 µl BCA solution prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A: B). The mixture was transferred to 96 well plate and incubated at 37°C for 30 minutes. The colorimetric reaction was measured by microplate reader at 562 nm.

4.3. CO-IMMUNOPRECIPITATION (CO-IP) ASSAYS

Immunoprecipitation is a technique that is widely used to analyze protein-protein interactions. This technique comprises precipitation of protein of interest out of a protein lysate in the presence of an antibody that specifically binds to target protein. Antibody-bound protein, then, can be isolated by using an antibody conjugated solid structure [138].

250 µg protein lysate prepared from SH-SY5Y human neuroblastoma cells was pre-cleared by 0.5 µg non-specific rabbit or mouse IgG for one hour at 4 °C. 50 µl of Protein A/G agarose bead slurry was added onto the samples and incubated on a rocking platform for 90 minutes at 4 °C. Protein/IgG/Protein A/G complex was precipitated by centrifugation and supernatant was kept. Precleared cell lysates then incubated with 1-2µg rabbit anti-Elk-1, mouse anti-Aurora A, mouse anti-Aurora B, mouse anti-Cdk1 and rabbit anti-Plk1 antibodies for two hours at 4 °C. 50 µl protein A/G agarose bead slurry was added into the protein lysate-antibody mixture and incubated for 2 hours at 4°C. Beads were washed with lysis buffer and dissolved in SDS loading buffer. Samples were analyzed with Western blotting.

4.3.1. Nocodazole Treatment

Nocodazole is a reversible anti-neoplastic compound which causes microtubule depolymerization by binding tubulin dimers and alters microtubule dynamics. It is frequently used to study microtubule functions and to synchronize cells by arresting cell-cycle at the G2/M phase [2].

In order to analyze the interaction between Elk-1 and mitotic kinases, 1.5 x 10^6 U-87 cells were seeded into the 10 cm tissue culture dishes and incubated for 24 hours for attachment. Cells were treated with 100 ng/ml nocodazole for 16 hours and after this time period, cells were released from nocodazole by incubating with growth medium and collected at 30-min intervals, 0, 30, 60 and 90 minutes, by mitotic shake-off. The cell pellet was lysed in RIPA buffer including phosphatase inhibitor cocktail and protease inhibitor cocktail and incubated 15 minutes on ice and then centrifuged at 14,000 rpm at 4 °C for 15 minutes.

Protein concentrations were determined by BCA assay and 250 µg total protein was incubated with 1-2µg rabbit anti-Elk-1, mouse anti-Aurora A, mouse anti-Aurora B, mouse anti-Cdk1 and rabbit anti-Plk1 antibodies for overnight at 4 °C. Protein A/G agarose beads were precleared by washing two times with PBS and two times with RIPA buffer and 50 µl protein A/G slurry was added into the protein lysate-antibody mixture and antibodies were pulled down for an additional 2 2 hours at 4 °C on a rotator. The tubes were centrifuged at 1000g for 2 minutes at 4 °C and supernatant was carefully remove. Beads were washed with RIPA for three times and resolved in SDS-loading buffer.

After the last wash, the supernatant was removed, and beads were dissolved in 20 µl SDS loading buffer. They were heated at 95 °C for 5 minutes and then centrifuged at 10.000g for 5 minutes to pellet the beads. The beads were stored at 4 °C until the Western blot.

4.3.2. Flag-Immunoprecipitation (IP)

SH-SY5Y were spread into 10 cm cell culture dishes at 1.5x106 cells/dish. After 24 hours, cells were transfected with either 10 µg of wild-type pCMV-Elk-1-Flag plasmid, mutant Elk-1 plasmids or with empty pCMV-Flag vector by PEI transfection reagent. After 2 days, cells were lysed in lysis buffer and 500 µg total protein was incubated with flag-garose beads overnight at 4°C. Beads were washed two times with lysis buffer 4and two times with wash buffer (50 mM Tris–HCl, 150 mM NaCl, pH7.4). After the last wash, beads were dissolved in 30 µl SDS loading buffer and heated at 95 °C for 5 minutes and then centrifuged at 10.000g for 5 minutes to pellet the beads. Beads were analyzed with Western blotting.

4.4. GST PULL-DOWN ASSAY

GST-pull down analysis can confirm the existence of a protein-protein interaction predicted by other techniques or identifies previously unknown protein-protein interactions. The technique relies on the fusion of the protein of interest to Glutathione-S-transferase (GST) protein. Glutathione-S-transferase (GST) protein is used to isolate target protein from a solution and isolated protein is released from the GST by the administration of glutathione which has a high binding affinity to Glutathione-S-transferase. BL21(DE) pLysS competent cells allow high-efficiency protein expression of target gene under the control of a T7 promoter.

BL21(DE) pLysS bacterial stock was thawed and made competent by $CaCl_2$ treatment. Either empty pGEX-4T2 plasmid or the carrying Elk-1 deletion GST fusions (obtained from Prof. A.D. Sharrocks) were transformed into BL21(DE) pLysS bacteria. 33 µg/ml chloramphenicol and 50-200 µg/ml ampicillin were used as a selection marker.

One colony from the plate containing GST-fused plasmids was inoculated into 10 ml LB medium with ampicillin and chloramphenicol for overnight at 37 ºC with 200 rpm shaking.

Next day, 300 ml of LB Broth containing ampicillin and chloramphenicol were inoculated with 3 ml of overnight culture and grown until OD_{600} is 0.5-0.7.

0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was used to induce protein expression and bacteria were grown at 37 ºC for 4 hours. After 4 hours of protein production, bacteria were collected by centrifugation at 5000 rpm for 10 minutes. Pellets were resuspended in ice-cold PBS containing 1:1000 protease inhibitor cocktail, 1 mM sodium orthovanadate and 1 mM PMSF as phosphatase inhibitors to prevent protein degradation, 1 per cent Triton X 100 and 1Mm DTT. Bacteria were lysed by sonication and protein lysate was cleared by centrifugation at 4 ºC, and 15000 rpm for 15 minutes. Protein lysate was stored at -80 ºC for further analysis.

Bacterial protein expression was confirmed by SDS-PAGE and further Coomassie staining of the gel. Subsequent to gel electrophoresis, protein gel was washed with distilled water and incubated with gel staining solution, described in Table 4.11., and incubated on the shaker for 30 minutes at room temperature. Coomassie-stained gel was de-stained by the boiling with distilled water for a few minutes and captured by Bio-Rad Chemi-Doc system.

Table 4.11. Gel staining solution

Component	**Final Concentration (%)**
Methanol	45
Sterile Water	45
Acetic Acid	10
Coomassie Brilliant Blue	0.2

GST-Agarose powder was reconstituted in sterile distilled water and incubated at room temperature for 30 minutes. 50 µl GST-Agarose beads were incubated 500 µg protein lysate at 4 ºC for overnight on the shaker. At the final step, beads were washed twice with ice-cold PBS and once with 0,1 per cent Triton X-100 in PBS. Beads were resuspended in SDS loading buffer and analyzed with Western blotting.

4.5. WESTERN BLOTTING

Western blotting is used for detection of specific proteins transferred to a protein membrane, mostly after an immunoprecipitation or kinase assay. Proteins that resolved in

SDS-PAGE gel is transferred to a nitrocellulose or PVDF membrane and blocked with BSA or non-fat dry milk. The blocked membrane will be incubated with primary and secondary antibodies captured by the chemiluminescent imaging system.

10 per cent resolving (lower part) and 5 per cent stacking gels (upper part) were prepared according to Table 4.12. and the gel was incubated at room temperature for 1 hour for polymerization.

Table 4.12. Components of resolving and stacking gel buffers

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

Protein samples were prepared in 3X SDS loading buffer and denatured at 95°C for 5 minutes. Protein gels were placed into SDS-PAGE tank filled with tank buffer (Tris-base, glycine and SDS) and denatured protein samples and protein marker were loaded on gels. Voltage was adjusted to 80V until the samples run through the stacking gel and then the voltage was increased to 120V. After size separation of proteins in SDS-PAGE, the gel was either stained with Coomassie brilliant blue or it is directly processed for Western blotting.

Protein gels were transferred to nitrocellulose or PVDF, activated by methanol, membranes in transfer tank filled with transfer buffer (Tris and Glycine) and 20 per cent methanol for 1 hour at 100V. Membranes then washed with distilled water and unoccupied protein binding sites on the membrane was blocked with 3 per cent BSA or 5 per cent non-fat dry milk for 1 hour at room temperature. Membranes further incubated with mouse anti-Elk-1 1:1000, mouse anti-Aurora A (1:1000), mouse anti-Aurora B (1:1000), mouse anti-Plk-1 (1:2000) or rabbit anti-Cdk-1 (1:5000) antibodies for overnight at 4°C. The following day, membranes were washed three times with TBS-T (1:1000 Tween-20) solution for 10 minutes. HRP (Horseradish peroxidase) conjugated secondary antibodies were prepared in

1X TBS-T solution as 1:5000 dilution ratio and incubated with membranes for 1 hour at room temperature. Membranes were washed twice in 1X TBS-T and once with TBS for 10 minutes. Finally, membranes were incubated with Crescendo Western HRP Substrate (Millipore) and analyzed by Image Lab software of Bio-Rad Chemi-Doc system.

4.6. PROTEIN KINASE ACTIVITY ASSAY

Protein kinases are a group of proteins that modifies other proteins by adding phosphate groups from a molecule of ATP on a serine, threonine or tyrosine residues, referred as phosphorylation and change their activity, cellular localization or interaction with other proteins. In vitro kinase assay is widely used to quantify this enzymatic activity [139].

$1.5x10^6$ SH-SY5Y cells were seeded on 10 cm tissue culture plate and incubated for overnight. Cells washed with ice-cold PBS and lysed in 1 ml Flag-buffer. Lysates were collected by centrifugation for 15 min at 4°C and 15000 rpm. 500 µg protein lysate was incubated with 1.5µl of rabbit anti-Elk1, rabbit anti-Cdk1 and mouse anti-Plk1 antibodies and the mixtures were rotated for 1 hour at room temperature. 25 µl of pre-cleared Protein A/G agarose beads were added to the mixture and again rotated for 1 hour at room temperature. 25 µl of pre-cleared Protein A/G agarose beads were added to the mixture and again rotated for 1 hour at room temperature. Beads were washed twice with Flag-buffer and once with kinase buffer (25 mm Hepes, pH 7.4, 25 mm MgCl2, 25 mm β-glycerophosphate, 0.1 mm Sodium orthovanadate) to remove nonspecifically bound proteins.

For non-radioactive detection of kinase activity, protein/bead complexes were incubated with 100 mM non-labelled ATP and 2 µg GST-Elk1/1-205 fusion protein which contains putative phosphorylation sites. The reaction was carried out at 30°C and terminated after 30 minutes by SDS sample buffer addition. These complexes were resolved by 10 per cent SDS-PAGE and analyzed by Western blot.

To perform a fluorometric kinase assay that measures ATP to ADP conversion which is directly proportional to kinase activity. Protein kinases transfer a phosphate group from ATP to acceptor amino acids and the activity of these phosphotransferase enzyme is monitored fluorometrically. The kinase reaction was set up with Elk-1 protein or specific peptides according to the Table 4.13. Serine 106, Threonine 108 and Serine 326 peptides were incubated with Plk1, Serine 198 was incubated with either Aurora A, Aurora B or

Plk1, Threonine 199 and Serine 200 were incubated with either Aurora A or Aurora B, Threonine 133, Serine 202, 303, 304 and 326 were incubated with Plk1at 37°C for 1 hour. Then, 20 µl kinase reaction was combined with 20 µl ADP sensor buffer and 10 µl ADP sensor composed of the mixture of ADP sensor I and II. The mixture was incubated in dark for 15 minutes and the fluorescence intensity was measured by spectrophotometry at 540 nm excitation and 590 nm emission.

Table 4.13. Components of fluorometric kinase assay reaction

Elk-1 Protein		**Elk-1 Peptides**	
Component	**Final Concentration**	**Component**	**Final Concentration**
Elk-1	0.1 µg	Peptides	1mg/ml
Kinases	0.2 µg	Kinases	0.2 µg
ATP	200 mM	ATP	200 mM
ADP Assay Buffer	50 µl	ADP Assay Buffer	25 µl

4.7. PHOSPHO-SPECIFIC ANTIBODY SYNTHESIS AND ANALYSIS

Antibodies that recognize and bind specific phosphorylated peptides are easily generated through structure-based engineering. To monitor the state of the cell and understand protein functions, phospho-specific antibody studies confer a functional area [140].

In order to analyze whether potential Aurora A, Cdk1 and Plk1 phosphorylation sites of Elk-1 indeed phosphorylated by these kinases or not, phospho-specific antibodies were custom-synthesized by Genscript (The USA) as listed in Table 4.14.

Lyophilized phospho and non-phospho peptides were dissolved in 2 ml distilled water resulted in 2,5 mg/ml stock solutions. Phospho-specific antibodies were reconstituted in distilled water at a final concentration of 0,5 mg/ml. Three of these phospho-specific antibodies are produced from two different animals, so they have two different variants; P-Thr133-Elk-1 (#7211 and #7212), P-Ser303-Elk-1 and P-Ser304-Elk-1 (#7205 and #7206).

Table 4.14. Peptide sequences of phospho-specific Elk-1 antibodies

Phospho Site	Phospho Peptide	Unmodified peptide	Catalog Number
Ser106	PQPEV (pSer) VTSTMPNC	PQPEVSVTSTMPNC	U6974CB220-1 U2399CB220-1
Thr108	PQPEVSV (pThr) STMPN	PQPEVSVTSTMPNC	U7201CB220-1 U4169CB220-1
Thr133	SGKPG (pThr) PKGAGMAC	SGKPGTPKGAGMAC	U9684CB220-1 U7761CB220-1
Ser198	SGSR (pSer) TSPSPLEAC	SGSRSTSPSPLEAC	219347-3 219347-8
Ser199	SGSRS (pThr) SPSPLEAC	SGSRSTSPSPLEAC	219347-7 219347-8
Ser200	SGSRT (pSer) PSPLEAC	SGSRSTSPSPLEAC	219347-11 219347-8
Ser202	GSRSTSP (pSer) PLEACLEC	GSRSTSPSPLEACLEC	U7818CB220-1 U3175CB220-1
Ser303	GHAA (pSer) SPEISQPQKC	GHAASSPEISQPQKC	U6557CB220-1 U1325CB220-1
Ser304	GGHAAS (pSer) PEISQPQC	GGHAASSPEISQPQC	U8137CB220-1 U9121CB220-1
Ser324	DLELPL (pSer) PSLLGGPC	DLELPLSPSLLGGPC	U4464CB220-1 U8689CB220-1
Ser326	ELPLSP (pSer) LLGGPGC	ELPLSPSLLGGPGPC	U8576CB220-1 U7509CB220-1

4.8. DOT BLOT ASSAY

Dot blot is a simplified version of the Western blot that quantitatively detects and identifies proteins, with the advantage of being fast, however disadvantage of being non-discriminate. For a fast screening of specificity of the synthesized phosphor-specific Elk-1 antibody, dot blot assay was performed. Small circles have been drawn on nitrocellulose membrane by pencil to indicate protein dropped regions. 4 µl protein sample (100ng) was spotted onto the membrane at the centre of the circle. After the membrane is dry, non-specific sites were blocked by 5 per cent of BSA-TBS-T incubation. Afterwards, membranes incubated with modified and non-modified peptides and/or primary antibodies (0.5 µg/ml), rabbit anti-Elk-1-P-Ser106, P-Thr108, P-Thr133, P-Ser198, P-Thr199, P-Ser200, P-Ser202, P-Ser303, P-Ser304, P-Ser324 and P-Ser326, dissolved in BSA/TBS-T for 30 min at room temperature. Membranes were washed two times with TBS-T and once with TBS for 5 minutes and incubated with secondary antibody conjugated with HRP (1:5000) for 30 minutes at room temperature. They have washed again three times with TBS-T for 5 minutes and once with TBS for 10 minutes. Membranes were treated with Crescendo Western HRP Substrate and analyzed by Bio-Rad Chemi-Doc system.

4.9. IMMUNOFLUORESCENCE (IF) ANALYSIS

Immunofluorescence is a powerful technique that relies on an antigen-antibody reaction where antibodies are attached to a fluorescent dye and antigen-antibody complex is visualized using a fluorescent microscope. Immunofluorescence is commonly used to detect the presence and amount of protein or to determine the localization of the protein of interest [141]. In order to identify the localization of total or phospho Elk-1 proteins during mitosis immunofluorescence analysis was used.

The 0.1 per cent (v/v) poly-L-lysine (Sigma) solution was diluted in 1:10 ratio by sterile distilled water to obtain 0.01 per cent (v/v) working solution. The coverslips were sterilized by autoclaving and autoclaved coverslips were placed into the wells of the 6-well plates. The diluted poly-L-lysine solution was added into the wells and incubated for 15 minutes at room temperature. The poly-l-lysine solution was discarded, and the coverslips remained in laminar flow cabinet overnight or at an oven at 65°C for 1 hour to let them dry.

U-87 cells were seeded onto the Poly-L-lysine coated coverslips at a density of $2.5x10^5$ in 3 mL of complete growth medium and incubated for 24 hours. Cells were either transfected with Elk-1 plasmids or directly proceeded to immunostaining. The medium on the cells was discarded and cells washed twice with PBS. Cells then fixed in ice-cold methanol for 10 minutes at -20°C. After three gentle wash with PBS, cells were permeabilized and blocked in 3 per cent BSA prepared in 0.1 per cent Triton–PBS solution for 1 hour. Afterwards, cells were incubated with primary antibodies in blocking solution for overnight at 4°C at the indicated dilutions: anti-P-S383-Elk-1 antibody (1:100), anti-total Elk-1 antibody (1:100), anti-β-tubulin antibody (1:50), anti-Flag antibody (1:200) and Elk-1 phospho-specific antibodies, P-Ser106, P-Thr108, P-Thr133, P-Ser198, P-Thr199, P-Ser200, P-Ser202, P-Ser303, P-Ser304, P-Ser324 and P-Ser326, (1:100).

Cells were washed three times with PBS-T and incubated with secondary antibodies, goat anti-rabbit IgG–Alexa Fluor 488 1:500 (Invitrogen), goat anti-rat IgG–Alexa Fluor 546 (1:500), goat anti-mouse IgG–Alexa Fluor 647 (1:500) in blocking solution for 1 hour at room temperature in dark. They were washed twice in PBS-T and once in PBS. 1mg/ml stock Propidium iodide was prepared in distilled water and diluted in PBS at 1:1000 working ratio. Cells incubated with Propidium iodide on the shaker for 15 minutes and washed once with PBS. At the last stage, Coverslips were mounted in Prolong Gold Antifade Reagent and analyzed under Zeiss Confocal Microscope.

4.10. CELL VIABILITY ASSAYS

4.10.1. Assessment of Cell Viability Using Flow Cytometry

In order to understand on which phase of the cell cycle of cells, DNA of the cells are stained with propidium iodide (PI) which is an intercalating agent and a fluorescent molecule that can be used to stain the nucleus of cells. When excited with 488 nm wavelength light, it fluoresces red and this fluorescence helps to identify cell viability and DNA content in cell cycle analysis by flow cytometry. PI is commonly used for identifying dead cells in a population. After PI staining of the cells, results are identified by using flow cytometry.

Mitotic index assay provides the determination of the percentage of cells that are undergoing mitosis within a specific cell population. To analyze such a mitotic profile of

cells, both Propidium iodide and Histone H3 pSer10 antibody is used. Propidium iodide staining allows to identify the portion of cells that have 4N DNA content, but it does not reveal the percentage of cells that have just completed DNA synthesis and what percentage are mitotic. To overcome this, cells are also stained with Histone H3 pSer10 antibody which is tightly related with chromosome condensation during mitosis. Thus, Histone H3 pSer10 signal measured by flow cytometry indicates a mitotic cell with condensed DNA.

3.10^5 SH-SY5Y and U-87 cells were seeded onto 6 well plates and transfected with 2 µg both wild-type and mutant Elk1 plasmids by PEI transfection reagent. Cells were collected by trypsin treatment and centrifuged at 500g for 5 minutes and supernatant was discarded. The Cell pellet was resuspended in 1 mL 1X PBS and centrifuged again at 500g for 5 minutes. The supernatant was discarded gently, and cells were dissolved in 400 resuspend in 400 µl PBS. Cells were treated with 100 µl 20 per cent paraformaldehyde for fixation and incubated for 10 minutes.

Cells were centrifuged, and paraformaldehyde was carefully removed from cell pellet. The pellet was resuspended in 1 mL 1X PBS and centrifuged again at 500g for 5 minutes. The supernatant was aspirated without disrupting the pellet and pellet was resuspended in 200 µl 0.5 per cent BSA prepared in 0.1 per cent Triton-X-100 solution for both blocking and permeabilization for 5 minutes at room temperature.

Cells were precipitated by centrifugation and supernatant was carefully removed. Alexa Fluor 488 conjugate anti- Histone H3 pSer10 antibody was diluted in 0.5 per cent BSA solution in a 1:100 dilution ratio and cell pellet were dissolved in this solution. The cells and antibody resuspension were incubated in dark for 45 minutes at room temperature.

1 ml blocking solution (0.5 per cent BSA+0.1 per cent Triton-X-100) was added into the cell/antibody complex and centrifuged at 500g for 5 minutes. PI staining solution was prepared according to Table 4.15, and cell pellet was dissolved in 1 ml PI staining solution. After 30 minutes incubation, cells were gently resuspended by pipetting up and down to dissolve cell aggregates and samples were run on flow cytometer at appropriate FSC (Forward Scatter) and SSC (Side Scatter) gates to exclude cell debris and aggregates.

Table 4.15. PI staining solution

Contents	Final Concentration
Triton-X-100	0.1 % (v/v)
Propidium Iodide (PI)	1 µg/ml
DNase-free RNase A	20 µg /ml

4.10.2. Assessment of Cell Viability Based on XTT Assay

Tetrazolium compounds have been widely used as detection reagents of viable cells. The most common tetrazolium dyes are MTT, XTT and WST-1 [142]. MTT is a positively charged compound and easily penetrates viable eukaryotic cells. Whereas, MTS, XTT, and WST-1 are negatively charged cannot so easily penetrate to cells and are typically used with an intermediate electron acceptor. These compounds can be effectively used in cell proliferation, cytotoxicity, and apoptosis assays [143].

SH-SY5Y, SK-N-BE (2) human neuroblastoma, U-87, A172 and T98G human glioblastoma cells were seeded on 96 well plates as 2.10^5 cells/well and transfected with various Elk-1 plasmids at a concentration of 1µg. Cells were incubated for 24 to express transfected genes and then 50 µl XTT solution was administrated to each well which is composed of XTT reagent and electron coupling solution at 1:50 volume ratio. Cells were incubated with XTT solution for 4 hours at 37°C in a CO_2 incubator and absorbance values were recorded at 450 nm by spectrophotometer.

4.10.3. Statistical Analysis

Statistical analyses were based on at least three independent series of experiments with triplets. One-way ANOVA and Tukey post hoc tests were used. To describe values levels of statistical significance asterisk was used, $P>0.05$ not significant, ns, $P= 0.01$ to 0.05 significant, *, $P= 0.0001$ to 0.01 very significant, **, and $P<0.001$ extremely significant, ***. Calculations were performed by Prism 5 GraphPad software.

4.11. *In silico* ANALYSIS OF PREDICTED PHOSPHORYLATION MOTIFS

Human Elk-1 protein sequence (NCBI ID NM_007922) was analyzed for potential phosphorylation motifs through a range of different online prediction tools, http://ppsp.biocuckoo.org/, http://kinasephos2.mbc.nctu.edu.tw, http://phosphosite.org. The prediction was done based on phosphor-Threonine, phosphor-Serine and phosphor-Tyrosine motifs and/or search of the protein sequence for consensus motifs for selected kinases (Auroras, Plks, Cdks etc). A unified prediction was formed through a combination of the results of the different searches.

5. RESULTS

5.1. PUTATIVE MITOTIC KINASE PHOSPHORYLATION MOTIFS ON ELK-1

It was previously demonstrated by our group that Elk-1 localizes different localization at various stages of the cell cycle; during interphase, Serine-383 phosphorylated Elk-1 was found at the nucleus, whereas through prophase to metaphase it translocates to the spindle poles, when cells proceed into anaphase, P-S383-Elk-1 transitioned from the spindle poles to the midzone, relocating to the spindle midbody in cytokinesis [7]. This peculiar localization of Elk-1was found to overlap with the localizations of main mitotic regulators, Aurora A, Cdk1 and Plk1 [9]. This localization similarity between Elk-1 and mitotic kinases raised the fundamental question underlying this thesis of whether these kinases are phosphorylating Elk-1 during mitosis or not.

To address that question, numerous online platforms were searched for prediction of putative phosphorylation sites *in silico*, as explained in the methods section. According to these bioinformatic tools, several putative sites for Aurora Kinases (Ser 77, Ser 149, Ser 166, Ser 198, Thr 199, Ser 200, Ser 355 and Ser394) Cdks (Thr133, Ser200, Ser202, 222, Ser303, Ser304, Ser324, Ser 336, Ser 363, Ser383, Ser389, Thr417) and Plks (Ser106, Thr108, Thr 126, Ser 308, Ser326, Ser 370, Ser 411) were determined as listed in Table 5.1. Those that gave the highest risk-difference score from these regions, namely Ser106, Thr108, Thr133, Ser198, Thr199, Ser200, Ser202, Ser303, Ser304, Ser324, Ser326, were defined as target residues in terms of phosphorylation by Aurora A, Cdk1 and Plk1 kinases. Ser 383 and Ser 389 residues were also analyzed in the same tools as positive controls, and as demonstrated in Table 5.1, they were correctly identified as MAPK sites. Interesting, Ser 383, Ser 389 and Thr 417, known phosphorylation sites of Elk-1, were also detected as Cdk target residues.

Table 5.1. Putative Phosphorylation Motifs of Elk-1. Prediction tools used for these predictions are presented in methods in more detail.

Position	Kinase	Peptide	Threshold	Risk-Difference
Ser 77	Aurora A	IRKV**S**GQKF	2.8	3.38
Ser 149	Aurora A/B	LARS**S**RNEY	3.2	4.63
Ser 166	Aurora A	FTIQ**S**LQPQ	2.8	3.23
Ser 198	Aurora A	SGSR**S**TSPS	2.8	4.85
Thr 199	Aurora A/B	GSRS**T**SPSP	3.2	4.03
Ser 200	Aurora A	SRSTSP**S**PL	2.8	6.26
Ser 355	Aurora A	ALTP**S**LLPT	2.8	3.75
Ser 394	Aurora B	PAKL**S**FQFP	2.8	3.34
Thr 133	Cdks	GKPG**T**PKGA	6	8.47
Ser 200	Cdks	SRST**S**PSPL	6	9.23
Ser 202	Cdks	STSP**S**PLEA	6	9.50
Ser 303	Cdks	GHHAA**S**SPE	6	8.23
Ser 304	Cdks	HAAS**S**PEIS	6	8.14
Ser 324	Cdks	ELPL**S**PSLL	6	9.08
Ser 336	Cdks	GPERTPG**S**G	6	7.24
Ser 383	Cdks	WSTL**S**PIAP	6	8.32
Thr 389	Cdks	IAPR**S**PAKL	6	10.28
Thr 417	Cdks	DGLS**T**PVVL	6	7.79
Ser 106	Plk	QPEV**S**VTST	1	3.94
Thr 108	Plk	EVSV**T**STMP	1	2.96
Thr 126	Plk	APGD**T**VSGK	1	1.08
Ser 308	Plk	SPEI**S**QPQK	1	2.17
Ser 326	Plk	PLSP**S**LLGG	1	2.72
Ser 370	Plk	LLTP**S**SLPP	1	1.76
Ser 411	Plk	IPSI**S**VDGL	1	1.74
Ser 383	MAPK	WSTL**S**PIAP	5.7	8.19
Thr 389	MAPK	IAPR**S**PAKL	5.7	10.15
Thr 417	MAPK	DGLS**T**PVVL	5.7	5.86

5.2. CLONING OF MITOTIC KINASES INTO MAMMALIAN EXPRESSION VECTORS

5.2.1. Cloning of Aurora Kinases

In order to analyze the interaction between Elk-1 and Aurora kinases, Aurora A and Aurora B coding sequences were cloned into pCMV-Myc mammalian expression vector. Aurora A and Aurora B specific primers were reconstituted into the water and gradient PCR was performed by Taq DNA polymerase to define annealing temperature of primer pairs. Annealing temperature was set as 62°C for Aurora A and this gene sequence was tried to be amplified by 3 different DNA polymerase enzymes that have proof-reading activity, as Finnzyme, Long and Pfu DNA polymerases. The 1212 base pair PCR product was obtained only by Long PCR enzyme reaction as shown in Figure 5.1a.

PCR product was purified from agarose gel by gel purification kit and both pCMV-Myc and isolated PCR product were cut by *Kpn*I and *Xho*I enzymes and further ligated by T4 DNA ligase at 1:3 insert/plasmid ratio. After transformation into *Escherichia coli* DH5α strain, recombinant bacteria was growth on LB broth medium and plasmids were isolated from bacteria via plasmid isolation kit. Diagnostic restriction screening was performed to select positive colonies and as seen in Figure 5.1b, only one colony out of 6 colonies was observed as carrying Aurora A gene product, P4 colony, the other ones were detected as empty vectors.

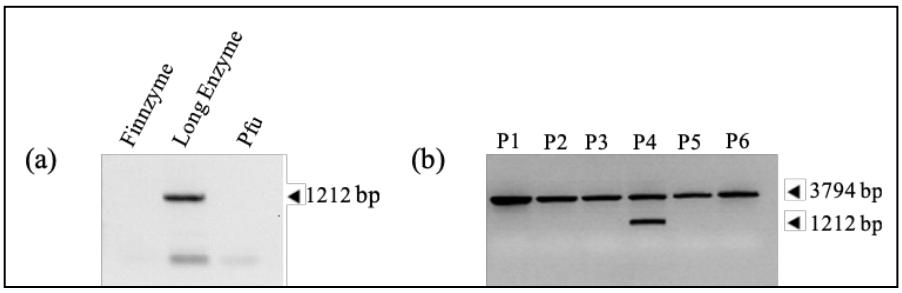


Figure 5.1. PCR amplification (a) and restriction digestion (b) results of Aurora A. P (plasmid) is the different bacterial colonies.

The optimum working temperature of Aurora B primer pairs was established as approximately 58°C as seen in Figure 5.2a. Following transformation of recombinant plasmids into DH5α strain, 8 bacterial colonies have been chosen and plasmids isolated

from these colonies. Plasmids were cut with EcoRI and XhoI restriction enzymes and to find out the recombinant plasmids. Figure 5.2b indicates that Aurora B coding sequence was successfully cloned into pCMV-Myc vector with an exception of P2 plasmid.

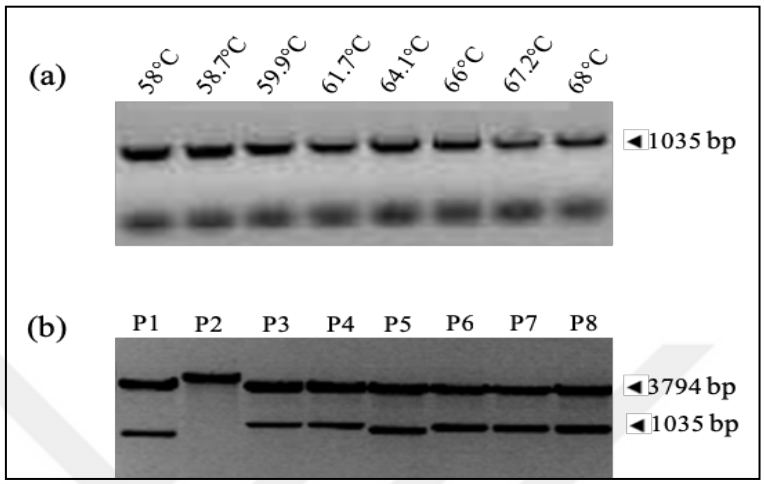


Figure 5.2. Gradient PCR (a) and restriction screening (b) results of Aurora B kinase. (P) represents different plasmids.

5.2.2. Expression Analysis of Aurora A Kinases

Aurora A and B carrying plasmids were sequenced with cloning primers and thereby the integrity of the gene sequence was confirmed. The expression level of Aurora A protein was confirmed by the transfection of 10 µg and 20 µg pCMV-Myc-Aurora-A recombinant plasmid into SH-SY5Y cells and Western blot was carried out. In Figure 5.3, 48 kDa protein band corresponding to Aurora A protein was detected by anti-Myc antibody which indicates efficient expression of the cloned gene.

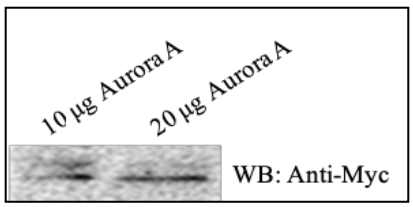


Figure 5.3. Expression analysis of Aurora A by Western blotting. WB: Western Blot.

5.2.3. Cloning of Cyclin-Dependent Kinases and Plk1

The coding sequences of Cyclin-dependent kinases 1, 2, 5 and Polo-like kinase 1 were cloned into mammalian expression vector pCMV-HA. The total RNA isolated from HEK293 human embryonic cells and RT-PCR was performed to obtain cDNA as a template for further PCR reactions. Gradient PCR was used to determine the optimal annealing temperatures of Cdk1, 2,5 and Plk1, 58.7°C, 66°C, 72°C and 61°C, respectively. pCMV-HA vector and PCR products of Plk1, Cdk1 and Cdk2 were cut with *EcoRI* and *XhoI*, Cdk5 was cut with *SalI* and *NotI* restriction endonucleases. Figure 5.4. demonstrates the diagnostic restriction enzyme digestion results of cloned Cdk1, 2, 5 and Plk1 carrying or empty pCMV-HA plasmids.

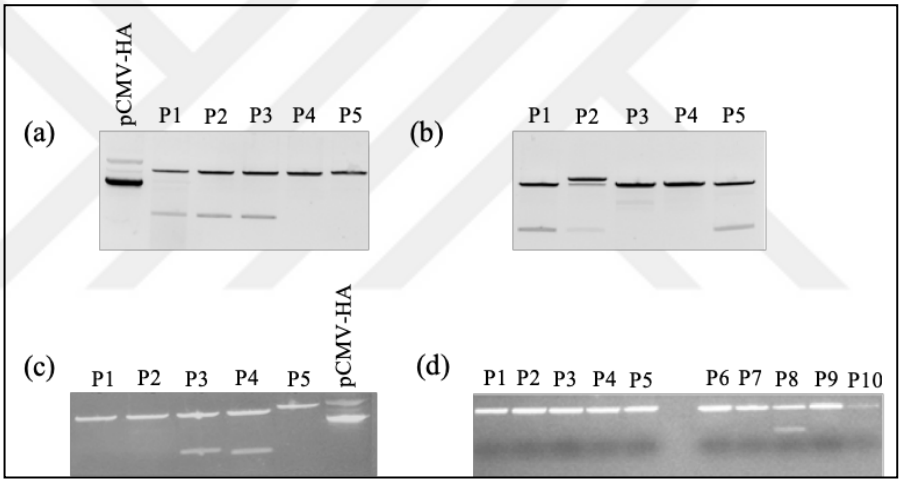


Figure 5.4. Restriction screening of ligated plasmids Cdk1 (a), Cdk2 (b), Cdk5 (c) and Plk1(d). (P) represents different plasmids.

Diagnostic restriction analysis revealed that 3 plasmids of Cdk1, P1, P2 and P3 (a), 2 plasmids of Cdk2, P1 and P5 (b), 2 plasmids of Cdk5, P3 and P4 (c), and 1 plasmid of Plk1, P8, are carrying inserts. These plasmids were further sequenced to control whether inserts completely cloned into plasmids or not.

5.2.4. Expression analysis of Cdk1, Cdk2, Cdk5 and Plk1 Kinases

SH-SY5Y cells were transfected with 10μg and 20 μg of target plasmids. Cells were also transfected with GFP (Green Fluorescent Protein) plasmid to evaluate transfection

efficiency and visualized by fluorescence microscopy as demonstrated in Figure 5.5. The green areas in the figure indicates cells carrying and expressing GFP.

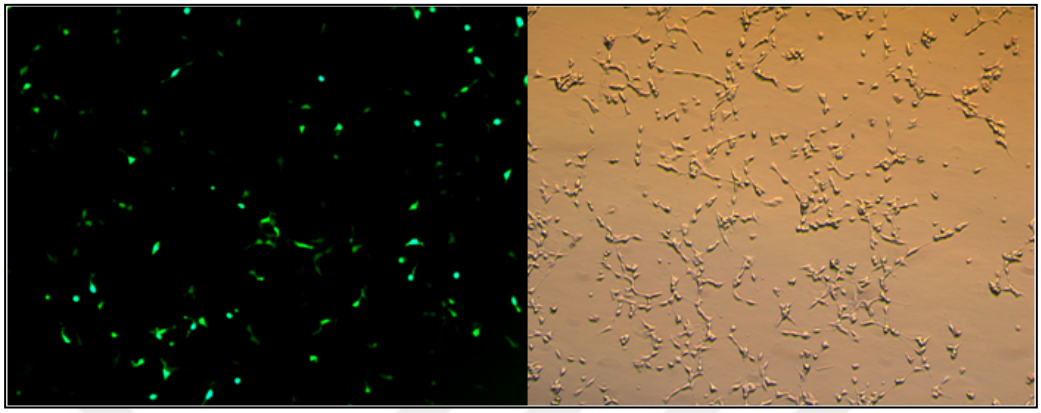


Figure 5.5. GFP Staining of SH-SY5Y cells. Fluorescent (left) and light (right) microscope images of GFP transfected cells (Magnification, 40X)

Proteins from these cells were isolated by RIPA lysis buffer and Western blotting was performed to control the expression of these kinases. Cdk1 (a), Cdk2 (b) and Cdk5 (c) protein expressions were confirmed by Anti-HA tagged protein detection by Western blot. β-Tubulin which is a housekeeping gene was used as internal control to confirm that protein loading is the same across the gel. Plk1 protein expression was not detected as seen in Figure 5.6d which may be originated from the amplification of target sequence by PCR or a problem in the cloning process. Therefore, Plk1 was supplied from Prof Wen H. Shen, Cornell University and the integrity of the gene sequence was confirmed by sequencing.

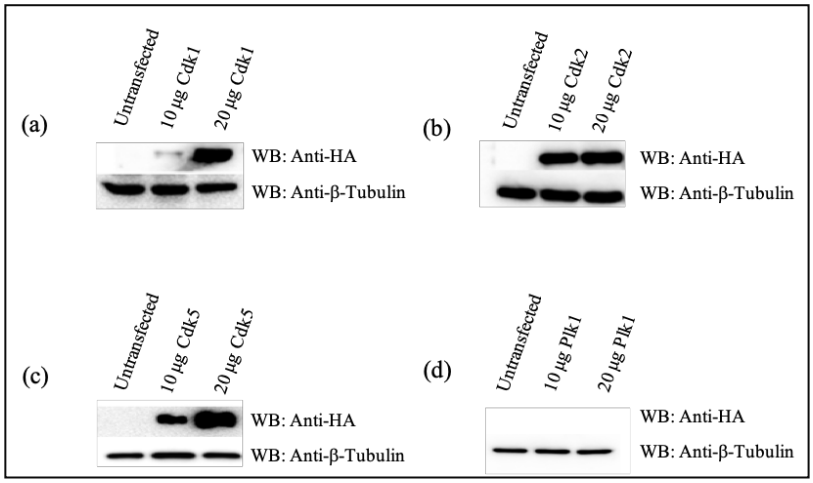


Figure 5.6. Expression analyses of Cdk1(a), Cdk2(b), Cdk5 (c) and Plk1(d) proteins.

Tetracyline and doxocyclin are commonly used as antibiotics as well as they are utilized to induce transcription of a target gene cloned into an inducible vector. Plk1 plasmid sent us from Prof Wen H. Shen into a tetracycline or doxocyclin inducible plasmid, pcDNA4-Tet-On-Plk1. To analyze the expression of Plk1 protein, empty pcDNA3 and pcDNA4-Tet-On-Plk1, Tet-On means tetracycline on or tetracycline induce expression of the target gene, into SH-SY5Y cells. 24 hours after transfection, cells were treated with 3 μg doxocyclin to induce protein expression for 24, 48 and 72 hours.

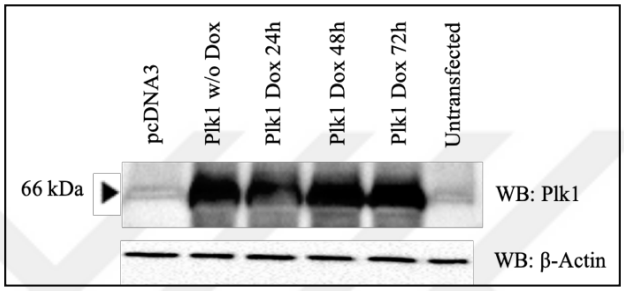


Figure 5.7. Expression control of Plk1 protein. Empty pcDNA3 plasmid was used as negative control and Untransfected protein lysate was used to detect endogenous Plk1 protein. Cells were treated with 3 μg doxocyclin for 24, 48 and 72 hours. Molecular weight of Plk1 protein is 66 kDa. Dox: Doxocyclin, WB: Western blot.

Western blot result demonstrated in Figure 5.7. indicates that the Plk1 protein expression increased due to the duration of treatment with doxycycline and reached the highest level after 72 hours. Empty pcDNA3 plasmid was used as negative control and since it does not contain Plk1 protein, Plk1 expression was quite low. Plk1 expression in untransfected or total cell lysate was considerably lower than that of doxocyclin-treated samples. β-Actin protein was used as a positive control and it was found that each sample was loaded an approximately equal amount of gel.

5.3. THE INTERACTION BETWEEN ELK-1 AND MITOTIC KINASES

The interaction between Elk-1 and Aurora A kinase has previously been shown [7] and repeated in this study as a positive control. To analyze whether a similar interaction exists between Elk-1 and other target mitotic kinases, namely Aurora B, Cdk1 and Plk1, co-immunoprecipitation assay has been used. The mechanism of Co-IP assay works by

choosing an antibody that is specific to the target (bait) protein in the total cell lysate. Precipitation of large complexes works when target proteins and the antibody bind to each other tightly [138].

Proteins were isolated from SH-SY5Y neuroblastoma cells and 250 µg total protein was incubated with anti-Elk-1 antibody. Protein/antibody complexes were precipitated with protein A/G slurry and analyzed by Western blot. Since the interaction between Elk-1 and Aurora A kinase was previously demonstrated, this interaction was used as a control and the interplay between Elk-1 and Aurora B, Cdk1 and Plk1 were also examined.

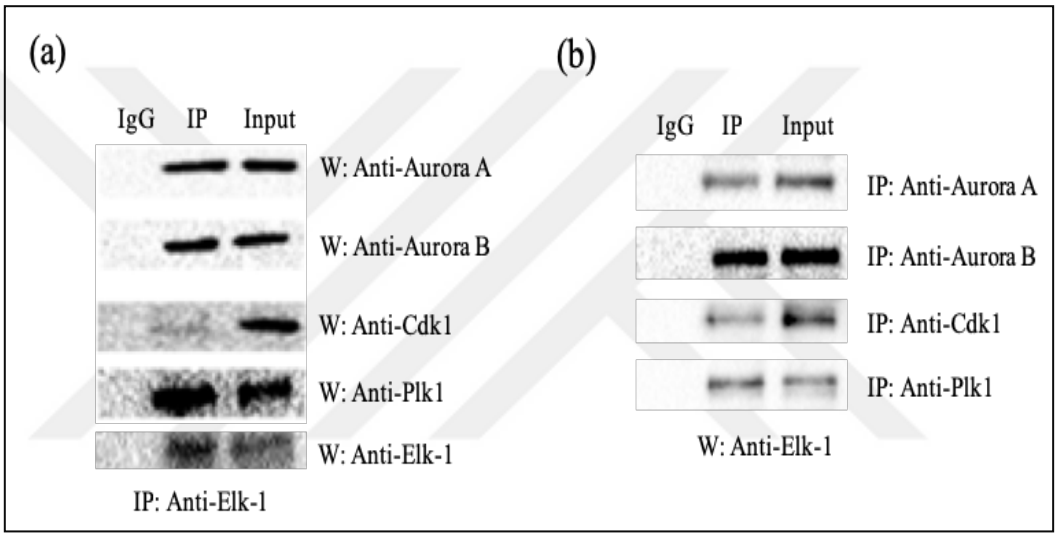


Figure 5.8. Interaction of Elk-1 with various mitotic kinases, Aurora-A, Aurora-B, Cdk1 and Plk1. (a) Immunoprecipitation (IP) of endogenous Elk-1 in SH-SY5Y cell lines, followed by Western blotting of the input and IP samples with antibodies specific for Aurora-A, Aurora-B, Cdk1 and Plk1. (b). To perform the reverse co-IP experiment, Aurora-A, Aurora-B, Cdk1 and Plk1kinases were precipitated and interaction between Elk-1 and these kinases were confirmed by Western blot (b). IgG was used as negative control and input is the total cell lysate.

Figure 5.8a indicates that Elk-1 is physically interacting with both Aurora A, Aurora B, Cdk1 and Plk1 kinases. Furthermore, this interaction was maintained when mitotic kinases were precipitated instead of Elk-1 and analyzed by Western blot incorporated with Elk-1 antibody as seen in Figure 5.8b. IgG was used as a negative control and no band was observed in these samples, whereas input or total cell lysate was used as a positive control to check the expression of indicated proteins.

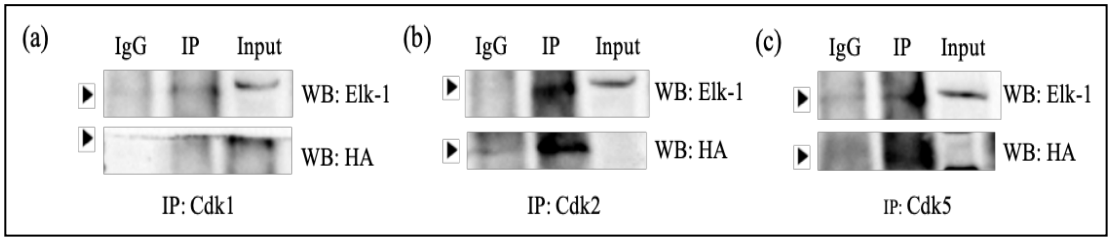


Figure 5.9. The interaction between Elk-1 and Cyclin dependent kinases; Cdk1, Cdk2 and Cdk5. HA-tag conjugate Cdk1(a), Cdk2 (b) and Cdk5 (c) proteins were precipitated from whole cell lysate by anti-HA antibody and analyzed by Western blot.

In addition to Cdk1, the interaction between Elk-1 and other cyclin dependent kinases, Cdk2 and Cdk5 was evaluated by immunoprecipitation and Western blot. SH-SY5Y cells transfected with Cdk1, as control, Cdk2 and Cdk5 kinases and these overexpressed kinases were precipitated from whole cell lysate by anti-HA antibody since they are cloned into pCMV-HA mammalian plasmid. According to the results in Figure 5.9, it was seen that Elk-1 interacts not only with Cdk1 but also with Cdk2 and Cdk5 kinases. To demonstrate these kinases could be precipitated from whole cell lysate, anti-HA antibody was used in Western blot and the bottom results in the figure proof the presence of these kinases. It should be noted that Cdk5 has previously been reported as the kinase that phosphorylates Threonine 417 residue of Elk-1 [135].

To analyze the interaction between Elk-1 and Aurora A, B, Cdk1 and Plk1 kinases more specifically, U-87 glioblastoma cells were blocked at prometaphase by the administration of 100 ng/ml nocodazole for 16 hours. Cells were released from nocodazole by incubating with growth medium and collected at 30-min time intervals by mitotic shake-off in an effort to collect cells in different stages of the cell cycle, prometaphase, metaphase, anaphase and telophase. Collected cells were lysed in cell lysis buffer (RIPA) containing protease and phosphatases inhibitors [7]. The cell lysate was incubated with normal rabbit IgG followed by protein A agarose bead incubation for pre-clearing. This pre-cleared cell lysate was incubated with rabbit polyclonal anti- Elk-1 antibody and protein-antibody complexes precipitated by protein A or G agarose bead slurry and then analyzed by Western blot.

Before Western blot analysis, to confirm that cells were arrested at G_2/M transition by nocodazole administration DNA of the cells were stained with propidium iodide (PI) which is an intercalating agent and that can be used to stain the nucleus of cells as depicted in

Figure 5.10a. When excited with 488 nm laser light, it emits red fluorescence which is used to identify cell viability and DNA content in cell cycle analysis. The PI staining solution that was used to stain cells is composed of PI, RNase A and Triton-X 100 as a permeabilization agent according to the concentrations given in table 4.14. The percentages of the cells in cell cycle were evaluated by FACS (Fluorescent activated cell sorting) analysis. According to FACS results, the first peak is corresponding to G_0/G_1, the second peak is G_2 and the area between two peaks is the S phase. The signals shown before the first peak represents apoptotic cells as shown Figure 5.10b.

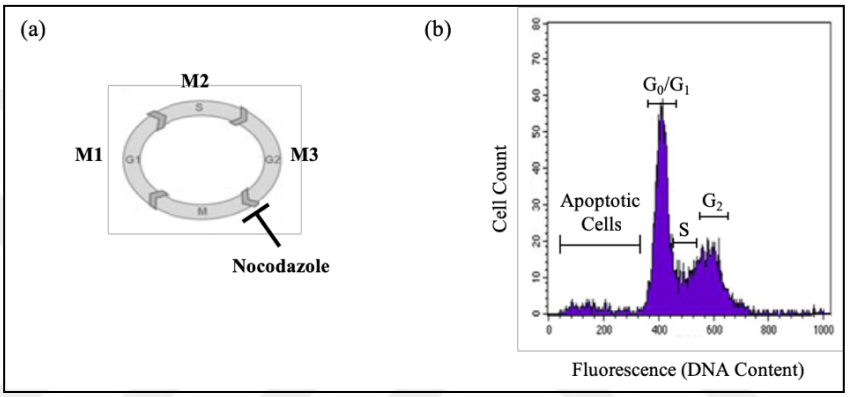


Figure 5.10. Schematic representations of a cell cycle (a) and the effect of Nocodazole (b) during the cell cycle

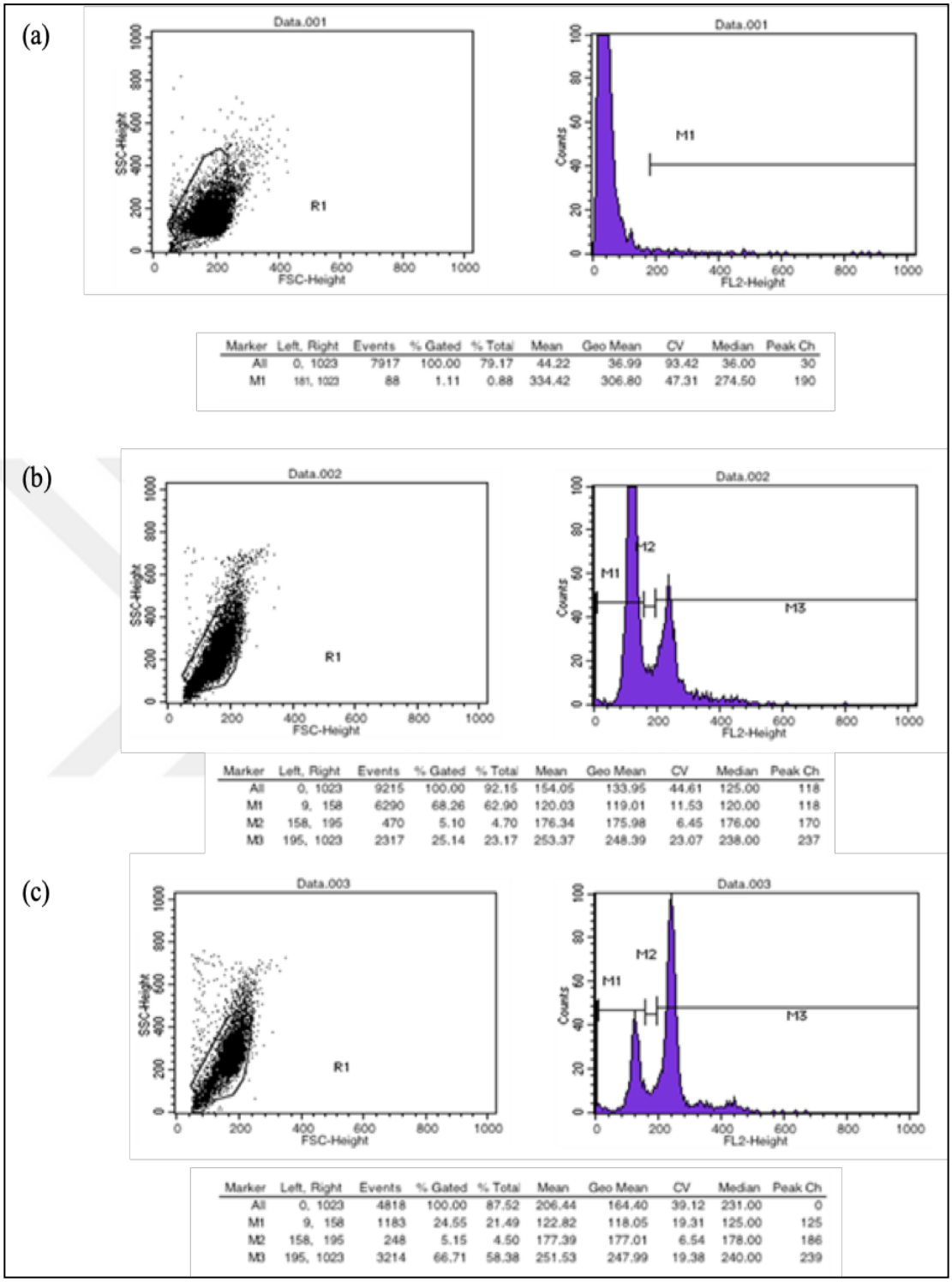


Figure 5.11. Flow cytometry analysis of the cells stained with propidium iodide (PI). Cell cycle profile of (a) unstained cell, used as negative control, (b) PI stained nocodazole-untreated cells and (c) PI stained and nocodazole-treated cells.

Figure 5.11a is the negative control which comprises the cells that were not stained with PI and mitotic phase discrimination has not been observed, as expected. Sample group that is not treated with nocodazole was used as control and when mitotic profiles of these cells were examined according to the Figure 5.11b, it was seen that 68.26 per cent was in G_0/G_1, 5.10 per cent in S phase and 25.14 per cent in G_2 phase. In the case of nocodazole-treated cells seen in Figure 5.11c, distribution of cells in mitosis was observed as 24,55 per cent in G_0/G_1 5,15 per cent in S and 66.71 per cent in G_2 phase. The cell density of the cells in G_2 was dramatically increased after nocodazole which proves that nocodazole administration successfully resulted in the increase of the cells in the G_2 phase.

After the confirmation of the cell cycle arrest at G/M transition as by nocodazole treatment as seen in Figure 5.11, the cell lysate was collected at thirty minutes time intervals as 0^{th}, 30^{th}, 60^{th} and 90^{th} minutes in an effort to arrest cells at different stages of the cell cycle, prometaphase, metaphase, anaphase and telophase, respectively. These protein lysates were used to examine the interaction between Elk-1 and Aurora A, Aurora B, Cdk1 and Plk1 kinases in a mitotic phase-dependent manner.

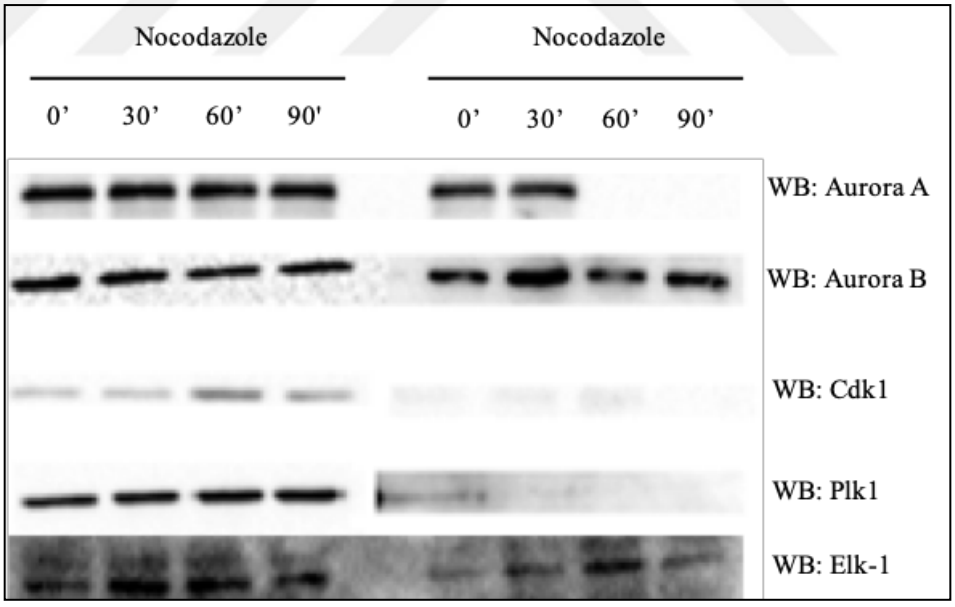


Figure 5.12. Interaction of Elk-1 with mitotic kinases, Aurora-A, Aurora-B, Cdk1 and Plk1 in nocodazole synchronized cells. U-87 cells were arrested with nocodazole treatment and released into mitosis at time zero and samples were collected at 30, 60, and 90 minutes after release.

As it is reported before, Elk-1 and Aurora-A kinase interact in the first 30 minutes of mitosis [7]. However, in this analysis, it was observed that Elk-1 binds Aurora B kinase

during all the phases of the cell cycle and this interaction is more prominent especially during metaphase and telophase as shown in Figure 5.12. Although the interaction with Cdk1 is also present in all time periods, Cdk1 staining is poor this may in part be attributed to the low amount of Cdk1 protein in the input. The interaction of Elk-1/Cdk1 is more likely to be weak because it is being expressed in close amounts to the Aurora-A amount. Elk-1 and Plk1 interact at the very beginning of mitosis, after the 30th minute this interaction is significantly reduced.

5.4. DETERMINATION OF EXACT BINDING MOTIFS OF MITOTIC KINASES ON ELK-1

GST (glutathione-S-transferase) pull-down assay is an effective technique used to examine interactions between a probe protein and its target proteins, so suspected interactions between probe and target can be confirmed *in vitro*. This protocol is based on the addition of a fusion tag for the GST enzyme, 26 kDa, at the N-terminus of the probe protein whose coding sequence is cloned into an IPTG inducible expression vector. The GST tagged fusion protein is expressed in bacteria and purified by affinity chromatography or glutathione-sepharose beads. Purified probe protein is generally exposed to incubation with cell lysate along with glutathione sepharose beads and this complex is resolved by SDS-PAGE and analyzed by Western blotting [144].

To determine the exact binding sites of target kinases on Elk-1 domain structure, GST-pull down assay was performed. Elk-1 deletion constructs shown in Figure 5.13. cloned into a GST fusion vector, pGEX-4T2, which are a generous gift from Prof Andrew D. Sharrocks. These constructs are comprised of 1-93, 1-205, 205-329, 205-428 and 349-428 amino acids and the predicted sizes of these constructs determined based on the amino acid composition as listed in Table 5.2. These plasmids were transformed into *Escherichia coli* BL21 (DE) pLysS bacteria strain which is deficient in *Lon* (cytoplasm) and *ompT* (outer membrane) proteases and designed to maximize the expression of fusion protein.

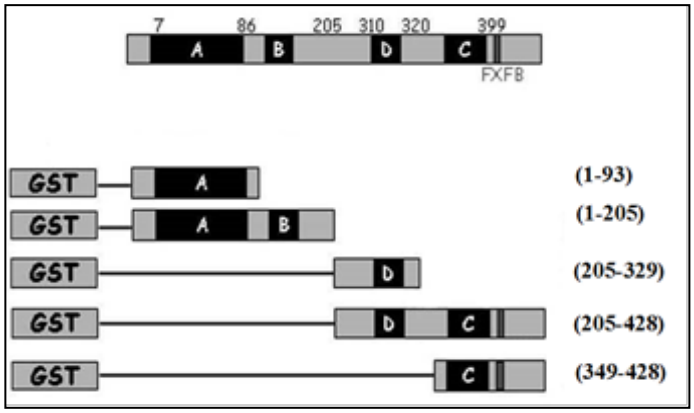


Figure 5.13. Deletion constructs of GST-Elk-1 fusion proteins.

One bacterial colony was picked up from the plate and inoculated into LB broth medium containing ampicillin and chloramphenicol as resistance markers. Then, bacteria were induced to start GST-fusion protein expression by IPTG administration and grown for 4 hours. After induction of fusion protein expression, bacteria were sonicated to obtain protein lysate.

Table 5.2. Predicted sizes of Elk-1 plasmid constructs and pGEX plasmid

Plasmid Construct	**Size (kDa)**
pGEX-4t2 (empty)	26
1-93	36
1-205	49
205-329	40
205-428	51
349-428	35

Bacterial proteins were stored in lysis buffer with bacterial protease inhibitor cocktail to prevent protein degradation after lysing bacteria. Proteins were loaded onto polyacrylamide gel (PAGE) and exposed to an applied electric field, electrophoresis. After the run, the gels were covered with staining solution composed of methanol, acetic acid, Coomassie blue and distilled water and incubated 30 minutes and de-stained by the boiling with distilled water and analyzed with Bio-Rad Chemi-Doc system.

Figure 5.14. demonstrates the protein expression of Elk-1 deletion constructs after 5 hours of IPTG induction. Since protein expression has not stimulated yet by IPTG administration at time zero, the expressions of these samples were quite weak. Rest of the samples were stimulated by IPTG at time intervals of one to five hours which resulted in gradually increased protein expression.

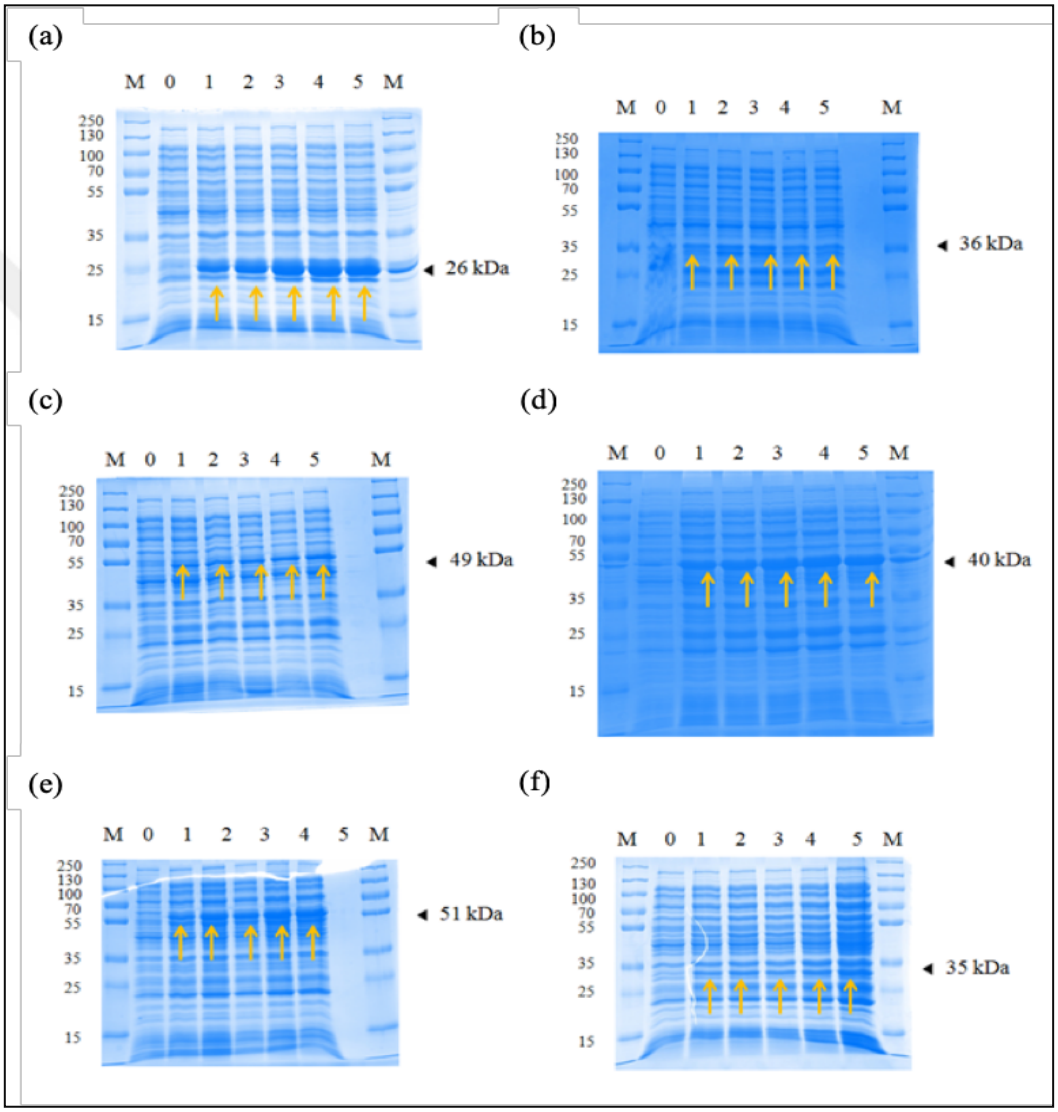


Figure 5.14. SDS-PAGE of GST-Elk-1 fusion proteins after IPTG induction. Empty pGEX-4T2 plasmid (a), pGEX Elk-1/1-93 (b), pGEX Elk-1/1-205 (c), pGEX Elk-1/205-329 (d), pGEX Elk-1/205-428 (e) and pGEX Elk-1/349-428 (f). M lines are the protein marker. Arrows indicate expression increments of each line. 0 to 5 indicates different time intervals (hours) after IPTG administration.

After the confirmation of IPTG induced increased protein expression by Coomassie staining, Western blot has been performed to control the expression of GST-tagged fusion proteins before and 5 hours after IPTG induction. Expression of Elk-1 deletion constructs was confirmed by the treatment of mouse monoclonal anti-GST and rabbit polyclonal anti-Elk-1 antibodies. Arrows in Figure 5.15. indicate the expression of GST and GST-fused Elk-1 which thereby points out the bacterial expression of Elk-1 deletion constructs. Protein expression before IPTG treatment is slightly lower than after 5-hour IPTG administration.

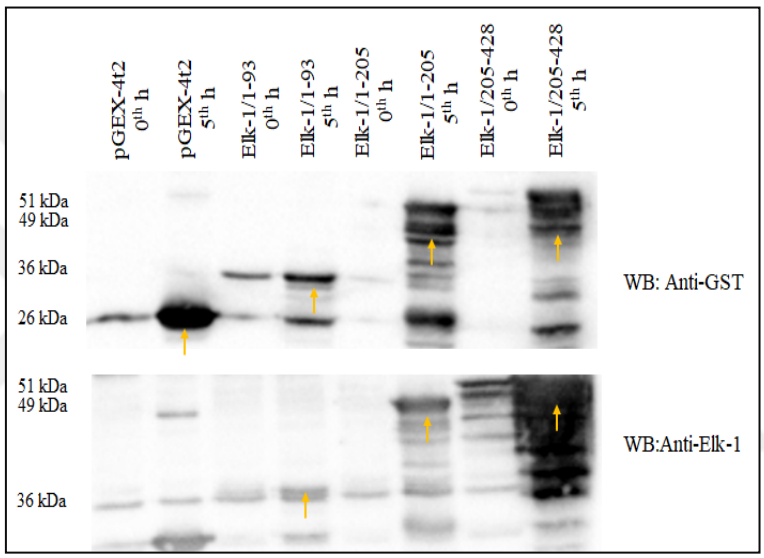


Figure 5.15. Western blot analysis of bacterial fusion proteins by anti-GST and Elk-1 antibody. Proteins isolated before (0) and 5-hours after (5h) IPTG induction.

Previously isolated bacterial protein lysate was incubated with Glutathione-sepharose beads that can bind GST-tagged fusion protein. Glutathione-sepharose bead/protein complex was incubated with U87 cell whole protein lysate, beads then were resuspended in SDS sample buffer and stored at 4° C until the Western blot experiment. In order to control whether Elk-1 deletions were pulled with GST beads, protein membranes were initially treated anti-Elk-1 antibody before initiating the interaction analyzes between Elk-1 with kinases.

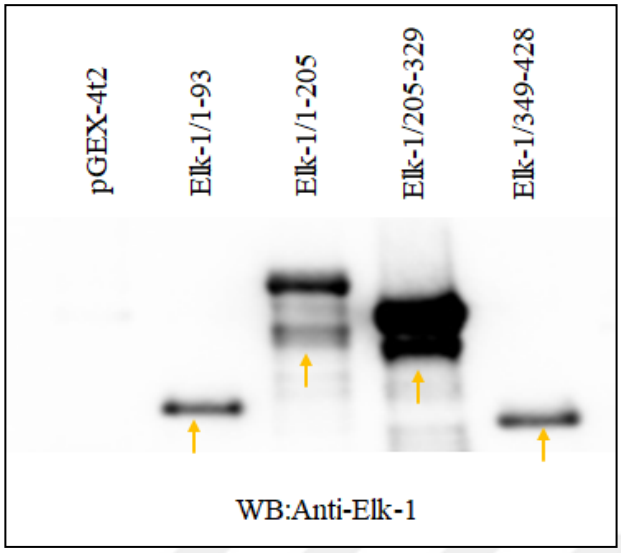


Figure 5.16. Western blot analysis of GST Pull down Assay. Empty pGEX plasmid was used as a negative control. GST-Elk-1/1-93, 1-205, 205-329 and 349-428 lines are deletion constructs fused to pGEX-4T2 plasmid.

Figure 5.16. confirmed that Elk-1 deletions were successfully pulled down by Glutathione-agarose beads. After this confirmation, GST pull-down samples were loaded on SDS-PAGE gels and interaction between Elk-1 and mitotic kinases was analyzed by Western blot. Western blot was performed to determine the binding parts of target mitotic kinases, Cdk1, Plk1 and Aurora B, on Elk-1 protein.

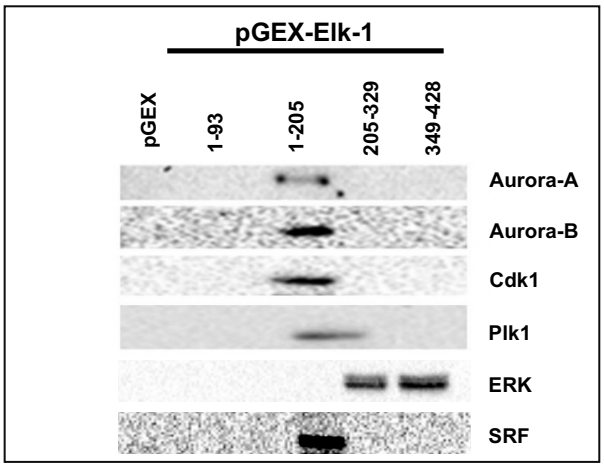


Figure 5.17. Western blot analysis of isolated bacterial proteins after GST-Pull down assay.

Empty pGEX vector was used as an experimental negative control and there was no band on this line, as expected. 1-93 deletion construct of Elk-1 is also used as a negative control since this part of Elk-1 is corresponds to A domain, DNA binding domain lies within the N-terminus end of the protein and is responsible for Elk-1 binding to DNA. It has been observed that both kinases, Aurora A, Aurora B, Cdk1 and Plk1 kinases bind Elk-1 through the first 205 amino acids of Elk-1 protein domain that corresponds to N-terminus site of Elk-1. As seen in Figure 5.17, ERK kinase binds Elk-1 protein from D domain, located at 213-328 amino acids, and DEF domain, located at the end of the C terminus. As the binding sites of ERK kinase on Elk-1 protein domain are known, it was used as a control of the experiment and in figure, it was shown that ERK binds to Elk-1 from 205-329 and 349-428 deletion constructs of Elk-1. SRF protein binds Elk-1 through B domain exclusive to it and it was shown that SRF binds Elk-1only through 1-205 deletion construct of Elk-1.

5.5. DEVELOPMENT OF PHOSPHO-ELK-1 ANTIBODIES

Physical interaction between Elk-1 and mitotic kinases, Aurora A, Aurora B, Cdk1 and Plk1, from N-terminus of Elk-1 has been confirmed by immunoprecipitation and GST-pull down assays. To analyze whether the predicted motifs were indeed phosphorylated and whether these motifs were phosphorylated by the mitotic kinases predicted, custom antibodies against selected phosphorylation sites predicted have been raised as listed in Table 4.13.

Phosphorylated proteins can be detected by phospho-specific antibodies in a complex protein mixture since phospho-specific antibodies recognize the phosphorylated amino acids of the surrounding amino acid sequence, thus assessment of the degree of the phosphorylation of a protein is possible via phospho-specific antibodies. To analyze phosphorylation status of Elk-1, custom phospho-specific antibodies have been developed by Genscript company.

To test the specificity of the phospho specific antibodies, initially, both phospho-peptides and unmodified peptides were dropped on the nitrocellulose membrane at a concentration of 100 ng/µl. Membranes further incubated with phospho-specific antibodies or their pre-serums which are collected from the rabbit before immunization with peptide used to elicit antibodies and visualized by enhanced chemiluminescence.

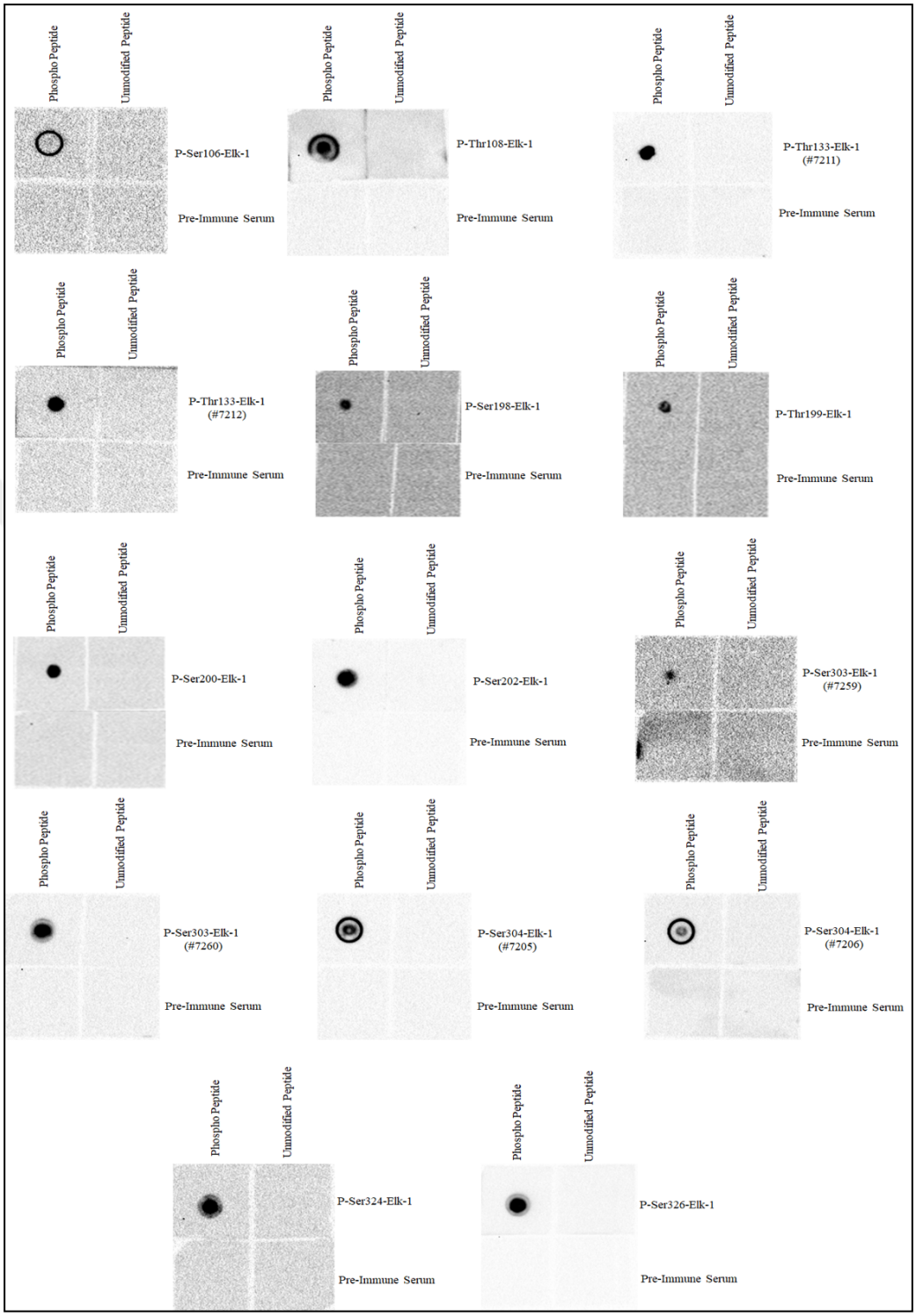


Figure 5.18. Dot Blot analysis of phospho-specific antibodies and their pre-serums with phospho and unmodified peptides.

Figure 5.18. demonstrates the specificity of all phospho-specific antibodies and their variants, which is tested via incubation of phospho and non-phospho peptides with both

antibodies and pre-immune serum. As seen in figure, all antibodies are specific to their related peptides, they are able to bind them, and any dot corresponds to binding of phospho-specific antibodies to unmodified peptides have not been observed.

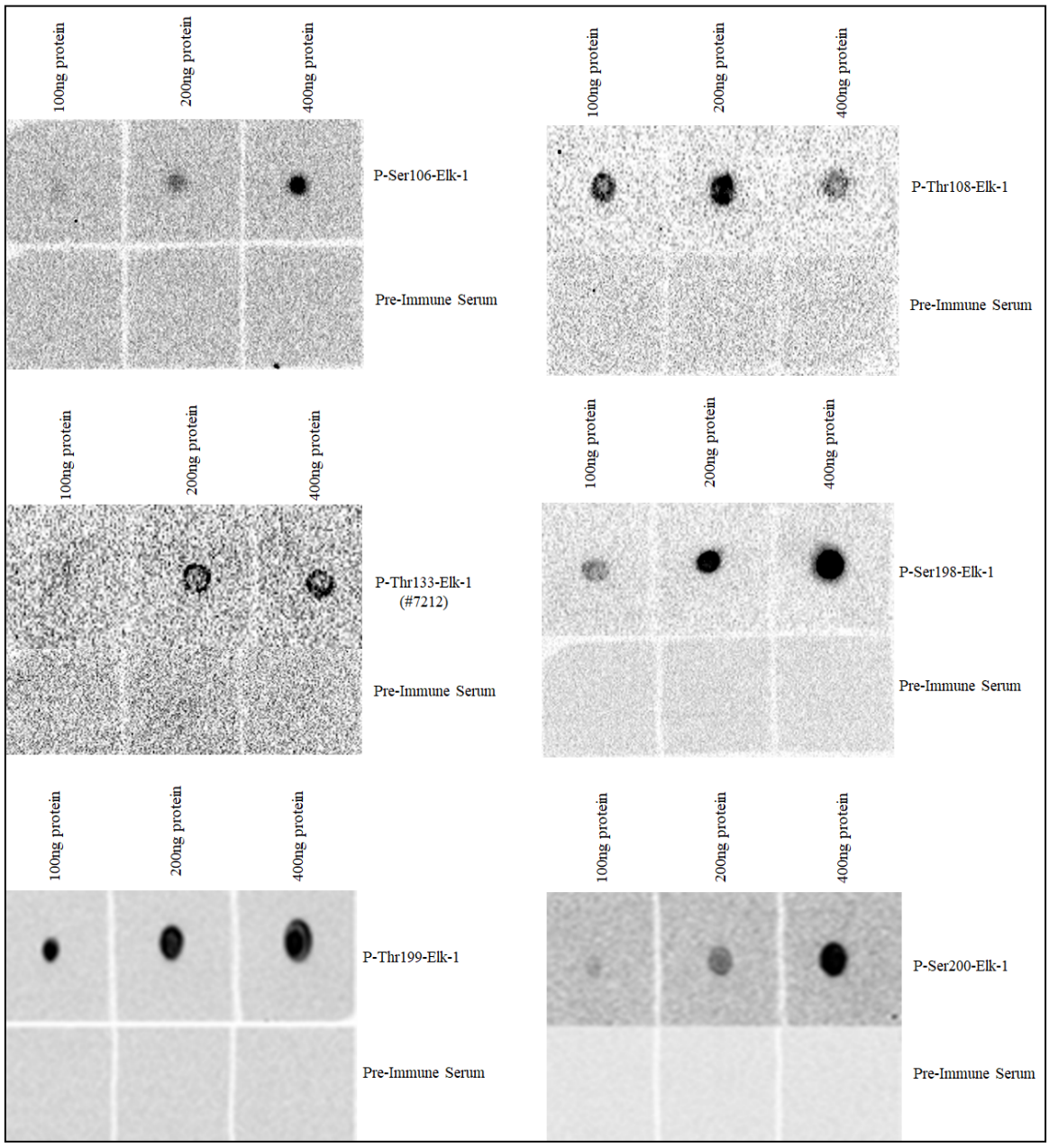


Figure 5.19. Dot Blot analysis of phospho specific antibodies and their pre-serums on total cell lysate

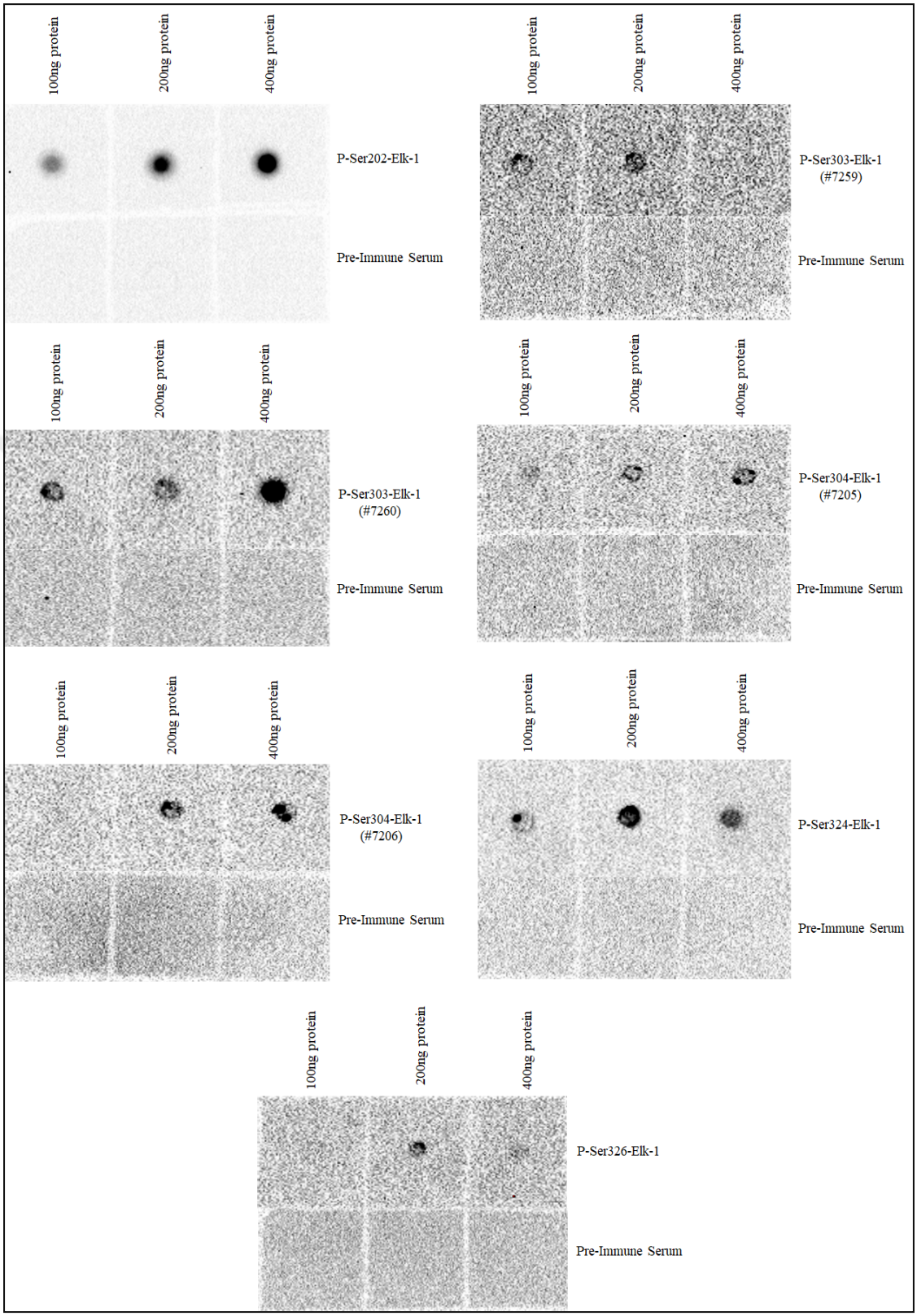


Figure 5.19. (continued) Dot Blot analysis of phospho specific antibodies and their pre-serums on total cell lysate

After confirming the efficiency of phospho-specific via specific peptides, they were also tested on total cell lysate to see whether they are able to bind specific sites in whole cell lysate or not. Protein was dropped on the membrane at increased concentrations as 100, 200 and 400 ng/µl to observe increased expression of phospho-specific antibodies. In addition to antibodies, protein samples were also incubated with pre-immune serums to validate the binding affinity of phospho-peptides.

Figure 5.19. demonstrates that, all of the phospho-specific antibodies are not only able to their special peptides synthesized by the company, they can also bind to their specific target proteins in cell lysates. Any interaction between pre-serums and proteins were not observed, as expected.

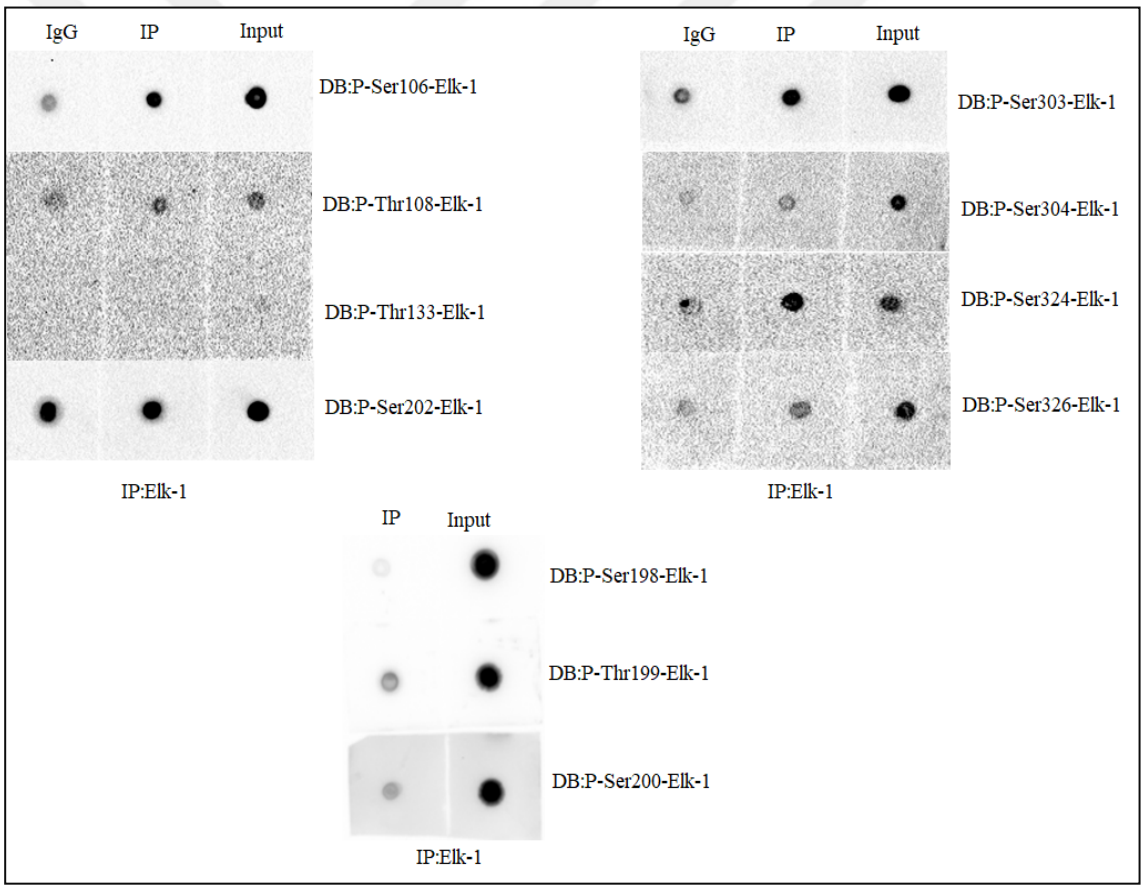


Figure 5.20. Dot Blot analysis of phospho specific antibodies on precipitated Elk-1 protein. IP: Immunoprecipitation, DB: Dot Blot, Input: Whole cell lysate

Expressions of phospho-specific antibodies were further tested on precipitated Elk-1 protein. Elk-1 was pulled down from protein lysate via an antibody/bead complex and dropped on nitrocellulose membrane along with anti-rabbit IgG as a negative control and

whole cell lysate, input, as a positive or loading control. Only low level of expression was detected on P-Thr133-Elk-1 antibody, whereas other antibodies displayed regular expressions when tested on the Elk-1 protein as seen in Figure 5.20.

In addition to dot blot assay, the expressions of the antibodies were also examined by Western blot analysis. To detect increased protein expression, 15 and 30µg of total U-87 proteins were loaded on an SDS-PAGE gel and analyzed by Western blot.

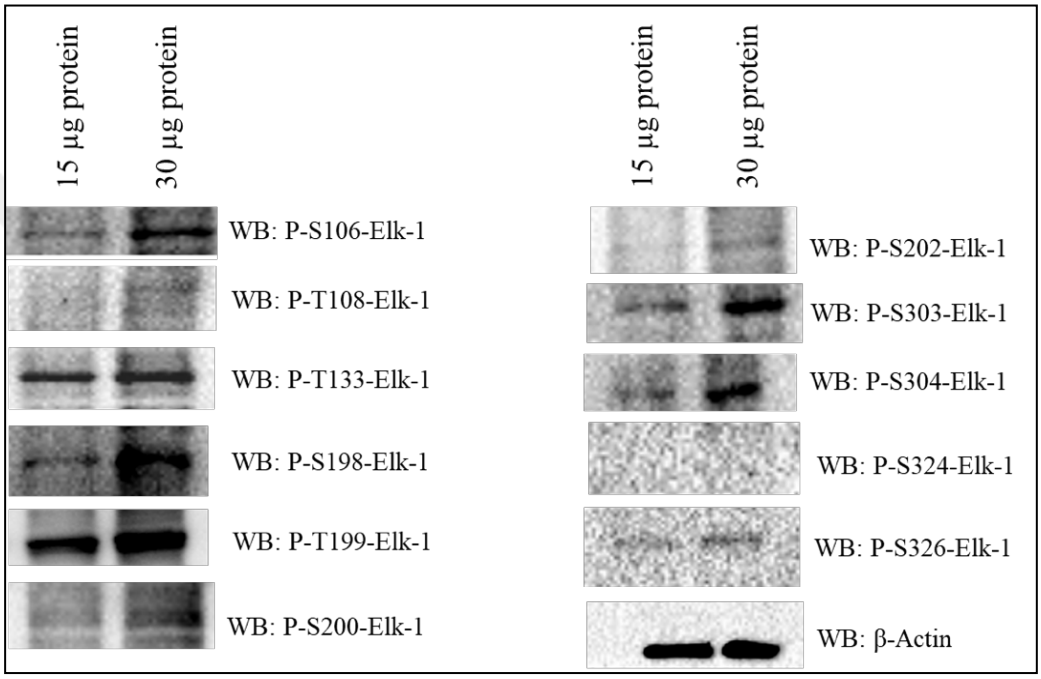


Figure 5.21. Expression of Phospho-specific antibodies. WB: Western Blot.

In the western blotting analysis shown in the Figure 5.21, approximately two-fold increased protein expression, Serine 106, 198, 303, 304 and Threonine 133, 199, has been observed due to the increased protein concentration. In other antibodies, protein expression was unclear, or increase is less than expected. The expression of β-actin was used to observe whether the protein had been loaded with an equal amount of gel in the initial stage and it was observed that an equal amount of protein was used as shown in figure.

Since low protein expression was observed in some antibodies such as Serine 324, 326, 200 and 202, the expression of antibodies was analyzed using three different protein concentrations as 10, 20 and 40µg.

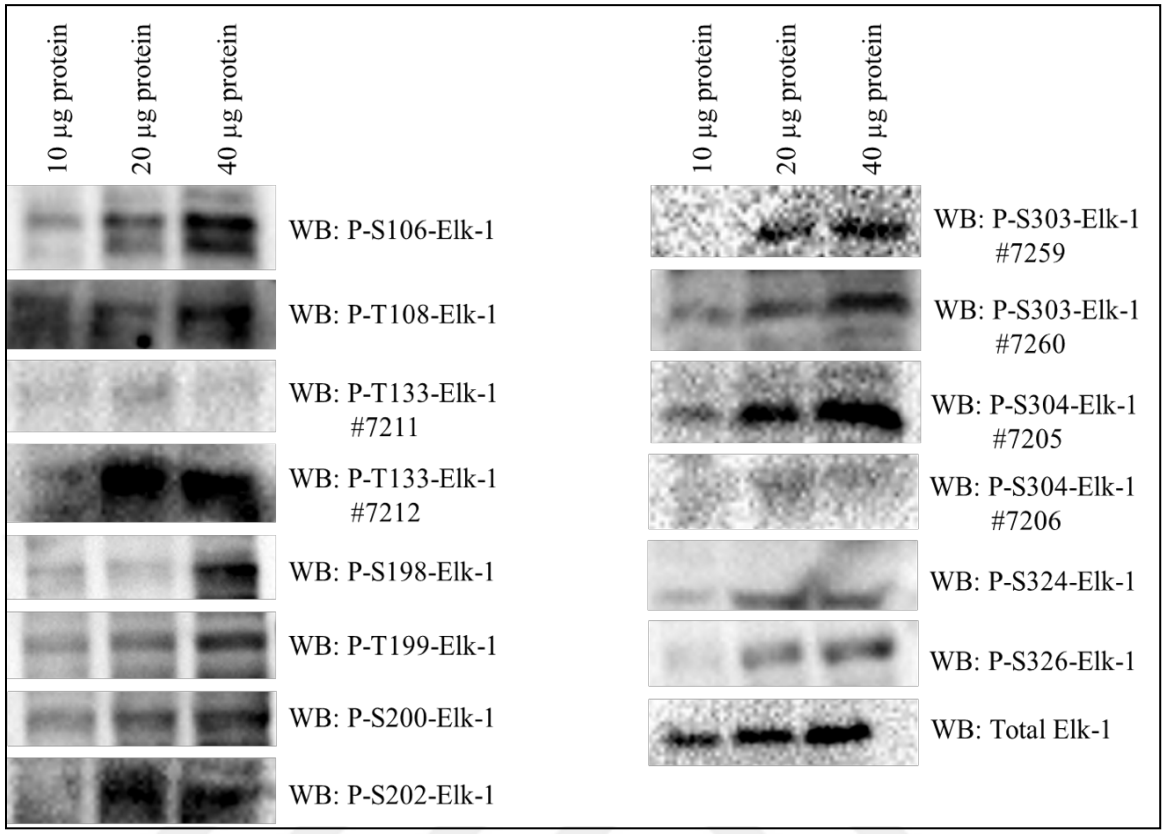


Figure 5.22. Expression Analysis of Phospho-Specific Antibodies. WB: Western Blot.

As seen in Figure 5.22, increased protein concentration and accordingly the specificity of the antibody were observed more clearly than the previous analysis. Some non-specific bands have also been shown since then the polyclonal antibodies can bind to multiple epitopes of the same antigen. Total Elk-1 antibody was used as positive control and increased expression of Elk-1 expression was also observed with increasing amount of protein.

The antibodies Threonine 133, Serine 303 and 304 were sent by the manufacturer as two different forms isolated from two different animals (rabbits), these different antibodies were also tested. #7212 Threonine 133, #7260 Serine 303 and #7205 Serine 304 antibodies were found to work better, and these antibodies were used in the further analyses.

Some of the antibodies that displayed vague or low expression were also analyzed by protein lysates transfected with both empty pCMV-Flag and pCMV-Elk-1 plasmids. SH-SY5Y cells were transfected with pCMV-Elk-1 and empty pCMV-Flag plasmids and then treated with medium containing 1, 10, and 20 per cent FBS to induce the activation of MAPK/ERK pathway and thereby the activation Elk-1. It is known that ERK pathway is

activated by serum stimulation, so the protein lysates treated with 1 per cent FBS were expected to have relatively low Elk-1 expression and relatively higher expression in those treated with 20 per cent FBS.

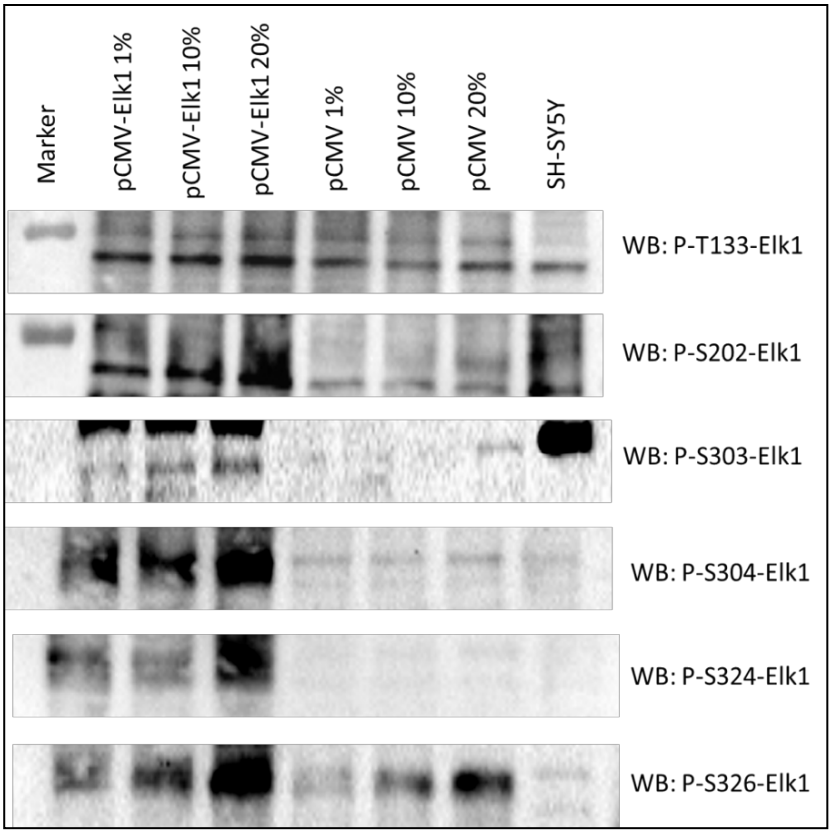


Figure 5.23. Expression analysis of Phospho-specific antibodies in pCMV-Flag and pCMV-Elk-1 plasmids transfected cells. WB: Western Blot, SH-SY5Y whole cell lysate.

Since Elk1 concentration is gradually increasing, increased phospho-antibodies levels expected to observe. Even though, protein level did not significantly change in T133 and S303-Elk1samples. Along with that, gradually increased phospho-Elk1 protein expression was observed when S304, S324 and S326-Elk1 antibodies were used. Since empty pCMV-Flag vector was used as a negative control, any significant protein expression was not detected on these protein samples as seen in Figure 5.23. Total SHSY5Y protein was used as a positive control to observe protein expression.

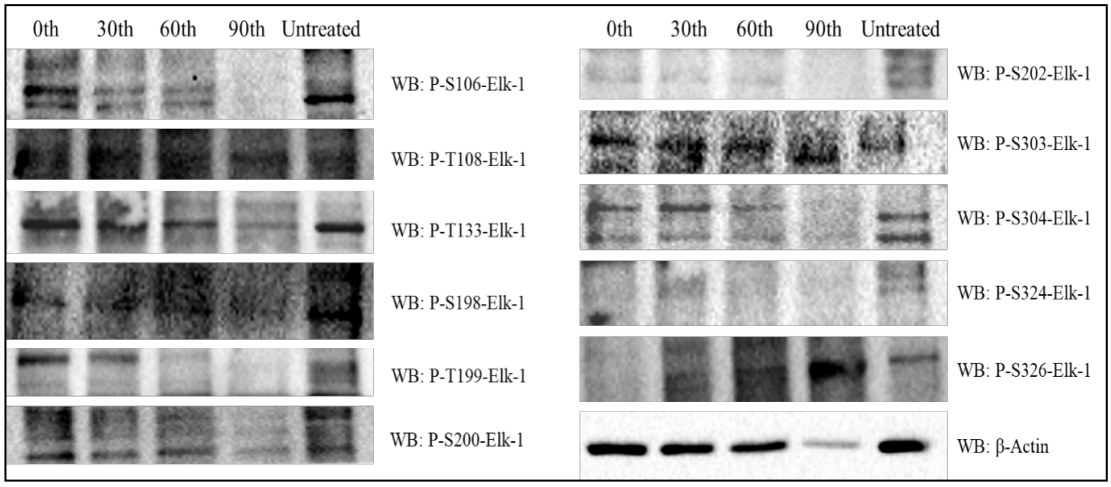


Figure 5.24. Expression analysis of Phospho-specific antibodies in nocodazole-treated cells. WB: Western blot. Untreated is the whole cell lysate.

Expressions of phospho-specific antibodies were further analyzed using protein lysates treated with nocodazole. Protein lysates were collected at 30 minutes time intervals after treatment with nocodazole, and protein expressions were analyzed by Western blotting. While the expression of some antibodies was clearly observed as in Serine 106, 200, 303, 304 and Threonine 133, low expression was observed due to the low protein concentration as demonstrated in Figure 5.24.

β-actin was used as a loading control and it was observed that protein lysate isolated at 90th minute was used with lower protein concentration than the other samples. This was probably due to a pipetting or similar error in protein concentration measurement. Therefore, it is expected to see the lower expression in protein lysate at 90th minute in the expression analysis of phospho-specific antibodies.

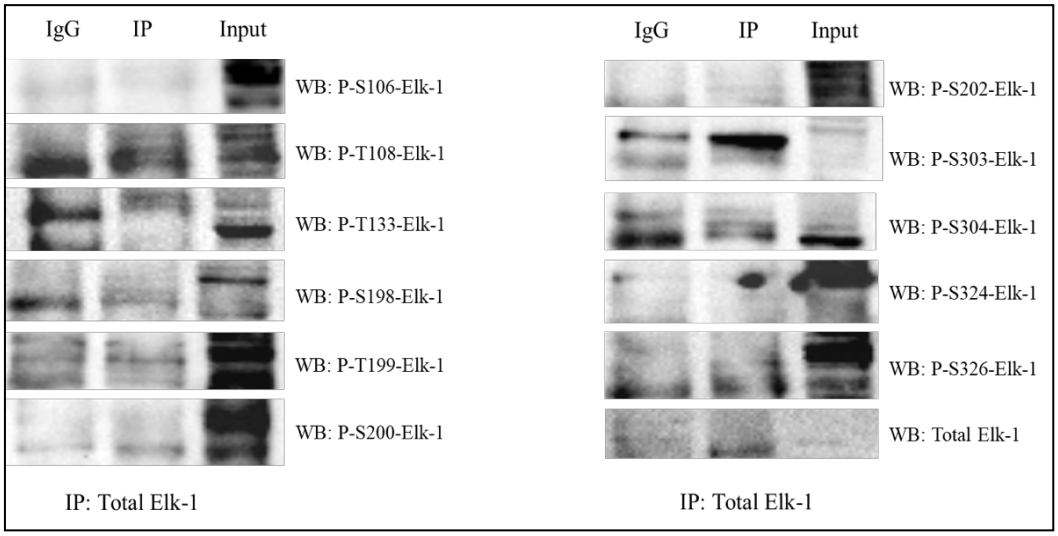


Figure 5.25. Expression analysis of Phospho-specific antibodies in immunoprecipitated Elk-1 protein. IgG; Immunoglobulin G, IP; Immunoprecipitation, Input; whole cell lysate, WB; Western blot.

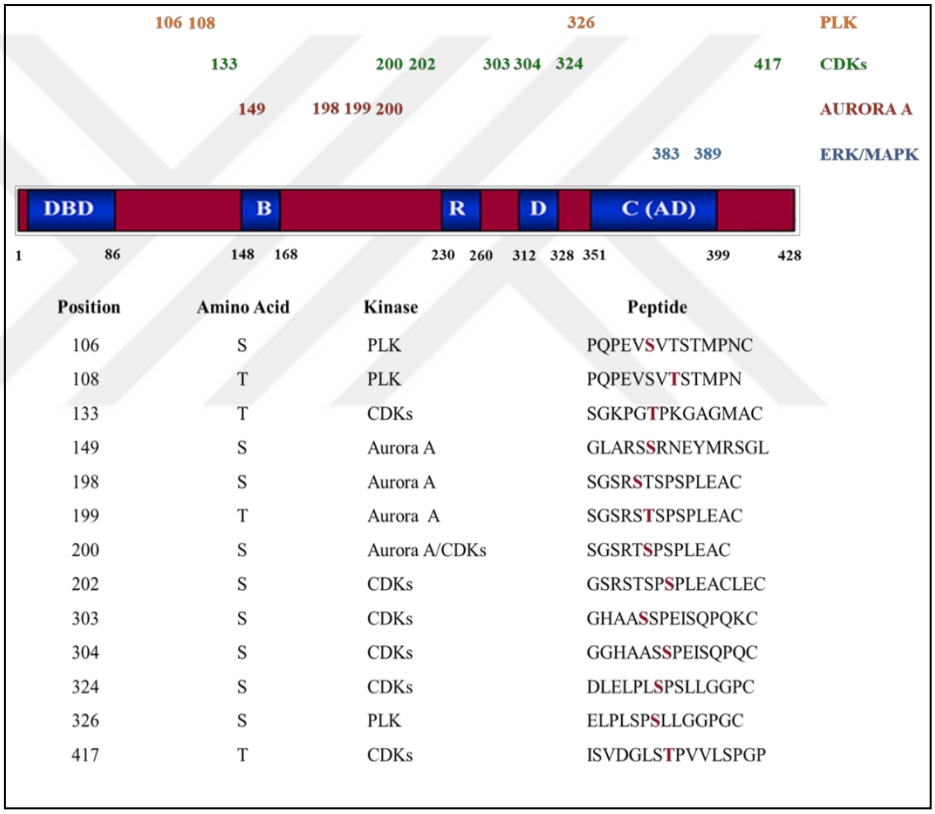
In addition to analyzes with total protein lysates, the analysis of phospho-specific antibodies has also been performed using immunoprecipitated Elk-1 protein. As seen in Figure 5.25, the expression of certain antibodies was detected as remarkably high, P-T108, P-S198, P-T199, P-S303, P-S304, whereas others displayed low protein expression. Immunoglobulin G (IgG) samples were used as negative control, while input or whole cell lysate was used as positive control. Elk-1 Western blot was performed to observe if the Elk-1 protein could be successfully precipitated from the total protein lysate or not and as it was demonstrated in figure, immunoprecipitation was carried out successfully.

5.6. CREATING PHOSPHO-MUTANTS BY SITE-DIRECTED MUTAGENESIS (SDM)

Site-directed mutagenesis (SDM) is a technique that is used to make specific and intended changes on a DNA sequence of a gene. SDM can be used to investigate the structural and biological activity of a gene or protein, protein-protein interactions, identification of binding domains of proteins or active sites of enzymes [145].

To analyze the effects of Elk-1 on different processes, site-directed mutagenesis is widely used. For that purpose, serine and threonine residues of the best-studied phosphorylation sites of Elk-1, Ser383, Ser389 and Thr417, are converted to alanine and aspartic (D) or

glutamic (E) acid residues to block potential phosphorylation sites or to create phospho-mimic mutations by SDM. pCMV-Elk-1-S383A plasmid was obtained from Prof. Andrew Sharrocks as a gift, however, pCMV-Elk-1-S383E, pCMV-Elk-1-S389A pCMV-Elk-1-S389E pCMV-Elk-1-T417A and pCMV-Elk-1-T417A mutations on Elk-1 protein sequence were generated by Q5 Site-directed mutagenesis kit. In addition to these known phosphorylation sites of Elk-1, putative Cdk1, Plk1 and Aurora A phosphorylation sites on Elk-1 protein sequence depicted in Figure 5.26, were converted to both alanine and glutamic acid residues to create phospho-null and phospho-mimic mutations by specific primer pairs listed in the methods section.



Position	Amino Acid	Kinase	Peptide
106	S	PLK	PQPEVSVTSTMPNC
108	T	PLK	PQPEVSVTSTMPN
133	T	CDKs	SGKPGTPKGAGMAC
149	S	Aurora A	GLARSSRNEYMRSGL
198	S	Aurora A	SGSRSTSPSPLEAC
199	T	Aurora A	SGSRSTSPSPLEAC
200	S	Aurora A/CDKs	SGSRTSPSPLEAC
202	S	CDKs	GSRSTSPSPLEACLEC
303	S	CDKs	GHAASSPEISQPQKC
304	S	CDKs	GGHAASSPEISQPQC
324	S	CDKs	DLELPLSPSLLGGPC
326	S	PLK	ELPLSPSLLGGPGC
417	T	CDKs	ISVDGLSTPVVLSPGP

Figure 5.26. Elk-1 protein sequence analysis in terms of potential phosphorylation sites. Red amino acids are putative phosphorylation sites of Elk-1.

Mitotic kinases are also phosphorylated by other protein kinases and these phosphorylations are crucial for their activation and function. Cdk1 kinase is phosphorylated from Threonine 161 residue [146], Plk1 kinase is phosphorylated from Threonine 210 residue [147] and Aurora A kinase is phosphorylated form Threonine 288 residue [148] by various kinases. If there is a phosphorylation link between Elk-1 and these kinases, phosphorylation of them also affects the function of Elk-1. To analyze such an

effect on Elk-1, kinase-dead and phospho-mimic, mutations on Cdk1, Plk1 and Aurora A kinases are required. Aurora A-T288A and T288D mutant plasmids are special gifts from Prof Hsu Shih-Lan, Taiwan. Wild-type Plk1, Plk1-T210A and Plk1-T210D plasmids are special gifts from Prof Wen H. Shen, Cornell University. These plasmids were also sequenced and the mutations on these sites were controlled by Workbench tool. Cdk1-T161A and T161E mutations were also performed on previously cloned pCMV-HA-Cdk1 vector and these mutations were also analyzed by sequencing and sequencing results are represented in Appendix A.1.

Kettenbach et al. analyzed potential targets of Aurora A and Polo-like kinase 1 (Plk1) during mitosis by inhibition of these kinases combined with large-scale quantitative phosphoproteomics. They identified 778 phosphorylation loci on 562 proteins from mitotic cells that are candidate targets of these kinases. This study revealed that Elk-1 is one of the targets of Aurora A and Polo-like kinase 1 during mitosis. Their mass phospho-proteomics studies demonstrated that Elk1 is phosphorylated from Serine 303, Serine 304, Serine 324 and Serine 326 [149]. In the light of this data, these sites also mutated to both alanine and glutamic acid residues for further analysis.

Elk-1 coding sequence was previously cloned into pCMV-5-Flag vector by Prof. Andrew Sharrocks and this vector was used to mutate putative phosphorylation sites. All Elk-1 phospho-mutations were generated by NEB Q5 site-directed mutagenesis kit and mutant plasmids were transformed into bacteria and isolated by plasmid isolation kit. To analyze whether mutations shown in Figure 5.27 were successfully created on desired residues or not, the mutated plasmids were sequenced by Macrogen sequencing service and results evaluated via Workbench tool. Sequencing results are depicted in Appendix. A.1.

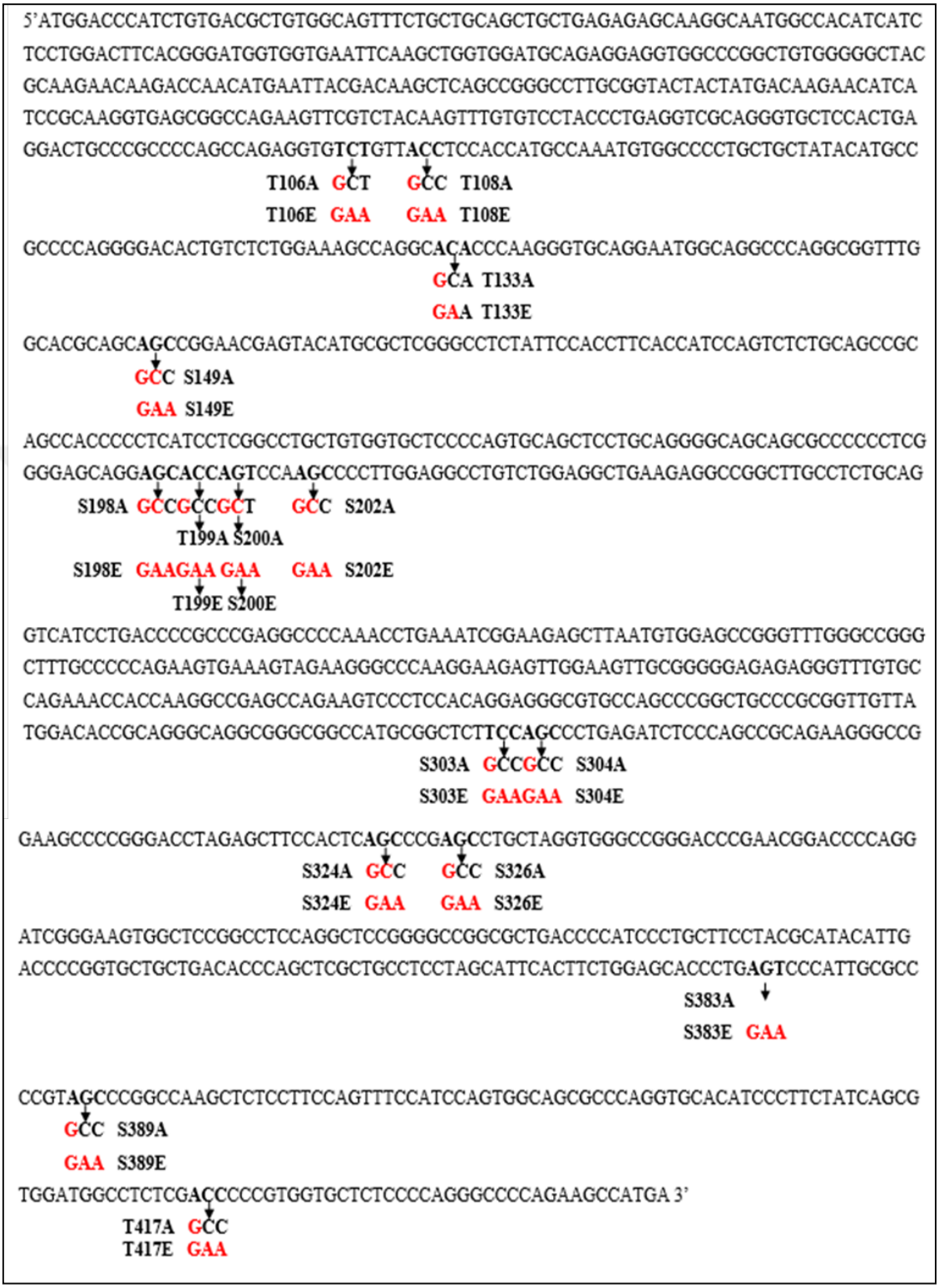


Figure 5.27. Created mutations on Elk-1 protein sequence. Red nucleotides on primer sequences indicate converted amino acids.

After the screening of the mutations on Elk-1 protein sequences corresponding putative Elk-1 phosphorylation sites by sequencing, expressions of these mutant plasmids were confirmed by immunoprecipitation and Western blot assays. To that end, S106A/E, T108A/E, T133A/E, S198A/E, T199A/E, S200A/E, S202A/E, wild-type Elk-1 and empty pCMV-Flag plasmids were transfected into SH-SY5Y neuroblastoma cells by PEI reagent. Proteins were isolated from cells bearing phospho-null and phospho-mimic mutations of Elk-1 and the expression of these plasmids were confirmed by Western blot.

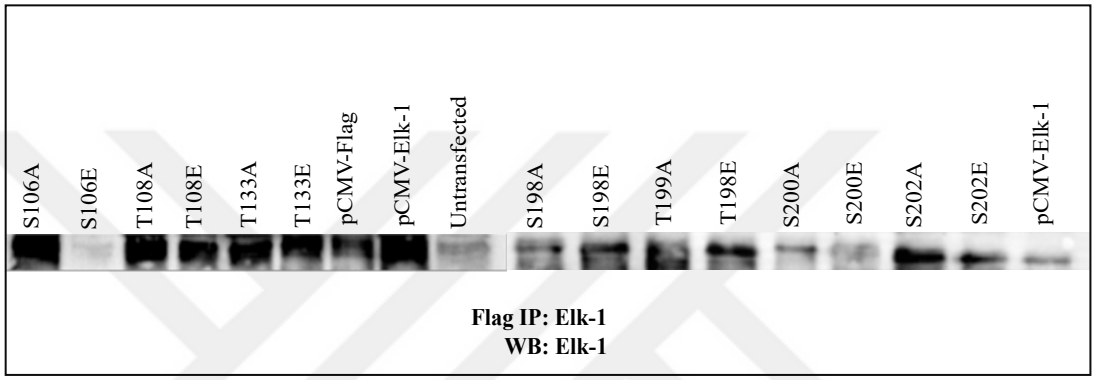


Figure 5.28. Expression control of wild-type and mutagenic Elk-1 plasmids.

Expression analysis of Elk-1 protein is depicted in Figure 5.28. According to this analysis, both mutagenic and wild-type Elk-1 protein were able to be expressed in SH-SY5Y human neuroblastoma cells. The protein expression seen in the empty plasmid was unexpected, but it can be ignored because it is very low compared to the wild-type Elk-1 expression. The untransfected sample represents total cell lysate which used to detect the exact position of Elk-1 protein on the membrane.

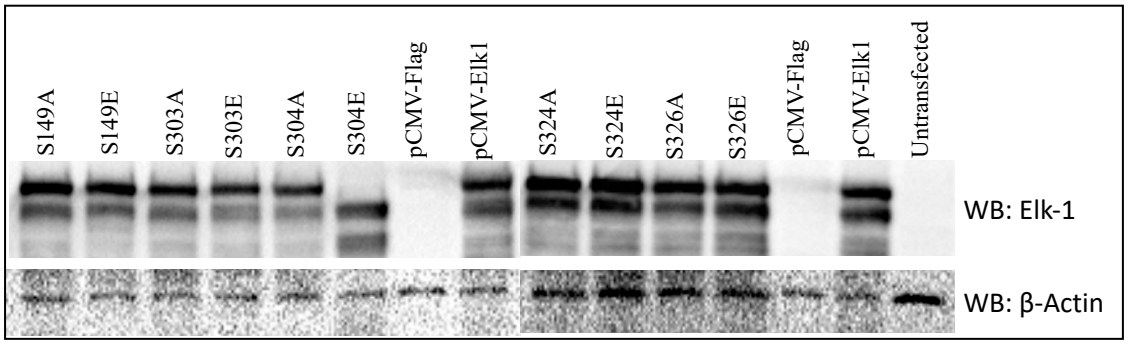


Figure 5.29. Expression control of various Elk-1 plasmids. Expression of wild-type and mutagenic Elk-1 proteins were evaluated by Western blot.

Expression of the second set of Elk-1 putative phosphorylation sites, Serine 149, 303, 304, 324 and 326 were also analyzed by Western blot. As in the previous analysis, it was observed that these set proteins were successfully expressed in SH-SY5Y cells as shown in Figure 5.29. In addition, in this experiment, protein expression was not seen in the empty pCMV-Flag plasmid, as expected. 'Untransfected' lane indicates the total cell lysate and used as positive control to confirm Elk-1 expression. β-actin was used as a loading or internal control since it belongs to a class of genes called housekeeping genes and β-actin expression in Figure 5.29. confirms that all proteins were loaded almost equal amounts to the gel.

Elk-1-S324E mutant was detected on the membrane at a lower level than the other samples. This brings to mind the question of whether this phosphorylation has a relation with SUMOylation of the protein or not. Additional experiments are required to precisely uncover the relationship between this phosphorylation site and SUMO modification.

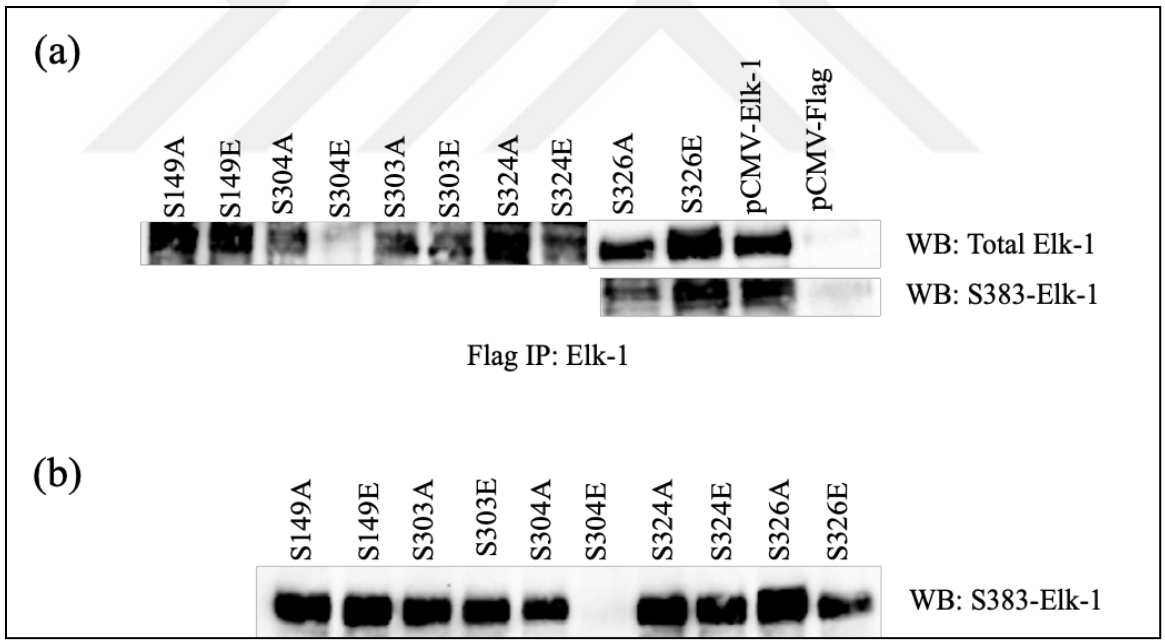


Figure 5.30. Effect of Elk-1 phosphorylation on Elk-1 Serine 383 phosphorylation. Proteins were precipitated by Flag antibody and analyzed by Western Blot (a). Serine 383-Elk1- expression was analyzed by Western Blot (b).

In order to further examine the expression of mutant and wild-type Elk-1 protein, these plasmids were transfected into SH-SY5Y cells by PEI reagents and proteins were isolated from the cells. 250 μg protein samples were incubated with Anti-flag M2 affinity agarose

gel (Sigma) and precipitated proteins were loaded on SDS-gel. Expression of these proteins was analyzed by the incubation of protein membranes with the anti-Elk-1 antibody.

When the protein expression in Figure 5.30a is examined, it can be observed that phosphorylation of Serine 149 and 303 do not have a significant effect on Elk-1 expression, whereas phospho-mimic mutations on Serine 304 and 324 residues resulted in a decrease in Elk-1 expression. However, the phospho-mimic mutation of Serine 326 residue promoted Elk-1 expression, and this indicates an enhancing effect of this residue on Elk-1 protein expression. In addition to Elk-1 expression, the effects of putative Elk-1 phosphorylation on Serine 383 phosphorylation of Elk-1 which is critical for Elk-1 transcriptional activity, was analyzed. Elk-1-Serine 383 expression, likewise, increased when Serine 326 phosphorylation is constitutively active.

In Figure 5.30b, total protein samples were loaded onto the gel and the effect of these potential phosphorylation sites of Elk-1 on phosphorylation of Serine 383 was investigated. In contrast to the IP samples, it was seen that the phosphorylation of Serine 326 had an inhibitory effect on the phosphorylation of Serine 383 and no significant difference was observed in the other phosphorylations.

5.7. ELK-1 LOCALIZATION DURING MITOSIS

Serine 383 phosphorylated Elk-1 localization during the cell cycle is found to be in mitotic spindle poles in prophase and metaphase, in midzone in anaphase and in spindle midbody in telophase cytokinesis transition. Although weaker staining was observed according to the Serine 383 phosphorylated Elk-1, total Elk-1 displayed a widespread staining during interphase and prophase and translocated to the spindle pole at metaphase. However, Threonine 417 phosphorylated Elk-1 was found to be predominantly nuclear in interphase, stack at DNA-bound as dot like structure throughout the different stages of mitosis, and it could not translocate to the spindle midbody during cytokinesis [7].

Based on this previous study, the localizations of developed phospho forms of Elk-1 in different phases of cell cycle has been investigated by immunostaining. U87 glioblastoma cells were spread onto poly-L lysine coated coverslips and were fixed in ice-cold methanol. After blocking with 10 per cent FBS-PBS solution they were incubated with β-tubulin and

phospho-specific Elk-1 antibodies, P-S106, P-T108, P-T133, P-S198, P-T199, P-S200, P-S202, P-S303, PS304, P-S324 and P-S326-Elk-1.To visualize cells on the microscope, cells were afterward stained with secondary antibodies and the localization of the cells during various stages of cell cycle was analyzed by confocal microscope.

Immunostaining results demonstrated that some of the phospho-specific antibodies were working well in immunostaining, while others did not give an apparent signal. For example, as seen in Figure 5.31, Serine 106 Elk-1 exhibited a remarkable staining, it was bound to DNA throughout the interphase/prometaphase, then passed to the spindle poles and remained there until the end of cytokinesis. While the Serine 106 Elk-1 was partially seen in the spindle poles and partly in the midzone, midbody localization of Serine 383 phosphorylation of Elk-1 was not observed in this phospho form.

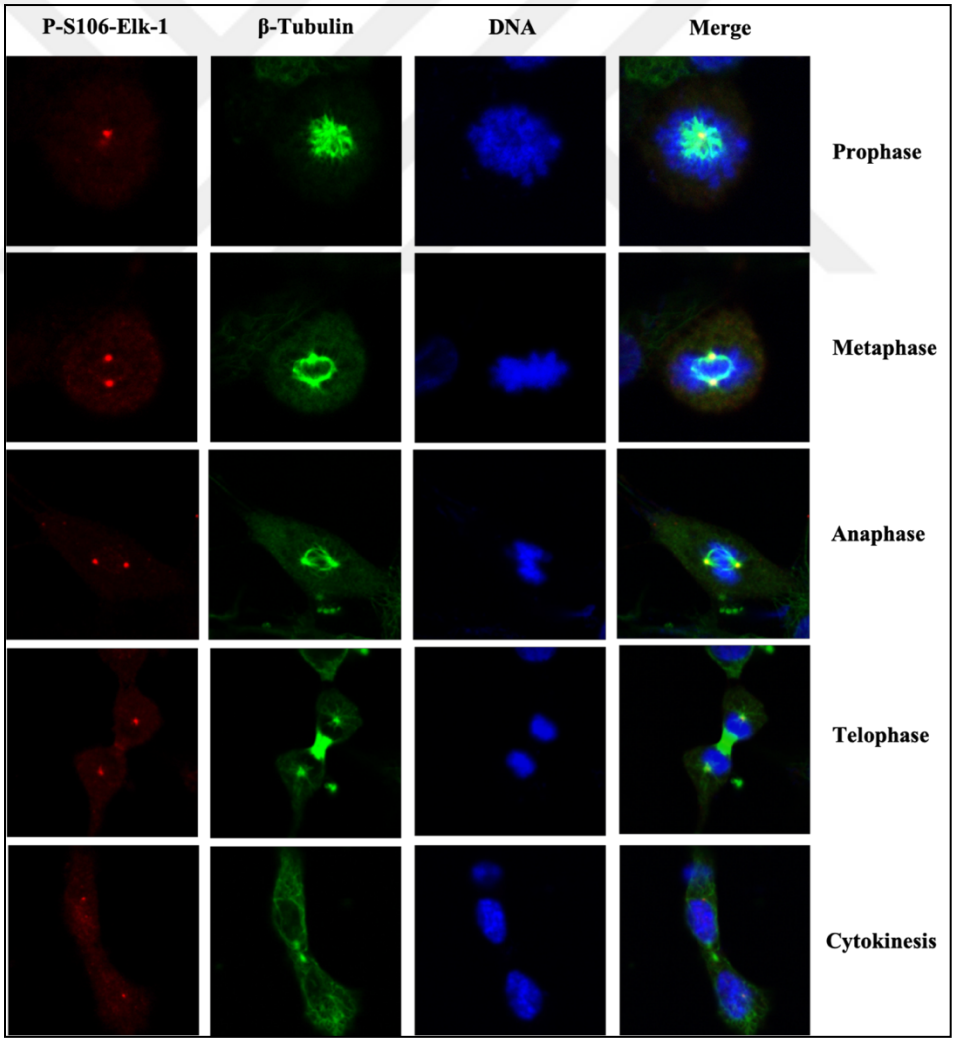


Figure 5.31. The localization of phospho-S106-Elk-1 during different stages of the cell cycle in U-87 cell lines (anti-P-S106-Elk-1: red; anti-β-tubulin: green, DNA: blue).

When Serine 324 phosphorylation of Elk-1 was analyzed in terms of mitotic localization, it was observed that this phospho-forms localizes prominently at spindle poles during prometaphase through anaphase likewise S-106-Ek-1 as seen in Figure 5.32. Although it was not as clear as the S-106-Elk-1, it was observed that P-S324-Elk-1remained at the spindle poles along the telophase and cytokinesis could not pass to the midbody region like P-S106-Elk-1.

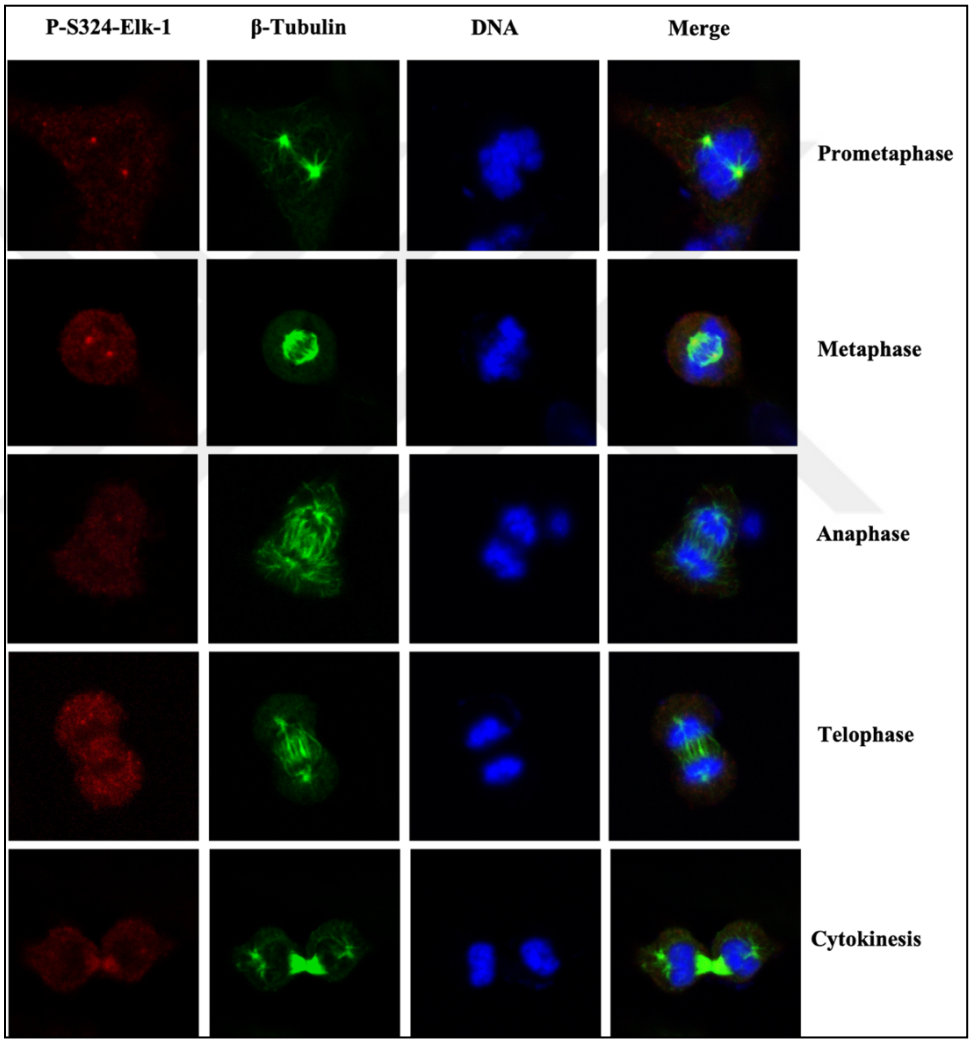


Figure 5.32. The localization of phospho-S324-Elk-1 during different stages of the cell cycle in U-87 cell lines (anti-P-S106-Elk-1: red; anti-β-tubulin: green, DNA: blue).

When mitotic localization of P-S108, P-T133, P-S202, P-S303, P-S304 and P-S326 phosphorylations of Elk-1 was examined, very faint staining was observed, and they were mainly, if any, localized at spindle poles (Appendix B). In addition to this, P-S198, P-T199

and P-S200-Elk-1 were mainly localized at spindle poles during metaphase and translocate to the midbody during cytokinesis. However, P-S200-Elk-1 staining which had a widespread localization during metaphase, similar to P-T417 form of Elk-1 (Appendix B).

In order to investigate the effect of Elk-1 phosphorylations on mitotic localization, the U-87 cells were transfected with phosho-null plasmids, S106A, T108A, T133A, S198A, T199A, S200A, S202A, S303A, S304A, S324A and S326-Elk-1, which block the phosphorylation sites. Cells, then, stained with β-tubulin, Flag and phospho-specific Elk-1 antibodies, P-S106, P-T108, P-T133, P-S198, P-T199, P-S200, P-S202, P-S303, PS304, P-S324 and P-S326-Elk-1. Afterwards, cells incubated with secondary antibodies and mounted onto glass slides with antifade reagent. The localization of the cells during various stages of cell cycle was analyzed by confocal microscope.

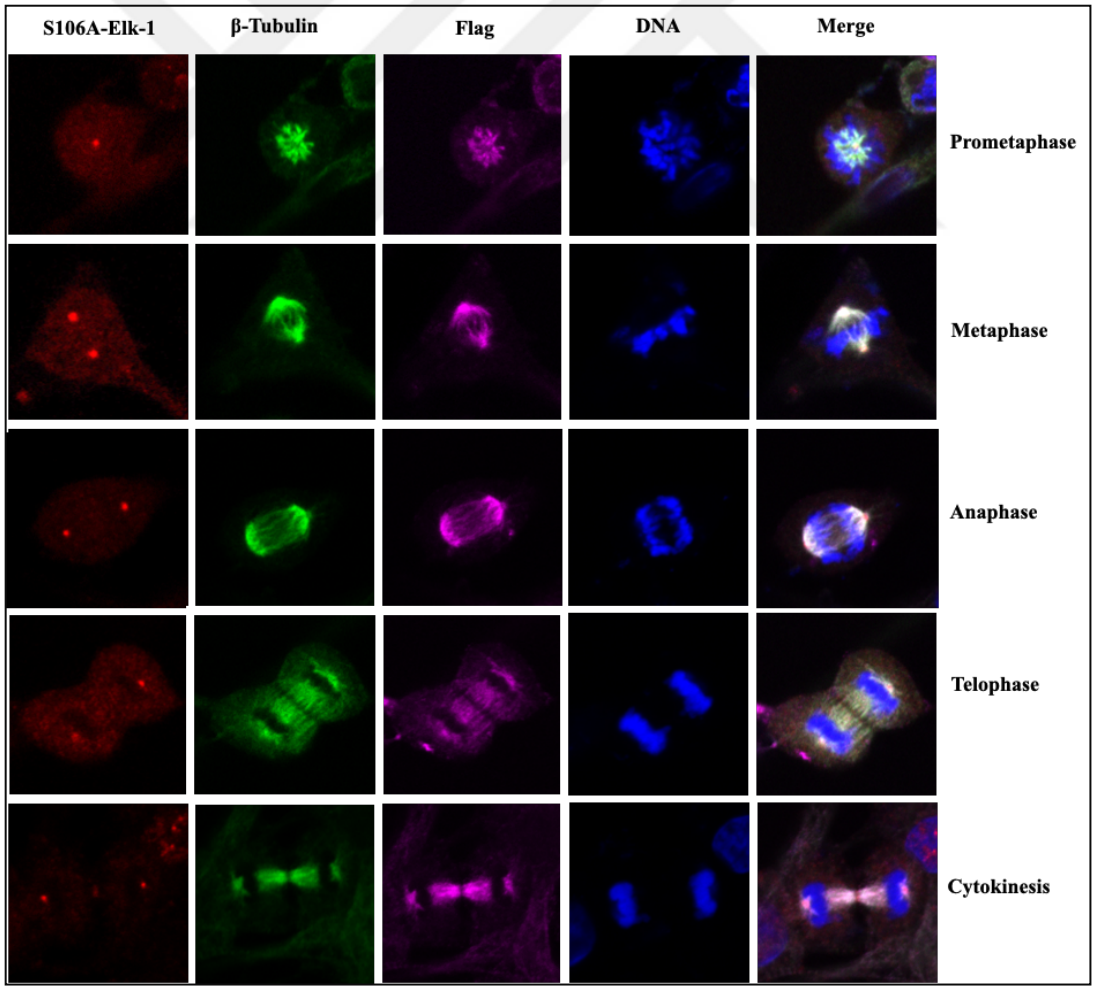


Figure 5.33. The localization of P-S106-Elk-1 after S106A transfection (anti-P-S106-Elk-1: red, anti-β-tubulin: green, anti-Flag: pink, DNA: blue).

When U-87 cells were transfected with phospho-null mutation, S106A-Elk-1, and P-S106-Elk-1 localization was examined, it was observed that the inhibition of phosphorylation of this residue did not significantly affect its localization; Elk-1 was able to settle in the spindle poles, unexpectedly, and maintained its localization through cytokines as seen in Figure 5.33. Effect of phospho-null mutation, S324A-Elk-1, on P-S324-Elk-1 localization was also evaluated by immunofluorescence and as it is demonstrated in Figure 5.34, spindle pole localization during metaphase was not affected significantly, whereas, during anaphase through cytokinesis, cells were unable to localize at spindle poles. Mitotic localizations of P-T133, P-S202, PS303 and P-S304-Elk-1 were also analyzed by phospho-null mutations but prominent localization changes were not observed as good as P-S106-Elk-1 and S324-Elk-1 (Appendix B).

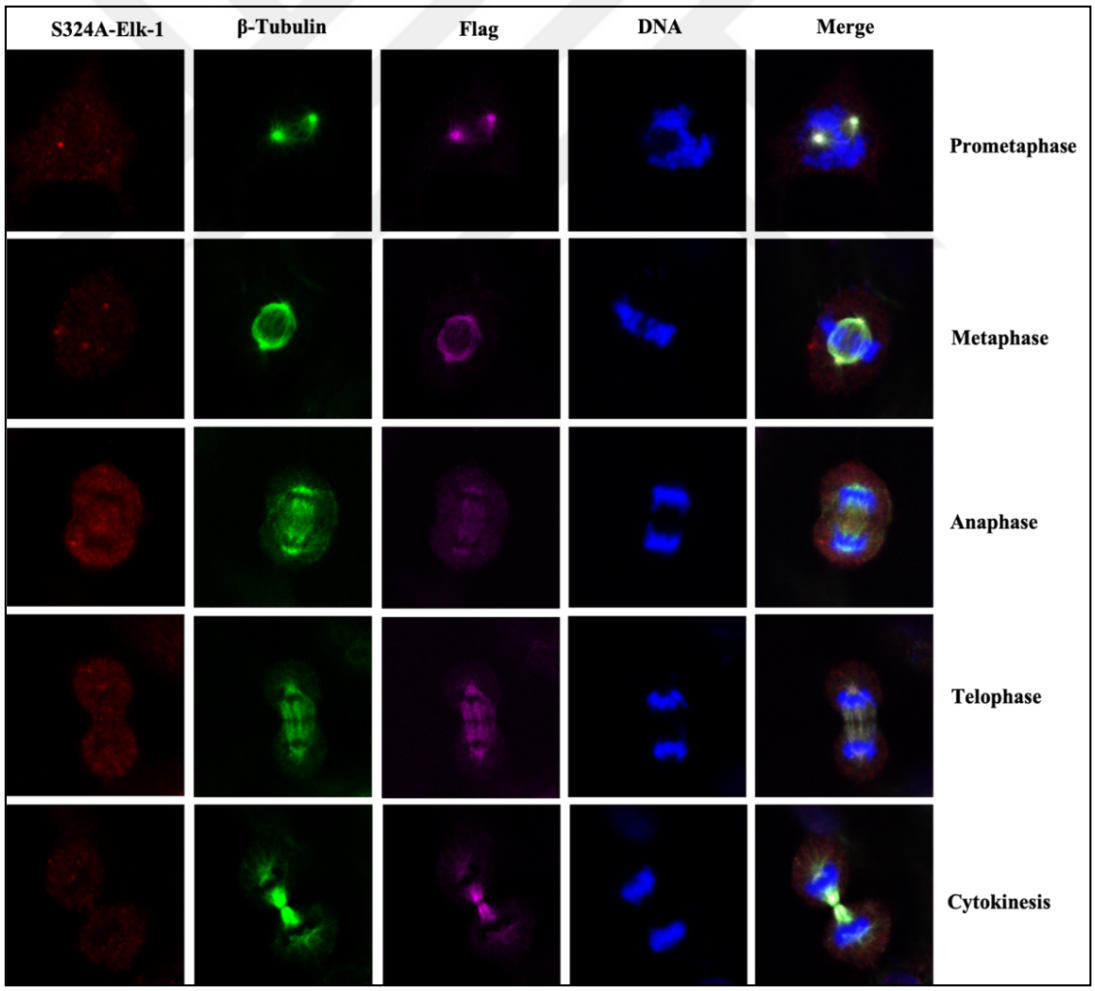


Figure 5.34. The localization of P-S324-Elk-1 after S324A transfection (anti-P-S106-Elk-1: red, anti-β-tubulin: green, anti-flag: pink, DNA: blue).

5.8. PHOSPHORYLATION STATUS OF ELK-1

Phosphorylation status of Elk-1 was evaluated by creating mutations on target residues that either continuously activates these regions, phospho-mimic, or block their phosphorylation, phospho-null, and detecting phosphorylation level by region-specific phospho-Elk-1 antibodies. Elk-1 phospho-mutants, S106A/E, T108A/E, T133A/E, S198A/E, T199A/E, S200A/E, S202A/E, S303A/E, S304A/E, S324A/ and S326A/E, wild-type, pCMV-Elk-1 and empty vectors, pCMV-Flag were transfected into the cells and following overexpression of these proteins, they were isolated from the cells. Each site-specific mutation was analyzed by Western blotting using antibodies specific for phosphorylation in the same region, for instance, Elk-1 Serine 106 phosphorylation was analyzed by the overexpression of Elk-1-S106A and Elk-1-S106E proteins and incubating these proteins with P-S106-Elk-1 antibody.

When the total protein Western blot results, in Figure 5.35, were examined, it can be seen that expression of phospho-specific antibodies, P-T108-Elk-1, P-S200-Elk-1, P-S202-Elk-1, P-S324-Elk-1, P-S326-Elk-1, were increased, phosphorylation of phospho-mimic mutations carrying proteins are higher than phospho-null carrying ones. This increase in protein expression indicates potential phosphorylation of these residues. There was no significant phosphorylation difference between phospho-null and phospho-mimic forms of other target residues.

According to the normalization results in Figure 5.36; about 0.5-fold increase in P-S198-Elk-1, about 2-fold increase in P-S202-Elk-, a 10-fold increase in P-S324-Elk-1 and 31.5-fold increase in P-S326-Elk-1 has been observed. Although there was an increase in P-S108-Elk-1 and P-S200-Elk-1 phosphorylation according to the Figure 5.35, 0.5 and 2-fold respectively, the reduction was observed in the results of normalization, which may have been due to the amount of protein used in the initial stage. In spite of that, there was not a significant increase in Western blot results, it was also seen that there was a 0.5-fold increase in P-S106-Elk-1 and about a 6-fold increase in P-T133-Elk-1 phosphorylation when the protein content was normalized with βeta-actin.

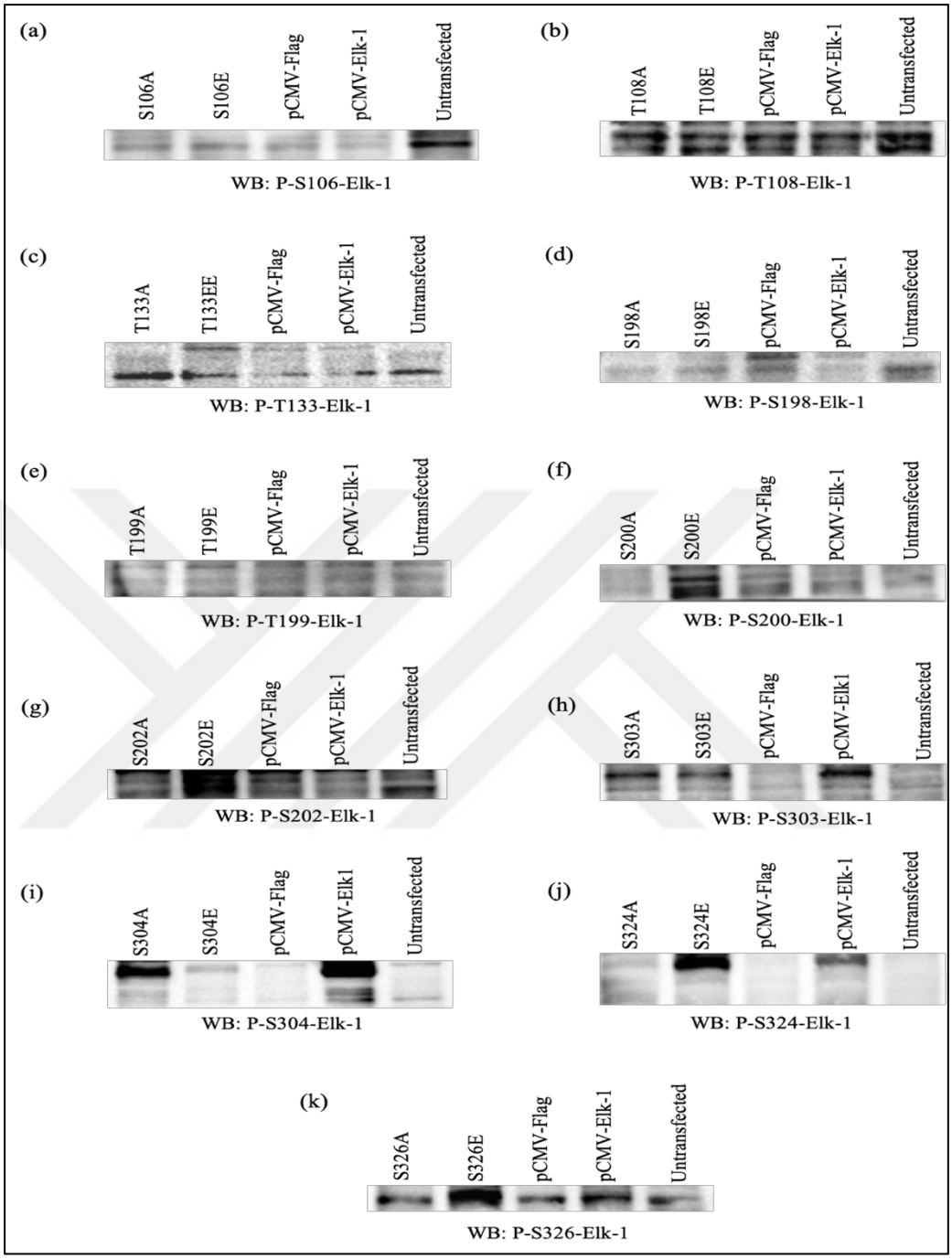


Figure 5.35. Phosphorylation analysis of Elk-1 target residues by Western blot. SH-SY5Y cells were transfected with specific mutagenic plasmids and wild-type and empty vector as controls, and phosphorylation of each residue analyzed by phospho-specific antibodies, P-S106-Elk-1 (a), P-T108-Elk-1 (b), P-T133-Elk-1 (c), P-S198-Elk-1 (d), P-T199-Elk-1 (e), P-S200-Elk-1 (f), P-S202-Elk-1 (g), P-S303-Elk-1 (h), P-S304-Elk-1 (i), P-S324-Elk-1 (j), and P-S326-Elk-1 (k).

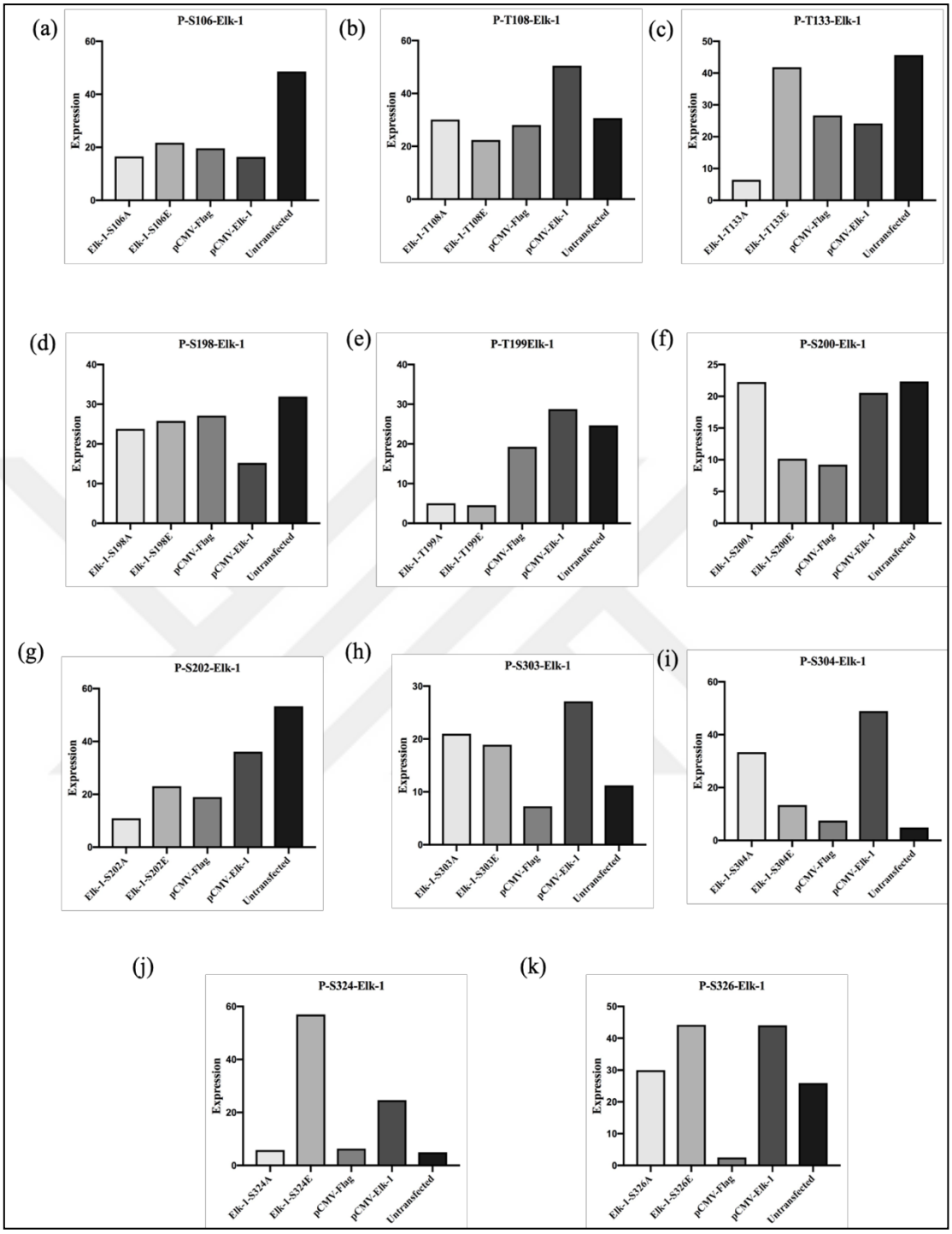


Figure 5.36. Normalizations of phosphorylation analysis of Elk-1 target residues by Western blot, P-S106-Elk-1 (a), P-T108-Elk-1 (b), P-T133-Elk-1 (c), P-S198-Elk-1 (d), P-T199-Elk-1 (e), P-S200-Elk-1 (f), P-S202-Elk-1 (g), P-S303-Elk-1 (h), P-S304-Elk-1 (i), P-S324-Elk-1 (j), and P-S326-Elk-1 (k). Western blot results were normalized by comparing band intensities to the intensity of total Elk-1 corresponding protein bands.

To analyze the phosphorylation status of Elk-1 by evaluating phosphorylation levels between phospho-null and phospho-mimic mutant proteins, total protein samples were used in the previous experiment. To determine Elk-1 phosphorylation in a more specific way, Elk-1 protein was pulled down from the total cell lysate by Flag-agarose beads, rather than analyzing Elk-1 along with other proteins in total protein lysate. Phospho-null and phospho-mimic forms of Elk-1 proteins stacked with flag agarose beads were loaded on protein gel and phosphorylation of these sites was assessed by specific phospho-Elk-1 antibodies as in the previous experiment.

In Figure 5.37, expression of phospho-specific antibodies, P-T198-Elk-1, P-T199-Elk-1, P-S202-Elk-1 and P-S324-Elk-1 were increased. Phosphorylation of phospho-mimic mutations carrying proteins is higher than phospho-null carrying ones likewise in previous results. This increase in protein expression indicates a potential phosphorylation of these residues. There was no significant phosphorylation difference between phospho-null and phospho-mimic forms of other target residues or increased phosphorylation of proteins containing phospho-null mutations, S303A-Elk-1, S304-Elk-1 and S326A-Elk-1, detected unexpectedly

To evaluate the results in Figure 5.37 numerically, normalization results in Figure 5.38; a has been used and it was observed P-S198-Elk-1 phosphorylation increased approximately 0.5-fold and P-T199-Elk-1 increased considerably high. Similarly, it was observed that P-S202-Elk-1, P-S303-Elk-1, P-S304-Elk-1, P-S324-Elk-1 P-S32-Elk-1 phosphorylation increased 4, 2, 2, 5.5 and 5-fold, respectively.

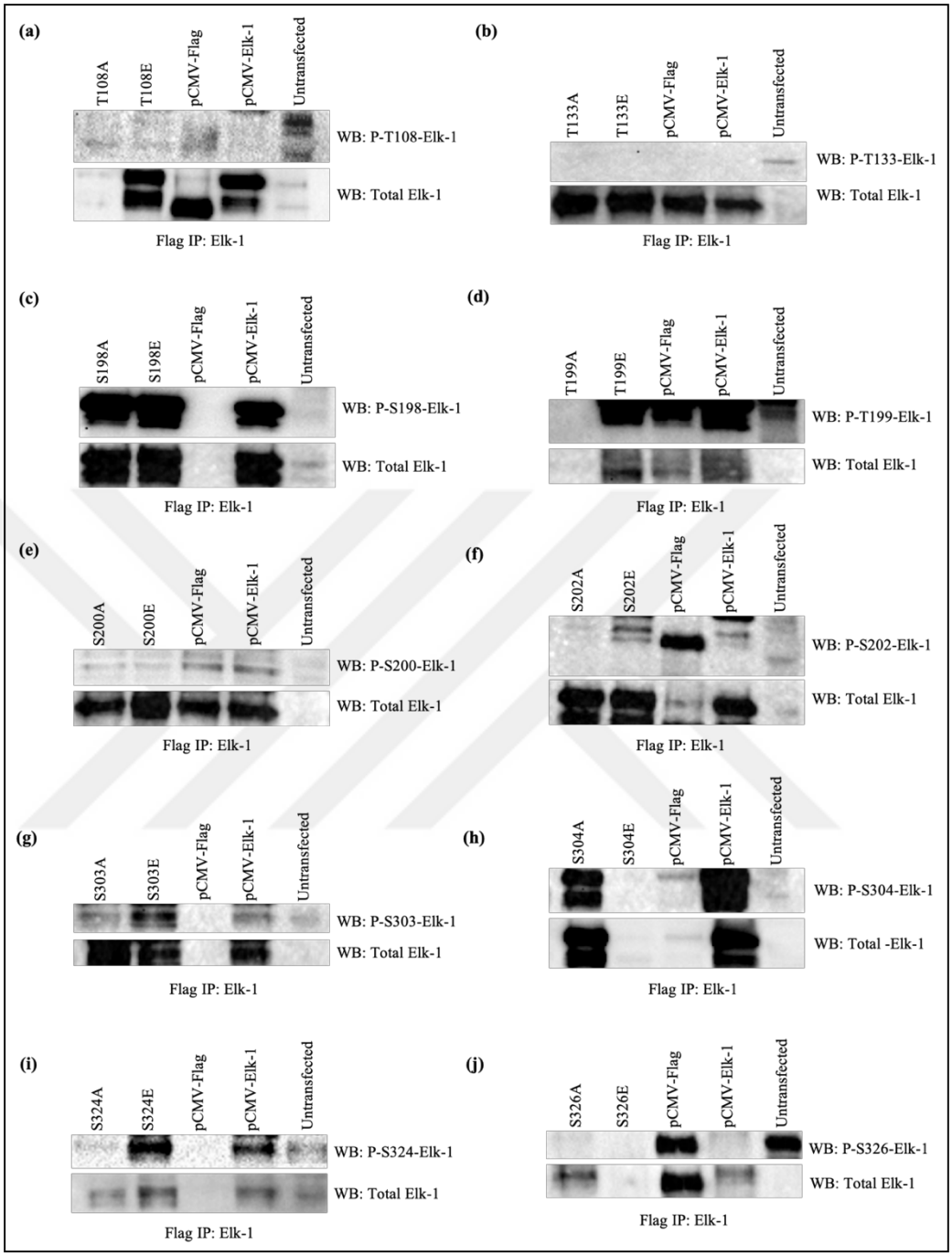


Figure 5.37. Phosphorylation analysis of Elk-1 target residues by immunoprecipitation followed by Western blot. SH-SY5Y cells were transfected with specific mutagenic plasmids and wild-type and empty vector as controls, and phosphorylation of each residue analyzed by Phospho-specific antibodies, P-T108-Elk-1 (a), P-T133-Elk-1 (b), P-S198-Elk-1 (c), P-T199-Elk-1 (d), P-S200-Elk-1 (e), P-S202-Elk-1 (f), P-S303-Elk-1 (g), P-S304-Elk-1 (h), P-S324-Elk-1 (i), and P-S326-Elk-1 (j).

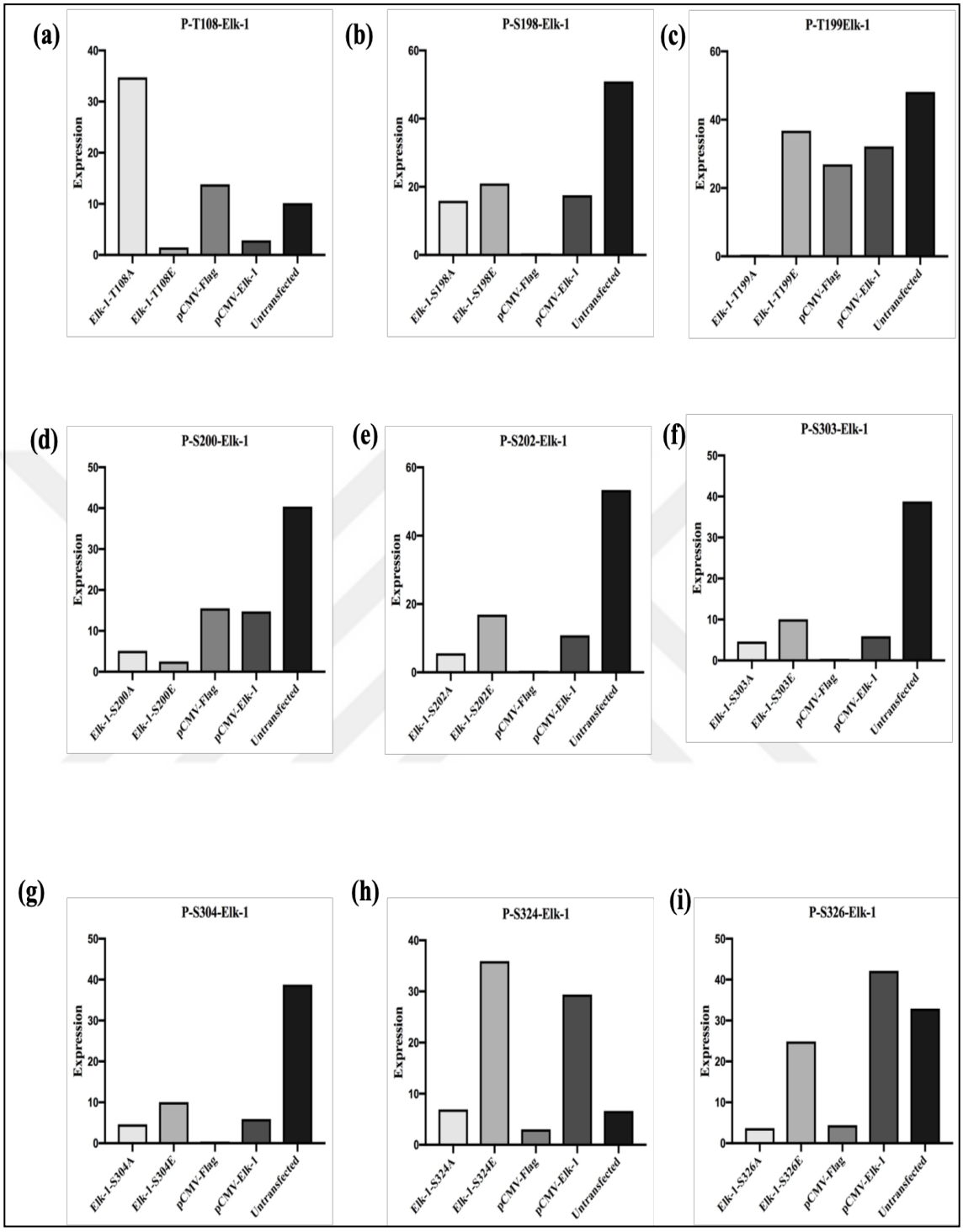


Figure 5.38. Normalizations of phosphorylation analysis of Elk-1 target residues by immunoprecipitation followed by Western blot P-T108-Elk-1 (a), P-S198-Elk-1 (b), P-T199-Elk-1 (c), P-S200-Elk-1 (d), P-S202-Elk-1 (e), P-S303-Elk-1 (f), P-S304-Elk-1 (g), P-S324-Elk-1 (h), and P-S326-Elk-1 (i).Western blot results were normalized by comparing band intensities to the intensity of total Elk-1 corresponding protein bands.

5.9. EFFECTS OF MITOTIC KINASES ON ELK-1 PHOSPHORYLATION

To analyze phosphorylation status of Elk-1, Western blot and immunoprecipitation assays were performed, initially. For the analysis of the first set of putative phosphorylation sites, SH-SY5Y human neuroblastoma cells were transfected with both S106A/E, T108A/E, T133A/E, S198A/E, T199A/E, S200A/E, S202A/E, wild-type pCMV-Elk1 and empty pCMV-Flag vectors. Flag-tagged proteins were isolated by RIPA buffer and 250 μg of protein was incubated with Flag-Agarose beads to attach flag tag of the wild-type and mutagenic Elk-1 proteins. Bead/protein complex was dissolved in SDS loading buffer and captured by Bio-Rad Chemi-Doc system.

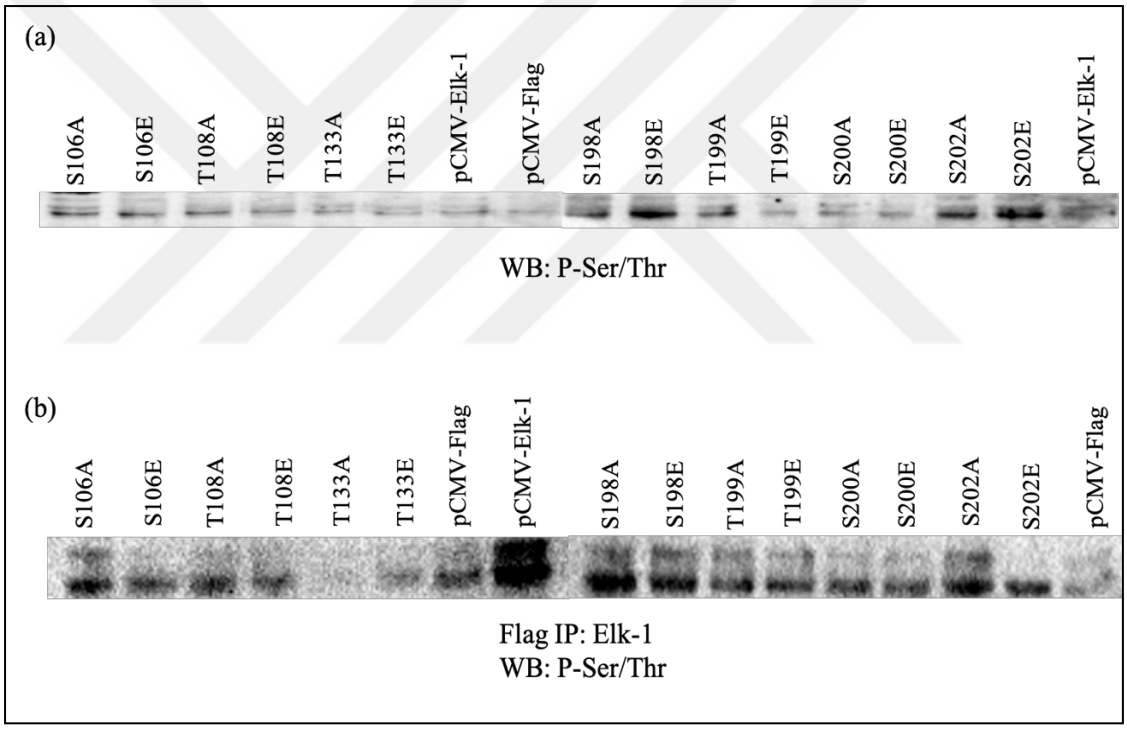


Figure 5.39. Phosphorylation analysis of putative Elk-1 phosphorylation sites by Western blot. SH-SY5Y cells were transfected with various Elk-1 plasmids and phosphorylation of Elk-1 analyzed by Western blot directly (a) or Immunoprecipitation followed by Western blot (b).

Phospho-(Ser/Thr) antibody was used to detect phospho-serine or threonine and it is widely used in phosphorylation analysis of proteins. In this experiment, phospho-null or phospho-mimic mutation carrying proteins were used and phosphorylation of these residues was investigated by Phospho-(Ser/Thr) antibody. It was expected to observe

increased Phospho-(Ser/Thr) expression on phospho-null residues, whereas, expression of phospho-mimic samples was expected to increase if these residues are indeed phosphorylated by target kinases.

In Figure 5.39a, total protein lysate was analyzed by Western blot, and increased phospho-serine activity observed in only constitutively active forms of Serine 198 and 202 residues which indicates a possible phosphorylation on these residues. On the other hand, phosphorylation is increased in the phospho-null form of Threonine 199 compared to fosfo-mimic residue, which is unexpected and may be due to differences in protein concentrations loaded on protein gel.

In addition to total protein lysate represented in Figure 5.39a, to analyze potential phosphorylation more specifically, mutagenic and wild-type Elk-1 protein were pulled-down from the protein lysate by flag agarose beads and analyzed by observing Phospho-(Ser/Thr) antibody expression on the membrane. According to the results obtained in Figure 5.39b, phosphorylation of Serine 198 residue was slightly increased, whereas Serine 106 phosphorylation is significantly reduced. In contrast to the total protein samples, phosphorylation of phospho-null form of Serine 202 residue was found to be increased compared to the phospho mimic version.

To examine the status of the second set of potential Elk-1 phosphorylation sites, phospho-mimic and phospho-null mutation carrying plasmids, S149A/E, S303A/E, S304A/E, S324A/E, S326A/E, wild-type pCMV-Elk1 and empty pCMV-Flag, were transfected into SH-SY5Y neuroblastoma cells as in the first set. Flag-tagged proteins were precipitated by Flag-agarose beads and phosphorylation of these residues was analyzed by Phospho-(Ser/Thr) antibody administration and Western blot.

In Figure 5.40a, as in the previous analysis, total cell lysate was used and if phosphorylated by the kinases, it is expected to see reduced Phospho-(Ser/Thr) antibody on the protein membrane on phospho-null mutations and just the opposite, increased Phospho-(Ser/Thr) antibody in phospho-mimic mutations. According to Figure 5.40a, increased Phospho-(Ser/Thr) antibody expression has not been detected in any of the phospho-mimic mutations. Nevertheless, fosfo-null mutation on Serine 303 residue gave rise to increased Phospho-(Ser/Thr) activity which is an indication of increased phosphorylation in the absence of Serine 303 phosphorylation of Elk-1.

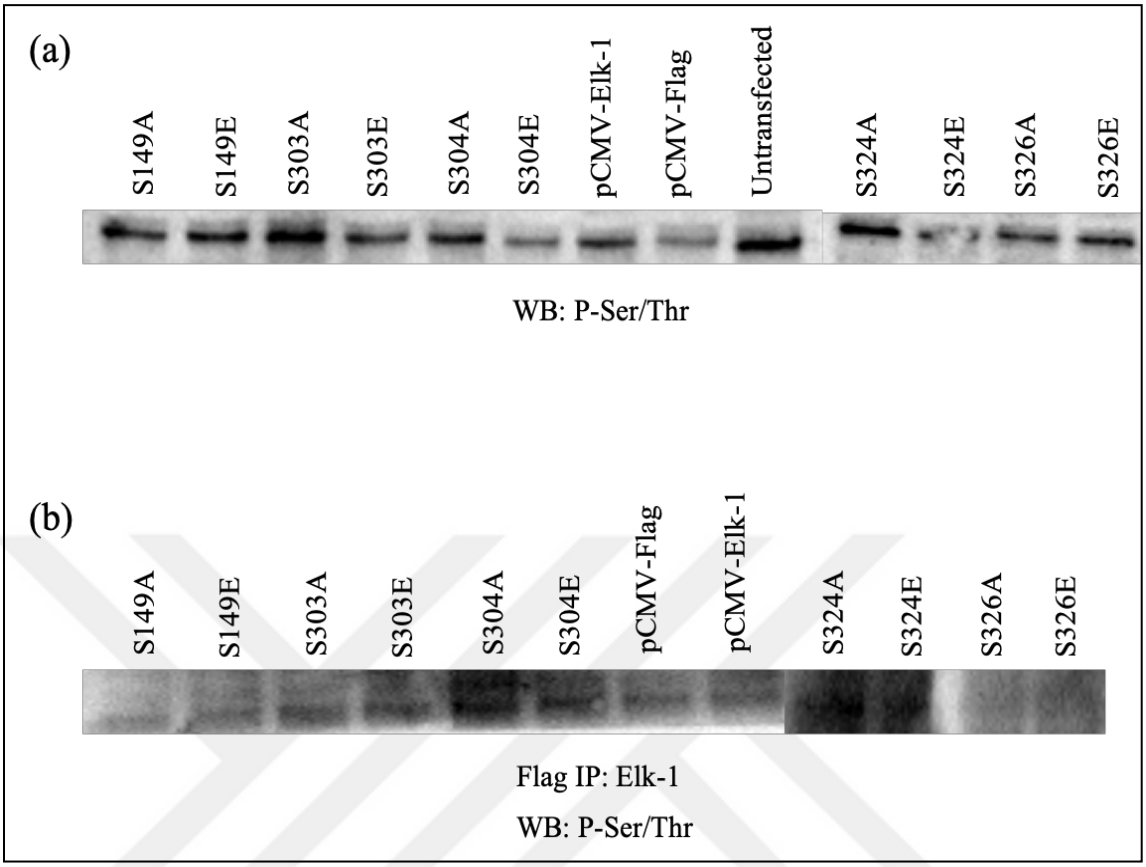


Figure 5.40. Phosphorylation analysis of certain Elk-1 phosphorylation sites by Western blot. SH-SY5Y cells were transfected with various Elk-1 plasmids and phosphorylation of Elk-1 analyzed by Western blot directly (a) or Immunoprecipitation followed by Western blot (b).

In Figure 5.40b, Phospho-(Ser/Thr) expression of mutagenic Elk-1 proteins was examined after immunoprecipitation of Elk-1 protein. As seen in the figure, Phospho-(Ser/Thr) activity was extremely low and uncertain to give a definitive conclusion, but it can be said that blocking of Serine 304 phosphorylation of Elk-1 resulted in increased phospho-serine or tyrosine activity.

5.10. ANALYSIS OF POTENTIAL PHOSPHORYLATION SITES OF ELK-1

Following analysis of the potential phosphorylation sites of Elk-1 by mutating these residues and detecting by region-specific phospho-Elk-1 antibody, the cells were again transfected with phospho-mimic and phospho-null mutant plasmids to analyze the effects of these phosphorylates, particularly Serine 303, 304, 324 and 326, to each other. Effect of

these mutations on Serine 303, 304, 324 and 326 phosphorylations was evaluated by P-S303-Elk-1 P-S304-Elk-, P-S324-Elk-1 and P-S326-Elk-1 antibodies.

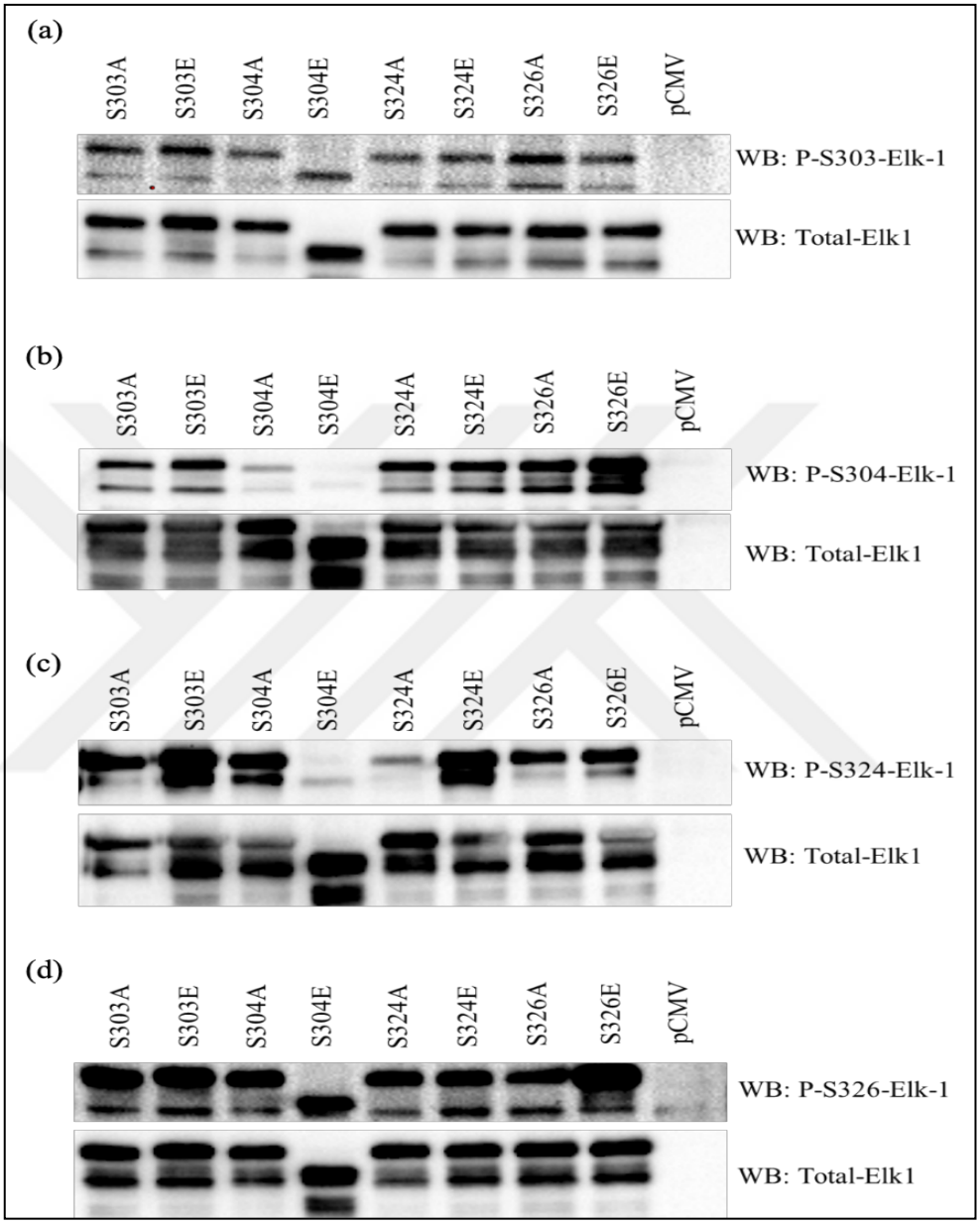


Figure 5.41. Phosphorylation analysis of various phospho-mutants of Elk-1. Cells were transfected with indicated Elk-1 phospho-mutants, and lysates were expressed for P-S303-Elk-1(a), P-S304-Elk-1(b), P-S324-Elk-1 (c) and P-S326-Elk-1 (d).

In this experiment, it was observed that when S303E-Elk-1 was overexpressed in cells, a modest increase in P-S303-Elk-1 antibody signal was observed as compared to S303A-Elk-1 overexpression. Blocking or constitutively activating Serine 303, 304, 324 or 326 residues did not significantly affect phosphorylation of P-S303-Elk-1 as in Figure 5.41a.

However, there is a slight effect of Serine 326 residue on P-S303-Elk-1, the reduction of Serine 326 phosphorylation has an enhancing effect on this phosphorylation since the Elk-1-S326A expression is stronger than Elk-1-S326E.

The effect of Serine 303, 324 and 326 potential phosphorylation sites of Elk-1 on P-S304-Elk-1 investigated in Figure 5.41b. While no significant difference was found between S303A and S304E which means Serine 303 phosphorylation is ineffective on Elk-1 S304 phosphorylation, S324 and S326 phosphorylation of Elk-1 had an enhancing effect this phosphorylation. The most striking point in the analysis of Figure 5.41c is that the phosphorylation of Serine 324 is clearly apparent, blocking of Serine 324 by alanine mutation resulted in decreased Serine 324 phosphorylation, in the meantime, creating a phospho-mimic version of this residue increased Serine 324 phosphorylation. In addition, it was observed that the Serine 303 and 326 phosphorylation of Elk-1 had a significantly increasing effect on Serine 324 phosphorylation, and especially the effect of Serine 303 phosphorylation was quite high. Overexpression of S326 phosphomimetic mutant, while yielding enhanced P-S326-Elk-1 staining as well as a modest increase in P-S324-Elk-1 band in cell lysates, resulted in decreased P-S303-Elk-1 staining, indicating a selectively negative regulatory function for this phosphorylation as demonstrated in Figure 5.41d.

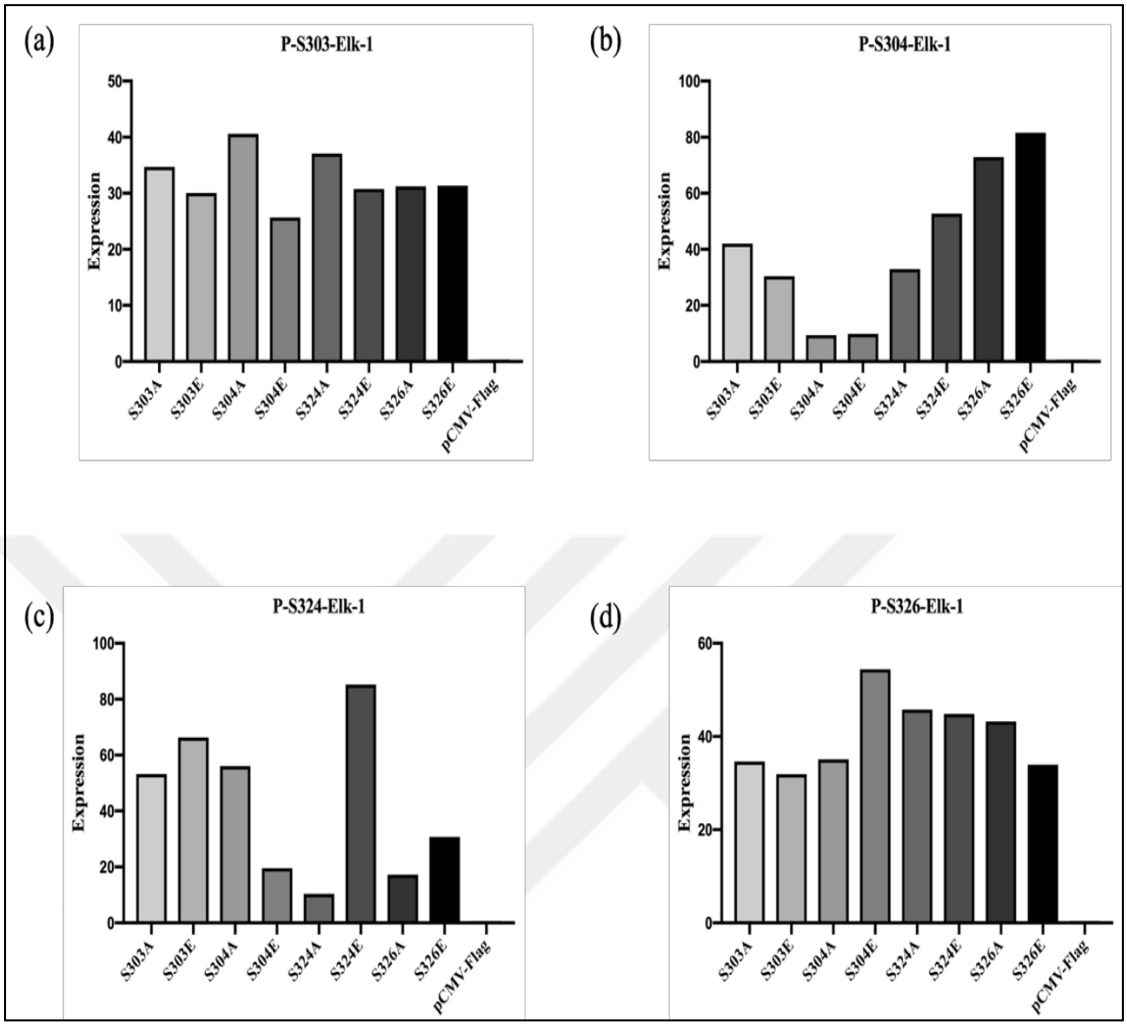


Figure 5.42. Normalizations of phosphorylation analysis of phospho-Elk-1 antibodies, P-S303-Elk-1(a), P-S304-Elk-1(b), P-S324-Elk-1 (c) and P-S326-Elk-1(d). Western blot results of Serine 303, 304, 324 and 326 phosphorylations were normalized by comparing band intensities to the intensity of total Elk-1 corresponding protein bands.

If the effects of the Elk-1 phosphorylations to each other, described above, are evaluated numerically according to Figure 5.41, inhibition of Serine 304 phosphorylation results in an increase of about 30 per cent on serine 304 phosphorylation compared to the phospho-mimic mutation. When Figure 5.42 was evaluated in terms of Serine 304 phosphorylation, it was found that both Serine 324 and Serine 326 caused a 20 per cent increase in Serine 304 phosphorylation of Elk-1. The graphics are shown in Figure 5.42c confirmed that the Elk-1 was indeed phosphorylated from Serine 324 residue since S324 phosphorylation of the S324E mutant increased by approximately 80 per cent compared to the S326A mutant. Serine 303 and Serine 326 phosphorylation resulted in an increase of about 10-20 per cent

on Serine 324 phosphorylation, whereas S304 phosphorylation decreased this phosphorylation by about 40 per cent. While Serine 303 and Serine 324 phosphorylations did not show a significant effect on Serine 326 phosphorylation of Elk-1, it was seen in Figure 5.42d that phosphorylation of Serine 304 increased P-S326-Elk-1 by 40 per cent.

5.11. EFFECTS OF PUTATIVE PHOSPHO-SITES ON MITOTIC KINASES

To analyze possible effects of putative Elk-1 phosphorylations on the mitotic kinases, Aurora A, Polo-like kinase 1 and Cyclin-dependent kinase, flag-immunoprecipitation assay has been performed. SH-SY5Y human neuroblastoma cells were transfected with S149A/E, S303A/E, S304A/E, S324/E, S326/E, pCMV-Elk1 and empty pCMV-flag vectors and proteins were isolated from these cells. Then, 250 µg total protein was incubated with 30µl Flag-Agarose beads and after 16-18 hours, beads were dissolved in 20 µl SDS loading buffer and analyzed by Western blot.

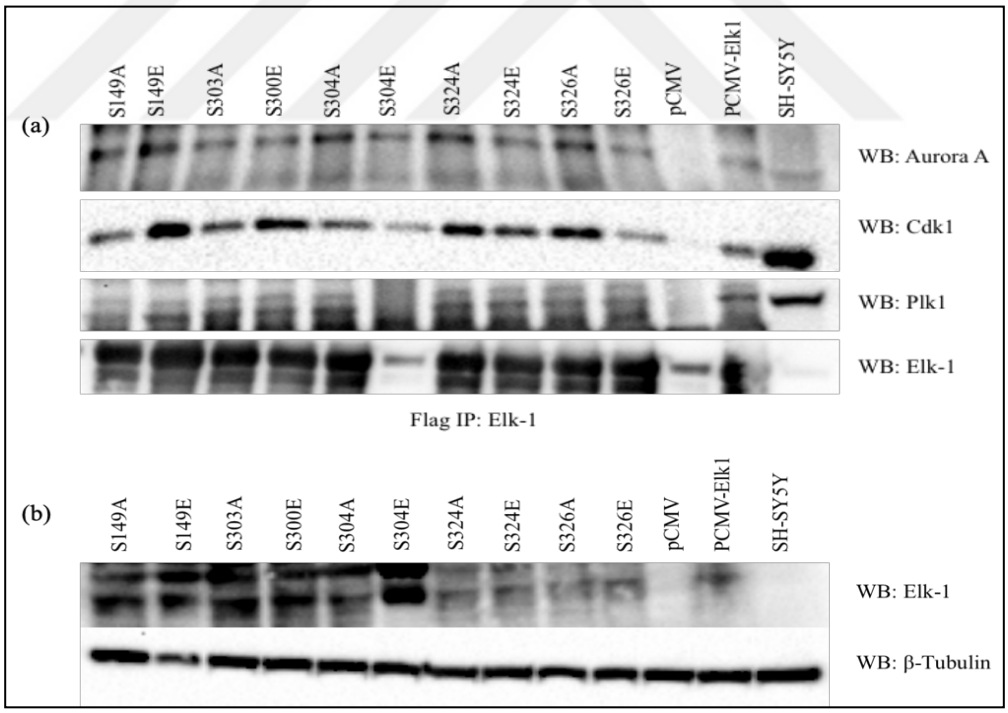


Figure 5.43. The interaction of Elk-1 phospho-mutants with mitotic kinases Aurora-A, Cdk1 and Plk1. Flag IP(a) and total protein lysate (b) analysis of Elk-1 phosphorylations. pCMV-Elk-1 was used as positive control to see the presence of Elk-1. pCMV-Flag indicates empty control plasmid, and untransfected denotes total cell lysate.

Figure 5.43 shows that Elk-1 phosphorylation does not have a significant effect on Aurora kinase expression. However, a phospho-mimic form of Serine 149 displayed a slight increase compared to phospho-null form of this phosphorylation on Aurora A expression. While Serine 303 phosphorylation of Elk-1 did not have a significant effect on Aurora A, Serine 304, 324 and 326 phospho-forms of Elk-1 have an inhibitory effect on the Aurora kinase, where Aurora A expression was increased when these residues were converted to Alanine.

When the effect of Elk-1 phosphorylation on Cdk1 kinase was examined, it was observed that Serine 149 and 303 phosphorylation of Elk-1 have an enhancing effect, whereas the phosphorylation of serine 304, 324 and 326 phosphorylations displayed a decreasing effect. Any significant effect of Elk-1 phosphorylation on Plk1 kinase was not found as seen in Figure 5.43.

In Figure 5.43b, the expression of the phospho-mutant forms of Elk was observed and it has been found that both phospho-mimic and phospho-null forms are successfully expressed in cells. β-tubulin was used as a loading control in order to compare the amount of protein loaded on the gel and there was no significant difference between protein concentrations.

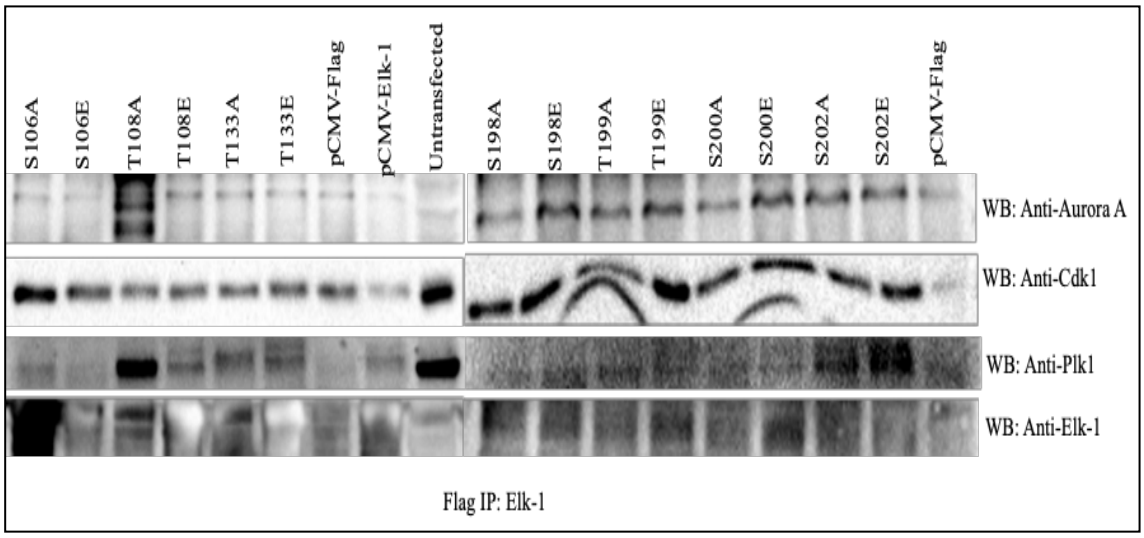


Figure 5.44. Interaction of various Elk-1 phospho-mutants with mitotic kinases Aurora-A, Cdk1 and Plk1. pCMV-Elk-1 was used as positive control to see the presence of Elk-1. pCMV-Flag indicates empty control plasmid, and untransfected denotes total cell lysate.

In addition to analysis of Serine 149, 303,304,324 and 326 phosphorylations of Elk-1 on the expression mitotic of mitotic kinases, other putative phosphorylation sites of Elk1 were also analyzed in this context. To that end, S106A/E, T108A/E, T133A/E, S198A/E, T199A/E, S200A/E, S202A/E, wild-type pCMV-Elk1 and empty pCMV-Flag plasmids were transfected into SH-SY5Y and effects of these mutations on potential phosphor-sites were analyzed by Western blot.

When Figure 5.44. were examined, it can be seen that only Serine 198 and Threonine 99 phosphorylations of Elk-1 were able to have a slightly enhancing effect on Aurora A kinase expression while no significant effect was observed in other regions. Similar to Aurora A kinase, Threonine 199 phosphorylation has an enhancing effect on Cdk1 kinase whereas phospho-null mutation on Serine 106 residue resulted in an increase on Cdk1 expression, therefore, phosphorylation may have an inhibitory effect on Cdk1. If the figure is examined in terms of Plk1 expression, it can be concluded that the phosphorylation of Serine 108 is inhibitory but the phosphorylation of 202 has an enhancing effect.

5.12. EFFECT OF THE INHIBITION OF MITOTIC KINASES ON ELK-1 PHOSPHORYLATION

To study phosphorylation status of a target protein, specific inhibitors against kinases have been widely used. Alisertib or MLN8237 is a small molecule inhibitor of Aurora A and shows higher selectivity for Aurora A than other Aurora kinase family members and does not have any significant effect on other known kinases [150]. BI-2536 is a potent and selective small molecule inhibitor of Plk1 and it inhibits Plk1 activity even at low nanomolar concentrations [151]. RO-3306 is a selective ATP competitor inhibitor of Cdk1 and it inhibits Cdk1/Cyclin activity [152].

To investigate phosphorylation changes of Elk-1 in the presence of selective kinase inhibitors, U-87 cells were treated with Alisertib, BI-2536 and RO-3306 to inhibit the activity of Aurora A, Plk1 and Cdk1 kinases, respectively. To determine the most effective dose and its incubation time, cells were treated with inhibitors at various dosages and time intervals. To inhibit Aurora A kinase activity, cells were treated with 0.1, 1 and 5 μm of Alisertib for 24, 48 and 72 hours. BI-2536 was used to inhibit Plk1 activity at 100, 500 and 1000 nM dosages and RO-3306 was used against Cdk1/Cyclin B1 complex at 2.5, 5 and 10 μm and BI-2536 for 24, 48 and 72 hours. After indicated time periods, cells were lysed,

and the effectiveness of the inhibitors was tested by Western blot compared to non-treated ones.

It has been demonstrated that Cdk1, Aurora A and Plk1 kinases bind and regulate the activity of CyclinB1, therefore, Cyclin B1 is used as a control since inhibition of these kinases also results in the reduction of Cyclin B1 activity. Phosphorylation of histone H3 at serine 10 (S10) is essential for the onset of mitosis and Histone H3 activity is regulated by a group mitotic protein including Aurora A, Cdk1 and Plk1. Therefore, any disturbance in the activity of these proteins also causes a reduction in histone activity [153].

When Figure 5.45. is examine it can be seen that 5 μm Alisertib treatment is sufficient for Aurora A inhibition for 48 hours. In order to decrease the Cdk1 kinase activity, the application of 10 μm RO-3306 for 72 hours is more suitable than the other doses and incubation times. BI-2536, which was used to inhibit the activation of P1k1, was found to be more effective at a concentration of 500 nM and 48 hours of administration was most effective. These concentrations and time intervals are also noted in Table 5.3. to be used for further analyzes.

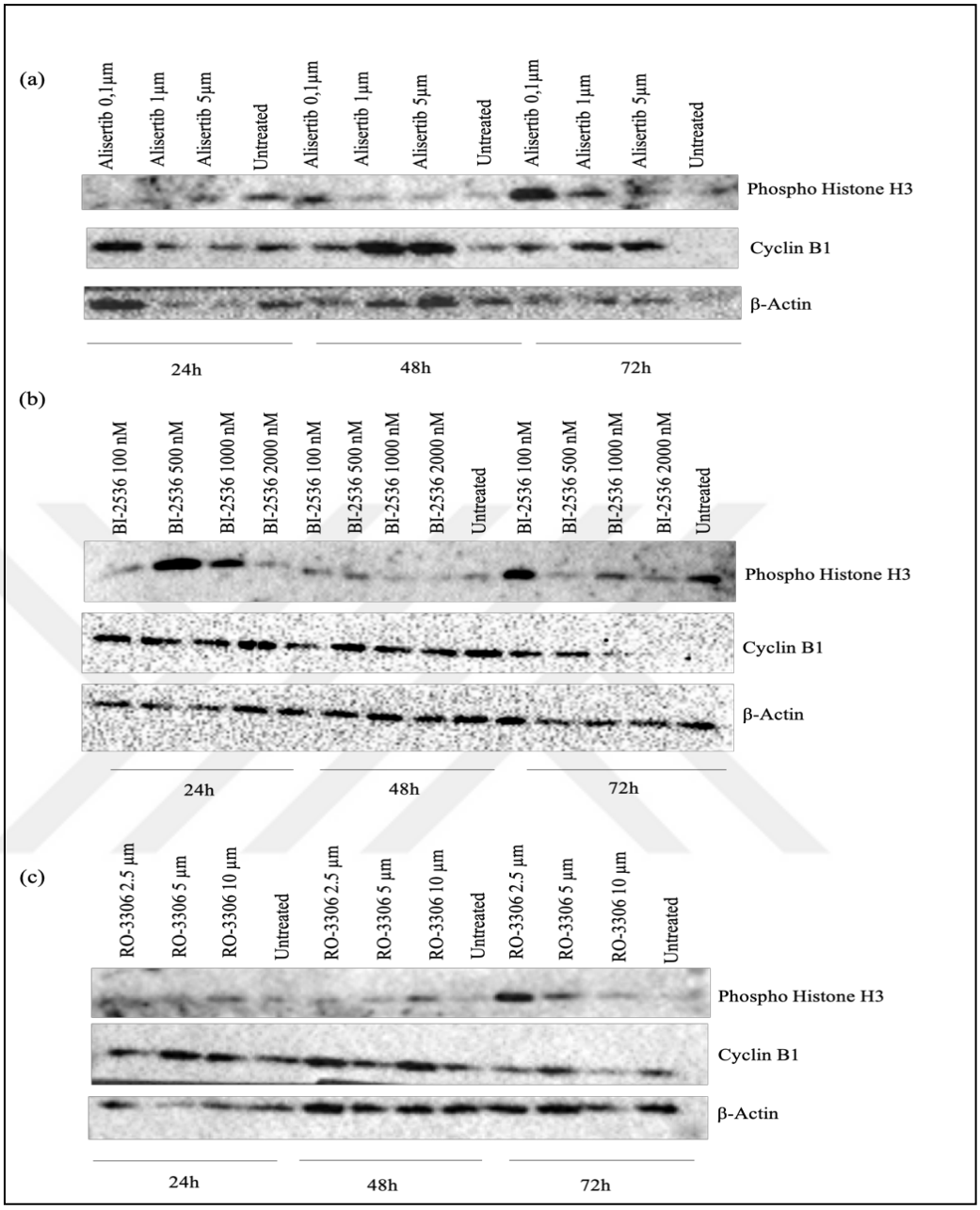


Figure 5.45. Western Blot analysis after inhibition of Aurora A, Cdk1 and Plk1 Kinases. (a) Alisertib was used to inhibit the Aurora A kinase at three different doses, 0.1, 1 and 5 µm. (b) RO-3306 was used at concentrations of 2.5, 5 and 10 µm to inhibit Cdk1 kinase activation. (c) BI-2536 was used to inhibit Plk1 kinase activity at concentrations of 100, 500 and 1000 nM.

Table 5.3. Inhibitors used against Aurora A, Cdk1 and Plk1

Inhibitor Name	Target Kinase	Dosage	Time (hour)
Alisertib	Aurora A	5 μM	48
BI-2536	Plk1	500 nM	48
RO-3306	Cdk1/Cyclin B1	10 μM	72

To determine which kinase phosphorylates the potential phosphorylation sites of Elk-1, as well as the effect of target kinases on other residues that are not their target sites, mitotic kinase inhibitors were utilized. To inhibit the activity of Aurora A, Cdk1/Cyclin B complex and Plk1 kinases, small molecule inhibitors, Alisertib, RO-3306 and BI-2536, respectively, were used. U-87 human glioblastoma cells were treated with Alisertib, BI-2536 and RO-3306 according to the dosage and time intervals listed in Table 5.3. Proteins were isolated after indicated time periods and phosphorylation of Elk-1 was assessed by the treatment of phospho-specific antibodies.

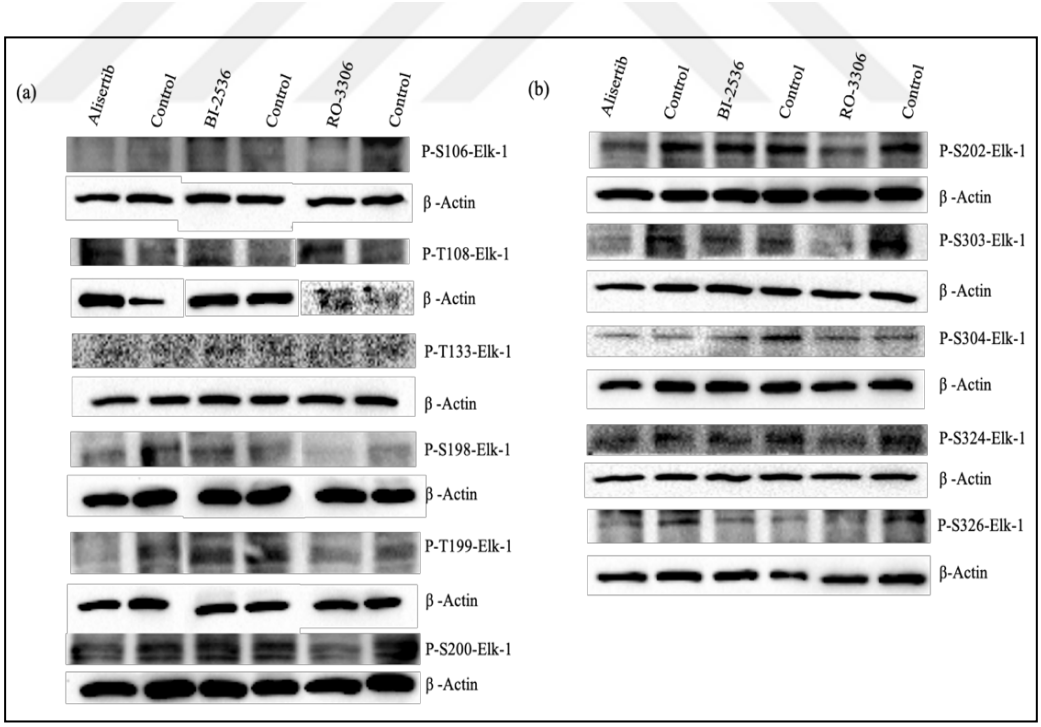


Figure 5.46. Western blot analysis of phospho-specific antibodies on kinase inhibitor-treated proteins. (a); P-S106-Elk-, P-T108-Elk-1, P-T133-Elk-1, P-S198-Elk-1, P-T199-Elk-1, P-S200-Elk-1, (b): P-S202-Elk-1, P-S303-Elk-, P-S304-Elk-1, P-S324-Elk-1 and P-S326-Elk-1.

To compare the relative abundance of proteins across the lanes of protein membranes, Western blot data given in Figure 5.46 were normalized. The signal intensities of proteins were calculated by Image Lab software of Chemi-Doc system and obtained values then normalized with respect to the signal intensity of the β-actin which is a housekeeping gene and expressed constitutively.

According to the Western blot, Figure 5.46, and normalization results, Figure 5.47, it was observed that phosphorylation of P-S106-Elk-1was decreased by 12 per cent in Alisertib-treated cells, whereas it was increased by 32 per cent in RO-3306 treated cells, while no significant change was observed in BI-2536 treated cells compared to those not treated. P-T108-Elk-1 phosphorylation of Elk-1 decreased by 57 per cent and 68 per cent depending on the treatment of Alisertib and BI-2536 respectively, but RO-3306 had no effect. In P-T133-Elk-1 phosphorylation, Alisertib and BI-2536 incubation of cells caused 31 per cent and 42 per cent reduction, respectively, whereas, RO-3306 incubation raised by 20 per cent. Therefore, Alisertib had an inhibitory effect on phosphorylation of both P-S106-Elk-1 and P-T108-Elk-1 and P-T133-Elk-1; BI-2536 was ineffective on both P-S106-Elk-1 and P-T108-Elk-1 but displayed an enhancing effect for the phosphorylation of P-T133-Elk-1; RO-3306 has an activatory effect on P-S106-Elk-1 while alleviated P-T108-Elk-1 and P-T133-Elk-1. According to these results, inhibition of Aurora A kinase significantly affected P-T108-Elk-1; inhibition of Plk1 kinase was not effective on P-S106-Elk-1 and P-T108-Elk-1 but increased P-T133-Elk-1; blocking of Cdk1/Cyclin B complex, resulting in an increase in P-S106-Elk-1 and significant reduction on P-T108-Elk-1 and P-T133-Elk-1.

After the inhibitor treatment, the phosphorylation of P-S198-Elk-1, P-T199-Elk-1, P-S200-Elk-1 and P-S202-Elk-1 was investigated; Alisertib administration reduced phosphorylation of P-S198-Elk-1, P-T199-Elk-1 and P-S202-Elk-1 by 17 per cent, 54 per cent and 11 per cent, respectively, however, P-S200-Elk-1 was raised by 19 per cent. BI-2536 treatment of cells increased P-S198-Elk-1, P-T199-Elk-1 and P-S202-Elk-1 phosphorylation with a ratio of 11 per cent, 11 per cent and 42 per cent, respectively, but decreased P-S200-Elk-1 by 10 per cent. When Cdk1 / cyclin complex was inhibited by RO-3306 administration, all these phosphorylation sites were affected negatively and phosphorylations were decreased by 30 per cent, 21 per cent, 39 per cent and 37 per cent. To summarize these results; inhibition of Aurora kinase showed an activating effect on P-S200-Elk-1, while reduction on P-S198-Elk-1, P-T199-Elk-1 and P-S202-Elk-1, was observed, inhibition of Plk1; resulted in reduction of P-S200-Elk-1 but it raised P-S198-

Elk-1, P-T199-Elk-1 and P-S202-Elk-1 phosphorylation, and inhibition of the activity of Cdk1 / cyclin B complex; showed an inhibitory effect on both of these phosphorylations, P-S198-Elk-1, P-T199-Elk-1, P-S200-Elk-1 and P-S202-Elk-1.

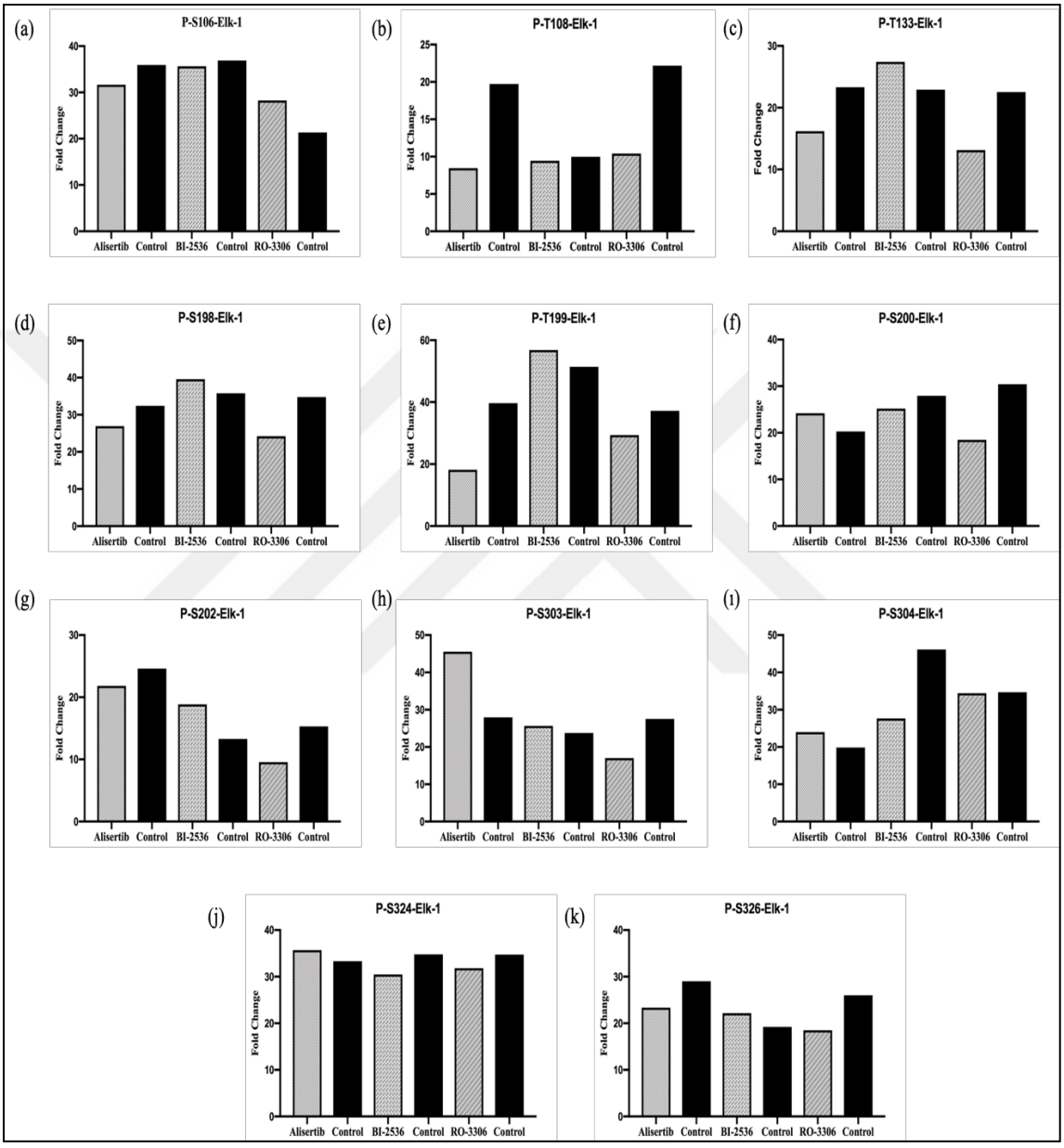


Figure 5.47. Normalizations of Western blot analysis of phospho-specific antibodies on kinase inhibitor-treated proteins, P-S106-Elk-1(a), P-T108-Elk-1(b), P-T133-Elk-1 (c), P-S198-Elk-1(d), P-T199-Elk-1(e), P-S200-Elk-1(f), P-S202-Elk-1(g), P-S303-Elk-1(h), P-S304-Elk-1(i), P-S324-Elk-1(j), P-S326-Elk-1(k). Western blot results were normalized by comparing band intensities to the intensity of β-actin corresponding protein bands.

If the status of phosphorylation of Elk-1 by Serine 303, 304, 324 and 324 is evaluated after treatment of specific small molecule inhibitors; Alisertib resulted in an increase in both P-S303-Elk-1, P-S304-Elk-1, P-S324-Elk-1, which was most noticeable on P-S303-Elk-1 with a ratio of 63 per cent, however it led to a 20 per cent reduction in P-S326-Elk-1; BI-2536 raised S303-Elk-1and P-S326-Elk-1 in small proportions, while it decreased P-S304-Elk-1 and P-S324-Elk-1 by 40 per cent and per cent12, respectively, inhibition of the activity of Cdk1 / cyclin B complex, likewise, showed an inhibitory effect on both phosphorylations, P-S198-Elk-1, P-T199-Elk-1, P-S200-Elk-1 and P-S202-Elk-1, this reduction was not significant on P-S304-Elk-1, P-S324-Elk-1, but S303-Elk-1and P-S326-Elk-1 phosphorylations were affected by 38 per cent and 29 per cent. According to these results; While the inhibition of Aurora A kinase had an activating role S303-Elk-1, P-S304-Elk-1, P-S324-Elk-1, it reduced phosphorylation of P-S326-Elk-1, inhibition of BI-2536 exerted an activatory effect on S303-Elk-1and P-S326-Elk-1, inhibitory effect on P-S304-Elk-1 and P-S324-Elk-1, and Cdk1/Cyclin B inhibition showed an inhibitory effect on both phosphorylations.

5.13. KINASE ASSAYS

Protein kinases transfer a phosphate group from a phosphate donor to an acceptor amino acid of the substrate protein which results in the alteration of the substrate conformation to either activate or inactivate it. The phosphorylated molecule can be another protein, the kinase itself (autophosphorylation) or any other molecule. Kinases are of great interest since they play a major role in cellular activation processes by phosphorylation. Most of the protein kinase assay kits are based on the ATP depletion or monitoring phospho-peptide formation. Kinase assay may be radioactive by labeling with a radiolabeled ATP, gamma ^{32}P-labeled ATP, or non-radioactive by utilizing phospho-specific antibodies [139].

To analyze phosphorylation of Elk-1 by mitotic kinases, non-radioactive *in vitro* kinase assay has been performed. U87 human glioblastoma cells were lysed in Flag-buffer and lysates were cleared by centrifugation. Then, Elk-1, Anti-Aurora A, Plk1 or Cdk1 as well as their kinase-active and kinase-dead forms, proteins were precipitated by specific antibodies and protein A or G agarose beads. This protein/antibody/bead complex was suspended in kinase buffer supplemented with 100 mM ATP and either 2µg GST-Elk-1/1–205 or GST-Elk-1/205-418 fusion proteins. GST fusion Elk-1 protein was chosen

according to the phospho-residue; for the one smaller than 205 amino acids, S106, T108, T133, S198, T199, S200 and S202, kinase reaction was carried out by GST-Elk-1/1–205 administration, while, GST-Elk-1/205-418 fusion protein was used for S303, S304, S324 and S326 target residues. After 25 minutes incubation at 30°C, the reaction is terminated by addition of SDS sample buffer and analyzed by Western blot.

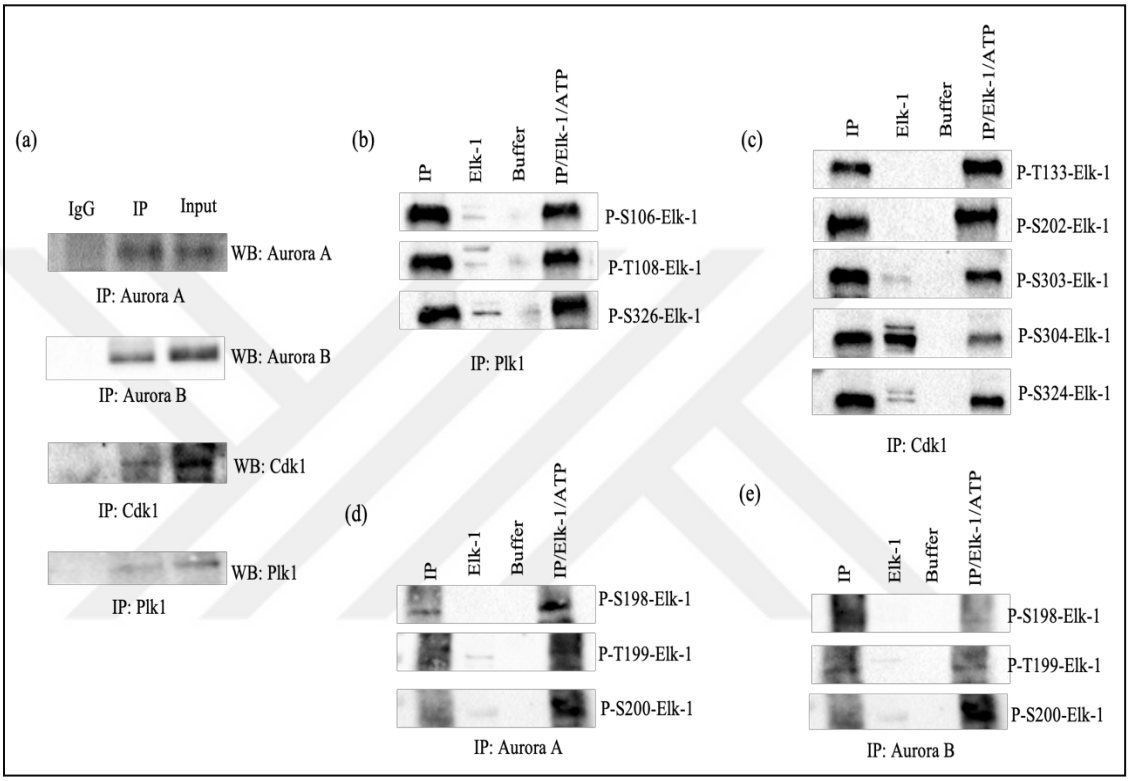


Figure 5.48. Pre-study of kinase analysis with GST-Elk-1 (1-205 and 205-428) fusion proteins. Immunoprecipitation control of Aurora A, Aurora B, Cdk1 and Plk1 kinases (a) and phosphorylation analysis of P-S106-Elk-1, P-T108-Elk-1, P-S326-Elk-1 (b); P-T133-Elk-1, P-S202-Elk-1, P-S303-Elk-1, P-S304-Elk-1, P-S324-Elk-1 (c), P-S198-Elk-1, P-T199-Elk-1 and P-S200-Elk-1 (d-e). IgG; Immunoglobulin G, Input; Total cell lysate, Elk-1/1-205 or Elk-1/205-428; GST-fused semi-purified protein; IP, immunoprecipitation.

In order to demonstrate that Aurora A, Aurora B, Cdk1 and Plk1 kinases were successfully pulled down from other proteins in cell lysate, specific antibodies against these proteins were used in both immunoprecipitation and Western blotting. As seen in Figure 5.48a, both proteins have been successfully pulled from the cell lysate and these proteins were subsequently used in the kinase assay.

To reveal whether Elk-1 is being phosphorylated by Aurora A, Aurora B, Cdk1 and Plk1 kinases from indicated residues, phosphor-specific antibodies were used along with kinase buffer as a buffering system, GST-fused Elk-1/1-205 protein and ATP. Since protein kinases transfer a phosphoryl group from ATP onto the target proteins, which is also known as phosphorylation, an increase in the expression of phosphor-forms was expected subsequent to ATP administration. Specificity of the assay is ensured by loading GST-Elk-1 fusion proteins and kinase buffer alone to the gel.

In Figure 5.48b, phosphorylation of Serine 106, Threonine 108 and Serine 326 were observed in both immunoprecipitated Plk1 protein alone and the kinase reaction containing Plk1 kinase, Elk-1 and ATP, and any significant increase in Serine 106 and Threonine 108 was not observed between these sample groups. However, it was found that Serine 326 phosphorylation of Elk-1 increased in the kinase reaction, containing ATP, kinase and GST-fused Elk-1/205-428. The band was not expected to appear in the kinase buffer since it doesn't contain any protein, but it was probably seen that kinase reaction sample loaded on the next well of kinase buffer was overloaded and flowed into the previous well.

Phosphorylation of Threonine 133, Serine 202, 303, 304 and 324 of Elk-1 by Cdk1 kinase was displayed in Figure 5.48c and according to these results an increase in the phosphorylation of Threonine 133 and Serine 202 of Elk-1 was observed in kinase assay reaction, while no further change was observed in the other phosphorylations. Since Serine 198, Threonine 199 and Serine 200 regions may be phosphorylated by Aurora A and Aurora B kinases, the phosphorylations of these regions were investigated by both Aurora A kinase and Aurora B kinases According to the kinase reactions, Serine 198 and Threonine 199 phosphorylations were found to be increased in the presence of Aurora A kinase and Serine 200 phosphorylation increased in the presence of both Aurora A and Aurora B kinases.

In order to investigate Aurora A and Plk1 phosphorylations of Elk-1 more deeply, second kinase assay reaction was designated; SH-SY5Y cells were transfected with either wild-type, Aurora A and Plk1, and kinase-dead, Aurora A-T288A or Plk1-T210A, plasmids and immunoprecipitation reaction of Aurora A or Plk1 has been performed from both protein lysates. Immunoprecipitated wild-type and kinase-dead mutation-bearing Aurora and Plk1 proteins were further used in kinase assay to detect the effect of inactive kinases on Elk-1 phosphorylations.

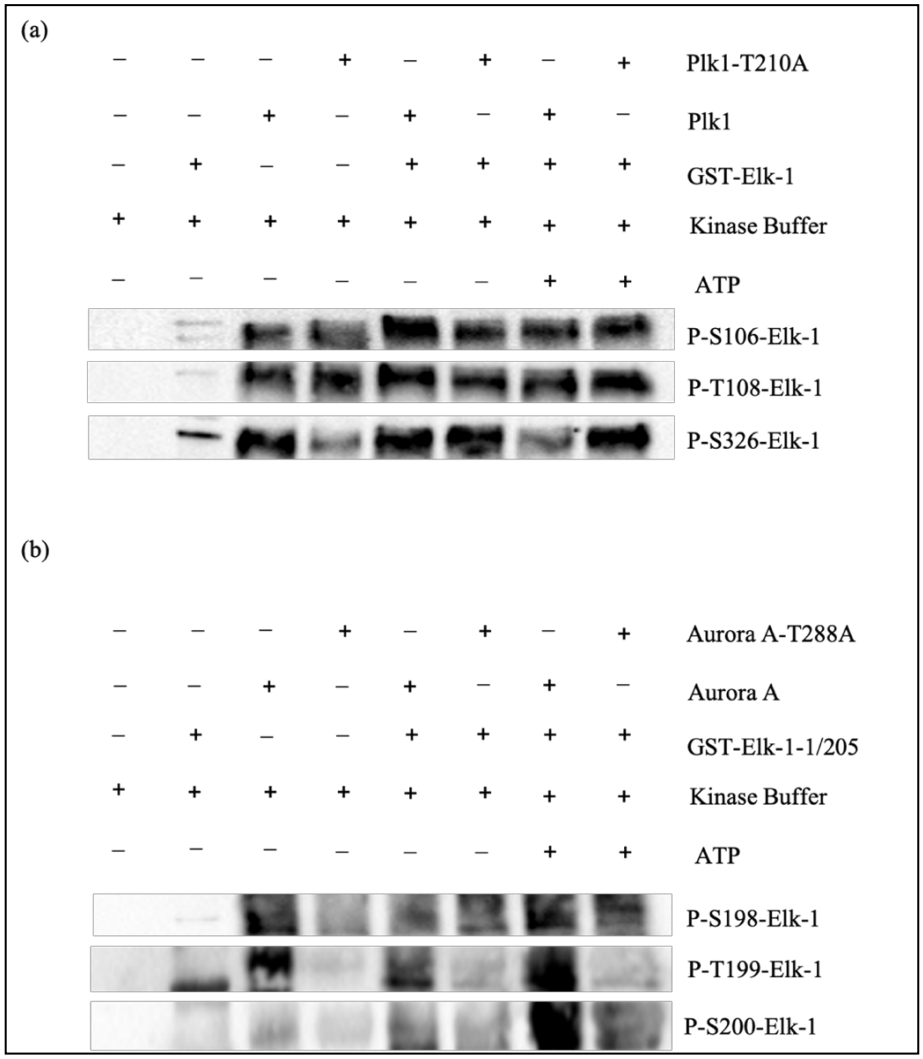


Figure 5.49. Phosphorylation analysis of certain Elk-1 residues); P-S106-Elk-1, P-T108-Elk-1, P-S326-Elk-1 (a), P-S198-Elk-1, P-T199-Elk-1, P-S200-Elk-1 (b). GST-Elk-1-1/205; GST-fused semi-purified protein. (+) indicates the component was included in the reaction condition and (-) indicates the component did not exist in the reaction.

Since phosphorylation of the Aurora A from Threonine 288 is very important for both its activation and its effect on its target proteins, inhibition of this phosphorylation by mutagenesis inhibits the functions of Aurora A, such as target protein phosphorylation. To analyze phosphorylation of Serine 106, Threonine 108, Serine 326, by Plk1 kinase, and Serine 198, Threonine 199 and Serine 200 residues by Aurora A kinase, kinase assay has been performed by custom-made phospho-specific antibodies likewise the previous kinase assay experiment. Specificity of the assay is ensured by performing the kinase reaction in the absence or presence of either GST-Elk1, wild-type or mutation carrying kinases or

ATP and the kinase reaction which contains all reagents together is supposed to give a strong signal.

As seen in Figure 5.49a, there was no difference between wild-type and kinase-dead forms of Plk1 in terms of Serine 106 and Threonine 108 phosphorylations. Interestingly, Serine 326 phosphorylation of Elk-1 increased when Plk1 kinase activity was inhibited by kinase-dead mutation, Plk1-T210A. However, In Figure 5.49b, a significant reduction in both phosphorylation of Serine 198, Threonine 199-Elk-1 and Serine 200-Elk-1 was observed when kinase assay was performed with mutant Aurora A-T288A, kinase-dead, kinase. Incubation of the GST-Elk-1-1/205 protein with the wild-type Aurora A protein in the presence of ATP, the Elk-1 protein was phosphorylated from both Serine 198, Threonine 199 and Serine 200 residues. These initial results demonstrate that the motifs we estimate are phosphorylated by Aurora-A kinase.

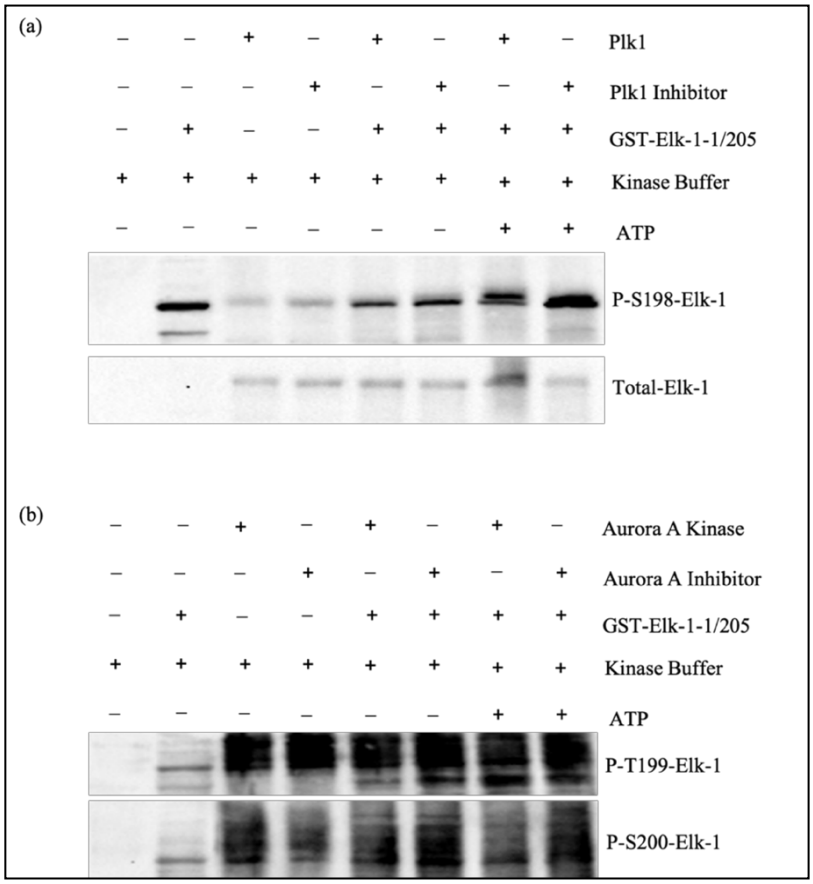


Figure 5.50. Serine 198, Threonine 199 and Serine 200 phosphorylations of Elk-1 by Plk1 (a) and Aurora A kinases (b).

In order to analyze Serine 198, Threonine 199 and Serine 200 phosphorylations of Elk-1 by Plk1 and Aurora A kinases more specifically, inhibitors against these kinases were used. Cells were treated with either 30 μM Alisertib or 500 nM BI-2536 to inhibit Aurora A and Plk1 kinase, respectively. Proteins were isolated from both inhibitor-treated cells and non-treated cells and Plk1 and Aurora A kinases were removed by immunoprecipitation from protein lysate. These isolated kinases were incubated with Elk-1/1-205 protein in the presence and absence of ATP likewise previous kinase assays. The kinase buffer was used as a negative control and as shown in this example, no bands were observed in this example. The specificity of the assay was achieved by performing a kinase reaction in the presence or absence of GST-Elk-1 immunoprecipitant or ATP. The kinase reaction containing only all the reagents together was expected to give a strong signal because the protein kinases add to the target molecules the phosphate group in the presence of ATP. For this reason, it is not expected to see the band in samples without ATP. However, the bands seen in these examples may have been observed due to the interaction with GST-Elk1-1 / 205 deletion with kinases precipitated by immunoprecipitation. As shown in Figure 5.50, the kinase reaction was carried out using both kinases that were precipitated from untreated cells and kinases treated with inhibitors. Both GST-Elk1-1 / 205 and ATP were added to all of these groups. In the kinase analysis performed with samples treated with these inhibitors, it was expected to see weaker bands since inhibition of kinase was in turn expected to result in blocked phosphorylation of Elk-1. As shown in the figure, however, no significant reduction in the indicated phosphorylation of Elk1 was observed when inhibitors were added.

In addition to the preliminary kinase assays performed to analyze Serine 198, Threonine199 and Serine 200 phosphorylation sites of Elk-1, the other phosphorylation sites were also investigated by kinase analysis. T133, S200, S202 and S324 residues were determined as Cdk1 target residues and T199 and S200 were determined as targets of Aurora A kinase. Since Serine 200 residue was shown as the target site of Cdk1 in some tools, and in some of the Aurora A, this region has been investigated in terms of both kinases.

To perform in vitro kinase assay, cells were treated with 5 μm of Alisertib for 48 hours or 10 μm of RO-3306 for 72 hours, non-treated cells were used as a control. Apart from the previous experiment, Elk-1 protein was precipitated from inhibitor-treated and non-treated cells rather than target kinases, and this protein was incubated with active Aurora A kinase

or Cdk1/Cyclin B protein. In addition to inhibitor-treated and non-treated Elk-1 and active kinases, kinase reaction has been designed with the addition of ATP or Lambda Phosphatase or not. Lambda Protein Phosphatase (Lambda PP) is a Mn^{2+} dependent protein phosphatase which targets phosphorylated serine, threonine and tyrosine residues and widely used in dephosphorylation sites. To dephosphorylate target serine or threonine residues, non-treated proteins were incubated with Lambda Phosphatase for 30 minutes and the reaction was terminated by heat inactivation at 65° C for 1 hour in the presence of 50 mM EDTA.

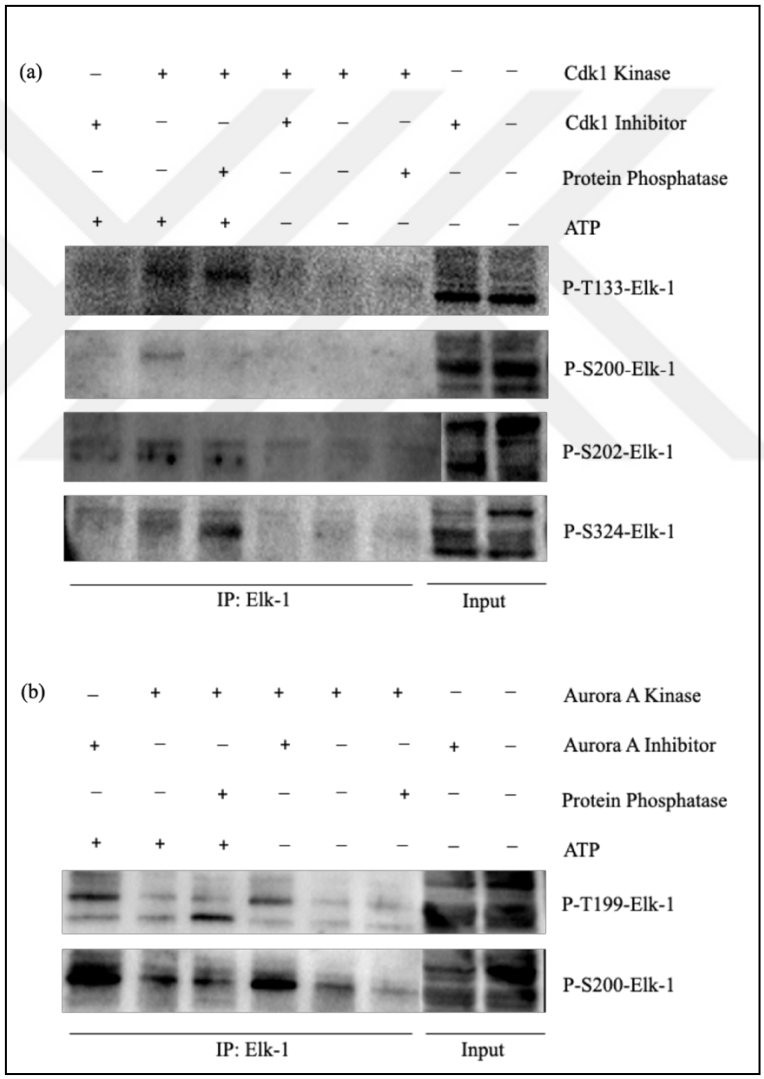


Figure 5.51. Threonine 133, Threonine 199, Serine 200, Serine 202 and Serine 324 phosphorylations of Elk-1 by Cdk1 (a) and Aurora A kinases (b). IP; Immunoprecipitation of Elk-1 protein, Input; total protein sample either inhibitor-treated or non-treated.

Figure 5.51a shows the kinase assay analysis of Threonine 133 and Serine 200, 202 and 324 residues which are thought to be phosphorylated by the Cdk1 kinase. It was expected to detect more intense bands when immunoprecipitated Elk-1 was incubated with Cdk1 and ATP, whereas, incubation of Elk-1, precipitated from protein lysate treated with Cdk1/Cyclin B inhibitor, RO-3306, or dephosphorylated with Lambda phosphatase, with Cdk1 and ATP would expect to give rise to none or low level of phosphorylation.

According to the results of Figure 5.51a, in the presence of ATP, the phosphorylation of Threonine 133, Serine 200 and Serine 324 in the samples treated with inhibitor decreased compared to those not treated, while the dephosphorylated samples did not change much. Phosphorylation was whether weak or undetectable in samples that are not incubated with ATP. In the case of Serine 200 phosphorylation by Cdk1, the only band was observed in sample incubated with un-treated Elk-1 protein and ATP. The fact that there is not too much change in the dephosphorylated samples indicates that the dephosphorylation process by Lambda phosphatase cannot be done completely or there may be a failure in another process, whereas, in the samples treated with inhibitor, the phosphorylation is lost or decreased. In Figure 5.50b, phosphorylation of Threonine 199 and Serine 200 was examined, and it was observed that phosphorylation of these residues was not noticeably changed when proteins were treated with inhibitor or diphosphatase, and even phosphorylation of inhibitor-treated proteins was increased. There was no significant difference in the presence or absence of ATP.

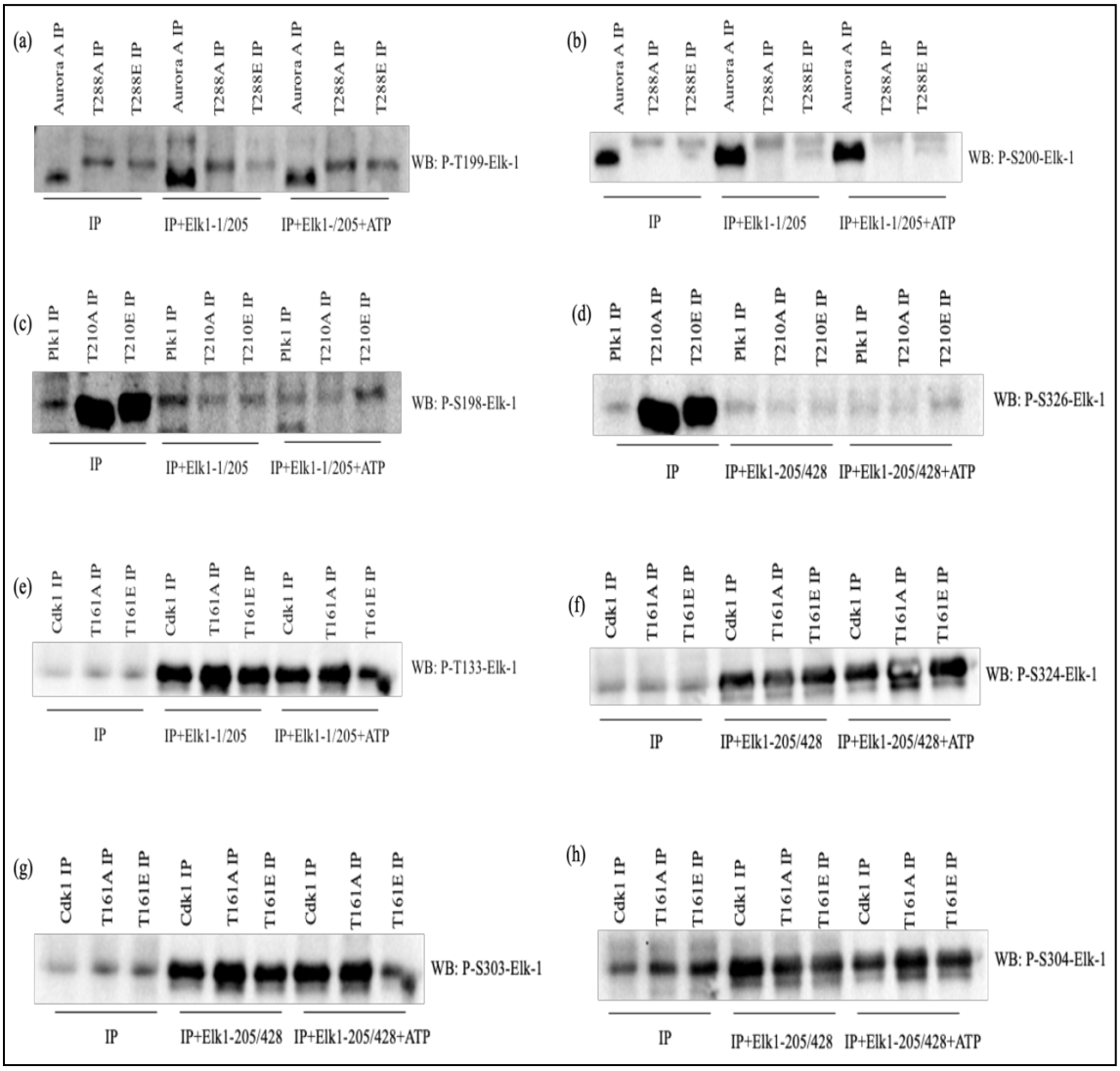


Figure 5.52. Kinase analysis of the effect of kinase-dead and kinase-active forms of Aurora A, Cdk1 and Plk1 kinase on Elk1 phosphorylation. Potential phosphorylation sites of Elk-1 analyzed by phospho-specific antibodies; Threonine 199 (a) Serine 200 (b), Serine 198 (c), Serine 326 (d), Threonine 133 (e), Serine 324 (f), Serine 303 (g) and Serine 304 (h).

Potential phosphorylation sites of Elk-1 thought to be phosphorylated by Aurora A, Cdk1 and Plk1 kinases were analyzed using kinase-dead and kinase-active forms of these kinases and phospho-specific antibodies. Since the kinases cannot be activated when kinase-dead forms of these target kinases are used, the target will not be able to add phosphate sites to the phosphorylation sites, which can be detected by means of phospho-specific antibodies. However, when the kinase-active forms of kinases are used, protein expression in the phosphorylation sites is expected to increase relative to the wild type. For this purpose, wild-type and mutant forms of Aurora kinase, T288A and T288D, Cdk1 kinase, T161A

and T161E, and Plk1 kinase, T210A and T210D, were transfected into SH-SY5Y cells and proteins were isolated from the cells. Proteins transfected with Aurora A were precipitated by the anti-Myc antibody, wild-type Cdk1, T161A and T161E transfected ones precipitated by anti-HA antibody and wild-type Plk1, T210A and T210D transfected proteins were isolated from the protein lysate by anti-Plk1 antibody and protein A/G agarose beads. Proteins transfected with T288A and T288D were removed from the cell lysate with flag agarose beads. These precipitated proteins were treated with GST-Elk1-1 / 205 protein for 30 minutes in the absence and presence of ATP to perform kinase analysis. After kinase analysis, the phosphorylation sites were analyzed by Western blotting using phospho-specific antibodies.

In the analysis of Figure 5.52, an increase in Serine 198 and Serine 326 phosphorylations were observed when kinase-active incubated with Elk1-1/205 and ATP compared to kinase-dead form of Plk1. Likewise, incubation of Cdk1 kinase-active form with Elk1-1 205 and ATP resulted in an increase in serine 324 phosphorylation according to the kinase-dead form of Cdk1. Any significant phosphorylation change has not been observed Elk1-1/205 in other phospho-forms of Elk-1.

In addition to indirect *in vitro* kinase assays, the activity of kinases studied in terms of Elk-1 phosphorylation was directly evaluated by fluorometric kinase assay kit. This assay is based on the measurement of ADP formation which is directly proportional to kinase activity. Protein kinases transfer a phosphate group from ATP to acceptor amino acids and the activity of these phosphotransferase enzymes is monitored fluorometrically.

To measure Elk-1 phosphorylation fluorometrically, the kinase reaction was set up with Elk-1 protein, active kinases, Aurora A, Aurora B, Plk1 and Cdk1, and ATP and incubated at 37°C for 1 hour. Then, kinase reaction was combined with ADP sensor buffer and ADP sensor and the mixture was incubated in dark for 15 minutes and the fluorescence intensity was measured by spectrophotometry at 540 nm excitation and 590 nm emission. As demonstrated in Figure 5.53 and Figure 5.54, kinase relative fluorescence unit (RFU) was measured at basal level, however, incubation of Elk-1 with target kinases increased this value significantly. The activity of both kinases was increased when they are incubated with Elk-1 in the presence of ATP.

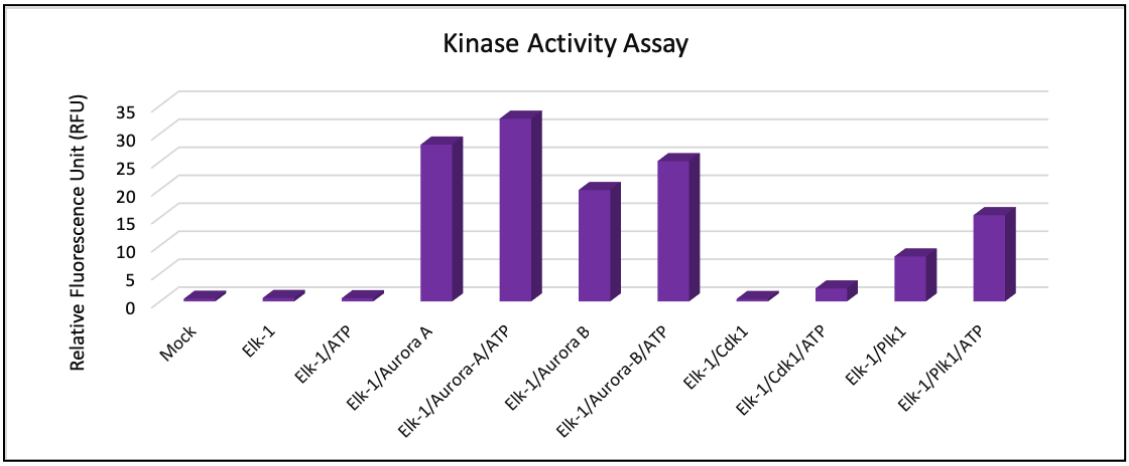


Figure 5.53 Fluorometric measurements of the activity of Aurora A, Aurora B, Cdk1 and Plk1 kinases.

To observe time-dependent changes in the activity of target kinases, the fluorescence intensity of all reactions was measured at spectrophotometry at 15-minute time intervals. In all of the kinase assay reactions, kinase activity increased compared to the reactions involving only Elk-1 or Elk-1 with ATP, and this increase was increased over time. While the conversion rate from ATP to ADP was very low in the reactions using Elk-1 only and in some samples with addition of ATP in addition to Elk-1, this ratio was observed to be increased and reached to the highest level in kinase reactions where kinases were also included in the reaction. The fact that the fluorometrically measured ADP ratio was increased in kinase reactions, containing both Elk-1, kinases and ATP, it can be concluded that Elk-1 is phosphorylated by all target kinases, albeit to different extents.

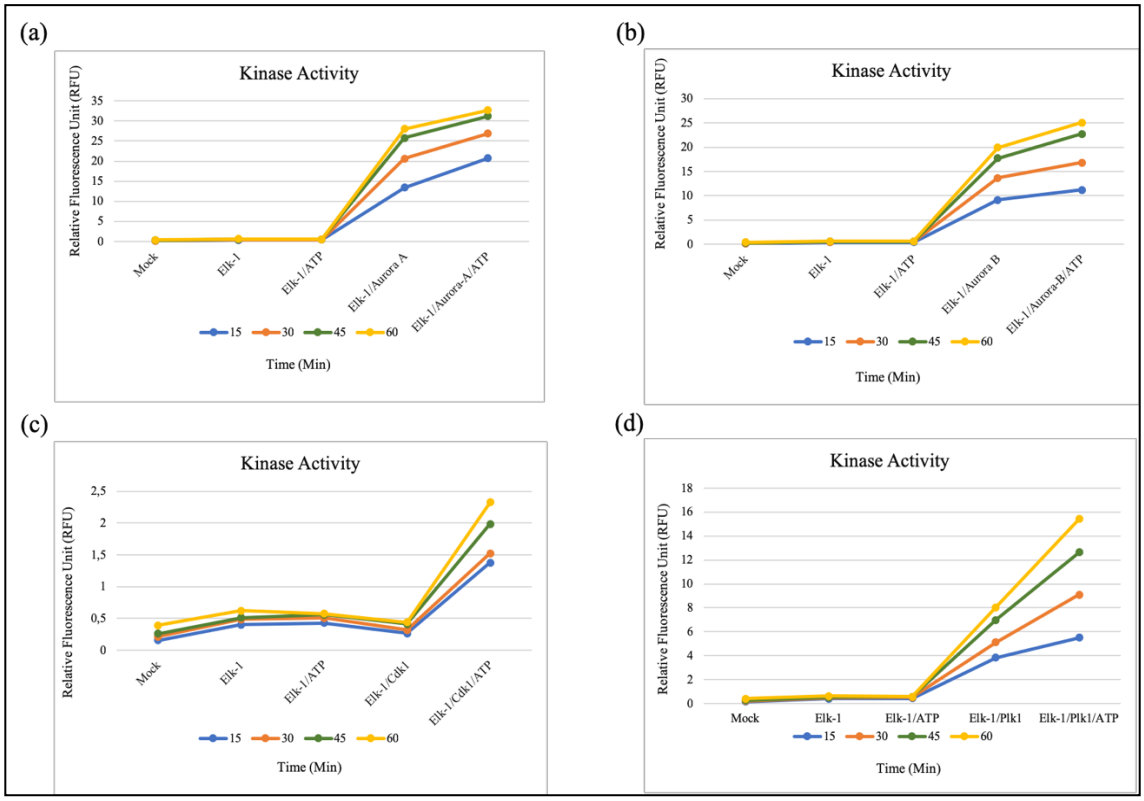


Figure 5.54. Time-dependent measurements of the activities of Aurora A (a), Aurora B (b), Cdk1 (c) and Plk1 (d) kinases. Mock reaction contains buffer only and used as a blank.

After the identification of Elk-1 phosphorylations by studied kinases, new set of kinase reactions have been set to determine whether putative Elk-1 phosphorylation sites are indeed phosphorylated by these kinases or not. To that, unmodified peptides listed in Table 4.14 which are specifically synthesized for each phospho-site were used instead of Elk-1 protein. Reactions were composed of either peptide alone, or peptides were either incubated with target kinase and ATP, as kinase assay reaction, or peptides with ATP only. According to the results of the kinase assay shown in Figure 5.55, incubation of Serine 106 and Ser326 peptides with Plk1 kinase and ATP resulted only a slight increase in measured ADP concentration with respect to reaction containing peptides and ATP but not Plk1 kinase. However, incubation of Threonine 108 peptide with Plk1 kinase in the presence of ATP, resulted in approximately 60 per cent increase in fluorescently measured ADP values compared to Threonine 108 peptide and ATP or peptide alone. In addition to Plk1 kinase phosphorylation, Serine 326 phosphorylation of Elk-1 was evaluated in terms of Cdk1 phosphorylation, but any significant change that indicates Cdk1 phosphorylation was not observed as demonstrated in Figure 5.55c.

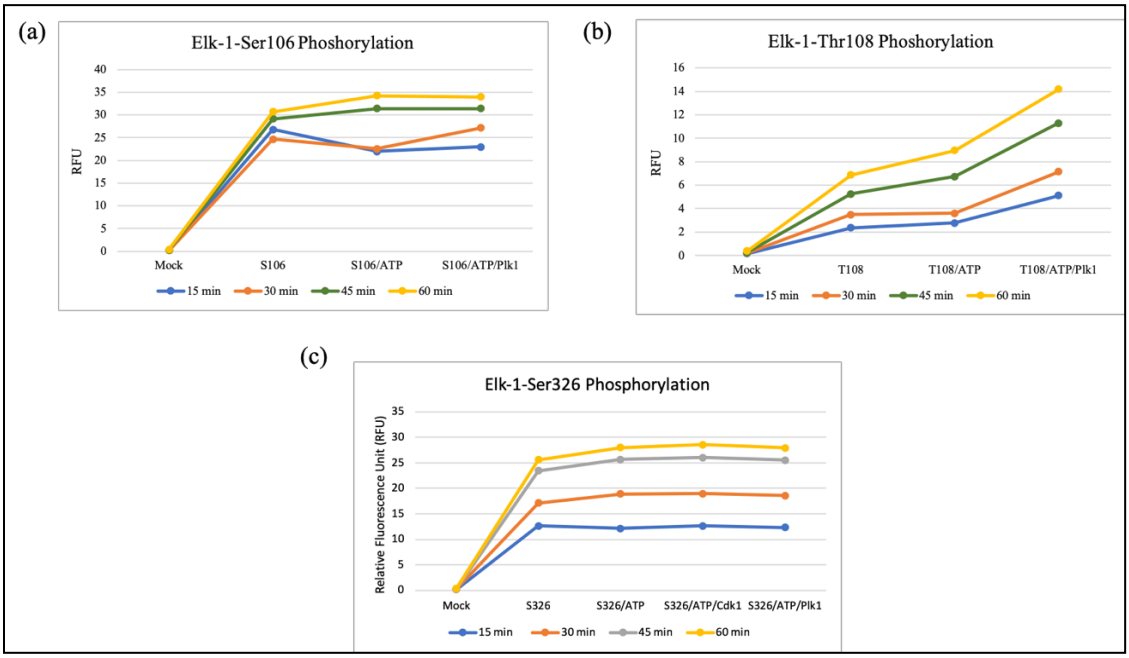


Figure 5.55. Fluorometric measurements of Serine 106 (a) and Threonine 108 (b) phosphorylations by Plk1 kinase and Serine 326 phosphorylation of Elk-1 by both Cdk1 and Plk1 kinases (c).

Phosphorylation of Elk-1 Threonine 133, Serine 202, 303, 304 and 324 residues by Cdk1 kinase was also evaluated by fluorometric kinase assay kit. Specific peptides were either incubated with Cdk1 kinase and ATP or ATP only and the conversion of ATP to ADP was measured by spectrophotometry. Incubation of Elk-1 Threonine 133, Serine 202, Serine 303 and Serine 304 peptides with ATP and Cdk1 kinase promoted ADP conversion by 70 per cent, 25 per cent, 22 per cent and 29 per cent, respectively, as seen in Figure 5.56.

In addition to Cdk1 phosphorylation, Serine 304 phosphorylation of Elk-1 was also evaluated by Aurora A kinase phosphorylation. The kinase reaction was set as incubation Serine 324 peptide with Aurora A kinase and ATP and it was observed that Serine 304 motif of Elk-1 is also phosphorylated by this kinase. As given in Figure 5.56d, Aurora A kinase reaction of this residue is more prominent than Cdk1 phosphorylation.

Serine 324 residue of Elk-1 was determined as putative Cdk1 phosphorylation site, but it was further defined that this site may be phosphorylated by also Plk1 kinase. Therefore, kinase assay reaction was prepared by the existence of Serine 324 peptide, ATP and either with Plk1 or Cdk1 kinases. As demonstrated in Figure 5.56e, phosphorylation of Serine

324 of Elk-1 increased by 2.6 and 3.2-fold by the treatment of both Cdk1 and Plk1 kinases, with Serine 324 peptide and ATP, respectively.

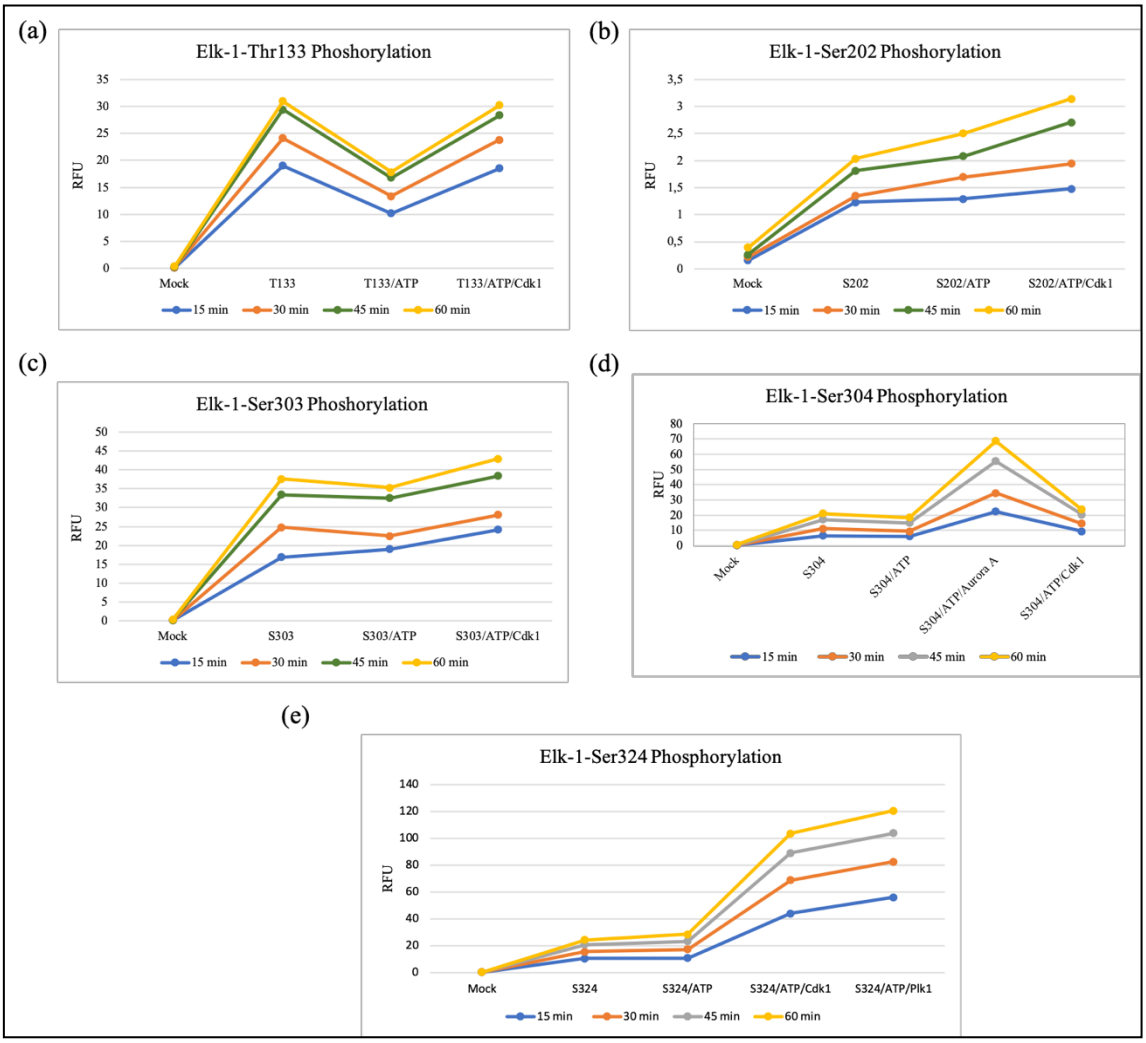


Figure 5.56. Fluorometric measurements of Threonine 133 (a), Serine 202 (b), Serine 303 (c) phosphorylations of Elk-1 by Cdk1 kinase, Serine 304 (d) phosphorylation by both Aurora A and Cdk1 kinases and Serine 324 phosphorylation of Elk-1 by both Cdk1 and Plk1 kinases.

As it was previously stated, Serine 198 residue of Elk-1 may be a target site for both Aurora A, Aurora B and Plk1 kinases, phosphorylation of this region by all these three kinases was analyzed. Measured fluorescence values were increased when Serine 198 peptide incubated with ATP increased, but addition of these target kinases did not significantly affect ADP conversion as demonstrated in Figure 5.57b. In order to precisely

identify the exact phosphorylating kinase of this residue, usage of various concentrations of peptide, Cdk1 and ATP may be necessary.

Phosphorylation of Threonine 199 residue by Aurora A and Aurora B residues was evaluated and it was observed that incubation of Threonine 133 specific unmodified peptide with both Aurora A and Aurora B kinases as well as ATP enhanced ADP conversion compared to the samples those not containing ATP. As seen in Figure 5.57b, incubation with Aurora B kinases increased fluorescently detected ADP ratio by two-fold.

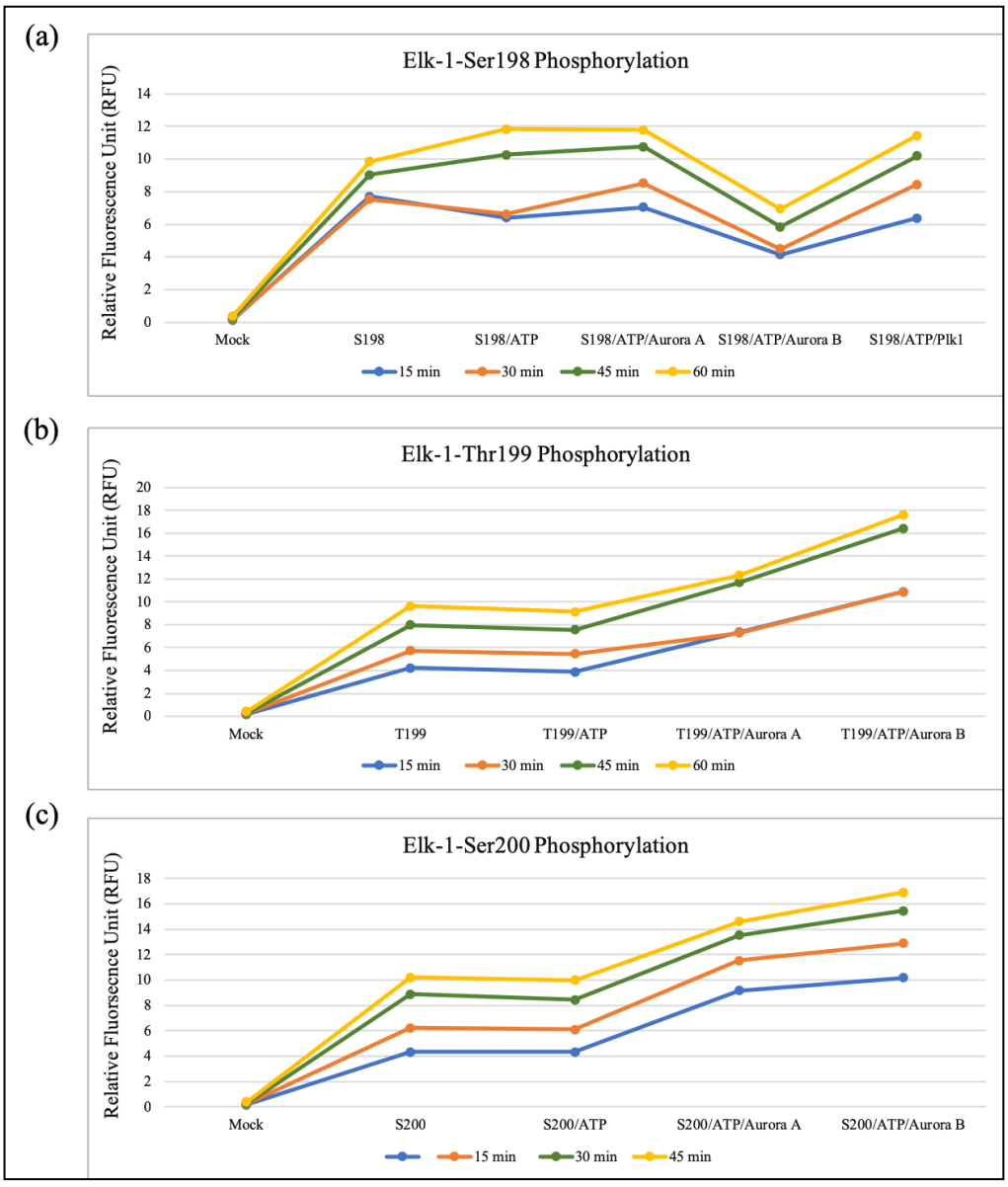


Figure 5.57. Fluorometric measurements of Serine 198 (a), Threonine 199 (b) and Serine 200 (c) phosphorylations of Elk-1 by various kinases.

In addition to Threonine 199 phosphorylation of Elk-1, Serine 200 phosphorylation was also evaluated in terms of Aurora A and Aurora B phosphorylation and it was observed that incubation of this peptide with indicated kinases and ATP increased fluorescence values by nearly 50 per cent and 70 per cent, respectively as depicted in Figure 5.57c.

5.14. EFFECTS OF ELK-1 ON CELL PROLIFERATION

To monitor the response of cells after treatment with various conditions, cellular proliferation, cell viability and cytotoxicity are usually monitored. There are numerous methods that can be used to test these parameters and proper choice of a method depends on the number, type of cells and stimulation conditions as well as the expected outcome [142]. Viable cells are able to convert the substrate to product resulting generation of a signal that is proportional to the number of viable cells present. Whereas, dead cells are not able to convert the substrate to product [143].

To test the effects of wild-type and phospho-mutant forms of Elk-1 on the proliferation of various brain tumor cells, tetrazolium reduction assays, MTT, WST-1 and XTT, were used. Signal intensity depends on several parameters including the concentration of the tetrazolium compound, the length of the incubation period, the number of viable cells and their metabolic activity. To generate optimum signal intensity, cells incubated with MTT, WST-1 or XTT at various time intervals as 1,2,3,4 and 5 hours and the signal was detected at different wavelengths, 440, 450 and 650 nm.

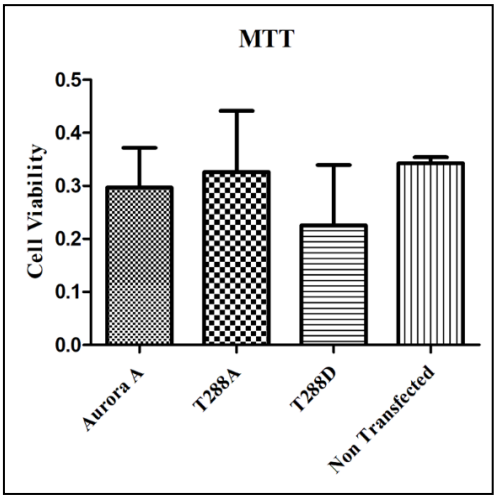


Figure 5.58. MTT assay results of Aurora Kinase transfected SH-SY5Y cells.

Aurora A kinase functions early in mitosis in mitotic spindle assembly and centrosome maturation and thereby plays a major role in cell cycle progression along with other mitotic kinases such as Plk1, Cdks and etc. Therefore, Aurora A kinase activity can be used as a marker of cellular proliferation and viability. Phosphorylation of Threonine 288 residue within the activation segment of Aurora A kinase is crucial for activity, so mutation on this residue is expected to diminish viability and proliferation of cells.

SH-SY5Y cells were transfected with wild-type and T288A and T288D phospho-mutant forms of Aurora A and cell viability were initially determined by MTT assay as seen in Figure 5.58. Since differences between different replicates are too high, scale bars are also high and then there is no significant changes between wild-type and mutant forms of Aurora in comparison with non-transfected cells.

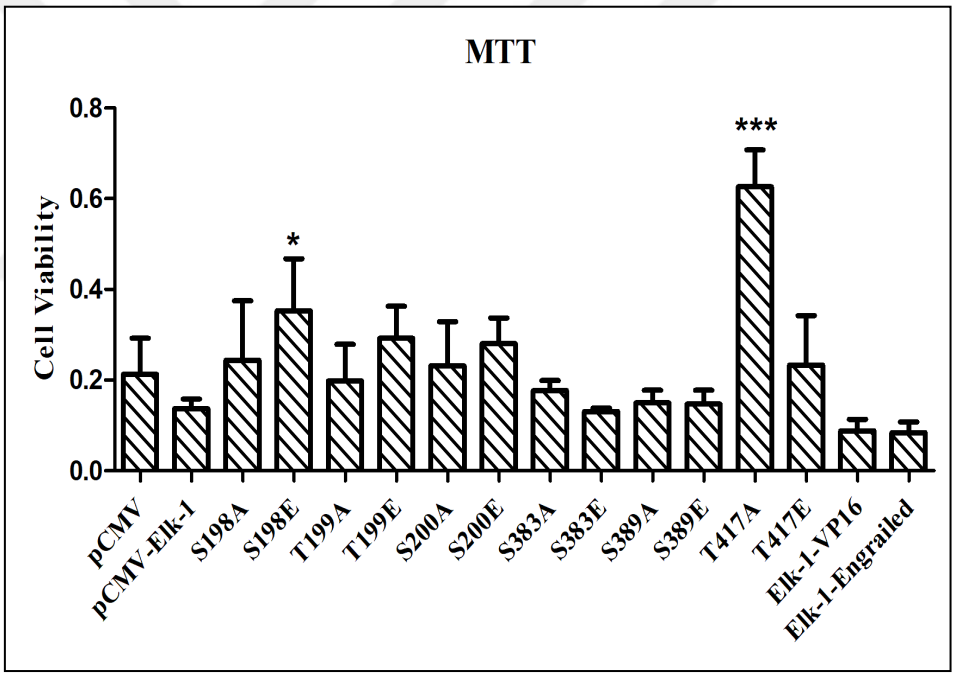


Figure 5.59. MTT assay results of various Elk1 plasmids transfected SH-SY5Y cells. Asterisk indicates significance levels, *** extremely significant, ** very significant, * significant and ns not significant.

SH-SY5Y cells were then transfected with various Elk-1 plasmids and cell viability was analyzed by MTT assay as shown in Figure 5.59. As in the previous result, the scale bars are too high to have a definite conclusion.

The formazan product of the MTT tetrazolium accumulates as an insoluble precipitate inside cells which needs to be solubilized prior to reading od absorbance by various solvents including isopropanol, DMSO, dimethylformamide, SDS and etc. Since solubility level of formazan crystals directly affects the level of cell viability, other assay technologies with much greater sensitivity than MTT has been developed such as XTT and WST-1.

Since XTT and WST-1 are water-soluble and there is no need to perform the solubilization step, they are sufficiently reduced by the cells in the presence of its substrates. Therefore, they are utilized to analyze viability of cells at different conditions instead of MTT.

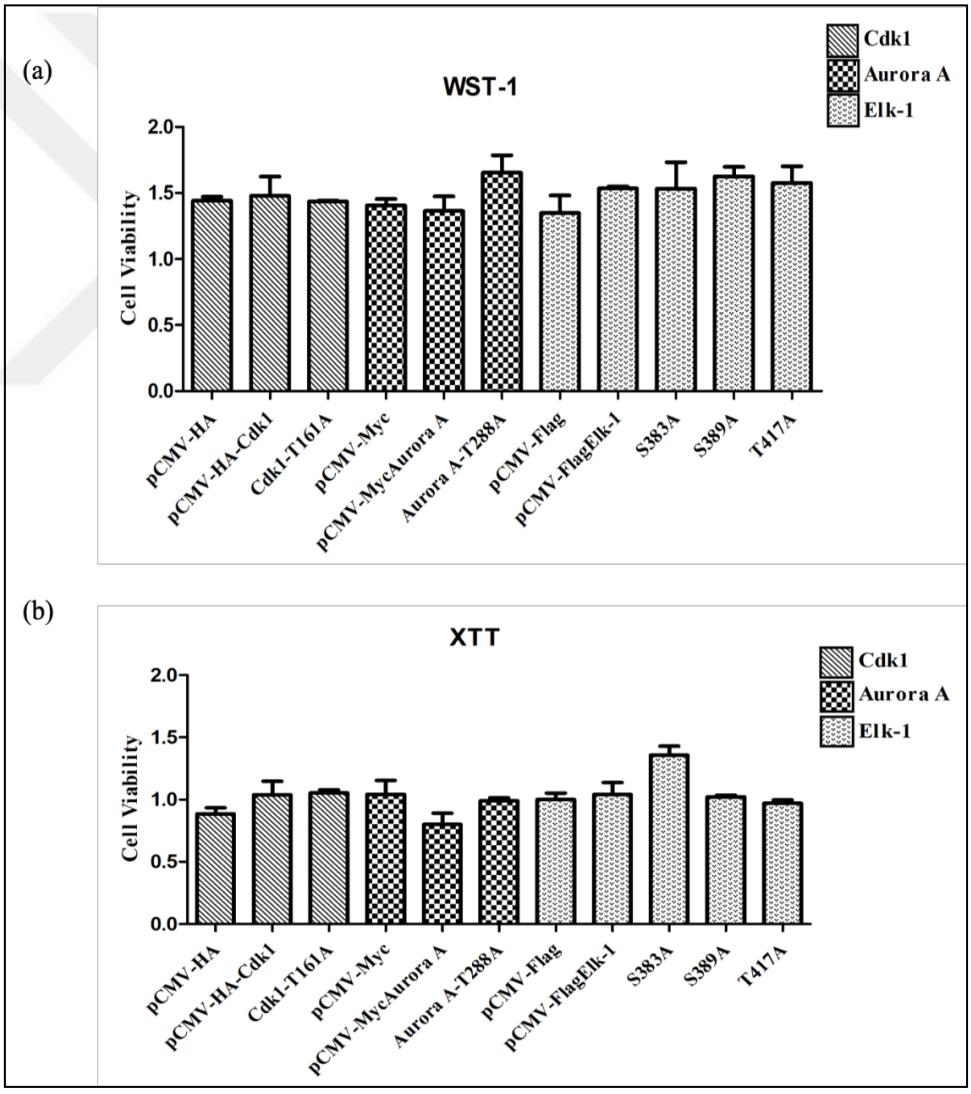


Figure 5.60. WST-1 (a) and XTT (b) assay results of transfected SH-SY5Y cells.

Effect of Cdk1, Aurora A kinase and Elk-1 on cell viability of SH-SY5Y cells were tested by WST-1 and XTT assays. SH-SY5Y cells were transfected with either empty, wild-type and mutant forms of Cdk1 and Aurora A, and Elk-1 plasmids. Similar to Aurora A kinase, Cdk1 is also a chief regulator of cell cycle and activated via Threonine 161 phosphorylation. According to the Figure 5.60, WST-1 and XTT results were similar, for instance, transfection of wild-type and kinase-dead forms of Cdk1 displayed similar effects on cell viability, but these differences between each sample were not significant according to the GraphPad analysis on both WST-1 and XTT assays. Interestingly, Serine 383 Alanine conversion of Elk-1 protein resulted in a significant increase in cell viability when compared to wild-type Since the differences between sample groups were more precise on XTT than WST-1 assay, rest of the viability analyses were evaluated by XTT assay.

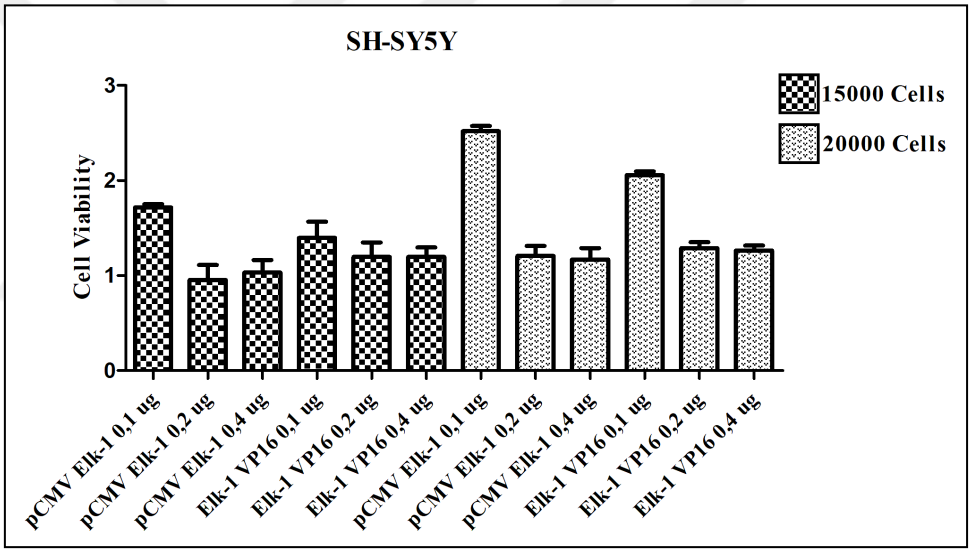


Figure 5.61. XTT assay results of various concentrations of pCMV-Elk-1 and Elk-1-VP16 plasmids.

For the determination of the optimum working concentration of plasmids and cell counts, $1.5.10^4$ and 2.10^4 SH-SY5Y cells were seeded on the plate. These cells were transfected with 0.1, 0.2 and 0.4 µg of pCMV-Elk-1 or Elk-1-VP16 plasmids. As seen in Figure 5.61, 0.1 µg plasmid transfected cells displayed a higher level of cell viability which indicates PEI transfection reagent has side effects on cells. Therefore, the use of low concentration of plasmid and PEI is more prominent in terms of analyzing cell viability. Cell counts of 2.10^4 also determined as the optimum count for XTT assay.

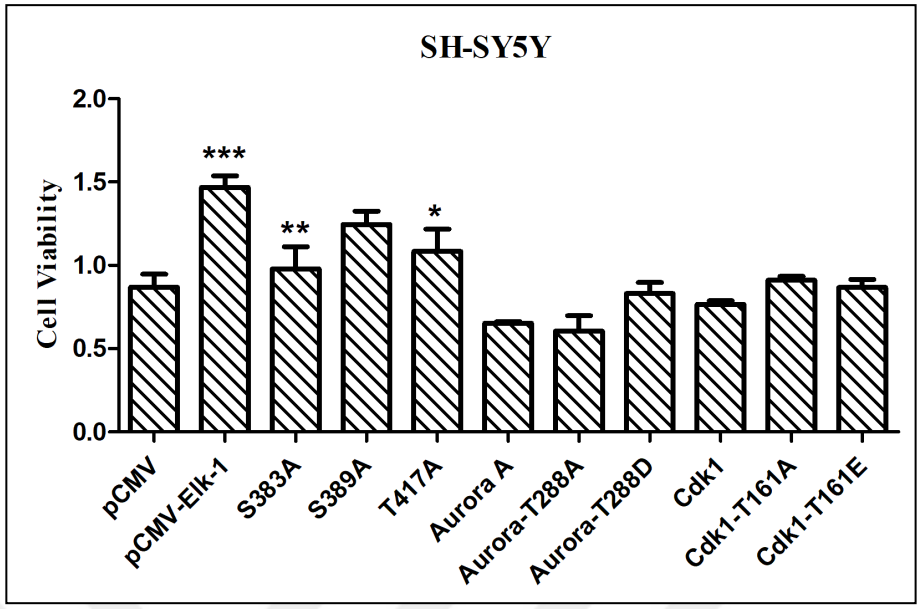


Figure 5.62. XTT assay results of Elk-1, Aurora A and Cdk1 transfected SH-SY5Y cells. Asterisk indicates significance levels, *** extremely significant, ** very significant, * significant and ns not significant.

SH-SY5Y cells were transfected with Elk-1, wild-type, Elk-1-S383A, Elk-1-S389A and Elk-1-T417A, Aurora A, wild-type, T288A, T288D and Cdk1 plasmids, wild-type, T161A, T161E, and their effects on cell proliferation were evaluated by XTT analysis as seen in Figure 5.62. Cells transfected with the wild-type pCMV-Elk-1 showed a significant increase in cell viability compared to the empty plasmid, whereas the cells transfected with the Elk-1-S383A and Elk-1-T417 mutants showed a decrease compared to the wild-type Elk-1. The kinase-active form of Aurora, T288D, increased cell viability, while kinase-dead, T288A, the form was decreased but these changes were not statistically significant. Among the kinase-active, T161D, and kinase-dead, T161A, forms of Cdk1, no significant change was observed.

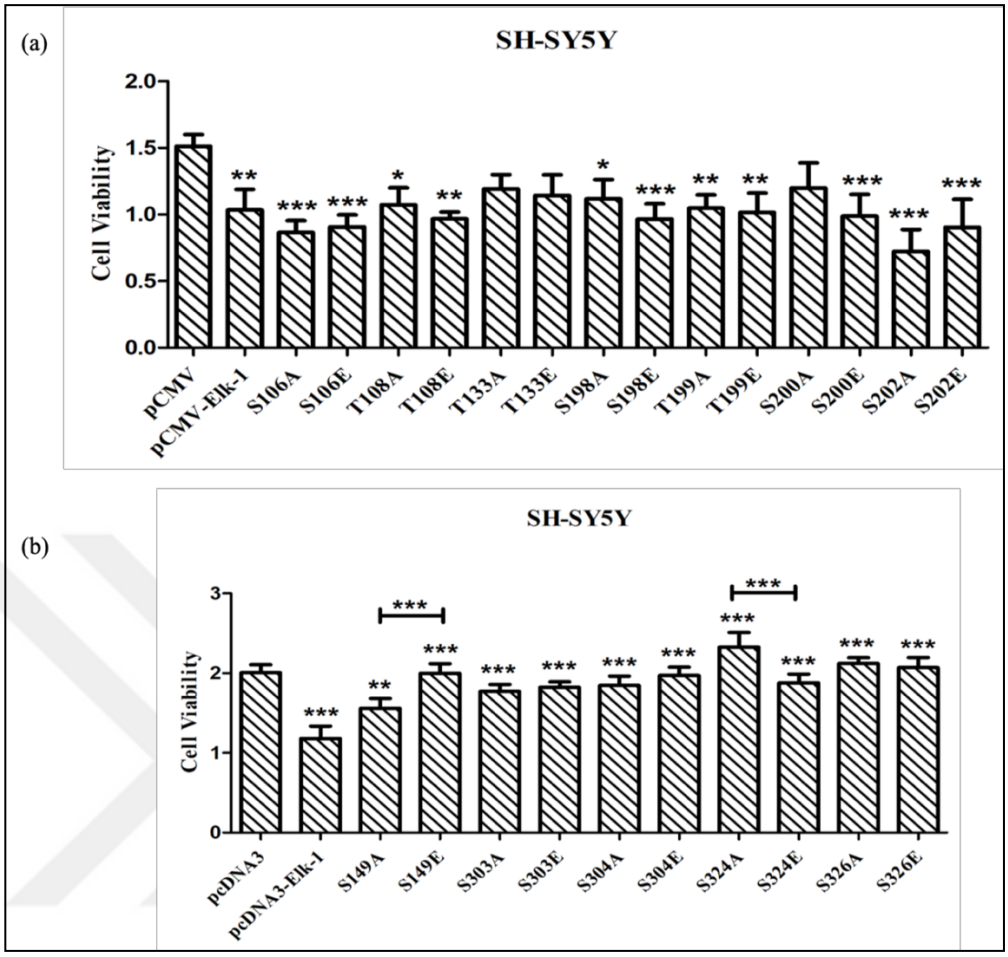


Figure 5.63. XTT assay results of wild-type and phospho-mutant Elk-1 transfected SH-SY5Y cells. Cells were transfected with either wild-type or mutagenic Elk-1 plasmids, S106A/E, T108A/E, T133A/E, S198A/E, T199A/E, S200A/E, S202A/E (a), S149A/E, S303A/E, S304A/E, S324A/E and S326A/E (b). Asterisk indicates significance levels, *** extremely significant, ** very significant, * significant and ns not significant.

The effect of putative Elk-1 phosphorylation sites on the cell viability of was assessed by XTT analysis using phospho-null and phospho-active forms of these residues. SH-SY5Y cells were transfected with empty, wild-type and mutagenic Elk-1 plasmids and fluorescence changes were measured by spectrophotometer. According to the results in Figure 5.63a, incorporation of wild-type Elk-1 into SH-SY5Y cells did not result in a significant change on cell viability; Ser198 and Ser200 phosphorylations have an inhibitory and S202 phosphorylation have an activatory effect on cell viability compared to wild-type Elk-1. When the kinase-active and kinase-dead forms were compared among themselves, Thr108, Thr199 and Ser202 phosphorylations were found to exhibit an

activatory effect on the cell viability in the kinase active forms and Thr133 phosphorylation has an inverse effect. In Figure 5.63b, it was observed that wild-type Elk-1 decreased cell viability, whereas all other mutants increased. When the mutations were evaluated among themselves, Ser149 phosphorylation was found to has an enhancing effect on cell viability and Ser324 has a reducing effect.

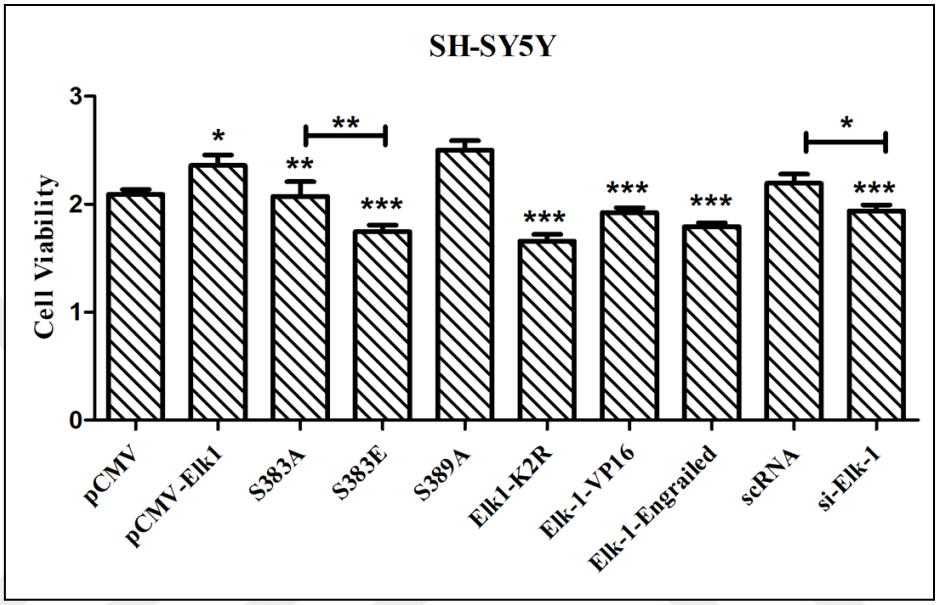


Figure 5.64. XTT assay results of various Elk-1 protein. Asterisk indicates significance levels, *** extremely significant, ** very significant, * significant and ns not significant.

The effects of various Elk-1 plasmids on cell viability were evaluated on SH-SY5Y cells. In this experiment, transfection of wild-type Elk-1 plasmid increased cell viability compared to the empty plasmid. It was observed that Serine 383 phosphorylation of Elk-1 has an inhibitory effect on cell viability, Elk-1-S383A transfection increased cell viability but Elk-1-S383E transfection decreased it. Serine 389 resides was not displayed any significant effect on cell proliferation. Effects of Elk1 on cell viability was also analyzed by using Elk-1-K2R, si-Elk-1, scrambled RNA, Elk-1-VP16 and Elk-1-Engrailed plasmids. Elk1-K2R plasmid was created for targeting Elk-1 protein by SUMOylation to negatively affect its protein function and as seen in Figure 5.64, cell viability decreased on Elk-1-K2R sample which means down-regulation of Elk-1 resulted in cell death. Similarly, si-Elk-1 was also used to inhibit Elk-1 function to see the effect of Elk1 on cell viability. Inhibition of Elk-1 caused decreased cell viability as Elk-1-K2R sample. Elk-1-VP16 is the constitutively active form of Elk-1 and Elk-1-Engrailed is the negative dominant form.

When compared these plasmids into each other, Elk-1-Eng plasmid of Elk1 caused a decrease in cell death.

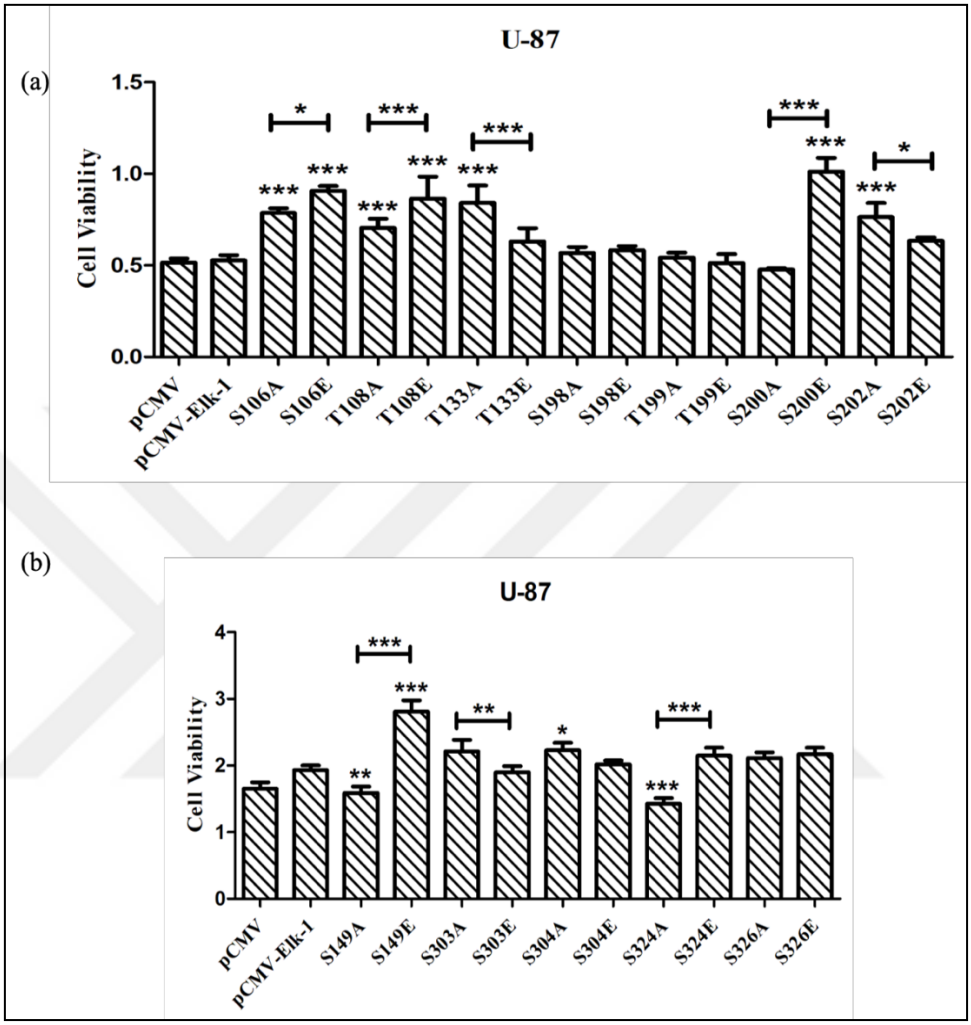


Figure 5.65. XTT assay results of wild-type and phospho-mutant Elk-1 transfected U-87 cells. Cells were transfected with either wild-type or mutagenic Elk-1 plasmids, S106A/E, T108A/E, T133A/E, S198A/E, T199A/E, S200A/E, S202A/E (a), S149A/E, S303A/E, S304A/E, S324A/E and S326A/E (b). Asterisk indicates significance levels, *** extremely significant, ** very significant, * significant and ns not significant.

Effect of Elk-1 phosphorylations on cell proliferation was also evaluated in U-87 human glioblastoma cells. While no significant difference was observed between Elk-1 and empty plasmid, it was observed that Ser 106, Thr 108, and Ser 200 phosphorylations had an enhancing effect on the proliferation of U-87 cells, whereas, on Thr 133 and Ser 202 phosphorylations, a reducing effect was observed in Figure 5.65a. The phosphorylation of particularly Serine 200 from these regions significantly increased cell viability. In Figure

5.65b, it was observed that phosphorylation of Ser 149 and Ser 324 increased cell viability and Serine 303 showed a decreasing effect on vitality. Serine 304 and especially Serine 326 did not have a significant effect on cell viability.

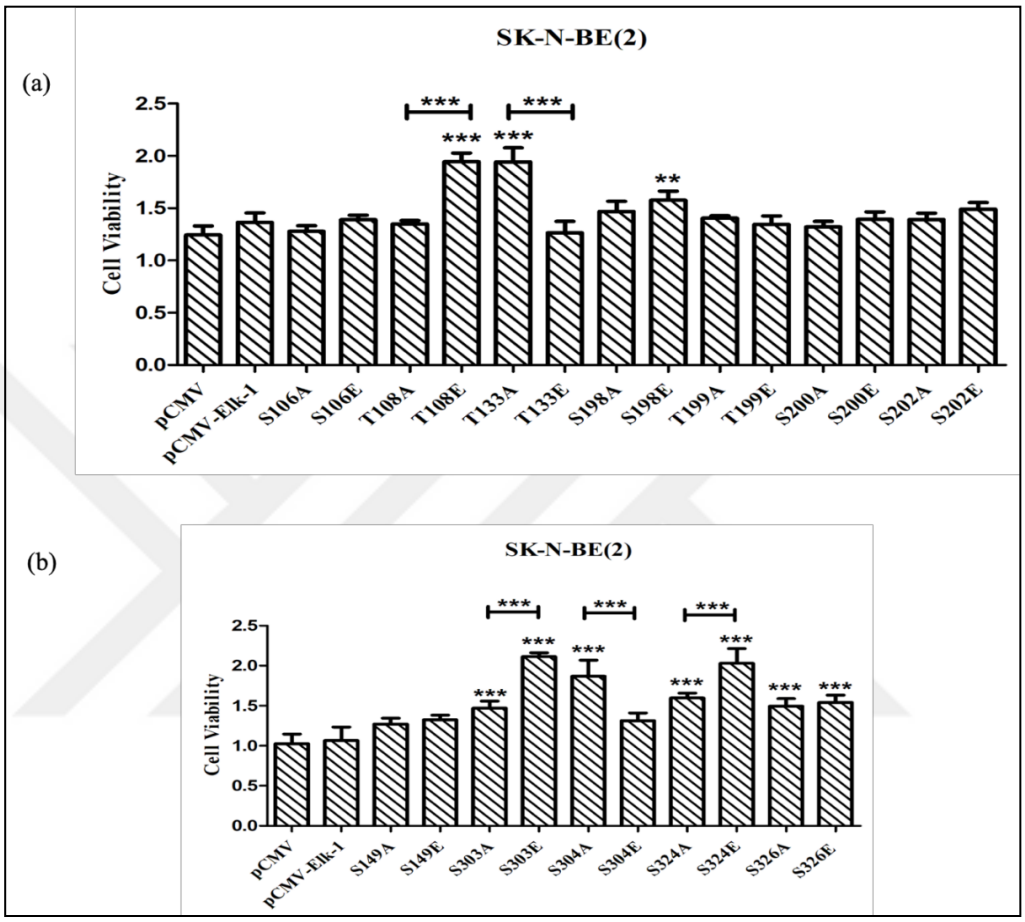


Figure 5.66. XTT assay results of wild-type and phospho-mutant Elk-1 transfected SK-N-BE (2) cells. Cells were transfected with either wild-type or mutagenic Elk-1 plasmids, S106A/E, T108A/E, T133A/E, S198A/E, T199A/E, S200A/E, S202A/E (a), S149A/E, S303A/E, S304A/E, S324A/E and S326A/E (b). Asterisk indicates significance levels, *** extremely significant, ** very significant, * significant and ns not significant.

The effects of wild-type and both phospho-null and phospho-active forms of Elk-1 were further tested on SK-N-BE (2) human neuroblastoma cell line. SK-N-BE (2) cells were transfected with indicated plasmids and the changes on cell viability after transfection of these plasmids were evaluated by XTT assay and subsequent statistical analysis. Overexpression of wild-type Elk-1 did not give rise to a remarkable change compared to the empty plasmid. According to the results represented in Figure 5.66a, Threonine 108 phosphorylation of Elk-1 has an enhancing, while Threonine 133 has an inhibitory effect

on cell viability. Overexpression of Serine 198 kinase active form resulted in increased cell viability, but there wasn't any significant change between phospho-null and phospho-active forms of this residue. In Figure 5.66b, it was observed that Serine 303 and Serine 324 phosphorylations increased cell proliferation, whereas, Serine 304 phosphorylation decreased it. While both phospho-null and phospho-active forms of Serine 326 phosphorylation increased cell viability, no significant effect was observed between each other.

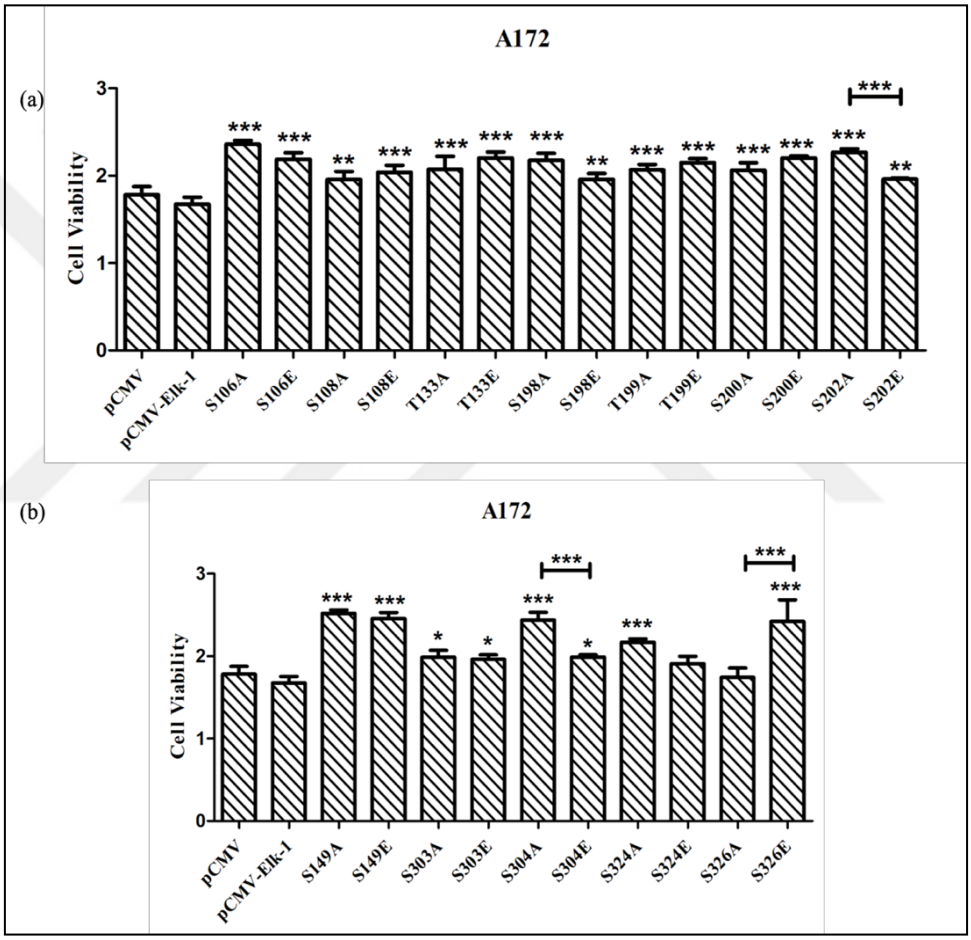


Figure 5.67. XTT assay results of wild-type and phospho-mutant Elk-1, transfected A172 cells. Cells were transfected with either wild-type or mutagenic Elk-1 plasmids, S106A/E, T108A/E, T133A/E, S198A/E, T199A/E, S200A/E, S202A/E (a), S149A/E, S303A/E, S304A/E, S324A/E and S326A/E (b). Asterisk indicates significance levels, *** extremely significant, ** very significant, * significant and ns not significant.

Human glioblastoma cell lines, T98 and A172 were also used in addition to the SH-SY5Y, SK-N-BE (2) and U87 cell lines in order to see whether the effects of Elk-1

phosphorylations on cell viability were cell-specific or not. These cells were a kind gift to the laboratory from Dr Tugba Bagci Onder, Koc University. According to the results represented in Figure 5.67, wild-type Elk-1 resulted in an increase in cell viability. Phosphorylation of Serine 106, Threonine 108, Serine 200 and Serine 324 displayed an enhancing effect on cell proliferation, while Serine 198, Serine 149, Serine 303 and Serine 326 has an inhibitory effect on it. Phosphorylation of Threonine 133, Threonine 199 and Serine 202 was ineffective on cell viability.

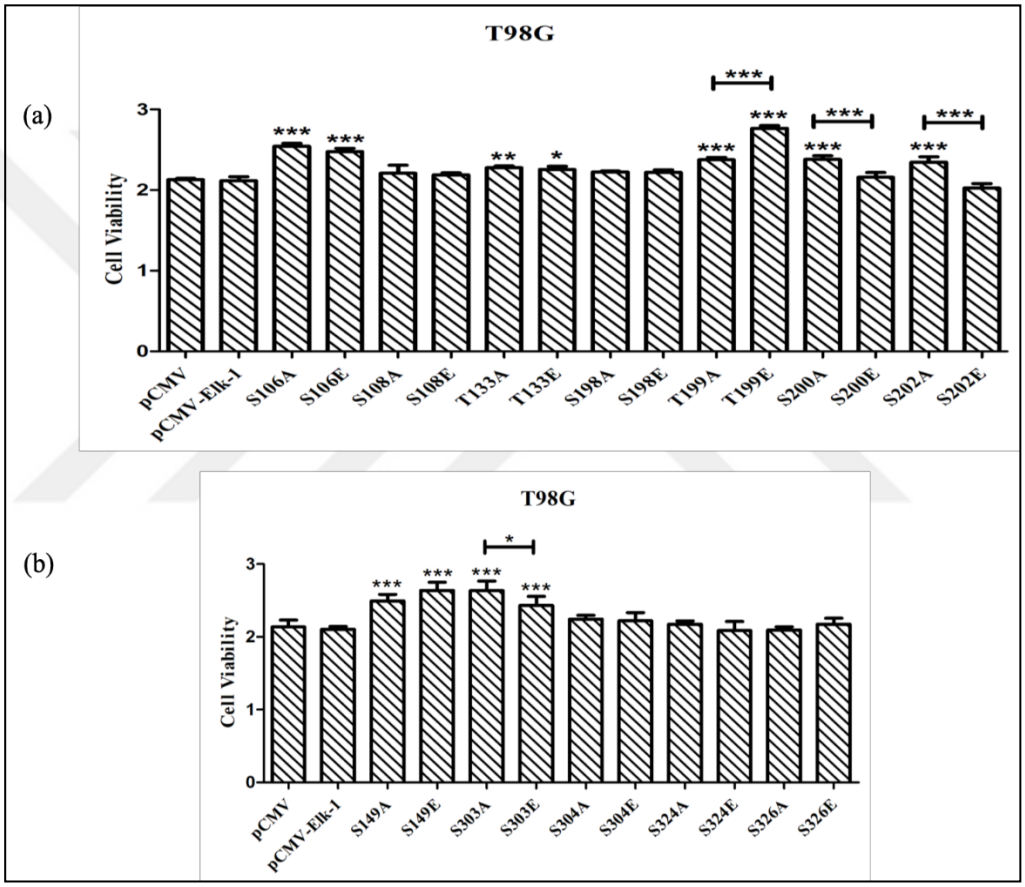


Figure 5.68. XTT assay results of wild-type and phospho-mutant Elk-1 transfected T98G cells. Cells were transfected with either wild-type or mutagenic Elk-1 plasmids, S106A/E, T108A/E, T133A/E, S198A/E, T199A/E, S200A/E, S202A/E (a), S149A/E, S303A/E, S304A/E, S324A/E and S326A/E (b). Asterisk indicates significance levels, *** extremely significant, ** very significant, * significant and ns not significant.

When the effect of Elk-1 phosphorylations on T98 cells was examined, it was seen that only Threonine 199 and Serine 149 phosphorylations had an enhancing effect, while Serine 200, Serine 202 and Serine 303 phosphorylations had a reducing effect on cell viability in

Figure 5.68. Both phospho-null and phospho-active forms of Serine 106 and Threonine 133 forms increased cell proliferation, but no significant difference was observed between these two forms.

5.15. EFFECTS OF ELK-1 ON MITOTIC PROFILE OF CELLS

In order to determine the phase of the cells during the cell cycle, DNA of the cells is generally stained with propidium iodide (PI) which is an intercalating agent and a fluorescent molecule that can be used to stain the nucleus of cells. When excited with 488 nm wavelength light, it fluoresces red and this fluorescence helps to identify cell viability and DNA content in cell cycle analysis by flow cytometry.

Serine 383 residue of Elk-1 is phosphorylated and thereby activated by the MAPK pathway and it is one of the best known and studied phosphorylation of Elk-1. In order to analyze the effect of this phosphorylation of Elk-1 on the mitotic profile of SH-SY5Y human neuroblastoma cells, phospho-null, Elk-1-S383A, and phospho-mimic, Elk-1-S383E, forms of Elk-1 were transfected into the cells. Then, cells were blocked at prometaphase by the administration of nocodazole that interferes polymerization of microtubules, for 16 hours [6]. Cells were stained with PI staining solution and mitotic profile of the cells was analyzed by FACS.

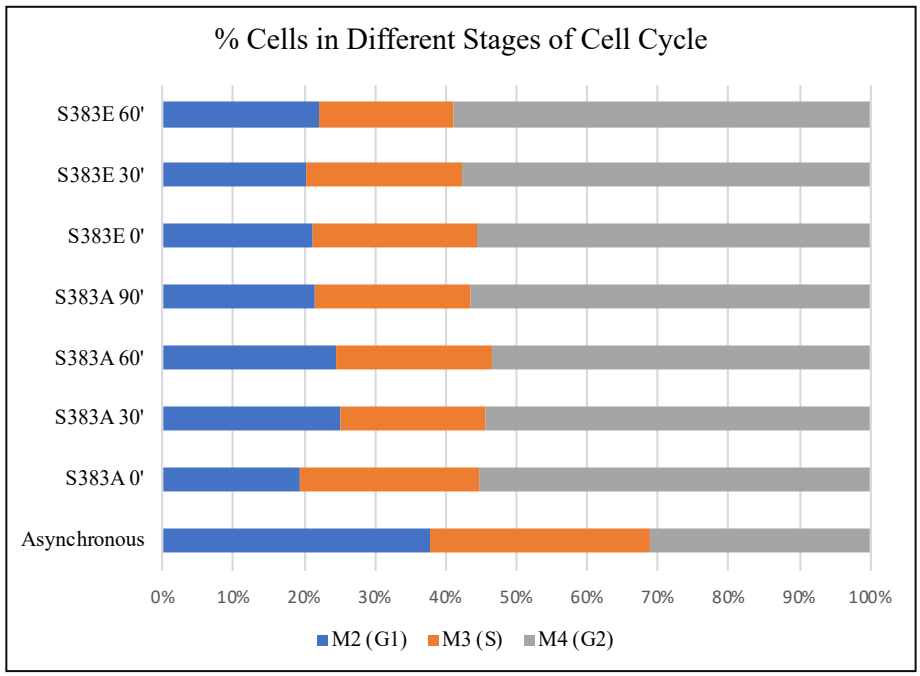


Figure 5.69. Effect of Serine 383 phosphorylation of Elk-1 on the mitotic profile.

After S383A-Elk-1 transfection and nocodazole treatment, most of the cells remained at G_2/M phase as seen in Figure 5.69. Serine 383E transfection also exhibited similar results, the majority of cells detected at the G_2/M phase, but the intensity of cells at the G_2/M phase decreased by time. In addition to Serine 383 phosphorylation of Elk-1, effects of Serine 149, Serine 303, Serine 304, Serine 324 and Serine 326 phosphorylations of Elk-1 on the mitotic profile was analyzed by flow cytometry. These phosphorylation sites were blocked by alanine mutations and these mutants were also transfected into SH-SY5Y cells. PI-stained cells were analyzed by flow cytometry. FACS results are represented in the Appendix.

To analyze Elk-1 phosphorylations on the mitotic profile of SH-SY5Y cells, these cells were transfected with indicated plasmids and then treated with nocodazole to synchronize them. After 16 hours, cells were released from nocodazole and collected at thirty minutes time intervals in an effort to catch cells at different stages as prometaphase, time zero, metaphase, 30th minutes, anaphase, 60th minutes, and telophase, 90th minutes. Collected cells then stained with PI and their mitotic profile were analyzed by fluorescence assisted cell sorting (FACS). Untransfected cells were also treated with nocodazole and stained with PI to compare results.

When the mitotic profile of the control, non-transfected cells in the graph, is examined, it is seen that almost 50 per cent of these cells are in G_1 phase. However, approximately 25 per cent of the cells in the S phase and 25 per cent in the G_2 phase were observed in Figure 5.70. The samples transfected with wild-type Elk-1 and empty plasmid were displayed a similar profile with non-transfected one, whereas mitotic profile of cells transfected with Elk-1 phospho-mutants quite different. For instance, when the cells transfected with in particular Elk-1-T108A, T199 and S200A phospho-null plasmids and harvested after 16 hours nocodazole treatment, it was found that the ratio of G_1 to these cells were dramatically decreased to less than 20 per cent, which was about 70 per cent of un-transfected cells.

In addition to the potent phosphorylation sites described above, the effects of Serine 149, 303, 304, 324 and 326 phosphorylations were also analyzed in a similar way in Figure 5.71. The mitotic profile of phospho-null plasmid transfected cells was significantly changed compared to untransfected cells. For instance, when the cells collected with the S326A plasmid and harvested for 16 hours with nocodazole were analyzed, it was found

that the ratio of G_1 to these cells decreased to less than 20 per cent and reached to a value of about 70 per cent. As can be seen from this conclusion, the cells transfected with plasmids such as S326A, 324A and 304A were retained in the G_2 phase of mitosis and could not pass into the cell division phase.

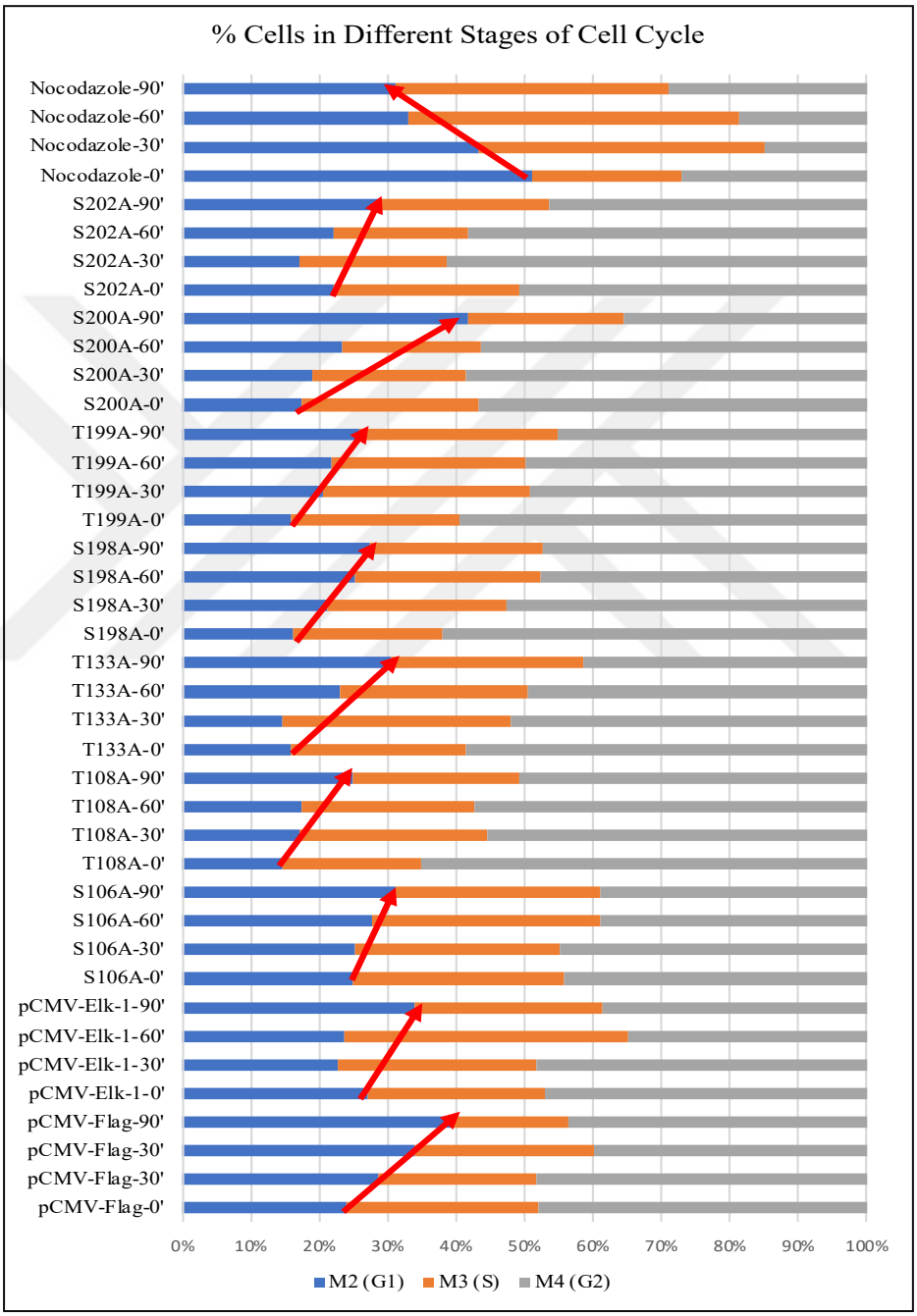


Figure 5.70. The percentage of cells in different phases of mitosis. The effect of various Elk-1 plasmids on the mitotic profile of SH-SY5Y cells. M2 (blue): G1 phase, M3 (orange): S phase, M4 (grey): G2 phase of the cell cycle. Red arrows pinpoint cell arrested at G1 phase.

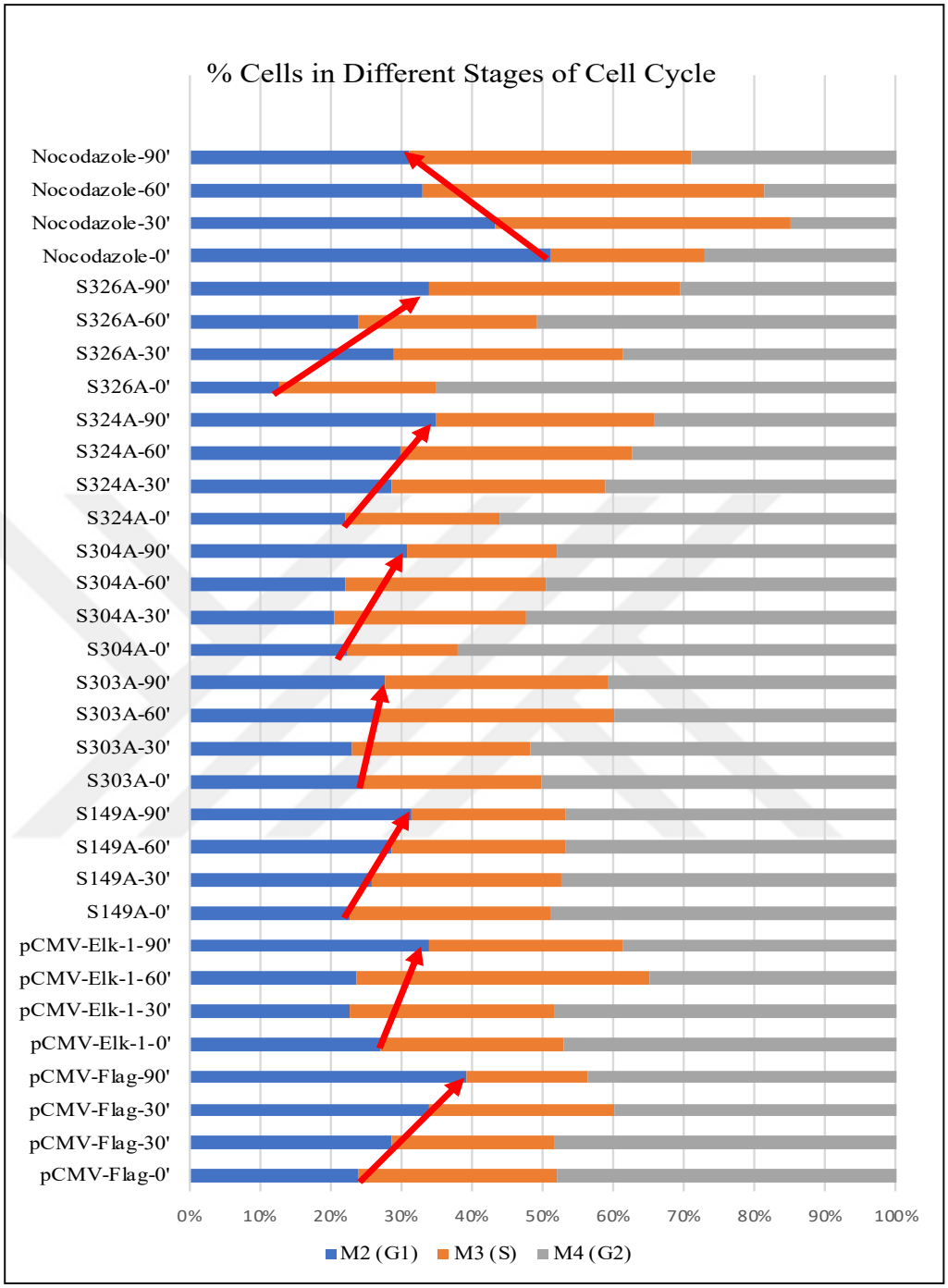


Figure 5.71. The percentage of cells in different phases of mitosis after transfection with various Elk-1 plasmids. M2 (blue): G1 phase, M3 (orange): S phase, M4 (grey): G2 phase of the cell cycle. Red arrows pinpoint cell arrested at G1 phase.

In addition to the analysis of the effect of Elk-1 phosphorylation on the mitotic profile of cells, the mitotic index of the cells was also analyzed by Histone H3 phospho Serine 10 staining. Histone H3 is one of the four core histone proteins, Histone 2A, Histone 2B,

Histone H3 and Histone H4, that pack DNA in nucleosomes. Histone H3 phosphorylation at Ser10 is correlated with chromosome condensation during mitosis. Ser10 phosphorylated Histone H3 signal indicates a mitotic cell with condensed DNA [154].

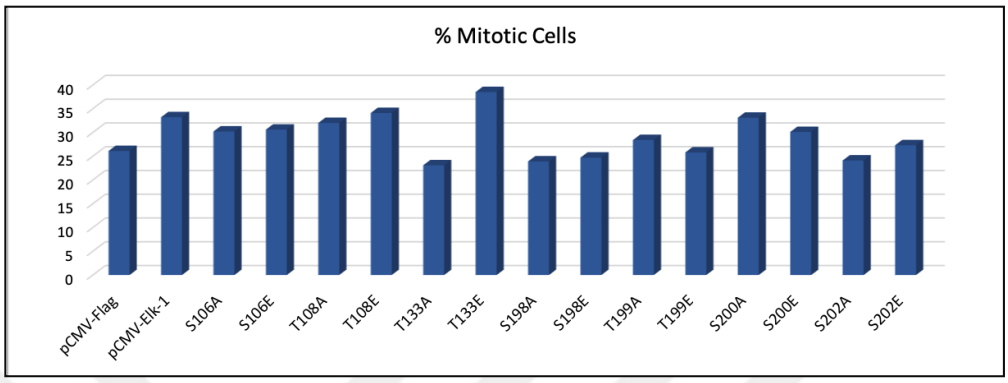


Figure 5.72. The percentage of mitotic cells transfected with wild-type, phospho-null and phospho-active forms of Elk-1. SH-SY5Y cells were transfected with empty, wild-type, S106A/E, T108A/E, T133A/E, S198A/E, T199A/E, S200A/E and S202A/E plasmids.

To analyze the effect of Elk-1 phosphorylation on mitotic profile in more detail, SH-SY5Y cells were transfected with target plasmids and stained with both PI and Histone H3 Ser10 antibody. Mitotic indexes, which is defined as the ratio between the number of cells in a population undergoing mitosis to the total number of cells in a population, were investigated by running samples on flow cytometer at appropriate FSC (Forward Scatter) and SSC (Side Scatter) gates to exclude cell debris and aggregates.

According to Figure 5.72, the samples transfected with Elk-1 showed that mitotic profiles, i.e. the total number of cells undergoing mitosis, increased by 1.27 times compared to empty plasmids. When compared with Elk-1, the most significant change in mitotic profile was seen in the phosphorylation of Threonine 133 of Elk-1, a 0.7-fold decrease in the mitotic index of the cells transfected with the phospho-null form, Elk-1-T133, and in the kinase-active form, Elk-1-T133E, the mitotic index increased by 1,16-fold. When the phospho-active and phospho-null forms of Threonine 133 phosphorylation were compared, the mitotic index increased by 1.66 times when the phospho-active form was given to the cells. No significant difference was observed between the phospho-null and phospho-active forms in the other phosphorylation sites. The FACS analysis results and table representing the numerical data of these results are in the Appendix section.

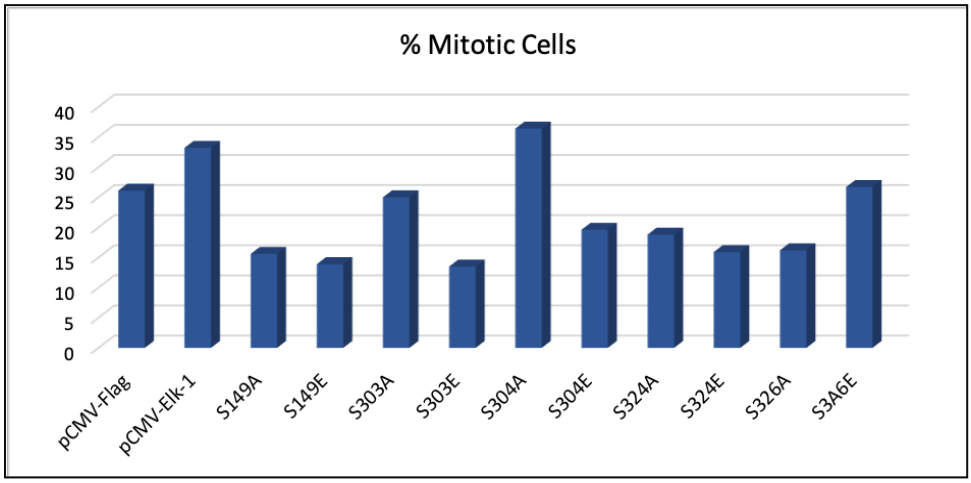


Figure 5.73. The percentage of mitotic cells transfected with various Elk-1 plasmids. SH-SY5Y cells were transfected with empty, wild-type, S149A/E, S303A/E, S304A/E, S324/E and S326A/E plasmids.

In addition to the Elk-1 phosphorylations shown in Figure 5.73, the effects of Serine 149, 303, 304, 324 and 326 phosphorylations on the mitotic profile were also investigated. While there was no significant difference between the phospho-null and phospho-active forms of Serine 149 and Serine 324 phosphorylations, blocking of Serine 303 and 304 phosphorylations reduced mitotic index. When compared to each other, blocking of Serine 326 phosphorylation of Elk-1 reduced mitotic profile, on the contrary, constitutively activation of this phosphorylation increased mitotic index.

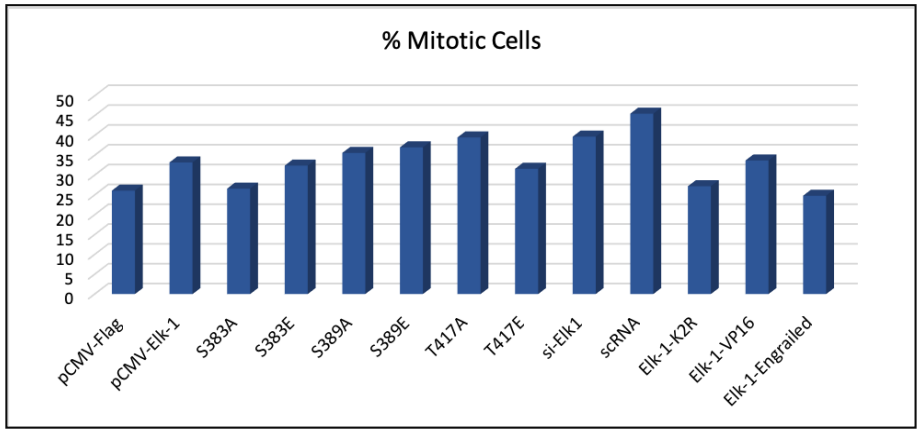


Figure 5.74. The percentage of mitotic cells transfected with wild-type and various mutagenic Elk-1 plasmids. SH-SY5Y cells were transfected with empty, wild-type, S106A/E, T108A/E, T133A/E, S198A/E, T199A/E, S200A/E and S202A/E plasmids.

In addition to the putative phosphorylation sites of Elk-1, the effects of Serine 383, 389 and Threonine 417 residues, which are the most studied and functionally best-known phosphorylation regions, to the mitotic index were also examined by FACS analysis. As shown in Figure 5.74, increasing the phosphorylation of Serine 383 resulted in a slight increase in the number of mitotic cells, while no significant effect of Serine 389 phosphorylation was observed. Threonine 417 phosphorylation had a mitigating effect on the mitotic index. Transfection of Elk-1-K2R, SUMOylation form of Elk-1, into SH-SY5Y cells also gave rise to a 0,82-fold decrease in terms of mitotic index. Elk-1-VP16 is the constitutively active form of Elk-1 and Elk-1-Engrailed is the negative dominant form. When compared the effects of these two forms of Elk-1 on the mitotic index, it was observed that constitutively active form of Elk-1 increased mitotic cell populated by 1,34-fold.

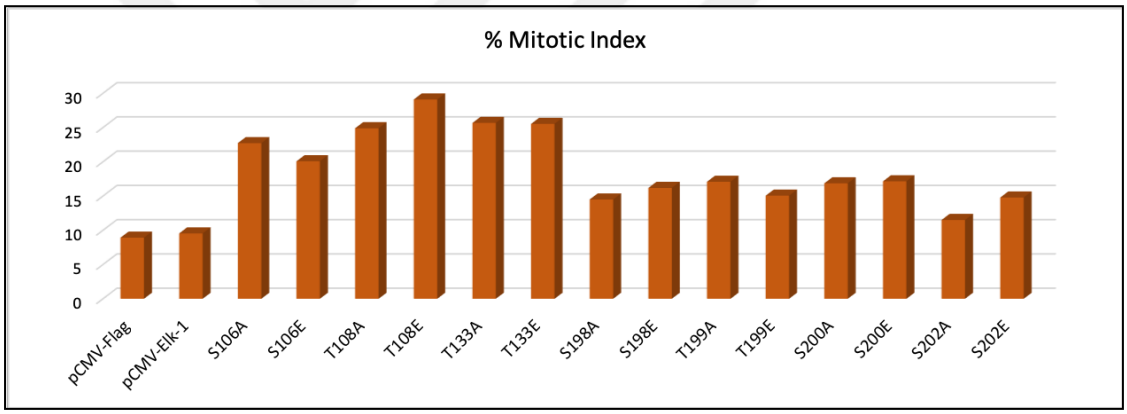


Figure 5.75. The mitotic percentage of U-87 cells transfected with wild-type, phospho-null and phospho-active forms of Elk-1. SH-SY5Y cells were transfected with empty, wild-type, S106A/E, T108A/E, T133A/E, S198A/E, T199A/E, S200A/E and S202A/E plasmids.

In addition to SH-SY5Y cells, the effect of Elk-1 phosphorylation on the mitotic index was also analyzed in U-87 cells. The phospho-null and phospho-active forms of the phosphorylation sites and the wild-type Elk-1 plasmid was transfected into U-87 cells and mitotic profiles of the cells were evaluated by FACS analysis after histone H3 phospho Serine 10 antibody and PI staining. In Figure 5.75, the mitotic indexes of Elk-1 overexpressed cells slightly increased compared to the empty pCMV-Flag plasmid. When the effects of putative phosphorylation sites on mitotic profiles of U-87 cells were examined; there was no significant change in phosphorylation of Threonine 133 and Serine 202, but it was observed that the phosphorylation of Serine 106 and Threonine 199 had an

enhancing and Threonine 108, Serine 198 and Serine 200 had an activatory effect. These changes were observed in a very mild manner.

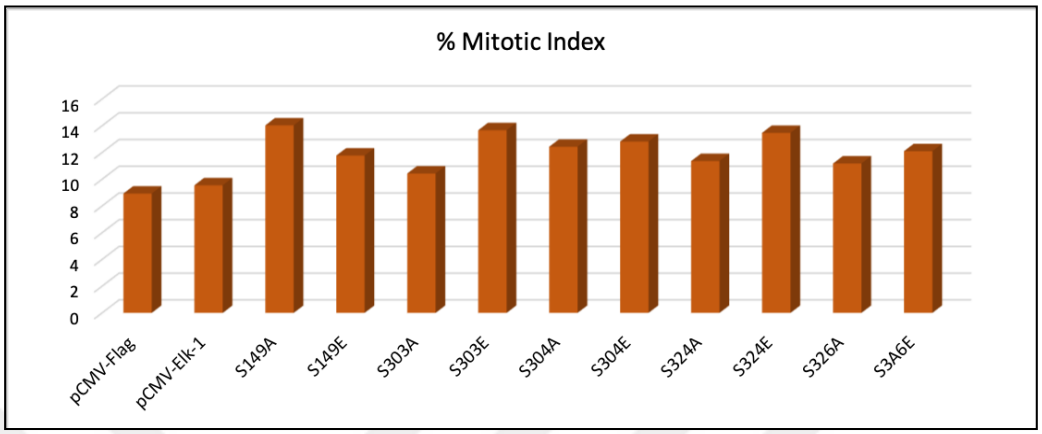


Figure 5.76. The mitotic percentages of U-87 cells transfected with Elk-1 phospho-mutant plasmids. U87 cells were transfected with empty, wild-type, S149A/E, S303A/E, S304A/E, S324/E and S326A/E plasmids.

The effects of Serine 149, 303, 304, 324 and 326 phosphorylations of Elk-1 on the mitotic cells of U-87 cells was also investigated as demonstrated in Figure 5.76. It was observed that Serine 303 and Serine 324 phosphorylation of Elk-1 significantly increased mitotic index dramatically, but the Serine 304 and 326 phosphorylation enhanced mitotic index slightly. On the contrary, it was observed that phosphorylation of Serine 149 reduced the number of mitotic cells.

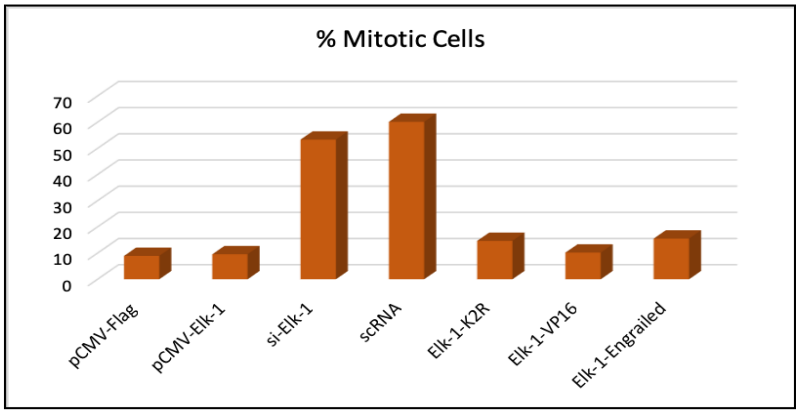


Figure 5.77. The mitotic percentages of U-87 cells transfected with various Elk-1 plasmids. U-87 cells were transfected with empty, wild-type, S149A/E, S303A/E, S304A/E, S324/E and S326A/E plasmids.

To analyze the effect of Elk-1, rather than phospho-mutant forms of it, U-87 cells were transfected with various types of Elk-1 plasmids, empty pCMV-Flag, pCMV-Elk-1, Si-Elk-1, scrambled RNA, Elk-1-K2R, Elk-1-VP16 and Elk-1-Engrailed. The mitotic percentage of these transfected cells were further stained with both PI and Histone H3 P-SER10-488 conjugate antibody and mitotic cells were detected by flow cytometry.

Transfected of cells with wild-type Elk-1 did not remarkably changed mitotic percentage of U-87 cells compared to empty plasmid as seen in Figure 5.77. However, silencing of Elk-1 by si-Elk-1 transfection resulted in an approximately 8 per cent reduction in the percentage of mitotic cells compared to those scrambled RNA transfected ones. Transfection of Elk-1-K2R, SUMOylation form of Elk-1, into U87 cells showed an increase in mitotic cell percentage according to the wild-type Elk-1. The constitutively active form of Elk-1 resulted in a nearly 6 per cent decrease in mitotic cells compared to the Elk-1-Engrailed, negative dominant form of Elk-1.

6. DISCUSSION

Demir and Kurnaz [7] had previously demonstrated that Elk-1 exhibits different mitotic spindle localizations at various stages of the cell cycle. Moreover, Elk-1 co-localizes and interacts with Aurora-A kinase in the first stages of mitosis. However, the peculiar mitotic localization of Elk-1 that overlaps with localizations of various mitotic kinases including Polo-like kinases (Plks) and Cyclin-dependent kinases (Cdks) has raised the question of whether Elk-1 itself is a target of these mitotic kinases in addition to Aurora-A kinase.

To address that question, an *in silico* search for the prediction of putative phosphorylation sites through several online platforms has been initially performed. According to these prediction tools, several putative sites for Plk1 (Serine 106, Threonine 108 and Serine 326), Cdks (Threonine 133, Serine 200, Serine 202, Serine 303, Serine 304 and Serine 324), Aurora kinases (Serine 149, Serine198, Threonine 199and Serine 200) have been identified in addition to the well-known Serine 389 and Serine 389 phosphorylations of Elk-1.

The presence of mitotic kinase phosphorylation motifs on the Elk-1 protein domain showed that the investigation of the relationship between Elk-1 and target kinases is crucial to understand the role of Elk-1 during mitosis and tumorigenesis. It has been previously demonstrated that Elk-1 interacts with Aurora-A in a mitotic stage-dependent manner [7]. To reveal such an interaction between Elk-1 and other mitotic kinases, Aurora B, Cdk1 and Plk1, immunoprecipitation assay has been performed. According to these results, a similar interaction between Elk-1 and Aurora A was also observed with Aurora B, Cdk1 and Plk1 kinases (Figure 5.8).

In addition to Cdk1 kinase, the relationship between other members of cyclin-dependent kinase family, Cdk2 and Cdk5, and Elk-1 has also been analyzed. Cyclin-dependent kinases regulate many aspects of cell division, so their action is crucial for accurate completion of the mitosis. They are involved in DNA replication in S phase, mitotic progression, regulation of mitotic checkpoints and cell senescence, transition between different phases, nuclear envelope breakdown, chromosome condensation and separation and cytokinesis [155]. In this study, it has been demonstrated that Elk-1 is not only able to interact with Cdk1 but also with Cdk2 mitotic kinase. Cdk1 is an important mitotic regulatory kinase and its function is required for S to G_2 transition and G_2 to M phase

transition along with its regulatory subunits, Cyclin A and Cyclin B, respectively. In addition to these, activity of Cdk1/Cyclin B complex is important for regulation of M checkpoint and mitotic spindle assembly [155]. Cdk2 is another regulatory mitotic kinase belongs to Cyclin dependent kinase family and it is implicated in initiation and progression of DNA synthesis ad transition between mitotic phases. Cdk2/Cyclin E complex mediates G_1 to S phase transition and regulates DNA and centrosome replication [156], while Cdk2/Cyclin A complex regulates progression through S phase [157]. It was previously reported that Cdk6, which functions as a cell-cycle initiator protein, regulates transcription of Elk-1. The well-coordinated actions of Cdk6/Cyclin D complex regulate transition and regulation of G_1 phase as well as involved in DNA damage response and repair, chromosome stability and cellular migration [158].

In addition to the interaction between mitotic related members of Cdk family, Elk-1 has been shown to interact with neuronal-specific kinase, Cyclin-dependent kinase 5. Cdk5 is a neural-specific member of Cyclin dependent kinase family and play key roles during the development of central nervous system. It is termed as an atypical cyclin-dependent kinase since it has not enhanced cell cycle specific functions as other members of Cdk family, but it is known that Cdk5 implicated in the suppression of cell cycle entry, which induces neuronal death. It functions in neuronal migration and differentiation, axon growth, synaptic plasticity, neurotransmission, cytoskeletal protein phosphorylation, neurite outgrowth, microtubule stability and axonal transport, neuronal migration and cortical lamination, cognitive functions and neuronal survival [159] Dysregulation of Cdk5 activity is associated with neuronal toxicity and therefore it is associated with numerous neurogenerative disorders such as ALS, Alzheimer disease (AD) and Parkinson's disease. Interestingly, Threonine 417 phosphorylation of Elk-1 is also related to neurodegenerative diseases and Cdk5 has been reported as the kinase that phosphorylates Elk-1 by this residue [135].

Since the activity of mitotic kinases are crucial for successful completion of the mitotic processes and accurate segregation of chromosomes into daughter cells, these kinases must be well-coordinated. Dysregulation of these mitotic kinases causes genome instability, and which is strongly associated with many cancer types. Since Elk-1 interacts with Aurora A, Aurora B, the members of Cdk family, Cdk1, Cdk2, Cdk6 and Cdk5, and Plk1, it is also likely to be involved in these processes. Not surprisingly, the disturbance of the Elk-1

function may be seen in the development and progression of various cancers as well as in neuronal disorders.

In a previous study, Elk-1 was shown to interact with Aurora-A in the first stages of mitosis [7]. To analyze whether similar stage-dependent interaction is true for other kinases, SH-SY5Y cells were arrested with nocodazole for 16 hours, after which cells were released into mitosis. Parallel to previous reports, Aurora A was found to interact with Elk-1 only at time 0 and 30 min, but not at 60 or 90 min, however, Aurora B interaction was present at all time points investigated (Figure 5.12). Some Plk interaction was visible at the onset of mitosis (0 min), but not at other time points analyzed, whereas a very low level of Cdk interaction was found at all time points albeit not to the same extent as found in asynchronous cells.

Serine 383 and Serine 389 phosphorylations have been largely studied and have shown to be important for its target DNA binding along with its transcriptional partner, SRF [137]. The N-terminal of Elk-1 harbors the consensus ETS DNA binding domain (residues 1-86) overlapping with Nuclear Localization Signal (NLS), and the SRF interacting B domain (residues 148-168) [20]. In the central segment of the protein, there is the repression domain (residues 230-260), where SUMOylation of K230, K249 and K254 was previously shown to be critical to the repression of Elk-1 activity, its nucleocytoplasmic shuttling, and PC12 differentiation [132]. On the C-terminal half of the protein, docking domain (D, residues 312-328) was formerly identified as the ERK/MAPK docking region of Elk-1, along with the FxF motif and the transactivation domain (TAD, or C domain, residues 351-399) was shown to be critical for the transactivation function of Elk-1 upon phosphorylation of Serine 383 and Serine 389 by ERK/MAPK [1].

After demonstration of Elk-1 and mitotic kinases Aurora-A, Aurora B, Plk1 and Cdk1 interact in cells in a mitotic stage-dependent manner, the exact binding sites of these kinases on Elk-1 protein domain was evaluated by GST-pull down assay. A series of Elk-1 deletion constructs as GST fusions, namely GST-Elk-1(1-93), GST-Elk-1(1-205), GST-Elk-1(205-329) and GST-Elk-1(349-328), have been expressed in bacteria and carried out pulldown assays with U87 cell lysates. When pull-down samples were analyzed for the presence of endogenous Aurora-A, Aurora-B, Cdk1, and Plk1, the interaction was only present in GST-Elk-1(1-205) construct (Figure 5.17). This region encompassing amino acids 1 – 205 of Elk-1 harbors the DNA binding ETS domain and was found to contain several binding consensus sites for Aurora A/Aurora B for Plk1 and for Cdk1 and these

predicted binding consensus for mitotic kinases were either overlapping with or in the vicinity of phosphorylation motifs of these kinases. It was therefore quite possible that the interaction with and phosphorylation by one mitotic kinase could affect the binding of another mitotic kinase in turn, which our results in fact indicate. Indeed, such dynamic interactions and inter-dependability have been shown for other protein-protein interactions.

Upon confirming physical interaction between Elk-1 protein and mitotic kinases studied and determining exact binding site on Elk-1 protein sequence, the next question to be addressed was whether the predicted motifs were indeed phosphorylated and whether these motifs were phosphorylated by the mitotic kinases predicted. To that end, custom antibodies have been raised against selected phosphorylation sites predicted, P-S106, P-T108, P-T133, P-S198, P-T199, P-S200, P-S202, P-S303, P-S304, P-S324 and P-S326-Elk-1. Initially, dot blots were performed to show specificity of the custom antibodies for phospho-peptides but not unmodified peptides, and in cell lysates. After this preliminary study, the antibodies that gave a specific signal in an increasing number of lysates were further used in Western blots to detect various phospho-Elk-1 species in U87 cells. Our results show the presence of P-S106-, P-198, -P199, -P200, P-S202-, P-S303-, P-S304-, P-S326-Elk-1 species in increasing amounts of cell lysates. On the other hand, P-T108-Elk-1, P-T133-Elk-1 and P-S324-Elk-1 antibodies did not yield a significant or specific signal (Figure 5.21). However, when cells were nocodazole-arrested and thereafter released into mitosis, differences in the mitotic stage-dependent expression of these phosphoforms were evident: P-S106-, P-T133, P-T199, P-S200, P-S202 (albeit very faint) and P-S304-Elk-1 antibodies gave positive result in the first stages of mitosis, whereas P-S326-Elk-1 signal was more intense in the second half of mitosis (Figure 5.24). In contrast, P-T108-, P-S198, P-S303-Elk-1 did not show a mitotic stage-dependent expression profile, and P-S324-Elk-1 band was visible only at 30 min after release from nocodazole arrest. It would be interesting to analyze whether these antibodies can indeed be used in patient-derived brain tumor samples and whether they can possess a diagnostic value.

It was previously demonstrated that during interphase, Serine 383 phosphorylated Elk-1 is found at the nucleus, whereas, through prophase to metaphase, predominantly localizes to the spindle poles. In anaphase, Elk-1 accumulates both at the spindle poles and midzone, relocates to the spindle midbody when the cells proceed to the cytokinesis. Serine to alanine mutation of 383 residues did not affect the spindle pole localization of Elk-1, but during telophase this mutant could not translocate to the midzone, remained throughout the

mitotic spindle. It was further demonstrated that, Elk-1 co-localizes with Aurora-A kinase during mitosis and when cells are treated with specific Aurora inhibitors, Elk-1 cannot localize to the poles and remains associated with DNA [6].

In order to see whether peculiar localization observed in Serine 383 phosphorylation of Elk-1 was also valid for other phospho forms, cells were stained with β-tubulin, DAPI and target phospho-antibodies and their localizations during in mitosis was monitored by confocal microscopy. Immunostaining results demonstrated that localization of new phospho-specific antibody of Elk-1 was similar to each other, whereas, some of the antibodies displayed a weak localization signaling and rest with a strong signal. Serine 106 and 324 Elk-1 exhibited a remarkable staining, it was bound to DNA throughout the interphase / prometaphase, then passed to the spindle poles and remained there until the end of cytokinesis (Figure 5.31-32). While the Serine 106 Elk-1 were partially seen in the spindle poles and partly in the midzone, midbody localization of Serine 383 phosphorylation of Elk-1 was not observed in this phospho forms.

To analyze the phosphorylation status of Elk-1, kinase assays were performed and fluorometric kinase assay demonstrated that Elk-1 is being phosphorylated from both of the kinases studies; the fluorometric signal intensity of Aurora A and Aurora B was quite high which signs a strong phosphorylation relation between Elk-1 and these kinases. It was found that Cdk1 and Plk1 are also phosphorylating Elk-1, although the ATP to ADP conversion, which is an indication of phosphorylation, of Cdk1 and Plk1 kinases was not as strong as Aurora kinases (Figure 5.53). Phospho-specific peptides were used to further analyze the phosphorylation sites of Elk-1 by target kinases. Peptide analyses revealed that Elk-1 is able to be phosphorylated from Threonine 108 and Serine 324 by Plk1, Threonine 199, Serine 200 and Serine 304 by Aurora A and B, Threonine 133, Serine 202, Serine 303, Serine 304 and Serine 324 by Cdk1 as illustrated in Figure 6.1. This data is consistent with a report performed by Sharma *et al*. showed the Threonine 133 and Serine 200 regions of Elk-1 may be target sites for phosphorylation but did not demonstrate a target kinase for the phosphorylation of these residues [135]. In a similar study mapped 778 phosphorylation sites on 562 proteins as mitotic substrates of Plk1 and Aurora-A kinase during mitosis, Elk-1-Ser304 and Elk-1-Ser324 phosphorylations being among them [149] and a quantitative mitotic phosphoproteome analysis has confirmed Elk-1 Ser 304, Ser324 and Ser326 phosphorylations to be specific for mitosis [155]. Our kinase assay data demonstrated that these residues are indeed phosphorylated by target kinases, but Serine

326 residue is not targeted by these kinases according to the fluorometric kinase assay. This indicates that Serine 326 residue may also be phosphorylated by kinases other than Cdk1 and Plk1.

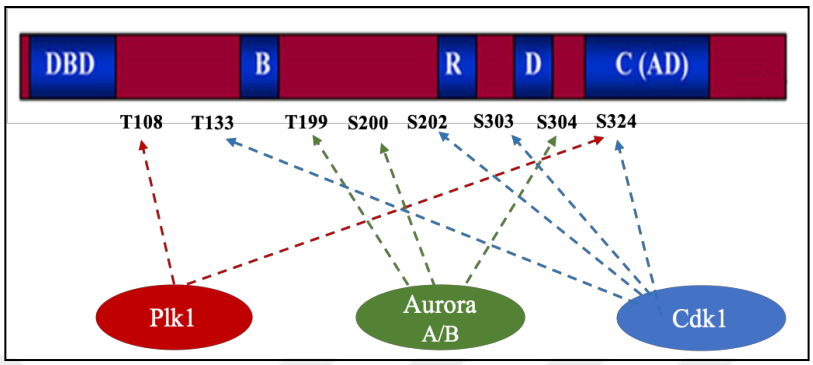


Figure 6.1. Elk-1 motifs phosphorylated by Aurora A/B, Cdk1 and Plk1 kinases. Elk-1 is found to be phosphorylated by Aurora A/B, Cdk1 and Plk1 kinases from indicated residues.

It has been shown that Elk-1 interacts with mitotic kinases in a mitotic stage-dependent manner, that different phospho-species of Elk-1 are detected in cell lysates in different stages of mitosis, and that mitotic kinase inhibitors do indeed affect the mitotic localization of P-S383-Elk-1 species, none of which in any way show whether phosphorylation of Elk-1 on these residues are important in mitotic progression. To address whether these specific phosphorylation motifs are directly relevant to the mitotic profile of cells, different phospho-mutants were overexpressed in U87 cells, nocodazole-arrested and synchronized the cells, and then released the cells into mitosis and carried out flow cytometry analysis. For this set of experiments, S303A, S304A, S324A and S326A phospho-mutants of Elk-1 were focused, which are mutants of residues identified as mitotic kinase Plk1 or Aurora kinases in previous phosphoproteome studies [160] and compared them to S149A-Elk-1 phospho-mutant and asynchronous cell population. When S149A mutant is overexpressed in cells after mitotic arrest (S149A, 0 min) and then analyzed 30, 60 and 90 min after release, the G_2 population was found to shift to the right, yielding a similar mitotic profile to the asynchronous cells (Figure 5.71). In overexpression of S303A and S326A mutants, the mitotic arrest was not prominent, and release into mitosis was delayed, whereas in overexpression of S304A and S324A mutants the mitotic arrest was similar to S149A mutant, however, there was almost no release into mitosis.

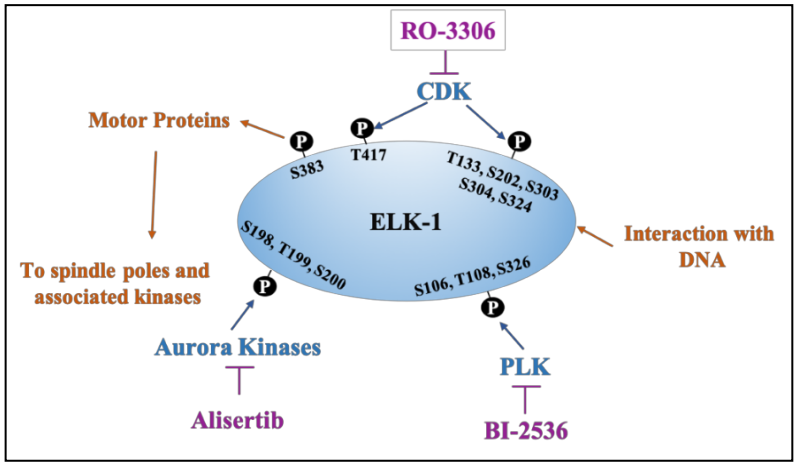


Figure 6.2. Phosphorylation of Elk-1 by mitotic kinases and the resulting interactions and effects. It is believed that Aurora A, Elk-1-S198, T199 and S200, Cdk1, Elk-1-T133, S202, S303, S304, S326 and T417, and Plk1, Elk-1-S106, T108 and S326, kinases phosphorylating Elk-1 on certain residues. Activity of Aurora A, Cdk1 and Plk1 kinases can be inhibited by specific kinase inhibitors, Alisertib, RO-3306 and BI-2536, respectively.

Targeting mitosis for anti-cancer therapy is an increasingly prominent strategy for the treatment of many cancers and kinase inhibitors, especially, are on the focal point of this therapy. Currently, more than 20 oncology drugs that selectively inhibit mitotic kinases have been approved and many of them are in various stages of clinical trials. As depicted in Figure 6.2, examples of such inhibitors include Alisertib, BI-2536 and RO-3306 that selectively inhibits the activity of Aurora A, Plk1 and Cdk1, respectively [150]. Our results indicate that specific mitotic-stage dependent phosphorylations of Elk-1 may indeed be important for mitotic progression, hence also open new avenues for potential brain tumor targets. In addition to these mitotic kinase-based therapies, the development of new drugs to inhibit the interaction between Elk-1 and mitotic kinases may be a new strategy to be used against these cancers.

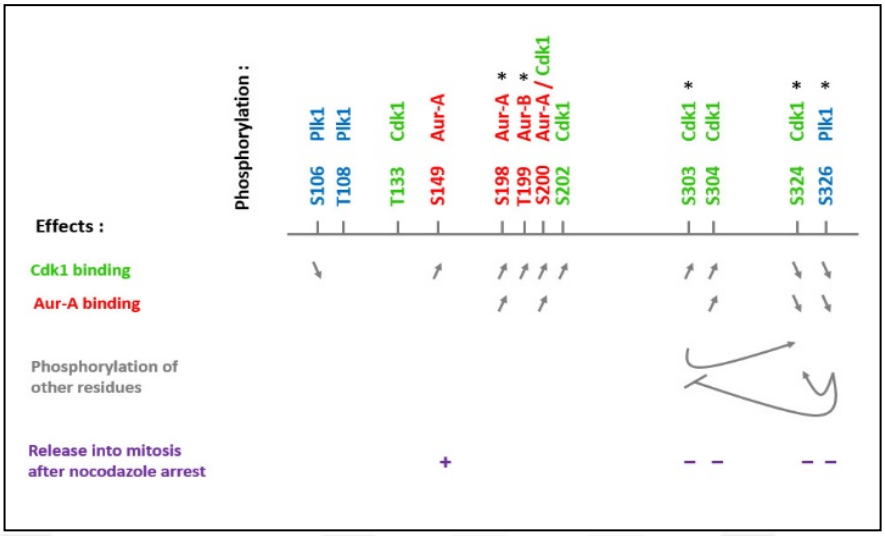


Figure 6.3. Schematic diagram of the effects of various phosphorylation sites on mitotic kinase binding Phosphorylated residues and the responsible mitotic kinases are indicated, where blue font denotes Plk1 phosphorylation (S106, T108 and S326), green font denotes Cdk1 phosphorylation (T133, S202, S303, S304, S324), and red denotes Aurora A and Aurora B phosphorylation (S149, S198, T199, S200); stars indicate phosphorylations confirmed by multiple approaches; On the left, effects of these phosphorylations (on Cdk1 and Aurora A binding, cross-phosphorylation of other residues, and release into mitosis) are listed; arrows pointing up indicate increase, arrows pointing down indicate decrease in binding; plus (+) indicates normal mitotic release profile, minus (-) indicates defective mitotic release profile.

Phosphorylation of S303, S324 and S326 residues of Elk-1 has been analyzed in the presence of phosphomimic or phospho-mutant overexpression, focusing on the phosphorylations previously identified in phosphoproteome analyses. In this experiment it was observed that when S303E-Elk-1 was overexpressed in cells, a modest increase in P-S303-Elk-1 antibody signal was observed as compared to S303A-Elk-1 overexpression, however P-S324-Elk-1 staining was similarly increased (S304A and S304E overexpression data were inconclusive due to a consistent mobility shift in S304E lysates; S324E-Elk-1 overexpression resulted in significantly higher P-S324-Elk-1 staining as compared to S324A-Elk-1 overexpression, confirming the specificity of the antibody, however, no significant alterations were observed in these mutants for either P-S303- or P-S326-Elk-1 staining, indicating this S324 residue does not significantly affect neighboring residue as illustrated in Figure 6.3. Consistent with previous results, however, overexpression of S326

phosphomimetic mutant, while yielding enhanced P-S326-Elk-1 staining as well as a modest increase in P-S324-Elk-1 band in cell lysates, resulted in decreased P-S303-Elk-1 staining, indicating a selectively negative regulatory function for this phosphorylation (Figure 5.41).

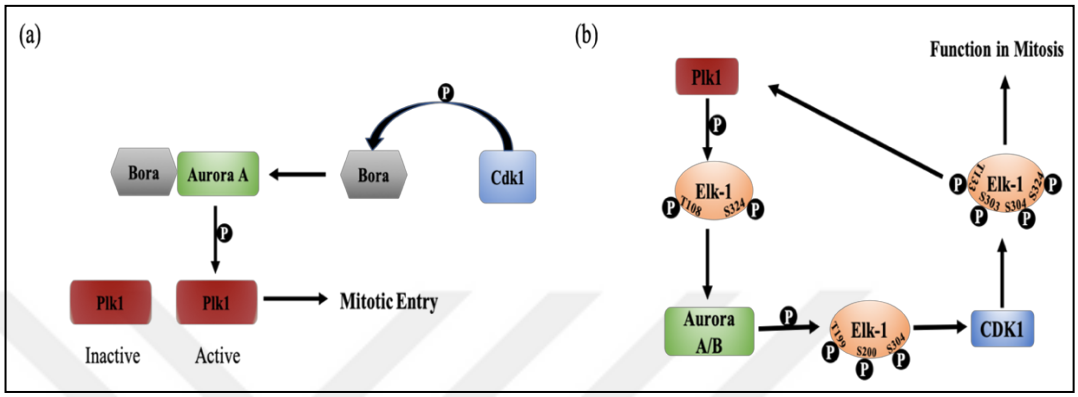


Figure 6.4. Dynamic phosphorylation relations during mitosis. Activation of Plk1 kinase by concurrent phosphorylations by Cdk1 and Aurora A kinases is required for mitotic entry (a). Such a dynamic phosphorylation dynamic may be viable for Elk-1 during mitosis (b).

Dynamic phosphorylation relations have been previously demonstrated for numerous proteins during biological processes. For instance, mitotic entry of a cell is regulated by concurrent interactions between Cdk1, Plk1 and Aurora kinases. As demonstrated in Figure 6.4a, Bora protein is phosphorylated by Cdk1 and this phosphorylation induce binding of Bora to Aurora A kinase. After Bora binding, Aurora A phosphorylates and activates Plk1to regulate mitotic entry [162]. Our results support the idea such a dynamic phosphorylation pattern may be viable for Elk-1 since binding of and phosphorylation by one mitotic kinase (positively or negatively) affects binding of another mitotic kinase. This would also explain how Elk-1 gets differentially phosphorylated in each step of mitosis. Phosphorylation of one residue may direct Elk-1 to another target kinase which in turn phosphorylate Elk1- and direct it to one another as illustrated in Figure 6.4b. Indeed, mitotic phosphoproteome studies have determined quite a number of mitosis-dependent phosphorylations, including 304, 324 and 326-Elk-1 phospho-species [149]. We propose that such sequential and highly regulated phosphorylations of Elk-1 is necessary for normal mitotic progression and that any disturbance in the mitotic phosphorylation homeostasis, hence of mitotic Elk-1 phosphorylations, would result in defective mitosis, as previously shown by Neumann et al. [137].

7. CONCLUSION

Mitosis is a well-orchestrated part of the cell cycle, and mitotic kinases, most notably Cdks, are key regulators of cell cycle progression; mitotic kinase Plk1 is required for mitotic entry, centrosome maturation, microtubule dynamics, and cytokinesis [161] and is itself known to require Aurora-A trigger; Aurora A and B, in return, is required for centrosome separation and maturation as well as cytokinesis [162]. It is therefore interesting that a transcription factor, Elk-1, harbors putative phosphorylation motifs for these mitotic kinases and colocalizes with Aurora-A during different phases of mitosis [7].

In this study, the interaction of Elk-1 with mitotic kinases Aurora A, Aurora B, Plk1 and Cyclin-dependent kinase family members, Cdk1, Cdk2, Cdk5, was shown, and all the putative phosphorylation motifs were analyzed in detail. It has been shown that S106 and S326 Plk1 motifs, as well as S324 Cdk1 phosphorylation motif, was inhibitory for Cdk1 binding, whereas S149, S198, S200 Aurora A motifs, T199 Aurora B motif, as well as S202, S303 and S304 Cdk1 motif is enhancing binding of Cdk1 kinase to Elk-1 transcription factor, whereas S324 Cdk1 motif and S326 Plk1 motif is inhibitory and S198, S200 Aurora A motifs are enhancing for Aurora-A binding to Elk-1.

Such mechanisms for cross-regulation of mitotic kinases has previously been shown in other contexts, Cdk1, for example, was shown previously to phosphorylate an Aurora A cofactor, which in turn facilitates binding to Plk1, triggering Aurora A-mediated activation of Plk1, while Plk1 inactivation resulted in decreased Aurora A phosphorylation in return [163]. In mitotic stage-dependent immunoprecipitation experiments, it has also been shown that the stage-specific interaction of these mitotic kinases with Elk-1 – Plk1 specifically interacting with Elk-1 at the onset of mitosis, while Aurora-A interacting with Elk-1 in the first half of mitosis; Aurora-B appears to interact with Elk-1 throughout mitosis, albeit in fluctuating levels, and Cdk1 is interacting with Elk-1 at extremely low levels throughout mitosis.

Cross-regulation of mitotic kinases has been shown to be critical for dynamic phosphorylation of substrates in mitosis. In a previous study, it was shown that Cdk1 and Erk2 phosphorylated a common substrate, Cep55, which was prerequisite for subsequent phosphorylation of an adjacent motif by Plk1 [164]. Using phosphor-specific antibodies and phosphomutants of Elk-1, we have studied mitotic stage-dependent phosphorylations of Elk-1 and whether the dynamic interaction of Elk-1 with mitotic kinases and subsequent

phosphorylation of one motif may affect another phosphorylation motif of a different kinase. Regarding cross-regulation of phosphorylation motifs, we have concentrated on S303, S304, S324 and S326 phosphomotifs, based on the mitotic phosphoproteomic results reported in the literature, where these particular phosphorylations of Elk-1 were identified among mitotic phosphoproteomes of Plk1, Aurora or Cdk1 kinases [155]. Cdk1 phosphorylation motif S303 was observed to increase phosphorylation of S324 residue; likewise, Plk1 motif S326 was observed to increase phosphorylation of S324, but inhibit phosphorylation of S303, indicating an interplay of mitotic kinases on these Elk-1 motifs previously shown to be phosphorylated in mitotic phosphoproteomes. The phosphomutants were overexpressed in U87 cells, nocodazole-arrested, synchronized and released into mitosis to see whether these four specific motifs observed in phosphoproteomes had an effect on mitotic progression. S149 Aurora A motif, which had not previously been identified in mitotic phosphoproteomes, was used as a control. Whereas Elk-1-S149A mutant was released normally into mitosis and restored normal cell cycle profile, all other mutants studied, namely S303A, S304A, S324A and S326A, was found to exhibit defects either in mitotic arrest or in the release into mitosis.

At this point, it is important to note that Aurora-A is overexpressed in glioma cells, and that its expression is reported to be correlated with patient outcome [165] and that PLK1 activity was elevated in CD133+ glioblastoma multiforme cells [166]. Together with the previous reports that Elk-1 is highly phosphorylated in brain tumor cells [167], it is interesting to further study the levels of the mitotic kinase-dependent phosphorylations of Elk-1 in brain tumor patients in different stages as compared to healthy controls. Such mitotic kinase phosphorylations of Elk-1 may present both as diagnostic markers for brain tumorigenesis, and also as a novel target for interfering with brain tumor proliferation and progression.

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APPENDIX A: SEQUENCE SCREENING OF MUTAGENIC PLASMIDS

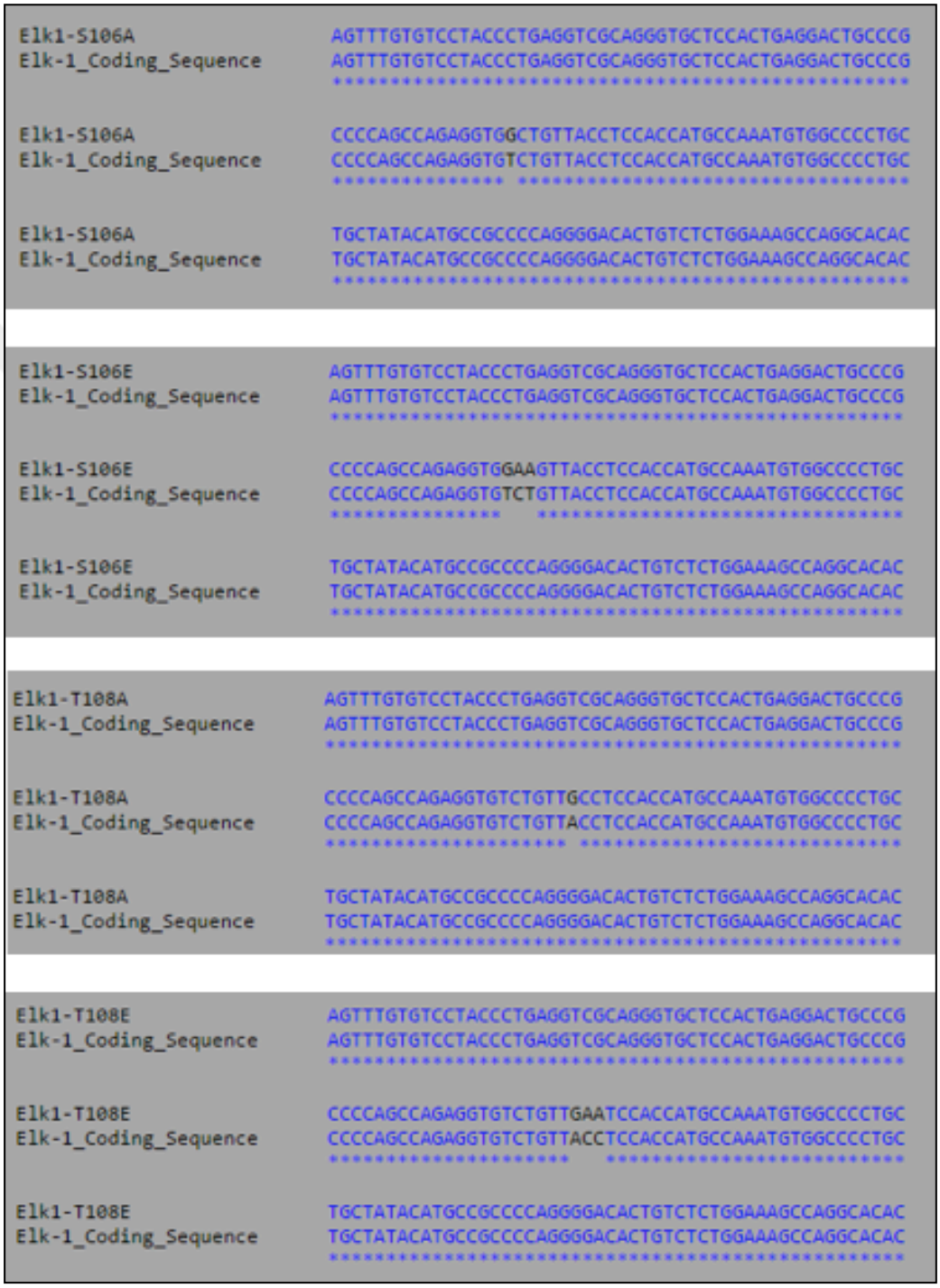

Figure A.1. Sequence screening of plasmids. Black nucleotides correspond to unmatched parts that indicates mutation sites.

Figure A.1. Continued.

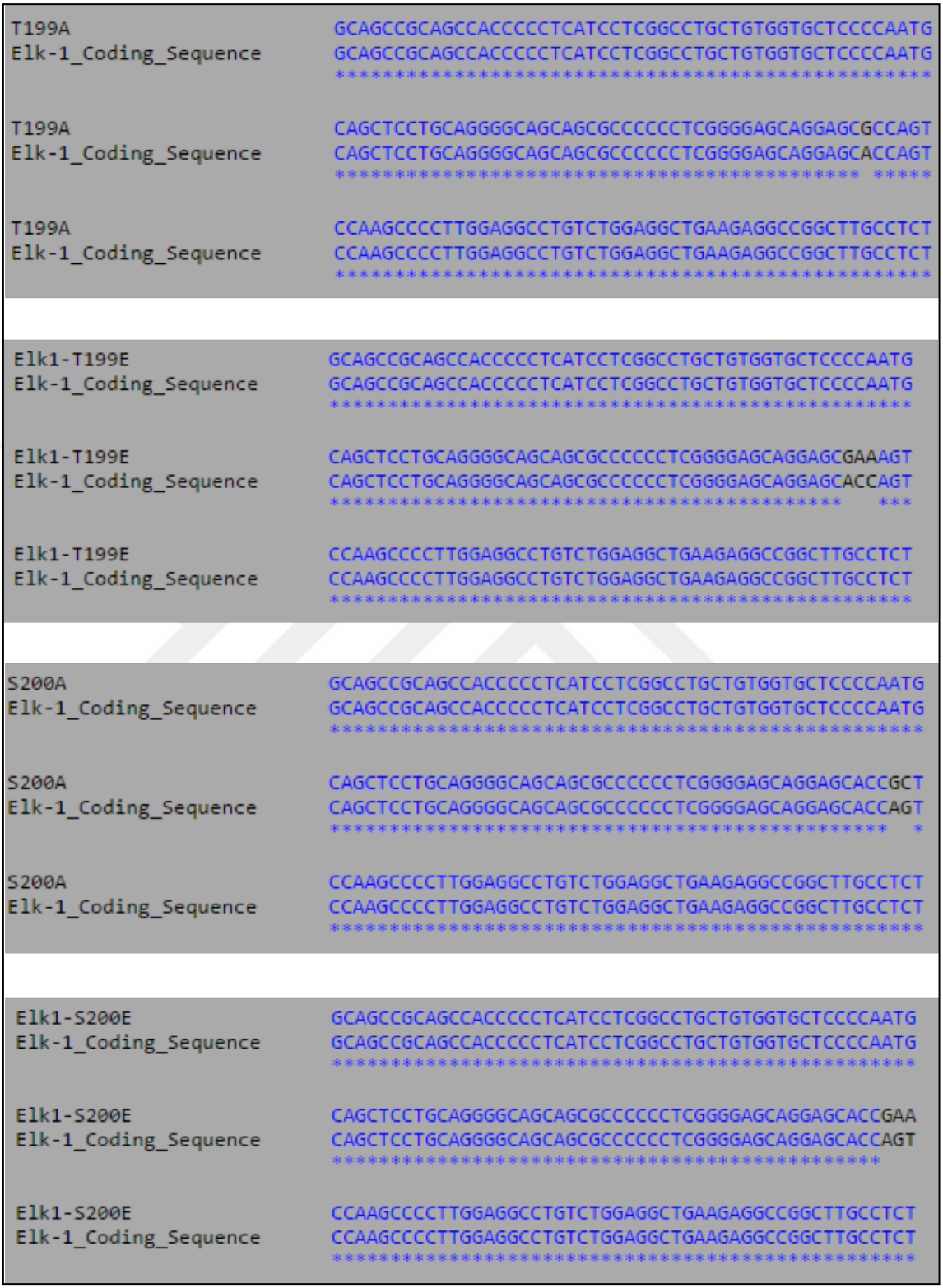

Figure A.1. Continued.

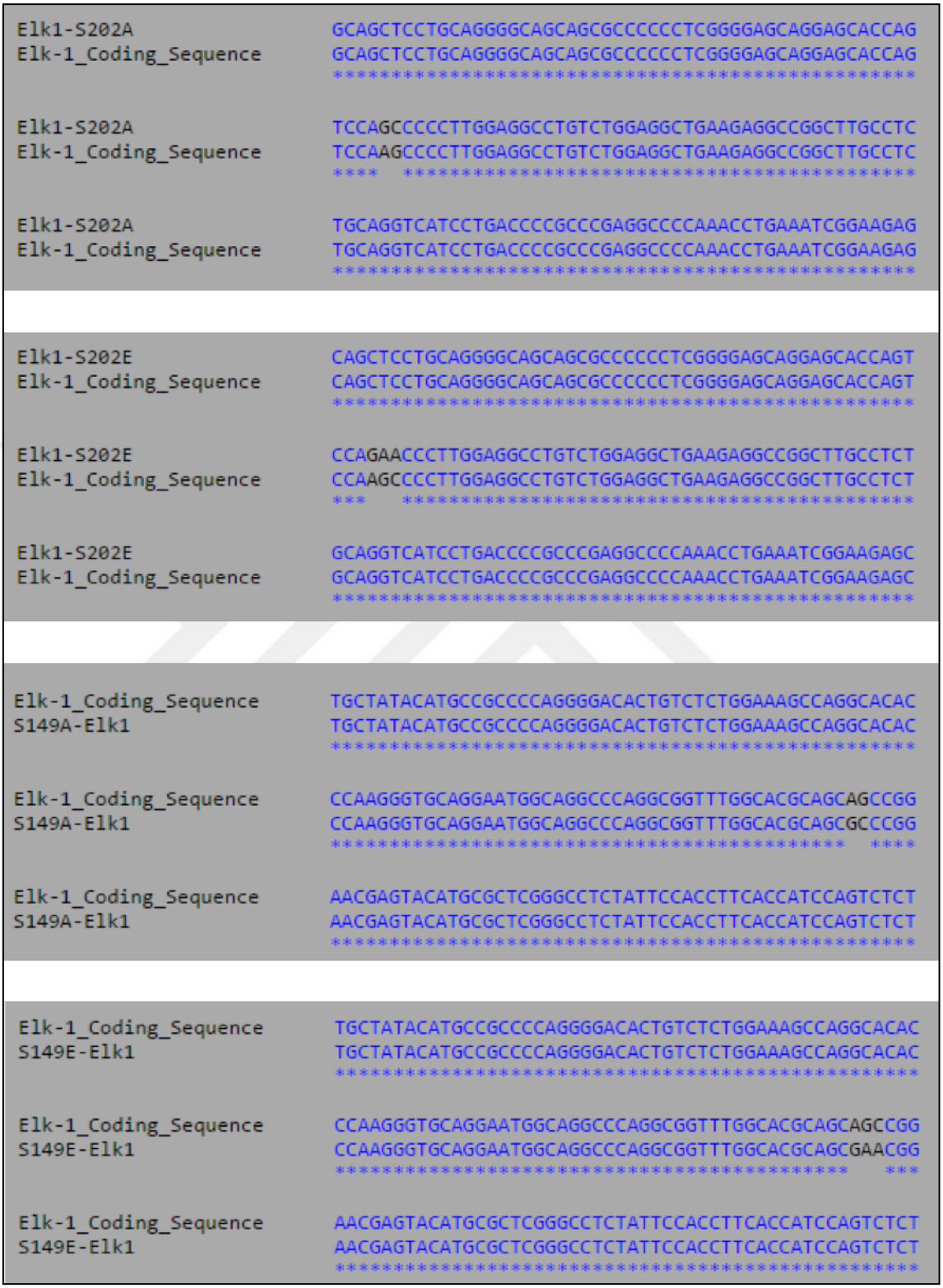

Figure A.1. Continued.

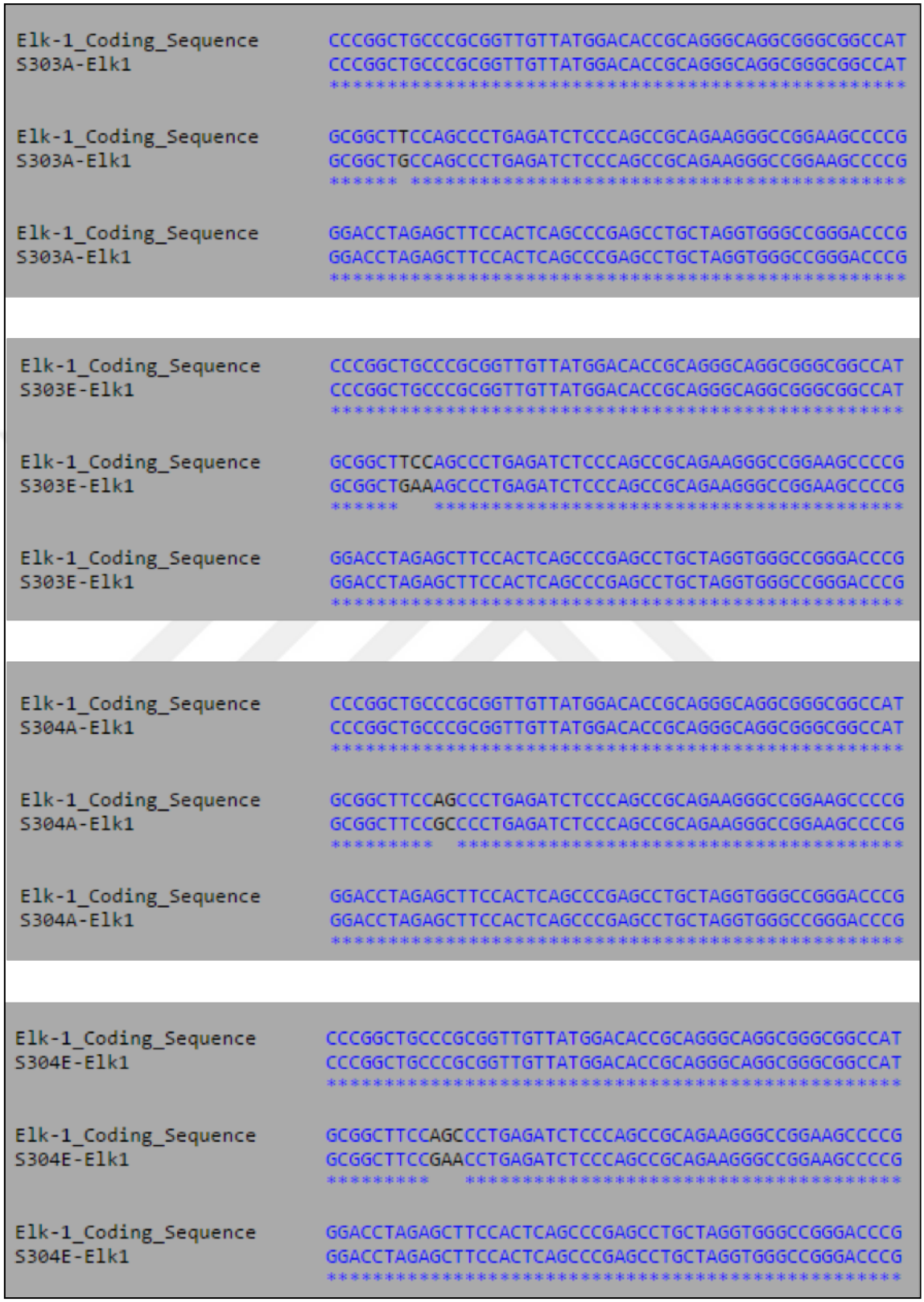

Figure A.1. Continued.

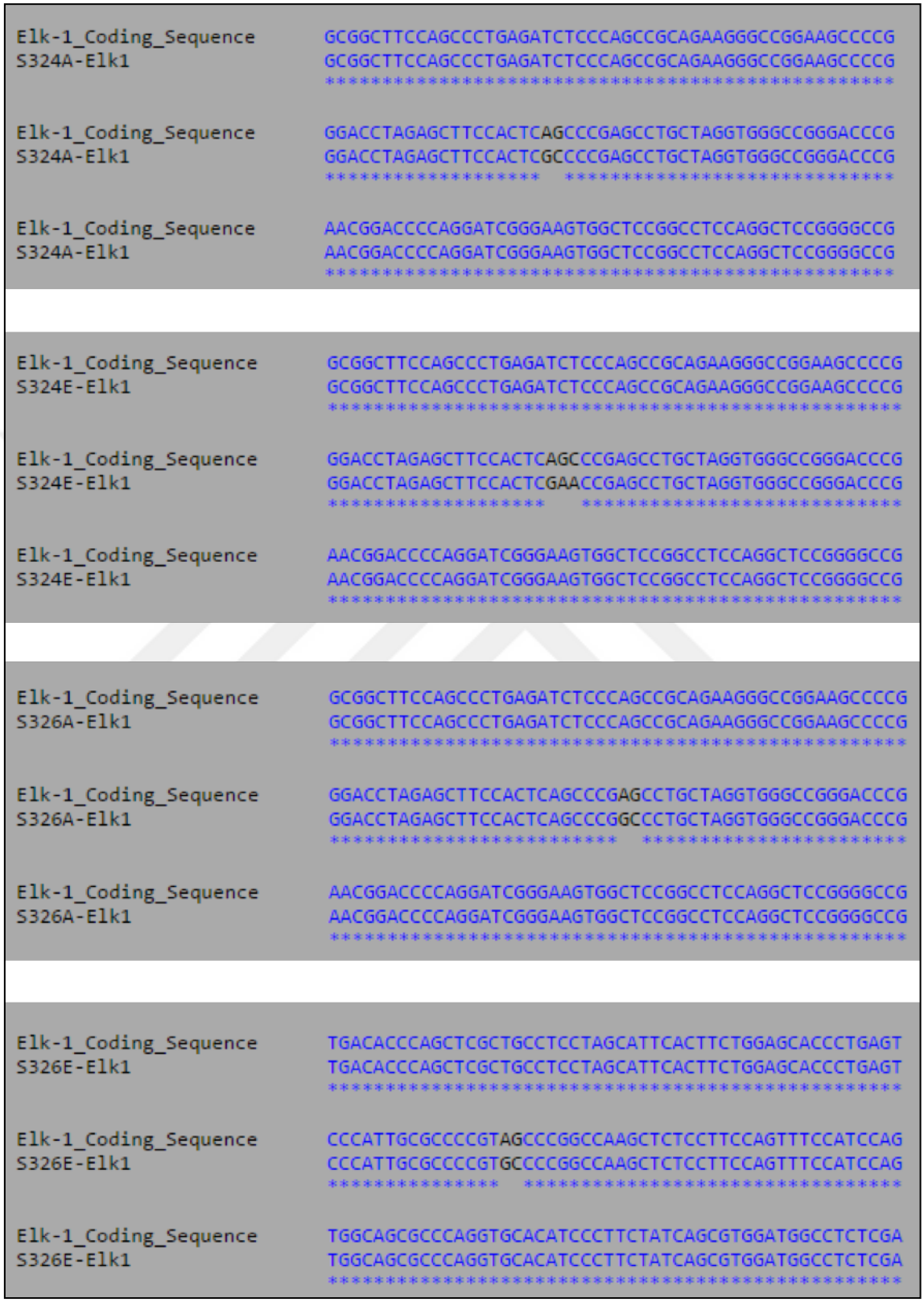

Figure A.1. Continued.

Figure A.1. Continued.

APPENDIX B: MITOTIC LOCALIZATION OF ELK-1

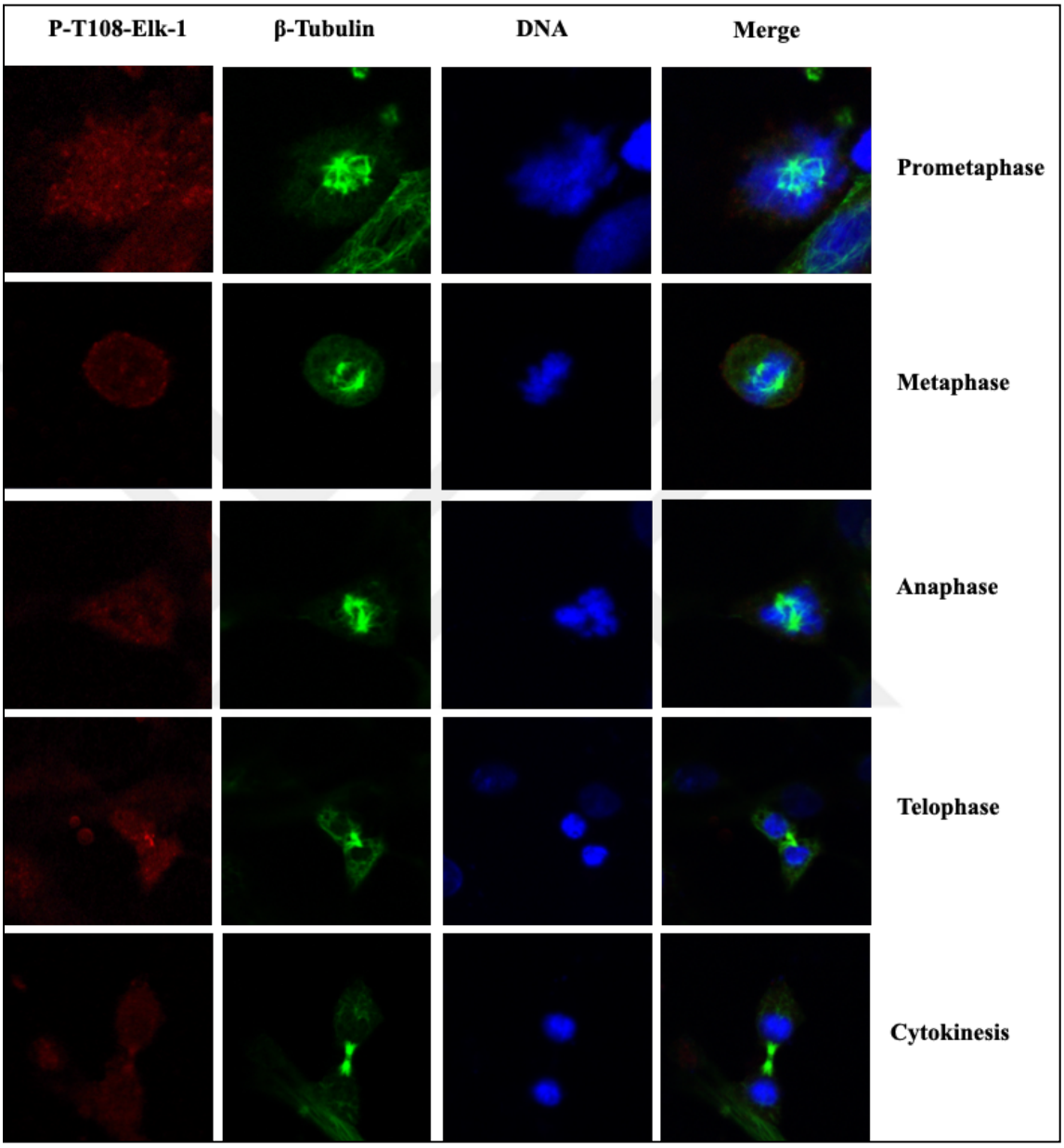

Figure B.1. The localization of phospho-T108-Elk-1 during different stages of the cell cycle in U-87 cell lines (anti-P-S198-Elk-1: red; anti-β-tubulin: green, DNA: blue).

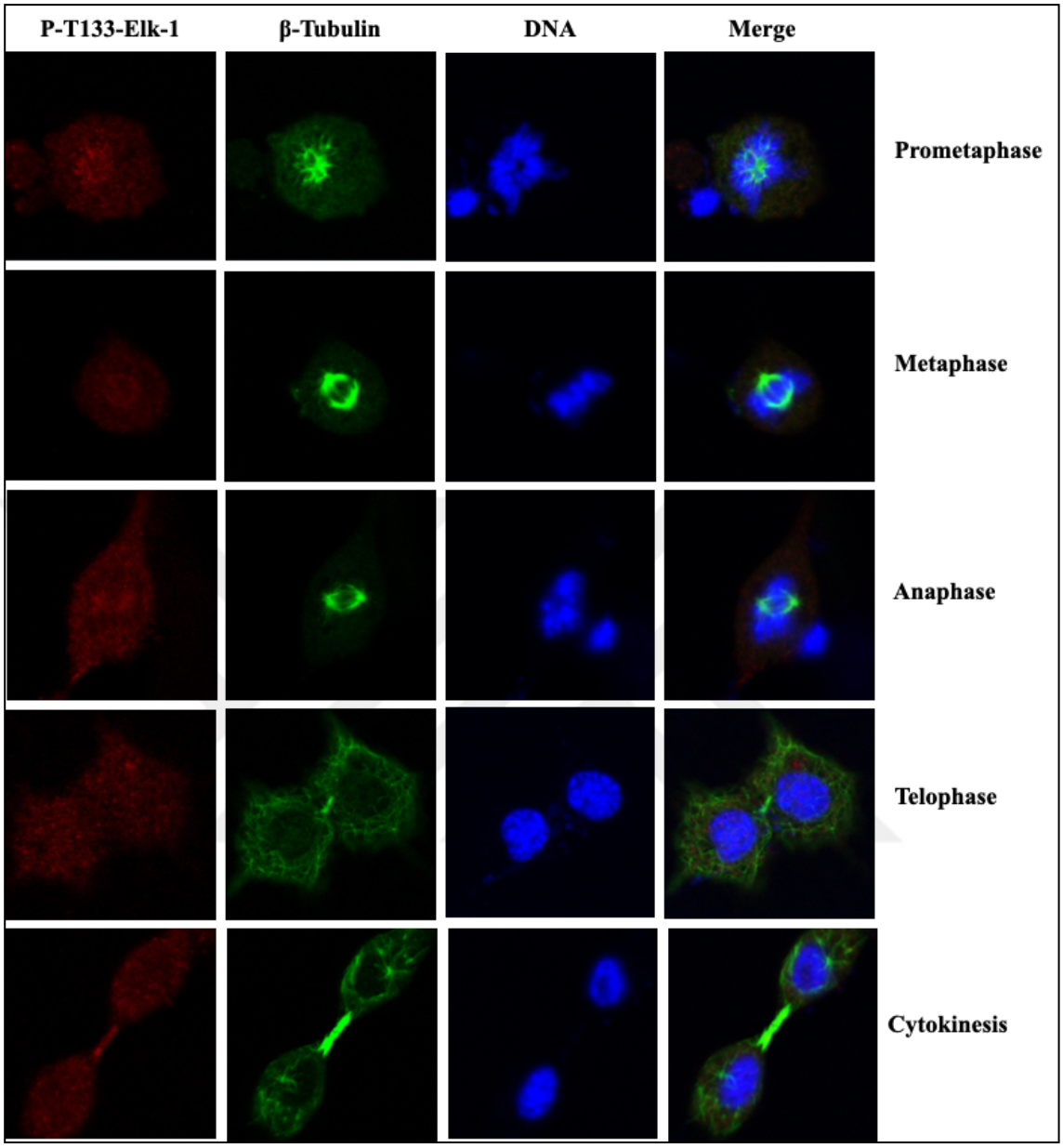

Figure B.2. The localization of phospho-T133-Elk-1 during different stages of the cell cycle in U-87 cell lines (anti-P-S198-Elk-1: red; anti-β-tubulin: green, DNA: blue).

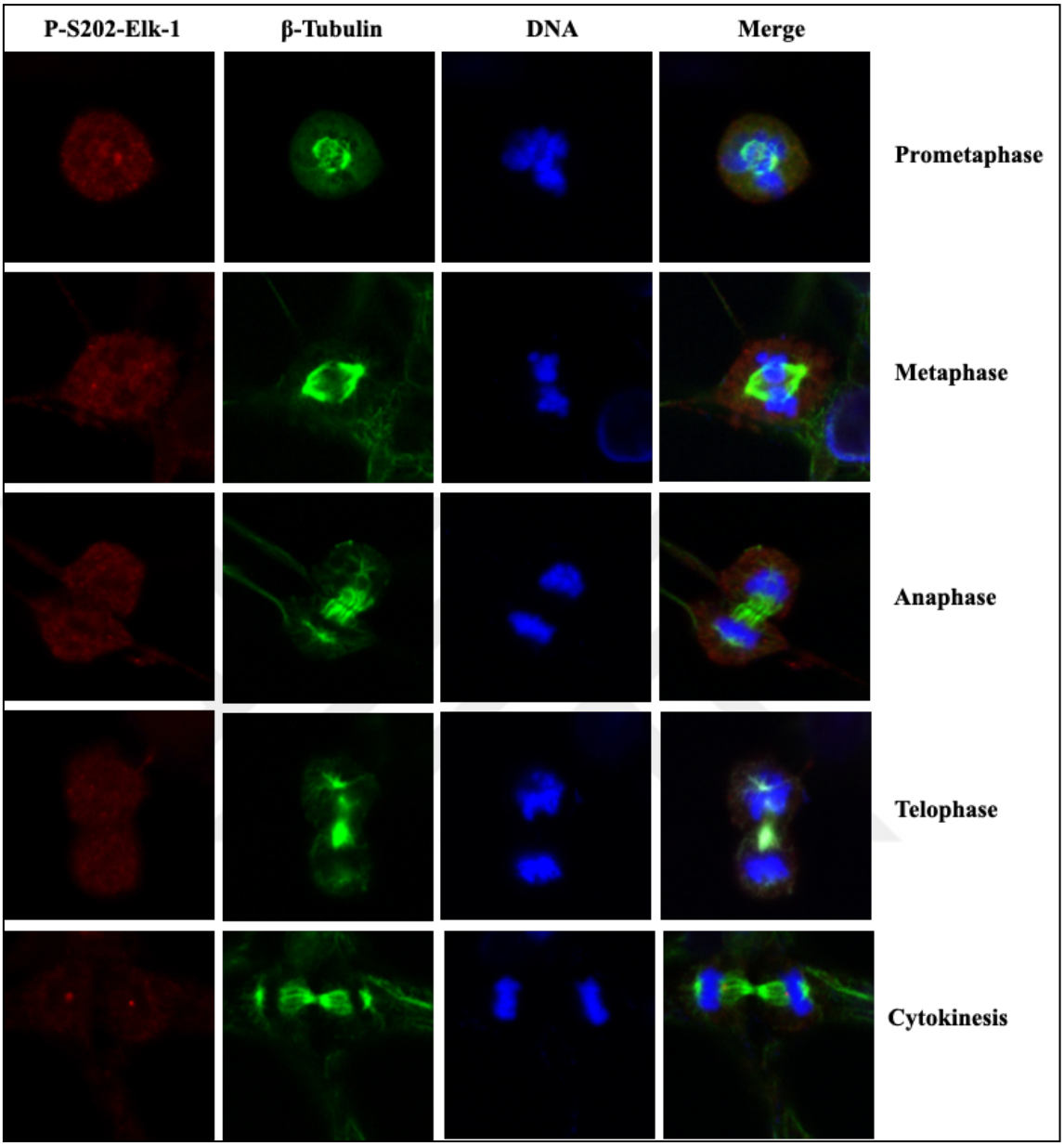

Figure B.3. The localization of phospho-S202-Elk-1 during different stages of the cell cycle in U-87 cell lines (anti-P-S198-Elk-1: red; anti-β-tubulin: green, DNA: blue).

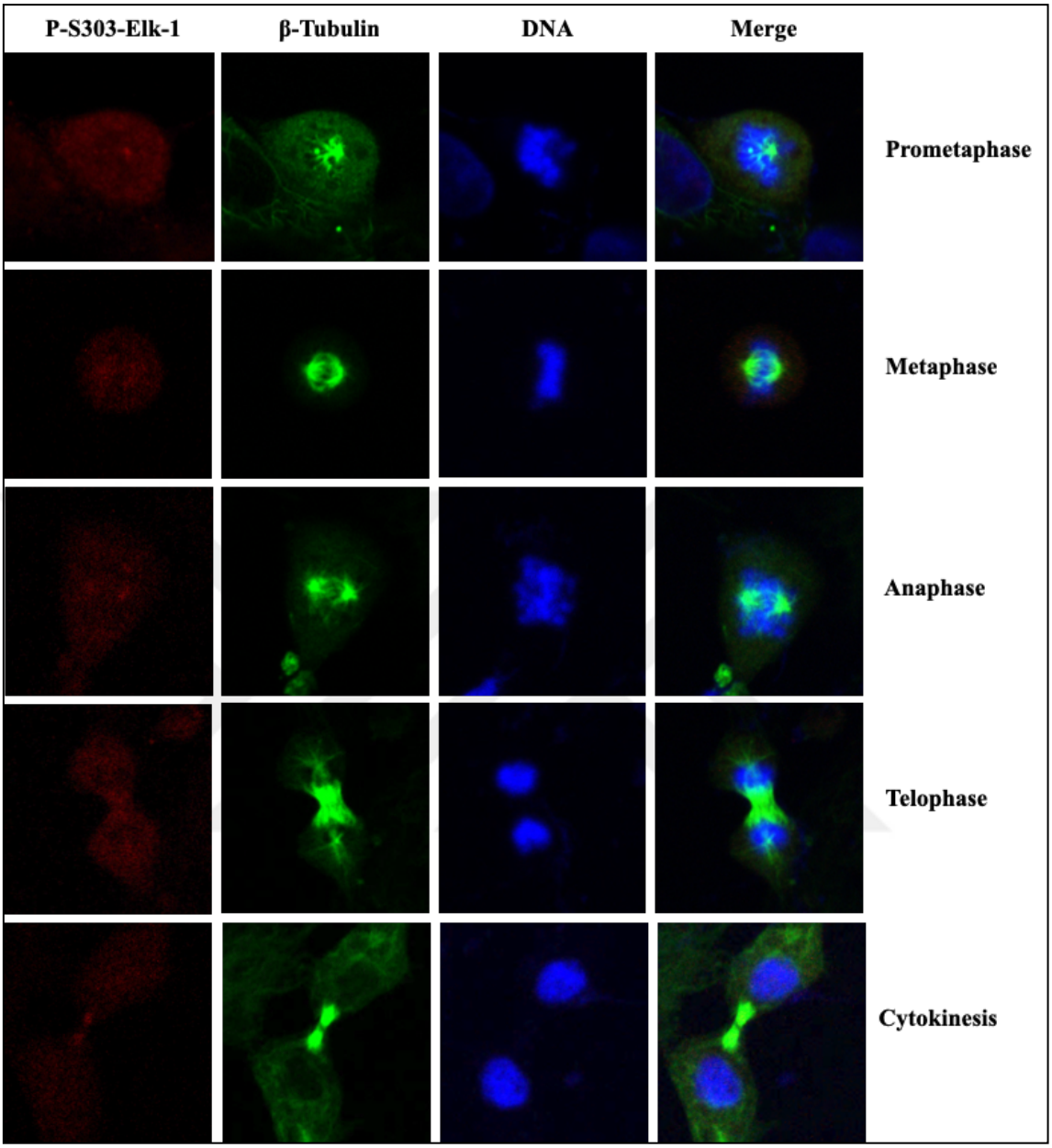

Figure B.4. The localization of phospho-S303-Elk-1 during different stages of the cell cycle in U-87 cell lines (anti-P-S198-Elk-1: red; anti-β-tubulin: green, DNA: blue).

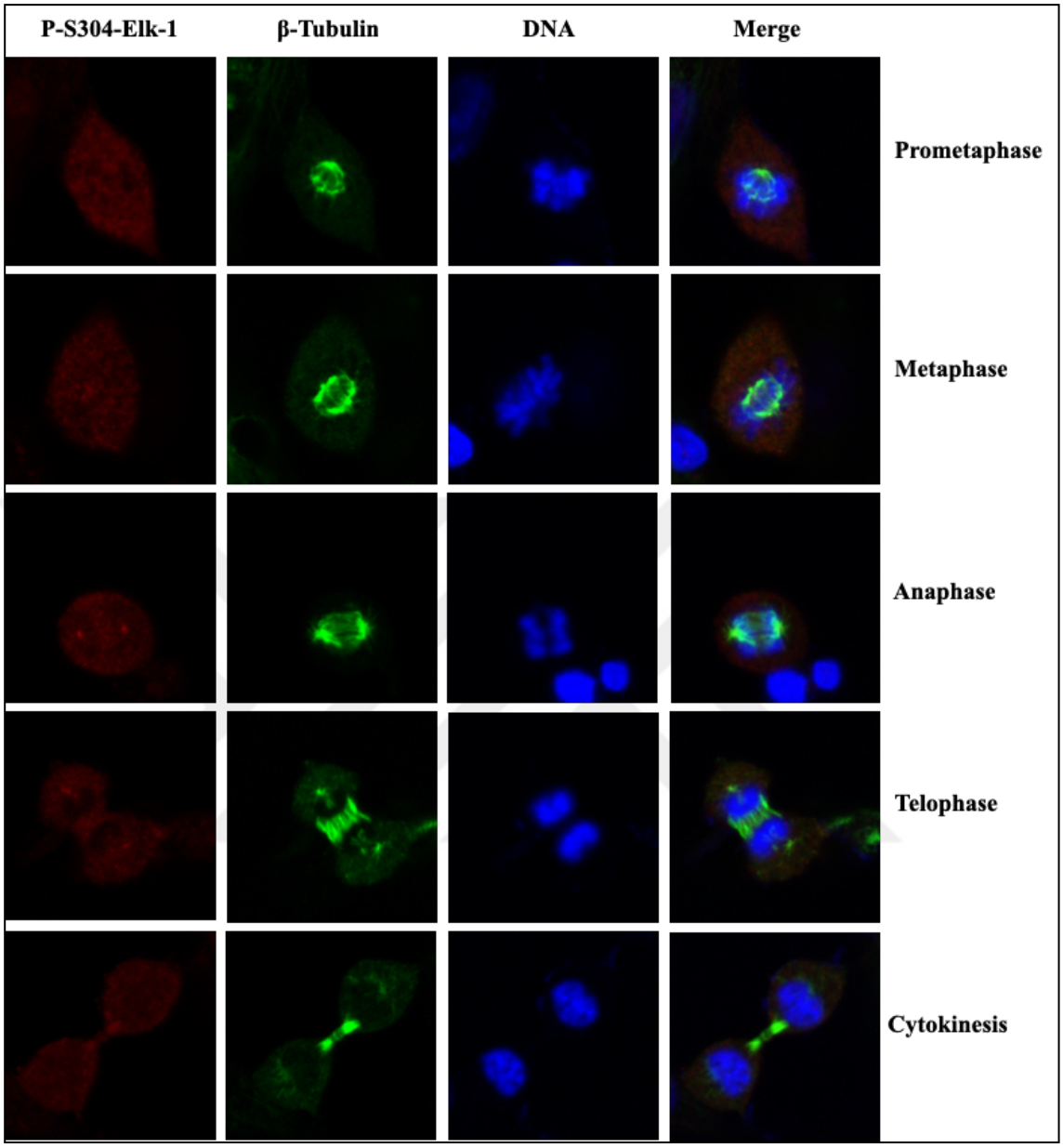

Figure B.5. The localization of phospho-S304-Elk-1 during different stages of the cell cycle in U-87 cell lines (anti-P-S198-Elk-1: red; anti-β-tubulin: green, DNA: blue).

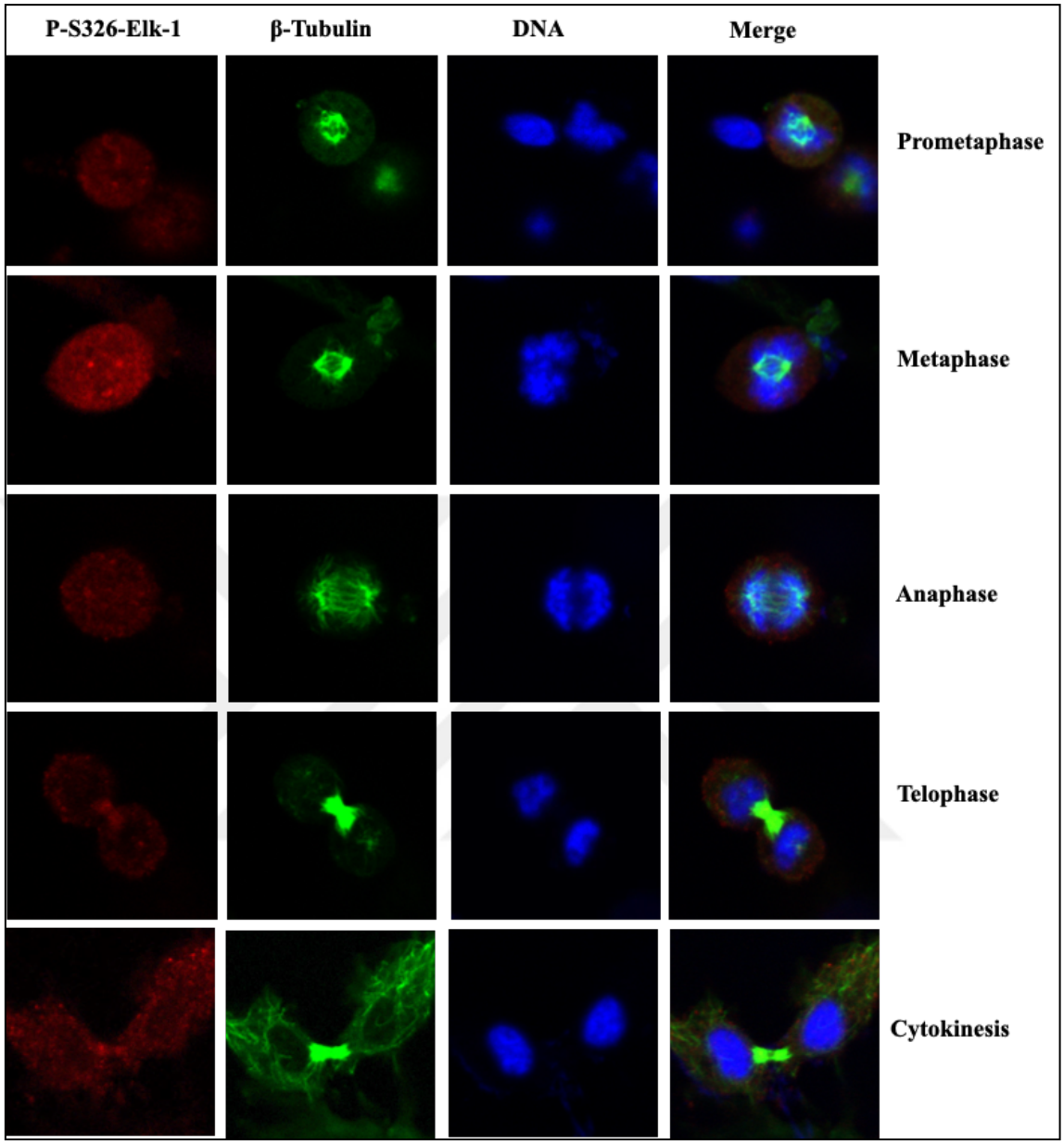

Figure B.6. The localization of phospho-S326-Elk-1 during different stages of the cell cycle in U-87 cell lines (anti-P-S198-Elk-1: red; anti-β-tubulin: green, DNA: blue).

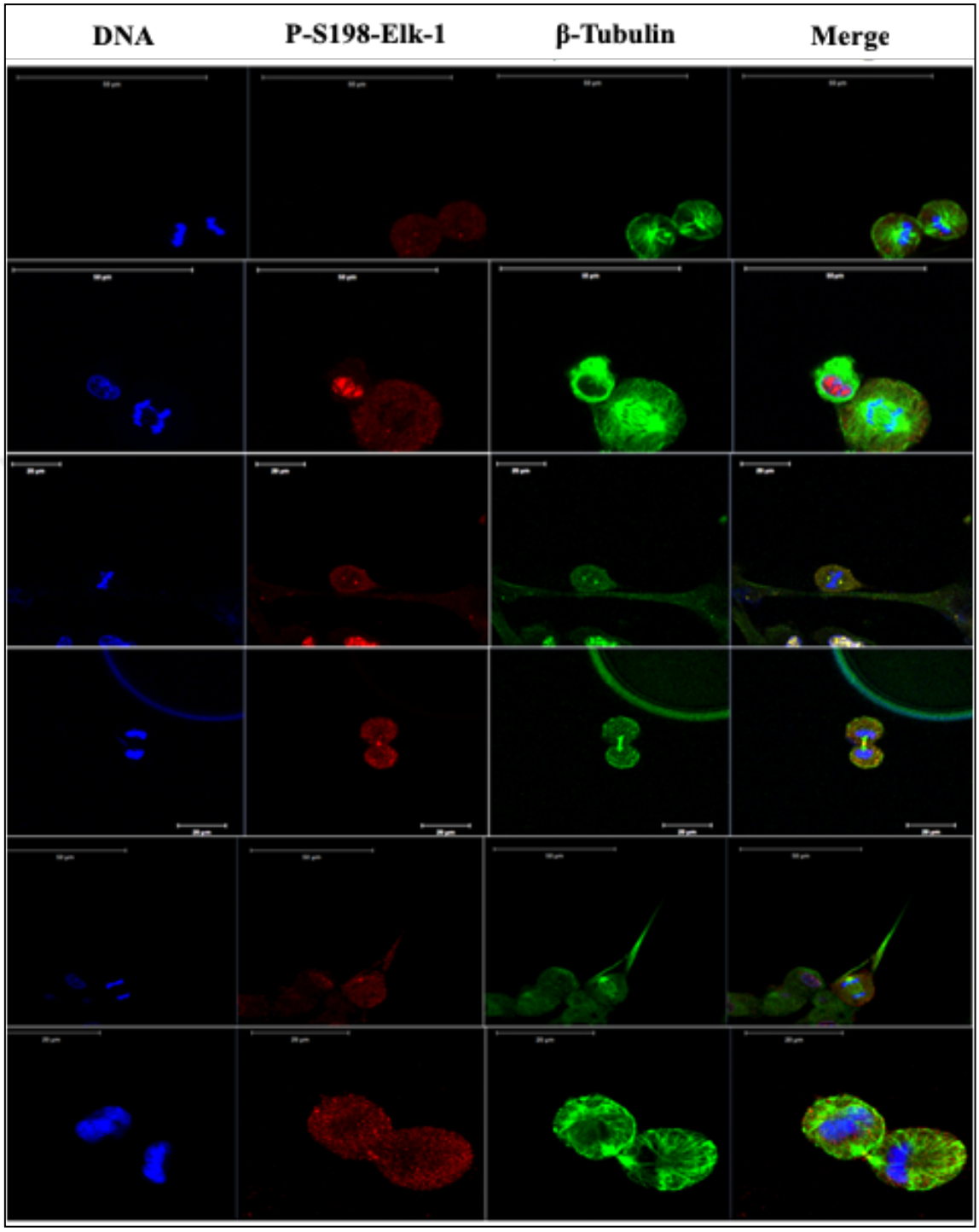

Figure B.7. The localization of phospho-S198-Elk-1 during different stages of the cell cycle in U-87 cell lines (anti-P-S198-Elk-1: red; anti-β-tubulin: green, DNA: blue).

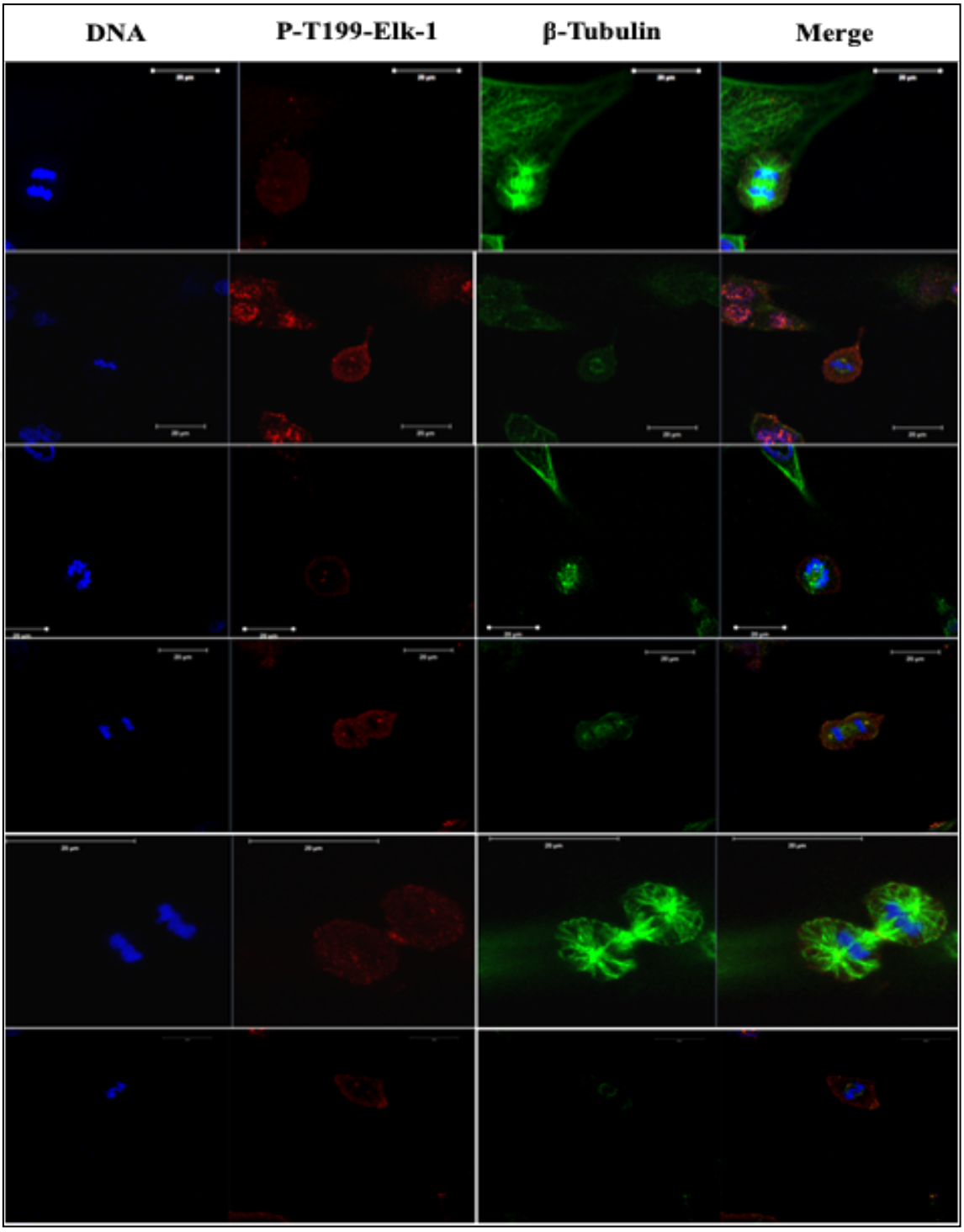

Figure B.8. The localization of phospho-T199-Elk-1 during different stages of the cell cycle in U-87 cell lines (anti-P-S198-Elk-1: red; anti-β-tubulin: green, DNA: blue).

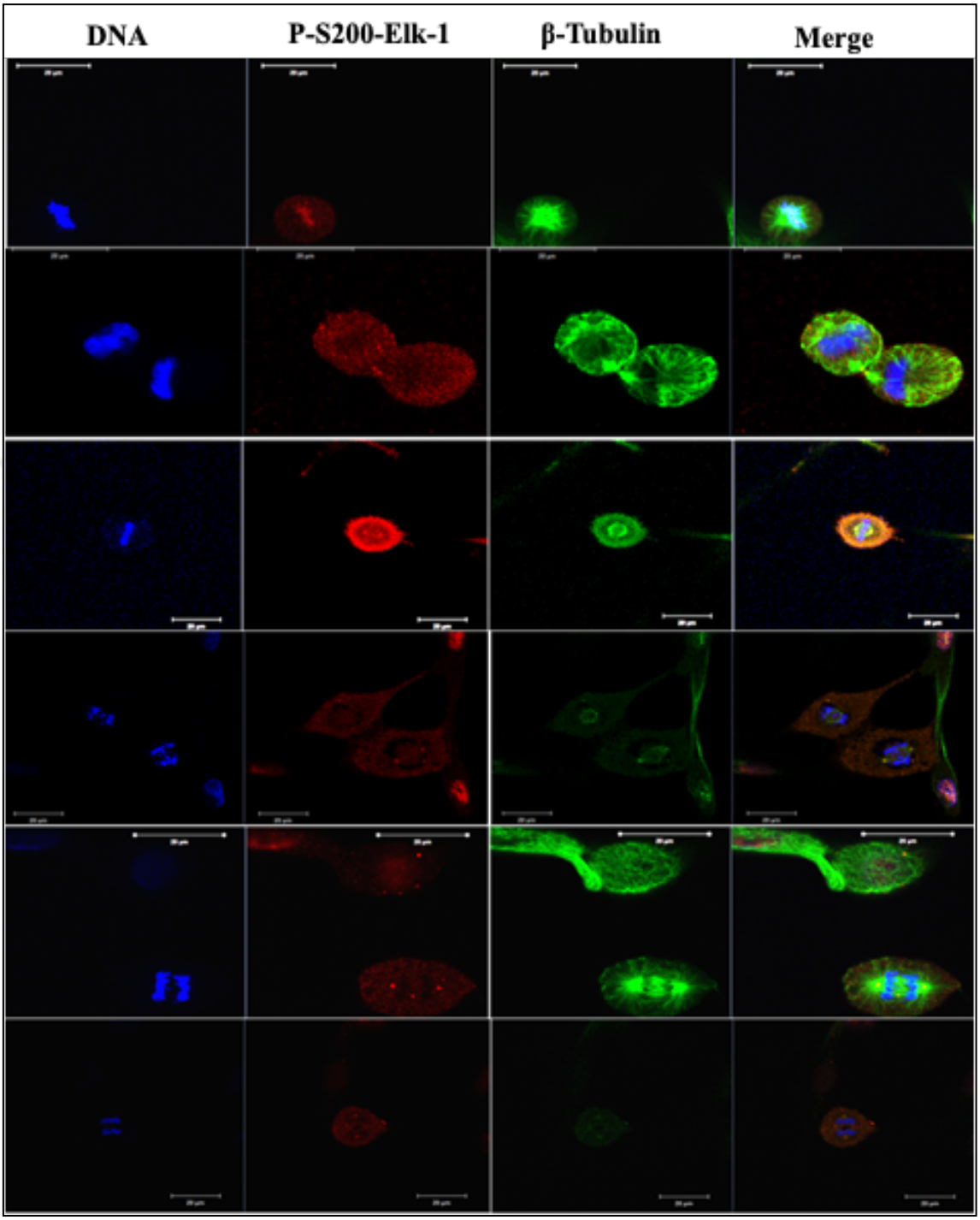

Figure B.9. The localization of phospho-S199-Elk-1 during different stages of the cell cycle in U-87 cell lines (anti-P-S198-Elk-1: red; anti-β-tubulin: green, DNA: blue).

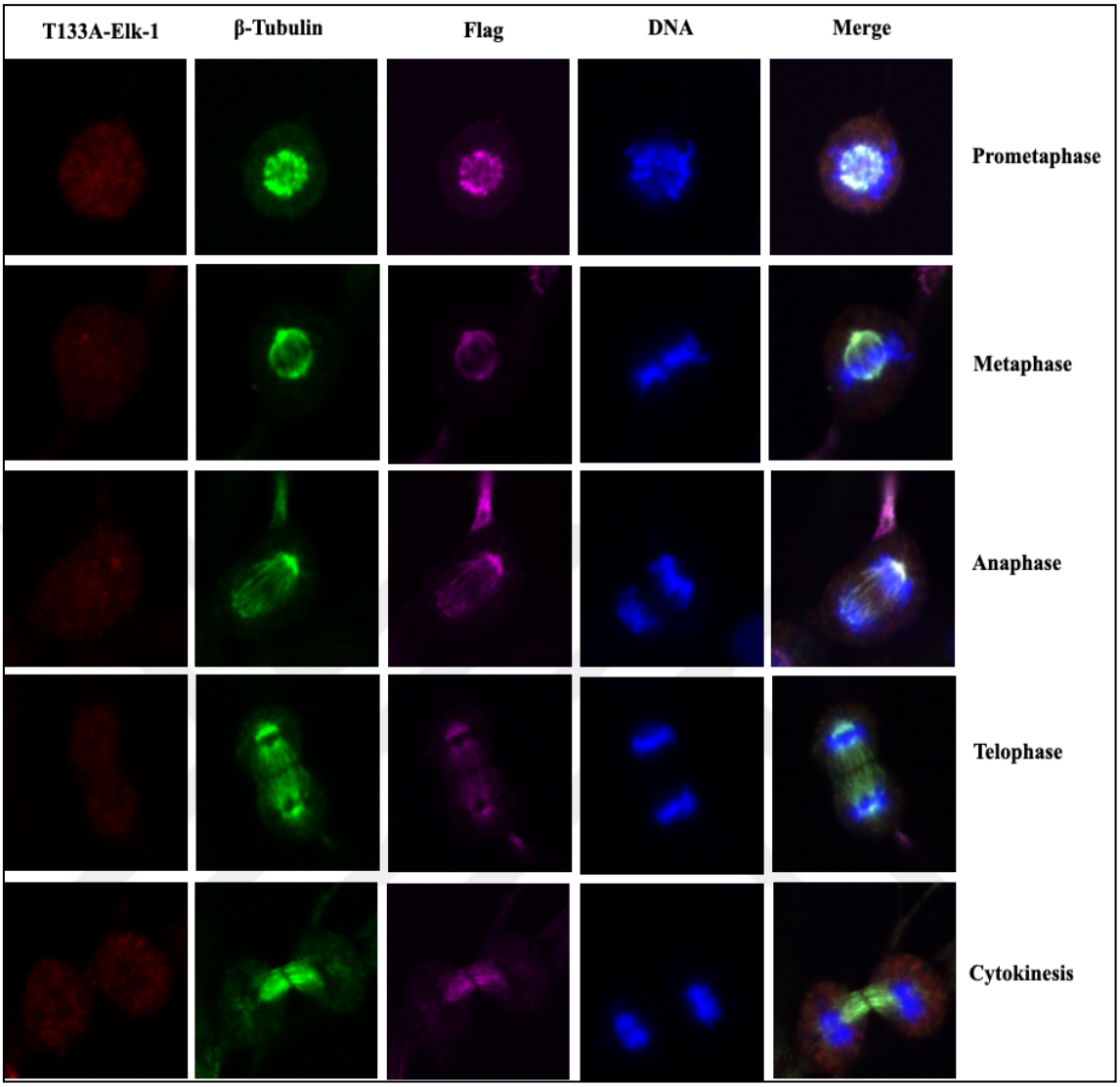

Figure B.10. The localization of P-T133-Elk-1 after S106A transfection (anti-P-S106-Elk-1: red, anti-β-tubulin: green, anti-flag: pink, DNA: blue).

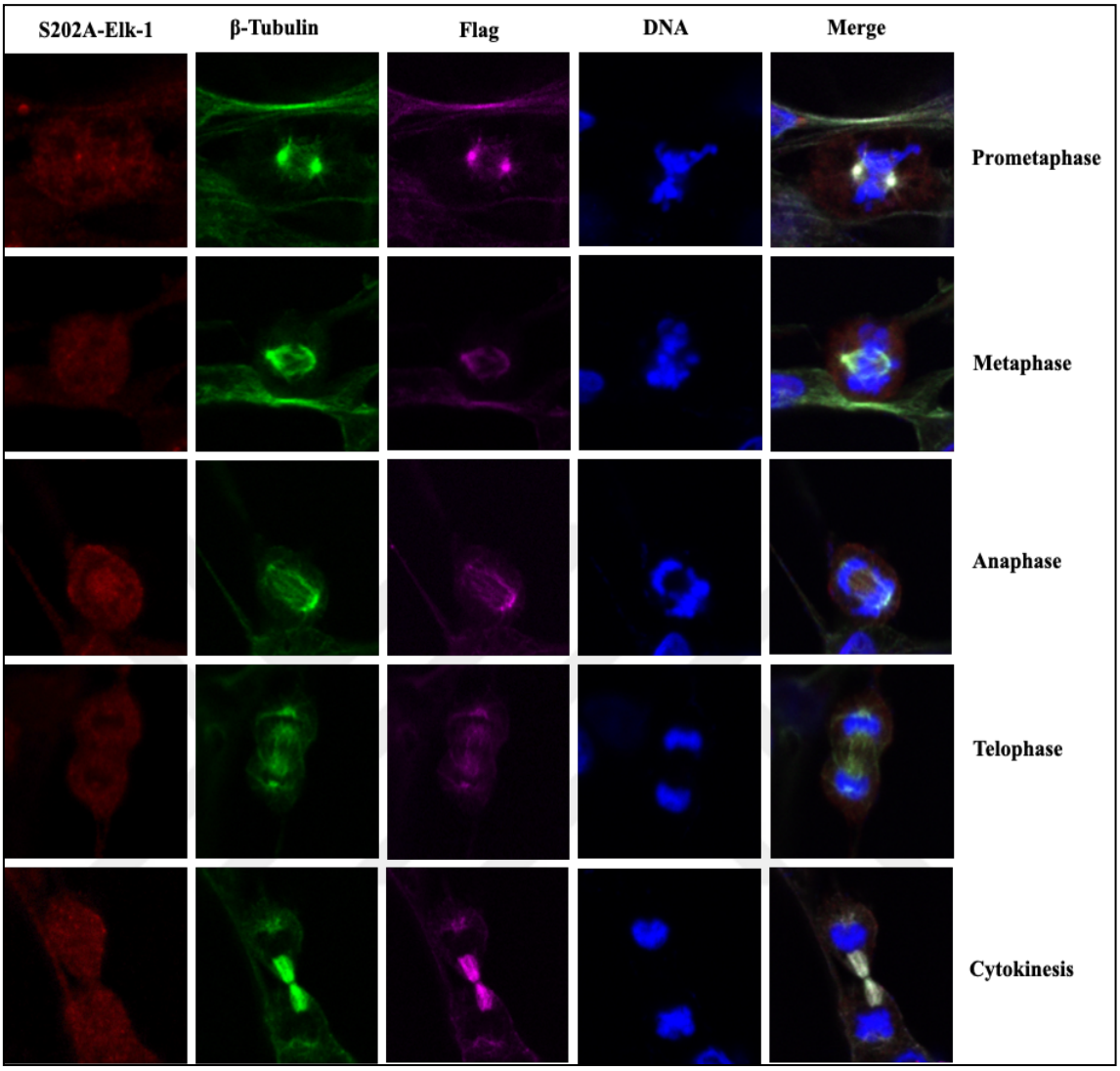

Figure B.11. The localization of P-S202-Elk-1 after S106A transfection (anti-P-S106-Elk-1: red, anti-β-tubulin: green, anti-flag: pink, DNA: blue).

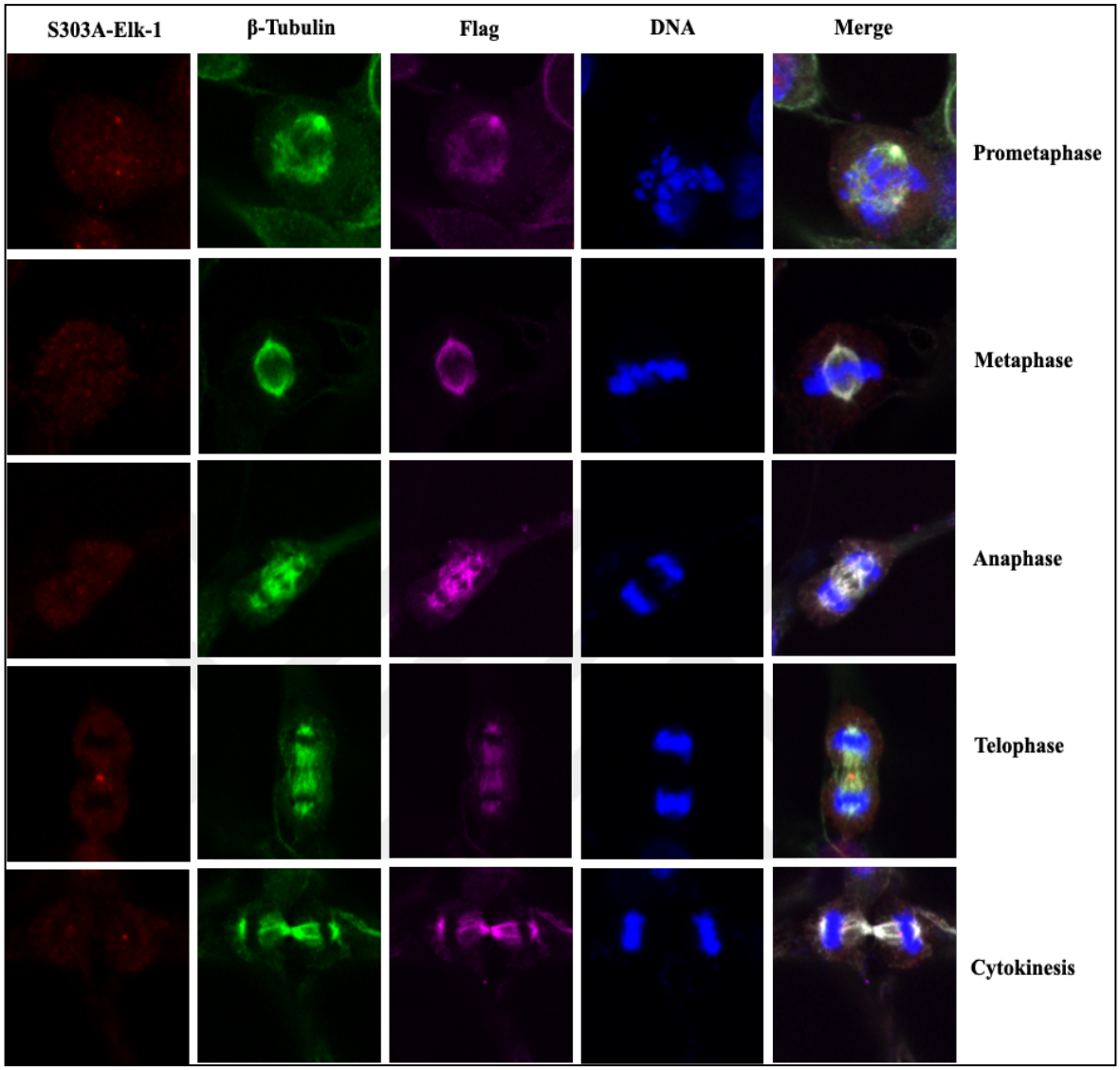

Figure B.12. The localization of P-S303-Elk-1 after S106A transfection (anti-P-S106-Elk-1: red, anti-β-tubulin: green, anti-flag: pink, DNA: blue).

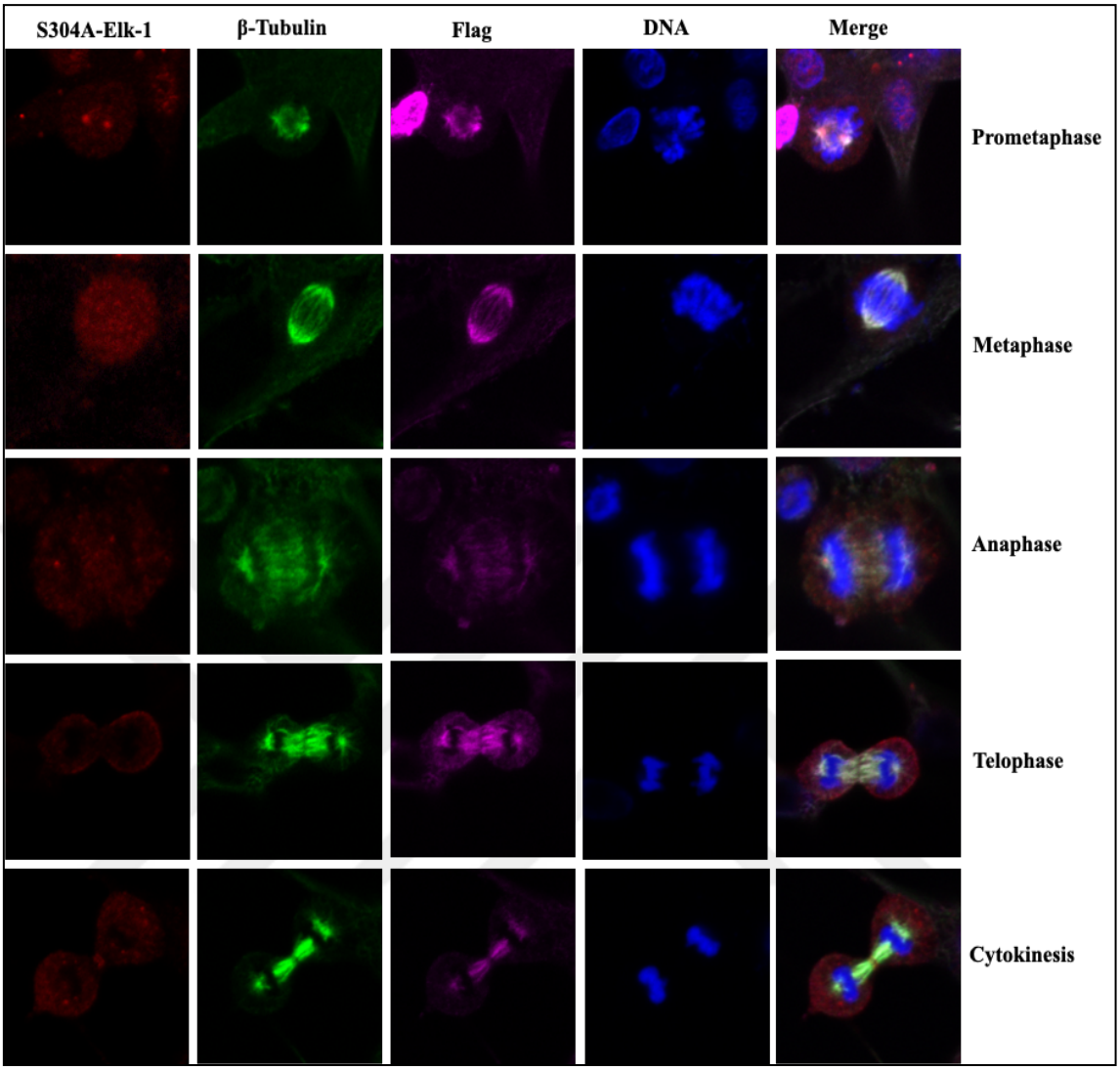

Figure B.13. The localization of P-S304-Elk-1 after S106A transfection (anti-P-S106-Elk-1: red, anti-β-tubulin: green, anti-flag: pink, DNA: blue).

APPENDIX C: FACS ANALYSES

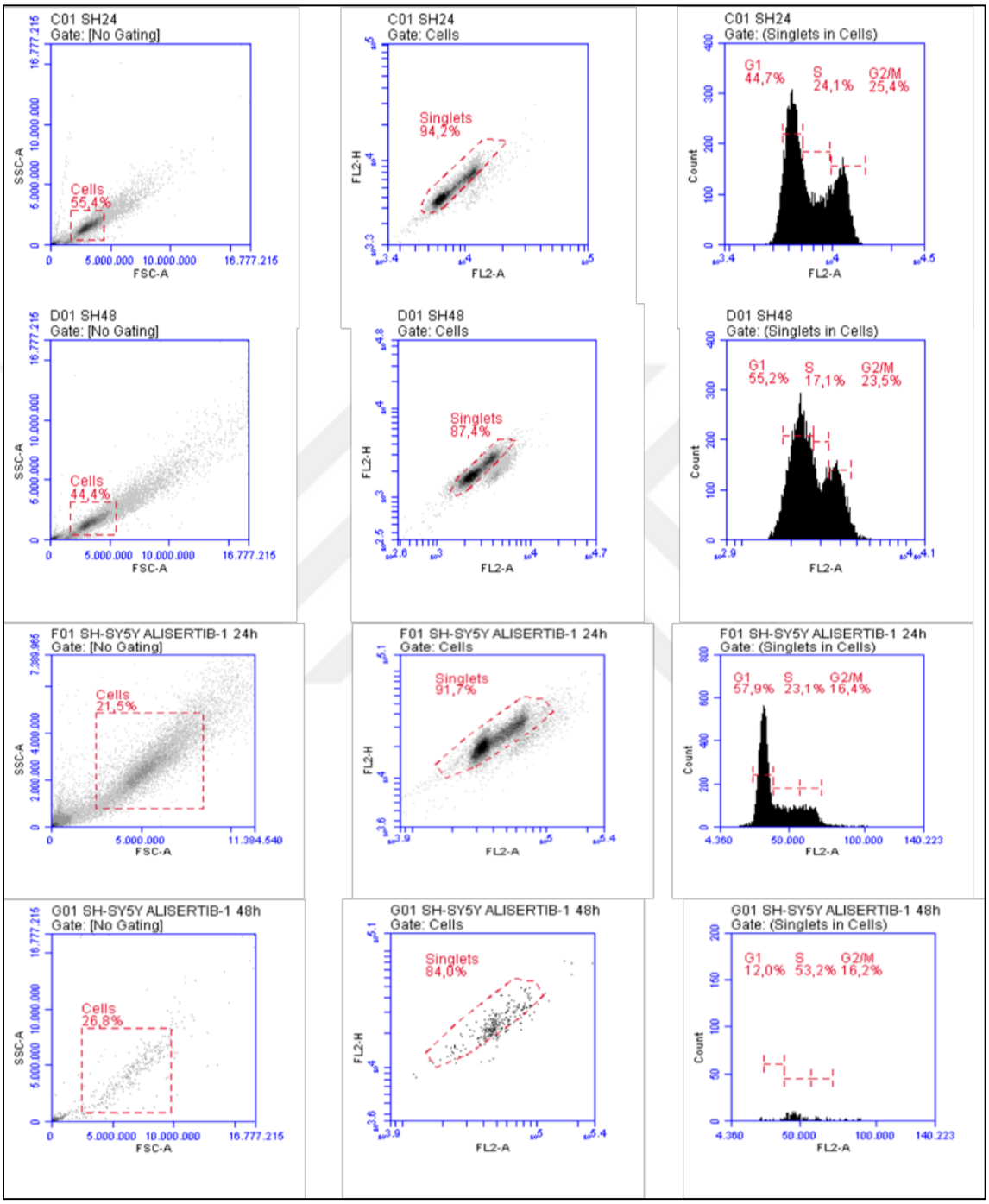

Figure C.1. Flow cytometry analysis of the kinase inhibitors on SH-SY5Y cells

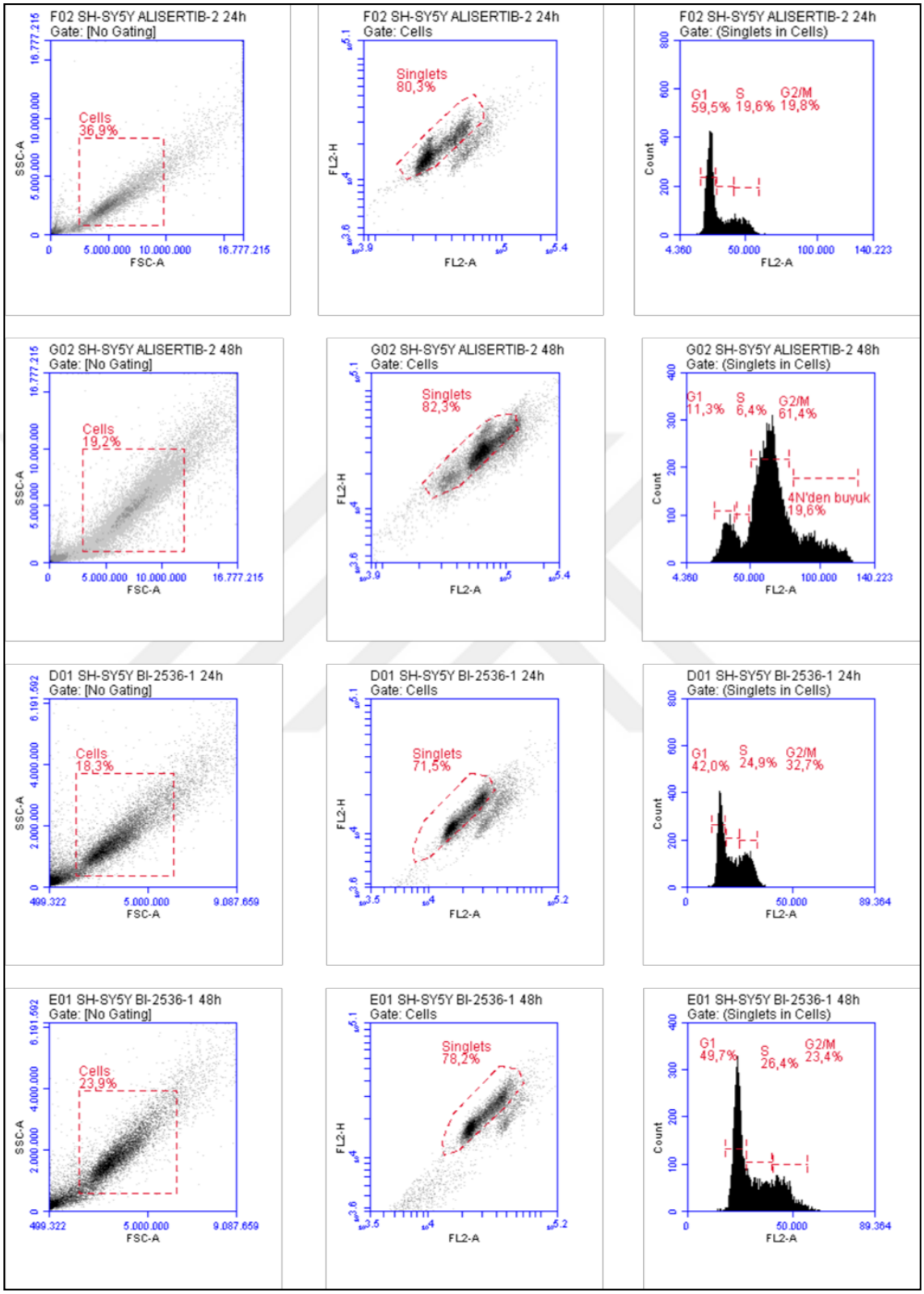

Figure C.1. Continued.

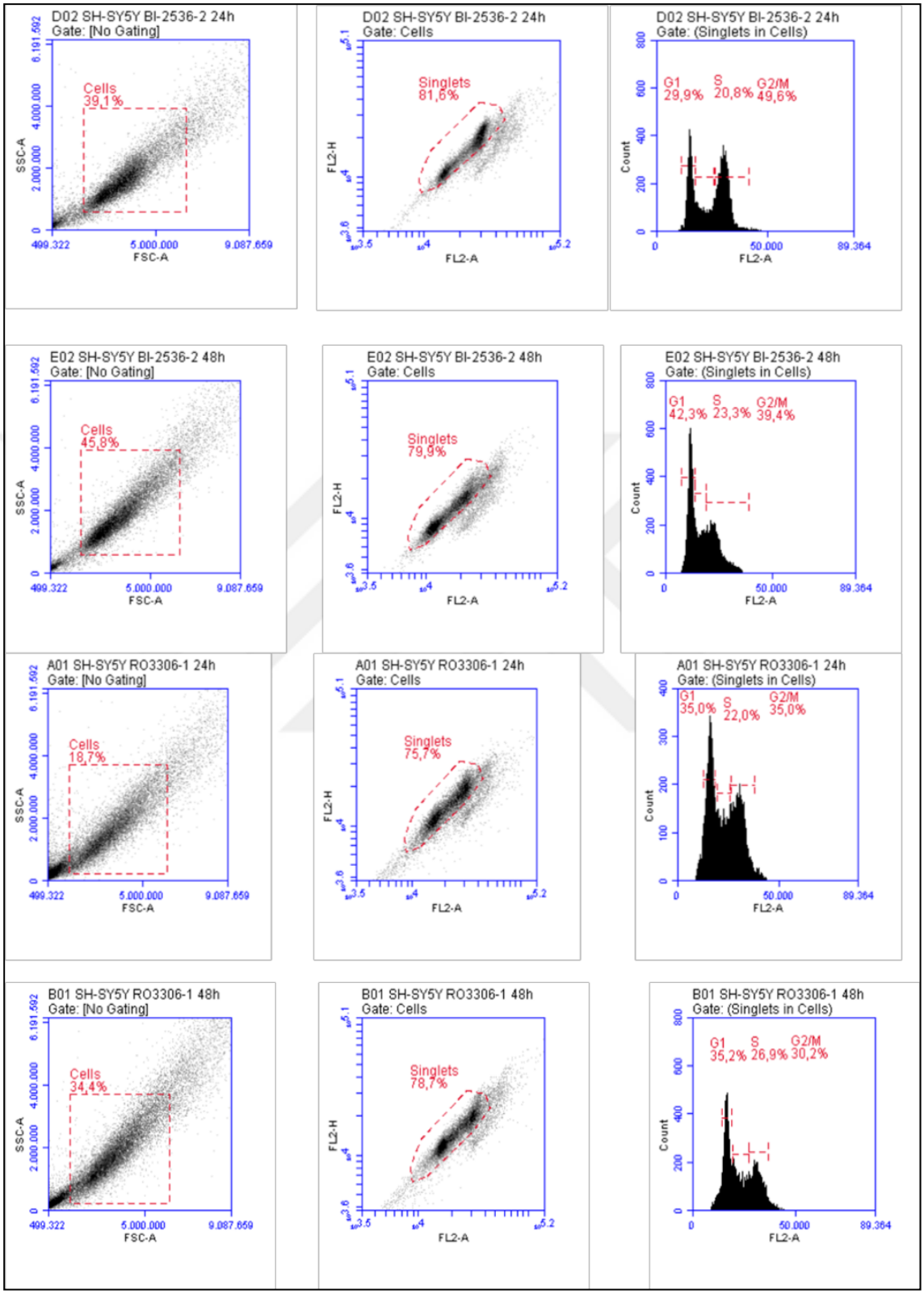

Figure C.1. Continued.

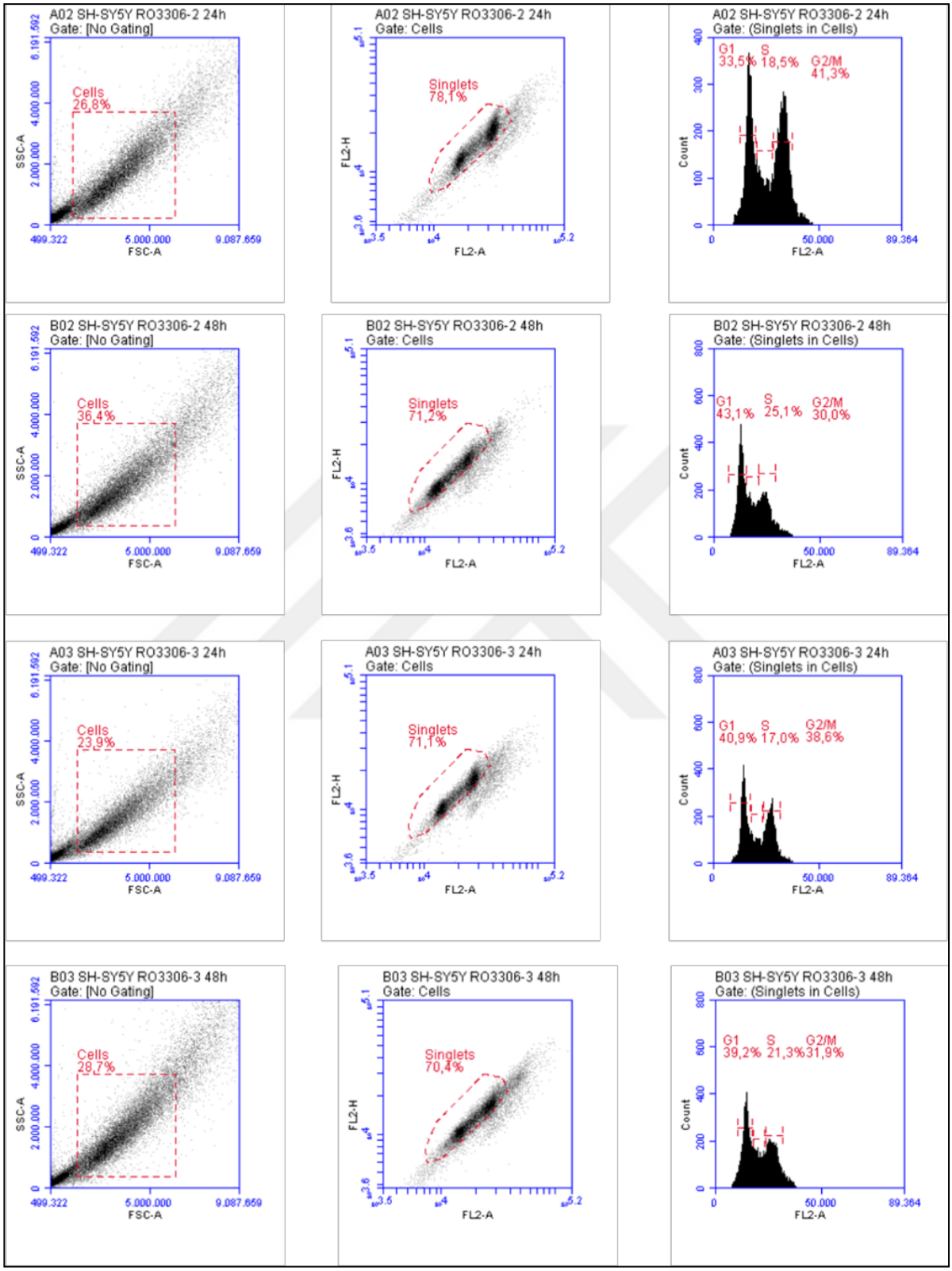

Figure C.1. Continued.

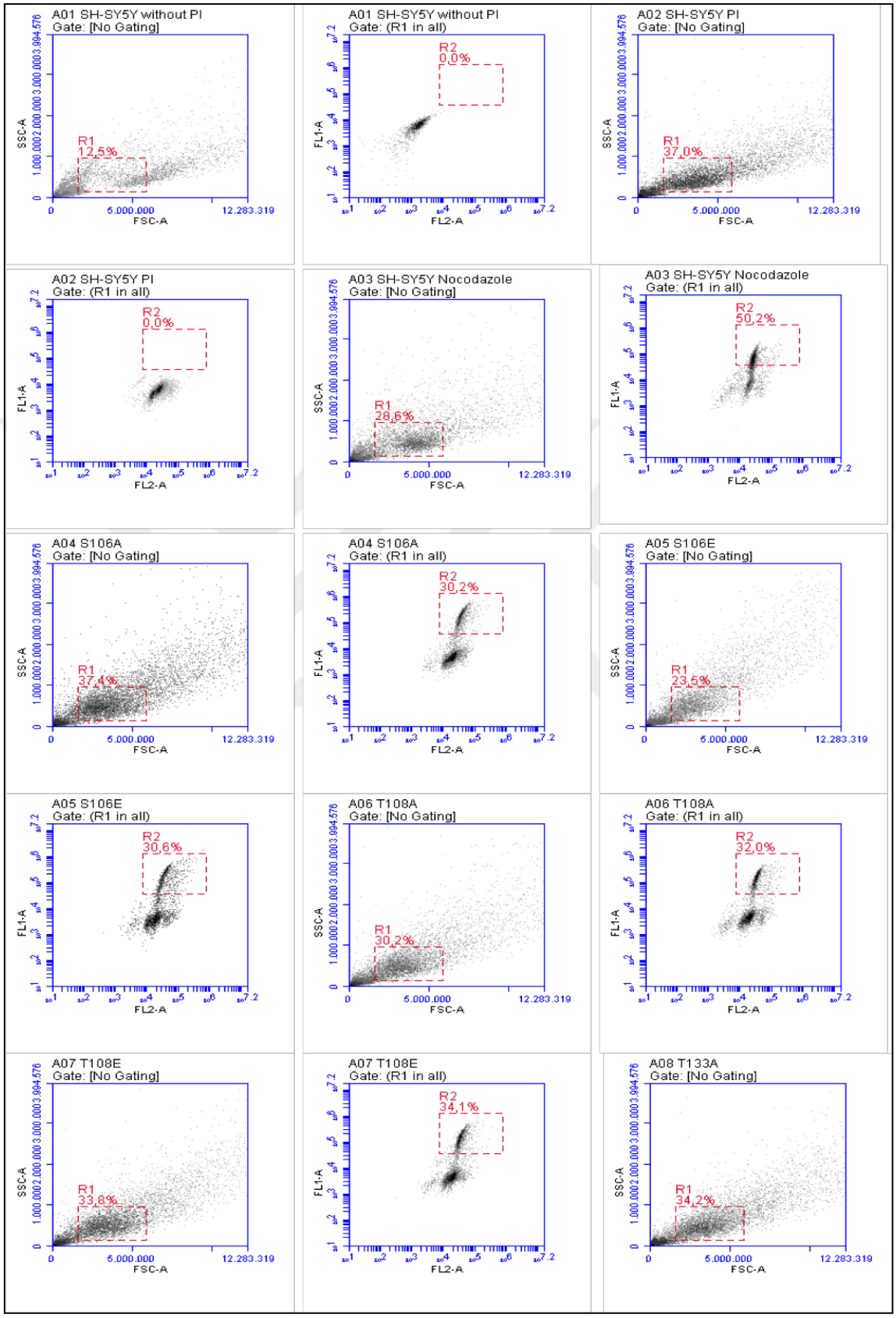

Figure C.2. Flow Cytometry Analysis of Cells After Histone H3 Staining

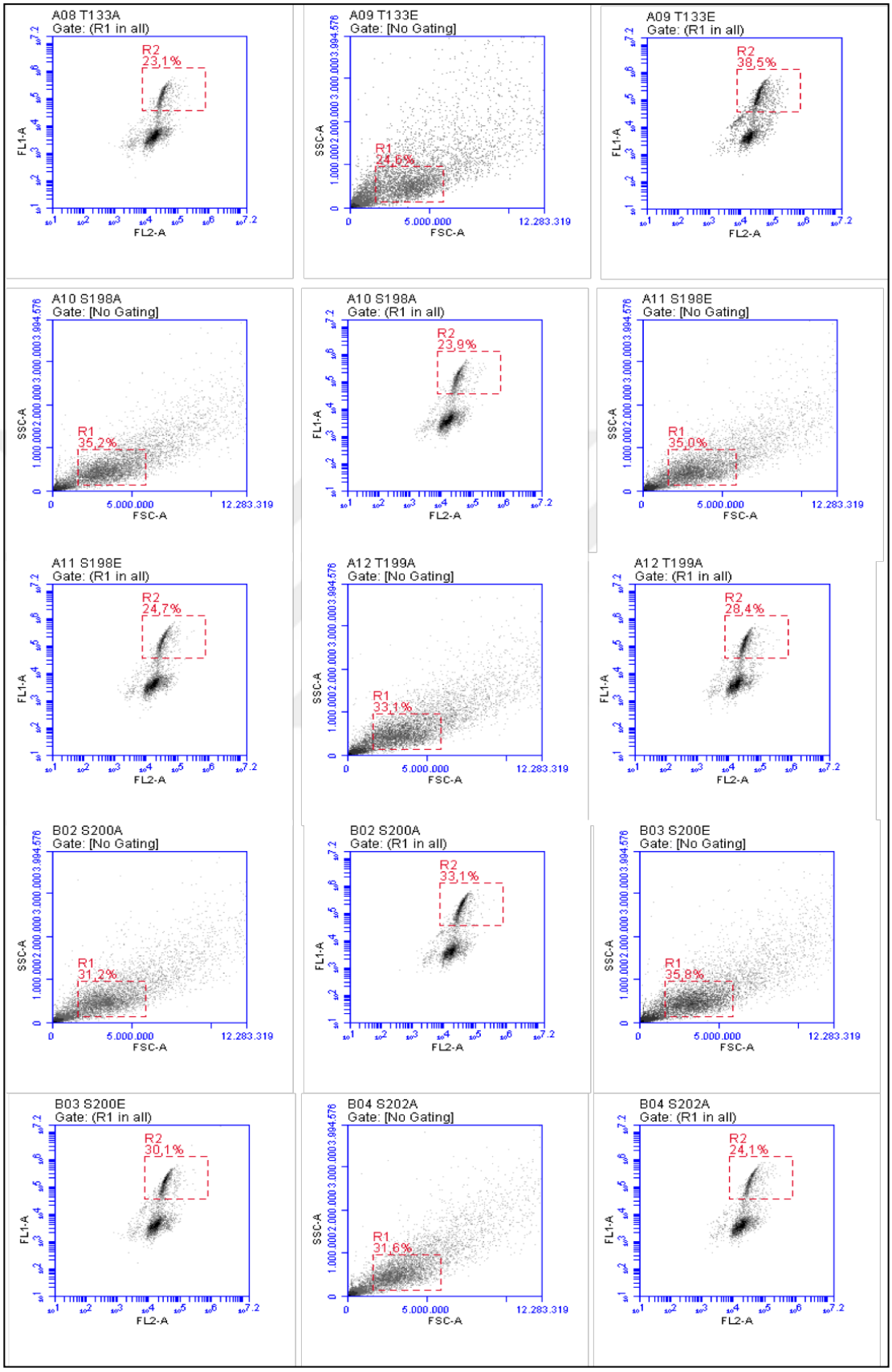

Figure C.2. Continued.

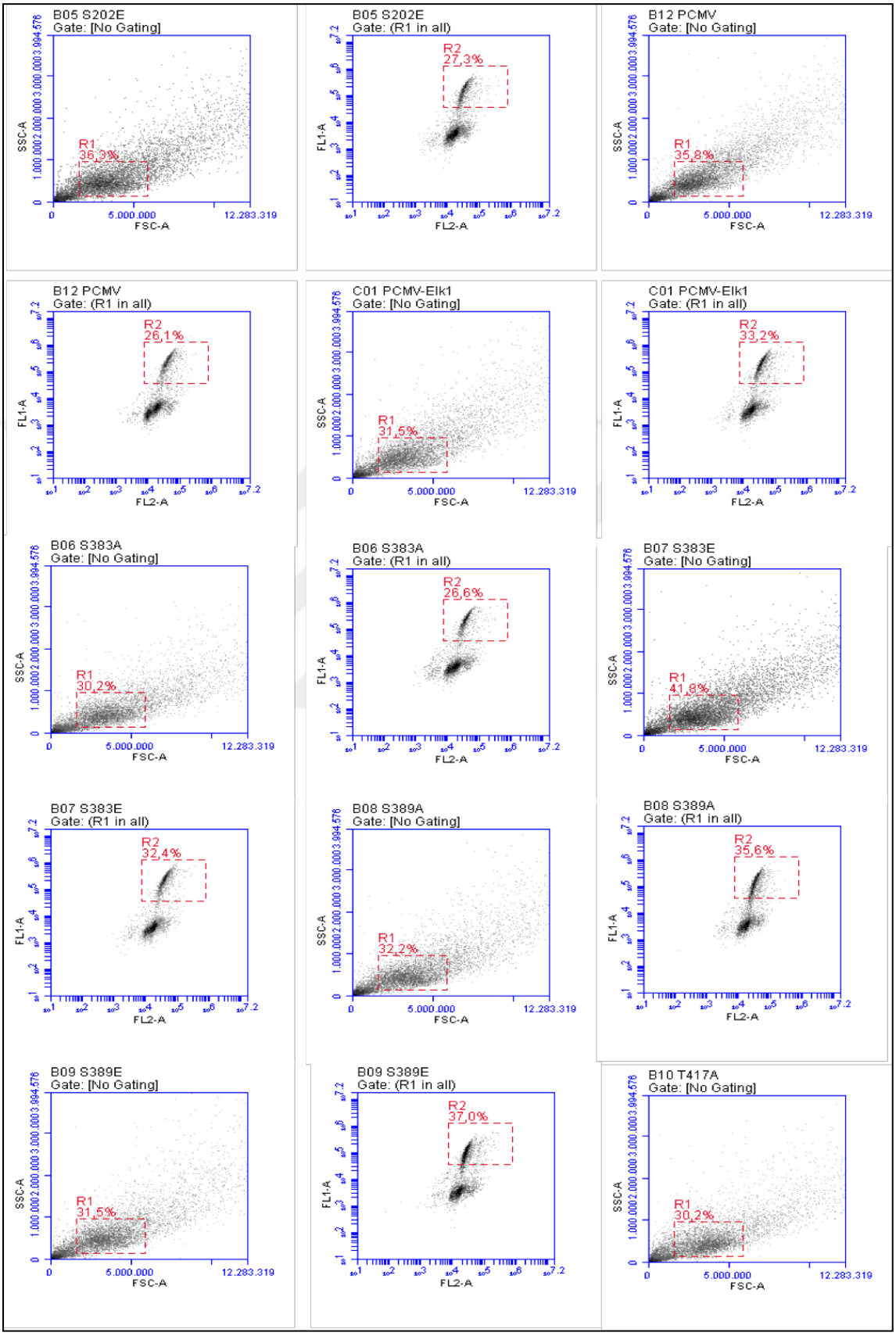

Figure C.2. Continued.

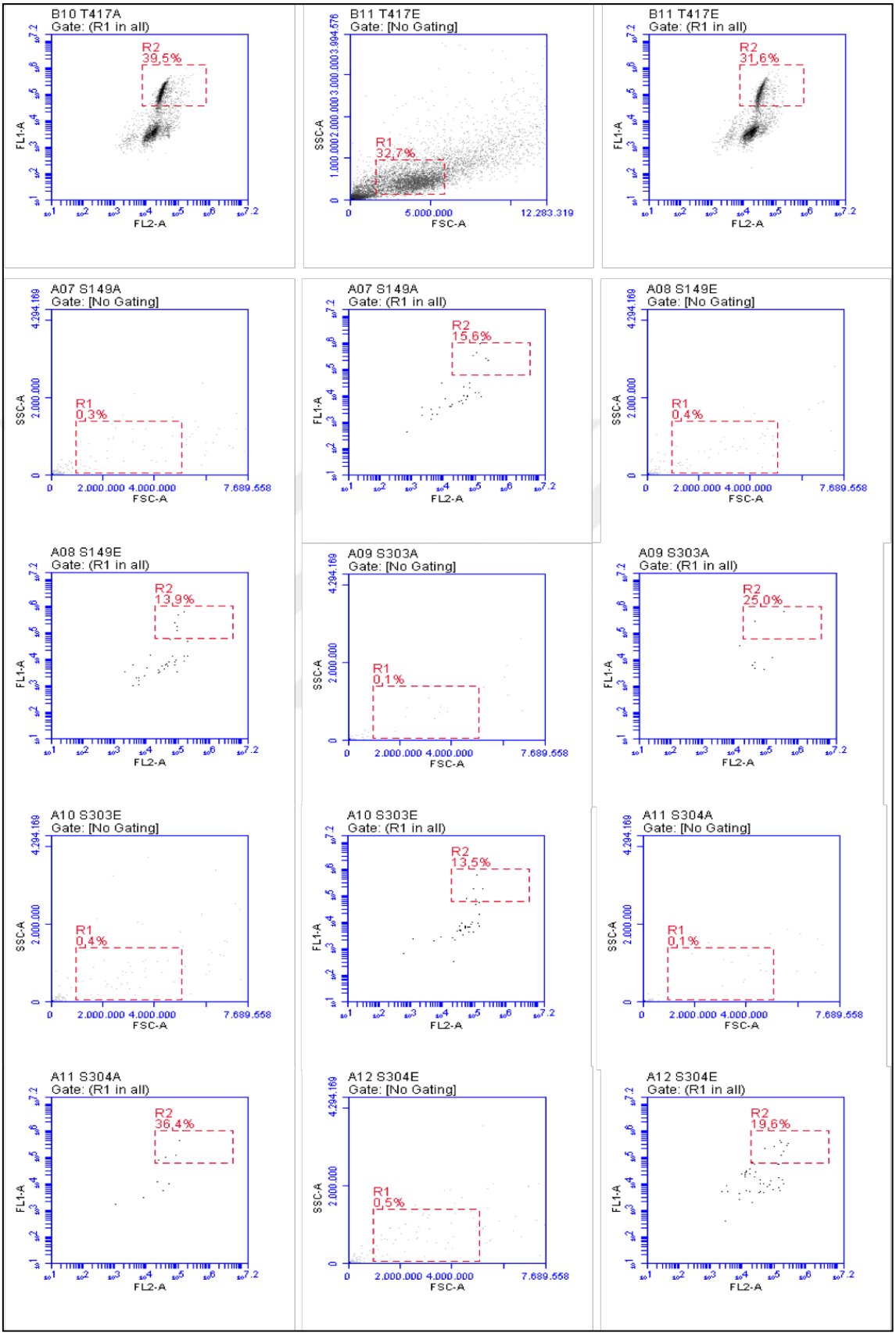

Figure C.2. Continued.

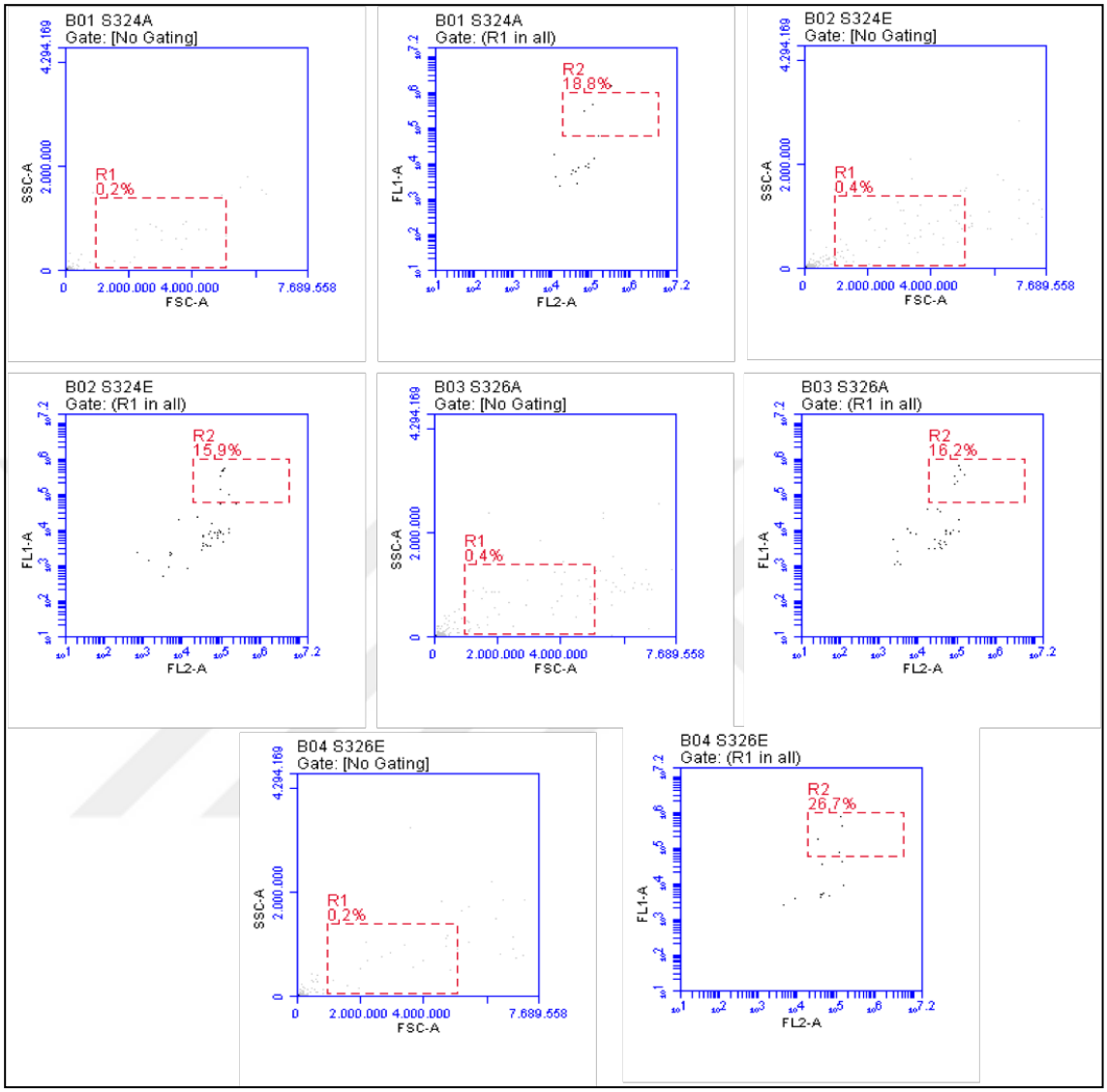

Figure C.2. Continued.

Table C.1. FACS Analysis of Histone H3 stained cells

Sample Name	**SH-SY5Y**		**U-87**	
Nocodazole Untreated	6,2	-	1,24	-
Nocodazole Treated	50	8,06	15,77	12,7
pCMV-Flag	26,1	-	8,93	-
pCMV-Elk1	33,2	1,27	9,55	1,07
S106A	30,2	0,91	22,73	2,38
S106E	30,6	0,92	20,08	2,1
T108A	32	1,05	24,90	2,61
T108E	34,1	1,03	29,1	3,05
T133A	23,1	0,7	25,68	2,69
T133E	38,5	1,16	25,54	2,67
S198A	23,9	0,72	14,50	1,52
S198E	24,7	0,74	16,19	1,7
T199A	28,4	0,86	17,09	1,58
T199E	25,8	0,78	15,06	1,77
S200A	33,1	0,99	16,86	1,8
S200E	30,1	0,91	17,15	1,2
S202A	24,1	0,73	11,51	1,55
S202E	27,3	0,82	14,79	1,55
S149A	15,6	0,47	14,03	1,47
S149E	13,9	0,42	11,77	1,23
S303A	25	0,75	10,43	1,09
S303E	13,5	0,41	13,67	1,43
S304A	36,4	1,1	12,43	1,30
S304E	19,6	0,59	12,82	1,34
S324A	18,8	0,57	11,36	1,19
S324E	15,9	0,48	13,47	1,41

S326A	16,2	0,49	11,18	1,17
S3A6E	26,7	0,80	12,10	1,27
S383A	26,6	0,80	-	-
S383E	32,4	0,98	-	-
S389A	35,6	1,07	-	-
S389E	37	1,11	-	-
T417A	39,5	1,19	-	-
T417E	31,6	0,95	-	-
si-Elk1	39,7	1,20	53,36	5,59
scRNA	45,5	1,37	60,13	6,30
Elk1-K2R	27,2	0,82	14,67	1,54
Elk1-VP16	33,7	1,01	10,18	1,07
Elk1-Eng	24,8	0,74	15,54	1,63