School of Biomedical Sciences

The role of monoubiquitylation in the regulation of the transcription factor Elk-1

by

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Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

September 2010

Contents

Abstract

Eukaryotic cells respond to extracellular stimuli by transmitting intracellular instructions via signalling pathways to coordinate appropriate responses. Mitogen-activated protein kinase (MAPK) pathways are often used to transmit these instructions to regulate gene expression, where Ternary Complex Factors (TCFs) are among their nuclear targets. Elk-1 is the founding member of the TCF family of transcription activators. The mechanism of function and regulation of Elk-1 has been extensively studied, therefore providing a paradigm for signal-induced transcription. The activity of Elk-1 is influenced by Post-Translational Modifications (PTMs), such as phosphorylation and sumoylation. Elk-1 ubiquitylation has also been reported *in vitro*, however little work has been done on this modification of Elk-1. This thesis sought to reveal the mechanism of regulation and function of Elk-1 ubiquitylation.

Elk-1 was demonstrated to be both monoubiquitylated and polyubiquitylated *in vitro* and in cells. Using size exclusion chromatography and the dominant negative nedd8 conjugating E2 enzyme Ubc12, several features of the Elk-1 specific E3 ligases have been revealed *in vitro*. In cells, ternary complex formation was shown to be important for monoubiquitylation. Furthermore monoubiquitylated Elk-1 is diminished following ERK-mediated phosphorylation, hence activation, in response to mitogen stimulation. It was also demonstrated that an Elk-1 derivative that exhibits strong monoubiquitylation level also exhibits a reduced capability to transactivate gene expression at the Serum Responsive Element (SRE), indicating a negative role of monoubiquitylation on Elk-1 transcriptional ability.

Acknowledgements

I would like to thank Professor Peter Shaw for his encouragement and support throughout this research project and in the preparation of this thesis. In addition, I would like to thank other members of Peter Shaw's research group including Li Li, Janice Saxton, Emma Evans, Sam Shelton and Thomas Strahl.

Thanks to my family and friends, in particularly David Carrier, James Dorrian, Paul Tisdales and Joanna Strachan for their support and encouragement throughout my studies and to the Biotechnology and Biological Sciences Research Council for their financial support. Without their support this work would not has been possible.

Abbreviations

ATP	adenosine triphosphate
BSA	bovine serum albumin
CBP	CREB binding protein
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CIAP	calf intestinal alkaline phosphatase
CRL	Cullin-RING Ligase
CXT	cytosolic extract
DM	DNA binding defective derivative
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DN	dominant negative
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
DTT	dithiothreitol
DUB	deubiquitinase
EBS	ETS binding site
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EMSA	Electrophoretic Mobility Shift Assay
ENaC	epithelial sodium channel
ERK	Extracellular signal-Regulated Kinase
ESCRT	Endosomal Sorting Complex Required for Transport
FCS	foetal calf serum

FPLC	Fast Performance Liquid Chromatography
HDAC	histone deacetylase
HECT	Homologous to the E6-AP Carboxyl Terminus
HLH	helix-loop-helix
IEG	Immediate Early Gene
IMAC	Immobilized Metal Affinity Chromatography
JAMM	Josphins and JAB1/MPN/MOV34 metalloenzyme
JNK	c-Jun N-terminal Kinase
Luc	luciferase
MAPK	Mitogen-Activated Protein Kinase
mRNA	messenger RNA
NXT	nuclear extract
OD	optical density
ONC	overnight culture
OUT	Ovarian Tumour Protease
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	polymerase chain reaction
PEI	polyethyleneimine
PKC	Protein Kinase C
PTM	post-translational modification
PVDF	polyvinylidene fluoride
RING	Really Interesting New Gene
RNA	ribonucleic acid
RT	room temperature

Sap-1	SRF accessory protein 1
SDS	sodium dodecyl sulphate
SM	SRF binding defective derivative
SRE	Serum Response Element
SRF	Serum Response Factor
SUMO	small ubiquitin-like modifier
TAE	tris-acetate EDTA
TBE	tris-borate EDTA
TBP	TATA binding protein
TBST	tris-buffered saline plus Tween
TC	tetracycline
TCF	Ternary Complex Factor
TE	tris-EDTA
TEMD	tetramethylethlenediamine
TNF	Tumour Necrosis Factor
TPA	12-O-tetradecanoylphorbol-13-acetate
UBA	Ubiquitin-Associated Domain
UBD	Ubiquitin-Binding Domain
UCH	Ubiquitin C-terminal Hydrolase
UIM	Ubiquitin-Interacting Motif
USP	Ubiquitin-Specific Protease
WCE	whole cell extract
WT	wild type
ZnF	Zinc Finger

1. Introduction

1.1. The ternary complex factor Elk-1

1.1.1. Overview

Accurate regulation of gene expression is vital for cell differentiation, proliferation, and the maintenance of cellular homeostasis. The expression of many genes is induced in response to specific stimuli to ensure precise regulation of cellular activity. In order for a regulated gene to be expressed, numerous molecular processes are coordinated in an appropriate sequence. RNA transcription is the first step of gene expression, where genetic information in DNA is copied into messenger RNA, which in turn is used as a template for protein translation. To initiate RNA transcription, sequence-specific transcription activators must bind to the promoter of the gene to be transcribed. These transcription activators work by recruiting proteins that alter the local chromatin structure to a more accessible conformation; and transcription mediators that in turn recruit the general transcription machinery to initiate RNA transcription (Thomas and Chiang 2006; Kornberg 2007).

Transcription activators with a similar conserved domain are often members of families, for example the ETS-domain protein family. The founding member of this family is ETS-1 which is homology to the v-ets oncoprotein of the avain erythroblastosis virus E26 (E Twenty Six), hence the name of this protein family (Leprince, Gegonne et al. 1983; Nunn, Seeburg et al. 1983). ETS-domain proteins comprise a large group of transcription activators that are fundamentally important in many cellular processes, such as cell proliferation, cell differentiation and apoptosis (Sharrocks 2001; Hsu, Trojanowska et al. 2004). The ETS-domain proteins can be subdivided based on sequence similarities in the ETS-domain and the

presence of further conserved domains. One subfamily is the Ternary Complex Factor (TCF) family. Ternary complex factors are characterised by their ability to form a ternary nucleoprotein complex with the Serum Response Factor (SRF) dimer at the Serum Responsive Element (SRE) to activate gene transcription. This ternary complex is formed upon activation of the Mitogen-Activated Protein Kinase (MAPK) signalling pathways which relay extracellular stimuli from cell surface receptors to the nucleus. Despite the diversity of biological functions of the ETS-domain proteins, many of their regulatory mechanisms are conserved among family members, particularly for the TCFs (Shaw and Saxton 2003; Buchwalter, Gross et al. 2004). Elk-1 is the founding member of the TCF subfamily. By studying Elk-1, the complex regulation of ETS-domain proteins can be revealed, therefore providing a paradigm for signal-induced transcriptional regulation.

1.1.2. Historical perspective

Elk-1 was found as part of a ternary complex in HeLa nuclear extract with the SRF dimer on the SRE of the *c-fos* promoter (Shaw, Schröter et al. 1989). It was initially named p62 due to its molecular weight and later shown to be identical to the previously cloned Elk-1, which was identified based on sequence homology with the v-ets protein (Rao, Huebner et al. 1989; Hipskind, Rao et al. 1991). Two structurally related proteins were subsequently identified, Sap-1 (SRF accessory protein 1) (Dalton and Treisman 1992) and Net/Sap-2/Erp (Giovane, Pintzas et al. 1994; Lopez, Oettgen et al. 1994; Nozaki, Onishi et al. 1996). Both proteins were shown to exhibit similar ability to form ternary complexes. In addition TCF homologues have also been identified in other vertebrates (Goldman, Sapru et al. 1998; Brown, Yang et al. 1999), however no TCF homologue has been identified in yeast despite the availability of the entire genomic sequence of *Saccharomyces cerevisiae*, indicating this family of proteins is only conserved among metazoan.

1.1.3. Domain structure of Elk-1

All TCFs share a similar domain arrangement with additional functional domains that are unique in family members to permit differential regulation and function (Figure 1-1). Four domains (A-D domains) were initially identified based on sequence comparison between Elk-1 and Sap-1 (Dalton and Treisman 1992). The N-terminal A-domain corresponds to the DNA-binding ETS-domain. Besides DNA binding, the ETS-domain has also been reported to exhibit repressive capability by recruiting the mSin3A-histone deacetylase 1 co-repressor (Yang, Vickers et al. 2001) and the DNA-binding inhibitor Id helix-loop-helix proteins (Yates, Atherton et al. 1999). The B-domain binds to the MADS-box transcription factor family member SRF to facilitate ternary complex formation (Shore and Sharrocks 1994). The C-terminal C-domain is a transactivational domain with multiple MAPK phosphorylation consensus sites (S/T)P, where phosphorylation activates its transcriptional ability (Janknecht, Ernst et al. 1993; Marais, Wynne et al. 1993; Gille, Kortenjann et al. 1995). The D-domain and the FXFP-motif within the C-domain are the MAPK docking sites (Yang, Whitmarsh et al. 1998; Yang, Yates et al. 1998; Jacobs, Glossip et al. 1999; Sharrocks, Yang et al. 2000). Beside these domains, which are shared among all TCF members, Elk-1 contains an additional R-domain for transcriptional repression (Yang, Bumpass et al. 2002). The R-domain contains three lysine residues, which can be sumoylated for the recruitment of the histone deacetylase 2 co-repressor (Yang, Jaffray et al. 2003; Yang and Sharrocks 2004).

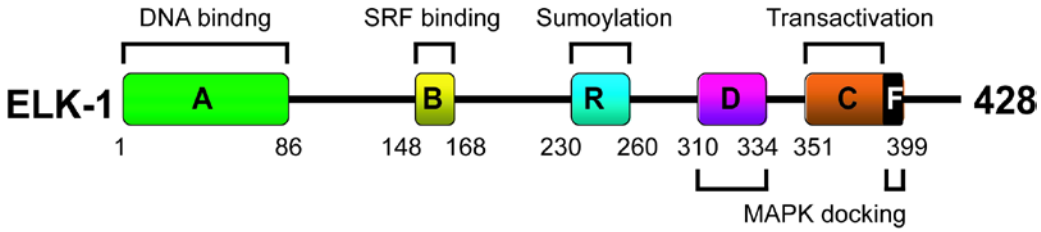

Figure 1-1, Schematic representation of the domain structure of Elk-1. Functions of the domains are as indicated. The amino acid residue locations of the domains are presented below the boxes.

1.1.4. Ternary complex formation

1.1.4.1. Serum Response Element

Despite the similarity between the TCFs, differential binding at the SRE has been reported between Elk-1 and Sap-1. Upon mitogen stimulation, Elk-1 cooperates with the SRF dimer to form a ternary complex at the SRE of the *c-fos* promoter (Gille, Sharrocks et al. 1992). Binding between Elk-1 and the SRE was not observed in absence of SRF (Shaw, Schröter et al. 1989; Hipskind, Rao et al. 1991), suggesting the SRF cooperative binding is essential for Elk-1 mediated ternary complex formation, whereas Sap-1 is able to interact with the SRE alone (Dalton and Treisman 1992). In addition, autonomous DNA binding of Elk-1 has also been reported with a high affinity ETS binding site (E74), however it has not been shown to be a physiological target (Sharrocks 1995; Mo, Vaessen et al. 1998; Mo, Vaessen et al. 2000).

The SRE are often found in the promoter of many Immediate Early Genes (IEGs), a set of genes that are rapidly and transiently induced following growth factor or mitogen stimulation (Treisman 1992; Treisman 1995). Besides IEGs, the SRE was also found in viral genomes (Cahill, Nordheim et al. 1994). The *c-fos* SRE consists of a weak ETS binding site (EBS) adjacent to two SRF binding sites (CArG-box). The EBS is characterised by a purine-rich

core sequence of GGA(A/T) surrounded by more variable sequences to allow differential binding specificity between ETS-domain proteins. The CArG-box consists of the sequences $CC(A/T)_6GG$, which interacts with the SRF dimer. It has been demonstrated that the spacing between the EBS and the CArG-box (an addition of 25 bps) and their relative orientation can be altered without a significant effect on ternary complex formation (Treisman, Marais et al. 1992).

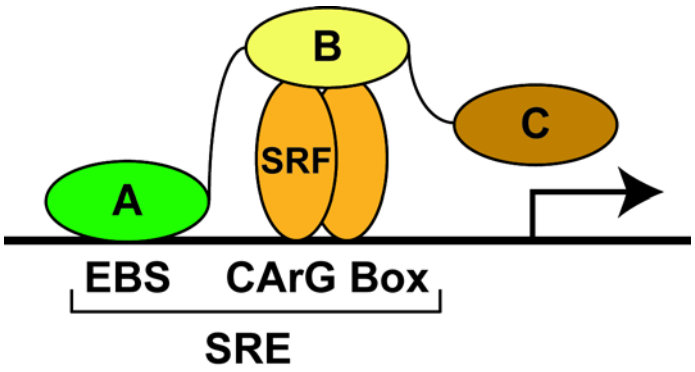

Figure 1-2 Diagram of ternary complex. The A-domain (ETS-domain) of a TCF binds to the EBS of the SRE whereas the B-domain mediates protein-protein interactions with the SRF dimer, which in turn binds to the CArG box of the SRE.

1.1.4.2. ETS/DNA binding

The ternary complex is formed by a combination of protein/DNA and protein/protein interactions (Figure 1-2). The DNA binding ETS-domain is 85 amino acids long and usually found at the C-termini of most ETS-proteins, whereas it is located at the N-termini of the TCFs (Sharrocks 2001; Hsu, Trojanowska et al. 2004). It adopts a winged Helix-Turn-Helix (wHTH) structure with three α-helices and four anti-parallel β-strands, where the wing is a

loop between adjacent β-strands (Figure 1-3 & Figure 1-4) (Donaldson, Petersen et al. 1994; Donaldson, Petersen et al. 1996; Mo, Vaessen et al. 2000).

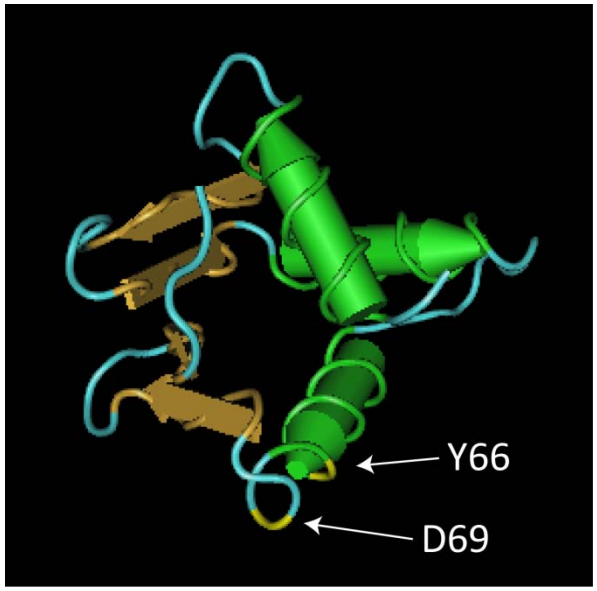

Figure 1-3 The 2.1Å crystal structure of the ETS-domain of Elk-1 (Mo, Vaessen et al. 2000). The image was generated using the Cn3D software (NCBI). α-helices and β-strands are presented as green cylindrical arrows and dark yellow flat arrows respectively. Asparagine 69 and tyrosine 66 are indicated by white arrows and coloured in light yellow.

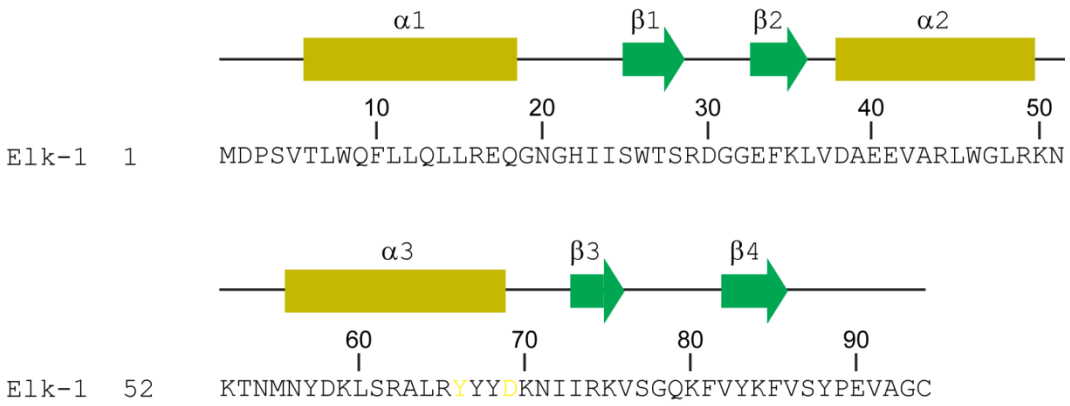

Figure 1-4 Schematic representation of the secondary structure of Elk-1 ETS-domain. α-helices and β-strands are presented as indicated. Asparagine 69 and tyrosine 66 are highlined in light yellow.

The crystal structure of the ETS-domain bound to the E74 duplex has revealed that the main residues contributing to the ETS/DNA binding are located in the α3 helix that lies in the major grove of DNA, with additional contacts mediated by the loop between α2 and α3 helices and the wing between β3 and β4 strands (Mo, Vaessen et al. 2000). It also revealed that differential DNA binding between Elk-1 and Sap-1 is due to the orientation of a conserved tyrosine residue in the DNA recognition α3 helix (Y66 for Elk-1 and Y65 for Sap-1), positioned by non-conserved residues distal from the helix (asparagine 69 for Elk-1 and valine 68 for Sap-1) (Mo, Vaessen et al. 1998; Mo, Vaessen et al. 2000). These two residues were previously described to be critical determinants for differential DNA binding between TCFs in another study and replacing asparagines 69 in Elk-1 with valine was able to confer the Sap-1 DNA binding specificity on Elk-1 (Shore, Whitmarsh et al. 1996).

1.1.4.3. TCF/SRF interaction

In addition to DNA/protein binding, protein/protein interactions are also essential for the ternary complex formation. It has been shown that the interaction between Elk-1 and SRF enhances their binding to the *c-fos* SRE (Mueller and Nordheim 1991; Shaw 1992). The B-domain (amino acids 137 - 169) of Elk-1 was shown to interact with an extended structure near the DNA binding domain of SRF (Janknecht and Nordheim 1992), where the B-domain forms an inducible helix to present a hydrophobic region to SRF for their interaction (Ling, West et al. 1998). Disruption of the B-domain completely abolishes ternary complex formation and SRE-dependent transcription (Hill, Marais et al. 1993). Subsequently direct interaction between Elk-1 and SRF in absence of the SRE has also been reported (Shore and Sharrocks 1994) and critical amino acids mediating this interaction have been identified (Ling, Lakey et al. 1997), namely three aromatic residues tyrosine 153, tyrosine 159 and Phenylalanine 162.

No structural study on Elk-1 ternary complex has been reported, however the crystal structure of a ternary complex containing Sap-1 has revealed and confirmed several observations (Hassler and Richmond 2001). (i) the B-domain adopts a partial α-helical structure (two short 3_{10} helices linked by a β-strand), where significant contacts were observed with the hydrophobic groove of SRF. (ii) the DNA-binding domain of Sap-1 and SRF are situated on opposite sides of DNA with some contacts with each other. (iii) the linker region between the B-domain and ETS-domain exhibits a loose structure to provide a flexible tether, supported by the evidence that the spacing and orientation between the EBS and the CArG-box in the *c-fos* SRE can be substantially altered with little impact on ternary complex formation (Treisman, Marais et al. 1992)

1.1.5. Regulation of Elk-1 by post-translational modifications

The transcriptional ability of Elk-1 is influenced by extracellular signal-induced post-translational modifications (PTMs). Phosphorylation of Elk-1 triggered by growth factors or mitogens has been shown to potentiate ternary complex formation and transactivation (Gille, Sharrocks et al. 1992; Janknecht, Ernst et al. 1993; Gille, Kortenjann et al. 1995; Sharrocks 1995), whereas sumoylation of Elk-1 represses its transcriptional capability through the recruitment of the histone deacetylase 2 co-repressor (Yang, Jaffray et al. 2003; Yang and Sharrocks 2004). Moreover PTMs often affect each other, for example Elk-1 phosphorylation represses its sumoylation (Yang, Jaffray et al. 2003). Elk-1 ubiquitylation has also been reported *in vitro* (Fuchs, Xie et al. 1997), however its regulation and function are poorly understood.

1.1.5.1. Elk-1 phosphorylation by MAPKs

Phosphorylation of Elk-1 is mediated by MAPKs (Janknecht, Ernst et al. 1993; Gille, Strahl et al. 1995; Whitmarsh, Shore et al. 1995; Price, Cruzalegui et al. 1996; Enslen, Raingeaud et al. 1998). MAPKs are a conserved family of serine/threonine kinases in all eukaryotes that transmit signals from cell surface receptors to the nucleus to control eukaryotic gene expression in response to extracellular stimuli (Yang, Sharrocks et al. 2003; Turjanski, Vaque et al. 2007). There are three major MAPK cascades; the ERK (ERK1 and ERK2; Extracellular signal Regulated Kinase); JNK (JNK1, JNK2 and JNK3; c-Jun N-terminal Kinase); and p38 (p38α, p38β, p38γ and p38δ) cascades. The ERK cascade is activated by growth stimuli, whereas the JNK and p38 cascades are activated in response to cellular stress and cytokines. Elk-1 is phosphorylated by all the three major MAPKs. ERK was the first MAPK shown to phosphorylate Elk-1 (Gille, Sharrocks et al. 1992; Marais, Wynne et al. 1993; Gille, Kortenjann et al. 1995; Cruzalegui, Cano et al. 1999). Subsequently, Elk-1 was

shown to be phosphorylated by JNK and P38 (Cavigelli, Dolfi et al. 1995; Gille, Strahl et al. 1995; Whitmarsh, Shore et al. 1995; Price, Cruzalegui et al. 1996; Enslen, Raingeaud et al. 1998).

To ensure signal specificity, MAPKs recognise their substrates via specific docking sites to facilitate phosphorylation (Sharrocks, Yang et al. 2000). The D-domain was the first identified ERK docking site in Elk-1 (Yang, Yates et al. 1998). It is located upstream from the C-domain that contains multiple MAPK phosphorylation sites. An additional docking site (FXFP-motif) for ERK was later discovered (Jacobs, Glossip et al. 1999). This motif is situated within the C-domain and shown to form a bipartite docking module. It was demonstrated that JNK also binds to Elk-1 via the D-domain (Yang, Whitmarsh et al. 1998), but does not interact with the FXFP-motif, whereas p38 seems to interact with Elk-1 through an unidentified region.

In addition to MAPK recruitment, the docking sites also direct MAPK-mediated phosphorylation on the MAPK consensus sites (S/T)P. Phosphorylation on serine 383 was shown to be directed by the FXFP-motif, whereas the D-domain mediates phosphorylation at other sites (Fantz, Jacobs et al. 2001). Phosphorylation at the serine 383 residue was shown to be most critical for Elk-1 transcriptional ability (Gille, Kortenjann et al. 1995). Despite the importance of serine 383 phosphorylation, other phospho-acceptor sites are modified to the same stoichiometry (Cruzalegui, Cano et al. 1999), however the functional role of other sites remains elusive.

1.1.5.2. The role of Elk-1 phosphorylation

Phosphorylation regulates several Elk-1 activities. First Elk-1 phosphorylation relieves its auto-inhibition by disrupting intra-molecular interaction between the ETS- and the C-domain

(Yang, Shore et al. 1999), resulting in the enhancement of its DNA binding capability (Gille, Sharrocks et al. 1992; Gille, Kortenjann et al. 1995; Sharrocks 1995).

Second, the Id helix-loop-helix (HLH) proteins inhibit Elk-1 DNA binding by masking the ETS-domain (Yates, Atherton et al. 1999). Id proteins usually interact with other HLH proteins to block their DNA binding. Phosphorylation of Elk-1 was shown to inhibit its interaction with the Id proteins, thereby enabling DNA binding.

Third, Elk-1 transactivational ability is potentiated by phosphorylation (Hill, Marais et al. 1993; Marais, Wynne et al. 1993; Gille, Kortenjann et al. 1995), presumably by enhancing its interactions with co-activators. The CREB binding protein (CBP) is a co-activator for Elk-1. CBP acetylates histones to relieve transcriptional repression. It was demonstrated that Elk-1 interacts with CBP on the *c-fos* SRE in a phosphorylation independent manner, however functional cooperation between the two proteins requires Elk-1 phosphorylation (Janknecht and Nordheim 1996; Nissen, Gelly et al. 2001). Furthermore, a similar histone acetyltransferase (P300) was reported to facilitate Elk-1 transactivation in the same manner (Li, Yang et al. 2003).

Fourth, Elk-1 phosphorylation is also implicated in the recruitment of transcription mediators (Boyer, Martin et al. 1999). The Srb mediator complex, consisting of 20 - 30 subunits, acts as a molecular bridge to connect transcription activators to the general transcription machinery to facilitate transcription (Kornberg 2005). A subunit (Sur-2) of the mediator complex has been shown be recruited to Elk-1 by phosphorylation (Stevens, Cantin et al. 2002).

Finally, besides contributing to transactivation, Elk-1 phosphorylation also mediates transcriptional repression. Phosphorylation of Elk-1 recruits the co-repressor mSin3A-HDAC, which exhibits histone deacetylase ability. Histone deacetylases alter local

chromatin conformation to repress transcription. Elk-1 mediates the temporal acute expression of many immediate early genes upon stimulation. The recruitment of mSin3A-HDAC correlates to the kinetics of target gene shutdown, thereby providing a mechanism by which Elk-1 phosphorylation mediates a transient burst of transactivation (Yang, Vickers et al. 2001).

1.1.6. Elk-1 target genes and biological function

Elk-1 was initially identified as part of the ternary complex with SRF at the *c-fos* SRE (Shaw, Schröter et al. 1989), which is commonly found in the promoters of many immediate early genes, a set of genes that are rapidly and transiently activated when cells are stimulated with growth factors or mitogens to mediate cell cycle entry from the G_0 to G_1 phase (Treisman 1990; Treisman 1992). Subsequently, other immediate early gene targets for Elk-1 such as *egr-1* and *jun-B* have been verified (Hipskind, Baccarini et al. 1994; Hodge, Liao et al. 1998). It has also been demonstrated that Elk-1 interacts with Pax5 on a composite element of the *mb-1* promoter (Fitzsimmons, Hodsdon et al. 1996) and binds to the ETS binding site in the TNFα promoter (Tsai, Falvo et al. 2000). To date, the full spectrum of Elk-1 target genes is not clear. Using an Elk-1 repressor fusion protein to suppress Elk-1 mediated transcription, it was demonstrated that Elk-1 target genes are critical for cell proliferation and apoptosis inhibition in a SRF dependent manner (Vickers, Kasza et al. 2004). In addition, a recent study has reported that Elk-1 also targets many SRF-independent genes and a high degree of redundancy between Elk-1 and other ETS-proteins in transcriptional activation was observed (Boros, O'Donnell et al. 2009).

A role in neuronal function of Elk-1 has been suggested based on its expression pattern (Sgambato, Vanhoutte et al. 1998). *Elk-1* mRNA was found in various adult rat brain

structures including soma, dendrite and axon terminals of neurons. It was demonstrated that Elk-1 promotes neuronal cell proliferation through activation of *c-fos* while a truncated isoform of Elk-1 plays an opposite role to full length Elk-1 in neuronal differentiation (Vanhoutte, Nissen et al. 2001). However, Elk-1 knockout mice are viable and do not exhibit strong phenotypical abnormalities (Cesari, Rennekampff et al. 2004), possibly due to the redundant activities of other ETS-proteins.

1.2. Ubiquitylation

1.2.1. Overview and historical perspective

Ubiquitylation was discovered in the early 1980's as a post-translational modification, in which a small protein (ubiquitin, 76 amino acids, ~8.5kDa) is attached to a protein substrate (Pickart 2001; Glickman and Ciechanover 2002; Fang and Weissman 2004). The conjugation of ubiquitin is found in a broad range of proteins and impacts in almost all cellular activities. Proteins can be conjugated with a single or multiple ubiquitin moieties (see chapter 1.2.7), which often confer different activity to the substrate once conjugated. Despite the initial finding that polyubiquitylation directs proteins for destruction by the 26S proteasome, non-proteolytic roles of ubiquitylation were subsequently revealed. In 2004, Aaron Ciechanover, Avram Hershko and Irwin Rose were awarded the Nobel Prize in chemistry for their discovery of the ubiquitin system (Giles 2004).

1.2.2. Mechanism of ubiquitylation

Ubiquitylation is performed by a three-step enzymatic cascade (Pickart 2001; Passmore and Barford 2004). In the first step, an E1 activating enzyme activates ubiquitin in an ATP-dependent manner forming an ubiquitin-E1 thiol ester between its catalytic cysteine and the carboxyl-terminal glycine of ubiquitin, thereby activating the ubiquitin for nucleophilic attack. In the second step, the activated ubiquitin is transferred to the catalytic cysteine of an E2 conjugating enzyme. The ubiquitin is consequently transferred to the ε-amino group of a substrate lysine via an isopeptide linkage, facilitated by an E3 ligase through two distinct mechanisms, depending on the type of E3 ligase involved. In rare cases ubiquitin is attached to the free α-amino group of the substrate instead of a lysine via a peptide linkage

(Breitschopf, Bengal et al. 1998; Aviel, Winberg et al. 2000; Reinstein, Scheffner et al. 2000; Kirisako, Kamei et al. 2006; Tokunaga, Sakata et al. 2009).

1.2.3. Ubiquitin enzymes

Ubiquitylation is a highly regulated process and specific ubiquitylation of substrates depends on sequential action of all three types of ubiquitin enzymes. The organization of these enzymes is hierarchical, where the human genome encodes two E1 activating enzymes, 38 E2 conjugating enzymes, and approximate 600 E3 ligases (Petroski and Deshaies 2005; Groettrup, Pelzer et al. 2008; Ye and Rape 2009). In this enzymatic cascade, the two types of E1 activating enzymes cooperate with a different but overlapping set of E2 conjugating enzymes, which in turn work with a much larger number of E3 ligases. E3 ligases can also cooperate with more than one E2 conjugating enzyme.

1.2.4. Ubiquitin E3 ligases

E3 ligases play a crucial role in ubiquitylation by selecting substrates (Ardley and Robinson 2005). There are two major types of E3 ligases, characterised by the presence of their catalytic HECT- or RING-domains which facilitate ubiquitylation through different enzymatic mechanisms (Figure 1-5). The HECT-domain E3 ligases initially transfer ubiquitin to a catalytic cysteine within the HECT-domain forming an E3-ubiquitin thioester and then transfer the ubiquitin to substrates (Kee and Huibregtse 2007). Conversely, RING-domains do not contain a catalytic cysteine and RING-domain E3 ligases merely act as a bridge orienting the E2-ubiquitin thioester and substrates in close proximity for direct ubiquitin transfer (Petroski and Deshaies 2005). In addition, two other domains (U-box and

PHD-domain) related to the RING-domain are also implicated in ubiquitylation (Aravind and Koonin 2000; Capili, Schultz et al. 2001).

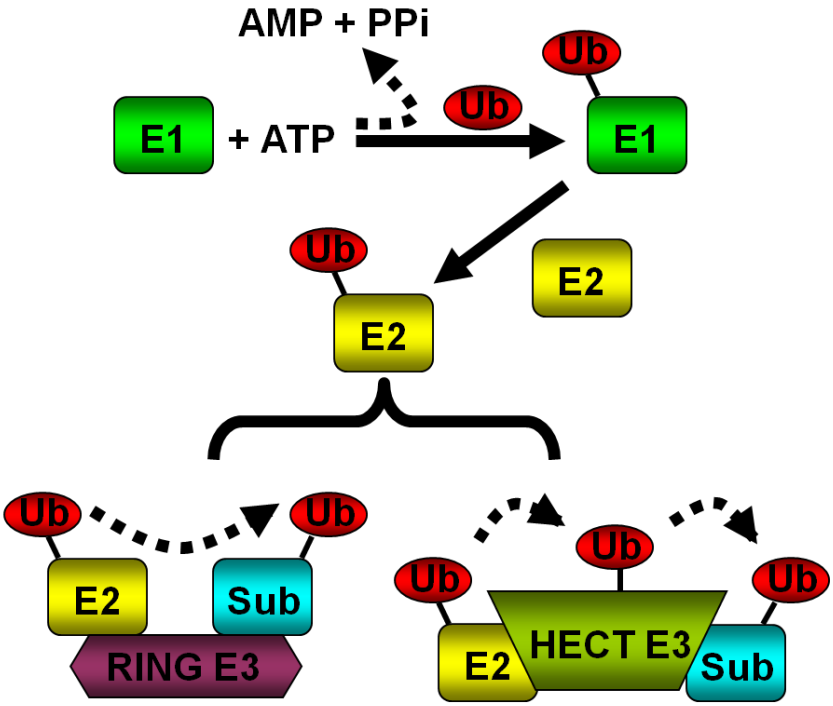

Figure 1-5 Mechanism of ubiquitylation mediated by HECT- and RING-domain E3 ligases. E1 activating enzyme utilises energy from ATP hydrolysis to activate ubiquitin. Ubiquitin is then carried as an E1- and E2-thioester and eventually transferred to substrates, facilitated by E3 ligases. HECT-domain E3 ligases form an ubiquitin thioester intermediate before substrate conjugation whereas RING-domain E3 ligases transfer ubiquitin from an E2 conjugating enzyme to a substrate directly.

1.2.4.1. RING-domain E3 ligases

RING-domain E3 ligases can exist either as monomers (e.g. Mdm2 and CBL), or as dimeric (e.g. RNF4) or multimeric protein complexes (e.g. the SCFs and the APC). Most RING-

domain E3 ligases are multimeric, the largest group of E3 ligases are Cullin-RING Ligases (CRLs). SCF^{Cdc4} is the archetype of CRLs and was first described in studies on cell division in *Saccharomyces cerevisiae* (Feldman, Correll et al. 1997; Skowyra, Craig et al. 1997). All SCFs contain the cullin scaffold CUL1 and their substrate recognition subunits are usually represented in superscript. Other CRLs are named after their cullin subunits and substrate recognition subunits. Characterisation of SCFs has revealed the typical components of CRLs which include a cullin family member, a RING-domain protein and a variable substrate receptor (either a single or multiple subunits) (Kamura, Koepp et al. 1999; Seol, Feldman et al. 1999; Tan, Fuchs et al. 1999).

The scaffold protein cullin is evolutionary conserved from yeast to mammals with the highest conservation in the C-terminal cullin homology domain. The human genome encodes seven cullins (CUL1, 2, 3, 4a, 4b, 5 and 7) and two other proteins that contain the cullin-homology domain (APC2 Subunit in the APC E3 ligase complex and PARC)(Yu, Peters et al. 1998; Zachariae, Shevchenko et al. 1998; Nikolaev, Li et al. 2003). Structural and sequence comparison studies revealed that all cullins adopt a curved structure with an N-terminal stalk that is comprised of three repeats of a five helix bundle (cullin repeat [CR] 1-3) and a C-terminal globular domain (Zheng, Schulman et al. 2002). The substrate receptor associates with the N-terminal CR1 region, while the C-terminal globular domain binds to the catalytic RING-subunit which in turn associates with an E2 conjugating enzyme.

The RING-domain is defined by a distinct arrangement of eight conserved cysteine and histidine residues that coordinate two zinc ions in a crossed-braced fashion (Borden and Freemont 1996). One common feature of CRLs is that most of them utilise the same catalytic RING-subunit. Most cullins bind to the same RING-subunit RBX1, except for CUL5, which interacts with RBX2 (Ohta, Michel et al. 1999). Despite the similarity in the catalytic core, the substrate receptors of CRLs are more diverse to accommodate their vast substrate

spectrum and often these receptors are comprised of an adaptor protein and a substrate recognition unit.

For the archetypal SCF^{SKP2}, the adaptor protein SKP1 associates with CUL1 via its N-terminal BTB-domain (Broad complex, Tramtrack and Bric-à-Brac domain), whereas its C-terminus binds to the substrate recognition F-box protein SKP2 (Schulman, Carrano et al. 2000; Zheng, Schulman et al. 2002). Besides CUL1, SKP1 can also recruit a substrate receptor to CUL7 (Dias, Dolios et al. 2002). Subsequently, other adaptor proteins have been shown to interact with cullins via a domain structurally similar to the F-box. For example, the adaptor protein Elongin C has been demonstrated to associate with CUL2 and CUL5 (Stebbins, Kaelin et al. 1999).

Substrate recognition units typically contain adaptor interacting domain at the N-terminus. Two adaptor interacting domains were reported (F-box or SOCS-box). The F-box was demonstrated to associate with Skp1, which in turn associates with CUL1 and CUL7 (Bai, Sen et al. 1996). The SOCS box was found to interact with Elongin C, which in turn binds to CUL2 and CUL5 (Kamura, Sato et al. 1998). Conversely, the receptor recognition unit of CUL3-based ligases is able to interact directly with CUL3 via its BTB-domain (Geyer, Wee et al. 2003). On the other hand, substrate binding is usually mediated by the WD40-motif or Leucine Rich Repeats (Kipreos and Pagano 2000).

1.2.4.2. CRLs regulation by neddylation

The activity of CRLs is regulated by several mechanisms, one of which is cullin neddylation (Merlet, Burger et al. 2009). Nedd8 is an ubiquitin-like modifier. The three-step enzymatic mechanism of neddylation is similar to ubiquitylation, mediated by the Nedd8 specific E1

activating enzyme (Ula1/Uba3), E2 conjugating enzyme (Ubc12) and E3 ligase (DCN1) (Gong and Yeh 1999; Kurz, Ozlu et al. 2005).

Neddylation is essential for CRL activity (Podust, Brownell et al. 2000; Read, Brownell et al. 2000; Wu, Chen et al. 2000). All known cullin proteins can be neddylated (Hori, Osaka et al. 1999) at a conserved lysine residue at the C-terminus (Osaka, Saeki et al. 2000). Neddylation was initially thought to activate CRLs by enhancing ubiquitin E2 recruitment (Kawakami, Chiba et al. 2001; Sakata, Yamaguchi et al. 2007). However, structural studies revealed a 50Å gap between the activated E2 and the substrate and some ubiquitin E2s (e.g. CDC34) simply lack the putative Nedd8 binding site, which draw doubts to the proposed mechanism of activation. It was later clear that neddylation activates CRLs by weakening the auto-inhibitory interaction between its E2-bound RING-domain and the cullin scaffold (Yamoah, Oashi et al. 2008), resulting in a flexibly linked E2-bound RING-domain to position E2-bound ubiquitin and the substrate in close proximity for ubiquitin conjugation (Duda, Borg et al. 2008; Saha and Deshaies 2008).

1.2.4.3. HECT-domain E3s

The first identified HECT-domain E3 ligase was E6-AP, which mediates p53 ubiquitylation in cells expressing the human papillomavirus E6 oncoprotein, resulting in p53 degradation by the 26S proteasome (Scheffner, Huibregtse et al. 1994). Subsequently, other proteins were found to share similarity with the C-terminus of E6-AP and such regions were named HECT (Homologous to the E6-AP Carboxyl Terminus) (Huibregtse, Scheffner et al. 1995).

There are approximately 50 HECT-domain E3 ligases in humans with a size range from 90 kDa to over 500 kDa (Kee and Huibregtse 2007; Rotin and Kumar 2009). The 350 amino acid long HECT domain is invariably located at the C-terminus of HECT-domain E3 ligases,

whereas their N-terminus contains determinants for substrate recognition and regulation. Structural studies have revealed that HECT is a bi-lobal domain (Huang, Kinnucan et al. 1999; Verdecia, Joazeiro et al. 2003; Ogunjimi, Briant et al. 2005) where the smaller C-terminal lobe contains the catalytic cysteine residue and the larger N-terminal lobe interacts with an E2 conjugating enzyme. The two lobes are connected by a short linker, which is critical for juxtaposing the two catalytic cysteine residues of an E2 conjugating enzyme and an E3 ligase for the transthiolation. The mechanism of ubiquitin transfer between HECT-domain E3 ligases and substrates is still largely unknown, however the phenylalanine residue, four residues from the end of the protein, was shown to be important in the positioning of E3-linked ubiquitin for substrate conjugation in most HECT-domain E3 ligases (Salvat, Jariel-Encontre et al. 1998).

1.2.5. E2 conjugating enzymes

Much of the investigations in ubiquitylation were carried out on E3 ligases due to their ability to confer substrate specificity and E2 conjugating enzymes were thought to play an auxiliary role as activated-ubiquitin carriers. However, increasing evidence has suggested that E2 conjugating enzymes play a crucial role in determining the processivity of the ubiquitin chain assembly and the topology and the length of ubiquitin chain (Ye and Rape 2009; David, Ziv et al. 2010). There are 13 different E2 conjugating enzymes (Ubc 1-13) in *Saccharomyces cerevisiae* and 38 in humans (van Wijk and Timmers 2010). Some of those facilitate ubiquitin-like modifier conjugation rather than ubiquitylation, for example Ubc9 and Ubc12 mediate sumoylation and neddylation respectively. All E2 conjugating enzymes share a 150 amino acid conserved ubiquitin-conjugating domain (UBC) that contains a catalytic cysteine residue. Ubiquitin E2 variants (UEV) also contain the UBC domain but lack this residue. The UBC domain adopts a structure of four α-helices, an anti-parallel β-

sheet formed by four strands and a short 3_{10}-helix (Lin, Hwang et al. 2002; Ozkan, Yu et al. 2005; Eddins, Carlile et al. 2006) where the catalytic cysteine is located in a shallow groove formed by a short loop linking the α2-helix with the α3-helix and a long loop proximal to the active site. In addition, it has been demonstrated that linkage specific E2 conjugating enzymes often function as heterodimers where one subunit positions the desired lysine in ubiquitin to another subunit to facilitate linkage-specific elongation (David, Ziv et al. 2010).

1.2.6. E1 activating enzymes

To date, two E1 activating enzymes for ubiquitylation have been identified (Groettrup, Pelzer et al. 2008). Traditionally Ube1 was thought to be the only E1 enzyme since its discovery in 1981. Recently another E1 activating enzyme (Uba6) has been demonstrated to activate both ubiquitin and the ubiquitin like modifier FAT 10 (Jin, Li et al. 2007; Pelzer, Kassner et al. 2007). The two E1 activating enzymes cooperate with only a partially overlapping pool of E2 conjugating enzymes, suggesting another level of regulation on protein ubiquitylation distinguished by different E1-mediated ubiquitin activation. However at present the redundancy and specificity of the two E1 activating enzymes or the dual functions of Uba6 are not clearly understood.

1.2.7. Ubiquitylation linkage

The ubiquitin system is involved in diverse cellular activities and ubiquitin conjugation can change the fate of the protein. To accommodate a plethora of different activities, protein ubiquitylation can exist in many forms to increase its versatility. Proteins can be monoubiquitylated with a single ubiquitin attachment at a single lysine residue, or multi-monoubiquitylated where several single ubiquitins are attached to multiple lysine residues.

Ubiquitin can also be self-conjugated, there are seven lysine residues in ubiquitin, each of which can serve as an acceptor site to form ubiquitin polymers. Furthermore ubiquitins can be linearly linked via their N-terminus and C-terminus (Kirisako, Kamei et al. 2006; Tokunaga, Sakata et al. 2009). To add to the complexity, ubiquitin polymer linkage can be homologous, heterogeneous or even branched, where two ubiquitin moieties are conjugated and branched out on a single ubiquitin (Komander 2009).

1.2.7.1. Multiple mono- and mono-ubiquitylation

Multiple mono- and mono-ubiquitylation generally leads to non-proteolytic activity. It has been reported that multiple monoubiquitylation of cell surface receptors triggers their internalisation and subsequent degradation in lysosomes or recycling to the cell surface (Haglund, Sigismund et al. 2003). This process is mediated by a multimeric protein complex, ESCRT (Endosomal Sorting Complex Required for Transport), which facilitates the sorting of endosomal cargos into vesicles of multivesicular bodies (Williams and Urbe 2007).

Monoubiquitylation has been shown to be impacted in translesion synthesis, protein trafficking and transcription (Bienko, Green et al. 2005; Ferdous, Sikder et al. 2007; Marchenko and Moll 2007). Most DNA lesions block the progression of replicative forks because replicative DNA polymerases are unable to accommodate damaged bases in their active site, resulting in the recruitment of a specialised set of DNA polymerases to carry out translesion synthesis (Friedberg, Lehmann et al. 2005; Lehmann, Niimi et al. 2007). DNA lesion induces monoubiquitylation of Proliferating Cell Nuclear Antigen (PCNA) which recruits the specialized DNA polymerase η via its ubiquitin binding domain to replicate past DNA lesion (Hoege, Pfander et al. 2002; Kannouche, Wing et al. 2004; Watanabe, Tateishi et al. 2004). Interestingly, monoubiquitylation can also play an opposite role in this process

depending on the modified substrate. Monoubiquitylation of DNA polymerase η was shown to induce an intramolecular interaction with its ubiquitin binding domain, inhibiting its PCNA interaction and translesion synthesis (Bienko, Green et al. 2009).

Monoubiquitylation also mediates p53 trafficking (Li, Brooks et al. 2003; Marchenko, Wolff et al. 2007). In unstressed cells, p53 is rapidly polyubiquitylated by Mdm2 and degraded (Kubbutat, Ludwig et al. 1998), whereas stress inhibits Mdm2-mediated p53 polyubiquitylation stabilising p53. At low level of Mdm2, p53 was shown to be monoubiquitylated resulting in its nuclear exportation (Li, Brooks et al. 2003). Furthermore following stress-induced stabilisation, a pool of monoubiquitylated p53 in the cytosol is translocated to the mitochondria, where it is deubiquitylated to become active to initiate mitochondrial apoptosis (Marchenko, Wolff et al. 2007).

Monoubiquitylation also impacts on transcriptional activation (Ferdous, Sikder et al. 2007; Archer, Burdine et al. 2008; Archer, Delahodde et al. 2008), where 26S proteasome ATPase mediated-destabilisation of the promoter-activator complex is prevented by monoubiquitylation of the transcription activator Gal4. This provides a model that monoubiquitylation of activators limits the lifetime of active transcription complexes until they are stripped off by ATPases or elongated to an ubiquitin polymer for destruction. Furthermore, monoubiquitylation also serves as a priming event for ubiquitin chain elongation (Hoege, Pfander et al. 2002; Rodrigo-Brenni and Morgan 2007; Windheim, Peggie et al. 2008).

1.2.7.2. Polyubiquitylation

Lysine 48- and lysine 63-linked polyubiquitylation are the most extensively studied forms of polyubiquitylation, ubiquitin polymers via other lysines (atypical linkages) were largely

neglected until recent years. A quantitative study of the ubiquitin polymer linkage of the yeast "ubiquitome" using mass spectrometry revealed the abundance of atypical linkages (Xu, Duong et al. 2009), indicating that they may play bigger roles in regulation of cellular activities than previously appreciated. The lysine 48 linkage was found to be the most abundant, accounting for 29 % of all ubiquitin linkages in yeast. Interestingly, the lysine 11 linkage was the second most abundant with the relative abundance of 28 %. Linkage via lysine 63 accounted for 17 %. Other linkage via lysine 6, lysine 27, lysine 29 and lysine 33 accounted for the relative abundance of 11 %, 9 %, 3 % and 3 % respectively.

1.2.7.2.1. Lysine 48-linked polyubiquitylation

Protein degradation is traditionally associated with the lysine 48-linked ubiquitin polymer. Lysine 48-linked polyubiquitylated proteins are rapidly degraded by the 26S proteasome (see chapter 1.2.8 for 26S proteasome). It has been demonstrated that tetra-ubiquitin is sufficient to trigger proteolysis by increasing proteasomal affinity (Thrower, Hoffman et al. 2000). Enzymes specific for lysine 48 linkage were described and analysed, for example the E3 ligase SCF^{Cdc4} cooperates with the E2 conjugating enzyme Cdc34 to attach lysine 48-linked ubiquitin polymer to the CDK inhibitor Sic1 (Petroski and Deshaies 2005).

1.2.7.2.2. Lysine 63-linked polyubiquitylation

Asides from protein degradation, polyubiquitylation can also lead to non-proteolytic activities. Numerous studies have demonstrated that lysine 63-linked polyubiquitylation occurs during endocytosis, DNA damage and kinase activation in signal transduction. A set of E2 conjugating enzymes (Ubc13/Uev1A) has been shown to specifically facilitate the

formation of lysine 63-linked ubiquitin polymers (Hofmann and Pickart 1999; Deng, Wang et al. 2000).

The most studied lysine 63 polyubiquitylation mediated activities are in the NFkB pathway, reviewed by (Skaug, Jiang et al. 2009). Stimulation of cells with cytokines such as Tumor Necrosis Factor α or interleukin 1 sequentially triggers activation of two kinase complexes, the TAK1 and the IKK complex. The TAK1 complex activates the IKK complex and eventually leads to the activation of the NFkB transcription factor. The TAK1 complex assembly is mediated by the lysine 63-specific ubiquitin binding domains of TAB2 and TAB3 and is dependent on TAK1 lysine 63-linked polyubiquitylation (Wang, Deng et al. 2001; Kanayama, Seth et al. 2004). Many other components within the receptor complexes , including TRAFs, RIP1 and IRAKs are also lysine 63-linked polyubiquitylated (Wang, Deng et al. 2001; Ea, Deng et al. 2006; Lamothe, Besse et al. 2007; Ordureau, Smith et al. 2008), which contributes to receptor complex assembly. In addition, the regulatory subunit IKKγ of the IKK complex was also shown to bind lysine 63 ubiquitin polymers for its receptor association (Wu, Conze et al. 2006).

Endocytosis was initially linked with mono- and multiple monoubiquitylation (see chapter 1.2.7.1), however increasing evidence has suggested lysine 63-linked polyubiquitylation is also implicated in these processes (Geetha, Jiang et al. 2005; Duncan, Piper et al. 2006; Kamsteeg, Hendriks et al. 2006). For example, lysine 63-linked polyubiquitylation of the nerve growth factor receptor Trka triggers its internalisation. Moreover a deubiquitinase specific for lysine 63 linkage has also been reported to mediate endocytosis, supporting the involvement of lysine 63 polyubiquitylation in endocytosis (McCullough, Clague et al. 2004).

Lysine 63 polyubiquitylation has also been shown to regulate DNA repair. Regulation of the DNA damage tolerance pathway known as post-replication repair is mediated by

ubiquitylation of PCNA. Monoubiquitylation of PCNA has been reported to trigger translesion synthesis, an error prone way for DNA replication (Hoege, Pfander et al. 2002). The single ubiquitin attachment can be extended via lysine 63, which switches DNA repair to the error free template switching pathway, reviewed by (Moldovan, Pfander et al. 2007). In addition, lysine 63 polyubiquitylation has been reported to mediate repair in DNA double-strand breaks (Panier and Durocher 2009). The E3 ligase RNF8 is recruited to damaged chromatin to monoubiquitylate histone H2A (Huen, Grant et al. 2007; Mailand, Bekker-Jensen et al. 2007). The E3 ligase RNF168 is in turn recruited by monoubiquitylated histone H2A to mediate lysine 63 chain extension, resulting in recruitment of the DNA repair proteins 53BP1 and BRCA1 (Doil, Mailand et al. 2009; Stewart, Panier et al. 2009).

1.2.7.2.3. Polyubiquitylation via other linkages

Ubiquitins can be linked through a peptide bond between the N-terminal α-amino group and the C-terminal carboxyl group. Such head to tail ubiquitin polymers are the source of cellular free ubiquitin encoded by four genes in the human genome (Kimura and Tanaka 2010), which need to be hydrolysed into single unit by deubiquitinases before substrate conjugation (Komander, Clague et al. 2009). Linear ubiquitin chain conjugation to a protein substrate has also been described in the IKK kinase complex activation of the NFkB pathway (Kirisako, Kamei et al. 2006; Tokunaga, Sakata et al. 2009).

Proteins implicated in diverse cellular processes were reported to be modified by lysine 11-linked ubiquitin polymer (Xu, Duong et al. 2009), indicating its physiological importance. Although protein degradation was commonly associated with lysine 48-linked polyubiquitylation, lysine 11-linked ubiquitin polymer can also serve as a potent degradative signal (Baboshina and Haas 1996; Jin, Williamson et al. 2008). Many studies on protein

degradation were done without any validation of the chain type. Together with the abundance of lysine 11-linked ubiquitylated proteins in yeast (Xu, Duong et al. 2009), this suggests that lysine 11 linkage may play a bigger role in proteolysis than previously thought.

In addition, assembly of heterogeneous and branched ubiquitin polymers was reported using certain sets of purified E2 conjugating enzymes and E3 ligases (Kim, Kim et al. 2007) Ubiquitylation is relatively promiscuous *in vitro* compared to that under physiological conditions, currently the *in vivo* abundance and relevance of these linkages are uncertain. However it is worth noting that branched ubiquitin polymers cannot be processed efficiently by the proteasome (Kim, Kim et al. 2009), suggesting their non-proteolytic roles. Branched ubiquitin polymer is also involved in E3 ligase activation. Autoubiquitylation of lysine 6/ lysine 27/ lysine 48-branched ubiquitin chains to the E3 complex Ring1B/BMI1 is essential for its ability to monoubiquitylate its physiological target histone H2A *in vitro* (Ben-Saadon, Zaaroor et al. 2006). To add to the complexity, cyclin B1 was shown to be conjugated with multiple mono- and short-polyubiquitin polymers leading to its degradation (Kirkpatrick, Hathaway et al. 2006)

1.2.8. 26S proteasome-mediated proteolysis

Degradation of lysine 48-linked polyubiquitylated proteins is mediated by the 26S proteasome, reviewed by (Glickman and Ciechanover 2002). The proteasome complex consists of a 19S regulatory cap and a 20S proteolytic core. The 19S cap in turn comprises of two subcomplexes; the lid, containing 10 subunits that relate to the Constitutively Photomorphogenic 9 (COP9) signalosome; and the base, composed of six AAA-type ATPases (Rpt1-6) and two non-ATPase subunits. Both the lid and base subcomplexes are responsible in the recognition of polyubiquitylated proteins (Elsasser, Chandler-Militello et al. 2004; Verma, Oania et al. 2004). Following recognition, the substrate is deubiquitylated

(Verma, Aravind et al. 2002; Guterman and Glickman 2004). The ATPases then unfold the substrate and mediate its translocation into the cavity of the 20S core to be degraded by the proteases in the proteolytic core or in some cases of protein processing, where protein precursors are partially degraded to create mature proteins (Benaroudj, Zwickl et al. 2003; Liu, Corboy et al. 2003).

1.2.9. Deubiquitylation

Deubiquitylation is a reversal process of ubiquitylation, carried out by about 100 deubiquitinases (DUBs) encoded in the human genome. DUB activity can be divided into three categories. Firstly, DUBs generate free ubiquitin from ubiquitin precursors, expressed from four ubiquitin genes. Secondly, ubiquitylation of protein substrates can be removed by DUBs, counteracting E3 ligase activity. Finally, DUBs are involved in ubiquitin chain linkage editing.

There are five classes of DUBs; Ubiquitin C-terminal Hydrolases (UCHs); Ubiquitin-Specific Proteases (USPs); Ovarian Tumor Proteases (OTUs); Josphins and JAB1/MPN/MOV34 (JAMM) metalloenzymes. A common feature of DUBs is the presence of Ubiquitin Binding Domains (UBDs), for example: the Zinc Finger Ubiquitin-specific Protease domain (ZnF-UBP-domain), the Ubiquitin-Interacting Motif (UIM) and the Ubiquitin Associated (UBA) domain. These UBDs directly regulate DUB activity or specificity. It has been demonstrated that the UIMs of DUBs (USP25, DUBA and Ataxin-3) are essential for their hydrolytic activity (Mao, Senic-Matuglia et al. 2005; Kayagaki, Phung et al. 2007; Meulmeester, Kunze et al. 2008). The UIM also imparts ubiquitin polymer linkage specificity. For example, the proteasome associated USP14 preferentially targets the

lysine 48 linkage, while Ataxin-3 has been shown to cleave specifically lysine 63 linkage (Winborn, Travis et al. 2008).

DUB activity is regulated by several mechanisms. For example, the IKK complex mediated phosphorylation of the DUB CYLD inhibits its capacity to deubiquitylate TRAF2, resulting in the activation of the NFkB pathway (Reiley, Zhang et al. 2005). Phosphorylation of another DUB, USP8, recruits the phospho-binding protein 14-3-3, resulting to the inhibition of its activity during interphase (Mizuno, Kitamura et al. 2007). In addition, most DUBs are cysteine proteases, therefore it is not surprising that reactive oxygen species regulates some DUB activity. For example, hydrogen peroxide inhibits deubiquitylating activity of the DUB Cezanne to RIP1, resulting in the activation of the NFkB pathway (Enesa, Ito et al. 2008). DUBs are also regulated through subcellular separation. For example, USP8 has been demonstrated to undergo epidermal growth factor (EGF)-dependent translocation to endosomes (Row, Prior et al. 2006).

1.3. Regulation of ubiquitylation

1.3.1. Regulation of ubiquitylation by phosphorylation

Ubiquitylation is often regulated by phosphorylation. Phosphorylation can affect ubiquitylation in three different ways. First, phosphorylation of substrates can create an inducible interaction surface to allow E3 ligase recognition, namely a phosphodegron. Second, phosphorylation of either substrates or ubiquitin enzymes may alter their subcellular localization, whereby restricting their interactions. Third, phosphorylation of ubiquitin enzymes can modulate their activity allosterically or through recruiting accessory proteins. Alternatively, similar regulations can be applied on deubiquitinases to regulate their hydrolytic ability.

1.3.1.1. Phosphodegron

Protein phosphorylation often leads to its instability. Many cell cycle regulators are sensitised for destruction by phosphorylation, for example; the G1 cyclin Cln2 and the CDK inhibitor Sic1p in *Saccharomyces cerevisiae* (Lanker, Valdivieso et al. 1996; Verma, Annan et al. 1997); cyclin E and the CDK inhibitor p27Kip1 in human (Clurman, Sheaff et al. 1996; Won and Reed 1996; Sheaff, Groudine et al. 1997). Phosphorylation at serine or threonine residues creates a phosphodegron, which can be utilised as a signal for ubiquitylation-mediated protein degradation. In most cases, such phosphorylation induced proteolysis is mediated by a subgroup of RING-domain E3 ligases, SCF (see chapter 1.2.4). These multimeric E3 ligases often contain a F-box substrate receptor to target specifically phosphorylated substrates (Kipreos and Pagano 2000). Although the majority of substrates recognised by SCFs are phosphorylated, a member of this family, $SCF^{\beta\text{-}TrCP}$, was also

reported to interact with a non-phosphorylated motif of the Cdc25A phosphatase (Kanemori, Uto et al. 2005).

Besides cell cycle regulation, phosphorylation inducible proteolysis is also observed in cytokine signalling pathways. In non-stimulated cells, NFkB proteins are sequestered by IkB in the cytosol. Upon stimulation, the IKK kinase complex is activated and phosphorylates two serine residues at the regulatory domain of the IkB protein creating a phosphodegron, resulting in its ubiquitin-dependent degradation and the release of the NFkB protein to the nucleus to elicit transcription (Yaron, Hatzubai et al. 1998; Karin and Ben-Neriah 2000).

Phosphorylation of substrates can sometimes converge responses from two signalling pathways. In other words, distinct phosphorylation of substrates triggered by different pathways may be required to facilitate phosphorylation-dependent degradation. For example, the interaction between the E3 ligase SCF^{Cdc4} and its substrate cyclin E requires phosphorylation at multiple sites mediated by the kinases CDK2 and GSK3 (Welcker, Singer et al. 2003). A similar regulatory mechanism has also been demonstrated on the hedgehog signalling effector Ci in *Drosophila melanogaster*, where phosphorylation by three different kinases is required for its ubiquitylation-dependent processing (Price and Kalderon 2002). Conversely, ubiquitylation of a substrate can be triggered by phosphorylation mediated by different kinases alone. For example, Gcn4 can be targeted for degradation by either Srb11-Srb10 or Pcl1/2Pho85-CDK mediated phosphorylation in *Saccharomyces cerevisiae* (Meimoun, Holtzman et al. 2000; Chi, Huddleston et al. 2001).

In some cases, multiple phosphorylation of a substrate is required for ubiquitylation. SCF^{Cdc4}-mediated degradation of the CDK inhibitor Sic1 is dependent on phosphorylation of Sic1 on at least six of its nine phosphorylation sites (Orlicky, Tang et al. 2003). Remarkably, the substrate receptor Cdc4 only contains a single phospho-epitope binding motif, thus such

multiple phosphorylation requirement cannot be explained by the traditional model of binding cooperation. This requirement was explained by a mathematical model that the probability of rebinding increases exponentially with the number of phosphodegrons on substrates (Klein, Pawson et al. 2003). This effect was coined 'allovalency', providing a mechanism by which accumulation of phosphorylation to a threshold on substrates acts as a molecular switch to determine interactions with their corresponding E3 ligases.

1.3.1.2. Regulation of ubiquitin enzymes by phosphorylation

Besides phosphodegrons, phosphorylation of E3 ligases can either enhance or inhibit their activity. Phosphorylation at different residues on the same E3 ligase can elicit different responses or phosphorylation of different E3 ligases by the same kinase can result in different outcome. For example, the HECT-domain E3 ligase Nedd 4-2 is responsible for regulating the level of epithelial sodium channel (ENaC). Nedd 4-2 mediated ENaC ubiquitylation leads to the degradation of this channel protein. Upon serum stimulation, the protein kinase SGK1 phosphorylates Nedd 4-2 in its WW domain, masking this substrate recognition domain by recruiting the phospho-binding protein 14-3-3, whereby inhibiting ENaC ubiquitylation and its subsequent degradation (Ichimura, Yamamura et al. 2005). Alternatively, Nedd 4-2 can also be phosphorylated by the stress-induced MAPK JNK at different residues which potentiates its E3 ligase activity to ubiquitylate ENaC for degradation (Hallows, Bhalla et al. 2010).

Apart from recruiting accessory proteins, phosphorylation can also elicit allosteric effects which change protein conformation that alters E3 ligase activity. The HECT-domain E3 ligase Itch facilitates T-cell activation through ubiquitylation of JunB. The activity of this E3 ligase is constitutively repressed by intramolecular interaction between its WW-domain and

the HECT-domain. JNK-mediated phosphorylation within the proline-rich region upstream of the WW-domain relieves this intramolecular repression, permitting its E3 ligase activity (Gallagher, Gao et al. 2006). JNK is recruited to Itch by D-domain like sequences within the N-lobe of the HECT-domain. The presence of D-domain like sequences in the HECT-domain provides a mechanism by which the MAPK pathways could regulate the catalytic activity of HECT-domain E3 ligases. Alternatively, phosphorylation of Itch by the kinase Fyn at another residue reduces its substrate (JunB) interaction, thereby decreasing ubiquitylation of its substrate (Yang, Zhou et al. 2006).

Beside E3 ligases, phosphorylation also regulates the activity of some E2 conjugating enzymes. Phosphorylation of the E2 conjugating enzyme Rad6 has been shown to be essential for its ability to facilitate histone H2B monoubiquitylation (Sarcevic, Mawson et al. 2002; Wood, Schneider et al. 2005).

1.3.2. Regulation of ubiquitylation by subcellular location

Phosphorylation of either E3 ligases or substrates can often alter their subcellular location, providing a mechanism by which spatial restriction regulates protein ubiquitylation. For example, phosphorylation of the CDK inhibitor p27kip1 promotes its nuclear export by CRM1 to permit its ubiquitylation-dependent degradation mediated by the E3 ligase complex KPC in the cytosol (Kamura, Hara et al. 2004; Besson, Gurian-West et al. 2006). Activation of the IKK complex in the NFkB pathway is also regulated by this manner in response to genotoxic stress. Upon DNA damage, the IKK regulatory subunit IKKγ is sumoylated, leading to its nuclear localisation. In the nucleus, IKKγ is phosphorylated by the kinase ATM, which triggers its monoubiquitylation, and in turn leads to the nuclear export of IKKγ and ATM, and eventually the activation of cytosolic IKK complex (Wu, Shi et al. 2006).

1.3.3. Regulation of ubiquitylation by complex formation

E3 ligases often target a selective pool of substrates as mentioned above that CRLs preferentially select phosphorylated substrates. It has been reported that complex formation may also facilitate ubiquitylation. Ubiquitylation of the CDK inhibitor Xic1 has been shown to require the formation of pre-replication complex in *Xenopus* (You, Harvey et al. 2002). In addition, ubiquitylation of p27 is dependent on both its phosphorylation and its trimeric complex with cyclin E and CDK2 (Montagnoli, Fiore et al. 1999).

1.4. Roles of ubiquitylation in transcription

1.4.1. Proteolysis-coupled transcription

In the past decade, mounting evidence has revealed the involvement of the ubiquitin system in the regulation of transcription, reviewed by (Conaway, Brower et al. 2002; Lipford and Deshaies 2003; Kodadek, Sikder et al. 2006; Leung, Geng et al. 2008). The most obvious connection between transcription and the ubiquitin system is the destruction of transcription activators when they are no longer needed for transcription. In this case, activator proteins are most abundant when they are active. For example, the tumour suppressor p53 level and activity are inhibited by the E3 ligase Mdm2 (Haupt, Maya et al. 1997; Fuchs, Adler et al. 1998). However this mode of regulation does not apply in all situations, studies in the past decade have revealed a more complex intervention, in which the ubiquitin system facilitates and regulates transcription. The oncogenic transcription activator Myc appears to be sensitive to proteolysis when it is activated. The region of Myc that mediates transcriptional activation is also found to be essential for its degradation (Salghetti, Kim et al. 1999). The overlapping feature of transactivation domains and degrons was later found to be a common feature in other transcription activators with transactivation domains rich in acidic residues, suggesting this functional connection is evolutionarily conserved (Salghetti, Muratani et al. 2000). Subsequently, about 30 transcription activators, for example: E2F-1, Fos, Gcn4, Jun and p53 were reported to contain such overlapping correlation.

It is now clear that transcription and degradation is intrinsically coupled for some cases. In those cases, transcription activators are degraded as part of the transcription process as a mechanism to restrict their ability to initiate unlimited rounds of transcription, therefore ensuring the acute regulation of activators. Many transcription activators have been demonstrated to possess such connection. For example, SREBP degradation was reported to be dependent on both its transactivation and promoter binding ability (Sundqvist and

Ericsson 2003). In addition, the phosphorylation dependent degradation of Gcn4 is triggered by Srb10, a kinase in the transcription mediator complex (Chi, Huddleston et al. 2001), supporting the notion that transcription and degradation are intrinsically connected.

It is logical that potent transcription activators are degraded to ensure timely regulation of transcription. Ubiquitin conjugation to substrates by E3 ligases can trigger their degradation, indicating the essential role of E3 ligases in the regulation of transcription. Deletion of the E3 ligase gene (*Met30*) inhibits its substrate (LexA-VP16) mediated transactivation and degradation (Salghetti, Caudy et al. 2001). Interestingly, fusing an ubiquitin protein at the N-terminus of LexA-VP16 was able to rescue both activities, indicating the sole purpose of Met30 in Lex-VP16 activity is to ubiquitylate it. Subsequently, a similar requirement of E3 ligases for transcription has been reported for other activators (von der Lehr, Johansson et al. 2003; Muratani, Kung et al. 2005) and co-activators (Kurosu and Peterlin 2004; Barboric, Zhang et al. 2005; Wu, Feng et al. 2007).

1.4.2. The roles of 26S proteasome in transcription

The 26S proteasome was first linked to transcription in yeast genetic studies. Deletion of the *Sug2/Rpt4* and *Sug1/Rpt6* genes allows the transcriptionally defective activator Gal4 to activate transcription (Swaffield, Bromberg et al. 1992; Russell, Sathyanarayana et al. 1996). They were initially thought to be transcription mediators, bridging the gap between transcription activators and the RNA Polymerase II (Kim, Bjorklund et al. 1994; Swaffield, Melcher et al. 1995). Subsequently, the discovery of Sug1 as a component of the 26S proteasome led to the idea that Sug1 mediates transcription indirectly by facilitating degradation of transcription regulatory proteins (Rubin, Coux et al. 1996). However it is now clear that the 26S proteasome facilitates transcription at multiple levels.

The 26S proteasome possesses both proteolytic and chaperone activity, conferred by its 20S core and 19S regulatory subunits respectively. The association of both proteolytic and non-proteolytic components of the 26S proteasome with active genes in a genome-wide study indicates both activities are essential for transcription (Auld, Brown et al. 2006).

For proteolysis-coupled transcription, protein degradation is required for RNA polymerase ll recruitment, transcription co-factor exchange and termination of transcription. Inhibition of the 26S proteasome was found to repress both progesterone receptor- and Gcn4-mediated transcription by reducing RNA polymerase ll recruitment to their cognate promoters (Dennis, Lonard et al. 2005; Lipford, Smith et al. 2005). Another involvement of proteolysis with transcription is facilitated through altering the composition of transcription complexes. In other words, the transcriptional co-factor exchange is often mediated by proteolysis (Kramer, Zhu et al. 2003; Metivier, Penot et al. 2003; Perissi, Aggarwal et al. 2004). For example, in LIM-HD-mediated transcription, destruction of the CLIM co-activator mediated by the RING-domain ligase RLIM (Ostendorff, Peirano et al. 2002). Furthermore, events following initiation also require proteolysis. For example, destruction of Gal4 ensures appropriate phosphorylation of RNA polymerase II, which is essential for transcription initiation and subsequent mRNA processing (Muratani, Kung et al. 2005). It is thought that proteolysis of Gal4 and co-factors post initiation allows RNA polymerase II to abandon its interacting proteins properly in order to recruit mRNA processing proteins. Finally, proteolysis has also been demonstrated to facilitate transcription termination. Inhibition of the proteasome leads to increased read through of termination sites and together with the presence of 20S proteasome at the 3'end of active genes indicate the importance of proteolysis in transcription termination (Gillette, Gonzalez et al. 2004).

There is also a significant amount of work demonstrating the importance of non-proteolytic function of the19S proteasome in transcription, particularly through its ATPase subunits. The

ATPases of the 19S regulatory complex possess the classic protein chaperone activity (Braun, Glickman et al. 1999), giving rise to the idea that ATPases may mediate transcription by facilitating complex assembly or disassembly. Like the 20S proteasome, the 19S proteasome facilities transcription at different levels. It has been demonstrated that the 19S proteins are important for transcription elongation (Ferdous, Gonzalez et al. 2001). Mutations of 19S proteins display phenotypes consistent with an elongation defect in yeast. Immuno-depletion of 19S proteins significantly inhibited the synthesis of long transcripts, but did not influence the rate of transcription initiation. Furthermore, 19S proteins were found to associate with elongation factor, supporting their role in transcription elongation.

Besides elongation, 19S proteins were also reported to facilitate histone H3 methylation, a signal commonly found at transcription active sites (Ezhkova and Tansey 2004). The ATPases Rpt4 and Rpt6 are recruited by histone H2B ubiquitylation to transcription active sites to facilitate subsequent histone H3 methylation.

Another role of 19S proteins in transcription is to recruit SAGA histone acetyltransferase complex recruitment (Lee, Ezhkova et al. 2005). The SAGA complex recruitment is one of the early steps in Gal4-mediated transcription. It was suggested that the ATPases utilise the energy from ATP hydrolyse to drive stable complex between Gal4 and SAGA.

1.4.3. Hypothesized mode of proteolysis coupled transcription initiation

A general consensus of how the ubiquitin system intervenes with transcription has now emerged. A licensing/black widow model has been proposed by several groups (Lipford and Deshaies 2003; Kodadek, Sikder et al. 2006; Leung, Geng et al. 2008). In this model, activators are responsible for recruiting co-activators, the general transcription machinery as well as ubiquitin ligases and the 26S proteasome to promoters. Concurrently with the initiation, the activators and co-activators are ubiquitylated and eventually degraded,

followed by promoter escape and transcription elongation. In addition, the proteasome also facilitates transcription at different stages following initiation of transcription.

Although the licensing model provides a general mechanism of proteolysis-coupled transcription, clearly it cannot explain situations in which activity of activators is inhibited by proteolysis. One explanation is that activators can be ubiquitylated by different E3 ligases under different conditions. In other words, sometimes protein degradation merely serves to down-regulate abundance thus is uncoupled from transcription, while others are integral to transcription. Gal4 has been shown to be regulated by two distinct F-box proteins (the substrate recognition unit of some CRL E3 ligases), Grr1 and Mdm30 under different conditions (Muratani, Kung et al. 2005). When Gal4 is inactive, its degradation is mediated by Grr1 with a sole purpose to keep Gal4 protein at low basal level. However, when Gal4 is activated, the Grr1 mediated proteolytic process is shut down. Gal4 degradation is now mediated by Mdm30 during transcription-coupled proteolysis.

1.5. Research aims and objectives

In response to extracellular signalling through the MAPK pathways, Elk-1 forms a ternary nucleoprotein complex with the SRF dimer at the SRE of the *c-fos* promoter to activate transcription. As a final effector of multiple signalling pathways and a regulator of gene promoters, Elk-1 represents a key element in the complex and dynamic regulation of transcription, therefore providing a paradigm for the study of signal-induced gene expression.

The molecular mechanisms that underlie the function and regulation of Elk-1 have been extensively studied. Elk-1 activity is regulated partly by PTMs. Phosphorylation of Elk-1 regulates its transcriptional ability through several mechanisms, such as relief from intramolecular inhibition, enhanced DNA binding and recruitment of transcription co-activators (Gille, Sharrocks et al. 1992; Hill, Marais et al. 1993; Marais, Wynne et al. 1993; Gille, Kortenjann et al. 1995; Sharrocks 1995; Yang, Shore et al. 1999; Stevens, Cantin et al. 2002). Sumoylation of Elk-1 is associated with transcription repression through recruitment of the histone deacetylase 2 co-represssor (Yang, Jaffray et al. 2003; Yang and Sharrocks 2004). Preliminary data from the group showed the presence of Elk-1 specific E3 ligase activity in HEK293 cell lysate and that Elk-1 is ubiquitylated in HEK293 cells. Elk-1 ubiquitylation has also been reported in vitro (Fuchs, Xie et al. 1997). This study sought to understand the nature of Elk-1 ubiquitylation by investigating its regulation, function and the enzymes involved in this modification.

2. Materials

2.1. Antibodies

Table 1. Antibodies used in this study

Antibody	Manufacturer	Manufacturer reference
αHA	Roche	11 867 423 001
αHis	AbD SeroTec	MCA1396GA
αElk-1(H-160)	Santa Cruz Biotechnology	SC22804
αP-Elk-1	Cell Signalling	9181
αERK2	Santa Cruz Biotechnology	SC154G
αP-ERK	Cell Signalling	9106S
αActin	Sigma Aldrich	A-2066
αTBP	Santa Cruz Biotechnology	SC204
αTubulin	Santa Cruz Biotechnology	SC8035
αPARP	Cell Signalling	9542
αRat HRP conjugated	Sigma Aldrich	A9037
αMouse HRP conjugated	Sigma Aldrich	A6782
αRabbit HRP conjugated	Sigma Aldrich	A0545
αGoat HRP conjugated	Sigma Aldrich	A5420

2.2. Chemicals and commercial kits

ECL detection reagent and ECL advance detection reagent (Amersham)

Emerald (Applied Biosystems)

Gel filtration molecular weight markers (Sigma-Aldrich)

MicroBio-Spin Chromatography Columns (Bio-Rad)

Page Ruler pre-stained protein ladder (Fermentas)

Protease inhibitor cocktail, complete (Roche)

Galacton (Applied Biosystems)

[^{35}S]-methionine (Perkin Elmer)

Quick Change Site-directed mutagenesis kit (Stratagene)

Superose 6 beads (GE Healthcare)

TNT T7 Quick Coupled Transcription/Translation System

U0126 (Sigma-Aldrich)

Ubiquitin aldehyde (Biomol)

Ubiquitin from bovine erythrocytes (Sigma-Aldrich)

SB202190 (Sigma-Aldrich)

2.3. Bacterial Strains

All bacterial works were undertaken using NM522 cells, and plasmids were propagated in this strain.

2.4. Plasmid Constructs

Table 2. Plasmids used in this study

Plasmid	Description	Constructed by/ Reference
pcDNA3.HA.Elk-1	Cell-free and mammalian expression vector for N-terminal HA-tagged human wild-type Elk-1 cDNA	(Janknecht and Nordheim 1992)
pcDNA3.HA.Elk-1 lysine substitution derivatives (see Figure 2-1)	Cell-free and mammalian expression vector for N-terminal HA-tagged human Elk-1 cDNA with the indicated substitutions of lysine with arginine	Leo Chow, this work
pcDNA3.HA.Elk-1.DM (DNA binding defective derivative)	Cell-free and mammalian expression vector for N-terminal HA-tagged human Elk-1 cDNA with the double amino acid mutation of R65A/Y66F	Leo Chow, this work
pcDNA3.HA.Elk-1.SM (SRF binding defective derivative)	Cell-free and mammalian expression vector for N-terminal HA-tagged human Elk-1 cDNA with the double amino acid mutation of L158P/Y159A	Leo Chow, this work
pCMV5.HA.Elk-1	Mammalian expression vector for N-terminal HA-tagged human wild-type Elk-1 cDNA	Peter Shaw's group
pCMV5.HA.Elk-1 phosphorylation site derivatives	Mammalian expression vector for N-terminal HA-tagged human Elk-1 cDNA with the indicated amino acid substitution	(Gille, Kortenjann et al. 1995)
pCMV5.HA.Elk-1.ΔD	Mammalian expression vector for N-terminal HA-tagged human Elk-1 cDNA with the deletion of amino acid residue 312 to321	(Zhang, Li et al. 2008)
pCMV5.HA.Elk-1.FXLA	Mammalian expression vector for N-terminal HA-tagged human Elk-1 cDNA with the mutation of F395L/Q396A	(Zhang, Li et al. 2008)

Plasmid	Description	Constructed by/ Reference
pCMV5.HA.Elk-1. ΔD/ FQLA	Mammalian expression vector for N-terminal HA-tagged human Elk-1 cDNA with the mutation of F395L/Q396A	(Zhang, Li et al. 2008)
pCMV5.Elk-1	Mammalian expression vector for human Elk-1 cDNA	Peter Shaw
pCMV5.Elk-1 lysine substitution derivatives (see Figure 2-1)	Mammalian expression vector for human Elk-1 cDNA with the indicated substitutions of lysine with arginine	Peter Shaw's group
pCMV5.Elk-1.DM (DNA binding defective derivative)	Mammalian expression vector for human Elk-1 cDNA with the double amino acid mutation of R65A/Y66F	Leo Chow this work
pCMV5.Elk-1.SM (SRF binding defective derivative)	Mammalian expression vector for human Elk-1 cDNA with the double amino acid mutation of L158P/Y159A	Leo Chow this work
pCMV5.HA.Sap-1a	Mammalian expression vector for N-terminal HA-tagged human wild-type Sap-1a cDNA	Peter Shaw's group
pCMV5.HA.Net	Mammalian expression vector for N-terminal HA-tagged human wild-type Net cDNA	Thomas Strahl
pCMV5.HA.Ub	Mammalian expression vector for N-terminal HA-tagged human wild-type ubiquitin cDNA	Peter Shaw's group
pCMV5.His6.Ub	Mammalian expression vector for N-terminal His-tagged human wild-type ubiquitin cDNA	Peter Shaw's group
pCMV5.His6.Ub.K0	Mammalian expression vector for N-terminal His-tagged human wild-type ubiquitin cDNA with all lysines substituted with arginines	Sam Shelton Peter Shaw's group

Plasmid	Description	Constructed by/ Reference
$p(SRE)_{x3}Luc$	SRE-dependent luciferase reporter vector	R.A. Hipskind
$p(E74)_{x3}Luc$	E74-dependent luciferase reporter vector	R. Janknecht
pCHll	β-galactosidase reporter vector	Peter Shaw's group

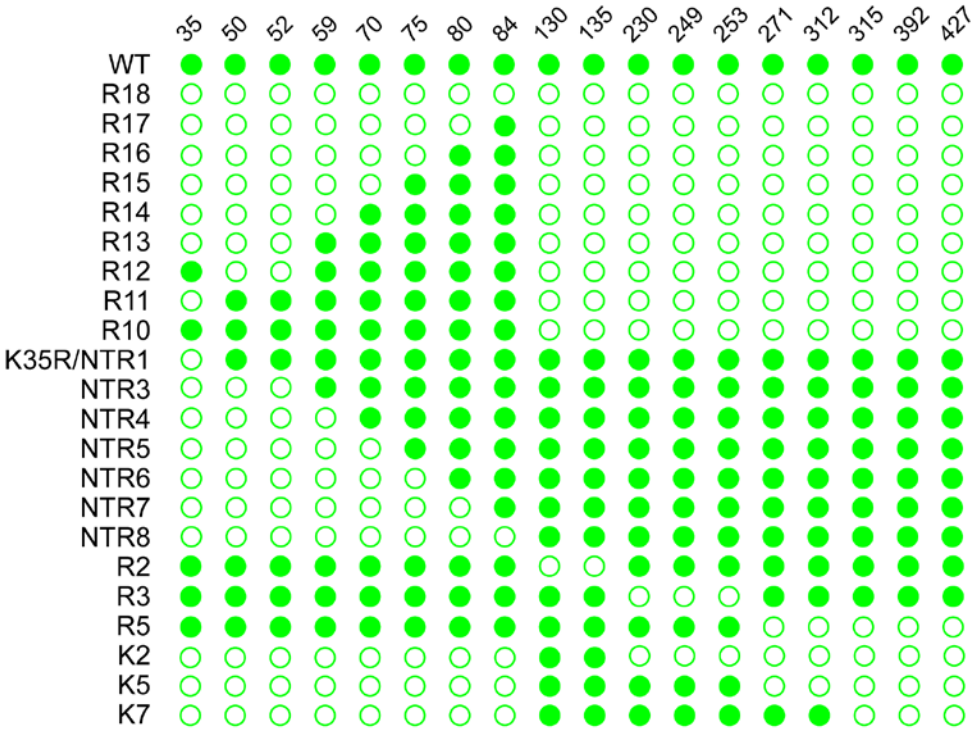

Figure 2-1 Table of the location of the substitutions in Elk-1. Lysine residues and arginine substitutions are represented with green circles and open circles respectively

2.5. Oligonucleotides

All oligonucleotides used in this study were synthesized at the Biopolymer synthesis and analysis unit (BSAU), School of Biomedical Sciences, the University of Nottingham

Table 3.Primers used in site-directed mutagenesis

Oligo name	Sequence	Base pairs
K35R For.	5′-ggatggtggtgaattccggctggtggatgcagagg-3′	35
K35R Rev.	5′-cctctgcatccaccagccggaattcaccaccatcc-3′	35
K50/52R For.	5′ggctgtggggactacgccggaaccggaccaacatgaattacg3′	42
K50/52R Rev.	5′cgtaattcatgttggtccggttccggcgtagtccccacagcc3′	42
K59R For.	5′-agaccaacatgaattacgacaggctcagccggg-3′	33
K59R Rev.	5′-cccggctgagcctgtcgtaattcatgttggtct-3′	33
K70R For.	5′-gcggtactactatgacaggaacatcatccgcaagg-3′	35
K70R Rev.	5′-ccttgcggatgatgttcctgtcatagtagtaccgc-3′	35
K75R For.	5′-aggaacatcatccgcagggtgagcggcc-3′	28
K75R Rev.	5′-ggccgctcaccctgcggatgatgttcct-3′	28
K80R For.	5′-gggtgagcggccagaggttcgtctacaagtt-3′	31
K80R Rev.	5′-aacttgtagacgaacctctggccgctcaccc-3′	31
K84R For.	5′-ccagaggttcgtctacaggtttgtgtcctaccctg-3′	35
K84R Rev.	5′-cagggtaggacacaaacctgtagacgaacctctgg-3′	35
R65A/Y66F For.	5′-gctcagccgggccttggcgttctactatgacaagaaca-3′	38
R65A/Y66F Rev.	5′-tgttcttgtcatagtagaacgccaaggcccggctgagc-3′	38
L158P/Y159A For.	5′-gtacatgcgctcgggccccgcttccaccttcaccatcc-3′	38
L158P/Y159A Rev.	5′-ggatggtgaaggtggaagcggggcccgagcgcatgtac-3′	38
Sp6 promoterFor.	5′-atttaggtgacactatag-3′	18
Sp6 promoterRev.	5′-ctatagtgtcacctaaat-3′	18
T7 promoter For.	5′-taatacgactcactataggg-3′	20
T7 promoter Rev.	5′-ccctatagtgagtcgtatta-3′	20

3. Methods

3.1. Nucleic Acid Techniques

3.1.1. DNA extraction

3.1.1.1. Small scale DNA preparation

A strain of bacteria, either from a single colony or glycerol stock, was inoculated into 5 ml Luria-Bertani broth (LB), supplemented with the appropriate antibiotic. The bacteria were cultivated overnight at 37^{o}C on a rotating wheel (New Brunswick Scientific). Two millilitres of the bacterial culture were collected by centrifugation at 7000g at room temperature for 2 mins (Eppendorf) The DNA was extracted using the Qiaprep miniprep kit (Qiagen), according to the manufacturer's instructions.

3.1.1.2. Large scale DNA preparation

Bacterial culture was set up as described (section 3.1.1.1) and cultivated for 8 hrs at 37^{o}C on a rotating wheel (New Brunswick scientific). The 5 ml bacterial culture was scaled up to 150 ml and cultivated overnight at 37^{o}C in a shaking incubator (New Brunswick Scientific). The bacteria were collected by centrifugation at 4200g at 4^{o}C for 20 mins (Sorvall). The DNA was extracted using the Nucleobond AX PC500 kit (Machery Nagal Germany), following to the manufacturer's instructions.

3.1.2. Restriction digestion of DNA

Digests of 1 µg – 2 µg of DNA were performed in a 10 µl reaction volume, using 5 U of restriction enzyme in the buffer recommended by the manufacturer, supplemented with bovine serum albumin (BSA) where required. The digests were incubated for 1 hr 30 mins at 37^{o}C in a heat incubator (New Brunswick Scientific).

3.1.3. Alkaline phosphatase treatment of DNA

Removal of the 5'end phosphate groups from digested vectors was performed using 1 U calf intestinal alkaline phosphatase (Fermentas) in a 10 µl reaction volume, supplemented with 1 µl of 1 M Tris HCl pH 9.5. The reaction mixtures were incubated at 37^{o}C for 20 mins in a heat incubator (New Brunswick Scientific). The enzyme was inactivated at 65^{o}C for 5 mins before subjecting the DNA to agarose gel electrophoresis (section 3.3.1.1).

3.1.4. Ligation of DNA

Ligation of DNA was performed using 1 U ligase (Fermentas Life Sciences) in 10 µl buffer provided by the manufacturer. Restriction digested recipient vector and DNA fragment insertion (section 3.1.2) were kept in a 1:2 ratio. The reaction was incubated overnight at 16^{o}C in a water bath. The entire ligation was transformed into competent NM522 bacteria (section 3.2.2).

3.1.5. Oligonucleotide ^{32}P [γ] ATP labelling

The 5'-terminal of oligonucleotide was labelled with the phosphate group from ^{32}P [γ] ATP. Two microlitres of 20 ng/µl oligonucleotide (40 ng) were mixed with 14 µl spermidine buffer (20 mM Tris-HCl pH9.5, 1 mM spermidine, 0.1 mM EDTA). The mixture was incubated at 95^{o}C for 2 mins, followed a pulse vortex and a 2 mins ice incubation to remove secondary structure from the oligonucleotide. The oligonucleotide was incorporated with ^{32}P by 10 U of T4 polynucleotide kinase (Promega) in 25 µl reaction volume in the presence of 2.5 µl kinase buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, 50 mM DTT and 50% glycerol) and 30-50 µCi ^{32}P [γ] ATP (Perkin Elmer). The reaction was incubated at 37^{o}C, followed by 10 mins 65^{o}C incubation to inactivate the kinase. The radio-labelled oligonucleotides were stored on ice for the production of ^{32}P labelled double stranded oligonucleotides (section 3.1.6).

3.1.6. Production of [^{32}P]-labelled double stranded oligonucleotides

Radio-labelled double stranded oligonucleotides were produced by annealing [^{32}P] γ ATP-labelled oligonucleotides (section 3.1.5) with the complementary oligonucleotides in a 1:50 ratio. The complementary oligonucleotides were made up in spermidine buffer (section 3.1.5) to 100 ng/µl. The complementary oligonucleotides were incubated at 95^{o}C for 2 mins, followed a pulse vortex and 2 mins ice incubation. Twenty microlitres of 100 ng/µl prepared complementary oligonucleotides (2 µg in total) were incubated with 40 ng of the ^{32}P [γ] ATP labelled oligonucleotides at 95^{o}C for 5 mins. The mixture was placed in 2 l boiling water and allowed to cool down to room temperature slowly.

Unincorporated ^{32}P [γ] ATP in the reaction mixture was eliminated using the nucleotide removal kit (Qiagen), according to the manufacturer's instructions. The ^{32}P labelled double

stranded oligonucleotides were eluted from the Qiagen column in 100 µl Qiagen EB buffer and stored at -20^{o}C.

3.1.7. Site-Directed Mutagenesis

Single or double nucleotide mutations were introduced into plasmids using the QuickChange Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer's protocol. The mutant plasmids were transformed into the NM522 competent cells (section 3.2.2) and produced by small scale DNA preparation (section 3.1.1.1). The desires mutations were examined by restriction digestion (section 3.1.2) and agarose gel analysis (section 3.3.1.1). The site specific mutations were verified by sequence analysis using the T7 promoter and SP6 promoter primers (Table 3).

3.2. Bacterial Techniques

3.2.1. Preparation of competent cells

NM522 competent cells used for transformation (section 3.2.2) were generated by chemical treatment. Bacteria from a glycerol stock was inoculated in 5 ml LB and cultivated at 37^{o}C overnight in a rotating wheel (New Brunswick Scientific).

A millilitre of overnight culture was used to seed a 100 ml culture. The bacteria were grown at 37^{o}C until mid-log phase, indicated by an optical density of 0.4 measured at a wavelength of 600 nm using a spectrophotometer (Pharmacia Biotech). The bacteria were collected by centrifugation at 4200g for 10 mins at 4^{o}C and incubated in 30 ml sterile transformation buffer 1 (10mM MOPS pH7, 10mM RbCl) for 10 mins on ice. The bacteria were collected by centrifugation under the same conditions as above and resuspended in 4 ml

transformation buffer 2 (100 mM MOPS pH6.5, 50 mM $CaCl_2$, 10 mM KCl, 15% glycerol). The competent cells were aliquoted into 200 µl and stored at -80^{o}C.

3.2.2. Transformation of competent cells with plasmid DNA

NM522 competence cells (section 3.2.1) were transformed with plasmid DNA by heat shock treatment. Either ligation reaction or intact plasmid DNA was mixed with 50 µl competent cells. The mixture was incubated on ice for 30 mins. The cells were heat shocked at 42^{o}C for 2 mins in a hot water bath, before returning to ice for 5 mins. The transformed cells were grown in 1 ml of LB at 37^{o}C for an hour in a bench top shaker (Eppendorf). Two different portions of the culture with at least 10-fold volume difference were evenly distributed to pre-warmed LB/agar plates containing the appropriate antibiotic. The plates were incubated at 37^{o}C overnight in a heat incubator (Sanyo).

3.3. Gel Electrophoresis

3.3.1. Agarose gel electrophoresis

3.3.1.1. DNA agarose gel

DNA was separated according to its size by electrophoresis in agarose gels, ranging from 0.8% (Biorad Laboratories) - 3% (2% Nusieve GTG agarose, Cambrex and 1% agarose, Biorad laboratories). Agarose was suspended in TAE buffer (40 mM Tris-acetate pH8, 1 mM EDTA) and solubilised by heat using a microwave. The solubilised agarose was casted into a Biorad gel tank (Biorad Laboratories), according to the manufacturer instructions.

DNA samples were mixed with 6X DNA loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF and 15% Ficoll in ddH_2O) before loading into the wells of the gel. The

DNA was subject to electrophoresis at 80-100V in TAE buffer for 40 - 60 mins, depending on the size of the DNA samples and the percentage of gels, until DNA fragments were resolved. The gels were equilibrated in an ethidium bromide bath (0.5 µg/ml in TAE) for 10 mins on a gentle shaker. The stained DNA was visualised using a UV gel documentation system (Syngene).

3.3.1.2. Extraction of DNA from agarose gels

DNA fragments from restriction digestion (section 3.1.2) were extracted for ligation (section 3.1.4). Separated fragments in agarose gels (section 3.3.1.1) were visualised using an UV light box. The regions of agarose gels containing the desire DNA were excised by a scalpel. The DNA was extracted using the Jetsorb gel extraction kit (Genomed), following the manufacturer's instructions.

3.3.2. Polyacrylamide gels

3.3.2.1. Non-denaturing polyacrylamide gel electrophoresis

Protein/DNA interactions were examined using Non-denaturing polyacrylamide gel electrophoresis. A 5% polyacrylamide gel (12.5 ml 29:1 acrylamide:bis-acrylamide [Sigma], 15 ml 5X TBE [445 mM Tris-HCl, 445 mM boric acid, 10 mM EDTA], 60 µl TEMED, 700 µl 10% APS, 47 ml H_2O) was pre-run in 1X TBE buffer at 20 mA for 30 mins. Samples were mixed with 6 X DNA loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF and 15% Ficoll in ddH_2O) before loading onto the gel. The gel was run in the same condition until the loading dye had migrated approximately 2/3 of the gel.

3.3.2.2. SDS polyacrylamide gel electrophoresis

Proteins were separated according to their molecular weight using SDS polyacrylamide gel electrophoresis (SDS-PAGE). Gels were casted and run based on the 'discontinuous' buffer method (Laemmli, 1970) using the Mini Protean II System (Biorad), set up according to the manufacturer's instructions. A 7.5% resolving gel (7.5% acylamide [29:1 acrylamide:bis-acrylamide, Sigma], 500 mM Tris-HCl pH 6.8, 0.4% SDS, 0.25% TEMED, 0.0375% APS) was polymerised under a layer of water-saturated butanol, which was washed off with distilled water before casting the stacking gel (4% acylamide [29:1 acrylamide:bis-acrylamide], 1.5 mM Tris-HCl pH 8.8, 0.4% SDS, 0.2% TEMED, 0.025% APS) on top of the resolving gel.

Protein samples were prepared by the addition of SDS loading buffer (3X SDS loading buffer: 150 mM Tris-HCl pH8, 6% SDS, 30% glycerol, 432 mM β-mercaptoethanol, 0.025% bromophenol blue, 6 mM EDTA) and heat incubation at 95^{o}C for 5 mins using a bench top heat block. Unsolubilised proteins were removed by centrifugation at 13000g at room temperature for 1 min. The supernatants were loaded onto the wells of the gels. The proteins were subjected to electrophoresis at 160 V in SDS buffer (25 mM Tris-HCl, 192 mM glycine, 1% SDS), until the dye reached the bottom of the gel.

3.4. Protein Techniques

3.4.1. Protein extraction

3.4.1.1. Preparation of whole cell extracts

Medium from each plate of 70% confluent HEK293 cells was removed. Adherent cells were washed once with ice-cold PBS and scraped off the dish in 1ml PBS. The cells were collected by centrifugation at 1000g for 2 mins at 4^{o}C. The Cell pellet was resuspended in 100 µl lysis buffer (0.1 M Tris-HCl pH 8, 0.1 M NaCl, 1% NP40, 1 mM DTT, 1 mM PMSF and 1X Protease Inhibitor Cocktail, Roche) and lysed on ice for 30 mins with 10 quick vortex pulses before and after the incubation. The cell lysate was then cleared by centrifugation at 13000g for 20 mins at 4^{o}C. The whole cell extract was frozen in liquid nitrogen and stored at -80^{o}C.

3.4.1.2. Preparation of HeLa cytosolic extracts from suspension culture

HeLa cytosolic extracts, provided by Mark Dear, were prepared as described below, modified from the method published by Dignam et al., 1983.

HeLa cells were cultivated to approximately 8 X 10^5 cells/ml in a 2.5 l suspension culture. The cells were washed twice with ice-cold PBS + V + F (PBS, 1 mM Na_3VO_4, 5 mM NaF) and collected by centrifugation at 1000g for 15 mins at 4^{o}C (Sorvall). The cell pellet was washed with buffer A, 5X volume (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl; supplemented just before use: 10 mM NaF, 2 mM Na_3Vo_4, 10 mM β-glycerophosphate, 10 mM *p*NPP, 0.5 mM DTT) and resuspended with the same buffer, 2X the volume, before 10 mins ice incubation. The cell membrane of the cells was disrupted using a dounce

homogeniser by ten repetitions of strokes. The nuclei were collected by centrifugation at 2500g for 10 mins at 4^{o}C (Heraeus). The supernatant was supplemented with buffer B, 0.11X the volume (300 mM HEPES pH 7.9, 30 mM $MgCl_2$, 1.4 M KCl) and cleared by centrifugation at 34,000rpm for 60 mins at 4^{o}C (Centrikon, T2070). HeLa cytosolic extracts were dialysed against buffer A (0.1 M Tris-HCl pH 8, 0.1 M NaCl, 1 mM DTT and 1 mM PMSF) as described in section 3.4.6. The HeLa cytosolic extracts were frozen in liquid nitrogen and stored at -80^{o}C.

3.4.1.3. Preparation of nuclear extracts

Medium from each plate of cells was removed. The adherent cells were washed with ice-cold PBS + V + F (PBS, 1 mM Na_3VO_4, 5 mM NaF) and hypotonic buffer (20 mM HEPES pH7.9, 20 mM NaF, 1 mM Na3VO4, 1 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM DTT, Protease Inhibitor Cocktail [Roche]) on the plates. The cells were lysed with 0.5 ml hypotonic buffer + 0.1% NP40 *in situ* and collected by centrifugation at 13000g for 20 s at 4^{o}C (Eppendorf). The supernatant of cytosolic extract was supplemented with 120 mM NaCl and 10% glycerol, before storing at -80^{o}C. The nuclear pellet was resuspended in 150 µl high salt buffer (1X hypotonic buffer, 420 mM NaCl, 20% glycerol) and rocked gently for 60 mins at 4^{o}C. The nuclear extract was cleared by centrifugation at 13000g for 20 mins at 4^{o}C (Eppendorf) and frozen in liquid nitrogen, before storing at -80^{o}C.

3.4.2. Transfer of proteins to Polyvinylidene Fluoride membrane

Proteins resolved by SDS-Polyacrylamide Gel Electrophoresis (section 3.3.2.2) were transferred onto 0.2 µm Polyvinylidene Fluoride (PVDF) membranes (Schleicher and Schuell) using the Semi-Dry Transfer System (Biorad). The PVDF membranes were wet

with methanol. The polyacrylamide gels, filter papers (Whatman) and the PVDF membranes were soaked in transfer buffer (24 mM Tris-HCl, 192 mM glycine, 20% methanol). The PVDF membranes were laid under the gels with three layers of filter papers on both the top and the bottom. The stack was laid on the platinum plate of the transfer system. Proteins were transferred at 100 mA/gel at 12 V for 1 hr 45 mins, before proceeding to western blot analysis (section 3.4.3).

3.4.3. Western blotting

PVDF membranes with transferred proteins (section 3.4.2) were blocked in 5% milk (5% non-fat milk powder[Marvel] and 1X TBST [50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween]) at room temperature for 1 hr. Primary antibodies, with concentration diluted in 5% milk according to the manufacturer's recommendation, were applied onto the membranes overnight at 4^{o}C. The membranes were washed three times in 1X TBST with gentle rocking at room temperature for 10 mins on the next day, before applying the corresponding secondary antibodies for an hour at room temperature. The membranes were washed with 1X TBST as described above, before applying the ECL Detection Reagent (GE Healthcare). The blots were developed using the LAS-3000 chemiluminescence camera (Fuji-Film).

3.4.4. Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts (section 3.4.1.3) and ^{32}P labelled double stranded oligonucleotides (section 3.1.6) were used to examine Protein/DNA interaction *in vitro* in the EMSAs. A 10 µl reaction in FPE buffer (35 mM KCl, 2 mM spermidine, 2.35 mM EDTA) containing nuclear extract, recombinant core SRF (provided by Prof. Peter Shaw, University of Nottingham), 6 µg salmon sperm (Amersham), 1.2 µg poly dI:dC (Amersham) were incubated on ice for 20

mins. A microlitre of radio-labelled double stranded oligonucleotides was added to the mixture and incubated at room temperature for 15 mins to allow protein/DNA complex formation. The mixture was subject to non-denaturing 5% polyacrylamide gel electrophoresis (section 3.3.2.1) after the addition of 2.5 µl 1.5X DNA loading buffer (0.0625% bromophenol blue, 0.0625% xylene cyanol FF and 3.75% Ficoll in ddH_2O). The gel was soaked in fixing buffer (10% glacial acetic acid, 40% methanol) for 10mins and dried at 90^oC for 45 mins using a vacuum gel drier (Bachoffer). Radioactivity from the gel was exposed to a phosphor imager plate overnight and visualised using the FLA-2000 phosphorimager (Fuji-Film).

3.4.5. Fast Performance Liquid Chromatography (FPLC)

Proteins of different sizes were separated by FPLC using a size exclusion chromatography column packed with superose 6 matrix (GE Healthcare). The column was pre-washed and run with filtered and degassed buffer (0.1 M NaCl, 0.1M Tris, pH7.5) at 0.2 ml/min using a high precision pump (Pharmacia). Gel filtration molecular weight markers (Sigma) were run through the column to create a molecular weight elution profile. Eluted proteins were detected by an UV detector (Pharmacia). Proteins were collected in 280 µl aliquots using a fraction collector (Amersham Biosciences). Size of eluted proteins was elucidated from the elution profile.

3.4.6. Dialysis

Cellulose dialysis membrane tubing with 3,500 Dal molecular weight cut off (Spectrum) was soaked in ddH_2O at room temperature for 30mins and rinsed thoroughly to remove glycerine coating. Sample was injected into the dialysis membrane tubing with either ends secured by

clamps. The sample was then placed in appropriate dialysis buffer with the volume 100X of the sample volume. The dialysis buffer was stirred gently for three hrs at 4^{o}C with fresh dialysis buffer replacement after the first 90 mins.

3.4.7. Immobilized metal affinity chromatography (IMAC)

Histidine-tagged proteins were retrieved from cells using IMAC. The cell lysate (section 3.8.3) was mixed with Ni^{2+} beads (Qiagen) for an hour at room temperature on a rotating wheel. The beads were collected by centrifugation at 13000g for 2 mins at room temperature and successively washed with washing buffer 1 to 5. Contents of the washing buffers are described below. Proteins on the beads were eluted by addition of 35 µl SDS elution buffer (200 mM imidazole 50 mM Tris-HCl pH8, 2% SDS, 10% glycerol, 144 mM β-mercaptoethanol, 0.008% bromophenol blue, 2 mM EDTA). The mixture was incubated at room temperature for 10 mins, followed by heat incubation at 95^{o}C for 5 mins. The elution was cleared by centrifugation at 13000g for 10 mins at room temperature, before subject to 7.5% SDS PAGE (section 3.3.2.2) and western blot analysis (section 3.4.3).

WB1: 6 M guanidinium-HCl, 0.1 M phosphate buffer pH8, 0.01 M tris pH8, 10 mM β-mercaptoethanol*. WB2: 8 M urea, 0.1 M phosphate buffer pH8, 0.01 M tris pH8, 10 mM β-mercaptoethanol*. WB3: 8 M urea, 0.1 M phosphate buffer pH6.3, 0.01 M tris pH6.3, 10 mM β-mercaptoethanol*. WB4: WB3 + 0.2% triton X-100. WB5: WB3 + 0.1% triton-X100. *β-mercaptoethanol was added just before use.

3.5. Eukaryotic cell culture

3.5.1. Determination of cell numbers

The density of cell suspensions was estimated using a haemocytometer (Hawksley) under a light microscope (Zeiss).

3.5.2. Eukaryotic cell culture

HEK293, HeLa and NIH3T3 cells were cultivated on 10 cm Falcon tissue culture dishes (BD Biosciences) in the low glucose (5.5 mM) Dulbecco's Modified Eagle Medium (Sigma Aldrich), supplemented with 10% foetal calf serum (Bioclear), 200 mM glutamine(Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma). Cells were passaged at a dilution of between 1:8 to 1:10 every 3 to 4 days and cultivated using a humidified incubator (Revco), set at 37^{o}C and 7.5% CO_2 for HEK293 and NIH3T3cells, 5% CO_2 for HeLa cells.

3.5.3. T-REx-293 cells with tetracycline-inducible expression of dnUbc12-HA

Tetracycline-inducible dnUbc12-HA T-Rex-293 cell line, provided by Thilo Hagen (Wolfson Digestive Disease Centre, University of Nottingham), was generated as described below (Chew, Poobalasingam et al. 2007).

Tetracycline-inducible dnUbc12-HA T-Rex-293 cell line was generated using the T-Rex System (Invitrogen). The plasmid pcDNA4/TO encoding the dnUBC12 cDNA with the C111S mutation, was stably transacted into T-Rex-293 cells. Successfully stable transfected cell was isolated using zeocin selection. Tetracycline-dependent protein expression was confirmed by western blot analysis.

3.6. Transfection of eukaryotic cells

3.6.1. Calcium phosphate

During this study, HEK293 cells were transfected using DNA $CaPO_4$ co-precipitation. Cells were passaged to 50% confluent the day before transfection. The culture medium on cells was replaced with fresh full medium 4hrs prior transfection. A total of 10 µg of plasmid DNA was diluted in 500 µl 300 mM $CaCl_2$ and incubated on ice for 10 mins. Following the incubation, 550 µl of 2X HEPES buffered saline (280 mM NaCl, 50 mM HEPES pH7) was added drop-wise to the mixture while mixing slowly using a vortex mixer. The mixture was incubated on ice for 30 mins before applying evenly onto the cells. The cells were serum starved or administrated with stimulator or inhibitor where required before harvesting, approximately 48 hrs post-transfection.

3.6.2. Polyethylenimine (PEI)

During this study, HeLa cells were transfected using PEI. Cells were passaged to 7 X 10^5 cells/plate the day before transfection. The culture medium on cells was replaced with fresh full medium 4 hrs prior transfection. A total of 10 µg of plasmid DNA and 30 µl of 20 mM PEI were diluted into 250 µl of PBS separately. The diluted PEI was added drop-wise to the diluted DNA while mixing slowly using a vortex mixer. The mixture was incubated at room temperature for 30 mins. During the incubation, the cells were washed and the full medium replaced with serum-free Optimem (Invitrogen) prior to applying the mixture evenly onto the cells. The optimem was replaced with full medium 5 hrs post transfection. The cells were serum starved or administrated with stimulator or inhibitor where required before harvesting, approximately 48 hrs post-transfection.

3.7. Luciferase reporter and β-galactosidase reporter gene assay

Elk-1 mediated transcription activity was examined using reporter gene assays in this study. NIH3T3 cells were passaged to 1.5 X 10^5 cells/well in 6 well plates (Corning Incorporated) the day before subjecting to PEI transfection (section 3.6.2). The transfection was done in triplicate for each experiment. The cells were collected by centrifugation at 13000g for 2 mins at 4^{o}C, 48 hrs post transfection and stored at -80^{o}C.

The cells were thawed for 10 mins and lysed in 65 µl lysis buffer (250 mM KCl, 50 mM Hepes pH7.5, 0.1% NP40 and 10% glycerol) for 25 mins on ice. The debris was removed by centrifugation at 14,000rpm for 10 min at 4^{o}C.

For the luciferase assays, 300 µl luciferin buffer (for 10 ml buffer: 0.5 ml 1 mM luciferin, 100 µl 0.2 M ATP, 100 µl 1 M MgCl2 and 25 mM gly-gly pH7.8) was injected into 10 µl lysate. For the β-galactosidase assays, 100 µl reaction buffer (for 5 ml buffer: 1 ml 0.5M $NaPO_4$ pH8, 5 µl 1M $MgCl_2$, 5µl Galacton [Tropix], 3.99 ml H_2O), followed by 300 µl enhancer buffer (for 15 ml of enhancer buffer: 1.5 ml 2 M NaOH, 750 µl Emerald enhancer [Tropix], 12.75 ml H_2O) was injected into 10 µl lysate using an automatic luminometer (Berthold). The intensity of emitted luminescence from both assays was measured in duplicate. The luciferase measurement was normalized to the β-galactosidase measurement.

3.8. Ubiquitylation assays

3.8.1. *In vitro* expression of L-[^{35}S]-methionine labelled protein

Radio-labelled Elk-1 was generated with the pcDNA3.HA.Elk-1 expression plasmid in presence of 20 µCi L-[^{35}S]-methionine (Perkin Elmer), using the TNT T7 Quick Coupled

Transcription/Translation System (Promega), following the manufacturer's instructions. Unincorporated L-[^{35}S]-methionine was eliminated using Micro Bio-spin P-6 chromatography columns (BioRad), pre-equilibrated in 25 mM Tris-HCl and 50 mM NaCl pH7.5.

3.8.2. *In vitro* ubiquitylation assay

In vitro ubiquitylation assays were performed in a 20 µl reaction volume. One microlitre L-[^{35}S]-methionine labelled Elk-1 (section 3.8.1), 850 ng Ubiquitin aldehyde (BIOMOL), 500 ng Ubiquitin (Sigma), 2 mM $MgCl_2$, 2 mM ATP, 0.5 mM DTT, HeLa cytosolic extract or HEK293 whole cell extract as a source of E3, 25 ng recombinant ubiquitin activation enzyme E1 and 25 ng ubiquitin conjugating enzyme E2 were incubated for 1 hr at 30^{o}C. Recombinant enzymes E1 and E2 were provided by Olivier Coux (Centre de Recherches de Biochimie Macromoléculaire, Montpellier). The reaction was terminated by addition of 10 µl 3X SDS loading buffer (150 mM Tris-HCl pH8, 6% SDS, 30% glycerol, 432 mM β-mercaptoethanol, 0.025% bromophenol blue, 6 mM EDTA), followed by heat incubation at 90^{o}C for 5 mins. Proteins in the mixture were then resolved using a 7.5% SDS-PAGE. The gels was dried and exposed to a phosphor imaging plate overnight and visualised using a phosphor imager (Fujifilm, FLA-2000).

3.8.3. *In vivo* ubiquitylation assay

Elk-1 ubiquitylation *in vivo* was examined using the *in vivo* ubiquitylation assay. Either HEK293 or HeLa cells were transfected as described (section 3.6.1 & 3.6.2). The cells were washed with PBS and collected by centrifugation at 14,000rpm for 1 min at 4^{o}C (Eppendorf). A tenth of cells was lysed in 3X SDS loading buffer (150 mM tris-HCl pH8, 6% SDS, 30%

glycerol, 432 mM β-mercaptoethanol, 0.025% bromophenol blue, 6 mM EDTA), followed by SDS-PAGE (section 3.3.2.2) and western blot analysis (section 3.4.3). The rest of cells were resuspended in lysis buffer (6 M guanidinium-HCl, 0.1 M phosphate buffer pH8, 0.01 M tris-HCl pH8, 5 mM imidazole, 10 mM β-mercaptoethanol) and sonicated for 5 s (Jencons). The lysate was subjected to IMAC (section 3.4.7), followed by SDS-PAGE (section 3.3.2.2) and western blot analysis (section 3.4.3).

4. Identification of Elk-1 specific E3 ligase(s)

4.1. Elk-1 specific ligase activity is presented in HEK293 cells.

To identify the Elk-1 specific E3 ligase(s), an ubiquitylation assay was established to reconstitute Elk-1 ubiquitylation *in vitro*. The assay was modified from an established method (Linares, Hengstermann et al. 2003). For efficient visualization, Elk-1 was synthesized *in vitro* and labelled with [^{35}S]-methionine. The E1 activating (Ube1) and E2 conjugating enzyme (UbcH5c) were recombinant proteins, initially provided by Olivier Coux (Centre de Recherches de Biochimie Macromoléculaire, Montpellier). UbcH5c belongs to the UbcH5 family and members of this family are known to interact with a broad range of E3 ligases with little specificity on E3 ligase partner selection (Jensen, Bates et al. 1995; Hatakeyama, Jensen et al. 1997; Lorick, Jensen et al. 1999; Brzovic and Klevit 2006; Brzovic, Lissounov et al. 2006; Kirkpatrick, Hathaway et al. 2006). Ube1 was the only identified ubiquitin E1 activating enzyme at the time and it cooperates with UbcH5c to facilitate protein ubiquitylation (Groettrup, Pelzer et al. 2008). Whole cell extracts (WCE) prepared from HEK293 cells were used to provide the E3 enzymatic activity. The essential components required for ubiquitylation were incubated to allow ubiquitin conjugation. The presence of Elk-1 specific E3 ligase activity in HEK293 WCE was examined.

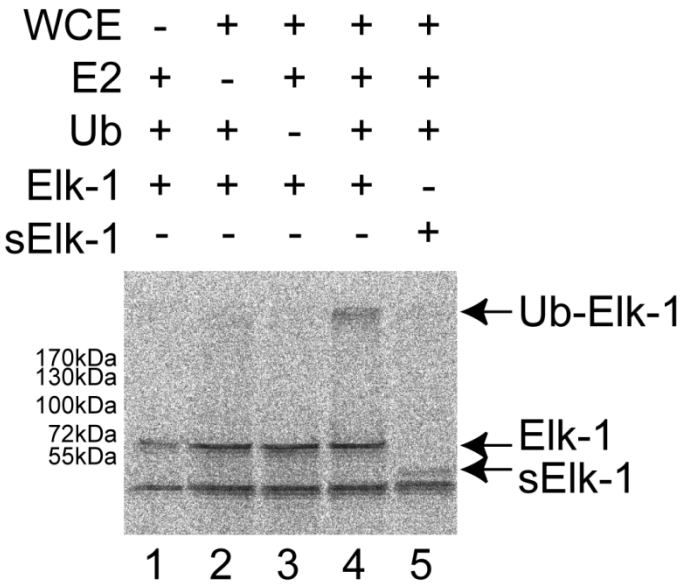

Figure 4-1 Elk-1 specific E3 ligase activity is present in HEK293 whole cell extracts. *In vitro* expressed [^{35}S]-labelled Elk-1, recombinant E1, E2, and HEK293 WCE as a source of E3 and ubiquitin were incubated under standard ubiquitylation conditions (see method) with different components missing as indicated. The reaction products were resolved by SDS-PAGE followed by autoradiography. Molecular weights are indicated on the left. Short Elk-1, an Elk-1 isoform lacking the first 54 amino acids, is presented as sElk-1.

Two species of [^{35}S]-labelled proteins with distinct molecular weights were visualised in all reactions. Elk-1 corresponds to the top species located just under the 72 kDa molecular weight marker (lanes 1 - 4, Figure 4-1), the bottom species may present a truncated version of Elk-1 due to either internal transcription initiation or premature termination. An Elk-1 isoform lacking the first 54 amino acids, sElk-1, appeared under the 55 kDa marker (lane 5, Figure 4-1). The size of the bottom species remained unchanged for sElk-1, suggesting the

bottom species came from internal transcription initiation as premature transcription termination would have yielded truncated proteins with size reduction in proportion to that of sElk-1. After an hour incubation of [^{35}S]-labelled Elk-1, a high molecular weight (M.W.) species of approximately 300 kDa accumulated (lane 4, Figure 4-1). Conversely, these high M.W. species did not appear when sELk-1 was incubated under the same condition (lane 5, Figure 4-1), suggesting the truncated region of sElk-1 is critical for the accumulation of this high M.W. species. Although sElk-1 was not expressed to the same level as the full length Elk-1, the absence of high M.W. species in lane 5 excludes the possibility that the high M.W. species observed are the modified forms of the bottom species and indicates the high M.W. species are modified Elk-1. Elk-1 remained unmodified when either ubiquitin, E2 conjugating enzyme or HEK293 whole cell extract was left out (lanes 1 - 3, Figure 4-1). Exclusion of each key component required for ubiquitylation abolished the modification of Elk-1 indicating that the high M.W. species is likely to be polyubiquitylated Elk-1. Despite the evidence indicating Elk-1 was modified with multiple ubiquitins, densitometry readings on either the top or the bottom species of [^{35}S]-labelled proteins did not show significant alteration (data not shown), suggesting only a small portion of Elk-1 was modified under the conditions of this *in vitro* assay.

4.2. Elk-1 specific ligase activity is present in HeLa cells

To identify Elk-1 specific E3 ligase(s), proteins in cell extracts were separated by size or biochemical properties into a number of fractions by Fast Performance Liquid Chromatography (FPLC). Enzymatic activity of ligase(s) in the fractions was followed using the *in vitro* ubiquitylation assay with the aim of obtaining a sufficiently purified active fraction(s) for protein identification by mass spectrometry.

The majority of E3 ligases in humans are multimeric protein complexes (Petroski and Deshaies 2005). To enable high preservation of the biological activity and protein complex formation of E3 ligases, size exclusion chromatography was first chosen as it is relatively gentle compared to other methods. Although size exclusion chromatography offers a gentle separation, there is an inevitable dilution of the protein concentration. To help retain sufficient ligase activity in the fractions, a good source of cell extract was used. These were obtained using a suspension HeLa cell culture system which had been previously established by members of our group to allow generation of cell extracts with high protein concentration. Nuclear (NXT) and cytosolic extracts (CXT) from HeLa cells following serum stimulation were already available. Separation of cytosolic and nuclear proteins was examined.

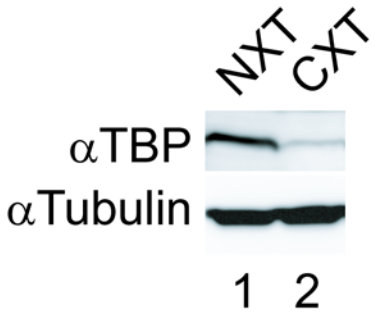

Figure 4-2 Protein extractions from the suspension HeLa cell culture system. Western blot analysis of nuclear and cytosolic extracts from HeLa cells stimulated with serum using the antibodies indicated. 50 µg of protein were examined in each lane.

A relatively large amount of TATA binding protein (TBP) was found in the NXT compared to a trace amount of TBP in the CXT (top panel, Figure 4-2), verifying the separation of

nuclear proteins from the CXT. The presence of tubulin in the nuclear extract indicates contamination of cytosolic proteins in the NXT (bottom panel, Figure 4-2).

Previous experiments conducted by Prof. Peter Shaw demonstrated the presence of the Elk-1 specific E3 ligase activity in both HeLa nucleus and cytosol (unpublished data). Transcription activators could be ubiquitylated in both the cytosol and the nucleus (Joseph, Zaika et al. 2003). To verify Elk-1 ubiquitylation in the cytosol, the presence of an Elk-1 specific E3 ligase activity in HeLa CXT was examined using the same *in vitro* ubiquitylation assay.

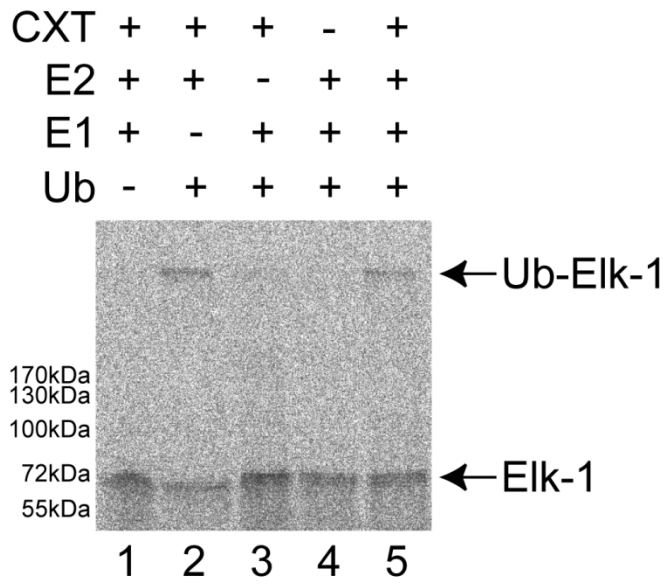

Figure 4-3 Elk-1 specific E3 ligase activity is present in HeLa cytosolic extract. *In vitro* expressed [^{35}S]-labelled Elk-1, recombinant E1, E2, HeLa CXT as a source of E3 and ubiquitin were incubated under standard ubiquitylation conditions (see method) with different components missing as indicated. The

reaction products were analysed by SDS-PAGE followed by autoradiography. Molecular weights are indicated on the left.

Similar results were obtained when HEK293 WCE was replaced with HeLa CXT, suggesting the presence of Elk-1 specific E3 ligase in HeLa cell cytosol (lanes 1 & 3 - 5, Figure 4-3). Interestingly, exclusion of the E1 activating enzyme did not affect Elk-1 ubiquitylation, which may indicate an abundance of endogenous E1 activating enzyme in the cytosol of HeLa cells (lane 2, Figure 4-3).

4.3. Elk-1 specific ligase(s) is approximately 29-50 kDa

After confirming the presence of Elk-1 specific E3 ligase activity in the HeLa CXT, proteins in the extract were separated using a size exclusion chromatography column of superose 6 matrix which has an optimal separation range of 5 - 5000 kDa. A set of markers were run through the column and detected at an optical density of 280 nm to generate a molecular weight elution profile. Proteins in the extract were separated into 30 fractions.

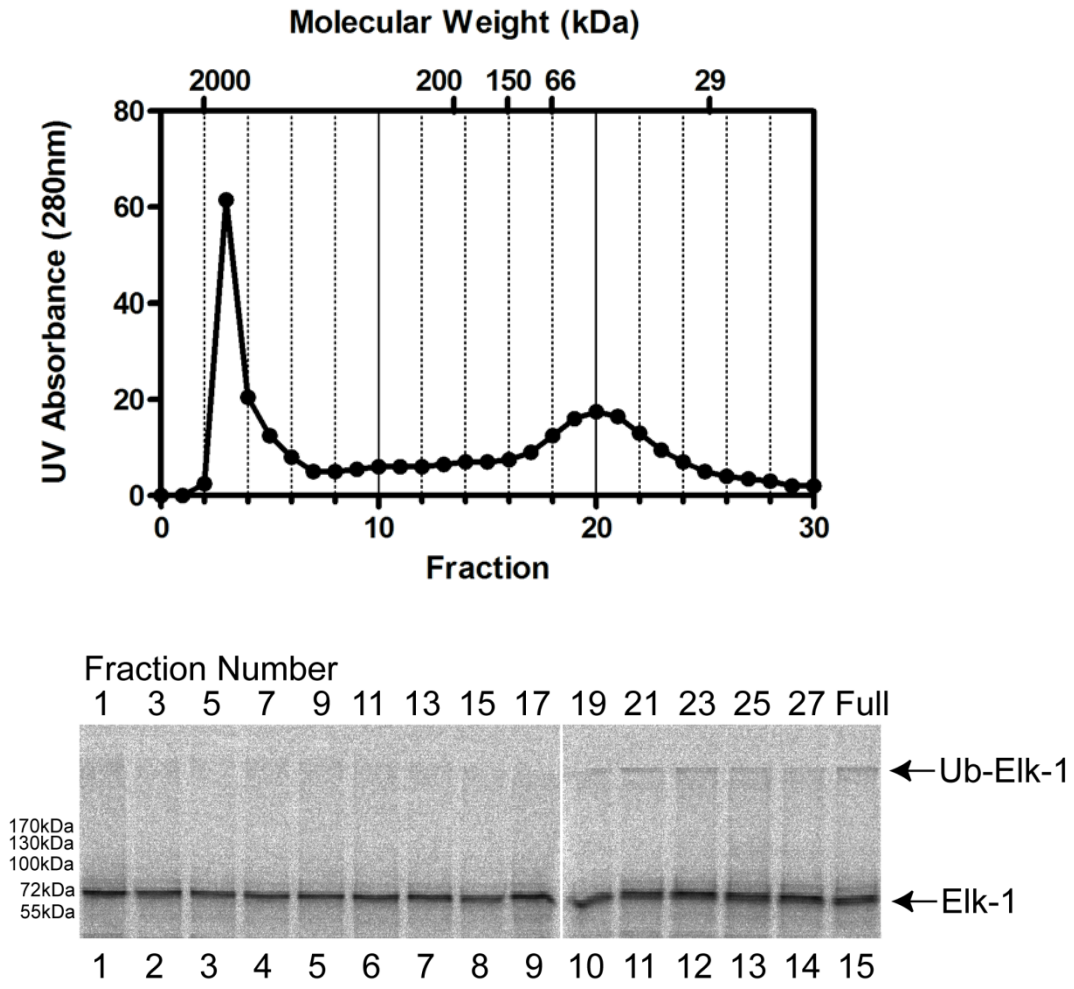

Figure 4-4 Elk-1 specific E3 ligase activity is present in HeLa cytosolic fractions containing proteins with molecular weight between 29 – 50 kDa. Top: HeLa CXT was fractionated by size exclusion chromatography, the curve represents the relative amount of eluted protein in different fractions with the average protein size indicated on the top of the graph; Bottom: *in vitro* expressed [^{35}S]-labelled Elk-1 and HeLa CXT/fractions were incubated under standard ubiquitylation conditions (see method). The reaction products were resolved by SDS-PAGE followed by

autoradiography. Molecular weights are indicated on the left. Lane 15 represents the assay with full (un-fractionated) HeLa CXT.

A relatively large amount of protein was present in fractions 2 - 4, which contains protein species of just under 2,000 kDa. The high molecular weight indicates that the species are likely to be multimeric protein complexes as the median length of proteins in the *Homo sapiens* proteome is 375 amino acids, which is equivalent to approximately 50 kDa (Brocchieri and Karlin 2005). This observation indicated that the intermolecular interactions between proteins were preserved during the size exclusion chromatographic separation.

Alternative fractions (odd numbers from 1 to 27) were examined using the *in vitro* ubiquitylation assay. The results indicated the presence of Elk-1 specific E3 ligase activity in fractions 21 - 25, which contain proteins of an estimated size range of 29 – 50 KDa (lanes 11 - 13, Figure 4-5). The peak of E3 ligase activity was found in fraction 23. To confirm this result, fractions 7, 15 and 23 were examined again.

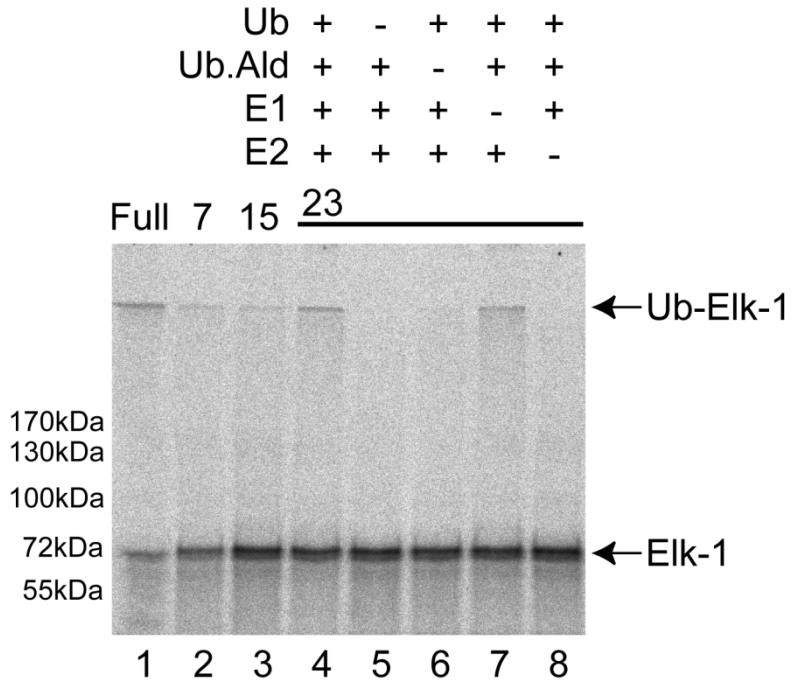

Figure 4-5 Confirmation of Elk-1 Specific E3 ligase activity in HeLa CXT fraction 23. *In vitro* expressed [^{35}S]-labelled Elk-1 and HeLa CXT/fractions were incubated under standard ubiquitylation conditions (see method). The reaction products were resolved by SDS-PAGE followed by autoradiography. Molecular weights are indicated on the left. Lane 1 represents the assay using full (un-fractionated) HeLa CXT, lane 2 - 4 represents the assay using fraction 32, 40 & 48 respectively and lane 5 - 8 represents the assay using fraction 48 with different components missing as indicated. This experiment was repeated three times.

Consistent with the previous results, the strongest Elk-1 E3 ligase activity was detected in fraction 23 (lane 4, Figure 4-4), whereas a relatively low level of ligase activity was observed in fractions 7 and 15 (lanes 2 & 3, Figure 4-5). Exclusion of E2 enzyme, ubiquitin

or ubiquitin aldehyde abolished polyubiquitylation (lanes 5, 6 & 8, Figure 4-4), confirming Elk-1 is polyubiquitylated.

Surprisingly, Elk-1 was still polyubiquitylated without the addition of E1 enzyme (lane 7, Figure 4-4). Both of the Ube1 and Uba6 E1 activating enzymes have a molecular weight of 118 kDa (Groettrup, Pelzer et al. 2008) and fraction 23 contains proteins with an estimated size range of 38 kDa – 44 kDa. Although size exclusion chromatography offers a gentle approach to protein separation by size, the separation is not precise. The estimated size range only represents the average protein size presented in the fractions. The abundance of E1 activating enzyme activity was already demonstrated in HeLa CXT (lane 2, Figure 4-3). It is possible that enough E1 activating enzyme was present in fraction 23 to support Elk-1 ubiquitylation, which could be verified by western blot analysis.

E3 ligases can be monomeric or multimeric. The multimeric Cullin-RING ligases (CRLs) comprise the largest group of ubiquitin E3 ligases (Petroski and Deshaies 2005; Merlet, Burger et al. 2009). All CRLs contain a cullin scaffold that has a molecular weight of approximately 90 kDa (Michel and Xiong 1998). Together with its variable substrate recognition module and RING-protein subunit, the size of CRLs considerably exceeds the estimated size range (38 kDa – 44 kDa) of proteins presented in fraction 23, therefore it is rational to exclude the possibility that the Elk-1 specific E3 ligase present in fraction 23 is a CRL.

Monomeric E3s characterize with either a 40 – 100 amino acid RING (~8 kDa) or a 350 amino acid HECT (~45 kDa) catalytic domain, together with additional substrate recognition and regulatory regions which can be varied in size (Fang and Weissman 2004). The HECT-domain alone reaches the estimated upper protein size limit of fraction 23. Given that a HECT-domain E3 ligase must contain regions for substrate interaction and activity regulation, it would easily exceed the estimated size range in fraction 23. On the other hand,

E3 ligases containing a small RING-domain would fit into this size range, therefore it is more likely that the potential Elk-1 specific E3 in fraction 23 is a monomeric RING-domain E3 ligase.

4.4. Elk-1 polyubiquitylation is inhibited by dominant negative Ubc12

In parallel with the identification of Elk-1 specific E3 ligase(s) using chromatographic approach, additional experiments were performed to examine whether a CRL is able to ubiquitylate Elk-1. It has been shown that neddylation, facilitated by the Nedd8 conjugating enzyme Ubc12 (Gong and Yeh 1999), is essential for the activity of CRLs (Podust, Brownell et al. 2000; Read, Brownell et al. 2000; Wu, Chen et al. 2000). Therefore, if the Elk-1 specific E3 ligase belongs to the class of CRLs, inhibiting neddylation would restrain Elk-1 ubiquitylation.

To examine this possibility, a cell line was utilized, kindly provided by Thilo Hagen (Department of Biochemistry, Nation University of Singapore), that expresses the HA-tagged dominant negative nedd8 conjugating enzyme (DN Ubc12) in order to block CRL activity (Chew, Poobalasingam et al. 2007). The DN Ubc12 contains a single C111S mutation which prevents cullin neddylation by sequestering Nedd8, thereby reducing CRL activity (Wada, Yeh et al. 2000). Expression of the HA-tagged DN Ubc12 was induced by treating cells with tetracycline (TC) and verified by western blot analysis (Figure 4-6). Whole cell extracts were prepared from cells with or without prior TC stimulation and examined for Elk-1 specific E3 ligase activity using the *in vitro* ubiquitylation assay.

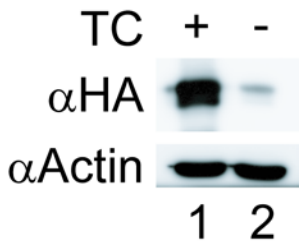

Figure 4-6 Tetracycline inducible HA-tagged dominant negative Ubc12 expression in HEK293 cells. Western blot analysis of WCE from HEK293 cells treated with or without TC.

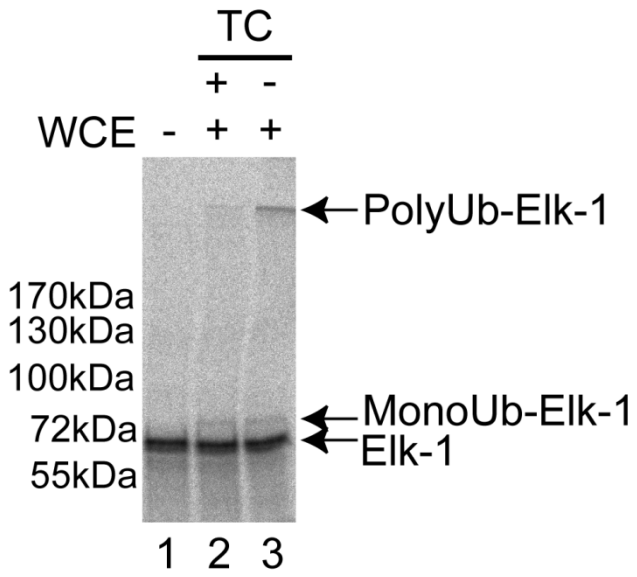

Figure 4-7 Dominant negative nedd8 conjugating enzyme inhibits Elk-1 polyubiquitylation. *In vitro* expressed [^{35}S]-labelled Elk-1 and HEK293 WCE with or without TC inducible dominant negative Nedd8 conjugating enzyme were incubated under standard ubiquitylation conditions. Lane 1 represents the assay without an addition of cell extract; lane 2 & 3 represents the assay using HEK 293 WCE with or without TC-induced DN

Ubc12. The experiment was repeated three times. Molecular weights are indicated on the left.

High molecular weight species representing polyubiquitylated Elk-1 accumulated when Elk-1 was incubated with HEK293 WCE, as previously observed in Figure 4-1 (lane 3, Figure 4-7), suggesting Elk-1 specific E3 ligase(s) is active in the cells. This modification was diminished when DN Ubc12 was present (lane 2, Figure 4-7), indicating that neddylation is essential for Elk-1 polyubiquitylation, hence at least a type of CRL is able to conjugate polyubiquitin to Elk-1 *in vitro*.

In addition to the high molecular weight species, a lower molecular weight species of 80 kDa also accumulated (lanes 2 & 3, Figure 4-7), regardless of the presence of DN Ubc12, suggesting that neddylation is not required for this modification. The approximate 8 kDa increase in molecular weight fits with a single ubiquitin attachment, however the above experiment cannot unequivocally confirm that the lower molecular weight species were monoubiquitylated Elk-1. This was later verified by additional experiments performed by another member of the group (Jürgen Handwerger) where essential components for ubiquitylation were omitted (data not shown). In contrast to earlier experiments (Figure 4-1), this lower molecular weight species did not appear where a different source of HEK293 WCE was used. The former WCE was prepared from normal growing HEK293 cells, whereas the later one was generated from HEK293 cells stably transfected with an expression cassette of inducible DN Ubc12 and treated with TC. The accumulation of this lower molecular weight species suggests the manipulation of HEK293 has altered Elk-1 monoubiquitylation sensitivity.

A single protein can be ubiquitylated by multiple E3 ligases under different circumstances, such as subcellular location or extracellular stimuli. For example, the CDK inhibitor p27kip1 is ubiquitylated by the SCF SKP2 E3 ligase in the nucleus, but by another E3 ligase KPC complex in the cytosol (Kamura, Hara et al. 2004). Previous analysis of the fractions of HeLa cytosol extract has ruled out the presence of Elk-1 specific CRL ligase in the cytosol of HeLa cells (Figure 4-5), whereas analysis on HEK293 WCE containing DN Ubc12 suggests Elk-1 can be ubiquitylated by a CRL. Nevertheless, given that a single protein can be ubiquitylated by multiple E3 ligases, it is possible that Elk-1 is ubiquitylated by a CRL in the nucleus while ubiquitylated by a different E3 ligase, other than a CRL, in the cytosol.

The *in vitro* ubiquitylation assay offers an efficient way to detect Elk-1 ubiquitylation, however there are disadvantages. The addition of ubiquitylation enzymes may restrict or enhance ubiquitylation. E2 conjugating enzymes play an important role in E3 ligase selection, ubiquitin chain initiation, processivity and specificity (Ye and Rape 2009; David, Ziv et al. 2010). The addition of the E2 conjugating enzyme UbcH5c enhanced Elk-1 polyubiquitylation *in vitro* (Figure 4-1 & Figure 4-2), however it was restricted to those facilitated by this particular E2 enzyme and its interacting E3 ligase partners, thereby limiting the observation from the whole spectrum of Elk-1 ubiquitylation. Although a reasonable level of Elk-1 polyubiquitylation was observed in early experiments, it was not possible to achieve the same intensity in later experiments. In order to process and to study Elk-1 ubiquitylation in a more physiological relevant condition, it was decided to adopt a cell-based ubiquitylation assay.

5. Elk-1 is mono- and polyubiquitylated in HEK293 cells

5.1. Elk-1 is polyubiquitylated in HEK293 cells

Previous experiments demonstrated the presence of Elk-1 specific E3 ligase activity in HEK293 cells *in vitro* (Figure 4-1), therefore it was rational to use these cells for a cell-based assay. HEK293 cells were co-transfected with expression vectors encoding C-terminal His-tagged Elk-1 and N-terminal HA-tagged ubiquitin. Whole cell extracts were prepared from the transfected cells treated with proteasome inhibitor (MG132) to inhibit proteasome-mediated proteolysis with the aim of stabilizing polyubiquitylated proteins destined for destruction. A portion of each whole cell extract was used to analyse exogenous protein expression levels. His-tagged Elk-1 was isolated from the rest of the whole cell extract by Immobilized Metal Affinity Chromatography (IMAC) using Ni^{2+} beads under denaturing conditions and HA-tagged ubiquitylated Elk-1 was visualised by immunoblot.

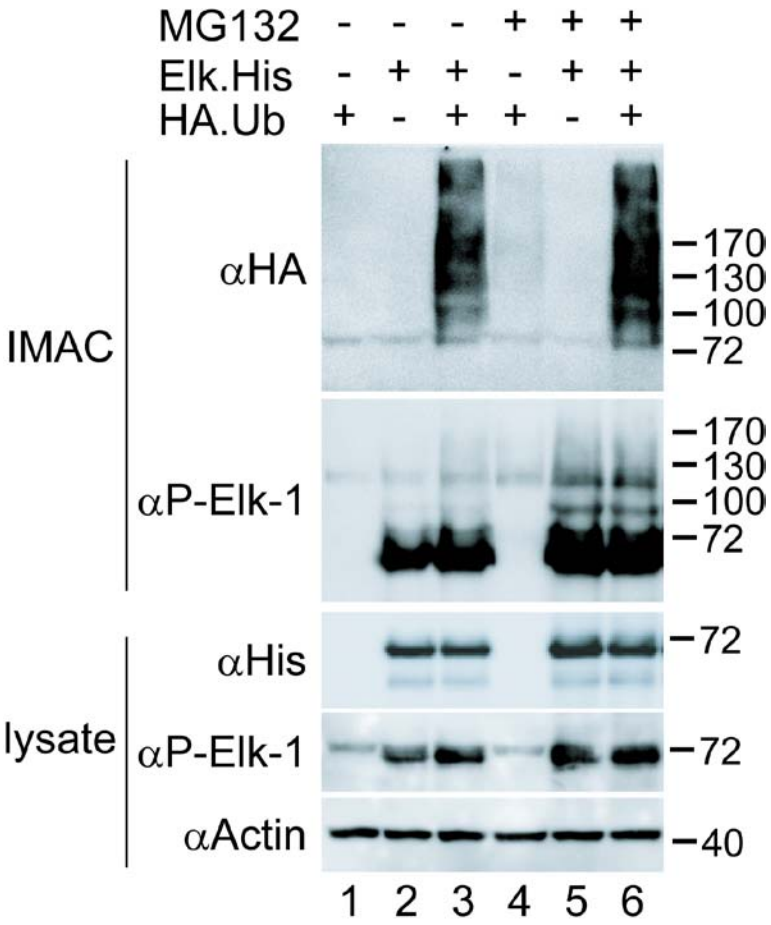

Figure 5-1 Elk-1 is polyubiquitylated in HEK293 cells. Upper panels (IMAC): Whole cell extracts from HEK293 cells transfected with expression vectors encoding His-tagged Elk-1 and HA-tagged ubiquitin and treated with MG132 as indicated were subjected to IMAC under denaturing conditions. Isolated proteins were washed, resolved by SDS-PAGE and analysed by immunoblotting with the antibodies indicated. Lower panels (lysate): Five percent of the whole cell extracts were resolved by SDS-PAGE for immunoblotting. The αP-Elk antibody targets phospho-serine 383 in Elk-1. Molecular weights are indicated on the right in kDa.

Elk-1 was covalently modified with ubiquitin in HEK293 cells (lane 3, top panel, Figure 5-1). The molecular weight of modified Elk-1 was between 72 to >300 kDa, which is equivalent to the range of a single to more than 30 units of ubiquitin attachment. The addition of MG132 mildly stabilized polyubiquitylated Elk-1 (compare lanes 3 with 6, top panel, Figure 5-1), suggesting a portion of polyubiquitylated Elk-1 was degraded by the proteasome. Interestingly, phosphorylated Elk-1 accumulated and exhibited slower SDS-PAGE mobility with proteasomal inhibition (compare lanes 2 & 3 with 5 & 6, second panel, Figure 5-1), suggesting that the proteasome may selectively mediate degradation of phosphorylated Elk-1.

5.2. Elk-1 is monoubiquitylated in HEK293 cells

To confirm Elk-1 ubiquitylation in HEK293 cells, a similar experiment to Figure 5-1 was performed with the substitution of C-terminal His-tagged Elk-1 and N-terminal HA-tagged ubiquitin with N-terminal HA-tagged Elk-1 and N-terminal His-tagged ubiquitin. In contrast to the previous experiment, total ubiquitylated proteins were retrieved by IMAC and modified Elk-1 was visualised by immunoblot.

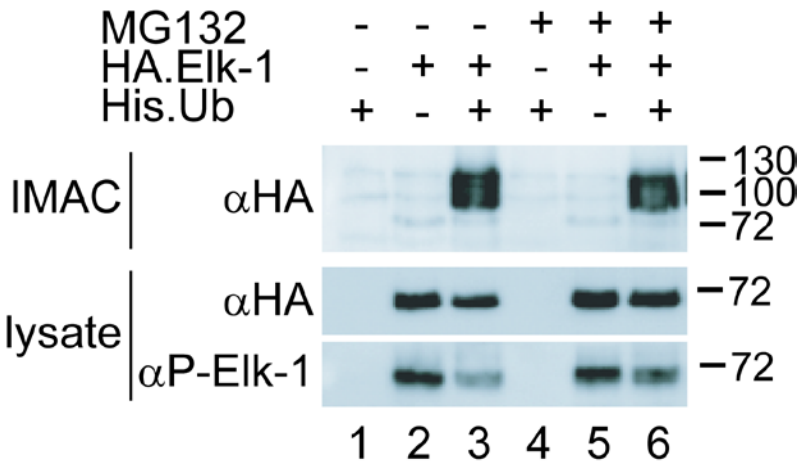

Figure 5-2 Elk-1 is monoubiquitylated in HEK293 cells. Upper panel (IMAC): Whole cell extracts from HEK293 cells transfected with expression vectors encoding HA-tagged Elk-1 and His-tagged ubiquitin and treated with MG132 as indicated were subjected to IMAC under denaturing conditions. Isolated proteins were washed, resolved by SDS-PAGE and analysed by immunoblotting with the indicated antibodies. Lower panels (lysate): Five percent of the whole cell extracts were resolved by SDS-PAGE for immunoblotting. The αP-Elk antibody targets phospho-serine 383 in Elk-1. Molecular weights are indicated on the right in kDa.

A different pattern of ubiquitylated Elk-1 was detected compared to that in Figure 5-1. The size of the retrieved Elk-1 was about 80 - 110 kDa, which is equivalent to 1 - 3 units of ubiquitin attachment (lane 3, upper panel, Figure 5-2). The level of the modified Elk-1 was not stabilised by proteasome inhibitor, suggesting its non-proteolytic role (compare lanes 3 with 6, upper panel, Figure 5-2). Elk-1 phosphorylation was significantly reduced when exogenous ubiquitin was co-expressed, which was inversely correlated to Elk-1

ubiquitylation, indicating phosphorylated Elk-1 may be preferentially ubiquitylated (compare lanes 2 & 3 with 5 & 6, upper and bottom panel Figure 5-2). Many proteins are ubiquitylated in a phosphorylation-dependent manner via phosphodegron (Won and Reed 1996; Verma, Annan et al. 1997). The effect of Elk-1 phosphorylation on its ubiquitylation is investigated in chapter 8.

Although the signals of Elk-1 attached with a single, double or triple ubiquitins were similar in this experiment, later experiments revealed that Elk-1 is predominantly modified with a single ubiquitin. To avoid confusion, the observed ubiquitylated Elk-1 species in Figure 5-2 are collectively termed monoubiquitylated Elk-1 in this thesis.

Distinct species of ubiquitylated Elk-1 were preferentially observed by the two experimental procedures (Figure 5-1 & Figure 5-2). The first experiment allows observation of a wider spectrum of ubiquitylated Elk-1 species from single to around 30 attachments, which may lead to either proteolytic or non-proteolytic functions (Figure 5-1). The second experiment detects a narrower scope of ubiquitylated Elk-1 species from single to up to three ubiquitin attachments, which only confers to non-proteolytic functions (Figure 5-2). An excess amount of Ni^{2+} beads were used in IMAC to retrieve His-tagged proteins in both experiments, therefore the dissimilar observations were unlikely due to different quantity of distinct ubiquitylated Elk-1 species, but more likely caused by the number of epitope tags attached on ubiquitylated Elk-1. In the first experiment (Figure 5-1), Elk-1 was isolated by IMAC and ubiquitylated Elk-1 was visualised by immunoblotting using antibodies against the epitope tag on each attached exogenous ubiquitin, therefore the more ubiquitin attachments the stronger the signal intensity. In other words, polyubiquitylated Elk-1 would have a stronger detection sensitivity compared to that of monoubiquitylated Elk-1 regardless of their quantity, thus the observed signal intensity doesn't proportionally represent the amount of ubiquitylated Elk-1. In the second experiment (Figure 5-2), total ubiquitylated proteins were

isolated and ubiquitylated Elk-1 was visualised by immunoblotting using the antibodies against the epitope tag on Elk-1. In this case, the signal intensity proportionally correlates to the quantity of ubiquitylated Elk-1 disregarding of the number of ubiquitin attachment. Therefore the majority of ubiquitylated Elk-1 is conjugated with a single ubiquitin.

It is worth noting that despite the relatively even signal of polyubiquitylated and monoubiquitylated Elk-1 observed using the first experimental procedure (Figure 5-1), only monoubiquitylated Elk-1 was detected using the second experimental procedure (Figure 5-2). This study was more interested in the non-proteolytic role of ubiquitylation to Elk-1. To avoid the ambiguity of mixed ubiquitylated species, attention was focused on the Elk-1 species with fewer ubiquitin attachments, preferentially observed by the second experimental procedure.

5.3. Mapping of Elk-1 monoubiquitylation site

To investigate the function of Elk-1 monoubiquitylation, one aim was to generate an Elk-1 derivative that is monoubiquitylation defective by eliminating the ubiquitin acceptor site(s). Lysine residues were substituted with arginine in combination in Elk-1 by site-directed mutagenesis with the aim of abolishing its ubiquitylation. The ubiquitylation sensitivity of Elk-1 derivatives was examined using the same cell-based ubiquitylation assay as described in Figure 5-2.

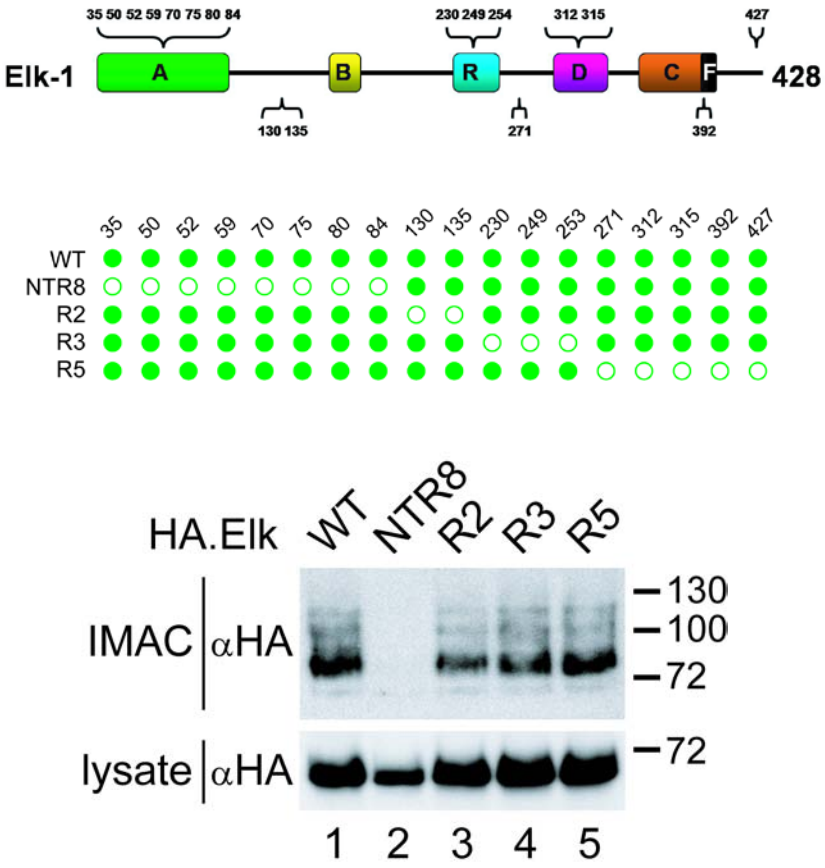

Figure 5-3 Elk-1 is monoubiquitylated in the ETS-domain. Top: Schematic representation of lysine residue distribution in Elk-1. Middle: Table of the location of the substitutions in Elk-1, lysine residues and arginine substitutions are represented with green circles and open circles respectively. Bottom: upper panel (IMAC): Whole cell extracts from HEK293 cells transfected with expression vectors encoding His-tagged ubiquitin and HA-tagged Elk-1 derivative as indicated were subjected to IMAC under denaturing conditions and resolved by SDS-PAGE for immunoblotting with the indicated antibodies. Bottom panel (lysate): Five percent of the whole cell extracts were resolved by SDS-PAGE for immunoblotting.

Wild type Elk-1 was monoubiquitylated similar to the previous observations in Figure 5-2 (lane 1, second panel, Figure 5-3). Replacing all eight lysine residues in the ETS-domain completely abolished ubiquitylation, indicating the ubiquitin acceptor residue may be located in the ETS-domain (lane 2, second panel, Figure 5-3). Although the NTR8 protein level was slightly lower than that of the wild type (compare lanes 1 with 2, bottom panel, Figure 5-3) the difference in protein level cannot be sufficient to cause the distinct ubiquitylation sensitivity between the wild type and NTR8.

The other Elk-1 derivatives with a combination of substitutions outside the ETS-domain had comparable monoubiquitylation levels to that of the wild type (lanes 3 - 5, second panel, Figure 5-3), indicating either that those lysine residues are not sites for Elk-1 monoubiquitylation or that multiple, redundant ubiquitin acceptors are present in the carboxyl-terminus. In a later experiment, it was shown that a derivative lacking all ten carboxyl-terminal lysines (R10) can still be monoubiquitylated (lane 10, top panel, Figure 5-5), eliminating the possibility of redundant carboxyl-terminal ubiquitin acceptor sites.

In addition to the above experiments, another approach was used to examine and confirm that the ubiquitin acceptor site(s) is located in the ETS-domain. In this case, all lysine residues in Elk-1 were substituted with arginine to abolish its monoubiquitylation. Lysine residues were then put back sequentially downstream from the ETS-domain with the aim to rescue Elk-1 monoubiquitylation.

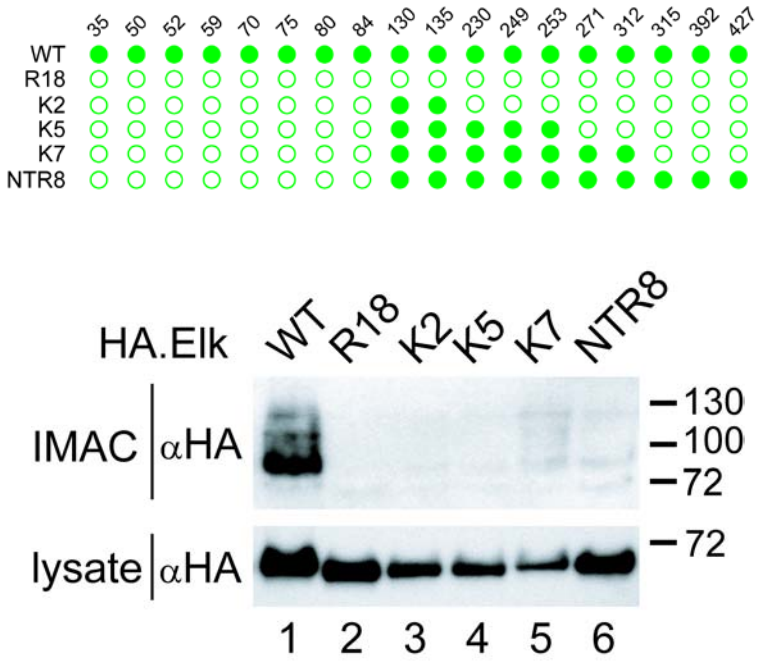

Figure 5-4 Confirmation of Elk-1 monoubiquitin acceptor site in the ETS-domain. Top: Table of the location of the substitutions in Elk-1, lysine residues and arginine substitutions are represented with green circles and open circles respectively. Bottom: Cell-based ubiquitylation assay of Elk-1 was preformed as described for Figure 5-3 using Elk-1 derivatives as indicated.

As expected, the substitution of all lysine residues to arginine in the R18 derivative completely abolished its monoubiquitylation (lane 2, upper panel, Figure 5-4). Putting back the lysine residues downstream of the ETS-domain did not rescue Elk-1 monoubiquitylation (lanes 3 - 6, upper panel, Figure 5-4), also consistent with the notion that the ubiquitin acceptor site is located in the ETS-domain.

To map the ubiquitin acceptor site(s) within the ETS-domain, another set of "put back" derivatives was used. This time, lysine residues were put back upstream from the ETS-domain. The ubiquitylation sensitivity of Elk-1 derivatives was examined using the same cell-based ubiquitylation assay.

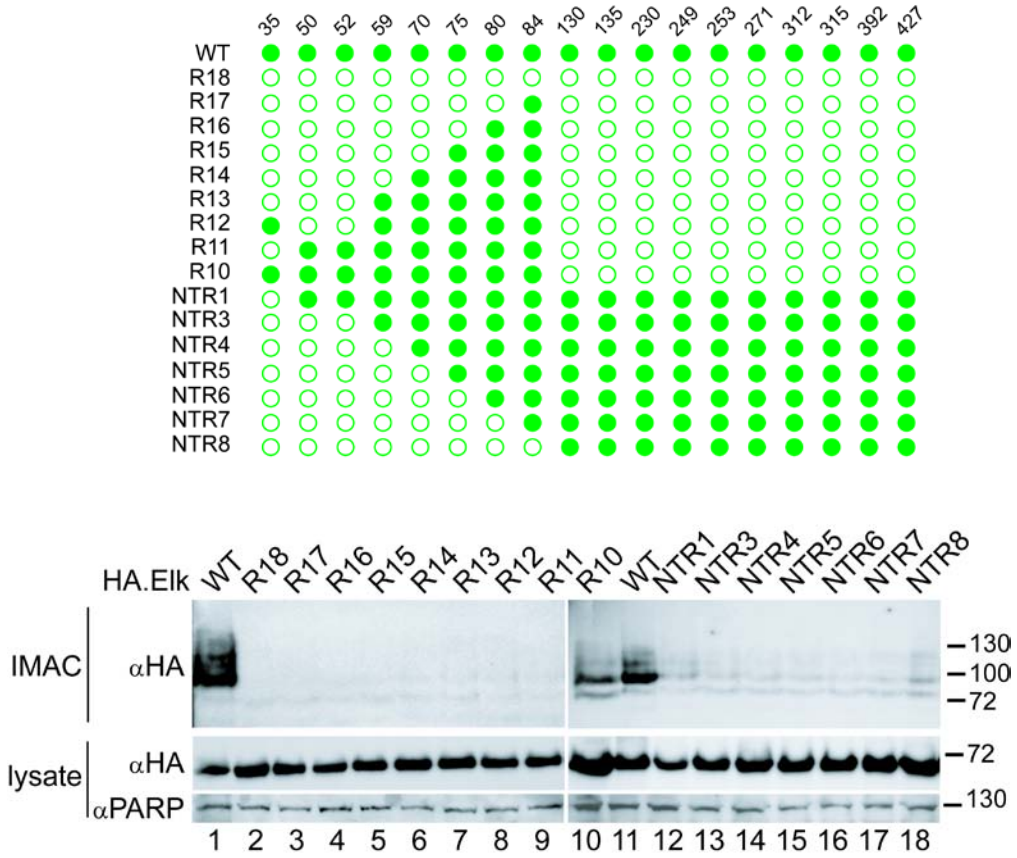

Figure 5-5 Lysines 35/50/52 are important for Elk-1 monoubiquitylation. Top: Table of the location of the substitutions in Elk-1, lysine residues and arginine substitutions are represented with green circles and open circles respectively. Bottom: Cell-based ubiquitylation assay of Elk-1 was performed as described for Figure 5-3 using Elk-1 derivatives as indicated.

Elk-1 derivatives lacking either lysine 35 or lysines 50/52 were not monoubiquitylated (lanes 2 - 9 & 12 - 18, top panel, Figure 5-5). Monoubiquitylation was only regained when lysines 35/50/52 were present in the R10 derivative (lane 10, top panel, Figure 5-5), suggesting these residues are essential for Elk-1 monoubiquitylation. Replacing any of the three amino-terminal lysine residues completely abolished monoubiquitylation (lanes 8 & 9, top panel, Figure 5-5) suggesting factors other than elimination of the ubiquitin acceptor site contributed to the loss of Elk-1 monoubiquitylation. In addition, the derivatives with or without lysine substitutions outside the ETS-domain did not differ in their ubiquitylation sensitivity (lanes 2 - 9 & 12 - 18, top panel, Figure 5-5), confirming those lysine residues are not involved in Elk-1 monoubiquitylation.

The ubiquitin acceptor site has been located within the ETS-domain (Figure 5-3 & Figure 5-4), where substituting any of the three amino-terminal lysine residues (K35/50/52) completely abolished Elk-1 monoubiquitylation (Figure 5-5). The ETS-domain is responsible for DNA binding, in which lysines 50 and 52 play a pivotal role (Mo, Vaessen et al. 2000). Loss of monoubiquitylation for Elk-1 derivatives lacking either lysine 35 or lysines 50/52 suggests all three residues are determinants for Elk-1 ubiquitylation. It is possible that replacing lysines 50 or 52 with arginine affects Elk-1 DNA binding, which in turn may be required for Elk-1 monoubiquitylation, despite both amino acids sharing similarity in charge and structure. This hypothesis prompted the investigation of the impact of lysine to arginine substitutions in Elk-1 on its DNA binding and the role of DNA binding on Elk-1 monoubiquitylation (chapter 7.3).

6. Elk-1 monoubiquitylation inhibits its transactivational ability

6.1. Elk-1 monoubiquitylation inhibits its transactivation on the SRE upon mitogen stimulation

Previous experiments have suggested that Elk-1 monoubiquitylation confers unknown non-proteolytic functions (Figure 5-2). Elk-1 is a transcription factor responsible for transactivation of genes under the control of the SRE in response to extracellular stimuli. It is possible that monoubiquitylation may influence the ability of Elk-1 to transactivate its target genes. The best studied SRE-induction is mediated through activation of TCFs via the MAPK signalling pathways. Among the three major MAPK pathways, the ERK pathway is activated by mitogens, whereas the JNK and p38 pathways are triggered by stress signals (Turjanski, Vaque et al. 2007). Initial experiments on Elk-1 monoubiquitylation were performed in HEK293 cells, however Elk-1 is constitutively active in these cells, as indicated by its high basal phosphorylation (Figure 5-1 & Figure 5-2). To overcome this problem, HeLa cells were used in which Elk-1 phosphorylation, hence activation can be manipulated by serum withdrawal followed by mitogen stimulation, demonstrated in Figure 9-1, Figure 9-2 and Figure 9-3. Initial experiments were performed by another member of the group (Li Li) in HeLa cells and low SRE-induction by exogenous Elk-1 in response to mitogen stimulation was observed due to high basal SRE-dependent transcription (data not shown), which obscures the observation of Elk-1 mediated transactivation. To improve the observation of Elk-1-mediated SRE-induction, a different cell line (NIH3T3) was chosen which had previously been used to study Elk-1 transactivation (Gille, Kortenjann et al. 1995).

The transactivational ability of exogenous Elk-1 derivatives was examined in NIH3T3 cells using a luciferase reporter gene assay system. The cells were co-transfected with three different types of expression vector encoding Elk-1 derivatives, luciferase reporter placed under control of triplicate SRE promoters and β-galactosidase as a control for transfection efficiency. Mitogens (either TPA or serum) were used to activate the ERK pathway to induce Elk-1 mediated SRE-dependent luciferase protein expression. Elk-1 transactivational activity was indicated by the amount of luciferase activity in the transfected cells, normalized to the β-galactosidase activity.

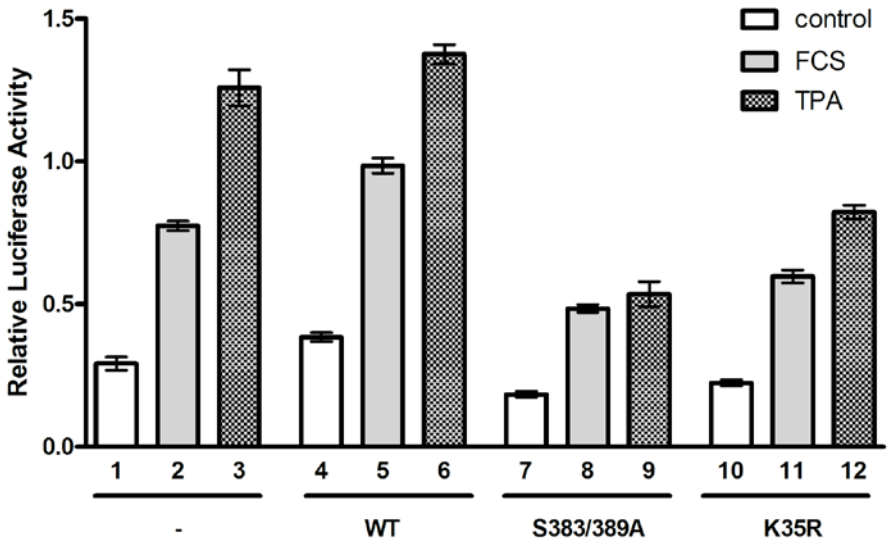

Figure 6-1 Replacing lysine 35 in the ETS-domain of Elk-1 confers dominant negative inhibition on SRE-induction upon mitogen stimulation. Whole cell extracts from NIH3T3 cells treated with either serum or TPA and co-transfected with Elk-1 derivative expression vectors, $(SRE)_3$-luciferase reporter vectors and β-galactosidase expression vectors were measured by a

luminometer. The ratio of luciferase/β-galactosidase activity was presented by the bars. Data shown are representative of triplicate repeats of a single experiment. Error bars represent standard deviation (n=3). The K35R derivative is hyper-monoubiquitylated in this experiment, which is explained in the text below and demonstrated in Figure 10-3.

SRE-dependent transcription represented by luciferase activity increased by two fold when cells were treated with serum (grey bars) (compare bars 1 with 2, Figure 6-1) and by five fold with TPA (dotted bars) (compare bars 1 with 3, Figure 6-1), indicating that endogenous proteins were able to facilitate SRE-dependent transcription in a mitogen inducible manner in NIH3T3 cells. Active ERK phosphorylates Elk-1, as well as other TCFs, Sap-1 and Net, to potentiate their transactivational ability at the SRE (Strahl, Gille et al. 1996; Ducret, Maira et al. 2000). In addition, serum can also induce SRE-dependent transcription, independent of TCFs (Graham and Gilman 1991; Johansen and Prywes 1994; Hill and Treisman 1995), through the RhoA-SRF pathway (Hill, Wynne et al. 1994; Hill, Wynne et al. 1995). Therefore the observed elevated SRE-dependent transcription was likely to be caused by a combination of TCF-dependent and TCF-independent SRE-induction. Compared to serum induction, TPA induced SRE-dependent transcription was more prominent (compare bars 2 with 3, Figure 6-1). TPA is a phorbol ester that activates PKCs (Blumberg 1988) which in turn activates at least three signalling pathways to converge on the SRE; the ERK, JNK and RhoA-SRF pathways (Soh, Lee et al. 1999). PKCs are known for their sustained activation (Cheng, Wung et al. 2001), which explains the higher elevation in SRE-induction by TPA compared to that by serum.

Exogenous expression of Elk-1 resulted in only a slight increase in the overall SRE-induction in basal, serum- and TPA-stimulated NIH3T3 cells (compare bars 1 - 3 with 4 - 6, Figure 6-1), suggesting Elk-1- mediated transcription may be responsible for a portion of the SRE-induction.

Phosphorylation at serine 383/389 is essential for Elk-1 transactivational activity (Gille, Kortenjann et al. 1995). Replacing these phospho-acceptor sites in Elk-1 reduced SRE-induction by approximately 60 % (compare bars 7 – 9 with 4 - 6, Figure 6-1). SRE-dependent transcription was reduced to a level below that of the untransfected cells (compare bars 1 – 3 with 7 - 9, Figure 6-1), indicating that the transcriptionally defective Elk-1 derivative (383/389A) possesses a dominant negative ability as observed in previous publications (Janknecht, Ernst et al. 1993; Gille, Kortenjann et al. 1995), possibly by competing with other activators for the SRE. Interestingly, the K35R derivative also exhibited a similar dominant negative inhibition on SRE-dependent transcription (compare bars 10 - 12, Figure 6-1).

Previous experiments have suggested that lysine 35 may be the site of Elk-1 monoubiquitylation ([NTR1 = K35R], lane 12, upper panel, Figure 5-5), therefore it was conceivable that Elk-1 monoubiquitylation may be required for its full transactivational ability at the SRE. However the location of the ubiquitin acceptor site was drawn into question due to artificial effects caused by the amino-terminal epitope tag on Elk-1 (chapter 10). In this experiment, the Elk-1 derivatives were expressed from the pCMV5.HA expression vectors instead of the pcDNA3.HA expression vectors which were used in previous experiments for the mapping of Elk-1 ubiquitylation site(s) (Figure 5-5). The Elk-1 derivatives generated from the two expression vectors contain a different linker for the HA tag (Figure 10-2) which alters their monoubiquitylation sensitivity. In this case, the K35R derivative exhibited higher monoubiquitylation level than the wild type (compare lanes 4

with 5, Figure 10-3), therefore the dominant negative inhibition on SRE-dependent transcription could be conferred by Elk-1 hyper-monoubiquitylation.

In later experiments, the substitution of lysine 35 with arginine was shown to mildly reduce the ability of Elk-1 to form ternary complexes at the SRE (compare lanes 3 with 6, left panel, Figure 7-3), which may contribute to the loss of Elk-1-mediated SRE-induction. However the K35R derivative must be able to bind to the SRE to elicit the dominant negative inhibition in SRE-induction, eliminating the possibility that the K35R derivative reduces SRE-induction simply by its weakened ability to form a ternary complex on the SRE, consistent with the notion that Elk-1 monoubiquitylation confers a dominant negative effect on SRE-mediated transcription.

In this experiment, the transcriptional activity of Elk-1 derivatives was estimated by the ratio of luciferase and β-galactosidase activity to ensure that the observed different transcriptional activity of Elk-1 derivatives was not due to uneven transfection efficiency or protein expression. However this cannot unequivocally demonstrate the level of Elk-1 derivatives are the same in each experiment. The protein level of Elk-1 derivatives was shown to be equal using western blot analysis in similar experiments (data not shown), however it is worth noting that the level of Elk-1 derivatives was not examined in this experiment.

6.2. Elk-1 monoubiquitylation inhibits its Raf-induced transactivational ability at the SRE

Both extracellular stimuli used in figure 7.1 are able to stimulate SRE-induction via multiple pathways both dependent and independent of TCFs, which may obscure the observation of TCF-mediated SRE-induction. To induce TCF-mediated SRE-dependent transcription specifically via the ERK pathway, NIH3T3 cells were transfected with an expression vector encoding

constitutively active c-Raf 259D, an upstream regulator of ERK (Morrison, Heidecker et al. 1993).

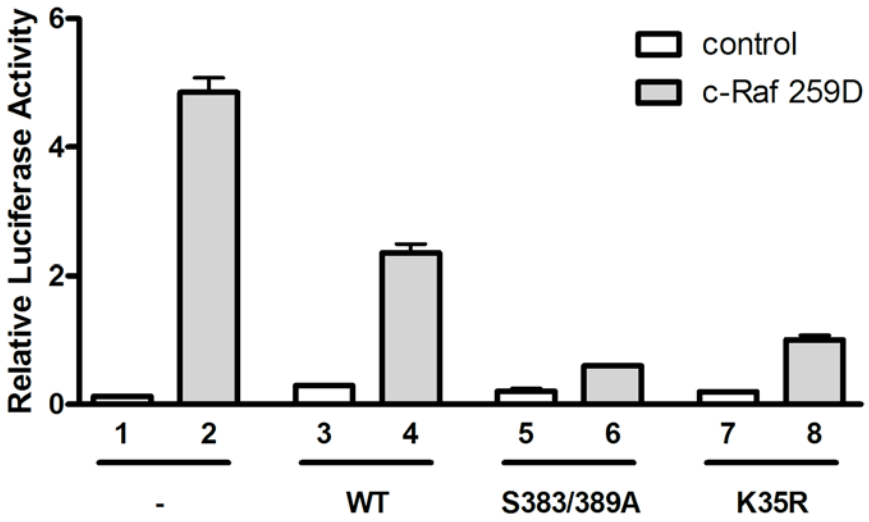

Figure 6-2 Replacing lysine 35 in the ETS-domain of Elk-1 confers dominant negative inhibition on Raf-induced SRE-induction. Whole cell extracts from NIH3T3 cells co-transfected with Elk-1 derivative expression vectors, $(SRE)_3$-luciferase reporter vectors β-galactosidase expression vectors and with/without Raf expression vector were measured by a luminometer. The ratio of luciferase/β-galactosidase activity is presented by the bars. Data shown are representative of triplicate repeats of a single experiment. Error bars represent standard deviation (n=3).

Elevated SRE-induction caused by endogenous proteins was observed with ectopic expression of c-Raf 259D (compare lanes 1 & 2, Figure 6-2). SRE-induction was also increased in the presence of exogenous Elk-1 (compare lanes 3 & 4, Figure 6-2). However the level of elevation was lower than that of the untransfected cells by two fold (compare lanes 1 & 2 with 3 & 4, Figure 6-2), suggesting exogenous Elk-1 was able to induce SRE-induction but inhibit SRE-induction mediated by endogenous proteins. This could explain the low level of SRE-induction by exogenous Elk-1 in response to mitogen stimulation in the previous experiments (compare bars 1 - 3 with 4 - 6, Figure 6-1). Consistent with the previous results (Figure 6-1), both the S383/389A and K35R derivatives displayed dominant negative inhibition on SRE-induction (compare lanes 3 & 4 with 5 & 6 and 7 & 8, Figure 6-2), consistent with the notion that Elk-1 monoubiquitylation exhibits dominant negative inhibition on SRE-induction.

6.3. Lysine 35 is critical for the Elk-1 fusion derivative transactivation on the E74

To facilitate efficient transactivation, Elk-1 must be able to bind to its cognate promoters as well as recruit other transcription mediators. The ETS-domain of Elk-1 is responsible for promoter binding, whereas recruitment of transcription mediators is mainly mediated by its transactivation domain. To focus on the role of Elk-1 monoubiquitylation on its promoter binding, constitutively active Elk-1 derivatives composed of the ETS-domain and the VP16 viral transactivation domain were generated. The VP16 transactivation domain was attached either to the C-terminus of full length Elk-1 (Elk-1-VP16) or in place of the Elk-1 transactivation domain (Elk-1(ΔApa)-VP16) (Kortenjann, Thomae et al. 1994). The luciferase reporter gene was placed under control of the E74 binding site, a stronger ETS-

domain binding site than the SRE, to which Elk-1 is able to bind directly without SRF (Sharrocks 1995). Transactivational ability of the Elk-1 fusion derivatives on the E74 were examined using the same luciferase reporter gene assay.

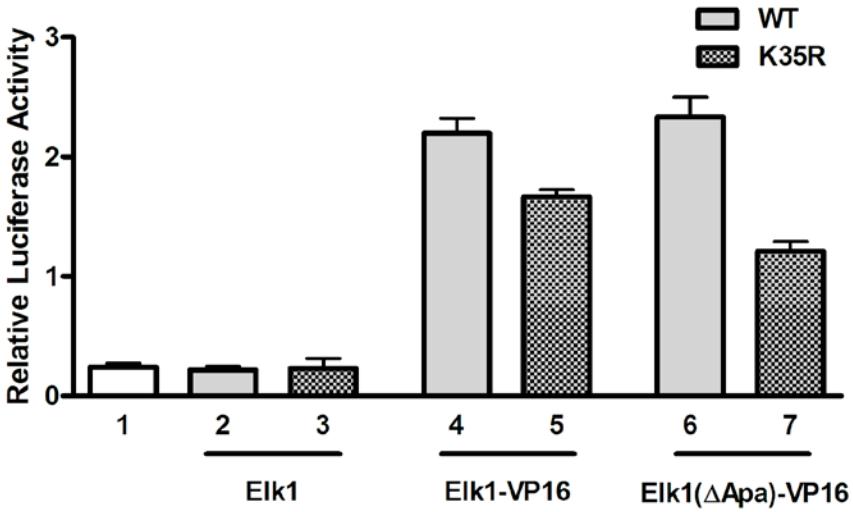

Figure 6-3 Lysine 35 is critical for the Elk-1 fusion derivative transactivation on the E74. Whole cell extracts from NIH3T3 cells co-transfected with Elk-1 derivative expression vectors, $(E74)_3$-luciferase reporter vectors and β-galactosidase expression vectors were measured by a luminometer. The ratio of luciferase/β-galactosidase activity is presented by the bars. Data shown are representative of triplicate repeats of a single experiment with error bars representing standard deviation (n=3).

The basal E74-dependent transcription in the presence of Elk-1 derivatives was low (lanes 2 & 3, Figure 6-3), indicating that the full length Elk-1 derivatives are not transcriptionally

active under normal growing condition in NIH3T3 cells. The attachment of the VP16 transactivation domain on full length Elk-1 significantly increased E74-dependent transcription, indicating this elevated activity was conferred by the VP16 transactivation domain (compare lanes 2 with 4, Figure 6-3).

A reduced E74-mediated transcription was observed for the K35R fusion derivative (compare lanes 4 with 5, Figure 6-3), suggesting this residue is important for Elk-1 binding to the E74. The K35R derivative expressed from the pCMV5.HA expression vector exhibits stronger level of monoubiquitylation (compare lanes 4 with 5, upper panel, Figure 10-3) which led to the thought that the high level monoubiquitylation of the K35R derivative inhibits binding to the E74. However the K35R derivative was later shown to possess significantly weaker intrinsic binding to the E74 (compare lanes 3 with 6, left panel, Figure 7-4), which suggests that the reduction in E74-mediated transcription was due to the loss of E74 binding, caused by the biochemical property alteration of the substitution of lysine 35 with arginine instead of the alteration of monoubiquitylation. Furthermore, a similar reduction on E74-induction was observed for Elk-1 fusion derivatives lacking their own transactivation domain (lanes 6 & 7, Figure 6-3).

7. Ternary complex formation is important for Elk-1 monoubiquitylation

Reporter gene assay data have shown that replacing lysine 35 in Elk-1 with arginine reduces its ability to transactivate gene expression (Chapter 6). The K35R derivative expressed from the pCMV5.HA expression vector exhibits stronger level of monoubiquitylation (compare lanes 4 with 5, upper panel, Figure 10-3), indicating Elk-1 monoubiquitylation may inhibit its transactivational ability. Lysine 35 is located in the ETS-domain which is responsible for DNA binding. Locations of lysine residues in respect to their proximity to the DNA interacting surface are illustrated in Figure 7-1. Although lysine 35 is situated away from the DNA recognition α3 helix, it is possibly that the substitution of lysine 35 may affect its binding to DNA allosterically. Therefore the effects of each point mutation of individual or multiple lysine residues in the ETS-domain of Elk-1 on DNA binding must be examined.

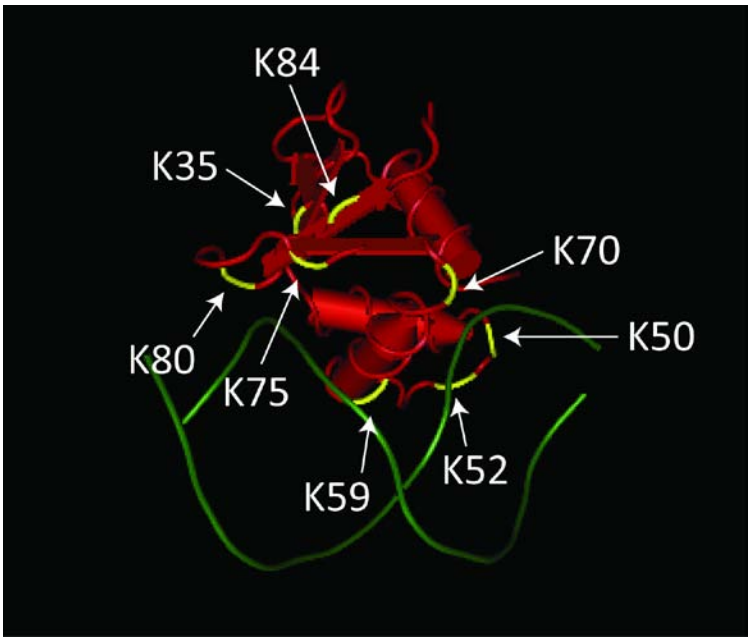

Figure 7-1 ETS/E74 binding. The 2.1Å crystal structure of the ETS-domain of Elk-1 bound to the E74 double-stranded oligonucleotide (Mo, Vaessen et al. 2000). The image was generated using the Cn3D software (NCBI).

Elk-1 binding to DNA was examined in two contexts in which Elk-1 exhibits distinct binding characteristics. The first where Elk-1 cooperates with SRF to form ternary complexes on the SRE (Shaw 1992) and the second where Elk-1 is able to bind to the E74 directly (Shore, Whitmarsh et al. 1996). In addition to single or double lysine to arginine substitutions in Elk-1, double amino acid substitutions of R65A/Y66F and L158P/Y159A were introduced in Elk-1 separately. Arginine 65 and tyrosine 66 are located in the DNA recognition α3 helix whereas leucine 158 and tyrosine 159 are situated in the SRF interacting B-domain. Mutations to these amino acids in Elk-1 were shown to disrupt its DNA or SRF interactions respectively and thus inhibit its binding to both the E74 and the SRE or the SRE alone (Ling, Lakey et al. 1997; Mo, Vaessen et al. 2000). The ability of Elk-1 derivatives to bind the SRE and the E74 were examined using Electrophoretic Mobility Shift Assay

(EMSA). Nuclear extracts containing exogenous Elk-1 derivatives were first prepared from HEK293 cells and examined by western blot analysis (Figure 7-2).

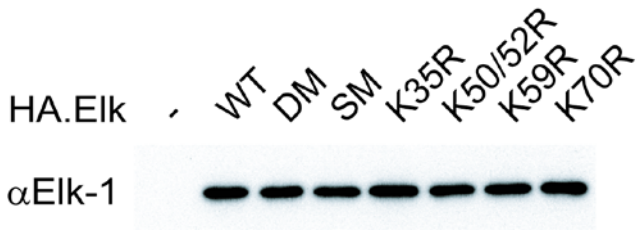

Figure 7-2 Exogenous Elk-1 derivatives used in the EMSAs. Western blot analysis of nuclear extracts from HEK293 cells transfected with expression vectors encoding HA-tagged Elk-1 derivatives. DM and SM represent DNA and SRF binding defective derivative respectively.

7.1. Elk-1 derivatives exhibit different binding to the SRE

Ternary complex formation was reconstituted *in vitro* with [^{32}P]-labelled double-stranded SRE oligonucleotide duplex; recombinant Core SRF, a minimal part of SRF required to form ternary complexes (Shaw 1992); and the obtained Elk-1 derivatives (Figure 7-2).

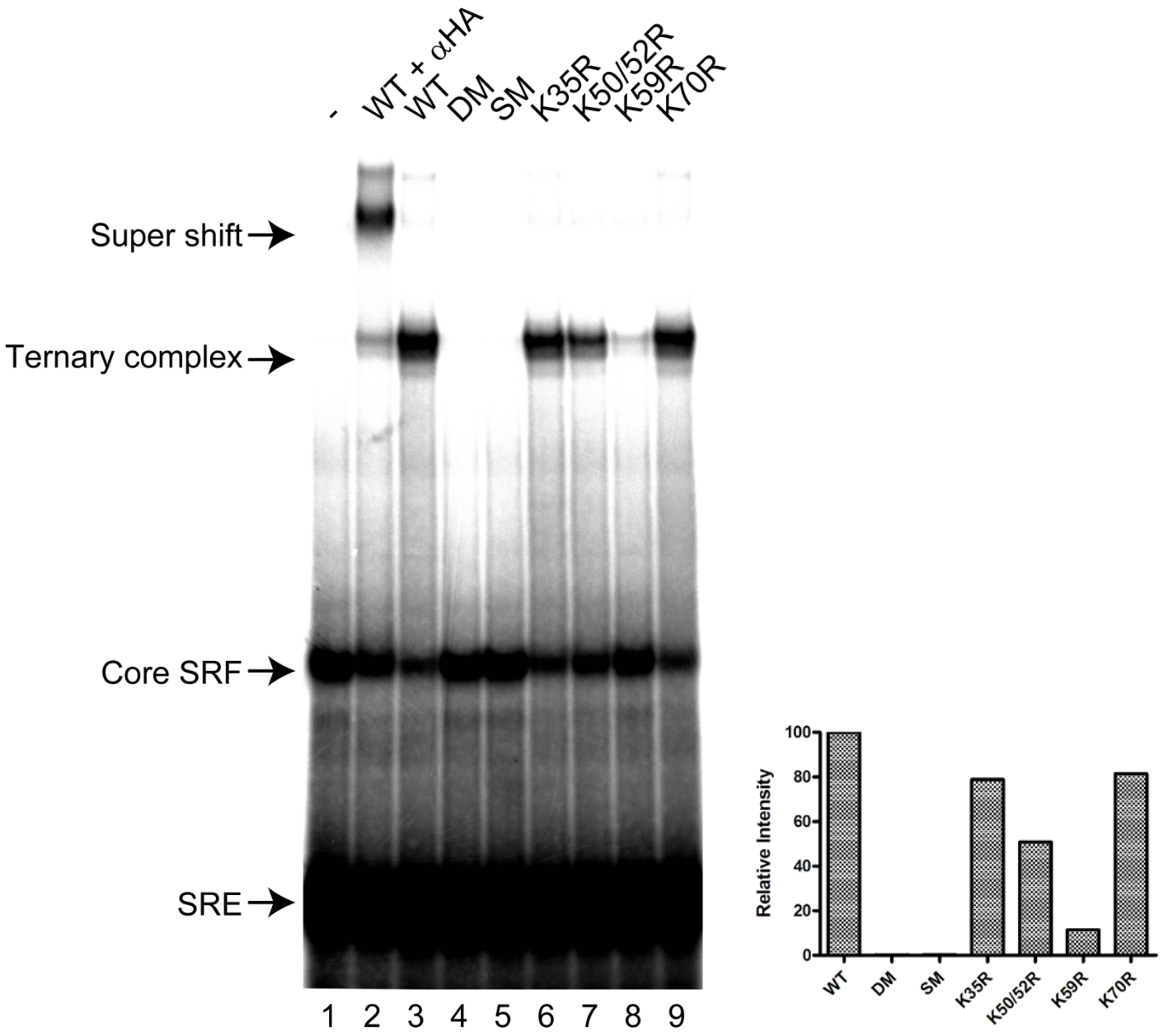

Figure 7-3 Elk-1 derivatives exhibit different binding ability to the SRE. Left: Nuclear extracts containing Elk-1 derivatives (Figure 7-2), [^{32}P]-labelled, double-stranded SRE oligonucleotide duplex and recombinant Core SRF were incubated for 15 mins at room temperature. The complexes were resolved on a non-denaturing gel and visualised by phosphor imager. Right:

Quantification of the level of Elk-1 derivatives binding to the SRE relative to the WT analysed using the Adia densitometry software.

Ternary complexes containing wild type Elk-1 and Core SRF on the SRE were observed (lane 3, Figure 7-3). These complexes were verified by the decreased gel mobility on addition of antibodies against HA-tagged Elk-1 and the diminished level of SRF bound SRE (lane 2, Figure 7-3). As expected, the SRF binding defective derivative (SM) and DNA binding defective derivative (DM) were unable to form ternary complexes (lanes 4 & 5, Figure 7-3). Substitution of either lysine 35 or 70, distal from the DNA recognition α3 helix, reduced ternary complex formation by approximately 20 % (lanes 6 & 9, Figure 7-3). In contrast, the K50/52R mutation in the adjacent loop to the α3 helix diminished the ternary complex formation by almost 50% (lane 7, Figure 7-3) and the K59R mutation in the α3 helix significantly by over 90 % (lane 8, Figure 7-3).

7.2. Elk-1 derivatives exhibit distinct binding to the E74 and the SRE

To examine Elk-1 interactions with DNA directly, i.e. independent of SRF co-operative SRE binding, the E74 binding site was used instead of the SRE (Shaw 1992; Shore, Whitmarsh et al. 1996). The same source of nuclear extracts was used as the previous experiment with the SRE in Figure 7-3 (Figure 7-2). Elk-1 derivatives binding to the E74 were examined using the same EMSA.

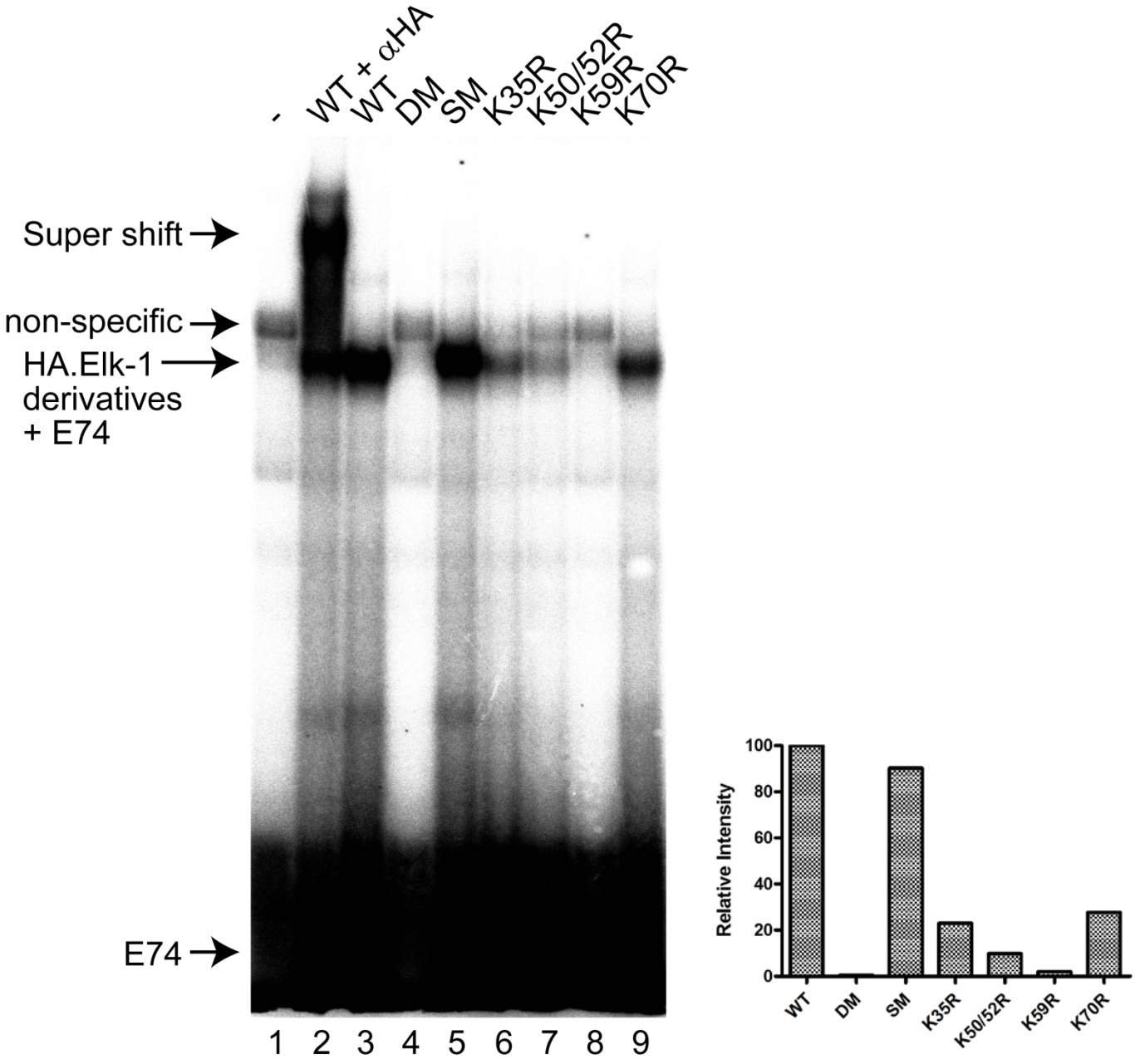

Figure 7-4 Elk-1 derivatives exhibit distinct affinity to the E74, different from that for the SRE promoter. Left: Nuclear extracts containing Elk-1 derivatives (Figure 7-2), [^{32}P]-labelled, double-stranded E74 oligonucleotide duplex were incubated for 15 mins at room temperature. The complexes were resolved on a non-

denaturing gel and visualised by phosphor imager. Right: Quantification of the level of Elk-1 derivatives binding to the E74 relative to that of the WT analysed using the Adia densitometry software.

Elk-1 derivatives exhibit different binding ability to the E74 compared to the SRE. Wild type Elk-1 and the SRF-binding defective derivative (SM) bound to the E74 to a similar level (lanes 3 & 5, Figure 7-4), whereas the DNA binding defective derivative (DM) was unable to bind to the E74 as expected (lane 4, Figure 7-4). Surprisingly, a single or double substitution of any amino-terminal lysine residues reduced Elk-1 binding to the E74 by at least 70 % (compare lanes with 3 with 6-9, Figure 7-4). The reductions were more severe compared to the SRE but retained a similar overall pattern in binding reduction, where replacing lysine residues close to the DNA recognition α3 helix had a more prominent reduction in DNA binding. The E74 is a stronger ETS-domain binding site compared to the SRE and it directly binds with the ETS-domain without SRF cooperative binding, therefore the E74 allows a more direct approach to examine the effect of each amino acid alteration in Elk-1 on its DNA binding. Although binding of the K35R and K70R derivatives to the SRE was only mildly weakened, significant reduction in the binding to the E74 revealed the importance of these residues in Elk-1 DNA binding. The different binding abilities of Elk-1 derivatives between the E74 and the SRE also suggest that SRF cooperative DNA binding compensate for the reduced binding ability when these lysine residues were replaced.

7.3. Ternary complex formation is essential for Elk-1 monoubiquitylation

Replacing any of the lysine residues 35/50/52 completely abolished Elk-1 monoubiquitylation (Figure 5-3, Figure 5-4 & Figure 5-5), suggesting factors other than eliminating the ubiquitin acceptor residue may contribute to the loss of Elk-1 monoubiquitylation. Lysine 50 and 52 are located in the major DNA interacting sites in Elk-1 (Figure 7-1). Replacing these two residues in Elk-1 significantly reduced ternary complex formation on the SRE (compare lanes 3 with 7, left panel, Figure 7-3). To investigate the role of DNA binding on Elk-1 monoubiquitylation, exogenous Elk-1 derivatives with different DNA binding properties were examined using the cell-based ubiquitylation assay.

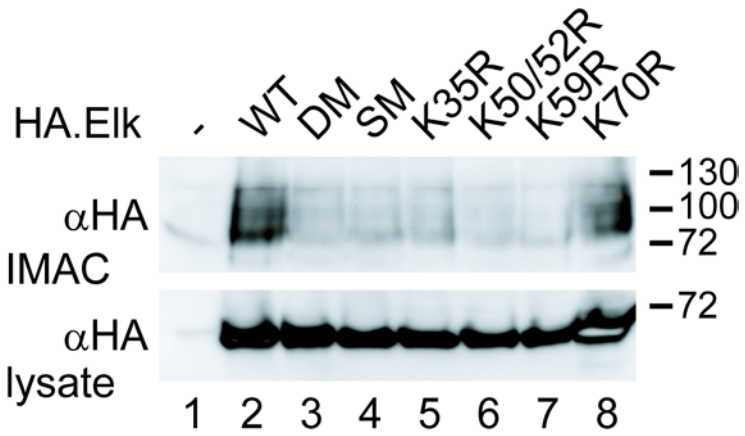

Figure 7-5 Ternary complex formation is essential for Elk-1 monoubiquitylation. Upper panel (IMAC): Whole cell extracts from HEK293 cells transfected with expression vectors encoding His-tagged ubiquitin and HA-tagged Elk-1 as indicated were subjected to IMAC under denaturing conditions. Isolated proteins were washed, resolved by SDS-PAGE and analysed by

immunoblotting with the indicated antibodies. Lower panel (lysate): Five percent of the whole cell extracts were resolved by SDS-PAGE for analysis by immunoblotting. Molecular weights are indicated on the right in kDa.

The DNA binding defective derivative (DM), unable to interact with both the SRE and E74 binding sites (lane 4, left panel, Figure 7-3; lane 4, left panel, Figure 7-4), was not monoubiquitylated (lane3, upper panel, Figure 7-5), indicating DNA binding is required for Elk-1 monoubiquitylation. Interestingly the SRF binding defective derivative (SM), able to interact with the E74 but unable to form a ternary complex on the SRE (lane 5, left panel, Figure 7-2; lane 5, left panel, Figure 7-3), was also not monoubiquitylated (lane 4, upper panel, Figure 7-5), suggesting ternary complex formation is important for Elk-1 monoubiquitylation .

Substitution of single or double lysine residues in the ETS-domain with arginine affected ternary complex formation and Elk-1 monoubiquitylation differently. Both the K35R and K70R derivatives possess a similar ability to form ternary complexes on the SRE as the wild type (compare lanes 3 with 6 & 9, left panel, Figure 7-3). The K35R derivative was not monoubiquitylated (lane 5, upper panel, Figure 7-5) whereas the K70R derivative was monoubiquitylated (lane 8, upper panel, Figure 7-5). This inhibition suggests lysine 35 is important for ubiquitin conjugation, possibly by serving as an ubiquitin acceptor site. Surprisingly the K50/52R substitution significantly weakened the ability of Elk-1 to form ternary complex and it was not monoubiquitylated (lane 7, upper panel, Figure 7-5), suggesting strong ternary complex formation is important for this modification. Moreover the K59R derivative, unable to form ternary complex, was not monoubiquitylated (lane 8,

upper panel, Figure 7-5), consistent with the notion that ternary complex formation is important for its monoubiquitylation.

8. Elk-1 monoubiquitylation is independent of ERK-mediated phosphorylation

8.1. Elk-1 monoubiquitylation is independent of single/double phosphorylation sites

Phosphorylation of proteins can create a phosphodegron, which often serves as a signal for E3 ligase recognition to mediate ubiquitylation (Lang, Janzen et al. 2003). Upon proteasomal inhibition, phosphorylated Elk-1 attained lower mobility in SDS-Page (Figure 5-1), suggesting phosphorylation of Elk-1 may trigger ubiquitylation-dependent proteasomal degradation, possibly mediated by inducible E3 ligase interactions via a phosphodegron. Furthermore a diminished level of phosphorylated Elk-1 coincided with co-expression of ubiquitin (Figure 5-2), supporting the notion that phosphorylated Elk-1 may be subjected to ubiquitylation-dependent proteasomal degradation. To investigate this hypothesis, serine or threonine residues at MAPK consensus sites in Elk-1 were substituted with alanine either individually or in pairs to abolish site specific phosphorylation (Gille, Kortenjann et al. 1995). The ubiquitylation sensitivity of the Elk-1 derivatives was examined using the same cell-based ubiquitylation assay described in Figure 5-2.

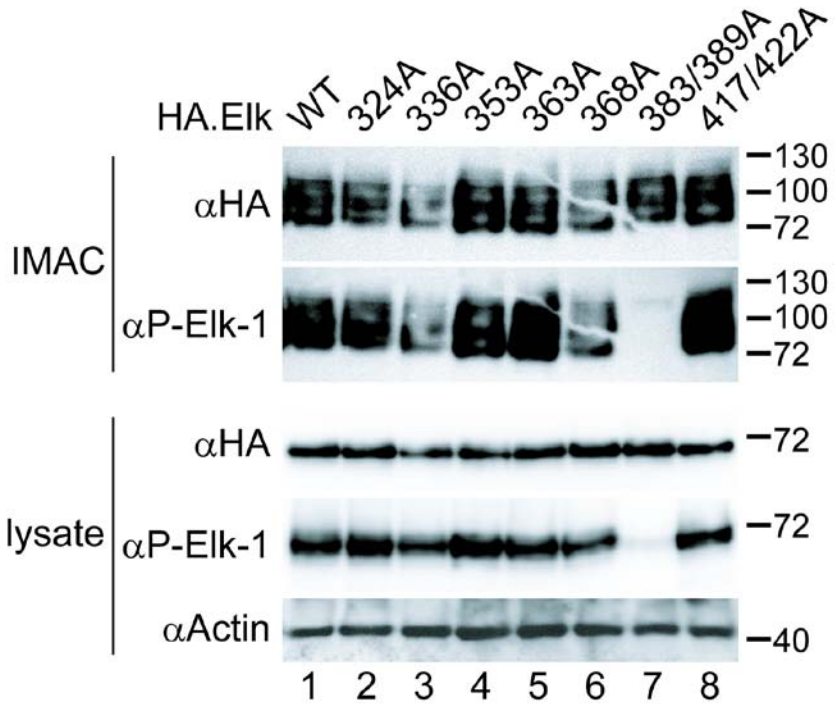

Figure 8-1 Elk-1 monoubiquitylation is independent of single/double phosphorylation site. Upper panels (IMAC): Whole cell extracts from HEK293 cells transfected with expression vectors encoding His-tagged ubiquitin and HA-tagged Elk-1 derivatives as indicated were subjected to IMAC under denaturing conditions. Isolated proteins were washed, resolved by SDS-PAGE and analysed by immunoblotting with the indicated antibodies. Lower panels (lysate): Five percent of the whole cell extracts were resolved by SDS-PAGE for immunoblotting. The αP-Elk antibody targets phospho-serine 383 in Elk-1. Molecular weights are indicated on the right in kDa.

Wild type Elk-1 and derivatives lacking either a single or double phosphorylation sites were all phosphorylated at serine 383 (lanes 1-6 & 8, fourth panel, Figure 8-1), except for

383/389A (lane 7, fourth panel, Figure 8-1). Antibodies targeting other phosphorylation sites in Elk-1 are not commercially available. Most Elk-1 derivatives exhibited monoubiquitylation levels comparable to that of the wild type (lanes 2 - 8, top panel, Figure 8-1), except for 336A and 368A which displayed a mild reduction (lanes 3 & 6, top panel, Figure 8-1). However as the protein levels of these two derivatives were low compared to the wild type (compare lanes 1 with 3 & 6, third & fourth panels, Figure 8-1), this could explain the reduction in their monoubiquitylation.

Moreover, phosphorylated Elk-1 was retrieved by IMAC, indicating that Elk-1 can be simultaneously monoubiquitylated and phosphorylated at serine 383 (lanes 1 - 6 & 8, second panel, Figure 8-1).

Protein ubiquitylation can be triggered by active transcription, where components of the transcription machinery facilitate ubiquitylation of transcription activators (Chi, Huddleston et al. 2001). Elk-1 phosphorylation at serine 383/389 correlates with its transactivational ability. Inhibiting phosphorylation on these residues did not have any effect on its monoubiquitylation, suggesting Elk-1 transactivation is not a prerequisite for its monoubiquitylation (lane 7, top panel, Figure 8-1).

8.2. Elk-1 monoubiquitylation is independent of ERK-mediated phosphorylation

Inhibition on a single or double phosphorylation did not halt Elk-1 monoubiquitylation, possibly due to redundancy of adjacent phosphorylation (Figure 8-1), therefore multiple phosphorylation alterations may be required to elicit the potential effect on Elk-1 monoubiquitylation sensitivity. Elk-1 is phosphorylated at multiple sites by the MAPK family members ERK and JNK (Gille, Kortenjann et al. 1995; Gille, Strahl et al. 1995;

Cruzalegui, Cano et al. 1999), dependent on two interaction sites in Elk-1, the D-domain and the FXFP-motif. ERK interacts with Elk-1 via both the D-domain and the FXFP-motif (Yang, Yates et al. 1998; Jacobs, Glossip et al. 1999), whereas JNK binds to Elk-1 via the D-domain only (Yang, Whitmarsh et al. 1998). Elk-1 is also phosphorylated by another MAPK p38, however its interaction site is currently unknown. To further elucidate the effect of Elk-1 phosphorylation on its monoubiquitylation, either a ten amino acid deletion in the D-domain (amino residues 312 to 321) or a double amino acid substitution of F395L/Q396A in the FXFP-motif was introduced in Elk-1 individually or together, aiming to abolish its MAPK interaction thus multiple phosphorylations (Zhang, Vougier et al. 2010). The ubiquitylation sensitivity of the Elk-1 derivatives was examined using the cell-based ubiquitylation assay.

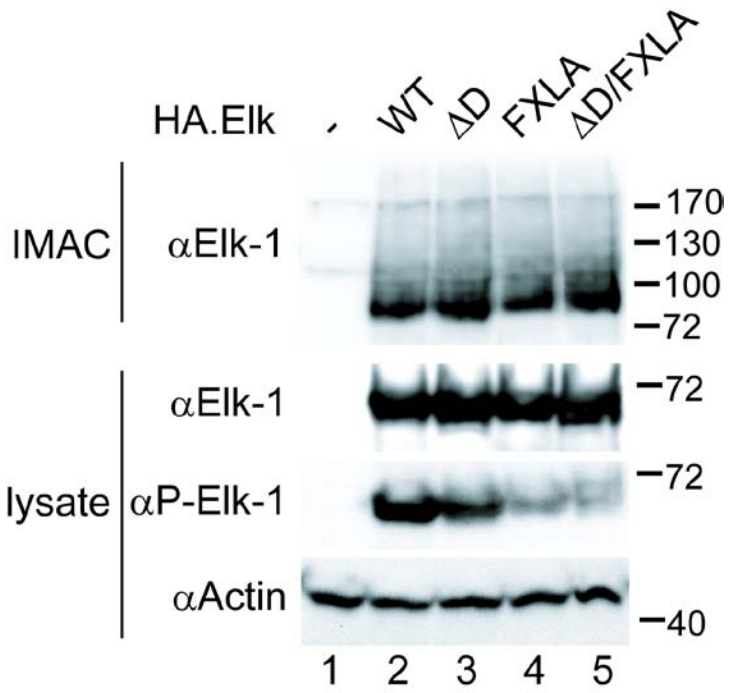

Figure 8-2 Elk-1 monoubiquitylation is independent of ERK-mediated phosphorylation. Upper panel (IMAC): Whole cell extracts from HEK293 cells transfected with expression vectors

encoding His-tagged ubiquitin and HA-tagged Elk-1 derivatives as indicated were subjected to IMAC under denaturing conditions. Isolated proteins were washed, resolved by SDS-PAGE and analysed by immunoblotting with the indicated antibodies. Lower panels (lysate): Five percent of the whole cell extracts were resolved by SDS-PAGE and analysed by immunoblotting.

Elk-1 derivatives lacking either a single or double MAPK binding sites exhibit a similar monoubiquitylation sensitivity compared to that of the wild type (top panel, Figure 8-2), indicating ERK interaction is not required for Elk-1 monoubiquitylation. Elk-1 specifically binds to ERK via both the FXFP-motif and the D-domain, where the FXFP-motif mediates phosphorylation at serine 383 and the D-domain directs phosphorylation at other sites (Fantz, Jacobs et al. 2001). Deletion of the D-domain slightly reduced Elk-1 phosphorylation at serine 383, (compare lanes 2 with 3, third panel, Figure 8-2) whereas mutation in the FXFP-motif significantly diminished this phosphorylation (compare lanes 2 with 4 & 5, third panel, Figure 8-2), confirming phosphorylation at serine 383 is primarily dependent on the FXFP-motif. It has been shown that ERK is able to phosphorylate at least six sites in Elk-1 (Cruzalegui, Cano et al. 1999). Currently, it is difficult to distinguish Elk-1 phosphorylation at other sites, however unaffected monoubiquitylation sensitivity between Elk-1 and Elk-1 derivatives that cannot interact with ERK indicates Elk-1 ubiquitylation is independent of ERK- mediated Elk-1 phosphorylation.

Elk-1 interacts with JNK only via its D-domain, deleting this interaction site did not abolish Elk-1 monoubiquitylation, suggesting the observed Elk-1 phosphorylation at serine 383 was not mediated by JNK. Moreover Elk-1 appeared to be unphosphorylated when the FXFP-

motif was replaced, supporting the notion that JNK was not responsible for Elk-1 phosphorylation in this experiment.

Previous experiments have ruled out the influence of specific single or double phosphorylation on Elk-1 monoubiquitylation (Figure 8-1) and this experiment has eliminated the effect of ERK-interaction and ERK-mediated Elk-1 phosphorylation on its monoubiquitylation (Figure 8-2).

9. Elk-1 monoubiquitylation is diminished in response to ERK activation

9.1. Elk-1 monoubiquitylation is diminished post TPA stimulation in HeLa cells

Protein ubiquitylation is often governed by extracellular stimuli (Gao and Karin 2005). To investigate the fate of monoubiquitylated Elk-1, exogenous Elk-1 was examined upon mitogen stimulation of HeLa cells. HeLa cells were co-transfected with expression vectors encoding N-terminal HA-tagged Elk-1 and N-terminal His-tagged ubiquitin. The cells were treated with TPA at different time intervals to induce Elk-1 phosphorylation and hence activation, the impact on Elk-1 ubiquitylation was examined using the cell-based ubiquitylation assay as described in Figure 5-2.

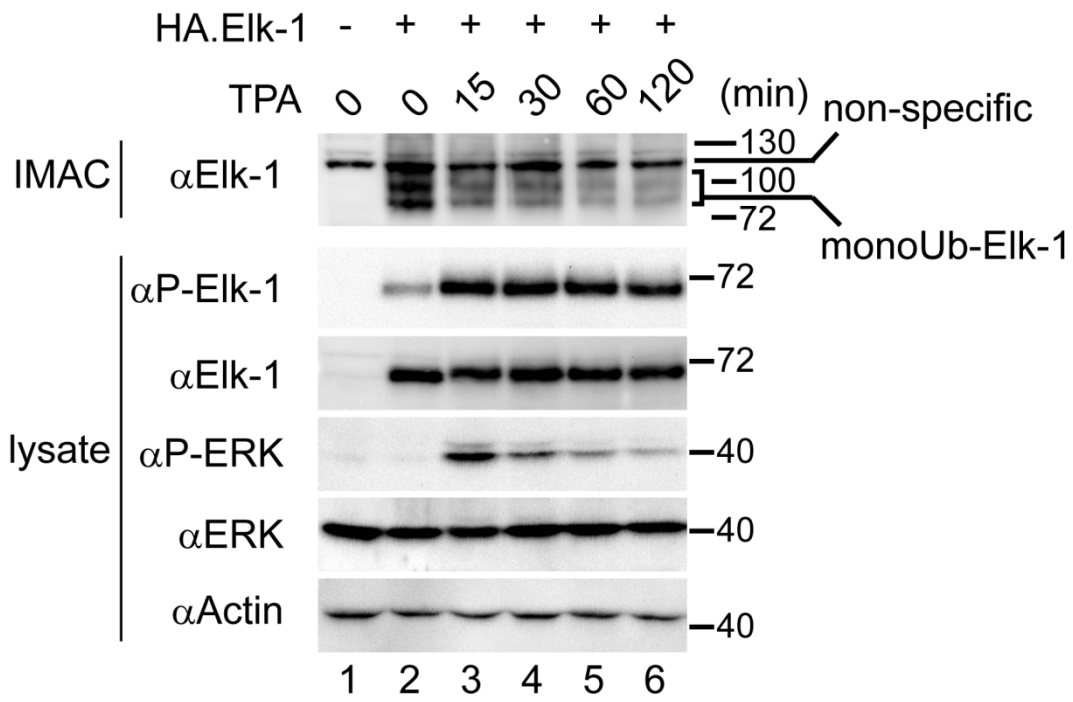

Figure 9-1. Elk-1 monoubiquitylation is diminished post TPA stimulation in HeLa cells. HeLa cells were transfected with expression vectors encoding His-tagged ubiquitin and HA-tagged Elk-1 as indicated, before being serum starved for 24 hours and stimulated with TPA for the indicated times. Upper panel (IMAC): Whole cell extracts from the cells were subjected to IMAC under denaturing conditions. Isolated proteins were washed, resolved by SDS-PAGE and analysed by immunoblotting. Lower panels (lysate): Five percent of the whole cell extracts were resolved by SDS-PAGE for analysis by immunoblotting.

Under serum starvation, Elk-1 monoubiquitylation was at its peak level (lane 2, upper panel, Figure 9-1), whilst the basal Elk-1 phosphorylation at serine 383 was low (lane 2, second panel, Figure 9-1). With TPA stimulation, Elk-1 monoubiquitylation gradually declined

(lanes 2 - 6, upper panel, Figure 9-1), while its phosphorylation was elevated and remained stable after 120 mins (lanes 2 - 6, second panel, Figure 9-1). Phosphorylation at serine 383 corresponds to Elk-1 transactivation (Gille, Kortenjann et al. 1995). This inverse correlation of Elk-1 phosphorylation and monoubiquitylation suggests Elk-1 activation, or Elk-1 phosphorylation per se, may be able to alter its interactions with ubiquitin enzymes (either E3 ligase or deubiquitylase), resulting in the net loss of Elk-1 monoubiquitylation. Alternatively, TPA-induced kinase activation may alter the catalytic activities of Elk-1 specific ubiquitin enzymes via phosphorylation, resulting in the diminished level of Elk-1 monoubiquitylation.

ERK is the best characterised kinase for Elk-1 and it is activated by phosphorylation (Crews, Alessandrini et al. 1992). ERK phosphorylation was elevated upon TPA stimulation and gradually declined (fourth panel, Figure 9-1), which does not completely correlate to the sustained Elk-1 phosphorylation (compare second panel with fourth panel, Figure 9-1). Besides ERK activation, TPA also activates another MAPK, JNK, which can in turn phosphorylate and activate Elk-1 (Soh, Lee et al. 1999). It is possible that other kinases may be activated by TPA in this experiment and Elk-1 may be phosphorylated by those kinases. It is clear that the level of Elk-1 monoubiquitylation is diminished in response to TPA stimulation in HeLa cells. To determine which signal pathway was responsible for the observed loss of Elk-1 monoubiquitylation in the above experiment, the role of ERK was examined in Figure 9-2.

9.2. The loss of Elk-1 monoubiquitylation is rescued by ERK inhibition

To examine whether the loss of Elk-1 monoubiquitylation was caused by ERK-activation, an inhibitor (U0126) was used to inhibit specifically the ERK pathway, aiming to abolish ERK-mediate phosphorylation to rescue Elk-1 monoubiquitylation. U0126 is a specific inhibitor for MEK, the upstream activator for ERK. Elk-1 can also be phosphorylated by stress-induced MAPKs, JNK and p38. To show Elk-1 phosphorylation is mainly mediated by ERK, an inhibitor specific for the p38 pathway (SB202190) was used as a negative control. HeLa cells were treated with MAPK inhibitors and TPA for an hour, when significant loss of Elk-1 ubiquitylation was observed (upper panel, Figure 9-1). Elk-1 monoubiquitylation was examined using the same cell-based ubiquitylation assay.

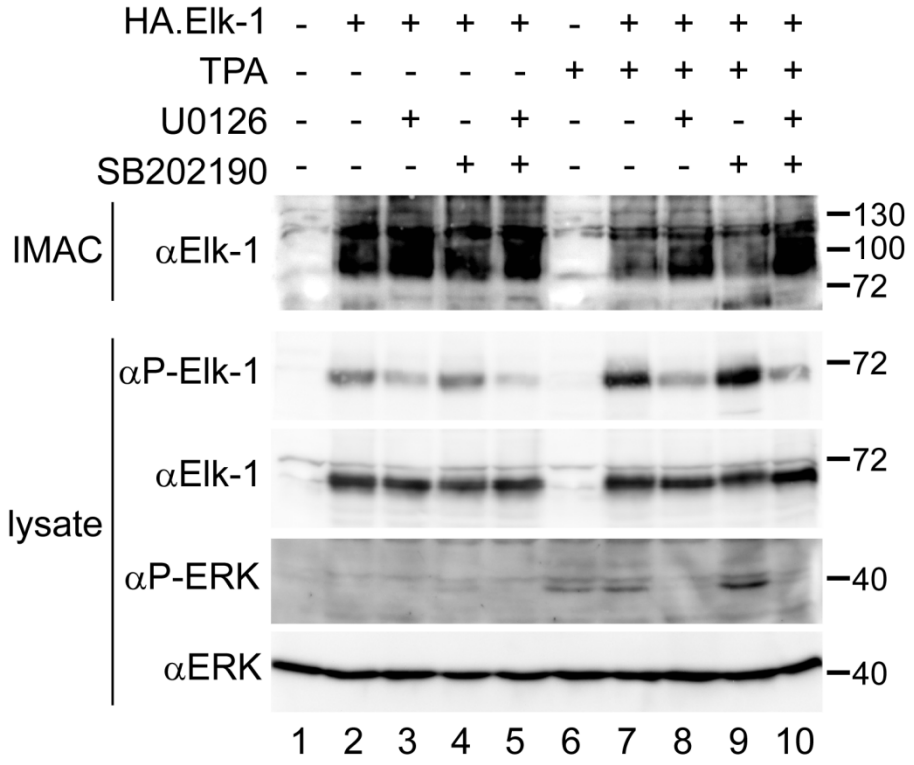

Figure 9-2 Elk-1 monoubiquitylation is rescued by ERK inhibitor post TPA stimulation. HeLa cells transfected with expression vectors encoding His-tagged ubiquitin and HA-tagged Elk-1 as indicated were serum starved for 24 hours and treated with TPA and MAPK inhibitors for one hour as indicated. Upper panel (IMAC): Whole cell extracts from those cells were subjected to IMAC under denaturing conditions. Isolated proteins were washed, resolved by SDS-PAGE and analysed by immunoblotting. Lower panels (lysate): Five percent of the whole cell extracts were resolved by SDS-PAGE for analysis by immunoblotting.

Under serum starvation, Elk-1 phosphorylation was minimal and significantly elevated phosphorylation was observed one hour post-TPA stimulation (compare lanes 2 with 7, second panel, Figure 9-2), whereas Elk-1 monoubiquitylation level was reduced as expected (compare lanes 2 with 7, top panel, Figure 9-2). Interestingly, the reduction of monoubiquitylation was rescued by the addition of U0126 (compare lanes 2 & 7 with 3 & 8, top panel, Figure 9-2) as Elk-1 phosphorylation remained at basal level (lanes 3 & 8, second panel, Figure 9-2), suggesting the reduction of Elk-1 monoubiquitylation and elevated Elk-1 phosphorylation was specifically facilitated by ERK. Addition of the p38-MAPK inhibitor did not affect Elk-1 phosphorylation or monoubiquitylation level (compare lanes 7 with 9, top and second panels, Figure 9-2), eliminating the role of p38 in TPA-induced Elk-1 phosphorylation in HeLa cells.

9.3. Monoubiquitylated Elk-1 is diminished post ERK-mediated phosphorylation

Loss of Elk-1 monoubiquitylation was observed following ERK-mediated activation (Figure 9-1 & Figure 9-2), however it is uncertain how Elk-1 monoubiquitylation was diminished. The decline of monoubiquitylated Elk-1 could be explained by four possible causes: 1) monoubiquitylated Elk-1 can be deubiquitylated, triggered by either Elk-1 activation or ERK-activated deubiquitylase activity; 2) Elk-1 can be deubiquitylated constitutively, TPA stimulation reduces Elk-1 specific E3 ligase activity, resulting in the net loss of Elk-1 monoubiquitylation; 3) the conjugated ubiquitin on Elk-1 can be elongated to generate an ubiquitin polymer for proteolysis or 4) monoubiquitylated Elk-1 can be polyubiquitylated at another lysine different from the monoubiquitylation site. To understand the basis of the loss of Elk-1 monoubiquitylation post ERK-mediated activation, an ubiquitin derivative (Ub.K0) was used to prevent polyubiquitin chain formation and proteolysis, therefore distinguishing if the reduction of Elk-1 monoubiquitylation was due to proteolysis (possible causes 3 & 4). All the lysine residues in this ubiquitin derivative were replaced by arginine to prevent self conjugation, acting as a chain terminator. Elk-1 was co-expressed with either the wild type (Ub) or the lysine-less ubiquitin (Ub.K0), its monoubiquitylation was examined at different TPA stimulation intervals using the cell-based ubiquitylation assay.

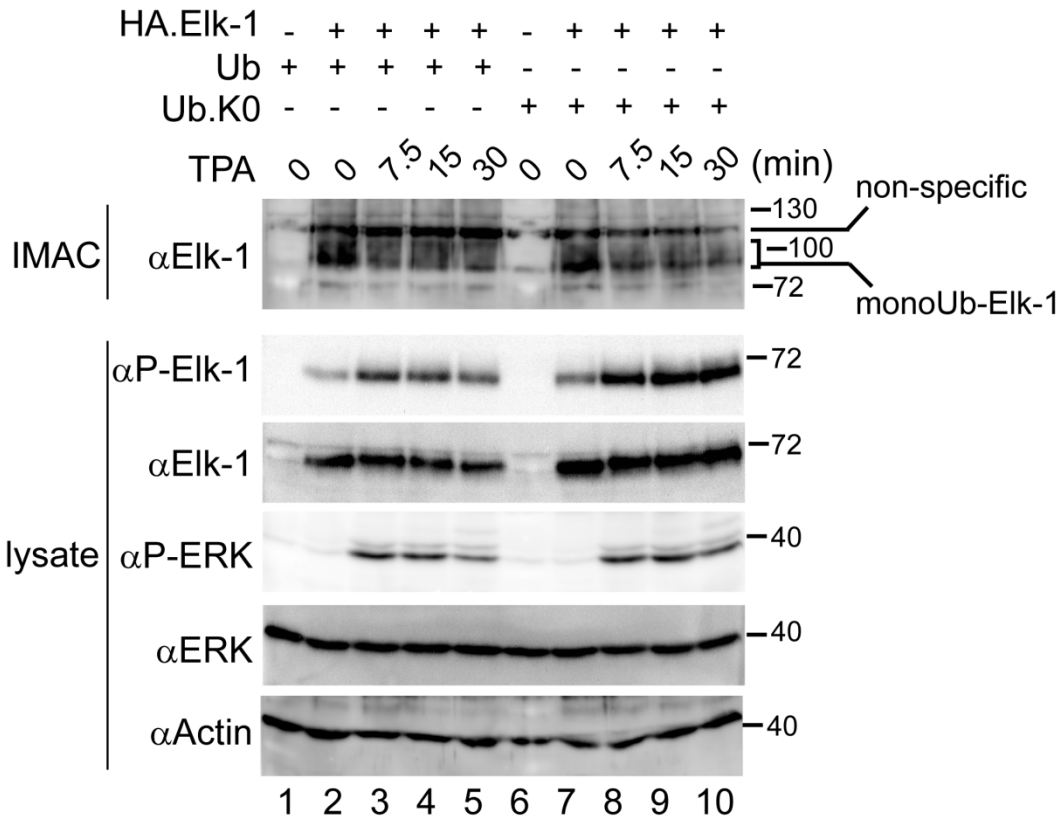

Figure 9-3. Monoubiquitylated Elk-1 is deubiquitylated post TPA-induced ERK-mediated phosphorylation. HeLa cells were transfected with expression vectors encoding HA-tagged Elk-1, His-tagged ubiquitin (Ub) or His-tagged lysine-less ubiquitin (Ub.K0) as indicated, before being serum starved for 24 hours and treated with TPA for times indicated. Upper panel (IMAC): Whole cell extracts from the cells were subjected to IMAC under denaturing conditions. Isolated proteins were washed, resolved by SDS-PAGE and analysed by immunoblotting. Lower panels (lysate): Five percent of the whole cell extracts were resolved by SDS-PAGE for analysis by immunoblotting.

As expected, Elk-1 monoubiquitylation declined upon TPA stimulation (lanes 2 - 5, top panel, Figure 9-3), in inverse correlation with Elk-1 phosphorylation as previously observed in Figure 9-1 (lanes 2 – 5, second panel, Figure 9-3). Interestingly, a similar result was obtained when wild type ubiquitin (Ub) was replaced with lysine-less ubiquitin (Ub.K0) (lanes 7 - 10, Figure 9-3). Given that polyubiquitylation was prevented by Ub.K0, Elk-1 monoubiquitylation would be retained if it was normally lost by polyubiquitylation and proteolysis following ERK-mediated activation. Moreover, the monoubiquitylated Elk-1 retrieved by IMAC consisted of His-tagged Ub.K0 which could not be extended. Therefore this result indicates that monoubiquitylated Elk-1 is not destined to degradation. However it remains uncertain whether the reduction of Elk-1 monoubiquitylation is caused by the increased activity of Elk-1 specific deubiquitylase or by the combination of the reduced activity of Elk-1 specific E3 ligase and the constitutive Elk-1 specific deubiquitylase activity.

Intriguingly, phosphorylated Elk-1 was stabilized in the presence of Ub.K0 (compare lanes 3 – 5 with 8 - 10, second panel, Figure 9-3), suggesting that Elk-1 phosphorylation leads to its degradation and coinciding with the previous observation that MG132 stabilizes phosphorylated Elk-1 (compare lanes 2 & 3 with 5 & 6, second panel, Figure 5-1).

10. N-terminal HA tag influences Elk-1 monoubiquitylation

10.1. The Ternary complex family member Net is ubiquitylated

Elk-1 was shown to be monoubiquitylated both *in vitro* and in cells (chapters 4 & 5). Elk-1 shares many structural and regulatory similarities with family members of the ternary complex factor (TCF), Sap-1a and Net (Shaw and Saxton 2003; Buchwalter, Gross et al. 2004). It is conceivable that Sap-1a and Net may also be subjected to monoubiquitylation. To investigate this hypothesis, a similar experiment was performed as described in Figure 5-2. HEK293 cells were co-transfected with expression vectors encoding N-terminal HA-tagged TCF and N-terminal His-tagged ubiquitin. The ubiquitylation sensitivity of exogenous Net and Sap-1a were examined using the same cell-based ubiquitylation assay.

In previous experiments, Elk-1 protein was generated from the pcDNA3.HA expression vector, which contains the T7 promoter for *in vitro* protein expression (chapter 4) and the CMV promoter for exogenous protein expression in mammalian cells (chapters 5, 7, 8 & 9). Sap-1a and Net were expressed from a different vector, pCMV5.HA, which utilizes the same CMV promoter to mediate protein expression in mammalian cells, but lacks the T7 promoter. To ensure consistency, Elk-1 was also expressed from the pCMV5.HA vector. All TCF proteins contain an N-terminal HA-tag.

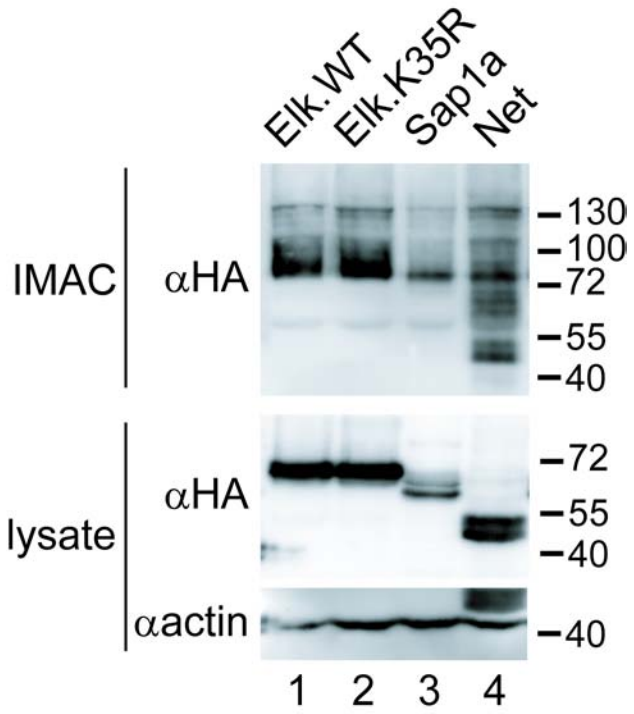

Figure 10-1 Sap-1 and Net are ubiquitylated in HEK293 cells. Upper panel (IMAC): Whole cell extracts from HEK293 cells transfected with expression vectors encoding HA-tagged TCFs and His-tagged ubiquitin as indicated were subjected to IMAC under denaturing conditions. Isolated proteins were washed, resolved by SDS-PAGE and analysed by immunoblotting with the indicated antibodies. Lower panels (lysate): Five percent of the whole cell extracts were resolved by SDS-PAGE for analysis by immunoblotting. The αactin immunoblot was previously used for αHA immunoblotting.

The 453 amino acid Sap-1a appeared as two species with molecular weights of approximately 65 and 62 kDa (lane 3, second panel, Figure 10-1). The lower molecular weight species was more abundant than the higher species. It is uncertain why Sap-1a

appeared as two species; however the N-terminal HA tag on Sap-1a eliminates the possibility of internal transcription initiation. It may have arisen from premature termination of transcription or post-translational modification of Sap-1a protein. Ubiquitylated protein was retrieved by IMAC and located at 80 kDa (lane 3, top panel, Figure 10-1). The approximate 20 kDa molecular weight increase indicates a possible attachment of two ubiquitins to Sap-1a. The level of the retrieved ubiquitylated protein was low and proteins with similar size were retrieved in all other lanes (lanes 1, 2 & 4, top panel, Figure 10-1). Without an appropriate negative control (an experiment without exogenous expression of TCF), it is difficult to verify the retrieved ubiquitylated species represent ubiquitylated Sap-1a.

The 409 amino acid Net also appeared as a duplex with molecular weights of 54 and 52 kDa respectively (lane 4, second panel, Figure 10-1). The abundance of the two species was similar. Ubiquitylated Net appeared in a smear from 56 kDa to 130 kDa, indicating Net was ubiquitylated with different ubiquitin moieties (lane 4, top panel, Figure 10-1).

Elk-1 was monoubiquitylated as previously observed in Figure 5-2 (lane 2, top panel, Figure 10-1). Surprisingly, the K35R derivative was also monoubiquitylated (lane 1, top panel, Figure 10-1), which contradicts the previous observations (Figure 5-5 & Figure 7-5). Elk-1 was generated from a different expression vector in this experiment compared to the previous experiments. This prompted the investigation of the difference between the two HA-tag Elk-1 proteins expressed from either pcDNA3.HA or pCMV5.HA on their monoubiquitylation.

10.2. N-terminal HA tag influences Elk-1 monoubiquitylation

The coding sequence of the N-terminal HA tag was grafted differently to Elk-1 in the two expression vectors, resulting in a different linker region (Figure 10-2). It appeared that the

two HA-tagged Elk-1 lacking lysine 35 exhibit distinct monoubiquitylation sensitivity (Figure 5-5, Figure 7-5 & Figure 10-1). To confirm this observation, either HA-tagged or untagged Elk-1 derivatives were examined in HEK293 cells using the cell-based ubiquitylation assay.

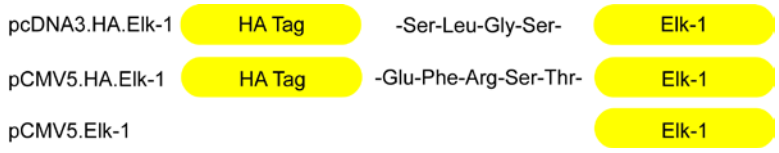

Figure 10-2 Schematic representation of a different linker region between the HA-tag and Elk-1.

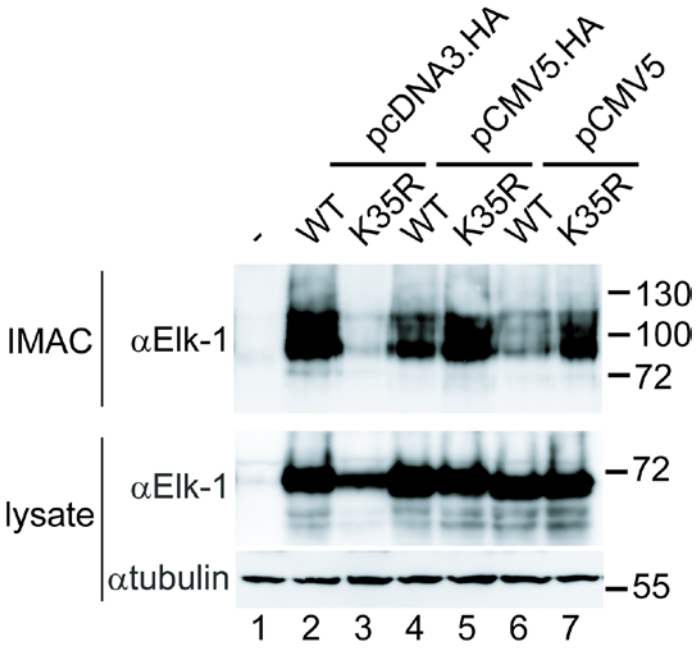

Figure 10-3 N-terminal HA-tag in Elk-1 derivatives alter their monoubiquitylation sensitivity. Upper panel (IMAC):

Whole cell extracts from HEK293 cells transfected with expression vectors encoding HA-tagged TCF and His-tagged ubiquitin as indicated were subjected to IMAC under denaturing conditions. Isolated proteins were washed, resolved by SDS-PAGE and analysed by immunoblotting with the indicated antibodies. Lower panels (lysate): Five percent of the whole cell extracts were resolved by SDS-PAGE for analysis by immunoblotting.

Wild type Elk-1 expressed from the pcDNA3.HA vector was monoubiquitylated, whereas the K35R derivative from the same vector was not monoubiquitylated as previously observed in Figure 5-5 & Figure 7-5 (lanes 2 & 3, upper panel, Figure 10-3). Although the K35R derivative protein level was relatively low compared to that of the wild type (compare lanes 2 with 3, second panel, Figure 10-3), previous experiments have clearly demonstrated the loss of monoubiquitylation was caused by the replacement of lysine 35 in this HA-tagged Elk-1 derivatives (Figure 5-5 & Figure 7-5).

Both wild type and the K35R derivative expressed from the pCMV5.HA vector were monoubiquitylated as obesrved in Figure 10-1 (lanes 4 & 5, upper panel, Figure 10-3). The monoubiquitylation level of the wild type was slightly lower than that of the K35R derivative (compare lanes 4 with 5, upper panel, Figure 10-3). Similar to this, untagged Elk-1 expressed from the pCMV5 vector exhibited a lower monoubquitylation level that increased significantly with the K35R substitution (compare lanes 6 with 7, upper panel, Figure 10-3).

The only difference between the two N-terminal tagged HA-tagged Elk-1 is the linker connecting the HA tag and Elk-1 protein. It is not certain why different linker contributes to the different monoubiquitylation sensitivity. The HA tag is attached to the N-terminus and different linkers may position the HA tag differently. Several lysine residues in Elk-1 (K35, K50, K52, K70, K75 & K84) are located close to the N-terminus (Figure 7-1). It is possible that the N-terminal linked HA tag can mask the ubiquitin acceptor residue, therefore influencing Elk-1 monoubiquitylation.

Elk-1 monoubiquitylation is dependent on ternary complex formation at the SRE (Figure 7-5 & Figure 10-6), therefore this modification must be carried out in the nucleus. However the linker region does not contain any obvious nuclear localisation or export signals. Nevertheless the above experiments have clearly demonstrated distinct monoubiquitylation sensitivity between the two N-terminal HA-tagged Elk-1 derivative expressed from different vectors.

10.3. Confirmation of the reduction of Elk-1 monoubiquitylation post ERK activation

Untagged Elk-1 is physiologically relevant compared to those with an immuno-tag attachment. Elk-1 was generated from the pcDNA3.HA expression vector in previous experiments and its N-terminal HA tag influences its monoubiquitylation sensitivity (Figure 10-3). To verify the previous observation that Elk-1 monoubiquitylation is diminished post ERK-mediated activation, exogenous untagged Elk-1 was examined using the same cell-based ubiquitylation assay. The experiment was performed as descibed in Figure 9-3, except for the replacement of the Elk-1 expression vector from pcDNA3.HA.Elk with pCMV5.Elk.

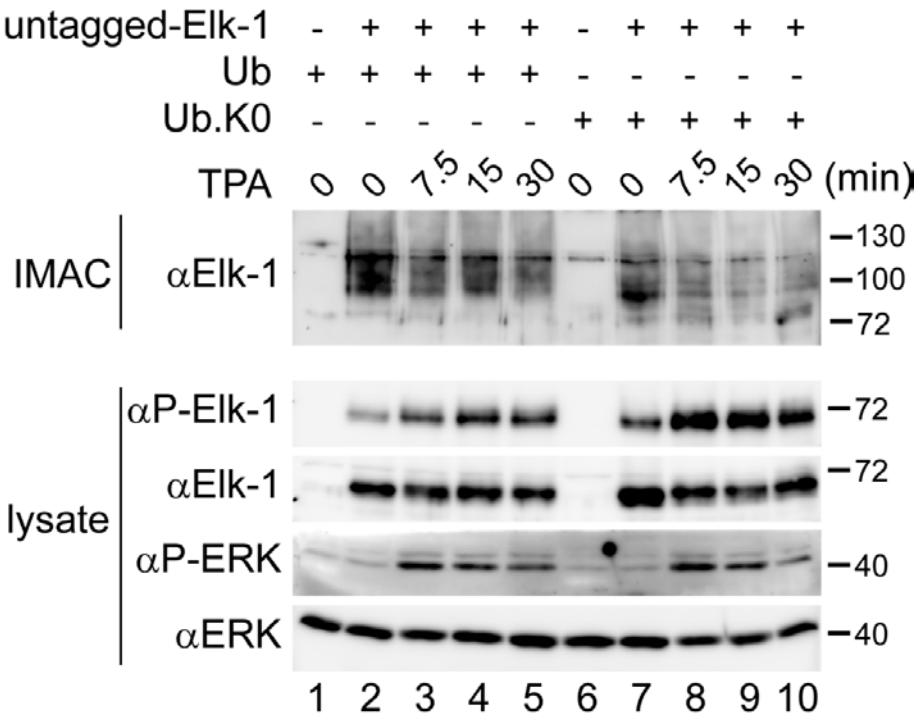

Figure 10-4 Verification of the reduction of Elk-1 monoubiquitylation post ERK-mediated phosphorylation. HeLa cells were transfected with expression vectors encoding untagged Elk-1, His-tagged ubiquitin (Ub) or His-tagged lysine-less ubiquitin (Ub.K0) as indicated, before serum starved for 24 hours and treated with TPA for the times indicated. Upper panel (IMAC): Whole cell extracts from the cells were subjected to IMAC under denaturing conditions. Isolated proteins were washed, resolved by SDS-PAGE and analysed by immunoblotting. Lower panels (lysate): Five percent of the whole cell extracts were resolved by SDS-PAGE for analysis by immunoblotting.

Untagged-Elk-1 expressed from the pCMV5 vector exhibited weaker monoubiquitylation level compared to those expressed from either the pcDNA3.HA or the pCMV5 vectors

(compare lanes 6 with 2 & 4, upper panel, Figure 10-3), which renders it harder to detect. Despite this downside, a reasonable level of untagged Elk-1 monoubiquitylation can still be detected (lane 2, top panel, Figure 10-4). Elk-1 monoubiquitylation was diminished upon mitogen stimulation with inverse correlation to ERK-mediated phosphorylation (lanes 2 - 5, top and second panels, Figure 10-4), consistent with the previous observation in Figure 9-1. Moreover, the level of Elk-1 monoubiquitylation was reduced in the presence of Ub.K0 upon TPA stimulation (lanes 6 - 10, top panel, Figure 10-4), again consistent with the idea that monoubiquitylated Elk-1 is not destined to degradation following TPA stimulation as previously observed (Figure 9-3).

10.4. Mapping of untagged Elk-1 monoubiquitylation site

Distinct monoubiquitylation sensitivity was observed on the K35R derivative with the differently attached N-terminal HA tag (Figure 10-4), suggesting lysine 35 is not the ubiquitin acceptor site in untagged Elk-1. To map the *bona fide* ubiquitin acceptor, the same approach to Figure 5-3 was used. Lysine residues were replaced with arginine residues in combination, aiming to abolish Elk-1 monoubiquitylation by eliminating the ubiquitin acceptor residue. The monoubiquitylation sensitivity of Elk-1 derivatives was examined using the cell-based ubiquitylation assay. HEK293 cells were initially used in the previous mapping experiments because of their high transfection efficiency (chapter 5.3), however Elk-1 was constitutively active in HEK293 cells. Functional studies of later experiments were performed using HeLa cells (chapter 9), in which Elk-1 activation can be manipulated. To ensure consistency, HeLa cells were used instead of HEK293 cells in the following mapping experiments.

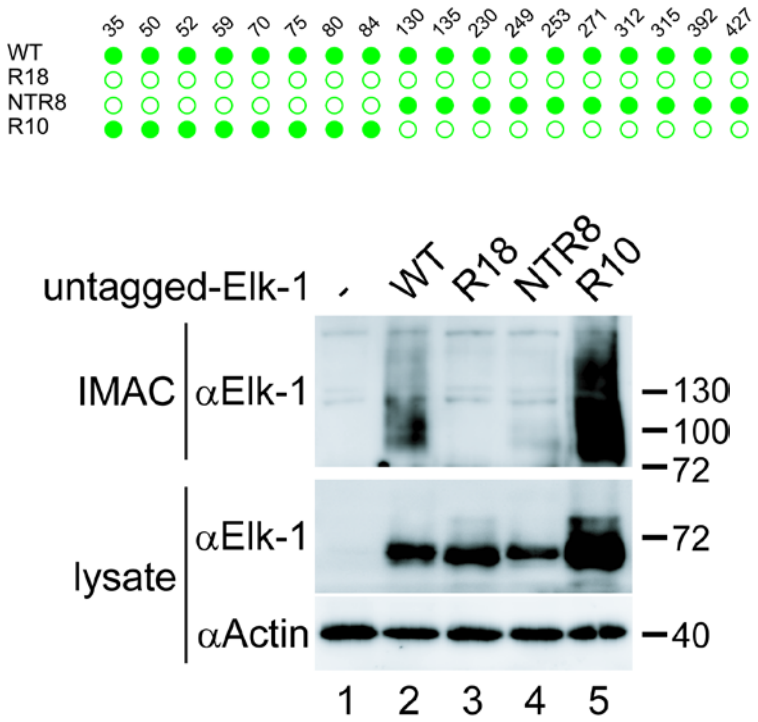

Figure 10-5 Verification of Elk-1 monoubiquitylated in the ETS-domain. Upper panel: Whole cell extracts from HeLa cells transfected with expression vectors encoding untagged Elk-1 derivatives and His-tagged ubiquitin as indicated were subjected to IMAC under denaturing conditions. Isolated proteins were washed, resolved by SDS-PAGE and analysed by immunoblotting with the indicated antibodies. Lower panels: Five percent of the whole cell extracts were resolved by SDS-PAGE for analysis by immunoblotting.

The protein levels of Elk-1 derivatives were different in HeLa cells (lanes 2 – 5, second panel, Figure 10-5), compared to those in HEK293 cells (chapter 5.3). The NTR8 derivative protein level was slightly lower compared to the wild type (compare lanes 2 with 4, second panel, Figure 10-5), whereas the R10 derivative protein level was relatively high (compare

lanes 2 with 5, second panel, Figure 10-5). One obvious explanation for this observation would be that lysine substitutions removed a polyubiquitylation site important for proteolysis, however the protein level of the R18 derivative lacking all lysine residues was comparable to the wild type. It is uncertain why these two Elk-1 derivatives exhibit different protein levels.

Wild type Elk-1 was weakly monoubiquitylated as previously observed in Figure 10-3 (lane 2, top panel, Figure 10-5), whereas the R18 derivative was not monoubiquitylated (lane 3, top panel, Figure 10-5). The R10 derivative, containing only the lysine residues in the ETS-domain, was strongly monoubiquitylated compared to the wild type (compare lanes 2 with 5, top panel, Figure 10-5), suggesting the acceptor site is situated in the ETS-domain. It is worth noting that the protein level of the R10 derivative was relatively high (compare lanes 2 with 5, second panel, Figure 10-5) which could contributes to the stronger level of Elk-1 monoubiquitylation. Replacing all the lysine residues in the ETS-domain in the NTR8 derivative completely abolished monoubiquitylation (lane 4, top panel, Figure 10-5), consistent with the notion that Elk-1 is monoubiquitylated within the ETS-domain.

10.5. Untagged Elk-1 monoubiquitylation is dependent on ternary complex formation.

To verify Elk-1 monoubiquitylation is dependent on ternary complex formation, a similar approach was used as described in Figure 7-5. Double amino acid mutations were introduced into Elk-1, aiming to inhibit its interactions with the SRE and hence inhibit ternary complex formation. The DNA binding (DM) and the SRF binding (SM) defective derivative contains the double mutations of R65A/Y66F and L158P/Y159A respectively. Their monoubiquitylation sensitivity was examined using the cell-based ubiquitylation assay.

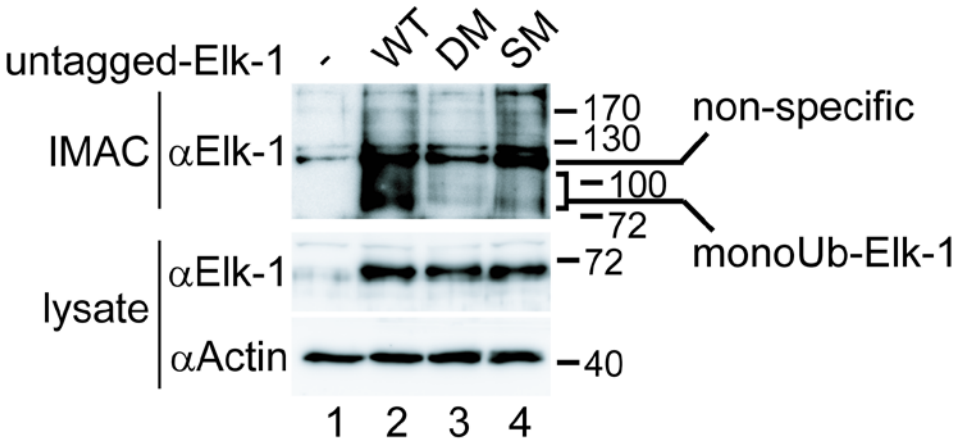

Figure 10-6 Verification of Elk-1 monoubiquitylation is dependent on ternary complex formation. Upper panel (IMAC): Whole cell extracts from HeLa cells transfected with expression vectors encoding HA-tagged Elk-1 derivatives and His-tagged ubiquitin as indicated were subjected to IMAC under denaturing conditions. Isolated proteins were washed, resolved by SDS-PAGE and analysed by immunoblotting with the indicated antibodies. Lower panels (lysate): Five percent of the whole cell extracts were resolved by SDS-PAGE for analysis by immunoblotting. DM and SM represent DNA and SRF binding defective derivative respectively.

Replacing critical DNA binding residues in Elk-1 abolished its monoubiquitylation (lane 3, upper panel, Figure 10-6), indicating that DNA binding is essential for Elk-1 monoubiquitylation. Inhibiting ternary complex formation by abolishing SRF interaction also inhibited Elk-1 monoubiquitylation (lane 4, upper panel, Figure 10-6), consistent with the previous observation that ternary complex formation is critical for Elk-1 monoubiquitylation (Figure 7-5). Although the N-terminal HA-tag influences the acceptor

site for Elk-1 monoubiquitylation, it does not appear to affect the regulation and other determinants of this modification.

11. Discussion

MAPK signalling pathways relay extracellular signals to the nucleus, where ternary complex factors (TCFs) are among their primary nuclear targets. The ternary complex factor Elk-1 is subjected to several post-translational modifications. Elk-1 phosphorylation potentiates its transactivational ability (Gille, Sharrocks et al. 1992; Janknecht, Ernst et al. 1993; Marais, Wynne et al. 1993; Gille, Kortenjann et al. 1995), whereas sumoylation represses it (Yang, Jaffray et al. 2003; Yang and Sharrocks 2004). It has also been shown that Elk-1 is ubiquitylated *in vitro* (Fuchs, Xie et al. 1997), however little work has been done on Elk-1 ubiquitylation.

This thesis has provided some insights into Elk-1 ubiquitylation. Several features of the Elk-1 specific E3 ligases have been revealed. It has also demonstrated that Elk-1 can be either monoubiquitylated or polyubiquitylated, where ternary complex formation at the SRE is important for monoubiquitylation. A strongly monoubiquitylated Elk-1 derivative was shown to exhibit reduced capability to transactivate gene expression at the SRE. Furthermore monoubiquitylated Elk-1 is diminished, triggered by ERK-mediated phosphorylation, hence activation. By investigating the regulation and function of Elk-1 monoubiquitylation, a clearer understanding can be drawn for Elk-1 mediated transcription, hereby providing a paradigm for signal-induced gene expression control.

11.1. Is Elk-1 ubiquitylated by multiple E3 ligases?

E3 ligases play a pivotal role in ubiquitylation by selecting substrates. E3 ligase activity specific for Elk-1 polyubiquitylation was detected in both HEK293 whole cell extracts and

HeLa cytosolic extracts *in vitro* (Figure 4-1 & Figure 4-3). Although the E3 ligase(s) responsible for this modification remains unknown, some features of Elk-1 specific E3 ligase(s) have been revealed. Cullin RING Ligases (CRLs) comprise the largest group of E3 ligases and neddylation is required for their activity (Podust, Brownell et al. 2000; Read, Brownell et al. 2000; Wu, Chen et al. 2000). Inhibition of neddylation by DN Ubc12 significantly reduced Elk-1 polyubiquitylation *in vitro* (Figure 4-7), indicating that at least one E3 ligase specific for Elk-1 polyubiquitylation is a CRL. A single substrate can be targeted by multiple ligases, as exemplified by the case of p53 (Brooks and Gu 2006) and different ligases often facilitate distinct ubiquitin attachments at different acceptor sites. Elk-1 monoubiquitylation levels remained unchanged in the presence of DN Ubc12 (Figure 4-7), suggesting that Elk-1 monoubiquitylation is facilitated by an E3 ligase other than a CRL. In addition, chromatographic separation of HeLa cytosolic extract revealed that the size of a Elk-1 specific E3 ligase is approximately between 29 and 50 kDa (Figure 4-4 & Figure 4-5), which is too small for a CRL that typically consists of a cullin (~776a.a.), a RING (~108 a.a.), an adaptor (~163a.a.) and substrate recognition unit (430 to >1000 a.a.) with an approximate size of >200kDa (Zheng, Schulman et al. 2002). This is consistent with the idea that Elk-1 mono- and polyubiquitylation are facilitated by different E3 ligases. E3 ligases for the same substrate can be situated in distinct subcellular locations, for example in either the cytosol or the nucleus (Kamura, Hara et al. 2004). From the above observations, it is conceivable that Elk-1 is regulated by multiple E3 ligases, where Elk-1 ubiquitylation is mediated by a CRL in the nucleus and by an E3 ligase other than a CRL, in the cytosol.

11.2. Patterns of Elk-1 ubiquitylation

11.2.1. Elk-1 polyubiquitylation

Elk-1 was monoubiquitylated and polyubiquitylated both *in vitro* (Chapter 4) and in cells (Chapter 5). Polyubiquitylated Elk-1 appeared at ~300 kDa *in vitro* (Figure 4-1, Figure 4-3, Figure 4-4, Figure 4-5 & Figure 4-7), indicating an attachment of around 30 units of ubiquitins (Elk-1 and ubiquitin are 62 kDa and 8 kDa respectively). In contrast, Elk-1 polyubiquitylation appeared as smear from 72 kDa to > 300 kDa in HEK293 cells (Figure 5-1). The cause of this different observation of Elk-1 polyubiquitylation is uncertain. Ubiquitylation is a continuous process in cells, which could be reversed by deubiquitinases (Komander, Clague et al. 2009). The addition of ubiquitin enzymes in the *in vitro* assay may shift the balance between ubiquitylation and deubiquitylation. Moreover both processes are tightly regulated by extracellular stimuli, for example: cytokine-induced phosphorylation of the deubiquitinase CYLD inhibits its activity (Reiley, Zhang et al. 2005), whereas phosphorylation of the E3 ligase Itch by JNK activates its activity in cells under stress stimuli (Gallagher, Gao et al. 2006). Monoubiquitylated Elk-1 is deubiquitylated following mitogen-induced activation (Figure 9-3 & Figure 10-4), suggesting an Elk-1 specific deubiquitinases(s) is also regulated by extracellular stimuli. In the absence of appropriate stimuli, the balanced activity between E3 ligases and deubiquitinases could be inclined toward to one side.

Ubiquitin can be self-conjugated via any of its seven lysine residues to form ubiquitin polymers with distinct linkage, which confer different activities to the attached proteins. It has been shown that a minimal chain of four ubiquitins via lysine 48 is sufficient to induce proteasome-mediated proteolysis (Thrower, Hoffman et al. 2000), whereas polyubiquitylation via lysine 63 linkage can lead to non-proteolytic functions, such as protein complex assembly (Hofmann and Pickart 1999; Deng, Wang et al. 2000). The

linkage of the observed ubiquitin polymer on Elk-1 is unknown. To unequivocally define the role of Elk-1 polyubiquitylation, the linkage of Elk-1 polyubiquitylation must be identified. Currently, several techniques can be employed to study ubiquitin chain linkage (Volk, Wang et al. 2005). Mass spectrometry has served as a powerful tool to investigate the "ubiquitome", i.e. linkage specificity of the total ubiquitylated protein population (Peng, Schwartz et al. 2003; Xu, Duong et al. 2009), as well as linkage specificity of individual proteins. For example, this technique has revealed that lysine 48- and lysine 29-linked polyubiquitylation by the HECT-domain ligase KIAA10 (Wang, Cheng et al. 2006) and the lysine 6 linkage by the RING-domain ligase BRCA1 (Nishikawa, Ooka et al. 2004). In addition, ubiquitin derivatives with lysine to arginine substitutions have also been used to provide insights into lysine 63-linked polyubiquitylation in IKK activation (Deng, Wang et al. 2000) and LUBAC-mediated linear ubiquitin chain (Kirisako, Kamei et al. 2006). Recently, linkage-specific antibodies against lysine 48-, lysine 63-linked and linear ubiquitin polymer have been developed (Newton, Matsumoto et al. 2008; Wang, Matsuzawa et al. 2008; Tokunaga, Sakata et al. 2009). Although linkage specificity for Elk-1 polyubiquitylation remained undefined, phosphorylated Elk-1 was stabilized when the 26S proteasome was inhibited (Figure 5-1), suggesting at least one role of Elk-1 polyubiquitylation is to direct Elk-1 for destruction.

11.2.2. Elk-1 monoubiquitylation

Elk-1 was conjugated with a single ubiquitin *in vitro* (Figure 4-7) and in both HEK293 and HeLa cells (Figure 5-2, Figure 9-1 & Figure 10-3). Albeit up to three ubiquitin attachments can be detected in these cells, Elk-1 was conjugated predominantly with a single ubiquitin (Figure 5-3, Figure 5-4 & Figure 5-5). E3 ligase activity specific for Elk-1 monoubiquitylation was detected in HEK293 whole cell extracts *in vitro* (Figure 4-7), but

not in HeLa cytosolic extracts (Figure 4-3), suggesting the E3 ligase for this modification is located in the nucleus. This was later verified by another member of the group (Jürgen Handwerger), where E3 ligase activity specific for Elk-1 monoubiquitylation was detected in HeLa nuclear extracts using the *in vitro* assay. Moreover ternary complex formation is important for Elk-1 monoubiquitylation in both HEK293 and HeLa cells (Figure 7-5 & Figure 10-6), consistent with the notion that the E3 ligase specific for Elk-1 monoubiquitylation is located in the nucleus.

11.3. Elk-1 monoubiquitylation acceptor site

To investigate the role of Elk-1 monoubiquitylation, the ubiquitin acceptor site must be identified. An initial attempt indicated that three amino-terminal lysines 35, 50 and 52 are critical for this modification of HA-tagged Elk-1 expressed from the pcDNA3.HA vector (Figure 5-5). This requirement of all three lysines for Elk-1 monoubiquitylation cannot be explained simply by the elimination of the ubiquitin acceptor residues. These three lysine residues are located in the ETS-domain (Figure 7-1), where lysine 35 lies in the β2 strand and lysines 50/52 lie in the loop between the α2 and the α3 helices. Each of those residues contribute to ternary complex formation at the SRE to different extents (Figure 7-3). Replacing lysine 35 with arginine had little effect on ternary complex formation (Figure 7-3), whereas replacing lysines 50 and 52 reduced complex formation by ~50%. It was later shown that ternary complex formation is important for Elk-1 monoubiquitylation (Figure 7-5), which provides an explanation for the absolute requirement of all three lysines for Elk-1 monoubiquitylation. For the HA-tagged Elk-1 expressed from the pcDNA3.HA vector, replacement of both lysines 50 and 52 inhibited Elk-1 monoubiquitylation by disrupting its ternary complex formation (Figure 5-5), whereas the replacement of lysine 35 eliminated the ubiquitin acceptor site of this HA-tagged Elk-1.

Elk-1 is a member of the ternary complex factor family and lysine 35 is conserved among family members, Sap-1a and Net, which led to the investigation of whether the same lysine also serves as an ubiquitin acceptor site in these two ternary complex factors. A pilot experiment to examine if Sap-1a and Net are ubiquitylated in HEK293 cells (Figure 10-1) revealed that HA-tagged Elk-1 with a different linker expressed from the pCMV5.HA vector exhibits different monoubiquitylation sensitivity. In this case, replacing lysine 35 did not abolish Elk-1 monoubiquitylation, indicating that ubiquitin is not conjugated to lysine 35 of the HA-tagged Elk-1 expressed from the pCMV5.HA vector, inconsistent with the previous observation that lysine 35 is the monoubiquitylation site in the HA-tagged Elk-1 expressed from the pcDNA3.HA vector (Figure 5-5, Figure 10-1 & Figure 10-3). Moreover, the K35R derivative expressed from the pCMV5.HA vector appeared to be monoubiquitylated more strongly compared to the wild type and the same effect was also observed with untagged Elk-1.

The only difference between the two HA-tagged Elk-1 is the linker connecting the HA tag to Elk-1 (Figure 10-2). Both linkers do not contain amino acid residues which may have an obvious influence on ubiquitylation or protein subcellular location, such as groups of positive-charged lysine and arginine or patterns of hydrophobic residues that are normally found in a nuclear localisation or an export signal. Therefore it is unlikely that the residues in these two linkers affect ubiquitylation directly or through restriction of subcelluar location. However the linkers may affect Elk-1 monoubiquitylation indirectly by determining the spatial position of the HA tag. The location of lysine 35 in the β2 strand is close to the N-terminal HA tag, so it is possible that the different linkers could position the HA tag differently, in turn restricting the availability of different lysine residues and resulting in a change in the preference of the acceptor site for Elk-1 monoubiquitylation. Lysines 35, 75 80 and 84 are located in proximity (Figure 11-1), where the side chains of lysines 35, 80 and 84

are all equally accessible, but not the side chain of lysine 75 which is hidden inside the ETS-domain. Restricted accessibility of one ubiquitin acceptor may lead to increased modification of other lysines.

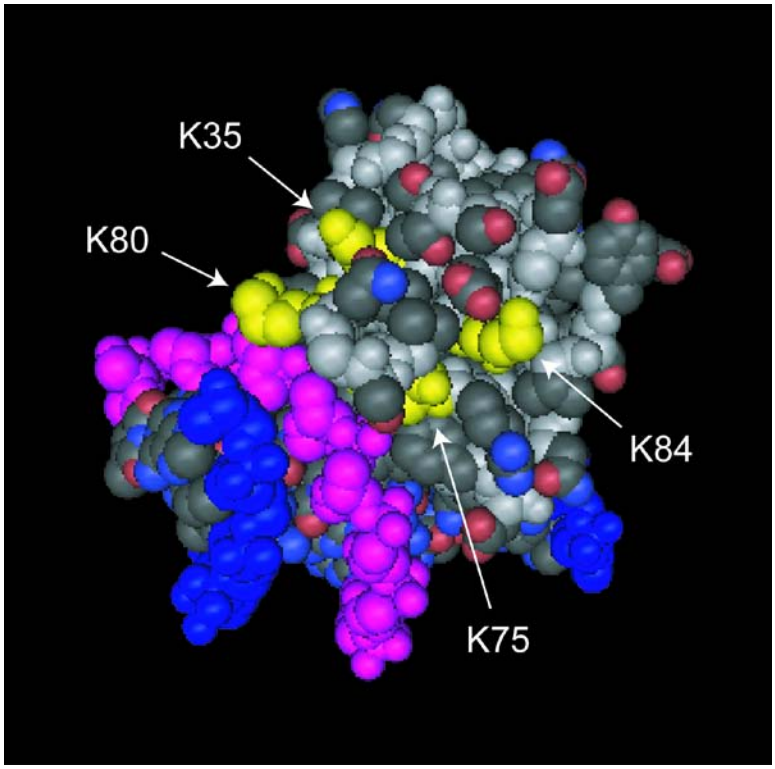

Figure 11-1 ETS/DNA binding. The 2.1Å crystal structure of the ETS-domain of Elk-1 bound to the E74 double-stranded oligonucleotides (Mo, Vaessen et al. 2000). The image was generated using the Cn3D software (NCBI). The ETS-domain is in light grey. lysines 35, 75 , 80 and 84 are in yellow. The E74 duplex is in purple and dark blue. Carbon, oxygen and nitrogen atom are in light grey, red and light blue respectively

To avoid the unexpected side effect of the N-terminal HA tag on Elk-1 monoubiquitylation, untagged Elk-1 was used for the mapping for Elk-1 monoubiquitylation acceptor residue and

other experiments assessing changes in Elk-1 monoubiquitylation. Consistent with the HA-tagged Elk-1, ubiquitin was shown to be attached to the ETS-domain (Figure 10-5), however the exact ubiquitin acceptor residue still remains to be identified.

The replacement of lysine residues with arginine in the ETS-domain affects both Elk-1 monoubiquitylation (Figure 5-5 & Figure 7-5) and ternary complex formation at the SRE (Figure 7-3). Ternary complex formation was shown to be essential for Elk-1 monoubiquitylation (Figure 7-5), therefore it is difficult to demonstrate unequivocally the role of lysine residues as ubiquitin acceptor using Elk-1 derivatives with lysine to arginine substitutions. Mass spectrometry would provide an alternative approach to locate the ubiquitin acceptor site, with which Elk-1 can be examined without alteration of lysine residues.

11.4. Regulation and function of Elk-1 monoubiquitylation

Phosphorylation triggers Elk-1 transactivation of SRE-dependent transcription (Janknecht, Ernst et al. 1993; Marais, Wynne et al. 1993; Gille, Kortenjann et al. 1995; Yang, Shore et al. 1999). Elk-1 was monoubiquitylated in serum-starved HeLa cells, inversely correlating with its phosphorylation (Figure 9-1), indicating that Elk-1 is monoubiquitylated when it is not transcriptionally active. Interestingly, Elk-1 monoubiquitylation was found to be dependent on its ability to form ternary complexes (Figure 7-5), suggesting Elk-1 monoubiquitylation is carried out at the SRE yet has a negative role in transcriptional regulation.

The K35R derivative expressed from the pCMV5.HA vector exhibits stronger monoubiquitylation level compared to the wild type. Luciferase reporter gene data demonstrated that this K35R derivative exhibits a dominant negative effect on SRE-dependent transcription, similar to the transcriptionally defective derivative S383/389A

(Figure 6-1 & Figure 6-2), indicating Elk-1 monoubiquitylation reduces its transactivational ability, in agreement with the observation that Elk-1 is monoubiquitylated when it is not transcriptionally active. Moreover the dominant negative effect on SRE-induction indicates that monoubiquitylated Elk-1 must be able to bind to the SRE.

The level of Elk-1 monoubiquitylation was diminished upon mitogen stimulation in HeLa cells, coinciding with the increase of its phosphorylation (Figure 9-1). The addition of the MEK inhibitor U0126 to inhibit the ERK/MAPK pathway clearly demonstrated that this Elk-1 phosphorylation was mediated by ERK (Figure 9-2). Although reduction of monoubiquitylation was observed, it was not completely abolished, indicating that Elk-1 can be simultaneously monoubiquitylated and phosphorylated, supported by the observation that phosphorylated Elk-1 was retrieved by IMAC against ubiquitin (Figure 8-1).

Using lysine-less ubiquitin to prevent ubiquitin self conjugation, monoubiquitylated Elk-1 was shown not to be destined to degradation following ERK activation (Figure 9-3). However the cause of the reduction of Elk-1 monoubiquitylation remains elusive. One possible answer is that Elk-1 is deubiquitylated post ERK-medicated phosphorylation, hence activation. Alternatively, monoubiquitylated Elk-1 may be deubiquitylated constitutively. Activation of ERK may inhibit Elk-1 specific E3 ligase activity, resulting in the net reduction of Elk-1 monoubiquitylation. Although it is widely reported that phosphorylation can create a binding surface (phosphodegron) for E3 ligase recognition (Sheaff, Groudine et al. 1997; Verma, Annan et al. 1997), phosphorylation-induced interactions with deubiquitinases have not been reported. Theoretically Elk-1 specific deubiquitinases could be recruited in this manner. Alternatively, Elk-1 phosphorylation can induce deubiquitylation indirectly. Elk-1 phosphorylation correlated to its transactivation, resulting in the recruitment of transcription mediators and the general transcription machinery. It was reported that the transcription mediator Med8 can recruit an E3 ligase to the transcription complex (Brower,

Sato et al. 2002), again the same mechanism may be utilised by deubiquitinases. Upon stimulation, ERK is recruited to the SRE by Elk-1 to phosphorylate other proteins in the vicinity (Zhang, Li et al. 2008). Activity of many ubiquitin enzymes was shown to be regulated by phosphorylation (Sarcevic, Mawson et al. 2002; Ichimura, Yamamura et al. 2005; Gallagher, Gao et al. 2006). There is an indication that monoubiquitylated and phosphorylated Elk-1 is located at the SRE, therefore ERK may also regulate Elk-1 deubiquitylation by mediating ubiquitin enzyme phosphorylation, thereby providing another possible regulatory dimension. Nevertheless, the intertwined relationship between Elk-1 monoubiquitylation and ERK-mediated phosphorylation remains to be explored.

11.5. The cycle of Elk-1 monoubiquitylation

To summarise, a model for Elk-1 monoubiquitylation cycle is proposed (Figure 11-2). In un-stimulated cells, Elk-1 is either monoubiquitylated at the SRE or unmodified. The monoubiquitylated Elk-1 is transcriptionally inactive and situated at the SRE waiting for an appropriate signal to activate transcription. Upon mitogen stimulation, monoubiquitylated Elk-1 is phosphorylated by ERK, but Elk-1 remains transcriptionally inactive. Phosphorylation of monoubiquitylated Elk-1 then triggers the recruitment of a deubiquitinase either directly or indirectly to remove its ubiquitin conjugation. The phosphorylated Elk-1 becomes transcriptionally active to mediate SRE-induction. Alternatively, transcriptional inactive Elk-1 conjugated with both ubiquitin and phosphate at the SRE can be replaced by phosphorylated Elk-1. Finally, phosphorylated Elk-1 is either polyubiquitylated for destruction or dephosphorylated to recycle for a new round of transcription.

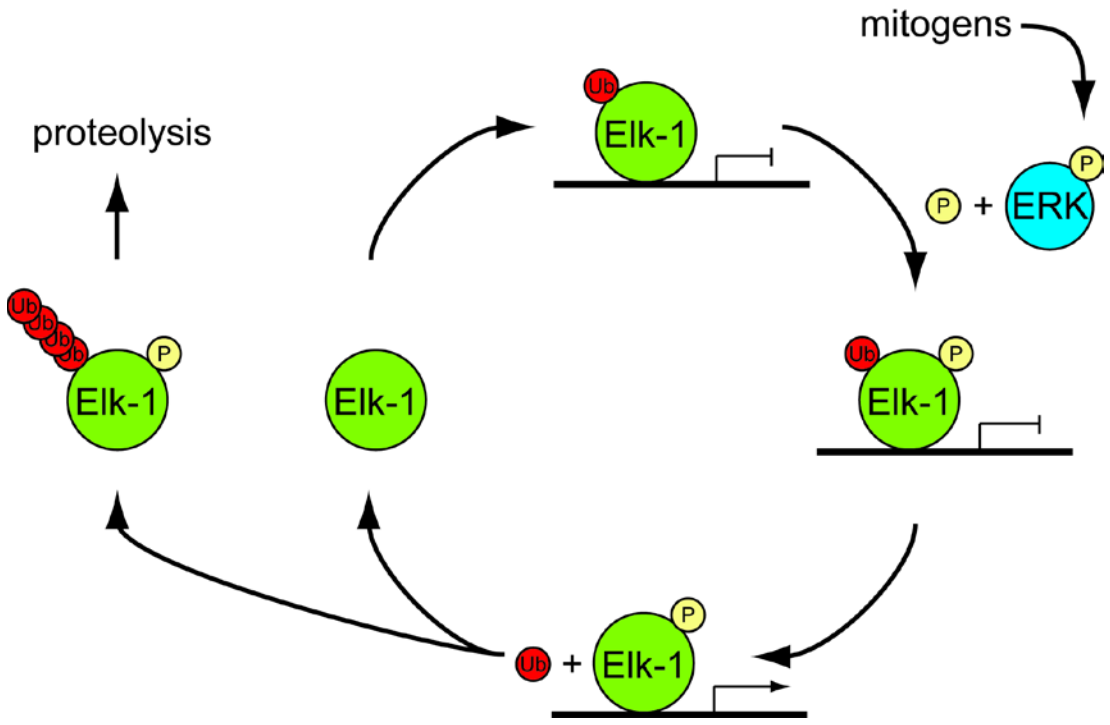

Figure 11-2 A proposed model for Elk-1 monoubiquitylation cycle at the SRE. For clarity reasons, multiple phosphorylations at Elk-1 and ERK are presented as one. The SRF dimer in the ternary complex is omitted

11.6. Experimental limitations

The addition of the E1 activating enzyme Ube1 and the E2 conjugating enzyme Ubc5Hc enhanced Elk-1 ubiquitylation in the *in vitro* assay (Figure 4-1, Figure 4-3 & Figure 4-5) but providing purified components limits ubiquitylation to those facilitated by this particular set of ubiquitin enzymes. E2 conjugating enzymes facilitate ubiquitylation partly by selecting their interacting partners, i.e. E1 activating enzymes and E3 ligases. The E1 activating enzymes select the appropriate protein modifier, while the E3 ligases determine substrate targets. Ube1 specifically activates ubiquitin protein and transfers activated ubiquitin to numerous ubiquitin E2 enzymes, but does not mediate the transfer of ubiquitin-like proteins (Groettrup, Pelzer et al. 2008). Ube1 was the only known E1 activating enzyme at the time

of the experiments, another E1 activating enzyme (Uba6) was later identified (Jin, Li et al. 2007; Pelzer, Kassner et al. 2007). The two E1 activating enzymes cooperate with only a partially overlapping pool of E2 conjugating enzymes, nevertheless Ube1 was able to transfer activated-ubiquitin to UbcH5c. UbcH5c belongs to the UbcH5 family and members of this family are able to interact with a wide range of E3 ligases, including both HECT- and RING-domain E3 ligases (Jensen, Bates et al. 1995; Hatakeyama, Jensen et al. 1997; Lorick, Jensen et al. 1999; Brzovic, Lissounov et al. 2006; Kirkpatrick, Hathaway et al. 2006). Nevertheless the limited observation of the whole spectrum of Elk-1 ubiquitylation would inevitably restrict the identification of the Elk-1 specific E3(s).

All experiments in this study were performed with either recombinant or exogenous proteins. Recombinant proteins used in chapter 4 were generated using the TNT Quick Coupled Transcription/Translation System. This system utilizes the translational machinery of rabbit reticulocyte lysate (immature red blood cells) for protein synthesis using messenger RNA transcribed by the T7 or T3 RNA polymerase as a template. Protein activities are often governed by extracellular stimuli via PTMs. Although this system offers a quick way for protein synthesis, recombinant proteins may not be modified with PTMs without appropriate signals, relevant to those physiologically. Furthermore enzymes responsible for such PTMs may not be present in the rabbit reticulocytes.

For all cell-based assays, Elk-1 derivatives were expressed ectopically. The use of exogenous proteins can increase the level of a particular protein in cells, and also allows alteration of the recombinant proteins, such as an addition of immune-tag or mutations in proteins. It is not clear how elevated expression of a protein would affect its activity or stability. In initial experiments, Elk-1 derivatives expressed from the pcDNA3.HA and the pCMV5.HA vectors contain an N-terminal HA-tag for immunoblotting, which was later demonstrated to confer an artificial effect on monoubiquitylation sensitivity, possibly by

altering the position of the monoubiquitylation acceptor site. The results with HA-tagged Elk-1 derivatives were later verified using untagged Elk-1 derivatives (Figure 10-4, Figure 10-5 & Figure 10-6).

11.7. Conclusion and future work

This thesis has clearly demonstrated that monoubiquitylated Elk-1 is reduced following ERK-mediated phosphorylation (Figure 10-4), the importance of ternary complex formation on Elk-1 monoubiquitylation (Figure 10-6), and that the monoubiquitylation acceptor site is located in the ETS-domain (Figure 10-5). Although the reporter gene data revealed that the strongly monoubiquitylated HA-tagged Elk-1 derivative displays a reduced ability for transactivation (Figure 6-1 & Figure 6-2), there is no direct evidence that this reduction is caused by monoubiquitylation. In order to confirm this, the ubiquitin acceptor of the untagged Elk-1 has to be identified and mutated to generate an Elk-1 monoubiquitylation-defective derivative. In addition, an Elk-1 fusion derivative with a single ubiquitin attachment via peptide linkage would provide a useful tool to examine the role of monoubiquitylation on Elk-1 DNA binding and transcriptional ability. An Elk-1 derivative with a single ubiquitin fused at the C-terminus generated in previous experiments was highly unstable in cells (Sam Shelton). As Elk-1 is monoubiquitylated within the ETS-domain in cells, it is possible that fusing an ubiquitin at the N-terminus may not render it unstable. Finally, to demonstrate unequivocally the role of Elk-1 monoubiquitylation, experiments have to be performed with endogenous Elk-1 to ensure the physiological relevance of this modification.

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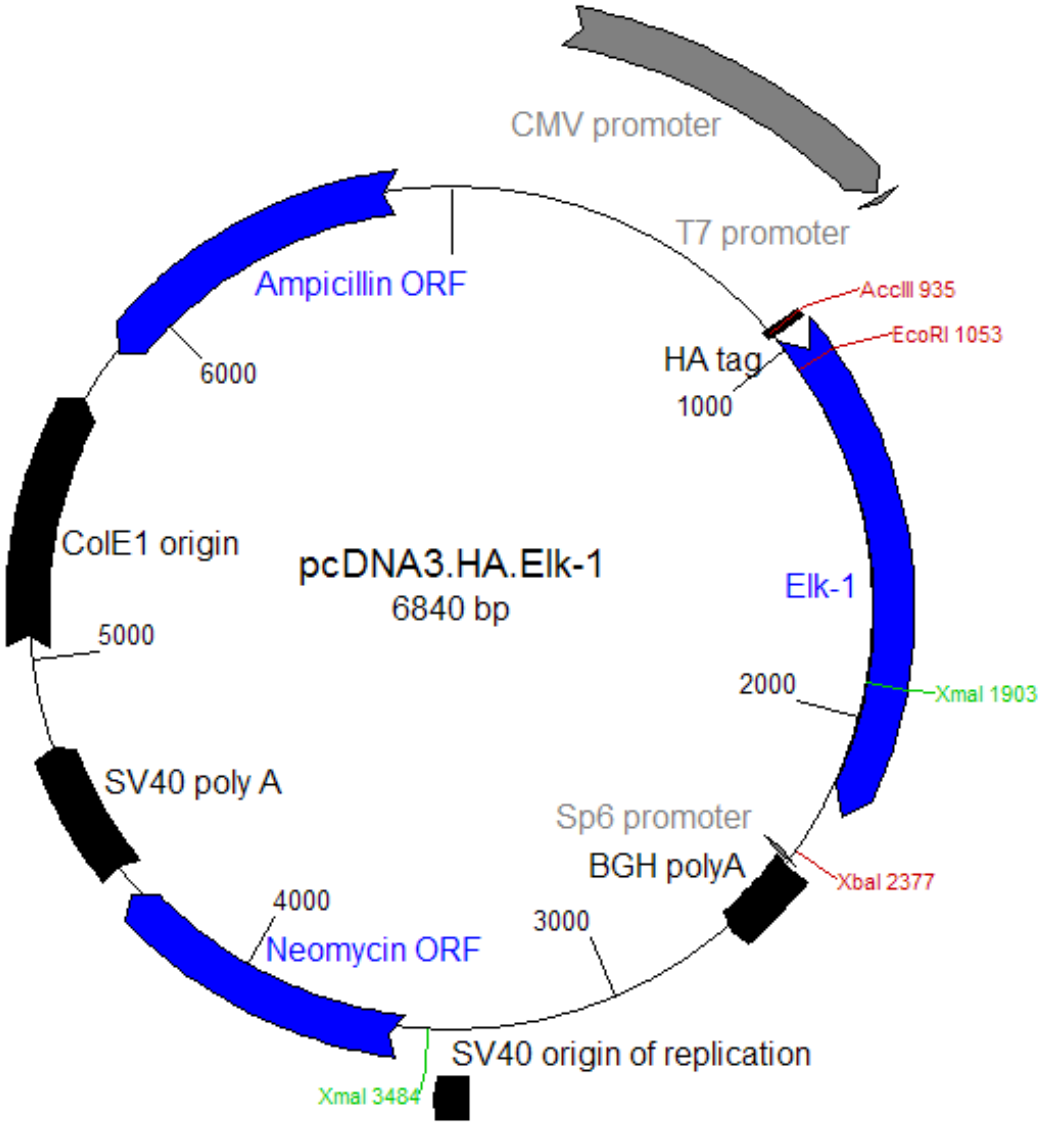
Appendices

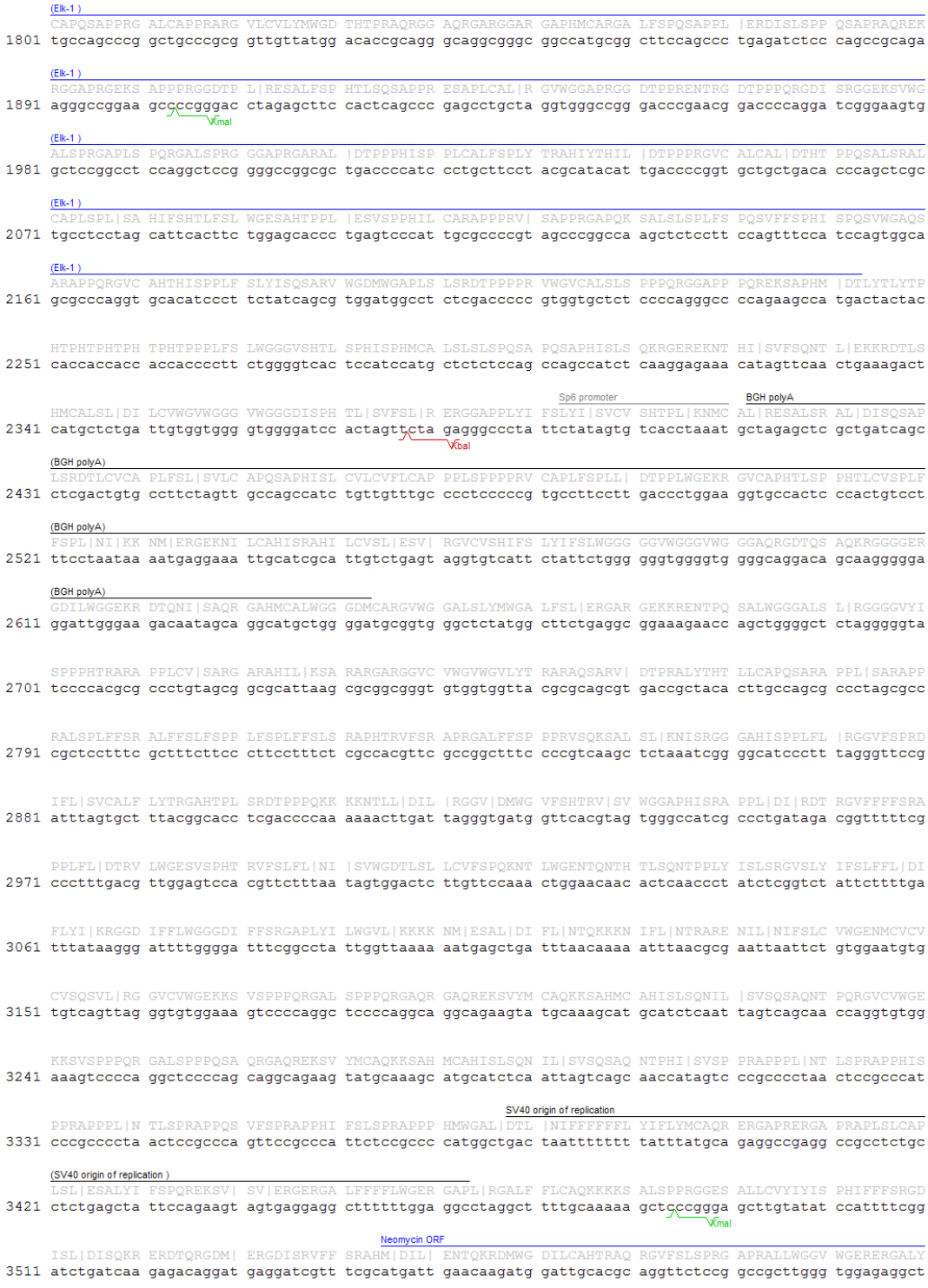
Clone chart

Plasmid:	pcDNA3.HA.Elk-1
Description:	Cell-free and mammalian expression vector for N-terminal HA-tagged human wild-type Elk-1 cDNA
Bacterial strain:	NM522
Resistances:	Ampicillin, neomycin

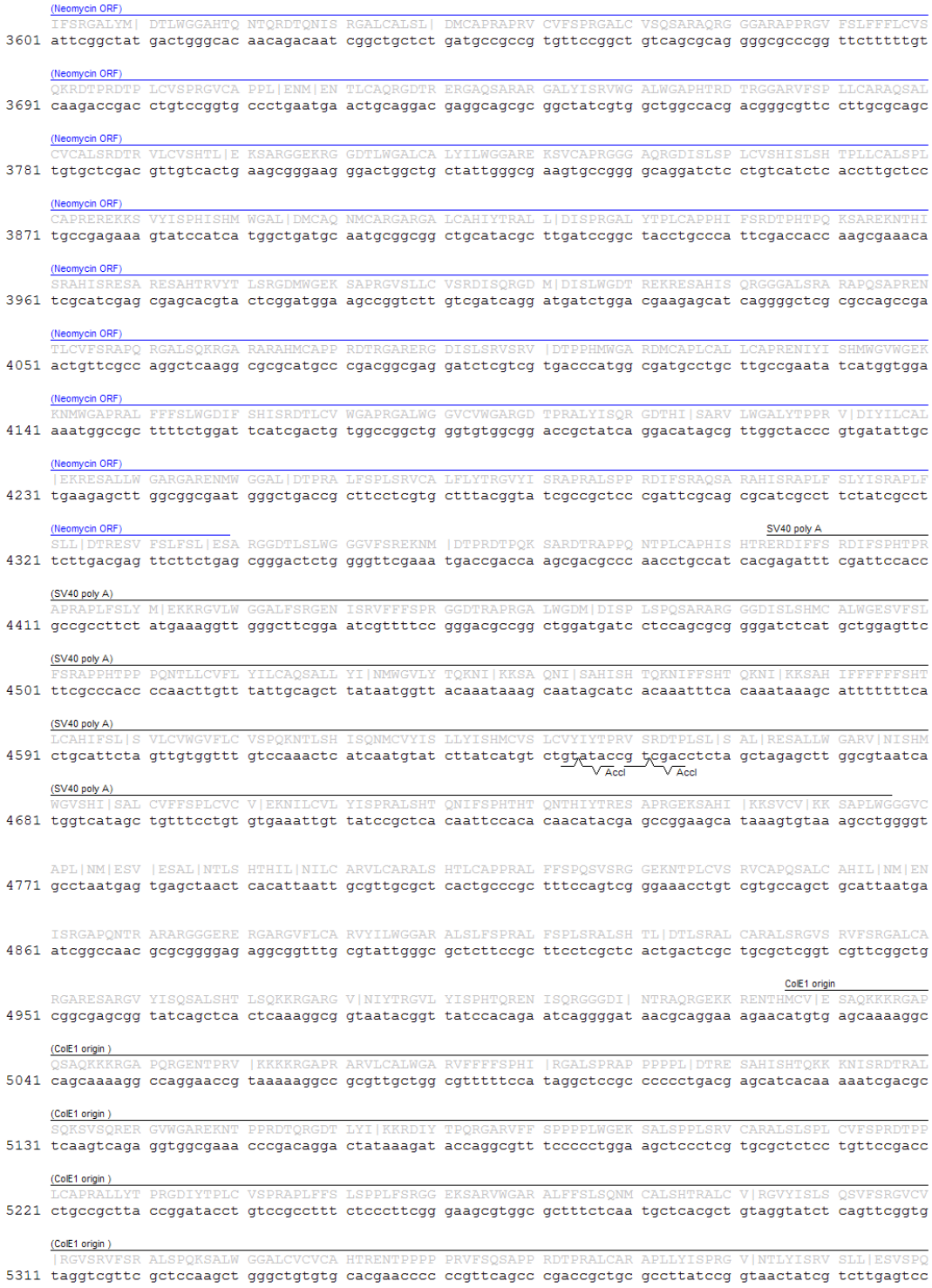


pcDNA3.HA.Elk-1

pcDNA3.HA.Elk-1

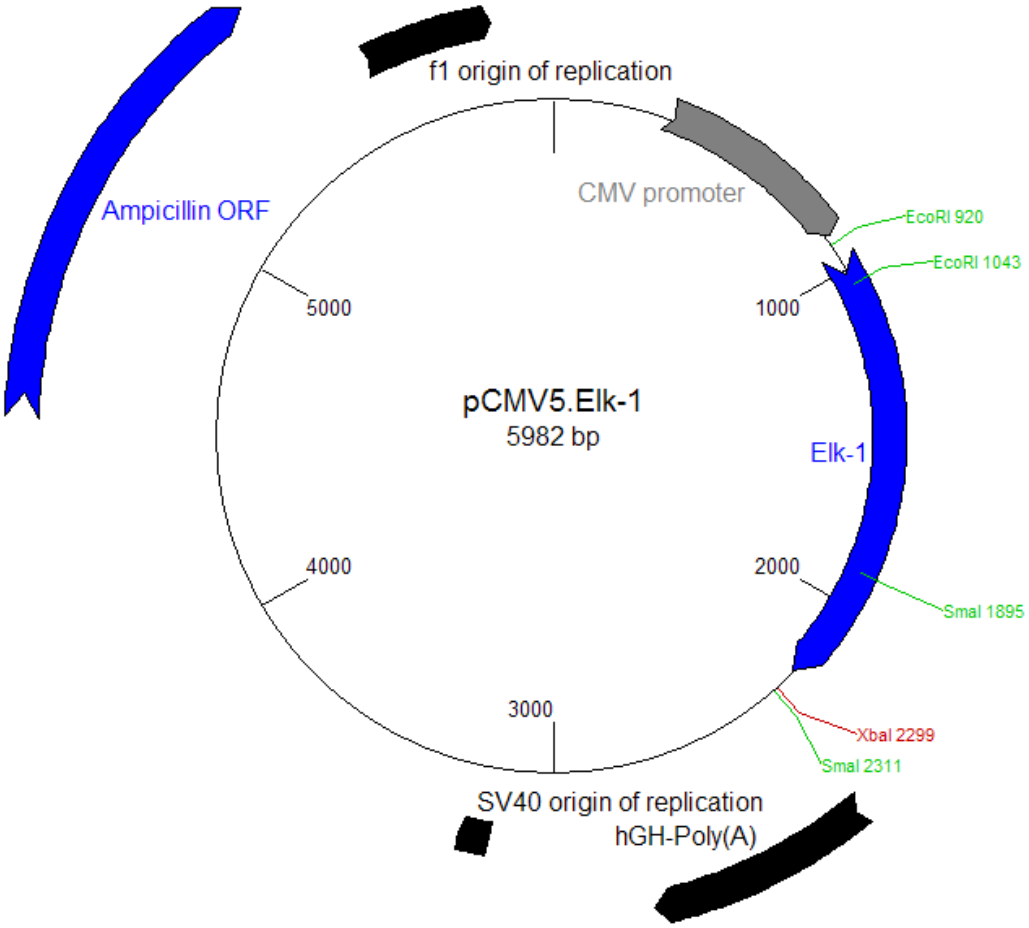


pcDNA3.HA.Elk-1

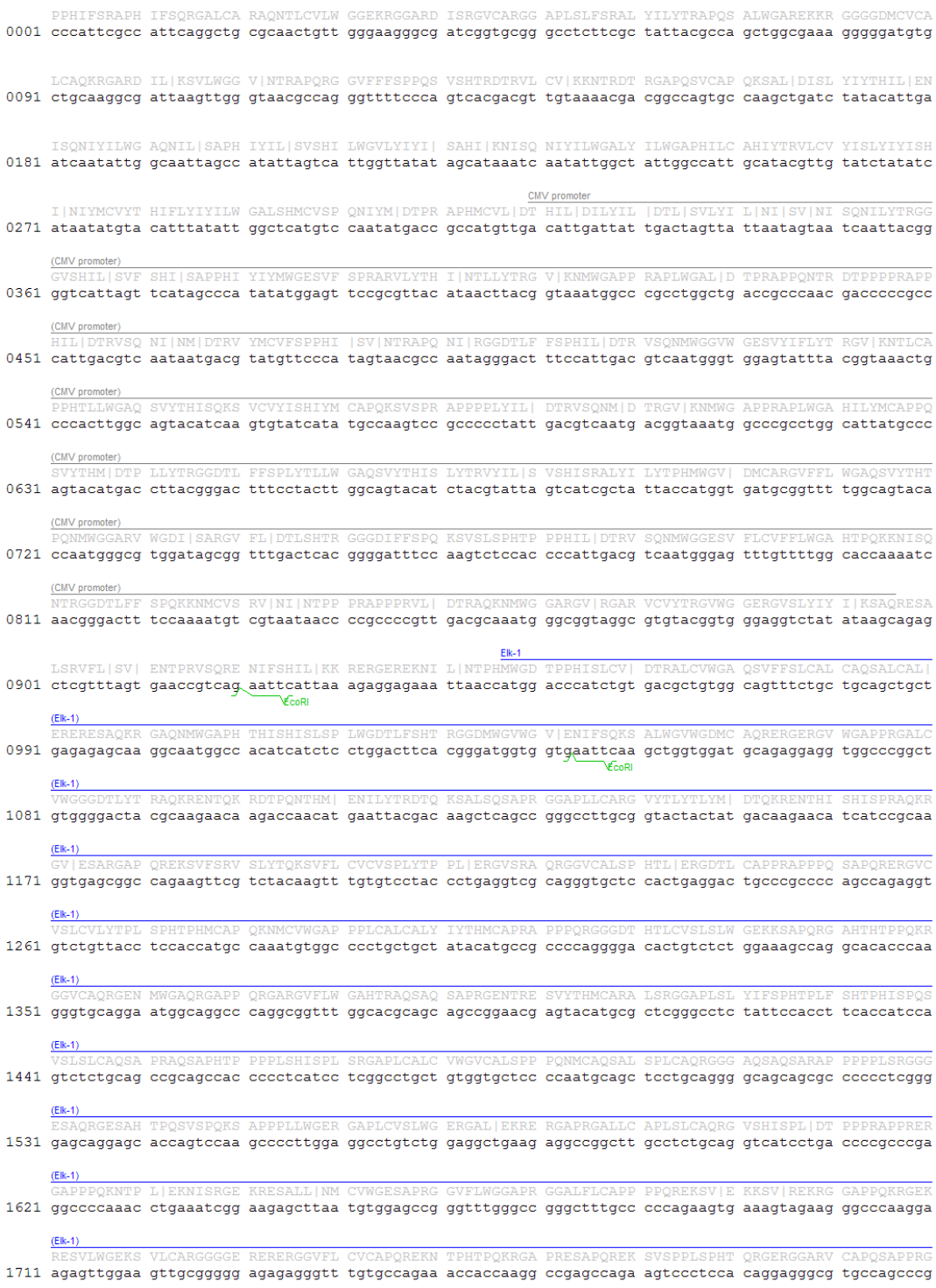


pcDNA.3.HA.Elk-1

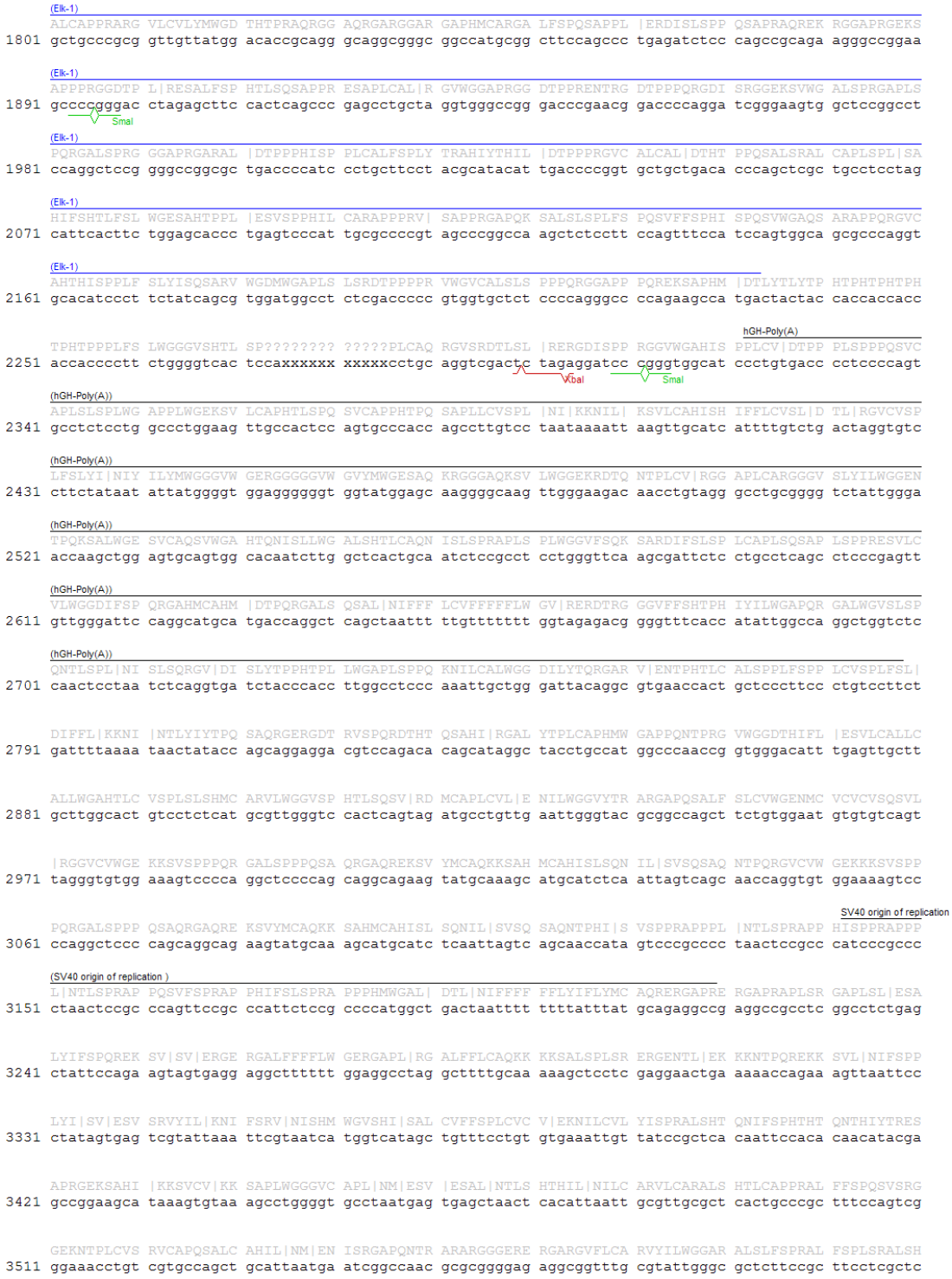
Plasmid:	pCMV5.Elk-1
Description:	Mammalian expression vector for human Elk-1 cDNA
Bacterial strain:	NM522
Resistances:	Ampicillin



pCMV5.Elk-1



pCMV5.Elk-1


(Elk-1)
ALCAPPRARG VLCVLYMWGD THTPRAQRGG AQRGARGGAR GAPHMCARGA LFSPQSAPPL |ERDISLSPP QSAPRAQREK RGGAPRGEKS
1801 gctgcccgcg gttgttatgg acaccgcagg gcaggcgggc ggccatgcgg cttccagccc tgagatctcc cagccgcaga agggccggaa
(Elk-1)
APPPRGGDTP L|RESALFSP HTLSQSAPPR ESAPLCAL|R GVWGGAPRGG DTPPRENTRG DTPPPQRGDI SRGGEKSVWG ALSPRGAPLS
1891 gccccgggac ctagagcttc cactcagccc gagcctgcta ggtgggccgg gacccgaacg gaccccagga tcgggaagtg gctccggcct
SmaI
(Elk-1)
PQRGALSPRG GGAPRGARAL |DTPPPHISP PLCALFSPLY TRAHIYTHIL |DTPPPRGVC ALCAL|DTHT PPQSALSRAL CAPLSPL|SA
1981 ccaggctccg gggccggcgc tgaccccatc cctgcttcct acgcatacat tgaccccggt gctgctgaca cccagctcgc tgcctcctag
(Elk-1)
HIFSHTLFSL WGESAHTPPL |ESVSPPHIL CARAPPPRV| SAPPRGAPQK SALSLSPLFS PQSVFFSPHI SPQSVWGAQS ARAPPQRGVC
2071 cattcacttc tggagcaccc tgagtcccat tgcgccccgt agcccggcca agctctcctt ccagtttcca tccagtggca gcgcccaggt
(Elk-1)
AHTHISPPLF SLYISQSARV WGDMWGAPLS LSRDTPPPPR VWGVCALSLS PPPQRGGAPP PQREKSAPHM |DTLYTLYTP HTPHTPHTPH
2161 gcacatccct tctatcagcg tggatggcct ctcgaccccc gtggtgctct ccccagggcc ccagaagcca tgactactac caccaccacc
hGH-Poly(A)
TPHTPPPLFS LWGGGVSHTL SP???????? ?????PLCAQ RGVSRDTLSL |RERGDISPP RGGVWGAHIS PPLCV|DTPP PLSPPPQSVC
2251 accacccctt ctggggtcac tccaxxxxxx xxxxxcctgc aggtcgactc tagaggatcc cgggtggcat ccctgtgacc cctccccagt
XbaI
SmaI
(hGH-Poly(A))
APLSLSPLWG APPLWGEKSV LCAPHTLSPQ SVCAPPHTPQ SAPLLCVSPL |NI|KKNIL| KSVLCAHISH IFFLCVSL|D TL|RGVCVSP
2341 gcctctcctg gccctggaag ttgccactcc agtgcccacc agccttgtcc taataaaatt aagttgcatc attttgtctg actaggtgtc
(hGH-Poly(A))
LFSLYI|NIY ILYMWGGGVW GERGGGGGVW GVYMWGESAQ KRGGGAQKSV LWGGEKRDTQ NTPLCV|RGG APLCARGGGV SLYILWGGEN
2431 cttctataat attatggggt ggaggggggt ggtatggagc aaggggcaag ttgggaagac aacctgtagg gcctgcgggg tctattggga
(hGH-Poly(A))
TPQKSALWGE SVCAQSVWGA HTQNISLLWG ALSHTLCAQN ISLSPRAPLS PLWGGVFSQK SARDIFSLSP LCAPLSQSAP LSPPRESVLC
2521 accaagctgg agtgcagtgg cacaatcttg gctcactgca atctccgcct cctgggttca agcgattctc ctgcctcagc ctcccgagtt
(hGH-Poly(A))
VLWGGDIFSP QRGAHMCAHM |DTPQRGALS QSAL|NIFFF LCVFFFFFLW GV|RERDTRG GGVFFSHTPH IYILWGAPQR GALWGVSLSP
2611 gttgggattc caggcatgca tgaccaggct cagctaattt ttgtttttt ggtagagacg gggtttcacc atattggcca ggctggtctc
(hGH-Poly(A))
QNTLSPL|NI SLSQRGV|DI SLYTPPHTPL LWGAPLSPPQ KNILCALWGG DILYTQRGAR V|ENTPHTLC ALSPPLFSPP LCVSPLFSL|
2701 caactcctaa tctcaggtga tctacccacc ttggcctccc aaattgctgg gattacaggc gtgaaccact gctcccttcc ctgtccttct
DIFFL|KKNI |NTLYIYTPQ SAQRGERGDT RVSPQRDTHT QSAHI|RGAL YTPLCAPHMW GAPPQNTPRG VWGGDTHIFL |ESVLCALLC
2791 gattttaaaa taactatacc agcaggagga cgtccagaca cagcataggc tacctgccat ggcccaaccg gtgggacatt tgagttgctt
ALLWGAHTLC VSPLSLSHMC ARVLWGGVSP HTLSQSV|RD MCAPLCVL|E NILWGGVYTR ARGAPQSALF SLCVWGENMC VCVCVSQSVL
2881 gcttggcact gtcctctcat gcgttgggtc cactcagtag atgcctgttg aattgggtac gcggccagct tctgtggaat gtgtgtcagt
|RGGVCVWGE KKSVSPPPQR GALSPPPQSA QRGAQREKSV YMCAQKKSAH MCAHISLSQN IL|SVSQSAQ NTPQRGVCVW GEKKKSVSPP
2971 tagggtgtgg aaagtcccca ggctccccag caggcagaag tatgcaaagc atgcatctca attagtcagc aaccaggtgt ggaaaagtcc
SV40 origin of replication
PQRGALSPPP QSAQRGAQRE KSVYMCAQKK SAHMCAHISL SQNIL|SVSQ SAQNTPHI|S VSPPRAPPPL |NTLSPRAPP HISPPRAPPP
3061 ccaggctccc cagcaggcag aagtatgcaa agcatgcatc tcaattagtc agcaaccata gtcccgcccc taactccgcc catcccgccc
(SV40 origin of replication)
L|NTLSPRAP PQSVFSPRAP PHIFSLSPRA PPPHMWGAL| DTL|NIFFFF FFLYIFLYMC AQRERGAPRE RGAPRAPLSR GAPLSL|ESA
3151 ctaactccgc ccagttccgc ccattctccg ccccatggct gactaatttt ttttatttat gcagaggccg aggccgcctc ggcctctgag
LYIFSPQREK SV|SV|ERGE RGALFFFFLW GERGAPL|RG ALFFLCAQKK KKSALSPLSR ERGENTL|EK KKNTPQREKK SVL|NIFSPP
3241 ctattccaga agtagtgagg aggctttttt ggaggcctag gcttttgcaa aaagctcctc gaggaactga aaaaccagaa agttaattcc
LYI|SV|ESV SRVYIL|KNI FSRV|NISHM WGVSHI|SAL CVFFSPLCVC V|EKNILCVL YISPRALSHT QNIFSPHTHT QNTHIYTRES
3331 ctatagtgag tcgtattaaa ttcgtaatca tggtcatagc tgtttcctgt gtgaaattgt tatccgctca caattccaca caacatacga
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3421 gccggaagca taaagtgtaa agcctggggt gcctaatgag tgagctaact cacattaatt gcgttgcgct cactgcccgc tttccagtcg
GEKNTPLCVS RVCAPQSALC AHIL|NM|EN ISRGAPQNTR ARARGGGERE RGARGVFLCA RVYILWGGAR ALSLFSPRAL FSPLSRALSH
3511 ggaaacctgt cgtgccagct gcattaatga atcggccaac gcgcggggag aggcggtttg cgtattgggc gctcttccgc ttcctcgctc

pCMV5.Elk-1

```
    TL|DTLSRAL CARALSRGVS RVFSRGALCA RGARESARGV YISQSALSHT LSQKKRGARG V|NIYTRGVL YISPHTQREN ISQRGGGDI|
3601 actgactcgc tgcgctcggt cgttcggctg cggcgagcgg tatcagctca ctcaaaggcg gtaatacggt tatccacaga atcaggggat

    NTRAQRGEKK RENTHMCV|E SAQKKKRGAP QSAQKKKRGA PQRGENTPRV |KKKRGAPRA RVLCALWGAR VFFFFSPHI| RGALSPRAPP
3691 aacgcaggaa agaacatgtg agcaaaaggc cagcaaaagg ccaggaaccg taaaaggccg cgttgctggc gtttttccat aggctccgcc

    PPPL|DTRES AHISHTQKKK NISRDTRALS QKSVSQRERG VWGAREKNTP PRDTQRGDTL YI|KKRDIYT PQRGARVFFS PPPPLWGEKS
3781 cccctgacga gcatcacaaa aatcgacgct caagtcagag gtggcgaaac ccgacaggac tataaagata ccaggcgttt ccccctggaa

    ALSPPLSRVC ARALSLSPLC VFSPRDTPPL CAPRALLYTP RGDIYTPLCV SPRAPLFFSL SPPLFSRGGE KSARVWGARA LFFSLSQNMC
3871 gctccctcgt gcgctctcct gttccgaccc tgccgcttac cggatacctg tccgcctttc tcccttcggg aagcgtggcg ctttctcaat

    ALSHTRALCV |RGVYISLSQ SVFSRGVCV| RGVSRVFSRA LSPQKSALWG GALCVCVCAH TRENTPPPPP RVFSQSAPPR DTPRALCARA
3961 gctcacgctg taggtatctc agttcggtgt aggtcgttcg ctccaagctg ggctgtgtgc acgaaccccc cgttcagccc gaccgctgcg

    PLLYISPRGV |NTLYISRVS LL|ESVSPQN TPPRGV|KRD THTRDTLLYI SRAPHTLWGA QSAQSAPHTL WGV|NTQRGD IL|SAQRESA
4051 ccttatccgg taactatcgt cttgagtcca acccggtaag acacgactta tcgccactgg cagcagccac tggtaacagg attagcagag

    RERGVYMCV| RGARGVCALY TQRESVFSLL |EKSVWGVWG APL|NTLYTR GALYTHTL|R EKRGDTQSVY IFLWGVYISL CARALSLCAL
4141 cgaggtatgt aggcggtgct acagagttct tgaagtggtg gcctaactac ggctacacta gaaggacagt atttggtatc tgcgctctgc

    |EKSAPQSVL YTPLFSRGEK KKKRESVLWG V|SALSLL|D ISPRGAQKNT QKNTPHTPRA LWGV|SARGV WGVFFFFLC VFLCAQKSAQ
4231 tgaagccagt taccttcgga aaaagagttg gtagctcttg atccggcaaa caaaccaccg ctggtagcgg tggttttttt gtttgcaagc

    SAQRDILYTR ARAQREKKKK KKRGDISLSQ KREKRDISPL FL|DISLFFF SLYTRGGGVS L|DTRALSQS VWGENTREKK NTLSHTRVL|
4321 agcagattac gcgcagaaaa aaaggatctc aagaagatcc tttgatcttt tctacggggt ctgacgctca gtggaacgaa aactcacgtt

    KRGGDIFFLW GVSHM|ERDI LYISQKKKKR GDISLFSHTP L|RDISPLFF L|KNIL|KKK NM|EKSVFFL |KNISQNISL |KKSVYIYIY
4411 aagggatttt ggtcatgaga ttatcaaaaa ggatcttcac ctagatcctt ttaaattaaa aatgaagttt taaatcaatc taaagtatat

                                Ampicillin ORF
    M|ESV|KNTL LWGVSL|DTQ SVLYTPQNMC ALL|NISQSV |ERGAHTPLY ISLSQSARDI SLCVSLYIFF SRVFSHISPH I|SVLCAPL|
4501 atgagtaaac ttggtctgac agttaccaat gcttaatcag tgaggcacct atctcagcga tctgtctatt tcgttcatcc atagttgcct

    (Ampicillin ORF)
    DTLSPPPRVS RVCV|RDI|N TLYTRDIYTR GGERGGALLY TPHISLWGAP PPQSVCALCA QNM|DIYTPR ARERDTPPHT RALSHTPRGA
4591 gactccccgt cgtgtagata actacgatac gggagggctt accatctggc cccagtgctg caatgatacc gcgagaccca cgctcaccgg

    (Ampicillin ORF)
    LSPQRDIFLY ISQSAQNI|K NTPQSAPQSA PRGEKRGGAP RESARAQREK SVWGVSPLCA QNTLFLYISP RAPLSPHISP QSVSLYIL|N
4681 ctccagattt atcagcaata aaccagccag ccggaagggc cgagcgcaga agtggtcctg caactttatc cgcctccatc cagtctatta

    (Ampicillin ORF)
    ILCVLCAPRG GEKSAL|RES V|KSV|SVFS RAPQSVL|NI |SVFLCARAQ NTRVLCVLCA PHILCALYTQ RGAHISRVWG VCVSHTRALS
4771 attgttgccg ggaagctaga gtaagtagtt cgccagttaa tagtttgcgc aacgttgttg ccattgctac aggcatcgtg gtgtcacgct

    (Ampicillin ORF)
    RVSRVFLWGV YMWGALFSHI FSQSALSPRG VFSPPQNTRD ISQKRGARES VLYTHM|DIS PPPPHMCVLC VCAQKKKKKS ARGVL|SALS
4861 cgtcgtttgg tatggcttca ttcagctccg gttcccaacg atcaaggcga gttacatgat cccccatgtt gtgcaaaaaa gcggttagct

    (Ampicillin ORF)
    PLFSRGVSPL SPRDISRVLC VSQREKSV|K SVLWGAPRAQ SVCVLYISHT LSHMWGVLYM WGAQSAHTLC AHI|NIFSLS LLYTLCVSHM
4951 ccttcggtcc tccgatcgtt gtcagaagta agttggccgc agtgttatca ctcatggtta tggcagcact gcataattct cttactgtca

    (Ampicillin ORF)
    CAPHISPRV| KRDMCALFFF SLCV|DTLWG V|ESVYTLSQ NTPQKSVSHI FSL|ERENI| SVCVYMCARG ARDTPRESVL CALSLLCAPP
5041 tgccatccgt aagatgcttt tctgtgactg gtgagtactc aaccaagtca ttctgagaat agtgtatgcg gcgaccgagt tgctcttgcc

    (Ampicillin ORF)
    RGARVSQNIY TRGGDI|NIY TPRARAPHTH I|SAQRENTL FL|KKKSVCA LSHISHILWG EKKNTRVFSL FSRGGGAREK KNTLSLSQKR
5131 cggcgtcaat acgggataat accgcgccac atagcagaac tttaaaagtg ctcatcattg gaaaacgttc ttcggggcga aaactctcaa

    (Ampicillin ORF)
    GDISLLYTPR ALCVL|ERDI SPQSVFSRDM CV|NTPPHTL SRVCAHTPPQ NTL|DISLFS QSAHISLFFL YTLFFSHTPQ SARVFFSLWG
5221 ggatcttacc gctgttgaga tccagttcga tgtaacccac tcgtgcaccc aactgatctt cagcatcttt tactttcacc agcgtttctg

    (Ampicillin ORF)
    GV|ESAQKKK NTQRGEKRGA QKKNMCAPRA QKKKKKRGGE NI|KRGGARD THTRGEKNMC VL|ENIYTLS HIYTLSLFSP LFFFFSQNIY
5311 ggtgagcaaa aacaggaagg caaaatgccg caaaaaaggg aataagggcg acacggaaat gttgaatact catactcttc ctttttcaat
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pCMV5.Elk-1

```
     ILYIL|EKSA HIFLYISQRG GVLYILCVSL SHM|ESARGD IYTHIYIFL| ENMCVYIFL| REKKKNI|KN TQKNI|RGGG VFSPRARAHT
5401 attattgaag catttatcag ggttattgtc tcatgagcgg atacatattt gaatgtattt agaaaaataa acaaataggg gttccgcgca

                                                f1 origin of replication
     HIFFSPPPRE KKKSVCAPHT PL|DTRARAP PLCV|SARGA RAHIL|KSAR ARGARGGVCV WGVWGVLYTR ARAQSARV|D TPRALYTHTL
5491 catttccccg aaaagtgcca cctgacgcgc cctgtagcgg cgcattaagc gcggcgggtg tggtggttac gcgcagcgtg accgctacac

     (f1 origin of replication)
     LCAPQSARAP PL|SARAPPR ALSPLFFSRA LFFSLFSPPL FSPLFFSLSR APHTRVFSRA PRGALFFSPP PRVSQKSALS L|KNISRGGG
5581 ttgccagcgc cctagcgccc gctcctttcg ctttcttccc ttcctttctc gccacgttcg ccggctttcc ccgtcaagct ctaaatcggg

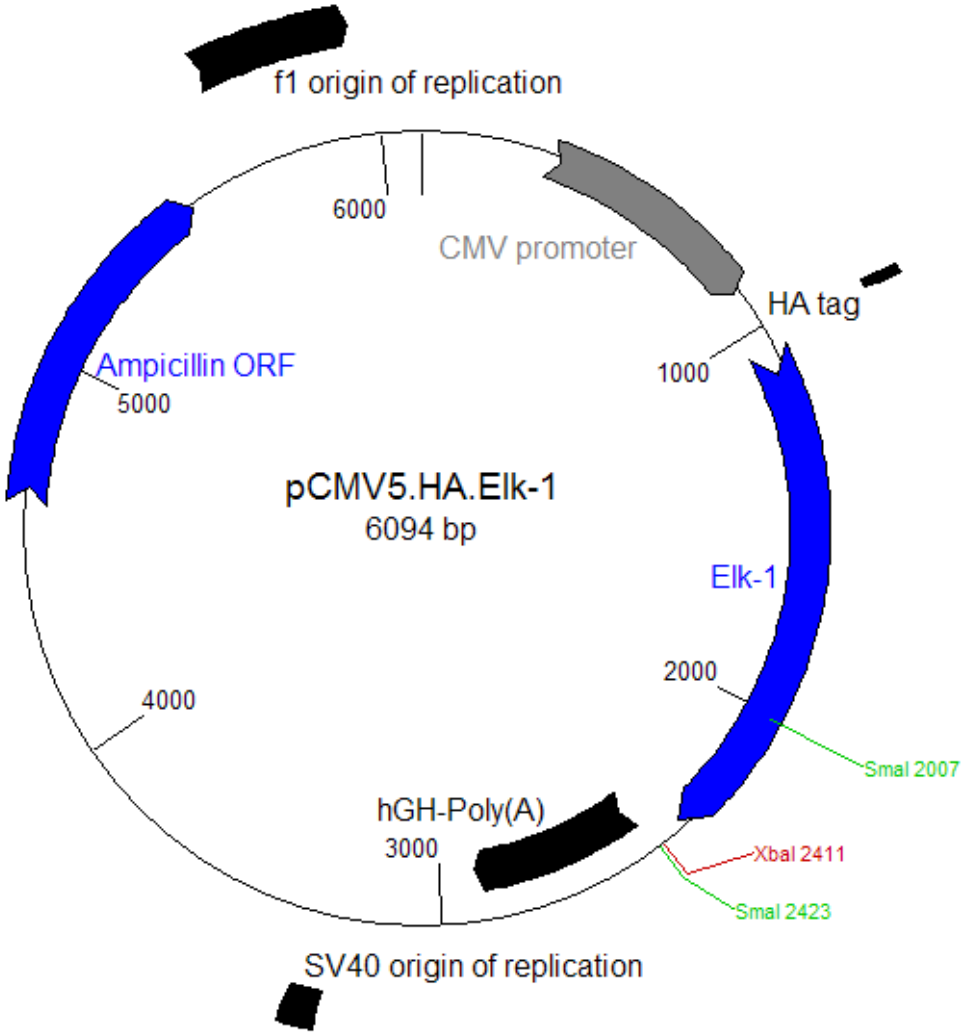
     (f1 origin of replication)
     AHISPPLFL| RGGVFSPRDI FL|SVCALFL YTRGAHTPLS RDTPPPQKKK KNTLL|DIL| RGGV|DMWGV FSHTRV|SVW GGAPHISRAP
5671 gcatcccttt agggttccga tttagtgctt tacggcacct cgaccccaaa aaacttgatt agggtgatgg ttcacgtagt gggccatcgc

     (f1 origin of replication)
     PL|DI|RDTR GVFFFFSRAP PLFL|DTRVL WGESVSPHTR VFSLFL|NI| SVWGDTLSLL CVFSPQKNTL WGENTQNTHT LSQNTPPLYI
5761 cctgatagac ggtttttcgc cctttgacgt tggagtccac gttctttaat agtggactct tgttccaaac tggaacaaca ctcaacccta

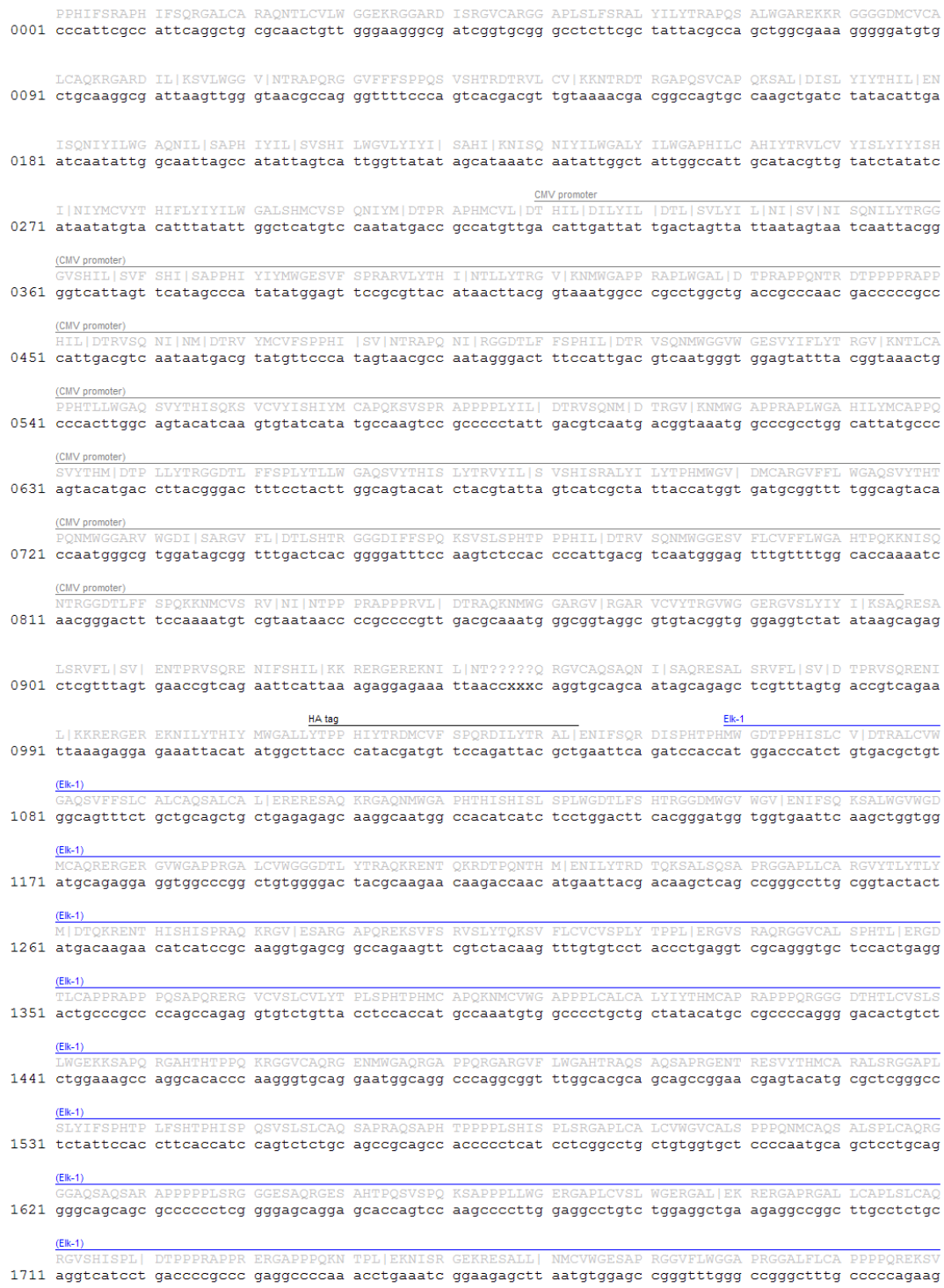
     SLSRGVSLYI FSLFFL|DIF LYI|KRGGDI FFLCAPRDIF FSRGAPLYIL WGVL|KKKKN M|ESAL|DIF L|NTQKKKNI FL|NTRAREN
5851 tctcggtcta ttcttttgat ttataaggga ttttgccgat ttcggcctat tggttaaaaa atgagctgat ttaacaaaaa tttaacgcga

     IFFL|NTQKK NIYIL|NTQK KNIYIL|NTR VFLYTQNIFF SP
5941 attttaacaa aatattaaca aaatattaac gtttacaatt tc
```

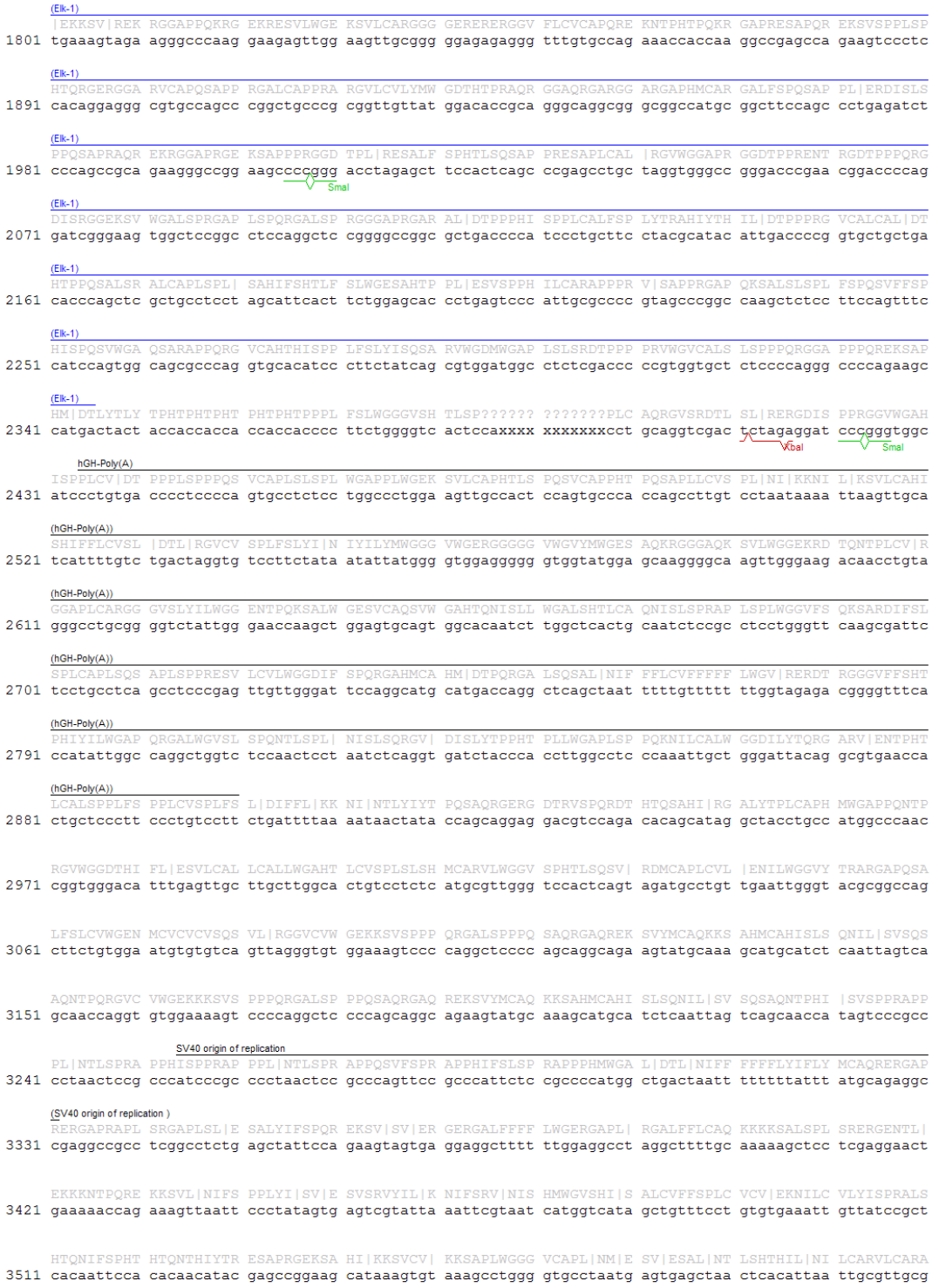
Plasmid:	pCMV5.HA.Elk-1
Description:	Mammalian expression vector for N-terminal HA-tagged human wild-type Elk-1 cDNA
Bacterial strain:	NM522
Resistances:	Ampicillin



pCMV5.HA.Elk-1



pCMV5.HA.Elk-1



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     LSHTLCAPPR ALFFSPQSVS RGGEKNTPLC VSRVCAPQSA LCAHIL|NM| ENISRGAPQN TRARARGGGE RERGARGVFL CARVYILWGG
3601 ctcactgccc gctttccagt cgggaaacct gtcgtgccag ctgcattaat gaatcggcca acgcgcgggg agaggcggtt tgcgtattgg

     ARALSLFSPR ALFSPLSRAL SHTL|DTLSR ALCARALSRG VSRVFSRGAL CARGARESAR GVYISQSALS HTLSQKKRGA RGV|NIYTRG
3691 gcgctcttcc gcttcctcgc tcactgactc gctgcgctcg gtcgttcggc tgcggcgagc ggtatcagct cactcaaagg cggtaatacg

     VLYISPHTQR ENISQRGGGD I|NTRAQRGE KKRENTHMCV |ESAQKKKRG APQSAQKKKR GAPQRGENTP RV|KKKRGAP RARVLCALWG
3781 gttatccaca gaatcagggg ataacgcagg aaagaacatg tgagcaaaag gccagcaaaa ggccaggaac cgtaaaaggc cgcgttgctg

     ARVFFFFSPH I|RGALSPRA PPPPPL|DTR ESAHISHTQK KKNISRDTRA LSQKSVSQRE RGVWGAREKN TPPRDTQRGD TLYI|KKRDI
3871 gcgtttttcc ataggctccg ccccccctgac gagcatcaca aaaatcgacg ctcaagtcag aggtggcgaa acccgacagg actataaaga

     YTPQRGARVF FSPPPPLWGE KSALSPPLSR VCARALSLSP LCVFSPRDTP PLCAPRALLY TPRGDIYTPL CVSPRAPLFF SLSPPLFSRG
3961 taccaggcgt ttccccctgg aagctccctc gtgcgctctc ctgttccgac cctgccgctt accggatacc tgtccgcctt tctcccttcg

     GEKSARVWGA RALFFSLSQN MCALSHTRAL CV|RGVYISL SQSVFSRGVC V|RGVSRVFS RALSPQKSAL WGGALCVCVC AHTRENTPPP
4051 ggaagcgtgg cgctttctca atgctcacgc tgtaggtatc tcagttcggt gtaggtcgtt cgctccaagc tgggctgtgt gcacgaaccc

     PPRVFSQSAP PRDTPRALCA RAPLLYISPR GV|NTLYISR VSLL|ESVSP QNTPPRGV|K RDTHTRDTLL YISRAPHTLW GAQSAQSAPH
4141 cccgttcagc ccgaccgctg cgccttatcc ggtaactatc gtcttgagtc caacccggta agacacgact tatcgccact ggcagcagcc

     TLWGV|NTQR GDIL|SAQRE SARERGVYMC V|RGARGVCA LYTQRESVFS LL|EKSVWGV WGAPL|NTLY TRGALYTHTL |REKRGDTQS
4231 actggtaaca ggattagcag agcgaggtat gtaggcggtg ctacagagtt cttgaagtgg tggcctaact acggctacac tagaaggaca

     VYIFLWGVYI SLCARALSLC AL|EKSAPQS VLYTPLFSRG EKKKKRESVL WGV|SALSLL |DISPRGAQK NTQKNTPHTP RALWGV|SAR
4321 gtatttggta tctgcgctct gctgaagcca gttaccttcg gaaaaagagt tggtagctct tgatccggca aacaaaccac cgctggtagc

     GVWGVFFFFF LCVFLCAQKS AQSAQRDILY TRARAQREKK KKKKRGDISL SQKREKRDIS PLFL|DISLF FFSLYTRGGG VSL|DTRALS
4411 ggtggtttttt ttgtttgcaa gcagcagatt acgcgcagaa aaaaaggatc tcaagaagat cctttgatct tttctacggg gtctgacgct

     QSVWGENTRE KKNTLSHTRV L|KRGGDIFF LWGVSHM|ER DILYISQKKK KRGDISLFSH TPL|RDISPL FFL|KNIL|K KKNM|EKSVF
4501 cagtggaacg aaaactcacg ttaagggatt ttggtcatga gattatcaaa aaggatcttc acctagatcc ttttaaatta aaaatgaagt

                                                                    Ampicillin ORF
     FL|KNISQNI SL|KKSVYIY IYM|ESV|KN TLLWGVSL|D TQSVLYTPQN MCALL|NISQ SV|ERGAHTP LYISLSQSAR DISLCVSLYI
4591 tttaaatcaa tctaaagtat atatgagtaa acttggtctg acagttacca atgcttaatc agtgaggcac ctatctcagc gatctgtcta

     (Ampicillin ORF)
     FFSRVFSHIS PHI|SVLCAP L|DTLSPPPR VSRVCV|RDI |NTLYTRDIY TRGGERGGAL LYTPHISLWG APPPQSVCAL CAQNM|DIYT
4681 tttcgttcat ccatagttgc ctgactcccc gtcgtgtaga taactacgat acgggagggc ttaccatctg gccccagtgc tgcaatgata

     (Ampicillin ORF)
     PRARERDTPP HTRALSHTPR GALSPQRDIF LYISQSAQNI |KNTPQSAPQ SAPRGEKRGG APRESARAQR EKSVWGVSPL CAQNTLFLYI
4771 ccgcgagacc cacgctcacc ggctccagat ttatcagcaa taaaccagcc agccggaagg gccgagcgca gaagtggtcc tgcaacttta

     (Ampicillin ORF)
     SPRAPLSPHI SPQSVSLYIL |NILCVLCAP RGGEKSAL|R ESV|KSV|SV FSRAPQSVL| NI|SVFLCAR AQNTRVLCVL CAPHILCALY
4861 tccgcctcca tccagtctat taattgttgc cgggaagcta gagtaagtag ttcgccagtt aatagtttgc gcaacgttgt tgccattgct

     (Ampicillin ORF)
     TQRGAHISRV WGVCVSHTRA LSRVSRVFLW GVYMWGALFS HIFSQSALSP RGVFSPPQNT RDISQKRGAR ESVLYTHM|D ISPPPPHMCV
4951 acaggcatcg tggtgtcacg ctcgtcgttt ggtatggctt cattcagctc cggttcccaa cgatcaaggc gagttacatg atcccccatg

     (Ampicillin ORF)
     LCVCAQKKKK KSARGVL|SA LSPLFSRGVS PLSPRDISRV LCVSQREKSV |KSVLWGAPR AQSVCVLYIS HTLSHMWGVL YMWGAQSAHT
5041 ttgtgcaaaa aagcggttag ctccttcggt cctccgatcg ttgtcagaag taagttggcc gcagtgttat cactcatggt tatggcagca

     (Ampicillin ORF)
     LCAHI|NIFS LSLLYTLCVS HMCAPHISPR V|KRDMCALF FFSLCV|DTL WGV|ESVYTL SQNTPQKSVS HIFSL|EREN I|SVCVYMCA
5131 ctgcataatt ctcttactgt catgccatcc gtaagatgct tttctgtgac tggtgagtac tcaaccaagt cattctgaga atagtgtatg

     (Ampicillin ORF)
     RGARDTPRES VLCALSLLCA PPRGARVSQN IYTRGGDI|N IYTPRARAPH THI|SAQREN TLFL|KKKSV CALSHISHIL WGEKKNTRVF
5221 cggcgaccga gttgctcttg cccggcgtca atacgggata ataccgcgcc acatagcaga actttaaaag tgctcatcat tggaaaacgt

     (Ampicillin ORF)
     SLFSRGGGAR EKKNTLSLSQ KRGDISLLYT PRALCVL|ER DISPQSVFSR DMCV|NTPPH TLSRVCAHTP PQNTL|DISL FSQSAHISLF
5311 tcttcggggc gaaaactctc aaggatctta ccgctgttga gatccagttc gatgtaaccc actcgtgcac ccaactgatc ttcagcatct
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pCMV5.HA.Elk-1