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**The regulatory role of ELK-1
ubiquitination in cell proliferation and
prostate cancer**

by

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A mi madre

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ABSTRACT

Early stage Prostate Cancer (PC) is usually androgen-dependent and androgen ablation therapies have been used with good results in the destruction of androgen-dependent cells. However, anti-androgens are associated with systemic side effects and selection for tumour cells that survive in the absence of androgens, leading to androgen-independent PC.

ELK-1 is a member of the ETS-domain transcription factor family. In response to mitogens, ERKs phosphorylate and activate ELK-1 to stimulate the expression of Immediate Early Genes (IEGs) such as *CFOS* and *EGR1*, thereby promoting cell cycle entry and proliferation. In PC cells, ELK-1 expression is frequently up-regulated and androgen receptor (AR) was found to function as a constitutive coactivator for ELK-1, up-regulating a major subset of its target genes involved in cell proliferation and migration. Removal or inactivation of ELK-1 could therefore serve to suppress PC tumour growth.

Mono-ubiquitination modulates ELK-1 activity. De-ubiquitination of ELK-1 by USP17, a de-ubiquitinase over-expressed in multiple tumour types, increases ELK-1 target gene expression and cell proliferation, indicating that mono-ubiquitination of ELK-1 suppresses its activity (Ducker et al., 2019).

This project aims to identify the ubiquitin E3 ligase responsible for ELK-1 mono-ubiquitination and evaluate its role in PC.

Using a combination of gene knockdown experiments and ubiquitination assays, as well as an *in silico* gene expression analysis to identify ubiquitin E3 ligases that were significantly down-regulated in PC cells, I have tested candidates including FBXW7, FBXO25, SPOP and UBR5, for their ability to modify ELK-1 and suppress its activity. Moreover, using the BioID screening I was able to identify ELK-1 partners under starved and mitogen-stimulated conditions.

My findings suggest that an ubiquitin E3 ligase previously reported to target ELK-1 is unlikely to promote ELK-1 ubiquitination and transactivation. Furthermore, the BioID analysis found some potential E3 ligase candidates that need to be evaluated to elucidate their role in promoting ELK-1 ubiquitination. Additionally, the BioID analysis concluded that ELK-1 interacts with transcriptional regulators to modulate ELK-1 target genes expression.

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ABBREVIATIONS

AR	Androgen receptor
ATP	Adenosine triphosphate
BCA	Bicinchoninic assay
BSA	Bovine serum albumin
CBP	CREB binding protein
CDK	Cyclin-dependent kinases
CHIP	C-terminus of Hsc70-interacting protein
CKI	Cyclin-dependent kinases inhibitors
Co-IP	Co-Immunoprecipitation
CREB	cAMP response element-binding
CTD	Carboxy-terminal domain
Cul	Cullin
DBD	DNA binding domain
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotides
DTT	Dithiothreitol
DUB	Deubiquitinating enzymes
EBS	ETS binding site
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGR1	Early growth response gene
ERG	ETS transcription factor
ERK	Extracellular Signal-Regulated Kinase
ES	Stem cells
ETS	E26 transformation-specific or E-twenty-six
FAND	FBXO25-associated nuclear domains
FBS	Foetal bovine serum
FBX	F-box only protein
FBXL	F-box and leucine-rich repeat protein
FBXO	F-box only protein
FBXW	F-box and WD repeat protein
FCS	Foetal calf serum
GSK3	Glycogen synthase kinase 3
HA	Hemagglutinin
HAT	Histone Acetylation
HBS	Hepes buffered saline
HDAC	Histone deacetylase
HECT	Homologous to the E6-AP Carboxyl Terminus

HRP	Horseradish peroxidase
IEG	Immediate early genes
IGF-1	Insulin-like growth factor-1
IGFBP	Insulin-like growth factor-1 binding proteins
IP	Immunoprecipitation
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
MAPK	Mitogen-Activated Protein Kinase
MDM2	Mouse double minute 2 homolog
MSK	Mitogen and stress-activated kinase
NES	Nuclear export signal
NLS	Nuclear localisation signal
NTD	N-terminal domain
PBS	Phosphate buffered saline
PC	Prostate cancer
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PIC	Pre-initiation complex
PSA	Prostate-specific antigen
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real-time polymerase chain reaction
Rb	Retinoblastoma
RBR	RING in Between RING
Rbx1	RING box protein 1
RING	Really Interesting new gene
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RNAPII	RNA polymerase II
RPMI	Roswell Park Memorial Institute medium
SBC	SPOP-binding consensus
SCF	Skp, Cullin, F-box containing complex
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
shRNA	Short-hairpin RNA
SKP1	S-phase kinase-associated protein 1
SKP2	S-phase kinase-associated protein 2
SPOP	Speckle-type POZ protein
SRE	Serum response element
SRF	Serum response factor
SUMO	Small ubiquitin-related modifier
TAD	Transcriptional activation domain
TAE	Tris-acetate EDTA

TBS	Tris-buffered saline
TCF	Ternary complex factor
TFIIH	Transcription factor IIH
TPA	12-O-Tetradecanoylphorbol-13-acetate
TRE	TPA responsive element
TRIM	Tripartite motif
UBQ	Ubiquitin
UPS	Ubiquitin-proteasome system
WCE	Whole cell extracts

1.0 INTRODUCTION

1.1 Uncontrolled proliferation in prostate cancer

Prostate cancer progression is associated with the deregulation of growth factors and their respective signalling pathway, which ultimately leads to uncontrolled proliferation. Uncontrolled proliferation is an important part of cancer development and progression.

In 1984, it was demonstrated that in mouse 3T3 cells, mitogens such as platelet-derived growth factor (PDGF) or epidermal growth factor (EGF) initiate the replicative response in the cells to become competent to synthesise DNA. These early G0/G1 events ultimately culminate in DNA synthesis and mitosis (Hendrickson and Scher, 1983). *CFOS* is the downstream target of the PDGF signalling axis, it is expressed within minutes after mitogenic exposure; this effect was considered the earliest described nuclear event that follows a mitogenic stimulus (Cochran et al., 1983, Cochran et al., 1984, Kruijer et al., 1984, Müller et al., 1984, Greenberg and Ziff, 1984), and promotes differentiation and proliferation through the activation of the MAPK pathway (Kerkhoff and Rapp, 1998). The regulation of cell proliferation by growth factors is mediated by the RAS/RAF/MEK/ERK cascade. By direct interaction the RAS protein targets the RAF kinase to the plasma membrane where it becomes activated. Subsequently, RAF stimulates MEK by phosphorylation, which then phosphorylates and activates ERK. ERK shuttles into the nucleus and induces the

activity of transcription factors (Mulcahy et al., 1985, Feig and Cooper, 1988, Kolch et al., 1991, Dobrowolski et al., 1994). In mammalian cells there are four major MAPK pathways, extracellular signal-related kinases (ERK 1/2), c-JUN amino-terminus kinases (JNK 1/2/3), p38 proteins (p38 α / β / γ / δ), and extracellular signal-regulated kinase 5 (ERK 5) (Chang and Karin, 2001). Several different targets of the RAS/RAF signalling pathway like cyclin D1, c-Myc and members of the ETS family like ETS-1, ETS-2 and ELK-1 have been described to mediate the proliferative response (Filmus et al., 1994, Kortgenjann et al., 1994, Liu et al., 1995, Gille et al., 1995a, Winston et al., 1996, Yang et al., 1996, McCarthy et al., 1997, Kerkhoff and Rapp, 1998)

Some studies indicated that insulin-like growth factor-1 (IGF-1) exerts a highly mitogenic activity in cells and is considered to be the most important positive growth factor (Cohen et al., 1991, Iwamura et al., 1993). Effective binding of IGF-1 ligand to the IGF-1 receptor leads to the activation of signalling pathways (Baserga et al., 2003). One function reported for the IGF-1 has suggested that in response to androgen stimulation, IGF-1 is produced by prostatic stromal cells and it stimulates the surrounding prostatic epithelial cells, resulting in increased proliferation (Moschos and Mantzoros, 2002, Bogdanos et al., 2003). In normal cells, the IGF-1 pathway is inhibited by the IGF binding proteins (IGFBP). IGFBPs bind to IGF-1 with high affinity, effectively sequestering IGF-1 and preventing pathway activation

through interaction with its receptor (Grimberg and Cohen, 2000, Stewart and Weigel, 2005).

Cancer progression and proliferation are associated with alterations in the control of the mammalian cell cycle. The cell cycle is divided into four phases. Two basic events in cell division take part in two phases. Generation of a single and faithful copy of its genetic material (the synthetic or S phase) and partitioning of all the cellular components between two identical daughter cells (mitosis or M phase), the two other phases of the cycle (G1 and G2) represent gap periods, during which cells prepare themselves for the subsequent completion of the S and M phases respectively (Norbury and Nurse, 1992).

The G1 phase of the cell cycle is the only time that a cell can respond to extracellular signals. During G1 phase, the cells prepare to initiate DNA synthesis. In the presence of extracellular signals like mitogens, progression into S phase occurs; by a complex process that involves at least three cyclin-dependent kinases CDKs (CDK4, CDK6 and CDK2) and their regulators. They include activators, mainly the cyclins and inhibitors known as CDK kinase inhibitors (CKIs) that comprise two groups, the INK4 family, which comprises p16 (INK4A), p15 (INK4B), p18 (INK4C) and p19 (INK4D) and the CIP and KIP family which comprises p21 (CDKN1a), p27 (CDKN1B) and p57 (CDKN1C) (Sherr and Roberts, 1999, Besson et al., 2008). CKIs exert their inhibitory activity by binding to the CDK4 and CDK6 kinases and

preventing their association with D-type cyclins (Sherr and Roberts, 1999).

Mitogenic signalling induces synthesis of the D-type cyclins (cyclins D1, D2 and D3) and transport to CDK4 and CDK6 to the nucleus. Nuclear export is promoted by glycogen synthase kinase 3- β (GSK3 β) a kinase that is inhibited by the RAS pathway (Diehl et al., 1998, Alt et al., 2000). The function of cyclin D is to promote cell proliferation as a regulatory partner for CDK4 or CDK6. In cancer, cyclin D-CDK4/6 activity can be increased by CDK4 and CDK6 amplification, mutation of CDK4 to an inhibitor resistant form, or loss of the INK4 inhibitors (Hall and Peters, 1996, Musgrove et al., 2011). Deregulation of cyclin D1 not only promotes mitogen-independent proliferation, but it also affects other cellular processes, both directly and indirectly, with oncogenic consequences. Oncogenic actions of cyclin D1 are predominantly nuclear (Kim and Diehl, 2009).

Active complexes of CDK4 and CDK6 and D-type cyclins phosphorylate members of the retinoblastoma (Rb) protein family, which includes pRb, p107 and p130 (Sherr and Roberts, 1999, Malumbres and Barbacid, 2001, Massagué, 2004). Rb proteins function to repress transcription by binding and modulating the activity of transcription factors, such as E2F family members, histone deacetylases and chromatin remodeling complexes, to ensure that they remain inactive during M and G0. (Cobrinik, 2005). CDK2 activity peaks in S and G2 phases, CDK1 activity is relatively low in S, increases in G2, and peaks in early mitosis to finally disappear during

anaphase (Frescas and Pagano, 2008). The transition from metaphase to anaphase which marks the end of mitosis is induced by the degradation of cyclins. Cyclin degradation is a crucial step governing exit from mitosis and progress into the next cell cycle. Degradation of cyclins involved during the cell cycle is mediated by the ubiquitin pathway. An N-terminus domain of cyclin is sufficient to confer cell-cycle-regulated degradation (Glutzer et al., 1991). Ubiquitin-mediated proteasomal degradation is an irreversible mechanism that guarantee the unidirectionality of the cell cycle. The ubiquitin pathway system (UPS) comprises two different steps, the covalent attachment of multiple ubiquitin molecules to the protein substrate and degradation of the poly-ubiquitinated protein by the 26S proteasome (Pickart, 2001, Pickart, 2004) (section 1.5). The E3 ubiquitin ligase enzyme specifically binds the substrate protein to mediate the transfer of ubiquitin to a lysine residue in the target (Hershko and Ciechanover, 1998) (section 1.5.2). Several E3 ligases have essential roles in cell cycle control. For example, decreased expression of p27 CKI is mediated by the S-phase-kinase-associated protein (SKP2), an F-box protein of the SCF complex that targets p27 for degradation. p27 is one of the main SKP2 target, however several CKIs such as p27, p21 and p57 have been reported to be degraded by SKP2. (Yu et al., 1998, Carrano et al., 1999, Sutterlüty et al., 1999, Kamura et al., 2003). There are many studies that show a role of the F-box protein β -TRCP (β -transducin repeat-containing protein)

targeting various cell cycle regulators for degradation such as EMI1/2 (Guardavaccaro et al., 2003, Margottin-Goguet et al., 2003), WEE1A (Watanabe et al., 2004) and CDC25A/B (Busino et al., 2003). For example, it has been reported that β -TrCP has a dual role in controlling CDK1 activity, in S phase and mitosis it can induce the degradation of CDC25A (cell division cycle 25A) and EMI1 (also known as F-box protein 5) that effect promotes a decreased in the CDK1 activity; on the other side, β -TRCP can promote degradation of claspin WEE1 at G2-M, that effect increase the activity of CDK1 (Frescas and Pagano, 2008).

The decrease in CDK2 activity at the G1/S transition is mainly controlled by the rapid degradation of the cyclin E by the SCF-FBXW7 ubiquitin ligase, which is inactivated in some cancer cell lines (Moberg et al., 2001, Strohmaier et al., 2001, Koepp et al., 2001). Moreover, there is evidence that p27 degradation is mediated by the E3 ligase SKP2 (S-phase kinase-associated protein 2). CDK-dependent phosphorylation of p27 on threonine 187 is present in proliferating cells and was reported as crucial for its degradation by SKP2 (Sheaff et al., 1997, Vlach et al., 1997, Carrano et al., 1999).

Additionally, c-Myc stability has been associated with FBXW7. Phosphorylation on threonine 58 and serine 62 are crucial to mediate FBXW7 recognition and subsequent degradation of c-Myc. (Yada et al., 2004, Welcker et al., 2004). Myc stabilisation has been linked to several E3 ligases, such as SKP2, FBX29, CHIP and TRIM32 (von der Lehr et al., 2003, Kim et al., 2003, Schwamborn et al., 2009, Koch

et al., 2007, Paul et al., 2013). Similarly to FBXW7, SKP2 expression is altered in some tumours cells (Gstaiger et al., 2001).

Together, this data indicates that deregulation of the ubiquitin proteasome system in the cell cycle control is linked to uncontrolled proliferation and cancer development.

1.2 Immediate early genes

Immediate early genes (IEG) were first identified in cells exposed to mitogens and have an important role in the regulation of the cell cycle (Greenberg and Ziff, 1984). Among the best characterised IEGs are FOS family genes, which include *CFOS*, *FOSB*, *FRA-1*, and *FRA-2*, induced within minutes of growth factor addition to quiescent cells (Herschman, 1991). The *CFOS* promoter is fully active within minutes of stimulation and thereafter is rapidly inactivated (Müller et al., 1984).

The FOS transcription factor is not independently active and must form a heterodimer with a member of the JUN family to form the active transcription factor activator protein (AP-1) (Curran et al., 1985, Bohmann et al., 1987, Halazonetis et al., 1988). FOS-JUN family proteins are members of a large group of transcription factors (the bZIP family) containing a highly conserved basic region involved in DNA binding and a heptad repeat of leucine residues, the leucine zipper, required for dimerization (Landschulz et al., 1988). All bZIP proteins form dimeric complexes through the leucine zipper that juxtapose the two basic regions to form a contiguous DNA-contact

interface in which each monomer interacts with the major groove of one half-site (Ellenberger et al., 1992, Glover and Harrison, 1995, Fujii et al., 2000).

AP-1 regulates the expression of target genes by binding the DNA at the consensus sequence 5'-TGA G/C TCA-3' known as the TPA (12-O-Tetradecanoylphorbol-13-acetate) responsive element (TRE), which is found within the promoter region of AP-1 target genes (Angel et al., 1987, Lee et al., 1987). The AP-1 transcription factor has been suggested to control cell proliferation and death through regulating the expression and function of cell cycle regulators, such as cyclin D1, p53, p21, p19 and p16 (Brown et al., 1998, Schreiber et al., 1999, Wisdom et al., 1999, Bakiri et al., 2000, Shaulian and Karin, 2001).

The regulatory role of *CFOS* in cell proliferation has been described as controversial, because some studies have suggested that it acts as a negative regulator of proliferation (Balsalobre and Jolicoeur, 1995, Mikula et al., 2003). However, several reports support a potential positive role for *CFOS* in tumorigenesis. Some evidence suggests that *CFOS* expression is required during all phases of the cell cycle in exponentially growing cells because it is a potent inducer of cell proliferation (Pai and Bird, 1994, Cosenza et al., 1994). The positive role of *CFOS* on proliferation has been demonstrated on hepatocyte proliferation. Overexpression of *CFOS* correlated with

high nuclear levels of inactive phosphorylated GSK- 3 β which in turns prevents cyclin D1 degradation (Güller et al., 2008).

Early growth response gene (*EGR-1*) is another member of the IEG family; its activation is very similar to that of *CFOS*. *EGR1* is also known as Zif268 and encodes a nuclear phosphoprotein, also known as Krox24 (Sukhatme et al., 1987, Sukhatme et al., 1988, Christy and Nathans, 1989, Janssen-Timmen et al., 1989).

ELK-1 phosphorylation by MAPK triggers the expression of the IEG, *CFOS*, *EGR-1* and *JUN B* (Hodge et al., 1998). Mitogenic stimuli promotes the assembly of a Ternary complex factor (TCF) on the serum response element (SRE) of the *CFOS* promoter (Shaw et al., 1989), by directly targeting the TCF transcription regulators; ELK-1, ELK-3 and ELK-4 ,SAP-2/ERP which in turn are all phosphorylated by ERK1/2 kinases (Dalton and Treisman, 1992, Gille et al., 1992, Janknecht et al., 1993, Marais et al., 1993, Lopez et al., 1994, Giovane et al., 1994, Hipkind et al., 1994, Price et al., 1995).

Induction of human *CFOS* transcription by serum growth factors is mediated by a common cis-acting enhancer element necessary for serum induction. This element is called the serum response element (SRE) (Treisman, 1985), which contains the binding site for the serum response factor (SRF) (Treisman, 1986, Norman et al., 1988), and the binding site for ELK-1 (Shaw et al., 1989). The induction of *CFOS* expression by serum has been demonstrated to be promoted by phosphorylation of ELK-1 which enhanced the formation of a ternary complex between ELK-1, SRF and the SRE (Gille et al., 1992, Marais

et al., 1993). The rapid induction of *CFOS* after growth factor stimulation demonstrated that it has a primary role in the control of cell proliferation (Müller et al., 1984, Greenberg and Ziff, 1984).

There are several pathways that lead to the activation of regulatory proteins involved in IEG expression, such as the RhoA-actin, ERK, p38 MAPK and PI3K pathways. These pathways lead to the phosphorylation and activation of regulatory proteins involved in IEG expression, such as members of the ETS-domain family for example the transcription factor ELK-1 and ETS1/2, which bind to the promoter of relevant genes and form a complex with lysine acetyltransferases. Furthermore, these pathways also lead to the activation of other regulatory factors that are essential for induction, such as the SRF and the Mediator complex (Galbraith et al., 2013, Janknecht and Nordheim, 1992).

Phosphorylated ELK-1 binds to SRE target sites and is associated with transcriptional co-activators like CREB-binding protein and/or p300 (Li et al., 2003b, O'Donnell et al., 2012). The p38 MAPK pathway can be stimulated by both growth factors and general stress, and leads to activation of the p38 MAPK kinase, which subsequently activates several transcription factors including ELK-1. ELK-1 promote the recruitment of MSKs to the promoter and thereby H3S10 phosphorylation (Zhang et al., 2008). The changes in histone acetylation lead to access of NF1 (nuclear factor 1) to a binding site occluded by the -1 nucleosome, and thereby transcriptional activation can take place. Also, PARP1 (poly ADP-ribose polymerase 1) is

recruited and can trigger the binding of additional regulators to the FOS promoter (Fig. 1). The role of PARP1 in FOS regulation is to enhance ERK-mediated ELK-1 phosphorylation (Cohen-Armon et al., 2007).

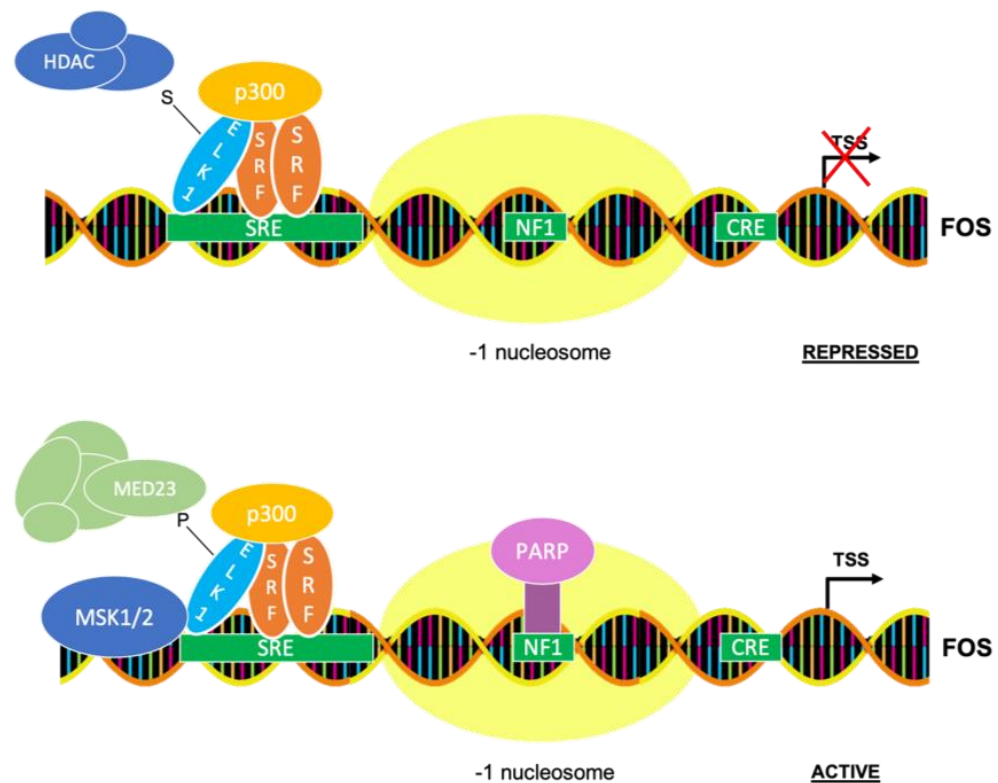


Figure 1. Transcriptional states of the FOS gene: basal expression, an activation phase, and a repression phase, respectively. The status of ERK pathway activity, the chromatin modifications around the TSS (transcriptional start site) and the transcriptional regulators associated with the promoter region are also shown. CRE (cAMP-response element), HDAC (histone deacetylase); NF1 (nuclear factor 1), PARP1 (poly ADP-ribose polymerase 1, SRE (serum response element), MSK1/2 (mitogen-and stress-activated kinase 1 and 2).

The nuclear kinase isoforms mitogen-and stress-activated kinase 1 and 2 (MSK1/2) are also downstream targets for activation by MAPK signalling. MSK1/2 targets several proteins for phosphorylation, including a number of transcription factors such as CREB and NF- κ B which regulate immediate early gene expression. MSK1/2 also phosphorylates histone H3 at serine 3 (phosH3S10) and serine 28 (phosH3S28) at the upstream promoter region of immediate early genes (Clayton and Mahadevan, 2003, Soloaga et al., 2003). It has been reported that MSK1/2 is essential for the transcription of IEG, knocking out or blocking MSK1/2 was found to reduce IEG expression (Soloaga et al., 2003).

1.3 Ternary complex formation

1.3.1 Serum response element

The SRE is located in the 5' flanking sequence of the *CFOS* RNA cap site. It functions independently of its orientation with respect to the gene, can move from its normal location and can activate a heterologous promoter. It is also considered as an enhancer-like element (Treisman, 1985). Furthermore, SRE sequences are found in promoters of several other immediate-early genes and viral genomes (Hipskind et al., 1994, Cahill et al., 1994).

The SRE DNA sequence is a point of convergence for different signal transduction pathways (Whitmarsh et al., 1995). SRE mutations that block SRF binding abolish the response of the SRE to a variety of stimuli (Treisman, 1986).

Formation of the ternary complex relies on protein-DNA and protein-protein interactions between ELK-1 and the SRE-SRF binary complex. ELK-1 makes direct contacts with the 5' flanking sequence of the SRE and it interacts with the C-terminus region of the SRF. (Shaw, 1992). The *CFOS* SRE contains an ETS binding site (EBS) characterised by a purine-rich core sequence of GGA(A/T), adjacent to two SRF binding sites or CARG box, as originally identified in the *CFOS* SRE. The juxtaposition of those two elements, promote the ternary complex formation (Treisman, 1986, Minty and Kedes, 1986, Taylor et al., 1989, Karim et al., 1990, Mueller and Nordheim, 1991, Treisman, 1992, Treisman et al., 1992).

1.3.2 ELK-1/SRF interaction

The SRF is a homodimeric protein composed of monomers of relative molecular mass of 67 kDa. Amino acids 133-222 on the SRF have been shown to constitute the minimal region necessary for the binary and ternary complex formation of SRF (Norman et al., 1988, Mueller and Nordheim, 1991). The crystal structure of the SRF specific interaction region (amino acids 133-222) has been described (Fig. 2). Two β -sheets (β 1, β 2) are located at the middle of the two α -helix (α 1,

$\alpha 2$) and are considered as the central element of the dimerisation interface (Fig. 2). The N-terminus of the $\alpha 1$ of each subunit makes direct contact with the DNA-SRE (Pellegrini et al., 1995).

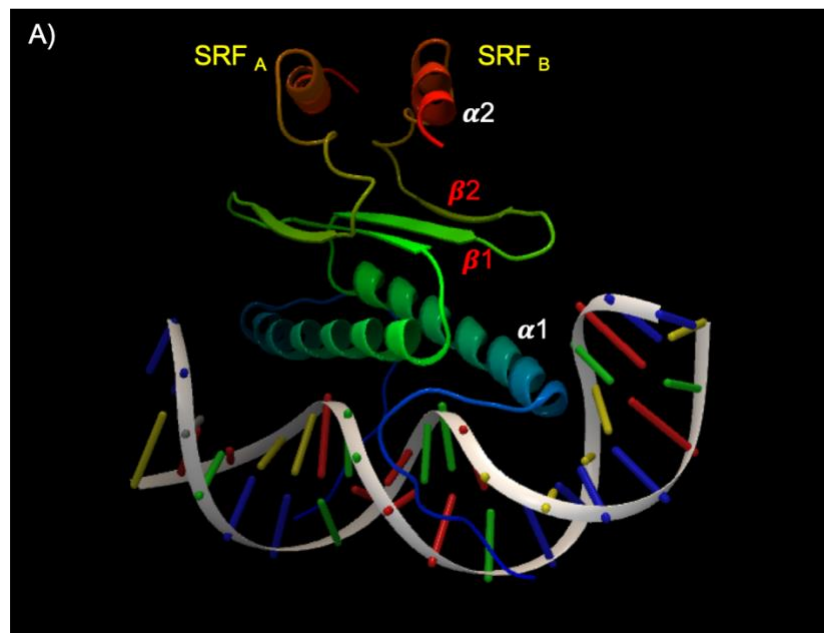


Figure 2. Crystal structure of the SRF (amino acids 132-22), bound to CArG box in the SRE at a resolution of 3.2 Å. A) α -helices of the SRF monomer B are indicated in white and β -sheets are indicated in red. Structure taken from (Pellegrini et al., 1995) PDB 1SRS - modified with CLC sequence viewer 7.

The interaction of ELK-1 with the SRE-SRF complex is dependent on both the ETS domain and amino acids 137-169 of ELK-1. Although, it was demonstrated that ELK-1 binding to the SRE-SRF complex does not increase the strength of interaction between the SRE and the SRF (Janknecht and Nordheim, 1992).

The efficiency of the ELK-SRF interaction relies on the residues Q190, V194, T196, T199, and I206 in the SRF, which form the interaction surface with ELK-1. In the absence of DNA, the mutations V194E, T196K, and T196E in SRF cause the greatest decrease in protein-protein interactions (Ling et al., 1998).

Another important structural domain which is linked to the amino-terminal ETS domain is the SRF-interaction domain (SID) or B-box, it functions as a linker to accommodate variations in the spacing between the two DNA elements. The formation of the TCF rely on the linker and the B-box (Ling et al., 1997, Sharrocks, 2002). The B-box consists of a β -strand flanked by two short helices. The β -strand aligns with those of the β -sheet that crossed the middle of the core SRF structure, while its β -helices contact with the core SRF and with phosphate groups of the DNA. A third major domain implicated in the regulation of TCFs by Mitogen-activate protein kinase (MAPK) is known as the activation domain or C-box; it is conserved among all three TCF at functional and sequence levels and it is located at the carboxy-terminal of each TCF (Fig. 3) (Shaw and Saxton, 2003).

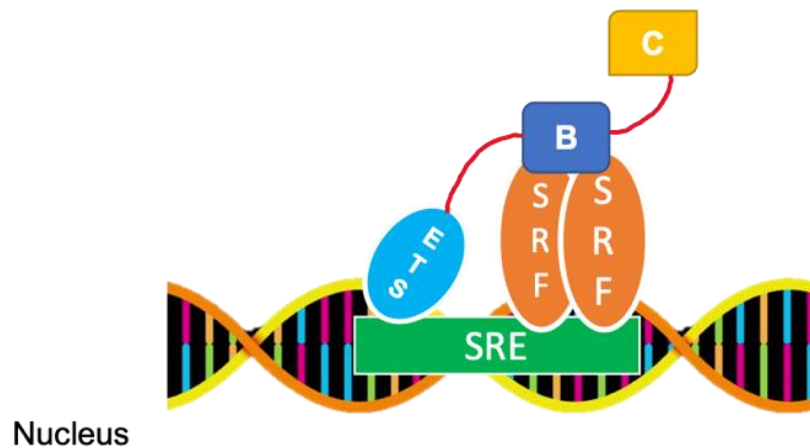


Figure 3. Schematic representation of the Ternary Complex. The ETS domain bound to an ETS site within the SRE and the B-box bound with a dimer of SRF which in turn its attached to a CARG box in the SRE.

1.4 ELK-1

ELK-1 was discovered on the SRE of the *CFOS* promoter of HeLa cell nuclear extracts as a protein of 62 kd, initially called p62 and described as an integral but physically separable part of a ternary complex formed with p67/SRF and the SRE. (Shaw et al., 1989).

The name p62_{TCF} was later replaced for ELK-1 because of the discovery that p62_{TCF} was the product of the ELK-1 gene. (Hipskind et al., 1991, Rao et al., 1989). Human ELK-1, ELK-3 and ELK-4 are members of the ETS family of transcription factors sharing a high degree of homology in three distinct domains, which include the amino-terminal ETS domain (region A), the SRF interaction domain

(region B), and the transactivation domain (region C) (Janknecht and Nordheim, 1992, Treisman et al., 1992)

1.4.1 Domain structure of ELK-1

The structure of ELK-1 belongs to the winged helix–turn–helix family of transcription factors. ETS domains contain three α -helices and four β sheets, the main protein-DNA contacts are from residues located in the third α -helix (Fig. 4) (Donaldson et al., 1996, Mo et al., 2000).

The ELK-1 domain A, localised in the N-terminus region corresponds to the ETS DNA Binding Domain (DBD) (Dalton and Treisman, 1992). This motif contains a nuclear localisation signal (NLS) and a nuclear export signal (NES) that is deleted in a smaller, neuronal-specific isoform of ELK-1 called short-ELK-1 (Fig. 4) (Besnard et al., 2011).

The B box, a region of 21 amino acids, which enables TCFs to interact with SRF and promotes ternary complex formation, is located downstream of the ETS domain (Shore and Sharrocks, 1994). The function associated with this domain has shown that it is required for the induction of DNA binding following ELK-1 phosphorylation (Fig.4) (Yang et al., 1999).

The D domain is located N-terminus from the C domain and it contains the MAPK docking sites (Yang et al., 1998b, Sharrocks et al., 2000, Jacobs et al., 1999, Yang et al., 1998a). ELK-1 contains an additional R-domain for transcriptional repression, it contains lysine residues that are SUMOylated for the recruitment of the histone deacetylase 2

co-repressor (Fig. 4) (Yang et al., 2003, Yang and Sharrocks, 2004).

The C-terminus region of ELK-1 corresponds to the transcriptional activation domain (TAD). ELK-1 C-terminus region contains nine (S/T)P motifs that form the MAP kinase sites, five of these lie within the conserved C-box (Gille et al., 1995a, Janknecht et al., 1993, Marais et al., 1993). The FXFP motif is located C-terminus from the TAD domain, it is specifically involved in docking to ERK (Fig. 4).

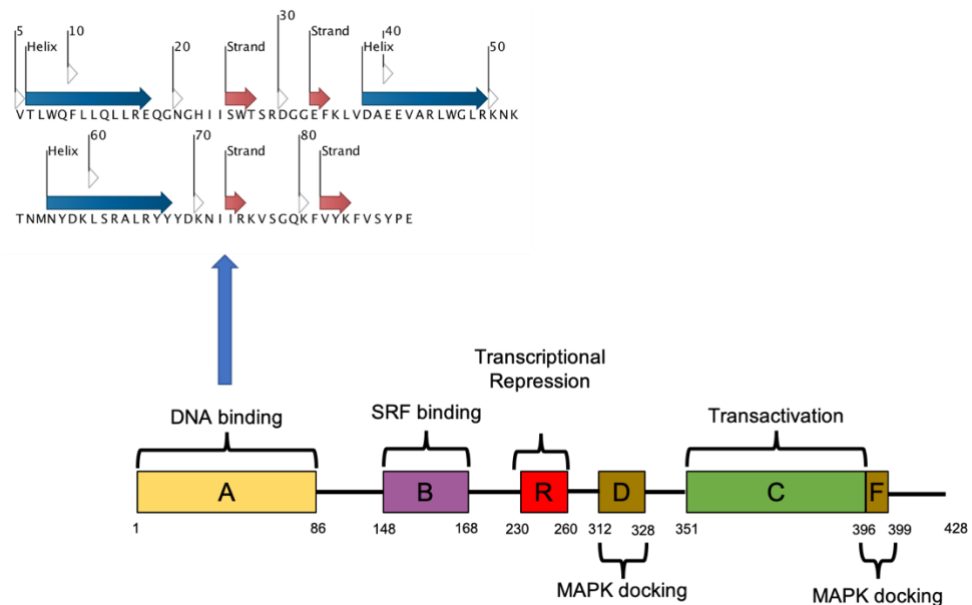


Figure 4. Diagram illustrating ELK-1 functional domains and schematic representation of the secondary structure of ELK-1 ETS domain. α -helices are indicated in blue and β -strands are indicated in red. Functions of the domains are indicated. The amino acid residue locations of the domains are presented below the boxes.

1.4.2 ETS / DNA binding

All ETS proteins share a highly conserved ETS DNA-binding domain of ~85 amino acids residues that mediates binding to a DNA target harboring a central core GGA sequence (Karim et al., 1990).

Structures of the ETS domain of ELK-1 bound to a DNA sequence that is identical to a natural target for the *Drosophila* E74 Ets protein, 5' -ACCGGAAGT-3' showed that the main residues contributing to the ETS/DNA binding are located in the $\alpha 3$ helix that lies in the major groove of DNA, with additional contacts mediated by the wing between $\beta 3$ and $\beta 4$ sheets, and the loop between $\alpha 2$ and $\alpha 3$ helices (Sharrocks et al., 1997). Mutagenesis and *in vitro* binding assays have shown that the ETS domains of ELK-1 and ELK-3 exhibit related but distinct DNA-binding specificities. Amino acid V68 of the ELK-1 ETS and the analogous D69 of the ELK-3 ETS domain are critical determinants for the differential binding specificities on the *CFOS* promoter. Creation of mutations D38Q and D69V is sufficient to confer ELK-3 DNA-binding specificity upon ELK-1 and thereby allow it to bind to a greater spectrum of sites (Shore et al., 1996).

However, crystal structures support a different role for the ELK-1-E74-DNA complex in promoter recognition. Crystal structures have revealed a high degree of sequence identity (~80%) within the ETS domain between ELK-3 and ELK-1, and complete sequence identity within the DNA recognition helix (Shore and Sharrocks, 1995, Mo et al., 1998). Although, ELK-3 and ELK-1 bind optimally to the E74

sequence, the target sequence found in the *CFOS* promoter, 5'-ACAGGATGT-3', binds with high affinity only to the ETS domain of ELK-3. The difference lies in the DNA recognition $\alpha 3$ helix corresponding to Y66 residue in ELK-1 and Y65 in ELK-3. Structural studies revealed that Y66 of ELK-1 does not make base-specific contacts to E74 DNA and also does not make base-specific interactions when ELK-1 binds independently to the *CFOS* DNA promoter. Asparagine 69, located C-terminus to the $\alpha 3$ DNA recognition helix of the ETS domain, mediates the rearrangement of conserved residues, especially Y66, which facilitates alternative protein-DNA contacts. Non-conserved residues distal to the protein-DNA interface also have a great impact influencing ETS domain-DNA interactions. (Fig. 5.) (Mo et al., 1998, Mo et al., 2000). Therefore, direct interactions with SRF facilitate the binding of ELK-1 to the *CFOS* DNA promoter after serum induction, ELK-1 is incapable of binding to the *CFOS* promoter in the absence of SRF and serum induction (Shaw et al., 1989).

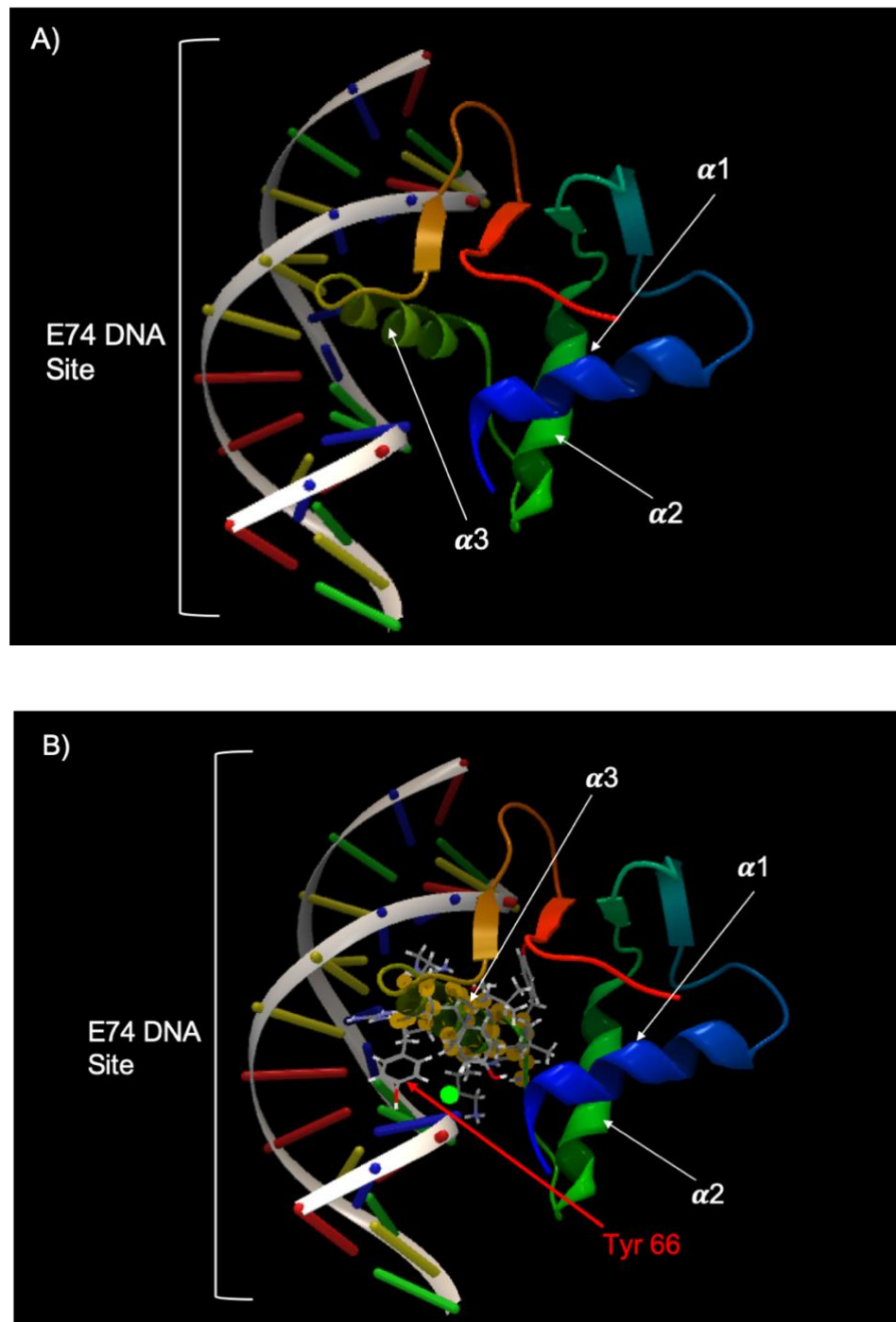


Figure 5. Crystal structure of the ETS domain of ELK-1 bound to E74 DNA high affinity site (residues 5-90) at a resolution of 2.1 Å. A) α -helices are indicated with white arrows and β -sheets are coloured. B) Y66 is indicated with the red arrow. Structure taken from (Mo et al., 2000) - PDB 1DUX - modified with CLC sequence viewer 7.

1.4.3 ELK-1 MAPK docking sites

ELK-1 transactivation domain transmits mitogenic stimuli to regulate gene expression. Phosphorylation of C-terminus ELK-1 by ERK2 occurs at multiple sites including serines 383 and 389 and has an effect regulating the capacity to form a ternary complex factor with the SRF and SRE (Gille et al., 1995a, Janknecht et al., 1993, Marais et al., 1993, Cruzalegui et al., 1999). C-terminus phosphorylation of ELK-1 causes a change in intramolecular interactions with either the ETS-domain or the C-terminus activation domain which in turn leads to activation of DNA binding by the N-terminus ETS-domain (Yang et al., 1999).

Stress signals are also able to cause phosphorylation of ELK-1. Under stress conditions the stress-activated protein kinases (also known as *c-JUN* N-terminus kinases; SAPKs or JNKs), which are a sub-group of the MAP kinase family, phosphorylate and activate ELK-1 promoting the expression of the *CFOS* gene (Gille et al., 1995b, Whitmarsh et al., 1997, Price et al., 1996, Cavigelli et al., 1995).

Hypothetically, unphosphorylated ELK-1 appears to have a 'closed' conformation. Phosphorylation of the C-domain of ELK-1 by ERK2 reverts to an open conformation in which the ETS-domain and B-domain become accessible to the DNA and SRF respectively (Fig. 6).

The D-domain was reported as essential for maximal phosphorylation and rapid activation of ELK-1 by ERK2 and JNK. Binding of ERK2 to ELK-1 through the D domain is a prerequisite for efficient phosphorylation and subsequent activation of ELK-1 in vitro and in vivo (Fig. 6) (Yang et al., 1998a, Yang et al., 1998b, Jacobs et al., 1999).

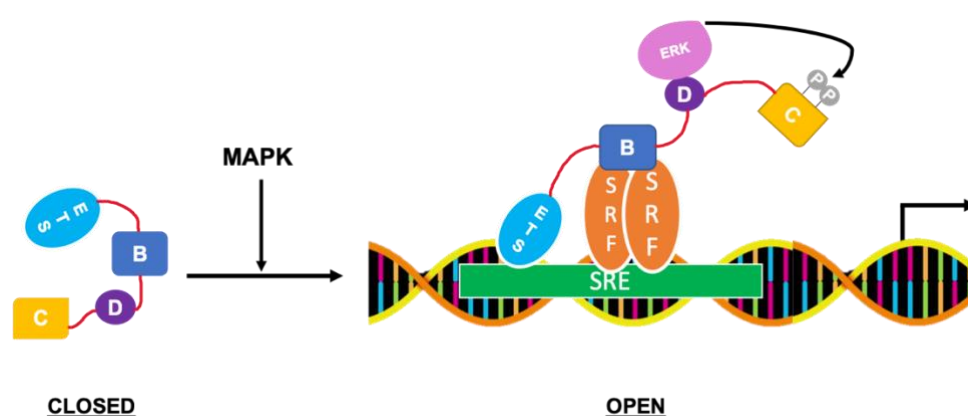


Figure 6. Diagrammatic representation of ELK-1 activation and phosphorylation by ERK2. In the unphosphorylated conformation, ELK-1 is inhibited from DNA binding and Ternary complex formation. Following phosphorylation of ELK-1 C-domain by ERK, the ETS-domain and B-domain become accessible to the DNA and SRF, respectively, promoting transcriptional activation (Sharrocks, 1995).

1.4.4 ELK-1 phosphorylation by MAPKS

The kinases ERK1 and ERK2 were reported as the principal nuclear protein kinases that phosphorylate and activate ELK-1 (Gille et al., 1995a, Gille et al., 1992, Marais et al., 1993). However, mitogenic stimulus are not the only stimulus able to activate ELK-1. ELK-1 responds to a wide variety of extracellular stimuli, it is a target for all three major MAPKs (ERK, JNK and p38) (Fig. 7) (Gille et al., 1995b, Gille et al., 1995a, Cavigelli et al., 1995, Price et al., 1996, Whitmarsh et al., 1997, Cruzalegui et al., 1999).

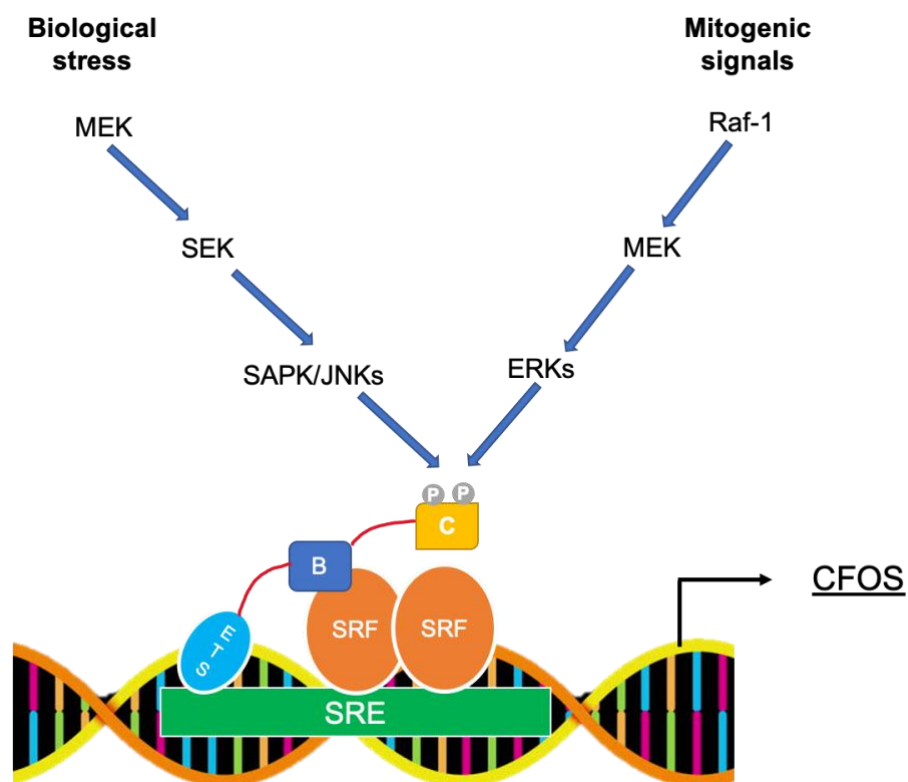


Figure 7. Diagrammatic representation of ELK-1 activation and phosphorylation by stress signals and mitogenic signals.

It has been reported that unphosphorylated ELK-1 occupies the *CFOS* SRE prior to stimulation and that phosphorylated ELK-1 is present transiently following mitogen stimulation (Herrera et al., 1989).

The CREB binding protein (CBP) has been demonstrated to be a coactivator for ELK-1. Activation of *CFOS* via ELK-1 requires phosphorylation of serine 383 and 389 on ELK-1 and phosphorylation of CBP by MAPK (Janknecht and Nordheim, 1996).

In addition, the histone acetyltransferase (p300) was reported to facilitate ELK-1 transactivation. ELK-1 phosphorylation on Ser383 and Ser389 increases its affinity for p300 which in turn increases its ability to transfer acetyl groups to the histones. Following ELK-1 phosphorylation, the histone acetyltransferase activity of the complex is activated, resulting in histone acetylation and changes in nucleosome structure. These conformational changes may also lead to interactions between p300 and the basal transcription machinery (Fig. 1) (Li et al., 2003b). Studies have shown that ELK-1 is able to recruit ERK and MSK (mitogen and stress-activated kinase) to the promoter of IEGs genes such as *CFOS* and *EGR1* through the D domain on ELK-1 (Zhang et al., 2008). The recruitment of ERK to chromatin in stimulated cells has demonstrated that ERK can play an important role phosphorylating additional substrates within pre-initiation complexes (PIC) that are formed on SRE promoters in mitogen-stimulated cells (Zhang et al., 2008).

1.4.5 ELK-1 interaction with Mediator

The Mediator has an essential involvement in regulation of transcription initiation across eukaryotes; it influences preinitiation complex (PIC) formation and stimulates phosphorylation of the RNA polymerase II (RNAPII) carboxy-terminal domain (CTD) by transcription factor IIH (TFIIH), and it interacts with activators and repressors to transfer regulatory information to the basal transcription machinery (Tsai et al., 2017, Ansari and Morse, 2013, Allen and Taatjes, 2015).

The PIC formed at gene promoters consists of the general transcription factors (TFIIA, TFIIB, TFIID, TFIIIE, TFIIF and TFIIH, RNA polymerase II (pol II), and Mediator (Knuesel et al., 2009a, Poss et al., 2013). The subunits of Mediator reside in four distinct modules termed the head, middle, tail, and a dissociable CDK8 kinase module. Components of each module are thought to be structurally and functionally connected (Table 1). The head, middle and tail modules form the core Mediator complex and, under normal conditions, are always found in association, while the CDK8 module association is less stable (Tsai et al., 2014, Ansari and Morse, 2013).

Table 1. Core Mediator complex subunits

HEAD	MIDDLE	TAIL
MED6	MED1	MED14
MED8	MED4	MED15
MED11	MED7	MED16
MED17	MED9	MED23
MED18	MED10	MED24
MED20	MED19	MED25
MED22	MED21	
MED27	MED31	
MED28	MED26	
MED29		
MED30		

Transcriptional regulation of ELK-1 target genes has been related to interactions with several Mediator components identified in SRE PICs. It was reported that ELK-1 is able to contact Mediator through phosphorylation-dependent interaction with subunit MED23 (Stevens et al., 2002).

Microarray analysis in mouse embryonic stem cells (ES) revealed that Mediator lacking the MED23 subunit can support near normal expression of the vast majority of genes (Stevens et al., 2002). However, *EGR1* is one of the genes most severely affected by loss of MED23 (Wang et al., 2005). The defect in *EGR1* transcription is due to the inability of the ELK-1 activation domain to interact with Mediator

and stimulate transcription (Stevens et al., 2002). It has been reported that serum treatment increased Mediator binding to the *EGR1* and *CFOS* promoter. The subsequent interactions between phospho-ELK-1 and the MED23 subunit stabilise the PIC, increasing the binding of Pol II, TFIIB, E and H in response to serum (Fig. 8) (Wang et al., 2005).

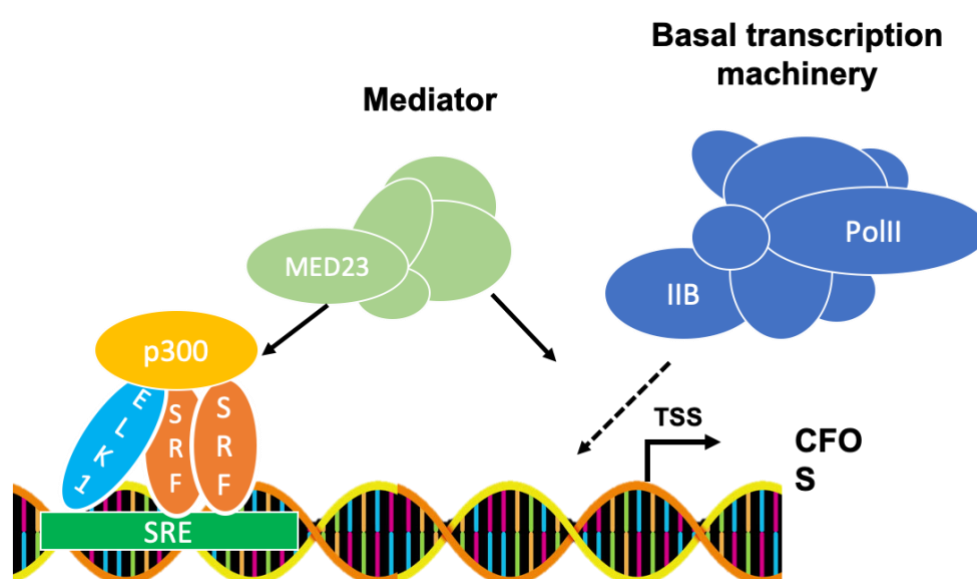


Figure 8. Model of the molecular events following ELK-1 interaction with the Mediator. Mediator can be recruited via ELK-1 binding and this can then facilitate basal transcription factor assembly, RNA polymerase recruitment and transcriptional initiation.

In contrast to the regulation of *EGR1* in ES, it has been shown that the lack of MED23 affects transcription differently in mouse ES and MEFs cells. Microarray analysis studies revealed that 793 genes are sensitive to the loss of MED23 in one cell type but not the other. The difference between ES and MEFs cells was reported to be due to cell-specific expression of ELK-1 and ELK-4 that each bind to *EGR1*

control elements, but the interaction with the mediator complex is different (Balamotis et al., 2009).

The participation of MED14 in the regulation of *CFOS* and *EGR1* was published. ELK-1 appears to be important to contact the mediator through MED14; MED23 does not contact phospho-ELK-1, whereas ELK-1 is able to contact Mediator independently of MED23 (Galbraith et al., 2013).

The CDK8 consists of the cyclin-dependent kinase CDK8, its partner CycC, MED12, and MED13. The characterisation of the complex has shown that both MED12 and MED13 mediate the assembly and stabilisation of the whole CDK8 module (Wang et al., 2013). It has been suggested that the subcomplex itself can regulate gene expression independently of Mediator in human cells and its kinase activity is dependent on MED12 (Knuesel et al., 2009b).

The human CDK8 sub-complex can have a direct or indirect impact on the activity of the Mediator, pol II and TFIIH. It can control Mediator-pol II interactions to help regulate transcription initiation and reinitiation events (Knuesel et al., 2009a).

Recent data showed that CDK8 is a positive regulator of IEGs. It was reported that serum triggers CDK8 accumulation at the promoter of ELK-1 target genes (*CFOS* and *EGR1*), promoting RNAPII CTD

phosphorylation, which is necessary for promoter clearance and elongation at serum responsive loci (Donner et al., 2010).

1.4.6 Role of ELK-1 in prostate cancer

The evidence reported about ELK-1 participation in prostate cancer has demonstrated that ELK-1 overexpression is highly important for the progression and adaptation of prostate cancer cells to undergo uncontrolled proliferation. In prostate cancer cells that are adapted to grow in the absence of hormone, the Androgen receptor (AR) apoprotein is localised in an active form in the nucleus. In such cells, the AR apoprotein activates a gene set that is distinct from genes that require androgen for activation in the same cells (Wang et al., 2009, Gonit et al., 2011, Chan et al., 2012). The mechanism by which the AR regulates those genes was demonstrated several years ago (Patki et al., 2013). ELK-1 recruits the AR and promotes the interaction of the N-terminus A/B domain (residues 1–559) of AR with ELK-1. This interaction promotes a sustained ELK-1 activation independent of ELK-1 phosphorylation and independent of the formation of the ternary complex (Patki et al., 2013). Using a mammalian two-hybrid assay, it was demonstrated that the two ELK-1 motifs required for association with AR (A/B) correspond to the two ERK-docking sites in ELK-1 (Rosati et al., 2016). Some examples of androgen-induced genes for which ELK-1 was required included DTL, CDC6, THBS1, CDCA3, MAP2, CCNB2, CDCA5, BMPR2, FEN1 and SLC7A11, which primarily support cell cycle progression and mitosis (Patki et al.,

2013). Recently, the compound quinone referred to KCI807 was reported to directly binds to AR and selectively block its association with ELK-1, inhibiting ELK-1-dependent transcriptional activity of AR. Substitutions of the hydroxyl groups 5, 7, 3', 4'- tetrahydroxyflavone with methoxy groups were crucial to find the selectivity of the compound against the ELK-1-AR interaction. KCI807 was shown to prevent recruitment of AR to chromatin by ELK1 and target the genes that were strongly related to cell cycle progression and mitosis (Rosati et al., 2018).

Together, these data suggest a new role for ELK-1 in prostate cancer that needs to be studied further.

1.4.7 ELK-1 repression

It has been shown that dephosphorylation of regulatory phosphoproteins is an important mechanism for the downregulation of *CFOS* promoter activity. It was reported that following stimulation of ERK, calcineurin translocates to the nucleus where it specifically dephosphorylates ELK-1 at phosphoserine 383 (Sugimoto et al., 1997, Tian and Karin, 1999). Small increases in intracellular Ca^{2+} have a negative effect on *CFOS* transcription through activation of calcineurin, however large increase in Ca^{2+} can stimulate *CFOS* transcription through activation of Ca^{2+} -responsive kinases. (Tian and Karin, 1999). The N-terminus ETS DNA-binding domain contained in ELK-1 exhibits repressive capability by recruiting the mSin3A-histone deacetylase 1 co-repressor and HDAC-1. It was published that

ELK-1 is rapidly activated by ERK phosphorylation at Ser383 following 15 to 30 min of EGF treatment and subsequently dephosphorylated by 60 min. Moreover, recruitment of mSin3A corepressor complex occurs after 30 min which overlaps the peak of ELK-1 phosphorylation but occurs immediately before *CFOS* expression is turned off. ERK phosphoacceptor sites in ELK-1 play a critical role in the ERK inducible recruitment of mSin3A (Yang et al., 2001).

Another mechanism described to repress ELK-1 transcriptional activity is related to the SUMO (small ubiquitin-related modifier) pathway. Four SUMO homologs have been described in mammals. SUMO-1 and SUMO-2/3 appear to modify both common and different substrates (Saitoh and Hinchev, 2000, Zhao et al., 2004). Posttranslational modification by SUMO has been widely related to regulate subcellular localisation of many targets including RANGAP, the first identified SUMO substrate (Matunis et al., 1996). However, another role for SUMO has been reported in the modulation of proteins controlling chromatin structure such as PML and DNA Topoisomerase I by SUMO-2/3 (Mao et al., 2000), and proteins involved in gene expression such as RNA polymerase I, II and III (Zhao et al., 2004). The distribution of charged residues on the surface of SUMO is very different from that of ubiquitin or other UbLs. Moreover, SUMO has an N-terminus extension not found in ubiquitin (Gill, 2004).

Ubiquitin and SUMO are each covalently attached to substrate proteins via an isopeptide bond between a C-terminus glycine in the UbL and a lysine residue in the substrate (Pickart, 2001). The E1 and E2 conjugating enzymes are sufficient for ATP-dependent SUMO modification of substrates *in vitro* (Bernier-Villamor et al., 2002). In contrast to the ubiquitin system, where dozens of E2 have been identified, UBC9 is the only known SUMO E2 conjugating enzyme, it recognises a consensus SUMO acceptor site containing the ϕ KXE motif, where ϕ represents a large hydrophobic amino acid that confers the ability to be SUMO-1 modified. *In vivo* modifications require the additional presence of a nuclear localization signal on its protein substrates which are targeted to the nucleus (Rodriguez et al., 2001). In the SUMO pathway, SUMO E3 ligases promote transfer of SUMO from the E2 to specific substrates. RANBP2, the PIAS proteins and the polycomb group protein Pc2 have been suggested to have SUMO E3 ligase activity. RANBP2 and the PIAS protein interact with Ubc9 and promote transfer of SUMO from UBC9 to substrate proteins. They localise in the nuclear pore complex, nucleoplasm and nuclear bodies (Kahyo et al., 2001, Johnson and Gupta, 2001, Sachdev et al., 2001, Pichler et al., 2002).

It has been reported that ELK-1 inhibition is dependent on the presence of two potential SUMO modification sites, K230 and K249 in the R motif (Fig. 9). This inhibition is reversed after ERK MAP kinase-mediated ELK-1 activation. (Yang et al., 2003).

The SUMO E3 ligase PIASx has been demonstrated to function as a coactivator to reverse SUMOylated ELK-1 in response to signaling through the ERK MAP kinase pathway. This coactivator activity is independent of its E3 ligase activity but still requires ELK-1 to be SUMO modified. PIASx α activates ELK-1 through altering the HDAC/HAT (Histone deacetylation and acetylation) complexes bound to ELK-1 and facilitating the removal of histone deacetylase (Fig. 9) (Yang and Sharrocks, 2005, Yang and Sharrocks, 2006). In 2019, it was demonstrated that ELK-1 is SUMOylated with SUMO1 by p38 MAPK pathway-dependent mechanisms in response to heat stress. PIAS2 acts as a coactivator upon the activation of ERK and has been shown to function as a corepressor upon its phosphorylation by p38 MAPK. Together, p38 MAPK pathway-dependent SUMOylation of ELK-1 and phosphorylation of PIAS2 correlate with the downregulation of ELK-1 (Chowdhury et al., 2019).

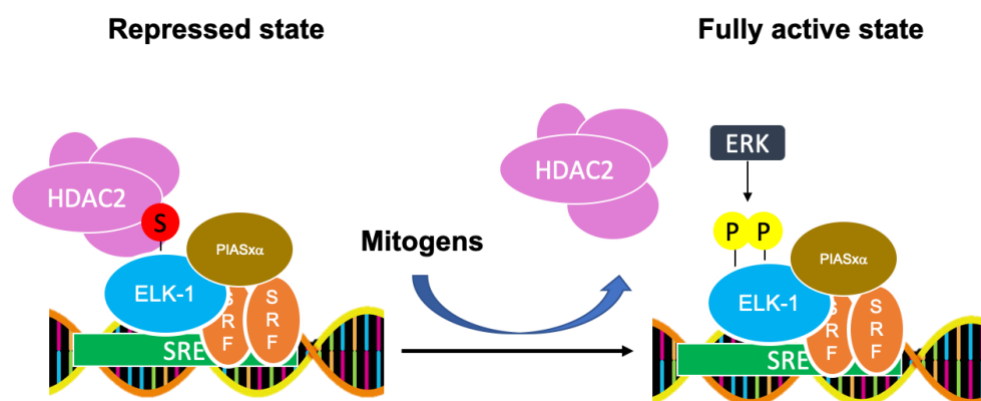


Figure 9. Model representation between the SUMO and MAPK pathways regulating the transcription of IEGs by ELK-1. In unstimulated cells Elk-1 is sumoylated (S) and recruits an HDAC2 co-repressor complex. Mitogenic stimuli cause the phosphorylation (P) of the ELK-1 Cterminus TAD leading to de-repression by the loss of both sumoylation and HDAC2 association. This is mediated by PIASx α which acts as a co-activator of ELK-1 (Yang et al., 2003, Yang and Sharrocks, 2005).

Accumulation of ELK-1 in the nucleus and its interaction with DNA have been widely studied. Studies have revealed that ELK-1 has dynamic interactions with DNA that contribute to its nuclear accumulation. This nuclear accumulation is exclusive of ELK-1 acting as a monomer. Moreover, ELK-1 can also accumulate in the cytoplasm where it can be stable due the capacity to homo-dimerize (Evans et al., 2011). Dimerisation of ELK-1 appears to confer cytoplasmic stability; the disruption of ELK-1 dimerisation has been shown to promote degradation of ELK-1 by the ubiquitin proteasome system.

The rapid turnover of dimerisation defective ELK-1 has been attributed to the presence of a cryptic degron (amino acids 167-216) adjacent to the B-box, which is a proline-rich hydrophobic motif. (Fig. 10) (Evans et al., 2011).

Recently, it was demonstrated that ELK-1 is mono-ubiquitinated within the ETS domain (K35) (Fig. 10). Mono-ubiquitination of ELK-1 decreases its transcriptional activity by impairing DNA binding and promoting its cytoplasmic re-localisation (Ducker et al., 2019).

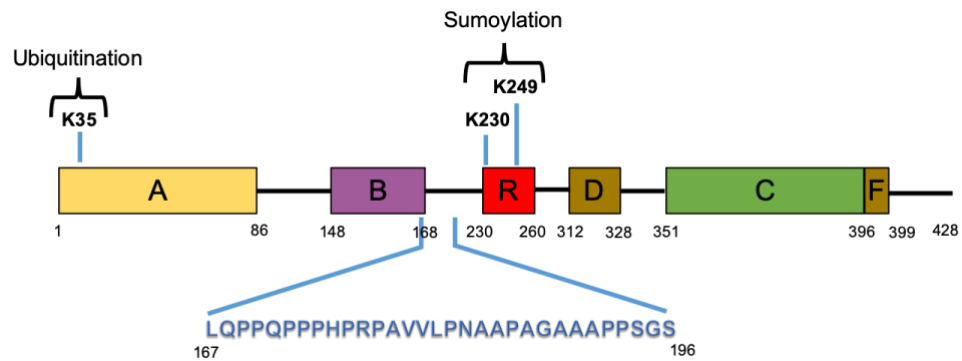


Figure 10. Schematic representation of ELK-1 degradation sites. Highlighted are the ubiquitination site (K35), the cryptic degron (amino acids 167-196) and sumoylation sites K230 and K249.

1.5 The ubiquitin system

Protein ubiquitination was discovered in the early 1980s as a post-translational modification, in which lysine residues are modified with the small protein ubiquitin, a 76 amino acid polypeptide of ~8500 Da. Initially, this modification was found to regulate the half-life of proteins, since a ubiquitinated protein would undergo rapid ATP-dependent degradation mediated by the proteasome (Ciechanover et al., 1984, Glickman and Ciechanover, 2002). However, in 2004 after the Nobel Prize in chemistry was awarded to Aaron Ciechanover, Avram Hershko and Irwin Rose for their discovery of the ubiquitin system, new discoveries were made (Giles, 2004).

The ubiquitin-proteasome system (UPS) consists of a series of essential components: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), ubiquitin ligase enzymes (E3s),

deubiquitinating enzymes (DUBs), and the 26S proteasome. There are about 50 E2 enzymes and more than 600 E3 enzymes that govern the activity of many downstream proteins (Pickart, 2001, Pickart, 2004, Deshaies and Joazeiro, 2009).

Ubiquitination begins with the covalent attachment of an ubiquitin to substrate lysines in a three-enzyme cascade catalyzed by E1, E2 and E3 enzymes, thus resulting in an isopeptide linkage between the C-terminus glycine of ubiquitin and the substrate ϵ -amino group of lysine (Pickart, 2001, Berndsen and Wolberger, 2014). Ubiquitin is activated in an ATP-dependent reaction by the E1 enzyme and then is transferred to the active site cysteine of the E2 enzyme that, in turn, generates an E2~Ub thioester intermediate. The E3 ligase binds to both the E2~Ub thioester and the substrate, catalysing transfer of the ubiquitin from the active site cysteine of the E2 to the substrate lysine or N terminus (Fig. 11) (Deshaies and Joazeiro, 2009).

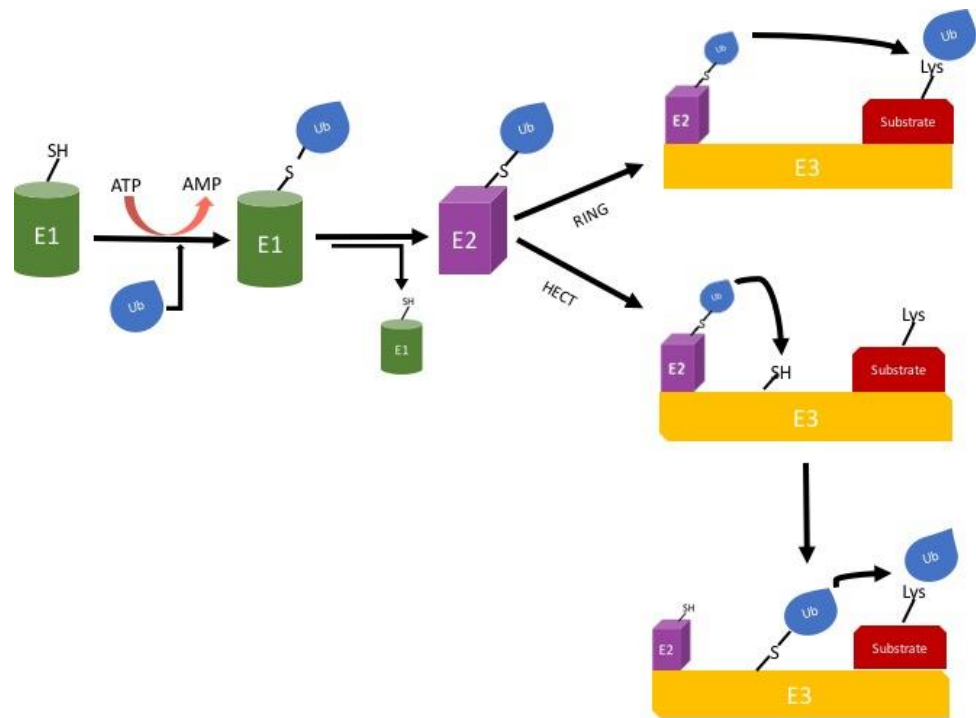


Figure 11. Mechanism of ubiquitination mediated by HECT and RING-domain E3 ligases. E1 activating enzyme utilises energy from ATP hydrolysis to activate ubiquitin. Ubiquitin is then carried as an E1 and E2-thioester and eventually transferred to substrates, facilitated by E3 ligases. HECT-domain E3 ligases form an ubiquitin thioester intermediate before substrate conjugation whereas RING- domain E3 ligases transfer ubiquitin from an E2 conjugating enzyme to a substrate directly.

A protein may be modified with a single Ub (mono-ubiquitination), several singles Ubs or by multiple Ubs that are covalently linked together via any of seven lysine residues (poly-ubiquitination) or the N-terminus of Ub (linear chains) (Komander, 2009).

1.5.1 Ubiquitin chain structure

Ubiquitin is a small protein that is very abundant in mammalian cells. It consists of 76 amino acids and a molecular weight of 8433 Da. Secondary structural features include three and one-half turns of α -helix, a short piece of 3_{10} -helix (a helix with three residues per turn instead of 3.6 for α -helices), a mixed β -sheet that contains five strands, and seven reverse turns. The α -helix contributes three hydrophobic residues, and 11 of the 13 hydrophobic residues in the β -sheet participate in the formation of the hydrophobic core. Only I44 and V70 are exposed. Other residues that contribute to this core include I36, L56 and I61 (Vijay-Kumar et al., 1987). Seven acceptor lysines (K6, K11, K27, K29, K33, K48, and K63) of the ubiquitin molecule can be involved in successive conjugation (Komander, 2009). Structural analysis of the available chain types has revealed that Lys₄₈ and Lys₆₃ linked chains have entirely different structures (Fig. 9). The hydrophobic Ile₄₄ patches of two linked ubiquitin moieties interact, and the linking residues are closely packed against the ubiquitin units. Lys₄₈-linked chains are not rigid, but are in dynamic equilibrium and can also adopt more open conformations (Tenno et al., 2004, Eddins et al., 2007). Lys₆₃-linked chains have an entirely different three-dimensional structure compared with Lys₄₈-linked chains. Lys₆₃-linked chains are extended and adopt an open conformation where the individual ubiquitin molecules are not interacting with each other (Fig. 12) (Tenno et al., 2004).

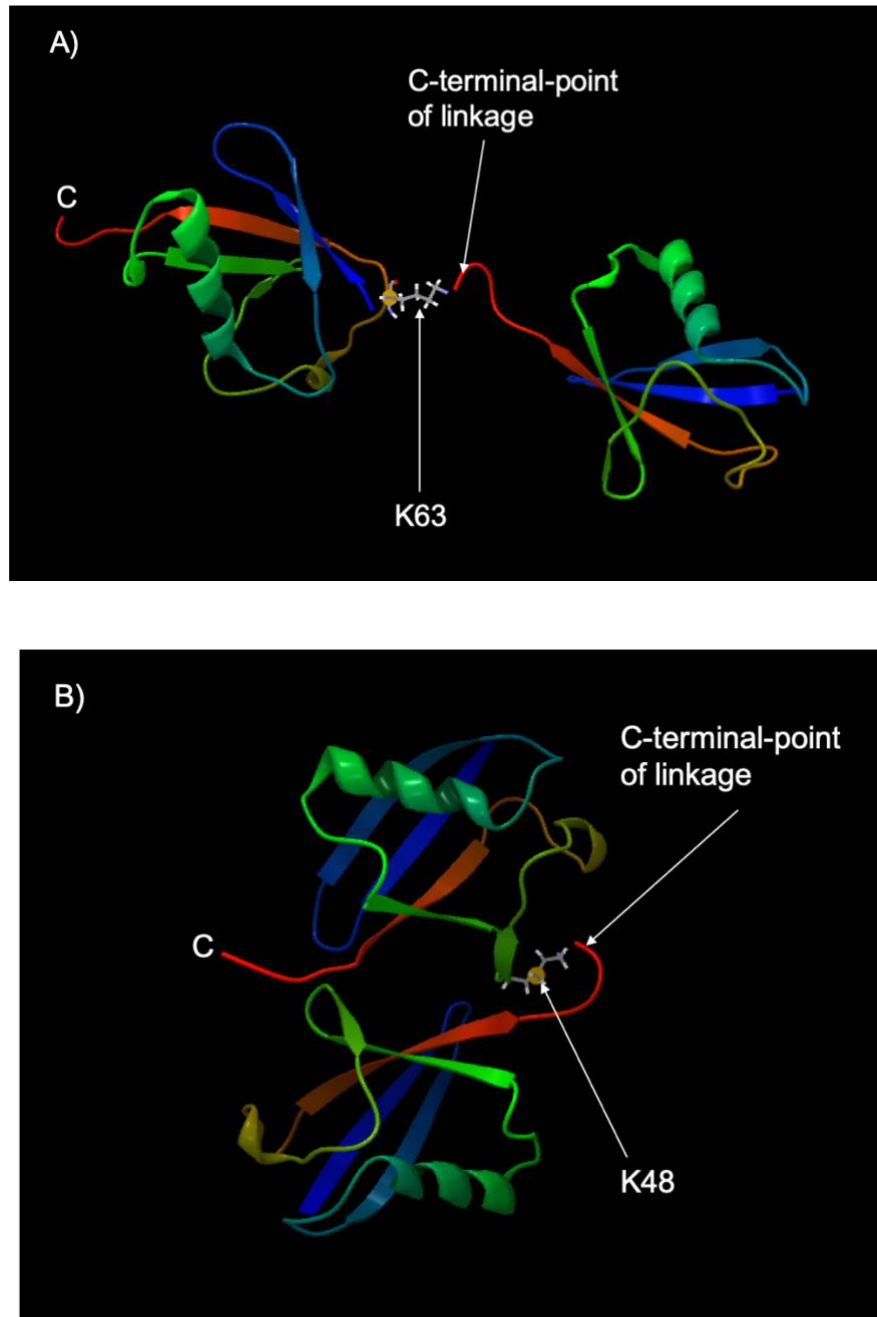


Figure 12. Diubiquitin chains. A) Crystal structure of a diubiquitin conjugate Lys63 at a resolution of 1.96 Å. The chain adopts a highly extended conformation in contrast with the compact globular fold of Lys48. Structure taken from (Datta et al., 2009) PDB 3HM3-modified with CLC sequence viewer 7. B) Crystal structure of a diubiquitin conjugate Lys48 at a resolution of 2.3 Å. Successive ubiquitins are linked by an isopeptide bond involving the side chain of Lys48 and the carboxyl group of Gly76. Structure taken from (Cook et al., 1992) PDB 1AAR-modified with CLC sequence viewer 7.

1.5.2 Ubiquitin E3 ligases

The first E3 to be characterised was the one that recognised the denatured lysozyme substrate used in early biochemical studies. It was called E3 α and required model substrates to carry a free α -amino group (Hershko et al., 1984). In 1986, studies by Varshavsky *et al*, in yeast, unexpectedly uncovered the complete relationship between the identity of the N-terminus amino acid and substrate stability, it was called the N-end rule (Bachmair et al., 1986). Further investigations of this mechanism showed that the orthologous yeast E3 (Ubr1) recognised this determinant, which together with a lysine residue subject to ubiquitination was termed an N-degron (Varshavsky, 1997). These studies provided the functional definition of a degron (an element that is both necessary and sufficient for substrate ubiquitination) and established the modular organisation that ultimately proved to apply to all E3s (Pickart, 2004).

E3 ligases are categorised into three classes based the type of transfer mechanism utilised: Really Interesting New Gene (RING)-type E3s, Homologous to the E6-AP Carboxyl Terminus (HECT)-type E3, and RING in Between RING (RBR) E3s (Dove and Klevit, 2017).

1.5.3 HECT ubiquitin ligases

HECT E3 ligases can be distinguished from other classes of ubiquitin E3 ligases in that they have an active-site cysteine that forms an intermediate thioester bond with ubiquitin before the ubiquitin is linked to its substrate (Fig. 11). In contrast to the RING E3s, HECT E3 ligases form an E3~Ub intermediate prior to the transfer of ubiquitin to the substrate (Fig. 11) (Sluimer and Distel, 2018).

The 28 human HECT E3 enzymes comprise between ~700 and ~4800 residues. They are characterised by a C-terminus HECT domain of ~40 kDa. The HECT domain consists of two lobes, a larger N-terminus lobe (N-lobe) and a smaller C-terminus lobe (C-Lobe) that are connected by a short linker. The C-lobe contains a catalytic residue, and it interacts with ubiquitin during the transfer reaction. While the N-lobe associates with E2 enzymes, the recognition of target proteins is typically mediated by domains or motifs within the extended regions N-terminus to the catalytic HECT domain (Fig. 13) (Lorenz, 2018, Sluimer and Distel, 2018).

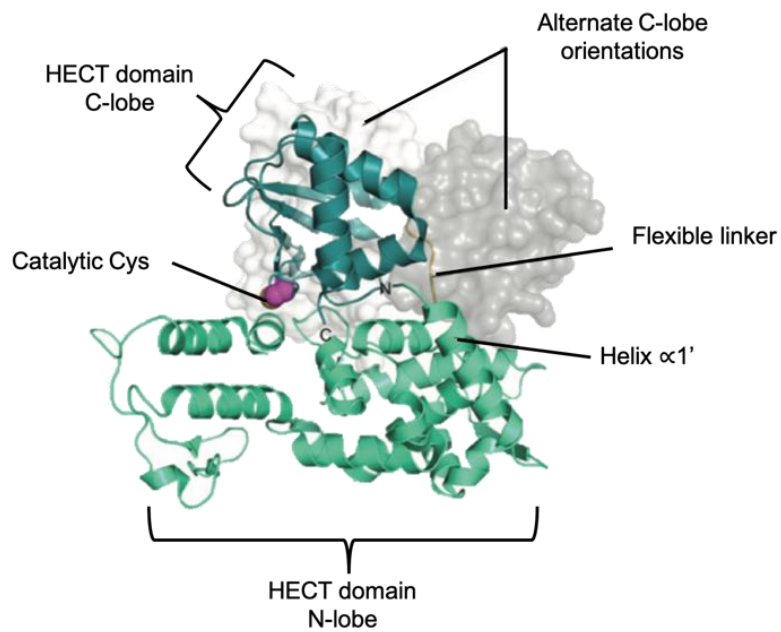


Figure 13. Architecture of the catalytic HECT domain. The side chain of the catalytic cysteine residue is displayed as balls; the flexible linker connecting the two lobes is highlighted in yellow. The N- and C-termini of the polypeptide chain are labeled. Image modified from (Lorenz, 2018).

1.5.4 RING ubiquitin ligases

Unlike HECT-type E3s, RING E3s can transfer ubiquitin directly from the ubiquitin-charged E2 enzyme to the substrate within a scaffold (Fig. 8). The RING-type subgroup contains the largest number of E3 ligases and reaches more than 600 in humans. (Deshaies and Joazeiro, 2009).

The standard RING domain has a RING fold structure with a zinc-binding site, while the U-box domain has the same RING fold but without zinc (Wang et al., 2017). The conserved cysteine and histidine residues are covered inside the domain's core. They function to maintain the overall structure through binding two atoms of zinc. Additional semi-conserved residues are implicated either in forming the domain's hydrophobic core or in recruiting other proteins (Borden et al., 1995).

Distinctly, either the RING domain or the U-box domain can assemble into monomers, homodimers, or heterodimers. It has been reported that only homodimers can bind two E2s simultaneously (Table 2) (Zhao and Sun, 2013).

Table 2. Classification of RING E3 ligases. Table modified from (Wang et al., 2017).

Types	Characteristic domains	Members	Subtypes	Characteristic domains of subtypes	Examples
RINGs	RING fold structure with/without zinc-binding site (RING/U-box)	About 600	Monomers	Monomeric RING/U-box	c-CBL, E4B, SIAH-1
			Homodimers	Homodimeric RING/U-box	cIAP, CHIP
			Heterodimers	Heterodimeric RING	Mdm2-mmdmX
			Cullin-RING-ligases	Cullin scaffold structure	SCF, CRL2s, CRL3s, CRL4s, CRL5s, Cullin 7/FBW8, FBXO25, FBXW7, SPOP

1.5.5 Mono-ubiquitination

The first protein identified over 40 years ago to be ubiquitinated was histone H2A (Goldknopf et al., 1975). Along with histone H2B, histone H2A is one of the most abundantly ubiquitinated nuclear proteins, with 5-15% of histone H2A being mono-ubiquitinated in human cells (Clague et al., 2008). Histone mono-ubiquitination is one of the processes that have been widely studied. Histones H2A and H2B form part of the core structure of nucleosomes and they are modified by monoubiquitin or short ubiquitin chains in their carboxy-terminal tails (Lys 119 on H2A; Lys 120 on H2B) (Hicke, 2001). Another example of histone mono-ubiquitination is the H1 histone, which is not part of the core nucleosome structure but is a 'linker' histone that binds to DNA, extending between neighbouring nucleosomes. It is mono-ubiquitinated in *Drosophila melanogaster* embryos. Its modification is dependent on a component of the general transcription machinery, TAF250 (a subunit of TFIID). Mutations in TAF250 have been demonstrated to inhibit its ubiquitination activity causing defects in transcriptional activation in *Drosophila* embryos (Pham and Sauer, 2000, Hicke, 2001).

Mono-ubiquitination also regulates the activity of proteins located at the plasma membrane. In mammalian cells, for example, ubiquitination of the growth hormone receptor promotes its endocytosis and subsequent lysosomal degradation (Strous et al., 1996). It has been found that most proteins ubiquitinated at the

plasma membrane are targeted for degradation in the lysosome (Strous et al., 1996, Staub et al., 1997, Hicke, 1999).

Mono-ubiquitination has been associated with diverse proteasome-independent cellular functions including intracellular protein movement. Mono-ubiquitination of receptor tyrosine kinases (RTKs) at multiple sites after ligand-induced activation, has become recognised as an important signal for their endocytosis and degradation in the lysosome (Haglund et al., 2003).

Mono-ubiquitination also regulates p53 trafficking in accordance with the levels of MDM2 activity. It has been shown that MDM2 catalyses both mono-and polyubiquitination of p53 in a dose dependent manner. MDM2 levels will depend on the severity of DNA damage. With high DNA damage that induces apoptosis, MDM2 levels are suppressed. Low MDM2 levels induce both mono-ubiquitination and cytoplasmic translocation of p53. (Li et al., 2003a, Rinaldo et al., 2007).

Mono-ubiquitination also plays a significant role in the nuclear/cytoplasmic shuttling of proteins. For example, the regulation of the transcription factor FOXO4 by mono-ubiquitination, in response to increased cellular oxidative stress, was reported to impact on its re-localisation to the nucleus and an increase in its transcriptional activity (van der Horst et al., 2006). Similar results were shown for the tumour suppressor PTEN. Nuclear localisation of PTEN is mediated by mono-ubiquitination, which is essential to control chromosomal integrity. Monoubiquitinated nuclear PTEN is stable, as opposed to

polyubiquitinated PTEN which rapidly degrades in the cytoplasm (Trotman et al., 2007).

Mono-ubiquitination also regulates transcriptional activity of ELK-1. Recently, it was demonstrated that ectopic expression of USP17 decreases ELK-1 mono-ubiquitination at K35, and up-regulates ELK-1 target genes promoting tumour cell proliferation. Conversely, USP17 depletion increased ELK-1 mono-ubiquitination levels. (Ducker et al., 2019).

1.5.6 Poly-ubiquitination

Poly-ubiquitination has been widely related to proteasome degradation of oncogenic proteins. For example, in prostate cancer, degradation of the androgen receptor (AR) can result in mitotic arrest or inhibition of its transcriptional activity. That effect has been attributed to several E3 ligases. It has been reported that low doses of an endogenous estrogen metabolite induce mitotic arrest. The mechanism involves degradation of the AR by two E3 ligases, CHIP and MDM2. In response to 2-methoxyestradiol, CHIP is the primary E3 ligase to promote degradation of the AR, low levels of phosphorylation favored CHIP over MDM2 the activation of which is dependent on PI3K/Akt phosphorylation. However, when silencing CHIP, the levels of MDM2 increased. Knockdown of CHIP or MDM2 individually or in combination reduced AR degradation and abrogated M phase arrest induced by 2-methoxyestradiol (Sarkar et al., 2014).

Moreover, poly-ubiquitination can also regulate the transcriptional activity of the AR by the E3 ligase SPOP (Speckle-type POZ protein). SPOP knockdown increased AR protein levels promoting its transcriptional activity by upregulating the expression of PSA and TMPRSS2 (AR transcriptional targets) (An, 2014).

Poly-ubiquitination also controls cell cycle transition through the degradation of the cyclin E following phosphorylation on threonine 380. The amount of cyclin E protein in the cells is controlled by multiple F-box proteins which contribute to cyclin E stability (Singer et al., 1999, Koepp et al., 2001, Strohmaier et al., 2001). Additionally, poly-ubiquitination also plays a role in controlling transcription initiation and re-initiation through the degradation of MED13. It has been published that the E3 ligase FBXW7 binds to CDK8–Mediator and targets MED13/13L for degradation following phosphorylation of T326. FBXW7 loss increases CDK8 module–Mediator association (Davis et al., 2013).

Besides protein degradation, poly-ubiquitination on K63 can also lead to DNA repair. For example, mono-ubiquitinated PCNA can be further poly-ubiquitinated by the E2 UBC13/MMS2 complex, in association with its E3 ligase RAD5 to generate a K63-linked poly-ubiquitin chain, which is important for error-free DNA repair (Bergink and Jentsch, 2009).

Lysine 63 polyubiquitination has also been shown to regulate endocytosis. For example, the yeast plasma membrane uracil permease undergoes cell-surface ubiquitination, which is dependent on the Npi1/Rsp5 ubiquitin-protein ligase. Ubiquitination of this permease signals endocytosis of the protein, leading to its subsequent vacuolar degradation. This process does not involve the proteasome, which binds and degrades ubiquitin-protein conjugates carrying Lys48-linked ubiquitin chains (Galan and Haguenauer-Tsapis, 1997).

Another example of lysine 63 polyubiquitination has been reported in the NF- κ B pathway. Stimulation of cells with interleukins causes the recruitment of the E3 ubiquitin ligase TRAF6 to the receptor. TRAF6 catalyses the synthesis of K63-linked polyubiquitin chains. The polyubiquitin chains function as a scaffold to recruit the TAK1 and IKK complexes through binding to the regulatory subunits, TAB2 and NEMO, respectively. Recruitment of the kinase complexes facilitates autophosphorylation of TAK1 and subsequent phosphorylation of IKK β by TAK1, leading to I κ B degradation and subsequent activation of NF- κ B (Tokunaga and Iwai, 2009, Skaug et al., 2009, Ikeda et al., 2011).

1.6 E3 Ligase SPOP

In the last years, the study of SPOP (Speckle-type POZ protein) in prostate cancer has provided strong evidence that it enhances the progression of cancer. The increased recognition of SPOP functions started to raise because it has been reported as a significantly mutated gene in human cancers such as breast, lung, ovarian and prostate (Kan et al., 2010). Subsequently, more studies revealed SPOP mutations in two out of seven tumour samples (Berger et al., 2011). Another study identified SPOP mutations in 6–13% of primary prostate adenocarcinomas and 14.5% of metastatic prostate cancers (Barbieri et al., 2012). The importance of mutated or overexpressed SPOP has been highly relevant in human cancers owing to its tumorigenic activity targeting substrates for ubiquitination and degradation which are principally regulators of cellular proliferation and apoptosis.

SPOP has also been implicated in modulating cell migration and invasion largely through governing ERG protein abundance (Gan et al., 2015, An et al., 2015). Additionally, there is evidence that suggests that N-terminus-truncated ERG proteins are resistant to SPOP mediated degradation (An et al., 2015).

1.6.1 SPOP domain structure

SPOP is a 374 residue protein that possesses a centrally located BTB domain that interacts with Cul3_{NTD}. Structurally, the 42 kDa protein SPOP comprises an N-terminus MATH domain (meprin and TRAF homology), a BTB/POZ domain (bric-a-brac, tramtrack and broad complex), a 3-box domain and a C-terminus nuclear localisation sequence (NLS) (Fig. 14). The MATH domain is primarily involved in substrate recognition and binding. Substrate binding involves characteristic amino acid residues Y87, F102, Y123, W131 and F133 present in the MATH domain of SPOP. The substrate proteins require the presence of a characteristic SPOP-binding consensus (SBC) motif ϕ - π -S-S/T-S/T (ϕ = nonpolar, π = polar) as a prerequisite for binding to SPOP (Mani, 2014, Zhuang et al., 2009).

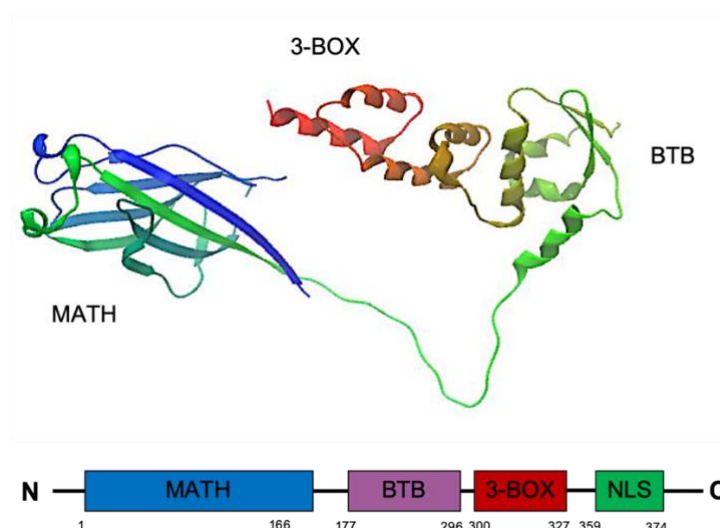


Figure 14. Schematic representation of the speckle-type POZ (pox virus and zinc finger protein) protein (SPOP) protein. The various domains are shown as boxes and indicated in SPOP crystal structure at a resolution of 2.62 Å. Structure taken from (Zhuang et al., 2009) PDB 3HQI-modified with CLC sequence viewer 7.

The MATH domain, present in a great number of proteins, is most frequently found linked to a C-terminus BTB domain. The $\alpha 3$ - $\beta 4$ loop consists of ten amino acid residues in the BTB domain and makes the biggest contribution to the SPOP-Cul3 interaction (Fig. 14). SPOP interaction surface of Cul3-NTD involves helices H2 and H5 (Errington et al., 2012). The $\alpha 3$ - $\beta 4$ loop in the BTB domain exhibit a motif φ -x-E (φ represents a hydrophobic residue, often Met or Leu, x represents any residue and E represents a glutamate residue) corresponding to residues M233, E234 and E235, which plays an important role for binding to the scaffold. More importantly, some studies reported that within the SPOP-BTB domain M233 makes a significant contribution to the SPOP-BTB/Cul3-NTD interaction (Errington et al., 2012). However, recent research demonstrated that the binding of SPOP to Cul3 might not be totally restricted to its BTB domain because an additional motif has been identified C-terminus to the BTB domain that extends the cullin-binding surface and enhances affinity of the complex. A pair of α -helices stretching beyond the BTB domain, called 3-box, enhance the binding with Cul3 (Mani, 2014, Marzahn et al., 2016, Errington et al., 2012).

The presence of two functional self-association domains in SPOP promotes its localisation to nuclear speckles. It has been shown that mutations in the BTB or BACK domain disrupt the self-association ability, resulting in diffuse localisation, supporting the idea that the localisation of SPOP depends on specific domain interactions,

principally driven by the BTB domain promoting the correct localisation of the protein (Marzahn et al., 2016).

1.6.2 SPOP substrates

In prostate cancer, studies have found recurrent SPOP mutations at a frequency of 6-15% across localised and advanced prostate tumours. The prostate cancer-associated SPOP mutations include Y87C, Y87N, F102C, S119N, F125V, W131G, F133L, and F133V located in the SPOP-MATH domain (Kan et al., 2010, Barbieri et al., 2012, Boysen et al., 2015). Further studies demonstrated that the AR is a *bona fide* substrate of SPOP, it promotes AR protein ubiquitination and proteasome degradation and inhibits prostate cancer cell growth in an AR-dependent manner. The ⁶⁴⁵ASSTT₆₄₉ motif functions as an AR degron, which is essential for SPOP binding and subsequent ubiquitin-dependent degradation of AR and Ser647 and Thr648 residues were reported as crucial for this function, while this effect was abolished with the prostate cancer-associated SPOP mutations (An, 2014).

In mammals, SPOP has been linked to ubiquitination of MacroH2A, to regulate its deposition on the inactive X chromosome, and Daxx to regulate transcription repression of proapoptotic proteins such as p53. The SPOP substrates reported for Puc, Daxx, Gli, Macro H2A and Ci bind to the SBC motif that perfectly match with the MATH central shallow groove (Fig. 15). Sequences outside the conserved motif

even from the same peptides do not superimpose with the SPOP binding site (Errington et al., 2012).

SPOP-MATH-SBC interactions are mainly supported by hydrophobic and polar interactions. Indeed, it has been reported that there are key residues for substrate recognition; single SPOP mutations in the MATH domain demonstrated that D130A and W131A substitutions are essential in the SBC (Fig. 12) (Puc, MacroH2A and Ci) (Zhuang et al., 2009).

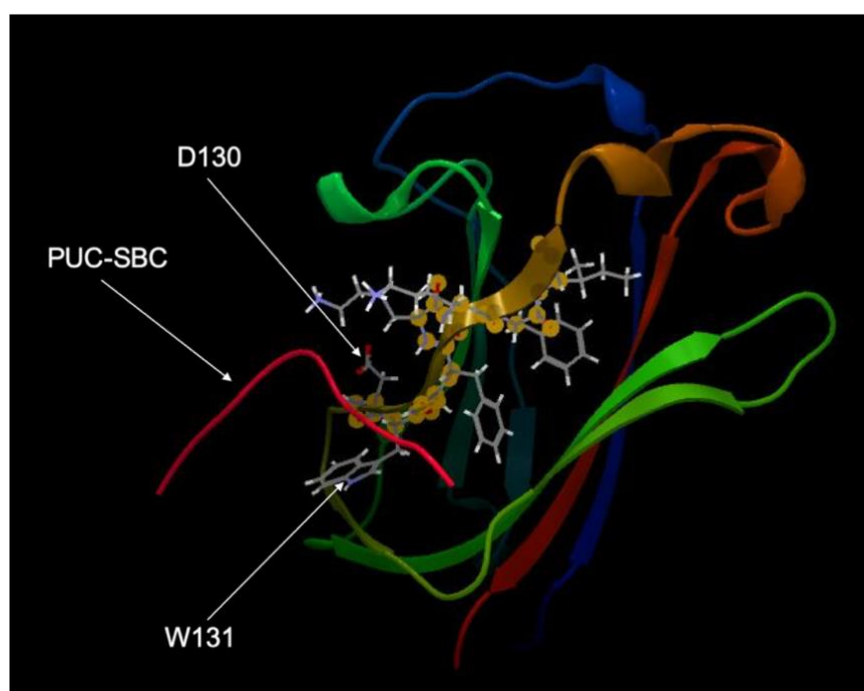


Figure 15. Crystal structure of SPOP-MATH/PUC-SBC interaction. SPOP-MATH critical residues for substrate recognition are indicated with white arrows. PUC-SBC is shown in red. Structure taken from (Zhuang et al., 2009) PDB 3IVV-modified with CLC sequence viewer.

1.7 The F-box protein family

F-box proteins can be categorised into three families, on the basis of the presence of recognizable domains beyond the F-box domain. In humans, the F-box and WD40 domain (FBXW) family is composed of 12 proteins, and the F-box and Leu-rich repeat (FBXL) family comprises 21 proteins. The remaining 37 F-box proteins were originally categorized as F-box only (FBXO) proteins (Jin et al., 2004). The CRL1 ligases, known as the Skp1/Cullin 1/F-box protein complex (SCF) are the best characterised. SCF complexes are assembled using the scaffold protein CUL1. CUL1 bridges two essential biochemical functions of the SCF complex. The carboxyl terminus of CUL1 recruits the small RING protein RBX1, which directs the E2 enzyme to the E3 ligase, and the amino terminus CUL1 binds the SKP1 adaptor and a variable F-box protein (Fig. 16). SKP1 recruits substrate receptors and the RING subunit recruits the ubiquitin-conjugating enzyme (E2) to form the active ligase complex (Zheng et al., 2002).

Sixty-nine human F-box proteins, each targeting multiple substrates, enable the core scaffold to select hundreds of proteins for degradation (Petroski and Deshaies, 2005, Jin et al., 2004).

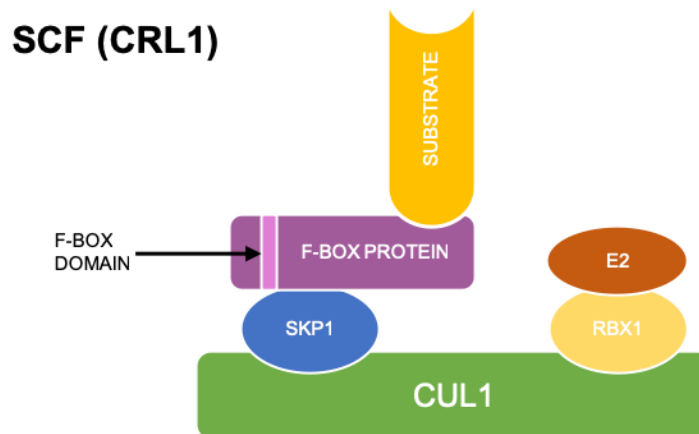


Figure 16. The SCF complex. Cullin 1 (CUL1) acts as a scaffold protein that brings the E2 enzyme in close proximity to the substrate. It binds to S-phase kinase-associated protein 1 (SKP1) at its amino terminus, and the E2 enzyme via RING-box protein 1 (RBX1) at its carboxyl terminus. The F-box protein functions as the interchangeable component that interacts with SKP1 via its F-box domain and with its cognate substrate through its specific substrate recognition domain (Skaar et al., 2013).

1.7.1 FBXO25

As mentioned before, FBXO25 was reported to be the E3 ligase for ELK-1 in HEK293T cells (Teixeira et al., 2013). The prevalent site of FBXO25 expression has been identified in the central nervous system, although intestine and kidney also show significant levels of FBXO25 expression (Maragno et al., 2006, Hagens et al., 2006). The subcellular localisation of FBXO25 has been described as predominantly in the nucleus of cultured cells and excluded from the nucleoli. It has been published that endogenous FBXO25 is associated with sub-nuclear structures named FANDs (FBXO25-associated nuclear domains) (Manfiolli et al., 2008).

1.7.1.1 FBXO25 substrates

The identification of FBXO25 substrates has been mainly related to nuclear proteins. It has been shown that nuclear β -actin is involved in the regulation of FANDs distribution. It was published that FBXO25 physically interacts with β -actin in order to regulate FAND formation (Teixeira et al., 2010). Recent data has shown that FBXO25 expression is mainly detected in cardiomyocytes in the presence of cardiac transcription factors. FBXO25 is reported to mediate ubiquitination of transcription factors including Nkx2-5, Tbx5, Isl1 and Hand1 (Jang et al., 2011). The gain or loss of function of FBXO25 disrupts cardiomyocyte differentiation. Thus, FBXO25 appears to play a crucial role in cardiac development (Jeong et al., 2015).

2.0 RESEARCH AIMS AND SCOPE

My project explores the molecular events that allow ELK-1 to accumulate in cancer cells and promote proliferation.

Recent findings in our laboratory have revealed that ELK-1 can be de-ubiquitinated by the deubiquitylase (DUB) USP17, increasing ELK-1 target gene expression and cell proliferation (Ducker et al., 2019). These findings indicate that ubiquitination of ELK-1 suppresses its activity. For these reasons, identifying the ELK-1 ubiquitin E3 ligase(s) in cancer cells and how they affect ELK-1 transcriptional activity is the aim of this project.

I began my project by exploring the role of FBXO25, which was already reported to target ELK-1 for degradation in HEK293T cells (Teixeira et al., 2013). Secondly, based on evidence reported for SPOP participation in PC, I explored the possibility that this E3 ligase might target ELK-1 for degradation. Thirdly, I undertook an unbiased bioinformatics study to identify ubiquitin E3 ligases that were down-regulated in PC cells in which ELK-1 expression was elevated. Lastly, using mass spectrometry and the BIOID technique I searched for E3 ligases that bind to ELK-1 under starved and stimulated conditions.

3.0 MATERIALS

3.1 Antibodies

Table 3. Antibodies used in this study

PROTEIN	AB TYPE	SOURCE
α ACTIN	Rabbit polyclonal	Sigma A2066
α ELK-1 (I20)	Rabbit polyclonal	Santa Cruz (sc-355)
α ELK-1 C-terminal	Rabbit polyclonal	Abcam E277
α ERK	Rabbit polyclonal	Santa Cruz (sc-154)
α FBXO25	Mouse monoclonal	Abcam AB57051
α FBXO25	Rabbit polyclonal	Life Span Biosciences c397856
α FBXW7	Rabbit polyclonal	Abcam 171961
α FLAG	Mouse monoclonal	Sigma Aldrich M2
α HA-HRP	Peroxidase	Roche 3F10
α HAX-1	Goat polyclonal	Novus Biologicals AF5458
α HIS	Mouse monoclonal	ADB Serotec MCA1396GA
α MED13	Mouse monoclonal	Santa Cruz (sc-515557)
α MYC (9E10)	Mouse monoclonal	Santa Cruz (sc-40)

α PARP	Rabbit polyclonal	Cell Signalling 9541
α pERK	Mouse monoclonal	Cell Signalling 9106
α SKP1	Rabbit polyclonal	Cell Signalling 2156T
α SPOP	Rabbit polyclonal	Abcam 137537
α SRF	Rabbit polyclonal	Santa Cruz (sc-335)
α USP17	Rabbit polyclonal	Sigma Aldrich SAB 1306139
α V5	Mouse monoclonal	Thermo Fisher R96-25
Mouse HRP	Peroxidase	Sigma Aldrich
Rabbit HRP	Peroxidase	Gene Tex gtx213110-01
Strep HRP	Peroxidase	Vector Laboratories SA-5004
Very blot for IP detection		Abcam 131366

3.2 Mammalian expression / knockdown vectors

Table 4. Plasmids used in this study

PLASMID	DESCRIPTION	CONSTRUCTED BY/REFERENCE
ELK-1-BirA-Ha	Mammalian expression pCDNA3.1 vector for C-terminal HA-tagged fused to a mutant biotin ligase (BirA) and human wild type ELK-1 cDNA.	Peter Shaw's group (Appendix 1).

Flag-FBXO25	Mammalian expression pCDNA3.1 vector for N-Terminal Flag-Strep-Strep tagged human wild type FBXO25 cDNA.	Baumann et al., 2014.
Flag-FBXW7-Myc	Mammalian expression pEGFP vector for N-terminal Flag- tagged and C-terminal Myc-tagged human wild type FBXW7 γ cDNA.	Abdolrahman Shams Nateri, University of Nottingham.
Flag-TRIM63	Mammalian expression pCMV3 vector for C-terminal Flag- tagged human wild type TRIM63 cDNA.	Sino Biological Inc.
HA-ELK-1	Mammalian expression pCMV5L vector for N-terminal HA- tagged human wild type ELK1 cDNA.	Peter Shaw's group.
HA-ELK-1 Δ 94-100	Mammalian expression pCMV5L vector for N-terminal HA- tagged human wild type ELK-1 cDNA with the amino acids 94-110 deleted.	Peter Shaw's group.
HA-UBQ	Mammalian expression pCMV5L vector for N-terminal HA- tagged human wild type ubiquitin cDNA.	Peter Shaw's group.
HA-UBR5	Mammalian expression pCDNA5 vector for N-Terminal Ha-Ha-Strep-Strep tagged human wild type UBR5 cDNA.	Mark Ditzel, University of Edinburgh.
HA-USP17	Mammalian expression pCMV5L vector for N-terminal HA- tagged human wild type USP17 cDNA.	Sara Q. Barceinas. (Appendix 2)
His-ELK-1	Mammalian expression pCMV5 vector for N-terminal His- tagged human wild type ELK1 cDNA.	Peter Shaw's group.

His-Hand1	Mammalian expression pCMV5 vector for N-terminal His- tagged human wild type HAND1 cDNA.	Ross Breckenridge, MRC National Institute for Medical Research.
His-UBQ-K0	Mammalian expression pCDNA3 vector for N-terminal His- tagged human wild type ubiquitin cDNA. with all lysines substituted by arginines.	Peter Shaw's group.
Myc-BirA ELK-1	Mammalian expression pCDNA3.1 vector for N-terminal Myc-tagged fused to a mutant biotin ligase (BirA) and human wild type ELK-1 cDNA.	Peter Shaw's group. (Appendix 3)
Myc-FBXO25 Δ F-box	Mammalian expression pCMV5m vector for N-terminal Myc-tagged human wild type FBXO25 cDNA with the amino acids 228-266 deleted (Δ F-box).	Sara Q. Barceinas. (Appendix 4)
Myc-FBXO25 WT	Mammalian expression pCMV5m vector for N-terminal Myc-tagged human wild type FBXO25 cDNA.	Peter Shaw's group.
Myc-SPOP	Mammalian expression pCMV beta vector for N-terminal Myc-tagged human wild type SPOP cDNA.	Haojie Huang, Mayo clinic.
Myc-SPOP D130A	Mammalian expression pCMV beta vector for N-terminal Myc-tagged human wild type SPOP cDNA with the amino acid Aspartic Acid 130 substitute for Alanine.	Sara Q. Barceinas. (Appendix 5)
Myc-SPOP F133V	Mammalian expression pCMV beta vector for N-terminal Myc-tagged human wild type SPOP cDNA with the amino acid Phenylalanine 133 substitute for Valine.	Sara Q. Barceinas (Appendix 6).

Myc-SPOP M233E	Mammalian expression pCMV beta vector for N-terminal Myc-tagged human wild type SPOP cDNA with the amino acid Methionine 233 substitute for Glutamic Acid.	Sara Q. Barceinas (Appendix 7).
Non tag- FBXO25	Mammalian expression pCDNA3.1 vector for human wild type FBXO25 cDNA.	Baumann et al., 2014.
V5-CHIP	Mammalian expression pCDNA4 vector for C-terminal V5-tagged human CHIP cDNA.	Vera Kalscheuer, Max Planck Institute.

3.3 shRNA target sequences

The shRNA preparation was done following the pSuper RNAi System (OligoEngine) manufacturer's protocol. The shRNA construct was verified by sequence analysis using the appropriate primers (Table 7).

Table 5. shRNA knockdown and gene target sequences used in this study.

PLASMID	DESCRIPTION	TARGET SEQUENCE, SENSE	CONSTRUCTED BY/ REFERENCE
shFBXO25 #1	Mammalian expression pSuper vector for human wild type FBXO25 coding region.	5'-GAGAGA ATAACC GTTGTAA-3'	Charles Ducker, Peter Shaw's group.

shFBXO25 #2	Mammalian expression pSuper vector for human wild type FBXO25 targeting the 3' end.	5'-GACACT AACGGC CAATAAT-3'	Charles Ducker, Peter Shaw's group.
shFBXW7 #1	Mammalian expression pSuper vector for human wild type FBXW7 coding region.	5'-GGTTCT GAGGTC CGCTCTT-3'	Sara Q. Barceinas. (Appendix 8)
shFBXW7 #2	Mammalian expression pSuper vector for human wild type FBXW7.	5'-GGCACC AGTCGT TAACAAG-3'	Sara Q. Barceinas. (Appendix 9)
shMED13	Mammalian expression pSuper vector for human wild type MED13 coding region.	5'-GCTCCA GACAGT CAAGTGA-3'	Sara Q. Barceinas. (Appendix 10)
shUSP17 #1	Mammalian expression pSuper vector for human wild type USP17 coding region.	5'GCAGGA AGATGC CCATGAA-3'	Daniele Guardavaccaro, Utrecht.
shUSP17 #2	Mammalian expression pSuper vector for human wild type USP17 coding region.	5'-GAATGT GCAATAT CCTGAG-3'	Daniele Guardavaccaro, Utrecht.

3.4 Oligonucleotides

Table 6. Primers used in site-directed mutagenesis in this study.

OLIGO NAME	SEQUENCE	BASE PAIRS
Myc-FBXO25 ΔF-box (25DFBX FOR)	5'-GCAAGTGAAC AATGGCCTCACC CAGCTGTGGAAGA AGCTTTGTCAG-3'	46
Myc-FBXO25 ΔF-box (25DFBX REV)	5'-CTGACAAAGCT TCTTCCACAGCTG GGTGAGGCCATT GTTCACTTGC-3'	46
Myc-SPOP D130A (POPD130 FOR)	5'-GTGCAAGGCAA AGCCTGGGGATT CAAG-3'	27
Myc-SPOP D130A (POPD130 REV)	5'-CTTGAATCCC CAGGCTTTGC CTTGAC-3'	27
Myc-SPOP F133V (POP_F133V FOR)	5'-GCAAAGACTGG GGAGTCAAGAAA TTCATC-3'	29
Myc-SPOP F133V (POP_F133V REV)	5'-GATGAATTTCT TGAATCCCCAG TCTTTGC-3'	29
Myc-SPOP M233E (POPM233 FOR)	5'-CCATGTTTGA ACATGAAGAGGA GGAGAGC-3'	29
Myc-SPOP M233E (POPM233 REV)	5'-GCTCTCCTCCT CTTCATGTTCAAA CATGG-3'	29

3.5 Sequencing primers

Table 7. Sequencing primers used in this study.

NAME	PRIMER SEQUENCE	BASE PAIRS
BGH rev	5'- TAG AAG GCA CAG TCG AGG -3'	18
CMV for	5'- CGC AAA TGG GCG GTA GGC GTG -3'	21
M13 F (20)	5'- GTA AAA CGA CGG CCA GT -3'	17

3.6 RT-PCR probes and primers

Table 8. TaqMan primers and probes used in this study.

GENE	FORWARD PRIMER	REVERSE PRIMER	TAQMAN PROBE
<i>CFOS</i>	5'-ACTACCA CTCACCC GCAGAC-3'	5'-GGTGGG AATGAAGT TGGCACT-3'	5'-CCTGTCA ACGCGCA GGACTTC TG-3'
<i>EGR1</i>	5'-CAGCACCT TCAACCCT CAG-3'	5'-CAGCACCT TCTCGTTGT TCA-3'	5'-CTACGA GCACCTGA CCGCAGA GTCTT-3'
<i>GAPDH</i>	5'-CTGCACC ACAACCTGCT TAG-3'	5'-ACAGTCTT CTGGGTGGC AGT-3'	5'-CCCTGGCC AAGGTCAT CCATG-3'

3.7 Buffer and solutions

Table 9. Buffer and solutions used in this study. All solutions were prepared in Milli-Q water unless otherwise specified.

NAME	COMPOSITION
2X HBS	▪ 50 mM HEPES ▪ 280 mM NaCl ▪ 1.5 mM Na₂HPO₄ ▪ pH 7.1
SDS-PAGE resolving gel 8% 5mL	▪ 2.3 mL H₂O ▪ 1.3 mL Acrylamide/Bis Acrylamide 30% ▪ 1.3 mL Tris-HCl 1.5M pH 8.8 ▪ 0.05 mL 10% SDS ▪ 0.05 mL 10% APS ▪ 0.003 mL TEMED
SDS-PAGE resolving gel 10% 5mL	▪ 1.9 mL H₂O ▪ 1.7 mL Acrylamide/Bis Acrylamide 30% ▪ 1.3 mL Tris-HCl 1.5M pH 8.8 ▪ 0.05 mL 10% SDS ▪ 0.05 mL 10% APS ▪ 0.002 mL TEMED
SDS-PAGE resolving gel 12% 5mL	▪ 1.6 mL H₂O ▪ 2.0 mL Acrylamide/Bis Acrylamide 30% ▪ 1.3 mL Tris-HCl 1.5M pH 8.8 ▪ 0.05 mL 10% SDS ▪ 0.05 mL 10% APS ▪ 0.002 mL TEMED
SDS-PAGE stacking gel 2mL	▪ 1.4 mL H₂O ▪ 0.33 mL Acrylamide/Bis Acrylamide 30% ▪ 0.25 mL Tris-HCl 1.0M pH 6.8 ▪ 0.02 mL 10% SDS ▪ 0.02 mL 10% APS ▪ 0.002 mL TEMED
10X SDS-PAGE Running buffer	▪ 250 mM Tris ▪ 1.92M Glycine ▪ 1% SDS

Laemmli Buffer (4X SDS buffer)	▪ 8 mL 10% SDS ▪ 4 mL Glycerol ▪ 2.4 mL Tris-HCl 1.0M pH 6.8 ▪ 2.8 mL H₂O ▪ Bromophenol Blue ▪ 4 mL β-mercaptoethanol
Western Blot transfer buffer	▪ 25 mM Tris ▪ 192 mM Glycine ▪ 20% Methanol
10X TBS	▪ 100 mM Tris-HCl pH 8.0 ▪ 9% NaCl
Immunoblot blocking buffer	▪ 5% milk powder/BSA ▪ 0.05% TBS-Tween
Western Blot Stripping buffer	▪ 62.5 mM Tris-HCl pH 6.7 ▪ 2% SDS ▪ 100 mM β-mercaptoethanol
RIPA Buffer	▪ 150 mM NaCl ▪ 1% NP-40 ▪ 0.5% DOC ▪ 0.1% SDS ▪ 50 mM Tris-HCl pH 7.5
2X Hypotonic buffer Cytoplasmic extracts	▪ 40 mM HEPES pH 7.9 ▪ 40 mM NaF ▪ 2 mM Na₃VO₄ ▪ 2 mM Na₄P₂O₇ ▪ 2 mM EDTA ▪ 0.4% NP-40 ▪ 2 mM DTT ▪ 2 mM PMSF ▪ 1X protease and phosphor inhibitors
High salt buffer Nuclear extracts	▪ 20 mM HEPES pH 7.9 ▪ 20 mM NaF ▪ 1 mM Na₃VO₄ ▪ 1 mM Na₄P₂O₇ ▪ 1 mM EDTA ▪ 0.2% NP-40 ▪ 1 mM DTT ▪ 1 mM PMSF ▪ 420 mM NaCl ▪ 20% Glycerol ▪ 1X protease and phosphor inhibitors

Co-IP Lysis buffer	▪ 40 mM HEPES pH 7.9 ▪ 1% NP-40 ▪ 150 mM KCl ▪ 5 mM MgCl₂ ▪ 5 mM NaF ▪ 0.5 mM EDTA ▪ 0.5 mM PMSF ▪ 0.5 mM Na₃VO₄ ▪ 0.5 mM DTT ▪ 10% Glycerol ▪ 1X protease and phosphor inhibitors
Triton Lysis buffer (Mass Spectrometry)	▪ 20 mM Tris-HCl pH7.4 ▪ 137 mM NaCl ▪ 2 mM EDTA ▪ 1% Triton X-100 ▪ 10% Glycerol ▪ 0.5 mM DTT ▪ 1X protease and phosphor inhibitors
Gel wash solution (Mass spectrometry)	▪ 50% (v/v) acetonitrile ▪ 25 mM NH₄HCO₃
Gel enzyme stock solution (Mass spectrometry)	▪ 12.5 mg/mL trypsin ▪ 25mM NH₄HCO₃
Gel extraction solution (Mass spectrometry)	▪ 50% (v/v) acetonitrile ▪ 1% (v/v) trifluoroacetic acid ▪ 49% H₂O Store at room temperature in dark glassware.
Lysis Buffer/Wash buffer 1 (Ubiquitination assay)	▪ 6 M Guanidinium-HCl ▪ 0.1 M Na₂HPO₄/NaH₂PO₄ pH 8.0 ▪ 0.01 M Tris-HCl pH 8.0 ▪ 10 mM β-mercaptoethanol ▪ 10 mM Imidazole
Wash Buffer 2 (Ubiquitination assay)	▪ 8 M Urea ▪ 0.1 M Na₂HPO₄/NaH₂PO₄ pH 8.0 ▪ 0.01 M Tris-HCl pH 8.0 ▪ 10 mM β-mercaptoethanol

Wash Buffer 3 (Ubiquitination assay)	▪ 8 M Urea ▪ 0.1 M Na₂HPO₄/NaH₂PO₄ pH 6.3 ▪ 0.01 M Tris-HCl pH 6.3 ▪ 10 mM β-mercaptoethanol
Wash Buffer 4 (Ubiquitination assay)	▪ 8 M Urea ▪ 0.1 M Na₂HPO₄/NaH₂PO₄ pH 6.3 ▪ 0.01 M Tris-HCl pH 6.3 ▪ 10 mM β-mercaptoethanol ▪ 0.2% Triton X-100
Wash Buffer 5 (Ubiquitination assay)	▪ 8 M Urea ▪ 0.1 M Na₂HPO₄/NaH₂PO₄ pH 6.3 ▪ 0.01 M Tris-HCl pH 6.3 ▪ 10 mM β-mercaptoethanol ▪ 0.1% Triton X-100
Urea Lysis Buffer (BirA experiment)	▪ 8 M Urea in 50mM Tris-HCl pH 7.4 ▪ 1 mM DTT ▪ 1X protease and phosphor inhibitors
Wash Buffer (BirA experiment)	▪ 8 M Urea in 50mM Tris-HCl pH 7.4
BSA blocking buffer (BirA experiment)	▪ 1% BSA ▪ 0.2% Triton X-100 ▪ Bring up to final volume with 1X PBS
ABS blocking buffer (BirA experiment)	▪ 10% ovalbumin (original recipe contains adult bovine serum) ▪ 1% Triton X-100
Coomasie Blue stain 1L	▪ 500 mL Methanol ▪ 200 mL Glacial acetic acid ▪ 1.2 g Coomassie Brilliant Blue R-250
Coomasie destain 1L	▪ 100 mL Methanol ▪ 100 mL Glacial acetic acid
TAE Buffer	▪ 40 mM Tris-acetate ▪ 1 mM EDTA pH 8.0

6X DNA Loading dye	▪ 0.25% bromophenol blue ▪ 0.25% xylene cyanol FF ▪ 15% Ficoll in ddH₂O
LB agar	▪ 10 g/L Tryptone ▪ 5 g/L Yeast extract ▪ 10 g/L NaCl ▪ 15 g/L Agar ▪ 2.5 g/L Select agar
Ethidium Bromide	▪ 60 µL Ethidium bromide ▪ 300 mL 1X TAE

4.0 Methods

4.1 Nucleic Acid Techniques

4.1.1. Polymerase chain reaction

The polymerase chain reaction (PCR) was used to amplify plasmid DNA for cloning purposes, following the next reaction:

- 2 µL dNTPs 10 mM
- 2.5 µL Forward primer 10 µM
- 2.5 µL Reverse primer 10 µM
- 10 ng Template plasmid DNA
- 10 µL 5X Q5 reaction buffer
- 1 µL Pfu/Phusion DNA polymerase (2 U/ µL)
- Nuclease-free water to 50 µL

Using a Sensoquet thermocycler, the thermal cycling conditions used were as follows:

- Initial denaturation: 98° for 1 minute
 - *Denaturation: 98° for 30 seconds
 - *Annealing: 62° for 40 seconds
 - *Extension: 72° for 90 seconds
 - Final extension: 74° for 5 minutes
- } 30X cycles

*Times were variable according to the PCR size amplicon. This example was used for a PCR product of ~1600 bp.

4.1.2 Restriction digests

The digestion was performed under the following conditions:

- 0.2 µg – 1 µg DNA
- 0.5 µL Restriction enzyme (12 U/ µL)
(either single or double digestion)
- 1.5 µL 10X Reaction buffer
- Nuclease free water to 15 µL

Reactions were incubated in a water bath at 37° for 1-2 hours.

4.1.3 DNA ligations

DNA ligations were performed in a total volume of 10 μL under the following conditions:

- 50 ng Vector DNA
- 150 ng Insert DNA (3:1 insert to vector molar ratio)
- 1 U T4 ligase
- 1 μL 10X Buffer
- Nuclease free water to 10 μL

Reactions were incubated overnight at 4° C.

4.1.4 Agarose gel electrophoresis

DNA was size-separated by electrophoresis through agarose gels. Agarose was dissolved in TAE buffer and solubilised by heat using an oven. Agarose concentrations ranged from 0.8% to 3%. DNA samples were mixed with 4 μL of 6X DNA loading dye before loading into the wells of the gel. The DNA was subjected to electrophoresis at 80-100V in TAE buffer for 40-60 minutes. The gel was equilibrated in an ethidium bromide bath (Table 9) for 10 mins on a gentle shaker. The stained DNA was visualised using a UV gel transilluminator.

4.1.5 Gel excision and extraction

DNA fragments from restriction digests (section 4.1.2) were extracted from agarose (section 4.1.4) for ligation (section 4.1.3). Separated fragments were excised from agarose gels (section 4.1.4) using a UV transilluminator and a clean scalpel. Gel extraction was performed following the QIAquick Gel Extraction (Quiagen) protocol, with a final elution of 30 μ L.

4.1.6 Site-Directed Mutagenesis

Single or double nucleotide mutations/fragment deletions were introduced into plasmids using the QuickChange Site-Directed Mutagenesis kit (Stratagene) following the manufacturer's protocol. The thermal cycling conditions used were as follow:

- Initial denaturation: 95° for 1 minute
 - Denaturation: 95° for 30 seconds
 - *Annealing: 62° for 1 minute
 - Extension: 68° for 18 minutes
 - Final extension: 68° for 14 minutes
- } 18X cycles

* To calculate the T_m , the following formula was used:

$$T_m = 81.5 + 0.41(\%GC) - 675/N$$

Where N did not include the bases inserted or deleted.

Product were transformed into competent cells (section 4.2.1). The DNA was extracted by small scale DNA preparation (section 4.2.2.1). The corresponding mutations were analysed by restriction digestion (section 4.1.2) and agarose gel (section 4.1.4). The site-specific mutations were verified by sequence analysis using the appropriate primers (Table 6).

4.2 Bacterial techniques

All microbiological techniques were undertake using *Escherichia coli* NM522 or XL-blue.

4.2.1 Plasmid DNA

Strains NM522 or XL-blue Competent cells were transformed with plasmid DNA by heat shock treatment. Either ligation reaction (10 μ L) or intact plasmid DNA (1 μ g) was mixed with 50-100 μ L competent cells. The mixture was incubated on ice for 30 minutes. Cells were then heat shocked for 45 seconds at 42°C in a water bath, before returning to ice for 2 minutes. The transformed cells were incubated with 1000 μ L of LB medium for 1 hour at 37°C and shaken vigorously. The cells were collected by centrifugation at 5500 rpm for 3 minutes, the supernatant was removed leaving 50-100 μ L behind. The pellet was resuspended; the suspension was plated out onto LB agar containing the appropriate antibiotic. The plates were incubated at 37°C overnight.

4.2.2 Plasmid purification

4.2.2.1 Small-scale (Miniprep)

Bacteria, either from a single colony or a glycerol stock were inoculated into 5 mL LB medium, supplemented with the appropriate antibiotic. The suspension was cultivated overnight at 37°C on a rotating wheel. The day after, two millilitres of the bacterial culture were collected by centrifugation at 7000 g at room temperature for 2 minutes. The DNA was extracted using the Qiaprep miniprep kit (Qiagen) following the manufacturer's protocol.

4.2.2.2 Large scale (Maxiprep)

Bacterial culture was prepared using 200 mL of LB medium inoculated either from a single colony or a glycerol stock containing the appropriate antibiotic. The bacteria were incubated overnight at 37°C in a shaking incubator. The day after, the culture was collected by centrifugation at 4500 g for 15 minutes at 4°C. The DNA was extracted using a Nucleobond AX PC500 kit (Machery-Nagel) following the manufacturer's protocol.

4.3 Eukaryotic cell culture

Cell lines were cultivated on 10 cm tissue culture dishes as specified in Table 10.

Table 10. Cell lines used in this study.

CELL LINE	PASSAGE	MEDIUM	TRANSFECTION METHOD
HEK293T (Human embryonic kidney)	Every 3-4 days. Ratio of 1:10.	DMEM low glucose, supplemented with 10% FBS, 100U/mL penicillin and 100µg/mL streptomycin.	Calcium Phosphate (section 4.3.2.1).
HeLa (Human cervical cancer cells)	Every 3-4 days. Ratio of 1:12.	DMEM low glucose, supplemented with 10% FBS, 100U/mL penicillin and 100µg/mL streptomycin.	No transfection needed.
LnCap (Human prostate cancer cells)	Every 3-4 days. Ratio of 1:3.	RPMI-1640, supplemented with 10% FBS, 100U/mL penicillin and 100µg/mL streptomycin.	Lipofectamine (Thermo Fisher's protocol)
MEFs (Mouse embryonic fibroblasts cells)	Every 3-4 days. Ratio of 1:40.	DMEM high glucose, supplemented with 10% FBS, 100U/mL penicillin and 100µg/mL streptomycin.	K2 transfection system (Biontex's protocol).

Cell lines were grown in a humidified incubator, set at 37°C and 7.5% CO₂ for HEK293T and 5% CO₂ for HeLa, LnCap and MEF cells.

4.3.1 Cell starving and mitogens stimulation

Serum starvation consisted of replaced the full medium for starving medium containing 0.5% FCS and 100 U/mL penicillin and 100 µg/mL

streptomycin, the times were variable according to every experiment. The mitogen stimulation condition consisted of replaced the full medium for fresh medium following the addition of 15% FCS and TPA (12-O-Tetradecanoylphorbol-13-acetate) 100 ng mL⁻¹ for 1h before harvesting.

4.3.2 Transient transfection

4.3.2.1 Calcium phosphate

HEK293T cells were transfected using DNA CaPO₄ co-precipitation. The day before transfection, confluent cells were passaged to a ratio of 1x10⁶ cells per plate. The day after, 4 h before the transfection, the medium was replaced with fresh full medium. A total volume of 50 µL solution containing, 10 µg of DNA and dH₂O water was prepared in a 1.5 mL Eppendorf tube. The mixture was transferred into a 5 mL round bottom tube and 500 µL of 0.25 M CaCl₂ were added, the DNA was vortexed for 3 seconds. The mixture was incubated 10 minutes on ice. Using a low speed vortex, 550 µL of 2X HBS were added dropwise, following by a further 20 minutes incubation on ice, before pipetted out evenly onto the plates, mixed, and returned to the incubator overnight. The day after, cells were washed twice with warm DMEM medium and replenished with fresh full or starving medium, depending on the experiment. Approximately 48 hours post-transfection, cells were harvested on ice, using ice cold PBS.

4.4 RNA techniques

4.4.1 Total RNA extraction-TRIzol

Medium was removed and adherent cells were harvested by scraping in 1 mL PBS on a 60mm dish. Cells were collected by centrifugation at 10000 rpm for 1 minute at room temperature. Supernatants were discarded and total RNA was isolated from cells using TRIzol (Sigma-Aldrich) in accordance with the manufacturer's instructions. All RNA was quantified using the Nanodrop 2000c UV/IV Spectrophotometer. RNA quality was first assessed using 260/280 and 260/230 ratios calculated using a Nanodrop 2000c UV/IV Spectrophotometer.

4.4.2 First strand DNA synthesis

First strand cDNA synthesis for taqman reactions were performed in a 20 μ L reaction volume. RNase-free water was first added to 2 μ g total RNA to reach a final volume of 20 μ L, before 3 μ L random primers (0.1 μ g/ μ l stock), 1 μ L 10 mM dNTPs were added and the reaction incubated at 65° for 5 minutes. The reactions were cooled down on ice for 1 minute, after that 4 μ L of 5X First strand buffer (Thermo Fisher), 1 μ L 0.1M DTT, 1 μ L RNase OUT (Thermo Fisher) and 1 μ L superscript III RT (Thermo Fisher) were added (total volume 27 μ L). Reactions were then incubated at 25° for 5 minutes to allow the primer to anneal, 50° for 1 hour for cDNA synthesis, and then 70° for 15 minutes to inactivate the reverse transcriptase. cDNA was stored at -80° until required.

4.4.3 Quantitative real-time polymerase chain reaction (qRT-PCR)

For the standard curve a series of 5 points was constructed for each set of cDNAs by first mixing 5 μ L of each cDNA sample (i.e. 15 μ L for 3 samples). This sample was referred as the “superneat”. The superneat was diluted 1:4 to obtain the “neat” (i.e. 15 μ L superneat + 45 μ L RNase-free water). A serial dilution of the neat was performed with a dilution factor of 4 to give dilutions 1:4 (i.e. 15 μ L neat + 45 μ L RNase-free water), 1:16 (i.e. 15 μ L (1:4) + 45 μ L RNase-free water), 1:64 (i.e. 15 μ L (1:16) + 45 μ L RNase-free water) and 1:256 (i.e. 15 μ L (1:64) + 45 μ L RNase-free water). TaqMan assays were carried out in a 96 well plate format. The total reaction volume per well was 13 μ L, the TaqMan assay mix was prepared as follows:

- 3 μ L diluted sample (1:10)
- 0.375 μ L 10 μ M Forward primer
- 0.375 μ L 10 μ M Reverse primer
- 0.25 μ L 10mM Probe
- 2.5 μ L RNase-free water
- 6.5 μ L TaqMan Fast Universal Master Mix (2X) (Thermo Fisher)

No template control (NTC) containing water instead of cDNA was considered as a control for each target gene and no reverse transcriptase control (NRTC) included a sample from first strand synthesis including no superscript III reverse transcriptase. Master

mix preparation considered samples from the standard curve and controls. PCR master mixes were prepared (minus cDNA), all samples were assayed in triplicate (i.e. 27 wells were considered for Master mix preparation for 3 samples for one gene: 15 wells for the standard curve (5 points were considered) + 6 wells for the controls (NTC and NRTC) + 9 wells (3 wells per sample). Ten μL aliquots were pipetted into 96 well plates, with cDNA subsequently added to the corresponding wells, starting with the standard curve, followed by the controls NTC and NTR, and the samples. The same protocol was repeated in order to analyse more than one gene.

Samples were run on a StepOnePlus thermocycler (Applied Biosystems) on fast (40 minutes), using the cycling conditions:

1. Denaturation: 95° for 20 seconds
2. Annealing: 95° for 1 second
3. Extension: 60° for 20 seconds

Step 2-3 was repeated 50 times.

4.5 Protein and proteomic techniques

4.5.1 Preparation of whole cell extracts (WCE) and quantification of protein

Cells were washed in cold PBS and lysed in 200-500 μ L RIPA buffer (supplemented with 0.1% v/v mammalian phosphatase and protease inhibitor cocktails [Sigma Aldrich]), rotated at 4°C for 30 minutes, and sonicated for 10 seconds at amplitude 25Hz and 60% power output., before being centrifuged at 14,000 rpm for 15 minutes at 4°C. After centrifugation, the supernatant was carefully transferred to fresh tubes. For evaluation of protein concentration, the Bicinchoninic assay (BCA, Pierce) was used following the manufacturer's protocol. OD₅₉₅ was measured on a Multiskan FC Microplate Photometer (Thermo Fisher).

4.5.1.2 Preparation of nuclear extracts

Before harvest, cells were washed twice with ice-cold PBS, followed by 1mL wash with ice-cold PBS containing 1 mM Na₃VO₄ and 5mM NaF. Before lysis, cells were rinsed with 2 mL of ice-cold hypotonic buffer (1X, no-NP-40) (Table 9). Cells were lysed directly in plate with 0.5 mL of ice-cold hypotonic buffer containing 0.2% NP-40 (Table 9). Lysates were transferred into 1.5 mL Eppendorf tube, centrifuged at 14000 rpm for 20 seconds. The supernatants (cytosol) were

transferred into 1.5 mL Eppendorf tubes. The nuclear pellets were resuspended in 150 μ L of high salt buffer (Table 9), followed by gentle rocking for 30 minutes at 4 °C and centrifuged for 20 minutes at 14000 rpm. Supernatants (nucleus) were collected and stored at -80°C. Cytosol samples were supplemented with 120 mM NaCl and 10% glycerol and centrifuged at 140000 rpm for 20 minutes.

4.5.2 SDS-PAGE and Immunoblot (Western blot)

Proteins were separated according to their relative molecular weight using SDS polyacrylamide gel electrophoresis (SDS-PAGE) on gels ranging from 8%-20% (Table 8). Protein samples were prepared by the addition of Laemmli buffer (4X SDS buffer [TABLE 9]), heated at 95°C for 5 minutes using a bench top heating block, loaded onto SDS-PAGE gel alongside 3 μ L BLUEye prestained protein ladder (Sigma Aldrich). Protein samples were subjected to electrophoresis at 90V until the dye front migrated to the resolving gel. Proteins in the resolving gel were resolved at 150V for 90 minutes. For qualitative evaluations of total protein content on gels, coomassie stain (Table 9) was performed for 1 hour, followed by destaining of the gel overnight. For immunoblot, proteins were transferred onto 0.2 μ m PVDF membrane (GE Healthcare) previously wetted with methanol and soaked in transfer buffer (Table 9). Using the Semi-Dry Transfer System (Biorad), the PVDF membranes were placed on top of three layers of 3mm Whatman paper, the gel was placed on top of the PVDF

membrane and three more layers of 3 mm Whitman paper were added on top. Proteins were transferred at 200 mA for 90 minutes.

Membranes were then blocked in immunoblot blocking buffer (Table 8) for 45 minutes, followed by the incubation with the corresponding primary antibodies (1:1000 in blocking buffer) for a minimum of 2 hours at room temperature or overnight at 4°C with gentle rocking. Membranes were then washed 3x for 10 minutes with 1X TBS-Tween (Table 9), and incubated with the secondary antibodies (1:10,000 in blocking buffer) for 1 hour at room temperature with gentle rocking. Membranes were washed with 1X TBS-Tween as described above, before applying the ECL Detection Reagent (GE Healthcare). The image was taken using the LAS-3000 chemiluminescence camera (Fuji-Film).

4.5.3 Immobilised Metal Affinity Chromatography (IMAC)

Cells were washed in cold PBS and 10% of the cells were transferred to a different tube and lysed with 50 µL RIPA buffer (supplemented with 0.1% v/v mammalian phosphatase and protease inhibitor cocktails [Table 9]). The other 90% was lysed in 900 µL lysis buffer/wash buffer 1 (Table 9) on rotator at 4°C for 1 hour, before twice sonicating for 10 seconds at amplitude 25Hz and 80% power output. Lysates were then centrifuged at 14,000 rpm for 15 minutes at 4°C. The supernatant was transferred to a fresh tube. Ni-NTA Agarose

beads (150 μ L per sample-50% slurry (Thermo Fisher) were washed with 1 mL PBS, before being centrifuged at 2000 rpm for 1 minute. The PBS was discarded and a second wash was performed with 1 mL of lysis buffer/wash buffer 1 (Table 9), followed by centrifugation as described above. The lysis buffer was discarded, and the beads were equilibrated with an equal volume of Lysis Buffer/Wash buffer 1 (i.e. 75 μ L beads + 75 μ L Lysis buffer per 150 μ L beads 50% slurry) the beads were placed on rotor at room temperature for 1 hour. Each sample containing 150 μ L of beads/lysis buffer suspension were placed on rotor at room temperature for 2 hours. The beads were then centrifuged as described above, the supernatant was discarded and the beads were washed sequentially for 5 minutes with 1 mL of wash buffer 1 on rotor at room temperature, followed by washes with wash buffer 2-5 (Table 9). Once the final wash buffer was removed, the beads were resuspended in 50 μ L of 2x Laemmli buffer supplemented with 200 mM imidazole, the tubes were heated at 95° for 5 minutes and centrifuged at 14000 rpm for 1 minute prior to SDS-PAGE (section 4.5.2).

4.5.4 Co-immunoprecipitation

HEK293T cells were washed with 5 mL ice-cold PBS, collected in 1 mL cold PBS and transferred to a 1.5 mL Eppendorf tube. The cells were centrifuged at 6000 g x 8 min, the PBS was discarded, and the cells were lysed in 0.5 mL cold co-IP lysis buffer (supplemented with 0.1% v/v mammalian phosphatase and protease inhibitor cocktails

[Table 8]). The tubes were placed on wheel at 4°C for 1 hour. The tubes were then centrifuged at 14000 rpm for 15 minutes. The supernatant was transferred to a fresh Eppendorf tube and 10% was saved for analysis of protein expression (input). Input was then centrifuged at 2500 rpm for 5 minutes and the pellet was resuspended in 50 µL of Laemmli buffer. For the pre-clearing, 20 µL of protein A/G plus-agarose beads (50% slurry) (Santa Cruz Biotechnology) were added and the tubes were rotated for 2 hours at 4°C. The samples were centrifuged at 4°C for 2 minutes at 4000 rpm, the supernatant was saved, and the beads were discarded. Subsequently, the corresponding antibody was added to the supernatant (10 µL). The tubes were rotated at 4°C overnight. The next day, 20 µL of protein A/G plus-agarose were added to each tube and rotated for 2 hours at 4°C. The tubes were centrifuged at 4°C for 2 minutes at 4000 rpm, the beads were collected, and the supernatant was discarded. The beads were washed four times with 1 mL cold lysis buffer (minus protease inhibitors) for 10 minutes at 4°C and centrifuged at 4000 rpm. Each pellet was supplemented with 40 µL of Laemmli buffer and the tubes were heated at 95°C for 5 minutes and centrifuged at 14000 rpm for 1 minute.

4.5.5 ELK-1 BirA partner interaction

Note: This protocol was modified from (Roux et al., 2018).

Grow to confluence HEK293T cells seeded on 10 cm dishes were passaged to a ratio of 1:8. Four dishes of cells for each experimental condition were considered (cells expressing BioID constructs or control cells). The day after, the cells were transfected following the calcium phosphate method (section 4.3.2.1). The next day, the cells were washed twice with DMEM low glucose medium, the medium was then replaced with starving or full medium, followed by the addition of 50 μ M biotin. The cells were incubated for 18-20 hours. After biotin incubation, the cells were washed twice with 5 mL PBS and harvested with 1 mL PBS. The cells were transferred to a fresh Eppendorf tube and centrifuged at 2500 rpm for 5 minutes, the PBS was discarded and 1 mL of PBS was added. 100 μ L per sample were taken and collected in the corresponding tube, the pellet was lysed in 20 μ L Laemmli buffer (Lysates) (Table 9). The 90% was lysed in 540 μ L of Urea lysis buffer (supplemented with 0.1% v/v mammalian phosphatase and protease inhibitor cocktails [Table 9]). The lysed cells were transferred to a 15 mL Falcon tube, and 120 μ L of 20% triton X-100 stock was added to each tube, the samples were vortex before being sonicated two times for 10 seconds at amplitude 25Hz and 80% power output. Pre chilled lysis buffer was added (2.52 mL) (Table 9) and one more sonication session was performed. The samples were evenly aliquoted into 2 mL Eppendorf tubes, followed by centrifugation at 16,500 g for 10 minutes at 4°C. During the

centrifugation, tubes containing 1 mL of Urea Lysis buffer (supplemented with 0.1% v/v mammalian phosphatase and protease inhibitor cocktails [Table 9]) were prepared. The streptavidin was mixed and 50 μ L of streptavidin beads were added to each tube containing the lysis buffer. The equilibrated beads were centrifuged for 2 minutes at 1000 g, the supernatant was removed once the centrifugation of the samples was completed, in order to prevent the beads from becoming dry. After sample centrifugation, 1 mL of the supernatant was transferred to the streptavidin tubes. The samples and beads were gently resuspended and transferred to a 15 mL Falcon tube, followed by incubation on a rotor at 4°C overnight. The next day, the cells were centrifuged for 5 minutes at 1000 g to collect streptavidin beads, the supernatant was discarded. The cells were washed by adding 1 mL of wash buffer (Table 9) to each tube and transferred to 1.5 mL Eppendorf tubes, followed by 8 minutes on a rotor at room temperature. The beads were centrifuged for 2 minutes at 1000 g and the supernatant was removed. The washes were repeated three more times. After last wash, the beads were resuspended with 1 mL wash buffer (Table 9) and 10% of resuspended beads were saved in a fresh tube. The samples were centrifuged (90%) and the supernatant was completely removed, the samples were resuspended in 1 mL of 50 mM Tris-HCl pH 7.4 solution to remove urea. The beads were collected by centrifugation, the supernatant was removed and 80 μ L of 4X SDS buffer was added to each tube (samples destined for mass spectrometry), the samples

were heated at 98°C for 5 minutes. The 10% samples were collected by centrifugation and 20 µL of 4X SDS buffer was added to each tube. The next day, the expression of ELK-1-BirA was assessed by Western blot. 15 µL of the samples and the lysates were loaded into a 10 % SDS-PAGE (section 4.5.2). The membrane was blocked with BSA blocking buffer (Table 9) and blotted against strep-HRP 1:10,000 for 1 hour in BSA blocking buffer. The membrane was washed two times with PBS and agitated in ABS blocking buffer for 5 minutes (Table 9). The membrane was washed three times with PBS and the ECL chemiluminescence was added. Following successful analysis of biotinylated proteins, the membrane was agitated for 20 minutes in 30% H₂O₂ to quench HRP signal. Subsequent immunoblot with anti-ELK-1 was carried out.

Following confirmation of biotinylation in control and expression of BioID fusion protein samples, the cells were prepared for mass spectrometry. Samples were loaded in a 12% polyacrylamide gel. Protein samples were subjected to electrophoresis at 90V, the gel was stopped once the low molecular weight proteins were separated. The gel was then placed in a glass container for subsequent stain with fresh Coomassie (Table 9) for 1 hour with gentle rocking and covered with cling film to avoid any contamination. The gel was destained (Table 9) overnight with gentle rocking and covered with cling film. The day after, the gel was washed twice with ddH₂O. Using a laboratory hood, the gel was placed on a glass plate, the streptavidin band from each sample was cut using a sterile razor blade. The gel

samples were sent for analysis of Mass spectrometry to Cambridge Centre for Proteomics.

4.5.6 Statistical Analysis

qRT-PCR data are expressed as mean $\pm$ SEM. Statistical analyses were performed with Student's *t*-test, using the software graph pad prism 8. Significance is reported in figures by **P* < 0.05, ***P* < 0.01.

5.0 RESULTS

5.1 ELK-1 ubiquitination affects its target genes expression

Previous studies demonstrated that ELK-1 is degraded by the proteasome system (Evans et al., 2011). Recently, it was reported that ELK-1 is mono-ubiquitinated and poly-ubiquitinated in HEK293T cells. ELK-1 mono-ubiquitination within the ETS domain at K35 was suggested to mediate ELK-1 cytoplasmic re-localisation (Ducker et al., 2019). It was also confirmed that ELK-1 mono-ubiquitination has a negative effect on expression of ELK-1 target genes (Ducker et al., 2019). This data indicated that ELK-1 specific E3 ligase activity is present in HEK293T cells. For that reason, I focused my research project on finding the E3 ligase responsible for ELK-1 poly-ubiquitination in HEK293T cells.

The experimental approach used in this project involved cell lysis under denaturing conditions and Immobilized Metal Affinity Chromatography IMAC (section 4.5.3) which rules out associations between ELK-1 and non-covalently bound poly-ubiquitin chains. Purification of His-tagged proteins is mediated by the interaction with Ni_2^+ immobilized on a matrix. The purified pool of ubiquitinated proteins can be analysed for the presence of a particular protein by western blot with specific antibodies (Bornhorst and Falke, 2000). The

main advantage of this strategy is the possibility to prepare the cell lysate in strong denaturing conditions that limit DUBs activities.

This method has been widely used to detect ubiquitination of a particular protein. (Treier et al., 1994, Xirodimas et al., 2001, Kaiser and Tagwerker, 2005).

Figure 17 shows a high molecular weight smear which represents multiubiquitin conjugates of ELK-1 that consist of mixtures of HA-tagged ubiquitin and the endogenous untagged ubiquitin with ubiquitin chains of variable length. This result demonstrates that ELK-1 is ubiquitinated when it is co-transfected with HA-tagged-ubiquitin (HA-UBQ) (Fig. 17, lane 3) which corroborated specific E3 ligase activity of ELK-1 in HEK293T cells. Moreover, it provides evidence that ELK-1 ubiquitination status can be modulated by suppressing the de-ubiquitinating activity of ELK-1 by USP17. This ubiquitination assay did not show bands > 58 kDa which would indicate ELK-1 mono-ubiquitination (Fig. 17).

Functionality of the shRNA in knocking down the expression of USP17 in HEK293T is shown in figure 18.

Figure 17 and 19 confirm the previous findings reported by (Ducker et al., 2019). USP17 depletion increases ELK-1 poly-ubiquitination and negatively modulates ELK-1 target genes.

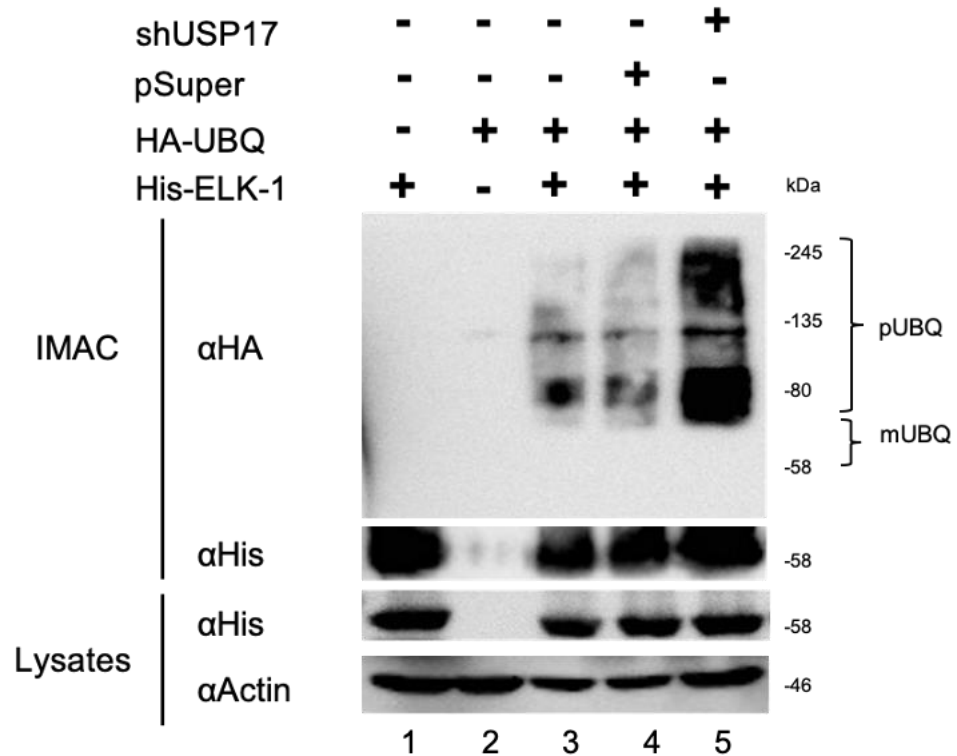


Figure 17. USP17 depletion increases ELK-1 poly-ubiquitination. After transfection, cells were kept on full normal medium. WCE from HEK293 cells transfected with expression vectors for His-ELK-1, HA-UBQ, pSuper and shUSP17#1 were subjected to IMAC. Isolated proteins were analysed by SDS- PAGE (8%) and immunoblotting with the antibodies indicated. Lower panels: WCEs analysed for His expression. The image represents three independent experiments.

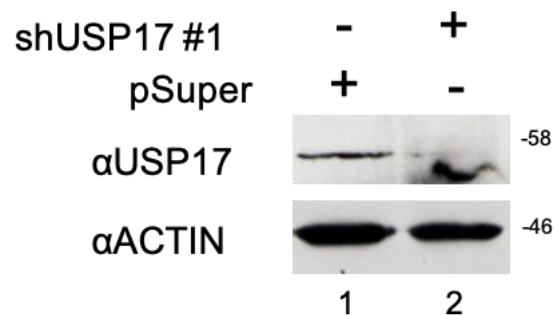


Figure 18. The shUSP17#1 decrease USP17 expression in HEK293T cells. HEK293T cells were transfected with USP17 sh#1 vector or vector control as indicated. USP17 levels were determined by SDS- PAGE (5–20%) and immunoblotting.

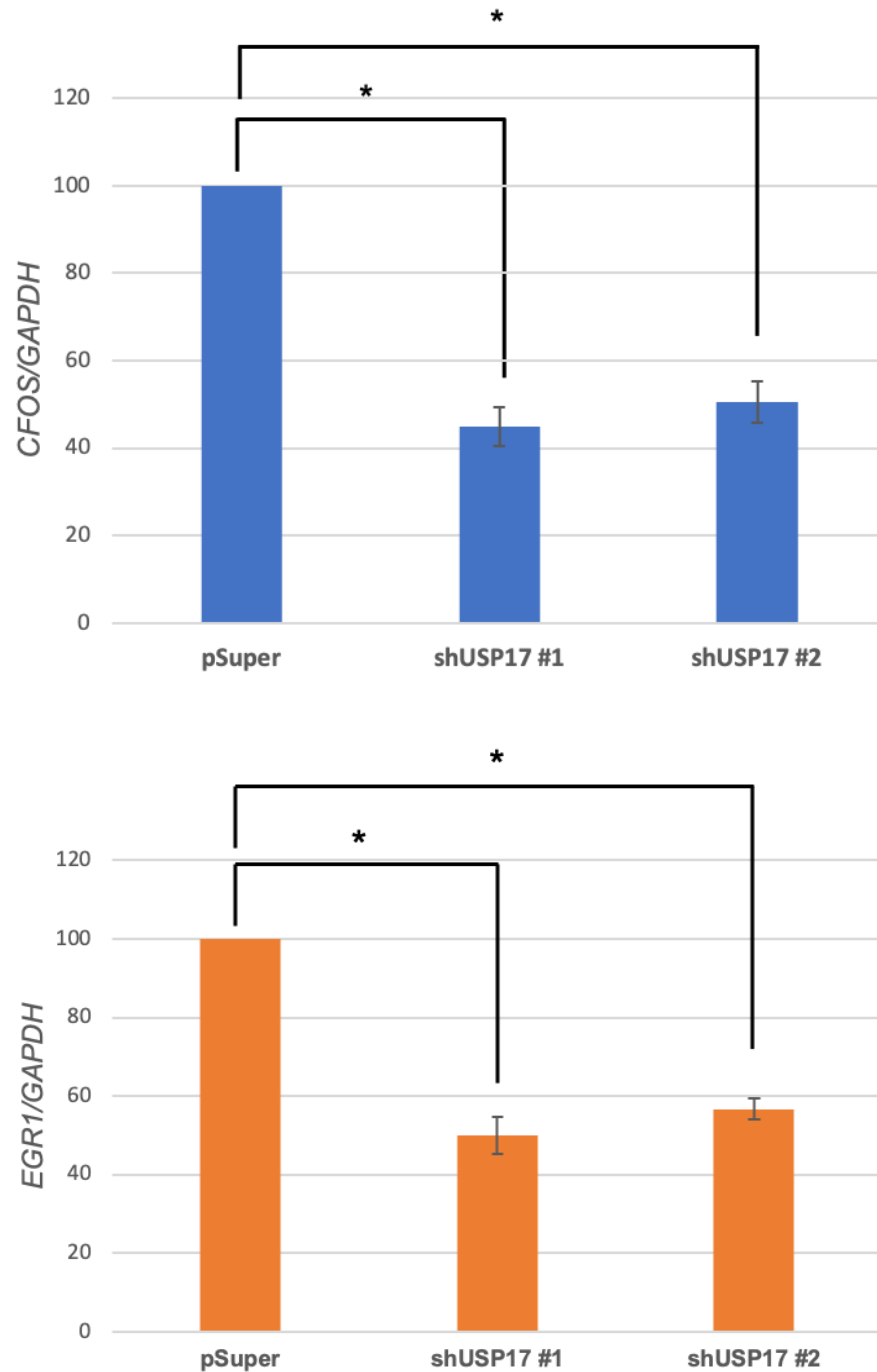


Figure 19. USP17 knockdown has a negative effect on ELK-1 target gene expression. HEK293T cells were transfected with shRNA vectors for USP17 or vector control as indicated. After 48 h, RNA was prepared and *CFOS* and *EGR1* mRNA were analysed by qRT-PCR. Levels are presented as fold ratios to *GAPDH* with controls normalized to 100. N=3. t-test $*P < 0.05$.

5.2 FBXO25 has no effect on ELK-1 ubiquitination

5.2.1 FBXO25 has no effect on ELK-1 poly-ubiquitination in HEK293T cells

In order to find the E3 ligase responsible for ELK-1 poly-ubiquitination, the first reasonable approach was to look in the literature for any publication related to ELK-1 poly-ubiquitination and degradation via the proteasome. In 2013, Teixeira *et al* reported that FBXO25 is the E3 ubiquitin ligase responsible for ELK-1 degradation in HEK293T cells (Teixeira et al., 2013). Unpublished studies in our lab confirmed that ELK-1 interacts with FBXO25 (Franziska Gehringer Master's thesis).

To investigate further the effect of FBXO25 on ELK-1 degradation, I used the Immobilized Metal Affinity Chromatography (IMAC) technique to test for ELK-1 poly-ubiquitination. My results show that ELK-1 is ubiquitinated when it is co-transfected with an HA-UBQ plasmid in HEK293T cells (Fig. 16, lane 3). However, overexpression of FBXO25 did not increase ELK-1 poly-ubiquitination (Fig. 16, lane 4).

Expression of His-ELK-1 by Ni-NTA agarose beads was higher in the IMAC compared to the lysates (Fig. 20), this effect is related to the

high efficiency of the Ni-His pull down and the transfection on HEK293T cells.

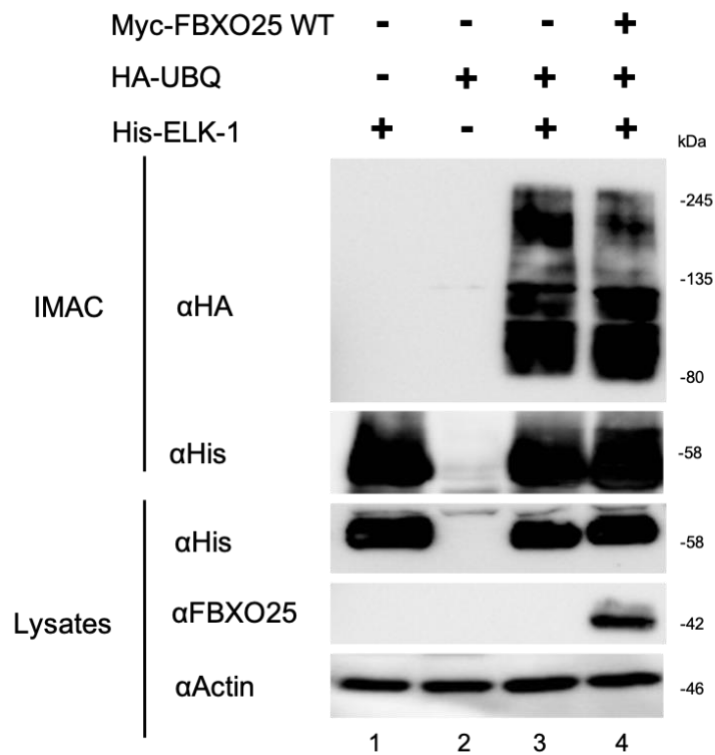


Figure 20. FBXO25 WT has no effect on ELK-1 poly-ubiquitination. After transfection, cells were kept on full normal medium. WCE from HEK293 cells transfected with expression vectors for His-ELK-1, HA-UBQ and FBXO25 WT were subjected to IMAC. Isolated proteins were analysed by SDS- PAGE (8%) and immunoblotting with the antibodies indicated. Lower panels: WCEs analysed for FBXO25 expression. The image represents three independent experiments.

On this basis, it is difficult to confirm that FBXO25 catalyses ELK-1 poly-ubiquitination in HEK293T cells.

5.2.2 FBXO25 mutant does not protect ELK-1 from degradation in HEK293T

It was reported that an Δ F-box mutant version of FBXO25 is not able to assemble with an active SCF1 complex nor able to promote ubiquitination and degradation of its substrates (Hagens et al., 2006). For that reason, I wanted to study the effect of an FBXO25 F-box deletion mutant (amino acids 228–266; Δ F) on ELK-1 poly-ubiquitination. My results show that the FBXO25 Δ F-box mutant did not interfere with ELK-1 poly-ubiquitination (Fig. 21, lane 3) and co-transfection with the FBXO25 WT did not increase ELK-1 poly-ubiquitination (Fig. 21, lane 2). However, functionality of the FBXO25 mutant need to be corroborated.

As previously mentioned, co-transfection of ELK-1 with HA-UBQ showed that ELK-1 is ubiquitinated in HEK293T cells (Fig. 21, lane 1).

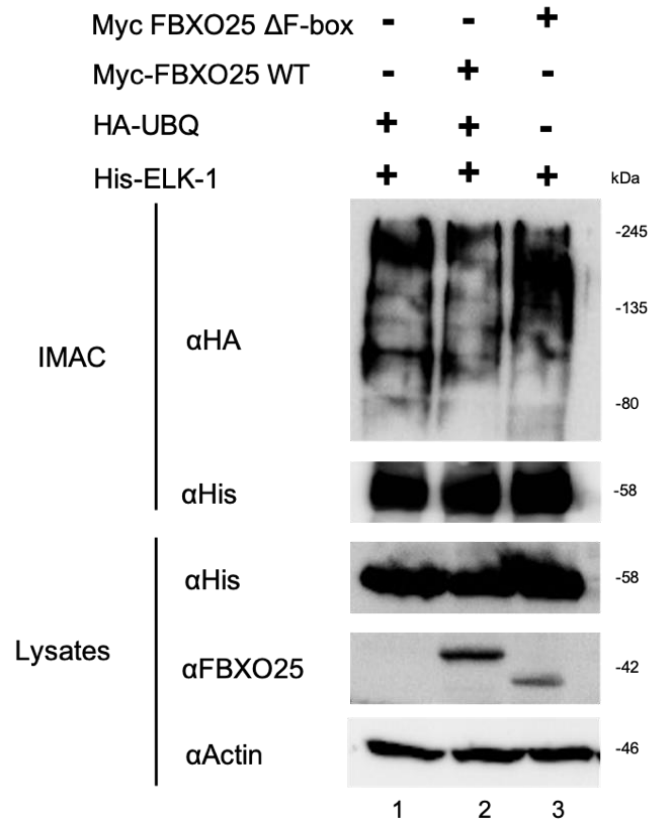


Figure 21. FBXO25 mutant does not protect ELK-1 from degradation. After transfection, cells were kept on full normal medium. WCE from HEK293 cells transfected with expression vectors for His-ELK-1, HA-UBQ, FBXO25 WT and FBXO25 Δ F-box mutant were subjected to IMAC. Isolated proteins were analysed by SDS- PAGE (8%) and immunoblotting with the antibodies indicated. Lower panels: WCEs analysed for FBXO25 expression. The image represents three independent experiments.

5.2.3 FBXO25 depletion does not reduce ELK-1 poly-ubiquitination

Depletion of USP17 was demonstrated to increase ELK-1 poly-ubiquitination in HEK293T cells (Ducker et al., 2019). As a consequence, I decided to investigate the effect of FBXO25 depletion on ELK-1 poly-ubiquitination.

I used shRNA (short hairpin RNA) to deplete FBXO25 levels in HEK293T cells. I checked the functionality of the shRNAs in knocking down the expression of FBXO25 in HEK293T. Figure 22 shows that both shRNAs successfully deplete the expression of FBXO25 in HEK293T.

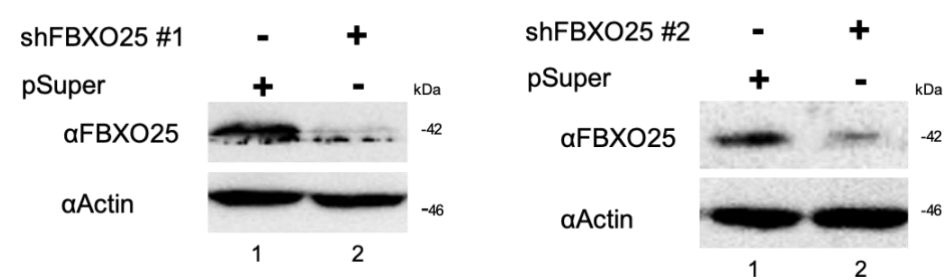


Figure 22. The shRNA#1 and #2 decrease FBXO25 expression in HEK293T cells. HEK293T cells were transfected with FBXO25 sh#1 and sh#2 vectors or vector control as indicated. FBXO25 levels were determined by SDS-PAGE (5–20%) and immunoblotting.

However, my results did not show a decrease in ELK-1 poly-ubiquitination after FBXO25 depletion (Fig. 23, compare lane 4, 5 and 6). Conversely, they show a slight increase in ELK-1 poly-ubiquitination. Furthermore, they suggest that poly-ubiquitination of ELK-1 is more likely to be mediated by a different E3 ligase present in HEK293T cells.

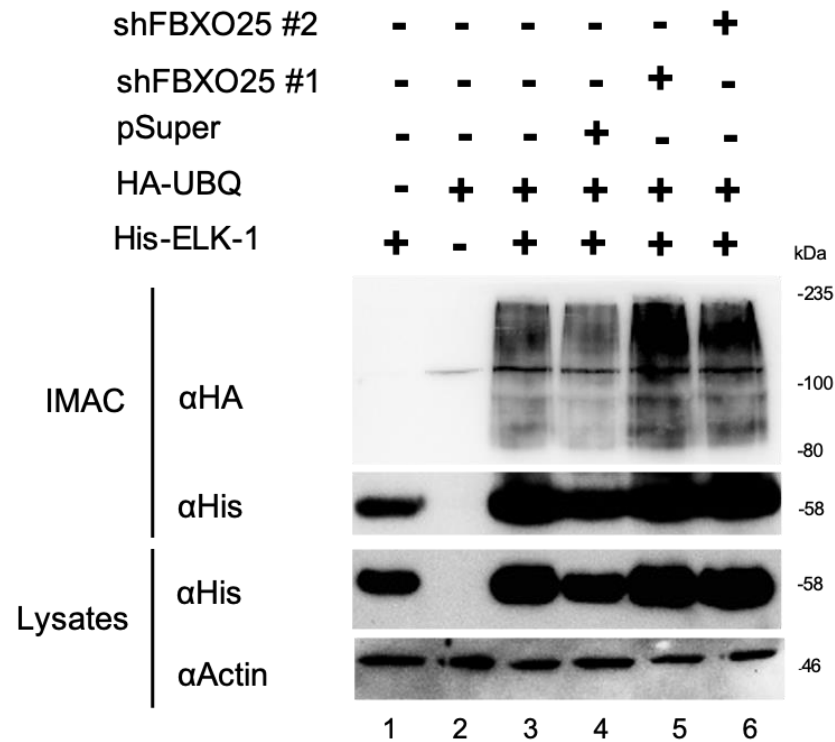


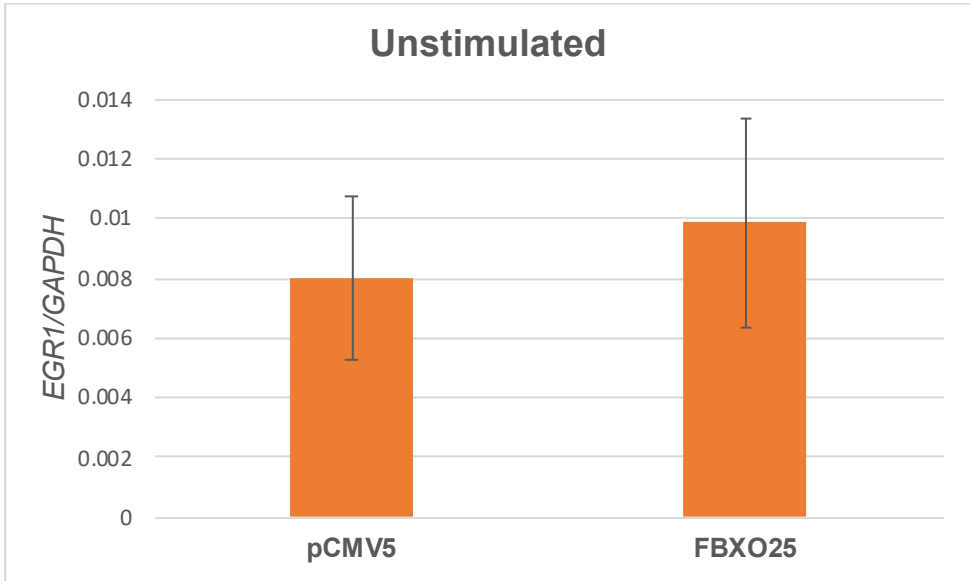
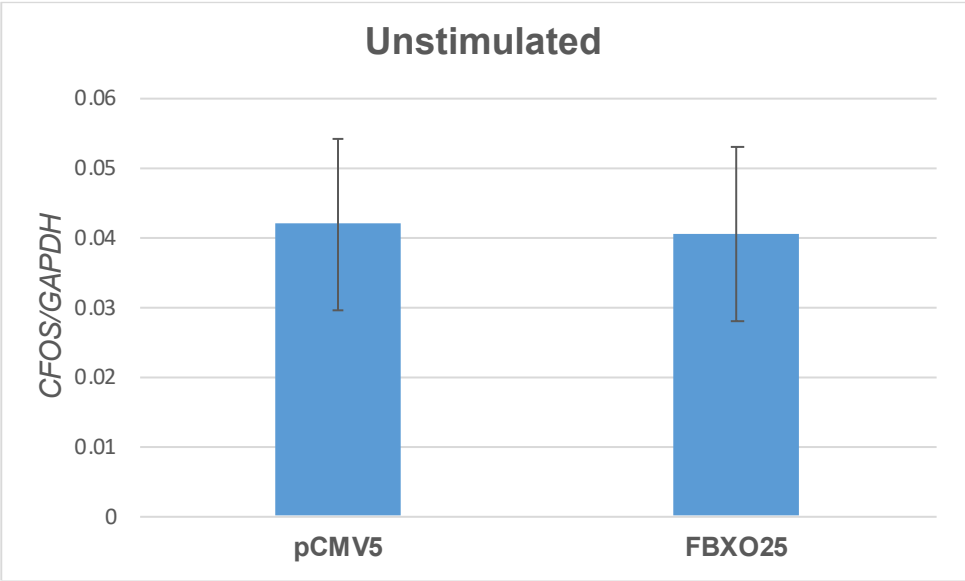
Figure 23. FBXO25 depletion does not decrease ELK-1 poly-ubiquitination. After transfection, cells were kept on full normal medium. WCE from HEK293 cells transfected with expression vectors for His-ELK-1, HA-UBQ, pSuper and FBXO25 sh#1 and sh#2 were subjected to IMAC. Isolated proteins were analysed by SDS- PAGE (8%) and immunoblotting with the antibodies indicated. Lower panels: WCEs analysed for FBXO25 expression. The image represents three independent experiments.

5.2.4 FBXO25 over-expression has no effect on ELK-1 target genes expression

According to Teixeira *et al*, overexpression of FBXO25 decreased *CFOS* and *EGR1* expression in HEK293T cells due to ELK-1 degradation (Teixeira et al., 2013). In an attempt to validate the idea that FBXO25 promotes the ubiquitin-dependent degradation of ELK-1 and modulates ELK-1 target genes expression, I

overexpressed FBXO25 and analysed *CFOS* and *EGR1* expression in unstimulated and mitogen-stimulated cells (Fig. 24).

Contrary to Teixeira *et al*, over-expression of FBXO25 and subsequent stimulation with TPA did not decrease *CFOS* and *EGR1* expression (Fig. 24). Control of transfection is shown in Figure 25.



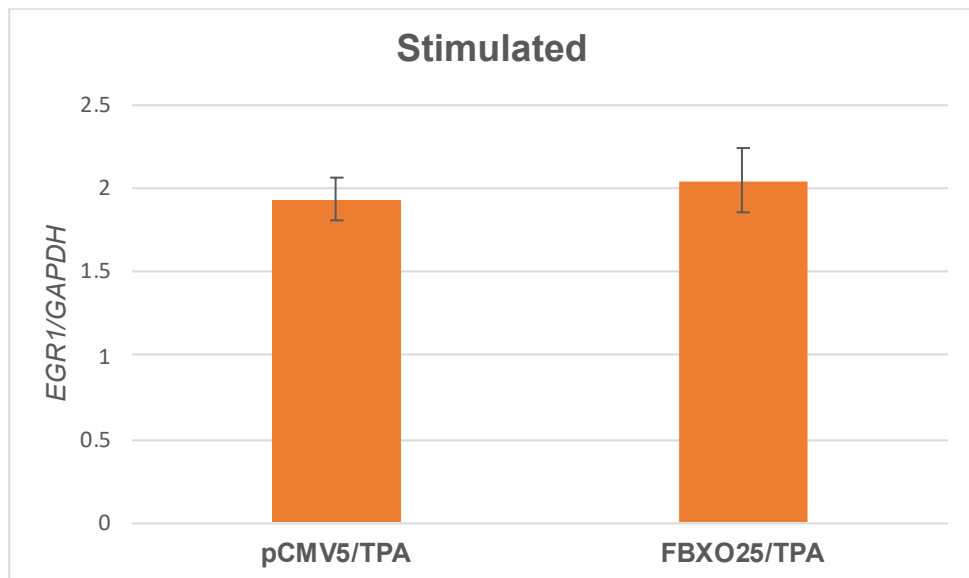
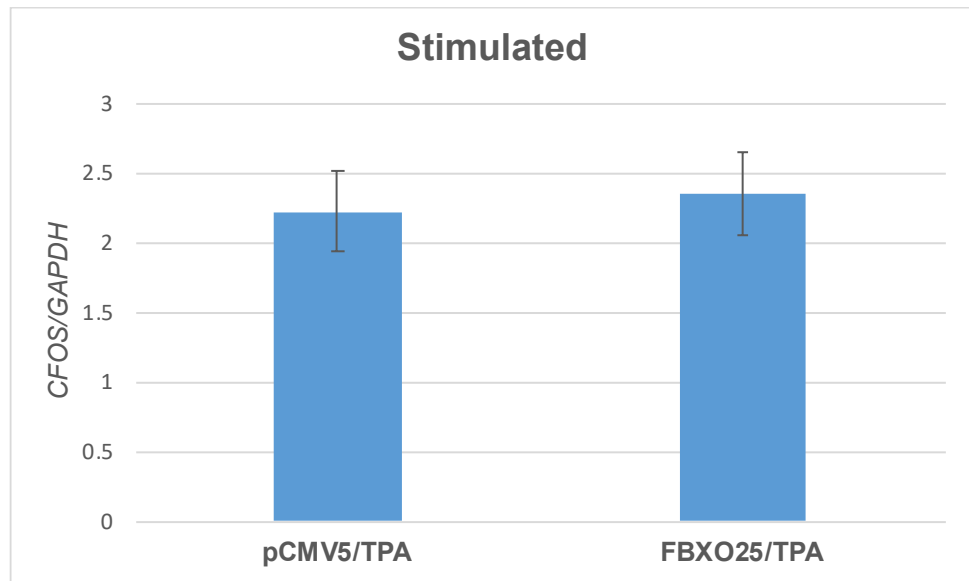


Figure 24. FBXO25 overexpression has no effect on ELK-1 target genes. HEK293T cells were transfected with vectors for FBXO25 or vector control as indicated. After 48 h cells were unstimulated/stimulated with TPA for 1h as indicated. RNA was prepared and *CFOS* and *EGR1* mRNA were analysed by qRT-PCR. Levels are presented as fold ratios to *GAPDH* with controls normalized to 100. N=3 t-test reveals no significance.

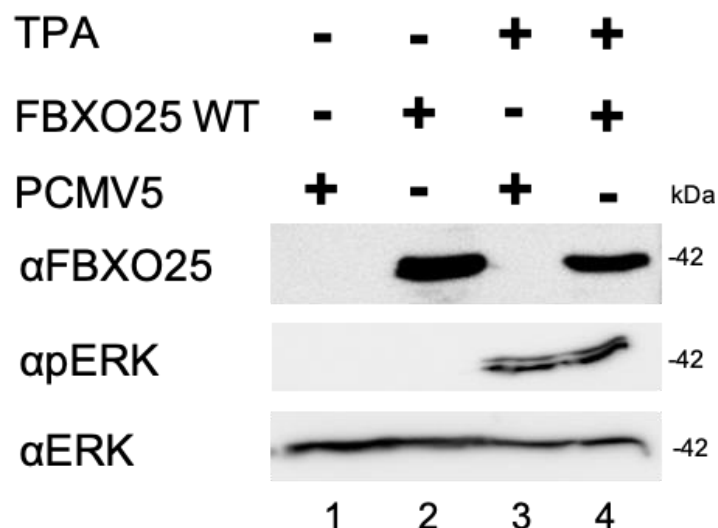


Figure. 25. HEK293T cells were transfected with expression vector for FBXO25 or vector control as indicated. After 48 h cells were untreated or stimulated with tetradecanoylphorbol acetate (TPA) for 1h as indicated. Whole cell extracts were prepared and analysed by SDS-PAGE and immunoblotting for FBXO25 expression and ERK phosphorylation as indicated.

My results suggest that knocking down FBXO25 has no effect on *CFOS* and *EGR1* expression. If ELK-1 is degraded by FBXO25, depletion of FBXO25 would have increased *CFOS* and *EGR1* expression. However, my results show that there is no significant increase on ELK-1 target genes expression after FBXO25 depletion in HEK293T (Fig. 26).

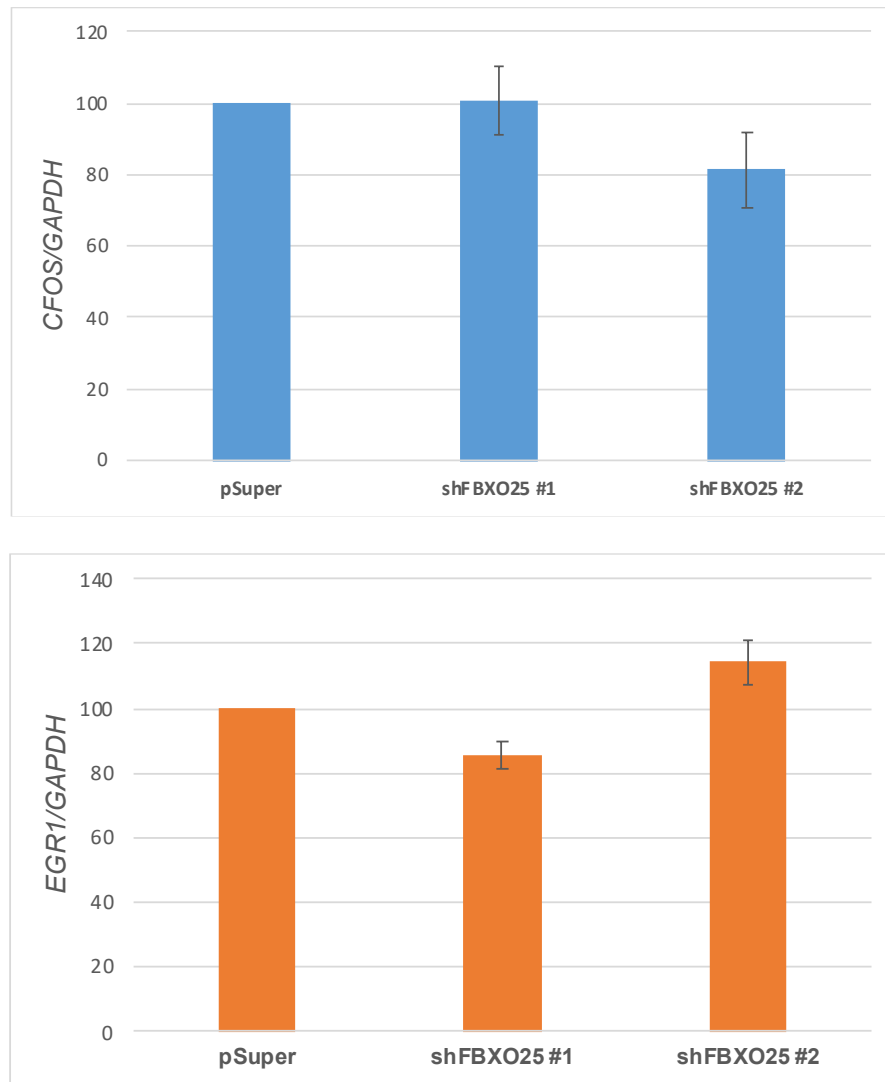


Figure 26. FBXO25 has no effect on ELK-1 target genes. HEK293T cells were transfected with shRNA vectors for FBXO25 or vector control as indicated. After 48 h, RNA was prepared and *CFOS* and *EGR1* mRNA were analysed by qRT-PCR. Levels are presented as fold ratios to *GAPDH* with controls normalized to 100. N=3. t-test reveals no significance.

My results are not in agreement with Teixeira *et al*, and I conclude that neither FBXO25 depletion nor FBXO25 overexpression modulate the expression of two ELK-1 target genes by promoting ELK-1 degradation or ELK-1 stabilisation. My results also suggest that FBXO25 has no effect modulating ELK-1 transactivation, but it might

have an indirect effect on ELK-1 independently of FBXO25 ubiquitin ligase activity (see discussion, section 6).

5.2.5 Is FBXO25 a functional E3 ligase?

As my generated data suggested that FBXO25 is unlikely to be the E3 ligase for ELK-1- conflicting with Teixeira *et al* report, the next logical approach to validate my results was to demonstrate that the FBXO25 plasmid(s) is able to ubiquitinate its target proteins when it is transfected into the cells. More important, it was necessary to demonstrate that the FBXO25 plasmid is able to bind to the SCF complex and it is active as an E3 ligase. Several attempts to demonstrate binding of FBXO25 to endogenous SKP1 in HEK293T cells were performed, however problems with the antibodies made really problematic the detection (data not shown).

FBXO25 has been reported to mediate ubiquitination of transcription factors including Nkx2-5, Tbx5, Isl-1 and Hand-1 (Jang et al., 2011, Jeong et al., 2015). For that reason, I decided to study the effect of FBXO25 on Hand-1. My results show an effect of FBXO25 on Hand-1 degradation. (Fig. 27, compare lane 3 and 4). Conversely, FBXO25 overexpression show a decrease on ELK-1 poly-ubiquitination (Fig. 27, compare lane 1 and 2). In agreement with my results, previous results in the lab demonstrated a greater effect of FBXO25 on Hand-1 degradation (Charles Ducker and Franziska Gehringer PhD and Master's thesis).

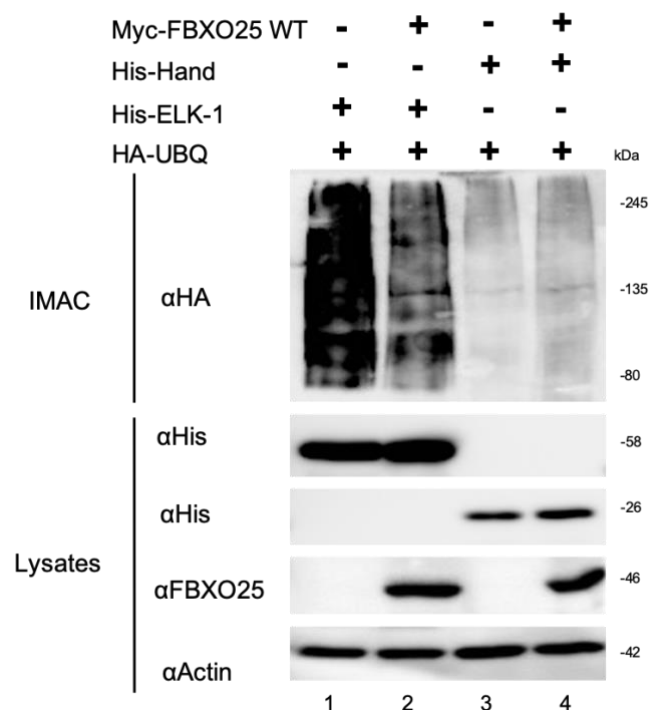


Figure 27. FBXO25 does not promote degradation of HAND-1. After transfection, cells were kept on full normal medium. WCE from HEK293 cells transfected with expression vectors for His-ELK-1, HA-UBQ, His-HAND-1 and FBXO25 WT were subjected to IMAC. Isolated proteins were analysed by SDS- PAGE (8%) and immunoblotting with the antibodies indicated. Lower panels: WCEs analysed for FBXO25 expression. The image represents two independent experiments.

In 2014 it was published that Hax-1 is a *bona fide* substrate of FBXO25, which it targets during apoptotic stress. The experiments described in the paper were performed in Mouse Embryonic Fibroblast cells (MEFs) and used etoposide to promote apoptosis (Baumann et al., 2014).

In an attempt to replicate the conditions published by (Baumann et al., 2014), the experiment was performed using MEFs. My results are in agreement with Baumann *et al*, observing a reduction in Hax-1 levels and controls after 24h etoposide treatment; comparison between

FBXO25 WT overexpression on treated cells vs untreated cells demonstrated a rapid Hax-1 destabilization. Destabilization of Hax-1 was inhibited by the proteasome inhibitor MG132 (Baumann et al., 2014). These results suggest that FBXO25 have an effect on Hax-1 degradation, but not on ELK-1 in MEFs (Fig. 28). However, more experiments such as ubiquitination assays or treatment with MG132 need to be performed in order to confirm that FBXO25 is not promoting ELK-1 degradation via the proteasome pathway in MEFs.

It is important to mention that the transfection conditions used in this experiment (Table 10) were not the same as used by Baumann *et al* (lentiviral transfection), which may have diminished the E3 ligase effect of FBXO25 on Hax-1, and could be the reason why FBXO25 WT overexpression in treated cells is not significant compare to the control (PCMV5). These results indicate that the FBXO25 mutant have an effect on Hax-1 steady state levels which might not involve the SCF complex (Fig. 28) (discussion section 6). However, it s still necessary to validate the functionality of the FBXO25 mutant.

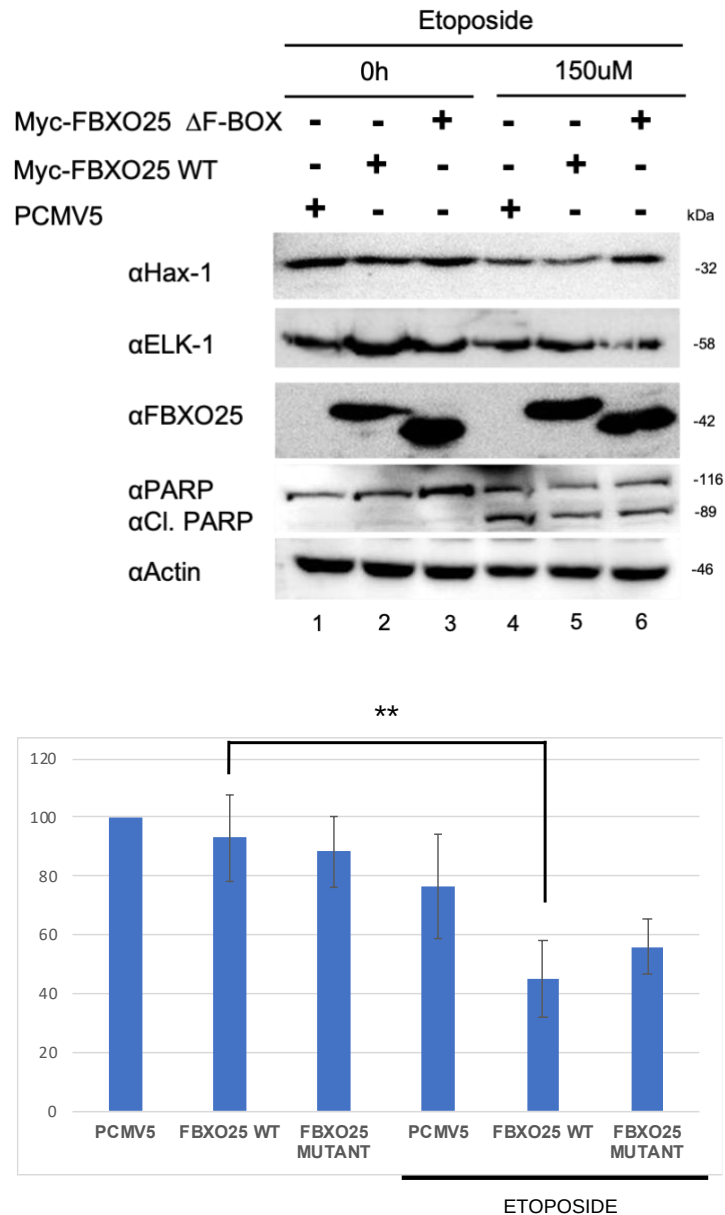


Figure 28. FBXO25 WT decreases Hax-1 levels in MEFs. Top) MEFs were transfected with vectors for FBXO25 WT and FBXO25 Δ F-box mutant and control as indicated. 48h post-transfection the cells were treated with etoposide for 24h as indicated. Hax-1, ELK-1 and FBXO25 levels were determined by SDS-PAGE (10%) and immunoblotting. Below) Quantification of Hax-1 protein expression levels. N=3 t-test ** $P < 0.01$.

Together, this data is consistent with the earlier report (Baumann et al., 2014) about FBXO25 able to promote degradation of Hax-1 in MEFs. However, with these experiments is difficult to conclude that FBXO25 does not have an effect on ELK-1 degradation in MEFs. Nevertheless, my results with Hand-1 corroborated that the FBXO25 WT vector is functional and it is unlikely to have an effect on ELK-1 degradation in HEK293T cells.

5.3. The effect of the E3 ubiquitin ligase SPOP on ELK-1

Based on reports of the E3 ligase SPOP targeting substrates which are principally regulators of cellular proliferation and apoptosis (Gan et al., 2015, Gao et al., 2015, Zhi et al., 2016, Zhang et al., 2017), I decided to investigate if SPOP has an effect on ELK-1 ubiquitination. ELK-1 has one potential SPOP binding consensus (SBC) motif between the A and B domains (100-110 residues) which could serve as a recognition site for the SPOP-MATH domain (Fig. 29) (Zhuang et al., 2009, Mani, 2014).

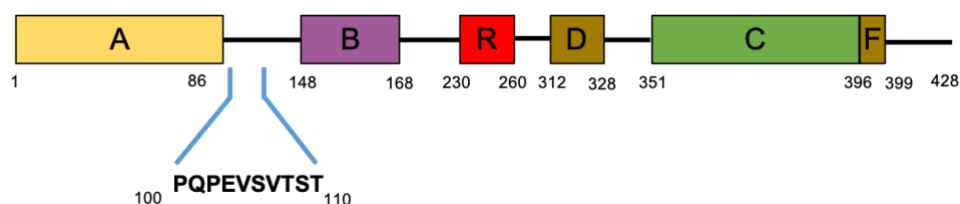


Figure 29. Schematic representation of ELK-1 SBC motif (black letters). SPOP-binding consensus (SBC) motif ϕ - π -S-S/T-S/T (ϕ = nonpolar, π = polar) (Zhuang et al., 2009, Mani, 2014)

5.3.1 SPOP interacts with ELK-1

In order to investigate if SPOP can interact with ELK-1, I performed co-immunoprecipitation assays using HEK293T cells co-transfected with ELK-1 and Myc-SPOP. My results show that SPOP co-immunoprecipitated with ELK-1 (Fig. 30A, lane 4). To confirm the result, I performed reverse co-immunoprecipitations using HEK293T cells transfected with the same plasmids (Fig. 30B, lane 4). In both assays, I observed an association between Myc-SPOP and ELK-1. Thus, my results indicate that SPOP and ELK-1 may be protein partners.

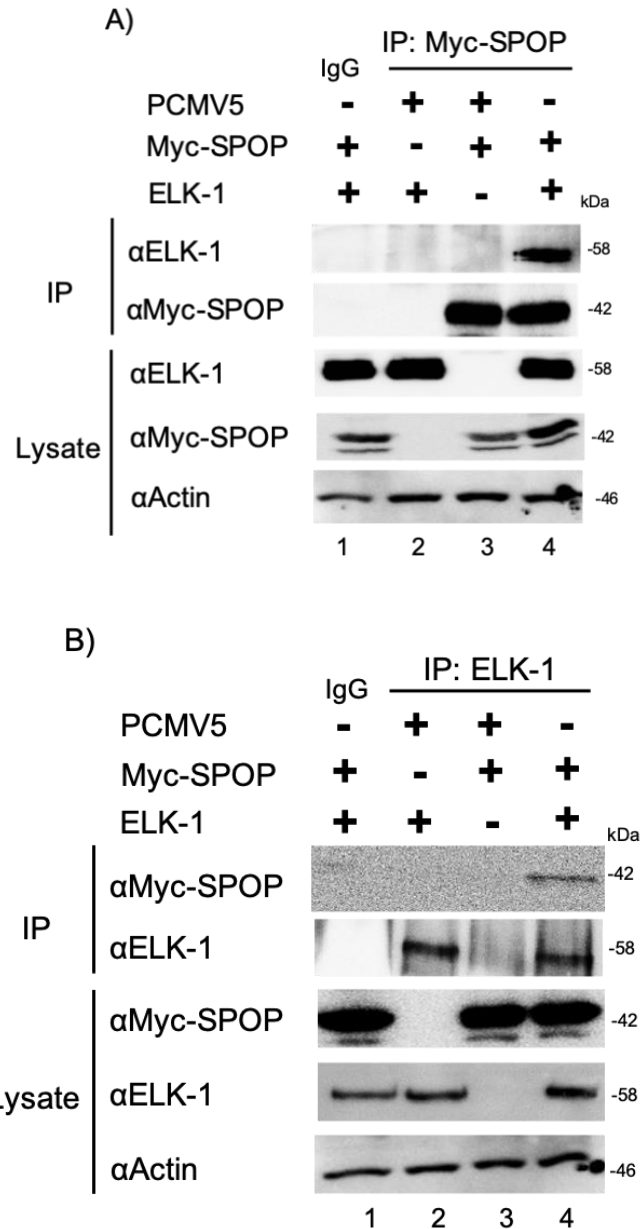


Figure 30. SPOP interacts with ELK-1. A) WCEs were prepared from HEK293T cells transfected with vectors for ELK-1 and Myc-SPOP. Cells were treated with MG132 20μM for 6 h prior to harvest. Immunoprecipitates were collected with control IgG, or αMyc antibody and analysed by SDS-PAGE (8%) and immunoblotting as indicated. (B) As in (A) except that immunoprecipitates were collected with control IgG, or αELK-1 antibody.

5.3.2 SPOP mutants disrupt the interaction with ELK-1

It was reported that SPOP interacts with its substrates through an SBC motif. Point mutations in the SPOP-MATH domain can completely disrupt the interaction of SPOP with its substrates, specifically mutations F133V and D130A (Zhuang et al., 2009).

The next approach was to test with co-immunoprecipitation assays if the mutations F133V and D130A are able to interrupt the interaction that I observed between SPOP and ELK-1 (Fig. 31).

I co-immunoprecipitated Myc-SPOP and evaluated the ability of ELK-1 to bind to the SPOP mutants. My results show that only SPOP WT was able to interact with ELK-1, whereas ELK-1 interaction with the SPOP mutants were disrupted (Fig. 31). This result suggests that the interaction seen between ELK-1 and SPOP involves the MATH domain.

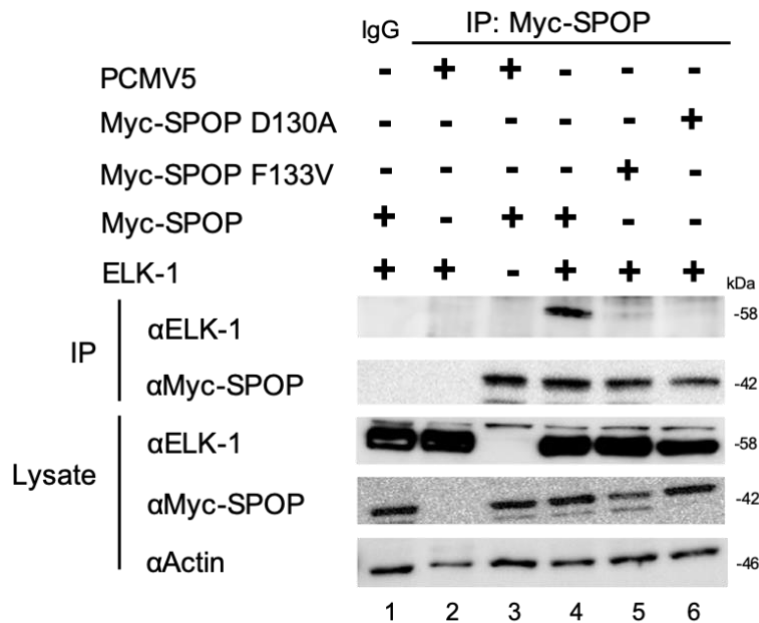


Figure 31. SPOP mutants disrupt the interaction with ELK-1. WCEs were prepared from HEK293T cells transfected with vectors for ELK-1, Myc-SPOP, SPOP F133V, SPOP D130A and SPOP M233E as indicated. Cells were treated with MG132 20μM for 8 h prior to harvest. Immunoprecipitates were collected with control IgG, or αMyc antibody and analysed by SDS-PAGE (8%) and immunoblotting as indicated.

5.3.3 The interaction SPOP-ELK-1 is not disrupted by ELK-1 mutant

My results demonstrated that ELK-1 was able to co-IP with Myc-SPOP WT. As a result, I wanted to investigate if a mutant version of ELK-1 with a deletion of the amino acids where the SBC is located (ELK-1 Δ94-110) can still interact with SPOP. My results indicate that there is a slight decrease on SPOP-ELK-1 interaction, but it is not completely disrupted using the ELK-1 mutant (Fig. 32, compare lane 4 and 5). This result highlights the idea that the interaction

SPOP-ELK-1 might not be a direct interaction and/or that the interaction SPOP-ELK-1 involves a second binding domain within ELK-1 (see discussion, section 6).

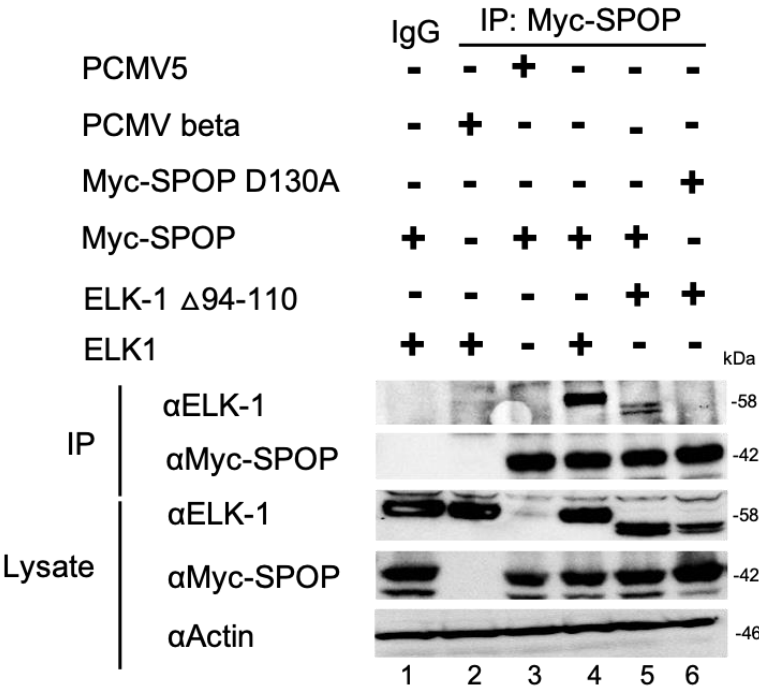


Figure. 32. ELK-1 mutant does not disrupt the interaction with SPOP. WCEs were prepared from HEK293T cells transfected with vectors for ELK-1, Myc-SPOP, SPOP D130A, ELK-1 Δ94-110 and controls as indicated. Cells were treated with MG132 20μM for 8 h prior to harvest. Immunoprecipitates were collected with control IgG, or αMyc antibody and analysed by SDS-PAGE (8%) and immunoblotting as indicated.

5.3.4 SPOP expression decreases the protein levels of ELK-1

I started to investigate if SPOP can decrease the protein levels of ELK-1 by overexpressing ELK-1 and SPOP (Fig. 33). I observed that the protein levels of ELK-1 decreased after overexpression of SPOP (Fig. 33, lane 2). In order to investigate if SPOP was promoting degradation of ELK-1 via the proteasome pathway, HEK293T cells were treated with the proteasome inhibitor MG132. Figure 29 shows a small increase in ELK-1 protein levels after the treatment with MG132 compare to untreated cells (Fig. 33, compare lanes 2 and 3).

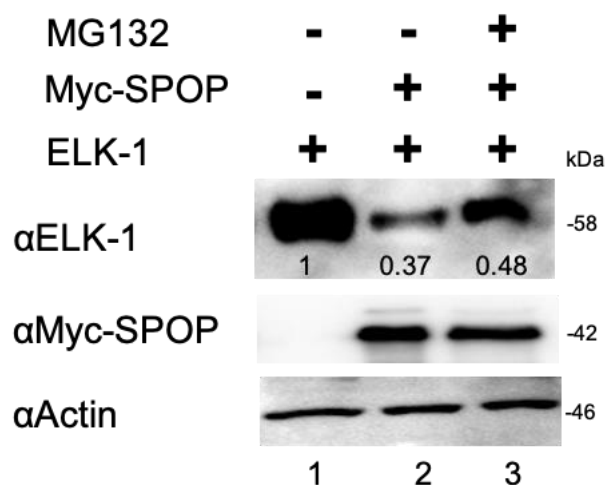


Figure 33. SPOP expression decreases the protein levels of ELK-1. HEK293T cells were transfected with vectors for Myc-SPOP and ELK-1 as indicated. Where indicated cells were treated with MG132 20 μ M for 6 h prior to harvest. ELK-1 and Myc-SPOP levels were determined by SDS-PAGE (8%) and immunoblotting. The densitometric values are shown below the ELK-1 gel.

5.3.5 SPOP mutants do not have any effect on ELK-1 degradation

Within the SPOP-BTB domain, it was reported that M233 makes the single largest contribution to the SPOP-BTB/CUL3-NTD interaction and the M233E substitution disrupts CUL3-NTD binding (Errington et al., 2012). As a result, I made a M233E SPOP clone, to study the effect of the SPOP mutants (F133V, D130A and M233E) on ELK-1 degradation. Figure 34 shows a reduction in the levels of ELK-1 when Myc-SPOP WT was overexpressed but an even greater decrease in the level of ELK-1 with the Myc-SPOP mutants (M233E, D130A and F133V).

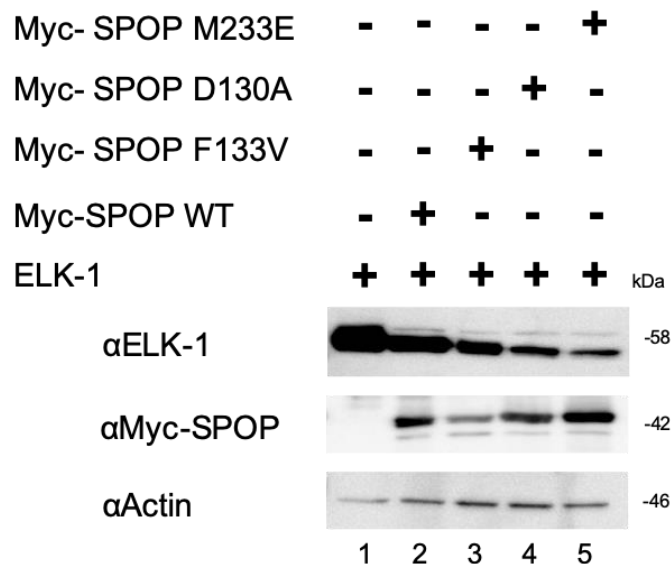


Figure 34. SPOP mutants do not have any effect on ELK-1 degradation. HEK293T cells were transfected with vectors for ELK-1, Myc-SPOP WT, SPOP F133V, SPOP D130A and SPOP M233E as indicated. ELK-1 and Myc-SPOP levels were determined by SDS-PAGE (8%) and immunoblotting.

This result did not confirm the evidence published regarding SPOP mutants and their effect on their substrates (Zhang et al., 2015). Moreover, figure 34 suggest that SPOP might be regulating ELK-1 indirectly by inducing degradation of an ELK-1 destabilising protein (see discussion, section 6). On the other hand, more experiments including controls for transfection using the PCMV beta plasmid are necessary in order to conclude that this effect is not related to a transfection problem (see discussion, section 6).

5.3.6 SPOP does not promote ELK-1 poly-ubiquitination

In order to demonstrate if SPOP can ubiquitinate ELK-1 and promote its degradation, I performed ubiquitination assays. My results indicate that SPOP WT does not increase ELK-1 poly-ubiquitination (Fig. 35, compare lanes 1 and 2,). Moreover, my results show that SPOP mutants have no effect on ELK-1 poly-ubiquitination (Fig.35).

To conclude, my results demonstrated that ELK-1 can interact with different E3 ligases but neither FBXO25 nor SPOP appears to promote ELK-1 poly-ubiquitination and degradation.

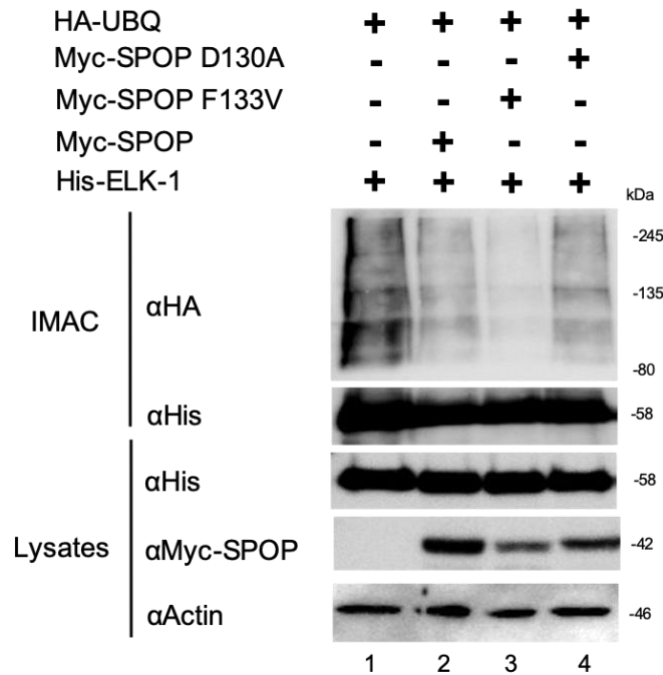
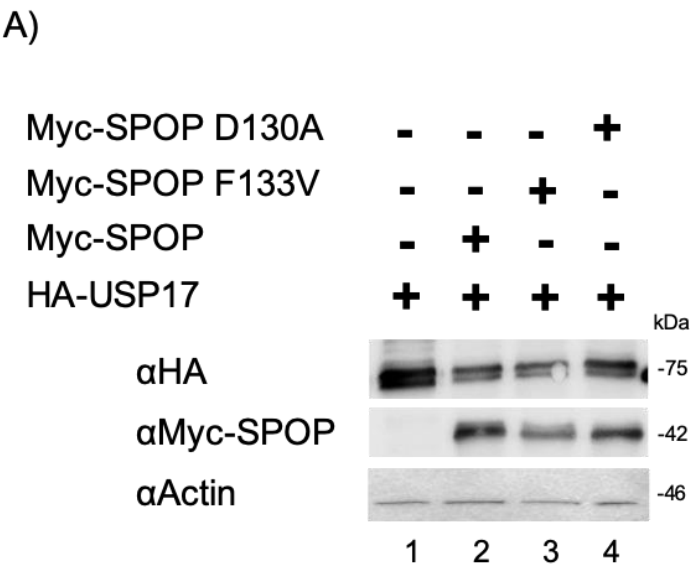


Figure 35. SPOP WT does not promote ELK-1 degradation. After transfection, cells were kept on full normal medium. WCE from HEK293 cells transfected with expression vectors for His-ELK-1, HA-UBQ, SPOP WT, SPOP F133V and SPOP D130A were subjected to IMAC. Isolated proteins were analysed by SDS- PAGE (8%) and immunoblotting with the antibodies indicated. Lower panels: WCEs analysed for SPOP expression.

5.3.7 The SBC is not a guarantee for SPOP recognition

I observed that the de-ubiquitinating enzyme USP17 has a perfect SBC motif located C terminus (amino acids 489-493). In order to test the hypothesis of the SBC (SPOP binding consensus) motif as prerequisite for SPOP recognition and degradation, I performed immunoprecipitation experiments and demonstrated that SPOP and USP17 do not co-immunoprecipitate, despite the SBC motif located in USP17 (data not shown).

My results show that the expression levels of USP17 decrease when co-transfected with SPOP WT or mutants (Fig. 36A). This effect was not reversed by the treatment with MG132 (Fig. 36B). To conclude, my results show that the SBC motif is not a guarantee for SPOP recognition and degradation. Moreover, I infer from my data that Myc-SPOP ectopic expression in HEK293T cells cannot be used as a method to study the E3 ligase activity of SPOP. However, controls for transfection are needed before ruling out any effect that SPOP might have on USP17.



B)

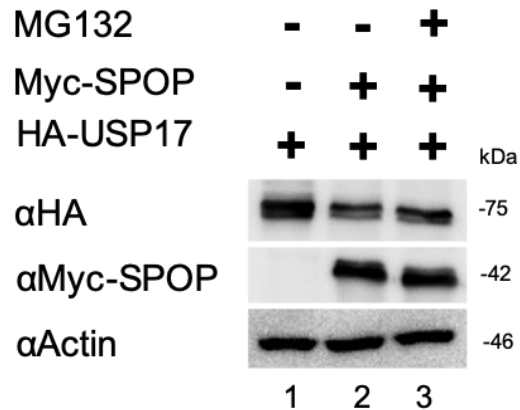


Figure 36. SPOP mutants do not have any effect on USP17 degradation. A-B) HEK293T cells were transfected with vectors for Myc-SPOP, SPOP D130A, SPOP F133V and HA-USP17 as indicated. Where indicated cells were treated with MG132 20 μ M for 6 h prior to harvest. HA-USP17 and Myc-SPOP levels were determined by SDS-PAGE (8%) and immunoblotting.

5.4 MED13 poly-ubiquitination regulates

ELK-1 target genes expression

5.4.1 FBXW7 depletion negatively regulates ELK-1 target genes expression

The mechanism that regulates ELK-1 interaction with the CDK8 complex is still unclear; there is evidence of CDK8 being a positive regulator of transcriptional elongation within the serum response genes (Donner et al., 2010). In addition, it was reported that the E3

ligase FBXW7 targets MED13 for degradation (Davis et al., 2013). Together, these reports indicate that FBXW7 might regulate ELK-1 target genes expression through MED13. For that reason, I made different shRNAs to knock down the expression of FBXW7 and MED13. Successful depletion of FBXW7 was achieved with the shRNA #1 and #2, however, I observed a greatest effect with the shRNA #2 in HEK293T cells (Fig. 37).

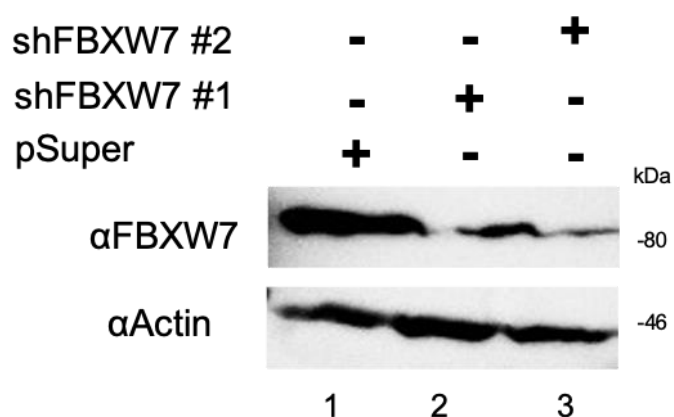


Figure 37. The shFBXW7#1 and shFBXW7#2 decrease FBXW7 levels in HEK293T cells. HEK293T cells were transfected with FBXW7 sh#1 and sh#2 vectors or vector control as indicated. FBXW7 levels were determined by SDS-PAGE (8%) and immunoblotting.

My results show that depletion of FBXW7 decrease *CFOS* and *EGR1* expression (Fig. 38). Therefore, they suggest that depletion of FBXW7 possibly enhance MED13 association with the mediator by avoiding its proteasome degradation mediated by FBXW7; this effect negatively influences ELK-1 target genes expression.

Additionally, my results suggest that ELK-1 target genes expression can be indirectly influenced by knocking down the expression of an E3 ligase.

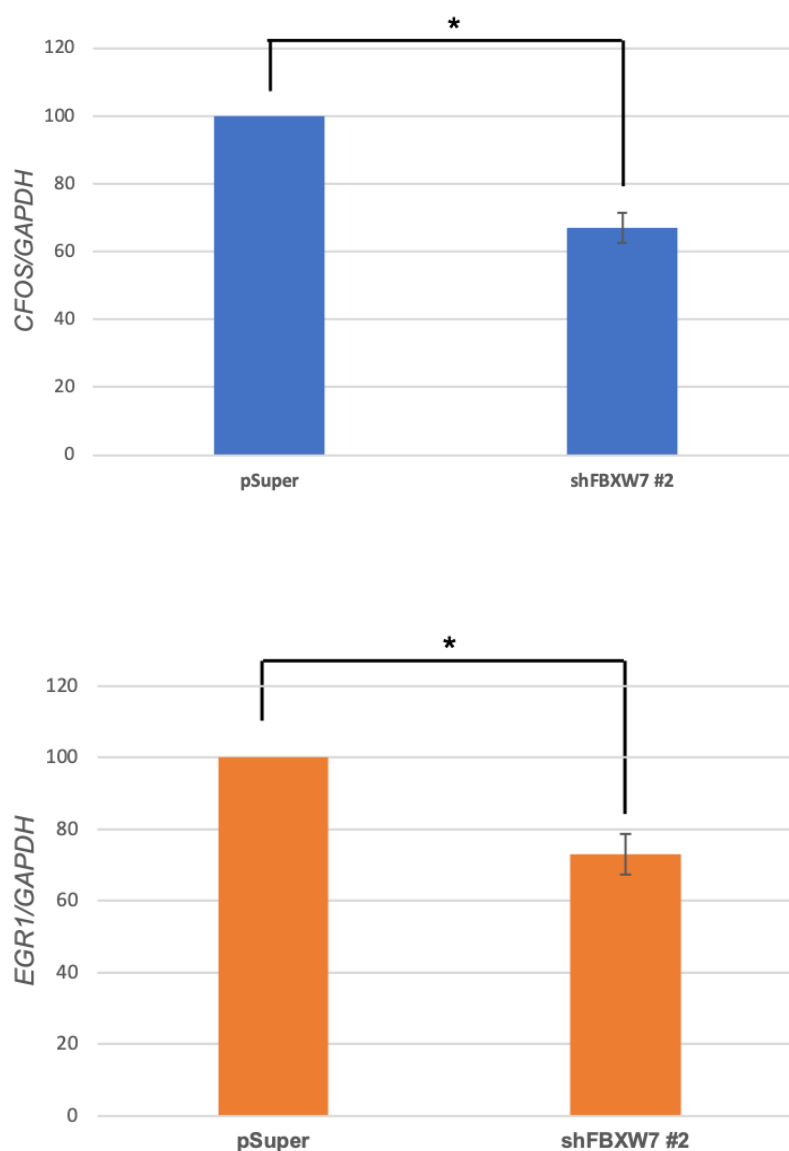


Figure 38. FBXW7 knockdown has a negative effect on ELK-1 target gene expression. HEK293T cells were transfected with shRNA vectors for FBXW7 or vector control as indicated. After 48 h, RNA was prepared and *CFOS* and *EGR1* mRNA were analysed by qRT-PCR. Levels are presented as fold ratios to *GAPDH* with controls normalized to 100. N=3 t-test * $P < 0.05$

5.4.2 ELK-1 target genes regulation by MED13

To investigate further the effect of MED13 on ELK-1, it was necessary to determinate if MED13 depletion has an effect on *CFOS* and *EGR1*.

Depletion of MED13 was achieved in HEK293T cells (Fig. 39).

My results show an increase in *CFOS* expression after 60 min of TPA stimulation, an effect which could not be demonstrated with *EGR1* (Fig. 40). These results are in partial agreement with the hypothesis that MED13 depletion activates ELK-1 target genes expression and corroborate the theory that MED13 poly-ubiquitination mediated by FBXW7 influence the levels of ELK-1 target genes.

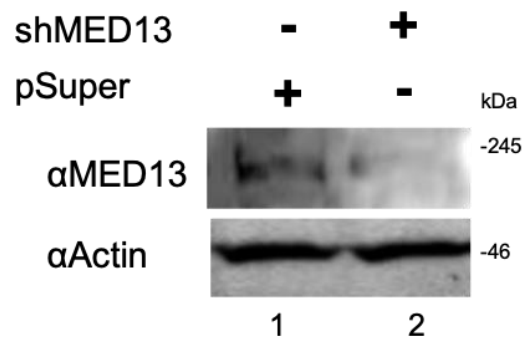


Figure 39. shMED13 decrease MED13 expression in HEK293T cells. HEK293T cells were transfected with shRNA vector for MED13 or vector control as indicated. MED13 levels were determined by SDS-PAGE (6%) and immunoblotting.

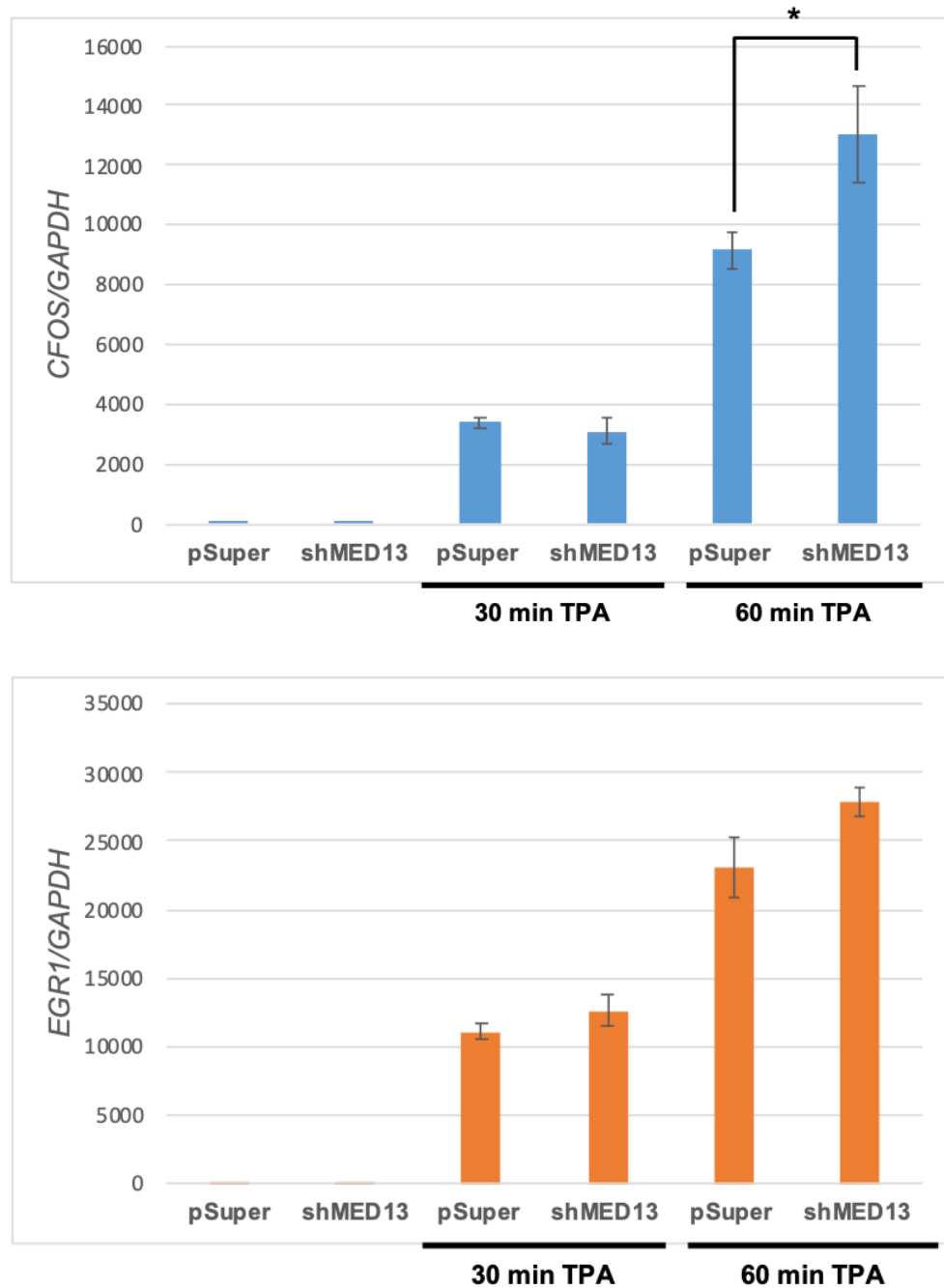


Figure 40. MED13 knockdown has a positive effect on ELK-1 target gene expression. HEK293T cells were transfected with shRNA vectors for MED13 or vector control as indicated. Prior to harvest, cells were stimulated with TPA for times indicated. RNA was prepared and *CFOS* and *EGR1* mRNA were analysed by qRT-PCR. Levels are presented as fold ratios to *GAPDH* with controls normalized to 100. N=3 t- test * $P < 0.05$.

5.5 ELK-1 *in silico* analysis in prostate cancer cells

To complement the experiments described above, I performed an *in silico* analysis with the aim of identifying novel candidate E3 ligases responsible for ELK-1 stabilization in Prostate Cancer (PC). Using the data base GEO (Gene Expression Omnibus), I searched for microarray data for PC biopsies hybridised with the microarray HG_U133 Plus 2.0 under the following conditions:

- Normal Prostate Tissue- No treatment
 - Prostate gland
 - Prostate Stroma
- Prostate Cancer tissue
 - Gleason grade: low, medium and high
- Prostate Cancer Cell Line
 - LNCAP

A total of 104 samples (microarrays) were found: Normal prostate tissue (31) Prostate cancer (49), LnCap (24).

5.5.1 Normalisation

Using Partek Genomic Suite, the samples were normalised using the RMA (mas) algorithm; it includes three steps (background correction, quantile normalisation and summarisation). Figure 41 shows a median intensity of 5.6 (log2). Samples with different values were not considered.

A total of 43 samples passed the quality controls. High grade PC (10), LnCap (23) and Normal gland (4), Normal Stroma (6) (sample information, Appendix 11).

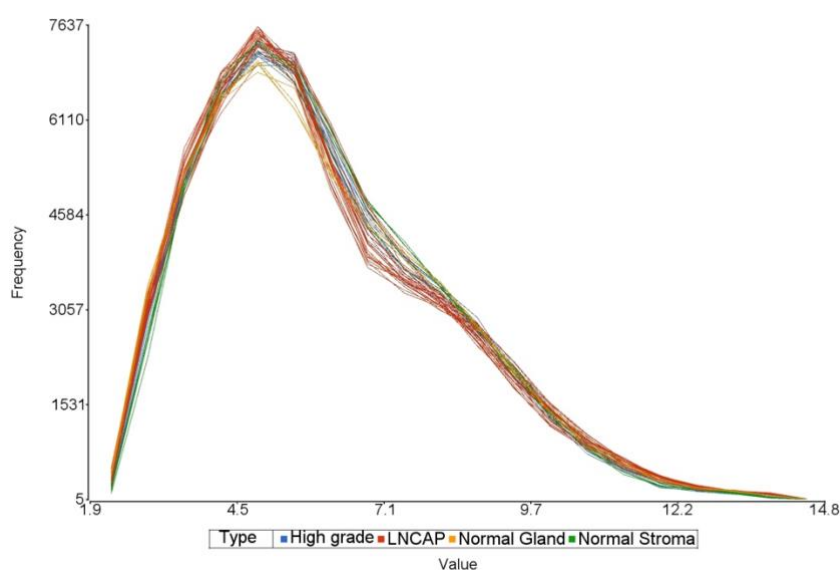


Figure 41. Frequency histogram following RMA analysis. The results are plotted as a frequency of signal vs log of the signal. In logarithmic scale (log2) values oscillate approximately between 2 for minor intensities and 14 for major intensities.

The principal components analysis (PCA) visualise similarities and differences between the samples in a data set. Figure 42 shows that after normalisation, the samples separated based on the category applied; there is a noticeable separation within category groups.

As a control group, normal gland and normal stroma were used, which are greatly separated into two groups (green and yellow spheres, fig. 42). The LnCap group shows more distribution among each sample, however, there are variations because the hybridisation method varies among laboratories (red sphere, fig. 39).

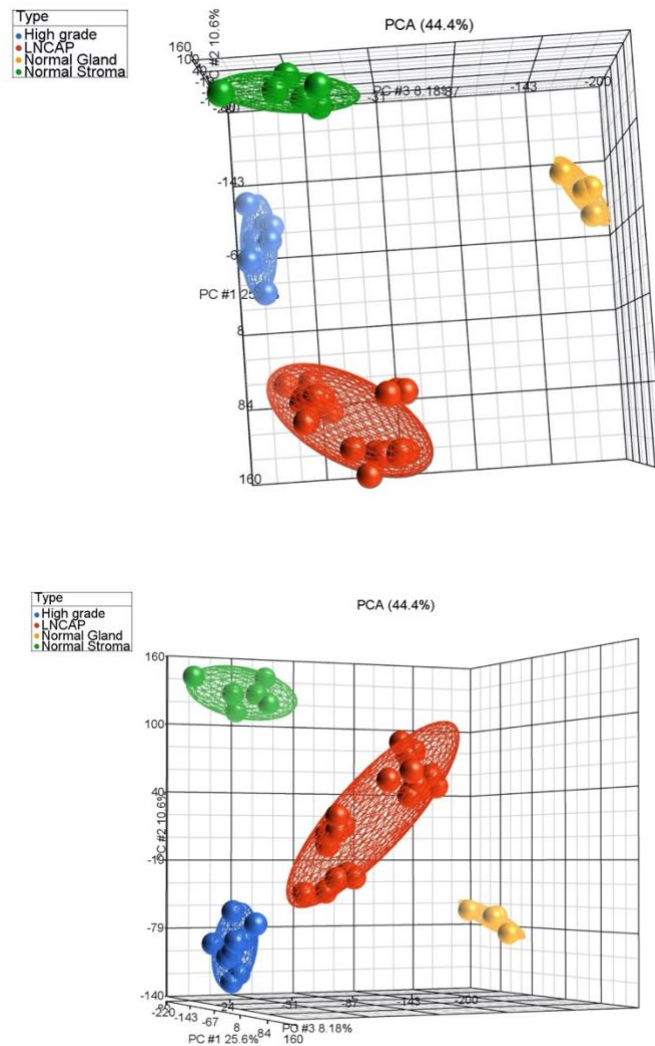


Figure 42. PCA following RMA analysis. The PCA shows each sample as a sphere, coloured by category group in a three-dimensional plot. The x, y, and z axes are the three principal components. The size of each axis is determined by the variance along the axis.

5.5.2 Differentially expressed genes

Using Partek Genomic Suite, detection of differentially expressed genes was performed using the ANOVA parametric test which studies dispersions between groups. Considering normal tissue as a control, genes that are upregulated in LnCAP or high-grade PC vs normal

gland or normal stroma were identified. With a fold change ≥ 2 changes in *ELK-1*, *ELK-3* and *ELK-4* expression levels were explored. The results show that *ELK-1* is upregulated in LnCap and *ELK-4* is upregulated in high-grade PC (Table 11).

Table 11. ELK level expression in LnCap and High-grade PC

	Combination	Condition	Fold Change
ELK-1	LnCap vs Normal Stroma	Upregulated	4.62
ELK-1	LnCap vs Normal Gland	Upregulated	2.01
ELK-1	LnCap vs High grade PC	Upregulated	3.61
ELK-4	LnCap vs Normal Stroma	Upregulated	2.30
ELK-4	LnCap vs Normal Gland	Downregulated	-9.38
ELK-4	High grade vs Normal Stroma	Upregulated	6.66
ELK-4	High grade vs Normal Gland	Downregulated	-2.78
ELK-3	LnCap vs Normal Stroma	Downregulated	-2.49

Some reports have suggested that the depletion of a specific E3 ligase increase mRNA levels of its substrates (Huang et al., 2019, Lan et al., 2019, Pedrazza et al., 2020). For example, it has been reported that SPOP depletion or overexpression of a dominant negative version of SPOP significantly raised mRNA and protein levels of target substrates such as GLYR1, TRIM24, and SETD2 in a cell type-specific manner (Lan et al., 2019). Considering the hypothesis of ELK-1 being upregulated because the E3 ligase is downregulated and unable to promote degradation of ELK-1 in LnCap, or prostate cancer for ELK-4, 2196 downregulated genes were found when ELK-1 is upregulated. (Fig. 43). For ELK-4, 5395 genes were downregulated when ELK-4 was upregulated (High-grade PC vs Normal stroma).

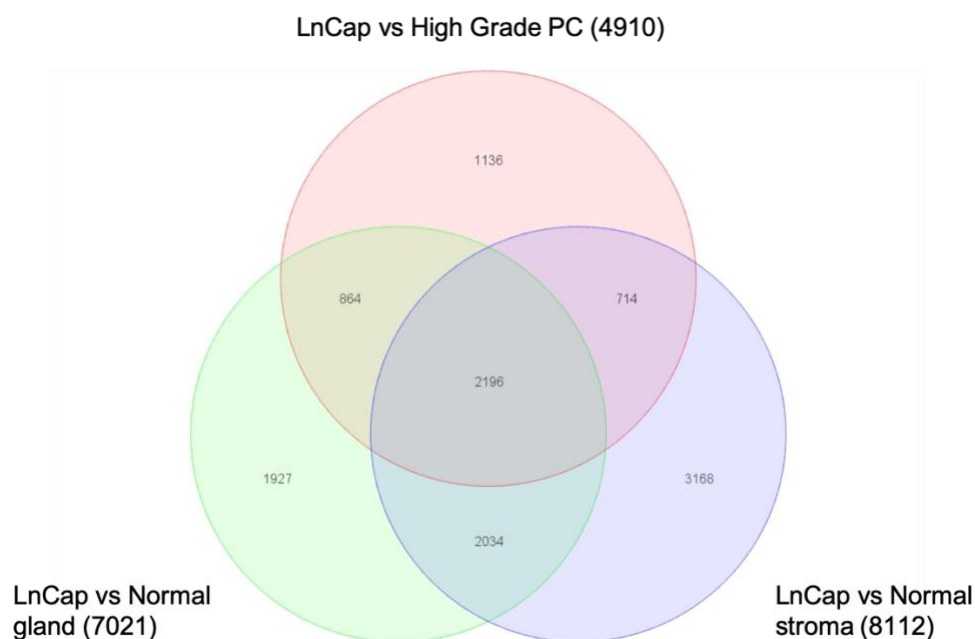


Figure 43. Venn diagram analysis of the genes that are downregulated when ELK-1 is upregulated. In the diagram: red circle represents LnCap vs high-grade PC; green circle represents LnCap vs Normal gland; blue circle represents LnCap vs Normal stroma. The total of downregulated genes for each condition is shown in brackets.

5.5.3 Functional annotation analysis

Using the open source software *DAVID Bioinformatic Resources* and the list of downregulated genes, the ubiquitination pathway was found using the Kegg pathway database, available online through the DAVID platform. The *in silico* analysis identified several E3 ubiquitin ligases as potentially downregulated in LnCaP when ELK-1 is upregulated (Fig. 44).

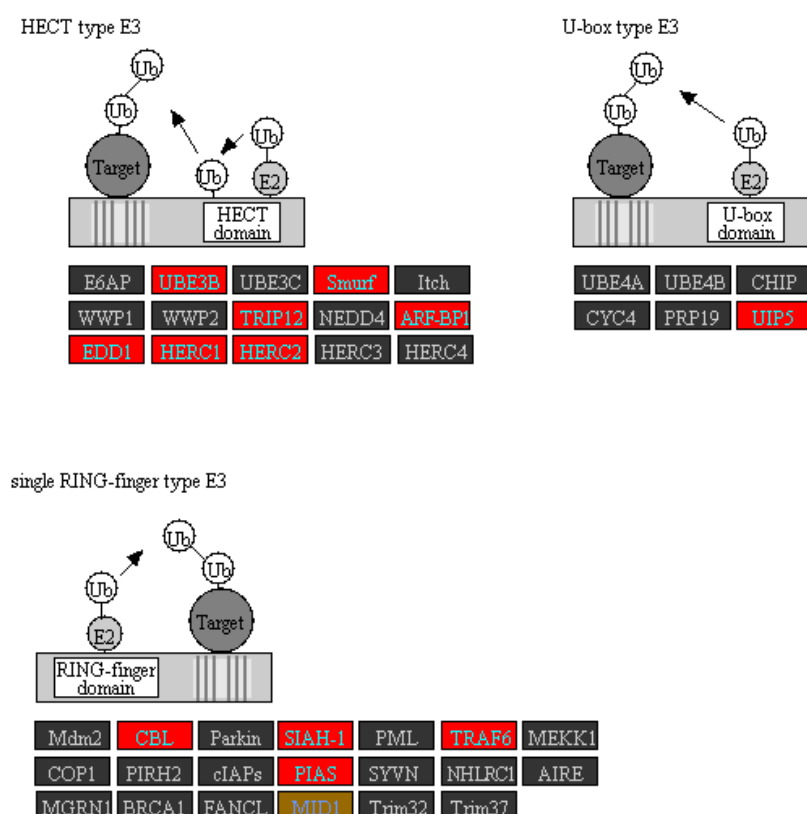


Figure 44. Representation of the candidate E3 ligases downregulated when ELK-1 is upregulated using the software Kegg pathway. The signalling analysis was performed on statistically differentially downregulated genes, with red indicating a higher fold change, brown indicating a lower fold change and black indicating insignificant change.

The same analysis was performed for ELK-4 in high-grade prostate cancer (Appendix 12). Using the previous results, search was focused on E3 ligases reported as modulators of proliferation or cell growth in cancer as well as located in the nucleus. SIAH-1 and EDD1 (UBR5) were identified as possible candidates with a notable participation promoting cell proliferation and suppressing cell apoptosis (McDonald et al., 2010, Hu et al., 2010, Brauckhoff et al., 2011, Malz et al., 2012).

5.5.4 E3 ligases screening experiments

Speculating that ELK-1 overexpression in prostate cancer is because the E3 ligase is downregulated and unable to ubiquitinate ELK-1, a new series of plasmids that express E3 ligases was used to search for changes in ELK-1 steady-state levels in the cell line LnCap. The results show that neither CHIP, TRIM63, SPOP or UBR5 have an effect on endogenous ELK-1, despite the fact that they are downregulated in LnCap. TRIM 63 is downregulated with a fold change of -2.23, SPOP -3.75, UBR5 -2.24 and FBXW7 -2.26 (Fig. 45). The E3 ligase CHIP is upregulated in LnCap.

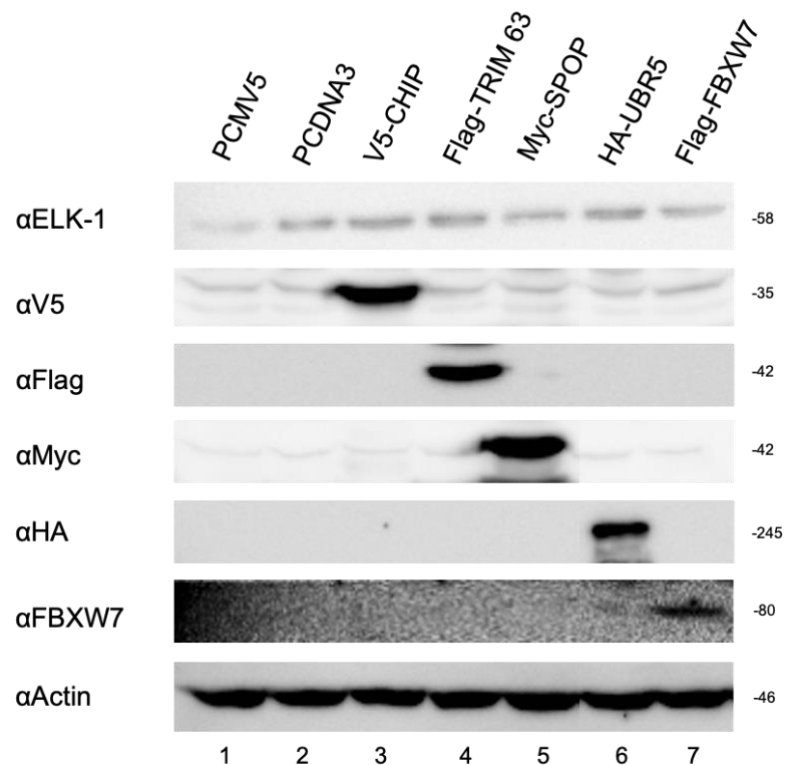


Figure 45. Downregulated E3 ligases in LnCap do not have any effect on ELK-1 degradation. LnCap cells were transfected with vectors for V5-CHIP, Flag-TRIM63, Myc-SPOP, Myc-FBXW7 and HA-UBR5 and controls as indicated. ELK-1, V5, Myc, Flag, HA and FBXW7 levels were determined by SDS-PAGE (5-10%) and immunoblotting.

The results obtained suggest that overexpression of ELK-1 in prostate cancer might not be related to E3 ligase downregulation. However, on this basis it is difficult to conclude that the ELK-1 specific E3 ligase is not downregulated in prostate cancer.

5.6 Mass Spectrometry as a method to find the ELK-1 E3 ligase

5.6.1 BioID screening

Considering all the information generated from my previous experiments, BioID was chosen as the method to find ELK-1 neighbours. The BioID analysis screens for potentially interacting proteins that are near-neighbours of the fusion protein; these proteins can be rapidly and transiently biotinylated and detected by mass spectrometry or immunoblotting (Roux et al., 2012). The ability to observe protein proximity can provide valuable information about protein dynamics, and allow the monitoring of post-translation modifications within a subpopulation of proteins (Roux, 2013).

The BioID method has been used to explore *in vivo* interactions in the nuclear lamina, the centrosome, the nuclear pore complex and more dynamic components, for example the NMD factors (Roux et al., 2012, Kim et al., 2014, Firat-Karalar and Stearns, 2015, Schweingruber et

al., 2016). Moreover, the BioID screening has been used as a method to identify ubiquitin E3 ligase substrates, for example, it has been published that the BioID screening was able to identify 163 unique proteins as part of the MIB1 E3 ligase interactome, 27 of which were previously identified as MIB1 partners using techniques such as AP-MS (Affinity purification-Mass Spectrometry), AP-Western or 2-Hybrid screens, 6 of which were reported as a direct interaction or ligase substrates. More important, the BioID screening identified 136 new MIB1 interactions and confirmed several proximity interactors as putative substrates of MIB1 E3 ligase activity. (Dho et al., 2019). The BioID approach was able to provide a map of MIB1 cellular localisation and a possible involvement within known functional complexes (Dho et al., 2019). Similarly, using the BioID screening, Coyaud *et al* identified 77 interactors as part of the SCF- β -TrCP1/2 E3 ligase interactome, 20 of which were substrates previously characterised (Coyaud et al., 2015).

In this study, two different constructs were made, N- and C- terminus fusions of BirA to ELK-1 (Myc-BirA-ELK-1 and ELK-1-BirA-HA respectively).

The constructs were tested for expression in HEK293T cells, which was corroborated by Western Blot (Fig. 46).

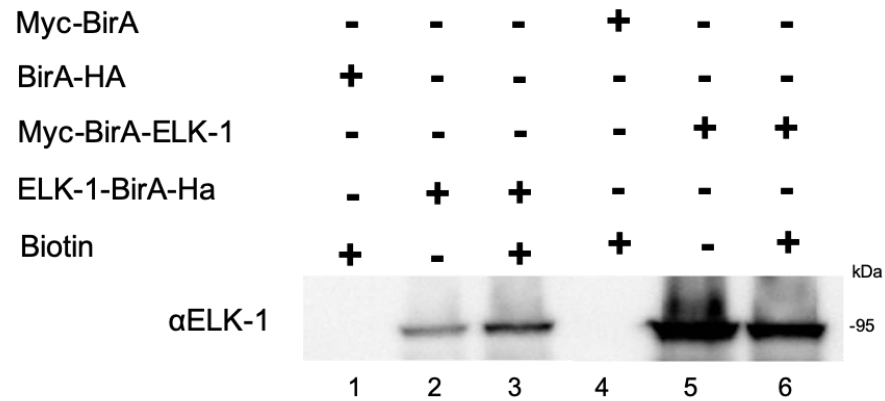


Figure 46. Expression of BioID-ELK-1 constructs in HEK293T cells. HEK293T cells were transfected with vectors for Myc-BirA-ELK-1, ELK-1-BirA-HA and controls as indicated. The day after transfection, cells were treated with 50 μ M biotin for 24 h. Cells were lysed with 200 μ L Laemmli buffer and 10% of the WCE was loaded. ELK-1 levels were determined by SDS-PAGE (8%) and immunoblotting.

The presence of biotinylated proteins was studied using two different conditions (starved and stimulated with TPA). My results demonstrate that only the construct with the BirA C-terminus to ELK-1 was able to biotinylate proteins (Fig. 47 lanes 3 and 6). The construct corresponding to the BirA N-terminus to ELK-1 (Myc-BirA-ELK-1) was not able to biotinylate proteins despite being successfully expressed (Fig. 46).

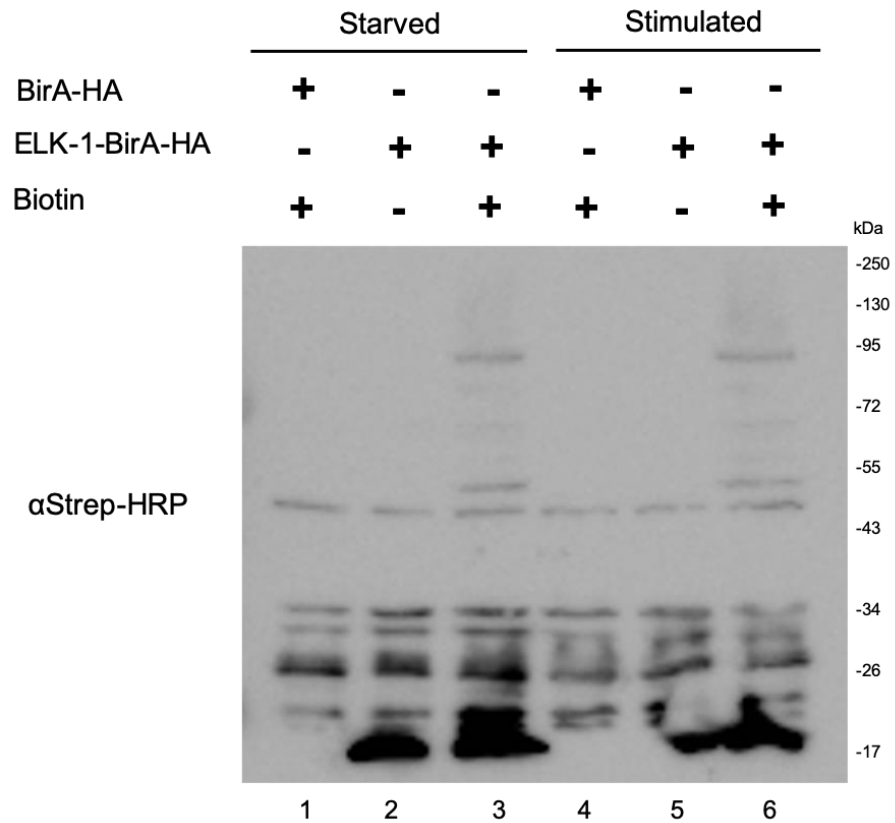


Figure 47. Expression of biotinylated proteins in HEK293T cells. HEK293T cells were transfected with vectors for ELK-1-BirA-HA and BirA-HA. The day after transfection, cells were treated with 50 μ M biotin for 24 h and stimulated or starved as indicated. Cells were harvested, 10% of the pellet was lysed with 20 μ L Laemmli buffer. Analysis was carried out by SDS-PAGE and immunoblotting with Strep-HRP antibody.

To guarantee the best conditions for the transfection, titration experiments using nuclear and cytoplasmic extracts were performed using the construct ELK-1-BirA-HA (Fig. 48).

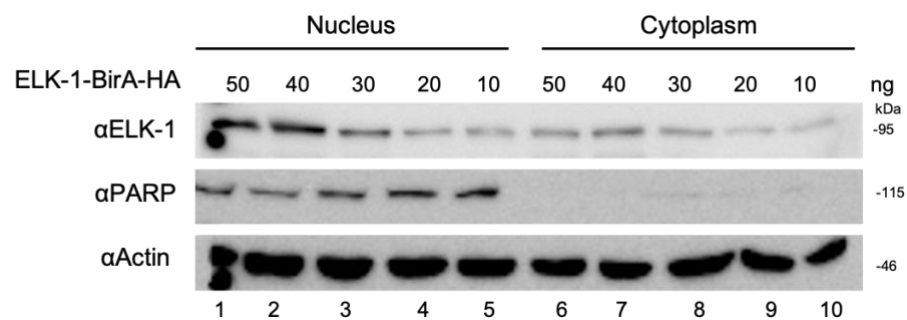


Figure 48. ELK-1-BirA-HA localises mainly in the nucleus. HEK293T cells were transfected with different amount of ELK-1-BirA-HA as indicated. Nuclear extraction was performed following the Gilman and Sadowski method (Sadowski and Gilman, 1993). ELK-1 levels were determined by SDS-PAGE (8%) and immunoblotting.

Samples were prepared for Mass Spectrometry by transfecting 40ng of ELK-1-BirA-HA under stimulated and starved conditions. Biotinylated proteins were collected using Streptavidin beads, 5% of the bound proteins were analyse by SDS-PAGE and the proteins were silver stained (Fig. 49). The 95% of proteins remaining were loaded in a 12% SDS-PAGE, proteins were stained with coomasie and the streptavidin band was removed prior to mass spectrometry analysis.

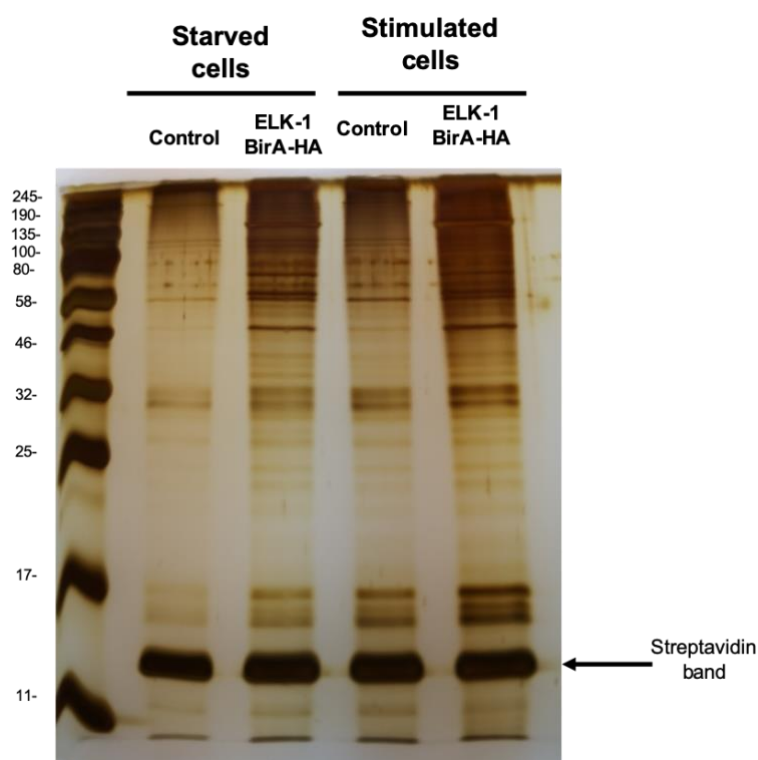


Figure 49. Expression of biotinylated proteins in HEK293T cells. HEK293T cells were transfected with vectors for ELK-1-BirA-HA and BirA-HA (control). The day after transfection, cells were treated with 50 μ M biotin for 24 h and stimulated or starved as indicated. Cells were harvested, 5% of the WCE was analysed by SDS-PAGE, proteins were silver stained.

A total of 1945 peptides were found in the MS analysis, 403 proteins were not present in the control and were distributed among the two conditions, ELK-1 starved (ELK-1 stv) and ELK-1 mitogen-stimulated cells (ELK-1 stm). Considering a minimum of 2 peptides difference between each condition, the proteins were distributed as follows: 102 proteins for ELK-1 stm, 122 proteins for ELK-1 stv and 179 common proteins (Appendix 13).

My results are in agreement with a previous paper that identified direct TCF-SRF cofactors involved in chromatin modification and transcription such as methyl-transferases, helicases, acetyl-transferases and histones. TCF-SRF direct genes were reported to be involved in the cell cycle, signalling networks and particularly transcription factors. (Esnault et al., 2017). The direct TCF-SRF target gene set was reported to include 54 different transcription factors which are sensitive to ERK activation such as Egr, AP-1 Ets, NF- κ B, Myc, p53 and STAT families (Gualdrini et al., 2016).

ELK-1 partners involved in transcription machinery identified using the BioID screening are shown in Table 12.

Table 12. Peptide count for transcription machinery peptides identified using Mass Spectrometry under ELK-1 mitogen-stimulated and starved conditions.

Identified Proteins	Alternate ID	Molecular Weight	Ctrl	Elk_stm	Elk_stv
Histone-lysine N-methyltransferase 2A	KMT2A	432 kDa	2	7	1
Cluster of Isoform 6 of Ankyrin repeat and KH domain-containing protein 1	CHD8	291 kDa	0	7	5
Cluster of Spliceosome RNA helicase DDX39B	DDX39B	49 kDa	14	22	19
Isoform 2 of Protein arginine N-methyltransferase 5	PRMT5	71 kDa	2	3	1
Bifunctional arginine demethylase and lysyl-hydroxylase JMJD6	JMJD6	39 kDa	0	2	1
Cluster of N-terminal Xaa-Pro-Lys N-methyltransferase 1	NTMT1	25 kDa	2	4	6
Histone deacetylase complex subunit SAP130	SAP130	111 kDa	0	2	6
Cluster of Histone deacetylase 1	HDAC1	55 kDa	0	5	3
Histone acetyltransferase type B catalytic subunit	HAT1	50 kDa	9	10	11
Cluster of Histone-binding protein RBBP7	RBBP7	48 kDa	0	10	3
Histone chaperone ASF1A OS=Homo sapiens	ASF1A	23 kDa	0	2	2
Histone deacetylase complex subunit SAP18	SAP18	18 kDa	8	11	9
Cluster of Histone H2A type 2-A	HIST2H2AA3	14 kDa	25	35	16
Histone H2A.Z OS=Homo sapiens	H2AFZ	14 kDa	5	7	6
Histone H4 OS=Homo sapiens	HIST1H4A	11 kDa	14	19	13
Mediator of RNA polymerase II transcription subunit 20	MED20	23 kDa	1	7	3
Mediator of RNA polymerase II transcription subunit 1	MED1	168 kDa	1	6	3
DNA-directed RNA polymerases I, II, and III subunit RPABC3	POLR2H	17 kDa	1	4	3
RNA polymerase-associated protein CTR9 homolog	CTR9	134 kDa	0	1	2
DNA polymerase	POLD1	126 kDa	0	4	2
Cluster of Poly(A) polymerase alpha	PAPOLA	83 kDa	0	0	4
DNA-directed RNA polymerase III subunit RPC5	POLR3E	76 kDa	0	0	2
RNA polymerase II-associated protein 3	RPAP3	76 kDa	0	6	5
DNA-directed RNA polymerase III subunit RPC3	POLR3C	46 kDa	1	2	4
BRCA2-interacting transcriptional repressor EMSY	EMSY	141 kDa	1	15	19
Transcription intermediary factor 1-alpha	TRIM24	117 kDa	0	3	4
General transcription factor 3C polypeptide 3	GTF3CE	101 kDa	0	1	3
General transcription factor 3C polypeptide 4	GTF3C4	92 kDa	0	4	6
Transcription intermediary factor 1-beta	TRIM28	89 kDa	13	16	20
Bcl-2-associated transcription factor 1	BCLAF1	83 kDa	0	6	5
Transcriptional repressor CTCF	CTCF	83 kDa	0	2	0
Transcription initiation factor TFIID subunit 6	TAF6	79 kDa	0	4	5
Transcriptional regulator Kaiso	ZBTB33	74 kDa	3	8	13
Transcription factor Sp2	SP2	65 kDa	0	2	5
Transcription factor AP-1	JUN	36 kDa	0	4	4
Transcription initiation factor IIE subunit beta	GTF2E2	33 kDa	3	5	5
Transcription factor E2F6	E2F6	32 kDa	0	1	2
General transcription factor IIF subunit 2	GTF2F2	28 kDa	2	1	4
RNA transcription, translation and transport factor protein	RTRAF	28 kDa	4	8	4
Cluster of Transcription initiation factor TFIID subunit 9B	TAF9B	28 kDa	0	5	7
General transcription factor IIE subunit 1	GTF2E1	14 kDa	0	0	2
Transcription initiation factor IIA subunit 1	GTF2A1	7 kDa	0	1	3
Signal transducer and activator of transcription	STAT1	83 kDa	0	3	5
Signal transducer and activator of transcription	STAT3	76 kDa	1	4	2
Isoform 2 of AFB4/FMR2 family member 4	AFF4	98 kDa	2	3	3

The Gene Ontology analysis for ELK-1 stm and ELK-1 stv grouped the proteins into 15 and 17 biological processes respectively (Fig. 50 and 51) (Appendix 14 and 15). Figure 50 and 51 show that apart from locomotion and cell population proliferation processes, the two conditions, mitogen-stimulated cells and starved cells shared the same biological processes but with different proteins in each condition. The major differences were found in the metabolic process, biological regulation and cellular process. In accordance with my results, it was reported that a growing number of ELK-1 SRF-independent genes were involved in nucleotide processing, metabolism and DNA-dependent transcription (Boros et al., 2009a, Boros et al., 2009b).

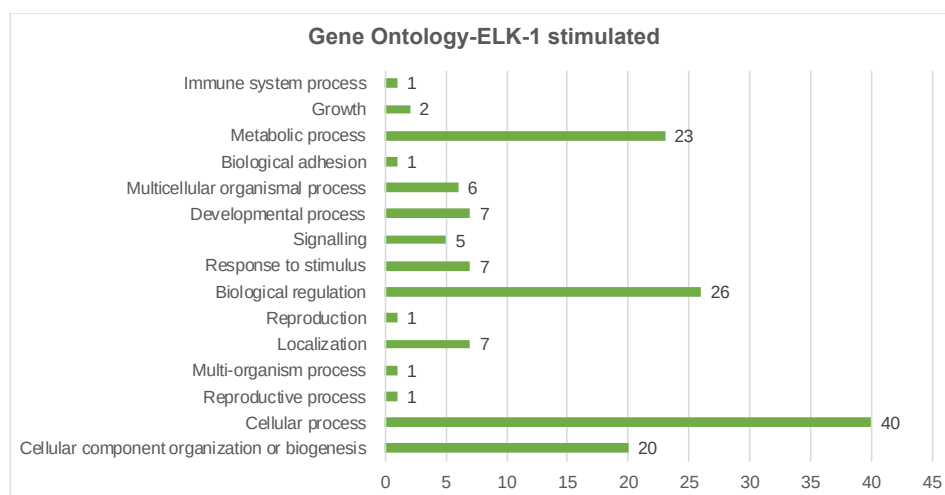


Figure 50. ELK-1 mitogen-stimulated cells, gene ontology analysis. 15 biological processes were found using <http://geneontology.org> tool. The number adjacent to the bar represents the number of proteins found.

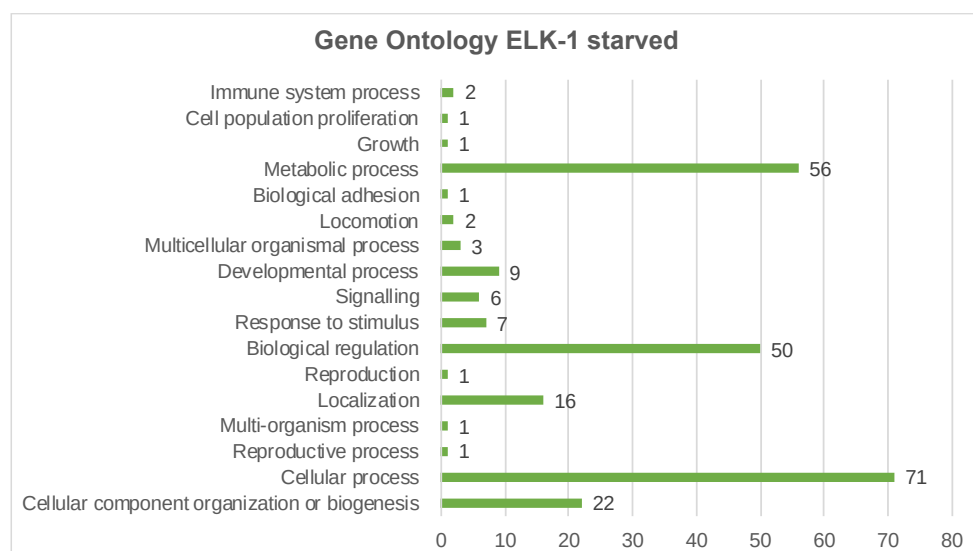


Figure 51. ELK-1 starved cells, gene ontology analysis. 17 biological processes were found using <http://geneontology.org> tool. The number adjacent to the bar represents the number of proteins found.

The SUMO and E3 ligases found in this experiment are shown in Table 13. The most significant peptide under mitogen-stimulated conditions corresponds to the SUMO E3 ligase RANBP2 which accounts for 30 peptides versus 18 peptides found in ELK-1 starved conditions. To date, there is no evidence that directly links ELK-1 with the SUMO E3 RANBP2, however I cannot rule out the possibility that RANBP2 may be a *bona fide* SUMO E3 ligase, its interaction with other transcription factor and/or co-activator or co-repressor related to ELK-1 might indicate a role in modulating ELK-1 repression by sumoylation (see discussion, section 6). The other SUMO and E3 ligases did not show a significant increase in the number of peptides compare to the control, for that reason they cannot be considered as specific ELK-1 E3/SUMO ligases.

Table 13. Peptide count for E3 ligases proteins identified using Mass Spectrometry under ELK-1 stimulated and starved conditions.

Identified Proteins	Alternate ID	Molecular Weight	Ctrl	Elk_stm	Elk_stv
Ubiquitin-protein ligase E3A	UBE3A	88 kDa	0	2	1
E3 ubiquitin-protein ligase BRE1B	RNF40	109 kDa	1	2	2
E3 ubiquitin-protein ligase MARCH5	MARCH5	31 kDa	1	2	1
E3 SUMO-protein ligase CBX4	CBX4	61 kDa	2	0	1
E3 ubiquitin/ISG15 ligase TRIM25	TRIM25	71 kDa	2	0	1
E3 UFM1-protein ligase 1	UFL1	90 kDa	3	4	2
Cluster of E3 SUMO-protein ligase RanBP2	RANBP2	358 kDa	4	30	18
Isoform 2 of E3 ubiquitin-protein ligase TRIP12	TRIP12	224 kDa	7	3	2
S-phase kinase-associated protein 1 (adaptor for SCF E3 ligases	SKP1	19 kDa	0	3	1
Isoform 2 of E3 ubiquitin-protein ligase ZNF598	ZNF598	98 kDa	9	6	6

Under stimulated conditions the ubiquitin conjugating enzyme UBE2O shows a significant number of peptides compare to the control and the starved conditions (Table 14). It has been reported that UBE2O possesses both E2 and E3 ligase activities and it is involved in mono-ubiquitination of transcription factors related to cell proliferation (Mashtalir et al., 2014). For that reason, it might be of great interest to study its participation in the ELK- ubiquitination pathway. The ubiquitin-conjugating enzyme UBE2Z is the most significant E2 enzyme found in starved conditions (Table 14). It has been reported as significantly essential to restrain tumour proliferation in human hepatocellular carcinoma by targeting ERK and STAT3 pathways (Shi et al., 2020). Nonetheless, there is no further information available regarding its interaction with E3 ligases and substrates. However, the BioID screening shows that it might be a *bona fide* ELK-1 partner.

Table 14. Peptide count for E2 conjugation proteins identified using Mass spectrometry under ELK-1 stimulated and starved conditions.

Identified Proteins	Alternate ID	Molecular Weight	Ctrl	Elk_stm	Elk_stv
NEDD8-conjugating enzyme Ubc12	UBE2M	21 kDa	3	6	6
Ubiquitin-conjugating enzyme E2 S	UBE2S	24 kDa	4	3	3
Ubiquitin-conjugating enzyme E2 K	UBE2K	22 kDa	2	6	6
E2 ubiquitin-conjugating enzyme	UBE2O	141 kDa	1	8	3
Ubiquitin-conjugating enzyme E2 D3	UBE2D3	17 kDa	1	2	2
Isoform 3 of Ubiquitin-conjugating enzyme E2	UBE2L3	24 kDa	2	4	4
Ubiquitin-conjugating enzyme E2 E1	UBE2E1	16 kDa	1	5	3
Ubiquitin-conjugating enzyme E2 N	UBE2N	17 kDa	1	3	3
Ubiquitin-conjugating enzyme E2 Z	UBE2Z	38 kDa	0	0	2

Additionally, it was shown that ELK-1 stimulated is ubiquitinated at K35, K52 and K59, which is consistent with published results regarding ELK-1 mono-ubiquitination sites and the identification of K35 as the predominant mono-ubiquitination site in ELK-1 (Ducker et al., 2019). De-ubiquitination of K35 was reported to be mediated by USP17 (Ducker et al., 2019). Table 14 shows the deubiquitinating enzymes found in this analysis. Under starved conditions the most significant deubiquitinating enzyme was USP10 (Table 15). The identification of USP10 provides more information about ELK-1 interacting partners involved in the ubiquitination and de-ubiquitination pathway.

Table 15. Peptide count for de-ubiquitinating proteins identified using Mass spectrometry under ELK-1 stimulated and starved conditions.

Identified Proteins	Alternate ID	Molecular Weight	Ctrl	Elk_stm	Elk_stv
Cluster of Ubiquitin carboxyl-terminal hydrolase 15	USP15	112 kDa	0	6	5
Cluster of Ubiquitin carboxyl-terminal hydrolase 7	USP7	106 kDa	0	1	2
Cluster of Ubiquitin carboxyl-terminal hydrolase 10	USP10	87 kDa	0	0	3
Ubiquitin carboxyl-terminal hydrolase 5	USP5	96 kDa	0	1	2
Cluster of Dual specificity protein phosphatase 3	DUSP3	20 kDa	1	3	3
RNA/RNP complex-1-interacting phosphatase	DUSP11	44 kDa	2	1	1
Ubiquitin carboxyl-terminal hydrolase 14	USP14	51 kDa	2	8	6
U4/U6.U5 tri-snRNP-associated protein 2	USP39	62 kDa	11	7	4

ELK-1 starved was found phosphorylated on S202, S303, S304, S324 and S326. Additionally, ELK-1 stimulated was found to be phosphorylated on S200, S202, S303, S304, S324 (Fig. 52). This result correlated with the ELK-1 phosphorylation sites described on the Phosphosite Database <https://www.phosphosite.org>. Moreover, new phosphorylation sites were found at S200 and S202. The S200 phosphorylation site is present under stimulated conditions (Fig. 50). And the S326 phosphorylation site is present under starved conditions (Fig. 52).

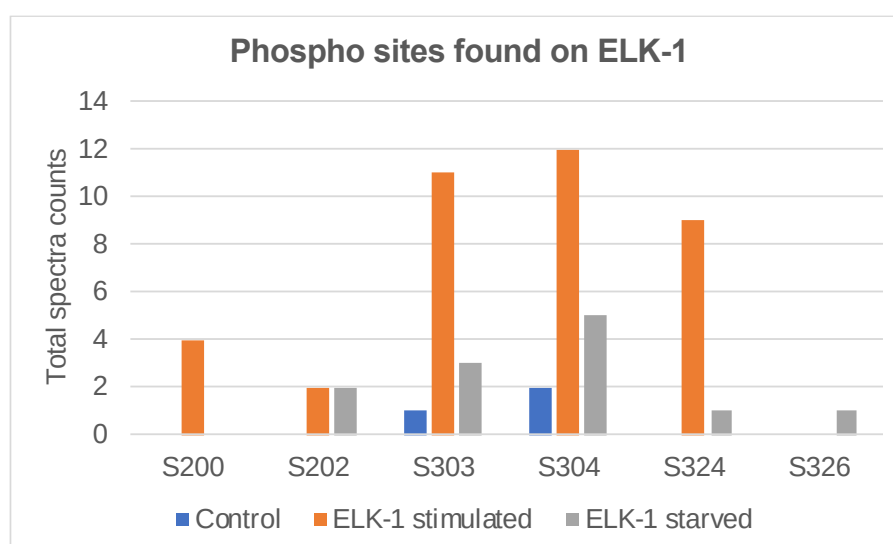


Figure 52. ELK-1 phosphorylation sites. Bars represent total spectral counts for each peptide from one analysis.

Table 16 shows the most significant protein kinases found in the analysis. BUB1 and GSK3 β are significant in ELK-1 stimulated, whereas HGS, CDK11B, CAMK2D, NUCKS1 and NEK6 are significant in ELK-1 starved.

Table 16. Peptide count for protein kinases identified using Mass Spectrometry under ELK-1 stimulated and starved conditions.

Identified Proteins	Alternate ID	Molecular Weight	Ctrl	Elk_stm	Elk_stv
A-kinase anchor protein 1, mitochondrial	AKAP1	97 kDa	0	6	3
Mitotic checkpoint serine/threonine-protein kinase BUB1	BUB1	122 kDa	0	7	0
Cluster of Serine/threonine-protein kinase PAK 2	PAK2	58 kDa	0	1	4
Inactive tyrosine-protein kinase 7	PTK7	118 kDa	0	3	0
Hepatocyte growth factor-regulated tyrosine kinase substrate	HGS	86 kDa	0	0	2
Isoform SV11 of Cyclin-dependent kinase 11B	CDK11B	64 kDa	0	0	2
Calcium/calmodulin-dependent protein kinase (CaM kinase) II delta	CAMK2D	56 kDa	0	0	2
Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1	NUCKS1	27 kDa	0	0	2
Serine/threonine-protein kinase Nek6	NEK6	23 kDa	0	0	2
Glycogen synthase kinase-3 beta	GSK3B	47 kDa	0	3	1

To conclude, the BioID screening did not detect any potential E3 ligase candidate for ELK-1. However, there is useful information which can lead to understanding more about ELK-1 regulation under cells stimulated with TPA and starved conditions.

6.0 DISCUSSION

The regulation of ELK-1 by mono-ubiquitination has identified new mechanisms that can modulate the expression of IEGs (Ducker et al., 2019). My results have revealed that ELK-1 can interact with different E3 ligases. Nevertheless, these interactions do not appear to modulate ELK-1 in a direct way, and ELK-1 target genes cannot be regulated by FBXO25 or SPOP.

It has been demonstrated that ELK-1 can be poly-ubiquitinated in HEK293T cells (Evans et al., 2011). These results indicate the presence of ELK-1-specific E3 ligase activity in HEK293T cells. However, the mechanism that leads to ELK-1 turnover by poly-ubiquitination is still unclear, and the published report about FBXO25 promoting ELK-1 degradation (Teixeira et al., 2013) is not supported by data presented in this research project (section 5.2).

6.1 ELK-1 ubiquitination

There are several ELK-1 features that ultimately reveal that ELK-1 turnover is mediated by the proteasome system. Firstly, ELK-1 can be considered as a short intracellular half-life protein, unpublished data from our lab have shown that ELK-1 has a half-life ($t_{1/2}$) of 3 hours (Franziszka Gehringer Master's thesis). In addition, there is more evidence that suggests that short-lived intracellular proteins such as

c-Myc, c-*JUN*, p53 and cyclin B are degraded by the ubiquitin pathway (Ciechanover et al., 1991, Glotzer et al., 1991, Treier et al., 1994, Shkedy et al., 1994). PEST sequences are found in a number of short-lived proteins. A PEST sequence is a peptide sequence that is rich in proline (P), glutamic acid (E), serine (S), and threonine (T) and they are associated with proteins that have a short intracellular half-life (Rogers et al., 1986). A PEST amino acid sequence in ELK-1 with a positive score (residues 197–230) was reported, however it did not show any effect destabilising ELK-1 N-terminus truncations (Evans et al., 2011). N-terminus truncations of ELK-1 lacking amino acids 1-24 including the $\alpha 1\beta 1$ loop were reported as crucial for the stability of ELK-1 in HEK293 cells, furthermore a proline-rich hydrophobic motif adjacent to the SRF interaction domain (amino acids 167-196) was demonstrated to serve as a cryptic degron to promote the turnover of destabilised N-terminus truncations of ELK-1 (Fig. 10) (Evans et al., 2011).

There is strong evidence that suggests that the degron is present in several transcription factors. For example, the transcription factor FOS has a degron which overlaps with the transcriptional activation domain (TAD) (Brown et al., 1995). This feature has been attributed to other unstable transcription factors, such as p53 and Myc (Fields and Jang, 1990, Salghetti et al., 2000). In the case of Myc, it was reported that there is a relationship between the degron and the TAD working together to promote the degradation of Myc (Salghetti et al.,

2000). The Myc degron function was demonstrated to be restricted to a subset of TADs enriched in acidic residues (Salghetti et al., 2000). To date, ELK-1 degron function has been related to N-terminus truncations; there is no evidence of its relationship with other ELK-1 domains. However, it is possible to speculate that it can be related to the ELK-1 TAD (D domain) because it plays an important role in regulating gene expression and proliferation. Furthermore, ELK-1 activation needs to be tightly connected with its destruction in order to maintain cellular homeostasis.

In this study, I examined the interaction of a mutant version of ELK-1 (ELK-1 Δ 94-110) which contains serine, threonine and proline residues (Fig. 32) and serves as potential SBC motif to interact with the SPOP ubiquitin ligase (Fig. 29). My results indicated that SPOP WT can interact with ELK-1 but the SBC motif on ELK-1 located within the amino acids 94-100 does not completely disrupted the interaction. ELK-1 contains another potential SPOP binding domain located within amino acids 190-200 (APPSGSRSTS). The presence of a second SPOP binding domain on ELK-1 which might be able to recruit and interact with SPOP could explain why the ELK-1 mutant (ELK-1 Δ 94-110) did not disrupt completely the interaction with SPOP (Fig. 32). It has been published that SPOP substrates Gli2 and Gli3, which are transcriptional regulators of the Hedgehog signaling pathway, contain several regions that are crucial for SPOP recognition (Zhang et al., 2009). Moreover, it has been discovered that SPOP can recognise and bind to weak motifs which lack a polar or non-polar amino acid or

contain a cysteine in a place of the serine that was previously suggested to be invariable based on the known SBC motif (Zhuang et al., 2009, Pierce et al., 2016). Each of the weak SPOP motifs have been demonstrated to bind the SPOP-MATH domain and contribute to ubiquitination by a mechanism that involves recruitment of CUL3 (Pierce et al., 2016). However, it was reported that recruitment of the substrate and ubiquitination efficiency was enhanced with SPOP homo-oligomers which is mediated through the presence of more than two perfect SBC motifs rather than dimerization of SPOP which takes place with two SBC motifs. (Errington et al., 2012, Pierce et al., 2016). On the other hand, substrates with only one SBC motif have been reported to undergo mono-ubiquitination (Pierce et al., 2016). More experiments are needed to demonstrate if the second potential weak motif on ELK-1 located within amino acids 197-200 is essential to mediate the SPOP interaction.

Figure 34 showed that SPOP WT and SPOP mutants decreased the levels of overexpressed ELK-1, suggesting that SPOP might indirectly regulate ELK-1 by a mechanism that involves degradation of an ELK-1 destabilising protein, probably by a degradation-independent mechanism. Consistent with my results, several SPOP targets have been identified in liver cancer, including cyclin dependent kinase inhibitor 2B (CDKN2B) and zinc-finger E-box binding homeobox 2 (ZEB2) whose modulation by SPOP has not yet been related to a proteasome-mediated degradation effect (Jia et al., 2014, Huang et

al., 2015). It has been published that overexpression of SPOP WT increases CDKN2B expression, whereas overexpression of the SPOP mutant S199N decreases CDKN2B expression in hepatoblastoma, those findings revealed one mechanism by which SPOP positively modulates stabilisation of a protein involving in cell proliferation (Jia et al., 2014).

Alternatively, more recently, SPOP has been shown to play a critical role in colorectal cancer tumorigenesis by regulating HDAC6 (histone deacetylase 6). It has been published that SPOP specifically binds to and promotes the poly-ubiquitination and degradation of HDAC6 in a deubiquitinase-dependent manner; cancer-derived SPOP mutations disrupted the binding between SPOP and HDAC6 and failed to promote HDAC6 poly-ubiquitination and subsequent degradation (Tan et al., 2017). Those results prompted the importance of SPOP in modulating cell proliferation and provide evidence to speculate that SPOP might attract several transcriptional repressors which can be positioned close enough to indirectly interact with ELK-1 and promote its repression.

A different approach to find an ELK-1 E3 ligase was used by performing an *in silico* analysis of prostate cancer biopsies. My results demonstrated that ELK-1 is upregulated in LnCap cells and ELK-4 is upregulated in high-grade prostate cancer using Normal Stroma as a control (Table 11). In agreement with my results, there is evidence that reported an increased in *ELK-4* expression in prostate cancers

relative to normal prostates, moreover ELK-4 was reported as a direct AR target in prostate cancer cells (Makkonen et al., 2008). To date, there is no evidence about ELK-4 mutations linked to PC. It would be of great interest to analyse what the levels of ELK-1 and ELK-4 are in low and medium prostate cancer in order to analyse their participation in tumour initiation and progression of prostate cancer. My rationale to find ELK-1 E3 ligase was hypothesising that upregulation of ELK-1 was related to its E3 ligase downregulation. My results demonstrated that thirteen E3 ligases were significantly downregulated when ELK-1 was upregulated (Fig. 44). I identified UBR5, SIAH-1 and TRIP12 as potential E3 ligases because of their participation in the proliferation process (Hu et al., 2010, Malz et al., 2012, Almeida et al., 2011). Immunoblotting was performed to evaluate changes in the steady-state levels of ELK-1 in LnCap cells (Fig. 45); other potential E3 ligases (CHIP and TRIM63) found in the *in silico* analysis as well and involved in the proliferation process were included in the immunoblot (Khan et al., 2018, Li et al., 2019). However, no changes were detected on ELK-1 steady-state levels in LnCap cells (Fig. 45). Changes on steady-state levels of ELK-1 would have indicated a potential effect of any E3 ligase on ELK-1 degradation, speculating that overexpression of the E3 ligase downregulated in LnCap would have been able to promote ELK-1 degradation. Around 200 E3 ligases were found in the analysis, more downregulated E3 ligases located in the nucleus and with a participation in the proliferation process were found, including the E3 ligase TRIM45 which acts as a transcriptional

repressor in mitogen-activated protein pathways (Wang et al., 2004), the ubiquitin E3 ligase BTRC that has been reported to degraded proteins in response to mitogens (Dorrello et al., 2006), TRIM33 that promotes the development of pre-cardiogenic mesoderm and plays an essential role in the control of cell proliferation (Xue et al., 2015, Rajderkar et al., 2019), UHRF2 that ubiquitinates PEST-containing nuclear protein and it is involved in cell cycle (Mori et al., 2002, Mori et al., 2004), and DCUN1D3 that it is associated with the serum response element (SRE) pathway activation (Ma et al., 2008) Notably, in agreement with published findings, the analysis showed that SPOP and FBXW7 are downregulated in prostate cancer (Welcker and Clurman, 2008, Mani, 2014). In conclusion, the result of the *in silico* analysis revealed new potential E3 ligases in prostate cancer that might be tested for their possible participation in ELK-1 ubiquitination.

6.2 FBXO25 and ELK-1 ubiquitination

The findings published by Teixeira *et al* indicated that FBXO25 promotes ELK-1 poly-ubiquitination and its degradation by the proteasome. Preliminary data in our lab confirmed that ELK-1 can interact with FBXO25. Furthermore, cycloheximide chase experiments demonstrated no effect on the half-life of ELK-1 ($t_{1/2}$ of 3h) after expression of FBXO25, and steady state levels of endogenous or ectopically express ELK-1 were not affected by FBXO25 expression (Franziszka Gehringer Master's thesis). A series

of experiments were completed following Teixeira's conditions and alternative approaches were adopted, such as using denaturing conditions to avoid non-covalently linked poly-ubiquitin chains. My results demonstrated no effect of FBXO25 modulating ELK-1 transcriptional activity and ubiquitination (Fig. 20, 21 and 24). More importantly, they showed no effect of FBXO25 in modulating ELK-1 target genes (Fig. 24). Whereas it has been shown that ELK-1 dependent gene expression changes in response to modulation of ELK-1 ubiquitination with USP17 (Fig. 17 and 19). The effect of ELK-1 ubiquitination has been published, and there is strong evidence to conclude that the ubiquitination of ELK-1 modulates expression of its target genes (Ducker et al., 2019).

Additionally, my results indicated that FBXO25 expression decreased ELK-1 poly-ubiquitination (Fig, 20 and 21). These results suggest that rather than a negative effect of FBXO25 modulating ELK-1 degradation, FBXO25 might have a positive effect modulating ELK-1 stabilisation through different mechanisms. Recently, studies related to SCF-independent function of F-box proteins started to highlight the importance of the F-box proteins as important transcriptional regulators. For example, it has been published that FBXO38 (MoKA) interacts with Kruppel-like transcription factor 7 (Klf7) through the F-box domain. This interaction is needed to support Klf7 function as a transcriptional cofactor at the promoter of the cell cycle inhibitor p21 (Smaldone et al., 2004). Moreover, it was published that F-box

proteins can have alternative functions that are not limited to simple binding interactions with other proteins, they also possess distinct and intrinsic enzymatic activities (Nelson et al., 2013). For example, FBXO18 has been shown to function as DNA helicase. It translocates in the 3' to 5' direction and interacts with SKP1 through the F-box domain. It was demonstrated that the DNA helicase activity of FBXO18 and the correct physiological function of the enzyme is mediated by the interaction with the SCF complex (Kim et al., 2002, Kim et al., 2004). Furthermore, it was demonstrated that FBXO7 function as a scaffold or chaperone to fold or stabilise cell cycle proteins. FBXO7 can interact with three active kinases, cyclin D3, CDK6 and p27, but it can distinguished among the CDKs to specifically interact with CDK6 and positively regulate it in a process that it is independent of its intrinsic ubiquitin ligase activity (Laman et al., 2005). For that reason, I can speculate that FBXO25 might have a positive effect on ELK-1 independent of the FBXO25 ubiquitin ligase function.

Figure 28 showed that FBXO25 overexpression promotes Hax-1 destabilisation. My results demonstrated that FBXO25 WT is able to decrease the steady state levels of Hax-1, in accordance with the results obtained by Baumann *et al.*, that effect was attributed to FBXO25 having an effect on Hax-1 degradation. However, with the experiment performed in this study I cannot demonstrated that FBXO25 is promoting Hax-1 poly-ubiquitination, more experiments

using the MG132 treatment and ubiquitination assays are needed to corroborate my results. Alternatively, figure 28 suggests that FBXO25 may be affecting Hax-1 levels indirectly by inducing degradation of a Hax-1 destabilising protein. It is possible that FBXO25 has a negative effect on Hax-1 independent of its interaction with the SCF complex, a possibility that might explain the effect observed with the FBXO25 deletion mutant which was not consistent with the effect reported by Baumann *et al.*, It has been published that about 12% of the human FBPs (F-box proteins) have SCF-independent roles, moreover it has been demonstrated that the presence of an F-box domain is not a guarantee that a protein will function as part of an SCF complex (Hermand, 2006, Nelson *et al.*, 2013). For example, it has been published that in fission yeast, the F-box protein Pof14 binds to inhibit the Erg9 squalene synthase, a key enzyme in ergosterol metabolism in response to oxidative stress, this effect was independently of its F-box domain or other SCF components. (Tafforeau *et al.*, 2006). In normal conditions the levels of Pof14 are very low due to an SCF complex regulating its own level; in the case of Pof14 it was reported that the F-box domain function more as a destruction box (Galan and Peter, 1999, Hermand, 2006).

Together, my results indicate that the effect observed on ELK-1 might not be related to FBXO25 ubiquitin ligase function, but it might have an effect on ELK-1 stabilisation by unknown mechanisms.

On the other side, my results demonstrated that it is not always possible to reproduce published experiments, possibly because the published data was incorrect. I have improved some experiments and added some additional controls. I have generated reproducible data which has been reproduced by other students in our laboratory.

I do not rule out the idea that an F-box protein can promote ELK-1 degradation, however I suggest that FBXO25 has no ubiquitin ligase effect on ELK-1. My results with FBXW7 (section 5.4) demonstrated that there are several F-box proteins interacting indirectly with ELK-1 and modulating its target genes expression through interactions with the PIC. My results demonstrated that the co-IP interaction of ELK-1 with SPOP and FBXO25 is not a guarantee of degradation, for that reason, the improvement in the detection of ELK-1-specific E3 ligases should be more stringent in order to avoid non-productive interactions which are not related to ELK-1 degradation.

6.3 Factors that define the protein levels of ELK-1

It has been published that SPOP targets the Androgen Receptor for degradation via the proteasome pathway, a decrease in the ectopic AR levels in HEK293T cells after SPOP overexpression was reported (An, 2014). The same ubiquitination effect was published with SPOP and the estrogen receptor- α (Zhang et al., 2015), the progesterone

receptor (Gao et al., 2015) and the steroid receptor co-activator-3 (Geng et al., 2013).

My results showed a reduction in the levels of ELK-1 when SPOP was overexpressed (Fig. 33). However, that effect was not reverted with the SPOP mutants as expected (Fig. 34). Figure 34 shows a reduction in the expression of ELK-1 levels when co-transfected with any SPOP plasmids; the reduction of the ELK-1 levels correlate with an increase in the SPOP levels. The same results were observed with SPOP and USP17 (Fig. 36). Co-transfection of SPOP decreased the levels of USP17 independent of any E3 ligase activity from SPOP on USP17.

ELK-1 expression levels could be partially recovered when cells were treated with the proteasome inhibitor MG132. For example, the IP experiments did not show a drastic reduction of ELK-1 levels when co-transfected with SPOP WT or SPOP mutants (Fig. 30, 31, 32). For that reason, I suggest that HEK293T cells might have a preference to express ectopic SPOP rather than ectopic ELK-1, or both proteins are expressed at the same level, but in any case, ELK-1 is being degraded and destabilised faster than SPOP in HEK293T cells. For future experiments, I suggest taking into consideration the issues associated with transfection; decreasing the amount of plasmid transfected could balance the transfection efficiency in the cells. Furthermore, I strongly suggest that the effect of any E3 ligase would be better evaluated by using shRNA models, stable cell lines or Crispr Cas technologies rather than ectopic expression.

6.4 BioID screening for ELK-1 neighbours

My results identified 403 proteins that were near ELK-1 using a ≥ 2 spectral count ratio (section 5.6.1). Table 12 demonstrates a high relevance on ELK-1 interacting with members of the transcription machinery. In agreement with my results, several new studies have identified ELK-1 simultaneous interactions with different transcriptional regulators that each influence transcription initiation and expression of ELK-1 target genes (Kim et al., 1998, Balamotis et al., 2009, Gualdrini et al., 2016, Esnault et al., 2017). For example, it was demonstrated that in the *CFOS* promoter the transcription factor TFII-I enhances the formation of binary and ternary complexes in vitro through an interaction with STAT1, STAT3 and SRF. Regulation of TFII-I was reported to be mediated by the RAS pathway which enhances its phosphorylation. Binding of endogenous TFII-I to the SRE is required for full transcriptional activity of the *CFOS* promoter (Kim et al., 1998).

Table 12 showed that ELK-1 interacts with STAT1, STAT3 and TFII. Interestingly, interaction with TFII can be detected under starved conditions while STAT1 and STAT3 peptides are detected in both conditions starved and mitogen-stimulated. I would suggest that under starved conditions there is a downstream activator of the RAS signalling that activates TFII and modulates ELK-1 target genes expression probably by forming a complex with STAT1 and STAT3. It has been reported that in ES, ELK-1 phosphorylation induces

mediator binds to phospho-ELK-1 through an interaction with MED23, this binding stimulates further Pol II assembly with GTF (General transcription factor) such as TFIIB, E and H into preinitiation complexes on EGR1 promoters and triggers post recruitment steps in transcription initiation (Stevens et al., 2002, Wang et al., 2005). Additionally, it was demonstrated that Mediator subunits MED1 and MED17 bind to the EGR1 promoter in ES and MEF cells under serum stimulation, however, the degree of mediator occupancy in response to serum was higher and maintained for a longer period in WT MEFs compared to WT ES cells; this difference was reported to be regulated by the differential expression of the three TCFs (ELK-1, ELK-3 and ELK-4) in MEFs and ES cells (Balamotis et al., 2009). Consistent with the findings mentioned above, my results demonstrated that MED1, MED20, TFIIE, TFIID and some other transcription factors (Table 12) are interacting with ELK-1 under mitogen-stimulated conditions. This strongly suggest that ELK-1 interacts simultaneously with several transcriptional regulators in response to mitogen stimuli to mediate transcription initiation and to regulate subsequent transcription events. The effect of ELK-1 interaction with the Mediator subunits has not been widely studied, in fact, only interactions with the tail module have been reported (Stevens et al., 2002, Galbraith et al., 2013). My results demonstrated that ELK-1 might be interacting with other Mediator modules, MED1 and MED20 belong to the middle and head modules respectively. It has been reported that the mammalian glucocorticoid receptor (GR) interact with several mediator subunits,

it is MED1 which provides the dominant GR-binding surface and MED14 which is involved in expression of some GR-regulated genes (Chen et al., 2006, Chen and Roeder, 2007). Moreover, it was reported that in *Saccharomyces cerevisiae*, Mediator subunits Med18, Med20 and Med19 are required for activation of transcription at the CYC1 promoter in response to environmental signals. These subunits of Mediator were reported to be essential in triggering an inactive polymerase at CYC1 into a productively elongating form by regulation recruitment and activity of the general transcription factor TFIID (Lee et al., 2013).

Figure 50 and 51 show that ELK-1 interaction partners can be categorised into 15 cellular processes. A different set of proteins under mitogen-stimulated cells and starved conditions were detected. My results also showed that the BioID screening identified a great number of ELK-1 substrates that are probably SRF-independent and categorised in the metabolic process (Fig. 50 and 451, Appendix 14 and 15). In agreement with that explanation, it was demonstrated that ELK-1 SRF-independent substrates are mostly involved in metabolism, nucleotide processing and DNA-dependent transcription. Co-occupancy of promoters by SRF and ELK-1 accounted for only 22% of the ELK-1 target genes, in contrast, co-occupancy was rather infrequent with approximately 78% promoter not co-bound by SRF (Boros et al., 2009a, Boros et al., 2009b).

Other proteins found in this study that they might interact with ELK-1 in an SRF-independent manner include proteins such as CALR, GSK3 β , DLG1, ZYX and GNA11 classified into the signalling category (Appendix 14), they are reported to act as signal transducer regulators. In the case of GNA11 (Guanine nucleotide binding protein subunit alpha 11) it has been reported that it upregulates the MAPK pathway in melanocytes when constitutively active (Gessi et al., 2013). On the other hand, under starved conditions in the signalling category, proteins such as EYA3, YAP1, TIPRL, NCLN and PAK2 were found (Appendix 15). It has been reported that YAP1 (Transcriptional coactivator) plays an essential role in organ size by restricting proliferation and promoting apoptosis (Zhao et al., 2007, Hao et al., 2008, Levy et al., 2008). For example, YAP1 is phosphorylated at position Y357 in response to DNA damage; phosphorylated YAP1 shows higher affinity to p73 and it selectively coactivates p73 proapoptotic target genes. YAP1 can switch between p73-mediated proapoptotic and growth arrest target genes based on its phosphorylation state (Levy et al., 2008).

Table 13 indicates that the SUMO E3 ligase RANBP2 might be an ELK-1 *bona fide* substrate which can lead to its repression. It has been reported that RANBP2/Nup358 has an essential role in nuclear transport (Vetter et al., 1999). Interaction of RANBP2 with the nucleoporin 214 (NUP214) provides a channel through which nucleocytoplasmic transport occurs. Nup214 mediates the attachment

of Nup358 to the Nuclear Pore Complex (NPC) (Bernad et al., 2004). The BioID results found seven NUP214 peptides present under mitogen-stimulated conditions compared to starved conditions (1 peptide) and it has been classified as a protein involved in localisation (Appendix 14). Several reports indicate that RANBP2 interacts with the E2 conjugating enzymes UBC9 and UBE2I (Pichler et al., 2002, Gareau et al., 2012).

The BioID results revealed a new potential E2 ubiquitin conjugating enzyme UBE2O (Table 14) which possesses both E2 and E3 ligase activities and has been involved in mono-ubiquitination of the tumour suppressor BAP1, which regulates cell proliferation (Mashtalir et al., 2014). Furthermore, it has also been reported that UBE2O interacts with AMPK α 2 (Vila et al., 2017), BMAL1 (Chen et al., 2018), c-Maf (Xu et al., 2017) and MLL protein (Liang et al., 2017), and promotes their poly-ubiquitination and proteasomal degradation. UBE2O has been recently related to tumour initiation and progression (Ullah et al., 2019). Together, these sets of data indicate that UBE2O might be a good candidate to investigate for a role in ELK-1 ubiquitination.

The BioID result demonstrated that ELK-1 is mono-ubiquitinated at K35, K52 and K59 (section 5.6.1), these results are in agreement with the recent findings which demonstrated that the major mono-ubiquitination site on ELK-1 is at K35 (Ducker et al., 2019). I suggest that when ELK-1 is overexpressed a portion of ELK-1 is mono-ubiquitinated at K35 and the other portion of ELK-1 is poly-ubiquitinated. In my experiments my cells were stimulated for 24 h,

which can be considered as a prolonged stimulus that leads to ELK-1 poly-ubiquitination.

Figure 52 showed that in mitogen-stimulated cells, ELK-1 is phosphorylated on S200, S202, S303, S304, S324 (section 5.6.1, fig. 52). ELK-1 phosphorylation sites S200 and S202 have not been reported before. They are located within a consensus amino acid sequence (Ser/Thr)XXX(PhosphoSer/Thr) reported as the most common target for phosphorylation by GSK3 (Frame et al., 2001, Xu et al., 2009). For that reason, it would be of interest to study the effect of GSK3 β on ELK-1 amino acids 196-202 to determine if there is any relation to ELK-1 degradation. Mutagenesis experiments would be useful to understand the role of S200 and S202 in ELK-1 degradation, as well as shRNA experiments with GSK3 β . It is well known that under serum stimulation GSK3 β is inhibited by phosphorylation mediated by the PI3K-Akt pathway (Cross et al., 1995). However, I suggest that under prolonged stimulation like the one used in this experiment (24h) an alternative pathway is activating GSK3 β and may be promoting degradation of ELK-1 by phosphorylation of S200 and/or S202.

Moreover, the BioID results showed that the other kinases PTK7, BUB1, AKAP1 and SKP1 have a significant number of peptides present in ELK-1 mitogen-stimulated cells compared to ELK-1 starved conditions (section 5.6.1, table 16) which may suggest that they can interact with ELK-1. To date, there is no information related about

ELK-1 interaction with PTK7, AKAP1, BUB1 and SKP1. SKP1 is an essential component of the SCF (SKP1-CUL1-F-box protein) ubiquitin ligase complex, which mediates the ubiquitination of proteins involved in cell cycle progression, signal transduction and transcription (Yada et al., 2004). It would be of great value to repeat the experiment and corroborate the result with SKP1 which links ELK-1 ubiquitination with an F-box protein.

Together, this analysis indicates that the BioID technique represents a valuable technique for the detection of ELK-1 neighbours and it can complement standard IP-based approaches for the identification of potential E3 ligases and ELK-1 interactions partners.

7.0 CONCLUSION AND FUTURE WORK

Firstly, my results indicate that the paper published by Teixeira *et al* may be incorrect. The results that I obtained with FBXO25 and ELK-1 do not confirm that FBXO25 is the E3 ligase for ELK-1.

Secondly, my results conclude that ELK-1 can interact with SPOP in HEK293T cells. Nonetheless, it appears unlikely that SPOP promotes ELK-1 degradation.

The different approaches used in this project to find the ELK-1 E3 ligase were valid. However, taking in consideration the suggestions mentioned above could help to improve the detection of potential E3 ligases. The BioID experiment was performed one time; it needs to be repeated at least three more times including the Myc-BirA-ELK-1 vector. Standard IP using HeLa or HEK293T cells should be included in order to improve the results obtained with the BioID technique.

8.0 APPENDICES

8.1 Appendix 1

Abbreviation: ELK-1-BirA-HA

Description: Eukaryotic expression vector for C-terminus HA-tagged BirA ELK-1 fusion

Size: Vector pCDNA3.1 6410 bp

Insert: 1200 bp

Bacterial Strain: XL- blue

Resistance: Ampicillin and kanamycin

Method: PCR amplify with primers:

ELK 5'AgeF

5'-CT GCA GCC **CAC CTT G**CC ACC **ATG** GAC CCA TCT GTG ACG

3' No-stop BamHI site

GG CCC CAG AAG CCA **TGT** CT**G GAT CCG** CGG CCG CCA CCG

-cut vector with AgeI and BamHI

Sequencing: ElkH300for

Sequence:

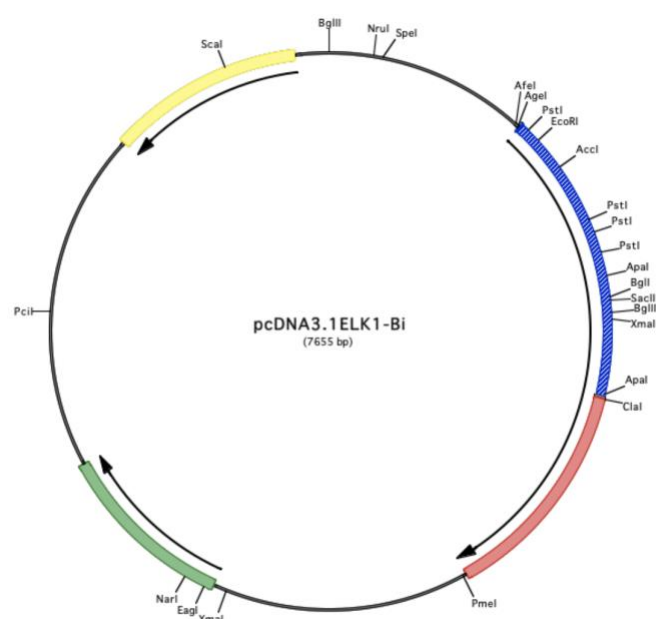
ATG= Start codon

GAATTC= AgeI site

TCTAGA= BamHI site

TGA= Stop codon

Clone chart:



8.2 Appendix 2

Abbreviation: HA-USP17

Description: Eukaryotic expression vector for HA-tagged USP17

Size: Vector pCMV5-HA 4639 bp

Insert: 1593 bp

Bacterial Strain: XL- blue

Resistance: Ampicillin

Method: PCR amplify with primers:

USP17Rlfor 5'-3'

GAAGAGGACGGA **GAATTC** GAGGACGACTCACTCTACTTGG (40 nt)

USP17XBArev 5'-3'

GGTGGGTGGTGG **TCTAGA** TCACTGGCACACAAGCAGAGCC (40 nt)

Cleave with EcoR1/Xba1; ligate into same sites in pCMV5L vector.

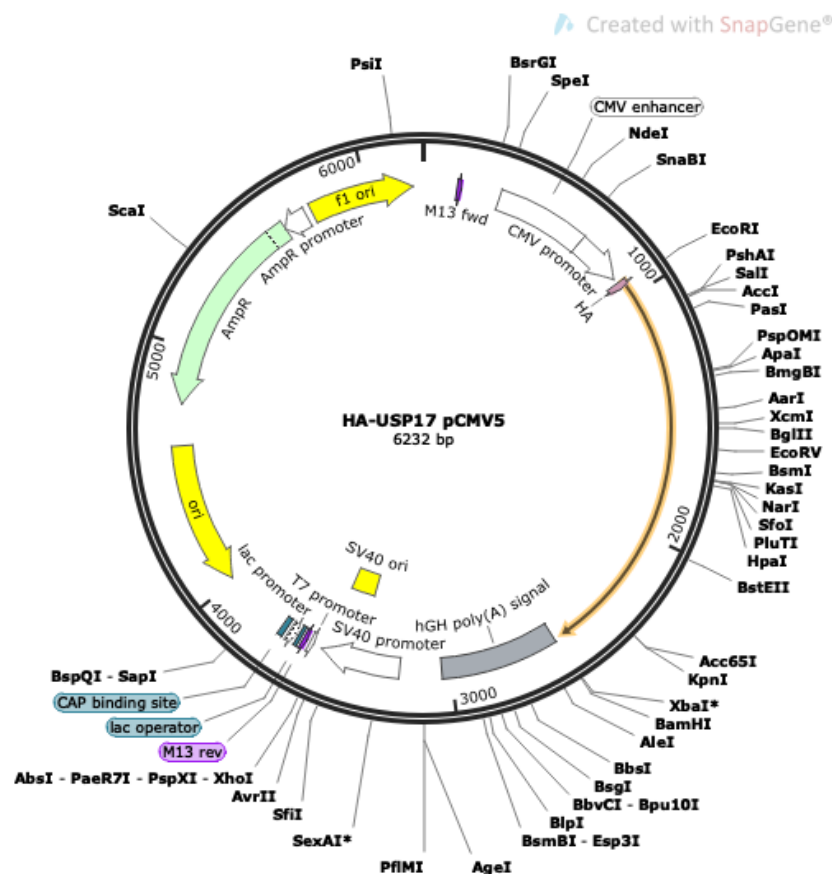
Sequencing: Primers CMVF and CMVR

Sequence:

GAATTC = EcoR1 site

TCTAGA = Xba1 site

Clone chart:



8.3 Appendix 3

Abbreviation: Myc-BirA-ELK-1

Description: Eukaryotic expression vector for N-terminus Myc-tagged BirA ELK-1 fusion

Size: Vector pCDNA3.1 6410 bp

Insert: 1200 bp

Bacterial Strain: XL- blue

Resistance: Ampicillin and kanamycin

Method: PCR amplify with primers:

ELK 5'MfeI

5'-G CAC GGA TTC **CAA TTG** GAC CCA TCT GTG ACG CTG

3' HindIII site

CCC CAG AAG CCA **TGA** CT**A AGC TT**G CGG CCG CCA CCG

-cut vector with EcoRI and HindIII

-ligate in insert with MfeI and EcoRI

Sequencing: ELK131HindR

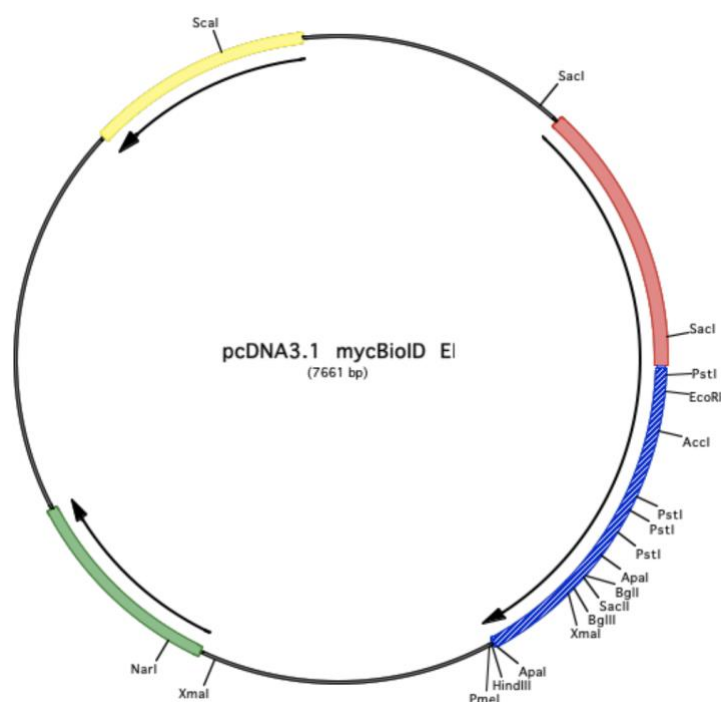
Sequence:

GAATTC = MfeI site

TCTAGA = HindIII site

TGA = Stop codon

Clone chart:



8.4 Appendix 4

Abbreviation: Myc-FBXO25 Δ F-box

Description: Eukaryotic expression vector for Myc-tagged FBXO25 deletion mutant (Δ F-box)

Size: Vector pCMV5m 4700 bp

Deletion: 117 bp

Bacterial Strain: XL- blue

Resistance: Ampicillin

Method: PCR amplify with primers:

25 Δ FBX fwd

5'- G caa gtg aac aat ggc ctc acc || cag ctg tgg aag aag ctt tgt cag -3'

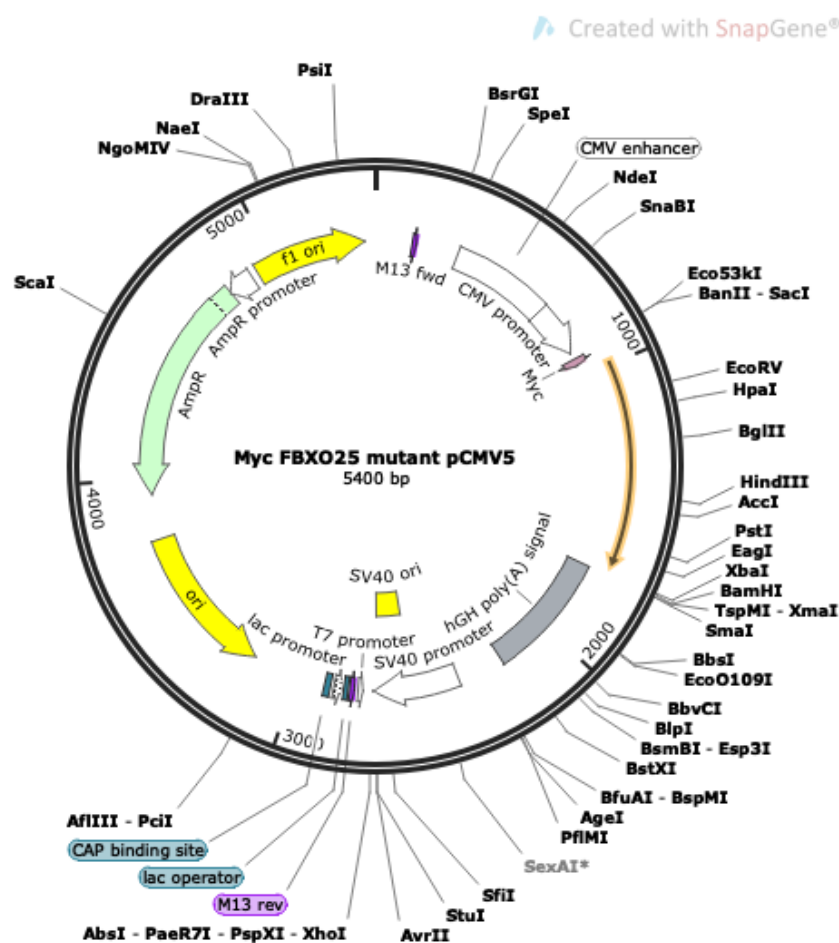
Q V N N G L T Q L W K K L C Q

25 Δ FBX rev

5'-ctg aca aag ctt ctt cca cag || ctg ggt gag gcc att gtt cac ttg c -3'

Q C L K K W L Q T L G N N V Q

Sequencing: Primers CMVF and CMVR



8.5 Appendix 5

Abbreviation: Myc-SPOP D130A

Description: Eukaryotic expression vector for Myc-tagged SPOP with Aspartate 130 substituted for Alanine.

Size: Vector pCMV5m 4700 bp

Bacterial Strain: NM522

Resistance: Ampicillin

Method: PCR amplify with primers:

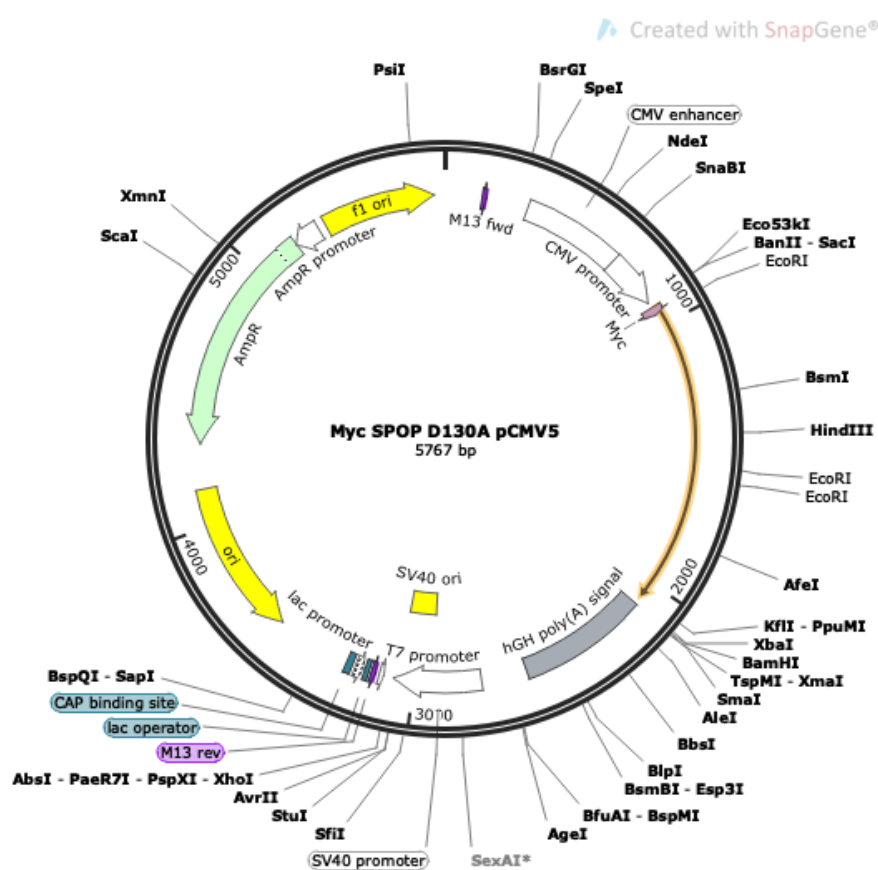
POP D130 for

5'-GTGCAAGGCAAAGCCTGGGGATTCAAG-3'

POP D130 rev

5'-CTTGAATCCCCAGGCTTTGCCTTGAC-3'

Sequencing: Primers CMVF



8.6 Appendix 6

Abbreviation: Myc-SPOP F133V

Description: Eukaryotic expression vector for Myc-tagged SPOP with Phenylalanine 133 substituted for Valine.

Size: Vector pCMV5m 4700 bp

Bacterial Strain: NM522

Resistance: Ampicillin

Method: PCR amplify with primers:

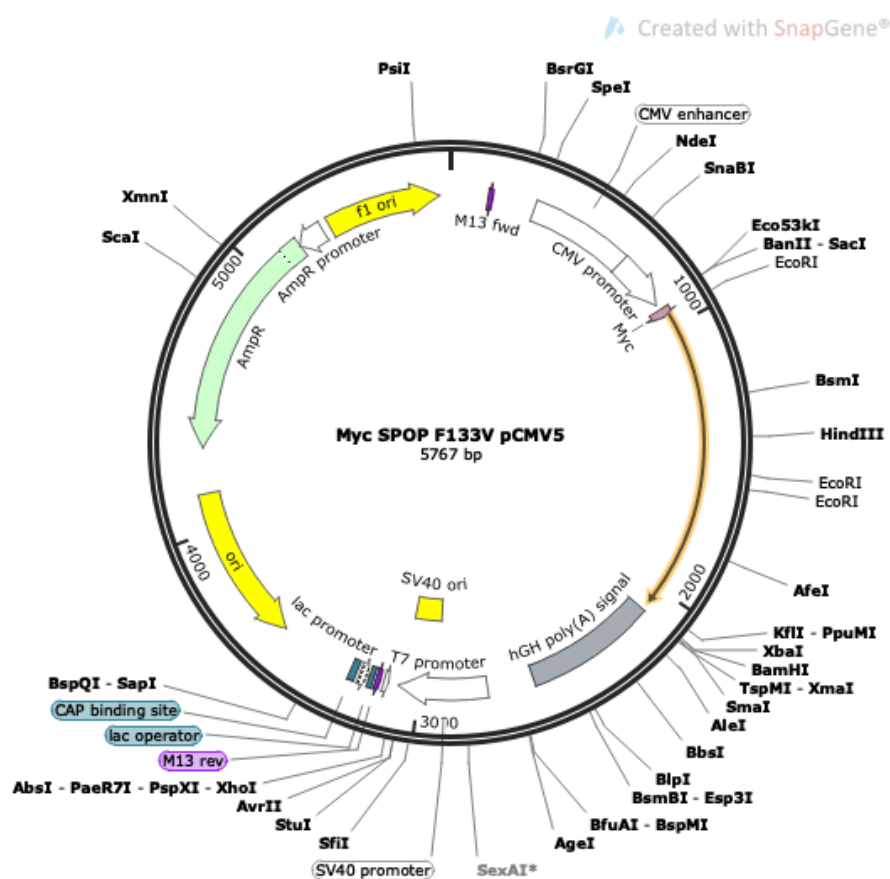
POP F133V for

5'-GCAAAGACTGGGGAGTCAAGAAATTCATC-3'

POP F133V rev

5'-GATGAATTTCTTGACTCCCCAGTCTTTGC-3'

Sequencing: Primers CMVF



8.7 Appendix 7

Abbreviation: Myc-SPOP M233E

Description: Eukaryotic expression vector for Myc-tagged SPOP with Methionine 233 substituted for Glutamate.

Size: Vector PCMV5m 4700 bp

Insert: 1642 bp

Bacterial Strain: NM522

Resistance: Ampicillin

Method: PCR amplify with primers:

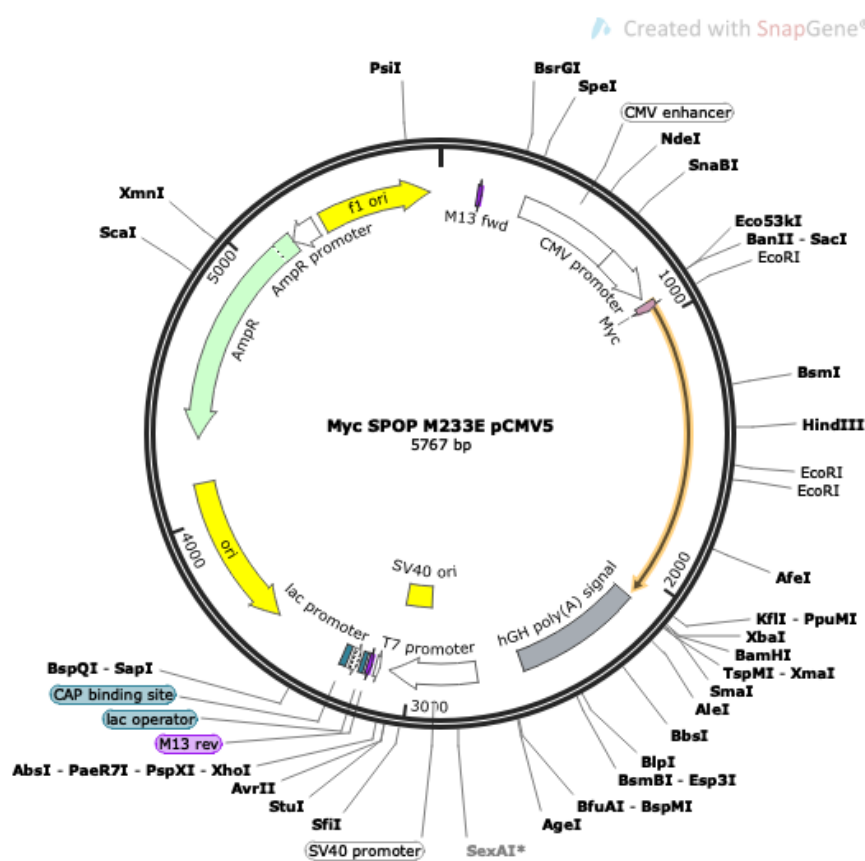
POP M233E for

5'-CCATGTTTGAACATGAAGAGGAGGAGAGC-3'

POP M233E rev

5'-GCTCTCCTCCTCTTCATGTTCAAACATGG-3'

Sequencing: Primers CMVF



8.8 Appendix 8

Abbreviation: shFBXW7 #1

Description: pSUPER FBXW7 sh#1 (5'-3')

Size: Vector pSUPER 3176 bp

Insert: 60 bp

Bacterial Strain: XL-blue

Resistance: Ampicillin

Method: Annealed oligos were ligated into pSUPER through Bgl II and HindIII.

Target sequence sense:

5'-GGTTCTGAGGTCCGCTCTT-3'

Target sequence antisense:

5' AAGAGCGGACCTCAGAACC- 3'

Hairpin:

5'-TTCAAGAGA-3'

8.9 Appendix 9

Abbreviation: shFBXW7 #2

Description: pSUPER FBXW7 sh#2 (5'-3')

Size: Vector pSUPER 3176 bp

Insert: 60 bp

Bacterial Strain: XL-blue

Resistance: Ampicillin

Method: Annealed oligos were ligated into pSUPER through Bgl II and HindIII.

Target sequence sense:

5'-GGCACCAGTCGTTAACAAG-3'

Target sequence antisense:

5' CTTGTTAACGACTGGRGCC- 3'

Hairpin:

5'-TTCAAGAGA-3'

8.10 Appendix 10

Abbreviation: shMED13

Description: pSUPER MED13 sh (5'-3')

Size: Vector pSUPER 3176 bp

Insert: 60 bp

Bacterial Strain: XL-blue

Resistance: Ampicillin

Method: Annealed oligos were ligated into pSUPER through Bgl II and HindIII.

Target sequence sense:

5'-GCTCCAGACAGTCAAGTGA-3'

Target sequence antisense:

5'-TCTCTTGACTGTCTGGAGC- 3'

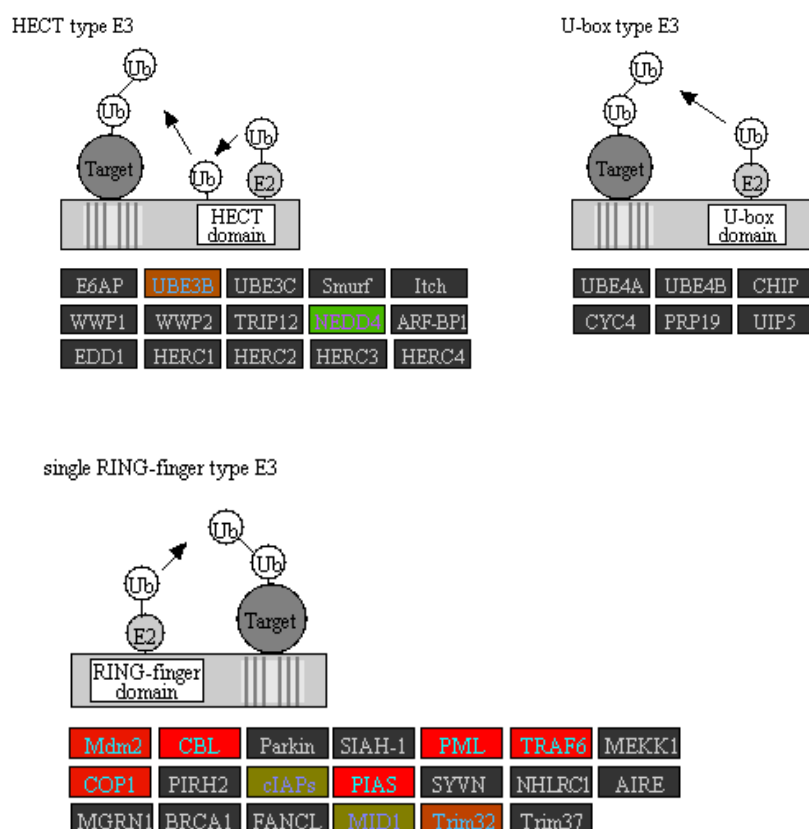
Hairpin:

5'-TTCAAGAGA-3'

8.11 Appendix 11

GEO Accession Number	Sample	Category
GSE 3325	GSM74875	Normal Prostate Gland
	GSM74876	Normal Prostate Gland
	GSM74877	Normal Prostate Gland
	GSM74878	Normal Prostate Gland
GSE 26910	GSM662756	Normal prostate stroma
	GSM662758	Normal prostate stroma
	GSM662760	Normal prostate stroma
	GSM662762	Normal prostate stroma
	GSM662764	Normal prostate stroma
	GSM662766	Normal prostate stroma
GSE 45016	GSM1095877	High grade prostate cancer
	GSM1095878	High grade prostate cancer
	GSM1095879	High grade prostate cancer
	GSM1095880	High grade prostate cancer
	GSM1095881	High grade prostate cancer
	GSM1095882	High grade prostate cancer
	GSM1095883	High grade prostate cancer
	GSM1095884	High grade prostate cancer
	GSM1095886	High grade prostate cancer
GSE 44905	GSM1093796	LNCap
	GSM1093797	LNCap
	GSM1093798	LNCap
GSE 43881	GSM1072826	LNCap
	GSM1072827	LNCap
GSE 30304	GSM751151	LNCap
	GSM751152	LNCap
	GSM751153	LNCap
GSE 27914	GSM689415	LNCap
	GSM689418	LNCap
	GSM689426	LNCap
	GSM689428	LNCap
	GSM689432	LNCap
	GSM689433	LNCap
	GSM689434	LNCap
	GSM689435	LNCap
GSE 22606	GSM560828	LNCap
	GSM560829	LNCap
	GSM560830	LNCap
GSE 17044	GSM426519	LNCap
	GSM426520	LNCap
GSE 7708	GSM87369	LNCap
	GSM87370	LNCap

8.12 Appendix 12



Appendix 12. Representation of the candidate E3 ligases downregulated when ELK-4 is upregulated using the software Kegg pathway. The signalling analysis was performed on statistically differentially downregulated genes, with red indicating a higher fold change, green indicating a lower fold change, with strength of colour representing the intensity of fold change and black insignificant.

8.13 Appendix 13

Biotinylated proteins detected using Mass Spectrometry under ELK-1 stimulated and ELK-1 starved conditions.

ELK-1 STM		ELK-1 STV		COMMON PROTEINS	
Alternate ID	Molecular weight	Alternate ID	Molecular weight	Alternate ID	Molecular weight
PDLIM1	36 kDa	YEATS2	151 kDa	CRKL	34 kDa
HAUS6	91 kDa	TIPRL	31 kDa	SPEN	402 kDa
RBBP7	48 kDa	CD2AP	71 kDa	RANBP3	53 kDa
ZYX	61 kDa	PPP6R3	94 kDa	PCNP	19 kDa
GATAD2B	63 kDa	RBM10	110 kDa	CNN3	36 kDa
CALD1	62 kDa	PFAS	145 kDa	SAR1B	22 kDa
ZMAT2	24 kDa	GABPA	51 kDa	RAB2B	17 kDa
CHD8	291 kDa	ARID3B	61 kDa	C15orf38-AP3S2	44 kDa
AKAP1	97 kDa	UNC45A	118 kDa	ACBD3	61 kDa
DCP1A	63 kDa	ARID1A	206 kDa	DYNCL1I1	57 kDa
ATXN2L	113 kDa	PUF60	57 kDa	ATF3	18 kDa
IGF2BP2	67 kDa	SF3A1	89 kDa	JMJD1C	263 kDa
DPYSL4	62 kDa	LIN54	79 kDa	CSTF2T	64 kDa
BUB1	122 kDa	DNAAF5	94 kDa	ESF1	99 kDa
RPLP2	12 kDa	CHAMP1	89 kDa	DVL3	78 kDa
SPAG5	134 kDa	EPS15L1	83 kDa	PELP1	125 kDa
TJP2	137 kDa	CBR3	31 kDa	SPDL1	70 kDa
SEC16A	249 kDa	TFCP2	57 kDa	RPAP3	76 kDa
NAP1L1	31 kDa	NCOA6	219 kDa	NUP50	50 kDa
ZNF318	251 kDa	CSDE1	89 kDa	USP15	112 kDa
SKP1	19 kDa	GTF3C4	92 kDa	FUBP1	69 kDa
BAG3	62 kDa	PPP4R3A	95 kDa	PPM1G	59 kDa
STAG2	141 kDa	THRAP3	109 kDa	JUN	36 kDa
SPC24	22 kDa	PRRC2B	166 kDa	CUX1	164 kDa
GPRASP2	94 kDa	STAT1	83 kDa	TJP1	188 kDa
EDC3	56 kDa	IPO8	120 kDa	TAF6	79 kDa
MMS19	113 kDa	ZNF516	124 kDa	SPART	73 kDa
TNRC6B	183 kDa	KRT31	47 kDa	NCAPH	82 kDa
TACC3	90 kDa	TBCD	137 kDa	CIC	258 kDa
BRD2	92 kDa	ZHX3	105 kDa	LYPLA2	25 kDa
PLIN3	47 kDa	SUGT1	41 kDa	ILKAP	43 kDa
TMEM189-UBE2V1	42 kDa	NR2C2	65 kDa	NCDN	79 kDa
DBNL	40 kDa	SMS	41 kDa	GEMIN4	119 kDa
PDAP1	21 kDa	SYMPK	141 kDa	GNA13	44 kDa
DCP1B	68 kDa	ZNF148	89 kDa	THOC1	76 kDa
C1orf52	21 kDa	SAP130	111 kDa	DCTN1	139 kDa
COTL1	16 kDa	ZW10	89 kDa	LMAN1	58 kDa
SMG7	132 kDa	GFPT1	79 kDa	CMBL	28 kDa
CYB5A	11 kDa	SEC31A	136 kDa	PPIL4	57 kDa
GNA11	42 kDa	ELF2	56 kDa	EXOSC8	30 kDa
BCORL1	191 kDa	WRNIP1	72 kDa	USP7	106 kDa
SNAP29	29 kDa	IFT27	20 kDa	TRIM24	117 kDa
GPRIN1	102 kDa	RUVBL2	51 kDa	PSMA5	26 kDa
RAI14	110 kDa	SWAP70	69 kDa	GMPS	77 kDa
APBB1	77 kDa	GTF2A1	7 kDa	DSG2	122 kDa
PHAX	44 kDa	FOXK1	75 kDa	RIC8A	60 kDa
DLG1	100 kDa	PAPOLA	83 kDa	MGEA5	103 kDa
CALR	48 kDa	RFX1	105 kDa	GEMIN5	169 kDa
XRCC1	66 kDa	NUP107	80 kDa	SEC13	32 kDa
ANKHD1	277 kDa	EYA3	63 kDa	CHMP5	20 kDa
HSPB1	23 kDa	NCOR1	259 kDa	MIS18A	26 kDa
SYAP1	40 kDa	PGLS	28 kDa	UBA6	118 kDa
EIF3I	37 kDa	LSS	83 kDa	COMMD7	23 kDa
COLGALT1	72 kDa	KPNA6	60 kDa	ZC3H4	96 kDa
AP5S1	23 kDa	UCHL5	29 kDa	CIP2A	102 kDa
TMEM199	23 kDa	WAPL	133 kDa	TPD52L2	20 kDa
CDIPT	26 kDa	PAK2	58 kDa	IDE	118 kDa
PRAF2	19 kDa	NCLN	55 kDa	TBC1D15	77 kDa
HSPB11	13 kDa	NELFE	43 kDa	VPS25	21 kDa
PPP6C	35 kDa	ZBTB21	119 kDa	TELO2	92 kDa

ELK-1 STM		ELK-1 STV		COMMON PROTEINS	
Alternate ID	Molecular weight	Alternate ID	Molecular weight	Alternate ID	Molecular weight
DPYSL5	61 kDa	SCPEP1	51 kDa	CPVL	54 kDa
PTK7	118 kDa	YAP1	50 kDa	GPATCH4	51 kDa
TP53BP2	126 kDa	SCYL2	104 kDa	PSEN1	42 kDa
PDCD5	14 kDa	GTF3C3	101 kDa	SAE1	15 kDa
HMMR	84 kDa	PIN1	18 kDa	CCDC43	26 kDa
CTCF	83 kDa	USP10	87 kDa	NAP1L4	43 kDa
SNRPC	20 kDa	XPO7	124 kDa	JAGN1	21 kDa
ATP1A1	113 kDa	YBX1	42 kDa	RUFY1	80 kDa
PSMD4	41 kDa	TMED1	10 kDa	EPB41L3	116 kDa
FANCI	149 kDa	CSTB	11 kDa	SEC11C	17 kDa
RREB1	153 kDa	TXN	9 kDa	MRI1	39 kDa
KIAA1143	17 kDa	ORC4	50 kDa	TDRKH	62 kDa
HAUS2	23 kDa	PPP5C	55 kDa	GSTO1	28 kDa
ZEB1	124 kDa	VPS72	41 kDa	EIF4ENIF1	106 kDa
SDCCAG3	19 kDa	PLPBP	30 kDa	FLYWCH2	16 kDa
SUN1	91 kDa	SIX4	83 kDa	MMAB	27 kDa
NDUFAF5	30 kDa	UBE2Z	38 kDa	MFAP1	52 kDa
GINS4	23 kDa	EXOC5	82 kDa	AGFG1	58 kDa
BCAS2	26 kDa	NUDT4	20 kDa	FAHD1	25 kDa
ISOC2	15 kDa	HGS	86 kDa	COA3	12 kDa
TIMMDC1	32 kDa	NRDC	139 kDa	ZNF503	63 kDa
TACC1	59 kDa	TFDP1	45 kDa	NDUFV3	51 kDa
ERBIN	158 kDa	PAIP1	40 kDa	TMUB1	18 kDa
BPTF	272 kDa	C9orf64	39 kDa	TACO1	32 kDa
GSK3B	47 kDa	PEF1	30 kDa	SYNGR1	18 kDa
PPP4C	35 kDa	TBC1D13	47 kDa	ALDH1A2	54 kDa
HDAC1	55 kDa	MRGBP	22 kDa	ANP32E	31 kDa
VCPIP1	134 kDa	BEND3	94 kDa	LIMA1	85 kDa
EFHD2	27 kDa	THG1L	35 kDa	TPD52	24 kDa
YEATS4	20 kDa	DBN1	71 kDa	FAM208A	117 kDa
FUBP3	62 kDa	ARPC5	16 kDa	EP400	340 kDa
NOLC1	74 kDa	NR2C1	47 kDa	EXOC4	111 kDa
IPO9	116 kDa	CDK11B	64 kDa	MRPS10	23 kDa
ZNF280C	83 kDa	CAMK2D	56 kDa	MTMR1	46 kDa
NUP214	215 kDa	GTF2E1	14 kDa	HIKESHI	22 kDa
CTTN	62 kDa	NUCKS1	27 kDa	GLG1	135 kDa
E7EVH7	84 kDa	NEK6	23 kDa	ZNF703	58 kDa
MRPS12	15 kDa	LANCL1	45 kDa	NSMCE1	31 kDa
POLD1	126 kDa	SERPINB12	46 kDa	PAFAH1B3	26 kDa
CLINT1	70 kDa	DPP3	84 kDa	COMMD8	21 kDa
NDUFA13	17 kDa	TNPO3	103 kDa	COPE	37 kDa
CDCA2	111 kDa	NELFCD	67 kDa	ANAPC10	21 kDa
		ZNF281	97 kDa	UBE3A	88 kDa
		SP2	65 kDa	TCF20	212 kDa
		PTPN11	68 kDa	IRF2BP2	59 kDa
		DACH1	79 kDa	BRAT1	88 kDa
		FDFT1	47 kDa	DKK	37 kDa
		MCMBP	73 kDa	XPNPEP1	62 kDa
		NACC1	57 kDa	BABAM2	32 kDa
		FAM49B	37 kDa	SCRIB	165 kDa
		P3H1	83 kDa	GOLGA2	113 kDa
		TAF9B	28 kDa	SLAIN1	62 kDa
		DNM1L	80 kDa	ZNF131	67 kDa
		RNPEP	73 kDa	L3MBTL3	88 kDa
		SRRT	101 kDa	LXN	26 kDa
		EHD4	61 kDa	TBX3	79 kDa
		PSME1	29 kDa	IST1	40 kDa
		CNDP2	53 kDa	USP5	96 kDa
		HEATR3	75 kDa	POLR3E	76 kDa
		SIN3A	145 kDa	CPNE1	60 kDa

ELK-1 STM		ELK-1 STV		COMMON PROTEINS	
Alternate ID	Molecular weight	Alternate ID	Molecular weight	Alternate ID	Molecular weight
		ACTR10	46 kDa	TMEM41A	30 kDa
		DPCD	23 kDa	ZBTB9	51 kDa
				STMN1	17 kDa
				SALL2	105 kDa
				CD59	12 kDa
				ESS2	53 kDa
				MAX	18 kDa
				BDH2	27 kDa
				MOCS2	21 kDa
				WAC	71 kDa
				PHC1	100 kDa
				CDC37	27 kDa
				SNX4	52 kDa
				QSOX2	78 kDa
				DCAF16	24 kDa
				IDH1	47 kDa
				ERGIC1	33 kDa
				E2F6	32 kDa
				DBT	53 kDa
				N4BP1	100 kDa
				ROCK1	158 kDa
				RAB3GAP2	156 kDa
				MCRIP2	18 kDa
				BCL2L13	54 kDa
				CAMSAP3	135 kDa
				PSPC1	46 kDa
				JMJD6	39 kDa
				PHC3	106 kDa
				PYCR1	30 kDa
				RPRD2	156 kDa
				GOLGA5	83 kDa
				DDRGK1	36 kDa
				ITPA	21 kDa
				AGTPBP1	143 kDa
				PIP4K2C	47 kDa
				TMEM9	21 kDa
				P4HB	57 kDa
				ADD1	44 kDa
				PARVA	47 kDa
				VTA1	31 kDa
				MTX1	36 kDa
				PFDN2	17 kDa
				CSTF2	47 kDa
				SAP30BP	36 kDa
				CLIC4	29 kDa
				NUDC	38 kDa
				AK6	20 kDa
				COMMD3-BMI1	53 kDa
				VTI1B	27 kDa
				NDUFB9	20 kDa
				ASF1A	23 kDa
				PCF11	89 kDa
				DLAT	51 kDa
				YY1	45 kDa
				CTR9	134 kDa
				SF3A3	59 kDa
				PSMA3	28 kDa
				SRSF1	28 kDa
				CARD19	21 kDa

8.14 Appendix 14

Biological adhesion	Cellular component organization or biogenesis
DLG1	BPTF
Biological regulation	SNRPC
BPTF	STAG2
CALR	PDLIM1
ZNF318	VCPIP1
GSK3B	GSK3B
RREB1	SMG7
SMG7	COTL1
APBB1	SEC16A
COTL1	MMS19
DCP1B	DBNL
ZNF280C	TACC1
CTCF	CTTN
SEC16A	TACC3
MMS19	SUN1
ATP1A1	HDAC1
DBNL	PSMD4
GATAD2B	ATXN2L
ZYX	BUB1
GNA11	EDC3
CTTN	Localization
DCP1A	IPO9
SUN1	NUP214
HDAC1	DLG1
TNRC6B	SEC16A
ATXN2L	ATP1A1
BUB1	AP5S1
EDC3	SUN1
Cellular process	Reproduction
BPTF	BUB1
DPYSL5	Response to stimulus
SNRPC	CALR
CALR	GIN54
ZNF318	GSK3B
STAG2	MMS19
DPYSL4	ZYX
ZMAT2	GNA11
GIN54	ANKHD1
PDLIM1	Signalling
VCPIP1	CALR
GSK3B	GSK3B
RREB1	DLG1
SMG7	ZYX
APBB1	GNA11
COTL1	Developmental process
DCP1B	PTK7
ZNF280C	PDLIM1
CTCF	GSK3B
DLG1	DLG1
SEC16A	DBNL
MMS19	TACC1
ATP1A1	TACC3
DBNL	Multicellular organismal process
SKP1	PDLIM1
GATAD2B	GSK3B
TACC1	DLG1
ZYX	DBNL
GNA11	TACC1
CTTN	TACC3
DCP1A	Metabolic process
TACC3	BPTF
SUN1	DPYSL5
HDAC1	SNRPC
TNRC6B	ZNF318
PSMD4	DPYSL4
ATXN2L	ZMAT2
BUB1	GIN54
EDC3	VCPIP1
Reproductive process	RREB1
BUB1	SMG7
Multi-organism process	APBB1
ANKHD1	DCP1B
Growth	ZNF280C
PTK7	CTCF
DBNL	MMS19
Immune system process	SKP1
ANKHD1	GATAD2B
	DCP1A
	HDAC1
	MRPS12
	TNRC6B
	PSMD4
	EDC3

8.15 Appendix 15

Biological adhesion	Cellular component organization or biogenesis
FAM49B	EYA3
Biological regulation	TMED1
EYA3	SEC31A
FAM49B	WAPL
ELF2	DNM1L
DACH1	ACTR10
LIN54	MRGBP
YAP1	DBN1
ZNF281	PSME1
WAPL	EXOC5
DNM1L	NUP107
TIPRL	HEATR3
NELFE	GTF2E1
SF3A1	CDK11B
NELFCD	ZW10
TFCP2	ARPC5
GABPA	VPS72
UBE2Z	TBCD
ZHX3	SIN3A
ZBTB21	RUVBL2
SAP130	ARID1A
SMS	PAK2
MRGBP	Growth
NCLN	DBN1
NR2C2	Cell population proliferation
DBN1	STAT1
SIX4	Immune system process
PSME1	FAM49B
STAT1	STAT1
NUP107	Localization
TFDP1	TMED1
RFX1	SEC31A
Cellular process	KPNA6
EYA3	WAPL
ORC4	DNM1L
SCPEP1	ACTR10
FAM49B	SMS
TMED1	TNPO3
ELF2	EXOC5
SEC31A	NUP107
DACH1	HEATR3
LIN54	TBC1D13
UCHL5	XPO7
YAP1	IPO8
ZNF281	ZW10
WAPL	ARPC5
DNM1L	Developmental process
TIPRL	EYA3
NELFE	ELF2
SF3A1	GABPA
ACTR10	NR2C2
NELFCD	DBN1
TFCP2	SIX4
GABPA	NR2C1
UBE2Z	PRRC2B
ZHX3	PAK2
ZBTB21	Locomotion
SAP130	SMS
UNC45A	ARPC5
SMS	Metabolic process
MRGBP	EYA3
NCLN	ORC4
NR2C2	SCPEP1
PGLS	ELF2
DBN1	DACH1
SIX4	LIN54
PSME1	UCHL5
STAT1	P3H1
GTF3C3	YAP1
EXOC5	ZNF281
NUP107	DNM1L
TFDP1	TIPRL
PAPOLA	NELFE
RFX1	SF3A1
WRNIP1	NELFCD
NUDT4	TFCP2
GTF2A1	GABPA
PPP6R3	ZHX3
GTF2E1	ZBTB21
CDK11B	SAP130
NR2C1	MRGBP
NCOR1	NR2C2
SP2	PGLS
PAIP1	SIX4
ZW10	PSME1
SMS	STAT1
MCMBP	GTF3C3
ARPC5	NUP107
VPS72	TFDP1
USP10	PAPOLA
TBCD	RFX1
ZNF148	WRNIP1
C9orf64	NUDT4
THG1L	GTF2A1
SIN3A	PPP6R3
RUVBL2	GTF2E1
ARID1A	CDK11B
FDFT1	NR2C1
SERPINB12	NCOR1
GFPT1	SP2
TAF9B	PAP1
NCOA6	SMS
PRRC2B	MCMBP
PAK2	USP10
Multi-organism process	ZNF148
STAT1	C9orf64
Multicellular organismal process	THG1L
NR2C2	SIN3A
DBN1	RUVBL2
PAK2	ARID1A
Reproduction	FDFT1
WAPL	SERPINB12
Response to stimulus	GFPT1
EYA3	TAF9B
YAP1	NCOA6
TIPRL	PAK2
NCLN	Signalling
STAT1	EYA3
WRNIP1	YAP1
PAK2	TIPRL
	NCLN
	STAT1
	PAK2

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