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An investigation into the functional roles of protein phosphatase 4 regulatory subunits PP4R3A and PP4R3B in leukemic T-cells

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Abstract

The serine/threonine protein phosphatase 4 holoenzyme consists of PP4 catalytic subunit (PP4c), which interacts with four different regulatory subunits (PP4R1, PP4R2, PP4R3, PP4R4). Previous studies have shown that PP4c acts as a tumour suppressor and controls cell fate of both leukemic T-cells and untransformed human peripheral blood T-cells. Emerging evidence suggests that PP4 regulatory subunits might also regulate cell fate independently of PP4c. In line with this, this study investigates the role of PP4R3A (SMEK1) and PP4R3B (SMEK2) in leukaemia.

SMEK1 and SMEK2 overexpression decreased cell growth, increased spontaneous apoptosis rate, and reduced colony forming ability of leukemic cells, while siRNA-mediated silencing of SMEK1 and SMEK2 led to increased short and long-term survival in these cells. Phospho-protein arrays analyses revealed that increased expression of SMEK1 and SMEK2 affected the phosphorylation of key proteins involved in five core cancer signalling pathways: MAPK3, AKT, JAK/STAT, NFκB and TGFβ. Most of the changes induced by SMEK1 and SMEK2 were mirrored in the cells overexpressing PP4c, apart from the effects on the TGFβ signalling pathway, which were SMEK1/2 specific. Eight transcription factors were confirmed to show changes in their phosphorylation in cells overexpressing SMEK1/2, suggesting a role for SMEK1/2 in the regulation of gene expression. Indeed, RNA sequencing confirmed the role of SMEK1/2 in the regulation of gene expression at epigenetic, transcriptional, and post-transcriptional levels. RNA sequencing also confirmed the tumour suppressor role of SMEK1 and the dual function of SMEK2 as an oncogene and tumour suppressor.

Taken together, this study suggests that both SMEK1/2 exert a key role in regulating cell fate of leukemic T cells, indicating that SMEK1/2 dysfunction may be important in the development and progression of leukaemia. The study also confirmed that both proteins have some non-overlapping functions and are not functionally redundant.

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As I submit this thesis it marks a decade of education and brings an end to my study in the UK. Words cannot express my greatest appreciation to my supervisor, Dr Mirna Mourtada-Maarabouni, who over the past five years has given me support and guidance and always went above and beyond her role as a supervisor. Therefore, my warmest wishes go to Mirna, a supervisor and friend whom I will be eternally grateful. I would also like to give special thanks to Dr Daniel Tonge for his role in supporting my studies, helping with data analysis and, his guidance, thank you!

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List of Acronyms

4E-BP1	4E-binding protein 1
53BP1	P53 binding protein 1
7-AAD	Dead cell marker 7-amino-actinomycin D
Ab1	Abelson interactor 1
AFT	Activating transcription factor
AIF	Apoptosis-inducing factor
AKT	Protein kinase B
AMH	Anti-Mullerian hormone
AML	Acute myeloid leukaemia
AMPK	AMP-activated protein kinase
Annexin V	Calcium-dependent phospholipid binding protein
AP-1	Transcription factor activator protein 1
APAF-1	Apoptotic peptides activating factor -1
ARHGAP11A	Rho GTPase activating protein 11A
ATF2	Activating Transcription Factor 2
ATM	ATM serine/threonine kinase
BAD	Bcl-2 associated agonist of cell death
BAFF	Lymphotoxin B and B cell-activation factor
BAK	Bcl-2 homologous antagonist/killer
BAX	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma
Bcl-xL	B-cell lymphoma-extra large
BCR	Breakpoint cluster region
bFGF	Basic fibroblast growth factor
BH3	BH3-only proteins
BMPs	Bone morphogenetic proteins
CDKs	Cyclin- dependent kinases
c-Jun	AP-1 transcription factor

c-MYC	V-Myc Avian Myelocytomatosis Viral Oncogene Homologue
CREB	cAMP response element-binding protein
c-Src	Proto-oncogene tyrosine-protein kinase cellular sarcoma
DCB1	Deleted in breast cancer 1
DDR	DNA damage response
DED	Death effector domain
DISC	Death-inducing signalling complex
DSB	DNA double strand break repair
DUSPs	Dual specific phosphatases
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eIF2a	Eukaryotic initiation factor 2A
EMT	Epithelial-to-mesenchymal transition
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
ERK1/2	Extracellular signal-regulated kinase1/2
ESCs	Embryonic stem cells
FADD	Fas-Associated Death Domain
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
FGFR2	Fibroblast growth factor receptor 2
Fos	Fos Proto-Oncogene
FOXO	Transcription factor Forkhead box O
GFP	Green Fluorescent Protein
Grb-2	Growth factor receptor-bound protein 2
GSK3A	Glycogen Synthase Kinase 3 Alpha
GSK3b	Glycogen Synthase Kinase 3 Beta
HDAC2	Histone Deacetylase 2

HDAC3	Histone deacetylase 3
HDAC4	Histone Deacetylase 4
HGS	Hepatocytes growth factor-regulated tyrosine kinase substrate
HIF-1α	Hypoxia-inducible factor 1
hprt	Hypoxanthine phosphoribosyl transferase
HR	Homologous recombination repair
HRAS	Proto-oncogene, GTPase
HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis protein
IFI27L1	Interferon alpha inducible protein 27 like 1
IGF	Insulin like growth factor
IkB	Inhibitor of NFκB
IkBa	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IKK	IkB kinase
IRS	Insulin receptor substrate
JAK/STAT	Janus kinases/signal transducer and activator of transcription
JAK1	Janus Kinase 1
JAK2	Janus Kinase 2
JNKs	c-Jun N-terminal kinases
KEGG	Kyoto 156, Encyclopedia of Genes and Genomes
lincRNAs	Long intergenic RNAs
lncRNAs	Long non-coding RNA
MAPK	Mitogen-activated protein kinase
MCC	Merkel cell carcinoma
Mcl-1	Myeloid cell leukemia-1
MCPyV	Merkel cell polyomavirus
MEK	Mitogen-activated protein-/ERK- kinase
miRNA	microRNA
MKK3	Mitogen-activated protein kinase 3

MMK6	Mitogen-activated protein kinase 6
MOMP	Mitochondria outer membrane permeabilization
MSK1	Mitogen- and stress-activated kinase 1
MSK2	Serine/threonine-protein kinase
mTOR	Mammalian target of rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NATs	Natural Antisense Transcripts
NEMO	NFκB essential modulator
NFκB	Nuclear factor kappa B
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B
NGS	Next Generation Sequencing
NHEJ	Non-homologous end joining repair
NIK	NFκB inducing kinase
NLS	Nuclear localization signal
NO	Nitric oxide
NODAL	Nodal growth differentiation factor
NPCs	Neural progenitor cells
NTRK1	Neurotrophic receptor tyrosine kinase 1
p27	Cyclin-dependent kinase inhibitor 1B
p38	p38 mitogen-activated protein kinase
P70S6K	p70 ribosomal S6 kinase
PBS	Phosphate buffered saline
PDAC	Pancreatic ductal adenocarcinoma
PDK1	Phosphoinositide-dependent kinase-1
PDK1	Pyruvate Dehydrogenase Kinase 1
PEA-15	Phosphoprotein enriched in Astrocytes
PH	Pleckstrin homology
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase

PIP_2	Phosphatidylinositol (3,4)-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PLC-γ1	Phospholipase Cγ1
PP2A	Protein phosphatase type 2A
PP4	Protein phosphatase 4
PP4c	PP4 catalytic subunit
PP4IP	Protein phosphatase 4 inhibitory protein
PP4R1	PP4 regulatory subunit 1
PP4R2	PP4 regulatory subunit 2
PP4R3 β or SMEK2	PP4 regulatory subunit 3 beta
PP4R3α or SMEK1	PP4 regulatory subunit 3 alpha
PP4R4	PP4 regulatory subunit 4
PPMs	Metal dependent protein phosphatases
PPPs	Phosphoprotein phosphatases
PRAS40	Proline-rich Akt substrate of 40kDa
preTCR	Pre-T cell antigen receptor
PS	Phosphatidylserine
PSMB3	Proteasome 20S subunit beta 3
PSPs	Protein serine/threonine phosphatases
PTEN	Phosphatase and tensin homolog, deleted on chromosome ten
PTPs	Protein tyrosine phosphatases
PVDF	Polyvinylidenedifluoride
RA	Nonenzymatic Ras association
RAF	Rapidly accelerated fibrosarcoma
RAF-1	RAF proto-oncogene serine/threonine-protein kinase
RAS	Rat sarcoma virus
RB	Retinoblastoma-associated
RIPA	Radioimmunoprecipitation assay buffer
RNAse A	Ribonuclease A
RPA2	Replication protein A2

RPS6	Phosphorylates the ribosomal protein S6
RPS9	Ribosomal protein S9
RQN	RNA Quality Number
rRNA	ribosomal RNA
RSK1	Ribosomal S6 kinase
RSK2	Ribosomal S6 kinase-2
R-SMAD	TGFβ receptor kinases
RTK	Receptor tyrosine kinase
Ryk	Receptor-like tyrosine kinase
SARA	SMAD anchor for receptor activation
SHP1	Src homology region 2 (SH-2) domain-containing phosphatase 1
SHP2	Src homology region 2 (SH2)-containing protein tyrosine phosphatase-2
siRNAs	Small interference RNAs
Smac	Second mitochondria-derived activator of caspase
SMAD	Suppressor of mothers against decapentaplegic
SMAD1	Mothers against decapentaplegic homolog 1
SMAD2	Mothers against decapentaplegic homolog 2
SMAD4	Mothers against decapentaplegic homolog 4
SMAD5	Mothers against decapentaplegic homolog 5
SMEK1/2	Suppressor of MEK: Mitogen-activated Protein/Extracellular Signal-regulated Kinase 1/2
snoRNAs	Small nucleolar RNAs
snRNPs	Small nuclear ribonucleoproteins
SOC	Super Optimal broth with Catabolite repression
SOCS	Suppressor of cytokine signalling
SOS 1	Son of Sevenless 1
Sprouty2	Protein sprout homolog 2
Src	Proto-oncogene tyrosine-protein kinase

ST	Small tumour antigen
Stat1	Signal Transducer and Activator Of Transcription 1
Stat2	Signal Transducer and Activator Of Transcription 2
Stat3	Signal Transducer and Activator Of Transcription 3
Stat5	Signal Transducer and Activator Of Transcription 5
Stat6	Signal Transducer and Activator Of Transcription 6
TAE	1X Tris-acetate-EDTA
TAK1	Mitogen-activated protein kinase kinase 7
TBK1	TANK associated kinase 1
TBS	Tris Buffer Saline
TCGA	Cancer Genome Atlas
TGF betaRI	TGFβ ligands to type I receptor
TGF betaRII	TGFβ ligands to type II receptor
TGF-beta	Transforming growth factor beta
TIPRL	TOR signalling pathway regulator
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TOMM6	Translocase of outer mitochondrial membrane 6
TP53	Tumour protein 53
TRADD	TNFR1-associated death domain protein
TRAF	Tumour necrosis factor receptor-associated factor proteins
TRAIL	TNF-related apoptosis-inducing ligand
TSC2	Tuberous sclerosis protein 2
TWIST1	Twist-related protein 1
TYK2	Tyrosine Kinase 2
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
XIAP	X-linked inhibitor of apoptosis protein
ZAP70	Zeta Chain of T Cell Receptor Associated Protein Kinase 70
γ-H2AX	Gamma H2A histone family member X

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Chapter One: Introduction

1.1 Hallmarks of Cancer

The development and progression of cancer are associated with a complicated interaction between the neoplastic cell and its neighbouring cells and the extracellular matrix (ECM). Moreover, the tumour cell acquires several characteristics; defined as the hallmarks of cancer as a result of deregulation of both the genome and the epigenome (Hanahan and Weinberg, 2011).

Six hallmarks of cancer have been identified and defined as common behaviour acquired by the neoplastic cells. These hallmarks include sustained proliferative signalling, evasion of growth suppressors, resisting cell death, induction of angiogenesis, activation invasion and metastasis and enabling replicative immortality (Hanahan and Weinberg, 2000). Through advances in research, another four hallmarks were identified: reprogramming energy metabolism, evading immune destruction, tumour promoting inflammation, and genomic instability (Hanahan and Weinberg, 2011).

1.1.2 Sustaining proliferative signalling, evading growth suppressors, and replicative immortality

In contrast to normal cells that require growth signals to grow and divide, cancer cells do not respond to the signals that regulate cell growth and division and instead maintain prolonged proliferation via autocrine signalling and without the need of any external growth signal (Hanahan and Weinberg, 2011). This results in the overproduction of growth factors such as epidermal growth factor (EGF), which in turn interacts with the tyrosine kinase cell-surface receptors such as epidermal growth factor receptor (EGFR). Activation of these

tyrosine kinase receptors leads to the constitutive activation of oncogenic signalling pathways such as: RAF-MEK-ERK, mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) and the interaction with multi-protein complex mammalian target of rapamycin (mTOR) (PI3K-AKT-mTOR) which drives uncontrolled cell proliferation (Populo, *et al*., 2012; Cornelia *et al.,* 2019). Moreover, the alterations in cell cycle in cancer cells due to the activation of these signalling pathways initiate uncontrolled cell division and growth (Cargnello and Roux, 2011).

At the same time, cancer cells can evade the anti-proliferative signals that suppress growth. The expression and activity of tumour suppressor genes which are responsible for the negative regulation of cell proliferation are lost in many, if not all cancers. These tumour suppressors include retinoblastoma-associated (RB), tumour protein 53 (TP53), Ste20-like kinase (Hippo) and PTEN (Pavlova and Thompson, 2016). RB plays an important role in the regulation of cell cycle progression by inhibiting cell progression through the restriction point in the G1 cell cycle phase (Aylon and Oren, 2011). Loss of RB activity in cancer cell leads to the removal of the cell cycle gatekeeper and their continuous proliferation (Aylon and Oren, 2011). Another tumour suppressor lost or mutated in cancer is p53, a transcription factor involved in DNA damage repairs and in initiating cell cycle arrest when DNA damage is detected (Williams and Schumacher, 2016). In the absence of p53, cells will progress through the cycle despite DNA damage (Williams and Schumacher, 2016).

Along with these, cancer cells have limitless replicative potential. Normal cells undergo limited numbers of cell division which, in part, is controlled by the loss of telomerase activity (the DNA polymerase responsible for the maintenance of the telomeric ends of chromosomes). In contrast, cancer cells have their telomerase reactivated leading to

indefinite divisions, resistance to senescence, and cell immortalization (Hanahan and Weinberg, 2011).

1.1.3 Resisting apoptosis

Apoptosis is a controlled programmed cell death which plays a vital role in normal development as well as tissue homeostasis through elimination of unwanted cells (Kerr *et al.,* 1972; Adams and Cory, 2007). The process is essential to maintain the balance between cell survival and cell death and thus evading apoptosis is an important hallmark of cancer (Hanahan and Weinberg, 2000; Negrini *et al.,* 2010). Cancer cells acquire mechanisms that help them to gain resistance to apoptosis. One of the most important sensors of DNA damage is p53 which activates apoptotic pathway in a cell with damaged DNA. In most types of cancer, p53 is deregulated or deleted, leading to the disturbance of the equilibrium, and favouring the anti-apoptotic machinery thereby promoting resistance to apoptosis (Adams and Cory, 2007; Hanahan and Weinberg, 2011).

The two apoptotic pathways known as the extrinsic and intrinsic pathways are regulated by two groups of molecules: the regulators (receptors and some caspases) and the downstream effectors (caspases) (Cragg *et al.,* 2009; Kelly and Strasser, 2011). In both pathways, absence or presence of extracellular/intracellular stimuli trigger the initiation of intracellular signalling cascades leading to the marking of apoptotic cell with “eat me” signals and their subsequent removal by phagocytosis (Dhanasekaran and Reddy, 2008).

The extrinsic pathway, also known as the death receptor pathway, is initiated by ligand-induced activation of Death Receptors which are members of the tumour necrosis factor receptor (TNFR) superfamily that includes Fas and TNF-related apoptosis-inducing

ligand (TRAIL) receptors (Marín-Rubio *et al.,* 2019). Best characterised death ligands that bind to death receptors include FAS (CD95, APO-1)-FasL, TNFR1-TNFα and TRAILR1 (DR4)-TRAIL (Pistritto *et al.,* 2016). Binding of death ligands to their correspondent death receptors leads to the trimerization of the receptor and the assembly of the death-inducing signalling complex (DISC). DISC assembly recruits pro-caspase-8 and Fas-Associated Death Domain (FADD) resulting in the cleavage of pro-caspase 8 and its activation. As a result, the active caspase-8 cleaves downstream effector caspases (caspases-3, -6 and -7) and activates them. Active caspases cleave key structural proteins and DNase proteins, such as poly (ADP-ribose) polymerase resulting in the blebbing and condensing of cells that ultimately leads to apoptosis (Dhanasekaran and Reddy, 2008).

The intrinsic apoptosis pathway, also known as the mitochondrial pathway, is initiated by intracellular death signals following DNA damage, growth factor starvation, oxidative stress, hypoxia, and hyperthermia (Campisi *et al.,* 2014; Radogna *et al.,* 2015). The mitochondrial pathway is activated when the outer mitochondrial membrane loses its potential (Δψ) leading to the disruption of the mitochondrial outer membrane (mitochondrial outer membrane permeability - MOMP) and the release of the apoptotic proteins that are normally confined in the intermembrane space into the cytosol. The release of the apoptogenic factors such cytochrome c, apoptosis-inducing factor (AIF) and second mitochondria-derived activator of caspase (Smac) into the cytosol leads to the formation of the apoptosome complex and the activation of caspase-9 (Plati *et al.,* 2011). The activated caspase 9 cleaves and activates the executioner caspases leading to apoptosis (Plati *et al.,* 2011). The intrinsic apoptosis pathway is regulated by the balance between the activity of pro- and anti-apoptotic members of the Bcl-2 family. Anti-apoptotic proteins such as Bcl-2, Bcl-xL, Bcl-w, and Mcl-1 inhibit apoptosis by binding to and suppressing two pro-apoptotic

activating proteins, Bax and Bak, which share a protein interaction domain called BH3 (Adams and Cory, 2007). Bak and Bax contribute to the disruption of the integrity of the outer mitochondria membrane leading to release of pro-apoptotic signalling proteins, and cytochrome c, further driving apoptosis (Willis and Adams ,2005; Adams and Cory, 2007) (Figure 1.1).

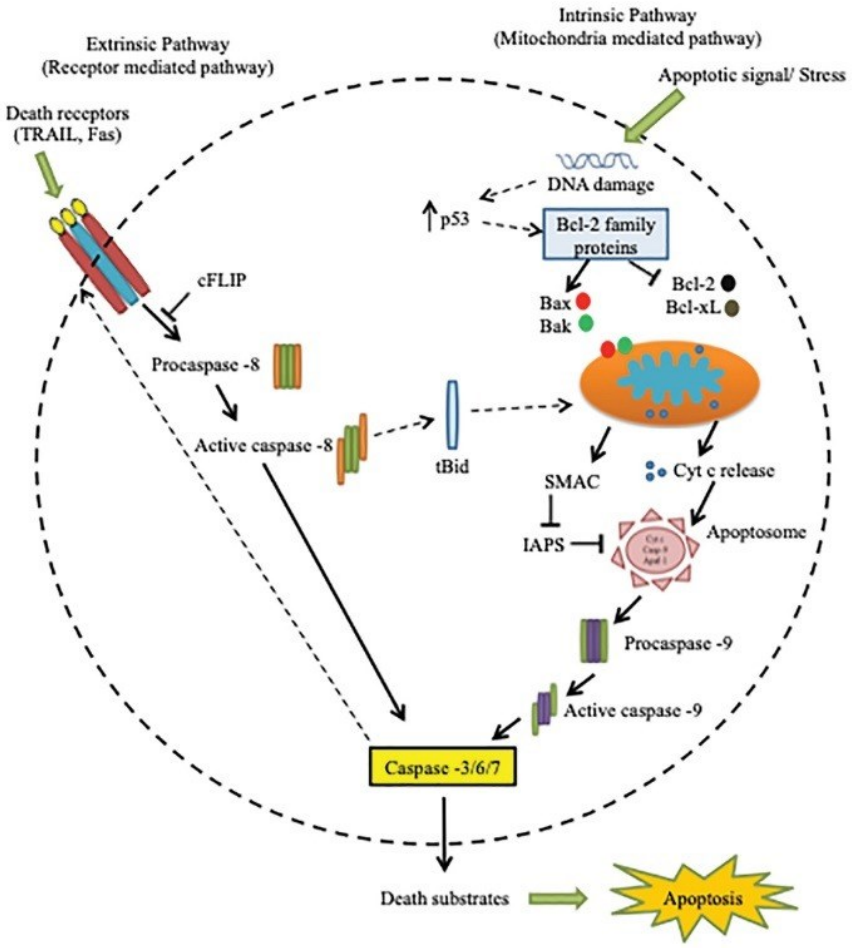


Figure 1.1 Apoptosis pathway induced by extrinsic and intrinsic pathway. Activation of the extrinsic pathway involves the transmission of signals through the tumour necrosis factor (TNF) family such as: TNF receptor 1 (TNF-R1), Fas, DR3, TNF-related apoptosis inducing ligand 1 (TRAIL-1) and TRAIL-2. Once bound, the intracellular signalling associated with recognition of the death domain of each receptor proceeds to implement the activation of adaptor proteins such as Fas-associated death domain (FADD) or TNFR1-associated death domain protein (TRADD) thereby recruiting pro-caspase 8 and form the death inducing complex (DISC). Of the caspases involved in delivering the execution of apoptosis and, proteolytic cleavage of pro-caspase 8 producing activated caspase 8 which can initiate downstream effects promoting cell degradation through the activated executioner caspases such as caspase 3/7. Activation of the intrinsic pathway increases levels and activity of pro-apoptotic proteins such as: Bax, Bak, and Bcl-xS. Bax and/or Bak oligomerization resulting MOMP and the release of cytochrome c. Cytochrome c activates apoptotic peptides activating factor-1 (APAF-1) which in turn activates pro-caspase-9, forming the apoptosome, the active caspase-9 then cleaves and activates caspase-3 leading to apoptosis (Adapted from Jan and Chaudhry, 2019).

1.1.4 Invasion and metastasis

Activating invasion and metastasis requires alterations to the cells ability to increase direct cell-cell adhesion as well as expansion into the (extracellular matrix) ECM. Loss of E-cadherin, known to be involved in serving as a key molecule in cell-cell adhesion, is lost in several carcinoma cells (Medeiros and Allan, 2019). E-cadherin can act as an antagonist of invasion and metastasis and therefore its expression is reduced under carcinoma cell expansion. Increasing the ability of epithelial cells to resist programmed cell death and to invade neighbouring cells is due to a process known as epithelial-to-mesenchymal transition (EMT). EMT allows tumour cells to acquire characteristics (potential of differentiation, immunoregulatory properties) of mesenchymal cells. The process is supported an inflammatory ECM through extracellular signalling of the TGFβ, NFκB and Wnt pathways (Tse *et al.,* 2007; Gule *et al.,* 2017). ERK1/2 activity is involved in the acceleration of EMT through its nuclear translocation, where the expression of EMT genes can be regulated (Forsyth *et al.,* 2002). The transcription factor Twist1 is known to induce EMT in a variety of tumours and to suppress oncogene-induced senescence and apoptosis (Cho *et al.,* 2013). Twist1-induced EMT enhances the invasive, metastatic and vasculogenic mimicry formation abilities of hepatocellular carcinoma cells (Sun *et al.,* 2009).

1.1.5 Inducing angiogenesis

Inducing angiogenesis involves switching the angiogenic switch into an activated sustained state that leads to the development of new blood vessels from quiescent vasculature neoplastic growth (Saman *et al.,* 2020). The process of angiogenesis is controlled

by factors that promote or oppose angiogenesis with various proteins acting as an angiogenic activators such as vascular endothelial growth factor (VEGF), angiogenin, basic fibroblast growth factor (bFGF), tumour necrosis factor-α (TNF-α), placental growth factor, interleukin-8, and EGF, or angiogenic inhibitors such as thrombospondin-1 (TSP-1) (Bergers and Benjamin, 2003; Baeriswyl and Christofori, 2009).

VEGF-A involvement in the regulation of angiogenesis and vascular permeability is via the activation of three tyrosine kinase receptors including VEGFR-1 (FIt-1), VEGFR-2 (KDR/FIk-1) and VEGFR-3 (Fit-4) (Gabhann and Popel, 2008; Ferrara, 2009). Additionally, other proangiogenic signals, including members of the fibroblast growth factor (FGF) family which has been involved in sustaining tumour angiogenesis once their expression is chronically increased (Baeriswyl and Christofori, 2009), whereas TSP-1 is expressed by endothelial cells in an effort to suppress proangiogenic stimuli (Kazerounian *et al.,* 2008).

The initiation of angiogenic switch during tumour progression is followed by different intensities of ongoing neovascularization, which is controlled by a complex biological regulator that involves cancer cells and their associated stromal microenvironment (Bergers and Benjamin, 2003; Baeriswyl and Christofori, 2009). In some tumours, activation of oncogenes such as Ras and Myc leads to the overexpression of angiogenic factors (Broustas and Lieberman, 2014).

1.2 Major Signalling Pathways in Cancer

Cancer is driven by genetic and epigenetic alterations that allows the uncontrolled growth and proliferation of cells, and therefore providing these cells with mechanisms to overcome the processes that normally control their survival and migration. Many of these

genetic alterations affect the signalling pathways that control cell growth and division, cell fate, and cell motility, and activation of many of these pathways is essential to fuel cancer progression (Sever and Brugge, 2015). Mutations that transform cellular proto-oncogenes to oncogenes often leads to the hyperactivation of these signalling pathways, and the loss of tumour suppressors activity results in the loss of the mechanisms that negatively regulate these signalling pathways (Sever and Brugge, 2015). This section provides a summary of the main signalling pathways involved in cancer.

1.2.1 MAPK Signalling Pathway

Mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases that can carry out autophosphorylation and the phosphorylation of other proteins leading to the activation or de-activation of their target. Accordingly, MAPKs are involved in regulating many important cellular functions including apoptosis, cell proliferation, and differentiation and immune defence (Dhanasekaran and Reddy, 2008). Activation of MAPK occurs through a cascade of a consecutive phosphorylation whereby following a previous stimulus, each MAPK is phosphorylated by an upstream MAPK. MAP3K is reported to activate MAP2K which in turn activates MAPK. MAPK activation can be inactivated by dephosphorylation of the phosphothreonine and phosphotyrosine residues, a reaction catalysed by MAPK protein phosphatases (MKPs) (Liu *et al.,* 2007; Pimienta and Pascual, 2007). Three subfamilies of MAPKs have been identified in mammalian cells, the p38 kinases, the extracellular signal-regulated kinases (ERKs), and the c-Jun N-terminal kinases (JNKs) (Zhang and Dong, 2007). All these three types of MAPKs are ubiquitously expressed and evolutionarily conserved in eukaryotes (Yarza *et al.,* 2016).

The MAPK signalling cascades start once growth factors bind to one member of the receptor tyrosine kinase (RTK) family, which leads to its phosphorylation and activation. The activated RTK will phosphorylate and recruit the adapter molecule such growth factor receptor-bound protein 2 (Grb-2) in the cell, the phosphorylated Grb-2 recruits nucleotide exchange factor Son of Sevenless 1 (SOS 1). SOS is involved in the activation of RAS (Vo *et al.,* 2016). Activated RAS leads to the activation of MAP kinase-ERK kinase (MEK) and extracellular signal-regulated kinases (ERK1/2). MEK and ERK regulate several processes such as cell survival, proliferation, and differentiation. In the nucleus, ERK1/2 can activate transcription factors including cAMP response element-binding protein (CREB), transcriptional regulator c-MYC and nuclear factor kappa B (NFκB) (Albensi, 2019) (Figure 1.2).

JNK1, JNK2, and JNK3 are three proteins of the JNK kinases family which are encoded by three distinct genes jnk1 (MAPK8), jnk2 (MAPK9), as well as jnk3 (MAPK3) which creates multiple splice variants of JNKs. Activated JNK in response to different stimuli induces phosphorylation events leading to specific cellular responses. JNK kinases activity is controlled via interaction with dual-specify phosphatase, scaffold proteins, and activated NFκB. Activated JNK by MAP2K phosphorylates several proteins such as transcription factor activator protein 1 (AP-1), Fos proteins (c-Fos, Fos B), activating transcription factor (AFT), c-MYC, and Bcl-2. These proteins regulate different cellular responses such as differentiation, proliferation, and apoptosis (Dhanasekaran and Reddy, 2008).

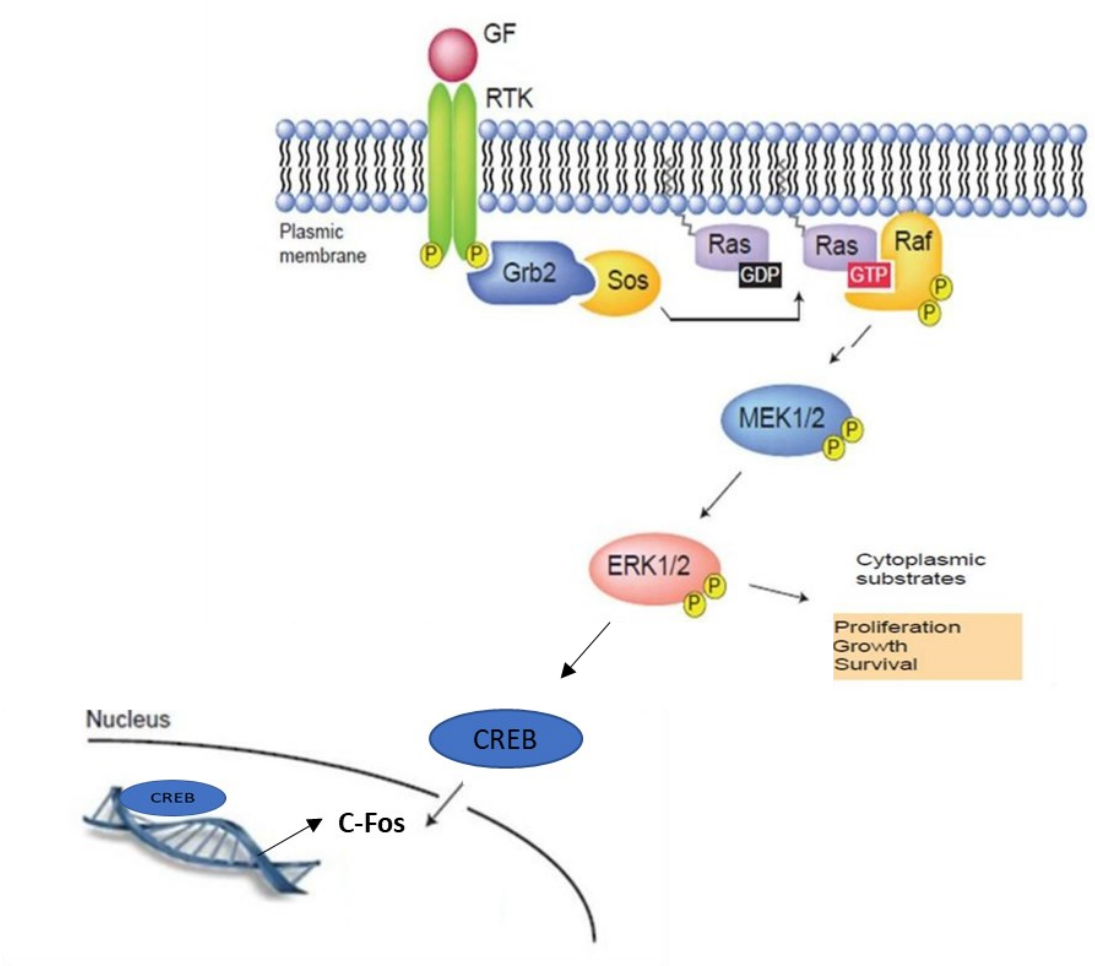


Figure 1.2 MAPK signalling pathway. Binding of growth factor (GF) to their correspondent tyrosine kinase receptor leads to the trans-autophosphorylation and activation of these receptors. Upon activation, these receptor tyrosine kinases exert downstream activation of RAS, RAF, MEK and ERK1/2 and EKR1/2 leading to the phosphorylation of several transcription factors as well as cAMP response element-binding protein (CREB) activation. Activated CREB promotes the transcription of target genes such as c-Fos (Adapted from Fremin and Meloche, 2010).

1.2.2 PI3K/AKT Signalling Pathway

The phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (AKT) signalling pathway is a key component in the regulation of cell growth and survival, proliferation, glucose metabolism, and neovascularization (Bellaocsa *et al.,* 2005). Many of the genes commonly mutated in cancer encode components or targets of the PI3K-AKT pathway. Normally PI3K/AKT pathway is transiently activated in response to growth factors however, genetic

mutations activating numerous PI3K-AKT pathway proteins can lead to constitutive activation even in the absence of growth factors (Sever and Brugge, 2015).

The PI3K-PKB/AKT pathway is highly conserved signalling pathway, and its activity is tightly regulated via a multistep process as shown in Figure 1.3. Binding of a ligand such as insulin or insulin like growth factor (IGF) to its tyrosine kinase receptor leads to the activation of the receptor and the stimulation of PI3K via an adapter molecule such as the insulin receptor substrate (IRS) proteins. Activated PI3K converts phosphatidylinositol (3,4)-bisphosphate (PIP_2) lipids to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). The generated PIP3 binds to proteins that contain pleckstrin homology domains and activates them including phosphoinositide-dependent kinase-1 (PDK1). PDK1 phosphorylates AKT at the threonine 308 residue (T308), this event facilitates the phosphorylation of AKT at serine 473 (S473) by the complex rictor/mTOR (mTORC2) leading to its activation (Song *et al.,* 2005; Altomare *et al.,* 2005). Activated AKT then phosphorylates and inactivates proline-rich AKT substrate of 40 kDa (PRAS40) and tuberous sclerosis protein 2 (TSC2) leading to the activation of mTORC1 (Inoki *et al.,* 2002; Vander Haar et al.,2007). Activated mTORC1 promotes the phosphorylation of its substrates including the eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), and ribosomal protein S6 kinase, 70 kDa polypeptide 1 (S6K1), which, in turn, phosphorylates the ribosomal protein S6 (S6/RPS6), promoting protein synthesis, cell survival, cell proliferation and inhibiting cell death (Liu *et al.,* 2009; Hers *et al.,* 2011). Additionally, AKT controls cell survival via inhibition of pro-apoptotic signals including BAD and the transcription factor Forkhead box O (FOXO), FOXO which functions as a tumour suppressor by regulating expression of genes involved in apoptosis and cell cycle (Jin *et al.,* 2016; Ouyang *et al.,* 2017). AKT activation also blocks the extrinsic apoptotic pathway by inhibition of the transcription

of proapoptotic proteins such as Fas (Pommier *et al.,* 2004) (Figure 1.3).

Genetic alterations that lead to the inactivation of the phosphatases which inhibit the PI3K-AKT pathway can also contribute to the constitutive activation of the pathway. Phosphatase and tensin homolog deleted on chromosome ten (PTEN) negatively regulates AKT function and its metabolic downstream effects. PTEN dephosphorylates PIP3 to form PIP2 leading to the inhibition of the pathway (Newton and Trotman, 2014). Mutation(s) in PTEN gene and its inactivation have been reported in many cancers including brain, prostate, and uterine cancer. These mutations lead to the enhancement of PI3K/AKT signalling and increase in cell proliferation and survival (Ouyang *et al.,* 2017).

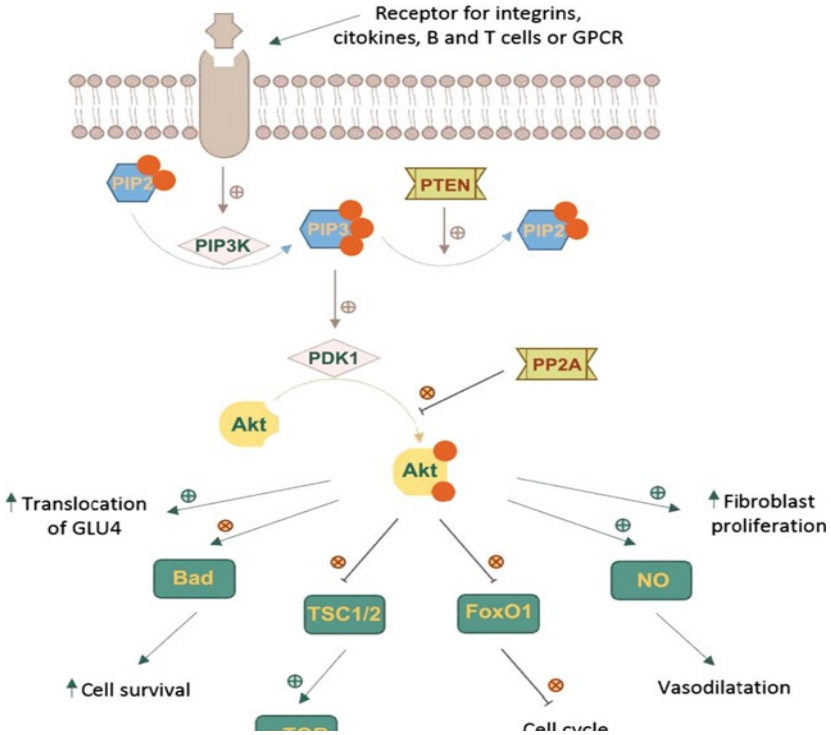


Figure 1.3 The PI3K/AKT signalling pathway. Activation of PDK1 through external stimuli such as cytokines and GPCRs (G protein coupled receptor) and subsequent production of PIP3 by PI3Ks results in AKT phosphorylation. Following AKT phosphorylation, this then targets several proteins, BAD, mTOR, FOXO1 and NO leading to promotion of cell survival, cell proliferation, and inhibition of apoptosis etc. Red circles represent de-activation and green circles represents activation (Adapted from Nitulescu *et al.,* 2018).

1.2.3 JAK/STAT Signalling Pathway

The JAK/STAT pathway is involved in the regulation of processes such as stem cell maintenance, inflammatory response, and haematopoiesis. In humans there are four JAK (Janus Kinase) family members: JAK1, JAK2, JAK3, and TYK2 (Tyrosine kinase 2) (Thomas *et al.,* 2015).

The pathway involves several transmembrane receptor families that activate upon stimuli from transducer signals such as interleukins, cytokines, and growth factors (Hu *et al.,* 2021). These receptors include intracellular domains known to be associated with the tyrosine kinase JAK. Binding of ligands causes receptor conformational alterations changing the alignment of receptor-associated JAKs, thereby leading to the phosphorylation and activation of JAKs (Brooks *et al.,* 2014). Active JAKs phosphorylate tyrosine residues in the cytoplasmic region of the receptor providing binding site to recruit and phosphorylate signal transducers and activators of transcriptions (STATs) (Thomas *et al.,* 2015). Phosphorylated STATs form dimers and translocate to the nucleus where they bind to target promoter and modulate transcription of genes involved in proliferation, differentiation, and apoptosis (Figure 1.4; Brooks *et al.,* 2014; Thomas *et al.,* 2015).

There are seven STAT family members including STAT1-4, STAT5A/B, and STAT6. Of these STAT members, STAT3 and STAT5 are reported to promote the expression of genes that enhance oncogenesis (Figure 1.4). In breast cancer, STAT3 drives the expression of epidermal growth factor, platelet-derived growth factor, hepatocyte growth factor, insulin, and insulin growth factor (Clevenger, 2004). Constitutive activation of STAT3 is reported to be important in progression of cancer in a various type of cancer such as breast cancer, head and neck

cancer, leukaemia, and lymphomas (Bowman *et al.,* 2000). Activated STAT3 accelerates the progression of cell cycle via enhancing the activity of cyclin-dependent kinases (CDKs) and enhances transcription of cell cycle positive regulators including cyclin D2 whilst decreasing transcription of CDK inhibitors including p21. STAT5 stimulates the expression of epidermal growth factor, platelet-derived growth factor, prolactin, and growth hormone (Clevenger, 2004). STAT5 has an anti-apoptotic activity via the activation of transcription of the anti-apoptotic protein Bcl-X_L (Thomas *et al.,* 2015). In contrast to STA3 and STAT5, STAT1 is generally considered to function as a tumour suppressor and reported to transcriptionally regulates the expression of cell cycle regulators, pro-apoptotic proteins and death-receptors and their ligands (Meissl *et al.,* 2017).

JAKs and STATs can be deactivated by dephosphorylation. Several phosphatases have the ability to dephosphorylate JAK and STAT proteins, thus rendering them inactive. Proteins involved in the inhibition of STATs include protein inhibitors of activated STATS (PIAS). These proteins control key aspects of STAT DNA binding, regulation of STAT locations and STAT post-translational modifications. JAKs are target of suppressor of cytokine signalling (SOCS) which inhibit their activity by binding to the phosphorylated tyrosine residue on JAK proteins preventing the recruitment and phosphorylation of STATs proteins (Thomas *et al.,* 2015).

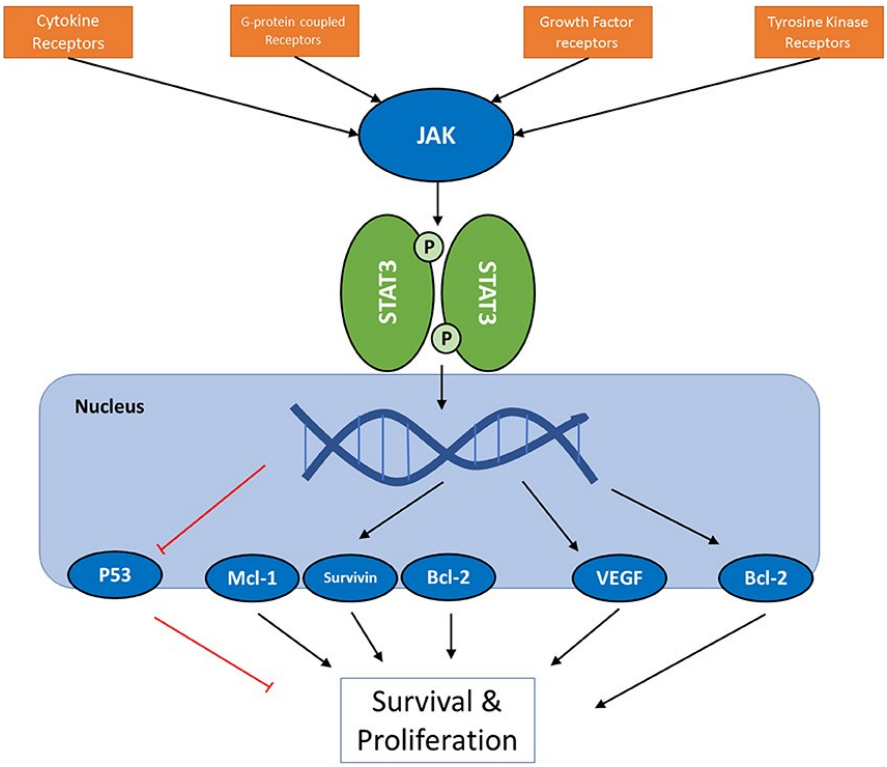


Figure 1.4 JAK/STAT signalling Pathway. Activation of the JAK/STAT pathway occurs through cytokines, G-coupled proteins-, growth factors and tyrosine kinase-receptors and leads to alterations in pro-survival genes involved in cell proliferation, anti-apoptosis, and angiogenesis. Inhibition of p53 aids in promoting survival and proliferation. Mcl-1, Survivin and Bcl-2 exert anti-apoptotic activity, and VEGF acts in promoting angiogenesis (tumour growth). Red line: inhibition effects of target protein (Bousoik and Aliabadi, 2018).

1.2.4 NFκB Signalling Pathway

NFκB (nuclear factor kappa light-chain-enhancer of activated B cells) represents a family of transcription factors involved in controlling gene expression, production of cytokines, inflammation, and cell survival (Yeh *et al.,* 2004). NFκB is usually located inactive in the cytoplasm bound to an inhibitory protein, IκBα. In response to multiple stimuli including cytokines, the IκB kinase enzyme IKK phosphorylates the inhibitory IκBα protein leading to its dissociation from NFκB and promoting its destruction by the proteasome pathway (Hoesel and Schmid, 2013). Unbound NFκB translocates to the nucleus where it binds to specific DNA sites activating the transcription of target genes involved in the immune response,

inflammation, cell growth and survival (Hoesel and Schmid, 2013). The NFκB family is composed of five different transcription factors, RelA, RelB, c-Rel, p52 and p50. Of these, RelA, RelB and c-Rel stimulate target gene expression whilst p52 and p50 repress transcription (Moynagh, 2005; Hoffman *et al.,* 2006).

The NFκB pathway can be activated via two signalling cascades, the canonical (classical pathway) and non-canonical (alternative pathway) however, both share the regulatory step of activation of IκB complex and NEMO (NFκB essential modulator). The classic pathway requires activation through cell surface receptor (such as a member of the Toll like receptor) binding to its ligand leading to recruitment of adaptors such as TRAF (Tumour necrosis factor receptor-associated factor proteins) leading to the phosphorylation and degradation of IκB inhibitor (Figure 1.5).

The alternative NFκB pathway is activated during the generation of B and T lymphocytes via lymphotoxin B and B cell-activation factor (BAFF). The IKK complex in this pathway contains two IKKα subunits, which is phosphorylated by NFκB inducing kinase (NIK) leading to activation and activation of the IKKα complex. In turn, the IKKα complex phosphorylates p100 leading to the processing and liberation of the p52/RelB active heterodimer and subsequent transcription of gene targets. Phosphorylation of NFκB p65 subunit also results in NFκB activation. For instance, protein kinase A phosphorylates p65 on Ser^{279} (Christian *et al.,* 2016). IKK complex is involved in phosphorylating p65 on Ser^{536}(Christian *et al.,* 2016), and tumour necrosis factor (TNF) phosphorylates P65 on Ser^{529} (Yang *et al.,* 2001; Krishnan *et al.,* 2015).

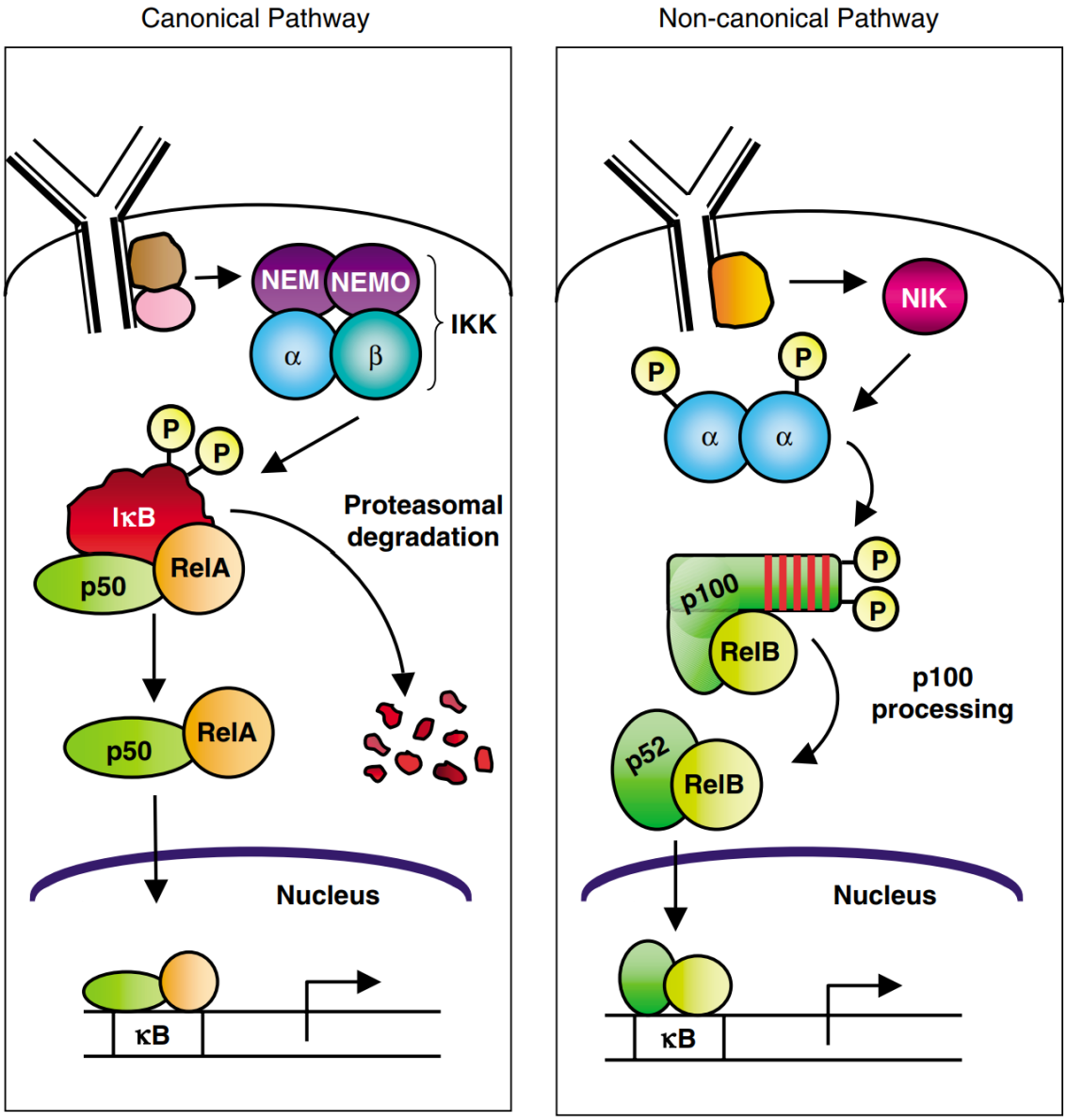


Figure 1.5 Classical and Alternative pathways in the activation of NFκB. In the classical pathway, IkBα inhibits the translocation of NFκB from the cytoplasm to the nucleus. Binding of ligands to cell surface receptors such as TNF-R results in the formation of IKK complex. The IKK complex then phosphorylates IkB leading to ubiquitination and degradation of K48 via proteasomal degradation. The alternative pathway is mainly activated during B- and T-cell organ development and relies on the activation of p100/RelB complex. Activation through specific ligand receptor binding such as BAFF, leads to activation of NFκB inducing kinase (NIK), thereby allowing the IKKa complex to form through phosphorylation. P100/RelB undergoes partial proteolysis allowing formation of p52/RelB complex that then translocates to the nucleus for subsequent targeting (Adapted from Gilmore, 2006).

1.2.5 TGFβ Signalling Pathway

The TGFβ (transforming growth factor-beta) signalling pathway is involved in regulation of cell growth, differentiation, invasion, apoptosis, extracellular matrix (ECM) production, immune response, and angiogenesis (Wakefield and Hill, 2013). TGF-β family comprises of 33 different members that share common characteristics and mechanisms of action. They include TGFβ1, TGFβ2, and TGFβ3, NODAL, growth and differentiation factors (GDF), anti-Mullerian hormone (AMH), and activins (Bierie and Moses, 2006; Watabe and Miyazono, 2009).

The stimulation of TGFβ pathway is initiated by the binding of TGFβ ligands to type II receptor (TGF betaRII). This binding leads to the activation of TGF betaRII by phosphorylation. The activated TGF betaRII then phosphorylates and activates type I receptor (TGF betaRI), Following phosphorylation, the type I receptor becomes activated resulting in activation of the canonical (dependent) pathway via phosphorylating the receptor regulated SMAD proteins (R-SMAD) (Figure 1.6). There are several SMAD isomers: SMAD1, SMAD2, SMAD3, SMAD4, SMAD5, and SMAD8. SMADS 1, 2, 3, 5 and 8 are termed R-SMADs. They contain SXS motif and are phosphorylated at their carboxy-termini by ligand activated TGFβ receptor kinases (Xu, 2006). Whilst SMAD2 and 3 only act downstream of TGFβ. Furthermore, SMAD1, 5 and 8 are only activated by Bone morphogenetic proteins (BMPs). In addition, SMAD 4 is termed as a co-SMAD as it is not phosphorylated at the carboxy-terminus. SMAD4 associates with other R-SMADs and relocates to the nucleus upon stimulation of TGFβ cytokines (Xu, 2006). SMAD 6 and 7 have been shown to inhibit TGFβ signalling (Xu, 2006). Two proteins involved in mediating the TGFβ pathway including, SARA (the SMAD anchor for receptor

activation) and HGS (hepatocytes growth factor-regulated tyrosine kinase substrate). SARA is involved in the recruitments of the R-SMAD by regulating its binding of the R-SMAD to the type I receptor (Moustakas, 2002). SARA is able to orientate the R-SMAD and is involved in the phosphorylation of serine residue on the R-SMAD causing a conformational change and thereby leading to its detachment from the receptor complex as well as SARA (Souchelnytskyi *et al,* 2001). These phosphorylated complexes translocate to the nucleus where they bind to transcription promoters, leading to the transcription of target genes. The activity of the TGFβ pathway depends on the several level of regulation such as ligand interaction with extracellular antagonists that inhibits their binding to receptors, variation of the stability of R-SMAD through phosphorylation via MAPKs and expression of inhibitory SMAD (I-SMAD), SMAD6 and SMAD7 (Wakefield and Hill, 2013).

In the non-canonical pathway, TGFβ signalling involves in the activation of SMAD-independent pathways including PI3K/AKT pathway, ERK, JNK, and p38 MAPK, c-Src, Ab1, FAK, and small GTPase including Rac1, Cdc42, and RhoA. Furthermore, TGFβ signalling benefits from extensive crosstalk with other signalling pathways including Notch, Wnt/β catenin, TNF-α, and EGF dependent pathways (Watabe and Miyazono, 2009, Sakaki-Yumoto *et al.,* 2013).

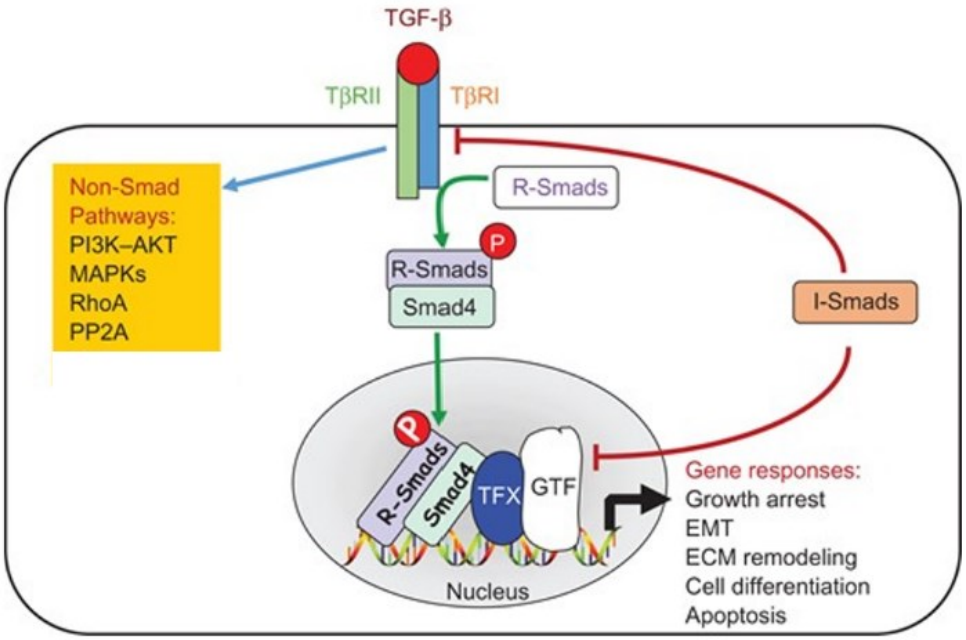


Figure 1.6 TGFβ pathway activation. Activation of the TGFβ pathway allows for the formation of a hetero-tetrameric complex between serine/threonine kinases, TβRI and TβRII via presentation of TGFβ to TβRII. Once this complex is formed, it recruits and phosphorylates SMAD2 and SMAD3 into their active states, leading to a SMAD2 or SMAD3 complex with SMAD4, thereby producing a heterotrimer. Increased levels of the SMAD complex results in an increase in transcription of a variety of genes such as transcription factors or cofactors. In addition to this, the TGFβ pathway always activates other signalling pathways such as PI3K/AKT, MAPKs, RhoA and PP2A. Inhibitory SMADs that are induced by TGFβ signalling act in a negative feedback loop (adapted from Gu and Feng, 2018).

1.3 Protein phosphorylation

Based on the physiological requirement of the cells, proteins shift from a phosphorylated to a dephosphorylated state or vice versa, a process controlled by protein kinases and phosphatases, respectively (Seshacharyulu *et al.,* 2013). Reversible phosphorylation is one of the most important and studied post-translational modifications. Regulation of reversible phosphorylation is critical to the cellular response to external and internal signals and many cellular processes including cell cycle, cell growth and apoptosis. Signalling molecules such as hormones and growth factors bind to their receptors leading to the activation of protein kinases, which in turn phosphorylate key proteins and thereby

stimulating relevant signalling pathways that directly modulate the cell physiological response to external stimuli. Protein phosphatases reverse the effects of kinases, and their role is essential for the tight regulation of the signalling pathways since kinase hyperactivity could result in too much phosphorylation and consequently uncontrolled activation or inactivation of target proteins (Bauman and Scott, 2002). Therefore, the balance between the activity of kinases and phosphatases is essential in maintaining tissue homoeostasis and for the regulation of cell growth and survival (Bauman and Scott, 2002).

The phosphorylation process by protein kinases involves the phosphate removal from an ATP prior to being attached to an amino acid on target protein, thus changing the conformation and activity of the protein. Protein Kinases have been grouped into those that phosphorylate serine/threonine residues (protein serine threonine kinases), those that phosphorylate the protein on tyrosine residues (protein tyrosine kinases) and thirdly the universal group that phosphorylate serine, threonine, and tyrosine (dual – specificity kinase) (Seshacharyulu *et al.,* 2013). The aberrant activity of many protein kinases has been determined to play a major role in the development and progression of cancer and many of these kinases have been identified to act as oncogenes (Perrotti and Neviani, 2013). Membrane-bound tyrosine kinases receptors such as EGFR, VEGFR and fibroblast growth factor receptor 2 5 (FGFR2) are examples of well-studied oncogenic kinases, as well as cytosolic protein kinases such v-raf murine sarcoma viral oncogene homolog B (B-Raf) (Levine and Broach, 1992; Perrotti and Neviani, 2013; Cicenas *et al.,* 2018). In fact, kinase gene amplifications could be used as a biomarker (prognostic, diagnostic, and predicative) in different types of cancer namely amplification and mutation in EGFR in colorectal, bladder, and breast cancers (Cicenas *et al.,* 2018). BRAF (V600E) is a well-known mutated kinase in several cancers including melanoma, colorectal cancer, and thyroid cancer (Cicenas *et al.,*

2018). Another example of an oncogenic kinase is BCR/ABL, the product of the Philadelphia chromosome (Ph) which results from the chromosomal translocation that causes a fusion of BCR gene (gene located on chromosome 9) with the ABL gene (gene encodes tyrosine kinase located on chromosome 22). The product of BCR/ABL is a constitutively active tyrosine kinase observed in 95% of patients with chronic myeloid leukaemia and signalling pathways activated by BCR/ABL are the driving force in the pathogenesis of CML (Cicenas *et al.,* 2018). The fact that many cancers are associated with constitutively active oncogenic kinases, it is logical to assume that the loss of the protein phosphatase activity which counteracts the effects of these kinases may also contribute to oncogenesis and that some of the phosphatases might functions as tumour suppressors. Accordingly, the loss of protein phosphatase 2A activity and mutations and deletion in PTEN gene have been identified in many cancer types (Ruediger *et al.,* 2001; Shin *et al.,* 2002).

1.4 Proteins Phosphatases

Contrary to kinases that phosphorylate proteins, phosphatases reverse the phosphorylation of cellular proteins, a process known as dephosphorylation (Seshacharyulu *et al.,* 2013). Phosphatases are classified into two major groups according to their substrate specificity and distinct catalytic mechanisms. Th ese two groups are protein serine/threonine phosphatases (PSPs) and the protein tyrosine phosphatases (PTPs) (Tonks, 2013; Brautigan, 2013). Dual specific phosphatases (DUSPs) are a subclass of the phosphatases, that can dephosphorylate phosphotyrosine as well as phosphoserine/phosphothreonine residues (Patterson *et al.,* 2009). In contrast to protein kinases which are encoded by more than 400 genes, a relatively smaller number of protein phosphatases genes are known to be present in

the biological system (Virshup and Shenolikar, 2009). Phosphatases can exist in active monomeric form which consists of active catalytic subunit alone, an active dimeric form (comprising of catalytic and regulatory subunit) or multimeric enzymes consisting of small number of catalytic subunits combining with many regulatory and scaffold subunits (Virshup and Shenolikar, 2009; Park and Lee, 2020). It is these combinations that regulate the activity of the phosphatases and allow coordinating and controlling many vital cellular functions (Park and Lee, 2020).

Protein serine/threonine phosphatases (PSPs) are further divided into three subfamilies, phosphoprotein phosphatases (PPPs), metal dependent protein phosphatases (PPMs) and aspartate-based phosphatases (Mumby and Walter, 1993, Barford, 1996; Moorhead *et al.,* 2009). PSPs are classified into three subclasses which include phosphoprotein phosphatases (PPPs) and aspartate-based phosphate and metal dependent protein phosphatases (PPMs/PP2C) (Shi, 2009). The mammalian serine/threonine phosphatase (PPP) group consists of the most abundant protein phosphatases and include PP1, Ca2+ -dependent PP2B, Mg2+ -dependent PP2c, PP2A and PP2A-like phosphatases such as PP6, PP5, PP7 and PP4 (Olsen *et al.,* 2006). PPPs are tightly regulated enzymes and their specificity, cellular localization, and regulation is achieved when their catalytic subunits interact with non-catalytic subunits to form multimeric holoenzymes. Each holoenzyme functions as a distinct signalling complex by controlling the activity of the catalytic subunit and establishing its substrate specificity. The association of the catalytic subunits with their partner regulatory subunits expand the number of functional phosphatases to several hundred (Shi, 2009). Figure 1.7 summarises the different PPP catalytic, regulatory, and scaffolding subunit genes and their isoforms.

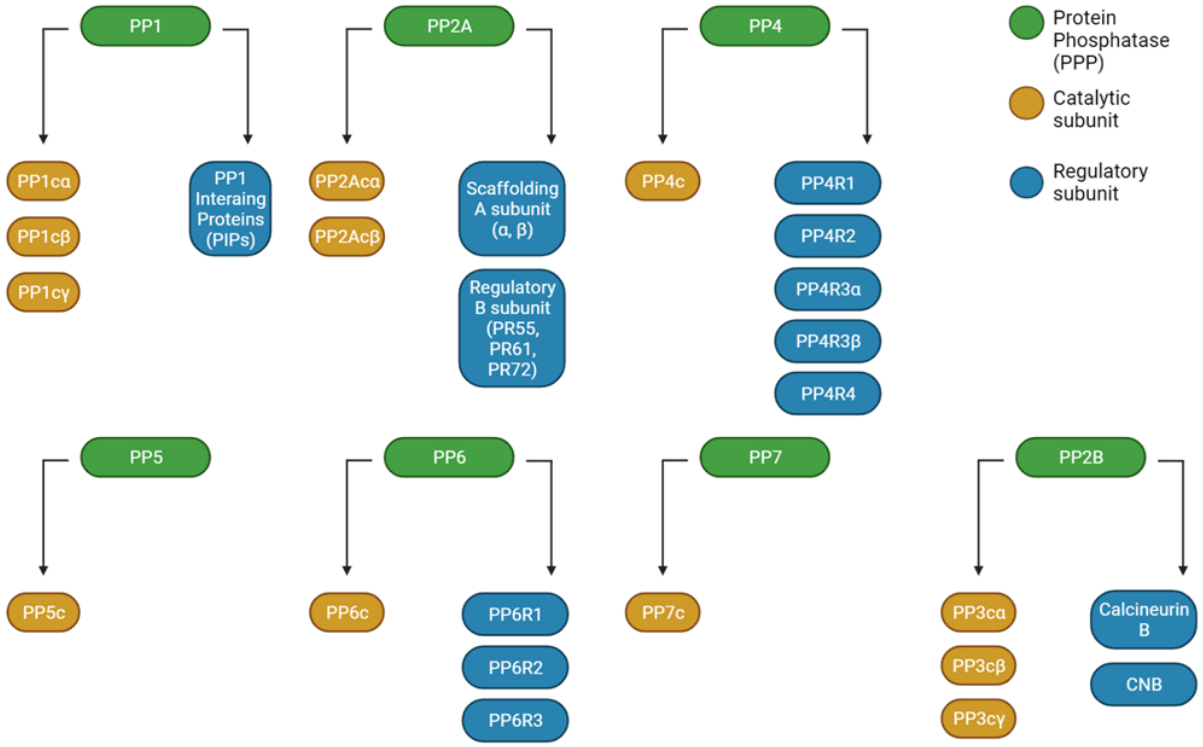


Figure 1.7 Catalytic and regulatory components of Ser/Thr phosphatases. Ser/Thr phosphatases can be categorized into phosphoprotein phosphatases (PPP) with each PPP sub divided dependent on their constituents. PPPs are classified into seven groups: PP1, PP2A, PP4, PP5, PP6, PP7 and, PP2B. Each phosphatase consists of catalytic subunit that can interact with different regulatory subunits. PP5 and PP7 consist of only one catalytic subunit (adapted from Park and Lee, 2020).

Protein phosphatase type 2A or PP2A is one of the major serine-threonine protein phosphatases in all eukaryotic cells that have been very well studied. It contributes to 0.3–1% of the total cellular protein in the mammalian cell (Chae *et al.,* 2011). PP2A a heterotrimeric complex that exists in dimeric ($PP2A_D$) and trimeric ($PP2A_T$) form (Chae *et al.,* 2011). The dimeric form consists of the catalytic and scaffold subunit, whereas the trimeric form is a complex composed of three subunits. So far, two PP2A catalytic (PP2Ac) consists of two isoforms (PP2Acα and PP2Acβ), two scaffolding subunits (PP2AA) and 15 regulatory subunits (PP2AR) have been characterised (Meeusen and Janssen, 2018). The role of PP2A in the regulation of major metabolic process, gene expression, apoptosis, cell proliferation and

cancer are well established (Seshacharyulu *et al.,* 2013). PP2A is reported to act as tumour suppressor or tumour promoter depending on the cell type (Bhardwaj *et al.,* 2011). Loss, mutations, and abnormal expression of PP2A catalytic, scaffold and regulatory subunits have been described in various human cancers including lung, breast, skin, prostate, and colon cancer, highlighting its role as a tumour suppressor (Bhardwaj *et al.,* 2011, Pandey *et al.,* 2013). On the other hand, some PP2A subunits have been implicated in tumour initiation, progression, and metastasis, underlining it role as an oncogene (Seshacharyulu *et al.,* 2013). PP2A has also been reported to regulate upstream members and regulatory proteins of the PI3K/AKT/mTOR pathway (Nakashima *et al.,* 2013). In addition, PP2A activity plays an inhibitory effect on the Wnt signalling pathway, in part, through dephosphorylation of β-catenin (Mitra *et al.,* 2012). PP2A has been shown to negatively regulate ERK/MAPK signalling by dephosphorylating key proteins in the pathway including protein sprout homolog 2 (sprouty2) and thereby disrupting activation of Ras (Bryant *et al.,* 2021).

Protein phosphatase 4 (PP4) is another member of the PPP family that has been reported to play important regulatory roles. The catalytic subunit of PP4 catalytic subunit (PP4c) is an evolutionary conserved form from yeast to human and shares more than 65% sequence identity with PP2Ac and possesses similar substrate selectivity and sensitivity towards the phosphatase inhibitors calyculin A and okadaic acid (Chen *et al.,* 2008). PP4c regulates various important biological processes independently of PP2Ac and no PP4c and PP2Ac overlapping function have been yet identified. Studies have shown that PP2Ac is unable to compensate the embryonic lethality of PP4c null mice (Shui et al., 2007). Cellular location of PP4c includes cytoplasm, nucleus, and centrosome (Chowdhury et al.,2008). Unlike the well-studied and characterised PP2Ac and its regulatory subunits, the underlying mechanism of PP4c action and the role of its regulatory subunits remains understudied.

1.5 Protein Phosphatase 4

PP4 is a serine/threonine-protein phosphate 4 also known as PPP4, PPX, and PPH3. It is a ubiquitous and highly conserved enzyme that exists as a holoenzyme composed of a highly conserved catalytic subunit (PP4c), and one or more regulatory subunits. The interaction of PP4c with different regulatory subunits controls the activity of the holoenzyme and confers its substrate specificity (Cohen *et al.,* 2005; Lillo *et al.,* 2014). The PP4 regulatory subunits include PP4 regulatory subunit 1 (PP4R1), PP4 regulatory subunit 2 (PP4R2), PP4 regulatory subunit 3 alpha (PP4R3α, PP43A or SMEK1), PP4 regulatory subunit 3 beta (PP4R3β, PP43B or SMEK2), PP4 regulatory subunit 4 (PP4R4), and alpha 4/ immunoglobulin (CD79A) binding protein 1 (α4/ IGBP1) (Hastie *et al.,* 2000; Carnegie *et al.,* 2003; Gingras *et al.,* 2005; Chen *et al.,* 2008). These regulatory subunits bind specifically to PP4c but not to its structurally related PP2Ac, except α4 that bind to both PP4c and PP2Ac (Murata *et al.,* 1997; Chen *et al.,* 1998).

PP4 plays an important role in several vital cellular processes including DNA damage repair, embryonic development, cell division and cell cycle progression (Liu and Feng, 2010). PP4 has gained much ground mostly due to the discovery of its critical role in DNA repair by dephosphorylating RPA2, and in centrosome maturation in *D. melanogaster, C. elegans* and humans (Sumiyoshi *et al.,* 2002). Empirical evidence also suggests that PP4 plays a crucial role in apoptosis (Mourtada-Maarabouni *et al.,* 2003; 2008; Mourtada-Maarabouni and Williams, 2009), tumour-necrosis factor- alpha signalling (Zhou *et al.,* 2002), activation of the c-Jun N-terminal kinase (Zhou *et al.,* 2002), histone acetylation (Zhang *et al.,* 2005), DNA damage response (Nakada *et al.,* 2008; Martin-Granados *et al.,* 2008) and the regulation of NFκB activity (Hu *et al.,* 1998).

1.5.1 PP4 Catalytic Subunit

The PP4 catalytic subunit (PP4c) gene is located on chromosome 16p11.2, a location mapped to mutations and translocations associated with acute leukaemia (Bastians *et al.,* 1997). Human PP4c protein consists of 307 amino acids with a molecular mass of 35 kDa (Cohen *et al.,* 2005). Human PP4c proteins differs from the rabbit PP4c by just two amino acids and shares 94% amino acid identity to *Drosophila melanogaster,* highlighting the high conservation of this protein during evolution (Brewis and Cohen, 1992). PP4c is ubiquitously expressed and located in centrosome, nucleus, and cytoplasm (Cohen *et al.,* 2005).

PP4c plays an important role in the regulation of many cellular functions and minimum expression level of this enzyme is necessary for cell survival and proliferation (Maarabouni and Williams, 2008). Previous studies suggest that both excessive and insufficient activity of PP4c could be lethal to the cells (Maarabouni and Williams, 2008). Many studies have implicated PP4c in various important cellular processes which range from microtubule organization, spliceosome assembly to apoptosis regulation and DNA Damage repair (Cohen *et al.,* 2005; Mourtada-Maarabouni *et al.,* 2003; 2008). PP4c plays major roles in many immune processes including cell lineage development, cytokine secretion, and immune cell receptor signalling with T-cell-specific elimination of PP4c causing deficient adaptive immunity, and ineffective T cell proliferation (Liao *et al.,* 2016). PP4c Knockout in mice is lethal, confirming a vital and non-redundant role for PP4c in ontogenesis and tissue development (Hu *et al.,* 2001; Shui *et al.,* 2007). In addition, PP4c gene deletion in the T-cell lineage resulted in abnormal thymocyte development and maturation, indicating that PP4c is essential for thymocyte development and pre-T cell antigen receptor (preTCR) signalling (Shui *et al.,* 2007). Knockout mice studies have also identified an essential role for PP4c in B cell

development (Su *et al.,* 2013). PP4c knockout mice showed a disruption in pro-B cell differentiation leading to a complete absence of mature B cells (Su *et al.,* 2013).

1.5.2 PP4c-interacting proteins

PP4c interacts with one or more of the PP4 regulatory subunits to form the PP4 holoenzyme and subsequent complexes. It is this interaction that regulate activity, mode of action, specificity, and location of the enzyme (Figure 1.8) (Cohen *et al.,* 2005). To date, a number of PP4c interacting proteins have been identified. These include PP4R1, PP4R2, PP4R3α or SMEK1, PP4R3β or SMEK2, PP4R4, and α4/ IGBP1 (Hastie *et al.,* 2000; Gingras *et al.,* 2005; Chen *et al.,* 2008). With exception of α4/ IGBP1, all the identified regulatory subunits bind specifically to PP4c but not to its structurally related PP2A (Murata *et al.,* 1997; Chen *et al.,* 1998).

PP4R1 (also known as MEG1 and $PP4R_{MEG}$) gene is mapped on chromosome 18p11.22 and has a molecular weight of 125 kDa (Chen *et al.,* 2008). PP4R1 is a distinctive non-catalytic regulatory subunit and was the first to be identified to interact with PP4c to form a complexPP4R1/PP4c (Kloeker and Wadzinski, 1999). Zhang *et al.,* (2005) described a PP4c/PP4R1 complex involved in regulating histone deacetylase 3 activity. PP4c/PP4R1 complex also acts as a negative regulator of NFκB in T lymphocytes (Brechmann *et al.,* 2012). PP4R1 protein has 14 heat repeats, like those found in the A regulatory subunit of PP2A (Kloeker and Wadzinski, 1999). HEAT repeats correspond to tandemly arranged bihelical structures that provide a form of flexible scaffolding to mediate protein-protein interactions (Abdul-Sada *et al.,* 2017).

The regulatory subunit PP4R2 is encoded by PP4R2 gene located on chromosome 3p13 and has a molecular weight of 55 kDa (Hastie *et al.,* 2000). PP4R2 gene is deleted in patients with acute myeloid leukemia (AML) and was reported to be required for DNA repair and to function as tumour suppressor protein (Herzig *et al.,* 2017). Immunological studies detected that PP4R2 is mainly located in the centrosomes indicating that it may target PP4c to this location (Hastie *et al.,* 2000). PP4R2 interacts with Gemin3 and Gemin4, components of the SMN (Survival of Motor Neuron) protein complex which is involved in spliceosome assembly and regeneration of spliceosomal components (Wada *et al.,* 2001). PP4R2 also promotes the temporal localization of newly formed snRNPs (small nuclear ribonucleoproteins) and modulates the differentiation and survival of neuronal cells (Carnegie *et al.,* 2003; Bosio *et al.,* 2012).

PP4R4 was first identified as a PP4c interacting subunit termed KIAA1622. The gene encoding PP4R4 is located on chromosome 14q32, and the protein consists of 873 amino acids and has a molecular mass of 100 kDa (Chen *et al.,* 2008). Sequence analysis of PP4R4 revealed the presence of 4 HEAT repeats PP4R4 exhibits high level of homology to the protein phosphatase 2A scaffolding A subunit (which contains 15 such repeats) (Chen *et al.,* 2008). PP4R4 also displays significant sequence homology PP4R1, which contains 13 HEAT repeats. PP4R4 is likely to interact with PP4c in a direct manner and independently of other subunits. PP4c-PP4R4 complex is reported to play role in dephosphorylation of γ-H2AX, a sensor of DNA damage (Chen *et al.,* 2008).

PP4R3 is another regulatory subunit of PP4 complex which is the focus of this thesis. There are two major isoforms of PPP4R3, PPP4R3A/B also known as SMEK1/2 (suppressor of MEK: Mitogen-activated Protein/Extracellular Signal-regulated Kinase) (Gingras *et al.,* 2005; Chowdhury *et al.,* 2008). In yeast and mammalian cells, PP4R3/SMEK forms a complex with

PP4c and PP4R2 resulting in a trimer PP4c-PP4R2-PP4R3 that have a conserved role in the resistance to cisplatin and other cytotoxic agents (Hastie *et al.,* 2000; Gingras *et al.,* 2005; Keogh *et al.,* 2006). Whereas another heterotrimeric PP4 complex PP4c-PP4R2-PP4R3β has been described to be involved in the DNA double strand break repair (DSB) (Lee *et al.,* 2010).

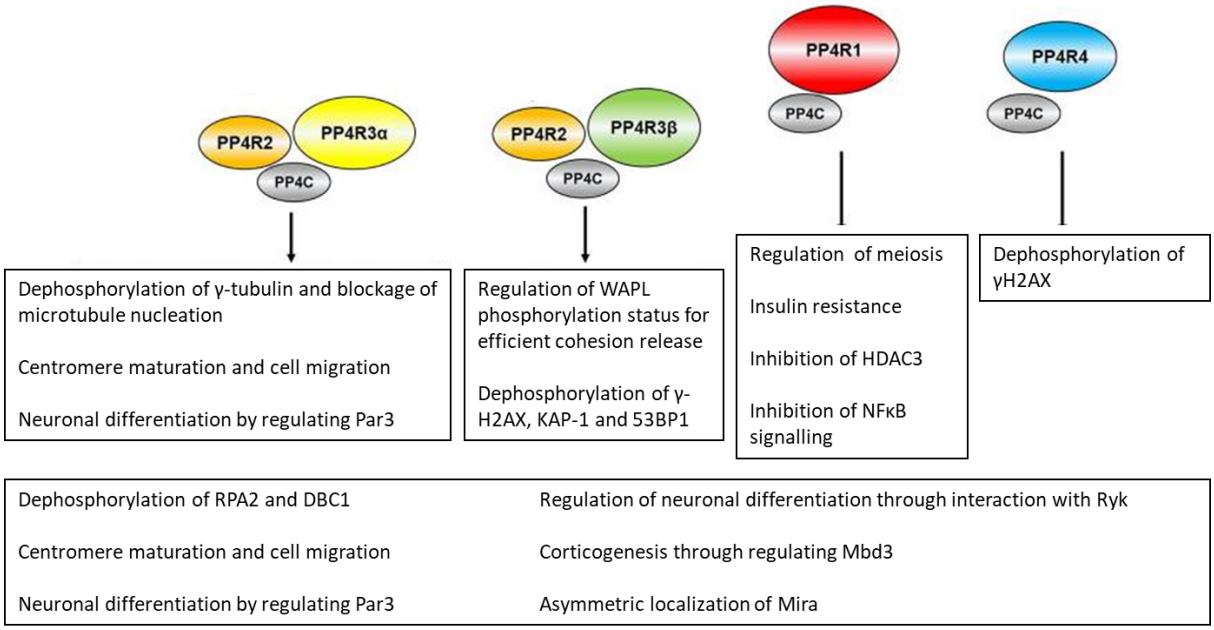


Figure 1.8 Protein phosphatase 4 complexes and their functions. The interaction of PP4c with PP4R1 or its regulatory subunits form a functional holoenzyme. PP4R1 is involved in the regulation of meiosis, insulin resistance, inhibition of Histone deacetylase 3 (HDAC3) and, inhibition of NFκB and its subsequent transcription targets. PP4c-PP4R4 compex dephosphorylates γ-H2AX, a sensor of DNA damage (adapted from Park and Lee, 2020).

1.5.3 PP4 role in DNA repair and cell cycle

PP4 has been shown to participate in several vital cellular processes including DNA damage repair, embryonic development, cell division and cell cycle progression (Liu and Feng, 2010). Investigations into the actions of PP4 have gained much interest, mostly due to the discovery of its critical role in efficient DNA repair and checkpoint control.

One of the hallmarks of oncogenesis is the loss of DNA repair systems leading to the loss of the repair of premutagenic lesions in the genome (Shimada and Nakanasi, 2013). DNA damage activates several cellular responses including, DNA damage response (DDR), DNA damage checkpoint, and initiation of apoptotic pathways (Shimada and Nakanishi, 2013). Mammalian cells employ two different pathways for DNA damage repair, the homologous recombination repair (HR) and the non-homologous end joining repair (NHEJ) (Davies and Chen, 2012). HR is involved in G_2 and S phases of cell cycle, while NHEJ mostly activated during G_1 phase of the cell cycle. Studies have reported that PP4 plays role in dephosphorylation of γ-H2AX (gamma H2A histone family member X) required for assembly of DNA repair proteins (Nakada *et al.,* 2008). Abnormal dephosphorylation of γ-H2AX by PP4c is involved in impairing DDR leading to genome instability (Sumiyoshi *et al.,* 2002; Chowdhury *et al.,* 2008). PP4c is also involved in dephosphorylation of RPA2 (replication protein A2), whose phosphorylation status is important for synthesis of DNA after DNA damage (Lee et al.,2010). Lee *et al.,* (2010) reported that PP4c regulates the phosphorylation of 53BP1 (p53 binding protein 1), that is one of the most important proteins in genome stability and tumorigenesis. During increased phosphorylation, 53BP1 is inhibited and therefore prevents NHEJ repairs (Lee et al., 2014). Studies have reported the presence of endogenous PP4c inhibitors, including dead box polypeptide 38 (DHX38), TOR signalling pathway regulator (TIPRL), and protein phosphatase 4 inhibitory protein (PP4IP) (Han *et al.,* 2015; Park *et al.,* 2019). DHX38 prevents activity of PP4 *in vitro* and *in vivo* and affects DNA DSB repairs, as well as synthesis of DNA after damage (Han *et al.,* 2015).

PP4c has been reported to regulate cell cycle. *In vitro* studies indicated a delay in G2 prior entry to the prophase in mouse embryonic fibroblast cells isolated from mice in which PP4c had been disturbed using Cre-loxP recombination (Toyo-oka *et al.,* 2008). Zhuang *et al.,*

(2014) demonstrated that siRNA mediated silencing of PP4c in HEK293 cells caused an increase in the cell populations in S phase (Zhuang *et al.,* 2014). Activity of PP4c shows dynamic alteration during the cell cycle with a peak in G1 phase and a reduction in the S phase, reaching the lowest amount in G2/M phase (Voss *et al.,* 2013). Huang *et al.,* (2013) reported that PP4c has dual function during cell proliferation with both over-expression and inhibition of PP4c leading to the prevention of cell proliferation (Huang *et al.,* 2013). PP4c over-expression inhibits the prometaphase/metaphase transition through induction of defects in chromosomes alignment and spindle assembly (Huang *et al.,* 2013), suggesting that PP4c is essential for recovery from a checkpoint induced arrest through dephosphorylation and inactivation of p53 in G1 phase (Shaltiel *et al.,* 2014).

1.5.4 PP4 function in JNK and NFκB signalling pathways

PP4c have been shown to activate Jun N-terminal kinase (JNK) pathway which is essential to relay TNF-α signalling (Zhou *et al.,* 2001). TNF-α is an important cytokine involved in inflammatory and immune responses and in many other important cellular processes, such as proliferation, differentiation, and apoptosis. Studies have shown that TNF- α increases PP4c phosphatase activity and its phosphorylation on serine and threonine residues in HEK293T embryonic kidney cells (Zhou *et al.,* 2001). The involvement of PP4c in TNF-α/JNK signalling was blocked by dominant negative mutant of PP4c, further demonstrating the involvement of PP4c in TNF-α induced activation of JNK pathway (Zhou *et al.,* 2001).

Studies have reported counteracting effects of PP4c on NFκB activation. PP4c is reported to act as a positive regulator of nuclear factor-kappa B (NFκB) activity in human cervical carcinoma SiHa (Yeh *et al.,* 2004). Whereas in T Lymphocytes, PP4c has been

characterised to have the opposite effects and negatively regulate the activity of NFκB activity (Brechmann *et al.,* 2012). Negative regulation of the activity of NFκB in T lymphocytes has been linked to PP4c-PP4R1 complex (Brechmann *et al.,* 2012). T cell receptor or tumour necrosis factor (TNF) receptor 1 (TNFR1) stimulation leads to the phosphorylation of NFκB kinase (IKK) complex followed by the proteasomal degradation of subsequent inhibitory kB proteins (IkBs) resulting in the translocation of NFκB proteins to the nucleus, where they bind to DNA and activate the transcription of NFκB-regulated genes (Hayden and Ghosh, 2006). Brechmann *et al.,* (2012) have showed that PP4R1 potentiates the activity of PP4c and allows its association with IKK complex leading to its dephosphorylation and inactivation and consequently the inhibition of NFκB activity. Their work indicated that in the absence of PP4R1, PP4c is unable to dephosphorylate the IKK complex, resulting in unrestrained IKK phosphorylation and abnormal NFκB activity (Brechmann *et al.,* 2012).

1.5.5 PP4 function in apoptosis and cancer

The involvement of PP4c in the regulation of apoptosis and cell proliferation in human cells was demonstrated in HEK239T, Jurkat and CEM-C7 leukemic T cells where studies revealed a tumour suppressive role for PP4c. These studies showed that PP4c overexpression reduced cell proliferation via the induction of apoptosis and cell cycle arrest in G1 phase (Mourtada-Maarabouni and Williams, 2008; Mourtada-Maarabouni and Williams, 2009). Proteomics approach indicated that changes in the expression levels of endogenous PP4c affects the phosphorylation status of proteins involved in cellular proliferation and apoptosis such as BAD, ERK1 and ERK2 (Extracellular signal-regulated kinase 1 and 2), Cofilin 1, Akt1, c-Fos, STAT3 (Signal transducer and activator of transcription 3) and PEA-15 (Phosphoprotein

enriched in Astrocytes (Xie *et al.,* 2013). Reduced levels of PP4c were associated with an increase in the phosphorylation of the BH3 -only pro-apoptotic Bcl-2 family protein BAD (Mourtada-Maarabouni and Williams, 2008). BAD phosphorylation inhibits dimerization with Bcl-x_L thereby preventing cytochrome c release and blocking activation of the execution caspase-3 (Jiang *et al.,* 2013). In addition, decreased expression of PP4c in leukemic and breast cancer cells was associated with an increased in the phosphorylation status of phosphoprotein enriched in astrocytes 15kDa (PEA15), another regulator of apoptosis (Mohammed *et al.,* 2016). PEA-15 is a member of death effector domain (DED) protein family that regulates cell proliferation as well as apoptosis (Renault, 2003). PEA-15 can act as either as a tumour suppressor or tumour promotor based on its phosphorylation status. Unphosphorylated form of PEA-15 binds to ERK1 and ERK2 and inhibits their nuclear translocation and their positive effects on proliferation (Krueger *et al.,* 2005). Phosphorylated form of PEA-15 is recruited to the DISC that prevents the extrinsic pathway of apoptosis.

One of the major causes of many types of human cancer is genomic instability which leads to an increase in the mutation rate and the accumulation of genetic changes resulting in the transformation of a normal cell into cancerous cell (Eshleman *et al.,* 1995). Studies have shown that PP4c over-expression reduces the mutation in the indicator gene, hypoxanthine phosphoribosyl transferase (hprt), whereas PP4c down regulation increased its mutation frequency (Mourtada-Maarabouni and Williams, 2008; 2009). These observations support the hypothesis that PP4c may function as a tumour suppressor (Krueger *et al.,* 2005).

While many studies describe PP4c to act as a tumour suppressor, other studies have suggested that PP4c may act as an oncogene (Wang *et al.,* 2008; Weng *et al.,* 2012). PP4c was reported to be overexpressed in human breast and lung tumours and decrease in its

expression was associated with an increased sensitivity to cisplatin treatment in breast and lung cancer cells (Wang *et al.,* 2008). PP4c was also reported to be over expressed in patient with pancreatic ductal adenocarcinoma (PDAC) and this overexpression was associated with poor prognosis and distant metastasis in patient with stage II PDAC (Weng *et al.,* 2012).

1.6 Protein Phosphatase 4 Regulatory Subunits 3

The focus of this thesis on the protein phosphatase 4 regulatory subunit 3 (PP4R3). Human PP4R3 exists in two isoforms PP4R3α/PP4R3A (termed SMEK1) and PP4R3β/PP4R3B (termed SMEK2) (Gingras *et al.,* 2005). The term SMEK stands for suppressor of MEK (Mitogen-activated Extracellular Signal regulated Kinase) (Yoon *et al.,* 2010). SMEK1 and SMEK2 are the terms used throughout the thesis to refer to PP4R3 regulatory subunits. Both proteins play a role in a different molecular process including enhancement of hepatic gluconeogenesis (Yoon *et al.,* 2010), cell cycle progression and dephosphorylation of the histone variant γ-H2AX in mammalian cells (Chowdhury *et al.,* 2008). Phosphorylation of H2AX to form γ-H2AX is one of the first step that occurs in DNA double strand break repair (DSB) (Chowdhury *et al.,* 2008). Starvation in *Dictyostelium* leads to the specific translocation of SMEK1 and SMEK2 to the nucleus and overexpression of both SMEKs results in the accumulation of PP4c in the nucleus, suggesting that both proteins regulate the localisation of PP4c to the nucleus (Mendoza *et al.,* 2005). SMEK1 and SMEK2 are described to form complex with PP4c-PP4R2. PP4c-PP4R2-SMEKs complex is the major form of PP4 holoenzyme that is evolutionary conserved from yeast to human (Lipinszki *et al.,* 2015). In yeast and mammalian cells, the trimeric complex of SMEK1, PP4c and PP4R2 has a conserved role in resistance to cisplatin and other cytotoxic agents

(Hastie *et al.,* 2000; Gingras *et al.,* 2005). Whereas another trimeric complex of SMEK2, PP4c and PP4R2 has been reported to be involved in the DSB (Lee *et al.,* 2010).

Both PP4R3 orthologues, SMEK1 and SMEK2, are conserved throughout evolution with similar domain architecture and have been found in a wide variety of organisms from yeasts to humans (Lipinszki *et al.,* 2015). SMEK1 and SMEK2 genes are located on distinct chromosomes with SMEK1 gene mapping to chromosome 14q32 while SMEK2 gene is located on chromosome 2p16. SMEK1 and SMEK2 proteins have a molecular mass of 95kDa and composed of 820 and 849 amino acids (aa), respectively (Figure 1.9).

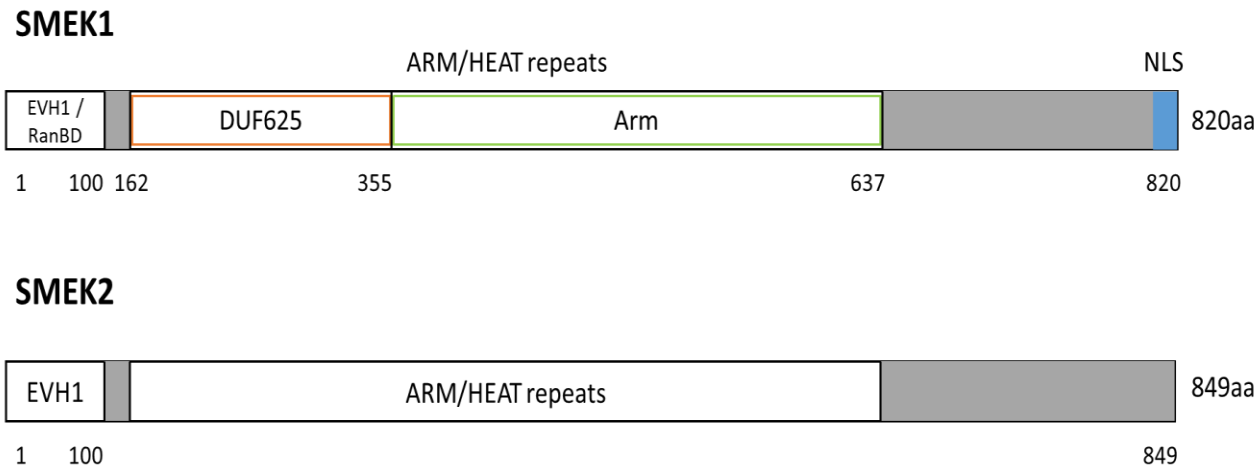


Figure 1.9 Schematic representation of SMEK1 and SMEK2 sequence and N-terminal domain. SMEK1 is composed of a RanBD/EVH1 N-terminal domain, a DUF625 region and Arm repeat, and a C-terminus NLS region. SMEK2 is composed of a EVH1 N-terminal domain, an ARM/HEAT repeats region and is 849 aa in length (adapted from Lyu et al., 2013; Ueki et al., 2019; 2020).

The similarity score between the two PP4R3 isoforms, SMEK1 and SMEK2 based on the T-Coffee alignment program (Di Tommaso et al., 2011) was 96, suggesting that these proteins might have similar function. SMEK proteins contain four conserved domains that are conserved between SMEK1 and SMEK2: EVH-1 (RanBD), domain of unknown function (DUF625), a variable number of ARM (armadillo/HEAT repeats) and nuclear localization signal

(NLS) (Lipinszki *et al.,* 2015). The carboxy-terminal unstructured tail contains low complexity region which varies in length between different species. SMEK1 has an additional Vitellogenin_N, domain, a conserved region found in several lipid transport proteins. The SMEK EVH-1 domain is necessary for cytoplasmic/cortical localization whereas the NLS is responsible for its nuclear localization (Fedorov *et al.,* 1999). EVH1 domains are structurally related to PH (Plekstrin Homology) domains but generally bind to proline-rich amino-acid sequences rather than phospholipids. The EVH1 domain of SMEK1 is evolutionary conserved from yeast to human. It contains sequence that allows the binding of PP4c to SMEK1 (Lyu *et al.,* 2013).

SMEKs binding to PP4c confers substrate specificity, sub-cellular localisation and holoenzyme stability and have been shown to be important in targeting PP4c to centromeres (Lipinszki *et al.,* 2015). Failure to do this results in some loss of integrity of the centromere during mitosis (Lipinszki *et al.,* 2015). Smk-1/DUF625 of SMEK appears interacts with the PP4 substrate Par3 (Partitioning-defective 3) in mouse neuronal differentiation (Lyu *et al.,* 2013) and to mediate the interaction with the receptor-like tyrosine kinase (Ryk), a Wnt receptor important for cell fate determination during corticogenesis (Lipinszki *et al.,* 2015). The Arm repeat domain consists of a multi-helical fold comprised of two curved layers of α-helices arranged in a regular right-handed superhelix (Ueki et al., 2020). This domain is required for the formation PP4c/SMEK1 complex and is important together with EVH1 and DUF625 domains for SMEK1 regulation of neuronal differentiation through PP4c (Lyu *et al.,* 2013).

SMEK proteins have been described to regulate Wnt signalling pathway, which known to regulate cell-fate differentiation, growth and proliferation, and homeostasis (Hall *et al.,* 2017). In embryonic stem cells (ESCs), the expression of development related Wnt responsive genes is normally silenced to maintain pluripotency and only activated during differentiation (Clevers, 2006). Transcriptional repression results from the deacetylation of histones mediated

by HDACs while acetylation of histones by histone acetyltransferases stimulates transcription. SMEKs proteins have been shown to promote histone deacetylation in ESCs and the subsequent silencing of the Wnt-responsive gene, *brachyury* (Lyu *et a.,* 2011). Activation of Wnt signalling pathway during differentiation results in the disruption of the SMEK-PP4c-HDAC1 complex leading to an increase in histones H3 and H4 acetylation at the *brachyury* gene locus. This provides evidence that SMEK-containing PP4 complex is essential for ESC pluripotency maintenance via the promotion of transcriptional repression of Wnt-responsive development-related genes through histone deacetylation (Lyu et al., 2011).

Previous studies have shown that SMEK1 is involved in neurogenesis. It promotes neuronal differentiation in the mouse forebrain neocortex and inhibits the proliferation of the multipotent Neural progenitor cells (NPCs) which can self-renew and differentiate into neurons and glial cells (Lyu *et al.,* 2013). SMEK1 inhibition of NPCs is mediated via its interaction with Par3, a negative regulator of neuronal differentiation. SMEK1, which is mainly located in the nucleus, translocates to the cytoplasm during mitosis where it interacts with cytoplasmic Par3 promoting its dephosphorylation by the catalytic subunit PP4c and suppressing its activity (Lyu *et al.,* 2013). Follow up studies showed that both SMEK1 and SMEK2 are involved in cortical neurogenesis and that SMEK1/SMEK2 double knockout mice displayed significant defects in the production of cortical neurons and an increase in the neural stem cell population (Chang *et al.,* 2017). The study showed that SMEK1 and SMEK2 regulate the localisation and function of the Wnt receptor RYK, which plays a significant role in cell fate determination during cortical neurogenesis. RYK activity and translocation to the nucleus are required for neuronal cell differentiation. SMEK1 and SMEK2 interacts with RYK and promotes its translocation to the nucleus. In the nucleus, both SMEKs and RYK binds to genes required for neural cell fate determination and regulate their transcription (Chang *et*

al., 2017). Screening studies have identified Mbd3, a potent epigenetic regulator, as a SMEK-interacting protein. During CNS neurogenesis, Mbd3 was found to be highly expressed in NPC populations in the ventricular zone, and it was predominantly expressed in the nucleus. SMEKs interacted directly with the Mbd3's Mbd domain, destabilizing Mbd3 protein and its interaction with NuRD components, and sequentially, preventing accumulation of the Mbd3/NuRD complex on target gene loci and promoting neuronal differentiation (Moon *et al.,* 2017).

Both SMEK1 and SMEK2 are involved in gluconeogenesis. Overexpression of SMEK1 and SMEK2 led to the increase in plasma glucose with increased hepatic gluconeogenic gene expression. Overexpression of both proteins induced the dephosphorylation of CRTC2 (cAMP-response element binding protein-regulated transcriptional coactivator 2) and the subsequent promotion of transcription of gluconeogenesis-related genes (Yoon *et al.,* 2010). Hepatic SMEK1/2 expression is up-regulated during fasting or in mouse models of insulin-resistant conditions (Yoon *et al.,* 2010). In addition, SMEK1 and SMEK2 are involved in the control of cell division. Both SMEKs are phosphorylated and inactivated in mitosis (Voss *et al.,* 2013; Huang *et al.,* 2016). SMEK2 phosphorylation is required for maintaining 53BP1 (tumour suppressor P53-binding protein 1), one of the main mediators of DDR phosphorylation and blocking premature 53BP1 foci formation in mitosis (Hwang *et al.,* 2016).

The role of SMEK2 in the regulation of cell survival has not been investigated however, several studies support a role for SMEK1 as a tumour suppressor and involved in the regulation of signalling pathway such as mTOR. TNF and PI3K/AKT (Kim *et al.,* 2016). SMEK1 has been shown to induce apoptosis mediated through the reduction of phosphorylated PDK1, mTOR and 4E-BP1 in ovarian tumour cells (Kim *et al.,* 2017). SMEK1 is reported to enhance the gemcitabine proapoptotic activity via activation of p53 expression (Byun *et al.,* 2012).

Furthermore, increase in expression level of SMEK1 have been shown to inhibit angiogenesis via suppressing VEGFR-2-mediated activation of PI3K/AKT/eNOS in human ovarian tumours (Byun *et al.,* 2012; Kim *et al.,* 2016). A study showed that the expression of BMI-1 (B lymphoma Mo-MLV insertion region 1), an oncogenic protein activated in different types of tumours, enhanced cell growth via reducing SMEK1-mediated apoptosis as well as suppressing p21 and p53 expression and increasing the expression of CDK4 and Bcl-2 (Kim *et al.,* 2016).

SMEK2 forms complexes with PP4R2 and PP4c and play a variety of roles in cell fate such as dephosphorylation of γ-H2AX, regulation of wings apart-like protein homolog (WAPL), dephosphorylation of the transcription regulator KAP-1 and in dephosphorylation of 53 binding protein 1. Figure 1.10 summarises the role pf SMEK1 and SMEK2.

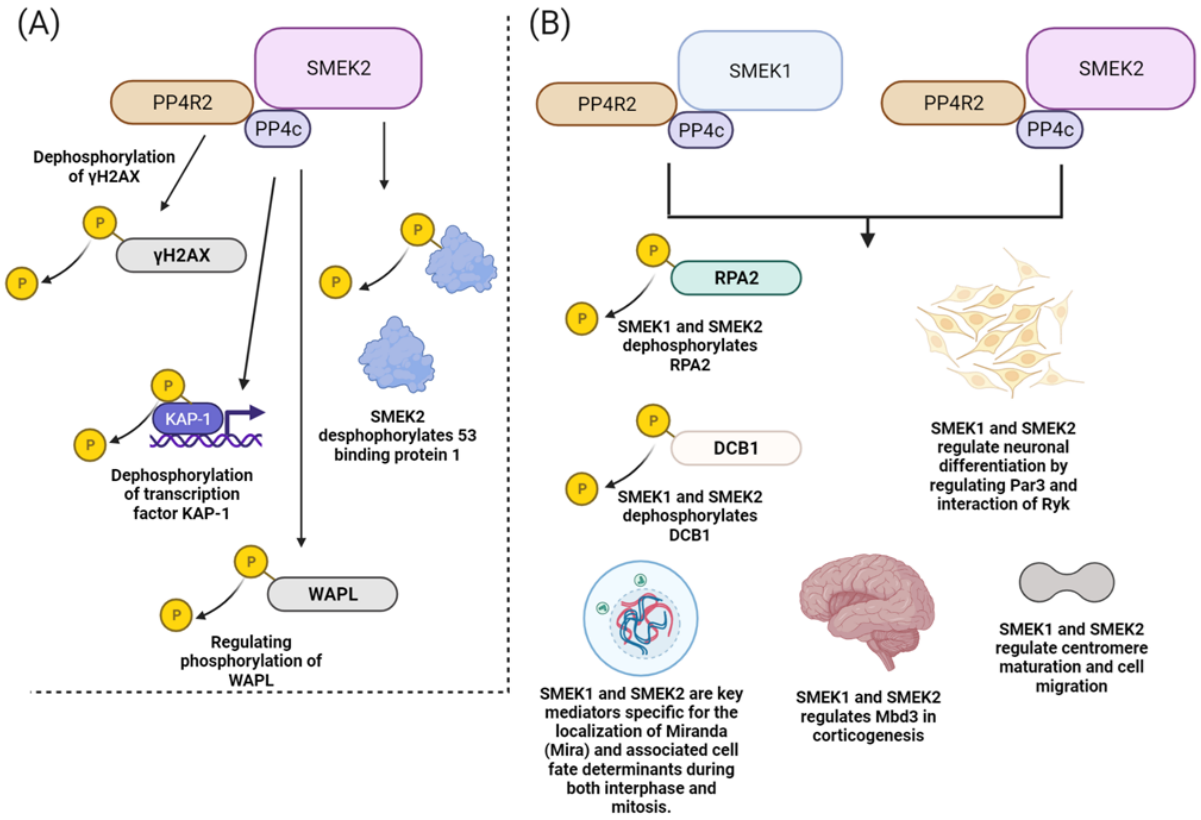


Figure 1.10 Role of SMEKs and associated complexes. (A) Specific role of SMEK2 in dephosphorylation of γH2AX, transcription factor KAP-1, regulation of WAPL and 53 binding protein. (B) Overlapping function of SMEK1 and SMEK2 in cell fate. In addition, both SMEK1 and SMEK2 dephosphorylate replication protein A2 (RPA2) and deleted in breast cancer 1 (DCB1), as well as mediating localization of Mira, regulation of Mbd3 and corticogenesis, regulation of neuronal differentiation through regulation of Par3 and RYK and centromere maturation and cell migration (adapted from Park and Lee, 2020).

1.7 Aims and Objectives

There is accumulating evidence that SMEK1 is a critical regulator of a number of physiological processes such as neuroinflammation (Duan *et al.,* 2021), neuronal development (Moon *et al.,* 2017) and differentiation and embryonic stem cell pluripotency maintenance by silencing Wnt-responsive development-related genes (Lyu *et al.,* 2011). Emerging evidence support a role for SMEK1 as a tumour suppressor. SMEK1 expression is reduced in a number of cancer cells and tissues (Byun et al.,2012). SMEK1 was

also reported to exert pro-apoptotic activities and to function as tumour suppressor in ovarian cancer cells where it was described to inhibit vascular endothelial growth factor (VEGF)-induced cell proliferation, migration, and angiogenesis via inhibition of the phosphorylation of the AKT signalling pathway (Kim *et al.,* 2016). The importance of SMEK1 in the regulation of ovarian cancer cell survival and proliferation suggests that SMEK1 might have a role in other types of cancer. Collectively, research so far reveals a complex role for SMEK1 in a physiology and pathophysiology and suggests that SMEK1 might have future clinical applications. Therefore, further research is needed to define the role of SMEK1 in cell fate decision and to determine its mechanisms of action.

To this end, the present study investigates the role of SMEK1 in leukaemia. It will specifically investigate the role of SMEK1 in the control of survival of leukemic T-cells and determine its mechanism of action. While many studies have focused on SMEK1, the role of SMEK2 is not well studied. Two studies so far confirmed that both SMEK1 and SMEK2 are functionally similar (Chang *et al.,* 2017; Yang *et al.,* 2020). This study will also extend to investigate the role of SMEK2 in the control of leukemic T-cell survival and to determine if SMEK1 and SMEK2 are functionally redundant.

Chapter Two: Materials and Methods

2.1 Cell culture

The human leukemic T cell lines, Jurkat and CEM-C7 (Schneider *et al.,* 1977; Zawydiwski *et al.,* 1983) were cultured at 37°C in 5% CO_2 humidified incubator in 25cm^2 and 75cm^2 tissue culture flasks in RPMI-1640 medium (Sigma Aldrich; #R0883) supplemented with 10% heat inactivated foetal bovine serum (FBS) (Sigma Aldrich; #F7524), 2mM L-glutamine (Thermo scientific; #25030081) and 200μg/ml gentamycin (Thermo scientific; #15750060) (labelled complete medium). All cell cultures and sub-cultures were carried out in a tissue culture hood with surfaces cleaned pre and post use with 70% Industrial methylated Spirit (IMS).

Cells were maintained at a density of 1x10^5 - 1x10^6 cells/ml, but not exceeding 3x10^6 cells/ml and were split twice a week fresh complete medium at a ratio of 1:10. Cell lines were replaced with fresh stocks after a defined number of passages usually lasting around 1-3 months dependent on thawed stock. All experiments were carried out using cells in the logarithmic growth phase.

2.1.1 Freezing/thawing cell lines

To maintain and replenish stocks of human leukemic T cell lines, Jurkat and CEM-C7, 1 x10^6 cells were collected and resuspended in 1ml of cryoprotectant medium containing 40% FBS, 10% DMSO and 50% complete RPMI-1640 medium. Cells were transferred into cryovials and placed into Mr Frosty ™ freezing container (ThermoFisher Scientific) containing

isopropanol and placed and stored at -80^{o}C for 4 hours (reducing 1^{o}C/min) transferred to liquid nitrogen (below -140^{o}C in vapour) for long term storage.

Thawing of cells was achieved by quickly heating cells at 37^{o}C for < 1 minutes and re-suspending in 9ml pre-warmed complete RPMI-1640 medium. Cells were then centrifuged at 1500 g for 5 minutes before being resuspended and transferred to a 25cm^{2} tissue culture flask. Once high density was achieved (assessed using light microscope), cells were transferred to a 75cm^{2} tissue culture flask and passaged for subsequent experiments.

2.2 Determination of short time cell survival

2.2.1 Vital dye staining

Total number of viable and non-viable cells was determined by vital dye staining using 0.4% trypan blue vital dye (Sigma Aldrich; # T8154). Cells were mixed with 0.4% Trypan blue at a ratio of 1:1 before being viewed under light microscope. Viable cells have the ability to exclude the blue dye from their cytoplasm and appear clear under microscope examination, whereas non-viable cells lose their ability to exclude the dye and retain a blue appearance. A haemocytometer was used to determine the cell number by counting each of the four corners of a sub-set of 16 squares (4x4), counting cells inside each square and that rested on the right and bottom lines, excluding cells that rested on the left and top lines.

The following equation was used to determine cells/ml:

$$Cells\ per\ m\text{l} = Average\ cell\ count \times 2 \times 10^{4}$$

2.2.2 Flow cytometry

Flow cytometry was used to determine cell count and viability using Muse® Count and Viability Kit (Luminex; #MCH100104) the Muse® Cell Analyser (Millipore). The Muse® Count and Viability assay allows determination of viable and non-viable cells by distinguishing the cells permeability to uptake two DNA binding dye present in the reagent. If the cells can uptake the dye and form a DNA-binding dye complex, then these cells have lost their membrane integrity and are labelled as dead or dying (non-viable). Those cells that have intact membranes do not uptake the dye and thus do not produce the DNA-binding dye complex and are labelled as viable. Further to this, a membrane-permeant DNA staining dye is also present in the solution which allows to differentiate between nucleated cells and debris. This membrane-permeant DNA dye stains all cells with a nucleus and accurately produces data that distinguishes nucleated cells from non-nucleated cells and cell debris (Muse protocol, 2019)

Cells were prepared following manufacturer's protocol. Samples were diluted (1:20) using Count & Viability Reagent and left to stain for 5 minutes at room temperature before assessed by the Muse® Cell Analyser. Data was expressed as total and viable cell number and percentage of viability (Figure 2.1).

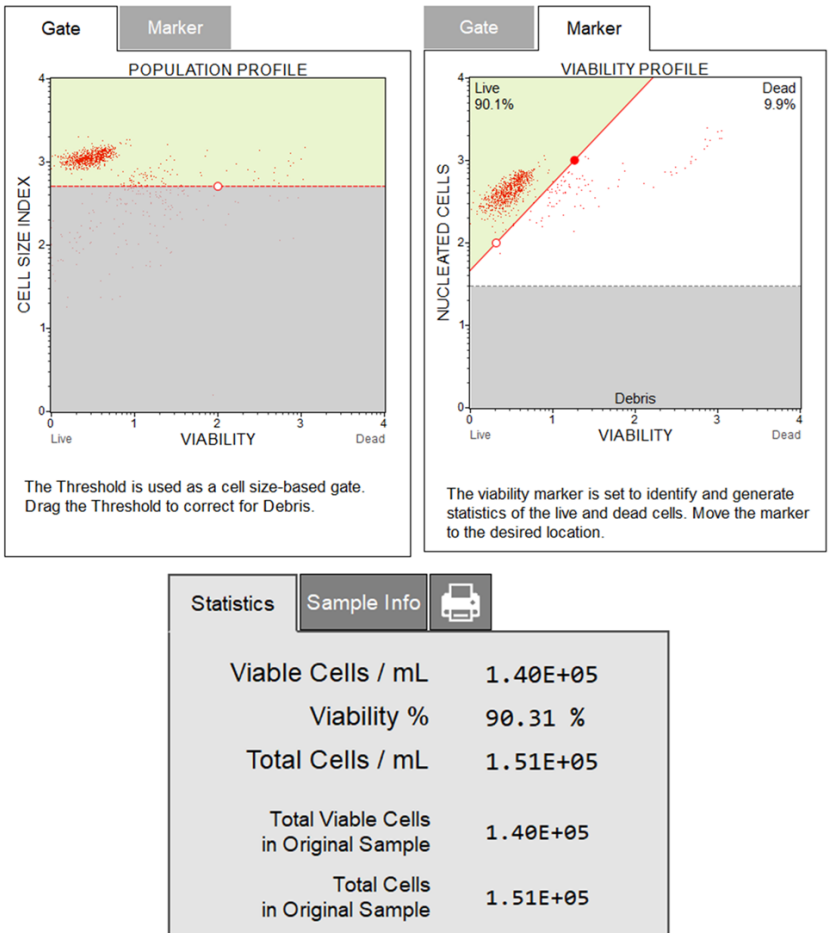


Figure 2.1 Representative image showing Cell Viability using flow cytometry. Jurkat cells analysed using Muse® Count and Viability Kit and the Muse® Cell Analyser. Total cell population profile captured via Muse® Cell Analyzer producing a percentage and number of viable and dead cell in the culture.

2.2.3 MTS cell viability assay

The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega; #G5421) was used to assess cell viability. The reagent contains 1.90mg/ml MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), which acts as an inner salt and 300µM PES (phenazine ethosulfate), which acts as an electron coupling reagent and enhances the chemical stability by forming a stable solution when combined with MTS. MTS tetrazolium is converted by NADPH-dependent dehydrogenases (NADPH or NADH) in living cells to a soluble formazan product. Formazan is soluble in cell

culture medium and the amount is directly proportional to the number of living cells in culture (Promega, 2012).

20µl of CellTiter 96® AQueous One Solution reagent was added to each well of a 96 well plate containing 200 µl of transfected cells. This was followed by incubating samples for 2 hours at 37°C in 5% CO_2 humidified incubator. Absorbance was measured at 490 nm using a Tecan Life Sciences Infinite M200 PRO plate reader with data expressed as percentage viability compared to control after subtracting the blank wells values from the values of all wells containing cell culture (% viability).

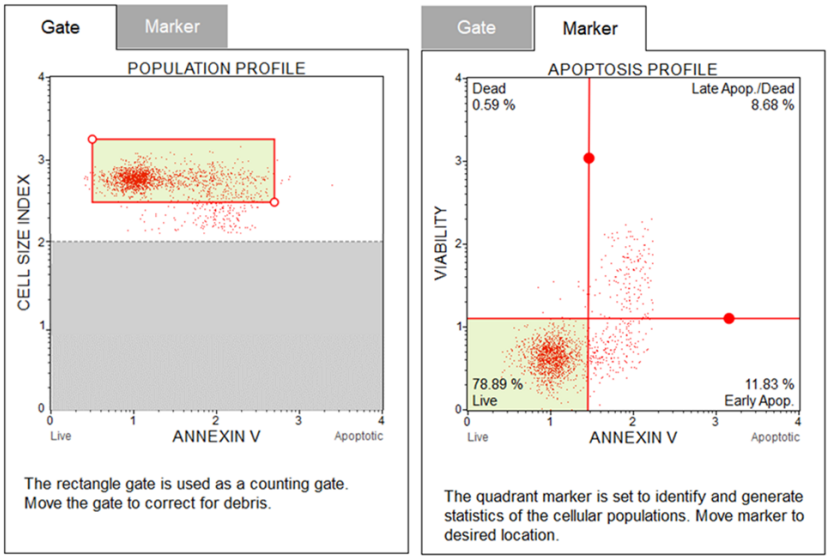
2.3 Determination of apoptosis

2.3.1 Determination of apoptosis using flow cytometry

The Muse® Annexin V and Dead Cell Assay (Luminex, #MCH100105) was used as a method to determine cellular apoptosis via flow cytometry. Apoptosis induces characteristic changes that includes alteration to the location of phosphatidylserine (PS), which can be found on the internal plasma membrane of healthy and viable cells (Muse protocol, 2016). During apoptosis, PS molecules located internally on the plasma membrane become translocated to the external plasma membrane of apoptotic cells (Muse Protocol, 2016). The Annexin V (calcium-dependent phospholipid binding protein) reagent is specifically designed to target the externally presented PS molecules and does so with a high affinity and is a first marker of apoptosis that can be detected. A dead cell marker 7-amino-actinomycin D (7-AAD) is also included in the Annexin V & Dead Cell Assay which can determine and identify cells with damaged membranes. Intact and viable cells which retails membrane integrity do not

allow entry of this marker. Cells treated with the Muse® Annexin V & Dead Cell Assay are categorized depending on the following markers: non-apoptotic cells: Annexin V (-) and 7-AAD (-), early apoptotic cells: Annexin V (+) and 7-AAD (-), late stage apoptotic and dead cells: Annexin V (+) and 7-ADD (+) and mostly nuclear debris: Annexin V (-) and 7-ADD (+) (Muse Protocol, 2016).

100 µl of cell suspension was added to a tube and mixed with 100 µl Muse Annexin V & Dead. Cell reagent in each tube was mixed by pipetting up and down. Samples were incubated at room temperature protected from light for 20 minutes. Following completion of the incubation, samples were analysed using the Muse® Cell Analyzer and data was expressed as total percentage mean of apoptosis (Figure 2.2).



Statistics | Sample Info

	Cell Conc (Cells / mL)	% Gated
Live (LL)	1.75E+05	78.89 %
Early Apoptotic (LR)	2.62E+04	11.83 %
Late Apop. / Dead (UR)	1.92E+04	8.68 %
Dead (UL)	1.32E+03	0.59 %
Total Apoptotic	4.55E+04	20.51 %

Figure 2.2 Representative image showing Annexin V and Dead Cell Assay using flow cytometry. Jurkat cells assessed to determine % apoptosis using Muse® Cell Analyzer and Annexin V and Dead Cell Assay. The figure shows % apoptosis profiles and viability in the Jurkat cell culture.

2.3.2 Determination of apoptosis using acridine orange staining

Acridine orange is a nucleic acid binding dye that emits green fluorescence when bound to double stranded DNA and red fluorescence when bound to RNA or single stranded DNA. Acridine orange has been used as a vital fluorescent stain to identify apoptotic cells which are characterised by the condensation and fragmentation of nuclear chromatin (Mpoke and Wolfe, 1997).

Staining with acridine orange (Sigma Aldrich, #235474) was used to determine apoptosis by assessment of nuclear morphology using fluorescence microscopy. Cells were

centrifuged at 1500 g for 5 minutes. The supernatant was discarded, and cells were re-suspended in 50 µl complete RPMI-1640 medium. 20 µl of cell suspension was mixed with acridine orange (25 µg/ml) at a ratio of 1:1 before loading onto a slide and coverslip added. Cells were viewed using an Invitrogen™ EVOS™ Digital Color Fluorescence Microscope and excited at 502nm with emissions collected at 525nm. Cells containing condensed or fragmented chromatin were scored as apoptotic. (Figure 2.3).

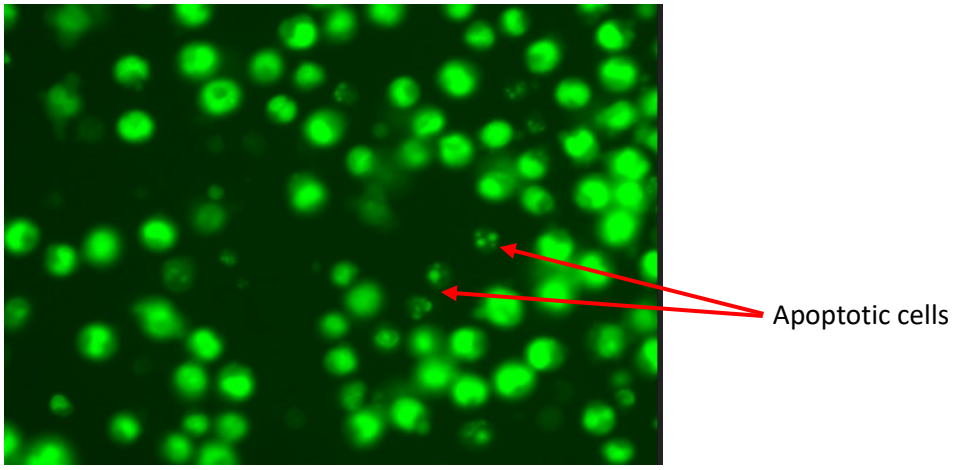


Figure 2.3 Representative image showing cells stained with acridine orange. Cells were mixed with Acridine Orange (25µg/ml) and analysed using an Invitrogen™ EVOS™ Digital Color Fluorescence Microscope, image magnification 400X. Red arrows show examples of apoptotic cells containing condensed/fragmented chromatin.

2.4 Clonogenic assay

Clonogenic assay was used to determine the ability of the cell to survive long-term in soft agar. Cancer cells have the ability to proliferate sufficiently to form visible colonies in an anchorage-independent manner. This characteristic of long-term survival can be assessed using colony forming assay (Borowicz *et al.,* 2014).

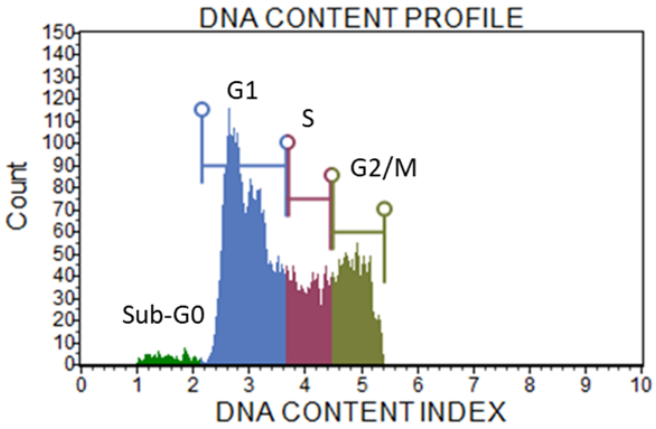
Long-term survival was assessed by the cells ability to form colonies on soft agar. An equal cell number proportion of cells was diluted in 3ml Iscove's medium (Sigma Aldrich, #12440053) containing 20% heat-inactivated FBS, 10% cell-specific conditioned medium and 10% noble agar solution (0.5% w/v, Difco) and plated in 6-well plates (Falcon #353046). Plates were left at room temperature under sterile conditions to allow the agar to solidify before adding an overlay consisting of 2ml Iscove's complete medium supplemented with 10% cell conditioned-medium. The number of colonies formed was counted following 2-3 weeks incubation at 37^{o}C in a 5% CO2 humidified incubator.

2.5 Cell cycle analysis using flow cytometry

The cell cycle is the series of events a cell undergoes before dividing to make new daughter cells. The Muse® Cell Cycle kit (Luminex, #MCH1001060) was used as a method to determine cell cycle using flow cytometry. The Muse® Cell Cycle premixed reagent was used which contains a mixture of propidium iodide (PI) (a nuclear DNA intercalating stain) and ribonuclease A (RNAse A) that cleaves single-stranded RNA, discriminates, and allow to measure the percentage of cells in the different stages of the cell cycle which include G0/G1, S, and G2/M phases of the cell cycle (Darzynkiewicz et al., 2001). In the first gap phase G0/G1, metabolic changes occur to allow the cell to grow physically larger, copies organelles, and makes the molecular building blocks required for cell division. The S phase is the DNA synthesis phase. Cells in the S phase will have more DNA than those in G1. These cells will take up proportionally more dye and will fluoresce more brightly than cells in G0/G1. In the second gap phase G2/M, the cell grows more, makes proteins and organelles, and begins to

prepare for cell division or mitosis. All cells in G2/M phase will have doubled their DNA and will take up proportionally more dye and will be approximately twice as bright as cells in G1.

Cell cycle analysis was carried out according to manufacturer's instructions. After 24 hours of re-plating cells following transfection, at least $5x10^5$ cells were collected, washed once with phosphate buffered saline (PBS) and centrifuged at 1500 g for 5 min. Cells were fixed in 1ml ice cold 70 % ethanol/30 % PBS and incubated at -20°C for at least 3 hours. After incubation, cells were resuspended in 250µl 1X PBS, centrifuged at 1500 g for a further 5 minutes before resuspending the cell pellet in 200µl Muse® Cell Cycle Reagent. They were then incubated for 30 minutes protected from light at room temperature before being transferred to a microcentrifuge tube for analysis using Muse® Cell Analyser. Data was represented as percentage cells in each phase: G0/G1, S, and G2/M phases (Figure 2.4).



Statistics | Sample Info

	G1	S	G2/M
% Gated	55.8	18.4	23.2
Mean Intensity	2977.5	4072.5	4883.7
% CV	11.2	5.7	4.9

Figure 2.4 Representative image showing cell cycle analysis using flow cytometry. Jurkat cells were collected 24 hours after replating. Cells were stained with Muse® Cell Cycle Reagent before assessing percentage of cells in the different phases of the cell cycle using Muse® Cell Analyzer.

2.6 Plasmid preparation and purification

The following plasmid vectors were purchased from GenScript: pcDNA3.1, pcDNA3.1-SMEK1 (Accession # NM_001284280), pcDNA3.1-SMEK2 (Accession # NM_001122964.2) and pcDNA3.1-PP4c (Accession #002720.2). The GenEZ ORF clones were delivered as 10µg lyophilized plasmid DNA. The vials were centrifuged at 6000 g for 1 minute at 4°C before being reconstituted in a volume of 100µl of molecular grade water.

All constructs were introduced into Alpha-Select Chemically Competent Bronze-efficiency *E.coli* cells (Bioline; #BIO-85025) via heat shock method. Chemically competent *E.coli* cells were thawed for a minimum of 15 minutes on ice prior to decanting 50µl of competent *E.coli* cells into a fresh sterile microcentrifuge tube. Subsequently, 300 ng of each vector was added to the 50µl of *E.coli* and gently mixed before incubating on ice for 30 minutes. After incubation on ice, cells were subjected to heat shock at 42^{o}C for 30 seconds. Cells were returned to ice for 2 minutes before adding 950µl of SOC (Super Optimal broth with Catabolite repression) medium and placed in a shaking incubator at 37^{o}C for 1 hour.

After transformation, bacterial cultures containing corresponding plasmid vectors were plated onto LB agar plates (10g SELECT Peptone 140, 5g SELECT Yeast Extract, 5g Sodium Chloride, 12g SELECT agar) containing ampicillin 100µg/ml. Plates were inverted and stored at 37^{o}C for 24 hours to allow ampicillin resistant colonies to form. Single colonies were inoculated into 5ml of LB Broth media (10g SELECT Peptone 140, 5g SELECT Yeast Extract, 5g Sodium Chloride) containing ampicillin (50µg/ml) and incubated for 8 hours at 37^{o}C with vigorous shaking (at 300 rpm).

The starter medium was then diluted in a ratio of 1:500-1:1000 in LB media containing 50µg/ml ampicillin and incubated at 37^{o}C for up to 16 hours with vigorous shaking (at 300 rpm) before the purification of plasmids using the EndoFree® Plasmid Maxi Kit (Qiagen, #12362). The purification protocol was based on an alkaline lysis procedure and anion-exchanged-based endotoxin free plasmid DNA purification. The EndoFree® Plasmid Maxi Kit allows the preparation and purification of transfection-grade plasmid. Briefly, 100ml of bacterial cultures were centrifuged at 6000 g for 15 minutes at 4^{o}C to pellet the bacteria. The cell pellet was resuspended in 10ml of re-suspension buffer 1 composed of 50mM Tris-Cl, pH

8.0; 10mM EDTA; 100µg/ml and RNase A which removes all RNA in the sample. A further 10ml of Buffer P2, a lysis buffer composed of 200mM NaOH and 1% SDS (w/v), was added and sample mixed by inverting 4-6 times before incubating at room temperature (15^{o}C – 25^{o}C) for 5 minutes. Plasmid DNA was then isolated by initially binding to a QIAGEN resin under low-salt and pH conditions by adding 10ml of P3 chilled neutralization buffer composed of 3.0M potassium acetate, pH 5.5, then the bacterial lysate was transferred to the barrel of the QIAfilter Cartridge and incubated at room temperature (15-25°C) for 10 minutes. The lysate was filtered into a 50ml tube. The column was washed twice using a medium-salt wash (1.0M NaCl; 50mM MOPS [free acid], pH 7.0; 15% isopropanol (v/v)) in order to remove all impurities including RNA, proteins, and low-molecular–weight particles. Plasmid DNA was then eluted with 15ml of a high-salt buffer (1.6M NaCl; 50mM MOPS, pH 7.0; 15% isopropanol (v/v)) and then concentrated and desalted by 10.5ml isopropanol precipitation followed by centrifugation ≥ 15000 g at 4°C for 30 min. The extracted plasmid DNA concentration and purity was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and samples with A260/A280 value that was less than 1.8 were discarded. The identity of the plasmid DNA constructs was verified by a diagnostic digest and sequence analysis (Eurofins Scientific). NCBI Blast was used to confirm the identity of the sequencing results.

2.7 Plasmid Transfection

Electroporation and nucleofection methods were used to transiently transfect the human leukemic T cell lines with pcDNA3.1, pcDNA3.1-SMEK1; pcDNA3.1-SMEK2 or pcDNA3.1-PP4c constructs. Green Fluorescent Protein (GFP)-expressing plasmid pmaxGFP (Lonza, #D-00066) was used to determine transfection efficiencies.

2.7.1 Plasmid transfection using electroporation

Key principles of electroporation involve the use of high-volt electric shocks to introduce DNA into cells. Applying electrical pulses to the cells permeabilizes the cell membranes allowing the uptake of the DNA (Bio Rad Protocol, 2000). The delivery of electrical pulses are defined by two parameters, the initial field strength (kV/cm) as well the time constant (t), and it is these parameters that are adjusted and optimised per cell type.

1 x10^7 cells were centrifuged at 1500 g for 5 minutes and resuspended in 20ml pre-warmed Opti-MEM® (Gibco®, #31985062) to wash cells from FBS containing medium. After washing, 1x10^7 cells were centrifuged at 1500 g for 5 minutes and resuspended in 400µl Opti-MEM® media in the presence of 5µg of plasmid. Cells were allowed to equilibrate for 10 minutes at room temperature before transferred to a 0.4cm gap electroporation cuvette (Bio-Rad, #1652088). The cuvette was placed in a Bio-Rad Gene pulser II and cells were electroporated at 248V (CEM-C7) and 293V (Jurkat) and 1050µF at room temperature. Cells were left for 10 minutes to allow plasmid entry to the cells via the membrane pores that was created as a result of electroporation before being transferred to a 6 well plate containing

5ml Iscoves medium supplemented with 2mM glutamine, 200μg/ml gentamycin and 20% heat-inactivated FBS. Subsequent experiments were carried out 48- and 72-hours following transfection.

Transfection efficiencies of cells transfected with 5μg pmaxGFP were routinely 70-80 % transfection efficiency in Jurkat cells (Figure 2.5).

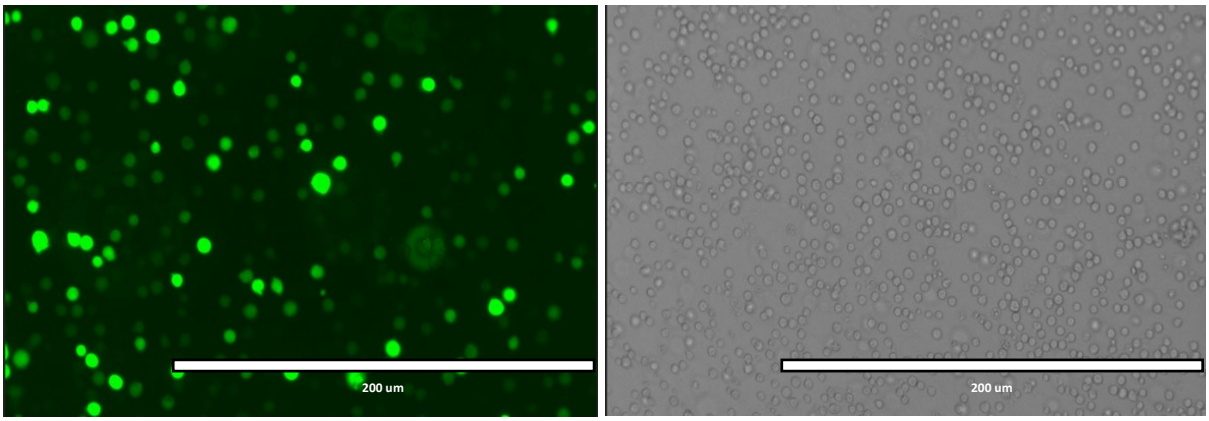


Figure 2.5 Representative image of Jurkat cells transfected with a GFP-encoding plasmid by electroporation. Cells were transfected with a pmaxGFP expressing plasmid using Bio-Rad Gene pulser II. After 24 hours cells were visualised using an Invitrogen™ EVOS™ Digital Color Fluorescence Microscope. Cells were counted and the percentage of GFP positive cells determined.

2.7.2 Plasmid transfection using nucleofection

Similar to electroporation, Nucleofection uses electrical impulses to introduce nucleic acid into a cell. Though the methods are the same, nucleofection aims to improve the high toxicity associated with traditional electroporation and thus increasing the efficiency of transfection. Using pre-determined Nucleofector® Technology of a combination of electrical parameters to cell-type as well as cell type-specific solutions, it allows for a more direct entry of the nucleic acids into cells and nucleus enabling more effective delivery (Lonza Protocol, 2009)

$1x10^6$ cells were centrifuged at 1500 g for 5 minutes before being resuspended in pre-warmed 100µl Ingenio® Solution (Mirus, #50108) and mixed with 2µg plasmid DNA before being transferred to a 0.2cm gap cuvette (Mirus, #50121) and left to incubated at room temperature for 10 minutes. Following incubation, cells were nucleofected using the Amaxa Nucleofector® II (program X-14 for Jurkat and program X-001 for CEM C7). After nucleofection, cells were transferred from the cuvettes using Ingenio® Cell Droppers (Mirus, #50125) into corresponding 6 well plates. Subsequent experiments were carried out after 48 hours following transfection.

Transfection efficiencies of Jurkat cells nucleofected with 2µg of pmaxGFP were routinely 50-60% (Figure 2.6).

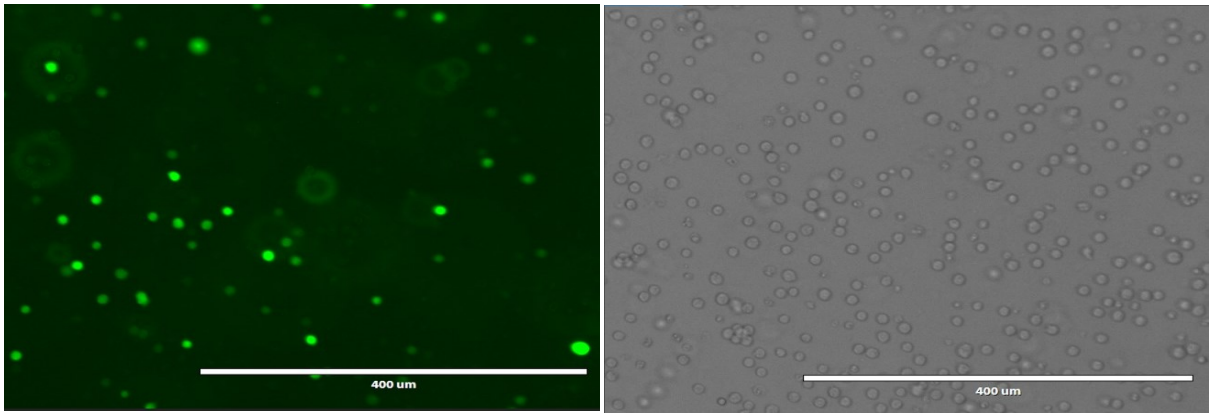


Figure 2.6 Representative image of Jurkat cells transfected with a GFP-encoding plasmid using nucleofection. Cells were nucleofected with a pmaxGFP expressing plasmid using Ingeno® electroporation kit and Amaxa ™ nucleofector™ II. After 24 hours cells were visualised using a EVOS fluorescent microscope.

2.8 RNA interference

The functional importance of endogenous SMEK1 and SMEK2 in CEM-C7 and Jurkat cells was investigated by determining the effects of their reduced expression on these cells using small interference RNAs (siRNAs). Silencer pre-designed siRNA to SMEK1 and SMEK2 (Table 2.1) along with negative scrambled siRNA control (Ambion, #4611) were used in these experiments. Cells were transfected with siRNA (20nM) by electroporation using a similar protocol to that described for plasmid transfection apart from allowing the cells to incubate at room temperature for 20 minutes post-transfection before transferring to 5ml of complete Iscoves medium in 6 well plates. 24h post-transfection, cells were split 1:1 with routine culture medium and maintained at 37^{o}C for 96 hours before use in subsequent experiments.

To determine transfection efficiency, parallel transfections were carried out using Cy3 labelled siRNA. siRNA labelling kit Cy™3 (Life Technologies®, #AM1632) was used to label scrambled negative siRNA following manufacturer's instructions. Labelling reaction was prepared by adding the components of the reaction as shown in Table 2.2 and incubated for 1 hour at 37^{o}C. Transfection efficiency was determined 24 hours post-transfection with the Cy3 labelled siRNA using fluorescent microscopy, as described for plasmid transfection. Transfection efficiencies were found to be routinely 70-80 % after visualizing using fluorescence microscope (Figure 2.7).

Table 2.1 Gene specific siRNAs used in the experiments.

Gene name	siRNA	Target exon	Catalogue number	Company
SMEK1	SMEK1 siRNA1	Exon 3	SI04294955	Qiagen
	SMEK1 siRNA5	Exon 4	SI05069960	Qiagen
SMEK2	SMEK2 siRNA3	Exon 2	SI04347252	Qiagen
	SMEK2 siRNA5	Exon 3	SI05096112	Qiagen

Table 2.2 Components of materials used in generating Cy™3 siRNA for the use in determining transfection efficiency

Reagent	Volume (µl)
Nuclease-free Water	18.3
10X labelling buffer	5
21-mer duplex siRNA at 20 µM (~5 µg)	19.2
Cy™3 or FAM™ Labelling Reagent	7.5

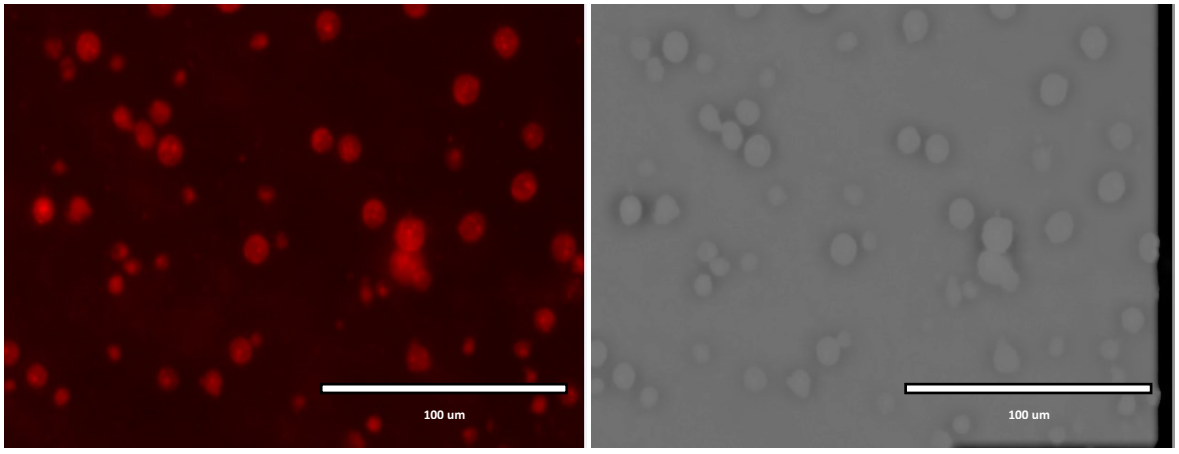


Figure 2.7 Representative image of Jurkat cells transfected with a Cy™3 labelled siRNA. Cells were transfected with Cy3 labelled siRNAs. After 24 hours cells were visualised using an Invitrogen™ EVOS™ Digital Color Fluorescence Microscope. Cells were counted and the percentage of Cy3 positive cells was determined.

2.9 Analysis of the level of protein expression by western blotting

Samples for protein analysis were collected from all transfected cells from every experiment in order to examine the level of SMEK1 and SMEK2 expression to ensure that the effects observed in the results can be attributed to the changes in SMEK1 and SMEK2 expression levels. Samples from transfected cells were also collected to validate the change in the phosphorylation of target proteins. A total of $1x10^6$ cells were harvested and washed in 1X PBS and centrifuged at 1500 g for 5 minutes. The pelleted cells were then further washed again in PBS to remove residual medium and centrifuged at 1500 g from 5 minutes. Following centrifugation, pellets were resuspended in a 30µl 1x RIPA buffer (radioimmunoprecipitation assay buffer; ThermoFisher Scientific, #89900) containing protease inhibitor (at a 1:100 dilution; Millipore, #535140) and incubated at 2-8^oC for 10 minutes. Following incubation, cells were centrifuged at 13000 g for 10 minutes to pellet the debris before transferring the supernatant to a 1.5ml microcentrifuge tube and the addition of 2X laemmli buffer (Bio Rad, #1610747) containing 10% β-mercaptoethanol (Sigma; # 60242), and incubation at 95^oC for 4 minutes.

Samples were loaded into 8%, 10% or 12% (dependent on the size of target protein) 10-well Mini-PROTEAN® TGX™ gels (Bio Rad, #456-1043) and were subjected to electrophoresis by using a Bio-Rad PowerPac High Current (HC) power supply at 150 Volts for 1 hour at room temperature in a 1X SDS-PAGE running buffer (0.025M Tris, 0.192M glycine, 0.1% SDS, pH8.5). A Plus Protein™ Dual Colour Standards (Bio Rad, #161-0374) was loaded alongside protein samples and used to confirm protein migration during analysis, as well as to confirm successful protein transfers during SDS-PAGE. Proteins were then electro-

transferred onto a polyvinylidenedifluoride (PVDF) membrane (Bio Rad), which had been pre-wetted in 100% methanol, using a wet transfer system at 30V, 90mA overnight at 4°C with stirring.

Following successful protein transfer, the PVDF blot was washed in excess 1X Tris Buffer Saline (TBS) (Tris Buffered Saline; 25mM Tris, 0.13 M NaCl) before incubating the membrane at room temperature in the blocking solution 5% non-fat milk (w/v 1X TBS containing 0.1% Tween (TBS-T) to block non-specific binding. The membrane was washed three times with TBS-T before adding the primary antibody (Table 2.3) at 4^{o}C for 16 hours. The membrane was then washed three times with TBS-T for 5 minutes each to remove residual primary antibody and then incubated with the appropriate secondary antibody for 1 hour (Table 2.3). Membrane was washed five times with TBS-T for 5 minutes before protein visualisation by enhanced chemiluminescence (ECL) using Clarity™ Western ECL Substrate (Bio Rad, #1705060S). Western Blots were imaged using either a C-DiGit® Blot Scanner (LI-COR®) and analysed using Image Studio™ Software (Version 3.1) or Bio Rad ChemiDoc MP

Imaging System and analysed using Image Lab 6.0.1 for Windows software. After analysis, and to determine equal loading, blots were stripped using Restore™ PLUS Western Blot Stripping Buffer (ThermoFisher Scientific, #46430) according to the manufacturer's instructions before being re-probed with a mouse-anti-β-actin antibody (1:5000 dilution), followed by an HRP-conjugated anti-mouse IgG secondary antibody (1:1000 dilution) with bands visualised as before. Table 2.3 contains a summary of the antibodies used in this study.

Table 2.3 – Primary and secondary antibodies

Primary antibody	Dilution	Secondary antibody and dilution
Anti-β-Actin Dako, #PO447	**1:5000**	**Goat Anti-Mouse HRP Conjugate (1:1000)** Thermo Scientific, #31430
Anti-SMEK1 Santa Cruz Biotechnology, #Sc-244184	**1:1000**	**Anti-Goat IgG-HRP conjugate (1:1000)** Thermo Scientific, #A32849
Anti-SMEK2 Abcam, #Ab70622	**1:2000**	**Anti-Rabbit IgG-HRP conjugate (1:1000)** Thermo Scientific, #A32731
Anti-PP4c (PPX/PP4 C-6) Santa Cruz Biotechnology, #Sc-374106	**1:1000**	**Anti-mouse IgG-HRP conjugate (1:1000)** Thermo Scientific, #31430
Anti-Caspase-3 Santa Cruz Biotechnology, #Sc-7272	**1:500**	**Anti-mouse IgG-HRP conjugate (1:1000)** Thermo Scientific, #31430
Anti-BAD Santa Cruz Biotechnology, #Sc-8044	**1:500**	**Anti-mouse IgG-HRP conjugate (1:1000)** Thermo Scientific, #31430
Anti-XIAP Santa Cruz Biotechnology, #Sc-55550	**1:500**	**Anti-mouse IgG-HRP conjugate (1:1000)** Thermo Scientific, #31430
Anti-Beclin-1 Cell Signalling, #59187	**1:1000**	**Anti-Rabbit IgG-HRP Conjugate (1:1000)** Thermo Scientific, #A32731
Anti- Phospho-Smad1 Cell Signalling, #12656	**1:1000**	**Anti-Rabbit IgG-HRP conjugate (1:1000)** Thermo Scientific, #A32731
Anti-Phospho-Smad5 Cell Signalling, #12656	**1:1000**	**Anti-Rabbit IgG-HRP conjugate (1:1000)** Thermo Scientific, #A32731
Anti-Smad4 Cell Signalling, #12656	**1:1000**	**Anti-Rabbit IgG-HRP conjugate (1:1000)** Thermo Scientific, #A32731
Anti-c-Jun Invitrogen, #C.238.2	**1:500**	**Anti-Rabbit IgG-HRP conjugate (1:1000)** Thermo Scientific, #A32731

Anti-p-Stat1 Santa Cruz Biotechnology, #Sc-8394	**1:500**	**Anti-mouse IgG-HRP conjugate (1:1000)** Thermo Scientific, #A32731
Anti-Phospho-Stat3 (Tyr705) Cell Signalling, #9914	**1:1000**	**Anti-Rabbit IgG-HRP Conjugate (1:1000)** Thermo Scientific, #A32731
Anti-p-Stat5a/b Santa Cruz Biotechnology, #Sc-81524	**1:500**	**Anti-mouse IgG-HRP conjugate (1:1000)** Thermo Scientific, #31430
Anti-p-NFκB Santa Cruz Biotechnology, #Sc-136548	**1:500**	**Anti-mouse IgG-HRP conjugate (1:1000)** Thermo Scientific, #31430

2.10 Phosphorylation pathway profiling array

The phosphorylation pathway profiling array was processed using the Human Phosphorylation Pathway Profiling Array C55 kit (Raybiotech, #AAH-PPP-1-2) following the manufacturer's instructions. The array was used to detect changes in the phosphorylation of proteins in five main signalling pathways MAPK, AKT, JAK/STAT, NFκB and TGFβ (Table 2.4) in Jurkat and HEK293T cells overexpressing SMEK1 and SMEK2.

Table 2.4 – Pathways and target proteins

Signalling Pathway	Primary Antibodies
Mitogen-Activated Protein Kinase **(MAPK)**	CREB, ERK1 ERK2, HSP27, JNK, MEK, MKK3, MMK6, MSK2, p38, P53, RSK1, RSK2
Protein kinase B **(AKT)**	AKT, AMPKa, BAD, GSK3a, GSK3b, mTOR, p27, P70S6K, PDK1, PRAS40, PTEN, RAF-1, RPS6
Janus kinase-signal transducer and activator of transcription **(JAK/STAT)**	EGFR, JAK1, JAK2, SHP1, SHP2, Src, Stat1, Stat2, Stat3, Stat5, Stat6, TYK2
Nuclear factor kappa light chain enhancer of activated B cells **(NFκB)**	ATM, eIF2a, HDAC2, HDAC4, MSK1, NFκB, TAK1, ZAP70
Transforming growth factor beta **(TGFβ)**	ATF2, C-Fos, c-Jun, SMAD1, SMAD2, SMAD4, SMAD5

Lysates of HEK293T cells transiently overexpressing SMEK1 (Reference Sequence NP_115949) and SMEK2 (Reference Sequence NP_06519) were purchased from OriGene Technologies GmbH. Lysates from cells transfected with the empty vector pcDNA3.1 was used as control. HEK293T cell lysates were used at a final concentration of 50µg total protein. Lysates from Jurkat T cells transiently expressing SMEK1 and SMEK2 were prepared as described in Section 2.9. Concentration of the total protein in the lysates was quantified using ThermoFisher Scientific Nanodrop™ 1000 spectrophotometer and a final concentration of 1000µg of total protein was used. Briefly, the membranes were incubated with blocking buffer at 25 °C for 30 min and then incubated with 1ml of 1:20 diluted cell lysates overnight at 4 °C and gently shaken. The membranes were washed twice with wash buffer and then incubated with Detection Antibody Cocktail at 4 °C for 16 hours and shaken. The membranes

were washed twice before being incubated with horseradish peroxidase (HRP)-labelled anti-rabbit secondary antibody at 25°C for 2 hours. Membranes were transferred to a sheet of chromatography paper, printed side up as to remove excess wash before being transferred to the provided plastic sheet. Detection Buffer was added to each membrane and incubated at room temperature for 2 minutes and signals were detected using a Bio-Rad ChemiDoc MP Imaging System. Spot intensities were analysed using Image Lab 6.0.1 for Windows software. The internal control signals of each protein array membrane were used for standardization. Positive controls on each membrane were averaged (6 positive spots) and global background noise deducted (average of the negative control spots (2-10 depending on array). All negative spots were satisfactory clear as according to manufacturer's specifications showing that all the membranes were acceptable for analysis. Once the positive spots of each of the membranes had been averaged and the negative background noise deducted, the fold changes in the phosphorylation was calculated after being normalised with cells transfected with pcDNA3.1.

2.11 RNA sequencing

2.11.1 RNA extraction

Total RNA was extracted from Jurkat T cells transfected with pcDNA3.1, pcDNA3.1-SMEK1 and pcDNA3.1-SMEK2. $1x10^6$ cells were collected 48 hours post-transfection using Direct-zol™ RNA miniprep (Zymo Research, #R2050). 3 volumes of TRIsure™ was added for every 1 volume of cell suspension, mixed and centrifuged at 13000 g for 15 minutes to pellet the debris and supernatant transferred into a new tube. TRIsure™ is a monophasic solution

comprising of phenol and guanidinium isothiocyanate which are involved in solubilization of biological material and protein denaturation. Equal volumes of 100 % ethanol were added to the solution containing RNA and mixed thoroughly. Samples were then transferred at a volume no greater than 700µL to a Zyom-Spin™ IICR column in a collection tube and centrifuged at 13000 g for 1 minute with the supernatant discarded. This process was repeated until all the solution had passed through the Zymo-Spin™ IICR column and the nucleic acids bound to the silica-based column matrix. The column containing RNA was transferred to a new Collection Tube and 400µl RNA Wash Buffer added, followed by centrifugation at 13000 g for 1 minute and flow-through discarded. DNase I was pre-mixed in Digestion buffer to a final concentration of 6U/µl before adding 80µl directly onto the column matrix and incubating at 20-30^{o}C for 15 minutes. The treatment of DNase I allows the removal and degradation of genomic DNA that may contaminate the samples.

After removal of genomic DNA, two lots of separate 400µl of Direct-zol™ RNA Prewash was added to the column and centrifuged at 13000 g for 1 minute with supernatant discarded, followed by the addition of 700µL RNA Wash Buffer and centrifuged once more for 1 minute as to remove residual contaminants. The flow through containing RNA Wash Buffer was discarded and the tube spun empty at 13000 g for 2 minutes as to remove any residual wash buffer before being transferred to a clean sterile RNase-free 1.5ml microcentrifuge tube. 30µL DNase/RNase-Free water added directly to the column matrix as to elute the samples when centrifuged. After RNA elution, samples were quantified and analysed using a ThermoFisher Scientific Nanodrop™ 1000 spectrophotometer to determine concentration of RNA. RNA samples with 260nm/280nm absorbance ratio between 1.8–2 was considered of high purity.

RNA integrity was also determined by running an aliquot of the RNA sample on an agarose gel stained with ethidium bromide. Intact total RNA run on an agarose gel are expected to have a clear 28S and 18S ribosomal RNA (rRNA) bands. 1µg of RNA was mixed with TriTrack DNA loading dye (ThermoFisher Scientific, #R1161) to a final concentration of 1X TriTrack/1µg RNA and samples loaded into a Midi-1% (w/v) agarose gel (ThermoFisher Scientific, #16500100) in 1X Tris-acetate-EDTA (TAE) Buffer (ThermoFisher Scientific, #15558042) containing 0.3% Ethidium Bromide. Gel was ran at 150 Volts for 45 minutes and was visualised using a Syngene™ U:Genius3 Transilluminator. Figure 2.8 shows a representative gel image showing 28S and 18S rRNA.

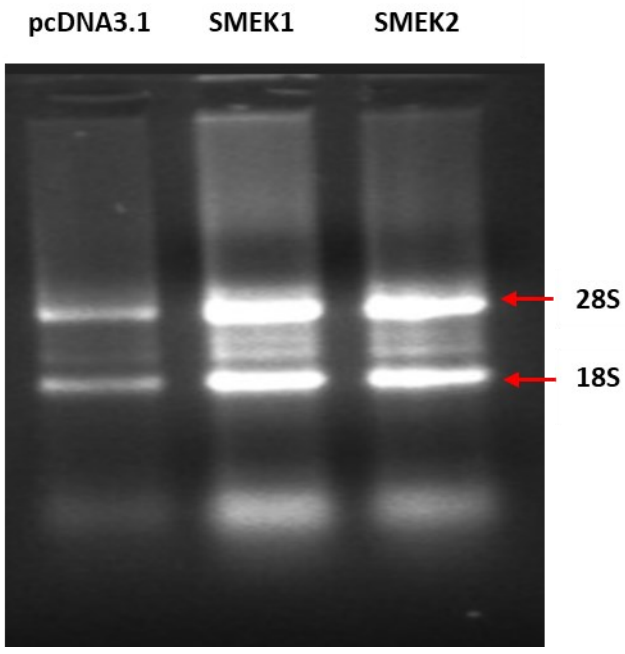


Figure 2.8 Representative image of RNA samples on agarose gel. Total RNA was extracted from Jurkat cell using Direct-zol™ RNA miniprep kit (Zymo research). RNA samples (1µg) were separated on a 1% agarose gel containing 0.3% ethidium bromide at 150 V for 45 minutes and visualised. The image shows a good 2:1 ratio of the 28S and 18S rRNA band.

2.11.2 RNA sequencing and Pathway analysis

Whole transcriptome sequencing was used to determine alterations in global gene responses in Jurkat cells in response to overexpressing SMEK1 and SMEK2. Total RNA was extracted as described above. The RNA samples passed the quality control and the RNA integrity in the samples was confirmed to be very good as seen from the RNA Quality Number (RQN) values of 9.2-9.6 (Appendix 1). Next generation sequencing was carried out by the Centre for Genomic Research at the University of Liverpool for Next Generation Sequencing (NGS). Sequencing libraries were prepared using the NEXTflex directional RNA-Seq Library Kit, and following stringent quality control measures, sequenced to a depth of approximately 30 million reads per sample, 150 bp PE read metric, on the HiSeq 4000 platform.

Raw sequencing data was trimmed of sequencing adapters and low-quality reads discarded using the Trimgalore package, a wrapper that incorporates CutAdapt and FastQC. Quality controlled reads were aligned to Human Genome build (hg19) using Tophat, a fast splice junction mapper for RNA-Seq reads. Transcripts were assembled using Cufflinks (with GTF support) and the number of reads mapping to each feature counted and expressed as FPKM using the CuffNorm package. Differentially expressed mRNAs were condensed into gene networks representing biological and disease processes using iPathwayGuide (Advaita Bioinformatics, Ann Arbor, MI, USA), with the aim of elucidating key mechanisms responsible for mediating the phenotypic effects of SMEK1 and SMEK2 overexpression.

2.12 Statistical analysis

GraphPad Prism 7 (GraphPad Software) was used to performs statistical analyses. Data are presented as the mean ± Standard Error Mean (SEM); the number of observations (n) refers to different experiment and each experiment being conducted on a separate culture of cells. Comparisons were made using an unpaired Student's t-test, one-way ANOVA with Tukey's post-test or two-way ANOVA with Bonferroni's multiple comparison test (MCT). Statistical significance was set at the 0.05 level. Differences were considered statistically significant when p-value was <0.05 (95% confidence intervals).

Chapter Three: The role of SMEK1 and SMEK2 in the regulation of leukemic T cell survival

3.1 Introduction

Serine/threonine-protein phosphate 4 (PP4) is a ubiquitous and highly conserved protein phosphatase that is essential in regulating several cellular processes, including DNA damage repair, genomic stability, immune response, and glucose homeostasis (Liu and Feng, 2010; Lipinszki *et al.,* 2015). PP4 exists as a holoenzyme composed of a catalytic subunit PP4c in association with various regulatory subunits. Several PP4-specific regulatory subunits were identified including PP4R1, PP4R2, PP4R3A (SMEK1), PP4R3B (SMEK2) and PP4R4. PP4R2 is reported to function as a scaffolding subunit that bridges SMEK1 or SMEK2 to PP4c (Lipinszki *et al.,* 2015). PP4R2 can also heterodimerizes with PP4c to direct substrate targeting independent of SMEK1 and SMEK2 (Lee *et al.,* 2010).

PP4c has been steadily studied over the past decade and was shown to control many vital functions in cells including the control of apoptosis, cell proliferation and mutation rate in human cells (Mourtada-Maarabouni and Williams, 2009). Several studies have implicated PP4c in tumorigenesis and suggested that PP4c is potential target for novel therapeutic applications in cancer (Park and Lee, 2020). Studies have also documented the importance of the interaction of PP4c with its different regulatory subunits in controlling the activity of the holoenzyme (Lipinszki *et al.,* 2015). Given the importance of PP4 regulatory subunits in controlling PP4c activity and dictating localisation and substrate specificity, molecular function and targets of these regulatory subunits have not been extensively studied the role of human PP4R3 regulatory subunits which remain to be addressed.

The human PP4R3 exists in two isoforms PP4R3A/SMEK1 and PP4R3B/SMEK2 encoded by two different genes which encode two proteins that share 70% sequence identity. SMEK1

is encoded by a gene located on chromosome 14q32.12 while SMEK2 gene is located on chromosome 2p16. Both regulatory subunits are shown to form complex with PP4c-PP4R2 which is the major form of the holoenzyme PP4 (Lipinszki *et al.,* 2015). SMEK1, reported to be the most abundant of the two isoforms, have been previously implicated in cancer and in controlling cancer cell survival. SMEK1 is reported to inhibit the proliferation of ovarian cancer cells OVCAR3 by binding and activating the tumour suppressor protein P53 (Byun *et al.,* 2012). SMEK1 expression levels are significantly reduced in tissues of patients with ovarian and cervical cancer (Byun *et al.,* 2012; Dong *et al.,* 2012). Studies have also showed that increased expression of SMEK1 prevents angiogenesis via suppressing VEGFR-2-mediated activation of PI3K/AKT pathway in human ovarian tumours (Byun *et al.,* 2012; Kim *et al.,* 2016). Emerging evidence has also highlighted important role for SMEK2, with Lee *et al.,* (2014) showing that SMEK2 can dephosphorylate p53 binding protein 1 (53BP1) independently from its interaction with PP4c. 53BP1 is well known for its role as a key DNA repair factor and regulator of p53-mediated transcription (Wrighton, 2016).

It is well established that PP4c exerts profound effects on the growth and survival of both normal and leukemic human T-cells, but it is not known if these effects are dependent on its interaction with SMEK1 and SMEK2 (Mourtada-Maarabouni and Williams, 2009). While previous studies have implicated SMEK1 in the regulation of a number of cellular processes that regulate cancer cell survival either by regulation PP4c activity or independently, the role of SMEK1 in the regulation of leukemic cell survival have not yet been investigated. In addition, studies so far concentrated on SMEK1 while it is not clear if SMEK2 also have a similar role in regulating cell fate. Therefore, it was of interest to study the functional effects of SMEK1 and SMEK2 on the survival of leukemic T cells.

The aims of this chapter are to study the functional effects of modulation of SMEK1 and SMEK2 expression on the cell survival of two leukemic T cell lines Jurkat and CEM-C7. Therefore, the specific aims are to determine the effects of overexpression and silencing of SMEK1 and SMEK2 on basal apoptosis, cell cycle and short- and long-term survival of leukemic T cells.

3.2 Materials and methods

Jurkat and CEM-C7 cell lines were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2mM L-glutamine and 200μg/ml gentamycin at 37°C in a humidified atmosphere of 5% CO_2 (Section 2.1).

To overexpress SMEK1, Jurkat and CEM-C7 cells were transfected with either pcDNA3.1 or pcDNA3.1-SMEK1 by electroporation (Section 2.7.1). For SMEK2 overexpression, pcDNA3.1-SMEK2 expression construct or vector only pcDNA3.1 were introduced into human leukemic T-cells Jurkat and CEM-C7 by nucleofection as described in section 2.7.2. In brief, cells subjected to electroporation were collected at a final concentration of $1x10^7$ cells, centrifuged at 1500 g for 5 minutes, washed in Opti-MEM® medium and collected by re-centrifugation at 1500 g before resuspended in fresh Opti-MEM® medium containing 5μg pcDNA3.1 or pcDNA3.1-SMEK1 and electroporated in a 0.4 cm gap cuvette at 248 V (CEM-C7) and 293 V (Jurkat) and 1050μF Capacitance. In some experiments, transfection was carried out using nucleofection. Cells subjected to nucleofection were collected at a final concentration of $1x10^6$ cells, centrifuged at 1500 g for 5 minutes, resuspended in 100μl Ingenio® Solution containing 2 μg pcDNA3.1 or pcDNA3.1-SMEK2, and nucleofected in a

0.2cm gap cuvette (program X-14 for Jurkat cells and X-001 for CEM-C7 cells). Levels of protein expression of the SMEK1 and SMEK2 was monitored by western blot analysis, as described in section 2.9.

SMEK1 and SMEK2 down-regulation was carried out in these cell lines using electroporation. Cells were transfected with either -siRNA, one of SMEK1 specific siRNAs: SMEK1s1 or SMEK1s5, or one of SMEK2 specific siRNAs: SMEK2s3 or SMEK2s5 as described above and in section 2.8. Western blotting was performed to determine the levels of protein expression and confirm reduced levels of SMEK1 and SMEK2 proteins.

Cell viability was assessed as described in section 2.2 using Muse® Count and Viability Kit and vital dye staining. Apoptosis was determined using Muse® Annexin V & Dead Cell Count (Section 2.3.1) and acridine orange staining (Section 2.3.2). Cell Cycle analysis was performed as described in Section 2.5 by flow cytometry using Muse® Cell Analyser and Muse® Cell Cycle Kit. Long term survival was determined by assessing the cell ability to form colonies over a period of 2-3 weeks (Section 2.4).

3.3 Results

3.3.1 The role of SMEK1 overexpression in Jurkat and CEM-C7 cells

The effects of SMEK1 overexpression on the leukemic Jurkat T cells were investigated and compared to control (vector only). Jurkat T Cells were transfected with pcDNA3.1-SMEK1 or pcDNA3.1 empty vector before examining the effects of SMEK1 over-expression on short- and long-term survival, apoptosis, and cell cycle profile.

The degree of overexpression of SMEK1 was determined by western blotting 48 hours post-transfection, with a 1.7-fold increase in the level of SMEK1 protein observed in Jurkat cells transfected with pcDNA3.1-SMEK1 as compared to mock transfected cells and cells transfected with pcDNA3.1 only (Figure 3.1, $p<0.05$ vs vector only transfection).

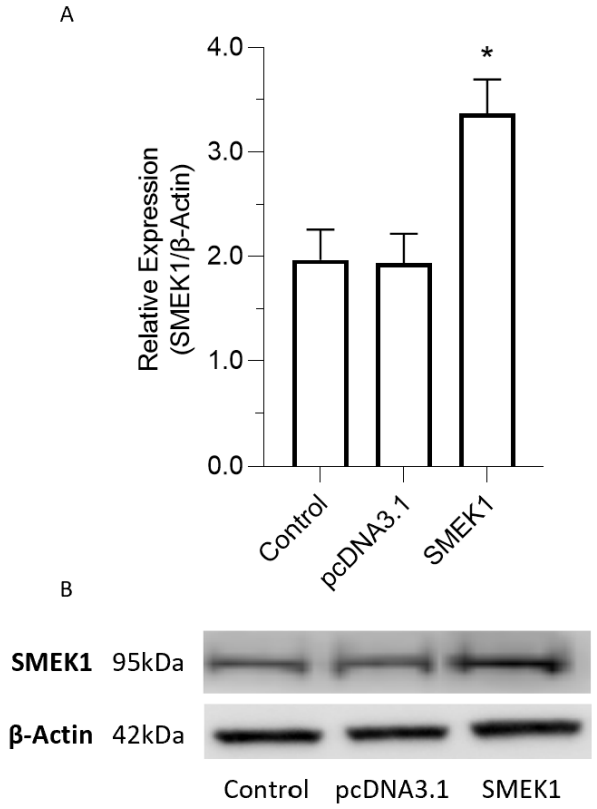


Figure 3.1 Effects of pcDNA3.1-SMEK1 transfection on endogenous SMEK1 protein expression levels in Jurkat cells. Cells lysates of transfected cells were separated by SDS-PAGE on 12% resolving gels followed by transfer to PVDF and detection of SMEK1. Blots were stripped and re-probed for β-actin. Western blotting and subsequent quantification show a significant elevation in the expression of SMEK1 in transfected cells as compared to control. (A) A bar graph represents three independent experiments expressed as mean ± SEM. Relative expression is the ratio of SMEK1 level versus β-actin as determined using a Bio-Rad ChemiDoc MP Imaging System. *$p<0.05$; one-way ANOVA test followed by Tukey's post-test. (B) Representative immunoblot image.

Overexpression of SMEK1 led to a significant increase in basal apoptosis of 2-fold compared to control as determined by fluorescence microscopy (Figure 3.2A). The results were confirmed when assessing apoptosis by Annexin V staining and flow cytometry where there was a 3.3-fold increase in basal apoptosis in cells overexpressing SMEK1 compared to control (Figure 3.2B).

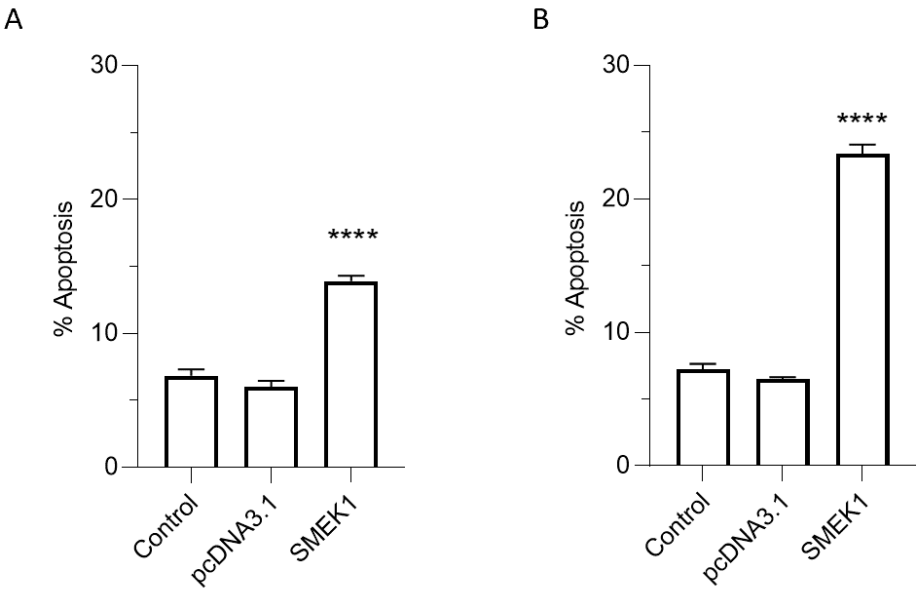


Figure 3.2 SMEK1 overexpression in Jurkat cell line increases basal apoptosis. Jurkat cells were transfected with pcDNA3.1-SMEK1 with or pcDNA3.1. Cells were assessed for basal apoptosis after 48 hours (A) % apoptosis as determined by acridine orange staining and fluorescence microscopy. (B) Total apoptosis using Annexin V staining and flow cytometry. Results represent means ± SEM with n=3. ****p<0.0001 relative to control as determined by one-way ANOVA and Tukey's post-test.

Overexpression of SMEK1 showed a significant decrease in both total and viable cells. There was approximately 50% decrease in the number of total and viable cells in the cells overexpressing SMEK1 compared to control (Figure 3.3A). The increase in total apoptosis and decrease in total and viable cell number caused by SMEK1 overexpression was associated with

significant reduction in the long-term survival of these cells as evidenced by a decrease in their colony forming ability (Figure 3.3B).

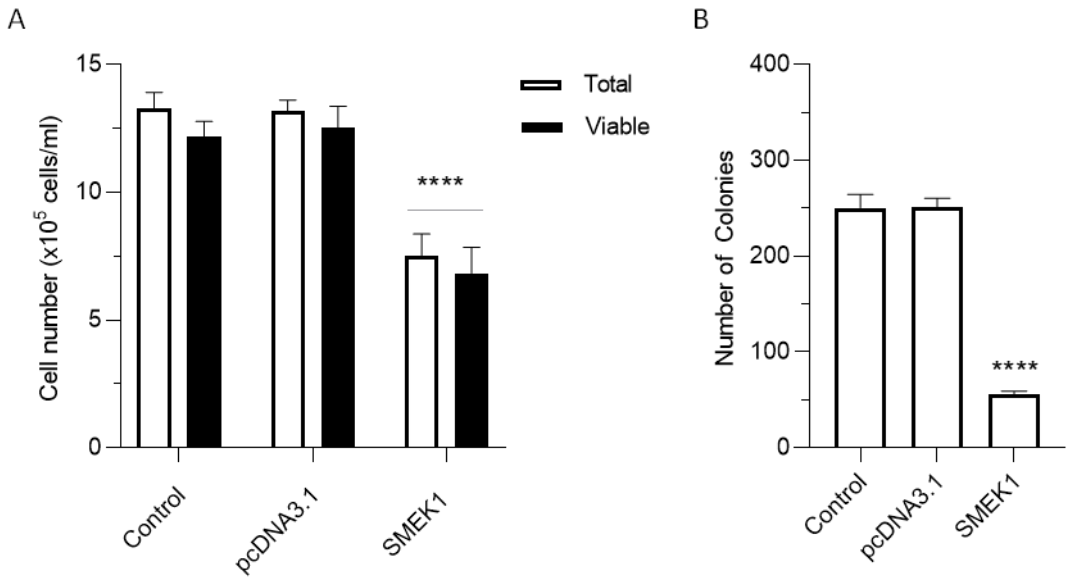


Figure 3.3 SMEK1 overexpression in Jurkat cells reduces short- and long-term survival. Jurkat cells were transfected with pcDNA3.1 or pcDNA3.1-SMEK1. (A) Total and viable cell number assessed using flow cytometry 48h post transfection. B) Colony forming assay. Results represent means ± SEM and are representative of data obtained from three independent experiments, ****p<0.0001 relative to control as determined by one-way ANOVA and Tukey's post-test.

To determine whether the growth suppression produced by SMEK1 were due to cell cycle arrest, cell cycle analysis was performed using propidium iodide staining and flow cytometry. The results revealed that the proportion of cells in the G1 and S phases in SMEK1 overexpressing cultures is consistently lower than that in the control. No change was observed in the G2/M phase compared to control (Figure 3.4). In addition, cultures of SMEK1 overexpressing cells showed a substantial increase in the proportion of cells in the Sub-G0 fraction, confirming that the apoptosis rate in these cells is indeed increased and suggesting that the growth suppression by SMEK1 was due to increased apoptosis (Figure 3.4).

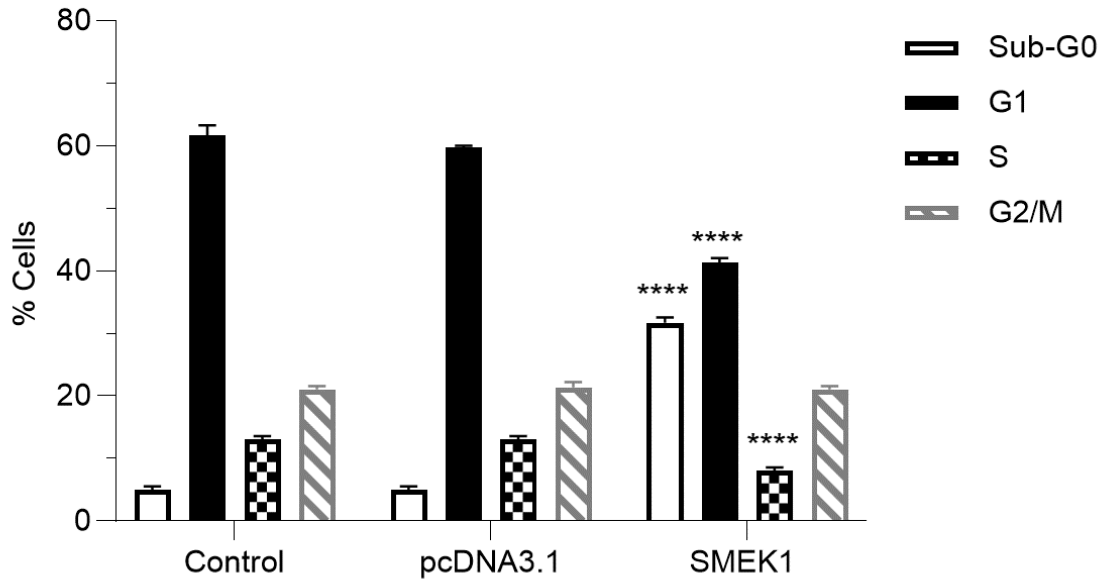


Figure 3.4 Effects of SMEK1 overexpression on the cell cycle profile of Jurkat T cells. Cell cycle analysis of Jurkat-pcDNA3.1 transfected cells and Jurkat-pcDNA3.1-SMEK1 transfected cells. DNA content was quantified by propidium iodide staining of fixed cells and flow cytometry. Results are represented as the mean ± SEM (n = 3). ****p<0.0001 relative to control as determined by two-way ANOVA and Bonferroni's post-test.

The results observed in Jurkat cells were further confirmed with CEM-C7 cells, confirming the growth inhibitory effects of SMEK1. SMEK1 transfection in CEM-C7 resulted in an approximate 2-fold increase in the level of SMEK1 protein expression (Figure 3.5). The increase in SMEK1 protein levels in CEM-C7 cells was associated with a decrease in total and viable cell number (Figure 3.6A), increased in basal apoptosis (Figure 3.6B) and reduction in long-term survival (Figure 3.6C), confirming the growth inhibitory role for SMEK1 in these leukemic T cells.

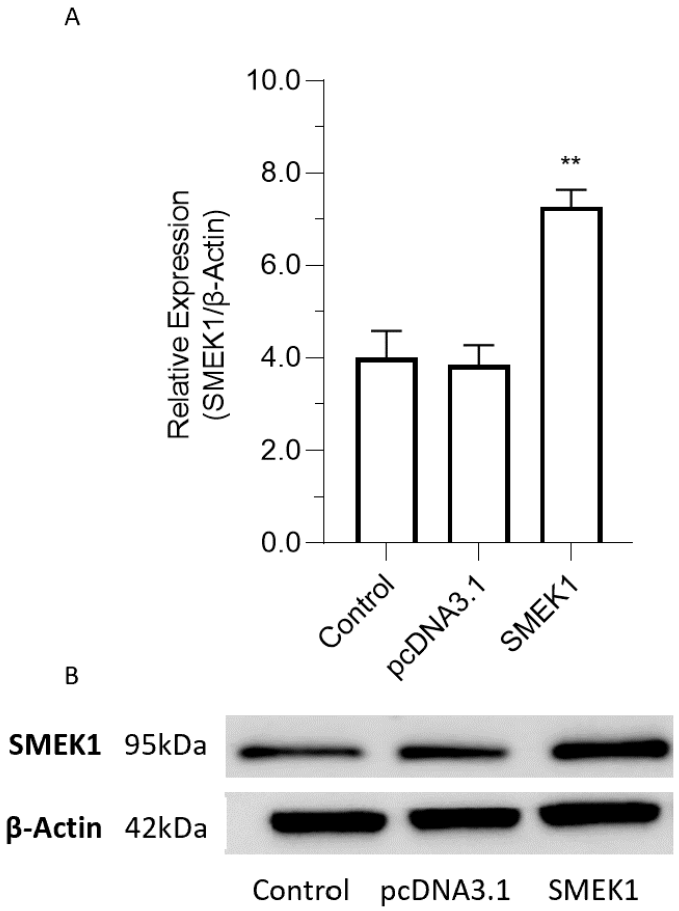


Figure 3.5 Effects of pcDNA3.1-SMEK1 transfection on endogenous levels of SMEK1 protein in CEM-C7 cells. CEM-C7 cells were transfected with pcDNA3.1-SMEK1 or pcDNA3.1. Cells lysates were prepared, and proteins were separated by SDS-PAGE on 12% resolving gels followed by transfer to PVDF and detection of PP4c. Blots were stripped and re-probed for β-actin. (A) A bar graph represents three independent experiments expressed as mean ± SEM. Relative expression is the ratio of SMEK1 level versus β-actin as determined using a Bio-Rad ChemiDoc MP Imaging System software (n=3). **p<0.01 relative to control as determined by one-way ANOVA and Tukey's post-test. (B) Representative immunoblot of SMEK1 protein expression.

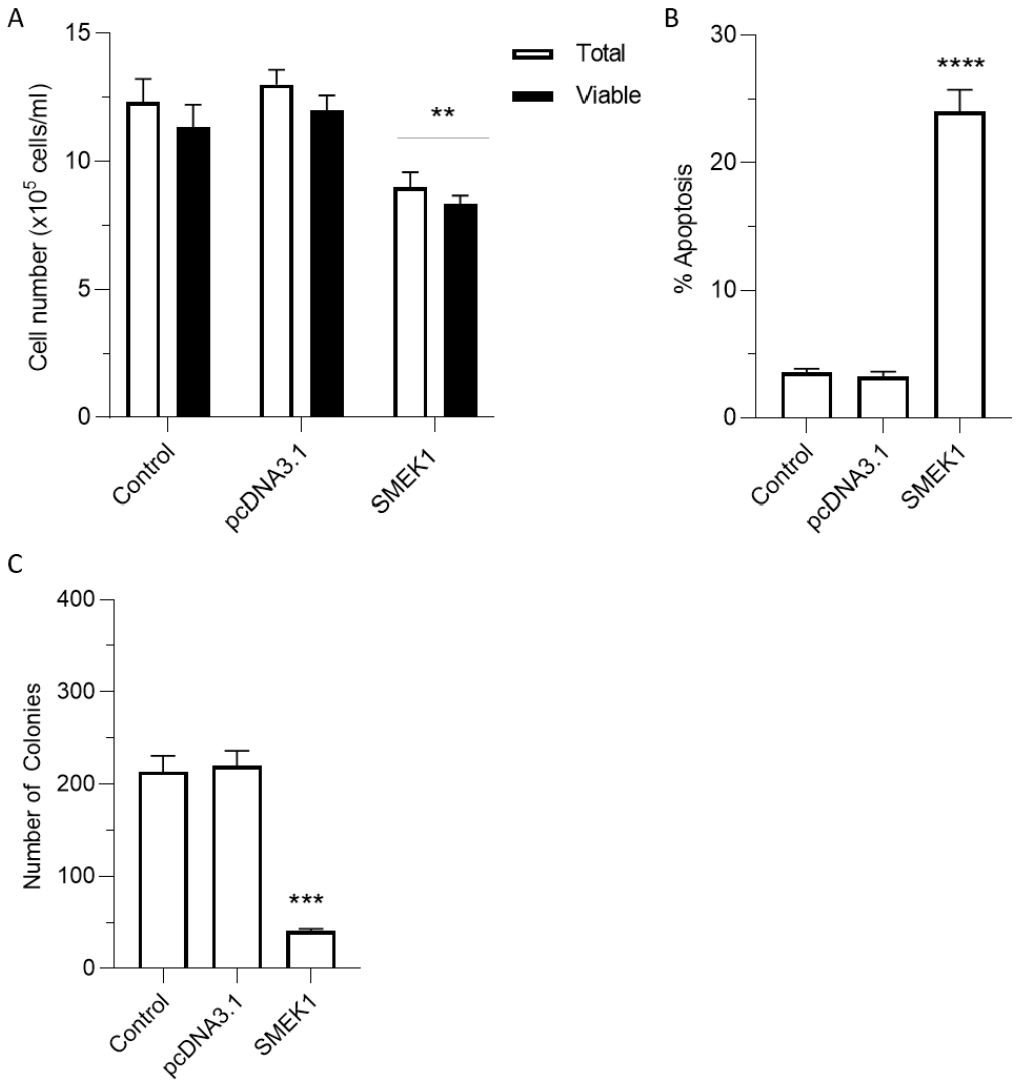


Figure 3.6 SMEK1 overexpression inhibits cell growth and increases apoptosis in CEM-C7 cells. CEM-C7 cells were transfected with either pcDNA3.1 or pcDNA3-SMEK1. (A) Total and viable cell number was determined using flow cytometry. (B) Total apoptosis was measured using the Annexin V staining and flow cytometry. (C) Long term survival assessed by colony forming assay. Results are represented as the means ± SEM (n = 3). **p<0.01, ***p<0.001, ****p<0.0001 relative to control as determined by one-way ANOVA and Tukey's post-test.

Cell cycle analysis for CEM-C7 cells transfected with SMEK1 confirmed the observations made in Jurkat overexpressing SMEK1 cells. The results showed that the proportion of cells in SMEK1 overexpressing cultures is consistently lower in G1 phase than that in the controls and the percentage of cells in S phase is also consistently lower with no changes in the percentage of cells in G2/M phase. Similar to Jurkat T cells, there was an increase in the proportion of cells in Sub-G0, further providing evidence of the pro-apoptotic role of SMEK1 in these cells.

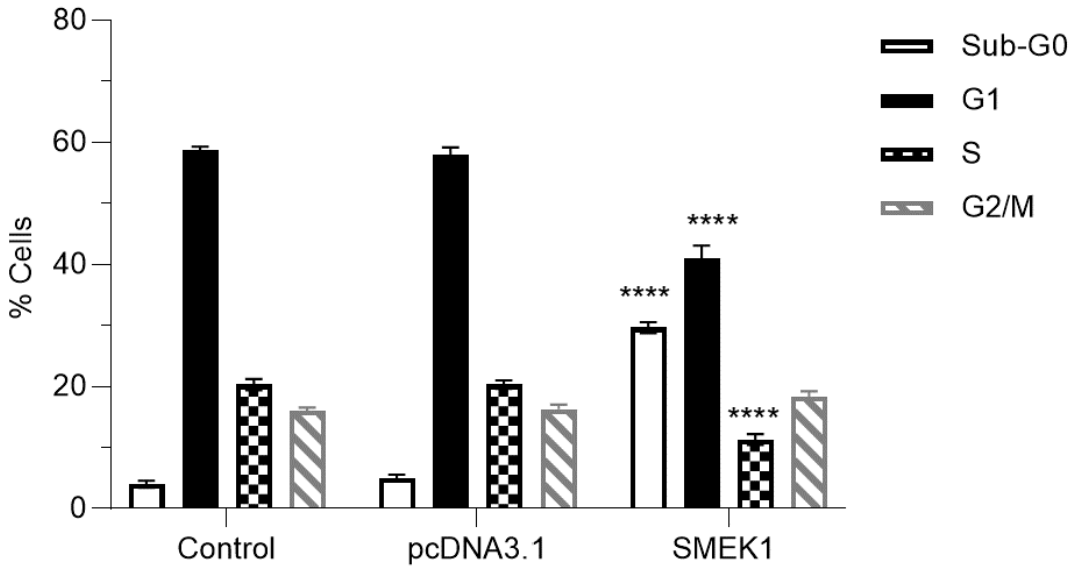


Figure 3.7 The effects of increased SMEK1 expression on the cell cycle profile of CEM-C7 cells. Cell cycle analysis of CEM-C7 cells transfected with pcDNA3.1 or pcDNA3.1- SMEK1. DNA content was quantified by propidium iodide staining of fixed cells and flow cytometry. Results are represented as the mean ± SEM (n = 3). ****p<0.0001 relative to control as determined by two-way ANOVA and Bonferroni's post-test.

Overall, the data presented strongly suggest that SMEK1 exerts a pro-apoptotic role in these two cells lines. Caspase 3 is an effector caspase that functions as a central regulator of apoptosis because of its role as an executioner caspase coordinating the destruction of cellular structures such as DNA fragmentation or degradation of cytoskeletal proteins (Hail *et al.,* 2006). Further experiments were carried out to investigate if SMEK1 overexpression affects the activity of caspase 3. Caspase 3 is present in healthy cells as inactive zymogens, but when stimulated it undergoes autolytic cleavage to become fully active. The cleaved caspase 3 can be detected by western blotting. The results confirmed that SMEK1 overexpression was associated with an increase in activated caspase 3 as shown by the increase in the level of cleaved caspase 3 in the cells overexpressing SMEK1. Results showed a 7.5-fold increase in the amount of cleaved caspase 3 compared to control in Jurkat T cells (Figure 3.8A and B) and 11-fold increase in CEM-C7 cells (Figure 3.8C and D), suggesting that SMEK1 induced-apoptosis involves activation of caspase 3. Caspase 3 activation occurs via the intrinsic and extrinsic apoptotic pathways. Intrinsic apoptosis is induced by cellular stress, leading to the activation of the proapoptotic members of Bcl-2 protein family. Bcl-2 Associated Agonist of Cell Death (BAD) is a pro-apoptotic member of the Bcl-2 family. Its effects are mediated by its ability to heterodimerize with survival proteins such as Bcl-$_{XL}$ leading to the promotion of cell death (Hardwick and Soane, 2013). The possibility that the increase in cell death and inhibition of cell survival induced by SMEK1 overexpression might be due to the change of BAD protein expression levels was also investigated. Results revealed that overexpressing SMEK1 in Jurkat and CEM-C7 cells for 48 hours led to significant increase in BAD expression relative to control in both cell lines. In both Jurkat and CEM-C7 cells, there was a 2-fold increase in BAD expression relative to control expression (Figure 3.9), implicating a role for BAD in SMEK1 induced apoptosis.

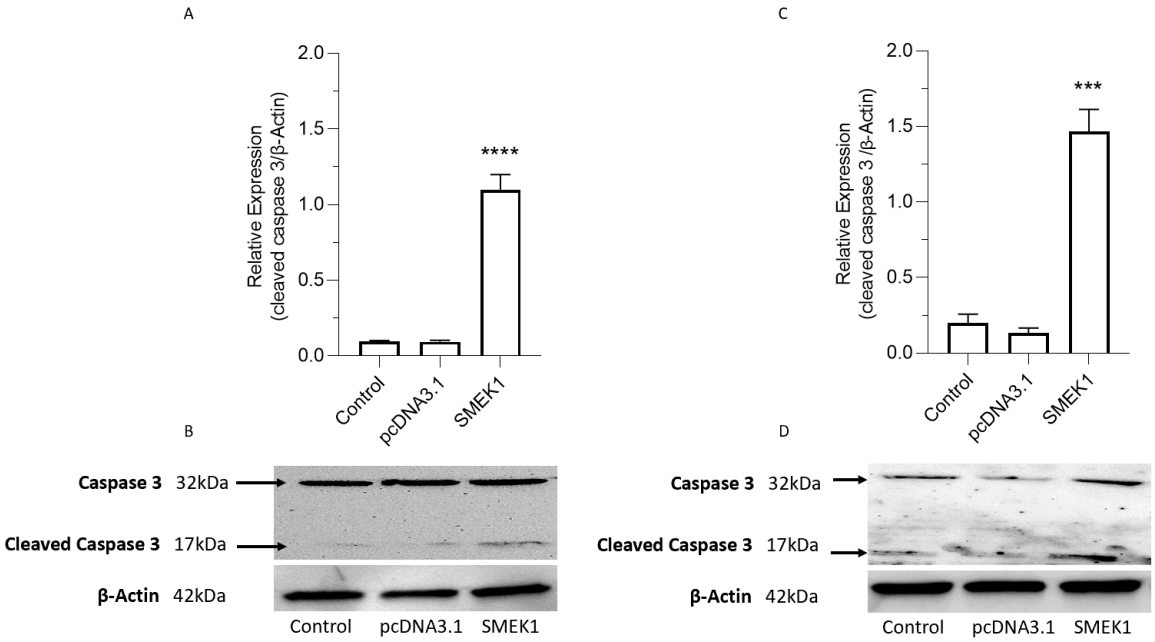


Figure 3.8 SMEK1 transfection increased the level of cleaved Caspase 3 in leukemic cells. Jurkat and CEM-C7 cells were transfected with pcDNA3.1-SMEK1 or pcDNA3.1. Lysates were prepared after 48h and used for western blotting. (A) Jurkat and (C) CEM-C7 Bar graphs showing ratio of cleaved caspase3 against relative to β-Actin. A bar graph represents three independent experiments expressed as mean ± SEM. ***p<0.001, ****p<0.0001 relative to control as determined by one-way ANOVA and Tukey's post-test. (B) and (D) Representative Western blot image collected using a Bio-Rad ChemiDoc MP Imaging System in Jurkat and CEM-C7 cells, respectively.

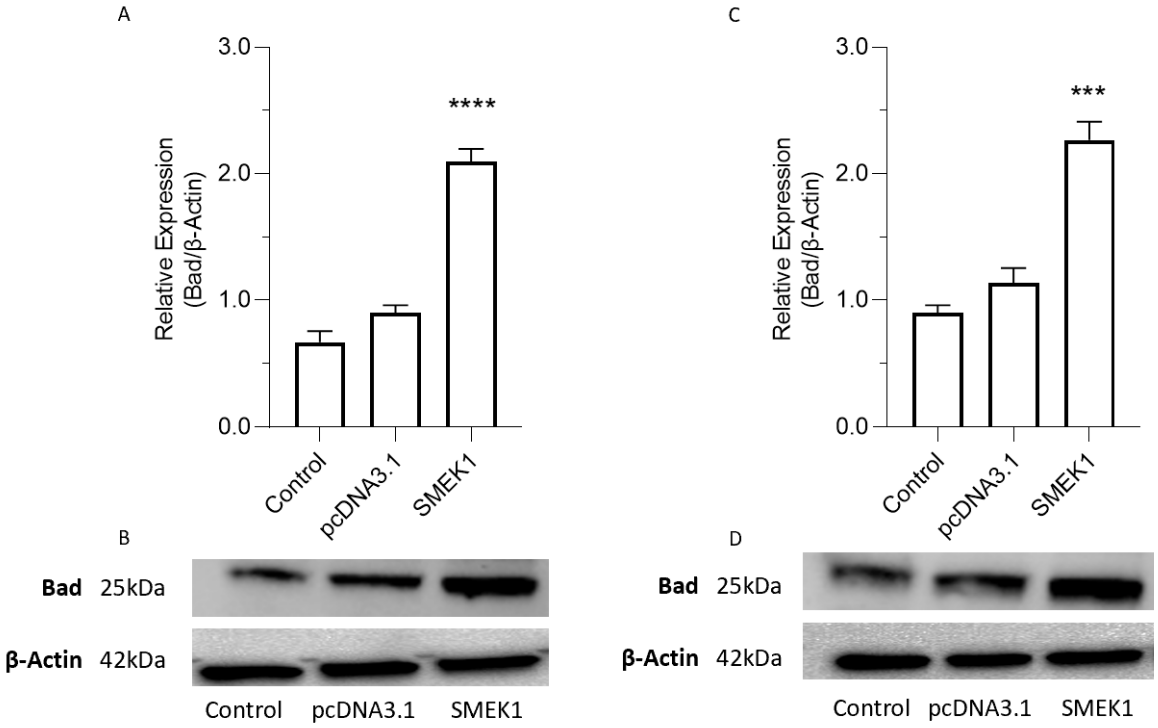


Figure 3.9 SMEK1 overexpression is associated with an increased expression in BAD protein level. Lysates were prepared from cells transfected with pcDNA3.1 or pcDNA3.1-SMEK1 and used for western blotting. (A) and (C) Bar charts present ratio of BAD protein against relative density of β-Actin for Jurkat and CEM-C7, respectively. Data are presented as ± SEM, n=3, ***p<0.001, ****p<0.0001 relative to control as determined by one-way ANOVA and Tukey's post-test. (B) and (D) Representative western blot image for Jurkat and CEM-C7 cells, respectively. Images collected using a Bio-Rad ChemiDoc MP Imaging System.

3.3.2 Effects of SMEK1 knockdown on leukemic cell survival

In order to investigate SMEK1 function further in CEM-C7 and Jurkat cells, specific SMEK1 siRNAs, SMEK1s1 and SMEK2s5, were used to inhibit endogenous PP4c expression in these cells. This strategy is particularly important to confirm the effects of overexpression of SMEK1 by an independent method and exclude possible artefacts. The efficiency of SMEK1 knockdown was determined by western blotting 48 hours post-transfection and the

specificity of the SMEK1 siRNAs was tested by comparison with the negative control siRNA (−siRNA).

Both SMEK1 specific siRNAs (SMEK1s1 and SMEK1s5) reduced SMEK1 protein levels in Jurkat and CEM-C7 cells by approximately 50% (Figure 3.10). SMEK1 knockdown did not affect basal apoptosis in both cell lines (results not shown). Reduced expression levels of SMEK1 in both cell lines was associated with an increased in total and viable cell number (Figure 3.11A and B) and an increase in their colony forming ability in both cell lines (Figure 3.11C and D). A cell cycle analysis was performed to study the effect of SMEK1 down-regulation on the cell cycle profile of Jurkat and CEM-C7 cells. Results showed that SMEK1 down-regulation had no effects on the cell cycle profile of Jurkat and CEM-C7 cells (results not shown). These findings support that SMEK1 regulates the survival of Leukemic cells.

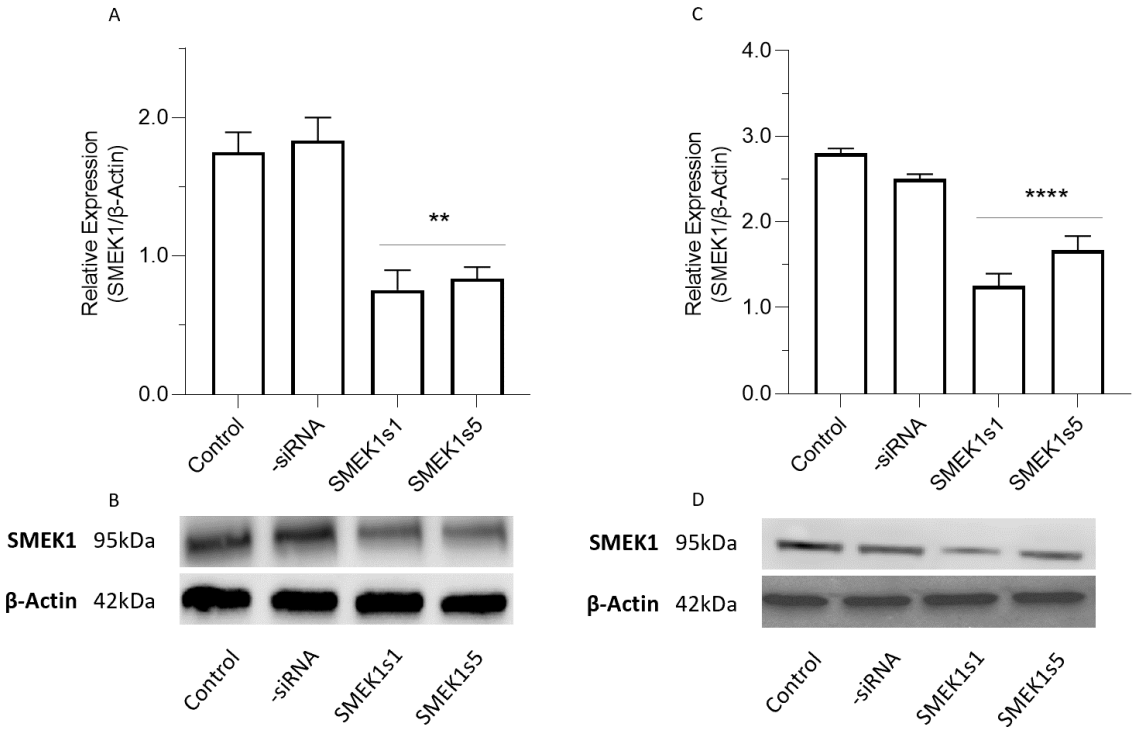


Figure 3.10 Effects of SMEK1 specific siRNAs on endogenous SMEK1 expression in leukemic cells. Cells lysates of transfected cells were separated by SDS-PAGE on 12% resolving gels followed by transferred to PVDF for detection of SMEK1. Blots were stripped and re-probed for β-actin. Western blotting and subsequent quantification show a significant reduction in the expression of SMEK1 in cells transfected with SMEK1 specific siRNAs compared to control (A) and (C) Relative expression is the ratio of SMEK1 to β-actin in Jurkat T cells (A) and CEM-C7 cells (C). Bar graphs represent three independent experiments expressed as mean ± SEM. **p<0.01, ****p<0.0001; one-way ANOVA test followed by Tukey's post-test). (B) and (D) Representative immunoblot images for Jurkat and CEM-C7 respectively.

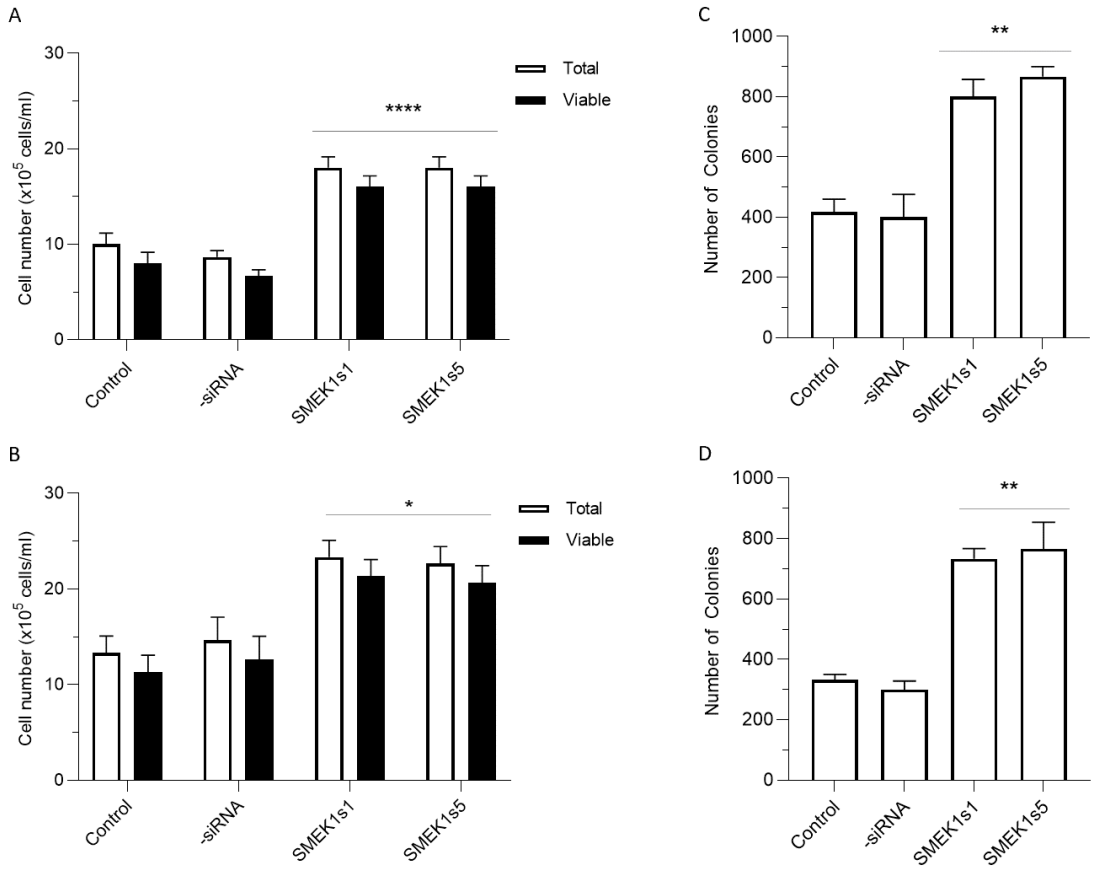


Figure 3.11 siRNAs mediated decrease in SMEK1 expression levels increases cell proliferation and survival of leukemic cells. CEM-C7 and Jurkat cells were transfected with SMEK1 specific siRNAs or with control −siRNA. Total and viable cell number for Jurkat (A) and CEM-C7 (B) was determined using flow cytometry. Long-term survival assessed by colony forming assay for Jurkat (C) and CEM-C7 (D). Results are represented as the means ± SEM (n = 3). *p<0.05, **p<0.01, ****p<0.0001 relative to control as determined by one-way ANOVA and Tukey's post-test**.**

Overall, the results show that reduced expression of SMEK1 resulted in increased survival of leukemic cells. Since SMEK1 overexpression induced apoptosis is linked to the activation of caspase 3, it was important to investigate if the anti-apoptotic effects of reduced SMEK1 expression involve X-linked inhibitor of apoptosis protein (XIAP). XIAP is the most potent member of the inhibitor of apoptosis protein (IAP) protein family in terms of its ability to inhibit caspase and suppress apoptosis (Hock *et al.,* 2001). Further experiments were therefore necessary to investigate whether the reduced level of SMEK1 influenced the levels of XIAP leading to the decrease in the basal apoptosis level and consequent increase in

survival. Indeed, the results showed that reduced SMEK1 protein levels were associated with an increased protein levels of XIAP in both Jurkat and CEM-C7 cells compared to control cells and cells transfected with (-) siRNA (Figure 3.12). There was a 3.5-3.7-fold increase in XIAP protein levels in Jurkat cells transfected with SMEK1 specific siRNAs and 2.2 -2.4-fold increase in CEM-C7 cells (Figure 3.12).

Previous studies reported that the stability/expression level of the catalytic subunit of PP4, PP4c, is influenced by its association with regulatory subunits. In addition, previous studies have shown that down-regulation of endogenous PP4c by 60–70% increased cell proliferation and Jurkat and CEM-C7 leukemic T cells, similar to the effects observed in cells with reduced SMEK1 levels. Therefore, we have investigated whether silencing SMEK1 affected PP4c protein level. As shown in Figure 3.13, protein levels of PP4c were significantly reduced in CEM-C7 and Jurkat cells transfected with SMEK1 siRNAs, suggesting that interaction with SMEK1 may maintain stability of PP4c.

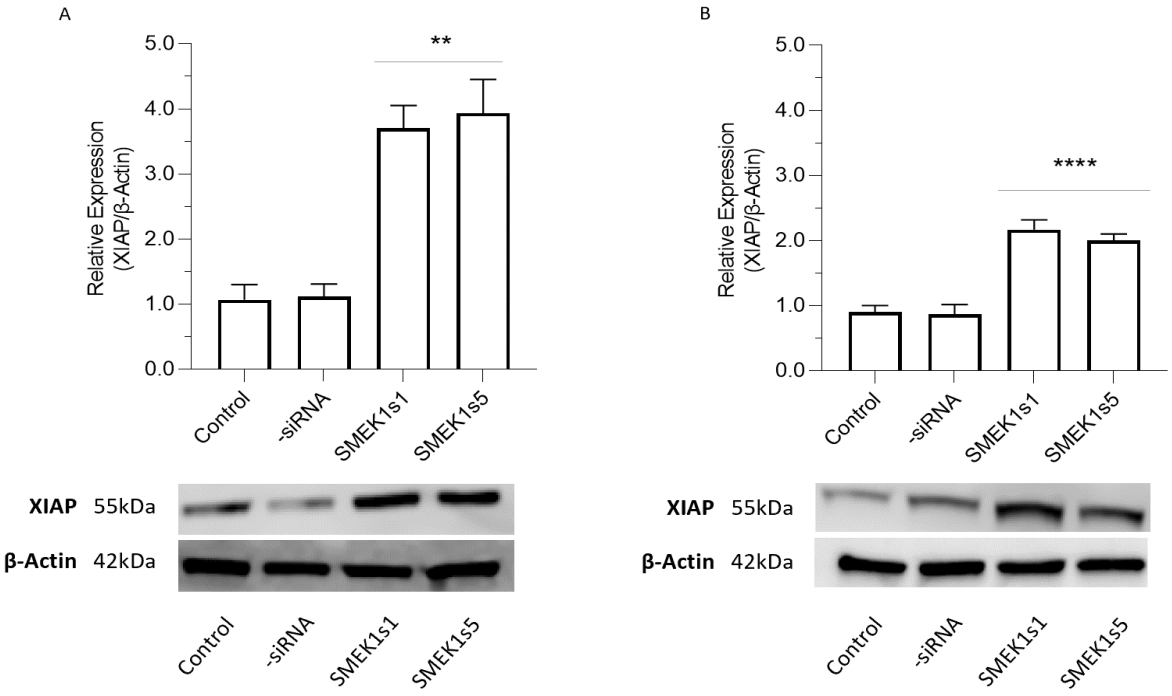


Figure 3.12 SMEK1 knockdown is associated with an increase in the protein level of XIAP. Jurkat and CEM-C7 cells were transfected with -siRNA or SMEK1 specific siRNAs. Cell lysates were prepared 72 hours post transfection. (A) and (C) Bar charts represent three independent experiments expressed as mean ± SEM. Relative expression is the ratio of XIAP level versus β-actin. (A) Jurkat, (B) CEM-C7. **p<0.01, ****p<0.0001 relative to control as determined by one-way ANOVA and Tukey's post-test. Representative Western blot images from Jurkat and CEM-C7 shown under its corresponding graph were collected using a Bio-Rad ChemiDoc MP Imaging System.

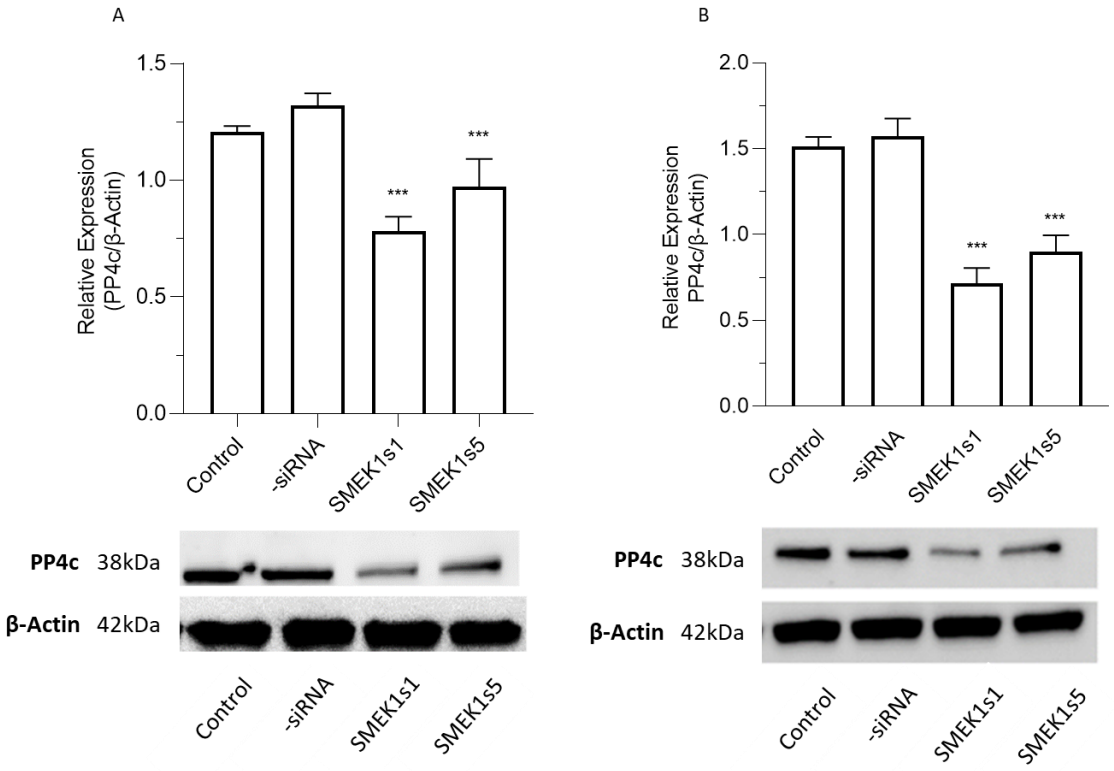


Figure 3.13 SMEK1 knockdown is associated with a decrease in the protein level of PP4c. Cell lysates were prepared 72h post transfection from Jurkat and CEM- C7 cells transfected with -siRNA or SMEK1 specific siRNAs (A) and (C) Bar charts represent three independent experiments expressed as mean ± SEM. Relative expression is the ratio of PP4c level versus β-actin. (A) Jurkat, (B) CEM-C7. ***p<0.001 relative to control as determined by one-way ANOVA and Tukey's post-test. Representative Western blot images collected from Jurkat and CEM-C7 using a Bio-Rad ChemiDoc MP Imaging System are shown under its corresponding graph.

3.3.3 Effects of modulation SMEK2 expression on the survival of Leukemic cells

SMEK1 and SMEK2 are isoforms of the PP4 regulatory subunit 3 PPP4R3. They both contain two highly conserved domains occupying the N-terminal region of the protein; an EVH1 domain, which belongs to the pleckstrin homology (PH) superfamily-like domains (Ball *et al.,* 2001), and a SMEK1 domain (DUF625 also known as domain of unknown function 625). SMEK1 domain/DUF625 have been implicated in affecting DAF-16-dependant regulation of

the aging process in *C.elegans* by regulating the transcriptional specificity of the conserved forkhead transcription factor DAF-16 (Wolff *et al.,* 2006). The results so far have established a growth inhibitory and proapoptotic role for SMEK1. It is not known if SMEK2 have any effect on cell fate decision and whether it has similar function in regulating Leukemic cell survival. Therefore, the effects of modulation SMEK2 expression on the survival of Leukemic T cells were investigated in this chapter. Jurkat and CEM-C7 cells were transfected with either pcDNA3.1 empty vector or pcDNA3.1-SMEK2 (cDNA of SMEK2 full length) before investigating the effects of SMEK2 overexpression on cell viability.

SMEK2 expression was analysed 48 hours post-transfection by western blotting and results revealed up to 2-fold increase in SMEK2 protein expression level in Jurkat and CEM-C7 cells (Figure 3.14). Similar to SMEK1, the number of total and viable cells in Jurkat and CEM-C7 cultures overexpressing SMEK2 was considerably decreased at 48 hours compared to cultures of untransfected cells and cells transfected with empty vector (Figure 3.15A and B) and total apoptosis increased (Figure 3.15C and D), as assessed using flow cytometry. Overexpression of SMEK2 caused a 1.8-fold decrease in Jurkat total and viable cell number and 2.3-fold decrease in CEM-C7 cells over-expressing SMEK2 compared to control and empty vector transfected cells (Figure 3.15A and B). Overexpression of SMEK2 also led to a 2-fold and 5.5-fold increase in apoptotic cells in Jurkat and CEM-C7 cells respectively, confirming the pro-apoptotic effect of over-expressing SMEK2 in these leukemic human cells (Figure 3.15C and D). Overall, the results confirmed a pro-apoptotic role for SMEK2 in these leukemic T cells.

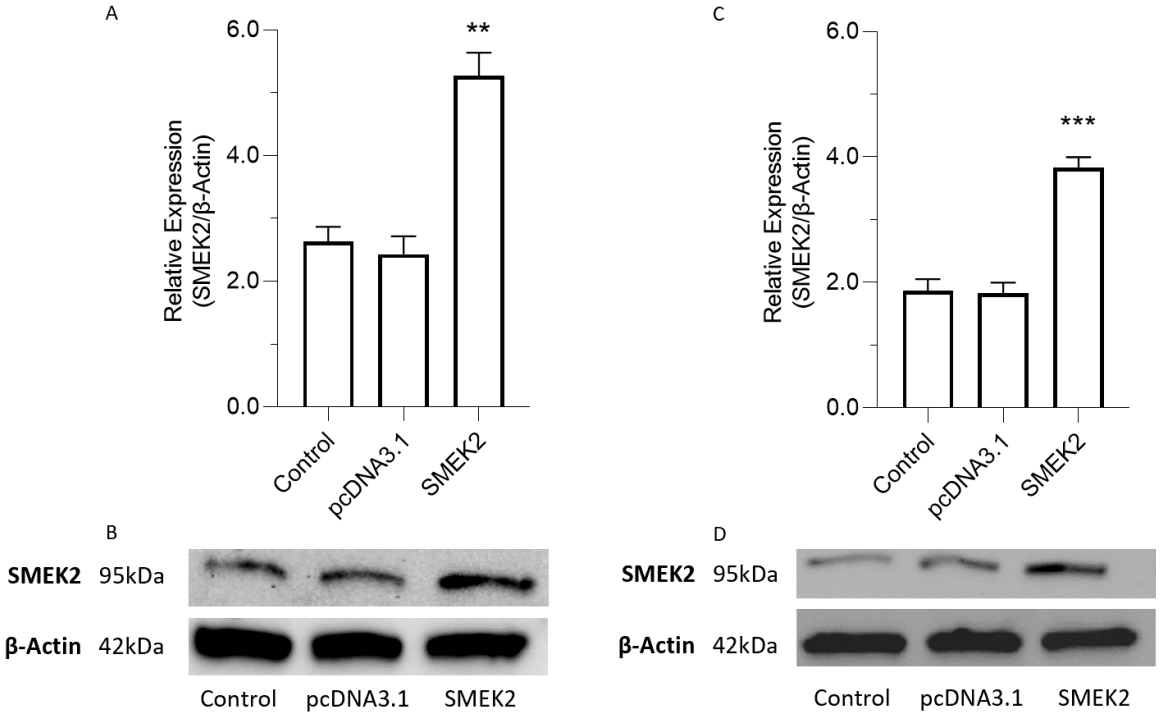


Figure 3.14 Effects of pcDNA3.1-SMEK2 transfection on endogenous expression level of SMEK2 in Jurkat and CEM-C7 cells. Cells lysates of transfected cells were separated by SDS-PAGE on 12% resolving gels followed by transfer to PVDF and detection of SMEK2. Blots were stripped and re-probed for β-actin. Western blotting and subsequent quantification show a significant elevation in the expression of SMEK2 in transfected cells as compared to control. (A) Jurkat and CEM-C7 (C) bar graph represents three independent experiments expressed as mean ± SEM. Relative expression is the ratio of SMEK2 level versus β-actin as determined using a Bio-Rad ChemiDoc MP Imaging System.; **p<0.01, ***p<0.001 one-way ANOVA test followed by Tukey's post-test. (B) Jurkat and (D) CEM-C7 representative immunoblot image.

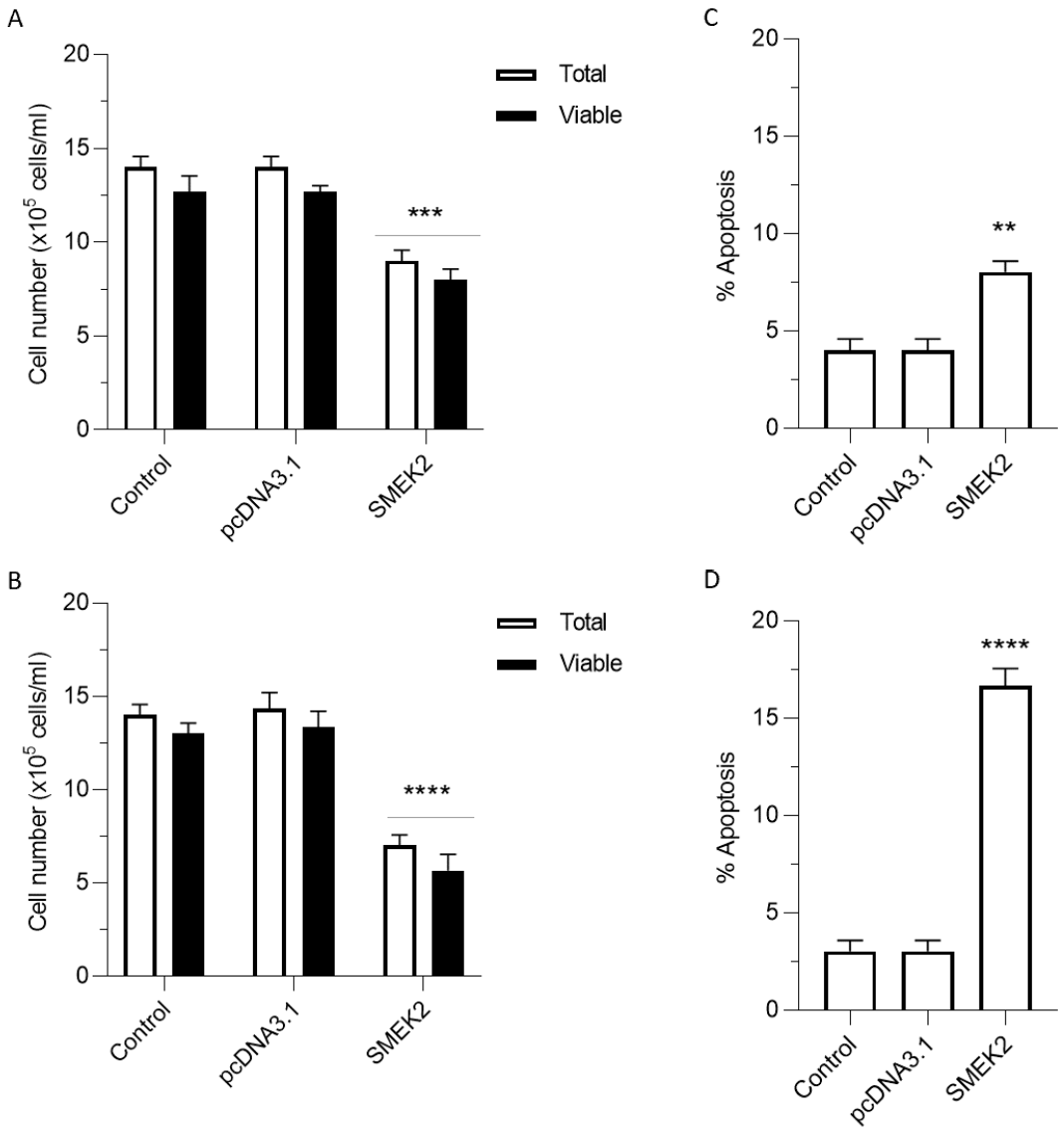


Figure 3.15 SMEK2 overexpression inhibits cell growth and increases apoptosis in Jurkat and CEM-C7 cells. Jurkat and CEM-C7 cells were transfected with either pcDNA3.1 or pcDNA3-SMEK2. (A) Jurkat and (B) CEM-C7 total and viable cell number was determined using flow cytometry. (C) Jurkat and (D) CEM-C7 total apoptosis was measured using the Annexin V staining and flow cytometry. Results are represented as the means ± SEM (n = 3). **p<0.01, ***p<0.001 ****p<0.0001 relative to control as determined by one-way ANOVA and Tukey's post-test.

Cell cycle analysis for both Jurkat and CEM-C7 cells revealed a considerable increase of cells in Sub-G0, confirming a proapoptotic role for SMEK2 similar to SMEK1 (Figure 3.16). The increase is the proportion of cells in Sub-G0 was associated with a decrease in the proportion of cells in G1 and S phase (Figure 3.16) and no changes were observed in G2 phase.

The above data strongly suggests and supports that it is likely that both isomers of the PP4R3 subunit exert similar effects.

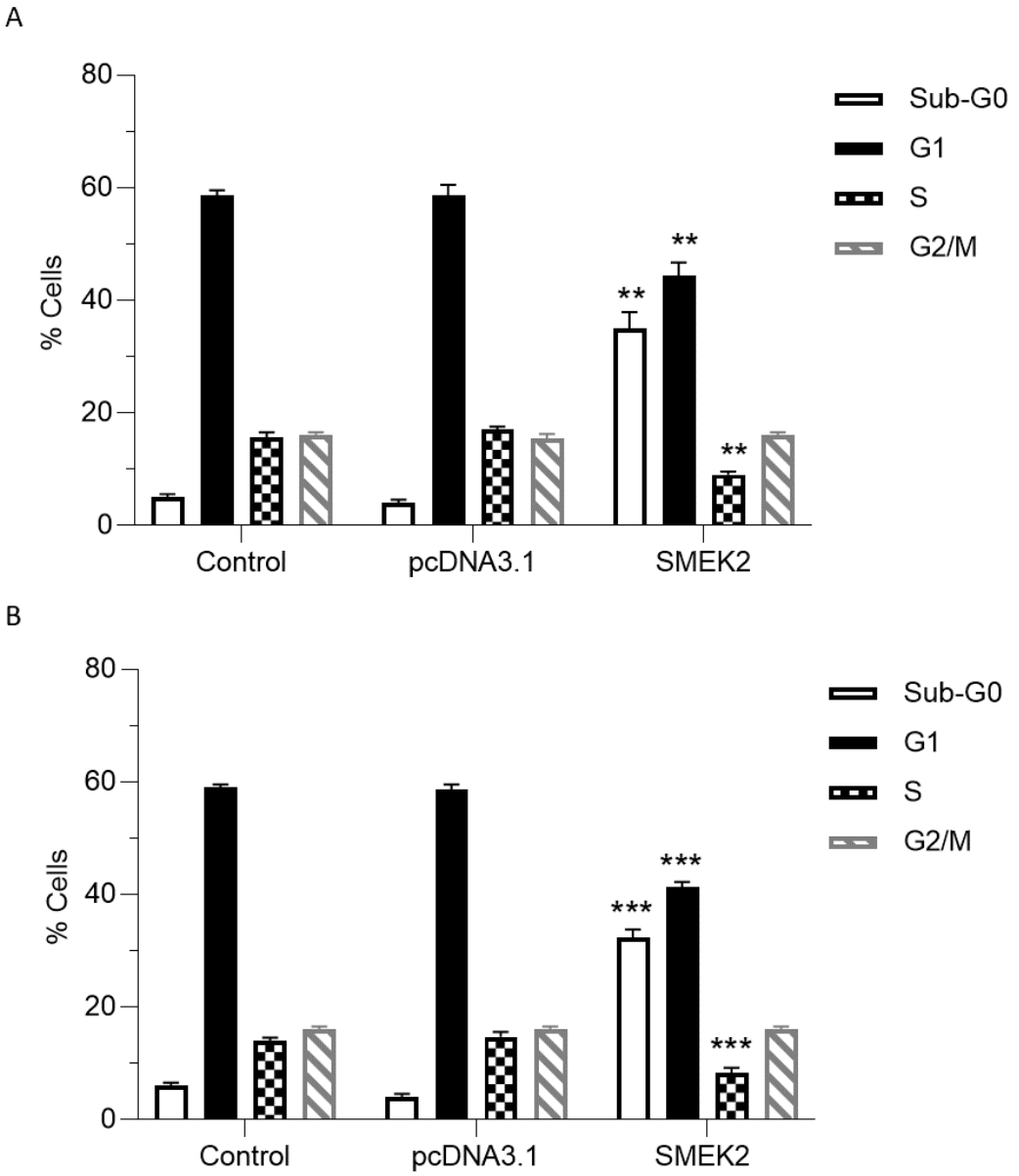


Figure 3.16 The effects of increased SMEK2 expression on cell cycle profile of leukemic cells. Jurkat (A) and CEM-C7 (B) cells were transfected with pcDNA3.1 or pcDNA3.1-SMEK2. DNA content was quantified by propidium iodide staining of fixed cells and flow cytometry. Results are represented as the mean ± SEM (n = 3). **p<0.01, ***p<0.001, relative to control as determined by two-way ANOVA and Bonferroni's post-test.

In order to investigate SMEK2 function further in CEM-C7 and Jurkat cells, we used SMEK2 specific siRNAs, SMEK2s3 and SMEK2s5, to inhibit endogenous SMEK2 expression in these cells. Transfection of SMEK2s3 and SMEK2s5 in both cell types led a significant decrease in the protein expression of the SMEK2 compared to un-transfected control and -siRNA transfected cells (Figure 3.17). SMEK2 reduced expression did not affect basal apoptosis level (results not shown). SMEK2 silencing was associated with an increase in the number of total and viable cells in both cells (Figure 3.18) but had no effects on the cell cycle profile (results not shown). These results show that reduction in the SMEK2 protein levels has the opposite effects seen with SMEK2 overexpression, confirming a role for SMEK2 in the regulation of Leukemic cell survival.

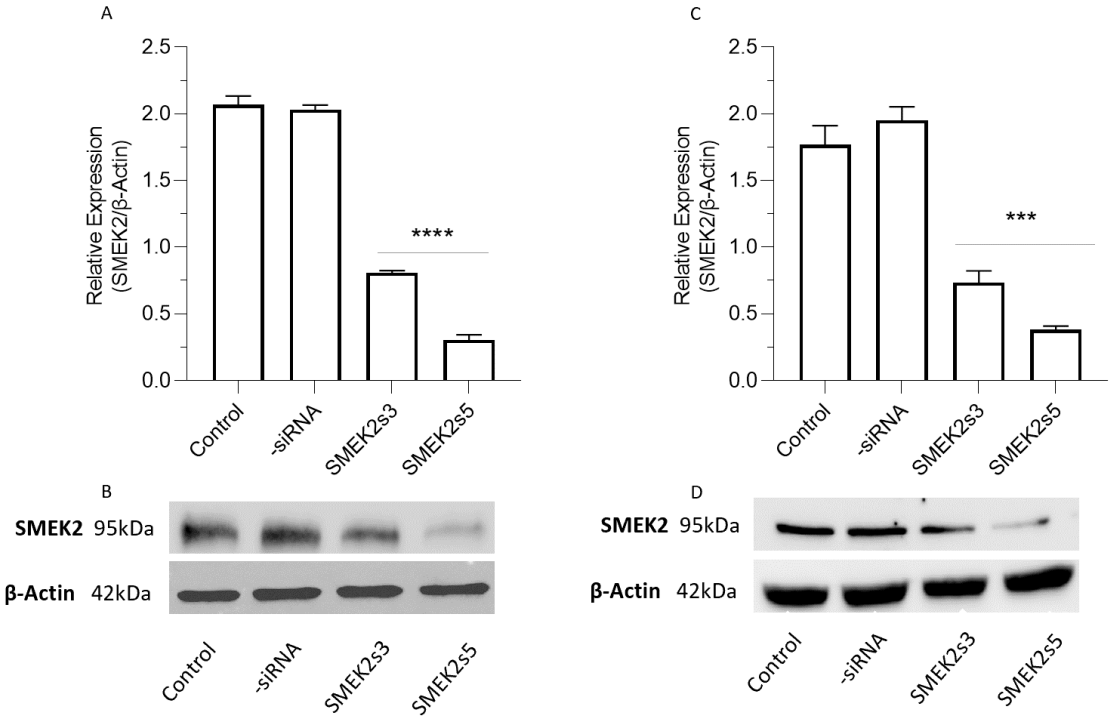


Figure 3.17 Effects of SMEK2 specific siRNAs on endogenous SMEK2 in leukemic cells. Cells lysates of transfected cells were separated by SDS-PAGE on 12% resolving gels followed by transfer to PVDF and detection of SMEK2. Blots were stripped and re-probed for β-actin. Western blotting and subsequent quantification show a significant reduction in the expression of SMEK2 in cells transfected with SMEK2 specific siRNAs compared to control (A) and (C). Relative expression is the ratio of SMEK2 to β-actin in Jurkat T cells (A) and CEM-C7 cells (C). Bar graphs represent three independent experiments expressed as mean ± SEM. ***p<0.001, ****p<0.0001; one-way ANOVA test followed by Tukey's post-test). (B) and (D) representative immunoblot images for Jurkat and CEM-C7 cells, respectively.

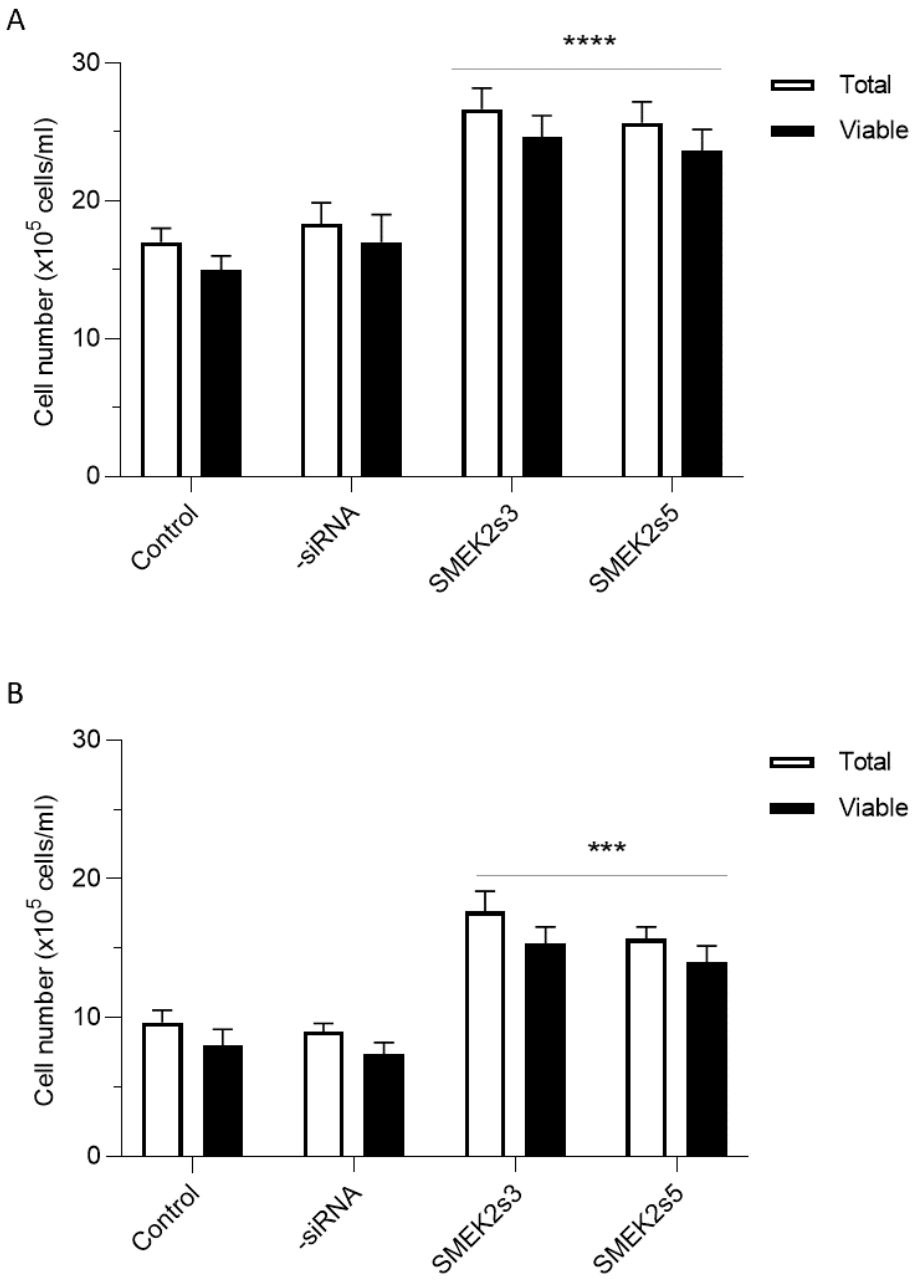


Figure 3.18 SMEK2 specific siRNAs increase cell proliferation and survival of leukemic cells. CEM-C7 and Jurkat cells were transfected with SMEK2 specific siRNAs or with control –siRNA. Total and viable cell number for Jurkat (A) and CEM-C7 (B) was determined using flow cytometry. Results are represented as the means ± SEM (n = 3). ***p<0.001, ****p<0.0001 relative to control as determined by one-way ANOVA and Tukey's post-test**.**

3.4 Discussion

Previous studies have implicated PP4c in regulating apoptosis and the growth and survival of both normal and leukemic human T-cells (Mourtada-Maarabouni and Williams, 2009). The data presented in this chapter confirmed that both SMEK1 and SMEK2 also exert profound and specific effects on the growth and survival of leukemic human T-cells. The results presented reveal that similar to the effects of PP4c, overexpression of SMEK1 and SMEK2 in the T-leukemic cell lines CEM-C7 and Jurkat increased basal apoptosis in the absence of extracellular apoptotic stimuli and decreased cell proliferation and survival. The effects of SMEK1 and SMEK2 on increasing cell death was also demonstrated by cell cycle analysis where overexpression of both proteins increased the percentage of cells in sub-G0 and consequently resulted in the decrease in the percentage of cells in G1 and S phases of the cycle. In support of the overexpression results, siRNA mediated silencing of SMEK1 and SMEK2 promoted short and long cell survival and the rate of cell proliferation in these leukemic T cell lines. Overall, the results suggest that both SMEK1 and SMEK2 might be acting as tumour suppressors.

The results presented in this study which confirm a role for SMEK1 in the control of survival of leukemic cells are consistent with previous studies performed in ovarian cancer cells (Dong *et al.,* 2012). Modulation of SMEK1 expression levels were shown to have effects on the level of cell proliferation and apoptosis in ovarian cancer cells (Dong *et al.,* 2012). SMEK1 overexpression in OVCAR3 ovarian cells sensitised the cells to the chemotherapeutic drug gemcitabine-induced apoptosis and increased the expression of proapoptotic proteins such p21, p27, and p53, and a reduction in the expression levels of anti-apoptotic proteins

such Bcl-2 and Bcl-$_{xL}$ (Dong *et al.,* 2012; Byun *et al.,* 2012). The results confirmed that SMEK1 overexpression led to an increase in basal apoptosis which was associated with the activation of caspase 3 and an increased expression of the proapoptotic protein BAD. Caspase 3 is key effector enzyme in inducing cell apoptosis and known as an executioner caspase in apoptosis because of its role in coordinating the processes that lead to the destruction of apoptotic cell which include DNA fragmentation and degradation of cytoskeletal proteins (Vickers *et al.,* 2013). BAD is a pro-apoptotic member of the Bcl-2 family. Its effects are mediated by its ability to heterodimerize with survival proteins such as Bcl-$_{xL}$ leading to the promotion of cell death (Kluck, 2010). The increase in protein expression levels of BAD and active caspase3 in SMEK1 overexpressing cells suggests that SMEK1 induced apoptosis is dependent on the activation of apoptotic intrinsic pathway leading to the activation of caspase 3. The involvement of caspase 3 in SMEK1 mediated cell death was further confirmed by the effects of SMEK1 down-regulation which resulted in an increase in short- and long-term survival that was related to the increase in X-inhibitory of apoptosis (XIAP) expression levels. XIAP is a potent enzymatic inhibitor of mammalian caspases in both the extrinsic and the intrinsic caspase pathway. ((Deveraux *et al.,* 1997; Carter *et al.,* 2013) and the increase in its protein expression levels in cells with down-regulated SMEK1 resulted in inhibition of cell death leading to increased short- and long-term survival. XIAP has been shown to be over-expressed in various cancers leading to poor overall survival and an increase level of XIAP was reported to inhibit apoptosis in glioblastoma (Tirapelli *et al.,* 2017). Therefore, the increase in XIAP protein expression in cells expressing low SMEK1 is significant for cancer cell survival and support a role for SMEK1 as a tumour suppressor.

Previous studies have reported that over-expression of SMEK1 causes cell cycle arrest in G1 phase by increasing transcription of P53 in OVCAR-3 cancer cells (Byun *et al.,* 2012). Cell

cycle analysis in the present study contrasts with Byun *et al.,* (2012) finding and showed that SMEK1 and SMEK2 overexpression causes an increase in the percentage of cells in Sub-G0 with a corresponding decrease in the percentage of cells in G1 and S phases. Such results suggest that SMEK1 is not affecting cell cycle progression and the decrease of viable and total cell number of SMEK1 overexpression in Jurkat and CEM-C7 cells could mainly be due to an increase in cell death. Such discrepancy may be due to the fact that both Jurkat and CEM-C7 T cells do not express functional p53 (Karpinich *et al.,* 2006). Cheng and Haas (1990) reported a heterozygous single-nucleotide substitution in codon 196 in Jurkat cells which leads to the production of shorter isoform of P53, and this mutation was suggested to be the leading cause of the oncogenic transformation of leukemic T cells (Cheng and Haas, 1990). Subsequent studies have confirmed the existence of the same heterozygous mutation in exon 6 of the TP53 gene and suggested that this mutation is likely responsible for the consistent reports of p53 dysfunction in leukemic T cells (Bending *et al.,* 2004).

The pro-apoptotic and growth inhibitory activities of SMEK1 have also been reported in endothelial cells. Studies have shown that SMEK1 overexpression inhibits angiogenesis by suppressing the proliferation of vascular endothelial growth factor (VEGF)-induced cell proliferation, migration, and the formation of capillary-like tubular structure in vitro (Kim *et al.,* 2015). The same study has shown that SMEK1 binds to vascular endothelial growth factor receptor 2 (VEGFR-2) inhibiting the phosphorylation of the signalling molecules involved in AKT signalling pathway, including phospholipase Cγ1 (PLC-γ1), 3-phosphoinositide-dependent protein kinase 1 (PDK1), endothelial nitric oxide synthase (eNOS), and hypoxia-inducible factor 1 (HIF-1α). The inhibitory effects of SMEK1 on angiogenesis was also confirmed in a xenograft human ovarian tumour model (Kim *et al.,* 2015). These results confirm SMEK1 controls endothelial cell function and suppress angiogenesis by blocking the activation of

VEGFR-2-mediated PI3K/Akt/eNOS signalling pathway. It is well documented that the PI3K/AKT signalling pathway plays an important role in T-cell growth and function. The activation of PI3K which results in the activation of Akt protects from Fas-mediated apoptosis in Jurkat and other T-cell lines (Xu *et al.,* 2002). It is therefore important to investigate the effects of SMEK1 silencing or over-expression on the PI3K/AKT signalling pathways and other signalling pathways in order to identify cellular pathways targeted by SMEK1 and SMEK2.

The interplay between the different targeting regulatory subunits of PP4 and PP4c appears to be central to the function of the PP4 enzyme. PP4c is targeted to its specific sites of action by its regulatory subunits and it is the interchange between the different regulatory subunits and binding proteins that plays a critical role in regulating the activity of PP4 complexes (Hastie *et al.,* 2000). The effects of modulation SMEK1 and SMEK2 on the survival of both types of leukemic T cells are similar to those observed when PP4c was overexpressed or silenced in these cells which suggest that SMEK1 and SMEK2 might mediate their action via their interaction with PP4c. SMEK1 and SMEK2 overexpression might stabilise PP4c leading to an increase in its phosphatase activity which is associated with growth inhibitory and proapoptotic effects in leukemic T cells (Mourtada-Maarabouni and Williams, 2009). Indeed, the results showed that down-regulation of SMEK1 in leukemic cell lines led to significant decrease in PP4c protein expression levels, suggesting that interaction with SMEK1 may maintain stability of PP4c increasing its activity. While overexpression of SMEK1 and PP4c caused similar growth inhibitory and proapoptotic effects in leukemic T cells, their effects on the cell cycle were different. PP4c overexpression in Jurkat and CEM-C7 cells induces cell cycle arrest in G1 (Mourtada-Maarabouni and Williams, 2009), whereas the effects of SMEK1 overexpression led to a significant increase in the percentage of cells in Sub-G0 and no cell cycle arrest in G1. Another difference is that PP4c overexpression leads to the

dephosphorylation of BAD without affecting its protein expression levels (Mourtada-Maarabouni and Williams, 2008). While SMEK1 overexpression results in an increased in BAD protein expression levels. Overall, the results suggest that SMEK1 regulate the survival of leukemic T cells via its interaction with PP4c and independently of PP4c.

The present report confirms that SMEK2 also regulate apoptosis in leukemic T cells. Overexpression of SMEK2 led to an increase in apoptosis and decrease in long- and short-term survival of leukemic T cells. The cell cycle analysis confirms the pro-apoptotic role of SMEK2 where overexpression led to an increase in percentage of cells in Sub-G0. These results were confirmed by the siRNA mediated silencing of SMEK2. Overall, the results suggest that SMEK2 and SMEK1 might be functionally similar for the regulation of the survival of leukemic T cells. Further investigation is required to confirm if the two proteins are functionally redundant.

It is clear that multiple cellular functions are regulated by SMEK1 and SMEK2 and that several of these are crucial to determining leukemic cell survival. The findings in this chapter indicate that the endogenous level of SMEK1 plays a critical role in maintaining the delicate balance between cell survival and cell death both in T-leukemic cells and acts through direct or indirect interaction with PP4c. These observations suggest that modulating SMEK1, SMEK2 may prove important in therapeutic strategies for the treatment of leukaemia.

3.5 Chapter highlights

1- Overexpression of SMEK1 in the T-leukemic cell lines CEM-C7 and Jurkat leads to the decrease in the short and long-term survival. These effects were associated with an increase in the rate of basal apoptosis levels and percentage in cell in Sub-G0, activation of caspase 3 and increased expression levels of BAD protein.

2- Down-regulation of endogenous SMEK1 increased the rate of cell proliferation. These effects were associated with an increased protein expression levels of the apoptosis inhibitor XIAP. PP4c protein expression levels in these cells were significantly reduced, suggesting a role for SMEK1 in maintaining the stability of PP4c and that SMEK1 might exerts its function at least in part, via its interaction with PP4c.

3- Modulation of SMEK2 expression in the T-leukemic cell lines CEM-C7 and Jurkat resulted in similar effects to SMEK1. Further experiments are required to determine if the two proteins are functionally redundant for the regulation of the survival of leukemic T cells.

Chapter Four: Investigation into the relationship between SMEK1 and SMEK2 and major signalling pathways involved in the regulation of cell survival

4.1 Introduction

The results presented in the previous chapter have elucidated the role of SMEK1 and SMEK2 in the regulation of survival and proliferation of T leukemic cells. Both SMEK1 and SMEK2 appears to have similar function as regulator of cell fate. Our studies have shown that both SMEK1 and SMEK2 have tumour suppressor activities. Overexpression of each of these proteins promoted apoptotic cell death and decreased cell survival. The proapoptotic and growth inhibitory effects of SMEK1 were accompanied by an increase in the activity of caspase 3 and protein levels of the proapoptotic protein BAD and a decrease in the protein levels of X-linked inhibitor of apoptosis protein (XIAP), suggesting that SMEK1 regulates signalling pathways involved in cell fate decision.

Both SMEK1 and SMEK2 are regulatory subunits of the serine/threonine phosphatase PP4 which is a key player in critical biological processes such as apoptosis, cell cycle, cell proliferation, DNA damage checkpoint, and TNFα (tumour necrosis factor alpha) signalling (Kathie et al., 2004). SMEK1 has been shown to bind to several intracellular proteins associated with these cellular processes (Chowdhury et al.,2008). Previous studies have also shown that SMEK1 interacts with components of the signalling pathways that regulate cell survival such as the mammalian target of rapamycin (mTOR) (Zhang et al., 2005). Overexpression of SMEK1 in the ovarian cancer cell line, OVCAR-3, caused a decrease in the phosphorylated mTOR (Kim et al., 2012). siRNA mediated knockdown of SMEK1 results in a decrease in the ability of IL-1β to activate NFκB and its subsequent downstream targets (Gewurz et al., 2012). These results imply that SMEK1 is involved in the regulation of both

mTOR and NFκB signalling pathways and suggest that SMEK1 might regulate other signalling pathways.

Many signalling pathways have been studied extensively for their roles in cell growth, cell fate decision and cancer. These include MAPK, AKT, JAK/STAT, NFκB, and TGFβ signalling pathways. MAPK signalling pathway regulates the expression of many proteins involved in the control of cell proliferation, differentiation, and apoptosis (Plotnikov et al., 2015). Alterations in MAPK signalling pathway and its targets have been shown to be involved in oncogenesis, progression of tumours, and drug resistance (Chapnick et al., 2011 and Plotnikov et al., 2015). AKT signalling pathway regulates several cellular functions including nutrient metabolism, cell growth, apoptosis, and survival (Yang *et al.,* 2017). JAK/ STAT pathway is central to signalling by cytokine receptors and regulates cell proliferation, survival, differentiation, and immune response. Several cancers, including blood malignancies, have been associated with constitutive activation of members of the STAT family, which usually require JAK-mediated tyrosine phosphorylation for transcriptional activation (Thomas et al., 2015). NFκB is an important signalling pathway involved in regulation of cell differentiation, proliferation, and apoptosis and plays an important role in the pathogenesis and treatment of cancers (Longzheng et al., 2018). NFκB signalling pathway controls the expression of genes, such as *TNFα, IL6, BCL-xL, BCL2* , *XIAP ,* and *VEGF* (Longzheng et al., 2018). TGFβ pathway has been studied extensively in different cancers including breast, prostate, colorectal and pancreatic (Padua and Massague, 2009). TGFβ signalling regulates cell proliferation, differentiation, apoptosis, cell plasticity, and migration. TGFβ signalling can be mediated by SMAD proteins or other signalling proteins such as MAP kinases and Akt (Jakowlew, 2006; Tian *et al.,* 2011). Deregulation of TGFβ signalling contributes to developmental defects and human diseases including cancer and liver and lung fibrosis (Jakowlew, 2006; Tian *et* al., 2011).

In this chapter, we have investigated the underlying mechanisms by which SMEK1 and SMEK2 affects the survival in Leukemic T cells. Protein phosphorylation regulates signalling pathways in multiple ways including regulation of biochemical activity of host proteins and reversible formation of protein complexes. We have therefore used Human Phosphorylation Pathway Profiling Array to assess the change in protein phosphorylation of 55 human proteins in the MAPK, AKT, JAK/STAT, NFκB, and TGFβ signalling pathways in cells transfected with empty vector and cells overexpressing SMEK1 or SMEK2.

4.2 Materials and methods

Lysates from HEK293T cells (immortalized human embryonic kidney cells) transfected with empty vector or overexpressing either SMEK1 or SMEK2 (Section 2.10.1) were purchased from OriGene Technologies (USA). Jurkat cells were nucleofected as described in section 2.7.2 with pcDNA3.1 or pcDNA3.1- SMEK1, pcDNA3.1-SMEK2 or pcDNA3.1-PP4c. Cells transfected with empty vector were used as control. In brief, cell lysates were prepared in RIPA/Protease inhibitor cocktail, incubated for 10 minutes, and centrifuged at 13000 g followed by quantification of total protein and stored at -20^{o}C until further use (Section 2.9). Proteins were collected to confirm overexpression of target protein using western blotting (Section 2.8.1).

RayBio ® C-Series Human Phosphorylation Pathway Profiling Array C55 for MAPK, AKT, JAK/STAT, NF-kB and TGFβ (Raybiotech, Inc.; Target proteins shown in Table 4.1), was used in these experiments, following the manufacturer's instructions (section 2.11). Briefly, the membranes were incubated with blocking buffer at 25°C for 30 min and then incubated with

1 ml of 1:20 diluted cell lysates overnight at 4 °C. The membranes were washed by wash buffer twice before they are incubated with Detection Antibody Cocktail at 25°C for 2 h. After washing twice, the membranes were incubated with horseradish peroxidase (HRP)-labelled anti-rabbit secondary antibody at 25°C for 2 h. Signals were collected using Bio-Rad ChemiDoc MP Imaging System and analysed using Image Lab 6.0.1 f or Windows software, with fold change in the phosphorylation calculated compared to control pcDNA3.1 transfected cells. Changes in the phosphorylation of transcription factors were further validated using western blotting.

Table 4.1 - Human Phosphorylation Pathway Profiling Array C55 target proteins

Signalling Pathway	Target proteins
Mitogen-activated protein kinase (MAPK)	**CREB**: cAMP response element-binding protein **ERK1/2**: extracellular signal-regulated kinase 1/2 **HSP27**: Heat shock protein 27 **JNK:** c-Jun N-terminal kinases **MEK:** Mitogen-activated protein-/ERK- kinase **MKK3:** Mitogen-activated protein kinase kinase 3 **MMK6**: Mitogen-activated protein kinase kinase 6 **MSK2:** Serine/threonine-protein kinase **p38:** p38 mitogen-activated protein kinase **p53:** tumor protein p53 **RSK1:** ribosomal S6 kinase **RSK2:** ribosomal S6 kinase-2 **Fos:** Fos Proto-Oncogene
AKT (Protein Kinase B)	**AKT:** AKR mouse strain **AMPKa**: AMP-activated protein kinase **BAD protein:** BCL2 Associated Agonist of Cell Death **GSK3A**: Glycogen Synthase Kinase 3 Alpha **GSK3b**: Glycogen Synthase Kinase 3 Beta **mTOR:** mammalian/mechanistic target of rapamycin **p27:** Cyclin-dependent kinase inhibitor 1B **P70S6K**: p70 ribosomal S6 kinase **PDK1**: Pyruvate Dehydrogenase Kinase 1 **PRAS40:** proline-rich Akt substrate of 40 kDa. **PTEN:** Phosphatase and Tensin homolog deleted on chromosome 10 **RAF-1**: RAF proto-oncogene serine/threonine-protein kinase **RPS6:** Ribosomal protein S6
Janus kinase-signal transducer and activator of transcription	**EGFR:** Epidermal growth factor receptor **JAK1:** Janus Kinase 1 **JAK2:** Janus Kinase 2 **SHP1:** Src homology region 2 (SH-2) domain-containing phosphatase 1

(JAK/STAT)	**SHP2:** Src homology region 2 (SH2)-containing protein tyrosine phosphatase-2 **Src:** Proto-oncogene tyrosine-protein kinase **Stat1:** Signal Transducer and Activator of Transcription 1 **Stat2:** Signal Transducer and Activator of Transcription 2 **Stat3:** Signal Transducer and Activator of Transcription 3 **Stat5:** Signal Transducer and Activator of Transcription 5 **Stat6:** Signal Transducer and Activator of Transcription 6 **TYK2:** Tyrosine Kinase 2
Nuclear factor kappa light chain enhancer of activated B cells (NFκB)	**ATM**: ATM serine/threonine kinase **eIF2a:** Eukaryotic Translation Initiation Factor 2A **HDAC2:** Histone Deacetylase 2 **HDAC4:** Histone Deacetylase 4 **MSK1:** Mitogen- and stress-activated kinase 1 **NFkB :** Nuclear Factor kappa-light-chain-enhancer of activated B **IkBa:** nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha **TAK1:** Mitogen-activated protein kinase kinase kinase 7 **ZAP70:** Zeta Chain of T Cell Receptor Associated Protein Kinase 70
Transforming growth factor beta (TGFβ)	**ATF2:** Activating Transcription Factor 2 **C-Fos:** Proto-oncogene c-Fos **c-Jun:** AP-1 transcription factor **SMAD1:** Mothers against decapentaplegic homolog 1 **SMAD2** : Mothers against decapentaplegic homolog 2 **SMAD4:** Mothers against decapentaplegic homolog 4 **SMAD5:** Mothers against decapentaplegic homolog 5

4.3 Results

4.3.1 Effects of SMEK1 and SMEK2 overexpression on MAPK, AKT, JAK/STAT, NFκB and TGFβ signalling pathways in HEK293T cells.

The first set of experiments which aimed to explore the effects of SMEK1 and SMEK2 overexpression on the signalling pathways were carried out using immortalised human embryonic kidney HEK293T cells. These cells have been used intensively to study different types of cell death, the role of PP4c in the regulation of cell fate decision and to identify several proteins that play significant roles in the regulation of cell survival by PP4c (Mourtada-Maarabouni and Williams, 2008). Lysates from HEK293T cells overexpressing SMEK1 or SMEK2 were obtained commercially together with lysates from cell transfected with empty

vector pcDNA3.1. HEK293T cells were subjected to the phosphorylation pathway profiling array before analysing the effects of SMEK1/2 overexpression on MAPK, AKT, JAK/STAT, NFκB and TGFβ signalling pathways. MAPK cascades transmit signals through sequential activation of three to five layers of protein kinases. Four MAPK cascades have been defined based on the components in the MAPK layer: ERK1/2, c-Jun N-terminal kinase (JNK), p38 MAPK and ERK5 (Plotnikov et al.,2010). Within each of the cascades, the signal is propagated by sequential phosphorylation and activations of the sequential kinases, and they eventually lead to the phosphorylation of target regulatory protein. The signals transmitted via these four cascades are transported across the nuclear envelope where they regulate gene expression by modulating the activity of transcription factors, transcription suppressors, and chromatin remodelling proteins (Matt, 2020). As shown in Figure 4.1A, SMEK1 overexpression did not affect MAPK pathway apart from a 2-fold decrease in the phosphorylation of Mitogen-and-stress-activated kinase 2 (MSK2) also called Ribosomal protein S6 kinase alpha-4. MSK2 is a nuclear protein activated by the ERK1/2 or p38 in the MAPK pathway. ERK1/2 and/or p38 phosphorylates three sites on MSK2, which activates the C-terminal kinase domain (Kathleen et al.,2016). SMEK2 overexpression in HEK293T significantly affected MAPK pathway by reducing the phosphorylation level of most of the proteins in the pathway including MSK2 that was affected by SMEK1 overexpression (Figure 4.1B). Figure 4.1B shows that overexpression of SMEK2 promoted a significant decrease in the phosphorylation of 12 out of 13 proteins in the pathway. These include ERK1/2, JNK, MEK, MMK3, MMK6, MSK2, p38, p53, RSK1 and RSK2 and the transcription factor CREB, a substrate of MSK2. Together the results suggest that an increased level of SMEK2 is associated with a reduction in the

phosphorylation level of the proteins involved in the MAPK signalling pathway leading to the inhibition of cell proliferation and increased cell death.

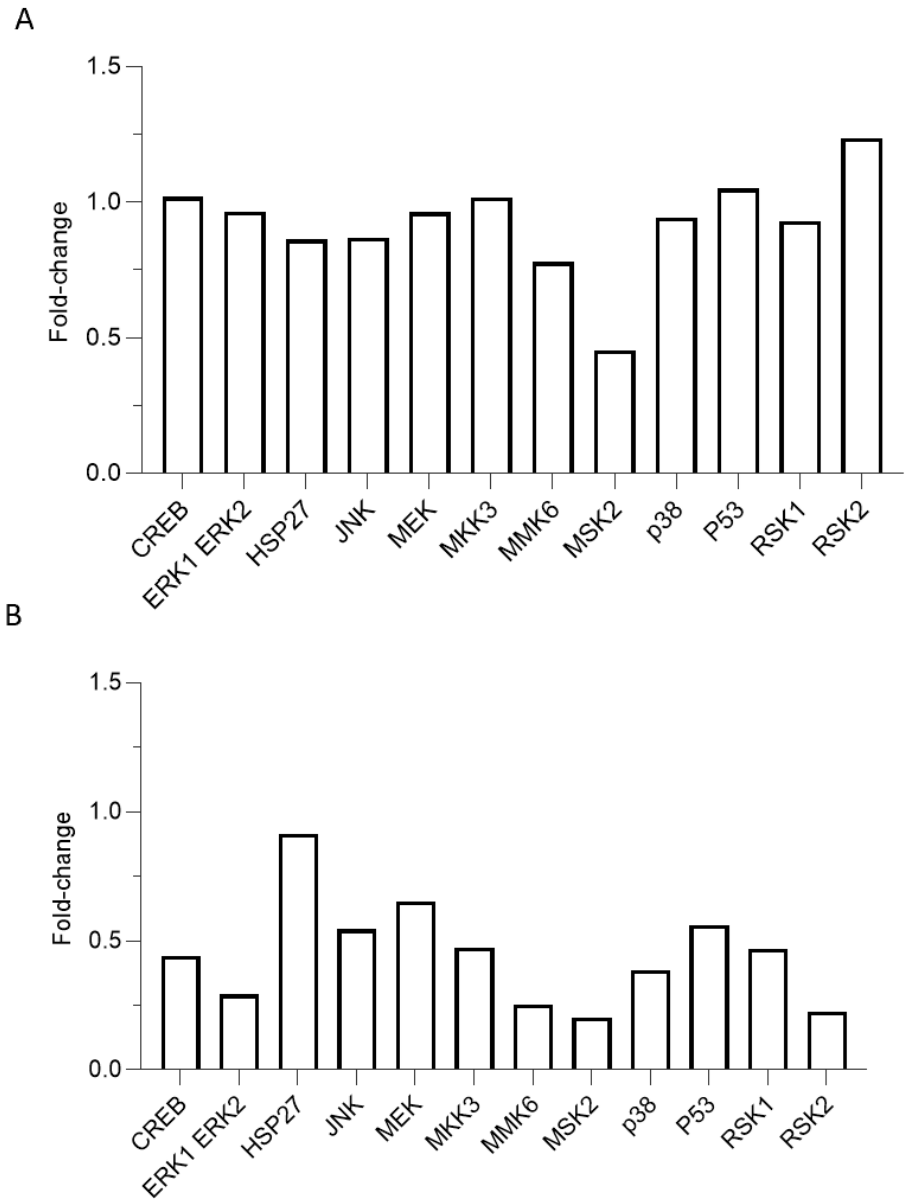


Figure 4.1 Effects of SMEK1 or SMEK2 overexpression in HEK293T cells on the phosphorylation of proteins involved in MAPK pathway. 50 µg of lysates from HEK239T cells containing pcDNA3.1 empty vector as a control or overexpressing SMEK1 (A) and SMEK2 (B) were used in the experiment. Changes in the phosphorylation level of protein involved in MAPK pathway were assessed using RayBio® human phosphorylation pathway profiling array with signals detected using a Bio-Rad ChemiDoc MP Imaging System and spot intensities normalized to control with results presented as fold change in the phosphorylation level.

Similar to many other protein kinases, AKT plays important role in multiple interconnected cell signalling mechanisms implicated in cell metabolism, growth and division, and apoptosis suppression. The AKT signalling pathway is activated by various signals including Receptor Tyrosine Kinases, integrins, B and T cell receptors, cytokine receptors and G-protein coupled receptors. A number of kinases phosphorylates AKT at the regulatory Serine 473 leading to the full activation of the enzyme (Nitulescu et al.,2018). Phosphorylated AKT contributes to the phosphorylation of downstream protein targets located either in the plasma membrane, the nucleus, and the cytosol, supporting cell growth and survival among other cellular effects (Porta et al.,2014). The ultimate effect of AKT activation is activation of mTOR, which is responsible for the increase of protein synthesis in cells (Irwin et al.,2010). Even though SMEK1 overexpression did not affect the phosphorylation of AKT and mTOR, it unexpectedly significantly increased the phosphorylation state of the mTOR substrates, eukaryotic initiation factor *4E*-binding protein 1 (4E-BP1) and p70 S6 kinase (P70S6K) (Figure 2A). Both 4E-BP1 and p70S6K are downstream effector of mTORC1 and their phosphorylation by mTORC1 leads to their activation driving protein synthesis (Wang et al.,2014). SMEK1 overexpression led to a significant reduction in the phosphorylation level of the proline-rich AKT substrate of 40 kDa (PRAS40) (Figure 2A). Phosphorylation of PRAS40 has been shown to contribute to the activation of the PI3K/AKT/mTOR signalling. The decrease in the phosphorylation of PRAS40 suggests that SMEK1 contributes to the inhibition of the AKT pathway. Figure 2B shows that similar to the effects shown in the cells overexpressing SMEK1, SMEK2 overexpression did not affect AKT and mTOR but led to considerable increase in the phosphorylation level of the mTOR substrate P70S6K and PDK-1. PDK-1 is a kinase that phosphorylates and activates AKT (Nitulescu et al.,2018). In contrast to the effects seen with SMEK1 overexpression, SMEK2 overexpression did not affect the phosphorylation of PRAS40

(Figure 2B). BAD, one of the BH3-only proapoptotic members, was found to be affected by overexpression of SMEK2. While SMEK1 overexpression did not cause significant changes in the phosphorylation level of BAD (Figure 4.2A), SMEK2 overexpression caused a significant decrease in the phosphorylated BAD (Figure 4.2B), BAD is activated through dephosphorylation leading to an increase in its apoptosis promoting activity (Kizilboga et al.,2019).

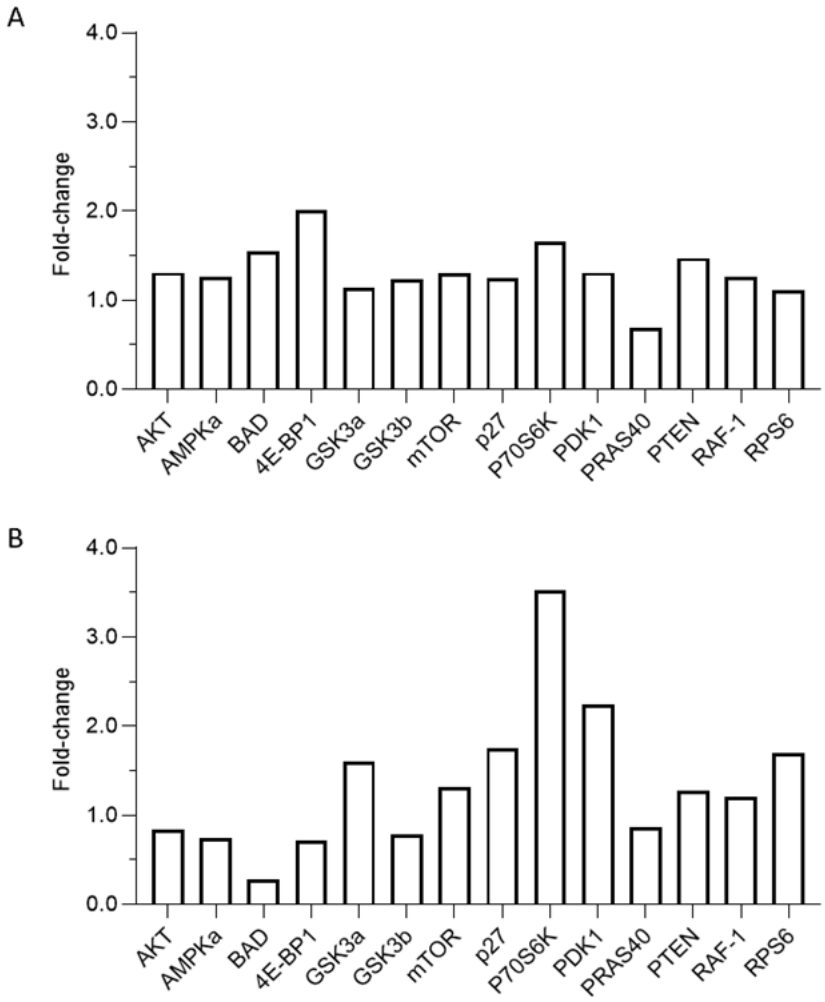


Figure 4.2 Effects of SMEK1 or SMEK2 overexpression in HEK293T cells on the phosphorylation of proteins involved in AKT pathway. 50 µg of lysates from HEK239T cells containing pcDNA3.1 empty vector as a control or overexpressing SMEK1 (A) and SMEK2 (B) were used in the experiment. Changes in the phosphorylation level of protein involved in AKT pathway were assessed using RayBio® human phosphorylation pathway profiling array with signals detected using a Bio-Rad ChemiDoc MP Imaging System and spot intensities normalized to control with results presented as fold change in the phosphorylation level.

The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway plays key role in development, proliferation, differentiation, and survival of cancer cell. Human JAK (Janus kinase) family belongs to a class of intracellular non-receptor tyrosine kinases and contains four kinases: JAK1, JAK2, JAK3 and TYK2 Tyrosine Kinase (Seif et al.,2017). STAT family is composed of seven members STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6, which mainly act as transcription factors (Seif et al.,2017). JAK are cytoplasmic tyrosine kinases associated with type I and II cytokine receptors. These receptors are activated when ligands bind to them leading to activation of JAK by promoting their autophosphorylation on tyrosine residues and subsequent recruitment of one or more STATs proteins to be phosphorylated (Seif et al., 2017). Phosphorylation of STATs lead to their dimerization and to their subsequent translocation to the nucleus where they regulate transcription of a number of target genes (Seif et al., 2017). JAK/ STAT pathway can also be activated by an activated EGFR (epidermal growth factor receptor which phosphorylates JAK and STATs on tyrosine residues (Andi et al., 2004). Figure 4.3A shows that SMEK1 overexpression did not affect the phosphorylation levels of key proteins in the JAK/STAT pathway. In contrast, SMEK2 overexpression resulted in the decrease in the phosphorylation in EGR receptor, JAK proteins including TYK2 and STAT 1, 3, 5 and 6 (Figure 3B), suggesting that SMEK2 overexpression is associated with the inactivation of this pathway leading to the inhibition of STATs- dependent gene transcription. SMEK2 overexpression led to a significant increase in the phosphorylation of SH2 containing protein tyrosine phosphatase-2 (SHP2) (Figure 4.3B). Studies have shown that JAK1 is negatively regulated by SHP2. The level of phosphorylated JAK1 and TYK2 is increased in SHP2 $^{-/-}$ fibroblasts after IFN-γ stimulation (Myers et al., 2001). Increases in the phosphorylation of SHP2 leads to an increase in the

activity of this phosphatase and the subsequent decrease in the phosphorylation of its targets including JAK and STAT proteins.

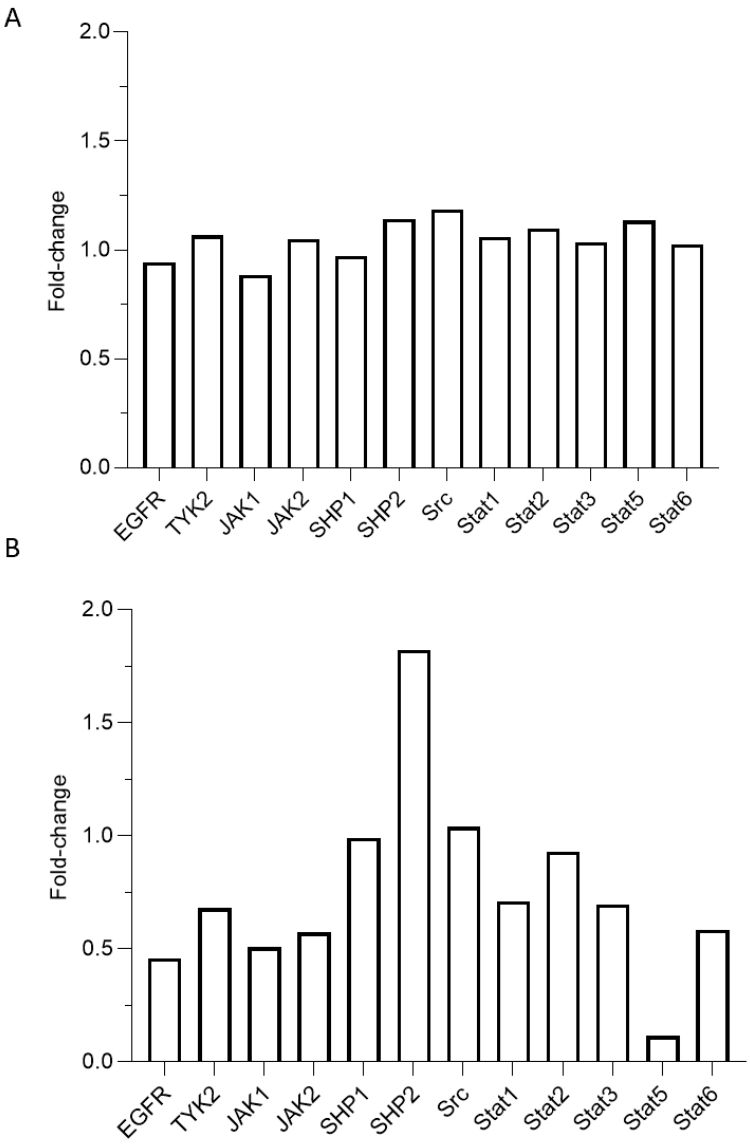


Figure 4.3 Effects of SMEK1 or SMEK2 overexpression in HEK293T cells on the phosphorylation of proteins involved in JAK/STAT pathway. Lysates from HEK239T cells transfected with pcDNA3.1 empty vector, SMEK1 (A) or SMEK2(B) were used in these experiments. Alteration in the phosphorylation level of protein involved in JAK/STAT pathway was assessed using RayBio® human phosphorylation pathway profiling array with signals detected using a Bio-Rad ChemiDoc MP Imaging System and spot intensities normalized to control with results presented as fold change in the phosphorylation level.

The next signalling pathway investigated was the nuclear factor kB (NFκB) pathway. NFκB encompasses an important family of inducible transcription factors, essential for the regulation of cell survival and to control gene expression in response to injury and inflammatory stimuli (Verzella et al.,2020). NFκB is a transcription factor consisting of a heterodimer that contains a p50 and a powerful transcriptional activation domain, p65 (Vermeulen et al., 2003). NFκB is usually kept inactive in the cytoplasm through binding of an inhibitory protein IκBα. Inducers, such as tumour necrosis factor (TNF) and interleukin-1 (IL-1) promotes the phosphorylation of the IκBα to increase its ubiquitylation and subsequent degradation by the 26S proteasome. This leads to the release of NFkB and its translocation to the nucleus to activate expression of various target genes (Verzella et al., 2020). Figure 4.4A shows that SMEK1 overexpression had no effects on the phosphorylation level of NFkB and two of the enzymes that activate it, MSK1 (Mitogen- and stress-activated kinase 1) and ATM (ataxia telangiectasia mutated). However, SMEK1 overexpression was associated with 8-fold decrease in the phosphorylation level of HDAC4 (Histone Deacetylase 4) and 2-fold decrease in the phosphorylation level of eIF2a (Eukaryotic initiation factor 2A), HDAC2 (Histone deacetylase 2) and TBK1 (TANK associated kinase 1) (Figure 4. 4B). Decrease in the phosphorylation of these proteins is associated with a decrease in the activation of NFκB (Verzella et al., 2020). SMEK2 overexpression caused a 6-fold decrease in the phosphorylation level of eIF2a, HDAC2 and HDAC4 and 2-fold decrease in the phosphorylation level of NFκB and MSK1 (Figure 4.4B). Overall, the results suggest that both SEMK1 and SMEK2 inhibit NFκB pathway but only SMEK2 overexpression is associated with the decrease in the phosphorylation of NFκB.

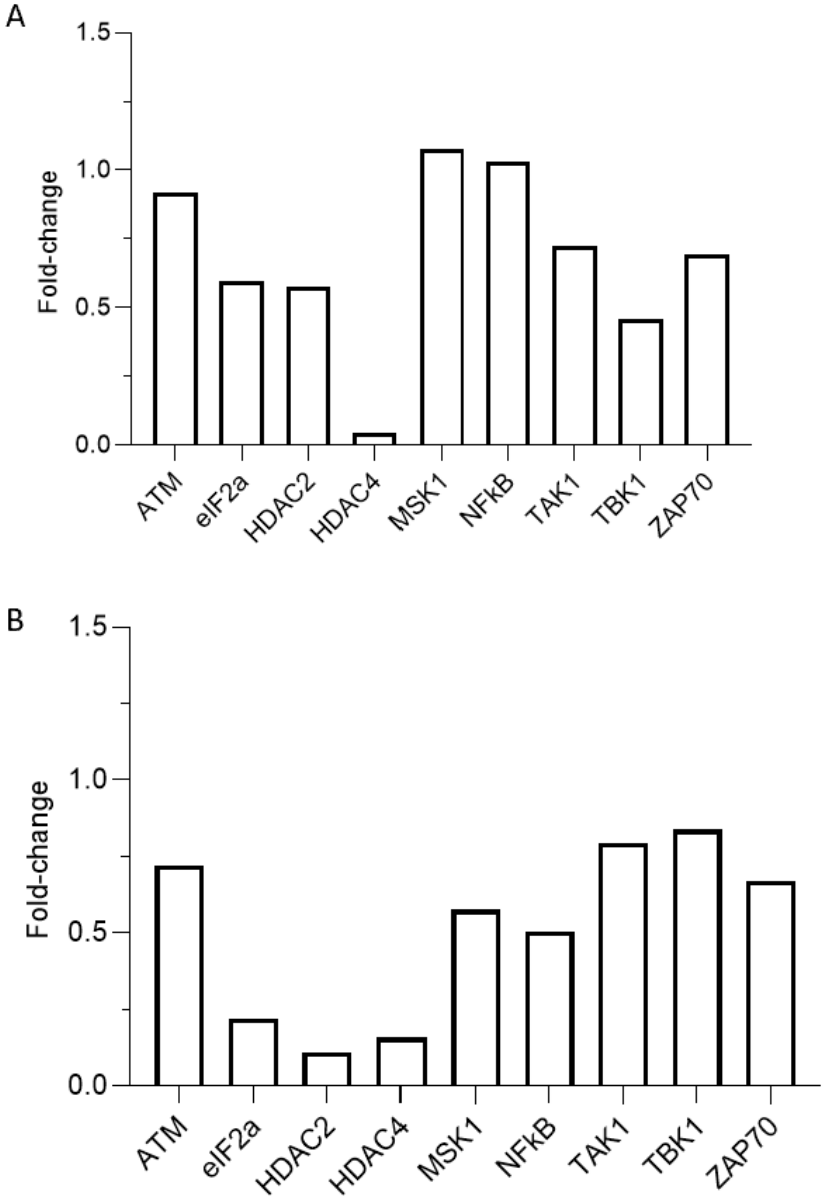


Figure 4.4 Effects of overexpressing SMEK1 or SMEK2 in HEK293T cells on protein phosphorylation in NFκB pathway. 50µg of HEK239T lystaes from cells transfected with pcDNA3.1, SMEK1(A) and SMEK2 (B). Changes in the phosphorylation level of proteins involved in NFkB pathway were assessed using RayBio® human phosphorylation pathway profiling array with signals detected using a Bio-Rad ChemiDoc MP Imaging System and spot intensities normalized to control with results presented as fold change in the phosphorylation level.

The last signalling pathway investigated was transforming growth factor-β (TGFβ) pathway. Signalling by TGFβ is initiated upon binding of one member of TGFβ superfamily to cell-surface serine/threonine kinase receptors (TGFβRI and TGFβRII), which mainly propagate the signal through intracellular mediators known as SMADs (Attisano and Lee-Hoeflich, 2001). Activation of SMADs results in their translocation from the cytoplasm into the nucleus, where they regulate gene expression by activating or repressing gene transcription, together with other transcription factors (Attisano and Lee-Hoeflich,2001). SMEK1 overexpression was associated with 1.4-fold increase in the phosphorylation level of SMAD1 and SMAD2 and 2-fold increase in the phosphorylation level of SMAD4 and SMAD5 (Figure 4.5A). SMEK1 overexpression was also associated with a decrease in the phosphorylation of members of activating protein 1 (AP-1) family, c-Fos, and c-Jun (Figure 4.5A). In contrast, SMEK2 overexpression was associated with a 2-fold decrease in phosphorylation of c-Fos and SMAD4 and 4-fold decrease in phosphorylation level of SMAD5 (Figure 4.5B). There was no detection of phosphorylated c-Jun, SMAD1 and SMAD2 in SMEK2 overexpression cells, suggesting that increased of SMEK2 has led to the dephosphorylation of these proteins (Figure 4.5B).

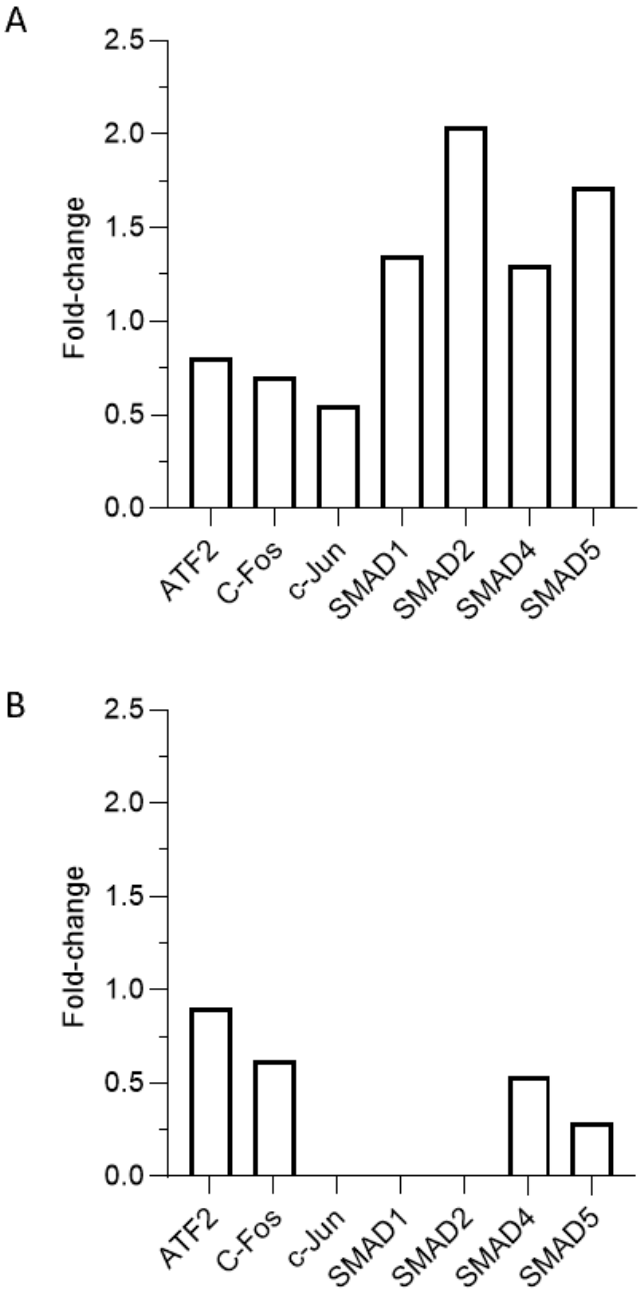


Figure 4.5 Effects of overexpressing SMEK1 or SMEK2 in HEK293T cells on protein phosphorylation in TGFβ pathway. HEK239T cells were transfected with pcDNA3.1 empty vector as a positive control or SMEK1(A) and SMEK2(B) and supplied as a lysate as 50µg**.** Alteration in the phosphorylation of proteins involved in TGFβ pathway was assessed using RayBio® human phosphorylation pathway profiling array C55 kit with signals detected using a Bio-Rad ChemiDoc MP Imaging System and spot intensities normalized to control with results presented as fold change in the phosphorylation level.

4.3.2 Effects of SMEK1 and SMEK2 overexpression on MAPK, AKT, JAK/STAT, NFκB and TGFβ signalling pathways in leukemic Jurkat T cells

The preliminary investigation using HEK293T cells have indicated that both SMEK1 and SMEK2 might be involved in the regulation of the MAPK, AKT, NFκB and TGFβ signalling pathways, whereas JAK/STATs pathway was only affected by SMEK2 overexpression. All these signalling pathways are involved in the regulation of gene expression, cell survival and proliferation. Since these pathways are dysregulated in cancer (Velloso *et al.,* 2017), it was therefore important to investigate whether SMEK1 and SMEK2 are implicated in the regulation of these major pathways in leukemic T cells. Cell lysates were prepared from Jurkat T cells 48 hours following the transient transfection of Jurkat T cells with pcDNA3.1 (control), pcDNA3.1-SMEK1 or pcDNA3.1-SMEK2. Cell lysates were then subjected to phosphorylation pathway profiling arrays. Unlike the effects observed with HEK293T where only SMEK2 significantly affected most of the proteins in MAPK pathway, both SMEK1 and SMEK2 overexpression in Jurkat T cells caused a similar effect on MAPK signalling pathway (Figure 4.6A and B). Overexpression of SMEK1 and SMEK2 led to the decrease in the phosphorylation of all the proteins involved in MAPK pathway, apart from MMK3 (Mitogen-activated Protein Kinase 3) which showed slight increase in the phosphorylation (Figure 6A and B). Collectively, the results suggest that SMEK1 and SMEK2 exert inhibitory effects on MAPKs and their substrates

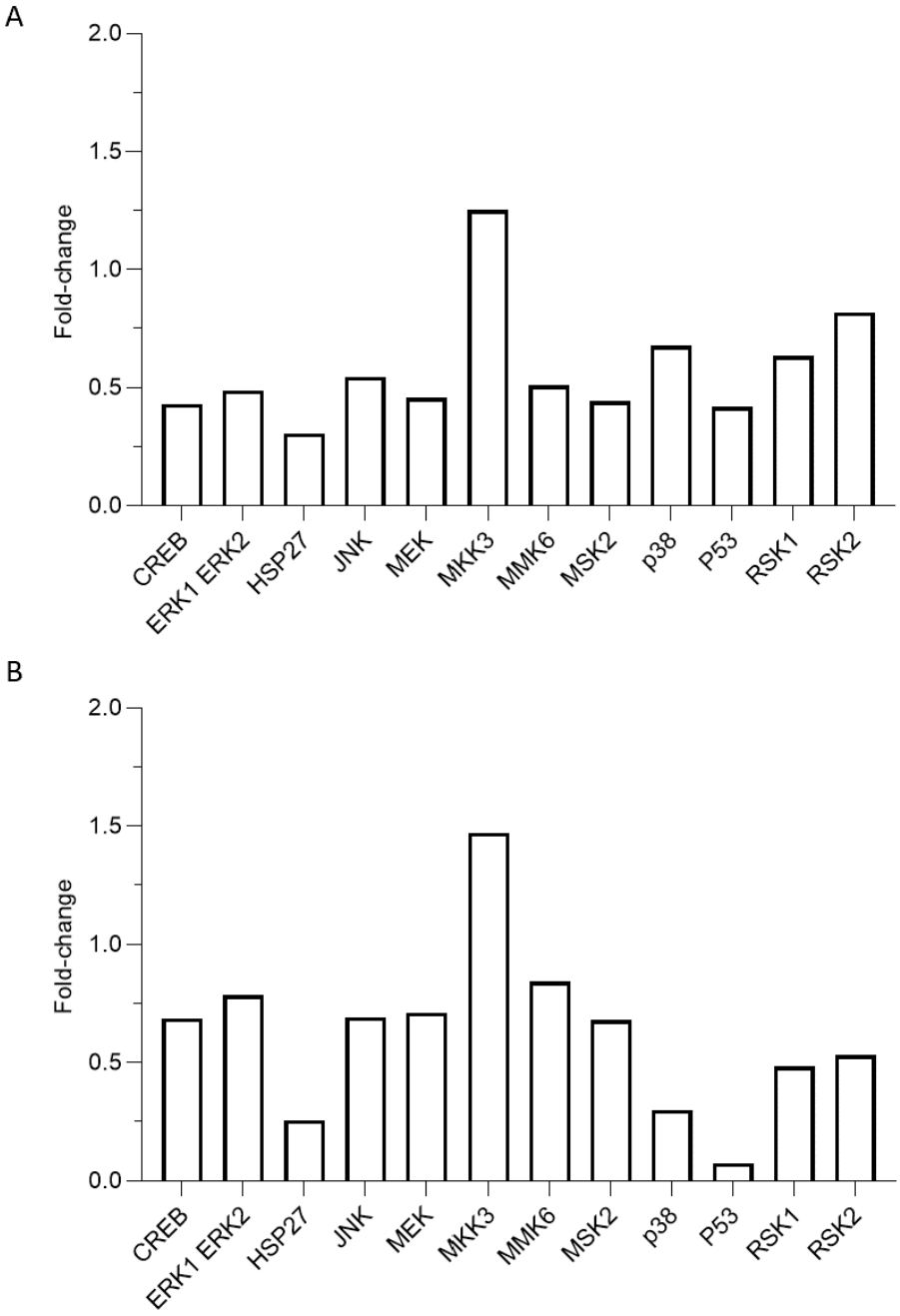


Figure 4.6 Effects of overexpressing SMEK1 and SMEK2 on MAPK pathway in Jurkat T cells. Effects of SMEK1 (A) and SMEK2 (B) overexpression on the phosphorylation of proteins involved in MAPK signalling pathway. Jurkat T cells were transfected with pcDNA3.1, SMEK1 or SMEK2. Cells lysates were used to detect alteration in protein phosphorylation using RayBio® human phosphorylation pathway profiling array. Signals were detected using a Bio-Rad ChemiDoc MP Imaging System and spot intensities normalized to control with results presented as fold change in the phosphorylation level.

Further experiments were carried out to investigate if the effects of SMEK1 and SMEK2 are also observed in PP4c overexpressing cells. This is important to establish if SMEK1 and SMEK2 exert their effects independently of the catalytic subunit of PP4, PP4c. Jurkat cells were transiently transfected with pcDNA3.1-PP4c and cell lysates were prepared 48 post transfection before being subjected to phosphorylation pathway profiling arrays. Figure 4.7A shows that overexpression of PP4c caused a significant decrease in all the proteins involved in MAPK pathway apart from MKK3. The effects observed with PP4c overexpression were similar to those observed in SMEK1 and SMEK2 overexpressing cells apart from MMK3 which showed no change in the phosphorylation level, as shown in the heatmap in Figure 4.7B.

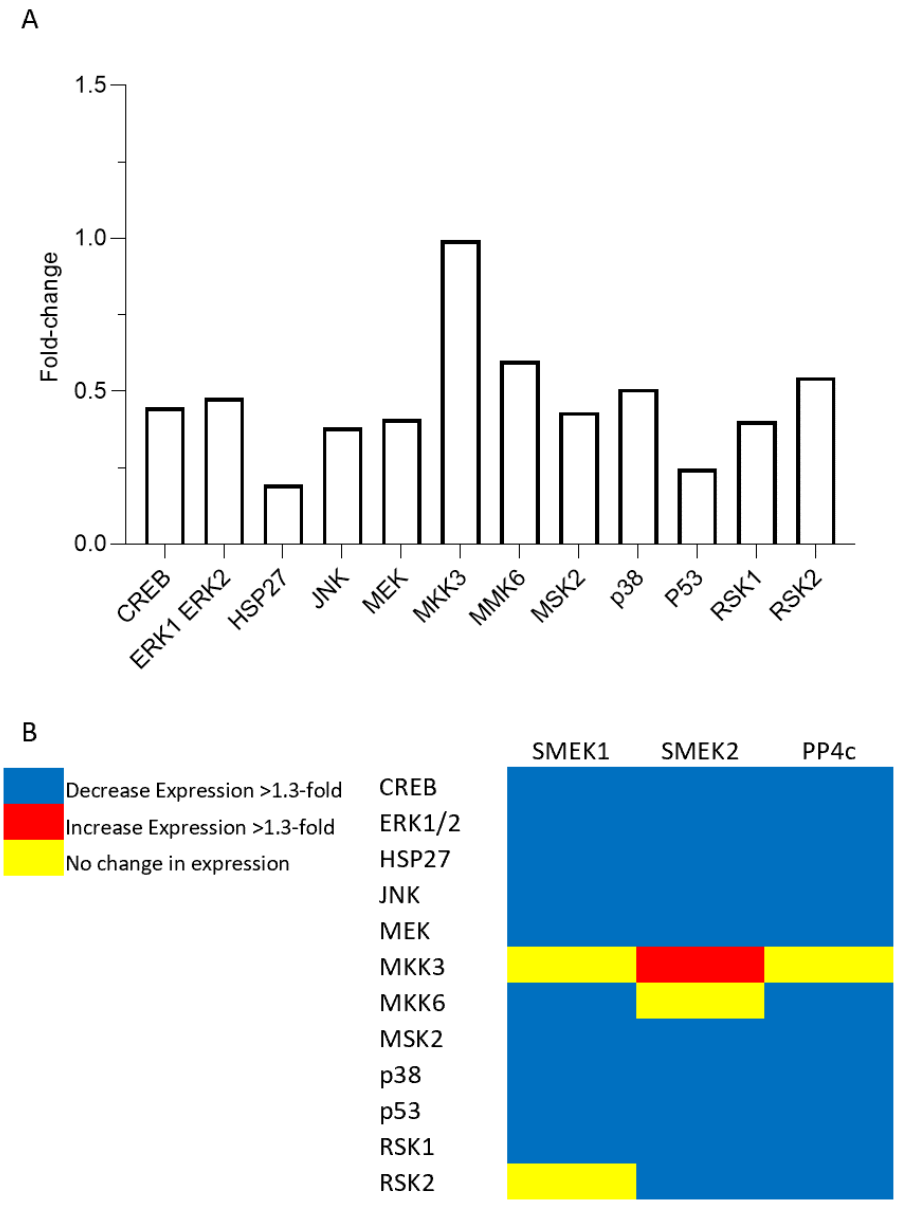


Figure 4.7 PP4c negatively regulates MAPK signalling in Jurkat T cells. Effect of PP4c overexpression on the phosphorylation of proteins involved in MAPK signalling pathway. Jurkat T cells were transfected with pcDNA3.1 and PP4c. Cells were collected after 48h for the preparation of cell lysates. Changes in protein phosphorylation were assessed using RayBio® human phosphorylation pathway profiling array with signals detected using a Bio-Rad ChemiDoc MP Imaging System and spot intensities normalized to control with results presented as fold change in the phosphorylation level. B) Heatmap comparing the effects of SMEK1, SMEK2 and PP4c.

` SMEK1 and SMEK2 overexpression in HEK293T cells was mostly associated with some increase in the phosphorylation of some of AKT and mTOR substrates. In contrast,

leukemic Jurkat T cells overexpressing SMEK1 and SMEK2 showed an overall reduction in all the proteins involved in AKT signalling pathway (Figure 4.8A and B).

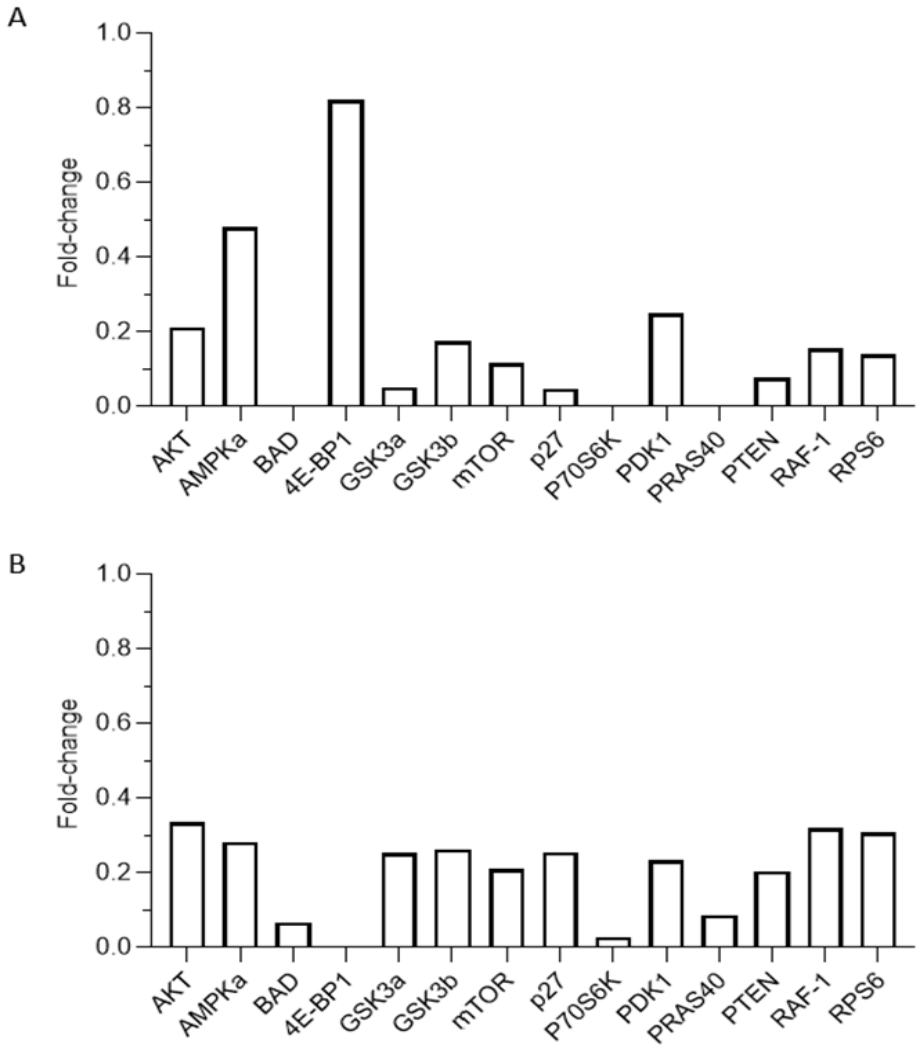


Figure 4.8 Effects of overexpressing SMEK1 and SMEK2 on AKT pathway in Jurkat T cells. Jurkat cells were transfected with pcDNA3.1-SMEK1 (A) or pcDNA3.1-SMEK2 (B). Cell lysates were collected 48 hours post transfection. Alteration in protein phosphorylation in AKT signalling pathway was assessed using RayBio® human phosphorylation pathway profiling array C55 kit. Signals were detected using a Bio-Rad ChemiDoc MP Imaging System and spot intensities were normalized to control with results presented as fold-change in the phosphorylation level compared to control.

Compared to the effects of SMEK1 and SMEK2 overexpression, overexpression of PP4c was also associated with an overall decrease in the phosphorylation levels of the proteins involved in AKT pathway (Figure 4.9A). Figure 4.9B clearly shows that increased levels of

SMEK1, SMEK2 and PP4c are associated with a decrease in the phosphorylation levels of proteins involved in AKT pathway.

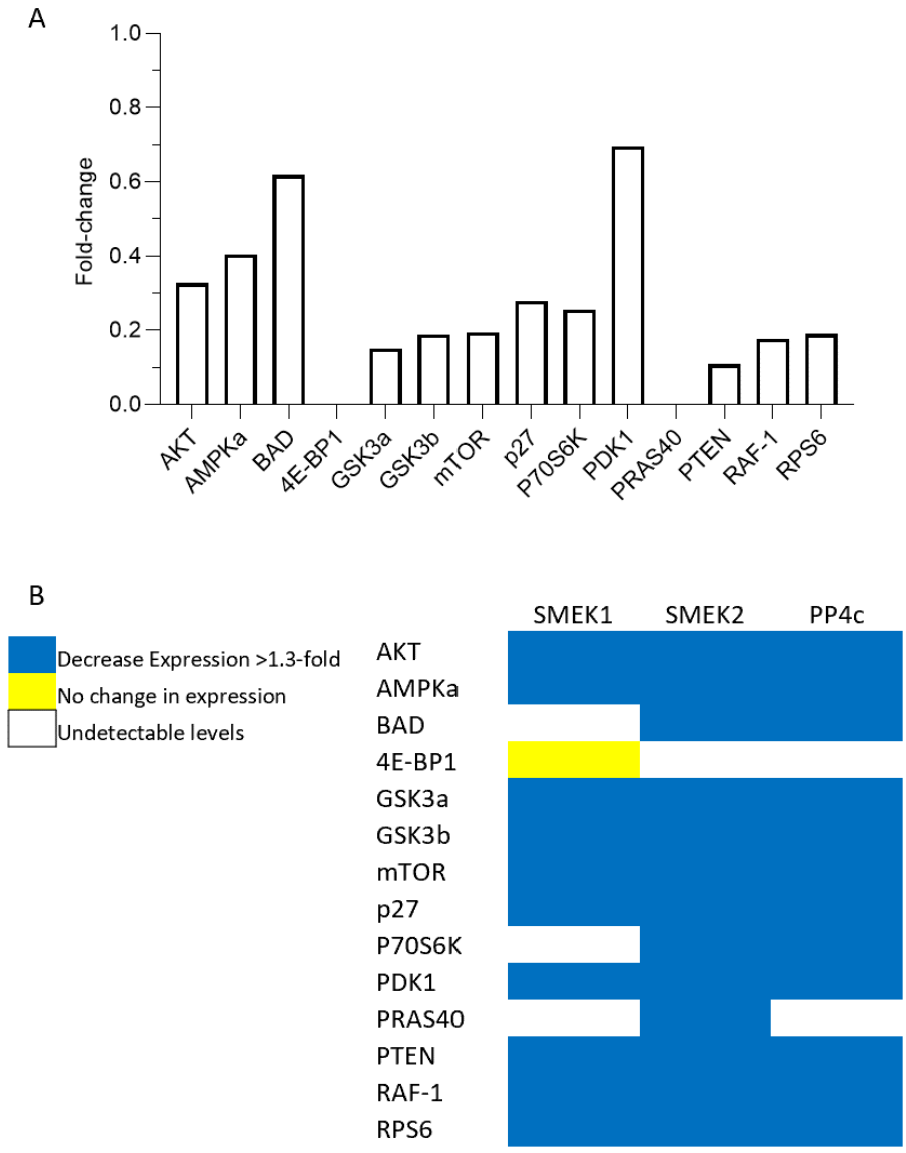


Figure 4.9 PP4c negatively regulates AKT signalling in Jurkat T cells. Effect of PP4c overexpression on the phosphorylation of proteins involved in AKT signalling pathway (A). Jurkat T cells were transfected with pcDNA3.1 and PP4c. Cells were collected after 48h for the preparation of cell lysates. Alteration in protein phosphorylation was assessed using RayBio® human phosphorylation pathway profiling array with signals detected using a Bio-Rad ChemiDoc MP Imaging System and spot intensities normalized to control with results presented as fold change in the phosphorylation level. B) Heatmap comparing the effects of SMEK1, SMEK2 and PP4c

A significant difference in the effects of SMEK1 and SMEK2 overexpression on JAK/STAT pathway was seen in Jurkat T cells compared to HEK293T cells. In HEK293T cells, only SMEK2 overexpression led to decrease in the phosphorylation level of EGR receptor, JAK proteins and STATs proteins and an increase in the phosphorylation of SH2 containing protein tyrosine phosphatase-2 (SHP2). In contrast, both SMEK1 and SMEK2 overexpression in Jurkat T cells was associated in significant reduction in the phosphorylation level in all the proteins in the JAK/STAT pathway including STAT2, 3, 5 and 6 and a significant increase in the phosphorylation level of STAT1 (Figure 4.10A and B).

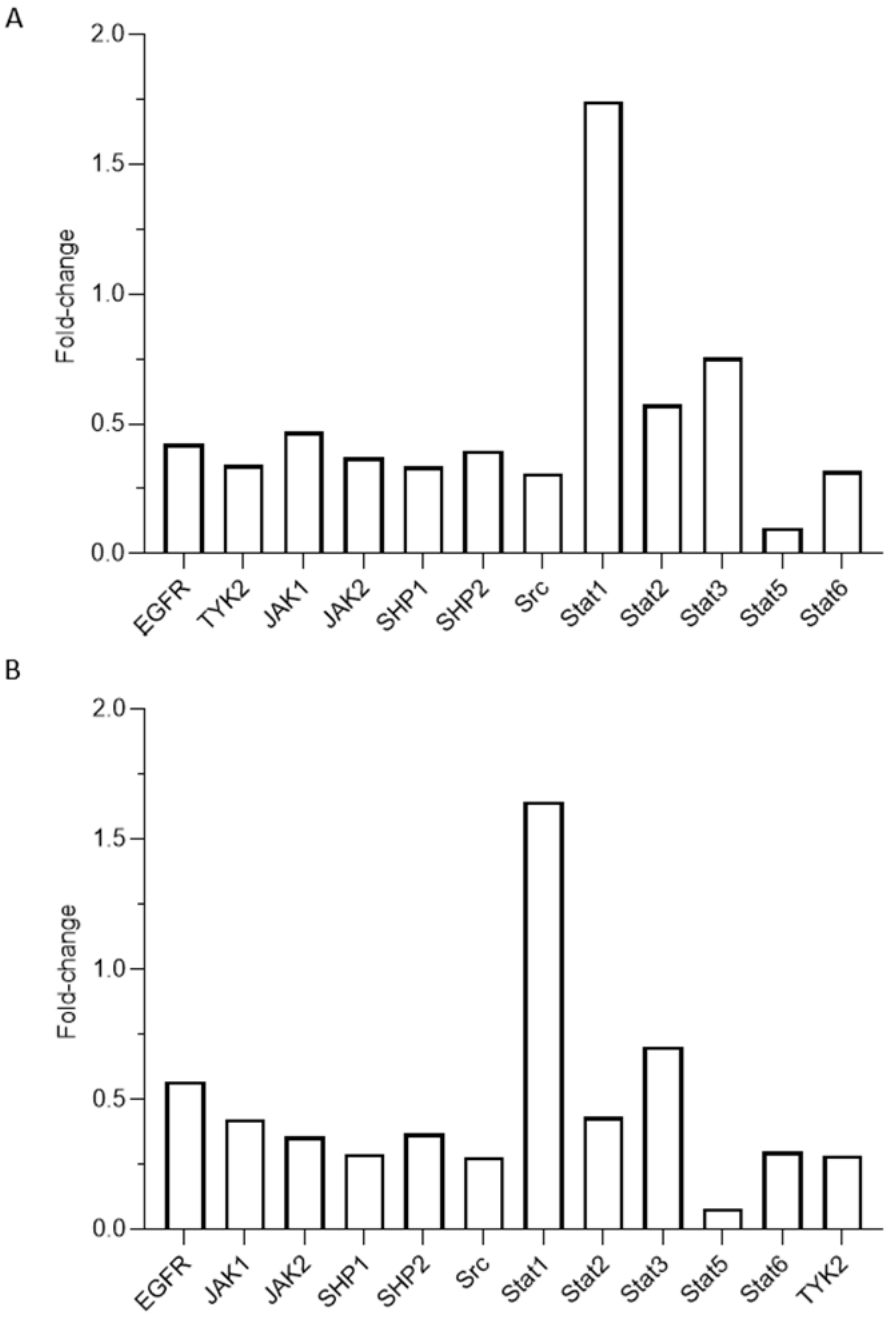


Figure 4.10 Effects of overexpressing SMEK1 and SMEK2 on protein phosphorylation in JAK/STAT pathway in Jurkat T cells. Jurkat cells were transfected with pcDNA3.1-SMEK1 (A) or pcDNA3,1-SMEK2 (B). Cell lysates were prepared after 48 hours. Alteration in phosphorylated proteins in JAK/STAT signalling pathway was assessed using RayBio® human phosphorylation pathway profiling array C55 kit with signals detected using a Bio-Rad ChemiDoc MP Imaging System and spot intensities normalized to control with results presented as fold change in phosphorylation compared to control.

PP4c overexpression also led to the decrease in the phosphorylation in the proteins involved in JAK/STAT pathway and an increase in STA1 phosphorylation (Figure 4.11A). Figure 4.11B shows that cells overexpressing SMEK1, SMEK2 and PP4c exert inhibitory effects on the JAK/STAT pathway by reducing the phosphorylation level of the JAK kinases and its substrates.

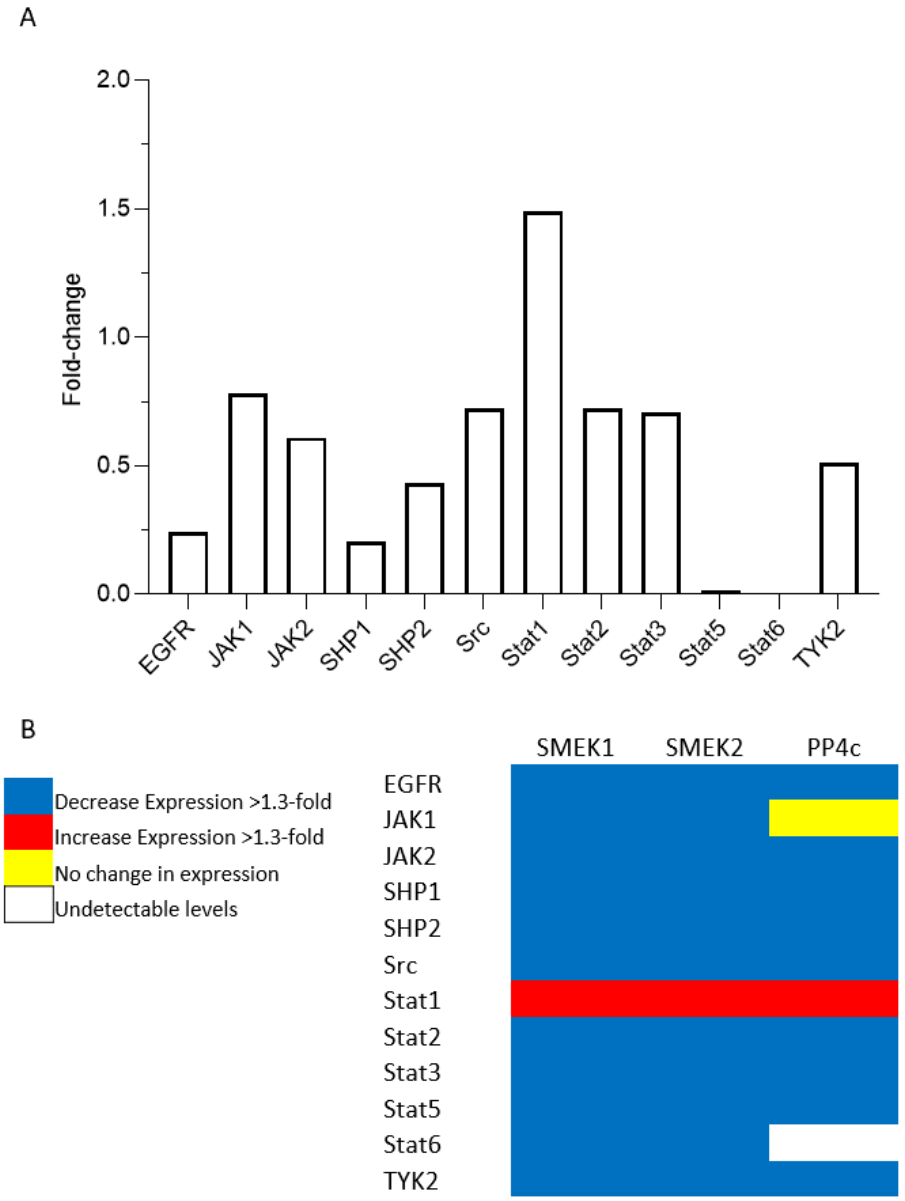


Figure 4.11 PP4c negatively regulates JAK/STAT signalling in Jurkat T cells. Effect of PP4c overexpression on the phosphorylation of proteins involved in JAK/STAT signalling pathway (A). Jurkat T cells were transfected with pcDNA3.1 and PP4c. Cells were collected after 48h for the preparation of cell lysates. Protein phosphorylation level was assessed using RayBio® human phosphorylation pathway profiling array with signals detected using a Bio-Rad ChemiDoc MP Imaging System and spot intensities normalized to control with results presented as fold change in the phosphorylation level. B) Heatmap comparing the effects of SMEK1, SMEK2 and PP4c.

NFκB pathway was negatively affected by overexpression of SMEK1 and SMEK2 in Jurkat T cells. All the proteins in the pathway showed decrease in the level of phosphorylation in Jurkat T cells, implying that they both exert inhibitory effect on the pathway (Figure 4.12A and B). The results were compared to the effects of overexpression of PP4c and it was revealed that increased levels of PP4c is also associated with decrease phosphorylation levels in all proteins in the NFκB pathway apart from HDAC4 (Figure 4.13A and B).

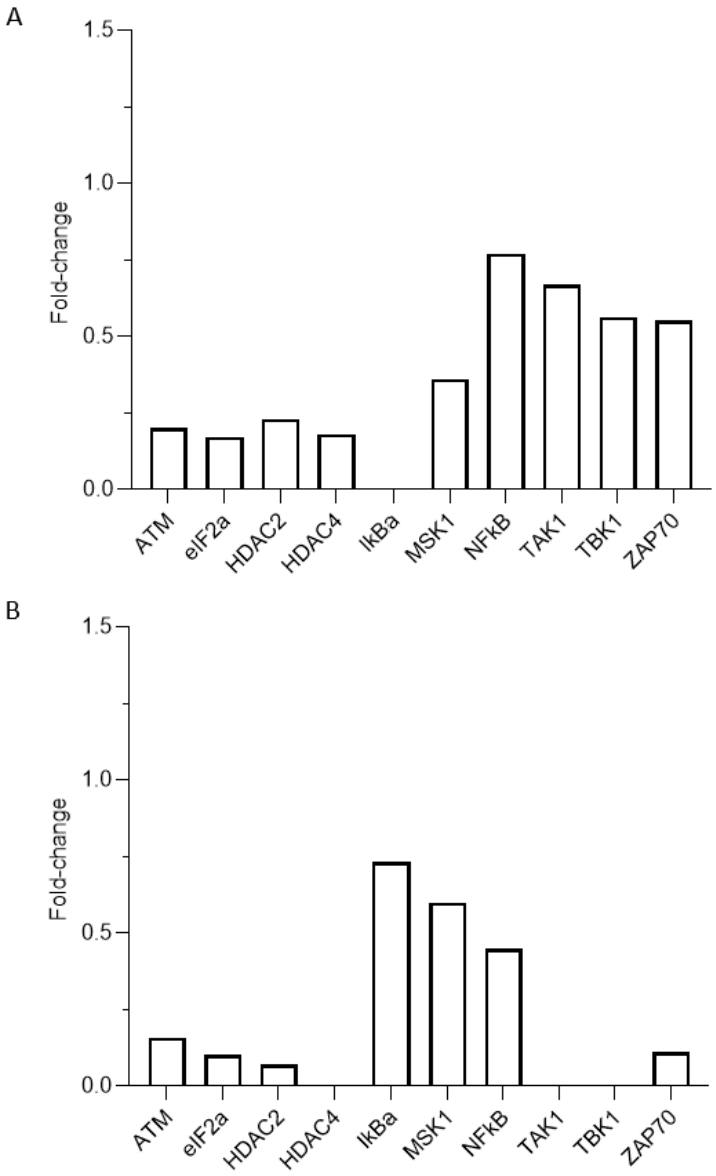


Figure 4.12 Effects of overexpressing SMEK1 and SMEK2 in Jurkat cells on NFκB signalling pathway. Jurkat cells were transfected with pcDNA3.1, SMEK1 (A) or SMEK2 (B). Cell lysates were collected 48 post transfections. Alterations in the level of protein phosphorylated in NFκB pathway were assessed using RayBio® human phosphorylation pathway profiling array C55 kit with signals detected using a Bio-Rad ChemiDoc MP Imaging System and spot intensities normalized to control with results presented as fold change.

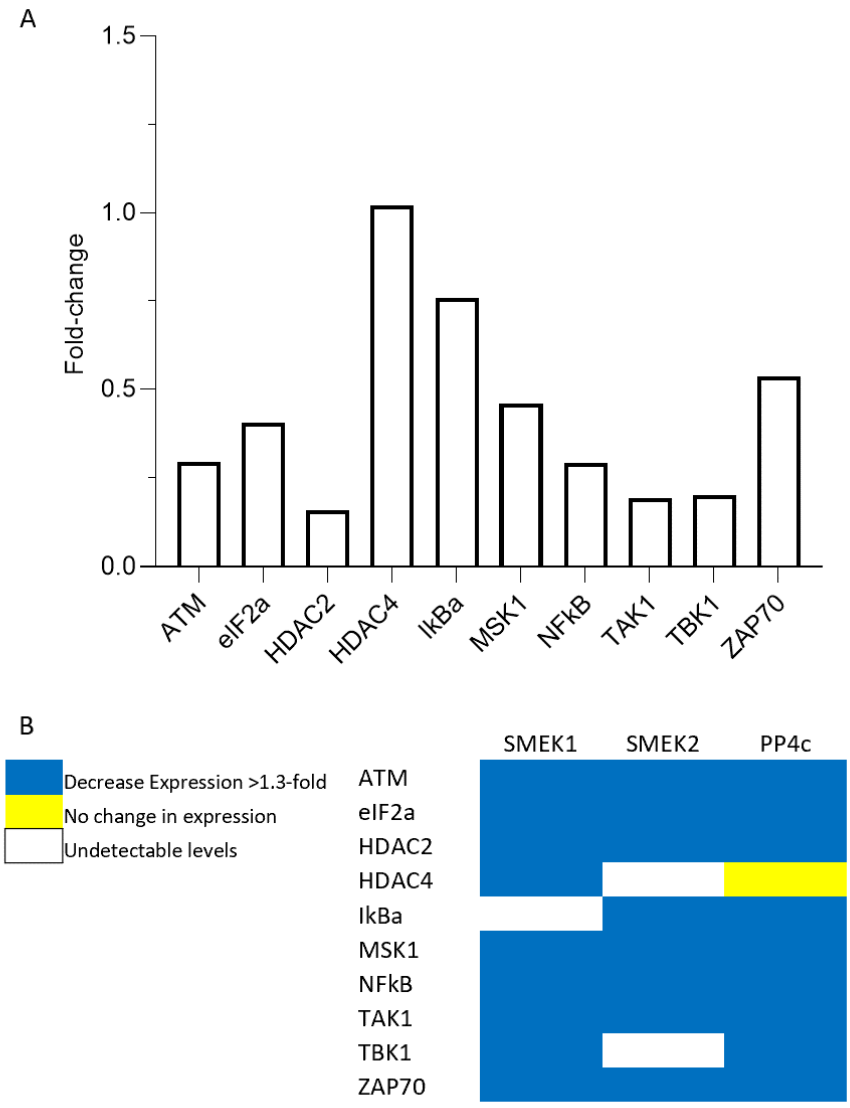


Figure 4.13 PP4c negatively regulates NFκB signalling in Jurkat T cells. Effect of PP4c overexpression on the phosphorylation of proteins involved in NFκB signalling pathway (A). Jurkat T cells were transfected with pcDNA3.1 and PP4c. Cells were collected after 48h for the preparation of cell lysates. Changes in protein phosphorylation were detected using RayBio® human phosphorylation pathway profiling array with signals detected using a Bio-Rad ChemiDoc MP Imaging System and spot intensities normalized to control with results presented as fold change in the phosphorylation level. B) Heatmap comparing the effects of SMEK1, SMEK2 and PP4c.

The effects of SMEK1 and SMEK2 overexpression on TGFβ pathway in Jurkat T cells was associated with an increase in the phosphorylation of SMADs proteins in the pathway (SMAD1, SMAD2 and SMAD5) (Figure 4.14 A and B). SMAD4 phosphorylation was not affected

by SMEK1 overexpression but was significantly increased in the cells overexpressing SMEK2. These effects were similar to those observed in HEK239T cells transfected with SMEK1 (Figure 4.5A). SMEK1 and SMEK2 overexpression was also associated with decrease in the phosphorylation of c-Fos and c-Jun (Figure 4.14A and B). Together c-Fos and c-Jun forms the dimeric transcription factor AP1 which is involved in inflammation, proliferation, differentiation, and apoptosis (Gazon et al., 2018). In contrast to the effects of SMEK1 and SMEK2 on SMAD proteins, there was no increase in the phosphorylation of SMADs in the cell overexpressing PP4c (Figure 4.15A). The heatmap in Figure 4.15B clearly highlights the difference between the effects of SMEK1 and SMEK2 and those observed in PP4c overexpressing cells.

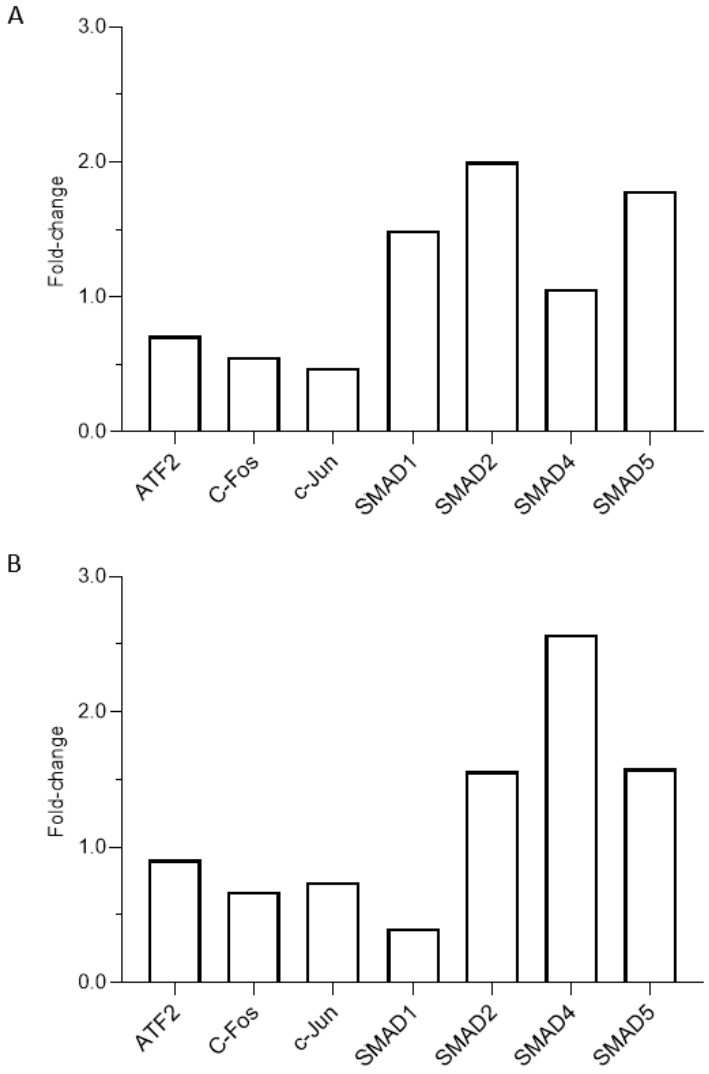


Figure 4.14 Effects of overexpressing SMEK1 and SMEK2 in Jurkat cells on TGFβ signalling pathway. Jurkat cells were transfected with pcDNA3.1-SMEK1 (A) or pcDNA3.1-SMEK2 (B). Cell lysates were collected 48 post transfections. Alteration in the level of protein phosphorylation in TGFβ pathway was assessed using RayBio® human phosphorylation pathway profiling array with signals detected using a Bio-Rad ChemiDoc MP Imaging System and spot intensities normalized to control with results presented as fold change.

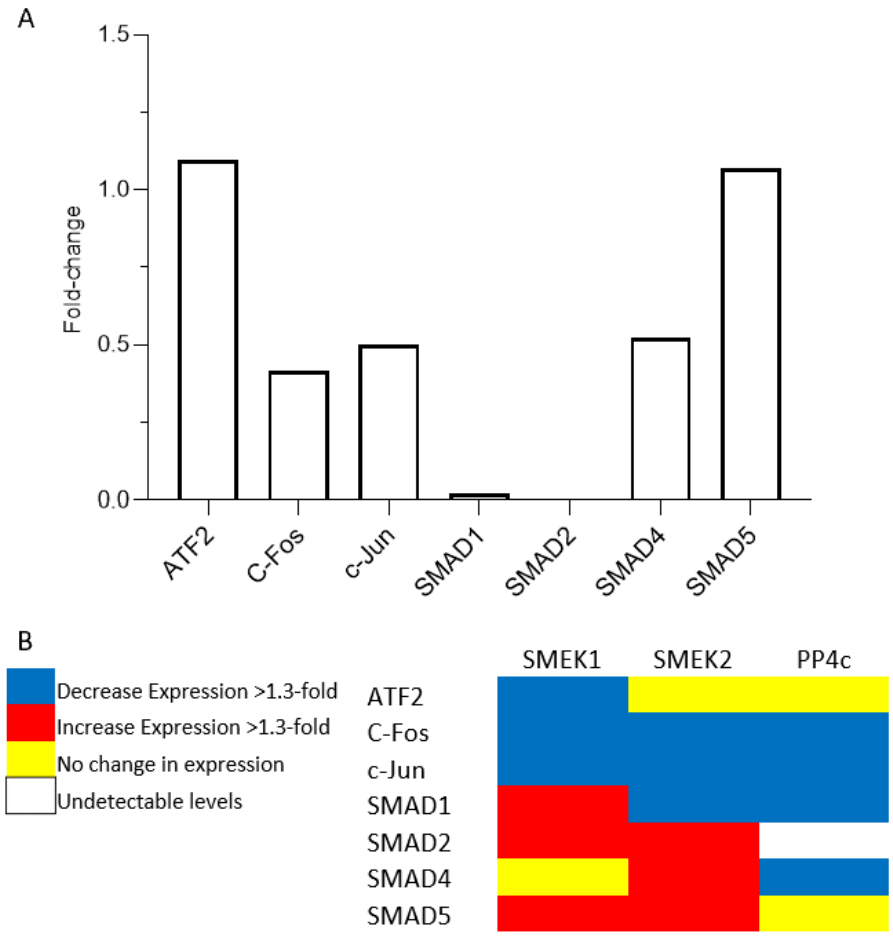


Figure 4.15 PP4c negatively regulates TGFβ signalling in Jurkat T cells. Effect of PP4c overexpression on the phosphorylation of proteins involved in TGFβ signalling pathway (A) Jurkat T cells were transfected with pcDNA3.1 and PP4c. Cells were collected after 48h for the preparation of cell lysates. Changes in protein phosphorylation were detected using RayBio® human phosphorylation pathway profiling array with signals detected using a Bio-Rad ChemiDoc MP Imaging System and spot intensities normalized to control with results presented as fold change in the phosphorylation level. (B) Heatmap comparing the effects of SMEK1, SMEK2 and PP4c.

Overall, the results indicated that SMEK1 and SMEK2 affects the phosphorylation of key proteins in the five major signalling pathways involved in the regulation of proliferation, survival, and cell death. Apart from the effects on SMADs proteins, the effects of SMEK1 and SMEK2 are similar to those caused by PP4c overexpression. These results suggests that SMEK1 and SMEK2 effects could be dependent on the activity of PP4c in regulating MAPK, AKT, JAK/STAT and NFκB signalling pathways and that both proteins affect TGFβ pathway independently of PP4c. Interestingly, an important observation emerged from these results is the effects of SMEK1 and SMEK2 on the phosphorylation of different transcription factors highlighting a role of SMEK1 and SMEK2 in the regulation of gene expression.

4.4.3 Confirmation of effects of SMEK1 and SMEK2 overexpression on the phosphorylation of transcription factors in Jurkat T cells

The results so far showed that SEMK1 and SMEK2 overexpression in Jurkat T cells affected the phosphorylation of key transcription factors mediating cell proliferation, differentiation, and survival. Transcription factors affected by SMEK1 and SMEK2 overexpression include NFκB, STATs, c-Jun and SMADs. Further experiments were carried out to confirm the effects of SMEK1 and SMEK2 on the phosphorylation levels of these transcription factors. The experiments used western blotting and the relevant phosphoantibody to validate the results obtained using the Phosphoarray results. Jurkat T cells were transfected with pcDNA3.1, pcDNA3.1-SMEK1 or pcDNA3.1-SMEK2 and cell lysates were prepared 48h post transfection. Cell lysates were used in western blotting.

NFκB (Nuclear Factor kappa-light-chain-enhancer of activated B) is one of the transcription factors that showed significant decrease in the phosphorylation level in SMEK1 and SMEK2 overexpressing cells. Western blotting analysis confirmed that SMEK1 and SMEK2 overexpression led to a reduction in the phosphorylation level of NFκB (Figure 4.16A and B). The results showed 2-fold decrease in the phosphorylation of NFκB in SMEK1 and SMEK2 overexpressing cells (Figure 4.16A and B).

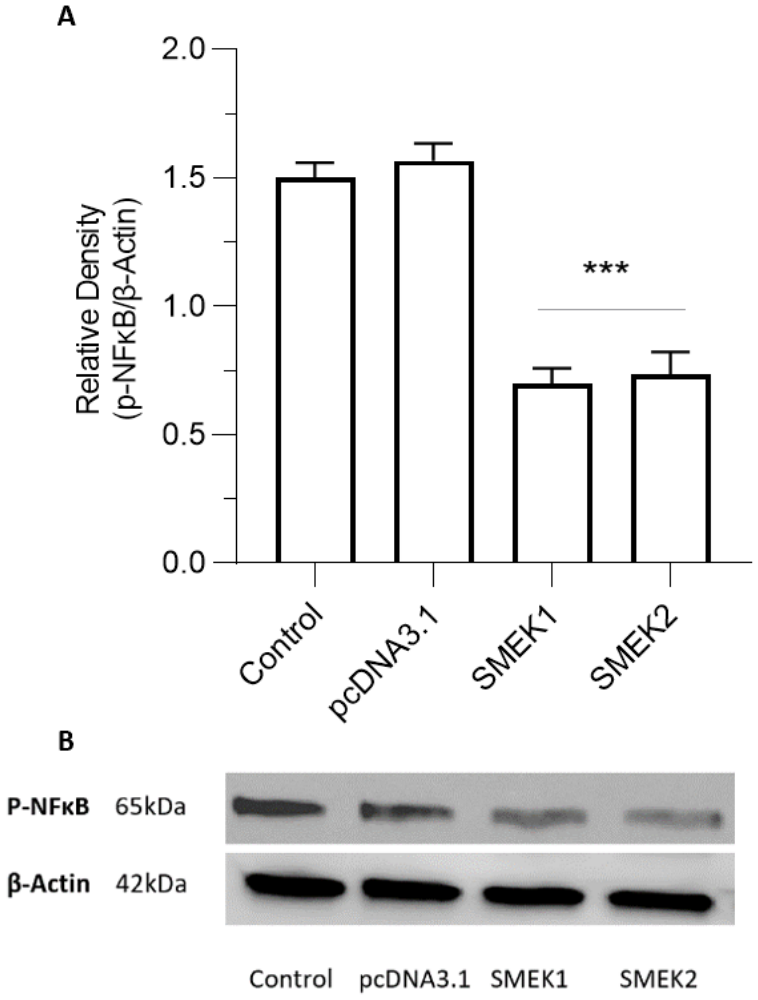


Figure 4.16 Effects of SMEK1 and SMEK2 overexpression on NFκB phosphorylation. Jurkat T cells were transiently transfected with SMEK1 and SMEK2. 48h post transfection, cell lysates were prepared and used for Western blotting. (A) Graph shows the ratio of p-NFκB against relative density of β-Actin, with data presented as mean ± SEM, n=3 ***p<0.001 relative to control as determined by one-way ANOVA and Tukey's post-test. (B) representative western blot image collected using a Bio-Rad ChemiDoc MP Imaging System.

Further experiments were carried out to assess the changes of phosphorylation of STATs proteins in the cells overexpressing SMEK1 and SMEK2. STATs (Signal Transducer and Activator of Transcription) are family of proteins that are activated by the upstream Janus kinases (JAK) family proteins and can integrate inputs from different signalling pathways (Yap Loh et al.,2019). The phosphorylation of five STATs proteins was shown to significantly

decreased in the cells overexpressing SMEK1 and SMEK2 as shown above in Figure 4.10. These include s STAT1, STAT2, STAT3, STAT5 and STAT6. Experiments using phospho-STAT2 and phospho-STAT6 antibodies were not successful in detecting phosphorylated STAT2 and STAT6. Results showed that STAT1 was increased in cells overexpressing SMEK1 and SMEK2 (Figure 4.17A and B).

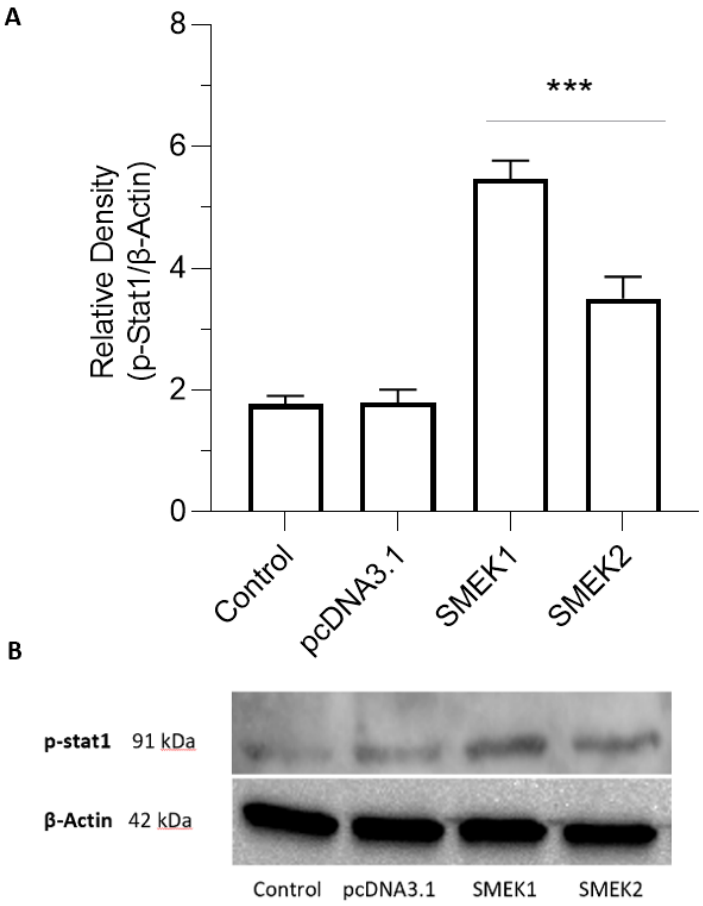


Figure 4.17 SMEK1 and SMEK2 overexpression increases the phosphorylation of STAT1. Jurkat cells were transfected with SMEK1 and SMEK2. Cell lysates were prepared after 48 hours and used for western blotting. (A) Graph shows ratio of p-stat1 against relative density of β-Actin. Data are presented as mean ± SEM (n=3) ***p<0.001 relative to control as determined by one-way ANOVA and Tukey's post-test. (B) Representative western blot image collected using a Bio-Rad ChemiDoc MP Imaging System.

Figure 4.18 shows that STAT3 phosphorylation level was significantly reduced in the cells overexpressing SMEK1 and SMEK2. There was 3-fold and 4.5-fold decrease in the phosphorylation levels of STAT3 in the cells overexpressing SMEK1 and SMEK2, respectively (Figure 4.18A and B).

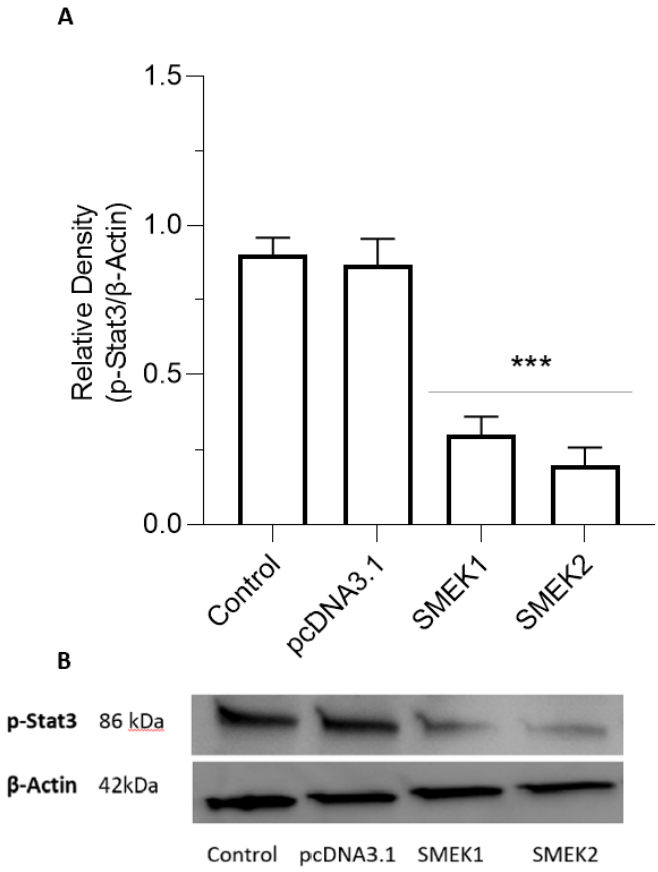


Figure 4.18 Overexpression of SMEK1 and SMEK2 leads to a decrease in the phosphorylation of STAT3. Cell lysates were prepared from Jurkat T cells 48h post SMEK1 and SMEK2 transfection. Lysates used for Western blotting. (A) Graph shows the ratio of p-stat3 against relative density of β-Actin, with data showing mean ± SEM, n=3 ***p<0.001 relative to control as determined by one-way ANOVA and Tukey's post-test. (B) representative Western blot image collected using a Bio-Rad ChemiDoc MP Imaging System.

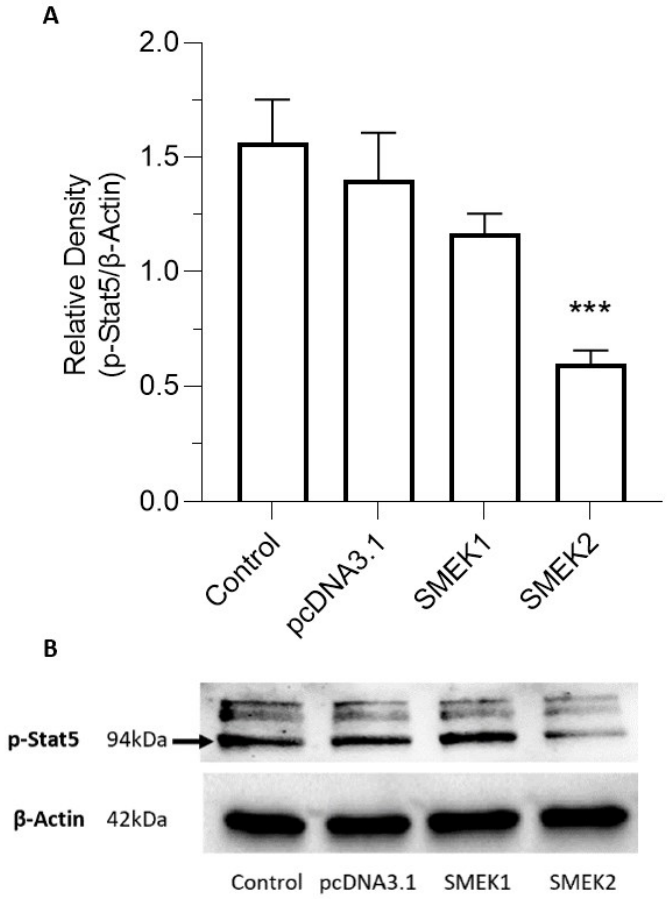


Figure 4.19 Effects of SMEK1 and SMEK2 on Stat5 phosphorylation in Jurkat T cells. Jurkat T cells were transfected with SMEK1, SMEK2 for 48 hours and cell lysates were prepared 48h post transfection and used for western blotting. (A) Graph showing the ratio of p-stat5 against relative density of β-Actin, with data presented as mean ± SEM, n=3 ***p<0.001 relative to control (untransfected) as determined by one-way ANOVA and Tukey's post-test. (B) Representative western blot image collected using a Bio-Rad ChemiDoc MP Imaging System.

There was no significant change in STAT5 phosphorylation level in the cells overexpressing SMEK1 (Figure 4.19A and B) but a significantly decreased level of phosphorylated STAT5 was detected in SMEK2 overexpressing cells (Figure 4.19 A and B).

Another transcription factor showed reduced phosphorylation in the cells expressing SMEK1 and SMEK2 is c-Jun. c-Jun is a protein of the activator protein-1 (AP-1) complex has been identified as a basic leucine zipper transcription factor that can acts as homo- or heterodimer, binding to DNA and regulating gene transcription (Meng and Xia, 2011). Western blotting analysis confirmed the inhibitory effects of SMEK1 and SMEK2 on the c-Jun (Figure 4.20A and B). Up-regulation of SMEK1 and SMEK2 was associated with 30% and 60% decrease in the phosphorylation level of c-Jun, respectively (Figure 4.20A and B).

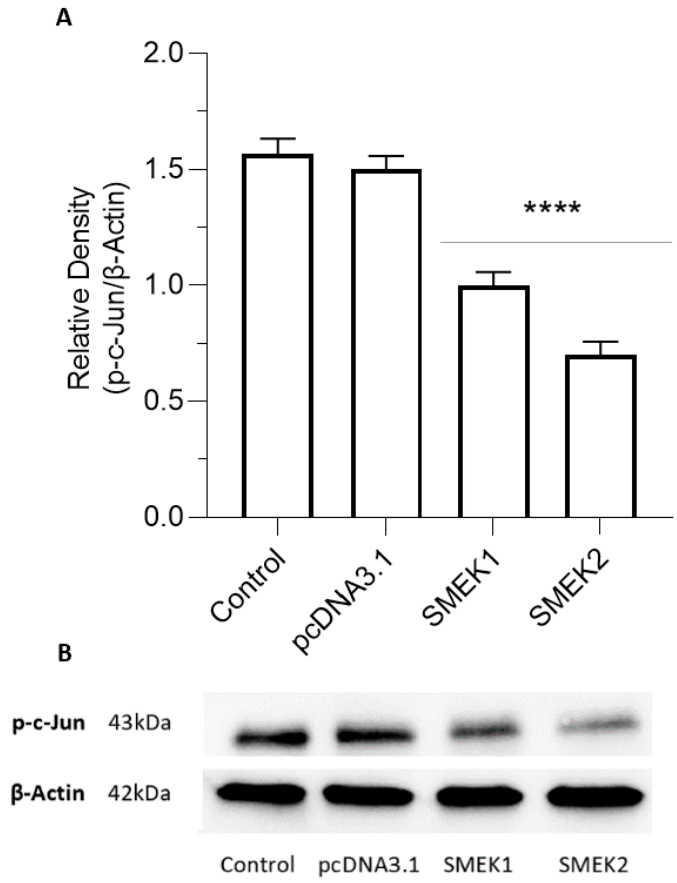


Figure 4.20 Effects of overexpressing SMEK1 and SMEK2 on the phosphorylation of c-jun. Jurkat cells were transiently transfected with pcDNA3.1-SMEK1 and pcDNA3.1-SMEK2 for 48 hours and cells harvested with lysates used for western blotting. (A) Graph presents ratio of p-c-Jun against relative density of β-Actin, with data are represented as mean ± SEM, n=3 ****p<0.0001 relative to control as determined by one-way ANOVA and Tukey's post-test. (B) Representative western blot image collected using a Bio-Rad ChemiDoc MP Imaging System.

SMADs are a family of structurally related transcription factor proteins involved in mediating the signal transduction induced by TGFβ transforming growth factor beta (TGFβ), which are critically important for regulating cell development and growth. SMAD1, SMAD2 and SMAD5 showed an increased phosphorylation level in Jurkat cells overexpressing SMEK1 (Figure 4.14A). SMEK2 overexpression was associated with an increase in the phosphorylation of SMAD2, SMAD4 and SMAD5 and a decrease in the phosphorylation of SMAD1 (Figure 4.14B). Western blotting analysis further confirmed these observations. Results showed that Jurkat cells overexpressing SMEK1 showed more than 2-fold increase in the amount of phosphorylated SMAD1 and SMAD5 but had no effect on the phosphorylated form of SMAD4 (Figure 4.21). SMEK2 overexpression in Jurkat cells was associated with 2-fold increase in the phosphorylation of SMAD4 and SMAD5 and 2- fold decrease in the phosphorylation of SMAD1 (Figure 4.22).

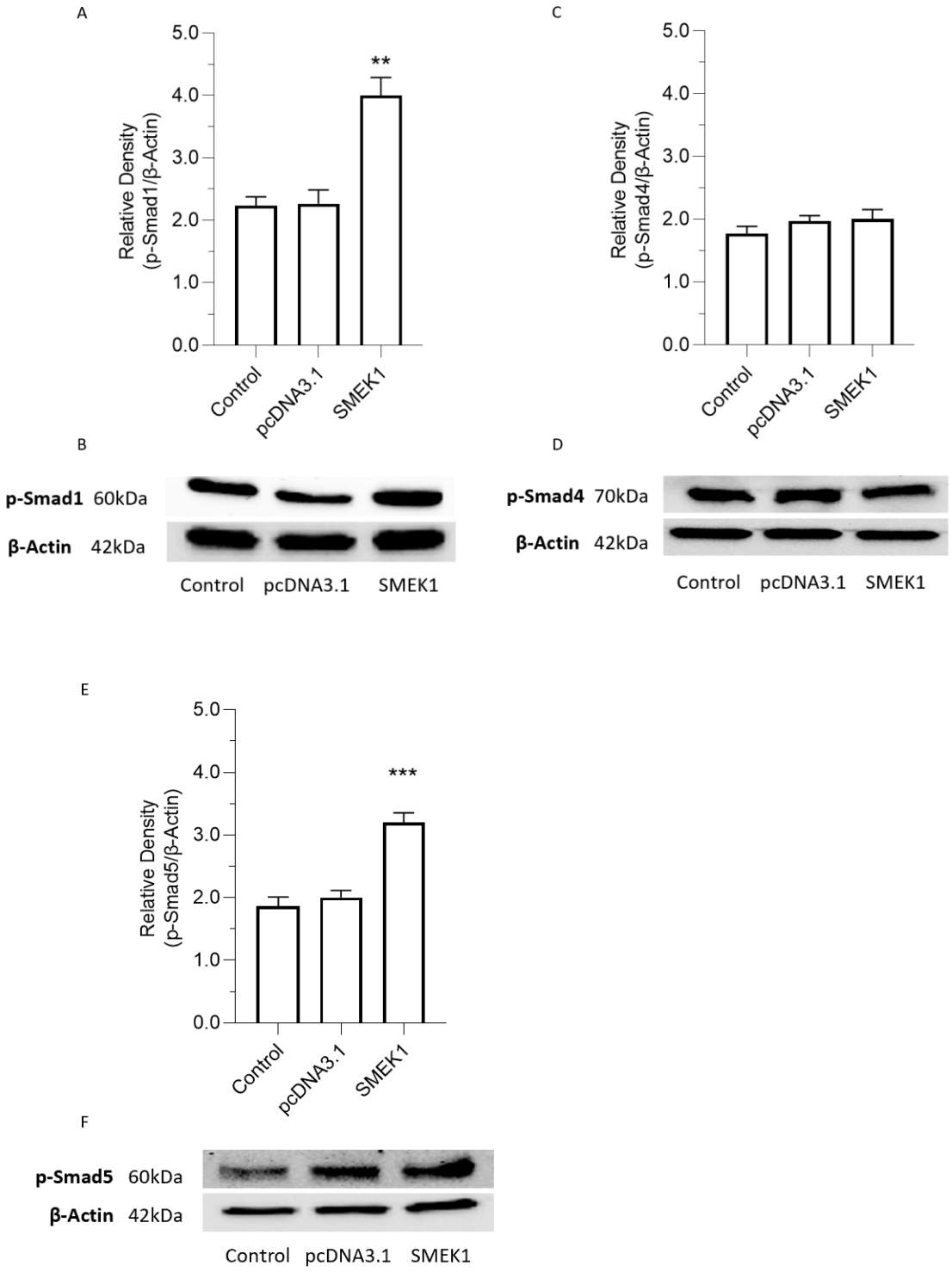


Figure 4.21 Effects of SMEK1 overexpression on the phosphorylation of SMAD proteins. Jurkat cells were transiently transfected with pcDNA3.1 or pcDNA3.1- SMEK1. Cell lysates were prepared 48 hours post transfection and used for western blotting. Graphs in (A), (C) and (E) show the ratio of phosphorylated target proteins against relative density of β-Actin. Data are presented as mean ± SEM, n=3, **p<0.01, ***p<0.001 relative to control un(transfected) as determined by one-way ANOVA and Tukey's post-test. (B), (D) and (F) show representative western blot images collected using a Bio-Rad ChemiDoc MP Imaging System.

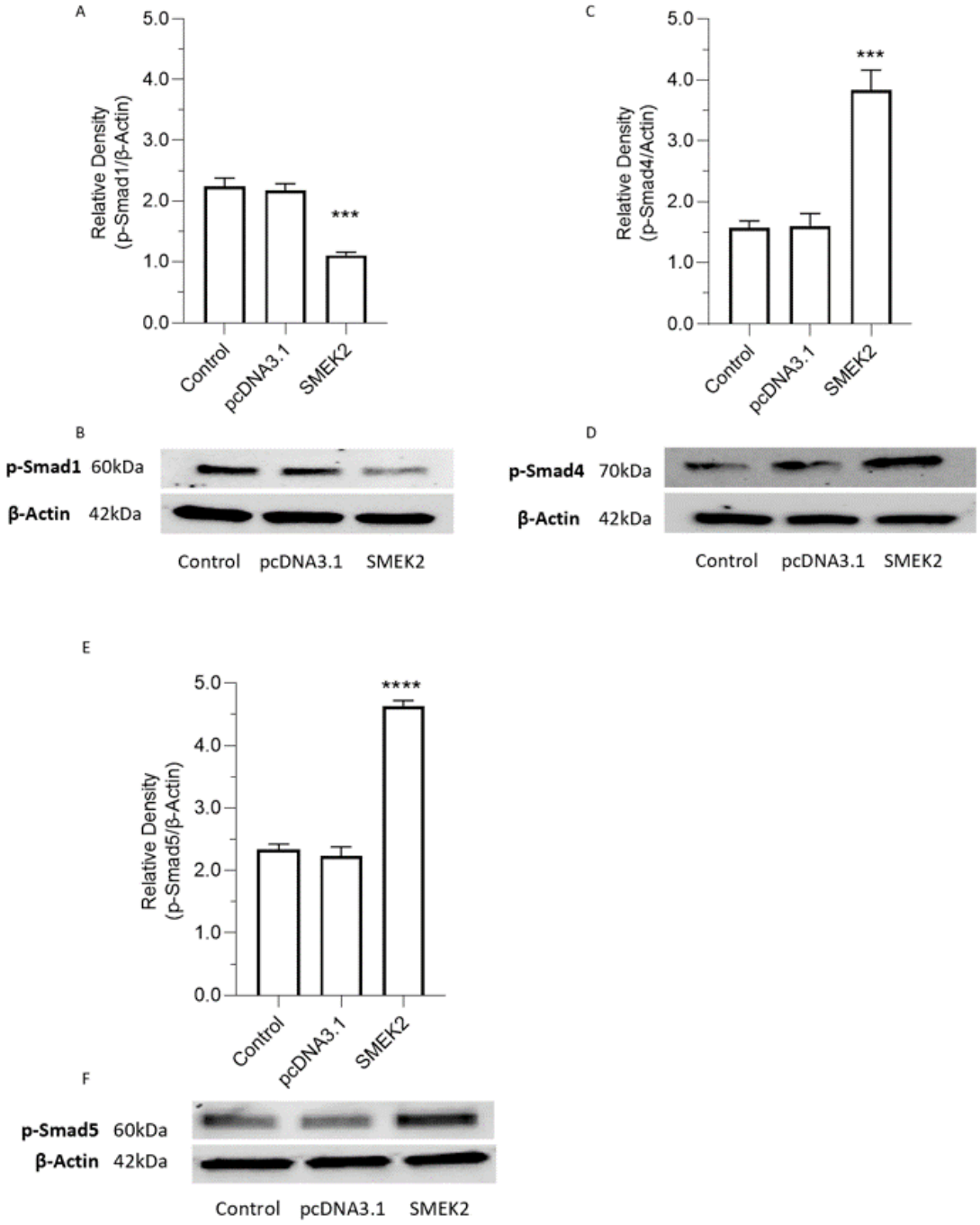


Figure 4.22 Effects of SMEK2 overexpression on the phosphorylation of SMAD proteins. Jurkat cells were transiently transfected with pcDNA3.1 or pcDNA3.1- SMEK1. Cell lysates were prepared 48 hours post transfection and used for western blotting. Graphs in (A), (C) and (E) show the ratio of phosphorylated target proteins against relative density of β-Actin. Data are presented as mean ± SEM, n=3, ***p<0.001, ****p<0.0001 relative to control un(transfected) as determined by one-way ANOVA and Tukey's post-test. (B), (D) and (F) show representative western blot images collected using a Bio-Rad ChemiDoc MP Imaging System.

4.4 Discussion

The work in Chapter 4 has implicated SMEK1 and SMEK2 in the regulation of proliferation and apoptosis of T leukemic cells. In this chapter, we have extended our analysis to identify specific signalling pathways regulated by them. To identify the cellular pathways targeted by SMEK1 and SMEK2, we employed phosphorylation pathway profiling array which detects phosphorylated proteins in five key signalling pathways MAPK, AKT, JAK/STAT, NFκB, and TGFβ. These pathways are involved in the regulation of cell growth, proliferation, and survival. Disruptions in these pathways are associated with cancer and other diseases such as diabetes, cardiovascular and neurological diseases (Guo and Wang, 2013). Protein phosphorylation is a mechanism of regulation that is extremely important as most of the components of these pathways are activated and deactivated via phosphorylation/dephosphorylation events due to specific kinases and phosphatases (Hornberg et al.,2005).

The initial experiments used human embryonic kidney 293T (HEK293T) cells which have been used previously to characterise PP4c role in regulating apoptosis and identifying proteins regulated by PP4c (Mourtada-Maarabouni and Williams, 2008). HEK293T cells have also been used to express and purify the PP4 holoenzyme consisting of PP4c, PPP4R2 and PP4R3A/ SMEK1 (Ueki et al.,2019). The results showed that several proteins involved in the five pathways exhibited changes, increases as well as reductions, in their phosphorylation state in the cells overexpressing SMEK1 and SMEK2, reflecting the complexity of the signalling pathways that are regulated by SMEK1 and SMEK2. The results also showed differences between the effects of SMEK1 and SMEK2 in HEK293T cells on the five signalling pathways.

MAPK signalling pathway converge in the amplification of one or more growth factors that sustain cell proliferation, growth, and survival processes. These growth factors such epidermal growth factor (EGFR) bind and activate their specific receptor tyrosine kinases leading to the activation of the signal transduction cascade through cytosolic intermediates, and finally the transcription or translation regulation of effector gene (Braicu et al.,2019). SMEK1 overexpression affected the phosphorylation state of one protein MSK2 in MAPK pathway. MSK2, mitogen- and stress-activated kinase 2, is a nuclear protein activated downstream of the p38 MAPK and Erk1/2 mitogen-activated protein kinases (Ananieva *et al.,* 2008). MSK2 activity is regulated by phosphorylation and activated MSK2 phosphorylates multiple substrates, including the transcription factor CREB (cAMP-response element binding protein) CREB (Ananieva *et al.,* 2008). Decrease in the phosphorylation of MSK2 inhibits its activity and the consequent reduction in the transcriptional activity of CREB (Kathleen et al.,2016), suggesting a role of SMEK1 in regulation the expression of CREB target genes which include genes that regulate cell proliferation and apoptosis (Kathleen et al.,2016). On the other hand, SMEK2 overexpression led to the decrease in the phosphorylation of 12 out of 13 proteins involved in MAPK pathway, suggesting that SMEK2 exerts inhibitory effect on the MAPK pathway. Interestingly, PP4c have been also implicated in the regulation of MAPK pathway. Merkel cell polyomavirus (MCPyV) -induced tumorigenesis which is associated with the aggressive skin cancer Merkel cell carcinoma (MCC) is largely dependent on the expression of the small tumour antigen (ST). MCPyV ST expression in the highly metastatic form of MCC promotes cell motility and migration by activating p38 MAPK signalling. Recent study has shown that MCPyV ST-mediated p38 MAPK signalling occurs as a result of interaction between MCPyV ST and PP4c. In these cells, PP4c was shown to inhibit MAPK signalling pathway, the interaction between MCPyV ST and PP4c leads to the inhibition of

PP4c and the consequent activation of MAPK pathway which in turn promotes cell motility and migration (Dobson *et al.,* 2020).

Another pathway that promotes survival and growth is the Akt signalling pathway. A number of kinases are known to phosphorylate and activate AKT which in turns to the phosphorylation of different proteins located either in the plasma membrane, in the nucleus or the cytosol, supporting cell growth and survival (Nitulescu et al., 2018). SMEK1 overexpression did not affect the phosphorylation of the main kinases in the pathway AKT and mTOR but led to an increase in the phosphorylation of mTOR substrates involved in protein synthesis 4E-BP1 and p70S6K. It is possible that SMEK1 either stimulated the activity of another kinase that phosphorylates these substrates or inhibited a phosphatase involved in the dephosphorylation of these proteins. SMEK1 overexpression also caused a significant reduction in the phosphorylation level of PRAS40. The reduction in the phosphorylation of PRAS40 suggest that SMEK1 contributes to the inhibition of the AKT pathway as it has been shown that the phosphorylated form of PRAS40 contribute to the activation of the PI3K/AKT/mTOR signalling (Wang et al.,2014). SMEK2 overexpression also did not affect AKT and mTOR despite an increase in the phosphorylation level of PDK-1 kinase which is an upstream activator of AKT (Dieterle et al.,2014). It is possible that SMEK2 is activating a phosphatase that counteracts the effects of PDK1 and therefore AKT phosphorylation is not changed. A very important effect of SMEK2 is the reduction in the phosphorylated level of the critical apoptosis regulator BAD (BCL-2–associated death promoter), a crucial BH3-only proapoptotic Bcl-2 family protein. BAD phosphorylation is critical to its role as a key regulator of cell death and survival. Phosphorylated form of BAD prevents its dimerization with Bcl-xL resulting in the inhibition of cytochrome c release to the cytosol and the subsequent caspase-3 activation, so leading to greater cell survival (Kizilboga *et al.,* 2019). In its unphosphorylated

form, BAD sequesters Bcl-xL, which results in BAK/BAX activation and apoptosis (Kizilboga *et al.,* 2019). The observation that SMEK2 overexpression leads to the decrease in the phosphorylated BAD is significant because PP4c has also been shown to dephosphorylate BAD, suggesting that SMEK2 effects on BAD might be mediated by its interaction with PP4c (Mourtada-Maarabouni and Willilams, 2008).

JAK/STAT signalling pathway was another pathway investigated in the HEK293T cells. The signalling pathway plays and important role in development, proliferation, differentiation, and survival of cancer cell (Vogelstein *et al.,* 2013). In fact, Vogelstein et al. (2013) have classified JAK/STAT pathway as one of 12 core cancer pathways. While SMEK1 overexpression had no effect on the phosphorylation levels of the proteins in the JAK/STAT pathway, SMEK2 overexpression led to a decrease in the phosphorylation in the receptor tyrosine kinase EGR, JAK proteins including JAK1, JAK2 and TYK2 and the transcription factors STAT 1, 3, 5 and 6. Phosphorylation of STATs proteins by activated JAK kinase is essential for their translocation to the nucleus where they regulate gene expression (Myers et al., 2001). SMEK2 overexpression led to a significant increase in the phosphorylation of the tyrosine phosphatase SHP2. Studies have shown that phosphorylation of SHP2 enhances its phosphatase activity leading to the dephosphorylation of its targets JAK and STAT proteins (Myers et al., 2001). These results suggest that SMEK2 overexpression is associated with the inactivation of JAK/STAT pathway and the subsequent inhibition of STATs- dependent gene transcription. Interestingly, STAT3 was also reported to show decrease in its phosphorylation levels in PP4c overexpressing cells (Mourtada-Maarabouni and Willilams, 2008), implying that SMEK2 effects on the JAK/STAT pathway could be mediated by its interaction with PP4c.

In contrast to the pathways discussed above, overexpression of both SMEK1 and SMEK2 led to a decrease in the phosphorylation of proteins involved in the NFkB signalling pathway, suggesting that both proteins can inhibit the NFkB dependent gene transcription. The complex PP4c-PP4R1 has also been shown to act as a negative regulator of NF-κB activity in T lymphocytes (Brechmann et al.,2012). Brechmann et al. (2012) have reported that PP4R1 potentiates the activity of PP4c and allows its association with IKK complex leading to its dephosphorylation and inactivation and consequently the inhibition of NF-κB signalling activity. Therefore, it is plausible that increased SMEK1 and SMEK2 expression increases efficiency of PP4 complex-mediated dephosphorylation of NFkB and other proteins involved in this pathway.

The last pathway investigated was TGFβ receptors pathway which signal through SMAD proteins that control the expression of hundreds of genes (Massague,2012). The effects of SMEK1 and SMEK2 overexpression on the proteins involved in TGFβ pathway indicated that the two proteins play different roles in HEK293T cells. SMEK1 overexpression was associated with an increase in the phosphorylation levels of SMAD1, 2, 4 and 5, whereas SMEK2 overexpression was associated with a reduction in the phosphorylation of SMAD4, SMAD5 and absence of the phosphorylated form of SMAD1 and SMAD2. The effects of SMEK1 and SMEK2 overexpression on SMADs proteins suggest that both play very important role in the regulation of signalling pathways associated with cell growth and survival. SMADs are a family of proteins that act as signal transducers and transcriptional modulators mediating TGFβ signalling pathway and other signalling pathways such as MAPK and JAK/STAT (Budi et al.,2017). SMAD phosphorylation is important for its translocation and accumulation in the nucleus to regulate target gene expression (Massague,2012). The results imply that increased

SMEK1 levels in HEK293T cells is associated with an increased activity of SMAD 1, 2, 4 and 5, while SMEK2 overexpression inhibits SMADs mediated target gene expression.

Overall, the results show that only SMEK2 overexpression in HEK2931 is associated with the inhibition of MAPK, AKT and JAK/STAT pathways. Both SMEK1 and SMEK2 overexpression led to the inhibition of NFkB, it is also possible that these effects are mediated by their interaction with PP4c. These results suggest that enhanced SMEK2 expression increased efficiency of PP4c mediated dephosphorylation of key proteins in MAPK, AKT and JAK/STAT pathways while increased SMEK1 and SMEK2 expression enhanced efficiency of PP4c mediated dephosphorylation of main proteins in NFkB pathway. Interestingly, increased expression of SMEK1 and SMEK2 in HEK293T cells exerted different effects on SMADs proteins in TGFβ pathway and these effects appear to be independent on their interaction of PP4c.

Further experiments were carried out using Jurkat leukemic T cells to confirm the roles of SMEK1 and SMEK2 in the regulation of these main pathways. The results showed a different role for SMEK1 from those observed in HEK293T cells. SMEK1 overexpression in Jurkat T cells caused similar effects to those shown in cells with increased expression of SMEK1, suggesting that both proteins have similar role in the regulation of these pathways. Both SMEK1 and SMEK2 overexpression in Jurkat T cell exerted inhibitory effects on MAPK, AKT, JAK/STAT and NFkB pathways. Most of the proteins involved in these pathways showed reduced levels of phosphorylation compared to control apart from STAT1 in JAK/STAT pathway. Increased expression of SMEK1 and SMEK2 was associated with an increased phosphorylation of STAT1 and decrease in the phosphorylation of STAT2,3,5 and 6. Phosphorylated STAT1 forms a homodimer before being transported to the nucleus where it promotes the expression of genes that enhance growth arrest and apoptosis (Bousoik et al.,2018). STAT1 activation leads

to an increased expression of several proapoptotic proteins including caspases (Caspase 2, 3 and 7), different death ligands and death receptors (Fas-ligand, FAS receptor and TRAIL) and different proteins involved in cell cycle arrest (p21 and p27) (Sironi et al.,2004). STAT1 activation results in the decrease in expression levels of genes associated with cell cycle progression and proliferation (Cyclin Dependent Kinases) and cell survival including BCL2 BCL-XL and the oncogene c-MYC (Bousoik et al.,2018). Increased expression of SMEK1 and SMEK2 resulted in the decrease in the phosphorylation levels of STAT3,5 and 6 which are involved in promoting the expression of antiapoptotic and cell cycle progression proteins including Bcl2, Bcl-xl, c-MYC and survivin (Bousoik et al.,2018). The growth inhibitory effects of SMEK1 and SMEK2 could be explained by their effects on STATs proteins. STAT1 phosphorylation by SMEK1 and SMEK2 leads to its activation which results in the increase in the expression of genes that mediate cell death and inhibiting cell growth (Bousoik et al.,2018). On the other hand, reduced phosphorylated levels of STAT3, 5 and 6 inhibit their activity leading to the inhibition of the expression of survival genes. Most of the effects of SMEK1 and SMEK2 overexpression in AKT, MAPK, JAK/STAT and NFkB pathways were nearly mirrored in the cells with increased expression in PP4c, suggesting that SMEKs proapoptotic effects on this pathway could be mediated via their interaction with PP4c. Increased expression of SMEK1 and SMEK2 expression in Jurkat T cells could lead to the enhancement of PP4c phosphatase activity and therefore leading to the dephosphorylation of protein targets in these pathways.

Unlike the effects of SMEK1 and SMEK2 in HEK293T cells, increase in the expression of both SMEK1 and SMEK2 in Jurkat T cells led to an increase in the phosphorylation of SMADs proteins of the TGFβ pathway. SMEK1 overexpression led to the increase in the phosphorylation of SMAD1, 2 and 5 and increased SMEK2 expression level was associated with an increase in the phosphorylation of SMAD2, 4 and 5. These effects were specific to

SMEK1 and SMEK2 and were not observed in the cells overexpressing PP4c, suggesting that the effects on SMADs were not dependent on SMEK1 and SMEK2 interaction with PP4c. There are eight different SMADs proteins, which fall into three functional classes receptor-regulated SMADs or R-SMADs, functional SMAD and inhibitory SMADs (SMAD6 and 7) (Ross et al.,2008). SMAD1, 2 and 5 are R-SMADs. Upon activation by TGFβ receptors and their subsequent phosphorylation, the R-SMADs form either homomeric or heteromeric complexes with the SMAD4, the only member in the second functional class of SMADs. SMAD complexes then accumulate in the nucleus and regulate the transcription of target genes in conjunction with other transcription factors (Ross et al.,2008). The SMAD pathway is of central importance for the way that TGFβ signals cell cycle arrest. Nuclear SMAD complexes regulate target genes leading to the suppression of mitogenic transcriptional signals such c-MYC and the induction of cell cycle inhibitor genes (p15, p21, p57) (Heldin et al., 2009; Massagué, 2004). Mitogenic transcription factors such as c-MYC are known to repress the expression of genes such as p15 and p21. SMADs repress c-MYC expression so that p15 and p21 can be expressed leading to the inhibition of cell cycle progression (Niimi et al. 2007). Studies have demonstrated that in Ras-transformed keratinocytes, TGFβ signalling mediated by SMAD3 directly induces expression of the tumour suppressor locus Ink4a/ARF leading to the production of p16ink4a and p19arf, a mechanism that explains the way that TGFβ can induce cell cycle arrest and eventually senescence in these cells (Vijayachandra et al. 2009). The growth inhibitory and proapoptotic effects of SMEK1 and SMEK2 in T-leukemic cells could be mediated, at least partly, through direct or indirect interactions with SMAD proteins leading to change in gene expression.

A common mechanism to control gene expression is by regulating the post-translational phosphorylation of transcription factors. These modifications either activate or

inhibit transcription factor activity in turning on gene expression. Protein phosphorylation and dephosphorylation can directly regulate transcription factor cellular localization, stability, protein-protein interactions and DNA binding (Whitmarsh and Davis 2000). The phosphorylation levels of eight transcription factors were confirmed to change in the cells overexpressing SMEK1 and SMEK2. These transcription factors include NFκB, STAT1, STAT3, STAT5, c-Jun, SMAD1, SMAD4 and SMAD5. In addition to their role in modulation gene expression, studies have shown that SMADs can recruit various epigenetic regulators to shape the transcriptome (Papageorgis et al.,2010). These data suggest that SMEK1 and SMEK2 regulate gene expression at both epigenetic level and transcriptional level.

The results presented in this chapter provide strong evidence that SMEK1 and SMEK2 play a crucial role in the control of apoptosis and cell proliferation in leukemic T cells by regulating critical signalling pathways such MAPK, AKT, JAK/STAT, NFkB and TGFβ. While the effects on MAPK, AKT, JAK/STAT, NFkB signalling pathways might be mediated by direct interactions with PP4c, the effects exerted on TGFβ pathway appear to be independent of PP4c. Although more studies are required to further clarify the signalling pathways regulated by SMEK1 and SMEK2, both proteins can regulate the activity of critical transcription factors and consequently regulate target gene expression.

4.5 Chapter highlights

1- In HEK293T cells, only SMEK2 overexpression in HEK2931 is associated with the decrease in the phosphorylation levels of most of the proteins involved in MAPK, AKT and JAK/STAT pathways. Both SMEK1 and SMEK2 overexpression led to the inhibition of NFkB pathway. Increased expression of SMEK1 and SMEK2 in HEK293T cells exerted different effects on SMADs proteins in TGFβ pathway.

2- The effects of SMEK1 and SMEK2 overexpression in Jurkat T cells were different from those observed in HEK293T cells. Increased endogenous levels of SMEK1 and SMEK2 Jurkat exerted inhibitory effects on MAPK, AKT, JAK/STAT and NFκB signalling pathways. Most of the proteins involved in these pathways showed reduced levels of phosphorylation compared to control apart from STAT1 in JAK/STAT pathway. These effects were mirrored in the cells overexpressing PP4c, suggesting that SMEK1 and SMEK2 effects on these signalling pathways might be mediated via their interactions with PP4c.

3- Increased levels of SMEK1 and SMEK2 in Jurkat T cells affected TGFβ signalling pathways which was evident by increasing the expression levels of SMADs proteins. SMEK1 and SMEK2 regulate TGFβ pathway independently of PP4c. Both proteins had different effects on the phosphorylation of SMADs. SMEK1 increased phosphorylation of SMAD1, 2 and 5, whilst SMEK2 increased phosphorylation of SMAD2, 4 and 5.

4- Increased endogenous levels of SMEK1 and SMEK2 in Jurkat cells affected the phosphorylation of eight transcription factors including NFκB, STAT1, STAT3, STAT5, c-Jun, SMAD1, 2, 4 and 5, confirming a role for SMEK1 and SMEK2 in the regulation of gene expression in leukemic T cells.

Chapter Five: The influence of SMEK1 and SMEK2 overexpression on gene expression in Jurkat leukemic T cells

5.1 Introduction

The lines of evidence presented in Chapter 3 support the tumour suppressive role of SMEK1 and SMEK2 given that overexpression of both proteins increases the levels of apoptotic cell death and decreases the short- and long-term survival of leukemic T cells and their down-regulation promoted cell proliferation and long-term survival. Mechanistic investigations presented in chapter 4 revealed that increased endogenous levels of SMEK1 and SMEK2 affect the phosphorylation and activity of key signalling proteins in signalling pathways involved in regulation of cell growth, apoptosis, and gene expression. These signalling pathways include MAPK, AKT, NFκB, JAK/STAT and TGFβ. Both SMEK1 and SMEK2 overexpression modulated cellular activities from upstream, which involved sequential changes in the phosphorylation levels of signalling molecules, to downstream where the phosphorylation states of transcription factors were also changed. Both SMEK1 and SMEK2 overexpression modulated the phosphorylation levels of critical transcription factors including NFκB, STAT1, STAT3, c-Jun, SMAD1 and SMAD5, while only SMEK2 overexpression led to change in phosphorylation of STAT5 and SMAD4.

One of the mechanisms used by the cells to regulate transcription factor activity and specificity is reversible phosphorylation. Reversible phosphorylation of transcription factors is a crucial link between cell signalling and the control of gene expression. Phosphorylation of transcription factors by protein kinases or dephosphorylation by protein phosphatases can either positively or negatively regulate their activity leading to a change in gene expression. Changes in transcription factors protein phosphorylation can also regulate their cellular localization, protein stability, protein-protein interaction and DNA binding resulting in

transcriptional regulation (Ying *et al.,* 2017). The fact that both SMEK1 and SMEK2 overexpression affected the phosphorylation of key transcription factors imply that both proteins may play a role in the regulation of gene expression. Therefore, the aims of this chapter are to investigate the effects of SMEK1 and SMEK2 overexpression on global gene expression in leukemic T cells using RNA sequencing.

5.2 Methods

Global gene expression changes in response to SMEK1 and SMEK2 overexpression were determined by sequencing the whole transcriptome, as detailed in section in Section 2.9.3.

In brief, Jurkat T cells were transfected with either pcDNA3.1, pcDNA3.1-SMEK1 or pcDNA3.1-SMEK2. Total RNA was extracted from transfected cells 48h post transfection as described in Section 2.9.1. RNA integrity was assessed by gel electrophoresis and nanodrop as described in Section (2.10.1). RNAs samples at a concentration of 5 μg of total RNA (100 ng/μl in 50 μl) were sent for sequencing at the Centre of Genomic Research at the University of Liverpool for Next Generation Sequencing (NGS). Quality controlled reads were aligned to Human Genome build (hg19) using Tophat, transcripts were assembled using Cufflinks (with GTF support) and the number of reads mapping to each feature counted and expressed as FPKM using the CuffNorm package. Differentially expressed RNAs were condensed into gene networks representing biological and disease processes using iPathwayGuide (Advaita Bioinformatics, Ann Arbor, MI, USA). The differential expression was measured and compared between Jurkat T cells transfected with the empty vector and Jurkat T cells transfected with

pcDNA3.1-SMEK1 or pcDNA3.1-SMEK2, and a cutoff threshold of 1.5-fold change (an equivalent 0.6 Log2 Fold Change) was applied. The Log2 Fold Change (Log2FC) is referred to as “fold change” in this chapter.

Meta-analysis to identify genes, pathways and gene ontology terms that may be in common in SMEK1 and SMEK2 or unique to each of them was carried out using Pathway Guide platform.

5.3 Results

5.3.1 RNA Sequencing reveals molecular and biological perturbations of gene expression pathways upon SMEK1 overexpression in Jurkat T cells

In order to investigate the role of SMEK1 in the regulation of gene expression and elucidate the molecular mechanisms through which SMEK1 exerts its biological effects, RNA sequencing was performed and the genes that exhibited the most pronounced expression changes in response to SMEK1 overexpression were identified. Transfection of Jurkat T cells with pcDNA3.1-SMEK1 resulted in a log2FC 0.75 increase in SMEK1/PP4R3A (equivalent to 1.7 fold increase) (Figure 5.1) and did not affect the expression levels of SMEK2, suggesting that SMEK1 overexpression does not affect the expression levels of SMEK2. The results also showed that SMEK1 overexpression did not affects the expression levels of PP4c and its other regulatory subunits. However, SMEK1 overexpression was associated with 1.5-1.8 fold decrease in the expression levels of protein phosphatase 1 regulatory subunits 32 and 11 (PPP1R32 and PPP1R11) and protein phosphatase 2 regulatory subunit 3B .

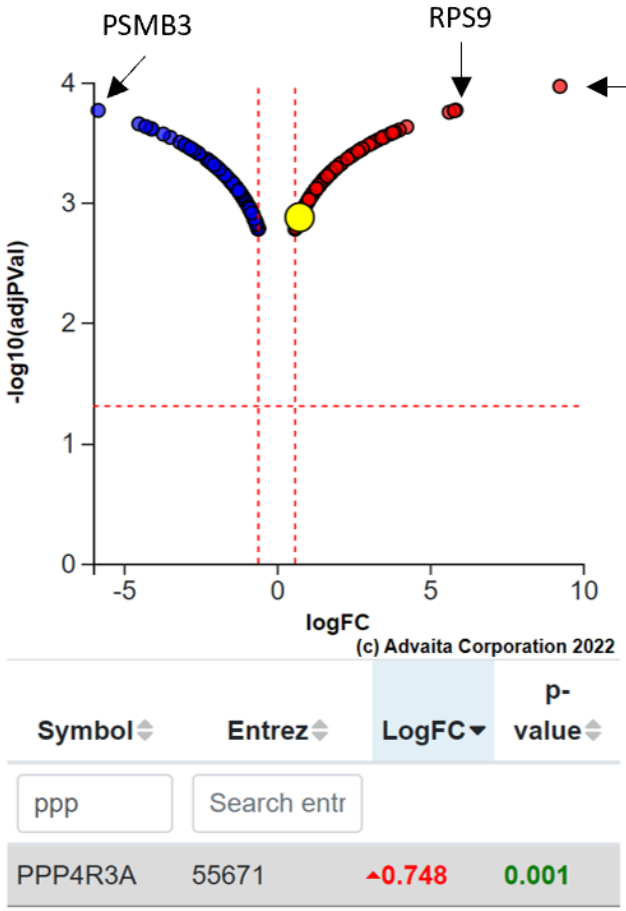


Figure 5.1 Visualization of RNA-Sequencing results using Volcano Plot. The dots represent the differentially expressed genes. The horizontal axis is the log fold change, and the vertical axis is the negative base -10 logarithm of the p-value. The red-dotted lines represent the threshold used to select the DE genes: Log FC 0.6 for expression change and 0.05 for significance. The upregulated genes (positive log fold) are shown in red, while the down-regulated genes are blue. Small nucleolar RNA, C/D box 3A (snoRND3A) and RPS9 (ribosomal protein S9) were the most highly upregulated genes while proteasome 20S subunit beta 3 (PSMB3) was the most down-regulated gene. The figure also shows the increase in SMEK1/PPP4R3a expression levels as a result of pcDNA3.1-SMEK1 transfection (Advaita 2022).

Following the confirmation of SMEK1 overexpression, we next investigated those genes that were deregulated by a factor of at least 1.5-fold (log2FC ≥ 0.6) between the control and SMEK1 transfected samples. 1512 differentially expressed genes were identified out of a total of 17199 genes with measured expression. These data were further analysed in the context of pathways obtained from the Kyoto 156, Encyclopaedia of Genes and Genomes

(KEGG) database (KEGG: 05206), gene ontologies from the Gene Ontology Consortium database, and miRNAs from the miRBase and TARGETSCAN (Targetscan version: 7.1) databases.

Top upregulated genes include small nucleolar RNA C/D box 3A and 3B-1 (SNORD3A and SNORD3B-1), ribosomal protein S9 (RPS9), RPS10-NUDT3 readthrough (read-through transcription between the neighbouring RPS10 (ribosomal protein S10) and NUDT3 (nudix (nucleoside diphosphate linked moiety X)-type motif 3) and RPS18 (ribosomal protein S18) (Figure 5.1 and Table 5.1). SnoRNAs are a class of non-coding RNAs belong to the small nucleolar RNAs family which are involved in the nucleolytic processing of ribosomal RNAs (rRNA) and function as guide RNAs in the post-transcriptional modifications of rRNAs and a number of them has been implicated in the control of cell survival and cancer (Kufel and Grzechnik,2018). Interestingly, the top upregulated gene in the cells overexpressing SMEK1 was SNORD3A (9-fold increase). More than 40 small nucleolar RNAs (snoRNAs) were also found to be overexpressed including SSNORD116-9 and SNORD20. The second top upregulated gene is RPS9 which is one of the first proteins that directly bind to the 18S rRNA and plays a crucial role in ribosome biogenesis (Sharma et al.,2019). Other ribosomal proteins have also showed increased expression as a result of SMEK1 overexpression. Ribosomal proteins (RPs) form the structural parts of the ribosome and their role is very important in the process of ribosome assembly and function (Kang *et al.,* 2021). Another upregulated gene includes SIN3-HDAC complex associated factor (SINHCAF3) which involved in the repression of genes involved in the TGFβ signalling pathway (Charles *et al.,* 2020),

Table 5.1 Top ten up-regulated genes in response to SMEK1 up-regulation in Jurkat cells

Gene symbol	Gene name	Function	Fold change
SNORD3A	Small nucleolar RNA, C/D box 3A	Small nucleolar RNA has a function in the processing of ribosomal RNA precursors.	9.25
RPS9	Ribosomal protein S9	This gene encodes a ribosomal protein which is a component of the 40S subunit. The protein product is a member of the S4P family of ribosomal proteins.	5.85
RPS10-NUDT3	RPS10-NUDT3 readthrough	This locus indicates readthrough transcription between the neighbouring ribosomal protein S10 (RPS10) and nucleoside diphosphate linked moiety X-type motif 3(NUDT3) genes on chromosome 6.	5.8 1
SNORD3B-1	Small nucleolar RNA, C/D box 3B-1	Small nucleolar RNA has a function in the processing of ribosomal RNA precursors.	5.637
CUTA	cutA divalent cation tolerance homolog	It is thought to enable copper ion binding activity as well as enzyme binding activity. It is also involved in protein localisation.	4.235
NAPRT	Nicotinate phosphoribosyl transferase	The protein product is an enzyme which catalyses the first and rate-limiting step in the biosynthesis of NAD from nicotinamide. It is also recognized as an essential factor in granulocyte-colony-stimulating factor-(G-CSF)-induced myeloid differentiation.	4
TOMM6	Translocase of outer mitochondrial membrane 6	The protein product is part of a complex of proteins found in the outer mitochondrial membrane of the mitochondria. It allows movement of proteins through this barrier and into the intermembrane space of the mitochondrion.	3.85
URGCP-MRPS24	URGCP-MRPS24 readthrough	This locus represents naturally occurring read-through transcription between the neighbouring URGCP (up-regulator of cell proliferation) and MRPS24 (mitochondrial ribosomal protein S24) genes on chromosome 7.	3.78
SINHCAF	SIN3-HDAC complex associated factor	Protein product facilitates local histone deacetylation and transcriptional repression.	3.71
RASSF7	Ras association domain family member 7	The protein product belongs to a family of proteins, many of which are tumour suppressor proteins.	3.47

Information regarding gene function were adapted from iPathwayGuide Advaita (2022).

The most down-regulated genes in SMEK1 overexpressing include proteasome 20S subunit beta 3 (PSMB3), which showed 5-fold decrease in expression levels. (Figure 5.1; Table 5.2). The gene encodes the 20S core subunit of the proteasome, a multi-catalytic proteinase complex involved in the ATP/ubiquitin-dependent process of peptide cleavage. The proteasome function is essential for the degradation of the intracellular proteins that have been targeted for degradation via polyubiquitination (Kors et al.,2019). Others top down-regulated genes include Rho GTPase activating protein 11A (ARHGAP11A), nuclear pore complex interacting protein family, member B13 (NPIPB13), interferon alpha inducible protein 27 like 1 (IFI27L1), casein kinase 2 beta (CSNK2B) and HRAS proto-oncogene, GTPase (HRAS) (Table 5.2). ARHGAP11A is reported to be overexpressed in a variety of tumours and to play an important role in the proliferation, invasion, metastasis, and cell cycle regulation of tumour cells (Fan et al,2021). Therefore, ARHGAP11A reduced expression in the cells overexpressing cells is consistent with tumour suppressor role for SMEK1. The significant observation is that SMEK1 overexpression led to the down-regulation of HRAS, a member of the RAS protooncogenes, which encodes a protein responsible for the regulation of growth, differentiation and survival of many cell types (Pazik *et al.,* 2021). HRAS plays a critical role in cellular processes such proliferation, survival, differentiation, motility, and transcription. Active HRAS pathway is one of the most deregulated pathways in human cancer and 30% of all tumours have an activating mutation in RAS genes (Harms *et al.,* 2021). HRAS significant decrease in SMEK1 overexpressing cells provides strong evidence of SMEK1 tumour suppressive role. In addition, SMEK1 overexpression was associated with a 2.8-fold decrease CSNK2B, the regulatory subunit of the serine/threonine protein kinase Casein kinase 2 whose activity is associated with apoptosis suppression (Ahmad *et al.,* 2007; Hashimoto *et al.,* 2018). CK2 regulates several signalling pathways including PI3K/AKT, NFκB and Wnt (Maira *et al.,*

2005) and is implicated in cancerous transformation and is a therapeutic target in anti-cancer therapy.

Table 5.2 - Top ten down-regulated genes in Jurkat cells overexpressing SMEK1

Gene Symbol	Gene name	Function	Fold Change
PSMB3	Proteasome 20S subunit beta 3	This gene encodes the 20S core beta subunit of the proteasome. It is a member of proteasome B-type family, also known as the T1B family.	5.83
HSPE1-MOB4	HSPE1-MOB4 readthrough	This locus represents naturally occurring read-through transcription between the neighbouring HSPE1 (heat shock 10kDa protein 1 (chaperonin 10)) and MOB4 (MOB family member 4) on chromosome 2.	4.5
NDUFC2-KCTD14	NDUFC2-KCTD14 readthrough	The neighbouring NDUFC2 (NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2, 14.5kDa) and KCTD14 (potassium channel tetramerization domain containing 14) genes on chromosome 11. The protein product is an accessory subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex1) which functions in the transfer of electrons from NADH to the respiratory chain.	4.28
ARHGAP11A	Rho GTPase activating protein 11A	This gene encodes a member of the Rho GTPase activating protein family. Increased expression promotes migration and invasion in colorectal cancer, glioblastoma, hepatocellular and pancreatic cancers.	4.12
NPIPB13	Nuclear pore complex interacting protein family, member B13	It is a protein coding gene that belongs to the nuclear complex interacting protein family. These proteins regulate the nuclear envelope and serve as highly selective transport gates that enable bi-directional macromolecule exchange between the nucleus and cytoplasm.	4.18
IFI27L1	Interferon alpha inducible protein 27 like 1	IFN α inducible protein 27 is an IFN-stimulated gene, belongs to the FAM14 which also include IFI6, IFI27, ISG12b, and ISG12c). Unknown function.	3.71
GGNBP2	Gametogenetin binding protein 2	Protein product is predicted to be involved in spermatogenesis. Predicted to regulate regulation of cell proliferation.	3.48
CXorf49	Chromosome X open reading frame 49	Unknown function.	3.16
HRAS	HRAS proto-oncogene, GTPase	This gene belongs to the Ras oncogene family, whose members are related to the transforming genes of mammalian sarcoma retroviruses. These proteins can bind GTP and GDP, and they have intrinsic GTPase activity. This protein undergoes a continuous cycle of de- and re-palmitoylation.	3.02

CHURC1-FNTB	CHURC1-FNTB readthrough	This locus represents naturally occurring read-through transcription between the neighbouring CHURC1 (churchill domain containing 1) and FNTB (farnesyltransferase, CAAX box, beta) on chromosome 14. The read-through transcript produces a fusion protein that shares sequence identity with each individual gene product.	3.01

Information regarding gene function were adapted from iPathwayGuide Advaita (2022).

More than 30 snoRNAs also showed reduced expression, highlighting the significant effects of SMEK1 on this class of non-coding RNAs. Three mitochondrial ribosomal proteins MRPS36, MRSP18B, and MRSP16 showed more than two-fold reduction in expression, implicating SMEK1 in mitochondrial translational machinery.

As an excess of 1500 genes were identified as being deregulated by at least 1.5 fold, we elected to condense the individual gene changes into common biological processes and pathways prior to interpretation. Pathway analysis revealed that several cellular processes were affected by the overexpression of SMEK1 with gene expression being the top process affected (Figure 5.2; Table 5.3). Among the perturbed cellular processes, numerous transcription related processes were significantly affected, including negative regulation of metabolic process (Figure 5.3), negative regulation of gene expression (Figure 5.3), RNA processing, post-transcriptional regulation of gene expression, epigenetic control of gene expression, gene silencing by RNA and miRNA and post-transcriptional gene silencing (Table 5.3).

Table 5.3 - Top perturbed Gene Ontology (GO) terms in response to SMEK1 overexpression in Jurkat T cells

Biological Process	Number of Gene (DE/ALL)
Gene expression	**254 /** 4719
Negative regulation of metabolic process	**129** / 2526
Negative regulation of gene expression	**102 /** 1795
RNA Processing	**87/**1119
Posttranscriptional regulation of gene expression	**66**/ 673
Defence response	**63/**973
Regulation of gene expression-epigenetic	**56** / 405
Gene silencing by RNA	**54** / 269
Gene silencing	**54**/ 340
Posttranscriptional gene silencing	**53** / 255
Gene silencing by miRNA	**87** / 1119

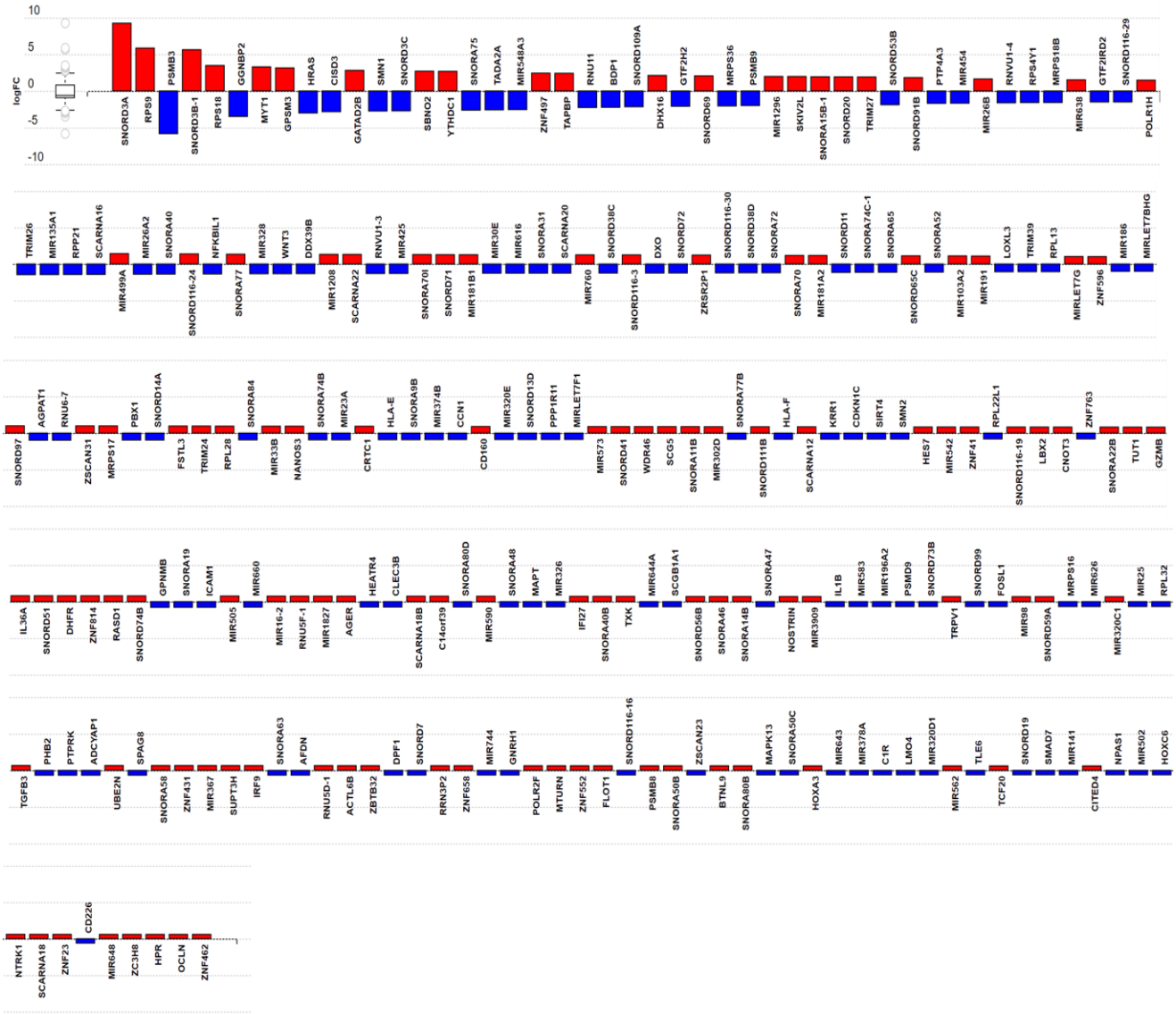


Figure 5.2 The expression of individual genes implicated in regulation of gene expression. Genes implicated in the biological process of gene expression, the top perturbed biological process as determined by sequencing and analysis of the whole transcriptome. Red coloured bars correspond to up-regulated gene expression, while blue coloured bars correspond to down-regulated gene expression. Data are the difference in expression between cells transfected with pcDNA3.1 and cells transfected with SMEK1, expressed as a normalised log2 fold change (log2FC). A threshold of 0.05 for statistical significance (p-value) and a log2 fold change of expression with an absolute value of at least 0.6 were applied.

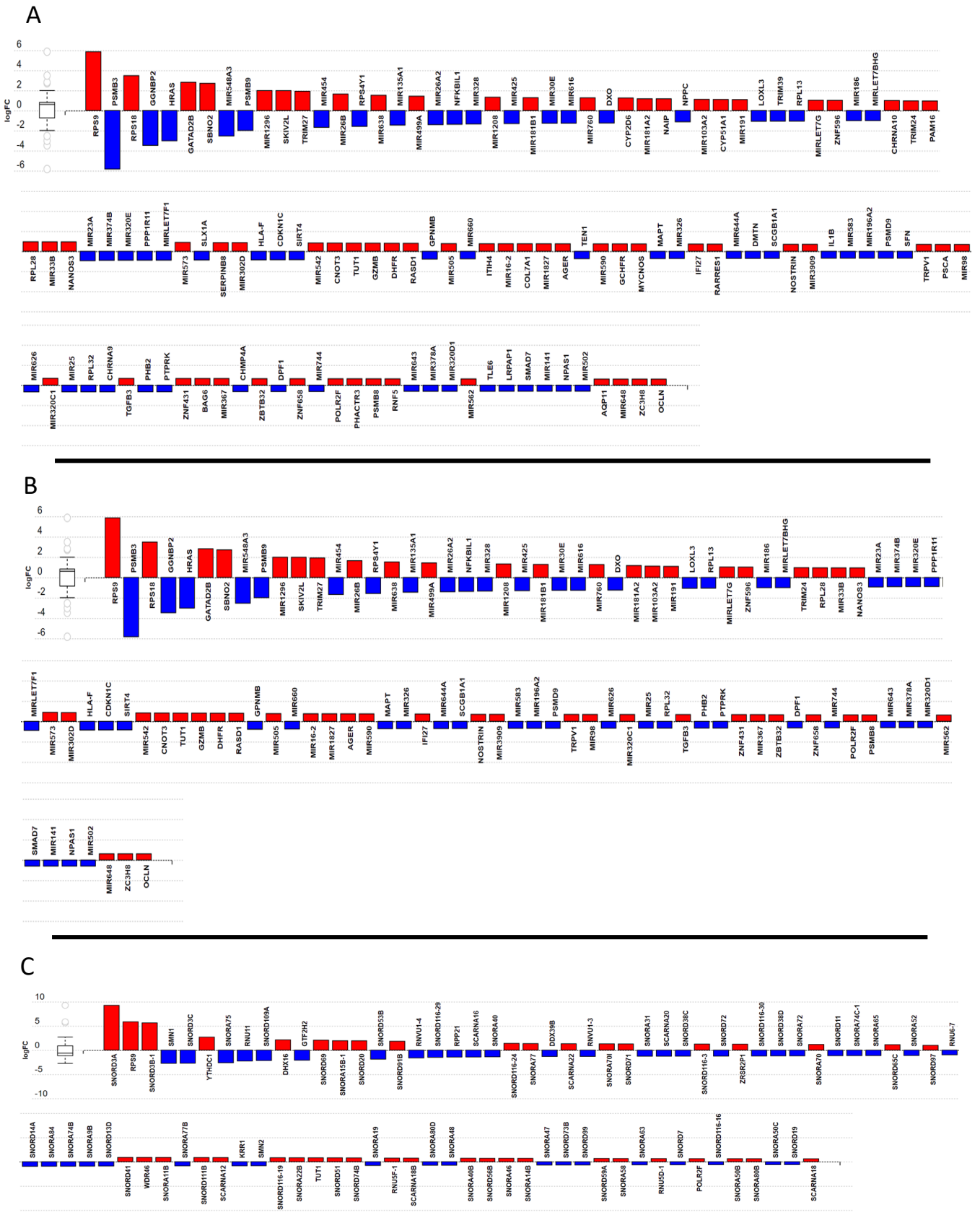


Figure 5.3 The expression of individual genes implicated in the negative regulation of metabolic processes, negative regulation of gene expression and RNA processing. Genes implicated in the biological process of negative regulation of metabolic processes (A), negative gene expression (B) and RNA processing (C), as determined by sequencing and analysis of the whole transcriptome. Red coloured bars correspond to up-regulated gene expression, while blue coloured bars correspond to down-regulated gene expression. Data are the difference in expression between cells transfected with pcDNA3.1 and cells transfected with SMEK1, expressed as a normalised log2 fold change (log2FC). A threshold of 0.05 for statistical significance (p-value) and a log2 fold change of expression with an absolute value of at least 0.6 were applied.

In addition, 120 Gene Ontology (GO) terms were significantly enriched in multiple molecular functions including signalling receptor binding, mRNA binding involved in posttranscriptional gene silencing, enzyme inhibitor activity, signalling receptor regulator activity, calcium signalling and structural constituent of ribosomes (Table 5.4). Interestingly, 12 differentially expressed genes were annotated to structural constituent of ribosome, highlighting the effects of SMEK1 on ribosomal structure and function (Figure 5.4).

Table 5.4 Top perturbed Gene Ontology (GO) terms (Molecular functions) in response to SMEK1 overexpression in Jurkat T cells

Molecular function	**Number of Gene (DE/**ALL**)**
Signalling receptor binding	**41 /** 890
mRNA binding	**27 /** 304
mRNA binding involved in posttranscriptional Gene silencing	**22 /** 52
RNA binding involved in posttranscriptional gene silencing	**22 /** 52
Posttranscriptional regulation of gene expression	**73**/ 673
Enzyme inhibitor activity	**14 /** 224
Signalling receptor activator activity	**13/**150
Structural constituent of ribosome	**12** / 159

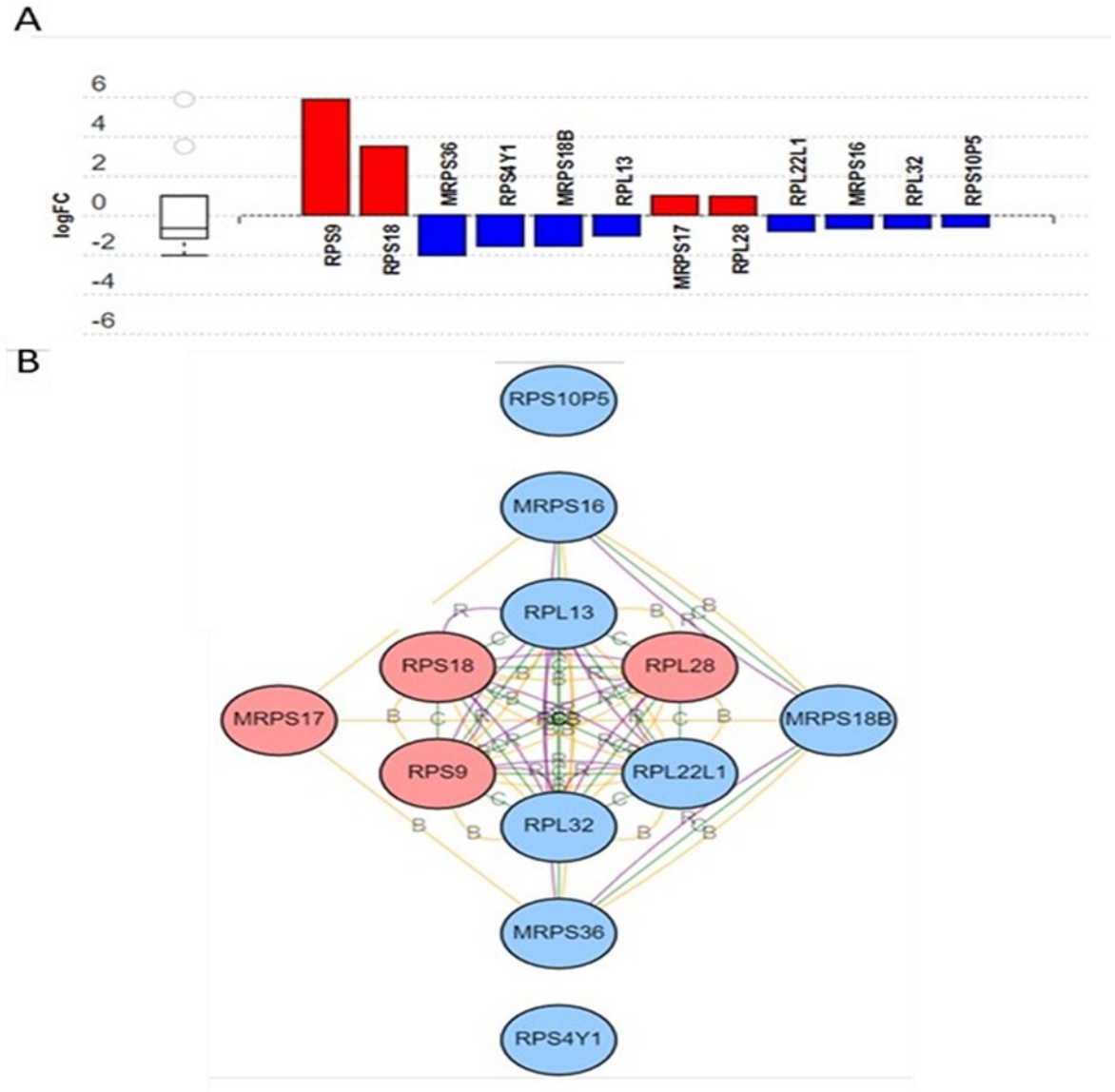


Figure 5.4 Effects of SMEK1 overexpression on genes annotated to structural constituent of ribosome. A) A bar chart showing the 12 differential expresses genes annotated to structural constituent of ribosome. Red bar are overexpressed genes. Blue represents the down-regulated genes. B) Network analysis of the 12 differentially expressed genes. B: binding; R: reaction and C: Catalysis expressed genes. B: binding (yellow); R: reaction(purple) and C: Catalysis (green). Genes upregulated: Mitochondrial Ribosomal Protein S17 (MRPS17), Ribosomal Protein S18 (RPS18), Ribosomal Protein S9 (RPS9), and Ribosomal Protein L28 (RPL28). Genes downregulated: Ribosomal Protein S10 Pseudogene 5 (RPS10P5), Mitochondrial Protein S 16 (MRPS16), Ribosomal Protein L13 (RPL13), Mitochondrial Ribosomal Protein 18B (MRPS18B), Ribosomal Protein L22 Like 1 (RPL22L1), Ribosomal Protein L32 (RPSL32), Mitochondrial Ribosomal Protein 36 (MRPS36), and Ribosomal Protein S4 Y-Linked 1 (RPS4Y1).

5.3.1.1 Impacted biological pathways in Jurkat T cells overexpressing SMEK1

An excess of 200 biological pathways was affected by overexpression of SMEK1. These included cancer independent biological pathways and cancer related processes. Cancer independent pathways include pathways of neurodegeneration, Alzheimer disease, Huntington disease, prion disease and amyotrophic lateral sclerosis. Cancer related pathways included microRNAs in cancer, pathways in cancer, gastric cancer, colorectal cancer and transcriptional misregulation in cancer. Perturbed signalling pathways related to cancer includes apoptosis, MAPK, mTOR, VGEF, ErbB, NFκB, TGFβ and RAS signalling pathways (Table 5.5). Differentially expressed genes that impacted the cancer related pathways are summarised in Table 5.6. These genes include HRAS, MAPK13 (mitogen-activated protein kinase 13), NTRK1 (neurotrophic receptor tyrosine kinase 1), CSNK2B (casein kinase 2 beta), WNT3 (Wnt family member 3), TGFβ3 (transforming growth factor beta 3) and PBX1 (PBX homeobox 1).

Table 5.5 Perturbed cancer related signalling pathways in cells overexpressing SMEK1

Signalling Pathway	Number of genes (Differentially Expressed/ALL)	Up-regulated genes	Down-regulated genes
Apoptosis	**4**/ 117	GZMB TUBA8 NTRK1	HRAS
RAS	**7**/146	NTRK1 CALML4	HRAS RALB GNG8 AFDN NTF4
mTOR	**2**/123	-	HRAS WNT3
MAPK	**7**/199	TGFβ3 NTRK1	HRAS MAPT IL-1β MAPK13
PI3K/AKT	**6**/206	COL9A3 NTRK1	HRAS PPP2R3B
NFκB	**3**/70	-	CSNK2B ICAM1 IL-1β
TGFβ	**2**/63	TGFβ3	SMAD7

Table 5.6 Differentially expressed genes that impacted cancer related pathways in Cells overexpressing SMEK1 and their biological relevance to cancer

Gene	Name of Gene	Fold-increase	Fold-decrease	Hallmark of Cancer
HRAS	HRAS proto-oncogene, GTPase		3.01	Angiogenesis, sustained proliferative signalling
WNT3	Wnt family member 3		1.34	Sustained proliferation, invasion and metastasis, resisting program cell death
COMMD3-BMI1	COMMD3-BMI1 readthrough		1.25	Sustaining proliferation signalling
NTRK1	Neurotrophic receptor tyrosine kinase 1	0.61		Avoiding immune destruction, sustain proliferative signalling, activating invasion and metastasis
CSNK2B	Casein kinase 2 beta		2.85	Sustain proliferative signalling.
MAPK13	Mitogen-activated protein kinase 13		0.63	Sustain proliferative signalling
TGFβ3	Transforming growth factor beta 3	0.67		Tumour suppressor. Promote cell death
RALB	RAS like proto-oncogene B		0.77	Invasion and metastasis
PBX1	PBX homeobox 1		0.97	Angiogenesis, Genome instability. Evading cell death, metabolic dysregulation
GZMB	Granzyme B	0.81		Promotes cell death
GZMH	Granzyme H	1.04		Promotes cell death
CALML4	calmodulin like 4	0.72		Not known
GNG8	G protein subunit gamma 8		0.70	Not known
GSTM3	glutathione S-transferase mu 3	0.68		Invasion and metastasis
NQO1	NAD(P)H quinone dehydrogenase 1		0.61	Metabolic dysregulation

Information regarding gene function were adapted from iPathwayGuide Advaita (2022).

Of the 200 impacted pathways, 50 pathways showed significant changes with 26 pathways do not have in common any differentially expressed gene. The dendrogram in

Figure 5.5A shows the significant results organised hierarchically based on overlap in associated DE genes in 24 impacted pathways. The results showed that all cancer related pathways have HRAS differentially expressed in common as one differentially expressed gene in common. The top nine affected pathways are shown in Figure 5.5B and they include microRNAs in cancer, graft-versus-host disease, allograft rejection, autoimmune thyroid disease, type I diabetes mellitus, oxytocin signalling pathway, Thyroid hormone signalling pathway, mTOR signalling pathway and bladder cancer in the cells overexpressing SMEK1 (Figure 5.6).

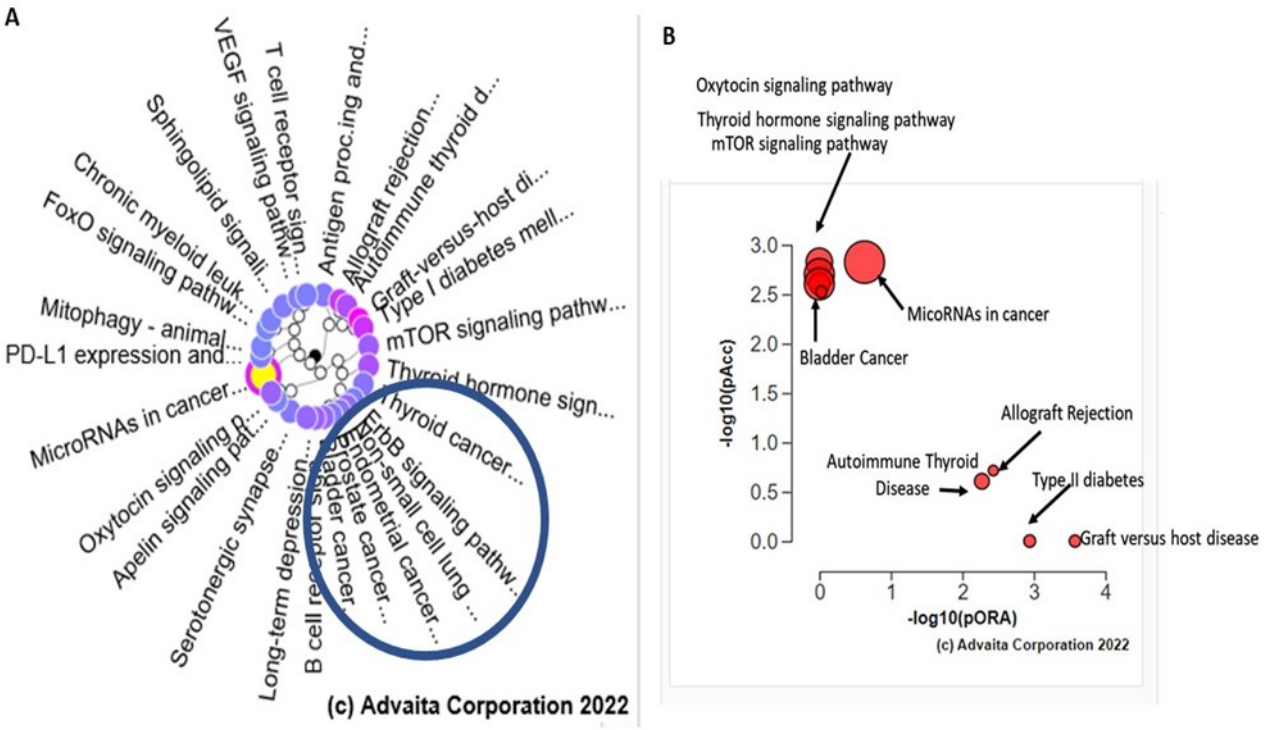


Figure 5.5 Perturbed pathways in Jurkat T cells overexpression SMEK1. A) Dendrogram showing significant pathways organised hierarchically based on overlap in associated differential expressed genes with the blue circle showing the cancer related pathways having one gene in common: HRAS. Node colours correspond to significance of the results (P value) with magenta circles representing more significant results and cyan circles representing lower significance. Yellow circles represent the top impacted pathway MicroRNAs in cancer. B) Perturbation vs over-representation: Top nine most significantly impacted pathways are shown, using negative log of the accumulation and over-representation p-values, along with the other most significant pathways (red circles). Pathways in red are significant, based on the combined uncorrected p-values. The size of each dot donates the total number of genes in the corresponding pathway.

MicroRNAs in cancer was the top perturbed biological pathway with 17 genes differentially expressed in the cells overexpressing SMEK1 (Figure 5.6). These genes include 13 miRNAs, HRAS, WNT3 and COMMD3-BMI with all of them reported to be involved in the regulation of different aspects of the hallmarks of cancer (Table 5.7). Six differentially expressed genes impacted graft-versus-host disease, allograft rejection, autoimmune thyroid disease, type I diabetes mellitus pathways. These genes include major histocompatibility complex genes (HLA): HLA-DMA, HLA-C, HLA-E and HLA-F, IL1B (interleukin 1 beta) and granzyme B (BGZMB).

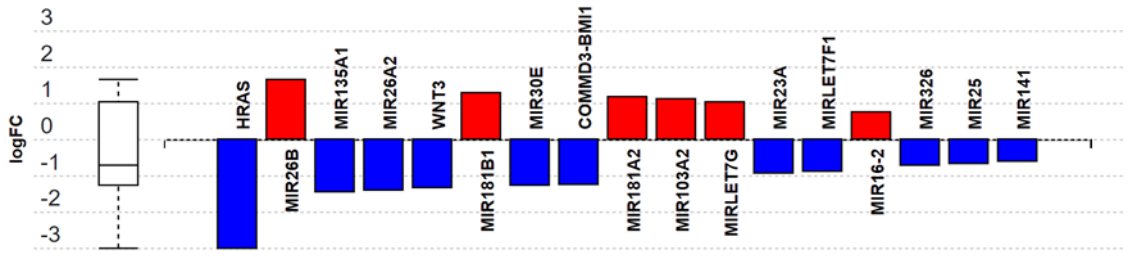


Figure 5.6 The expression of individual genes in microRNAs in cancer pathway. The graph shows the expression of the 17 differentially expressed genes in microRNAs in cancer pathway, as determined by sequencing and analysis of the whole transcriptome. Red coloured bars correspond to up-regulated gene expression, while blue coloured bars correspond to down-regulated gene. Data are the difference in expression between cells transfected with pcDNA3.1 and cells transfected with SMEK1. A threshold of 0.05 for statistical significance (p-value) and a log2 fold change of expression with an absolute value of at least 0.6 were applied.

Table 5.7 Differentially expressed genes in the top perturbed pathway, microRNAs in cancer and their biological relevance to cancer.

Gene	Name of Gene	Fold-increase	Fold-decrease	Hallmark of Cancer
HRAS	HRAS proto-oncogene, GTPase		3.016	Angiogenesis, sustaining proliferative signalling
MIR26B	microRNA 26b	1.645		Inhibits proliferation. Migration and invasion. Promote apoptosis.
MIR135A1	microRNA 135a-1		1.456	Proliferative signalling, migration and invasion
MIR26A2	microRNA 26a-2		1.410	Sustain proliferative, activating invasion and metastasis, angiogenesis.
WNT3	Wnt family member 3		1.340	Sustain proliferative activating, invasion and metastasis, resisting program cell death.
MIR181B1	microRNA 181b-1	1.280		Tumour suppressor. Inhibits apoptosis. Target TGFβR1/Smad signalling.
MIR30E	microRNA 30e		1.274	Sustain proliferative signalling, inhibits cell death.
COMMD3-BMI1	COMMD3-BMI1 readthrough		1.253	Protein Coding gene. Among its related pathways are targets of C-MYC transcriptional activation.
MIR181A2	microRNA 181a-2	1.171		Activating invasion and metastasis, inhibits cell death.
MIR103A2	microRNA 103a-2	1.113		Sustain proliferative signalling, inhibits cell death.
MIRLET7G	microRNA let-7g	1.027		Sustain proliferative signalling, activating invasion and metastasis.
MIR23A	microRNA 23a		0.939	Angiogenesis, activating invasion and metastasis.
MIRLET7F1	microRNA let-7f1		0.889	Activating invasion and metastasis.
MIR16-2	microRNA 16-2	0.750		Tumour suppressor
MIR326	microRNA 326		0.723	Evading immune response
MIR25	microRNA 25		0.675	Sustaining proliferative signalling
MIR141	microRNA 141		0.612	Invasion and metastasis

Information regarding gene function were adapted from iPathwayGuide Advaita (2022).

5.3.1.2 RNA sequencing reveals perturbations of the non-coding elements upon SMEK1 overexpression in Jurkat T cells

The RNA sequencing analysis revealed that apart from the perturbations in protein-coding genes and pathways, numerous and significant changes in expression were observed in the non-coding repertoire, as well. miRNAs and snoRNAs are two types of small non-coding RNAs that have significant role in the regulation of gene expression and RNA processing, respectively. A total of 40 snoRNAs and 202 miRNAs was predicted to be perturbed in response to SMEK1 overexpression (Table 5.8 and 5.9).

Table 5.8 Top perturbed snoRNAs in cells overexpression SMEK1

Gene symbol	Gene name	Fold increase
SNORD3A	small nucleolar RNA, C/D box 3A	9.25
SNORD3B-1	small nucleolar RNA, C/D box 3B-1	5.64
SNORD69	small nucleolar RNA, C/D box 69	2.06
SNORA15B-1	small nucleolar RNA, H/ACA box 15B-1	1.94
SNORD20	small nucleolar RNA, C/D box 20	1.932
		Fold decrease
SNORD3C	small nucleolar RNA, C/D box 3C	2.71
SNORA75	small nucleolar RNA, H/ACA box 75	2.62
SNORD109A	small nucleolar RNA, C/D box 109A	2.15
SNORD53B	small nucleolar RNA, C/D box 53B	1.88
SNORA63D	small nucleolar RNA, H/ACA box 63D	1.67

Table 5.9 Top perturbed miRNAs in the cells overexpressing SMEK1

Gene symbol	Gene name	Fold increase
MIR1296	microRNA 1296	1.98
MIR4427	microRNA 4427	1.78
MIR4265	microRNA 4265	1.75
MIR1976	microRNA 1976	1.73
MIR3125	microRNA 3125	1.72
		Fold decrease
MIR548A3	microRNA 548a-3	2.54
MIR3134	microRNA 3134	1.81
MIR6814	microRNA 6814	1.73
MIR454	microRNA 454	1.68
MIR4484	microRNA 4484	1.61

Natural Antisense Transcripts (NATs) are group of long non-coding RNAs (lncRNAs) transcribed from the opposite strands of protein-coding genes by RNA polymerase II (Hany *et al.,* 2017). They are reported to interfere with the expression of their corresponding sense transcript (Hany *et al.,* 2017). They have been implicated in a wide variety of biological and pathological processes, including tumorigenesis and oncogenic progression (Krappinger *et al.,* 2021). The expression of 76 NATs was found to be deregulated in cells overexpression SMEK1 compared to cells transfected with pcDNA3.1 and some of these have been implicated in oncogenesis and had their expression perturbed in many cancers. For example, the top up-regulated NAT, PXN-AS1 (1.5 fold increase) has been attributed a dual role, depending on the cancer type (Yan *et al.,* 2019). PITPNA-AS1 (PITPNA antisense RNA 1) was the most down-regulated gene in cells overexpressing SMEK1 (1.5 Fold decrease). PITPNA-AS1 have been

shown to increase proliferation and migration of lung squamous cell carcinoma cells by stabilize the oncogene High mobility group box 3 (HMGB3) (Ren e*t al.,* 2020).

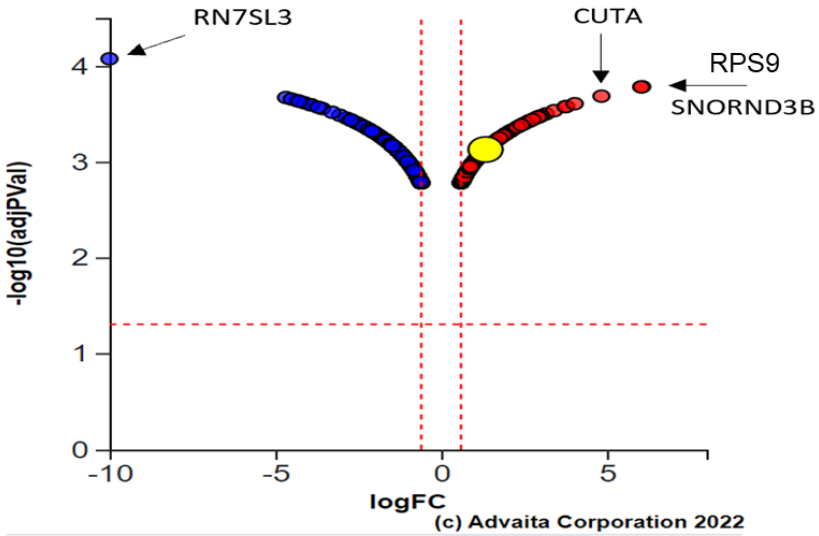
Long intergenic RNAs (lincRNAs) are a class of transcribed lncRNAs that is known to be enriched in the nucleus (Tuck et al.,2018). The expression of 30 lincRNAs were found to be deregulated in the cells overexpressing SMEK1. The function of the differentially expressed lincRNAs is not established. However, it is well known that most of lincRNAs regulate gene expression and this regulation might occur in cis involving neighbouring genes or in trans involving distant genes on another chromosomes (Tuck *et al.,* 2018). Another class of long non-coding RNAs is Intronic RNAs that have been reported to play an important role in inflammation and immune response (Laurent *et al.,* 2012). Twenty intronic RNAs have shown perturbed expression in the cells with increased SMEK1 expression highlighting the effects of SMEK1 on the expression of different classes of long non-coding RNAs.

Pseudogenes are defined as sequences of DNA that have high homology to known functional genes but are not coded into proteins. Emerging evidence is implicating a growing number of these pseudogenes in important biological roles and pathogenesis. An excess of 150 pseudogenes were differentially expressed in the cells overexpressing SMEK1. These pseudogenes include 27 different ribosomal proteins pseudogenes, six mitochondrial ribosomal proteins pseudogenes, 46 RNA,7SL (RN7SL) pseudogenes, RNA polymerase II subunit K pseudogene 1, DNA polymerase eta pseudogene, Ras homolog family member Q pseudogene 2 and Ras homolog family member T1 pseudogene 1.

5.3.2 Effects of SMEK2 on global gene expression as determined by RNA sequencing

The effects of increased expression of SMEK2 on gene expression in Jurkat T cells were also investigated. RNA sequencing of cells transfected with SMEK2 have shown significant changes in the transcriptome compared to cells transfected with pcDNA3.1. Transfection of SMEK2 into Jurkat T cells resulted in a 1.34 $\log_2$ fold of expression in SMEK2 RNA levels which is equivalent of 2.5 fold increase (Figure 5.7). There was no change in the expression of SMEK1, confirming that SMEK2 overexpression increased the expression levels of SMEK2 specifically. The increased levels of SMEK2 did not affect PP4c or its regulatory subunits apart from PPP4R4 which displayed 1.5 fold decrease in its RNA expression levels. The increase in SMEK2 expression was associated with 1.8-fold decrease in protein phosphatase 1 regulatory subunits PPP1R18 expression levels and 1.5-fold increase in the RNA levels of PPP1R11.

Analysis of the RNA sequencing data was carried similar to the analysis of SMEK1. A total of 1651 genes were found to be differentially expressed out of 17199 genes with measured expression. Similar to SMEK1 data, the SMEK2 RNA sequencing data were further analysed in the context of pathways obtained from the Kyoto 156, Encyclopedia of Genes and Genomes (KEGG) database (KEGG: 05206), gene ontologies from the Gene Ontology Consortium database, and miRNAs from the miRBase and TARGETSCAN (Targetscan version: 7.1) databases.



Symbol	Entrez	LogFC	p-value
PPP	Search entr		
PPP4R3B	57223	▲1.338	7.466e-4

Figure 5.7 Visualization of RNA-Sequencing results using Volcano Plot. The dots represent the differentially expressed genes. The horizontal axis is the log fold change, and the vertical axis is the negative base -10 logarithm of the p-value. The red-dotted lines represent the threshold used to select the DE genes: Log FC 0.6 for expression change and 0.05 for significance. The upregulated genes (positive log fold) are shown in red, while the down-regulated genes are blue. Ribosomal protein 9 (RPS9), Small nucleolar RNA, C/D box 3B (snoRND3A) and CutA Divalent Cation Tolerance Homolog (CUTA) were the most highly upregulated genes while RNA component of signal recognition particle 3 (RN7SL3) was the most down-regulated gene. The figure also shows the increase in SMEK2/PPP4R3B expression levels as a result of pcDNA3.1-SMEK2 transfection (Advaita 2022).

Top up-regulated genes included Small nucleolar RNA, C/S box 3B-1 (SNORD3B-1) and Ribosomal protein S9 (RSP9) both showed 6-fold increase in the RNA expression levels compared to control. Both genes showed same levels of increased expression in the cells transfected with SMEK1. Other upregulated genes in the cells overexpressing SMEK2 include CUTA which showed 4.8 fold increase compared to 4.2 fold increase caused by SMEK1 overexpression. The expression of translocase of outer mitochondrial membrane 6 (TOMM6) was shown to be increased by 4-fold similar to SMEK1. TOMM6 is a part of the mitochondrial

translocase of the outer membrane (TOM) complex responsible for the bulk of mitochondrial protein import (Pitt and Buchanan, 2021). Interestingly, similar to SMEK1, SIN3-HDAC complex associated factor (SINHCAF3) which is involved in the repression of genes involved in the TGFb signalling pathway (Charles *et al.,*2020) was also upregulated. Other upregulated genes included, gasdemin D (GSDMD), NADH ubiquinone oxidoreductase subunit A6 (NDUFA6) a subunit of NADH dehydrogenase (ubiquinone) involved in electron transport chain, myelin transcription factor 1 (MYT1), G protein signalling modulator 3 (GPSM3) which is linked to autoimmune diseases and transcription factor 19 (TCF19) associated with type I and II diabetes (Table 5.10).

Table 5.10 - Top ten up-regulated genes in Jurkat cells overexpressing SMEK2

Gene Symbol	Gene name	Function	Fold change
RPS9	Ribosomal protein S9	This gene encodes a ribosomal protein that is a component of the 40S subunit.	6.06
SNORD3B-1	Small nucleolar RNA, C/D box 3B-1	SNORD3B-1 (Small Nucleolar RNA, C/D Box 3B-1) is a C/D snoRNA involved in ribosomal RNA modification	6.03
CUTA	CutA divalent cation tolerance homolog	Protein product enables enzyme binding activity. Involved in protein localization. Located in membrane.	4.84
TOMM6	Translocase of outer mitochondrial membrane 6	Protein product is part of mitochondrial protein import machinery, TOM complex.	4.03
SINHCAF	SIN3-HDAC complex associated factor	Protein product functions by deacetylating histones, condensing chromatin, and modulating gene expression.	3.77
GSDMD	Gasdermin D	Gasdermin D is a member of the gasdermin family. Protein product plays a role in regulation of epithelial proliferation and have been suggested to act as a tumour suppressor or oncogene.	3.75
NDUFA6	NADH:ubiquinone oxidoreductase subunit A6	The encoded protein is an accessory subunit of NADH: ubiquinone oxidorerductase (Complex I), which is the largest enzyme of the mitochondrial membrane respiratory chain.	3.41
MYT1	Myelin transcription factor 1	The protein encoded by this gene is a member of a family of neural specific, zinc finger-containing DNA-binding proteins. The protein plays a role in the developing nervous system.	3.16
GPSM3	G protein signalling modulator 3	Protein product regulates G protein activity and is involved in positive regulation of inflammatory response.	3.01
TCF19	Transcription factor 19	Transcription factor 19 (TCF19) has been reported as a type 1 diabetes-associated locus involved in maintenance of pancreatic β cells through a fine-tuned regulation of cell proliferation and apoptosis.	2.95

Information regarding gene function were adapted from iPathwayGuide Advaita (2022).

The top down-regulated gene in the cells overexpressing SMEK2 was RNA component of signal recognition particle 7SL3 (RN7SL3) which showed 10-fold decrease compared to control. Interestingly, the expression of RN7SL3 was not downregulated in SMEK1 overexpressing cells, instead it showed 0.9 fold increase in its expression, highlighting a significant difference in the effects of SMEK1 and SMEK2 on Jurkat T cells. Table 5.11 shows the top ten down-regulated genes as a result of SMEK2 overexpression. There was 5-fold decrease in Lymphocyte antigen 6 family member E (LY6E) expression which is reported to be overexpressed in gastric cancer and to promote cancer cell growth and metastasis (Lv et al.,2018). Similar to SMEK1 effects, SMEK2 overexpression was associated with a 4-fold decrease in Rho GTPase activating protein 11A (ARHGAP11A). It functions as oncogene and act as prognostic biomarker in gastric cancer (Biao et al.,2021). Ubiquitin protein ligase E3 component n-recognin 7 (UBR7) was also down-regulated (3.9 fold decrease). UBR7 is transcriptional target of NOTCH1 and overexpressed in NOTCH1-driven T cell acute lymphoblastic leukaemia (T-ALL) (SRIVASTAVA et al.,2021). Two genes also reported to promote survival showed decrease expression compared to control which include interferon alpha inducible protein 27 like protein 1 (1IFI27L1) and Allograft inflammatory factor 1 (Yang *et al.,* 2005). Similar to SMEK1, SMEK2 overexpression also led to a 2.8-fold decrease in the expression levels of casein kinase 2 beta (CSNK2B), the regulatory subunit of CK2.

Table 5.11 - Top ten down-regulated genes in Jurkat cells overexpressing SMEK2

Gene Symbol	Gene name	Function	Fold change
RN7SL3	RNA component of signal recognition particle 7SL3	The signal recognition particle (SRP) is a cytoplasmic ribonucleoprotein complex and consists of 6 polypeptides (e.g., SRP19; MIM 182175) and a 7SL RNA molecule, such as RN7SL3.	10
LY6E	Lymphocyte antigen 6 family member E	Human LY6 genes represent novel biomarkers for poor cancer prognosis and are required for cancer progression in addition to playing an important role in immune escape.	4.68
HSPE1-MOB4	HSPE1-MOB4 readthrough	This locus represents naturally occurring read-through transcription between the neighbouring HSPE1 (heat shock 10kDa protein 1 (chaperonin 10) and MOB4 (MOB family member 4) genes on chromosome 2.	4.50
CLIC1	Chloride intracellular channel 1	Protein product is a member of the p64 family; the protein localizes principally to the cell nucleus and exhibits both nuclear and plasma membrane chloride ion channel activity.	4.34
NDUFC2-KCTD14	NDUFC2-KCTD14 readthrough	This locus represents naturally occurring read-through transcription between the neighboring NDUFC2 (NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2, 14.5kDa) and KCTD14 (potassium channel tetramerisation domain containing 14) genes on chromosome 11.	4.28
ARHGAP11A	Rho GTPase activating protein 11A	This gene encodes a member of the Rho GTPase activating protein family. Overexpressed in a number of tumours and may act as an oncogene.	4.12
UBR7	Ubiquitin protein ligase E3 component n-recognin 7	The encoded protein recognizes N-degrons, the destabilizing N-terminal residues of short-lived proteins, which results in ubiquitinylation, and proteolysis via the proteasome.	3.94
RNH1	Ribonuclease/angiogenin inhibitor 1	RNase inhibitor binds to both intracellular and extracellular RNases. In addition to control of intracellular RNases, RNH1 may have a role in the regulation of angiogenin also known as ribonuclease 5.	3.91
IFI27L1	Interferon alpha inducible protein 27 like 1	Belongs to a family of small, interferon alpha-inducible genes. The specific function of IFI27 is still unclear but evidence emerging that it functions as oncogene in different cancer.	3.7
AIF1	Allograft inflammatory factor 1	This gene encodes a protein that binds actin and calcium and may promote macrophage activation and growth of vascular smooth muscle cells and T-lymphocytes.	3.59

Information regarding gene function were adapted from iPathwayGuide Advaita (2022).

Individual genes that were identified as being deregulated by at least 1.5-fold were condensed into common biological processes and pathways for further interpretation. Pathway analysis revealed that several cellular processes were affected by the overexpression of SMEK2 with macromolecule metabolic process being the top process affected (Table 5.12). Pathway analysis revealed that several cellular processes were affected by the overexpression of SMEK2 with macromolecule metabolic process being the top process affected showing deregulated expression in 382 genes (Figure 5.8; Table 5.12). Among the perturbed cellular processes, numerous transcription related processes were significantly affected, including gene expression (Figure 5.9), RNA processing, negative regulation of gene expression, RNA processing, post-transcriptional regulation of gene expression, epigenetic regulation of gene expression, defense response, gene silencing by RNA and post-transcriptional gene silencing (Figure 5.10; Table 5.12).

Table 5.12 - Top ten perturbed Gene Ontology (GO) terms in response to SMEK2 overexpression in Jurkat T cells

Biological Process	Number of Gene (DE/ALL)
macromolecule metabolic process	**382** /7324
Gene expression	**292 /**4719
RNA Processing	**112/**1119
Negative regulation of gene expression	**73/**1795
Posttranscriptional regulation of gene expression	**73**/673
Regulation of gene expression-epigenetic	**67/**405
Défense response	**66 /**973
Gene silencing	**65**/269
Gene silencing by RNA	**63**/1119
Posttranscriptional gene silencing	**63**/255

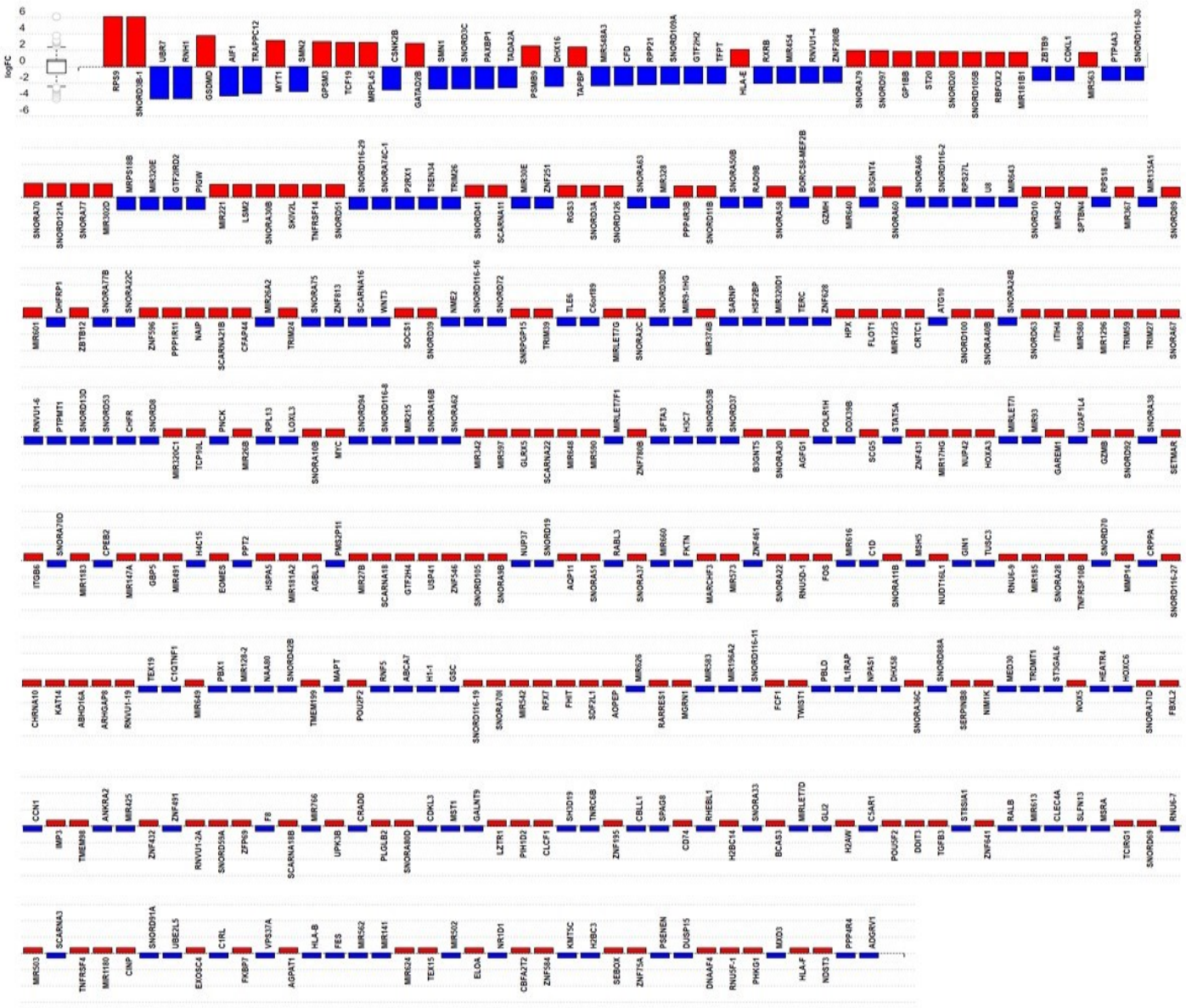


Figure 5.8 The expression of individual genes implicated in regulation of macromolecule metabolic process. Genes implicated in the biological process of gene expression, the top perturbed biological process as determined by sequencing and analysis of the whole transcriptome. Red coloured bars correspond to up-regulated gene expression, while blue coloured bars correspond to down-regulated gene expression. Data are the difference in expression between cells transfected with pcDNA3.1 and cells transfected with SMEK2, expressed as a normalised log2 fold change (log2FC). A threshold of 0.05 for statistical significance (p-value) and a log2 fold change of expression with an absolute value of at least 0.6 were applied.

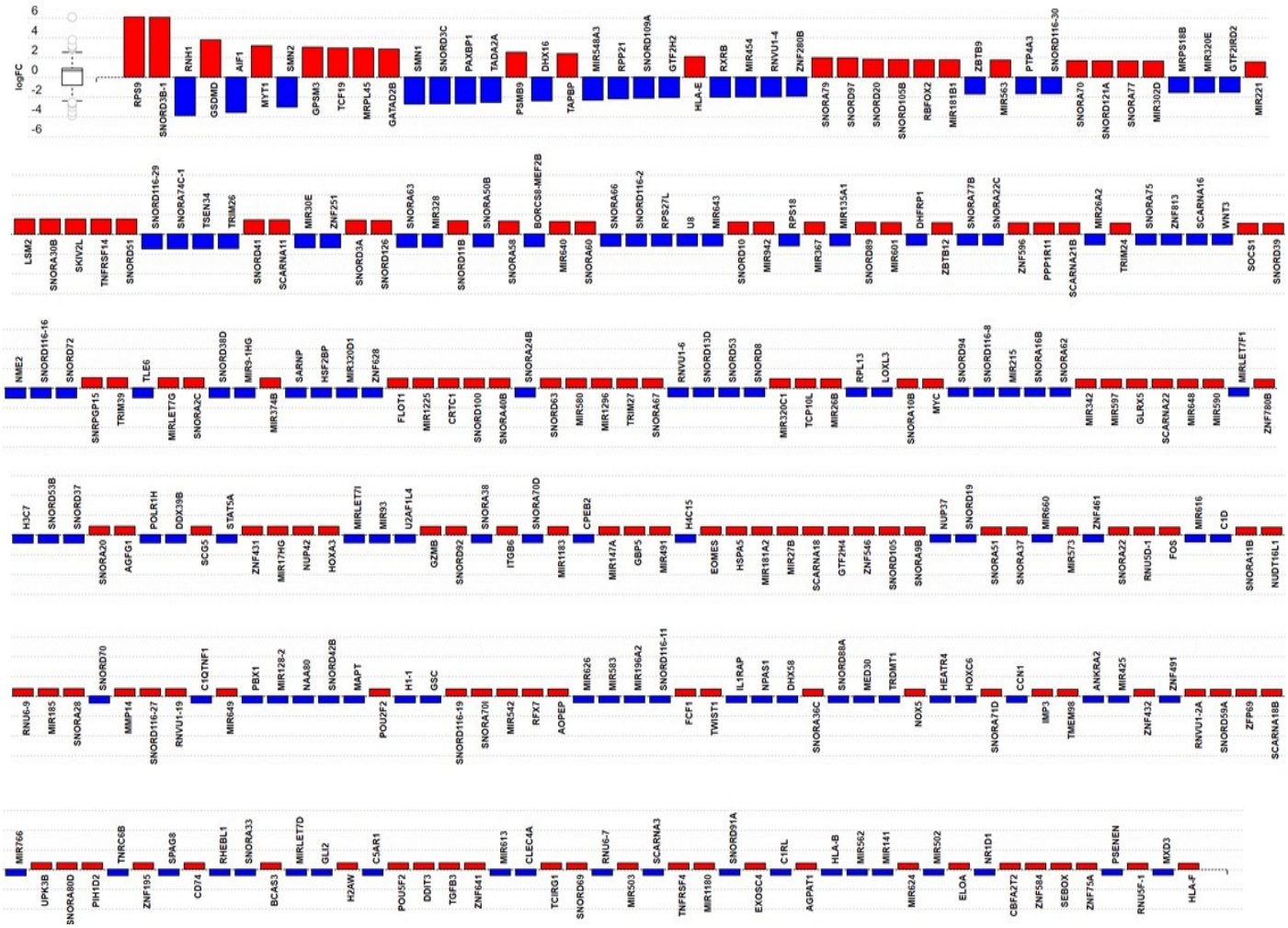


Figure 5.9 The expression of individual genes implicated in regulation of gene expression. Genes implicated in the biological process of gene expression, the top perturbed biological process as determined by sequencing and analysis of the whole transcriptome. Red coloured bars correspond to up-regulated gene expression, while blue coloured bars correspond to down-regulated gene expression. Data are the difference in expression between cells transfected with pcDNA3.1 and cells transfected with SMEK2, expressed as a normalised log2 fold change (log2FC). A threshold of 0.05 for statistical significance (p-value) and a log2 fold change of expression with an absolute value of at least 0.6 were applied.

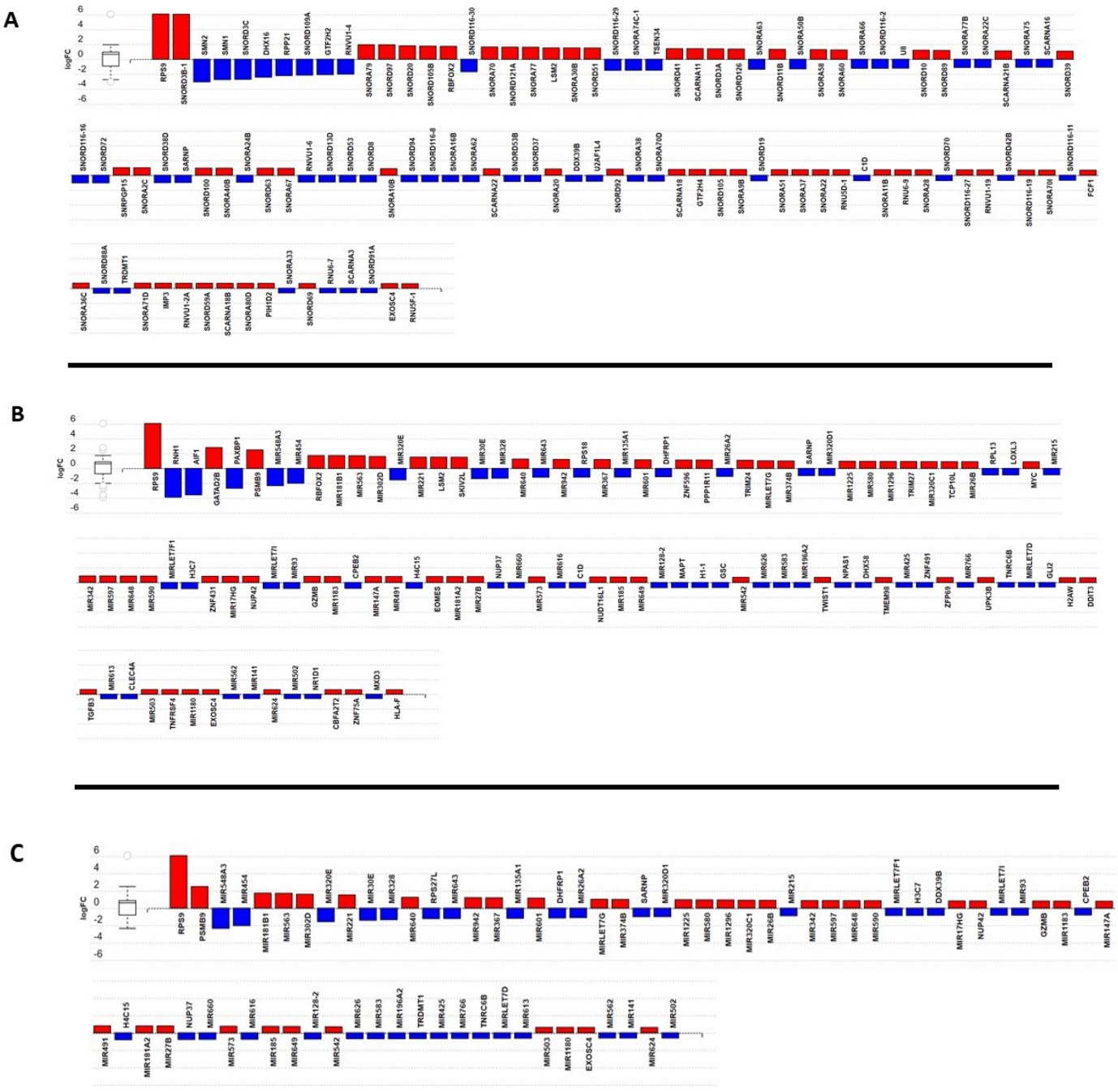


Figure 5.10 The expression of individual genes implicated in the RNA processing, negative regulation of gene expression and posttranscriptional regulation of gene expression. Genes implicated in the biological process of RNA processing (A), negative gene expression (B) and posttranscriptional regulation of gene expression (C), as determined by sequencing and analysis of the whole transcriptome. Red coloured bars correspond to up-regulated gene expression, while blue coloured bars correspond to down-regulated gene expression. Data are the difference in expression between cells transfected with pcDNA3.1 and cells transfected with SMEK2, expressed as a normalised log2 fold change (log2FC). A threshold of 0.05 for statistical significance (p-value) and a log2 fold change of expression with an absolute value of at least 0.6 were applied.

In addition, 90 Gene Ontology (GO) terms were significantly enriched in multiple molecular functions including signalling receptor binding, transporter activity, transmembrane transporter activity, signalling receptor activity mRNA binding in posttranscriptional gene silencing and ion channel activity (Table 5.13).

Table 5.13 Top ten Gene Ontology (GO) terms (Molecular functions) in response to SMEK2 overexpression in Jurkat T cells

Molecular function	Number of Gene (DE/ALL)
Signalling receptor binding	**46/**890
Transporter activity	**36/**666
Transmembrane transporter activity	**34/**571
mRNA binding	**30/**304
Molecular transducer activity	**26/**444
Signalling receptor activity	**26/**444
mRNA binding involved in posttranscriptional Gene silencing	**30/**304
RNA binding involved in posttranscriptional gene silencing	**26/**52
Inorganic molecular entity transmembrane transporter activity	**24**/419
Transmembrane signalling receptor activity	**20/**322

5.3.2.1 Impacted biological pathways in Jurkat T cells overexpressing SMEK2

An excess of 200 biological pathways was affected by overexpression of SMEK2. These included cancer independent biological pathways and cancer related processes. Cancer independent pathways include Herpes simplex virus 1 infection, RNA transport, pathways of neurodegeneration multiple diseases, Amyotrophic lateral sclerosis, Alzheimer disease, Huntington disease and prion disease. Cancer related pathways included microRNAs in cancer, pathways in cancer, Human T-cell leukaemia virus 1 infection, gastric cancer, colorectal cancer and transcriptional misregulation in cancer. Perturbed signalling pathways related to cancer includes pathways in cancer, MAPK, PI3K/AKT, RAS, HIPPO and wingless/Integrated **(**WNT) and peroxisome-proliferator-activated receptors (PPAR) (Table

5.14). Differentially expressed genes that impacted the cancer related pathways are summarised in Table 5.15. These genes include MYC, F***os*** Proto-Oncogene, MAPT (microtubule associated protein tau, CSNK2B (casein kinase 2 beta), WNT3 (Wnt family member 3), TGFb3 (transforming growth factor beta 3), ITGB6 (Integrin subunit beta 6) and RXRB (retinoid X receptor beta). TERC (telomerase RNA component), STAT5 and RALB (RAS like proto-oncogene B).

Table 5.14 Perturbed cancer related signalling pathways in cells overexpressing SMEK2

Pathway name	**Number of genes (Differentially Expressed/**All**)**	**Gene Symbol (Upregulated)**	**Gene Symbol (Down-regulated)**
Pathways in cancer	**11**/351	MYC, FOS, TGFB3, GSTM2	RXRB, WNT3, TREC, STAT5, GNG6, GLI2, RALB
MAPK signalling pathway	**9**/199	PLA2G4B, MYC, FOS, CACNA1A, DDIT3, TGFb3	JMJD7-PLA2G4B, MAPT, IL1RAP
PI3K/AKT signalling pathway	**5**/206	MYC, TNXB, ITGB6	GNG8, COL9A2
RAS signalling	**4**/146	PLA2G4B	JMJD7-PLA2G4B, GNG8, RALB
Hippo signalling pathway	**4**/107	MYC, TGFb3	WNT3, GLI2
WNT signalling pathway	**3**/103	MYC	CSNK2B, WNT3
PPAR	**2**/41	FABP6	RXRB

Table 5.15 Cells overexpressing SMEK1 and impact on gene expression in cancer pathways

Gene Symbol	Name of Gene	Fold-increase	Fold-decrease	Hallmark of Cancer
COMMD3-BMI1	COMMD3-BMI1 readthrough		1.253	A gene fusion between a transcription repressor (BMI1) and transcriptional factor (COMMD3). Promotes proliferation and significantly increased in prostate cancer.
WNT3	Wnt family member 3		1.085	Sustains proliferative activating invasion and metastasis, resisting program cell death.
SOCS1	suppressor of cytokine signalling 1	1.082		Supress Inflammation.
MYC	MYC proto-oncogene	0.893		Sustains proliferative activating. Genome instability Inhibits programmed cell death.
TNXB	Tenascin XB	0.816		Promotes proliferation. Oncogene.
DDIT3	DNA damage inducible transcript 3	0.637		Promotes apoptosis.
RXRB	Retinoid X receptor beta		2.047	Regulates gene expression and cell proliferation.
GZMB	Granzyme B	0.82		Promotes cell death.
H3C7	H3 clustered histone 7		0.854	Regulate DNA replication and cell cycle.
STAT5A	Signal transducer and activator of transcription 5A		0.833	Prevents metastatic dissemination. Enhanced expression and activity in peripheral T-cell lymphoma.
FHIT	Fragile histidine triad diadenosine triphosphatase	0.692		Tumour suppressor, as loss of its activity results in replication stress and DNA damage.
PLA2G4B	Phospholipase A2 group IVB	1.006		Regulates cell death.
JMJD7-PLA2G4B	JMJD7-PLA2G4B readthrough		0.842	An oncogenic fusion gene. Promotes proliferation.
FOS	Fos proto-oncogene, AP-1 transcription factor subunit	0.747		Regulator of cell proliferation, differentiation, and transformation.
RALB	RAS like proto-oncogene B		0.635	Promotes proliferation
CSNK2B	Casein kinase 2 beta		2.848	Contributes to tumour development, proliferation, and suppression of apoptosis.
NUDT16L1	Nudix hydrolase 16 like 1	0.740		Oncogene.
TWIST1	Twist family bHLH transcription factor 1	0.688		Promotes tumour cell invasion and metastasis, as well as metastatic recurrence.

GSTM3	Glutathione S-transferase mu 2	0.615		Tumour suppressor.
GLI2	GLI family zinc finger 2		0.638	Transcription factor. Promotes proliferation.
TERC	Telomerase RNA component		0.9	RNA component of Telomerase. Replicative immortality

Information regarding gene function were adapted from iPathwayGuide Advaita (2022).

Interestingly, overexpression of SMEK2 was associated in an increase expression of both oncogenes and tumour suppressors genes. Overexpressed oncogenes include the transcription factors MYC, TFC19, FOS and TWIST1 (Table 5.15). Tumour suppressors with increased expression in SMEK2 cells include GZMB, SOCS1 and GSTM2 (Table 5.15). On the other hand, SMEK2 overexpression was associated with the decreased expression of known oncogenes such as RALB, CSNK2B, GLI2, RXRB and the oncogenic fusion JMJD7-PLA2G4B (Table 5.15).

Of the 250 impacted pathways, the top nine affected pathways are shown in Figure 5.11, and they include microRNAs in cancer, graft-versus-host disease, allograft rejection, autoimmune thyroid disease, type I diabetes mellitus, PPAR signalling pathway, mRNA surveillance pathway and antigen processing and presentation in the cells overexpressing SMEK2.

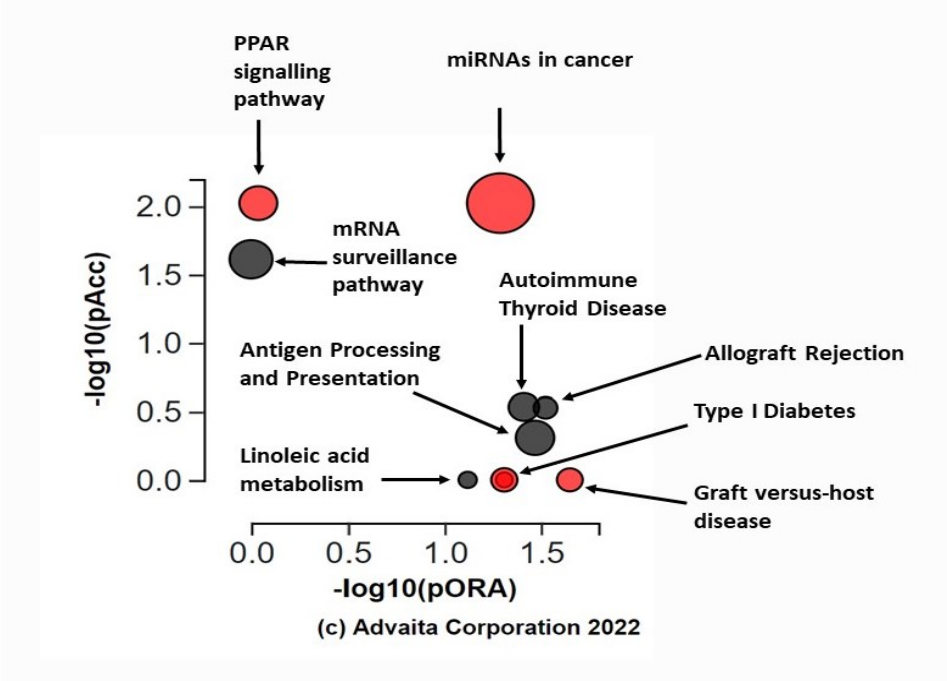


Figure 5.11 Perturbed pathways in Jurkat T cells overexpression SMEK2. Perturbation vs over-representation: Top nine most significantly impacted pathways are shown, using negative log of the accumulation and over-representation p-values, along with the other most significant pathways (red circles). Pathways in red are significant, based on the combined uncorrected p-values. The size of each dot donates the total number of genes in the corresponding pathway.

MicroRNAs in cancer was the top perturbed biological pathway with 22 genes differentially expressed in the cells overexpressing SMEK2 (Figure 5.11). These genes include 17 miRNAs, MYC, WNT3 and COMMD3-BMI, SOCS1 and TNXB with all of them reported to be involved in the regulation of different aspects of the hallmarks of cancer (Table 5.16). Eight differentially expressed genes impacted antigen processing and presentation (Figure 5.13). These include TAP binding proteins TAPBP and TAP2, major histocompatibility complex HLA-E, HLA-B and HLA-F, heat shock protein family A (Hsp70) member 5 (HSPA5), `CD8B and CD74. Four differentially genes impacted graft-versus-host disease, allograft rejection, autoimmune thyroid disease and type I diabetes mellitus pathways. These genes include major histocompatibility complex genes (HLA): HLA-B, HLA-E and HLA-F and granzyme B (GZMB).

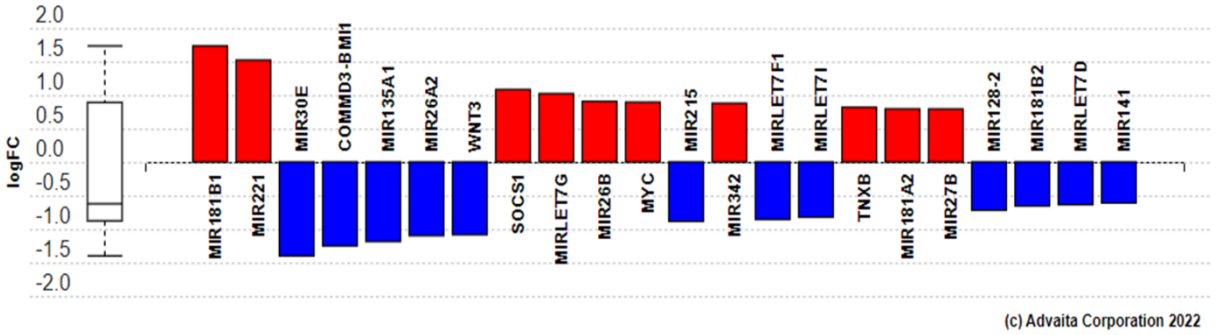


Figure 5.12 The expression of individual genes in microRNAs in cancer pathway. The graph shows the expression of the 22 differentially expressed genes in microRNAs in cancer pathway, as determined by sequencing and analysis of the whole transcriptome. Red coloured bars correspond to up-regulated gene expression, while blue coloured bars correspond to down-regulated gene. Data are the difference in expression between cells transfected with pcDNA3.1 and cells transfected with SMEK2. A threshold of 0.05 for statistical significance (p-value) and a log2 fold change of expression with an absolute value of at least 0.6 were applied.

Table 5.16 Differentially expressed genes in the top perturbed pathway, microRNAs in cancer and their biological relevance in cancer

Gene Symbol	Name of Gene	Fold-increase	Fold-decrease	Hallmark of Cancer
MIR181B1	microRNA 181b-1	1.732		Activating invasion and metastasis, inhibits cell death.
MIR221	microRNA 221	1.521		Activating invasion and metastasis, inhibits cell death.
MIR30E	microRNA 30e		1.402	Suppresses proliferation, migration and invasion, Promotes apoptosis.
COMMD3-BMI1	COMMD3-BMI1 readthrough		1.253	Oncogenic fusion. Promotes proliferation
MIR135A1	microRNA 135a-1		1.187	improves proliferation, and promotes migration and invasion
MIR26A2	microRNA 26a-2		1.102	Promotes cell survival and proliferation.
WNT3	Wnt family member 3		1.085	Sustain proliferative activating invasion and metastasis, resisting program cell death.
SOCS1	Suppressor of cytokine signalling 1	1.082		Avoiding immune destruction.
MIRLET7G	microRNA let-7g	1.020		Tumour suppressor
MIR26B	microRNA 26b	0.905		Tumour suppressor- Inhibits proliferation
MYC	MYC proto-oncogene,	0.893		Sustain proliferative signalling. Promotes genome instability and mutation.
MIR215	microRNA 215		0.887	Mediates repression of cell cycle
MIR342	microRNA 342	0.875		Tumour suppressor
MIRLEFT7F1	microRNA let-7f1		0.858	Activating invasion and metastasis.
MIRLET7I	microRNA let-7I		0.824	Tumour suppressor
TNXB	tenascin XB	0.816		Oncogene
MIR181A2	microRNA 181a-2	0.792		Activating invasion and metastasis, inhibits cell death
MIR27B	microRNA 27b	0.790		Inhibits cell proliferation and invasion
MIR128-2	microRNA 128-2		0.721	Regulates apoptosis
MIR181B2	microRNA 181b-2		0.657	Regulates cell proliferation and migration
MIRLET7D	microRNA let-7d		0.638	Regulates cell proliferation
MIR141	microRNA 141		0.612	Invasion and metastasis

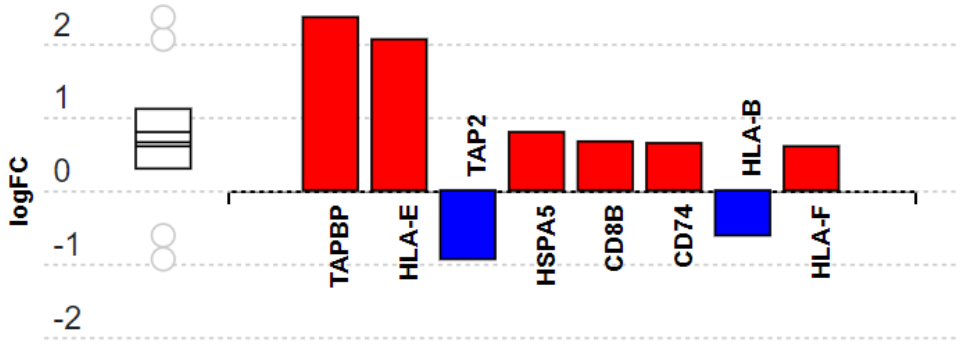


Figure 5.13 Differentially expressed pathway genes in antigen processing and presentation The graph shows the expression of the 22 differentially expressed genes in antigen processing and presentation, as determined by sequencing and analysis of the whole transcriptome. Red coloured bars correspond to up-regulated gene expression, while blue coloured bars correspond to down-regulated gene. Data are the difference in expression between cells transfected with pcDNA3.1 and cells transfected with SMEK2. A threshold of 0.05 for statistical significance (p-value) and a log2 fold change of expression with an absolute value of at least 0.6 were applied.

5.3.2.2 SMEK2 overexpression is associated with perturbations of the non-coding repertoires in Jurkat T cells

In addition to the perturbations in protein coding genes and pathways, RNA sequencing analysis revealed that SMEK2 overexpression also caused numerous and significant changes in expression of the non-coding elements of the transcriptome. A total of 85 snoRNAs and 224 miRNAs was predicted to be perturbed in response to SMEK2 overexpression (Table 5.17 and 5.18).

Table 5.17 Top ten differentially expressed small nucleolar RNAs in Jurkat cells overexpressing SMEK2

Gene symbol	Gene name	Fold-change
SNORD3B-1	small nucleolar RNA, C/D box 3B-1	6.031
SNORD116-9	small nucleolar RNA, C/D box 116-9	1.94
SNORD97	small nucleolar RNA, C/D box 97	1.79
SNORD20	small nucleolar RNA, C/D box 20	1.76
SNORD105B	small nucleolar RNA, C/D box 105B	1.62
		Fold decrease
SNORD3C	small nucleolar RNA, C/D box 3C	2.71
SNORD109A	small nucleolar RNA, C/D box 109A	2.15
SNORD116-30	small nucleolar RNA, C/D box 116-30	1.68
SNORD116-29	small nucleolar RNA, C/D box 116-29	1.51
SNORD116-2	small nucleolar RNA, C/D box 116-2	1.23

Table 5.18 Top ten differentially expressed microRNAs in Jurkat cells overexpressing SMEK2

Gene symbol	Gene name	Fold Increase
MIR4660	microRNA 4660	2.09
MIR6857	microRNA 6857	2.06
MIR6165	microRNA 6165	1.97
MIR4444-2	microRNA 4444-2	1.77
MIR1289-1	microRNA 1289-1	1.77
		Fold decrease
MIR548A3	microRNA 548a-3	2.35
MIR6801	MicroRNA 6801	2.08
MIR454	microRNA 454	2.02
MIR320E	microRNA 320e	1.57
MIR6842	microRNA 6842	1.55

Expression of long non-coding RNAs was also affected by the increase in SMEK2 expression level. The expression of 116 NATs was perturbed by SMEK 2 overexpression and some of these have been implicated in oncogenesis and immune response and had their expression perturbed in many cancers. For example, both PSMB8-AS1 and ITGB2-AS1 which showed 1.3-fold increase and 1.6-fold decrease respectively, have been described as biomarkers for pancreatic cancer (Fard et al.,2021). LncRNA PITPNA-AS1, down-regulated by 1.3-fold, is a potential diagnostic marker and therapeutic target and promotes hepatocellular carcinoma progression (Wang et al.,2021). NEXN-AS1 (nexilin F-actin binding protein antisense), another down-regulated gene by 1.3 fold, have been shown to increase the proliferation and migration of vascular smooth muscle cells (Wu et al.,2019). Interestingly, XIAP-AS1 (XIAP antisense RNA 1) also showed 1.3 fold decrease in its RNA level in SMEK2 overexpressing cells. XIAP-AS1 have been shown to promote XIAP transcription by interacting with the transcription Factor Sp1 in gastric cancer cells (Cai et al.,2017). Therefore, SMEK2 overexpression effects on reducing XIAP-AS1 expression levels could lead to the decrease in XIAP levels and the increase in apoptosis levels observed in SMEK2 overexpressing cells.

The expression of several lincRNAs and intronic transcripts were also affected in the cells with increased levels of SMEK2. 25 lincRNAs and 15 intronic RNA transcripts showed perturbed expression in SMEK2 overexpressing cells. An excess of 300 pseudogenes have showed differential expression in SMEK2 overexpressing cells. These include 30 ribosomal protein pseudogenes, 50 ribosomal RNA pseudogenes, 68 RNA,7SL (RN7SL) pseudogenes and 11 DNAJ Heat protein family pseudogenes have shown change in the expression in the cells overexpressing SMEK2, confirming that SMEK2 overexpression affect the gene expression of both protein coding and non-protein coding genes.

5.3.3 Meta-Analysis of differentially expressed genes in Jurkat cells overexpressing SMEK1 and SMEK2

iPathwayGuide platform was used to carry out a meta-analysis across SMEK1 and SMEK2 overexpression RNA expression datasets. The platform allows the identification of genes, pathways, miRNAs and GO terms that may be in common or unique across SMEK1 and SMEK2 data. The analysis showed that 638 genes were common differentially expressed genes in both SMEK1 and SMEK2 overexpressing cells. 874 genes were differentially expressed only in SMEK1 overexpressing cells and the expression of 1031 genes were SMEK2 specific (Figure 5.14). Table 5.19 and 5.20 list the top ten differentially expressed genes where the direction of gene expression changes was concordant in both SMEK1 and SMEK 2 overexpressing cells. Interestingly, Overexpression of both SMEK1 and SMEK2 led to an increase in the expression levels of transforming growth factor beta 3 (TGFβ3), the secreted ligand of the TGFβ receptor, implicating them both in TGFb signalling.

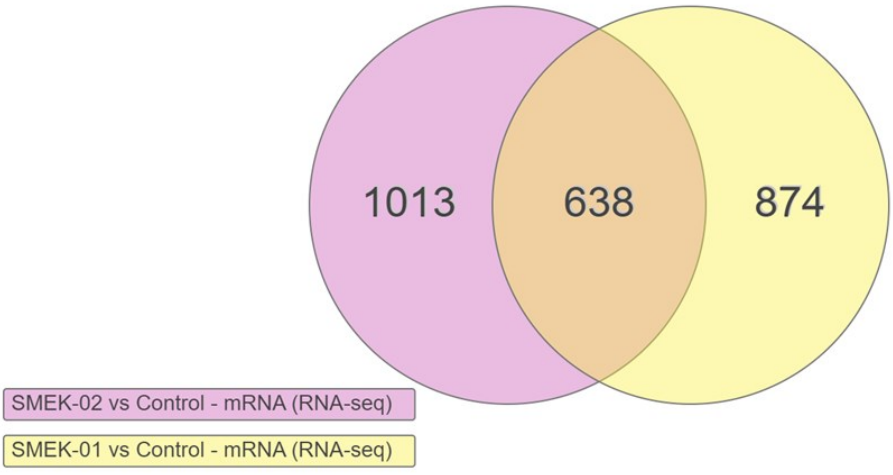


Figure 5.14 Meta-analysis gene summary. Venn diagram representing the intersections of differentially expressed gene sets associated to SMEK1 and SMEK2 RNA sequencing data. Pink shows the number of differentially expressed genes only in SMEK2 overexpressing cells, orange represents the number of differentially expressed genes in both SMEK1 and SMEK2 overexpressing cells, and yellow represent differentially expressed genes specific to SMEK1.

Table 5.19 Top ten up-regulated genes that showed similar changes in both SMEK1 and SMEK2 overexpressing cells.

Gene Symbol	Gene name	2-Fold change in SMEK2	Fold change in SMEK1
RPS9	ribosomal protein S9	6.063	5.853
SNORD3B-1	small nucleolar RNA, C/D box 3B-1	6.031	5.637
CUTA	cutA divalent cation tolerance homolog	4.835	4.245
TOMM6	translocase of outer mitochondrial membrane 6	4.029	3.855
SINHCAF	SIN3-HDAC complex associated factor	3.773	3.706
GSDMD	gasdermin D	3.748	3.748
NDUFA6	NADH:ubiquinone oxidoreductase subunit A6	3.408	3.473
MYT1	myelin transcription factor 1	3.157	3.293
GPSM3	G protein signalling modulator 3	3.011	3.151
GUSBP14	GUSB pseudogene 14	2.947	3.121

Table 5.20 Most down-regulated genes that showed similar changes in both SMEK1 and SMEK2 overexpressing cells.

Gene Symbol	Gene name	Fold change in SMEK2	Fold change in SMEK1
HSPE1-MOB4	HSPE1-MOB4 readthrough	4.504	4.504
NDUFC2-KCTD14	NDUFC2-KCTD14 readthrough	4.279	4.279
ARHGAP11A	Rho GTPase activating protein 11A	4.122	4.122
IFI27L1	interferon alpha inducible protein 27 like 1	3.705	3.705
SMN2	survival of motor neuron 2, centromeric	3.050	0.840
CSNK2B	casein kinase 2 beta	2.848	2.848
SMN1	survival of motor neuron 1, telomeric	2.741	2.741
SNORD3C	small nucleolar RNA, C/D box 3C	2.714	2.714
TADA2A	transcriptional adaptor 2A	2.578	2.578
MIR548A3	microRNA 548a-3	2.541	5.8

Among the 638 genes, 44 deregulated genes showed different direction of gene expression changes while the rest of gene expression changes was concordant in term the direction and the fold change apart from one snoRNA, SNORD3A. SONRD3A is the top upregulated gene is SMEk1 (9-fold increase) showed only 1.4-fold increase in SMEK2 overexpressing cells (Figure 5.15A). The most important observation is that most down-regulated gene in *RN7SL3* (10-fold decrease) in SMEK2 overexpressing cells showed 1-fold increase in cells with increased SMEK1 expression. These results are significant and implies that only SMEK2 overexpression leads to the significant down-regulation of the RNA component of signal recognition particle which mainly is involved in coupling the synthesis of nascent proteins to their proper secretory pathway and membrane localisation (Gencheva et al.,2010) (Figure 5.15B). Another gene showed different direction of gene expression was DHX16 (DEAH-box helicase 16), its protein product is required for pre-mRNA splicing (Gencheva *et al.,* 2010). The expression level of DHX16 was decreased by two folds in SMEK2 overexpression and increased by two folds on SMEK1 overexpressing cells (Figure 5.15C). RSP18 (Ribosomal protein S18) was also found to be down-regulated in SMEK2 by 1.2 fold and upregulated in SMEK2 overexpressing cells by 3.5 fold (Figure 5.15D). The expression of CFD (complement factor D), a serine protease that regulates a key step in the activation of the alternative complement pathway, is decreased by 2.3 fold in SMEK2 overexpressing cells and increased by 2.5 fold in SMEK2 overexpressing cells (Figure 5.15E). The expression of PSMB9 (Proteasome 20S subunit beta 9) was also not concordant displaying 2.5-fold increase in the cells with increased expression of SMEK2 and 2-fold decrease in SMEK1 overexpressing cells (Figure 5.15F). PSMB9 is part of the immunoproteasomes and its synthesis is driven by driven by interferon gamma (IFN-γ) via the JAK/STAT pathway (Morozov and Karpov, 2019).

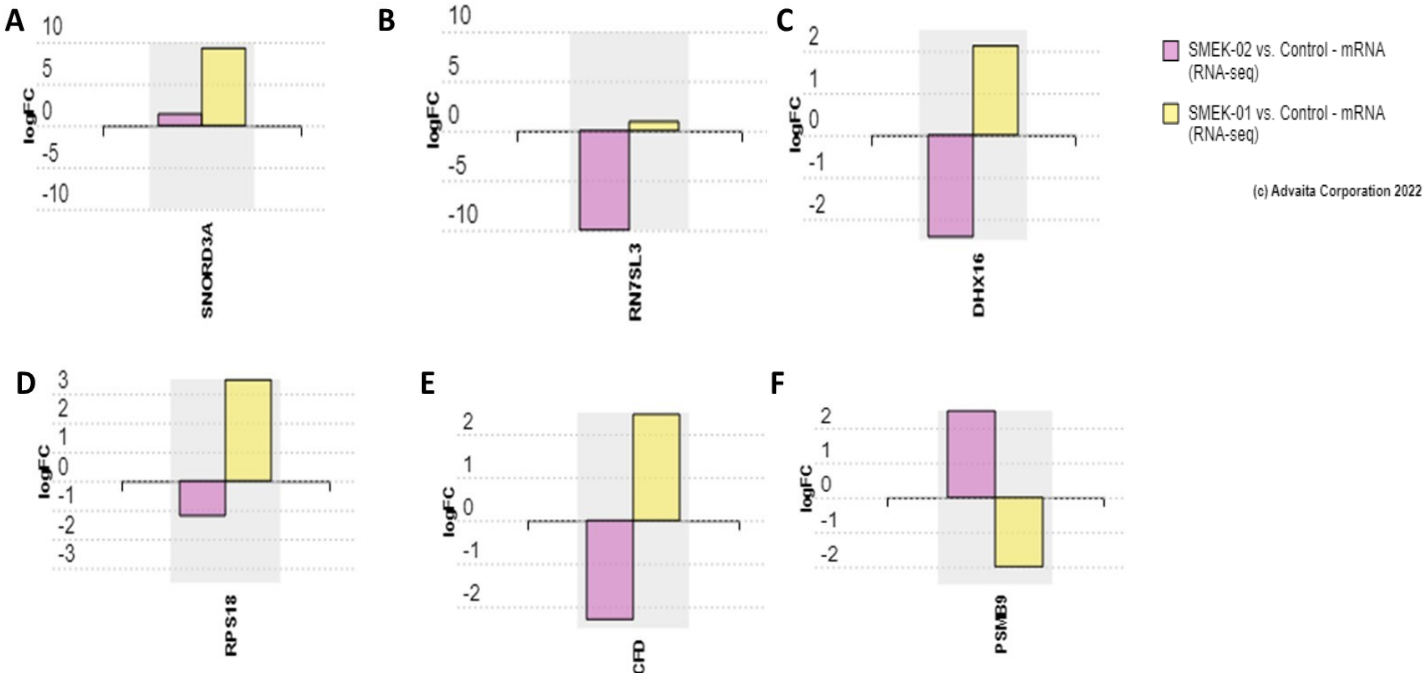


Figure 5.15 Differentially expressed genes that showed changes in the fold expression and in the different direction in Jurkat cells overexpressing SMEK2. Bar plot that displays the fold of the selected genes. Pink bar shows the expression levels in SMEK2 overexpressing cells and the yellow bar showed the expression levels in SMEK1 overexpressing cells. Expression change by contrast A) SNORDA3A (snoRNA D3A), B) RN7SL (RNA Component of Signal Recognition Particle 7SL), C) DHX16 (DEAH-box helicase 16), D) RPS19 (Ribosomal protein S18), E) CFD (Complement factor D) and E) PSBM9 (Proteasome 20S subunit beta 9.

Meta-analysis of GO term summary revealed that changes in 123 biological processes were common between SMEK1 and SMEK2, whereas 248 biological pathways were specific to SMEK1 and 191 specific to SMEK2 (Figure 5.16).

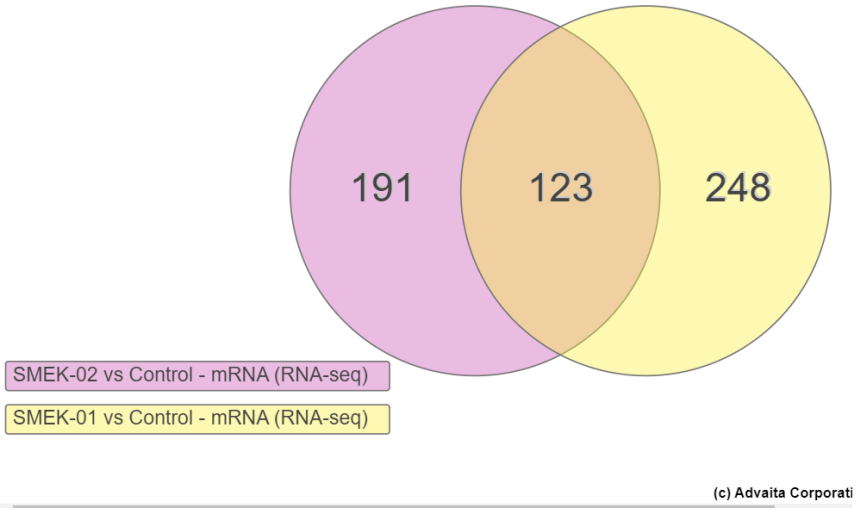


Figure 5.16 Meta-analysis GO terms summary. Venn diagram representing the intersections of GO terms associated to SMEK1 and SMEK2. Pink SMEK2 specific changes, yellow SMEK1 specific changes and orange common changes between SMEK1 and SMEK2.

Top common perturbed biological processes shared between SMEK1 and SMEK2 include gene, RNA processing expression, negative regulation of gene expression, posttranscriptional regulation of gene expression, epigenetic control of gene expression, defense response, gene silencing by RNA and miRNA, posttranscriptional gene silencing, regulation of cytokine production, adaptive immune response, inflammatory response and lymphocyte mediated immunity. Top common pathways affected by both SMEK1 and SMEK2 was miRNAs in cancer. Other common pathways include type I diabetes, antigen processing and presentation, graft-versus-host disease, allograft rejection and autoimmune thyroid disease.

5.3.3.1 Differentially expressed genes, perturbed biological pathways and molecular functions specific to SMEK1

Among the total 1512 genes that were differentially expressed in SMEK1 overexpressing cells, 874 showed SMEK1 specific changes. The top ten upregulated genes are listed in Table 5.21. These include two naturally occurring transcripts generated by read-through transcription between two neighbouring genes, RPS10-NUDT3 and URGCP-MRPS24. RPS10-NUDT3 represents naturally occurring read-through transcription between the neighbouring RPS10 (ribosomal protein S10) and NUDT3 (nudix (nucleoside diphosphate linked moiety X)-type motif 3) genes on chromosome 6. The read-through transcript produces a fusion protein that shares sequence identity with each individual gene product. The role of RPS10-NUDT3 protein product has not yet been identified, however, it was recently identified as one of the candidate genes that might play essential roles in obesity associated type 2 diabetes mellitus (Prashanth *et al.,* 2021). URGCP-MRPS24 represents naturally occurring read-through transcription between the neighboring URGCP (upregulator of cell proliferation) and MRPS24 (mitochondrial ribosomal protein S24) genes on chromosome 7. The read-through transcript is predicted to encode a protein that shares sequence identity with the upstream gene product but its C-terminal region is distinct due to a frameshift relative to the downstream gene. While the function of URGCP-MRPS24 has not yet been characterised, it was identified by computational analysis to be one of the hub genes related to the progression of type 1 diabetes by computational analysis (Prashanth *et al.,* 2021). The enzyme involved in NAD synthesis NAPRT was one of the SMEK1 specific differentially expressed genes (4-fold increase). Studies have shown that NAPRT plays a key role in inflammation (Duarte-Pereira *et al.,* 2021). Most normal tissues express NAPRT while a significant proportion of

malignant cells do not as epigenetic silencing of NAPRT have been detected in a number of cancers including glioma and lung cancer (Cole *et al.,* 2017). Other differentially expressed genes include a snoRNA (SNORA15B-1) and a pseudogene HSPE1P26. Among the top ten SMEK1 specific differentially expressed genes include SBNO2 involved in RNA processing and splicing and YTHDC1 and ZNF497 involved in regulation of transcription. RASSF7 was another SMEK1 specific genes that showed overexpression by 3.5-fold. RASSF7 belongs to the RASSF family of proteins that contain RAS domain, a domain of about 100 residues that interacts with Ras and other small GTPases. RASSF genes are frequently suppressed by DNA hypermethylation in human cancers (Prashanth *et al.,* 2021). RASSF7 specifically interact with core kinases of the tumour suppressor Hippo pathway (Iwasa *et al.,* 2018).

Top down-regulated SMEK1 specific genes, listed in Table 5.22, include PSMB3 which encode a subunit required for the assembly of 20S proteasome complex, a pivotal component for the ubiquitin-proteasome system (Zhang et al.,2017). In fact, SMEK1 overexpression resulted in perturbed expression of 4 different genes involved in proteasome assembly and ultimately proteasomal function. These include PSMB3, PSMB9, PSMB8 and PSMD9 (proteasome 26S subunit, non-ATPase 9).

Table 5.21 Top ten up-regulated genes that are specific to SMEK1

Gene Symbol	Gene name	Function	Fold change
RPS10-NUDT3	RPS10-NUDT3 readthrough	This locus represents naturally occurring read-through transcription between the neighboring RPS10 (ribosomal protein S10) and NUDT3 (nudix (nucleoside diphosphate linked moiety X)-type motif 3) genes on chromosome 6.	5.81
NAPRT	Nicotinate phosphoribosyltransferase	The protein product is an enzyme that catalyses the first and rate-limiting step in the biosynthesis of nicotinamide adenine dinucleotide (NAD) from nicotinamide. Endogenous ligand for TLR4 and a mediator of inflammation.	4.00
URGCP-MRPS24	URGCP-MRPS24 readthrough	This locus represents naturally occurring read-through transcription between the neighboring URGCP (upregulator of cell proliferation) and MRPS24 (mitochondrial ribosomal protein S24) genes on chromosome 7.	3.78
RASSF7	Ras association domain family member 7	Protein product has Ras association (RA) domain that can potentially interact with the Ras GTPase family of proteins. Predicted to be involved in apoptotic process; regulation of microtubule cytoskeleton organization; and signal transduction. Located in centriolar satellite.	3.47
ITPK1	inositol-tetrakisphosphate 1-kinase	Protein product is kinase that can phosphorylate various inositol polyphosphate such as Ins(3,4,5,6)P4 or Ins(1,3,4)P3.	2.75
SBNO2	strawberry notch homolog 2	Protein product act as a transcriptional coregulator, that can have both coactivator and corepressor functions. Involved in cellular response to IL^ and activation of macrophage activation involved in immune response	2.71
YTHDC1	YTH domain containing 1	Protein product is a regulator of mRNA alternative splicing and processing.	2.69
ZNF497	zinc finger protein 497	Its protein product is involved in transcriptional regulation.	2.44
SNORA15B-1	Small nucleolar RNA, H/ACA box 15B-1	modification, maturation, and stabilization of rRNA	2.07
HSPE1P26	heat shock protein family E (Hsp10) member 1 pseudogene 26	A pseudogene. Not known.	2.07

Information regarding gene function were adapted from iPathwayGuide Advaita (2022).

Another SMEK1 gene among the ten most down-regulated is GGNBP2 (Gametogenetin binding protein 2). A decreased expression of GGNBP2 is reported to regulate cell cycle, cell proliferation, invasion, and migration of prostate cancer PC-3 cells

(Yang *et al.,* 2020). CISD2 (CDGSH iron sulfur domain 2) is another SMEK1 specific down-regulated gene by 2.76-fold. CISD2 is reported to be highly expressed in several cancers and deficiency inhibits cell proliferation and induces cell differentiation of neuroblastoma (Li et al.,2019). BDP1 and GON7 are also SMEK1 specific down-regulated genes. BDP1 encodes a subunit of RNA polymerase III transcription initiation factor IIIB involved in transcription and mutations in its sequence is reported to be associated with lung, breast, and colorectal cancers (Li et al.,2019). GON7 is a subunit of KEOPS complex (Kinase, Endopeptidase and Other Proteins of Small size) and is reported to be required for tRNAs modifications (Beenstock *et al.,* 2021). Interestingly, SMEK1 overexpression was associated with a 3-fold decrease in HRAS expression level. HRAS is one of the three human RAS oncogenes, KRAS, NRAS, and HRAS which encode four different RAS proteins that belong to the protein family of small GTPases (Kessler *et al.,* 2019) involved in the regulation of cell growth. Activating mutations in RAS proteins are among the most common oncogenic drivers in human cancers, with KRAS being the most frequently mutated oncogene (Kessler *et al.,* 2019). The mitochondrial ribosomal protein S36, MRPS6, also showed decrease level in SMEK1 overexpressing cells by 2-fold. Another two mitochondrial ribosomal proteins genes showed perturbed expression by increased SMEK1 levels, MRPS16 and 17, highlight the role of SMEK1 in maintaining the functional integrity of the mitochondrial ribosome. More SMEK1 specific changes include perturbed expression of 33 snoRNAs, 100 miRNAs, more than 80 pseudogenes, 6 intronic lncRNAs, 40 NATs and 20 lincRNAs.

Table 5.22 Top ten down-regulated genes that are specific to SMEK1

Gene symbol	Gene name	Function	Fold change
PSMB3	Proteasome 20S subunit beta 3	One of the core subunits of the proteasome.	5.83
GGNBP2	Gametogenetin binding protein 2	Protein product involved in spermatogenesis . Knockdown of ZNF403 inhibits cell proliferation and induces G2/M arrest by modulating cell-cycle.	3.48
CXorf49	Chromosome X open reading frame 49	Its protein is a product of an open reading frame identified in various cell and tissue proteomes.	3.16
HRAS	HRAS proto-oncogene, GTPase	Protein product is oncogene that belongs to the Ras oncogene family whose members are related to the transforming genes of mammalian sarcoma retroviruses.	3.01
CHURC1-FNTB	CHURC1-FNTB readthrough	This locus represents naturally occurring read-through transcription between the neighbouring CHURC1 (churchill domain containing 1) and FNTB (farnesyltransferase, CAAX box, beta) on chromosome 14. Functional importance is unclear.	3.01
CISD3	CDGSH iron sulfur domain 3	CISD3 is a member of the CDGSH domain-containing family, which may play a role in regulating electron transport and oxidative phosphorylation.	2.83
CCDC92	Coiled-coil domain containing 92	Enables identical protein binding activity. Located in centriole; centrosome; and nucleoplasm.	2.76
GON7	GON7 subunit of KEOPS complex	Protein product is a subunit of KEOPS (Kinase, Endopeptidase and Other Proteins of Small size). Required for tRNAs modifications.	2.35
BDP1	B double prime 1, subunit of RNA polymerase III transcription initiation factor IIIB	Protein product is a subunit of the TFIIIB transcription initiation complex, which recruits RNA polymerase III to target promoters in order to initiate transcription.	2.34
MRPS36	Mitochondrial ribosomal protein S36	This gene encodes a 28S subunit protein of mitochondrial ribosomal protein.	2.14

Information regarding gene function were adapted from iPathwayGuide Advaita (2022).

Among the 248 perturbed biological processes that are SMEK1 specific, 110 perturbed processes were associated with one or two differentially genes whereas the other 138 biological processes were also affected by SMEK2 but involved different differentially

expressed genes. The same for molecular functions, the only SMEK1 specific perturbed molecular function was structural constituent of ribosome. All the impacted pathways were common in both SMEK1 and SMEK2 overexpression but involved another set of differentially expressed genes.

5.3.3.2 Differentially expressed genes, perturbed biological pathways and molecular functions specific to SMEK2

An excess of 1013 genes were differentially expressed specifically in the cells overexpressing SMEK2. The top ten SMEK2 specific upregulated genes are shown in Table 5.23. These include three proapoptotic genes GSDMD (Gasdermin D) (3.75-fold increase), MIR4660 (2-fold increase) and MIR6165 (2-fold increase). GSDMD is involved the process of pyroptosis, the process of inflammatory cell death (Burdette *et al.,* 2021). MIR44660 has been recently shown to inhibit osteosarcoma cells by binding to 3'UTR of the mRNA of the oncogene MAFG (v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog G) forms a heterodimer with Nrf2 (NF-E2-related factor 2) and inhibiting its expression (Shan *et al.,* 2021). MIR6165 has been shown to downregulate insulin growth factor-1 expression and promote apoptosis in the human colon adenocarcinoma cells SW480 (Hassanlou *et al.,* 2020) On the other hand, two antiapoptotic genes TFC19 (Transcription factor 19) showed 3- fold increase in SMEK2 overexpressing cells. An increased expression of TFC19 is associated with the occurrence of distant metastasis and poor prognosis of colorectal cancer (Du *et al.,* 2020) and to promote cell-cycle progression of non-small-cell lung cancer cells (Zhou *et al.,* 2019). Three SMEK2 differentially expressed genes included three pseudogenes. They include PANK4, an inactive form or pseudogene of the pantothenate kinase (Yao *et al.,* 2019), RNU6-

450P and RNU7-141P (Pseudogenes of U6 and U& RNA components of small nuclear ribonucleoproteins). Two snoRNAs, SNORNA79 and SNORD116 were also among the top ten differentially expressed genes in SMEK2 overexpressing cells. Another 50 snoRNAs were also differentially expressed in SME2 overexpressing cells and not in cells with increased expression of SMEK1.

Table 5.23 Top ten SMEK2 specific up-regulated genes in Jurkat cells

Gene symbol	Gene name	Function	Fold change
GSDMD	Gasdermin D	Gasdermin D is a member of the gasdermin family. Members of this family appear to play a role in regulation of epithelial proliferation. Gasdermin D has been suggested to act as a tumor suppressor.	3.75
TCF19	Transcription factor 19	This gene encodes a protein that contains a PHD-type zinc finger domain and likely functions as a transcription factor. The encoded protein plays a role proliferation and apoptosis of pancreatic beta cells.	2.93
MRPL45	Mitochondrial ribosomal protein L45	Mammalian mitochondrial ribosomal proteins are encoded by nuclear genes and help in protein synthesis within the mitochondrion.	2.93
PANK4	Pantothenate kinase 4	This gene encodes an inactive form of the pantothenate kinase enzyme. Pantothenate kinase is a key regulatory enzyme in the biosynthesis of coenzyme A (CoA).	2.66
RNU6-450P	RNA, U6 small nuclear 450, pseudogene	Inactive form of U6 snRNA. U6 snRNA forms much of the catalytic core of the spliceosome and is highly dynamic, interacting with three snRNAs, the pre-mRNA substrate, and >25 protein partners throughout the splicing cycle.	2.29
MIR4660	microRNA 4660	A novel miRNA reported to inhibit cell proliferation	2.09
MIR6165	microRNA 6165	Suppresses gastric cancer migration and invasion and enhances apoptosis.	1.97
RNU7-141P	RNA, U7 small nuclear 141 pseudogene	An inactive form of U7 RNA. U& RNA is an RNA molecule and a component of the small nuclear ribonucleoprotein complex (U7 snRNP). The U7 snRNA is required for histone pre-mRNA processing.	1.95
SNORA79	Small nucleolar RNA, H/ACA box 79	Belongs to the H/ACA box class of snoRNAs involved in ribosomal RNA modification.	1.94
SNORD116	Small nucleolar RNA, C/D box 116-9	Belongs to C/D box snoRNAs involved in ribosomal RNA modification.	1.94

Information regarding gene function were adapted from iPathwayGuide Advaita (2022).

The top ten down-regulated genes that are specific to SMEK2 includes three genes LY6E, CLIC1 and UBR, which are reported to have antiapoptotic and pro-survival activity. LY6E (Lymphocyte antigen 6 complex, locus E) is a member of the lymphostromal cell membrane Ly6 superfamily protein. Studies have shown that LY6E is a critical antiviral immune effector and overexpressed in gastric cancer (Lvy *et al.,* 2018). LY6E overexpression in gastric cancer cells promotes cancer cell growth and metastasis (Lvy *et al.,* 2018). CLIC1 is a membrane-targeted chloride intracellular channel. 1. CLIC1 is upregulated in human hepatocellular carcinoma (HCC) and associated with tumour invasiveness, metastasis, and poor prognosis (Giu *et al.,* 2020). Among the top ten down-regulated genes that are specific to SMEK2 includes LY6E, CLIC1 and UBR7. LY6E (Lymphocyte antigen 6 complex, locus E) is a member of the lymphostromal cell membrane Ly6 superfamily protein and have shown to be a critical antiviral immune effector and overexpressed in gastric cancer. LY6E overexpression in gastric cancer cells promotes cancer cell growth and metastasis (Lvy *et al.,* 2018). CLIC1 which showed 4.5-fold decrease in its expression, is a membrane-targeted chloride intracellular channel. 1. CLIC1 is upregulated in human hepatocellular carcinoma (HCC) and associated with tumour invasiveness, metastasis, and poor prognosis (Qiu *et al.,* 2021). CLIC1 also promotes the progression of oral squamous cell carcinoma via integrins/ERK pathway (Feng *et al.,* 2019). UBR7 (Ubiquitin protein ligase E3 component n-recognin 7) is as a transcriptional target of NOTCH1 and was shown to be overexpressed in NOTCH1-driven T cell acute lymphoblastic leukaemia (T-ALL) (Feng *et al.,* 2019; Srivastav *et al.,* 2021). Another SMEK2 specific down-regulated gene is RNH1 (ribonuclease inhibitor 1) (3.9-fold decrease), a part of the protein translation machinery and its role is to protect mRNAs from extracellular RNases. AIF1 (Allograft inflammatory factor 1) also showed more than 3.5-fold decreased expression, has been identified as a modulator of inflammatory response, a major player in

breast cancer progression by promoting crosstalk between breast cancer cells and infiltrating immune cells (Slim *et al.,* 2018) and recently published studies have shown its increased expression in hepatocellular and oesophageal carcinoma (Sarangdhar and Allam, 2021). TRAPPC12 was another SMEK2 down-regulated gene (3.3- fold). TRAPPC12 is a component of the Transport Protein Particle complexes (TRAPP) complex, regulator of membrane trafficking, and involved in endoplasmic reticulum to Golgi apparatus protein trafficking (Harries et al.,2021). Paxbp1 is SMEK2 specific down-regulated gene (2.7- fold) which was identified as a nuclear Pax7-binding protein. Loss of Paxbp1 in muscle cells induces apoptosis and lead to cell cycle arrest (Zhou et al.,2021). DENND11 (DENN domain containing 11) is another gene down-regulated in SMEK2 overexpressing cells by 2.55-fold. DENND11 is reported to promote the exchange of GDP to GTP leading to the conversion of inactive GDP-bound small GTPases into their active GTP-bound form (Zhou et al.,2021). TUBGCP5 (Tubulin gamma complex associated protein 5) was also found to be down-regulated only in SMEK2 overexpression cells (2.5-fold). The protein product of TUBGCP5 is a part of γ-Tubulin complex which is essential for microtubule nucleation and plays a role in cell-cycle-related functions (Chumova et al.,2018). It is worth noting that SMEK2 overexpression was associated with a decrease in STAT5 expression levels (0.9-fold). The results in chapter 4 have shown that STAT5 phosphorylation levels were reduced by SMEK2 overexpression not SMEK1 which implies that the reduction in phosphorylation levels was due to the reduction in the expression of STAT5. In addition, the meta-analysis also showed SMEK2 specific changes in genes encoding non-coding RNAs. These changes include the differential expression of 122 miRNAs, 50 snoRNAs, 75 NATs, 22 lincRNAs and more than 200 pseudogenes.

Among the 191 perturbed biological pathways that are specific to SMEK2 (Figure 5.16), only production of molecular mediator of immune response, immunoglobulin production,

immunoglobulin mediated immune response, positive regulation of immune effector process and regulation of endothelial cell migration showed perturbations that are specific to SMEK2. The rest of the pathways were also affected by SMEK1 but by different set of differentially expressed genes. Among the 54 perturbed molecular functions, only 4 showed SMEK2 specific changes death receptor activity, tumour necrosis factor-activated receptor activity, chaperone binding and MHC protein binding.

Table 5.24 Top ten SMEK2 specific down-regulated genes in Jurkat cells

Gene symbol	Gene name	Function	Fold change
LY6E	Lymphocyte antigen 6 family member E	This gene belongs to the human Ly6 gene family. Protein product is and encodes a glycosylphosphatidyl-inositol (GPI)-anchored cell surface protein. The protein plays an important role in T cell physiology, oncogenesis and immunological regulation.	4.68
CLIC1	Chloride intracellular channel 1	Protein product is chloride channel regulates cellular processes including stabilization of cell membrane potential, maintenance of intracellular pH, and regulation of cell volume.	4.34
UBR7	Ubiquitin protein ligase E3 component n-recognin 7	This protein product is involved in the pathway protein ubiquitination, which is part of protein modification.	3.93
RNH1	Ribonuclease inhibitor 1	Protein product is RNase inhibitor that occur in many tissues and bind to both intracellular and extracellular RNases	3.91
AIF1	Allograft inflammatory factor 1	This gene encodes a protein that binds actin and calcium. May promote macrophage activation and growth of vascular smooth muscle cells and T-lymphocytes.	3.59
TRAPPC12	Trafficking protein particle complex subunit 12	Protein product is involved in protein trafficking from endoplasmic reticulum to Golgi apparatus.	3.29
PAXBP1	PAX3 and PAX7 binding protein 1	This gene encodes a protein that may bind to GC-rich DNA sequences, which suggests its involvement in the regulation of transcription.	2.70
DENND11	DENN domain containing 11	Protein product may promote the exchange of GDP to GTP, converting inactive GDP-bound small GTPases into their active GTP-bound form.	2.55
TUBGCP5	Tubulin gamma complex associated protein 5	Protein product is part of the gamma tubulin complex, which required form microtubule nucleation at the centrosome.	2.47
RXRB	Retinoid X receptor beta	This gene encodes a member of the retinoid X receptor (RXR) family of nuclear receptors which are involved in mediating the effects of retinoic acid (RA). The encoded protein forms homodimers with the retinoic acid, thyroid hormone, and vitamin D receptors, increasing both DNA binding and transcriptional function on their respective response elements.	2.16

Information regarding gene function were adapted from iPathwayGuide Advaita (2022).

5.4 Discussion

In the light of evidence presented in Chapter 3 and 4 suggesting the involvement of SMEK1 and SMEK2 in the regulation of leukemic cell survival and basal apoptosis and the regulation of the phosphorylation levels of important transcription factors, the current chapter aimed at expanding the knowledge on the modes of action of SMEK1 and SMEK2 in leukemic cells by studying their effects on gene expression. The analysis of the RNA sequencing on cells overexpressing SMEK1 and SMEK2 provided some interesting insights. Both SMEK1 and SMEK2 overexpression modulate the regulation of metabolic processes, gene expression and RNA processing-related processes including posttranscriptional regulation of gene expression, epigenetic regulation of gene expression and gene silencing by miRNA. Moreover, increased in the expression levels of SMEK1 and SMEK2 affected many genes involved in cancer, apoptosis, and important cancer related signalling pathways. The most affected pathway by the increased levels of SMEK1 and SMEK2 was miRNA in cancer, further confirming the roles of SMEK1 and SMEK2 in regulating gene expression. Increased expression of SMEK1 and SMEK2 can also alter the non-coding RNA landscape leading to the alteration of expression of snoRNAs, miRNAs, transcribed pseudogenes and lncRNAs and many of these have been associated with oncogenesis.

The RNA sequencing demonstrated that a striking 1512 genes were differentially expressed in the cells overexpressing SMEK1, and some of these genes are associated with seven cancer related pathways. Interestingly, two of the top upregulated genes were the C/D box small nucleolar RNAs SNORD3A and SNOD3B1. While the role of SNORD3B1 is not characterised, an increased expression of SNORD3A, which was the top upregulated gene (9-

fold increase), has been recently reported to sensitize breast cancer cells to 5-fluorouracil (5-FU) in vitro and in vivo and was associated with a better outcome in breast cancer patients receiving 5-FU-based chemotherapy (Luo et al., 2020). SNORDA was also found to be down-regulated in cervical cancer (Roychowdhury et al.,2020).

The RNA sequencing analysis supported a tumour suppressive role for SMEK1 as the increased in SMEK1 expression levels was associated with the overexpression of tumour suppressors genes. Proapoptotic and tumour suppressor genes that showed an increased expression including Transforming growth factor beta 3 (TGFβ3), graenzyme B and H (GZMB and GZMBH), Ras association domain family member 7 (RASSF7) and glutathione S-transferase mu 3 (GSTM3). TGFβ3 activates signalling pathway that plays important role during embryogenesis, including differentiation, proliferation and migration (Kaartinen et al.,1995; Zimmerman et al.,2000). Similar to the other TGF signalling molecules, TGFβ3 signals through binding to TGFβ tyrosine kinase receptor leading to the phosphorylation and activation of SMAD transcription factors and the activation of other downstream signalling pathways (Moustakas et al.,2001). Results in chapter 4 showed that increased in both SMEK1 and SMEK2 led to increase phosphorylation of SMADs. The increased in SMAD phosphorylation could be related to the increased expression levels of TGFβ3 which would subsequently lead to an increase in the activity of the TGFβ3 receptors and the subsequent phosphorylation/activation of SMADs. GZMB and GZMH belong to the family of human serine proteases that are secreted by Natural killer and cytotoxic T cells. Cleaved active GZMB and GZMH enzymes induce apoptosis in target cells (Kiselevsky et al.,2020). Contrary to GZMB, cell death by GZMH does not involve the activation of executioner caspases nor the release of cytochrome c (Fellows et al.,2007). RASSF7 is a member of the nonenzymatic Ras

association (RA) domain protein family which have one RA domain. A number of studies have demonstrated that these proteins are tumour suppressor since the expression of these proteins is suppressed in human cancers and that the suppression of each of them is correlated with tumour progression (Iwasa et al.,2018). Some of RASSF proteins interact with mammalian Ste20-like kinases which is the core kinases of the tumour suppressor Hippo pathway and promote its activity while others interact with MDM2 and inhibits the MDM2-mediated degradation of p53 (Iwasa et al.,2018). RASSF7, in particular, regulate cell proliferation by modulating protein–protein interactions. RASSF7 is reported to inhibit cMYC-mediated c-MYC–mediated oncogenic transformation in HEK293T and HeLa cell lines (Kumarasmy et al.,2018). RASSF7 was shown to destabilize the c-MYC protein in these cells by promoting its polyubiquitination and degradation. RASSF7 also compete with MYC-associated factor X (MAX) in the formation of a heterodimeric complex with c-MYC and therefore inhibit the transcription of c-MYC target genes (Kumaraswamy et al.,2018). The study also showed an inverse correlation between the expression levels of the RASSF7 and c-MYC gene in human cancers (Kumaraswamy et al.,2018). GSTM3 belongs to the family of Phase II detoxification enzymes that protects cellular component by carrying out detoxification of electrophilic compounds including carcinogens, and products of oxidative stress (Cheng et al.,2021). Loss of GSTM3 (GSTM3) plays a crucial role in tumour progression in various cancers (Wang et al.,2020). Studies have shown that patients with oesophageal squamous cell carcinoma showed low expression of GSTM3 which was associated with poor survival and was suggested to function as a tumour suppressor and to be a potential novel prognostic biomarker for disease-free survival in oesophageal squamous cell carcinoma (Yang et al.,2021). Other studies have implicated GSTM3 in pancreatic cancer where low expression of GSTM3 was detected in pancreatic ductal adenocarcinoma tissues compared to normal

cancer-*adjacent tissues.* High expression of GSTM3 xenograft animal models was associated with good overall survival, lower cell proliferation and increased apoptosis and its silencing caused the opposite effects (Wang et al.,2020).

In addition to the upregulation of tumour suppressor genes, RNA sequencing results revealed the down-regulation of oncogenes, further supporting a tumour suppressive role for SMEK1. Interestingly, increased expression of SMEK1 was associated with a deregulated expression of a member of Rho family of small GTPases (Rho GTPases) HRAS (HRAS proto-oncogene, GTPase) and a member of Rho GTPase activating proteins family (RhoGAP), ARHGAP11A. Rho GTPases are a subfamily of the Ras superfamily proteins which play key roles in many biological processes including cell proliferation and survival, migration and invasion (Porter et al.,2016; Muller et al.,2020). RhoGAPs functions as non-enzymatic upstream regulators of Rho GTPases and have been described to be frequently dysregulated in many types of cancers (Muller et al.,2020). Rho GTPases genes encode three small GTPases RAS proteins HRAS, KRAS and NRAS, that function as molecular switches by cycling between the “on” and “off” conformations via binding of GTP and GDP, respectively (Munoz-Maldonado et al.,2019). The three RAS proteins share high homology apart from their hypervariable C-terminus region which is thought to confer the specific function of each protein (Munoz-Maldonado et al.,2019). The oncogenic RAS signalling network was identified by the Cancer Genome Atlas (TCGA) project as the most commonly altered oncogenic network in cancer. RAS signalling network is reported to be deregulated in 46% of all cancer samples and to contribute to 20-30% of all human cancers (Gimple and Wang,2019). Previous studies have shown that SMEK1 expression levels is strongly decreased in ovarian cancer patient tissues and that overexpression in OVCAR-3 ovarian cancer cells sensitised the cells to the chemotherapy drug gemcitabine and inhibited the phosphorylation of PDK1 and AKT (Dong

et al.,2012; Byun et al.,2012). The current findings which show that the increased expression of SMEK1 causes a decrease in HRAS expression level suggests that the effects of SMEK1 in these ovarian cancer cells could be mediated via its effects on RAS expression levels since PDK1 and AKT are downstream effectors of RAS signalling. The transition between both the active and inactive states of Rho GTPases is regulated by guanine nucleotide exchange factors (GEFs) which promote activation by GDP dissociation and GTP binding and by RhoGAPs which stimulate RAS intrinsic GTPase activity to inactivate the protein (Munoz-Maldonado et al.,2019). RhoGAPs were presumed to function as tumour suppressors because they are involved in the inactivation of RAS proteins. However, many studies have identified that RhoGAPs are overexpressed in many cancers and function as oncogenes (Lawson et al.,2016). In this context, ARHGAP11A, a member of these RhoGAPs, which was found to be down-regulated in cells overexpressing SMEK1 has been reported to be up regulated in liver cancer and involved in promoting the proliferation and migration of hepatocellular carcinoma MHCC97-H cell (Dai et al.,2018). Expression of ARHGAP11A was also found to be increased in basal-like breast cancer patient tissues compared to control and its knockdown in breast cancer cell lines inhibited proliferation and caused cell cycle arrest in G1 (Fan et al.,2021). High expression of ARHGAP11A was also found in gastric cancer with lymph node metastasis and has been suggested to play a key role in lymph node metastasis of gastric cancer (Fan et al.,2021). The top down-regulated gene was PSMB3, a non-catalytic subunit of the multi-catalytic 20S core proteasome complex involved in the proteolytic degradation of most intracellular proteins. While PSBM3 has not been described to function as an oncogene, its gene maps to the same chromosomal location as the oncogene ERBB2 which is amplified in 10-40% of breast tumours and is co-expressed with ERBB2 in breast cancer biopsies (Dressman et al.,2003).

Altered gene expression is the main molecular mechanism responsible for the pathological processes of cancer and other human diseases. Cellular processes involved in the alteration of gene expression were perturbed in the cells overexpressing SMEK1, implicating SMEK1 in the regulation of gene expression. These processes include negative regulation of gene expression, RNA processing, post-transcriptional regulation of gene expression, epigenetic control of gene expression, gene silencing by RNA and miRNA and post-transcriptional gene silencing. Two biological pathways processes associated with regulation of gene expression, miRNAs in cancer and transcriptional mis-regulation in cancer, were also perturbed in the cells with increased levels of SMEK1, further supporting a role for SMEK1 in regulation gene expression at both transcriptional and post-transcriptional levels by miRNAs. miRNAs (microRNAs) are endogenous small non-coding RNAs consisting of approximately 22 nucleotides and function as key regulators in many biological processes. They are involved in the regulation of gene expression at the post-transcriptional level by binding to 3' UTR of their target messenger RNA (mRNA) stimulating the suppression of their expression (Liu et al.,2018). Dysfunction of miRNAs alters the expression of oncogenes or tumour suppressor genes, contributing to oncogenesis and the expression of many miRNAs is deregulated in cancer pathogenesis. As such, a large number of miRNAs have been found to be downregulated or upregulated in human cancers and many identified to function as oncomiRs or oncosuppressor miRs (Liu et al.,2018). The expression of 202 miRNAs was deregulated by the overexpression of SMEK1 suggesting that the effects of SMEK1 on gene expression and regulating survival could have resulted from the changes of expression of these miRNAs. Other molecular processes affected includes could explain the profound effects SMEK1 have on the alteration of gene expression. Previous studies have also implicated SMEK1 in transcriptional regulation. Wnt-responsive development-related genes in embryonic stem

cells are silenced to maintain pluripotency and their expression is activated during differentiation (Lyu et al.,2011). Studies have shown that SMEK1 represses transcription of Wnt-responsive development-related genes through histone deacetylation (Lyu et al.,2011). SMEK1 mediates recruitment of PP4c and HDAC1 to the binding site of the Wnt-responsive development-related gene brachyury promoter and inhibits its expression in ESC (Lyu et al.,2011). Moon et al. (2017) showed that double knockout of *SMEK1/2* led to a severe defect in neurogenesis both in vivo and in vitro, a decrease in the number of differentiated neurones and an increase in the number of neural stem cells. The study showed SMEK1/2 interacts directly with methyl-CpG–binding domain 3 (Mbd3), a core component of the repressive nucleosome remodelling and deacetylase (NuRD) complex which mediates epigenetic silencing of target genes during neural development. SMEK1/2 interaction destabilises the protein and prevents its accumulation on target gene loci functioning in neurogenesis and consequently drives gene expression (Moon et al. 2017).

In addition of the perturbed signalling pathways related to cancer such as apoptosis, MAPK, mTOR, VGEF, ErbB, NFκB, TGFβ and RAS, increased level of SMEK1 affected the expression of 12 ribosomal proteins genes that were annotated to structural constituent of ribosomes. In addition to their roles as ribosomal components, ribosomal proteins play an essential role in ribosome biogenesis by acting as RNA chaperones stabilising rRNAs and promoting their correct folding for the assembly of ribosomal subunits (Xu et al.,2016).

Deregulation of any step in the process of ribosome biogenesis activates nucleolar stress followed by the loss of nucleolar integrity leading to a halt to cell proliferation (Boulon et al., 2010). Studies have reported that ribosomal proteins carry out several extra-ribosomal functions which include regulation of apoptosis, cell cycle, cell proliferation, cell migration

and invasion, and DNA damage repair (Anderson et al., 2007; Da Costa et al., 2003; Du et al., 2005). Expression of genes that impacted graft-versus-host disease, allograft rejection, autoimmune thyroid disease, type I diabetes mellitus pathways were also impacted. These observations agree with a recent study which reported a role of SMEK1 in neuroinflammation (Duan et al.,2021). Expression of SMEK1 was decreased in Multiple Sclerosis patients, suggesting that decreased level of SMEK1 could contribute to the susceptibility to CNS autoimmune diseases (Duan et al.,2021). Indeed, SMEK1 was confirmed to play a protective role in autoimmune demyelination pathogenesis via immune suppression and inflammation regulation in both the immune system and the central nervous system (Duan et al, 2021).

SMEK2 overexpression had a profound effect on gene expression with a total 1651 genes showing differential expression. The top ten upregulated genes in SMEK2 overexpressing cells were also among the top upregulated genes affected by SMEK1 increased levels. These include RPS9, SNORD3B-1, CUTA, TOMM6, SINHCAF and MYT1. Interestingly, Gasdermin D (GSDMD) was one of the top upregulated gene that did not show any changes in SMEK1 overexpressing cells. GSDMD is a member of the gasdermin family, the critical effectors of inflammasomes-induced programmed cell death, pyroptosis (He et al.,2015). Transcription factor 19 (TFC19) was also another gene that was only upregulated in SMEK2 overexpressing cells. Genome wide association studies have identified TFC19 as a gene associated with type 1 diabetes and type 2 diabetes and studies have shown that TCF19 knockdown halts β-cell proliferation and increases their apoptosis (Yang et al.,2021). The changes in the expression of five of the top down-regulated genes were specific to SMEK2 overexpressing. RN7SL3, the top down-regulated gene in SMEK2 overexpressing cells (10-fold decrease) showed 0.9 fold increase in its expression in the cells with increased SMEK1 expression, highlighting a significant difference in the effects of SMEk1 and SMEK2. RN7SL3 is

the backbone RNA molecule of the signal recognition particle (SRP), a cytoplasmic ribonucleoprotein complex which consists of 6 polypeptides and a 7SL RNA (Bradshaw and Walter, 2007). SRP mediates the mediates the targeted delivery of ∼30% of the proteome and is involved in the protection of mRNAs of secretory proteins from degradation, suggesting a specific role for SMEK2 in protein targeting and mRNA protection (Bradshaw and Walter, 2007). RN7SL3 is reported to be overexpressed in many cancers and to promotes growth through repressing p53 translation (Yang *et al.* 2016). LY6E was one of the top ten down-regulated genes in SMEK2 overexpressing cells but not changed in SMEK1. LY6E is a cell surface protein that regulates T-lymphocytes proliferation, differentiation and has been shown to modulate viral entry to the cell and infection in a cell type-dependent manner and to be a critical antiviral immune effector (Yu et al.,2019). Studies have shown that LY6E is a is overexpressed in gastric cancer (Lv et al.,2018). LY6E overexpression in gastric cancer cells promotes cancer cell growth and metastasis (Lv et al.,2018). Other SMEK2 specific down-regulated genes include AIF1 (allograft inflammatory factor 1) an interferon inducible cytoplasmic protein cloned from activated macrophages in human and has been identified as a modulator of inflammatory response (Elizondo et al.,2019) and RNH1 (Ribonuclease Inhibitor), a leucine-rich repeat protein which protects RNAs by binding ribonucleases rendering them inactive and has been reported to regulate inflammatory response (Elizondo et al.,2019). The importance of RNH1 function is highlighted by the fact that complete deletion of RNH1 in mice is lethal (Sarangdhar and Allam, 2021). Overall, the top SMEK2 specific differentially expressed genes suggest that SMEK2 might play a key role in in protein targeting, mRNA protection, pyroptosis and inflammatory response.

While RNA sequencing results supported a tumour suppressive role for SMEK1, the molecular picture for SMEK2 overexpression is not very clear. Overexpression of SMEK2 was

associated with an increased expression levels of a number of proapoptotic and tumour suppressor genes including TGFb3, GZMB, SOCS1 and GSTM2 and a decreased expression levels of oncogenes such RXRB (Retinoid X receptor beta), CSNK2B, RALB, GLI2 (GLI family zinc finger 2, also called glioma-associated oncogene homolog) (Rimkus et al.,2016), ARHGAP11A and TERC (Telomerase RNA component) (Flacco et al.,2015), suggesting a tumour suppressive role for SMEK2. However, increased levels of SMEK2 also led to an increased expression of oncogenes such as c-MYC, TFC19, GSDMD and Twist1, casting doubt on its role as a tumour suppressor. Aberrations in c-MYC expression and function occur in the vast majority of cancers and cancer with c-MYC activation acquires many of the hallmarks required for autonomous neoplastic growth (Dhanasekaran et al.,2021). TFC19 (Transcription factor 19) has been implicated in cell proliferation of hepatic carcinoma and non-small cell lung carcinoma (Mondal et al.,2019) and its increased expression was shown to correlate with the occurrence of distant metastasis and poor prognosis of progression of colorectal cancer (Du et al., 2020). Twist1 is a transcription factor that promotes cell migration and invasion during embryonic development and in cancer cells and is expressed in multiple types of invasive cancer cells (Honeth et al.,2008; Sarrio et al.,2008). GSDMD is as an essential mediator of pyroptosis, a process associated with the destruction of membranes and the release of cellular contents triggering immune response and inflammation (Gao et al.,2018). Pyroptosis can inhibit cancer cell proliferation and tumorigenesis, however this type of proinflammatory death have been linked to the formation of a suitable microenvironment that promote tumour growth (Gao et al.,2018). Indeed, GSDMD is linked to worse prognosis in lung adenocarcinoma and osteosarcoma (Lin et al.,2020). It is significantly upregulated in non-small cell lung cancer and its expression is associated with larger tumour size and metastasis (Wu et al.,2020). In addition, high GSDMD expression indicated a poor prognosis

in lung adenocarcinoma and colorectal cancer (Du et al.,2020; Wang et al.,2021). c-MYC is often deregulated in inflammation (Sipos et al.,2016), therefore its increase levels might be related to the increase in GSDMD expression and its effects on inflammatory pathways.

RNA sequencing analysis revealed a role for SMEK2 in immune response, mRNA transport, viral infection, regulation of gene expression at transcriptional and post-transcriptional levels. Out of 250 pathways affected by SMEK2 overexpression, the top nine impacted pathways include microRNAs in cancer, graft-versus-host disease, allograft rejection, autoimmune thyroid disease, type I diabetes mellitus, PPAR signalling pathway, mRNA surveillance pathway, and antigen processing and presentation. SMEK2 overexpression also led to perturbation in biological pathways such Herpes simplex virus 1 infection, RNA transport and pathways of neurodegeneration multiple diseases. In addition to miRNAs in cancer, other perturbed cancer related biological pathways included Human T-cell leukemia virus 1 infection, gastric cancer, colorectal cancer, transcriptional misregulation in cancer and relevant signalling pathways such as MAPK, PI3K/AKT, HIPPO, WNT and peroxisome-proliferator-activated receptors (PPAR), further highlighting a key role for SMEK2 in the regulation of a number biological and cellular processes including cancer. Similar to SMEK1 effects, increased in SMEK2 expression levels affected the expression of short and long non-coding RNAs. The expression of more than 80 snoRNAs and 200 miRNAs were perturbed by SMEK2 overexpression in addition to different types of lncRNAs and pseudogenes, further highlighting a role for SMEK2 in regulation of gene expression.

Meta-analysis across SMEK1 and SMEK2 overexpression RNA expression datasets revealed that the function of SMEK1 and SMEK2 is partially similar and both genes are not functionally redundant. Only 638 differentially expressed genes were common between

SMEK1 and SMEK2 overexpressing cells and 44 of these deregulated genes showed different direction of gene expression. 874 differentially genes were SMEK1 specific, and the expression of 1031 genes were SMEK2 specific, highlighting a significant difference between the effects of SMEK1 and SMEK2. Analysis of SMEK1 specific differentially expressed genes revealed a role for SMEK1 in maintaining the functional integrity of the mitochondrial ribosome, RNA processing and splicing and regulation of transcription, proteasome function and ribosomal structure. SMEK2 specific perturbed pathways include production of molecular mediator of immune response, immunoglobulin production, immunoglobulin mediated immune response, positive regulation of immune effector process and regulation of endothelial cell migration. Assessing SMEK2 specific deregulated genes revealed a role for SMEK2 in RNA protection, inflammatory response, immune response and viral infection. Increased in the expression of both SMEK1 and SMEK2 affected same biological pathways but through different genes. The most striking difference relevant to cancer is the down-regulation of RAS oncogene in the cells overexpressing SMEK1 and the overexpression of the oncogene c-MYC and other oncogenes only in the cells overexpressing SMEK2, suggesting that a different role for SMEK1 and SMEK2 in oncogenesis.

5.5 Chapter highlights

1- SMEK1 overexpression triggers the differential expression of 1512 genes and affects 200 biological pathways. These pathways include cancer dependent and independent pathways. Cancer related pathways include microRNAs in cancer, pathways in cancer, gastric cancer, colorectal cancer and transcriptional misregulation in cancer. Perturbed signalling pathways related to cancer include apoptosis, MAPK, mTOR, VGEF, ErbB, NFκB, TGFβ and RAS signalling pathways.

2- SMEK1 overexpression is also associated with an increased expression level of tumour suppressor genes and decreased expression of known oncogenes including HRAS, supporting its role as a tumour suppressor.

3- Increased expression of SMEK2 leads to the differential expression of 1651 genes and affected and affects an excess of 200 biological pathways. Cancer and non-cancer related pathways are affected. Cancer related pathways include microRNAs in cancer, pathways in cancer, Human T-cell leukaemia virus 1 infection, gastric cancer, colorectal cancer and transcriptional misregulation in cancer. Perturbed signalling pathways related to cancer include pathways in cancer, MAPK, PI3K/AKT, RAS, HIPPO and WNT and PPAR.

4- SMEK2 overexpression is also associated with the decreased expression of oncogenes and increased expression of both tumour suppressor and oncogenes, implying that SMEK2 is a gene with dual biological functions, oncogenic and tumour suppressor.

5- Both SMEK1 and SMEK2 overexpression induce changes in the non-coding landscape leading to the differential expression of many miRNAs, snoRNAs, pseudogenes and lncRNAs.

6- RNA sequencing revealed that both SMEK1 and SMEK2 regulate gene expression at the transcriptional and post-transcriptional levels. Top common perturbed biological processes between SMEK1 and SMEK2 include RNA processing, negative regulation of gene expression, posttranscriptional regulation of gene expression, epigenetic control of gene expression, gene silencing by RNA and miRNA and posttranscriptional gene silencing.

7- Metanalysis across SMEK1 and SMEK2 overexpression RNA expression datasets confirms that both SMEK1 and SMEK2 exert some overlapping and non-overlapping functions and supported the concept that both genes are not functionally redundant.

Chapter Six: General Discussion and Concluding remarks

Reversible protein phosphorylation is a key mechanism in the control of cellular processes including proliferation, differentiation and apoptosis (Moorhead et al., 2007). The well-balanced opposing operation of protein kinases and phosphatases is therefore critical for the control of cell death and survival. Many oncogenes encode protein kinases and changes in their activity contribute to the process of tumorigenic transformation (Calabretta and Perrotti, 2004). The role played by protein phosphatases is equally important and crucial to the activity of many of the target proteins involved in the control of cell death and survival (Tonks, 2006; Ventura and Nebreda, 2006). One such protein phosphatase which is increasingly recognised as of importance in cancer biology is PP4. PP4 is a member of the serine/threonine phosphatase (PPP) family that regulates many cellular processes including microtubule growth (Hastie et al., 2000), centrosome maturation (Sumiyoshi et al., 2002), DNA damage repair (Lee et al., 2007), and NFκB and tumour necrosis factor signalling (Yeh et al., 2004; Mihindukulasuriya et al.,2004; Brechmann et al.,2012).

The PP4 core enzyme is composed of a catalytic subunit (PP4c) that associates with different regulatory proteins (PP4R1, PP4R2, PP4R3α/SMEK1, PP4R3β/SMEK2, and PP4R4) giving rise to a diverse collection of distinct PP4 holoenzymes (Gingras et al., 2005; Lee et al., 2010). Interaction with these regulatory proteins determines function, substrate selectivity and subcellular localisation of the catalytic subunit (Janssens and Goris, 2001). The role of PP4c in the regulation of cell fate is well established. Studies have shown that PP4c is a critical component of several pathways controlling cell survival and proliferation in different cell types including mouse thymocytes (Shui et al., 2007), adenocarcinoma human alveolar basal epithelial cells A549 and HeLa cells (Theobald et al., 2013), HEK 293T and both leukemic T-cells and primary human T-cells (Mourtada-Maarabouni and Williams, 2008; 2009). However, the functions of its regulatory subunits in controlling cell survival remain understudied.

PP4R3 or Suppressor of Mek null (SMEK), an evolutionary conserved protein family, consists of two isoforms, SMEK1 (PP4R3A, PP4R3α) and SMEK2 (PP4R3B, PP4R3β). SMEKs were first reported to play an important a role in the formation of a holoenzyme with PP4c, PP4R1, and PP4R2 complex (Chen et al.,2008). Initially SMEKs were identified in *Dictyostelium discoideum* and reported to regulate cell polarity, chemotaxis, and gene expression (Chen et al.,2008; Mendoza et al.,2005). In *Caenorhabditis elegans*, SMEKs are implicated in longevity by modulating DAF-16/FOXO3a transcriptional activity (Sen et al.,2020). SMEKs are involved in DNA repair through dephosphorylation of H2AX during DNA replication (Chowdhury et al.,2008), and glucose metabolism by controlling cAMP-response element binding protein (CREB)-regulated gene expression (Yoon et al.,2010). In higher eukaryotes, SMEKs have a critical role cell-fate determination. In *Drosophila* neuroblasts, Falafel, the highly conserved orthologous of SMEKs, regulates cell division by controlling localization of Miranda, a protein involved in neuroblast division (Lyu et al.,2011).

Both SMEK1 and SMEK2 have shown overlapping functions in mammalian cells. Both proteins are involved in neurogenesis and neuronal cell differentiation in mice (Lyu et al.,2013; Moon et al.,2017; Cheng et al.,2017). In mice, both SMEK proteins regulate cell differentiation via suppression of the expression of Wnt-responsive gene brachyury in embryonic stem cells (ESCs) (Chang et al.,2017). Whereas SMEK1, especially, promotes neuronal stem cell differentiation by negatively regulating Par3, a negative regulator of neuronal differentiation (Lyu et al.,2013). However, only SMEK1 has been described to regulate cell fate decision and to function as tumour suppressor gene in prostate and ovarian cancer (Kim et al.,2015). Studies have shown that SMEK1 expression level is decreased in ovarian and cervical tumour tissues and ovarian cell lines, and its gene was found hypermethylated in ovarian cancer (Kim et al.,2015). SMEK1 has been reported to interact

with BLU tumour suppressor and increases its proapoptotic activity (Dong et al.,2012). In addition, SMEK1 specific roles include microtubule organization, DNA damage checkpoint and TNF signalling (Bertram et al.,2000; Chowdhury et al.,2008; Nakada et al.,2008). The evidence presented so far suggests growth inhibitory and tumour suppressive roles for SMEK1 in ovarian and prostate cancers, however further studies are required to investigate its role in leukaemia. In addition, it is not clear if SMEK2 has any effect on the regulation of cell survival and whether it has the same function as SMEK1. Therefore, the present study aimed to investigate the role of SMEK1 and SMEK2 in the regulation of leukemic cell fate decision and whether the two proteins are functionally redundant.

6.1 Effects of the modulation of SMEK1 and SMEK2 expression levels on cell fate decision in leukemic T cells.

Previous studies have supported a tumour suppressive role for SMEK1. SMEK1 RNA expression level is significantly decreased in human primary ovarian and cervical cancer when compared to normal tissues (Dong et al.,2012). Further studies showed that SMEK1 expression is silenced in these tumours due to the fact that SMEK1 promoter is hypermethylated in both primary ovarian and cervical tumours (Dong et al.,2012). SMEK1 was found to enhance the pro-apoptotic activity of the tumour suppressor BLU. The expression of both genes correlated in cancer and both proteins were reported to inhibit ovarian and cervical cancer cell growth (Dong et al.,2012). In addition to its proapoptotic and anti-growth activity, overexpression of SMEK1 was also shown to increase ovarian cancer cell response to gemcitabine by promoting apoptosis associated with increased Bax protein expression, and reduced expression of the anti-apoptotic proteins Bcl-xL and Bcl-2 (Byun et al.,2012).

The functional analyses carried out in Chapter 3 have confirmed the tumour suppressive nature of SMEK1 for the first time in leukemic cells. SMEK1 overexpression led to an inhibition of leukemic cell growth and increase in apoptotic cell death. SMEK1 proapoptotic effects was caspase dependent as it was associated with the activation of caspase 3. SMEK1-induced apoptosis appeared to be dependent on the activation of apoptotic intrinsic pathway as SMEK1 overexpression led an increased expression of the proapoptotic protein BAD, which is known to induce cell death by binding to survival proteins such as Bcl-XL and BCL-2 and inhibiting their activity (Kluck and Dewson, 2010). The proapoptotic effects of SMEK1 were further confirmed in the siRNA mediated silencing studies. SMEK1 down-regulation led to an increase in short- and long-term survival. The effects of decreased expression of SMEK1 led to an increase in the expression levels of XIAP which inhibits mammalian caspases activity, further confirming that SMEK1 induced apoptosis is caspase dependent (Deveraux et al., 1997; Carter et al., 2013). The increase in XIAP protein expression in cells expressing low SMEK1 is significant for cancer as XIAP has been shown to be over-expressed in various cancers leading to poor overall survival (Tu and Costa,2020). Collectively, Chapter 3 results confirm that SMEK1 has a tumour suppressor role and plays an important role in the regulation of leukemic cell survival.

Results described in Chapter 3 have also confirmed for the first time that the modulation of SMEK2 expression have significant effects on the survival of leukemic cells. Overexpression of SMEK2 led to an increase in basal apoptosis and decrease in long- and short-term survival of leukemic T cells, although the obtained magnitude of the effect was slightly lower compared to SMEK1 mediated effects. siRNA mediated silencing of SMEK2 also confirmed its growth inhibitory role. Decreased expression levels of SMEK2 resulted in an increase in short- and long-term survival of leukemic T cells. Overall, these findings suggest

that both SMEK2 and SMEK1 might be functionally similar for the regulation of the survival of leukemic T cells.

The role of SMEK2 in the regulation of cell survival has not been studied yet. However, similar effects in concordance with SMEK1 results herein described have already been reported in the literature. Increased SMEK1 expression inhibited cell growth and angiogenesis *in vitro* human umbilical vein endothelial cells, SKOV-3 ovarian cancer cells, and xenograft human ovarian tumour model (Kim et al.,2015). In this study, SMEK1 was shown to suppress the growth, survival and cell migration effects of VEGF (Kim et al., 2015). SMEK1 inhibited VEGF signalling by binding to the receptor, VEGFR-2 leading to the inhibition of the phosphorylation of the signalling molecules involved in AKT signalling pathway such as PDK1, endothelial nitric oxide synthase (eNOS), and hypoxia-inducible factor 1 (HIF-1α), implicating SMEK1 in the regulation of VEGFR-2-mediated activation of PI3K/Akt/eNOS signalling pathway. Other studies have shown that SMEK1 enhanced the pro-apoptotic activity of chemotherapeutic drugs in aggressive ovarian carcinoma cells and mouse xenograft models. SMEK1 overexpression enhanced the cytotoxic activity of chemotherapeutic drugs and exerted an additive effect on the inhibition of ovarian cancer cell growth by inducing cell cycle arrest and apoptosis and affecting related gene expression levels and survival proteins activities such as suppression of RAS homolog enriched in the brain (Rheb) and mTOR (Kim et al.,2014), altogether suggesting a rather universally crucial role of SMEK1 in the regulation of cell fate decision.

6.2 Effects of SMEK1 and SMEK2 overexpression on five of the core cancer signalling pathways

Given the magnitude of the SMEK1 and SMEK2 regulated effects described in Chapter 3, it was hypothesised that both proteins would be involved in signalling pathways and networks of great significance in the regulation of cell fate and cancer settings, therefore, that its fluctuations would heavily impact significant signalling pathways linked with the regulation of cell survival, carcinogenesis, and cancer progression. Protein phosphorylation and dephosphorylation play a central role in controlling the activity of signalling pathways. To this end, we have used Human Phosphorylation Pathway Profiling Array to investigate the effects of SMEK1 and SMEK2 overexpression on the phosphorylation levels of proteins involved in five signalling pathways with deregulated functions in cancer.

As presented in Chapter 4, the results of the Phosphorylation Pathway Profiling Array were highly enlightening in terms of modifications associated with SMEK1 and SMEK2 levels. Initial experiments using non-malignant H293T cells revealed that a striking difference between the effects of SMEK1 and SMEK2 on the phosphorylation levels of proteins involved in core cancer signalling pathways. Only SMEK2 overexpression led to a change in the phosphorylation of proteins involved in MAPK, AKT and JAK/STAT signalling pathways, providing evidence that in some settings SMEK1 and SMEK2 have a non-overlapping function. Interestingly, SMEK2 overexpression had profound effects on the phosphorylation of key proteins in JAK/STAT pathway, one of the 12 core cancer signalling pathways activated by cytokines (Sever and Brugge, 2015). Increased expression levels in SMEK2 expression levels decreased the phosphorylation levels of key signalling proteins such as EGR, JAK and STATs

proteins and an increase in the phosphorylation of SH2 containing protein tyrosine phosphatase-2 (SHP2), suggesting an inhibitory role for SMEK2 on the activity of this pathway. SHP2 plays an essential role in hematopoietic cell development and had stimulatory and inhibitory functions in the JAK/STAT pathway depending on the acting sites (Xu and Qu,2008). Loss or mutation of JAK/STAT signalling pathway components is associated with many human diseases including cancer, as it does constitute one of the central communication nodes in the cell function. JAK/STAT mediated signalling involves more than 50 cytokines and growth factors including interferons (IFN), interleukins (ILs), and colony-stimulating factors (Hu et al.,2021). JAK/STAT downstream signalling regulates hematopoietic cell development, immune response, tissue repair, inflammation and apoptosis (Hu et al.,2021). Previous results have reported a crosstalk between JAK/STAT signalling and MAPK and AKT signalling pathways (Rädler et al.,2017). Studies have reported that JAK/STAT signalling cascades regulate cell date decision of mammary epithelial cells by regulating the expression and functionality of some MAPK and PI3 kinase subunits and AKT (Rädler et al.,2017). It is possible that the inhibitory effects induced by SMEK2 on the activation of MAPK and AKT signalling pathways is mediated via the effects of SMEK2 on the dephosphorylation of JAK/STAT protein components and the consequence inhibition of the pathway and its crosstalk with other signalling pathways. Overall, the results suggest a regulatory axis involving SMEK2-JAK/STAT signalling pathway affecting MAPK and AKT pathways which warranties further investigation.

The effects of SMEK1 and SMEK2 on the NFκB pathway in 293T cells also revealed a difference between both proteins. Both SMEK1 and SMEK2 affected the phosphorylation of different proteins in the pathway with only SMEK2 causing a decrease in the phosphorylation of NFκB. More evidence was obtained to support a distinct role for SMEK1 and SMEK2 in the regulation of TGFβ signalling pathway. SMEK1 overexpression was associated with an

increase of the phosphorylation levels of the transcription factors SMAD1, 2, 4 and 5 , whereas increased expression of SMEK2 led to a decreased in the phosphorylation levels of SMAD1, 2, 4 and 5. SMAD phosphorylation is important for its translocation and accumulation in the nucleus to regulate target gene expression (Massague,2012). These results suggest that in HEK293T cells, SMEK1 and SMEK2 exert different effects on SMADs transcription factors with SMEK1 exerting stimulatory effects on SMADs, while SMEK2 effects being inhibitory. A balance between the activity of SMEK1 and SMEK2 could be important for cell death or survival in non-cancerous cells.

In Jurkat leukemic T cells, SMEK1 exerted similar effects to those caused by increased expression of SMEK2, suggesting a different role of SMEK1 in cancer cells. Both SMEK1 and SMEK2 overexpression in Jurkat T cell exerted inhibitory effects on MAPK, AKT, JAK/STAT and NFκB pathways, as shown by the decrease in the phosphorylation levels of Key proteins involved in these pathways. Both SMEK1 and SMEK decreased the phosphorylation levels of STAT proteins in JAK/STAT pathway except for STAT1 which showed significant increase in its phosphorylation levels in cells overexpressing SMEK1 or SMEK2. This is significant as it implies that the growth inhibitory effects of SMEK1 and SMEK2 could be mediated by their effects on STATs proteins. STAT1 phosphorylation leads to its activation which results in the increase in the expression of genes that mediate cell death and inhibiting cell growth (Bousoik et al.,2018; Sironi et al.,2004) and inhibits the expression of genes associated with cell cycle progression and proliferation including the oncogene c-MYC (Bousoik et al.,2018). Whereas the reduced phosphorylated levels of STAT3, 5 and 6 inhibit their activity leading to the inhibition of the expression of survival genes and cell cycle progression proteins including Bcl2, Bcl-xl, c-MYC and survivin (Bousoik et al.,2018). Interestingly, most of the effects of SMEK1 and SMEK2 overexpression on AKT, MAPK, JAK/STAT and NFκB pathways were nearly mirrored in the cells

overexpressing PP4c, suggesting that SMEK1 and SMEK2 effects on these pathways could be mediated at least partly via their interaction with PP4c. It is likely that the increased expression of SMEK1 and SMEK2 in Jurkat T cells could lead to the enhancement of PP4c phosphatase activity or stability leading to the dephosphorylation of target proteins in these pathways.

Some of the changes induced by SMEK1 and SMEK2 overexpression in Jurkat cells on TGFβ signalling pathway were mirrored by increased expression of PP4c, apart from the effects on the increased on the phosphorylation levels of SMADs transcription factors. The effects on SMADs transcription factors were specific to SMEK1 and SMEK2, implying that the two proteins are acting independently from PP4c. The results indicate that both SMEK1 and SMEK2 regulate the TGFβ signalling pathway via their effects on SMADs phosphorylation and confirm that both proteins have specific targets within the pathway. TGFβ signalling regulates cell growth, survival and death pathways and has protective and cytostatic effects in normal cells and early stages of cancer (Lebrun, 2012). Many types of cancer are associated with a mutations and deletions in the genes coding for the proteins involved in TGFβ signalling components. Therefore, as tumours develop and progress, the tumour suppressor effects of TGFβ are often lost and TGFβ signalling then shifts to promote cancer progression, invasion, and metastasis (Lebrun, 2012). Activated SMAD2 is reported to inhibits the action of ubiquitous E3 ligase, leading to stabilising the levels of the cell cycle inhibitor p27 during cell cycle arrest in endometrial carcinoma cells (Lecanda et al., 2009). Activated SMADs regulate the promoter of the translational and proliferation inhibitor 4E-BP1 gene leading to higher levels of this protein and thus resulting in a halt in cell proliferation in epithelial and mesenchymal cells (Azar et al., 2009). It is likely that the growth inhibitory effects of SMEK1

and SMEK2 could be mediated by promoting SMADs phosphorylation leading to the activation of SMADs – mediated tumour suppressor activity of TGFβ signalling pathway.

The result presented in chapter 4 indicated a distinct role of SMEK1 and SMEK2 in non-cancerous and cancerous cells. The results also confirmed that both SMEK1 and SMEK2 have overlapping and non-overlapping functions. In Jurkat T cells, SMEK1 and SMEK2 affected the phosphorylation of key proteins in the five major signalling pathways involved in the regulation of proliferation, survival and cell death. Apart from the effects on SMADs proteins in TGFβ signalling pathway, the effects of SMEK1 and SMEK2 are similar to those caused by PP4c overexpression, suggesting that SMEK1 and SMEK2 effects could be dependent on the activity of PP4c in regulating MAPK, AKT, JAK/STAT and NFκB signalling pathways and that both proteins affect TGFβ pathway independently of PP4c.

In addition to revealing a role of SMEK1 and SMEK2 in the regulation of the activity of five cores cancer signalling pathways, the results in chapter 4 highlighted that overexpression of SMEK1 and SMEK2 affected the phosphorylation of important transcription factors. Post-translational phosphorylation of transcription factors is a common control mechanism for the regulation of their activity in regulating gene expression. Phosphorylation or dephosphorylation of transcription factors determine the transcription factor cellular localization, stability, protein-protein interactions and DNA binding and can either activate or inhibit their activity in turning on gene expression (Whitmarsh and Davis 2000). The phosphorylation state of eight transcription were confirmed to be altered by the increased expression of SMEK1 and SMEK2. These transcription factors include NFκB, STAT1, STAT3, STAT5, c-JUN, SMAD1, SMAD4 and SMAD5. The phosphorylation level of NFκB and c-JUN was significantly reduced in the cells overexpressing SMEK1 and SMEK2. STAT1 and SMAD5

transcription factors showed an increased phosphorylation levels in the cells with an increased level of SMEK1 and SMEK2. SMEK1 specific changes included an increase in the phosphorylation levels of SMAD1. SMEK2 specific changes included a decreased level in the phosphorylation of STAT5 and SMAD1 and an increase in the phosphorylation of levels of SMAD4. SMADs proteins are not just transcription factors, they are also involved in the epigenetic regulation through the recruitment of different epigenetic regulators, such as chromatin remodelers, histone modifiers and DNA modifiers to the target gene regulatory elements (Papageorgis et al., 2010). Altogether, the results suggest that SMEK1 and SMEK2 regulate gene expression at both epigenetic and transcriptional levels.

6.3 Effects of SMEK1 and SMEK2 overexpression on gene expression

Given SMEK1 and SMEK2 effects described in Chapter 3 and 4, it was hypothesised that both proteins would be involved in changing gene expression and the regulation of molecular pathways and networks of great significance in cancer settings. To this end, RNA sequencing approach was adopted to investigate the molecular mechanisms underpinning the effects observed upon the overexpression of SMEK1 and SMEK2,

As presented in Chapter 5, the results of the RNA sequencing were highly illuminating in terms of molecular modifications associated with SMEK1 and SMEK2 levels. The results strongly implicated SMEK1 and SMEK2 in regulation of gene expression, epigenetic control and RNA processing. Deregulated processes that were found to be associated with increased expression of SMEK1 and SMEK2 included gene expression, post-transcriptional regulation of

gene expression, epigenetic regulation of gene expression, transcriptional mis-regulation in cancer and gene silencing by miRNA. The results in chapter 4 suggested that SMEK1 and SMEK2 regulate gene expression via their effects on the phosphorylation of transcription factors. RNA sequencing revealed that increased levels of SMEK1 and SMEK2 was associated with the differential expression of more than 200 miRNAs and the deregulation of processes of gene silencing by miRNA and miRNAs. These results implies that SMEK1 and SMEK2 regulate two important types of gene expression regulators, transcription factors and miRNAs. Transcription factors bind to promoter regions and regulate gene expression at the transcriptional level, while miRNAs act at the post-transcriptional level by binding the 3' untranslated regions (Zhao et al.,2016). Interestingly, a role for the plant orthologue of SMEK1, PP4R3A, in regulating the transcription of miRNA genes have been reported in *Arabidopsis thaliana*, a model organism in plant biology (Su et al.,2017). PP4R3A together with the catalytic subunits PPX1 and PPX2 form a complex that dephosphorylates and activates the Hyponastic Leaves 1, HYL1, which has a role in promoting miRNA biogenesis (Su et al.,2017). PP4R3A also interacts with RNA polymerase II and recruits it to the promoters of miRNA-encoding genes (*Wang et al., 2019*). The expression of many snoRNAs, another class of small non-coding RNAs, was deregulated with some being the top down- or up-regulated genes by SMEK1 and SMEK2, adding another layer of complexity to the role of SMEK1/2. snoRNAs have been implicated in post-transcriptional processes such as rRNA acetylation, RNA splicing, control of mRNA abundance and translational efficiency (*Wang et al.,2019*), suggesting that SMEK1 and SMEK2 could be also implicated in regulation of gene expression at posttranscriptional level.

Moreover, it is noteworthy that the overexpression of SMEK1 and SMEK2, on top of changing the molecular landscape on a pathway and on a miRNA level, also led to

perturbation of the long non-coding RNA landscape with many pseudogenes and different types of lncRNAs being deregulated. Types of differentially expressed lncRNAs include NATs and lincRNAs. Among them PXN-AS1 which has been attributed a dual role, depending on the cancer type (Yan et al.,2019) and PITPNA-AS1 (PITPNA antisense RNA 1) which have been shown to increase proliferation and migration of lung squamous cell carcinoma cells by stabilize the oncogene High mobility group box 3 (HMGB3) (Ren et al.,2020). Other lncRNA differentially expressed included MYCN opposite strand (MYCNOS), a gene located on the antisense strand to MYCN and its expression reported to be deregulated in a number of cancer (O'Brien et al.,2018) and XIAP-*AS1*, transcribed from the first intron of the complementary strand of the *XIAP* gene and reported to regulate XIAP expression (O'Brien et al.,2018). The present results agree with previous study which reported effects of SMEK1 silencing on the lncRNAome, where the expression of lncRNAs and their regulatory network was significantly altered during SMEK1/2 Knockout mouse neural stem cells differentiation (Yang et al.,2020).

Moreover, the RNA sequencing revealed that SMEK1 regulates the expression of many genes involved in cancer, apoptosis, and important cancer related signalling pathways. Cancer related processes affected by SMEK1 increased levels include microRNAs in cancer, pathways in cancer, gastric cancer, colorectal cancer and transcriptional misregulation in cancer. Perturbed signalling pathways related to cancer resulted by SMEK1 overexpression include apoptosis, MAPK, mTOR, VGEF, ErbB, NFκB, TGFβ and RAS. Several cancer related genes were differentially expressed in SMEK1 including tumour suppressors that showed increased expression and oncogenes that were downregulated, supporting a tumour suppressor role for SMEK1. One of the significant findings is that the downregulation of the oncogene HRAS in SMEK1 overexpressing cells, further confirming a tumour suppressive role for SMEK1.

RNA sequencing revealed a significant and complex role for SMEK2 in cell death and cancer. A number of cancer related pathways were perturbed in SMEK2 overexpressing cells. These include miRNAs in cancer, Human T-cell leukaemia virus 1 infection, gastric cancer, colorectal cancer, transcriptional misregulation in cancer and relevant signalling pathways such as MAPK, PI3K/AKT, HIPPO and wingless/Integrated **(**WNT) and peroxisome-proliferator-activated receptors (PPAR). An increased expression levels of a number of proapoptotic and tumour suppressor genes including TGFβ3, GZMB, SOCS1 and GSTM2 and a decreased expression levels of oncogenes such RXRB, CSNK2B, RALB, GLI2, ARHGAP11A and TERC, were observed in SMEK2 overexpressing cells, suggesting a tumour suppressive role for SMEK2. However, some oncogenes also showed increased expression in SMEK2 cells such as c-MYC, TFC19, and Twist1, casting doubt on the role of SMEK2 as a tumour suppressor. More importantly, SMEK2 overexpression was associated with an increased levels for GSDMD, the effector of the inflammatory form of cell death, pyropotosis (Gao et al.,2018). Some studies have showed that pyroptosis promotes inflammatory cell death of cancer and inhibits proliferation and migration of cancer cells (Xia et al.,2019), however, emerging evidence implicates pyroptosis in the formation of a suitable microenvironment that promotes tumour growth (Gao et al.,2018).

In addition to cancer related processes, SMEK1 overexpression affected genes annotated to structural constituent of ribosomes, graft-versus-host disease, allograft rejection, autoimmune thyroid disease and type I diabetes mellitus pathways. The RNA sequencing results also implicated SMEK2 in processes such as immune response, mRNA transport and stability, viral infection, regulation of gene expression at transcriptional and post-transcriptional levels, graft-versus-host disease, allograft rejection, autoimmune thyroid disease, type I diabetes mellitus, PPAR signalling pathway, mRNA surveillance pathway, and

antigen processing and presentation. SMEK2 overexpression also led to perturbation in biological pathways such Herpes simplex virus 1 infection, RNA transport and pathways of neurodegeneration multiple diseases.

The metanalysis of across SMEK1 and SMEK2 overexpression RNA expression datasets further confirms that both SMEK1 and SMEK2 exert some overlapping and non-overlapping effects and supported the concept that both genes are not functionally redundant. Analysis of SMEK1 specific differentially expressed genes revealed a role for SMEK1 in maintaining the functional integrity of the mitochondrial ribosome, RNA processing and splicing, regulation of transcription, proteasome function and ribosomal structure. On the other hand, SMEK2 specific deregulated genes revealed a role for SMEK2 in RNA protection, inflammatory response, immune response and viral infection. SMEK2 role in oncogenesis appears to be different from the tumour suppressive role of SMEK1. In addition to the increase expression levels of tumour suppressors and decrease expression of oncogenes, overexpression of SMEK2 was also associated the overexpression of some oncogenes including c-MYC. Interestingly, Data available on Protein Atlas showed that SMEK2 is a prognostic marker in renal cancer where its high expression is associated with poor prognosis (Protein Atlas; 2021). It is possible that SMEK2 acts as dual functional gene and have both oncogenic and tumour-suppressor function.

6.4 Concluding Remarks and Future Perspectives

The present study investigated the role of SMEK1 and SMEK2 in the regulation of cell fate decision of leukemic cells. The study has presented novel findings implicating both

proteins in the regulation of 1) cell death and survival, 2) regulation of the activity of five core cancer signalling pathways and 3) gene expression at transcriptional, post-transcriptional and epigenetic levels. Some of the effects of SMEK1 and SMEK2 appeared to be mediated via their interaction with PP4c, while other functions were independent of PP4c. In addition, the present study confirmed that both SMEK1 and SMEK2 have overlapping, and non-overlapping functions and they do not seem to be functionally redundant.

Together, findings form the present and previous studies suggest that both SMEK1 and SMEK2 play multifaceted roles which control a variety of cellular phenomena. In the light of the significant impact of SMEK1 and SMEK2 in leukemic and other cells, it is obvious that a more intensive study should be pursued to achieve better understanding their molecular function and their involvement in cancer and other diseases. Further studies are required to investigate the regulatory axis involving SMEK2-JAK/STAT and SMEK1/2 -TGFβ signalling pathways. More studies are required to understand the role of SMEK2 in oncogenesis and its dual function as tumour suppressor and oncogene.

The observation that SMEK1 overexpression leads to the decrease expression of the oncogene HRAS, one of the most frequently mutated gene family in cancers and described as 'undruggable' could be harnessed for the development of therapeutic materials to target HRAS. For example, discovering the part of SMEK1 that interacts with RAS could lead to the development of molecules that mimics SMEK1 effects. Understanding the interaction between SMEK1 and HRAS could lead to the development of synthetic inhibitor specific to HRAS.

The present study confirmed the tumour suppressive role for SMEK1 and previous studies have reported the loss of SMEK1 expression in many types of cancer. Future studies

should offer transformative insights into the underlying mechanisms that lead to the loss of the expression and the function of SMEK1 in cancer.

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Appendices

Appendix A: List of Publications

Frank F, Kavousi N, Bountali A, Dammer EB, Mourtada-Maarabouni M, Ortlund EA. 2020. The lncRNA Growth Arrest Specific 5 Regulates Cell Survival via Distinct Structural Modules with Independent Functions. Cell Rep, 107933, vol. 32(3).

Kavousi, N. and Mourtada-Maarabouni, M. (2019) Protein Phosphatase 4 Regulatory Subunit 3A regulates leukemic cell survival independent of PP4 catalytic subunit. National Cancer Research Institute. Available from https://abstracts.ncri.org.uk/abstract/protein-phosphatase-4-regulatory-subunit-3a-regulates-leukemic-cell-survival-independent-of-pp4-catalytic-subunit/

Appendix B: Clonogenic Assay Images

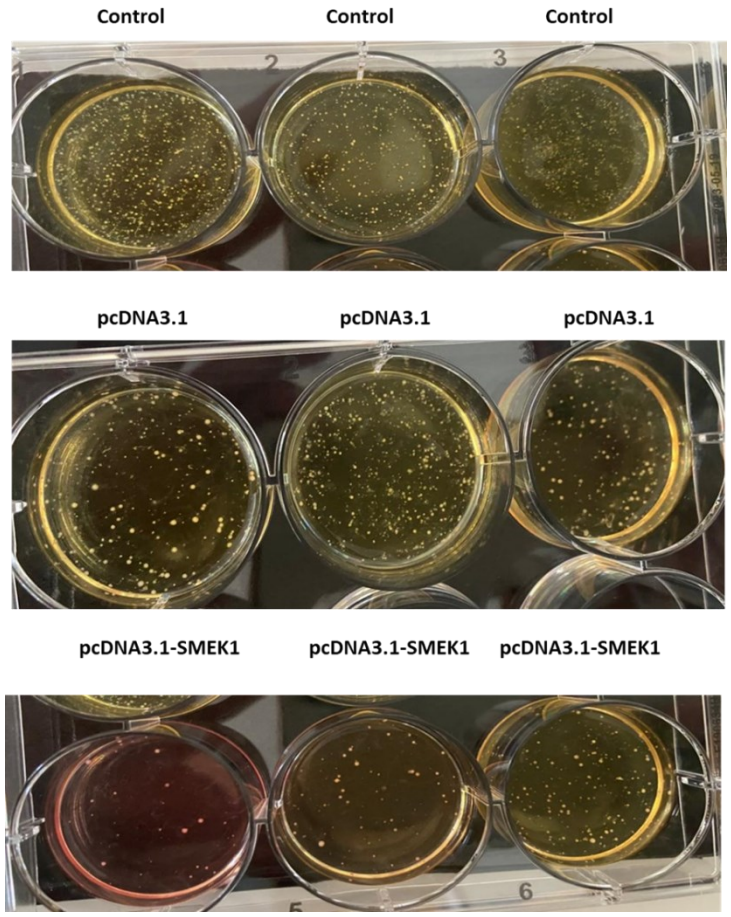

Figure B.1 SMEK1 overexpression decreases colony-forming ability in Jurkat cells. An example image of colony forming assay plate in cells transfected with pcDNA3.1-SMEK1. 24 hours after transfection, cells were cloned in soft agar and long-term survival and proliferation of Jurkat cells which were transfected with mock transfected cells (control), pcDNA3.1 only and pcDNA3.1-SMEK1 and were assessed after incubating for 2-3 weeks.

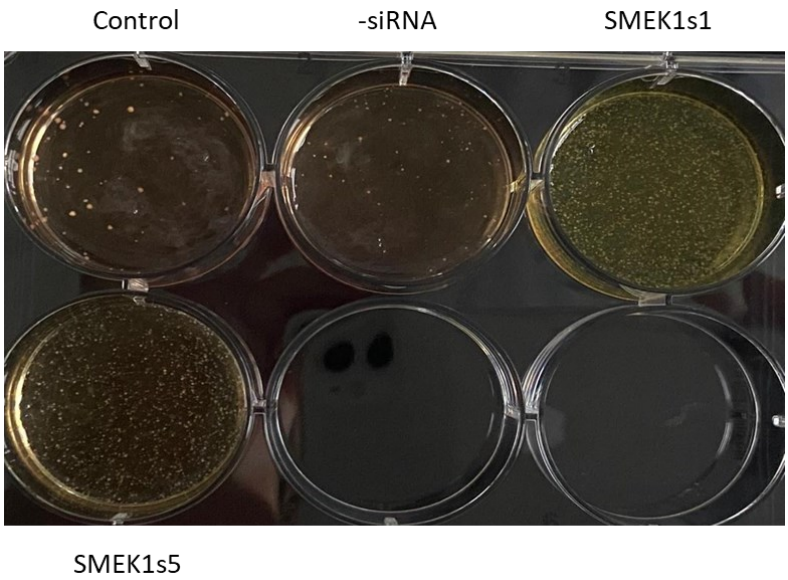

Figure B.2 SMEK1 down regulation increases colony-forming ability of Jurkat cells. An example image of colony forming assay plate using SMEK specific RNA. 24 hours after transfection, cells were cloned in soft agar and long-term survival and proliferation of Jurkat cells which were transfected with mock transfected cells (control), control cells received negative siRNA (-siRNA), SMEK1s1 and SMEK1s5 siRNAs were assessed after incubating for 2-3 weeks.

Appendix C: RNA quality control

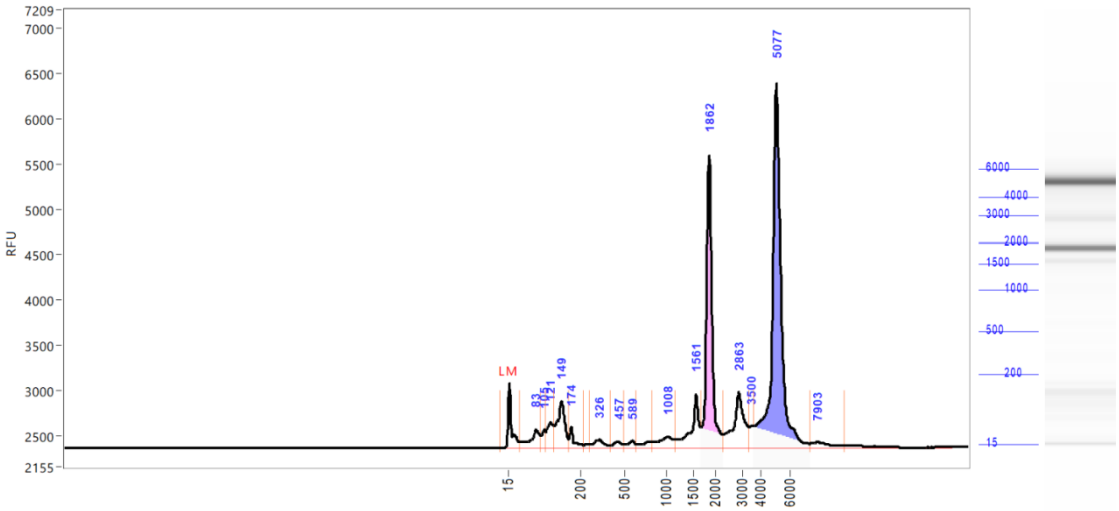

Figure C.1 Relative intensity of 28s/18s rRNA in Jurkat cells transfected with pcDNA3.1. Total RNA was isolated using Direct-zol RNA miniprep kit (Zymo research). 1µg RNA samples were sent for RNA sequencing with samples tested prior. Image shows relative fluorescence units for 28s/18s with a ratio of 2.0, suggesting good quality rRNA.

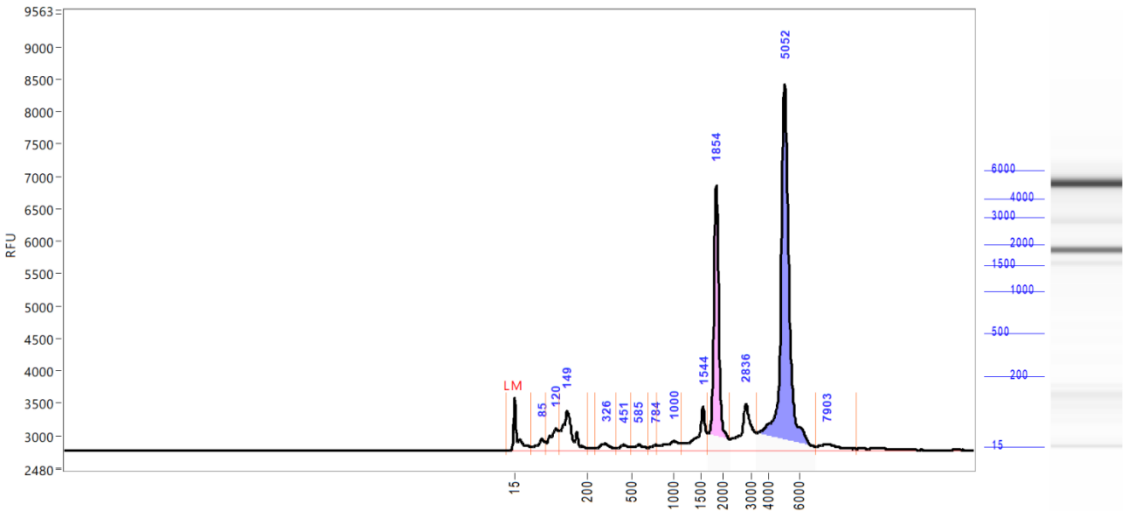

Figure C.2 Relative intensity of 28s/18s rRNA in Jurkat cells transfected with pcDNA3.1-SMEK1. Total RNA was isolated using Direct-zol RNA miniprep kit (Zymo research). 1µg RNA samples were sent for RNA sequencing with samples tested prior. Image shows relative fluorescence units for 28s/18s with a ratio of 2.1, suggesting good quality rRNA.

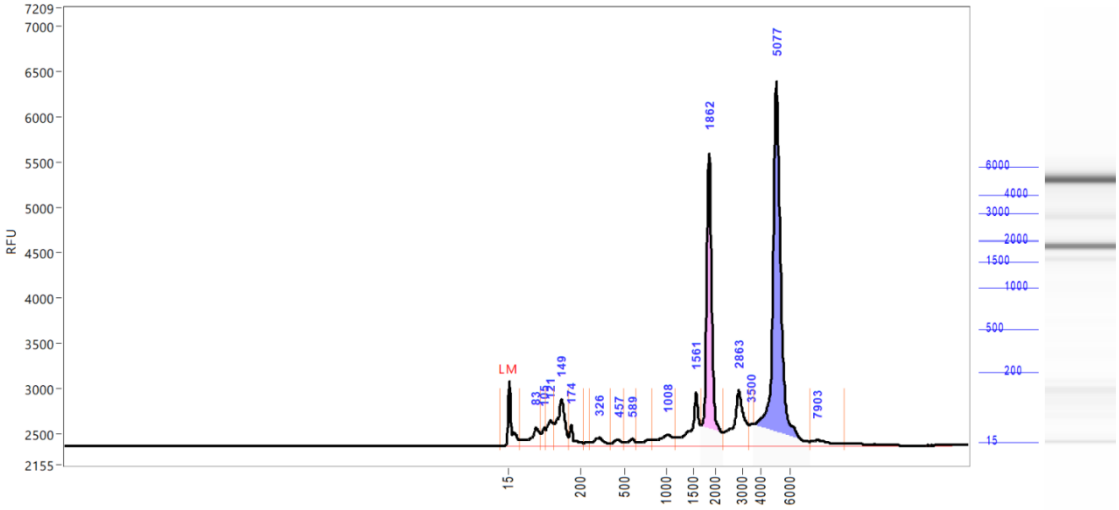

Figure C.3 Relative intensity of 28s/18s rRNA in Jurkat cells transfected with pcDNA3.1-SMEK2. Total RNA was isolated using Direct-zol RNA miniprep kit (Zymo research). 1µg RNA samples were sent for RNA sequencing with samples tested prior. Image shows relative fluorescence units for 28s/18s with a ratio of 1.8, suggesting good quality rRNA.