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New Insights into the functional role of protein phosphatase 4 regulatory subunit PP4R3A/SMEK1 in the regulation of leukemic cell fate

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Abstract

The serine/threonine protein phosphatase 4 holoenzyme consists of a PP4 catalytic subunit (PP4c), which interacts with four different regulatory subunits. Previous studies have shown that PP4c acts as a tumour suppressor. Emerging evidence suggests that the protein phosphatase 4 regulatory subunits might regulate cell fate independently of PP4c. To this end, we investigated the role of PP4R3A (SMEK1) in Jurkat and CEM-C7 leukemic cell lines. SMEK1 overexpression decreased cell growth, increased spontaneous apoptosis, and reduced the colony forming ability of leukemic cells. Conversely, siRNA-mediated silencing of SMEK1 led to increased short and long-term survival in these cells. Phospho-protein arrays revealed that increased expression of SMEK1 affected the phosphorylation of key proteins involved in MAPK3, AKT, JAK/STAT, NFκB and TGFβ signalling pathways. These proteins include transcription factors such NFκB, STAT3, c-JUN, SMAD1, and SMAD5, suggesting a role for SMEK1 in the regulation of gene expression. RNA sequencing confirmed the role of SMEK1 in the regulation of gene expression. RNA sequencing also confirmed the tumour suppressor role of SMEK1. Taken together, this study shows that SMEK1 regulates leukemic T cell survival, indicating that SMEK1 dysfunction may be important in the development and progression of

leukaemia.

Key words: PP4R3A; SMEK1; apoptosis; phosphorylation arrays; RNA sequencing; TGFβ pathway; leukaemia

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 [1,2]. PP4 exists as a holoenzyme composed of a catalytic subunit (PP4c) in association with various regulatory subunits. Several PP4-specific regulatory subunits have been identified including PP4R1, PP4R2, PP4R3, PP4R3B and PP4R4 [2].

PP4c has been steadily studied over the past decade and was shown to undertake many vital functions in human cells including the control of apoptosis, cell proliferation and mutation rate [3,4]. Several studies have implicated PP4c in tumorigenesis and suggested that PP4c is a potential novel therapeutic target in cancer [5]. Studies have also documented the importance of the interaction of PP4c with its different regulatory subunits in controlling the activity of the holoenzyme [2]. Given the importance of PP4 regulatory subunits in controlling PP4c activity and dictating localisation and substrate specificity, it is surprising that the molecular function and targets of these regulatory subunits have not been extensively studied to date.

Human PP4R3 exists in two isoforms: PP4R3A/SMEK1 and PP4R3B/SMEK2, encoded by two different genes which in turn encode two proteins that share ~70% sequence identity. 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 [2]. SMEK1, the most abundant of the two isoforms, is encoded by a gene located on chromosome 14q32.12 while SMEK2 gene is located on chromosome 2p16. SMEK1 and SMEK2 proteins have a molecular mass of 95kDa and are composed of 820 and 849 amino acids (aa), respectively [2]. Both regulatory subunits are shown to form complexes with PP4c-PP4R2, the major form of the holoenzyme PP4 [2]. Binding of both proteins to PP4c confers substrate specificity, sub-cellular localisation and holoenzyme stability and has been shown to be important in targeting PP4c to the centromeres [2]. 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 [6,7].

There is accumulating evidence that SMEK1 is a critical regulator of several physiological processes such as neuroinflammation [8], neuronal development and differentiation [9], and embryonic stem cell pluripotency maintenance by silencing Wnt-responsive development-related genes [10]. Emerging evidence supports a role for SMEK1 as a tumour suppressor in ovarian cancer. SMEK1 expression levels are significantly reduced in the tissues of patients with ovarian and cervical cancer [11,12]. SMEK1 is reported to inhibit the proliferation of ovarian cancer cells OVCAR3 by binding and activating the tumour suppressor protein P53 [11]. 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 too.

Collectively, research so far reveals a complex role for SMEK1 in 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. While it is well-established that PP4c exerts profound effects on the growth and survival of both normal and

leukemic human T-cells [4] and previous studies have implicated SMEK1 in the regulation of several cellular processes that regulate cancer cell survival, the role of SMEK1 in the regulation of leukemic cell fate has not yet been investigated.

This study investigates the functional effects of SMEK1 on the survival of leukemic T cells. Through functional analysis, we investigated the role of SMEK1 in the regulation of both short- and long-term cell survival and cell death. Using Phosphorylation Pathway Profiling Arrays, we examined the change in the phosphorylation levels of proteins involved in five core cancer pathways in response to altered SMEK1 expression. Furthermore, RNA sequencing of the whole transcriptome was used to investigate gene expression changes and identify key molecular pathways modulated in cells following SMEK1 overexpression.

1. Materials and methods

2.1 Cell culture

The human leukemic T cell lines, Jurkat and CEM-C7 [13,14] were cultured at 37°C in a 5% CO_2 humidified incubator 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 per week with fresh complete medium at a ratio of 1:10. All experiments were carried out using cells in their logarithmic growth phase.

2.2 Plasmid transfection

The following plasmid vectors were purchased from GenScript: pcDNA3.1, pcDNA3.1- SMEK1 (Accession # NM_001284280), and pcDNA3.1-PP4c (Accession #002720.2). pcDNA3.1-SMEK1, pcDNA3.1-PP4c expression constructs, or the vector alone pcDNA3.1, were introduced into 10^7 human leukemic T-cells by electroporation (5 µg DNA at 248 V (CEM-C7) and 293 V (Jurkat), 1050 µF in 0.4 cm cuvettes (Biorad) at room temperature [4]. Efficiency of transfection was 65–75% for CEM-C7 and Jurkat cells, respectably. The level of expression of the transfected gene was monitored by western blot analysis in all cases (see 2.4, below).

2.3 RNA interference

The experiments were performed using electroporation as a method of transfection [4]. The siRNAs used included the Silencer© Negative control siRNA (Ambion, #4611), and two different SMEK1 specific siRNAs: SMEK1 siRNA1 targeting exon 3 (Qiagen # SI04294955) and SMEK1 siRNA5 targeting exon 4 (Qiagen # SI05069960). Cells were transfected with 20 nM siRNA 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 5 ml of complete Iscoves medium supplemented with 20% heat inactivated FBS, 2 mM L-glutamine and 200 µg/ml gentamycin (Sigma). 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. Efficiency of transfection was 65–75% as determined by Cy3 labelling [4]. The level of SMEK1 expression was assessed after 48 h by western analysis (see 2.4, below).

2.4 Analysis of protein levels expression by western blotting

10^6 cells were washed twice in PBS and lysed in 50 µl RIPA buffer (radioimmunoprecipitation assay buffer; ThermoFisher Scientific, #89900) containing

protease inhibitor (at a 1:100 dilution; Millipore, #535140) and incubated on ice for 30 min before centrifugation at 10,000 × g for 10 min. The protein content of the supernatant was quantified using the Coomassie Plus™ protein assay reagent (PIERCE; # 1856210). Protein samples (50 µg) were boiled for 10 min in 2X laemmli buffer (Bio Rad, #1610747) containing 10% β-mercaptoethanol (Sigma; # 60242) and incubated at 95^{o}C for 4 minutes. Samples were loaded on 10% or 12% (dependent on the size of target protein; Biorad Mini-PROTEAN® TGX™ Precast Protein Gels # 4561033 and 4561043, respectively) and then electrotransferred onto a polyvinylidenedifluoride membrane (Biorad). The blots were probed with either the anti-SMEK1 (1:1000; Santa Cruz Biotechnology, #Sc-244184), anti-PP4c (PPX/PP4 C-6; 1:1000; Santa Cruz Biotechnology, #Sc-376410) or with anti-Bad antibody (1:500; Santa Cruz Biotechnology # Sc8044), followed by the appropriate horse-radish peroxidase-conjugated secondary antibodies. Blots were stripped using ReBlot Plus Kit (Chemicon; # 2500) and re-probed with anti-β-actin antibody (diluted 1:5000; Sigma, # A5441) for the purpose of normalisation. The secondary antibodies used were anti-Goat IgG HRP conjugate (diluted 1:1000; Thermo Scientific #A32849), anti-mouse immunoglobulin (diluted 1:800; Dako, # P0447). Protein bands were visualised by enhanced chemiluminescence (ECL) using Clarity™ Western ECL Substrate according to the manufacturer's instructions (Bio Rad, #1705060S). Western Blots were imaged using either a C-DiGit® Blot Scanner (LICOR®) 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.

2.5 Determination of cell survival and apoptosis

At 24h post-transfection with plasmids or at 72h post-transfection with siRNAs, cells were cultured for 24 and 48h before being used to determine cell viability and apoptosis. Flow cytometry was used to determine cell count and viability using the Muse® Count and Viability Kit (Luminex; #MCH100104) and the Muse® Cell Analyser (Merck Millipore, Darmstadt, Germany), as described previously [15]. Apoptosis was routinely determined by assessment of nuclear morphology by fluorescence microscopy after staining with acridine orange (25 µg/ml); cells containing condensed or fragmented chromatin were scored as apoptotic. Apoptosis was also measured by flow cytometry using a Muse annexin V and 'dead cell assay kit, according to the manufacturer's protocol (Luminex, #MCH100105). Long-term survival of cells transfected with SMEK1, or with SMEK1 siRNAs, was assessed by the ability of the cells to form colonies in soft agar as described previously [4].

2.6 Cell cycle analysis

Cell cycle analysis was assessed by flow cytometry following nuclear propidium iodide staining using the Muse cell cycle kit (Luminex, #MCH1001060), as described previously [15]. Transfected cells were plated in fresh medium at 5×10^5 cells/well in 4 ml medium in 6-well plates. Following incubation for 24h, cells (~ 10^6) were suspended in 200 µl phosphate buffered saline (PBS), and fixed in 1 ml ice-cold 70% ethanol/30% PBS. Cells were incubated at − 20 °C for at least 3 h. Cells were then re-suspended in 200 µl of Muse™ Cell Cycle Reagent and incubated for 30 min in the dark before data was acquired using the Muse Cell analyser.

2.7 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 in Jurkat and HEK293T cells overexpressing SMEK1. Samples were lysates of HEK293T cells transiently overexpressing SMEK1 (Reference Sequence NP_115949) purchased from OriGene Technologies GmbH. Lysates from cells transfected with the empty vector pcDNA3.1 was used as control. Lysates from Jurkat T cells transfected with SMEK1 construct or empty vector pcDNA3.1 expressing SMEK1 were prepared as described in Section 2.4. 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. signals were detected using a Bio-Rad ChemiDoc MP Imaging System. Spot intensities were analysed using Image Lab 6.0.1 for Windows software.

2.8 RNA sequencing

Global gene expression changes in response to SMEK1 overexpression were determined by sequencing of the whole transcriptome. Total RNA was extracted from cells using the Direct-zol™ RNA MiniPrep kit (ZYMO RESEARCH, Cat # R2050), according to the manufacturer's standard protocol. The assessment of RNA purity and the quantity was performed by spectrophotometric analysis (NanoDrop™ 1000, ThermoFisher Scientific). Samples with NanoDrop 260nm/280nm absorbance ratio between1.8-2 and with RNA Quality Number (RQN) values of 9.2-9.6 were considered of high quality. 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 sequenced with 150 bp PE read metric, on the

HiSeq 4000 platform to a depth of approximately 30 million reads per sample, Raw sequencing data was trimmed of sequencing adapters and low-quality reads discarded using the Trimmomatic package package (Galaxy version 0.38.1), a wrapper that incorporates CutAdapt and FastQC: sliding window = 4, average quality threshold = 20. Quality controlled reads were aligned to Human Genome build (hg38) using HISAT2 (Galaxy version 2.1.0), a fast splice junction mapper for RNA reads. Transcripts were assembled using Stringtie (with GTF support from hg38.ens.Gene.gtf) and the number of reads mapping to each feature counted and expressed as FPKM. 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 overexpression.

Statistical Analysis

GraphPad Prism 7 (GraphPad Software) was used to perform statistical analyses. Data were presented as the mean ± Standard Error Mean (SEM); the number of observations (n) refers to different experiments with each experiment being conducted on a separate culture of cells. Data analysis was by one-way analysis of variance with Bonferroni's multiple comparison test (MCT). Differences were considered statistically significant when p-value was <0.05.

Results

<u>Modulation of SMEK1 expression levels MIAT knockdown affects basal apoptosis and cell survival in leukemic T cells</u>

To investigate the role of SMEK1 in the regulation of T cell survival, SMEK1 was over-expressed in leukemic Jurkat T cells by transfection with pcDNA3.1-SMEK1

Accession # NM_001284280) or pcDNA3.1 empty vector. The effects of SMEK1 overexpression on short-and long-term survival, apoptosis, and cell cycle profile were determined. Transfection of SMEK1 into Jurkat leukemic T cells resulted in a 2-fold increase in SMEK1 protein levels (Figure 1a). The increase in SMEK1 protein levels was associated with a significant 3-fold increase in basal apoptosis (Figure 1b). Overexpression of SMEK1 also resulted in a significant decrease in both total and viable cells (~ 50% decrease in the number of total and viable cells in the cells overexpressing SMEK1 compared to control (Figure 1c)). The increase in apoptosis and decrease in total and viable cell number caused by SMEK1 overexpression was associated with a significant reduction in the long-term survival of these cells as evidenced by a decrease in their colony forming ability (Figure 1d).

To determine whether the growth suppression produced by SMEK1 was due to cell cycle arrest, cell cycle analysis was performed using propidium iodide staining and flow cytometry. Results revealed that the proportion of cells in the G1, S and G2 phases in SMEK1 overexpressing cultures was consistently lower than that in the control (Figure 1e). 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 was indeed increased and suggesting that the growth suppression by SMEK1 was due to increased apoptosis.

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 2a). The 2-fold increase in SMEK1 protein levels in CEM-C7 cells was associated with an increased in basal apoptosis (Figure 2b), a decrease in total and viable cell number (Figure 2c), and reduction in long-term survival (Figure 2d),

confirming the growth inhibitory role for SMEK1 in these leukemic T cells. Cell cycle analysis for CEM-C7 cells transfected with SMEK1 confirmed the observations made in Jurkat T cells. The results showed that the proportion of cells in SMEK1 overexpressing cultures was consistently lower in G1, S and G2/M phases (Figure 2e). In line our observations in 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 2e).

Intrinsic apoptosis is induced by cellular stress, leading to the activation of the pro-apoptotic members of the 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 [16]. Previous studies have reported that the increased in protein expression levels of the catalytic subunit of PP4 (PP4c) lead to a decrease in the phosphorylation levels of the pro-apoptotic protein BAD but with no effect on the BAD total protein levels. We investigated the possibility that the increase in cell death and inhibition of cell survival induced by SMEK1 overexpression might be due to a change of Bad protein expression levels. Results revealed that overexpressing SMEK1 in Jurkat and CEM-C7 cells led to significant 2-fold increase in Bad protein expression levels relative to control in both cell lines (Figure 3a and b), highlighting a difference in the effects of PP4c and SMEK1 and implicating BAD in SMEK1-induced apoptosis.

To further investigate SMEK1 function in CEM-C7 and Jurkat leukemic T cells, specific SMEK1 siRNAs, SMEK1s1 and SMEK2s5, were used to inhibit endogenous SMEK1 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. Both SMEK1 specific siRNAs (SMEK1s1 and SMEK1s5) reduced SMEK1 protein levels in Jurkat and CEM-C7 cells by ~50% (Figure 4a and b). SMEK1 knockdown did not affect basal apoptosis in either cell line (results not shown) but was associated with an increase in total and viable cell number (Figure 4c and d) and an increase in colony forming ability in both cell lines (Figure 4e and f). SMEK1 down-regulation had no effect on the cell cycle profile of Jurkat and CEM-C7 T cells (results not shown). These findings further confirm the role of SMEK1 in the regulation of the survival of Leukemic cells.

Previous studies reported that the stability/expression level of 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 in Jurkat and CEM-C7 leukemic T cells, similar to the effects observed in cells with reduced SMEK1 levels. Therefore, we investigated whether silencing SMEK1 affected PP4c protein levels. As shown in Figure 5a and b, protein levels of PP4c were significantly reduced in CEM-C7 and Jurkat T cells transfected with SMEK1 siRNAs, suggesting that interaction with SMEK1 maintained stability of PP4c.

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

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 [17]. Protein phosphorylation regulates the activity of these signalling pathways in multiple ways including regulation of biochemical activity of host proteins and reversible formation of protein complexes. We therefore investigated the

underlying mechanisms by which SMEK1 modulated the survival in Leukemic T cells using a Human Phosphorylation Pathway Profiling Array. This phosphorylation array permits assessment of 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.

The first set of experiments which aimed to explore the effects of SMEK1 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 [3]. Lysates from HEK293T cells overexpressing SMEK1 (Reference Sequence NP_115949) were obtained commercially together with lysates from cells transfected with empty vector pcDNA3.1 (purchased from OriGene Technologies GmbH). HEK293T cell lysates were applied to the phosphorylation pathway profiling array before analysing the effects of SMEK1 overexpression on MAPK, AKT, JAK/STAT, NFκB and TGFβ signalling pathways. SMEK1 overexpression did not affect the phosphorylation of proteins involved in the MAPK pathway apart from a 2-fold decrease in the phosphorylation of Mitogen-and-stress-activated kinase 2 (MSK2), also known as Ribosomal protein S6 kinase alpha-4 (Figure 6a). 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 [18]. SMEK1 overexpression affected the AKT signalling pathway as shown by the significant reduction in the phosphorylation level of the proline-rich AKT substrate of 40kDa (PRAS40) (Figure 6b). 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 6c shows that SMEK1 overexpression did not affect the phosphorylation levels of key proteins in the JAK/STAT. In contrast, SMEK1 overexpression had significant effects on the NFkB pathway (Figure 6d). Figure 6C shows that SMEK1 overexpression had no effects on the phosphorylation level of NFκB 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 3-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 6d). Decrease in the phosphorylation of these proteins is associated with a decrease in the activation of NFκB [19]. 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 propagates the signal through intracellular mediators known as SMADs [20]. 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 6D). 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 6e).

The preliminary investigation using HEK293T cells have indicated that SMEK1 had no effect on the regulation of JAK/STAT pathway but is involved in the regulation of the MAPK, AKT, NFκB and TGFβ signalling pathways. All these signalling pathways are involved in the regulation of gene expression, cell survival and proliferation and

are dysregulated in cancer [21]. Further experiments were carried out to investigate whether SMEK1 is implicated in the regulation of these major pathways in leukemic T cells. The results showed that the effects of SMEK1 overexpression on these major signalling pathways in leukemic T cells were different from those observed in HEK293T cells. Overexpression of SMEK1 in Jurkat T cells was associated with an overall reduction in the phosphorylation of all the proteins involved in MAPK and AKT signalling pathways (Figure 7A and B). Unlike the effects observed in HEK293T, SMEK1 overexpression in Jurkat T cells was associated with significant changes in phosphorylation levels of proteins involved in the JAK/STAT pathway. There was a significant increase in the phosphorylation levels of STAT1 and a reduction in the phosphorylation levels of the other proteins in the pathway including STAT2, 3, 5 and 6 (Figure 7C). NFκB pathway was also negatively affected by overexpression of SMEK1 in Jurkat T cells. All the proteins in the pathway showed decrease in the level of phosphorylation in Jurkat T cells, implying that SMEK1 exerts inhibitory effects on this pathway (Figure 7D). The effects of SMEK1 overexpression on TGFβ pathway in Jurkat T cells was associated with an increase in the phosphorylation of Suppressor of Mothers against Decapentaplegic **(**SMAD) proteins in the pathway (SMAD1, SMAD2 and SMAD5) (Figure 7E). SMEK1 overexpression was also associated with decrease in the phosphorylation of the activating transcription factor 2 (ATF2) factor phosphorylation of c-Fos and c-Jun (Figure 7E). Together c-Fos and c-Jun forms the dimeric transcription factor AP1 which is involved in inflammation, proliferation, differentiation, and apoptosis [22].

Further experiments were carried out to investigate if the effects of SMEK1 on the signalling pathways were also observed in PP4c overexpressing cells. This is important to establish if SMEK1 exerts its effects independently of the catalytic

subunit of PP4, PP4c. Jurkat cells were transiently transfected with pcDNA3.1-PP4c and cell lysates were prepared 48-hours post transfection before being applied to phosphorylation pathway profiling arrays. The results showed that the effects of PP4c overexpression on MAPK, AKT, JAK/STAT and NFkB were similar to those observed in SMEK1 overexpressing cells (Supplementary Figure1 a,b,c,d). There were some differences between the effects of SMEK1 and PP4C overexpression effects on TGFβ pathway. Like the changes observed with SMEK1, PP4c overexpression led to a decrease in the phosphorylation of c-Fos and c-Jun (Supplementary Figure 1e). In contrast to the effects of SMEK1 on SMAD proteins, there was no increase in the phosphorylation of SMADs in the cell overexpressing PP4c (Supplementary Figure 1e).

Overall, the results indicated that SMEK1 affects the phosphorylation of key proteins in the five major signalling pathways involved in the regulation of proliferation, survival and cell death. With the exception of the effects on SMADs proteins, the effects of SMEK1 are similar to those resulting from PP4c overexpression. These results suggests that SMEK1 function could be dependent on the activity of PP4c in regulating MAPK, AKT, JAK/STAT and NFκB signalling pathways and that SMEK1 affects the TGFβ pathway independently of PP4c. Interestingly, an important observation emerged from these results is the effects of SMEK1 on the phosphorylation of different transcription factors including NFkB, STATs, c-jun and SMADs proteins, highlighting a role of SMEK1 in the regulation of gene expression. These observations were further confirmed using western blotting using and the relevant phosphoantibody. The results confirmed that SMEK1 overexpression was associated with a decrease in the phosphorylated levels of NFκB (Figure 8a), STAT3 (Figure 8b) and c-Jun (Figure 8c) and an increase in the phosphorylation levels of

SMAD1 (Figure 8d) and SMAD5 (Figure 8e).

RNA Sequencing reveals cancer and transcription regulation -related differentially expressed pathways and perturbed biological processes following 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 might excert its biological effects, RNA sequencing was performed and the genes that exhibited the most pronounced expression changes in response to SMEK1 overexpression identified. Transfection of Jurkat T cells with pcDNA3.1-SMEK1 resulted in a 1.7 fold increase in SMEK1/PP4R3A (Supplementary Figure 2). Following confirmation of SMEK1 overexpression, we next investigated those genes that were deregulated by a factor of at least 1.5-fold ($\log2 \geq 0.58$) between the control and SMEK1 transfected samples. 6274 differentially expressed genes were identified out of a total of 22954 genes with measured expression. There was no effect on the expression levels of its PP4R3 isoform SMEK2, PP4c nor the other PP4 regulatory subunits. Interestingly, top upregulated genes include small nucleolar RNA C/D box 3A and 3B-1 (SNORD3A and SNORD3B-1), ribosomal proteins S9 and S18 (RPS9, RPS18), and SIN3-HDAC complex associated factor (SINHCAF3) which is involved in the repression of genes involved in the TGFβ signalling pathway [23], (Supplementary Figure 2 and Supplementary Table 1). Among the most down-regulated genes in SMEK1 overexpressing cells included proteasome 20S subunit beta 3 (PSMB3) which encodes the 20S core subunit of the proteasome, and three genes associated with oncogenic activities and promotion of cell survival: Rho GTPase activating protein 11A (ARHGAP11A), nuclear pore complex interacting protein family, casein

kinase 2 beta (CSNK2B) and HRAS proto-oncogene, GTPase (HRAS) (Supplementary Figure 2 and supplementary Table 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 [24]. 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 [25,26]. CK2 regulates several signalling pathways including PI3K/AKT, NFκB and Wnt [27] and is implicated in cancerous transformation and is a therapeutic target in anti-cancer therapy. HRAS, a member of the RAS protooncogenes, 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 [28,29]. HRAS significant decrease in SMEK1 overexpressing cells provides strong evidence of SMEK1 tumour suppressive role. 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. Other non-coding RNAs affected include 76 Natural Antisense Transcripts (NATs), 30 long intergenic RNAs (lincRNAs) and 150 pseudogenes.

As an excess of 6274 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 impacted process (Figure 9; Table 1). Among the perturbed cellular processes, numerous transcription related processes were significantly affected, including negative regulation of metabolic process, 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 (Figure 9; Table 1).

Table 1. Top perturbed Gene Ontology terms in response to SMEK1 overexpression in Jurkat T cells. Number of differentially expressed genes annotated to the listed biological processes.

Biological Process	**Number of Genes (Differentially Expressed/All genes in the pathway)**
Macromolecule metabolic process	382 / 7324
Gene expression	254 / 4719
Negative regulation of metabolic process	129 / 2526
Negative regulation of gene expression	111 / 1795
RNA Processing	112/1119
Posttranscriptional regulation of gene expression	73/ 673
Defence response	66/973
Regulation of gene expression-epigenetic	67 / 405
Gene silencing by RNA	65 / 269
Gene silencing	65/ 340
Posttranscriptional gene silencing	53 / 255

Gene silencing by miRNA	87 / 1119

An excess of 200 biological pathways was affected by overexpression of SMEK1. The top nine affected pathways 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 (Figure 10a)). microRNAs in cancer was the top perturbed biological pathway with 17 genes differentially expressed (Figure 10b). Perturbed signalling pathways related to cancer includes apoptosis, MAPK, mTOR, NFκB, TGFβ and RAS signalling pathways (Table 2). Differentially expressed genes that impacted cancer related pathways 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 2. Perturbed cancer related signalling pathways in cells overexpressing SMEK1. GZMB: granzyme B; TUB8A: tubulin alpha 8; NTRK1: neurotrophic receptor tyrosine kinase 1; HRAS: HRas proto-oncogene, GTPase; CALML4: calmodulin like 4; RALB: RAS like proto-oncogene B; GNG8: G protein subunit gamma 8; AFDN: adherens junction formation factor; NTF4: neurotrophin 4; WNT3: Wnt family member 3;TGFB3: transforming growth factor beta 3; MAPT: microtubule associated protein tau; IL1B: interleukin 1 beta; MAPK13: mitogen-activated protein kinase 13; COL9A3: collagen type IX alpha 3 chain; PP2R3B : protein phosphatase 2 regulatory subunit B"beta; CSNK2B : casein kinase 2 beta; ICAM1: intercellular adhesion molecule 1; SMAD7: SMAD family member 7

Signalling Pathway	Up regulated genes	Down-regulated genes
Apoptosis	GZMB TUBA8 NTRK1	HRAS
RAS	NTRK1 CALML4	HRAS RALB GNG8 ADDN NTF4
mTOR	-	HRAS WNT3
MAPK	TGFB3 NTRK1	HRAS MAPT IL1β MAPK13
PI3K/AKT	COL9A3 NTRK1	HRAS PPP2R3B
NFkB	-	CSNK2B ICAM1 IL1β
TGFβ	TGFB3	SMAD7

Discussion

PP4R3 or Suppressor of Mek Null (SMEK), an evolutionary conserved protein family, consists of two isoforms, SMEK1 (PP4R3A, PP4R3α) and SMEK2 (PP4R3B,

PP4R3β). Both SMEKs play an important a role in the formation of a holoenzyme with PP4c, PP4R1, and PP4R2 complex [30]. SMEK1 has been shown to regulate cell fate decisions and to function as a tumour suppressor gene in prostate and ovarian cancer [31]. Studies have shown that SMEK1 expression level is decreased in ovarian and cervical tumour tissues and ovarian cell lines, and its gene found to be hypermethylated in ovarian cancer [31]. 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. Therefore, the present study aimed to investigate the role of SMEK1 in the regulation of leukemic cell fate decision.

Functional analyses 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-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 [32]. 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. Collectively, the results confirm that SMEK1 has a tumour suppressor role and plays an important role in the regulation of leukemic cell survival. These effects agree with other studies investigating the role of SMEK1 in cancer. Kim et al (2015) reported that increased SMEK1 expression inhibited VEGF mediated cell growth and angiogenesis in human umbilical vein endothelial cells, SKOV-3 ovarian cancer cells, and xenograft human ovarian tumour model. Other studies have shown that SMEK1 enhanced the pro-

apoptotic activity of chemotherapeutic drugs in aggressive ovarian carcinoma cells and mouse xenograft models and exerted an additive effect on the inhibition of ovarian cancer cell growth by inducing apoptosis and affecting related gene expression levels and survival proteins activities such as suppression of RAS homolog enriched in the brain (Rheb) and mTOR [33]. Altogether suggesting a rather universally crucial role of SMEK1 in the regulation of cell fate decision.

The results of the Phosphorylation Pathway Profiling Array have provided evidence of a role for SMEK1 in the regulation of five core cancer signalling pathways. The initial experiments used HEK293T cells which have been used to express and purify the PP4 holoenzyme consisting of PP4c, PPP4R2 and PP4R3A/ SMEK1 [34]. The results showed that SMEK1 overexpression had no effect on the phosphorylation levels of the proteins involved in the JAK/STAT pathway, however the expression of several proteins involved in MAPK, AKT, NFκB and TGFβ signalling pathways were modulated or their phosphorylation state in the cells overexpressing SMEK1 altered, reflecting the complexity of the signalling pathways that are regulated by SMEK1. Experiments using Jurkat leukemic T cells revealed a different role for SMEK1 from those observed in HEK293T cells. SMEK1 overexpression exerted inhibitory effects on the MAPK, AKT, and NFκB pathways where most of the proteins showed reduced levels of phosphorylation compared to control. The effects of SMEK1 on the AKT pathway has been previously reported where an increased expression of SMEK1 was shown to prevent angiogenesis via the suppression of VEGFR-2-mediated activation of PI3K/AKT pathway in human ovarian tumours [35,36]. For the JAK/STAT pathway, an increased expression of SMEK1 was associated with enhanced phosphorylation of STAT1 and a 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 [37]. 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) [38]. 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 BCL-2, BCL-xL and the oncogene c-Myc [37]. Increased expression of SMEK1 resulted in a 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 [37]. The growth inhibitory effects of SMEK1 could be explained by its effects on the phosphorylation of STATs proteins. STAT1 phosphorylation by SMEK1 leads to its activation which results in the increase in the expression of genes that mediate cell death and cell growth inhibition [37]. 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 overexpression in AKT, MAPK, JAK/STAT and NFκB pathways were mirrored in the cells with increased expression in PP4c, suggesting that SMEK1 proapoptotic effects on these pathways could be mediated via their interaction with PP4c. These observations and the decrease in PP4c protein levels in the cells with reduced SMEK1 expression suggest that increased expression of SMEK1 in Jurkat T cells could lead to the enhancement of PP4c phosphatase activity and therefore to the dephosphorylation of protein targets in these pathways. Interestingly, PP4c has also been implicated in the regulation of MAPK pathway in Merkel cell polyomavirus (MCPyV) -induced tumorigenesis which is associated with

the aggressive skin cancer Merkel cell carcinoma (MCC). Expression of the small tumour antigen (ST) of MCPyV in the highly metastatic form of MCC promotes cell motility and migration by activating p38 MAPK signalling. Dobson et al., (2020) has shown that MCPyV ST-mediated p38 MAPK signalling occurs because of its interaction with 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 [39]. PP4c has also been shown to act as a negative regulator of NFκB activity in T lymphocytes [40].

Some of the changes induced by SMEK1 overexpression in Jurkat cells on TGFβ signalling pathway were mirrored by increased expression of PP4c, apart from the increased in the phosphorylation levels of SMAD1, 2 and 5, suggesting that the effects on SMADs were not dependent on SMEK1 interaction with PP4c. 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) [41,42]. 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 [43]. Activated SMAD2 is reported to inhibit the action of ubiquitous E3 ligase, leading to stabilisation of the cell cycle inhibitor p27 during cell cycle arrest in endometrial carcinoma cells [44]. 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 [45]. 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 [46]. The growth inhibitory and proapoptotic effects of SMEK1 in T-leukemic cells could be mediated, at least partly, through direct or indirect interactions with SMAD proteins leading to change in gene expression.

In addition to revealing a role of SMEK1 in the regulation of the activity of five cores cancer signalling pathways, the results highlighted that overexpression of SMEK1 saffected 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 determines cellular localization, stability, protein-protein interactions, and DNA binding and can either activate or inhibit their activity in turning on gene expression [47]. The phosphorylation state of some transcription factors were altered by the increased expression of SMEK1. These include NFκB, STAT3, c-JUN, SMAD1, and SMAD5. 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 [48]. Altogether, the results suggest that SMEK1 regulates gene expression at both epigenetic and transcriptional levels.

RNA sequencing results provided very useful and diverse insights into the role of SMEK1 in the regulation of gene expression and in a variety of cancer-related biological pathways. The results strongly implicated SMEK1 in the regulation of gene

expression, epigenetic control and RNA processing. Deregulated processes that were found to be associated with increased expression of SMEK1 included gene expression, post-transcriptional regulation of gene expression, epigenetic regulation of gene expression, transcriptional mis-regulation in cancer and gene silencing by miRNA. RNA sequencing revealed that increased levels of SMEK1 was associated with the differential expression of miRNAs and the deregulation of processes of gene silencing by miRNA. These results implied that SMEK1 regulates 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 [49]. 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 [50]. 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 [50]. PP4R3A also interacts with RNA polymerase II and recruits it to the promoters of miRNA encoding genes [51]. The expression of many snoRNAs, another class of small non-coding RNAs, was deregulated with several appearing in the most deregulated genes by SMEK1, adding another layer of complexity to the role of SMEK1. 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 could be also implicated in regulation of gene expression at posttranscriptional level.

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 mis-regulation in cancer. Perturbed signalling pathways related to cancer following SMEK1 overexpression included apoptosis, MAPK, mTOR, VGEF, ErbB, NFκB, TGFβ and RAS. Several cancer related genes were differentially expressed following SMEK1 over-expression including tumour suppressors that showed increased expression and oncogenes that were downregulated, supporting a tumour suppressor role for SMEK1. One of the most significant findings is that the downregulation of the tumour suppressor gene HRAS in SMEK1 overexpressing cells, further confirming a tumour suppressive role for SMEK1.

In conclusion, the current study has presented novel findings implicating SMEK1 in the regulation of 1) cell death and survival, 2) the activity of five core cancer signalling pathways and 3) gene expression at transcriptional, post-transcriptional and epigenetic levels. In the light of the significant impact of SMEK1 on leukemic T cells, it is obvious that a more intensive study should be pursued to achieve better understanding of its molecular function and involvement in cancer and other diseases. Further studies are required to investigate the regulatory axis involving SMEK1 -TGFβ signalling pathways and SMEK1-HRAS. The observation that SMEK1 overexpression leads to the decreased expression of the oncogene HRAS, one of the most frequently mutated gene families in cancers and presently described as 'undruggable' could be harnessed for the development of therapeutic materials to target HRAS.

Declarations of interest

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Figure 1: SMEK1 over-expression inhibits short-and long-term survival, and increases apoptosis of Jurkat cells. Jurkat cells were transfected with

pcDNA3.1-SMEK1 with or pcDNA3.1. Cellular levels of SMEK1 are increased in the cells transfected with the SMEK1 expression construct as determined by western blotting analysis; β-actin as a loading control. Representative immunoblots are presented to the right of the bar chart (a). The proportion of apoptotic cells, determined by annexin V staining and flow cytometry, is increased in PP4c transfected cells (b). Total and viable cell counts, as determined by flow cytometry, are reduced in cells at 48 h post-transfection with SMEK1 (c). Clonogenic assay demonstrates that long-term survival of Jurkat cells is compromised after transfection with the SMEK1 construct (d). Cell cycle analysis revealed that SMEK1 overexpression reduces the proportions of cells in G1-, S- and G2 phases, while increasing the sub-G0 cell count (e). The bar graphs represent means ± SEM from four independent experiments. *p<0.05, **p<0.01, ***p < <0.0001 versus cells transfected with pcDNA3.1 alone (One-way ANOVA and Tukey's post-test).

Figure 2: SMEK1 induces apoptosis and inhibits cell growth, colony-forming ability short in CEM-C7 leukemic cells. CEM-C7 cells were transfected with pcDNA3.1-SMEK1 or empty vector pcDNA3.1. SMEK1 protein levels are increased in cells transfected with the SMEK1 expression construct, as determined at 24 h post transfection by western blotting analysis; β-actin as a loading control. Representative immunoblots are presented to the right of the bar chart (a). The percentage of apoptotic cells, as determined by annexin V staining and flow cytometry, is increased at 48 h post transfection with SMEK1 (b). Total and viable cell counts, determined by flow cytometry, are decreased in cells transfected with SMEK1 (c). Clonogenic activity of CEM-C7 is significantly decreased after transfection with SMEK1

construct (d). SMEK1 overexpression disturbs the cell cycle profile of CEM-C7 cells, causes an increase in cell count in the sub-G0 phase and a concomitant decrease in proliferating cells in G1, S and G2/M phases (e) . The bar graphs represent means ± SEM from four independent experiments. 1*p<0.05, **p<0.01, ****p < <0.0001 versus cells transfected with pcDNA3.1 alone (One-way ANOVA and Tukey's post-test).

Figure 3: 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<0.01, ****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.

Figure 4: SMEK1-specific siRNAs inhibit basal apoptosis and increase the short- and long-term survival of Leukemic T cells. Jurkat and CEM-C7 cells were transfected with control (−) siRNA or with SMEK1-specific siRNA. Cells were harvested at 72 h post-transfection, and re-plated for assessment of cell survival after a further 48 h. Expression of SMEK1 protein levels in Jurkat (a) and CEM-C7 (b) was determined by western blotting 72 h after transfection and equivalent loading was demonstrated using anti-β-actin antibody. Representative immunoblots are presented to the right of the bar chart. Down-regulation of SMEK1 is associated with an increase in total and viable cell numbers, as determined by flow cytometry (c and d). Colony forming ability is

enhanced in the cells transfected with SMEK1 siRNAs (e and f). The bar graphs represent means ± SEM from four independent experiments. **p<0.01, ****p < <0.0001 versus cells transfected with (−)siRNA control (one-way ANOVA and Bonferroni's MCT).

Figure 5: 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. Bar charts represent three independent experiments expressed as mean ± SEM. Relative expression is the ratio of PP4c level versus β-actin. Jurkat (a), CEM-C7 (b) . *** p<0.001 relative to control (one-way ANOVA and Bonferroni's MCT). Representative Western blot images collected from Jurkat and CEM-C7 using a Bio-Rad ChemiDoc MP Imaging System are shown under its corresponding graph.

Figure 6: SMEK1 overexpression in HEK293T cells alter the phosphorylation of proteins involved in five signalling pathways. Cell lysates from HEK239T cells containing pcDNA3.1 empty vector as a control or overexpressing SMEK1 were used in the experiment. Changes in the phosphorylation level of proteins involved in MAPK (a), AKT (b), JAK/STAT (c), NFκB (d) and TGFβ (e) pathways 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. Red coloured bars correspond to decreased protein phosphorylation, while green coloured bars correspond to increased protein phosphorylation.

Figure 7: Increased SMEK1 expression levels is associated with an alteration

of phosphorylation levels of proteins involved in five core cancer signalling pathways. Jurkat cells were transfected with pcDNA3.1-SMEK1. Cell lysates were collected 48 hours post transfection. Alteration in protein phosphorylation of proteins involved in MAPK (a), AKT (b), JAK/STAT (c), NFκB (d) and TGFβ (e) signalling pathways 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. Red coloured bars correspond to decreased protein phosphorylation, while green coloured bars correspond to increased protein phosphorylation.

Figure 8: Increased SMEK1 levels alters the phosphorylation levels of five transcription factors. Jurkat cells were transfected with SMEK1. Cell lysates were prepared after 48 hours and used for western blotting. Graphs show ratio of p-NFkB (a), p-STAT3 (b), p-c-Jun (c), p-SMAD1 (d) and p-SMAD5 (e), relative density of β-Actin. Data are presented as mean ± SEM (n=3). **p<0.01, ***p<0.001, ****p < <0.0001 versus cells transfected with (−)siRNA control (one-way ANOVA and Bonferroni's MCT).

Figure 9: The expression of individual genes implicated in regulation of gene expression and RNA processing. Genes implicated in the biological process of regulation of gene expression (a), negative regulation of 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.

Figure 10 Perturbed pathways in Jurkat T cells overexpression SMEK1 as determined by sequencing and analysis of the whole transcriptome. 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 (a). Perturbation vs overrepresentation: Top nine most significantly impacted pathways are shown, using negative log of the accumulation and overrepresentation 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 (b). (c) The graph shows the expression of the 17 differentially expressed genes in microRNAs in cancer pathway. 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.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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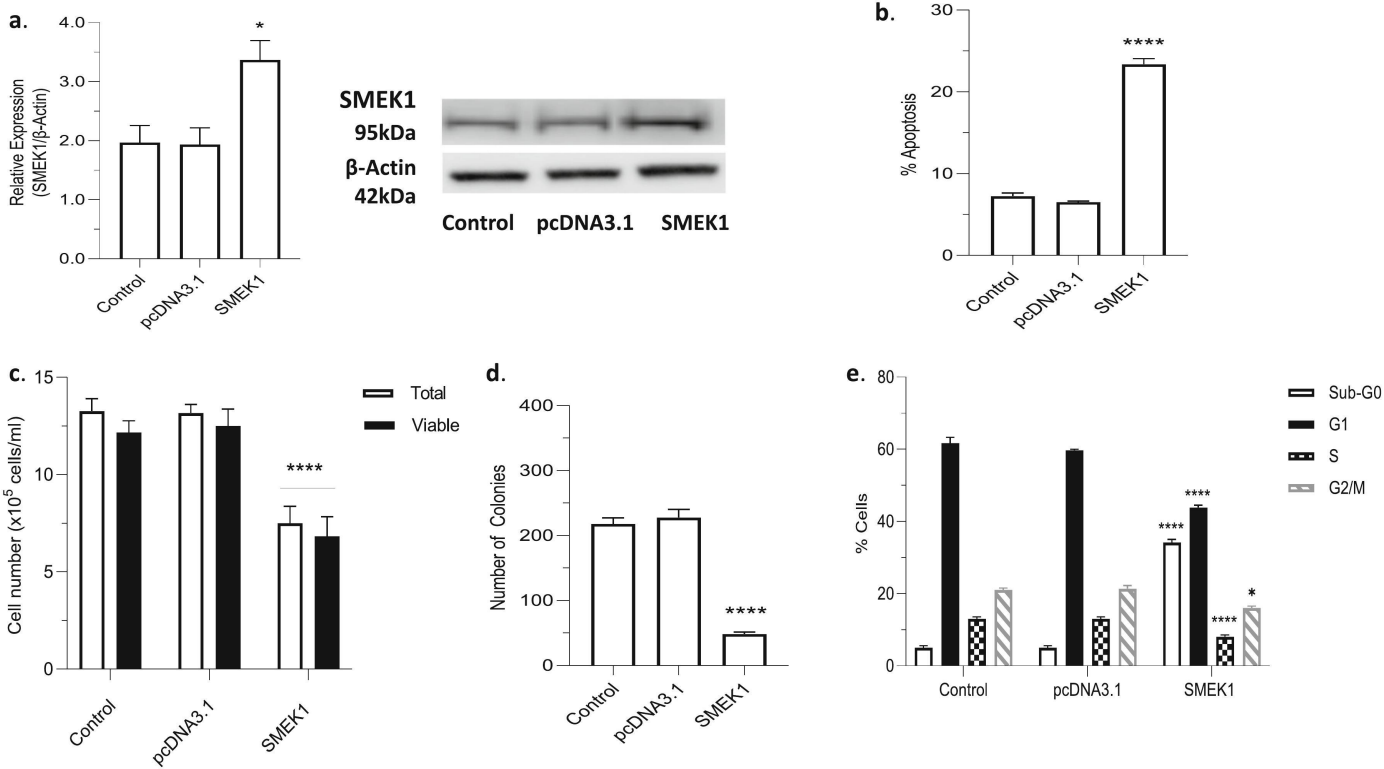

a.
Relative Expression (SMEK1/β-Actin)
4.0
3.0
2.0
1.0
0.0
*
Control
pcDNA3.1
SMEK1
SMEK1
95kDa
β-Actin
42kDa
Control
pcDNA3.1
SMEK1
b.
% Apoptosis
30
20
10
0
****
Control
pcDNA3.1
SMEK1
c.
Cell number (x10^5 cells/ml)
15
10
5
0
Total
Viable
****
Control
pcDNA3.1
SMEK1
d.
Number of Colonies
400
300
200
100
0
****
Control
pcDNA3.1
SMEK1
e.
% Cells
80
60
40
20
0
Sub-G0
G1
S
G2/M
****
****
****
****
*
Control
pcDNA3.1
SMEK1

Figure 1

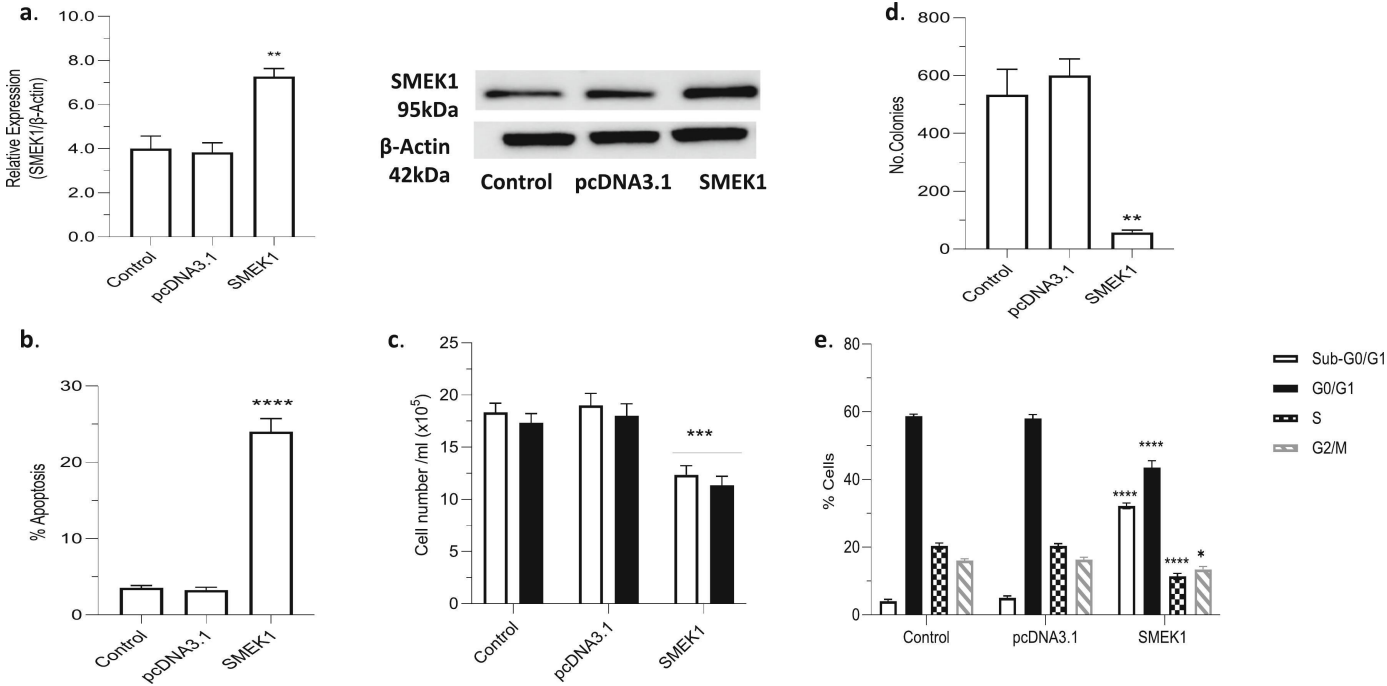

a.
Relative Expression (SMEK1/β-Actin)
10.0
8.0
6.0
4.0
2.0
0.0
**
Control
pcDNA3.1
SMEK1
SMEK1 95kDa
β-Actin 42kDa
Control
pcDNA3.1
SMEK1
d.
No.Colonies
800
600
400
200
0
**
Control
pcDNA3.1
SMEK1
b.
% Apoptosis
30
20
10
0
*****
Control
pcDNA3.1
SMEK1
c.
Cell number /ml (x10^5)
25
20
15
10
5
0
***
Control
pcDNA3.1
SMEK1
e.
% Cells
80
60
40
20
0
****
****
****
*
Control
pcDNA3.1
SMEK1
Sub-G0/G1
G0/G1
S
G2/M

Figure 2

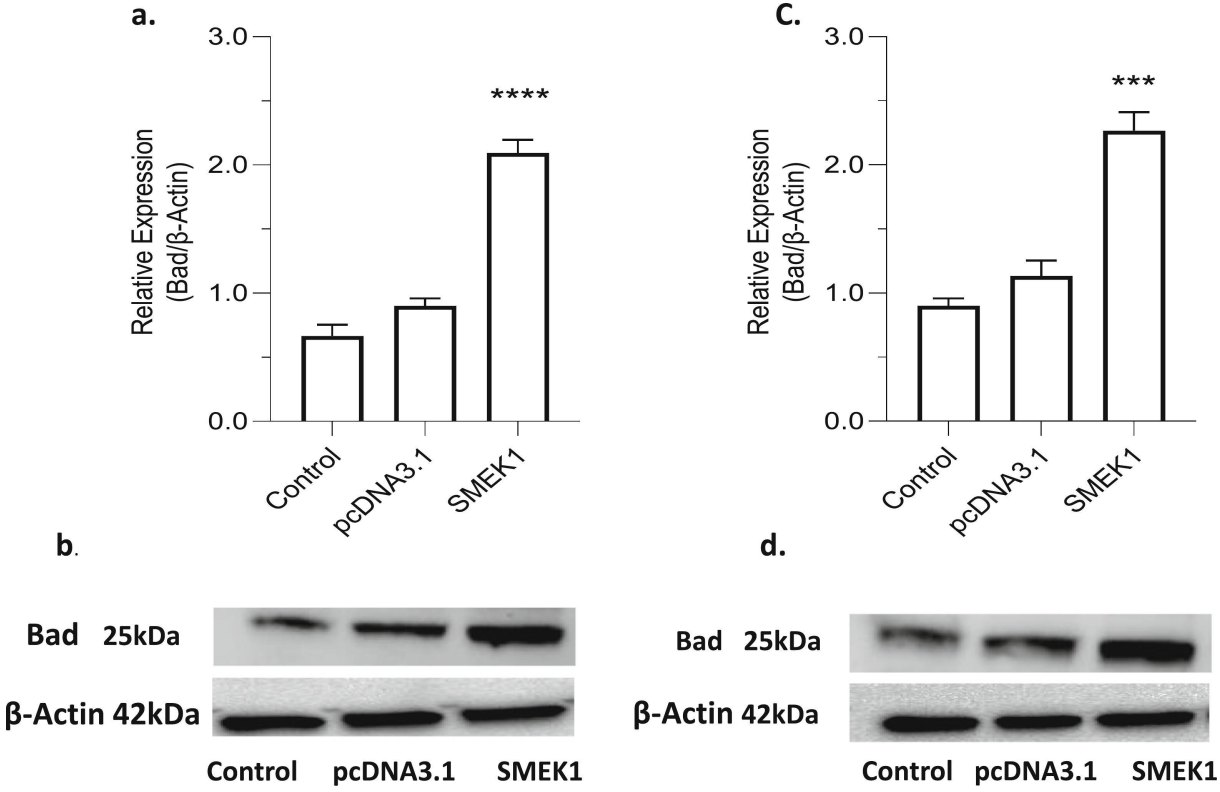

a.
3.0
2.0
1.0
0.0
Relative Expression
(Bad/β-Actin)
****
Control
pcDNA3.1
SMEK1
C.
3.0
2.0
1.0
0.0
Relative Expression
(Bad/β-Actin)
***
Control
pcDNA3.1
SMEK1
b.
Bad 25kDa
β-Actin 42kDa
Control
pcDNA3.1
SMEK1
d.
Bad 25kDa
β-Actin 42kDa
Control
pcDNA3.1
SMEK1

Figure 3

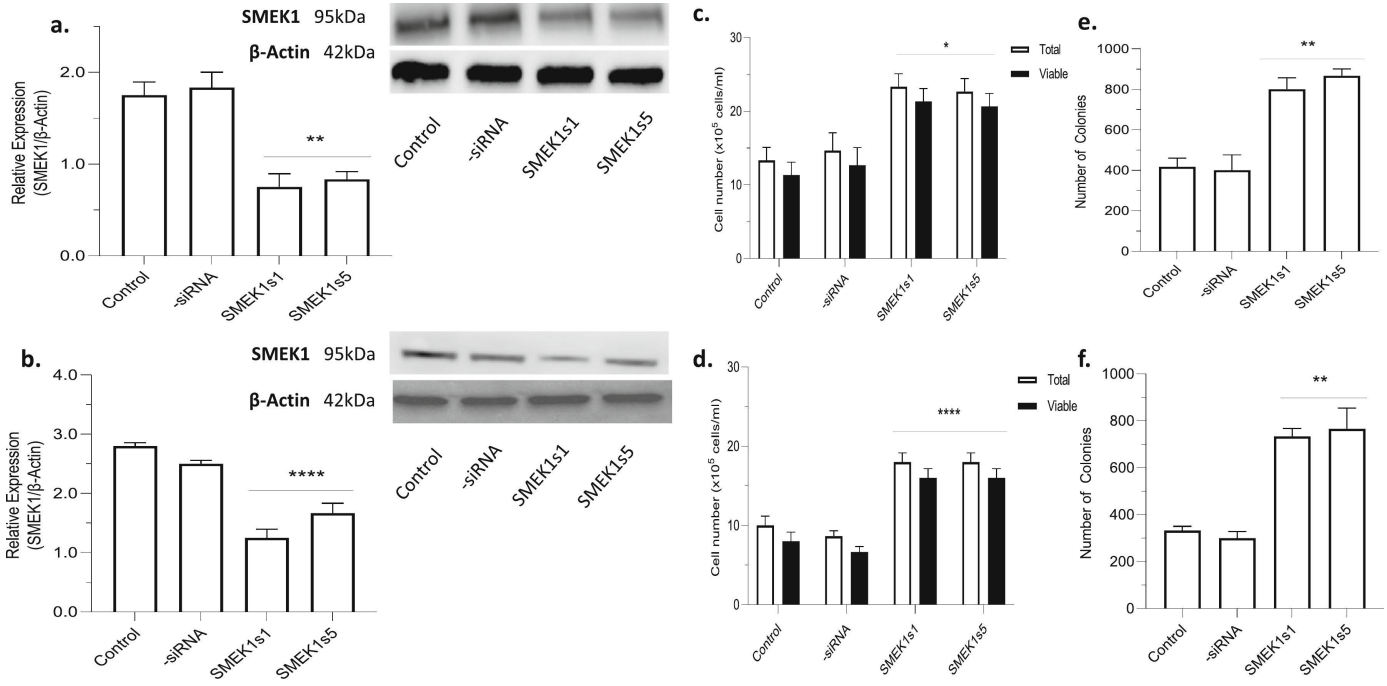

a.
SMEK1 95kDa
β-Actin 42kDa
Relative Expression (SMEK1/β-Actin)
2.0
1.0
0.0
**
Control
-siRNA
SMEK1s1
SMEK1s5
b.
SMEK1 95kDa
β-Actin 42kDa
Relative Expression (SMEK1/β-Actin)
4.0
3.0
2.0
1.0
0.0
****
Control
-siRNA
SMEK1s1
SMEK1s5
c.
Cell number (×10^5 cells/ml)
30
20
10
0
*
Total
Viable
Control
-siRNA
SMEK1s1
SMEK1s5
d.
Cell number (×10^5 cells/ml)
30
20
10
0
****
Total
Viable
Control
-siRNA
SMEK1s1
SMEK1s5
e.
Number of Colonies
1000
800
600
400
200
0
**
Control
-siRNA
SMEK1s1
SMEK1s5
f.
Number of Colonies
1000
800
600
400
200
0
**
Control
-siRNA
SMEK1s1
SMEK1s5

Figure 4

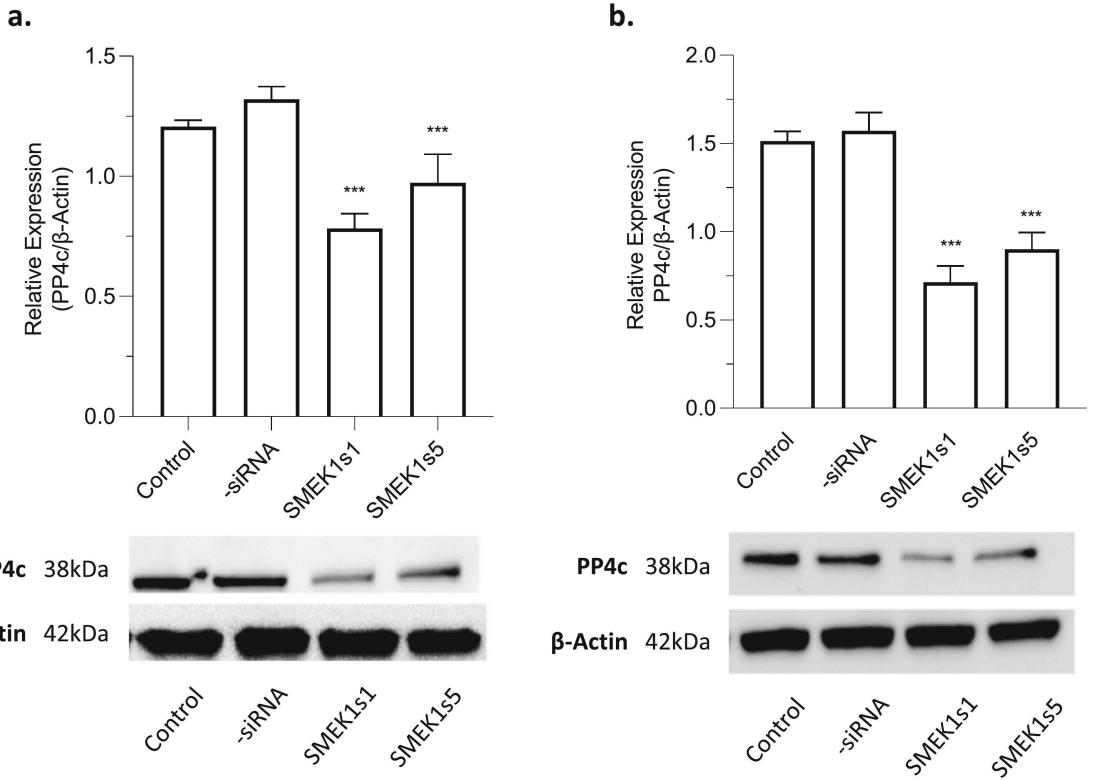

a.
Relative Expression (PP4c/β-Actin)
1.5
1.0
0.5
0.0
***
***
Control
-siRNA
SMEK1s1
SMEK1s5
PP4c 38kDa
β-Actin 42kDa
Control
-siRNA
SMEK1s1
SMEK1s5
b.
Relative Expression PP4c/β-Actin)
2.0
1.5
1.0
0.5
0.0
***
***
Control
-siRNA
SMEK1s1
SMEK1s5
PP4c 38kDa
β-Actin 42kDa
Control
-siRNA
SMEK1s1
SMEK1s5

Figure 5

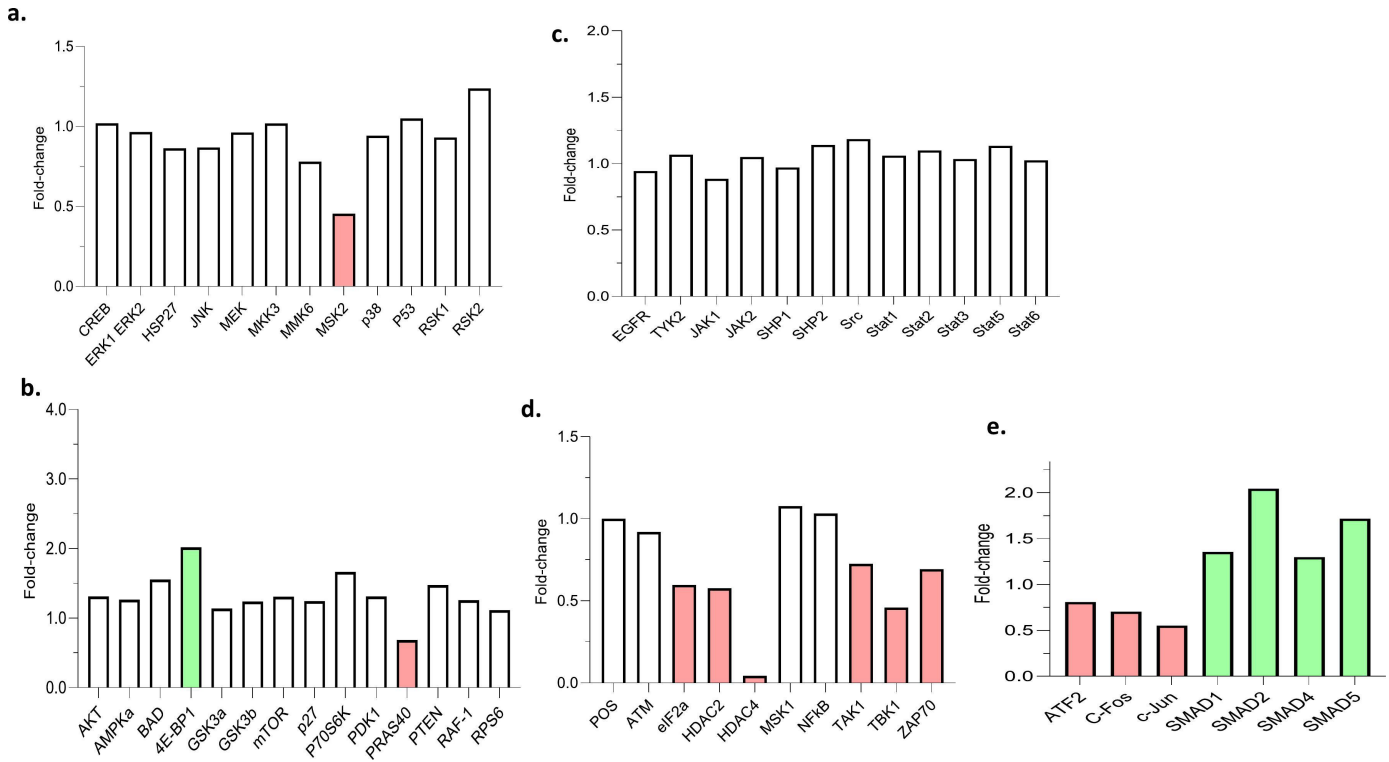

a.
Fold-change
1.5
1.0
0.5
0.0
CREB
ERK1 ERK2
HSP27
JNK
MEK
MKK3
MMK6
MSK2
p38
P53
RSK1
RSK2
c.
Fold-change
2.0
1.5
1.0
0.5
0.0
EGFR
TYK2
JAK1
JAK2
SHP1
SHP2
Src
Stat1
Stat2
Stat3
Stat5
Stat6
b.
Fold-change
4.0
3.0
2.0
1.0
0.0
AKT
AMPKa
BAD
4E-BP1
GSK3a
GSK3b
mTOR
p27
P70S6K
PDK1
PRAS40
PTEN
RAF-1
RPS6
d.
Fold-change
1.5
1.0
0.5
0.0
POS
ATM
eIF2a
HDAC2
HDAC4
MSK1
NFkB
TAK1
TBK1
ZAP70
e.
Fold-change
2.0
1.5
1.0
0.5
0.0
ATF2
C-Fos
c-Jun
SMAD1
SMAD2
SMAD4
SMAD5

Figure 6

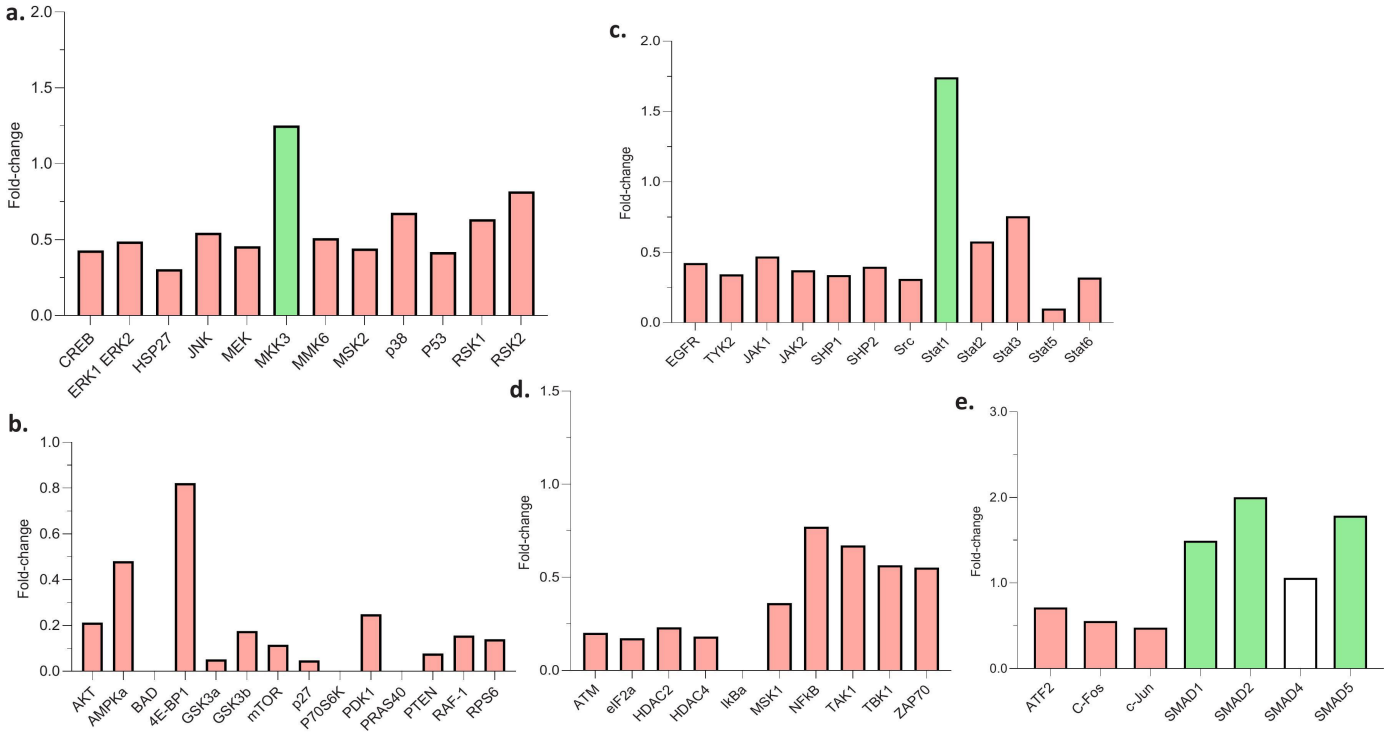

a.
Fold-change
2.0
1.5
1.0
0.5
0.0
CREB
ERK1 ERK2
HSP27
JNK
MEK
MKK3
MMK6
MSK2
p38
P53
RSK1
RSK2
b.
Fold-change
1.0
0.8
0.6
0.4
0.2
0.0
AKT
AMPKa
BAD
4E-BP1
GSK3a
GSK3b
mTOR
p27
P70S6K
PDK1
PRAS40
PTEN
RAF-1
RPS6
c.
Fold-change
2.0
1.5
1.0
0.5
0.0
EGFR
TYK2
JAK1
JAK2
SHP1
SHP2
Src
Stat1
Stat2
Stat3
Stat5
Stat6
d.
Fold-change
1.5
1.0
0.5
0.0
ATM
eIF2a
HDAC2
HDAC4
IkBa
MSK1
NFkB
TAK1
TBK1
ZAP70
e.
Fold-change
3.0
2.0
1.0
0.0
ATF2
C-Fos
c-Jun
SMAD1
SMAD2
SMAD4
SMAD5

Figure 7

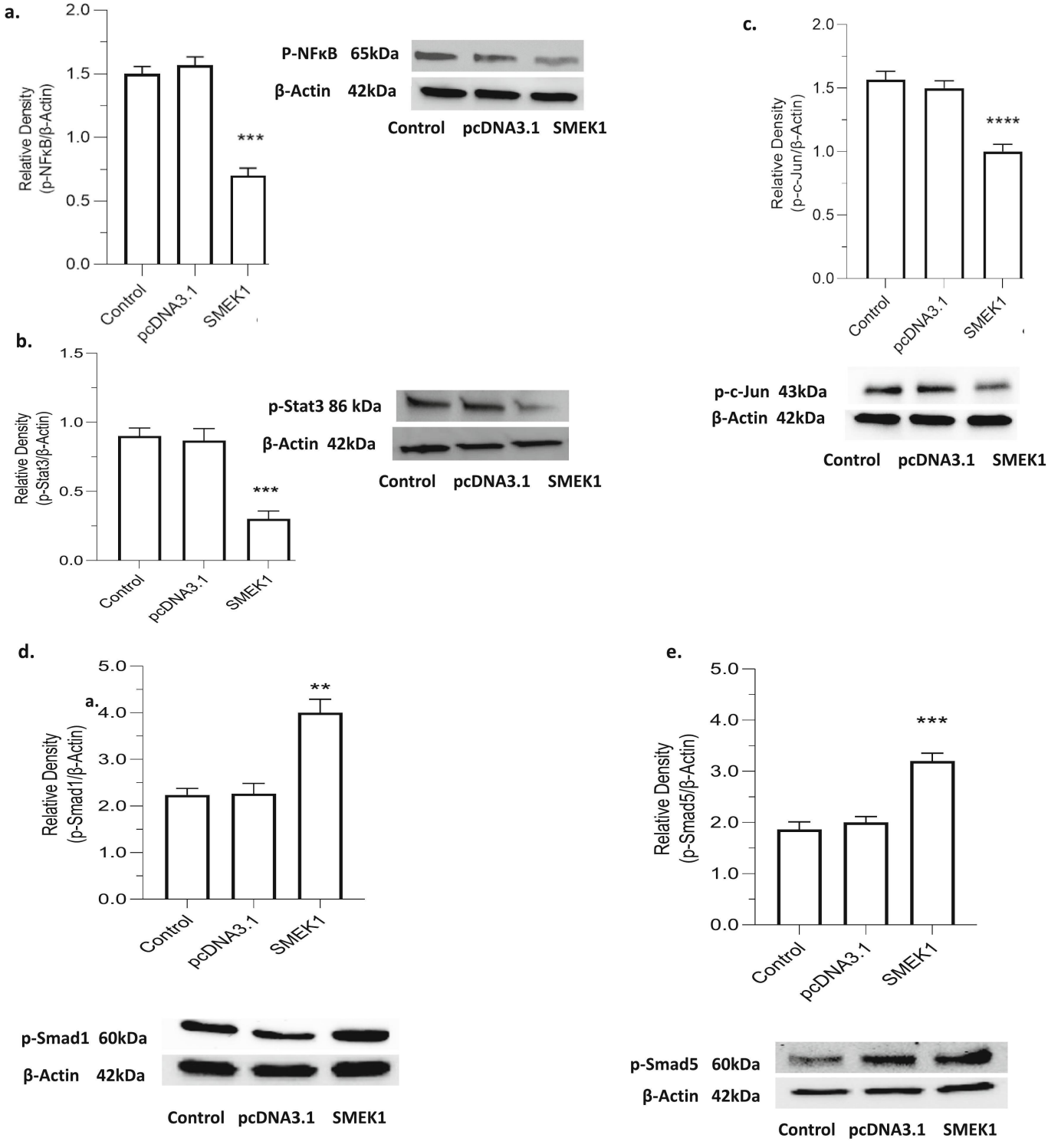

a.
Relative Density (p-NFκB/β-Actin)
2.0
1.5
1.0
0.5
0.0
***
Control
pcDNA3.1
SMEK1
P-NFκB 65kDa
β-Actin 42kDa
Control pcDNA3.1 SMEK1
b.
Relative Density (p-Stat3/β-Actin)
1.5
1.0
0.5
0.0
***
Control
pcDNA3.1
SMEK1
p-Stat3 86 kDa
β-Actin 42kDa
Control pcDNA3.1 SMEK1
c.
Relative Density (p-c-Jun/β-Actin)
2.0
1.5
1.0
0.5
0.0
****
Control
pcDNA3.1
SMEK1
p-c-Jun 43kDa
β-Actin 42kDa
Control pcDNA3.1 SMEK1
d.
a.
Relative Density (p-Smad1/β-Actin)
5.0
4.0
3.0
2.0
1.0
0.0
**
Control
pcDNA3.1
SMEK1
p-Smad1 60kDa
β-Actin 42kDa
Control pcDNA3.1 SMEK1
e.
Relative Density (p-Smad5/β-Actin)
5.0
4.0
3.0
2.0
1.0
0.0
***
Control
pcDNA3.1
SMEK1
p-Smad5 60kDa
β-Actin 42kDa
Control pcDNA3.1 SMEK1

Figure 8

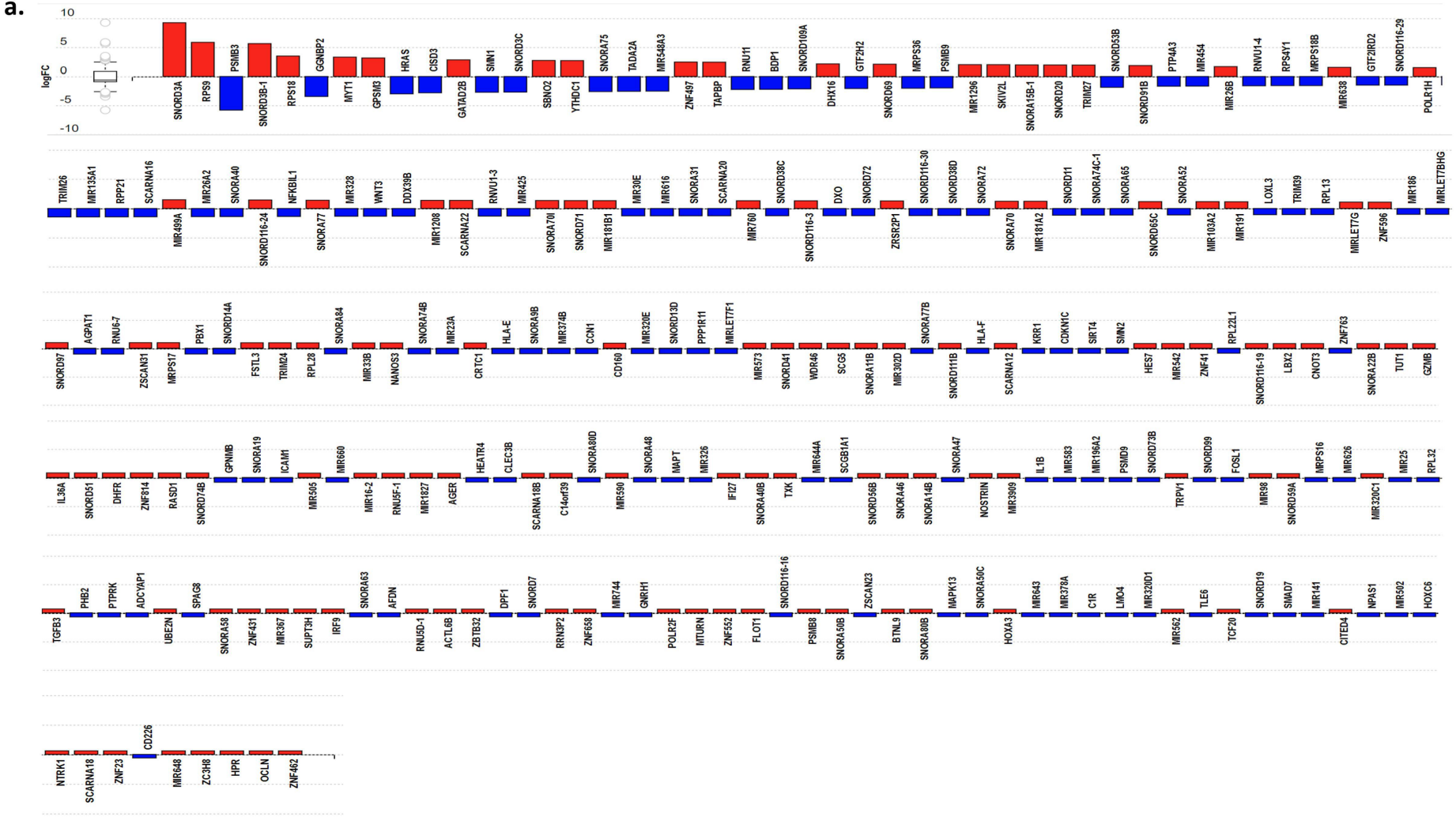
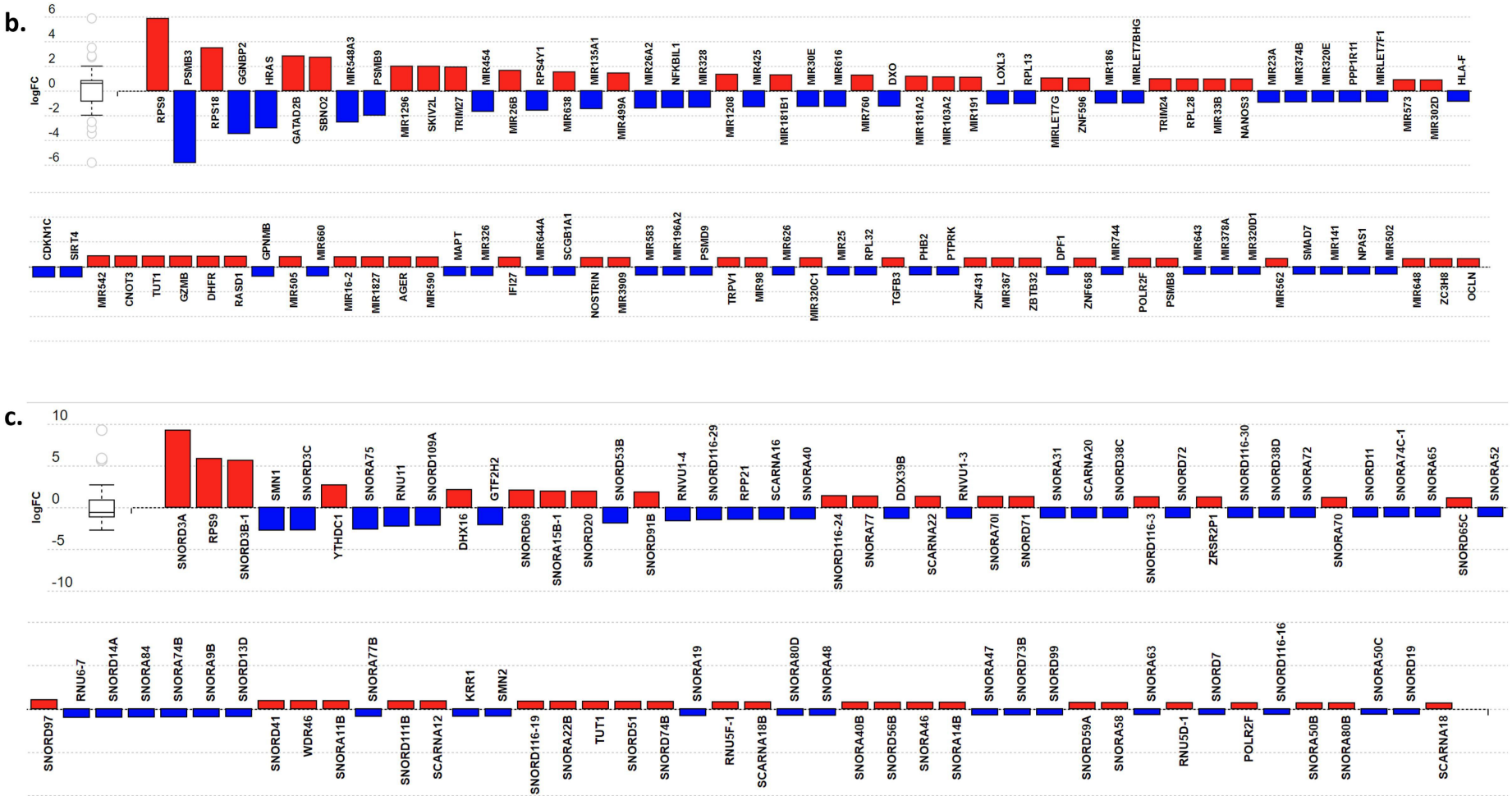

a.
logFC
10
5
0
-5
-10
SNORD3A
RPS9
PSMB3
SNORD3B-1
RPS18
GGNBP2
MYT1
GPSM3
HRAS
CISD3
GATAD2B
SMN1
SNORD3C
SBNO2
YTHDC1
SNORA75
TADA2A
MIR548A3
ZNF497
TAPBP
RNU11
BDP1
SNORD109A
DHX16
GTF2H2
SNORD69
MRPS36
PSMB9
MIR1296
SKIV2L
SNORA15B-1
SNORD20
TRIM27
SNORD53B
SNORD91B
PTP4A3
MIR454
MIR28B
RNVU1-4
RPS4Y1
MRPS18B
MIR638
GTF2IRD2
SNORD116-29
POLR1H
TRIM26
MIR135A1
RPP21
SCARNA16
MIR499A
MIR26A2
SNORA40
SNORD116-24
NFKBIL1
SNORA77
MIR328
WNT3
DDX39B
MIR1208
SCARNA22
RNVU1-3
MIR425
SNORA70E
SNORD71
MIR181B1
MIR30E
MIR616
SNORA31
SCARNA20
MIR760
SNORD38C
SNORD116-3
DXO
SNORD72
ZRSR2P1
SNORD116-30
SNORD38D
SNORA72
SNORA70
MIR181A2
SNORD11
SNORA74C-1
SNORA65
SNORD65C
SNORA52
MIR103A2
MIR191
LOXL3
TRIM39
RPL13
MIRLET7G
ZNF596
MIR186
MIRLET7BHG
SNORD97
AGPAT1
RNU6-7
ZSCAN31
MRPS17
PBX1
SNORD14A
FSTL3
TRIM24
RPL28
SNORA84
MIR33B
NANOS3
SNORA74B
MIR23A
CRTC1
HLA-E
SNORA9B
MIR374B
CCN1
CD160
MIR320E
SNORD13D
PPP1R11
MIRLET7F1
MIR573
SNORD41
WDR46
SCG5
SNORA11B
MIR302D
SNORA77B
SNORD111B
HLA-F
SCARNA12
KRR1
CDKN1C
SIRT4
SIN2
HES7
MIR542
ZNF41
RPL22L1
SNORD116-19
LBX2
CNOT3
ZNF763
SNORA22B
TUT1
GZMB
IL36A
SNORD51
DHFR
ZNF814
RASD1
SNORD74B
GPNMB
SNORA19
ICAM1
MIR505
MIR660
MIR16-2
RNU5F-1
MIR1827
AGER
HEATR4
CLEC3B
SCARNA18B
C14orf39
SNORA80D
MIR590
SNORA48
MAPT
MIR326
IFI27
SNORA40B
TXK
MIR644A
SCGB1A1
SNORD56B
SNORA46
SNORA14B
SNORA47
NOSTRIN
MIR3909
IL1B
MIR583
MIR196A2
PSMD9
SNORD73B
TRPV1
SNORD99
FOSL1
MIR98
SNORD59A
MRPS16
MIR626
MIR320C1
MIR25
RPL32
TGFB3
PHB2
PTPRK
ADCYAP1
UBE2N
SPAG8
SNORA58
ZNF431
MIR367
SUPT3H
IRF9
SNORA63
AFDN
RNU5D-1
ACTL6B
ZBTB32
DPF1
SNORD7
RRN3P2
ZNF658
MIR744
GNRH1
POLR2F
MTURN
ZNF552
FLOT1
SNORD116-16
PSMB8
SNORA50B
ZSCAN23
BTNL9
SNORA80B
MAPK13
SNORA50C
HOXA3
MIR643
MIR378A
C1R
LMO4
MIR320D1
MIR562
TLE6
TGFZP
SNORD19
SMAD7
MIR141
CITED4
NPAS1
MIR502
HOXC6
NTRK1
SCARNA18
ZNF23
CD226
MIR648
ZC3H8
HPR
OCLN
ZNF462

Figure 9a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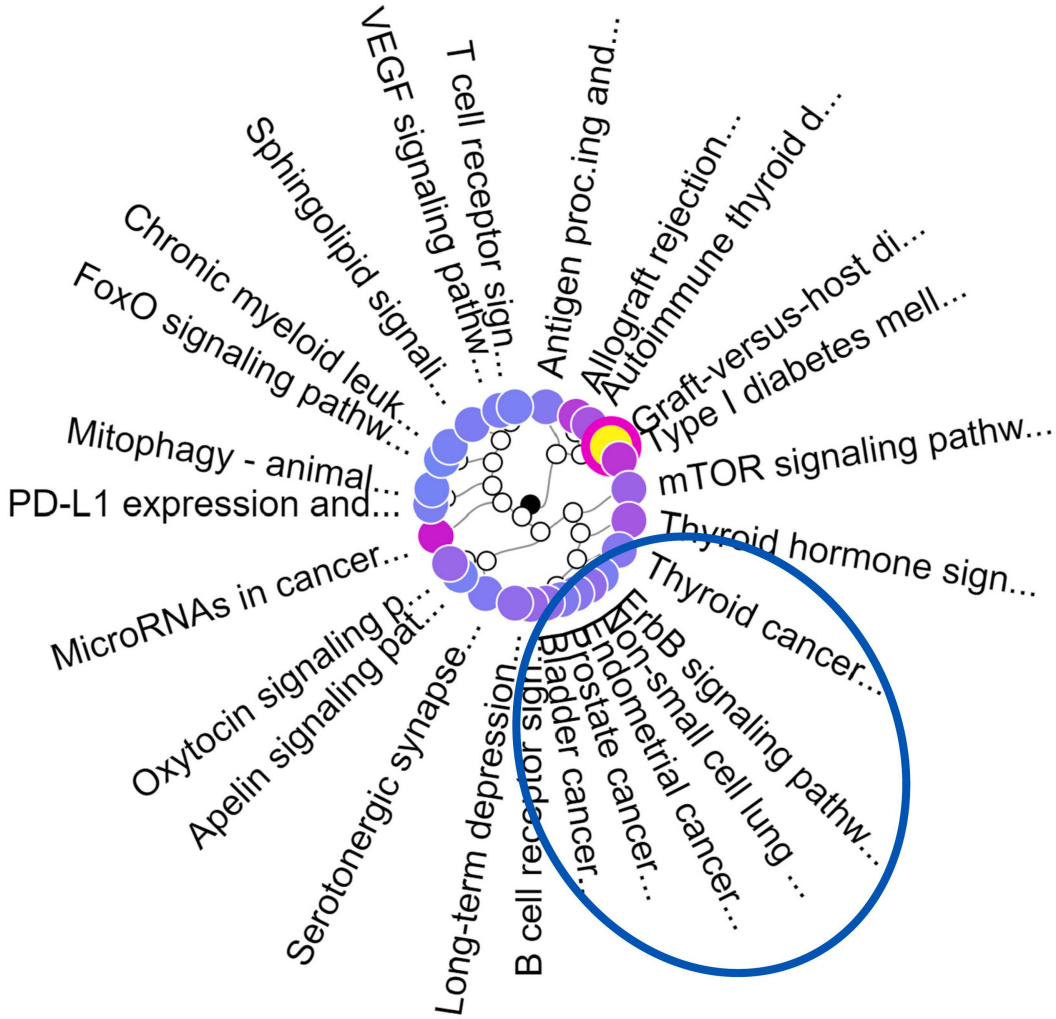
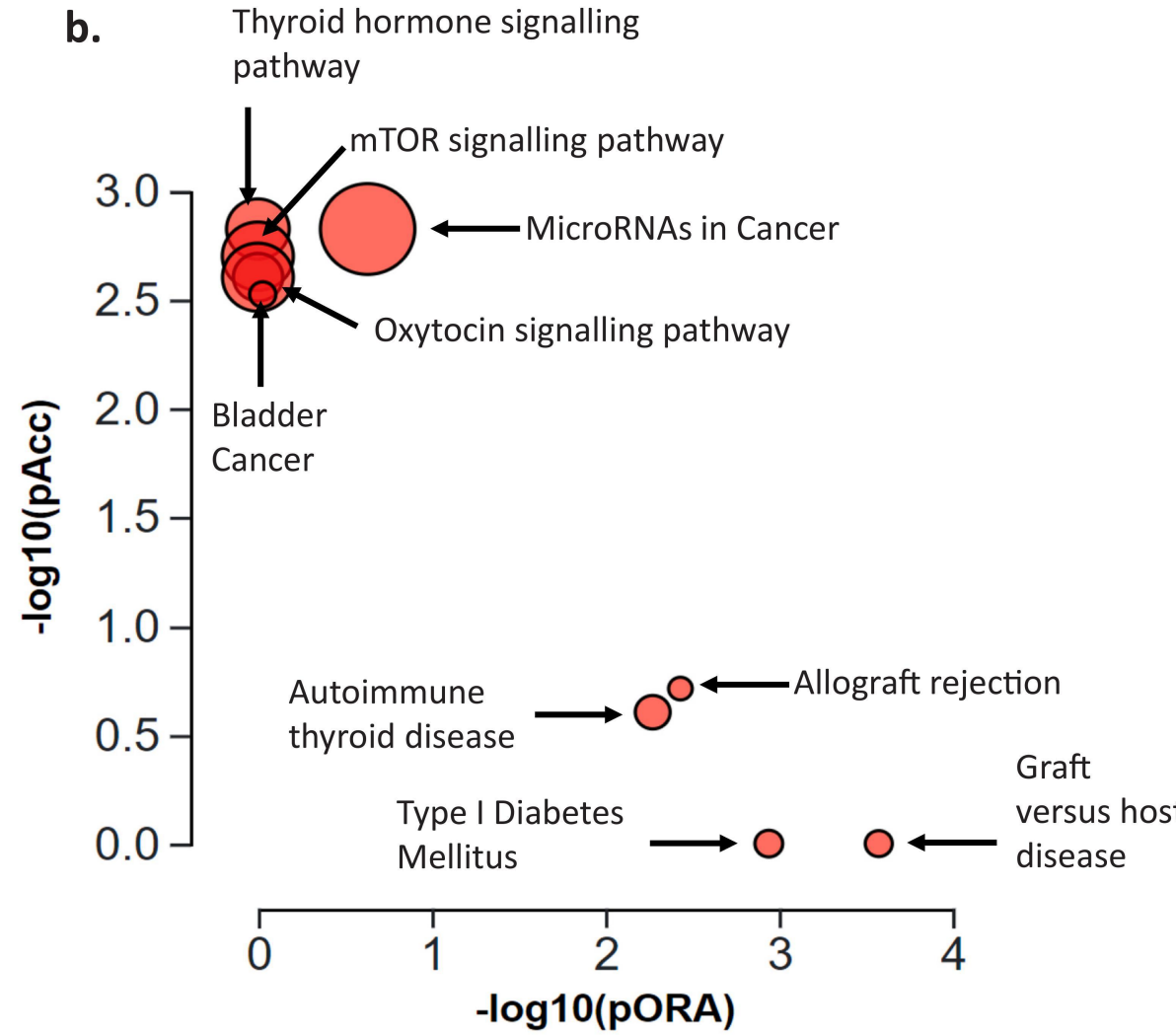
logFC
6
4
2
0
-2
-4
-6
RPS9
PSMB3
RPS18
GGNBP2
HRAS
GATAD2B
SBNO2
MIR548A3
PSMB9
MIR1296
SKIV2L
TRIM27
MIR454
MIR26B
RPS4Y1
MIR638
MIR135A1
MIR499A
MIR26A2
NFKBIL1
MIR328
MIR1208
MIR425
MIR181B1
MIR30E
MIR616
MIR760
DXO
MIR181A2
MIR103A2
MIR191
LOXL3
RPL13
MIRLET7G
ZNF596
MIR186
MIRLET7BHG
TRIM24
RPL28
MIR33B
NANOS3
MIR23A
MIR374B
MIR320E
PPP1R11
MIRLET7F1
MIR573
MIR302D
HLA-F
CDKN1C
SIRT4
MIR542
CNOT3
TUT1
GZMB
DHFR
RASD1
GPNMB
MIR505
MIR660
MIR16-2
MIR1827
AGER
MIR590
MAPT
MIR326
IFI27
MIR644A
SCGB1A1
NOSTRIN
MIR3909
MIR583
MIR196A2
PSMD9
TRPV1
MIR98
MIR626
MIR320C1
MIR25
RPL32
TGFB3
PHB2
PTPRK
ZNF431
MIR367
ZBTB32
DPF1
ZNF658
MIR744
POLR2F
PSMB8
MIR643
MIR378A
MIR320D1
MIR562
SMAD7
MIR141
NPAS1
MIR502
MIR648
ZC3H8
OCLN
c.
logFC
10
5
0
-5
-10
SNORD3A
RPS9
SNORD3B-1
SMN1
SNORD3C
YTHDC1
SNORA75
RNU11
SNORD109A
DHX16
GTF2H2
SNORD69
SNORA15B-1
SNORD20
SNORD53B
SNORD91B
RNVU1-4
SNORD116-29
RPP21
SCARNA16
SNORA40
SNORD116-24
SNORA77
DDX39B
SCARNA22
RNVU1-3
SNORA70I
SNORD71
SNORA31
SCARNA20
SNORD38C
SNORD116-3
SNORD72
ZRSR2P1
SNORD116-30
SNORD38D
SNORA72
SNORA70
SNORD11
SNORA74C-1
SNORA65
SNORD65C
SNORA52
SNORD97
RNU6-7
SNORD14A
SNORA84
SNORA74B
SNORA9B
SNORD13D
SNORD41
WDR46
SNORA11B
SNORA77B
SNORD111B
SCARNA12
KRR1
SMN2
SNORD116-19
SNORA22B
TUT1
SNORD51
SNORD74B
SNORA19
RNU5F-1
SCARNA18B
SNORA80D
SNORA48
SNORA40B
SNORD56B
SNORA46
SNORA14B
SNORA47
SNORD73B
SNORD99
SNORD59A
SNORA58
SNORA63
RNU5D-1
SNORD7
POLR2F
SNORD116-16
SNORA50B
SNORA80B
SNORA50C
SNORD19
SCARNA18

Figure 9bc

a.



b.



c.

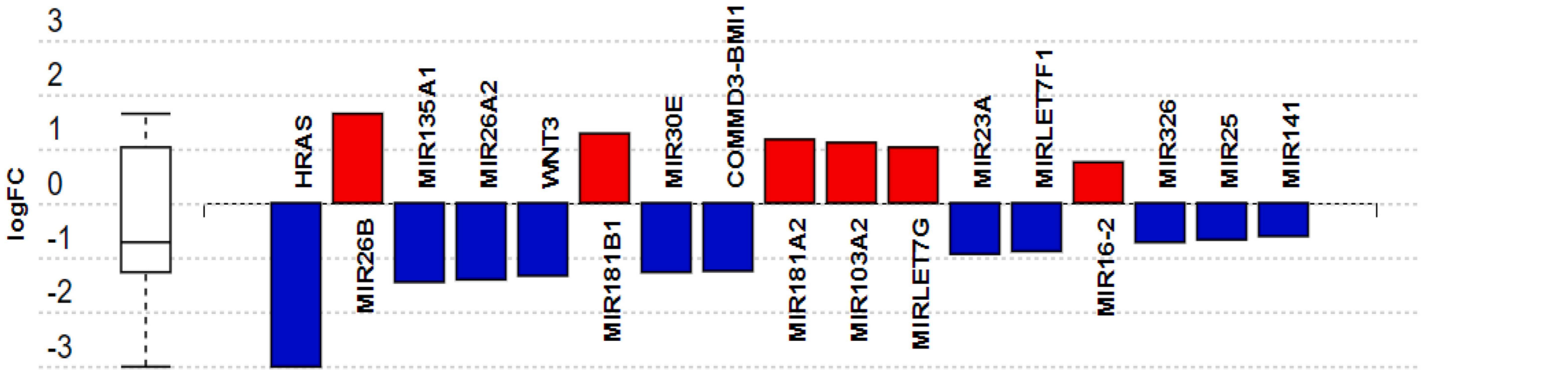


Figure 10