Salt-inducible Kinase 2 Regulates Mitotic Progression and Transcription in Prostate Cancer

Hélène Bon1, Karan Wadhwa1, Alexander Schreiner2, Michelle Osborne3, Thomas Carroll4, Antonio Ramos-Montoya1, Helen Ross-Adams1, Matthieu Visser5, Ralf Hoffmann6, Ahmed Ashour Ahmed7, David E. Neal1,8,9#, Ian G. Mills1,10,11,12*#

1. Uro-oncology Research Group, Cambridge Research Institute, Cambridge, CB2 0RE, UK
2. Microscopy and Imaging Core, Cambridge Research Institute, Cambridge, CB2 0RE, UK
3. Genomics Core, Cambridge Research Institute, Cambridge, CB2 0RE, UK
4. Bioinformatics Core, Cambridge Research Institute, Cambridge, CB2 0RE, UK
5. Health Care Innovation, Philips Research, Eindhoven, Netherlands
6. Molecular Diagnostics, Philips Research, Eindhoven, Netherlands
7. Weatherall Institute of Molecular Medicine, University of Oxford, OX3 9DS and Nuffield Department of Obstetrics and Gynaecology, University of Oxford, OX3 9DU, UK
8. Department of Urology, Addenbrooke’s Hospital, Cambridge, CB2 2QQ, UK
9. Department of Oncology, University of Cambridge, Cambridge, CB2 2QQ, UK
10. Department of Urology, Oslo University Hospital, 0424 Oslo, Norway
11. Department of Cancer Prevention, Oslo University Hospital, 0424 Oslo, Norway
12. Prostate Cancer Research Group, Centre for Molecular Medicine Norway, University of Oslo and Oslo University Hospital, N-0349, Oslo, Norway

# These authors contributed equally
*correspondence to be addressed to: ian.mills@cruk.cam.ac.uk; ian.mills@ncmm.uio.no, Tel: +47-22840767

Conflicts of Interest statement:

The authors declare that there no conflicts of interest associate with this study.
Abstract

SIK2 is a multifunctional kinase of the AMPK family which plays a role in CREB1-mediated gene transcription and was recently reported to have therapeutic potential in ovarian cancer. The expression of this kinase was investigated in prostate cancer clinical specimens. Interestingly, auto-antibodies against SIK2 were increased in the plasma of patients with aggressive disease. Examination of SIK2 in prostate cancer cells found that it functions both as a positive regulator of cell cycle progression and a negative regulator of CREB1 activity. Knockdown of SIK2 inhibited cell growth, delayed cell cycle progression, induced cell death and enhanced CREB1 activity. Expression of a kinase-dead mutant of SIK2 also inhibited cell growth, induced cell death and enhanced CREB1 activity. Treatment with a small molecule SIK2 inhibitor (ARN-3236), currently in preclinical development, also led to enhanced CREB1 activity in a dose- and time-dependent manner. Since CREB1 is a transcription factor and proto-oncogene, it was posited that the effects of SIK2 on cell proliferation and viability might be mediated by changes in gene expression. To test this, gene expression array profiling was performed and whilst SIK2 knockdown or over-expression of the kinase-dead mutant affected established CREB1 target genes; the overlap with transcripts regulated by forskolin (FSK), the adenylate cyclase/CREB1 pathway activator, was incomplete.

Implications

This study demonstrates that targeting SIK2 genetically or therapeutically will have pleiotropic effects on cell cycle progression and transcription factor activation, which should be accounted for when characterizing SIK2 inhibitors.
Keywords: Prostate cancer, salt-inducible kinase 2, cell cycle, cAMP response element-binding protein, transducers of regulated CREB1 activity, endoplasmic reticulum stress response
Introduction

Prostate cancer is the most common cancer diagnosed in men and the second most common cause of cancer death after lung cancer, with around 40,000 men diagnosed and 10,000 deaths annually in the UK. [1] If diagnosed early, it is generally successfully treated using radical prostatectomy. However, 10 to 15% of the patients are diagnosed after their cancer has spread and present with advanced or inoperable cancer. [2] Since androgens have been shown to play an important role in the progression of prostate cancer, removing or blocking the action of androgens using hormonal therapy, also referred to androgen deprivation, is the treatment of choice for patients at this stage. It usually results in a favorable clinical response with the initial regression of at least 80% of hormone-sensitive prostate cancers. However, 10 to 20% of men eventually fail this therapy since tumour cells acquire the capability to grow in the absence of androgens, and the disease eventually recurs with fatal hormone refractory prostate cancer (HRPC). [3] Consequently, new approaches are needed for the treatment of prostate cancer so biomarkers for diagnosis and novel targets for therapeutic intervention are urgently required.

Salt-inducible kinase 2 (SIK2) is a serine/threonine kinase that belongs to the calcium calmodulin kinases (CaM) superfamily and the AMP-activated protein kinases (AMPK) subfamily. The members of this family act as sensors of cellular energy changes, and are known to regulate physiological processes that consume or generate ATP in order to maintain energy homeostasis in the cells. [4] Several studies report that SIK2 is activated in refeeding from starvation and modulates homeostasis in order to help the cells to adapt to metabolic stresses via the regulation of CREB1-mediated gene transcription. [5-11] cAMP response element-binding protein (CREB1) is a transcription factor which binds to DNA sequences called cAMP response elements (CRE), thereby stimulating gene expression once phosphorylated at Ser133. [12] The activity of CREB1 is enhanced by the transducers of regulated CREB1
activity (TORC) via a phosphorylation-independent interaction with the bZIP DNA binding/dimerization domain of CREB1. [13] The TORC family is composed of TORC1, TORC2 and TORC3 and all three TORC isoforms contain the putative SIK2 phosphorylation site and are downstream targets of SIK2 in the regulation of CREB1-mediated gene expression. [14]

Few studies have provided evidence of a role for SIK2 in cancer. SIK2 gene is located in the commonly deleted region of breast cancer at 11q23.1. [15-17] In contrast, this region is amplified in diffuse large B cell lymphoma (DLBCL) which results in increased SIK2 expression at the RNA and protein levels in tumours, where SIK2 was found to regulate survival and glucose metabolism, suggesting its role in DLBCL progression. [18] In addition, SIK2 has recently been identified as a centrosome kinase controlling the G2/M phase transition in ovarian cancer. It localises with the centrosomes where it phosphorylates the centrosome linker c-Nap1 at Ser2392, resulting in its translocation from the centrosomes to the cytoplasm at the onset of mitosis to facilitate loss of centriole cohesion as an initial step in centrosome separation. [19]

In the present study, we found high levels of auto-antibodies against SIK2 in plasma of patients with prostate cancer. We show for the first time that SIK2 is present in prostate cancer cells, and that its depletion inhibits growth and induces cell cycle arrest and apoptosis. Using immuno-precipitation, reporter assays and cellular fractionation methods, we found that SIK2 interacts with the CREB1 regulator TORC1 and negatively regulates CREB1 activity by interfering with TORC1 nuclear translocation. Using a whole genome profiling approach, we then identified the genes and networks regulated by SIK2.
Material and Methods

Protein array profiling of plasma samples

The study was performed on a patient cohort comprising men who have received prostatectomy and for which there was at least 5 years follow-up and assembled as part of the ProtecT Study (Prostate testing for cancer and Treatment). The disease of men with likely indolent cancer is characterised as follows: PSA<10 ng/ml; pStage <=T2; prostate cancer volume <=0.5 ml, and no PSA recurrence >5 years follow-up, while the disease is classified as likely aggressive disease if there has been clinical/PSA recurrence, or if in the post-surgery pathology pGleason >6, pStage >T2 or prostate cancer volume >0.5 ml [20]. Plasma samples were analysed using ProtoPlexTM immune response assays as previously described [21]. All experimental work was performed at the service laboratory of the array supplier (Invitogen, Carlsbad, CA, USA).

Cell culture and reagents

LNCaP cells were obtained from ATCC. C4-2 cells and C4-2b were obtained from MD Anderson Cancer Center, Houston, TX. LNCaP-Bic cells were a kind gift from Dr Zoran Culig (General Hospital Feldkirch). DuCaP cells were a kind gift from Philips Research (Eindhoven, The Netherlands). SKOv3 cells were a kind gift from Dr. James Brenton (University of Cambridge). LNCaP, C4-2, C4-2b and DuCaP cells were maintained at 37°C in RPMI 1640 (Invitrogen) containing L-Glutamine and supplemented with 10 % fetal bovine serum (Fbs) (Gibco) in a humidified atmosphere supplied with 5 % CO₂. LNCaP-Bic cells were cultured under the same conditions but medium was supplemented with 1 μM Bicalutamide (Enzo Life Science). Cells were routinely sub-cultured 1:4 using 0.25 % Trypsin-EDTA (Invitrogen) when 80-90 % confluency was reached.
Forskolin was obtained from Sigma-Aldrich. A SIK2 inhibitor, ARN3236, was obtained from Arrien Pharmaceuticals with reported low nanomolar IC50 for SIK2 both in activity assays and cell-line experiments. [22] SIK2 siRNA sequences were obtained from Dharmaco. [19] The non-targeting siRNA was obtained from Dharmaco. The pCMV6-Entry-myc/flag construct was obtained from Origene. The pCMV6-Entry-WT-SIK2-myc/flag and pCMV6-Entry-SIK2-EOS-KI-myc/flag were generated as previously described. [19]

**Cell transfection**

**AMAXA nucleofection**

For AMAXA nucleofection, 2x10⁶ cells were mixed with 100 µL Nucleofector solution R (Lonza) and 10 µg of DNA construct or 1 µM of siRNA duplexes. An electrical current was then applied to the cells (Nucleofector programme T-09) to deliver the DNA or siRNAs into the nucleus. Cells were then transferred into culture dishes containing RPMI 1640 supplemented with 10 % Fbs.

**Lipofectamine transfection**

For each reaction, siRNA duplexes at 40 nM were mixed with 6 µl of Lipofectamine™ RNAi Max (Invitrogen) and 500 µl of Optimem (Gibco). Mixtures were incubated for 20 min at room temperature (RT) and then dispensed in a 6-well plate before addition of 200,000 cells in 2 ml of RPMI 1640 supplemented with 10 % Fbs.

**DNA preparation**

All the DNA constructs used in this study were expanded using the XL10-Gold ultracompetent *Escherichia coli* cells (Agilent Technologies) and were purified using HiSpeed Plasmid Midi Kit (Qiagen) according to manufacturer’s recommendations.

**Cell counting and Cell viability**
Cells were seeded in triplicate at a density of 300,000 cells per well in a 6-well plate. At each time point, the supernatant was harvested to include dead or detached cells and live cells were harvested using 0.25 % Trypsin-EDTA (Invitrogen). Dead cells and live cells were then pooled together, pelleted, resuspended in 500 μl 1X PBS and transferred to a vial for cell counting and estimation of cell viability using a Beckman Coulter™ Vi-Cell.

**IncuCyte growth assays**

Cells were seeded in four replicates at a density of 20,000 cells per well in a 48-well plate. Plates were placed in the IncuCyte™ and nine time-lapse images of each well were taken at 3 hour intervals for seven days. IncuCyte™ 2010A software was used to assess changes in cell confluence as a surrogate for change in cell number.

**MTS Cell proliferation assay**

Cells were seeded in four replicates at a density of 10,000 cells per well in a 96-well plate. At each time point, 20 μl of CellTiter 96® AQsous Assay reagent (Promega) were added directly to each well with minimal exposure to light. Plates were incubated for 1 h at 37°C, 5 % CO₂. Formazan absorption was measured at 490 nm using an Infinite M200 spectrophotometer (Tecan). The mean absorbance of wells was displayed as optical density to estimate proliferation status.

**Soft agar colony formation assay**

Cell were resuspended in DMEM (Cell Biolabs) supplemented with 6 % Fbs and containing 0.4 % agar. They were then seeded in six replicates at a density of 1,000 cells per well in a 96-well plate containing a bottom layer of DMEM supplemented with 10 % Fbs and containing 0.6 % agar. Cell-agar suspension was overlayed with media containing 10 % Fbs and cultured for seven days. After seven days, the soft agar layer was solubilised, cells were lysed and number of colonies was determined using the CyQuant GR dye and measure of
fluorescence at 520 nm. To measure colony formation of cells after transient knock-down, cells were transfected with siRNA, trypsinised 24 h later and 10,000 cells were reseeded in soft agar as described above.

**Cell cycle analysis**

For DNA content analysis, cells were seeded in triplicate at a density of 300,000 cells per well in a 6-well plate and were grown for 48 h or 72 h. At each time point, cells were trypsinized using 0.25 % Trypsin-EDTA (Invitrogen), were washed in 1X PBS and were fixed with 1 % paraformaldehyde (Electron Microscopy Science) for 1 h at 4°C. Cells were then washed in cold 1X PBS (Gibco), resuspended in 80 % ice cold methanol and stored at -20°C until staining. Methanol-fixed cells were treated with 3 μM DAPI (Sigma-Aldrich) overnight at 4°C. Fluorescence activated cell sorting (FACS) analysis was carried out using a BD LSRII instrument (Becton&Dickinson, San Jose, CA) and data acquisition was performed using BD FACSDiva software (v.5.0.3.). The fluorescence emitted by DAPI was collected using a UV-450/50 bandpass filter. Data were analysed after doublet discrimination [23] using the FlowJo software (Tree Star, v.8.8.4.) and applying the curve-fitting algorithm contained in the software.

**Annexin V Apoptosis assay**

Cells were seeded in triplicate at a density of 300,000 cells per well in a 6-well plate. At each time point, the supernatant was harvested to include dead or detached cells and live cells were harvested using 0.25 % Trypsin-EDTA (Invitrogen). Dead cells and live cells were then pooled together, washed in 1X PBS, resuspended in Annexin V binding buffer (BioLegend) and stained with 5 μg/ml PI (Sigma-Aldrich) and Annexin V-AlexaFluor647 (BioLegend) for 15 min. Fluorescence activated cell sorting (FACS) analysis was carried out using a BD LSRII instrument (Becton&Dickinson) and data acquisition was performed using BD
FACSDiva software (v.5.0.3.). The fluorescence emitted by PI and AlexaFluor647 was collected using a Blue-575/26 and a Red-660/20 bandpass filters. Data were analysed after doublet discrimination [23] using the FlowJo software (Tree Star, v.8.8.4.).

**Immunofluorescence**

Cells were seeded at a density of 50,000 cells per well in a 24-well plate with coverslips. After at least 48 h to allow adherence, cells were washed in 1X PBS and were fixed with 4 % paraformaldehyde (Sigma-Aldrich) for 3 min. Cells were then washed in cold 1X PBS (Gibco), treated with 80 % ice cold ethanol and stored at -20°C until staining. For staining, cells were washed in 1X TBS-0.2 % Triton-0.04 % SDS, blocked for 30 min with 1X TBS supplemented with 1.5 % bovine serum albumin (BSA) (Sigma-Aldrich) and incubated for 1 h with a SIK2 (BioLegend) or γ-Tubulin (Sigma-Aldrich) primary antibody followed by an appropriate AlexaFluor488® or AlexaFluor594® conjugated secondary antibody (Invitrogen). Cover slips were then mounted onto slides with Vectashield solution containing DAPI (Vectashield). Images were taken using a Leica Tandem confocal microscope.

**Time lapse imaging**

Cells were seeded at a density of 40,000 cells per well in a 15 µ-Slide 8 well plate (Ibidi). After 36 h, the plate was placed in an incubator in a humidified atmosphere supplied with 5 % CO$_2$ connected to a Nikon Eclipse TE 2000 microscope (Nikon Instruments Europe). Images were obtained using 40X lens and were collected every 5 min for 48 h from 4 different positions per well using NIS-Elements AR 3.2 software (NIS Elements). The time from the start of pro-metaphase, as evidence by the cells detaching and rounding-up, to the end of cytokinesis, as evidence by the generation of two daughter cells, was scored for individual cells. The exact start time of pro-metaphase for each cell was recorded to avoid duplication of measurement.
Preparation of total cell extracts

Cells were washed, scraped in ice cold 1X PBS, pelleted and resuspended in lysis buffer containing 20 mM Hepes (pH 7.5), 150 mM Sodium Chloride, 1% NP-40, 0.25% Sodium Deoxycholate, 10% Glycerol, 40 mM Sodium Fluoride, 12 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.5 mM EDTA, 2.5 mM EGTA, a protease inhibitor cocktail (Calbiochem) and a phosphatase inhibitor mix (Sigma-Aldrich). The extracts were cleared by centrifugation (8,000 g for 10 min at 4°C) and supernatants were collected. Protein quantification was performed using the Quant-iT Protein Assay according to manufacturer’s recommendations (Invitrogen). Total cell lysates were boiled at 70°C in 5X SDS sample buffer for 10 min and immunoblot analyses were performed as described below.

For treatment of the extracts with a phosphatase, the same procedure was followed but the extracts were prepared in NEBuffer 3 (New England Biolabs) and were sonicated for 90 sec before being treated with Alkaline Phosphatase Calf Intestinal (New England Biolabs) at 1 unit per µg of protein for 2 hours at 37°C.

Preparation of cellular and nuclear extracts

Cells were washed, scraped in ice cold 1X PBS, pelleted and resuspended for exactly 10 min in lysis buffer containing 10 mM Hepes (pH 7.9), 10 mM Potassium Chloride, 1.5 mM Magnesium Chloride, 0.34 M Sucrose, 10% Glycerol, 1 mM Dithiothreitol, 0.1% Triton, a protease inhibitor cocktail (Calbiochem) and a phosphatase inhibitor mix (Sigma-Aldrich). The extracts were cleared by centrifugation (1,300 g for 4 min at 4°C) and the cytoplasmic fractions were collected and cleared by centrifugation (20,000 g for 15 min at 4°C). The nuclei were washed twice in lysis buffer containing 10 mM Hepes (pH 7.9), 10 mM Potassium Chloride, 1.5 mM Magnesium Chloride, 0.34 M Sucrose, 10% Glycerol, 0.1% Triton, a protease inhibitor cocktail (Calbiochem) and a phosphatase inhibitor mix (Sigma-
Aldrich). They were then resuspended in lysis buffer containing 3 mM EDTA, 0.2 mM EGTA, 1 mM Dithiothreitol, 0.1 % Triton, a protease inhibitor cocktail (Calbiochem) and a phosphatase inhibitor mix (Sigma-Aldrich), sonicated for 90 sec and cleared by centrifugation (20,000 g for 15 min at 4°C) to obtain the nuclear fractions. Protein quantification was performed using the Quant-iT Protein Assay according to manufacturer’s recommendations (Invitrogen). Cellular and nuclear extracts were boiled at 70°C in 5X SDS sample buffer for 10 min and immunoblot analyses were performed as described below.

**Immuno-precipitation**

10 µg of SIK2 (Cell Signaling) or IgG Rabbit (Vector) antibody were incubated with 50 µL Protein A Dynabeads (Novex by Life Technologies) for 4 h at 4°C and subsequently cross-linked with 20 mM Dimethyl pimelimidate in 0.2 M triethanolamine pH 8.2 at room temperature. For each condition, 75 million cells were lysed and cellular and nuclear extracts prepared as indicated in the sections above. Each fraction was then incubated with the antibody bound beads overnight at 4°C with rotational mixing. Beads were washed 3 times in 1 % RIPA and then placed in 1X Laemli buffer and heated at 70°C for 10 min. Beads were removed on a magnet and immunoblot analyses on the IP products were performed as described below.

**Western blotting**

Immunoblot analyses were performed using a range between 20 µg and 100 µg of lysates depending on the abundance of the protein of interest. The material was electrophoretically resolved on denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels ranging from 6 % to 16 % acrylamide and was transferred to nitrocellulose Immobilon-P membranes (Invitrogen) using the iBlot™ Dry Blotting System (Invitrogen). Membranes were then blocked in 5 % w/v Marvel™ milk or 5 % BSA (Sigma-Aldrich) w/v
in 1X TBS-0.1 % Tween for 30 min at RT and were immunoblotted with β-Actin (Abcam), β-Tubulin (Abcam), Bak (Cell Signaling), Bax (Cell Signaling), Bid (Cell Signaling), CREB1 (Cell Signaling), pCREB1 (Cell Signaling), Cyclin D1 (Cell Signaling), Histone H3 (Abcam), p21 (Cell Signaling), p27 (Cell Signaling), PARP (Cell Signaling), SIK2 (BioLegend, Sigma-Aldrich or Cell Signaling), TORC1 (Cell Signaling), TORC2 (Cell Signaling) and TORC3 (Cell Signaling) primary antibodies overnight at 4°C. Membranes were then washed 5 times in 1X TBS-0.1 % Tween and were incubated with an appropriated HRP-conjugated secondary antibody (Dako) for 1 h at RT. Membranes were then washed 5 times in 1X TBS-0.1 % Tween and proteins were visualised using ECL Plus Western Blotting Detection System (GE Healthcare).

**RNA extraction and cDNA synthesis**

Cells were washed, scraped in ice cold 1X PBS, pelleted and resuspended in 1 ml Trizol reagent (Sigma-Aldrich). Samples were incubated for 5 min at RT, mixed with 200 μl of chloroform and cleared by centrifugation (12,000 g for 15 min at 4°C). The aqueous upper phase was saved and incubated with 500 μl isopropanol for 10 min at RT. Samples were centrifuged (12,000 g for 15 min at 4°C), supernatants were removed and pellets were washed with 1 ml 75 % ethanol. Ethanol was discarded after centrifugation and pellets were resuspended in RNase free H2O. RNA quantification was assessed using NanoDrop™ 1000 (Thermo Scientific). 1 μg of total RNA was reverse transcribed using the High Capacity RNA-to-cDNA MasterMix (Applied Biosystems) according to manufacturer’s recommendations.

**Quantitative Real-Time PCR (qRT-PCR)**

Reactions were performed in triplicate using 5 μl of FAST SYBR GreenPCRMaster Mix (Applied Biosystems), 2 pmol of primers (Table S1) and 1 μl of cDNA in a total volume of
10 μl. The cycling conditions were 20 sec at 95°C, 40 cycles of 1 sec at 95°C, and 20 sec at 60°C. All reactions were performed on the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) and data were analysed with Applied Biosystems Sequence Detection Software (v.2.3.).

Relative expression levels were calculated using the following formula:

\[
\frac{(\text{efficiency of test PCR}^{(\text{control sample Ct} - \text{test sample Ct})})}{(\text{efficiency of control PCR}^{(\text{control sample Ct} - \text{test sample Ct})})}
\]

with PCR efficiency \(= 10^{\left(\frac{1}{\text{standard curve slope}}\right)} = 2\)

Ct represents the number of cycles required before an arbitrary threshold is reached. Test PCR represents the gene of interest. Control PCR represents the control genes. Test sample represents the sample of interest (e.g. cell line or condition). Control sample represents the reference sample (e.g. parental cell line or cells at t=0).

Data were normalized to housekeeping genes β-Actin and SDH, and the ratio between the sample of interest and the reference sample was calculated.

**Illumina HumanHT-12 v4 Expression BeadChip**

Cells were washed, scraped in ice cold 1X PBS, pelleted and resuspended in 1 ml TRI reagent solution (Ambion). RNAs were extracted using RiboPure™ Kit according to manufacturer's instructions (Ambion). RNA quantification was assessed using NanoDrop™ 1000 (Thermo Scientific) and RNA integrity by microanalysis (Agilent Bioanalyzer, Palo Alto, CA). RNAs were diluted to 22.7 ng/μl and 250 ng were used as the input for cRNA conversion which includes amplification and biotin labelling, using the Illumina TotalPrep-96 Kit (Ambion). Purified, quality controlled (Bioanalyser and Nanodrop spectrophotometer) and normalised cRNA was hybridized to arrays according to the Illumina protocol (Illumina, WGGX DirectHyb Assay Guide 11286331 RevA). Raw image files were processed and analysed using the Beadarray package from Bioconductor. [24]
**Reporter assays**

For reporter assays, cells were transfected with 2 µg of CRE(1) reporter construct (Panomics) using Amaxa nucleofection as described above. Cells were then seeded in 12 replicates at a density of 150,000 cells per well in a 24-well plate. At each time point, cells were scraped directly in culture medium, transferred into a 96-well plate, pelleted, washed in ice cold 1X PBS and resuspended in 20 µl 1X lysis buffer (Promega). Lysates were then transferred into a dark 96-well plate and luciferase assays were performed using a Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) and an automatic injection luminometer instrument (PHERAstar FS, BMG Labtech). 100 µl of Dual-Glo® Luciferase reagent was injected to each well, mixed and Firefly luciferase activity was measured 10 min later. Then, 100 µl of Dual-Glo® Stop&Glo® reagent was added to each well, mixed and *Renilla* luciferase activity was measured 10 min later. Activity was determined by calculating the ratio of Firefly luciferase to *Renilla* luciferase for each well and replicates were averaged.
Results

SIK2 is found in plasma and is a marker for prostate cancer.

In order to identify novel biomarkers for prostate cancer in biological fluids, we conducted a study using protein arrays in order to detect the immune reactivity of plasma samples obtained from 21 patients with indolent disease and 23 with aggressive disease. [21] Auto-antibody responses were able to discriminate between indolent and aggressive cases and identified salt-inducible kinase 2 (SIK2) as over-expressed in patients with aggressive disease compared to patients with indolent disease (p=2.32E-04). (Figure 1A) The same samples were used to generate a receiver operating characteristic (ROC) curve [25] for SIK2 in plasma and confirmed that SIK2 auto-antibodies could be a good blood marker for prostate cancer. (Figure 1B)

SIK2 knock-down and kinase-dead SIK2 reduce proliferation and viability of LNCaP cells.

In order to assess the role of SIK2 in prostate cancer, we transiently over-expressed a construct containing the wild-type sequence of the kinase (pCMV6-Entry-SIK2-myc/flag) and the corresponding construct containing the kinase-dead sequence (pCMV6-Entry-SIK2-EOS-KI-myc/flag) in LNCaP cells (Figure 2A). The N-terminal of SIK2 contains the serine/threonine kinase domain with lysine 49 (K49) located at the putative ATP binding site that is essential for SIK2 kinase activity. The SIK2 mutant construct used had this lysine replaced with a methionine (K49M), so the activity of the kinase was abolished. [26]

Cell proliferation and viability assays were then conducted using a Beckman Coulter™ Vi-Cell and Trypan Blue staining. Reduced cell growth could be observed after 4 and 7 days of growth when the kinase-dead construct was over-expressed in the LNCaP cell line compared to the wild-type and empty vectors. (Figure 2B) In addition, LNCaP cells over-expressing
the kinase-dead mutant had reduced cell viability compared to the empty vector and wild-type SIK2 (75.6 %, 91.3 % and 92.0 % viable cells respectively after 4 days of growth). (Figure 2C) To compensate for limitations on cell numbers and variable transfection efficiencies following transient transfection, we went on to generate LNCaP cells stably over-expressing the same constructs and confirmed these phenotypic effects. (Supplementary Figure 1) However, the very low proliferation rate of the kinase-dead over-expressing stable cell line resulted in the de-selection of the cells over-expressing the construct over time (data now shown).

We then used multiple siRNA sequences [19] to transiently knock-down SIK2 in LNCaP cells. Cell proliferation and viability after 4 and 7 days of knock-down were measured using a Beckman Coulter™ Vi-Cell and Trypan Blue staining. SIK2 knock-down induced a significant reduction in cell proliferation and cell viability with up to 70 % cell death after 7 days of growth. The magnitude of inhibition of cell proliferation and viability correlated with that of SIK2 depletion. The cytotoxic effect was the most pronounced for siRNA C, which was also the siRNA which gave the most marked phenotype in ovarian cells and vise a versa for siRNA A. [19] (Figure 2B and C) The phenotypic effects observed after SIK2 knock-down in LNCaP cells were also observed using other methods to assess cell proliferation (Supplementary Figure 1) and in a selection of other model prostate cancer cell lines. (Supplementary Figure 2)

**SIK2 knock-down induces apoptosis in LNCaP cells.**

The significant reduction in cell viability observed after knock-down of SIK2 in LNCaP cells prompted us to consider whether it has a role in apoptosis. We carried-out Annexin V assays on LNCaP cells transfected with all three SIK2-siRNA sets (A, B and C) and the non-targeting siRNA (NT). PI and Annexin V stainings were performed on the cells at day 2, 3, 4
and 7 after knock-down, and the percentages of viable, necrotic and apoptotic cells were evaluated using flow cytometry and an appropriate gating strategy. **Figure 3A** shows the percentage of apoptotic cells along the time course following transfection with all three SIK2 siRNAs and the non-targeting siRNA. Significant differences were observed when the cells were transfected with all three SIK2-siRNAs, showing a clear induction of apoptosis after SIK2 knock-down (up to 60 % with siRNAs B and C, and 40 % with siRNA A). Interestingly, when the cells were transfected with siRNA C, apoptosis occurred faster, with 40 % of cells apoptotic only two days after knock-down. Representative FACS profiles for the non-targeting siRNA and SIK2-siRNA C are shown in **Figure 3B**.

To gain further insight into the mechanisms involved, we then looked at the levels of the key apoptosis regulators Bid, Bax, Bak, Caspase-3 and PARP in the LNCaP cell line knocked-down for SIK2. (**Figure 3C**) SIK2 immunoblotting confirmed the efficiency of the knock-down with all three siRNAs and the equal amount of β-actin in all cell extracts confirmed that cellular integrity was unaffected. Bid levels were found to be decreased after SIK2 knock-down, which suggested it had been cleaved into its truncated form tBid, although the lower molecular weight band could not be detected (15 kDa). Bax and Bad levels were increased after knock-down with all three siRNAs, and a clear reduction in the levels of Caspase-3 and PARP accompanied by the appearance of lower molecular weight bands (17 kDa and 89 kDa respectively) were observed after knock-down with SIK2-siRNA C, which suggests that Caspase-3 and PARP were cleaved. These results indicate that SIK2 knock-down induces apoptosis in LNCaP cells via a mechanism involving the pro-apoptotic proteins Bid, Bax and Bak, and the subsequent cleavage of Caspase-3 and PARP. Again, the effects seen were the most apparent with siRNA C and least apparent with the less efficient siRNA A.
SIK2 knock-down induces a cell cycle arrest in G1 phase and delays mitotic progression of LNCaP cells.

Following on from Ahmed et al. (2010) showing that SIK2 is a centrosome kinase required for cell cycle progression from G1 to S and for the initiation of mitosis [19], we questioned whether the effects observed on cell proliferation after SIK2 knock-down in LNCaP cells were a consequence of its implication in cell cycle regulation.

The population distribution in G1, S and G2/M phases of the cell cycle 48 h and 72 h after SIK2 knock-down in LNCaP cells was assessed using flow cytometry and DAPI staining. There was a significant increase in the proportion of cells in G1 phase when SIK2 was knocked-down with all three siRNAs versus the non-targeting siRNA control (68.9 %, 69.9 %, 75.3 % cells in G1 for siRNAs A, B and C respectively versus 65.5 % for NT-siRNA at 48 h) (Figure 4A and B). This indicated that SIK2 inhibition efficiently inhibited the progression of the cells through the cell cycle by arresting them in G1 phase. Consistent with this phenotype was an increase in p21 expression with all three siRNAs. (Figure 4C) There was also a decrease in Cyclin D1 level with siRNA C and an increase in p27 was observed after knock-down with siRNA A, reflecting the cells inability to exit G1 phase after SIK2 knock-down. (Supplementary Figure 3) These data support the phenotype observed using flow cytometry, indicating that SIK2 knock-down induces a cell cycle arrest in G1 via the up-regulation of p21 and p27 and the down-regulation of Cyclin D1.

A G1-cell cycle arrest can be a consequence of the inability of the cells to enter mitosis and then escaping into G1. Given that SIK2 has been shown to play a key role in the initiation of the G2/M phase transition by localising at the centrosomes in ovarian cancer cells [19], we decided to test whether this was also the case in prostate cancer cells. γ-Tubulin is a protein member of the microtubule organising center (MTOC), which plays a role in the nucleation and polar orientation of microtubules. It localises at the centrosome and is commonly used as
a centrosome marker. [27] Therefore, we used SIK2 and γ-Tubulin co-stainings and immunofluorescence to look at a potential co-localisation between the kinase and the centrosomes in our cells. LNCaP cells were stained using antibodies for SIK2 and γ-Tubulin. Staining was also performed on the ovarian cancer cell line SKOv3 for a positive control. Images are shown in Supplementary Figure 4A. We could reproduce the data published by Ahmed et al. (2010) showing a clear co-localization of SIK2 at the centrosomes in ovarian cancer cells in interphase and in mitosis. [19] We also observed co-localisation between SIK2 and γ-Tubulin in LNCaP cells, providing some evidence of SIK2 co-localization at the centrosomes and a potential role in the regulation of mitosis in prostate cancer cells. This co-localisation was particularly apparent in metaphase cells, and to a lesser extent in anaphase and interphase cells. (Supplementary Figure 4B) The predominant detection of a single band using Western blotting which was enhanced after over-expression of the wild-type or kinase-dead (KI) constructs and dissipated after siRNA depletion confirmed the specificity of the antibody as previously reported. [19] (Supplementary Figure 5)

The co-localisation of SIK2 with the centrosomes in prostate cancer cells strongly suggests that SIK2 might be involved in the initiation of centrosome splitting and the subsequent induction of cell entry into mitosis as it was described in ovarian cancer cells. [19] We consequently used bright field microscopy and time lapse imaging to test whether cells knocked-down for SIK2 had delayed mitotic progression. LNCaP cells were transfected with all three SIK2-siRNA sets (A, B and C) and the non-targeting siRNA (NT), and were grown for 36 h before being monitored for 48 h using time lapse imaging. The time from the start of pro-metaphase, as evidence by the cells detaching and rounding-up, to the end of cytokinesis, as evidence by the generation of two daughter cells, was scored for 20 individual cells per condition. Results showed that loss of SIK2 resulted in a dramatic increase in the mean mitotic progression time (52 min in control cells transfected with non-targeting siRNA versus
112, 183, and 437 min following SIK2 knock-down. (Figure 4D) Importantly, many of the SIK2 depleted cells either did not exit mitosis during the time of recording or failed to undergo cytokinesis so exited into G1 or entered apoptosis (data now shown). This was particularly apparent with siRNA C where 15 cells out of the 20 counted underwent apoptosis, which represents 75% of the cells which corroborates the data shown in Figure 3A. Interestingly, out of those 15 cells which underwent apoptosis, 2 managed to complete mitosis but entered apoptosis as soon as cytokinesis was completed (data not shown). These data strongly suggest that SIK2 is involved in the initiation of cell entry into mitosis (G2 to M) or in the progression through mitosis as it was described in ovarian cancer cells. [19] Therefore, the cell cycle arrest observed in G1 after SIK2 depletion (Figure 4A) is the result of cells failing to complete mitosis and escaping into G1.

**SIK2 regulates CREB1 activity via the phosphorylation of TORC1 and its sequestration in the cytoplasm.**

The importance of SIK2 in prostate cancer cells growth and survival and the significant impact of the kinase-dead mutant on the cells phenotype implicate SIK2 and its downstream targets in prostate cancer progression. Several studies have pointed to a role for SIK2 in the regulation of metabolic and survival pathways via the repression of CREB1 (cAMP response element-binding protein)-dependent transcription following the phosphorylation of the transducers of regulated CREB1 activity (TORC). [5-11] Prostate cancer therapies typically involve the imposition of stress on cancer cells through hormone deprivation and related approaches. To provide a more complete understanding of the impact of SIK2 on prostate cancer cells, we tested whether changing SIK2 expression levels might affect CREB1-dependent gene transcription via TORC phosphorylation and degradation or cellular re-localisation in our cell lines. Firstly, we used reporter assays and nuclear fractionation
methods to test whether CREB1 activity and TORC1 phosphorylation and degradation and/or localisation were under the control of SIK2 in LNCaP cells.

Using a luciferase reporter construct containing the specific DNA binding sequences of CREB1 (cAMP response elements (CRE)) and a constitutively expressing Renilla luciferase construct, we monitored the activity of CREB1 24 h following SIK2 knock-down and 12 h following over-expression of wild-type and kinase-dead SIK2. CREB1 activity also was assessed 12 h after treatment with Forskolin, an inducer of CREB1 which raises the intracellular levels of cAMP and induces CREB1 activity through the activation of cAMP-dependent protein kinase (PKA), and the subsequent phosphorylation of CREB1 at Ser133.

As expected, the control Forskolin induced a dramatic increase of CREB1 activity. SIK2 depletion using three independent siRNAs or ectopic expression of kinase-dead SIK2 (KI) resulted in a significant increase in CREB1 activity. Conversely, CREB1 activity was significantly reduced when the wild-type kinase was over-expressed. (Figure 5A) We also observed a dose-dependent increase in CREB1 activity along time when using the SIK2 inhibitor ARN3236 (Supplementary Figure 6) which confirmed the role of the kinase in CREB1 regulation.

Based on the observation of a potential regulation of CREB1 activity by SIK2, we tested whether we could validate a direct interaction between the kinase and its well-known substrate, the transducers of regulated CREB1 activity (TORC), TORC1. [14] We could demonstrate that SIK2 and TORC1 directly interact by co-immunoprecipitating both proteins in C4-2b, a prostate cancer cell line expressing high endogenous levels of the kinase. (Figure 5B)

Protein extracts of LNCaP cells transfected with EV, WT and KI were then prepared in order to examine the effects of the kinase on the expression levels of TORC1. Western blots showed that over-expression of the wild-type kinase increased the steady-state levels of
TORC1 but also revealed the presence of a double band when the cells were transfected with the wild-type kinase (Figure 5C, (-CIP)). This prompted us to explore whether this was the result of TORC1 phosphorylation by SIK2. The same extracts were therefore also treated with a phosphatase alkaline (Figure 5C, (+CIP)), and the disappearance of the upper band after treatment with the phosphatase confirmed the phosphorylation status of TORC1 following over-expression of the wild-type kinase. This confirmed the findings of others depicting role of SIK2 in the prevention of TORC degradation via its phosphorylation. [6-9, 11] Furthermore, we observed that the levels of phospho-CREB1 were unchanged following over-expression of the wild-type and kinase-dead SIK2, which suggests that CREB1 activity is regulated via a phosphorylation-independent mechanism.

Isolated cytoplasmic and nuclear fractions for EV, WT and KI were then prepared in order to examine the effects of wild-type and kinase-dead SIK2 on the cellular distribution of TORC1. Western blots performed on lysates obtained after cellular fractionation confirmed the presence of the transcription factor CREB1 and its phosphorylated form pCREB1 in the nucleus. They also showed a clear accumulation of TORC1 in the cytoplasmic fraction when the cells were transfected with wild-type SIK2. Conversely, lower levels of TORC1 were found in the nuclear fractions when the cells were transfected with the wild-type kinase compared to the empty vector or the kinase-dead mutant. The change in TORC1 localisation after over-expression of wild-type and kinase-dead SIK2 suggests it relates to the activity of the kinase and not merely its presence, since the kinase-dead mutant was not able to maintain TORC1 in the cytoplasm. Surprisingly, this mutant does not appear to induce more TORC1 translocation to the nucleus than the empty vector (similar levels of TORC1 were found in the nucleus with KI compared to EV) but there was a clear induction of CREB1 activity with kinase-dead SIK2 in the reporter assay. Interestingly, the cellular localisation of the wild-type and kinase-dead SIK2 was also investigated and high levels of the mutant protein were found
in the nuclear fractions compared to the wild-type. This would indicate that kinase-dead SIK2 does not only permit TORC1 translocation to the nucleus but also participates in its trans-activation. (Figure 5D)

Whilst we could nicely confirm increased steady-state levels of the other two TORC isoforms – TORC2 and TORC3 – (Supplementary Figure 6B -CIP), and their phosphorylation following over-expression of the wild-type kinase (Supplementary Figure 6B +CIP), unfortunately, the examination of their cellular distribution has been more difficult since TORC2 and TORC3 were not discernible in the nuclear fractions despite our efforts to increase the concentrations of the lysates. When we could see a strong accumulation of TORC2 and TORC3 in the cytoplasmic fraction when the cells were transfected with wild-type SIK2, the two isoforms could not be detected in the nuclear fractions. (Supplementary Figure 6C) This issue can be due to a combination of TORC2 and TORC3 being far less abundant than TORC1 in our cell line and/or the quality of the antibodies used. (Supplementary Figure 5) Therefore, at that stage, we could not confirm their role in CREB1 regulation since their presence in the nucleus remained unproven.

**SIK2 regulates a cell cycle network and a ER stress network via CREB1-dependent transcription.**

After discovering a possible role for the transcription factor CREB1 and its co-activators, TORCs downstream of SIK2, we performed gene expression profiling using microarray in order to define genes and networks regulated by the SIK2. Based on the reporter assay, this approach was used in follow-up to provide an unbiased assessment of the impact on gene expression including but also extending beyond established CREB1 target genes.

For comparability the experiment was conducted under the same conditions as the reporter assay and cellular fractionation experiments. LNCaP cells were transiently transfected with
the wild-type SIK2 construct, the kinase-dead SIK2 construct (KI) and siRNA C (SIK2si) since this was the siRNA that gave the strongest phenotype in all the functional work performed. These conditions allowed us to identify a list of SIK2-regulated genes and pathways. We also included the condition where the cells were stimulated with Forskolin (Forsk.) in order to generate a signature of CREB1-regulated genes. The entire list of differentially expressed genes (DEG) identified in each condition are shown in Table S2.

We first looked at the DEG in the conditions where SIK2 was knocked-down (SIK2si), kinase-dead SIK2 was over-expressed (KI) and when the cells were treated with Forskolin (Forsk.) since all three conditions induced CREB1 activity. (Figure 5A) By comparing the gene signatures in those conditions, we aimed to identify SIK2-regulated genes dependent on CREB1 activity. A number of publications have defined CREB1 target genes based on chromatin immunoprecipitation and expression array profiling under conditions of Forskolin treatment and information is available in addition from a searchable online database, http://natural.salk.edu/CREB/. We therefore compared the genes that were differentially expressed in our cell lines with those reported to be CREB1 targets in other studies [29, 30] and selected inhibitor of DNA binding 1 (ID1), nuclear receptor subfamily 4 group A member 1 (NR4A1), homeobox A5 (HOXA5) and salt inducible kinase 1 (SIK1) for validation. Of these genes, ID1 was increased in expression by Forskolin treatment but also by knock-down of SIK2 and over-expression of the kinase-dead mutant. By contrast the other genes were either only induced by Forskolin treatment (e.g. SIK1) or indeed were inhibited in all conditions (e.g. HOXA5). This suggests that whilst CREB1 activity is enhanced by all three treatments, the impact of SIK2 knock-down or over-expression of kinase-dead SIK2 is pleiotropic and extends to other transcriptional regulators. Beyond that, the difference in Forskolin response in our study versus others, for example in respect to HOXA5 expression, suggests cell type dependency also plays a role and that systematic unraveling of the CREB1
regulome and SIK2-dependency will require subsequent in-depth study in prostate cells. Cell type dependency in the CREB1 regulome has been previously reported with Zhang et al. (2005), demonstrating on overlap of less than 5%.

We then went on to select a number of additional genes for validation based on their consistent pattern of expression change in the array data under all three conditions. These additional genes were CCR4 carbon catabolite repression 4-like (CCRN4L), DnaJ (Hsp40) homolog, subfamily C, member 12 (DNAJC12), ERBB receptor feedback inhibitor 1 (ERRFI1) and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta (NFKBIZ)), which were up-regulated in all three conditions, and homo sapiens chromosome 1 open reading frame 112 (C1orf112), forkead box O3 (FOXO3), and protein tyrosine phosphatase, receptor type, F (PTPRF)), which were down-regulated in the same conditions. (Figure 6A) Interestingly none of these genes have been reported to be CREB1 target genes in the database. We used quantitative real-time PCR in order to validate those findings and confirmed that FOXO3, HOXA5 and PTPRF were significantly down-regulated in all three conditions while ERRFI1 was significantly up-regulated. (Supplementary Figure 7)

Around half of the total number of genes down-regulated after SIK2 knock-down overlapped with the condition where the cells were treated with Forskolin (46 %) which confirmed that SIK2 regulates gene expression via CREB1, but surprisingly, only 12 % of the genes up-regulated by LNCaP-SIK2-KI and 10 % of the genes down-regulated were similar to the genes differentially expressed after Forskolin treatment. This suggests that the impact of the kinase-dead mutant on gene transcription probably involves transcription factors other than CREB1. (Figure 6A) The detailed gene lists are shown in Table S3.

The small number of SIK2-regulated genes dependent on CREB1 activity identified in our study is not amenable to a pathway analysis but transcriptional regulation is an over-
represented function when considering all eight using the DAVID gene ontology web tool. Four fall into this functional classification (FOXO3 [31], HOXA5 [32], NFKBIZ [33] and CCRN4L [34]). In addition two of the eight genes have been reported to be negative regulators of receptor tyrosine kinases (ERRFI1 and PTPRF) [35, 36]. Clearly therefore, although the number of genes is small, their impact on gene regulation and signaling is potentially significant.

In order to get a broader insight into the genes and networks downstream of SIK2, we submitted to the gene ontology data base Ingenuity® (IPA) the complete list of DEG after wild-type SIK2 and kinase-dead SIK2 over-expression and after SIK2 knock-down. The top five enriched networks associated with the categories “diseases and disorders” and “molecular and cellular functions” for each condition are shown in Supplementary Figure 8A. It is clear that networks related to cancer were considerably affected by triggering SIK2 in our prostate cancer cell line (cancer was the top disease in 4 out of 6 gene lists). This is particularly important since only one study to date has demonstrated a role for SIK2 in cancer progression. [19] The molecular and cellular functions repeatedly associated with the DEG identified in this study related to cellular growth and proliferation, cell death and survival, cell cycle and transcription. These findings agree with those of our functional study showing the involvement of SIK2 in prostate cancer cell growth, cell cycle and survival, as well as gene transcription via the regulation of the TORC/CREB1 transcriptional pathway.

We then interrogated the gene ontology data base GeneCodis [37, 38] to identify the enriched biological processes and the common transcriptional mediators underpinning the molecular function “cell cycle” after SIK2 knock-down and “cell death and survival” after over-expression of kinase-dead SIK2. The list of 211 genes down-regulated after SIK2 knock-down was submitted to the data base and the top 10 biological processes associated with this
gene list were identified. (Supplementary Figure 8B) It is clear that processes associated with cell cycle progression and especially mitotic progression, were over-represented. The gene lists of the top four biological processes were then submitted for DNA motif analysis in promoters within 2 kb of the transcription start site. There was a particular enrichment in E2F binding motifs, indicating that a majority of these genes are regulated by the E2F family of transcription factors. Interestingly motif enrichment using the CREB1 binding sites mapped by Zhang et al. (2005) by chromatin immuno-precipitation also reported that E2F motifs were the most significantly over-represented in those sites (p value $10^{-42}$). [29, 30]

The same procedure was followed using the 152 genes up-regulated after over-expression of kinase-dead SIK2. We identified a particular enrichment for processes related to response to stress with pathways relating to ER stress, unfolded protein response (UPR) and protein folding. The induction of a set of genes involved in stress response might be an unattended effect following the ectopic expression of a recombinant protein into the cells. Despite this, pathways relating to protein folding and response to stress were not enriched after over-expression of the wild-type kinase (Supplementary Figure 8A) and from the gene lists generated, a subset of those stress genes were distinctly being expressed after over-expression of the kinase-dead protein versus the wild-type (DNAJA1, DNAJB9, DNAJC12, DNAJC3, HSP90AA1, HSP90AB1, HSP90AB3P, HSP90AB4P, HPSA4, HSPA4L, HSPA5, HSPB1, HSPD1) (Table S2) which reflects the distinct effect of the mutant kinase on ER stress genes transcription. This was further supported by an enrichment for transcription factors such as heat shock factor (HSF), a transcriptional activator of heat shock genes, interferon regulatory factor 3 (IRF), a transcription factor involved in the regulation of interferon regulated genes, and nuclear factor kappa-light-chain-enhancer of activated B (NFκB), a transcriptional activator involved in cellular responses to stimuli such as stress, when the kinase-dead protein was over-expressed. (Supplementary Figure 8B)
The detailed lists of genes involved in the processes of "mitotic cell cycle", "protein folding" and "response to stress" are shown in Table S4. Owing to the profound impact of SIK2 knock-down in reducing cell proliferation and viability it was not possible to perform rescue experiments with SIK2 overexpression constructs against a knock-down background. Further dissection of these processes will therefore be pursued in future studies.

Taken together, these data suggest a hypothetical mechanism in which when SIK2 is present and catalytically active, it phosphorylates TORC1 which results in its sequestration in the cytoplasm. When in the cytoplasm, TORC1 is stabilised and not degraded as described in other studies, but its translocation into the nucleus is inhibited so CREB1-dependent gene transcription is repressed which results in cell cycle progression and ultimately, cell survival. (Figure 6B.i) When SIK2 is present but unable to phosphorylate TORC1, it is translocated to the nucleus with TORC1, probably as a complex. Once in the nucleus, the TORC1-SIK2-KI complex could act as a CREB1 trans-activator and also triggers the activation of other transcription factors such as HSF, IRF and NFκB, which results ER stress response and ultimately, cell death. (Figure 6B.ii)
Discussion

This is the first study to report that SIK2 auto-antibodies are highly expressed in patients with aggressive disease and poor prognosis and that they could be a good blood marker for the diagnosis of prostate cancer. Currently no single cohort exists with sufficient clinical follow-up as well as matched blood and tissue samples in which to test for SIK2 auto-antibody levels, SIK2 tissue expression and prognosis. Biobanks are being developed that will allow this to be addressed in the longer term future and this analysis will, from a clinical perspective, be vital in determining whether SIK2 inhibitors really should be introduced in prostate cancer patients. The case for developing SIK2 inhibitors was made earlier in ovarian cancer by Ahmed et al. (2010) [19]. They showed that that SIK2 is a centrosome kinase required for the initiation of mitosis so we first considered a potential role for SIK2 in cell growth and cell cycle regulation in our cells.

In our study, we characterised the phenotypic effects of SIK2 over-expression and knock-down on prostate cancer cell lines for the first time. Our results showed that SIK2 knock-down induces a pronounced reduction in cell growth in a selection of prostate cancer cell lines (LNCaP, LNCaP-Bic, C4-2 and DuCaP) and this is accompanied by a G1 cell cycle arrest involving the cell cycle regulators p21, p27 and Cyclin D1 in LNCaP cells. We could reproduce the findings from research into ovarian cancer cells using our prostate cancer cell lines and showed that SIK2 co-localises at the centrosomes in cells in metaphase and anaphase and regulates cells entry or progression into mitosis. The most intriguing aspect of our findings was the pronounced inhibition of cell proliferation observed when the activity of the kinase was abolished (over-expression of a kinase-dead mutant of SIK2) in LNCaP cells. This was sufficiently potent that it led to a de-selection of cells transfected with the kinase-dead mutant during our attempts to derive stable cell-lines expressing the mutant. The significance of the kinase-dead mutant on cell growth has not been reported before and indicates the
importance of SIK2 downstream targets in prostate cancer progression. SIK2 mutations are detectable in TCGA (The Cancer Genome Atlas) data [39] and in the Cancer Cell-Line Encyclopedia (CCLE) [40] however none have been find in the catalytic site with the exception of a Lysine-49 to threonine mutation reported in an intestinal cell-line in the CCLE dataset. This residue is the same one that was mutated to create the kinase-dead mutant, SIK2-KI. Several studies have identified a role for SIK2 and its downstream target TORC in the regulation of cellular energy homeostasis under certain stress conditions. [5-11] It is important to take into account that SIK2 is a member of the AMPK family which are metabolic master kinases involved in the regulation of a number of metabolic processes including β-oxidation of fatty acids, lipogenesis, protein and cholesterol synthesis, as well as apoptosis. [41, 42] It was therefore crucial to consider that SIK2 is not only a centrosome kinase but it may also be implicated in the regulation of biological processes essential for the survival of prostate cancer cells. Indeed, a new function for SIK2 described in our work and previously unreported in other cancer types is its role in cell survival. We showed that SIK2 depletion induces cell death and that inhibition of its kinase activity also dramatically affects cell viability. This supports findings by Ahmed et al. (2010), who suggested that SIK2 could present novel avenues for the development of novel cancer therapies. [19] The authors suggest that SIK2 inhibitors could be used in combination with paclitaxel, a drug of category of the taxanes, which disrupts microtubules function so inhibits the process of cell division. Drugs which interfere with the mitotic process are commonly used for the treatment of many types of cancers. [43] For instance, docetaxel, another taxane family member, is used for the treatment of advanced prostate cancer. The cytotoxic activity of this type of drug in combination with SIK2 inhibitors might be exerted in prostate cancer compared to other cancer types since our data indicate that SIK2 not only interferes with cell cycle progression but also induces caspase-dependent apoptosis.
We then demonstrated that SIK2 regulates the activity of the transcription factor CREB1 by interacting with its trans-activator TORC1 and altering its cellular distribution via its phosphorylation. (Figure 5B, C and D) Over-expression of a kinase-dead mutant reduced steady-state levels but also led to a loss of a band doublet which could also be removed by phosphatase treatment indicating that it represented the phosphorylated form of the protein. (Figure 5C) These results confirmed findings published by others showing that SIK2 phosphorylates TORCs which results in their sequestration in the cytoplasm, but they did not corroborate the findings of Koo et al. (2005), Dentin et al. (2007), Wang et al. (2008), Ryu et al. (2009) or Sasaki et al. (2011) who reported that accumulation of TORC within the cytoplasm was followed by its degradation. [6-9, 11] Interestingly, we also showed that the cellular localisation of the kinase-dead mutant was nuclear compared to the wild-type kinase. This suggests that SIK2 regulation of CREB1-mediated transcription might be direct. Only SIK1 has been shown to act directly on CREB1 in the nucleus and all studies which report a role for SIK2 in CREB1 mediated transcription have implicated the trans-activators TORCs. However, Horike et al. (2003) have shown that SIK2 was mostly present in the cytoplasm in mouse adipose 3T3-L1 cells, but could be translocated to the nucleus when its phosphorylatable Ser587 was replaced with Alanine (S587A). The authors suggested that SIK2 could act as a direct repressor for CREB1-mediated gene transcription, as was the case with SIK1. [26] Here, we suggest that kinase-dead SIK2 might be part of a complex with TORC which directly induces CREB1 trans-activation in the nucleus. We used a gene expression profiling approach in order to define the genes and networks regulated by the SIK2/TORC/CREB1 transcriptional pathway. We compared the genes differentially expressed in our study with two published studies in which CREB1 target genes were identified. [29, 30] Out of the genes we identified, HOXA5 was a known CREB1-regulated gene [29] which makes it an interesting candidate involved in SIK2 signaling via CREB1-
mediated gene transcription. However, it was down-regulated in response to Forskolin treatment, SIK2 depletion and kinase-dead SIK2 over-expression which did not corroborate the enhanced CREB1 activity observed in our reporter assay. Zhang et al. (2005) further reported that certain CREB1 targets are silenced by CpG island hypermethylation, suggesting that other genomic factors can affect patterns of these genes [30]. One of the genes we selected for validation based on Rajvenskar et al.’s (2007) study, ID1 [29], was reported to be never hypermethylated in Zhang et al.’s (2005) and was robustly induced in our experiment [30].

We also found that about only 10% of the genes regulated by LNCaP-SIK2-KI were similar to the genes differentially expressed after Forskolin treatment, which suggests that the impact of the kinase-dead mutant on gene transcription probably involves transcription factors other than CREB1. Consistent with this was the very different network of genes identified following over-expression of kinase-dead SIK2 and depletion of the kinase (very few overlaps between both conditions). This was further supported by the motif analysis which revealed that transcription factors such as E2F, HSF, IRF, NFκB were enriched after over-expression of the kinase-dead protein.

Knock-down of SIK2 and over-expression of the kinase-dead mutant both affect cell cycle progression. Consequently the differential expression of cell cycle/E2F associated genes may not be a direct consequence of the effect of targeting SIK2 on transcription, but rather a by-product of the impact on cell cycle progression. Equally the over-expression of stress response genes when the cells were transfected with the kinase-dead mutant may be due to the stress associated with ectopic expression of high levels of a non-native protein in the cells rather than a direct regulatory impact of the mutant on a particular transcription factors or group of transcription factors. Clearly dissecting cause versus effect within these gene expression data sets will require significant additional studies that are beyond the scope of the
current paper. In particular, given that SIK2 and TORCs undergo nuclear-cytoplasmic translocation, it is of interest in future work to map their associated proteomes in these compartments. A recent method, rapid immuno-precipitation mass spectrometry of endogenous proteins (RIME) also provides the possibility to enrich CREB1 or indeed TORCs/SIK2 together with chromatin and begin to define the chromatin-associated protein complexes [44]. This method has revealed new co-regulators for estrogen receptor alpha and may uncover important differences in the transcription factors associating with the kinase-dead or wild-type kinase [45]. These points are of fundamental importance also because of the rapid progress that is being made to develop drugs that inhibit SIK2 kinase activity, based on the previous paper in ovarian cancer, with the aim of arresting cell cycle progression. In prostate cancer, enhanced CREB1 activity has been reported to promote prostate cancer progression and cell survival. [46] In part this has been suggested to occur because CREB1 importantly also enhances the expression of a number of anti-apoptotic genes including Bcl-2 in other experimental models. [47, 48] In this setting it is also worth noting that CREB1 activity is also enhanced by other pathways and enzymes that have been associated with progression to castrate-resistant disease including CAMKII activation. [49] This is important neuronal survival and memory potentiation but the kinase also has a role in promoting cell progression in conditions of androgen deprivation. [50] Consequently in prostate cancer the possibility exists that whilst inhibiting SIK2 may inhibit cell cycle progression by the same mechanism that was reported in ovarian cancer, there may be a counteracting pressure towards survival and ultimately cancer progression through enhanced CREB1 activation.

The impact of SIK2 on cellular phenotypes, as with many kinases, can be through their kinase activity but also through protein-protein interactions. [51] By over-expressing the kinase-dead mutant we are potentially sequestering SIK2 interacting proteins in complexes that are no longer dynamically regulated by SIK2 kinase activity whereas with SIK2 knock-
down, we are disrupting processes that require both SIK2-mediated kinase and SIK2-mediated protein-protein interactions. The impact of the SIK2 kinase-dead mutant on network of genes and pathways involved in cell growth and survival under certain stress conditions indicates that SIK2 protein-protein interactions are important regulators of viability. SIK2 inhibitors are under development to treat ovarian cancer based on the role of SIK2 as a regulator of mitosis. We have tested one such inhibitor with low nanomolar IC50 values in activity assays and preclinical models, ARN-3236 [22]. In reporter assays, this drug induced CREB activation in a similar manner to the kinase-dead mutant (SIK2-KI) (Supplementary Figure 6A) underscoring the importance of considering the impact on CREB signaling in evaluating responses to these novel agents. In conclusion, our study argues for further dissection of the mechanism of action of SIK2 in prostate cancer, particularly given the metabolic features of localised disease and the cell cycle dysregulation associated with metastatic progression.

Acknowledgements

H.B was supported by a BBSRC/CASE industrial PhD studentship. We are grateful to study volunteers for their participation and to staff at the Welcome Trust Clinical Research Facility, Addenbrooke's Clinical Research Centre, Cambridge for their help in conducting the study. We also acknowledge the support of the NIHR Cambridge Biomedical Research Centre, the DOH HTA (ProtecT grant) and the NCRI / MRC (ProMPT grant) for help with the bio-repository. We also thank Cancer Research UK for funding. None of the authors have any competing financial interests. All expression array data has been deposited on GEO (GSE45711).


Figure legends

Figure 1 – SIK2 autoantibodies are found in plasma and are a blood marker for prostate cancer.

(A) SIK2 autoantibodies were detected using protein arrays (ProtoPlexTM, Invitrogen) in plasma samples of two patient cohorts comprising 21 patients with indolent prostate cancer and 23 patients with aggressive prostate cancer (n = 44; n=23 in positive group and n=21 in the negative group). [20, 21]

(B) The same samples were used to generate a receiver operating characteristic (ROC) curve [25] for plasma SIK2. Area under the curve (AUC) for PSA is 0.8 with a p-value of <0.0001.

Figure 2 – Kinase-dead SIK2 and SIK2 knock-down reduce proliferation and viability of LNCaP cells.

(A) SIK2 over-expression and knock-down were confirmed by Western blot at day 4 and day 7 after transfection with a wild-type SIK2-Myc construct, a kinase-dead SIK2-Myc construct (KI) or the corresponding empty-vector construct (EV), and after transfection with the control non-targeting siRNA (NT) or SIK2-siRNAs (A, B, C). Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for SIK2 (Sigma-Aldrich). β-Actin (Abcam) was used as loading control.

(B) Cell proliferation of LNCaP cells over-expressing wild-type or kinase-dead SIK2 or knocked-down for SIK2 was assessed by counting the cells using a Beckman Coulter™ Vi-Cell and Trypan Blue staining. The number of viable cells at day 0, day 4 and day 7 is shown. (n=3; error bars represent mean ± SEM; *p<0.05, **p<0.01, ***p<0.001)

(C) Cell viability was assessed with a Beckman Coulter™ Vi-Cell and Trypan Blue staining. The percentage of viable cells at day 0, day 4 and day 7 is shown. (n=3; error bars represent mean ± SEM; *p<0.05, **p<0.01, ***p<0.001)
**Figure 3 – SIK2 knock-down induces apoptosis in LNCaP cells.**

(A) Apoptosis of LNCaP cells knocked-down for SIK2 was assessed using flow cytometry by simultaneously staining the cells with an Annexin V antibody (BioLegend) and the DNA dye PI (Sigma-Aldrich). The percentage of apoptotic cells at day 2, day 3, day 4 and day 7 after transfection with the non-targeting siRNA (NT) and SIK2-siRNAs (A, B, C) is shown. (n=3; error bars represent mean ± SD; *p<0.05, **p<0.01, ***p<0.001).

(B) Dot-plots are representative of the gating strategy used on LNCaP cells transfected with the control non-targeting siRNA (NT) and SIK2-siRNA C. Staining allows the viable cells (double negative), the apoptotic cells (Annexin V positive) and the necrotic cells (Annexin V and PI double positive) to be distinguished.

(C) Bid, Bak, Bax, PARP and Caspase-3 protein expression was assessed by Western blot four days after transfection of LNCaP cells with the control non-targeting siRNA (NT) and SIK2-siRNAs (A, B, C). Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for Bid, Bak, Bax, PARP and Caspase-3 (Cell Signaling). SIK2 levels after knock-down are also shown (Sigma-Aldrich). β-Actin (Abcam) was used as loading control. Cleaved PARP and Caspase-3 are indicated with arrows.

**Figure 4 – SIK2 knock-down induces a cell cycle arrest in G1 phase and delays mitotic progression of LNCaP cells.**

(A) The population distribution of LNCaP cells transiently knocked-down for SIK2 was assessed by cell cycle analysis using DAPI staining and flow cytometry. The percentage of cells in G1, S and G2/M phases of the cell cycle 48 h and 72 h after transfection with the control non-targeting siRNA (NT) and SIK2-siRNAs (A, B, C) is shown. (n=3; error bars represent mean ± SD; *p<0.05, **p<0.01, ***p<0.001).
(B) Representative flow cytometry profiles for each condition.

(C) p21 protein expression was assessed by Western blot 48 h after transfection of LNCaP cells with the control non-targeting siRNA (NT) and SIK2-siRNAs (A, B, C). Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for p21 (Cell Signaling). SIK2 levels after knock-down are also shown (Sigma-Aldrich). β-Actin (Abcam) was used as loading control.

(D) LNCaP cells were transiently knocked-down for SIK2 for 36 h and monitored for 48 h (one image every 5 min) using bright-field phase-contrast microscopy. The time interval from the start of pro-metaphase (cells detaching and rounding-up) to the end of cytokinesis (generation of two daughter cells) was estimated for 20 individual cells per condition. The mean mitotic progression time after transfection with the control non-targeting siRNA (NT) and SIK2-siRNAs (A, B, C) is shown. (n=20; error bars represent mean ± SD; *p<0.05, **p<0.01, ***p<0.001)

Figure 5 – SIK2 regulates CREB1 activity via the phosphorylation of TORC1 and its sequestration in the cytoplasm.

(A) LNCaP cells were transiently transfected with SIK2-siRNAs (A, B, C), the control non-targeting siRNA (NT), a wild-type SIK2-Myc construct, a kinase-dead SIK2-Myc construct (KI) and the corresponding empty-vector construct (EV). A control Forskolin treatment (Forsk., 10 μM) (+) or vehicle (DMSO) (-) was also included. CREB1 activity was assessed using a CRE(1) reporter construct (Affymetrix) and a dual-luciferase reporter assay system (Promega) 12 h after Forskolin treatment or SIK2 over-expression and 24 h after SIK2 knock-down. (n=12; error bars represent mean ± SD; *p<0.05, **p<0.01, ***p<0.001)

(B) SIK2 was immunoprecipitated (Cell Signaling) in C4-2b cells total protein lysates and immunoblot analyses of TORC1 were performed. Total cell lysates (input) and
immunoprecipitation products (SP and IP) were resolved by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for TORC1 (Cell Signaling). SIK2 levels after immunoprecipitation are also shown (Sigma-Aldrich).

(C) Phosphorylation status of CREB1, pCREB1 and TORC1 were checked after treatment of total protein lysates obtained from LNCaP cells 12 h after transfection with EV, WT, and KI using an alkaline phosphatase (New England Biolabs) (+ CIP). Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for CREB1, pCREB1 and TORC1 (Cell Signaling). SIK2 levels after over-expression are also shown (Sigma-Aldrich). β-Actin (Abcam) was used as loading control.

(D) Cellular fractionation was performed on LNCaP cells 12 h after transfection with EV, WT, and KI. CREB1, pCREB1 and TORC1 protein expression in cytoplasmic (cytop.) and nuclear (nucl.) fractions was assessed by Western blot. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for CREB1, pCREB1 and TORC1 (Cell Signaling). SIK2 localisation is also shown (Sigma-Aldrich). β-Tubulin and Histone H3 (Abcam) were used to confirm the purity of the fractionation.

Figure 6 – SIK2 regulates cell cycle and a ER stress response via CREB1-dependent transcription in prostate cancer cells.

(A) Venn diagrams summarise how many genes were found to be down-regulated or up-regulated in LNCaP cells after SIK2 knock-down (SIK2si), over-expression of kinase-dead SIK2 (KI) or treatment with Forskolin (Forsk.). Overlaps between the 3 conditions are also shown. 78 % of the genes down-regulated after SIK2 knock-down are dependent on CREB1 activity. 10 % and 12 % of the genes down- and up-regulated after over-expression of kinase-dead SIK2 are dependent on CREB1 activity.
(B) Proposed mechanism. (i) Wild-type SIK2 phosphorylates TORC, which results in TORC sequestration and accumulation in the cytoplasm. TORC translocation to the nucleus is inhibited, so CREB1 activity is repressed, which results in cell cycle progression and cell survival. (ii) Kinase-dead SIK2 (KI) cannot phosphorylate TORC. SIK2-KI and TORC form a complex which translocates to the nucleus where it activates CREB1 and other transcription factors such as HSF, IRF and NFκB, which results in ER stress response and cell death.
**Figure 2**

A. 

<table>
<thead>
<tr>
<th></th>
<th>EV</th>
<th>WT</th>
<th>KI</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIK2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Day 4     Day 7

MW (kDa) 38 102

B. 

**Cell proliferation**

<table>
<thead>
<tr>
<th></th>
<th>EV</th>
<th>WT</th>
<th>KI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Day 0     Day 4     Day 7

**Number of viable cells (x10^6)**

C. 

**Cell viability**

<table>
<thead>
<tr>
<th></th>
<th>EV</th>
<th>WT</th>
<th>KI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Day 0     Day 4     Day 7

**Percentage of viable cells (%)**
Figure 3

A. Apoptosis

B. Day 2

C.

<table>
<thead>
<tr>
<th>MW (kDa)</th>
<th>NT</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIK2</td>
<td>38</td>
<td>17</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>Bid</td>
<td></td>
<td></td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>Bak</td>
<td></td>
<td></td>
<td>31</td>
<td>17</td>
</tr>
<tr>
<td>Bax</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PARP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**A.** Apoptosis

**B.** Day 2

**C.**

Downloaded from mcr.aacrjournals.org on July 4, 2017. © 2014 American Association for Cancer Research.
Figure 4

A.

Cell cycle

48h

Cell cycle

72h

Proportion of total cell number (%)

NT  A  B  C

%G1  %S  %G2/M

B.

48h

72h

NT-siRNA

SIK2-siRNA A

SIK2-siRNA B

SIK2-siRNA C

Cell cycle

Proportion of total cell number (%)

%G1  %S  %G2/M

C.

MW (kDa)

NT  A  B  C

β-Actin  - 38

SIK2  - 102

p21  - 17

D.

Time lapse

Mean mitotic progression time (min)

NT  A  B  C

p=8.82E-05

p=3.75E-02

p=4.22E-02
Figure 5

A. Reporter assay CRE(1)

B. SP

IP

MW (kDa)

SIK2

TORC1

C. - CIP

+ CIP

MW (kDa)

SIK2

TORC1

D. Cytopl. Nucl.

MW (kDa)

β-Tubulin

Histone H3

CREB1

pCREB1

TORC1

SIK2
A. 

Down-regulated genes  

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forsk.</th>
<th>KI</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1orf112, FOXO3, HOXA5, PTPRF</td>
<td>563</td>
<td>19</td>
</tr>
<tr>
<td>SIK2si</td>
<td>243</td>
<td>4</td>
</tr>
</tbody>
</table>

10% 

Up-regulated genes  

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forsk.</th>
<th>KI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCRN4L, DNAJC12, ERF11, NFKB1Z</td>
<td>646</td>
<td>52</td>
</tr>
<tr>
<td>SIK2si</td>
<td>420</td>
<td>8</td>
</tr>
</tbody>
</table>

12% 

B. 

(i) 

WT - SIK2/TORC complex  

TORC phosphorylation  

Repression of cell death and cell cycle arrest genes 

(ii) 

SIK2-KI/TORC complex translocation  

Activation of cell death and cell cycle arrest genes
Molecular Cancer Research

Salt-inducible Kinase 2 Regulates Mitotic Progression and Transcription in Prostate Cancer

Helene Bon, Karan Wadhwa, Alexander Schreiner, et al.

Mol Cancer Res  Published OnlineFirst December 29, 2014.

Updated version  Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-13-0182-T

Supplementary Material  Access the most recent supplemental material at: http://mcr.aacrjournals.org/content/suppl/2015/01/06/1541-7786.MCR-13-0182-T.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.