The role of p90 ribosomal S6 kinases (RSKs) in Steroid signalling

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Abstract

The p90 ribosomal S6 kinases (RSKs) are a family of serine/threonine kinases consisting of four isoforms (RSK1-4), which regulate key cellular processes including cell cycle, proliferation, motility and survival. Among the several transcription factors targeted by RSKs, several studies have identified the Steroid Receptors (SRs) as substrates of the RSKs. SRs are a subfamily of the nuclear receptor superfamily consisting of five proteins (androgen, glucocorticoid, estrogen, progesterone and mineralocorticoid receptors). These proteins regulate gene expression thus are involved in crucial biological processes, including organ development and maintenance, the immune system, neuroprotection and metabolic homeostasis. Importantly, SRs are the main drivers of hormone driven cancers. Therefore, we hypothesised that the RSKs could play an important part in SR signalling within hormone driven cancer development and progression.

This was investigated using recombinant DNA techniques to incorporate the RSKs genes into the mammalian expression vector PCDNA 3.1(+) and to produce phospho-mimetic mutants. Luciferase assays were used to determine optimal hormone and DNA concentrations, the role of RSKs on SR activity, the activity of the endogenous MAPK pathway and the significance of phosphorylation state. The PMA experiments indicated that in most cases PMA does not induce a significant change to RSK activity, thus the endogenous MAPK pathway is sufficiently active. The mutant experiments suggested that the phospho-mimetic mutation used does not cause constitutive activation of the RSKs. Together considering all experiments it was illustrated that the RSKs have differential effects on SR signalling. All the RSKs increased AR and GR activity despite some contradictions within the data. RSK4 significantly decreased ERα activity and RSK3 significantly increased PR activity.

Statement of Originality

I declare that the work within this document is of my own unless stated within the text.

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Abbreviations

ACTH – Adrenocorticotropic hormone
ADT – Androgen deprivation therapy
AF – Activation function
AKT – Protein kinase B
AP2 – Adipose specific fatty acid binding protein
APC – Anaphase promoting complex
AR – Androgen receptor
ARE – Androgen response element
ARG – Androgen responsive gene
As_2O_3 – Arsenic trioxide
ATF – Activating transcription factor
ATP – adenosine triphosphate
Aβ – Amyloid β
BAD – BCL-2 associated death protein
BAG-1L – BAG- 1 long
BAT – Brown adipose tissue
BAX – BCL-2 associated X protein
BBB – Blood brain barrier
BBS – Borate buffered saline
BCa – Breast Cancer
BCL-2 – B-cell lymphoma 2
BDNF – Brain derived neurotrophic factor
BID – BH3 interacting-domain death agonist
BIMEL – BCL-2 interacting mediator of death – extra long
Bub1 – Budding uninhibited by benzimidazoles 1
C/EBPβ – Ccaat/ Enhancer binding protein β
C_aCl_2 – Calcium chloride
CAMK – Calcium/Calmodulin- dependent protein kinase
cAMP – Cyclic adenosine monophosphate
CBP – CREB binding protein
CDC – Cell division cycle kinase
CDC25C - M-phase inducer phosphatase 3
CDK – Cyclin dependent kinase
CHIF – Channel inducing factor
Chip – Chromatin immunoprecipitation
CHIP – C-terminus HSC70 – interacting protein
Chk1 – Checkpoint kinase 1
CK2 – Casein kinase II
CLDN2 – Claudin 2
cmk – chloromethylketone
COPD – Chronic obstructive pulmonary disease
COUP-TFII – COUP transcription factor 2
CREB – cAMP responsive element binding protein
CRF – Corticotropin release factor
CRPC – Castrate resistant prostate cancer
CTKD – C-terminal kinase domain
CUEDC1 – CUE Domain Containing 1
CXCR4 – C-X-C chemokine receptor type 4
DAG – Diacylglycerol
DAPK – Death associated protein kinase

DBD – DNA binding domain
D-d – ERK docking domain
DHT – Dihydrotestosterone
DMEM - Dulbecco's modified eagle's medium
DNA – Deoxynucleotide acid
E2 – 17β – estradiol
E6AP – E6-associated protein
ECM – Extracellular matrix
EGF – Epidermal growth factor
EGFR – Epidermal growth factor receptor
eIF4F – heterotrimeric eukaryotic initiation factor 4F
Emi2 – Early mitotic inhibitor 2
ENac – Epithelial Na channel
ERE – Estrogen response element
ERK – extracellular signal-regulated kinase
ERα – Estrogen receptor α
ERβ – Estrogen receptor β
ETSV1 – E twenty six variant 1
FGR1 – Fibroblast growth factor receptor 1
FLT3 – FMS – Like tyrosine kinase
FOXO3a – Forkhead box O3
FRA1 – Fos related antigen 1
G6PD – Glucose-6-phosphate dehydrogenase
GAP – Guanine nucleotide activating protein
GC – Glucocorticoids
GCR – Glucocorticoid receptor
GDP - Guanosine diphosphate
GPCR – G-protein coupled receptor
GR – Glucocorticoid receptor
Grb2 – Growth factor receptor bound protein 2
GRE – Glucocorticoid response element
GSK3β – Glycogen synthase kinase 3 β
GTP - Guanosine triphosphate
HD – Hemidemisomes
HDAC – Histone deacetylase
HDL – High density lipoproteins
HM – Hydrophobic motif
HMECs – Human mammary epithelial cells
HPA – Hypothalamo-pituitary-adrenal axis
HRE – hexamer response element
HSP – Heat shock protein
IEG – Immediate early genes
Ihh – Indian hedgehog
IkBα/β – NFkB inhibitor α/β
IL-1B – Interleukin 1B
IL-23R – Interleukin-23 receptor
IP_3 – Inositol triphosphate
ITD – Internal tandem duplication
JNK – c-JUN N-terminal kinase
KKK – IkB kinase
KO – knockout

LB – Luria broth
LBD – Ligand binding domain
LDL – Low density lipoproteins
LPA - Lysophosphatidic acid
LPL – Lipoprotein lipase
LTK – Leukaemia tyrosine kinase
MAD-1 – Mitotic arrest deficient protein 1
MAF – Musculoaponeurotic fibrosarcoma oncogene homolog
MAPK – Mitogen activated protein kinase
MCP1 – Monocyte chemoattractant protein 1
MCP-1 – Monocyte Chemoattractant-1
MCR – Mineralocorticoid receptor
Mdm2 – Mouse double minute homolog
MEK – MAPK/ERK kinase
MKK3 – Mitogen-activated protein kinase kinase 3
mTOR – Mechanistic target of rapamycin
mTORC – Mammalian target of rapamycin
Myt1 – Myelin transcription factor 1
NADPH – Nicotinamide adenine dinucleotide phosphate
Nedd4-2 – developmentally down-regulated receptor 4
NFkB – Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS – Nuclear localisation signal
NR – Nuclear receptor
NSCLC – Non-small cell lung cancer
NTD – N-terminal domain
NTKD – N-terminal kinase domain
ORM1 – Orosomucoid 1
p160 – Nuclear receptor coactivator 2
P21 – Cyclin-dependent kinase inhibitor 1
$P27^{KIP1}$ – Cyclin-dependent kinase inhibitor 1B
P300 – E1A-associated protein
p42 – Papyrus 42
p44 – Mitogen-activated protein kinase 3
PABP-1 – Polyadenylate-binding protein 1
PAL-1 – Plasminogen activator-1
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PDK1 – Phosphoinositide-dependent kinase 1
PI3K – Phosphoinositide 3 Kinase
PIAS1 – Protein inhibitor of activated STAT1
PIF – PDK1 interacting fragment
PKC – Protein Kinase C
PKCθ – Protein kinase C θ
PLD – Phospholipase D
PMA – Phorbol 12-myristate 13-acetate
PPAR – Peroxisome proliferator activated receptor
PR – Progesterone receptor
PRb - Retinoblastoma protein
PSA – Prostate specific antigen
PTMs – Post-translational modifications
PVDF – Polyvinylidene difluoride

RA – Rheumatoid arthritis
RAPTOR – Regulatory associated protein of mTOR
RNA – Ribonucleic acid
ROS – Reactive oxygen species
RPS6 – Ribosomal protein S6
RSK – p90 ribosomal S6 kinase
RT – Room temperature
RTK – Receptor tyrosine kinase
S6K – Ribosomal S6 kinase
SAGE – Serial analysis of gene expression
SCa – Skin cancer
SDS-PAGE – SDS polyacrylamide gel electrophoresis
SGK1 – Serum/Glucocorticoid Regulated Kinase 1
siRNA – Short interfering RNA
SOD – Super oxide dismutase
SOS – Sons of sevenless
SR – Steroid Receptor
SRC – Steroid receptor co-activator
SRE – Serum response element
SRF – Serum response factor
STAT3 – Signal transducer and activator of transcription 3
TAT-LUC – TAT-GRE-EIB-LUC-1
TBI – Traumatic brain injury
TFIIH – Transcription factor IIH
TGFβ – Transforming growth factor β
TIA-1 – Cytotoxic granule-associated RNA binding protein
TIF2 – Transcription intermediate factor 2
TIF-IA – Transcription initiation factor IA
TLR – Toll like receptor
TM – Turn motif
TMBCa – Triple negative breast cancer
TMPRSS2 – Transmembrane protease serine 2
TSC2 – Tuberous sclerosis complex 2
Unc93b1 – Unc-93 homolog B1
VA – Vasopressin
VMH – Ventromedial nucleus of the hypothalamus
YB1 – Ybox binding protein 1
β-GAL - PDM-LAC-Z-β-GAL
βTrCp – F-box/WD repeat-containing protein 1A

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1.1 - RSK Structure and Expression

The 90 kDa ribosomal S6 kinases (RSKs) are a family of serine/threonine kinases composed of four isoforms (RSK 1-4). The isoforms range from 733-745 amino acids long and share between 73-80% total sequence identity (Figure 1.1.1). The general structure of the RSKs is characterised with two key kinase domains, the C-terminal kinase domain (CTKD) which is closely related to the calcium/calmodulin-dependent protein kinases (CAMK) and the N-terminal kinase domain (NTKD) which is closely related to AGC kinases. This unique protein structure originates from a gene fusion event (Jones *et al.* 1988). The two kinase domains have high sequence identity among the 4 isoforms, while the N- and C-terminus are not conserved. The CTKD and NTKD are joined together by a linker region of approximately 100 amino acids containing the turn motif (TM) and the hydrophobic motif (HM), which are key regulatory domains, with a reduced sequence identity (49-67%). The C-terminus contains the ERK D-domain (D-d) required for ERK1/2 docking which is indispensable for protein activation (Lara *et al.* 2013).

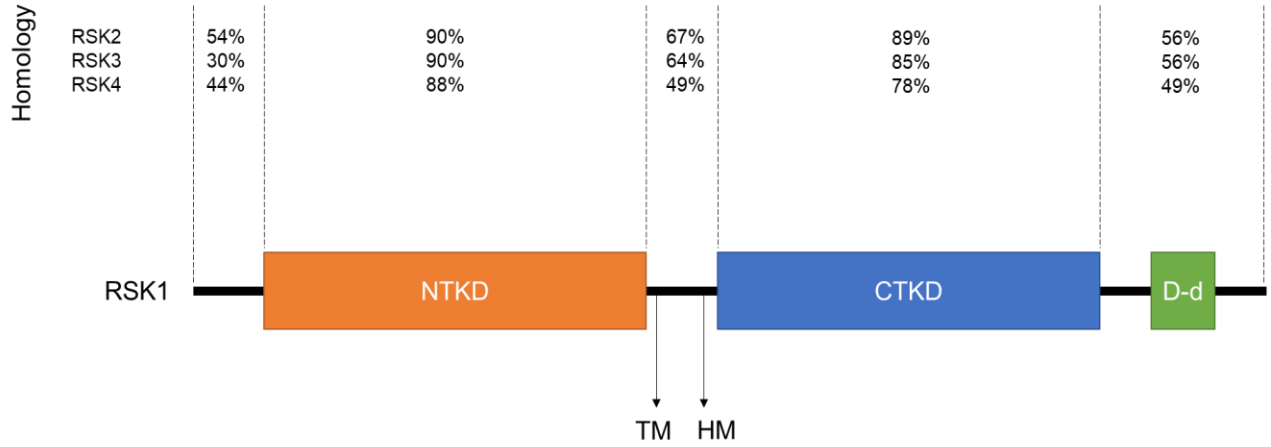

Figure 1.1.1 Schematic representation of RSK functional domains and homology between the kinases. Above schematic is a percentage of the amino acid sequence identity shared between the different RSK isoforms at different domains across the shared structure compared to RSK1. The N-terminal Kinase Domain (NTKD) is preceded by the variable N-terminus. Following the NTKD in the hinger region which possesses the Turn Motif (TM) and the Hydrophobic Motif (HM). Following the hinger region is the C-terminal kinase domain CTKD which proceeds into the ERK docking domain (D-d) (adapted from Lara *et al.,* 2013).

The RSKs are ubiquitously expressed across the adult human body but the levels of expression vary dependent on tissues type. RSK1 exhibits higher expression in the hematopoietic system, gastrointestinal tract and the female sex organs but also shows reduced expression in adipose and soft tissue. RSK2 has relatively high expression across most tissues but shows a reduction in expression in adipose and soft tissue. RSK3 has moderate expression across most tissues but shows enhanced expression in endocrine tissues and reduced expression in adipose and soft tissue. RSK4 has moderate expression across most tissues but displays reduced expression in muscle tissues (Uhlén *et al.* 2015).

1.2 - Cellular Localisation

In non-dividing cells RSK1-3 mostly reside in the cytoplasm awaiting activation. Once growth factor stimulation occurs the RSKs become activated by ERK the downstream activator of the MAPK pathway (Chen *et al.* 1992). Once activated the RSKs translocate to the nucleus via an unknown mechanism. RSK3 is the only isoform to possess a Nuclear Localisation Sequence (NLS), however its functionality has never been tested. Once in the nucleus the RSKs phosphorylate their corresponding substrate targets. RSK2 has also been shown to translocate to stress granules when under oxidative stress. Differently from the other RSKs, RSK4 is growth factor independent and is predominantly a cytoplasmic protein (Cargnello & Roux 2011).

1.3 - RSK Activation

Recent evidences have shown that different RSK isoforms are activated in different ways, with RSK1-3 sharing a relatively similar mechanism and RSK4 deviating from this canonical mechanism. As such, RSK4 will be discussed separately. RSK 1-3 are primarily downstream effectors of the mitogen activated protein kinases (MAPK) signalling cascade. The extracellular signal can be produced by several growth factors or hormones, which bind to and activate Receptor Tyrosine Kinases (RTKs) present on the extracellular surface of the cell membrane (Hilger *et al.* 2002). The stimulated RTK dimerises leading to autophosphorylation of the receptors cytoplasmic domain and initiation of the intrinsic signal. Presentation of phosphorylated tyrosines on the RTK cytoplasmic domain are recognised by growth-factor-receptor-bound-protein 2 (Grb2) which in turn recruits Sons Of Sevenless (SOS). SOS is a guanine exchange factor which causes the GDP/GTP exchange in membrane bound Ras causing a structural change into its active conformation (Hilger *et al.* 2002). Active Ras then binds to the kinase Raf (most commonly to the B-Raf isoform), causing its translocation to the intracellular surface of the cell membrane and activation. Raf then phosphorylates two serine residues on the kinase MEK1/2 activation loop. MEK1/2 then phosphorylates ERK (Kolch., 2000). Unusually inactive ERK is found in a pre-existing complex with inactive RSK1-4 in the cytoplasm, bound via the D-domain of the RSKs (Hsiao *et al.* 1994) although the function of this complex is unknown. The docking of ERK to the D-domain of RSKs requires a minimal sequence LAQRR which is conserved across all isoforms (Smith *et al.* 1999). In a broader sense the docking of ERK requires a cluster of positive amino acid residues encompassed by hydrophobic residues (Tanoue & Nishida 2003). When ERK is activated it phosphorylates the CTKD of RSK 1-3 at amino acids Thr573, Thr577 and Thr570 respectively. The activated CTKD then auto-phosphorylates RSK 1-3 in the HM at amino acids Ser380, Ser386 and Ser377 respectively. The HM phosphorylation is essential for activation of the RSKs. This phosphorylation creates a docking site that facilitates the interaction between the HM and PDK1 via the PDK1 interacting fragment (PIF). This docking site is most likely created by conformational

changes induced by the HM phosphorylation in conjunction with the previous CTKD phosphorylation, which exposes previously concealed amino acids. This is supported by experimental data in unstimulated cells, where a RSK HM/N-terminus construct was phosphorylated but a RSK HM/C-terminus construct and full length RSK were not phosphorylated, suggesting that unphosphorylated CTKD conceals the HM (Frödin *et al.* 2000). Alongside the HM phosphorylation two further phosphorylations occur in the linker region in the TM for RSK 1-3 at amino acids (Thr359, Ser363), (Thr365, Ser369) and (Thr356, Ser360) respectively. The kinase responsible for these phosphorylations has been shown to be a MAPK due to the specific recognition sequences (Dalby *et al.* 1998). Due to previous ERK involvement in RSK activation it is hypothesised to be ERK, but the exact mechanism is still to be elucidated. The purpose of these two phosphorylations is to enhance the interaction of HM and PDK1, by assisting C-terminal conformational changes (Pearce *et al.* 2010). Once PDK1 is recruited to RSK 1-3, its kinase activity is stimulated by the phosphorylated HM (most likely by inducing conformational changes in PDK1 that stimulate autophosphorylation) (Frödin *et al.* 2000), which is stabilised by the phosphorylated TM. PDK1 is then able to phosphorylate the NTKD of RSK 1-3 at amino acids Ser221, Ser227, Ser218 respectively. PDK1 then dissociates in a fully active with the ability to phosphorylate other substrates. The now catalytically active NTKD auto-phosphorylates the C-terminus just outside of the D-domain in RSK1 and RSK2 at amino acids Ser732 and Ser715 respectively. These phosphorylations create an auto-inhibitory effect reducing the affinity to ERK and producing a negative feeback loop for RSK activation (Romeo *et al.* 2012). RSK3 does not have this phosphorylation which could be linked to ERK remaining bound for longer than RSK1/2 (Roux *et al.* 2003). The RSKs are then inactivated by phosphatases and return to an inactive state (Romeo *et al.* 2012).

RSK4 activation mechanism is largely uncharacterised, but initial evidences suggest it differs from RSK1-3 mechanism. Inactive ERK is found in a pre-formed complex with inactive RSK4. ERK is stimulated and phosphorylates RSK4 on the CTKD at amino acid Thr581. The CTKD then auto-phosphorylates the HM at amino acid Ser389 while ERK phosphorylates

the TM only in one position (in contrast to two positions with RSK1-3) at amino acid Ser372. The NTKD is phosphorylated in a PDK1-independent manner most likely by an autophosphorylation event at amino acid Ser232 (Dümmler *et al.* 2005). The NTKD then autophosphorylates the C-terminal just outside the D-domain at amino acid Ser742 to create an auto inhibitory effect, reducing affinity to ERK. The significant difference with RSK4 to the other RSK isoforms is that it found constitutively active across most cells even in serum-starved cells. The serum starved cells are not stimulated by growth factor meaning the MAPK pathway is inactive, thus RSK4 activation is growth factor independent. This is because the expression of RSK4 is so low that the basal concentrations of ERK are sufficient enough to cause maximal phosphorylation of ERK sites. This, in combination with the PDK1-independent NTKD phosphorylation, allows activation of RSK4 independently of growth factor (Dümmler *et al.* 2005). The general mechanism of action which was described in-depth previously and the cellular outcomes of RSK activation have been illustrated (Figure 1.3.1).

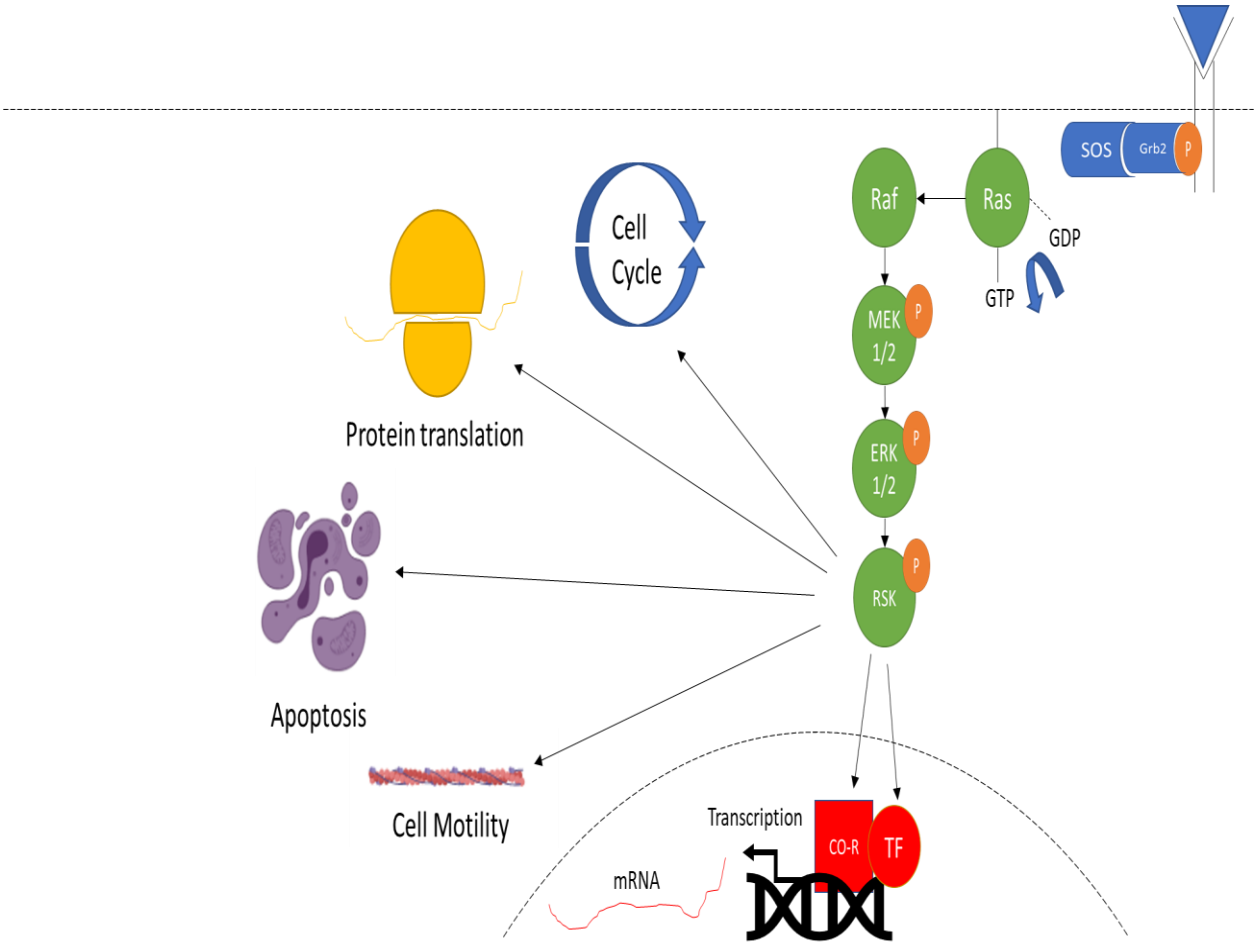

Figure 1.3.1 – Schematic representation of MAPK signalling pathway. The RSKs are activated by the activation of the MAPK pathway via a phosphorylation cascade. The RSKs can then translocate to the nucleus and phosphorylate Transcription factors and/or Co-regulators to influence the transcriptional program resulting in production in mRNA. Additionally, the RSKs phosphorylate several cytosolic proteins which regulate cellular processes like the cell cycle, protein translation, apoptosis and cell motility.

1.4 - Cellular Roles

The RSKs are the most downstream effectors of the MAPK pathway. The activation of these proteins allows phosphorylation of a range of substrates, which leads to changes in several cellular processes. The RSKs have been shown to have roles in transcription and translation regulation, cell cycle and proliferation, cell survival, and cell migration (Figure 1.3.1).

1.4.1 - Regulation of Transcription

In response to mitogenic signal and translocation to the nucleus, RSKs phosphorylate specific transcription factors, which result in the transcription of the Immediate Early Genes (IEGs). RSK2 phosphorylates cAMP Responsive Element Binding protein (CREB) in response to epidermal growth factor (EGF) causing binding to its recognition site within the C-fos promoter and transcription of IEG product (Bruning *et al.* 2000). RSK2 also phosphorylates Serum Response Factor (SRF) at Ser103 increasing it affinity for the Serum Response Element (SRE) within the promoter of C-fos leading to its transcription (Rivera *et al.* 1993). RSKs have also been shown to directly phosphorylate the IEG products. In fact, RSK1/2 phosphorylates C-fos at Ser326 cooperatively with ERK which phosphorylates C-fos at Ser374 (Chen *et al.* 1993). These phosphorylations stabilise C-fos and increase its transactivation activity (Chen *et al.* 1996). The IEGs act as molecular sensors, which allows for differentiation between transient and sustained mitogenic signal. The initial signal may be long enough for RSK/ERK to phosphorylate transcription factors such as SRF or CREB to produce IEG products such as C-fos but if the signal is not sustained ERK/RSK are inactivated and are unable to phosphorylate C-fos before it is degraded. A sustained signal causes ERK/RSK to remain active, phosphorylate the IEG products such as C-fos, which stabilises the protein. Additionally, these phosphorylations create new sites within C-fos which allow further phosphorylation events to occur (Murphy *et al.* 2002). C-fos then positively regulates the expression of Cyclin D-1, which is necessary for G_1/S phase transition.

The RSKs regulate transcription of other important cellular processes via different transcription factors. For example, RSK1/2 cooperatively phosphorylates Transcription Initiation Factor - IA (TIF-IA) with ERK. The RSK phosphorylation of Ser649 precedes ERK phosphorylation of Ser633 which then leads to initiation of transcription of RNA polymerase I (Zhao *et al.* 2003), the polymerase necessary for RNA synthesis. RSK1/2 also phosphorylate CREB at Ser133 in response to Brain Derived Neurotrophic Factor (BDNF) and growth hormones. CREB then binds to promoters of CREB responsive genes and transcribes pro-survival gene bcl-2 which promotes cell survival of neurons (Bonni *et al.* 1999). Similarly, RSK1/2 also phosphorylate the transcription factor FRA1 at Ser252 which controls the expression of cell motility genes these, phosphorylations also stabilise the FRA1 protein in order to sustain expression of its target genes (Doehn *et al.* 2009). RSK substrate phosphorylation can even induce degradation of the target. For example, it has been shown that RSK1 can form a complex with IkBα/β (inhibitors of NF-kB) and Nuclear Factor kappa-light-chain-enhancer of activated B cells (NFkB). Once stimulated RSK1 phosphorylates IkBα on unknown residues and IkBβ on Ser19 and Ser23 causing ubiquitination and degradation of the proteins. This removes the previous inhibition of NFkB. NF-kB can then induce expression of its target genes to combat inflammation (Xu *et al.* 2006). Similarly, RSK1 phosphorylates the transcription factor Myc inhibitor Mitotic Arrest-Deficient protein 1 (MAD1) on Ser145. This phosphorylation causes the ubiquitination and degradation of MAD1 leading to enhanced Myc transcription of proliferative genes (Zhu *et al.* 2008).

1.4.2 - Regulation of Protein Translation

RSKs were initially thought to be the primary kinases for Ribosomal Protein S6 kinase (RPS6) a component of the ribosomal 40S subunit, (Erikson & Maller 1986). It was later shown that ribosomal S6 Kinase 1 and 2 (S6K1/2) are the primary kinases for RPS6, which phosphorylate five clustered serines at the C-terminus of RPS6 in comparison to RSK1/2, which only phosphorylate RPS6 on Ser235 and Ser236. These RSK1/2 mediated phosphorylations are in response to MAPK pathway stimulation via several different stimuli and occur in a mTORC independent manner. Once RPS6 is phosphorylated at these specific residues the affinity of RPS6 for the 7-methylguanosine cap complex is increased promoting recruitment of the translational complex, highlighting the importance of RSK1/2 in the formation of the preinitiation complex necessary for protein translation (Roux *et al.* 2007). As part of the preinitiation complex, there are several different eukaryotic initiation factors (eIFs), which recruit the ribosome to mRNA and initiate translation. The heterotrimeric eukaryotic Initiation Factor – 4F (eIF4F) consists of a cap binding protein eIF4E, a scaffolding protein eIF4G and a helicase eIF4A. RSK1/2 phosphorylates eIF4B on Ser422 in response to serum stimulation of the MAPK pathway, this enhances cap dependent translation by stimulating the helicase activity of eIF4A to unwind the structure of mRNA ready for translation. This translational stimulation occurs in a convergent manner, the MAPK pathway produces a transient signal through RSK1/2 alongside a sustained signal from the phosphoinositide 3 kinase (PI3K) pathway through S6K (Shahbazian *et al.* 2006). It has also been shown that RSK2 can increase protein production by phosphorylating the Glycogen Synthase Kinase 3β (GSK3β) at Ser9, which inhibit its kinase activity and alleviates its inhibition on eIF2B, resulting in increased translation (Sutherland *et al.* 1993). RSKs are also implicated in regulation of protein synthesis by regulating mTOR, the master regulator of protein synthesis, which in humans exists in two complexes, mTORC1 and mTORC2. Tuberous sclerosis complex 2 (TSC2) is a complex formed of tuberin and hamartin, which inhibits mTOR signalling. RSK1/2 phosphorylates TSC2 at Ser1798 and Ser1799, key regulatory sites, removing the inhibition invoked by its Guanine nucleotide-

Activating Protein (GAP) activity thus allowing mTOR signalling via S6K1, resulting in increased protein translation (Roux *et al.* 2004). RSKs can also interact directly with mTOR via phosphorylation of Regulatory Associated Protein of mTOR (RAPTOR), an important mTOR scaffolding protein. RSK1/2 were found to phosphorylate RAPTOR on three residues Ser719, Ser721 and Ser722, which directly stimulates the kinase activity of mTOR, which in turn phosphorylates S6K2. Interestingly, there are significant variances with these three phosphorylation sites: Ser721 is a classical recognition phosphorylation site of RSK1/2; Ser719 has the minimal consensus sequence of a RSK phosphorylation site; while Ser722 is located +1 position of the classical recognition sequence for RSKs (Carrière *et al.* 2008).

1.4.3 - Regulation of Cell cycle and Cell Proliferation

RSKs can regulate cell proliferation via regulation of cell cycle components. $P27^{Kip1}$ is a Cyclin Dependent Kinase (CDK) inhibitor that sequesters and inactivates cyclin A/E – CDK2 complexes, and its own activity is regulated by concentration and cellular localisation. RSK1/2 phosphorylate $P27^{Kip1}$ on Thr198, stimulating its binding to 14-3-3 scaffolding proteins. Once bound to the scaffolding proteins $P27^{Kip1}$ remains localised to the cytoplasm preventing translocation to the nucleus and sequestering of cyclin A/E, thus allowing A/E – CDK2 complexes to form and G_1 / S phase transition to occur (Fujita *et al.* 2003). In addition to regulation of G_1/S phase, RSKs have been shown to regulate the transition from G_2 to Meiosis in oocytes, which are naturally arrested in late G_2 phase awaiting progesterone stimulation for transition. Once stimulated with progesterone, transition into the M phase begins and a kinase, Mos, is synthesised and activates the MAPK pathway. RSK2 then phosphorylates Myt1. Cell Division Cycle 2 kinase (CDC2), a CDK, is responsible for this transition in meiosis, but is normally inhibited by Myt1. Phosphorylation by RSK2 on Myt1 attenuates inhibition on CDC2 allowing cell cycle progression (Nebreda & Ferby 2000). In addition, RSK2 aids in activation of CDC2 by phosphorylating CDC25C an enzyme involved in activated the CDC2/ cyclin B complex (Wang *et al.* 2010). Furthermore, RSK1/2 phosphorylates the Anaphase Promoting Complex (APC) inhibitor, Bub1 kinase, causing metaphase II arrest (Schwab *et al.* 2001). Additionally, RSK1/2 phosphorylate early mitotic inhibitor 2 (Emi2) another APC inhibitor, promoting its association with a phosphatase which removes all phosphorylations and activates the protein, allowing its inhibition of the APC and continued anaphase arrest (Nishiyama *et al.* 2007). RSK4 has been shown to cause $G_0 – G_1$ cell cycle arrest. This occurs by RSK4 causing hypophosphorylation of pRB and increased expression of p21 but by an unknown mechanism (Thakur *et al.* 2008). RSK3 was shown to reduce proliferation in cancer cells by causing cell cycle arrest in G_1 phase by an unknown mechanism (Cargnello & Roux 2011).

1.4.4 - Cell Motility

RSK1/2 were first shown to be involved in cell motility by phosphorylating cytoskeleton protein filamin A on Ser2152 in response to growth factors. After phosphorylation filamin A was shown to migrate at a significant rate and this phosphorylation is essential for membrane ruffling (Woo *et al.* 2004). RSK1/2 also regulate a range of motility functions in epithelial cells; cell scattering, wound healing, cell multilayering, chemotaxis, 3D organoid to 2D migration and 3D ECM invasion. RSK1/2 were also found to participate in an autocrine feedback loop (Doehn *et al.* 2009). Hemidemisomes (HDs) are junctional complexes that aid adhesion of epithelial cells to basal membrane. The hemidemisomes are formed of intergrin α6β4, plectin, pemphigoid antigens and tetraspanin CD151. Following growth factor stimulation of keratinocytes RSK1/2 phosphorylate the β4 subunit of intergrin at Ser1356 and Ser1364 which hinders the interaction between the β4 subunit and plectin causing a reduction in HDs and an increase in cell motility due to a decrease in strength of cell adhesion (Frijns *et al.* 2010).

1.4.5 - Cell Survival

The RSKs also have roles in the regulation of cell survival, most of which occur by post-translational modifications and result in inhibition of a pro-apoptotic protein saving the cells from apoptosis, cell death programme. RSK1/2 phosphorylates Bcl-2 Associated Death protein (BAD) on Ser112 (Bonni *et al.* 1999) which causes BAD previous dimerization with Bcl-xl to become reversed. Once the Bcl-xl is free and no longer inhibited by BAD it can carry out its pro-survival function. The phosphorylated BAD binds to 14-3-3 scaffolding proteins sequestering it from further inhibition of Bcl-xl (Shimamura *et al.* 2000). In a similar manner RSK1/2 phosphorylates Death Associated Protein Kinase (DAPK) on Ser289 causing inhibition of its pro-apoptotic activity and a significant reduction in cell death (Anjum *et al.* 2005). It has also been shown that RSK1 phosphorylates Ccaat/Enhancer Binding Proteinβ (C/EBPβ) on Thr217. This causes the creation of a functional XEXD caspase substrate/inhibitor box due which binds to and inhibits the caspases 1 and 8 preventing them from activating effector procaspases that execute the apoptosis programme, allowing survival of the stellate cells (Buck *et al.* 2001).

1.5 - RSKs and Cancer

In the last 10 years, the RSKs have been connected to cancer mostly due to their involvement in key cellular processes. These processes in cancer often become dysregulated due to abnormal expression or activity of RSKs allowing the cancerous cells to uncontrollably proliferate. However, the story is complicated by the fact that different RSK isoforms perform different, sometime opposing, functions in different cancer types

1.5.1 - Breast Cancer (BCa)

BCa has many subsets with varying expression of different receptors. In a study focused on BCa with high expression of Fibroblast Growth Factor Receptor 1 (FGFR1), it was shown that RSK1 can regulate the proliferation in these cells to some degree by an unknown mechanism. This was elucidated by treating three different BCa cell lines with RSK1 inhibitor chloromethylketone (cmk) which caused a reduction in cell proliferation, suppressed colony formation and decreased survival of the FGFR1 expressing cells (Xian *et al.* 2009). RSK2 have also been shown to interact with estrogen Receptor alpha (ERα) in a two-step mechanism, firstly RSK2 binds to the Ligand Binding Domain (LBD) between residues 326-394 which influences the activity of the AF2 domain causes conformational change which exposes Ser167 for phosphorylation by RSK2 which influences the Activation Function 1 (AF1) domain and enhances the transcriptional activity of ERα (Clark *et al.* 2001). This phosphorylation is present in hormone driven BCa and is used as a prognostic for patients to determine if they are suitable for endocrine therapy after relapse as it increase the chances of overall survival (Yamashita *et al.* 2008). RSK2 can also promote cell survival in BCa by the formation of stress granules, which reduces the translation of selective mRNAs. RSK2 facilitates the formation of stress granules by direct interaction and sequestering of TIA-1 (an RNA binding protein that acts as a translational inhibitor) and PABP-1 a stress granule marker to form the granule. The mechanism which causes the cell survival is unknown but is shown by siRNA silencing of RSK2 of the BCa cells resulted in increased apoptosis (Eisinger-Mathason *et al.* 2008). RSK1/2 were also shown to phosphorylate YB1 at Ser102

resulting in increased expression of EGF receptor (EGFR) which in turn results in increased proliferative signal due to an increased amount of growth hormone receptor (Stratford *et al.* 2008). RSK2 was also shown to regulate its expression of cyclin D1 in BCa, use of RSK inhibitors in MCF-7 cells caused a reduction in both protein and mRNA levels of cyclin D1, cyclin D1 itself is an oncogene promoting proliferation (Eisinger-Mathason *et al.* 2008). Differently, RSK4 acts as a tumour suppressor in BCa by causing an overall reduction in cell metastasis, reduction in cell proliferation. RSK4 achieves inhibition of cell metastasis most likely by regulation of CXCR4 and CLDN2, of which induced decreased and increased expression respectively. This change in expression would mediate a reduction in metastasis as CXCR4 specifically induces metastasis and CLDN2 is a transmembrane protein which is also a component of tight junctions which maintain integrity of epithelial tissues, when altered results in mesenchymal transition (Thakur *et al.* 2008).

1.5.2 - Prostate Cancer (PCa)

RSK2 regulates proliferation of prostate cancer by enhancing the expression of prostate specific antigen (PSA) and regulates androgen receptor mediated expression most likely by indirect phosphorylation (Clark *et al.* 2005). RSK1 has also been implicated in prostate cancer by phosphorylating the transcription factor Y-box Binding protein 1 (YB1), which increases androgen receptor (AR) transcription in response to AR degradation caused by androgen deprivation therapy used to induce apoptosis (Shiota *et al.* 2014).

1.5.3 - Ovarian Cancer (OvCa)

RSK3 has been shown to repress growth in multiple OvCa cell lines, this is further potentiated by overexpression of RSK3 which provided simultaneous results (Eisinger-Mathason *et al.* 2010).

1.5.4 - Lung Cancer (LCa)

A short interfering RNA (siRNA) study showed that RSK1 knock down in lung adenocarcinomas resulted in enhanced metastatic potential with an increase in cell migration and invasion suggesting tumour suppressor properties of RSK1 in this type of cancer. In the same study RSK2 and RSK4 were also investigated, in a converse manner these knock downs showed inhibition of cell metastasis suggesting that they are metastatic promoters in lung adenocarcinoma (Lara *et al.* 2011). In Non-Small Cell Lung Cancer (NSCLC) RSK1 phosphorylates BAD, as previously mentioned, and promotes cell survival (Hurbin *et al.* 2005). By an alternative mechanism RSK1/2 also promote cell survival in NSCLC by phosphorylating Bcl-2 Interacting Mediator of death – Extra Long (BIMEL) (one of three BIM splice variants). BIMEL regulates the Bcl-2 associated X protein (Bax) mediated apoptosis, BIMEL when phosphorylated on Ser69 interacts with F-box protein bTrCP causing its degradation. This degradation prevents BIMEL from stabilising Bax and BCL from permeabilising the mitochondrial membrane and releasing cytochrome C, which induces the apoptotic program. Ultimately allowing the cancerous cells to escape cancer therapy induced apoptosis (Dehan *et al.* 2009).

1.5.5 - Acute Myeloid Leukaemia (AML)

AML is treated with arsenic trioxide (As_2O_3) to target the cancerous cells however chemoresistance to this treatment arises. This chemoresistance arises from activation of RSK1 in response to As_2O_3 and phosphorylation of Bad at Ser112 causing an anti-apoptotic signal that allows the cancerous cells to survive (Galvin *et al.* 2013). RSK2 is also involved in Leukaemia by a different mechanism; Leukaemogenic tyrosine kinases (LTKs) activate RSK2 in leukaemia cells to generate a myeloproliferative neoplasm signal causing expansion of neutrophils. The LTKs that activate RSK2 are BCR-ABL (a fusion protein) and FMS-like tyrosine kinase 3 (FLT3) - internal tandem duplication (ITD) mutant (FLT3-ITD). However in response to inhibition of RSK2, only cells expressing the FLT3-ITD showed increased apoptosis suggesting RSK2 is dispensable in BCR-ABL positive cells (Elf *et al.* 2011).

1.5.6 - Head and Neck Squamous Cell Carcinoma (HNSCC)

In HNSCC RSK2 invokes metastasis by phosphorylating two substrates. RSK2 phosphorylates CREB at Ser133, which most likely cause a transcriptional dependent signalling cascade which results in the expression of pro-metastatic genes. RSK2 also phosphorylates Heat Shock Protein 27 (HSP27) at Ser78 and Ser82 to regulate actin filaments by controlling their state of polymerisation (Kang *et al.* 2010).

1.5.7 - Melanoma

In skin cancer (SCa) melanoma drugs such as cisplatin often cause an initial response in cancerous cells causing apoptosis. This occurs by activation ataxia telangiectasia mutated- and RAD3-related protein, which in turn phosphorylates checkpoint kinase 1 (Chk1) at Ser317 and Ser345 enabling its activity. Chk1 then goes onto rapidly degrade CDC25 phosphatase leading to cell cycle arrest for DNA repair before mitosis. However, chemoresistance often builds up due to RSK1/2 phosphorylation of Chk1 at Ser280 inhibiting the protein and allowing the cells to proceed through the cell cycle and silencing of the G2 DNA repair mechanism in tumour promoting manner (Ray-David *et al.* 2013). RSK1 also promotes cell survival in melanoma by phosphorylating BAD at Ser75 causing its inactivation and removal of the apoptotic signal by the previous mechanism mentioned (Eisenmann *et al.* 2003).

1.6 - RSK Inhibitors

As demonstrated above the RSKs are involved in a range of metabolic processes by a variety of mechanisms. When studying these proteins, whether to prove a function or for potential use as a cancer therapeutic, the use of inhibitors is essential. There are two different ways in which inhibitors affect the RSKs, both of which affect their kinase domains, by target of the CTKD or the NTKD. BI-D1870 competes for the ATP binding site of the NTKD in a reversible manner and is highly selective for RSKs relative to other AGC kinases, the IC_{50} is 15-30nM at ATP concentration 100μM. This inhibitor shows higher propensity to inhibit RSK4 out of the four isoforms but still inhibits all four isoforms significantly. SL0101 is another inhibitor that competes for the ATP binding site of the NTKD in a reversible manner but does so in a less potent manner when comparing to BI-D1870 with an IC_{50} of 90nM at ATP concentration 10μM. Fmk is another inhibitor but interacts with RSKs differently, fmk contains an electrophile which enables inhibition to occur via covalently linking to the RSK in an irreversible manner. This occurs via covalent addition of its chloromethlyketone group to a thiol group from a Cys residue near the ATP binding site of the CTKD. Despite inhibiting via a covalent modification this inhibitor is still highly selective for the RSKs with reasonable potency IC_{50} of 1.2μM. However, fmk cannot inhibit RSK3 because it possesses a methionine at the 'gatekeeper' residue of the ATP pocket which prevents the entry of fmk into the pocket. The different mechanism of inhibition between the two types means that BI-D1870 and SL0101 inhibit phosphorylation of substrates of the RSKs by interacting with NTKD whereas fmk inhibits activation of the CTKD thus maximal activation of the proteins (Nguyen 2008). These inhibitors are now considered the traditional inhibitors of the RSKs, however in recent years new and more potent inhibitors have been designed. Two next generation inhibitors, LJH685 and LJI308, inhibit and bind to the RSKs via the NTKD ATP site. Both inhibitors have high selectivity and potency for the RSKs and inhibit *in vivo* and *in vitro* with an IC_{50} of 0.2-0.3μM/L. Furthermore, the mechanism of inhibition is unusual by forming a non-polar conformation which aids in its selectivity (Aronchik *et al.* 2014).

1.7 - Steroid Receptor Structure and Activation

1.7.1 - Structure

Steroid Receptors (SRs) are transcription factors which bind to their cognate ligand and additional regulators (cofactors) to control gene expression. The primary ligand for each receptor is testosterone/dihydrotestosterone (DHT) for the Androgen Receptor (AR), 17β-estradiol (E2) for the Estrogen Receptor alpha (ERα), cortisol for the Glucocorticoid Receptor (GR), progesterone for the Progesterone Receptor (PR) and aldosterone for the Mineralocorticoid Receptor (MCR). SRs are a subfamily of the superfamily Nuclear Receptors (NRs). They share this superfamily with three other subfamilies; thyroid/retinoid/vitamin D receptors, peroxisome proliferator activated receptors and the orphan receptors.

All NRs have a degree of sequence homology and share a general structure (Figure 1.7.1.1). This shared structure consists of two main features; the DNA Binding Domain (DBD) which is positioned roughly in the middle of the protein and the Ligand Binding Domain (LBD) which is positioned towards the C-terminus. These two domains are connected by a hinge region (Kumar & Thompson 1999). The DBD is composed of a conserved 66 amino acid central core followed immediately by a short unconserved extension into the hinge region. The conformation of the DBD creates two alpha helices and two zinc binding groups. One of the alpha helices is responsible for DNA recognition via the major groove, whilst the second is essential for hetero/homodimerisation (Rastinejad 2001). Additionally, the DBD is responsible for the selectivity of which DNA response element it binds to. Properties like N-terminal flexibility, length of the hinge region and amino acid sequence determines positioning on DNA and in turn selectivity. Furthermore, the extension into the hinge region stabilises DNA binding by interacting with the minor groove of DNA (Helsen & Claessens 2014).

The LBD is composed of a three layered alpha helical structure consisting of 12 α-helices (H1-H12), forming an ‘α-helical sandwich’. Ligand binding to the LBD causes a conformational change which dictates which coregulators will be recruited, co-activators or co-repressors (co-regulatory proteins that either enhance or repress receptor activity), thus whether the protein is transcriptionally active or inactive. The conformational changes from ligand binding creates a hydrophobic surface for coregulators to bind (Greschik *et al.* 2002).

NRs also have two Activation Functions: (AF) AF-1 and AF-2. AF-1 is located inside the highly flexible N-terminus, this domain varies in length and sequence and is recognised by co-regulators. AF-2 is located to the LBD where it is induced by ligand binding to create the interaction surface for co-regulators. In addition to their structural features the SRs possess positions in their structure which allow for post-translation modifications. These modifications can also influence their transcriptional activity (Lavery & Mcewan 2005).

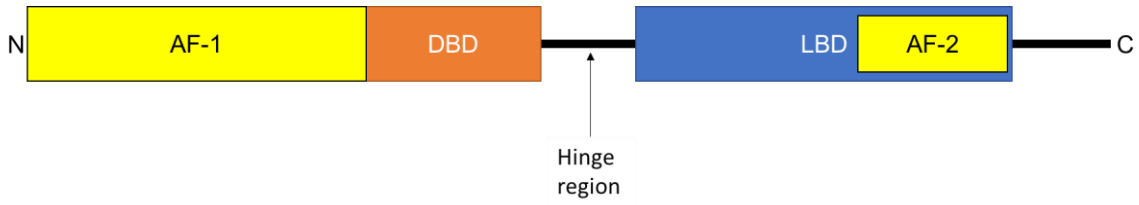

Figure 1.7.1.1 - Schematic representation of SR functional domains. The activation function 1 (AF-1) is present in the N-terminus of the SR proceeded by the DNA binding domain (DBD), the DBD extends into the hinge region which proceeds into the Ligand binding domain (LBD) which contains the activation function 2 (AF-2) followed by an unstructured C-terminus.

1.7.2 - Mechanisms of action

1.7.2.1 - Classical steroid receptor signalling

This classical method of SR activation involves the binding of hormone to intracellular SRs. Signalling is initiated when the relevant hormone, diffusing through the phospholipid bilayer, bind to the receptor. Hormones can diffuse across the cell membrane as they are small lipophilic molecules. The hormone then comes into proximity with the SR usually in the cytoplasm and causes an allosteric change due to its chemical structure. The allosteric change occurs via the H12 α-helix. The H12 helix switches from a previous dynamic state becomes fixed in one position, closing the ligand binding pocket and further stabilising the ligand (Nagy & Schwabe 2004). The new positioning of H12 helix facilitates AF-2 function and allows co-activators to interact with the newly formed coactivator interaction groove (Rastinejad *et al.* 2013). Co-activators interact with this groove via LXXLL (L represents leucine and X represents any amino acid) motifs. The LXXLL motifs interact with the SR via a hydrophobic cleft formed from H3, H4 and H12 helices. The SR is now capable to interact with DNA, dependent on the SR they translocate to the nucleus if not already present there (Gronemeyer *et al.* 2004). Once in proximity of DNA one of the zinc-finger like modules of the DBD interacts with the major groove of DNA. This is the recognition helix which interacts with hexamer response elements (HREs) via specific DNA half sites (Schwabe *et al.* 1993). AR, GR, PR and MR recognise 5'-AGGACA-3' where as ERα recognises 5'-AGGTCA-3' half sites. The half sites are found by utilising a series of hydrophilic residues which read the DNA sequence until recognition of the specific response element. As an added layer of selectivity to ensure correct recognition, in a DNA-dependent manner the SRs cooperatively form homodimers to account for orientation and space between half sites. The SRs form these homodimers via the second zinc finger like module present in the DBD (Roemer *et al.* 2006).

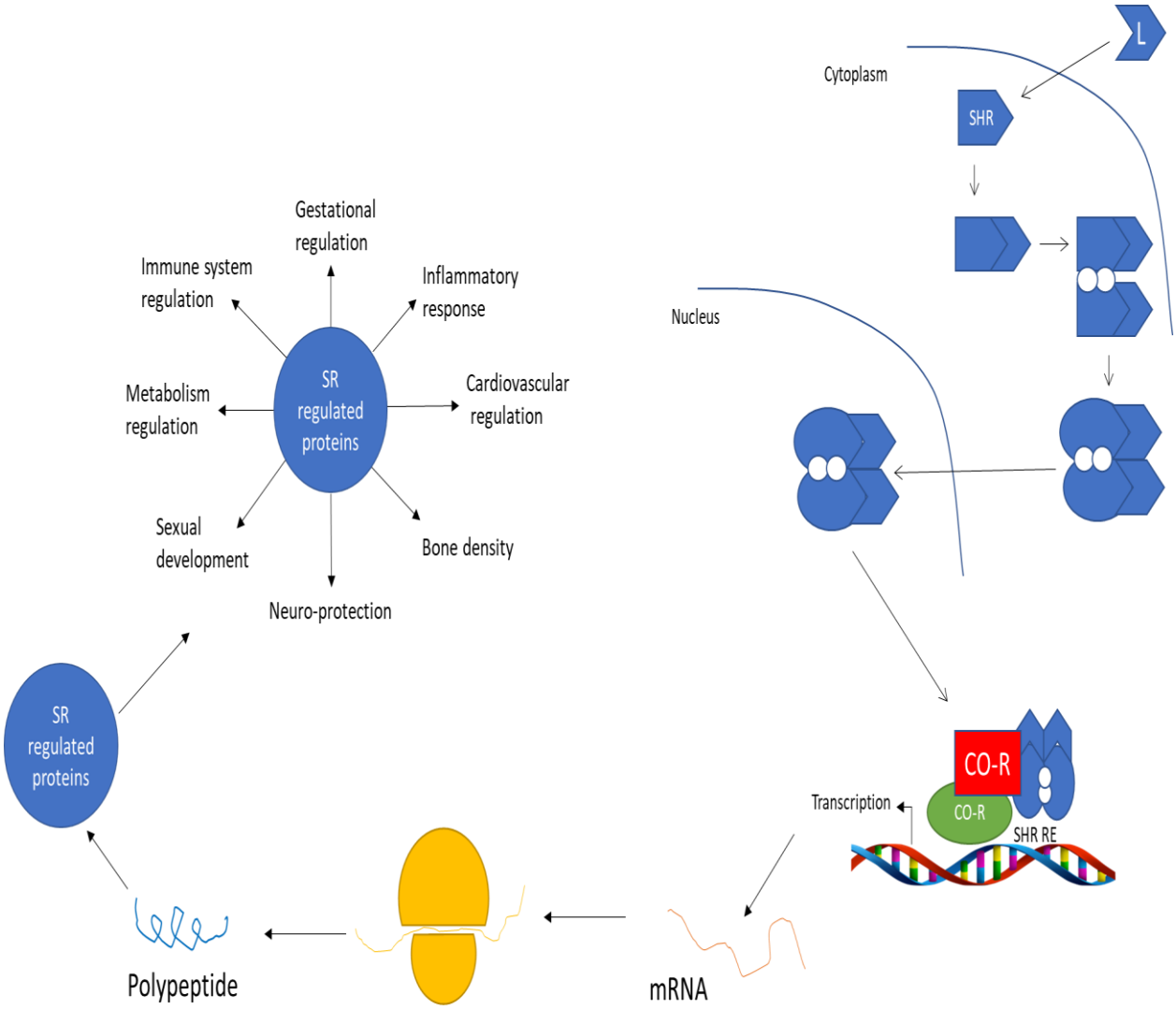

Figure 1.7.2.1.1 – Schematic representation of SR signalling pathway.
Ligand diffuses across the phospho-lipid bilayer, binds to its SR which causes homodimerization and translocation to the nucleus. In the nucleus SR bind to are influenced by Co-regulators to determine the transcriptional program. mRNA is produced and translated into a polypeptide which then folds into the SR regulated protein which then carries out its relative function. The SR regulate proteins then regulate a variety of cellular processes such as: neuro-protection, sexual development, metabolism, immune system, gestation, inflammatory response, cardiovascular system and bone density.

1.7.2.2 - Non-classical steroid receptor signalling

The non-classical mechanism of action occurs via the activation of membrane-bound SRs. Activation via this mechanism can cause a range of signalling events which can then alter other signalling pathways. The most frequently activated pathways are the MAPK pathway, the PI3-AKt pathway and second messengers such as IP_3 and cAMP can also be activated (Lösel & Wehling 2003). The mechanisms non-classical signalling seen across all the SRs is not as transferable as the classical activation, but examples can be shown. For example, activation of membrane bound ERα leads to activation of G-protein coupled receptors (GPCRs) which in turn leads to the activation of PLC and the production of DAG and IP_3. These molecules then cause an influx of Ca^{2+} and the activation of PKC (Chaudhri *et al.* 2014). PKC then activates downstream MAPKs which influence gene expression, promoting proliferation. Additionally, E2 activation can induce anti-apoptotic effects and promote cell survival via PI3K. Activation of PLD at the membrane leads to the production of LPA which in turn activates PI3K. PI3K then stops the caspase cascade which inhibits apoptosis (Schwartz *et al.* 2016). Similarly, treatment with progesterone was shown to affect the cell cycle by causing cell cycle arrest in G_1. This was done via the MAPK pathway which targeted cyclin D1. Loss of cyclins A/B and reduction in cyclin D1, D3 and E were also seen (Garg *et al.* 2017). Within one minute of treatment with androgen, cells show a Ca^{2+} influx which leads to cytoskeleton rearrangements and PSA expression secretion within ten minutes. This occurs rapidly in comparison to classical signalling (Rahman & Christian 2007).

1.8 - Steroid Receptor Expression and Function

The SRs are widely expressed throughout the body, as they regulate gene transcription they serve many physiological functions. However, the SRs have different expression profiles and transcribe different genes resulting in varied effects and phenotypes across tissues.

1.8.1 - Androgen Receptor (AR)

The genes transcribed by the AR determines the outcome of that cell, however this varies dependent on tissue and conditions. Immunohistochemistry has shown the AR is expressed primarily in the male and female sexual organs; the vagina, cervix, ovaries and breast in women; accessory sex organs, the testes and the skeletal muscles surrounding the male reproductive organs. Additionally AR was detected in sweat glands (Ruizeveld De Winter *et al.* 1991). Within the primary reproductive organs of men, AR function is mainly related to the development and maintenance of these organs. However, due to expression and gene regulation in several tissues, a distinct function of the AR cannot be applied. Several studies have used genetically modified mice to produce a tissue specific global Knock Out (KO) model to investigate key changes in phenotype when AR is diminished (Davey & Grossmann 2016). It yielded substantial results across several tissues and highlights the widespread expression and function of AR, despite being secondary expression sites. In the AR expressing non-reproductive organs in males, AR regulated transcription contributes to; bone density (Yeh *et al.* 2002), sexual drive and aggression (Sato *et al.* 2004), development of the heart (Ikeda *et al.* 2005), metabolism homeostasis (Rana *et al.* 2011), muscle formation (MacLean *et al.* 2008) and regulation of the haemopoietic immune system (Lai *et al.* 2012). The same principle was applied to female mice but was limited to the reproductive organs. AR transcription was shown to contribute to folliculogenesis, mammary gland development, and uterine morphology and development (Walters *et al.* 2009).

The AR receptor facilitates the expression of numerous Androgen Response Elements

(AREs), where the AR binds to and facilitates transcription, throughout the genome (DePrimo *et al.* 2002). The composition and context of these sites differ between different genes allowing AR to navigate several different regulatory functions within one nucleus. Expression profiling analysis on prostate cells showed 205 Androgen Responsive Genes (ARGs) and chromatin immunoprecipitation (Chip) validated 524 AREs. Two thirds of the ARE sites were within 50kb of an ARG transcription start site. However, most ARGs were linked to more than one ARE. An additional level regulation was also present, gene clusters some of which consisted of 13 AREs and 12 ARGs. Furthermore, many AREs were shown to be adjacent to other binding motifs of other transcriptional regulators (Bolton *et al.* 2007). This highlights the complexity AR transcriptional regulation, but this is further regulated by the recruitment of nuclear co-regulators and transcription factors. In the absence of ligand, the AR resides in the cytoplasm until ligand binding. Prior to this, the Hsp90 complex is bound to AR in the cytoplasm along with other chaperones to increase AR stability. The BAG-1L component was shown to enhance transactivation of AF-2 by aiding in the correct folding of the AR when ligand binds exposing its Nuclear Localisation Sequence (NLS) (Froesch *et al.* 1998). Phosphorylation of the transcription factor STAT3 allowed its interaction with the AR and enhancement of its transcriptional activity (Chen *et al.* 2000).

1.8.2 – The Glucocorticoid Receptor (GR)

The GR is expressed in almost all tissues in the body and primarily resides in the cytoplasm. Despite being almost ubiquitously expressed there are two primary functions of the GR; metabolic homeostasis and response to stress through the immune system for example, inflammation. These processes extend across the whole body and are facilitated through hormone communication, but the mechanism varies dependent on tissue type. The production of glucocorticoids themselves are under control of a negative feedback loop by the hypothalamo-pituitary-adrenal axis (HPA) (Kudielka & Kirschbaum 2005). The hypothalamus is activated and secretes vasopressin (VA) and corticotropin releasing factor (CRF), which is controlled by the amygdala and hippocampus. These hormones then stimulate the anterior pituitary gland which secretes adrenocorticotropic hormone (ACTH) which in turn stimulates the adrenal cortex to produce glucocorticoids (GCs). The GCs then complete the negative feedback loop by inhibiting the production of ACTH and CRF. In response to stress, physical or mental (for example danger), the HPA axis becomes activated and increases the production of GCs which executes a variety of tissue specific effects. For example, GC's cause the mobilisation of energy sources through GR mediated transcription of gluconeogenic genes. The enzymes phosphoenolpyruvate carboxy kinase, glucose-6-pase, serine dehydratase and tyrosine amino-transferase are transcribed in hepatocytes, lipolysis is increased in the adipocytes and glucose uptake is reduced in peripheral tissues resulting in large scale relocation of glucose (Tronche *et al.* 1998).

The GR regulates inflammatory effects through the immune system. The immune system has two layers of defence. The initial response comes from the innate immune system which utilises the invariant pattern recognition receptors on the surface of macrophages, dendritic cells and other specialised cells. Through signalling pathways, the adaptive immune system becomes active and recruits T and B lymphocytes, which causes inflammation at the site. It is here the GR regulates inflammation at a cellular and transcriptional level (Oakley & Cidlowski 2011). On a cellular level the GR can cause apoptosis of the lymphocytes and

other specialised cells to reduce inflammation. The most common way GR regulates inflammation through transcription is by regulating the expression of Activating Protein-1 (AP-1) and Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NFkB). AP-1 is a key regulator of the inflammatory response, acting as a homo/heterodimer of leucine zipper transcription factors of which there are many; the Fos family, the Jun family, the ATF family and the MAF family. However, the most common form in response to downstream cytokine signalling caused by inflammation is the Fos/c-Jun heterodimer. The dimer formation is dependent on the phosphorylation of c-Jun by c-Jun N-terminal Kinase (JNK). The AP-1 complex then regulates the transcription of several pro-inflammatory genes via AP-1 response elements (Cruz-Topete & Cidlowski 2014). The GR interferes with AP-1 by three different mechanisms; (i) GR can be tethered to the c-Jun subunit which represses the activity of AP-1 thus inhibits pro-inflammatory gene transcription; (ii) GR binds to Glucocorticoid Responsive Element (GRE) whilst simultaneously tethering c-Jun thus repressing transcriptional activity of AP-1; (iii) GR induces transcription of MKP-1, a phosphatase which can dephosphorylate JNK thus removing AP-1 activation. Under normal conditions NFkB is retained in the cytoplasm due to the inhibitor IkB (Smith & Cidlowski 2010). Under inflammatory conditions the IkB kinase (IKK) is activated and phosphorylates IkB which causes its degradation. The degradation of IkB removes its previous inhibition of NFkB and allows its translocation to the nucleus. Once in the nucleus NFkB can bind to its response elements and induce transcription of several pro-inflammatory genes. The mechanism in which the GR represses the transcriptional activity of NFkB is unknown but it thought to be similar to that of AP-1 (Busillo & Cidlowski 2013). Following the downregulation of inflammation there is a resolution stage, GR regulates transcription of genes which promote phagocytosis of apoptotic cells and cell debris in macrophages and monocytes. Additionally, GR induces transcription of the anti-inflammatory cytokines Transforming Growth Factor β (TGFβ) and Interleukin-10 (IL-10). Furthermore GR induces the transcription of lipoxin A4 receptor, a pro-resolving factor, to sensitise the surrounding cells to stimulation of resolving factors (Cain & Cidlowski 2017).

1.8.3 – Estrogen Receptor α

The ERα is primarily expressed in the reproductive organs and its primary function is the development and maintenance of those organs. However, ERα is also expressed in most tissue types around the body and regulates various functions, including metabolic, cardiovascular, musculoskeletal, immune and in the nervous system. The role of ERα in metabolic homeostasis has been shown through KO models in mice and rats. The ERα KO mice showed increased adiposity, primarily mediated through the reduction in energy expenditure and an increase in food intake (Brown & Clegg 2010). This coincided with another study which used viral RNA interference vectors to reduce ERα expression in a specific region of the brain, the Ventromedial Nucleus of the Hypothalamus (VMH), which had a subsequent effect upon body weight, but no mechanism was elucidated (Musatov *et al.* 2007). More recent studies have identified some components of metabolic homeostasis regulated by the ERα. Through the independent expression of intracellular and extracellular ERα, it was shown that of both were necessary to suppress visceral fat development. Mice which lacked either localised ERα showed increased visceral fat retention and weight gain (Pedram *et al.* 2016).

ERα has been shown to regulate several components of the immune system. For example, ERα was shown to induce the expression of Unc93b1, an endoplasmic reticulum membrane protein which regulates the trafficking of Toll Like Receptors (TLRs). TLRs are key components of the innate immune system recognising foreign entities and are expressed on the membranes of monocytes (Panchanathan *et al.* 2013). ERα was also shown to regulate the expression of the cytokine IL-23R in response to TLR stimulation from external stimuli in dendritic cells and macrophages (Cunningham *et al.* 2012). ERα has also been shown to repress NFkB transcriptional activity by displacing its co-activator CBP at the MCP1 promoter. The ERα further regulates the transcriptional activity of NFkB via different mechanisms and also regulates AP-1 by similar mechanisms to the GR (Kovats 2015).

ERα was also shown to have protective cardiovascular effects via regulation of gene expression. ERα induces the expression of the vasodilatory enzymes prostacyclin synthase, endothelial nitric oxide synthase and regulate genes which promote growth and repair in response to vascular injury (Mendlesohn & Karas 1999). Additionally, ERα regulates serum lipid concentrations, increasing High Density Lipoproteins (HDLs) and triglycerides, decreasing Low Density Lipoproteins (LDLs), therefore reducing the accumulation of lipids on the artery wall. Furthermore, ERα regulates expression of coagulation and fibrinolytic enzymes (Mendelsohn 2002). ERα was also shown to be pivotal in bone growth both longitudinally and radially through the expression of insulin like growth factor 1 in KO mouse models (Vidal 2000).

ERα also exhibits neuroprotective functions. When acute injury is sustained in the brain, aromatase expression is stimulated to increase the levels of estradiol. Estradiol then stimulates ERα mediated transcription of cell survival genes and promotes neuronal survival (Dubal *et al.* 2006). Additionally, the estradiol production stimulates the astrocytes and microglia cells to reduce the immediate inflammatory response via ERα (Spence *et al.* 2013). This is mediated through activation of the PI3K pathway which inhibits NFkB activation and translocation to the nucleus thus represses inflammatory gene expression (Arevalo *et al.* 2015).

1.8.4 – The Progesterone Receptor

The PR is primarily expressed in the reproductive organs and serves the purpose of development and maintenance of these organs. The PR is additionally expressed in the brain, pancreas and bone. The PR is predominantly localised to the nucleus, irrelevant of ligand-binding status (Scarpin 2009). The PR is vital for the initiation and progression of pregnancy within the uterus. The PR works in combination with the other ovarian hormone receptor ERα to facilitate gene expression changes throughout pregnancy. The importance of the PR in reproduction was demonstrated through PR KO mice models, which resulted in fertility defects, such as follicles which rupture the ovaries and failure of embryo implantation and decidualisation (Lydon *et al.* 1995). As early pregnancy progresses PR expression increases which inhibits E2 mediated proliferation of the luminal epithelium to allow for embryo implantation represses recruitment of leukocytes and macrophages to the uterus which is essential for survival of the embryo (Tibbetts *et al.* 1999). Furthermore, during the implantation stage of pregnancy, PR was shown to be responsible for the expression of the Indian hedgehog (Ihh) gene, a signalling pathway molecule which promotes the correct development of skeletal, vascular and gastrointestinal tract systems (Dyer *et al.* 2001). The PR also induces the expression of COUP-TFII, which in combination with Ihh is essential for implantation through paracrine signalling and progression to decidualisation but is also essential for cell differentiation and tissue development (Kurihara *et al.* 2007).

The PR has been shown to play an important regulatory role in neuroprotection. In response to Traumatic Brain Injury (TBI) the body tries to protect the brain by invoking inflammation, swelling (edema) and delayed neuronal death, however TBI remains the leading injury related cause of death and severe disability. By an unknown mechanism, the PR was shown to mediate a reduction in vasogenic and cytotoxic edema (Wright *et al.* 2001). Additionally, PR mediates a reduction in lipid peroxidation to approximately 1/3 of its levels in the absence of ligand. Lipid peroxidation is a major contributor to secondary damage of the Blood Brain Barrier (BBB) after TBI, thus a reduction in this process reduces damage (Roof

& Hoffman 1997). Furthermore, the PR was shown to enhance the expression of superoxide dismutase (SOD), an antioxidant enzyme which removes harmful reactive oxygen species, stabilising the BBB and in turn promoting cell survival following TBI (Moorthy *et al.* 2005). The PR has been shown to promote cell survival in response to TBI by regulating the immune system via repression of cytokines Interleukin 1β (IL-1β) and Tumour Necrosis Factor α (TNFα). The PR was also demonstrated to inhibit apoptosis via its signalling components cytochrome C, caspase-3 and B Cell Lymphoma-2 (BCL-2) and upregulation of antiapoptotic proteins such as ERK (Wei & Xiao 2013). In a similar way PR was shown to inhibit the β-amyloid peptide 25-35 (Aβ) induced apoptosis program pathway in Alzheimer mice models. This was done synergistically by PR repression on the Aβ induced expression of JNK, the upstream kinase of the Aβ induced apoptosis program, and through non-classical inhibition of the apoptotic pathway (Qin *et al.* 2015). These findings can be further potentiated by the results of a genomic study which additionally implicated the PR with regards to TBI. This study was run over an extended 7 day period, in contrast to similar studies, and showed continued low dosage of progesterone resulted in the differential expression of 551 genes compared to vehicle. These genes were associated with increased proliferation, immune response, anti-apoptosis and blood vessel remodelling (Anderson *et al.* 2011).

1.8.5 – The Mineralocorticoid Receptor

The Mineralocorticoid Receptor (MCR), like the other SRs, is expressed across most of the body and resides primarily in the cytoplasm. When first discovered, functions of the MCR were thought to be almost exclusive to polarised epithelial tissues like the kidneys and the colon where it regulated salt balance and water homeostasis (Viengchareun 2007). The MCR was shown to induce transcription of ionic transporters of the cell membrane, which are responsible for the unidirectional transport of sodium from the lumen to the interstitium. The MCR expresses the amiloride-sensitive Epithelial Na Channel (ENaC) subunits (Rossier *et al.* 2002), the basolateral Na+/K+ - ATPase pump subunits (Horisberger *et al.* 1991) and the pump regulatory protein Channel Inducing Factor (CHIF) (Brennan & Fuller 1999). Additionally, the MCR also induces transcription of the Serum and Glucocorticoid receptor Kinase-1 (SGK1) which phosphorylates ubiquitin ligase Nedd4-2. This inhibits the binding of Nedd4-2 to ENaC which promotes its degradation by the proteasome, thus allowing a pool of channels to reach the membrane and facilitate transport (Bhargava *et al.* 2001)

The MCR exhibits regulation within the brain, MCR transcription was able to rescue cells from GR induced apoptosis in hippocampal neurons (Crochemore *et al.* 2005). The mechanism of apoptosis inhibition was shown to be through the activation function in the NTD of the MCR. When the MCR is introduced into Glucocorticoid sensitive pre-B lymphoma cell line 697, the cells exhibit a dramatic change in the expression profile. Increases in expression of BCL-2 and Bfl-1 and reductions in expression of BAX and BID occur. This happens because the MCR-NTD directly interacts with the GR, altering its transcriptional activity thus causes attenuation of the apoptotic program and caspase formation (Planey *et al.* 2002). The significance of MCR in the brain is further potentiated by a genomic study which used Serial Analysis of Gene Expression (SAGE) to determine the hippocampal genes regulated by GR and/or MCR in response to adrenal corticosteroids (Datson *et al.* 2002). This study shows that there are 98 MCR-regulated genes and 33 genes that

regulated by both MCR and GR. The functions of these genes include metabolism, protein synthesis and signal transduction (Datson *et al.* 2002).

The first evidence highlighting the MCR in fat regulation utilised the hibernoma cell line T37i. The study shows MCR induces adipogenesis by increasing expression of adipogenic enzymes such as lipoprotein lipase (LPL), Peroxisome Proliferator-Activated Receptor (PPAR) and Adipocyte-specific fatty acid binding Protein (AP2) which contribute to triglyceride accumulation. Together these changes in expression were indicative of very early stage Brown Adipose Tissue (BAT) differentiation (Penfornis *et al.* 2000). This is further potentiated with additional research which shows when MCR is inhibited by antagonist Drospirenone clonal expansion of preadipocytes is reduced, expression of PPAR decreases, differentiation is inhibited and an overall reduction in triglyceride accumulation (Caprio *et al.* 2011).

Obesity causes chronic low-grade inflammation increasing expression of cytokines TNFα, Monocyte Chemoattractant-1 (MCP-1) and Plasminogen Activator Inhibitor 1 (PAL-1). Additionally, in a diabetes mouse model CD-68 and leptin expression increases (in addition to the cytokines mentioned) paired with a decrease in expression of PPAR and adiponectin. These changes in gene expression were reversed following a 16-week treatment with the MCR antagonist eplerenone, highlighting the significant role of the MCR mediated transcription in obesity and diabetes (Marzolla *et al.* 2012).

In endothelial cells of the vascular system, MCR activation has detrimental effects. MCR induces the expression of NADPH oxidases which are responsible for creating Reactive Oxygen Species (ROS) thus increases oxidative stress (Caprio *et al.* 2008). MCR also decreases expression and activity of Glucose-6-Phosphate Dehydrogenase (G6PD) which is a regulator of the intracellular redox state thus increases oxidative stress. MCR similarly reduces the expression and activity of nitric oxide synthases which are responsible for

combating the harmful effects of ROS thus increases oxidative stress. In combination these transcriptional changes lead an inhibition of vasodilation thereby increasing hypertension (McCurley & Jaffe 2012).

1.9 - Steroid Receptor Post-Translational Modifications

As demonstrated SRs can mediate a vast array of biological functions through classical and non-classical signalling following ligand binding. There is an additional layer of regulation to this process via post-translation modifications (PTMs) by other proteins. There are five main PTMs that modulate SR activity: phosphorylation, ubiquitination, acetylation, and sumoylation.

1.9.1 - Phosphorylation

The PTM of phosphorylation has diverse effects on the SRs transcriptional activity. The AR has been shown to have eight phosphorylation sites, some of which modulate its transcriptional activity. For example, CDK1, 5 and 9 phosphorylate the AR on Ser81, which increases its transcriptional activity and stabilises the protein (Koryakina *et al.* 2014). Similarly, the GR possesses eight sites and is phosphorylated by CDK5 at Ser203 and 211 which diminished the recruitment of co-factors and repressed transcription (Kino *et al.* 2007). The ERα is phosphorylated on Ser118 and Ser167, by RSK1/2, in response to mitogen stimulation. These phosphorylation events occur in the AF1 and result in an increase in transcriptional activity (Lannigan 2003). The PR is phosphorylated by CDK2 at Ser25, 162, 190, 213, 400, 554, 676 and Thr430, some of these are yet to be studied but some have been shown to modulate transcriptional activity (Hagan *et al.* 2012). The MCR is phosphorylated by CDK5 on Ser128, Ser250 and Thr159 which negatively regulate receptor activity (Kino *et al.* 2010).

1.9.2 - Ubiquitination

The PTM of poly-ubiquitination on the SRs causes their degradation via the proteasome pathway. However, the specific sites that are ubiquitinated and lead to degradation have not been elucidated. This degradation serves a biological function; rapid degradation is needed to sustain and tightly regulate a transcriptional signal. This process utilises three types of proteins E-1 activating enzyme, E-2 conjugating enzymes and E-3 ligases (Faus & Haendler

2006). AR interacts with the Mouse double minute 2 homolog (Mdm2) E3 ligase and a hinge region phosphorylation is necessary to recruit another E3 ligase C terminus of HSC70-Interacting Protein (CHIP), both of which leads to its degradation (Rees *et al.* 2006). ERα was shown to interact with the ubiquitin conjugase Ubc4 (Luo *et al.* 2005), but additionally Mdm2 and human papillomavirus E6-Associated Protein (E6-AP) (an E3 ligase) dependent on ERα Ser118 phosphorylation, which leads to its degradation (Valley *et al.* 2005). PR interacts with neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4) E3 ligase in a Ser294 phosphorylation-dependent manner which leads to its degradation (Qiu & Lange 2003). CHIP was shown to target the GR for proteasomal degradation via ubiquitination (Wang & DeFranco 2005). Like the GR, the MCR also interacts with CHIP which leads to its proteasomal degradation (Faresse *et al.* 2010).

1.9.3 - Acetylation

The PTM of acetylation regulates the affinity of co-factors to the SRs, and this is modulated by Histone modifying proteins. Histone Acetyltransferases (HATs) introduce acetyl groups whereas Histone Deactylases (HDACs) remove them. Both modifications mediate histone conformation and change DNA accessibility. The AR is acetylated on Lys630, 632 and 633 by p300 and a p300/cAMP-response element-binding protein complex and mutation of these sites inhibits hormone induced AR transcription thus illustrating the transcriptional enhancement of these modifications (Fu *et al.* 2000). HDAC2 was shown to deacetylate the GR on Lys494 and 495, in response to hormone which in turn regulates NFkB signalling through GR-NFkB interactions (Ito *et al.* 2006). The ERα has several acetylation sites Lys266, 268, 299, 302, 303 and all are mediated by p300. Of these sites Lys266 and 268 directly regulate DNA binding and transcriptional activity of ERα in response to hormone (Kim *et al.* 2006). The PR is acetylated at resides 638-641 within its hinge region in response to hormone and this causes a temporary repression of transcription by hindering translocation to the nucleus (Daniel *et al.* 2010). The MCR is deacetylated at Lys677 by HDAC3, this enhances the transcription of genes which contribute to hypertension. Inhibition

of HDAC3, causes acetylation of Lys677 and represses the expression of hypertension genes (Lee *et al.* 2013).

1.9.4 - Sumoylation

The PTM of Sumoylation covalently attaches a SUMO chain to lysine residues. This can have a significant effect on the fate of the protein, affecting the proteins subcellular location, DNA binding and transcriptional activity. Like ubiquitination, it occurs through E1 activating enzymes, E2 conjugating enzymes and E3 ligase enzymes (Gill 2003). The sumoylation sites of the AR are Lys386 and 520, which occur in a hormone dependent manner and approximately 10% of AR are sumoylated in steady state cells. This is mediated through the E3 ligase Protein Inhibitor of Activated STAT (PIAS1) and PIASxα which both show propensity towards the AR. Sumoylation of Lys386 reduces AR transcriptional activity (Kaikkonen *et al.* 2009). Three sumoylation sites have been identified within GR: Lys277 and 293 which reside in the AF1 region and Lys703 which resides in the LBD. The Lys277 and 293 sumoylations within the AF1 influence selectivity promoter site binding and increase transcriptional activity. However, Lys 703 had no apparent effect (Duma *et al.* 2006). Two sumoylation sites have been identified in the ERα hinge region, Lys266 and 268 which occur in a hormone dependent manner. PIAS1 and PIAS3 were shown to mediate the sumoylation events and with the addition of the E3 ligase Ubc9 they were shown to enhance the transcriptional activity of the ERα (Sentis *et al.* 2005). The PR has three sumoylation sites at Lys7, 388 and 531 and that modification of 7 and 531 are dependent on sumoylation of 388. PIAS3 was shown to mediate sumoylation at these sites and significantly reduces PR transcriptional activity in the presence of hormone (Abdel-Hafiz & Horwitz 2014). PIAS1 was shown to sumoylate the MCR in five positions; Lys89, 399, 428, 494 and 953. Of these Lys953 was in the LBD and the rest were in the NTD. These sumoylations were shown to repress target gene expression dependent on promoter context (Faresse 2014).

1.10 - Steroid Receptor Roles in Cancer and Disease

As demonstrated the SRs have an array of functions across several tissue types. As a result, alterations in SR signalling has been linked to the development of multiple disease.

1.10.1 - Breast Cancer

In BCa there is involvement of ERα, AR and PR within its complex carcinogenic profile. The ERα is the primary SR linked with BCa and is known to be crucial in the development, growth, reproduction and maintenance of the breast organs (Lee *et al.* 2012). Sustained exposure of E2 is thought to be a key stimulatory event in the development BCa, mediated via the ERα (Yue *et al.* 2005). The exact mechanism by which E2 signalling drives tumorigenesis is unclear, but there are two main hypotheses' – (i) E2 stimulates cells within the mammary gland which causes proliferation and the subcellular processes which cause tumorigenesis. This includes DNA synthesis, where mutational errors can occur and disrupt cellular processes. Under sustained E2 the proliferation continues and causes the accumulation of mutations which ultimately leads to cancer. (ii) E2 metabolism leads to the production of genotoxic by-products that directly mutate DNA and once again accumulate under sustained E2. A study concluded that it is likely that both hypotheses occur and act synergistically (Yue *et al.* 2005). In addition to the carcinogenic effects induced by E2, these effects are further propagated by PTMs of the ERα. Primarily phosphorylation events within the AF1 domain enhance transcriptional activity and expression of tumour promoting genes. Two phosphorylation sites, Ser118 and Ser167, are targeted by several different kinases in the presence of E2. Ser118 can also be phosphorylated independently of E2 when Epidermal Growth Factor (EGF) stimulates the MAPK pathway. For example, CDK7 phosphorylates this site in response to both stimuli. This results in increased association with co-factors such as the p160 family. In clinical terms, this phosphorylation results in more differentiated cells with better prognosis and a better response to early tamoxifen treatment (Anbalagan & Rowan 2015). However, if the MAPK stimuli continues it can promote hormone-independent cancer. In such a situation, phosphorylation of Ser167 increases the

binding of the receptor to DNA and Steroid Receptor Co-activator 3 (SRC3), resulting in enhanced transcription. For example the gene CUEDC1 is an ERα target gene which is essential for BCa proliferation (Lopes *et al.* 2018).

Treatment of ERα+ BCa is primarily through anti-estrogen therapy such as tamoxifen which competes with E2 for the ERα. Additionally aromatase inhibitors are regularly used to attenuate conversion of androgens to estrogens thus depleting E2 levels in the patient (Droog *et al.* 2013). The AR is also largely expressed in BCa irrelevant of ERα expression status. In AR+/ERα- BCa, the AR gene expression profile resembles that of ERα in ERα+ BCa, therefore demonstrating that in some cases, the AR can also drive BCa growth. Further, AR overexpression has been shown to contribute to estrogen-based therapy resistance in BCa model systems (De Amicis *et al.* 2010). The role of AR in both ERα+ and AR+/ERα- BCa remains unclear. However, some mechanisms of AR gene regulation have started to emerge; AR was shown to interact with the MAPK pathway via ErbB2, regulating ERK phosphorylation and its downstream effectors the RSKs and in turn Elk-1 and c-Fos in ERα-/AR+ BCa (Chia *et al.* 2015). Anti-androgen therapy would be the logical treatment for ERα-/AR+ BCa and enzalutamide, a potent AR antagonist, has been shown to reduce the proliferation of both ERα+/AR+ and ERα-/AR+ BCa (Cochrane *et al.* 2014).

The role of PR is controversial in BCa with some studies showing proliferative effects and other repressive. PR was shown to increase the responsiveness of MCF7 cells to E2 by transcribing a subset of ERα target genes. Cells stably expressing PR also showed enhanced ERα S167 phosphorylation and recruitment of several co-factors to EREs which in the case of ERα+ BCa led to increased expression of target genes (Daniel *et al.* 2015). Furthermore, knockdown of endogenous PR in multiple BCa cell lines was shown to reduce expression of these target genes, reduced growth in soft agar and partially restored tamoxifen sensitivity, therefore highlighting the receptors proliferative attributes (Daniel *et al.* 2015). Conversely PR was shown to reduce tumour volume in MCF-7 mouse xenografts in

comparison to the ERα+ xenografts which exhibited tumour growth. PR also inhibits growth in primary breast tumour explants. CHIP analysis was performed in the presence of E2 and progesterone, this reveals 3603 differential ERα binding events than with E2 alone, suggesting that PR influences the selectivity of ERα binding (Mohammed *et al.* 2015).

1.10.2 - Prostate Cancer

The AR is the primary SR concerning PCa. Testosterone enters prostate cells where it is metabolised into its more potent form, DHT, which induces the transcription of target genes, such as Prostate Specific Antigen (PSA) and transmembrane protease serine 2 (TMPRSS2). This regulation of transcription promotes cell growth and survival (Tan *et al.* 2015). Development and progression of PCa is dependent on androgen stimulation. Therefore, the primary treatment for PCa is Androgen Deprivation Therapy (ADT) or inhibition of receptor function. However, this is a short-term solution, as relapse occurs due to therapy resistance and development of castrate resistant prostate cancer (CRPC) (Litwin & Tan 2017).

PCa is initiated by activation growth promoting pathways, for example AR upregulates fusion proteins such as E-Twenty-Six Variant 1 (ETSV1) which causes cell cycle progression (Rubin *et al.* 2011). Additionally dysregulation of proliferative pathways such as MAPK and PI3K have been shown to contribute to initiation and disease progression (Taylor *et al.* 2010). The NTD of the AR contains two types of repeated structure: polyglutamine (CAG) and polyglycine (GGG) repeats. It has been shown that men with CAG repeats shorter than 21/22 are at a higher risk of developing PCa. This is because these repeats affect the transcriptional activity of AR, with shorter repeat lengths leading to a more transcriptionally active AR (Neto *et al.* 2008). Several mechanisms have been implicated in CRPC development. During ADT therapy, AR levels are significantly reduced, therefore the AR gene is often amplified to increase the sensitivity of the cell to the current androgen concentrations and allowing for proliferation to again occur. This is present in approximately 30% of CRPC patients (Yuan *et al.* 2014). Mutations in the AR have also been linked to CRPC and cause the AR to have increased affinity to androgen or to respond to other steroid hormones (Taplin *et al.* 2003). Increased expression of co-factors has been demonstrated in the development of CRPC. The dysregulated expression of co-factors SRC1 and Transcriptional Intermediate Factor 2 (TIF2) enhance AR transcriptional activity and increase proliferation (Gregory *et al.* 2001). The AR is also activated in a ligand-

independent manner via PTMs which regulate its transcriptional activity, most commonly by phosphorylations (Katzenwadel & Wolf 2015).

New therapies are being developed as a treatment option for CRPC. For example, drugs that target the NTD of the AR have been developed. However the development of such drugs has proved more difficult than current drugs that target the LBD as there is less intrinsic order in this domain of the protein with few secondary structures (Assi *et al.* 2016). Although not considerable, ERα has been shown to contribute to prostatic growth and development. KO models have shown ERα as an important mediator of prostate carcinogenesis and treatment with anti-estrogens significantly decreased early PCa progression (Jia *et al.* 2015).

1.10.3 - Ovarian Cancer

As with BCa and PCa, ERα target genes can result in the upregulation of proliferative genes which promotes the development of, and drives the progression of, ovarian cancer. The treatment for OvCa is limited due to a poor response to the widely used ERα antagonist tamoxifen with approximately 10-15% of patients responding. This means often cytoreductive surgery is necessary (De Stefano *et al.* 2011). OvCa mainly regards the primary ER isoform ERα. The isoform ERβ plays a significant tumour suppressive role in the ovaries and is the primarily expressed isoform but is seen to be significantly downregulated in this disease. In addition, the proliferative isoform ERα shows the inverse relationship, weaker expression in normal tissues but upregulated in cancer. These changes in ERa expression appear to be regulated by the tumour suppressor microRNA 206 (miR206), which is downregulated in ovarian cancer. Interestingly, reintroduction of this miR in the ovarian cancer cell lines CAOV−3 and BG−1 inhibits proliferation (Li *et al.* 2014). Conversely, agonistic activation of ERβ was shown to reduce proliferation in the OV2008 cell line which has an unbalanced ratio of ERα:ERβ 14:1, highlighting the strong suppressive effects of ERβ (Chan *et al.* 2014).

1.10.4 - Glucocorticoid Resistance

As mentioned previously, the GR is pivotal in the regulation of the immune system, as such GCs are regularly used to alleviate inflammation. However, long term use of GC can lead to GC resistance (GCR), resulting in a return of the disease symptoms such as asthma, rheumatoid arthritis (RA) and Chronic Obstructive Pulmonary Disease (COPD) (Vandewalle *et al.* 2018). The underlying mechanism which causes GCR is caused by the inflammatory responses, mostly cytokines, that the GCs themselves are trying to inhibit. Inhibition of these molecules has been shown to reverse GCR, for example inhibition of TNFα restores GC sensitivity in asthma mouse models (Dejager *et al.* 2015) and inhibition of the IL-2 returned sensitivity to inflammatory bowel disease patients (Creed *et al.* 2006). In a converse manner activation of proteins negatively regulated by GCR can alleviate the symptoms. Inflammatory

stimuli have been shown to reduce activity of HDAC2, reactivation via theophylline restored GC sensitivity (Ford *et al.* 2010). Additionally, stress has also been implicated in GCR, the study concluded that long term stressors can cause the development of GCR and interferes with inflammation (Cohen *et al.* 2012).

1.10.5 - Heart Disease

Myocardial Infarction (MI) rodent models were used to investigate the relationship between the MCR and heart disease. Inactivation of MCR was shown to attenuate left ventricle dilation, cardiac hypertrophy and the development of Heart Failure (HF). Whereas MCR overexpression causes these phenotypes. Expression of MCR is upregulated in the MI mouse models in response to a high-salt diet (de Resende *et al.* 2006). Additionally the presence of aldosterone and salt induces an inflammatory response causing the upregulation of cytokines such as TNFα (Martín-Fernández *et al.* 2014). These effects can be counteracted with MCR directed therapy, which reached clinical trials. Across three randomised clinical trials, treatment with a MCR antagonist in patients with HF showed significantly reduced mortality and morbidity across all causes of death in comparison to the control (Bauersachs *et al.* 2015).

1.11 - Summary

To conclude, RSKs are the most downstream kinases of the MAPK pathway and the SRs are nuclear transcription factors which mediate essential and diverse functions throughout the body. Both families of proteins have been shown to interact and have been implicated in several diseases, including cancer. However, little is known about the cross-talk between these families. As such I aim to complete a comprehensive study of the interactions between the RSKs and SRs. I plan to do this by using recombinant DNA techniques to clone all proteins into mammalian expression vectors. The proteins will then be co-overexpressed, and luciferase reporter assays performed to quantify changes in SR activity in response to RSK signalling.

1.12 – Hypothesis, Aims and objectives

The hypothesis of this project is that different RSK isoforms regulate different SRs, which contributes to development and progression of hormone driven cancers. As such the aims of this project are to: i) Determine which RSKs regulate which SRs, ii) Determine if PMA enhances RSK activation of the SRs, iii) Determine if RSK phosphorylation state effects regulation of SR activity. This will be achieved by the following objectives: i) Cloning all RSKs into PCDNA3.1 (+) for luciferase assays. ii) Use site directed mutagenesis to create RSK mutants, iii) Use Luciferase assays to achieve each aim.

Chapter 2 - Methods and Materials

2.1 - Molecular Cloning

2.1.1 - PCR Primer design

The RSK and MCR DNA sequences were obtained from Uniprot (Consortium, 2017) a protein database. From these sequences primers were designed for DNA amplification via PCR. Primers physical properties were estimated using an online TM and GC calculator (Tm Calculator). The primer pairs were designed to have similar Tm values to increase annealing efficiency. GC content was relatively high at 46% and above to increase binding efficiency of primers to DNA template. Similar sequences were avoided to prevent primer dimer formation (Table 2.1.1.1)

Table 2.1.1.1 Properties of Primers used for PCR of RSK1-4, PCR of MCR and HA annealing

Tm and GC% were calculated with an online bioinformatic tool (Tm Calculator). Forward primers are indicated by (Fwd) and reverse primers are indicated by (Rev).

Primer	Sequence	Length	Tm	GC%
HA tag NheI (Fwd)	5'-CTAGCGATGTACCCATACGATGTTCCAGATTACGCTA-3'	37	66	46
HA tag HindIII (Rev)	5'-AGCTTAGCGTAATCTGGAACATCGTATGGGTACATCG-3'	37	66	46
RSK1 NOTI (Rev)	5'-GGAATTCGCGGCCGCTCACAGGGTGGTGGATGG-3'	33	72	67
RSK1 KPNI (Fwd)	5'-GCAATTCGGTACCATGCCGCTCGCCCAGCT-3'	30	68	63
RSK2 NOTI (Rev)	5'-GGAATTCGCGGCCGCTTACAGGGCTGTTGAGGTGA-3'	35	70	60
RSK2 KPNI (Fwd)	5'-GCAATTCGGTACCATGCCGCTGGCGCAGCTGGC-3'	33	72	67
RSK3 NOTI (Rev)	5'-GGAATTCGCGGCCGCTCACAGCCGCGT-3'	27	69	70
RSK3 KPNI (Fwd)	5'-GCAATTCGGTACCATGGACCTGAGCATGAAGAA-3'	33	64	48
RSK4 NOTI (Rev)	5'-GGAATTCGCGGCCGCTTACAGGCCAGTTGATGTTCG-3'	36	70	58
RSK4 KNPI (Fwd)	5'-GCAATTCGGTACCATGCTACCATTCGCTCCTCAG-3'	34	67	53
MCR XmaI (Fwd)	5'-CCCGGGATGGAGACCAAA-3'	18	53	61
MCR BglII (Rev)	5'-AGATCTTCACTTCCGGTGGAAGTAGA-3'	26	58	46

2.1.2 - Transformation of E.coli DH5α for DNA plasmid amplification

1 µl of plasmid DNA or 5 µl of Ligation mixture was added to 50 µl of DH5α *E. coli* competent cells (Thermo scientific) and incubated on ice for 30 minutes. The cells were then heat-shocked in a water bath at 42^{o}C for 45 seconds. The cells were then incubated on ice for 2-5 minutes. 500 µl of Luria Broth (LB) (or 200 µl for ligation) were then added to the cells, followed by an hour incubation at 37^{o}C with shaking. Cells were then diluted in 10ml of LB or, for ligation, spread on LB agar plates; 100µg/ml Ampicillin of LB for both PCDNA 3.1 (+) and PSG5 vectors. The cultures/plates were incubated overnight at 37^{o}C. Any steps that include exposing the cells to naked air were done in a class II microbiological safety cabinet to reduce the likelihood of contamination.

2.1.3 - Plasmid Miniprep

Minipreps were completed using the Thermo Scientific GeneJet Plasmid miniprep kit.
Cell cultures were centrifuged for 10 minutes at 4000G in a Thermo Scientific Heraeus Megafuge 40R bench top centrifuge. The supernatant was discarded and the pellet was re-suspended in 250 µl of resuspension buffer, lysed with 250 µl of Lysis buffer and 350 µl of neutralisation buffer added. The mixture was centrifuged at 11,400G for 10 minutes in a Thermo Scientific minispin microcentrifuge. The supernatant was then transferred to a miniprep column and centrifuged for one minute at 11,400g. 500 µl of wash buffer was added to the column and centrifuged for one minute at 11,400g. The wash was repeated once more. The column was then centrifuged for 90 seconds at 11,400g to remove the residual wash buffer and transferred to an Eppendorf microcentrifuge tube. 40 µl of water was added and the column left to stand at RT for 1-2 minutes before centrifugation at 11,400G for one minute to elute the DNA.

2.1.4 - PCR DNA amplification

PCR was used to amplify RSK1-4 DNA and MCR DNA (Table 1.1.1). (Table 2.1.4.1) shows the volumes of each component of a PCR reaction, (Table 2.1.4.2) shows the conditions used and (Table 2.1.4.3) shows the variable annealing temperature for the RSKs and MCR.

Table 2.1.4.1 PCR reaction mixture, volumes and concentrations of reagents

Volume	Component
10 µl	5X Phusion DNA Polymerase GC buffer (Thermo Scientific)
5 µl	Dimethyl sulfoxide (DMSO) 100%
1 µl	dNTPs mix (Thermo Scientific, 200mM)
1 µl	Primer mix (250ng/µl)
1 µl	DNA template (100-250ng/µl)
1 µl	Phusion DNA Polymerase (Thermo Scientific, 2U/µl)
31 µl	H_2O

Table 2.1.4.2 PCR program used. X depicts variable annealing temperatures dependent on gene of interest.

Temperature (°C)	Time (minutes)	Process	Number of cycles
98	10	Initial denaturation	1
98	1	Denaturation	30
X	1	Annealing	
72	1	Extension	
72	10	Final extension	1

Table 2.1.4.3 Annealing temperatures of RSKs and MCR.

Annealing Temperature (°C)	Gene of interest
60	RSK1-4
54	MCR

2.1.5 HA annealing

The HA forward and reverse primers were annealed in a buffer containing 10mM Tris (pH 7.5), 50mM NaCl and 10mM to create the HA tag. Reaction mixture and annealing program are shown by (Table 2.1.5.1 and 2.1.5.2) respectively.

Table 2.1.5.1 HA annealing reaction mixture, volumes and concentrations of reagents

Volume	Component
2.5 µl	Primer 1 (100µM)
2.5 µl	Primer 2 (100µM)
45 µl	Buffer

Table 2.1.5.2 HA annealing program used

Temperature (°C)	Time (minutes)	Process
98	2	Initial denaturation
25	45	Annealing

2.1.6 - Agarose Gel Electrophoresis

RSK and MCR PCR products were purified on 2% and 1.5% w/v agarose gels respectively. Double digested RSK and MCR DNA, PCDNA 3.1(+) and PSG5 plasmids were purified on a 1% w/v agarose gel. Ligation double diagnostic digests were run on 1% w/v agarose gels. 1 µl of Gel Red Nucleic acid stain (50,000X) was added per 50ml of agarose. Thermo Scientific 1Kb Gene ruler and 100bp DNA ladders (0.5µg/ µl) were also used in lanes to check the size of DNA. Electrophoresis was carried out at 100V from 40-60 minutes. DNA bands were visualised using a Syngene UV illuminator.

2.1.7 - Gel Extraction

DNA was extracted from agarose gels and purified using the GeneJET Gel Extraction Kit (Thermo Scientific). All centrifugation steps were carried out on a Thermo Scientific minispin microcentrifuge at 11,400 G. DNA was solubilised by adding one volume of solubilisation buffer to one volume of gel (i.e. 100 µl of buffer per 100mg of gel) and heated at 55^{o}C for 10 minutes, vortexing the sample every two minutes. The solution was then transferred to a gel extraction column, centrifuged for one minute and the flow through discarded. An additional 100 µl of binding buffer were added to the column and centrifuged for one minute. 700 µl of wash buffer were added and then centrifuged for one minute. The microcentrifuge tube was centrifuged for 90 seconds, to remove the residual wash buffer, and the column transferred to an Eppendorf microcentrifuge tube. 40 µl of water was added to the column, left to incubate at RT for 1-2 minutes before centrifugation for one minute to elute the DNA.

2.1.8 - Double digests of RSKs, PCDNA 3.1 (+), MCR, PSG5 and HA tag

To generate sticky ends, RSKs and PCDNA 3.1 (+) vector were digested with *Kpn*I and *Not*I. MCR and PSG5 vector were digested with *Xma*I and *Bgl*II. The HA tag was digested by *Xho*I at two positions. Reaction mixtures (Table 2.1.8.1) were incubated at 37^{o}C.

Table 2.1.8.1 Restriction enzyme double digest, volumes and concentrations of reagents

Volume	Component
5 µl	10X FD buffer green (Thermo Scientific)
1 µl	1st RE (Thermo Scientific, 10U/µl)
1 µl	2nd RE (Thermo Scientific, 10U/µl)
35 µl / 20 µl	Insert DNA/ Vector DNA (30-100ng/µl)
Make up to 50 µl	H_2O

2.1.9 - Ligation of RSKS into PCNDA 3.1(+) vector, MCR into PSG5 vector and HA tag into PCDNA 3.1(+)

Digested vectors and inserts were ligated together using T4 DNA ligase. To increase success of ligation, excess insert concentration to plasmid was used (Table 2.1.9.1). The plasmids were transformed into competent bacteria (section 2.1.2). The resulting colonies were grown in 10ml LB culture with 100µg/ml Ampicillin and left to grow overnight. Ligation product was then purified using Plasmid Miniprep (section 2.1.3).

Table 2.1.9.1 Ligation mixture volumes and concentrations of reagents

Volume	Component
3 µl	10X T4 ligase buffer (Thermo Scientific)
1 µl	Vector DNA (100-300ng/µl)
25 µl	Insert DNA (50-100ng/µl)
1 µl	T4 DNA ligase (Thermo Scientific)

2.1.10 - Diagnostic digest check

Recombinant DNA was double digested to confirm successful ligation. (Table 2.1.10.1) shows the components of a double digest check. Samples were incubated for 3 hours at 3^{o}C and then run on a 1% agarose gel at 100V for 45 minutes.

Table 2.1.10.1 Diagnostic digest check volumes and concentrations of reagents

Volume	Component
1 µl	Plasmid (1-2µg)
1 µl	1st Restriction Enzyme (Thermo Scientific, 10U/µl)
1 µl	2nd Restriction Enzyme (Thermo Scientific, 10U/µl)
1 µl	buffer
6 µl	H_2O

2.1.11 - Site directed mutagenesis

Primers were designed to incorporate a single amino acid mutation on the N-terminal phosphorylation site of each RSK isoform. The primers were designed using an online tool by Agilent (Agilent 2002), primer length and Tm were kept constant so that all newly synthesised DNA is identical (Table 2.1.11.1-4). The primers were then mixed with additional components (Table 2.1.11.5) to create a mutagenesis reaction mixture which was then put through a designated mutagenesis program to the mutants (Table 2.1.11.6).

Table 2.1.11.1 Primers properties used for Site directed mutagenesis of RSK1

Primers for RSK1 S221E	Length (bp)	Tm	Duplex energy at 68°C	Energy cost of mismatches
5'-ccacgagaagaaggcctatgaattctgcgggacagtggag-3'	40	78.65 ℃	-58.33 kcal/mol	0.77%
5'-ctccactgtcccgcagaattcataggccttcttctcgtgg-3'	40	78.65 ℃	-56.50 kcal/mol	1.74%

Table 2.1.11.2 Primers properties used for Site directed mutagenesis of RSK2

Primers for RSK2 S227E	Length (bp)	Tm	Duplex energy at 68°C	Energy cost of mismatches
5'-tctattgaccatgaaaagaaggcatat gaattttgtggaactgtggagtatatggc-3'	56	78.73℃	-58.60 kcal/mol	.77%
5'-gccatatactccacagttccacaaaatt catatgccttcttttcatggtcaataga-3'	56	78.73℃	-60.05 kcal/mol	1.64%

Table 2.1.11.3 Primers properties used for Site directed mutagenesis of RSK3

Primers for RSK3 S218E	Length (bp)	Tm	Duplex energy at 68°C	Energy cost of mismatches
5'-ctcgatcgtcccgcagaattcgtacgctctcttgtcgtg-3'	39	78.58 ℃	-53.16 kcal/mol	10.28%
5'-cacgacaagagagcgtacgaattctgcgggacgatcgag-3'	39	78.58 ℃	-55.70 kcal/mol	9.55%

Table 2.1.11.4 Primers properties used for Site directed mutagenesis of RSK4

Primers of RSK4 S232E	Length (bp)	Tm	Duplex energy at 68°C	Energy cost of mismatches
5'-catatactctactgtaccacaaaattcgtaagccttcttttcttgatctac-3'	51	78.01 ℃	-46.73 kcal/mol	6.35%
5'-gtagatcaagaaaagaaggcttacgaattttgtggtacagtagagtatatg-3'	51	78.01 ℃	-50.06 kcal/mol	2.76%

Table 2.1.11.5 Mutagenesis reaction volumes, concentrations and mass of reagents

Volume/mass	Component
10 µl	5X Phusion DNA Polymerase GC buffer (Thermo Scientific)
1 µl	200mM dNTPs mix (Thermo Scientific)
1 µl	250ng/µl Primer mix
50ng	DNA template
1 µl	Phusion DNA Polymerase (Thermo Scientific)
Make to 50 µl	H_2O

Table 2.1.11.6 Mutagenesis program used

Temperature (°C)	Time (minutes)	Process	Number of cycles
98	3	Initial denaturation	1
98	1	Denaturation	18
55	1	Annealing	
68	3	Extension	
68	10	Final extension	1

2.1.12 - Sequence analysis

Purified plasmids were sequenced by Eurofins to check if the insert had successfully been ligated into the vector with no point mutations or to check if site directed mutagenesis was successful and contained no additional undesired mutations. DNA sequences were translated into amino acids using the online Expasy Translate tool (Gasteiger *et al.* 2003). The Clustal Omega Multiple sequence alignment tool (Goujon *et al.* 2010) was then used to align the sequencing results with sequences obtained from uniprot database (Consortium 2017).

2.1.13 - DNA midi prep

RBC Bioscience Fast Ion Plasmid Midi Advanced Kit was used to generated high-purity plasmids for mammalian cell transfection. DH5α cells were transformed and grown ON in 10 ml LB. This pre-culture was then diluted in 200ml of LB containing 100µg/ml of ampicillin and incubated at 37ºC until the OD_{600} reached a value between 2-4 The cells were then aliquoted into four sterile 50ml falcon tubes and centrifuged at 6000G for 15 minutes at 4ºC. The supernatant was discarded and the cells resuspended in 10ml of PM1 buffer. The resuspended cells were transferred to a 30ml centrifuge tube and 10ml of PM2 buffer added. The mixture was inverted 10-15 times before being left to stand at RT for five minutes. In the meantime, a PMI column was equilibrated with 10ml of PEQ buffer. Once the lysate had cleared 10ml of PM3 buffer was added to the solution, inverted 10-15 times and left to stand at RT for five minutes. The solution was then centrifuged at 15,000G for 15 minutes at 4ºC. The supernatant was transferred to the column and then washed with 15ml of PWA buffer. 10ml of PEL buffer was added to the column and the eluted DNA was collected in a new sterile 30ml centrifuge tube. 7.5ml of isopropanol was added, the tube inverted 10-15 times and then left to stand at RT for 2-5 minutes. The mixture was centrifuged at 20,000G for 30 minutes at 4ºC. The liquid was carefully removed prior to the addition of 5ml of 70% ethanol and the tube centrifuged at 20,000G for 10 minutes at 4ºC. The supernatant was again discarded. The DNA pellet was left to stand at room temperature until dry. Dependent on pellet size an appropriate volume of sterile water was used to dissolve the DNA and this was transferred to a sterile 1.5ml Eppendorf.

2.2 - Cell Culture

Monkey COS-1 cells were maintained in DMEM or stripped DMEM media, supplemented with 5% Foetal Bovine Serum (FBS) or 2% double charcoal stripped FBS respectively. Both variations of DMEM were also supplemented with Penicillin (100 U) - Streptomycin (0.1 mg/mL) - L-Glutamine (2 mM) (PSG). All manipulations were performed under sterile conditions inside a Class II microbiological cabinet and cells were incubated in humidified air containing 5% CO_2 at 37^{o}C.

2.2.1 - Passaging cells

Cells were passaged every time they reached 70-80% confluency. Old media was removed, the cells were then washed with Phosphate Buffered Saline (PBS) and, dependent on flask size, 0.75-1.5ml of Trypsin/EDTA was added. The cells were incubated for a few minutes at 37^{o}C to detach the cells from the flask surface. The trypsinised cells were then resuspended in 10ml of media and the desired volume of cells were added to a new flask containing preheated media and placed back into the incubator.

2.2.2 - Plating cells

Cells were passaged every time they reached 70-80% confluency. Old media was removed, the cells were then washed with Phosphate Buffered Saline (PBS) and, dependent on flask size, 0.75-1.5ml of Trypsin/EDTA was added. The cells were incubated for a few minutes at 37^{o}C to detach the cells from the flask surface. The trypsinised cells were then resuspended in 10ml of media and the desired volume of cells were added to a new flask containing preheated media and placed back into the incubator.

2.2.3 - Calcium Phosphate Transfections

100 µl of transfection mixture was pipetted dropwise into each well of a 24 well plate and incubated for 20-24 hours. The transfection mixtures varied dependent on experimental design. All transfection mixtures contained a set combination of DNA plasmids at set concentrations in addition to $CaCl_2$ and Borate Buffered Saline (BBS). The DNA plasmids were added first, 2M $CaCl_2$ was then added in a swirling manner followed by bubbling to mix the solution, BBS was added last in the same manner. The mixtures were left to incubate at RT for 15 minutes before being transferred onto cells. (Table 2.2.3.1-5) highlight the components of all transfection mixes for different experiment sets.

Table 2.2.3.1 Hormone optimisation experiments - volumes and concentrations of reagents added per well

Volume per well	Component
0.5 µl	Steroid receptor plasmid (100ng/µl)
2 µl	Luciferase plasmid (500ng/µl)
1 µl	B-gal plasmid (10ng/µl)
41.5 µl	H_2O
5 µl	2M $CaCl_2$
50 µl	BBS

Table 2.2.3.2 DNA optimisation experiments – volumes and concentrations of reagents added per well

Volume	Component
0.5 µl	Steroid receptor plasmid (100ng/µl)
2 µl	RSK plasmid (0/100/200ng/µl)
2 µl	Luciferase plasmid (500ng/µl)
1 µl	B-gal plasmid (10ng/µl)
39.5 µl	H_2O
5 µl	2M $CaCl_2$
50 µl	BBS

Table 2.2.3.3 Volumes of plasmid used to create the different DNA concentrations.

	RSK condition		
	0ng	100ng	200ng
Empty vector (100ng/µl)	9 µl	4.5 µl	0 µl
RSK DNA (100ng/µl)	0 µl	4.5 µl	9 µl
Total Volume	9 µl	9 µl	9 µl

Table 2.2.3.4 PMA induction experiments – volumes and concentrations of reagents added per well

Volume	Component
0.5 µl	Steroid receptor plasmid (100ng/µl)
1 µl	RSK plasmid (100ng/µl)
2 µl	Luciferase plasmid (500ng/µl)
1 µl	B-gal plasmid (10ng/µl)
40.5 µl	H_2O
5 µl	2M $CaCl_2$
50 µl	BBS

Table 2.2.3.5 Phospho-mimetic experiments – volumes and concentrations of reagents added per well

Volume	Component
0.5 µl	Steroid receptor plasmid (100ng/µl)
1 µl	RSK plasmid (100ng
2 µl	Luciferase plasmid (500ng/µl)
1 µl	B-gal plasmid (10ng/µl)
40.5 µl	H_2O
5 µl	2M $CaCl_2$
50 µl	BBS

2.2.4 - Hormone/drug treatments

COS-1 cells were treated with preheated hormone-depleted DMEM media and hormones/drugs 20-24 hours after they were transfected. Stock hormone concentrations were prepared 1000x more concentrated than the desired final concentration to allow a dilution factor of 1:1000. Prior to treatment, cells were washed two times with 0.5ml of preheated hormone-depleted DMEM media. The hormone optimisation experiments used varied concentrations (0, 0.1, 1, 10, 100, 1000nM) of the corresponding hormone to steroid receptor. The DNA optimisation experiments used a set concentration of hormone 1nM, 10nM, 10nM, 1nM with regards to AR, GR, ER, PR respectively. In addition to this there was a 100% ethanol control for each condition which was also diluted in preheated hormone-depleted DMEM at the same ratio as hormone. The DNA optimisation experiment conditions were used for the PMA induction experiments except 200nM of PMA was also added to corresponding wells. The DNA optimisation experiment conditions were used for the phosphor-mimetic experiments.

*2.2.5 - Cell lysis, **β**-galactosidase and Luciferase assays*

All media was removed from the 24 well plate and cells were washed 2 times with 0.5ml of PBS. 60 µl of Promega Cell Culture Lysis Reagent was added to each well and the plate was frozen for 15 minutes at -80^{o}C. The plates were left to thaw and kept on ice for the duration of the assay. For the luciferase assays, 20 µl of cell lysate was taken from each well and added to individual wells of a 96 well plate. 20 µl of Promega Luciferase Substrate was added to each well and the plate was covered with foil and rotationally mixed for 15 minutes at room temperature. The plate was again read on a FLUOstar Omega Plate Reader.

For the **β**-galactosidase assay, 5 µl of cell lysate was taken from each well and added to individual wells of a 96 well plate. 50 µl of Tropix Galacton-Plus was then added to each well, the plate was covered with foil and rotationally mixed for one hour at room temperature. 75 µl of Tropix Accelerator II was added to each well, the plate was re-covered with foil and mixed rotationally for 15 minutes at room temperature. The plates were then read immediately on a FLUOstar Omega Plate Reader. Luciferase data were normalised using the **β**-galactosidase results.

2.3 - SDS-PAGE

Protein concentration was measured on a Nanodrop ND-1000 Spectrophotometer on the protein A280 setting. In addition to these readings known concentrations of BSA were measured and a standard curve generated. 4.17 µl of Lamelli loading dye was added to 40µg of each sample and lysis buffer was added to make all samples to the same final volume. Samples were boiled for ten minutes at 90^{o}C and immediately placed on ice. The samples were loaded onto a 12% SDS-PAGE (Table 2.3.1). 4 µl of Thermofisher Prestained Protein PageRuler Ladder was added to one well and 4 µl of laemmeli loading dye was added to any empty wells. The gel was then run in running buffer (Table 2.3.1) at 180V until the dye front ran off the gel.

Once the SDS-PAGE was completed the proteins present were transferred onto a PVDF membrane. Five pieces of electrode paper were soaked in transfer buffer (Table 2.3) and placed on the BIO-RAD trans-blot semi-dry transfer cell. A piece of membrane was cut to similar size as the electrode paper and soaked in methanol. The membrane was then soaked in transfer buffer and placed on top of the PDVF membrane. The SDS-PAGE was then placed on top of the membrane. The last 5 pieces of PDVF paper were then soaked in transfer buffer, placed on top of the SDS-PAGE and rolled to remove any potential bubbles. The electrode plates were then dampened with transfer buffer to ensure current flow. The SDS-PAGE was then transferred for two hours at 15V and 100mA.

Table 2.3.1 Reagents used for SDS-PAGE

Reagent	Components
SDS-PAGE (12%)	Per gel, a 12 % resolving gel was made; 4mL Acrylamide/Bis-acrylamide 30 % solution (Sigma-Aldrich), 2.6 mL of 1.5 M Tris/HCl at pH 8.8, 3.2 mL of ddH2O and 50 µL of 10 % SDS. Immediately prior to pouring, 10 µL of 10 % APS stock and 2.5 µL N,N,N',N'Tetramethylethylenediamine (TEMED, Sigma-Aldrich) were added. Additionally a stacking gel was made; 670 µL of Acrylamide/Bis-acrylamide 30 % solution (Sigma-Aldrich), 1.25mL of 0.5 M Tris/HCl at pH 6.8, 3 mL of ddH2O and 50 µL of 10 % SDS. Immediately prior to pouring, 10 µL of 10 % APS and 5 µL TEMED were added.
Running buffer	3 g of Tris base (25 mM, Fisher Scientific), 14.45 g of Glycine (0.2 M, Fisher Scientific) and 0.5 g of SDS (0.05 %, Fisher Scientific) were dissolved in a total volume of 1 L ddH2O.
Transfer buffer	5.63 g Glycine (150 mM, Fisher Scientific), 1.22 g Tris base (20 mM) and 100 ml of Methanol (20 %, MeOH, Fisher Scientific), dissolved in a total volume of 500 mL ddH2O.

2.4 - Immunoblotting

1g of powdered milk was dissolved in 20ml of PBS-0.1% tween (PBST) creating a 5% PBST-milk solution. The 5% PBST-milk was added to the PVDF membrane and incubated at room temperature for 30 minutes. The block was removed and primary antibodies were added. The primary antibodies (Table 2.4.1) were prepared in 5% PBST-milk and incubated over night at 4^{o}C with rotation. The following day the primary antibody was removed and the membrane washed three with 5ml of PBST (5min with agitation). Membranes were blocked with 5% PBST-milk for 15 minutes on a roller at room temperature. Secondary antibodies (1:2000) were then added and the membrane left for one hour on a rolling machine at RT. The wash steps were repeated and a final wash with PBS for five minutes was performed on a roller at RT. After the final PBS wash, 350 µl of Luminata Forte Western HRP substrate was continuously pipetted over the membrane for 3 minutes. The membrane was placed in a plastic film and bands visualied using a Fusion FX Vilber Lourmat.

Table 2.4.1 Antibodies used for western blots, their targets and the dilutions used.

Target	Primary Antibody code	Dilution used	Secondary antibody (1:2000)
AR	Ab74272	1:1000	Anti-Rabbit
ERa	IR084	1:1000	Anti-Rabbit
GR	Sc-393232	1:500	Anti-Rabbit
MCR	Sc-53000	1:1000	Anti-Rabbit
PR	Sc-166169	1:1000	Anti-Mouse
RSK1-4	Ab71113	1:100	Anti-Rabbit
Actin	Ab8227	1:1000	Anti-Rabbit

Chapter 3. Results

3.1 Cloning of the RSKs and Steroid Receptors

It has been demonstrated that some RSKs can regulate the transcriptional activity of certain SRs, however not all are characterised. To overcome this a comprehensive study is needed to fill the knowledge gap, therefore some of the RSKs and the MCR need to be cloned into the appropriate mammalian expression vectors.

The RSKs and MCR were amplified by PCR and isolated via agarose gel electrophoresis. The RSKs were amplified and visible at approximately 2000bp which is the correct size. Additionally, MCR was amplified and a single band is visible at approximately 3000bp which is the correct size (Figure 3.1.1). The genes were then digested with the relevant restriction enzymes to create sticky ends for ligation, ligated into the relevant vector and sequence verified. Prior to incorporation of the RSK genes into PCDNA 3.1(+), a HA tag was first incorporated into the vector to facilitate simple western blotting in later experiments. Both the HA tag and PCDNA 3.1(+) were digested with Restriction Enzymes (REs) NheI and HindIII, ligated together and sequence verified via sanger sequencing. HA tag containing PCDNA 3.1 (+) and the RSKs were then digested by NOTI and KPNI, ligated together and sequence verified. This positioned the RSK gene directly after the HA tag within the plasmid (Figure 3.1.2). Through use of several primers the complete gene was sequenced with no mutations, including the HA tag at the start of the gene. RSK1 and RSK4 genes were synthesised and sequence verified by an external company TWIST, therefore there are no sequence alignments. The MCR and PSG5 were digested with XmaI and BglII, however MCR was not successfully ligated into PSG5 vector.

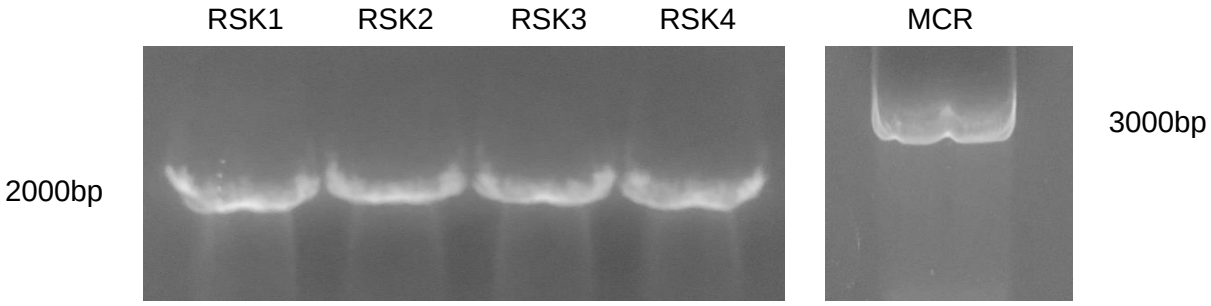

Figure 3.1.1 Agarose gel electrophoresis of RSK1-4 and MCR fragmentssite

RSK1-4 and MCR were amplified by PCR. Loading dye was added to PCR product of RSK1-4 and MCR and loaded into wells of a 2% agarose gel. The gel underwent electrophoresis at 100V for 60 minutes, viewed under a Syngene illuminator and images were taken on a mobile phone camera.

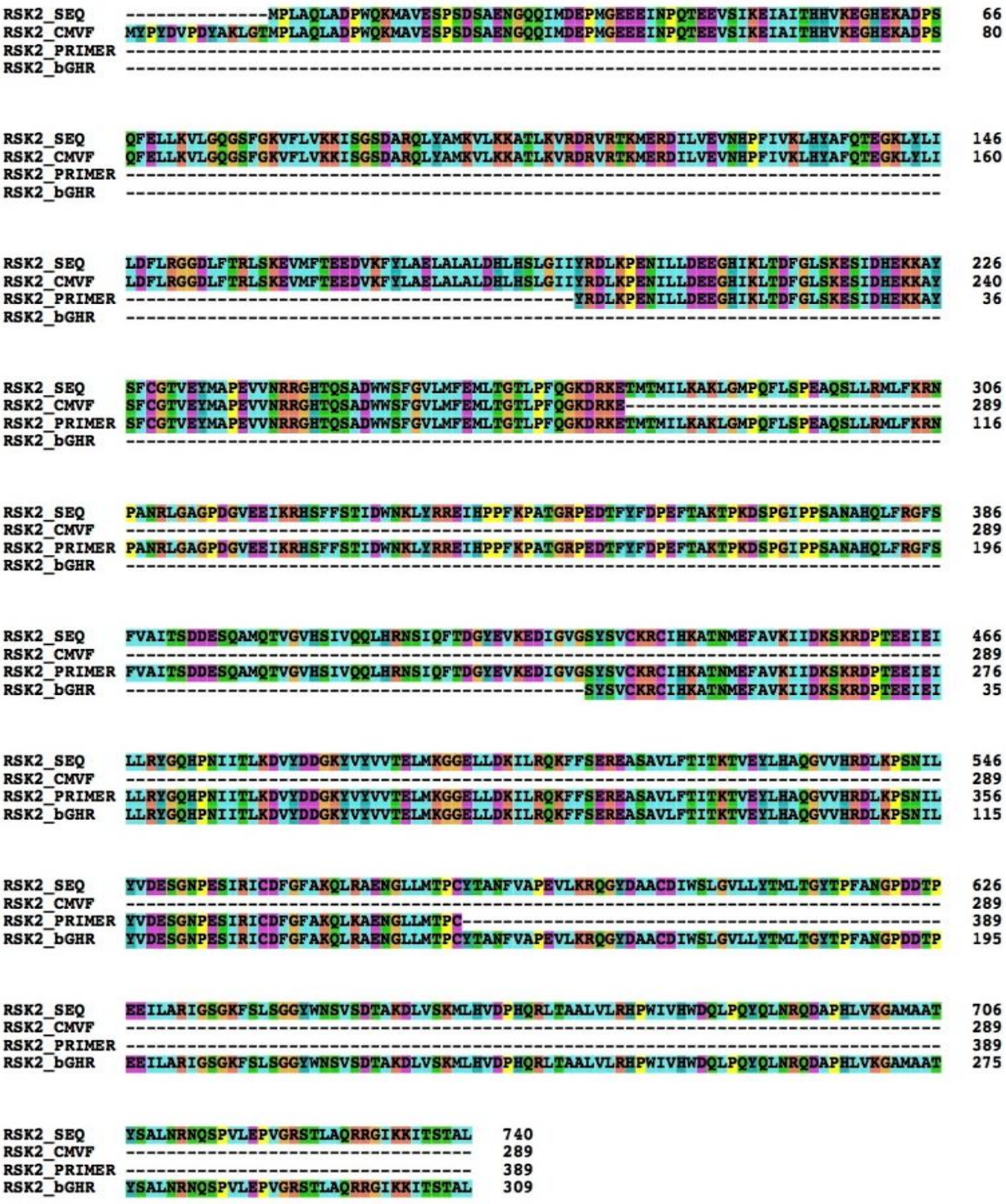

Figure 3.1.2 Multiple sequence alignment of RSK2.

DNA samples were sent to Eurofins for sanger sequencing. Upon return sequences were manipulated to produce one file which shows the composition of DNA sequence. RSK_SEQ is the RSK DNA sequence obtained from uniprot, RSK_CMVF is a company primer used to sequence the beginning of the gene, RSK_PRIMER is a manually created and synthesised primer used to sequence the middle of the gene and RSK_bGHR is a company primer used to sequence the end of the gene. Combined use of these primers led to a complete sequence for both RSK2 with no mutations.

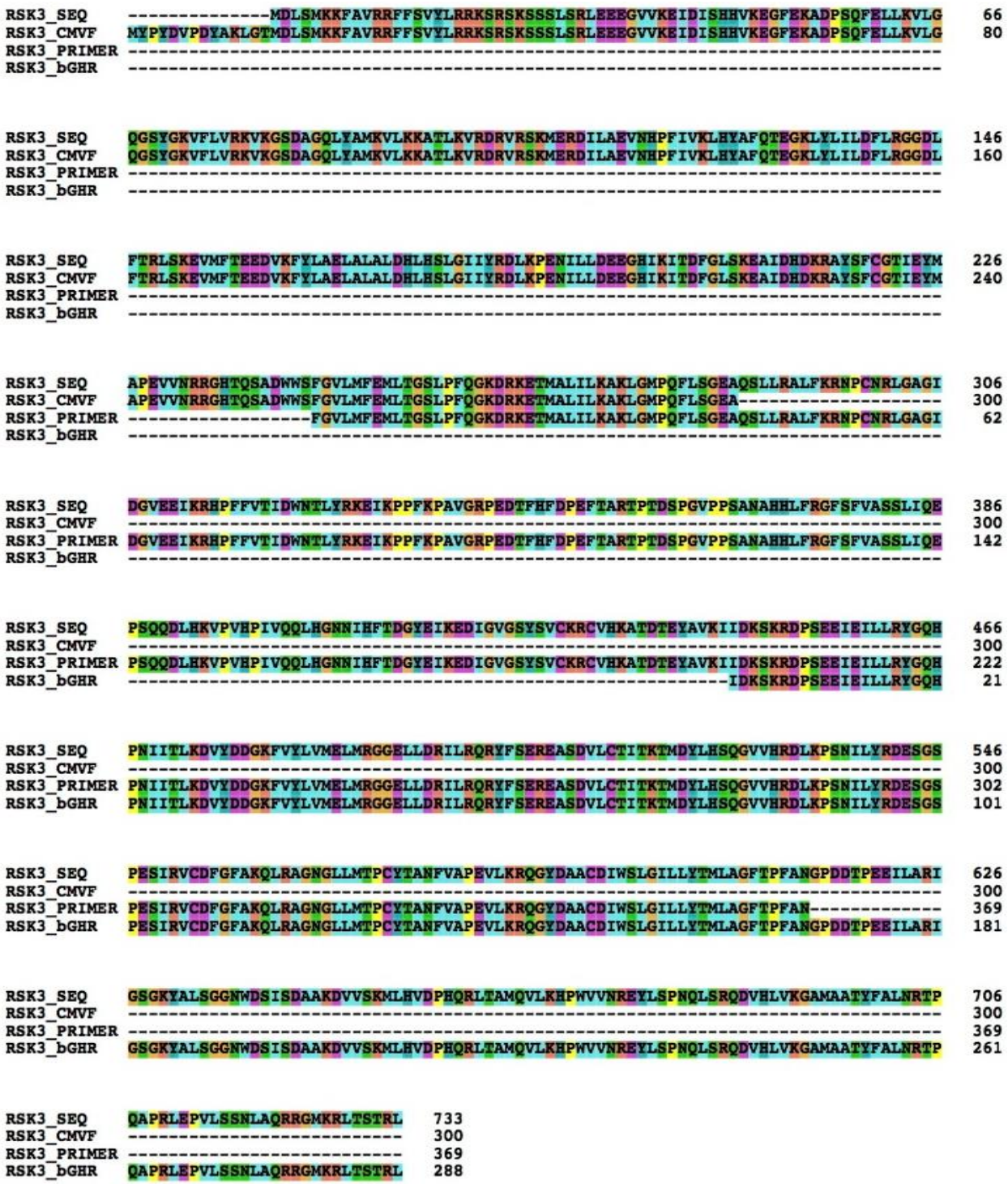

Figure 3.1.3 Multiple sequence alignment of RSK3.

DNA samples were sent to Eurofins for sanger sequencing. Upon return sequences were manipulated to produce one file which shows the composition of DNA sequence. RSK_SEQ is the RSK DNA sequence obtained from uniprot, RSK_CMVF is a company primer used to sequence the beginning of the gene, RSK_PRIMER is a manually created and synthesised primer used to sequence the middle of the gene and RSK_bGHR is a company primer used to sequence the end of the gene. Combined use of these primers led to a complete sequence for both RSK3 with no mutations.

3.2 Site directed mutagenesis of the RSKs to introduce a phospho-mimetic mutation

The RSKs are the downstream effectors of the MAPK pathway, which is physiologically activated by growth factors. However, this may not be sufficient to fully activate the overexpressed RSKs. As such, site directed mutagenesis was used to create a phospho-mimetic mutation in the RSKs activation loop of the N-terminal domain. The purpose of these mutations was to create constitutively active RSK mutants for use in luciferase reporter assays. Comparisons between wildtype and the phospho-mimetic has been used to infer if the endogenous MAPK pathway of the COS-1 cell were activating all the overexpressed RSKs. The position of the N-terminal phosphorylation changes between the isoforms due to varied isoform sequence and length (Figure 1.1.1). In all isoforms the wildtype Serine (S) in the position of the NTKD phosphorylation was mutated into a Glutamic acid (E) all of which used the same DNA triplet codon GAA. Glutamic acid was chosen for the mutation as it structurally mimics that of a phosphate group. To achieve this, pairs of primers were created for each isoform using Agilent site directed mutagenesis primer tool.

The mutagenesis reactions were transformed into bacteria and single colonies were picked, transformed, purified and sequence verified. The sequence was analysed to check if the mutation was present and if there were any additional undesired mutations. There is a single amino acid mutation of S to E in RSK2, RSK3 and RSK4 at their respective positions 227, 218 and 232 (Figure 3.2.1). Unfortunately, RSK1 was never successfully mutated without additional undesired mutations.

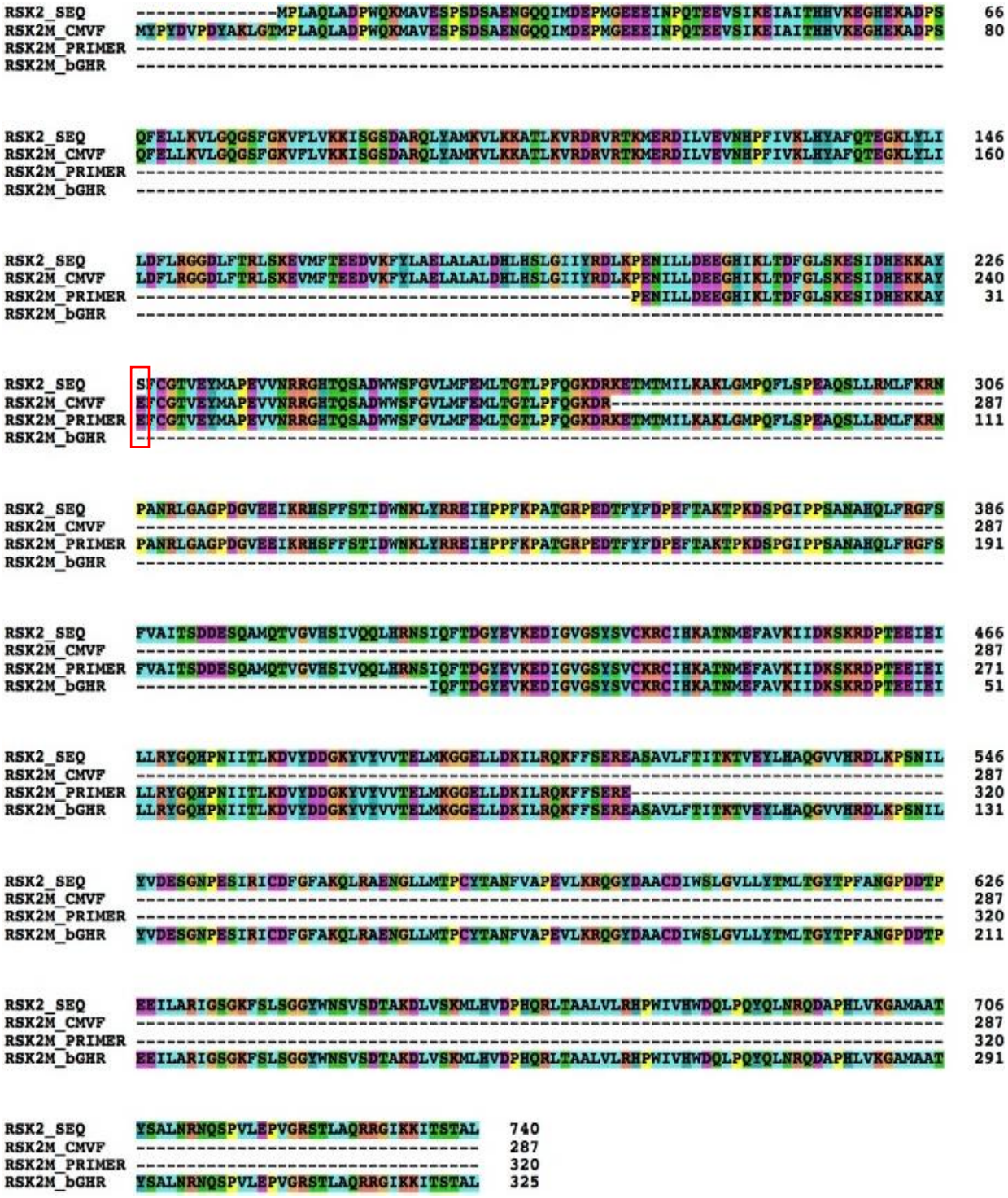

Figure 3.2.1 Multiple sequence alignments of RSK2 mutant 2M.

Site directed mutagenesis was performed on RSK2 in a thermocycler. The mutated DNA was amplified, purified and sent to Eurofins for sanger sequencing. Upon return sequences were analysed and manipulated. RSK_SEQ is the RSK DNA sequence obtained from uniprot, RSK_CMVF is a company primer used to sequence the beginning of the gene, RSK_PRIMER is a manually created and synthesised primer used to sequence the middle of the gene and RSK_bGHR is a company primer used to sequence the end of the gene. Combined use of these primers led to a complete sequence for 2M with the desired mutations and no additional mutations. Red box highlights the mutation.

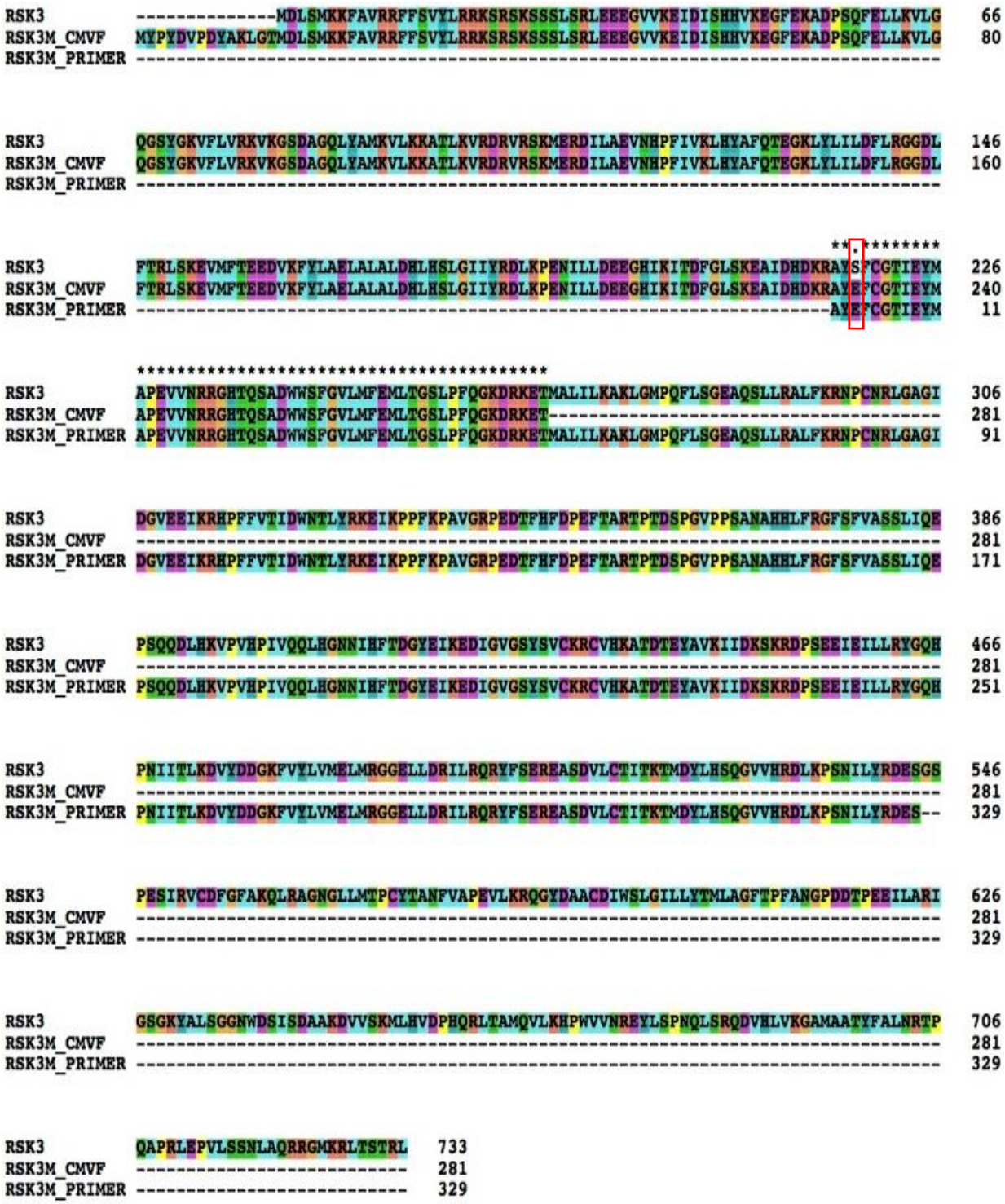

Figure 3.2.2 Multiple sequence alignments of RSK3 mutant 3M.

Site directed mutagenesis was performed on RSK3 in a thermocycler. The mutated DNA was amplified, purified and sent to Eurofins for sanger sequencing. Upon return sequences were analysed and manipulated. RSK_SEQ is the RSK DNA sequence obtained from uniprot, RSK_CMVF is a company primer used to sequence the beginning of the gene, RSK_PRIMER is a manually created and synthesised primer used to sequence the middle of the gene and RSK_bGHR is a company primer used to sequence the end of the gene. Combined use of these primers led to a complete sequence for 3M with the desired mutations and no additional mutations. Red box highlights the mutation.

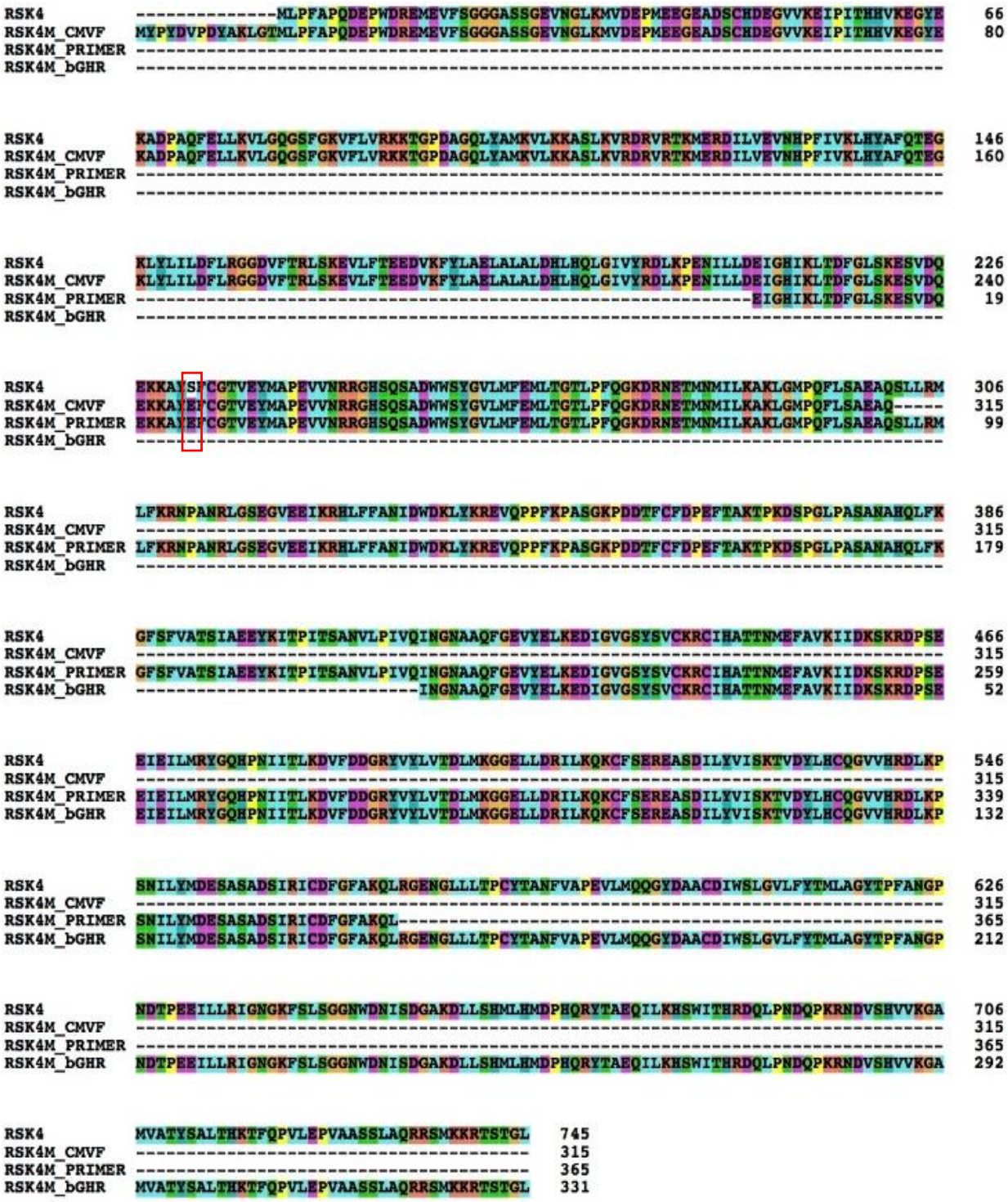

Figure 3.2.3 Multiple sequence alignments of RSK4 mutant 4M.

Site directed mutagenesis was performed on RSK4 in a thermocycler. The mutated DNA was amplified, purified and sent to Eurofins for sanger sequencing. Upon return sequences were analysed and manipulated. RSK_SEQ is the RSK DNA sequence obtained from uniprot, RSK_CMVF is a company primer used to sequence the beginning of the gene, RSK_PRIMER is a manually created and synthesised primer used to sequence the middle of the gene and RSK_bGHR is a company primer used to sequence the end of the gene. Combined use of these primers led to a complete sequence for 4M with the desired mutations and no additional mutations. Red box highlights the mutation.

3.3 Luciferase reporter assays determining interactions between RSKs and SRs

The relationship between SRs and RSKs has been characterised in a few combinations for example RSK2 was shown to phosphorylate ERα at Ser167 which enhances its transcriptional activity (Clark *et al.* 2001). To create a comprehensive understanding of all relationships between SR and RSKs luciferase reporter assays were performed. This technique was carried out in COS-1 cells because they are negative for all steroid receptors. The vectors pCDNA 3.1 (+) and PSG5 were both chosen because they are mammalian expression vectors thus are applicable for use with the COS-1 cells.

3.3.1 Hormone optimisation experiments of steroid receptors

Prior to directly investigating the effects of RSKs and SRs, the optimal concentration of each hormone was identified. The optimal hormone concentration was set to allow for a sub-maximal level of receptor activity which would allow for both an increase or decrease in activity when investigating the effects of RSKs on said receptors. COS-1 cells were transfected with plasmids encoding AR or GR or ERα or PR and their corresponding responsive luciferase reporters ERE-LUC in the case of ER or TAT-GRE-EIB-LUC-1(TAT-LUC) in the case of AR, GR, PR. COS-1 cells were also transfected with a control plasmid encoding the gene β-galactosidase PDM-LAC-Z-β-GAL (β-GAL). The cells were incubated for 20-24 hours post transfection and treated with either EtOH or a dose range (0-1000nM) of the receptors cognate hormone: mibolerone (AR), dexamethasone (GR), oestradiol (ERα) or progesterone (PR). Receptor activity was measured use luciferase assays and the data normalised to β-galactosidase expression.

The results show a consistent trend across all the SR hormone optimisation experiments (Figure 3.3.1.1 A-D). As hormone concentration increases, SR activity also increases. This effect plateaus at the highest concentrations of the hormones. All data points are statistically significant when compared to activity of SR without hormone (Figure 3.3.1.1 A-D). From this data, it was decided that 1nM MIB, 10nM DEX, 10nM E2 and 1nM PROG would be used for future experiments. To confirm successful expression of the steroid receptors, western blotting was performed (Figure 3E). AR, ERα and GR expression was confirmed, however the PR antibody did not appear to work (data not shown). The AR and GR expression increased as hormone concentration was increased. The expression of ERα was present at all concentrations, highest expression was seen in response to 0.1nM E2 and was similar at 1, 10 and 100nM E2. Minimal expression was seen in absence of hormone and at the highest concentration of E2 (Figure 3.3.1.1 E).

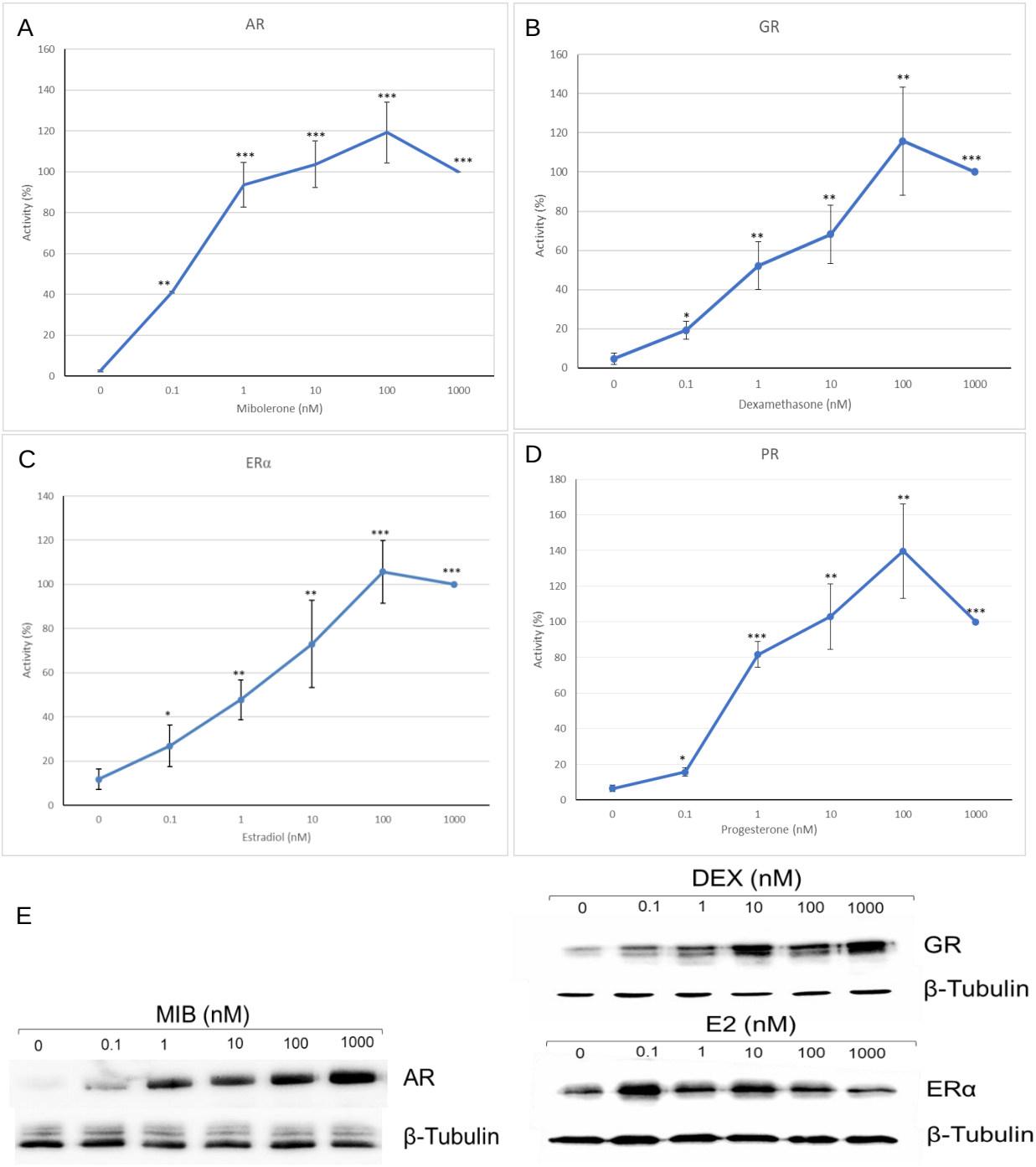

Figure 3.3.1.1 Steroid Receptor activity and expression in response to hormone dose range

COS-1 cells were transfected with expression vectors for **(A)** AR and TAT-LUC or **(B)** GR and TAT-LUC or **(C)** ERα and ERE-LUC or **(D)** PR and TAT-LUC. COS-1 cells were also transfected with a β-GAL expression plasmid. Cells were treated with either EtOH or a dose range (0.1-1000nM) of **(A)** mibolerone, **(B)** dexamethasone, **(C)** 17-β-oestradiol or **(D)** progesterone for 20-24 hours. SR activity was measured using luciferase and normalised to β-GAL activity. Mean of 4 independent duplicates ±SE. T-tests were used to compare receptor activity at each hormone concentration to receptor activity in the absence of hormone. *P<0.05, **P<0.005, ***P<0.0005. **(E)** Western blotting was conducted to assess AR, ERα and GR expression with β-tubulin included as a loading control.

3.3.2 RSK DNA optimisation experiments

Following the hormone optimisation experiments, DNA optimisation experiments were undertaken, the optimal concentration of RSK DNA was identified. The optimal DNA concentration was set to allow for a sub-maximal level of receptor activity which would allow for both an increase or decrease in activity when investigating the effects of PMA on RSK induced changes on steroid receptor activity.

3.3.2.1 RSK DNA optimisation experiment of the AR

COS-1 cells were transfected with plasmid DNA encoding the AR, its corresponding responsive luciferase reporter gene TAT-LUC and control plasmid β-GAL. The cells were also transfected with different concentrations of RSK DNA plasmid with empty plasmid used to ensure that equal concentrations of DNA were transfected. Cells were treated with either the control EtOH or 1nM MIB and incubated for 20-24 hours. Receptor activity was measured using luciferase assays and normalised to β-GAL expression.

In the presence of androgen, 200ng RSK1 significantly increased AR activity to approximately 450%. RSK2 caused an increase in AR activity at the 100ng concentration. RSK3 caused no significant change in AR activity. In contrast to RSK1/2, RSK4 reduced AR activity by 20% at the 100ng concentration (Figure 3.3.2.1.1). It was therefore decided that a concentration of 100ng of the RSK plasmids would be used for future experiments.

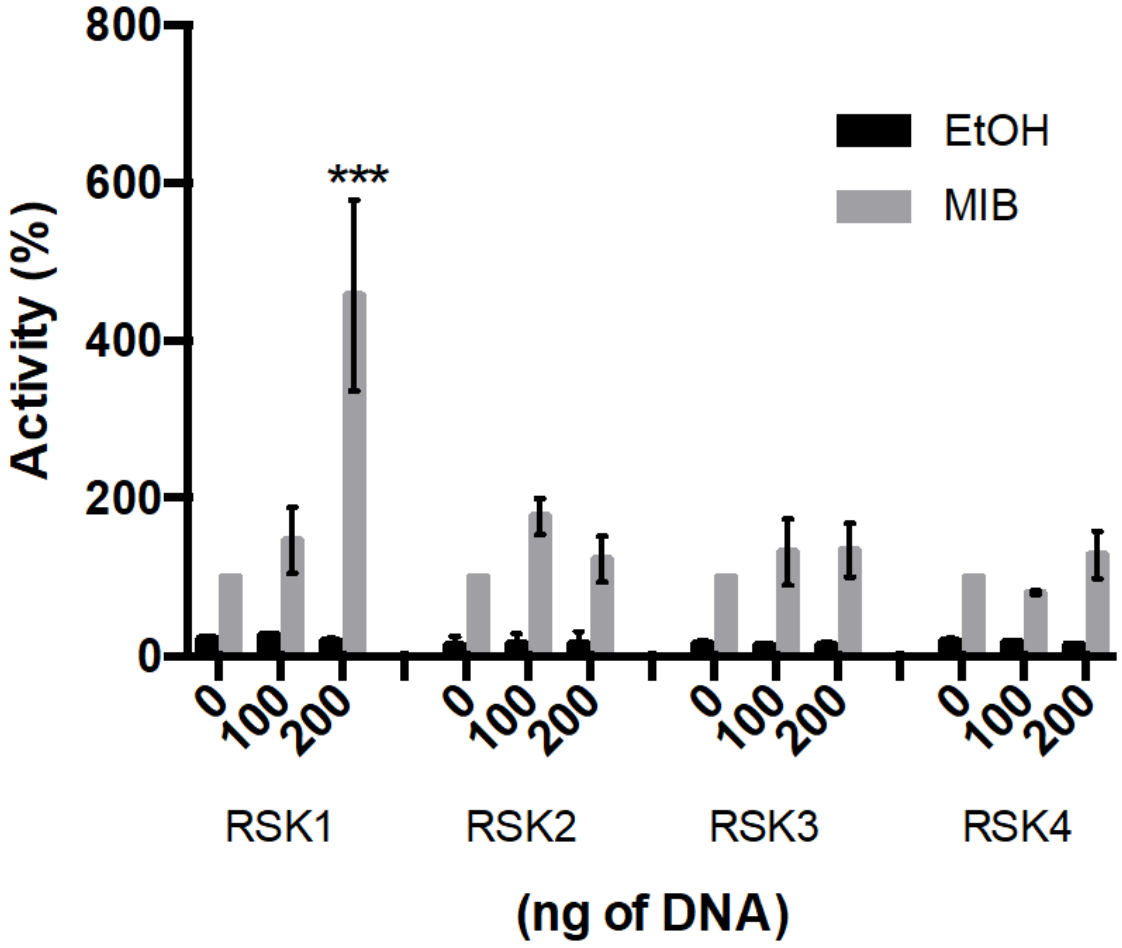

Figure 3.3.2.1.1 Androgen Receptor activity in response to varied RSK DNA concentrations

COS-1 cells were transfected with plasmids encoding for the AR, TAT-LUC, β-GAL and RSK1-4 and incubated for 20-24 hours. Cells were treated with EtOH or 1nM of Mibolerone for 20-24 hours. SR activity was measured using luciferase and normalised to β-GAL activity. Mean of 5 independent duplicates ±SE. Two-way ANNOVA test was used to compare receptor activity at 100ng and 200ng DNA concentrations to receptor activity at 0ng of RSK in the presence of hormone. ***P<0.0005.

3.3.2.2 RSK DNA optimisation experiment of the GR

COS-1 cells were transfected with plasmid DNA encoding the GR, its corresponding responsive luciferase reporter gene TAT-LUC and control plasmid β-GAL. The cells were also transfected with different concentrations of RSK DNA plasmid with empty plasmid used to ensure that equal concentrations of DNA were transfected. Cells were treated with either EtOH or 10nM DEX and incubated for 20-24 hours. Receptor activity was measured using luciferase assays and normalised to β-GAL expression.

In the presence of dexamethasone, RSK1 caused a significant increase in GR activity at both DNA concentrations. RSK2/3 caused modest increases in GR activity, more noticeably seen with RSK3 at the 100ng concentration. RSK4 also caused a significant increase in GR activity at the 200ng DNA concentration to approximately 195% (Figure 3.3.2.2.1). It was therefore decided that a concentration of 100ng of the RSK plasmids would be used for future experiments.

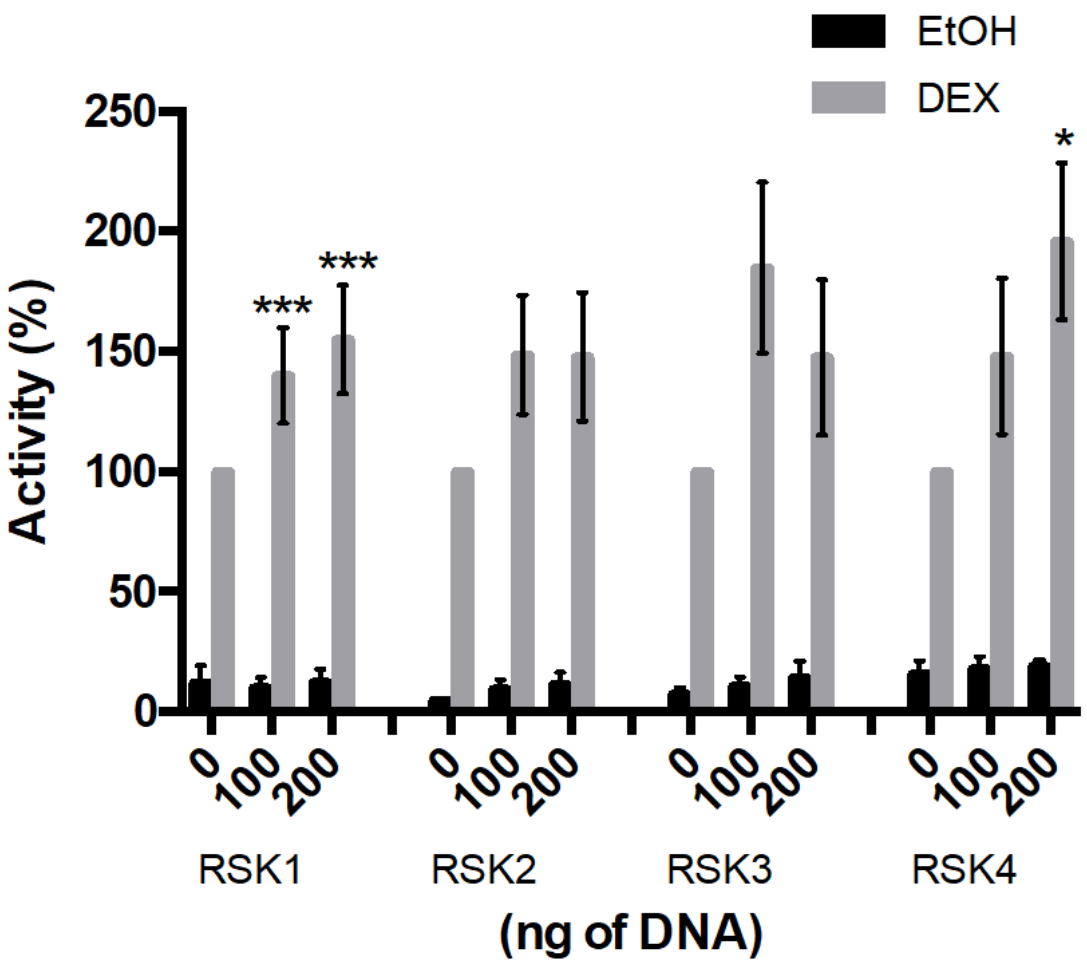

Figure 3.3.2.2.1 Glucocorticoid Receptor activity in response to varied RSK DNA concentrations

COS-1 cells were transfected with plasmids encoding for the GR, TAT-LUC, β-GAL and RSK1-4 and incubated for 20-24 hours. Cells were treated with EtOH or 1nM of Dexamethasone for 20-24 hours. SR activity was measured using luciferase and normalised to β-GAL activity. Mean of 4 independent duplicates ±SE. Two-way ANNOVA test was used to compare receptor activity at 100ng and 200ng DNA concentrations to receptor activity at 0ng of RSK in the presence of hormone. *P<0.05, ***P<0.0005.

3.3.2.3 RSK DNA optimisation experiment of the ERα

COS-1 cells were transfected with plasmid DNA encoding the ERα, its corresponding responsive luciferase reporter gene ERE-LUC and control plasmid β-GAL. The cells were also transfected with different concentrations of RSK DNA plasmid with empty plasmid used to ensure that equal concentrations of DNA were transfected. Cells were treated with either EtOH or 10nM E2 and incubated for 20-24 hours. Receptor activity was measured using luciferase assays and normalised to β-GAL expression.

In the presence of 17β-estradiol, RSK4 caused a significant decrease in ERα activity at the 200ng DNA concentrations, to approximately to 25% whilst additional modest decreases were caused by RSK1/3 (Figure 3.3.2.3.1). RSK2 caused no significant change in ERα activity at both concentrations. Despite the 200ng concentrations causing a more profound change in activity to allow for additional changes in later experiments the 100ng concentration was chosen.

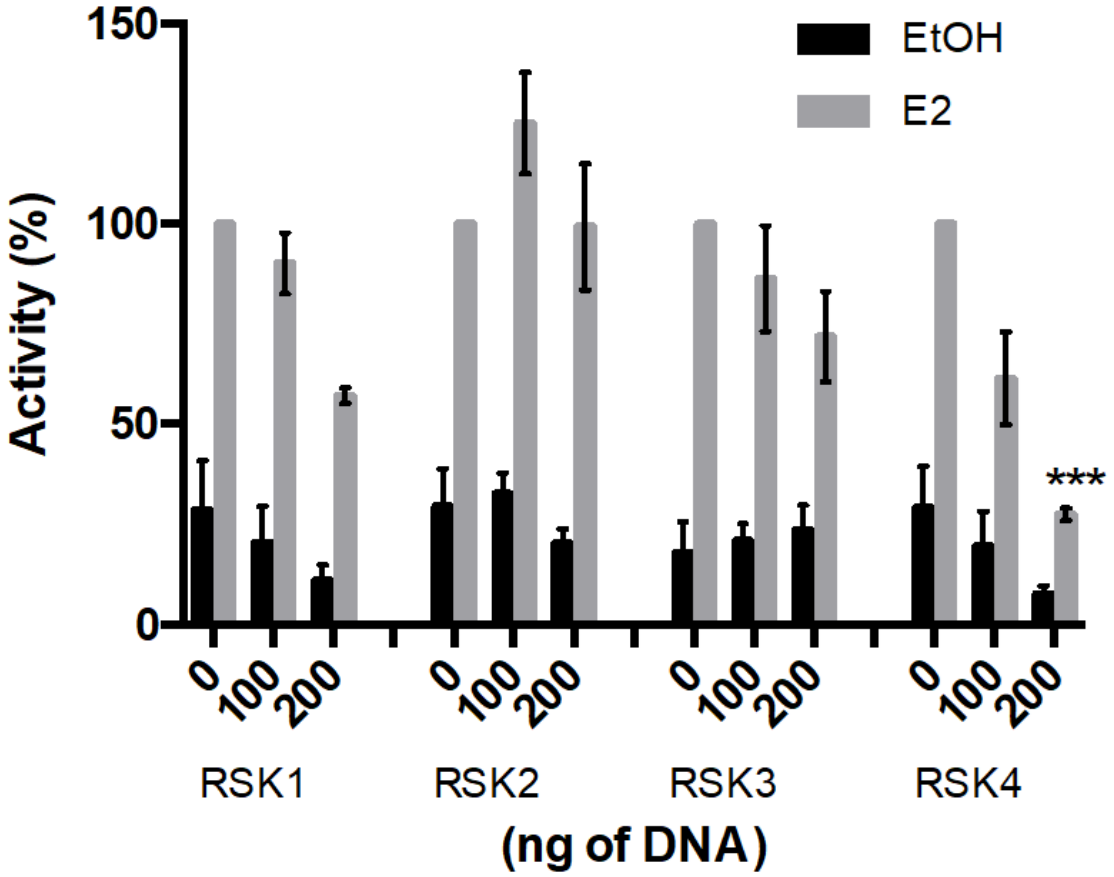

Figure 3.3.2.3.1 Estrogen Receptor alpha activity in response to varied RSK DNA concentrations

COS-1 cells were transfected with plasmids encoding for the ERα, ERE-LUC, β-GAL and RSK1-4 and incubated for 20-24 hours. Cells were treated with EtOH or 10nM of 17β-estradiol for 20-24 hours. SR activity was measured using luciferase and normalised to β-GAL activity. Mean of 3 independent duplicates ±SE. Two-way ANNOVA test was used to compare receptor activity at 100ng and 200ng DNA concentrations to receptor activity at 0ng of RSK in the presence of hormone. ***P<0.0005.

3.3.2.4 RSK DNA optimisation experiment of the PR

COS-1 cells were transfected with plasmid DNA encoding the PR, its corresponding responsive luciferase reporter gene TAT-LUC and control plasmid β-GAL. The cells were also transfected with different concentrations of RSK DNA plasmid with empty plasmid used to ensure that equal concentrations of DNA were transfected. Cells were treated with either EtOH or 1nM PROG and incubated for 20-24 hours. Receptor activity was measured using luciferase assays and normalised to β-GAL expression.

In the presence of progesterone, RSK1 caused no change to PR activity. RSK2 caused a modest increase in PR activity at the 200ng concentration whilst RSK3 caused a significant increase in PR activity at the 200ng concentration. RSK4 caused a slight decrease in PR activity at the 200ng concentration (Figure 3.3.2.4.1). To remain consistent with other receptors it was decided that a concentration of 100ng of the RSK plasmids would be used for future experiments.

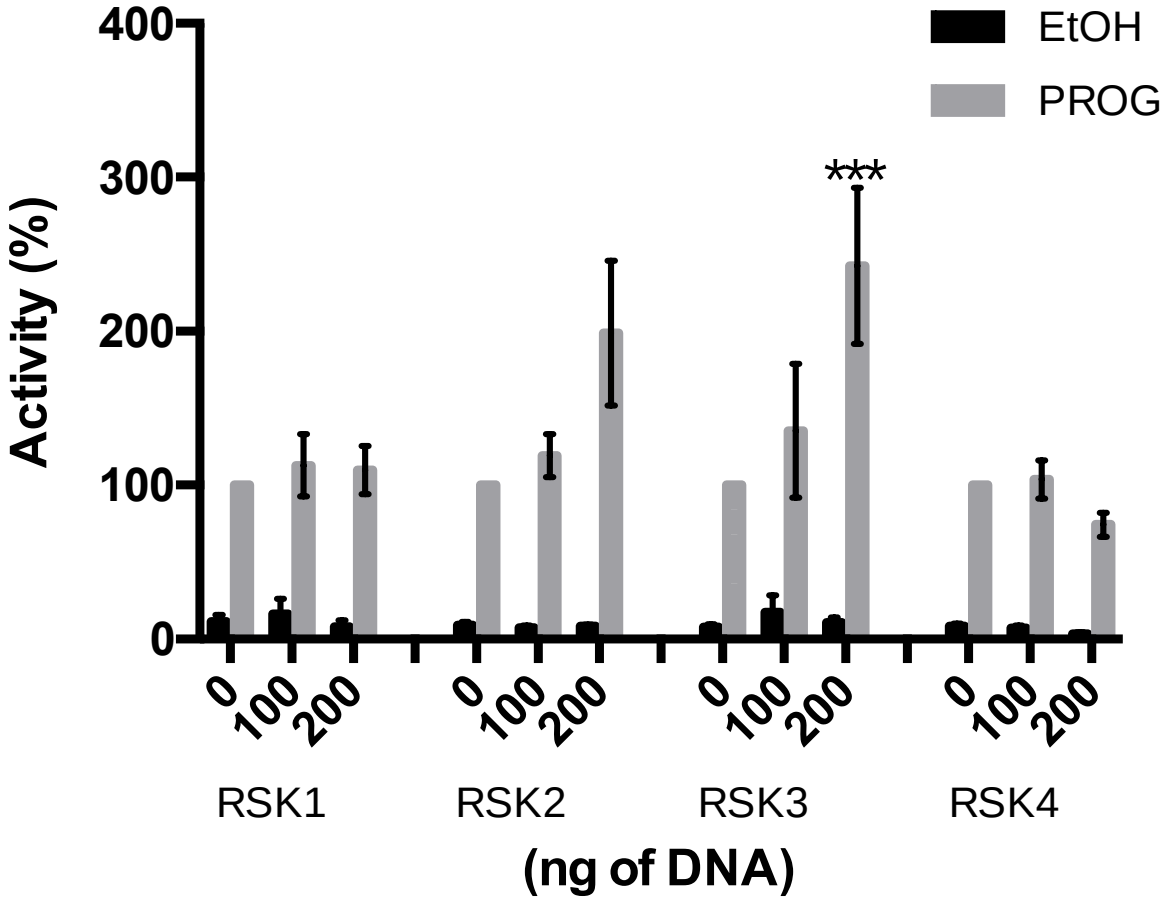

Figure 3.3.2.4.1. Progesterone Receptor activity in response to varied RSK DNA concentrations

COS-1 cells were transfected with plasmids encoding for the PR, TAT-LUC, β-GAL and RSK1-4 and incubated for 20-24 hours. Cells were treated with EtOH or 10nM of progesterone for 20-24 hours. SR activity was measured using luciferase and normalised to β-GAL activity. Mean of 5 independent duplicates ±SE. Two-way ANNOVA test was used to compare receptor activity at 100ng and 200ng DNA concentrations to receptor activity at 0ng of RSK in the presence of hormone. ***P<0.0005.

3.3.3 PMA induction experiments

Following the DNA optimisation experiments, experiments directly investigating the effects of RSKs on SR activity were undertaken. The RSKs are downstream effectors of the MAPK pathway. To promote maximal activation of the overexpressed RSK, the MAPK activator Phorbol Miseryl Acetate (PMA) was used.

3.3.3.1 PMA induction experiment of AR

COS-1 cells were transfected with plasmid DNA encoding the AR, its corresponding responsive luciferase reporter gene TAT-LUC and control plasmid β-GAL. The cells were also transfected with 100ng of RSK DNA plasmid. Cells were treated with either EtOH or 1nM MIB, with either 1 µl of PMA or untreated and incubated for 20-24 hours. Receptor activity was measured using luciferase assays and normalised to β-GAL expression.

RSK1/3/4 caused a significant increase in AR activity with RSK1/4 causing the most effect increasing AR activity by approximately 6-fold. Whilst RSK1/2/4 caused a significant increase in AR activity in the presence of PMA. However, PMA only significantly enhanced RSK1 activation of AR whilst modestly enhancing RSK2/4 activation of AR activity and slightly reducing RSK3 activation of AR (Figure 3.3.3.1.1)

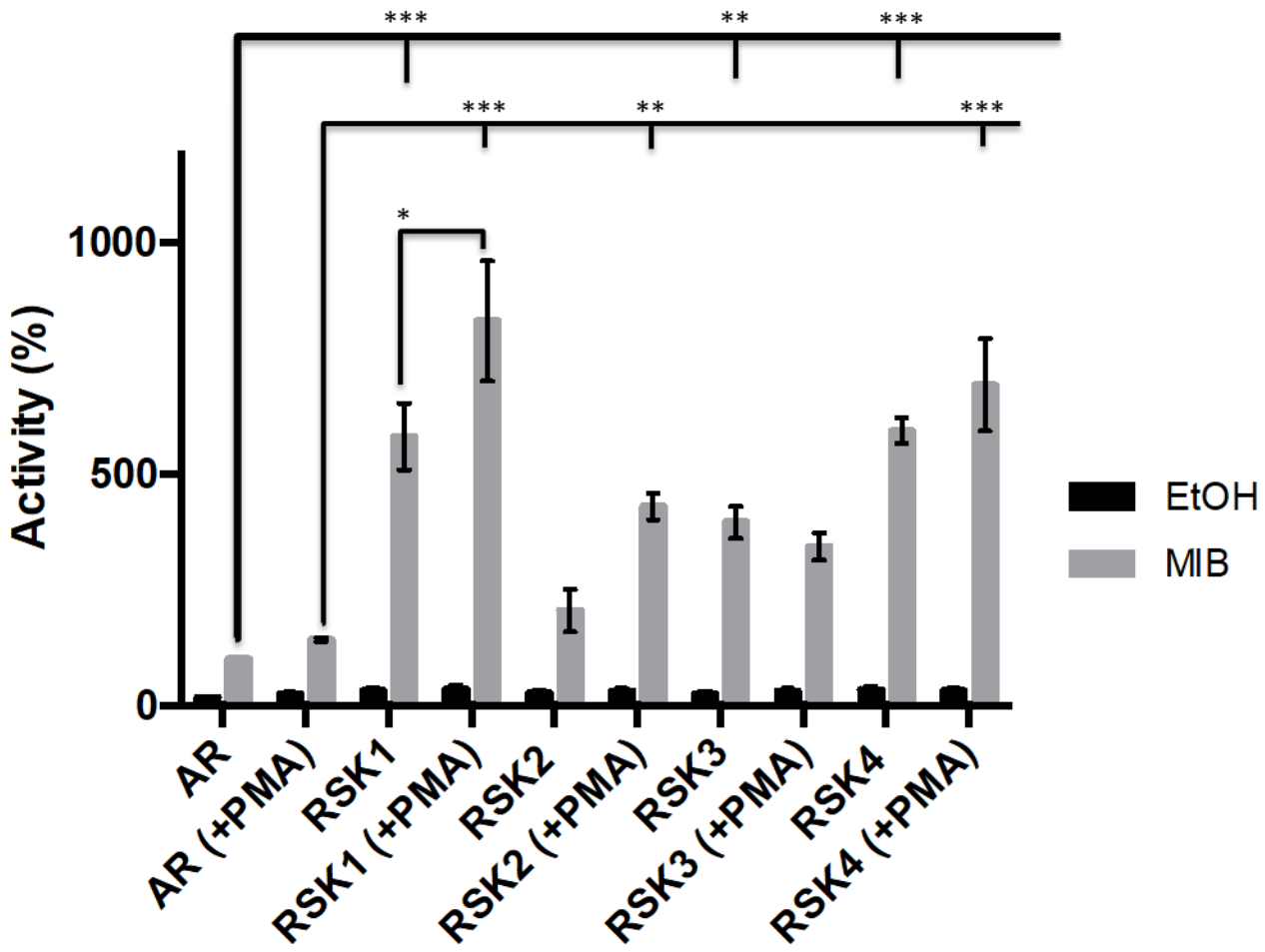

Figure 3.3.3.1.1 Androgen Receptor activity in response to PMA induced RSKs

COS-1 cells were transfected with plasmids encoding AR, TAT-LUC, β-GAL, RSK1-4 and incubated for 20-24 hours. Cells were treated with either EtOH or 1nM of Mibolerone and ± 1 µl of [PMA]. SR activity was measured using luciferase assays and data normalised to β-GAL expression. Mean of 5 independent duplicates ±SE. Two-way ANNOVA test was used to compare receptor activity to receptor activity and RSK in the presence of hormone, to compare receptor activity to receptor activity and RSK in presence of hormone and PMA and to compare differences between SR and RSK in the absence/presence of PMA. *P<0.05, **P<0.005, ***P<0.0005.

3.3.3.2 PMA induction experiment of GR

COS-1 cells were transfected with plasmid DNA encoding the GR, its corresponding responsive luciferase reporter gene TAT-LUC and control plasmid β-GAL. The cells were also transfected with 100ng of RSK DNA plasmid. Cells were treated with either EtOH or 10nM DEX, with either 1 µl of PMA or untreated and incubated for 20-24 hours. Receptor activity was measured using luciferase assays and normalised to β-GAL expression.

RSK2/3 caused a significant increase in GR activity in the absence and presence of PMA, whilst RSK1/4 caused a reasonable increase. The addition of PMA modestly enhanced RSK2 activation of GR increasing its activity approximately 0.5-fold whilst causing subtle changes to RSK1/3/4 (Figure 3.3.3.2.1).

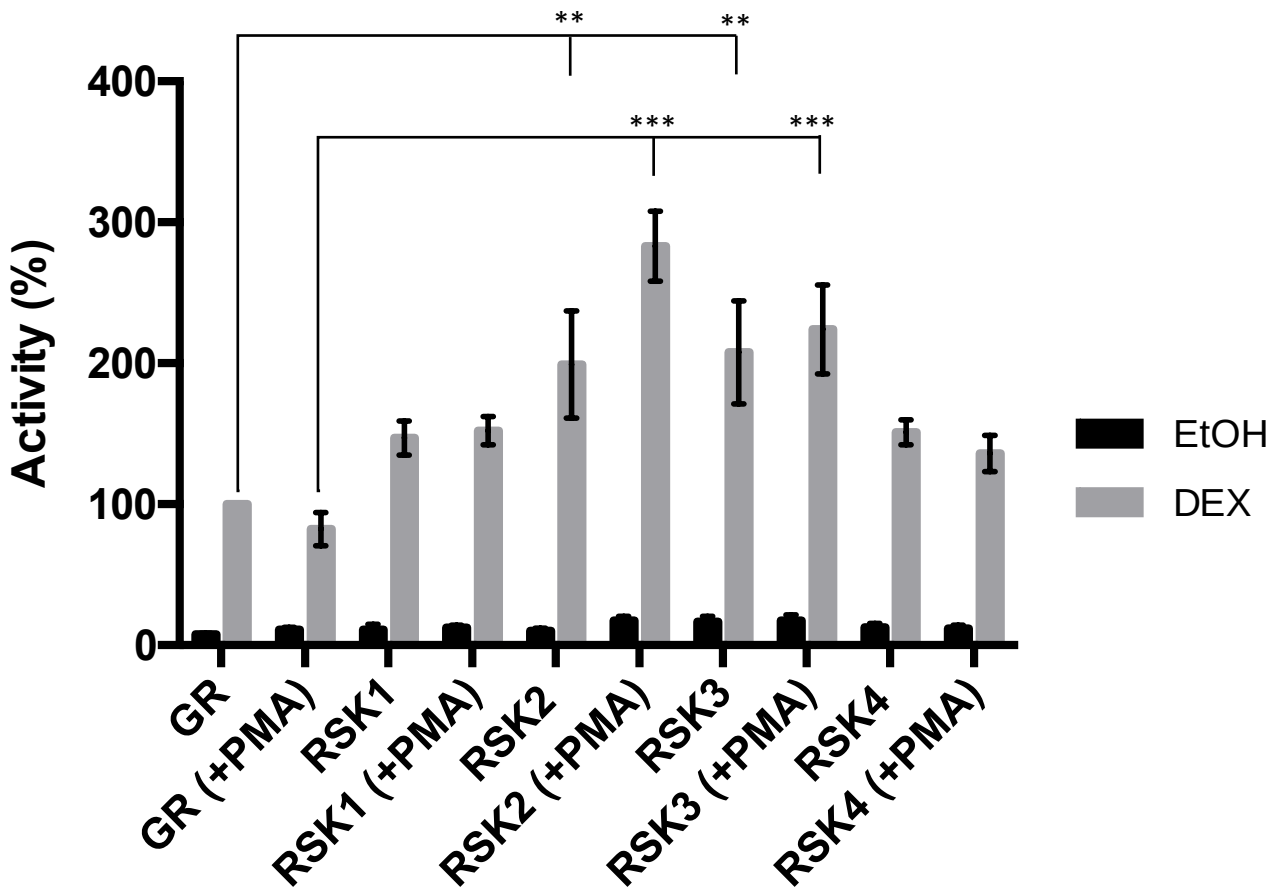

Figure 3.3.3.2.1 Glucocorticoid Receptor activity in response to PMA induced RSKs

COS-1 cells were transfected with plasmids encoding GR, TAT-LUC, β-GAL, RSK1-4 and incubated for 20-24 hours. Cells were treated with either EtOH or 10nM of Dexamethasone and ± 1 µl of [PMA]. SR activity was measured using luciferase assays and data normalised to β-GAL expression. Mean of 5 independent duplicates ±SE. Two-way ANNOVA test was used to compare receptor activity to receptor activity and RSK in the presence of hormone, to compare receptor activity to receptor activity and RSK in presence of hormone and PMA and to compare differences between SR and RSK in the absence/presence of PMA. **P<0.005, ***P<0.0005.

3.3.3.3 PMA induction experiment of ERα

COS-1 cells were transfected with plasmid DNA encoding the ERα, its corresponding responsive luciferase reporter gene ERE-LUC and control plasmid β-GAL. The cells were also transfected with 100ng of RSK DNA plasmid. Cells were treated with either EtOH or 10nM E2, with either 1 µl of PMA or untreated and incubated for 20-24 hours. Receptor activity was measured using luciferase assays and normalised to β-GAL expression.

RSK4 causes a significant decrease in ERα activity in both the absence and presence of PMA whilst RSK1 causes a minor decrease in ERα activity in the absence of PMA. The addition of PMA causes no significant change in ERα activity but does cause a moderate increase in activity for RSK1/4 returning ERα activity towards basal activity (Figure 3.3.3.3.1).

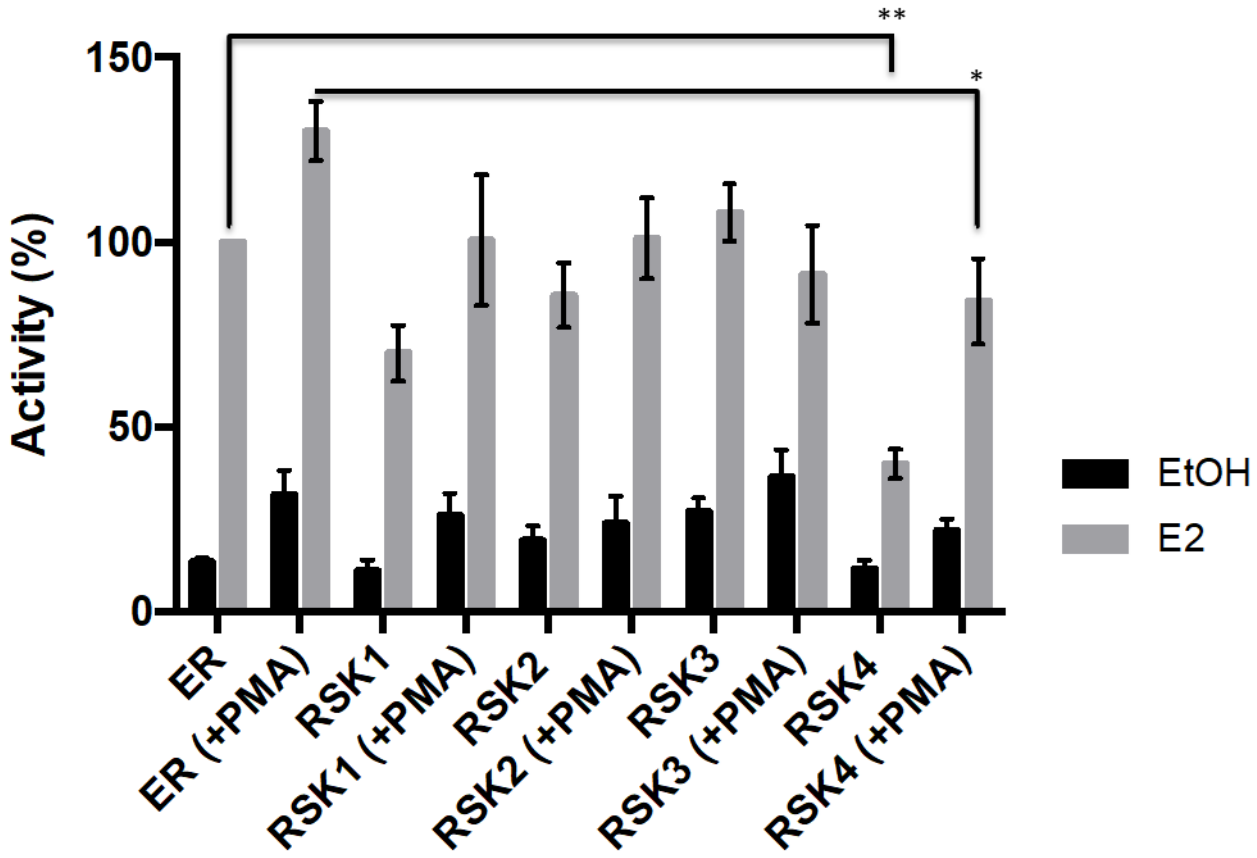

Figure 3.3.3.3.1 Estrogen Receptor α activity in response to PMA induced RSKs

COS-1 cells were transfected with plasmids encoding ER, ERE-LUC, β-GAL, RSK1-4 and incubated for 20-24 hours. Cells were treated with either EtOH or 10nM of 17β-estradiol and ± 1 µl of [PMA]. SR activity was measured using luciferase assays and data normalised to β-GAL expression. Mean of 7 independent duplicates ±SE. Two-way ANNOVA test was used to compare receptor activity to receptor activity and RSK in the presence of hormone, to compare receptor activity to receptor activity and RSK in presence of hormone and PMA and to compare differences between SR and RSK in the absence/presence of PMA. *P<0.05, **P<0.005.

3.3.3.4 PMA induction experiment of PR

COS-1 cells were transfected with plasmid DNA encoding the PR, its corresponding responsive luciferase reporter gene TAT-LUC and control plasmid β-GAL. The cells were also transfected with 100ng of RSK DNA plasmid. Cells were treated with either EtOH or 10nM E2, with either 1 µl of PMA or untreated and incubated for 20-24 hours. Receptor activity was measured using luciferase assays and normalised to β-GAL expression.

In the absence of PMA RSK3 causes a significant increase in PR whilst RSK2 causes a minor reduction in PR activity. The addition of PMA significantly enhances the reduction in RSK3 activation of PR returning activity towards basal levels, additionally RSK1/2/4 activation of PR activity is repressed marginally in the presence of PMA. (Figure 3.3.3.4.1).

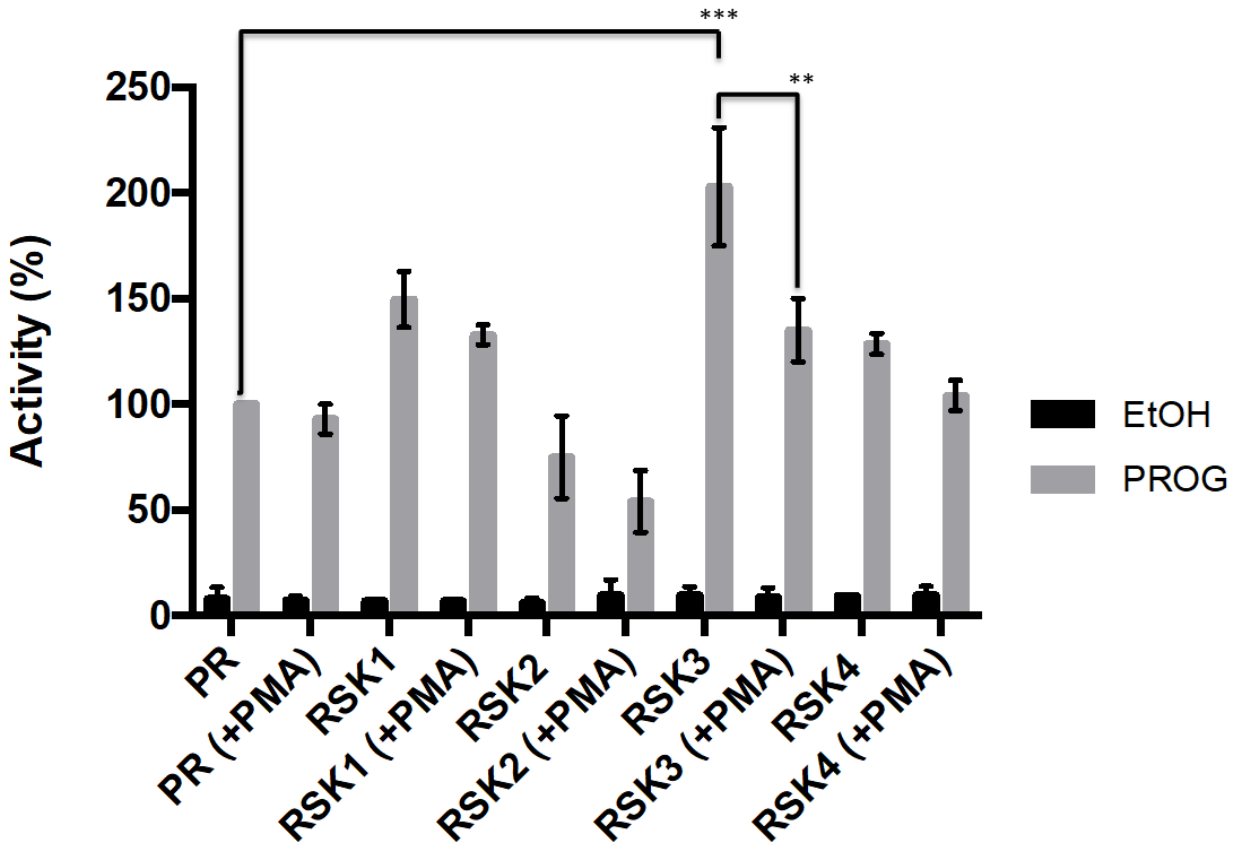

Figure 3.3.3.4.1 Progestrone Receptor activity in response to PMA induced RSKs

COS-1 cells were transfected with plasmids encoding PR, TAT-LUC, β-GAL, RSK1-4 and incubated for 20-24 hours. Cells were treated with either EtOH or 10nM of progesterone and ± 1 µl of [PMA]. SR activity was measured using luciferase assays and data normalised to β-GAL expression. Mean of 7 independent duplicates ±SE. Two-way ANNOVA test was used to compare receptor activity to receptor activity and RSK in the presence of hormone, to compare receptor activity to receptor activity and RSK in presence of hormone and PMA and to compare differences between SR and RSK in the absence/presence of PMA. **P<0.005, ***P<0.0005.

3.3.4 Phospho-mimetic experiments

Following the PMA induction experiments, experiments investigating the activation state of the endogenous MAPK pathway for each SR were undertaken. This was done by creating constitutively active phosho-mimetic mutants of the RSKs at their N-terminal phosphorylation site which were compared to wildtype to infer the activation state.

MIB and incubated for 20-24 hours. Receptor activity was measured using luciferase assays and normalised to β-GAL expression.

3.3.4.1 Phospho-mimetic experiment of AR

COS-1 cells were transfected with plasmid DNA encoding the AR, its corresponding responsive luciferase reporter gene TAT-LUC and control plasmid β-GAL. The cells were also transfected with 100ng of RSK DNA plasmid or 100ng of RSK-Mutant (RSKM) DNA. Cells were treated with either EtOH or 1nM MIB and incubated for 20-24 hours. Receptor activity was measured using luciferase assays and normalised to β-GAL expression (Figure 3.3.4.1.1).

Wildtype RSK4 causes a significant increase in AR activity by approximately 2-fold, whilst RSK2 causes a moderate increase AR activity. Similarly, 4M causes a significant increase in AR activity whilst 2M represses the activity AR. Wildtype RSK2 causes significantly higher activity on the AR than its mutant counterpart suggesting the endogenous MAPK pathway is sufficiently active (Figure 3.3.4.1.1).

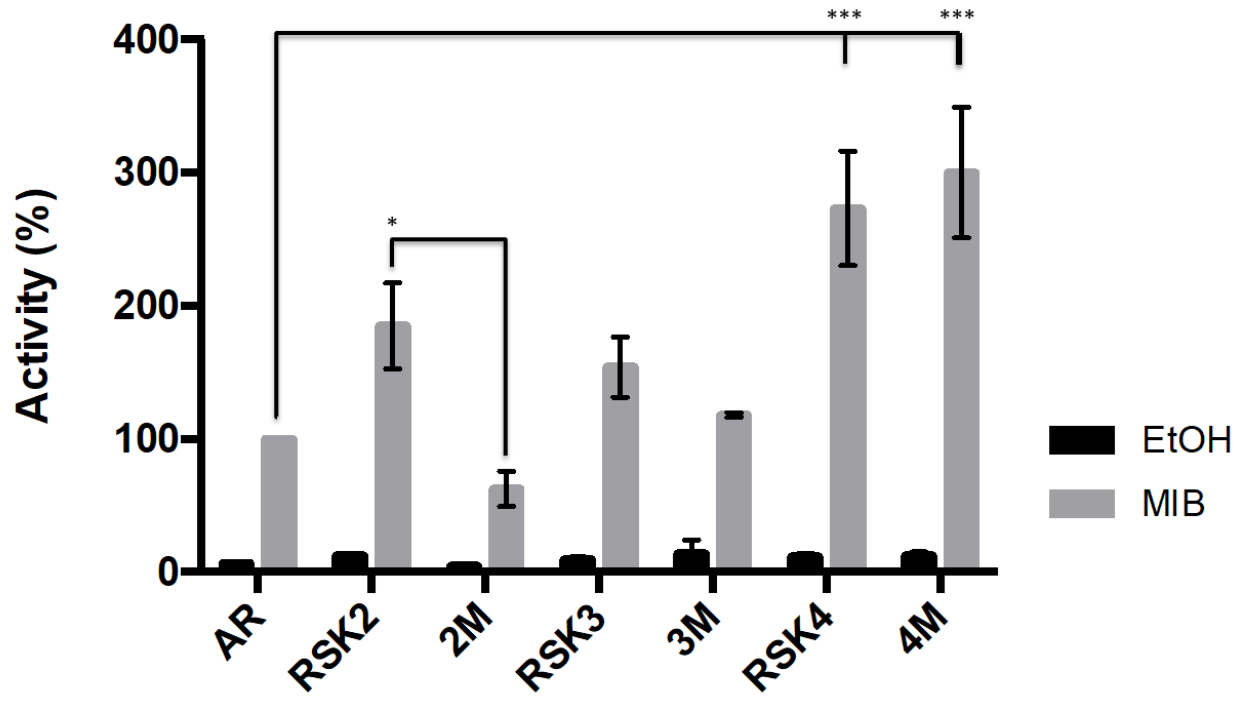

Figure 3.3.4.1.1 Androgen Receptor activity in response to RSKs and RSK mutants

COS-1 cells were transfected with plasmids encoding AR, TAT-LUC, β-GAL, either RSK2-4 or RSK2-4M and incubated for 20-24 hours. Cells were treated with either EtOH or 1nM of mibolerone. SR activity was measured using luciferase assays and data normalised to β-GAL expression. Mean of 4 independent duplicates ±SE. Two-way ANNOVA test was used to compare SR to SR with RSKs or RSK mutants in the presence of hormone and to compare the RSKs to their corresponding mutants in the presence of hormone. *P<0.05, ***P<0.0005.

3.3.4.2 Phospho-mimetic experiment of GR

COS-1 cells were transfected with plasmid DNA encoding the GR, its corresponding responsive luciferase reporter gene TAT-LUC and control plasmid β-GAL. The cells were also transfected with 100ng of RSK DNA plasmid or 100ng of RSK-Mutant (RSKM) DNA. Cells were treated with either EtOH or 10nM DEX and incubated for 20-24 hours. Receptor activity was measured using luciferase assays and normalised to β-GAL expression (Figure 3.3.4.2.1).

Wildtype RSK2/4 cause a significant increase in GR activity, RSK4 causing the most effect, increasing GR activity by approximately 2-fold. Additionally, 4M caused a significant increase in GR activity. Wildtype RSK2 induces a significantly higher activity on the AR than its mutant counterpart suggesting the endogenous MAPK pathway is active sufficiently (Figure 3.3.4.2.1).

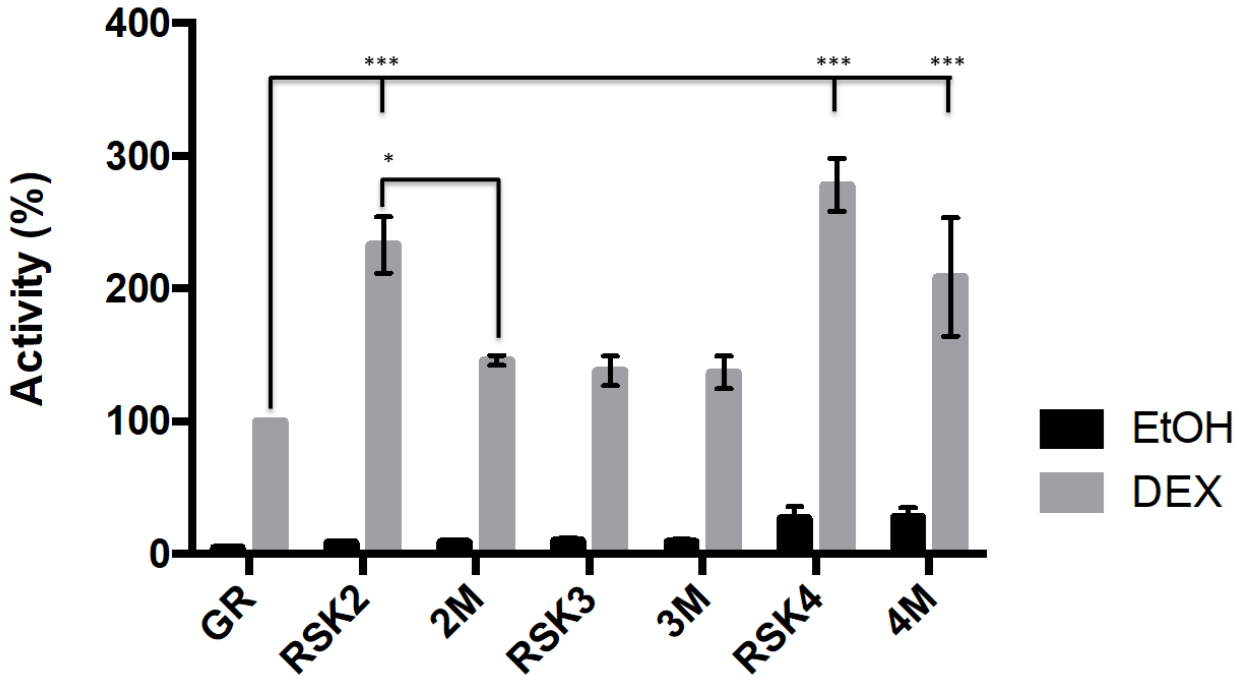

Figure 3.3.4.2.1. Glucocorticoid Receptor activity in response to RSKs and RSK mutants

COS-1 cells were transfected with plasmids encoding GR, TAT-LUC, β-GAL, either RSK2-4 or RSK2-4M and incubated for 20-24 hours. Cells were treated with either EtOH or 10nM of dexamethasone. SR activity was measured using luciferase assays and data normalised to β-GAL expression. Mean of 4 independent duplicates ±SE. Two-way ANNOVA test was used to compare SR to SR with RSKs or RSK mutants in the presence of hormone and to compare the RSKs to their corresponding mutants in the presence of hormone. *P<0.05, ***P<0.0005.

3.3.4.3 Phospho-mimetic experiment of ERα

COS-1 cells were transfected with plasmid DNA encoding the ERα, its corresponding responsive luciferase reporter gene ERE-LUC and control plasmid β-GAL. The cells were also transfected with 100ng of RSK DNA plasmid or 100ng of RSK-Mutant (RSKM) DNA. Cells were treated with either EtOH or 10nM E2 and incubated for 20-24 hours. Receptor activity was measured using luciferase assays and normalised to β-GAL expression (Figure 3.3.4.3.1).

All Wildtype RSKs caused no significant change in ERα activity but RSK4 causes a reasonable decrease in ERα activity by approximately 0.5-fold. All RSK mutants caused a significant decrease in ERα. However, there are no significant differences between the wildtype and mutant RSKs suggesting the endogenous pathway is sufficiently active (Figure 3.3.4.3.1).

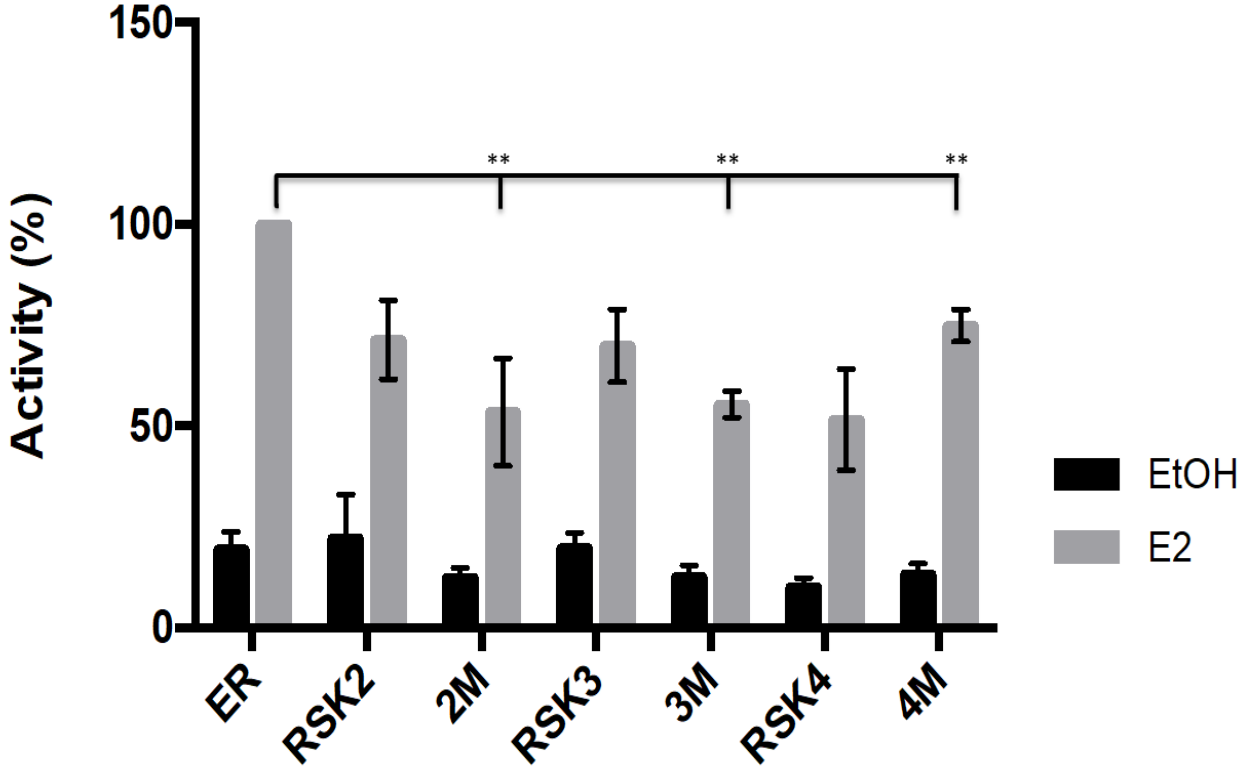

Figure 3.3.4.3.1 Estrogen Receptor alpha activity in response RSKs and RSK mutants

COS-1 cells were transfected with plasmids encoding ERα, ERE-LUC, β-GAL, either RSK2-4 or RSK2-4M and incubated for 20-24 hours. Cells were treated with either EtOH or 10nM of 17β-estradiol. SR activity was measured using luciferase assays and data normalised to β-GAL expression. Mean of 4 independent duplicates ±SE. Two-way ANNOVA test was used to compare SR to SR with RSKs or RSK mutants in the presence of hormone and to compare the RSKs to their corresponding mutants in the presence of hormone. **P<0.005.

3.3.4.4 Phospho-mimetic experiment of PR

COS-1 cells were transfected with plasmid DNA encoding the PR, its corresponding responsive luciferase reporter gene TAT-LUC and control plasmid β-GAL. The cells were also transfected with 100ng of RSK DNA plasmid or 100ng of RSK-Mutant (RSKM) DNA. Cells were treated with either EtOH or 1nM PROG and incubated for 20-24 hours. Receptor activity was measured using luciferase assays and normalised to β-GAL expression (Figure 3.3.4.4.1).

Wildtype RSK3/4 cause a significant increase in PR where as RSK2 causes a marginal decrease in PR activity. 2M/4M caused a significant decrease in PR and 3M causes a moderate decrease in PR activity. Wildtype RSK3/4 cause a significantly higher increase in PR activity than their mutant counterparts suggesting the endogenous pathway is sufficiently active (Figure 3.3.4.4.1).

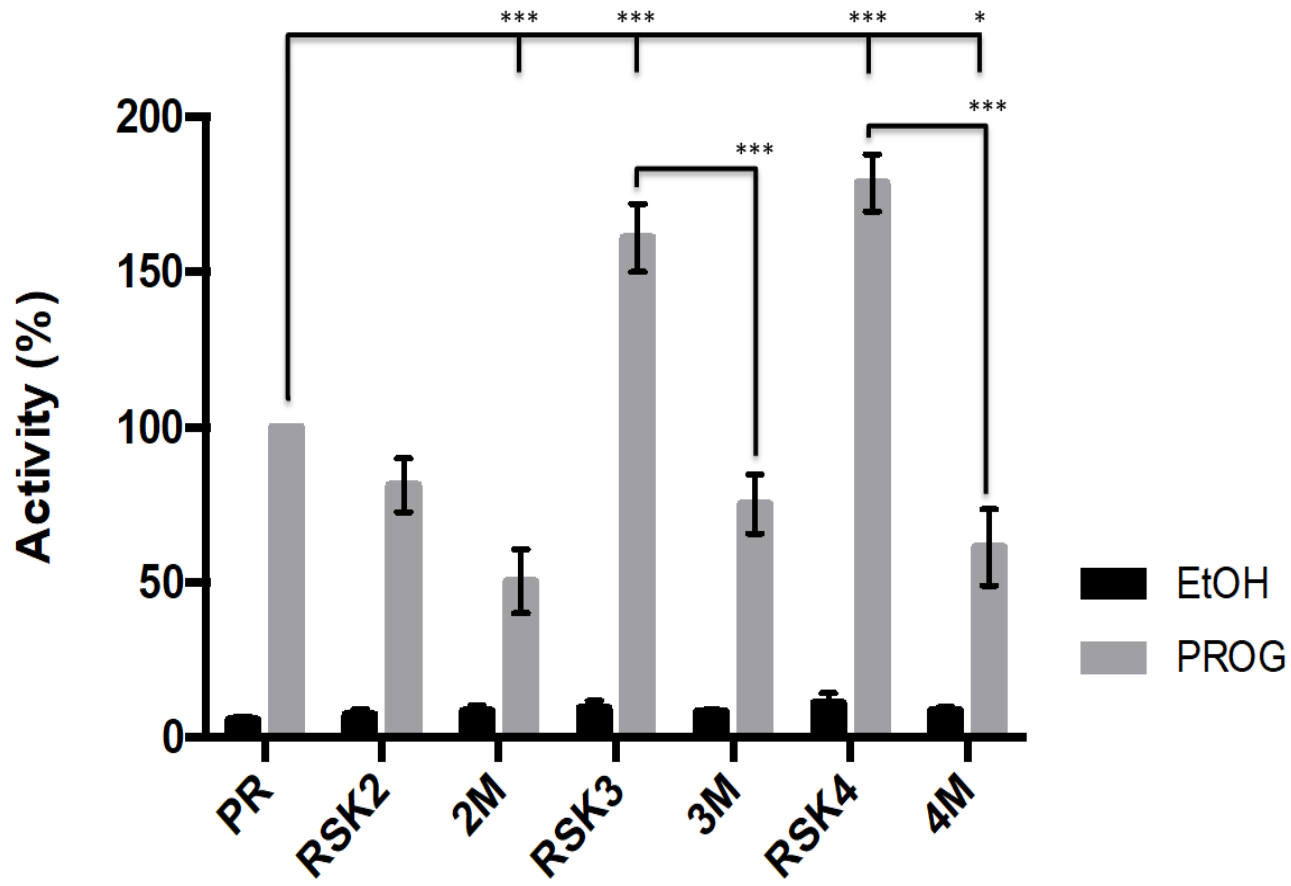

Figure 3.3.4.4.1 Progesterone Receptor activity in response to RSKs and RSK mutants

COS-1 cells were transfected with plasmids encoding PR, TAT-LUC, β-GAL, either RSK2-4 or RSK2-4M and incubated for 20-24 hours. Cells were treated with either EtOH or 1nM of progesterone. SR activity was measured using luciferase assays and data normalised to β-GAL expression. Mean of 4 independent duplicates ±SE. Two-way ANNOVA test was used to compare SR to SR with RSKs or RSK mutants in the presence of hormone and to compare the RSKs to their corresponding mutants in the presence of hormone. *P<0.05, ***P<0.0005.

3.3.5 Summary of Luciferase reporter assays

(Table 3.3.5.1) shows a summary of the RSK induced changes in SR activity and the significant differences between the pairs of data

Table 3.3.5.1 Summary of RSK effects on Steroid Receptor activity.
↑ represents an increase in SR activity 200≥, ↑↑ represents an increase in SR activity <500, ↑↑↑ represents an increase in SR activity >500. ↓ represents a decrease in SR activity ≥75, ↓↓ represents a decrease in SR activity ≥50, ↓↓↓ represents a decrease in SR activity <50. B.L means basal levels of activity.

AR	RSK1		RSK2		RSK3		RSK4	
DNA	↑↑		↑		↑		↓	
-PMA	↑↑↑	*	↑↑		↑↑		↑↑↑	
+PMA	↑↑↑		↑↑↑		↑↑		↑↑↑	
WT			↑	*	↑		↑↑	
M			↓↓		↑		↑↑	

GR	RSK1		RSK2		RSK3		RSK4	
DNA	↑		↑		↑		↑	
-PMA	↑		↑		↑↑		↑	
+PMA	↑		↑↑		↑↑		↑	
WT			↑↑	*	↑		↑↑	
M			↑		↑		↑↑	

ERα	RSK1		RSK2		RSK3		RSK4	
DNA	↓↓		↑		↓		↓↓↓	
-PMA	↓↓		↓		↑		↓↓↓	
+PMA	B.L		B.L		↓		↓	
WT			↓↓		↓↓		↓↓	
M			↓↓		↓↓		↓↓	

PR	RSK1		RSK2		RSK3		RSK4	
DNA	↑		↑		↑↑		↓	
-PMA	↑		↑		↑↑	*	↑	
+PMA	↑		↓↓		↑		↑	
WT			↓		↑	*	↑	*
M			↓↓		↓		↓↓	

There is a common condition across all three experiments, 100ng of RSK DNA with the set concentration of hormone relative to its receptor. It can be seen from this table and within the data, there are some discrepancies with the RSK effects induced on the same SR across the three experiments which could question the reproducibility. There are also examples which show adverse effects from the mutant RSK which could question the constitutive activation of the RSK mutants, especially when comparing to +PMA.

Chapter 4 - Discussion

The RSKs are a group of Serine/Threonine kinases which are the terminal effectors of the MAPK pathway. This pathway and by extension the RSKs, regulates and drives essential cellular processes such as cell proliferation, cell survival and cell motility. Cancerous cells can exploit this pathway to favour growth and proliferation, resulting in RSKs being implicated in several cancerogenic processes. Specifically, the RSKs have roles in BCa and PCa progression, which are tumours driven by steroids and their respective steroid receptors. Unfortunately, the number of characterised interactions between RSKs and the steroid receptors is limited.

Due to the level of similarity, RSK1 and RSK2 have been considered the prototypical RSKs and RSK3 and RSK4 have been largely neglected. However, an increasing number of studies have indicated that RSK isoform function differently and have sometime opposing roles in different cancer types. In fact, an increasing number of evidences suggest that RSK1/2 are not always inducing cancer growth and RSK3/4 are not always tumour suppressor genes. For example, RSK2 was shown to promote head and neck squamous cell carcinoma (HNSCC) metastasis whereas RSK1 has no effect on HNSCC metastasis (Kang *et al.* 2010), but was shown to negatively regulate metastasis in non-small cell lung cancer (Lara *et al.* 2011). Additionally, RSK3/4 facilitate proliferation in BCa through inhibition of the apoptotic pathway in response to PI3K inhibitors (Serra *et al.* 2013). This suggests that the effect RSKs have varied activities dependent on tissue and/or cancer type. Considering the number of cancer types the RSKs are implicated in and the varied effects they have on different tissue types, this project aimed to fill the lack of knowledge regarding the relationships between the RSKs and SRs. Through this project the use of recombinant DNA techniques enabled a series of reporter assays to show changes in SR activity in response to the RSK signalling. RSKs genes were amplified and incorporated into the mammalian expression vector PCDNA 3.1+ with a HA tag, which was successful for RSK2/3 (Figure 3.1.1 and 3.1.2). This was not necessary for RSK1/4 as synthetic clones were purchased.

Site directed mutagenesis was used to create mutant RSKs, which was successful for RSK2/3/4 (Figure 3.2.1). Luciferase experiments were performed on mammalian COS-1 cells as they do not express SRs. The hormone optimisation experiments (Figure 3.3.1) were used to determine the concentration of hormone to use for each SR which would allow for a suitable increase or decrease in SR activity when under the influence of the RSKs. 1nM, 10nM, 10nM and 1nM were chosen for AR, GR, ERα and PR respectively. The DNA optimisation experiments (Figures 3.3.2.1-4.1) were used to determine the concentration of DNA to use for all RSKs which would allow for a suitable increase or decrease in SR activity (100ng was chosen). Cells were treated with PMA (Figures 3.3.3.1-4.1) to induce activation of all RSKs expressed thus to determine if the endogenous MAPK pathway was active sufficiently to activate all RSKs over-expressed within the COS-1 cells. The mutagenesis experiments (Figures 3.3.4.1-4.1) were used to determine if the phosphorylation state of the RSKs is necessary for SR activation. Overall, all RSK isoforms cause an increase in AR and GR activity despite not all being statistically significant, whereas RSK4 significantly decreased ERα activity and RSK3 significantly increased PR activity.

4.1 – The Androgen Receptor

The AR plays a significant role in both PCa and BCa. In PCa activated AR transcribes genes which promote cell growth and survival. As such, PCa is often treated with anti-androgen treatments, which block the formation and circulation of AR ligand (Litwin & Tan 2017). However, a significant proportion of patients relapse over time by developing Castration Resistant Prostate Cancer (CRPC), which occurs by several proposed mechanisms all of which revolve around the AR (Tan *et al.* 2015). BCa is predominantly ERα driven, however there is also evidence of the AR contributing to BCa. The AR has been shown to increase tamoxifen resistance in ERα+ BCa (De Amicis *et al.* 2010) and be the driving force in ERα-/- BCa. The AR drives a positive feedback loop, regulating the activity of ERK and the RSKs to regulate gene expression and drive growth (Chia *et al.* 2015). Unfortunately, resistance to anti-estrogen therapy occurs often in BCa. However, treatment with enzalutamide was shown to decrease the proliferation of both ERα+/+ and ERα-/- BCa highlighting the significant role that AR plays (Cochrane *et al.* 2014).

From this project the luciferase assays showed the effect the RSKs have on the AR. Although there are some discrepancies, considering the data across all experiments it is apparent that RSK1-4 cause an increase in AR activity. The DNA optimisation experiments (Figure 3.3.2.1.1) showed that RSK1 causes a significant increase in AR activity. The PMA induction experiments (Figure 3.3.3.1.1) showed that RSK1/3/4 cause a significant increase in AR activity in the absence of PMA whilst RSK2 causes a significant increase in AR activity in the presence of PMA. However, only RSK1 caused a statistically significant difference between the +PMA and -PMA conditions suggesting that the endogenous MAPK pathway is sufficiently active. The phospho-mimetic experiments using RSK mutants (Figure 3.3.4.1.1) showed that the RSK4 causes a significant increase in AR activity. Additionally, when comparing the wildtype RSK2 with 2M there is a statistically significant difference between the two conditions. In conjunction with this, when considering all RSK2 experiments with comparable conditions there is a relatively similar luciferase output however the 2M output is

considerably lower. This could suggest that the mutation did not cause constitutive activation. In fact, treatment with PMA generates a signal 7-fold higher than the mutant, while we would have expected a similar or higher signal by a constitutively active mutant.

Although the data illustrated the effect of the RSKs on SR activity there is no insight by which mechanism these effects are caused. When considering the AR and the RSKs this could occur in two ways, (i) direct regulation via phosphorylation of the AR, or (ii) indirect regulation via the phosphorylation of another protein which in turn regulates the AR. (i) It is well established that AR can be directly phosphorylated by a several kinases to regulate gene expression. The AR has been shown to be phosphorylated at the Serine residues 16, 81, 94, 213, 256, 293, 308, 424, 515, 578, 650, 791 and on Threonine residue 282 (Gioeli & Paschal 2012). CDK9 phosphorylates the AR on Ser81 which in turn causes changes in gene expression and increased cell growth in both LHS and LaCP4 cell lines. Most AR canonical target genes remained unchanged or had insignificant changes in gene expression when Ser was substituted for Ala. However, two genes TMPRSS2 and ORM1, had reduced transcription when compared to the AR wt. This suggests that the CDK9 phosphorylation of Ser81 regulates the AR selectivity of promoters and transcriptional program (Gordon *et al.* 2010). Conversely, in LNCaPs and C4-2 PCa cell lines, CDK1 phosphorylates the AR on Ser81 and increases AR expression but had no effect on its transcriptional activity. Furthermore, CDK1 increases the stability of the AR by an unknown mechanism, which could potentially be due to an additional phosphorylation. As such, CDK1 activation would support CRPC, as an overall increase in AR is achieved, thus increasing sensitivity to ligand (Chen *et al.* 2006). Additionally, CDK7 was shown to phosphorylate the AR on Ser515. This phosphorylation is caused by recruitment of the transcription factor complex TFIIH, which contains a CDK7 subunit, to the PSA promoter. After phosphorylation further coactivators are recruited, transcription occurs and the AR gets ubiquitinated for degradation to ensure cyclical activation and transcription of the gene (Chymkowitch *et al.* 2011). (ii) Previous data show that RSK1 regulates the AR by in an indirect mechanism.

LNCaP cells were androgen deprived and treated with enzalutamide causing activation of RSK1, which in turn activates the transcription factor YB1. YB1 then induces the transcriptional activity of the AR. This then went on to contribute to CRPC (Shiota *et al.* 2014). There is also evidence to show RSK2 involvement in PCa however the mechanism was not fully elucidated. RSK2 causes an increase in expression of the PCa diagnostic marker PSA. The increase in PSA is caused by association of RSK2 with the transcriptional coactivator p300. Through RNAi it was shown that the AR was involved, but the exact mechanism was not characterised (Clark *et al.* 2005).

While no direct RSKs phosphorylation of AR has been reported, there is a possibility of cross talk between the CDKs and the RSKs. RSK2 has been shown to indirectly regulate CDK2 by phosphorylating and localising $P27^{KIP1}$ to the cytoplasm which then allows for the formation of A/E CKD2 complexes and progression of the cell cycle (Fujita *et al.* 2003). While it is possible to hypothesise that the RSKs is regulating the AR an indirect mechanism (see future work) acting on other transcription factor or kinase, it is unlikely. Despite the previous data showing indirect regulation, the increases in AR activity were so large that it is unlikely to be via indirect regulation, which would only increase endogenous AR expression or could only moderately increase over-expressed AR activity.

These results also show that the RSKs are a potential therapeutic target for BCa and PCa. In fact, RSK-YB1 signalling was shown to be active in Triple Negative Breast Cancer (TNBCa). Human Mammary Epithelial Cells (HMECs) were treated with RSK inhibitors BI-D1870, the next generation inhibitor LJI308, and Luteolin to reveal promising results. LJI308 was most effective repressing growth and causing apoptosis in the HMECs, in addition the inhibitor targeted transformed cells preferentially over parental HMECs which observed little effect from the inhibitor, supporting RSKs as a therapeutic target for TNBCa (Davies *et al.* 2015). To build on this the RSK-YB1 signalling was also observed in the PCa cell line LNCaP therefore it would follow that RSK inhibitors would also show promise in PCa. Finally,

when considering that all the RSKs induced an increase in AR activity it could be possible that phosphorylating the AR is a redundant function across all the isoforms.

4.2 – The Glucocorticoid Receptor

The GR is implicated in diseases such as Chron's disease, Asthma, Arthritis and several more. Glucocorticoids (GCs) have been used for decades as anti-inflammatory drugs and to control autoimmune diseases. The GR is ubiquitously expressed but retains cell type dependent specificity, however, this means that adverse systemic side effects can occur. In addition, the development of GC resistance (GCR) can arise from long term use GC based drugs (Caratti *et al.* 2015). The GR is also implicated in Cancer but has adverse effects dependent on the cancer type. For example, the GR is responsible for activating the apoptotic program in Haematological Malignancies and aids prevention of skin tumour promotion. Conversely, the GR has been shown to repress chemotherapy-induced apoptosis in BCa and Skin Cancer (SCa). Furthermore, PCa GCs are used for inhibition as part of chemotherapy in advanced CRPC, but PCa patients which have been on Androgen deprivation therapy show increased proliferation (Sundahl *et al.* 2016).

Results from this project showed the effect the RSKs have on the GR. The DNA optimisation experiments (Figure 3.3.2.2.1) show that RSK1/4 cause a significant increase in GR activity. The PMA induction experiments (Figure 3.3.3.2.1) show that RSK2/3 cause a significant increase in GR activity and that the endogenous pathway of the COS-1 cells was active sufficiently to activate all expressed RSKs. The phospho-mimetic experiments (Figure 3.3.4.2.1) show that RSK2/4 cause a significant increase in GR activity. Like the AR, RSK2 induces a statistically significant difference between wildtype and its mutant counterpart suggesting that the RSKs may not be constitutively active. Although there are some discrepancies, considering the data across all experiments it is apparent that RSK1-4 cause an increase in GR activity despite some contradictory results between experiment types.

Although the data illustrated the effect of the RSKs on GR activity, there is no insight by which mechanism these effects are caused. Like the conclusion drawn for AR, when considering the GR and the RSKs interplay, this could occur in two ways, (i) direct regulation (ii) or indirect regulation. To present there is no data concerning the RSKs relationship with the GR. However, how other proteins regulate the GR can show insight as to how the RSKs might also regulate the GR. (i) GR can be phosphorylated in several sites, some of which have been identified on Ser113, Ser134, Ser141, Ser203, Ser211, Ser226, and Ser404 (Ismaili & Garabedian 2004). Additionally, potential phosphorylation sites which have not been tested for function are Ser45, Ser234 and Ser267 (Vandevyver *et al.* 2014). A CDK5/p35 complex was shown to bind to the LBD and then phosphorylate the GR at Ser203 and Ser211, both of which are in the AF1 domain of the NTD. These phosphorylations cause a halt in recruitment of transcriptional co-factors to GR when it is bound to Glucocorticoid Responsive Elements (GREs) of MMTV in HCT116 and COS-7 cells and SGK promoters in rat neuronal cortical cells. This further illustrates the diversity of GR signalling and supports the premise of regulation of genes and promoters is cell type specific (Kino *et al.* 2007). GSK3-β was also shown to phosphorylate the GR at Ser404. This phosphorylation resulted in a large-scale change in transcriptional activity of the GR. For example, the gene IL-4 was expressed 2-fold higher and the gene STAT4 was expressed 6-fold higher when Ser404 was phosphorylated under hormone conditions than when under normal cellular conditions (Galliher-Beckley *et al.* 2008). c-Jun N-terminal Kinase (JNK) was also shown to phosphorylate the GR at Ser226. This phosphorylation causes the early export of GR from the nucleus once withdrawal of ligand occurs. This export then causes inhibition of transcription of GR target genes (Itoh *et al.* 2002). Similarly, p38, once activated by MKK3, was also shown to phosphorylate the GR at Ser211. These events were induced by the GR mediated apoptosis pathway which resulted in apoptosis of lymphoid cells. The Ser211 phosphorylation in this case was shown to induce the GRE-dependent transcription of the p38 kinase MKK3 (Miller *et al.* 2005). (ii) However, at this stage, we cannot exclude that RSKs increased activation of GR it could be from an indirect mechanism. However,

differently from AR, there are no available data regarding transcription factors regulating GR expression, which are targeted by RSKs. These results again provide further evidence contesting the previous perception of set roles of the RSKs.

Although not directly linked to GR activity, studies have investigated the effect of RSK inhibitors on SCa, which, as mentioned before, is a type of cancer driven by GR. Nearly every relapse of melanoma has a mutation within B-Raf which causes constitutive activation of the MAPK pathway, which in turn activates cell survival and promotes resistance to MAPK inhibitors. Inhibition of the RSKs with BI-D1870 and LJH-685 caused a reduction in growth of the drug-resistant melanoma cells, which becomes sensitive again to the MAPK inhibitors. It is not known if this positive effect is achieved through indirect inhibition of GR, which is however a hypothesis worth exploring considering the preliminary data collected in this study. However, this shows promise for the use of RSK inhibitors independently and in combination with MAPK inhibitors for the treatment of melanomas and advanced resistant melanomas (Kosnopfel *et al.* 2017).

4.3 – The Estrogen Receptor α

The ERα has been shown to participate in BCa, OvCa and PCa (Jia *et al.* 2015). ERα is pivotal in BCa as it drives tumorigenesis and disease progression. Anti-estrogen therapy is the primary treatment for BCa to reduce hormone activated proliferation, the use of aromatase inhibitors is also implemented to reduce the conversion of androgens to estrogens. However, often patients experience relapses due to resistance to the therapy (Droog *et al.* 2013). In OvCa ERα drives cell proliferation, invasion and therapy resistance. Anti-estrogen therapy would seem favourable however the response is modest (Chan *et al.* 2014). The ERαα also contributes to carcinogenesis in PCa by producing E2 inside the cells which unbalances hormone concentrations in the cellular environment and drives growth. Anti-estrogen therapy has been shown to be effective in PCa early stages (Ricke *et al.* 2007).

Results from this project showed the effect the RSKs have on the ERα. Differently from AR and GR, ERα activity seems to be RSK-isoform dependent. The DNA optimisation experiments (Figure 3.3.2.3.1) show that RSK4 causes a significant decrease in ERα activity. The PMA induction experiments (Figure 3.3.3.3.1) show that RSK4 causes a significant decrease in ERα activity in the absence and presence of PMA. The phospho-mimetic experiments (Figure 3.3.4.3.1) show that 2/3/4M cause a significant decrease in ERα activity whilst all wildtype RSKs cause a moderate reduction in ERα activity. Considering all of the data RSK4 causes a significant decrease in ERα activity whilst RSK1/3 may need further investigation as they caused a moderate decrease in ERα activity, but they were not statistically significant.

The conflicting results of RSK2 on ERα activity will require further attention (see future work), considering that several studies have shown direct interaction of RSK2 with ERα. From literature it has been shown that RSK2 binds to the LBD associating with residues 326-394 and then phosphorylate the ERα at Ser167, enhancing the AF1 transcriptional activity (Clark *et al.* 2001). However, this conflicts with the data obtained (Figure 3.3.2.3.1). RSK1/2 were also shown to phosphorylate ERα at Ser167 and Ser118, both of which enhanced the transcriptional activity of AF1 (Joel *et al.* 1998), which again contradicts with the results presented here. This was shown to be through a co-operation between the MAPK pathway and the mTORC pathway which leads to the development of therapy resistance, with Ser167 phosphorylation being diagnostic to determine whether to proceed with endocrine or anti-estrogen therapy (Yamnik & Holz 2010). Data on RSK3 are in line with previous observation that showing that RSK3 is a tumour suppressor in OvCa. RSK3 was shown to be monoallelically expressed in normal ovary cells but this single expression resulted in a loss of function in cancer cells. Reintroduction of RSK3 into cancer cell lines resulted in reduced proliferation, suppressed growth, G1 cell cycle arrest leading to apoptosis. The mechanism by which this occurred was not elucidated (Bignone *et al.* 2007). Considering that ERα and RSK3 have been implicated in OvCa it could be possible that these two proteins interact to mediate the effects seen, especially in conjunction with the results where RSK3 caused a decrease in ERα activity. Similarly RSK4 was shown to possess tumour suppressor properties in breast adenocarcinoma cells as downregulation by several methods lead to cancer cell proliferation and invasion (Zhu *et al.* 2015) which coincides with the data presented.

It is therefore tempting to speculate that both RSK3 and RSK4 play a tumour suppressor function in ERα-driven cancer by interacting directly with ERα.

This is further reinforced by studies that shows how different kinases regulates ERα by directly phosphorylating it. PKCθ was shown to regulate the expression of ERα via an indirect mechanism. In normal mammary gland cells PKCθ and AKT activity is low which

causes the ERα/p27^{KIP} transcription factor FOXO3a to remain unphosphorylated and transcribe the ERα and p27^{KIP}. In turn the ERα then repressed the transcription factor NFkB c-Rel and p27^{KIP} to inhibits G_1/S transition. However, in most primary BCas NFkB c-Rel is upregulated by PKCθ activation which in turn phosphorylated AKT which then phosphorylates FOXO3a causing it translocation to the cytoplasm. The translocation of FOXO3a results in repression of the ERα which can then no longer repress NFkB c-Rel. Additionally, it decreases in expression of P27^{KIP} and alleviates cell cycle arrest in G_1. NFkB c-Rel is then able to transcribe its target genes c-MYC, Cyclin D1, BCL-XL, which promote cell growth and cell survival, and NFkB RelB which promotes an invasive cancer phenotype (Belguise *et al.* 2007). Furthermore, the c-Rel transcriptional product RelB negatively regulates the expression of ERα in most primary ERα positive BCa's. RelB transcribes BCL-2 which in turn associates and activates Ras leads to the expression of a zinc finger repressor Blimp1. Blimp1 then binds upstream of a ERα promoter and reduces expression. The reduction of ERα expression in turn results in reduced transcription of the relevant target genes which encode E-cadherin and γ-catenin which promotes cancer migration (Wang *et al.* 2009). There is also evidence of additional direct phosphorylations to the ERα to regulate its transcriptional activity. In response to hormone it has been shown that ERα is phosphorylated on Ser104 and Ser106 by CDK2 both of which reside in the AF1 domain and enhances its transcriptional activity (Rogatsky *et al.* 1999). Additionally, Protein Kinase A (PKA) was shown to phosphorylate the ERα on Ser236 within the DNA binding domain which negatively regulates transcription by inhibiting ERα binding to DNA (Chen *et al.* 1999). AKt was also shown to phosphorylate the ERα on Ser167 within its AF1 domain and cause transcription of the PS2 gene, BCL-2 and macrophage inhibitory cytokine to contribute to chemotherapy resistance (Campbell *et al.* 2001).

4.4 – The Progesterone Receptor

The PR has primarily been implicated in BCa. PR plays a key role in BCa and in most cases possesses tumour suppressing properties. For example, progesterone treatment has been shown to reduce proliferation in BCa cell lines and synthetic progestogens have been shown to oppose estrogen stimulate growth in ERα+/PR+ xenografts. Additionally, exogenous expression of PR was shown to block ERα mediated proliferation and transcription. Due to such involvement of PR in regulating the effects of ERα it is used for a positive prognostic marker for disease outcome, with higher expression in early stage disease being highly correlated with increased responsiveness to endocrine therapy (Mohammed *et al.* 2015).

The data obtained from the luciferase assays showed the effect the RSKs have on PR. The DNA optimisation experiments (Figure 3.3.2.4.1) show that RSK3 causes a significant increase in PR activity. The PMA induction experiments (Figure 3.3.3.4.1) show that RSK3 causes a significant increase in PR activity. Furthermore, RSK3 generated a statistically significant higher signal without PMA treatment than in its presence in addition to RSK1/2/4 following the same pattern in a modest manner. This suggests that somehow PMA treatment has a negative effect on PR activity and that the endogenous MAPK pathway is sufficiently active. The mutant experiments (Figure 3.3.4.4.1) show that wildtype RSK3/4 caused a significant increase in PR activity and all three mutant isoforms showed a major decrease in PR activity, of which 2/4M were statistically significant. Considering this in conjunction with a statistical significance being present between RSK3/4 and their mutant counterparts it again suggests that the mutants are not constitutively active, and the endogenous MAPK pathway was able to fully activate the over-expressed RSKs. When considering all the data RSK3 causes a significant increase in PR activity whilst RSK4 may require some further investigation.

There are several discrepancies within the different experiments, which do not fully clarify the role of RSKs in PR activity. It is however possible to detect a link between RSKs and PR activity, which is novel, since no studies have focused on the relationship between RSK and PR. Unlike the other SRs, the PR has four basal phosphorylations which occur in a ligand independent manner, but are increased after hormone treatment, Ser81, Ser162, Ser190 and Ser400. These phosphorylations results in the transcription of a small set of genes (Abdel-Hafiz & Horwitz 2014). CDK2 was shown to phosphorylate the PR on Ser400 independently of hormone, being the kinases responsible for this basal phosphorylation. This resulted in the increased localisation of the PR to the nucleus and increased its transcriptional activity. However, the phosphorylation was increased in response to hormone and mitogens (Pierson-Mullany & Lange 2004). CDK2 has also been shown to phosphorylate the PR at several additional positions Ser25, 162, and 190, 213, 294, 554, 676 and Thr430 highlighting its highly regulatory role with PR. Of these Ser190, 554 and 676 have been shown to regulate the transcriptional activity of PR in a hormone dependent manner (Hagan *et al.* 2012). In contrast, it was shown that elimination of CDK2 phosphorylation sites resulted in minimal change to PR activation. Additionally, it was shown that PR interacts with cyclin A and recruits CDK2 to form the cyclin A/CDK2 at PR responsive promoters to facilitate transcription. This was also dependent on recruitment of Steroid receptor coactivator 1 (SRC-1) and its phosphorylation state to acetylate DNA at the promoter (Narayanan *et al.* 2005). Although CDK2 was shown to phosphorylate several sites on the PR, these sites are not exclusively phosphorylated by it. Casein Kinase II (CK2) was shown to phosphorylate PR on Ser81 both in the presence of hormone and in the absence of hormone during the cell cycle G_1/S transition point. The phosphorylation in the presence of hormone was however, considerably faster. Through mutational experiments it was shown that the Ser81 phosphorylation was vital for the recruitment of PR and CK2 to progestin responsive element 1 and transcription of a gene subset. Furthermore, CK2 is a ubiquitously expressed protein and is overexpressed in all cancers known to date and is associated with pro-survival and proliferative phenotypes therefore could be contributing towards BCa

(Hagan *et al.* 2011). The MAPKs p42 and p44 were shown to phosphorylate the PR on Ser294 after hormone treatment. This caused the PR to become downregulated, which was due to a Ser294 phosphorylation dependent degradation by the 26S proteasome. Additionally, in a ligand independent manner the 26S proteasome was shown to degrade immature PR intermediates by an unknown mechanism (Lange *et al.* 2000). MEK was also shown to phosphorylate the PR at Ser345 in response to hormone. This occurs via the non-classical mechanism, PR activated the EGFR, c-SRC and MAPK signalling which resulted in the Ser345 phosphorylation. This phosphorylation then caused the PR to strongly associate and tether with the transcription factor Sp1, resulting in the transcription of p21 and EGFR. This transcription occurred on promoter which are not progestin response elements and produced a positive feed-forward loop (Faivre *et al.* 2008). Although undetermined through this study, the non-classical signalling of PR could have a large influence on BCa. This would occur by upregulation of proliferative pathways such as the MAPK. Furthermore there is also evidence of ER signalling similarly activating the MAPK pathway through non-classical mechanisms in BCa (Santen *et al.* 2001).

Considering that PR can be phosphorylated by other MAPKs, the previously described cross-talk with CDKs, it is tempting to speculate that RSKs regulates PR activity by directly phosphorylating it.

4.5 - Future Work

There are few relationships characterised between the RSKs and the SRs however, both are often implicated in hormone driven cancers. The uncharacterised relationships leave a knowledge gap across several cancers types concerning the role of RSKs in steroid signalling. Furthermore, these cancers cause many deaths each year globally therefore it is extremely important to elucidate the RSK/SR relationships. In doing so, it could lead to a breakthrough in the understanding in steroid signalling and provide new approaches to therapeutic treatments. From this project there were some aspects of work which were unsuccessful or due to time constraints could not be started or completed.

To make this a comprehensive study, the mineralocorticoid receptor needs to be incorporated into the mammalian expression vector PSG5 for the use in the same luciferase assays. This would then reveal the full extent as to how all RSKs cause changes in all SR activities. Additionally, mutagenesis experiments would need to continue for RSK1 to provide a full set of RSK mutants. These two processes both failed in laboratory for unknown reasons yet were successful for other components, therefore as a precautionary step new reagents and primers will be purchased to remove this possibility. Additionally, previous notes and protocols will be inspected to check for any inconsistencies or errors.

Throughout the luciferase assays there has been one condition which is comparable between the three experiment types, 100ng concentration of RSK DNA under a constant hormone concentration. However, across most of the experiments there are inconsistencies between these values. For example, RSK2/3 with the AR have considerably higher values in the PMA induction experiments in the -PMA condition than the comparable conditions across the other two experiment types. These kinds of discrepancies can also be seen with AR-RSK1 and GR-RSK4. This could be due to several different variables which can be overcome; different batches of DNA used throughout project, different batches of COS-1 cells used throughout project, different batches of $CaCl_2$ and BBS used throughout project and varied freezer incubation times. To overcome these problems fresh DNA for each RSK

isoform, SR and the additional luciferase components will be made from the same large culture. One new fresh batch of COS-1 cells will be used and the health of the cells will be monitored with high attention. One large batch of both $CaCl_2$ and BBS will be made. Luciferase assays will be conducted on the day and plates will not be frozen longer than mentioned on the protocol.

In terms of furthering the depth of this project, inhibitor experiments will be created to validate the effects caused on SR activity by the RSKs. As with the hormone and DNA concentrations, inhibitor optimisation experiments will first be conducted to determine the concentration of inhibitor that will not destroy RSK activity but that will allow for a visible reduction/increase that can be used to validate RSK effects seen. Mammalian 2 hybrid assays will be carried out to determine the mechanism by which the RSKs regulate SRs activity. Fusion proteins will be created of both the RSKs and the SRs. This will be done by recombinant DNA techniques to incorporate the RSKs and SRs into different domains of the Gal4 protein. Gal4 has two different domains, the binding domain (BD) which binds to DNA and the activating domain (AD) which is necessary for activating the transcription of the reporter gene. If the AD is removed from the BD no transcription occurs, conversely when the two domains are near, transcription of the reporter gene occurs. The RSKs will be fused to the BD as they don't possess transcriptional activity and will not interfere with the reporter gene thus the SRs will be fused to the AD. This will then provide evidence as to which mechanism the proteins interact. If the proteins interact it would indicate that the RSK phosphorylates the SR and if they don't it indicated the RSKs cause this effect via indirect mechanisms. In the case of direct phosphorylation there are several additional experiments that can be done to further dissect the interaction. The kinase kinetics could be measured with a kinase kit. The phosphorylation position could be probed for using phospho-antibodies as most phosphorylation site on the SRs are known and are most likely commercially available.

4.6 - Conclusion

To conclude, this study used recombinant DNA techniques to prepare the RSKs and SRs for a series of luciferase assays. The luciferase assays were used to show the changes in SR activity induce by RSKs. RSK1-4 cause an increase in AR and GR activity whilst RSK4 significantly decreased ERα activity and RSK3 significantly increased PR activity. The use of PMA to invoke maximal activity through MAPK pathway activation caused no significant change except for 2 isoforms across all PMA induction experiments suggesting the endogenous pathway is sufficiently active to activate all over expressed RSKs. The phospho-mimetic experiments suggested that the mutation was not sufficient to cause a constitutively active protein. These interactions will be further investigated primarily through mammalian 2 hybrid assays which will elucidate the mechanism of regulation between each RSK isoform and SR of interest. Inhibitor optimisation experiments, followed by inhibitor experiments will be used to validate the effects seen by inhibiting the RSKs. Once this data is finished it could provide new insight into RSK-SR signalling.

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