THE EFFECT OF HYPOXIA ON ALTERNATIVE SPLICING IN PROSTATE CANCER CELL LINES

ELIZABETH BOWLER

Thesis submitted in partial fulfilment of the requirements of the University of the West of England, Bristol for the degree of Doctor of Philosophy

Faculty of Applied Sciences, University of the West of England, Bristol

2016

ACKNOWLEDGEMENTS

First and foremost I would like to thank my primary supervisor Dr. Michael Ladomery. His encouragement and support throughout my PhD has helped me achieve some excellent results and has really built my confidence as a researcher. I am incredibly grateful for all the guidance he has provided through this project. Without Mike's help and supervision this thesis would not be possible.

I would also like to thank my secondary supervisors, Prof. John Hancock and Dr. Ian Wilson. John has been a great support during my time at UWE and has gone above and beyond to offer his help and expertise. Ian's guidance with statistics was both helpful and insightful.

In addition, I would like to acknowledge the collaborators that are linked to this project, Dr. Silvia Pastorekova (Slovak Academy of Sciences, Slovakia) has provided expertise for the hypoxia element of this PhD, and Dr. Roscoe Klick and his team at RNomics Platform Sherbrooke University, Canada) who conducted the high-throughput PCR work.

I offer my thanks to the technicians in CRIB laboratory at UWE, especially David Corry who provided assistance with the confocal microscope. Furthermore, I greatly appreciate the encouragement and laughs provided by the Ladomery laboratory team as well as all the other PhD students in the CRIB laboratory. As expected in research, there have been many ups and downs and it has been a pleasure to share them with you all!

Next, I would personally like to thank my family. My mum, dad and sister have always been incredibly supportive, encouraging and loving throughout my entire life and I am supremely appreciative for everything that they have done for me. I am exceptionally grateful to my partner Aydin who has provided me with a lot of love, support and reassurance through this period.

This project was funded by the UWE RAE2008 QR Fund and the Doctoral Completion Bursary 2015 -2016 (UWE).

ABSTRACT

Hypoxia is defined as the state in which the availability or delivery of oxygen is insufficient to meet tissue demand. It occurs particularly in aggressive, fast-growing tumours in which the rate of new blood vessel formation (angiogenesis) cannot match the growth rate of tumour cells. Cellular stresses such as hypoxia can cause cells to undergo apoptosis; however some tumour cells adapt to hypoxic conditions and evade apoptosis. Tumour hypoxia has been linked to poor prognosis and to greater resistance to existing cancer therapies. This thesis provides evidence that alterations in alternative splicing patterns of key genes is one method tumour cells adapt to hypoxia.

A hypoxic-induced change in the alternative splicing of *carbonic anhydrase IX* (*CA IX*) is confirmed. CA IX is one of the best studied hypoxia markers, involved in maintaining an intracellular pH that favours tumour cell growth. Furthermore, evidence is provided here that in the PC3 prostate cancer cell line, the regulation of *CA IX* splicing involves the scaffold attachment factor B 1 (SAFB1) and pre-mRNA-processing-splicing factor-8 (PRPF8) splice factors. However, SAFB1 expression is shown to decrease in hypoxia.

Alternative splicing patterns of previously documented cancer-associated genes are altered in hypoxia in the PC3, VCaP and PNT2 prostate cell lines. There is evidence of significant changes in the alternative splicing of several cancer-associated genes in hypoxia, which have varied roles in the hallmarks of cancer: Apoptosis (*APAF1, caspase-9, Bcl-x and survivin*); Immune tolerance (*BTN2A2*); Cellular motility and

invasion (*CDC42BPA, FGFR1OP and UTRN*); Alternative splicing *(PUF60*); Proliferation (*RAP1GDS1*) (proliferation); and those with an unknown function but linked to cancers (*MBP, PTPN13* and *TTC23).* Most notably, there was a higher proportion of the pro-oncogenic isoforms of *APAF1, Bcl-x, survivin, BTN2A2* and *RAP1GDS1* in hypoxia than in normoxia. The mRNA expression of splice factors (*SRSF1, SRSF2, SRSF3, SAM68, HuR* and *hnRNP A1*) and SRSF1 protein production were shown to significantly increase in hypoxia. Phosphorylation of SRSF4 and SRSF5 was demonstrated to increase in hypoxia indication that hypoxia may alter alternative splicing patterns.

The mRNA expression of the CLK1 and SRPK1 splice factor kinases also increased in hypoxia; however only CLK1 protein production was shown to also increase in hypoxia. There were no significant changes to alternative splicing when SRPK1 was knocked down or inhibited (using SPHINX) suggesting that SRPK1 was not involved in the alteration of alternative splicing of the cancer-associated genes studied. . However, siRNA knockdown and chemical inhibition of CLK1 (using TG003) suggested a shift in *FGFR1OP* splicing that mirrored the effect of hypoxia on *FGFR1OP* splicing. This suggests that CLK1 activity is inhibited in hypoxia. This conflicts the finding that CLK1 production increases in hypoxia and suggests that there are more mechanisms concerned in the regulation of CLK1 during hypoxia.

This work has provided an insight into mechanisms that are involved in alternative splicing changes in hypoxia in mammalian cell lines. These novel research findings may aid in the understanding of how cells adapt to hypoxia especially in regards to alternative splicing and may offer future therapeutic targets in hypoxic tumours.

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ABBREVIATIONS

ADC	Adenocarcinoma
ADT	Androgen deprivation therapy
ASO	Short antisense oligonucleotide
BHD	Bcl-2 homology domain
BSA	Bovine serum albumin
CA IX	Carbonic anhydrase 9
cDNA	Complementary DNA
chIP	Chromatin immunoprecipitation
CLK	CDC-like kinase
CT values	Threshold cycle values
DAPI	4',6-diamidino-2-phenylindole
DiO	3,3'-Dioctadecyloxacarbocyanine Perchlorate
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide
DTT	Dithiothreitol
ECACC	European collection of authenticated cell cultures
EDTA	Ethylenediaminetetraacetic acid
EGTA,	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EMT	Epithelial-to-mesenchymal transition
ESE	Exonic splicing enhancer
ESS	Exonic splicing silencer
FBS	Fetal bovine serum
GO	Gene ontology
H_2O_2	Hydrogen peroxide
HAF	Hypoxia associated factor
HAP	Hypoxia activated prodrug
HCC	Hepatocellular carcinoma
HCl	Hydrochloric acid
HIF	Hypoxia inducible factor
hnRNP	Heterogenous nuclear ribonucleoprotein
HNSCC	Head and neck squamous cell carcinoma
HRE	HIF response element
HRP	Horseradish peroxidise
HUVEC	Human umbilical vein endothelial cells
IC Tail	Intracellular tail
IRE	Iron-response element
IRES	Internal ribosome entry site

IRP	Iron-regulatory protein
ISE	Intronic splicing enhancer
ISS	Intronic splicing silencer
KCl	Potassium chloride
$MgCl_2$	Magnesium chloride
MIC-101	Modular incubator chamber-101
MOC	Manders' overlap coefficients
mRNA	Messenger RNA
N_2	Nitrogen
NaCl	Sodium chloride
$NaHCO_3$	sodium bicarbonate
NHE1	Na^+/H^+ exchanger 1
NL	Nuclear lysis buffer
NMD	Nonsense-mediated decay
NSCLC	Non small cell lung carcinoma
O_2	Oxygen
oligoDT	Oligodeoxythymidylic acid
ORF	Open reading frame
PAS domain	PER-ARNT-SIM domain
PBS	Phosphate buffered saline
PC3 cells	Prostate cancer-3 cells
PCR	Polymerase chain reaction
pH_i	Intracellular pH
PI tablet	Phosphatase inhibitor tablet
PSA	Prostate specific antigen
PVDF	Polyvinyldene fluoride
RBP	RNA binding protein
RCC	Renal clear cell carcinoma
RIPA buffer	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
ROS	Reactive oxygen species
RRM	RNA recognition motif
RS domain	Serine/arginine domain
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SF	Subcellular fractionation
siRNAs	Small interfering ribonucleic acid

SMD	Spinal muscular atrophy
SmgGDS	Smg GDP dissociation stimulator
SMN	Survival motor neuron
SNB	Stress-induced sub-nuclear bodies
snRNP	Small nuclear ribonucleoparticle
SPHINX	((5-methyl-N-[2-(morpholin-4-yl)-5-(tri-fluoromethyl)phenyl]furan-2-carboxamide))
SR protein	Serine-arginine rich protein
SRE	Splicing regulatory element
SRPK1	SRSF protein kinase 1
SRSF	Serine/arginine-rich splice factor
SSO	Splice switching antisense oligonucleotide
TBS-T	Tris-buffered saline-Tween20
TEMED	Tetramethylethylenediamine
TG003	1-(3-ethyl-5-methoxy-2(3H)-benzothiazolylidene)-2-propanone
TH	Tyrosine hydroxylase
TM region	Transmembrane region
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
tRNA	Transfer RNA
TSS	Transcription start site
UTR	Untranslated region
VCaP cells	Vertebral-cancer of the prostate cells
VHL	von Hippel-Lindau
WCRF	World cancer research fund

1.0 INTRODUCTION

1.1 Prostate Cancer

1.1.1 Incidence

Over the past ten years there has been an increase in prostate cancer incidence but at the same time a decrease in prostate cancer mortality in England and Wales; with an average annual percentage change of 3.8% and -1.4% respectively (Center *et al.,* 2012). This suggests that prostate cancer treatment and detection in the UK is much more successful than ten years ago. Despite this, in 2012 prostate cancer was reported to be the fifth leading cause of cancer death and the second most common cause of cancer among men worldwide (Ferlay *et al.,* 2012). Therefore, additional research into causes, prevention and treatment of the disease is still vital.

1.1.2 Risk factors for prostate cancer

Previous research has uncovered certain factors associated with a higher risk of developing prostate cancer. The most well-established risk factors for prostate cancer include: older age, black-African heritage, a family history of the malignancy, and certain genetic polymorphisms such as those seen in the homeobox gene, *HOXB13* (Zhou *et al.,* 2016; Platz and Giovannucci, 2006; Ewing *et al.,* 2012). Interestingly, most of the well-established risk factors suggest a genetic

predisposition to the disease, although there is now emerging evidence that there could also be environmental risk factors that cause prostate cancer.

As there is generally an increased risk of prostate cancer incidence in the Western world, environmental risk factors that are associated with a western lifestyle have been proposed. A report by the World Cancer Research Fund (WCRF; 2014) has provided strong evidence that obesity, a tall frame and the consumption of beta-carotene increases the risk for developing prostate cancer. The report also suggests that increased consumption of dairy products and a high intake of calcium could be classed as prostate cancer risk factors (WCRF, 2014), although due to the limited evidence obtained further research has to be conducted to confirm these findings.

1.1.3 Current prostate cancer therapies

Men are advised about the best treatment option through an assessment that encompasses a range of different factors, such as; patient age, clinical tumour stage and serum PSA to name a few. Many localised low-grade prostate cancers that usually occur in older males tend to be less aggressive and are harmless. Therefore, active surveillance may be considered as a more beneficial option for the patient than other therapies, which in particular may put older patients in distress and discomfort (Parker, 2004). In active surveillance, patients diagnosed with prostate cancer are monitored regularly for any changes in the tumour. It has been shown that the 10 year prostate cancer-specific survival rate is at 97% (Klotz *et al.,* 2010), which suggests that for some patients, particularly those with less-aggressive

prostate cancer, active surveillance may be a more viable treatment than invasive treatments.

For patients with aggressive prostate cancers, there are a number of different treatment options available. This is particularly useful for younger men whodevelop early onset prostate cancer (<55 years) which tend to have a more aggressive phenotype with a poorer prognosis (Salinas *et al.,* 2014). Radical prostatectomy surgery has been shown to be effective for localised tumours, with a survival percentage at 12 years of 67% compared to 60% for active surveillance (Bill-Axelson *et al.,* 2011). However, surgery is associated with greater incontinence and erectile dysfunction (Wilt *et al.,* 2012) and therefore suggested to be beneficial only to those with a higher-risk disease.

Focal therapy uses a tissue sparing approach whereby only the cancerous area of the prostate is targeted for treatment. In terms of how aggressive the treatment is, it is regarded as midway between active surveillance and more radical treatments, such as radical prostatectomy and radiotherapy (Onik *et al.,* 2008). Methods of focal therapy include; cryotherapy, high-intensity focused ultrasound and laser ablation to name a few (Perera *et al.,* 2016). A study by Lambert *et al.* (2007) showed that 84% of patients that underwent focal cryotherapy had no evidence of biochemical recurrence of the disease after 2.5 years. In addition, 74% of patients retained erectile function and no patient experienced any worsening of urinary symptoms, which makes focal cryotherapy a more attractive option for treating localised prostate cancer (Lambert *et al.,* 2007). Proton beam therapy is a newly established type of focal therapy whereby proton particles are targeted to a

tumour. Unlike electron or photon beam therapies, protons do not transverse the target which spares healthy surrounding tissue (Yamoah and Johnstone, 2016). However, the therapy is expensive and no more advantageous than the conventional intensity-modulated radiation therapy, which delivers precise radiation does to specific areas within a tumour (Yamoah and Johnstone, 2016).

More advanced cancers that have either metastasised or recurred are treated with androgen deprivation therapy (Saraon, Jarvi and Diamandis, 2011). Androgens activate the androgen receptor transcription factor, which in turn can activate and regulate the expression of many genes involved in growth and proliferation (Chodak *et al.,* 1992; Ruizeveld de Winter *et al.,* 1991; Sadi, Walsh and Barrack, 1991). This includes the *kallikrein-related peptidase 3 (KLK3)* gene that encodes prostate specific antigen (PSA), which is widely used as a biomarker for prostate cancer (de Winter *et al.,* 1991). Furthermore, in the presence of low androgen levels, the androgen receptor has also been found in the cytoplasm to function in non-genomic signalling. This is achieved through interaction with Src and p85α, which activate the MAPK and Akt pathways, respectively, which enhance cellular proliferation and survival (Leung and Sadar, 2017).

Androgen deprivation therapy reduces the amount of androgens available for activation of androgen receptor transcription of genes that are involved in cellular growth; and in the control of signalling pathways, such as ERK/MAPK and B cell receptor signalling (Toropainen *et al.,* 2016). However, after some time, cancer cell growth is not limited by the absence of androgens and most patients will eventually become tolerant to androgen deprivation therapy; this is known as androgen-

independent prostate cancer (Chodak *et al.,* 1992). In a study that investigated differences between androgen independent and androgen sensitive prostate cancer cell lines, it was found that the AMP-activated-protein kinase, interleukin-3 and androgen receptor signalling pathways were the top deregulated signalling pathways in androgen-independent cell lines (Tonry, Armstrong and Pennington, 2017). The AMP-activated-protein kinase signalling pathway suppresses androgen receptor mRNA expression and promotes androgen receptor protein degradation, which ultimately decreases androgen receptor protein levels (Ge *et al.,* 2015; Shen *et al.,* 2014). The interleukin-3 signalling pathway has been linked to tumour cell proliferation and migration. Furthermore, blockage of interleukin-3 has been shown to prevent tumour growth and angiogenesis (Dentelli *et al.,* 2011). This suggests that androgen-independent prostate cancer cells may have drive to proliferate and migrate through the interleukin signalling pathway.

Surprisingly, the androgen receptor is over-expressed at the RNA and protein level in many androgen-independent prostate cancer cases (Brown *et al.,* 2002; Edwards *et al.,* 2003; Koivisto *et al.,* 1997). However, it has also been shown that in untreated prostate cancer samples, androgen receptor expression is not increased which suggests that hormonal therapy causes an increase in androgen receptor expression (Saraon, Jarvi and Diamandis, 2011). In addition, it has been found that androgen receptor mutations can also account for androgen independent prostate cancer--mediated cancer progression (Taplin *et al.,* 1995). Many androgen receptor mutations result in decreased ligand specificity of the androgen receptor, which allows other hormones such as progesterone, oestrogen and anti-androgens to

activate the receptor. Therefore, during androgen ablation, cancer cells harbouring these mutations are able to continue proliferating (Saraon, Jarvi and Diamandis, 2011).

The *androgen receptor* gene contains a NH_2-terminal domain followed by a DNA binding domain, a hinge region and a COOH-terminal domain . A ligand binding domain forms part of the COOH-terminal domain between exons 5-8 (Dehm and Tindall, 2011). There are over 20 reported splice variants of the *androgen receptor* gene that have been reported in human prostate cancer cell models and clinical samples (Cao *et al.,* 2016) (Figure 1.1). AR45 contains a truncated N-terminal domain, which results in a loss of *trans*-activating ability. AR45 acts as an inhibitor of full-length androgen receptor function (Ahrens-Fath *et al.,* 2005). Most of the variants contain an intact DNA-binding domain, which is required for transcription to occur. AR8 does not harbour a DNA-binding domain and so cannot perform as a transcription factor (Yang *et al.,* 2011). AR-V3 and AR-V23 both contain the exons 2 and 3, which make up the DNA-binding domain (exons 2 and 3) but have sections of sequence in between the coding exons. Despite this, AR-V3 is still able to bind to DNA and activate promoters that respond to the androgen receptor (Dehm *et al.,* 2008); however, AR-V23 has been shown to have cytoplasmic functions instead (Jagla *et al.,* 2007). Some variants are continually active, such as AR-V7 and AR-V12 (Hu *et al.,* 2011), which can drive androgen receptor transcription of genes and therefore can contribute to the progression of prostate cancer. Furthermore, elevated expression of alternatively spliced androgen receptor isoforms are associated with disease recurrence after radical prostatectomy (Guo *et al.,* 2009;

Welsbie *et al.,* 2009). Therefore, alternative splicing of the *androgen receptor* is thought to be another method in which prostate cancer can progress after castration (Sun *et al.,* 2010).

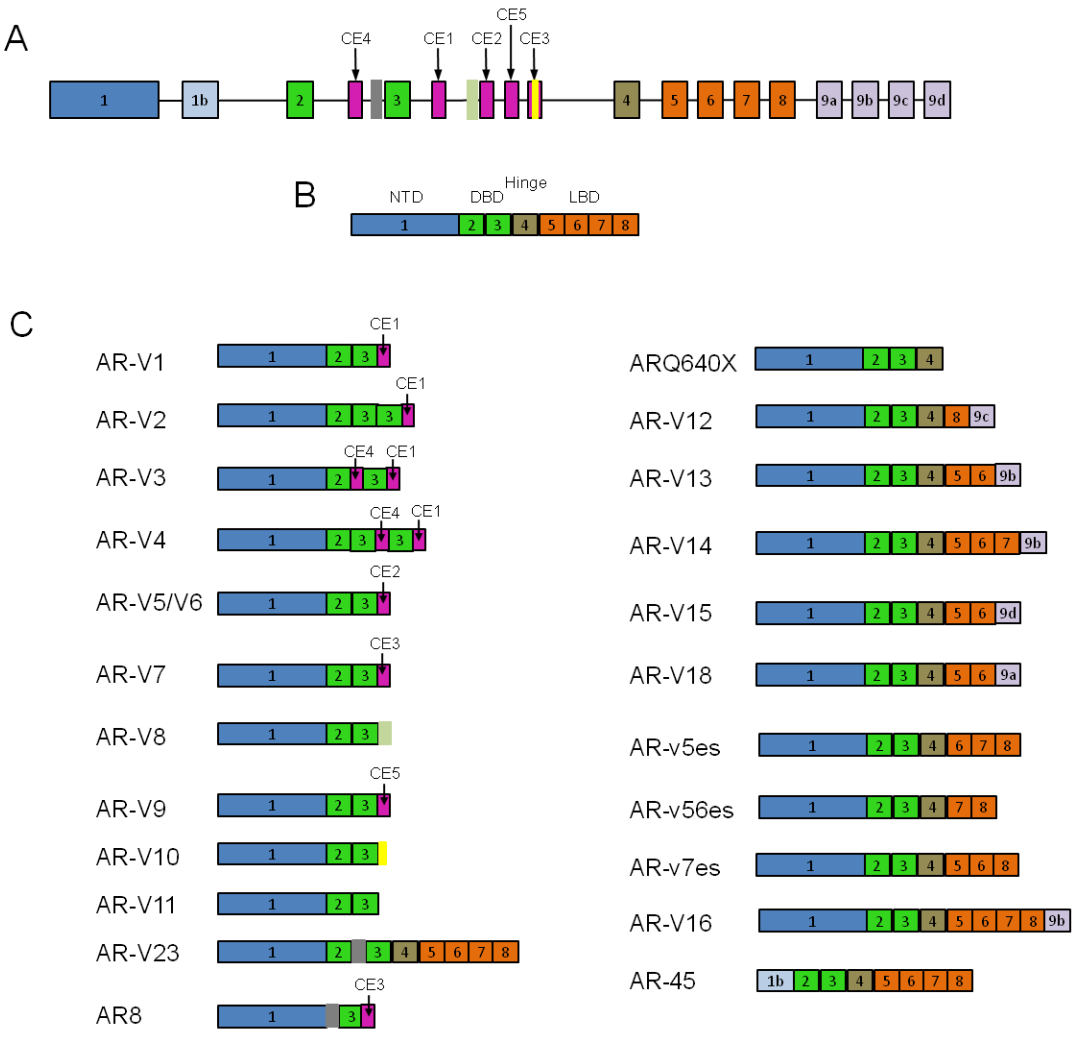


Figure 1.1: Schematic representation of the structure of the androgen receptor gene and respective splice variants (A) Canonical exons and cryptic exons (CE) of the androgen receptor gene. (B) Full-length androgen receptor mRNA showing exons belonging to the N-terminal domain, DNA-binding domain, hinge region and ligand-binding domain. (C) mRNA structures of currently reported splice variants derived from the androgen receptor gene. Illustration is adapted from Cao *et al.* (2016) and used with permission from the author.

External-beam radiotherapy is an effective treatment that is found to significantly improve 10 year mortality when used in conjunction with hormonal treatment

compared to hormonal treatment alone (Widmark *et al.,* 2009). A similar study by The National Cancer Institute of Canada (Warde *et al.,* 2011) also found that radiotherapy used in combination with androgen deprivation reduced 7-year mortality when compared to androgen deprivation alone. These two studies have led to radiotherapy in addition to androgen deprivation therapy as treatment for high-risk and locally advanced prostate cancer patients.

Metastatic androgen-independent prostate cancers can be targeted by chemotherapeutic drugs to prolong survival. Docetaxel is a first line chemotherapeutic drug that prolongs life by 2.9 months on average (Tannock *et al.,* 2004; Berthold *et al*., 2008). Other chemotherapeutic drugs, such as Sipuleucil-T (Kantoff *et al.,* 2010) and Enzalutamide (Scher *et al.,* 2012) have a greater average gain in survival (4.1 months and 4.8 months respectively), but are much more expensive as they are covered by a patent and so not as widely available to patients (Attard *et al.,* 2015).

Particularly aggressive cancers can contain regions that have an inadequate oxygen supply, which are referred to as hypoxic. These areas can reduce the effectiveness of radiotherapy and chemotherapy. Therefore, there has been extensive research by several groups to try and combat this problem; and these are outlined below.

1.2 Hypoxia

Hypoxia is defined as the state in which the availability or delivery of oxygen is insufficient to meet tissue demand (Loboda and Dulak, 2012). This occurs particularly in aggressive cancers (Höckel *et al.,* 1999; Evans *et al.,* 2004), as vascular formation cannot keep up with the rate of cancer growth. Cells at the centre of a tumour are particularly susceptible to hypoxia as there is a lack of necessary local vasculature, which causes cellular stress. In severe hypoxic conditions and in the absence of nutrients, apoptosis can be triggered (Semenza, 2000; Carmeliet *et al.,* 1998); however, some cells are able to cheat cell death and survive and thrive.

1.2.1 Hypoxia and cancer therapy

Since the 1950s, it has been well established that hypoxia has a significant effect on the effectiveness of radiotherapy on tumours (Gray *et al.,* 1953; Evans and Koch, 2003; Vaupel and Mayer*,* 2007; Wilson and Hay, 2011). Radiotherapy works by directing high-energy photons to the tumour site. Photons can interact with molecules within the body and form radicals, which can then damage DNA. Interaction with a water molecule produces a hydroxyl radical ($HO^{\cdot}$) through the loss of a proton which can cause DNA-base damage. However, this is a common type of DNA damage and is usually repairable allowing the cancer cell to survive and thrive. If however, there is an interaction with an oxygen molecule, a peroxy radical ($HO_2^{\cdot}$) is produced which damages DNA beyond repair, known as 'fixing' and

therefore cancer cells undergo apoptosis causing the tumour to shrink (Grimes and Partridge, 2015). Therefore, in hypoxia where there is a low level of oxygen molecules, radiotherapy is not a very effective form of treatment.

Tumour hypoxia can also provide resistance to chemotherapeutic drugs (Zhou *et al.,* 2016; Hoang *et al.,* 2016). This is due to several reasons. Firstly, as chemotherapeutic drugs travel around the bloodstream, they will be at their highest potency around the surrounding blood vessels and at a much lower concentration near the embedded hypoxic regions of a tumour. Therefore the drug may not be at the desired concentration for cell killing when it has reached the hypoxic region; in fact it may not even make it to some areas of the hypoxia region if at all (Brown, 1999). Secondly, hypoxic cells generally progress through the cell cycle much more slowly than the rapidly proliferating cells that most chemotherapeutic drugs target, and may even induce cell cycle arrest (Brown, 1999). HIF-1α dependent transcription of p21 and p27, which are involved in negative regulation of the cell cycle, have been particularly attributed to hypoxic initiation of cell cycle arrest (Koshiji *et al.,* 2004; Goda *et al.,* 2003). In effect, the hypoxic oncogenic cells are able to 'hide' from the anti-oncogenic drugs. Thirdly, the expression of proteins that are involved in drug resistance may be up-regulated, providing protection to the cancer cell from anticancer drugs, such as: metallothionein-IIA (MT-IIA) in cisplatin resistance (Murphy *et al.,* 1994), and periostin in arsenic trioxide resistance (Liu *et al.,* 2017).

As tumour hypoxia limits the effectiveness of therapies, it has been linked to poor prognosis (Bos *et al.,* 2003; Evans and Koch, 2003) and therefore there has been

research into new methods to treat hypoxic tumours. Interestingly, a recent publication by Koyama *et al.* (2017) has shown that induction of the hypoxia inductible factor pathway (Figure 1.2) through chemical inhibition of the prolyl hydroxylase (PHD) family improved blood vessel abnormalities in hypoxic tumours, and ultimately, improved sensitivity to chemotherapeutic drugs. This is thought to be due to activation of the HIF pathway results in the transcription of genes required to withstand the harsh hypoxic tumour microenvironment, including genes involved in angiogenesis. Ongoing research into the tumour microenvironment (fluctuating hypoxia, low pH and nutrient deprivation) could also uncover potential therapeutic targets.

1.2.2 Hypoxic tumour microenvironment

Tumour cells have been observed to acquire energy mainly from glucose through glycolysis even in aerobic conditions (Warburg, 1956). This property, termed the 'Warburg Effect' (Warburg, 1956) is well established and forms part of the reprogrammed energy metabolism hallmark of cancer (Hsu and Sabatini, 2008; Hanahan and Weinburg, 2011). Solid tumours also activate other non-glucose dependent pathways, such as glutaminolysis (Deberardinis and Cheng, 2010). Both of these metabolic pathways produce lactic acid, which contributes to metabolic acidosis commonly found in solid cancers (Feron, 2009). The Warburg effect is a lot less energy efficient than the conventional oxidative phosphorylation pathway executed by healthy cells (Hsu and Sabatini, 2008) and therefore many researchers

have speculated advantageous reasons for cancer cells to appoint this type of energy production.

One theory focuses on the increased proliferative property of cancer cells (Vander Heiden, Cantley and Thompson, 2009). Incomplete glucose utilisation exhibited by cancer cells results in less carbon being converted into carbon dioxide. Carbon helps to form biological macromolecules and therefore is able to form building blocks required for rapid proliferation, such as fatty acids and ribose for nucleotides (Vander Heiden, Cantley and Thompson, 2009). Another proposal for the Warburg effect is that acidification of the micro-environment by lactic acid selects cells with an acid-resistant phenotype (Gatenby and Gillies, 2004). These cells have a growth advantage that encourages cellular proliferation and invasion. In addition, lactic acid is found to increase the production of interleukin-8, which promotes angiogenesis and therefore drives the growth of new blood vessels to the proliferating cells (Polet and Feron, 2013). Moreover, it is now also suggested that the function of immune cells can be impeded by an acidic environment (Lardner, 2001). Calcinotto *et al.* (2012) showed that acidifying the microenvironment to a pH that is generally found in tumours (pH6.0 – 6.5), can lead to the loss of T-cell function, but this effect can be reversed at physiological levels. Therefore, it is suggested that perhaps lactate is produced to inhibit the immune response against oncogenic cells in order to promote their survival (Choi *et al.,* 2013).

1.2.3 The hypoxia inducible factor (HIF) pathway

The hypoxia inducible factor (HIF) pathway is activated during hypoxia. It activates the transcription of genes that enable the cell to withstand the hypoxic tumour microenvironment. These include genes that regulate the intracellular pH (pH_i) such as the carbonic anhydrases (Pastorekova, Parkkila and Zavada, 2006), bicarbonate transporters (Karumanchi *et al.*, 2001), and monocarboxylate transporters which can carry lactate across biological membranes (Halestrap and Meredith, 2004). In addition, genes involved in increasing oxygen delivery to hypoxic areas are also targeted by the HIF pathway, such as vascular endothelial growth factor (VEGF) that drives angiogenesis (Maxwell *et al.,* 1997) and erythropoietin (EPO) that stimulates red blood cell production (erythropoiesis) (Watowich, 2011).

The HIF complex is comprised of two subunits: an alpha (α) subunit that is sensitive to oxygen levels and a beta (β) subunit, which is ubiquitously expressed. Under normoxic conditions, HIF-α is synthesised but is rapidly degraded by the proteasome. The degradation of HIF-α begins with the hydroxylation of proline residues (Pro^{402} and Pro^{564}) by the prolyl hydroxylase domain protein 2 (PHD2), which allows the von Hippel-Lindau (VHL) tumour suppressor protein to bind to the subunit (Semenza, 2007). This allows an interaction with the Elongin C protein which recruits an E3 ubiquitin-protein ligase complex that ubiquinates the subunit, thereby targeting it for degradation by the proteasome (Figure 1.2).

During hypoxia, the degradation of HIF-α is inhibited through reduced activity of PHD2. As oxygen is one of the substrates of PHD2, during hypoxic conditions PHD2

activity is limited (Epstein *et al*., 2001). In addition, it has been shown that the catalytic centre of PHD2 is inhibited by reactive oxygen species (ROS) that are generated by complex III of the mitochondrial respiratory chain (Semenza, 2007). This allows the HIF-α subunit to accumulate and dimerise with the HIF-β subunit. The complex is then able to translocate to the nucleus and bind to *cis*-acting hypoxia response elements (HREs) in target genes, which leads to increased transcription and subsequent elevated expression of genes required in the HIF response (Jewer, Findlay and Postovit, 2012) (Figure 1.2). It is through this mechanism that HIF-1α regulates the expression of a spectrum of genes with a variety of functions.

HIF transcriptional activity is further regulated by factor inhibiting HIF-1 (FIH-1). During normoxia FIH-1 hydroxylates an asparagine residue in the *c*-terminal transactivation domain of HIF-α. This prevents the formation of the DNA binding complex necessary for transcription, including a reduced interaction with p300/CBP transcription co-activators (Mahon *et al.,* 2001; Lando *et al.,* 2002). This mechanism is particularly useful when normoxia is restored after a period of hypoxia as it inhibits further transcription of hypoxia-associated genes by the activated HIF complex.

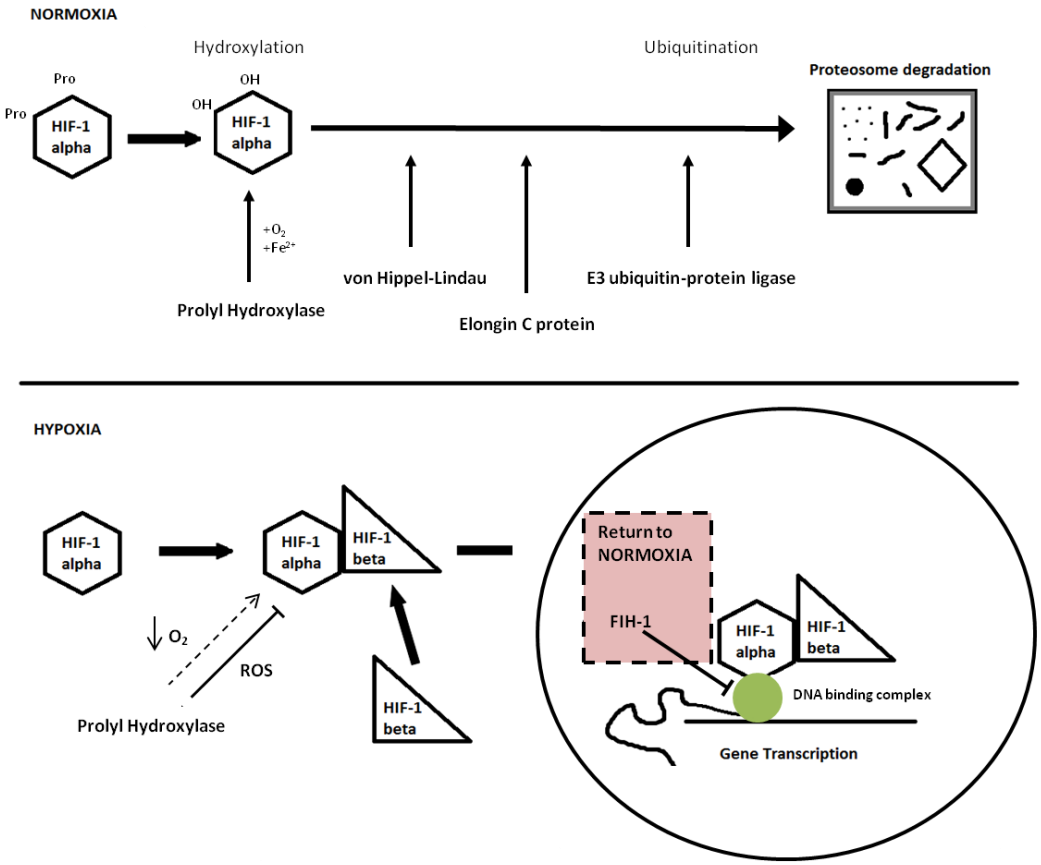


Figure 1.2: The HIF-α pathway during normoxia and hypoxia. During normoxia HIF-α proline residues are hydroxylated by prolyl hydroxylase which allows the binding of VHL and ubiquitin. This signals the protein for degradation by the proteasome. During hypoxia, low oxygen levels prevent maximum prolyl hydroxylase activity, and prolyl hydroxylase is inhibited by ROS generated by the mitochondria. HIF-α is therefore stabilised and able to form a dimer with HIF-β. The HIF complex translocates to the nucleus for transcription of genes required in the HIF response. Figure illustrated using text from Semenza (2007).

1.2.4 Genes expressed as a result of Hypoxia Inducible Factor (HIF) activation

The first gene that was found to be activated by HIF-1 was *Erythropoietin* (Semenza and Wang, 1992). Erythropoietin is a glycoprotein hormone that is important for the proliferation and differentiation of erythroid progenitor cells into mature erythrocytes (Krantz, 1991). This aids cellular survival during hypoxic stress, as an influx of mature erythrocytes can transport more oxygen to the hypoxic areas through newly synthesised blood vessels that are created via angiogenesis. Indeed,

HIF-1α also activates genes involved in angiogenesis, such as *VEGF* (Maxwell *et al.,* 1997) and therefore HIF-1α is able to up-regulate genes that aid in reoxygenation and provide nutrients to deprived cells.

Carbonic anhydrase IX (*CA IX*) is also activated by HIF-1α during hypoxia (Wykoff *et al.,* 2000), and is involved in the cellular response to the acidic microenvironment that is generated by the lactic acid produced during aerobic glycolysis (Chiche *et al.,* 2009). Cells require a neutral to fairly alkaline internal pH for efficient biological reactions and survival. Therefore, hypoxia up-regulates the export of lactate and protons out of the cell via transmembrane ion transporters, such as monocarboxylate transporter (MCT4) and Na^+/H^+ exchanger 1 (NHE1) (Yilmaz and Christofori, 2010; Parks, Chiche and Pouyssegur, 2011). In addition, bicarbonate is imported into the cell via bicarbonate transporters, which help to buffer the internal pH to the required level for survival. Eventually however, the bicarbonate ions become depleted in the increasing acidic microenvironment (Svastova and Pastorekova, 2013). CA IX plays a vital role in solving this problem.

Carbonic anhydrase IX forms metabolons with bicarbonate transporters; AE1, AE2 and AE3. In the metabolons, CA IX catalyses the hydration of CO_2 found in the microenvironment (Morgan *et al.,* 2007). This reaction produces a high level of bicarbonate ions, which are then transported into the cell by the bicarbonate transporters (Pastorekova, Ratcliffe and Pastorek, 2008). This neutralises the intracellular environment, which is required for survival. Protons are produced as by-products of the CO_2 hydration reaction, which further acidifies the microenvironment and supports tumour cell invasiveness (Ditte *et al.,* 2011).

In fact, CA IX is found to be expressed in only a few healthy tissues (such as the stomach mucosa and intestinal crypts) whereas it is expressed in solid tumours all over the body, particularly those with an aggressive phenotype (Svastova and Pastorekova, 2013). A number of studies have linked CA IX expression with cellular migration and invasion. CA IX over-expression promoted cell migration and invasion whereas suppression of CA IX activity reduced these processes (Svastova *et al.,* 2012; Radvak *et al.,* 2013; Sansone *et al.,* 2009). It is thought that the role of CA IX in the acid-base reaction could aid tumour cells for invasion, migration and ultimately metastasis.

CA IX is argued to be a more appropriate marker for hypoxia than HIF-1α, as the latter is lost rapidly upon re-oxygenation after hypoxia. In contrast, CA IX expression remains stable upon re-oxygenation and is only lost upon cell death or division (Sobhanifar *et al.,* 2005). Therefore, CA IX was used as the hypoxia marker of choice in this project.

In addition to increased expression of some genes, there are also a few genes found to be down-regulated by hypoxia, and it was shown using HIF-1α -/- cells that 17% of these genes are HIF-1α dependant (Greijer *et al.,* 2005). These include genes involved in DNA repair (REV3), mRNA processing (hnRNP H1) and cytoskeleton maintenance (Rho kinase) (Greijer *et al.,* 2005).

1.2.5 HIF-α subunits

Three types of HIF alpha subunits have been discovered; HIF-1α, HIF-2α and HIF-3α. HIF-1α and HIF-2α share 48% amino acid sequence homology and both are able to dimerise to HIF-1β. Both types of HIF complex are able to bind to the same HIF response element sequence and initiate transcription of target genes (Tian, McKnight and Russell, 1997). Genetic inactivation studies have indicated differences in the genes that are targeted by the two subunits, and previous studies have identified ubiquitously expressed HIF-1α to be the dominant subunit for hypoxic-induced transcription (Hu *et al.,* 2003; Park *et al.,* 2003; Sowter *et al.,* 2003). Furthermore, only HIF-1α has been found to target genes that are involved in the glycolytic pathway (Hu *et al.,* 2003; Raval *et al.,* 2005).

HIF-2α expression was previously thought to be more restricted to cell type, with abundant HIF-2α expression found in blood vessels. This led to the theory that HIF-2α regulated endothelial cell function, which was supported by a correlation between HIF-2α and VEGF expression (Tian, McKnight and Russell, 1997). However, there is now evidence for hypoxic-induced HIF-2α expression in a number of organs in the rat, including the brain, heart, lung, kidney, liver, pancreas and intestine (Wiesener *et al.,* 2003). In addition, other groups have also identified HIF-2α as an initiator of gene transcription during the hypoxic response (Hu *et al.,* 2003).

HIF-1α and HIF-2α have been shown to have opposing effects on the same hypoxic-inducible genes. For example, it was shown in renal clear cell carcinoma (RCC) cells that HIF-1α over-expression restricted tumour growth, whilst an elevation of HIF-2α

expression drove tumour progression (Raval *et al.,* 2005). Furthermore, HIF-1α has been shown to inhibit the function of the cell cycle progression transcription factor *MYC* during hypoxia and there is also evidence that HIF-1α promotes the degradation of *MYC* under chronic hypoxia (Keith, Johnson and Simon*,* 2012). In contrast, HIF-2α has been shown to enhance *MYC* activity and promotes cell cycle progression in hypoxic cells (Gordan *et al.,* 2007). Therefore, the activity of HIF-dependent cellular responses during tumour hypoxia may be governed by the levels of HIF-1α and HIF-2α complexes. There is evidence to suggest that HIF-1α may control the acute hypoxic response, whilst HIF-2α may dominate chronic hypoxia activity (Henze and Acker, 2010). It is thought that hypoxia-associated factor (HAF) is responsible for the switch in alpha subunit between acute and chronic hypoxia (Koh *et al.,* 2011).

Less is known about the HIF-3α subunit, although human HIF-3α has been shown to be alternatively spliced to form at least seven different splice variants (Heikkila *et al.,* 2011). There is evidence to show that HIF-1α and hypoxia respectively, up-regulate the expression of all HIF-3α isoforms (Pasanen *et al.,* 2010). Some HIF-3α isoforms are reported to be able to promote HIF-1α and HIF-2α activity, whereas other isoforms inhibit activity of the subunits (Keith, Johnson and Simon, 2012). It is therefore suggested that HIF-3α regulates HIF-1 and HIF-2 activity; and may also be regulated by hypoxia, although more research is needed to confirm this possibility.

1.3 Change in post transcriptional regulation in hypoxia

Gene transcription is not the only process that has been found to be regulated by hypoxia. There are a number of post-transcriptional processes that have also been shown to be modified during hypoxia, such as translation and alternative splicing.

1.3.1 Translation: Internal ribosome entry sites (IRES)

Conventional translation initiation begins with the recruitment of the eIF4F complex to the cap on the 5' end of the mRNA. This in turn attracts the 43S pre-initiation complex, formed of the small ribosomal subunit, tRNA, GTP and the elongation initiation factor- 2 (eIF2), to the start codon of mRNA (Galban and Gorospe, 2009). However, during cellular stress such as hypoxia, cap-dependent translation is inhibited in order to conserve energy for cellular survival. In fact, cells exposed to acute hypoxia were found to decrease overall translation by 60-70% (Kraggerud, Sandvik and Pettersen, 1995).

Cap-dependent translation inhibition is regulated during hypoxia via two main mechanisms. The first involves the inactivation of mTOR, which results in the hypo-phosphorylation of eIF4E binding proteins (4E-BP) and subsequently increases their affinity for the eIF-4E cap-binding protein. This results in a reduced rate of cap-dependent translation (Richter and Sonenberg, 2005). A second mechanism requires the activation of the protein-kinase-RNA-like-endoplasmic-reticulum-kinase (PERK) by hypoxia, which inhibits cap-dependent translation through phosphorylation of the translation initiation factor eIF2-α (Koumenis and Wouters, 2006).

To overcome this, some mRNAs, particularly those that function during periods of cellular stress, contain internal ribosome entry site (IRES) sequences that allow the initiation for translation at a location that is a substantial distance from the cap structure (Morfoisse *et al.,* 2015). Indeed, HIF-1α contains an IRES sequence, which allows cap-independent translation of the alpha subunit during hypoxia (Lang *et al.,* 2002). Other mRNAs associated with cancer progression and the hypoxic response have also been identified to have IRES sequences. Vascular endothelial growth factor-A (VEGF-A) (Huez *et al.,* 1998) and basic fibroblast growth factor (Vagner *et al.,* 1995) both contain IRES sequences in their mRNA and are of particular interest as they are involved in angiogenesis and therefore are heavily implicated in both the hypoxic response and tumour progression.

It has also recently been reported that vascular endothelial growth factor-C (VEGF-C) mRNA contains an IRES sequence, which is increased during hypoxia (Morfoisse *et al.,* 2015). VEGF-C is involved in lymphangiogenesis, which enhances tumour migration, and ultimately metastases. Therefore it can be proposed that the cap-independent translation of VEGF-C may be a mechanism through which hypoxia can drive tumour migration.

1.3.2 Pre-mRNA splicing

After gene transcription, splicing of eukaryotic pre-mRNAs involves the excision of introns and joining together of exons, which produces mature mRNA for translation (Zhou and Fu, 2013). Pre-mRNA splicing must be precise in order to avoid errors

that could disrupt the open reading frame (ORF), which could produce truncated proteins (Biamonti *et al.,* 2012). This is achieved by the spliceosome, a structure comprised of protein and RNA that undergoes several structural changes throughout the reaction to ensure that only proper splice sites are selected (Biamonti *et al.,* 2012). The spliceosome is made up of five small nuclear ribonucleoparticles (snRNPs); U1, U2, U4, U5 and U6 (Figure 1.3), and 100-200 non-snRNPS, which can be sub-categorised into RNA-binding proteins; U2 auxiliary factor (U2AF), splice factor 1 (SF1) and serine-arginine rich proteins (SR proteins), and enzymes; RNA helicases, protein kinases and phosphatases (Biamonti *et al.,* 2012).

To begin, the 5' and 3' splice sites of an intron have to be recognised. This is achieved by the E complex, which is formed of the U1 snRNP at the 5' splice site; and SF1, U2AF65 and U2AF35 at the branch-point, polypyrimidine tract and 3' splice site respectively. The consensus sequence at the 5' end of the intron ('*CAG GURAGU*' in humans) is bound by the U1 snRNP through base-pairing interactions (Wu and Manley, 1989; Elliott and Ladomery, 2011). At around the same time, SF1 binds to the branch point sequence ('*YNYURAC*' in humans) and the downstream polypyrimidine sequence of the branch point is bound by the 65kDa U2 auxiliary factor (U2AF65) (Graveley, 2000). In addition, the U2AF35 subunit of the U2AF hetero-dimer binds to an AG dinucleotide in the 3' splice site. Following this, the U2 snRNP displaces the SF1/BBP protein at the branch point sequence, which forms the A complex (Graveley, 2000). This interaction is stabilised by an arginine-serine-rich domain in the U2AF65 protein (Valcarcel *et al.,* 1996), and through protein

complexes within the U2 snRNP itself, particularly with the SF3a and SF3b subunits (Gozani *et al.,* 1996). Next, the U5.U4/U6 tri-snRNP is recruited to the U1 snRNP, which forms the B complex. The B complex undergoes conformational and compositional rearrangements, which activate its catalytic function for the first transesterification reaction (Jurica and Moore, 2003). The activated B complex is known as the B* complex and requires the release of U1 and U4 snRNPs. The activated spliceosome undergoes the first transesterification step at the 5' splice site which generates an intron lariat-3' exon and a 'free' 5' exon (C complex). An additional transesterification reaction at the 3' splice site releases the 3' exon and this leads to exon ligation and excision of the looped shaped intermediate, known as the lariat, which contains the intron and U4, U5 and U6 snRNPs. The snRNPs released during the splicing reaction are recycled for additional splicing reactions (Wahl, Will and Lührmann, 2009; Jurica and Moore, 2003) and the spliced mature mRNA transcript is translated into protein (Figure 1.3).

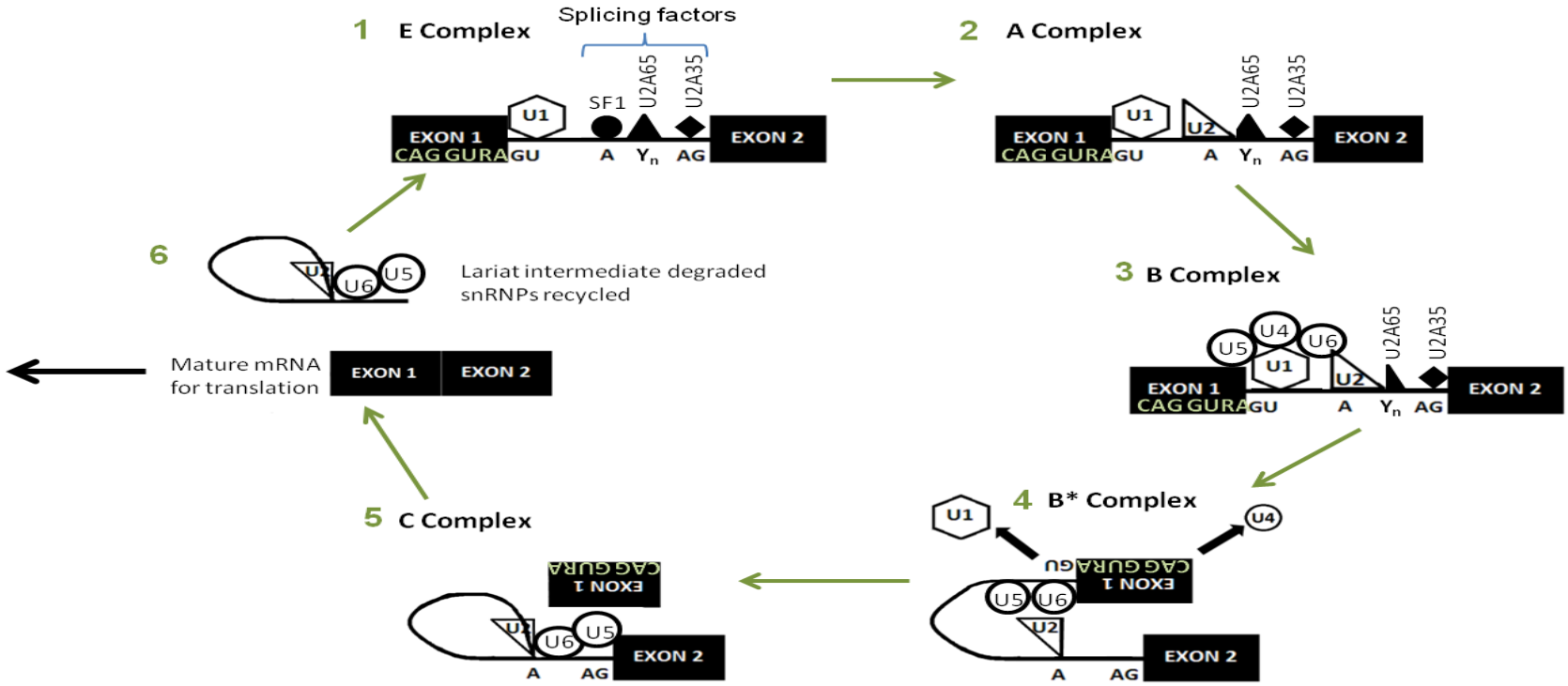


Figure 1.3: The Spliceosome Cycle for Splicing of Pre-mRNAs . 1. Complex E formation: U1 snRNP binds to the '*CAG GURAGU*' sequence at the 5' splice site on the intron. SF1, U2A65 and U2A35 bind to the branch-point, polypyrimadine tract and 3' splice site AG dinucleotide respectively. **2. Complex A formation:** The U2 snRNP displaces SF1 at the branchpoint sequence. **3. B complex formation:** The U5. U4/U6 tri-snRNP is recruited to the U1 snRNP. The B complex undergoes conformational and compositional rearrangements that activate its catalytic function. **4. B* complex formation:** U1 and U4 snRNPs are released and the activated B* complex undergoes the first transesterification reaction at the 5' splice site. **5. C complex formation:** This generates an intron lariat-3' exon and a free 5' exon. **6.** A second transesterification reaction at the 3' splice site releases the 3' exon and this leads to excision of the lariat intron which is degraded. The exons are ligated together and the mature mRNA is ready for translation. The snRNPs are recycled for additional splicing reactions.

1.3.3 Alternative splicing

Alternative splicing allows the formation of multiple mRNA products to be generated from a single pre-mRNA. More than 94% of pre-mRNAs are alternatively spliced in humans, which gives rise to at least 100,000 distinct proteins (Biamonti *et al.,* 2012), despite there only being an estimated 20,000-25,000 genes (International Human Genome Sequencing Consortium, 2004). Alternatively spliced isoforms that arise from the same gene can have distinct properties and can even have opposing functions (Biamonti *et al.,* 2012). For example, there are a number of apoptotic genes that can produce anti- or pro-apoptotic isoforms through alternative splicing of the pre-mRNA (Schwerk and Schulze-Osthoff, 2005). Other spliced transcripts may house premature stop codons, which signal mRNAs for nonsense-mediated decay (NMD) (Schwerk and Schulze-Osthoff, 2005). The NMD pathway prevents truncated proteins from being translated; however, both of these serve to negatively regulate the expression of the main protein (Chang, Imam and Wilkinson, 2007).

There are five major alternative splicing patterns (Figure 1.4), which materialise through the recognition of short *cis*-acting sequences (frequently referred to as silencers and enhancers) by *trans*-acting factors, also known as RNA binding proteins (RBPs), at the intron-exon boundaries (Biamonti *et al.,* 2012).

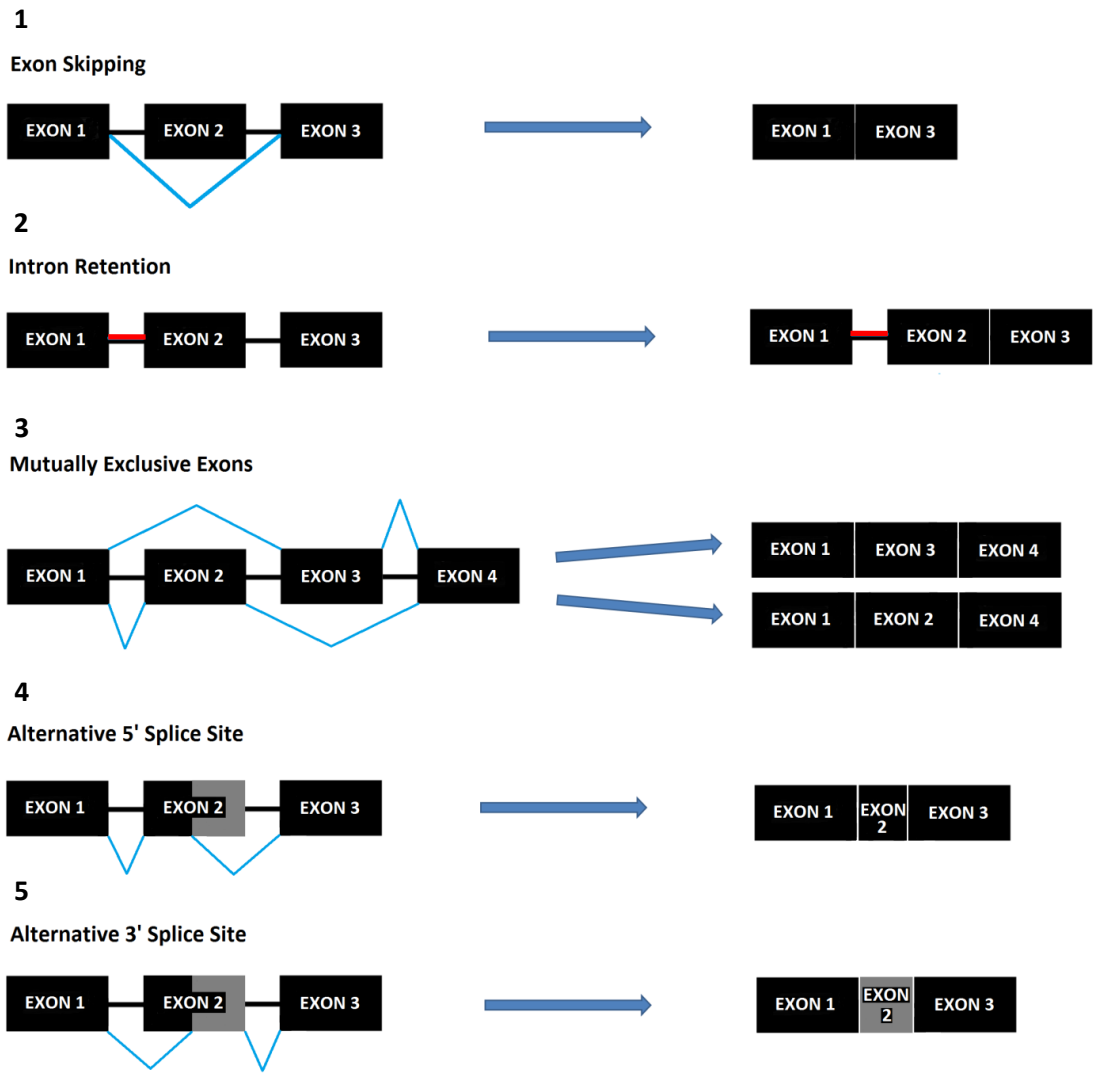


Figure 1.4: The five major alternative splicing patterns, which can arise through various combinations of donor and acceptor sites of exons. (1) Exon skipping: Exons are skipped and therefore not present in the exon sequence that is spliced together. **(2)** Intron retention: An intron is retained in the exonic sequence (shown in red). If this occurs in the coding region, this could lead to a change in the protein produced, which could result in a non-functional protein. **(3)** Mutually exclusive exons: Only one of two exons is selected for splicing, but not both, which can lead to different proteins being produced from the same pre-mRNA sequence. **(4)** Alternative 5' splice site: An alternative 5' splice site is selected, resulting in the change of the 3' splice site, and therefore splicing out some of the exonic sequence from the exon involved. **(5)** Alternative 3' splice site: An alternative 3' splice site is used, which changes the location of the 5' splice site and splices out some of the sequence including the original 5' splice site. Figure adapted from Biamonti *et al.* (2012) and used with permission from the author.

1.3.4 Regulation of alternative splicing

RNA binding proteins (RBPs) influence where splicing occurs in the pre-mRNA and ultimately which sections of pre-mRNA are included in the mature spliced transcript (Chen and Manley, 2009; Nilsen and Graveley, 2010). Alternative splicing can therefore be regulated by changes in the levels and activity of RBPs (David and Manley, 2010). There are many different RBPs, but the two main studied groups are serine/arginine rich (SR) proteins and heterogenous nuclear ribonucleoproteins (hnRNPs).

RBPs regulate constitutive and alternative splicing by promoting splice site selection via cis-acting splicing regulatory elements (SREs), which are grouped depending on where in the pre-mRNA the RBP is binding (exon or intron) and the net effect this has on splicing (enhance or inhibit). This results in four categories of SREs; exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs) (Matlin, Clark and Smith, 2005).

The SR proteins serine/arginine-rich splice factor – 1 and -2 (SRSF1 and SRSF2) are essential regulators of constitutive and alternative splicing as they promote the binding of U1 and U2 snRNPs to the 5’ and 3’ splice site, respectively (Corkery *et al.,* 2015). As SR proteins are key players in the initiation of splicing, the binding of SR proteins to pre-mRNA is heavily regulated through phosphorylation events from SR protein kinases such as those found in the CDC-like-kinase (CLK) and SRSF-protein-kinase (SRPK) protein kinase families (Ghosh and Adams, 2011). It has been shown

that the arginine/serine (RS) domains of SRSF1 have to be hyper-phosphorylated in order to facilitate the binding of the U1 snRNP to the 5' splice site (Cho *et al.,* 2011), and de-phosphorylation of SRSF1 is necessary for the catalysis of the first transesterification reaction (Cao*,* Jamison and Garcia-Blanco, 1997). Therefore, there has been great interest in the regulation of SR proteins through phosphorylation by SR protein kinases.

The CLK and SRPK protein kinase families

Two of the most established splice factor kinase families are the CLKs and SRPKs. It has been suggested that CLKs play an important role during development, as mutations in the Drosophila CLK homologue, Darkener of Apricot (DOA) are embryonic lethal and lead to segmentation abnormalities, eye formation and neuronal development (Yun *et al.,* 1994). The CLK family consists of four members (CLKs 1-4) which all share the *C*-terminal kinase domain that contains the '*EHLAMMERILG*' signature motif and are therefore referred to as LAMMER kinases (Yun *et al.,* 1994). The *N*-terminus of CLKs contain a nuclear localisation signal and RS dipeptides where SR proteins bind (reviewed in Bates *et al.,* 2016). CLKs are documented to be dual-specificity kinases, which are able to phosphorylate their substrates on serine and threonine residues, but are also able to auto-phosphorylate themselves on serine, threonine and tyrosine residues (reviewed in Bates *et al.,* 2016). Alternatively spliced isoforms that encode truncated proteins

which lack the kinase domain have been discovered for all four CLK family members and are suggested to regulate CLK activity (Duncan *et al.,* 1995; Hanes *et al.,* 1994).

The SRPK family (SRPK 1-3) is characterised by a well conserved bipartite catalytic domain which is separated by a non-conserved spacer sequence (Ghosh and Adams, 2011). It has been shown that SRPKs prefer to phosphorylate on serine residues (Wang *et al.,* 1998), and on average SRPK1 phosphorylates five to eight of the available 12 serines in the RS1 domain of SRSF1 (Ghosh and Adams, 2011). SRPK family members are constitutively active (Ngo *et al.,* 2007; Ghosh and Adams, 2011), and so they do not require post-translational modifications for stimulation.

Although SRPK1 has been shown to be active in both the cytoplasm and nucleus, the localisation of the protein may be one way that its activity is regulated. Ding *et al.* (2006) provided evidence that the spacer sequence encodes for a cytoplasmic localisation signal. They showed that deletion of the spacer sequence altered the cellular distribution of SRPK1 from nuclear-cytoplasmic to completely nuclear. In the cytoplasm phosphorylation of SR proteins facilitates nuclear import (outlined below).

Regulation of SR proteins by SR protein kinases

SR proteins have N-terminal domains consisting of RNA recognition motifs (RRMs) that bind RNA and C-terminal arginine-serine rich (RS) domains, which are the sites of phosphorylation (Long and Caceres, 2009). SRSF1 contains two RS domains consisting of arginine/serine repeats. When the RS domain of SRSF1 is in a hypo-

phosphorylated state, it stimulates interaction with its own RRM (Cho *et al.,* 2011). Phosphorylation releases the RS domain and promotes spliceosome assembly through the binding of the available RRM with the U1 snRNP (Cho *et al.,* 2011). It has been suggested that the first RS domain of SRSF1 is targeted by SRPK1 phosphorylation in the cytoplasm, which facilitates binding of SRSF1 to a transportin protein, (Tr-SR) (Yeakley *et al.,* 1999; Lai *et al.,* 2000). This allows for the export of SRSF1 into the nucleus where it is stored as nuclear speckles. Subsequent phosphorylation of the RS2 domain of SRSF1 by CLK1 results in the release of the SR protein from nuclear speckle storage in order to regulate splicing (reviewed in Naro and Sette, 2013). In accordance with this mechanism, it has been shown that SRPK1 is predominantly found in the cytoplasm and nucleus, whereas CLK1 is thought to be a nuclear protein (Aubol *et al.,* 2013).

After splicing is completed, SR proteins are released from mRNA through de-phosphorylation by nuclear phosphatases, such as protein phosphatases 1 and 2A (PP1 and PP2A) (Naro and Sette, 2013). Once released, they are either exported into the cytoplasm to assist in other duties such as the regulation of translation or are re-phosphorylated and stored once again as nuclear speckles for the next round of splicing (reviewed in Naro and Sette, 2013) (Figure 1.5).

<u>The competition between SR and hnRNP families of splice factors for SREs</u>

It has been found that proteins in the heterogenous nuclear ribonucleoprotein (hnRNP) A/B family and SR proteins can antagonise one another when binding to

SREs (reviewed in Kedzierska *et al.,* 2017). In general, SR proteins promote exon inclusion by binding to exonic splicing enhancers (ESEs), whereas hnRNPs bind to exonic and intronic splicing silencers (ESS and ISS) thereby promoting exon exclusion (reviewed in Jean-Philippe, Paz and Caputi, 2013). Therefore, changes in the molar ratio of hnRNP protein to SR protein in the nucleus can affect splicing.

In addition to the regulation of splicing, RBPs have also been found to have roles outside of the splicing mechanism. For example, SRSF1 is involved in nuclear export regulation (Huang *et al.,* 2003), mRNA stability (Lemaire *et al.,* 2002), microRNA processing (Wu *et al.,* 2010), translation (Sanford *et al.,* 2004) and nonsense-mediated mRNA decay (Zhang and Krainer, 2004).

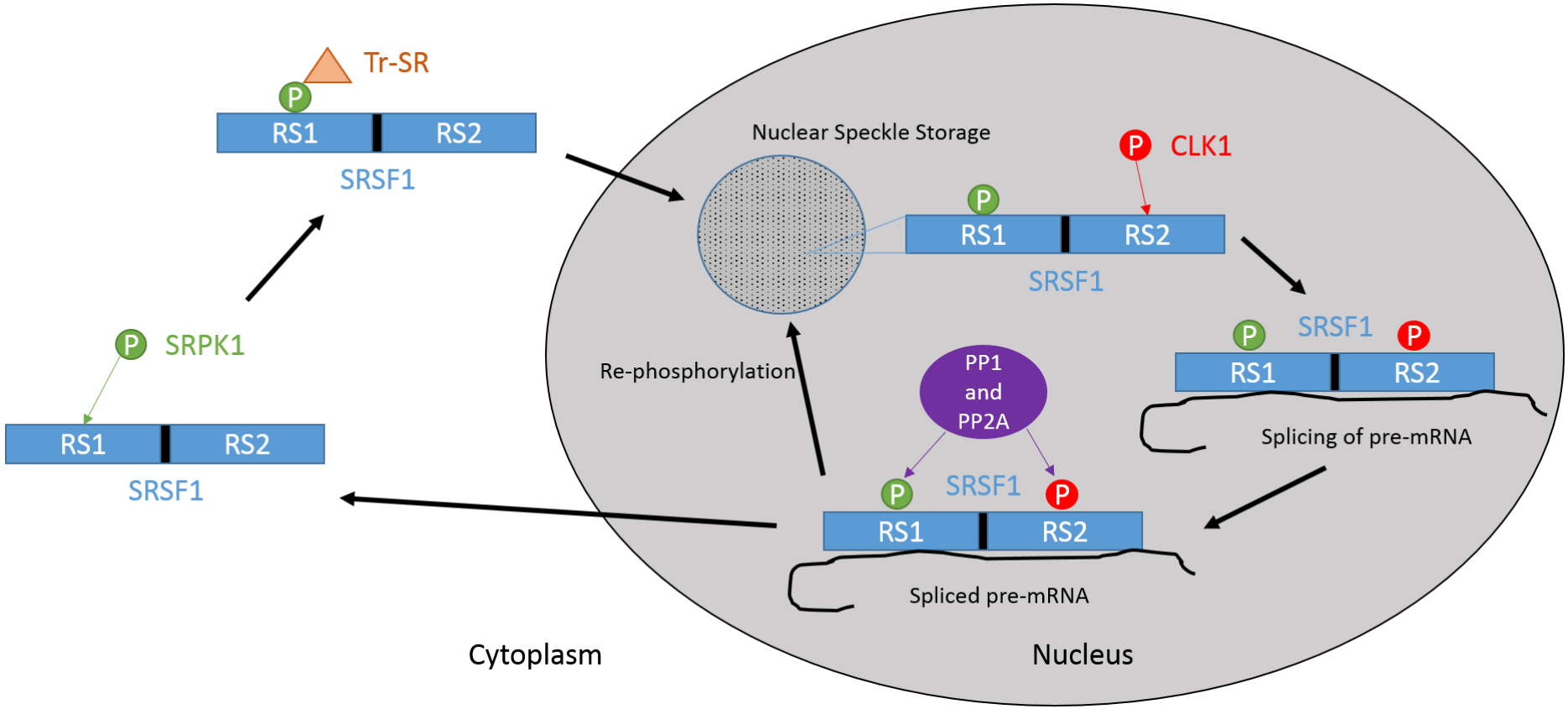


Figure 1.5: Change in localisation of SRSF1 through phosphorylation by SRPK1 and CLK1 splice factor kinases. SRPK1 phosphorylates the first SRSF1 RS domain, which enables the TR-SR transportin protein to bind and translocate SRSF1 into nuclear speckle storage in the nucleus. CLK1 phosphorylation releases SRSF1 from nuclear speckle storage and SRSF1 is able to take part in the splicing of pre-mRNA. PP1 and PP2A phosphatases dephosphorylate SRSF1 and it then either moves back to the cytoplasm or it is re-phosphorylated and stored as nuclear speckles.

1.3.5 Alternative splicing during cellular stress

Stress causes alterations in gene expression in order to allow cells to adapt to their changed envirionment. In accordance with this, alternative pre-mRNA splicing patterns can be modified to aid cellular survival during cellular stress. Conversely, in the late 1980s to early 1990s, it was documented that severe heat shock inhibits splicing in eukaryotic cells (Yost and Lindquist, 1986; Bond, 1988; Shukla *et al.,* 1990). Furthermore, a study by Shalgi *et al.* (2014) also revealed that severe heat shock inhibits the splicing of more than 1700 genes in mouse fibroblast cells.

However, some heat shock proteins (Hsp), such as Hsp90a (Jolly *et al.,* 1999), Hsp47 (Takechi, 1994) and Hsp105 (Yasuda *et al.,* 1999) are alternatively spliced during heat shock. Furthermore, recently Yamamoto *et al.* (2016) also provided evidence of 4,260 genes that are alternatively spliced during mild heat stress at 42°C. Using gene ontology analysis, the team showed that alternative splicing of genes involved in the cell cycle, ubiquitination, mRNA metabolism and the stress response were affected duing heat stress. Alternative splicing of the retained intron variant of CLK1 was also shown to be induced during mild heat stress, which echoes the previous finding by Ninomiya *et al.* (2011), which also provided evidence that osmotic stress also induced expression of the intron retained variant of CLK1. The study by Yamamoto *et al.* (2016) also revealed that SRSF10, SRSF2 and hnRNP K are all involved in alternative splicing regulation during heat stress, which suggests that RNA binding proteins influence alternative splicing during heat shock stress.

In light of the above, it is proposed that severe heat shock inhibits splicing, whereas mild heat shock allows alternative splicing of certain genes to take place in order to allow cells to survive. Perhaps there is a gradual diminish in splicing with increased heat shock temperature. In agreement with this, it was shown that the U4. U5. U6 snRNP complex was disrupted at both 43 and 46°C; whereas the U1 and U2 snRNPs were unaffected at 43°C but were affected at 46°C (Bond, 1988).

Furthermore, other types of cellular stress have also been documented to alter alternative splicing. Osmotic shock induced by sorbitol treatment has been shown to increase SRPK1 nuclear localisation (Zhong *et al.,* 2009). It could be postulated that a higher level of SRPK1 in the nucleus would encourage splicing; however it has been shown to hampen splicing. This is because excessive amounts of SRPK1 can cause hyperphosphorylation of SR proteins, which inhibits their splicing activity. In contrast, osmotic stress and UV radiation respectively, lead to phosphorylation and relocalisation of HnRNP A1 from the nucleus to stress granules in the cytoplasm (Guil, Long and Caceres, 2006). This suggests that hnRNP A1 mediated splicing is reduced during osmotic stress.

Genotoxic stress has also been shown to affect splicing. Chalfant *et al.,* (2002) provided evidence that the chemotherapeutic agent, gemicitabine induced apoptosis in A549 lung adenocarcinoma cells through the formation of Bcl-xs and caspase-9a pro-apoptotic splice variants. Furthermore, the group also observed that gemicitabine caused a down-regulation of the Bcl-xL and caspase-9b splice variants, which inhibit apoptosis. This provides evidence that chemotherapeutic drugs alter splicing to favour cell death.

Moreover, UV irradiation has also been documented to alter splicing. Both UVB and UVC induced DNA damage and promoted alternative splicing of *human-double-minute-oncogene-2* (*hdm2*). Full-length hdm*2* encodes a protein, which functions as a negative regulator of the tumour suppressor, p53 (Tong and Wu, 2015). P53 transcribes genes required for DNA damage repair, cell cycle arrest, apoptosis and senescence (Levine and Oren, 2009; Vousden and Prives, 2009). On the other hand, the alternatively spliced variant of *hdm2* lacks most of the p53 binding domain and so cannot interact with p53, which suggests that UV irradiation promotes apoptosis through a change in splicing of *hdm2.*

Interestingly, *p53* is itself alternatively spliced, and 13 human p53 splice isoforms have been identified (Marcel *et al.,* 2011). The human protein ∆133p53 forms through transcription of an alternative p53 promoter in intron 4 in response to UV irradiation, and can antagonise p53-mediated apoptosis (Chen *et al.,* 2009; Marcel *et al.,* 2010; Aoubala *et al.,* 2011). Furthermore, ∆133p53 represses cell replication, senescence and promotes tumour progression and angiogenesis (Fujita *et al.,* 2009; Bernard *et al.,* 2013). Gong *et al.* (2015) suggests that both p53 and ∆133p53 have roles in the DNA damage response. The paper suggests that as ∆133p53 promotes DNA double strand break repair, it targets cells with repairable DNA damage, whereas full-length p53 induces apoptosis in cells with severe DNA damage. As alternative splicing has been shown to be altered under the influence of different types of cellular stress, it is no surprise that hypoxic stress has also been shown to change splicing patterns. As hypoxia is a common feature observed in aggressive

cancers, a number of studies that have investigated changes in alternative splicing that influenced by hypoxic stress in cancers.

The effect of hypoxia on alternative splicing in cancer

There have been a number of recent papers that have investigated the genome-wide effect of hypoxia on differential splicing in normal endothelial cells (Hang *et al.,* 2009; Weigand *et al.,* 2012), cartilage endplate-derived stem cells (Yao *et al.,* 2016), Hep3B lung cancer cells (Sena *et al.,* 2014), and most recently, MCF7 breast cancer cells (Han *et al.,* 2017). Using exon microarrays, Weigand *et al.* (2012) provided evidence for altered expression of 294 genes in hypoxia in endothelial HUVEC cells. The group reported an up-regulation of genes associated with glucose metabolism and angiogenesis and a down-regulation of genes that are linked to the cell cycle and DNA repair. This suggests that hypoxia favours cancer progression.

Cancer is profoundly affected by alternative splicing. Changes in splicing of cancer-associated genes linked to various hallmarks of cancer can favour splice isoforms which promote the oncogenic phenotype (Hanahan and Weinberg, 2000, 2011; Biamonti *et al.,* 2012; Ghigna, Valacca and Biamonti, 2008; David and Manley, 2010). Therefore, the manipulation of these alternative splicing events may present future targets for cancer therapies and biomarkers for prognosis (Dales *et al.,* 2010).

Vascular Endothelial Growth Factor (*VEGF*) (Salton, Voss and Misteli, 2014)) and *carbonic anhydrase IX* (*CA IX*) (Barathova *et al.*, 2008) are two cancer-associated

genes that have been found to be affected by alternative splicing in hypoxia. These examples provide evidence that hypoxia can influence shifts in alternative splicing to favour isoforms that promote tumour progression, as follows.

Hypoxia and VEGF splicing

Hypoxia has been linked to a higher expression of VEGF (Liao and Johnson, 2007) which is a key player in angiogenesis. *VEGF* is alternatively spliced due to an alternative, proximal and distal 3' splice site in terminal exon eight (Figure 1.6). The result is a change in a mere six amino-acids at the C terminus of the growth factor. There are also cassette exons in the *VEGF* pre-mRNA, which produce isoforms of differing lengths, for example one of the most abundant in humans is $VEGF_{165}$ where 165 refers to the number of amino-acids in the isoform. The novel splice isoforms due to the distal 3' splice site in exon eight are labelled 'b', hence $VEGF_{165}b$; the b isoforms are anti-angiogenic respectively (Ladomery, Harper and Bates, 2007). The b isoforms are more abundantly expressed in normal adult tissues, such as the renal cortex (Bevan *et al.,* 2008), and found to be down-regulated in oncogenic tissues, such as renal cancers (Bates and Harper, 2002) and prostate cancers (Woolard *et al.,* 2004). It is therefore no surprise that expression of VEGF in oncogenic tissues is derived from the $VEGF_{165}$ form rather than in the anti-angiogenic form. This suggests there is a switch in the splicing of *VEGF* during cancer progression to favour the pro-angiogenic isoform over the anti-angiogenic

isoform in order to produce vasculature that will deliver nutrients to the oncogenic tissue (Bates and Harper, 2002; Woolard *et al.,* 2004).

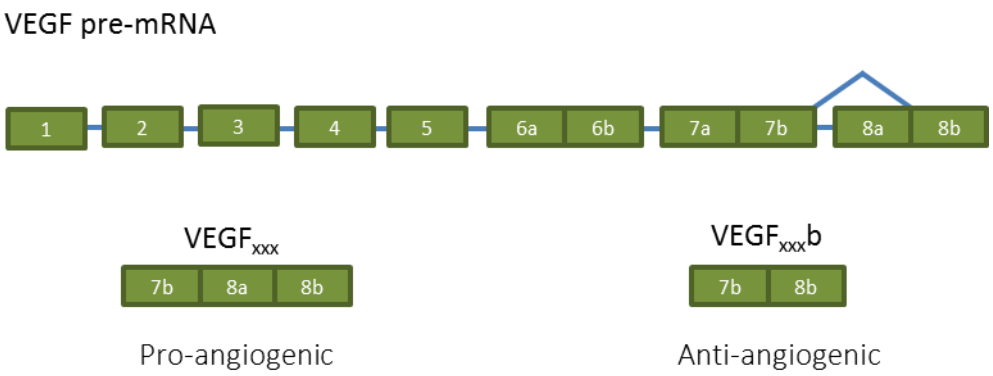


Figure 1.6: VEGF pre-mRNA splicing of exon eight. *$VEGF_{xxx}$ and* $VEGF_{xxx}$b are formed through alternative proximal and distal 3' splice site in terminal exon 8, respectively. *$VEGF_{xxx}$* isoforms are angiogenic, whereas *$VEGF_{xxx}b$* isoforms are considered anti-angiogenic. Exons are shown in green boxes and introns are shown as blue lines. Figure adapted from Ladomery, Harper and Bates (2007) and used with permission from the author.

CA IX: alternative splicing

To date, two alternatively spliced variants of *carbonic anhydrous IX* (*CA IX*) have been reported; *full length CA IX* (*CA IX FL*) and a shorter *alternatively spliced CA IX* isoform (*CA IX AS*) (Malentacci *et al.,* 2009) (Figure 1.7). CA IX FL is localised to the plasma membrane and contains the whole of the coding sequence, whereas CA IX AS does not contain exons eight and nine. The truncated *CA IX AS* splice isoform lacks the *c*-terminal part of the catalytic domain; the transmembrane (TM) region and the intracellular tail (IC). The altered localisation of the AS variant to the intracellular space is suggested to be due to the absence of the TM and IC regions. The AS variant is also reported to have diminished catalytic activity and therefore

has reduced extracellular acidification capacity compared to CA IX FL. Furthermore, it is thought that when CA IX FL levels are low, such as during moderate hypoxia, CA IX AS may interact with CA IX FL (Barathova *et al.* 2008). In addition, CA IX AS is more prevalent in normal tissues and its expression is independent of hypoxia, which can therefore produce false positives (Malentacci *et al.,* 2009). As CA IX will be used as a marker for hypoxia in this study, the expression of both splice variants will therefore be assessed in order to deduce whether the treatment has been successful.

Weigand *et al.* (2012) provided further evidence of alternative splicing changes in various genes in hypoxia through an exon array in endothelial HUVEC cells. Six of the genes are involved in angiogenesis-mediated cytoskeleton remodelling (*cask, itsn1, larp6, sptan1, tpm1* and *robo1);* one is involved in membrane anchor synthesis (*pign*); and two are involved in gene expression regulation (*cugbp1* and *max*).

Furthermore, in Hep3B lung cancer and MCF7 breast cancer cells, hypoxia was found to alter differential splicing of cancer-associated genes that are involved in a number of processes, including; cytoskeleton oraganisation, apoptosis, angiogenesis and cell growth (Sena *et al.,* 2014; Han *et al.,* 2017). This provides further evidence that hypoxia drives cancer progression.

As 95% of human genes are alternatively spliced (Pan *et al.,* 2008) and in light of the evidence provided by Weigand *et al.* (2012), Sena *et al.* (2014) and Han *et al.* (2017), it is hypothesised that hypoxia may promote the expression of splice

isoforms of other cancer-associated genes to favour cancer progression. This is the first study that aims to investigate changes in differential exonic splicing in a prostate cancer cell lines.

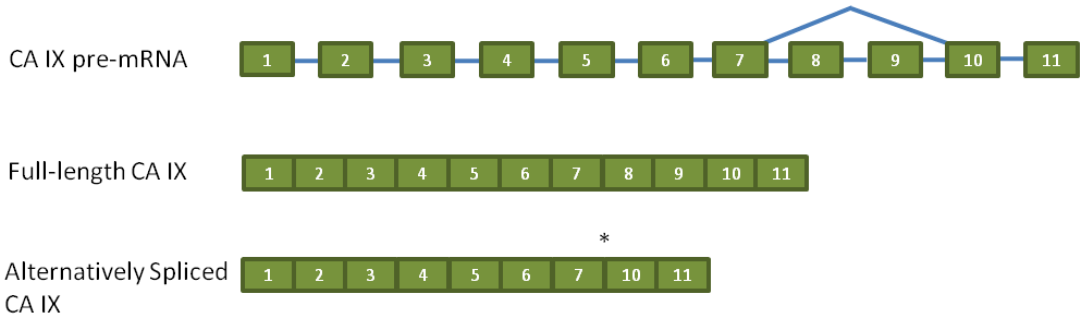


Figure 1.7: *CA IX* pre-mRNA splicing of exons 8 and 9. The excision of introns produces the full-length isoform containing all 11 exons. The shorter alternatively spliced variant is formed from additional exon skipping of exons 8 and 9 (notated by *).

1.4 Hypotheses, Aims and Objectives

A working model of the main aims of this project can be found in Figure 1.8.

1.4.1 Hypothesis 1

Cells respond to hypoxia by altering the expression of genes. Therefore, it was proposed that hypoxia might also alter alternative splicing of cancer-associated genes.

Aim

Changes in alternative splicing patterns of cancer-associated genes during hypoxia in prostate cancer cell lines was confirmed.

Objectives:-

- RT-PCR was used to examine the effect of hypoxia on the alternative splicing of apoptosis-associated genes.

- A high-throughput PCR approach was used to investigate more widely the expression of alternatively spliced isoforms of cancer-associated genes.

1.4.2 Hypothesis 2

Hypoxia changes the expression and activity of splice factors and splice-factor kinases.

Aim

Changes in expression and localisation of splice factors and splice factor kinases in hypoxia was examined.

Objectives:-

- qPCR and western blotting were used to investigate changes in mRNA expression and protein production of splice factors and splice factor kinases. The effect of hypoxia on the phosphorylation rates of splice factors were also examined using western blotting.

- Immunoflurorescence was used to investigate the localisation of splice factors and splice factor kinases in hypoxia.

1.4.3 Hypothesis 3

The splice factors and splice factor kinases whose expression and localisation altered during hypoxia contribute to the changes in alternative splicing of cancer-associated genes in hypoxia.

Aim

The role of splice factors and splice factor kinases on the expression of hypoxia-associated splice isoforms of cancer-associated genes was investigated.

Objectives:-

- Chemical inhibition of splice factor kinases was used to assess the effect on the alternative splicing of splice isoforms associated with hypoxia.
- Knockdown of affected splice factors was used to examine the effect on the alternative splicing of splice isoforms associated with hypoxia.

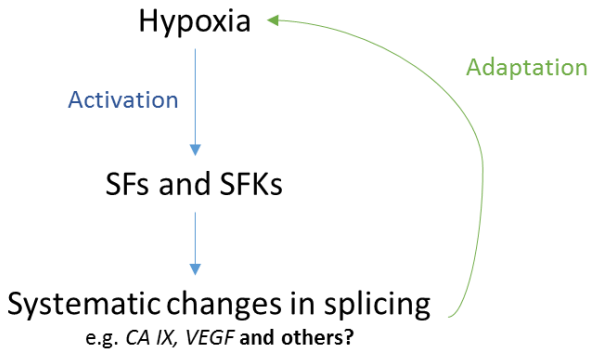


Figure 1.8: Working model for this study. Hypoxia activates splice factors (SFs) and splice factor kinases (SFKs), which cause systematic changes in splicing that allow the cell to adapt to hypoxia. Hypoxia has already been shown to switch splicing of *CA IX* and *VEGF,* and this project aims to identify other genes whose splicing is altered by hypoxia. The effect of hypoxia on mRNA expression of select SFs and SFKs will also be examined.

2.0 METHODS AND MATERIALS

2.1 Cell lines

Prostate cancer cell lines; prostate cancer-3 (PC3) (grade IV androgen-independent prostate cancer cell line derived from metastatic bone cancer in a 62 year old Caucasian male) (Sigma-aldrich, 2017a); and Vertebral-Cancer of the Prostate (VCaP) (androgen-dependent prostate cancer cell line harvested from vertebral metastasis of a patient) (Sigma-aldrich, 2017b), along with the immortalised normal prostate epithelium cell line PNT2 (harvested from normal prostatic epithelial cells of a 33 year old male) (Sigma-aldrich, 2017c) were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) (4,500 mg/L-glucose, 110 mg sodium pyruvate/ L-pyridoxine. HCl and $NaHCO_3$ without L-glutamine), supplemented with 10% FBS and 1% L-glutamine-penicillin-streptomycin. The VCaP cell line required a 20% (v/v) FBS final concentration. All cell media reagents were sourced from Sigma-Aldrich. All cell lines were provided from previous cryopreserved laboratory stocks (Sigma-Aldrich).

2.2 Trypsinisation of adherent cells

Once cells became 80% confluent, they were split to ensure that the cell mass did not outgrow the T_{75} area. This is particularly important in hypoxia studies, as cells that are over-confluent become hypoxic (Sheta *et al.*, 2001). Media was removed and replaced with 5 ml phosphate buffered saline (PBS wash). 1 ml of 1x trypsin-

EDTA (Sigma) was added to each flask to dissociate the cells. The flasks were gently rocked to allow the trypsin to sufficiently cover the surface and then stored in the incubator at 37^{o}C for 10 mins. The flasks were checked to see if the cells had detached. 2 ml of modified DMEM medium was added to each flask, containing FBS, which inactivates trypsin. The mixtures was then transferred to a 15 ml or 50 ml falcon tube and spun down in the Universal 320 Hettich Zentrifugen centrifuge for 5 mins at 1000 rpm (106 g). The supernatant was aspirated off and the pellet was resuspended in modified DMEM medium. If splitting the cells, the pellets were resuspended in complete media at a ratio of 1:4 and 1 ml cell mixture was added to 10 ml complete media into a new T_{75} flask.

For cell counts, 2 ml of media was added for every T_{75} flask used to resuspend the cell pellet. 30 µl of cells were added to an Eppendorf tube, where 0.4% trypan blue solution (Sigma) was also added at a ratio of 1:1. Using a haemotcytometer, the live cells were counted. The dark blue dead cells stained with trypan blue were disregarded in the cell count. 6- well plates were seeded with approximately 300,000 cells per well for experiments.

2.3 Cryopreserving cells

Cells were cryopreserved to provide a stock of early passages. Cells were trypsinised and 4 ml of modified DMEM media was added to them. The cell mixture was then placed in a 15 ml falcon tube and centrifuged (Universal 320 Hettich Zentrifugen)) at 1000 rpm (106 g) for 5 min. The supernatant was then aspirated

off, leaving the cell pellet. Cells were resuspended in freezing media (10% (v/v) dimethyl sulfoxide [DMSO], 20% (v/v) FBS, 70% (v/v) Dulbecco's Modified Eagle Medium [DMEM]) and counted. Freezing media was added to the pellet to provide a concentration of 1 million cells per ml. Aliquots of 1 ml of cell resuspension was then placed into cryovials and transferred to a cryopreserving chamber (Mr Frosty), which contained isopropanol. The chamber was stored in the -80^{o}C freezer, where it decreased the temperature at 1^{o}C per min and left overnight, after which the cryovials were transferred into the liquid nitrogen cell storage chamber.

2.4 Thawing cryo-preserved cells

A cryovial of stored cells were taken out of liquid nitrogen and thawed in a 37^{o}C water bath. The defrosted cells were then transferred into a T_{75} culture flask in 10 ml complete media and stored in the incubator at 37^{o}C. After a few hours, once the cells had attached to the surface, the media was aspirated off and replaced with 10 ml fresh media. The purpose of the latter was to decrease the amount of DMSO exposure to the cells. Cells were split at 80% confluency.

2.5 Hypoxia treatment

Prior to treatment, PC3 and PNT2 cells were seeded approximately at 3 x 10^5 per well and VCaP cells at 1x10^6 per well in 6-well plates and allowed to adhere

overnight. Media was aspirated and replenished with 2 ml media prior to treatment.

A MIC-101 hypoxia chamber was used throughout this study to induce hypoxic stress. The chamber containing cells is initially flooded with hypoxic gas and then is sealed and stored in the 37°C incubator for the desired treatment time. The main issue with this type of hypoxia treatment is that for the duration of the experiment, oxygen in the chamber will be metabolised by the cells and therefore the oxygen concentration will diminish throughout the experiment. In addition, carbon dioxide will be produced which will accumulate during the treatment. Hypoxia workstations release a steady flow of the desired oxygen concentration, whilst also keeping carbon dioxide at a required level in an incubated environment to combat this problem.

However, hypoxia workstations are much more expensive when compared to the MIC-101 hypoxia chamber, which make the MIC-101 chamber an attractive option to researchers that are new to the field of hypoxia or are not intending to conduct many hypoxic experiments. Furthermore, it can be argued that the MIC-101 chamber may create a more physiologically viable hypoxic system. Hypoxic areas of a tumour will contain a reduced blood supply which will lead to an accumulation of carbon dioxide and a steady rate of reduced oxygen concentration. Therefore, the diminishing oxygen concentration and accumulation of waste products throughout a hypoxic treatment in a MIC-101 chamber may provide a more suited model than a hypoxia workstation to mimic hypoxic conditions in the tissues. Scientific publications have utilised the MIC-101 chamber for hypoxia experiments (Buras *et*

al., 2000; Ezashi, Das and Roberts, 2005; Li *et al.,* 2007) which shows that the system is accepted in the scientific community for the initiation of hypoxia.

Cell culture plates were placed in a MIC-101 chamber (Billups-Rothenberg) and flooded with a hypoxic gas mixture. In tumours, the mean oxygen concentration is around 1.5% (Jiang *et al.*, 1996) compared to 3 - 7.4% in normal tissues (McKeown, 2014) so therefore a mixture containing; 1% O_2 / 5% CO_2 / 94% N_2 (BOC, Bristol, UK) was used. The sealed chamber along with a normoxic control was stored in an incubator for the desired treatment time at 37^{o}C in 5% CO_2.

2.6 Chemical inhibition of splice factor kinases

1-(3-ethyl-5-methoxy-2(3H)-benzothiazolylidene)-2-propanone (TG003) is a competitive inhibitor of CLK1 (Muraki *et al.,* 2004), and SPHINX ((5-methyl-N-[2-(morpholin-4-yl)-5-(tri-fluoromethyl)phenyl]furan-2-carboxamide)) is a competitive inhibitor of SRPK1 (Gammons *et al.,* 2013); both inhibit at concentrations up to 10 μM. TG003 was kindly provided by Prof. Masatoshi Hagiwara (Kyoto University, Japan) and SPHINX by Dr. Sebastian Oltean (Bristol University, UK). 0.1 M TG003 and 50 mM SPHINX stock solutions were prepared in DMSO, wrapped in parafilm and stored at -20°C. Cells were treated for 48 hours with 10 μM TG003 or SPHINX. A DMSO control was also prepared. At the end of the 48h treatment, three wells for each treatment were pooled together to extract RNA or protein.

2.7 Knockdowns by RNA interference (RNAi)

A cherry-pick siGENOME siRNA library customised plate containing 0.5 nmol siRNAs against SRPK1, CLK1, PRPF8 and SAFB1 was obtained from GE Healthcare (Cardiff, UK) (including four independent siRNAs against each gene) (Table 2.1). There were also a set of siRNAs that targeted the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) which were used as a positive control of siRNA treatment. A pool of non-targeting siRNAs were also used to measure baseline cellular response to siRNA application as a negative control. The plate was spun down and the foil cover was wiped with 70% ethanol as instructed in the protocol. The plate was opened and each siRNA was resuspended in 50 µl nuclease free water and transferred to eppendorfs to give 10 µM stocks. The stocks were wrapped in parafilm and stored at -20°C.

In the knock-down experiments, 10 µl of each siRNA for a given gene was mixed together to give a 10 µM pooled stock. 6-well plates were seeded with 300,000 PC3 cells per well and covered with 2 ml fresh growth medium prior to siRNA transfections. A transfection mix was made up for each siRNA using the pooled 10 µM stock and adding 1.6 ml jetPRIME buffer (Polyplus, France). This was mixed by pipetting up and down. 32 µl of jetPRIME reagent (Polyplus, France) was added and the mixture was vortexed for 10 sec and then incubated at room temperature for 10 min. 200 µl transfection mix was added to each well drop-wise and the plates were rocked gently back and forth. The final concentration of siRNAs was 25 nM. Controls included media only and transfection reagent only. Liu *et al.,* (2003)

showed that 48h siRNA treatment successfully knocked-down CLK1, and as hypoxia treatment was also given for 48h, protein and RNA samples were extracted for each treatment 48h after siRNA transfection.

2.8 RNA extraction

After washing cells in 1 ml ice cold PBS per well, RNA was extracted using Tri Reagent (Sigma-Aldrich). The cell lysate was passed through a pipette tip several times, before vortexing thoroughly for 15 sec and incubated at room temperature for 5 min. The cell lysate was then centrifuged (Beckman Allegra x-22R, Rotor: F2402H) at 9,650 g for 10 min 4°C. After centrifugation, the pellet was discarded and 10 % chloroform (Fisher Scientific) was added to the supernatant. Samples were vortexed for 15 seconds before incubating at room temperature for 3 min and then a subsequent centrifugation at 9,650 g for 30 min at 4°C. The chloroform separated out the samples into three phases. The upper aqueous phase was kept and 0.25 ml isopropanol (Fisher Scientific) was added to each sample before incubating at room temperature for 10 min, followed by a centrifugation at 9,650 g for 10 min at 4°C. The interphase and lower phase were discarded. Supernatants were discarded and the pellets were washed twice in 0.5 ml 70% ethanol with a centrifugation both times at 3,400 g for 5 min at 4°C. After the second wash centrifugation, all ethanol was removed to allow RNA pellets to air dry for 10 min. Pellets were then dissolved in 30 μl nuclease free water and stored at -80°C in parafilm-wrapped eppendorf tubes.

Genomic DNA was removed from the RNA using the Precision DNase kit as per manufacturer's instructions (Primerdesign, UK). Total RNA concentration was determined using Nanodrop spectrophotometer (Thermo Fisher Scientific, Deleware, USA).

2.9 cDNA synthesis

The GoScript Reverse Transcription System (Promega, Southampton, UK) was used for synthesising cDNA as per manufacturer's instructions. Total RNA was reverse transcribed using 1 µl of a mix of random primers and oligodT at a 1:1 ratio. Nuclease free water used in the assay was obtained from Promega (Southampton, UK).

2.10 Standard PCR

The GoTaq Hot Start Polymerase kit from Promega (Southampton, UK) was used to perform standard PCR reactions using concentrations as per the manufacturer's guidelines (final concentrations were 0.95x of 5x Green GoTaq Flexibuffer, 3mM $MgCl_2$, 0.25mM of each dNTP, 0.5µM upstream primer, 0.5µM downstream primer, 1.05U GoTaq Hotstart Polymerase, 0.01µg/50µl Template DNA). Hot start polymerases reduce non-specific amplification during the initial stages of PCR. cDNA samples of concentration 100 ng/µl were diluted 1:19 with nuclease free water prior to the addition of the master mix. Standard PCR conditions were as

follows; initial denaturation at 94^{o}C for 2 min, denaturation at 94^{o}C for 30 sec, annealing at 58^{o}C for 30 sec, extension at 72^{o}C for 30 sec, final extension at 72^{o}C for 5 min. The denaturation, annealing and extension steps were cycled 30-39 times depending on the genes being amplified. Primer sequences are shown in Table 2.2. PCR samples along with hyperladder II or V were run on 2% agarose gels for 1 hour at 95V and imaged using FluorChem Q software on the Alpha Innotech MultiImage III apparatus.

2.10.1 Calculating percentage exon inclusion for alternatively spliced genes

Optical density peak percentage values were generated from gel images for individual amplicons using Image J software. Excel software was used to calculate the percentage (%) of exon inclusion after normoxia and hypoxia treatment using the optical density peak percentage values. The formula [L/(S+L) x100] was used to generate PSI values, where L represents the full length (FL) isoform and S represents the shorter isoform with exon skipping.

2.11 Quantitative (real-time) PCR (qPCR)

qPCR was used to analyse gene expression using the protocol suggested by PrimerDesign (Southampton, UK). cDNA was diluted with nuclease-free water to a concentration of 5 ng/ul. One reaction well used 1µl resuspended primer mix (PrimerDesign, Southampton, UK), 10µl SensiFAST Sybr Hi-Rox (Bioline, London,

UK), 4µl nuclease-free water and 5µl of diluted cDNA). Three technical repeats were used for each experimental sample. A negative control of 20µl nuclease-free water was also used for each primer and repeated twice.

The qPCRs were run on an ABI (Applied Biosciences) 7300 qPCR thermal cycler. PCR conditions were as follows; 95^{o}C for 10 min, followed by 95^{o}C for 15 sec and 60^{o}C for 1 min for 40 cycles. Melting curve conditions were 95^{o}C for 15 sec, followed by 60^{o}C for 1 min and 95^{o}C for 15 sec. The primers used are shown in Table 2.3. CT values were reverted back to standard numbers using the formula 2^CT before normalisation to the *Ubiquitin C* (*UBC*) housekeeping gene; which was shown to be stable in prostate cancer cell lines during hypoxia in a study conducted by Vajda *et al.,* 2013). All results were calculated using Excel software (Microsoft).

2.12 High-throughput PCR

2.12.1 High-throughput PCR of cassette exon inclusion in cancer-associated genes in hypoxic PC3 cells

RNA extracted from normoxia–treated and hypoxia-treated PC3 cells was sent to Sherbrooke University, Canada for high-throughput PCR analysis. RNA quality was re-assessed by the Sherbrooke team using an Agilent 2100 Bioanalyzer (Agilent Technologies). 1.5 µg RNA was reverse transcribed using transcriptor reverse transcriptase, random hexamers, dNTPs (Roche Diagnostics) and 10 units of RNAseOUT (Invitrogen) and performed to the manufacturer's instructions in a final

volume of 20 µl. Individual 20-100 µM stock solutions for forward and reverse primers were made up in Tris-EDTA buffer and diluted as a primer pair using RNase DNase-free water to 1.2 µM.

10 ng cDNA was used for PCR reactions in a final volume of 10 µl containing 0.1 mmol/L of each dNTP, 1.5 mmol/L $MgCl_2$, 0.6 µmol/L of each primer and 0.2 units of Platinum Taq DNA polymerase (Invitrogen). PCR reactions were carried out on the GeneAmp PCR System 9700 (ABI) thermocycler and PCR cycles were as follows:- initial incubation at 95°C for 2 min, then 35 cycles of denaturation at 94^{o}C for 30 sec, annealing at 55^{o}C for 30 sec, extension at 72^{o}C for 1 min. The final extension was at 72^{o}C for 2 min. Completed PCR products were analysed on Caliper LC-90 instruments (Caliper LifeSciences) using automated chip-based microcapillary electrophoresis. Quantification and amplicon sizing were performed by the manufacturer's software. The primer pairs for cancer-associated genes used were originally selected by Dr. Julian Venables at Sherbrooke University. The primer pairs with the best hits can be found in Table 2.2.

2.12.2 High-throughput PCR analysis of the effect of splice factor knockdowns on *CA IX* alternative splicing

The effect of splice factor knockdowns on the expression of *CA IX* splice isoforms; *CA IX-FL* and *CA IX AS* was investigated by the Sherbrooke team using a set of cDNAs obtained from systematic splice factor knockdowns in MCF7 breast cancer cells. The MCF7 cell line is an adherent cell line derived from a breast adenocarcinoma of a 69

year old female. The set of splice factors knocked down in this set were selected from previous papers published by the Sherbrooke University group (Venables *et al.,* 2008; 2013). The Sherbrooke team designed the *CA IX* primer sequences. Changes in *CA IX* splicing shown on electropherograms extracted from the Sherbrooke online Palace program were selected. Isoform expression ratio values were then picked out for the SAFB1 and PRPF8 isoforms, along with their corresponding lipofectamine control ratio values and graphs were created depicting the values using Excel software.

2.13 Protein extraction and quantification

PC3 cells were washed in ice cold PBS, before the addition of 200 µl lysis buffer per well of a 6-well plate. Lysis buffer was made up of RIPA buffer (Sigma) and phosphatase inhibitor tablets (cOmplete, Mini, EDTA-free PI tablets; Roche Diagnostics, UK) (1 tablet per 10 ml RIPA buffer). Cells were scraped off whilst in the buffer, transferred to 1.5 ml Eppendorf tubes and left to incubate on ice for 20 min. Protein samples were diluted with distilled water at a 1:1 ratio in order to bring the protein concentration into the range of the quantification assay. If the concentration was still too high for quantification, more distilled water was added to bring the protein concentration into the desired range of detection. Protein quantification was performed using the Pierce BCA assay (Thermo Fisher Scientific, Delaware, USA). Five standards were prepared using albumin at the following concentrations; 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml and 0.125 mg/ml. The

working reagent was made up using the recommendations in the Pierce BCA protocol of 50:1 (BCA reagent A: BCA reagent B). 200 µl of the working reagent was added to each well of a 96 well plate and then the plate was incubated in the dark at 37^{o}C for 30 min and cooled to room temperature (RT). Two repeated readings were made for each standard, sample and negative control. The plate was read on the Anthos htll plate reader at 562nm absorbance. A standard curve was generated using Excel (Microsoft) and the protein sample concentrations were determined by using the graph equation generated. Protein samples were mixed with 2x Laemmli buffer (Sigma) (1:1) and then incubated at 100^{o}C for 5 min before being chilled on ice.

2.14 Western blot analysis

2.14.1 Acrylamide gels

Using short glass plates from Bio-Rad and a 1 mm Bio-Rad mini-protean gel system, a 10% acrylamide separating gel was made using 30% (v/v) acrylamide mix (29:1 acrylamide: bis-acrylamide) (Sigma-Aldrich), buffer A, distilled water, 10% (w/v) ammonium persulfate (Sigma-Aldrich) and TEMED (tetramethylethylenediamine) solution (Sigma-Aldrich). Isopropanol was added to the separating gel to level out any unevenness and prevent it drying out. Once the separating gel was set, the isopropanol was drained off and the excess soaked up with blotting paper. A 5% acrylamide stacking gel was added to the separating gel and a 10 or 15 well comb was inserted into the top of the gel and allowed to set.

2.14.2 SDS PAGE

The Criterion Electrophoresis Cell apparatus (Bio-Rad, UK), was assembled with the gel plates and sealed. The middle chamber and wells were filled with running buffer (25 mM Tris, 190 mM Glycine, 0.1% (w/v) SDS, pH 8.3) to check for any leaks. Once it was confirmed that the apparatus was sealed, the comb was removed and 5 µl of pre-stained protein marker (Cell Signalling Technology, Massachusetts, USA) and 35 µl protein samples were loaded carefully into the wells. More running buffer was then poured to top up the middle chamber and about 3 cm of running buffer was added to the outer chamber. The gel was run at 70V through the stacking gel for 30 min and then at 90V for 1 hour.

2.14.3 Transfer of proteins onto a membrane

Polyvinyldene fluoride (PVDF) membrane (Hybond-P, GE Healthcare, Buckinghamshire, UK) and six pieces of blotting paper were cut to size (9 x 6 cm). The PVDF membrane activated in methanol for 5 min and then along with the blotting paper and cassette sponges was washed in ice cold transfer buffer (25 mM Tris, 190 mM glycine, 0.2% methanol and made up to 1 L with distilled water). The stacking gel was removed from the acrylamide gel and placed in the wet cassette along with the sponges, blotting paper and PVDF membrane into the transfer tank. The transfer tank was assembled with an ice pack, filled with transfer buffer and placed on a stirrer. The transfer tank was run at 50V for at least 2 hours.

2.14.4 Detection of antigens

After transfer, the membrane was blocked for 1 hour at room temperature with 5% (w/v) blocking solution, made up of 5% milk powder or BSA in 1 x TBS-T (50 mM Tris, 150 mM NaCl, 0.5% (v/v) Tween-20, pH 7.6. Afterwards, the blocking reagent was replaced with the primary antibody solution made up in blocking solution and incubated at 4^{o}C overnight on a rocker. A list of antibodies used in this project can be found in Table 2.4. Dilutions of the primary antibody varied depending on the primary antibody in the range of 1:200 to 1:5000. The membrane was removed from the solution and washed three times for 5 min in 1 x TBS-T solution. The secondary antibody was diluted 1:5000 in blocking solution (Table 2.4), added to the membrane and incubated for 1 hour at RT. The membrane was then washed three times for 5 min with 1 x TBS-T solution. β-actin was used as a loading control.

2.14.5 Image acquisition

The PVDF membrane was taken out of the TBS-T wash solution and covered in 2 ml Laminata Forte Western HRP substrate (Millipore, UK) for 2 min. Membranes were then transferred to cassettes and CL-XPosure Film (5 x 7in) (Thermoscientific) was placed on top of the membrane in the dark for 5 minutes to begin with. The film was then placed into developer solution (Ilford PQ Universal) and when the image began to appear, the film was quickly washed in water before being placed into fixer solution (Tetenal Superfix Plus). The film was then washed again in water and left to air dry. If the image was overexposed, another piece of film was exposed to

the membrane for a shorter amount of time. If the image was underexposed, the time of exposure was increased. The film was scanned using the canon c2020i photocopier scanner and imported into this thesis.

2.14.6 Scaffold attachment factor B1 (SAFB1) normalisation to β-actin

Image J was used to generate optical density peak percentage values from the film images of the scaffold attachment factor B1 (SAFB1) and β-actin western blots. All β-actin values were normalised to the largest β-actin expression value in order to get the values into percentages in correspondence to one another. Then the SAFB1 values were normalised to each corresponding normalised β-actin value. Values were then displayed on a graph.

2.15 Cellular localisation of CLK1 protein

2.15.1 Nuclear and nuclear-free cellular fractions

The fractionation assay was performed using the protocol published by Yu, Huang and Lung (2013) with some minor alterations, as follows. Cells grown in 6-well plates were washed twice with ice-cold PBS. 1 cOmplete Mini, EDTA-free PI tablet was dissolved in 10 ml double-distilled water to form the stock PI cocktail. 250 µl of the PI cocktail was added into 10 ml subcellular fractionation (SF) buffer (250 mM Sucrose, 20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT). To extract the protein lysate, 50 µl of complete SF buffer was

added per well and a cell scraper was used to detach cells from the plate surface. The lysates were transferred into 1.5 ml Eppendorf tubes and kept on ice.

The lysates were then agitated on a tube roller at 4^{o}C for 30 min. After this, the tubes were centrifuged at 720 g for 5 min at 4^{o}C. The supernatant (crude cytoplasmic fraction) was then transferred into another 1.5 ml Eppendorf tube and the crude nuclear pellet was washed and dispersed in 500 µl of complete SF buffer.

Following this, the dispersed pellet was subjected to another round of centrifugation at 720 g for 10 min at 4^{o}C. The nuclear pellets were re-suspended in 100 µl nuclear lysis (NL) buffer [250 µl stock PI cocktail; 50 mM Tris HCl (pH 8); 150 mM NaCl; 1% (v/v) NP-40; 0.5% sodium deoxycholate (v/v); 0.1% SDS (w/v); 10% glycerol (v/v)], agitated and incubated at 4^{o}C for 15 min.

The crude cytosolic supernatants were centrifuged at 10,000 g for 10 min at 4^{o}C. After centrifugation, the supernatant was transferred to a fresh 1.5 ml Eppendorf tube. The cytosolic and membrane fractions were not separated using the ultracentrifuge as this proved problematic and so the assay provided nuclear fractions and crude cytosolic fractions, the latter including membrane proteins. The proteins were then quantified, separated using SDS-PAGE and western blotted. HSP90 protein was blotted as a cytoplasmic control. Blots of RNA-polymerase-II-subunit-B2 were attempted as a nuclear controls; however, despite many attempts the nuclear control was not successfully seen on the western blots.

2.15.2 Immunofluorescence analysis of CLK1 splice factor kinase subcellular localisation to the cytoplasm

CLK1 splice factor kinase localisation was determined using immunofluorescence coupled with microscopy (Nikon Eclipse 80i microscope) which allowed observeations by eye; and immunoflurescence using a confocal microscope (Perkin Elmer Ultraview ERS FRET-H microscope) which allowed Manders' Overlap Coefficients to be calculated.. Media was aspirated from each well of a 6-well plate containing PC3 cells. Each well was washed in 1 ml ice-cold PBS twice. 2 ml of 50:50 methanol: acetone (Fisher Scientific) fixative was added carefully to the edge of each well and left to incubate for 10 min. For confocal images, 1 ml per well of 4% (w/v) paraformaldehyde (Sigma-Aldrich) was used as a fixative instead. Paraformaldyhyde is a more hazardous fixing agent and so was only used when necessary. A lipid stain was used as a crude cytoplasmic stain for the confocal slides. Alcohol based fixatives, such as 50:50 methanol: acetone would disrupt the lipids in the cytoplasm of the cells, and so use of 4% paraformaldehyde as a fixative instead of the alcohol based fixative prevented lipid disruption during fixing. The fixative was aspirated and each well was once again washed twice in ice-cold PBS. To permeabilise the cell membranes, 1 ml of 0.5% Triton X (Sigma) made up in PBS was added to each well and incubated for 5 min. For confocal images, 1 ml 0.3% (v/v) Tween 20 (Sigma) made up in PBS for 3 x 5 min was used instead. This was because Tween 20 is a much milder membrane solubiliser and so would have less of an effect on cytoplasmic lipids. The wells were washed in ice-cold PBS before 1 ml

of blocking reagent (3% (w/v) BSA made up in PBS) was added to each well and left to incubate for 30 min on a rocker at room temperature.

Using a razor and a pair of forceps, each cover slip was carefully prised out of each well and placed on top of 80 μl of 1:25 dilution of CLK1 antibody (table 2.4) made up in 3% (w/v) BSA buffer. PC3 cells were incubated for 1 hour at room temperature in a humidified chamber formed of a roll of damp tissue inside a box. Each of the coverslips were placed back into another 6-well plate and washed three times for 5 min with 1 ml ice-cold PBS per well on the rocker. The coverslips were then once again carefully prised out of each well and placed on top of 80 μl of 1:500 dilution of goat anti-rabbit IgG Heavy and Light (Alexa Fluor 594) (Abcam, UK) immunofluorescent secondary antibody made up in 3% (w/v) BSA buffer. The cells were incubated for 1 hour at room temperature in a humidified chamber. A secondary antibody control slide was also generated. After 1 hour, the coverslips were placed into a fresh 6-well plate and once again washed three times for 5 min in ice-cold PBS on a rocker. Ice cold PBS was used to preserve the sample.

For the immunofluorescence images, the coverslips were carefully prised from each well and placed on top of a drop of 1 μL/mL DAPI solution made up in PBS (Sigma), which was placed on a labelled slide. Images were then taken from the slides using the NiS-Elements BR3.1 computer programme and x40 lens on the Nikon Eclipse 80i microscope. For the confocal images, after the PBS washes, the coverslips were placed on top of 80 μl Vybrant DiO (Emission ~501 nm), a green lipid stain made up in PBS for 30 min in a humidified chamber. Lipid dye control coverslips were also generated. A subsequent PBS wash was made afterwards and the coverslips were

mounted onto slides with a drop of Vectashield containing DAPI (Vector Laboratories). Slides were incubated in the fridge overnight at 4°C, and wrapped in foil to minimise exposure to light. Images from the slides were generated with the help from the head technician at the University of the West of England, David Corry, using the Perkin Elmer Ultraview ERS FRET-H microscope and ImageSuite computer program at x63 magnification. Manders' overlap coefficients (MOC), M1 and M2 were calculated using the Volocity Colocalisation software, which effectively calculated the percentage of colocalisation between CLK1 and either DAPI or DiO lipid stain. The values were plotted on a graph using Excel software.

2.16 Statistical Analysis

All results were calculated using Microsoft Excel, and were first subjected to a Shapiro-Wilks test in order to deduce whether or not the data was normally distributed. After this, a Bartlett's test was conducted in order to deduce if the data was of equal variance. If the data was found to be both normally distributed and of equal variance, a parametric test was conducted. For the inhibitor treatment results (section 2.6), the parametric test conducted was a one-way ANOVA, which allowed multiple comparisons to be made between the different treatments. This was followed by a Tukey-Kramer test, which deduced any respective significant changes. For the effect of hypoxia on alternative splicing of cancer-assocaited genes using standard PCR (section 2.10) and the effect of hypoxia on the expression of splice

factors and splice factor kinases using qPCR (section 2.11), a student's T – test was conducted to assess the statistical significance of the data.

However, if the Shapiro-Wilks and Bartlett's test revealed that the data was not normally distributed and/or heteroschedastic, a non-parametric test was applied. For the TG003 inhibitor treatment assay (section 2.6), this was in the form of a Dunn's test. However, the TG003 data was based on n=3, and the Dunn's test requires a lot more repeats to be conducted, so a statistical test could not be performed on this set of data. For the SPHINX treatment data, results were based on n=2 and so a statistical test could not be applied. For the effect of hypoxia on the alternative splicing of cancer-associated genes using standard PCR and the effect of hypoxia on the expression of splice factors and splice factor kinases using qPCR, a Mann Whitney U non-parametric test was performed to assess the significance of the data. For the qPCR data, it must be noted that the CT values were close together, which indicated precise pipetting technique, and any variation found was between experimental replicates. The means of the data were calculated from the results, plotted on bar charts and error bars were added using the 95% confidence interval.

siRNA Target	Sequences	Target Sequence
PRPF8 Accession Number: NM_006445 Species: Human	UGAAGCAUCUCAUCUAUUA	1937-1955 (Exon 13)
	GCAGAUGGAUUGCAGUAUA	1837-1855 (Exon 13)
	GGAAGAAGCUAACUAAUGC	4658-4676 (Exon 29)
	GAUAAGGGCAGGCGUGUCA	4411-4429 (Exon 27)
SAFB1 Accession Number: NM_002967 Species: Human	GGACCAAGAUGAUCAGAAA	1782-1800 (Exon 12)
	GGAAGAGGGUGAUUUAGAU	969-987 (Exon 7)
	GGACUGUAGUAAUGGAUAA	1856-1874 (Exon 13)
	UCAAAGAGGUCUAGCAAAG	427-445 (Exon 2)
CLK1 Accession Number: NM_004071 Species: Human	GAACGCACCUUAAUAAAUC	1118-1136 (Exon 9)
	UGAAUACUAUCUUGGGUUU	1291-1309 (Exon 10)
	GGAGUAUGAUCCAGCCAAA	1555-1573 (Exon 13)
	UAGAGCACCUGAAGUUAUU	1216-1234 (Exon 9)
SRPK1 Accession Number: NM_003137 Species: Human	GAAGUCAGUUCGCAAUUCA	508-526 (Exons 5-6)
	GAACACAUAUCUGCAUGGU	597-615 (Exons 6-7)
	UCACGAAGCUGAAACCUUG	1923-1941 (Exon 16)
	GUUACAGGGUCUUGAUUAU	712 -730 (Exon 8)
GAPDH control Species: Human	UGGUUUACAUGUUCCAAUA	
Non-targeting controls Species: Human	UAGCGACUAAACACAUCAA	
	UAAGGCUAUGAAGAGAUAC	
	AUGUAUUGGCCUGUAUUAG	
	AUGAACGUGAAUUGCUCAA	

Table 2.1: siRNA sequences and their target sequences. siRNA sequences were determined and produced by GE Healthcare (Cardiff, UK).

Gene Name	Primer Sequences	Target Site	Reference
CA IX NM_001216	**F:** CTCTGACTACACCGCCCTGTG **R:** GCGGTAGCTCACACCCCCTTT	1031-1051 (Exon 7) 1392-1372 (Exon 10)	Barathova *et al.* (2008)
Caspase-9 NM_001229	**F:** GCTCTTCCTTTGTTCATCTCC **R:** CATCTGGCTCGGGGTTACTGC	450-470 (Exon 2) 1191-1171 (Exon 7)	Hagen *et al.* (2013)
β-actin NM_001101	**F:** CCTGGCACCCAGCACAAT **R:** GCCGATCCACACGGAGTACT	1041-1058 (Exon 5) 1110-1091 (Exon 6)	Designed by M. Ladomery
BIRC5 (Survivin) NM_001168	**F:** GCATGGGTGCCCCGACGTTG **R:** GCTCCGGCCAGAGGCCTCAA	120-139 (Exon 1) 566-547 (Exon 4)	
Bcl-x NM_138578	**F:** CATGGCAGCAGTGAAGCAAG **R:** GCATTGTTCCCGTAGAGATCC	857-876 (Exon 2) 1207-1191 (Exon 3)	
Mcl-1 NM_012960	**F:** GTGCAGCGCAACCACGAGAC **R:** GCAGCACATTTCTGATGCCG	866-885 (Exon 1) 1205-1187 (Exon 3)	
BTN2A2 NM_006995	**F:** CCTGCTCCTCCTCCTTCTCAGC **R:** CAGCCAGATGCTCCCATCCTC	165-186 (Exon 2) 612-592 (Exon 4)	Venables *et al.* (2008); Venables *et al.* (2012)
INSR NM_000208	**F:** TGAGGATTACCTGCACAACG **R:** GCTGGTCGAGGAAGTGTTG	2607-2626 (Exon 10) 2772-2754 (Exon 12)	
UTRN NM_007124	**F:** CAAACACCCTCGACTTGGTT **R:** TGGCAATACTGCTGGATGAG	9524-9543 (Exon 65) 9793-9774 (Exon 69)	
FGFR1OP NM_007045	**F:** CTGTGGGTGGACCCTTATTATTAG **R:** GACACTTGTATCACTCTGATTGGC	461-484 (Exon 5) 714-691 (Exon 8)	
CDC42BPA NM_003607	**F:** GCTAATGCTGTGAGGCAAGAAC **R:** GCTCACTCTGTTCACGTAGCTT	2489-2510 (Exon 12) 2903-2882 (Exon 14)	
SYNE2 NM_182914	**F:** CTCACGAAGAGGACGAGGAG **R:** TTGCTTGTAGTGATGCTCGG	19519-19538 (Exon 106) 19706-19687 (Exon 108)	
APAF1F1 NM_181861	**F:** CTTTGGGATGCGACATCAGCAA **R:** CCACCTTTGAACGTGAGTCTGT	2882-2903 (Exon 17) 3074-3053 (Exon 18)	
APAF1F8 NM_181861	**F:** GTGAAGTGTTGTTCGTGGTCTG **R:** CATCACACCATGAACCCAAC	2978-2999 (Exon 17) 3244-3225 (Exon 18)	
PUF60 NM_078480	**F:** GCCAAGAAGTACGCCATGG **R:** GTAGACGCGGCACATGATG	291-309 (Exon 4) 479-461 (Exon 6)	
MBP NM_001025081	**F:** CTCGCACACCACCCCCGTC **R:** TCGACTCCCTTGAATCCCTTGT	459-477 (Exon 4) 606-585 (Exon 6)	
LHX6 NM_014368	**F:** TCCGACGACATCCACTACAC **R:** TGGTGGGTTCTGGTTCTCAG	1051-1070 (Exon 8) 1441-1422 (Exon 10)	
PTPN13 NM_080685	**F:** GACTCCTCATCCATTGAAGACC **R:** CCAAGCCATACTTTGCATCTTT	3604-3625 (Exon 19) 3793-3772 (Exon 21)	
RAP1GDS1 NM_001100426	**F:** TCCATGTGTGGATGCTGGATTG **R:** TGCATTTTGGCAGTGGATGC	442-463 (Exon 4) 781-762 (Exon 6)	
TTC23 NM_001288615	**F:** AAGGAGTGATTGAGTGTCAAAGGA **R:** CGTGGGTTCAGCTTTCTAGGTC	304-327 (Exon 1) 654-633 (Exon 3)	
CLK1 (Exon 4) NM_004071	**F:** CAAGGATGTGAACCTGGACATCGC **R:** CTCCTTCACCTAAAGTATCAAC	425-448 (Exon 3) 692-671 (Exon 5)	
CLK1 (Intron 4) NM_004071	**F:** GGAGGGTCACCTGATCTGTCAG **R:** CTGCTACATGTCTACCTCCCGC	616-637 (Exon 4) 749-728 (Exon 6)	Designed by M. Ladomery

Table 2.2: Forward (F) and reverse (r) primer sequences for all human genes amplified using standard PCR. The target sites of the primers, including the exonic locations are indicated.

Gene Name	Primer Sequences	Target Site	References
β-actin NM_001101	**F:** CCTGGCACCCAGCACAAT **R:** GCCGATCCACACGGAGTACT	1041-1058 (Exon 5) 1110-1091 (Exon 6)	Designed by M. Ladomery
SRSF1 NM_006924	**F:** GATGGAATTGTGTTTTGCGTTTT **R:** CATCTACTCGTGCTGAATCCTT	2611-2633 (Exon 4) 2711-2690 (Exon 4)	Sequences determined by PrimerDesign UK
SRSF2 NM_003016	**F:** GTGCTTGGCTGTTTCCTGTTT **R:** CACTGTATGCTCCGTTATTTATATGC	2579-2599 (Exon 2) 2728-3703 (Exon 2)	
SRSF3 NM_003017	**F:** GTGAGAGAGTTGGTTGGTGTTG **R:** AAATGCGGCGGCTCAAATC	75-96 (Exon 1) 152-134 (Exon 1)	
SAM68 NM_006559	**F:** GCCACAGCCTCGGTCAAG **R:** AGTCTCCTTTCTGAATCTTCTCAATT	573-590 (Exon 1) 711-686 (Exon 2)	
HuR NM_001419	**F**: TCGTCAACTACCTCCCTCAGA **R:** GCTGTGTCCTGCTACTTTATCC	235-255 (Exon 2) 347-326 (Exon 3)	
HnRNP A1 NM_002136	**F:** CTTCATCCAGCCAAAGAGGTC **R:** AAGTTGTCATTCCCACCGAAAC	684-704 (Exon 5) 765-744 (Exon 6)	
CLK1 NM_004071	**F:** TGAATACTATCTTGGGTTTACCGTAT **R:** CGTTTCCTGGTTTTCTGTATCATAT	1291-1316 (Exon 10) 1407-1383 (Exon 12)	
SRPK1 NM_003137	**F:** TGGCCACAGGTGACTATTTG **R:** CCCAAGGTTTCAGCTTCGT	1755-1774 (Exon 14) 1943-1925 (Exon 16)	Designed by M. Ladomery

Table 2.3: Forward and reverse primer sequences for all human genes amplified using qPCR.

Antibody, Source and Species	Original Concentration	Dilution used	Secondary Antibody used for Western Blotting	Secondary Antibody used for Immunofluorescence
Anti-β-actin Abcam (*ab8226*). Mouse	1mg/ml	1:10000	Horse anti-mouse IgG HRP-linked antibody. Cell Signalling (7076S)	
Anti-CA IX (M75 monoclonal antibody) kindly gifted from Dr. Pastorekova (Slovak Academy of Sciences, Slovakia). Mouse		1:3		
Anti-SAFB1 GeneTex (*GTX49227*). Mouse	1mg/ml	1:10000		
Anti-HSP90 Abcam (ab13492). Mouse	1mg/ml			
Anti-SR (1H4) SantaCruz Biotechnology (sc-13509). Mouse	200µg/ml	1:500		
Anti-SRPK1 SantaCruz Biotechnology (sc-100443). Mouse	200µg/ml	1:1000		Donkey Anti-mouse IgG H&L (Alexa Fluor 488) Abcam (ab150105)
Anti-SRSF1 SantaCruz Biotechnology (sc-33652). Mouse	200µg/ml	1:500		
Anti-CLK1 Abiocode (R1471-1s). Rabbit	1mg/ml	1:5000	Goat anti-rabbit IgG HRP-linked antibody. Cell Signalling (7074)	Goat Anti-rabbit IgG H&L (Alexa Fluor 594) Abcam (ab150080)
Anti-VE Cadherin Sigma (V1514-200UL). Rabbit	0.08 - 1.2 mg/ml	1:500		
Anti-PRPF8 kindly gifted by Sergey Bessonov (GWDG, Germany). Rabbit	Unknown	1:1000		

Table 2.4: Antibodies used for western blotting and immunofluorescence. The source of the antibodies and the dilutions used are also stated.

CHAPTER 3:

Examining Alternative Splicing Changes in Hypoxia in a Prostate Cancer Cell Line Model

3.1 Background

Hypoxia is a common attribute of solid cancers, particularly those deemed aggressive. Hypoxia stabilises hypoxia-inducible-factor-1α (HIF-1α), which facilitates the transcription of genes that promote survival. Recently, there has been a few papers that have studied the genome-wide effect of hypoxia on alternative splicing in non-cancerous epithelial and cartilage endplate-derived stem cells (Hang *et al.,* 2009; Weigand *et al.,* 2012; Yao *et al.,* 2016); and lung and breast cancer cell lines (Sena *et al.,* 2014; Han *et al.,* 2017). These papers have provided evidence to show that hypoxia changes the alternative splicing of genes implicated in cancer, such as genes involved in angiogenesis, the cell cycle and DNA repair. However, this is the first study to investigate the effect of hypoxia on alternative splicing in prostate cancer cells.

This chapter aims to identify cancer-associated genes that switch their splicing during hypoxia in PC3 and VCaP prostate cancer cell lines, and the PNT2 normal prostate epithelium cell line. Androgens are required by prostate cells for growth and survival. Prostate cancer cells therefore utilise androgens for cancer progression (Livermore, 2016; Mills, 2014). Androgens bind to the androgen receptor in the cytoplasm, which causes translocation of the androgen receptor into the nucleus where it begins gene transcription (reviewed in Munkley *et al.,* 2017). In prostate cancer cells, the androgen receptor is thought to transcriptionally control in the order of 700 genes (Munkley *et al.,* 2016).

The *androgen receptor* itself is alternatively spliced forming around twenty splice variants that lack androgen control, and therefore cannot be targeted by androgen deprivation therapies (Lu and Luo, 2013). The most common *androgen receptor* splice isoform, *ARv7,* includes a cryptic exon within intron three. This results in the formation of a truncated androgen receptor protein that lacks the ligand-binding domain, which is required for binding to androgens for activation, and instead produces a variant that is constitutively active that drives cancer progression (reviewed in Munkley *et al.,* 2017). In addition to affecting gene expression, androgen hormones have also been shown to affect splicing patterns. Rajan *et al.* (2008) observed an increase in exon skipping from transcripts derived from a *CD44* minigene, which housed a steroid-responsive promoter in the presence of both the androgen receptor and androgens.

Exon microarrays that have probed the entire transcriptome for androgen-dependent splice isoforms have identified an activated cassette exon in the *ZNF121* gene, which encodes a zinc finger protein. The study also uncovered a repressed cassette exon in the *NDUFV3* gene, which encodes a mitochondrial respiratory protein (Rajan *et al.,* 2011). However, the clinical importance of both these types of splicing is not yet known (Munkley *et al.,* 2017). Rajan *et al.* (2011) also uncovered androgen-dependent expression of an alternative isoform of the *tuberous sclerosis-2* gene, which arises through use of an alternative promoter. Full-length tuberous sclerosis protein represses cell growth, whereas the alternatively spliced isoform promotes cell growth (Munkley *et al.,* 2014). Furthermore, a splice variant of the *STBGALNAC1* gene has been identified in androgen-regulated prostate cancer

(Munkley *et al.,* 2015). The *STBGALNAC1* gene encodes a protein that is involved in the synthesis of the sialyl-Tn antigen, which is associated with cancer. The shorter, alternatively spliced isoform is thought to have enhanced translation, resulting in increased *ST6GALNAC1* expression and ultimately more synthesis of sialyl-Tn antigen (Munkley *et al.,* 2016) leading to a more cancerous phenotype. The studies above suggest that androgens affect alternative splicing in prostate cancer. Therefore, it is proposed that there may be a difference in alternative splicing patterns exhibited in androgen-dependent and androgen-independent prostate cancers.

This is the first study that compares the effect of hypoxia on alternative splicing patterns of cancerous genes in both androgen-independent (PC3) and androgen-dependent (VCaP) prostate cancer cells; and in the normal prostate epithelial cell line, PNT2. However, changes in alternative splicing may also be cell line specific and so it cannot be deduced that any differences are due to whether the cell line responds to androgens or not.

3.2 Confirming that hypoxia treatment was successful through use of the hypoxia marker carbonic anyhydrase 9 (CA IX)

Carbonic anhydrase 9 (CA IX) is an established marker of hypoxia that is deemed to be more reliable that HIF-1α, as HIF-1α is lost upon re-oxygenation whereas CA IX is not (Sobhanifar *et al.,* 2005). Therefore, it was important that the expression of CA IX was assessed at RNA and protein levels in order to evaluate whether the hypoxia

method used was working. Results showed that CA IX protein expression was markedly elevated in hypoxia, with a greater elevation at 48 hour hypoxic treatment (85% increase) when compared to 24 hour treatment (almost 60% increase) (Figure 3.1). 72h hypoxia was also conducted; however, the level of cell death was very high, and so 72h treatment was deemed unsatisfactory for experiments. Therefore, 48 hour hypoxia treatment was chosen for subsequent experiments.

ImageJ software was used to calculate optical density peak values from RT-PCR gel data. The percentage splicing index (PSI) was applied to the optical density peak values in order deduce whether or not there was a significant alteration in alternative splicing. PSI values were calculated by assessing the average percentage inclusion level of exons. In other words, the percentage of expression of the full-length isoform that does not contain any skipped exons. The following calculation was used for PSI values: [Expression of the full-length isoform / Total expression of the full-length isoform and exons-skipped isoform] *100.

There was a significant increase in CA IX exon inclusion in the PC3 cell line after hypoxia treatment (Figure 3.2B and C). The alternatively spliced isoform was not detected in the VCaP and PNT2 cell lines (Figure 3.2 D and F), and so the percentage of exon inclusion could not be determined. Therefore, the expression of the larger CA IX isoform, CA IX-FL was normalised to the β-actin loading control in order ot see if the expression of CA IX-FL was affected by hypoxia (Figure 3.2 E and G). There was not a significant change in CA IX-FL mRNA expression observed during hypoxia in the VCaP and PNT2 cell lines. However, the error bar in the VCaP graph (Figure 3.2

E) is quite long. This is due to the third repeat experiment where the expression of CA IX-FL is shown to decrease, which contradicts the first two repeats that show a clear increase in CA IX-FL expression in hypoxia. With more repeats, it is hoped that this result will be found to be significant.

In the PNT2 cell line, the Shapiro-Wilks test showed that the data was not normally distributed, and so a Mann Whitney U test was applied to the data in order to assess whether or not it was statistically signiciant (Figure 3.2 G). However, there were not enough data points to complete the Mann Whitney U test and so the statistical significance of the data is unknown. A student's T-test did reveal a statistical significance of $p<0.001$, which suggests that the results may be significant. However, without the correct statistical test this cannot be confirmed. The gel image does however give a good indication that the expression of CA IX-FL increases during hypoxia in the PNT2 cell line (Figure 3.2F). Therefore, hypoxia treatment for 48h duration at 1% oxygen was deemed appropriate in all three cell lines.

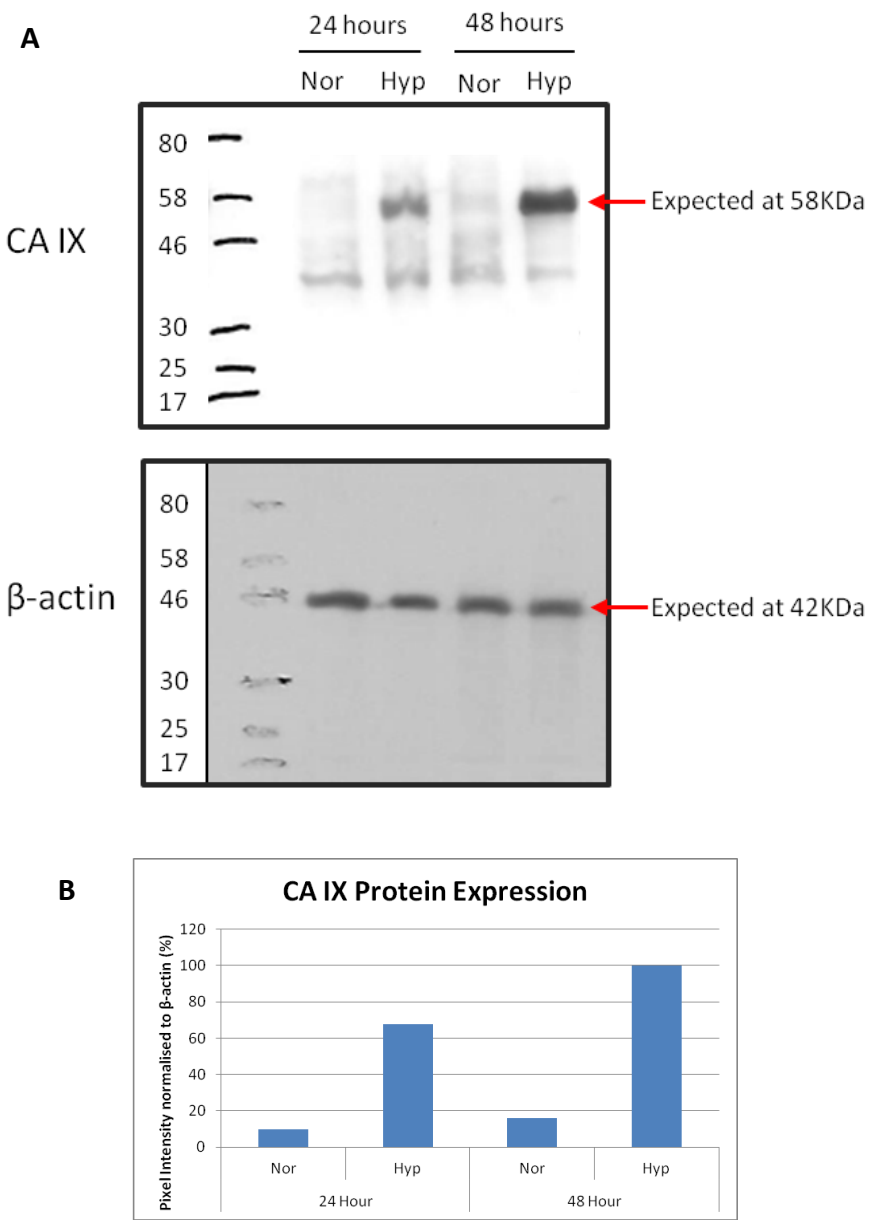


Figure 3.1: CA IX expression after 24h and 48h hypoxia treatment. (A) Western blot image of CA IX protein expression along with the β-actin loading control. (B) CA IX protein expression normalised to the β-actin loading control. PC3 cells were cultured in 24h and 48h hypoxic conditions using 1% oxygen gas. Mouse monoclonal M75 antibody was used to detect CA IX expression at a 1:3 dilution with blocking reagent. Anti-mouse IgG was used as a secondary antibody. n=1.

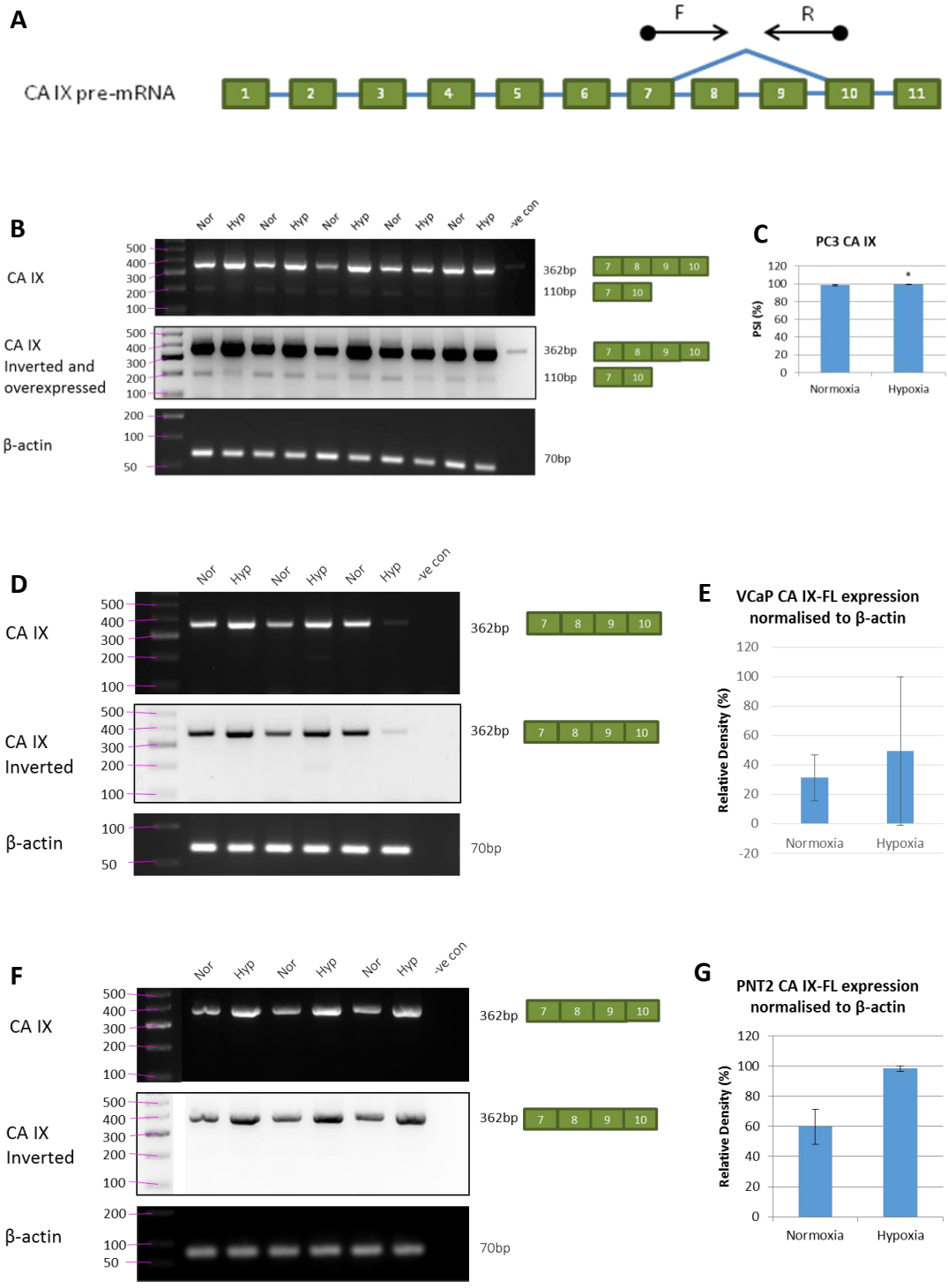


Figure 3.2: Expression of *CA IX* isoforms in hypoxia in prostate cancer cell lines. (A) Illustration of *CA IX* pre-mRNA showing exons (green boxes), introns (blue lines) and location of the primers (F = forward; R = reverse). The alternative splicing event whereby exons 8 and 9 are included or skipped is shown. Cells seeded from prostate cancer cell lines were cultured in normoxic or hypoxic conditions. RTPCR was performed using the following conditions: 94^{o}C for 2 min, 94^{o}C for 30 sec, 58^{o}C for 30 sec, 72^{o}C for 30 sec, 72^{o}C for 5 min with 40 cycles. **(B)** *CA IX* isoform expression with **(C)** respective average percentage inclusion level of exons 8 and 9, also known as the percentage splicing index (PSI %) after normoxic or hypoxic

treatment in the PC3 cell line (n=5). Expression of the *CA IX-FL* isoform with respective normalisation to the β-actin loading control in the **(D and E)** VCaP cell line (n=3) and **(F and G)** PNT2 cell line (n=3). ImageJ software was used to generate optical density peaks from the gel isoform expression. Excel was used to normalise to the β-actin loading control, and then calculate the average proportion of exons 8 and 9 inclusion (%). * = $p<0.05$. Statistical tests were as follows, where SW stands for Shapiro-Wilks test, BT stands for Bartlett's test, MWU stands for Mann-Whitney U test and TT stands for student's T test. In the PC3 and VCaP cell lines, SW showed that the data was normally distributed, BT showed that the data was homoschedastic, and so a TT was applied to the data to assess whether it was statistically significant. In the VCaP cell line, the p value obtained in the TT was just outside what is deemed significant. The error bar for the hypoxia data is very long, and so it is suggested that with more results, the error bar would decrease resulting in a significant result. In the PNT2 cell line, SW showed that the data was not normally distributed, BT showed that the data was homoschedastic, and so a MWU test was applied to the data to access whether it was statistically significant. However, there were not enough data points to perform a MWU. A TT was applied to the data which had a statistical significance of $p<0.001$. This suggests that the data may be significant, although without the correct test (MWU) this can not be confirmed.

3.3 Examining the effect of hypoxia on alternative splicing in genes involved in apoptosis

In order to test the hypothesis that hypoxia alters alternative splicing, a selection of genes involved in apoptosis were examined for changes in alternative splicing after hypoxia treatment. The genes assessed were: *caspase-9, survivin, Mcl-1 and Bcl-x*. The anti- or pro – apoptotic nature of each splice variant is shown in Table 3.1. The expression of their different splice variants were determined in PC3, VCaP and PNT2 prostate cancer cell lines.

Hypoxia significantly increased the PSI of *caspase-9* in both the PC3 and PNT2 cell lines respectively (Figure 3.3 B, C, F and G). This means that hypoxia favoured the pro-apoptotic full-length splice isoform of *caspase-9* in the PC3 and PNT2 cell lines, and therefore suggests that hypoxia may signal apoptosis through regulation of *caspase-9* splicing.

Hypoxia significantly favoured an increase in proportion of the *wild-type survivin* (*survivin-WT*) isoform in the PC3 cell line (Figure 3.4 C). This result is not obvious from looking at the RT-PCR gel data, as the gel image appears to show a decrease in expression of the *survivin-WT* isoform with hypoxia treatment (Figure 3.4 B; however, the expression of the other two *survivin* isoforms also decrease in expression with hypoxia treatment. Therefore, although the expression of all three *survivin* isoforms decrease with hypoxia treatment, the overall proportion of the *survivin-WT* isoform increases significantly. Survivin is an anti-apoptotic gene (Table

3.1), and so the fact that all three isoforms appear to decrease in expression suggests that apoptosis is favoured during hypoxia.

The PSI value for *BCL-x* increased significantly in hypoxia in the PNT2 cell line (Figure 3.5 G), suggesting that hypoxia favours the expression of the full-length isoform, which is anti-apoptotic. Similiarly to the RT-PCR gel image observed for *survivin,* the full-length *BCL-x* splice isoform also appears to contradict the PSI value calculated (Figure 3.5 F), as it decreases in expression slightly with hypoxia treatment. However, the faint expression of the exon 2b skipped (*BCL-xs)* splice variant also decreases, which increases the overall proportion of the full-length splice variant. The expression of the *BCL-xs* splice variant is very faint and so although this result is shown to be significant, further work is required to deduce whether this is actually the case.

Hypoxia was not found to significantly alter the PSI value of *MCL-1* in the PC3 cell line (Figure 3.6 C). The *MCL-s* splice isoform was not detected in the VCaP and PNT2 cell lines (Figure 3.6 D and F), and so the expression of the *MCL-1L* amplicon was normalised to the β-actin loading control and the results are displayed in Figures 3.6 E and G. There was no significant change in the expression of *MCL-1L* in the VCaP and PNT2 cell lines.

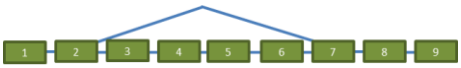
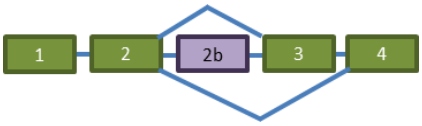
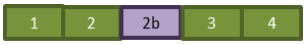
Gene	Isoform Apoptotic Nature	References
Caspase-9 	*Caspase 9a*: Pro-apoptotic  *Caspase-9b*: Anti-apoptotic 	Soel and Billiar, 1999 Srinivasula *et al.*, 1999
Survivin 	*Survivin-WT*: Anti-apoptotic  *Survivin-ΔEx3*: Anti-apoptotic  *Survivin-2B*: Reduced anti-apoptotic potential 	Mahotka *et al.*, 1999
Bcl-X 	*Bcl-xL*: Anti-apoptotic  *Bcl-xS*: Pro-apoptotic 	Boise *et al.*, 1993
Mcl-1 	*Mcl-1L*: Anti-apoptotic  *MCL-1S*: Pro-apoptotic 	Bae *et al.*, 2000

Table 3.1: The apoptotic nature of caspase-9, survivin, Bcl-x and Mcl-1 isoforms. Illustrations of the pre-mRNA and mRNA structures are also shown (exons – green boxes; introns – blue lines). The *survivin-ΔEx3* isoform has a loss of exon 3 resulting in a frame shift with extension of the reading frame into the open reading frame (ORF) of the 3' untranslated region.

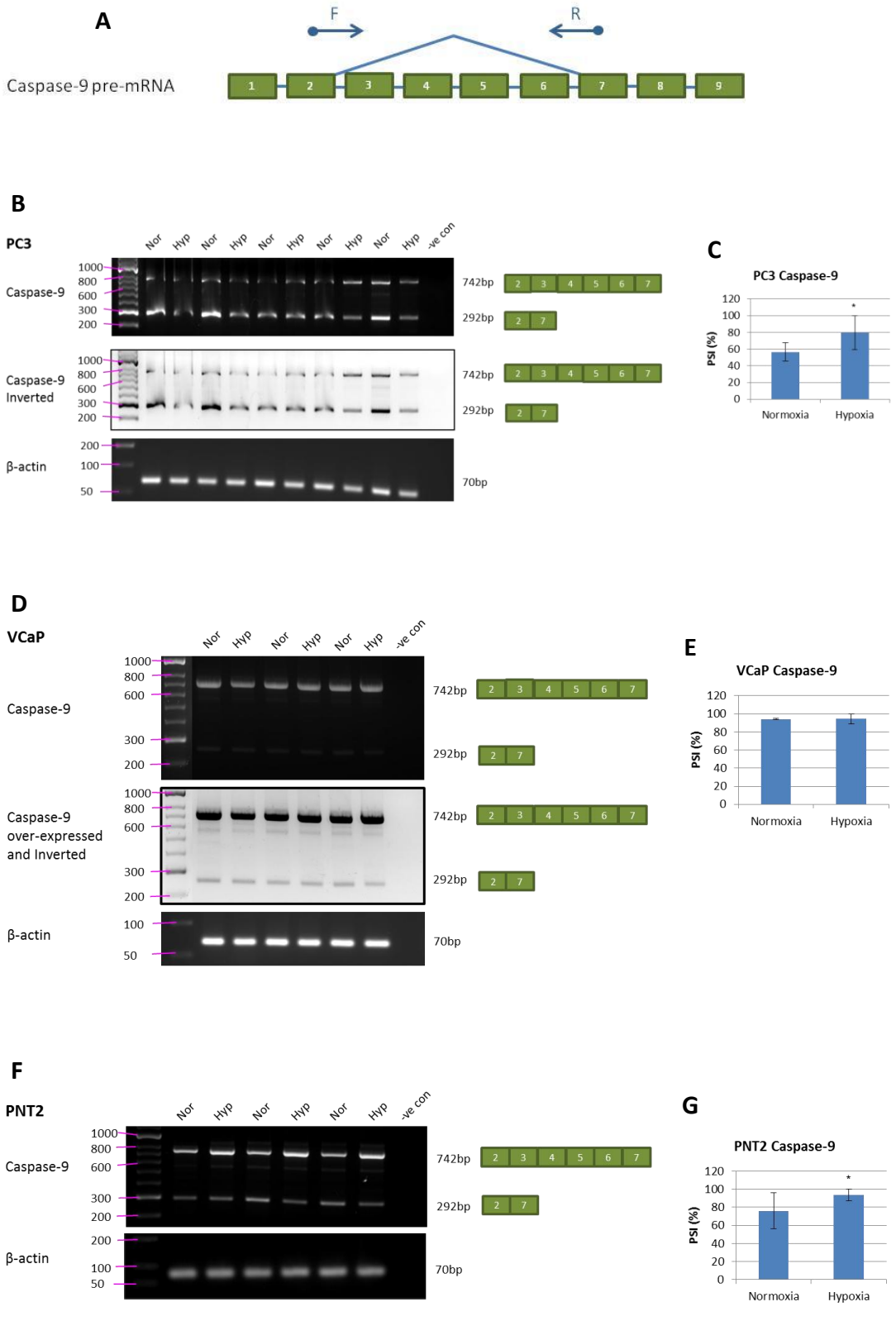


Figure 3.3: Proportion of exon inclusion in the *caspase-9* gene after hypoxia in prostate cancer cell lines. (A) Illustration of *caspase-9* pre-mRNA showing exons (green boxes), introns (blue lines) and location of the primers (F = forward primer; R = reverse primer). Cassette exon splicing of exons 3, 4, 5 and 6 is also shown. Cells seeded from prostate cancer cell lines were cultured in normoxic or hypoxic

conditions. RTPCR was performed using the following conditions: 94^{o}C for 2 min, 94^{o}C for 30 sec, 58^{o}C for 30 sec, 72^{o}C for 30 sec, 72^{o}C for 5 min with 35 cycles. *Caspase-9* isoform expression with respective average percentage inclusion level of exons 3, 4, 5, and 6, also known as percentage splicing index (PSI %) after normoxic or hypoxic treatment in the **(B and C)** PC3 cell line (n=5); **(D and E)** VCaP cell line (n=3); **(F and G)** PNT2 cell line (n=3). ImageJ software was used to generate optical density peaks from the gel isoform expression. Excel was used to normalise to the β-actin loading control, and then calculate the average percentage of exons 3, 4, 5 and 6 inclusion (%). * = $p<0.05$. In all three cell lines, Shapiro-Wilks test showed that the data was normally distributed, Bartlett's test showed that the data was homoschedastic, and so student's T-tests were applied to the data in order to assess the statistical significance of the data.

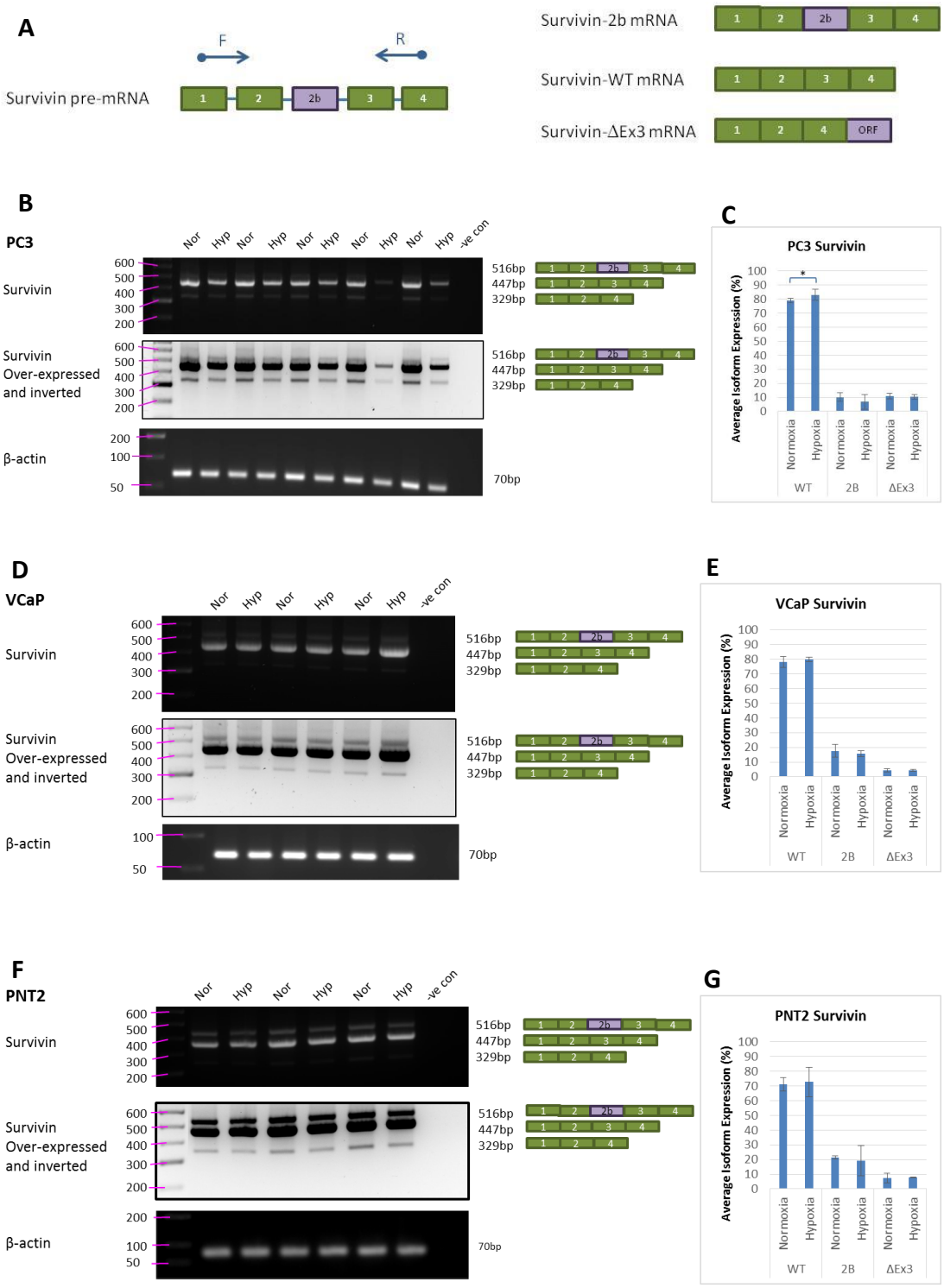


Figure 3.4: Proportion of *survivin* isoforms after hypoxia in prostate cancer cell lines. (A) Illustration of *survivin* pre-mRNA showing exons (green boxes), introns (blue lines) and location of the primers (F = forward; R = reverse). The three different types of *survivin* mRNA splice isoforms (*survivin-2b, survivin-WT* and *survivin-ΔEx3*) are also shown. It can be noted that the *survivin-ΔEx3* isoform has a loss of exon 3, resulting in a frame shift with extension of the reading frame into the open reading frame (ORF) of the 3' untranslated region. Cells seeded from prostate cancer cell lines were cultured in normoxic or hypoxic conditions.

RTPCR was performed using the following conditions: 94^{O}C for 2 min, 94^{O}C for 30 sec, 58^{O}C for 30 sec, 72^{O}C for 30 sec, 72^{O}C for 5 min with 35 cycles. *Survivin* isoform expression with respective average percentage (%) isoform expression after normoxic or hypoxic treatment in the **(B and C)** PC3 cell line (n=5); **(D and E)** VCaP cell line (n=3); **(F and G)** PNT2 cell line (n=3). ImageJ software was used to generate optical density peaks from the gel isoform expression. Excel was used to normalise to the β-actin loading control, and then calculate the average proportion of *survivin* isoforms (%). Calculations were as follows; (1) WT/total*100; (2) 2b/total*100; (3) ΔEx3/total*100. * = $p<0.05$. In all statistical tests, apart from *survivin-ΔEx3* data in the PNT2 cell line, Shapiro-Wilks test showed that the data was normally distributed, Bartlett's test showed that the data was homoschedastic and so student's T-tests were applied to the data to assess the statistical significance. In the *survivin-ΔEx3* data, Shaprio-Wilks test showed that the data was normally distributed, but the Bartlett's test showed that the data was heteroschedastic and so a Mann Whitney U test was applied to the data. However, there were not enough data points to complete the Mann Whitney U test successfully.

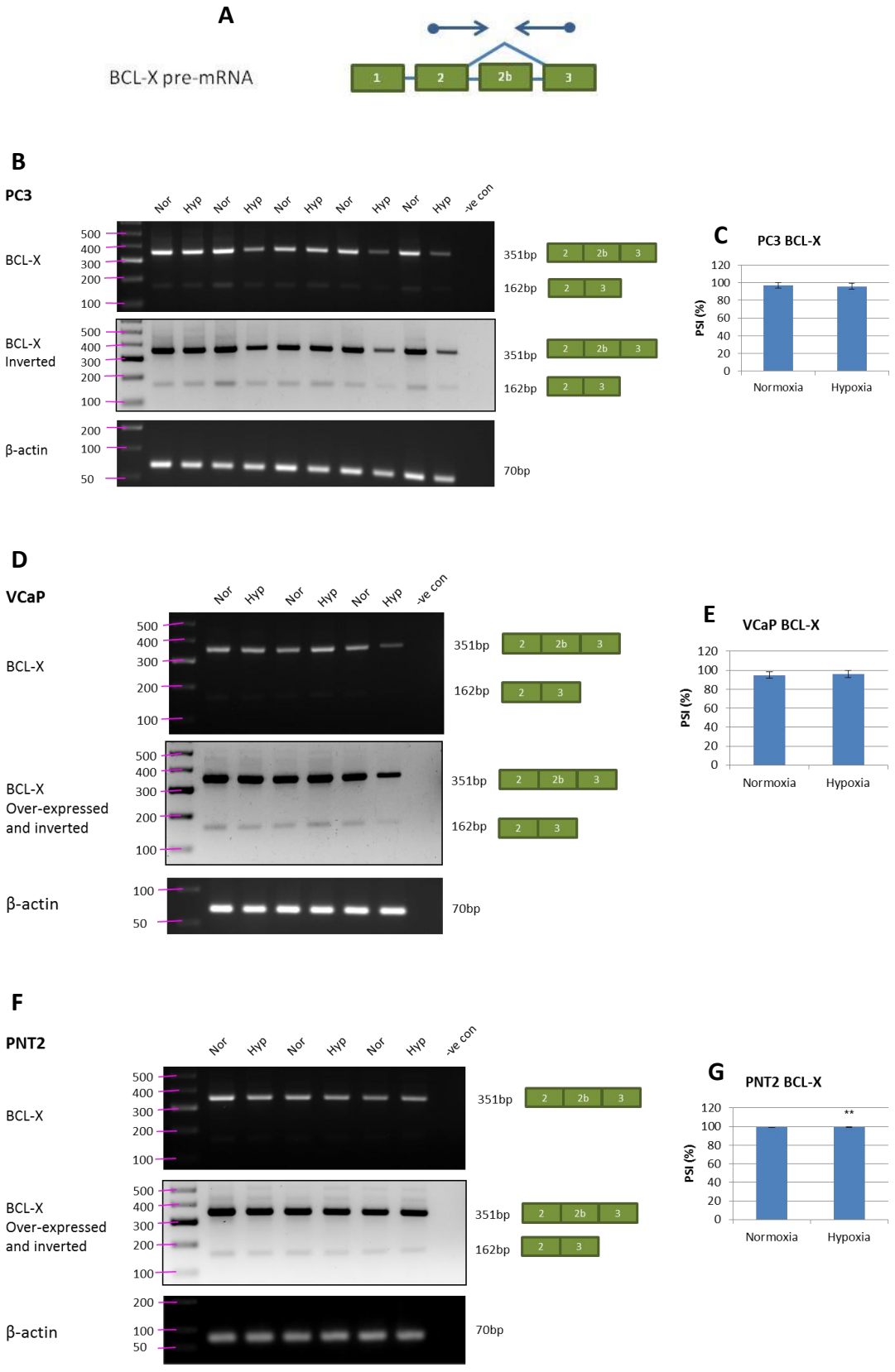


Figure 3.5: Proportion of exon inclusion in the *Bcl-x* gene after hypoxia in prostate cancer cell lines. (A) Illustration of *Bcl-x* pre-mRNA showing exons (green boxes), introns (blue lines) and location of the primers (F = forward primer; R = reverse primer). The exon 2b inclusion

and skipping alternative splicing event is also shown. Cells seeded from prostate cancer cell lines were cultured in normoxic or hypoxic conditions. RTPCR was performed using the following conditions: 94^{o}C for 2 min, 94^{o}C for 30 sec, 58^{o}C for 30 sec, 72^{o}C for 30 sec, 72^{o}C for 5 min with 35 cycles. *Bcl-x* isoform expression with respective average percentage inclusion level of exon 2b, also known as percentage splicing index (PSI %) in the **(B and C)** C3 cell line (n=5); **(D and E)** VCaP cell line (n=3); **(F and G)** PNT2 cell line (n=3). ImageJ software was used to generate optical density peaks from the gel isoform expression. Excel was used to normalise to the β-actin loading control and then calculate the average percentage of exon 2b inclusion (%). ** = $p<0.01$. In all cell lines, the Shapiro-Wilks test showed that the data was normally distributed, Bartlett's test showed that the data was homoschedastic, and so student's T test were applied to the data in order to assess whether it was statistically significant.

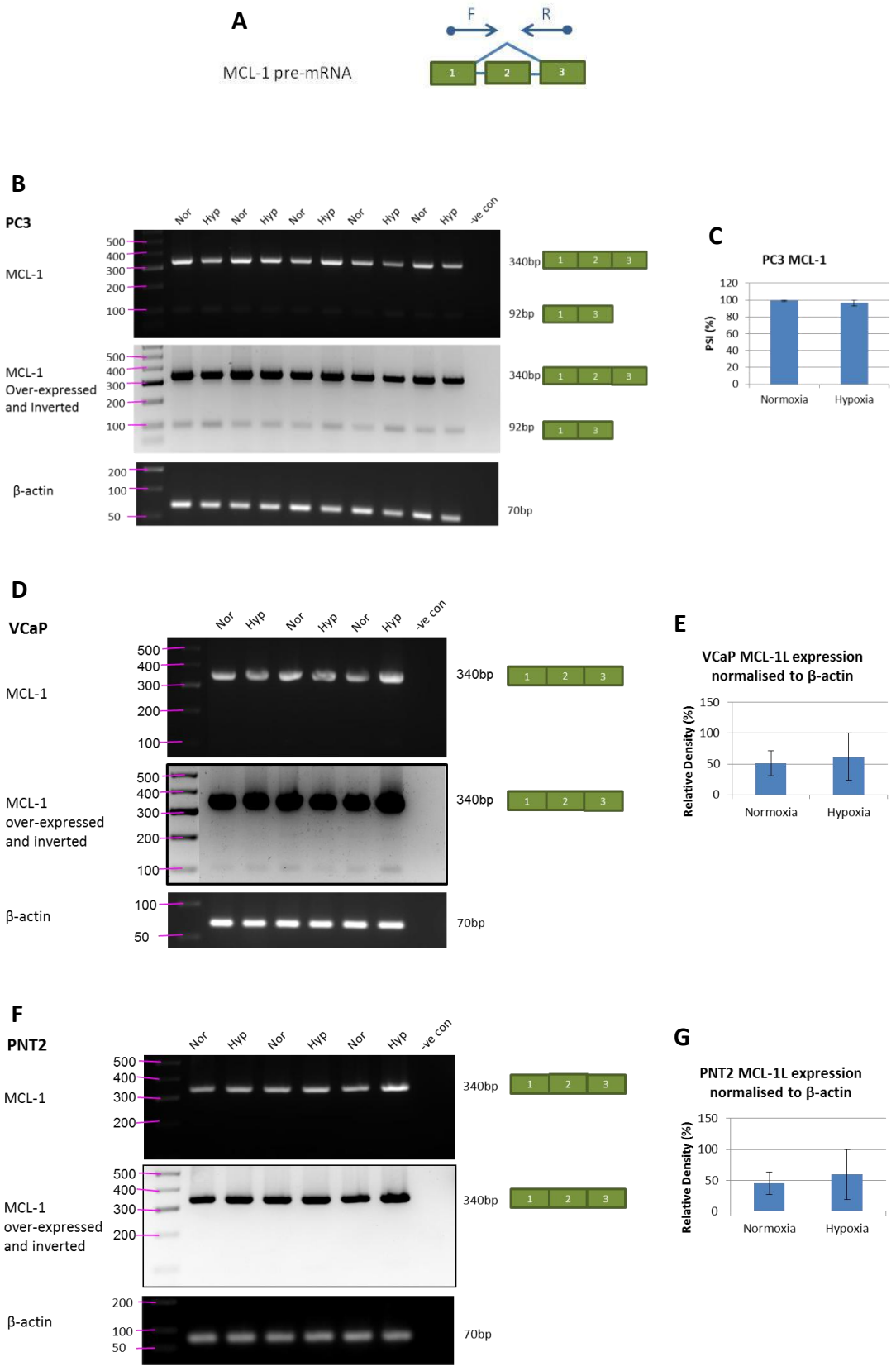


Figure 3.6: Proportion of exon inclusion in the *Mcl-1* gene after hypoxia in prostate cancer cell lines. (A) Illustration of *Mcl-1* pre-mRNA showing exons (green boxes), introns (blue lines) and location of the primers (F = forward; R = reverse). The exon 2 inclusion or skipping

alternative splicing event is also shown. Cells seeded from prostate cancer cell lines were cultured in normoxic or hypoxic conditions. RTPCR was performed using the following conditions: 94^{O}C for 2 min, 94^{O}C for 30 sec, 58^{O}C for 30 sec, 72^{O}C for 30 sec, 72^{O}C for 5 min with 35 cycles. **(B and C)** *Mcl-1* isoform expression with respective average percentage inclusion level of exon 2, also known as the percentage splicing index (PSI %) in the PC3 cell line (n=5). *Mcl-1L* mRNA expression with respective normalisation to β-actin loading control in the **(D and E)** VCaP cell line (n=3), and **(F and G)** PNT2 cell line (n=3). ImageJ software was used to generate optical density peaks from the gel isoform expression. Excel was used to normalise to the β-actin loading control and calculate the average percentage of exon 2 inclusion (%). In the PC3 cell line, Shapiro-Wilks test showed that the data was not normally distributed, and Bartlett's test showed that the data was heteroschedastic and so a Mann Whitney U test was applied to the data. In both the VCaP and PNT2 cell lines, the Shapiro-Wilks test showed that the data was normally distributed, Bartlett's test showed that the data was heteroschedastic, and so student's T tests were applied to the data.

3.4 High-throughput PCR of exon inclusion in cancer-associated genes

A normoxic and a hypoxic sample generated in the PC3 cell line were sent to collaborators (Dr. Roscoe Klinck and his team at the RNomics platform at Sherbrooke University, Canada) for high-throughput PCR analysis. 238 alternatively spliced cancer-associated genes were analysed in the PCR screen to assess the effect of hypoxia on the alternative splicing of cassette exons linked to cancer. The top 12 genes that had a change in the percentage of exon inclusion, also known as the percentage splicing index (PSI) value were selected for further analysis (Table 3.2). PSI is calculated using the following equation; [expression of exon inclusion isoform/total expression of isoforms] * 100. It must be noted that the APAF1 gene features in table 3.2 twice due to the use of two different sets of primers (F1 and F8). The location of these primers are shown later in figures 3.7 and 3.8, but both sets of primers concern exon 17a splicing.

In addition the *Insulin receptor* (*INSR*) and *LIM/homeobox protein 6* (*LHX6*) genes were also selected. The *INSR* gene was chosen as it has been previously shown to increase its expression during hypoxia in glioma cells (Minchenko *et al.,* 2013), and so an investigation into whether this also occurred in PC3 cells was also conducted. *LHX6* regulates genes involved in proliferation, apoptosis, the cell cycle and cell migration (Liu *et al.,* 2013). Therefore, *LHX6* is of interest as it is involved in several hallmarks of cancer (Hanahan and Weinberg, 2011).

Table 3.2 suggests that hypoxia may alter the expression of splice isoforms of some of the cancer-associated genes more than others. For example, *MBP* is shown to have a PSI value of 36.3%, whereas *TTC23* is shown to have a PSI value of 26.4%. This suggests that hypoxia may alter the splicing *MBP* more than *TTC23,* and it could imply that *MBP* has more of a role to play during hypoxia. However, this table is based on n=1, and so more repeats were conducted, which are analysed in the next section.

Gene	PSI (%)	Function
MBP	36.3	Formation of the myelin sheath. Elevated in breast cancer and lung cancer patients with brain metastasis (Shaharabani *et al.,* 2016; Liu *et al.,* 2015; Salh, 1998). **Isoform function:** Unknown but believed to have a role in development (Mathisen *et al.,* 1993; Kruger *et al.,* 1999).
APAF1F8	35.5	Formation of the apoptosome for apoptosis (Bao *et al.,* 2007). **Isoform function:** Without Exon 17a, the apoptosome cannot form to initiate apoptosis (Benedict *et al.,* 2000).
PUF60	33.9	Modulates alternative splicing through recognition of 3' splice sites. Regulates *c-myc* transcription (Hastings *et al.,* 2007; Page-McCaw, Amonlirdviman and Sharp, 1999; Liu *et al.,* 2006). **Isoform function:** Exon 5 deletion forms the FIR protein, which is a *c-myc* repressor (Kano *et al.,* 2016; Matsushita *et al.,* 2014).
APAF1F1	33.6	See APAF1F8 above.
SYNE2	32.3	Shape and migration of the cell (King *et al.,* 2014). **Isoform function:** Absence of scaffolding for protein-protein interactions (Rajgor and Shanahan, 2012).
CDC42BPA	32.1	Reorganisation of the cytoskeleton, formation of filopodia and assignment of cellular polarity. Implicated in cancer cell motility and invasion (Gomes *et al.,* 2005; Tan *et al.,* 2008; Nobes *et al.,* 1995; Kozma *et al.,* 1995; Etienne-Manneville and Hall, 2002). **Isoform function:** Unknown.
FGFR1OP	31.1	Ciliogenesis, cellular motility, cell growth and cell cycle progression (Lee and Stearns, 2013). **Isoform function:** Unknown.
BTN2A2	30.8	Immune tolerance of cancers (Smith *et al.,* 2010). **Isoform function:** Negatively regulates the expression of *FL-BTN2A2.*
UTRN	30.4	Maintaining the cytoskeleton (Li *et al.,* 2007). **Isoform function:** Unknown.
RAP1GDS1	26.6	Activates multiple small GTPases in the Rho and Ras families (Quilliam, Rebhun and Castro, 2002). **Isoform function:** Exon 5 skipped variant plays a greater role in proliferation and NFκB activity than the full-length splice variant (Hause *et al.,* 2013).
PTPN13	26.5	Conflicting roles as a tumour suppressor and oncogene (Freiss and Chalbos, 2012). **Isoform function:** Unknown.
TTC23	26.4	Unknown function, but expression found linked to cervical, bladder and prostate cancers.
INSR	22.6	Stimulates transcription of insulin-related genes (Leibiger *et al.,* 2001). **Isoform function:** Exon 11 inclusion transcribes *glucagon*. Exon 11 skipping transcribes the *insulin* gene which is found elevated in some cancers (Seino *et al.,* 1989; Leibiger *et al.,* 2001).
LHX6	20.8	Differentiation and development of neural and lymphoid cells, interneurone migration. Tumour suppressor gene (Liodis *et al.,* 2007; Jung *et al.,* 2010; Liu *et al.,* 2013). **Isoform function:** Unknown function but exon 9 skipping produces a truncated protein (Kimura *et al.,* 1999).

Table 3.2: Genes selected for further investigation after high-throughput PCR analysis. The top 12 genes with a change in the percentage of exon inclusion ([full length isoform expression/total expression]*100), also known as the percentage splicing index (PSI %) after 48h hypoxia compared to normoxia were selected for further investigation. The *INSR* and *LHX6* genes were also selected for further investigation (highlighted in orange), as *INSR* has been previously shown to be affected by hypoxia in glioma cells (Minchenko *et al.,* 2013) and *LHX6* is involved in many different hallmarks of cancer (Liu *et al.,* 2013). Known gene and splice isoform functions are also shown.

3.5 Verification of high-throughput PCR results

As the high-throughput PCR results were based on n=1, the selected gene set was further analysed with more repeats in the PC3 cell line. In addition, the effect of hypoxia on isoform expression was also examined in the PNT2 and VCaP cell lines.

APAF1F1 and *APAF1F8* both used primers that spanned across exons 17-19 and detected exon 17a inclusion or exclusion. The *APAF1F1* primer set found a significant decrease in exon inclusion in the PC3 cell line after hypoxia but did not find a significant difference in the VCaP or PNT2 cell lines (Figures 3.7). The *APAF1F8* primer set showed a similar pattern of decreased exon inclusion in the PC3 cell line; however, the long error bars prevented this result form being significant (Figure 3.8).

In addition, there was also a significant decrease in PSI value of the *PTPN13* gene in the PC3 cell line, but this was not observed in the VCaP or PNT2 cell lines (Figure 3.9). There was a significant decrease in PSI value with hypoxia treatment in the *CDC42BPA* and *FGFR1OP* genes in the PC3 and PNT2 cell lines; and also in the VCaP cell line for *CDC42BPA* (Figures 3.10 and 3.11). A Shapiro-Wilks test revealed that the data collected for *FGFR1OP* isoform expression in the VCaP cell line was not normally distributed which ruled out the use of a student's T test for data analysis of significance. However, a Mann Whitney U test could not be conducted successfully as there were not enough data points, and so the significance of this data is unknown. With further repeats a Mann Whitney U test could be conducted to find out if hypoxia significantly alters the splicing of *FGFR1OP* in the VCaP cell

line. However, there is no indication from the gel image that hypoxia has an effect on exon inclusion in *FGFR1OP* in the VCaP cell line. There was a significant decrease in exon inclusion in the *RAP1GDS1* and *UTRN* genes after hypoxia in the PC3 and VCaP cell lines (Figures 3.12 B and C and 3.13 B and C). However, a change in exon inclusion was not observed in the PNT2 cell line for both genes (Figures 3.12 F and G and 3.13 F and G).

Hypoxia significantly decreased the PSI value of *PUF60* exon inclusion in the PC3 cell line (Figure 3.14B and C). A larger isoform was also detected in the VCaP and PNT2 cell lines, and so the expression of each isoform was calculated as a percentage of the overall proportion. The calculation was as follows: (Expression of isoform of interest / total isoform expression) * 100. In both the VCaP and PNT2 cell lines, the proportion of exon 5 skipping of *PUF60* significantly increased during hypoxia. Taking into account the results for *PUF60* splicing in all three cell lines, a conclusion can be made that hypoxia favours the exon 5 skipped splice variant of *PUF60.*

Hypoxia was found to significantly incease exon inclusion in the *MBP* gene after in the PNT2 cell line (Figure 3.15 F and G). There was not a significant change in splicing of the *MBP* mRNA during hypoxia in the PC3 and VCaP cell lines. This result was quite surprising as the change in PSI value for *MBP* splicing was the highest in the high-throughput PCR experiment.

Hypoxia was found to significantly favour exon inclusion in the *BTN2A2* and *TTC23* genes in the PC3 and PNT2 cell lines (Figures 3.16 B, C, F and G and 3.17 B, C, F and G). There was no significant difference in exon inclusion found in the *INSR, LHX6*

and *SYNE2* genes after hypoxia (Figures 3.18, 3.19 and 3.20). However, there was an indication from gel PCR images that hypoxia decreased the amount of exon inclusion in the *LHX6* and *SYNE2* genes in the PNT2 and PC3 cell lines, respectively (Figures 3.19 F and 3.20 B). The error bars for both of these results are quite long and overlap, which is why a significant result may not have been found. Table 3.3 concludes the significant effect of hypoxia on exon inclusion of the genes selected from the high-throughput PCR gene set in the PC3, VCaP and PNT2 cell lines.

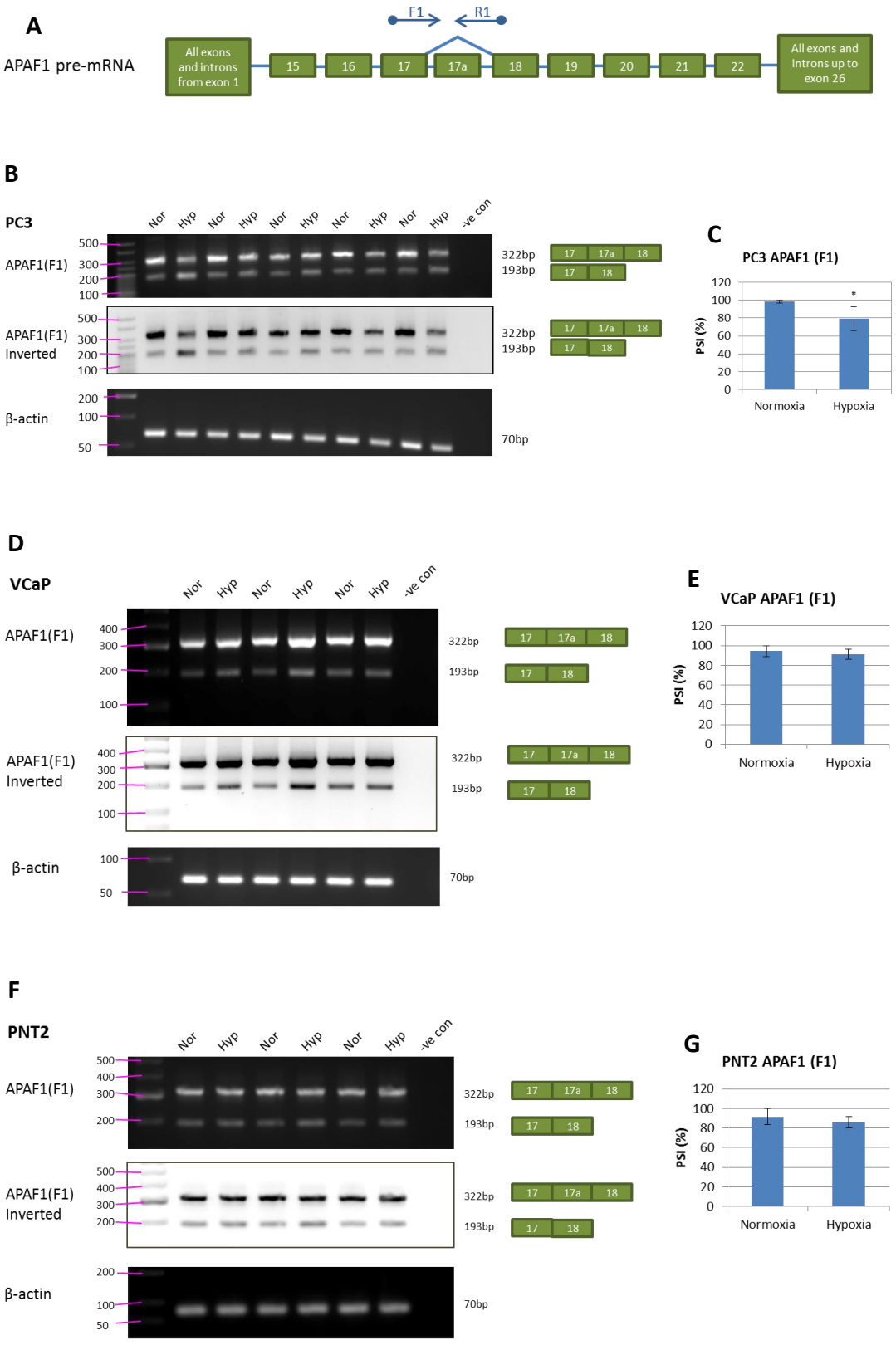


Figure 3.7: Proportion of exon inclusion in the *APAF1* gene using F1 primers after hypoxia in prostate cancer cell lines. (A) Illustration of *APAF1* pre-mRNA showing exons (green boxes), introns (blue lines), and location of the primers (F= forward primer; R= reverse primer). The

exon 17a inclusion or skipping alternative splicing even is also shown. Cells seeded from prostate cancer cell lines were cultured in normoxic or hypoxic conditions. RTPCR was performed using the following conditions: 94^{o}C for 2 min, 94^{o}C for 30 sec, 58^{o}C for 30 sec, 72^{o}C for 30 sec, 72^{o}C for 5 min with 35 cycles. *APAF1* isoform expression with respective average percentage inclusion of exon 17a, also known as the percentage splicing index (PSI %) after normoxic or hypoxic treatment in the **(B and C)** PC3 cell line (n=5); **(D and E)** VCaP cell line (n=3); **(F and G)** PNT2 cell lines (n=3). ImageJ software was used to generate optical density peaks from the gel isoform expression. Excel was used to normalise to the β-actin loading control, and then calculate the average percentage of exon 17a inclusion (%). * = $p<0.05$. Statistical tests used were as follows, where SW stands for shapiro wilks test, BT stands for Bartlett's test, MWU stands for Mann-Whitney U and TT stands for student's T-test. PC3: SW showed data was not normally distributed, BT showed that the data was heteroschedastic and so a MWU test was used to test statistical significance of the data. For both VCaP and PNT2 data: SW showed data was normally distributed, BT showed data was homoschedastic, and so a TT was used to test statistical significance of the data.

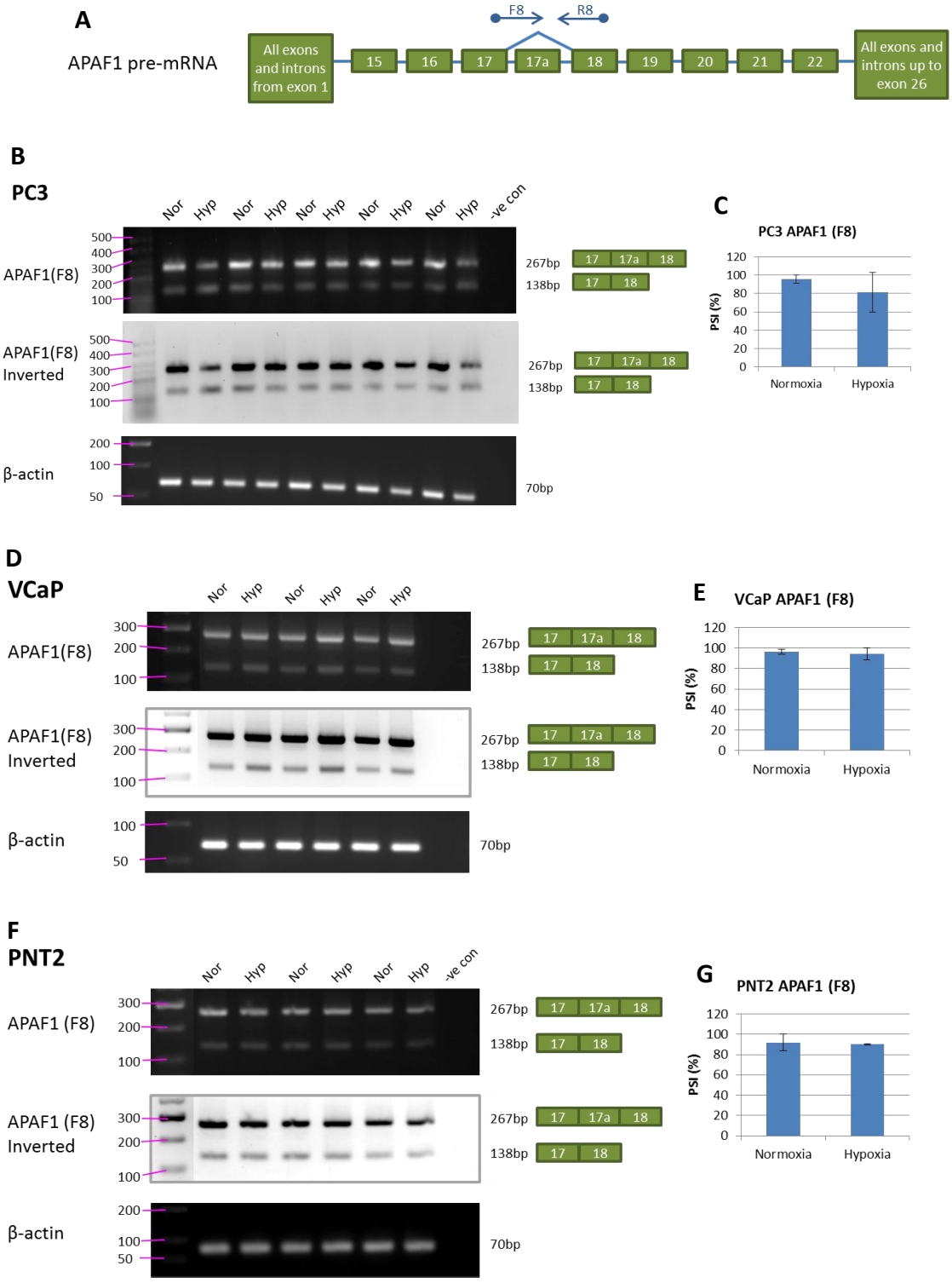


Figure 3.8: Proportion of exon inclusion in the *APAF1* gene using F8 primers after hypoxia in prostate cancer cell lines. (A) Illustration of *APAF1* pre-mRNA showing exons (green boxes), introns (blue lines), and location of the primers (F= forward primer; R=reverse primer). The exon 17a inclusion or skipping alternative splicing event is also shown. Cells seeded from prostate cancer cell lines were cultured in normoxic or hypoxic conditions. RTPCR was performed using the following conditions: 94^{o}C for 2 min, 94^{o}C for 30 sec, 58^{o}C for 30 sec,

72^{o}C for 30 sec, 72^{o}C for 5 min with 35 cycles. *APAF1* isoform expression with respective average percentage inclusion level of exon 17a, also known as the percentage splicing index (PSI %) after normoxic or hypoxic treatment in the **(B and C)** PC3 cell line (n=5); **(D and E)** VCaP cell line (n=3); **(F and G)** PNT2 cell lines (n=3). ImageJ software was used to generate optical density peaks from the gel isoform expression. Excel was used to normalise to the β-actin loading control, and then calculate the average percentage of exon 17a inclusion (%). Statistical tests used were as follows, where SW stands for shapiro wilks test, BT stands for Bartlett's test, MWU stands for Mann-Whitney U and TT stands for student's T-test. PC3: SW showed data was not normally distributed, BT showed that the data was heteroschedastic and so a MWU test was used to test statistical significance of the data. For both VCaP and PNT2: SW showed data was normally distributed, BT showed data was homoschedastic, and so a TT was used to test statistical significance of the data.

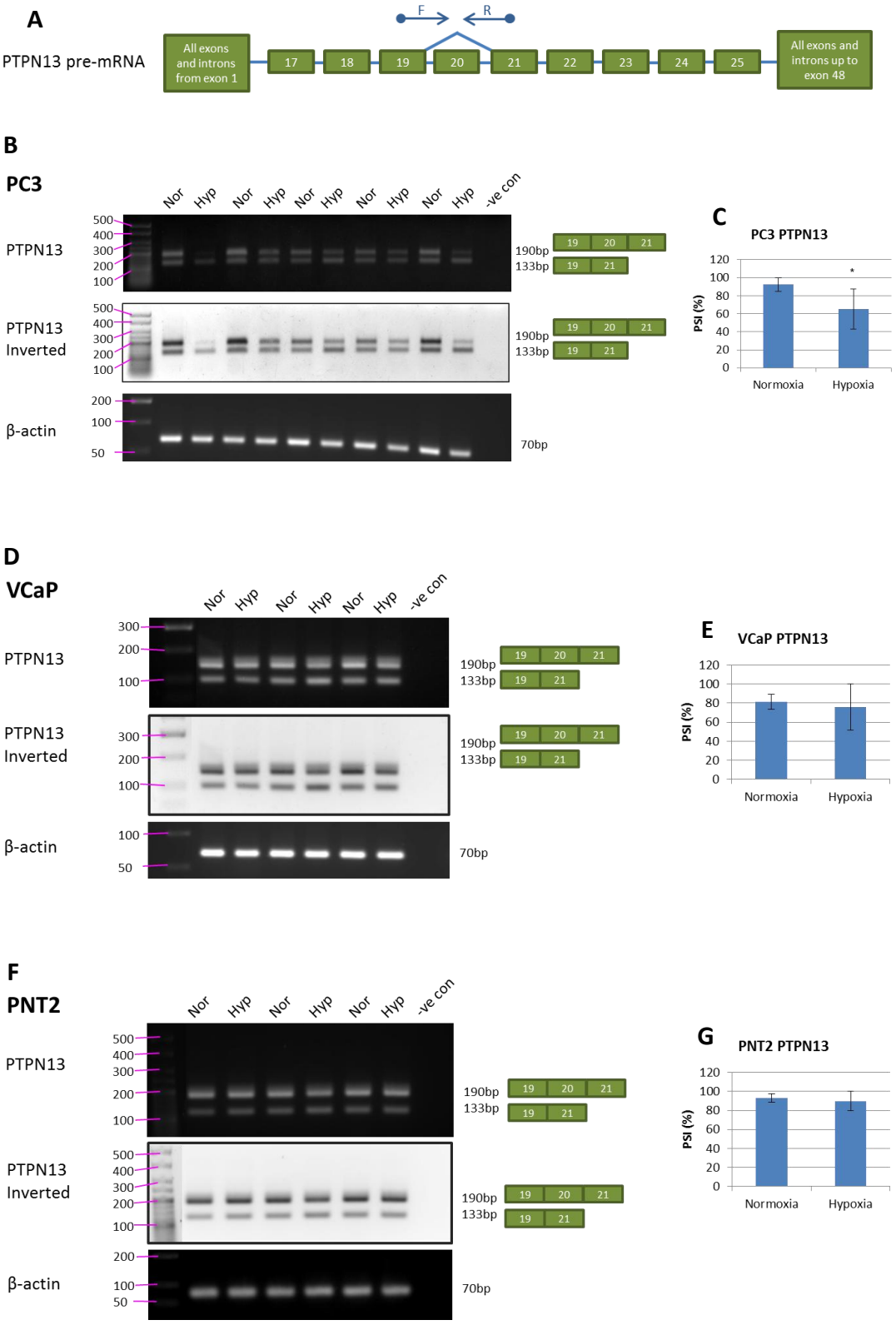


Figure 3.9: Proportion of exon inclusion in the *PTPN13* gene after hypoxia in prostate cancer cell lines. (A) Illustration of *PTPN13* pre-mRNA showing exons (green boxes), introns (blue lines) and location of the primers (F= forward primer; R= reverse primer). The exon 20

inclusion or skipping alternative splicing event is also shown. Cells seeded from prostate cancer cell lines were cultured in normoxic or hypoxic conditions. RTPCR was performed using the following conditions: 94^{o}C for 2 min, 94^{o}C for 30 sec, 58^{o}C for 30 sec, 72^{o}C for 30 sec, 72^{o}C for 5 min with 35 cycles. *PTPN13* isoform expression with respective average percentage inclusion level of exon 20, also known as the percentage splicing index (PSI %) after normoxic or hypoxic treatment in the **(B and C)** PC3 cell line (n=5); **(D and E)** VCaP cell line (n=3); **(F and G)** PNT2 cell lines (n=3). ImageJ software was used to generate optical density peaks from the gel isoform expression. Excel was used to normalise to the β-actin loading control, and then calculate the average percentage of exon 20 inclusion (%). * = $p<0.05$. Statistical tests used were as follows, where SW stands for Shapiro-Wilks test, BT stands for Bartlett's test, MWU stands for Mann-Whitney U test and TT stands for student's T-test. In all three cell lines, SW showed data was normally distributed, BT showed that the data was homoschedastic, and so TTs were used to test statistical significance of the data.

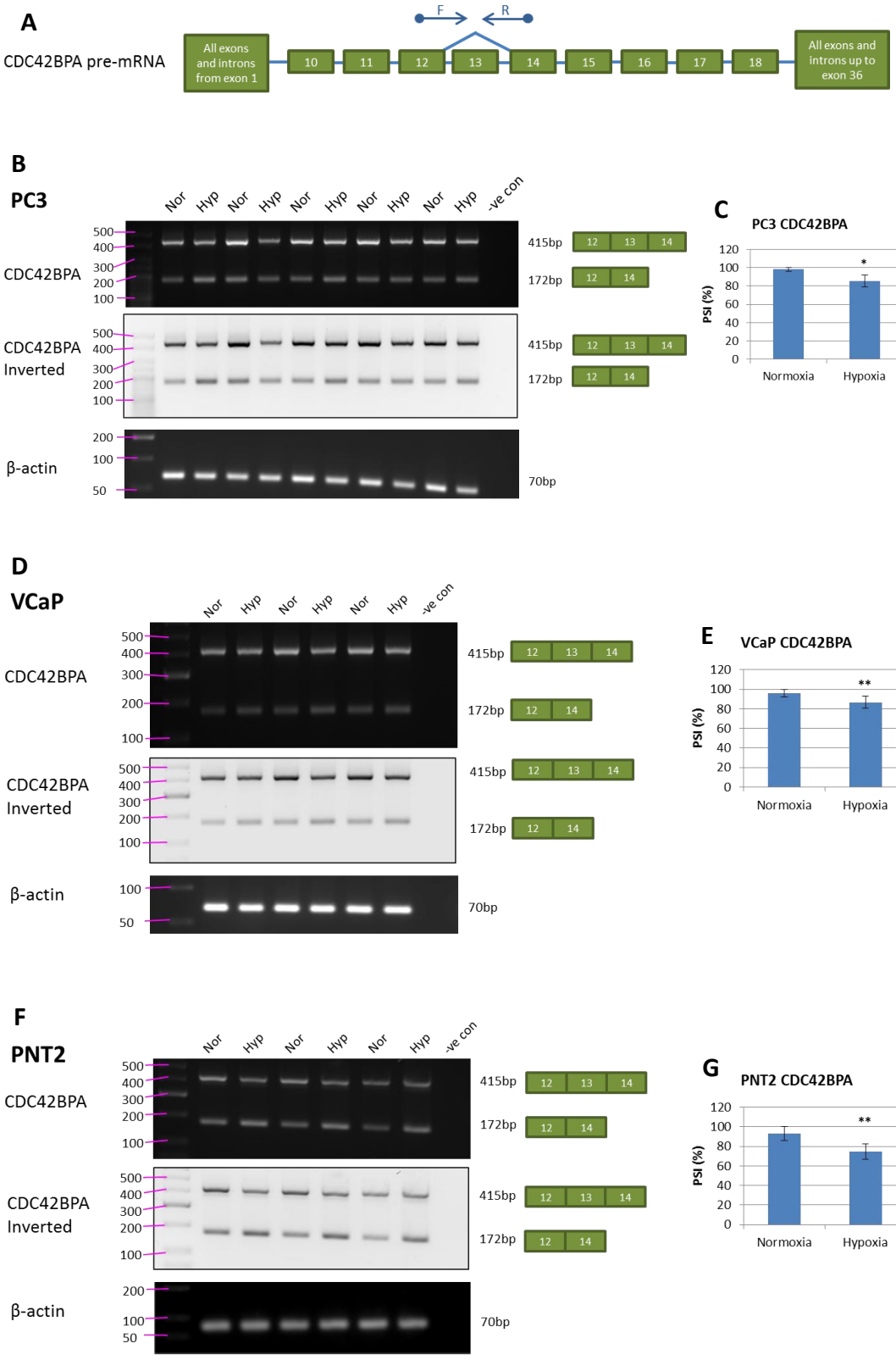


Figure 3.10: Proportion of exon inclusion in the *CDC42BPA* gene after hypoxia in prostate cancer cell lines. (A) Illustration of *CDC42BPA* pre-mRNA showing exons (green boxes), introns (blue lines) and location of the primers (F = forward primer; R = reverse primer). The

exon 13 inclusion or exclusion alternative splicing event is also shown. Cells seeded from prostate cancer cell lines were cultured in normoxic or hypoxic conditions. RTPCR was performed using the following conditions: 94^{o}C for 2 min, 94^{o}C for 30 sec, 58^{o}C for 30 sec, 72^{o}C for 30 sec, 72^{o}C for 5 min with 35 cycles. *CDC42BPA* isoform expression with the respective average percentage inclusion level of exon 13, also known as the percentage splicing index (PSI %) after normoxic or hypoxic treatment in the **(B and C)** PC3 cell line (n=5); **(D and E)** VCaP cell line (n=3); **(F and G)** PNT2 cell line (n=3). ImageJ software was used to generate optical density peaks from the gel isoform expression. Excel was used to normalise to the β-actin loading control, and then calculate the average percentage of *CDC42BPA* exon inclusion (%). *=p<0.05; ** = p<0.01. Statistical tests used were as follows, where SW stands for Shapiro-Wilks test, BT stands for Bartlett's test, MWU stands for Mann-Whitney U test and TT stands for student's T-test. PC3: SW showed data was normally distrubuted, BT showed that the data was heteroschedastic and so a MWU test was used to test statistical significance of the data. In both the VCaP and PNT2 cell lines, SW showed the data was normally distributed, BT showed that the data was homoschedastic and so a TT was used to test the statistical significance of the data.

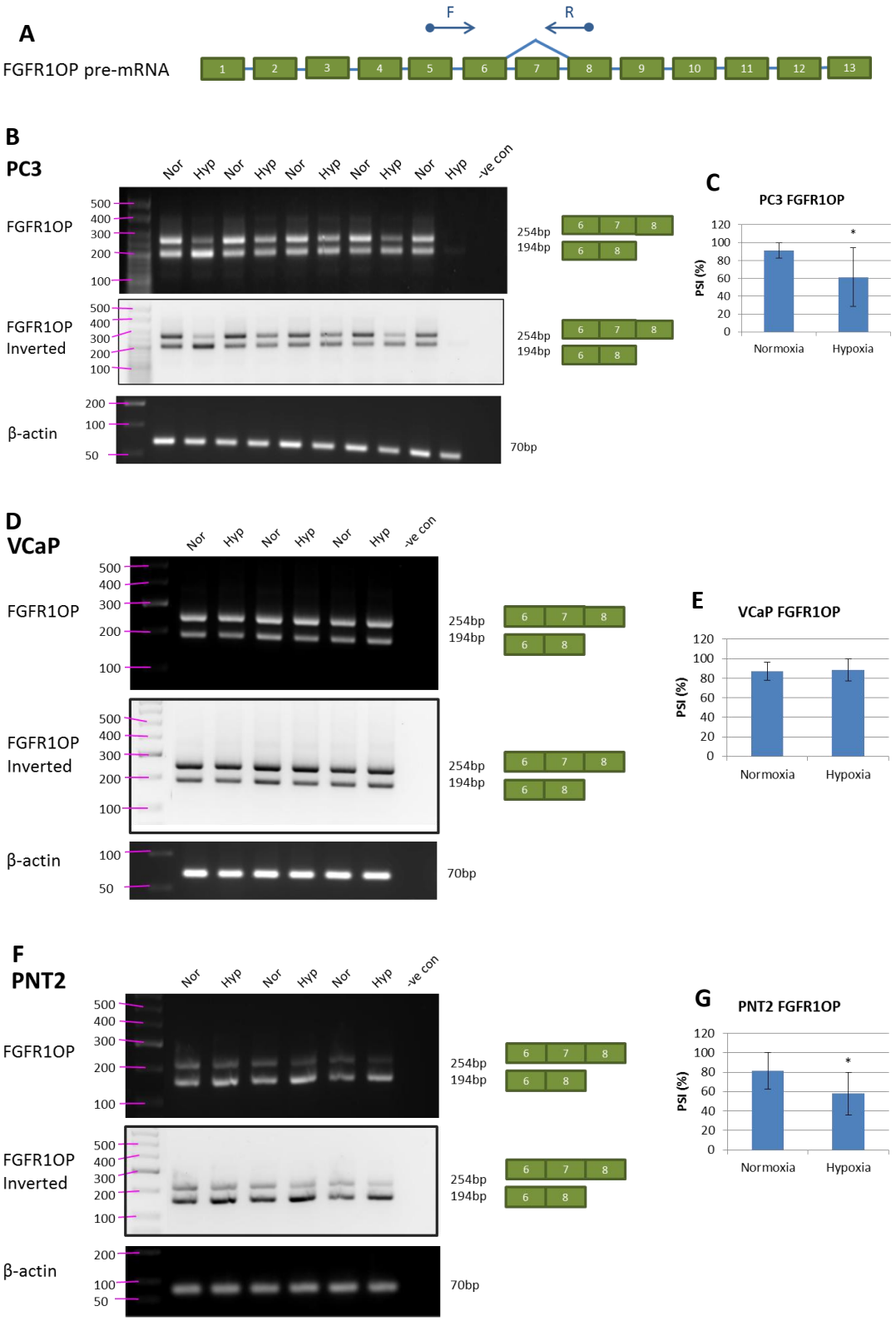


Figure 3.11: Proportion of exon inclusion in the *FGFR1OP* gene after hypoxia in prostate cancer cell lines. (A) Illustration of *FGFR1OP* pre-mRNA showing exons (green boxes), introns (blue lines) and location of the primers (F = forward primer; R = reverse primer). The exon 7 inclusion or skipping alternative splicing event is also shown. Cells seeded from prostate

cancer cell lines were cultured in normoxic or hypoxic conditions. RTPCR was performed using the following conditions: 94^{O}C for 2 min, 94^{O}C for 30 sec, 58^{O}C for 30 sec, 72^{O}C for 30 sec, 72^{O}C for 5 min with 35 cycles. *FGFR1OP* isoform expression with respective average percentage inclusion level of exon 7, also known as the percentage splicing index (PSI %) after normoxic or hypoxic treatment in the **(B and C)** PC3 cell line (n=5); **(D and E)** VCaP cell line (n=3); **(F and G)** PNT2 cell line (n=3). ImageJ software was used to generate optical density peaks from the gel isoform expression. Excel was used to normalise to the β-actin loading control, and then calculate the average percentage of exon 7 inclusion (%). * = $p<0.05$. Statistical tests used were as follows, where SW stands for Shapiro-Wilks test, BT stands for Bartlett's test, MWU stands for Mann-Whitney U test and TT stands for student's T-test. In both the PC3 and PNT2 cell lines, SW showed that the data was normally distributed, BT showed that the data was homoschedastic, and so TT were used to test the statistical significance of the data. In the VCaP cell line, SW showed that the data was not normally distributed, BT showed that the data was homoschedastic, and so a MWU test was attempted to test the statistical significance of the data. However, due to there only being three data points, the test was unsuccessful.

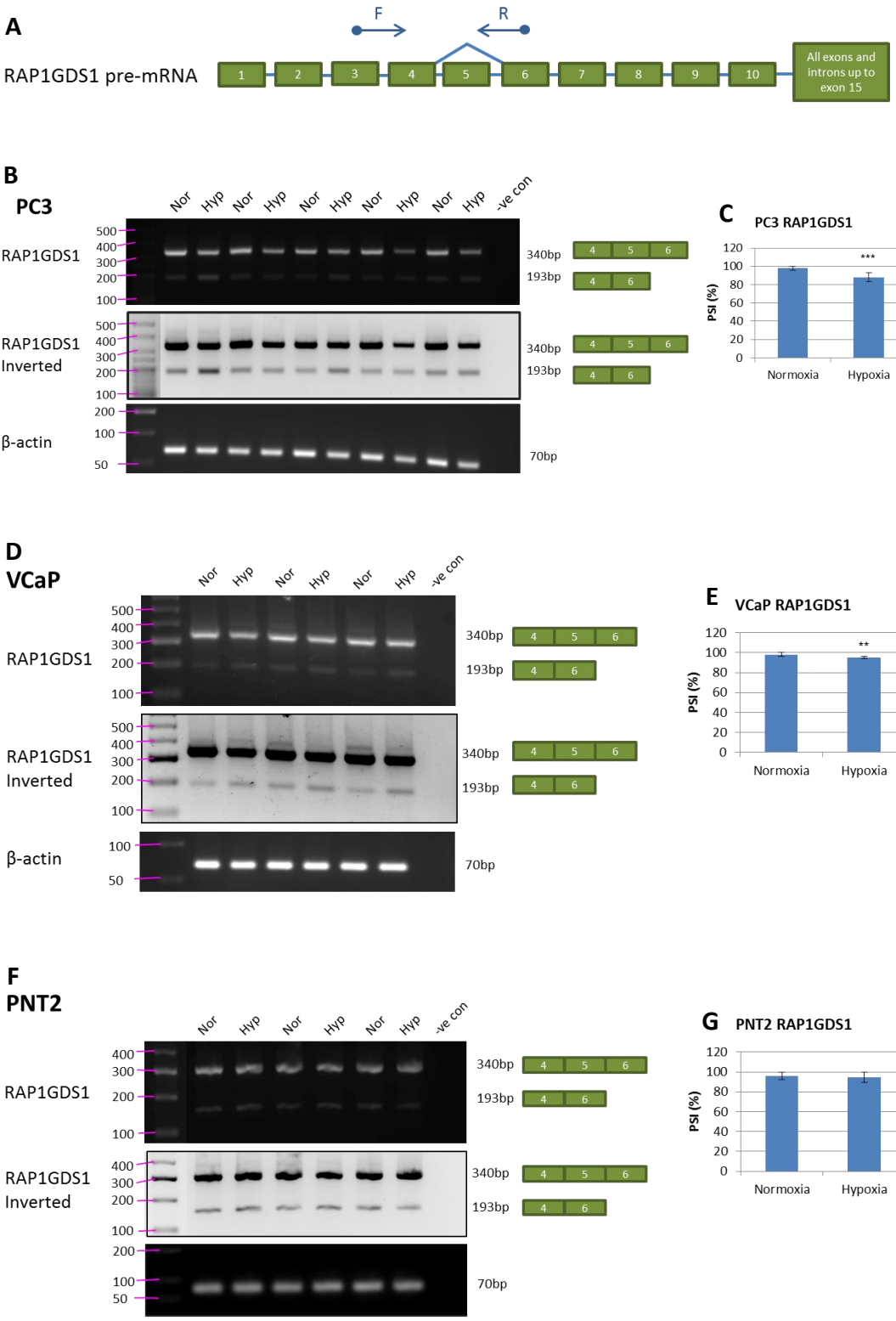


Figure 3.12: Proportion of exon inclusion in the *RAP1GDS1* gene after hypoxia in prostate cancer cell lines. Illustration of *RAP1GDS1* pre-mRNA showing exons (green boxes), introns (blue lines) and location of the primers (F = forward primer; R = reverse primer). The exon 5 inclusion of skipping alternative splicing event is also shown. Cells seeded from prostate

cancer cell lines were cultured in normoxic or hypoxic conditions. RTPCR was performed using the following conditions: 94^{O}C for 2 min, 94^{O}C for 30 sec, 58^{O}C for 30 sec, 72^{O}C for 30 sec, 72^{O}C for 5 min with 35 cycles. *RAP1GDS1* isoform expression with respective average percentage inclusion level of exon 5, also known as the percentage splicing index (PSI %) after normoxic or hypoxic treatment in the **(B and C)** PC3 cell line (n=5); **(D and E)** VCaP cell line (n=3); **(F and G)** PNT2 cell lines (n=3). ImageJ software was used to generate optical density peaks from the gel isoform expression. Excel was used to normalise to the β-actin loading control, and then calculate the average percentage of exon 5 inclusion (%). ** = $p<0.01$; *** = $p<0.001$. In all three cell lines, the Shapiro-Wilks test showed that the data was normally distributed, Bartlett's Test showed that the data was homoschedastic and so student's T-tests were used to assess the statistical significance of the data.

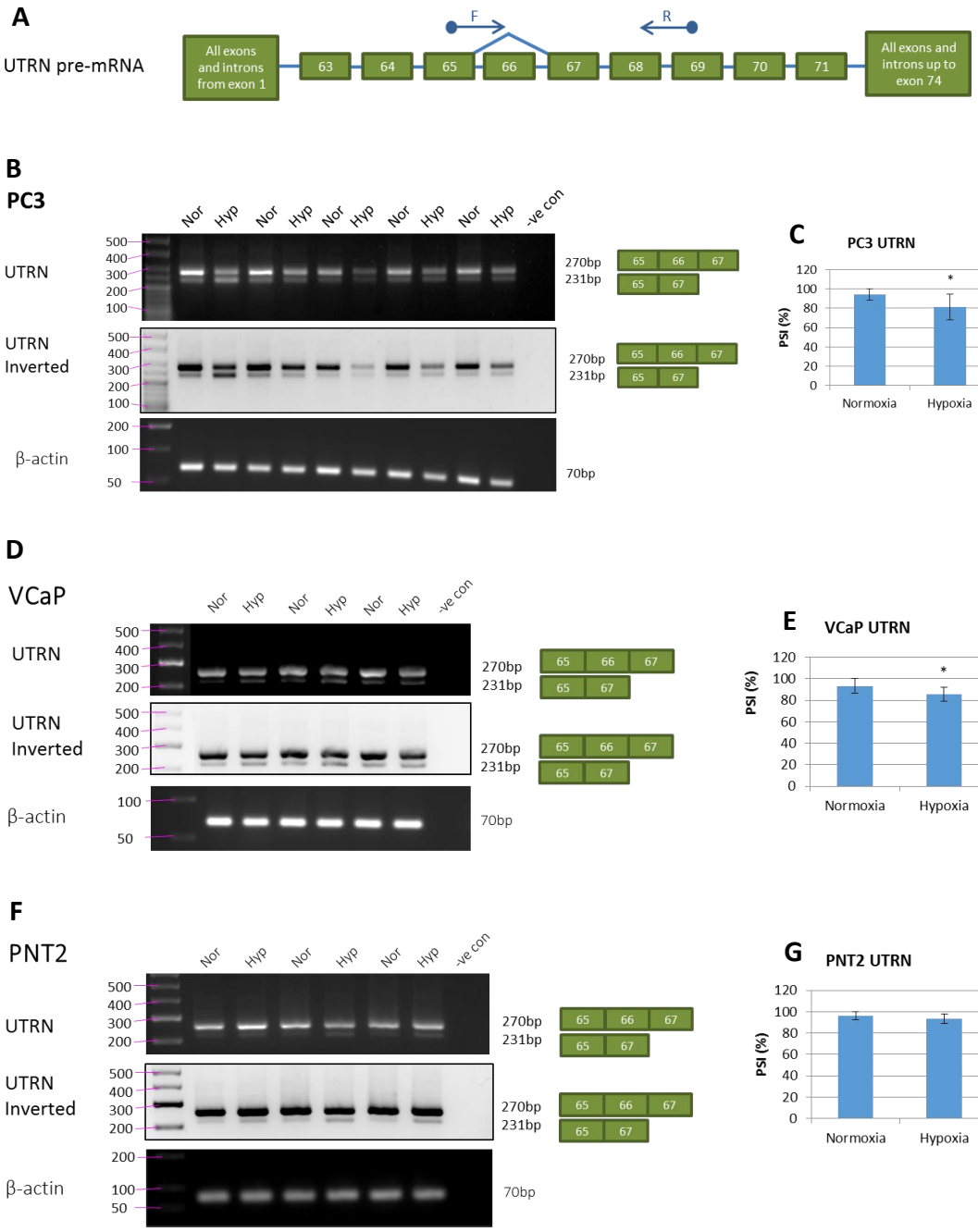


Figure 3.13: Proportion of exon inclusion in the *UTRN* gene after hypoxia in prostate cancer cell lines. (A) Illustration of *URTN* pre-mRNA showing exons (green boxes), introns (blue lines), and location of the primers (F = forward primer; R = reverse primer). The exon 66 inclusion or skipping alternative splicing event is also shown. Cells seeded from prostate cancer cell lines were cultured normoxic or hypoxic conditions. RTPCR was performed using the following conditions: 94^{o}C for 2 min, 94^{o}C for 30 sec, 58^{o}C for 30 sec, 72^{o}C for 30 sec, 72^{o}C for 5 min with 35 cycles. *UTRN* isoform expression with respective average percentage inclusion level of exon 66, also known as the percentage splicing index (PSI %) after normoxic or hypoxic treatment in the **(B and C)** PC3 cell line (n=5); **(D and E)** VCaP cell line (n=3); **(F and G)** PNT2 cell line (n=3). ImageJ software was used to generate optical density peaks from the gel isoform expression. Excel was used to normalise to the β-actin loading control, and then

calculate the average percentage of exon 66 inclusion (%). * = $p<0.05$. Statistical tests used were as follows, where SW stands for Shapiro-Wilks test, BT stands for Bartlett's test, MWU stands for Mann-Whitney U test and TT stands for student's T-test. PC3: SW showed that the data was not normally distributed, BT showed that the data was homoschedastic, and so a MWU test was used to assess the statistical significance of the data. In both the VCaP and PNT2 cell lines, SW showed that the data were normally distributed, BT showed that the data were homoschedastic, and so TT were used.

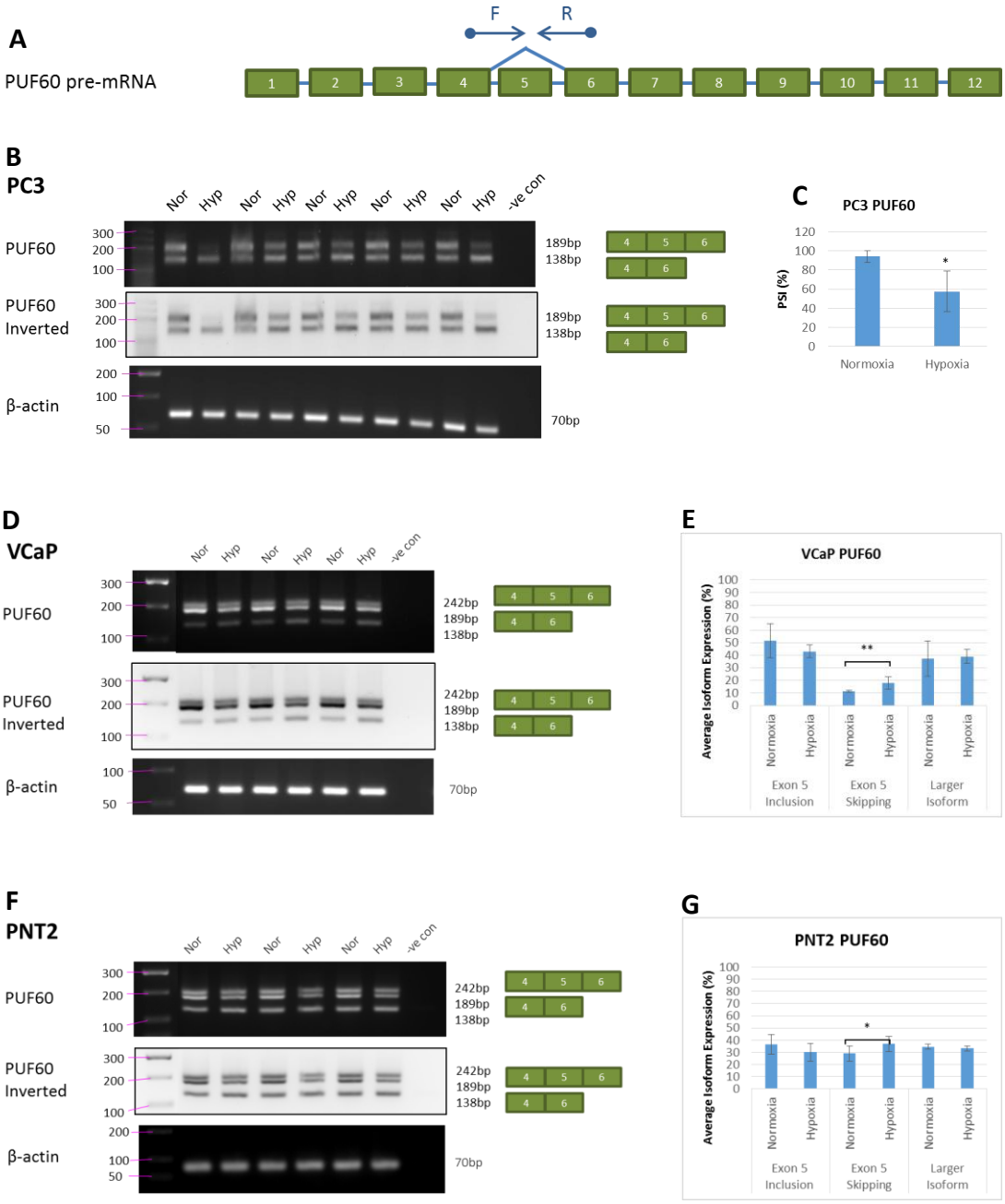


Figure 3.14: Proportion of *PUF60* isoforms after hypoxia in prostate cancer cell lines. (A) Illustration of *PUF60* pre-mRNA showing exons (green boxes), introns (blue lines) and the location of the primers (F = forward primer; R = reverse primer). The exon 5 inclusion or skipping alternative splicing event is also shown. Cells seeded from prostate cancer cell lines were cultured in normoxic or hypoxic conditions. RTPCR was performed using the following conditions: 94^{o}C for 2 min, 94^{o}C for 30 sec, 58^{o}C for 30 sec, 72^{o}C for 30 sec, 72^{o}C for 5 min with 35 cycles. *PUF60* isoform expression with respective average percentage inclusion level of exon 5, also known as the percentage splicing index (PSI %) after normoxic or hypoxic treatment in the **(B and C)** PC3 cell line (n=5); **(D and E)** VCaP cell line (n=3); **(F and G)** PNT2 cell line (n=3). A larger isoform was also detected in the VCaP and PNT2 cell lines and so the percentage of each of the isoforms detected are shown in the graphs for those cell lines.

ImageJ software was used to generate optical density peaks from the gel isoform expression. Excel was used to normalise to the β-actin loading control, and then calculate the average percentage proportion of *PUF60* isoforms (%). Calculations were as follows; (1) exon 5 inclusion/total*100; (2) exon 5 skipping/total*100; (3) larger isoform/total*100. * = $p<0.05$; ** = $p<0.01$. Statistical tests used were as follows, where SW stands for Shapiro-Wilks test, BT stands for Bartlett's test, MWU stands for Mann-Whitney U test and TT stands for student's T-test. In the PC3 cell line, SW showed that the data was normally distributed, BT showed that the data was heteroschedastic, and so a MWU test was used to assess the statistical significance of the data. In all tests conducted in the VCaP and PNT2 cell lines, SWs showed that the data were normally distributed, BTs showed that the data were homoschedastic, and so TTs were used to assess the statistical significance of the data.

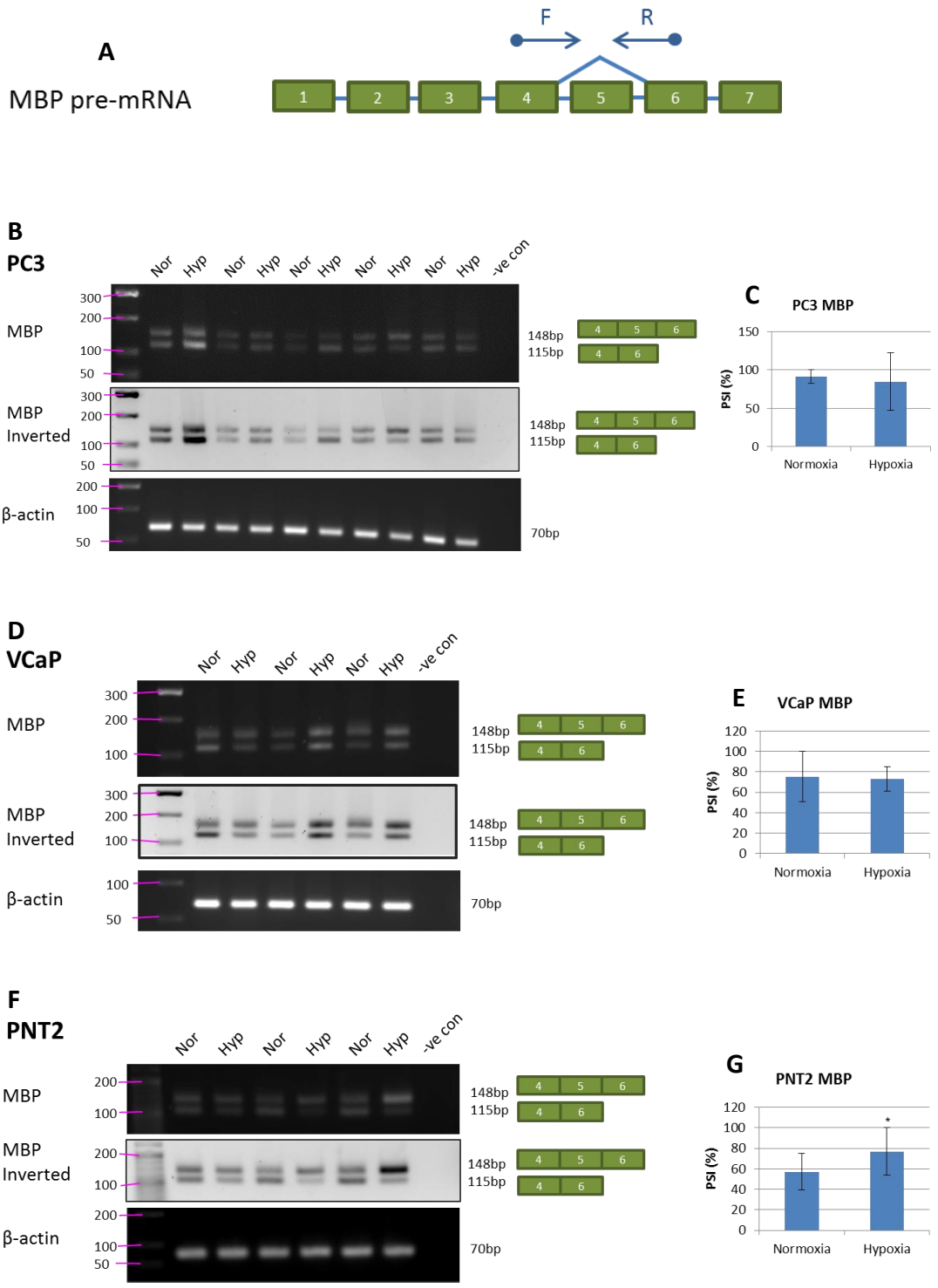


Figure 3.15: Proportion of exon inclusion in the *MBP* gene after hypoxia in prostate cancer cell lines. (A) Illustration of *MBP* pre-mRNA showing exons (green boxes), introns (blue lines) and location of the primers (F = forward primer; R = reverse primer). The exon 5 inclusion or skipping alternative splicing event is also shown. Cells seeded from prostate cancer cell lines were cultured in normoxic or hypoxic conditions. RTPCR was performed using the following conditions: 94^{o}C for 2 min, 94^{o}C for 30 sec, 58^{o}C for 30 sec, 72^{o}C for 30 sec, 72^{o}C for 5 min

with 35 cycles. *MBP* isoform expression with respective average percentage inclusion level of exon 5, also known as the percentage splicing index (PSI %) after normoxic or hypoxic treatment in the **(B and C)** PC3 cell line (n=5); **(D and E)** VCaP cell line (n=3); **(F and G)** PNT2 cell lines (n=3). ImageJ software was used to generate optical density peaks from the gel isoform expression. Excel was used to normalise to the β-actin loading control, and then calculate the average percentage of exon 5 inclusion (%). * = $p<0.05$. Statistical tests used were as follows, where SW stands for Shapiro-Wilks test, BT stands for Bartlett's test, MWU stands for Mann-Whitney U test and TT stands for student's T-test. PC3: SW showed that the data was normally distributed, BT showed that the data was heteroschedastic, and so a MWU was used to assess the statistical significance of the data. In both the VCaP and PNT2 cell lines, SW tests showed that the data were normally distributed, BTs showed that the data were homoschedastic, and so TTs were used to assess the statistical significance of the data.

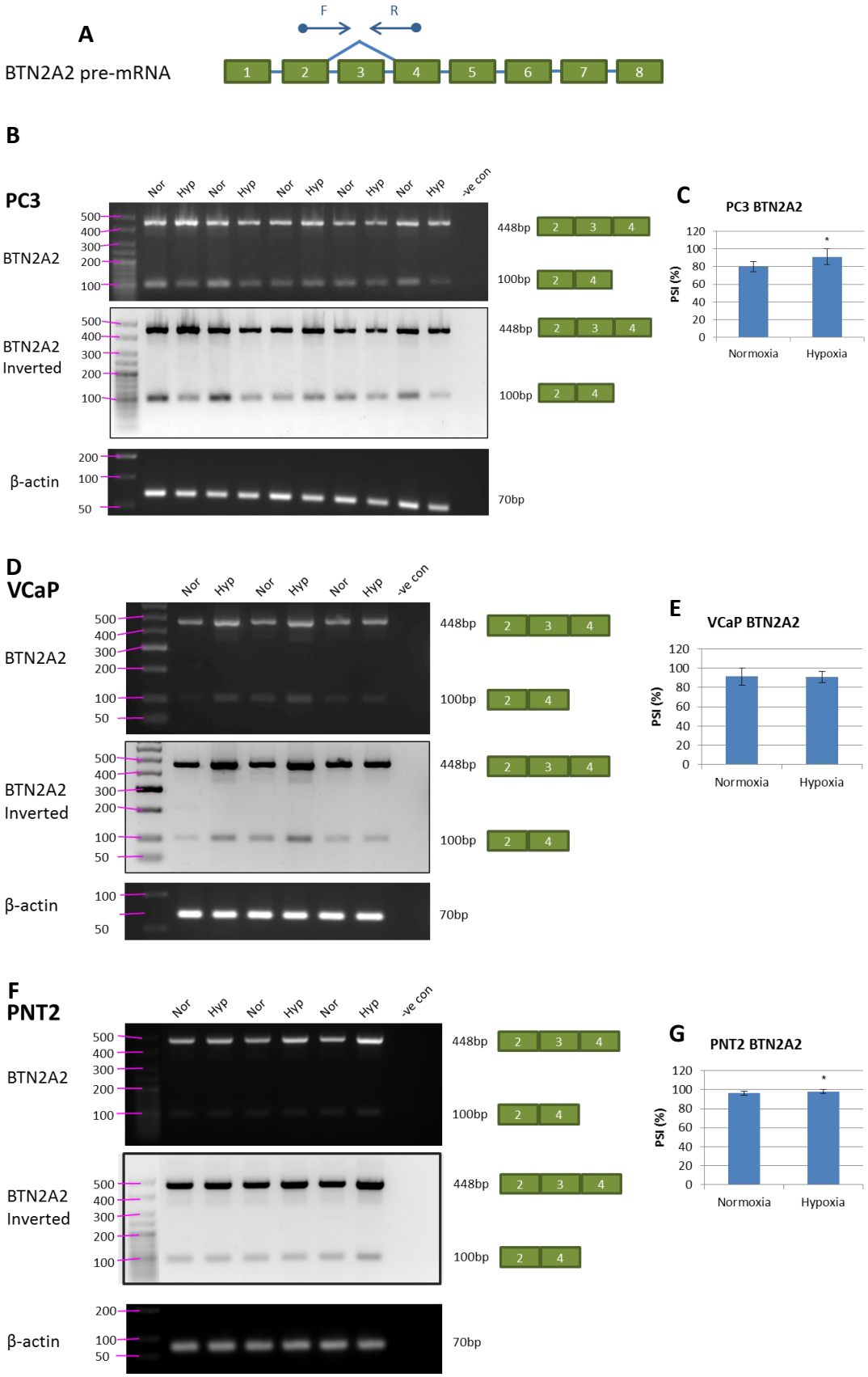


Figure 3.16: Proportion of exon inclusion in the *BTN2A2* gene after hypoxia in prostate cancer cell lines. (A) Illustration of *BTN2A2* pre-mRNA showing exons (green boxes), introns (blue lines) and

location of the primers (F = forward primer; R = reverse primer). The exon 3 inclusion or skipping alternative splicing event is also shown. Cells seeded from prostate cancer cell lines were cultured in normoxic or hypoxic conditions. RTPCR was performed using the following conditions: 94^{O}C for 2 min, 94^{O}C for 30 sec, 58^{O}C for 30 sec, 72^{O}C for 30 sec, 72^{O}C for 5 min with 35 cycles. *BTN2A2* isoform expression with respective average percentage inclusion level of exon 3, also known as the percentage splicing index (PSI %) after normoxic or hypoxic treatment in the **(B and C)** PC3 cell line (n=5); **(D and E)** VCaP cell line (n=3); **(F and G)** PNT2 cell line (n=3). ImageJ software was used to generate optical density peaks from the gel isoform expression. Excel was used to normalise to the β-actin loading control, and then calculate the average percentage of exon 3 inclusion (%). * = $p<0.05$. In all three cell lines, statistical tests were as follows. Shapiro-Wilks test showed that the data was normally distributed, Bartlett's test showed that the data was homoschedastic, and so student's T tests were used to asses the statistical significance of the data.

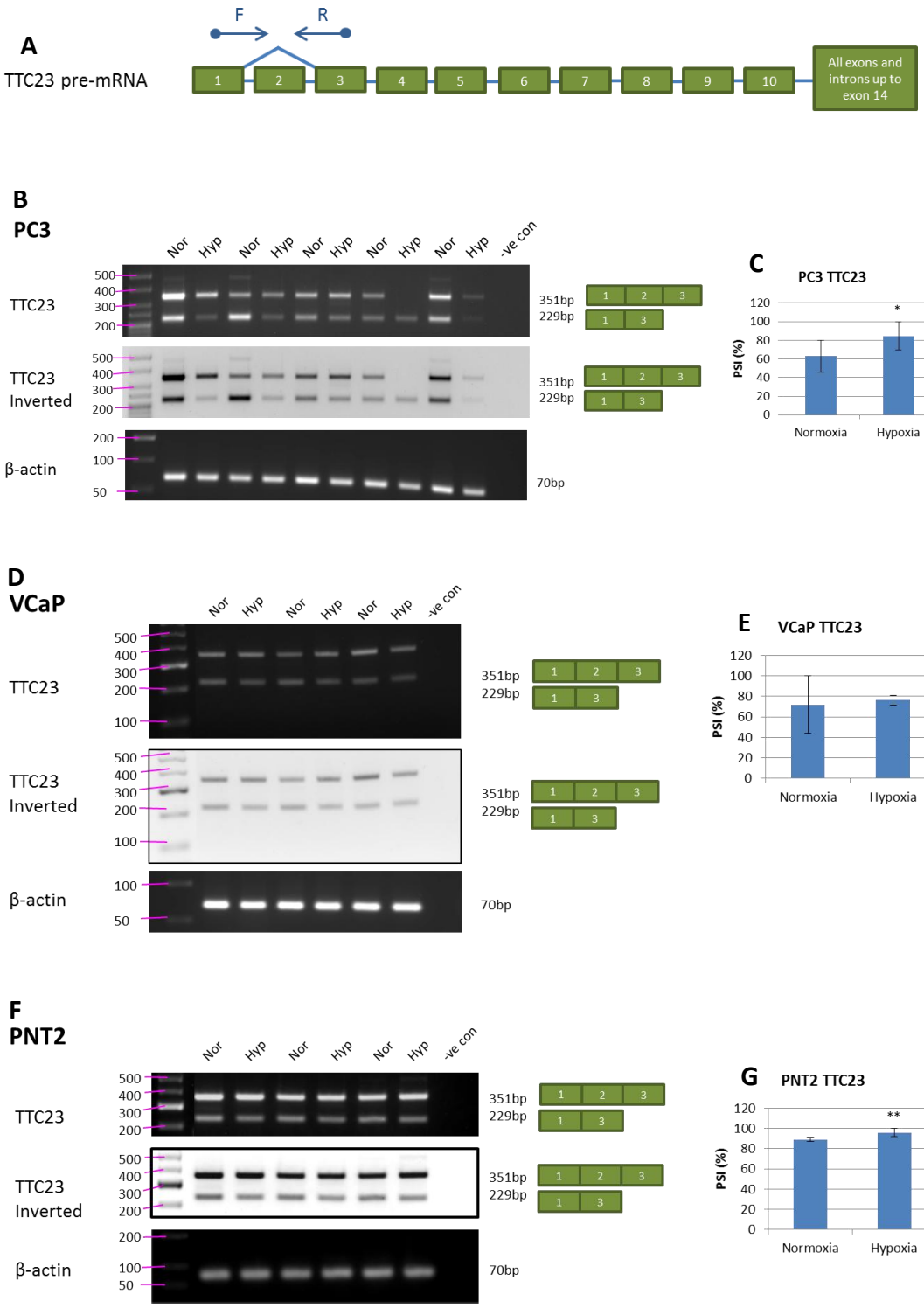


Figure 3.17: Proportion of exon inclusion in the *TTC23* gene after hypoxia in prostate cancer cell lines. (A) Illustration of *TTC23* pre-mRNA showing exons (green boxes), introns (blue lines) and location of the primers (F = forward primer; R = reverse primer). The exon 2 inclusion or skipping alternative splicing event is also shown. Cells seeded from prostate cancer cell lines were cultured in normoxic or hypoxic conditions. RTPCR was performed using the following conditions: 94^{o}C for 2 min, 94^{o}C for 30 sec, 58^{o}C for 30 sec, 72^{o}C for 30 sec,

72^{o}C for 5 min with 35 cycles. *TTC23* isoform expression with respective average percentage inclusion level of exon 2, also known as the percentage splicing index (PSI %) after normoxic or hypoxic treatment in the **(B and C)** PC3 cell line (n=5); **(D and E)** VCaP cell line (n=3); **(F and G)** PNT2 cell line (n=3). ImageJ software was used to generate optical density peaks from the gel isoform expression. Excel was used to normalise to the β-actin loading control, and then calculate the average percentage of exon 2 inclusion (Ψ). * = $p<0.05$; ** = $p<0.01$. Statistical tests used were as follows for all three cell lines. Shapiro-Wilks test showed that the data was normally distributed, Bartlett's test showed that the data was homoschedastic, and so student's T tests were used to assess the statistical significance of the data.

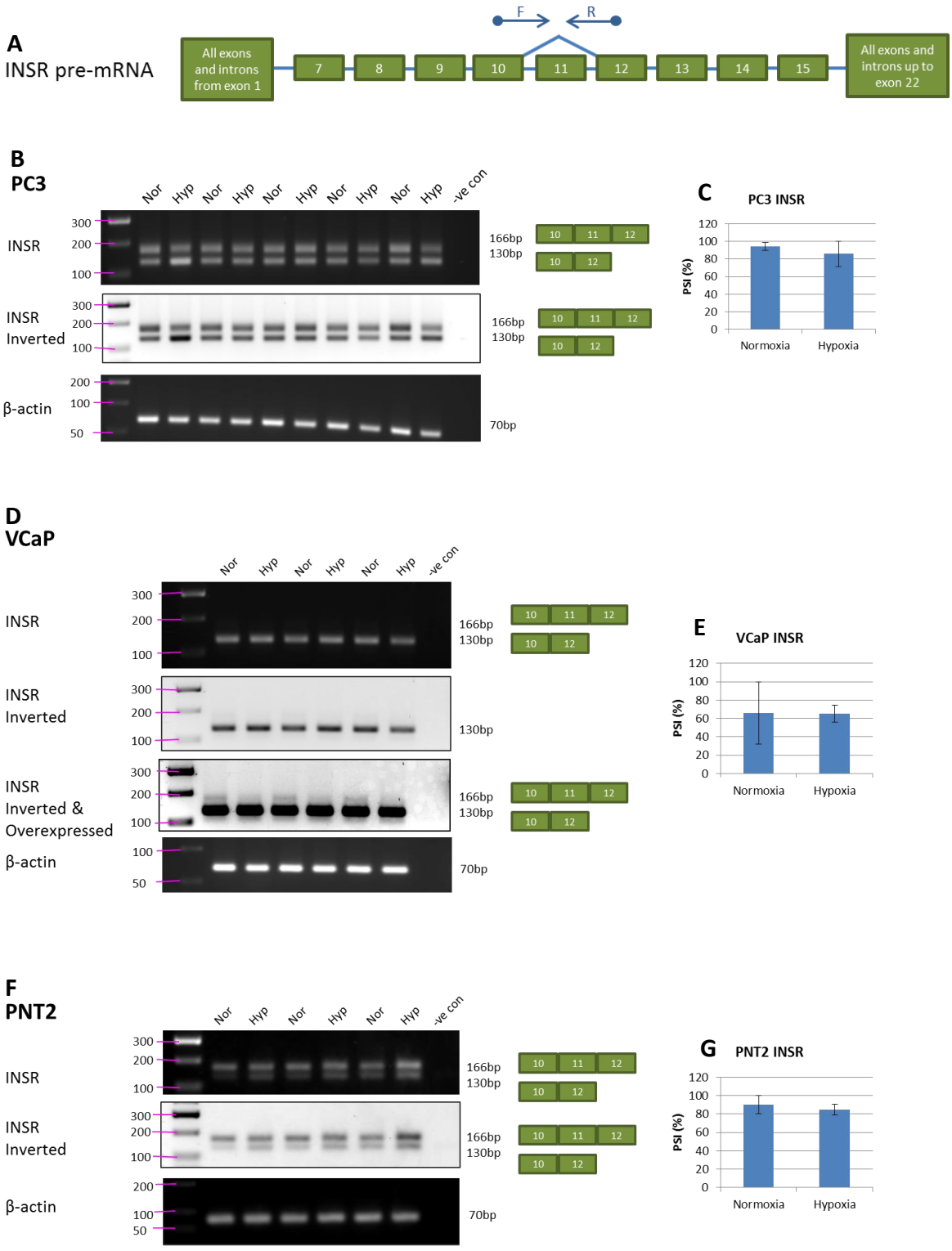


Figure 3.18: Proportion of exon inclusion in the *INSR* gene after hypoxia in prostate cancer cell lines. (A) Illustration of *INSR* pre-mRNA showing exons (green boxes), introns (blue lines), and the location of the primers (F = forward primer; R = reverse primer). The exon 11 inclusion or skipping alternative splicing event is also shown. Cells seeded from prostate cancer cell lines were cultured in normoxic or hypoxic conditions. RTPCR was performed using

the following conditions: 94^{o}C for 2 min, 94^{o}C for 30 sec, 58^{o}C for 30 sec, 72^{o}C for 30 sec, 72^{o}C for 5 min with 35 cycles. *INSR* isoform expression with respective average percentage inclusion level of exon 11, also known as the percentage splicing index (PSI %) after normoxic or hypoxic treatment in the **(B and C)** PC3 cell line (n=5); **(D and E)** VCaP cell line (n=3); **(F and G)** PNT2 cell line (n=3). ImageJ software was used to generate optical density peaks from the gel isoform expression. Excel was used to normalise to the β-actin loading control, and then calculate the average percentage of exon 11 inclusion (%). Statistical tests used were as follows, where SW stands for Shapiro-Wilks test, BT stands for Bartlett's test, MWU stands for Mann-Whitney U test and TT stands for student's T-test. PC3: SW showed that the data was not normally distributed, BT showed that the data was heteroschedastic, and so a MWU test was used to assess the statistical significance of the data, In both the VCaP and PNT2 cell lines: SW showed that the data was normally distributed, BT showed that the data was homoschedastic, and so TT were used to assess the statistical significance of the data.

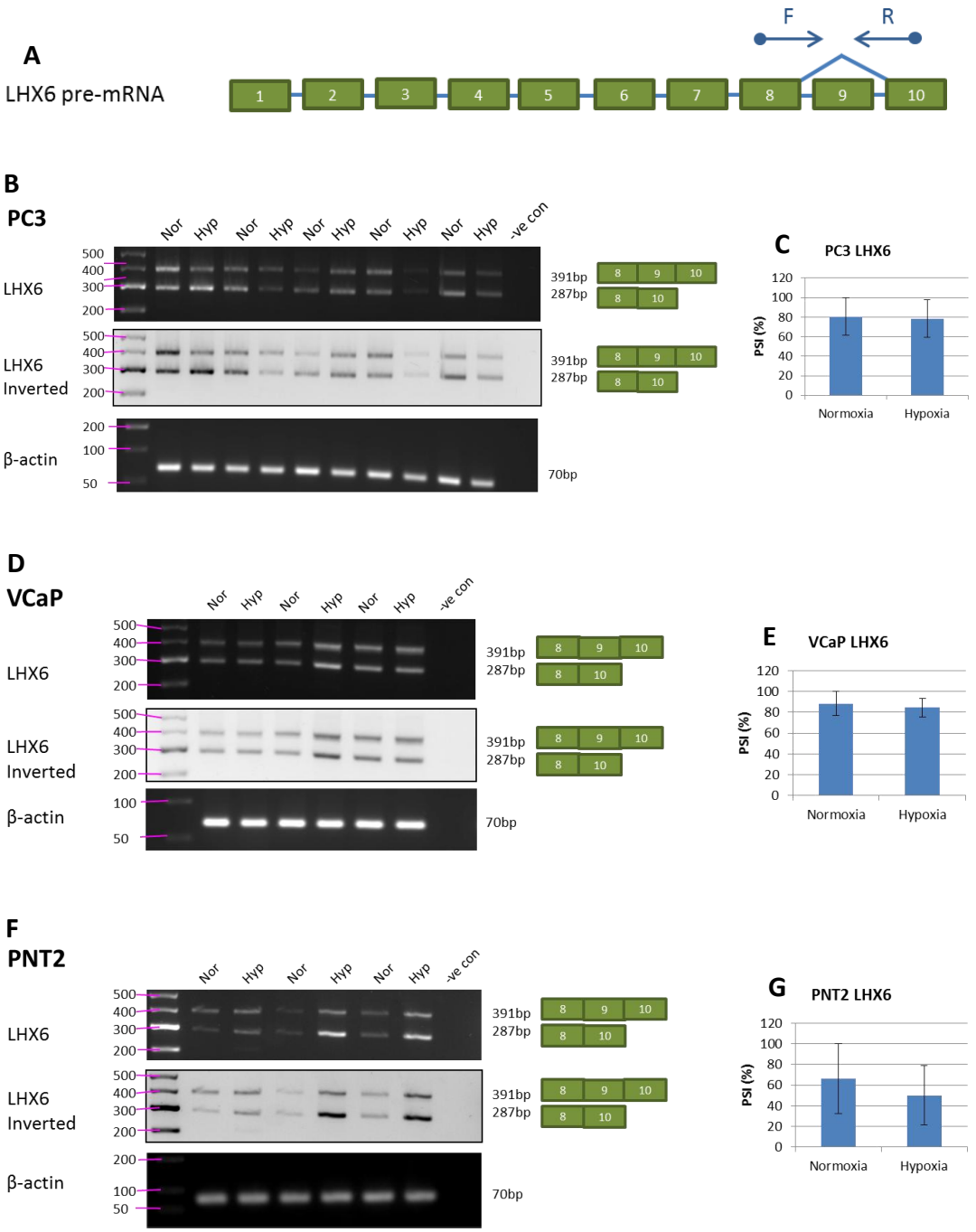


Figure 3.19: Proportion of exon inclusion in the *LHX6* gene after hypoxia in prostate cancer cell lines. (A) Illustration of *LHX6* pre-mRNA showing exons (green boxes), introns (blue lines) and location of the primers (F = forward primer; R = reverse primer). The exon 9 inclusion and skipping alternative splicing event is also shown. Cells seeded from prostate cancer cell lines were cultured in normoxic or hypoxic conditions. RTPCR was performed using the following conditions: 94^{o}C for 2 min, 94^{o}C for 30 seconds, 58^{o}C for 30 seconds, 72^{o}C for 30 seconds, 72^{o}C for 5 minutes with 35 cycles. *LHX6* isoform expression with respective average percentage inclusion level of exon 9, also known as the percentage splicing index (PSI %) after normoxia or hypoxia treatment in the **(B and C)** PC3 cell line (n=5); **(D and E)** VCaP cell line (n=3); **(F and G)** PNT2 cell line (n=3). ImageJ software was used to generate optical density peaks from the gel isoform expression. Excel was used to normalise to the β-actin loading

control, and then calculate the average percentage of exon 9 inclusion (%). Statistical tests used were as follows, where SW stands for Shapiro-Wilks test, BT stands for Bartlett's test, MWU stands for Mann-Whitney U test and TT stands for student's T-test. In both the PC3 and PNT2 cell lines, SW showed that the data was normally distributed, BT showed that the data was homoschedastic, and so TTs were used to assess the statistical significance of the data. In the VCaP cell line, SW showed that the data was not normally distributed, BT showed that the data was homoschedastic, and so a MWU test was attempted to assess the statistical significance of the data. However, due to there only being 3 data points, a MWU test could not be completed successfully.

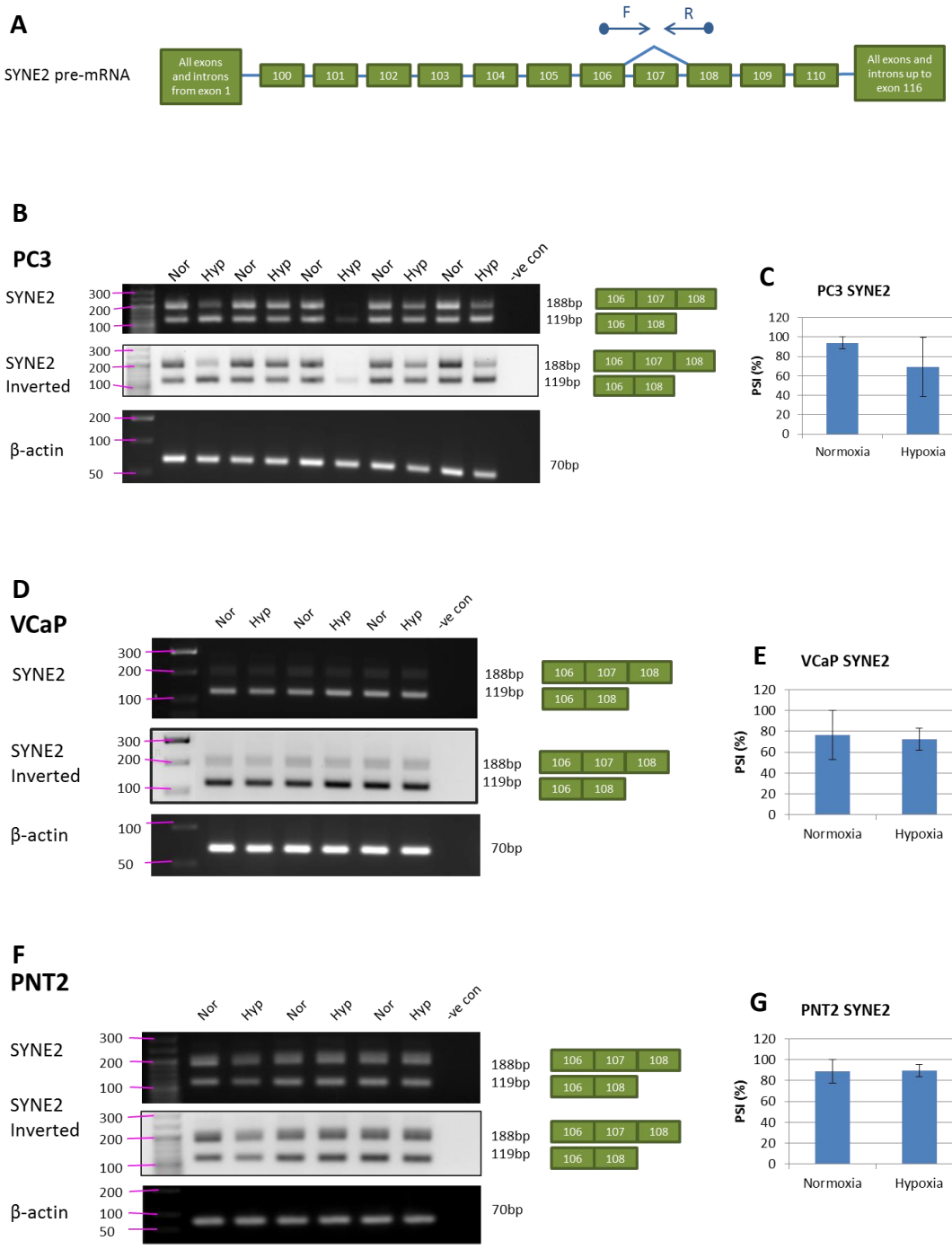


Figure 3.20: Proportion of exon inclusion in the *SYNE2* gene after hypoxia in prostate cancer cell lines. (A) Illustration of *SYNE2* pre-mRNA showing exons (green boxes), introns (blue lines) and location of the primers (F = forward primer; R = reverse primer). The exon 107 inclusion or exclusion alternative splicing event is also shown. Cells seeded from prostate cancer cell lines were cultured in normoxic or hypoxic conditions. RTPCR was performed using the following conditions: 94^{o}C for 2 min, 94^{o}C for 30 sec, 58^{o}C for 30 sec, 72^{o}C for 30 sec, 72^{o}C for 5 min with 35 cycles. *SYNE2* isoform expression with respective average percentage inclusion level of exon 107, also known as the percentage splicing index (PSI %) after normoxic or hypoxic treatment in the **(B and C)** PC3 cell line (n=5); **(D and E)** VCaP cell line

(n=3); **(F and G)** PNT2 cell lines (n=3). ImageJ software was used to generate optical density peaks from the gel isoform expression. Excel was used to normalise to the β-actin loading control, and then calculate the average percentage of exon 107 inclusion (%). Statistical tests used were as follows, where SW stands for Shapiro-Wilks test, BT stands for Bartlett's test, MWU stands for Mann-Whitney U test and TT stands for student's T-test. In the PC3 cell line, SW showed that the data was not normally distributed, BT showed that the data was heteroschedastic, and so a MWU test was used to assess the statistical significance of the data. In both the VCaP and PNT2 cell lines, SW tests showed that the data were normally distributed, BTs showed that the data was homoschedastic, and so TTs were used to assess the statistical significance of the data.

Gene	PC3	VCaP	PNT2
APAF1(F1)	↓		
APAF1(F8)			
BTN2A2	↑		↑
CDC42BPA	↓	↓	↓
FGFR1OP	↓		↓
INSR			
LHX6			
MBP			↑
PTPN13	↓		
PUF60	↓	Exon 5 skipping significantly increased	Exon 5 skipping significantly increased
RAP1GDS1	↓	↓	
SYNE2			
TTC23	↑		↑
UTRN	↓	↓	

Table 3.3: Summary of significant changes in splicing of cancer-associated genes (in alphabetical order) in hypoxia in prostate cancer cell lines. Blue downwards arrow denotes decreased exon inclusion (increased exon skipping); orange upwards arrow denotes increased exon inclusion.

3.6 Discussion

3.6.1 *CA IX*

It was important to evaluate that hypoxia was achieved with the treatment of 1% oxygen. CA IX is argued to be a more stable marker of hypoxia than HIF-1α because CA IX is not lost upon re-oxygenation (Sobhanifar *et al.,* 2005). Instead, *CA IX* mRNA expression remains stable and is only lost upon cell division or cell death (Sobhanifar *et al.* 2005). *CA IX* is alternatively spliced to form a truncated protein lacking exons 8 and 9 which interferes with full length (FL) CA IX (Barathova *et al.,* 2008); and so the expression of both isoforms was assessed. A treatment time of 48 hours at a concentration of 1% oxygen had a more profound effect on CA IX protein expression than 24 hours of treatment in the PC3 cell line (Figure 3.1). Therefore, 1% oxygen treatment for 48 hours was deemed appropriate for achieving hypoxia in this cell line. In addition, it was found that hypoxia significantly favoured the expression of the *FL CA IX* isoform in the PC3 cell line (Figure 3.2 B and C). The alternatively spliced variant was not detected in the VCaP and PNT2 cell lines. Therefore, the percentage of exon inclusion could not be calculated; however, the *FL CA IX* splice variant was normalised to the *β-actin* loading control in these cell lines (Figure 3.2 E and G). A significant change in *CA IX FL* expression during hypoxia was not found in the VCaP cell line. However, this is thought to be due to a long overlapping error bar. The data obtained for the PNT2 cell line was found not to be normally distributed; however, a Mann Whitney U statistical test could not be applied to the data collected from the PNT2 cell line as there were not enough data points. A student's T test was applied to the data, which showed a significant

change in *CA IX FL* expression during hypoxia ($p<0.001$). Although this is not the correct statistical test, it suggests that there may be a significant change in *CA IX FL* expression during hypoxia in the PNT2 cell line. It is proposed that with more repeats, hypoxia will be found to significantly alter the expression of *CA IX-FL* in the VCaP and PNT2 cell lines. The PCR gel images showed an indication that hypoxia was increasing the expression of the *CA IX FL,* in both cell lines (Figures 3.2 D and F). Furthermore, the graphs for the VCaP and PNT2 cell lines show a marked increase in percentage expression of *CA IX FL* of approximately 20% and 40% respectively (Figures 3.2 E and G). Therefore, the hypoxia treatment was deemed successful in the PC3 cell line. In the VCaP and PNT2 cell lines, there is an indication that the hypoxia treatment was successful; however, further repeats are required to determine this.

3.6.2 Alternative splicing of apoptotic genes

Caspase-9 is recruited to the apoptosome upon a cell death stimulus. This in turn triggers the cleavage of down-stream effector pro-caspases, which ultimately leads to apoptosis. As seen in figure 3.3A, *caspase-9a* and *caspase-9b* mRNAs are formed from the inclusion or exclusion of a 4-exon cassette, which comprises of exons 3, 4, 5 and 6. The full length isoform, *caspase-9a* conducts full protease activity, whereas *caspase-9b* lacks a huge section of the catalytic domain, and so cannot cleave downstream pro-caspase-3 and pro-caspase-7 into active forms for apoptosis execution (reviewed in Li *et al.,* 2017). *Caspase-9b* mRNA does however possess

interaction domains, and so can inhibit *caspase-9a* attachment to the apoptosome, which suppresses the caspase apoptosis cascade (Vu *et al.,* 2013).

During hypoxia, it was found that the PSI value increased significantly in the PC3 and PTN2 cell lines, which indicates that the ratio of full-length isoform was increased. Therefore, it can be proposed that hypoxia promotes apoptosis through increasing the proportion of expression of the full-length *caspase-9a* isoform in the PC3 and PNT2 cell lines. This result supports the idea that hypoxic stress can cause cells to apoptose (Semenza, 2000).

Survivin is encoded by the *BIRC5* gene, which can be described as an oncogene as it drives forward mitosis at the G2/M check-point. To date, there have been five confirmed additional splice variants to the wild-type isoform i.e.; ΔEx3, 2B, 3B, 2α and 3α (Figure 3.21) (Mahotka *et al.,* 2002; Caldas *et al.,* 2007; Sampath *et al.,* 2007). This study forcused on the effect of hypoxia on expression of wild-type, ΔEx3, 2B mRNAs. The ΔEx3 isoform has been associated with poor clinical outcome and prognosis in cancer (Necochea-campion *et al.,* 2013). Studies of the 2B isoform have unearthed conflicting data on the cancerous function of the isoform. The 2B isoform has been associated with poor survival and aggravated disease, but other studies have shown an association of 2B with less severe disease (Suga *et al.,* 2005; Antonacopoulou *et al.,* 2010). However, there is a general agreement that the ΔEx3 variant is anti-apoptotic and the 2B isoform has an attenuated anti-apoptotic function (reviewed in Sah *et al.,* 2015).

Although the gel image for the PC3 cell line shows that the expression of the full-length *survivin* isoform decreases with hypoxia, the proportion of the full-length *survivin* isoform is increased significantly (Figures 3.4 B and C). This is because of a general decrease in expression in all *survivin* isoforms was found after hypoxia, but the expression of the full-length isoform did not decrease as much as the other isoforms. The percentage proportion of exon inclusion is not found to be altered in the VCaP and PNT2 cell lines (Figures 3.4 E and G), which suggest that this result could be cell line specific. Therefore, it is suggested that hypoxia may evade apoptosis through this mechanism in the PC3 cell line.

Survivin has been shown to up-regulate VEGF expression, and knockdown of Survivin in glioma has been shown to inhibit angiogenesis (Wang *et al.,* 2012; Fernandez *et al.,* 2014). Therefore, it is suggested that perhaps Survivin-WT may also aid in angiogenesis during hypoxia in PC3 cells. Furthermore, survivin has also been found to inhibit caspase-9 activity (O'Connor *et al.* 2004; Dohi *et al.,* 2004). This may offer another mechanism in which cells evade apoptosis during hypoxia despite the increase in the pro-apoptotic *caspase-9a* variant during hypoxia.

Bcl-x is alternatively spliced to produce an anti-apoptotic long isoform, Bcl-xL, and a shorter, pro-apoptotic isoform, Bcl-xs (Boise *et al.,* 1993; Lindenboim, Yuan and Stein, 2000). Bcl-xL evades apoptosis by preventing mitochondria from releasing cytochrome *c*, which is needed to form the apoptosome, and this in turn is required to facilitate apoptosis (Kim, 2005).

A study by Xiao *et al.* (2012) showed that Bcl-x splicing was altered after hypoxia-ischemia in neonatal rat brains, with increased expression of the Bcl-xs variant relative to the Bcl-xL variant. This suggests that hypoxia may favour the smaller Bcl-xs isoform in order to facilitate apoptosis. The group also found that Bcl-xs knockdowns prior to hypoxia-ischemia treatment resulted in reduction of brain injury, which is probably due to less brain cell death.

Hypoxia was found to significantly favour the longer anti-apoptotic Bcl-xL isoform in the PNT2 cell line (figure 3.5G), which suggests that hypoxic cells may evade apoptosis through this mechanism. The gel PCR image for the PC3 cell line (figure 3.5B) showed a suggestion that the shorter isoform was favoured in the PC3 cell line; however, this was not found to be significant due to the spread of the values (figure 3.5C). With greater repeats however this may be found to be significant which would conflict the results found for the PNT2 cell line.

Mcl-1 has been identified as an anti-apoptotic member of the Bcl-2 family that heterodimerises with other Bcl-2 proteins to protect against cell death (Mott *et al.,* 2007). Bae *et al.* (2000) found that there are two splice variants of *Mcl-1*; *Mcl-1L* and *Mcl-1S* (Figure 3.6A). The shorter variant (*Mcl-1S*) arises due to the skipping of exon 2 and lacks Bcl-2 homology domains 1 and 2. This prevents interaction between Mcl-1S with the Bcl2 protein family and induces apoptosis. In addition, Mcl-1S dimerises with Mcl-1L, which results in higher levels of apoptosis (Bae *et al.,* 2000). Recently, splice switching oligonucleotides (SSOs) have been developed to shift the isoform ratio of *Mcl-1* towards the pro-apoptotic *Mcl-1S* splice variant. The results from these studies support previous findings that Mcl-1S triggers apoptosis

and inhibits tumour development (Shieh *et al.,* 2009; Kim *et al.,* 2011; Morciano *et al.,* 2015). In this study, hypoxia was not shown to alter the splicing of *Mcl-1* (Figure 3.6). The full length anti-apoptotic isoform, *MCL-1L* was the dominant isoform in all samples studied.

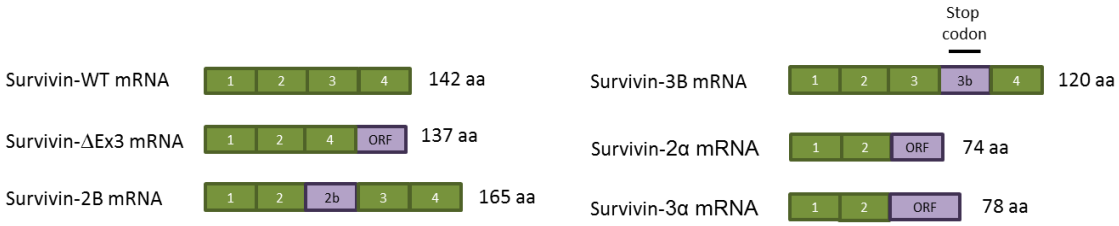


Figure 3.21: Confirmed splice variants of Survivin encoded by the *BIRC5* gene. Schematic representation of alternatively spliced mRNA transcripts derived from the *BIRC5* gene. WT mRNA contains is coded by exons 1-4. ΔEx3 is formed from a deletion of exon 3, which results in a frame shift with extension of the reading frame into the open reading frame (ORF) of the 3' untranslated region. An additional exon of 69bp between exons 2 and 3 forms the 2B variant. 3B is formed from an additional seven amino acids (aa) (denoted as exon 3b) at the c-terminal. Variants 2α and 3α contain exons 1 and 2 followed by a 3' UTR, and are 74 and 78 aa in length respectively. Figure adapted from Garg *et al.* (2016) and used with permission from the author.

3.6.3 Alternative splicing of cancer-associated genes

Normoxic and hypoxic samples generated in the PC3 cell line were sent to collaborators (Dr. Roscoe Klinck and his team at the RNomics platform at Sherbrooke University, Canada) for high-throughput PCR analysis using 238 alternatively spliced cancer-associated genes. The top 12 genes with a change in splicing after hypoxia were then studied with further repeats in the PC3 cell line, and also in the VCaP and PNT2 prostate cancer cell lines. A summary of the known splice isoform functions of the selected gene set is shown in Table 3.4; and a summary of the overall effect of hypoxia on the gene set can be found in Table 3.5.

It must be noted that the linear range of the PCRs were not tested in this study; however, the RNOmics platform (Sherbrooke University, Canada) used 35 PCR cycles for the high-throughput PCR work, and so it was assumed that 35 cycles was within the linear range of the PCR.

Hypoxia favoured the alternative splicing of pro-oncogenic isoforms in three genes selected by high-throughput PCR; *APAF1*, *RAP1GDS1* and *BTN2A2*. APAF1 is involved in the formation of the apoptosome, which functions in the apoptotic pathway. The smaller isoform does not contain a WDR insert, which is crucial for the binding of cytochrome-*c* to APAF1, and without which formation of the apoptosome cannot occur (Bao *et al.,* 2007).

There were two separate sets of primers that showed a change in *APAF1* splicing in the high-throughput experiments, and so both sets of primers were once again tested using n=5. With use of the APAF1-F1 primer set*,* it was found that the hypoxia decreased the splicing PSI value significantly in the PC3 cell line (Figure 3.7 B and C), which shows that the proportion of the larger *APAF1* isoform significantly decreased with hypoxia. This therefore suggests that the smaller *APAF1* isoform has a larger proportion of overall isoform expression during hypoxia, which may aid cells in avoiding apoptosis during hypoxia. Wang *et al.* (2016) provided evidence that hypoxia increased APAF-1 expression. Although expression of the splice isoforms were not investigated, the paper provided evidence that hypoxia increased apoptosis in H9c2 rat ventrivular cardiomyocytes, which suggests that expression of the full-length *APAF1* isoform was increased in their model. The results shown in this study however contradict these findings, and so it is thought

that these changes could be cell line specific. In support of this, hypoxia did not significantly change in *APAF1* splicing in the VCaP or PNT2 cell lines (Figure 3.7 E and G).

There was no signficant change in splicing during hypoxia when using the APAF1F8 primers in any of the cell lines examined (Figure 3.8. However, in the PC3 cell line a pattern can be observed in the gel PCR image (Figure 3.8 B), and although not significant, there is an overall decrease in the proportion of *APAF1-FL* during hypoxia (Figure 3.8C). The error bar is quite long in the figure 3.8C, and so more repeats may show this result to be significant.

The *RAP1GDS1* gene produces the SmgGDS protein, which activates multiple small GTPases in the Rho and Ras families (Quilliam, Rebhun and Castro, 2002) and is therefore involved in multiple signalling pathways. Furthermore, SmgGDS expression is also elevated in prostate cancer tissues and non-small cell lung carcinoma (Zhi *et al.,* 2009; Tew *et al.,* 2008). *RAP1GDS1* is spliced to give the long SmgGDS-607 and the short SmgGDS-558 isoforms (Berg *et al.* 2010). Hypoxia significantly decreased the proportion of exon inclusion of *RAP1GDS1* in the PC3 and VCaP cell lines (Figures 3.12 B, C, D and E), and so encouraged splicing of the SmgGDS-558 splice variant. Studies have shown that SmgGDS-558 plays a greater role than SmgGDS-607 in promoting proliferation and nuclear-factor-kappa-light-chain-enhancer-of-activated-B-cells (NFκB) activity (Hauser *et al.,* 2013), as well as promoting non-small-cell-lung carcinoma (NSCLC) colony formation (Berg *et al.,* 2010) and regulation of the cell cycle (Schuld *et al.,* 2014). Hypoxia was not found to alter splicing of *RAP1GDS1* in the PNT2 cell line, which suggests that changes in

splicing of *RAP1GDS1* during hypoxia may only occur in cancer cell lines. As SmgGDS has been found to be involved in cell proliferation and migration in both androgen-dependent (DU145, LNCaP) and androgen-independent (PC3) cell lines (Zhi *et al.,* 2009), it is suggested that the alterationa in splicing in both types of cell line is also similar in order to carry out these functions, as observed in this study.

BTN2A2 binds to activated T-cells and suppresses T-cell metabolism (Smith *et al.,* 2010). It is reported to inhibit the proliferation of CD4 T-helper cells, and CD8 killer T-cells, and blocks cytokine production (Smith *et al.,* 2010). Therefore, it is thought to promote the immune tolerance of tumours. Recently, *BTN2A2* mRNA expression was shown to be increased in ulcerative colitis, and linked to inflammation (Lebrero-Fernandez *et al.,* 2016). Rhodes *et al.* (2001) identified a splice variant of *BTN2A2* that encodes a truncated protein, which negatively regulates the expression of full-length *BTN2A2.* Hypoxia increased the percentage of exon inclusion in the PC3 and PNT2 cell lines, which favoured the expression of the full-length isoform (Figure 3.16 B, C, F and G). This suggests that through increasing the proportion of the full-length isoform, hypoxia drives immune tolerance, which therefore protects cancer cells from immune-mediated cell death. There was not a significant change in exon inclusion during hypoxia in the VCaP cell line (Figure 3.16 E). However, the gel PCR shows a suggestion that expression of the larger isoform was increased in hypoxia in the first two repeats; but this was not shown in the the third repeat (Figure 3.16 D). Therefore, further repeats may find that the percentage of exon inclusion in the VCaP cell line is also significant.

Hypoxia was found to change the alternative splicing of some cancer-associated genes whose splice isoforms have not yet been assigned a function. Interestingly, two of these genes (*UTRN* and *CDC42BPA*) are found to have a role in the shape and maintenance of the cytoskeleton and its reorganisation, respectively (Gomes *et al.,* 2005; Wilkinson *et al.,* 2005; Li *et al.,* 2007; Tan *et al.,* 2008). A meta-analysis of two studies conducted in prostate cancer identified *UTRN* as one of eight key genes differentially expressed with progression of prostate cancer (Wang *et al.,* 2013). In this study, hypoxia was found to significantly decrease the percentage exon inclusion in *UTRN* mRNA in the PC3 and VCaP prostate cancer cell lines (Figure 3.13 B, C, D and E), suggesting an increase in the proportion of the smaller isoform. Hypoxia did not alter alternative splicing in the PNT2 cell line (Figure 3.13), which is harvested from normal prostatic epithelial cells. As hypoxia altered splicing of *UTRN* in both cancer cell lines, but did not alter splicing in the non-cancerous PNT2 cell line, it is suggested that the study mentioned above by Wang *et al.* (2013) may have detected the smaller isoform of *UTRN*. Therefore, it is proposed that the smaller isoform is involved in the progression of prostate cancer. However, further studies will be required to investigate this hypothesis.

Further to its role in reorganisation of the cytoskeleton, *CDC42BPA* is also shown to; induce the formation of filopodia, be involved in the assignment of cell polarity, and initiates cancer cell motility and invasion (Nobes and Hall. 1995; Kozma *et al.,* 1995; Etienne-Manneville and Hall. 2002; Olson and Sahai. 2009; Unbekandt *et al.,* 2014). Hypoxia increased the percentage of exon skipping of *CDC42BPA*; however, the relevance of this is not yet known. A microarray that investigated the profile of

alternatively splice mRNA isoforms in prostate cancer identified a *CDC42BPA* splice variant as a top prostate cancer marker (Zhang *et al.,* 2006). The variant decreased in expression in prostate cancer tissue compared to normal prostate tissue. The paper states that details of the splice variant can be accessed via the online Manually Annotated Alternatively Spliced Events (MAASE) database; however, access to this information was problematic. Nevertheless, this does provide evidence that *CDC42BPA* splicing is altered in prostate cancer. Hypoxia siginificantly decreased the percentage of *CDC42BPA* exon inclusion in all three cell lines, which suggests that this event is not exclusive to cancerous prostate cells.

Hypoxia also favours exon skipping in *FGFR1OP.* FGFR1OP protein is involved in ciliogenesis (Lee and Stearns, 2013) and mediates centrosome functions (Maxim *et al.,* 2015). In addition, Mano *et al.* (2007) found that *FGFR1OP* was overexpressed in lung cancer and showed evidence of its role in cellular motility, cell growth and progression. Furthermore, FGFR1OP has been reported to be involved in G1/S transition, which therefore implicates the protein in cell-cycle progression and survival (Acquaviva *et al.,* 2009). Popovici *et al.* (1999) discovered *FGFR1OP* when confirming it as a fusion partner gene to *FGFR1,* the resulting fusion protein of which is implicated in stem cell myeloproliferative disorder, a disease that generally progresses to acute myeloid leukaemia. The group identified alternatively spliced transcripts arising from exon 7 or 11 skipping; however the function of the splice isoforms was not investigated. In fact, no papers have come to light on the significance or function of any *FGFR1OP* splice variants. The primers used in this study focus on the alternative splicing of exon 7. Furthermore, *FGFR1OP* has also

been implicated in other cancers; it forms a fusion protein with *RET* that is involved in chronic myelomonocytic leukaemia (Mulligan, 2014), and single nucleotide polymorphisms in *FGFR1OP* have shown a significant connection between the gene and lung cancer risk (Kang *et al.,* 2016). In this study, hypoxia significantly decreased the percentage of exon 7 inclusion in *FGFR1OP* in the PC3 and PNT2 cell lines (Figures 3.11 C and G). Hypoxia did not affect splicing of *FGFR1OP* in the VCaP cell line (Figure 3.11 E), which suggests that this event may be cell line specific. As the PNT2 cell line is derived from normal prostatic epithelial cells, it is possible that this event occurs in normal tissues.

MBP was expressed at low levels in all three prostate cell lines. Therefore, as the bands were fainter and more difficult to analyse the results may not be an accurate representation of a change in splicing. MBP is a main component of the myelin sheath that coats neural axons (Shaharabani *et al.,* 2016), and therefore the prostate may not require much of this protein. It would be interesting to examine whether there is a change in *MBP* splicing with hypoxia in Schwann cells, where *MBP* is more highly expressed. Exon 5 skipped isoforms are expressed during embryonic development, but are no longer expressed during the post natal stage of development (Mathisen *et al.,* 1993; Kruger *et al.,* 1999), which suggests that the splice variant has a developmental function. Furthermore, Nakajima *et al.* (1993) revealed a role for the exons skipped variant of *MBP* in differentiation of maturing oligodendrocytes. Results showed a significant increase in exon 5 inclusion in the PNT2 cell line (Figure 3.15 G); however, a significant change in splicing of *MBP* was

not observed in the PC3 or VCaP cell lines. This suggests that hypoxic regulation of *MBP* splicing may be cell line specific, or that it does not occur in cancerous cells.

PTPN13 has been shown to have conflicting roles as a tumour suppressor and an oncogene (Freiss and Chalbos, 2012). PTPL1, which is the protein encoded by the *PTPN13* gene, induces apoptosis in breast cancer cell lines (Revillion *et al.,* 2009). However in contrast, the protein has been implicated in cell motility and cellular adhesion processes, which are associated with invasion and metastasis (Bompard *et al.,* 2003; Kimber *et al.,* 2003). The conflicting oncogenic and tumour suppressive roles stated for *PTPN13* could be due to different isoforms of the gene, which may give rise to isoforms with opposing functions. This highlights the importance of studying the alternative splicing of genes in diseases rather than just the entire gene expression. To date, four splice variants of *PTPN13* have been identified (Albaan and Toretsky, 2008); however, despite an in-depth literature search on the significance of cassette exon 20 exclusion, no information has been found, and so it is concluded that a function has not yet been assigned to this isoform. Hypoxia was found to significantly decrease exon inclusion of *PTPN13* mRNA in the PC3 cell line (Figure 3.9 C).

Not much is documented about TTC23 and so a limited amount of information can be found on PubMed and google scholar about this protein. A thesis by Akawi (2012) provided evidence for a possible role for TTC23 in human cognition and there is some evidence to suggest that TTC23 is linked to cancer. TTC23 has been described as the cervical cancer proto-oncogene 8 protein that is associated with cervical cancer and cervicitis in the genecards database (Akawi, 2012). In addition,

Warnatz *et al.* (2011) showed that *TTC23* expression is transcriptionally repressed by BTB-and-CNC-homology-1 (BACH1), which is interesting as BACH1 has been shown to be down-regulated in some cancers. Furthermore, a thesis by Han (2015) has also linked loss of methylation of *TTC23* to bladder cancer and Shukeir *et al.* (2015) found an association between hypermethylated *TTC23* and prostate and cervical cancers. In bladder cancer, TTC23 has also been shown to form a fusion protein with IGF1R, usig exons 1 and 2 from IGF1R joined to exons 8-13 of TTC23 (Kekeeva *et al.,* 2016). Hypoxia was found to favour exon 2 inclusion in *TTC23* splice in the PC3 and PNT2 cell lines (Figure 3.17 C and G).

PUF60 encodes a splicing factor that is involved in the modulation of alternative splicing of pre-mRNAs through the recognition of 3' splice sites (Hastings *et al.,* 2007; Page-McCaw, Amonlirdviman and Sharp, 1999). *PUF60* is also reported to regulate *c-Myc* transcription, which plays a critical role in cell proliferation, tumorigenesis, apoptosis and cell-cycle regulation (Liu *et al.,* 2006). FIR is a splice variant of *PUF60* that lacks exon 5 and is a *c-Myc* transcription repressor. This results in interference with the DNA damage response pathway, cellular proliferation and the cell cycle (Kano *et al.,* 2016; Matsushita *et al.,* 2015; Rahmutulla *et al.,* 2014; Matsushita *et al.,* 2013; Matsushita *et al.,* 2012). Tanaka *et al.,* (2015) evaluated the anti-tumour effects of FIR using a fusion gene-deleted Sendai virus as a vector against head and neck squamous cell carcinoma (HNSCC). They found that the vector suppressed *c* –myc, induced apoptosis and exerted anti-tumour effects. Hypoxia significantly decreased the proportion of the full-length and larger isoform, and significantly increased the proportion of the FIR isoform in

the PC3 cell line (Figure 3.14 C). Another splice variant that is slightly larger than the full-length *PUF60* isoform was also detected in the VCaP and PNT2 cell lines (Figure 3.14 D and F). The proportion of the full-length isoform was therefore calculated as a percentage of all three isoforms. Hypoxia was shown to significantly increase exon 5 skipping in the VCaP and PNT2 cell lines (Figure 3.14 E and G). This therefore suggests that hypoxia favours the FIR isoform.

Although in PC3 cells the high-throughput PCR analysis identified a change in the proportion of exon inclusion of 22.6%, 20.8% and 32.3% in the *INSR, LHX6* and *SYNE2* genes respectively in response to hypoxia, this was not found to be significant in further repeats in the PC3, PNT2 or VCaP cell lines (Figures 3.18, 3.19 and 3.20). However, this is not necessarily ruling out these genes as having a change in splicing during hypoxia as only a limited number of repeats could be conducted due to time constraints. In fact, the results showed that the proportion of exon inclusion of *SYNE2* decreased in every repeat with hypoxia treatment in the PC3 cell line (Figure 3.20 B), but because of the large standard error this was not found to be significant. With a greater number of repeats, the standard error may decrease and produce a more significant result for *SYNE2.* Therefore this method can only confirm genes that are shown to significantly alter splicing in response to hypoxia and cannot rule any out which are shown not to be significant. This also applies to all other alternative splicing event studied in this section that may not have shown a change after hypoxia. In addition, standard PCR is a semi-quantitative method and therefore through use of qPCR, a greater level of accuracy could be achieved and more significant results may be found.

Upon revisiting the high-throughput PCR data, it was found that there were two genes missed off the list that had a change in the proportion of exon inclusion in line with the genes studied in this chapter. *Adenomatous-polyposis-coli* (*APC*) is a tumour suppression gene that inhibits the WNT pathway and has been found to have many roles. These include cellular migration, adhesion and apoptosis (Hanson and Miller, 2005). *Low-density-lipoprotein-receptor-related-protein-8* (*LRP8*) codes for a receptor that has been shown to have a role in melanoma cancer endothelial cell migration and angiogenesis (Pencheva *et al.,* 2012), and also to function in a growth signal pathway for triple negative breast cancer (Shiang *et al.,* 2011). Therefore, it would be interesting to see how hypoxia may affect the splicing of the *APC* and *LRP8* genes in the future.

Recent studies examining the genome-wide effect of hypoxia on alternative splicing in both non-cancerous and cancerous cell lines have identified various changes to splicing patterns in genes associated with cancer (Hang *et al.,* 2009; Weigand *et al.,* 2012; Yao *et al.,* 2016; Sena *et al.,* 2014; Han *et al.,* 2017). These include genes implicated in angiogenesis, apoptosis, the cell cycle and DNA repair, as well as others. Furthermore, investigations conducted in the Hep3B liver cancer cell line confirmed altered splicing patterns in *CA IX, RAP1GDS1* and *MBP* genes, which supports findings in this chapter. This is the first study to confirm systematic changes in alternative splicing in response to hypoxia in prostate cancer cell lines. However, the literature so far suggests that hypoxic alteration of splicing is not isolated to one cell line or tissue, but it must be noted that splicing profiles may vary depending on the cell line or tissue type.

Genome-wide exon microarrays have identified an activated cassette exon in the *ZNF121* gene, which encodes a zinc finger protein, and a repressed cassette exon in the *NDUFV3* gene, which encodes a mitochondrial respiratory protein respectively (Rajan *et al.,* 2011). The study also uncovered an alternative isoform of the *tuberous sclerosis-2* gene, which arises through use of an alternative promoter. Furthermore, a splice variant of the *STBGALNAC1* gene has been identified in androgen-regulated prostate cancer (Munkley *et al.,* 2015). The protein encoded by the *STBGALNAC1* gene is involved in the synthesis of the sialyl-Tn antigen, which is associated with cancer. The shorter, alternatively spliced isoform is thought to have enhanced translation, resulting in increased synthesis of sialyl-Tn antigen (Munkley *et al.,* 2016) leading to a more cancerous phenotype. These studies indicate that androgens can alter alternative splicing in prostate cancer. However, none of these studies have also incorporated the effect of hypoxia on the androgen-independent or androgen-dependent cell lines. The supplementary tables from the Munkley *et al.* (2016) paper, showed that *PUF60* expression was up-regulated in response to treatment with R1881, a synthetic androgen. Furthermore, the expression of *BTN2A2, SYNE2* and *RAP1GDS1* was found to decrease in response to R1881 treatment. Furthermore, *SYNE2* was also shown to be regulated by androgens in the Rajan *et al.* (2011) paper. This suggests that these genes are regulated by androgens, and therefore it was thought that perhaps the alternative splicing of these genes may also alter differentially in response to hypoxia depending on the type of cell line used. However, this was not found to be the case. Splicing patterns that altered in the PNT2 cell line generally also altered in the PC3 cell line (apart

from MBP, which only altered in the PNT2 cell line). As the PC3 cell line is androgen independent, it suggests that changes to alternative splicing patterns derived from these genes during hypoxia are not regulated by androgens. Furthermore, all changes to splicing pattens shown in the VCaP cell line, were also shown in the PC3 cell line, which also provides evidence that androgens do not play a role in the regulation of alternative splicing during hypoxia in these genes. It must be noted, that hypoxia decreased exon inclusion in *RAP1GDS1* mRNA in both cancerous cell lines, which suggests that this change in splicing could be cancer specific. Therefore, *RAP1GDS1* splicing may be a candidate for future cancer therapy.

In conclusion, it is evident that hypoxia alters the splicing of cancer-associated genes. The majority of the alternatively spliced changes occur in the PC3 cell line, which is likely to be due to the fact that the high-throughput PCR analysis was conducted on samples generated in the PC3 cell line. There is the possibility that there are other cell line specific genes that are affected by hypoxia in the VCaP or PNT2 cell lines but are not affected in the PC3 cell line. However, as the majority of the splicing changes found in this chapter have been in the PC3 cell line, subsequent experiments will be conducted in the PC3 cell line.

Gene	Change in isoform expression after hypoxia
APAF1	Exon 17a skipping is favoured by hypoxia, which produces a pro-oncogenic splice isoform.
BTN2A2	Increased expression of the larger amplicon, which promotes immune tolerance
CDC42BPA	Decrease in exon inclusion in hypoxia. No function has been assigned to the isoform where exon 13 is skipped.
FGFR1OP	Exon skipping favoured by hypoxia. A function has not yet been assigned to the exon 7 skipping isoform.
INSR	No significant change in the proportion of exon inclusion after hypoxia treatment.
LHX6	No significant change in the proportion of exon inclusion after hypoxia treatment.
MBP	Decrease in exon inclusion found in the PC3 cell line; Increase in exon inclusion found in the PNT2 cell line.
PTPN13	Decrease in exon inclusion with hypoxia treatment. No assigned function to isoforms.
PUF60	Anti-oncogenic *FIR* isoform favoured by hypoxia, which arises due to the skipping of exon 5.
RAP1GDS1	Hypoxia favours the smgGDS-558 isoform which is pro-oncogenic
SYNE2	No significant change in the proportion of exon inclusion after hypoxia treatment.
TTC23	Exon inclusion favoured by hypoxia.
UTRN	Hypoxia favours the smaller isoform. No function has been assigned to the smaller isoform.

Table 3.4: Summary of changes to alternative splicing of genes in hypoxia. Genes that were identified by the high-throughput PCR assay as changing alternative splicing patterns in response to hypoxia treatment were tested with further repeats. A summary of findings is displayed in the table above.

CHAPTER 4:

Changes in expression and localisation of splice factors and splice factor kinases in response to hypoxia

4.1 Background

Chapter three found that hypoxia switched the splicing of several cancer-associated genes. Some of the isoforms favoured by hypoxia promoted apoptosis, but others supported cell survival. Those involved in cell survival are of particular interest as those genes may enable cancers to survive and thrive during hypoxic conditions. Splice factors are heavily involved in the regulation of alternative splicing and splice factor kinases regulate the activity and localisation of splice factors via phosphorylation.

4.1.1 Role of splice factors and splice factor kinases in cancer

As aberrant alternative splicing has been identified as a hallmark of cancer (Ladomery, 2013), it is not surprising that splice factors and splice factor kinases have been linked to different cancers. *SRSF1* and *SRSF3* have been identified as proto-oncogenes (Jia *et al.,* 2010; Anczukow *et al.,* 2012; Das and Krainer, 2014) that are linked to a number of oncogenic phenotypes; with evidence of an anti-apoptotic nature and promoting cell growth and tumour formation in nude mice (Ajiro *et al.,* 2015). Both splice factors have been shown to increase their expression in a variety of cancers, including: colon; lung; breast; stomach; skin; bladder; liver; cervix; and lymphoma cells (Ajiro *et al.* 2015).

Iborra *et al.* (2013) also provided evidence of an increased expression of both serine/arginine-rich (SR) proteins in primary ovarian tumours. Specifically,

overexpression of SR splice factor-2 (SRSF2) has also been reported in ovarian cancer (Fischer *et al.,* 2004). In addition, phosphorylated SRSF2 was shown to correlate with larger tumour size and higher tumour stage in lung adenocarcinoma (ADC) (Gout *et al.,* 2012). However, it must be noted that decreased expression of a splice factor can also result in a cancerous phenotype. For example, recently a decreased expression of SRSF2 was found in renal cancer (Kedzierska *et al.,* 2016). The group found evidence in this study that decreased expression of SRSF2 inhibited apoptosis, driving cancer progression. Furthermore, the group found that a decrease in SRSF2 expression affected differential splicing of apoptotic genes, including some of the apoptotic genes studied in chapter 3 of this thesis; *caspase-9*, *survivin*, and *MCL-1* (Kedzierska *et al.,* 2016). Therefore, it must be kept in mind that an alteration in the level of any splice factor, whether it is increased or decreased can have an effect on alternative splicing patterns.

SRPK1 has been suggested as a potential oncogene by Zhou *et al.* (2013) as it has been shown to affect proliferation, malignancy and tumour growth in hepatocellular carcinoma. SRPK1 expression has been found to be elevated in breast, colon, pancreatic, lung, melanoma and prostate cancers. Similarly *CLK1* has been found to be overexpressed in erythroleukaemia (Hayes, Carrigan and Miller, 2007; Gout *et al.,* 2012; Gammons *et al.,* 2014; Mavrou *et al.,* 2014; Garcia-Sacristan *et al.*, 2005).

Members of the heterogenous nuclear ribonucleoprotein (hnRNP) A/B family and SR protein family can antagonise one another when binding to SREs (reviewed in Kedzierska *et al.,* 2017). In general, SR proteins bind to exonic splicing enhancers

(ESEs), which promote exon inclusion, whereas hnRNPs bind to exonic and intronic splicing silencers (ESS and ISS) to promote exon exclusion (reviewed in Jean-Philippe, Paz and Caputi, 2013). Therefore, changes in the molar ratio of hnRNP protein to SR protein in the nucleus can affect splicing. HnRNP A1 has been linked to cancer progression. A recent paper has implicated hnRNP A1 in migration (Wang *et al.,* 2017), and Zerbe *et al.* (2004) showed an elevated level of nuclear hnRNP A1 protein in tumours compared to surrounding non-neoplastic cells using an *in vivo* model of mouse lung tumorigenesis. Furthermore, increased hnRNP A1 expression has been found in a number of cancers; including glioma and colorectal cancers (David *et al*., 2010; Park *et al.,* 2016). Therefore, it is thought that hnRNP A1 is involved in the promotion of cancer.

Src-associated substrate in mitosis of 68kDa (SAM68) is a splice factor that is implicated in various cancer-driving phenotypes, including; cell cycle progression, apoptosis and tumourigenesis (Frisone *et al.,* 2015). SAM68 expression was found to be elevated in breast cancer where it is found to have a role in cell proliferation and invasiveness (Song *et al.,* 2010), and in colon cancer, SAM68 is found to have a role in epithelial-to-mesenchymal transition through a mechanism that involves SRSF1 (Valacca *et al.,* 2010). Recently, SAM68 has been identified as a regulator of *survivin-ΔEx3* splicing (Gaytan-Cervantes *et al.,* 2017), which as discussed in chapter 3 is an anti-apoptotic isoform. Furthermore, SAM68 along with hnRNP A1 has been linked to regulation of *BCL-x* splicing in prostate and breast cancers (Bielli *et al.,* 2014), and therefore may regulate splicing of other cancer-associated genes in this study.

Human antigen R (HuR) is a RNA binding protein that is found to be up-regulated in various cancers, such as non-small cell lung cancer (Wang *et al.,* 2009); Glioma (Filippova *et al.,* 2011); ovarian cancer (Huang *et al.,* 2016); meningioma (Gauchotte *et al.,* 2017). HuR regulates many cancer-driving processes, such as; proliferation, angiogenesis, migration, invasion, metastasis (Dormoy-Raclet *et al.,* 2007; Wang *et al.,* 2009; Kakuguchi *et al.,* 2010; Muralidharan *et al.,* 2014; Muralidharan *et al*., 2015; Huang *et al*., 2016; Muralidharan *et al.,* 2017). Furthermore, HuR is thought to play a role in chemotherapeutic resistance (Romeo *et al.,* 2016; Blanco *et al.,* 2016); and inhibition of HuR has been shown to radiosensitise human breast cancer cells (Mehta *et al.*, 2016), which suggests that targeting of HuR could aid in improving existing cancer therapies.

4.1.2 Role of RNA binding proteins during cellular stress

It has been shown previously that environmental stresses can affect splicing. Ischemia treatment using an inadequate blood supply for one hour and osmotic shock have both been shown to alter splice site selection and lead to changes in the cytoplasmic localisation of hnRNP A1 and SAM68 (Daoud, 2002). More recently, HuR and hnRNP A1 have been shown to leave the nucleus and co-localise into cytoplasmic stress granules in response to heat stress (Papadopoulou *et al.,* 2013). In contrast, cellular stress has also been shown to increase nuclear localisation of SRPK1, which can induce nuclear speckle enlargement (Zhong *et al.,* 2009). In addition, there is evidence that heat shock and osmotic stress inhibits pre-mRNA

splicing and causes de-phosphorylation of SR proteins, which affects their protein-protein and protein-RNA interactions, intracellular localisation and trafficking, and ultimately the splicing of pre-mRNA (Shin *et al.,* 2004; Shi and Manley, 2007; Naro and Sette, 2013).

This chapter will focus on the effect of hypoxic stress on the expression of a selection of key splice factors and kinases that have been associated with cancer. SRPK1 and CLK1 are able to regulate the localisation of SR proteins, such as SRSF1 and so the localisation of SRSF1, SRPK1 and CLK1 will also be assessed.

4.2 Examination of the promoters of splice factors and splice factor kinases for Hypoxia Inducible Factor (HIF) response elements

Several publications have identified a core HIF response element 5'-RCGTG-3' that is conserved from mammals (Wenger, Stiehl and Camenisch, 2005; Schödel *et al.,* 2011) to zebrafish (Greenald *et al.,* 2015). Therefore, the promoters of a number of selected splice factors and splice factor kinases were examined for HIF response elements. The Eukaryotic Promoter database was used with parameters set to -1000 to +100 of the transcription start site as HIF-1 was found to mostly bind close to the transcription start site (Schödel *et al.,* 2011). The position of the HIF response elements in comparison of the transcription start site is illustrated in Figure 4.1. It was found that all of the genes analysed apart from SRPK1 had at least one HIF response element element within -1000 and +100 of the transcription start site.

SRSF1 and *SAM68* were of particular interest as these genes had five and four HIF response elements in their promoter regions respectively.

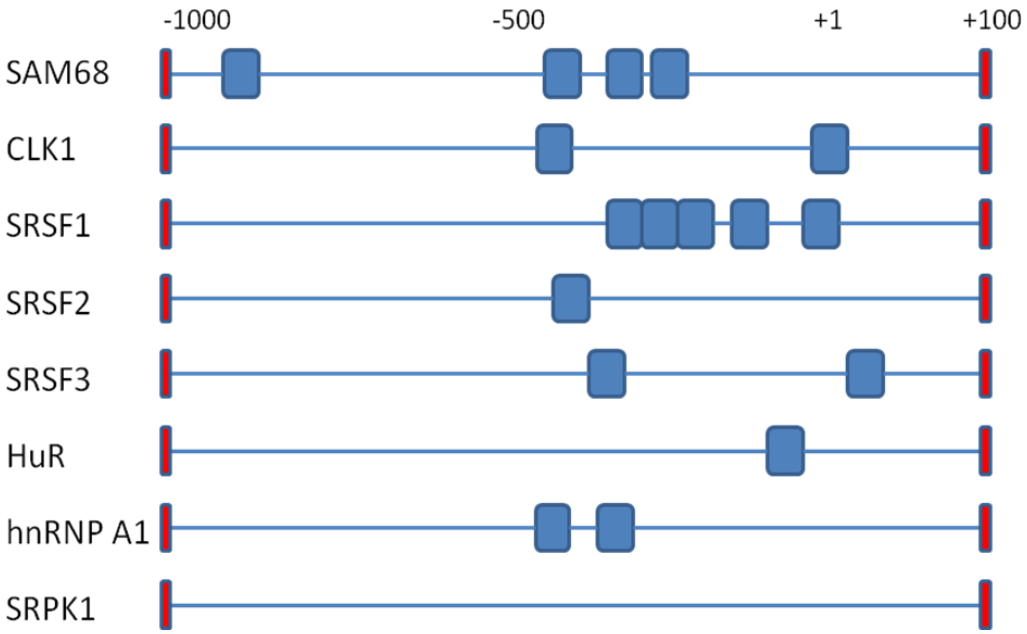


Figure 4.1: Illustration of core hypoxia Inducible factor (HIF) response elements 5'-RCGTG-3' in the promoters of a selection of key splice factor and splice factor kinase genes. HIF response element sequences are depicted by the blue boxes. Red boxes signify the beginning and end of the promoter sequence studied positioned -1000 and +100 around the transcription start site.

4.3 The effect of hypoxia on the expression of splice factors and kinases

4.3.1 Examining the changes in expression of splice factors and splice factor kinases at the RNA level in hypoxia

Normoxia and hypoxia samples were generated in the PC3 cell line and analysed to examine the effect of hypoxia on the expression of the splice factors and splice factor kinases (see Section 4.1) (*SRSF1, SRSF2*, *SRSF3, SAM68, HuR, HnRNP A1, CLK1* and *SRPK1*). Their expression was normalised to the *Ubiquitin C* (*UBC*) housekeeping gene and changes in gene expression were calculated.. It was found that the expression of all the genes analysed significantly increased during hypoxia, with most genes increasing by approximately 2-fold, with the exceptions of *CLK1* (1.5-fold), *HnRNP A1* (4-fold) and *SRSF3* (4-fold) (Figure 4.2). This included *SRPK1* despite the absence of consensus HRE elements in the promoter region analysed (Figure 4.1).

4.3.2 Examining protein production of CLK1, SRPK1 and SRSF1 in hypoxia

The protein production of CLK1, SRPK1 and SRSF1 was examined in the PC3 cell line. CLK1 and SRSF1 protein production was found to increase in hypoxia in both experimental replicates (Figure 4.3A, B and C). SRPK1 protein production did not change at 24 and 48 hour hypoxia (Figure 4.3D), which suggests that hypoxia does not affect SRPK1 protein expression. However, it must be noted that as results are

not based on n=3, statistical analysis or robust conclusions can be made on this data; it can only give an indication.

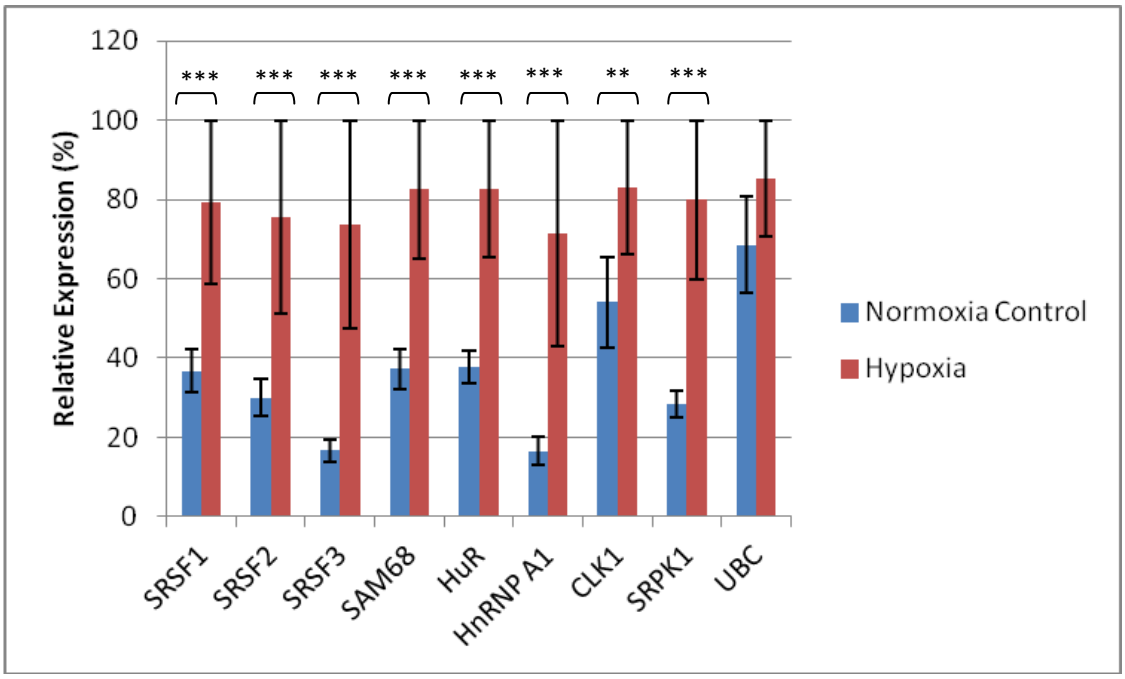


Figure 4.2: The effect of hypoxia on gene expression of SR proteins and SR protein kinases determined using qPCR. The expression of splice factors (*SRSF1, SRSF2, SRSF3, SAM68, HuR* and *HnRNP* A1) and splice factor kinases (*SRPK1* and *CLK1*) was normalised to the *UBC* housekeeping gene. All genes apart from the *UBC* housekeeping gene were found to not be either normally distributed or showed no homogeneity of variance using the Shapiro-Wilks and Bartlett's tests, respectively. The Mann-Whitney U test was therefore applied to data obtained. A student's T-test was applied to the data obtained for the *UBC* housekeeping gene. There were three technical replicates for each gene at each treatment in the qPCR plate and five experimental repeats. The error bars show 95% confidence intervals for the data. Treatment was for 48h and 1% oxygen was used for hypoxia treatment. ** = $p<0.01$; *** = $p<0.001$.

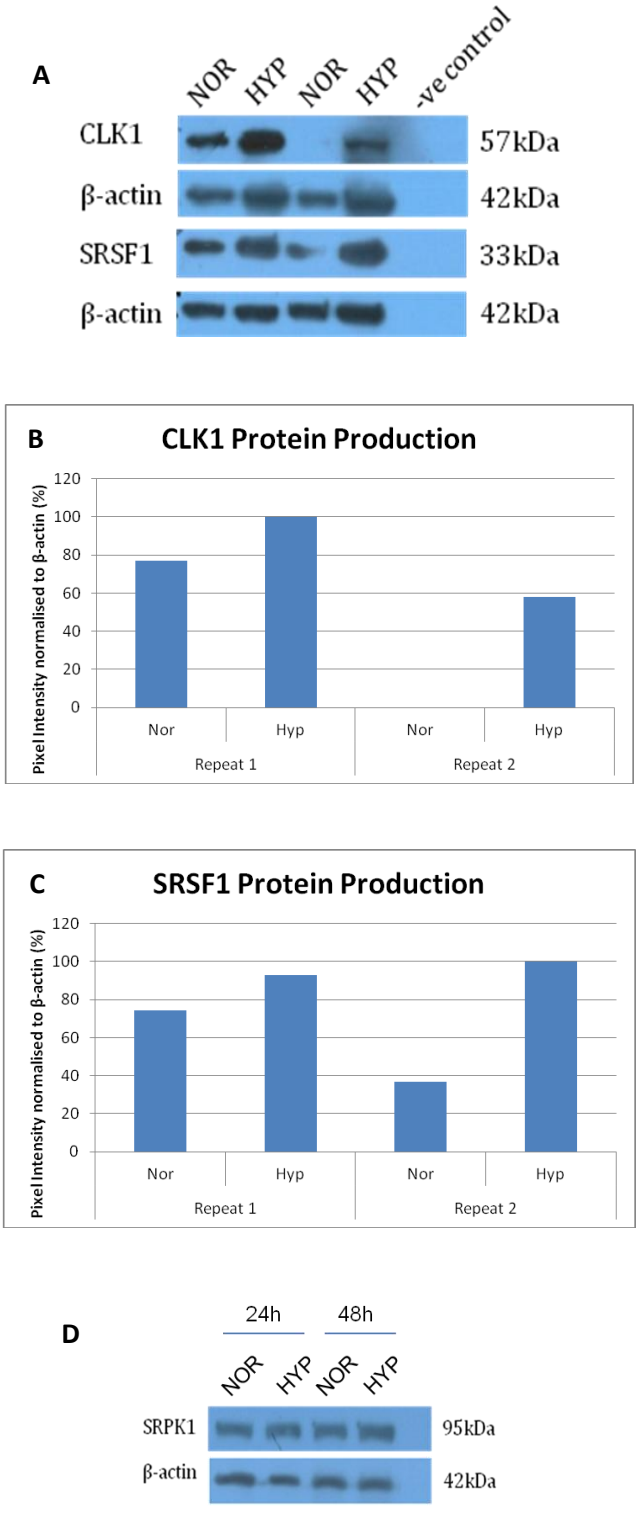


Figure 4.3: CLK1, SRSF1, CA IX and SRPK1 protein production after hypoxia treatment in PC3 cells. (A) CLK1 and SRSF1 protein production after 48h hypoxia treatment. (n=2). (B) CLK1 and (C) SRSF1 normalisation to the β-actin loading control. (D) SRPK1 protein expression after 24h and 48h hypoxia treatment respectively (n=1). β-actin was used as a loading control and 1% oxygen was used to achieve hypoxia.

4.3.3 The effect of hypoxia on *CLK1* alternative splicing

CLK1 has been found to be alternatively spliced to produce two isoforms in addition to the full-length transcript (Figure 4.4A). An isoform which skips exon 4 produces a truncated non-functional CLK1 and is targeted by the nonsense-mediated decay pathway (Duncan *et al.,* 1997). A more recently discovered isoform that retains introns 3 and 4 has been shown to be induced by heat shock and osmotic stress; intron retention also results in non-functional CLK1 (Ninomiya, Kataoka and Hagiwara, 2011). A reduction in intron retention is thought to be involved in the reinstatement of splicing after environmental stress and rapid phosphorylation recovery of SR proteins (Ninomiya, Kataoka and Hagiwara, 2011). Therefore, the effect of hypoxia on the splicing of *CLK1* was investigated.

The same normoxia and hypoxia samples that were used in Chapter three were amplified with *CLK1* primers specific for the investigation of exon 4 skipping or intron 4 retention. There was no indication that hypoxia affected intron 4 inclusion or skipping (Figure 4.4).

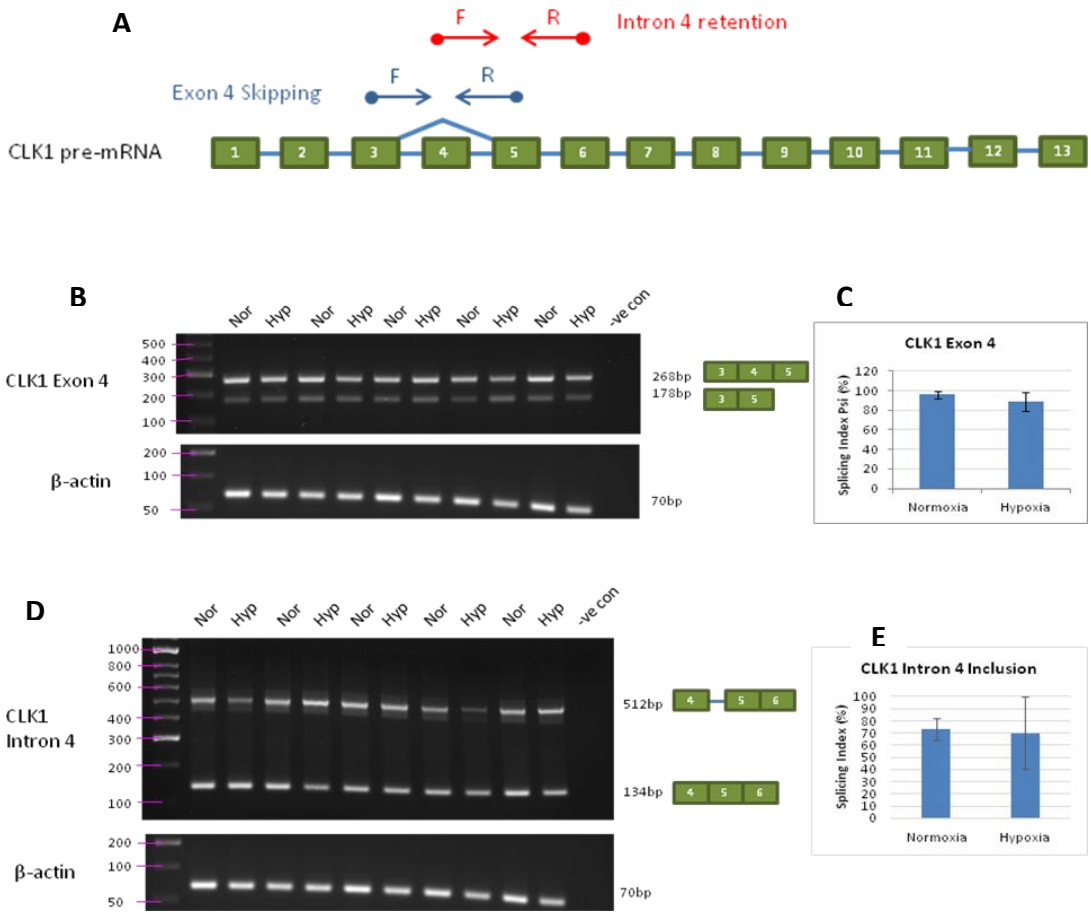


Figure 4.4: The effect of hypoxia on the alternative splicing of *CLK1*. (A) Schematic illustration of the full-length, exon 4 skipping and intron 3 & 4 retention pre-mRNAs of *CLK1* along with exons (green boxes), introns (blue lines) and location of the primers (F = forward primer; R = reverse primer), **(B)** Exon 4 cassette splicing PCR gel and **(C)** respective average percentage exon inclusion (%ψ) after normoxic or hypoxic treatment (n=5). **(D)** Intron 4 inclusion and exclusion splice isoform expression PCR gel and **(E)** respective average percentage intron 4 inclusion (%ψ) after normoxic or hypoxic treatment (n=5). Standard PCR was performed using the following conditions; 94^{o}C for 2 min, 94^{o}C for 30 sec, 58^{o}C for 30 sec, 72^{o}C for 30 sec, 72^{o}C for 5 minutes with 35 cycles. Primer sequences can be found in the methods section. Treatment was for 48h and hypoxia was performed at 1% oxygen. The 95% confidence interval is displayed as error bars. The Shapiro-Wilks test showed that the exon 4 data was not normally distributed, the Bartlett's test showed that it was homoschedastic, and so a Mann Whitney U test was conducted to test the statistical significance of the data. The data was not found to be significant at the 5% level but the test revealed that the data was significant at the 10% level, suggesting that with further repeats exon 4 splicing may be found to be significant. Shapiro-Wilks test showed that the intron 4 data was normally distributed, the Bartlett's test showed that the data was heteroschedastic, and so a Mann Whitney U test was applied to the data. Hypoxic induction of intron 4 was found not to be significant.

4.3.4 The effect of hypoxia on Serine/arginine-rich (SR) protein phosphorylation

The binding of serine/arginine-rich (SR) proteins to pre-mRNA is heavily regulated by phosphorylation of the serine/arginine domain (RS domain) (Xiao and Manley, 1998). Therefore, the effect of hypoxia on the phosphorylation state of SR proteins was investigated using the SR (1H4) monoclonal antibody (Santa Cruz Biotechnology, U.S.A), which is specific for detection of phosphorylated SR proteins. Bands were detected which were of the right size for SRSF4, SRSF5 and SRSF6; however as this was based on n=1, the results have to be taken with caution. Therefore, these results show a suggestion that the phosphorylation status of SRSF4 and SRSF5 is increased in hypoxia, and the phosphorylation of SRSF6 is not affected (Figure 4.5), but further repeats need to be conducted in order to deduce whether this is the case.

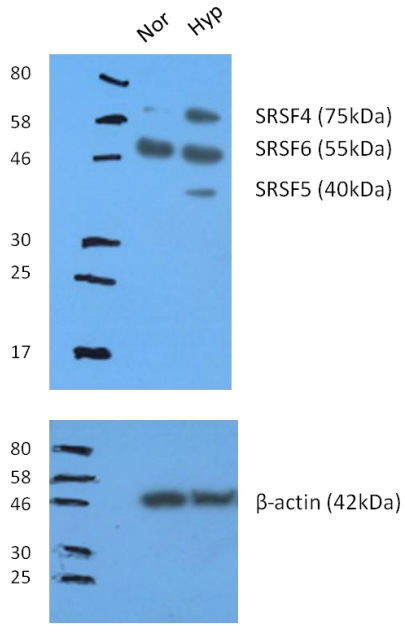


Figure 4.5: The effect of hypoxia on the phosphorylation status of SR proteins in PC3 cells. Bands of the right size for SRSF4, SRSF5 and SRSF6 were detected with the SR (1H4) monoclonal antibody (Santa Cruz Biotechnology, U.S.A). Hypoxia treatment time was 48h at 1% oxygen. β-actin was used as a loading control (n=1).

4.4 The effect of hypoxia on the localisation of CLK1, SRPK1 and SRSF1

SRSF1 is thought to be translocated from the cytoplasm to the nucleus through phosphorylation by SRPK1; and in the nucleus CLK1 promotes its hyper-phosphorylation and release from nuclear speckles (see Section 1.3.3). Therefore, the localisation of CLK1, SRPK1 and SRSF1 in hypoxia was investigated.

4.4.1 Protein fractionation to examine intracellular localisation

Protein fractionation was used to examine the presence of each protein in nuclear or crude cytoplasmic extracts, which also contained the cell membrane. It was found that hypoxia did not change the nuclear localisation of SRSF1 (Figure 4.6A). SRPK1 expression was found mainly in the cytoplasm during normoxia but there was an increase in nuclear SRPK1 expression in hypoxia suggesting that the protein may be found more evenly distributed throughout the nucleus and cytoplasm during hypoxia (Figure 4.6B). Although the majority of the literature has reported CLK1 to be localised mainly in the nucleus, the results here show that CLK1 is localised to the cytoplasm during both treatments (Figure 4.6C). HSP90 was used as a cytoplasmic control; however although several attempts were made to show RNA-polymerase-II-subunit-B2 expression as a nuclear control, it was not detected and therefore could not be included in the results.

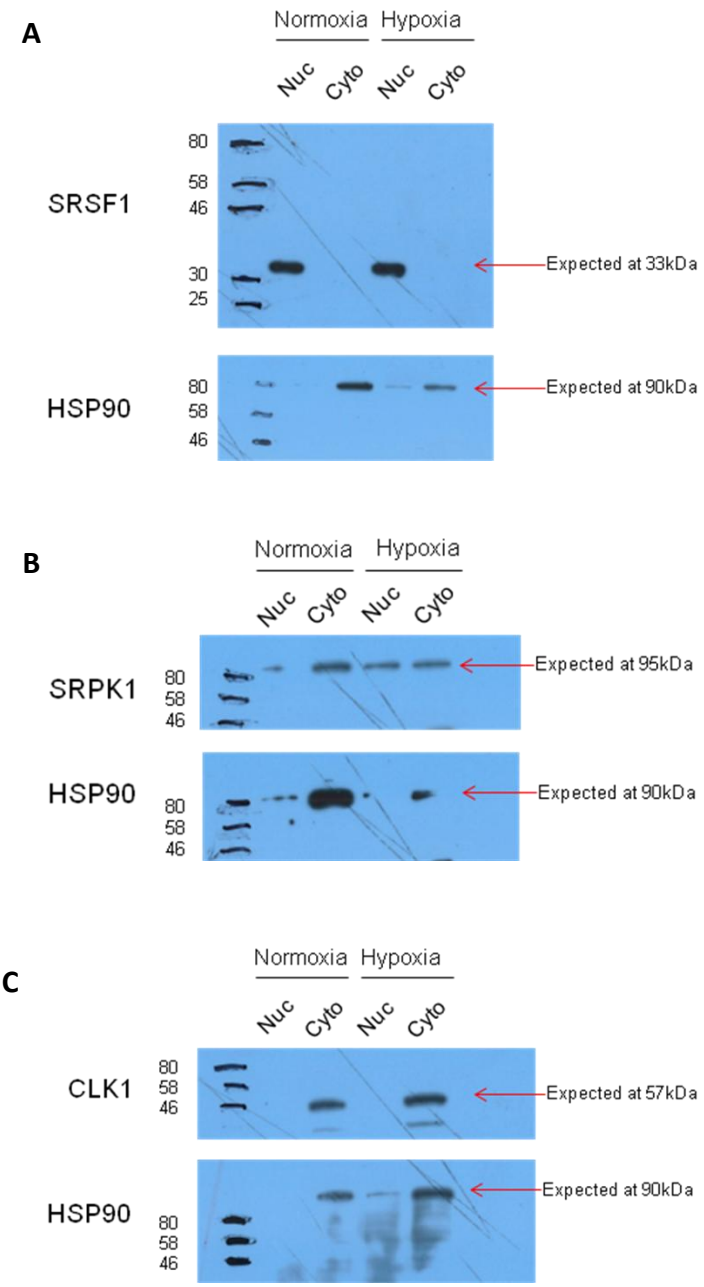


Figure 4.6: Protein expression of (A) SRSF1, (B) SRPK1 and (C) CLK1 in nucleic and crude cytoplasmic fractions in normoxia and hypoxia. HSP90 was used as a cytoplasmic control. Hypoxia treatment time was 48h at 1% oxygen (n=1).

4.4.2 Analysis of intracellular localisation of CLK1 via immunofluorescence

As the protein fractionation assay unexpectedly showed that CLK1 is localised to the cytoplasm, further analysis of CLK1 localisation was conducted using immunocytochemistry using the same CLK1 antibody. Figure 4.7 shows that CLK1 is found mostly outside the nucleus, although in the hypoxia sample there appeared to be some bright red spots in the middle of the some of the nuclei (Figure 4.7).

Confocal microscopy was used to provide further evidence for the localisation of CLK1 in response to hypoxia. Common to the first immunocytochemistry assay, DAPI was used to stain the cell nuclei, but in the confocal slides the lipid dye Vybrant DiO was also used as a crude control for the cytoplasm, as described in Section 2.16. Manders' overlap coefficients (MOC), M1 and M2 were calculated using the Volocity Colocalisation software to assess the percentage of colocalisation between CLK1 and either DAPI stain or the lipid stain.

DAPI was shown to co-localise to DiO, with most values above 50% co-localisation. This suggests a degree of overlap between the DAPI and DiO stain. The DiO stain was used to stain lipids in the plasma membrane as a crude stain for the cytoplasm. However, the stain targets lipids, which can be also found in intracellular membranes, such as those that encapsulate the Golgi body, mitochondria and the nucleus. Therefore, co-localisation between the DAPI and lipid stains could be due to localisation of DAPI to the nucleus and lipid stain localisation to the Golgi body or nuclear membrane, which may be in close proximity to one another. When it is also taken into consideration that some of the lipid signals were oversaturated, a high

level of colocalisation between DAPI and the lipid dyes can be explained. Due to the crude nature of the control DiO lipid stain for the cytoplasm, CLK1 localisation can only be stated as inside or outside of the nucleus.

An illustration depicting CLK1 localisation using the confocal can be found in figure 4.8A. During normoxia, it was found that an average of 65% of CLK1 was co-localised with the DiO stain compared to only 10% that co-localised with DAPI (Figure 4.8B). The rest of the CLK1 signal was either localised elsewhere or due to background. This suggests that CLK1 is mainly found outside the nucleus during normoxia. During hypoxia, an average of 54% of CLK1 was found localised to DiO, whereas 12% was found localised to DAPI (Figure 4.8B). This indicates that CLK1 is also predominantly found outside the nucleus during hypoxia.

SRSF1 and SRPK1 cellular distribution was also examined using the same antibodies as in the protein fractionation assay, and a DiI lipid stain. However, the background was too high and so these results are not included in this study.

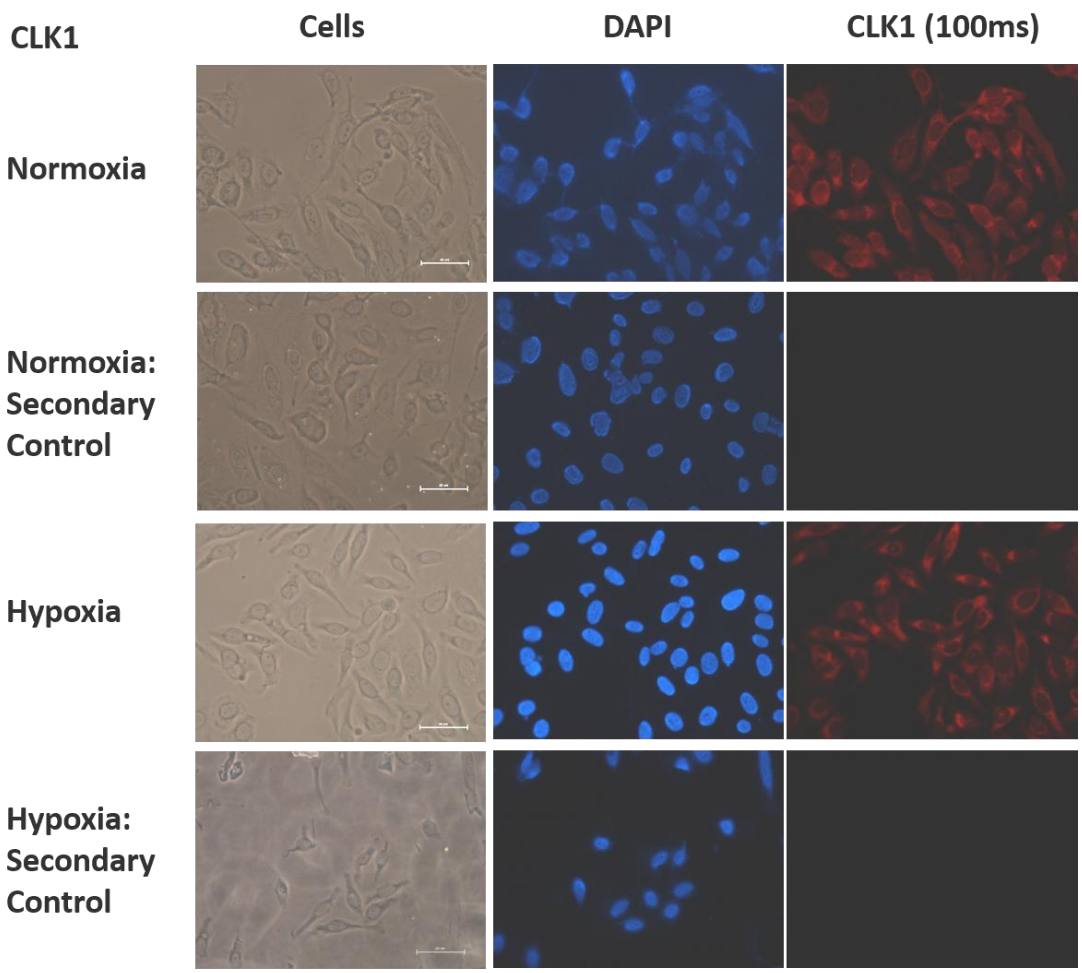


Figure 4.7: Localisation of CLK1 during hypoxia using immunofluorescence coupled with microscopy. Hypoxia treatment was for 48h at 1% oxygen. DAPI stain was used to illustrate the cell nuclei in the immunofluorescence assay. Antibodies used were; CLK1 (Abiocode: R1471-1) and anti-rabbit IgG HRP-linked antibody (Cell Signalling: ab150080).

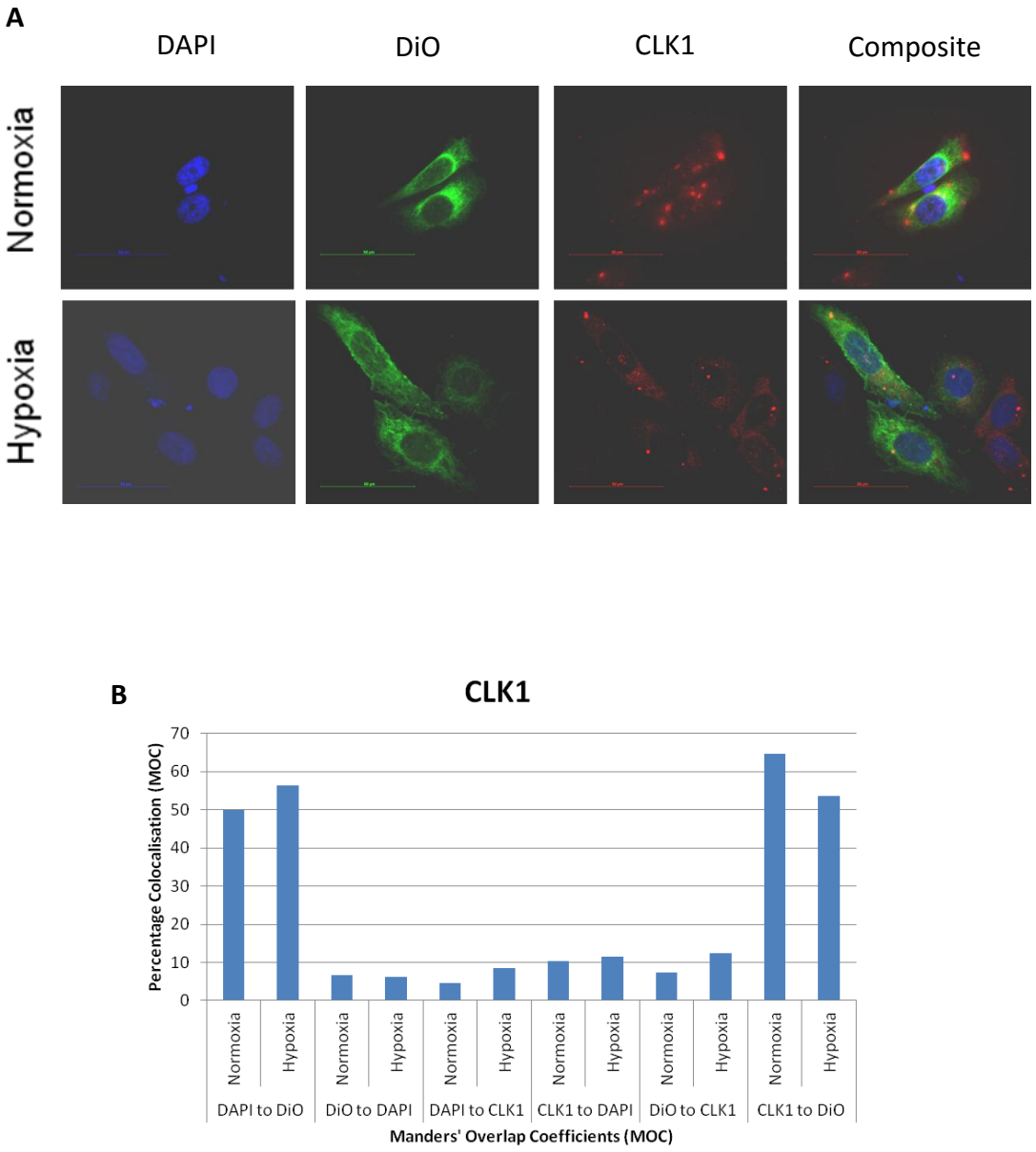


Figure 4.8: Colocalisation of CLK1 with the nucleus (DAPI) or DiO lipid stain. (A) Illustration depicting confocal localisation of CLK1. DAPI stain was used to show the cell nucleus and DiO lipid stain was used to show the cell lipid membranes and act as a crude cytoplasmic control. Hypoxia samples were incubated for 48h in 1% oxygen. Scale bars show 50µm. (B) Percentage colocalisation values obtained from Volocity software using Manders' Overlap Coefficients (MOC). Antibodies used were CLK1 (Abiocode: R1471-1) and anti-rabbit IgG HRP-linked antibody (Cell Signalling: ab150080). Average calculations were based on 3 seperate images taken from a CLK1 normoxia slide and 4 seperate images taken from CLK1 hypoxia slide. Images were taken from one entire experiment and so significance cannot be deduced.

4.5 Discussion

4.5.1 HIF Response Elements (HREs)

The promoters of a selection of key SR proteins and SR protein kinases were examined using the Eukaryotic Promoter Database for the core HIF response element (HRE) 5'-RCGTG-3'. This HRE has been reported to be highly conserved by various groups (Wenger, Stiehl and Camenisch, 2005; Schödel *et al.,* 2011; Greenald *et al.,* 2015). It was found that all of the genes investigated bar *SRPK1* had at least one HRE in their promoters, when the parameters were set to -1000 to +100 of the transcription start site (TSS) (Figure 4.1).

It must be noted that although the HIF response element motif is present in the promoter of these genes, it is not concrete evidence that HIF will bind to these promoters and initiate transcription from them, but just gives an indication that this is a possibility. This is because HIF elements have been reported to act from further away than the TSS, and have even been reported to skip the consecutive gene and activate another more distant promoter (Schödel *et al.,* 2011). This may be explained by the fact that in reality, DNA in three-dimensional form allows some genes to be closer than perceived in two-dimensional form and therefore HREs may act on genes that are adjacent to one another.

In addition, some of the genes contained more than one HRE in their promoter, as seen with *SRSF1* that contained five possible HREs. This suggests that there is a higher probability that *SRSF1* has at least one coding HRE for HIF binding, but again this remains to be determined. Schodel *et al.* (2011) highlighted that identifying

potential HIF-binding sites through distance from the TSS and mammalian conservation of the motif is limited as this approach does not allow any mechanistic insight into the factors, which may prevent HIF from binding to the motif. Also there may be other conditions that dictate HIF-dependent transcription in addition to the HRE motif during hypoxia which may not have yet been revealed. This can be resolved using a chromatin immunoprecipitation (chIP) assay, which determines protein-DNA interactions at particular locations within the genome (Nelson *et al.,* 2006), and therefore whether HIF binds to particular HRE locations at projected locations in the genome.

Jakubauskiene *et al.* (2015) reported five potential HIF binding sites in the *CLK1* promoter region, but the group used the motif 5'-NCGTG-3'. 'N' stands for any preceding nucleotide to the '*CGTG*' motif, whereas this analysis used 'R', which stands for a preceding purine (A or G) nucleotide as also used in papers by Wenger, Stiehl and Camenisch (2005), Schödel *et al.* (2011) and Greenald *et al.* (2015). This narrowed down the five potential HIF binding sites to two potential HREs. The same group performed chIP analysis to deduce whether enhanced CLK1 expression in hypoxia was HIF-dependent and found that HIF-1α was recruited to the *CLK1* promoter during hypoxia (Jakubauskiene *et al.,* 2015). A similar investigation into whether HIF-1α is recruited to the promoters of the other genes studied during hypoxia would be of interest.

An interesting finding in the literature is that HIF does not play a direct role in gene down-regulation. This is evidenced by a lack of HIF binding sites in hypoxia-down-regulated genes (Ortiz-Barahona *et al.,* 2010) and also the result from a chIP-chip

study which showed non-association between HIF-binding and transcriptional downregulation (Mole *et al.,* 2009). It is suggested by Ortiz-Barahona *et al.* (2010) that observed transcriptional downregulation triggered by hypoxia is either HIF-independent or as a result of activation by a downstream effector of HIF.

4.5.2 Using an appropriate housekeeping gene for normalisation in qPCR studies

Quantitative real-time PCR is a very sensitive method for deducing a change in the expression of a given gene. Therefore, it is important to use a reliable housekeeping gene for normalisation, whose expression remains stable during treatment. Several studies have highlighted the issue that a given housekeeping gene may be stable in some treatments but vary in others, and some have even shown that housekeeping gene stability can vary between cell lines (Caradec *et al.,* 2010; Vajda *et al.,* 2013; Powell *et al.,* 2014; van den Bergen *et al.,* 2009; Cinar *et al.,* 2013). There are numerous housekeeping gene assays available to be able to pinpoint the best housekeeping gene for each individual experiment. In addition, RNA molecules generated synthetically or cloned from another species can be incorporated into the qPCR mix, which is known as a spike (Huggett *et al.,* 2005). Plant RNA is one of the most widely used methods of spiking samples for normalisation. The rationale behind normalisation to a spike is that foreign RNA will not alter when exposed to treatment that may affect housekeeping genes (Huggett *et al.,* 2005). Although spiking is considered the most accurate method for normalisation, a lot of time is required to generate the spike, and so housekeeping genes are still widely used by research groups but with caution. Melt curves can provide information on the

specificity of the primers used. All melt curves showed just one peak, which showed that the primers used were specific.

Vajda *et al.* (2013) investigated the effect of hypoxia on a range of housekeeping genes in a variety of prostate cancer cell lines and found that *UBC* remained the most stable housekeeping gene, followed by *β-actin.* Therefore, samples were normalised to *UBC* and it was found that the gene expression of all the splice factors and splice factor kinases studied were significantly increased during hypoxia (Figure 4.2). This is consistent with the previous finding that all genes except *SRPK1* contained HREs in the promoter region, which suggests that these genes are directly targeted by HIF during hypoxia. *SRPK1* does not contain a HRE in the promoter region, which suggests that this gene could be activated downstream of the HIF pathway or another pathway that is activated during the hypoxic response. Another reason could be that the HRE element that is not in the promoter region of SRPK1. As stated before it is reported that the HRE could even be situated in the promoter of the preceeding gene (Schödel *et al.,* 2011).

4.5.3 Hypoxia increased the expression of selected splice factors in PC3 cells

Recent studies have also uncovered alterations in splice factor expression during hypoxia. HuR protein expression was increased during hypoxia in meningioma, and HuR knockdown was shown to decrease cell growth (Gauchotte *et al.,* 2017). This correlates with previous research, which shows that HuR mediates cell proliferation in cancer cell lines (Kullmann *et al.,* 2002), and builds on the finding that hypoxia

correlates with HuR expression in a meningioma cell line (Sakuma *et al.,* 2008). Furthermore, the group also reported a positive correlation between HuR expression and tumour grade, and concluded that cytoplasmic HuR could be a marker of poor prognosis in meningioma (Gauchotte *et al.,* 2017). In this thesis chapter, HuR mRNA expression is increased in hypoxia in PC3 prostate cancer cells, which provides more evidence that HuR expression is increased during hypoxia. However, whether or not the increased HuR expression is due to nuclear or cytoplasmic HuR remains to be elucidated. The finding of an increased cytoplasmic HuR during hypoxia in meningioma (Gauchotte *et al.,* 2017) suggests that it is likely that an increase in cytoplasmic HuR is occurring in the PC3 cell line. Furthermore, there has been evidence to show that during heat stress, HuR exits the nucleus to co-localise into cytoplasmic stress granules (Papadopoulou *et al.,* 2013), and so it is suggested that this also occurs during hypoxic stress.

Moreover, increased expression of SAM68 and hnRNP A1 during hypoxia has also been measured in HeLa cells (Bebee *et al.,* 2012). Like HuR, both of these proteins have been shown to relocate to stress granules during oxidative stress, which is suggested to be due to additional functions of the proteins in RNA metabolism (Guil, Long and Caceres, 2006; Henao-Mejia and He, 2009; Papdopoulou *et al.,* 2013).

In contrast to the evidence displayed in this chapter, there has been evidence in HeLa cells that hypoxia does not alter SR protein expression at the mRNA or protein level; in fact if anything hypoxia appeared to decrease the expression of some SR proteins (Jakubauskiene *et al.,* 2015). This could be due to differences in hypoxic

treatment time and/or cell line; Jakubauskiene *et al.* (2015) used 24h hypoxia treatment in HeLa cells, whereas this study used 48h hypoxia treatment in PC3 cells. The paper did however state evidence that SR protein phosphorylation is increased in hypoxia, which suggests that SR protein activity is increased during hypoxia. The discussion on the effect of hypoxia on phosphorylation status of SR proteins appears later in this chaper. Recently, in pluripotent stem cell-derived cardiomyocytes, Stoehr *et al.* (2016) provided evidence for increased stabilisation of SRSF2 during treatment with the prolyl hydroxylation inhibitor, dimethyloxalylglycine (DMOG). During hypoxia, prolyl hydroxylation is inhibited, which stabilises HIF-mediated transcription. Therefore, this suggests that the expression of SR proteins during hypoxia may be specific to a given cell type.

4.5.4 The effect of hypoxia on CLK1, SRPK1 and SRSF1 protein expression

It was found that CLK1 and SRSF1 protein expression increased in hypoxia, whereas SRPK1 protein expression was unaffected by hypoxia (Figure 4.3 A, B and C). Eisenreich *et al.* (2013) provides supportive evidence in A459 lung cells that the expression of CLK1 increases at both the mRNA and protein level after two hours of hypoxia treatment using 3% oxygen. Furthermore, Jakubauskiene *et al.* (2015) increased expression of CLK1, SRPK1 and SRPK2 at both the mRNA and protein levels in HeLa cells, which backs up the finding that CLK1 expression is elevated during hypoxia. However, in this study in PC3 cells, SRPK1 expression was shown to be significantly up-regulated at the mRNA level, but not at the protein level, which

suggests that hypoxic regulation of SRPK1 protein may be cell type specific. The Jakubauskiene group used a treatment time of 24h hypoxia, whereas this study used 48h hypoxia conditioning, which may also explain the difference in results. Furthermore, in glioma cells hypoxia resulted in lower mRNA expression of *SRPK1* and *SRSF1* (Wu *et al.,* 2013), which suggests differential expression of *SRPK1* depending on cell type. The fact that hypoxia caused the gene expression of *SRPK1* to significantly increase but the protein level was unchanged could be explained by post-translational modifications to SRPK1, such as an increase in nonsense-mediated decay of the mRNA transcripts or RNA interference of the mRNA.

4.5.5 The effect of hypoxia on splicing of CLK1

CLK1 is alternatively spliced to produce two further transcripts in addition to the full-length isoform (Figure 4.4A). The skipping of exon four produces a truncated isoform, which is thought to regulate the amount of active CLK1 protein (Duncan *et al.,* 1997). It was found that over-expression of full-length *CLK1* promoted exon 4 skipping, but an elevated level of the truncated protein promoted the inclusion of exon 4 and therefore increased the amount of full-length CLK1 (Duncan *et al.,* 1997). Ninomiya, Kataoka and Hagiwara (2011) showed that the retention of introns 3 and 4 produces a protein that is thought to be involved in rapid recovery of the splicing reaction and fast phosphorylation of SR proteins after heat shock or osmotic environmental stresses. The group showed that this is achieved through step-wise splicing of the introns to produce the mature active form of the protein.

The qPCR primers for *CLK1* spanned across exons 9 and 10 and therefore only picked up the total expression of all *CLK1* isoforms. This is good practice for qPCR in order for accurate measurement of expression. Therefore, it was thought that perhaps the increase in CLK1 protein expression could be due to a change in the alternative splicing of *CLK1*. However, there was no indication that hypoxia had an effect on exon skipping or intron retention (Figure 4.4).

4.5.6 The effect of hypoxia on the phosphorylation of SR proteins

The phosphorylation of SR proteins enables them to form the spliceosome and bind to transcription sites in the pre-mRNA for the initiation of splicing. In addition, the phosphorylation status of SR proteins can affect the cellular localisation of the proteins (reviewed in Naro and Sette, 2013). Heat shock and osmotic stress have been shown to cause de-phosphorylation of SR proteins SRSF4 and SRSF10 (Shin *et al.,* 2004; Shi *et al.,* 2006; Ninomiya, Kataoka and Hagiwara, 2011). In addition, there is evidence to show that CLK1 and CLK4 are involved in the re-phosphorylation of SRSF4, SRSF5, SRSF6 and SRSF10 after cellular stress (Ninomiya, Karaoka and Hagiwara, 2011). Therefore, the effect of hypoxia on SR protein phosphorylation was investigated.

The SR (1H4) antibody (Santa Cruz Biotechnology), which is reactive to phosphorylated RS domains was used to examine levels of phosphorylated SR proteins. The antibody detected bands, which are the correct size for SRSF4, SRSF5 and SRSF6. There was a suggestion that the phosphorylation of SRSF4 and SRSF5

was increased during hypoxia (Figure 4.5); however as this observation is based on n=1, futher investigations need to be executed to conclude if this is the case. Eisenreich *et al.* (2013) also found that hypoxia induced phosphorylation of SRSF4. Therefore, it can be suggested that SRSF4 and SRSF5 phosphorylation may be involved in the hypoxia response. Furthermore, Jakubauskiene *et al.* (2015) provided evidence that SR proteins were hyper-phosphorylated and interacted more strongly with RNA during hypoxia, which suggests an increased in SR protein-mediated regulation of splicing during hypoxia.

4.5.7 The effect of hypoxia on the localisation of CLK1, SRPK1 and SRSF1

Results obtained for this section are based on n=1 and so only crude assumptions can be made about these results and further experimental repeats need to be conducted in order to determine whether the conclusions made are true. Therefore, these conclusions need to be treated with caution. The protein fractionation assay suggested a nuclear localisation for SRSF1 during both normoxia and hypoxia (Figure 4.6A). It is suggested that SRSF1 may be localised to the nucleus during hypoxia, as SRSF1 is found to be a HIF-regulated gene and therefore nuclear localisation would allow the protein to regulate splicing to favour isoforms that are required during oxygen deprivation.

Protein fractionation suggested that SRPK1 was localised to the cytoplasm during normoxia, but during hypoxia SRPK1 localisation was split between the nucleus and the cytoplasm (Figure 4.6B). The literature provides evidence that SRPK1 resides in

the cytoplasm until the cell is stimulated by a signal (Ding *et al.,* 2006). Zhong *et al.* (2009) showed that SRPKs are anchored to the cytoplasm by molecular chaperones, but osmotic stress is able to translocate SRPKs to the nucleus to regulate SR protein phosphorylation and ultimately alternative splicing. The results displayed here for suggest that SRPK1 translocates into the nucleus during hypoxia. When considering the results of the study by Zhong *et al.* (2009) along with the results displayed in this chapter, it can be suggested that cellular stress causes SRPK1 to translocate to the nucleus. Furthermore, it has been shown that AKT is able to bind to SRPK1 and stimulate auto-phosphorylation of the SRPK1 protein, which ultimately leads to its nuclear translocation where it can perform its regulatory function of SR proteins (Zhou *et al.,* 2012). SRPK1 protein levels were not found to change during hypoxia (Figure 4.3D); however, this mechanism would increase SRPK1 expression in the nucleus, without the need to increase overall expression.

It was suggested in the confocal assay that there may be an overlap between the DAPI and lipid stains, as DAPI was found to colocalise with both lipid stains in the majority of experiments. However, this could also be explained by the fact that some of the lipid signals were found to be saturated. This would cause a glow like effect around the signal which could be mistaken for colocalisation with DAPI.

The confocal assay provides localisation information for all the proteins throughout the cell, whereas the immunofluorescence assay alone assesses the localisation of the proteins at a particular cross-section of the cell. Therefore, if a protein resides in the nucleus but the cross-section examined also contains cytoplasm that is just above that nucleus, it could appear that the protein is in the cytoplasm or vice-

versa. Therefore, confocal imaging is thought to be more accurate in determining the localisation of a protein. However, due to time constraints, the confocal assay was only run once and a few images were created from each slide generated. Some images were not viable for analysis and so for some of the protein treatments only one set of values was calculated. Therefore, results have to be interpreted with some caution.

A surprising observation was shown with the localisation of the CLK1 protein using protein fractionation coupled with western blotting and immunofluorescence coupled with microscopy. CLK1 has previously been described throughout the literature as a nuclear protein that phosphorylates the RS2 domain of SRSF1 releasing it from nuclear speckle storage to regulate gene splicing (Aubol *et al.,* 2013; Corkery *et al.,* 2015). However, results displayed in this chapter have shown CLK1 to reside in the cytoplasm during normoxia and hypoxia (Figures 4.6C, 4.7 and 4.8). Previous studies that localise CLK1 to nuclear speckles have been conducted on over-expressed CLK1 protein and therefore could misrepresent the localisation of the protein (Menegay *et al.*, 2000). However, CLK1 is also found to possess motifs for nuclear localisation in its N-terminus (Menegay *et al.,* 2000). As mentioned previously, CLK1 is alternatively spliced, and it was found through subcellular fractionation of NIH-3T3 cells that mature CLK1 resided in the cytoplasm, whereas intron-retained CLK1 was localised to the nucleus (Ninomiya, Kataoka and Hagiwara, 2011). Therefore, previous studies may have detected the intron-retained version of CLK1, rather than the active form of the protein. The same CLK1 antibody was used for the immunoblot and immunocytochemistry

experiments, and so it is no surprise that a similar subcellular localisation of CLK1 was observed in both assays. Other previous studies that declare CLK1 as a nuclear protein may have used different antibodies and so further investigations are required with use of other CLK1 antibodies in different cell lines.

Menegay *et al.* (2000) provides some insight into these conflicting results, after the group also found CLK1 to reside in the cytoplasm of PC12 cells when using immunohistochemical staining. The group investigated various fixation agents for immunostaining, and found that whilst methanol and light (0.4%) paraformaldehyde fixing agents resulted in nuclear staining of CLK1, fixing in Bouin's fixative or 4% paraformaldehyde resulted in cytoplasmic staining of the proteins. This is due to the fact that fixation in methanol precipitates proteins but does not cross-link them, whereas Bouin's fixative and 4% paraformaldehyde both precipitates and cross-links proteins and therefore prevents any movement of the protein during the fixation process (Menegay *et al.,* 2000). Although the results in this chapter agree with the Menegay *et al.* (2000) paper that CLK1 is a cytoplasmic protein, the fixative used in the immunofluorescence assay is a methanol/acetone mix, which disagrees with the conclusion that methanol based fixatives show CLK1 to be localised to the nucleus. As all localisation assays show that CLK1 has cytoplasmic localisation in normoxia and hypoxia, it can be deduced that CLK1 is most likely a cytoplasmic protein, at least in the cell lines used here. More generally these results suggest that it might be worth investigating in more detail the possibility that CLK1 may contribute to substrate phosphorylation in the cytoplasm as well as in the nucleus.

CHAPTER 5:

The involvement of SAFB1, PRPF8 and CLK1 in the regulation of alternative splicing in hypoxia.

5.1 Background

Carbonic anhydrase 9 (CA IX) is a stable marker for hypoxia (Sobhanifar *et al.,* 2005) that has been utilised throughout this project as a hypoxia control. As discussed in Section 1.2.4, CA IX is implicated in cellular invasion, migration and ultimately metastasis (Svastova and Pastorekova, 2013). A number of groups have conducted research into the development of CA IX inhibitors in the hope of producing a novel therapeutic for hypoxic tumours (Ahlskog *et al.,* 2009; Nocentini *et al.,* 2015; Pastorek and Pastorekova, 2015). CA IX is alternatively spliced to produce an isoform that skips exons eight and nine in addition to the full-length isoform (Malentacci *et al,* 2009). Unlike the full-length isoform, the shorter isoform produces a truncated protein that is expressed independently of hypoxia (Malentacci *et al.,* 2009). Therefore, it is possible that shifting the splicing of CA IX to favour the hypoxia-independent isoform could provide an additional cancer therapy for hypoxic tumours. For this reason, an investigation into the splice factors that control the alternative splicing of CA IX was conducted.

In Chapter four of this thesis, the splice factor kinase CLK1 and splice factor SRSF1 were both found to have increased mRNA and protein expression after hypoxia treatment. CLK1 phosphorylates the RS domains of SR proteins, which are then able to facilitate pre-mRNA splicing. Therefore, it is proposed that an increase in expression of CLK1 may increase the phosphorylation of some SR proteins, such as SRSF1 and therefore influence alternative splicing to produce splice isoforms that enable the cell to cope with the low oxygen levels during hypoxia.

5.2 Identifying potential splice factor regulators of *CA IX*

A library of 57 splice factor knockdowns, created in the Michigan-Cancer-Foundation-7 breast cancer cell line by our collaborators (Dr. Roscoe Klinck and his team at the RNomics platform at Sherbrooke University, Canada) were utilised to assess the effect on *CA IX* splicing. This enabled the determination of possible splice factors involved in the regulation of *CA IX* alternative splicing. High-throughput analysis suggested that scaffold-attachment-factor-B1 (SAFB1) and pre-mRNA-processing-splicing-factor-8 (PRPF8) splice factor knockdowns switched the splicing of *CA IX* (Figure 5.1). Knockdown of SAFB1 altered the ratio of the full-length *CA IX* isoform to the shorter *CA IX* isoform. CA IX-FL was found to be the dominant isoform in the lipofectamine control, but knockdown of SAFB1 caused a shift in splicing of *CA IX*, which favoured the shorter *CA IX* splice variant (lipofectamine control: 80% *CA IX-FL,* 20% *CA IX-AS*; SAFB1 siRNA: 30% *CA IX-FL,* 70% *CA IX-AS*).

Similarly, PRPF8 knockdown also altered splicing of *CA* IX. Although the full-length variant of *CA IX* was shown to be the dominant splice variant in both the control and PRPF8 knockdown samples, expression of the shorter splice variant of *CA IX* was introduced when PRPF8 was knocked down. This suggests that PRPF8 favours splicing of the *CA IX-FL* isoform. The results abovesuggest that both SAFB1 and PRPF8 are required for the expression of the longer *CA IX* splice variant (Figure 5.1). However, as these findings are based on a single experiment, further investigation is required to deduce whether or not this is the case. Therefore, siRNAs for PRPF8

and SAFB1 were applied to PC3 cells in order to examine whether or not these two splice factors affected the splicing of *CA IX* in a prostate cancer cell line.

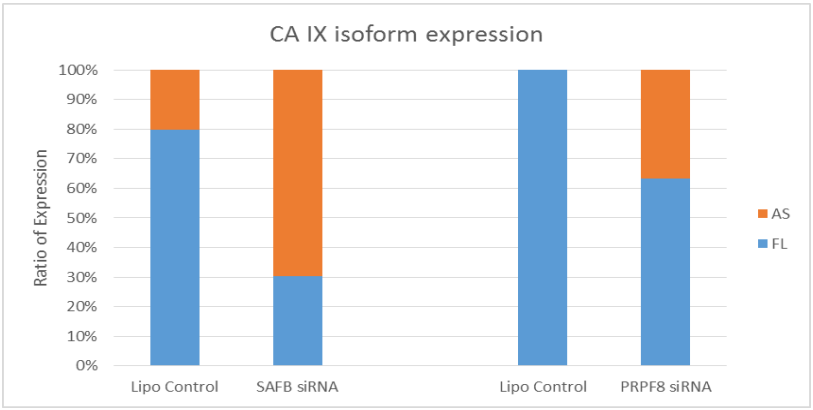


Figure 5.1: Effect of SAFB1 and PRPF8 siRNA on the alternative splicing of *CA IX*. Collaborators at Sherbrooke University, Canada subjected a sample library of 57 siRNA splice factor knockdowns in the MCF-7 breast cancer cell line for high-throughput PCR across the *CA IX* cassetted exons 8 & 9. Lipofectamine was used as a vector control n=1. SAFB1 and PRPF8 were identified as splice factors that may change splicing of *CA IX*. **AS =** Alternatively Spliced variant lacking exons 8 & 9, **FL** = Full-length variant.

In order to verify whether or not the siRNA knockdowns of PRPF8 and SAFB1 were successful, western blots were performed to check the expression level of the proteins. The PRPF8 siRNA western blot assay proved to be problematic and although many attempts were made to produce a western blot showing control and knockdown bands of the large 220kDa PRPF8 protein, a result was not obtained. In order to check whether the protein had transferred, the blot was re-probed with a slightly smaller protein, VE cadherin**.** As can be seen in figure 5.2, VE cadherin did provide a band of the required size providing evidence of a successful transfer of protein to the western blot membrane. As it was not possible to verify the PRPF8 knockdown, it was not possible to confirm its involvement in *CA IX* alternative splicing, and therefore this be investigated in future work.

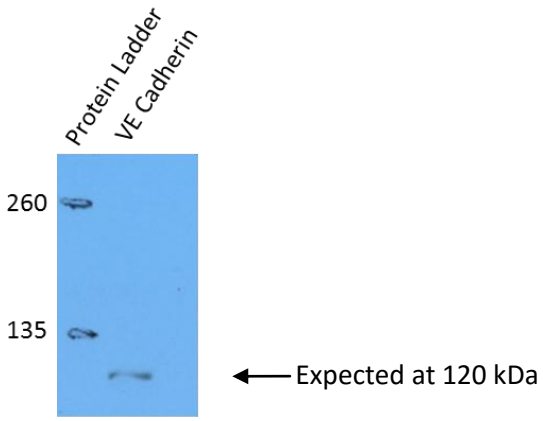
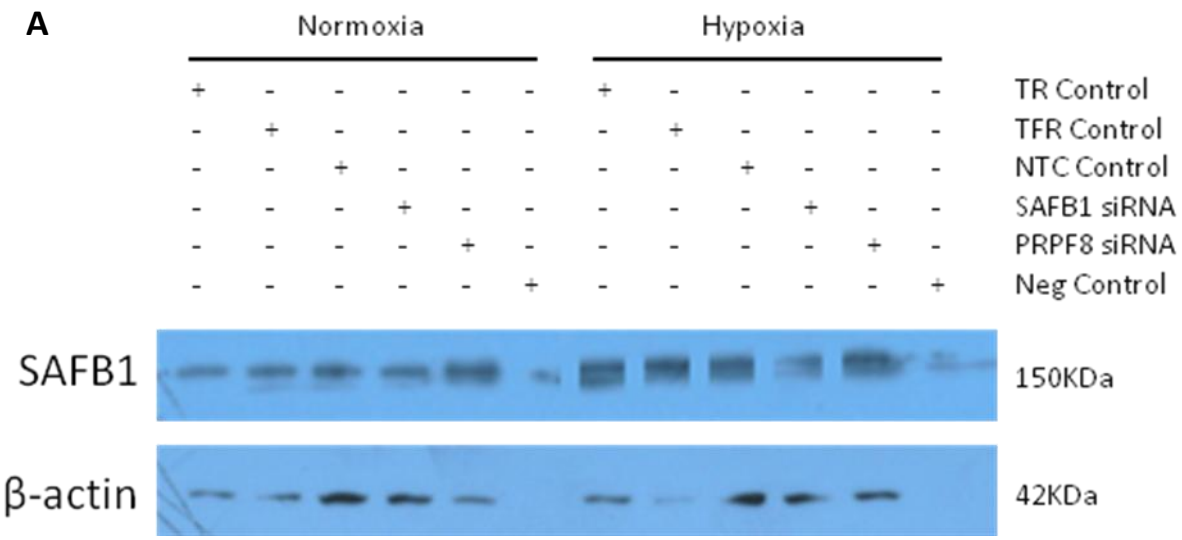


Figure 5.2: Confirmation of successful protein transfer using VE Cadherin. PRPF8 antibody was not able to detect PRPF8 protein (220KDa) and so VE Cadherin (120KDa) was used to re-probe the membrane to determine whether there was a successful transfer of protein to the blot in order to rule out any experimental error.

Knockdown of SAFB1 using siRNAs was found to be unsuccessful when normalised to the β-actin control , as the expression of SAFB1 protein did not deviate much from the control samples that did not contain SAFB1 siRNA. A few other attempts were made to try and knockdown SAFB1 but they were also without success. A representative western blot is shown in figure 5.3.

In order to shed some more light on the behaviour of SAFB1 during hypoxia, the expression and localisation of SAFB1 during normoxia and hypoxia was examined. It was found that SAFB1 protein expression decreased during hypoxia treatment in two experimental repeats (Figure 5.4A), which suggests that SAFB1 may not be required for the hypoxic response. Conversly, protein fractionation coupled with western blotting found that there was a higher expression of SAFB1 protein in the nucleus after both normoxia and hypoxia treatment (Figure 5.4B), which suggests

that SAFB1 may be translocated into the nucleus to regulate splicing during hypoxia. It is proposed that SAFB1 may regulate splicing of some genes during the hypoxic response, which is why it is translocated into the nucleus during hypoxia; however, perhaps there is a down-regulation of SAFB1 in order for the cell to conserve some energy during hypoxic stress. Further experimentation will be required to deduce the effect of hypoxia on SAFB1 activity, but this study provides an indication that SAFB1 expression and localisation are affected by hypoxia.



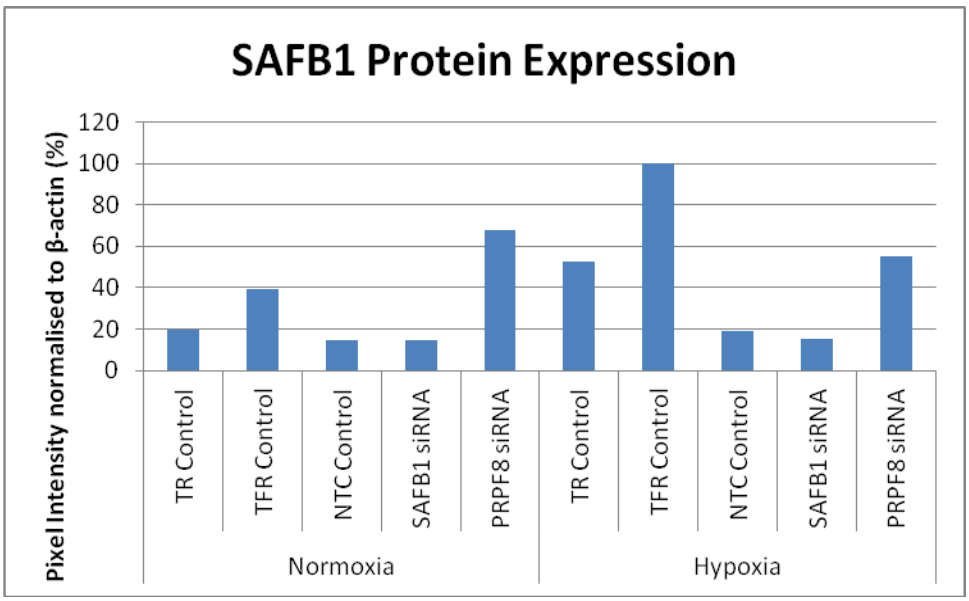


Figure 5.3: SAFB1 knockdown with siRNA. A western blot was conducted to show whether the SAFB1 siRNA knockdown was successful. **(A)** SAFB1 and β-actin protein expression. **(B)** SAFB1 protein expression was normalised to the β-actin control using EXCEL software n=1. Hypoxic treatment was for 48h at 1% oxygen in a MIC-101 hypoxia chamber at 37°.

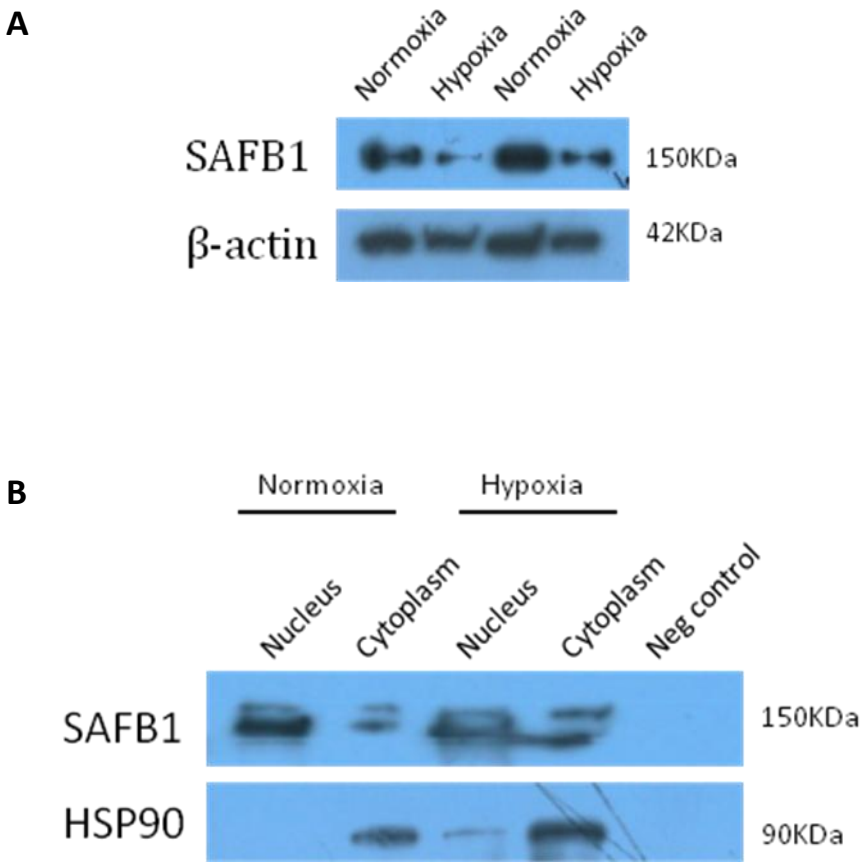


Figure 5.4: Effect of hypoxia on SAFB1 protein expression and localisation. (A) SAFB1 protein expression after 48h normoxia and hypoxia treatment at 1% oxygen concentration using a MIC-101 hypoxia chamber incubated at 37°C. β-actin (42KDa) was used as a loading control. **(B)** SAFB1 protein localisation after 48h normoxia and hypoxia treatment under the same conditions. Protein fractionation produced a nuclear and crude cytoplasmic sample which were probed for SAFB1 expression n=2. HSP90 (90KDa) was used as a cytoplasmic control.

5.3 Examining the effect of chemical inhibitors of CLK1 and SRPK1 on CA IX expression

In order to investigate whether the splice factor kinases CLK1 and SRPK1 had an effect on CA IX expression, 1-(3-ethyl-5-methoxy-2(3H)-benzothiazolylidene)-2-propanone, (known as TG003, a CLK1 inhibitor) and SPHINX (a SRPK1 inhibitor) were used to inhibit the activity of the protein kinases, respectively. Muraki *et al.*

(2004) confirmed that 10 μM TG003 was sufficient to inhibit CLK1 activity and Gammons *et al.* (2013) showed that 10 μM SPHINX inhibited SRPK1 activity so these concentrations were used for experiments. TG003 and SPHINX were dissolved in DMSO and therefore a DMSO control was also included. In figure 5.5A, DMSO appeared to increase the expression of the full-length isoform of *CA IX* (*CAIX-FL*). However, there was no indication that the same amount of DMSO had an effect on *CA IX-FL* mRNA expression in figure 5.5C. More experiments are required in order to deduce whether DMSO affects *CA IX-FL* mRNA expression. The PCR gel and western blot provided no clear evidence that TG003 had an effect on *CA IX* splicing or protein expression (Figure 5.5A and B). In addition, there was no indication that SPHINX inhibition of SRPK1 had an effect on *CA IX* splicing (Figure 5.5C). However, as results are based on a single experiment, further repeats are required to deduce whether or not this is the case.

Ideally, a positive control would be used to confirm that the inhibitors were working. In later experiments, TG003 (CLK1 inhibitor) was shown to change *FGFR1OP* splicing (section 5.5); however, SPHINX (SRPK1 inhibitor) was not shown to alter splicing of any of the genes studied. Expression of the *vascular endothelial growth factor A* (*VEGFA*) variant $VEGF_{165}$ has been shown to decrease with SPHINX treatment (Gammons *et al.,* 2013), and therefore could act as a positive control for future experiments.

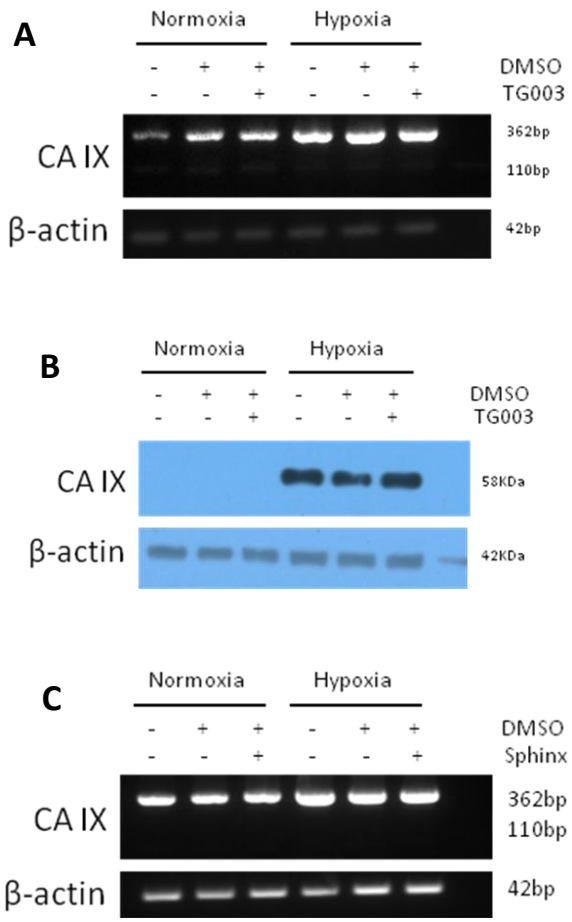


Figure 5.5: Effect of CLK1 and SRPK1 inhibition on CA IX expression. (A) *CA IX* isoform expression, and **(B)** average percentage exon inclusion of exons 8 & 9 after treatment with the CLK1 inhibitor, TG003 (n=3). Shapiro wilks test and Bartlett's test were applied to the data, and found to not to normally distributed, but homoschedastic. Therefore, a Dunn's test would ideally be applied to the data; however, there were not enough experimental repeats to conduct a Dunn's test and so the statistical significance is unknown. **(C)** protein expression of CA IX after treatment with the CLK1 inhibitor TG003. **(D)** mRNA expression of *CA IX* after treatment with the SRPK1 inhibitor SPHINX. Treatment was for 48h at 37°C with 10 µM of TG003 or SPHINX, respectively. Hypoxia was achieved using 1% oxygen during the 48h inhibition in a MIC-101 hypoxia chamber. β-actin was used as a loading control n=1.

5.4 The effect of CLK1 and SRPK1 siRNA knockdowns on the alternative splicing of cancer-associated genes

Chapter four presented evidence that CLK1 mRNA and protein expression increased with hypoxia treatment, and therefore the effect of CLK1 siRNAs on the alternative splicing of the cancer-associated genes identified in Chapter three was investigated. In addition, the effect of an SRPK1 siRNA knockdown on the alternative splicing of the same cancer-associated genes was also investigated. Evidence was provided for successful SRPK1 knockdown (Figure 5.6A), but the western blot of the knockdown of CLK1 was unable to show conclusively that CLK1 knockdown had been achieved. Therefore, the PCR results displayed for the CLK1 knockdown have to be considered with caution.

RNA extractions taken at the same time as the protein extractions for the siRNA experiments were used to generate RT-PCRs. This allowed assessment of the effect of SRPK1 and unconfirmed CLK1 knockdown on the alternative splicing of cancer-associated genes (Figure 5.6B). Due to a lack of experimental repeats (n=1), statistical analysis could not be conducted for changes in splicing with SRPK1 and CLK1 siRNA treatment. However, there was an indication that the unconfirmed CLK1 siRNA reduced the percentage of exon inclusion and therefore altered splicing in the *FGFR1OP* and *UTRN* genes (Figure 5.6C and D). Again, this observation has to be taken with caution, as the CLK1 siRNA was not confirmed and there is a lack of repeats. Further experimental repeats with confirmed successful CLK1 knockdown are required to support these observations.

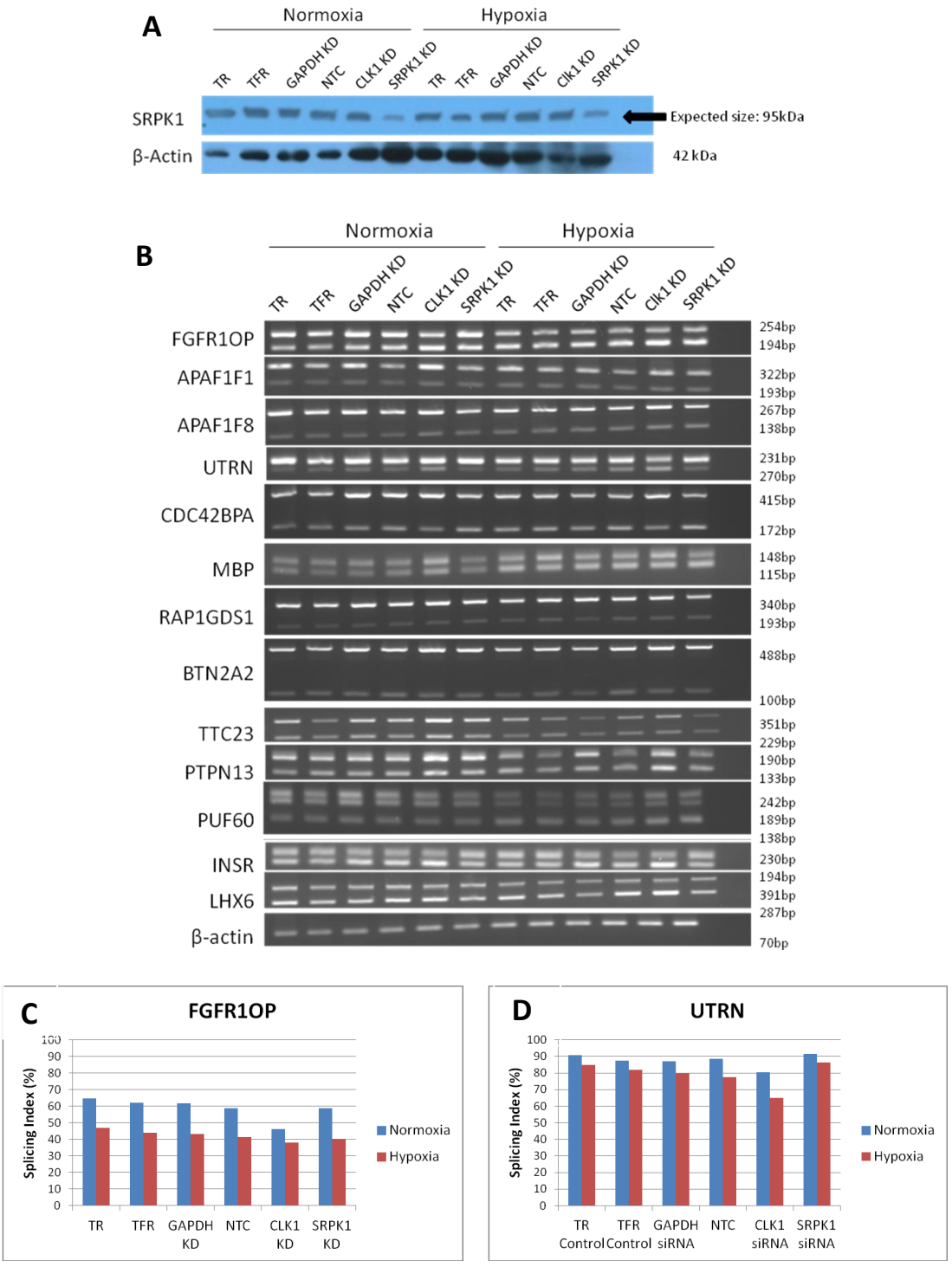


Figure 5.6: Effect of CLK1 and SRPK1 siRNA on the alternative splicing of cancer-associated genes. Cancer-associated genes identified with a change in splicing in response to hypoxia were subjected to CLK1 and SRPK1 knock-downs (KD) using siRNAs. Hypoxia treatment was for 48h at 1% oxygen in a MIC-101 hypoxia chamber at 37°C. **TR** = Treatment Control (either normoxia or hypoxia only), **TRF** = Transfection Reagent Control, **GAPDH siRNA** = control for siRNA treatment, **NTC** = Non-targeted Control. β-actin was used as a loading control n=1. **(A)** Confirmation of SRPK1 knockdown. Evidence of **(B)** Gel PCR of cancer-associated genes showing alternatively spliced isoforms. Evidence of **(C)** *FGFR1OP* and **(D)** *UTRN* alternative splicing changes with CLK1 siRNA treatment using ψ analysis.

5.5 Examining the effect of chemical inhibitors of CLK1 and SRPK1 on the alternative splicing of cancer-associated genes

As the knockdown for CLK1 was unconfirmed, TG003 was used to chemically inhibit CLK1 to see if impaired function of CLK1 had any effect on the alternative splicing of cancer-associated genes. RNAs were extracted for CLK1 treated and untreated samples respectively, during 48 hours of normoxia or hypoxia treatment. In addition, SPHINX, a chemical inhibitor of SRPK1 was used to assess any changes in the splicing of the same cancer-associated genes. Statistical analysis was based upon three experimental repeats for TG003 examination, and was in the form of a one-way ANOVA for all genes studied apart from *FGFR1OP*, which was found not to be normally distributed. In this instance a Dunn's test would usually be conducted to test for significance; however this requires more repeats and so the statistical significance of *FGFR1OP* splicing under TG003 treatment is not known. However. There was an indication from the gel PCR that TG003 treatment did have an effect on *FGFR1OP* splicing (Figure 5.7 and 5.8); favouring the smaller *FGFR1OP* splice isoform. TG003 did not significantly alter the splicing of any of the other genes studied (Figure 5.8). Statistical analysis could not be applied to the SPHINX experiments as there were two experimental repeats for SPHINX; however there was no indication from the gel PCR images that SPHINX altered splicing of any of the cancer-associated genes studied (Figure 5.9 and 5.10).

A positive control is required to show that the CLK1 chemical inhibitor is working. Ongoing work in the laboratory by Simon Uzor has shown to significantly increase

apoptosis and reduce proliferation using 1-10μM TG003 treatment in PC3 cells, which shows that the compound has an effect at the same concentration used in this study. Furthermore, the fact the TG003 appeared to dramatically alter the splicing of *FGFR1OP*, but did not affect differential splicing of any other genes suggests that the inhibitor was working due to the selective nature of the effects. *FGFR1OP* could therefore also act as a positive control for future experiments.

As already mentioned in section 5.3, a positive control for SPHINX inhibition would allow confirmation that the SRPK1 inhibitor is working. Expression of the $VEGF_{165}$ splice variant has been shown to decrease with SPHINX treatment (Gammons *et al.,* 2013), and so future experiments should utilise a decrease in $VEGF_{165}$ mRNA expression as a positive control for SPHINX treatment. In light of the results obtained from the SRPK1 knockdown experiment, it can be suggested that SRPK1 does not affect alternative splicing of the cancer-associated genes studied in hypoxia. However, these results are based on n=1 and so must be treated with caution.

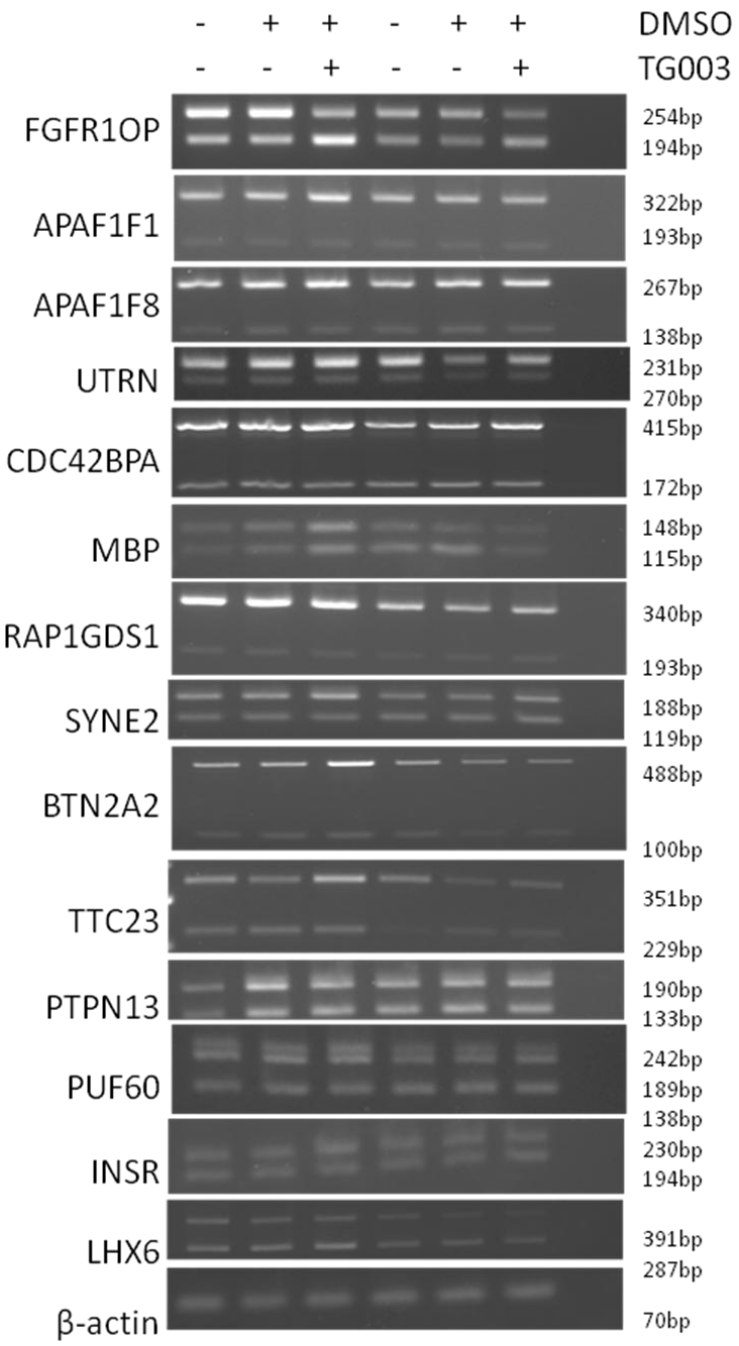


Figure 5.7: Effect of chemical inhibition of CLK1 using TG003 on the alternative splicing of cancer-associated genes. Representative PCR gels showing the effect of normoxia, hypoxia and 10μM TG003 on the expression of splice isoforms of cancer-associated genes. TG003 was dissolved into DMSO and so DMSO was also used as a control. For *BTN2A2, FGFR1OP, PTPN13, RAP1GDS1, SYNE2, TTC23, UTRN, INSR, CDC42BPA, MBP* and *LHX6,* n=3. For *APAF1F1, APAF1F8 and PUF60,* n=2. Hypoxic conditions were for 48h at 1% oxygen in a sealed MIC-101 hypoxia chamber at 37°C.

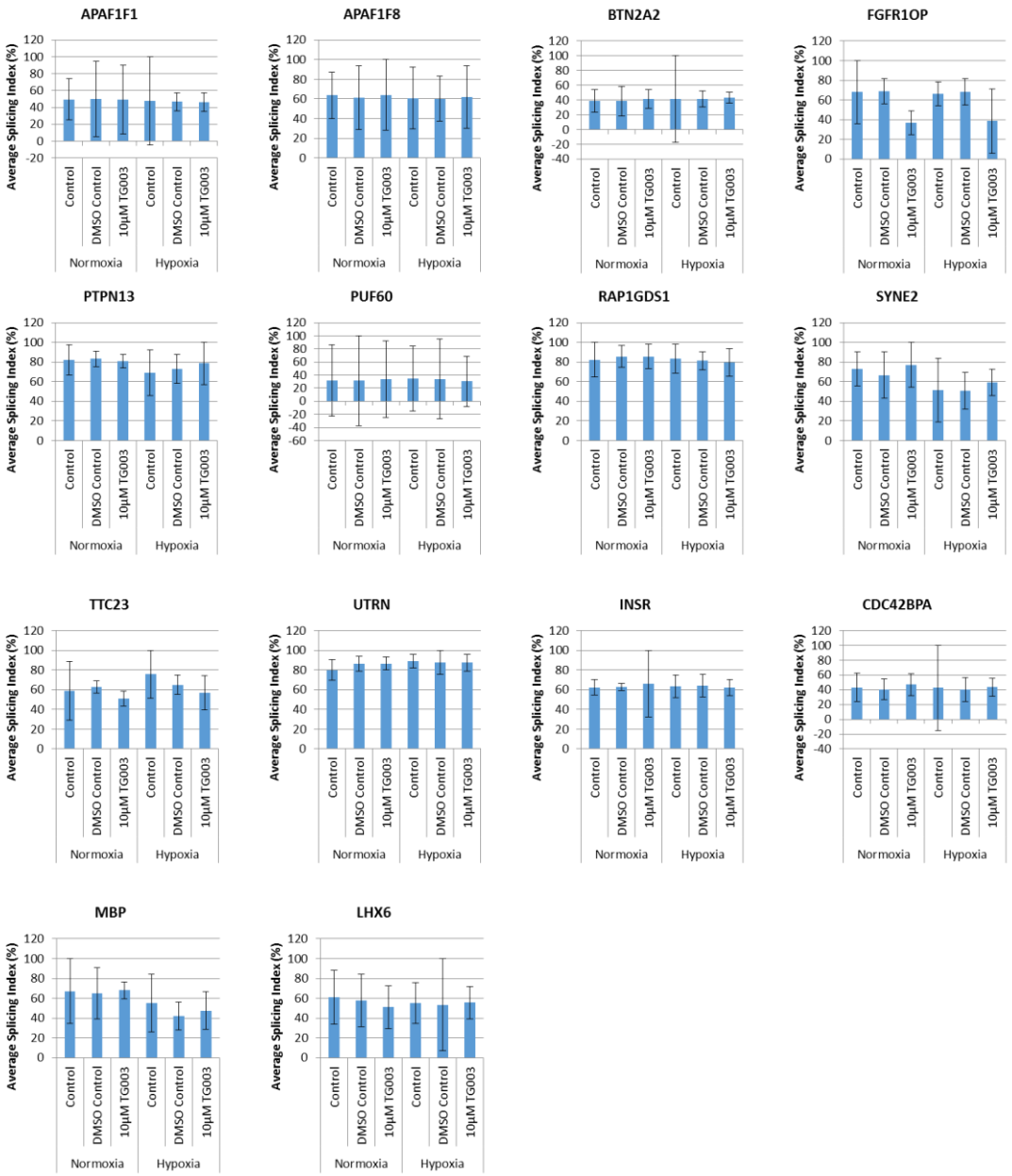


Figure 5.8: Effect of TG003 on percentage exon inclusion (%) in cancer-associated genes. DMSO was used as a vehicle control. Calculations for *BTN2A2, FGFR1OP, PTPN13, RAP1GDS1, SYNE2, TTC23, UTRN, INSR, CDC42BPA, MBP* and *LHX6* were based on n=3. For *APAF1F1, APAF1F8 and PUF60,* n=2. Where n=3, Shapiro-Wilks and Bartlett's tests were applied to the data. If the data was normally distributed and homoschedastic, a one-way ANOVA was applied to the data to determine statistical significance. Ideally, a Dunn's test would be applied to the data if it was found to be not normally distributed and / or heteroschedastic. *FGFR1OP* was the only gene with n=3 where this applied. However, there were not enough experimental repeats to conduct a Dunn's test and so the statistical significance of the *FGFR1OP* results are unknown. Hypoxic conditions were for 48h at 1% oxygen in a sealed MIC-101 hypoxia chamber at 37°C.

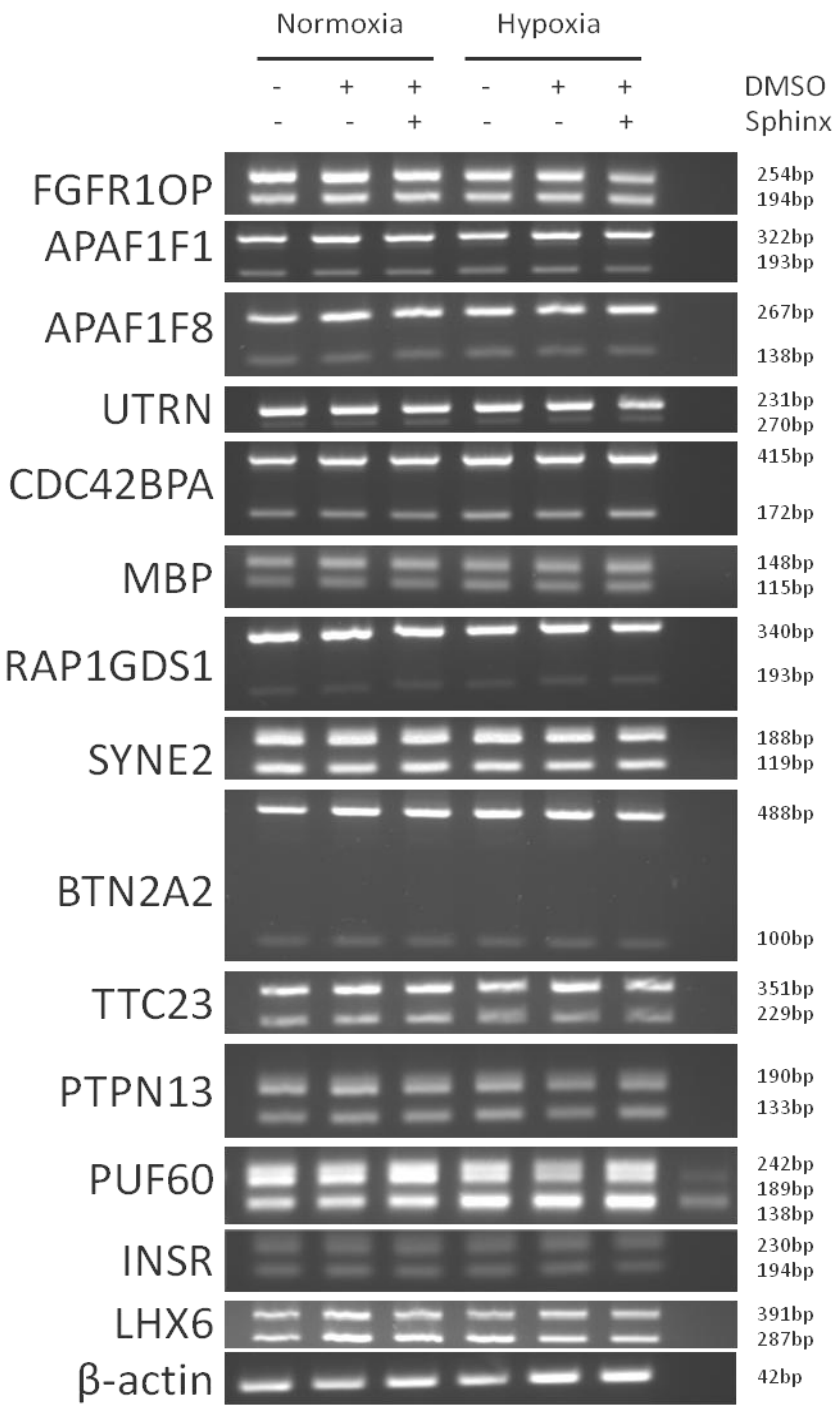


Figure 5.9: Effect of chemical inhibition of SRPK1 using SPHINX on the alternative splicing of cancer-associated genes. Representative PCR gels showing normoxia, hypoxia and 10 μM SPHINX on the expression of splice isoforms of cancer-associated genes. SPHINX was dissolved in DMSO and so DMSO was also used as a control. β-actin was used as a loading control. Hypoxic conditions were for 48h at 1% oxygen in a sealed MIC-101 hypoxia chamber at 37°C, n=2.

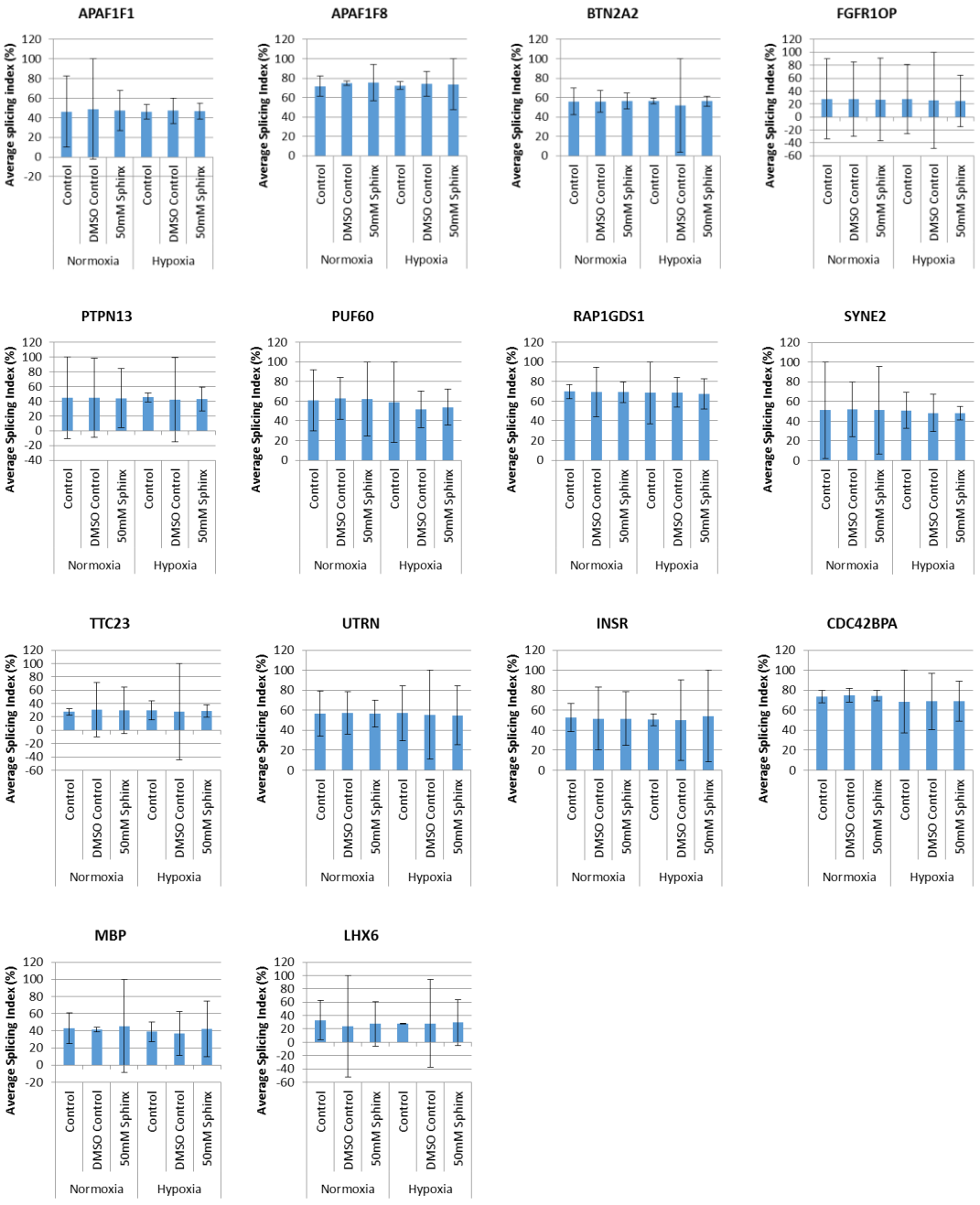


Figure 5.10: Effect of SPHINX on the average % change in exon inclusion of cancer-associated genes. DMSO was used as a vehicle control. β-actin was used as a loading control. Hypoxic conditions were for 48h at 1% oxygen in a sealed MIC-101 hypoxia chamber at 37°C. As averages were based on n=2, statistical tests could not be applied to the data.

5.6 Discussion

5.6.1 PRPF8 and SAFB1 splice factors regulate *CA IX* splicing

A library of 57 splice factor knockdowns identified pre-mRNA-processing-splicing factor 8 (PRPF8) and Scaffold-attachment-factor B1 (SAFB1) as splice factors that potentially help regulate the alternative splicing of *carbonic anhydrase 9* (*CA IX*) (Figure 5.1). CA IX is a newly established hypoxia marker that is involved in cellular invasion, migration and metastasis (Svastova and Pastorekova, 2013). The PRPF8 knockdown was found to induce some expression of the truncated shorter version of *CA IX*; however the *CA IX-FL* isoform was still the more predominantly expressed splice isoform. In contrast, the lipofectamine control for PRPF8 (which expresses PRPF8) favoured the expression of *CA IX-FL.* These results suggest that PRPF8 favours the expression of the *CA IX-FL* isoform, which is the isoform that is induced during hypoxia.

PRPF8 forms part of the catalytic core of the spliceosome and is therefore heavily implicated in a number of events during the splicing mechanism. PRPF8 is found to form part of the U5 snRNP and U5.U4/U6 snRNP complexes, and is present during the two transesterification steps of splicing and also in the excised lariat complex (Grainger and Beggs, 2005). Furthermore, PRPF8 is shown to cross-link to the 5' and 3' splice sites respectively, and also to the branch point sequence, which indicates PRPF8 as a major player throughout the splicing mechanism. PRPF8 is the largest and most highly evolutionary conserved protein in the human genome, sharing 61%

homology with its yeast counterpart (Grainger and Beggs, 2005). This highlights the importance of this protein to life.

Kurtovic-Kozaric *et al* (2015) showed that PRPF8 mutations have a widespread effect on alternative splicing. In fact, the group reported that the alternative splicing of hundreds of genes are affected, and exon inclusion and exclusion events are both affected. The group observed that alternative splice sites were activated when PRPF8 was mutated or haploinsufficient, which heavily implicates PRPF8 in the regulation of alternative splicing. In addition, the study also provided evidence that PRPF8 lesions in myeloid malignancy resulted in increased cellular proliferation and abnormal spliceosomal activity, which produces a more aggressive phenotype. Furthermore, PRPF8 knockdowns by RNAi was shown to alter alternative splicing across 10-30% of 96 alternative splicing events, which further implicate the protein in alternative splicing regulation (Tanackovic *et al.,* 2011). Wichramasinghe *et al.* (2015) provided evidence that restricted PRPF8 expression correlates with the selection of weak 5' splice sites, which identifies a mechanism through which PRPF8 may alter alternative splicing. This suggests that weaker 5' splice sites in *CA IX* may be utilised when PRPF8 is knocked down, forming the alternatively spliced variant.

Unfortunately, follow-up experiments on PRPF8 proved to be problematic. As PRPF8 is a very large protein, it was possible that the protein had not transferred, and so the membrane was re-probed for a similar sized protein, VE-cadherin. A band of the required size for VE-cadherin was present on the blot, which suggests that the blot had transferred successfully. A possibility for the undetection of a protein is that the protein may be expressed at low levels or not at all; however,

this is highly unlikely, as PRPF8 is heavily involved in the spliceosome machinery. Therefore, it is suggested that the PRPF8 antibody did not work and there was insufficient time to test alternatives. However, the indication that PRPF8 might be involved is an intriguing new finding that warrants further investigation.

Scaffold attachment factor B1 (SAFB1) is a large ubiquitously expressed, multifunctional protein that is involved in a number of cellular process, such as chromatin organisation, transcriptional regulation, RNA splicing and the stress response (Garee and Oesterreich, 2010). This chapter provides evidence for SAFB1 in the regulation of *CA IX* splicing, which encompasses three of the above known functions of SAFB1.

The high-throughput SAFB1 siRNA knockdown was shown to favour the alternatively spliced truncated variant of *CA IX,* whereas the lipofectamine control promoted the expression of full-length *CA IX*. This suggests that during hypoxia, the increase in the full-length *CA IX* isoform could be due to a switch in splicing mediated at least in part by SAFB1. Most previous studies have identified SAFB1 as a repressor of promoter activity. Oesterreich *et al.* (1997) showed that SAFB1 repressed *heat shock protein 27* (*HSP27*) promoter activity, Lin *et al.*(2008) showed a negative regulation of *xanthine oxidoreductase* (*XOR*) by SAFB1, and Hammerich-Hill *et al*. (2010) provided evidence that SAFB1 repressed the expression of target genes involved in the immune system and apoptosis. However, Omura *et al.* (2009) showed that SAFB1 positively regulated the promoter of *sterol-regulatory-element-binding-protein* (*SREBP1c*), which plays a role in the regulation of lipogenesis in the

liver. This therefore demonstrates that SAFB1 can also activate promoter activity. There is also a possibility that SAFB1 may influence *CA IX* splicing indirectly.

SAFB1 has been identified as a hnRNP protein and has therefore been shown to bind to RNA and interact with RNA processing proteins (Weighardt, 1999; Garee and Osterreich, 2009). As previously stated in Chapter 1 of this thesis, hnRNP proteins bind to exonic and intronic splicing silencers, which promotes exon exclusion (reviewed in David and Manley, 2010) and so SAFB1 may either be an exception to this rule, or SAFB1 may negatively regulate another splice factor that is involved in the repression of the *CA IX-FL* promoter. In fact, a number of groups have shown that SAFB1 interacts with and represses transcription factors such as estrogen receptor alpha (ERα) and p53 via its C-terminal repression domain (Oesterreich *et al.,* 2000a, Oesterreich *et al.,* 2000b; Townson *et al.,* 2004; Peidis *et al.,* 2011).

Furthermore, a recent paper by Rivers *et al.* (2015) provided evidence that SAFB1 is directly implicated in the regulation of alternative splicing of a number of genes that have a role in neuronal function (*NCAM1, ANK3, ANKS1B, SAP97, ADD2, KIF16B* and *ELK3).* Therefore, it is speculated that SAFB1 may be directly involved in the regulation of alternative splicing of cancer-associated genes, in addition to *CA IX*. Using gene ontology (GO) analysis, the group also showed that SAFB1 is likely to regulate genes that have roles in controlling cellular responses to stress, which implies that SAFB1 may be involved in the hypoxic stress response; however further research is required to provide evidence for this hypothesis.

Rivers *et al.* (2015) also showed that SAFB1 knockdown increased the expression of the microRNA cluster miR-17-92, which when repressed has been found to function in hypoxia-induced apoptosis (Yan *et al.,* 2009). Moreover, over-expression of miR-17-92 was reported to inhibit hypoxia-induced apoptosis (Yan *et al.,* 2009). This suggests that SAFB1 may be involved in hypoxia-induced apoptosis through the regulation of miR-17-92. It is therefore suggested that the decrease in SAFB1 expression in hypoxia (Figure 5.4A) may serve to up-regulate miR-17-92 in order to inhibit hypoxia-induced apoptosis and ultimately promote the survival of hypoxic PC3 cells.

SAFB1 has been implicated to play a role in the stress response. Chiodi *et al.* (2000) provided evidence for the relocation of SAFB1 to stress-induced sub-nuclear bodies (SNBs) upon genotoxic treatment with cadmium sulphate or heat shock treatment. It has been suggested that SNBs may control post-transcriptional modification of RNA and regulate protein activity (Denegri *et al.,* 2001). After 48h hypoxia treatment, SAFB1 expression was found to decrease compared to the normoxic control (Figure 5.4A). The previous studies mentioned above have not provided evidence on whether SAFB1 protein expression increases or decreases with stress treatment and so it may be that this is a common feature for SAFB1 in all types of implicated cellular stress. Whether or not SAFB1 resides into SNBs during hypoxic stress remains to be elucidated.

A number of studies have identified SAFB1 as a tumour suppressor. SAFB1 over-expression in breast cancer cells resulted in a reduced cell number, whereas a SAFB1 knockdown resulted in an increase in cell growth (Townson *et al.,* 2000;

Garee and Osterreich, 2009). In addition, studies in mouse embryonic fibroblasts taken from SAFB1 germline knockout mice (SAFB1 $^{-/-}$) resulted in an increase in immortalsation and a lack of senescence (Dobrzycka *et al.,* 2006). The results provided in this chapter show that SAFB1 expression decreases during hypoxia (Figure 5.4A), which supports the proposal that hypoxia produces a more oncogenic phenotype. The finding that SAFB1 favours the splicing of the full-length *CA IX* isoform (Figure 5.1) is surprising as CA IX is implicated in tumour cell invasion, migration and metastasis (Svastova *et al.,* 2012; Radvak *et al.,* 2013; Sansone *et al.,* 2009). However, this mechanism may be due to a variety of reasons already explained and in addition, there has not been any research into the effect of SAFB1 on cellular invasion, migration and metastasis.

5.6.2 The effect of CLK1 and SRPK1 chemical inhibition on *CA IX* mRNA isoform and protein expression

TG003 and SPHINX were used to chemically inhibit splice factor kinases; CLK1 and SRPK1, respectively in order to see if there was an effect on *CA IX* expression. Jakubauskienne *et al.* (2015) have recently provided evidence that TG003 treatment decreased *CA IX-FL* mRNA expression. In addition, the group reported that CLK1 knockdowns caused a decrease in CA IX-FL expression at the mRNA and protein level. However, the results provided here suggest that TG003 inhibition of CLK1 does not have an effect on *CA IX* splicing at the mRNA or protein level, at least in the cell line models examined (Section 5.5). Jakubauskienne *et al.* (2015) used a

concentration of 10μM TG003 to treat cells in 1% oxygen, which is the same as the concentrations used in this chapter; however the group used a treatment time of 24h, which suggests that the potency of TG003 may diminish during a longer treatment time and therefore restore CLK1 phosphorylation to the natural level. The half-life of TG003 is not documented and so this would need to be investigated further. Jakubauskienne *et al.* (2015) utilised HeLa cells as a model, whereas our study was conducted in PC3 cells and so there is also the possibility that the effect of TG003 on *CA IX* isoform and protein expression is cell-line or tissue specific.

The group that developed TG003 recently published findings that TG003 itself is a general exon skipping enhancer (Sakuma, Lida and Hagiwara, 2015). This highlights that the results of chemical inhibition may not only be due to the inhibited function of the targeted protein, but other mechanisms may also be affected. SPHINX did not alter the mRNA expression of *CA IX* (Section 5.5) and there is no previous published evidence to show that SPHINX has any effect on *CA IX* expression. Therefore, it is suggested that SRPK1 is not involved in the regulation of *CA IX* splicing.

5.6.3 The effect of SAFB1, SRPK1 and CLK1 siRNA knockdowns on the alternative splicing of cancer-associated genes

siRNAs were used to knockdown SAFB1, SRPK1 and CLK1 proteins in order to assess the effect on the alternative splicing of the cancer-associated gene set from Chapter four. To confirm whether the knockdowns were successful, western

blotting was used to detect the expression of the proteins of interest in control and knockdown samples respectively. The SRPK1 knockdown was shown to be successful (Figure 5.6A). However, SAFB1 and CLK1 knockdowns were not confirmed. There are a number of reasons that could explain the inability to knockdown these two proteins.

SRPK1 and CLK1 knockdowns were the first knockdowns attempted in this study and were conducted at the same time. Optimisation of the CLK1 antibody proved to be problematic, as milk was used in the blocking reagent for CLK1 blots, which denatured the CLK1 protein producing blots with a number of bands that were not at the required size. However, it was found that using BSA in the blocking reagent produced a band of the required size for CLK1. This is because CLK1 is a phosphorylated protein, and milk contains a number of proteins which include the phosphorylated protein casein. This can lead to a higher background and non-specific binding to phosphorylated proteins present in the milk. Before this realisation, a number of attempts were made to detect the CLK1 knockdown, and therefore the CLK1 siRNA sample was depleted before correct CLK1 detection was seen.

An experimental error occurred when attempting a siRNA knockdown for SAFB1 and a subsequent CLK1 knockdown, which resulted in the depletion of an entire SAFB1 siRNA stock and CLK1 siRNA stock, respectively. After realising the mistake, a subsequent knockdown for the two proteins was attempted with the remaining three siRNAs. It is likely that these knockdowns were not successful as they were conducted without a full set of siRNAs. Another reason for the unsuccessful CLK1

and SAFB1 knockdowns could be due to a poor transfection (Elbashir *et al.,* 2002), or having a transfection time that is too long. Chen *et al.* (2007) reported a higher rate of siRNA knockdown success using rapid transfection in OCT-3/4 cells. The group reported a 90% success rate when transfecting the cells 4h after seeding compared to a 40% success rate with overnight seeding. However, as the SRPK1 siRNA knockdown was shown to be successful, and in light of the mistake outlined above, it is most likely that the unsuccessful CLK1 and SAFB1 siRNA knockdowns are due to the utilisation of an incomplete set of siRNAs.

Samples generated from the first siRNA experiment that aimed to knockdown SRPK1 and CLK1 were used to assess the effect of SRPK1 and CLK1 on the alternative splicing of the cancer-associated gene set. The SRPK1 knockdown was confirmed by western blot (Figure 5.6A), but there was no indication that the SRPK1 had an effect on alternative splicing. Although the CLK1 knockdown was conducted at the same time as the SRPK1 knockdown, it was not confirmed and therefore these results have to be taken with caution. There was strong evidence that CLK1 siRNA favoured the exon-skipped splice variants of the cytoskeletal proteins, *FGFR1OP* and *UTRN.* This therefore suggests that CLK1 may favour the full-length *FGFR1OP* and *UTRN* splice isoforms. *FGFR1OP* is implicated in ciliogenesis and has been found to be overexpressed in lung cancer, with evidence of a role in cellular motility, growth and progression (Lee and Stearns, 2013; Mano *et al.,* 2007). The function of the shorter exon-skipped variant has not yet been identified, but it is postulated that the shorter variant may regulate the function of the full-length variant (Popovici *et al.,* 1999). Full-length UTRN protein has been deemed a tumour

suppressor and is involved in maintenance of the cytoskeleton. The smaller isoform encodes a truncated protein that has been detected in neuroblastoma (Li *et al.,* 2007). *FGFR1OP* and *UTRN* both switch splicing to favour the smaller isoform during hypoxia. As Chapter 4 showed that CLK1 increases in mRNA and protein expression during hypoxia, it is suggested that perhaps CLK1 activity is inhibited during hypoxia.

5.6.4 The effect of SRPK1 and CLK1 chemical inhibition on the alternative splicing of cancer-associated genes

To investigate the effect of SRPK1 and CLK1 on alternative splicing further, SPHINX and TG003 were used to help confirm whether the two splice factors are involved in the alternative splicing of the cancer-associated gene set. It was found that SPHINX did not alter the alternative splicing of any of the genes examined. During hypoxia, the HIF complex has been shown to transcribe the Vascular Endothelial Growth Factor (VEGF) protein, which increases angiogenesis allowing the transport of nutrients and oxygen to the hypoxic area. SRPK1 has been found to favour the $VEGF_{165}$ isoform, which is pro-angiogenic and reduction in SRPK1 activity through knockdown and inhibition, have been shown to promote expression of the anti-angiogenic $VEGF_{165}b$ isoform in melanoma and colon carcinomas (Gammons *et al.,* 2014; Amin *et al.,* 2011; Oltean *et al.,* 2012). Furthermore, Mavrou *et al.* (2014) provided evidence for the regulation of angiogenesis and tumour growth by SRPK1-mediated control of *VEGF* splice isoform expression in prostate cancer. Therefore, it

was surprising that a reduction in SRPK1 activity through the siRNA knockdown and SPHINX inhibition of SRPK1 phosphorylation activity did not alter the splicing of any of the cancer-associated genes studied. In accordance with this, results in Chapter four showed that SRPK1 was found to increase in mRNA expression but not at the protein level after 48h hypoxia treatment. Therefore, these results do not provide any evidence that SRPK1 expression is elevated during hypoxia, and it is proposed that SRPK1 is not required in the hypoxic response. It may be that *VEGF* alternative splicing is regulated through a HIF induced mechanism which does not contain SRPK1 or that SRPK1 is required at a much earlier stage of hypoxia stress. There is also a possibility that SRPK1 could alter the splicing of other cancer-associated genes in other tissues during hypoxia, or that SRPK1 is post-translationally modified in hypoxia, such as through Akt-mediated phosphorylation (see Section 4.5.5); however further investigation is required to determine this.

TG003 was used to provide evidence on whether CLK1 had an effect on the splicing of cancer-associated genes during hypoxia. CLK1 is a known splice factor kinase that regulates the activity and localisation of splice factors, such as SRSF1 through hyperphosphorylation (Colwill *et al.,* 1996; Duncan *et al.,* 1997). Therefore, changes in CLK1 activity can indirectly alter alternative splicing.

PCR gel images showed that TG003 treatment switched the splicing of *FGFR1OP* to favour the smaller isoform. This echos the effect of the CLK1 siRNA treatment on the alternative splicing of *FGFR1OP*, and suggests that CLK1 may be inhibited during hypoxia. Statistical analysis of the results revealed that there was no significant difference between data sets; however, this was based on three experimental

repeats using a semi-quantitative method and therefore it is hoped that with a greater number of repeats, the results would be deemed significant.

In addition to CLK1, TG003 is also shown to inhibit CLK4, which is closely related to CLK1 (Ninomiya, Kataoka and Hagiwara, 2011). At this current time, there are no alternative inhibitors of CLK1 that offer a higher specificity. A further CLK1 knockdown experiment and an additional CLK4 knockdown experiment would confirm whether the change of *FGFR1OP* splicing during hypoxia is dependent on CLK1 and/or CLK4 activity. Despite these caveats and the need for further experiments, it seems clear that CLK1 has a prominent role in determining alternative splicing changes in hypoxia.

6.0 DISCUSSION AND FUTURE WORK

6.1 Technical issues associated with hypoxia treatments

It is important that both the normoxic and hypoxic conditions for experimental treatment are relevant to physiological conditions. In this section, the relevance of the normoxic and hypoxic conditions used in this study will be discussed. In addition, another form of hypoxia known as intermittent hypoxia will be explained.

6.1.1 Normoxia treatment

In the human body, oxygen concentrations vary in healthy tissues but range between 1 to 12%, which is much lower than the 21% oxygen concentration in the atmosphere (Bates, 2012). Therefore it is suggested that control samples regarded as 'normoxic' which are incubated in atmospheric oxygen for the duration of treatment, are actually in a state of hyperoxia. This is likely to have an effect on cellular physiology and behaviour. For example, Parrinello and Samper (2003) showed that culturing mouse embryonic fibroblasts (MEFs) in 3% oxygen averted cellular senescence that usually occurs after 28 days in 20% oxygen. Furthermore, a number of research groups have provided evidence that culturing cells in a lower oxygen concentration to that of atmospheric oxygen provide cells with more suited conditions including; increased cell growth and life span; reduced senescence and DNA damage; and most relevant to this thesis, reduced cellular stress (Richter *et al.,*

1972; Packer *et al.,* 1977; Parrinello and Samper, 2003; Atkuri *et al.,* 2007; Bates, 2012).

Most published hypoxia research has been conducted using a normoxic control that has been incubated at atmospheric oxygen, which allows for comparisons between studies but also may provide some incorrect findings. Therefore it is proposed that not only is it vital for future hypoxia studies to incubate normoxic control samples at the desired oxygen concentration required by the cell type, but also that any research involving cell culture should also adhere to the oxygen needs of the cell type used.

6.1.2 Issue of hypoxia treatment using the modular incubator chamber (MIC-101)

As discussed in section 2.5 there were concerns with using a MIC-101 hypoxia chamber induce hypoxic stress. The main issues being with the diminishing oxygen concentration and accumulation of carbon dioxide during the experiment due to metabolising cells. However, it can be argued that the MIC-101 chamber may create a more physiologically viable hypoxic system as hypoxic areas of a tumour will contain a reduced blood supply which will lead to an accumulation of carbon dioxide and a steady rate of reduced oxygen concentration. Furthermore, as there have been scientific publications which have utilised the MIC-101 chamber for hypoxia experiments (Buras *et al.,* 2000; Ezashi, Das and Roberts, 2005; Li *et al.,* 2007) it can be deduced that the MIC-101 hypoxia chamber is accepted in the scientific community for the initiation of hypoxia.

6.1.3 Intermittent hypoxia

The experiments used throughout this thesis have used 48h continuous hypoxia treatment to mimic a chronic hypoxic environment. Chronic hypoxia is defined as a limitation in the diffusion of oxygen (Brown 1990). Oxygen is able to diffuse 100-150 μm into normal and malignant tissues from the blood vessels, and above this distance cells become hypoxic (Brown, 1990). Intermittent hypoxia is another form of hypoxia that is caused by a fluctuation in blood flow which can produce short cycling periods of hypoxia and re-oxygenation (Brown, 1990; Chaplin, Durand and Olive, 1986; Chaplin, Olive and Durand, 1987). Blood vessels found in tumours are usually tortuous with excessive branching and many dead ends, which can cause irregular blood flow (Bergers and Benjamin, 2003). In addition, pressure from the ever-growing tumour mass can compress the tumour vessels and also impede blood flow (Morikawa *et al.,* 2002; Padera *et al.,* 2004; Baudele *et al.,* 2006). Studies in different tumour types have shown that the duration of the blood flow fluctuations can vary between several minutes to more than 1 hour (Chaplin and Hill, 1995; Bennewith and Durand, 2004; Braun, Lanzen and Dewhirst, 1999; Hill *et al.,* 1996; Pigott *et al.,* 1996; Carins, Kalliomaki and Hill, 2001; Cardenas-Navia *et al.,* 2004). Like chronic hypoxia, intermittent hypoxia can also induce HIF-1α stabilisation and transcriptional activity (Toffoli and Michiels, 2008).

There is evidence to show that the pathways that regulate HIF-1 activity during chronic and intermittent hypoxia are different, with the requirement of phosphoinositide-3-kinase (PI3K) and extracellular signal-related kinase 1/2 (ERK1/2) for HIF-1 function during chronic hypoxia; and protein kinase A (PKA) for

HIF-1 activity during intermittent hypoxia (Toffoli and Michiels, 2008). Although both intermittent and chronic hypoxia share some similarities, the fact that both types of hypoxia activate HIF-1α through different pathways suggests that other genes may also be transcribed. It would be therefore worthwhile to examine the effect of intermittent hypoxia on the alternative splicing of cancer-associated genes, such as the ones investigated in this thesis (see Sections 3.5 and 3.6).

6.1.4 HIF-1α regulation by reactive oxygen species (ROS)

A number of groups have shown that mitochondrial reactive oxygen species (mROS) generated from complex III in the electron transport chain play a role in the stabilisation of the HIF-α subunit (Chandel *et al.,* 2000; Guzy *et al.,* 2005; Brunelle *et al.,* 2005; Bell *et al.,* 2007). The finding of the involvement of mROS in the stabilisation of HIF-α is currently fuelling research for further investigation, and although the exact mechanism is not yet determined, there is evidence that mROS inhibit PHD2 which therefore prevents the degradation of the HIF-α subunit (Bell *et al.*, 2007). Furthermore, reactive oxygen species (ROS) have been shown to be generated during the re-oxygenation periods of intermittent hypoxia (Toffoli and Michiels, 2009), which can also stabilise HIF-α, and can regulate transcription factors, such as activator protein-1 (AP-1) and nuclear factor kappa-B (NFκB) which are discussed below.

Yuan *et al.* (2004) provided evidence that AP-1 activation is correlated with ROS production during intermittent hypoxia in PC12 cells. The group showed that

intermittent hypoxia activates *c-fos,* which is a constituent of the AP-1 transcription factor complex and this in turn increased expression of a downstream AP-1 regulated gene, *tyrosine hydroxylase* (*TH*). In addition, the group showed intermittent hypoxia decreased aconitase activity and increased H_2O_2 production, which provided evidence that ROS levels were increased. Finally the group showed that AP-1 activation was regulated by ROS in intermittent hypoxia using an $O_2.^-$ scavenger, Manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP), which prevented activation of the AP-1 complex. AP-1 is involved in the regulation of the cell cycle; down-regulation of tumour suppressor genes; and tumour promotion, transformation, progression and invasion (Matthews, Colburn and Young, 2007), which are all pathways associated with the pathogenesis of cancer.

NFκB induces the transcription of genes involved in apoptosis inhibition, proliferation, angiogenesis and the degradation of the extracellular matrix (Bond *et al.,* 1998; Guttridge *et al.,* 1999; Huang *et al.,* 2000; Karin *et al.,* 2002). In resting cells, the N-terminus of NFκB is bound to I-kappa-B (IKB), which masks the nuclear localisation signal keeping NFκB in the cytoplasm (Hayden and Ghosh, 2004). However, upon stimulation by ROS for example (Mohan and Meltz, 1994), upstream kinases such as the I-kappa-B-kinase (IKK) become activated and phosphorylate IKB, which cause ubiquitination and subsequent proteosomal degradation of the protein (Wu *et al.,* 2013). This reveals the nuclear localisation signal in the NFκB protein, which leads to the translocation of the protein into the

nucleus where it binds to specific sequences in the promoter regions of NFκB targeted genes (Hayden and Ghosh, 2004).

The HIF pathway has been implicated in the control of mechanisms that produce ROS (Görlach, 2003), which suggests that the HIF pathway is also involved in other systems in addition to the hypoxic response. Indeed, Rabbani *et al.* (2010) provided evidence in irradiated Fischer 344 rat lungs that expression of hypoxia markers (HIF-1α, CA IX and pimonidazole) correlated with the expression of markers for oxidative stress (8-OHdG); angiogenesis (VEGF and CD 105); and inflammation (ED-1, NFκB and TGFβ1). In addition, using the MnTMPyP O_2^- scavenger the group showed a decrease in HIF-1α expression and oxidative stress and concluded that the increase in HIF-1α expression and ROS during radiation-induced lung injury may be linked. As irradiation has been shown to affect HIF-1α expression, it would be worthwhile investigating whether the alternative splicing of the same cancer-associated genes examined in this thesis are also affected by irradiation, or whether the alternative splicing of other genes is affected.

6.2 Discussion of key findings

Hypoxia is defined as when the availability of oxygen is insufficient to meet tissue demand (Loboda and Dulak, 2012). Approximately 50-60% of human tumours contain hypoxic regions, which are difficult to treat with radio- and chemo-therapies; the presence of hypoxic regions is indicative of a poor prognosis (Bayer and Vaupel, 2012). Therefore, there has been much research into the physiology of

hypoxic tumours and attempts have been made to develop new methods to treat them.

Hypoxia has been shown to affect the alternative splicing of genes involved in the various hallmarks of cancer (Weigand *et al.,* 2012; Sena *et al.,* 2014; Han *et al.,* 2017). For example, *vascular endothelial growth factor (VEGF)* and *carbonic anhydrase 9 (CA IX)* are involved in angiogenesis and cellular migration and invasion, respectively (Hanahan and Weinberg, 2000, 2011; Ladomery, Harper and Bates, 2007; Barathova *et al.,* 2008). Therefore, this thesis was aimed first and foremost to determine whether or not the alternative splicing of other cancer-associated genes was affected during hypoxia.

6.2.1 Effect of hypoxia on the alternative splicing of cancer-associated genes

The results from this thesis show that alternative splicing of the apoptotic-associated genes; *caspase-9, Bcl-X* and *survivin* was altered during hypoxia (Section 3.4). The protein encoded by the full length isoform of caspase-9, *caspase-9a* is involved in the apoptotic response and activated upon DNA damage. The smaller isoform, *caspase-9b* does not have any catalytic activity but is able to inhibit caspase-9 activity and is therefore classified as an anti-apoptotic isoform (Seol and Billiar, 1999; Srinivasula *et al.,* 1999). Caspase-9 expression was shown to increase during hypoxia in cerebral cortical tissue of newborn piglets that were exposed to 5-7% oxygen for 1 hour; however, the isoforms involved were not investigated (Khurana *et al.,* 2002). This thesis provides evidence that hypoxia favours the pro-

apoptotic full-length *caspase-9a* splice isoform during hypoxia in the PC3 cell line, indicating that apoptosis is induced through the manipulation of caspase-9 alternative splicing.

Bcl-xL prevents mitochondria from releasing cytochrome *c,* which is needed to form the apoptosome for the initiation of apoptosis and is therefore classed as an anti-apoptotic isoform (Kim, 2005). The shorter alternatively spliced isoform, *Bcl-xS* lacks two of four Bcl-2 homology domains (BHDs) (Boise *et al.,* 1993). Members of the Bcl-2 protein family share one or more BHDs, which are required for protein function. The *Bcl-xS* splice variant is suggested to encode a pro-apoptotic protein that inhibits Bcl-2 and Bcl-xL function (Boise *et al.,* 1993; Minn *et al.,* 1996). Hypoxia was shown to significantly favour the longer *Bcl-xL* isoform in the PNT2 cell line, although this was a marginal increase (Figure 3.5). Shimizu *et al.* (1996) showed that hypoxia favours the anti-apoptotic *Bcl-xL* isoform in PC-12 cells potentially preventing the cell from undergoing apoptosis. Therefore, it is suggested that hypoxia favours the anti-apoptotic *Bcl-xL* splice variant; however, the nature of Bcl-x alternative splicing may be cell line specific.

Survivin drives mitosis at the G2/M check-point which promotes cellular proliferation (Ito *et al.,* 2000). The *survivin* isoforms studied in this thesis (*Full-length survivin, survivin-ΔEX3* and *survivin-2B*) all possess anti-apoptotic properties; however *survivin-2B,* which contains a cryptic exon with intron 2, has been reported to have markedly reduced anti-apoptotic ability when compared to the other two splice isoforms (Mahotka *et al.,* 1999). Hypoxia decreased the expression of all three *survivin* splice isoforms in the PC3 cell line; however, the ratio of the full-

length wild-type isoform was found to increase when compared to the other two splice isoforms (Figure 3.4). Therefore, it is suggested that hypoxia tumour cells that evade apoptosis may do so through altering the alternative splicing of *survivin* to favour the full-length splice isoform. Furthermore, Peng *et al.* (2006) showed that HIF-1α increases survivin expression and reduces apoptosis, which suggests that survivin is involved in the HIF-1 hypoxic response. Moreover, Zhu *et al.* (2001) provided evidence that *survivin* expression is linked to the formation of blood vessels after hypoxic preconditioning. This group showed that a period of hypoxia followed by re-oxygenation, known as hypoxic preconditioning, increased survivin expression in a NFκB and PI3 kinase dependent mechanism. This was shown using antagonists of the nuclear transcription factor, NFκB (SN50) and the PI3 kinase (LY294002). Furthermore, the same inhibitors decreased blood vessel formation suggesting that there is a link between the PI3-kinase/Akt/ NFκB /survivin pathway and blood vessel construction during hypoxia. Additionally survivin has been shown to inhibit caspase-9 activity (Chandele *et al.* 2004; Dohi *et al.,* 2004), which may offer a further mechanism in which cells may evade apoptosis during hypoxia despite the increase observed in the pro-apoptotic *caspase-9a* splice variant in this thesis. Therefore, in light of the above it is suggested that cancer cells that are able to survive in hypoxic conditions may have an elevated level of survivin, and there is evidence provided from this thesis that the full-length anti-apoptotic splice variant of *survivin* may be elevated in particular. It is suggested that during normal hypoxia, cells undergo splicing changes that favour apoptosis, such as those seen in *caspase-*

9; however, some tumour cells may subvert the pro-apoptotic splicing changes and instead favour anti-apoptotic splice isoforms.

High-throughput PCR of cassette exon inclusion in cancer-associated genes in hypoxic PC3 cells revealed a number of cancer-associated genes that changed splicing during hypoxia. The expression of oncogenic isoforms of *APAF1, BTN2A2,* and *RAP1GDS1*, which are involved in apoptosis, immune tolerance and proliferation respectively, were favoured by hypoxia treatment (Figures 3.7, 3.17 and 3.9). In contrast, hypoxia was found to promote the expression of the anti-cancer smaller isoform of *PUF60* that encodes the FIR protein (Figure 3.16). FIR is a transcriptional repressor of the oncogene, *c-Myc*.

In addition, hypoxia was found to change the alternative splicing of some cancer-associated genes to favour the expression of isoforms that have not yet been assigned a function; *SYNE2, UTRN, FGFR1OP, CDC42BPA, MBP, TTC23 and PTPN13* (Table 3.3). The first four of these genes are involved in the shape and maintenance of the cytoskeleton. Furthermore, *carbonic anhydrase 9 (CA IX)* is an established alternatively spliced hypoxia marker that has been implicated in cellular migration and invasion by a number of groups (Svastova *et al.,* 2012; Radvak *et al.,* 2013; Sansone *et al.,* 2009) and was confirmed in this study to increase in hypoxia (Section 3.2). It is therefore proposed that through changes in alternative splicing, hypoxia may regulate cellular motility and invasion and encourage cells to migrate to areas where there is a greater availability of nutrients, and where the environment is less acidic.

6.2.2 Splice factors and splice factor kinases implicated in the hypoxic response

Splice factors are instrumental in the regulation of alternative splicing and splice factor kinases are able to affect the activity of splice factors. Therefore, the mRNA expression of a number of splice factors and splice factor kinases was examined (see Section 4.3). There was evidence that the following splice factors and splice factor kinases increase in mRNA expression during hypoxia; *SRSF1, SRSF2, SRSF3, SAM68, HuR, HnRNP A1, CLK1* and *SRPK1*. In addition, it was shown by western blot that CLK1 and SRSF1 protein expression increased during hypoxic stress (Figure 4.3A, B and C). This implies that a number of splice factors and kinases may be involved in the cellular response to hypoxia. The effect of splice factor kinases on alternative splicing was therefore investigated further using siRNA knockdowns and chemical inhibitors: SPHINX for SRPK1 inhibition; TG003 for CLK1 inhibition.

There was no indication of a change in splicing of any of the cancer-associated genes with SRPK1 knockdown or inhibition (Figures 5.6 and 5.8). It must be noted that SPHINX inhibition results were based on n=2, and so further experimental repeats need to be conducted in order to assess the statistical significance of SRPK1 inhibition with SPHINX treatment on alternative splicing of the genes studied. However, SRPK1 protein expression was also unaffected by hypoxia (Figure 4.3D), which suggests that SRPK1 does not play a role in the hypoxic response in the PC3 cell line.

Although not found to be significant, the effect of CLK1 siRNA knockdown and inhibition provided an indication at the PCR gel level that CLK1 was involved in the

regulation of splicing of *FGFR1OP* (Sections 5.4 and 5.5). However, there was a suggestion that CLK1 knockdown and inhibition favoured the smaller splice variant of *FRFG1OP*, which implies that CLK1 favours the full-length isoform. This is a surprising result, as hypoxia was found to promote the shorter *FGFR1OP* splice variant and suggests that CLK1 activity is diminished during hypoxia. However, as already mentioned, CLK1 expression is increased during hypoxia (Figure 4.3). TG003 is also reported to inhibit CLK4 (Ninomiya, Kataoka and Hagiwara, 2011), and therefore it is possible that the change in *FGFR1OP* alternative splicing shown with TG003 treatment may be due to an inhibition of CLK4 activity.

CLK1 was found to be localised outside of the nucleus (Section 4.4), despite previous research showing that CLK1 is a nuclear protein (Aubol *et al.,* 2013). It is possible that CLK1 may have a cytoplasmic role during hypoxia which is not yet documented. Another explanation for this unexpected result is that CLK1 may be removed from the nucleus in order to reduce its effects there, whilst also being in ready supply once required again. The fact that the alternative splicing pattern of *FGFR1OP* with TG003 treatment (Section 5.3) mirrors the effect of hypoxia on *FGFR1OP* splicing (Section 3.6) suggests that CLK1 activity is inhibited during hypoxia. The relocation of CLK1 away from the nucleus may be one method in which the cell achieves this.

6.2.3 Regulation of *CA IX* splicing

CA IX is an established hypoxia marker that is linked to tumour cellular migration and invasion through its acid-base regulatory function (Svastova *et al.,* 2012; Radvak *et al.,* 2013; Sansone *et al.,* 2009). *CA IX* is itself alternatively spliced, which produces a variant that skips exons 8 and 9 and encodes for a truncated protein. The full-length isoform encodes a fully functional CA IX protein and is increased during hypoxia (Malentacci *et al.,* 2009).

Jakubauskienne *et al.* (2015) provided evidence in HeLa cells that TG003 treatment decreased *CA IX-FL* expression, which suggests that CLK1 or CLK4 may regulate *CA IX* splicing. However, an investigation into the effect of TG003 treatment on *CA IX* splicing in PC3 cells has not shown any changes in *CA IX* isoform expression (see Section 5.3). Jakubauskienne *et al.* (2015) used a treatment time of 24h compared to a 48h treatment time in the PC3 cells and so it is suggested that perhaps the effect of TG003 on the splicing of *CA IX* becomes diminished during longer treatment times. In addition, two different cell lines were used; HeLa (cervical cancer cell line) and PC3 (prostate cancer cell line), and therefore it may be that this effect is tissue or cell line specific.

Therefore, an investigation into the splice factors that may control splicing of *CA IX* in PC3 cells was investigated and there was evidence to show that SAFB1 and PRPF8 splice factors promote expression of the full-length CA IX isoform (see Section 5.2). Further investigation provided evidence that SAFB1 protein expression decreased after 48h hypoxia (Section 5.2). Rivers *et al.* (2015) showed that knockdown of

SAFB1 expression increased the expression of the microRNA cluster miR-17-92, the over-expression of which has been shown to inhibit hypoxia-induced apoptosis (Yan *et al.,* 2009). Therefore, a decrease in SAFB1 expression would favour miR-17-92 mediated inhibition of hypoxia-induced apoptosis. This shows that although a decrease in SAFB1 expression during hypoxia would favour the expression of the alternative spliced variant of *CA IX*, down-regulation of SAFB1 expression may be required for other mechanisms that are regulated by SAFB1. Furthermore, it is suggested that promotion of the FL splice variant of *CA IX* during hypoxia may be achieved through another mechanism, such as through regulation by PRPF8. However, this remains to be elucidated.

Furthermore, SAFB1 has been shown to interact with several splice factors, including SRSF1 (Denegri *et al.,* 2001), SAM68 (Sergeant *et al.,* 2007) and hnRNP A1 (Denegri *et al.,* 2002) which were all shown to increase in mRNA expression significantly during hypoxia (Section 4.3). Additionally, the protein expression of SRSF1 was also shown to increase in hypoxia (Section 4.3.2). SNBs may function to isolate splicing factors in order to down-regulate their expression and decrease normal cellular splicing in order to conserve energy for the processing of transcripts required by the stress response (Weighardt *et al.,* 1999; Biamonti, 2010). Perhaps SAFB1 expression is decreased during hypoxia in order to release the splice factors that are shown to interact with SAFB1 from SNBs as they may be needed by the hypoxic response. Further research is required to investigate whether the formation of SNBs is favoured or not by hypoxic stress.

6.3 Potential implications for therapy

Hypoxic solid tumours tend to be more aggressive and difficult to treat than those that have a sufficient supply of oxygen, which therefore results in a poor prognosis. There are a number of approaches that have been investigated to combat hypoxic tumours, which include; gene therapy (such as the use of short antisense oligonucleotides), hypoxia-activated prodrugs, and the targeting of the HIF pathway, as follows.

6.3.1 Splice switching antisense oligonucleotides (SSOs) that target hypoxia-induced isoforms

Short antisense oligonucleotides (ASOs) are synthetic molecules that are able to complementarily bind to specific pre-mRNA sequences through base-pairing. Splice switching antisense oligonucleotides (SSOs) are a type of ASO that bind to the pre-mRNA and block the binding of splice factors, which can disrupt the intended splicing of the pre-mRNA. SSOs can therefore be used therapeutically to switch splicing in order to favour splice isoforms that do not promote disease progression (Havens and Hastings, 2016). In addition, the nucleotides of SSOs are chemically modified to prevent the degradation of the pre-mRNA/SSO complex by the RNA-cleaving enzyme, RNase H (Summerton, 1999; Rigo, Seth and Bennett, 2014). This allows SSOs to alter the splicing of the pre-mRNA without provoking the degradation of the selected pre-mRNA (Havens and Hastings, 2016). However, SSOs are a novel discovery that are still under investigation as potential therapeutics.

SSOs targeted to treat Duchenne Muscular Dystrophy (DMD) and Spinal Muscular Atrophy (SMD) are currently in Phase 3 clinical trials (Mendell *et al.,* 2016; Hache *et al.,* 2016; Havens and Hastings, 2016) and highlight an important aspect of SSOs. As some genes are spliced to produce isoforms that result in a dampened response, some SSOs may only be able to provide a less harmful form of the disease. For example, the SSO for DMD favours the production of a partially functional protein that reduces the severity of DMD; however the SSO for SMD results in the promotion of the functional full-length *SMN* transcript. Therefore although SSOs may be beneficial forms of therapy in the future, they will not be able to cure all diseases (Havens and Hastings, 2016). Despite a lot of research into SSOs, clinical progress and utilisation has been hindered due to the cost of producing the molecules and challenges involving the delivery without toxicity to selected sites of treatment (Sridharan and Gogtay, 2016; Juliano, 2016).

Chemical modifications to SSOs, such as changes to the phosphate backbone and/or sugar unit have enabled improved binding affinity, stability and pharmacodynamic properties. A popular adjustment is the phosphorothiate (PS) backbone modification, which has improved stability and reduced binding affinities. Furthermore, renal clearance of the drug has been reduced as PS ASOs can bind to proteins in plasma, and so this allows broad distribution; however the risk of toxicity is also increased. (Eckstein, 2014; Geary, 2009). However, a problem with PS ASOs is that they are not resistant to RNAse H, and so without additional modifications, they can be degraded. RNAse H resistance can be achieved through modifications at the 2' sugar position; the most common of which are 2'-O-methyl

(2'-OME) and 2'-O-methoxyethyl (2'-MOE). Locked nucleic acid (LNA) is another version, which bridges the furanose ring and causes increased binding affinity, allowing for the use of shorter SSO sequences. This advantage of this is that there are less unwanted off-targets as a shorter sequence decreases the probability that the sequence will bind partially to the wrong site. Consequently, many ASOs containing a PS backbone and modifications at the 2' sugar position are currently being developed as potential therapeutics targeting a range of diseases, including; Hutchinson-Gilford progeria (Lee *et al.,* 2016); Usher syndrome (Lentz *et al.,* 2013); Alzheimer's disease (Hinrich *et al.,* 2016); spinal muscular atrophy (Hache *et al.,* 2016; Chiriboga *et al.,* 2016); Huntington disease (Disterer *et al.,* 2013), as well as others. A more detailed list can be found in the review paper by Havens and Hastings, (2016).

One of the most common type of SSO are the phophorodiamidate morpholinos (PMO), which are characterised by a neutral phosphorodiamidate backbone and the namesake morpholine ring in place of the furanose ring that is found in natural nucleic acids (Summerton, 1999). The neutral backbone offers both an advantage and disadvantage; they have a low binding of plasma proteins, but rapid clearance by the kidneys results in a low accumulation in tissues when compared to a PS backbone (Geary, 2009). Therefore, high doses of PMOs may be required for the desired response (Yokota *et al.,* 2009; Rigo *et al.,* 2014), and so further modifications have been developed to improve the effectiveness of PMOs. One such modification enhances cellular uptake through the addition of cell penetrating peptides or octaguanidine dendrimers (known as Vivo-morpholinos, VPMO) to the

2’ position of the sugar (Moulton and Jiang, 2009; Betts *et al.,* 2012; El Andaloussi *et al.,* 2012). PMOs are currently being developed for the following diseases; cardiomyopathy (Gramlich *et al.,* 2015); duchenne muscular dystrophy (Mendell *et al.,* 2015; Voit *et al.,* 2014); Nijmegen breakage syndrome (Salewsky *et al.,* 2016); Ebola (Panchal *et al.,* 2014); Pompe disease (Clayton *et al.,* 2014), to name a few.

Moreover, SSOs are currently being developed to combat cancers, including; a 2’MOE/PS SSO aimed at targeting an alternative 5’ splice site in exon 2 of the *BCL2L1* gene (151); a LNA targeted at skipping exon 26 of the *ERBB4* gene (152); A PMO aimed at skipping exon 6 of the *MDM4* gene (153). Furthermore, Shieh *et al.,* (2009) showed successful alterations in splicing of the *MCL-1* gene using a morpholino SSO targeted at exon 2 skipping in basal cell carcinoma cells and AGS cells (derived from human gastric adenocarcinoma epithelial cells). This promoted apoptosis, and has potential as a future therapeutic strategy for inducing apopotosis in cancer cells. In addition, Bauman *et al.,* (2010) used a 2’-O-MOE-phosphorothiate SSO to successfully switch splicing of the *BCL-x* gene to favour the *BCL-xs* pro-apoptotic splice variant in mouse melanoma cells. More recently, Wu, Mau and Ming (2016) have used a 2’-O-Me-phosphorothiate SSO to also successfully alter splicing of *BCL-x* to favour the pro-apoptotic *BCL-xs* splice isoform in primary human hematopoietic stem cells and hepatocytes with a view to reducing apoptosis in liver fibrosis.

It is hoped that in the future, SSOs may be generated against genes found to switch splicing during hypoxia to favour oncogenic isoforms, such as *Survivin, APAF1, RAP1GDS1* and *BTN2A2,* which were identified in this study. However, as the

alternative isoforms of *Survivin* and *RAP1GDS1* are less oncogenic, this type of therapy may only dampen the anti-oncogenic properties of these genes. Therefore, this type of therapy may be more useful during hypoxia for genes such as *APAF1* and *BTN2A2* where the isoforms are strictly pro- or anti-oncogenic. A schematic representation of how this might occur is shown in figure 6.1.

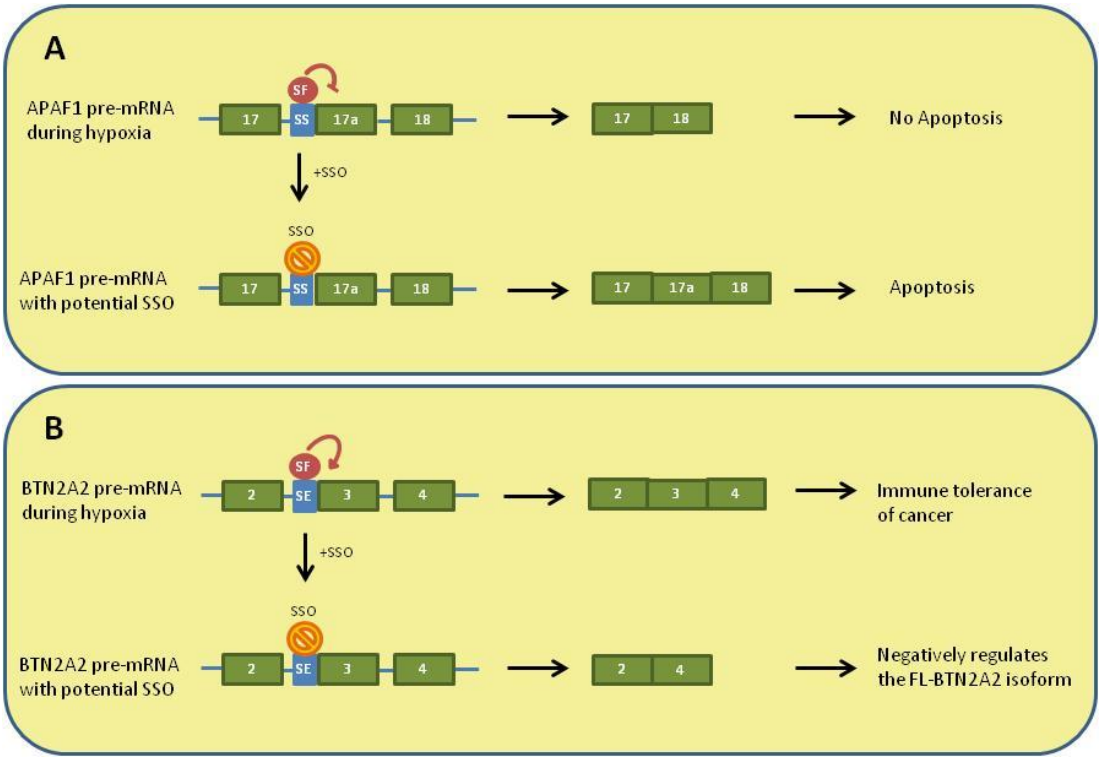


Figure 6.1: Schematic representation of potential splice switching oligonucleotide (SSO) targeting strategy to therapeutically switch splicing of (A) *APAF1* and (B) *BTN2A2* during hypoxia to favour pro-cancerous isoforms. Green boxes are exons and blue lines are introns. SS stands for splicing silencer and SE stands for splicing enhancer. The splice factor (SF) is prevented from binding to the SS or SE by the SSO. Whether or not the SS and SE is found in the exon or intron remains to be elucidated.

6.3.2 Hypoxia activated prodrugs (HAPs)

Hypoxia activated prodrugs (HAPs) are inactive under normoxic conditions, but become activated in hypoxic regions. This leads to a reduction in adverse effects in

the normoxic region (Ikeda *et al.,* 2016). Hypoxic tumour tissues overexpress a number of endogenous reductive enzymes, (such as NADPH-cytochrome P450 reductase, DT-disphorase, xanthine oxidase/xanthine dehydrogenase and cytochrome b_5 reductase), and therefore several HAPs have been designed to become active upon reduction (gain of electrons) (Chen and Hu, 2009). Reducing enzymes present in normoxic areas are also able to activate prodrugs; however, molecular oxygen is able to rapidly oxidise the prodrugs back to an inactive state (Chen and Hu 2009). As the concentration of oxygen is much lower in hypoxic areas, prodrugs are able to remain in an active state for longer and can therefore exert their effects.

A major issue found with HAPs is the delivery of the drug to the required region. As already discussed, the vasculature found in solid tumours is usually disordered and can cause many problems with drug administration. A further problem is found with targeting hypoxic areas, as the drug is required to be able to penetrate into these distant areas (Ikeda *et al.,* 2016). In addition to the obvious problem of increased distance to hypoxic regions, Minchinton and Tannock (2006) highlighted several other factors that can account for poor penetration of HAPs, which include administration of a limited dose due to the associated toxicity of the drug and physiochemical properties of the drug (e.g. high molecular weight or aqueous solubility). TH-302 is a HAP that has showed promising results for combating pancreatic and soft tissue sarcoma when used in combination with gemcitabine and doxorubicin, respectively; and is currently undergoing phase III clinical trials (Borad *et al.,* 2015; Chawla *et al,* 2014; Wigerup, Pahlman and Bexell, 2016). Cancer-

associated genes that are found to alter splicing patterns during hypoxia may in the future fuel the development of HAPs, which target those particular genes. The HAPs may be in the form of SSOs (as described above) which would allow the splicing pattern of a particular gene to be targeted, whilst also only becoming active in hypoxic areas. This would decrease non-specific targeting of the drug; however, this kind of therapy is in the distant future as much more research is required before this can occur.

6.3.3 Targeting of the HIF Pathway

The hypoxia inducible factor (HIF) pathway becomes activated during hypoxia and results in the transcription of genes required in the HIF response. Therefore as shown in the sections below, there has been research conducted by a number of groups into manipulating the HIF pathway to prevent the transcription of genes that may aid in cell survival in hypoxic tumours.

Inhibition of the PI3/AKT/mTOR pathway

Lee *et al.* (2015) have provided evidence that glyceollins can inhibit the PI3K/AKT/mTOR pathway under hypoxic conditions, which leads to the blocking of HIF-1α translation. Glyceollins are derived from the soy plant and are a biologically active type of phytoalexin (Graham and Graham, 1991). Phytoalexins are synthesised in plants in response to infection or stress and have been shown to

have anti-oncogenic properties (Darvill and Albersheim, 1984; Lee *et al.,* 2015). The PI3K/AKT/mTOR pathway is up-stream of the HIF pathway and therefore targeting of these molecules can indirectly inhibit HIF expression. Furthermore, a number of groups have provided evidence that mTOR inhibition decreases HIF-1α and HIF-2α expression during normoxia and hypoxia (Hudson *et al.,* 2002; Majumder *et al.,* 2004; Thomas *et al.,* 2006; Mohlin *et al.,* 2015).

Inhibition of HIF dimerisation

HIF subunits each contain two PER-ARNT-SIM (PAS) domains, designated PAS-A and PAS-B domains. During hypoxia, the HIF subunits dimerise to form the HIF complex, which then translocates into the nucleus for the initiation of transcription. The HIF PAS domains are required for heterodimer formation (Yang *et al.,* 2005). Inhibitors that target the PAS domains of the HIF subunits prevent the HIF complex from forming. An example of this is acriflavine, which is a potent inhibitor of the HIF-α subunit and prevents the assembly of HIF-1 and HIF-2 respectively (Lee *et al.,* 2009). Acriflavine treatment reduced tumour growth and vascularisation in prostate and xenograft models (Lee *et al.,* 2009).

HIF-2α Translational Inhibitors

Unbound iron can generate ROS such as $OH^{\cdot}$, which can cause oxidative stress to cells. Therefore, the amount of free iron must be kept as low as possible and this is

achieved through various mechanisms, such as; increased iron uptake and utilisation; and increased storage and export of iron (Cairo and Recalcati, 2007). Iron-regulatory proteins (IRPs) are involved in the control of these processes through interaction with iron-response elements (IREs) found in 5' and 3' untranslated regions (UTRs) of mRNAs (Ray, Huang and Tsuji, 2012). HIF-2α mRNA contains an iron-responsive element (IRE) in its 5' UTR end (Sanchez *et al.,* 2007). Binding of IRP1 to the HIF-2α IRE results in the repression of HIF-2α translation. During hypoxia, the binding of IRP1 to the HIF-2α IRE is repressed, which allows the translation of HIF-2α (Wigerup *et al.,* 2016). Zimmer *et al.* (2008) have identified compounds that promote IRP1 binding to the IRE of HIF-2α and suggest that these compounds may be able to repress HIF-2α translation.

Inhibition of HIF Translational Activity

The HIF complex has to bind to DNA in order to transcribe HIF target genes. Echinomycin is an established DNA intercalator that has been shown to inhibit the binding of HIF-1 to the VEGF HIF response element (HRE) resulting in hampered VEGF expression (Kong *et al.,* 2005). It has been shown that although during hypoxia echinomycin inhibits HIF-1α activity, echinomycin increases HIF-1 activity in normoxia, which in turn increases the expression of HIF-1 target genes (Vlaminck *et al.,* 2007). Therefore, more research needs to be completed on the drug before it is established as a therapy.

It is proposed that the above methods of targeting the HIF pathway may also be of use when investigating whether an alternative splicing event in a cancer-associated gene is HIF-dependent or –independent. Inhibitors of the PI3/AKT/mTOR pathway when used in conjunction with inhibitors of HIF dimerization and translational activity could provide insight into whether or not alternative splicing changes that occur during hypoxia in cancer-associated genes are HIF-dependent. In an instance where an alternative splicing change is not HIF-dependent, it can be deduced whether the PI3/AKT/mTOR pathway is involved but may exert alternative splicing effects through a different mechanism that does not include HIF. Furthermore, HIF-2α inhibitors may be used to assess whether HIF-2α is involved in the regulation of alternative splicing of cancer-associated genes.

6.4 Concluding remarks

Hypoxic tumours are indicative of a poor prognosis due to the increase in pro-survival proteins and the difficulty to treat hypoxic regions with current therapies. The hypoxic response is very complicated and encompasses many of the hallmarks of cancer (Ruan, Song and Ouyang, 2009; Hanahan and Weinberg, 2011). Ladomery (2013) proposed aberrant alternative splicing as another hallmark of cancer. Therefore, this thesis aimed to explore whether the hypoxic response exerted changes to the alternative splicing of cancer-associated genes and looked for potential clues into mechanisms that may be involved.

Evidence has been provided for changes in alternative splicing of apoptotic genes, *caspase-9, Bcl-x* and *survivin*; and in other cancer-associated genes, *APAF1, BTN2A2, CDC42BPA, FGFR1OP, MBP, PTPN13, PUF60, RAP1GDS1, TTC23* and *UTRN* which are implicated in various arms of the hallmarks of cancer (Figure 6.2). In addition, an increase in mRNA expression of splice factors (*SRSF1, SRSF2, SRSF3, SAM68, HuR* and *hnRNP A1*) and splice factor kinases (*CLK1* and *SRPK1*) during hypoxia were also shown, which implicates these splice factors and splice factors in the hypoxic response and may offer targets for future therapies.

In addition, the CLK1 splice factor kinase has been implicated in the regulation of *FGFR1OP* alternative splicing. TG003, a chemical inhibitor of CLK1 was used to show that the inhibition of CLK1 mimicked the effect of hypoxia on the alternative splicing of *FGRF1OP* and therefore it is thought that a down-regulation in CLK1 activity may be responsible for this effect. Therefore, it is suggested that CLK1 may be inhibited during hypoxia by an endogenous protein. This could explain how inhibition of CLK1 results in an alternative splicing pattern of *FGFR1OP* that mirrors the pattern found in hypoxia, despite an increase in CLK1 expression in hypoxia. TG003 also inhibits CLK4 activity, and so a CLK4 knockdown is required to deduce whether the change in *FGFR1OP* splicing under the influence of TG003 is due to CLK4 inhibition.

There is an indication that the PRPF8 and SAFB1 splice factors favour the *CA IX FL* isoform, which is an established marker for hypoxia. Further research revealed that SAFB1 expression is decreased in hypoxia, and it is thought that this may be due to the need of other factors for the hypoxic response that may be negatively regulated

by SAFB1. For example, a decrease in SAFB1 expression is proposed to elevate miR-17-92 expression, which is involved in the inhibition of hypoxia-induced apoptosis; however, this remains to be elucidated.

A number of potential therapies to combat hypoxic tumours have been discussed in this thesis. It is hoped that in the future splice switching oligonucleotides (SSOs) will be developed that are able revert the splicing of pro-oncogenic isoforms, such as *APAF-1, BTN2A2, RAP1GDS1* and *CA IX*, back to the non-oncogenic isoforms during hypoxic conditions. In addition, if it is possible for the proposed SSOs to only become active in hypoxic conditions, like hypoxia activated prodrugs (HAPs), this would provide a specific targeted therapy for hypoxic tumours. At the very least, this thesis has provided an insight into other mechanisms and uncovered factors that are involved in alternative splicing changes during hypoxia (Figure 6.2).

6.5 Future work

6.5.1 Investigate the effect of intermittent hypoxia on the alternative splicing of cancer-associated genes

As discussed in Section 6.1.3, Toffoli and Michiels (2008) provided evidence that chronic and intermittent hypoxia activate HIF-1α through different pathways. Their paper showed that PI3K and ERK1/2 are required for HIF-1α activation during chronic hypoxia, whilst PKA is involved in stimulating HIF-1α during intermittent hypoxia. It is possible therefore that the two such pathways may be involved in

targeting different genes for transcription and each may alter distinct alternative splicing patterns. Therefore as this thesis concentrated on the effect of 48h chronic hypoxia on the alternative splicing of cancer-associated genes, it would be important to also examine the effect of intermittent hypoxia on the alternative splicing of cancer-associated genes, such as the ones investigated in this thesis (Sections 3.5 and 3.6). Furthermore, the effect of intermittent hypoxia on the expression of splice factors and splice factor kinases, such as the ones investigated in Section 4.3.1 would also provide an insight into whether intermittent and chronic hypoxia activate distinct alternative splicing mechanisms. This is especially important when it is considered that intermittent hypoxia is of physiological relevance to many tumour types, as discussed in Section 6.1.3.

6.5.2 Examine the effect of irradiation on the alternative splicing of cancer-associated genes

Rabbani *et al.* (2010) showed that irradiation correlated with the expression of hypoxia markers: HIF-1α, CA IX and pimonidazole (Section 6.1.4). This provides evidence that other factors may regulate the HIF response. Therefore, it would be prudent to examine whether or not irradiation is able to activate genes associated with the hypoxic response, such as EPO; and whether the same alternative splicing alterations shown to be regulated by hypoxia in this thesis are also affected by irradiation. This would be especially important when it is considered that irradiation

is used as a cancer treatment. Knowledge of the down-stream effects of such treatment may be important for its future use.

6.5.3 Development of a more specific CLK1 inhibitor

TG003 was used in the work reported in this thesis to inhibit CLK1 expression, which in turn was able to provide evidence that CLK1 is involved in the alternative splicing regulation of *FGFR1OP* (Section 5.4). However, TG003 is documented to also inhibit CLK4, and therefore there is the possibility that CLK4 may regulate the alternative splicing of *FGFR1OP.* Although a CLK1 siRNA knockdown provided an additional suggestion that CLK1 is involved in the regulation of *FGFR1OP* alternative splicing, this was not shown to be significant. Therefore, there is a possibility that CLK4 may also be involved in the regulation of *FGFR1OP* alternative splicing. The development of a more specific inhibitor of CLK1 would be beneficial as this would be able to address any uncertainties in regards to whether CLK1 and/or CLK4 were involved in the splicing changes exhibited by *FGFR1OP* during hypoxia. SiRNAs targeted towards CLK1 and CLK4 could also be used to knockdown the expression of the proteins in order to examine the effect on *FGFR1OP* expression; however, these would not target the activity of the proteins. The development of a new CLK1 inhibitor may also open up new avenues for future therapeutic treatments.

6.5.4 Examine the effect of successful SAFB1 and PRPF8 knockdowns on the alternative splicing of cancer-associated genes

Section 5.2 of this thesis intended to knock-down *SAFB1* and *PRPF8* in order to further assess their effect on the alternative splicing regulation of CA IX. However, this was not successful and although the RNomics platform performed by our collaborators in the MCF7 breast cancer cell line provided evidence that *CA IX* splicing is regulated by SAFB1 and PRPF8, it would be beneficial to confirm this further using successful *SAFB1* and *PRPF8* siRNA knockdowns in the PC3 prostate cancer cell line. Moreover, the effect of the knockdowns on the alternative splicing of the cancer-associated gene set used in this thesis could also be investigated.

6.5.5 The effect of hypoxia on the formation of SNBs

As discussed in Section 5.6.1, SAFB1 is involved in the formation of SNBs and has also been shown to interact with SRSF1, SAM68 and hnRNP A1 (Denegri *et al.,* 2001; Sergeant *et al.,* 2007; Rivers *et al.,* 2015), which were all shown to increase in mRNA expression during hypoxia. SNBs have been suggested to sequester splice factors away from sites of transcription in certain conditions (Weighardt *et al.,* 1999; Biamonti *et al.,* 2010). As the expression of SAFB1 was shown to decrease in hypoxia, it suggests that a reduced amount of SNBs are formed. This would allow splice factors, such as SRSF1, SAM68 and hnRNP A1 to regulate the alternative splicing of genes required for the hypoxic response. Therefore, it would be

worthwhile to investigate this hypothesis and examine whether or not the formation of SNBs is favoured by hypoxic stress.

6.5.6 Development of an SSO with HAP technology for the targeting of hypoxia-mediated alternative splicing of oncogenes

SSOs bind to specific pre-mRNA sequences and block the binding of splice factors, which disrupts the intended splicing of the pre-mRNA. Therefore, SSOs can be used to switch splicing to favour splice isoforms that are not associated with disease progression (Havens and Hastings, 2016) (Section 6.3.1). HAPs are drugs that have been designed to be inactive in normoxic conditions, but become active in hypoxic conditions (as described in Section 6.3.2). Therefore, the development of an SSO with HAP technology would allow the specific targeting of hypoxia-mediated alternative splicing changes, such as *APAF-1, BTN2A2, RAP1GDS1* and *CA IX,* which are shown in this thesis, whilst only targeting hypoxic areas.

The ultimate aim of any future work would be to enable a better understanding of how alternative splicing contributes to the onset and maintenance of tumours with the view to using splicing pathways as a focus for the development of new therapeutic strategies. The development of HAP technology may be a good first step, which may one day help in the battle against cancer.

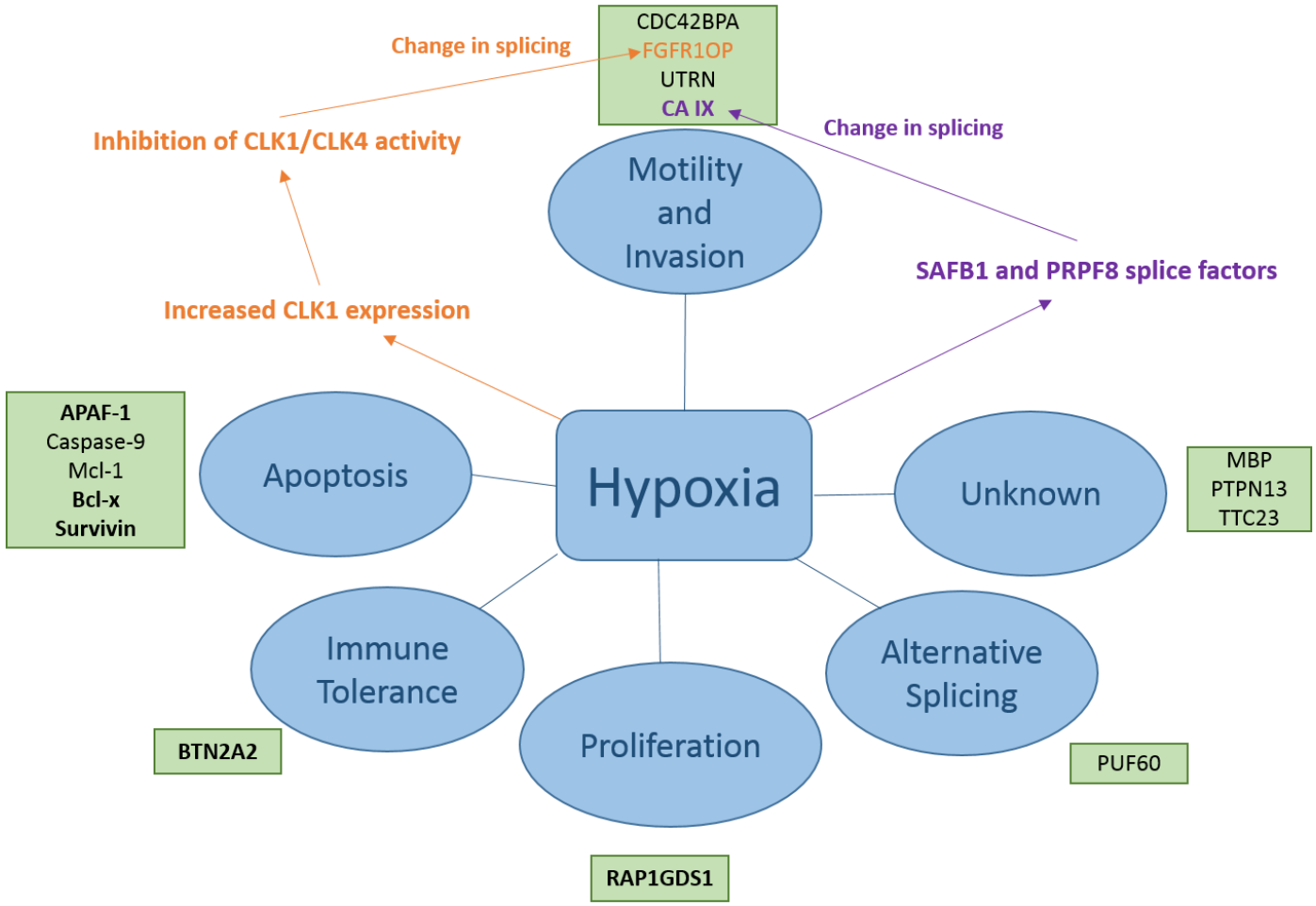


Figure 6.2 – Summary of key findings. Cancer-associated genes identified as having a change in mRNA alternative splicing during hypoxia are shown (green boxes) next to the hallmark of cancer that is affiliated with them (blue circles). mRNAs that favour oncogenic isoforms under the influence of hypoxia are shown in bold if known. The mechanism for hypoxic-mediated change in *FGFR1OP* splicing is shown in orange. Splice factors involved in the change in *CA IX* splicing are shown in purple.

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