Characterising novel phosphatase enzymes important in regulating Androgen Receptor function in the progression of Castrate Resistant Prostate Cancer

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Abstract

Prostate cancer (PC) is the most common oncogenic malignancy in men in the UK, and is expected to affect 1 in 4 men throughout their lifetime. Whilst the treatment for organ confined PC is initially very effective, no successful therapies exist for patients where the disease has progressed to an advanced stage, and is reflected by the poor 5-year survival rate of 30%. Resistance to current treatment modalities, aimed at disrupting the androgen signalling axis, renders the disease what is termed castrate resistant PC (CRPC). Crucially, the primary target in the treatment of PC, the androgen receptor (AR), remains a key driver of disease survival and differentiation throughout disease progression.

Activation of the AR at the post-translational level by aberrant co-activator activity is a well-established resistance mechanism, however, the role of phosphatase enzymes on AR function represents a significant knowledge gap in AR regulation. To address this issue, phosphatase enzymes identified from a human phosphatome RNAi screen in the androgen responsive PC cell line, LNCaP, were characterised within the context of AR regulation. As such, myosin phosphatase (MLCP) was identified as a novel negative regulator of AR activity. Robust molecular biology techniques revealed that MLCP repressed AR function via indirect mechanisms involving the activation of the clinically relevant tumour suppressors RB1 and NF2. With this in mind, it was possible to identify the endogenous MLCP inhibitors, PPP1R14C and NUAK 1/2, as novel AR activators and potential therapeutic targets in both PC and CRPC. Disruption of either PPP1R14C or NUAK 1/2, via RNAi or small molecule inhibition, respectively, repressed AR transcriptional activity, characterised by reduced protein stability and impaired ligand induced nuclear translocation, culminating in reduced PC cell cycle progression, migration and proliferation, providing significant evidence for a novel, and therapeutically exploitable, AR regulatory mechanism.

Acknowledgement

First of all I would like to thank my supervisor, Professor Craig Robson, for his fundamental role throughout my doctoral research project. Despite making some costly mistakes at the beginning of my research project, Craig gave me the freedom, and financial backing, to develop my own independent research ideas and implement them within the lab, whilst at the same time contributing with invaluable feedback. Craig's support extended beyond the lab to career advice, culminating in the award of an external fellowship position, and my convenient departure to Rotterdam. Similarly, this research project would not have been as successful without the ever-available advice of my co-supervisor Dr Luke Gaughan. Our daily 'scientific' chats over coffee will always be a highlight of the project. I am also very thankful to all members of NICR surgical oncology group, particularly Dom, Lew and Mark, for their guidance, advice and patience when teaching me new experimental techniques. Their presence is a constant reminder that successful science is based on collaborations.

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Contents

Abbreviations

Activation Function 1 (AF-1)

Activation Function 2 (AF-2)

Adenosine Monophosphate-Activated Protein Kinase (AMPK)

Androgen Deprivation Therapy (ADT)

Androgen Receptor (AR)

Androgen Response Element (ARE)

Ataxia Telangiectasia Mutated (ATM)

Carboxy-Terminal Extension (CET)

Castrate Resistant Prostate Cancer (CRPC)

C-Jun N-Terminal Kinase 1 (JNK)

C-Terminal Domain (CTD)

Cyclin Dependent Kinase (CDK)

Death-Associated Protein Kinase 3 (ZIPK)

Dihydrotestosterone (DHT)

Double Minute 2 Protein (MDM2)

DNA Binding Domain (DBD)

Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit (EZH2)

Epidermal Growth Factor (EGF)

Epithelial and Endothelial Tyrosine Kinase (ETK)

ETS Variant 1 (ETV1)

Eukaryotic Translation Initiation Factor 2A (eIF2α)

Fluorescence Activated Cytometry (FACs)

FK506 Binding Protein 5 (FKBP5)

Follicle Stimulating Hormone (FSH)

Gene Set Enrichment Analysis (GSEA)

G-Protein Coupled Receptor (GPCR)

Heat Shock Protein (HSP)

Hes Family BHLH Transcription Factor 6 (HES6)

Histone Deacetylase (HDAC)

Insulin Like Growth Factor 1 (IGF-1)

Integrin Linked Kinase (ILK)

Large Tumour Suppressor Kinase 1 (LATS1)

Ligand-Binding Domain (LBD)

LNCaP-Androgen Independent (LNCaP-AI)

LNCaP-Casodex Resistant (LNCaP-CdxR)

LNCaP-Enzalutamide Resistant (LNCaP-EnzR)

Low-Molecular Weight Protein Tyrosine Phosphatase (LMWPTP)

Luteinising Hormone (LH)

Luteinising Hormone Releasing Hormone (LHRH)

Lysine Demethylase 1 (LSD1)

Lysine N-Methyltransferase 7 (SET9)

Mechanistic Target of Rapamycin Complex 1 (MTORC1)

Merlin (NF2)

Metastatic Castrate Resistant Prostate Cancer (mCRPC)

Mitogen-Activated Protein Kinase (MAPK)

Molecular Signature Database (MSigDB)

Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT)

Myosin Phosphatase (MLCP)

Myosin Light Chain Kinase (MLCK)

Nuclear Localization Signal (NLS)

N-Terminal Domain (NTD)

N-Terminal Domain-C-Terminal Domain Interaction (N/C Interaction)

NUAK Family Kinase (NUAK)

Phosphatase and Tensin Homolog (PTEN)

Phosphate Buffered Saline (PBS)

Phosphoinositide 3-Kinase (PI3K)

Phosphorylated Myosin Light Chain Serine 19 (pMLC-S19)

Pim-1 Proto-Oncogene, Serine/Threonine Kinase (PIM1)

Platelet-Derived Growth Factor

Polo Like Kinase 1 (PLK1)

Poly-Glutamine (Poly-Gln)

Poly-Glycine (Poly-Gly)

Post-Translational Modification (PTM)

Propidium Iodide (PI)

Prostate Cancer (PC)

Prostate-specific Antigen (PSA)

Protein Kinase A (PKA)

Protein Kinase B (AKT)

Protein Kinase C (PKC)

Protein Kinase G (PKG)

Protein Kinase N (PKN)

Protein Phosphatase 1 (PP1)

Protein Phosphatase Holoenzyme Inhibitory Domain (PHIN Domain)

Protein Phosphatase 1 Regulatory Inhibitor Subunit 14A (PPP1R14A)

Protein Phosphatase 1 Regulatory Inhibitor Subunit 14C (PPP1R14C)

Protein Phosphatase 1 Regulatory Subunit 12A (PPP1R12A)

Protein Phosphatase 1 Regulatory Subunit 15B (PPP1R15B)

Protein Phosphatase 2A (PP2A)

Protein Phosphatase 2 Regulatory Subunit B (PPP2R2C)

Protein Tyrosine Phosphatase of Regenerating liver 3 (PRL-3)

Protein Tyrosine Phosphatase 1B (PTP1B)

P21 Protein-Activated Kinase 6 (PAK6)

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Retinoblastoma 1 (RB1)

Rho Associated Coiled-Coil Containing Protein Kinase (ROCK)

Ring Finger Protein 6 (RNF6)

RNA Integrity Number (RIN)

Serine/Threonine Kinase 24 (MST3)

Serine/Threonine Kinase 26 (MST4)

Seven In Abstentia Homolog 2 (SIAH2)

Small C-Terminal Domain Phosphatase 2 (SCP2)

Small Ubiquitin-Like Modifier (SUMO)

Solute Carrier Family 45 member 3 (SLC45A3)

SRC Proto-Oncogene Non-Receptor Tyrosine Kinase (SRC)

STIP1 Homology and U-Box Containing Protein 1 (CHIP)

Transcriptional Activation Unit 1 (TAU1)

Transcriptional Activation Unit 5 (TAU5)

Tumour-Nodes-Metastasis (TNM)

Tyrosine Kinase FER (FER)

Tyrosine Kinase Non-Receptor 2 (ACK)

Ubiquitin Specific Peptidase (USP)

Vascular Endothelial Growth Factor B (VEGFB)

V-Myc Avian Myelocytomatosis Viral Oncogene Homolog (MYC)

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Materials and Methods

Myosin Phosphatase is a Dynamic Regulator of Androgen Receptor Transcriptional Activity

Characterisation and Validation of a PPP1R14C RNAi Gene Signature Obtained by RNA Sequencing

Myosin Phosphatase Modulation Impacts on the Phosphorylation Status of the Androgen Receptor

Investigating the Therapeutic Potential of PPP1R14C Inhibition

Chapter 1.

Introduction

1.1 Prostate Cancer

1.1.1 Prostate Cancer Incidence

Prostate cancer (PC) is the most common oncogenic malignancy in men in the UK (excluding non-melanoma skin cancer), accounting for approximately 25% of male diagnosed cancers (CRUK, 2016). This equates to 47,000 new cases being diagnosed each year, with current opinion suggesting that 1 in 8 men will be diagnosed with PC within their lifetime (CRUK, 2016; PCUK, 2016b). Diagnosis of PC has more than tripled since 1975, as demonstrated in figure 1.1A. Amongst others, the advent and wide-spread implementation of the prostate-specific antigen (PSA) test, as well as an aging population, are considered major contributors to this phenomena (Schroder *et al.*, 2014). Whilst little is known regarding risk factors for PC, incidence is significantly coupled with age, with the median age of diagnosis being between 70 and 74 years. The rate of PC incidence for different age groups within the UK can be visualized in figure 1.1B. Indeed, the prevalence of histological PC upon post-mortem is significantly higher than the number of diagnosed cases (Klotz and Emberton, 2014), suggesting PC may be more prevalent in the elderly than currently thought. Unlike other cancer types, currently no highly penetrant inherited genes conferring the PC phenotype have been identified. However, men with a family history of PC are 2-3 times more likely to be diagnosed with PC. Furthermore, men with a mutation in BRCA2, a major genetic risk factor for breast and ovarian cancer, are up to 7 times more likely to be diagnosed with PC (Ostrander and Udler, 2008). Globally, rates vary dramatically as depicted in figure 1.2, and indeed, black British males are twice as likely to be diagnosed with PC as white British males (Ben-Shlomo *et al.*, 2008), whereas British males of Asian descent are half as likely (CRUK, 2016), suggesting

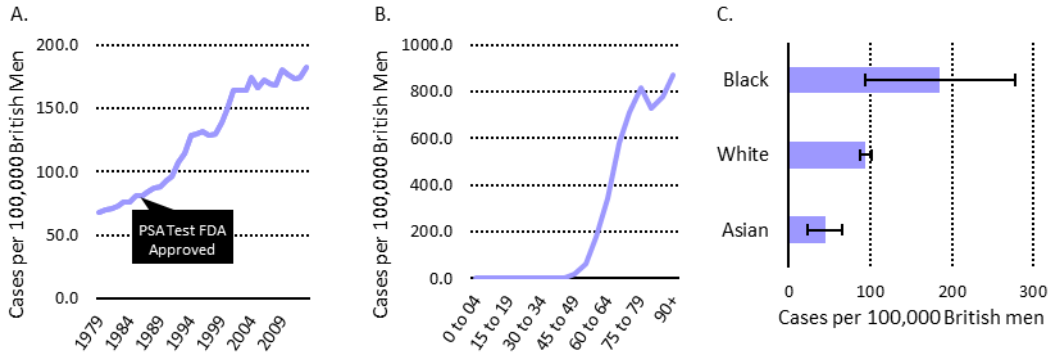


Figure *1.1* ***–Rate of Prostate Cancer Incidence*** *A. Rate of PC incidence in the UK 1979-2011 B. Rate of PC incidence in the UK across different age groups C. Rate of PC incidence in the UK across different ethnicities. Data obtained from Cancer Research UK.*

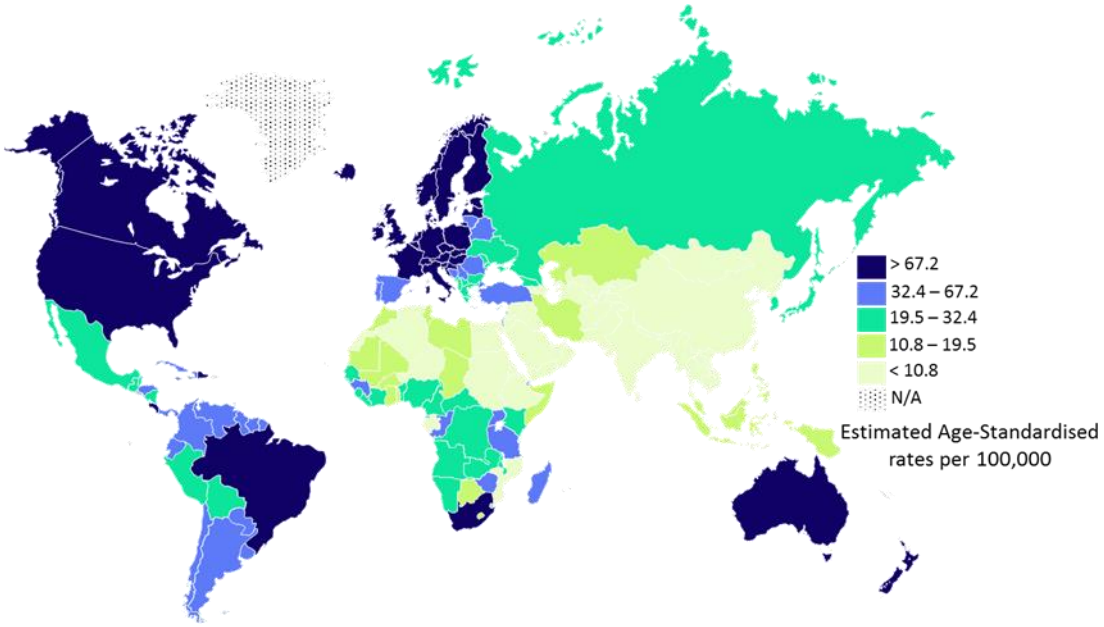


Figure 1.2 - ***Age standardised rate of PC across the world***. *Data obtained from GLOBOCAN 2012(Ferlay J, 2013). Figure generated using publicly available medical art from www.servier.com.*

a genetic predisposition to PC development exists. The difference in PC incidence between British males of different ethnicity is represented in figure 1.1C.

1.1.2 Prostate Cancer Grading and Staging

Upon diagnosis of PC, the cancer is staged and graded in order to ascertain the risk of disease progression and to implement the appropriate disease management regime. Cancers are graded using the Gleason grading system, which is a morphological assessment of the cancer cells, performed microscopically by a pathologist, based on the extent of cancer cell differentiation from a normal prostate cell (CRUK, 2016; PCUK, 2016a). This is used to determine the aggressiveness of the cancer. The pattern is given a grade from 1-5, with scores of 1-2 being considered normal tissue, and 5 being the most differentiated cancer. Multiple tumour biopsies are taken, and the Gleason score is calculated from adding the most common biopsy grade with the highest biopsy grade, therefore PC must be defined by a Gleason score of between 6 and 10. The Gleason score is then used in combination with the stage and the PSA level of the patient to dictate treatment modality. Staging of the cancer is an assessment of how far the cancer has spread. Although different staging methods exist, the Tumour-Nodes-Metastasis (TNM) system is employed in the UK and is represented in figure 1.3. This system determines the size and localization of the primary tumour, and the extent of metastasis to lymph nodes and/or distant sites. Taken together, tumour grade and stage allows patients to be stratified into 3 main categories; localized disease, locally advanced

disease, and advanced metastatic disease. The five year survival rate for patients classified into these cohorts is >90%, 70-80% and 30%, respectively, highlighting the need for early detection, appropriate risk stratification and more effective treatment options for advanced metastatic patients.

T1	Organ confined microscopic	**NX**	Lymph nodes not looked at/scans unclear	**MX**	Spread of the cancer not looked at/scans unclear
T2	Organ confined visible	**N0**	No cancer detected in the lymph nodes	**M0**	No cancer detected at distant sites of the body
T2a	Cancer is in <half of one lobe	**N1**	Cancer detected in the lymph nodes	**M1**	Cancer detected at distant sites of the body
T2b	Cancer is in >half of one lobe				
T2c	Cancer is in both lobes				
T3a	Cancer has broken through the capsule of the prostate				
T3b	Cancer has broken through the capsule of the prostate and spread to the seminal vesicles				
T4	Cancer has spread to nearby organs				

Figure *1.3* – ***TNM staging of PC**. Data obtained from PCUK and table produced independently. PC is staged according to a TNM scoring system based on the extent of tumour spread and dissemination.*

1.1.3 Localized Prostate Cancer

For localized prostate cancer the primary tumour is confined within the prostate organ. The majority of patients present with localized PC upon diagnosis, which are then further stratified according to their risk of disease progression (Klotz and Emberton, 2014). As localised PC is very often slow growing and has a low metastatic potential, it is crucial to identify which patients may benefit from treatment, and which patients may not require treatment at all. Low-risk PC is characterized by a PSA value lower than 10ng/ml and a Gleason score of 6 or less, whilst high-risk PC is defined by having a PSA level of 20ng/ml or higher, or, a Gleason score of 7 and above, or, if the stage is T2C, T3 or T4 (T3 and T4 staging means the tumour has broken through the prostate capsule, and as such will be reclassified as locally advanced). Radical prostatectomy has been the primary treatment option for all newly diagnosed PC cases over the last 30 years, however, following the introduction of the PSA test, a dramatic increase in the diagnosis of low-risk PC was observed, and as such, a large proportion of patients were over-diagnosed and subsequently over-treated. Recently, the general consensus for low-risk patients is to take a more cautious treatment approach termed active

surveillance (Draisma *et al.*, 2009). Active surveillance consists of serial PSA assessments and repeat biopsies and has been considered a successful intervention for risk reclassification, as the lead time between diagnosis and disease progression is often very long, or of no detrimental consequence. However, approximately one third of patients undergoing active surveillance will be reclassified as high risk and offered treatment (Klotz and Emberton, 2014). Again, avoidance of radical procedures is an emerging theme, and men will typically be offered focal therapy where applicable (Giannarini *et al.*, 2014). Focal therapy involves excision of a solitary tumour target (>1.3cm^3) following MRI guided identification, which may be performed in conjunction with repeat biopsies, resulting in minimally invasive surgery, and subsequently reduced morbidity. In cases where multiple positive cores have been identified, radical prostatectomy and/or radiotherapy will be recommended. Reclassification of low-risk PC to high-risk PC may occur following rising levels of PSA, or through increased tumour grading at repeat biopsy, or alternatively, up to 15% of patients present with high-risk disease at diagnosis (Chang *et al.*, 2014).

1.1.4 Locally Advanced Prostate Cancer

Patients that undergo tumour reclassification from low-risk to high-risk PC are managed in the same way as patients diagnosed with locally advanced disease, whereby treatment of the primary tumour is crucial in delaying disease progression and gaining local control. Currently, a multi modal approach is considered the most efficacious including neo-adjuvant androgen deprivation therapy (ADT) with radical prostatectomy and/or external beam radiation (Bolla *et al.*, 2002). Radical prostatectomy requires complete removal of the gland itself, confirmation intraoperatively that surgical margins are negative for cancer, and more recently extended pelvic lymph node dissection. There are very few differences between prostatectomy and radiotherapy in relation to study end-points, and current practice is often dictated by the overall health and age of the patient. In patients where PSA continues to rise following radical treatment, salvage external beam radiation is employed and has shown significant survival advantages (Stephenson *et al.*, 2007). Despite a 5-year survival rate of 70-80% for patients with locally advanced PC, many patients will develop non-castrate metastasis, or rising PSA castrate resistant PC (CRPC), which will invariably lead to the development of castrate resistant metastatic PC (mCRPC), and ultimately death.

1.1.5 Advanced Metastatic Prostate Cancer

Initially metastatic PC is characterized by a period of responsiveness to more advanced anti-androgens, however, this period is short lived and the transition in disease progression to mCRPC is inevitable. The most frequent site of CRPC metastasis observed in patients is at the bone. This often results in what is termed 'skeletal-related events', consisting of bone pain, fracture and spinal cord compression, often resulting in the need for additional radiotherapy and/or orthopaedic surgery (Attard *et al.*, 2016). Two drugs are currently employed to aid in the reduction of skeletal-related events, the first being zoledronic acid (Saad *et al.*, 2002). Zoledronic acid is a bisphosphonate that inhibits osteoclast mediated bone resorption by approximately 10%. The second is the human monoclonal antibody denosumab that targets the RANKL protein, impeding osteoclast function (Fizazi *et al.*, 2011). The addition of radium-223 into treatment regimens has also proven to improve overall survival whilst reducing skeletal-related events in patients with mCRPC (Parker *et al.*, 2013). However, currently no effective treatment regimens exist that significantly impede disease progression, with current therapy resulting in modest increases in survival and symptomatic relief (Gartrell *et al.*, 2015). A number of cytotoxic agents have been investigated in the treatment of mCRPC, but only

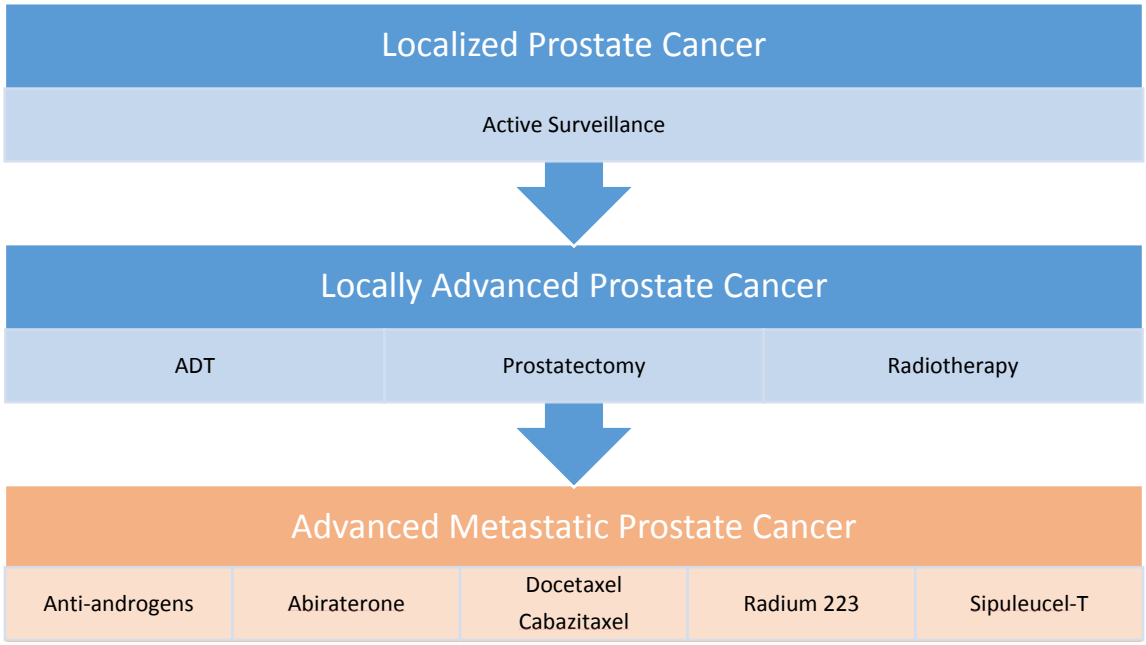


Figure *1.4* – ***Current Treatment Regimen of Prostate Cancer.*** *Localized PC is initially monitored by active surveillance including regular PSA testing. Locally Advanced PC is primarily treated by radical prostatectomy, and/or radiotherapy, followed by androgen deprivation therapy. Advanced metastatic PC is treated with advanced anti-androgens in combination with androgen biosynthesis inhibitor abiraterone. Radium 223 is frequently employed to combat associated bone metastasis and pain, whilst the implementation of cytotoxic agents, docetaxel or cabazitaxel, are employed as a last line of therapy. More recently, the immunotherapy sipuleucel-T has been in the treatment of advanced metastatic PC and shows modest benefit.*

docetaxel and cabazitaxel have been FDA approved based on modest survival advantages (Attard *et al.*, 2016). The implementation of immunotherapy in patient treatment is emerging, but remains highly costly, approximately £60,000/month(Simpson *et al.*, 2015). Sipuleucel-T is an FDA approved active cellular immunotherapy with a reported 4.1 month survival advantage over placebo (Kantoff *et al.*, 2010). The current treatment regimen employed within the UK throughout the progression of PC is represented in figure 1.4.

1.1.6 Treatment Rationale

The dependency of PC on circulating androgens was first demonstrated by Huggins *et al* in 1941, where it was observed that tumour regression could be induced following the reduction of serum testosterone either by orchiectomy, or through the administration of exogenous estrogens (Huggins *et al.*, 1941). Indeed, it was this discovery that provided the first successful treatment for PC, and prompted the emergence of therapeutics aimed at disrupting the androgen signalling axis. In 1971 Andrew Schally *et al* successfully characterised the luteinising hormone releasing hormone (LHRH) and his subsequent research focused on the development of LHRH agonists (Schally *et al.*, 1971). Administration of LHRH agonists leads to stimulation of the hypothalamic signalling axis, resulting in the release of luteinising hormone (LH) and follicle stimulating hormone (FSH) and subsequent production of testosterone in the testes. However, when administered chronically, LHRH agonists render the pituitary gland refractory to further stimulation through down regulation of LHRH receptors, resulting in castrate levels of serum testosterone (Sandow *et al.*, 1978). Due to the initial hyper-stimulation of LH release, use of LHRH agonists is associated with a surge in serum testosterone termed 'testosterone flare', which is associated with a number of unwanted side effects in patients such as hot flushes, fatigue and loss of libido (Rick *et al.*, 2013). Nevertheless, LHRH agonists are associated with less morbidity than orchiectomy or estrogen administration whilst maintaining similar survival outcomes, and remain a crucial component of current ADT regimens (Byar and Corle, 1988; Ferraldeschi *et al.*, 2015). More recently, LHRH antagonists have been developed that display very similar efficacy in relation to LHRH receptor downregulation, but do not exhibit the initial testosterone flare, thus providing a more favourable toxicity profile, at least in the short term (Wong *et al.*, 2014). As such, it is likely LHRH antagonists will continue to be developed and may replace the use of LHRH agonists as the predominant agent in ADT (Klotz *et al.*, 2014). In parallel, the discovery and

characterisation of the androgen receptor (AR) was being performed throughout the 1960's (Anderson and Liao, 1968; Bruchovsky and Wilson, 1968; Mainwaring, 1969). As the understanding of AR regulation increased, the first generation of anti-androgens were developed. Cyproterone acetate is considered a 'pure' steroidal anti-androgen and acts as an AR antagonist, blocking the binding of endogenous AR ligands testosterone or the more potent metabolite dihydrotestosterone (DHT) (Isurugi *et al.*, 1980; Pavone-Macaluso *et al.*, 1986). Cyproterone acetate was successfully used in PC patients resulting in tumour regression, reduction in serum testosterone, LH and FSH, and displayed similar survival advantages to estrogen administration. However, cyproterone acetate had affinity for other steroidal receptors and resulted in systemic progestational side effects including loss of libido and impotence, therefore the development of non-steroidal anti-androgens ensued (Wong *et al.*, 2014). In the 1970s flutamide was characterised by Shutsung Liao and colleagues as a competitive non-steroidal antiandrogen, and received FDA approval for use in advanced PC patients in 1989 (Liao *et al.*, 1974; Labrie *et al.*, 1982; FDA, 1989). Subsequent compounds were generated with higher affinity for the ligand binding domain of the AR including bicalutamide (Casodex) which is still widely used today (Rathkopf and Scher, 2013). However, as sequencing techniques advanced, it emerged that as many as 30% of patients present with mutations within the AR ligand binding domain following treatment courses, allowing for anti-androgens to act as agonists, and thus stimulate the AR signalling axis (Taplin *et al.*, 2003). In addition, first-generation anti-androgens exhibit significantly lower binding affinity for the AR than endogenous androgens, and have recently been outclassed by second-generation anti-androgens (Simard *et al.*, 1997; Kolvenbag *et al.*, 1998). In 2010, enzalutamide, formerly MDV-3100, was developed by Jung *et al,* which through further characterisation demonstrated a significantly higher AR binding affinity over bicalutamide of approximately seven-fold and resulted in xenograft tumour shrinkage as opposed to retardation (Tran *et al.*, 2009; Jung *et al.*, 2010). Enzalutamide showed considerable efficacy in phase I and II trials, including over a 50% reduction in PSA levels in 43-56% of patients, enabling it to rapidly progress to phase III trials (Scher *et al.*, 2010). In the phase III trial AFFIRM, enzalutamide treatment significantly increased patient survival (*18.4 months vs. 13.6 months*, *HR 0.63, 95% CI 0.53–0.75; P <0.001*) as well as displaying superiority in all secondary end-points (Scher *et al.*, 2012), and was subsequently FDA approved for the treatment of CRPC in patients with prior administration of chemotherapy (FDA, 2012). It has since been included in trials for use prior to

chemotherapy, notably the PREVAIL trial (Beer *et al.*, 2014). The rapid progression of enzalutamide through clinical trials, combined with the high demand for increased treatment efficacy in CRPC, has driven the development of a number of additional AR targeting agents currently in preliminary trials. One such compound is ARN-509, an AR antagonist derived from the same chemical series as enzalutamide (Bambury and Rathkopf, 2015) that provides comparable *in-vitro* efficacy characterised by AR degradation and impaired nuclear localization. Another AR antagonist is ODM-201, but this compound has a distinct structure to enzalutamide and ARN-509, and does not cross the blood brain barrier. Both novel AR antagonists have demonstrated promising results in phase I/II trials and are currently being employed in phase III trials (clinicaltrials.gov, 2016a; Clinicaltrials.gov, 2016b). Similarly other regions of the AR protein are crucial for AR transactivation, and may represent a novel approach to treating patients where resistance has arisen, or delaying resistance in the first instance. EPI-001 targets the N-terminal domain of the AR as opposed to the C-terminal ligand binding domain for which all previously mentioned AR antagonists have affinity. Development of EPI-001 remains in very early stages, but it is hoped this compound or a sister compound will progress to further studies shortly (Brand *et al.*, 2015). Similarly EZN-4176 works by targeting the AR via a distinct mechanism, the AR mRNA (Bianchini *et al.*, 2013). EZN-4176 is considered a third-generation anti-androgen and is in fact a locked anti-sense oligonucleotide that binds the hinge region of AR mRNA leading to degradation and is currently in phase I trials. Directly targeting the AR has proven an effective strategy thus far, but similarly to the very first effective PC treatments, minimising circulating androgens provides an equally successful approach, and as such our greater understanding of testosterone production and conversion has led to the development of additional androgen biosynthesis inhibitors. Despite the effectiveness of LHRH agonists and antagonists at reducing circulating androgens produced in the testes, the adrenal glands are still capable of producing circulating pro-androgens through two independently regulated reactions catalysed by the cytochrome P450 enzyme CYP17. Firstly, CYP17 is responsible for the 17-α-hydroxylation of progesterone and pregnenolone, and secondly the lyase cleavage of 17-α-hydroxyprogesterone and 17-α-hydroxypregnenolone resulting in the formation of androstenedione and DHEA respectively (Attard *et al.*, 2005). These two products can then be converted to testosterone via a reaction catalysed by the enzyme 17-keto reductase (Hellerstedt and Pienta, 2002). Understanding this route of androgen production led to the development of the highly selective and potent CYP17

inhibitor abiraterone, a compound of similar structure to pregnenolone that has a binding affinity for CYP17 of <1nM (Attard *et al.*, 2009). Abiraterone was approved for treatment of mCRPC patients in 2011(FDA, 2011) following success in a phase III trial where it displayed a significant increase in overall survival (*14.8 months vs. 10.9 months; HR 0.65; 95% CI 0.54 to 0.77; P<0.001*) (de Bono *et al.*, 2011). Novel compounds targeting the androgen biosynthesis pathway include but are not limited to TAK-700, TOK-001 and VT-464 and are under clinical development demonstrating that exploitation of the androgen biosynthesis pathway remains an approach of continued refinement (Molina and Belldegrun, 2011; Gomez *et al.*, 2015; Bird and Abbott, 2016). As such, targeting the androgen signalling axis has proven to be a successful intervention in the treatment of PC over the last 60 years, and crucially, through the development of more advanced molecular biology techniques, data from clinical and experimental model systems not only demonstrate that the AR is still essential for driving disease progression under castrate conditions, but remains a viable therapeutic target in the future treatment of both PC and CRPC (Buchanan *et al.*, 2001; Feldman and Feldman, 2001), and thus there is a great need to enhance our knowledge on AR regulation and function.

1.2 The Androgen Receptor

1.2.1 The Androgen Receptor Structure and Function

In order to continue developing more effective AR targeting agents it is crucial we enhance our knowledge on the intricate regulation of the AR. The *AR* gene is located on chromosome Xq11-12 in males, existing as a single allele (Gelmann, 2002). Whilst not being developmentally lethal, complete loss of the *AR* gene results in complete androgen insensitivity syndrome, whereas missense mutations that result in impaired activity result in partial androgen insensitivity syndrome. Phenotypically, retardation or loss of male sexual development is observed despite the production of normal physiological levels of androgens (Quigley *et al.*, 1995). This is due to the fact that androgens require a biologically active AR in order to exert their effects at a cellular level. The AR is therefore defined as a member of the steroid hormone receptor family of transcription factors (Heinlein and Chang, 2004). Furthermore, it has been shown that the conversion of testosterone to DHT is essential for complete prostate morphogenesis, confirming DHT as the more potent AR ligand (Wilson *et al.*, 1993). Aside from normal prostate development, the AR is essential for cell differentiation,

secretory function, metabolism, morphology, proliferation and survival (Dehm and Tindall, 2006). The AR is expressed in both the prostate epithelial and stromal cells, with androgen depletion resulting in apoptosis of the prostate epithelia (Litvinov *et al.*, 2003). This process can be reversed following the reintroduction of androgens, resulting in the rapid proliferation and differentiation of basal epithelial stem cells, and subsequently reinstating the secretory function of the prostate (Kurita *et al.*, 2001). In turn, transcription of the *AR* gene is regulated by androgens amongst other steroid hormones, as well as being shown to be tissue and age specific. Studies of the *AR* gene promotor have revealed a broad range of transcription factor binding sites including palindromic DNA binding sites recognized by the AR, the glucocorticoid receptor and the progesterone receptor (Baarends *et al.*, 1990). Interestingly, the androgen signalling axis forms an auto-regulatory negative feedback loop whereby androgen stimulation results in reduced *AR* mRNA expression, and castration results in increased *AR* mRNA expression (Quarmby *et al.*, 1990). Like other steroid hormone receptors, the exons of the *AR* gene code for functionally distinct domains of the ~919 amino acid AR protein, with exon 1 coding for the N-terminal domain (NTD), exons 2 and 3 coding for the DNA binding domain (DBD), exon 4 the hinge region, and exons 5 through 8 coding for the C-terminal domain (CTD) which contains the ligand-binding domain (LBD) (Lubahn *et al.*, 1988; Jenster *et al.*, 1992; Bain *et al.*, 2007). Additionally, the NTD and CTD harbour co-activator binding

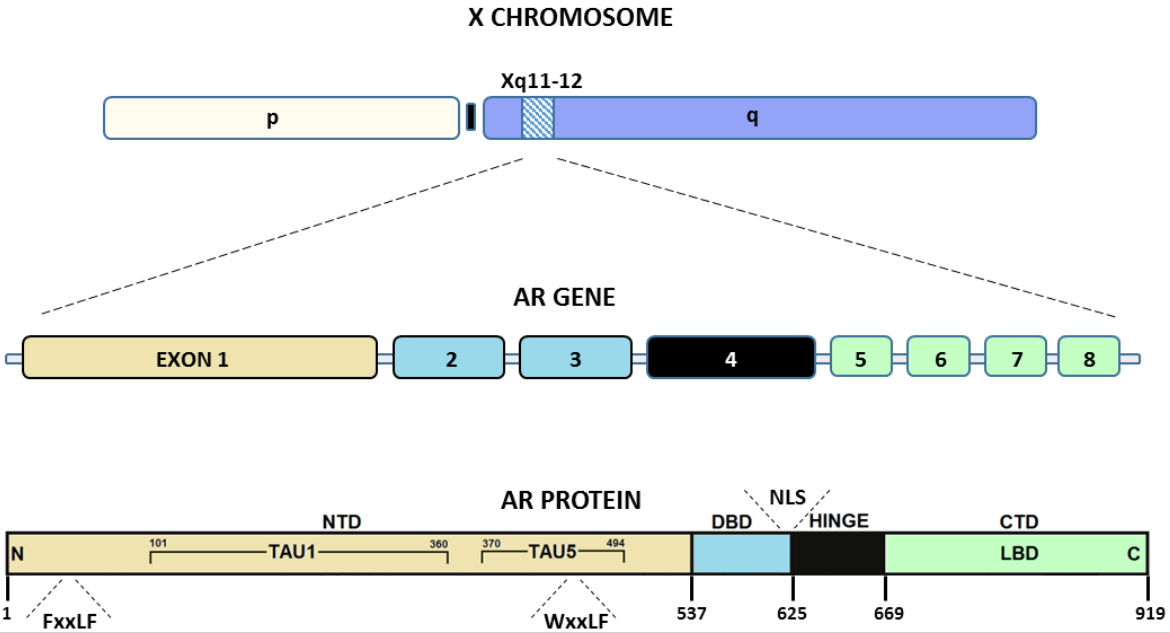


Figure *1.5* – ***Schematic representation of the Androgen Receptor gene and protein.*** *The AR gene is located on chromosome Xq11-12 and spans 8 exons. The AR protein possesses 4 distinct domains, the N-terminal domain, DNA binding domain, hinge region, and the C-terminal domain. The N and C-terminal domains can functionally interact through the 'FxxLF' and 'WxxLF' motifs located in the N-terminus. The nuclear localization signal (NLS) spans both the DNA binding domain and the hinge region.*

grooves termed transcriptional activation function 1 (AF-1) and transcriptional activation function 2 (AF-2) respectively (Jenster *et al.*, 1995; Dehm and Tindall, 2006). A schematic diagram representing the genomic and protein structure of the AR can be found in figure 1.5.

Despite being highly conserved throughout evolution, the complexity of the structural and functional elements of the AR remain to be fully elucidated (Thornton and Kelley, 1998). Whilst it has been possible to solve the crystal structures of the CTD and DBD, the same cannot be said for the NTD (Matias *et al.*, 2000; Sack *et al.*, 2001). The AR CTD is organised as three antiparallel helical sheets, comprised of 11 rather than 12 α-helices like other hormone receptors (Sack *et al.*, 2001). Each of the α-helices contains 18 amino acids that form a central LBD cavity, and create direct interactions with the ligand. Upon ligand binding, the ultimate α-helix of the LBD undergoes a conformational change, causing it to stabilize the ligand within the cavity. This conformational change leads to the formation and exposure of the AF-2 coactivator binding surface (Dehm and Tindall, 2006). Exposure of the hydrophobic AF-2 groove allows for docking of proteins containing an 'LxxLL' motif (Hur *et al.*, 2004). Despite extensive crystallography, the consequence of such interactions remains unclear, with AR peptide based studies revealing the AR CTD displays minimal intrinsic transcriptional activity (Bevan *et al.*, 1999; He *et al.*, 1999; Hur *et al.*, 2004). As such, subsequent studies identified that the CTD was capable of binding 'FxxLF' and 'WxxLF' peptide sequences present in the AR NTD (He *et al.*, 2000). Furthermore, the CTD displayed significantly higher binding affinities for NTD derived peptides compared with 'LxxLL' containing proteins. Additional studies were able to demonstrate that the NTD-CTD (N/C) interaction was more commonly associated with mobile AR following androgen stimulation, but was also lost upon DNA binding, suggesting the N/C interaction may serve as a means of preventing protein binding until the AR is deposited at the chromatin (Li *et al.*, 2006; van Royen *et al.*, 2007). The DBD of the AR is highly conserved with other nuclear hormone receptors and plays an important role in mediating AR dimerization, nuclear localization and DNA binding (Shaffer *et al.*, 2004). This domain encompasses 2 zinc fingers and a loosely structured carboxy-terminal extension (CET) (Dehm and Tindall, 2006). The first zinc finger is termed the P-box, which forms the recognition helix that binds the DNA major groove at androgen response elements (AREs). Interestingly, the P-box shares perfect homology with that found in the glucocorticoid receptor, progesterone receptor and mineralocorticoid receptor, and thus the AR constitutes a member of the Class I

of nuclear hormone receptors (Claessens *et al.*, 1996; Schoenmakers *et al.*, 2000; Verrijdt *et al.*, 2000). AREs are hexameric half-sites arranged as either direct-repeat or inverted-repeat sequences split by a 3bp spacer recognised by AR homodimers arranged in a head to head orientation (Claessens *et al.*, 2008). The head to head dimerization is unique to the AR amongst the steroid receptor family and is mediated through D-box, the second zinc finger, interactions (Shaffer *et al.*, 2004). However, this does raise fundamental issues on the specificity of AR DNA binding, which to date is still unclear. There are speculations that the CET or an additional interface results in increased AR/ARE stability. In addition to harbouring the P-box, D-box and CET, the DBD also encompasses a proportion of the nuclear localization sequence (NLS). The NLS also spans into the hinge region and comprises the bipartite sequence 617-RKCYEAGMTLGARKLKK-634 that possesses 2 basic motifs at either flank (Jenster *et al.*, 1993). The NLS is believed to be exposed upon ligand binding, allowing for the binding of α-importin and nuclear translocation (Ni *et al.*, 2013). In addition to containing the NLS, mutagenesis studies have revealed that the hinge region also has roles in AR dimerization and ultimately the transcriptional output of the AR (Haelens *et al.*, 2007; Clinckemalie *et al.*, 2012). The NTD accounts for over 50% of the total AR protein and is encoded entirely by exon 1. Within the NTD are two poly-amino acid stretches of significantly variable length throughout the human population. These are made up of poly-glutamine (poly-Gln) and poly-glycine (poly-Gly) sequences respectively (Chang *et al.*, 1988; Hsing *et al.*, 2000; Sasaki *et al.*, 2003). The exact function of these repeats is largely unknown, but there is considerable evidence for them acting as docking sites for protein-protein interactions (Palazzolo *et al.*, 2008). Additionally it has been shown that the length of the poly-Gln repeat inversely correlates with AR transcriptional activity and may in fact be a risk factor for the development of PC (Chamberlain *et al.*, 1994). Indeed, there is modest evidence for males of African-American descent possessing shorter poly-Gln stretches and an increased incidence of PC compared to white American males (Platz *et al.*, 2000). As previously mentioned, the NTD is structurally disordered and described as possessing a molten-globule like structure and therefore likely to be heavily influenced through protein-protein interactions, adding to the complexity of its role and function, as well as adding dynamic context specific factors to its regulation (Lavery and McEwan, 2006). Independently of this, the NTD has been shown to be a potent transcriptional activator in the absence of the CTD (Reid *et al.*, 2002). This transcriptional activity was mapped to two distinct regions within the AF-1 binding groove termed transcriptional activation unit

1 (TAU1) and transcriptional activation unit 5 (TAU5) (Callewaert *et al.*, 2006). Both TAU regions have been shown to be indispensable for AR transcriptional activity through mutagenesis studies, whilst TAU1 and TAU5 also possess the 'FQNLF' and 'WHTLF' motifs respectively, required for the ligand dependent N/C interaction (Doesburg *et al.*, 1997; He *et al.*, 2000; Wilson, 2011).

1.2.2 Androgen Receptor Transactivation

In the absence of ligand the AR is found predominantly in the cytoplasm (Cardozo *et al.*, 2003), maintained in complex with a range of heat shock (HSP), chaperone and co-chaperone proteins, in a high ligand binding affinity conformation (Cano *et al.*, 2013). Circulating androgens can freely diffuse through the cell membrane where testosterone is metabolized to the more potent AR binding metabolite, DHT. Ligand binding then triggers a series of conformational changes within the AR and partial dissociation from the unbound protein complex (Cano *et al.*, 2013). The conformational changes lead to exposure of the NLS and subsequent nuclear translocation via a classical import mechanism involving recognition by importin α and β, followed by movement through the nuclear pore complex (Black and Paschal, 2004). The AR homodimer is then capable of engaging its transcriptional program through

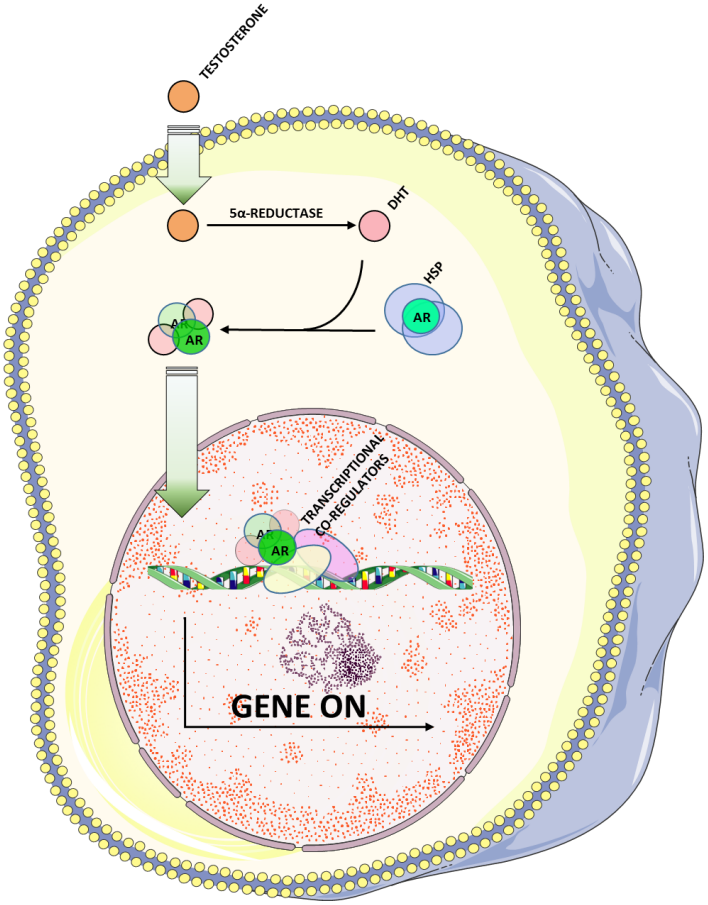


Figure *1.6* - ***AR Transactivation.*** *Testosterone freely enters prostate cells by passive diffusion where it is metabolised to the more potent metabolite, DHT, by 5α-reductase. DHT then binds to the AR causing dissociation of the AR from the foldosome and leading to homodimerization. AR homodimers then translocate to the nucleus where they are able to bind to AREs, recruit additional transcriptional regulators, and initiate gene transcription.*

association with AREs and interaction with co-activators and co-repressors. Figure 1.6 represents a simplified visualization of AR transactivation. However, post-translational modifications (PTMs) of the AR, including phosphorylation, ubiquitination, sumoylation and acetylation invariably add extra layers to the structural and functional complexity of the AR (van der Steen *et al.*, 2013), and as such represent an invaluable area to expand our knowledge on AR regulation.

1.2.3 Post-Translational Modifications of the Androgen Receptor

It has been well documented in the literature that AR activity is heavily influenced by the activity of co-activators and co-repressors in a dynamic and context specific manner, but crucially, many of these co-activators and repressors impact AR function through direct PTM. Indeed, PTM of the AR is capable of regulating protein stability, interactions with additional proteins, subcellular localization and structure (Coffey and Robson, 2012). Many of these modifications have been extensively studied and their functional consequences identified, and as such, their respective modulators have been evaluated as novel therapeutic targets in diseases where aberrant AR activation plays a significant role in disease initiation and progression. This section will introduce the impact of acetylation, methylation, ubiquitination and sumoylation (depicted in figure 1.7) on AR function, but will focus primarily on phosphorylation of the AR.

As a transcription factor, the impact of histone acetylation on the AR transcriptional program has been extensively studied (Li *et al.*, 2005), but it wasn't until 2000 when acetylation was first described as a direct modification of the AR (Fu *et al.*, 2000). Specifically, three lysines within the 'KLKK' motif flanking the DBD (K630, K632, K633) were shown to be acetylated by histone acetyltransferase P300 and lysine acetyltransferase 2B. Subsequent research also identified lysine acetyltransferase 5 and N-acetyltransferase arrest-defect 1 protein as direct acetyl transferases of the AR (Gaughan *et al.*, 2002; Wang *et al.*, 2012). Through the implementation of mutagenesis studies, it was shown that acetylation of the AR plays a pro-androgenic role, contributing to the transactivation of the AR through the recruitment of additional co-activators (Gaughan *et al.*, 2002). It was shown that the AR can be acetylated in response to androgen, but also through additional stimuli such as bombesin and interleukin 4 (Fu *et al.*, 2000; Gong *et al.*, 2006; Lee *et al.*, 2009). In turn, acetylated AR can be de-acetylated by histone deacetylase (HDAC) enzymes, thus providing a context specific means of AR

modulation. Indeed, upon inhibition of HDAC1, an increase in acetylation of the AR is observed, and this correlates with increases in AR transcriptional activity (Gaughan *et al.*, 2002; Faus and Haendler, 2008). Conversely, overexpression of the HDAC sirtuin 1 reduces AR transactivation, and it was identified that de-acetylation of the AR within the 'KLKK' motif disrupts the AR N/C terminal interaction and subsequent nuclear translocation and chromatin binding, providing substantial evidence for the impact of AR acetylation on receptor structural rearrangements and interactions (Fu *et al.*, 2006).

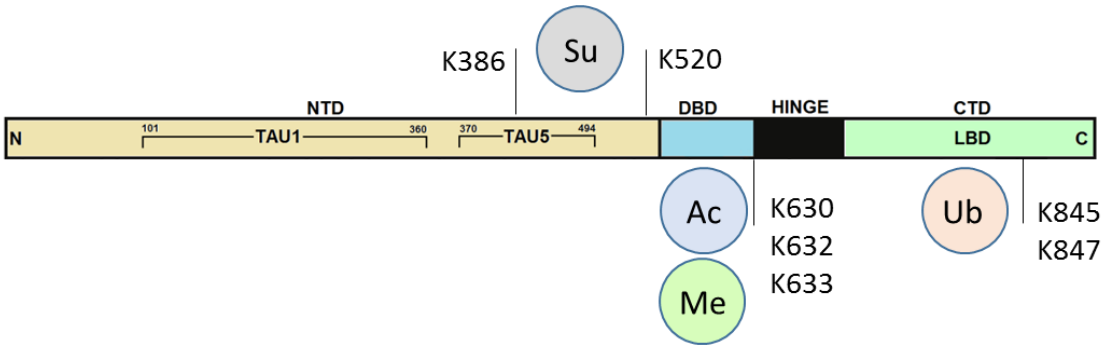


Figure 1.7 – ***Acetylation, Methylation, Ubiquitination and Sumoylation of the AR.*** *The AR can be acetylated and methylated at residues K630, K632 and K633, whilst ubiquitination occurs on resideus K845 and K847 and sumoylation occurs on K386 and K520.*

The 'KLKK' motif within the AR known to be acetylated also displays significant sequence homology with the consensus sequence for methylation by SET9. Despite strong but conflicting evidence for the exact residue of modification, it was shown that lysine N-methyltransferase 7 (SET9) is capable of methylating the AR by two independent research groups (Gaughan *et al.*, 2011; Ko *et al.*, 2011). Similarly to acetylation, methylation of the 'KLKK' motif was shown to be important for N/C interaction and subsequent transcriptional activity of the AR. Expression of SET9 in clinical PC samples demonstrated elevated expression in cancerous tissue vs non-cancerous tissue and provides evidence of its activity as an AR co-activator in the progression of PC (Gaughan *et al.*, 2011). Similarly, methylation is a reversible process, and the characterisation of de-methylation enzymes on AR activity has ensued (Coffey *et al.*, 2013).

Ubiquitination is the process of covalently modifying lysine residues with the addition of ubiquitin, a small 8.5 kDa protein. Ubiquitination has been linked to signal transduction as well as protein recognition and degradation. Ring finger protein 6 (RNF6) and seven in abstentia

homolog 2 (SIAH2) have been shown to promote the mono-ubiquitination of the AR on residues K845 and K847 resulting in increased transcriptional activity, whereas poly-ubiquitination mediated by double minute 2 protein (MDM2) and STIP1 homology and u-box containing protein 1 (CHIP) have been shown to promote AR protein turnover (Lin *et al.*, 2002; Xu *et al.*, 2009; Qi *et al.*, 2013; Sarkar *et al.*, 2014). Preventing de-ubiquitination of the AR by the ubiquitin specific peptidase (USP) family of proteins has emerged as a promising therapeutic approach to destabilize the AR protein. USP7 and USP12 have both been described as AR co-activators and are capable of influencing the ability of the AR to bind to AREs (Burska *et al.*, 2013; Chen *et al.*, 2015).

Similarly to ubiquitination, sumoylation is the process of covalently modifying lysine residues with the addition of a small protein, in this instance, small ubiquitin-like modifier (SUMO). There are 4 SUMO protein members, with SUMO 1 being the most frequently observed AR sumoylation conjugate (van der Steen *et al.*, 2013). AR sumoylation was first described in 2000 after the sumo conjugating enzyme ubiquitin conjugating enzyme E2 I was co-immunoprecipitated with the AR (Poukka *et al.*, 2000). The AR contains 2 sumolyation motifs, (I-L-V)-K-x-D/E, encompassing K386 and K520 residues. Androgen stimulation leads to increased sumoylation and was shown to be inhibitory to AR activity by impeding the AR N/C interaction (Kaikkonen *et al.*, 2009). It also became apparent that sumoylation of the AR elicits a distinct transcriptional program to non-sumoylated AR (Sutinen *et al.*, 2014). Despite the induction of sumoylation of the AR by androgens, and the distinct downstream effects of gene transcription, sumoylation does not appear to be localization dependent and sumoylated AR exists equally between the nucleus and cytoplasm (Nishida and Yasuda, 2002). Phosphorylation is the most extensively studied PTM of the AR to date, and many of the phosphorylation sites have been functionally annotated and their respective kinase identified (Gioeli and Paschal, 2012). Phosphorylation occurs on serine, threonine and tyrosine residues across all of the AR's functional domains, and these have been shown to occur in both the presence and absence of androgen, and indeed through the stimulation of alternative signalling cascades, demonstrating the complexity of AR regulation. This becomes of increasing relevance in relation to the function of the AR throughout the progression of PC to CRPC, and through further understanding of the biological consequences of AR phosphorylation, it may be possible to identify novel therapeutic targets for the treatment of

patients where resistance to antiandrogens has emerged. Figure 1.7 illustrates the phosphorylation sites identified thus far and are subsequently described below.

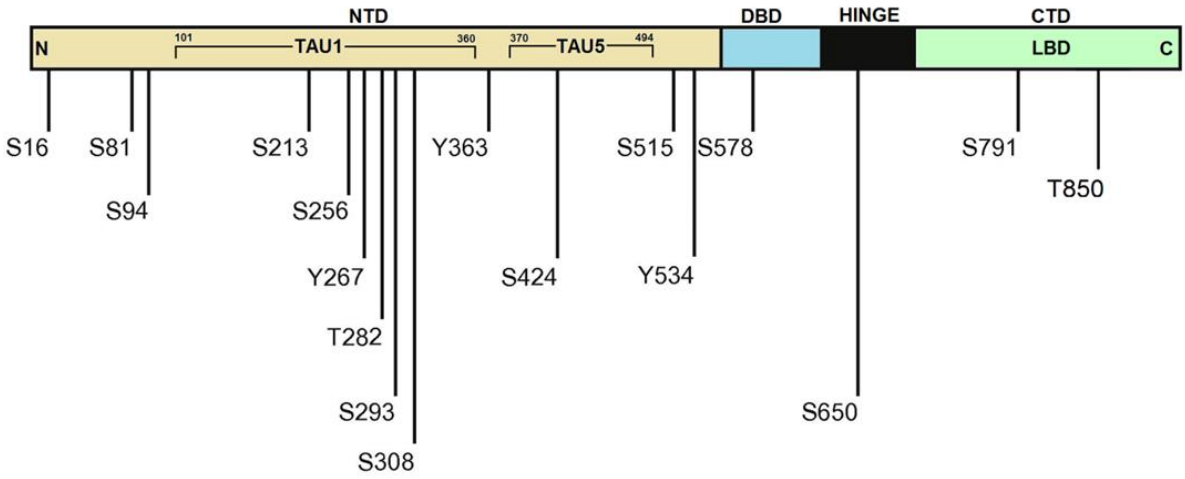


Figure *1.8* - ***Phosphorylation sites of the AR.*** *The AR has been described to be phosphorylated on 17 sites thus far spanning all four domains of the AR protein. These have been shown to result in distinct regulatory outcomes in relation to AR function. Phosphorylation has been attributed to a range of kinases from distinct signalling cascades.*

Serine 16 was found to be phosphorylated in response to androgen and lies within a consensus sequence for both protein kinase A (PKA) and calcium calmodulin II (Gioeli *et al.*, 2002). However, upon stimulation of PKA with forskolin no observable increase in the phosphorylation of serine 16 was observed, raising some doubts as to the responsible kinase. Interestingly, upon deletion of the LBD, serine 16 was phosphorylated in the absence of androgens suggesting it may play a role in the cross-talk between domains and may be of functional importance in androgen splice variants that lack the LBD (Gioeli *et al.*, 2002).

Serine 81 was found to display the highest stoichiometric phosphorylation in response to androgen, and also displayed the most prolonged phosphorylation (Gioeli *et al.*, 2002). Multiple kinases belonging to the cyclin dependent family of kinases (CDK) have been described to phosphorylate this site, each under distinct contexts. CDK1 has been shown to phosphorylate the AR in a cell cycle specific manner, whereas CDK9 has been shown to phosphorylate the AR in the nucleus during transcription (Chen *et al.*, 2006; Gordon *et al.*, 2010). In addition it was reported that CDK5 is also responsible for the phosphorylation of the AR at this residue (Hsu *et al.*, 2011). Functionally, it was found that CDK1 stabilizes the AR protein throughout the G2/M phase of the cell cycle, when CDK1 is most active, and had the

most profound impact on AR stabilization in the absence of androgen (Chen *et al.*, 2006). Interestingly, CDK1 was also found to be expressed at higher levels in androgen independent tumours, suggesting a potential role in AR activation under androgen depleted conditions. However, it was also observed that serine 81 is phosphorylated outside of the G2/M phase of the cell cycle when CDK1 is inactive, leading to the observations that CDK5 and CDK9 were also capable of phosphorylating this site. Upon over-expression of CDK9, a marked increase in serine 81 phosphorylation was observed, and this coincided with an increase in AR nuclear localization, chromatin deposition and retention, and gene transcription (Chen *et al.*, 2012). Similarly, upon over-expression of CDK5, increases in serine 81 phosphorylation were identified, correlating with increases in AR transcriptional activity. Conversely, siRNA depletion of CDK5 leads to a reduction in the phosphorylation of serine 81, and subsequent repression of AR regulated gene transcription (Hsu *et al.*, 2011). Crucially, it has been demonstrated through mutagenesis studies that loss of serine 81 phosphorylation limits PC cell growth (Gordon *et al.*, 2010).

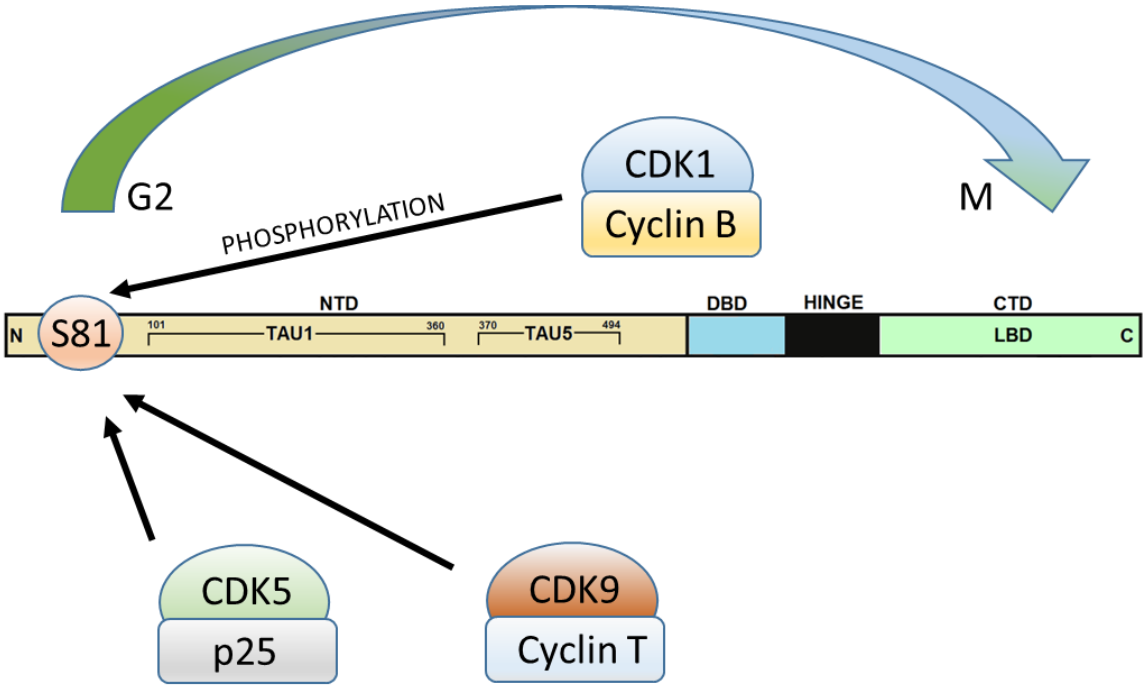


Figure 1.9 – ***AR serine 81 phosphorylation.*** *Phosphorylation of AR serine 81 by CDK1 occurs in a cell cycle dependent manner, specifically during G2/M phase of the cell cycle when CDK1 is most active. In addition, CDK5 and CDK9 have been shown to phosphorylate the AR in the nucleus throughout all phases of the cell cycle. Phosphorylation of serine 81 has been shown to induce AR transcriptional activity characterised by increased nuclear localization and chromatin binding.*

There is evidence for serine 94 being constitutively phosphorylated and does not increase in response to androgen (Yang *et al.*, 2007). Currently the kinase responsible for modification of this residue and its biological relevance remains unknown.

The impact of serine 213 phosphorylation on AR function has been extensively studied, and a number of kinases have been identified as the conjugating enzymes responsible for this modification. It has emerged that phosphorylation of this site by the distinct kinases results in different functional outcomes (Koryakina *et al.*, 2014). Indeed, the kinases responsible for the phosphorylation of this site belong to independent signalling cascades, both of which have been identified as possessing clinical significance in the progression of PC. The first kinase, protein kinase B (AKT), was demonstrated to induce serine 213 phosphorylation following stimulation of the phosphoinositide 3-kinase (PI3K) pathway, whilst inhibition of this pathway diminishes phosphorylation of serine 213 (Lin *et al.*, 2001; Palazzolo *et al.*, 2007). Subsequent *in-vitro* phosphorylation assays went on to prove that AKT was capable of phosphorylating this site. Functionally, phosphorylation by AKT results in the recruitment of MDM2, followed by subsequent ubiquitination and degradation, and thus is regarded as a repressive phosphorylation site (Lin *et al.*, 2002). Indeed, it was demonstrated that the AR forms a complex with both AKT and MDM2 upon phosphorylation of serine 213 (Deep *et al.*, 2008). The AKT mediated AR degradation can be rescued following addition of the proteasomal inhibitor MG132 confirming phosphorylation of serine 213 results in proteasomal degradation. Despite the repressive impact of AKT on AR signalling observed throughout these studies, the PI3K pathway is frequently dysregulated in advanced PC due to genomic deletion of the PI3K repressor phosphatase and tensin homolog (PTEN), and as such the interplay between these two signalling cascades may be more complex than initially thought (Lin *et al.*, 2004). Indeed, AKT is also capable of phosphorylating serine 791 resulting in the proteasomal degradation of the AR via the same mechanisms involving MDM2 (Gioeli *et al.*, 2002). Pim-1 proto-oncogene, serine/threonine kinase (PIM1) is also capable of phosphorylating the AR at serine 213, but its two transcript variants, PIM1-S and PIM1-L differentially impact on AR function (Ha *et al.*, 2013). PIM1-S induces similar functional outcomes whereby upon phosphorylation of S213 MDM2 is recruited and the AR undergoes proteasomal degradation, and as a result AR transcriptional activity is reduced. PIM1-L on the other hand does not impact on AR proteasomal degradation, and over-expression of this kinase results in increased

transcriptional activity despite increased phosphorylation of serine 213. However, PIM1-L is also capable of phosphorylating threonine 850 which is associated with increased activity, suggesting there may be some degree of cross-talk between the two events. Phosphorylation of threonine 850 by PIM1-L leads to AR stabilization and increased transcriptional activity, particularly in the presence of low concentrations of androgens. This was found to be due to the recruitment of RNF6 as opposed to MDM2, leading to subsequent ubiquitination, but favoured enhanced stability as opposed to degradation. In the presence of androgens the interaction between PIM1-L and the AR is enhanced (Linn *et al.*, 2012).

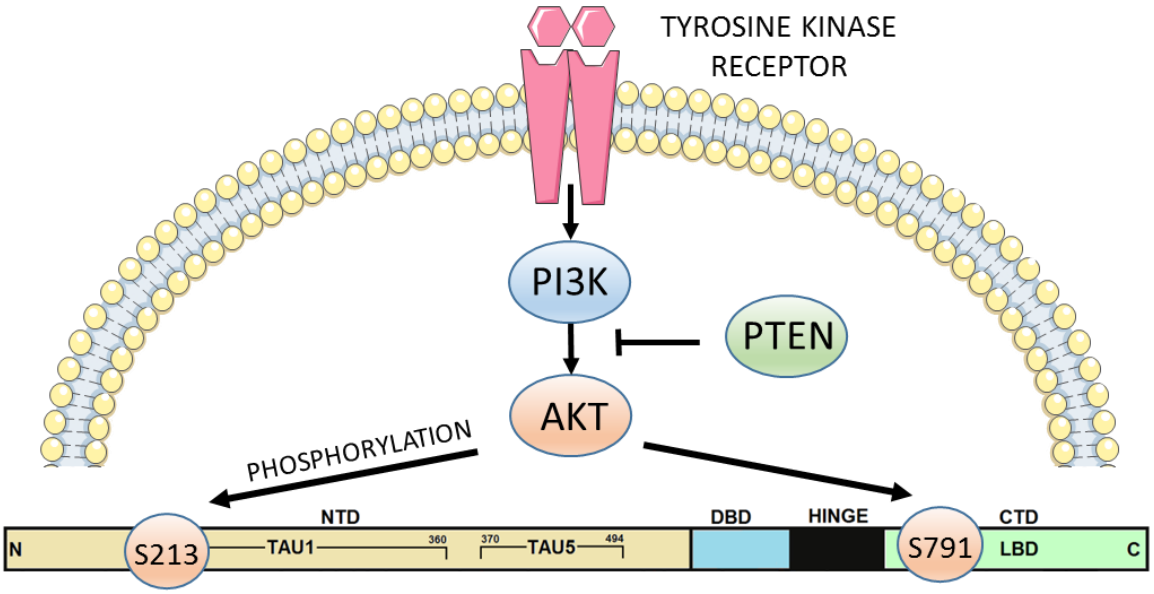


Figure 1.10 – ***AR serine 213 and serine 791 phosphorylation.*** *Activation of tyrosine kinase receptors by growth factors leads to the initiation of the PI3K signalling cascade, culminating in the activation of AKT. AKT is then capable of phosphorylating the AR on serine 213 and serine 791. Phosphorylation of these residues leads to the recruitment of MDM2, subsequent AR ubiquitination and ultimately proteasomal degradation.*

Another phosphorylation site with unknown functional consequence is serine 256, which is phosphorylated in response to androgen stimulation and lies within a calcium calmodulin kinase II consensus sequence (Gioeli and Paschal, 2012).

Aurora A is a kinase that has been implicated with the progression of PC to CRPC and is responsible for the phosphorylation of 2 residues in the AR, threonine 280 and serine 291 (Shu *et al.*, 2010). Both sites were demonstrated to be phosphorylated in the presence and absence of androgen leading to the potentiation of AR transcriptional activity through enhanced DNA binding. Aurora A expression has been shown to increase between localized PC and advanced

PC in clinical samples, and correlates with PSA expression, Gleason score, and survival (Buschhorn *et al.*, 2005; Furukawa *et al.*, 2007; Beltran *et al.*, 2011; Mosquera *et al.*, 2013), reinforcing its viability as a potential therapeutic target.

Serine 308 is phosphorylated by CDK11^{P58} throughout the G2/M phase of the cell cycle (Zong *et al.*, 2007; Chi *et al.*, 2011). Modification of this residue results in repression of the AR signalling axis and repression of cell growth. Upon siRNA depletion of CDK11^{P58}, increases in AR transcriptional activity are observed, whereas overexpression results in repression of AR transactivation. Indeed, expression of CDK11^{P58} inversely correlates with the Gleason score and proliferative capacity of PC when quantified by Ki67 staining (Olshavsky *et al.*, 2008). More recently, it was shown that CDK1 also phosphorylates serine 308, which raises the possibility that CDK1 acts as both a co-activator and co-repressor (Koryakina *et al.*, 2015). The authors went on to show that phosphorylation of serine 308 directed the AR to a distinct subset of androgen regulated genes which would suggest that the AR is capable of driving distinct transcriptional programs throughout different phases of the cell cycle, based on the stimulation and activity of co-activators.

Genomic sequencing of a male with AIS led to the discovery of serine 405 as a phosphorylation site (Lagarde *et al.*, 2012). Mutation of this residue to alanine leads to impaired AR activity and in the case of this patient, AIS.

Serine 424 is another residue identified as being androgen dependent, but the functional impact of this modification remains to be elucidated. The kinase responsible also remains unknown (Gioeli *et al.*, 2002).

Serine 515 is another residue where multiple kinases have been successfully identified as modulators of this residue. CDK7 was proven to phosphorylate serine 515 through *in-vitro* biochemical assays, further compounded through the incorporation of serine-515-alanine mutants in to *in-vitro* phosphorylation assays (Chymkowitch *et al.*, 2011). Functionally, it was shown that phosphorylation by CDK7 occurred at the promotor regions of androgen target genes, resulting in the recruitment of the ubiquitin ligase MDM2, and promoting AR turnover. However, impaired phosphorylation of this residue by CDK7 was shown to reduce the overall transactivation potential of the AR, suggesting AR protein turnover is a crucial step in the androgen signalling axis. Secondly, significant evidence for the phosphorylation of serine 515

by the mitogen-activated protein kinase (MAPK) signalling cascade exists (Gregory *et al.*, 2004; Mellinghoff *et al.*, 2004). It was demonstrated that following epidermal growth factor (EGF) stimulation, an increase in AR transcriptional activity is observed, and this is recapitulated by an increase in PC cell growth. Upon treatment with MAPK inhibitors, a clear reduction in the phosphorylation of serine 515 is observed. Indeed, future mutagenesis studies successfully identified serine 515 as the site of AR phosphorylation in response to EGF (Ponguta *et al.*, 2008). In contrast, a more recent study demonstrated that elevated levels of phosphorylated serine 515 at diagnosis by immuno-histochemistry correlated with PSA expression and a decreased time to biochemical relapse (Willder *et al.*, 2013). However, phosphorylation of serine 515 did not correlate with MAPK or phosphorylated MAPK expression, but rather, CDK1 and phosphorylated CDK1 expression. Additional *in-vitro* studies using the pan-CDK inhibitor roscovitine demonstrated that a reduction in the phosphorylation of CDK1 correlated with a reduction in the phosphorylation of serine 515. Furthermore, phosphorylation of serine 515 appears to be co-regulated with phosphorylation of serine 578 following EGF stimulation. Mutagenesis studies revealed that phosphorylation of serine 515 is maximal when serine 578 is not phosphorylated (Ponguta *et al.*, 2008). This cross-talk becomes increasingly complex when it was identified that both protein kinase C (PKC) and p21 protein-activated kinase 6 (PAK6) can phosphorylate serine 578 (Ponguta *et al.*, 2008; Liu *et al.*, 2013). Phosphorylation by PKC drives increased transcriptional activity and mediates cross-talk with serine 515 phosphorylation, whereas phosphorylation by PAK6 results in the recruitment of MDM2 and repression of AR transactivation. As such, the impact of EGF stimulation on AR function remains a point of interest for future research as it is also well documented that the MAPK signalling cascade is frequently dysregulated in the progression of PC and poses a viable therapeutic target (Gioeli *et al.*, 1999; Kinkade *et al.*, 2008).

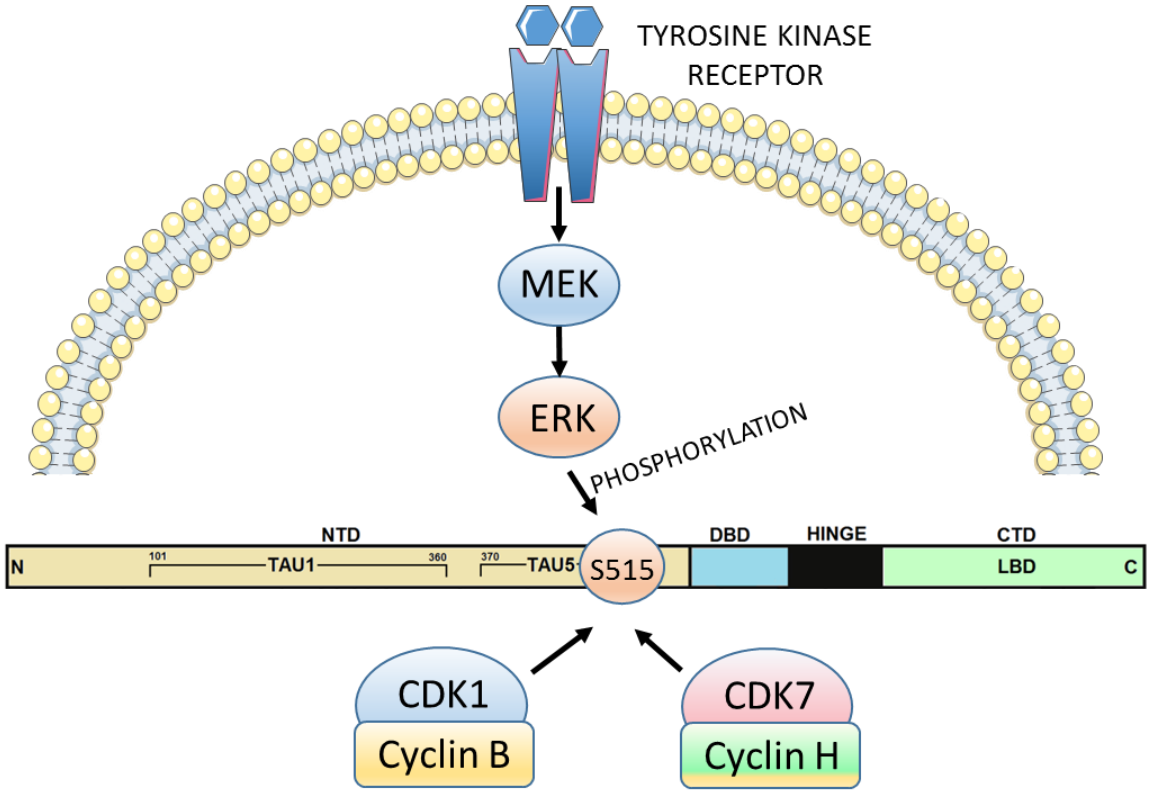


*Figure 1.11 – **AR serine 515 phosphorylation.** Activation of tyrosine kinase receptors by growth factors leads to the induction of the MAPK signalling cascade culminating in activation of the ERK kinases. The ERK kinases are then capable of phosphorylating the AR on serine 515 resulting in enhanced nuclear localisation and transcriptional activity. In addition, CDK1 and CDK7 can also phosphorylate serine 515.*

Another signalling cascade capable of impacting on AR phosphorylation is that of the stress-activated protein kinase pathway, highlighting once again the convergence of multiple pathways on the AR (Gioeli *et al.*, 2006). Both C-Jun N-terminal kinase 1 (JNK) and p38 MAPK were shown to phosphorylate the AR on serine 650 upon activation with phorbol 12-myristate 13-acetate. Conversely, inhibition of JNK and p38 MAPK with SP600125 and SB203580 respectively resulted in a reduction of phosphorylated serine 650. Phosphorylation of serine 650 was demonstrated to be repressive to AR function when either kinase was over-expressed, whereas increases in AR transcriptional activity was observed following siRNA depletion of either kinase. Repression of AR activity following phosphorylation of this mark was attributed to enhanced nuclear export.

Similar to threonine and serine, there are a number of tyrosine residues that are able to be phosphorylated. Once again, the phosphorylation of these residues is mediated through a number of independent signalling cascades. Tyrosine 267 and tyrosine 363 were identified as

being phosphorylated by tyrosine kinase non-receptor 2 (ACK) through mass spectrometry and site directed mutagenesis (Mahajan *et al.*, 2007). Mutation of both tyrosine residues independently with phenylalanine reduced AR transcription and chromatin binding in both the presence and absence of androgen. It was also shown that activated ACK stimulated growth of PC xenograft tumours, whilst mutation of tyrosine-267-phenylalanine blocked the growth of castrate resistant xenografts. In addition, it was shown that stimulation of ACK with EGF, heregulin and Gas6 also resulted in increased tyrosine phosphorylation. More recently, it was shown that stimulation of ACK with EGF directed the tyrosine phosphorylated species of AR to distinct gene promotors when compared to androgen stimulation, specifically the ATM gene promotor (Mahajan *et al.*, 2012). This not only highlights the ability of ACK to phosphorylate and stimulate AR activity, but also the ability of ACK to direct a distinct transcriptional program, even in the absence of androgen, whilst also implicating a role for ACK in driving resistance to radiotherapy in PC.

Tyrosine 534 has been identified as a SRC proto-oncogene non-receptor tyrosine kinase (SRC) phosphorylation site (Guo *et al.*, 2006). This residue is phosphorylated by SRC following stimulation by EGF, bombesin and interleukin 6. Site-directed mutagenesis demonstrated that phosphorylation of tyrosine 534 enhances AR transcriptional activity and the growth of PC xenografts. Immunohistochemical analysis also demonstrated that levels of phosphorylated tyrosine 534 correlated with SRC activity as well as disease progression. Interestingly, it was observed that tyrosine 534 phosphorylation also correlated with epithelial and endothelial tyrosine kinase (ETK) expression in human prostate tumours (Dai *et al.*, 2010). Over-expression of either ETK or SRC results in increased Y534 phosphorylation and subsequent AR stabilization (DaSilva *et al.*, 2009). Although similar observations can be made between AR tyrosine phosphorylation by SRC and ETK, it is likely their impact remains stimulus and context dependent.

Finally, it has also been shown that the tyrosine kinase FER (FER) phosphorylates tyrosine 223 in the presence of interleukin 6, leading to the formation of AR-FER complexes in the nucleus, resulting in enhanced AR gene transcription (Rocha *et al.*, 2013).

Crucially, phosphorylation of the AR is a highly complex regulatory mechanism, with many of the modulators in question regarded as oncogenes both in the context of PC and in additional

cancers. As described, phosphorylation events can occur both in the presence and absence of androgen, are both enhancing and repressive to AR function, and can be stimulated by alternative signalling cascades. With this in mind, elucidating the role of phosphatases on AR dephosphorylation poses an unmet need in our understanding of AR regulation.

The role of phosphatases on AR function represents a significant knowledge gap when compared to the role of kinases on AR function, and as such there are only a handful of known phosphatases capable of impacting on AR function both directly and indirectly. PTEN is one of the most widely studied prognostic markers for PC. Loss of PTEN is known to drive PC progression to a more aggressive and castrate resistant phenotype, and genomic loss is observed in up to 40% of mCRPC cases (Shen and Abate-Shen, 2007; Dan *et al.*, 2015). Loss of PTEN has been shown to be independently associated with increased risk of lethal progression and decreased survival (Ahearn *et al.*, 2016). Through de-repression of the PI3K-AKT pathway, PTEN loss results in a reduction in AR transcriptional activity, characterised by increased AKT mediated phosphorylation and degradation (Carver *et al.*, 2011b). With the prominent role of PTEN loss established within the progression of PC, a number of additional phosphatases that feed into the PI3K-AKT pathway have also been identified within the context of PC progression. These include the AKT phosphatases PH domain and leucine rich repeat protein phosphatase (PHLPP) 1 and PHLPP2, capable of antagonising the PI3K pathway by direct dephosphorylation of AKT, and inositol polyphosphate-4 phosphatase type II B, a phosphatase capable of dephosphorylating the phosphatidylinositol (3,4,5)-trisphosphate that accumulates following PTEN loss (Chen *et al.*, 2011; Kofuji *et al.*, 2015). All 3 of these phosphatases have been shown to be down-regulated in the progression of PC (Chen *et al.*, 2014; Rynkiewicz *et al.*, 2015). Protein tyrosine phosphatase 1B (PTP1B) is a well-established regulator of metabolic signalling, and has recently been identified as being an androgen regulated gene (Lessard *et al.*, 2012). Although its reciprocal role in AR signalling has not been assessed, PTP1B was shown to be required for optimal cell migration of PC cells in an androgen independent manner. 2 additional phosphatases, protein tyrosine phosphatase of regenerating liver 3 (PRL-3) and low-molecular weight protein tyrosine phosphatase (LMWPTP), have also recently been shown to positively impact on PC proliferation and migration (Ruela-de-Sousa *et al.*, 2016; Vandsemb *et al.*, 2016).

However, to date only 3 phosphatases have been described to directly interact with the AR. The first, small CTD phosphatase 2 (SCP2), interacts with the AR, but no evidence exists for direct modulation of the AR phosphorylation status by SCP2 (Thompson *et al.*, 2006). Rather, SCP2 binds to the AR and translocates with the AR upon androgen stimulation to the AREs of androgen regulated genes, such as PSA, where it is able to repress AR transcriptional activity. It is believed that repression of AR transcriptional activity occurs through dephosphorylation of the recruited transcriptional machinery, such as RNA polymerase II. The second phosphatase known to interact with the AR is protein phosphatase 2A (PP2A) (Yang *et al.*, 2005a). PP2A binds to the AR in a ligand-dependent manner, suggesting phosphorylation of androgen responsive residues enhances the affinity of PP2A for the AR. Preliminary evidence would suggest that PP2A is capable of dephosphorylating serine 81, serine 256, serine 308 and serine 424, subsequently reducing AR transcriptional activity. As such, PP2A is considered an AR co-repressor. Indeed, ectopic expression of the PP2A catalytic subunit, PPP2CA, prevents epithelial to mesenchymal transition, and potently supresses PC tumour growth and metastasis *in-vivo* (Bhardwaj *et al.*, 2014). Furthermore, protein phosphatase 2 regulatory subunit B (PPP2R2C), the substrate specifying subunit of PP2A, is frequently lost in PC (Bluemn *et al.*, 2013). It was found that down-regulation of PPP2R2C promotes androgen-independent growth of PC cells, albeit not by AR mediated mechanisms, and is tightly correlated with the increased likelihood of disease recurrence, and ultimately PC-specific mortality.

Protein phosphatase 1 (PP1) on the other hand, first identified to directly interact with the AR in 2009, is considered an AR co-activator (Chen *et al.*, 2009). Initial studies first demonstrated that inhibition of PP1 with okadaic acid led to cell type-dependent effects on AR activity and expression. Subsequent studies using the more specific PP1 inhibitor tautomycin, or indeed RNAi depletion of the catalytic subunit of PP1, PP1α, led to enhanced proteasomal degradation of the AR. Conversely, over-expression of PP1α led to increases in AR protein expression and transcriptional activity. Using phospho-specific antibodies, depletion of PP1α resulted in a marked increase in the phosphorylation status of the AR at serine 650. As previously described, phosphorylation of serine 650 by either JNK or p38 MAPK leads to reduced AR transcriptional activity characterised by reduced nuclear localisation and enhanced degradation. In support of this, PP1α depletion led to significant impairment in the nuclear localisation of a wild-type AR construct, but not a serine 650-alanine mutant construct.

Subsequent studies have identified that PP1 also mediates AR stabilisation through additional mechanisms. PP1 is capable of dephosphorylating and inhibiting the AR ubiquitin ligase MDM2, and it was demonstrated that PP1 contributes to enhanced AR stabilisation, particularly in the absence of androgen, via this mechanism leading to the prevention of proteasomal degradation (Liu *et al.*, 2016a; Liu *et al.*, 2016b). Recently, it was also demonstrated that the opposing impact on AR stabilization between PP1 and AKT is also extended to the constitutively active AR splice variant, AR-V7 (Li *et al.*, 2015). It was shown that PP1 enhanced AR-V7 stabilization via a reduction in the phosphorylation of serine 213, however, no evidence was presented for the direct dephosphorylation of serine 213 by PP1, suggesting the impact is likely to be mediated through repression of either AKT or MDM2 as previously described (Xiao *et al.*, 2010; Liu *et al.*, 2016a).

The lack of understanding into the role of phosphatases in PC disease progression, and more specifically, the contribution of phosphatase enzymes to AR activity, led our research group to investigate the role of phosphatase enzymes on AR transcriptional activity. In order to gain a greater insight into the role of phosphatase enzymes on the regulation of AR activity, both directly and indirectly, our research group performed an RNAi screen targeting 291 phosphatase enzymes and phosphatase interacting proteins with 3 individual RNAi oligos, in the androgen responsive, AR positive cell line, LNCaP-7B7 (Clayton, 2011). The LNCaP-7B7 cell line used also contained a chromosomally integrated luciferase reporter under the control of the ARE3 element from the PSA promotor. This enabled subsequent luciferase activity measurements to be used as a surrogate for AR activity and ranking of the RNAi targets as AR co-activators or co-repressors. As the targets were depleted through RNAi transfection, increases in luciferase activity were considered to be attributed to the depletion of a repressive phosphatase, whereas a reduction in luciferase activity was considered to be attributed to the depletion of a pro-androgenic phosphatase, when compared to the scrambled control.

Although potential hits were observed throughout all of the major classes of phosphatases, the impact of PP1 regulatory subunit depletion particularly stood out from what is known in the literature. PP1 belongs to the serine/threonine protein phosphatase family (Virshup and Shenolikar), which collectively have been described to catalyse approximately 90% of all eukaryotic dephosphorylation events, reversing the modifications imposed by hundreds of

serine/threonine kinases (Heroes *et al.*, 2013). Indeed, PP1 is encoded by 3 independent genes, collectively resulting in the formation of 6 alternatively spliced isoforms, all displaying ~90% amino acid sequence homology and resulting in very broad yet highly overlapping substrate specificity *in-vitro* (Cohen, 1988; Sasaki *et al.*, 1990; Dombradi *et al.*, 1993; Cohen, 2002). However, it is now becoming widely understood that PP1 catalytic subunits rely on the hundreds of mutually exclusive PP1 interacting proteins to govern their promiscuous activity in a spatio-temporal manner, thus allowing for the independent regulation of protein dephosphorylation (Korrodi-Gregorio *et al.*, 2014). As such, PP1 catalytic subunits never exist as monomeric subunits *in-vivo,* but through association with their regulatory subunits, form multimeric PP1 holoenzymes with distinct subcellular localizations, substrate specificity and catalytic activity (Peti *et al.*, 2013). A graphical representation of this process is depicted in figure 1.12. Furthermore, the association of PP1 with its regulatory subunits is a dynamic

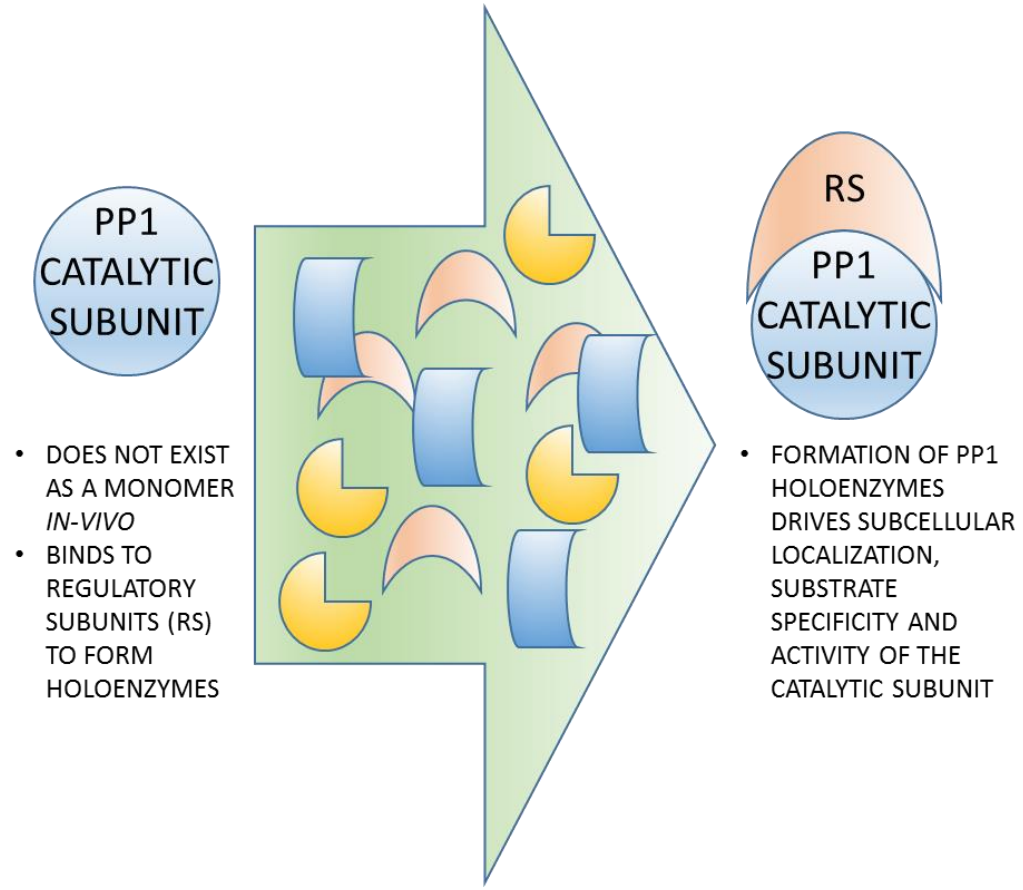


Figure 1.12 – ***Regulation of the PP1 catalytic subunit.*** *The PP1 catalytic subunit displays very broad substrate specificity in-vitro, however, does not exist as a monomeric subunit in-vivo. The PP1 catalytic subunit associates with upto 100 mutually exclusive regulatory subunits, all of which have an affinity for the catalytic subunit in the nM range. The association of the catalytic subunit with regulatory subunits results in the formation of functionally distinct PP1 holoenzymes, all of which possess distinct subcellular localizations, substrate specific, and catalytic activity.*

process, governed by the intracellular concentration of the regulatory subunits, which varies significantly between cell type, and their independent affinity for PP1, which in turn can also be modulated at the post-translational level, allowing for signalling cues to impact on dephosphorylation events, creating the pathway sensitivity required to counteract the action of serine/threonine kinases in a biologically relevant manner (Shi, 2009; Virshup and Shenolikar, 2009; Bollen *et al.*, 2010; Choy *et al.*, 2012). With this in mind, a highly variable response in AR activity following RNAi depletion of specific PP1 regulatory subunits was observed upon analysis of the RNAi screen, depicted in figure 1.13. This is in contrast to what is currently known in the literature regarding the role of PP1 on AR activity. Crucially, it was the identification of protein phosphatase 1 regulatory inhibitor subunit 14C (PPP1R14C) as a potent activator of the AR (luciferase activity vs scrambled control, 0.4 fold-change), and that of protein phosphatase 1 regulatory subunit 12A (PPP1R12A) as a repressor of the AR (luciferase activity vs scrambled control, 1.38 fold-change) that led to the subsequent characterisation and validation studies presented in this thesis. The rationale underlying the pursuit of PPP1R14C and PPP1R12A characterisation is that both proteins are components of the same PP1 holoenzyme, myosin phosphatase (MLCP), providing preliminary evidence for cross-talk between the dynamic regulation of MLCP activity and AR transactivation.

Regulatory Subunit	Luciferase Activity
PPP1R11	1.64
PPP1R9B	1.48
PPP1R12A	1.38
PPP1R13B	1.29
PPP1R16A	1.22
PPP1R15A	1.19
PPP1R1A	1.09
PPP1R14A	1.07
PPP1R12B	0.96
PPP1R14B	0.96
PPP1R3D	0.85
PPP1R14D	0.75
PPP1R8	0.68
PPP1R10	0.66
PPP1R9A	0.63
PPP1R16B	0.61
PPP1R3C	0.60
PPP1R1B	0.60
PPP1R2	0.60
PPP1R3A	0.59
PPP1R12C	0.56
PPP1R7	0.52
PPP1R3B	0.50
PPP1R14C	0.40
PPP1R15B	0.13

*Figure 1.13 – **The impact of PP1 regulatory subunit RNAi depletion on AR transcriptional activity.** PP1 regulatory subunits were depleted by RNAi as part of a phosphatase RNAi screen (Clayton, et al.). Luciferase activity (Fold-Change vs Scrambled control) was measured as a surrogate for AR activity. Analysis revealed that depletion of PP1 regulatory subunits results in distinct outcomes in relation to AR transcriptional activity (Clayton, 2011).*

Chapter 2.

Materials and Methods

2.1 Mammalian cell culture and storage

2.1.1 Cell Lines

Cell lines purchased from ATCC (Virginia, US):

LNCaP (ATCC® CRL-1740™) – First isolated from the lymph node metastasis of a 50 year old male in 1980 (Horoszewicz *et al.*, 1980), the LNCaP cell line represents an androgen-responsive model of PC.

CWR22RV1 (ATCC® CRL-2505™) – Is a subclone of the CWR22 cell line serially propagated in mice under castrate conditions. This cell line expresses AR splice variants and represents an androgen-independent model of PC.

HEK293T (ATCC® CRL-3216™) – Originally derived from human embryonic kidney cells, this cell line is readily transfected and as such will be used for over-expression studies within this thesis.

LNCaP, CWR-22RV1 and HEK293T cells were maintained in RPMI-1640 (R5886, Sigma Aldrich) supplemented with 10% (v/v) foetal bovine serum (HyClone) and 2 mM L-glutamine (Sigma Aldrich), and will be referred to as full media throughout this thesis. For steroid-depleted media, RPMI-1640 was supplemented with 10% (v/v) dextran-coated charcoal-stripped foetal bovine serum (HyClone) and 2 mM L-glutamine and is referred to as DCC media. Cell lines were cultured at 37°C in a 5% CO_2 humidified incubator (MCO-20AIC, Sanyo).

Cell line subclones generated in-house:

LNCaP-Androgen Independent (LNCaP-AI) – Generated from serial passage of the parental LNCaP cell line under steroid-depleted conditions. LNCaP-AI cells are maintained in DCC media. The LNCaP-AI cell line represents a model of androgen-independence.

LNCaP-Casodex Resistant (LNCaP-CdxR) – Generated from serial passage of the parental LNCaP cell line in full media and escalating doses of casodex until a final concentration of 10µM is reached. LNCaP-CdxR cells are maintained in full media + 10µM casodex. The LNCaP-CdxR cell line represents a model of resistance to current anti-androgens.

LNCaP-Enzalutamide Resistant (LNCaP-EnzR) – Generated from serial passage of the parental LNCaP cell line in full media and escalating doses of enzalutamide until a final concentration

of 10µM is reached. LNCaP-EnzR cells are maintained in full media + 10µM enzalutamide. The LNCaP-EnzR cell line represents a model of resistance to next generation anti-androgens.

2.1.2 Cell Passaging

Cell culture was carried out in a BioMat class II microbiological safety cabinet. To passage cells, culture media was removed from the culture flask and cells gently washed two times with phosphate buffered saline (PBS) (Invitrogen) and incubated with 1 x Trypsin-EDTA (Sigma Aldrich) solution and incubated for 5 minutes at 37°C. Following cell detachment, the trypsin was neutralised by the addition of culture media to the flask and the cell suspension was transferred to a sterile universal tube (Thermo scientific) and centrifuged at 400 x g for 5 minutes. The cell pellet was resuspended and cells seeded into new flasks at a 1:3 to 1:10 dilution. Mycoplasma tests were carried out every 2 months in-house.

2.1.3 Cell Storage

When required, cells would be stored at -80°C following resuspension in full media spiked with 10% DMSO in 1ml cryovial aliquots. If cells needed to be thawed, cryovials were warmed to 37°C in a water bath and added to 10ml of 37°C full media. The cells were then centrifuged at 400 x g for 5 mins, resuspended in 10ml of 37°C full media and transferred to a T25 cell culture flask.

2.1.4 Cell Counting

Cells were detached from culture flasks as previously described and resuspended in fresh 37°C media. 10µl of the cell suspension was then pipetted onto a haemocytometer (Fisher Scientific) and the cells within a $1mm^2$ area were counted and then multiplied by 10,000 in order to obtain cells/ml. Cells would then be diluted as required.

2.2 Compounds

2.2.1 5α-Dihydrotestosterone (DHT)

Dihydrotestosterone (DHT), a potent androgen receptor agonist (K_d=0.25-0.5nM (Ferner, 2012)), was supplied from Sigma Aldrich in powder form, resuspended in ethanol (Fisher Chemicals) at a concentration of 10 mM, and subsequently stored at -80°C.

2.2.2 Casodex

N-[4-cyano-3-(trifluoromethyl)phenyl]-3-[(4-fluorophenyl)sulfonyl]-2-hydroxy-2-methylpropanamide is an AR antagonist (IC50=160nM) that is marketed under the name casodex and developed by AstraZeneca. Casodex was supplied by AstraZeneca in powder form which was dissolved in endotoxin free anhydrous DMSO (Sigma Aldrich) to a stock concentration of 20mM. Stock solutions of casodex were stored at -80^{o}C and dilutions were made in DMSO and stored at -20^{o}C.

2.2.3 Enzalutamide

Enzalutamide (chemical name: 4-[3-[4-cyano-3-(trifluoromethyl)phenyl]-5,5-dimethyl-4-oxo-2-thioxo-1-imidazolidinyl]-2-fluoro-N-methyl-benzamide, brand name Xtandi), a potent anti-androgen (IC50=36nM) was purchased in powder form (Selleckchem), resuspended in dimethyl sulfoxide (DMSO) (Sigma Aldrich) at a concentration of 30 mM and stored at -80°C for no more than 6 months.

2.2.4 Cycloheximide

Cycloheximide (chemical name: 3-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide), an antibiotic that inhibits translation, was purchased in powder form (Sigma Aldrich) and suspended in molecular grade water (Life Technologies) to a final concentration of 5 mg/ml. Cycloheximide solution was made fresh when required.

2.2.5 MG-132

MG-132 (chemical name: benzyl (S)-4-methyl-1-1((S)-4-methyl-1-((S)-4-methyl-1-oxopentan-2-ylamino)-1-oxopentan-2-ylamino)-1-oxopentan-2-ylcarbarmate), a membrane permeable proteasome inhibitor (IC50=100nM) was purchased as a readymade solution in DMSO at a concentration of 10 mM (Sigma Aldrich) and stored at -20°C.

2.2.6 MK2206

MK2206 (chemical name: 8-(4-(1-aminocyclobutyl)phenyl)-9-phenyl-[1,2,4]triazolo[3,4-f][1,6]naphthyridin-3(2H)-one), a potent AKT1/2/3 inhibitor (IC50=8nM, 12nM, 65nM respectively, Selleckchem) and suspended in DMSO at a concentration of 5mM and stored at -80°C for no more than 6 months.

2.2.7 ML-7

ML-7 (chemical name: Hexahydro-1-[(5-iodo-1-naphthalenyl)sulfonyl]-1*H*-1,4-diazepine hydrochloride) is a potent inhibitor of myosin light chain kinase (MLCK, IC50=300nM) purchased in powder form (TOCRIS), resuspended in DMSO at a final concentration of 5mM, and stored at -80°C for no more than 6 months.

2.2.8 Y-27632

Y-27632 (chemical name: (1R,4r)-4-((R)-1-aminoethyl)-N-(pyridin-4-yl)cyclohexanecarboxamide dihydrochloride) is a selective Rho associated coiled-coil containing protein kinase (ROCK) I/II inhibitor (IC50=140nM, 300nM respectively) purchased in powder form (Selleckchem), resuspended in DMSO at a final concentration of 5mM, and stored at -80°C for no more than 6 months.

2.2.9 WZ-4003

WZ-4003 (chemical name: Propanamide, N-[3-[[5-chloro-2-[[2-methoxy-4-(4-methyl-1-piperazinyl)phenyl]amino]-4-pyrimidinyl]oxy]phenyl]-) is a selective NUAK family kinase (NUAK)1/2 inhibitor (IC50=20nM, 100nM respectively) purchased in powder form (Selleckchem), resuspended in DMSO at a final concentration of 10mM, and stored at -80°C for no more than 6 months.

2.3 RNAi Transfection

The RNAi sequences used throughout this thesis have been generated according to Tuschl's rules of design and have been cross checked using the siRNA check software available from the National Cancer Institute. RNAi sequences used are presented in Table 2.1. RNAi oligos were stored at 20°C in aliquots at a concentration of 50µM. Lipofectamine® RNAiMAX transfection reagent (Thermo Fisher) was used for delivery of RNAi into cell lines. Transfection mixes were prepared in Eppendorf tubes under sterile conditions by adding the appropriate amount of siRNA to give a final concentration of 25nM in the desired cell culture vessel. Dilution was performed using basal media (RPMI-1640 + 2mM L-glutamine, no serum). Transfection mixtures were incubated for 30 minutes at room temperature and then added directly to the cell culture plate in a dropwise fashion over pre-seeded cells. Pre-seeded cells were typically grown for 24 hours prior to transfection. Transfections were incubated for 24-96 hours at 37°C to acquire optimal gene knockdown.

Table 2.1 - RNAi Targets and Sequences

RNAi Target	RNAi sense sequence
PPP1R12A	AGUACUCAACCAUAAUUAATT
PPP1R14C-1	GAUAUCAUGACUCUAGCCATT
PPP1R14C-2	CAAAGGAGGUGGACACUCATT
PPP1R14C-3	CAGCCUAACCAAGGAUUAUTT
AR	CCAUCUUUCUGAAUGUCCU
Scrambled Control (Non-targeting RNAi)	UUCUCCGAACGUGUCACGUTT

2.4 Plasmid DNA Transfection

Mammalian expression plasmids were transfected into cells using TransIT®-LT1 transfection reagent (Mirus) according to the manufacturer's protocol. The required amount of plasmid DNA was added to basal media in an Eppendorf tube. 3µl of TransIT®-LT1 transfection reagent was added to the tube for every 1µg plasmid DNA to be transfected. The TransIT®-LT1 reagent and DNA mixture was incubated at room temperature for 30 minutes. TransIT®-LT1 reagent:DNA complexes were added in a dropwise fashion over pre-seeded cells. Cells were typically grown for 24 hours prior to transfection. The transfection was typically incubated for 48-96 hours before harvesting for the required assay.

The AR plasmid DNA was a kind gift from Professor Ralf Janknecht, University of Oklahoma. The PPP1R14C cDNA construct was purchased from the Harvard Medical School plasmid repository.

2.5 Bacterial Transformation of Plasmid DNA

Propagation of the PPP1R12A plasmid DNA was carried out by transforming NEB® 5-alpha *E. coli* chemically competent cells (New England BioLabs). Competent cells were stored at -80°C before gently thawing out on ice for 10 minutes when required. Approximately 500 ng of plasmid DNA was added to the competent cells, very gently mixed, and incubated on ice for 30 minutes before a 30 second 42°C heat-shock. Cells were placed back on ice for 2-5 minutes followed by the addition of 950 µl SOC medium (2% (w/v) peptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 20 mM glucose, 10 mM $MgCl_2$, 2.5 mM KCl, 10 mM $MgSO_4$) and incubated at 37°C, with agitation (200 rpm in a Thermotron incubator shaker (Infors HT)) for 1 hour. Cell

cultures were gently mixed and then 50-200µl culture was spread onto pre-warmed LB agar plates (1% (w/v) NaCl, 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1.5% (w/v) agar) containing 100 µg/ml ampicillin.

2.6 Culture of Transformed Bacteria

For DNA mini-prep cultures, single bacterial colonies were picked from LB agar plates using sterile pipette tips and incubated in 3 ml LB medium (1% (w/v) NaCl, 1% (w/v) tryptone, 0.5% (w/v) yeast extract) containing 100 µg/ml ampicillin for selection overnight at 37°C with rotation at 200 rpm. For maxi-prep cultures, a single bacterial colony was incubated in 5 ml LB medium containing 100 µg/ml ampicillin for 8 hours at 37°C with rotation at 200 rpm and then transferred to a conical flask containing 200 ml of pre-warmed antibiotic-containing LB medium and incubated overnight with rotation at 37°C.

2.7 Plasmid DNA Extraction

Plasmid DNA extraction from bacteria was performed using commercially available extraction and purification kits. For mini-prep cultures, the GenElute™ Plasmid Miniprep Kit (Sigma Aldrich) was used according to manufacturer's instruction. For maxi-prep cultures, the PureLink® HiPure Plasmid Filter Maxiprep Kit (Life Sciences, Invitrogen) was used according to the manufacturer's protocol. Extracted and purified DNA was resuspended in a suitable volume of TE buffer and DNA concentration measured using a Nanodrop spectrophotometer (Thermo Scientific).

2.8 SDS-PAGE

Cell lysates were generated by adding SDS sample buffer (125 mM Tris-HCl, pH6.8, 5% SDS, 10% glycerol, 10% β-mercaptoethanol and 0.01% bromophenol blue) directly to cultured cells washed with PBS. Samples were then boiled for 10 minutes at 100°C prior to loading into a gel. Various percentage gels were made using the quantities outlined in Table 2.2 using a mini-PROTEAN® tetra casting system (Bio-Rad) dependent upon the size of the protein of interest. Gels were loaded with pre-boiled protein samples alongside a prestained gel ladder mixture of recombinant protein of known sizes (Spectra Multicolor Broad Range Protein Ladder, Thermo). Protein samples were separated using a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad) filled with running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS) at a voltage between 100-200V.

*Table 2.2 - **SDS-PAGE Gel Recipes***

	10% Gel	15% Gel	
	Running	Running	Stacking
Acrylamide (30%)	3.33ml	5ml	1.25ml
Water (distilled)	1.67ml	-	1.25ml
2X Buffer A (750mM Tris-HCl, pH8.8, 0.2% SDS)	5ml	5ml	-
2X Buffer B (250mM Tris-HCl, pH 6.8, 0.2% SDS)	-	-	2.5ml
N,N,N',N'-tetramethylethane-1,2-diamine (TEMED)	20µl	20µl	6µl
Ammonium persulphate (10%)	100µl	100µl	50µl

2.9 Western blotting

Following protein separation by gel electrophoresis, proteins were transferred to Hybond ECL Nitrocellulose membrane (GE healthcare) using the Mini Trans-Blot Electrophoretic Transfer Cell system (Bio-Rad) according to manufacturer's instruction. The transfer was carried out using transfer buffer (25 mM Tris-HCl, pH8.3, 150 mM glycine, 10% methanol) at 100V for 1 hour or 30V overnight. Following the transfer of proteins from the acrylamide gel to nitrocellulose, the membrane was blocked using 5% (w/v) milk (Marvel)/ TBS (500 mM NaCl, 200 mM Tris-HCl pH7.5) for 1 hour at room temperature or overnight at 4°C with gentle rocking. Primary antibodies outline in Table 2.3 were typically made up to a final concentration of 200ng/ml. Membranes were then washed 3 times in TTBS (500 mM NaCl, 200 mM Tris-HCl, pH7.5, 0.001% Tween-20) for 5 minutes with gentle rocking and then incubated with specific primary antibody diluted in a volume of 4 ml 1% (w/v) milk/TTBS for 1 hour at room temperature or overnight at 4°C. The following day, membranes were washed 3 times for a total of 15 minutes with TTBS followed by incubation with a horse-radish peroxidase-conjugated secondary antibody, raised against the species of the primary antibody, for an hour at room temperature. After incubation, membranes were washed twice with TTBS for 5 minutes each and once in TBS for 10 minutes and then incubated with prepared ECL western blotting detection reagent (GE healthcare) for one minute with gentle agitation prior to X-ray

film (FujiFilm, SuperRX) exposure for signal detection. Films were developed using an automatic X-ray film processor model JP-33 (JPI Healthcare).

*Table 2.3 - **List of Antibodies, species, suppliers and applications***

Antibody	**Species**	**Supplier**	**Applications**
AR (N-20) (sc-816)	Rabbit	Santa Cruz	WB, IP, IF
Phosphorylated AR S81 (07-1375)	Rabbit	Merck Millipore	WB
Phosphorylated AR S213 (ab47562)	Rabbit	Abcam	WB
Phosphorylated AR S515	Rabbit	Gift from Dr Joanne Edwards University of Glasgow	WB
PPP1R12A (DU34962)	Sheep	University of Dundee - commercial	WB
Phosphorylated PPP1R12A S472 (S509C)	Sheep	University of Dundee- commercial	WB
Phosphorylated Myosin Light Chain S19 (#3675)	Mouse	Cell Signaling	WB, IF
PARP-1 (H-250) (sc-7150)	Rabbit	Santa Cruz	WB
MAPK1 (D-2) (sc-1647)	Mouse	Santa Cruz	WB
Phosphorylated MAPK1/MAPK3 T202/Y204 (sc-101760)	Rabbit	Santa Cruz	WB
α-tubulin (T9026)	Mouse	Sigma Aldrich	WB
Phosphorylated AKT S473 (sc-7985)	Rabbit	Santa Cruz	WB
AKT (H-136) (sc-8312)	Rabbit	Santa Cruz	WB
NF2 (A-19) (sc-331)	Rabbit	Santa Cruz	WB
Phosphorylated PLK-1 T210 (sc-135706)	Rabbit	Santa Cruz	WB
PLK-1 (F-8) (sc-17783)	Mouse	Santa Cruz	WB
Phosphorylated RB1 S807/811 (9308)	Rabbit	Cell Signaling	WB
RB1 (554140)	Mouse	BD Pharmingen	WB
Anti-Mouse-HRP	Rabbit	Dako	WB

Anti-Rabbit-HRP	Swine	Dako	WB
Anti-Sheep-HRP	Rabbit	ThermoFisher Scientific	WB
Anti-Rabbit-HRP	Swine	Dako	WB
Rabbit IgG	Rabbit	Diagenode	IP
Anti-Rabbit-Alexa Fluor 488 (ab150077)	Goat	Abcam	IF
Anti-Mouse-Alexa Fluor 594 (ab150116)	Goat	Abcam	IF

2.10 Live cell imaging

Cell growth was measured using the IncuCyte® ZOOM system (Essen BioSciences) which incorporates live cell imaging and analysis to provide quantification of cell proliferation over a given time period. As long as the morphology of the cells following treatment remains consistent, nor do the cells reach 100% confluency, this method of quantification provides an accurate surrogate for cell proliferation. Briefly, cells were seeded in the appropriate culture vessel and allowed to adhere for 24 hours where they would be 5-10% confluent. Following seeding, cells would be subjected to DNA/siRNA transfection or compound treatments and placed into the IncuCyte® ZOOM system. Images would then be taken from multiple fields per well every 4 hours over the desired period of time required for the assay. Proliferation was calculated by a measure of cell confluency and provided as a percentage. Raw percentage was normalised to the confluency of time point zero for each particular well and then calculated as fold changes.

2.11 Cell cycle analysis

Cell cycle analysis was carried out using fluorescence activated flow cytometry (FACs) in order to assess the percentage of cells in a particular phase of the cell cycle following target modulation. Propidium iodide (PI), a DNA intercalating dye, is incorporated into cells and upon excitation produces a quantifiable fluorescent signal corresponding to cellular DNA content as a result of stoichiometric binding. As such, cells in G2/M phase will have twice as much DNA as cells in G0/G1 phase, whilst cells in S-phase will possess an intermediary value. Excitation and fluorescent measurement of samples was carried out using the FACSCalibur™ (BD biosciences) with subsequent data analysis performed using Cyflogic 1.2.1 (CyFlo Ltd.). Cells

were seeded onto 6-well plates at an appropriate confluency that they would be exponentially growing at the time of harvest.

2.11.1 Cell harvest and staining

At the time of harvest, culture media was transferred to 5 ml round-bottom tubes (BD biosciences), cells washed once in PBS, retaining the PBS and adding to the previously removed culture media in tubes. Cells were detached as described in section 2.1.2 and added to the aforementioned tubes. Tubes were centrifuged at 400 x g for 5 minutes at 4°C and cell pellets washed with PBS before a second round of centrifugation after which the pellet was resuspended in 100 µl citrate buffer (250 mM sucrose, 40 mM sodium citrate, pH7.6). 400 µl of DNA staining buffer (20 µg/ml PI, 0.5 mM EDTA, 0.5% NP40, 10 µg/µl RNase A) was added to the cell suspension mix and incubated for 1 hour at 4°C in the dark.

2.11.2 Flow cytometry and data analysis

Samples were briefly mixed and passed through a Microlance hypodermic needle, 21G (BD biosciences) and loaded into the FACSCalibur™ machine using BD CellQuest™ to acquire data of 10,000 events (cells). Cell aggregates were discriminated and removed from analysis by gating only single cell populations. Histogram plots were generated using cell counts Vs FL2-A (fluorescent emission from a cell) and % of cells in cell cycle phases analysed from the histogram by the Cyflogic software.

2.12 RNA extraction

RNA was isolated from cells using TRIzol® Reagent (Life Sciences, Invitrogen) according to the manufacturer's protocol. Briefly, media was removed from the culture vessel (typically a 6-well plate) and cells washed once in PBS. 1 ml of TRIzol® Reagent was added directly to the cells and the homogenized sample was transferred to an Eppendorf tube and incubated at room temperature for 5 minutes. 0.2 ml of chloroform (Sigma Aldrich) was then added to each sample and shaken vigorously and incubated at room temperature for 3 minutes before centrifugation for 15 minutes at 12,000 x g at 4°C. The upper aqueous phase was transferred to a fresh tube and 0.5 ml of isopropanol (Fisher Chemicals) was added followed by incubation at room temperature for 10 minutes before centrifugation at 12,000 x g for 10 minutes at 4°C. The resultant RNA pellet was washed in 75% ethanol in nuclease-free water (Life Sciences, Invitrogen) and centrifuged at 7,500 x g for 5 minutes at 4°C. The ethanol wash was removed

and the RNA pellet was allowed to air dry. The pellet was resuspended in 20 µl of water and incubated at 55°C for 10 minutes to remove any secondary structures. RNA concentration was then quantified using the Nanodrop (Thermo Scientific) prior to reverse transcription. RNA was stored at -20°C.

2.13 Reverse transcription

RNA was reverse transcribed to cDNA using moloney murine leukaemia virus reverse transcriptase (M-MLV RT) (Promega) according to the manufacturer's protocol. Briefly, 1 µg RNA was made up to a final volume of 12.7µl with DEPC water. Seperately, a reverse transcription master mix was made up for the appropriate number of samples by adding 4 µl M-MLV 5X reaction buffer, 2µl 4mM dNTPs, 100µg Oligo(dT) and 0.3µl M-MLV RT enzyme to a final volume of 7.3µl. Samples were then incubated with the reverse transcription master mix for 1 hour at 37°C, followed by 5 minutes at 95°C to inactivate the RT enzyme. Resultant cDNA was further diluted 1:5 with nuclease-free water. Samples were stored at -20°C until required.

2.14 Quantitative real time polymerase chain reaction (qPCR)

Gene expression was quantified by subjecting cDNA samples to qPCR in 384-well format using a 7900HT Fast Real-Time PCR thermocycler (Applied Biosystems). Samples were run in triplicate using PCR reaction master mixes prepared for each gene target of interest in a total volume of 9 µl per well with the following components: 5µl Fast SYBR Green Master Mix (2X) (Thermo Fisher), 0.4µl forward primer (see Table 2.4), 0.4µl reverse primer and 3.2µl DEPC water.

Reaction master mixes were loaded onto a 384-well plate (Applied biosciences), followed by 1µl of sample cDNA. Using an absolute quantification method, a standard curve was generated by loading master mix reaction followed by the addition of serial dilutions of a cDNA sample (1, 0.5, 0.05, 0.005, 0.005 and a H_2O (no template only control) onto the plate. The prepared microplate was covered with an optical adhesive cover, briefly centrifuged (Labnet MPS 1000) and ran using the following PCR parameters: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute.

Table 2.4 ***Primer sequences used for qPCR****. Primers were diluted to a concentration of 25ng/µl in nuclease free water prior to qPCR reaction setup.*

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
PSA	GCAGCATTGAACCAGAGGAG	AGAACTGGGGAGGCTTGAG
TMPRSS2	CTGCTGGATTTCCGGGTG	TTCTGAGGTCTTCCCTTTCTCCT
KLK2	AGCATCGAACCAGAGGAGTTCT	TGGAGGCTCACACACCTGAAGA
PPP1R14C	GGAAGAAGAAATGCCAGAGGTAGA	GCATCAAGAAGATCATCAATGTCAAT
AR	AAGAGAAGTACCTGTGCGCC	TTCAGATTACCAAGTTTCTTCAG
HPRT1	TTGCTTTCCTTGGTCAGGCA	AGCTTGCGACCTTGACCATCT

Data acquired was analysed using Sequence Detection System (SDS) software version 2.3 (Applied Biosystems). Relative quantities for each gene of interest was calculated from the standard curve generated, these values were subsequently normalised using the relative quantity *HPRT1* gene expression values for each sample.

2.15 Luciferase reporter assay

Luciferase reporter assays were performed in HEK293T cells due to their high transfection efficiency. Cells were seeded at a density of $2x10^4$ per well of 24-well plate in DCC media and were reverse transfected with 100ng/well p3xARE-luc, 100ng/well pCMV-β-galactosidase construct and 50ng/well androgen receptor expressing plasmids. Cells were the incubated with the transfection mix for 48 hours with DHT treatments (if required) applied for an additional 24 hours prior to harvesting in 100µl 1x reporter lysis buffer (Promega) per well. Plates were incubated at 37°C for 15 minutes and then stored at -80°C until required. When needed, cells were thawed on ice, scraped, and pipetted up and down. 10µl of lysate was transferred to a 96 well white, flat bottom microplate (Greiner bio-one) containing 40µl DEPC water, which was then inserted into a FLUOstar Omega plate reader (BMG Labtech). The plate reader injected and mixed 50 µl luciferase assay substrate (Promega) into each well and measured luminescence before injecting into the next well. Data acquired was presented as light counts per second (LCPS). The transfection efficiency between experimental arms was measured using a β-galactosidase assay. To measure enzymatic activity, 10 µl of cell lysates

were transferred to a clear bottom 96 well plate (Corning) and mixed with 10 µl of β-galactosidase substrate (2mM $MgCl_2$, 100mM β-mercaptoethanol, 1.33mg/ml o-nitrophenyl-β-D- galactopyranoside, 100mM sodium phosphate buffer, pH7.3) at 37°C for 5 minutes until the lysate/substrate mix turned from colourless to yellow. 50 µl of 1M Na_2CO_3 was added to terminate the enzymatic reaction and the plate was then read on a microplate reader (Model 680, Bio-Rad) at Abs_{480}. Results of luciferase assays were presented as normalised LCPS (LCPS/β-gal).

2.16 Immunofluorescence

LNCaP cells were grown in 6-well plate format (Corning) upon glass coverslips. Following RNAi modulation, cells were fixed with 2mls paraformaldehyde (2%v/v) overnight at 4°C. The next day cells were washed twice with PBS prior to being permeabilised with 0.1% Trition-X-100 (PBS) for 15 minutes. Cells were then blocked for 1 hour at room temperature in 0.1% Triton-X-100, 1% BSA – PBS. Coverslips were then incubated with blocking solution containing 4µg/ml primary antibody overnight at 4°C. Coverslips were then washed 3 times with room temperature PBS before addition of the secondary antibody, 1µg/ml in blocking solution, for 1 hour at room temperature. Coverslips were then washed twice with PBS and mounted to glass slides with DAPI mounting medium (Vectashield), air-dried, and visualised using a Leica DMR fluorescent microscope.

2.17 Immunoprecipitation

HEK293T cells were cultured as described in section 2.1.2 and underwent AR plasmid DNA transfection as described in section 2.4. Following a 48 hour period, cells were washed with 4°C PBS and lysed with 1ml immunoprecipitation buffer (0.5ml 1M Tris-HCl pH7.5, 0.375ml 4M NaCl, 100µl 0.1M phenylmethane sulfonyl fluoride (in methanol), 1X cOmplete mini protease inhibitor tablet (Roche), 10µl 1M dithiothreitol, 100µl 10mM sodium orthavanadate, 100µl nonyl phenoxypolyethoxylethanol, 8.9ml water). Cells were scraped and collected in an Eppendorf Tube and gently agitated for 30 minutes by rotation at 4°C. In parallel, protein-G sepharose (PGS) beads were prepared by resuspending 20µl of PGS slurry in 500µl immunoprecipitation lysis buffer, followed by centrifugation at 12,000 x g for 3 minutes. The supernatant was then discarded and the PGS beads resuspended in 500µl immunoprecipitation buffer. This wash process was repeated 3 times and the PGS beads were then stored at 4°C. 50µl cell lysate was then removed to be used as an input control for

western blot analysis. The remaining 950µl was then incubated with 20µl of the prepared PGS solution at 4°C for 1 hour. The samples were then centrifuged for 3 minutes at 12,000 x g, with the supernatant being split evenly (475µl per tube) between fresh Eppendorf tubes containing 10µg of either AR (N20) antibody, or Rabbit IgG. Samples were then gently rotated overnight at 4°C. The following day samples were centrifuged for 3 minutes at 12,000 x g and the supernatant discarded. PGS Samples were washed once with immunoprecipitation wash buffer A once, and twice with wish buffer B, centrifuged at 12,000 x g for 3 minutes between each wash. Final PGS samples were then boiled for 10 minutes in 100µl SDS sample buffer. Immunoprecipitation samples were then ready to be analysed by western blot.

2.18 Boyden Chamber Assay

A migration assay employing the Boyden chamber transwell approach was performed as previously described (Chen, 2005). Briefly, LNCaP cells were grown as described in section 2.1.1 and transfected with siPPP1R14C-1 as previously described for 48 hours. Cells were collected and resuspended in basal media prior to counting. $10x10^2$ cells in 50µl basal media were then placed within the Boyden chamber (8µm pore size polycarbonate membrane, Merck Millipore) in 24 well plate format positioned within 250µl full media and incubated for 24 hours at 37°C. Cells were then fixed with 0.5mls paraformaldehyde (2%v/v) for 30 minutes at room temperature prior to washing 3 times with PBS. Migrated cells were then stained with crystal violet and visualized using an EVOS XL Core Cell Imaging System (Thermo Scientific). The cells within 8 independent fields of view were counted for each experimental repeat.

2.19 Nuclear-Cytoplasmic Fractionation

Nuclear-cytoplasmic fractionation was performed using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) according to the manufacturer's protocol. Briefly, LNCaP cells were cultured as previously described in DCC media and transfected with siPPP1R14C-1 or the scrambled control RNAi oligo. Cells were incubated for 72 hours prior to a 30 minute 10nM DHT stimulation. Cells were then harvested according to the manufacturer's protocol, with the isolated nuclear and cytoplasmic lysates stored at -20°C until analysis by western blot.

2.20 RNA sequencing

LNCaP cells were cultured as previously described in section 2.1.1 and underwent RNAi depletion as described in section 2.3. RNAi knockdown was performed in LNCaP cells grown

for at least 5 passages independently of each other. Briefly, RNA extraction was performed by column filtration using the commercially available RNeasy Plus Kit (Qiagen) according to manufacturer's protocol. Isolated RNA was then quantified by Nanodrop (Thermo Scientific) and validated by qPCR as described in sections 2.13 and 2.14. Once the level of knockdown for each sample was established, RNA samples were subject to analysis through use of the 2100 Bioanalyzer (Agilent Technologies) to ascertain the integrity of the RNA sample. The RNA integrity number (RIN) value ranges between 0-10, with 10 representing the maximum integrity. Only samples with a RIN value >9 were taken forward for RNA sequencing. RNA sequencing was performed by Aros Applied Biotechnology, Denmark on the Illumina HiSeq 2500 platform. RNA samples were prepared using Stranded Total RNA Sample Prep kit (Illumina), depleted of ribosomal RNA using the RiboZero Gold kit (Illumina) and sequenced with 100bp paired end reads resulting in an estimated ~80 million reads per sample. Reads were mapped to human genome hg19 using the RNA-sequence alignment tool STAR (Dobin *et al.*, 2013). Raw read counts were then calculated for each gene using HTSeq (Anders *et al.*, 2015) prior to differential gene expression analysis using DEseq2 (Love *et al.*, 2014). Differential gene expression enrichment analysis was performed using the gene set enrichment analysis (GSEA) software (Broad Institute) and publicly available datasets from the molecular signatures database (MSigDB, Broad Institute).

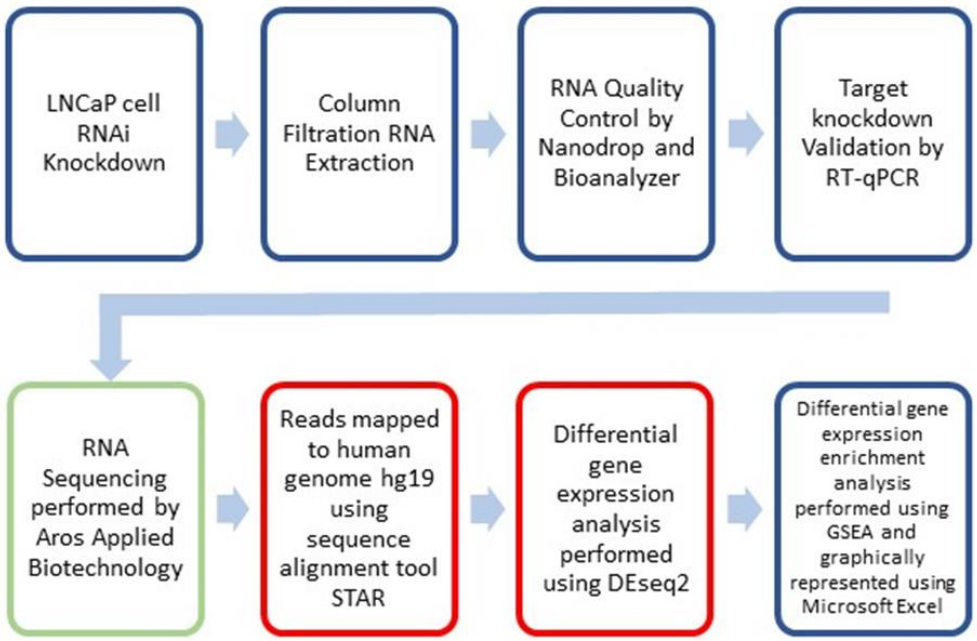


Figure 2.1 – ***RNA sequencing work-flow.*** *The work-flow for generating the PPP1R14C RNAi depletion dataset was performed as shown above. Tasks bordered in blue were performed by myself, whilst tasks bordered in red were performed by the in-house bioinformatition Dr. S. Nakjang, and the task in green performed by Aros Applied Biotechnology.*

2.21 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, California, USA) implementing paired and unpaired student t-tests where applicable.

Chapter 3.

Myosin Phosphatase is a Dynamic Regulator of Androgen Receptor Transcriptional Activity

3.1 Introduction

Myosin Phosphatase (MLCP) is heterotrimeric protein comprising the PP1δ catalytic subunit isoform, PPP1R12A regulatory subunit, and M20, a small 20 kDa protein that binds to the C-terminus of PPP1R12A (Ito *et al.*, 2004). It has been demonstrated that M20 does not impact on the phosphatase activity of MLCP, but as of yet, its exact role remains to be elucidated (Hartshorne *et al.*, 1998). For this reason, the implication of M20 will not be discussed in this thesis in the context of AR signalling. PPP1R12A is a 110 kDa protein expressed by a single gene on chromosome 12q15-q21.2 (Takahashi *et al.*, 1997). The N-terminus of PPP1R12A contains the PP1 binding motif (K/R-I/V-x-F/W) and a 7 ankyrin repeat domain, both of which have been shown to be involved in the interaction between PP1δ and PPP1R12A (Hirano *et al.*, 1997; Tanaka *et al.*, 1998; Toth *et al.*, 2000). A schematic representation of the PPP1R12A protein can be found in *figure* 3.1. Mutation of the 'KVKF' sequence in PPP1R12A demonstrated that this motif is essential for PP1δ binding. Using PPP1R12A peptide fragments of varying lengths it was demonstrated that MLCP had enhanced affinity for its substrate, phosphorylated myosin light chain serine 19 (pMLC-S19), compared to purified PP1δ, and that the NTD was sufficient to induce allosteric regulation of the catalytic activity (Johnson *et al.*, 1997). In addition, inclusion of the ankyrin repeat domain was shown to be required for maximal affinity toward pMLC-S19 (Tanaka *et al.*, 1998). Indeed, the ankyrin repeat domain has since been reported to act as a docking platform for interacting proteins and additional substrates (Ito *et al.*, 2004). PPP1R12A also contains 2 nuclear localization signals in the C-terminus, and whilst unbound to PP1δ it is predominantly found in the nucleus, however,

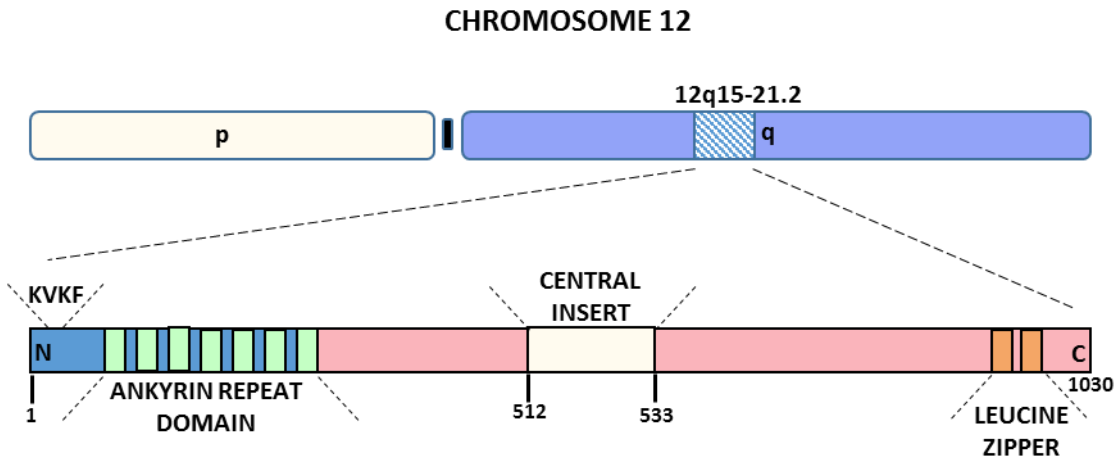


Figure *3.1* – ***Schematic Representation of the PPP1R12A Protein.*** *The PPP1R12A gene is located on chromosome 12q15-21.2 and encodes the 110kDa PPP1R12A protein. PPP1R12A contains the PP1 binding motif 'KVKF' at its N-terminus adjacent to the ankyrin repeat domain, whilst the C-terminus possesses a leucine zipper.*

when in complex as MLCP, it is found equally distributed throughout all cellular compartments (Wu *et al.*, 2005). PPP1R12A can be expressed as 2 alternatively spliced isoforms, representing the presence or absence of a 123 nucleotide centrally located exon (Shimizu *et al.*, 1994). The inclusion or exclusion of this exon dictates the presence or absence of 2 leucine zipper motifs in the C-terminus. Presence of this exon leads to a frameshift, leading to the inclusion of a premature stop codon and exclusion of the leucine zipper motifs (Khatri *et al.*, 2001). It was demonstrated that the presence of the leucine zipper motifs did not mediate an interaction between MLCP and protein kinase G (PKG), but did result in repressive modulation of MLCP in a cyclic guanosine monophosphate and PKG dependent manner (Huang *et al.*, 2004).

The catalytic activity of MLCP is heavily influenced through a number of different mechanisms, principally PTM and through association with endogenous inhibitory proteins (Eto *et al.*, 2007; Matsumura and Hartshorne, 2008). In this section I will introduce the concept of MLCP inhibition via interaction with endogenous inhibitory proteins, whilst PTM of MLCP will be discussed in section 9.1.

Protein phosphatase regulatory inhibitor subunit 14A (PPP1R14A) was the first MLCP inhibitory protein to be identified, and it was demonstrated to act as a signal transduction protein, responding to G-protein coupled receptor (GPCR) stimulation, and resulting in MLCP inhibition (Eto *et al.*, 1995). Subsequent studies identified PPP1R14-B, C, D through sequence homology and were successfully characterised as MLCP inhibitors (Eto *et al.*, 1999; Liu *et al.*, 2002; Liu *et al.*, 2004). Indeed, there is a very high degree of sequence homology between all MLCP inhibitory proteins, but no sequence homology with any other known PP1 inhibitory proteins (e.g. Inhibitor-1) despite similar mechanisms of inhibition (Eto *et al.*, 2007). All the members contain a conserved central PP1 holoenzyme inhibitory (PHIN) domain containing a pseudo palindromic motif incorporating an inhibitory threonine (Eto *et al.*, 2007). Solution NMR studies of PPP1R14A has revealed the 3D structure of the PHIN domain when both phosphorylated and dephosphorylated (Eto *et al.*, 2007). The PHIN domain consists of a loop structure surrounding the inhibitory threonine termed the ‘P loop’ followed by a 4-helix bundle that acts to stabilize the ‘P loop’. Upon phosphorylation of the inhibitory threonine a conformational change occurs whereby a hydrophobic core is created, causing the phospho-threonine to be displayed on the surface of the protein. In addition, a tyrosine residue flanking the threonine anchors the phospho-threonine in place. Mutagenesis studies proved that the

tyrosine residue is essential in the prevention of hydrolysis of the phosphorylated threonine (Hayashi *et al.*, 2001). As such, a stable complex is formed between MLCP and the inhibitory protein, resulting in transient inhibition of MLCP catalytic activity (Eto *et al.*, 2004). Indeed, the electrostatic surface potentials from MLCP inhibitory proteins appear to complement the acidic cluster formed by PP1δ and the PPP1R12A ankyrin repeat domain providing evidence for interactions between the inhibitory proteins with both the catalytic and regulatory subunits of MLCP (Terrak *et al.*, 2004). Furthermore, the MLCP inhibitory proteins can be dephosphorylated by purified PP1δ and indeed PP1δ containing holoenzymes, and as such, their specificity towards MLCP is more a question if they behave as inhibitors or substrates (Eto, 2009).

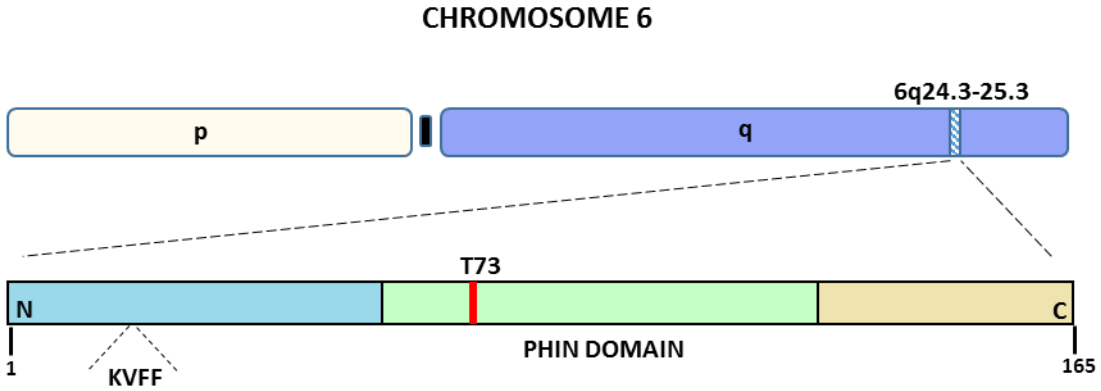


Figure *3.2* – ***Schematic Representation of the PPP1R14C Protein.*** *The PPP1R14C gene is located on chromosome 6q24.3-25.3 and spans 4 exons. The encoded protein contains an N-terminal PP1 binding motif, 'KVFF', and a central phosphatase inhibitory (PHIN) domain. Within the PHIN domain resides threonine 73, which upon phosphorylation, enhances the affinity of PPP1R14C for myosin phosphatase by 600-fold, where it behaves as a pseudosubstrate and inhibits subsequent myosin phosphatase activity.*

More specifically, the *PPP1R14C* gene is located on chromosome 6q24.3-q25.3 and spans 4 exons coding for a 165 amino acid protein with a mass of 19kDa as depicted in *figure* 3.2 (Liu *et al.*, 2002). Phosphorylation of threonine 73, located within the PHIN domain, dramatically increases the binding affinity of PPP1R14C to MLCP by 600-fold, resulting in an IC50 of 0.1nM (Eto and Brautigan, 2012). Multiple kinases have been demonstrated to directly phosphorylate PPP1R14C, or have been inferred through sequence homology surrounding the inhibitory threonine in the PHIN domain of PPP1R14A. These include PKC, integrin linked kinase (ILK), death-associated protein kinase 3 (ZIPK), rho-associated coiled-coil containing protein kinase (ROCK) I/II, protein kinase N (PKN) and p21-activated kinase (Hamaguchi *et al.*, 2000; Koyama *et al.*, 2000; MacDonald *et al.*, 2001; Deng *et al.*, 2002; Liu *et al.*, 2002; Takizawa *et al.*, 2002;

Erdodi *et al.*, 2003; Liu *et al.*, 2013). Crucially, phosphorylation of threonine 73 by any of the aforementioned kinases results in the same functional output, i.e. inhibition of MLCP. In addition, PPP1R14C also contains the classical 'KVFF' PP1 binding motif within its N-terminus, and has been shown to bind PP1δ when dephosphorylated, albeit with a highly reduced affinity (Eto and Brautigan, 2012). However, this cannot rule out additional mechanisms of inhibition or indeed additional signalling roles. PPP1R14C was initially identified as an MLCP inhibitory protein regulated by morphine, and the understanding of its role in cancer remains limited (Liu *et al.*, 2002). However, ectopic expression of PPP1R14C in the breast cancer cell line MCF-7 results in activation of the MAPK signalling cascade, a pathway frequently dysregulated in various malignancies (Wenzel *et al.*, 2007). A more recent study successfully identified PPP1R14C as the substrate for serine/threonine kinase 24 (MST3) and serine/threonine kinase 26 (MST4), resulting in phosphorylation of threonine 73, inhibition of MLCP and reduced cancer cell migration (Madsen *et al.*, 2015). The authors also went on to show that PPP1R14C was rapidly phosphorylated in response to serum stimulation, promoting its inhibitory impact on MLCP. In a separate study, PPP1R14C was shown to translocate to the nucleus upon phosphorylation, resulting in impaired dephosphorylation of the tumour suppressor retinoblastoma 1 (RB1), a known MLCP substrate (Kiss *et al.*, 2008; Dedinszki *et al.*, 2015). Although limited, preliminary evidence exists for PPP1R14C to act as a signal transduction molecule for a number of signalling cascades that may become of significance within the context of this thesis. Besides the role of PPP1R14C in the inhibition of MLCP, no other functions have been identified, and as such, the interplay between PPP1R12A and PPP1R14C in the regulation of MLCP activity will be assessed in the context of AR activity. In this chapter I aim to validate the impact of PPP1R14C and PPP1R12A RNAi depletion on AR transcriptional activity identified from the phosphatase RNAi screen.

3.2 Results

3.2.1 PPP1R14C depletion reduces androgen receptor transcriptional activity

Following RNAi transfection of LNCaP cells for 72 hours with 3 independent RNAi oligos targetting PPP1R14C (denoted siPPP1R14C-1, 2 and 3), a significant reduction (p-value <0.05) of approximately 80% in the mRNA expression of PPP1R14C was observed with all RNAi duplex sequences and is depicted in figure 3.3. LNCaP cells were stimulated with 10nM DHT for 24 hours in order to elicit an androgenic response prior to RNA extraction. Knockdown of

PPP1R14C with all 3 oligos resulted in statistically significant repression of the AR target genes *PSA, TMPRSS2* and *KLK2* (p-value < 0.05). siPPP1R14C-1 had the most pronounced effect on *PSA* mRNA expression, reducing its relative quantity normalised to HPRT1 by 90% compared to the scrambled control. siPPP1R14C-2 had the most profound impact on *TMPRSS2* mRNA expression, again, reducing gene expression by approximately 90% when compared to the scrambled control. siPPP1R14C-3 was capable of statistically reducing all 3 AR target genes by over 50% compared to the scrambled control. Crucially, all 3 RNAi oligos recapitualte the impact observed in relation to AR activity within the RNAi screen described in section 3.2 where PPP1R14C RNAi depletion resulted in a 60% reduction in luciferase activity compared to the scrambled control. Due to the consistency of knockdown and repression of AR target genes, particularly *PSA*, siPPP1R14C-1 was taken forward for future characterisation assays.

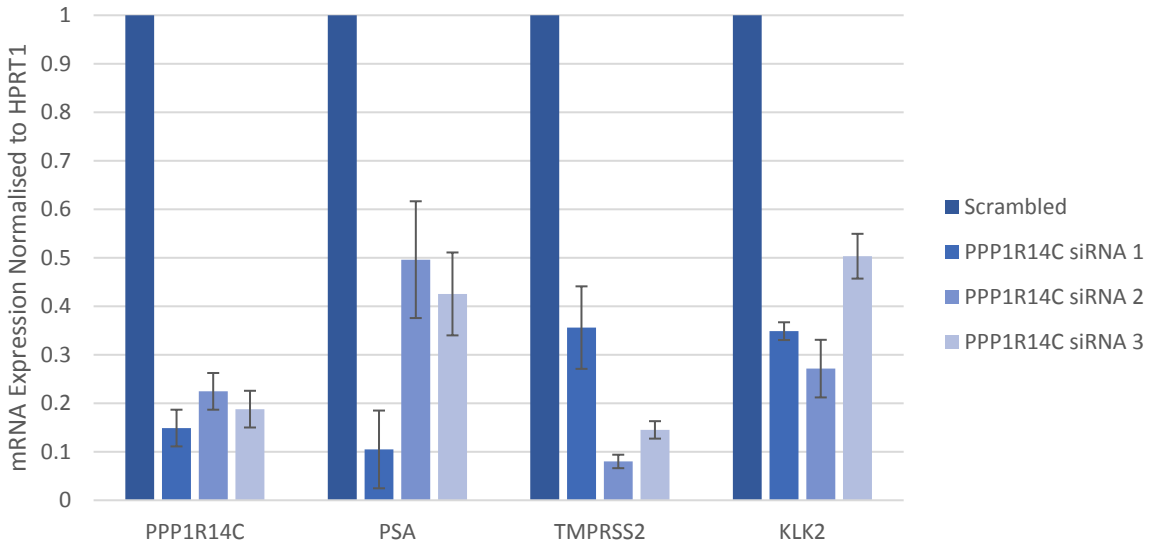


Figure *3.3 –* ***PPP1R14C Depletion Reduces AR Transcriptional Activity in the Presence of Androgen****. PPP1R14C was depleted in LNCaP cells using 3 independent RNAi oligos for 48 hours in DCC media prior to 24 hour 10nM DHT stimulation. AR regulated gene mRNA expression was quantified by RT-qPCR. Data represents n=3 mean ± sem. Unpaired student t test performed using graphpad..*

In the absence of androgen, depicted in figure 3.4, RNAi knockdown of PPP1R14C resulted in statistically significant repression of AR target gene mRNA expression (p-value < 0.05). In contrast to the androgen stimulated arm, all 3 target genes were repressed by approximately 80% when compared to the scrambled control. Unfortunately it was not possible to succesfully quantify the extent of PPP1R14C knockdown at the protein level. However, following RNAi depletion of PPP1R14C, it was possible to evaluate any alterations in MLCP activity through

the detection of phosphorylated MLC-S19. As such, in figure 3.5 a modest reduction in the detection of pMLC-S19 is observed when quantified by western blot using a phospho-specific antibody regardless of the presence or absence of androgen. This would suggest that the activity of MLCP has increased in response to the depletion of its endogenous inhibitory protein, PPP1R14C. It was also possible to quantify the protein expression of both the AR and PPP1R12A following knockdown, and no significant difference was observed compared to the scrambled control, albeit with the expected stabilization of the AR following androgen stimulation compared to the androgen deprived arm.

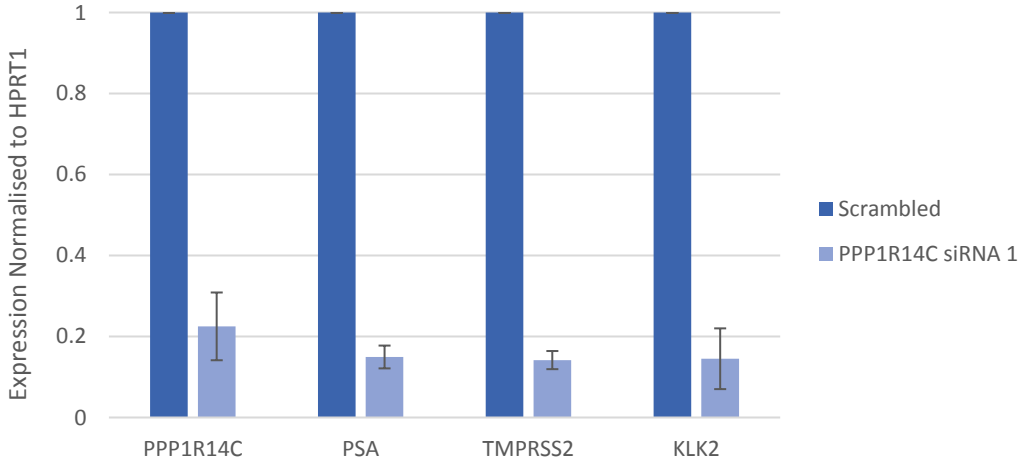


Figure *3.4* - ***PPP1R14C Depletion Reduces AR Transcriptional Activity in the Absence of Androgen***. *PPP1R14C was depleted in LNCaP cells for 72 hours in DCC media. AR regulated gene mRNA expression was quantified by RT-qPCR. Data represents n=3 mean ± sem. Unpaired student t test performed using graphpad.*

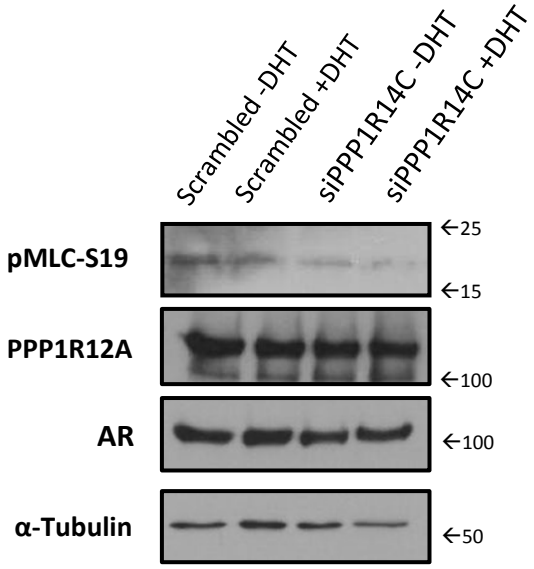


Figure *3.5* - ***PPP1R14C Depletion Increases the Activity of Myosin Phosphatase.*** *The impact of PPP1R14C depletion on MLCP activity was assessed by western blot analysis of the MLCP substrate pMLC20 in both the presence and absence of androgen.*

3.2.2 PPP1R12A depletion enhances androgen receptor transcriptional activity

Conversely, RNAi knockdown of PPP1R12A was quantifiable at the protein level, and at 72 hours post-transfection the protein expression of PPP1R12A were greatly reduced as observed in figures 3.6B and 3.6D. Indeed, only upon long exposure of the x-ray film were endogenous levels detected. Interestingly, AR protein levels increased dramatically following siPPP1R12A transfection, particularly in the absence of androgen (figure 3.6D). AR mRNA expression also increased significantly following PPP1R12A depletion. In the presence of androgen, AR mRNA expression increased by ~2.5-fold (p-value < 0.05, figure 3.6A), however, in the absence of androgen, a dramatic ~15-fold (p-value < 0.05, figure 3.6C) increase is observed and as such, AR mRNA levels complement AR protein expression. When investigating the impact of

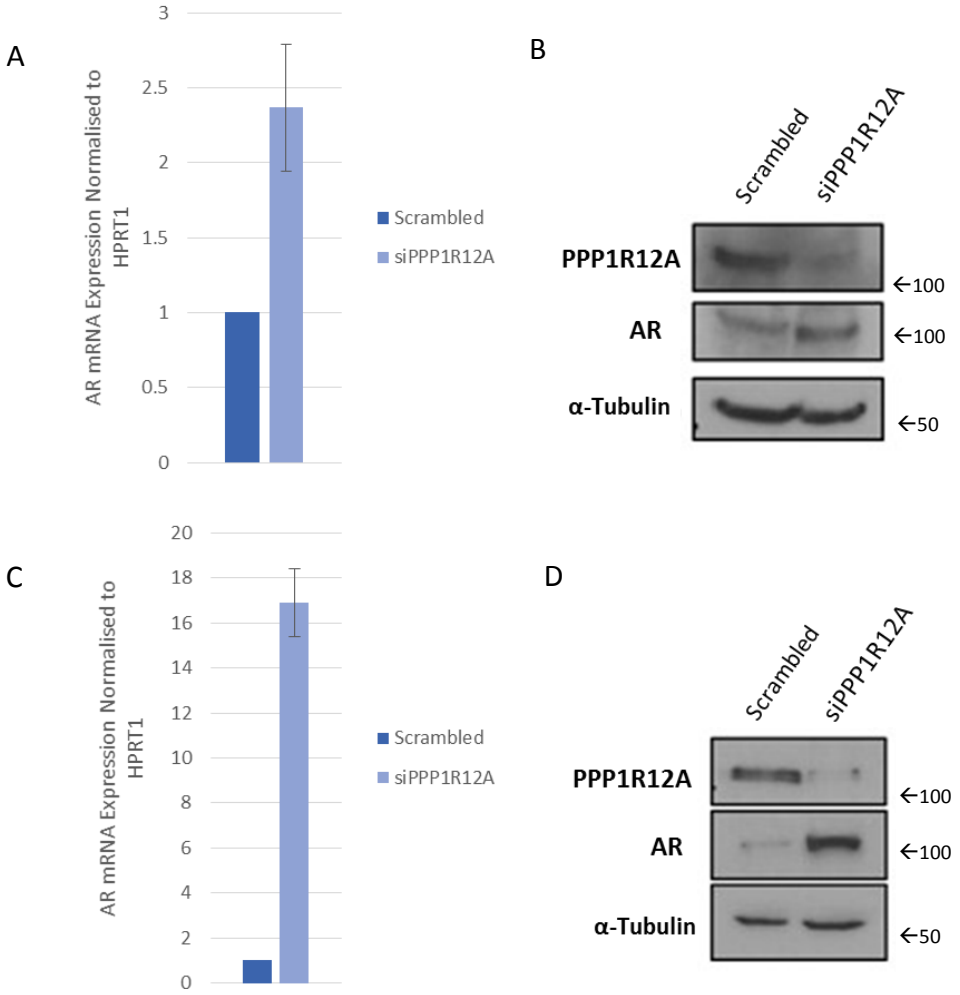


Figure *3.6 -* ***PPP1R12A Depletion Increases AR mRNA and Protein Expression in the Presence and Absence of Androgen****. A-B) PPP1R12A was depleted by RNAi knockdown in LNCaP cells stimulated with 10nM DHT for 24 hours. C-D) PPP1R12A was depleted by RNAi knockdown in LNCaP cells in the absence of androgen. mRNA expression was quantified by RT-qPCR. Data represents n=3 mean ± sem. Unpaired student t test performed using graphpad.*

PPP1R12A RNAi knockdown on AR transcriptional activity in the presence of androgen (figure 3.7A), an increase in *PSA, TMPRSS2* and *KLK2* mRNA expression was observed, albeit with only *PSA* mRNA expression increasing statistically significantly (p-value < 0.05) by approximately 4-fold. However, this increase in AR targetted gene transcription falls in line with the observations made from the RNAi screen. In contrast, the induction of AR activity in relation to the mRNA expression of *PSA, TMPRSS2* and *KLK2* in the absence of androgen wa significantly enhanced (p-value < 0.05). A 2-fold induction of *TMPRSS2*, 7-fold induction of *KLK2*, and a 15-fold induction of *PSA* mRNA expression is observed in figure 3.7B.

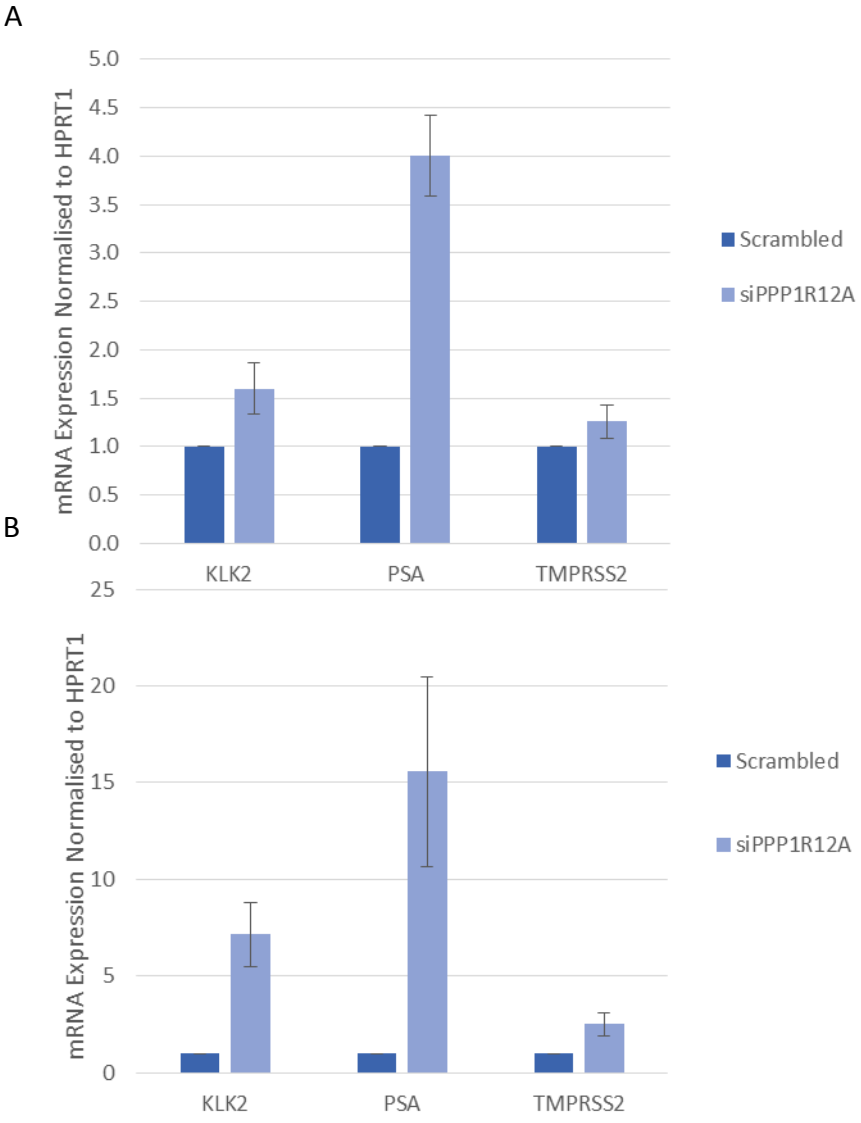


Figure *3.7* - ***PPP1R12A Depletion Increases AR Transcriptional Activity in the Presence and Absence of Androgen****. A) PPP1R12A was depleted by RNAi knockdown in LNCaP cells stimulated with 10nM DHT for 24 hours. B) PPP1R12A was depleted by RNAi knockdown in LNCaP cells in the absence of androgen. mRNA expression was quantified by RT-qPCR. Data represents n=3 mean ± sem. Unpaired student t test performed using graphpad.*

3.2.3 PPP1R14C increases androgen receptor transcriptional activity

Over-expression of PPP1R14C was performed in HEK293T cells as described in section 2.4 and quantified by luciferase reporter assay as described in section 2.15. In the presence of androgen, AR activity increased non significantly in correlation with increasing amounts of transfected PPP1R14C (figure 3.8A). In contrast, transfection of both 50ng and 100ng PPP1R14C in the absence of androgen (figure 3.8B), significantly increases the transcriptional activity of the AR compared to the 0ng transfected arm, reciprocating the results observed in figure 3.7B following PPP1R12A knockdown.

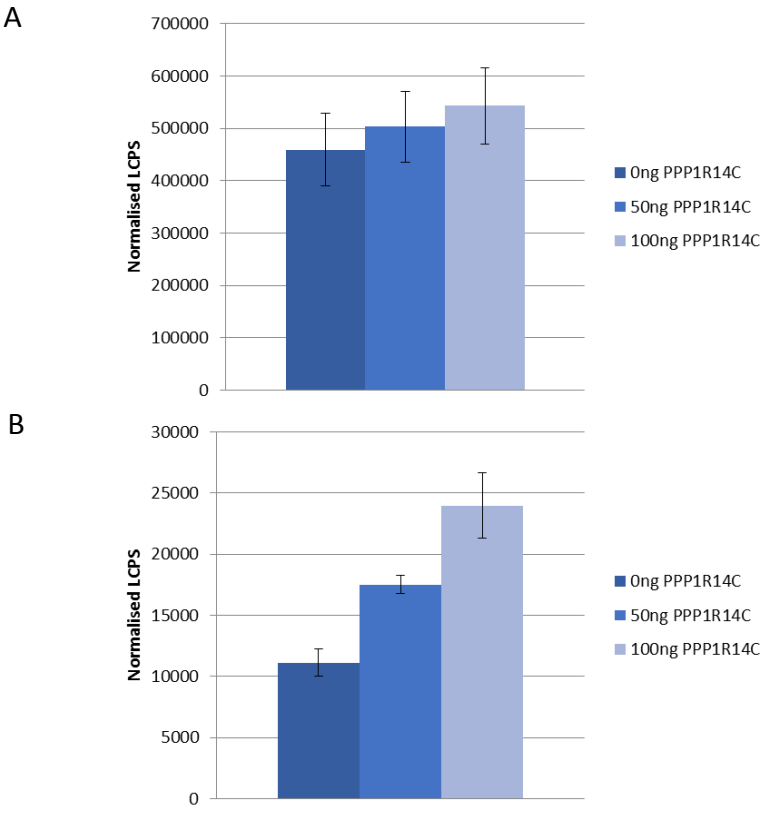


Figure *3.8 -* ***PPP1R14C Over-Expression Increases AR Transcriptional Activity****. A) In a luciferase reporter assay, HEK293T cells were transfected with increasing amounts of PPP1R14C plasmid DNA and stimulated with 10nM DHT for 24 hours prior to analysis. B) In a luciferase reporter assay, HEK293T cells were transfected with increasing amounts of PPP1R14C plasmid DNA in DCC for 72 hours. Data represents n=3 mean ± sd. Unpaired student t test performed using graphpad.*

Interestingly it was observed that PPP1R14C mRNA expression increased following 10nM DHT treatment in LNCaP cells over a 96 hour time period (figure 3.9). Maximal induction of PPP1R14C mRNA expression was observed at the 72 hour time point, corresponding to a 4.5

fold increase (p-value < 0.05). PSA mRNA expression increased rapidly as expected, reaching maximal induction of approximately 14-fold 48 hours post 10nM DHT stimulation. Similarly, upon RNAi knockdown of the AR, confirmed in figure 3.10B, PPP1R14C mRNA expression was significantly reduced at the 72 hour time-point (p-value < 0.05, figure 3.10A). The mRNA expression of PSA was also quantified to use as a surrogate for AR activity, and a significant reduction (p-value < 0.05) in PSA mRNA expression is observed as early as 24 hours, whilst PPP1R14C mRNA expression remained largely unchanged. Together this would suggest PPP1R14C is androgen regulated, but may represent a secondary effect of androgen signalling.

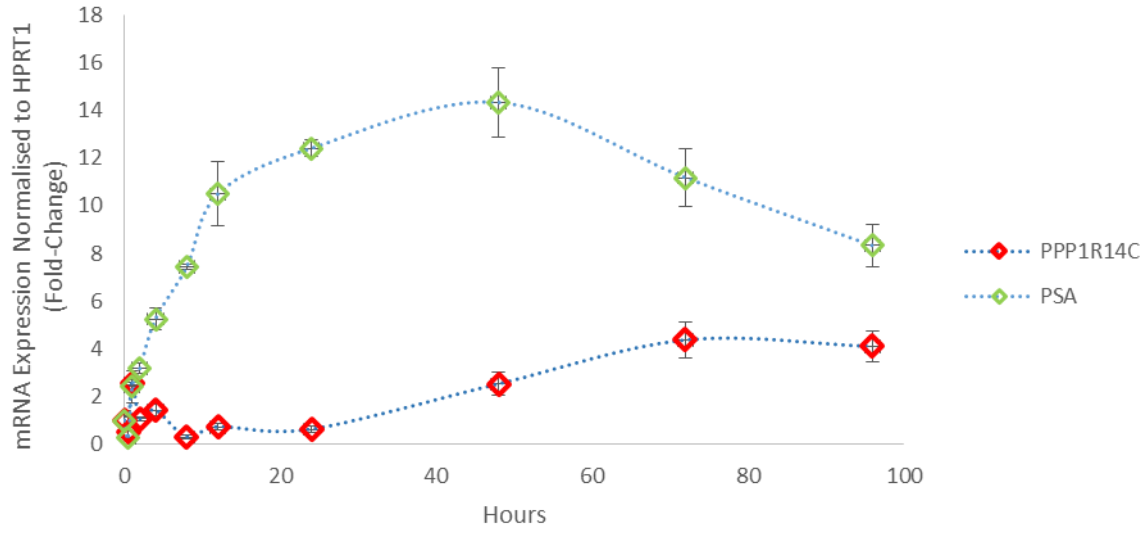


Figure 3.9 - ***PPP1R14C mRNA Expression Increases Following DHT Stimulation***. *LNCaP cells were stimulated with 10nM DHT and subject to RNA extraction at 0, 0.5, 1, 2, 4, 8, 12, 24, 48, 72 and 96 hour time-points. mRNA expression was quantified by RT-qPCR. Data represents n=3 mean ± sem. Unpaired student t test performed using graphpad.*

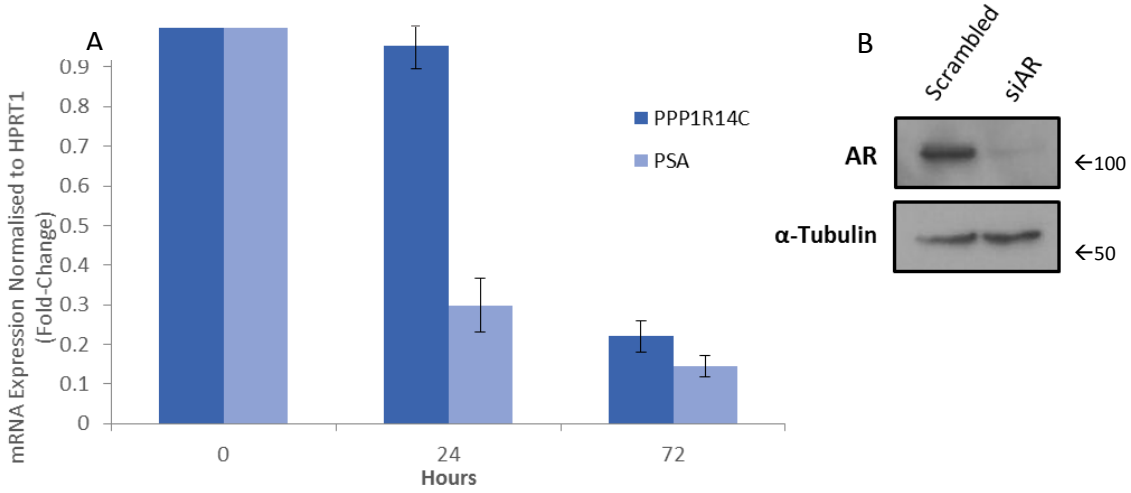


Figure 3.10 - ***AR Depletion Reduces PPP1R14C mRNA Expression***. *A) LNCaP cells were transfected with AR RNAi for 72 hours in full media and subsequently harvested for RT-qPCR analysis. B) Western blot analysis of AR RNAi depletion. mRNA expression was quantified by RT-qPCR. Data represents n=3 mean ± sem. Unpaired student t test performed using graphpad.*

3.3 Discussion

As discussed in section 1.2.1, the cellular effects of androgens are mediated through transactivation of the AR and its subsequent impact on gene transcription. In turn, prostate metabolism, survival, growth and differentiation are dependent upon the transcriptional activity of the AR. As such, targeting the AR has proven to be an effective strategy in the treatment of PC. ADT is initially very effective at preventing the progression of PC by limiting the availability of circulating ligands for the AR, however, invariably the disease progresses from an initial state of sensitivity to a state termed CRPC. However, it has been proven that the AR signalling axis remains active and is capable of driving disease progression, and as such, studies have proven that disruption of AR transactivation remains a viable therapeutic option, and indeed, 2nd generation antiandrogens display modest efficacy. Despite positive responses in a large proportion of patients, this too invariably fails. It is known however, that co-regulators of the AR are frequently dysregulated in advanced PC and are capable of inducing AR activity in both the presence and absence of androgens, and such are likely to contribute to treatment resistance, representing potential therapeutic targets. In this section, an AR inducing PP1 regulatory subunit, PPP1R14C, identified from an RNAi screen described in chapter 1, was validated at the qPCR level with 3 independent RNAi oligos (figure 3.3). Similarly, an AR repressing PP1 regulatory subunit, PPP1R12A, was also validated (figure 3.6). Crucially, these two subunits are components of the same PP1 holoenzyme, MLCP. PPP1R12A is the regulatory subunit that provides substrate specificity to PP1, whilst PPP1R14C is an inhibitory subunit that upon phosphorylation by a range of independent signalling effectors, behaves as a pseudo-substrate and transiently inhibits MLCP activity. In the context of AR signalling, when MLCP activity is retained through the RNAi depletion of PPP1R14C, a dramatic reduction in the expression of AR regulated gene expression was observed in both the presence and absence of androgens (figure 3.3 and figure 3.4). This provides an initial piece of evidence for the ligand independent regulation of the AR by MLCP. Furthermore, it is demonstrated that PPP1R14C mRNA expression increases following androgen stimulation, albeit at prolonged time-points compared to primary AR target genes such as PSA (figure 3.9). This may suggest that PPP1R14C expression increases as the levels of intracellular androgens are reduced. With the half-life of DHT being 24 hours, it is plausible that PPP1R14C mRNA expression increases under androgen depleted conditions to enhance AR transcriptional activity under diminished androgen levels. In addition, ectopic expression of PPP1R14C

enhanced the transcriptional activity of the AR, particularly in the absence of androgen (figure 3.8). Interestingly, the maximum induction of PPP1R14C mRNA expression coincided with the negative expression of PSA mRNA (figure 3.9). This would suggest that PPP1R14C may be involved in a delayed positive feedback loop, potentiating the AR signalling axis under reduced androgen levels. Interrogation of the most recent publicly available PC datasets using CBioportal has revealed that PPP1R14C is frequently dysregulated in PC samples. PPP1R14C was found to undergo both genomic amplification and deletion, but the most common form of dysregulation was increased mRNA expression, suggesting increased expression of PPP1R14C may play a role in disease progression. PPP1R14C was found to be dysregulated in 6-26% of patients from 6 publicly available datasets (Barbieri *et al.*, 2012; Baca *et al.*, 2013; 'The Molecular Taxonomy of Primary Prostate Cancer,' 2015; Dan *et al.*, 2015; Beltran *et al.*, 2016; Kumar *et al.*, 2016)

Conversely, when the formation of MLCP was prevented through RNAi knockdown of PPP1R12A, a dramatic increase in AR transcriptional activity was observed (figure 3.6). This too was more pronounced in the absence of androgen. It could be possible that AR activity in the presence of androgen is already maximal and therefore any induction following PPP1R12A depletion may in fact not be physiologically possible, hence explaining in part why the effect is less pronounced following androgen stimulation. However, the extent of AR transcriptional induction from both arms does appear to correlate with the level of induction of AR mRNA and protein expression (figure 3.7). As such, the ratio of AR mRNA and protein expression following PPP1R12A RNAi knockdown compared to the scrambled control is much higher in the absence of androgen than in the presence of androgen. However, despite the increases in AR protein expression, it may be expected that any induction in AR activity in the absence of androgens would be minimal, due to LNCaP cells being androgen responsive. This adds additional evidence that MLCP plays a role in AR regulation in a ligand independent manner. Furthermore, in contrast to the impact on AR expression by PPP1R12A knockdown, no observable impact on AR protein expression was witnessed following PPP1R14C knockdown (figure 3.5). This would suggest that MLCP is capable of regulating both AR expression, and activity at the post-translational level. A schematic diagram representing this chapters findings can be found in figure 3.12.

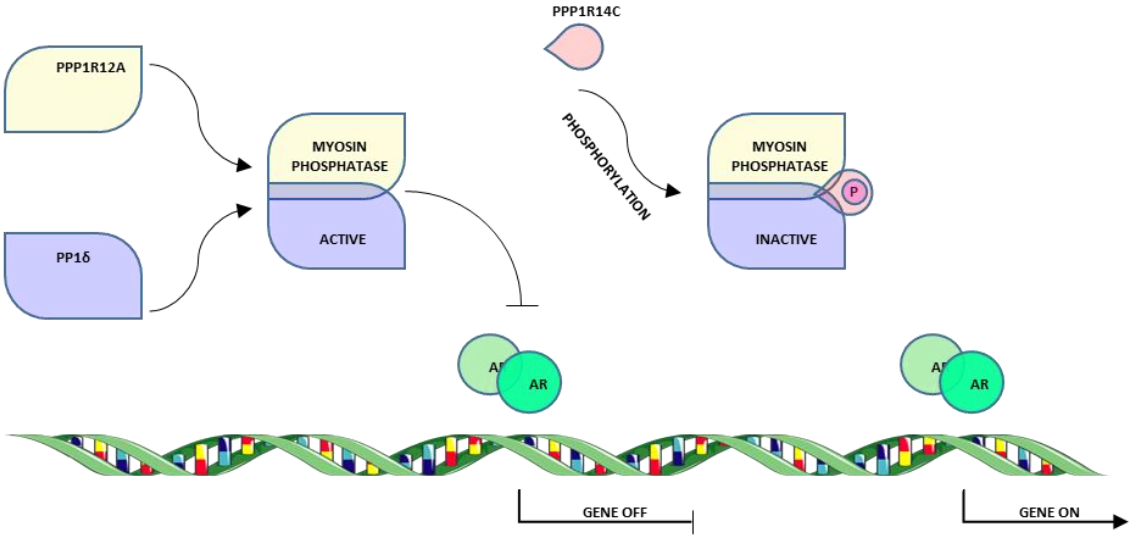


Figure *3.12* – ***Schematic Summary of Section 3 Results.*** *The PP1 holoenzyme MLCP was found to be a negative regulator of AR function, whilst the endogenous MLCP inhibitory subunit, PPP1R14C, was found to be a positive regulator of AR function. RNAi depletion of PPP1R14C resulted in enhanced MLCP activity, which correlated with a reduction in AR transcriptional activity. Conversely, upon RNAi depletion of PPP1R12A, AR transcriptional activity increased.*

Chapter 4.

Characterisation and Validation of a PPP1R14C RNAi Gene Signature Obtained by RNA sequencing

4.1 Introduction

Depletion of PPP1R14C by RNAi transfection has proven to result in significant repression of AR transcriptional activity. However, the underlying mechanisms of enhanced MLCP activity on independent signalling cascades in the context of this project remain unknown, and as such, known substrates of MLCP will be discussed in this section, and their relevance discussed in correlation with a global gene signature obtained by RNA sequencing following PPP1R14C RNAi depletion in LNCaP cells.

Classically, MLCP has been investigated within the context of actomyosin contractility. Increases in intracellular Ca^{2+} leads to the activation of calmodulin and subsequent activation of myosin light chain kinase (MLCK) (Allen and Walsh, 1994). MLCK specifically phosphorylates MLC-S19, resulting in cross bridging between myosin and actin filaments, and ultimately contractile force (Allen and Walsh, 1994). Reversible phosphorylation of MLC-S19 is essential for cytoskeletal rearrangements (Somlyo and Somlyo, 1994). pMLC-S19 dephosphorylation is catalysed by MLCP (Ito *et al.*, 2004). However, it is emerging that MLCP plays a critical role in the dephosphorylation of a number of clinically important proteins, particularly in relation to oncogenic malignancies.

Initial studies observed that PP1 was capable of dephosphorylating the tumour suppressor RB1 *in-vitro*, with subsequent research demonstrating that inhibition of PP1 with either okadaic acid or tautomycin led to sustained C-terminal hyperphosphorylation of RB1 (Alberts *et al.*, 1993; Ludlow *et al.*, 1993). More recent studies were able to confirm that PP1 and indeed PPP1R12A co-immunoprecipitate with RB1 and co-localize together in the nucleus (Vietri *et al.*, 2006; Kiss *et al.*, 2008). Furthermore, structural analysis of the PP1-RB1 interaction revealed that the binding of PP1 to RB1 blocked kinase access to the CDK docking sites on RB1 whilst retaining RB1 activity (Hirschi *et al.*, 2010). However, the most relevant publication to date demonstrated that over-expression of PPP1R14C results in the maintenance of hyperphosphorylated RB1, and upon stimulation with the PKC activator, PMA, increases in the phosphorylation of the inhibitory threonine of PPP1R14C, threonine 73, correlate with increases in the phosphorylation of RB1 (Dedinszki *et al.*, 2015). It should be noted that the phospho-specific antibody used to detect the phosphorylation of PPP1R14C is

in fact an antibody raised against phosphorylated PPP1R14A and although the peptide sequences are highly homologous, specificity should be a concern, particularly as a suitable negative control was not presented. Crucially, it is known that dephosphorylation of RB1 leads to enhanced activity (Manning and Dyson, 2011), and as such, MLCP is a positive regulator of RB1 tumour suppressor function.

Another tumour suppressor known to be dephosphorylated and subsequently activated by MLCP is merlin (NF2) (Jin *et al.*, 2006). The authors went on to show that MLCP specifically dephosphorylates the inhibitory phosphorylation residue serine 518, and that over-expression of the MLCP inhibitory protein PPP1R14A is sufficient to induce tumorigenic transformation in NIH 3T3 mouse embryonic fibroblast cells. NF2 is a well-documented tumour suppressor and is known to repress multiple oncogenic signalling cascades including the RAS, MTOR and HIPPO pathways (Morrison *et al.*, 2007; James *et al.*, 2009; Yu and Guan, 2013).

Additional MLCP substrates include polo like kinase 1 (PLK1) and HDAC7 (Parra *et al.*, 2007; Yamashiro *et al.*, 2008). During mitosis PPP1R12A binds to PLK1, antagonising its catalytic activity at centrosomes. Depletion of PPP1R12A leads to a pronounced increase in the phosphorylation of the PLK1 activating phospho-residue threonine 210, resulting in aberrant cell cycle progression (Yamashiro *et al.*, 2008). HDAC7 is a known AR transcriptional repressor (Karvonen *et al.*, 2006) and it was demonstrated that MLCP is responsible for the dephosphorylation of HDAC7 leading to enhanced nuclear localization and transcriptional repression (Parra *et al.*, 2007).

4.2 Results

4.2.1 PPP1R14C depletion leads to the differential expression of 826 genes ± 2-fold

To determine the impact of sustained MLCP activity, through PPP1R14C RNAi depletion, on the global LNCaP transcriptional program, RNA sequencing was performed using the Illumina HiSeq 2500 platform following 3 independent transfections of LNCaP cells, maintained separately for a minimum of 5 passages, with either siPPP1R14C-1 or the non-silencing scrambled control. RNA sequencing was employed as it results in low background signal, no hybridization issues and ultimately possesses the capacity to quantify absolute transcript expression levels as well as relative expression levels (Wang *et al.*, 2009). In order to maintain relativity to the RT-qPCR and luciferase data presented in section 3, LNCaP cells were cultured, maintained and siRNA transfected in the same manner as previously described in sections 2.1 and 2.3. Quality control and reads per lane can be found in supplementary figure 1.

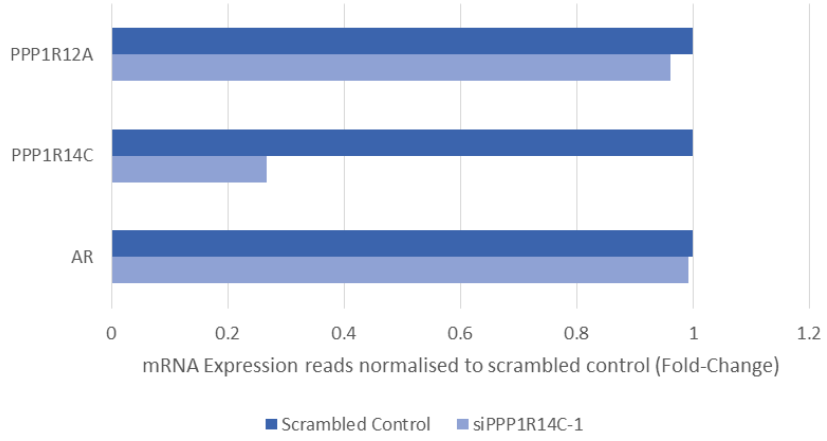


Figure *4.1* - ***PPP1R14C is significantly down-regulated following RNAi knockdown as quantified by RNA Sequencing***. *PPP1R12A, PPP1R14C and AR mRNA expression in LNCaP cells following PPP1R14C depletion and subsequent analysis by RNA sequencing*

Significant depletion of *PPP1R14C* was confirmed by RNA sequencing following RNAi transfection of LNCaP cells when compared to the scrambled control as presented in figure 4.1. RNAi knockdown of PPP1R14C resulted in no significant alterations in the mRNA expression of *PPP1R12A* or the *AR* as also depicted in figure 4.1. In a global context, PPP1R14C knockdown resulted in the statistically significant differential expression of 826 genes ± 2-fold compared to the scrambled control (figure 4.2). Crucially, the AR regulated genes *PSA* (KLK3), *KLK2* and *TMPRSS2* were all significantly down-regulated, and indeed, were amongst the top 20 down-regulated genes depicted in figure 4.3A. This validated the results obtained by RT-

qPCR and presented in section 3. Interestingly, there were more significantly up-regulated genes compared to down-regulated genes, with the top 20 up-regulated genes presented in figure 4.3B. This would suggest that PPP1R14C depletion does not impact on cellular transcription as a whole, but rather, impacts on a subset of transcriptional programs.

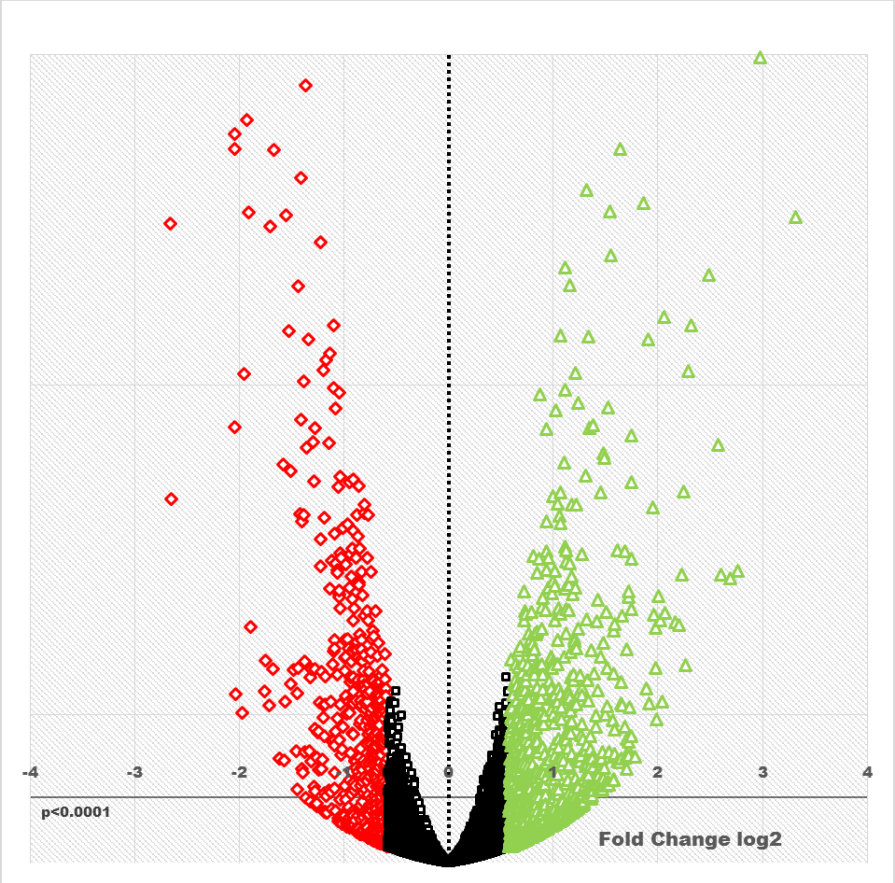


Figure ***4.2 - PPP1R14C Depletion leads to the Differential Expression of 826 Genes ± 2-Fold.*** *Figure generated using Microsoft Excel*

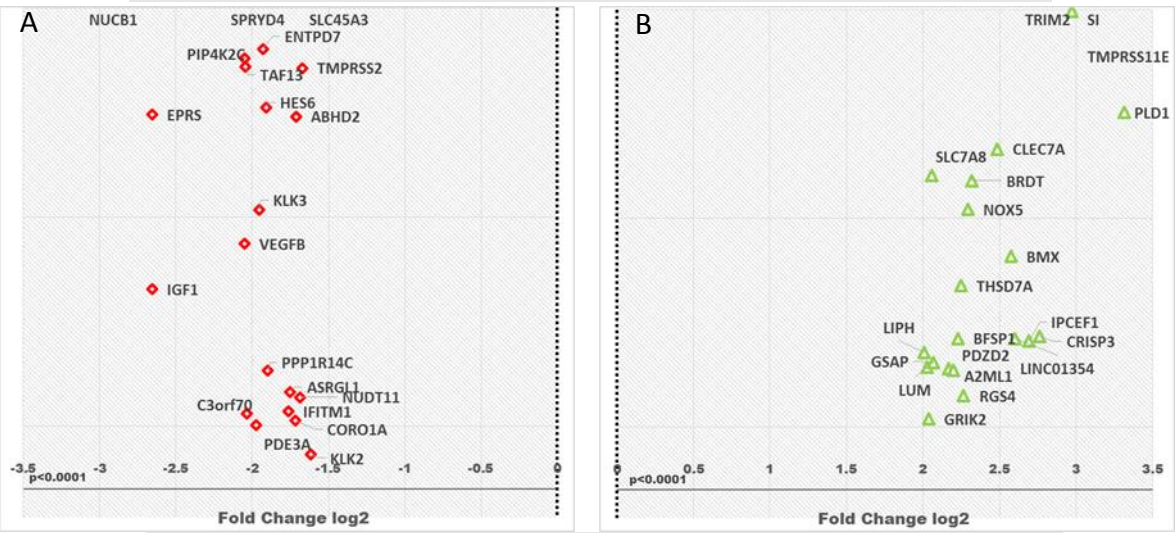
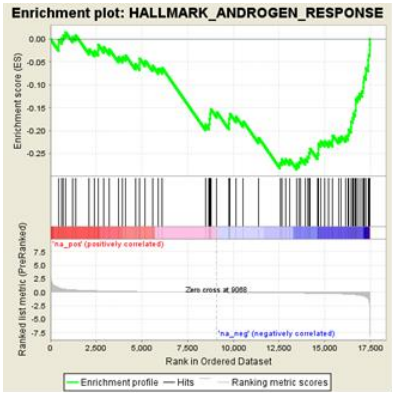


Figure ***4.3 - PPP1R14C Depletion leads to the significant induction and repression of clinically relevant proteins.*** *A) Top 20 Down-Regulated genes following PPP1R14C depletion in LNCaP cells B) Top 20 Up-Regulated genes following PPP1R14C depletion in LNCaP cells.*



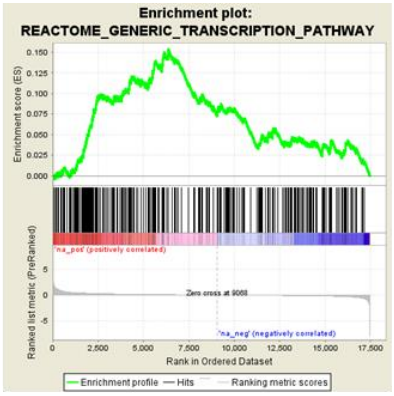


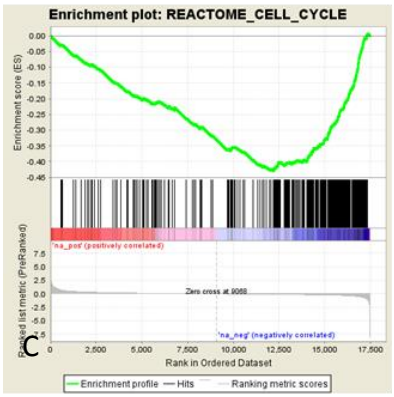
Figure *4.4* - ***Gene Set Enrichment Analysis of the PPP1R14C Knockdown Gene Signature I*** *A) GSEA Hallmark Androgen Response B) REACTOME Generic Transcription C) REACTOME Cell Cycle D) REACTOME G1-S Transition E) REACTOME M-G1 Transition. Gene set sizes can be found in brackets under the gene set name.*

A

Gene Set	HALLMARK ANDROGEN RESPONSE (101)
Enrichment Score (ES)	-0.2847436
Normalized Enrichment Score (NES)	-2.9946814
Nominal p-value	<0.001
FDR q-value	<0.001

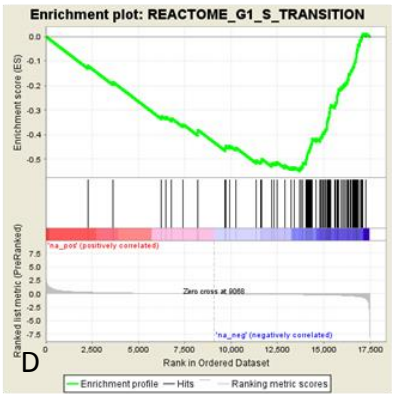
B

Gene Set	REACTOME GENERIC TRANSCRIPTION PATHWAY (352)
Enrichment Score (ES)	0.15347415
Normalized Enrichment Score (NES)	3.0385175
Nominal p-value	<0.001
FDR q-value	<0.001



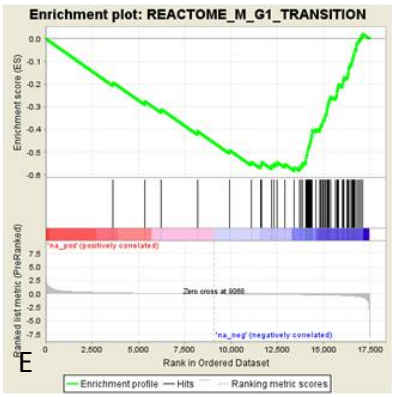
C

Gene Set	REACTOME CELL CYCLE (421)
Enrichment Score (ES)	-0.42977363
Normalized Enrichment Score (NES)	-8.842511
Nominal p-value	<0.001
FDR q-value	<0.001



D

Gene Set	REACTOME G1 S TRANSITION (112)
Enrichment Score (ES)	-0.5478876
Normalized Enrichment Score (NES)	-6.056714
Nominal p-value	<0.001
FDR q-value	<0.001

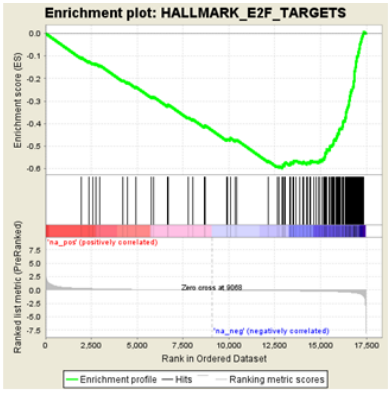


E

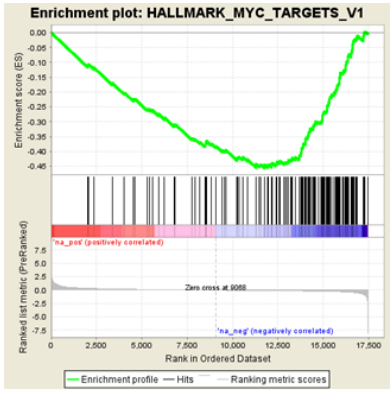
Gene Set	REACTOME M G1 TRANSITION (81)
Enrichment Score (ES)	-0.5829419
Normalized Enrichment Score (NES)	-5.5102086
Nominal p-value	<0.001
FDR q-value	<0.001

4.2.2 Myosin phosphatase is a negative regulator of the cell cycle

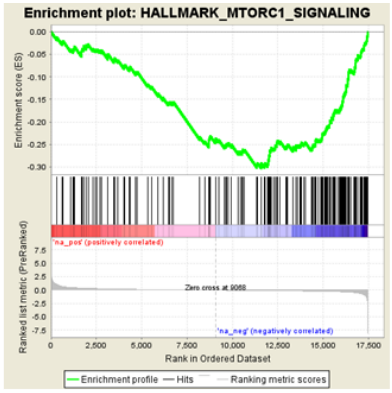
In order to elucidate the cellular pathways impacted, gene set enrichment analysis (GSEA) was performed using annotated gene sets from 'The Molecular Signatures Database'. Again, following GSEA it was possible to confirm the impact of PPP1R14C depletion on androgen signalling. The siPPP1R14C-1 data set negatively correlated with the hallmark 'Androgen Response' gene set, resulting in a normalised enrichment score (NES) of -2.99, and a false discover rate (FDR) q-value of <0.001 (figure 4.4A). First, GSEA was performed against REACTOME datasets in the attempt to identify the most significantly impacted cellular pathways. The top 50 positively and negatively correlating datasets can be found in supplementary tables 1 and 2, however, it was clear that the positively correlating datasets were much less significant and possessed higher FDRs compared to the negatively correlating datasets, suggesting the down-regulated genes were of more biological significance. Indeed, the only pathway of note that positively correlated with the siPPP1R14C-1 dataset was the 'generic transcription' pathway (NES=3.04, FDR<0.001, figure 4.4B), confirming that cellular transcription was not being impeded at a global level and cells were still capable of driving active transcriptional programs. Many pathways negatively correlate with the siPPP1R14C-1 gene set, particularly those involved in cell cycle progression. The REACTOME cell cycle pathway as a whole had a NES of -8.84, p value <0.001 and FDR <0.001 (figure 4.4C). More specifically, genes from the REACTOME G1-S transition dataset are repressed, possessing a NES of -6.05, p value <0.001 and FDR <0.001 (figure 4.4D). Similarly, genes from the REACTOME M-G1 transition dataset are highly repressed, resulting in a NES of -5.51, p value <0.001 and FDR <0.001 (figure 4.4E). This strongly suggest that the increase in MLCP activity is significantly repressing the expression of genes required for cell cycle progression, or indeed, the activity of proteins required for cell cycle progression. Further interrogation of the GSEA hallmark datasets (supplementary table 3) revealed that E2F transcription factor target genes represent the most significantly repressed genes from the siPPP1R14C-1 data set (figure 4.5A). Enrichment plots result in a NES of -9.325, p value <0.001 and FDR <0.001. Other significantly enriched hallmark datasets were v-Myc avian myelocytomatosis viral oncogene homolog (MYC) and mechanistic target of rapamycin complex 1 (MTORC1) target genes, represented in figure 4.5B and 4.5C respectively. The next GSEA collection to be interrogated was 'oncogenic signatures'; data sets defined directly from



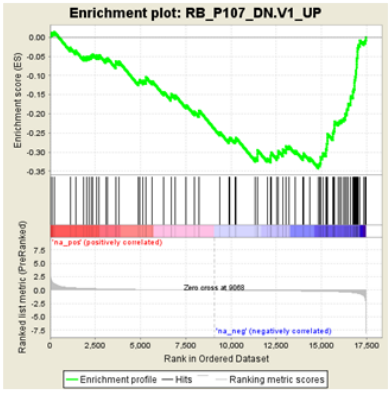
A	Gene Set	HALLMARK E2F TARGETS (200)
	Enrichment Score (ES)	-0.59757894
	Normalized Enrichment Score (NES)	-9.325936
	Nominal p-value	<0.001
	FDR q-value	<0.001



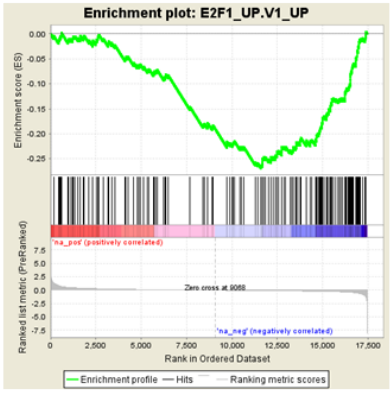
B	Gene Set	HALLMARK MYC TARGETS V1 (200)
	Enrichment Score (ES)	-0.4565567
	Normalized Enrichment Score (NES)	-6.753558
	Nominal p-value	<0.001
	FDR q-value	<0.001



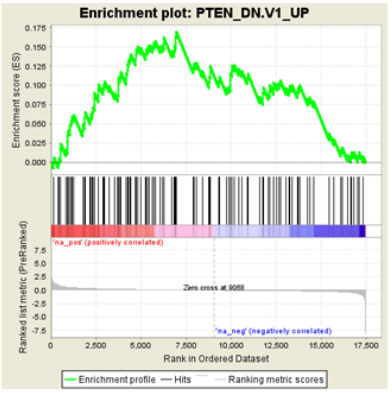
C	Gene Set	HALLMARK MTORC1 SIGNALING (200)
	Enrichment Score (ES)	-0.3026658
	Normalized Enrichment Score (NES)	-4.5127535
	Nominal p-value	<0.001
	FDR q-value	<0.001



D	Gene Set	RB P107 DN.V1 UP (140)
	Enrichment Score (ES)	-0.34232354
	Normalized Enrichment Score (NES)	-4.2409663
	Nominal p-value	<0.001
	FDR q-value	<0.001



E	Gene Set	E2F1 UP.V1 UP (189)
	Enrichment Score (ES)	-0.26901853
	Normalized Enrichment Score (NES)	-4.004147
	Nominal p-value	<0.001
	FDR q-value	<0.001



F	Gene Set	PTEN DN.V1 UP (191)
	Enrichment Score (ES)	0.16940895
	Normalized Enrichment Score (NES)	2.2683954
	Nominal p-value	<0.001
	FDR q-value	0.020674372

Figure *4.5* - ***Gene Set Enrichment Analysis of the PPP1R14C Knockdown Gene Signature II*** *A) Hallmark E2F Targets B) Hallmark MYC Targets V1 C) Hallmark MTORC1 Targets D) RB P107 DN.V1 UP E) E2F1 UP.V1 UP F) PTEN DN.V1 UP*

microarray gene expression data from cancer gene perturbations. Interestingly, the siPPP1R14C-1 dataset positively correlated with genes up-regulated following PTEN RNAi depletion in A431, HCC827 and SKBR-3 cell lines (epidermoid carcinoma, lung adenocarcinoma and breast carcinoma, respectively, figure 4.5F). As previously mentioned, LNCaP cells are PTEN null, and therefore it is of some reassurance that a gene set obtained from LNCaP cells positively correlates with gene sets obtained following RNAi knockdown of PTEN. However, it is the negatively correlating data sets that represent the most promising insight into MLCP function in the context of this thesis. The siPPP1R14C-1 dataset negatively correlates with genes up-regulated following RB1 and RB-like 1 knockdown in keratinocytes (NES=-4.24, p value <0.001, FDR <0.001, figure 4.5D), whilst also negatively correlating with genes up-regulated following over-expression of E2F1 in mouse fibroblasts (NES=-4.00, p value <0.001, FDR <0.001, figure 4.5E), strongly suggesting PPP1R14C depletion impacts on the negative regulation of E2F1 by RB1. Increases in gene expression following over-expression of E2F3, enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2), MYC, platelet-derived growth factor (PDGF), interleukin 15 and MTOR also negatively correlated with the gene set obtained following PPP1R14C RNAi depletion and are outlined in supplementary table 4. Interrogation of 'motif based gene sets', based on the conserved cis-regulatory motifs from a comparative analysis of the human, mouse, rat and dog genomes, revealed that 15 of the top 20 negatively correlating data sets belonged to genes containing E2F binding motifs (supplementary table 5), again reinforcing the negative impact of PPP1R14C depletion on the activity of the E2F family of transcription factors.

4.2.3 PPP1R14C depletion restores a non-malignant gene expression profile

Finally, the siPPP1R14C-1 data set was run against gene sets obtained from studies investigating differential gene expression between benign prostate tissue and PC tissue. Genes found to be down-regulated in PC samples vs matched benign samples from two independent studies positively correlated with the PPP1R14C RNAi knockdown gene set. These are represented in figure 4.6. Conversely, 5 independent gene sets representing genes over-expressed in PC tissue vs benign prostate tissue all negatively correlated with the gene set following PPP1R14C depletion. These too are represented in figure 4.6.

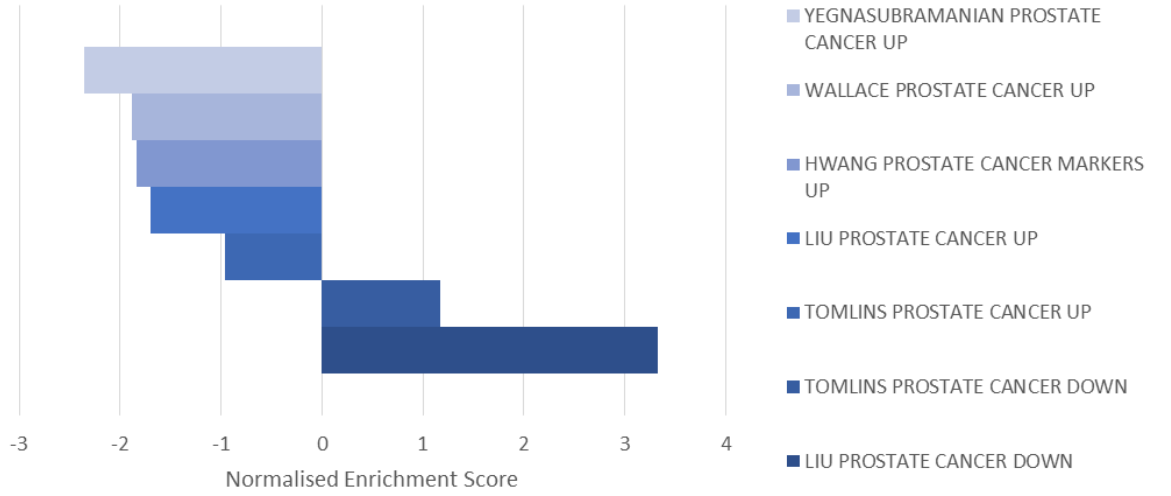


Figure ***4.6 - PPP1R14C Depletion Restores a Non-Malignant Prostate Gene Expression Profile.*** *Following knockdown of PPP1R14C by RNAi, genes identified as being up-regulated in PC vs Normal (matched tissue) were down-regulated. Conversely, genes identified as being down-regulated in PC vs Normal (matched tissue) were found to be up-regulated following PPP1R14C RNAi depletion. Normalised enrichment scores were calculated following gene set enrichment analysis.*

4.2.4 PPP1R14C depletion enhances the dephosphorylation of RB1, NF2 and PLK1 by myosin phosphatase

In order to validate the impact of PPP1R14C depletion on the phosphorylation status of MLCP substrates, cell lysates were harvested from LNCaP cells transfected as previously described. Cells were stimulated with 10nM DHT for 24 hours following 48 hours of exposure to the siPPP1R14C transfection mix, as performed for previous assays. Using phospho-specific antibodies, the phosphorylation status of RB1 serine 807/811 and PLK1 threonine 210 were investigated by western blot. The phosphorylation status of NF2 was also investigated by western blot, but was analysed by a shift in protein migration as previously described (Jin *et al.*, 2006). In figure 4.7A it is possible to observe a pronounced reduction in the phosphorylation of RB1 serine 807/811 following PPP1R14C depletion compared to the scrambled control. Figures 4.7A also demonstrates that there is a reduction in the protein levels of E2F1, contributing to the repression of E2F1 target genes. Figure 4.8B demonstrates that the phosphorylation of PLK1 at its activational residue, threonine 210, was reduced upon PPP1R14C RNAi depletion. Using a total NF2 antibody it is possible to demonstrate a shift in

the signal of NF2 from the phosphorylated band to the dephosphorylated band, depicted in figure 4.8A, confirming that NF2 undergoes enhanced dephosphorylation following PPP1R14C knockdown.

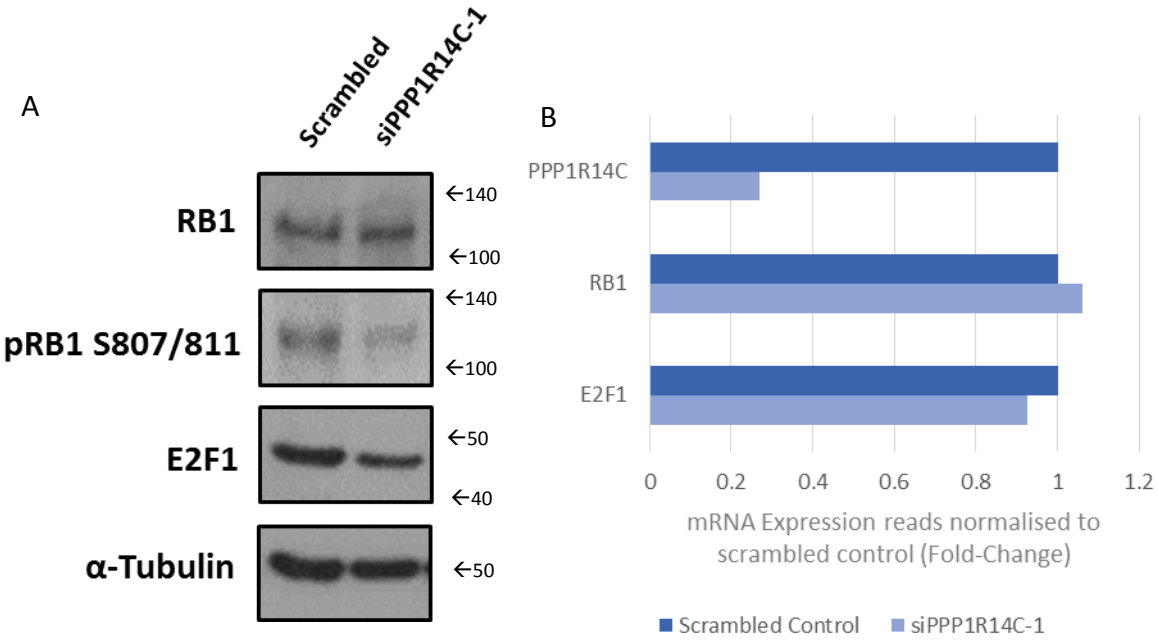


Figure *4.7 -* ***PPP1R14C Depletion Reduces the Phosphorylation Status of RB1*** *A) Western Blot analysis reveals a reduction in RB1 phosphorylation status at serine 807/811 following PPP1R14C depletion in LNCaP cells B) RB1 and E2F1 mRNA expression following PPP1R14C depletion in LNCaP cells quantified by RNA sequencing*

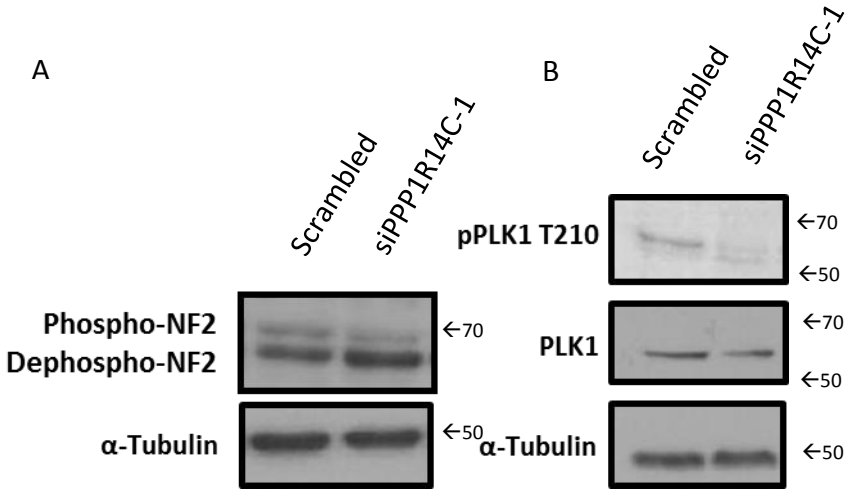


Figure *4.8 –* ***PPP1R14C Depletion Reduces the Phosphorylation Status of NF2 and PLK1*** *A) Western Blot analysis reveals reduced NF2 phosphorylation in LNCaP cells following PPP1R14C depletion B) Western Blot analysis reveals reduced PLK1 threonine 210 phosphorylation status following PPP1R14C depletion*

4.2.5 PPP1R14C depletion results in G1 cell cycle arrest

Finally, the impact of PPP1R14C on cell cycle progression was investigated by FACs analysis as previously described and is presented in figure 4.9. A statistically significant increase of cells in the G1 phase of the cell cycle was observed following PPP1R14C depletion (mean ± sem, 69±1.22% vs. 84±0.46%, p-value < 0.05), coinciding with a statistically significant reduction of cells in the G2/M phase of the cell cycle (mean ± sem, 21±0.96% vs. 9±0.54%, p-value < 0.05). No significant alteration in the number of cells in sub G1 was observed (mean, 4.6% vs. 5.8%). This would suggest that depletion of PPP1R14C is cytostatic as opposed to cytotoxic, falling in line with the down-regulation of cell cycle associated gene sets observed in figures 4.4 C-E.

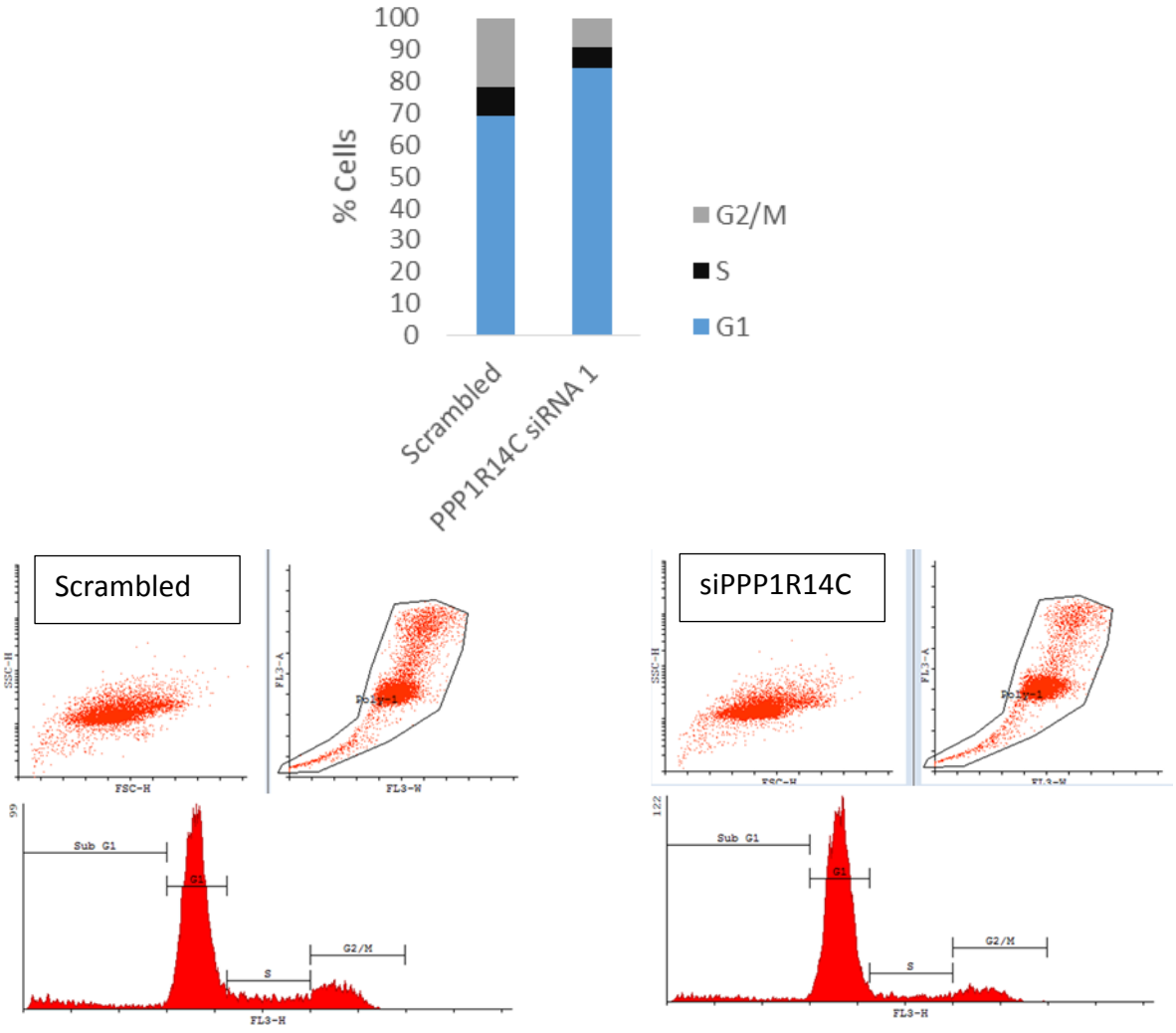


Figure *4.9* - ***PPP1R14C Depletion Causes G1 Cell Cycle Arrest****. Cells depleted of PPP1R14C were subject to FACS analysis as previously described.* Figure *represents mean n=3. PPP1R14C RNAi depletion results in G1 cell cycle arrest. Representative gating examples are also provided.*

4.3 Discussion

Crucially, within this section it was possible to confirm RNAi depletion of PPP1R14C by RNA sequencing, thus validating the results obtained by RT-qPCR in section 4. In addition, no significant impact on the mRNA expression of PPP1R12A and AR was observed, confirming any alterations in the activity of MLCP and indeed the AR must be imposed at the post-translational level. Importantly, down-regulation of *PSA, TMPRSS2* and *KLK2* mRNA expression was also observed, and indeed, they were within the top 20 most significantly repressed genes following PPP1R14C depletion. Considering it has been demonstrated that MLCP does not directly interact with the AR, to achieve such a dramatic reduction in AR transactivation would suggest the increase in MLCP activity is repressing pathways crucial for AR function. Indeed, the hallmark androgen response gene set negatively correlated with the PPP1R14C knockdown gene set, possessing a highly significant NES of -2.99, confirming MLCP as a negative regulator of the global AR transcriptional program.

Upon taking a more general approach to investigating the role of MLCP in AR signalling, it became apparent that the cell cycle associated pathways were significantly down-regulated. These include some of the more general cell cycle associated gene sets from REACTOME such as 'Cell Cycle', 'Mitotic Cell Cycle' and 'Cell Cycle Checkpoints', as well as more specific gene sets from phases of the cell cycle including 'S-Phase', 'G1-S Transition' and 'M-G1 Transition' (Supplementary Table 2). Crucially, this can be recapitulated phenotypically following FACs analysis of LNCaP cells depleted of PPP1R14C. LNCaP cells are predominantly found in the G1 phase of the cell cycle, and following PPP1R14C RNAi knockdown, a significant increase in G1 cell cycle arrest is observed when compared to the scrambled control (69% vs. 84%, p-value < 0.05). The increase in cells within the G1 phase of the cell cycle appear to be predominantly drawn from the G2/M phase of the cell cycle, which is significantly reduced, whilst the percentage of cells within the sub G1 gating remains consistent, suggesting knockdown of PPP1R14C is cytostatic as opposed to cytotoxic. More specific analysis revealed that E2F target genes are significantly down-regulated, coinciding with repression of genes known to be up-regulated following E2F1 over-expression as well as genes up-regulated following RB1 and P107 knockdown. As introduced in this section, RB1 is dephosphorylated and activated by MLCP. Indeed, confirmation of a reduction in the phosphorylation of the inhibitory phospho-

sites RB1 serine 807/811, was observed by western blot following knockdown of PPP1R14C in both the presence and absence of androgen. The opposing impact of PPP1R12A knockdown on RB1 phosphorylation was not investigated in this thesis, but has been published by another research group, where significant induction of RB1 serine 807/811 phosphorylation upon depletion of PPP1R12A is observed, further confirming the involvement of PPP1R14C in RB1 regulation (Cho *et al.*, 2011). In addition, no significant alteration in the mRNA expression of RB1 and E2F1 was detected by RNA sequencing. RB1 is a well-studied tumour suppressor and is known to be implicated in a number of human malignancies, including PC (Macleod, 2010). A recent multi-collaborative study identified loss of RB1 in 21% of metastatic CRPC, whilst previous studies have suggested loss of heterozygosity can be observed in up to 60% of PC patients (Phillips *et al.*, 1994; Dan *et al.*, 2015). RB1 plays a key role in supressing the transcriptional activity of E2F transcription factors, which in turn, are best known for their regulation of cell cycle related genes (Macleod, 2010). Phosphorylation of RB1 is mediated by CDKs in a cell cycle/cyclin dependent manner. Indeed, over-expression of CDK2 and CDK4 as well as cyclin D1 is also frequently observed in PC, suggesting inhibition of RB1 occurs at both the genomic and post-translational level (Dan *et al.*, 2015). However, the activity and expression of the AR is intrinsically linked to the activity of the E2F transcription factors, and as such, RB1. In 2010, it was reported that depletion of RB1 led to enhanced AR mRNA and protein expression, increased AR transactivation and dramatic increases of PC cell and tumour growth both *in-vitro* and *in-vivo* (Sharma *et al.*, 2010). Crucially, it was demonstrated that the increases in AR signalling occurred in the absence of androgen, and was mediated by E2F1, strongly suggesting RB1 inhibition is a major contributor to castrate resistant disease. Importantly, the increases in AR mRNA, protein and transcriptional activity observed following PPP1R12A RNAi depletion correlate with the data presented by Sharma *et al* following RB1 depletion, suggesting the impact of MLCP activity on AR signalling is in part mediated by RB1.

Using an antibody previously described to investigate the phosphorylation status of NF2, it was possible to identify increased levels of NF2 dephosphorylation following PPP1R14C knockdown by western blot (Jin *et al.*, 2006). The role of NF2 in PC progression is relatively understudied but preliminary evidence exists for its genomic loss and/or mutational inactivation (Kawana *et al.*, 2002; Malhotra *et al.*, 2013). Indeed, the expression of NF2 is low in a number of PC cell lines including LNCaP cells, and is often found in a hyperphosphorylated

state (Horiguchi *et al.*, 2008). Activation of the PI3K/AKT/MTOR pathway is frequently observed in PC, particularly at advanced stages (approximately 50% metastatic CRPC), and is predominantly characterised by PTEN loss (Dan *et al.*, 2015). As such, there is growing interest in targeting the PI3K pathway alongside ADT (Bitting and Armstrong, 2013). Following PPP1R14C RNAi knockdown, MTOR target gene expression is significantly down-regulated, coinciding with the increase in activated NF2. NF2 is a known MTORC1 inhibitor, however, the underlying mechanism remains to be elucidated (James *et al.*, 2009). Interestingly, it was found that NF2 inhibits MTORC1 downstream from AKT, partially explaining why AKT phosphorylation levels were not reduced by increased NF2 activity. Indeed, the increase in phosphorylated AKT observed following PPP1R14C knockdown in section 5 cannot be explained by any impact on the expression of the AKT phosphatases PHLPP1 and PHLPP2 as these were unchanged following PPP1R14C knockdown as quantified by RNA sequencing (data not shown). However, it has recently been reported that PHLPP mediated dephosphorylation of AKT requires the presence of a scaffolding protein called FK506 binding protein 5 (FKBP5), which was shown to be down-regulated following PPP1R14C depletion (Log2FC -0.225, p-value < 0.05)(Pei *et al.*, 2009). This would suggest that the increase in AKT phosphorylation may be mediated through down-regulation of FKBP5 in addition to de-repression of the negative feedback loop between the PI3K and MAPK signalling cascades, however, destabilization of the PHLPPs at the protein level cannot be ruled out at this stage and may require further investigation.

As mentioned in the introduction to this section, PLK1 is a substrate for MLCP, and dephosphorylation of PLK1 threonine 210 by MLCP results in inhibition of PLK1 activity (Yamashiro *et al.*, 2008). With this in mind, it was possible to demonstrate that through depletion of PPP1R14C, sustained MLCP activity has led to increased dephosphorylation of PLK1 observed in figure 6.8B possibly contributing to the repression of AR target genes, as well as the inhibition of E2F1 target genes. Therefore a more in depth investigation into the impact of PPP1R14C knockdown on PLK1 activity is warranted.

As such, it has been possible to confirm that PPP1R14C knockdown results in significant repression of the AR, E2F1 and MTOR signalling cascades amongst others, highlighting possible routes of AR modulation by MLCP. Crucially, genes up-regulated following PPP1R14C depletion positively correlated with genes down-regulated in PC vs benign prostate tissue,

whilst genes down-regulated following PPP1R14C depletion negatively correlated with genes up-regulated in PC vs benign prostate tissue. This would suggest that knockdown of PPP1R14C may partially restore a non-malignant genotype in PC cells, further reinforcing its validity as a therapeutic target.

In conclusion, characterising and validating a global LNCaP gene signature following PPP1R14C RNAi knockdown has confirmed repression of AR transactivation, has demonstrated increased RB1 activity through enhanced dephosphorylation leading to significant repression of the E2F1 transcriptional program, as well as resulting in significant repression of the MTOR signalling pathway characterised by an increase in the dephosphorylation of NF2. Collectively this has resulted in significant repression of cell cycle associated genes, in turn, leading to G1 cell cycle arrest. The involvement of PLK1 dephosphorylation was confirmed but its role in repression of the AR signalling cascade remains to be fully elucidated. Thus the data presented in this section convincingly implicates enhanced dephosphorylation of MLCP substrates in the regulation of AR function, contributing to the partial restoration of a non-malignant genotype associated with benign prostate tissue compared to PC. Figure 4.10 depicts a schematic summary of this chapters findings.

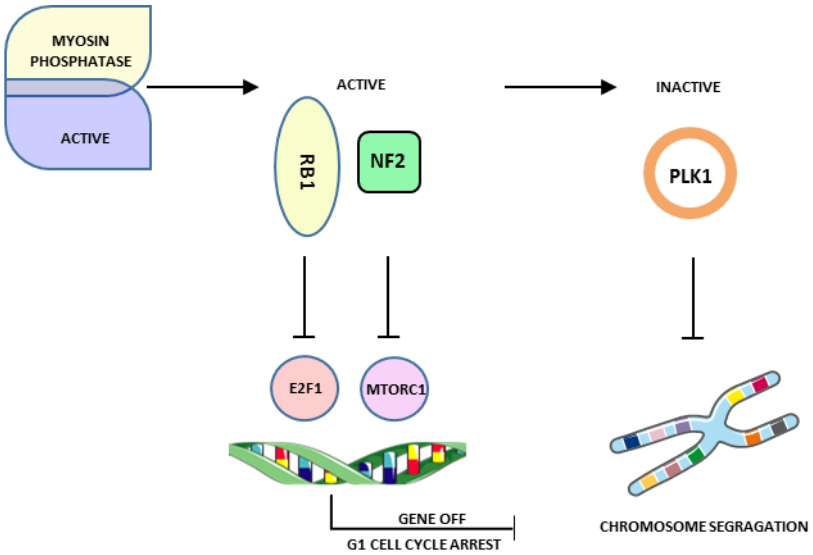


Figure *4.10* – ***Schematic Summary of Section 6 Results.*** *Sustained MLCP activity following PPP1R14C RNAi depletion leads to enhanced dephosphorylation of the MLCP substrates RB1, NF2 and PLK1. RNA sequencing confirms down-regulation of the AR signalling axis as well as E2F1 and MTORC1 activity. Depletion of PPP1R14C leads to G1 cell cycle arrest, partially contributed by the repression of PLK1.*

Chapter 5.

Myosin Phosphatase Modulation Impacts on the Phosphorylation Status of the Androgen Receptor

5.1 Introduction

As described in section 1.2.3, the AR is a phosphoprotein heavily influenced by the action of kinases, and as such, phosphatases. Phosphorylation of the AR has been described to occur in both the absence and presence of androgens. Crucially, a number of phosphorylation sites have been shown to be induced by androgen stimulation, and functionally enhance transactivation of the AR. Conversely, there are phospho-residues that have been shown to be phosphorylated by non-androgenic signalling cascades that are capable of driving increased AR activity, and thus are of interest in a castrate-resistant setting. In this section I will discuss the impact of MLCP modulation on AR phosphorylation status, and its subsequent functional outcome.

5.2 Results

5.2.1 Myosin phosphatase does not interact with the androgen receptor

In order to attempt to dissect the mechanism of regulation between MLCP and the AR, it was necessary to identify any potential interactions to elucidate if the means of regulation was direct or indirect. Any interaction would suggest that MLCP may act to dephosphorylate the AR directly and enhance our knowledge on the interplay between AR modifying kinases and phosphatases. As such, exogenous AR cDNA was transfected into the AR-null HEK293T cell line so that the non-transfected arm of the experiment could be used as a negative control. The AR was then immunoprecipitated as described in section 2.17 with an AR N-terminal targeting antibody (N-20) and purified using protein-G sepharose beads. The collected lysate was then probed by western blot for both AR and PPP1R12A. Results from this experiment, depicted in figure 5.1, demonstrate that indeed the protein levels of AR were enriched following immunoprecipitation compared to the input controls. It was also possible to identify the presence of endogenous PPP1R12A within the input samples, however, no apparent PPP1R12A was co-immunoprecipitated with the AR, regardless of the presence or absence of androgens. This suggests that the role MLCP plays within AR regulation is indirect, or extremely transient, and as such, no endogenous interactions were pursued.

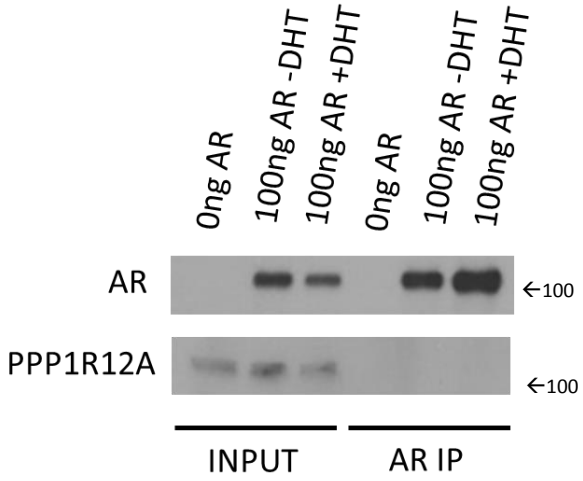


Figure *5.1* - ***Myosin Phosphatase does not interact with the AR***. *HEK293T cells were transfected with AR plasmid DNA and immunoprecipitated as previously described. AR-PPP1R12A interactions were then investigated by western blot.*

Despite no direct interaction between MLCP and the AR being observed, characterisation of the phosphorylation status of the AR was performed to gain a greater insight into the functional outcome of MLCP modulation on AR PTM. This in turn would provide valuable knowledge on any potential upstream signalling cascades impacted by MLCP modulation, and as such, aid in elucidating to some extent the underlying mechanisms involved.

5.2.2 PPP1R14C depletion alters the phosphorylation status of the androgen receptor

Following PPP1R14C RNAi transfection, endogenous AR was immunoprecipitated from LNCaP cells by protein-G sepharose as described in section 2.17, and the phosphorylation status of 3 residues, serine 81, serine 213 and serine 515 of the AR, were investigated using two commercially available phospho-specific antibodies (serine 81 and serine 213) and one custom phospho-specific antibody (serine 515, a kind gift from Dr. Joanne Edwards). Due to difficulties in detecting the phosphorylation status of the AR by western blot using whole cell lysates as previously described (McEwan *et al.*, 2010). AR protein levels were enriched by immunoprecipitation prior to western blot analysis. Again, enrichment of the AR protein was observed following immunoprecipitation when compared to the input samples, presented in figure 5.2. Secondly, a dramatic reduction was observed in the phosphorylation status of serine 81 following PPP1R14C RNAi knockdown compared to the scrambled control, to the point where no level of phosphorylation can be detected. Similarly, a reduction in the phosphorylation of serine 515 was observed in the siPPP1R14C-1 experimental arm, albeit to

a lesser extent that serine 81. Conversely, enhanced phosphorylation of serine 213 was observed following RNAi depletion of PPP1R14C. As described in section 1.2.3, phosphorylation of these 3 residues is known to impact dramatically on the transactivation of the AR, and as such, enabled subsequent AR functionality assays to be designed.

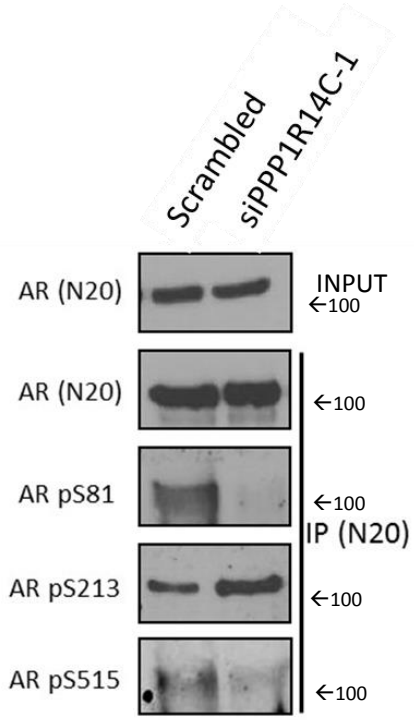


Figure 5.2 - ***PPP1R14C Depletion Impacts on the Phosphorylation Status of the AR.*** *Endogenous AR was immunoprecipitated from LNCaP cells depleted of PPP1R14C and analysed by western blot using phospho-specific antibodies.*

5.2.3 PPP1R14C depletion impairs ligand induced nuclear translocation of the androgen receptor

Serine 81 and serine 515 phosphorylation is considered to enhance AR transactivation, mediated through enhanced nuclear localization, chromatin binding and ultimately increased transcriptional activity (Gioeli *et al.*, 2002; Chen *et al.*, 2012; Willder *et al.*, 2013). As described in section 4.2, RNAi depletion of PPP1R14C resulted in a statistically significant repression of AR regulated gene transcription. This correlated with the reduction in the phosphorylation status of serines 81 and 515 observed in figure 5.2. In complement of this, figure 5.3 demonstrates AR nuclear/cytoplasmic localization following transfection with siPPP1R14C-1 compared to the scrambled control in the presence of androgen. Indeed, the AR was found in both the cytoplasm and nucleus in the scrambled arm of the assay as expected (Kuiper *et al.*,

1993), but in the PPP1R14C knockdown arm, the AR was predominantly found in the cytoplasm, with minimal levels detected in the nucleus. This correlates with the literature surrounding phosphorylation of serine 81 and 515, as well as reinforcing initial findings from section 4.2. Similarly, LNCaP cells transfected with siPPP1R14C-1 were subjected to immunofluorescent analysis 30 minutes post 10nM DHT stimulation (figure 5.4). Crucially, a reduction in nuclear AR was detected following androgen stimulation in the siPPP1R14C-1 treated cells compared to the scrambled arm, further reinforcing the results obtained following nuclear-cytoplasmic fractionation. It was also possible to visually detect pMLC-S19 by immunofluorescence and a reduction in this mark was detected following depletion of PPP1R14C, consistent with enhanced MLCP activity. However, what was not evident by western blot in figure 4.5, was that androgen stimulation dramatically increases phosphorylation of MLC-S19 at the cellular periphery, an effect that is abolished following PPP1R14C knockdown. This would suggest that androgen stimulation leads to rapid phosphorylation of MLC-S19 mediated by the inhibition of MLCP by PPP1R14C.

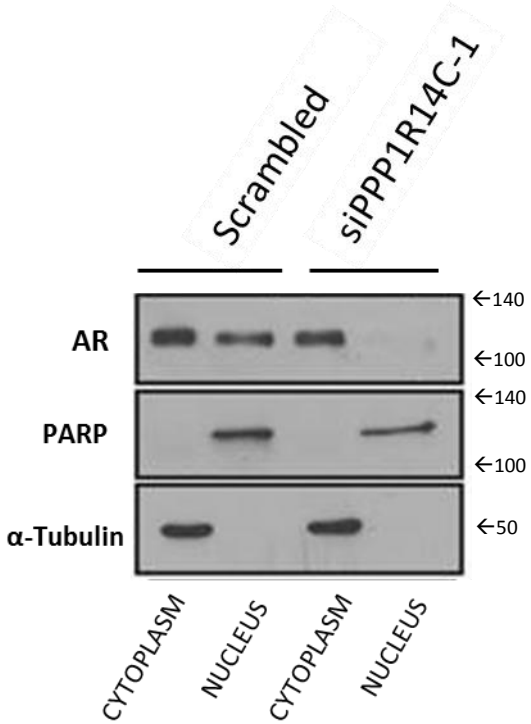


Figure 5.3 - ***PPP1R14C Depletion Impairs AR Nuclear Translocation***. *LNCaP cells were depleted of PPP1R14C as previously described and subjected to 30 mins 10nM DHT stimulation prior to nuclear-cytoplasmic fractionation. Knockdown of PPP1R14C resulted in impaired androgen induced AR nuclear translocation vs the scrambled control.*

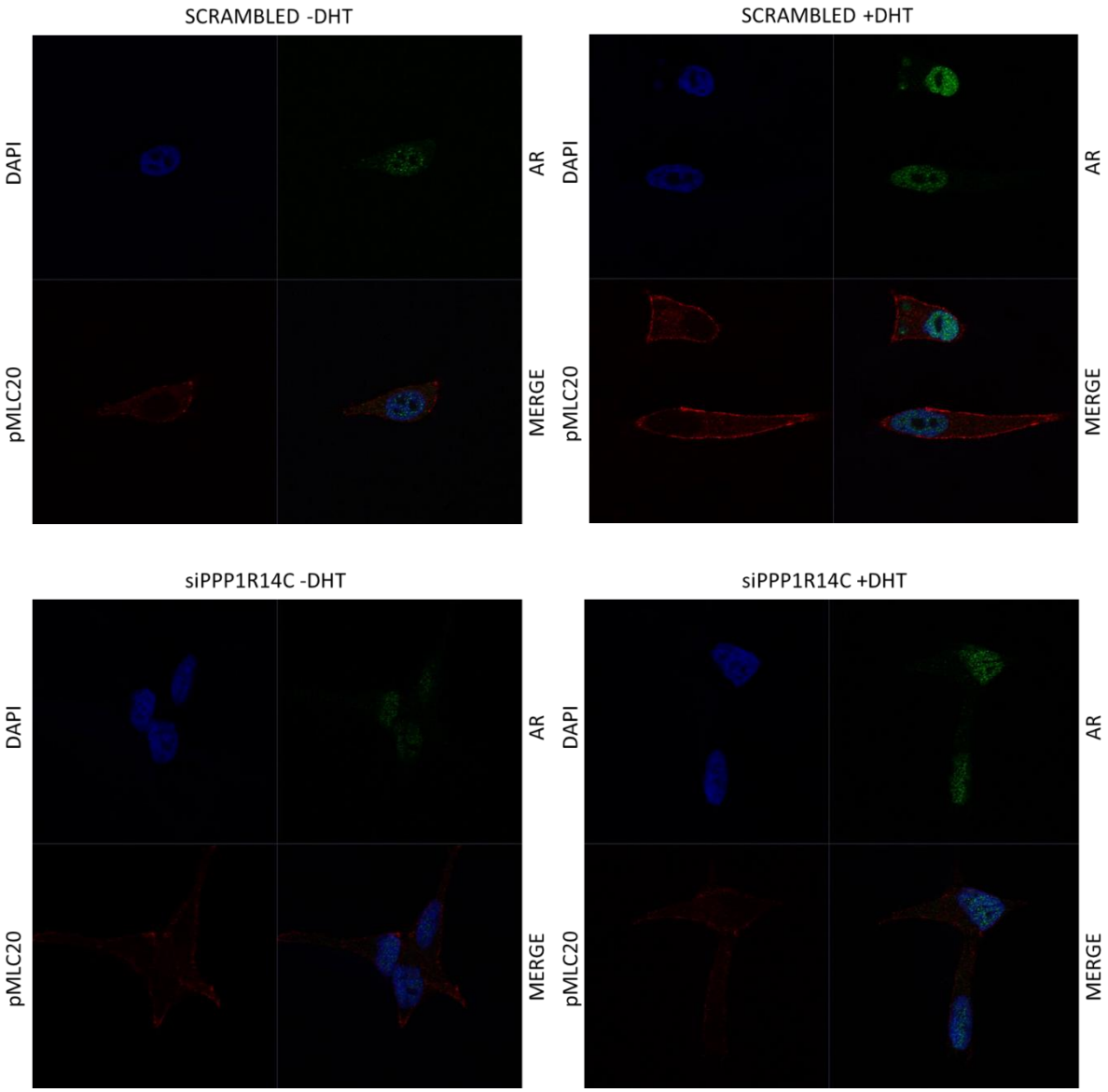


Figure 5.4 - ***PPP1R14C Depletion Prevents Androgen Mediated MLC20 Phosphorylation***. *LNCaP cells were depleted of PPP1R14C as previously described and subjected to 30 mins 10nM DHT stimulation prior to immunofluorescent analysis. Cell nuclei were stained with DAPI, AR (Green) and pMLC20 (Red) were detected using alexa-fluor conjugated secondary antibodies. PPP1R14C depletion prevents androgen induced MLC20 phosphorylation and reduces AR nuclear translocation compared to the scrambled control.*

5.2.4 PPP1R14C depletion reduces MAPK activation

As it has been previously reported that MAPK signalling is in part responsible for the phosphorylation of AR serine 515, the impact of PPP1R14C depletion on MAPK activation was investigated. LNCaP cells depleted of PPP1R14C as previously described were subject to 10ng/ml EGF stimulation for 0, 5, 10 and 15 minutes prior to lysis and western blot analysis. Figure 5.5 demonstrates that a marked reduction in the phosphorylation of MAPK1/3 occurs in response to EGF stimulation following PPP1R14C knockdown compared to the scrambled control arm of the experiment, however, PPP1R14C depletion also leads to a marginal increase in the basal phosphorylation status of MAPK1/3.

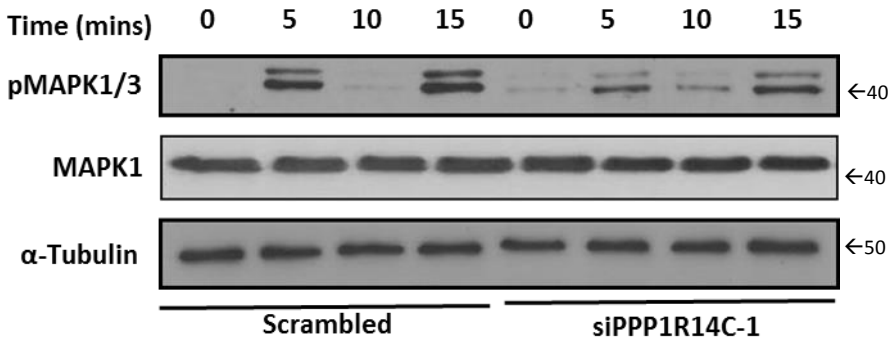


Figure 5.5- ***PPP1R14C Depletion Reduces MAPK Activation Following EGF Stimulation***. *LNCaP cells were depleted of PPP1R14C as previously described and stimulated with 10ng/ml EGF. LNCaP cells were harvested at 0, 5, 10 and 15 minutes post stimulation and analysed by western blot. Depletion of PPP1R14C reduces EGF induced MAPK phosphorylation compared to the scrambled control.*

5.2.5 PPP1R14C depletion results in enhanced AKT mediated proteasomal degradation of the androgen receptor

The other phosphorylation site investigated was serine 213. This is a repressive phospho-mark described to lead to enhanced MDM2 mediated proteasomal degradation, and as such, repression of AR target gene expression. Indeed, figure 5.6 demonstrates that the AR undergoes enhanced degradation over an 8-hour period in the presence of cycloheximide following RNAi depletion of PPP1R14C compared to the scrambled control. AR protein levels decreased over time as expected in the scrambled arm of the assay, confirming the accelerated AR degradation observed in the siPPP1R14C-1 arm is legitimate. In further support of this, LNCaP cells transfected with siPPP1R14C-1 were exposed to 1µM of the proteasomal inhibitor MG132 and AR regulated gene expression was quantified by RT-qPCR. Figure 5.7

demonstrates that *PPP1R14C* gene expression is indeed repressed following RNAi depletion compared to the scrambled control, and as in section 4.2, significant repression of the AR target gene *PSA* was observed. However, upon addition of MG132, AR transcriptional activity can be partially rescued, confirming that in part some degree of the repressive regulation of

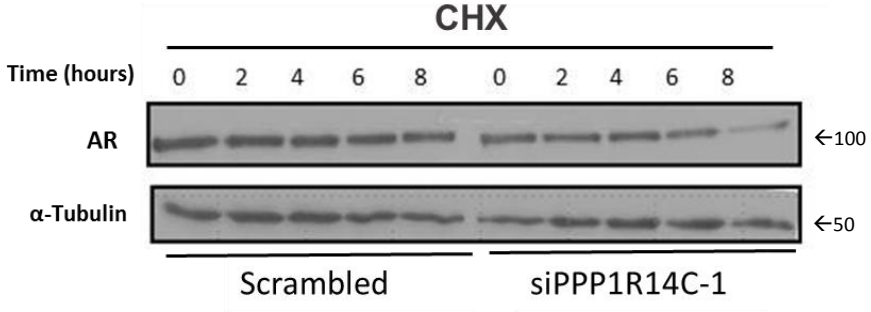


Figure 5.6 - ***PPP1R14C Depletion Accelerates AR Degradation***. *LNCaP cells were depleted of PPP1R14C and incubated with 20µg/ml cycloheximide (CHX) for increasing time-periods. Cell lysates were analysed by western blot.*

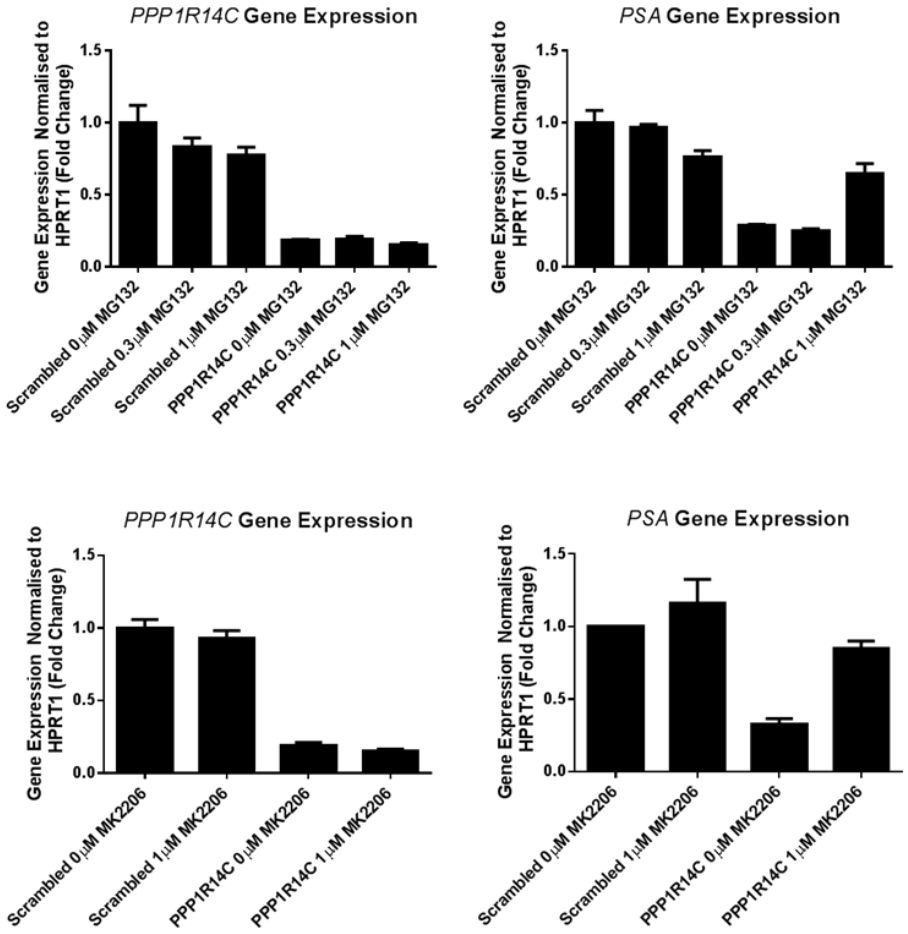


Figure 5.7 - ***AKT and Proteasomal Inhibitors rescue AR Transcriptional Activity following PPP1R14C Depletion***. *A) LNCaP cells depleted of PPP1R14C were exposed to increasing doses of MG132 and underwent RT-qPCR analysis. B) LNCaP cells depleted of PPP1R14C were exposed to increasing doses of MK2206 and underwent RT-qPCR analysis. Data represents n=3 mean ± sem.*

the AR by MLCP is indeed mediated through enhanced proteasomal degradation. Furthermore, the increase in serine 213 phosphorylation observed in figure 5.2 would suggest that the accelerated proteasomal degradation is mediated by AKT. Indeed, figure 5.7 demonstrates that upon exposure to the AKT inhibitor MK2206, partial rescue of *PSA* mRNA expression was observed following RNAi depletion of PPP1R14C.

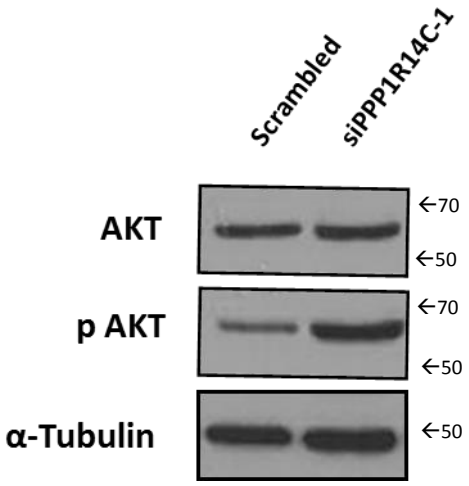


Figure *5.8* - ***PPP1R14C Depletion Increases AKT Phosphorylation***. *LNCaP cells depleted of PPP1R14C for 72 hours in full media were subject to western blot analysis using an AKT phospho-specific antibody. Depletion of PPP1R14C results in increased AKT phosphorylation.*

As an increase in AR serine 213 phosphorylation was observed, the phosphorylation status of AKT 1/2/3 serine 473 was investigated by western blot using a phospho-specific antibody following PPP1R14C depletion. Figure 5.8 demonstrates that an increase in the phosphorylation status of AKT was observed following PPP1R14C knockdown compared to the scrambled control, correlating with the increase in AR serine 213 phosphorylation

5.3 Discussion

Deciphering the role PP1 regulatory subunits play in the regulation of the AR will be crucial in building on the current literature surrounding the impact of PP1 on the AR and ultimately generating more targeted approaches to targeting PP1 in the treatment of AR driven diseases including PC. Indeed, PP1 has been shown to directly interact with the AR (Chen *et al.*, 2009), but as of yet no PP1 regulatory subunits have been described to interact with the AR. As such, PPP1R12A is the substrate specifying subunit of MLCP, and no interaction with the AR was detectable through co-immunoprecipitation assays, strongly suggesting that MLCP does not

interact directly with the AR (figure 5.1). Unfortunately it has not been possible to validate a commercially available PPP1R14C antibody, and as such, it cannot be ruled out that PPP1R14C interacts with the AR and is capable of imposing some form of regulation via this mechanism. However, further characterisation of the phosphorylation status of the AR following PPP1R14C RNAi depletion revealed that 2 pro-androgenic phospho-residues are down-regulated, whilst 1 repressive phospho-residue is induced, suggesting that indeed MLCP regulates the AR indirectly via upstream mechanisms, which correlates with the results from the co-immunoprecipitation assay (figure 5.2).

As described in section 1.2.3, serine 81 phosphorylation has been shown to be induced by androgen (Gioeli *et al.*, 2002) and in the scrambled arm from figure 5.2 it was possible to detect this phosphorylation mark. Phosphorylation of serine 81 was described to stabilize the AR, contributing to enhanced AR transcriptional activity. Following PPP1R14C RNAi depletion, reduced AR stability and transcriptional activity was observed, correlating with a reduction in the phosphorylation of serine 81 (figure 5.2). In addition, phosphorylation of serine 81 also correlates with enhanced nuclear localization and chromatin binding. Figure 5.3 highlights that PPP1R14C RNAi knockdown also leads to a pronounced reduction in the ability of the AR to translocate to the nucleus following androgen induction. This in turn provides a rationale for the reduced AR transcriptional activity observed in section 4.2. Indeed, chromatin immunoprecipitation assays have not been performed to confirm decreased deposition of the AR at AREs following MLCP modulation, but figures 5.3 and 5.4 provide sufficiently strong enough evidence to suggest that as AR nuclear localization is significantly impaired, this too would be the case. The kinases responsible for modification of serine 81 have been identified as CDK1, CDK5 and CDK9 (Gioeli *et al.*, 2002; Chen *et al.*, 2006; Gordon *et al.*, 2010; Hsu *et al.*, 2011). Crucially, it has been demonstrated that phosphorylation of serine 81 predominantly occurs in the nucleus (Kesler *et al.*, 2007; Gordon *et al.*, 2010). Due to the 30 minute androgen stimulation time-scale used in the nuclear-cytoplasmic extraction and AR immunoprecipitation assays, the results obtained in relation to serine 81 phosphorylation and impaired nuclear localization following PPP1R14C RNAi knockdown would strongly suggest that MLCP modulation prevents androgen induced nuclear translocation prior to phosphorylation by the CDKs in the nucleus (figure 5.2 and figure 5.3), which would suggest the activity of CDK1 towards the AR is being reduced. Indeed, as described in chapter 4, RNAi

depletion of PPP1R14C resulted in G1 cell cycle arrest, characterised by a reduction in the phosphorylation status of RB1. As CDK1 is most active in the G2/M phase of the cell cycle, the reduction in serine 81 phosphorylation may be attributed to the G1 arrest observed. Furthermore, due to the time-scale used, enhanced nuclear export is unlikely to impact on serine 81 phosphorylation, but may contribute to the overall reduction in AR transcriptional activity following siPPP1R14C-1 transfection and therefore should be interrogated. Similarly serine 515 is also phosphorylated in the nucleus, also by CDK1 as well as CDK7 (Chymkowitch *et al.*, 2011; Willder *et al.*, 2013). The reduction in phosphorylation of this residue following PPP1R14C depletion may also be as a result of increased G1 cell cycle arrest and subsequent down-regulation of CDK1 activity. Again, phosphorylation of this residue is reported to increase AR transcriptional activity. Therefore it would appear that the reduction in serine 515 phosphorylation following PPP1R14C knockdown is also likely to be caused by a reduction in the nuclear translocation of the AR. However, the reduction in the phosphorylation of serine 515 following MLCP modulation compared to the scrambled control was less pronounced than the reduction in serine 81 phosphorylation, and is still in fact detectable (figure 5.2). This could be attributed to the other kinases known to phosphorylate this residue, MAPK1 and MAPK3 (Gregory *et al.*, 2004; Mellinghoff *et al.*, 2004; Ponguta *et al.*, 2008). Indeed, androgen stimulation has been documented to induce the phosphorylation and subsequent activation of MAPK1 and MAPK3 within sub-minute time-scales, providing a rationale for serine 515 phosphorylation observed in figure 5.2 (Foradori *et al.*, 2008). Identification of MAPK activation by androgen in the sub-minute time-scale proved difficult to identify, however, activation by the canonical EGFR pathway following EGF stimulation was detectable. As such, following PPP1R1C RNAi depletion, a pronounced reduction in the phosphorylation of MAPK1/3 was observed in figure 5.5, suggesting that MLCP activity also plays a repressive role in MAPK signalling. The results would suggest that the MAPK pathway is still capable of being activated, albeit to a lesser extent, and such still capable of phosphorylating AR serine 515. Indeed, it has been previously reported that ectopic expression of PPP1R14C in the breast cancer cell line MCF-7 induces MAPK activation but the authors draw no conclusion on MLCP activity, nor any specific role for PPP1R14C (Wenzel *et al.*, 2007). However, as described in Chapter 4, PPP1R14C depletion results in the enhanced dephosphorylation of the tumour suppressor NF2. NF2 has been reported to repress the MAPK signalling cascade by directly inhibiting the activity of Ras (Garcia-Rendueles *et al.*, 2015; Riecken *et al.*, 2016) Most

importantly, both serine 81 and serine 515 phosphorylation are tightly associated with PC growth, correlate with disease progression and have been shown to hypersensitize PC cells to low levels of androgens. In turn, this is reflected by the frequent observation that CDK1 and MAPKs are dysregulated in advanced PC and represent viable therapeutic options within their own right. Crucially, it was observed following MLCP modulation that the downstream impact of CDK and MAPK action on AR phosphorylation is abolished, whilst an overall reduction in activation of the MAPK signalling cascade is also reduced, suggesting disruption of the MLCP-PPP1R14C interaction may pose a viable therapeutic option in both PC and CRPC. It will be of great significance to investigate the direct impact of MLCP modulation on CDK activity to gain a greater insight into the molecular mechanisms underlying the results obtained in relation to serine 81 and 515 phosphorylation.

Serine 213 was identified as a repressive phosphorylation site of the AR modified by AKT and PIM1-S/L (Lin *et al.*, 2001; Linn *et al.*, 2012). Figure 5.2 demonstrates that there was a considerable increase in the phosphorylation of serine 213 following PPP1R14C RNAi knockdown compared to the scrambled control. As phosphorylation of this site leads to the recruitment and ubiquitination of the AR by MDM2 and subsequent proteasomal degradation, AR protein stability in the presence of cycloheximide was investigated. Correlating with the increase in phosphorylation, accelerated AR protein degradation was observed over an 8 hour period (figure 5.6). This in turn will be partly responsible for the reduction in AR transcriptional activity observed. Phosphorylation of serine 213 by AKT predominantly occurs in the cytoplasm and is known to impede nuclear translocation (Palazzolo *et al.*, 2007). This would correlate with the impaired nuclear localization observed in figure 5.3, however, it would be difficult to suggest if the increase in phosphorylation is a result of impaired nuclear translocation, or the cause of impaired nuclear translocation without interrogating the experimental setup with the inclusion of AR serine 213 mutants. Furthermore, LNCaP cells are PTEN null, and it would be expected that AKT is constitutively phosphorylated, and as such, activated (Li *et al.*, 1997). In the scrambled arm of figure 5.8 it was possible to detect high levels of AKT phosphorylation, however, this was increased following PPP1R14C RNAi knockdown, correlating with the increase in AR serine 213 observed. The cross-talk between independent signalling cascades has emerged as an important area of research, particularly in relation to disease resistance mechanisms. As such, there is significant evidence for reciprocal

regulation between the PI3K and androgen signalling pathways. It was shown that AR repression leads to enhanced AKT phosphorylation through downregulation of the AKT phosphatase, PHLPP (Carver *et al.*, 2011a). As pronounced AR inhibition is observed following PPP1R14C depletion (figure 4.2), downregulation of PHLPP remains a plausible reason for increased AKT phosphorylation, which in turn would lead to enhanced AR degradation via serine 213 phosphorylation, potentiating this negative feedback loop. Similarly, inhibition of the MAPK signalling cascade has been shown to induce activity of the PI3K pathway characterised by increased AKT phosphorylation (Turke *et al.*, 2012). As a reduction in the phosphorylation of MAPK1/3 is observed in figure 5.5, this too is likely to contribute to increased AKT phosphorylation. Serine 213 phosphorylation is also mediated by both isoforms of PIM-1, however, these were not interrogated throughout this project, and hence their involvement in the modification of serine 213 in the context of MLCP modulation cannot be excluded. Nevertheless, phosphorylation of serine 213 by PIM1-S results in proteasomal degradation mediated by MDM2 in very much the same manner as AKT, and therefore would support the data presented in figures 5.2 and 5.6. Phosphorylation by PIM1-L on the other hand results in increased nuclear localization, stability and nuclear localization, and as such, would conflict with the data presented in this section.

Finally, immunofluorescence analysis of LNCaP cells transfected with PPP1R14C RNAi not only confirmed impaired nuclear translocation of the AR, but provided significant evidence for the rapid involvement of PPP1R14C mediated MLCP inhibition in response to androgen stimulation (figure 5.4). PPP1R14C phosphorylation has previously been reported to be enhanced in response to serum (Madsen *et al.*, 2015), and here it is possible to show that this induction is extended to androgen stimulation. Indeed, this finding could be further enhanced using phospho-specific antibodies raised against PPP1R14C threonine 73, but as of yet a specific antibody does not exist, and the use of a phosphorylated PPP1R14A threonine 43 antibody would raise specificity issues. The MLCP inhibitor protein family display significant similarities both in terms of structure and regulation, and it has been previously reported that PPP1R14A is phosphorylated in response to androgen, further suggesting that androgen mediated phosphorylation of PPP1R14C is plausible (Song *et al.*, 2010). Indeed, this raises the possibility that disruption of androgen mediated MLC20 phosphorylation prevents efficient AR nuclear translocation. A number of cytoskeletal associated proteins have been associated

with co-regulation of AR activity, one of which, filamin, has been associated with a crucial role in AR nuclear translocation (Ozanne *et al.*, 2000). As such, the direct involvement of MLCP in AR cytoplasmic retention cannot be ruled out.

In conclusion, MLCP impacts on the phosphorylation status of the AR at a number of different biologically important phospho-residues, resulting in impaired ligand induced nuclear translocation and accelerated proteasomal degradation. These can be in part explained by the repression and induction of the MAPK and PI3K signalling cascades respectively, but crucially, suggests that MLCP modulation is having a profound impact on a number of independent signalling cascades, and ultimately exerting repressive regulation of the AR via indirect mechanisms. Indeed, the impaired nuclear localization and enhanced degradation of the AR correlate with the reduction in AR mediated gene transcription described in section 4.2. Functionally, it is now possible to understand how MLCP modulation impacts on the cellular role of the AR, however, the underlying mechanisms linking MLCP activity to AR repression remain unknown. Therefore it is necessary to gain a greater understanding of MLCP function, and in particular, a greater understanding of its therapeutic relevance in order to assess MLCP modulation as a viable modality in the treatment of PC. A schematic diagram summarising the findings from this chapter can be found in figure 5.9.

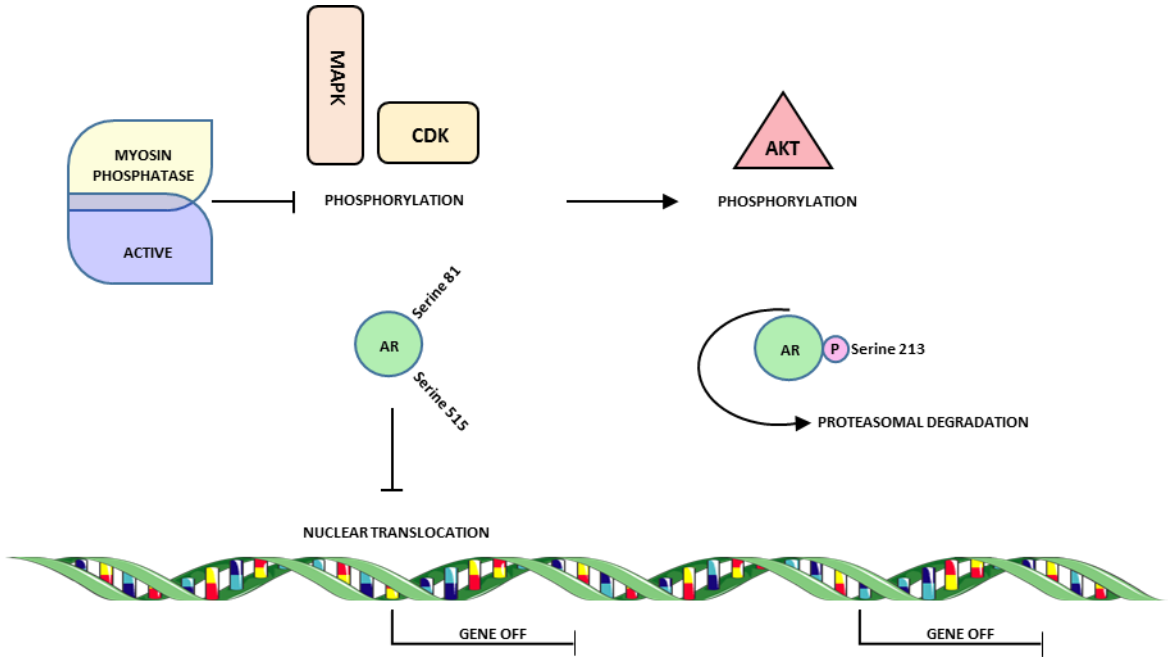


Figure *5.9* – ***Schematic Summary of Section 5 Results.*** *Enhanced MLCP activity via depletion of PPP1R14C reduces MAPK and CDK mediated phosphorylation of AR serine 81 and serine 515 resulting in impaired ligand induced nuclear translocation. Conversely, phosphorylation of the AR at serine 213 by AKT is enhanced following PPP1R14C depletion, resulting in enhanced proteasomal degradation.*

Chapter 6.

Investigating the Therapeutic Potential of PPP1R14C Inhibition

6.1 Introduction

As described in section 1.1.6, preventing transactivation of the AR has remained a crucial treatment modality in PC therapy for over 40 years. Although drugs have advanced pharmacologically, or indeed, novel approaches of targeting the androgen signalling axis have developed in parallel with our improved understanding of AR signalling, ultimately, the aim of preventing the biological role of the AR in the treatment of PC has remained the same. Indeed, disruption of the AR either by small molecule inhibition, ligand withdrawal or genetic modification represses PC cell and tumour growth. As the genotypic impact of PPP1R14C RNAi knockdown has been established in relation to the activation of clinically relevant tumour suppressors, and its involvement in cell cycle progression assessed, the impact of PPP1R14C modulation on LNCaP cell growth and migration was investigated using live cell imaging techniques and a Boyden chamber assay respectively. Similarly, the depletion of PPP1R14C was assessed in cell line models of treatment resistance. Since the discovery by Huggins *et al* (Huggins *et al.*, 1941) in 1941 that PC is highly dependent upon the action of androgens for survival, growth and progression, targeting the AR signalling axis has been the mainstay of treatment for localised, advanced, and metastatic PC. As described in section 1.1.6, this form of treatment is initially very effective, achieving response rates in up to 90% of patients, however, after a median time of 2-3 years, it invariably fails, rendering the cancer what is termed castrate resistant. Whether ADT resistant clones exist prior to treatment is currently unknown, but undoubtedly, therapy with ADT applies a selection pressure upon the cancer, favouring survival and propagation of resistant cells.

Although novel cytotoxic agents, AR targeting agents, and immunotherapies have been developed, demonstrating modest increases in survival outcomes, CRPC invariably progresses in the absence of any effective treatments, characterised by rising PSA levels, metastasis, and ultimately death after a median period of 16-18 months (Chen *et al.*, 2004). The rising expression of PSA, combined with initial responses to novel anti-androgens, suggests CRPC tumours still possess an active androgen signalling axis and are sensitive to androgen blockade, and therefore the AR remains a viable therapeutic target. Indeed, a number of resistance mechanisms that allow continued AR signalling in CRPC have been identified. These include incomplete blockade of androgen biosynthesis pathways, AR amplification, AR mutation, aberrant AR co-activator activity, and the emergence of AR splice variants.

Emerging evidence exists that intratumoral androgen biosynthesis pathways are up-regulated in CRPC, enabling PC cells to increase androgen availability both within the primary tumour and at distant metastatic sites (Mohler *et al.*, 2004; Locke *et al.*, 2008; Montgomery *et al.*, 2008). Sensitization of the AR to low levels of androgens also occurs through over-expression of the AR. Indeed AR amplification has been observed in up to 50% of mCRPC cases (Dan *et al.*, 2015). In addition to gene amplification, DNA hypermethylation has been shown to lead to reduced binding of the AR suppressor binding complex, resulting in enhanced AR mRNA expression (Perry *et al.*, 2010). Multiple mutations have been detected within the AR LBD. Furthermore, these mutations have been demonstrated to convert anti-androgen antagonism to AR agonism, enhancing AR transcriptional activity (Zhou *et al.*, 2010). Similarly, mutation of the AR LBD has also been documented to allow AR activation following the binding of alternative steroidal hormones such as progesterone and corticosteroids (Culig *et al.*, 1993; Zhao *et al.*, 2000). Finally, the emergence of AR splice-variants has been heavily linked to the progression of CRPC. There are a number of splice variants encoding an AR protein lacking the LBD, resulting in a constitutively active and nuclear localized AR molecule (Dehm *et al.*, 2008; Hu *et al.*, 2009).

In this section, an investigation into the impact of PPP1R14C depletion on the AR signalling axis from anti-androgen resistant and steroid deprived resistant LNCaP cells is performed. Resistant and steroid deprived LNCaP cells were generated in house and have been shown to possess an active androgen signalling axis (O'Neill *et al*.).

6.2 Results

6.2.1 PPP1R14C depletion reduces LNCaP cell growth

In order to investigate the impact of PPP1R14C RNAi depletion on cell growth, LNCaP cells were monitored by live cell imaging for 96 hours following transfection. Images were taken every 4 hours, and the percentage confluency calculated using the Incucyte Zoom software. Figure 6.2 shows representative images taken at 96 hours of LNCaP cells transfected with scrambled and siPPP1R14C-1 RNAi oligos, respectively. It is clear from the images that depletion of PPP1R14C resulted in impaired cellular proliferation whilst maintaining a physiological phenotype. Figure 6.1 highlights the rate of proliferation for the two experimental arms. LNCaP cells transfected with the scrambled control siRNA increased in confluency by 2.8-fold over a 96 hour period, whilst cells transfected with the siPPP1R14C-1 achieved a 2-fold increase in confluency over the same time period. This is the equivalent of a 45% reduction in cell proliferation.

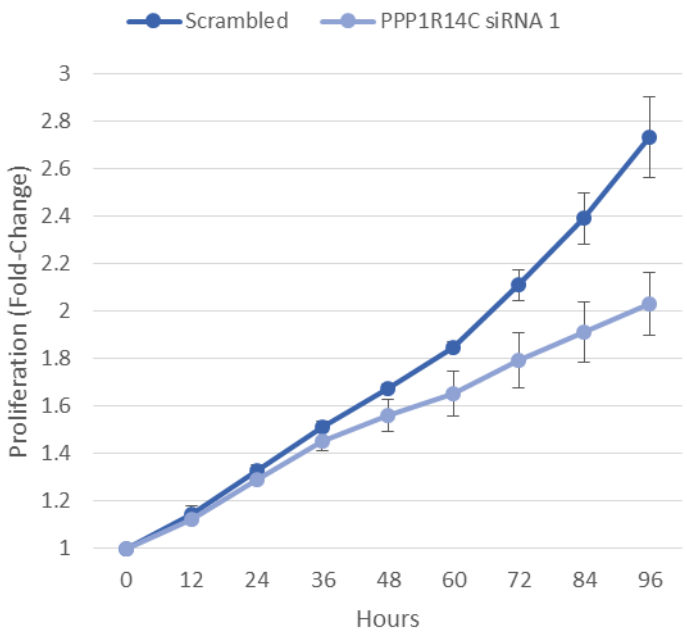


Figure *6.1* - ***PPP1R14C Depletion Reduces LNCaP Cell Growth***. *LNCaP cells depleted of PPP1R14C were cultured in full media for 96 hours. Growth was measured by live cell imaging and calculated from percentage confluency. Data represents n=3 mean ± sd.*

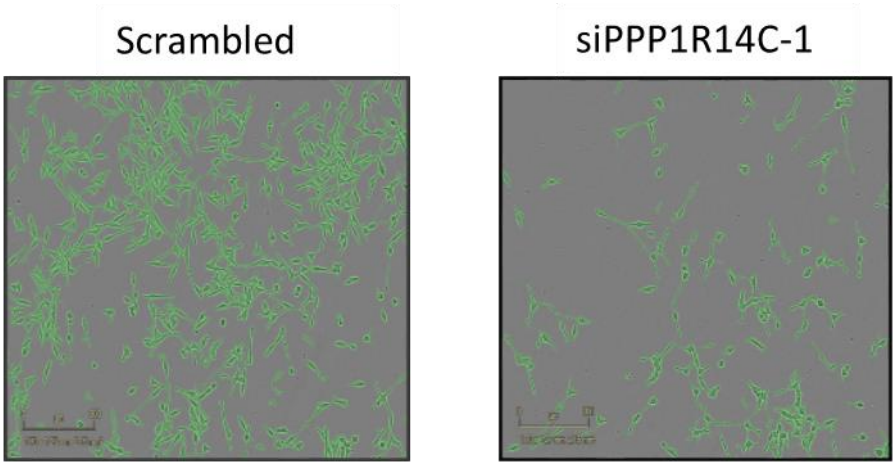


Figure *6.2* - ***PPP1R14C Depletion Doesn't Impact on the Morphology of LNCaP Cells.*** *LNCaP cells depleted of PPP1R14C and were imaged every 4 hours. This figure is a representative image of LNCaP cell growth and morphology at the 96 hour time-point.*

6.2.2 PPP1R14C depletion reduces LNCaP cell migration

To assess the impact of PPP1R14C depletion on cell migration a Boyden chamber assay was implemented as described in section 2.18. Figure 6.3 contains representative images from each of the experimental repeats for both siPPP1R14C-1 and scrambled RNAi transfection. Following cell fixation and staining, cells were counted and plotted in a box-plot chart including maximum value, minimum value, median, and 1st and 3rd quartiles. Knockdown of PPP1R14C resulted in a dramatic reduction of LNCaP cell migration compared to the scrambled control of approximately 80% (median, p-value).

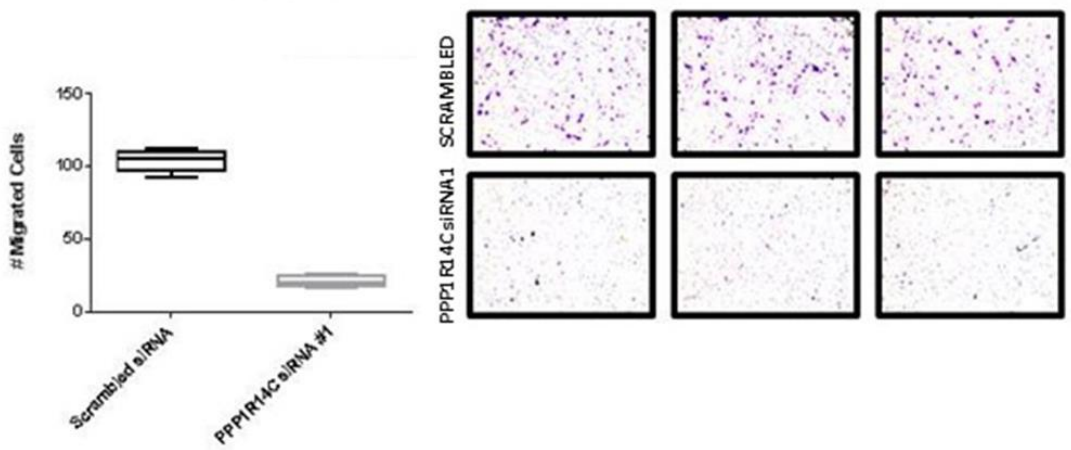


Figure ***6.3 - PPP1R14C Depletion Reduces LNCaP Cell Migration.*** *LNCaP cells were depleted of PPP1R14C as previously described for 48 hours. Cells were then trypsinised and counted (N=10,000) before being placed in the upper chamber of a Boyden chamber in DCC media. The lower chamber contained full media. Migrated cells were fixed, stained and counted after a period of 24 hours. Data represents n=3 maximum, minimum, median, 1st and 3rd quartile values.*

6.2.3 PPP1R14C depletion reduces androgen receptor transcriptional activity in distinct cell line models of treatment resistance

RNAi depletion of PPP1R14C in LNCaP-AI cells resulted in a statistically significant mean reduction in PPP1R14C mRNA expression of approximately 90%, as depicted in figure 6.4. In parallel with the parental LNCaP cell line, this resulted in a statistically significant repression of the AR target genes PSA and TMPRSS2 at the mRNA level as quantified by RT-qPCR. Indeed both genes were repressed by over 70%, reinforcing that the AR is still active in the absence of androgen in the LNCaP-AI cell line, and is sensitive to repression by MLCP activity.

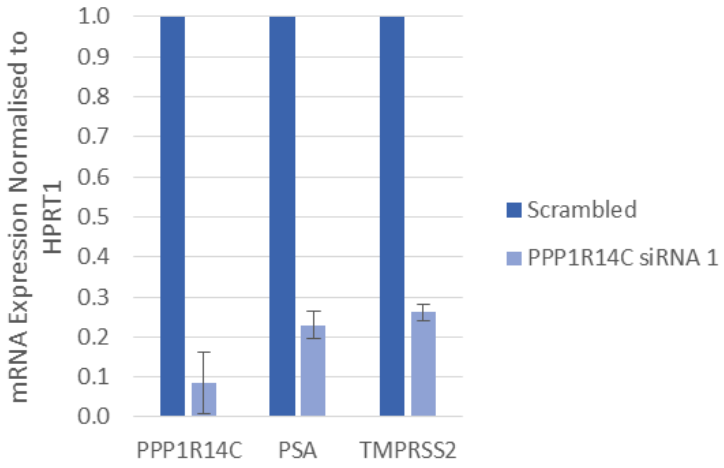


Figure *6.4* - ***PPP1R14C Depletion Reduces AR Transcriptional Activity in Androgen Independent LNCaP Cells****. PPP1R14C was depleted in LNCaP-AI cells for 72 hours in DCC media. AR regulated gene mRNA expression was quantified by RT-qPCR. Data represents n=3 mean ± sem. Unpaired student t test performed using graphpad*

Subsequent RNAi investigations took place in the anti-androgen resistant cell lines LNCaP-CdxR and LNCaP-EnzR in the presence of the respective anti-androgen. Successful knockdown of approximately 80% following siPPP1R14C-1 transfection can be observed in both LNCaP-CdxR and LNCaP-EnzR in figure 6.5 and figure 6.6 respectively. In the LNCaP-CdxR cell line this resulted in inhibition of both PSA and TMPRSS2 mRNA expression of over 60%. In the enzalutamide resistant LNCaP cell line, LNCaP-EnzR, PPP1R14C knockdown resulted in a 90% reduction in PSA expression, and an approximately 60% reduction in TMPRSS2 expression. Again, this reinforces that both resistant cells possess active AR signalling cascades in the

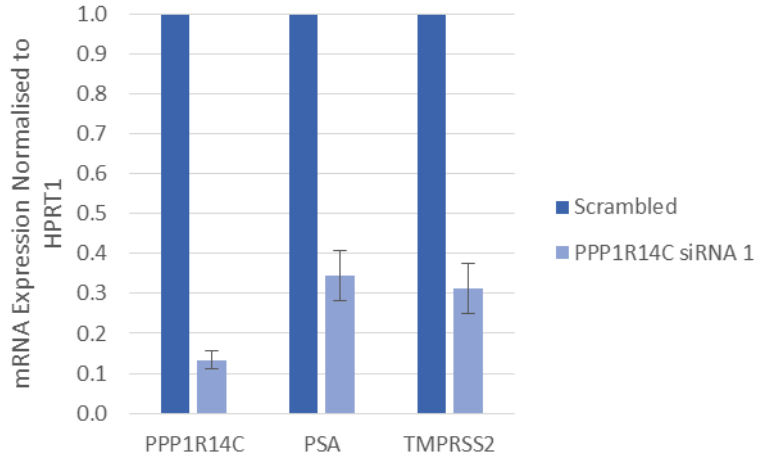


Figure *6.5* - ***PPP1R14C Depletion Reduces AR Transcriptional Activity in Casodex Resistant LNCaP Cells***. *PPP1R14C was depleted in LNCaP-CdxR cells for 72 hours in FM media + 10μM casodex. AR regulated gene mRNA expression was quantified by RT-qPCR. Data represents n=3 mean ± sem. Unpaired student t test performed using graphpad*

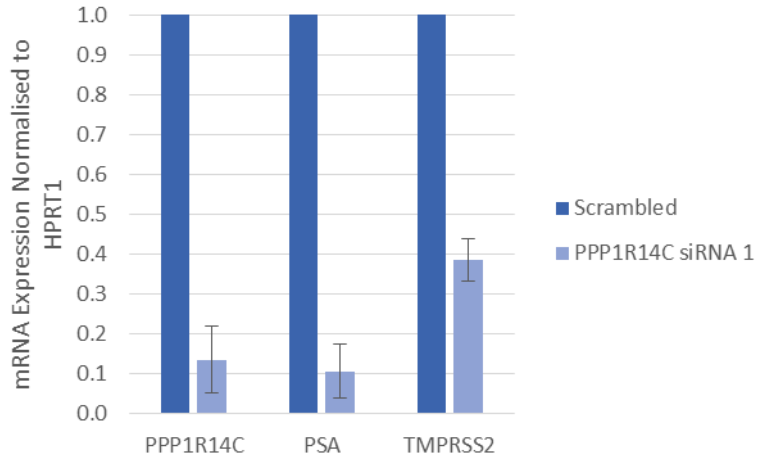


Figure *6.6* - ***PPP1R14C Depletion Reduces AR Transcriptional Activity in Enzalutamide Resistant LNCaP Cells***. *PPP1R14C was depleted in LNCaP-EnzR cells for 72 hours in FM media + 10μM enzalutamide. AR regulated gene mRNA expression was quantified by RT-qPCR. Data represents n=3 mean ± sem. Unpaired student t test performed using graphpad*

presence of their respective anti-androgen, and can be repressed following modulation of PPP1R14C expression.

Figure 6.7 highlights the ability of PPP1R14C to impact on the cell growth of the LNCaP-AI cell line. Over a 96 hour period, cells within the scrambled control arm of the assay increased in confluency by 4.5-fold. In contrast, LNCaP-AI cells depleted of PPP1R14C increased in confluency by less than 3-fold, representing a 50% reduction in cell proliferation.

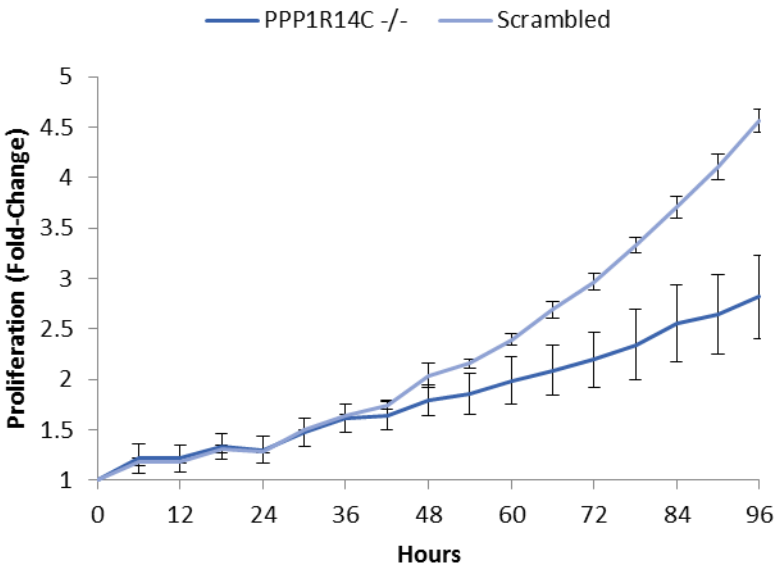


Figure *6.7* - ***PPP1R14C Depletion Reduces Androgen Independent LNCaP Cell Growth***. *LNCaP-AI cells depleted of PPP1R14C were cultured in DCC media for 96 hours. Growth was measured by live cell imaging and calculated from percentage confluency. Data represents n=3 mean ± sd.*

7.3 Discussion

In this section it was possible to identify the impact of PPP1R14C depletion on LNCaP cell proliferation and migration. LNCaP cells are androgen dependent and it has been previously shown that inhibition of the AR via multiple mechanisms is capable of reducing LNCaP cell growth and migration (Yang *et al.*, 2005b; Zhu and Kyprianou, 2010; Guerrero *et al.*, 2013). In previous sections it was demonstrated that PPP1R14C depletion leads to significant inhibition of the AR signalling cascade as well as impairing cell cycle progression, this now correlates with a 45% reduction in LNCaP cell proliferation over a 96-hour time-period. Indeed, detecting down-regulation of PPP1R14C at the protein level has proven difficult throughout this project

and therefore a direct relationship between PPP1R14C protein levels and proliferation cannot be concluded. However, it has been possible to quantify depletion of *PPP1R14C* at the mRNA level at both the 72 and 96 hour time points, confirming repression of *PPP1R14C* mRNA is still persistent at these-time points. One negative factor of this assay would be the time-point at which PPP1R14C mRNA depletion is first observed is unknown, and indeed, this should be incorporated into future assays. If this was known it might be possible to draw a more accurate conclusion between PPP1R14C mRNA expression and LNCaP cell growth. In this assay, confluency has been used as the measurement for cell growth, which provides a suitable surrogate for proliferation as long as cells do not reach such a high density that they begin to grow in layers, and indeed that the morphology of the cells does not change following modulation. As can be observed in figure 6.2 the morphology of the LNCaP cells following PPP1R14C depletion remains consistent with the morphology of the LNCaP cells transfected with the non-silencing control, suggesting there is no significant detrimental effect on essential cellular pathways. Furthermore, the cells in either arm do not reach a confluency forcing overlapping growth. It is also possible to observe that there is no evidence of increased cell death, which is consistent with the cell cycle data presented in section 4 where no significant difference in the population of LNCaP cells in the sub-G1 phase was detected, reinforcing that PPP1R14C does not appear to be cytotoxic, but rather, cytostatic.

Following implementation of the Boyden chamber assay, a profound reduction in the migration of LNCaP cells is observed following depletion of PPP1R14C of approximately 80% compared to the scrambled control. Therapeutically this is very advantageous, particularly as death from PC is typically associated with metastasis from the primary tumour to distant sites. PC is regarded as a slow growing cancer, and more recently the preferred treatment option for low-risk patients is active surveillance, therefore by combining a reduction in proliferation with a pronounced inhibition of migration would be well suited characteristics of future treatment modalities. Whilst the reduction in cell proliferation following PPP1R14C depletion may be associated with enhanced cell cycle regulation, the impact on migration is more likely to be associated with alternative signalling cascades. Cell motility is dictated via modulation of the actin cytoskeleton, and whilst actin polymerization pushes the plasma membrane forward, both the trailing membrane and stress fibres are pulled in through actomyosin contraction, a process that requires the reversible phosphorylation of MLC20, mediated by

both MLCK and MLCP (Watanabe *et al.*, 2007). Whilst neither the phosphorylation nor dephosphorylation of MLC20 has been inhibited, it could be possible that enhanced MLCP activity following depletion of PPP1R14C prevents hyperphosphorylation of MLC20, and as such reduces the rate at which MLC20 phosphorylation is cycled, thus slowing the migratory rate of LNCaP cells. Indeed, the implication of PPP1R14C on lamellipodia formation has been previously published (Madsen *et al.*, 2015). The lamellipodium is an essential cellular structure for cell migration and has been demonstrated to play a key role in PC invasion and metastasis. Upon migration, cells produce sheet-like protrusions, the lamellipodium, at the leading edge in order to generate cellular-matrix interactions, which upon cycling of the MLC20 phosphorylation status, leads to cellular contraction and motility (Kato *et al.*, 2014). Upon depletion of PPP1R14C, the authors noted a reduction in both the length and the peak angle of the lamellipodia in MDA-MDB-231 cells (breast adenocarcinoma), resulting in fewer, more broader extensions characterised by a reduction in pMLC20, and conclude that this phenotype leads to a reduction in intra-cellular hydrostatic pressure, preventing migration through confined spaces (Madsen *et al.*, 2015). This would directly implicate PPP1R14C depletion in the repression of cell migration via increased dephosphorylation of pMLC20, but it should also be noted that the tumour suppressor NF2 plays a crucial role in cell motility, providing secondary, indirect, evidence for the repression of migration by PPP1R14C knockdown. Both lamellipodia formation and MLC20 phosphorylation is mediated in a Rho/Rac1 GTPase dependent manner (Nobes and Hall; Kimura *et al.*, 1996). NF2 is a potent repressor of both Rho and Rac1 through inhibition of guanine nucleotide exchange factors, a crucial step in activation of both Rho and Rac1 (Morrison *et al.*, 2007). Thus, increased dephosphorylation and activation of NF2 by MLCP following PPP1R14C depletion, will undoubtedly play a repressive role in the reduction of LNCaP cell migration observed in figure 6.3. It should also be noted that cells are at their most migratory during the G1 phase of the cell cycle, and despite the increase in cells found in G1 following PPP1R14C depletion, a dramatic reduction in the migration of LNCaP cells is observed, making this finding more significant. In conclusion, a significant reduction in the proliferation of PC cells can be attributed to the increase in G1 cell cycle arrest observed in section 6, whilst a dramatic reduction in PC cell migration can be linked to increased dephosphorylation of both MLC20 and NF2 by MLCP, providing novel evidence that PPP1R14C depletion represents a novel approach to reduce both LNCaP cell proliferation and migration as well as AR transactivation.

The ability of the AR to function in the presence of next-generation anti-androgens and under castrate resistant conditions has become a hallmark feature of CRPC. Whilst next generation AR targeting agents only show modest efficacy in CRPC patients, it has become evident that targeting the AR signalling axis remains a viable therapeutic approach. In this section it has been possible to demonstrate that depletion of PPP1R14C results in the significant repression of AR transactivation as quantified by the mRNA expression of its target genes PSA and TMPRSS2 in 3 LNCaP derived cell lines, capable of continued AR signalling in the absence of androgen, and in the presence of casodex and enzalutamide, respectively. Furthermore, PPP1R14C knockdown reduces LNCaP-AI cell growth by 50%, demonstrating that this cell line is still sensitive to disruption of the AR signalling axis. In section 3 it was possible to demonstrate that depletion of PPP1R14C was capable of reducing AR transcriptional activity in both the presence and absence of androgen. Conversely, depletion of PPP1R12A resulted in increased AR transcriptional activity in both the presence and absence of androgen. This strongly suggests that MLCP is intrinsically repressive to AR function, but moreover, in a ligand independent manner. This is further confirmed in this section, as all 3 resistance models represent ligand independent resistance mechanisms, and are sensitive to depletion of PPP1R14C. The underlying mechanisms of resistance within each cell line remains unknown, and therefore it is not possible to make any direct conclusions regarding the impact of PPP1R14C depletion on the particular resistance mechanisms each cell line possess. However, as knockdown of PPP1R14C plays a pivotal role in the repression of upstream signalling cascades required for AR expression and signalling in both the presence and absence of androgen, it is likely these too are involved within the context of this section. Indeed, despite a reduction in AR function in both the LNCaP-CdxR and LNCaP-EnzR cell lines following PPP1R14C depletion, ultimately the impact on cell growth has not been assessed and as such requires future interrogation. Furthermore, it has been demonstrated that mutations within the AR LBD, enabling antagonists to act as agonists, induce a distinct transcriptional program to wild type AR, and as such, additional AR target gene expression should be investigated, particularly in relation to mutant AR target genes (O'Neill *et al.*, 2015). This chapters findings are schematically represented in figure 8.5.

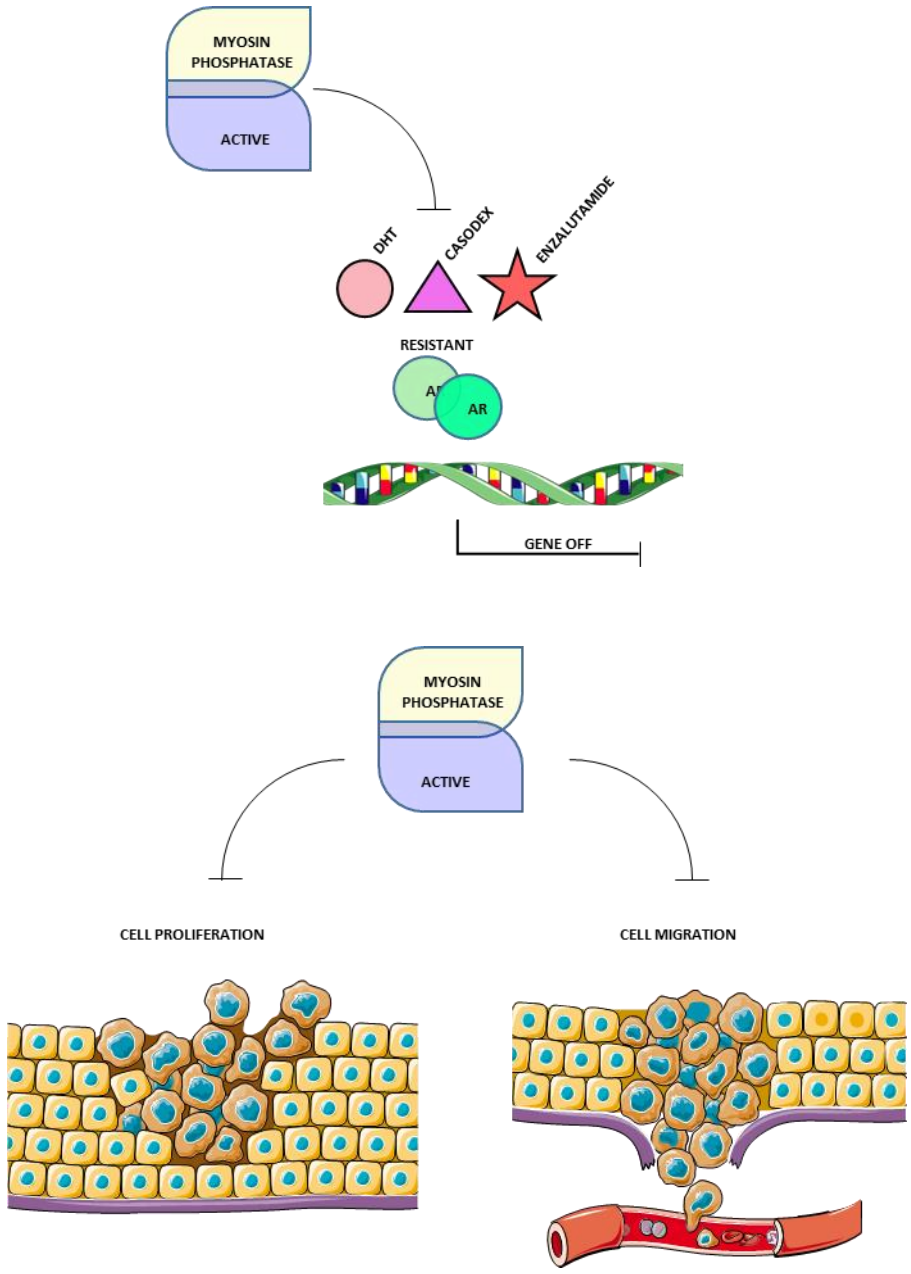


Figure *6.8* – ***Schematic Summary of Section 6 Results.*** *RNAi depletion of PPP1R14C reduces LNCaP cell proliferation and migration. Furthermore, PPP1R14C RNAi depletion reduces AR transcriptional activity in LNCaP-AI, LNCaP-CdxR and LNCaP-EnzR cell lines providing significant evidence for viability of PPP1R14C as a therapeutic target.*

Chapter 7.

NUAK1/2 Represent Novel Therapeutic Targets in the Treatment of Prostate Cancer

7.1 Introduction

Throughout this thesis a thorough analysis into the impact of MLCP activity on AR function has been performed following RNAi depletion of PPP1R14C, providing significant evidence for its viability as a therapeutic target in PC. However, limited availability of commercial reagents against PPP1R14C, and indeed insufficient knowledge on the therapeutic disruption of PP1 holoenzymes, currently render PPP1R14C untargetable. As such, investigation into additional endogenous MLCP inhibitors represents an attractive route of further reinforcing previous findings within this thesis, whilst identifying potential novel therapeutic targets in the treatment of PC. With this in mind, PTMs of MLCP will be introduced in this chapter, with an emphasis on MLCP inhibition.

As described in section 4, PPP1R12A is the substrate specifying subunit for the PP1 holoenzyme MLCP, and is subject to a number of PTMs capable of influencing its activity. These include phosphorylation, ubiquitination and methylation. Phosphorylation of PPP1R12A occurs in response to a number of physiological stimuli, many of which result in specific spatio-temporal regulation of MLCP activity. It has been demonstrated that PPP1R12A is phosphorylated specifically during mitosis at 3 serine residues; serine 432, serine 473 and serine 601 (Yamashiro *et al.*, 2008). This was shown to be mediated by proline directed kinases, including CDK1. The authors went on to show that phosphorylation of serine 473 results in the formation of a PLK1 binding motif, and indeed, phosphorylated PPP1R12A at serine 473 co-immunoprecipitates with PLK1, whilst a PPP1R12A 473A mutant does not. Further characterisation revealed that this interaction mediates dephosphorylation of the PLK1 activation site, threonine 210, by MLCP. A more recent study went on to show that phosphorylation of PPP1R12A serine 445 by LATS1 was crucial for PLK1 inactivation by MLCP, suggesting there is significant interplay between the different phosphorylation sites on PPP1R12A (Chiyoda *et al.*, 2012). Crucially, phosphorylation of serine 445 by large tumour suppressor kinase 1 (LATS1) occurred in an ataxia telangiectasia mutated (ATM) dependent manner following DNA damage, resulting in repression of PLK1 activity and attenuation of mitotic entry. Another site capable of enhancing MLCP activity upon phosphorylation is serine 695, mediated by PKA and PKG (Wooldridge *et al.*, 2004). It was demonstrated that phosphorylation of serine 695 prevents phosphorylation at threonine 696, the most characterised inhibitory site of PPP1R12A. Phosphorylation of threonine 696 has been

extensively studied in the context of cell motility and actomoyosin contractility, and has been demonstrated to account for RhoA mediated Ca^{2+} sensitization in smooth muscle. More in depth studies successfully demonstrated that the RhoA-associated kinases ROCKI/II were responsible for phosphorylation and inhibition of PPP1R12A at threonine 696 and serine 854, and as such these 2 residues have since been regarded as the PPP1R12A inhibitory phosphorylation sites (Kimura *et al.*, 1996; Feng *et al.*, 1999). Subsequent studies have shown that many kinases are capable of phosphorylating PPP1R12A at threonine 696, and indeed inhibit MLCP activity. These include RAF1, myotonic dystrophy kinase-related CDC42-binding kinase, myotonic dystrophy protein kinase, ILK and ZIPK (Muranyi *et al.*, 2001; Broustas *et al.*, 2002; Kiss *et al.*, 2002; Wilkinson *et al.*, 2005; Takamoto *et al.*, 2006).

Methylation of PPP1R12A at lysine 442 has been shown to impact on its stability both *in-vitro* and *in-vivo* (Cho *et al.*, 2011). Methylation was demonstrated to be mediated by SET9, which upon RNAi depletion, led to a pronounced destabilization of the PPP1R12A protein. Conversely, demethylation has been shown to be mediated by lysine demethylase 1 (LSD1), which upon RNAi depletion leads to enhanced PPP1R12A stability, and is characterised by increased dephosphorylation of MLCP substrates, specifically RB1 serine 807/811. Degradation of PPP1R12A has been reported to occur in an SIAH2 dependent manner (Twomey *et al.*, 2010). PPP1R12A contains an SIAH2 consensus sequence, 'RLAYVAP', within its CTD, and has been shown to interact through this motif with SIAH2. As such, PPP1R12A acts as a signalling hub for a number of signalling pathways and plays a crucial role in modulating cell motility, and a number of down-stream signalling cascades associated with the activity of its substrates.

However, it is the role of the adenosine monophosphate-activated protein kinase (AMPK)-related kinases NUAK1 and NUAK2 that were pursued within the context of MLCP inhibition within this thesis. NUAK1/2 were identified as AMPK-related kinases through sequence homology with the AMPK catalytic domain (Manning *et al.*, 2002). Indeed, NUAK1/2 can be activated via phosphorylation by LKB1 like AMPK (Lizcano *et al.*, 2004). However, the known AMPK inducers, AICAR, phenformin and metformin, failed to induce NUAK1/2 activity, suggesting different regulatory mechanisms exist for NUAK1/2 (Lefebvre and Rosen, 2005). Both NUAKs can be activated in response to osmotic stress and nutrient deprivation including glucose and glutamine withdrawal, whilst NUAK2 activity can also be induced by DNA damage

(Suzuki *et al.*, 2003a; Suzuki *et al.*, 2003b). Crucially, both members have been associated with tumorigenesis. NUAK1 has been shown to induce tumour cell survival and suppress cell death during nutrient starvation, as well as being identified as an important component of AKT-dependent cancer cell survival and migration (Suzuki *et al.*, 2003b; Kusakai *et al.*, 2004). Furthermore, over-expression of NUAK1 has been demonstrated to induce cellular aneuploidy and senescence (Humbert *et al.*, 2010). Similarly, NUAK2 has been implicated with the migration and proliferation of melanoma cells, with NUAK2 expression being a significant risk factor for patient relapse (Namiki *et al.*, 2011). In fact, NUAK2 gene amplification has recently been identified in a number of human cancers (Monteverde *et al.*, 2015). Interestingly, PPP1R12A is the only known substrate for NUAK2 (Yamamoto *et al.*, 2008). NUAK1 is also capable of phosphorylating PPP1R12A in addition to LATS1, an MLCP enhancer, contributing to the cell migratory and detachment roles of the NUAK kinases (Humbert *et al.*, 2010; Zagorska *et al.*, 2010).

Phosphorylation of PPP1R12A by NUAK1/2 occurs on serine 445, serine 472 and serine 910 (Zagorska *et al.*, 2010). Crucially, phosphorylation at serine 472, triggers the binding of 14-3-3 and inhibits the catalytic activity of MLCP (Koga and Ikebe, 2008). Over-expression of 14-3-3 significantly impairs MLCP activity through enhanced binding to PPP1R12A characterised by an increase in the phosphorylation status of MLC20. 14-3-3ζ has also been demonstrated to induce AR transcriptional activity in the absence of androgens in an AR dependent manner (Quayle and Sadar, 2007). A subsequent study demonstrated that 14-3-3ε was involved in a positive feed-forward loop with the AR and capable of driving AR nuclear localization in the absence of androgens, whilst enhancing MAPK induced AR activity in CRPC (Titus *et al.*, 2009). More recently 14-3-3ζ has been confirmed as an AR regulated gene capable of activating AR transcriptional activity, enhances PC cell survival and proliferation, and facilitates the progression of PC to CRPC, drawing considerable similarities to the impact of PPP1R14C on AR transactivation (Murata *et al.*, 2012).

With this in mind, this section aims to elucidate the impact of endogenous MLCP inhibitors on the AR-signalling axis using commercially available small molecule inhibitors. NUAK1/2, ROCKI/II and MLCK inhibitors will be employed to elucidate the means by which MLCP inhibition enhances AR activity. NUAK1/2 and ROCKI/II phosphorylate different PPP1R12A phospho-residues resulting in distinct mechanisms of MLCP inhibition, whilst MLCK directly

phosphorylates the MLCP substrate MLC20 serine 19, potentially revealing a novel route of AR regulation. Affirmation of the impact of MLCP modulation on the AR signalling cascade will reinforce previous findings presented in this thesis, whilst simultaneously providing additional therapeutic targets in the treatment of PC.

7.2 Results

7.2.1 Myosin light chain kinase inhibition enhances androgen receptor transcriptional activity

To examine if direct phosphorylation of MLC20 contributes to the regulation of AR function by MLCP, LNCaP cells were exposed to 5µM ML-7, a specific inhibitor of MLCK. An initial concentration of 5µM was used as previously described in a panel of human cancer cell lines (Barkan *et al.*, 2008). Inhibition of MLCK would be expected to result in impaired phosphorylation of MLC20, mirroring an increase in MLCP activity following PPP1R14C depletion. However, upon inhibition of MLCK with ML-7, a significant induction of both *PSA* and *TMPRSS2* mRNA expression quantified by RT-qPCR is observed in LNCaP cells in the presence of androgen, contrasting to the repression of AR target gene expression observed following PPP1R14C knockdown. Figure 9.1 demonstrates that PSA and TMPRSS2 mRNA expression was increased by 1.6 and 2.5-fold, respectively.

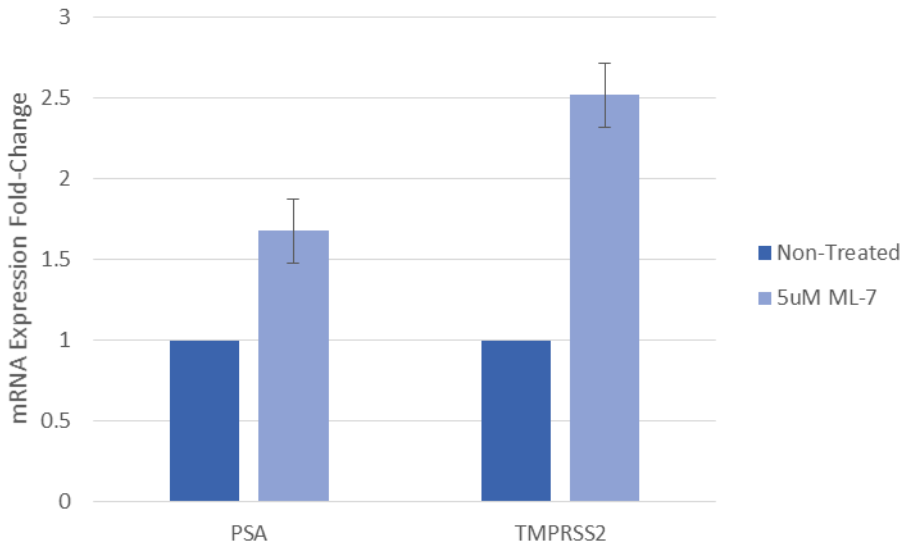


Figure *7.1* - ***Inhibition of Myosin Light Chain Kinase Increases AR Transcriptional Activity.*** *LNCaP cells were subject to inhibition of MLCK with 5µM ML-7 for a period of 24 hours. mRNA expression was quantified by RT-qPCR. Data represents n=3 mean ± sd. Unpaired student t test performed using graphpad*

7.2.2 ROCK I/II inhibition enhances androgen receptor transcriptional activity

ROCK I/II is the known RhoA effector responsible for increased MLC20 phosphorylation. ROCK I/II is capable of directly phosphorylating MLC20 serine 19 as well as phosphorylating PPP1R12A on threonine 696 and serine 854, inhibiting MLCP activity towards phosphorylated MLC20. Similar to the inhibition of MLCK, upon addition of 10µM Y-27632, a specific ROCK I/II inhibitor, increases in AR regulated gene expression in the presence of androgen was observed as demonstrated in figure 7.2. 10µM Y-27632 was used as previously described for the LNCaP cell line (Xiao *et al.*, 2009). Whilst the induction of PSA mRNA expression was not statistically significant, TMPRSS2 mRNA expression was significantly increased 2-fold. This supports the data presented following MLCK inhibition, and strongly suggests modulation of the phosphorylation status of MLC20 does not contribute to the repressive regulation of the AR by MLCP, but rather may suggest a positive role in AR function.

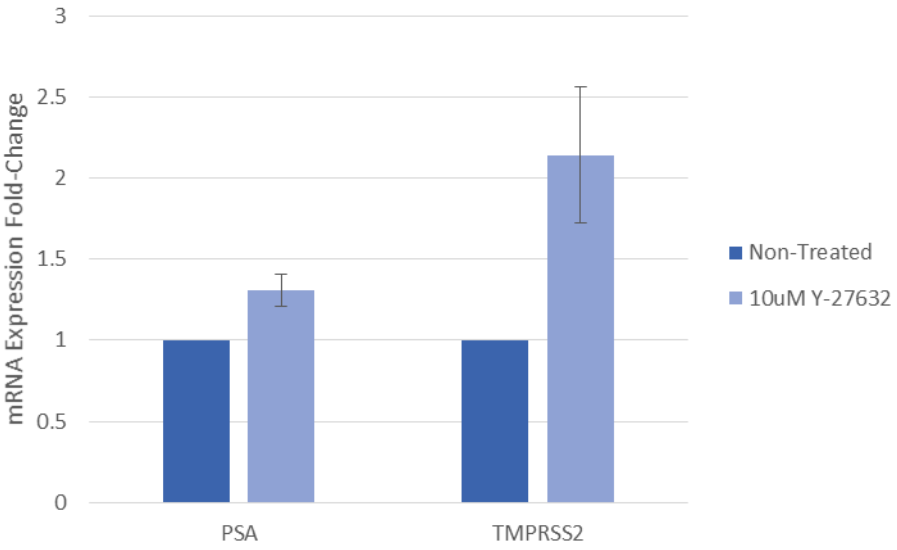


Figure *7.2* - ***Inhibition of ROCK I/II Increases AR Transcriptional Activity.*** *LNCaP cells were subject to inhibition of ROCK I/II with 10µM Y-27632 for a period of 24 hours. mRNA expression was quantified by RT-qPCR. Data represents n=3 mean ± sd. Unpaired student t test performed using graphpad*

7.2.3 NUAK 1/2 inhibition represses androgen receptor transcriptional activity

Following on from this, LNCaP cells were exposed to NUAK1/2 inhibition in the presence of androgen through the addition of 100nM WZ-4003, a specific NUAK 1 and NUAK2 inhibitor (IC50 20nM and 100nM respectively). WZ-4003 IC50 values were calculated for both the LNCaP and CWR-22RV1 cell lines (6.5µM and 14µM, respectively), as depicted in supplementary figure 2. However, it became apparent that the AR signalling axis was significantly repressed at much lower concentrations of WZ-4003. Figure 7.3 demonstrates

that 100nM WZ-4003 was capable of significantly reducing AR transcriptional activity in relation to the mRNA expression of PSA and TMPRSS2. PSA mRNA expression was reduced by 60% whilst TMPRSS2 expression was reduced by 40%. Further reinforcing the positive impact of NUAK1/2 inhibition on AR activity, a dose-dependent reduction in LNCaP cell growth can be observed in figure 7.4. Interestingly, at the 100nM dose shown to significantly repress AR transactivation, only a modest reduction in cell proliferation was observed. However, statistically significant repression of cell proliferation was observed upon the addition of 300nM and 1000nM WZ-4003, respectively. Analysis of MLCP activity following NUAK1/2 inhibition was then investigated by western blot using phospho-specific antibodies against the MLCP substrates phosphorylated MLC20 serine 19 and phosphorylated RB1 serine 807/811.

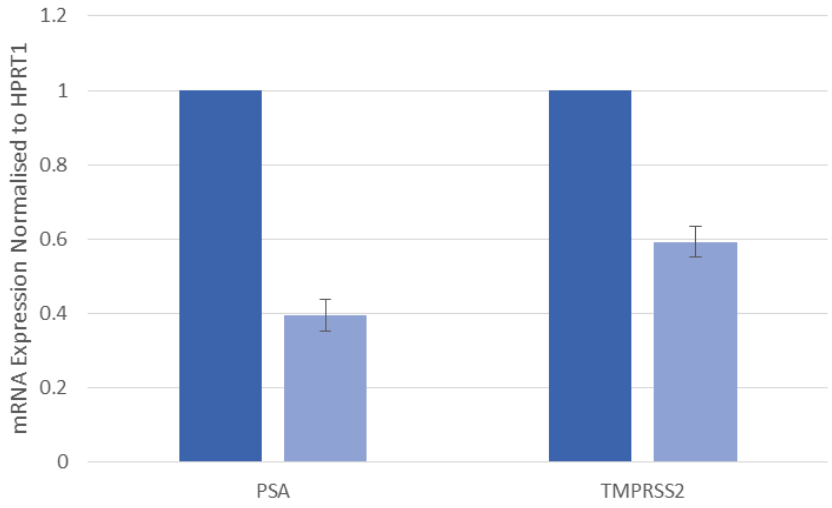


Figure 7.3 - ***Inhibition of NUAK 1/2 Reduces AR Transcriptional Activity in LNCaP Cells.*** *LNCaP cells were subject to inhibition of NUAK 1/2 with 100nM WZ-4003 for a period of 24 hours. mRNA expression was quantified by RT-qPCR. Data represents n=3 mean ± sd. Unpaired student t test performed using graphpad*

Similarly the phosphorylation status of PPP1R12A at serine 472 was investigated using a phospho-specific antibody. Figure 7.5 demonstrates that a modest reduction in the phosphorylation status of PPP1R12A serine 472 occurred in a dose dependent manner, with the largest reduction occurring following the addition of 300nM WZ-4003. This correlated with a pronounced reduction in the phosphorylation status of both RB1 serine 807/811 and MLC20 serine 19. No observable reduction in total PPP1R12A was observed, suggesting the increase in MLCP activity occurs at the post-translational level.

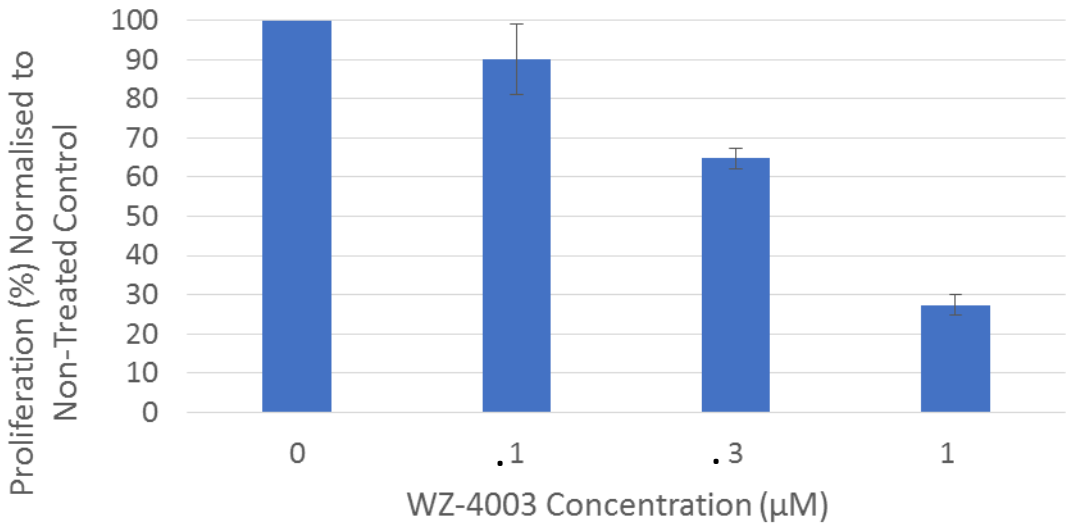


Figure *7.4* - ***Inhibition of NUAK 1/2 Reduces LNCaP Cell Growth.*** *LNCaP cells were subject to inhibition of NUAK 1/2 with 0, 100, 300 and 1000nM WZ-4003 for a period of 96 hours. Cell growth was measured by live cell imaging. Data represents n=3 mean ± sd.*

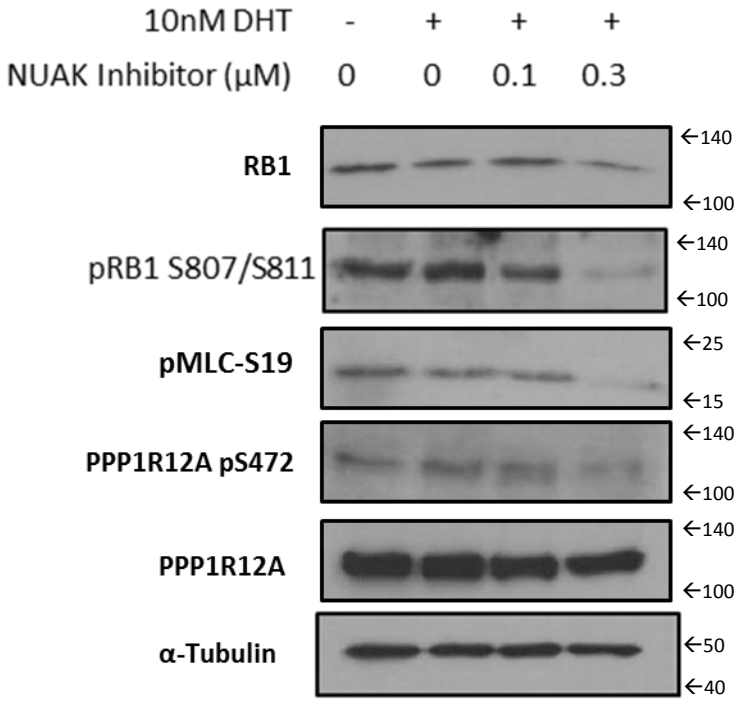


Figure *7.5-* ***Inhibition of NUAK 1/2 Increases Myosin Phosphatase Activity.*** *LNCaP cells were subject to inhibition of NUAK 1/2 with 0, 0.1, 0.3 µM WZ-4003 for a period of 24 hours. Cell lysates were then analysed by western blot. A reduction in the phosphorylation status of MLCP substrates RB1 and pMLC20 is observed following the incubation of LNCaP cells with WZ-4003 in a dose dependent manner. This correlates with a reduction in the phosphorylation status of PPP1R12A serine 472.*

7.2.4 NUAK 1/2 inhibition represses constitutively active androgen receptor variant transcriptional activity

The CWR-22RV1 cell line express constitutively active AR splice variants, particularly the AR-V7 and AR-1/2/3/2B variants (Tepper *et al.*, 2002). As described in section 8, the emergence of AR variants represents a significant prognostic factor in disease progression and treatment resistance. Unfortunately, the CWR-22RV1 cell line do not express detectable PPP1R14C and therefore it has not been possible to investigate the impact of PPP1R14C depletion on AR splice variant activity. However, they do express NUAK1 and NUAK2, therefore using the small molecule inhibitor WZ-4003 it has been possible to investigate the impact of MLCP modulation on AR variant transcriptional activity. Figure 7.6 represents the impact of 100nM WZ-4003 exposure on AR transcriptional activity in the CWR-22RV1 cell line cultured in full media. Upon inhibition of NUAK1/2 with WZ-4003, a statistically significant reduction in the mRNA expression of both PSA and TMPRSS2 was observed of 60% and 50% respectively. This correlated with a significant repression in CWR-22RV1 cell growth in the presence of 1µM WZ-4003 of 40% as depicted in figure 7.7. This was less pronounced than the 75% reduction in proliferation observed for the LNCaP cell line. Another treatment resistant cell line employed within this section was the LNCaP-EnzR cell line described in section 6. Again, this cell line proved to be sensitive to the inhibition of NUAK1/2, resulting in a 60% reduction in cell growth.

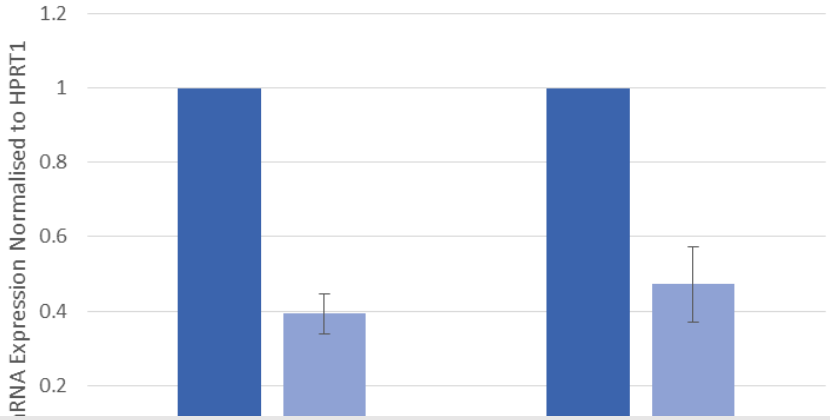


Figure *7.6* - ***Inhibition of NUAK 1/2 Reduces AR Transcriptional Activity in CWR22RV1 Cells.****CWR22RV1 cells were subject to inhibition of NUAK 1/2 with 100nM WZ-4003 for a period of 24 hours. mRNA expression was quantified by RT-qPCR. Data represents n=3 mean ± sd. Unpaired student t test performed using graphpad*

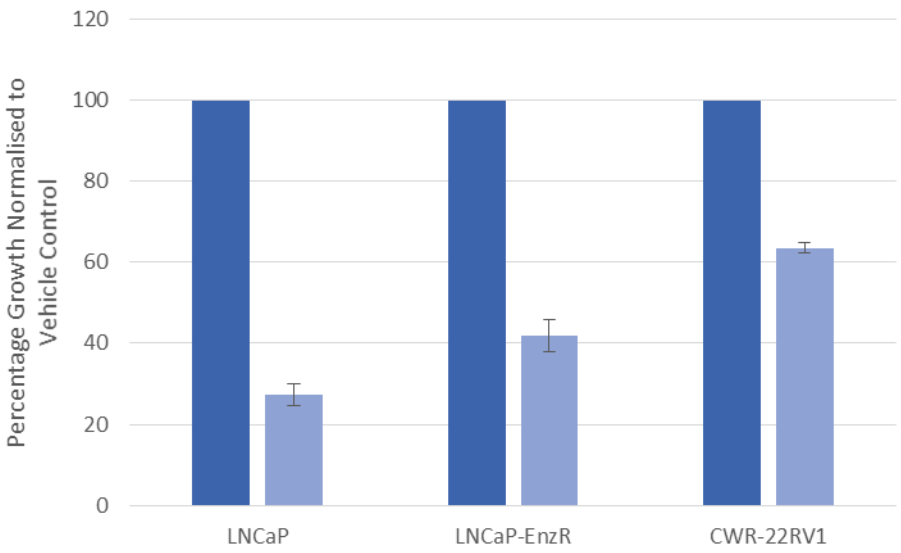


Figure 7.7 - ***Inhibition of NUAK 1/2 Reduces Enzalutamide Resistant LNCaP and CWR22RV1 Cell Growth.*** *Cell lines were subject to inhibition of NUAK 1/2 1μM WZ-4003 for a period of 96 hours. Cell growth was measured by live cell imaging. Data represents n=3 mean ± sd.*

7.3 Discussion

In this section the role of endogenous MLCP modulators was assessed in relation to AR regulation. The aim was to identify potential underlying mechanisms supporting the repressive role of MLCP on AR regulation previously identified in this thesis, and as such, to identify novel AR co-activating targets. As MLCP has been extensively studied in the context of MLC20 serine 19 dephosphorlyation, and MLCK directly phosphorylates MLC20 serine 19, inhibition of this kinase with the small molecule ML-7 would enable evaluation of this modification in the context of AR signalling. As observed in figure 7.1, MLCK inhibition results in enhanced AR transcriptional activity, suggesting that dephosphorylation of MLC20 serine 19 by MLCP is not crucial for the repression of AR activity. In the literature, MLCK inhibition with ML-7 has been demonstrated to enhance the cytotoxic activity of etoposide in PC cells, and independently reduce the growth of PC tumours *in-vivo* as a single agent (Gu *et al.*, 2006). However, the impact of MLCK inhibition on AR signalling was not evaluated and as such requires further interrogation. Similarly, ROCK I/II plays a pivotal role in the phosphorylation of MLC20 serine 19 by both direct phosphorylation, and via the inhibitory phosphorylation of MLCP. Inhibition of ROCK I/II with Y-27632 has been demonstrated to attenuate PC motility

and proliferation in the AR-null PC cell line, PC3 (Zhang *et al.*, 2014). As such, both MLCK and ROCK I/II inhibition have been independently identified as potential therapeutic targets in the treatment of PC, without a thorough investigation into the impact on AR activity. In this section it became evident that both inhibition of MLCK and ROCK I/II in fact induce AR transcriptional activity, and as such, may not represent suitable targets in the treatment of PC. Whilst no studies investigating the impact of MLCK on AR activity have been published, one study has identified that MLCK expression is down-regulated in response to androgen in an AR dependent manner (Leveille *et al.*, 2009). One hypothesis as to why MLCK inhibition can increase AR transcriptional activity would be that both MLCK and the AR form a negative feedback loop, and upon MLCK inhibition, AR expression and ultimately activity is increased. The relationship between ROCK I/II and AR activity is also understudied. However, one study also documents down-regulation of ROCK I/II in an AR mediated manner, again raising the possibility of another negative feedback loop (Kroiss *et al.*, 2015). But more compelling is that Y-27632, the ROCK I/II inhibitor employed in this section, dramatically enhances signalling of the EGFR pathway (Nakashima *et al.*, 2011). This results in significant induction in the activity of MAPK1 and MAPK3, kinases capable of inducing AR activity in both the presence and absence of androgens (Gioeli *et al.*, 1999). The induction of MAPK activity with Y-27632 may be responsible for the increase in AR transactivation observed in figure 7.2. As such, preventing phosphorylation of MLC20 serine 19, either directly or indirectly, may contribute to the repression of PC cell growth and migration, but it would appear that it does not contribute to the repression of AR activity, suggesting additional MLCP substrates are responsible for the repression of AR function observed throughout this thesis.

In contrast to MLCK and ROCK I/II inhibition, exposure of LNCaP cells to the NUAK1/2 inhibitor WZ-4003 appears to contribute to MLCP mediated repression of the AR signalling cascade through reduced phosphorylation of PPP1R12A. In parallel with PPP1R14C depletion, NUAK1/2 inhibition results in enhanced dephosphorylation of phosphorylated MLC20 serine 19, but crucially, also results in dephosphorylation of the MLCP substrate RB1 at serine 807/811, suggesting NUAK1/2 phosphorylation of PPP1R12A contributes to RB1 repression. Furthermore, NUAK1/2 inhibition prevents transactivation of the androgen independent AR variants in the CWR-22RV1 cell line, further reinforcing that MLCP represses AR function in a ligand independent manner. As discussed in the introduction to this section, phosphorylation

of PPP1R12A at serine 472 mediates the interaction between MLCP and the AR inducer 14-3-3 resulting in subsequent inhibition of MLCP activity. As a reduction in PPP1R12A serine 472 phosphorylation is observed following NUAK1/2 inhibition, it is likely the interaction between MLCP and 14-3-3 has been diminished, allowing for enhanced activity towards the additional substrates including RB1, NF2 and PLK1 outlined in section 4.

Crucially, inhibition of NUAK1/2, like RNAi depletion of PPP1R14C, results in increased dephosphorylation of MLC20 serine 19. However, through the inhibition of MLCK and ROCK I/II, this has been shown to not represent a route of AR repression, and therefore, MLCP must impose its repressive role on AR function through the dephosphorylation of additional substrates. Fundamentally, AR repression by MLCP may be mediated by the distinct spatio-temporal localization of its endogenous modulators. Both MLCK and ROCK I/II are localized to the cell periphery where they are able to respond to extracellular cues and signalling cascades, and ultimately modulate the phosphorylation of MLC20 directly and indirectly. Conversely, PPP1R14C has been shown to translocate to the nucleus upon phosphorylation following serum stimulation (Kiss *et al.*, 2008; Madsen *et al.*, 2015). Indeed, preliminary evidence presented in section 5 would suggest that PPP1R14C is capable of mediating a rapid response to androgen stimulation in the context of MLCP inhibition. In addition, NUAK1/2 have both been shown to predominantly localize to the nucleus and the cytoplasm, whilst PPP1R12A also localizes within both the cytoplasm and nucleus, targeting MLCP to its distinct substrates throughout varying subcellular localizations. Crucially, the MLCP substrates identified as being differentially phosphorylated following PPP1R14C RNAi depletion in section 4 all localize to the nucleus as well as the cytoplasm. Therefore, it is likely that PPP1R14C and NUAK1/2 contribute to the repression of MLCP at subcellular locations where MLCP substrates important for AR regulation co-localize.

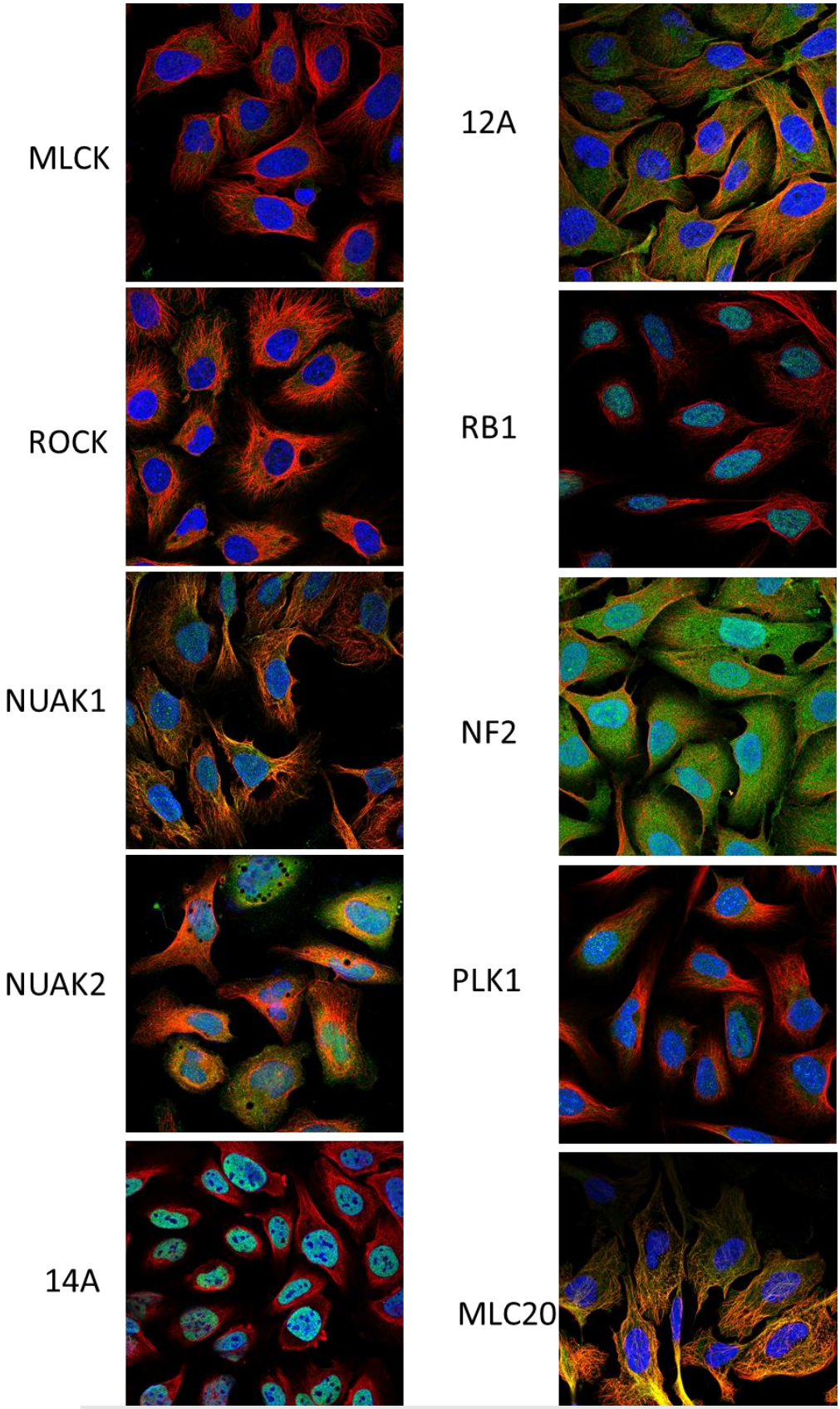


Figure ***7.8 - Distinct Subcellular Localizations of Endogenous MLCP Inhibitors and Substrates*** *– collated from Human Protein Atlas. Myosin Phosphatase is distributed throughout distinct subcellular localizations, whilst its substrates and endogenous inhibitors tend to localize to specific subcellular localizations, which in turn may provide spatio-temporal regulation of myosin phosphatase activity.*

Finally, investigation into the expression of NUAK1 and NUAK2 in publicly available datasets revealed that both kinases undergo gene amplification in PC. In the most recent publicly available dataset (Beltran *et al.*, 2016), NUAK1 was found to undergo gene amplification in 21% of cases (16/77), reinforcing its viability as a candidate therapeutic target. NUAK2 was found to be genomically amplified in 34% of cases (26/77), also confirming its validity as a target. Interestingly, NUAK2 amplification has been associated with PTEN deficiency in melanoma, promoting disease progression (Namiki *et al.*, 2015). As described in section 1.2.3, PTEN loss is a major prognostic marker for PC and is frequently observed in up to 40% of cases. Therefore it will be of interest to see if the association between NUAK2 and PTEN observed in melanoma extrapolates to PC. Furthermore, NUAK2 is located on chromosome 1q32, a gene locus found to be amplified in 45% of advanced PC samples but unaltered in primary tumours (Holcomb *et al.*, 2008). In a subsequent study, 1q32 was found to be amplified in 50% of metastatic PC samples (Holcomb *et al.*, 2009). As such, NUAK2 not only represents a valuable biomarker, but warrants further interrogation as a potential therapeutic target in the treatment of PC. This chapters findings are schematically represented in figure 7.9.

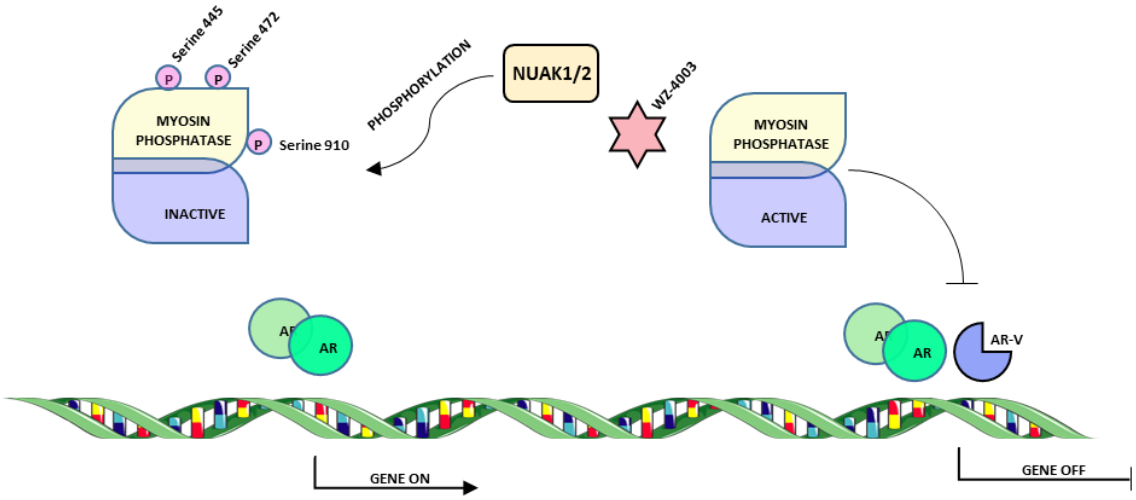


Figure *7.9* – ***Schematic Summary of Section 9 Results.*** *Inhibition of NUAK 1/2 mediated MLCP phosphorylation through the addition of WZ-4003results in repression of AR and AR-V transcriptional activity characterised by increased MLCP activity.*

Chapter 8.

Concluding Remarks

Disruption of the androgen signalling axis is initially a very effective and essential strategy in the treatment of localized and locally advanced PC. Unfortunately, patient responses to this form of treatment invariably fail after a median period of 2 years, with the disease progressing to castrate resistance. Crucially, it has been demonstrated that the AR is still responsible for driving disease progression and maintaining an active androgen signalling axis. Furthermore, the addition of next generation anti-androgens, such as enzalutamide, to the treatment regimens of CRPC patients, elicit partial responses and convey a modest survival advantage over placebo, demonstrating that the AR remains a viable therapeutic target even under androgen ablation. Indeed, resistance to next generation anti-androgens has been heavily linked to aberrant activation of the AR signalling axis, including AR mutation, emergence of AR splice variants and dysregulation of AR co-activators. The activity of AR co-activators, particularly those that are capable of directly modifying the AR at the post-translational level, are often found to positively correlate with disease progression, and reciprocally, post-translationally modified AR species can be indicative of a poor disease prognosis. With this in mind, a significant knowledge gap was identified surrounding the role of phosphatase enzymes on AR function in the progression of PC. To address this issue, an RNAi screen targeting 291 phosphatase enzymes and phosphatase related proteins was performed in the androgen responsive PC cell line, LNCaP, using AR transcriptional activity as an experimental end-point. This resulted in the identification of both AR co-activators and co-repressors, representing potential therapeutic targets and biomarkers, and providing a novel insight into the role of phosphatase enzymes on AR transcriptional activity. Of particular interest was the impact of PP1 regulatory subunit depletion on AR function, providing compelling evidence for the differential regulation of AR activity by distinct PP1 holoenzymes. In contrast to the current literature surrounding the positive impact of PP1 on AR function, it has been possible to demonstrate for the first time that PP1 is a negative regulator of AR activity. Furthermore, both the positive and negative regulation of the AR by PP1 is dictated by association with its mutually exclusive regulatory subunits, revealing a novel layer of complexity to the underlying mechanisms of regulation previously established. Whilst a number of regulatory subunits were depleted in the RNAi screen, to date over 200 PP1 interacting proteins have been identified, resulting in the formation of hundreds of PP1 holoenzymes, each with distinct subcellular localizations, substrate specificity and catalytic activity, emphasising the current lack of knowledge regarding AR regulation by PP1

holoenzymes. Conversely, this thesis highlights the opportunity to further dissect the role of PP1 regulatory subunits on AR function, allowing for the identification of specific PP1 holoenzymes involved in the androgen signalling axis, providing a novel route of therapeutically targeting the activity of PP1. As such, it was identified within this thesis that MLCP is a multimeric PP1 holoenzyme intrinsically repressive to AR function. Depletion of PPP1R12A, the substrate specifying regulatory subunit of MLCP, significantly enhanced AR transcriptional activity in both the presence and absence of androgen, characterised by a dramatic increase in AR mRNA and protein expression. This provides novel evidence for 2 things; firstly, the identification of a specific PP1 holoenzyme capable of modulating AR activity, and secondly, a repressive role for PP1 in the regulation of AR function. Due to the fact that MLCP appears to be intrinsically repressive to AR function in a ligand independent manner, it might be expected that PPP1R12A could be subject to genomic loss or mutation throughout the progression of PC, particularly in a castrate resistant setting. However, upon interrogation of a publicly available dataset containing 150 sequenced mCRPC samples (Dan *et al.*, 2015) 0% of samples harboured genomic loss of the PPP1R12A gene, whilst only 0.75% (1/150) of samples harboured a mutation (fusion gene) within the PPP1R12A gene. This strongly suggests that PPP1R12A is essential for cell viability, despite being identified as a repressive regulator of AR function. Upon dissection of the literature, it is apparent that MLCP plays a crucial role in cytoskeletal rearrangements, impacting on cell migration, cell cycle progression and ultimately cellular proliferation. However, it also becomes increasingly apparent that the activity of MLCP is heavily influenced at the post-translational level by a number of different signalling cascades in a very sensitive spatio-temporal manner. With this in mind it was possible to identify PPP1R14C, an endogenous inhibitory protein for MLCP, as an activator of AR transcriptional activity. Characterisation of the role of MLCP on AR functionality using robust molecular biology techniques revealed that RNAi depletion of PPP1R14C leads to enhanced AR proteasomal degradation and impaired ligand induced nuclear translocation, characterised by a repressive phosphorylation status, culminating in significantly reduced AR transcriptional activity in both the presence and absence of androgen. Phenotypically, RNAi knockdown of PPP1R14C causes G1 cell cycle arrest whilst dramatically reducing cell migration, but crucially, represses the proliferation of PC cells within distinct models of castrate resistance. However, with the increased availability of enhanced sequencing techniques, it is becoming increasingly apparent that the genomic landscape of CRPC is highly heterogeneous

and extremely complex. Aberrations within the PI3K signalling cascade and the cell cycle machinery are amongst some of the most frequently observed abnormalities in CRPC, undoubtedly contributing to disease resistance and driving disease progression. Crucially, it was established that MLCP inhibition of AR activity partly occurs via an indirect mechanism, incorporating the activation of 2 clinically relevant tumour suppressors, RB1 and NF2. Increasing the activity of both RB1 and NF2, via the depletion of endogenous MLCP inhibitory proteins, provides a novel route of repressing E2F1 and MTORC1 respectively, both independent PC therapeutic targets within their own right. An additional advantage of increasing MLCP activity via PPP1R14C depletion is increased dephosphorylation of PLK1, another promising therapeutic target in the treatment of PC. Targeting specific PP1 regulatory subunits has recently emerged as a viable route of modulating PP1 activity, providing significant reassurance that PPP1R14C remains a viable therapeutic target for the future. However, currently no tool compounds exist to directly enhance the activity of MLCP via disruption of endogenous inhibitory proteins. This provided the project with a rationale to investigate additional MLCP inhibitors where small molecule inhibitors may be available. As previously mentioned, PPP1R12A is heavily influenced at the post-translational level by a number of different signalling cascades in a specific and sensitive manner. Using a candidate based approach it was possible to identify NUAK1/2 as novel AR co-regulators and potential therapeutic targets in the treatment of PC. Inhibition of NUAK1/2 with the small molecule WZ-4003 prevented the repressive phosphorylation of PPP1R12A by NUAK1/2, characterised by an increase in MLCP activity, leading to repression of AR transcriptional activity, and resulting in a reduction in PC cell growth. Furthermore, inhibition of NUAK1/2 was capable of repressing AR variant transcriptional activity in the CWR22RV1 cell line, reinforcing the finding that MLCP is a ligand independent repressor of the AR. In conclusion, MLCP has been characterised as a novel PP1 holoenzyme capable of significantly repressing castrate resistant AR transcriptional activity, with modulation of 2 distinct MLCP inhibitory mechanisms providing compelling therapeutic potential, ultimately warranting further scientific investigation.

Appendix

Gene Set	NES	p-Value	FDR q-Value
REACTOME_GENERIC_TRANSCRIPTION_PATHWAY	3.04	0	0
REACTOME_BIOLOGICAL_OXIDATIONS	2.76	0	0.001
REACTOME_PHASE1_FUNCTIONALIZATION_OF_COMPOUNDS	2.63	0	0.004
REACTOME_CYTOCHROME_P450_ARRANGED_BY_SUBSTRATE_TYPE	2.54	0	0.005
REACTOME_INTEGRIN_CELL_SURFACE_INTERACTIONS	2.51	0.002	0.005
REACTOME_INTEGRIN_ALPHAIIB_BETA3_SIGNALING	2.44	0	0.007
REACTOME_AXON_GUIDANCE	2.44	0	0.006
REACTOME_GPCR_DOWNSTREAM_SIGNALING	2.38	0	0.007
REACTOME_EFFECTS_OF_PIP2_HYDROLYSIS	2.31	0	0.01
REACTOME_SIGNALING_BY_RHO_GTPASES	2.27	0	0.012
REACTOME_HS_GAG_BIOSYNTHESIS	2.12	0.002	0.03
REACTOME_PLATELET_AGGREGATION_PLUG_FORMATION	2.01	0	0.054
REACTOME_SIGNALING_BY_ROBO_RECEPTOR	1.95	0.01	0.07
REACTOME_ION_CHANNEL_TRANSPORT	1.94	0.01	0.071
REACTOME_BRANCHED_CHAIN_AMINO_ACID_CATABOLISM	1.94	0.002	0.067
REACTOME_ION_TRANSPORT_BY_P_TYPE_ATPASES	1.9	0.01	0.077
REACTOME_NUCLEAR_RECEPTOR_TRANSCRIPTION_PATHWAY	1.82	0.008	0.111
REACTOME_SIGNALING_BY_GPCR	1.8	0.02	0.115
REACTOME_NCAM_SIGNALING_FOR_NEURITE_OUT_GROWTH	1.8	0.01	0.11
REACTOME_HEPARAN_SULFATE_HEPARIN_HS_GAG_METABOLISM	1.8	0.017	0.107
REACTOME_CGMP_EFFECTS	1.79	0.012	0.105
REACTOME_NCAM1_INTERACTIONS	1.78	0.025	0.109
REACTOME_NITRIC_OXIDE_STIMULATES_GUANYLATE_CYCLASE	1.77	0.023	0.105
REACTOME_DEVELOPMENTAL_BIOLOGY	1.77	0.02	0.101
REACTOME_CLASS_A1_RHODOPSIN_LIKE_RECEPTORS	1.75	0.032	0.108
REACTOME_METABOLISM_OF_STEROID_HORMONES_AND_VITAMINS_A_AND_D	1.73	0.027	0.116
REACTOME_HS_GAG_DEGRADATION	1.72	0.029	0.117
REACTOME_OLFACTORY_SIGNALING_PATHWAY	1.72	0.025	0.115
REACTOME_CELL_JUNCTION_ORGANIZATION	1.66	0.029	0.152
REACTOME_APOPTOTIC_CLEAVAGE_OF_CELLULAR_PROTEINS	1.64	0.051	0.161
REACTOME_NRAGE_SIGNALS_DEATH_THROUGH_JNK	1.62	0.042	0.172
REACTOME_PEPTIDE_LIGAND_BINDING_RECEPTORS	1.59	0.055	0.19
REACTOME_COLLAGEN_FORMATION	1.57	0.059	0.199
REACTOME_NETRIN1_SIGNALING	1.55	0.047	0.212
REACTOME_ADHERENS_JUNCTIONS_INTERACTIONS	1.55	0.056	0.208
REACTOME_STEROID_HORMONES	1.55	0.057	0.207
REACTOME_GPCR_LIGAND_BINDING	1.53	0.059	0.215
REACTOME_L1CAM_INTERACTIONS	1.5	0.072	0.243
REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES	1.49	0.077	0.243
REACTOME_PHASE_II_CONJUGATION	1.47	0.093	0.268
REACTOME_G_ALPHA_Q_SIGNALLING_EVENTS	1.46	0.072	0.264

Supplementary Table 1 – ***Top 50 positively enriched REACTOME gene sets following GSEA***

Gene Set	NES	p-Value	FDR q-Value
REACTOME_CELL_CYCLE	-8.84	0	0
REACTOME_CELL_CYCLE_MITOTIC	-7.97	0	0
REACTOME_DNA_REPLICATION	-6.96	0	0
REACTOME_MITOTIC_M_M_G1_PHASES	-6.72	0	0
REACTOME_METABOLISM_OF_RNA	-6.6	0	0
REACTOME_CELL_CYCLE_CHECKPOINTS	-6.49	0	0
REACTOME_METABOLISM_OF_MRNA	-6.32	0	0
REACTOME_S_PHASE	-6.26	0	0
REACTOME_MITOTIC_G1_G1_S_PHASES	-6.13	0	0
REACTOME_G1_S_TRANSITION	-6.06	0	0
REACTOME_METABOLISM_OF_PROTEINS	-6.03	0	0
REACTOME_SYNTHESIS_OF_DNA	-5.91	0	0
REACTOME_TRANSLATION	-5.63	0	0
REACTOME_M_G1_TRANSITION	-5.51	0	0
REACTOME_TELOMERE_MAINTENANCE	-5.49	0	0
REACTOME_CHROMOSOME_MAINTENANCE	-5.13	0	0
REACTOME_ER_PHAGOSOME_PATHWAY	-5.04	0	0
REACTOME_REGULATION_OF_MITOTIC_CELL_CYCLE	-5.02	0	0
REACTOME_SRP_DEPENDENT_COTRANSLATIONAL_PROTEIN_TARGETING_TO_MEMBRANE	-4.97	0	0
REACTOME_G2_M_CHECKPOINTS	-4.88	0	0
REACTOME_APC_C_CDC20_MEDIATED_DEGRADATION_OF_MITOTIC_PROTEINS	-4.84	0	0
REACTOME_APC_C_CDH1_MEDIATED_DEGRADATION_OF_CDC20_AND_OTHER_APC_C_CDH1_TARGETED_PROTEINS_IN_LATE_MITOSIS_EARLY_G1	-4.84	0	0
REACTOME_MRNA_PROCESSING	-4.82	0	0
REACTOME_MRNA_SPLICING	-4.81	0	0
REACTOME_PROCESSING_OF_CAPPED_INTRON_CONTAINING_PRE_MRNA	-4.79	0	0
REACTOME_ASSEMBLY_OF_THE_PRE_REPLICATIVE_COMPLEX	-4.78	0	0
REACTOME_INFLUENZA_LIFE_CYCLE	-4.74	0	0
REACTOME_ORC1_REMOVAL_FROM_CHROMATIN	-4.73	0	0
REACTOME_3_UTR_MEDIATED_TRANSLATIONAL_REGULATION	-4.69	0	0
REACTOME_P53_DEPENDENT_G1_DNA_DAMAGE_RESPONSE	-4.69	0	0
REACTOME_REGULATION_OF_MRNA_STABILITY_BY_PROTEINS_THAT_BIND_AU_RICH_ELEMENTS	-4.69	0	0
REACTOME_DNA_STRAND_ELONGATION	-4.56	0	0
REACTOME_CDT1_ASSOCIATION_WITH_THE_CDC6_ORC_ORIGIN_COMPLEX	-4.54	0	0
REACTOME_ACTIVATION_OF_ATR_IN_RESPONSE_TO_REPLICATION_STRESS	-4.5	0	0
REACTOME_SCFSKP2_MEDIATED_DEGRADATION_OF_P27_P21	-4.47	0	0
REACTOME_AUTODEGRADATION_OF_CDH1_BY_CDH1_APC_C	-4.43	0	0
REACTOME_EXTENSION_OF_TELOMERES	-4.43	0	0
REACTOME_CYCLIN_E_ASSOCIATED_EVENTS_DURING_G1_S_TRANSITION_	-4.42	0	0
REACTOME_CDK_MEDIATED_PHOSPHORYLATION_AND_REMOVAL_OF_CDC6	-4.41	0	0
REACTOME_CLASS_I_MHC_MEDIATED_ANTIGEN_PROCESSING_PRESENTATION	-4.39	0	0

Supplementary Table 2 – ***Top 50 negatively enriched REACTOME gene sets following GSEA***

Gene Set	NES	p-Value	FDR q-Value
HALLMARK_E2F_TARGETS	-9.33	0	0
HALLMARK_G2M_CHECKPOINT	-7.53	0	0
HALLMARK_MYC_TARGETS_V1	-6.75	0	0
HALLMARK_MTORC1_SIGNALING	-4.51	0	0
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	-4.09	0	0
HALLMARK_DNA_REPAIR	-3.58	0	0
HALLMARK_MYC_TARGETS_V2	-3.43	0	0
HALLMARK_OXIDATIVE_PHOSPHORYLATION	-3.36	0	0
HALLMARK_ANDROGEN_RESPONSE	-2.99	0	0
HALLMARK_UV_RESPONSE_UP	-2.58	0	0
HALLMARK_MITOTIC_SPINDLE	-2.43	0	0.001
HALLMARK_PI3K_AKT_MTOR_SIGNALING	-2.18	0.002	0.004
HALLMARK_NOTCH_SIGNALING	-1.78	0.024	0.038
HALLMARK_WNT_BETA_CATENIN_SIGNALING	-1.73	0.029	0.045
HALLMARK_GLYCOLYSIS	-1.44	0.108	0.164
HALLMARK_TGF_BETA_SIGNALING	-1.17	0.257	0.434
HALLMARK_CHOLESTEROL_HOMEOSTASIS	-1.11	0.303	0.496
HALLMARK_P53_PATHWAY	-1.06	0.347	0.536
HALLMARK_ALLOGRAFT_REJECTION	-1.05	0.373	0.525
HALLMARK_PROTEIN_SECRETION	-0.99	0.457	0.611
HALLMARK_HEME_METABOLISM	-0.98	0.449	0.584
HALLMARK_SPERMATOGENESIS	-0.98	0.436	0.559
HALLMARK_REACTIVE_OXIGEN_SPECIES_PATHWAY	-0.94	0.503	0.602
HALLMARK_ADIPOGENESIS	-0.85	0.645	0.727
HALLMARK_APOPTOSIS	-0.84	0.662	0.704
HALLMARK_HEDGEHOG_SIGNALING	-0.84	0.658	0.677
HALLMARK_APICAL_SURFACE	-0.72	0.809	0.823

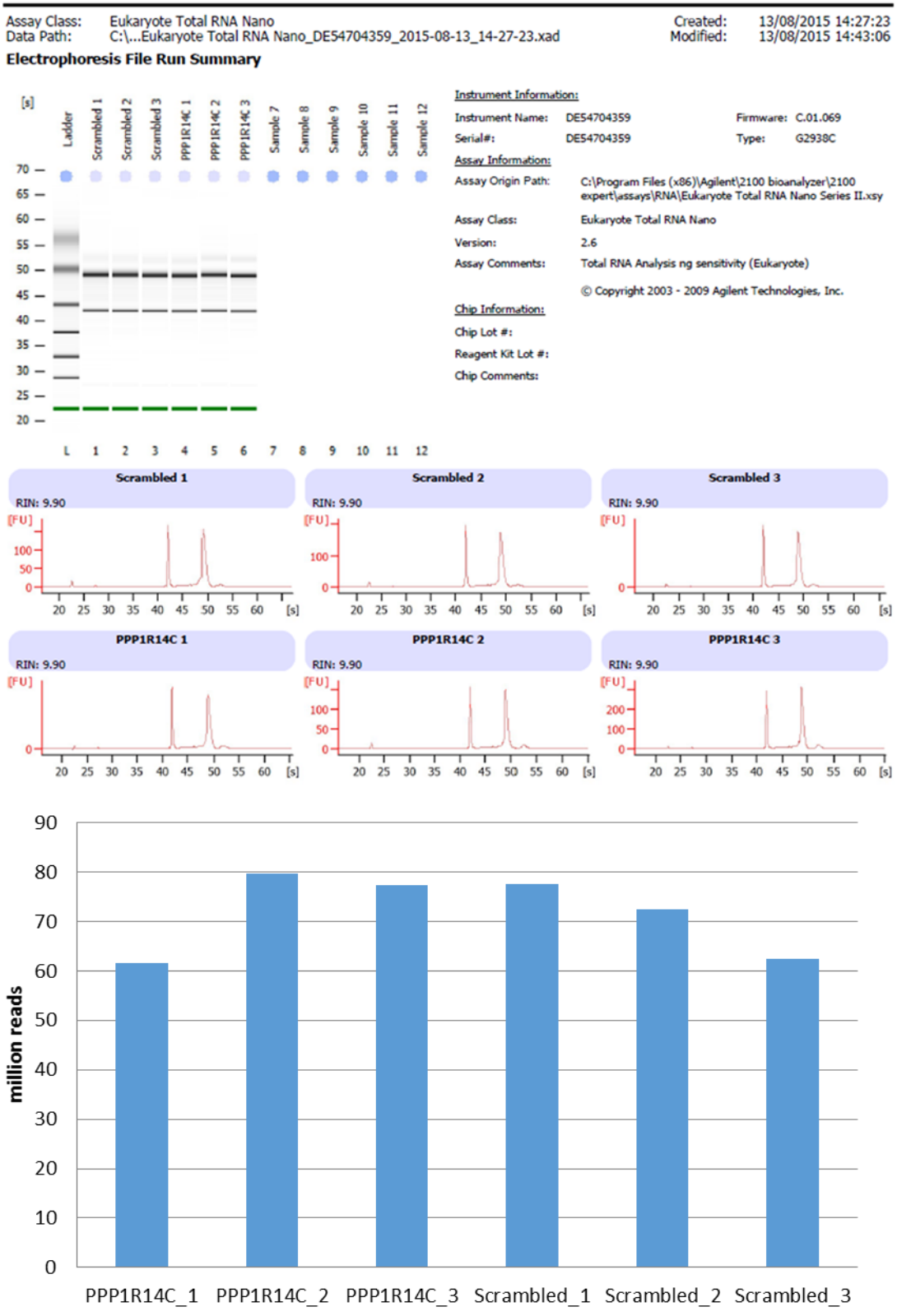
Supplementary Table 3 – ***Most significant negatively enriched MSigDB 'Hallmark' gene sets following GSEA***

Gene Set	NES	p-Value	FDR q-Value
CSR_LATE_UP.V1_UP	-4.9	0	0
RB_P107_DN.V1_UP	-4.24	0	0
E2F1_UP.V1_UP	-4	0	0
GCNP_SHH_UP_LATE.V1_UP	-3.46	0	0
GCNP_SHH_UP_EARLY.V1_UP	-3.33	0	0
CAMP_UP.V1_UP	-3.29	0	0
E2F3_UP.V1_UP	-2.86	0	0
HOXA9_DN.V1_DN	-2.85	0	0
PRC2_EDD_UP.V1_UP	-2.77	0	0
RPS14_DN.V1_DN	-2.71	0	0
PRC2_EZH2_UP.V1_UP	-2.65	0	0
PTEN_DN.V2_DN	-2.6	0	0
VEGF_A_UP.V1_DN	-2.57	0	0
MYC_UP.V1_UP	-2.51	0	0.001
RB_DN.V1_UP	-2.43	0	0.001
TBK1.DF_DN	-2.38	0	0.002
PDGF_UP.V1_UP	-2.34	0	0.003
RB_P130_DN.V1_UP	-2.24	0	0.005
EIF4E_UP	-2.11	0	0.011
SNF5_DN.V1_UP	-1.93	0.008	0.031
CORDENONSI_YAP_CONSERVED_SIGNATURE	-1.91	0.008	0.031
ESC_J1_UP_LATE.V1_DN	-1.9	0.01	0.031
TBK1.DF_UP	-1.88	0.01	0.034
CSR_EARLY_UP.V1_UP	-1.87	0.01	0.034
TBK1.DN.48HRS_DN	-1.76	0.017	0.062
IL15_UP.V1_UP	-1.74	0.025	0.064
PDGF_ERK_DN.V1_DN	-1.73	0.022	0.065
LTE2_UP.V1_UP	-1.7	0.018	0.074
RB_P107_DN.V1_DN	-1.7	0.017	0.072
NRL_DN.V1_UP	-1.66	0.049	0.086
TBK1.DN.48HRS_UP	-1.6	0.036	0.111
MTOR_UP.N4.V1_UP	-1.5	0.085	0.171

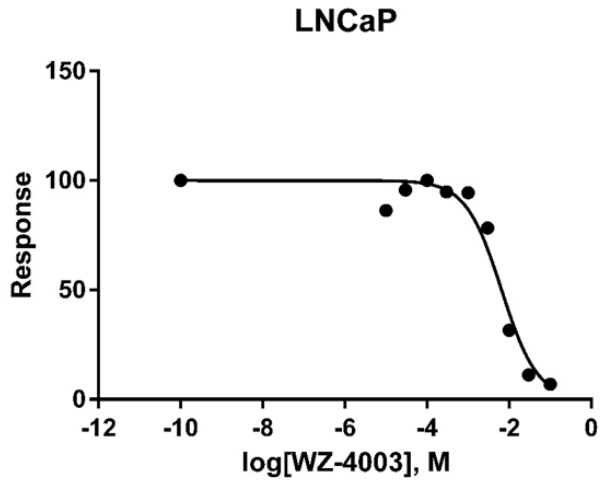
Supplementary Table 4 – ***Most significant negatively enriched MSigDB 'Cancer Gene Perturbations' gene sets following GSEA***

Gene Set	NES	p-Value	FDR q-Value
GGGCGGR_V$SP1_Q6	-6.96	0	0
SCGGAAGY_V$ELK1_02	-6.31	0	0
V$E2F4DP1_01	-5.69	0	0
V$E2F1_Q6	-5.59	0	0
GCCATNTTG_V$YY1_Q6	-5.53	0	0
V$E2F1DP1RB_01	-5.53	0	0
V$E2F_Q4	-5.45	0	0
V$E2F_Q6	-5.45	0	0
V$E2F_03	-5.39	0	0
V$E2F1DP1_01	-5.34	0	0
RCGCANGCGY_V$NRF1_Q6	-5.32	0	0
V$E2F1DP2_01	-5.31	0	0
V$E2F4DP2_01	-5.31	0	0
V$E2F_02	-5.29	0	0
GATTGGY_V$NFY_Q6_01	-5.2	0	0
V$E2F_Q4_01	-5.08	0	0
V$E2F1_Q6_01	-5.05	0	0
V$E2F_Q3	-4.96	0	0
V$E2F_Q6_01	-4.95	0	0
V$E2F1_Q4_01	-4.91	0	0
V$E2F1_Q3	-4.86	0	0
CACGTG_V$MYC_Q2	-4.79	0	0
SGCGSSAAA_V$E2F1DP2_01	-4.75	0	0
V$E2F_Q3_01	-4.48	0	0
MGGAAGTG_V$GABP_B	-4.06	0	0
V$NFMUE1_Q6	-3.94	0	0
GTGACGY_V$E4F1_Q6	-3.9	0	0
V$E2F1_Q3_01	-3.83	0	0
V$E2F1_Q4	-3.82	0	0

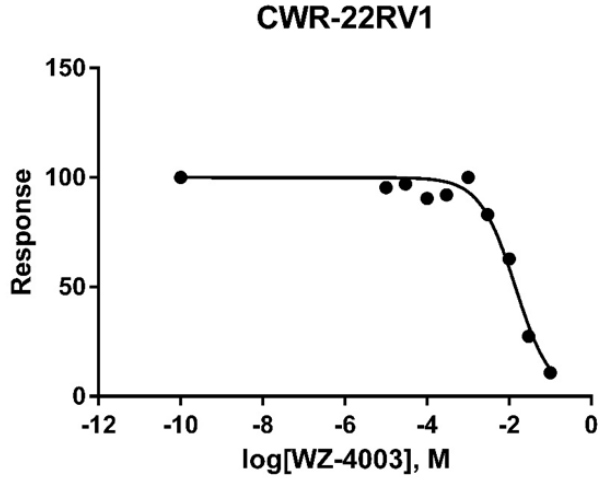
Supplementary Table 5 – ***Most significant negatively enriched MSigDB 'Motif Based' gene sets following GSEA***



Supplementary figure 1 – ***Results from RNA integrity analysis and reads per sample upon RNA sequencing.*** *Extracted RNA was subjected to anaylsis using the bioanalyzer as described in chapter 2. All samples possess a RIN value > 9 and were therefore taken forward for RNA sequencing. Upon sequencing, the amount of reads per sample was calculated by Aros Biotechnology and can be observed above.*



LogIC50	-2.182
IC50	0.006573
Std. Error	
LogIC50	0.08228
95% Confidence Intervals	
LogIC50	-2.368 to -1.996
IC50	0.004282 to 0.01009



LogIC50	-1.845
IC50	0.01427
Std. Error	
LogIC50	0.05754
95% Confidence Intervals	
LogIC50	-1.976 to -1.715
IC50	0.01058 to 0.01926

*Supplementary figure 2 – **WZ-4003 IC50 in LNCaP and CWR-22RV1 cell lines.** LNCaP and CWR-22RV1 cells were exposed to increasing concentrations of WZ-4003 over a 120 hour period. Cell growth was measured using the BioEssen Incucyte live cell imager as described in chapter 2. IC50 values were calculated using GraphPad Prism.*

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